Universitat Autònoma de Barcelona

# Molecular basis of innate immune activation pathways as regulators of susceptibility and clinical evolution of viral infections

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# Molecular basis of innate immune activation pathways as regulators of susceptibility and clinical evolution of viral infections.

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Certifiquen:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral titulada "Molecular basis of innate immune activation pathways as regulators of susceptibility and clinical evolution of viral infections" han estat realitzades per Maria Pujantell Graell sota la seva direcció i considera que és apta per a ser presentada per optar al grau de Doctora en Immunologia Avançada per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signen aquest document a Badalona,

17 de Juliol de 2020.

Dr. Bonaventura Clotet Sala

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To my parents, that just as ADAR1,

their absence triggers a cascade of events

that push me to my highest potential.

# TABLE OF CONTENTS

## SUMMARY

The innate immune system is the first to respond against a pathogen invasion that threatens the host. An innate immune response is characterized by inducing innate immune activation, IFN signaling and inflammatory pathways that generate an antiviral state able to limit pathogen spread and induce cell death of damaged cells. The identification of HIV host restriction factors, such as SAMHD1, has provided insights linking nucleotide metabolism, innate immune activation and viral pathogenesis. Hence, this thesis evaluates the relationship between viral and host factors that strongly affect innate immune modulation and IFN pathways that have the potential to alter viral outcome and progression of associated diseases.

Through the study of HIV infection, we have identified that HIV induces an innate antiviral mechanism associated to IFN-I production and interferon stimulated gene activation. Moreover, HIV induces a cell cycle arrest at G2/M mediated by p21 leading to elevated levels of cell death in monocyte derived macrophages. On the other hand, accessory protein Vpx from HIV-2/SIV, which targets the downregulation of SAMHD1 and TASOR, also triggers innate immune activation signatures characterized by induction of IFNs and expression of ISGs which may hold key in designing strategies targeting reactivation of latent HIV.

The discovery that mutations in *SAMHD1* and *TREX1* are associated to autoimmune disease Aicardi-Goutières syndrome (AGS) and the fact that both genes have a role in HIV replication, suggests that evaluation of nucleic acid sensors in AGS may have potential for identifying host factors regulating viral replication. Indeed, a screening of AGS genes in primary macrophages has identified ADAR1 as a negative regulator of RIG-I like receptor RLR-MAVS signaling pathway contributing to innate immune activation and IFN production. ADAR1 downregulated cells show increase expression of RNA sensors, MDA5 and RIG-I, enhanced phosphorylation of transcriptional factor IRF7 and increase type I IFN production. ADAR1 *in-vitro* evaluation have shown a proviral role in HIV, but an antiviral role in HCV and HPV, suggesting ADAR1 role depends on the specific virus host combination. Finally, *ADAR1 in-vivo* genetic variations are associated to poor clinical outcomes and affect treatment response and disease progression.

In summary, this thesis demonstrates that dysfunction of intracellular nucleic acid sensing pathways that trigger strong innate immune activation and IFN signatures hold promising in the design of novel therapeutic strategies aiming to modulate viral replication and disease progression and infection outcomes.

## RESUM

El sistema immune innat és el primer en respondre davant patògens que amenacen l'hoste. La resposta immune innata es caracteritza per induir una activació immune innata, iniciar la senyalització d'IFNs i activar vies inflamatòries que generen un estat antiviral capaç de limitar la propagació de patògens i induir la mort cel·lular de cèl·lules afectades.

La identificació de factors de restricció del VIH, com SAMHD1, ha proporcionat coneixements que han relacionat el metabolisme dels nucleòtids, l'activació immune innata i la patogènesi del virus. Per tant, aquesta tesi avalua la relació entre factors virals i de l'hoste que poden afectar la regulació del sistema immune innat i activació de vies d'interferó on potencialment poden controlar la replicació viral i progressió de malalties associades.

Gràcies a l'estudi *in-vitro* de la infecció del VIH, hem identificat que el virus indueix un mecanisme antiviral innat associat a la producció d'interferó de tipus I i activació de gens estimulats per interferó (ISGs). D'altra banda, el VIH indueix una aturada en el cicle cel·lular a la fase G2/M, mediada per p21, que causa un augment en els nivells de mort cel·lular en macròfags derivats de monòcits (MDMs). D'altra banda, la proteïna accessòria Vpx de VIH-2/SIV, té com a objectiu disminuir l'expressió de SAMHD1 i TASOR. Al mateix temps, Vpx inicia una cascada de senyalització d'activació immune innata caracteritzada per la inducció d'IFNs i expressió d'ISGs que poden ser claus en el disseny d'estratègies dirigides a la reactivació latent del VIH.

El descobriment que mutacions en *SAMHD1* i *TREX1* estan associades a la malaltia autoimmune del síndrome d'Aicardi-Goutières (AGS) i el fet que ambdós gens tinguin un paper en la replicació del VIH, suggereix que la avaluació de sensors d'àcid nucleic en base a AGS pot potencialment identificar factors de l'hoste que regulen la replicació viral. De fet, un cribratge de gens d'AGS en macròfags primaris ha identificat ADAR1 com un regulador negatiu de la via de senyalització RLR-MAVS que contribueix a l'activació immune innata i la producció d'IFNs. Les cèl·lules on s'ha disminuït l'expressió d'ADAR1 mostren un augment de l'expressió de sensors d'ARN com MDA5 i RIG-I, un augment en la fosforilació del factor de transcripció IRF7 i un augment en la producció d'IFNs de tipus I. L'avaluació *in-vitro* d'ADAR1, ha mostrat que ADAR1 té un efecte proviral davant el VIH o antiviral davant de VHC i VPH, suggerint que el rol d'ADAR1 depèn de la combinació específica entre virus i hoste. Finalment, les variacions genètiques d'ADAR1 *in-vivo* s'associen a mal pronòstic clínic i afecten a la resposta del tractament i a la progressió de la malaltia.

En resum, aquesta tesi demostra que la disfunció en vies de detecció d'àcids nucleics intracel·lulars, que desencadenen a una forta activació immune innata i les signatures IFN, són prometedores pel disseny de noves estratègies terapèutiques que tenen com a objectiu modular la replicació viral, alterar la progressió de la malaltia i afectar el resultat final de la infecció.

## RESUMEN

El sistema inmune innato es el primero en responder ante patógenos que amenazan el huésped. La respuesta inmune innata se caracteriza por inducir una activación inmune innata, iniciar la señalización de IFNs y activar vías inflamatorias que generan un estado antiviral capaz de limitar la propagación del patógeno e inducir la muerte celular de células afectadas.

La identificación de factores de restricción del VIH, como SAMHD1, ha proporcionado conocimientos que han relacionado el metabolismo de los nucleótidos, la activación inmune innata y la patogénesis del virus. Por lo tanto, esta tesis quiere evaluar la relación entre factores virales y del huésped que pueden afectar a la regulación del sistema inmune innato y activación de vías de interferón donde potencialmente pueden controlar la replicación viral y progresión de enfermedades asociadas.

Gracias al estudio *in-vitro* de la infección del VIH, hemos identificado que el virus induce un mecanismo antiviral innato asociado a la producción de interferón de tipo I y activación de genes estimulados por interferón (ISGs). Por otra parte, el VIH induce una parada en el ciclo celular en la fase G2 / M, mediada por p21, que causa un aumento en los niveles de muerte celular en macrófagos derivados de monocitos (MDMS). Por otra parte, la proteína accesoria Vpx de VIH-2/SIV, tiene como objetivo disminuir la expresión de SAMHD1 y TASOR. Al mismo tiempo, VPX inicia una cascada de señalización de activación inmune innata caracterizada por la inducción de IFNs y expresión de ISGs que pueden ser claves en el diseño de estrategias dirigidas a la reactivación latente del VIH.

El descubrimiento que mutaciones en SAMHD1 y TREX1 están asociadas a la enfermedad autoinmune del síndrome de Aicardi-Goutières (AGS) y el hecho de que ambos tengan un papel en la replicación del VIH, sugiere que la evaluación de sensores de ácido nucleico en base a AGS puede potencialmente identificar factores del huésped que regulan la replicación viral. De hecho, un cribado de genes de AGS en macrófagos primarios ha identificado ADAR1 como un regulador negativo de la vía de señalización RLR-MAVS que contribuye a la activación inmune innata y la producción de IFNs. Las células donde se ha disminuido la expresión de ADAR1 muestran un aumento de la expresión de sensores de ARN como MDA5 y RIG-I, un aumento en la fosforilación del factor de transcripción IRF7 y un aumento en la producción de IFNs de tipo I. La evaluación *in-vitro* de ADAR1, ha mostrado que ADAR1 tiene un efecto proviral ante el VIH o antiviral ante VHC y VPH, sugiriendo que el rol de ADAR1 depende de la combinación específica entre virus y huésped. Finalmente, las variaciones genéticas de ADAR1 *in-vivo* se asocian a mal pronóstico clínico y afectan a la respuesta del tratamiento y la progresión de la enfermedad.

En resumen, esta tesis demuestra que la disfunción en vías de detección de ácidos nucleicos intracelulares, que desencadenan una fuerte activación inmune innata y patrón de IFNs, son prometedoras para el diseño de nuevas estrategias terapéuticas que tienen como objetivo modular la replicación viral, alterar la progresión de la enfermedad y afectar el resultado final de la infección.

# ABBREVIATIONS

| | |
|---|---|
| DCV | Daclatasvir |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| IL | Interleukin |
| IN | Integrase |
| IRFs | IFN-regulatory factors |
| ISGs | IFN-stimulated genes |
| JAKs | Janus-activated kinases |
| LOD | Limit of detection |
| MAVS | Mitochondrial antiviral-signaling protein |
| MCSF | Macrophage colony-stimulating factor |
| MDA5 | Melanoma Differentiation-Associated antigen 5 |
| MHC | Major histocompatibility complex |
| MV | Measles virus |
| NFKB | Nuclear factor-kB |
| NK | Natural killer cell |
| NLRs | NOD-like receptor |
| PAMPS | Pathogen-associated molecular patterns |
| PEG IFN/riba | Pegylated interferon alpha and ribavirin |
| PKR | Protein kinase R |
| PNB | Panobinostat |
| PR | Protease |
| PRRs | Pattern recognition receptors |
| RIG-I | Retinoic acid-Inducible Gene I |
| RLR | Rig-I-like receptor |
| RT | Reverse transcriptase |
| SAMHD1 | Sterile Alpha Motif and Histidine Aspartate domain-containing protein 1 |
| SIV | Simian immunodeficiency virus |
| SNP | Single nucleotide polymorphisms |
| ssDNA | Single stranded DNA |
| ssRNA | Single stranded RNA |

Abbreviations

| | |
|---|---|
| STAT1 | Signal transducer and activator of transcription 1 |
| SVR | Sustained virological response |
| TBK1 | Serine/threonine-protein kinase |
| TCR | T-cell receptors |
| TLR | Toll-like receptor |
| TRAF | TNF receptor-associated factor |
| VL | Viral load |
| Vpr | Viral protein r |
| Vpu | Viral protein u |
| Vpx | Viral protein x |
| VSV | Vesicular stomatitis virus |
| WHO | World health organization |

# INTRODUCTION

# 1. THE IMMUNE SYSTEM

The immune system is responsible for recognizing, neutralizing and eliminating pathogens, damaged cells or nucleic acids detected as "foreign" [1] that pose a threat for our health status and body homeostasis.

All organisms share certain immune characteristics and traits. However, time and evolution resulted in different layers of immune complexity that distinguish them - the more complex the organism the higher level of immune protection developed. Harmful pathogens affecting humans can be classified in five categories: bacteria, fungi, protozoa, worms and virus. This thesis will focus on the immune response toward viruses and the different human immune mechanisms to effectively eliminate them.

Host immunity comprises the innate and the adaptive immune systems. **The innate immune system** is present long before any pathogen is recognized. It is the first to respond after sensing the presence of a pathogen or identifying an alarm signal from damaged or injured cells. The innate immune response is unspecific or generic, meaning the response is the same independently of the trigger and it does not confer long-lasting immunity against the pathogen. On the other hand, **the adaptive immune system** is only developed after the recognition of a pathogen. It is highly specific and involves specialized immune cells and processes, producing a stronger response that takes days to develop. The adaptive response mounts antigen-specific responses against "foreign" antigens and develops immune memory that builds long-lasting immunity.

There are different types of cells pertaining to the innate and/or adaptive immune response, which will be described in detail in the following section. However, immune cells from the innate as well as adaptive immune system must interact and develop a coordinated immune response. Innate immunity has the capacity to restrict antigens by inducing an inflammatory response [2] triggered by different mechanisms such as identifying specific structural components from pathogens or discerning self- and non-self-nucleic acids. Specialized innate immune cells engulf foreign pathogens or damaged cells and present antigens to adaptive immune cells. The adaptive immune response recruit effectors cells to produce antibodies, specific to the pathogen and develops memory cells capable of controlling future pathogen invasions.

## 1.1. CELLS OF THE IMMUNE RESPONSE

Immune cells form in the bone marrow, mature, and migrate to peripheral tissues. All immune cells originally derive from the same **progenitor cell**, the hematopoietic pluripotent stem cell, that further differentiates into a variety of specialized cells which will be part of the innate or adaptive immune system. The progenitor cell gives rise to two distinct cell lineages: the common lymphoid progenitor and the common myeloid progenitor (figure 1).

The **common lymphoid progenitor** develops into B and T lymphocytes, natural killer (NK) and NK-T cells. B and T cells differ on their site of differentiation (T cells differentiate in the thymus; B cells differentiate in the bone marrow) and also on their cell surface phenotypes (T cells express TCR and B cells express B cell receptors). NK cells are morphologically distinguished by large granular lymphocytes, do not express TCR or surface immunoglobulins (Ig), and recognize virus-infected or tumor cell targets using cell surface receptors. Finally, NK-T cells share characteristics from NK and T cells, expressing surface markers characteristic from both NK and conventional T cells[2,3].

On the other hand, the **common myeloid progenitor** gives rise to granulocytes, megakaryocytes and erythrocytes. Cells from the granulocyte lineage include neutrophils, eosinophils, basophils, and monocytes. Monocytes will further give rise to mast cells, macrophages and dendritic cells[2,4]. **The granulocyte lineage consists of p**hagocytic cells that engulf marked cells for clearance and repair of tissue injury[2]**.** These cells persist for long periods of time at sites of chronic inflammation and infection and are distinguished by the active molecules they produce and accumulate in specific pathological conditions.

Moreover, cells from the monocyte/macrophage lineage take antigens and process them through proteolysis (digestion to peptide fragments), to subsequently present these processed antigens to T cells and activate adaptive immune response, which take days to develop. In critical tissues subjective to pathogen invasion, specialized cells are responsible for this process, for instance in the epidermis there are the Langerhans cells; in the liver there are the Kupffer cells; and in the central nervous system the microglia[2].

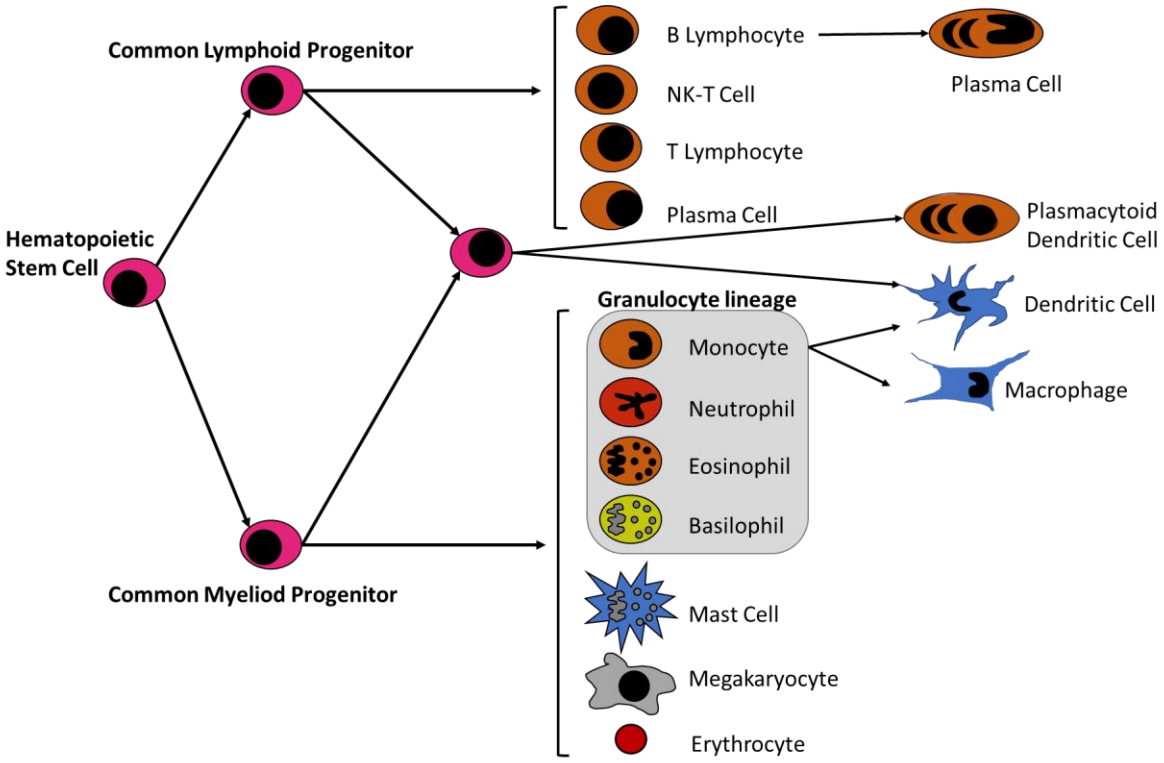


**Figure 1. Schematic representation of hematopoietic stem cell derived cell lineages.** The hematopoietic stem cell gives rise to two different progenitor cells: common lymphoid and myeloid progenitors. These then further develop to highly specialized cells that make up the immune system. Figure modified from[2].

## 1.2. INNATE IMMUNE RESPONSE

Innate immunity is the first defense against pathogens or damaged host cells by the immune system. An innate immune response is composed of physical, chemical and cellular barriers that restrict the spread of a pathogen or host aberrant cells through the induction of an inflammatory response able to restrict its spread and limit host damage[5]. Innate immunity is ubiquitously present in multicellular organisms, plants and animals[6], and it is quite conserved across species from plants to mammals[7] .

In humans, **physical barriers** responsible for restricting viral entry are the skin, mucous membranes, gastrointestinal (GI) track, urinary track and endothelial membranes throughout the body[1].

Also, **chemical responses** such as changes in pH (acidic pH in skin and gastric acid) are hostile to foreign pathogens, and chemical agents as antimicrobial molecules such as lysozyme, IgA and beta-defensins also contribute to the neutralization and cleavage of bacterial, fungi, parasites and virus.

The **cellular response** of the innate immune system is tackled by phagocytic cells, dendritic cells and NK cells. Phagocytes are cells that constantly ingest harmful or foreign particles, bacteria and dead

cells from the environment as a defense mechanism. There are three types of phagocytic cells: macrophage, granulocytes and dendritic cells. Dendritic cells have different functions, also process and present antigens to the adaptive immune cells to produce antibodies and stimulate T cell responses. On the other hand, NK cells are considered professional cells that recognize virus-infected or tumor cells, NK cells induce the release of cytokines that cause apoptosis or lysing of stressed cells that are not well-functioning. Moreover, NK cells are in the midst of the innate and adaptive immune response.

### 1.2.1 Macrophages

In this thesis, monocyte derived macrophages have been widely used as a key cell model to study the relationship between the innate immune responses and viral infections in humans.

Macrophages have been described in all tissues of the body. These cells are plastic and have heterogeneous phenotypes that adapt according to the tissue and organ in which they reside. In general, macrophages have been traditionally classified by their polarization type in M0, M1 or M2. Polarization refers to how macrophages have been activated at a specific point in space and time [8]. M0 macrophages are considered naïve macrophages that have not been exposed to any pro- or anti-inflammatory stimuli. M1 macrophages, also referred as classically activated macrophages (CAMs), are thought to display a pro-inflammatory phenotype. M2 polarization, also referred as alternatively activated macrophages (AAMs), are considered to display anti-inflammatory properties.

To induce M1 macrophages simulating an inflammatory environment by TLR (toll-like-receptors) and IFN signaling, cells can be induced *in vitro* by granulocyte-macrophage colony-stimulating factor (GM-CSF) or type II IFN and/or TLR agonist. M1 macrophage exhibit high level of phagocytic activity and guide acute inflammatory responses. These are stimulated by Th1 cytokines, which display pro-inflammatory properties[9].

On the other hand, M2 polarized macrophages are involved in parasitic, helminthic, and fungal infections. M2-like phenotype is induced in response to Th2 cytokines, which display anti-inflammatory properties, macrophage colony-stimulating factor (M-CSF), IL-4, IL-10, IL-13, or a combination of these factors[9]. Different subsets have been described within M2 category (M2a-like, M2b-like, and M2c-like)[8] and are differentiated by their activation stimulus. M2a are induced by IL-

4 or IL-13; M2b induced by poly I:C, TLR or IL-1R agonists; M2c are induced by IL-10 and glucocorticoids[10].

As mentioned, these cells are plastic and therefore moldable depending on the stimuli encountered. Polarization status can switch from M1 to M2 or vice versa in response to environmental stimuli. In fact, the properties assigned to each type are not as simplistic as classically described and if looked in-depth can be much more complex.

There are different types of tissue-resident macrophages, each type is responsible for specific functions needed within the tissue. For instance, Kupffer cells are macrophages resident in the liver, essential for clearance of microorganisms and cell debris from the blood. Langerhans cells are found in the skin, interact with T lymphocytes and act as sentinel cells. Microglia are found in the central nervous system, promote neuronal survival and are involved in immune surveillance. Finally, alveolar macrophages are found in the lungs and are responsible for immune surveillance[11].

### 1.2.2 Coordination between innate and adaptive response

Macrophages, as well as dendritic cells, exert also important functions as innate and adaptive immunity effectors by presenting antigens and thus, play a key role in the outcome of the overall immune response.

Innate and adaptive immune responses must work together for a stronger and more complex immune response. Innate immune cells act first, after the detection of a pathogen, and present antigens to adaptive cells that start the process of antibody-specific responses (figure 2). Four to seven days after the innate immune system is activated, the specific adaptive immune response takes place. Adaptive immunity targets the pathogen more specifically by using antibodies and developing memory cells that will respond faster in the case of a reinfection.

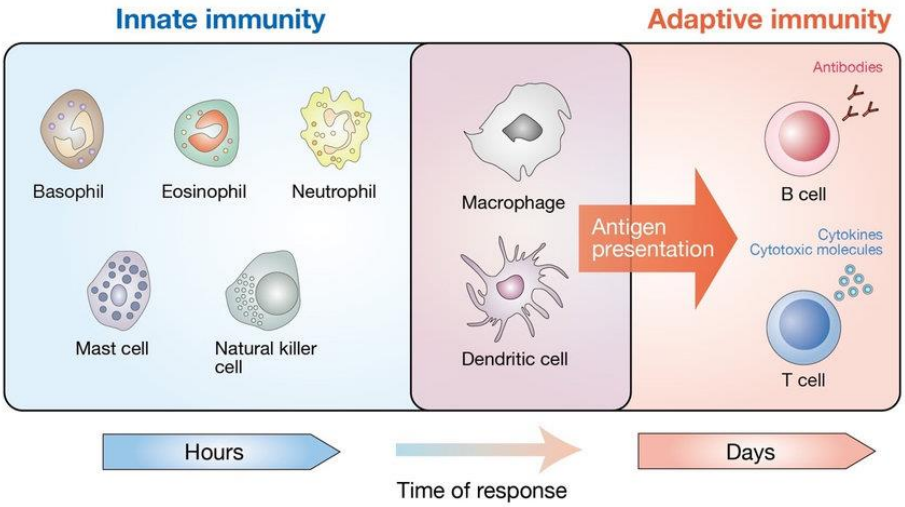


**Figure 2. Coordination between innate and adaptive cells to mount an effective immune response**. Innate and adaptive immune cells interact to present antigens and develop cytokines and antibodies necessary for the development of an inflammatory and antiviral response to respond to a foreign pathogen and an approximate timeline of the responses (Figure from[12] under Creative Commons Attribution License).

As described above, when a pathogen enters the body, the immune system tries to eliminate it using phagocytes or scavenger cells, specialized cells that travel through the body to detect, uptake and digest pathogens or foreign particles, into smaller pieces called antigens. Upon contact with the antigen, phagocytes such as macrophages and dendritic cells, mature to antigen presenting cells (APC), although most APC are dendritic cells. The maturation process involves losing the ability to further phagocyte and alter surface expression of major histocompatibility complex (MHC) and other co-stimulatory molecules and develop the ability to produce increased levels of cytokines.

APC bind their antigens to MHC (major histocompatibility complex) proteins that express on the cell surface and present the epitope (the part of an antigen that binds to a receptor) to naive T lymphocytes in the lymph nodes. Depending on the pathogen, different signals trigger distinct DC (dendritic cells) maturation profiles leading to different activation of T-cell subsets (Th1, Th2, Treg, CTL), and the subsequent modulation of immune response. Finally, B cells will start the antibody production[13,14] that will lead to a directed antibody inhibition of the pathogen.

Many infectious agents as viruses and bacteria have evolved different protection mechanisms, such as encapsulations, that enable them to go undetected by macrophages and other phagocytic cells from the innate immune system that rely on membrane-bound receptors to detect pathogens. However, dendritic cells are constantly digesting and taking up extracellular material by macropinocytosis, a nonreceptor-dependent process that will unmask infectious agents once digested[13].

A key element of adaptive immunity are T-cell receptors (TCR), protein complexes found on the surface of T cells, that are responsible for recognizing fragments of antigens bound to MHC molecules. The binding between TCR and antigen peptides is of relatively low affinity and degenerate, that is, many TCRs recognize the same antigen peptide and many antigen peptides are recognized by the same TCR. In addition, each T cell expresses clonal TCRs which recognize a specific peptide loaded on a MHC molecule. Therefore, the specificity of each lymphocyte is different but collectively, the millions of lymphocytes in the body carry millions of different antigen receptor specificities, the so-called lymphocyte receptor repertoire of the individual. During a lifetime of a person, the lymphocytes that encounter an antigen whose receptor binds to, will be activated to proliferate, and differentiate to an effector cell. An effector cell is an activated cell that divides to produce identical clones, what is referred as clonal expansion, which will secrete clonotypic antibodies with a specificity identical to that of the initial activated lymphocyte[15].

At this stage, the adaptive immune system supports the innate immune response by producing specific antibodies to fight the pathogen[13].

### 1.3. PATTERN RECOGNITION RECEPTORS (PRRs)

Innate immune cells recognize pathogens through intracellular mechanisms such as pattern recognition receptors (PRRs). PRRs are germline-encoded receptors, expressed predominantly in immune cells and specialized to recognize certain features not found in the host cell. These receptors recognize specific characteristics from harmful microbes, referred as pathogen-associated molecular patterns (PAMPS); or aberrant cells, referred as damage associated molecular patterns (DAMPs). There are 4 types of PRRs, each of them with specific intracellular localization and specialized to recognize certain PAMPS or DAMPS (table 1).

**Table 1. Characteristics of PRRs and corresponding PAMPs and DAMPs.**

| PRR | Subcategory | Location | PAMPs /DAMPs |
|---|---|---|---|
| **Toll-like receptors (TLRs)** | TLRs 1, 2, 4, 5, 6 and 10 | Cell membrane[16] | Lipids, lipoproteins, proteins, nucleic acids, peptidoglycans, lipotechoic acids and flagellin[16,17]. |
| | TLRs 3, 7, 8, and 9 | Intracellular compartments[16] | Nucleic acids dsRNA, ssRNA, Poly(I:C), CpG and DNA[17]. |
| **RIG-I-like receptor (RLRs)** | RIG-I (DDX58)<br>MDA5 (IFIH1)<br>LGP2 | Cytoplasm | dsRNA and ssRNA[18,19] |
| **NOD-like receptor (NLRs)** | NLRA<br>NLRB<br>NLRC<br>NLRP | Cytoplasm | Peptidoglycan, flagellin, viral RNA, fungal hyphae[20] |
| **C-type lectin-like receptors (CLR)** | Group I<br>Group II | Cell membrane | Glycoprotein, mannose, β-glucans[21] |

Activation of PRRs trigger diverse signaling pathways (figure 3) each of them being distinct, although all lead to transcription of IFNs and other genes with antiproliferative, antiviral or antibacterial properties that later induce production of interferon-stimulated genes.

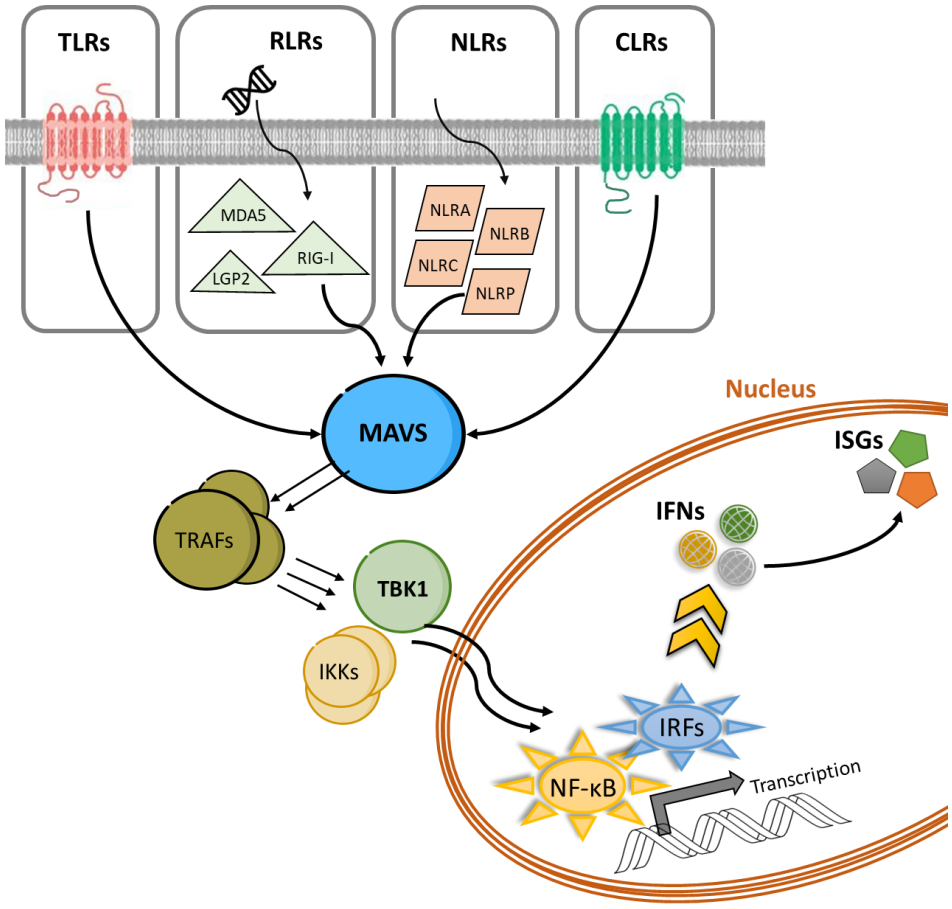


**Figure 3. Common signaling pathway stimulated by PRRs.** Activated PRRs signal through MAVS signaling pathway, which lead to activation of transcription factors NF-kB and IRFs that induce expression of IFNs and ISGs. PRRs can also activate additional cascades.

Common effector proteins shared in all pathways are mitochondrial antiviral-signaling protein (MAVS also referred as IPS-1, CARDIF or VISA), TNF receptor-associated factor (TRAF), serine/threonine-protein kinase (TBK1), and inhibitor of nuclear factor kappa-B kinase complexes (IKK -γ, -α, -β). MAVS is found in the outer membrane of mitochondria, peroxisomes or endoplasmic reticulum and is triggered after the binding of cytosolic proteins, which activate TRAF proteins[22]. TRAF proteins are present in the cytoplasm and act as signaling adaptor proteins and E3 ubiquitin ligase to activate downstream signaling events. TRAF recruits TBK1 and IKK complexes that will trigger expression of transcriptional factors in the nucleus. TBK1 and IKK complexes are highly homologous, specially between TBK1 and IKKε, these adaptor proteins phosphorylate transcriptional factors that subsequently induce expression of IFNs[23,24], which at its turn, induce IFN-stimulated genes (ISGs)[25].

Diverse transcriptional factors are involved, including nuclear factor-kB (NFKB), IFN-regulatory factors (IRFs) and many others taking part in the signaling cascade.

## 1.4. SENSING OF NUCLEIC ACIDS

As mentioned above, endogenous sensing by PRRs is key in the recognition of intracellular pathogen replication or malfunctioning cells. Induction of type I interferons is a hallmark of immune sensing of nucleic acids by the innate immune system[25] that distinguishes between "self" and "non-self" nucleic acids and induce an antiviral environment when needed.

Nucleic acids sensing is based on three criteria[25], although the combination of the three determines the recognition and induction of an innate immune response:

1- Presence and availability of nucleic acid ligands at a local concentration, conditioned by the degradation rate and concentration of nucleases at that site.
2- Localization of the nucleic acid ligand: outside cell membrane, inside endosome/lysosome compartment or at the cytosol.
3- Structure of nucleic acid ligands determined by repetitive sequence motifs and chemical modifications

PRRs sensing nucleic acids can be distinguished by the class of nucleic acid sensed, RNA or DNA. These PRRs focus on the identification of nucleic acids and signal the presence of aberrant or foreign replication.

### 1.4.1. RNA sensing receptors

RNA sensors detect host and foreign ribonucleic acids within the cell and signal the presence of foreign RNAs by inducing expression of IFNs and ISGs that trigger gene transcription necessary for an antiviral response. There are many RNA sensors, although TLRs and RLR families have been extensively studied in terms of RNA sensing (table1, figure 4).

TLRs are expressed on different immune cells as macrophages, dendritic cells, B cells, and certain types of T cells. And in nonimmune cells as fibroblasts and epithelial cells[17]. In terms of RNA sensors, TLR3, TLR7 and TLR8 recognize ssRNA and dsRNA and play a critical role in activation of downstream signaling molecules leading to transcription of type I interferons and inflammatory cytokines[25].

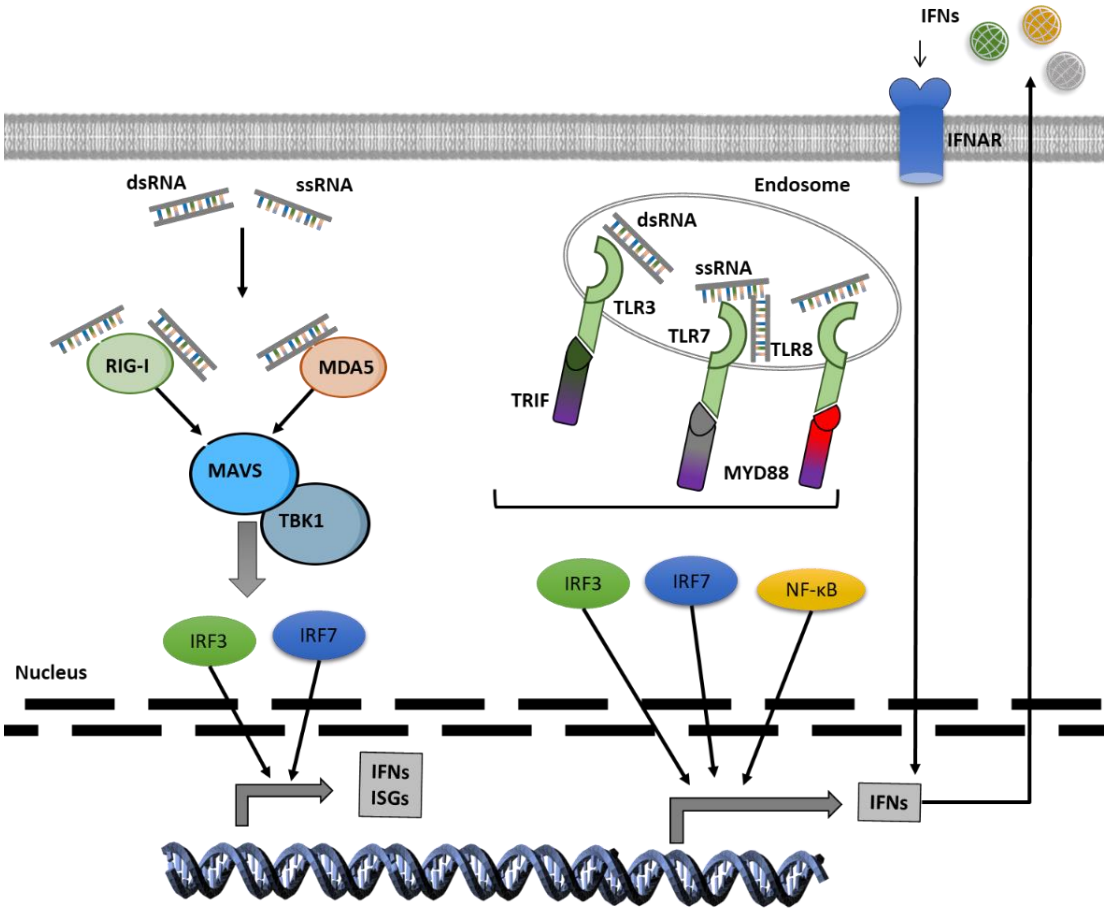


**Figure 4. RNA sensors found in the cytoplasm or membranes**. TLRs present in the lysosome act as RNA sensors. TLR3 senses dsRNA, TLR7 senses both ssRNA and dsRNA species and TLR8 can sense ssRNA, TLRs are reported to induce expression of IFNs through transcriptional factors IRFs and NF-κB. In the cytosol, RIG-I and MDA5 also sense different species of RNAs and activate cascade mediated by MAVS, TBK1 and transcriptional factors IRFs that induce expression of IFNs and ISGs. Figure modified from[25]**.**

### 1.4.1.1. RIG-I-LIKE receptors

There are 3 different RIG-I-like receptors (RLRs), all of which share an helicase domain that is able to recognize and unwind RNA[26]:

- RIG-I (Retinoic acid-Inducible Gene I) coded by the *DDX58* gene
- MDA5 (Melanoma Differentiation-Associated antigen 5) coded by the *IFIH1* gene
- LGP2 coded by the *DHX58* gene

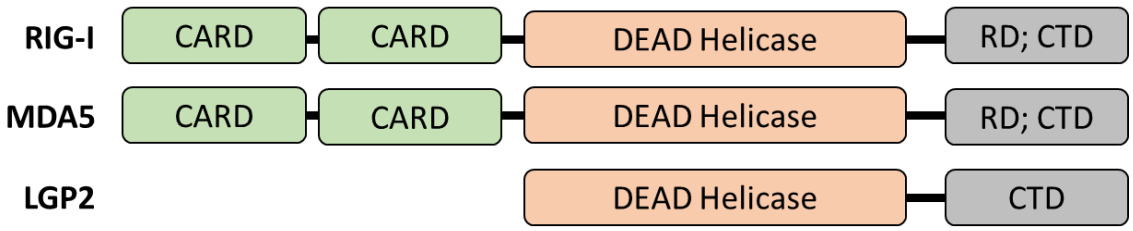


**Figure 5. Structural representation of Rig-I-Like receptors**. Rig-I-like receptors (RLR) share DEAD helicase and CTD domains. RIG-I and MDA5 also share two CARD domains (figure modified from[27]).

All three RLRs share a DEAD helicase domain followed by a CTD motif on the C-terminal domain that are involved in nucleic acid recognition of non-self RNAs (figure 5). RIG-I and MDA5 are cytoplasmic sensors critical for antiviral defense. These two sensors share similar sequences with two N-terminal caspase recruitment domains (CARDs), a DExD/H-box helicase domain, and a C-terminal domain (CTD, also known as the regulatory or repressor domain)[26]. Even though their similarities, they sense different RNA structures.

The differences in binding to dsRNAs between RIG-I and MDA5 are determined by the specific cooperation between the helicase and CTD domains of each protein[26]. RIG-I adopts a specific conformation similar to a closed circle in the cytosol until activated by RNAs. In contrast, MDA5 forms a C shape between the two domains and displays a flexible but inactive conformation until activation by RNAs. MDA5 recognizes the internal duplex structure, and RIG-I recognizes the terminal end of dsRNA having different CTD flexibilities for dsRNA binding[28].

In general, RIG-I prefers head-to-tail packing on long dsRNA, in comparison to MDA5 that senses shorter sequences of RNA with a head-head configuration. RIG-I and MDA5 through their CARD domains signal via MAVS leading to the activation of interferon kinases as TBK1 and IKKε that can phosphorylate IRF3 and IRF7 transcriptional factors, subsequently triggering type I IFNs after RNA recognition[29] (figure 6).

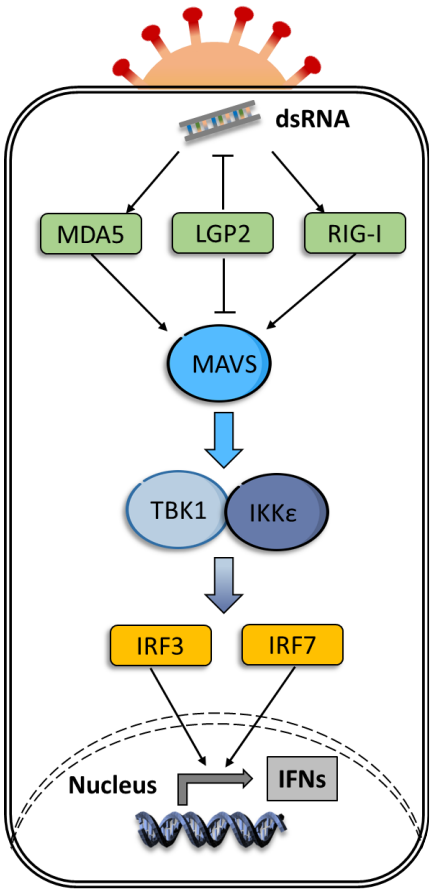


**Figure 6 Signaling pathway triggered by RIG-I-like receptors.** RIG-I and MDA5 sense dsRNAs and activate MAVS, which signals through TBK1 and IKKε, and consequently phosphorylate IRF3 and IRF7 that move to the nucleus and initiate transcription of type I IFNs and ISG.

On the other hand, LGP2 protein (probable ATP-dependent RNA helicase DHX58) also senses dsRNA molecules but does not contain any N-terminal CARD, therefore cannot transduce RLR signals. The functional role of LGP2 within RLR pathway is still not fully understood. LGP2 has been reported to be a negative inducer of RIG-I and MDA5 signaling[30–32], as well as a positive inducer[33] (figure 6). In addition, LGP2 has also been reported to play a role in inducing apoptosis and apoptotic regulatory genes through the binding of TRBP and miRNAs during viral infection[34].

### 1.4.2. DNA sensing receptors

DNA sensors detect ssDNA and dsDNA in aberrant locations. DNA should only be present in the nucleus, therefore the presence of DNA in endoplasmic compartments or the cytosol induces an immune response leading to induction of IFNs further immune activation and in some cases induction of apoptosis. Sensors need to discriminate between foreign and self-DNA and signal through different adaptor proteins depending on their location.

