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TESI DI DOTTORATO DI RICERCA

IMMUNOMODULATORY EFFECTS OF NITAZOXANIDE AND RELATED MOLECULES

Settore MED/04 - Patologia Generale

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ABSTRACT

Background: Nitazoxanide (Alinia®, NTZ) and its active circulating metabolite tizoxanide (TIZ) belong to a new class of anti-infective agents active against parasites, anaerobic bacteria, and viruses. Nowadays, NTZ is licensed in the United States for the treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia* in adults and children older than twelve months of age. The amplitude of the spectrum of pathogens targeted by NTZ and new-generation non-nitro thiazolides, makes it very unlikely that the action of these compounds is mediated by a pathogen-specific mechanism(s), suggesting instead that thiazolides act as immunomodulants. To date, the potential effect of these compounds on immune responses has nevertheless not been analysed. In particular, because innate immunity and type 1 interferons are pivotal in early and effective antiviral immune responses that are not antigen-restricted, it is plausible to hypothesize that thiazolides could potentiate this arm of the immune system. To verify this possibility, we performed extensive *in vitro* analyses on the immunomodulatory effects of TIZ and the second generation non-nitro thiazolide RM4848 using two different models of viral infection: Influenza and HIV-1.

Methods*:* Peripheral blood mononuclear cells (PBMCs) from 20 healthy donors were stimulated with influenza virus antigen (FLU) or infected with HIV-1_{BaL} strain and cultured in presence/absence of TIZ or RM4848. Thiazolide effects on innate immunity were examined by evaluating TLRs expression on monocytes, IFN-secretion by dendritic cells, cytokine and chemokine production, mRNA expression of multiple genes involved in TLR, type I IFN and in cholesterol metabolism pathways.

Results: Thiazolides are associated with strong immunomodulatory effects. Notably, these compounds, both in FLU-stimulated and in HIV-1-infected cells, significantly increase: 1) TLR-expression on monocytes, 2) IFN production, 3) chemokine and cytokine production, 4) mRNA expression of different genes operating in the TLR and type I IFN pathways, 5) genes involved in cholesterol metabolism and efflux.

Conclusions: Data herein show that thiazolides are potent type I IFN inducers, triggering a selective activation of several IFN-stimulated gene (ISG) pathway.

Thus, increased expression of innate antiviral factors and the different modulation of genes involved in cholesterol metabolism and efflux suggest a new mechanism of action mediated by thiazolides.

SOMMARIO

Introduzione: La nitazoxanide (Alinia®, NTZ) ed il suo metabolita circolante attivo tizoxanide (TIZ), appartengono ad una nuova classe di farmaci dotati di comprovata attività contro parassiti, batteri anaerobi e virus. Ad oggi, la NTZ è approvata negli Stati Uniti per il trattamento di infezioni causate da *Cryptosporidium parvum* e *Giardia lamblia* nei bambini di età superiore ai 12 mesi e negli adulti. L'ampio spettro di patogeni verso cui questi farmaci risultano efficaci, lascia presupporre che il loro meccanismo d'azione non sia patogeno-specifico ma che agiscano attraverso la modulazione del sistema immunitario. Tuttavia, i potenziali effetti mediati da questi composti a livello della risposta immunitaria devono ancora essere chiariti. In particolare, vista l'importanza dell'immunità innata e degli Interferoni di tipo I nella risposta antivirale antigene-non specifica, è plausibile ipotizzare che i tiazolidi agiscano potenziando questo ramo del sistema immune. Per verificare questa ipotesi, abbiamo effettuato un'approfondita analisi *in vitro* degli effetti immunomodulanti esercitati dalla TIZ e dal RM4848, composto antivirale di seconda generazione, usando due differenti modelli di infezione virale: Influenza e HIV-1.

Metodi: Cellule mononucleate isolate dal sangue periferico (PBMCs) di 20 donatori sani sono state stimolate con antigeni di influenza virus o infettate con HIV-1_{BaL} in presenza o in assenza di TIZ o RM4848. Gli effetti esercitati dai tiazolidi sull'induzione dell'immunità innata sono stati valutati attraverso la quantificazione proteica dei TLR nei monociti, di IFN in cellule dendritiche plasmacitoidi e la produzione di citochine e chemochine. Infine, è stata valutata, utilizzando Real Time PCR array, l'espressione di geni coinvolti nel pathway dei TLR, degli IFN e del metabolismo del colesterolo intracellulare.

Risultati: Complessivamente i tiazolidi mostrano un potente effetto immunomodulante. In particolare, tali composti, aumentano significativamente: 1) l'espressione dei TLR nei monociti, 2) la produzione di IFN, 3) la produzione di chemochine e citochine, 4) l'espressione di diversi geni coinvolti nel pathway dei TLR e in quello degli IFN, 5) l'espressione di geni coinvolti nel metabolismo del colesterolo.

Conclusioni: I risultati ottenuti dimostrano che i tiazolidi sono potenti induttori degli IFN di tipo I e innescano una selettiva attivazione di diversi geni IFN-dipendenti. Pertanto, l'aumentata espressione di fattori antivirali appartenenti all'immunità innata e la diversa modulazione di geni coinvolti nel metabolismo e nell'efflusso del colesterolo intracellulare suggeriscono un meccanismo d'azione del tutto nuovo mediato dai tiazolidi.

LIST OF ABBREVIATION

7-AAD: 7-aminoactinomicyn D
ABC: ATP-binding cassette
AGS: Aicardi-Goutières Syndrome
AIDS: acquired immune deficiency syndrome
AP: activator protein
Apo: apolipoprotein
ATP: adenosine tri-phosphate
CARD: caspase activation and recruitment
CCR: CC chemokine receptor
cEVR: complete EVR
CMV: cytomegalovirus
Ct: threshold cycle
CTLs: cytotoxic T lymphocytes
CXCR: CXC chemokine receptor
DAMP(s): damage-associated molecular pattern molecule(s)
DC(s): dendritic cell(s)
dNTP(s): deoxy-nucleotide triphosphate(s)
dsRNA: double-stranded RNA
eIF: eukaryotic translation initiation factor
ETR: end of treatment response
EVR: early virologic response
FADD: Fas-associated protein with death domain
FBS: Fetal Bovine Serum
FLU: influenza
GAS: gamma interferon activation site
GSTP: glutathione S-transferase pi gene
HA: hemagglutinin
HAART: Highly Active Antiretroviral Therapy
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
HSV: herpes simplex virus
IFIT: interferon induced protein with tetratricopeptide repeat motifs
IFITM: IFN-induced transmembrane protein
IFN(s): interferon(s)
IFNAR: interferon alpha receptor
IFNGR: interferon gamm receptor
IL: interleukine
INSIG: insuline-induced gene
IPS-1: interferon-b promoter stimulator 1
IRAK: interleukin-1 receptor-associated kinase
IRF: interferon regulatory transcription factor
ISG(s): interferon-stimulated gene(s)
ISRE(s): interferon-sensitive reponse element(s)
JAK(s): janus kinase(s)
JNK(s): c-Jun N-terminal kinase(s)
LPS: lipopolysaccharide

LRE: LXR response element
LRR: leuchine-rich repeat
LXR(s): liver X receptor(s)
MAPK(s): mitogen-activated protein kinase(s)
MAVS: mitochondrial antiviral signaling protein
MDA5: melanoma differentiation-associated gene 5
MFI: man fluorescence intensity
MHC: Major Histocompatibility Complex
MIP-1α and β: macrophage inflammatory protein-1 α and β
MTT: tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5diphenyl formazan
MX1 or MX2: Interferon-induced GTP-binding protein 1 or 2
MyD88: myeloid differentiation primary response 88
NA: neuraminidase
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NK(s): natural killer(s)
NLRs: NOD-like receptor(s)
NNRTIs: non-nucleoside reverse transcriptase inhibitors
NOD: nucleotide-binding oligomerization domain
NRTIs: nucleoside reverse transcriptase inhibitors
NTZ: nitazoxanide
P/S: Penicillin and streptomycin
PAMP(s): pathogen-associated molecular pattern molecule(s)
PBMC(s): peripheral blood mononuclear cell(s)
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PFOR: pyruvate-ferredoxin oxidoreductase
PHA: phytohemagglutinin
PI: protease inhibitors
PKC: protein kinase C
PRR(s): pattern recognition receptor(s)
PYD: pyridine domain
RANTES: regulated on activation, normal T cell expressed and secreted
RD: repressor domain
RIG-I: retinoic acid-inducible gene 1
RLR(s): RIG-I-like receptor(s)
ROS: reactive oxygen species
RT: reverse transcriptase
RVR: rapid virological response
RXR(s): retinoid X receptor(s)
SBE: STAT-binding element
SCAP: (SREBP) cleavage-activating protein
SOC: standard of care
SREBP: sterol regulatory element–binding protein
ssRNA: single-stranded RNA
STAT: signal transducers and activators of transcription
STING: stimulator of interferon genes
SVR: sustained virological response
TAK1: transforming growth factor-β-activated protein kinase 1
$TCID_{50}$: tissue culture infectious dose

TICAM: toll-like receptor adaptor molecule
TIZ: tizoxanide
TLR(s): Toll Like receptor(s)
TNF: tumor necrosis factor
TNFR: tumor necrosis factor receptor
TRADD: TNFR-associated death domain
TRAF: TNF-receptor associated factor
TRAP: translocation-associated protein
TRIM5α: tripartite motif 5α
TZD(s): thiazolide(s)

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INTRODUCTION

1. THIAZOLIDES

1.1. History

The discovery of thiazolides finds its origin in the mid-1970s [1]. During that period, nitroimidazoles were developed for treatment of parasitic diseases, including trichomoniasis, giardias and amebiasis, with metronidazole as the main compound [2]. Afterwards, benzimidazole carbamate derivatives, as mebendazole and albendazole, profoundly improved the treatment of intestinal parasitic infection caused by nematodes [3]. The discovery and development of praziquantel provided the first broad spectrum anthelminthic compound, effective against schistosomes and intestinal cestode infections [4].
In developing countries, the need for antiparasitic drugs with broad spectrum activity against protozoa and helminthes arose from the difficult diagnose due to the simultaneous infection with several intestinal pathogens. A product provided with this multi-functional characteristics did not exist. Albendazole was eventually discovered to be effective in treating giardiasis, but not until the mid-1980s [5]. Thus, Rossignol et all. of the Romark Institute for Medical Research (Tampa, Florida) used the scaffold niclosamide, a commercially available cestocidal anthelminthic, to design nitazoxanide (NTZ), originally known as PH5776 (**fig. 1**) [1].