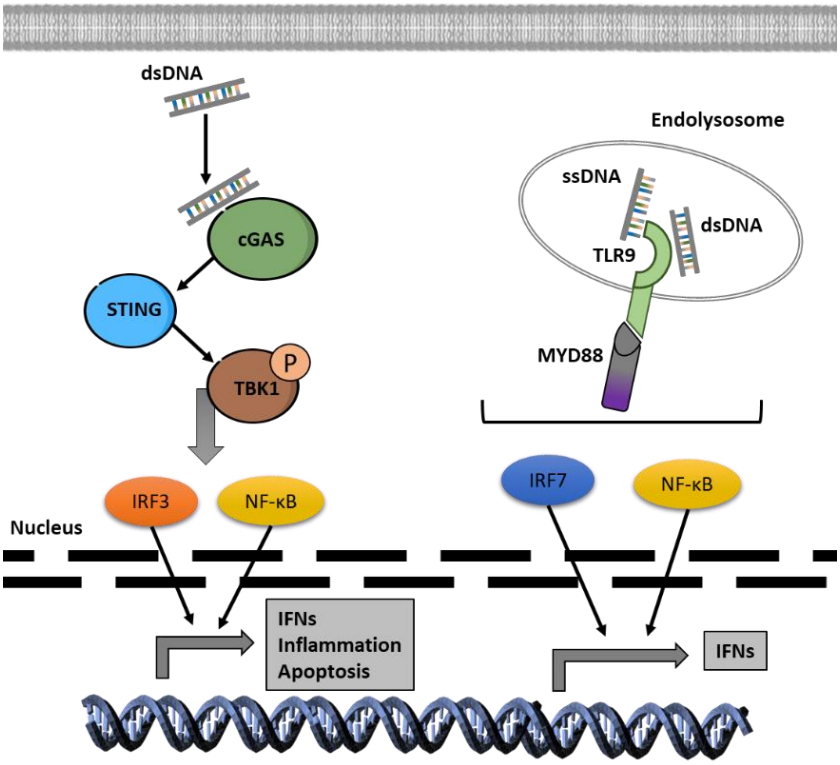


**Figure 7. Schematic representation of DNA sensors.** TLR9 located in the lysosome is reported to sense ssDNA and dsDNA molecules and activate through MYD88 mediator the activation of transcriptional factors IRFs and NF-κB that induce expression of IFNs. In the cytosol, cGAS is activated after sensing dsDNA molecules that signal through STING and TBK1, which activate transcriptional factors IRFs and NF-κB to induce expression of IFNs and induction of inflammation and apoptosis. Figure modified from[25].

There are many DNA sensors, either bound to the cellular membrane or in the cytoplasm (table 1, figure 7). TLR9 is present in the membrane of endolysosomes, which recognizes unmethylated cytidine-phosphate-guanosine (CpG) common in bacteria, and upon activation recrutes signaling molecules leading to production of proinflammatory IFNs and cytokines. In contrast, in the cytosol there is cyclic GMP-AMP synthase, or cGAS, detects dsDNA. cGAS has been described to sense viral DNA during HIV-1 infection and trigger type I IFN response[35,36]. cGAS sythetizes cGAMP, used as a second messenger, and activates adaptor protein STING. STING then induces phosphorylation of TBK1 and IRF3 that triggers transcription of IFNs and inflammatory genes inducing immune activation and apoptosis.

## 1.5. ANTIVIRAL INNATE IMMUNITY

The consequence of activation of the innate immune response is the production of different molecules with antiviral properties particularly for intracellular pathogens, where IFNs play a prominent role[37].

There are three types of IFNs: type I IFNs, present in all cell types but mostly expressed in immune cells such as lymphocytes and macrophages[38]; type II IFNs (IFN-γ) expressed in activated immune cells, specially NK and T cells[37,39]; and type III IFNs (IFN-λ) mostly expressed in epithelial surfaces[40,41]. Cells express very low levels of IFNs in the absence of pathogens. Hence, upon innate immune activation the signaling pathway is quickly amplified[42,43].

Type I IFNs are constituted by IFN-α, IFN-β, IFN-ω and IFN-τ. However, IFN-α and IFN-β are the most well characterized due to its wider tissue expression[37] and are mostly expressed after viral or bacterial infections[39]. All type I IFNs share a variable degree of structural homology but have no significant resemblance to type II IFN-γ[39]. It is important to note the role of plasmocytoid dendritic cells during an antiviral immune response, these cells produce large amounts of type I IFNs that strongly affect their environment and are key drivers in the generation of a systemic response to viral infection[44,45]. Irrespective of their nature, all type I IFN classes bind to the same heterodimeric receptor composed of IFNAR1 and IFNAR2 chains. These receptors signal through Janus-activated kinases (JAKs) TYK2 and JAK1[39,46,47] (figure 8).

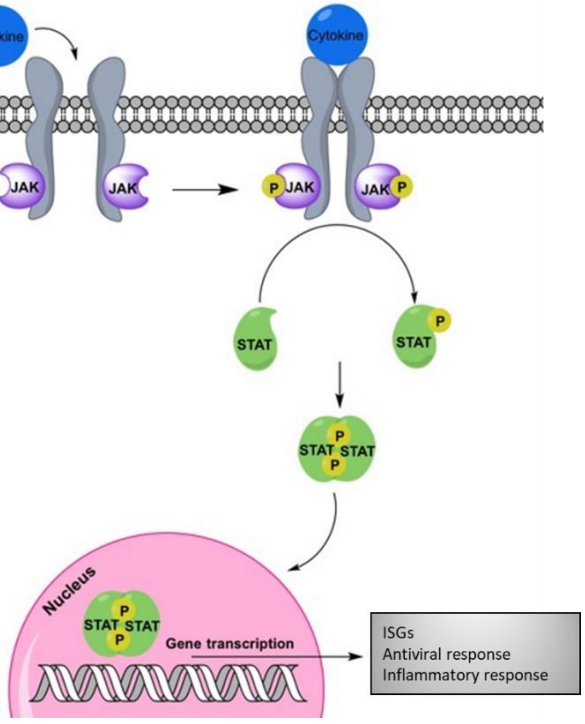


**Figure 8. JAK-STAT signaling pathway.** Cytokines such as interferons and interleukins induce janus kinases (JAKs) to dimerize and phosphorylate each other. Signal transducer and activator of transcription proteins (STATs) bind to the receptor and are phosphorylated. STATs then dissociate and form hetero- or homodimers that translocate to the nucleus and induce gene transcription of interferon stimulated genes (ISGs) leading to antiviral and inflammatory responses. Figure from Wikicommon, under Creative Commons Attribution-Share Alike 4.0 International license, gene transcription text was included.

IFN functions include (i) inhibition of proliferation of virus-infected cells, (ii) increasing MHC class I expression therefore enhancing antigen recognition[38], (iii) participate in the regulation of the immune system directly or indirectly by regulating the function and differentiation of NK cells[48], DC[49], monocyte and macrophage[50], CD4+ and CD8+ T cells[51] and (iv) can also enhance the primary antibody response to soluble antigen, stimulating the production of all subtypes of immunoglobulin G (IgG) and inducing long-lived antibody production and immunologic memory[38].

Activation of JAKs by phosphorylation induces reciprocal phosphorylation of tyrosine STAT1, STAT2 and STAT3 depending on the combination. The three different STAT complexes control different expression patterns[39,47].

Expression of IFNs induce transcription of many genes including ISGs and cytokines that amplify the antiviral response. The number and variety of ISG is extremely high, each playing a distinct role in the host immune response. For instance, ISG proteins as PKR, tetherin, viperin, MX1 or APOBEC3; ISG cytokines IL1, IL6, IL10 or TNF-α; or ISG chemokines as CXCL9, CXCL10 and CXCL11. In addition, depending on the cell type, intensity and time-frame of stimulus the different ISG are stimulated at different degrees[52,53].

Once transcription is activated by type I IFNs there are mechanisms that activate a positive-feedback loop consisting of activation of transcriptional factors, which leads to a second wave of gene transcription[46,54–56]. On the other hand, the tight regulation of the IFN pathways includes also transcriptional repressors that function as inhibitors of excessive putatively damaging type I IFN signaling such as IRF2[57]. The triggering of IFNs and cytokines with inflammatory properties can malfunction, which lead to so called “cytokine storm”, were excessive production of cytokines is released and can be very damaging and toxic leading to death[58].

The interplay between production of type I IFNs and activation of innate immune cells as neutrophils and inflammatory monocytes is critical for the understanding of mechanisms that respond to viral infections[59] (figure 9). It has been seen that early expression of antiviral molecules after an infection takes place, correlates with higher production and activation of type I IFNs. Hence, indicating that differences in the activation pattern during an infection is related to differences in the response and its outcomes, which may also be beneficial or detrimental depending on the specific pathogen and cell type.

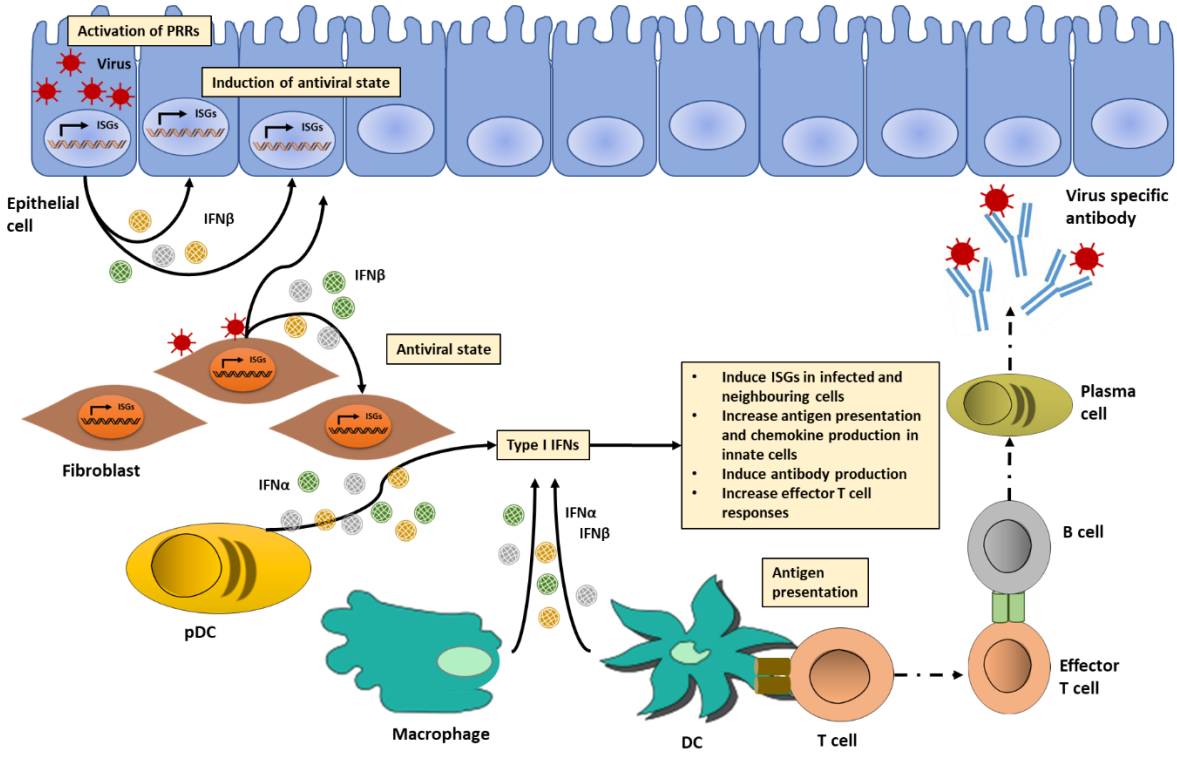


**Figure 9. Type I interferon controls innate and adaptive immunity mechanisms.** Upon pathogen detection, infected cells produce type I interferons (IFNs). Innate immune cells, as macrophages and dendritic cells (DCs), produce type I IFNs after sensing pathogens through pattern-recognition receptors (PRRs).Plasmacytoid DCs (pDCs) produce large quantities of IFNα. Innate immune cells respond to type I IFNs by enhancing antigen presentation and production of immune response mediators, as cytokines and chemokines. Figure modified from[47] with vectors freely accessible from Wikimedia commons licensed under the Creative Commons Attribution-Share Alike 4.0 International, 3.0 Unported, 2.5 Generic, 2.0 Generic and 1.0 Generic license.

# 2. AUTOIMMUNE DISEASE

Autoimmune diseases are a set of disorders caused by abnormal functioning of the immune system. In most cases, the immune system displays an uncommon high activity attacking and damaging its own tissues and cells. Currently, around 80 different types of autoimmune diseases have been clinically described; however, the cause is still not known for most of them. For certain autoimmune disease, the genetic component is clear[60,61], but also can be affected by other factors such as infections[62,63] or other environmental factors[64] that contribute to disease onset and evolution.

There is a subset of autoimmune disorders, that are characterized by enhanced signaling and production of type I IFNs and ISGs referred as "type I interferonopathies". This group of disorders are characterized by an abnormal response to intracellular or cytosolic nucleic acid sensing and defective regulation of protein degradation, leading to an extremely high production of IFN[65]. Most type I interferonopathies are genetically determined and in several cases the causative gene mutations have been identified[66–68].

Despite the high diversity in the conditions involved as well as the diverse genetic determinants that have been linked to the different conditions, all type I interferonopathies share similar characteristics in terms of systemic autoinflammation with signs of autoimmunity or immunodeficiency. Although the specific determinants are still unknown in most cases, an aberrant accumulation of self or non-self DNA seems to be the common link and shared condition causing severe disease inducing immune activation and overproduction of IFNs and inflammatory cytokines.

## 2.1. AICARDI-GOUTIÈRES SYNDROME

The clinical and molecular characterization of Aicardi-Goutières syndrome (AGS) led to the definition of the concept of type I interferonopathies described above to refer to a group of Mendelian disorders in which a constitutive upregulation of type I IFN production is directly linked to pathogenesis[69].

From a clinical point of view, AGS was first defined as an early onset progressive brain disease that is associated with increased numbers of white blood cells in the cerebrospinal fluid (CSF), suggestive of an inflammatory process. Throughout time, other features have been described as skin lesions known as chilblains, raised intraocular pressure (glaucoma) and, in some cases, an overlap with the autoimmune disorder systemic lupus erythematosus (SLE).

From a molecular point of view, AGS can be caused by mutations in distinct genes responsible for diverse functions and regulatory pathways (figure 10), hence the real range of associated clinical phenotypes might be broader than currently appreciated[69]. Currently, mutations in *TREX1*, *RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1* and *IFIH1* have been associated to AGS (table 2)[69]. Genes identified as causative of AGS must be involved in induction of type I interferon signaling, although based on their described functions the link is not direct for some of them.

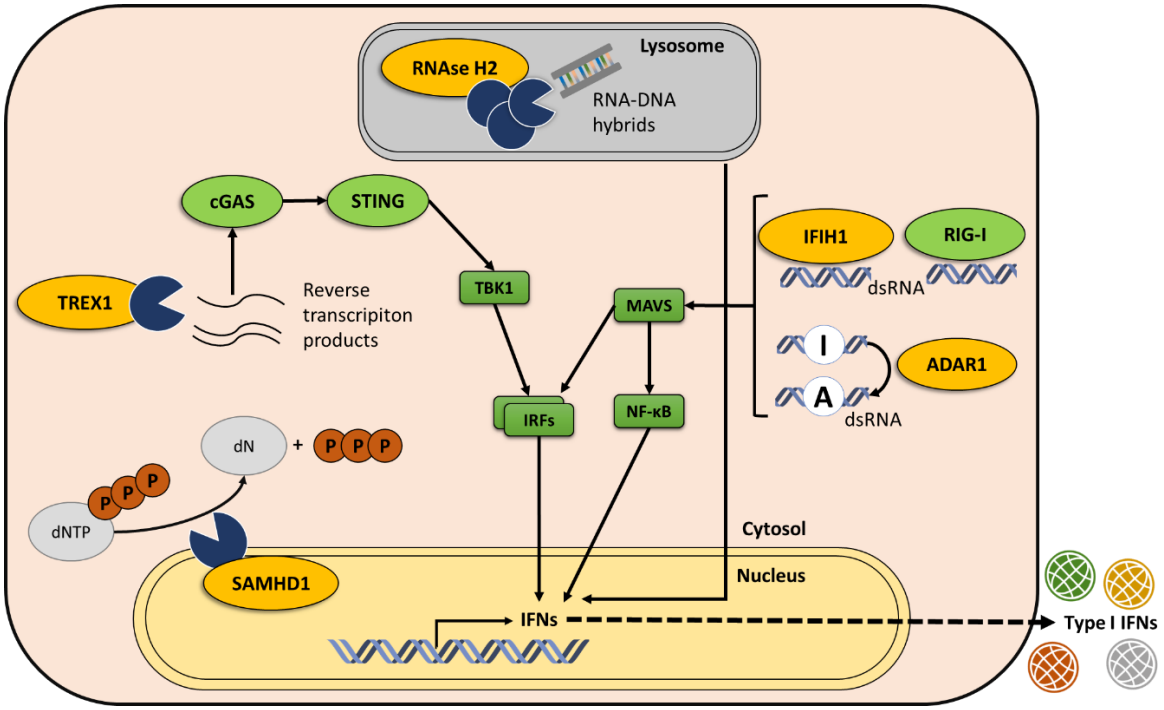


**Figure 10. Aicardi–Goutières syndrome (AGS) genes and their molecular function associated to production of type I interferons**. TREX1 degrades ssDNA and dsDNA products of reverse transcription that are sensed by cGAS and lead to production of type I interferons. IFIH1 senses exogenous viral dsRNA, similar to RIG-I, and triggers type I IFNs. ADAR1 deaminates adenosine to inosines in dsRNA inhibiting type I IFN responses. SAMHD1 mutations lead to IFN induction. RNase H2 (Ribonuclease H2) degrades RNA in RNA–DNA heteroduplexes. Mutations in RNase H2 subunits lead to IFN induction. Figure modified from[69].

After the description of mutations in nucleic acid sensing genes as *TREX1, RNASEH2, SAMHD1, ADAR* and *IFIH1*[69] known to regulate and discern self- and non-self-nucleic acids within the cell, it has been speculated that disturbance of endogenous nucleic acid pathways triggers an innate immune response more normally induced by exogenous nucleic acids. Aberrant sensing of nucleic acids therefore will cause immune dysregulation affecting the body systemically.

**Table 2. Genes associated to AGS.**

| Gene | Location | Function | Ref |
|---|---|---|---|
| ***TREX1*** | Cytosol | 3' - 5' DNA exonuclease | 70,71,72,73,74, 75 |
| ***RNaseH2A*** | Nucleus and cytosol | Degradation of RNA from RNA:DNA hybrids | 76, 77, 78, 74, 75 |
| ***RNaseH2B*** | Nucleus | Degradation of RNA from RNA:DNA hybrids | 76, 77, 78, 74, 75 |
| ***RNaseH2C*** | Nucleus | Degradation of RNA from RNA:DNA hybrids | 76, 77, 78, 74, 75 |
| ***SAMHD1*** | Nucleus | dNTP triphosphohydorlase | 79, 80, 81, 82, 83, 84, 74, 75 |
| ***ADAR1*** | Nucleus and cytosol | Conversion of adenosine to inosines on RNA (A-to-I editing) | 85, 86, 74, 75 |
| ***IFIH1*** | Cytosol | Cytoplasmic sensor of RNA | 87, 88, 89, 74, 75 |

The present thesis has focused in the study of *ADAR1* gene. Thus, an in depth description its role in innate immune response is described in the following section.

### 2.1.1. Adenosine deaminase acting on RNA 1, ADAR1

ADAR proteins are enzymes that bind to RNA and convert adenosines to inosines by deamination. There are three different ADAR proteins in the human genome (ADAR1-3). ADAR1 and ADAR2 are found in all tissues and have catalytic activity, whereas ADAR3 is only found in the brain and is catalytically inactive.

ADAR1 was the first ADAR protein identified and it is essential for normal development[90]. *ADAR1* gene is located in chromosome 1 at position 21.3 and two different isoforms have been described: ADAR1p110 and ADAR1p150. p110 isoform is exclusively found in the nucleus, whereas p150 isoform is located both in the cytoplasm and in the nucleus. Moreover, the p150 isoform expression is regulated by IFN stimulation.

Biochemically, ADAR1 binds to dsRNA and converts adenosines to inosines by hydrolytic deamination, a process referred as A-to-I editing, (figure 11). The change of adenosine (A) to inosine (I) is interpreted as a guanosine (G) by the translation and splicing machinery of the cell, therefore altering RNA sequences[91].

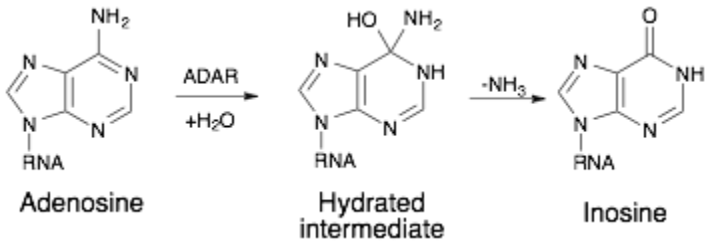


**Figure 11. Hydrolytic deamination by ADAR1**. Chemical reaction of ADARs from adenosine to inosine (A-to-I) by hydrolytic deamination. Figure from Wikicommon, licensed under Creative Commons Attribution-Share Alike 3.0 Unported license.

A-to-I modifications at mRNAs can alter amino acid sequence of proteins; change pre-mRNA splicing by affecting splice site recognition sequences; or affect RNA stability by changing sequences involved in nuclease recognition.

ADAR1 binding and editing is sequence-specific[92–95], and has shown preference for 5' target adenosines with certain 5' and 3' neighbors[96]. A-to-I modification is the most common post-transcriptional modification of mRNA in mammals, with hundreds of A-to-I editing events reported in humans found in coding, as well as, non-coding regions[85] and in Alu-repeats[86], demonstrating the important biological regulatory role of this protein.

Indeed, ADAR1 editing has been linked to the regulation of innate immune responses. A-to-I modifications marks RNAs as "self" compared to "non-self"[97] which may trigger production of IFNs and consequent innate immune activation and inflammation (figure 12). Non-self RNAs are detected, in a general way, by RLR sensors which include RIG-I and MDA5. However, it has been described that in the absence of ADAR1, non-edited endogenous RNAs are sensed specifically by the cytoplasmic RNA sensor MDA5 (coded by *IFIH1* gene) and signal via MAVS adaptor protein, highlighting MDA5 protein as the main sensor of ADAR1 products[98,99]. Deletion or malfunction of ADAR1 leads to induction of RLR pathway and triggers IFN-induced signaling pathway, inducing transcription of IFNs and ISG.

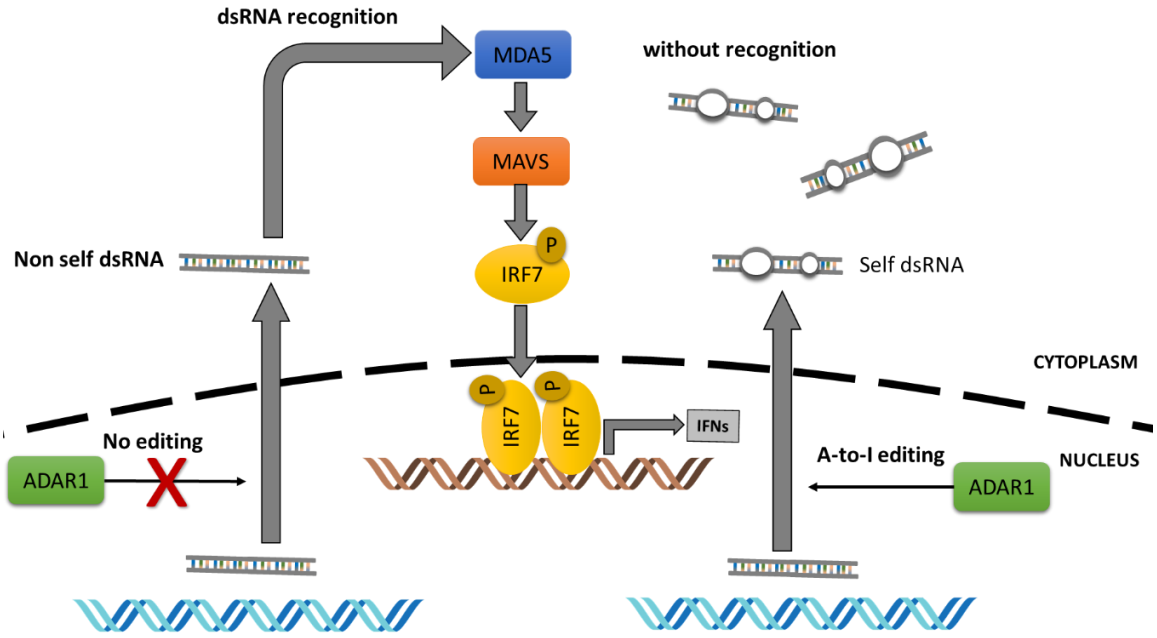


**Figure 12. Representation of A-to-I editing effects by ADAR1.** ADAR1 edits endogenous dsRNA that mark as self RNA and does not initiate immunogenic recognition by MDA5 sensor present in the cytoplasm. In contrast, ADAR1 loss of function induces the recognition of endogenous dsRNA as non self RNAs by MDA5. MDA5 then activates MAVS-dependent phosphorylation of IFN regulatory factor 7 (IRF7) and induces expression of IFNs. Figure modified from[97].

The relationship of ADAR1 function and its regulation of IFN responses highlights RNA editing of ADAR1 as a key intracellular defense mechanism. The induction of IFN-related pathways and expression of ISGs has the potential to regulate and limit a pathogenic invasion. Moreover, the cell has developed a balanced mechanism of ADAR1 editing to maintain self-tolerance and prevent autoimmunity. Indeed, ADAR1 function has long been linked to different viral infections such as hepatitis C virus (HCV[100]), vesicular stomatitis virus (VSV[101]), measles virus(MV[102]) and hepatitis delta virus(HDV[103]) having both proviral or antiviral effects depending on the specific modification and the virus affected[104].

# 3. VIRAL INFECTIONS

This thesis has focused on the study of innate immune activation in human immunodeficiency virus (HIV) and common coinfections as hepatitis C virus (HCV) and human papilloma virus (HPV). The following section includes a brief description of the most relevant features of each virus.

## 3.1. HUMAN IMMUNODEFICIENCY VIRUS

HIV is an RNA virus that harbors two identical copies of positive (+) single stranded RNA and belongs to the family of *Retroviridae,* member of the genus Lentivirus.

HIV has two different subtypes HIV-1 and HIV-2. Both viruses are the causatives agents of the acquired immunodeficiency syndrome (AIDS). HIV-1 is distributed worldwide and it is the main contributor of the epidemic due to higher virulence compared to HIV-2, that is mainly found in Central and West Africa[105–108]. Based on the latest WHO report, there are approximately 37.9 million of people living with HIV as of 2018 reported by WHO.

### 3.1.1. Molecular mechanisms

The genome of the HIV virus is 9.8 kb long and has overlapping open reading frames that encode for several genes coding for structural, regulatory and accessory proteins[109–111].

Structural genes:

- Gag: Encodes for the precursor of p24 (CA, capsid), p17 (MA, matrix), p7 (NC, nucleocapsid) and p6 proteins, before being processed by the viral protease.
- Env: Encodes for the gp160 precursor that, after being processed, becomes the envelope glycoproteins gp120 (SU, surface protein) and gp41 (TM, transmembrane protein).
- Pol: Encodes for a precursor of the viral enzymes PR (protease), RT (reverse transcriptase) and IN (integrase).

Regulatory genes:

- Tat: Enhances viral transcription initiation and elongation.
- Rev: Allows the export of unspliced viral mRNA from the nucleus to the cytoplasm.

Non-essential accessory HIV genes:

- Vpr: Plays a role in transport of the viral pre-integration complex (PIC) to the nucleus. Induces G2/M cell cycle arrest and apoptosis. Targets the degradation of host proteins.
- Vif: Involved in stability of the nucleoprotein core of HIV and contributes to G2 cell cycle arrest. Also targets APOBEC3F and APOBEC3G restriction factors for degradation.
- Vpu*/Vpx: Vpu enhances virion budding by targeting for degradation host CD4 and viral restriction factor tetherin. Vpx plays a role in nuclear translocation of PIC, and it is necessary for the virus to infect non-dividing cells. Vpx also increases viral replication by inhibiting restriction factors such as SAMHD1.
- Nef: Important factor that contributes to infectivity and pathogenicity of the virus. Alters different pathways of T lymphocytes function and downregulates immunity surface molecules to evade immunity.

HIV-2 shows significant genomic differences compared to HIV-1 in the nucleotide and amino acid sequences, that alter envelope and protein genes. SIV and HIV-2 express *Vpx* instead of *Vpu* found only in HIV-1. Vpx is thought to have arised from a tandem duplication of *Vpr* gene, due to its similar structure and function.

### 3.1.2. HIV life cycle

The HIV life cycle consists of eight steps (figure 13):

- Virus entry and attachment: The virion attaches to CD4 cell by coreceptor CCR5 or CCRX4 and HIV envelope proteins gp41 and gp120. The attachment releases the capsid and other proteins from inside the virion into the cell.
- Reverse transcription: Viral proteins from the virion are released within the cell and start reverse transcription by viral reverse transcriptase or RT that changes the single strand of RNA into a double strand of DNA.
- Integration: The double stranded DNA from HIV enters the nucleus and integrates in the cell genome.
- Transcription: The infected cell transcribes genes by using host machinery, as any other host gene, and produces mRNAs.
- Translation: viral transcripts are translated by host machinery

- Cleavage: Proteins are further processed by viral proteases that cut and assemble the virions.
- Assembly: Viral proteins and viral RNAs are assembled into virions.
- Budding and release: The newly formed virions leave the cell.

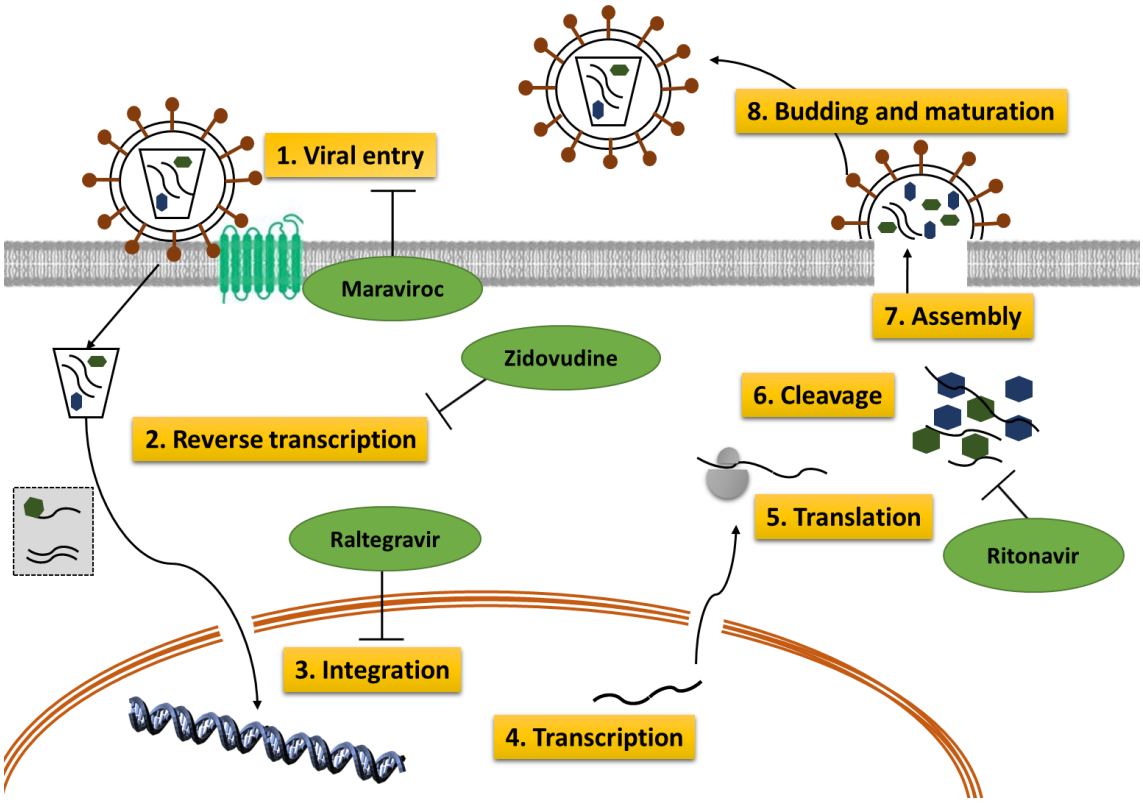


**Figure 13. Life cycle of human immunodeficiency virus.** HIV replication consists of 8 life cycle steps including viral entry, reverse transcription of RNA, integration to host genome, transcription of viral genes, translation and cleavage of viral proteins, assembly and budding of new virions that must mature before being able to infect. Figure modified from[112].

Nowadays, antiviral drugs that can inhibit different steps of the viral cell cycle have been developed and used in the clinic. For instance, fusion or entry inhibitors as maraviroc; reverse transcription inhibitors, as zidovudine, tenofovir or efavirenz; integration inhibitors as raltegravir; and protease inhibitors as ritonavir or atazanavir.

Successful HIV replication depends on the resources of the host-cell. The virus must affect and overcome certain regulatory mechanisms of the host to replicate. These are primordially immune and cell cycle regulatory mechanisms, important for immune escape while being able to regulate viral transcription and translation or even initiate the establishment of latency.

### 3.1.4. HIV host restriction factors

Restriction factors are a group of proteins with diverse structures and functions that share the ability to limit diverse steps of the viral replication cycle. All restriction factors are induced by IFNs and most are part of innate immune mechanisms that target viral components, suppress viral transcription and/or induce degradation of viral RNA transcripts. The presence of restriction factors forces the virus to develop proteins to counteract their activity, reveling the complex interplay between host and pathogens (figure 14, Reviewed in[113–116]).

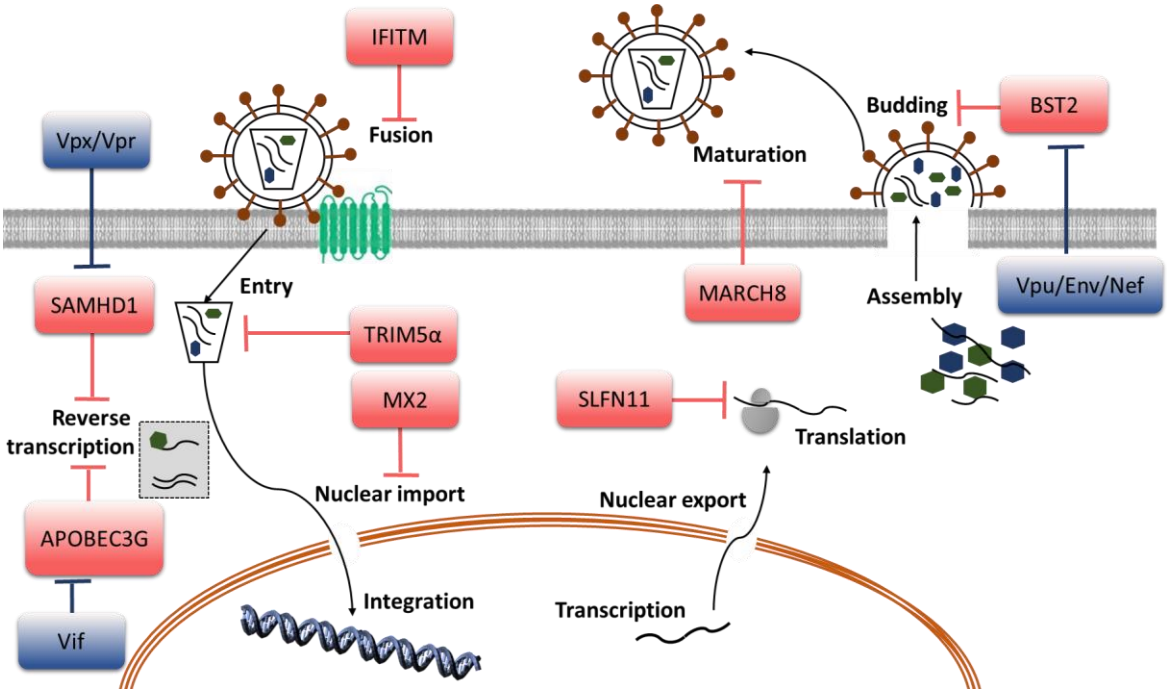


**Figure 14. Schematic representation of HIV restriction factors and counteraction by viral proteins**. Restriction factors are anti-viral proteins, considered the first line of defense against virus, prevent different steps of HIV replication cycle. Some viral proteins from HIV also counteract restriction from host restriction factors. Figure modified from[117].

#### 3.1.4.1. SAMHD1

Sterile Alpha Motif and Histidine Aspartate domain-containing protein 1, SAMHD1, is a protein that regulates the levels of cellular dNTPs by its deoxynucleotide triphosphohydrolase function. SAMHD1 is expressed in both lymphoid and myeloid cells and is able to limit HIV replication by depleting the dNTP pool affecting the reverse transcription and replication step (figure 15)[118]. Moreover, SAMHD1 has also a role in DNA repair that promotes DNA end resection and facilitates double stranded break repair by homologous recombination[79] therefore preventing interferon induction by promoting the degradation of nascent DNA at stalled replication forks[119].

SAMHD1 phosphorylated state and cell cycle progression are tightly regulated due to SAMHD1 ability to limit intracellular dNTPs, which directly affects the replicative capacity of the cell. SAMHD1 phosphorylation is mediated by cyclin-dependent kinases[120,121], in particular CDK2 and cyclinE, that also control cell cycle progression. Hence, there is a link between SAMHD1 regulation and control of host and viral replication (review in[122]) with further implications in cancer and other disease[123].

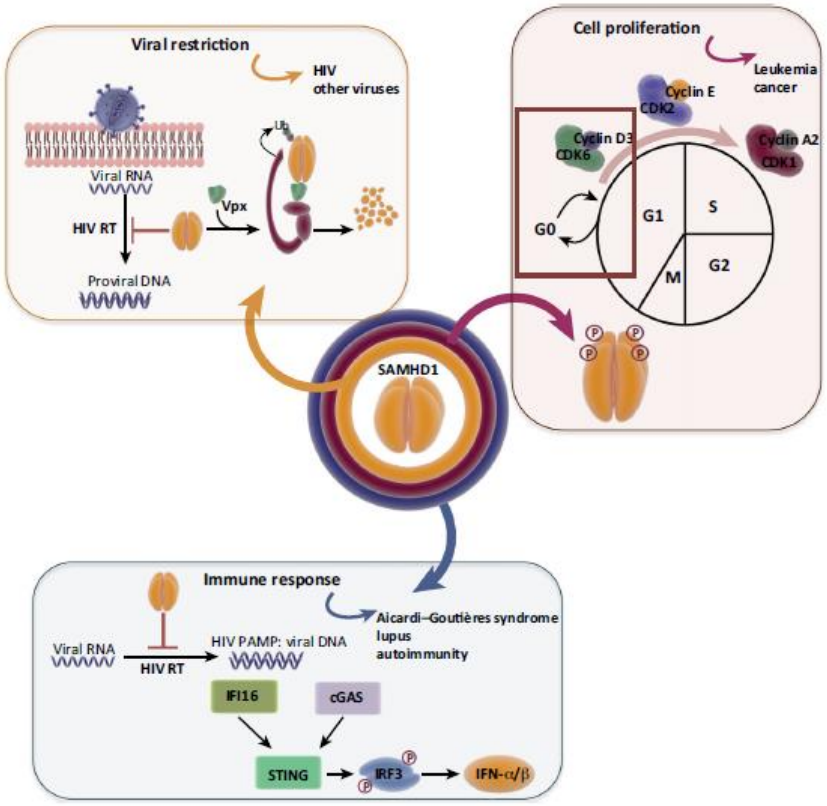


**Figure 15. SAMHD1 functions.** SAMHD1 is a triphosphohydrolase that controls intracellular levels of deoxynucleoside triphosphates (dNTPs), playing an essential role in cell-cycle progression and cell proliferation. The activation or inactivation through phosphorylation of SAMHD1 is tightly controlled by cyclins and cyclin-dependent kinases regulating cell cycle, hence SAMHD1 function can be associated to aberrant cell proliferation, leukemia and cancer. The regulation of dNTPs makes SAMHD1 a regulator of virus and retrovirus replication. HIV-2 and SIV virus have counteracted SAMHD1 function through viral protein x (Vpx) that induces its degradation through the proteasome. Mutations in SAMHD1 have been associated with clinical manifestations that resemble congenital viral infections characterized by a high expression of type I IFN such as the Aicardi–Goutières syndrome (AGS). Thus, SAMHD1 is at the crossroads between multiple metabolic processes that play a prominent role in health and disease. Figure from[122].

Moreover, SAMHD1 is counteracted by Vpx accessory protein from HIV-2 and other simian strains such as SIVsm and SIVmac[80]. Vpx targets SAMHD1 for proteasomal degradation through cullin4A-RING E3 ubiquitin ligase (CRL4)[124] (figure 16), counteracting SAMHD1 restriction on viral replication.

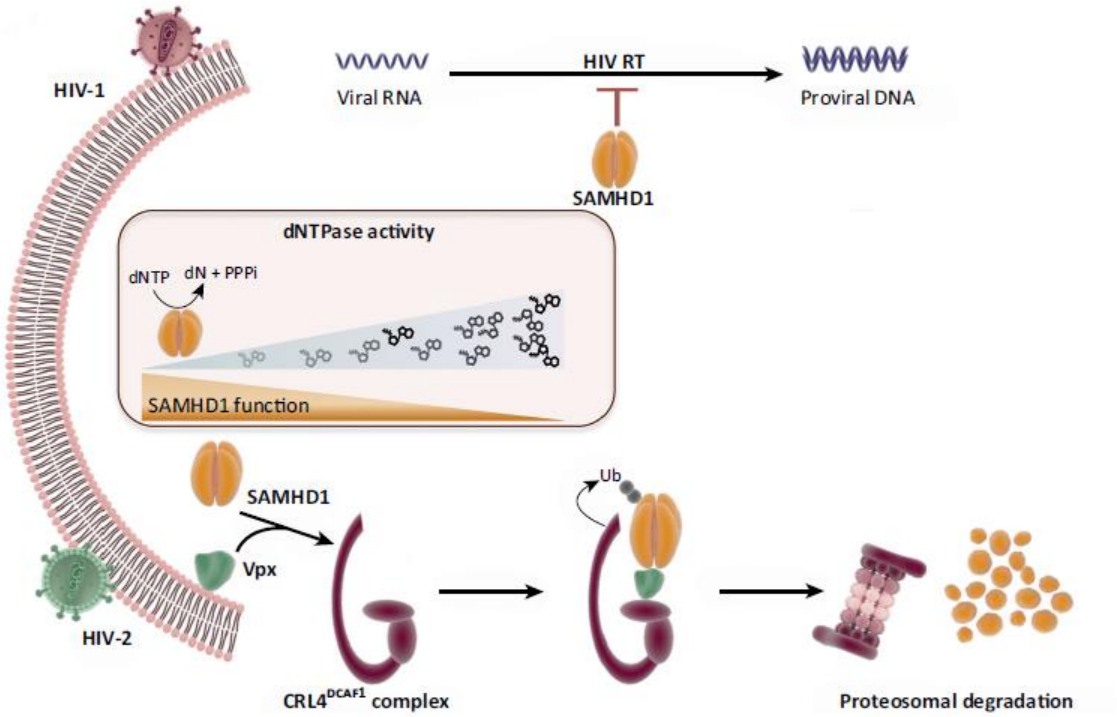


**Figure 16. SAMHD1 dNTPase function and HIV restriction.** Proposed model for SAMHD1-dependent restriction of HIV-1. HIV-2 vpx promotes SAMHD1 degradation by recruiting it to the E3 ligase complex (CRL4DCAF1), inducing its ubiquitination and degradation by the proteasome. Figure modified from[122].

### 3.1.4.5. HUSH complex

Recently, the HUSH complex, has been highlighted as a potential restriction factor that controls viral expression and is counteracted by accessory protein Vpx[125]. The HUSH complex is composed of three proteins: TASOR, MPP8 and periphilin. The complex is reported to maintain transcriptional silencing by recruiting methyl transferase SETDB1 that silences gene expression by methylation of heterochromatin[126]. Showing the importance of this factor in regulating gene expression and viral latency.

TASOR protein is encoded by *FAM208A* gene. This gene from the HUSH complex has been identified in a screen as an HIV antiviral factors[127].

The characterization of the components of the HUSH complex as putative restriction factors have identified a post-integration block during HIV infection that affects transcription regulation and more importantly limits the establishment of HIV latency.

### 3.1.5. Clinical course of HIV infection

HIV-1 infects preferentially CD4+ T cells but is also found in other immune cells as dendritic cells, macrophages, Langerhans cells, B cells, and granulocytes that in turn spread the infection through the lymph nodes and, finally, to the bloodstream. The natural course of HIV-1 infection can be classified in 3 stages: acute infection, chronic phase and acquired Immunodeficiency syndrome (AIDS) (figure 17).

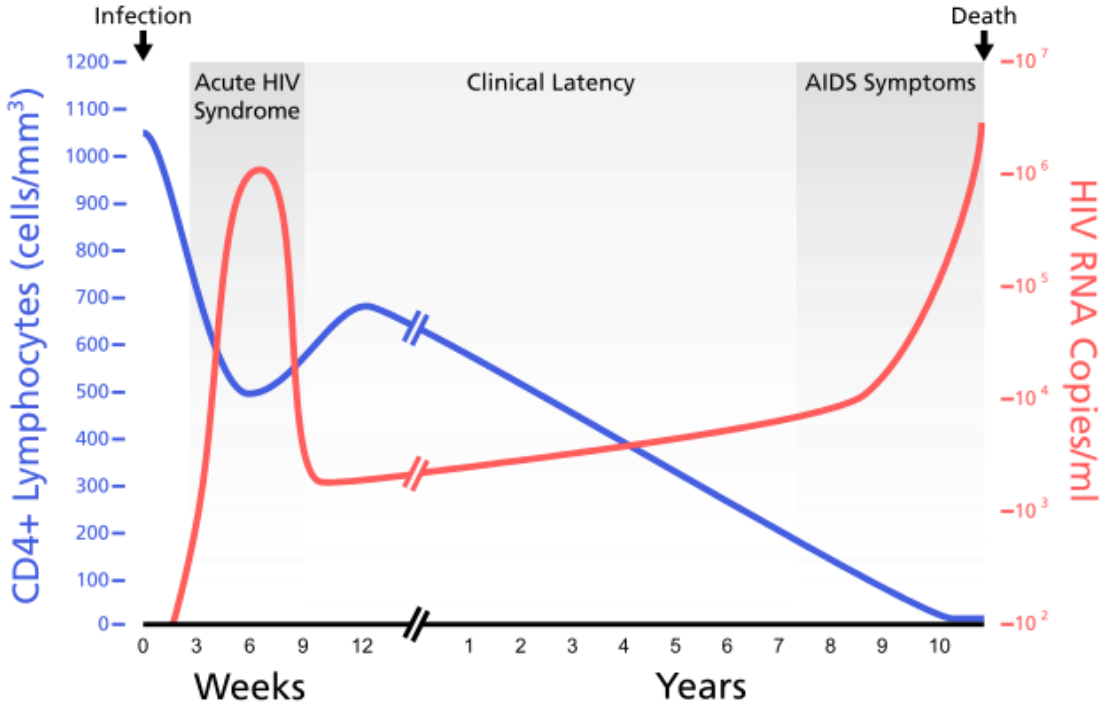


**Figure 17. Typical course of HIV-1 infection.** In the absence of treatment, HIV-1 infection can be classified in 3 phases: (1) Acute infection, characterized by a sudden increase on HIV-1 viremia and a decrease of the CD4+ T cell count; (2) Chronic phase, characterized by a decrease of HIV-1 viremia due to the host immune system, and a partial recovery of the CD4+ T cells at the beginning, it can last for years, though CD4+ T count will decrease steadily without treatment; and (3) AIDS phase, characterized by a low CD4+ T count, a raise in HIV-1 viremia and the apparition of opportunistic infections, which will ultimately lead to the death of the infected individual[128].

#### Acute phase

After the initial exposure and transmission, the virus infects and replicates in the cells of the lymphatic tissue and lymph nodes[129] before reaching the bloodstream. Those infected cells can either actively replicate the virus, contributing to the dissemination of the infection, or establish a latent infection, constituting the first cells of the HIV-1 latent reservoir[130]. During this early infection, there is a gap of 10 to 12 days where the virus cannot be detected in the plasma, the eclipse phase[131],

followed by a rapid increase of viral RNA in the blood ($10^5$ – $10^9$ copies/mL). This increase in viremia is accompanied by a decrease of the immune CD4+ T cells, which is maintained until the body is able to generate an immune response to partially control the viremia. During this phase, infected patients may present certain symptoms including fever, fatigue, rash, myalgia, lymphadenopathy and oral or genital ulcers. Although these symptoms might last for more than 10 days, the duration rarely exceeds 14 days[132].

**Chronic phase**

At the beginning of the chronic phase of HIV-1 infection (also called asymptomatic HIV-1 infection or clinical latency) there is a decrease in the viral load (VL) driven by the humoral and cellular immune responses, including the generation of antibodies and the activity of CD8+ cytotoxic T lymphocytes (CTL)[133,134]. The VL keeps decreasing until it reaches a steady level, the viral set point, within 2 months after the primary exposure to HIV-1[135]. The establishment of this viral set point is also accompanied by an increase of the CD4+ T cells, although at lower levels than those present before the infection.

During this asymptomatic infection, HIV-1 establishes a low but persistent infection, leading to a chronic HIV-associated systemic inflammation and immune activation. As the HIV-1 infection persists, peaks of viremia can be detected even without the presence of HIV-related symptoms. The persistent infection also leads to a steady decrease of the CD4+ T cell count, weakening the immune system, which marks the end of the clinical latency. At this point, viral replication and chronic activation of immune cells promote the destruction of the lymphoid tissue architecture, which leads to an increased viral replication and diffusion, and the subsequent depletion of CD4+ T cells. There are some patients, however, that are able to control the infection, having a non-detectable viremia for years: the so-called “elite controllers”[136].

**Acquired Immunodeficiency Syndrome (AIDS)**

The last stage of HIV-1 infection is characterized by extremely low levels of CD4+ T cells (<200cells/µL) in blood and the dramatic increase of the VL. The CD4+ T cell count can even reach very low levels, being the lowest count the CD4+ nadir. During this phase, the organism becomes susceptible to opportunistic infections by other viruses, bacteria, fungi and parasites, and tumors, which define the AIDS stage[108,137]. Typical progressors may reach the AIDS phase 8-10 years after

HIV-1 primary infection in the absence of therapy. However, there are records of non-typical progressors who can reach this phase earlier or later[138].

Since the FDA approval of the first antiretroviral compound, AZT[139], in 1987, advances in antiretroviral therapy (ART) have changed the perspective of HIV-1 infection from a lethal illness to a somewhat manageable chronic disease[140]. Nowadays, the use of combination therapy suppresses viral load below the limit of detection (LOD) (<50 copies of viral RNA/mL) following a four-phase decay of viremia, reaching stable level of viremia below LOD, that ideally should last for an unlimited period of time [141,142].

This decreased replication allows the treated individuals to control viremia, delay disease progression, prevent transmission and partially recover the CD4+ T cell count indefinitely[143]. However, upon treatment failure, virus replication increases again and CD4+ T cell count plummets, as observed in the HIV-1 acute phase. Treatment failure is caused by the acquired resistance to treatment due to the high mutagenesis rate of the RT, resulting in the appearance of resistant quasispecies in the presence of ART[144]. In an effort to avoid this drug resistance, current combination therapy uses three antiretrovirals targeting at least two different steps of the viral cycle, thus, the chances of a virus to evolve and become resistant to the three drugs are decreased[145].

Current antiretroviral combination therapy blocks HIV replication at different steps. And consists in the combination of three drugs from two different classes that include:

- Nucleoside reverse transcriptase inhibitors (NRTIs)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Integrase strand transfer inhibitors (INSTIs)
- Protease inhibitors (PIs)
- Fusion inhibitors
- CCR5 antagonists
- Post-attachment inhibitors (PAIs)

Combination therapy must be taken in combination and without interruption for a long-lasting effect. This therapy reduces the appearance of mutations, in the presence of two different classes of antiretrovirals, if one escape mutation arises, the other antiretroviral drug continues suppressing its replication and reduces its emergence as a quasispecie. Although current ART is able to successfully control viral replication, it is unable to target those cells in which HIV-1 remains silent,

the HIV-1 latently infected cells[146]. Due to this incomplete clearance of the infection, HIV-1 is able to rebound after ART discontinuation, independently of the time spent under treatment.

Long-term ART is also linked to a persistent immune activation and inflammation[147] as well as to toxicities associated with the treatment. Therefore, there is a need to develop novel strategies to achieve an effective HIV-1 cure, defined as a treatment that should be able to induce a sustained remission of the virus after ART discontinuation.

### 3.1.6. Innate immunity and HIV infection

Early treatments by IFNα therapy were very promising due to the antiviral therapeutic potential of IFNs blocking many viral infections *in-vitro*. Unfortunately, IFNα therapy was soon proven innefficient and therapy was directed in favor to ART. Although, interferon appears to be a double-edge sword during HIV infection, IFNs show to be beneficial during acute infection, but disadvantageous during chronic infection since sustain IFN induction and contribute to immunological exhaustion[148].

In a molecular basis, the host cell senses HIV at different stages through PRR, especially nucleic acids sensing, that enhance IFNs and cytokines expression and induce antiviral responses, finally leading to apoptosis. Host restriction factors also affect the virus by limiting replication at different viral stages. In contrast, HIV can also potentiate and activate the immune responses in a matter not yet understood, but thought to enhance transcription and immune activation which aids viral replication, elucidating a long coevolution between the virus and the host.

## 3.2. COMORBIDITIES OF HIV

ART treatment allows for a partial recovery of the immune system. However, the extended disease exposure may increase the risk of developing other conditions not associated to HIV or AIDS, such as comorbidities and opportunistic infections. Moreover, HIV patients seem to “age” faster demonstrated by earlier appearance of certain geriatric syndromes and frailty in HIV patients[149]. Aging is thought to be caused partly from antiretroviral and other drug toxicities, immune dysfunction and inflammation.

It is importantly to note that people living with HIV on effective ART treatment still maintain persistent chronic immune activation and inflammation due to underlying viral replication that will directly or indirectly affect different aspects throughout the body. Indeed, immune activation and

inflammation leads to increase production of pro-inflammatory cytokines[150] and thymus dysfunction[151], among others, which enhance the risk of morbidity and mortality[152,153].

Comorbidities associated to HIV+ people include disease in the cardiovascular system, kidneys, liver, cognitive function, malignancies, and metabolic bone disease[154]. On the other hand, common opportunistic infections within the HIV community are fungal, bacterial and viral, and the development of cancer due to an already weakened immune system[155,156].

On the other hand, 36.7 million people world-wide are estimated to be co-infected with HCV and HIV according to the World Health Organization (WHO). Moreover, there is a higher risk and prevalence of HPV-related cancers within HIV infected people[157] and the prevalence of HIV and HPV coinfection is estimated to be as high as 93% in HIV+ men[158]. The presence of coinfections are associated to worsen health outcomes, complex clinical management and increased health care costs[159].

## 3.3. HEPATITIS C VIRUS

Hepatitis C virus, or HCV, is a positive-sense, single-stranded enveloped RNA virus, whose genome is 9600 nucleotides in length and belongs to the family *Flaviviridae*. There are 6 different genotypes of HCV, HCV-1 to HCV-6, that can vary up to 30% in nucleotide sequence between them[160]. HCV has high genetic diversity and a tendency for immune and drug resistance mutations due to its error prone RNA polymerase[161].

In 2019, there were an estimate of 71.1 million individuals chronically infected by HCV in the world. HCV transmission is most commonly associated with direct percutaneous exposure to blood, via blood transfusions, health-care-related injections, and injecting drug use[162]. HCV infection can be asymptomatic during years until the virus damages the liver and causes liver disease. Approximately 10–20% of individuals who are chronically infected with HCV develop complications, such as cirrhosis, liver failure, and hepatocellular carcinoma over a period of 20–30 years.

### 3.3.1. HCV life cycle

HCV genome codes for 10 proteins, that are classified in structural – non-structural depending on their function[163].

Structural proteins of HCV consist of the following:

- Nucleocapsid (C) that forms HCV core. The HCV core is composed of the viral nucleocapsid protein and has many functionalities, these include RNA binding, immune modulation, cell signaling, oncogenic potential and autophagy. The core protein of HCV also associates with the lipid droplets where HCV assembly also takes place.
- Two envelope glycoproteins (E1 and E2). HCV E1/E2 are glycosylated envelope glycoproteins that surround the viral particles. HCV envelope is targeted by virus neutralizing antibody selection pressure with high degree of sequence variation that may render antibody responses ineffective and contributes to HCV persistence.

Nonstructural proteins composed of:

- p7 viroporin, small ion channel protein, is required for viral assembly and release
- Two transmembrane proteins (NS1 and NS2): Involved in viral assembly
- Metalloprotease serine protease RNA helicase (NS3): Encodes for protease and helicase-NTPase, have a role in HCV processing and is a target for HCV antiviral drugs.
- Cofactors (NS4A and NS4B): Involved in membranous web formation
- IFN-resistance protein (NS5A): Binds to viral RNA and host factors
- RNA-polymerase (NS5B): Encodes for the polymerase enzyme and it is also a target for HCV antiviral drugs

Collectively, these proteins also contribute to various aspects of HCV life cycle, including viral attachment, entry and fusion, HCV RNA translation, posttranslational processing, HCV replication, virus assembly and release.

HCV replication cycle consist of seven steps (figure 18):

1) Attachment. The viral particle is surrounded by lipoproteins and binds to the target cells by interacting with cell surface receptors.

2) Viral entry. The virus enters through a clathrin-mediated endocytosis.

3) Uncoating. Cellular and viral membranes fuse and the capsid is disorganized after encounters low pH from the endosome. After uncoating the positive strand RNA genome is released into the cytoplasm.

4) Translation. Genomic RNA is directly translated to a polyprotein precursor that is then cleaved into single proteins by both host and viral proteases.

5) Translation of the non-structural proteins. The replication complex synthesized from multiple copies of the HCV RNA genome via a minus-strand replicative intermediate, translates non-structural proteins.

6) Assembly and maturation. It takes place in the endoplasmic reticulum where the virion acquires the envelope with E1 and E2 glycoproteins. Then, endogenous lipoproteins maturate and associate with the virion to form the lipoviral particles.

7) Release. Virions are released by exocytosis or transmitted to other cells via cell-free mechanism[164].

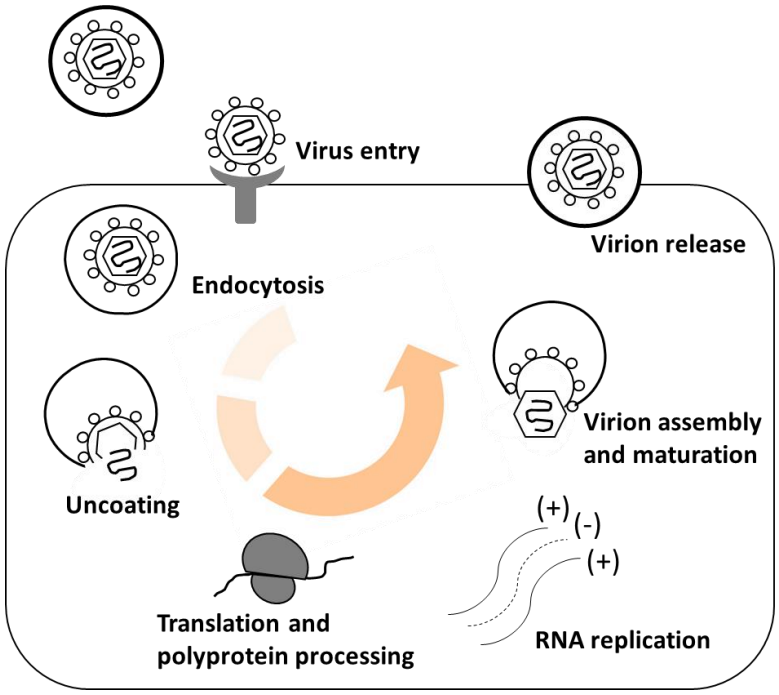


**Figure 18. Life cycle of hepatitis C virus.** HCV enters through several receptors and enters through endocytosis. Cellular and viral membranes fuse and uncoates, where the positive strand of RNA is released. Genomic RNA is translated and polyplorteins are processed, HCV proteins are synthesized. Assembly and maturation follow and the new virion is released. Figure modified from[164].

### 3.3.2. Innate immunity and HCV infection

After viral entry and replication hepatocytes express IFN-stimulated genes (ISGs) as a response to restrict viral replication and spread[165].

It is reported that HCV is sensed by PRRs. HCV ssRNA triggers RIG-I and PKR that activates MAVS and IRF3 signaling. On the other hand, dsRNA sequences found as a result of the secondary structure of HCV genome also triggers Toll-like receptor-3 (TLR3) mediated signaling and signaling via TIR domain-containing adaptor inducing IFN-β (TRIF) through IRF3 and NFkB transcription factors. Therefore, RNA from HCV induce innate immune responses via IRF3 and NFkB signaling that induce expression of IFNs and ISGs that can inhibit viral replication (figure 19).

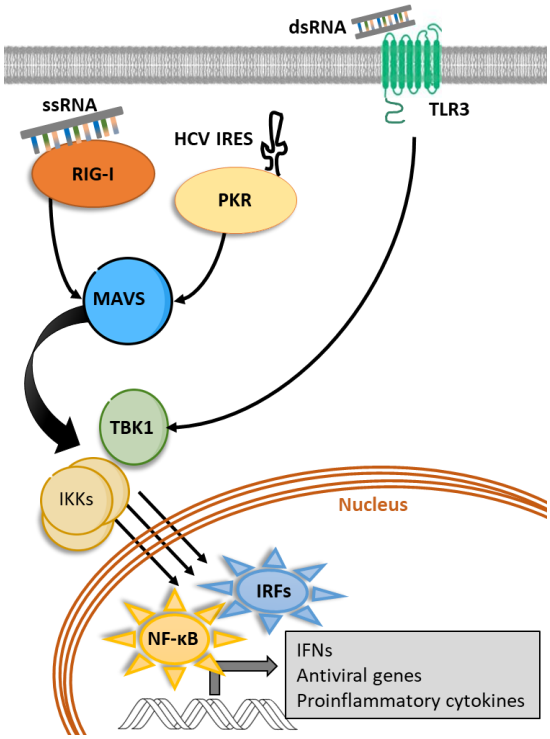


**Figure 19. Innate immune signaling in response to HCV infection.** Presence of HCV is detected by PRRs RIG-I, PKR, and TLR3 and leads to downstream signaling to activate transcriptional factors NF-κB and IRFs that lead to transcription of IFNs, antiviral genes and proinflammatory cytokines that limit infection. Figure modified from[166].

### 3.3.3. HCV treatment

Hepatitis C infection is nowadays treated with direct acting antiviral (DAA) medications that cure more than 90% of people. This medication is taken for 8 to 12 weeks depending on the HCV genotype, presence of liver damage, evaluation of other medical conditions and/or history of

previous treatments. To be effective, the course of the treatment by DAAs must be fully completed to ensure total clearance of HCV. There are three types of DAA medications that target multiple mechanisms of HCV life cycle: NS3/4A protease inhibitors, NS5A polymerase inhibitors and NS5B polymerase inhibitors. Moreover, certain types of hepatitis C can be treated by using a combination of DAAs taken in the form of tablets[167]. As a result of DAAs, patients show fewer side effects from the medication, shorter treatment types and have better outcomes.

The standard of care for HCV+ patients until 2014 was a dual therapy with pegylated interferon alpha and ribavirin (PEG IFN/riba). However, this therapy was only beneficial for genotypes 2 and 3 with reports showing 80% sustained virological response (SVR), whereas for genotype 1 only 50% of achieved SVR. Moreover, the cost of the treatment was elevated and the treatment has several adverse effects.

## 3.4. HUMAN PAPILLOMA VIRUS

Human papillomaviruses (HPV) are small, double-stranded, circular DNA viruses from the family of *Papillomaviridae*. Human papilloma virus (HPV) prevalence is very high worldwide[168]. There are more than 100 different types of HPV[168]; however, only 14 of these are considered high risk types referring to its potential oncogenic characteristics. HPV-related cancer types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58[169].

Most HPV infections are asymptomatic and completely cleared by the immune system; however, oncogenic HPV subtypes have been associated to a higher prevalence of different types of cancer such as cervical cancer, anal cancer and head-and-neck cancer[170].

### 3.4.1. HPV life cycle

HPV enters through microabrasions or epithelial wounds and infect epithelial cells. These infected cells then have the viral genome in episomal form. As cells divide, the infected cells enter different stages of differentiation leading to desquamated host cells that release virions. HPV can be present for decades in a latent state, utilizing the cell machinery to replicate. However, HPV infected cells can undergo malignant transformation. Specifically, E6 and E7 oncoproteins are responsible for malignant transformation. These oncoproteins are known to inactivate tumor suppressor protein p53 and pRb, along with other cellular mechanisms as viral integration into host DNA is a critical factor that affect carcinogenesis of HPV[171]. In normal stratified epithelia, the only actively dividing cells are located in the basal and parabasal layers of the cervix. As basal cells divide, one daughter

cell becomes a new basal cell, while the other migrates away and begins to differentiate. Differentiating cells exit the cell cycle and undergo a complex series of changes in gene expression, eventually resulting in cell death and desquamation[171] (figure 20).

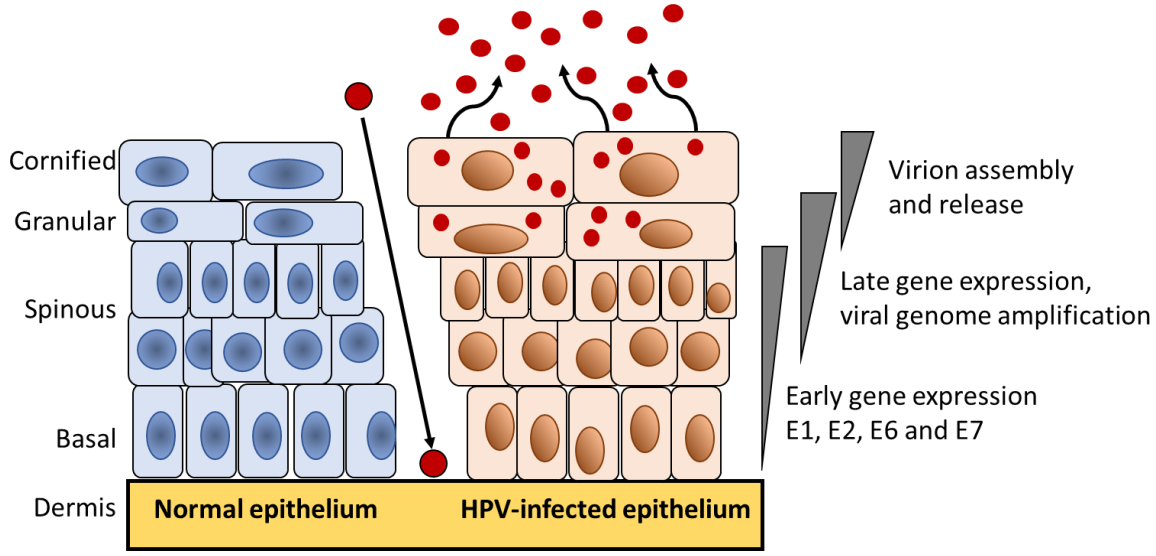


**Figure 20. Representation of human papilloma virus life cycle**. HPV infects basal layer of keratinocytes, probably through wounds. HPV growth is dependent on keratinocytes differentiation. Early HPV oncogenic genes are expressed in the basal layers, whereas virion assembly and release is only found in the upper layers of epithelium. Modified from[172].

HPV is known to replicate at a low rate (50-100 copies per host cell) when infecting the basal layer of the squamous epithelium, hence a key feature in evading the host immune system during early stages of the infection. In the later stages, when the cell has differentiated to keratinocyte and is found in the superficial layer of the epithelium, there is a substantial amount of viral replication and upregulation of genes, including E6 and E7. At this stage the copy number of viral replication has increased and the infected keratinocyte is released along with other desquamated cells. The release of virions along with other cell debris helps evading adaptive immune response and since the virus is released from the squamous epithelium, HPV is not exposed to the lymphatic system[173].

### 3.4.2. Innate immunity and HPV infection

HPV infection is able to evade the innate immune recognition, a process that is not associated to inflammation[174]. HPV, through E5, E6, and E7 proteins, can interfere and regulate host immune responses. These oncoproteins dysregulate gene expression, protein-protein interactions, posttranslational modifications, and cellular trafficking of critical host immune modulators[175], such

as interferences with the IFN pathway, modulation of antigen presentation, inhibition of interleukin-18 activity and downregulation of the major histocompatibility class I on infected cells[176].

Focusing on IFN regulation, it is know that HPV oncoproteins E6 and E7 can directly interfere with mediators of the IFN signaling pathways as transcriptional factors IRFs and JAK-STAT signaling[177]. For instance, HPV E7 protein can inhibit IFN-α-induced transcription through blocking the formation of ISGF-3 complex[176,178]. In addition, the HPV E6 protein has also been shown to decrease the expression of IFN-α, IFN-β, and STAT1 by binding to IRF1[174]. HPV has the ability to alter the function of other immunostimulatory cytokines, including interleukin (IL)-18, while enhancing the expression of immunosuppressive cytokines[177,179].

### 3.4.3. HPV treatment

There is no treatment for HPV infection itself. Although, there are treatment options directed towards HPV lesions such as genital warts.

On the other hand, HPV vaccines have been developed as a prophylactic cure using the major capsid protein L1 of the viral particle. These vaccines are used to prevent healthy patients from HPV infections and also to prevent re-infections. HPV vaccine was first licensed in 2016. Nowadays it prevents infection of HPV-6, -11, -16, -18, -31, -33, -45, -52, -58 high-risk oncogenic strains that induce 90% of cervical cancers. Vaccination is recommended as routine vaccination by the WHO, and in Spain the standard guidelines are girls between 9 to 12 years old should vaccinate with 2 to 3 doses, depending on started age, of the selected HPV vaccine. It is recommended shots should be taken within 6-month timeline.

Once HPV infection is already established, treatment depends on the nature of infection. In the case of HPV lesions that preclude cancer such as anal intraepithelial dysplasia (AID) or high-grade squamous intraepithelial lesion (HSIL) in the anus or the cervix[180], treatment consist in infrared laser ablation or surgery to remove infected cells.

# HYPOTHESIS AND OBJECTIVES

Innate immunity is the first acting player after a pathogen invasion, its effectiveness is key in regulating and limiting pathogen spread. Effective recognition of infection by pattern recognition receptors (PRRs) is key for initiaton of IFN signaling and appropiate activate inflammatory pathways. This pro-inflammatory process contributes to cell stress and cell death, therefore linking host factors with the capacity to trigger an inflammatory state able to affect viability of the infected cell. At its turn, pathogens, as HIV, have developed mechanisms that inhibit inflammation within the host to evade host immune responses and limit viral recognition.

The discovery of SAMHD1 as an HIV restriction factor, a protein also tightly linked to innate immunity, provided novel insights linking nucleotide metabolism, innate immune activation and viral pathogenesis that might be useful to identify novel potential therapeutic approaches affecting the outcome of viral infections.

The main objective of the thesis was to study the relationship between viral and host factors that strongly affect the innate immune modulation and IFN stimulation pathways, thus having the potential to alter viral outcome and progression of associated diseases.

The specific objectives are:

1. To evaluate the effects of HIV infection on innate immunity in primary immune cells
    a. To study effects of long-term HIV-1 infection on cell cycle, innate immune response and regulation of cell death
    b. To evaluate the potential of HIV-2 accessory protein Vpx (HIV-2/SIV) as a modulator of innate immunity and its effect on acute and/or latent HIV infection.
2. To identify host factors that modulate immune activation and affect viral susceptibility, as a putative strategy that can impact infection outcome.
    a. Screening of genes involved in Aicardi-Goutières syndrome through the generation of loss of function models in primary macrophages: the case of ADAR1
    b. Evaluate the role of ADAR1 in HIV-1 infection
    c. Evaluate the role of ADAR1 in HIV coinfections: HCV and HPV

# MATERIALS AND METHODS

**Cell lines.**

The main cell lines and culture conditions used in the present work can be found in table 3. If not otherwise specified, cells were cultures in DMEM or RPMI (Gibco, Madrid, Spain) supplemented with 10% heat-inactivated FCS (Gibco, Madrid, Spain) and penicillin/streptomycin (Life Technologies).

**Table 3. Cell line characteristics**

| Cell line | Provider | Culture method |
|---|---|---|
| **TZM-bl cells** | AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA | DMEM +10% FCS +Pen/Strep |
| **Huh7 and Huh7.5 cells**<br>*Huh7.5 is a derivative from Huh7 cells expressing RFP-NLS-IPS and a constitutive mitochondrial marker, mito-EGFP | Dr. Charles M. Rice, The Rockefeller University, NY, USA | DMEM +10% FCS (Hyclone, Cultek) +4.5 g/l Glucose (Invitrogen) +2mM L-Glutamine (Sigma-Aldrich) +1mM Sodium pyruvate (Sigma-Aldrich) +1% MEM NEEA (Sigma-Aldrich) |
| **SiHa cells**<br>***HPV16 positive** | Kindly provided by Dr. Berdasco Menendez from Bellvitge Biomedical Research Institute (IDIBELL, Spain) | DMEM +10% FCS +Pen/Strep |
| **HaCaT**<br>***HPV16 negative** | Eucellbank (Banco de Células Eucariotas Celltech, UB) | DMEM +10% FCS +Pen/Strep |
| **MT4 cells** | AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA | RPMI 1640 L-Glutamine medium +10% FCS |
| **Jurkat cells** | AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA | RPMI 1640 L-Glutamine medium +10% FCS |
| **ACH2 cells** | AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA | RPMI 1640 L-Glutamine medium +10% FCS |

## Materials and methods

### Primary cells.

PBMC were obtained from blood of healthy donors using a Ficoll-Paque density gradient centrifugation and CD4+ T lymphocytes (#17952, StemCell Technologies) or monocytes (#19359, StemCell Technologies) were purified using negative selection antibody cocktails, following the manufacturer protocol. Monocytes were cultured in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (Gibco) and differentiated to monocyte derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) or granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech) both at 100 ng/ml, or alternatively to monocyte derived dendritic cells (moDC) for 5 day in the presence of GM-CSF at 20ng/ml and IL-4 at 2ng/ml. CD4+ T cells were activated with anti-CD3 and anti-CD28 (at 1µg/ml each, StemCell technologies) for 3 days. The protocol was approved by the scientific committee of Institut de Recerca de la Sida - IrsiCaixa. Buffy coats were purchased from the Catalan Banc de Sang i Teixits (http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease

### RNA interference.

RNA interference was used to downregulated different genes, whose function was being explored through the distinct section points. In a general way, isolated monocytes were transfected[181120121] with 50 pmol of the corresponding siRNA using a Monocyte Amaxa Nucleofection kit (Lonza, Basel, Switzerland) following manufacturer instructions. Monocytes were left untreated overnight and then differentiated to macrophages or monocyte derived dentritic cells as described above.

For activated CD4+ T cells, 9 pmol of the corresponding siRNA with 0.2 µl of siGuard RNase inhibitor (Genlantis) per 1 million of CD4+ T cells were transfected using Amaxa Human T Cell Nucleofector Kit (Lonza, Basel, Switzerland) following manufacturer's recommendations. CD4+ T cells were left untreated for 20 hours and harvested afterwards for HIV-1 infection or processed for mRNA or protein expression.

In the case of adherent cells lines, RNA interference was performed in Huh7, SiHa and HaCaT cells with 50pmol siRNA (or 25pmol for each siRNA in double knockdown experiments) or 2 µg of Poly(I:C) were mixed with Lipofectamine 2000 reagent (11668027, Invitrogen) at a final concentration of 100

nM. Cells were seeded 2x$10^5$ cells for Huh7, or 1.25 $10^5$ cells for SiHa and HaCaT in 24-well plates in the absence of serum, using OPTIMEM medium (Invitrogen) as described[182]. For Huh7, medium with serum was added and phenotype assessed 24 hours later. RNA and protein lysates from SiHa and HaCaT were collected 64 h post-transfection for siRNA and 16 h post-transfection for Poly(I:C).

siRNA used are listed in the following table 4, all siRNAs were ON-TARGET plus purchased from Dharmacon, Waltham, USA.

**Table 4. Knockdown siRNA**

| siRNA | Reference |
|---|---|
| **Non-targeting siRNA Pool** | D-001810-10 |
| **Human ADAR1 siRNA-SMARTpool** | L-008630-00 |
| **Human IFIH1 siRNA-SMARTpool** | L-013041-00 |
| **Human SAMHD1 siRNA-SMARTpool** | L-013950-01 |
| **Human FAM208A siRNA-SMARTpool** | L-020306-02 |
| **Human TREX1 siRNA-SMARTpool** | L-013239-02 |
| **Human RNASEH2B siRNA-SMARTpool** | L-014369-01 |
| **Human RNASEH2C siRNA-SMARTpool** | L-014801-00 |
| **Human ADAR1 siRNA-siGENOME** | M-008630- 01 |
| **Human DDX58 siRNA-siGENOME** | M-012511-01 |

**Compounds.**

Small molecules specifically targeting different cellular or viral proteins were used to inhibit different procedures that were under study. In the following table 5, compounds are listed according cellular or viral targets.

**Table 5. Compound list and their targets**

| Name | Function | Provider |
|---|---|---|
| **3-Azido-3-deoxythymidine (zidovudine, AZT)** | HIV reverse transcription inhibitor | Sigma-Aldrich (Madrid, Spain) |
| **Raltegravir (RAL)** | HIV integrase inhibitor | Merck Sharp and Dome (Kenilworth, USA) |

| | | |
|---|---|---|
| **TAK-779** | HIV entry inhibitor | NIH AIDS Research and Reference Reagent Program (Germantown, USA) |
| **Daclatasvir (DCV)** | Inhibitor of NS54 from HCV | Selleckchem (Houston, USA) |
| **Panobinostat** | Non-selective histone deacetylase inhibitor (HDAC inhibitor) | LC Laboratories (Woburn, USA) |
| **Lipopolisaccaride (LPS)** | Endotoxin, induces pro-inflammatory responses | Sigma-Aldrich (Madrid, Spain) |
| **Polyinosinic:polycytidylic acid (Poly I:C)** | TLR2 and RLR agonist | Sigma-Aldrich (Madrid, Spain) |
| **MRT67307** | Pharmacological inhibitor of IKKϵ and TBK1 | Selleckchem (Houston, USA) |
| **Chaetocin** | Nonspecific inhibitor of histone lysine methyltransferases | Abcam (Cambridge, UK) |

**Viruses and infections.**

Envelope-deficient HIV-1 NL4-3 clone encoding IRES-luciferase was pseudotyped with VSV-G by cotransfection of HEK293T cells using polyethylenimine (Polysciences) as previously described[183]. Three days after transfection, supernatants were harvested, filtered and stored at -80 ºC. Viral stocks were concentrated using Lenti-X concentrator (Clontech). Viruses were titrated by infection of TZM cells followed by GFP quantification by flow cytometry, or titrated by luciferase measurement with a lumunometer for Huh7 infections.

Primary macrophages and dendritic cells were infected with VSV-pseudotyped HIV-1 NL4-3-GFP[81]. Viral replication was measured in all cases two days after infection by flow cytometry (LSRII, BD Biosciences). Measurement of cell cytotoxicity was performed by flow cytometry, i.e., cells were gated as living or dead, according to flow cytometry FSC and SSC parameters. In all cases, antiviral drugs were added simultaneously with the virus as controls.

R5-tropic HIV-1 strain BaL was grown in stimulated PBMC and titrated for its use in MDM. MDM were infected with the R5 HIV-1 strain BaL at day 4 after differentiation in the presence of M-CSF. Four days after infection, half of the culture supernatant were replaced by fresh medium containing M-CSF and drugs when corresponding. HIV production was analyzed at day 7 post-infection by ELISA HIV-p24 Ag detection (Bio-Rad Laboratories, Hercules, CA) in culture supernatants and by

intracellular p24 staining followed by flow cytometry (LSRII, BD Biosciences). In all cases, antiviral drugs were added simultaneously with the virus.

Huh7 cells were infected with VSV-pseudotyped NL4-3-luciferase virus and viral replication was measured two days later by quantification of luciferase production in a luminometer.

For HCV infection, we used the HCV Jc1 virus (kind gift of Dr. Charles M. Rice (The Rockefeller University). Transfected Huh7 cells were infected with HCV Jc1 at a MOI of 0.02 and viral replication was measured five days later by quantification of HCV RNA in infected cells. Briefly, total cellular RNA of HCV infected cells was extracted with the Quickextract™ RNA extraction kit protocol (EPICENTRE Biotechnologies) following manufacters' protocol, including the DNAseI treatment step and extracted RNA was stored at -30ºC until analyzed. Then, 1 mg of total RNA was retro-transcribed to cDNA with a PrimeScript RT master mix (Takara Bio, Clontech, Inc.). cDNA was used for Taqman quantitative PCR (Premix Ex Taq (perfect real time) from Takara Bio, Clontech, Inc.) of total viral RNA using HCV 5'NC6 primers and probe (kindly provided by Dr. Juana Díez), normalized with the transcript GAPDH (Hs99999905_m1, Taqman Gene Expression Assay, Applied Biosystems) according to manufacturer's protocol.

SiHa cells, HPV16+[184], were evaluated after siRNA transfection or stimuli addition, RNA and protein lysates were collected and evaluated 16h post-stimuli or 64h post-transfection and evaluated for the presence of increase expression of *HPV16-E1* (Pa03453396) as previously described[185] and *HPV16-E7* primers and probe (forward primer 5'-AGAACCGGACAGAGCCCATTAC-3', reversed primer 5'-GCCCATTAACAGGTCTTCCAAAG-3' and probe 6FAM-CGCACAACCGAAGCGTAGAGTCACACTT-TAMRA). Protein expression was evaluated by Western blot sing the following antibodies: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce) and anti-HPV16 E7 (1:1000; ab82601; Abcam).

**VLP-Vpx variants.**

VLP-Vpx lipoparticles were used to target the degradation of SAMHD1 and TASOR. Production of viral-like particles carrying Vpx (VLP-Vpx) with HEK293T cells were cotransfected with pSIV3+[186] or pSIVrcm[187] provided by Dr.Oliver T. Keppler or commercially available pLpcx-GFP[187], coated with a VSV-G–expressing plasmid. Three days after transfection, supernatants were harvested, filtered,

and stored at −80°C. Cells were treated with different VLPs for 24h, where RNA and/or protein extraction was performed.

**Cell cycle and proliferation staining by Flow cytometry.**

Cell proliferation was measured by intracellular Ki-67 staining. Cells were fixed for 3 min with fixation buffer (FIX & PERM; Life Technologies) before adding precooled 50% methanol for 10 min at 4°C. Cells were washed in PBS with 5% FBS and incubated for 30 min with the Ki-67 FITC (1:10; clone B56; BD Biosciences) diluted in permeabilization buffer. For cell cycle analysis, cells were suspended in 0.03% saponin (Sigma-Aldrich) in PBS and then incubated in 20 mM 7-aminoactinomycin D (7AAD; Sigma-Aldrich) for 30 min at room temperature in the dark followed by 5 min at 4ºC. Then, Pyronin Y (Sigma-Aldrich) was added at a final concentration of 1.5 µg/ml and cells were further incubated at 4ºC for 15 min and prior to FACS using a LSRII flow cytometer(BD Biosciences). The data were analyzed using *FlowJo* software. In order to correct the overestimation of G2/M population by miss discrimination of cellular doublets, FL2W versus FL2A of the 7AAD dye was plotted before gating for the distinct cell cycle phases as reported.

**Production and reactivation of J-HIG cells**

Latently infected cells (J-HIG) were generated following a modified protocol[188]. Briefly, cells were generated after acute infection of CD4+ Jurkat cells with HIG and maintained in culture for 10 days to allow for the attrition of productively infected cells. Latently infected primary CD4+ T cells were generated according to the cytokine-polarized primary T cells model of latency[189,190] with few modifications. Briefly, naïve CD4+ T cells were activated with αCD3/αCD28 antibodies (1 µg/mL each; BD, Madrid, Spain) and supplemented with TGFβ1 (10 µg/mL, Peprotech), αIL-12 (2 µg/mL) and αIL-4 (1 µg/mL, Peprotech). Medium supplemented with rIL-2 (6.5 IU/mL, Roche) was replaced every 3 days. After 7 days of activation, CD4+ T cells were infected with NL4-3*Vpx by spinoculation (1200×g, 1 h 30 min at 37 °C). Three days later, GFP negative cells containing both latently infected and uninfected.

J-HIG cells were incubated for 24h with different VLPs variants. Reactivation of HIV was monitored as the percentage of living GFP+ positive cells according to forward and side laser light scatter flow cytometry analysis in a FACS LSRII flow cytometer (BD Biosciences). LRA panobinostat (PNB) was

used as a positive control at a concentration of 0.4uM for HIV-1 reactivation. The data were analyzed using the FlowJo software.

**RNA extraction and quantitative RT-polymerase chain reaction (qRT-PCR).**

RNA was extracted using the NucleoSpin RNA II kit (Magerey-Nagel), as recommended by the manufacturer, including the DNase I treatment step. Reverse transcriptase was performed using the PrimeScript™ RT-PCR Kit (Takara). mRNA relative levels of the genes of interest were measured by two- step quantitative RT-PCR and normalized to GAPDH mRNA expression using the DDCt method. TaqMan gene expression assays were used to evaluate gene expression, all purchased from Life Technologies table 6.

**Table 6. List of TaqMan gene expression assays**

| Gene | Reference |
|---|---|
| ***SAMHD1*** | Hs00210019 |
| ***TASOR*** | Hs00961781 |
| ***CXCL10*** | Hs00171042 |
| ***ISG15*** | Hs01921425 |
| ***IFI27*** | Hs01086373 |
| ***IFNB1*** | Hs01077958 |
| ***IRF3*** | Hs01547283 |
| ***IRF7*** | Hs01014809 |
| ***DDX58*** | Hs01061436 |
| ***IFIH1*** | Hs00223420 |
| ***TMEM173*** | Hs00736955 |
| ***MB21D1*** | Hs00403553 |
| ***ADAR1*** | Hs00241666 |
| ***RNASEH2B*** | Hs00608793 |
| ***RNASEH2C*** | Hs00611049 |

## Materials and methods

### Quantification of proviral DNA, HIV-1 integration and HIV transcription in primary cells.

Infected MDM with HIV-1 BaL were stopped after 16h to measure proviral DNA, 20h to measure viral integration and 40h to evaluate viral transcription as described before[121,181]. DNA was extracted using a DNA extraction kit (Qiagen) and RNA extracted using NucleoSpin RNA II kit (740955, Macherey-Nagel), as recommended by the manufacturers.

Proviral DNA quantifications were performed using Gag amplification using the following primers and probe: forward 5'-CAAGCAGCCATGCAAATGTT-3', reverse 5'-TGCACTGGATGCAATCTATCC-3', and probe FAM 5'-AAAGAGACCATCAATGAGGAAGCTGCAGA-3' TAMRA.

HIV-1 integration levels were evaluated by a pre-amplification of Alu-Gag (HIV group-specific antigen) after recommendation of the manufacturer by using the following primers: forward 5'-GCCTCCCAAAGTGCTGGGATTACAG-3', and reverse 5'AGGGTTCCTTTGGTCCTTGT-3. Afterwards, samples were then followed by a Gag amplification by using the following primers and probe: forward 5'-CAAGCAGCCATGCAAATGTT-3', reverse 5'-TGCACTGGATGCAATCTATCC-3', and probe FAM 5'-AAAGAGACCATCAATGAGGAAGCTGCAGA-3' TAMRA. Ct values for proviral DNA and integrated viral DNA were normalized using RNaseP as housekeeping gene by the ΔΔCt method. Infections were normalized to an untreated control. To ensure that measured proviral DNA was the product of infection and not result from DNA contamination of the viral stocks samples treated with RT inhibitor AZT (1 μM) were run in parallel. Raltegravir (2 μM) was used to control for post-RT steps.