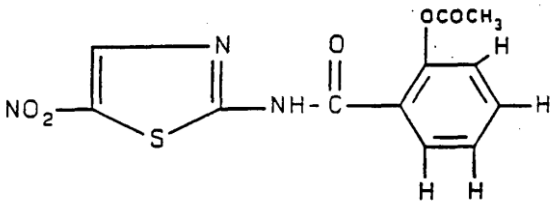


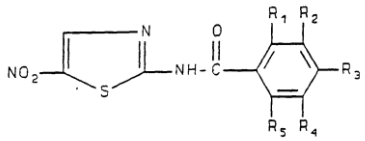
Figure 1 *NTZ structure* [Rossignol J.F., *United States patent* 1976]

A nitrothiazole ring was selected to resemble a nitroimidazole derivative with the expectation to be effective against protozoa whereas the salicylate portion of the molecule was left unchanged [1].
Extensive *in vitro* tests of NTZ, confirmed its efficacy against nine different intestinal parasites of mice, cats, dogs and sheep [6]. Moreover, NTZ was found to be effective *in vitro* and in animal models against *Trichomonas vaginalis* and *Entamoeba histolyca* [7]. Development of NTZ was set aside in the early 1980s, and over the next 10 years antiparasitic drug development focused on albendazole, a single dose anthelmintic with exceptional activity against intestinal nematodes and some cestodes [8]. In the attempt to address the emergence of new opportunistic protozoan infection in AIDS (Acquired Immunodeficiency Syndrome) patients, mainly *Cryptosporidium parvum*, the development of NTZ started again in early 1990s. After demonstrating its efficacy against *C. parvum* in cell cultures and in several animal models [9], NTZ received regulatory approval in the US in 2002 for treatment of *Criptosporidium parvum* and *Giardia intestinalis* infections in non-immunodeficient children and adults [10].
In vitro analysis for antiviral activity led to the discovery of NTZ's efficacy against a wide range of DNA and RNA virus including Hepatitis B Virus (HBV) and Hepatitis

C Virus (HVC) [11-12]. Results from clinical studies concerning the role of NTZ in viral infections treatment motivated the search for new compounds with exclusive activity against viruses. Extensive studies about the mechanism of action of this compound identified the nitro group as responsible for the anti-protozoa and –bacteria properties [13]. Thus, the substitution of the nitro group in the 5-position of the thiazole ring by an electrophilic non-reducible function led to the synthesis of second-generation thiazolides with higher efficacy against viruses [14-15]. To date, NTZ and new compounds are extensively tested in human clinical trials for the treatment of different pathological conditions.

1.2. Pharmacology of thiazolides

NTZ is a synthetic nitrothiazolyl-salicylamide derivative. Chemically it is 2-acetyloxyl-N-(5-nitro-2 thiazolyl) benzamide, composed of a nitrothiazole-ring and a salicylic acid moiety which are linked together by an amide bond. Derivatives of NTZ are those in which the acyloxy group, represented in **tab.1** by the R_1, R_2, R_3, R_4 and R_5 symbols, is an acetoxy or propionoxy group, whereas the alkaloxy group is the methoxy group and the halogen is chlorine or bromine [15].



		Thiazole Ring Substituents		Benzene Ring Substituents			
Agent	MWt	X	Y	R_1	R_2	R_3	R_4
NTZ	307.3	NO_2	H	OAc	H	H	H
TIZ	265.2	NO_2	H	OH	H	H	H
RM5038	296.7	Cl	H	OAc	H	H	H
RM4848	254.7	Cl	H	OH	H	H	H

Table 1 *– Chemical structures of NTZ and some halogeno thiazolide agents*

Thus, the elimination of the nitro group in second generation thiazolides resulted in a significant advance for this class of drugs, given its poor reputation from a toxicological perspective.

NTZ, commercialized with the brand name Alinia® (Romarck Laboratories) is formulated as 500 mg tablets and as a 100 mg per 5ml suspension for oral administration (**fig. 2**). The recommended dosage is: 100 mg (by oral suspension) every 12 hours for 3 days for children aged 1 to 3 years; 200 mg (by oral suspension) every 12 hours for 3 days, for children aged 4 to 11 years; 500 mg (oral suspension or oral tablets) every 12 hours for 3 days, for children older than 12 years or adults [16].

Clinical pharmacology studies showed that NTZ was actually well absorbed in humans [17-19]. A study conducted in six human volunteers receiving a single 500 mg p.o. dose of ^{14}C-labeled NTZ in capsules in fasted conditions showed that 33% of the drug was excreted in urine whereas the rest was excreted with feces over a period of 240 h [18]. Notably, it was demonstrated that the administration of NTZ tablets with food increases the oral absorption by ~50% [18].

Figure 2 *– Alinia® Romark Lab. (Tampa, Florida)*

Moreover, NTZ revealed good metabolic stability. The drug undergoes rapid deacetylation in plasma (half-life of 6 minutes) to form tizoxanide (TIZ), which is at least as active as NTZ against anaerobes and viruses [17-19]. TIZ is glucurono-conjugated in the liver, and the drug is excreted as TIZ or TIZ-glucuronide in urine and bile. TIZ-glucuronide albeit it is essentially an excretion metabolite, retains some moderate antimicrobial properties of the parental compound [18]. Plasma concentration of TIZ and TIZ-glucuronide achieves maximum between 1 to 4 hours following ingestion [17-19]. Active metabolites are essentially completely protein bound (>99%), whereby caution should be used when administering NTZ simultaneously with other highly plasma protein-bound drugs, as competition for binding sites may occur (e.g., warfarin) [17-19]. *In vitro* metabolic studies have demonstrated that TIZ has no significant inhibitory effect on cytochrome P450 enzymes [19-20]. Thus, it is expected that no significant interaction would occur when NTZ is administered with drugs that are metabolized by or inhibit this enzymes. NTZ is generally well tolerated, and no significant adverse events have been recorded in human trials. Side effects are mild and transitory and principally related to the gastrointestinal tract, such as abdominal pain, diarrhea, and nausea. In addition, no significant changes have been reported in relation to electrocardiography, vital signs, or hematologic, clinical chemistry, or urinalysis parameters in patients treated with NTZ [20].

In subacute and chronic toxicity studies, NTZ showed no toxicity in rats when administered at doses < 450 mg/kg/day for 6 months, 30 times the proposed human dose [20-21]. In a 28-day subacute toxicity study, doses of < 2700 mg/kg/day p.o. determined significant and limiting toxicity for the gastrointestinal tract. Interestingly, these dose levels did not produce systemic toxicity [20-21]. Subsequently, a 9-month chronic study showed that administration of 60 mg/kg/day, 4 times the proposed human dose, did not produce toxicity in dogs [Romarck Laboratories, pers. commun.]. No embryotoxic or teratogenic effects resulted from studies in different animal models, and the drug received approval in the US with a pregnancy category B classification [21].

NTZ and TIZ showed weak positive results in the Ames test using TA_{100} strain of *Salmonella typhimurium*, but neither NTZ nor TIZ were found to be mutagenic [21].

New generation thiazolides show a very similar metabolic profile. These drugs are rapidly hydrolyzed in plasma to form their active de-acetylated metabolites.

Like NTZ and TIZ, second generation thiazolides family members are highly bound to plasma proteins and do not interact with the important CYP enzymes, suggesting a low potential for drug interaction (Romark Lab, pers. Commun.).
Preliminary subacute toxicity studies in rats and dogs with the second generation thiazolide RM4865, have shown very little systemic toxicity and better tolerability in dogs than that observed with NTZ. As with other thiazolides, RM4865 is also not mutagenic in the Ames test (Romarck Laboratories, pers. Commun.).

1.3. In vitro activity

1.3.1. Protozoa and anaerobic bacteria

In humans, NTZ has been reported to be effective against a broad range of parasites, including *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Trichomonas vaginalis*, *Vittaforma corneae*, *Encephalitozoon intestinalis*, *Isospora belli*, *Blastocystis hominis*, *Balantidium coli*, *Enterocytozoon bieneusi*, *Ascaris lumbricoides*, *Trichuris trichura*, *Taenia saginata*, *Hymenolepis nana*, and *Fasciola hepatica* [2,7,9,10,22-32]. *In vitro* studies have also shown antimicrobial activity against numerous gram-positive and gram-negative anaerobic bacteria and against aerobic gram-positive bacteria [33-34].
Studies of protozoa and anaerobic bacteria have shown that NTZ interferes with pyruvate-ferrodoxin oxidoreductase (PFOR) enzyme dependent electron transfer reaction which is important for anaerobic glucose energy metabolism [13-14]. This results in cell swelling, membrane damage and vacuole injury and in the consequently dysfunction of the parasite. As this activity depends on the nitro group, second generation thiazolides might not be effective against anaerobic bacteria and protozoa that affects human [14]. Nevertheless, inhibition of PFOR enzyme might not be the only pathway by which NTZ exhibits antiprotozoal activity, and the mechanism of NTZ's activity against helminthes is still unknown.
In vitro studies have shown NTZ ability to inhibit the growth of sporozoites of *C. Parvum* on its own, and have also established a combined *in vitro* activity with both azithromycin and rifampin, against *C. Parvum* by 83.9% and 79.8%, respectively, compared with 56.1% when used alone [26]. Similarly, *in vitro* studies of NTZ and TIZ, revealed greater efficacy than metronidazole against *G. intestinalis* [26]. The antimicrobial properties of NTZ and TIZ have been verified against 241 anaerobes, the majority of which were inhibited *in vitro*, with an MIC90 between 0.06 mg/L and 4 mg/L [33]. NTZ has also shown *in vitro* and *in vivo* antimicrobial activity against *Clostridium difficile* [32] and both metronidazole-susceptible and metronidazole-resistant strains of *H. pylori* [34,35].