Last, HIV-1 RNA transcription was quantified by extracting RNA from MDM 40 hours post infection and measuring multiple spliced viral RNA as described before[182]. RNA was extracted and reverse transcription was performed as previously mentioned, and the following set of primers and probe were used to amplify spliced *tat/rev/nef* mRNA: forward 5'-GGATCTGTCTCTGTCTCTCTCTCCACC-3', reverse 5'-ACAGTCAGACTCATCAAGTTTCTCTATCAAAGCA-3' and the dual-labeled fluorescent probe FAM'5-TTCCTTCGGGCCTGTCGGGTCCC-3'TAMRA. mRNA relative levels were measured by two-step quantitative RT-PCR, normalized to *GAPDH* mRNA expression using the DDCt method[191].

### Determination of ADAR1 function by direct sequencing.

ADAR1 A-to-I deamination is measured by direct sequencing of mRNA transcripts and scanning of the presence of I, which is read as G by conventional Sanger sequencing. A control gene, where

ADAR1 modification has been very well described, was selected as a positive control. ADAR1 deaminase function was determined by sequencing *NEIL1* or 5' UTR of HIV-1 RNA regions previously described to be modified by ADAR1[93,192]. Total RNA was extracted and reversed transcribed to obtain cDNA as described above. *NEIL1* was amplified first using primers N1-TCOF 5'-TCCAGACCTGCTGGAGCTAT-3', and N1-TCOR 5'-GGCCTTGGATTTCTTTTTG-3', followed by a nested PCR with Forward N1-TCIF 5'-CCCAAGGAAGTGGTCCAGTTGG-3', and Reverse 1-TCIR 5'-CTGGAACCAGATGGTACGGCC-3' primers, as described in[93]. PCR conditions were as follows 1µL cDNA with thermocycler conditions of 98 °C for 30 s hot start, followed by 40 cycles of 98 °C for 30 s, 55°C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min. The nested PCR conditions with 1µL of input PCR products were 98 °C for 30 s hot start, followed by 30 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min final extension, and were run on a 2% agarose gel to confirm band size of approximately 150bp.

5' UTR of HIV-1 RNA was evaluated since previous reports have identified A-to-I modification by ADAR1 in such region[192], amplification was performed by using the following primers: forward 5'-GGGTCTCTCTGGTTAGA-3', and reverse 5'-GGGTTCCCTAGTTAG-3'[193].

To screen for putative ADAR1 edited sites in HPV genome, we first determined the expression of different transcripts in SiHA cells. Then, HPV primers were designed based on HPV16 genome sequence (GenBank accession number AF001599.1) using Primer3 software (*bioinfo.ut.ee/primer3-0.4.0/)*. Primer pairs used for sequencing HPV transcripts expressed in SiHa cells were as follows: HPV16 E6 5'-TTCATGTATAAAACTAAGGGCGTAA-3', 5'-CAGCTGGGTTTCTCTACGTGT-3' (546 bp); HPV16 E7 5'-CATGGAGATACACCTACATTGC-3', 5'-CTGAGAACAGATGGGGCACA-3' (285 bp); HPV16 E1_a 5'-TGATCCTGCAGGTACCAATG-3', 5'-TGCTGCCTTTGCATTACTAGTTT-3' (613bp); HPV16 E1_b 5'-GGGGAGAGGGTGTTAGTGAA-3', 5'-CACATTGTTGCACAATCCTT-3' (700 bp); HPV16 E2_a 5'-AGATGATGGAGGTGATTGGA-3', 5'-CACATTTAAACGTTGGCAAAGA-3' (623 bp); HPV16 E2_b 5'-CTCAAGGACGTGGTCCAGAT-3', 5'-AGTAGACATACTGGGTTATCTGAGG-3' (590 bp). PCR conditions for HPV16 transcripts were 95 °C for 5min hot start, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min final extension.

PCR products were run on a 2% agarose gel to confirm band size of approximately 150bp for *NEIL1* and 180bp for 5' UTR of HIV-1 before analyzing them by direct sequencing. HPV transcript bands were purified using QIAquick PCR purification kit (QIAGEN). NEIL1 and HPV16 SiHa amplified

products were treated with illustra™ ExoProStar™ (GEUS78210, Sigma) as described by the manufacturer. Samples were sequenced in a ABI3730XL system (Applied Biosystems).

Relative ADAR1 editing efficiency in transcripts was estimated calculating the ratio of edited peak height (height of edited peak divided by the total height of edited and non-edited peak heights) for each sequence, and calculate the mean for the specific site for each independent experiment. Mean ratio of at least three independent experiments were calculated.

**SNP Genotyping.**

Genomic DNA from HIV/HCV and HIV/HPV cohorts were extracted using the QuickExtract™ DNA Extraction Solution 1.0 (QE09050, Epicentre Biotechnologies) from blood samples in the HCV study and from anus or cervical samples in the HPV study. Briefly, $1x10^6$ cells were resuspended in 0.5 mL of QuickExtract DNA Solution, incubated at 65ºC for 15 min, followed by an incubation of 2 min at 98ºC, and then stored at -30ºC. 2 ml of the extracted DNA were used for the genotyping.

For HCV study, two ADAR1 SNPs (rs2229857 and rs1127326) were genotyped using TaqMan SNP genotyping assay (Assay num: C_1235929_10 and C_8724402_20, respectively, Applied Biosystems). And in the HPV study, six ADAR1 SNPs (rs6699729, rs3766927, rs3766925, rs3766924, rs9616 and rs9427097) were selected based on linkage disequilibrium testing and cover all described variation in ADAR1 gene in the HPV study. The variants were typed using TaqMan SNP genotyping assay (Assay num: C__30114879_10, C__11259682_10, C___222942_10, C__25800598_10, C___8724401_10 and C__303121822_10, respectively, Applied Biosystems) following manufacturer's protocol. Reactions were analyzed on an ABI PRISM 7500 (Applied Biosystems) and allele calling was performed by AutoCaller Software v 1.1 (Applied Biosystems).

**Western blot.**

Cells were rinsed in ice-cold phosphate-buffered saline (PBS) and extracts prepared in lysis buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM Na Pyrophosphate, 270 mM sucrose and 1% Triton X-100) supplemented with protease inhibitor (Roche) and 1 mM phenylmethylsulfonyl fluoride. Lysates were subjected to SDS-

PAGE and transferred to a PVDF membrane (ImmunolonP, Thermo and Trans-Blot Turbo RTA, BioRad).

The following antibodies were used for immunoblotting: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce); anti-FAM208A (1:500; HPA006735, SIGMA); anti-human Hsp90 (1:1000; 610418, BD Biosciences); RRM2 (1:1000; 11661-1-AP, Proteintech); anti human Caspase3 (BD Pharmigen); anti-SAMHD1 (1:2,500; ab67820), anti-GAPDH (1:2500; ab9485), anti-HPV16 E7 (1:1000; ab82601), anti-RNase H2C (1:1000, ab89726), anti-RNase H2B (1:1000, ab122619), and anti-HIV-Gag (1:1000), all from Abcam; anti phospho-CDK2 (Thr160; 2561), anti-CDK2 (2546), anti-CDK6 (3136), anti CDK4 (D9G3E), anti CDK1 (77055), anti-Rb (9309) anti-phospho-Rb (Ser807/811, 9308) anti-E2F1 (3742), anti-ADAR1 (14175), anti-RIG-I (3743l), anti-MDA5 (5321), anti-PARP (9532), anti-cleaved Caspase-3 (9664), anti-phospho-NF-κB p65 (3033) anti-MAVS (3993) IRF3 (11904), anti-IRF7 (4920), anti-PKR (12297), anti-TREX1 (16712215S), anti-phospho-SAMHD1 Thr592 and anti-phospho-Stat1 (9167) all 1:1000 from Cell Signaling.

**Cytokine Array.**

Cytokine expression was evaluated by using the commercial TaqMan Human Cytokine Network array (4414255, Life Technologies), which included primers and probes for 28 different cytokine genes. mRNA relative levels of all cytokine genes were measured by two-step quantitative RT-PCR and normalized to GAPDH mRNA expression using the DDCt method.

**Quantification by ELISA of CXCL10 and HIV-1 p24 in culture supernatants.**

CXCL10 was quantified in macrophage culture supernatant and in baseline plasma samples from HCV/HIV-1 coinfected patients (in HCV study) using human IP-10 ELISA Kit SimpleStep (ab173194, Abcam). Macrophage supernatants were quantified at day 4-post differentiation and/or day 7-post infection following manufacturer instructions.

Production of HIV-1 p24 present in culture supernatants was analyzed in MDMs seven days post infection using ELISA HIV-p24 antigen detection (BioRad, Barcelona, Spain).

## Materials and methods

### Antiviral activity of IFN-β antibody.

Transfected macrophages were treated with a dose-dependent amounts of anti-human IFN-β antibody (LEAF Purified anti-human IFN-b, 514004, BioLegend) or the isotype control (LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody, 400123, BioLegend). Treated macrophages were infected with VSV-pseudotyped NL4-3-GFP and viral replication was measured two days later by flow cytometry (LSRII, BD Biosciences).

### Antiviral activity of culture supernatants.

Supernatants from transfected macrophages were collected 4 days post-transfection and stored at -30ªC. Non-transfected monocytes from a distinct donor were differentiated to macrophages in 96 well plates for 4 days. Macrophages were incubated with different amounts of culture supernatants (150 µl and dilutions 5-fold), followed by infection with VSV-pseudotyped NL4-3-GFP and viral replication was measured two days later by flow cytometry (LSRII, BD Biosciences).

### RNA-seq and bioinformatic analysis.

RNA sequencing was carried out in collaboration with Macrogen sequencing facility (Macrogen, South Korea). Total RNA was extracted using NucleoSpin RNA II kit (Magerey-Nagel), as recommended by the manufacturer, including the DNase I treatment step. RNA integrity was evaluated using Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA). Library was constructed using Illumina TruSeq stranded mRNA Library. Sequencing coverage was 2x100bp by Novaseq and 30M reads/samples with 100bp read length.Sequenced raw reads quality control was evaluated and overall reads' quality, total bases, total reads, GC (%) and basic statistics were assessed. To reduce biases in analysis, artifacts such as low quality reads, adaptor sequence, contaminant DNA, or PCR duplicates were removed. Trimmed reads were mapped to reference genome with HISAT2, splice-aware aligner. Transcripts were assembled by StringTie with aligned reads. Expression profiles were represented as normalization value which is based on transcript length and depth of coverage. The FPKM (Fragments Per Kilobase of transcript per Million Mapped reads) value or the RPKM (Reads Per Kilobase of transcript per Million mapped reads) value were used as expression profile.

In groups with different conditions, genes or transcripts that express differentially were filtered out through statistical hypothesis testing. In case of known gene annotation, functional annotation and gene-set enrichment analysis were performed using GO and KEGG database on differentially expressed genes.

**Statistical analysis.**

Experimental data are presented as mean ± SD. Paired Student's t test was used for comparison between two groups, using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). P-values lower than 0.05 were considered significant.

***HIV/HCV genetic association study.***

Treatment baseline covariates were analysed with chi-squared test, unpaired t-test and the Mann-Whitney U test (GraphPad Prism, version 4.00; GraphPad Software Inc., San Diego, California, USA).

**Genetic association studies**

The association of each SNP and odds ratio and CI were computed using a stratified analysis by gender with a logistic regression model adjusted by HCV viral load and IL28B-rs12979860 and HCV genotype[194]. We consider four inheritance models (codominant, dominant, recessive model, and log-additive), and akaike information criterion (AIC) was considered for estimation of the relative quality of statistical models. P values were derived from likelihood ratio tests. In all cases, the reference class was set as the homozygosis for the major allele among controls. The level of significance was set up equal to 0.0083 (0.05/6=0.0083) based on Bonferroni correction for multiple comparisons. We considered gender and the two considered outcomes (response to treatment and fibrosis). Since two assessed variants are highly correlated (r2=0.99), Bonferroni correction for two variants was considered too conservative and not considered[195]. All analyses were carried out using the SNPassoc R library[194]. Experimental data are presented as mean ± SD. Paired Student's t test was used for comparison between two groups, using the GraphPad Prism software. p-values lower than 0.05 were considered significant.

## Materials and methods

### *HPV/HPVgenetic association study.*

Genetic association analysis was computed using a logistic regression model[194] for recurrent HPV dysplasia adjusted by age and sex, number of months since the first proctologist evaluation, CD4 nadir, number and type of HPV genotypes. We consider four inheritance models (codominant, dominant, recessive model, and log-additive), and the akaike information criterion (AIC) was considered for estimation of the relative value of statistical models. The level of significance was set up equal to 0.0083 (0.05/6 = 0.0083) based on Bonferroni correction for multiple comparisons. These analyses were carried out using the SNPassoc R library[194]. Haplotype estimation and association analysis for recurrent HPV dysplasia was performed using the *haplo.stats* R package. P-values were corrected by permutation after 10,000 permutations using the score test implemented in *haplostats*[196]. No formal sample size was calculated. The sample was defined as all HIV- infected with biopsy proven AIN2 or AIN-3, CIN2 or CIN3 confirmed by HRA or colposcopy.

# RESULTS

# CHAPTER 1. EVALUATION OF THE EFFECTS OF HIV INFECTION ON INNATE IMMUNITY IN PRIMARY IMMUNE CELLS

## Summary

In this first chapter of results, we aimed at evaluating the effect of HIV infection on innate immunity, taking into special account the interplay between infection, cell cycle progression and host and viral factors affecting transcription, cell death and reactivation of provirus.

To do so, we first focused on the evolution and effects during a long-term HIV-1 infection in monocyte derived macrophages. Through an in-depth evaluation of the different viral stages of the infection and its effects on cell cycle regulation, we have linked susceptibility to the HIV-1 infection and innate immune response to inflammation and cell death regulation. Host factors such as SAMHD1 have a pivotal role regulating susceptibility to HIV infection and regulating the innate immune response. The fact that non-cycling cells have active SAMHD1, which prevents the infection, shows the value in understanding host and viral factors and their relationship to the innate immune response and cell cycle. Thus, we evaluated the role of the accessory protein Vpx from HIV-2/SIV that counteracts host factors regulating transcription and cell replication and its influence on the IFN-mediated immune response and regulation of cell death in acute and latent models of HIV infection.

## 1.1 Long-term HIV-1 infection induces an antiviral state in primary macrophages

In this section of results, we will evaluate the effects on cell cycle progression and innate immune responses after acute HIV infection on primary macrophages.

We study cell cycle regulation by evaluating expression of CDKs and other regulatory proteins that act as checkpoints in the progression of cell cycle. Then, we describe the innate immune response and immune players affected during the infection and evaluate immune activation and transcription of IFN-related genes. Finally, we assess cell death to determine whether the immune response is predominantly destroying infected cells.

### 1.1.1. M-CSF induces MDM cell proliferation and entry in cell cycle, determining HIV-1 susceptibility.

To investigate the effect of HIV-1 infection on cell cycle, MDM from healthy donors were differentiated with M-CSF during four days and then infected with the R5-tropic HIV-1 strain BaL for seven additional days in the presence of M-CSF. When adding M-CSF, monocytes begin to proliferate and differentiate as previously reported[121] as seen by the increment in intracellular Ki67+ staining that is negative in monocytes as well as by the entry into cell cycle reflected by the increment in the RNA and DNA content (figure 21A). Entry into cell cycle determines the susceptibility of MDM to HIV-1 infection as measured by the presence of intracellular p24 antigen and secreted in the supernatant of the infected cells. As expected, p24 antigen was not detected when raltegravir was added at the time of infection and was clearly diminished after addition of the CCR5 antagonist TAK-779 twenty hours after infection to simulate a single cycle of replication (figure 21B).

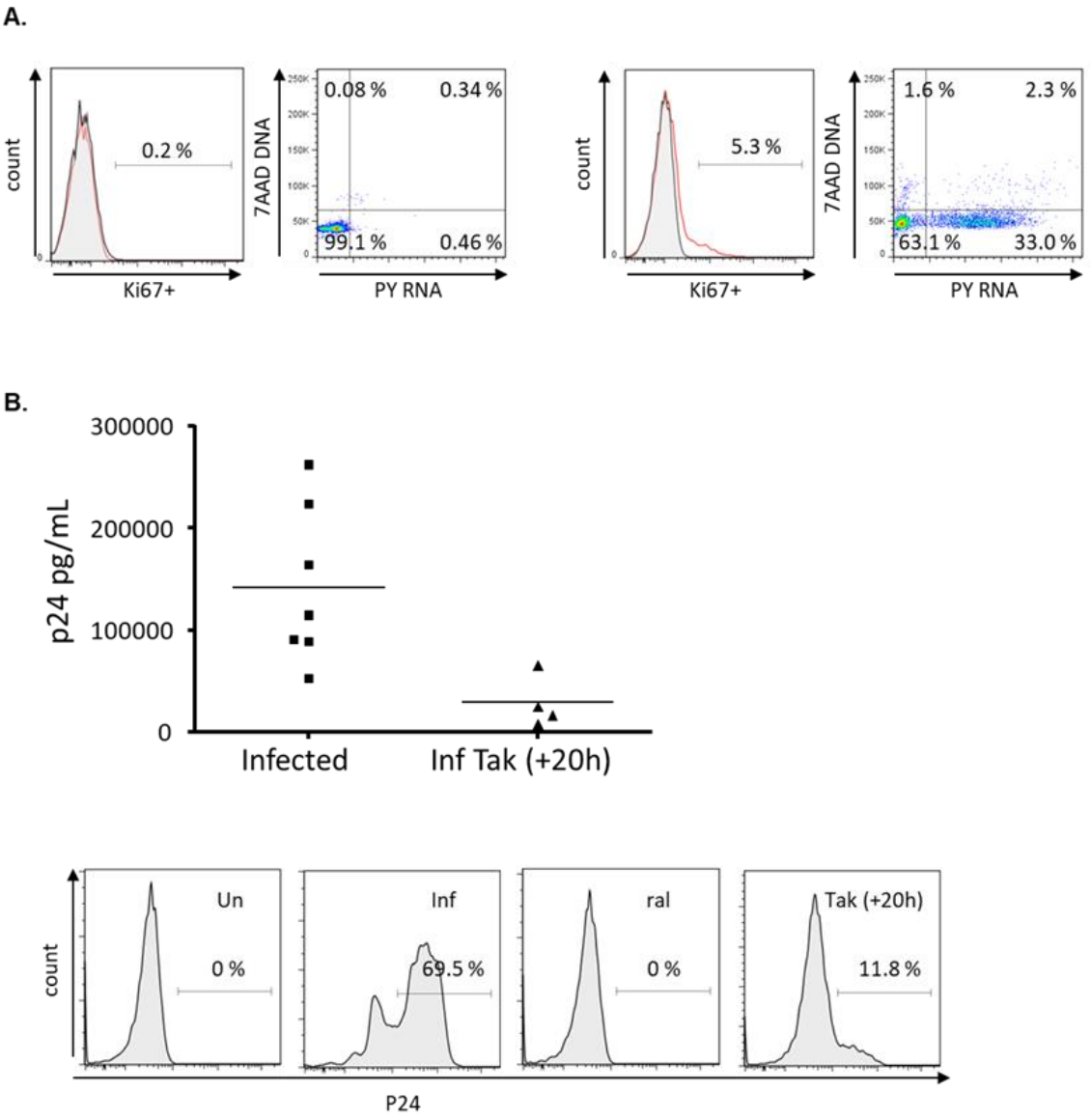


**Figure 21. M-CSF induced MDM cell proliferation and entry in cell cycle determining HIV-1 susceptibility.** (A) Intracellular Ki67+ and 7AAD/ Pyronin Y (PY) staining showing that before stimulation with M-CSF (left), monocytes show absence of proliferation and most majority of cells in G0 cell cycle stage. 4 days after M-CSF addition, MDM begin to proliferate as measured by positive Ki67 staining and entry into the cell cycle (right). A representative donor out of three is shown. (B) HIV-1 infection measured by production of CAp24 antigen in the culture supernatant at day seven post-infection in the absence of drugs (Infected) or in the presence of TAK-779 added twenty hours post-infection to ensure a single cycle of replication (Inf Tak (+20h)). Absolute values and mean of seven independent donors are shown (upper panels). Histograms showing intracellular CAp24 staining of a representative donor are shown (lower panels). Un, uninfected; Inf, infected; ral, infected in the presence of raltegravir; Tak (+20h), infected in the presence of TAK-779 added twenty hours post infection.

### 1.1.2. HIV-1 infection induces cell cycle arrest at G2/M.

To investigate the effect of HIV-1 infection on proliferation status and cell cycle, intracellular Ki67 staining was evaluated at day three and seven post infection. Intracellular Ki67+ cells were 2-fold higher ($p<0.05$) in HIV-1 infected MDM compared with uninfected controls three days after infection. This increment in Ki67+ cells was not maintained when cells were infected in the presence of the integrase inhibitor raltegravir, but could still be observed ($p<0.05$), when TAK-779 was added twenty hours after infection to ensure a single cycle of replication (figure 22A (upper) and B (left)). Seven days post-infection, cell proliferation was drastically diminished in both the uninfected controls and the HIV-1 infected MDM. However, the drop of Ki67+ cells was more drastic in infected MDM, compared to uninfected controls (3-fold reduction $p<0.0001$). Addition of TAK-779 after twenty hours of infection also significantly reduced ($p<0.005$) cell proliferation compared with uninfected controls although to a lesser extent than in HIV-1 infected cells in the absence of drugs (figure 22A (lower) and B (right)).

Cell cycle profile analysis indicated that the percentage of cells arrested in G2/M increased ($p<0.005$) in HIV-1 infected MDM compared to uninfected controls, as previously suggested[197,198]. As cell cycle analysis relies on DNA and RNA cell content and HIV infection induces the formation of syncitia with increased amount of nucleic acid per cell, the CCR5 antagonist TAK-779 added 20 hours after infection provided an important control to avoid syncytium formation. In addition, the discrimination module of the cytometer than discriminates doublets from singlets was used in all the analysis. In the presence of TAK-779, the percentage of cells in G2/M compared with uninfected controls was slightly higher (figure 21B, $p=0.06$), suggesting that the observed cell cycle arrest was due to HIV-1 infection per se, rather than accumulation of DNA from different cells.

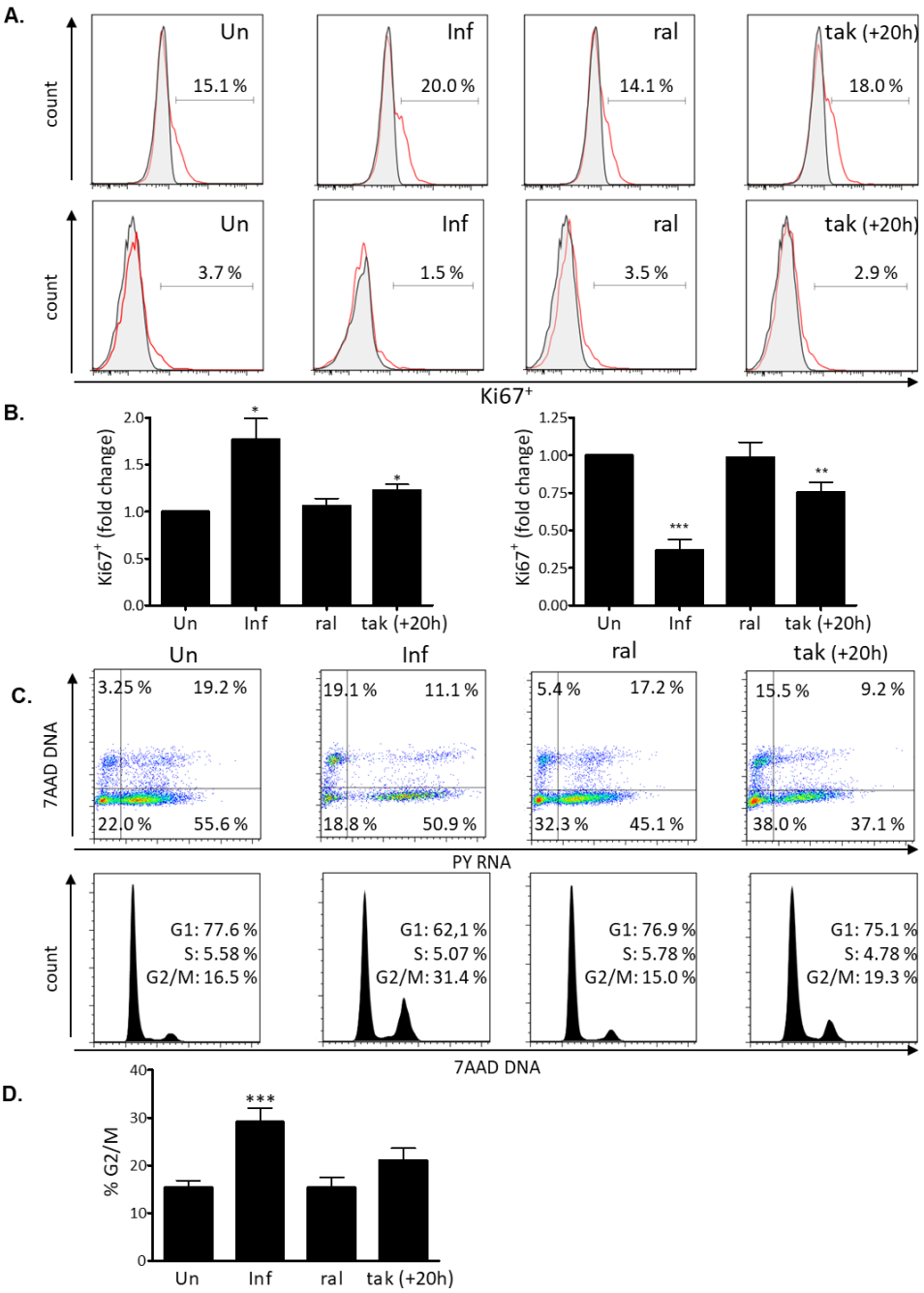


**Figure 22. HIV-1 infection induces cell proliferation and cell cycle arrest at G2/M.** (A) Percentage of Ki67+ cells in uninfected (Un), infected (Inf), treated with raltegravir (ral) or TAK-779 added twenty hours after infection (tak +20h) MDM, three days post infection (upper panels, 3dpi) and seven days post infection (lower panels, 7dpi). Isotype staining was used as a control. A representative donor out of three is shown. (B) Fold change in Ki67+ values in HIV-1 infected MDM compared to uninfected controls three days (left) and seven days after infection (right). Means ± SD of three independent donors is shown. (C) Dot blots of cell cycle profile showing 7AAD DNA and Pyronin Y (PY) RNA staining content quantified by Flow cytometry analysis. Corresponding histograms showing DNA content of a representative experiment is shown. (D) Percentage of cells in G2/M at day seven post-infection showing mean and ± SD values of three independent donors. Un, uninfected; Inf, infected; ral, infected in the presence of raltegravir; tak (+20h), infected in the presence of TAK-779 added twenty hours post infection. *p<0.05, **p<0.005, ***P<0.0001.

### 1.1.3. The HIV-1 induced cell cycle arrest is driven by a p21-dependent CDK inactivation signaling pathway.

HIV-1 is proposed to cause cell arrest at G2/M trough different mechanisms, several of them involving regulation of cyclin-dependent kinase by p21/Waf1/Cip1[199,200]. Consistent with this, we found overexpression of p21 in HIV-1 infected MDM, but not in the uninfected controls or in HIV-1 infected cells in the presence of raltegravir (figure 23A and B). Conversely, we did not observe a significant change in the expression of a different cell cycle regulator such as p16INK4a (p16) neither at the protein nor at the mRNA level (figure 23A and B). p21 is a potent cyclin-dependent kinase inhibitor that binds to and inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, and thus functions as a regulator of cell cycle progression. In HIV-1 infected MDM, CDK6 and CDK4 protein levels remained unchanged (figure 23A). However, in infected cells there was a marked decline in CDK2 activity, as measured by the lack of CDK2 phosphorylation. Consistent with CDK2 inactivation, pRb became hypo-phosphorylated in HIV-1 infected MDM, but not in the uninfected controls or in HIV-1 infected cells in the presence of raltegravir (figure 23A). As a consequence of the hypo-phosphorylated active estate of pRb there was a marked downregulation of both mRNA and protein of the autoregulated[201] transcription factor E2F1 and the E2F-dependent transcription of the ribonucleotide reductase subunit R2 (RNR2) (figure 23A and B). In addition, and consistent with the increment of p21, CDK1 expression was downregulated only in the HIV-1 infected cells both at the protein and the mRNA levels, which halts cell cycle progression at G2/M. In the single cycle infected MDM, in which TAK-779 was added twenty hours after infection, downregulation of the proteins that control cell cycle progression was only barely observed due to the low percentage of infected cells, but remained statistically significant when measured by p21 and RNR2 mRNA expression ($p<0.05$) (figure 23A and B).

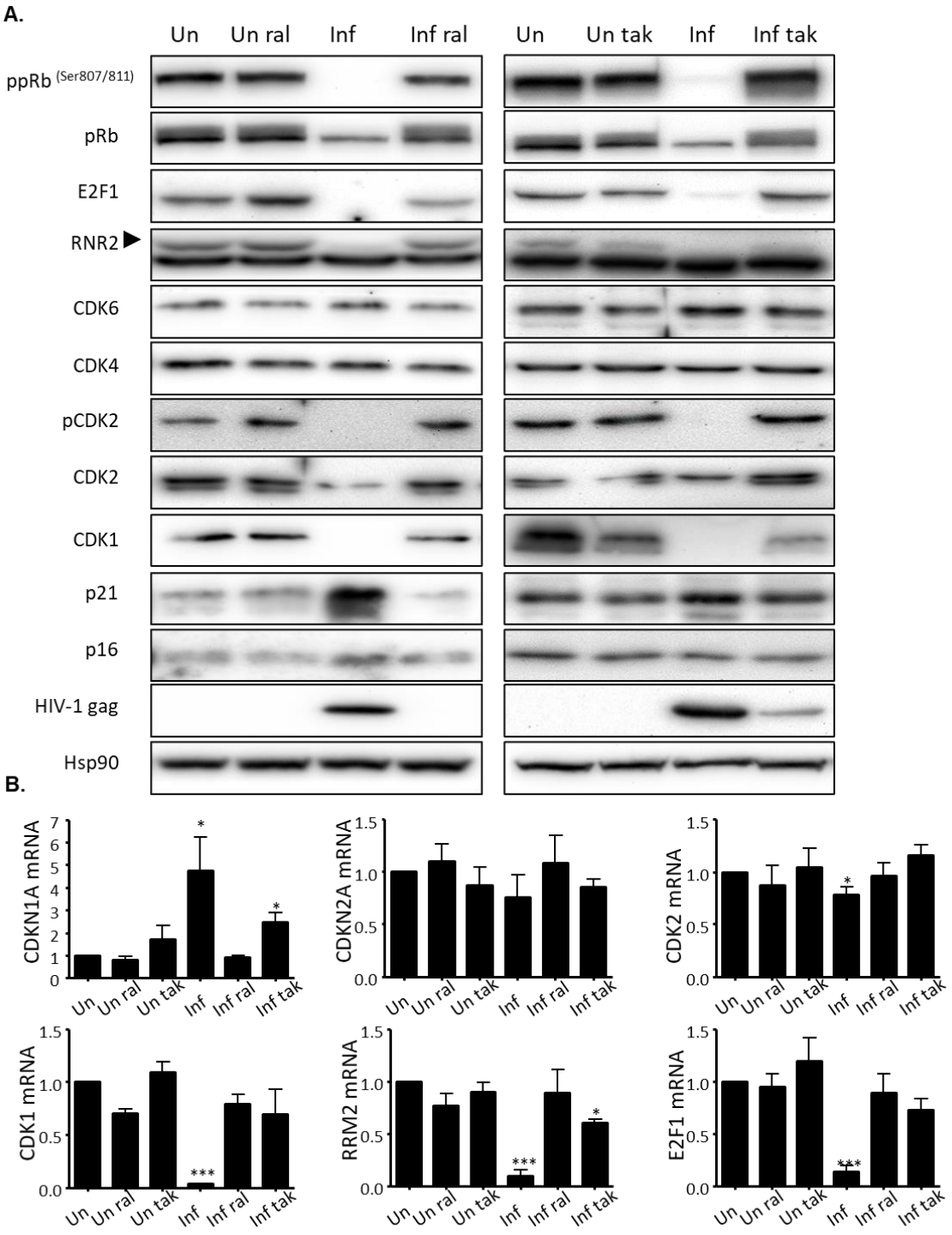


**Figure 23. p21 drives HIV-1 induced cellular arrest through CDK inactivation signaling.** (A) Lysates of MDM were collected at day seven post-infection, subjected to SDS-PAGE and blotted with the indicated antibodies against different cell cycle control proteins. Hsp90 was used as loading control. Representative blots of two donors are shown. (B) mRNA of the indicated genes were measured by quantitative PCR and normalized to GAPDH expression. mRNA levels are shown compared to uninfected MDM at day seven post-infection. Mean ± SD values of three independent experiments are shown. Un, uninfected; Inf, infected; ral, MDM in the presence of raltegravir; tak, MDM in the presence of TAK-779. *p<0.01; **p<0.001; ***p<0.0001.

### 1.1.4. HIV-1 infection in human MDM leads to type I interferon expression and activation of ISGs.

Previous studies reflected opposite results concerning the production of interferon and activation of interferon stimulated genes induced by HIV-1. To evaluate whether or not HIV-1 induces features of innate immune activation in MDM, we assessed mRNA levels of IFNB1 gene by quantitative PCR. HIV-1 infected MDM showed increased levels of IFNB1 mRNA (figure 24A) and CXCL10 in the supernatant (figure 24B) compared with uninfected controls. In addition, activation of the transcription of interferon-stimulated genes as measured by STAT1 phosphorylation was also observed (figure 24C). Of note, whereas the MDM infected in the presence of raltegravir did not show features of immune activation, in the presence of TAK779, infected MDM showed comparable levels of IFNB1 mRNA and STAT1 phosphorylation than untreated, infected MDM, suggesting that a single round of viral infection is sufficient to trigger activation of innate immunity.

To confirm HIV-1 sensing associated to the production IFN-1β we evaluated the expression of pattern recognition receptors in MDM (figure 24D). Infection with HIV-1 BaL led to overexpression of MDA5 and RIG-I and downregulation of MAVS, the adaptor protein that acts downstream of RIG-I and MDA5[202]. This effect was overturned by inhibition of viral DNA formation and virus replication by AZT (figure 24E and F).

Taken together these results indicate that wild type infection may be sensed by MDM, leading to activation of an antiviral and inflammatory response.

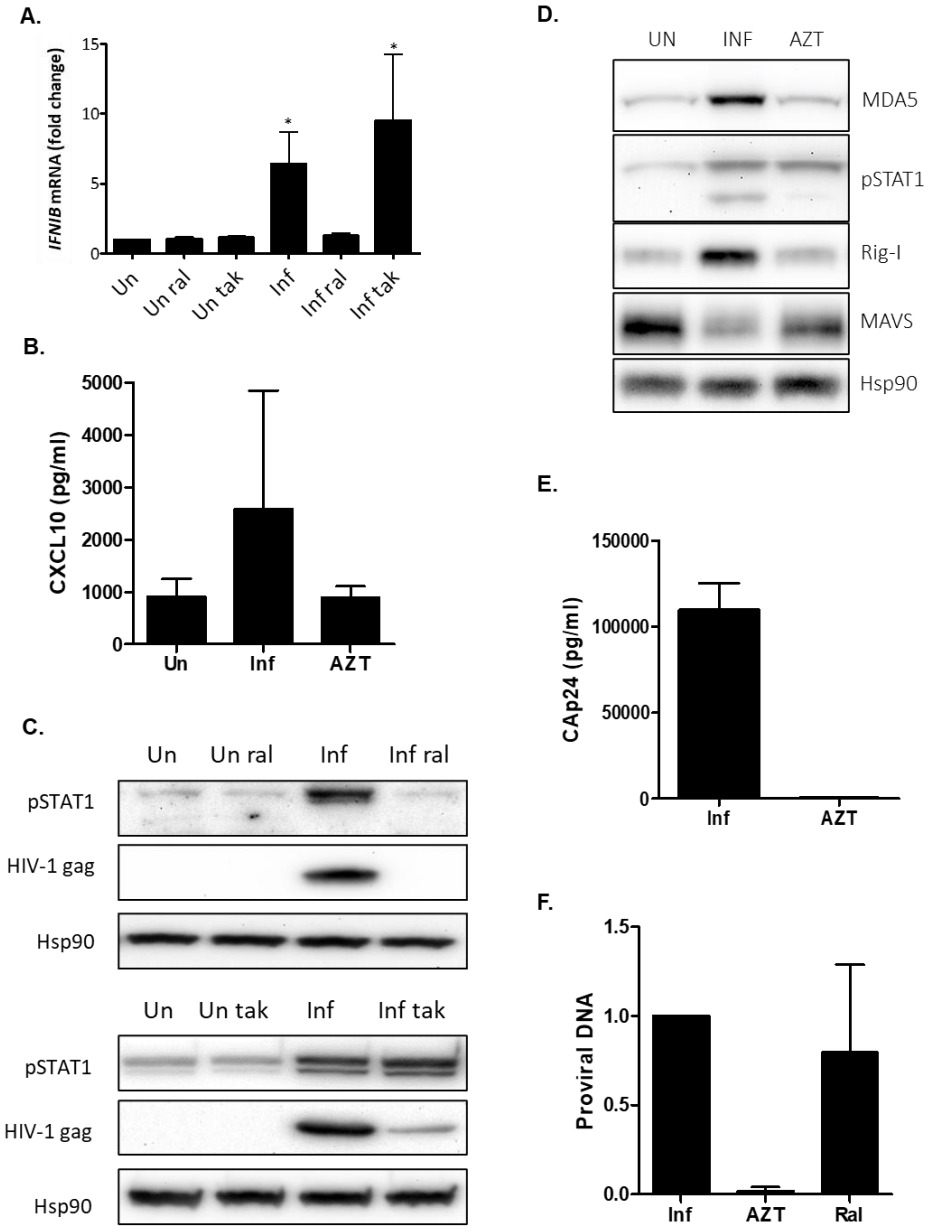


**Figure 24. HIV-1 infected MDM show features of innate immune activation, pathogen pattern recognition and IFN-I production.** (A) IFNB1 expression measured by qPCR showing mRNA levels of infected MDM in the absence of drugs (Inf), or in the presence of raltegravir (Inf ral) or TAK-779 added twenty hours post infection (Inf tak) compared with uninfected controls. Mean ±SD values of three independent donor is shown. (B) CXCL10 production in the supernatant measured by ELISA at day seven post infection in uninfected, infected and AZT (1µg/ml) treated MDM. Mean ± SD of four different donors is shown. (C) Western Blot of two representative donors showing pSTAT1 expression. (D) Western Blot of MDM collected at day seven post-infection, showing innate immune proteins involved in RLR signaling pathway. Hsp90 was used as loading control. A representative blot is shown. (E) HIV-1 infection measured by production of CAp24 antigen in the culture supernatant at day seven post-infection in the absence of drugs (Inf) or in the presence of 1 µg/ml AZT. Mean ± SD of four different donors performed in triplicate is shown. (F) Proviral DNA formation after 16h infection with HIV-1 BaL of MDM untreated or treated with AZT (1µg/ml) or raltegravir (Ral; 1µg/ml). Total viral DNA was normalized to infected macrophages. Mean ± SD of three different donors is shown. Un; uninfected, Inf; infected, AZT; treated 1µg/ml. *p<0.001.

### 1.1.5. HIV-1 infection increments the levels of cell death in primary MDM.

Control of intracellular levels of deoxyribonucleoside triphosphates (dNTPs) is achieved by the concerted action of a group of enzymes, such as RNR2 and the triphosphohydrolase enzyme SAMHD1, which plays also a role in innate immune sensing. Moreover, SAMHD1 activity is controlled by cyclin-dependent kinases tightly regulated during cell cycle (reviewed in[122]). Accordingly, with the observation that in HIV-1 infected MDM CDK2 activity is abolished and CDK1 expression is downregulated, we found reactivation of SAMHD1, as measured by the lack of phosphorylation at residue T592 compared with the uninfected controls or infected in the presence of raltegravir controls (figure 25A). When TAK-779 was added twenty hours after infection, activity of SAMHD1 was only partial, accordingly with the low percentage of single cycle infected cells (figure 25A). In concordance with previous studies suggesting that the activity of SAMHD1 triggers an innate immune response resulting in cell death, we observe a significant increment ($p<0.005$) in the percentage of dead cells in the HIV-1 infected MDM compared with uninfected controls (figure 25B, upper). In addition, the ratio between active versus pro-active form of Caspase 3 was significantly higher ($p<0.005$) in MDM infected with HIV-1 (figure 25B, lower), suggesting that activation of classical apoptotic pathways is the mechanism underlying HIV-1 induced cell death.

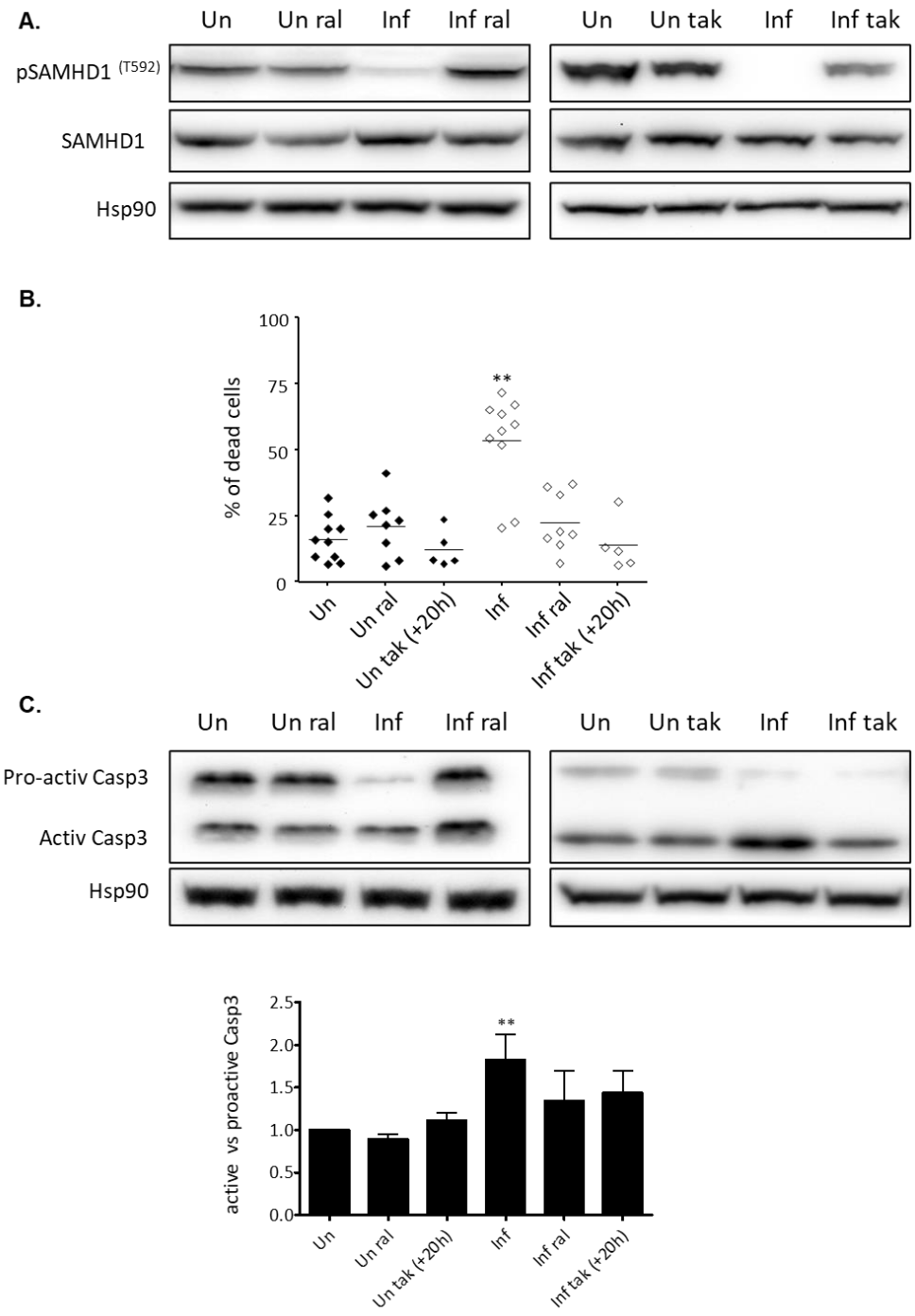


**Figure 25. Increased cellular death induced by HIV-1 infection.** (A) Western blot of two representative donors showing SAMHD1 expression and activation. Hsp90 as loading control (B) Percentage of cell death measured by forward-side scatter parameter flow cytometry in uninfected and infected MDM in the presence or not of raltegravir or TAK-779. Mean values of at least five donors is shown. (C) Western blot of two representative donors showing expression of activate and pro-active Caspase 3 and Hsp90 as loading control. Bar graphs (lower panel) showing quantification of active Caspase3 relative to the uninfected control (Un) as measured by the ratio between the density of the active Casp3 and the pro-active Casp3 bands. Mean ± SD values of three donors is shown. Un, uninfected; Inf, infected; ral, raltegravir; tak (+20h), TAK-779 added twenty hours after infection. **p<0.001.

### 1.2 Vpx induces innate immune activation and increases cell death

Based on previous results suggesting HIV infection induces an IFN-mediated antiviral response in primary macrophages, where SAMHD1 might play a role (figure 25). We aimed to further study the role of SAMHD1 by evaluating the effects of Vpx-induced degradation of SAMHD1 and innate immune signatures.

Interestingly, recent data showed that both Vpx and Vpr also counteract an intracellular transcriptional repressor, the HUSH complex[203], by specifically targeting for proteasomal degradation TASOR, one of its components. TASOR has been described to limit retrotransposons and retroviruses[204,205], affect transcriptionally active genes by epigenetic silencing[127] and induce replication and reactivation of HIV/SIV[206]. These data add an additional layer of complexity to the study of Vpx induced changes in the context of HIV infection and merited further evaluation.

Taking this into account, we characterized innate immune activation and IFN-mediated responses in primary macrophages treated with Vpx by whole transcriptome profiling. Our results demonstrate enhanced innate immune activation following Vpx treatment and linked to the degradation of both SAMHD1 and TASOR. Moreover, further characterization of Vpx-induced immune activation led to the description of its effects in both acute and latent HIV infections.

#### 1.2.1 Vpx-SIVmac triggers an IFN-mediated response in primary macrophages

To evaluate the transcriptional effects of Vpx on primary macrophages, we performed whole transcriptome profiling of Vpx-treated and non-treated MDM in two different donors.

Each donor was either transduced or not with HIV-2 Vpx for 12h before RNA extraction and transcriptional analysis was performed. Presence or absence of SAMHD1, a known target of Vpx, was confirmed and validated by western blot in all conditions (data not shown).

The expression level of 41 genes was significantly different between the two conditions (fold change ≥2, figure 26A), 27 upregulated genes and 14 downregulated genes (figure 26B, Addendum table 1 ) represented on the heat map (figure 26C). Interestingly, 14 upregulated genes (52% of the upregulated genes) were found to be IFN-related suggesting a link between Vpx function and IFN-mediated immune response in primary macrophages.

Three upregulated genes *CXCL10* (p=0.0006), *IFNB1A* (p=0.0019) and *IRF7* (p=0.0063) were validated in additional donors (figure 26D).

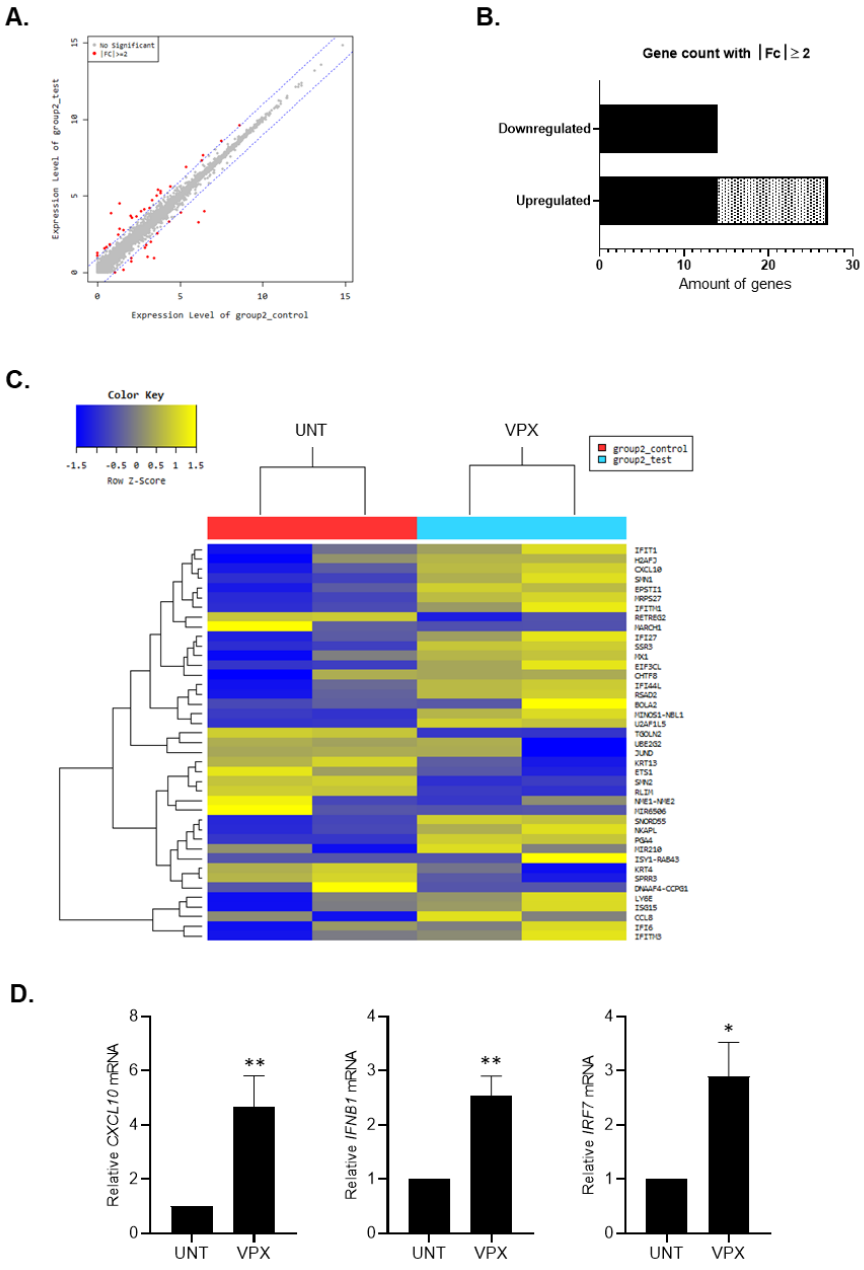


**Figure 26. Vpx-treated primary macrophages significantly upregulate IFN-stimulated genes (ISG).** (A) Scatter plot showing expression level between groups analysed. Correlation of gene expression between control and test groups. Red dots represent >2-fold change (FC) and grey dots indicate <2-fold change in gene expression levels between untreated and VPX-treated groups. (B) Representation of total number of genes significantly upregulated and downregulated after vpx-transduction. 27 genes were upregualted >2-fold change, of which 52% are interferon inducible genes (dotted region). 14 genes were downregualted >2-fold change. (C) Heatmap shows result of hierarchical clustering analysis (Euclidean Method, Complete Linkage) which clusters genes and samples similarity by expression level (normalized value) from significant list. 41 genes were significantly expressed after vpx-transduction, upregulated (yellow) and downregulated (blue) with >2-fold change in genes expression. Group control (in red, left side) with untreated macrophages and test group (in blue, right side) treated with VPX. Two independent donors were used. (D) Upregulation of *CXCL10, IFNB1* and *IRF7* gene expression was confirmed in additional donors after RNAseq analysis. Data represents mean±SD of three independent experiments. *p<0,01; **p<0,001.

### 1.2.2. Innate immune activation in primary macrophages is dependent on Vpx-SIVmac lineage

To investigate the contribution of SAMHD1 into the observed innate immune activation, we selected two different Vpx (vpx-SIVmac251 and -SIVrcm), based on their distinct capacity to degrade cellular factors SAMHD1 and TASOR[203,206] and explore their role in mediating an antiviral IFN response in primary macrophages.

Vpx-SIVmac VLP induces degradation of SAMHD1 and TASOR (figure 27C), whereas Vpx-SIVrcm or heat inactivated Vpx-SIVmac or Vpx-SIVrcm VLPs do not affect SAMHD1 or TASOR protein expression levels (figure 27C).

Primary macrophages transduced with Vpx-SIVmac VLP show significant increase in expression of different ISGs: *ISG15* (figure 27A, p=0,0205)*, CXCL10* (figure 27A, p=0.0046); *IFI27* (figure 27A, p<0.0001); RNA sensor *DDX58* (figure 27A, p<0.0001); RNA sensor *IFIH1* (figure 27A, p=0.0002); DNA sensor *cGAS* (figure 27A, p=0.0367); and transcriptional factor *IRF7* (figure 27A, p=0.0071) compared to GFP-VLP or untreated control. *CXCL10* was further confirmed by quantification of IP-10 by ELISA, as a secreted chemokine in the supernatant of cultured cells (figure 27B, p= 0,0168).

In contrast, transduction with Vpx-SIVrcm VLP did not significantly alter gene expression in any ISGs or immune players (RNA and DNA sensors or transcriptional factors) compared to GFP-VLP or untreated control (figure 27A). Similarly, heat inactivated Vpx-SIVmac (hiSIVmac) and Vpx-SIVrcm (hiSIVrcm) VLPs, did not affect gene expression of ISGs or immune players when compared to VLP-GFP or untreated control (data not shown), suggesting that SAMHD1 might have a role in triggering expression of IFNs and ISGs in MDMs.

Protein expression confirmed gene expression data, i. e., transduction with Vpx-SIVmac VLP increased expression of innate immune RNA sensors MDA5 and RIG-I (figure 27C) and phosphorylation of STAT1 (figure 27C), compared to GFP-VLP or untreated control.

It is well described that Vpx-mediated degradation of SAMHD1 enhanced HIV-1 replication, due to the increased in intracellular dNTPs availability for RT. On the other hand, increased IFN and ISG might induce an antiviral state putatively blocking viral infection of untransduced bystander cells. To test whether enhanced ISG expression is affecting viral replication, antiviral activity of supernatants collected from Vpx-treated MDMs was measured. Supernatants from Vpx-SIVmac or

Vpx-SIVrcm treated cells did not alter HIV susceptibility of MT4 cells (data not shown), suggesting limited antiviral properties of IFN expression triggered after Vpx transduction.

Thus, although a degree of innate immune activation was observed after Vpx-SIVmac transduction, it does not affect viral replication. It is important to note that innate immune activation only occurred in Vpx-SIVmac treated cells, suggesting a role of proteins targeted to degradation by VPX-SIVmac, SAMHD1 and TASOR in regulating the innate immune activation in MDMs.

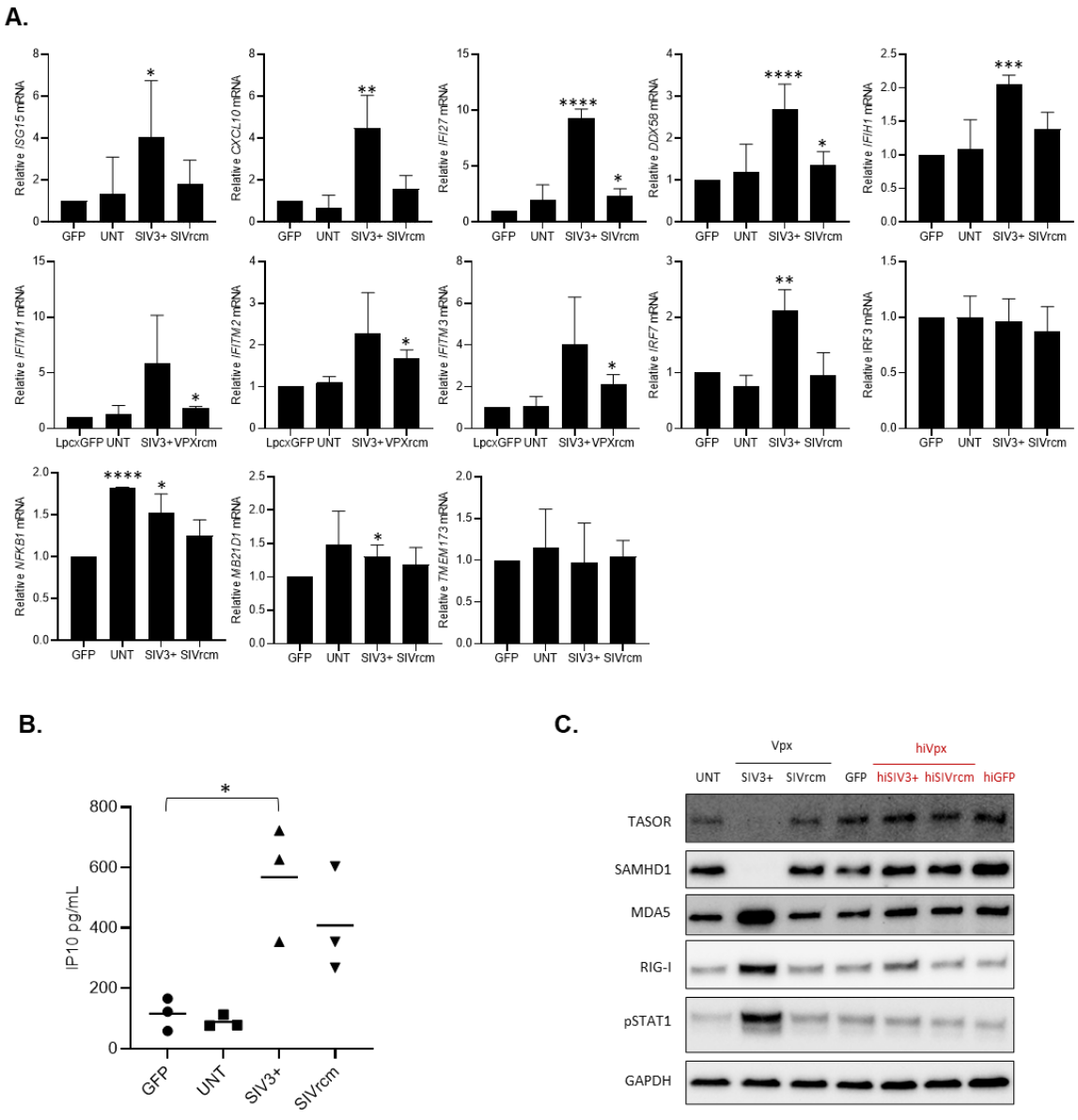


**Figure 27. Vpx triggers an IFN-stimulated response in MDMs.** (A) ISG induction by Vpx-SIV3+ from SIVmac251. Relative gene expression of *ISG15, CXCL10, IFI27, DDX58, IFIH1, IFITM1, IFITM2, IFITM3, IRF7, IRF3, NFKB1, MB21D1, TMEM173* 24h-post trasduction with VLP-vpx. Expression measured by quantitative PCR and normalized to GAPDH expression. Data represents mean±SD of at least 3 different donors and it is normalized

to VLP-GFP transduced M-CSF macrophages. (B) IP10 induction by vpx from SIVmac251. Quantification of IP10 in the supernatant of transduced M-CSF macrophages 24h-post transduction with VLP-vpx. Total quantification represents mean of two duplicates from three independent experiments evaluated. (C) Protein expression of MDMs transfected with different VLP-vpx. Western blot of TASOR, SAMHD1, RNA sensors MDA5 and RIG-I, and phosphorylated STAT1 (pSTAT1). Vpx from SIVmac251 induces degradation of TASOR and SAMHD1, induces RNA sensors MDA5 and RIG-I, and induces pSTAT1 24h-post transfection. GAPDH was used as loading control. A representative donor is shown. * p<0,01; ** p<0,001; *** p<0,0001; **** p<0,00001.

### 1.2.3 SAMHD1 or TASOR alone does not recapitulate IFN-response induced by Vpx transduction.

To determine the relative contribution of SAMHD1 and TASOR in immune activation by Vpx-SIVmac, we downregulated either SAMHD1 (siSAMHD1) or TASOR (siTASOR) by RNA interference in MDMs.

Downregulation of *SAMHD1* and *FAM208* (TASOR) was confirmed at mRNA and protein levels (figure 28A and D, p<0.0001) compared to non-targeting siRNA or mock-transfected controls.

Downregulation of si*SAMHD1* or siTASOR did not affect IFN (*IFNB1)* or ISGs gene expression *IFI27, ISG15* or *IRF7* (figure 28B), nor expression of innate immune RNA sensors *DDX58* (RIG-I) *and IFIH1*(MDA5)*,* or DNA sensors *MB21D1* (cGAS) and *TMEM73* (STING) (figure 28C) compared to non-targeting or mock controls. siSAMHD1 and siTASOR in MDMs show no changes in protein levels of RNA sensors (RIG-I or MDA5) or phosphorylation of STAT1, compared to non-targeting or mock controls (figure 28D).

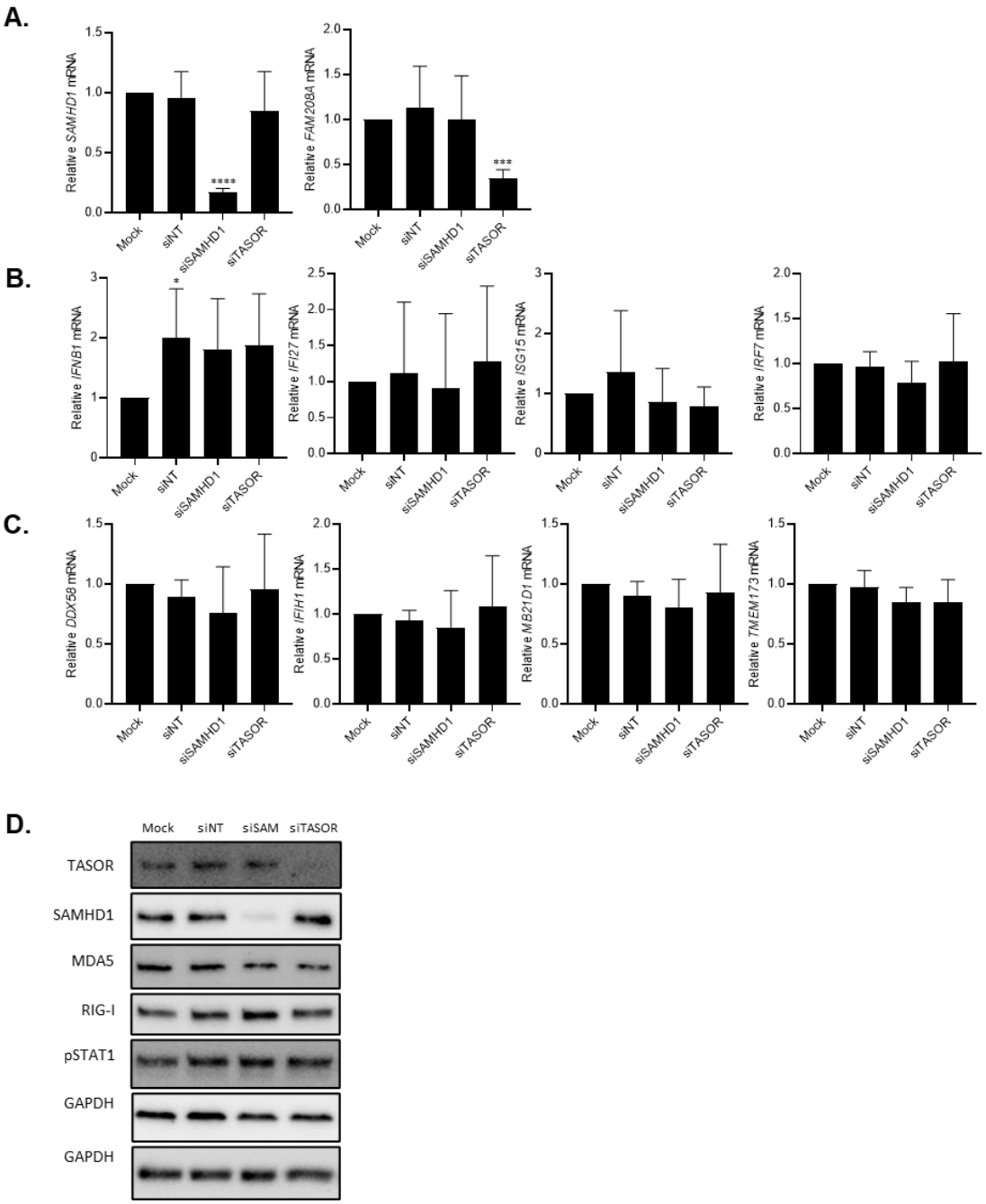


**Figure 28. SAMHD1 and TASOR single downregulation does not trigger IFN-mediated innate immune response in MDMs.** (A, B, C) Gene expression of *SAMHD1, FAM208A, IFNB1, IFI27, ISG15, IRF7, DDX58, IFIH1, MB21D1* and *TMEM173* in knockdown macrophages. Relative mRNA expression was measured by quantitative PCR and normalized to GAPDH expression. Data represents mean±SD of at least 5 different donors and it is normalized to mock-transfected M-CSF macrophages. (D) Protein expression in SAMHD1 and TASOR knockdown macrophages. Western blot of TASOR, SAMHD1, RNA sensors MDA5 and RIG-I, phosphorylation of STAT1 (pSTAT1) in siRNA-treated M-CSF macrophages. Complete knockdown was achived and no changes in protein expression is seen in siSAMHD1 and siTASOR knockdown macrophages compared to the corresponding non-targeting siRNA (siNT) or mock-transfected macrophages. GAPDH was used as loading control. A representative donor is shown. Mock; siNT-siRNA-Non Target; siSAMHD1 siRNA-SAMHD1; siTASOR siRNA-FAM28A; * p<0,01; **** p<0,00001.

### 1.2.4. Vpx-SIVmac degrades TASOR and reactivates JHIG latent cells.

NF-κB transcriptional factor has been extensively described as a transcriptional regulator of the inflammatory response, and other processes such as cell survival, proliferation, and differentiation (Reviewed in[207–215]). Indeed, Vpx-SIVmac-treated cells show an increase in phosphorylation of NF-κB p65 (Ser536) (data not shown), which indicates activation of the NF-κB pathway and overall induction of cell transcription processes.

Taking into account the role of the HUSH complex in gene transcription and its putative role in inducing transcription of latent HIV, we evaluated the effect of Vpx transduction in a latent model of HIV infection. First, the reactivation capacity of Vpx-SIVmac and Vpx-SIVrcm was assessed in a non-clonal HIV latency cell line model that expresses GFP upon transcription and do not express SAMHD1 (J-HIG) (figure 29A).

As a positive control, 0.4μM of panobinostat (PNB), a known LRA, was used in the reactivation assays. Panobinostat was able to reactivate J-HIG 3.75-fold (figure 29B, p=0.0001) compared to untreated condition or heat inactivated lentivirus, which loses its ability to degrade TASOR (figure 27C). As expected, transduction with Vpx-SIVmac VLP moderately reactivates JHIG 1.2-fold higher (figure 29B, p=0.0028) compared to untreated condition (UNT) or heat inactivated lentivirus (hiVpx-SIVmac) as previously reported[203]. In contrast, transduction with Vpx-SIVrcm VLP, a Vpx that does not degrade TASOR (figure 27C), did not significantly reactivate J-HIG (figure 29A) compared to untreated condition (UNT) or heat inactivated lentivirus (hiSIVrcm).

These results suggest that provirus reactivation in SAMHD1-negative non-clonal HIV latency cell line model depends on degradation of TASOR and its effect upon transcription.

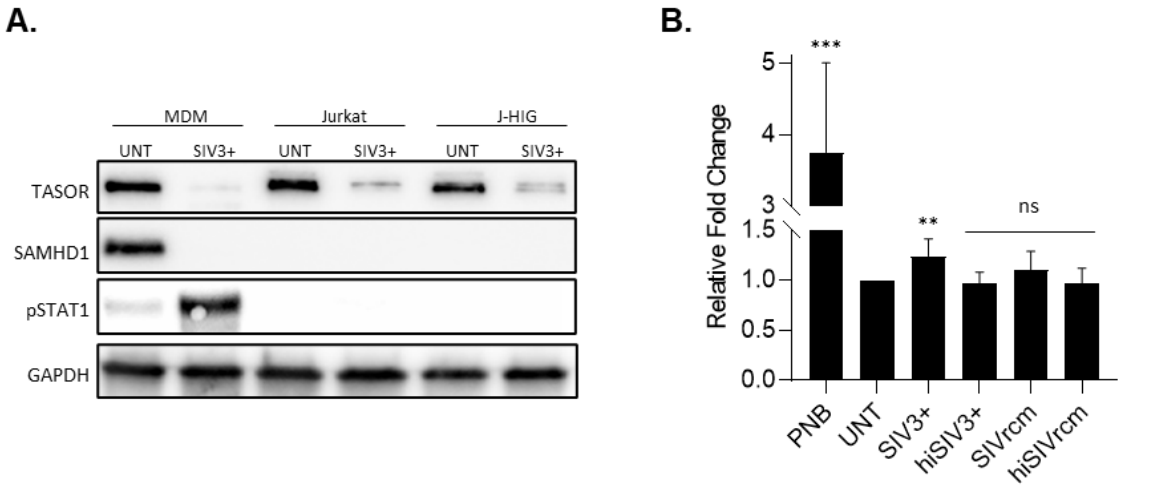


**Figure 29. VLP-vpx from SIVmac251 induces reactivation in JHIG (SAMHD1-) latency cell model.** (A) Protein expression of TASOR, SAMHD1 and pSTAT1 in MDM, Jurkat and J-HIG cells. VPX downregulates expression of TASOR and SAMHD1, and induces phosphorylation of STAT1 in cells expressing basal protein levels. Cells were treated for 24h. GAPDH was used as loading control. A representative donor is shown. (B) Vpx from SIVmac251 transduction induces reactivation of HIV provirus in JHIG cell line model 24h-post transduction. Reactivation in JHIG cells (SAMHD1-) only depend on TASOR downregulation by vpx. Relative fold change in reactivation is calculated by measuring GFP percentage relative to untreated condition (UNT). Mean ±SD of 7 independent experiments were performed in duplicates. PNB panobinostat 0,4µM; UNT, untreated; SIV3+; VLP-vpx from SIVmac251; hiSIV3+, VLP-vpx from SIVmac251 denaturated; SIVrcm, VLP-vpx from SIVrcm; hiSIVrcm, VLP-vpx from SIVrcm denaturated; ns, not significant; ** p>0,001; *** p>0,0001.

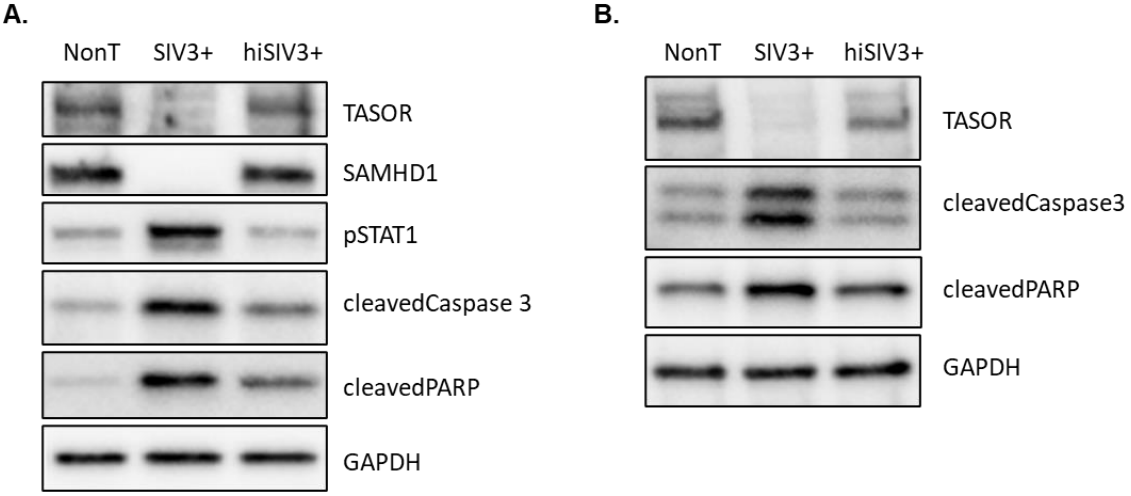


**Figure 30. VPX from SIVmac251 induces markers of apoptosis in macrophages and latent cell line model.** Protein expression of MDMs (A) and JHIG (B) treated with chaetocin and transduced with different VLP-vpx. Western blot of TASOR, SAMHD1, phosphorylated STAT1 (pSTAT1), cleaved Caspase 3 and cleaved PARP. Vpx from SIVmac251 induces cleavage of Caspase 3 and PARP apoptotic markers in both cell lines compared to NonT. In (A) MDMs increase pSTAT1 and degrade TASOR and SAMHD1 compared to NonT. In JHIG (B) degrades TASOR compared to NonT. GAPDH was used as loading control. A representative donor is shown for each western blot. NonT Non-transduced condition; SIVmac251 VLP-VPX from SIVmac251; hiSIVmac251 VLP-VPX from SIVmac251 heat inactivated.

### 1.2.5. Vpx from SIVmac251 lentivirus induces apoptosis in primary macrophages and JHIG CD4+ T latency cell model

Given the fact that Vpx-SIVmac251 affects immune inflammatory genes, promotes transcription and expression of provirus Vpx might also influence cell processes that could trigger cell death mechanisms. Therefore, we investigated whether the induction of an innate immune response, reactivation of HIV latent cells and induction of transcription was correlated with an increase in cell death[188,216]. Thus, we evaluated expression of apoptosis cell markers to identify the relationship between apoptotic cell markers expression and presence of antiviral signaling (as reviewed in[216]).

MDMs and J-HIG were pre-treated with chaetocin, a stimulator of oxidative stress to induce apoptosis and cell cycle arrest[217] before transduction with Vpx-SIVmac, VLP-heat inactivated Vpx-SIVmac (hiVpx-SIVmac) with non-functional Vpx and a non-transduced (NonT) condition as a reference for basal levels of apoptotic markers.

As expected MDMs transduced with Vpx-SIVmac show no expression of TASOR or SAMHD1 (figure 30A)[125,187,203], but induces phosphorylation of STAT1 and cleavage of caspase 3 and PARP (figure 30A), two markers of apoptosis, compared to non-transduced condition (NonT) or heat inactivated Vpx-SIVmac (hiSIVmac).

Similarly, transduction of Vpx-SIVmac251 in latently infected JHIG cells[203,206] (figure 29A) also induced expression of cleaved caspase 3 and PARP (figure 30B), compared to non-transduced condition (NonT) or heat inactivated Vpx-SIVmac (hiSIVmac).

These results suggest that SAMHD1 and TASOR mediated degradation by Vpx induces apoptosis putatively as a consequence of induction of innate immune activation

# CHAPTER 2. EVALUATION OF ADAR1 ROLE IN VIRAL INFECTIONS

## Summary

The identification of SAMHD1 as an HIV restriction factor represented a major breakthrough for a better comprehension of virus-host interactions. Mutations in SAMHD1 were first identified as causative of AGS; however after its recognition as an HIV restriction factor, SAMHD1 function and regulation has been the center of interest to virologist and immunologists. Interestingly,
other genes linked to AGS syndrome such as TREX1, have also been linked to. AGS syndrome, pointing towards a novel pathway that might influence HIV pathogenesis and infection outcome.

Based on this, this chapter of the thesis was dedicated to the study and characterization of AGS-linked genes in the context of HIV replication. We identified two genes, *ADAR1* and *IFIH1* as putatively relevant factors affecting replication of distinct viruses, including HIV, HCV and HPV.

## 2.1 Evaluation of the contribution of genes associated to AGS as cell factors affecting HIV replication

First, the contribution of genes linked to AGS on HIV infection and replication was assessed in loss of function studies. All genes with mutations associated to AGS and not previously associated to HIV infection were downregulated by siRNA in primary macrophages, including *ADAR1, IFIH1, RNASEH2B* and *RNASEH2C.*

Significant downregulation of mRNA expression was achieved for each of the genes (figure 31A) relative to mock treated cells (Mock). Interestingly, a significant inhibition of HIV-1 replication was observed only in ADAR1 downregulated cells (80% inhibition; p<0.0001), when compared to infected mock-transfected cells. No effect was observed for the other genes evaluated (figure 31B).

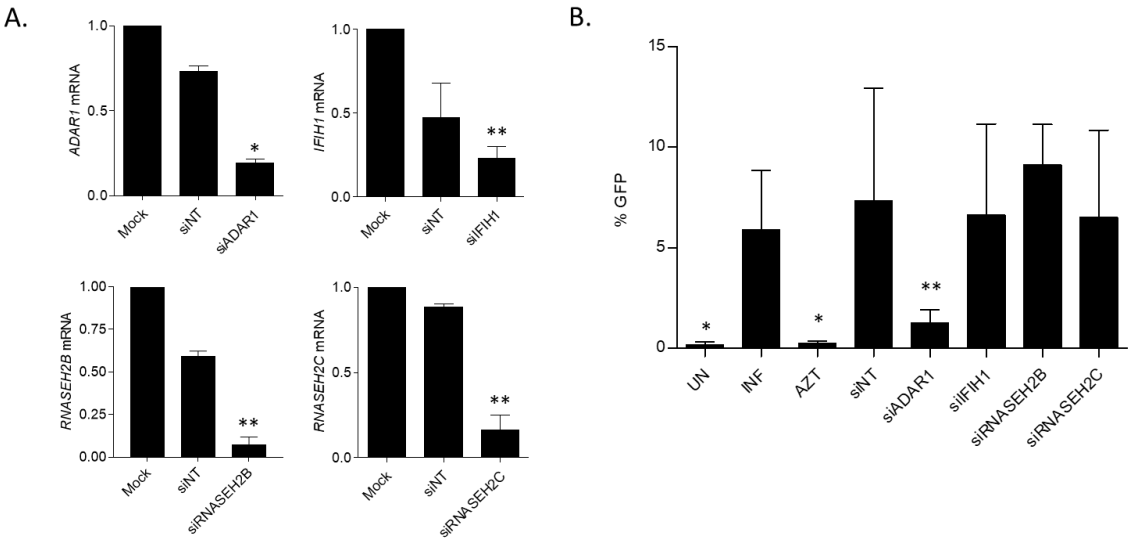


**Figure 31. Screening of AGS genes by siRNA and HIV susceptibility.** (A) Downregulation of AGS genes: ADAR1, IFIH1, RNASEH2B and RNASEH2C. All genes were effectively downregulated using siRNAs. Expression was measured by quantitative real time PCR at day 4 post-differentiation. Data was normalized to untreated cells. Mean ± SD of at least 2 independent donors is shown. *p<0.0004; **p<0.0058. (B) Percentage of HIV-1 infection in siRNA treated MDM. Cells where infected at day 4 post-differentiation and infection was assessed 48h later measuring GFP+ cells by flow cytometry. Mean ± SD of at least 3 independent donors is shown. Mock are UN, INF and AZT. siNT; non-targeting siRNA, UN; uninfected, INF; infected, AZT; zidovudine at 1µM. *p<0.0027; **p<0.00297.

Based on these screening data and due to the known relationship between IFIH1 and ADAR1[98,99], we focused our attention to the evaluation and characterization of *ADAR1* and IFIH1 function in viral infections.

## 2.2 RNA editing by ADAR1 regulates innate and antiviral immune function in primary macrophages

We aim to determine the role of *ADAR1* as a regulator of innate immune activation and modifier of HIV susceptibility. First, we identified and characterized the innate immune signaling pathway regulated by ADAR1 function and assessed ADAR1 role in the HIV-1 replication cycle.

### 2.2.1. ADAR1 function regulates type I IFN and innate immune activation in primary macrophages

To investigate the regulation of the innate immune response we used monocyte derived macrophages. Effective and specific downregulation of *ADAR1* and *IFIH1* (MDA5) was achieved at both mRNA and protein level in macrophages (figure 32A and B). ADAR1 downregulation led to a significantly enhanced expression of the cytosolic RNA sensor MDA5, both at mRNA (5.5-fold change, $p = 0.0001$) and protein levels (figure 32A and B), as previously suggested[99]. ADAR1 knockdown macrophages also showed increased production of IFNβ (7.5-fold change, $p = 0.0388$, figure 32C) and the IFN stimulated gene (ISG) CXCL10, measured by mRNA expression (50-fold change, $p = 0.049$, figure 32D) and as secreted chemokine in the culture supernatant (2.6-fold change, $p = 0.01$, figure 32D). In addition, increased phosphorylation of STAT1 (pSTAT1), another well-recognized marker of type I IFN induction, was observed in ADAR1 knockdown macrophages (figure 32B). In contrast, no effect was seen in MDA5 knockdown macrophages (figure 32A–D). Importantly, confirmatory siRNA sequences targeting ADAR1 showed similar effects in activation of type I IFN response (figure 32E). These data are suggestive of a role of ADAR1 as a negative regulator of innate immune response in primary macrophages.

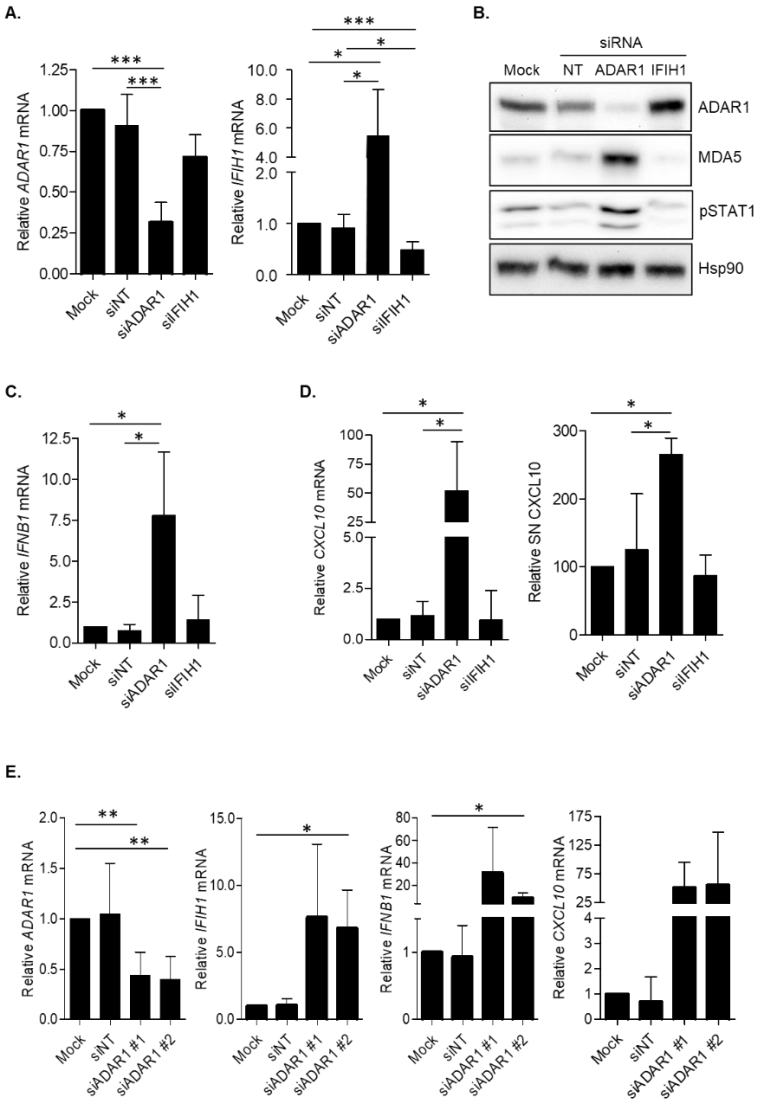


**Figure 32. ADAR1 knockdown boosts type I IFN response in primary macrophages.** (A) Gene expression of ADAR1 (left panel) and IFIH1 (right panel) knockdown macrophages. Relative mRNA expression of ADAR1 and IFIH1 was measured by quantitative PCR and normalized to GAPDH expression. Data represents mean ± SD of 5 different donors and is normalized to mock-transfected M-CSF macrophages. (B) Protein expression in ADAR1 and IFIH1 knockdown macrophages. Western blot of ADAR1, MDA5 and phosphorylation of STAT1 (pSTAT1) in siRNA-treated M-CSF macrophages. MDA5 and pSTAT1 are increased in ADAR1 knockdown macrophages compared to the corresponding non-targeting siRNA (NT). Hsp90 was used as loading control. A representative donor is shown. (C) Relative mRNA expression of IFNβ in siRNA-treated macrophages measured by quantitative PCR and normalized to GAPDH expression. IFNB1 gene expression was significantly enhanced in ADAR1 knockdown macrophages. Data represents mean ± SD of 5 different donors and is normalized to mock-transfected M-CSF macrophages. (D) CXCL10 mRNA (left panel) and protein expression in the supernatant (right panel) in siRNA treated macrophages. Relative mRNA expression of CXCL10 was measured by quantitative PCR and normalized to GAPDH expression. CXCL10 protein in the culture supernatants was measured by ELISA. (E) Effective downregulation of *ADAR1* by two different siRNA (left panel) and relative mRNA expression of *IFIH1*, *IFNB1* and *CXCL10* in ADAR1 knockdown macrophages. Relative mRNA expression was measured by quantitative PCR and normalized to GAPDH expression. Data represents mean ± SD of 3 different donors and is normalized to Mock-transfected macrophages. * p<0.05; ** p<0.005; *** p<0.0005.

### 2.2.2. ADAR1 downregulation blocks HIV-1 transcription in primary macrophages

The role of ADAR1 on HIV-1 susceptibility of primary macrophages was evaluated by testing the capacity of siRNA-treated macrophages to support HIV-1 replication. Significant inhibition of HIV-1 replication was seen in ADAR1 knockdown macrophages either using a VSV-pseudotyped single cycle GFP-expressing HIV-1 (roughly 75% inhibition, $p = 0.0001$, figure 33A) or a full replicative R5 HIV-1 BaL (roughly 85% inhibition, $p = 0.0001$, figure 33B). Confirmatory siRNA sequences targeting ADAR1 showed comparable inhibition of HIV-1 replication (figure 33D). Conversely, inhibition of *IFIH1* (MDA5) expression did not have any effect on HIV-1 infection (figure 33A and B). Thus, we investigated the specific step of viral replication cycle affected by ADAR1 knockdown. For this purpose, the levels of total viral DNA, integrated proviral DNA and multispliced viral transcripts were quantified by qPCR. No significant changes were observed in proviral DNA formation or integration in *ADAR1* or *IFIH1* knockdown macrophages (figure 33C, left and middle panels). As expected, the HIV-1 reverse transcriptase inhibitor AZT completely blocked viral DNA formation, while the HIV-1 integrase inhibitor raltegravir (RAL) did not have any effect on viral DNA formation, but inhibited viral integration (figure 33C, left and middle panels). Conversely, HIV-1 transcription was significantly inhibited in ADAR1 knockdown macrophages ($p=0.0007$, figure 33C, right panel), indicating that ADAR1 function is necessary for appropriate HIV-1 transcription.