1.3.2. Virus

NTZ, TIZ and new generation thiazolides have been shown to inhibit replication of rotavirus, HBV and HCV in a selective and dose-dependent manner [11,12]. Thiazolides have also been reported to be active against influenza A virus, Sendai virus, respiratory syncytial virus, coronavirus, vescicular stomatitis virus, adenovirus and herpes simplex virus type I [36,37].
The antiviral activity of the thiazolides is distinct from the one against anaerobic protozoa and bacteria that has been related to interference with PFOR enzyme. Although the mechanism remains to be clarified, these drugs showed interesting

characteristics in laboratory studies that indicate a novel device that could be complementary to other antiviral drugs.
Studies in rotavirus have shown that NTZ is cytoprotective [38]. Studies in HBV have demonstrated that NTZ inhibits production of the HBV surface antigens [11]. NTZ is also synergistic with lamivudine and adefovir against HBV, and it is active against lamivudine- and adefovir-resistant isolates of HBV [11]. Studies in HCV replicons have shown that NTZ is synergistic with interferon alpha, telaprevir (a protease inhibitor) and 2'C-methyl cytidine (a polymerase inhibitor) and that it is active against telaprevir- and 2'C-methyl cytidine-resistant mutants [11,39]. Interestingly, HCV replicons exposed to NTZ and subsequently treated with interferon show significantly greater sensitivity to the interferon antiviral effects [11, 40]. Studies in HCV replicons have shown that NTZ does not induce viral mutations that could promote resistance development [40]. Efforts to select HCV replicons resistant to NTZ resulted in a significant increase in cytotoxic concentrations suggesting a mechanism of action that is host cell-mediated [40].
Finally, NTZ induces phosphorylation of eukaryotic initiation factor-2α (eIF2α) via activation of the protein kinase activated by double-stranded RNA (PKR), a critical IFN-induced antiviral response mediators [41].
Teams of researchers are studying the mechanism of antiviral activity of thiazolides. Preliminary findings about the potent anti-influenza A activity exerted by thiazolides suggest that they act a post-translational level by selectively blocking hemagglutinin (HA) maturation, without directly affecting virus infectivity [37]. It was demonstrated that TIZ impairs HA trafficking between the ER and the Golgi complex, preventing its transport and insertion into the host cell plasma membrane and blocking the exit of mature virions [37]. Targeting the maturation of the viral HA offers the opportunity to interfere with viral particle production acting at a different level from the currently available anti-influenza drugs.

1.3.3. Cancer

Although NTZ was originally designed as an anti-microbial drug, anti-cancer properties have also been observed. It has been established that NTZ and/or bromo-thiazolide RM4819 treatment inhibits colon cancer cells proliferation *in* vitro, by binding to glutathione S-transferase P1 protein (GSTP1) [42]. GSTP1 protein expression has been recently correlated to tumor tissue and ovarian cancer prognosis [43]. In particular high levels of GSTP1 in tumor cells may greatly limit the efficacy of antitumor chemotherapy [43].
Furthermore, NTZ has shown also anti-Myc activity [44].The c-Myc oncogene is overexpressed and amplified in the majority of human cancers and and it is inversely correlated with prognosis. c-Myc controls cell proliferation and is critically involved in the regulation of many growth-promoting signal transduction pathways [44]. In particular, it is established its role in complex inflammatory responses leading to the recruitment of various inflammatory cells with pro-tumorigenic behavior. In breast cancer xenograft mouse models, NTZ significantly suppressed tumor growth by inhibiting c-Myc and inducing apoptosis [44]. These findings support NTZ's potential as a new, anti-tumor agent for the inhibition of c-Myc associated neoplasia.

1.4. NTZ for the treatment of viral infections: human experience [Romark Lab. personal communications]

The safety and tolerability of NTZ in humans has been documented by >10 years of its commercial use. More than 20 million people have been treated with NTZ in post-marketing experience, most of them for relatively short durations ranging from 3 to 10 days. In clinical trials > 700 patients have received much longer treatments without significant drug-related side effects.

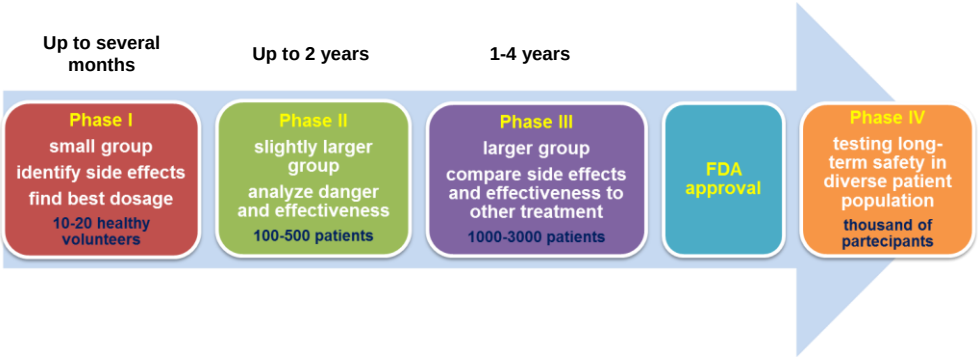


Figure 3 *– Clinical trial phases*

1.4.1. Completed Studies

1.4.1.1. Hepatitis C Virus infection treatment: STEALT C Clinical Development Program and OPTIMA HCN program

The first Phase II randomized, controlled trial is included in the STEALTH C (Studies to Evaluate Alinia for Treatment of Hepatitis C) clinical development program, designed to estimate the safety and efficacy of NTZ tablets in combination with peg-interferon or peg-interferon and ribavirin (standard of care, SOC) in HCV patients. In this trial, conducted at two centers in Egypt, 96 treatment-naive patients with HCV genotype 4 were randomly assigned into three groups to receive either 48 weeks of SOC treatment (n=40), 12 weeks of NTZ followed by 36 weeks of NTZ plus peginterferon (a dual regimen, n=28), or 12 weeks of NTZ followed by 36 weeks of NTZ plus SOC treatment (a triple regimen, n=28). An additional 24 patients previously interferon-treated were randomized to receive 12 weeks of NTZ followed by either the dual regimen (n=12) or the triple regimen (n=12) for 36 weeks.

Naïve patients receiving the triple regimen showed a significantly higher sustained virological response (SVR: HCV RNA < 10 IU/mL, Abbott m2000) than the group receiving the SOC regimen (79% vs. 43%, respectively) (p=0.006) 12 weeks after the end of the treatment. At the same time, patients treated with the dual regimen showed a SVR not inferior to SOC (68% vs. 43%, respectively) (+25%; 95% CI: -1%, +47%). Of 24 treatment-experienced patients, the triple regimen (n=12) resulted in an SVR of 25% at week 12 post-treatment, and the dual regimen group (n=12) had an SVR of 8%.

STEALTH C1[2] phase II trial was) was planned to evaluate the possibility to reduce the lead-in phase from 12 to 4 weeks. Therefore, 44 patients (40 with HCV

genotype 4; 3 with HCV genotype 1; and 1 with HCV genotype 2) received 4 weeks of NTZ 500 mg twice daily followed by Pegasys® (peginterferon alfa-2a) and NTZ for 36 weeks. STEALTH C1 trial was used as an historical control. The 80% (44) of patients treated with a 4-week lead-in phase of NTZ followed by the addition of peginterferon for 36 weeks reached a SVR 12 weeks after the end of treatment compared to 50% in the SOC historical control group (*P*=0.004), 61% in patients receiving a 12-week lead-in with NTZ followed by 36 weeks of NTZ plus peginterferon, and 79% in patients receiving a 12-week lead-in with NTZ followed by 36 weeks of NTZ plus SOC. Of the 44 patients in the study, 78% (n=40) of patients with HCV genotype 4, 100% (n=3) of patients with HCV genotype 1, and 100% (n=1) of HCV genotype 2, had undetectable virus at 12 weeks after the end of treatment.

STEALTH C2 double-blind, placebo controlled trial enrolled US patients with genotype 1 chronic hepatitis C who had previously failed to respond (never achieved undetectable HCV RNA) to peginterferon plus ribavirin. 64 patients were randomized 2:1 to receive NTZ 500 mg twice daily for 4 weeks followed by 48 weeks of NTZ plus standard therapy (Pegasys® and Copegus®, F. Hoffman LaRoche) or placebo for 4 weeks followed by 48 weeks of placebo plus standard therapy. The 7% (3/42) of patients treated with NTZ plus standard therapy reached SVR 24 week following the end of treatment compared to 0% (0/22) of patients treated with placebo plus standard therapy.

Results from others endpoints concerning rapid virological response (RVR), complete early virologic response (cEVR), early virological response (EVR) and end of treatment response (ETR) are shown in **Tab. 2**. No serious adverse event has been related to NTZ.

Subjects	RVR	cEVR	EVR	ETR	SVR
NTZ + PR (n=42)	2 (5%)[1]	3 (7%)	16 (38%)	6 (14%)	3 (7%)[2]
Placebo + PR (n=21)[3]	0	0	6 (29%)	0	0

PR= Peginterferon alfa-2a (Pegasys®) and Ribavirin (Copegus®); RVR= serum HCV RNA <10 IU/mL after 4 weeks of combination therapy; cEVR= serum HCV RNA <10 IU/mL after 12 weeks of combination therapy; EVR= 2 log10 decrease in HCV RNA after 12 weeks of combination therapy; ETR= serum HCV RNA <10 IU/mL at end of treatment.
[1]One patient with RVR showed a 1.83 log10 decrease in HCV RNA during 4 weeks of monotherapy.
[2]All 3 patients with SVR had a cEVR, 2 of whom had an RVR;
[3]One subject was a prior relapser and excluded from efficacy analysis.

Table 2 *– STEALTH-C2 study: results [Romark pers. comm.]*

The last of this series of clinical trials is the randomized double-blind placebo controlled trial STEALTH C3. This randomized, double-blind, placebo controlled trial enrolled patients with genotype 1 chronic hepatitis C, 35% of whom had advanced stage 3 or 4 fibrosis from thirteen centers in the United States 112 patients were randomized to receive either NTZ (500 mg twice daily) plus peginterferon alfa-2a (Pegasys®, F. Hoffman LaRoche) and ribavirin (Copegus®, F. Hoffman LaRoche) (n=75) or placebo plus Pegasys® and Copegus® (n=37). SVR 24 week after the end of the treatment occurred in 44% of patients treated with NTZ plus standard therapy for 48 weeks versus 32% of patients treated with placebo plus standard therapy.

In patients with high baseline viral load 41% vs. 29%) and in African Americans (38% vs. 20%), SVR rates were consistently higher. Moreover, the rate of serious adverse event were similar for the NTZ and placebo treatment groups.
Subsequently, Romark Lab., performed two different trials, as part of Romark's OPTIMA HCN (OPTImizing Management of Hepatitis C with NTZ) program. OPTIMA HCN-1, a ramdomized, double blind crossover phase 1 study enrolled a total of 12 healthy adults volunteers in order to study pharmacokinetic following oral administration of 675 mg or 1350 mg of NTZ twice daily with food for seven days.
This two different doses of controlled release NTZ produced 3x and 12x plasma concentrations of TIZ compared to that observed in historical studies using a standard NTZ 500 mg tablet. Controlled release NTZ showed favorable safety and tolerability during the course of the study, with slight to moderate adverse events reported.
Phase II study, OPTIMA HCN-2, a total of 41 treatment-naïve patients with chronic hepatitis C genotype 4 were randomized to receive NTZ at 675 mg (n=17), NTZ at 1350 mg (n=16) or placebo (n=8) twice daily for four weeks followed by the same regimen plus SOC with peginterferon alfa-2a (Pegasys®; 180 micrograms once per week) and ribavirin (Copegus®; 1,000 or 1,200 mg daily according to body weight) for 36 weeks (48 weeks for the placebo arm). 59% and 63% of patients treated with the low and the high dose of NTZ showed RVR (HCV RNA <12 IU/mL after 4 weeks of combination therapy), compare with 50% for the placebo group; 82% and 100%, respectively, showed cEVR (HCV RNA <12 IU/mL after 12 weeks of combination therapy) compared with 63% for the placebo group; 88% and 100%, respectively, showed early virologic response (EVR, =2 log10 decline in HCV RNA after 12 weeks of combination therapy) with 63% for the placebo.
Moreover, a dose-related decrease in serum HCV RNA was observed starting on day 4 of combination therapy and was maintained till week 16. No serious adverse events have been reported following NTZ treatment.

1.4.1.2. Influenza Virus treatment: clinical trial

Two Phase 2 clinical trials, one in pediatric patients age 1 to 11 years, and another in adults and adolescents age 12 – 65 years, were conducted in Cajamarca, Peru. Patients were enrolled based upon influenza symptoms (fever, at least one respiratory symptom and one constitutional symptom) and randomized to receive treatment with NTZ or placebo in double-blind manner.
One hundred children (median age 3 years) enrolled in the pediatric trial (50 per treatment group), received 100 mg NTZ (age 12 – 47 months), 200 mg NTZ (age 4–11 years) or placebo twice daily for five days as an oral suspension.
Heighty-six adults and adolescents enrolled in the second trial (43 per treatment group)received 500 mg NTZ or placebo twice daily for five days as an oral tablet.
A statistically significant reduction in symptom duration has been obtained following treatment with NTZ compared to placebo, in both trials. In the pediatric study, median time from first dose to alleviation of symptoms was 4 days for the NTZ treatment group compared to >7 days for the placebo treatment group (P<0.0001). In the study involving adults and adolescents, median time from first dose to alleviation of symptoms was 4 days compared to 7 days for the placebo treatment group (P=0.037). In the pediatric study, 60% of the patients in the placebo treatment group required post-study antibiotics compared to only 8% in the NTZ

treatment group (P<0.0001). In the adult/adolescent study, 36% of the patients in the placebo treatment group required post-study antibiotics compared to 18% in the NTZ treatment group (P=0.13). In each study, adverse events were similar between the treatment groups.

1.4.2. Ongoing studies

A global Phase 3 clinical trial of NTZ-300 for the treatment of acute uncomplicatedhas been started in April 2013 in the United Stated, in order to provide data for marketing approval. This study will extend in Australia and New Zealand and is expected to be completed during the 2013-2014 FLU season in the northern hemisphere. Moreover, the aim is also to combine treatment with NT-300 and oseltamivir in an effort to improve treatment and to mitigate the risk of oseltamivir resistance.

The clinical trial will enroll a total of 1400 patients with fever and other symptoms of influenza. Patients are being randomized to receive treatment with either NT-300, placebo (sugar pill), oseltamivir (Tamiflu®) or NT-300 plus oseltamivir. The primary objectives of the study are to evaluate the reduction of symptoms duration. Currently, there are only two classes of drugs approved in the United States for treating influenza, and only the neuraminidase inhibitors, oral Tamiflu® (oseltamivir) and inhaled Relenza® (zanamivir), are recommended for use. New drugs with different mechanisms of action could be important in overcoming drug resistance and providing better treatment for patients with influenza. The Phase 3 clinical trial of NT-300 will be fully funded by the U.S. Department of Health and Human Services (HHS)/Office of the Assistant Secretary of Preparedness and Response (ASPR)/Biomedical Advanced Research and Development Authority (BARDA).

2. VIRAL INFECTIONS

2.1. Influenza (FLU) virus

Influenza viruses are among the most common causes of human respiratory infections [45] with an high morbidity and mortality. Up to 50% of the population can be infected in a single pandemic year, and the number of deaths caused by influenza can dramatically exceed what is normally expected [46].
Influenza viruses, of the family *Orthomyxoviridae,* are enveloped negative-strand RNA viruses with segmented genomes containing seven to eight gene segments [47] (**fig. 4**).

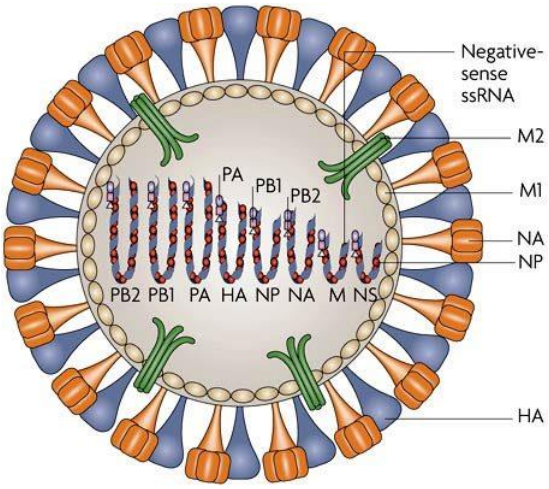


Figure 4 *- The structure of influenza A virus* [Nelson M.I., *Nat Rev Gen*, 2007]

One genus includes influenza A and B viruses, and the other comprises influenza C viruses. The three virus types differ in host range and pathogenicity. Type B and C influenza viruses are isolated almost exclusively from humans, although influenza B viruses have been isolated from seals and influenza C viruses have been isolated from pigs and dogs.
Influenza A and B viruses have a similar structure, whereas influenza C is more divergent. The virus particle is 80–12 nm in diameter and usually roughly spherical, although filamentous forms can occur. These filamentous forms are more common in influenza C, which can form cordlike structures up to 500 micrometres long on the surfaces of infected cells.
A and B type viruses contain eight discrete gene segments, each of them coding for at least one protein, and are covered with projections of three proteins: hemagglutining (HA), neuraminidase (NA) and M2. For example, the influenza A genome contains 11 genes on eight pieces of RNA, encoding for 11 proteins: HA, NA, NP, M1, M2, NS1, NS2, PA, PB1, PB1-F2 and PB2. Influenza C viruses have seven segments and only one surface glycoprotein (**fig.5**).