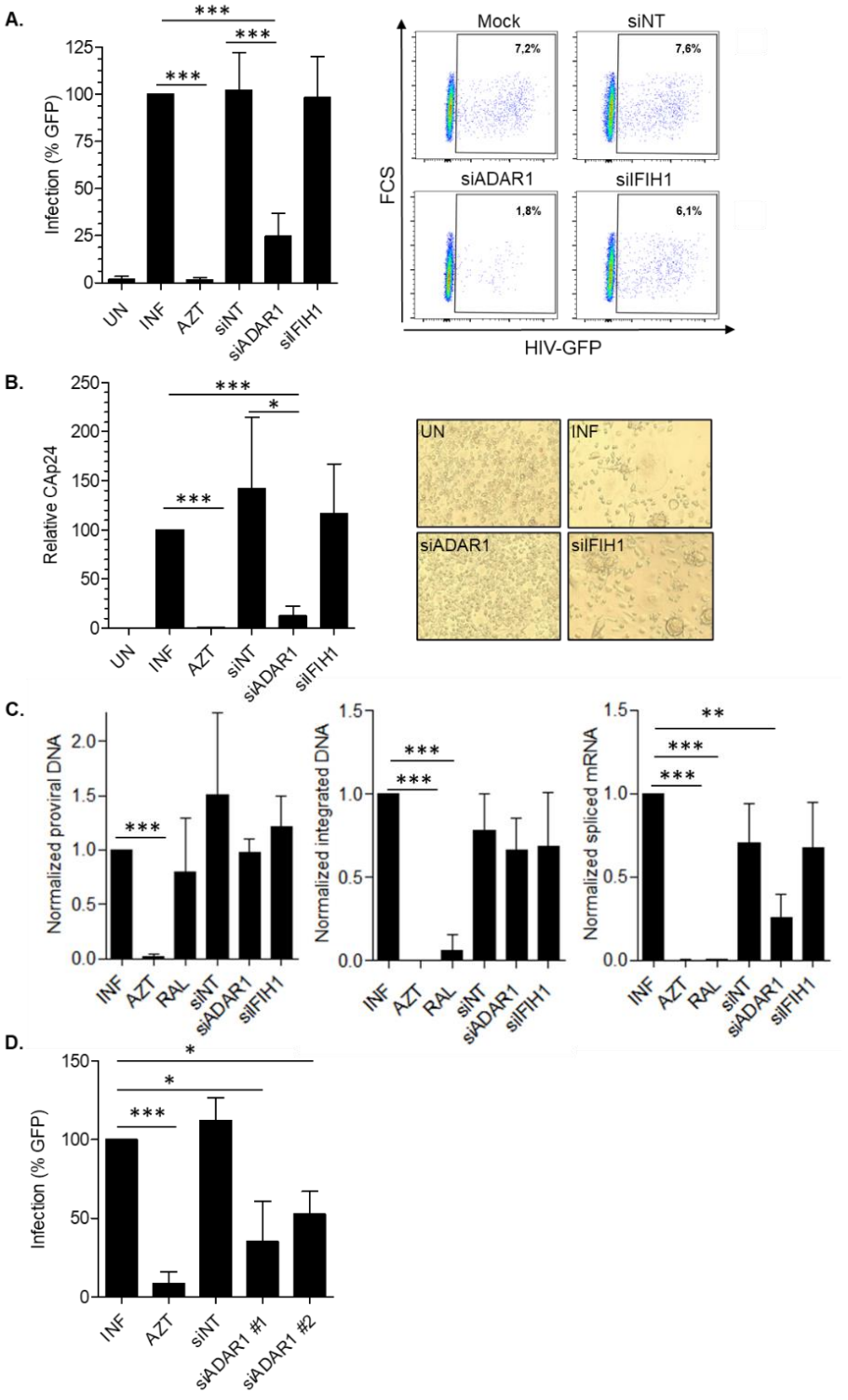


**Figure 33. ADAR1 knockdown blocks HIV-1 transcription in primary macrophages.** (A) HIV-1 replication in ADAR1 and IFIH1 knockdown M-CSF macrophages, infected with a VSV-pseudotyped, GFP-expressing HIV-1. Infection was measured 72 h later by flow cytometry. Data represent percentage replication relative to mock transfected macrophages (left panel). A representative flow cytometry dot plot showing infected macrophages is also depicted (right panel). Mean ± SD of at least 5 different donors performed in triplicate is shown. (B) HIV-1 replication in ADAR1 and IFIH1 knockdown M-CSF macrophages, infected with a full

replicative HIV-1 BaL strain. Differentiated macrophages were infected for 7 days and CAp24 production was measured in culture supernatant by ELISA. Mean ± SD of 3 different donors performed in triplicate is shown. (C) Proviral DNA formation (left panel), viral integration (middle panel) and viral transcription (right panel) in ADAR1 and IFIH1 knockdown macrophages. siRNA treated and subsequently differentiated macrophages were infected with HIV-1 BaL for 16 h (for proviral DNA formation), 20 h (for viral DNA integration) or 40 h (for viral transcription) before DNA or RNA extraction. All determinations were normalized to mock-treated infected macrophages and AZT (3μM) or raltegravir (RAL; 2μM), were included as controls. Mean ± SD of at least 3 different donors is shown. (D) HIV-1 replication in ADAR1 knockdown by two different siRNA in M-CSF macrophages, infected with a VSV-pseudotyped, GFP-expressing HIV-1. Data represent percentage replication relative to mock-transfected macrophages. Mean ± SD of 3 different donors performed in triplicate is shown. In all panels, isolated monocytes were transfected with the corresponding siRNA and differentiated to macrophages with M-CSF for 4 days, prior to infection with HIV-1. *p<0.05; **p<0.005; ***p<0.0005.

ADAR1 may be affecting viral replication by directly A-to-I editing of HIV-1 mRNAs. However, no A-to-I editing was detected in five different HIV-1 mRNA predicted editing sites, while editing of cellular mRNAs was clearly seen in siNT macrophages but not in ADAR1 knockdown macrophages (figure 34), demonstrating that ADAR1 is functional in primary macrophages. Besides its catalytic function, ADAR1 can also serve an editing-independent role that relies on the inhibition of PKR, an IFN-induced protein reported to inhibit mRNA translation[218]. As expected, PKR expression was enhanced in ADAR1 knockdown macrophages (figure 35A), validating previous data[218]. However, PKR knockdown did not have any effect on HIV-1 replication or innate immune function (figure 35B-D).

Altogether, these results demonstrate that direct A-to-I posttranscriptional editing of viral mRNA or PKR induction may not be the underlying mechanisms of ADAR1 effect on HIV replication; pointing towards the regulation of innate immune function as the key process affecting viral replication.

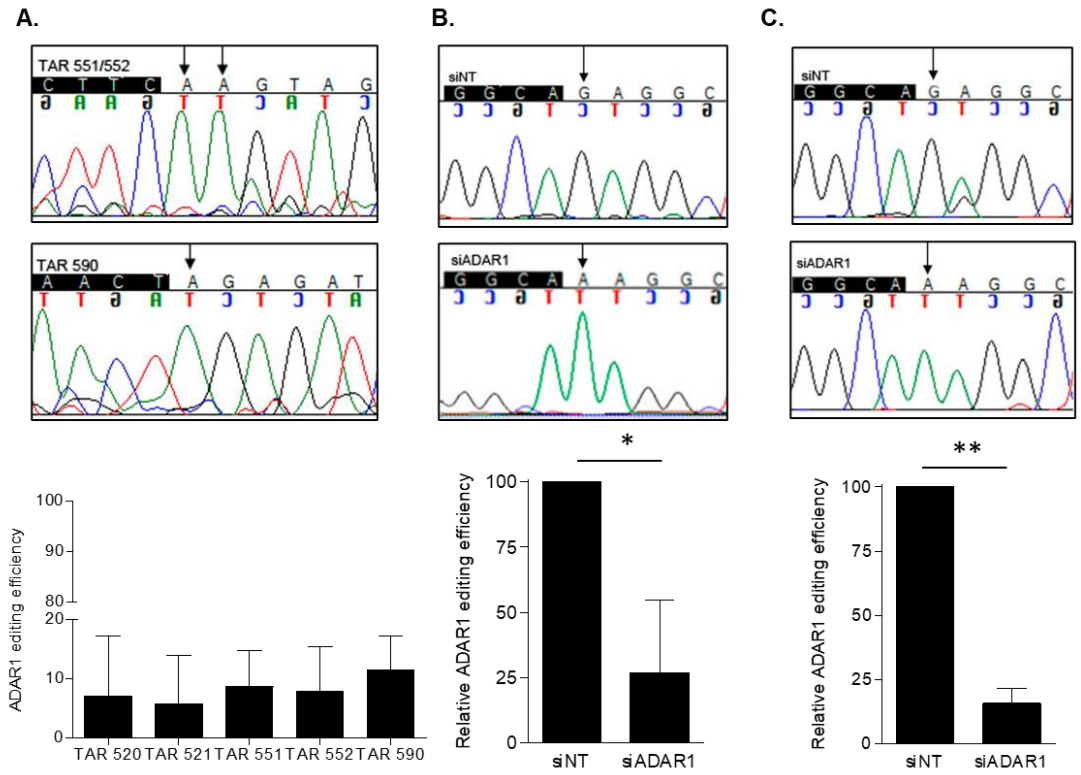


**Figure 34. ADAR1 efficiently edits host *NEIL1* transcript, but does not edit HIV-1 *TAR* transcript.** (A) Representative DNA sequencing chromatogram of RT-PCR products of 5' UTR HIV-1 mRNA transcript and the estimated percentage of editing efficiency at positions TAR520/521, TAR551/552 and TAR590. (B-C) Representative DNA sequencing chromatograms of of *NEIL1* transcripts in M-CSF (B) and GM-CSF (C). MDMs of siNT and siADAR1, and relative ADAR1 editing efficiency at edited site. 5' UTR HIV-1 could not be amplified in si*ADAR1* sample due to restricted infection. Edited Adenosines to Inosine are detected as G by direct sequencing. *p<0.05; **p<0.0005.

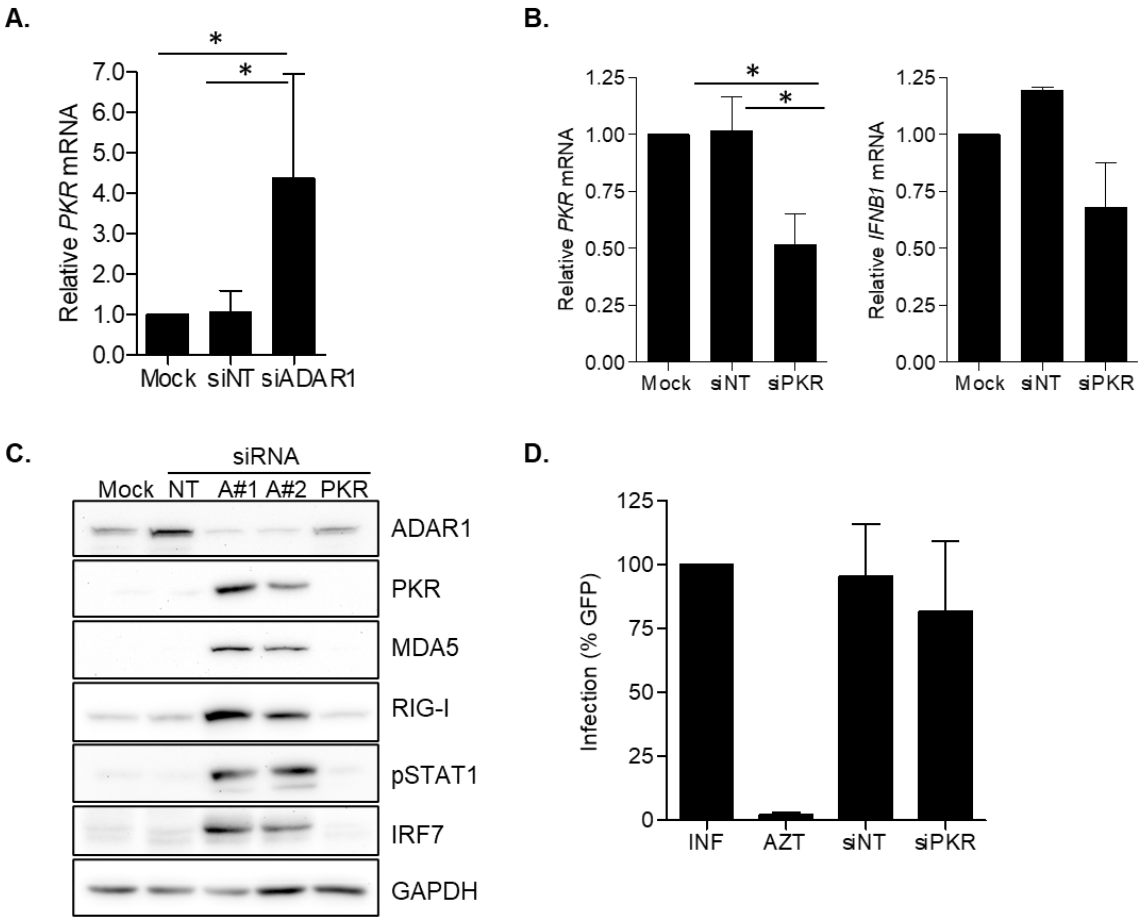


**Figure 35. PKR knockdown by siRNA does not affect HIV-1 replication or innate immune function** (A) Relative PKR mRNA expression is enhanced in ADAR1 knockdown macrophages. (B) Effective downregulation of PKR by siRNA does not affect mRNA expression of *IFNB1*. (C) Western blot showing PKR downregulation and innate immune proteins in PKR knockdown macrophages. GAPDH was used as loading control. A representative donor is shown. (D) HIV-1 replication in PKR knockdown M-CSF macrophages, infected with a VSV-pseudotyped, GFP-expressing HIV-1. Data represent percentage replication relative to mock-transfected macrophages. Mean ± SD of 3 different donors performed in triplicate is shown. mRNA expression data represents mean ± SD of 3 different donors and is normalized to Mock-transfected macrophages. * $p<0.05$.

### 2.2.3. ADAR1-mediated innate immune activation and block of HIV-1 infection is specific of macrophages

M-CSF macrophages represent an *in vitro* model of an HIV-1 target cell, relevant for innate immune function. However, the innate immune system is dependent on other cell types. Thus, we aimed at investigating the role of ADAR1 in other primary cells from the myeloid compartment and CD4+ T-cells. Protein expression of ADAR1, MDA5 and phosphorylation of STAT1 (pSTAT1) were evaluated in M-CSF and GM-CSF monocyte-derived-macrophages, monocyte derived dendritic cells (moDC)

and resting and activated PBMCs. All cell types showed comparable expression of ADAR1, MDA5 and pSTAT1, except for the higher expression of ADAR1 and pSTAT1, observed in activated PBMCs (figure 36A). Then, ADAR1 expression was downregulated using siRNA and the effect on type I IFN expression and susceptibility to HIV-1 infection was evaluated. Effective and specific downregulation of ADAR1 was achieved in all cell types tested (GM-CSF macrophages, moDC and CD4+ T-cells, figure 3B–D, left panels). However, induction of MDA5 expression or phosphorylation of STAT1 after ADAR1 knockdown was only observed in GM-CSF macrophages, and slightly in CD4+ T cells, but not in other cell types (figure 36B-D, middle panels). Indeed, HIV-1 infection was only significantly inhibited in GM-CSF macrophages (roughly 60% inhibition, $p = 0.0019$, figure. 36B, right panel), whereas no change in HIV-1 susceptibility was seen in moDC or CD4+ T lymphocytes (figure 36C and D, right panels). Therefore, ADAR1-mediated innate immune regulation appears to be especially relevant in macrophages, but not in other cell types.

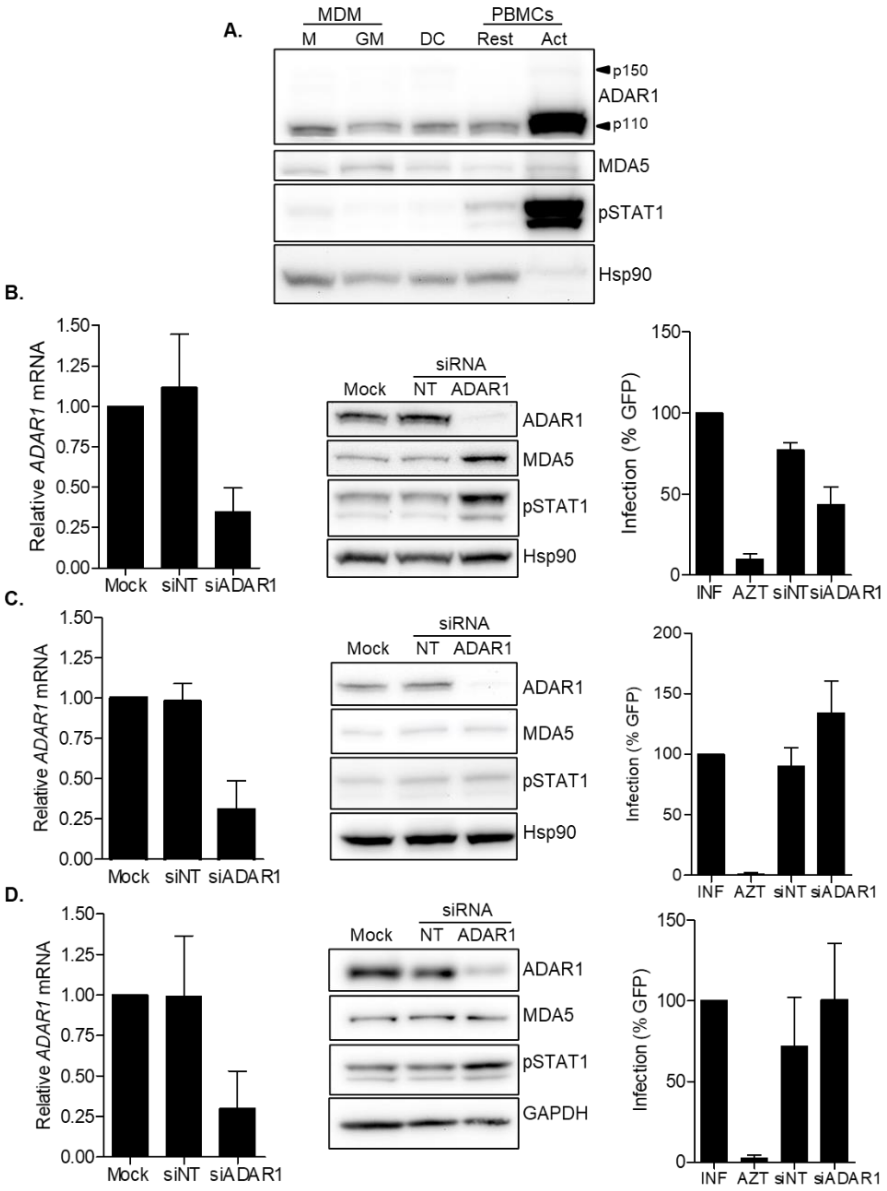


**Figure 36. ADAR1-mediated regulation of innate immune activation and HIV-1 infection is specific of macrophages.** ADAR1-mediated regulation of innate immune activation and HIV-1 infection is specific of macrophages. (a) Evaluation of ADAR, MDA5 and pSTAT1 protein expression in different myeloid and lymphoid primary cells. ADAR1 and pSTAT1 expression were increased in activated PBMCs compared to other cell types. M; M-CSF macrophages, GM; GM-CSF macrophages, DC; monocyte derived dendritic cells, Rest; Resting PBMCs and Act, Activated PBMCs. Hsp90 was used as loading control. A representative experiment is shown. (b–d) Evaluation of ADAR1 knockdown in GM-CSF macrophages (b), monocyte-derived dendritic cells (c) and CD4+ T cells (d). Downregulation of ADAR1 mRNA expression by qPCR (left panels), western blot showing protein expression (middle panels) and susceptibility to HIV-1 infection (right panels) are shown. Effective ADAR1 mRNA inhibition was achieved in all cell types (B-D, left panels). Upregulation of MDA5 and pSTAT1 was only observed in ADAR1 knockdown GM-CSF macrophages (b, middle panel), which correlated with inhibition of HIV-1 replication (B, right panel). No significant differences were observed in protein expression or HIV-1 infection in dendritic cells or CD4+ T cells, following ADAR1 knockdown (C and D, middle and right panels respectively). Data from mRNA expression and HIV-1 infection represent the mean ± SD of 3 different donors. A representative western blot is shown in each case. *p<0.05; **p<0.005.

### 2.2.4. ADAR1 is a negative regulator of the RIG-I like receptor (RLRs)-MAVS signaling pathway

As demonstrated above, ADAR1 is a cofactor of HIV-1 replication in primary macrophages which presumably acts as a negative regulator of innate immune response. Therefore, we aimed to further explore the intracellular signaling pathway that leads to innate immune activation following ADAR1 knockdown.

ADAR1 knockdown led to a significant increase in the RNA sensors *IFIH1* (MDA5) and *DDX58* (RIG-I) and the interferon regulatory factor IRF7, compared to mock transfected macrophages or macrophages treated with a non-targeting siRNA (siNT) ($p = 0.0129$ and $p = 0.0024$ respectively, figure 37A). Conversely, no changes in mRNA expression of DNA cytosolic sensors cGAS and STING or the downstream effectors MAVS, TBK1 and IRF3 were observed (figure 37A). There was a clear correlation between ADAR1 knockdown and overexpression of MDA5, RIG-I, IRF7 protein expression and the phosphorylation of STAT1 (figure 37B, 35C). Stimulation of macrophages with LPS or poly I:C showed a similar pattern of protein overexpression (figure 37C and 38D), suggesting that ADAR1 is a regulator of the RLRs-MAVS canonical pathway of innate immune activation leading to type I IFN production.

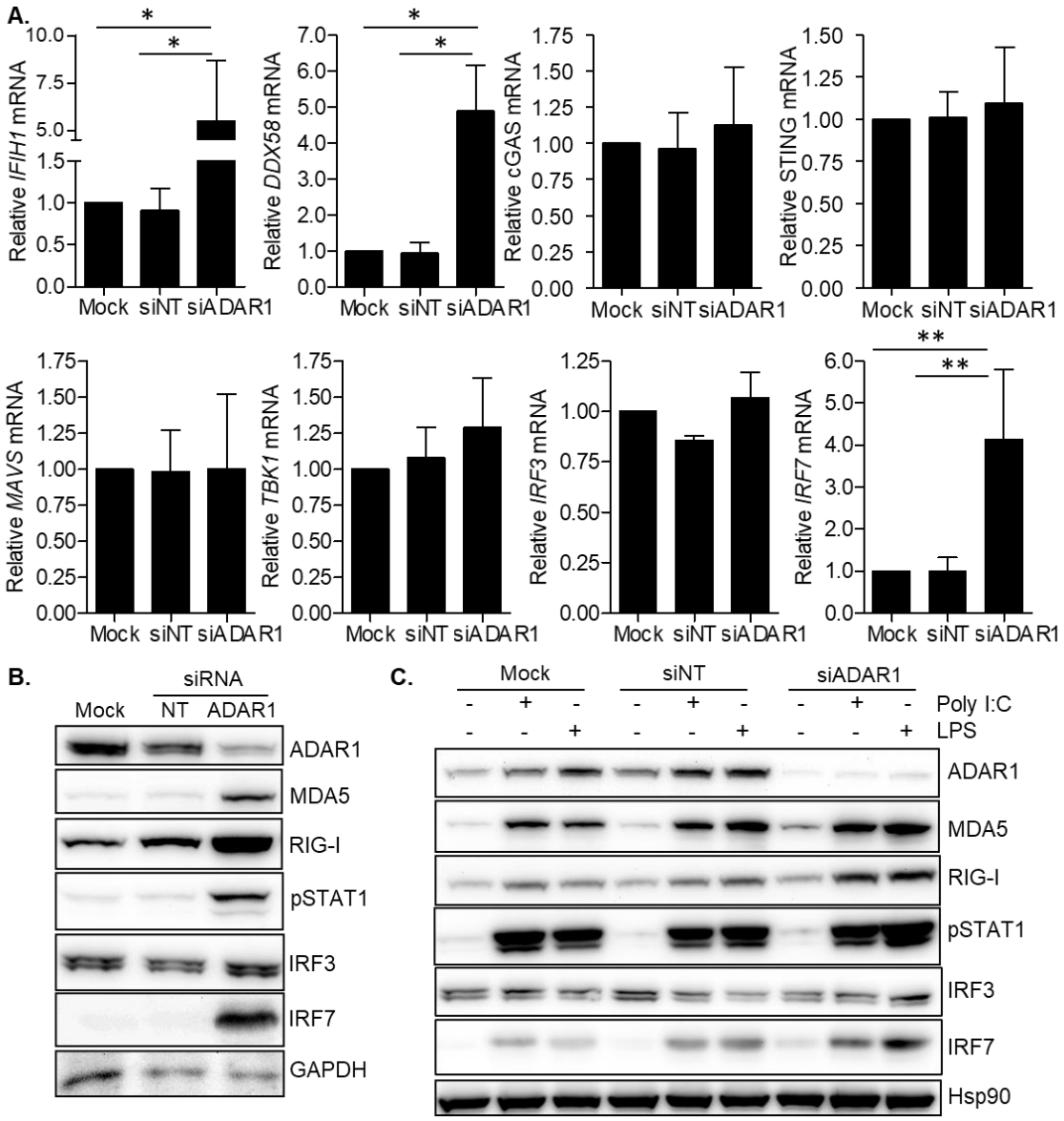


**Figure 37. ADAR1 specifically regulates de RLRs-MAVS signaling pathway.** ADAR1 specifically regulates the RLRs-MAVS signaling pathway. (a) Relative mRNA expression of RLRs (IFIH1 and DDX58), DNA sensors (cGAS and STING), downstream signaling proteins (MAVS, TBK1) and transcription factors (IRF3 and IRF7) in ADAR1 knockdown macrophages. Data represents mean ± SD of at least 4 different donors and is normalized to Mock-transfected macrophages. *p<0.05; **p<0.005. (b) Protein expression of RLRs and related proteins in macrophages, showing overexpression of MDA5, RIGI, pSTAT1 and IRF7 consequence of ADAR1 inhibition. A representative donor is shown. (c) Western blot showing protein expression pattern in transfected macrophages, stimulated or not with LPS (100ng/ml) or Poly I:C (10µg/ml) to resemble canonical TLR-mediated activation of type I IFN response. A representative donor is shown. *p<0.05; **p<0.005.

MRT67307, an inhibitor of IKKϵ and TBK1, has been shown to prevent the phosphorylation of IRF3 and the production of IFNβ in macrophages[219]. Treatment of ADAR1 knockdown macrophages with MRT67307 limited the effects of ADAR1 depletion by significantly reducing overexpression of IRF7 and pSTAT1 and to a lesser extent RLR (figure 38A), and its effect was dose-dependent (figure 38D).

As expected, a decrease in the amount of secreted CXCL10 in siADAR1 macrophages in the presence of MRT67307 was also observed (figure 38B), indicating that MRT67307 limits innate immune activation associated to ADAR1 downregulation. Moreover, although MRT67307 was able to partially inhibit HIV-1 infection in mock or siNT macrophages, this was not observed in ADAR1 knockdown cells (figure 38C). These data suggest that ADAR1 function specifically regulates de RLRs-MAVS signaling pathway and that innate immune activation consequence of ADAR1 inhibition is the responsible of the block in HIV-1 replication.

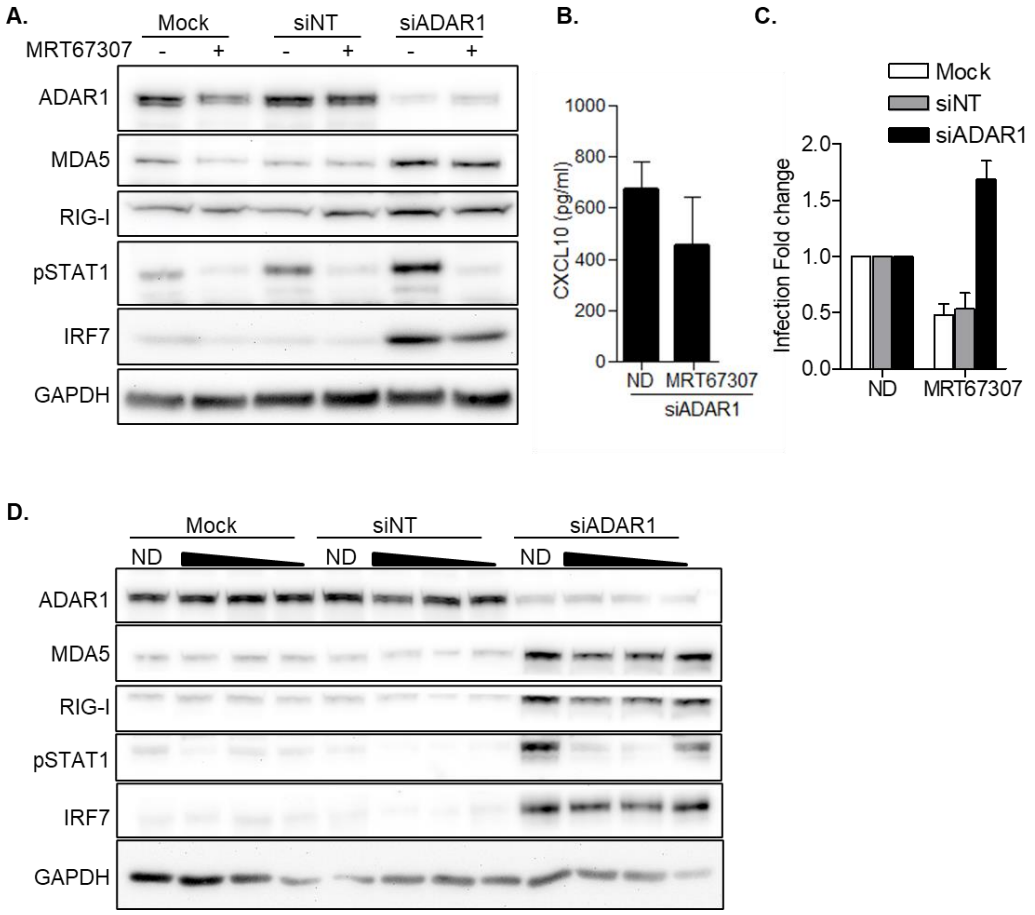


**Figure 38. Blockade of TBK1 function restores protein expression phenotype observed in ADAR1 expressing macrophages.** (A) Protein expression in ADAR knockdown macrophages treated with the TBK1 inhibitor MRT67307 (5µM). Blocking TBK1 function partially restores protein expression phenotype observed in Mock- or siNT transfected macrophages. A representative donor is shown. (B) CXCL10 protein expression in the supernatant in ADAR1 knockdown macrophages, treated or not with MRT67307 (5µM). CXCL10 protein in the culture supernatants was measured by ELISA. Data represents mean ± SD of 3 different donors. (C) Change in HIV-1 replication of macrophages treated with the TBK1 inhibitor MRT67307 (5µM). Fold change of HIV-1 infection in macrophages treated or not with MRT67307. Infection is normalized to the corresponding untreated condition. Data represents mean ± SD of 3 different donors performed in triplicate. (D) Protein expression in ADAR knockdown macrophages treated with growing concentrations of the TBK1 inhibitor MRT67307 (5 µM, 1 µM, 0.2 µM and 0.04 µM). A representative donor is shown. ND, no drug. *p<0.05; **p<0.005.

### 2.2.5. Immune activation in siADAR1 macrophages is pro-inflammatory and inhibits HIV replication in bystander macrophages

Once transcriptional activation of type I IFN genes has taken place, IFN and cytokines are produced and secreted. Secreted IFNs may act as autocrine and paracrine factors and initiate a tissue-wide, systemic signaling through the cellular Jak-STAT pathway, inducing the transcription of hundreds of ISG[220]. Therefore, the type of cytokines produced by ADAR1 knockdown macrophages was further characterized and their paracrine function in terms of antiviral activity was evaluated.

We evaluated the contribution of IFNβ to ADAR1-mediated inhibition of HIV-1 replication in the presence of a blocking antibody targeting IFNβ. However, no change in HIV-1 susceptibility was observed in ADAR1 knockdown macrophages, in mock-transfected macrophages or macrophages transfected with a non-targeting siRNA (figure 39D). However, apart from *IFNB1*, four other genes (*IFNG, IL12A, IL1A and IL6*) showed enhanced expression in ADAR1 knockdown macrophages (at least 2.5 fold difference compared to siNT-treated macrophages, figure 39A), an effect that was further confirmed in six additional donors (figure 39B). Upregulated cytokines are suggestive of a pro-inflammatory macrophage phenotype with an inhibitory effect on HIV-1 replication. Thus, antiviral activity of culture supernatants from transfected macrophages was evaluated in primary macrophages. Culture supernatants from ADAR1 knockdown macrophages inhibited HIV-1 replication in a dose-dependent manner (roughly 35% inhibition at highest concentration, $p = 0.05$, figure 39C), whereas no effect was seen in mock or siNT transfected macrophages. Although the inhibitory capacity of supernatants from ADAR1 knockdown macrophages is modest compared to ADAR1 depletion, these results indicate that pro-inflammatory cytokines from ADAR1 knockdown macrophages are secreted and can function as paracrine factors that contribute to HIV-1 susceptibility.

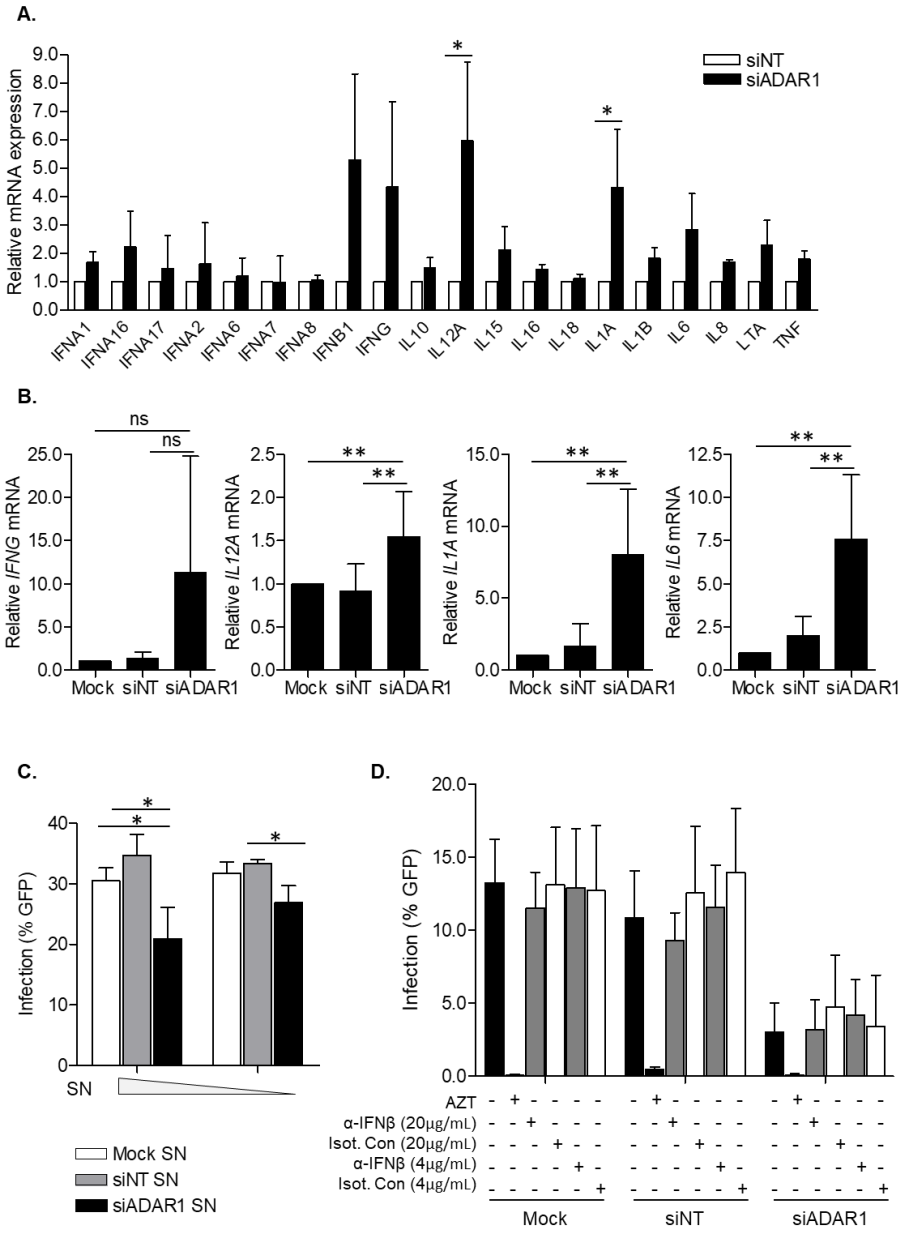


**Figure 39. ADAR1 knockdown macrophages present a pro-inflammatory phenotype that inhibits HIV-1 replication of bystander cells and it is not affected by secreted IFNβ.** (A) Cytokine expression profile of ADAR1 knockdown macrophages. Relative mRNA expression of the different cytokines normalized to siNT-transfected macrophages is depicted. Data represents mean ± SD of 3 different donors. (B) Relative mRNA expression of cytokines identified as upregulated in ADAR1 knockdown macrophages (IFNG, IL12A, IL1A and IL6). Data represents mean ± SD of 6 independent donors and is normalized to mock-transfected macrophages. (C) Antiviral activity of culture supernatants from ADAR1 knockdown macrophages. Percentage infection of untreated macrophages incubated with different concentrations of culture supernatants from mock- (white bars), siNT (grey bars) and siADAR1- (black bars) treated macrophages. (D) Percentage HIV-1 infection in ADAR1 knockdown macrophages, untreated (black bars) or treated with different concentrations of anti-IFNβ1 (grey bars) or an isotype control (white bars). No change in HIV-1 susceptibility was observed neither in ADAR1 nor in mock-transfected macrophages or macrophages transfected with a non-targeting siRNA. Data represents mean ± SD of 3 different donors performed in triplicate. SN; supernatant, ns; non-significant, *p<0.05, **p < 0.005.

## 2.3 ADAR1 is a regulator of innate and antiviral immune function in HCV infection

Given the identification of ADAR1 role during an HIV infection and the fact that ADAR1 has been associated to other viral infections as a proviral or antiviral factor, we further evaluated ADAR1 role in the context of HCV / HIV coinfections. In this section, we describe ADAR1 role as a regulator of innate and antiviral immune function in HCV infection, both *in-vitro* and in patients.

First, we performed a genetic association study including of *ADAR1* polymorphysms in a cohort of HIV/HCV coinfected patients from our clinical unit. Then, we evaluated in *in-vitro* cell models the innate immune pathways regulated by ADAR1, in the context of HCV infection, in an attempt to further elucidate the role of ADAR1 in HCV/HIV coinfection.

### 2.3.1 Polymorphisms in ADAR1 are associated to HCV infection progression in HCV/HIV-1 coinfected patients.

The IFN-alpha pathway is crucial in achieving HCV viral clearance and thus, genes involved in this pathway may alter disease progression and treatment response, including ADAR1.

A cohort of 155 patients with chronic HCV/ HIV-1 coinfection, who had a standard course of treatment with PEGIFNα/Ribavirin (RBV) with known virological response status at 24 weeks post-therapy were included in this study. Cohort characteristics are summarized in table 7 and 8. Treatment success (i. e. sustained-IFN viral response, SVR) was defined as undetectable HCV RNA in plasma 24 weeks after finishing therapy. Severe fibrosis was considered in patients with a METAVIR score≥F3. HCV viral load, HCV genotype, HIV-1 viral load, CD4+ T cell count and transaminases levels were determined with conventional methods as described before [221]. The protocol for the study was approved by the scientific committee of Hospital Universitari Germans Trias I Pujol. Written informed consent was obtained from each patient who participated in the study. All methods were carried out in accordance with relevant guidelines and regulations and to the ethical principles suggested in the Declaration of Helsinki.

HCV/HIV coinfected patients were genotyped for ADAR1 polymorphisms rs2229857 and tested for association to treatment response. rs2229857 codes for a nonsynonymous substitution, K384R, located in exon 2, between the third double-stranded RNA (dsRNA)-binding domain (dsRBD) and the first ZDNA-binding region. ADAR1 rs2229857 SNP was significantly associated to IFN plus ribavirin SVR in males (adjusted p-value=0.007, T risk allele), suggesting that ADAR1 may have a role in HCV

infection outcome and response to treatment (table 9). ADAR1 SNPs were also significantly associated to advanced fibrosis in females (table 10, adjusted p-value=0.001, T risk allele). Association results were replicated with an additional SNP rs1127326, which is in complete linkage disequilibrium with rs2229857 (data not shown), i. e., rs2229857 alleles are non-randomly associated to rs1127326 alleles.

**Table 7. Clinical characteristics of 155 HCV/HIV-1 coinfected patients with known response to the IFN/RBV therapy.**

| | Sustained-IFN viral response | Non-Sustained IFN viral response | p-value |
|---|---|---|---|
| **Patients, n (%)** | 60 (38,7) | 95 (61,3) | - |
| **Age, yr (median, IQR)** | 47,5 (45-52) | 47 (44-49,5) | 0.1245 |
| **Gender, n (%)** | | | |
| **Female** | 20 (33,3) | 27 (28,4) | |
| **Male** | 40 (66,6) | 68 (71,6) | 0.52 |
| **CD4 Absolute (cells/mm3) (mean±SEM)** | 587,6 ± 32,6 | 589,4 ± 27,34 | 0.97 |
| **HCV genotype, n (%)** | | | |
| **1** | 25 (41,6) | 59 (62,1) | |
| **3** | 27 (45) | 18 (18,9) | |
| **4** | 8 (13,3) | 18 (18,9) | **0.0023** |
| **Fibrosis stage, n (%)** | | | |
| **F0-F2** | 36 (60) | 59 (62.1) | |
| **F3-F4** | 18 (30) | 32 (33.7) | |
| **Unknown** | 6 (10) | 4 (4.2) | 0.82 |
| **ALT (U/L) (mean±SEM)** | 90.68 ± 11.16 | 89.43 ± 5.052 | 0.9088 |
| **AST (U/L) (mean±SEM)** | 60.48 ± 6.688 | 66.09 ± 3.406 | 0.4121 |
| **Baseline HCV RNA Viral load (IU/ml) log10 (median ,IQR)** | 5,84 (5,48-6,02) | 5,93 (5,7-6,33) | **0.0097** |
| **Undetectable HIV-1, n (%)** | 46 (76,6) | 79 (83,1) | 0.32 |
| **On Antiretroviral treatment, n (%)** | 58 (96,6) | 93 (97,9) | 0.64 |

CD4+, ALT, AST, unpaired t test; BL HCV VL, age, Mann-Whitney; HCV genotype, fibrosis, Gender, undetectable HIV-1, on ART, Chi-square test. Graph Pad Prism 4.0.

**Table 8. Clinical characteristics of HCV/HIV-1 coinfected patients with different liver fibrosis stages (n=147).**

| | | Non-cirrhosis (F0 to F3) | Cirrhosis (F4) | p-value |
|---|---|---|---|---|
| **Patients, n (%)** | | 130 (88.5) | 17(11.6) | - |
| **Age, yr (median, IQR)** | | 47 (44.5-50) | 47 (44.5-53) | 0.7284 |
| **Gender, n (%)** | | | | |
| | **Female** | 38 (29.2) | 6 (35.3) | |
| | **Male** | 92 (70.7) | 11 (64.7) | 0.6077 |
| **CD4+ Absolute (cells/mm3) (mean ± SEM)** | | 588.4 ± 22.57 | 570.7 ± 71.66 | 0.7931 |
| **HCV genotype, n (%)** | | | | |
| | **1** | 74 (56.9) | 7 (41.2) | |
| | **3** | 35 (26.9) | 6 (35.3) | |
| | **4** | 21 (16.1) | 4 (23.5) | 0.4641 |
| **ALT (U/L) (mean ± SEM)** | | 87.56 ± 5.87 | 103.9 ± 14 | 0.3378 |
| **AST (U/L) (mean ± SEM)** | | 61.78 ± 3.459 | 82.18 ± 13.39 | 0.0586 |
| **Baseline HCV RNA Viral load log10 (IU/ml) (median, IQR)** | | 5.94 (5.7-6.10) | 5.8 (5.55-6.6) | 0.9050 |
| **Undetectable HIV-1, n (%)** | | 103 (79.2) | 15 (88.2) | 0.58 |
| **On Antiretroviral treatment, n (%)** | | 128 (98.4) | 17 (100) | 0.61 |

CD4+, ALT, AST, unpaired t test; Baseline HCV RNA Viral Load, Age, Mann-Whitney; HCV genotype, Gender, undetectable HIV-1, on ART, Chi-square test (GraphPad Prism 4.0). Eight patients without liver fibrosis data were excluded from the analysis.