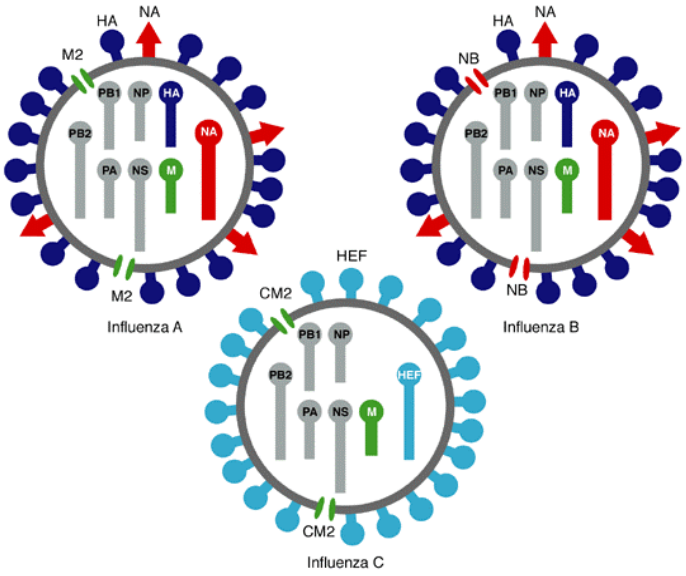


Figure 5 *- Genome organization of FLU viruses* [Kumar V., *Robbins Basic Pathology*, 2007]

HA and NA are the two large glycoproteins on the outside of the viral particles. HA is an integral membrane glycoprotein, constituted by three identical monomers constructed into a central α-helix coil; three spherical heads contain the sialic acid binding sites. HA monomers are synthesized as precursors (HA0) that are then cleaved into two smaller polypeptides: the HA1 and HA2 subunits. HA has two functions. Firstly, it allows the recognition of target cells, accomplished through the binding of these cells sialic acid-containing receptors. Secondly, once bound, it facilitates the entry of the viral genome into the target cells by causing the fusion of host endosomal membrane with the viral membrane [48].
NA exists as a projection on the surface of the influenza virus. It has a head consisting of four subunits, and a hydrophobic region that is embedded within the interior of the virus membrane. NA catalyzes the hydrolysis of terminal sialic acid residues from the newly formed virions and from the host cell receptors.
Viruses can only replicate in living cells. Influenza infection and replication is a multi-step process [49] (**fig. 6**). Influenza viruses bind through HA to sialic acid residues on the surface of its target cells. Upon binding, receptor-mediated endocytosis occurs and the virus enters the host cell in an endosome. The cell then attempts to begin digesting the contents of the endosome by acidifying its interior and transforming it into a lysosome. The low pH induces a conformational change in HA0, leading to maintenance of the HA1 receptor-binding domain but exposing the HA2 fusion peptide. This fusion peptide inserts itself into the endosomal membrane, bringing both the viral and endosomal membranes into contact with each other and causing the two to fuse together.
Finally the M2 ion channel allows protons to move through the viral envelope and acidify the core of the virus, which causes the release of viral RNA, accessory proteins and RNA-dependent RNA polymerase into the cytoplasm. These proteins

and viral RNA are transported into the cell nucleus, where the RNA-dependent RNA polymerase begins transcribing complementary positive-sense viral RNA. Viral RNA is either exported into the cytoplasm, and translated, or remains in the nucleus. Newly synthesised viral proteins are either secreted onto the cell surface or transported back into the nucleus to bind viral RNA and form new viral genome particles. Negative-sense viral RNAs that form the genomes of future viruses, RNA-dependent RNA polymerase, and other viral proteins are assembled into a virion.

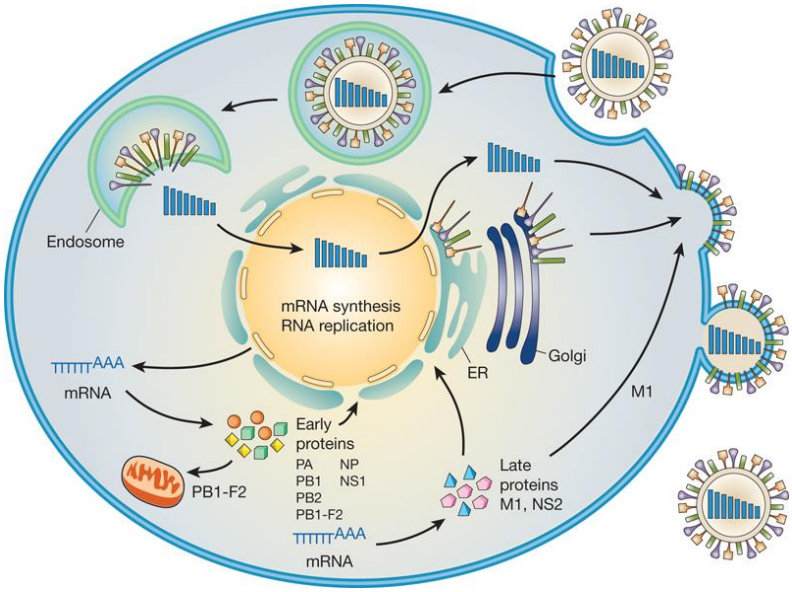


Figure 6 – *Influenza virus life-cycle* [Neumann G., *Nature*, 2009]

HA and NA molecules cluster into a bulge in the cell membrane. The viral RNA and viral core proteins leave the nucleus and enter this membrane protrusion. The mature virus buds off from the cell in a sphere of host phospholipid membrane, acquiring HA and NA with this membrane coat. NA promotes the release of progeny viruses and the spread of the virus from the host cell to uninfected surrounding cells. It also cleaves sialic acid residues from viral proteins, preventing aggregation of viruses (**fig. 7**). Thus, its activities include assistance in the mobility of virus particles through the respiratory tract mucus and in the elution of virion progeny from the infected cells. After the release of new influenza viruses, the host cell dies.

Influenza viruses accumulate point mutations during replication because their RNA polymerase complex has no proofreading activity. Their genes have high mutation rates (ranging from approximately 1×10^3 to 8×10^3 substitutions per site per year). Mutations that change amino acids in the antigenic portions of surface glycoproteins may produce selective advantages for viral strains by allowing them to evade pre-existing immunity.

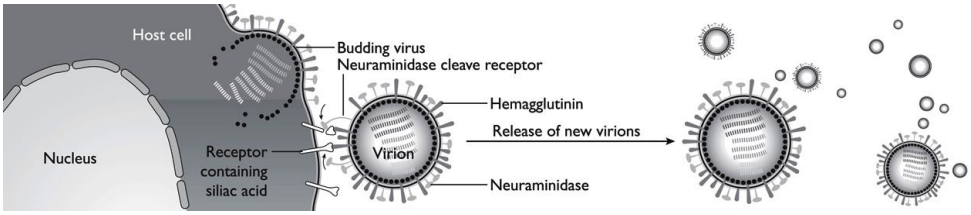


Figure 7 – *Influenza virus neuraminidase activity* [Moscona A., *N Engl J Med*, 2005]

The HA molecule initiates infection by binding to receptors on specific host cells. Antibodies against the HA protein prevent receptor binding and are effective at preventing re-infection with the same strain. The HA and NA can evade previously acquired immunity by either antigenic drift, in which mutations limit or prevent antibody binding, or antigenic shift, in which the virus acquires HA of a new subtype by re-assortment between two influenza A viruses [50]. Antigenic drift occurs in all types of influenza including influenza virus A, influenza B and influenza C. Antigenic shift, however, occurs only in influenza virus A because it infects more than just humans. Affected species include other mammals and birds, giving influenza A the opportunity for a major reorganization of surface antigens. Influenza B and C principally infect humans, minimizing the chance that a re-assortment will change its phenotype drastically. Although an antigenically novel HA subtype is a likely requirement for the emergence of an influenza pandemic, human infections with animal-adapted influenza virus of novel HA subtype have been observed in which the virus is not transmitted efficiently from person to person, suggesting that stable adaptation to humans by re-assortment or whole genome adaptation is required for the emergence of a pandemic strain.

Influenza is an acute respiratory disease characterized in its full form by the sudden onset of high fever, nasal congestion, cough, headache, prostration, malaise and inflammation of the upper respiratory tree and trachea. In most cases, pneumonic involvement is not clinically prominent. Acute symptoms and fever often persist for 7 to 10 days. Weakness and fatigue may linger for weeks.

People with chronic pulmonary or cardiac disease, or diabetes mellitus, are at high risk of developing severe complications from influenza A viruses, which may include hemorrhagic bronchitis, pneumonia (primary viral or secondary bacterial) and death. Fulminant fatal influenza viral pneumonia occasionally occurs; dyspnea, cyanosis, hemoptysis, pulmonary edema and death may proceed in as little as 48 hours after the onset of symptoms.

The mechanisms by which influenza infection causes symptoms in humans have been studied intensively. Knowing which genes are carried by a particular strain can help predict how well it will infect humans and how severe this infection will be [51] from influenza-infected cells. For instance, part of the process that allows influenza viruses to invade cells is the cleavage of the viral HA protein by any one of several human proteases. In mild and avirulent viruses, the HA can only be cleaved by proteases found in the throat and lungs, so these viruses cannot infect other tissues. However, in highly virulent strains, such as H5N1, the HA can be cleaved by a wide variety of proteases, allowing the virus to spread throughout the body. Strains that are easily transmitted between people have HA proteins that

bind to receptors in the upper part of the respiratory tract, such as in the nose, throat and mouth. In contrast, the highly lethal H5N1 strain binds to receptors that are mostly found deep in the lungs. This difference in the site of infection may be part of the reason why the H5N1 strain causes severe viral pneumonia in the lungs, but is not easily transmitted by people coughing and sneezing.
Common symptoms of influenza such as fever, headaches, and fatigue are the result of the huge amounts of proinflammatory cytokines and chemokines (such as IFN-γ and TNF-α) produced from influenza-infected cells [52]. Influenza does cause tissue damage, so symptoms are due to either a massive immune response that produce a life-threatening cytokine storm or the massive levels of viral replication inside the infected cells.
Effective measures against influenza A and B diseases include prevention of infection by either vaccination with inactivated or live attenuated vaccines or administration of antiviral drugs prophylactically or therapeutically. Chemically inactivated influenza vaccines consist of detergent-split virion subunits composed of HA and lesser amounts of other virion proteins including NA. This inactivated, split vaccine is not completely effective, particularly in the elderly. The efficacy of the vaccine is significantly compromised when circulating viruses do not have a good match with vaccine strains due to antigenic drift or inaccurate epidemiological predictions. A live, attenuated influenza virus vaccine (FluMist®) is also licensed for seasonal influenza and is intended for intranasal administration to people 6 months to 49 years of age. Current influenza vaccines normally protect only for a matter of months; in any case, continuous viral antigenic drift of influenza A viruses makes once effective vaccines ineffective after few time. Furthermore, they do not generate broadly neutralizing antibodies to multiple strains of influenza and must be re-formulated annually to match the predominant virus strains circulating each year. Antiviral drugs can have both therapeutic and prophylactic effects, but to prevent disease they must be administered continuously at times of high influenza activity. Matrix 2 ion channel blockers are effective against influenza A viruses, but resistant viral strains develop rapidly and have been recognized in approximately one-third of treated patients. The more recently developed NA inhibitors are effective against both influenza A and B viruses. Both classes of drugs are effective in preventing influenza when administered prophylactically [53]. However, the importance of predicting the emergence of new circulating influenza strains for subsequent annual vaccine development cannot be underestimated.

2.2. Human immunodeficiency virus (HIV)

HIV remains one of the leading causes of mortality and morbidity worldwide. The virus infects 15.000 people every day and causes millions of death every years.
HIV is a member of the family *Lentiviridae*. Two closely related types of HIV, designated HIV-1 and HIV-2, have been identified. HIV-1 is by far the most common cause of AIDS, but HIV-2, which differs in genomic structure and antigenicity, causes a similar clinical syndrome [54].
An infectious HIV particle consists of two identical strands of RNA packaged within a core of viral proteins and surrounded by a phospholipid bilayer envelope derived from the host cell membrane but including virally encoded membrane proteins (**fig. 8**). The RNA genome of HIV is approximately 9.2 kb long and has the basic arrangement of nucleic acid sequences characteristic of all known retroviruses.

LTRs at each end of the genome regulate viral gene expression, viral integration into the host genome, and viral replication. The *gag* sequences encode core structural proteins. The *env* sequences encode the envelope glycoproteins gp120 and gp41, which are required for infection of cells. The *pol* sequences encode reverse transcriptase, integrase, and viral protease enzymes required for viral replication. In addition to these typical retrovirus genes, HIV-1 also includes six other regulatory genes, namely, the *tat, rev, vif, nef, vpr,* and *vpu* genes, whose products regulate viral reproduction in various ways.

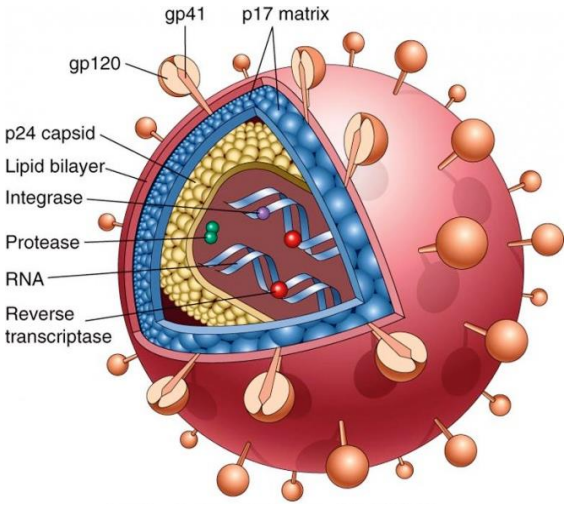


Figure 8 - *HIV-1 virion* [Kumar V., *Robbins Basic Pathology*, 2007]

HIV infection of cells begins when the envelope glycoprotein (Env) of a viral particle binds to both CD4 and a coreceptor that is a member of the chemokine receptor family. The viral particles that initiate infection are usually in the blood, semen, or other body fluids of one individual and are introduced into another individual by sexual contact, needle stick, or transplacental passage. Env is a complex composed of a transmembrane gp41 subunit and an external, noncovalently associated gp120 subunit. The Env complex is expressed as a trimeric structure of three gp120/gp41 pairs. This complex mediates a multistep process of fusion of the virion envelope with the membrane of the target cell.
The first step of this process is the binding of gp120 subunits to CD4 molecules, which induces a conformational change that promotes secondary gp120 binding to a chemokine coreceptor. This process induces a conformational change in gp41 that exposes a hydrophobic region, called the fusion peptide, which inserts into the cell membrane and enables the viral membrane to fuse with the target cell membrane (**fig. 9**).
Once an HIV virion enters a cell, the nucleoprotein core of the virus becomes disrupted, the RNA genome of HIV is transcribed into a double-stranded DNA form by viral reverse transcriptase and the viral DNA enters the nucleus. The viral integrase also enters the nucleus and catalyzes the integration of viral DNA into the host cell genome. The integrated HIV DNA is called provirus and may remain

transcriptionally inactive for months or years, with little or no production of new viral proteins or virions. Transcription of the genes of the provirus is regulated by the LTRs upstream of the viral structural genes, and cytokines or other physiologic stimuli to T-cells and macrophages enhance viral gene transcription. The LTRs contain polyadenylation signal sequences, the TATA box promoter sequence and binding sites for two host cell transcription factors, NF-κB and SP1. Initiation of HIV gene transcription in T-cells is linked to activation of the T-cells by antigens or cytokines.

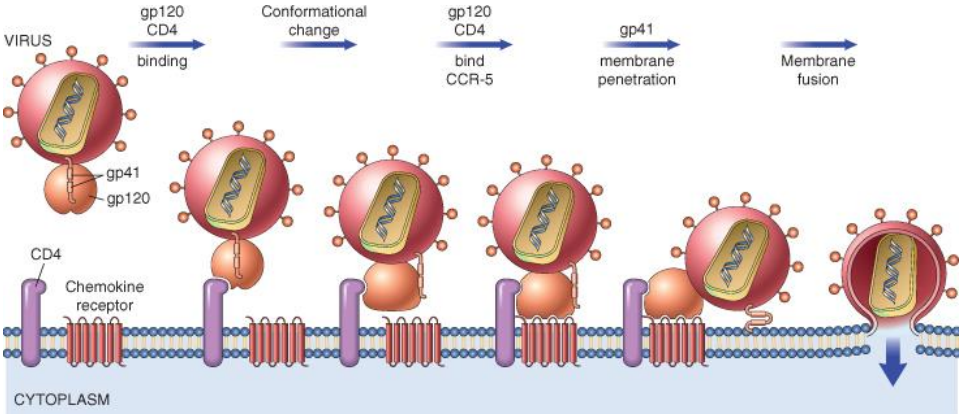


Figure 9 *- HIV-1 entry in host cells* [Kumar V., *Robbins Basic Pathology*, 2007]

Tat protein is required for HIV gene expression and acts by enhancing the production of complete viral mRNA transcripts. Tat binds to the nascent mRNA and increases the "processivity" of RNA polymerase by several hundred-fold.
The mRNAs encoding the various HIV proteins are derived from a single full-genome-length transcript by differential splicing events (**fig. 10**).
The Rev, Tat, and Nef proteins are early gene products encoded by fully spliced mRNAs that are exported from the nucleus and translated into proteins in the cytoplasm soon after infection of a cell. Late genes include *env, gag,* and *pol,* which encode the structural components of the virus and are translated from singly spliced or un-spliced RNA. The Rev protein initiates the switch from early to late gene expression by promoting the export of these incompletely spliced late gene RNAs out of the nucleus. The *pol* gene product is a precursor protein that is sequentially cleaved to form reverse transcriptase, protease, ribonuclease, and integrase enzymes. The *gag* gene encodes a 55-kD protein that is proteolytically cleaved by the viral protease into p24, p17 and p15 polypeptides, which are the core proteins required for assembly of infectious viral particles. The primary product of the *env* gene is a 160-kD glycoprotein (gp160) that is cleaved by cellular proteases within the endoplasmic reticulum into the gp120 and gp41 proteins required for HIV binding to cells.
Assembly of infectious viral particles then begins by packaging full-length RNA transcripts of the proviral genome within a nucleoprotein complex that includes the *gag* core proteins and the *pol*-encoded enzymes required for the next cycle of integration. This nucleoprotein complex is then enclosed within a membrane

envelope and released from the cell by a process of budding from the plasma membrane. The rate of virus production can reach sufficiently high levels to cause cell death. In addition, gp120 and gp41, which are expressed on the plasma membrane of infected cells before virus is released, can mediate cell-cell fusion with an uninfected cell that expresses CD4 and coreceptors, and HIV genomes can then be passed between the fused cells directly.