**Table 9. Association of ADAR1 rs2229857 SNP with Non-Sustained IFN viral response in HCV/HIV-1 coinfected patients treated with IFN plus Ribavirin.**

| Gene | SNP | Gender | Genetic model | Non-Sustained IFN viral Response | % | Sustained IFN Viral Response | % | OR | 95%CI | p-value | AIC | p-value2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | All (n=150) | **Codominant** | | | | | | | | | |
| | | | C/C | 44 | 47,3 | 27 | 47,4 | 1 | | 0,26 | 181,4 | 0,1 |
| | | | T/C | 31 | 33,3 | 25 | 43,9 | 1,5 | 0.67-3.38 | | | |
| | | | T/T | 18 | 19,4 | 5 | 8,8 | 0,56 | 0.16-1.93 | | | |
| | | | **Dominant** | | | | | | | | | |
| | | | C/C | 44 | 47,3 | 27 | 47,4 | 1 | | 0,67 | 181,9 | 0,83 |
| | | | T/C-T/T | 49 | 52,7 | 30 | 52,6 | 1,17 | 0.55-2.48 | | | |
| | | | **Recessive** | | | | | | | | | |
| | | | C/C-T/C | 75 | 80,6 | 52 | 91,2 | 1 | | 0,19 | 180,4 | 0,06 |
| | | | T/T | 18 | 19,4 | 5 | 8,8 | 0,47 | 0.14-1.53 | | | |
| | | | **log-Additive** | | | | | | | | | |
| | | | 0,1,2 | 93 | 62 | 57 | 38 | 0,92 | 0.54-1.55 | 0,74 | 181,9 | 0,45 |
| ADAR1 | rs2229857 | Male (n=104) | **Codominant** | | | | | | | | | |
| | | | C/C | 29 | 43,9 | 19 | 50 | 1 | | **0,021** | 121,7 | **0.0031*** |
| | | | T/C | 21 | 31,8 | 18 | 47,4 | 1,48 | 0.55-3.96 | | | |
| | | | T/T | 16 | 24,2 | 1 | 2,6 | 0,11 | 0.01-1.02 | | | |
| | | | **Dominant** | | | | | | | | | |
| | | | C/C | 29 | 43,9 | 19 | 50 | 1 | | 0,83 | 127,4 | 0,84 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T/C-T/T | 37 | 56,1 | 19 | 50 | 0,9 | 0.36-2.26 | | | |
| | **Recessive** | | | | | | | | | |
| | C/C-T/C | 50 | 75,8 | 37 | 97,4 | 1 | | **0.007*** | 120,3 | **0.0012*** |
| | T/T | 16 | 24,2 | 1 | 2,6 | 0,09 | 0.01-0.83 | | | |
| | **log-Additive** | | | | | | | | | |
| | 0,1,2 | 66 | 63,5 | 38 | 36,5 | 0,63 | 0.32-1.25 | 0,18 | 125,6 | 0,1 |
| Female (n=46) | **Codominant** | | | | | | | | | |
| | C/C | 15 | 55,6 | 8 | 42,1 | 1 | | 0,35 | 62,6 | 0,43 |
| | T/C | 10 | 37 | 7 | 36,8 | 1,68 | 0.37-7.58 | | | |
| | T/T | 2 | 7,4 | 4 | 21,1 | 5,51 | 0.43-71.16 | | | |
| | **Dominant** | | | | | | | | | |
| | C/C | 15 | 55,6 | 8 | 42,1 | 1 | | 0,27 | 61,5 | 0,47 |
| | T/C-T/T | 12 | 44,4 | 11 | 57,9 | 2,17 | 0.53-8.91 | | | |
| | **Recessive** | | | | | | | | | |
| | C/C-T/C | 25 | 92,6 | 15 | 78,9 | 1 | | 0,2 | 61,1 | 0,2 |
| | T/T | 2 | 7,4 | 4 | 21,1 | 4,36 | 0.38-50.36 | | | |
| | **log-Additive** | | | | | | | | | |
| | 0,1,2 | 27 | 58,7 | 19 | 41,3 | 2,08 | 0.71-6.05 | 0,16 | 60,7 | 0,26 |

*Bonferroni correction threshold is considered at 0.0083, correcting for strata and outcome (IFN viral response). Only patients with all clinical data available were included (n=150). P-value: p-value obtained from logistic regression adjusting by HCV genotype, log10 HCV RNA viral load and IL28B-rs12979860. P-value2: p-value obtained from logistic regression without adjustment.

**Table 10. Association of ADAR1 rs2229857 SNP with liver disease progression in HCV/HIV-1 coinfected patients.**

| Gene | SNP | Gender | Genetic model | Non-Cirrhosis (F0 to F3) | % | Cirrhosis (F4) | % | OR | 95%CI | p-value | AIC | p-value2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | All (n=142) | **Codominant** | | | | | | | | | |
| | | | C/C | 61 | 48,4 | 5 | 31,2 | 1 | | 0,32 | 109,1 | 0,27 |
| | | | T/C | 45 | 35,7 | 8 | 50 | 2,41 | 0.72-8.05 | | | |
| | | | T/T | 20 | 15,9 | 3 | 18,8 | 2,09 | 0.44-10 | | | |
| | | | **Dominant** | | | | | | | | | |
| | | | C/C | 61 | 48,4 | 5 | 31,2 | 1 | | 0,13 | 107,1 | 0,11 |
| | | | T/C-T/T | 65 | 51,6 | 11 | 68,8 | 2,31 | 0.74-7.19 | | | |
| | | | **Recessive** | | | | | | | | | |
| | | | C/C-T/C | 106 | 84,1 | 13 | 81,2 | 1 | | 0,67 | 109,2 | 0,81 |
| | | | T/T | 20 | 15,9 | 3 | 18,8 | 1,35 | 0.34-5.43 | | | |
| | | | **log-Additive** | | | | | | | | | |
| | | | 0,1,2 | 126 | 88,7 | 16 | 11,3 | 1,55 | 0.76-3.15 | 0,22 | 107,9 | 0,24 |
| ADAR1 | rs2229857 | Male (n=99) | **Codominant** | | | | | | | | | |
| | | | C/C | 40 | 44,9 | 5 | 50 | 1 | | 0,91 | 76,6 | 0,98 |
| | | | T/C | 34 | 38,2 | 3 | 30 | 0,78 | 0.17-3.68 | | | |
| | | | T/T | 15 | 16,9 | 2 | 20 | 1,17 | 0.19-7.13 | | | |
| | | | **Dominant** | | | | | | | | | |
| | | | C/C | 40 | 44,9 | 5 | 50 | 1 | | 0,88 | 74,7 | 0,99 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T/C-T/T | 49 | 55,1 | 5 | 50 | 0,9 | 0.24-3.45 | | | |
| | **Recessive** | | | | | | | | | |
| | C/C-T/C | 74 | 83,1 | 8 | 80 | 1 | | 0,77 | 74,7 | 0,87 |
| | T/T | 15 | 16,9 | 2 | 20 | 1,28 | 0.23-7.17 | | | |
| | **log-Additive** | | | | | | | | | |
| | 0,1,2 | 89 | 89,9 | 10 | 10,1 | 1,02 | 0.41-2.51 | 0,96 | 74,7 | 0,93 |
| Female (n=43) | **Codominant** | | | | | | | | | |
| | C/C | 21 | 56,8 | 0 | 0 | 1 | | **0.005*** | 38,3 | **0.008*** |
| | T/C | 11 | 29,7 | 5 | 83,3 | NA | NA | | | |
| | T/T | 5 | 13,5 | 1 | 16,7 | NA | NA | | | |
| | **Dominant** | | | | | | | | | |
| | C/C | 21 | 56,8 | 0 | 0 | 1 | | **0.001*** | 36,8 | **0,021** |
| | T/C-T/T | 16 | 43,2 | 6 | 100 | NA | NA | | | |
| | **Recessive** | | | | | | | | | |
| | C/C-T/C | 32 | 86,5 | 5 | 83,3 | 1 | | 0,84 | 46,7 | 0,82 |
| | T/T | 5 | 13,5 | 1 | 16,7 | 1,29 | 0.1-16.41 | | | |
| | **log-Additive** | | | | | | | | | |
| | 0,1,2 | 37 | 86 | 6 | 14 | 3,93 | 0.96-16.06 | **0,0411** | 42,6 | **0.008*** |

*Bonferroni correction threshold is considered at 0.0083, correcting for strata and outcome (cirrhosis). Only patients with fibrosis measurements were included (n=142) P-value: p-value obtained from logistic regression adjusting by HCV genotype, log10 HCV RNA viral load and IL28B-rs12979860. P-value2: p-value obtained from logistic regression without adjustment.

### 2.3.2. Differential expression of ADAR1 and RNA sensors determines HIV and HCV viral susceptibility in cell lines.

To investigate the differences in the innate immune signaling pathway that might be controlled by ADAR1, expression of ADAR1 and its related RNA sensors was evaluated in different cell lines, including HuH7.5 and its parental cell line HuH7 hepatoma cell that have been extensively used for evaluating HCV infection in vitro. ADAR1 total mRNA expression was relatively stable in all cell lines tested (figure 40A, left panel). However, MDA5 and RIG-I expression was significantly higher in HuH7 cells, compared to the others, including the hepatoma cell line HuH7.5 (figure 40A and B), which are known to be RIG-I null function-wise. Interestingly, a significant difference was also observed in IFNβ and CXCL10 expression in HuH7 cells compared to Huh7.5, suggesting higher innate immune activation and type I IFN production in HuH7 cells (figure 40C). Then, susceptibility to HCV viral infection was evaluated in the two hepatoma cell lines and as expected, HuH7.5 cells showed 100-fold higher susceptibility to HCV infection (figure 40D). Susceptibility to HIV-1 infection was also assessed obtaining similar results to HCV infection (figure 40E). Overall, these data suggest that innate immune effectors may be significantly modulating susceptibility to viral infection.

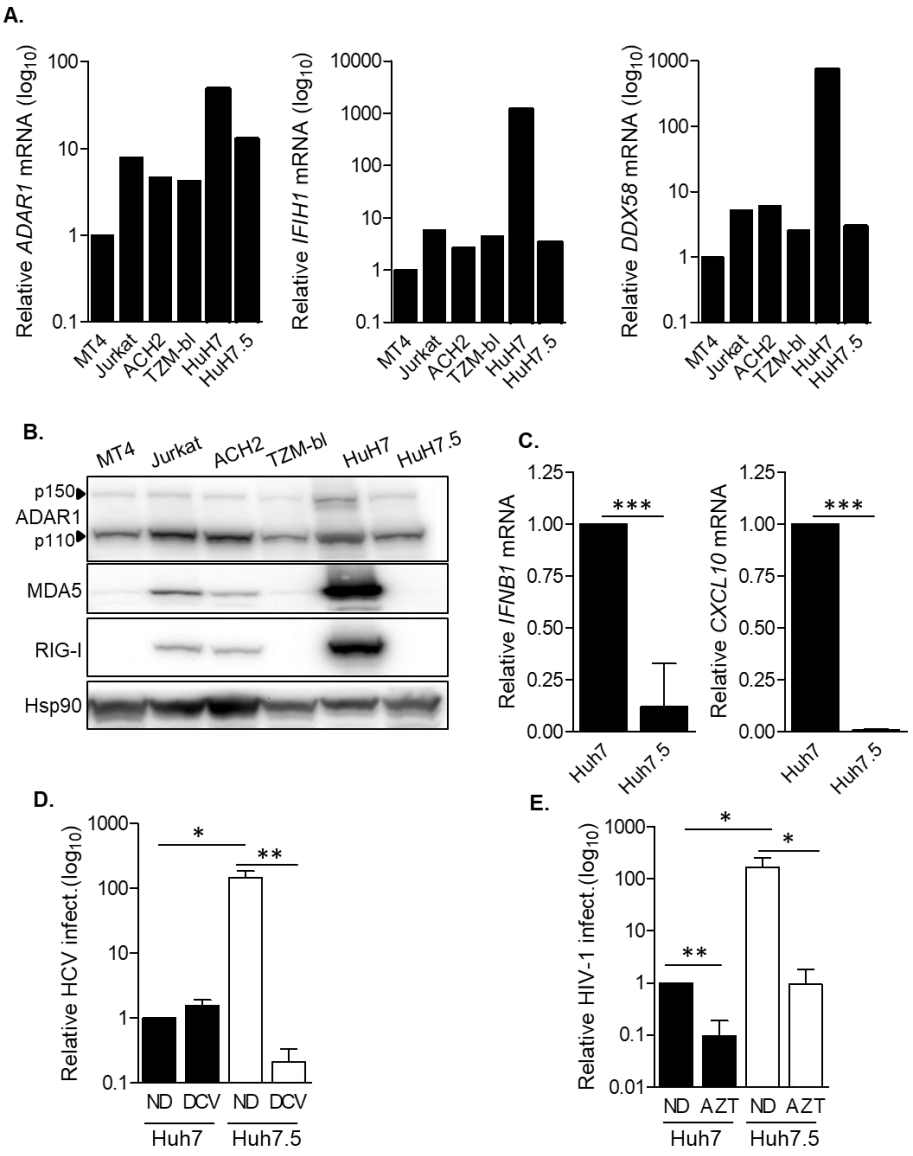


**Figure 40. Differential expression of ADAR1 and RNA sensors determines HIV and HCV viral susceptibility in cell lines.** (A) Relative mRNA expression of ADAR1, IFIH1 (MDA5) and DDX58 (RIG-I) in different cell lines measured by quantitative PCR and normalized to GAPDH expression. Data is normalized to MT4 cell line. (B) Protein expression of ADAR1, MDA5 and RIG-I in different cell lines. Significant expression differences were observed between HuH7 and HuH7.5 cell lines. A representative blot is shown. (C) Relative mRNA expression of IFNB1 and CXCL10 in hepatoma cells measured by quantitative PCR and normalized to GAPDH expression. IFNB1 and CXCL10 gene expression is significantly higher in Huh7 cells. Data represents mean ± SD of 3different determinations. (D) Susceptibility to HIV-1 infection in HuH7 and HuH7.5 cells. Relative HIV-1 infection measured as luciferase expression is shown. AZT, zidovudine (1 µM). Data represents mean ± SD of 3 different experiments performed in triplicate and is normalized to HuH7 cells. (E) Susceptibility to HCV infection in HuH7 and HuH7.5 cells. Relative HCV infection measured by quantitative PCR is shown. DCV, daclatasvir (25 nM). Data represents mean ± SD of 3 different experiments performed in triplicate and is normalized to HuH7 cells. *p<0.05; **p<0.005; ***p<0.0005.

### 2.3.3. ADAR1 is a negative regulator of innate immune pathway in the HuH7 hepatoma cell line.

In contrast to Huh7.5 cells that express undetectable levels of the RNA sensors RIG-I and MDA5, HuH7 cells have a preserved expression of innate immune effectors, a feature that may determine the outcome of HCV infection. Therefore, the role of ADAR1 and MDA5 was further investigated in HuH7 cells. Effective and specific downregulation of ADAR1 and MDA5 was achieved in HuH7 cells (figure 41A). ADAR1 downregulation was linked to increased IFIH1 (MDA5) and DDX58 (RIG-I) expression as described for macrophages in the previous chapter (figure 41A, middle and right panels), whereas no significant change was observed in MDA5 knockdown cells or when using a non-targeting control (figure 41A, left and middle panels). In addition, ADAR1 knockdown in HuH7 cells correlated also with increased production of IFNβ and increased expression of the IFN stimulated gene (ISG) CXCL10 (figure 41B). Importantly, the increase in IFN stimulated genes consequence of ADAR1 knockdown was also observed at protein level (figure 41C), showing enhanced expression of RIG-I and increased phosphorylation of IRF7, indicative of its activation as the transcription factor responsible for IFN production. We did not observe changes in IRF3 expression or phosphorylation. Increased RIG-I expression was also observed in MDA5 knockdown cells, suggesting a coordinated regulation between both RNA sensors. Conversely, no effect was seen when ADAR1 was inhibited in cells that do not express RNA sensors MDA5 and RIG-I such as Huh7.5 cells (data not shown). Overall, these data indicate that ADAR1 regulates innate immune activation and IFN production in hepatocyte-like cells and thus, can have an effect on HCV viral infection.

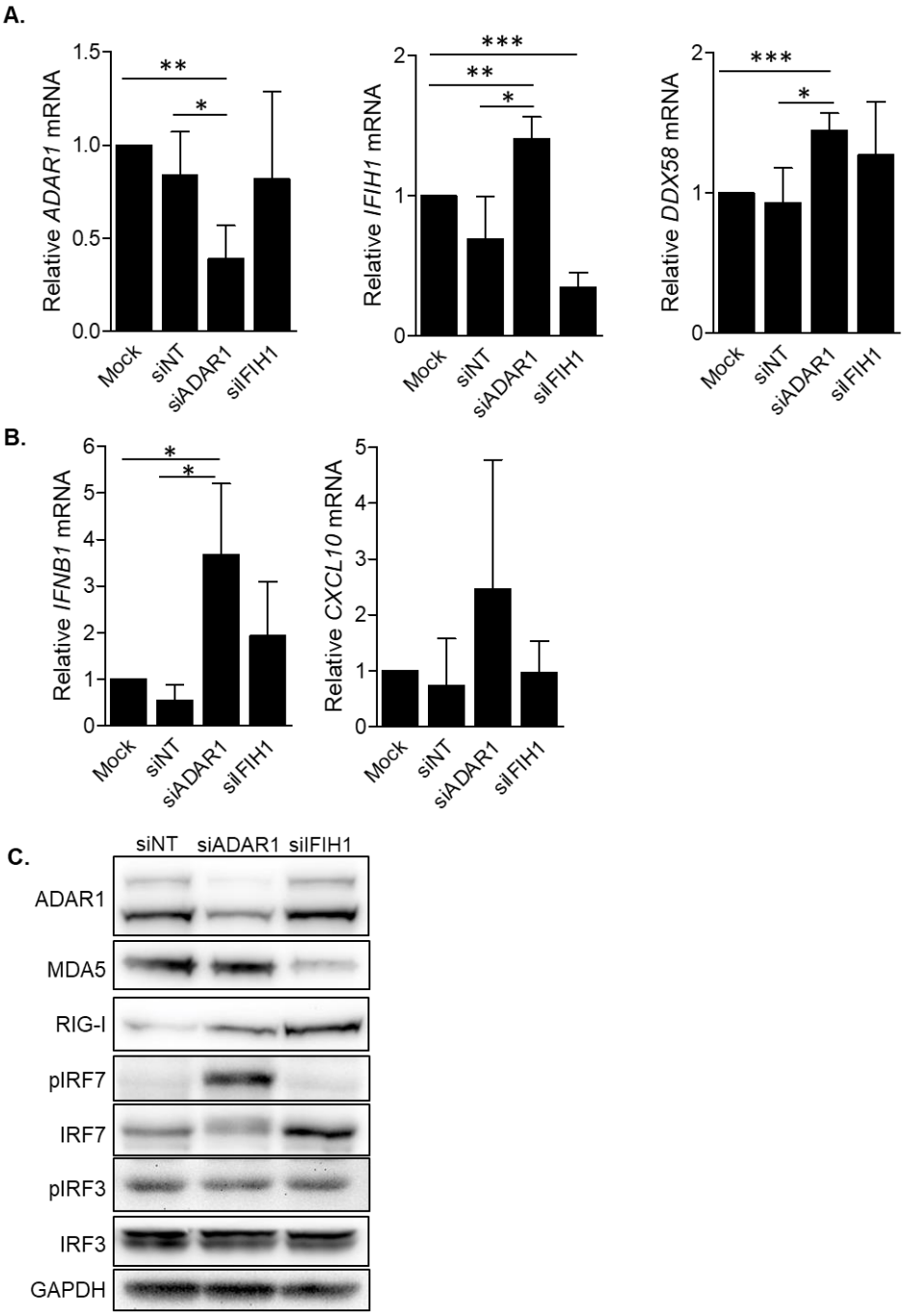


**Figure 41. ADAR1 regulates de RLRs signaling pathway in HuH7 cells.** (A) Relative mRNA expression of ADAR1, IFIH1 (MDA5) and DDX58 (RIG-I) in ADAR1 and IFIH1 downregulated HuH7 cells. Gene expression is measured by quantitative PCR and normalized to GAPDH expression. ADAR1 knockdown enhanced IFIH1 and DDX58 gene expression. Data represents mean ± SD of at least 3 independent experiments and is normalized to Mock-transfected cells. (B) Relative mRNA expression of IFNB1 and CXCL10 in siRNA-treated HuH7 cells measured by quantitative PCR and normalized to GAPDH expression. IFNB1 and CXCL10 gene expression was enhanced in ADAR1 knockdown cells. Data represents mean ± SD of 3 different experiments and is normalized to Mock-transfected cells. (C) Protein expression of RLRs and related proteins in HuH7 cells, showing overexpression of MDA5, RIG-I, IRF7 and increased IRF7 phosphorylation consequence of ADAR1 inhibition. A representative experiment is shown. *p<0.05; **p<0.005; ***p<0.0005.

### 2.3.4. ADAR1 knockdown in hepatoma cells stimulates HCV infection.

The role of ADAR1 and MDA5 on viral susceptibility was evaluated by testing the capacity of siRNA-treated HuH7 cells to support HCV replication. HuH7 cells were refractory to HCV infection as observed by the lack of inhibition of viral replication with DCV (figure 42). Knockdown of ADAR1 but not MDA5 (IFIH1) was able to bypass the restriction on HCV replication, supporting the notion that ADAR1 acts as an antiviral factor in HCV infection (figure 42).

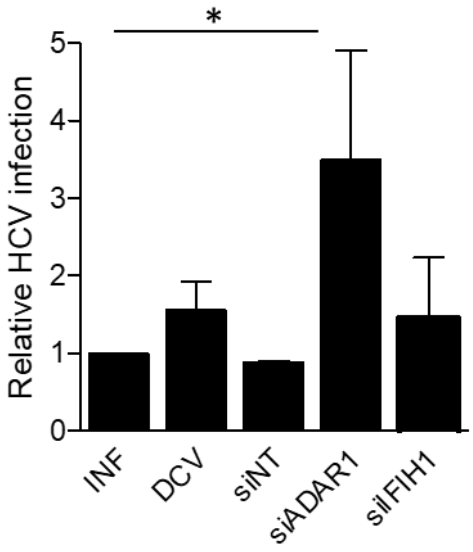


**Figure 42. ADAR1 knockdown in hepatoma cells stimulates HCV infection.** Susceptibility to HCV infection in HuH7 cells treated or not with siRNA. Relative HCV infection measured by quantitative PCR is shown. Data represents mean ± SD of 3 different experiments performed in triplicate and is normalized to infected mock-transfected HuH7 cells. INF, infected; DCV, daclatasvir. *p<0.05.

## 2.4 ADAR1 function affects HPV replication and is associated to recurrent human papillomavirus-induced dysplasia in HIV coinfected individuals

In this section, we investigate whether ADAR1-mediated innate immune activation may influence HPV disease outcome by studying HPV+ cell line *in-vitro* and evaluating genetic variants of *ADAR1* in a patient cohort from our clinic. First, we characterized the innate immune activation and signaling cascade of ADAR1 in keratinocyte cell lines susceptible to HPV infection and related cancers. We will assess the profile of cytokine production consequence of ADAR1, and the consecutive changes in HPV genes and protein expression by ADAR1. To rule out ADAR1 editing function influencing HPV replication, we identify putative A-to-I editing sites on HPV RNAs by InosinePredict software. Finally, we will study genetic association of *ADAR1* in our HPV / HIV patient cohort.

### 2.4.1. ADAR1 regulates type I IFN and innate immune activation in keratinocytes cell lines.

Protein expression of innate immune effectors was first characterized in two keratinocyte cell lines SiHa HPV16+ and HaCaT HPV- (figure 43). ADAR1 and MDA5 protein expression is similar between the two cell lines, however SiHa cells have higher RIG-I and phosphorylated STAT1 protein expression compared to HaCaT cells. As expected, expression of oncoprotein HPV16 E7 was only present in SiHa cells.

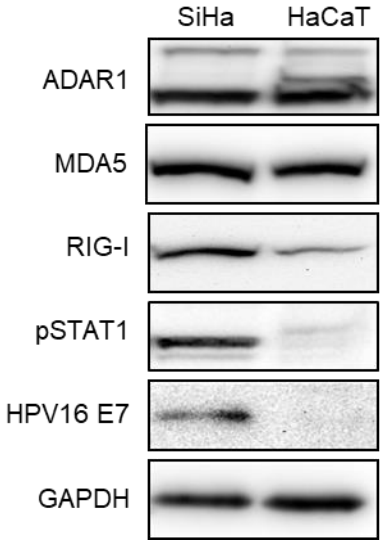


**Figure 43. Protein expression profile of players in the innate immune response between SiHa and HaCaT cell lines.** Western blot of lysates from SiHa (HPV+) and HaCaT (HPV-) cell lines shows higher protein expression of RIG-I and phosphorylated STAT1 in SiHa (HPV16+) compared to HaCaT (HPV16-) cell line. Expression of MDA5, another cytoplasmic RNA sensor, and ADAR1 remained similar between SiHa and HaCaT. HPV16 E7 protein is only present in SiHa cell line, as expected. A representative western blot is shown.

To investigate the role of ADAR1 in cells susceptible to HPV infection, ADAR1 was downregulated in both keratinocytes cell lines using RNA interference as an *in vitro* model for evaluating its contribution to HPV replication. In both cell lines, ADAR1 expression was significantly downregulated at the mRNA and protein level (figure 44A, p = 0.0001 and figure 44B ADAR1). Interestingly, ADAR1 downregulation led to a significant increase in *DDX58* (figure 44C, p = 0.0133) and *IFIH1* (figure 44D, p = 0.0024) mRNA, as well as protein expression (figure 44B, RIG-I and MDA5, respectively). Furthermore, ADAR1 downregulation induced the expression of *IFNB1* (figure 44E) and the ISG *CXCL10* (figure 44F). Accordingly, siRNA-ADAR1 overexpressed transcriptional factor IRF7 at the mRNA level (figure 44G) and increased phosphorylation of IRF7 and STAT1 as seen by Western blot (figure 44B, pIRF7 and pSTAT1), indicating activation of the innate immune response.

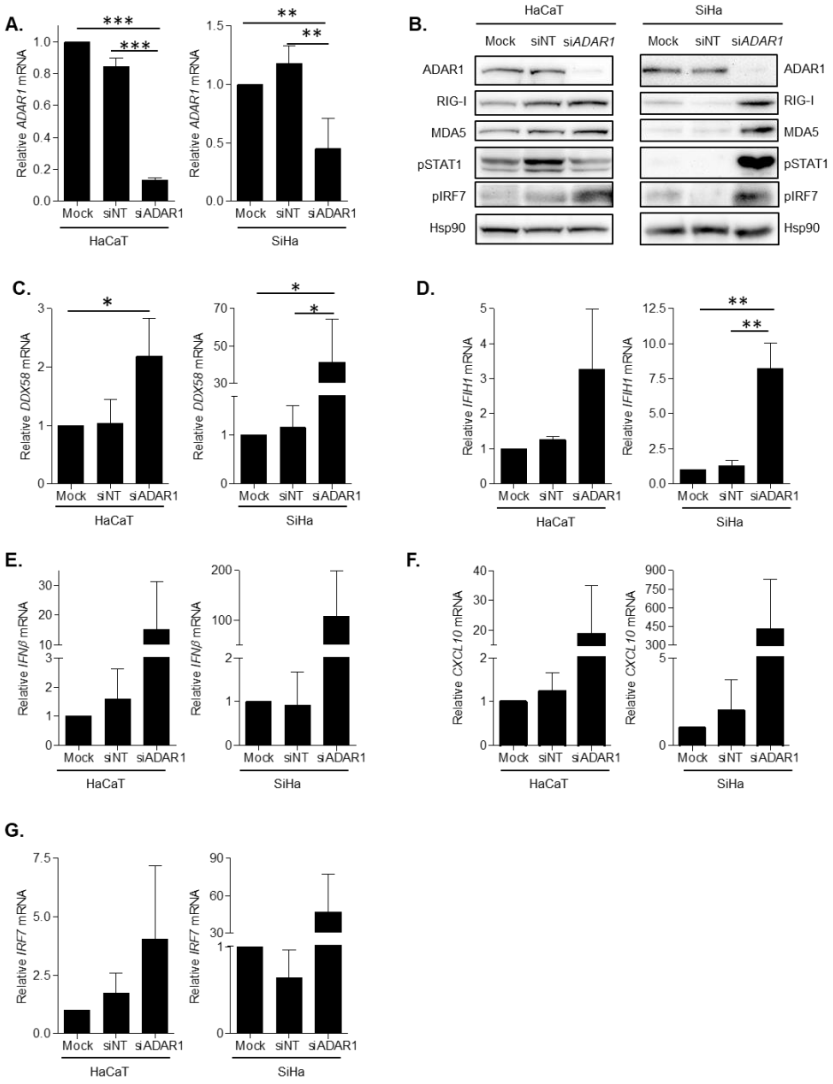


**Figure 44. Characterization of ADAR1 knockdown profile in HaCaT and SiHa cell line.** (A) Relative mRNA of ADAR1 measured by quantitative PCR. ADAR1 expression was significantly downregulated in siADAR1, whereas expression level did not change in siNT. (B) Protein expression of innate immune factors involved in sensing of nucleic acids and IFN-I response in siRNA-treated cells. Downregulation of ADAR1p150 correlates with upregulation of RIG-I and MDA5, increased phosphorylation of STAT1 and transcriptional factor IRF7 in HaCaT and SiHa cell line. A representative Western blot is shown. (C) mRNA expression of DDX58 in siRNA-treated cells measured by qPCR. DDX58 expression was significantly upregulated in siADAR1, whereas expression level did not change in siNT. (D) Relative mRNA expression of IFIH1 in siRNA-treated HaCaT and SiHa cells measured by quantitative PCR and normalized to GAPDH expression. IFIH1 gene expression was upregulated in siADAR1, whereas expression level in siNT did not change in both cell lines. (E) Relative mRNA expression of IFNB1 in siRNA-treated cells measured by qPCR and normalized to GAPDH expression. IFNB1 gene expression was upregulated in siADAR1, whereas expression level in siNT did not change in any cell line. (F) Relative mRNA expression of CXCL10 in siRNA-treated cells measured by qPCR and normalized to GAPDH expression. CXCL10 gene expression was upregulated in siADAR1, whereas expression level in siNT did not change in both cell lines. (G) Relative mRNA expression of IRF7 in siRNA-treated cells measured by qPCR and normalized to GAPDH expression. IRF7 gene expression was upregulated in siADAR1, whereas expression level in siNT did not change in both cell lines. Left side graphs represent HaCaT and right side SiHa cell line. Data represents mean ± SD of at least 5 independent experiments and is normalized to Mock-transfected SiHa or HaCaT cells. *p<0.05; **p<0.005; ***p<0.0005.

### 2.4.2. ADAR1 knockdown in SiHa cells induces a pro-inflammatory phenotype.

To understand the microenvironment induced by ADAR1 knockdown, cytokine production was evaluated in HPV16 + SiHa cells, which harbor integrated copies of HPV. Significant upregulation of nine different IFNs and cytokine genes were observed in siRNA-ADAR1 cells (IFNA2 (p = 0.0341); IFNA7 (p = 0.0365); IFNB1 (p = 0.0006); IL12A (p = 0.0005); IL12B (p = 0.0252); IL15 (p = 0.0292); IL5 (p = 0.0011); IL6 (p = 0.0015); IL8 (p = 0.0025); LTA (p = 0.0004)) compared to siRNA-NT (figure 45). These results suggest ADAR1 knockdown is inducing a pro-inflammatory phenotype, not limited to the increase in IFN, and a significant innate immune activation that may alter the cell microenvironment and their response to viral infection.

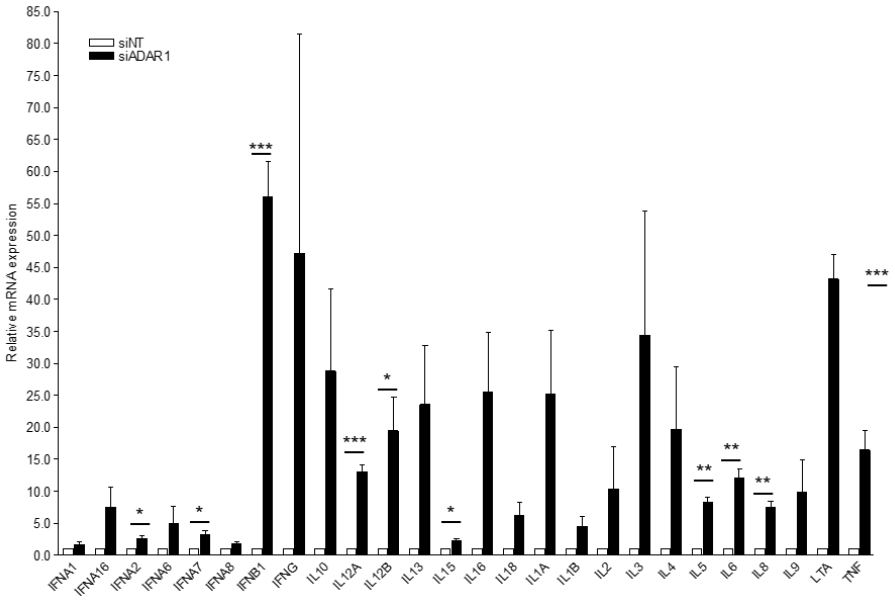


**Figure 45. Cytokine induction in treated siRNA-ADAR1 SiHa (HPV+) cells.** Cytokine expression profile of ADAR1 knockdown in SiHa cell line. Relative mRNA expression of the different cytokines normalized to siNT-transfected cells is depicted. Data represents mean ± SD of 3 different experiments. *p<0.05; **p<0.005; ***p<0.0005.

### 2.4.3. ADAR1 regulates HPV expression in SiHa cell line.

The role of ADAR1 on HPV replication was evaluated in HPV16 + SiHa cells, containing the HPV genome integrated in chromosome 13q21[184]. HPV viral RNA and protein expression was measured in an ADAR1 loss-of-function cell model. Interestingly, downregulation of ADAR1 significantly upregulated mRNA expression of HPV16 E1 and HPV16 E7 (figure 46A, p=0.0068 and p=0.0098,

respectively). Similarly, HPV16 E7 protein expression was also increased, as measured by Western blot (figure 46B, HPV16 E7). As expected, siRNA-NT did not have any effect on HPV mRNA nor protein expression in SiHa cells (figure 46A, B). Transfection of SiHa cells with PolyI:C, a potent inducer of innate immune activation, enhanced also HPV viral RNA expression, indicating that ADAR1 innate immune activation might explain enhanced HPV replication (figure 46C and D).

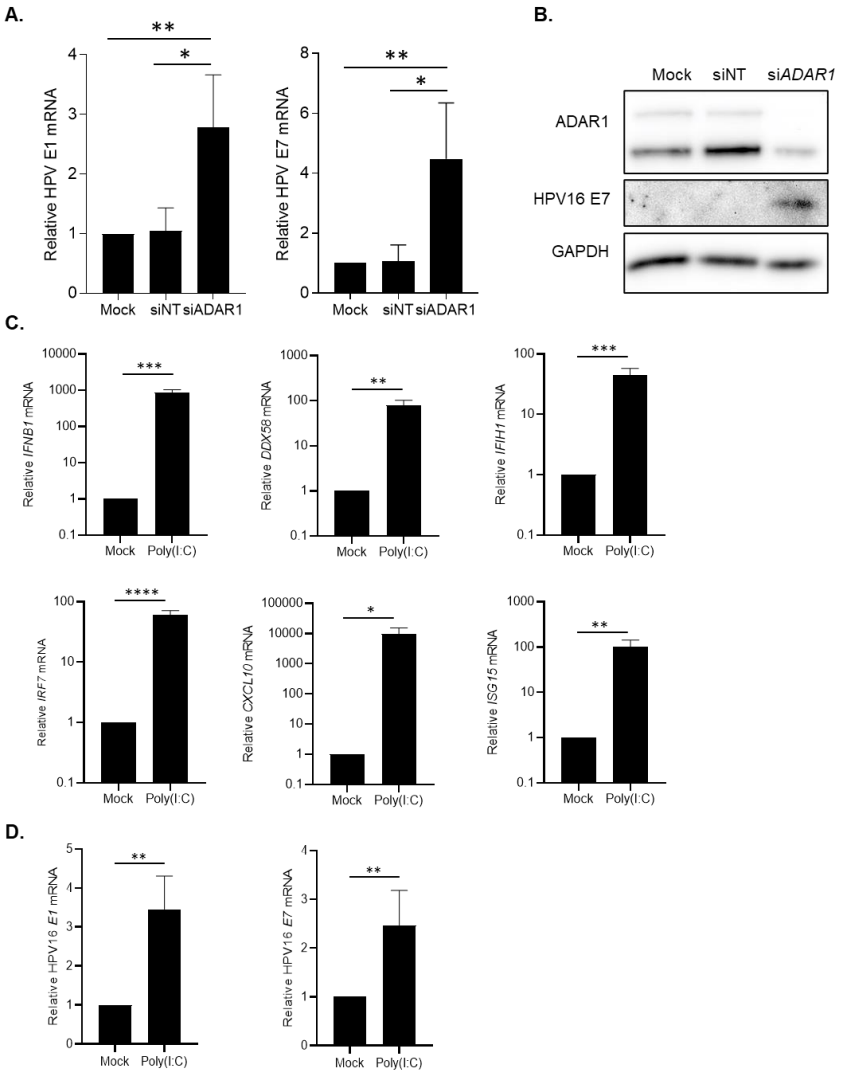


**Figure 46. ADAR1 knockdown triggers innate immune activation similar to poly(I:C) and induces HPV16 expression in SiHa cell line.** (A) Relative expression of HPV16 E1 and HPV16 E7 mRNA in siRNA-treated SiHa cells. ADAR1 downregulation induces 2.7-fold HPV16 E1 expression and 4.4-fold HPV16 E7 expression compared to Mock transfected SiHa cells. (B) Protein expression of ADAR1 and HPV E7 in siRNA treated SiHa cells. Downregulation of ADAR1 in SiHa cell line induces HPV16 E7 protein expression. GAPDH was used as loading control. A representative western blot is shown. (C) Relative mRNA expression of interferon stimulated genes (*IFNB1, DDX58, IFIH1, IRF7, CXCL10* and *ISG15*) and (D) HPV16 E1 and HPV16 E7 genes, 16h post-transfection with 2µg of poly(I:C) per 1,25 x105 SiHa cells. Gene expression was assessed by quantitative PCR and normalized to GAPDH expression. Data represents mean ± SD of at least 3 independent experiments and is normalized to Mock-transfected cells. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.

To further delineate the pathway underlying ADAR1-mediated regulation of HPV infection, RLR expression was knockdown in wild-type or ADAR1-depleted SiHa cells. As expected, RIG-I and MDA5 knockdown did neither increase innate immunity nor HPV transcription in ADAR1-expressing cells (figure 47). Importantly, ADAR1-mediated induction of innate immunity was limited in RIG-I or MDA5-depleted cells, suggesting that ADAR1 effect is dependent on RLR function. Overall, these results suggest an innate immune mediated antiviral role of ADAR1 function in HPV infection in vitro.

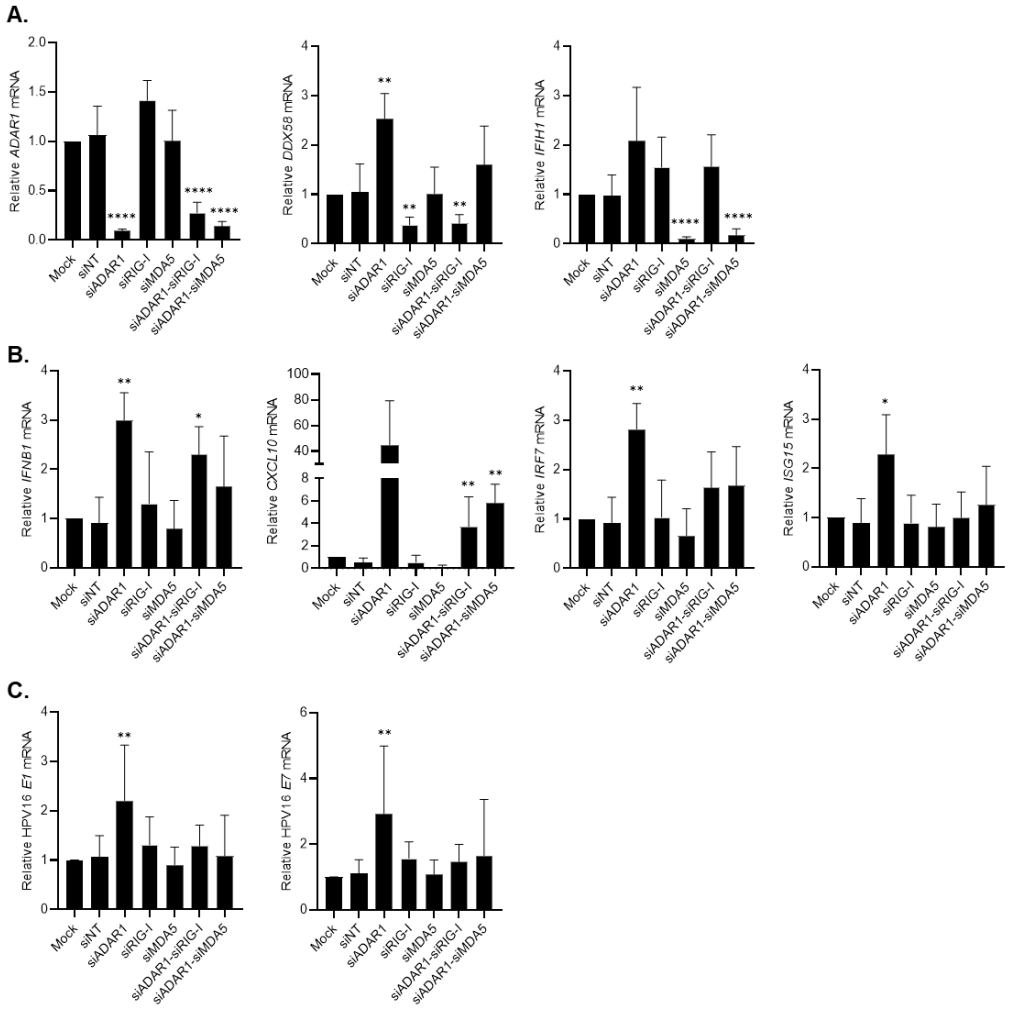


**Figure 47. Gene expression profile of ADAR1, RIG-I and MDA5 knockdowns and ADAR1-RIG-I and ADAR1-MDA5 double knockdowns**. (A) Significant downregulation of *ADAR1*, RIG-I (*DDX58)* and MDA5 (*IFIH1*) in all conditions. (B) Significant increase in gene expression of interferon stimulated genes (*IFNB1, CXCL10, IRF7 and ISG15)* in siRNA-ADAR1 knockdown cells, enhanced *IFNB1* in siADAR1-siRIG-I double knockdown and increase *CXCL10* expression in siADAR1-siRIG-I and siADAR1-siMDA5 double knockdowns. (C) Significant increase in gene expression of HPV16 *E1* and HPV16 *E7* only in siRNA-ADAR1 knockdown. 64h post-transfection with 50pmol of siRNA, or 25pmol of each siRNA for double knockdown, per 1,25 x$10^5$ SiHa cells. Gene expression was assessed by quantitative PCR and normalized to GAPDH expression. Data represents mean ± SD of at least 3 independent experiments and is normalized to Mock-transfected cells. *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001.

### 2.4.4. A-to-I editing by ADAR1 is not present in HPV transcripts in SiHa cell line.

To determine the role of ADAR1-mediated-A-to-I editing involved in the regulation of HPV expression, we evaluated A-to-I editing sites in HPV16 transcripts from SiHa cell line. InosinePredict software was used to estimate putative A-to-I editing sites and primers were designed to amplify HPV16 transcript fragments (figure 48A). 148 putative ADAR1 modification sites were estimated (figure 48A). HPV16 transcripts were amplified, sequenced and scanned for base changes that could be the result of adenosine deamination in mRNA. Although all detectable transcripts in SiHa cells were amplified and scanned, no A-to-I differences were found in siNT vs siADAR1 cells (figure 48B). As a control, we evaluated A-to-I editing in the known ADAR1-target NEIL1. As expected, A-to-I editing was present in NEIL1, whereas in siRNA-ADAR1 samples the site was not edited (figure 48C). Moreover, we determined the relative A-to-I editing efficiency at the NEIL1 site, ADAR1 knockdown cells showed a significant reduction of approximately 70% inhibition (figure 48D, $p = 0.0043$), confirming editing function of ADAR1 is decreased in ADAR1 knockdown cells. In summary, our results suggest that ADAR1 effect over HPV infection is not determined by direct A-to-I editing of HPV genome.

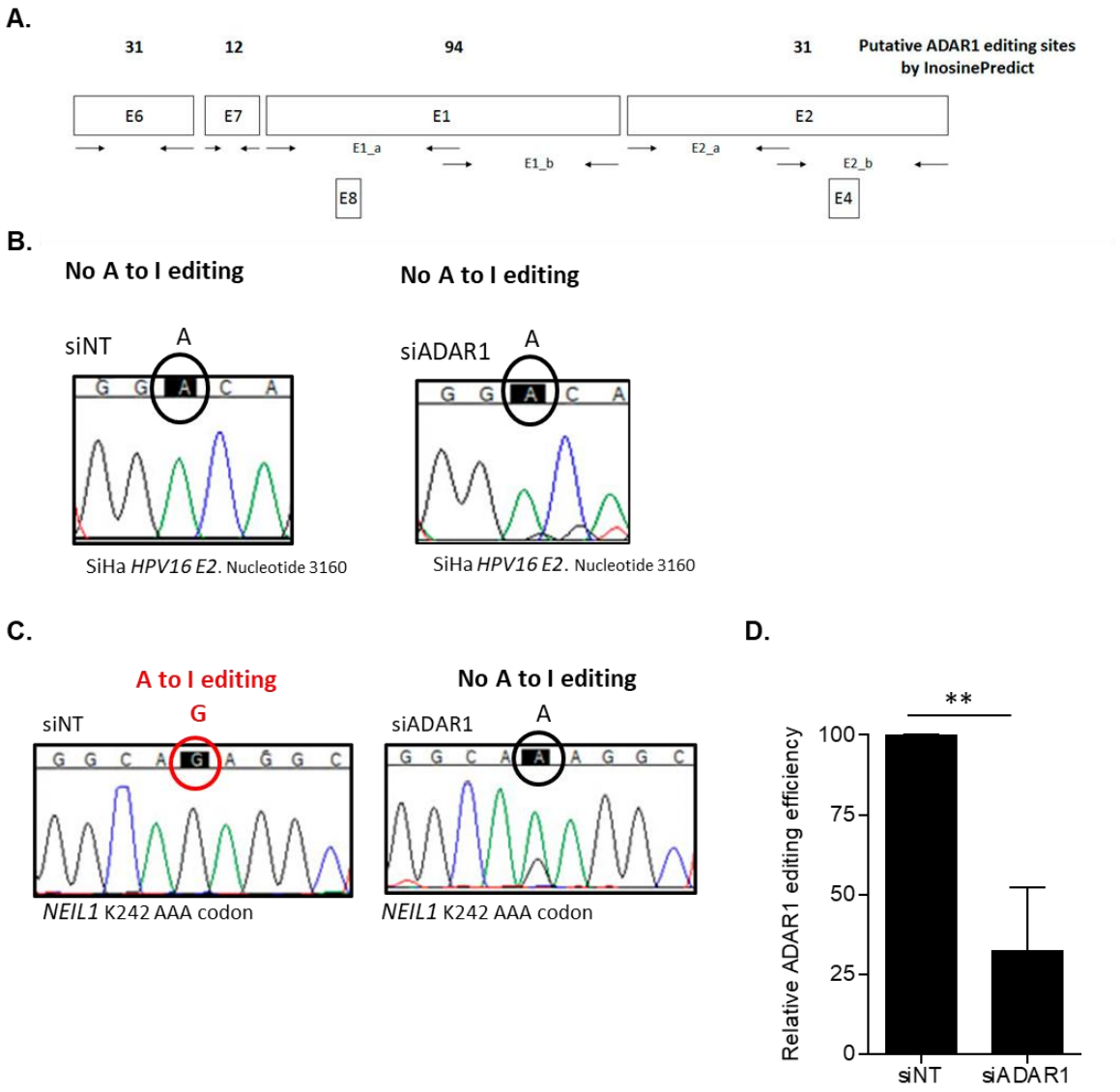


**Figure 48. Putative ADAR1-mediated A-to-I editing sites in HPV16 and NEIL1 transcripts.** Schematic representation of designed primers and putative A-to-I editing sites of HPV16 integrated sequence in SiHa cell line. InosinePredict was used to estimate site and number of ADAR1 A-to-I editing sites. (B) Representative DNA sequencing chromatogram of HPV16 E2 putative editing site with no A-to-I editing detected in nucleotide 3160 in SiHa-derived transcripts. HPV16 SiHa transcripts were amplified from RQPCR using primers represented in (A), sequenced and aligned siNT and siADAR1 sequences to detect A-to-I editing events. siNT and siADAR1 chromatograms are from the same experiment. (C) Representative DNA sequencing chromatogram of NEIL1 transcripts showing A-to-I editing at known K242 codon in siNT, but not in siADAR1 SiHa transcripts. A-to-I editing function is confirmed in siNT SiHa transcripts. siNT and siADAR1 chromatograms are from the same experiment. (D) Relative ADAR1 editing efficiency in SiHa NEIL1 transcript. Reduced edited peak in siADAR1 was relativized to edited peak in siNT. Edited adenosines to inosines are detected as Gs by direct sequencing. Data represents mean ± SD of at least 3 independent experiments. A representative chromatogram is shown. **p<0.005.

### 2.4.5. The ADAR1 haplotype AACCAT is associated with recurrent dysplasia in HPV patients.

To study the effect of ADAR1 in the development of HPV infection, a genetic association study was designed using a cohort of HPV/HIV coinfected individuals (n = 173).

**HPV/HIV cohort description.**

A single-center, prospective cohort of 173 HIV+ individuals annually assessed for HPV infection at anal, genital, and oral sites attending the Outpatient HIV Clinic of the Hospital Germans Trias i Pujol (Can Ruti Hospital, Badalona, Spain), were described in[223,224]. Clinical characteristics of patients are shown in table 11. The study was approved by the Ethics committee of Hospital Universitari Germans Trias i Pujol. Written informed consent was obtained from patients participating in the study. All methods were carried out in accordance with relevant guidelines and regulations and to the ethical principles suggested in the Declaration of Helsinki.

Individuals in the study were those suspected for anal intraepitlelial dysplasia (AID, n: 162) or cervical high-grade squamous intraepithelial lesions (HSIL, n: 11) following a cytology. If lesions were visualized, during high resolution anoscopy (HRA) or cervical colposcopy, a directed biopsy was performed for histological analysis. All patients with biopsy-proven of anal intraepithelial neoplasia-2 or -3 were treated with infrared ablation or surgery. A surgical resection (conization) was proposed in case of HSIL diagnosed confirmed by histology (CIN2 or CIN3). Cytologic changes were classified according to the Bethesda System: normal (no cell changes), atypical squamous cells of uncertain significance (ASCUS) or low- or high-grade squamous intraepithelial lesions (LSIL or HSIL, respectively). Overall recurrent dysplasia was defined as detection of at least two biopsy proven AIN 2-3 or CIN 2-3 at any time point during follow-up at the treated location or at new site[225]. Anal samples were obtained introducing a cytobrush (Eurogine SL, Spain) 3 cm into the anal canal and gently rotating it for 30 to 45 seconds. The cytobrush was introduced into and shaken in a 20 mL of PreservCyt/Thin solution (Cytyc Iberia SL, Spain) for 30 seconds and assessed by the PAP method. Cervical citology samples were obtained from the exocervix with an Avre spatula and from the endocervix with a cotton swab and was assessed by the Pap method41. For HPV detection, the same samples were used to determine HPV genotype using the AnyplexTM II HPV28 real-time PCR (Seegene, Seoul, Korea) as described before[226]. HPV genotypes were classified as High (genotypes

16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and low (6, 11, 40, 42, 43, 44, 54, 61 and 70) oncogenic risk as described[226].

Six ADAR1 polymorphisms covering all known genetic variation in ADAR1 gene (rs6699729, rs3766927, rs3766925, rs3766924, rs9616 and rs9427097) were genotyped and tested for association to recurrence of HPV associated dysplasia (relapse in HPV associated dysplasia). Selected demographic and clinical information are summarized in table 11. Genetic association of each SNP was adjusted by age, sex, time since first cytology and HIV infection time, CD4 nadir, and oncogenic HPV genotypes in a logistic regression model. When testing for single SNP-association, no significant association was found to HPV recurrence (p-values > 0.05).

To further explore whether ADAR1 may influence recurrence in HPV-induced dysplasia, estimation of haplotype frequencies was performed. Haplotype-based analysis represent a valuable resource for investigating the genetic basis of disease susceptibility, albeit the identification of the functional variant has proven challenging. When we estimated ADAR1 haplotypes to HPV-dysplasia recurrence, we found the low frequency haplotype AACCAT was significantly associated with recurrent HPV dysplasia (2% and 8% of non-recurrent and recurrent HPV dysplasia respectively) (psim= 0.0064) (table 12), suggesting that ADAR1 plays a role in HPV infection *in vivo*. Importantly, the number of malignancy-associated HPV types identified was not significantly different (p > 0.05) between groups of recurrent and non-recurrent HPV-associated dysplasia, indicating that ADAR1 was an independent factor from higher prevalence of oncogenic HPV types

**Table 11. Clinical characteristics of HPV/HIV-1 coinfected patients.**

| | **All** | **Recurrent dysplasia** | **Non- Recurrent dysplasia** | **p** |
|---|---|---|---|---|
| | | | | |
| **Individuals, n (%, CI)** | 173 (100) | 52 (30%, **95%CI: 24-37%**) | 121 (70%, **95%CI: 63-76%**) | - |
| **Gender, female/male (% of total)** | 32 (18.5) /141 (81.5) | 10 (19)/ 42 (81) | 22 (18)/ 99 (82) | NS |
| **Age, years** median (range) | 45 (23-77) | 46 (24-77) | 43 (23-52) | NS |
| **Follow-up time since first cytology,** median (years) | 5.8 | 5.6 | 6.1 | NS |
| **Time to first dysplasia,** median years (range) | 2.6 (0.7-10) | 1.6 (0.7-10) | 2.2 (0.1-6.9) | NS |
| **Time since first visit to HIV clinic** (years) | 12.8 | 10.7 | 13.3 | NS |
| **HPV genotypes,** n | | | | |
| **Low oncogenic type** | | 0 | 13 | - |
| **High oncogenic type** | | 52 | 108 | - |
| **CD4 Nadir,** median (range) | 240 (22-993) | 225 (2-576) | 248 (80-993) | NS |

CI: confidence interval, NS: not significant after Student's T test. Time to first dysplasia calculated from the time to first cytology. Samples were considered high oncogenic type with one or more high risk oncogenic HPV types detected.

**Table 12. Haplotype association results for recurrent dysplasia adjusted by sex, age, HIV infection, CD4 nadir and HPV oncogenic types.**

| Haplotype | | | | | | Frequency | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| rs6699729 | rs3766925 | rs3766927 | rs3766924 | rs9616 | rs9427097 | All | Non-recurrent dysplasia | Recurrent dysplasia | P-value | P-value_sim |
| A | A | C | C | A | T | 0.041 | 0.022 | 0.081 | **0.0084** | **0.0064** |
| A | A | T | C | A | T | 0.249 | 0.271 | 0.204 | 0.075 | 0.084 |
| T | T | C | C | A | G | 0.139 | 0.132 | 0.151 | 0.336 | 0.338 |
| A | T | T | C | A | T | 0.069 | 0.061 | 0.089 | 0.349 | 0.361 |
| A | T | C | C | A | G | 0.015 | 0.019 | 0.008 | 0.525 | 0.592 |
| T | T | C | C | T | T | 0.252 | 0.256 | 0.24 | 0.647 | 0.656 |
| A | T | C | T | A | T | 0.177 | 0.179 | 0.177 | 0.879 | 0.886 |

Haplotype frequencies are shown for all the patients and for dysplasia groups. *P-value_sim: corrected p-value after 10,000 permutations in the score test implemented in haplo.stats R package[196].

# DISCUSSION AND PERSPECTIVES

The genetic and molecular dissection of rare Mendelian disorders associated with constitutive overproduction of type I IFN has provided unique insight into cell-intrinsic disease mechanisms that initiate and sustain autoinflammation and autoimmunity and that are caused by disturbances in the intracellular nucleic acid metabolism or in cytosolic nucleic acid–sensing pathways. Collectively, these findings have greatly advanced our understanding of mechanisms that protect the organism against inappropriate immune activation triggered by self nucleic acids while maintaining a prompt and efficient immune response to foreign nucleic acids derived from invading pathogens. This thesis takes advantage of knowledge generated in the context of one of these disorders, AGS syndrome, to understanding the nature and regulation of innate mechanisms in the context of HIV infection with the long term goal to identify novel therapeutic approaches or targets able to modulate innate immune activation critical in the development, progression, and outcome of many diseases. Based on that, one of the most important objectives of this thesis was to evaluate the effects of HIV infection on innate immunity in primary immune cells, especially in macrophages.

Macrophages have long been considered important immune effector cells[227], involved in innate immune response to pathogens and thought to initiate inflammation. Also, macrophages play a critical role in transmission, viral spread and act as viral reservoir of human immunodeficiency virus (HIV)[228–230]. Moreover, the limited lifespan of primary macrophages, compared to immortalized cell lines that unlimitedly proliferate, make these cells a unique model for the evaluation of cell cycle progression and innate immune responses in the context of viral infections.

We found that HIV susceptibility in primary macrophages was dependent on the proliferation status and the ability to enter cell cycle, two variables tightly linked that affect viral replication. The association between viral susceptibility, regulation of cell cycle progression and expression of host proteins is not new, but it has gained special interest after the description of SAMHD1 role in regulating susceptibility to viral infections[118,122,181,231]. SAMHD1 is a key regulator of cellular nucleotide metabolism. It is expressed in all cells, and importantly, its triphosphohydrolase function is regulated by phosphorylation by cyclin-dependent kinases, either CDK1 or CDK2[122,183], which inactivates SAMHD1, providing a link between the HIV restriction activity and cell cycle.

We demonstrate that M-CSF differentiation of monocytes into macrophages induces cell proliferation and entry into cell cycle, in line with the previously reported proliferative effect of M-CSF[232]. Moreover, the proliferation status of the cell, correlates with expression level of

phosphorylated inactive SAMHD1, determining susceptibility to HIV-1 infection in macrophages, in clear contrast to monocytes. We also characterized alterations in the molecular pathway leading to cell cycle arrest after HIV-1 infection in M-CSF MDMs. Previous studies identified G2/M cell cycle arrest in infected MDMs dependent on Vpr accessory protein expression[197] through a possible replication stress dependent pathway[233], although the exact mechanism is not completely understood besides the involvement of the VPRBP-DDB1-CUL4 E3-ligase complex[234]. Most reports show that HIV induced G2/M arrest is mediated by inactivation of CDK1 by a cascade of reversible phosphorylations[235]. Our results not only demonstrate a strong downregulation of CDK1 as one of the inducers of G2/M arrest in infected MDMs, but more importantly a complete dysregulation of the molecular pathway controlling cell cycle progression. Elevated levels of p21 induced the inhibition of CDK2 activity preventing the phosphorylation of RB protein and the subsequent release of E2F1 transcription of genes required for cell-cycle progression that are in line with previous reports describing the role of Vpr[200]. Also, low expression levels of E2F1 are consistent with the known auto-regulatory transcription of E2F1[201].

When evaluating macrophage immune function in the context of HIV-1 infection, our results clearly show that HIV-1 infected MDMs induce innate immune activation with elevated levels of *IFN1B* mRNA and ISG expression, including *CXCL10*, upregulation of RLR expression and increased phosphorylation of STAT1. The activation of the innate immune system through expression of type I IFNs reinforces the cell cycle arrest produced by HIV-1. Indeed, STAT1 expression is reported to mediate growth inhibition by enhanced p21 transcription[236]. Upregulation of MDA5 and RIG-I, and induction of ISGs following HIV-1 infection in MDMs are in line with previous reports[237–240].

In fact, immune activation is described to be linked to cell death through activation of NF-κB transcription factor which regulates expression of many regulatory genes involved in inflammation, apoptosis and viral replication[241], suggesting a connection between immune activation and inflammation with viral replication and cell death that is mediated by regulatory transcriptional factors, such as NF-κB and others. We characterized that cell death in primary macrophages is mediated by caspase 3 through modulation of the transcription factor IRF3[242], which links the induction of innate immune response to cell death of infected cells.

On the other hand, the close monitoring of SAMHD1 expression and function concomitant to cell cycle progression and infection showed that SAMHD1 shifted to an active form in long-term cultures of HIV-1 infected macrophages, indicating that apart from CD4 downregulation by HIV,

SAMHD1 acts as a factor preventing HIV-1 reinfection and/or super-infection. Infected MDMs also show reduced levels of E2F1-dependent RNR2, an indispensable enzyme for dNTP biosynthesis[243]. The concomitant downmodulation of RNR2 and reactivation of SAMHD1 in infected MDMs leads to the reduction of the dNTP pool necessary for cell cycle progression. Limiting availability of dNTPs affects the completion of reverse transcription by HIV-1 that may be associated to cell death by activation of caspases[244,245].

The study of host restriction factors in HIV-1 has identified additional functions involved in the regulation of intrinsic immunity, cell death and establishment of latency that critically affects the virus. Therefore, understanding differences between human and primate lentiviruses can also help us to identify key features and discrete factors altered by biological pressure that affect viral and host regulatory mechanisms otherwise difficult to discern. On the other hand, aberrant immune activation or over-stimulation of the innate immune signaling often affects immune-, metabolic- and neurodegenerative- disorders that could be modulated with the identification of immune factors controlling critical pathways leading to improved therapeutic approaches. In this scenario and based on data obtained from macrophage infection, we aimed at evaluating the potential of HIV-2 accessory protein Vpx (HIV-2/SIV) as a modulator of innate immunity and its efficacy on acute and/or latent HIV infection.

As suggested elsewhere[246,247], the combination of viral and host factors contributes to the molecular and clinical differences between HIV-2 and HIV-1 infections. HIV-2 has a lower rate of infectivity and is less virulent compared to HIV-1. Genetic differences between HIV-1 and HIV-2 are mainly based on accessory proteins Vpx and Vpu that could explain certain clinical and molecular features. For instance, viral accessory proteins are used by the virus to evade intrinsic antiviral responses[248], both Vpx and Vpr have been previously described to facilitate HIV replication in myeloid cells although the mechanisms are not completely elucidated (reviewed in[249]).

We show that Vpx from SIVmac251 targets the degradation of SAMHD1 and TASOR, contrary to Vpx from SIVrcm, confirming an additional Vpx function reported[125,203,206,250]. Whether the difference in a single viral protein significantly affects diverse biological processes important for viral replication and maintenance is still controversial, albeit there is no doubt that the concerted action of each viral factor contributes to the dysregulation of immune and transcriptional pathways.

By whole transcriptome profiling, we showed that in MDMs, Vpx treatment induces an IFN-mediated antiviral immune response characterized by enhanced ISG expression. The enhanced

immune response is able to reactivate latent HIV in a non-clonal cell line model and at the same time induce apoptosis in treated cells. Our results, although preliminar, are in line with previous reports by Decalf et al.,2017 showing that MDMs are able to trigger an IFN response and induce ISGs after sensing HIV. Decalf et al 2017 demonstrates MDM sensing during an HIV infection is independent from the glycoprotein used in fusion or the delivery of viral genomic material [251]. Even though we did not use HIV virions as in Decalf et al[251], both reports used VSV-G-pseudotyped lentiviral preparations produced by transfected HEK 293T for the experiments, allowing for the comparison of the two reports. Contrarily to Decalf et al[251], our results highlight that it is not HIV sensing but the function of Vpx from SIVmac251 that triggers type I IFNs, since evaluation of various Vpx lineages and the loss of Vpx function by heat inactivation does not have the same effect.

Also, the RNA seq data presented here is similar to Decalf et al[251] microarray of whole-transcript cDNA, furthermore most of the genes reported are IFN-induced genes, similarly to our report. We demonstrate IFNs and ISG induction is specific to Vpx from SIVmac251, a lineage that induces proteasomal degradation of SAMHD1 and TASOR, suggesting that either both proteins or one of them might play a relevant role in antiviral immune response. In this sense, the link between SAMHD1 and innate immunity is far more clear than that of TASOR. Mutations in SAMHD1 are reported to cause Aicardi-Goutières syndrome, a genetic disease characterized by the activation of an innate immune response via cytosolic nucleic acid sensors[66]. In an attempt to confirm SAMHD1 prominent role in antiviral immune response, we could not recapitulate the innate immune activation described by *in-vitro* Vpx transduction in single siSAMHD1 or siTASOR transient knockdowns by siRNA. A major drawback of our study is the inability to completely knockdown by siRNA primary macrophages, which might help solving this issue. Moreover, due to limitations of primary cells, we cannot discard that siRNA might not achieve a full knock-out of SAMHD1 compared to Vpx-induced degradation. Thus, it is unknown whether the induction of innate immune responses is a direct effect of the lack of both proteins or it is caused by an unknown additional function of Vpx.

Given the induction of IFNs after treatment with Vpx, it is surprising that viral replication is not restricted. In fact, Vpx-mediated degradation of SAMHD1 enhances replication suggesting that the increased availability of dNTPs is far more favorable in viral replication than the limiting effect exerted from antiviral immunity. However, the overproduction of IFNs may have a different effect upon HIV reactivation due to the transcriptional effects associated to IFNs. Vpx treatment in non-clonal HIV latent cell line model induces reactivation of HIV provirus in a SAMHD1-independent manner, since our model does not express SAMHD1, hence induction of

provirus transcription is only dependent on TASOR expression level. Unfortunately, our data is far from describing the exact contribution of TASOR in relationship to HIV reactivation and establishment of latency, and further research is needed to extract clear conclusions regarding these aspects. It would be interesting to describe the role of TASOR on HIV transcription and whether TASOR has a cell-dependent effect. It is important to continue working on the mechanisms that induce and regulate provirus transcription and the establishment of latency, as the identification and characterization of novel players might help to develop novel therapeutic strategies in the future.

Keeping in mind the need to identify additional host factors that might play a role in the intersection between innate immune activation and HIV pathogenesis, we designed a strategy to screen the effect of AGS causing genes and pathways in primary cells. Different reports have suggested AGS patients are less susceptible to HIV infection in $CD4^{+}$T cells, in particular AGS caused by *SAMHD1* and *ADAR1* mutations[252,253]. Moreover, *TREX1* and *SAMHD1* genes, two of six genes described to cause AGS, have already been described as modifiers of HIV replication, suggesting that the study of immune disease such as AGS may be a strategy to identify additional host factors involved in viral replication.

Indeed, our loss of function screening identified only ADAR1 as putatively affecting HIV replication. Interestingly, ADAR1 downregulation lead to increased expression of MDA5, also mutated in AGS patients, suggesting that MDA5 function is also influencing HIV replication, presumably as a consequence of ADAR1 function. Taking into account existing data from SAMHD1 and TREX1 together with the identification of ADAR1 as a host factor affecting HIV replication, it is plausible to think that the dysfunction of cellular pathways involved in AGS might be relevant in HIV pathogenesis and associated coinfections.

One of the main focus of the present work has been the study of ADAR1, including the characterization of its function in primary macrophages and the effects of its dysfunction on viral replication and pathogenesis. Overall and from a functional point of view, we have found that *ADAR1* inhibition induced a high IFN response, which was in accordance with previously proposed ADAR1 function, including the suppression of IFN responses and cell protective role from excessive IFN signaling[254]. In loss of function models, we identified that ADAR1 induces innate immune activation through type I IFN signaling, induction of RLR pathway, leading to enhanced ISG expression and production of pro-inflammatory cytokines in primary macrophages, hepatocyte, and cervical cell line *in-vitro* models, clearly indicating that ADAR1 contributes to the modulation of the innate immune activation. Moreover, our results are also

in accordance with described phenotype hallmarks of AGS patients but also from *in-vivo* KO mice models, including high levels of IFN in cerebrospinal fluid and serum[69], increased levels of ISGs in peripheral blood[255–257], and other characteristics[99,254,256].

On the other hand, when ADAR1 knockdown models have been challenged with different virus, our data suggested that the contribution of ADAR1 to viral replication can be mainly assigned to its capacity of regulating innate immune responses and inducing type I IFNs. We have seen that ADAR1-induced regulation of innate immune activation is strong enough to alter the outcome of a diverse set of viral infections evaluated *in-vitro*. However, ADAR1 inhibition displays controversial effects depending on the virus tested, as we identify a proviral or antiviral effects depending on the specific virus-host combination being studied.

Innate immune activation signatures and enhanced expression of proinflammatory cytokines are present in all *ADAR1* downregulated cells independent of tissue type. Although, the effect of *ADAR1* downregulation is different depending on the particular virus-host combination. For instance, *ADAR1* downregulation in HIV infection in primary macrophages displays an antiviral role, able to limit viral replication when *ADAR1* is downregulated in both infected cells and bystander cells, a result which confirms previous reports[192,253,258]. On the other hand, *ADAR1* downregulation displays a proviral role in hepatocyte and cervical cell lines where HCV and HPV replication has respectively been evaluated, indicating that there is increased viral transcription when *ADAR1* expression is downregulated in these cells. These controversial results highlight the possibility of being virus- or cell-dependent effects, which alter the outcome of *in-vitro* infections. On one hand, these effects might be due to the different life cycle of each virus as different nucleic acids forms are present in the distinct infection cycles: from reverse transcription and integration needs, in the case of HIV, replication of circular DNA genome, in HCV, to host cell differentiation needs, in HPV. On the other hand, it may also depend on cell type characteristics, for instance primary macrophages being involved in detection of pathogens might need to maintain a virus-free environment, whereas other cell types might rely on immune cells for pathogens clearance. Hence, mechanisms against virus or other pathogens depending on cell type may hold critical differences explaining these controversial effects. Furthermore, the effect of AGS gene deficiency seems to be both tissue and cell specific[259], putatively explaining the differences observed after ADAR1 downregulation between the distinct cell models.

The understanding of ADAR1-virus interplay leads to the consideration of the more appropriate *in vitro* model needed to evaluate both innate immune activation and viral replication effects. It

is essential to also consider the innate immune signaling and transcription factor expression patterns of the particular cell model being evaluated and its contribution to nucleic acid immunity[260]. In this work, innate immune activation signatures and subsequent inhibition of HIV-1 replication observed in macrophages (both M-CSF and GM-CSF derived) were not observed when ADAR1 was downregulated in DC or only partially observed in CD4+ T cells. This data is in contrast with previous data from ADAR1-deficient CD4+ T lymphocytes from AGS patients[253], which presented high type I IFN and ISG expression and were also refractory to HIV-1 infection. The lack of innate immune activation in our CD4+ T cell cultures compared to that observed in CD4+ T cells from AGS patients may account for the observed differences[253], reinforcing the idea that ADAR1-mediated block of HIV-1 replication is mainly dependent on innate immune activation.

In addition, antiviral evaluation of ADAR1 downregulation in HCV also pointed out the importance of expression and function of innate immune effectors, as RNA sensors, and downstream signaling mediators. In HCV, Huh7 cells resembles innate immune activation features observed in AGS compared to altered Huh7.5 cells. Huh7.5 cell line are more permissive than Huh7 to HCV infection owing to RIG-I gene (*DDX58*) mutation that inactivates its function[222]. Indeed, our HCV infection data demonstrates that complete innate immune signaling pathway is necessary for ADAR1 effect, presenting a similar profile to that observed in primary macrophages and ADAR1-associated AGS patients and KO mouse models[99,254,256]. Only limited mechanistic studies have been described regarding the link between ADAR1 and host response to HCV infection[261], although our data confirms previous reports showing inhibition of ADAR1 stimulate HCV viral RNA production[100].

Because of the unavailability of a stable HPV infection model, and HPV life cycle being completely dependent on cell differentiation, mechanistic evaluation of HPV is difficult. ADAR1 downregulated cells induced immune activation that can activate transcription of many genes including HPV16 E6 expression. Unfortunately, our model is unable to fully elucidate ADAR1 effects and many questions remain to be answered.

The exact mechanism by which ADAR1 functions is still far from understood, despite intensive research particularly in ADAR1 genetically modified mice[99,262,263]. Liddicoat *et al*. linked RNA editing to RNA recognition exclusively by cytosolic sensor MDA5, based on the fact that MDA5 deficiency could rescue the phenotype of Adar1E861A/E861A editing-deficient mice[99]. However, two major sensors of cytosolic dsRNA exist, MDA5 and RIG-I, with different RNA chain preferences[264–266]. The type of dsRNA described by Liddicoat *et al*. does not fit into the

knowledge of dsRNA preference by RIG-I and MDA5. Moreover, an Adar1 knockout model indicated that Mavs deficiency, an essential adaptor of both RIG-I and MDA5, was not able to totally revert the Adar1 knockout phenotype[267], by turning off the signaling cascade. These results suggest that apart from the MDA5-MAVS axis, additional mechanisms mediate innate immune response in the absence of ADAR1. In our cell culture models, both cytosolic dsRNA receptors MDA5 and RIG-I are similarly affected by ADAR1 knockdown, comparable to LPS or poly (I:C) induction seen in primary macrophages, indicating that the effect might not be exclusively driven by MDA5. Moreover, pharmacological blockade of TBK1[219] partially rescued the phenotype observed in ADAR1 knockdown macrophages, indicating that ADAR1 deficiency may signal through MAVS-TBK1. In addition, considering that ADAR1 function can affect numerous host and viral RNA substrates, our data indicates that RNA editing by ADAR1 is a key process for marking dsRNA as self, therefore, preventing innate immune recognition by cytosolic sensors and, in consequence, autoreactivity and the development of inflammation and/or autoimmune disease[254].

When taking into account viral infections, the underlying mechanism of ADAR1 effect on viral replication is also still controversial, and conflicting mechanisms has been suggested, ranging from direct editing of viral mRNA, towards editing-independent effects either as a consequence of the antiviral environment or mediated by the IFN-inducible protein PKR affecting viral translation[268,269]. In the present work, although an extensive search of putative A-to-I editing sites has been done, we did not detect A-to-I editing in HIV-1 or HPV16 viral transcripts, as previously suspected by others[192,262]. However, we cannot exclude the existence of edited sites under the limit of detection of our experimental settings or in other viral genomic regions as well as the existence of a cell dependent-effect, as reported elsewhere[192].

ADAR1-mediated effects have also been linked to the function of PKR, a protein that inhibits mRNA translation and has reported to affect measles virus, vesicular stomatitis virus, HTLV-1 infection, HCV and HIV-1[258,270–274]. In contrast to previous data, our results do not support a direct role of PKR in HIV-1 or HCV replication. In macrophages, PKR inhibition is not affecting HIV-1 replication and we found that ADAR1 knockdown blocks HIV-1 at viral transcription, prior to viral RNA translation, ruling out a major effect of PKR in our model.

To further study the putative role of ADAR1 *in-vivo*, we designed two genetic association studies in two different cohorts of HIV-1 coinfections, i. e. HIV/HCV and HIV/HPV. In both studies, we found that SNPs in ADAR1 gene were associated to both HCV and HPV infections outcome in

coinfected subjects, suggesting that the regulation of innate immune activation is key in the development and clinical outcome of HCV and HPV infection.

In particular in HCV/HIV-1 coinfected patients, ADAR1 SNPs were found significantly associated to poor clinical outcome to IFN-RBV therapy and advanced liver fibrosis. Interestingly, SNP rs2229857 (p-value 0.007) codes for a missense mutation (K384R) that may have an impact on ADAR1 function or regulation, suggesting that a dysfunctional ADAR1 might negatively impact viral infection outcome. Our results are in accordance to previous reports where ADAR1 polymorphisms are associated to improved HCV liver fibrosis prognostic and treatment[275,276]. The phenotype associated to ADAR1 TT allele was different between male and female individuals. Previous analysis of polymorphisms in several genes of the IFN system rs2229857 and rs1127326 revealed that host genetic factors affect HCV responsiveness to IFN therapy, in addition to other factors including HCV virus genotype, age, gender and cirrhosis status[276,277]. Indeed, successful response to IFN therapy in HCV infection has been linked to SNPs in interferon-α pathway genes[276]. HCV/HIV-1 coinfected women have more favorable HCV virological and clinical profile than men, particularly when referring to liver disease progression. Thus, the distinct association of ADAR1 SNP in males and females may be reflecting the complex interaction of patient genetic background with viral and host factors that altogether trigger and control the outcome of HCV infection. In the case of HPV/HIV coinfected individuals, we identified a low frequency haplotype in *ADAR1* associated with recurrent relapse in HPV dysplasia, suggesting a putative role of ADAR1 in HPV infection outcome, similar to the finding in HCV, where *ADAR1* generic variations are associated to poor clinical outcomes.

On the other hand, ADAR1 overexpression in the cytoplasm has been linked to better prognosis in individuals with oral squamous cells carcinoma[278] and cervical cancer progression and angiogenesis[279], providing further support to our data and pointing towards the involvement of epitranscriptomic modifications as determinants of HPV-associated pathogenesis. In fact, ADAR1 has previously been linked to cancer progression, where aberrant editing of RNAs have been described to promote transition from normal to malignant cancer cells. Indeed, ADAR1 overexpression is present in breast, lung, liver and esophageal cancer as well as in chronic myelogenous leukemia, where it promotes cancer progression (reviewed in[280]). Hence, increased expression of ADAR1 translates to aberrant editing and reduced IFN immune responses due to ADAR1 presence, whereas low expression of ADAR1 would translate to enhanced IFN responses and increased cell death pointing towards a putative therapeutic option.

Genetic association studies aim to identify the putative link of a given gene to a certain phenotype without informing on the specific molecular determinants of the association, thus, further studies are needed to better delineate the exact contribution of ADAR1 function to HCV and HPV clinical course. In addition, whether differences in ADAR1 expression in our cohorts accounts for the effect seen in HCV-infection and HPV-associated dysplasia cannot be rule out from our study. Finally, it has to be taken into account that from our data we cannot exclude that HIV infection might have a role in the genetic association found in both HCV and HPV cohorts, and thus, data from additional infected cohorts must be analyzed to solve this issue.

In summary, this thesis highlights the value of understanding intrinsic mechanisms of immune regulation which identify key proteins affecting the outcome of viral infections. Based on the pathogenesis of a rare autoimmune disease like Aicardi-Goutères syndrome, this work identified a series of molecular mechanisms, intracellular regulators of nucleic acid metabolism and recognition system that contribute to viral infections such HIV, HCV and HPV. The better knowledge of theses pathways can suggest new therapeutic options directed towards the inhibition of specific steps in nucleic acid metabolism that can impact not only the course of infectious disease but also autoimmunity and cancer treatments. Indeed, a better understanding of the intersection between nucleic acid metabolism and innate immunity can guide the way towards novel therapies for a group of human diseases characterized by nucleotide pool imbalances and aberrant immune signaling that have increasing incidence.

# CONCLUSIONS

1. In-vitro long-term HIV-1 infection induced an antiviral immune response associated to IFN-type I production, interferon stimulated gene activation, and p21-mediated G2/M arrest leading to enhanced cell death in primary macrophages.

2. Whole transcriptome profiling of primary macrophages transduced with Vpx show signatures of innate immune activation that resemble AGS phenotype. Vpx induced innate immune activation may affect reactivation of latent HIV through the degradation of SAMHD1 and TASOR.

3. ADAR1 dysfunction enhances innate immune activation through RIG-I like receptor (RLR)-MAVS signaling pathway, characterized by expression of the RNA sensors, MDA5 and RIG-I, enhanced expression and phosphorylation of IRF7 transcription factor, type I IFN production and induction of IFN-stimulated genes.

4. ADAR1 differently affects viral replication depending on the specific virus–host interaction, acts as a proviral factor in HIV infection, but as antiviral factor in HCV and HPV infections. However, no A-to-I editing in viral RNAs has been detected in our experimental models, suggesting that ADAR1 mediated effect on viral replication is mainly dependent on innate immune activation.

5. Genetic association studies in HIV/HCV and HIV/HPV coinfected cohorts showed that ADAR1 genetic variation is associated to poor clinical outcomes. In the case of HCV, ADAR1 SNPs are linked to non-sustained IFN viral response and liver disease progression. In HPV, the ADAR1 haplotype AACCAT is associated with recurrent HPV-associated dysplasia.

6. Overall, our data indicate that the dysfunction of cellular pathways regulating innate immune activation as those regulated by ADAR1 and SAMHD1 might be relevant in HIV pathogenesis and associated coinfections.

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# ADDENDUM

| **Addendum table 1. List of significant genes after whole transcriptome profiling of Vpx treated MDMs.** | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| | **Gene ID** | **Transcript ID** | **Gene Symbol** | **Description** | **Fc** |
| **Upregulated** | 100532736 | NM_001204088,NM_001204089 | MINOS1-NBL1 | MINOS1-NBL1 readthrough | **8,957241** |
| | 102724594 | NM_001320646,NM_001320648,NM_001320650,NM_001320651 | U2AF1L5 | U2 small nuclear RNA auxiliary factor 1 like 5 | **8,050780** |
| | 6747 | NM_001308197,NM_001308204,NM_001308205,NM_007107 | SSR3 | signal sequence receptor subunit 3 | **3,274068** |
| | 3627 | NM_001565 | CXCL10 | C-X-C motif chemokine ligand 10 | **3,143131** |
| | 10964 | NM_006820 | IFI44L | interferon induced protein 44 like | **2,912268** |
| | 9636 | NM_005101 | ISG15 | ISG15 ubiquitin-like modifier | **2,879171** |
| | 3429 | NM_001130080,NM_001288952,NM_001288954,NM_001288956,NM_0012 88957,NM_001288958,NM_001288959,NM_001288960,NM_001288 995,NM_005532 | IFI27 | interferon alpha inducible protein 27 | **2,770290** |
| | 6606 | NM_000344,NM_001297715,NM_022874 | SMN1 | survival of motor neuron 1, telomeric | **2,759709** |
| | 4599 | NM_001144925,NM_001178046,NM_001282920,NM_002462 | MX1 | MX dynamin like GTPase 1 | **2,731212** |
| | 8519 | NM_003641 | IFITM1 | interferon induced transmembrane protein 1 | **2,619142** |
| | 3434 | NM_001270927,NM_001270928,NM_001270929,NM_001270930,NM_001548 | IFIT1 | interferon induced protein with tetratricopeptide repeats 1 | **2,593367** |
| | 54921 | NM_001039690,NM_001040146,NR_033227 | CHTF8 | chromosome transmission fidelity factor 8 | **2,519729** |
| | 643847 | NM_001079808 | PGA4 | pepsinogen 4, group I (pepsinogen A) | **2,445662** |
| | 23107 | NM_001286748,NM_001286751,NM_015084 | MRPS27 | mitochondrial ribosomal protein S27 | **2,412453** |
| | 55766 | NM_177925,NR_027716 | H2AFJ | H2A histone family member J | **2,405246** |
| | 4061 | NM_001127213,NM_002346 | LY6E | lymphocyte antigen 6 family member E | **2,363089** |

| | | | | | |
|---|---|---|---|---|---|
| | 552900 | NM_001031827,NM_001320579,NR_135304 | BOLA2 | bolA family member 2 | **2,285176** |
| | 91543 | NM_080657 | RSAD2 | radical S-adenosyl methionine domain containing 2 | **2,265230** |
| | 728689 | NM_001099661,NM_001317856,NM_001317857 | EIF3CL | eukaryotic translation initiation factor 3 subunit C like | **2,254579** |
| | 222698 | NM_001007531 | NKAPL | NFKB activating protein like | **2,211527** |
| | 26811 | NR_000015 | SNORD55 | small nucleolar RNA, C/D box 55 | **2,178979** |
| | 100534599 | NM_001204890 | ISY1-RAB43 | ISY1-RAB43 readthrough | **2,127744** |
| | 406992 | NR_029623 | MIR210 | microRNA 210 | **2,122289** |
| | 10410 | NM_021034,NR_049759 | IFITM3 | interferon induced transmembrane protein 3 | **2,121942** |
| | 94240 | NM_001002264,NM_001330543,NM_001331228,NM_033255 | EPSTI1 | epithelial stromal interaction 1 | **2,070398** |
| | 6355 | NM_005623 | CCL8 | C-C motif chemokine ligand 8 | **2,030195** |
| | 2537 | NM_002038,NM_022872,NM_022873 | IFI6 | interferon alpha inducible protein 6 | **2,019397** |
| **Downregulated** | 79137 | NM_001321109,NM_001321110,NM_024293 | RETREG2 | reticulophagy regulator family member 2 | **-2,002860** |
| | 55016 | NM_001166373,NM_017923 | MARCH1 | membrane associated ring-CH-type finger 1 | **-2,120066** |
| | 100533483 | NR_037923 | DNAAF4-CCPG1 | DNAAF4-CCPG1 readthrough (NMD candidate) | **-2,121238** |
| | 3851 | NM_002272 | KRT4 | keratin 4 | **-2,127041** |
| | 7327 | NM_001202489,NM_003343,NM_182688 | UBE2G2 | ubiquitin conjugating enzyme E2 G2 | **-2,207590** |
| | 2113 | NM_001143820,NM_001162422,NM_001330451,NM_005238 | ETS1 | ETS proto-oncogene 1, transcription factor | **-2,269019** |
| | 3860 | NM_002274,NM_153490 | KRT13 | keratin 13 | **-2,382999** |
| | 654364 | NM_001018136,NR_037149 | NME1-NME2 | NME1-NME2 readthrough | **-2,399360** |
| | 6707 | NM_001097589,NM_005416 | SPRR3 | small proline rich protein 3 | **-2,497050** |
| | 102465252 | NR_106761 | MIR6506 | microRNA 6506 | **-2,619781** |

| | | | | | |
|---|---|---|---|---|---|
| | 51132 | NM_016120,NM_183353 | RLIM | ring finger protein, LIM domain interacting | **-3,964455** |
| | 6607 | NM_017411,NM_022875,NM_022876,NM_022877 | SMN2 | survival of motor neuron 2, centromeric | **-5,507686** |
| | 10618 | NM_001206840,NM_001206841,NM_001206844,NM_006464 | TGOLN2 | trans-golgi network protein 2 | **-5,591449** |
| | 3727 | NM_001286968,NM_005354 | JUND | JunD proto-oncogene, AP-1 transcription factor subunit | **-7,184083** |

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