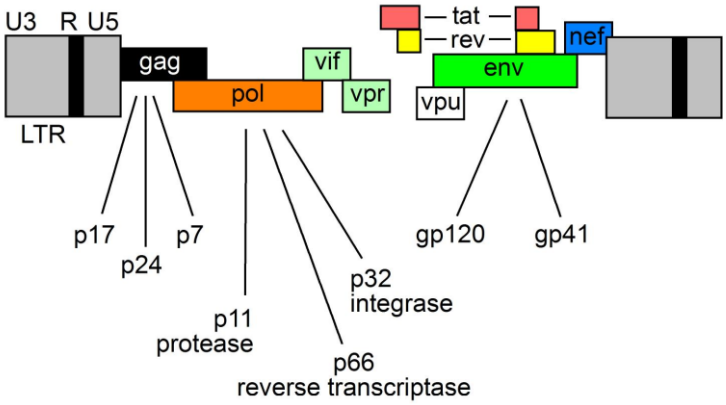


Figure 10 – *Genomic structure of HIV-1* [Hoffmann C, *HIVbook*]

HIV is transmitted via mucosal surfaces. Major groups at risk for the development of AIDS include homosexual or bisexual persons, intravenous drug abusers, heterosexual partners of members of other risk groups and babies born of infected mothers. Health care workers have a small increased risk for infection.
Different isolates of HIV have distinct tropisms for different cell populations that are related to the specificity of gp120 variants for different chemokine receptors. All HIV strains can infect and replicate in freshly isolated human $CD4^+$ T-cells that are activated in vitro. In contrast, some strains will infect primary cultures of human macrophages but not continuous T-cell lines (macrophage-tropic, or M-tropic, virus), whereas other strains will infect T-cell lines but not macrophages (T-tropic virus). Some virus strains also infect both T-cell lines and macrophages (dual-tropic virus). Macrophage-tropic virus isolates express a gp120 that binds to CCR5, which is expressed on macrophages (and some memory T-cells), whereas T cell-tropic viruses bind to CXCR4, which is expressed on T-cell lines. In many HIV-infected individuals, there is a change from the production of virus that uses CCR5 and is predominantly macrophage tropic early in the disease to virus that binds to CXCR4 and is T-cell line tropic late in the disease. The T-tropic strains tend to be more virulent, presumably because they infect and deplete T-cells more than do M-tropic strains.
HIV disease begins with acute infection, which is only partly controlled by the adaptive immune response, and advances to chronic progressive infection of peripheral lymphoid tissues (**fig. 11**). Acute infection is characterized by infection of memory $CD4^+/CCR5^+$ T-cells in mucosal lymphoid tissues, and death of many infected cells. Because the mucosal tissues are the largest reservoir of T cells in

the body, and the major site of residence of memory T-cells, this local loss is reflected in considerable depletion of lymphocytes. The transition from the acute phase to a chronic phase of infection is characterized by dissemination of the virus, viremia and the development of host immune responses. DCs in epithelia at sites of virus entry capture the virus and then migrate into the LNs. DCs express a protein with a mannose-binding lectin domain that may be particularly important in binding the HIV envelope and transporting the virus. Once in lymphoid tissues, DCs may pass HIV on to $CD4^+$ T-cells through direct cell-cell contact. Within days after the first exposure to HIV, viral replication can be detected in the LNs. This replication leads to viremia, during which large numbers of HIV particles are present in the patient's blood, accompanied by an acute HIV syndrome that includes a variety of nonspecific signs and symptoms typical of many viral diseases.

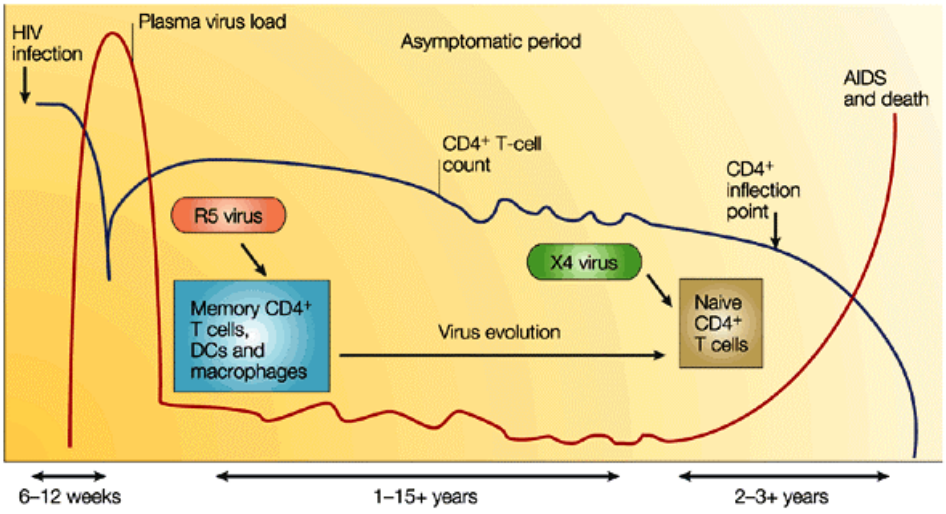


Figure 11 - *Typical course of HIV infection* [Rowland-Jones S.L., *Nat Rev Imm,* 2003]

The viremia allows the virus to disseminate throughout the body and to infect helper T-cells, macrophages and DCs in peripheral lymphoid tissues. As the HIV infection spreads, the adaptive immune system mounts both humoral and cell-mediated immune responses directed at viral antigens. These immune responses partially control the infection and viral production, and such control is reflected by a drop in viremia to low but detectable levels by approximately 12 weeks after the primary exposure. In the next, chronic phase of the disease, LNs and the spleen are sites of continuous HIV replication and cell destruction. Although the majority of peripheral blood T-cells does not harbour the virus, destruction of $CD4^+$ T-cells within lymphoid tissues steadily progresses during the latent period, and the number of circulating blood $CD4^+$ T-cells steadily declines. As the disease progresses, patients become susceptible to other infections, and immune responses to these infections may stimulate HIV production and accelerate the destruction of lymphoid tissues. HIV disease progresses to the final lethal phase,

called AIDS, when the blood $CD4^+$ T-cell count drops below 200 cells/mm^3. HIV viremia may climb dramatically as viral replication in other reservoirs accelerates unchecked. Patients with AIDS suffer from combinations of opportunistic infections, neoplasms, cachexia (HIV wasting syndrome), kidney failure (HIV nephropathy), and CNS degeneration (AIDS encephalopathy).
Macrophages express much lower levels of CD4 than helper T-cells do, but they do express CCR5 coreceptors and are susceptible to HIV infection. However, because macrophages can be infected but are relatively resistant to the cytopathic effects of HIV, they may become a reservoir for the virus. DCs can also be infected by HIV but are not directly injured by HIV infection. However, these cells form intimate contact with naïve T-cells during the course of antigen presentation and may thus be an important pathway for T-cell injury. FDCs in the germinal centres of LNs and the spleen trap large amounts of HIV on their surfaces, in part by Fc receptor-mediated binding of antibody-coated virus, and can infect macrophages and $CD4^+$ T-cells in the LNs. HIV infection results in impaired function of both the adaptive and innate immune systems.
An important cause of the loss of $CD4^+$ T-cells in HIV-infected people is the direct cytopathic effect of infection of these cells by HIV. Mechanisms in addition to direct lysis of infected $CD4^+$ T-cells by virus have been proposed for the depletion and loss of function of these cells in HIV-infected individuals.
Chronic activation of the T-cells may predispose the cells to apoptosis and apoptotic death of activated lymphocytes may account for the observation that the loss of T-cells greatly exceeds the numbers of HIV-infected cells. HIV-specific CTLs are present in many patients with AIDS, and these cells can kill infected $CD4^+$ T-cells. In addition, antibodies against HIV envelope proteins may bind to HIV-infected $CD4^+$ T-cells and target the cells for ADCC. Binding of gp120 to newly synthesized intracellular CD4 may interfere with normal protein processing in the endoplasmic reticulum and block cell surface expression of CD4, making the cells incapable of responding to antigenic stimulation. HIV-specific humoral and cell-mediated immune responses develop following infection but generally provide limited protection, primarily because of the depletion and functional inhibition of the $CD4^+$ T-cells. The initial adaptive immune response to HIV infection is characterized by expansion of $CD8^+$ T-cells specific for HIV peptides, which nevertheless prove ineffective because of the emergence of viral escape mutants (variants with mutated antigens).
Antibody responses to a variety of HIV antigens are detectable within 6 to 9 weeks after infection. The most immunogenic HIV molecules that elicit antibody responses appear to be the envelope glycoproteins, and high titers of anti-gp120 and anti-gp41 antibodies are present in most HIV-infected individuals. Other anti-HIV antibodies found frequently in patients' sera include antibodies to p24, reverse transcriptase, and *gag* and *pol* products. The early antibodies are not neutralizing, and are generally poor inhibitors of viral infectivity or cytopathic effects. Neutralizing antibodies against gp120 develop 2 to 3 months after primary infection, but even these antibodies cannot cope with a virus that is able to rapidly change the most immunodominant epitopes of its envelope glycoproteins.
The failure of cell-mediated and humoral immune responses to eradicate HIV infection is probably due to several factors. Notably HIV has an extremely high mutation rate because of error-prone reverse transcription, and in this way it may

evade detection by antibodies or T-cells generated in response to viral proteins. Active research efforts have been aimed at developing reagents that interfere with the viral life cycle. Treatment of HIV infection and AIDS now includes the administration of three classes of antiviral drugs, used in combination, that target viral molecules for which no human homologues exist.
The first type of drug to be widely used consists of nucleoside analogues that inhibit reverse-transcriptase activity. These drugs include deoxythymidine nucleoside analogues such as 3'-azido-3'-deoxythymidine (AZT), deoxycytidine nucleoside analogues, and deoxyadenosine analogues. When these drugs are used alone, they are often effective in significantly reducing plasma HIV RNA levels for several months to years. They usually do not halt progression of HIV-induced disease, largely because of the evolution of virus with mutated forms of reverse transcriptase that are resistant to the drugs.
More recently, viral protease inhibitors have been developed that block the processing of precursor proteins into mature viral capsid and core proteins. When these protease inhibitors are used alone, mutant viruses resistant to their effects rapidly emerge. However, protease inhibitors are now being used in combination with two different reverse-transcriptase inhibitors. This new triple-drug therapy, commonly referred to as HAART (highly active antiretroviral therapy), has proved to be remarkably effective in reducing plasma viral RNA to undetectable levels in most treated patients for up to 3 years. An integrase inhibitor is also now used as part of anti-viral therapy. Although anti-retroviral therapy has reduced viral titers to below detection for up to 10 years in some patients, it is unlikely that such treatment can eliminate the virus from all reservoirs, especially long-lived infected cells, and resistance to the drugs may ultimately develop. Other problems associated with these new drug therapies, which will impair their effective use in many parts of the world, include high expense, complicated administration schedules and serious side effects. Although there have been numerous approaches for generating HIV vaccines in the past 20 years, to date there is no approved vaccine on the market.

2.3. Innate Immunity to viral infections

Virus invasion initially activate innate immune system that set up robust antiviral responses. Host cells detect, through pattern-recognition receptors (PRRs), specific viral components, such as genomic DNA, single-strand RNA (ssRNA), double-strand RNA (dsRNA), RNA with 5'-triphosphate ends and viral proteins [55]. Currently, three classes of PRRs are implicated in viral component recognition, Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (**fig. 12**). Among these receptors, TLRs and RLRs are important for the production of type I interferons (IFNs) and cytokines, whereas NLRs are involved in the production of interleukin-1β (IL-1β) [56].
Intracellular signaling cascades activated by type I IFNs results in the regulation of a wide range of IFN-inducible genes (ISGs), involved in eliminating viral components from infected cells, inducing apoptosis of infected cells and conferring resistance to viral infection on uninfected cells. Type I IFNs are produced not only by professional innate immune cells, including dendritic cells (DCs) and macrophages, but also by non-professional cells. Proinflammatory cytokines and

chemokines are also critical in eliminating virus infection by causing inflammation and recruiting innate and acquired immune cells. Costimulatory molecules are essential for T cell activation leading to acquired immune responses.

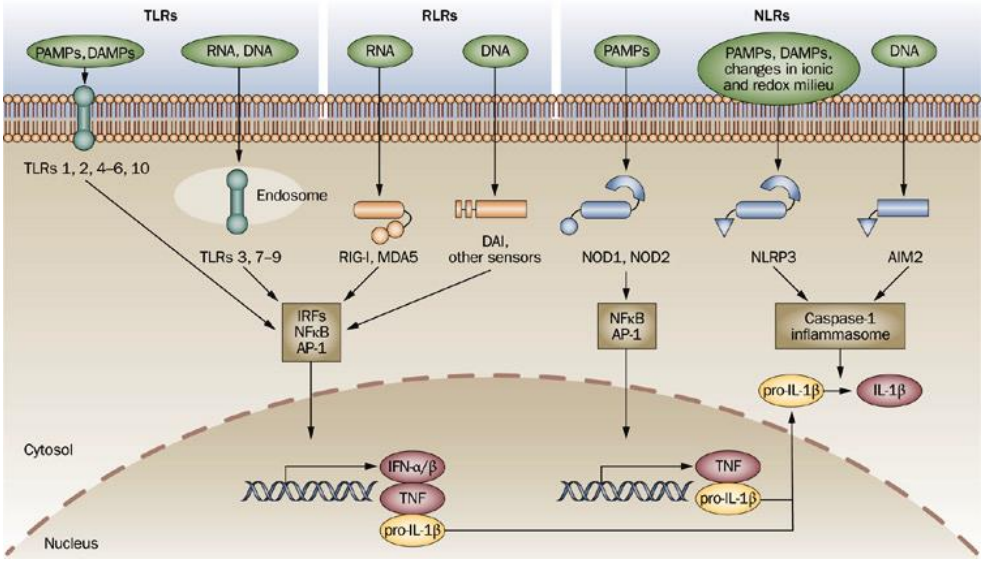


Figure 12 *- Innate immune sensors* [Theofilpoulus A.N., *Nature Reviews Rheum*, 2010]

2.3.1. Innate immunity sensors

2.3.1.1. The RLR signaling pathway

RLRs consist of a family of cytoplasmic protein comprising of three members, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (LGP2) [57]. RIG-I and MDA5 consist of two N-terminal caspase-recruitment domains (CARDs), a DExD/H box RNA helicase domain, and a C-terminal repressor domain (RD), although LGP2 lacks the CARD-like region [58].
RNA virus infection leads to the generation of dsRNA and ssRNA with 5'-triphosphate ends in infected cells, not usually present in host cells. Thus, RLRs located in the cytoplasm, recognize viral RNA.
The recognition of these RNAs is mediated by the helicase domain and the RD while the CARDs are essential for eliciting downstream signaling pathway. The LGP2 domain, lacking a CARD, seems to function as a negative regulator [59, 60].
RIG-I and MDA5 interact with viral RNA with different ligand specificity and elicit signaling leading to the transcription of type I IFN and inflammatory cytokines. In response to detection of viral RNAs, RIG-I and MDA5 associate with the adapter protein IFNβ promoter stimulator-I (IPS-1), also known as mitochondrial antiviral signaling (MAVS) [61, 62] (**fig. 13**).

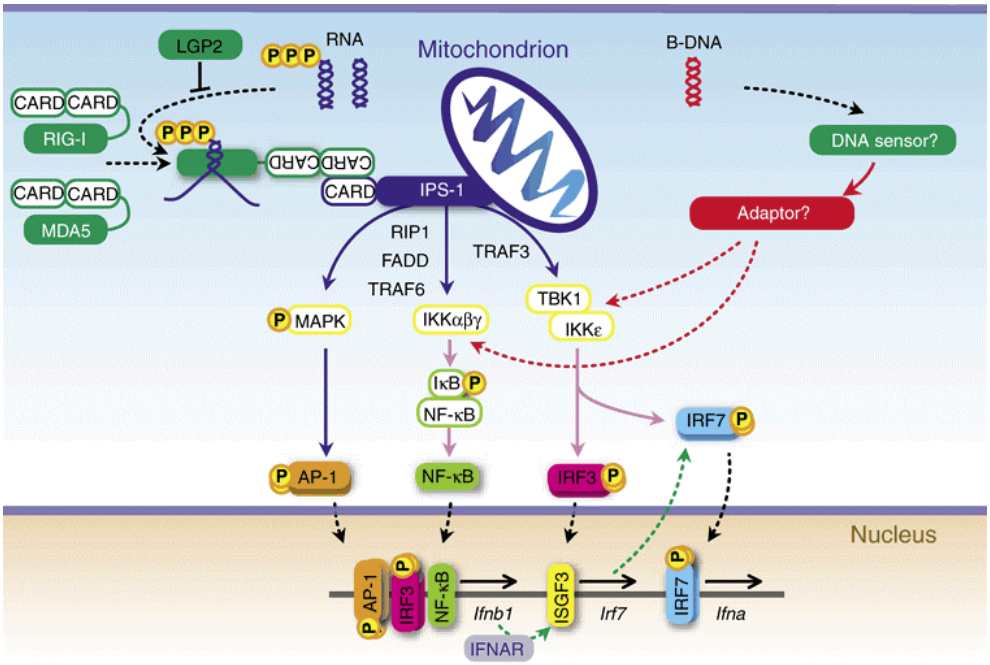


Figure 13 – *RIG-I-like receptor pathway [Baccala R., Nature Med. 2007]*

Virus-specific RNA species specifically bind to RIG-I through its RNA binding domain. This complex in presence of ATP induce a conformational change of RIG-I that allows the CARD domain to bind IPS-1 on the outer membrane of mitochondria [63]. Tumor necrosis factor receptor (TNFR)-associated death domain (TRADD) protein is recruited to IPS-1 upon stimulation and forms a complex with Fas-associated death domain-containing protein (FADD) and a death domain kinase receptor-interacting protein 1 (RIP1), in addition to TNF-receptor associated factor 3 (TRAF3) [64, 65].

Downstream of TRAF3, two IκB kinases (IKK)-related kinases, TKB1 (TANK-binding kinase 1) and inducible IκB kinase (IKK-i), phosphorylate the IFN-regulatory factor-3 (IRF3) and IRF7 [66, 67]. The activation of IRF3/7 by these kinases induces the formation of homodimers and/or heterodimers that translocate into the nucleus and bind to IFN-sensitive response elements (ISREs), resulting in the expression of type I IFNs and a set of IFN-inducible genes [68].

The RLR signaling pathway activates another transcription factor, NF-κB for the expression of pro-inflammatory genes. IPS-1, TRADD and FADD are important for activating both IRFs and NF-κB [69]. FADD interacts with caspase-8/-10, and the catalytic activities of these caspases are critical for the subsequent nuclear translocation and activation of NF-κB.

A recent study identified a novel protein named stimulator of IFN genes (STING) as an important molecule for RIG-I/MDA5 signaling [70]. Overexpression of STING activated NF-κB and ISRE via TBK1/IKK-i.

2.3.1.2. Recognition of viral component by TLR system

TLRs represent a group of PPRs located on cell surface or within endosomes suitable for detecting viral components outside of cells as well as in cytoplasmic vacuoles after phagocytosis or endocytosis [71]. TLRs are type I integral membrane receptors and comprise an ectodomain, which contains leucine-rich repeats that mediate the recognition of pathogen-associated molecular patterns (PAMPs), a transmembrane region, and cytosolic Toll-IL-1 receptor (TIR) domains that activate downstream signaling pathway [55]. To date, ten TLRs have been identified in humans. Each TLR detects distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi and parasites. These include lipoproteins (recognized by TLR1, TLR2 and TLR6), dsRNA (TLR3), lipopolysaccharide (LPS) (TLR4), bacterial flagellin (TLR5), viral or bacterial ssRNA (TLRs 7 and 8), and CpG-rich un methylated DNA (TLR9) [72]. TLR1, TLR2, TLR4, TLR5, and TLR6 are localized on the cell surface and largely recognize microbial membrane components whereas TLR3, TLR7, TLR8, and TLR9 are expressed within intracellular vesicles and recognize nucleic acids [73]. The intracellular localization enables TLRs to recognize nucleic acids delivered to the intracellular compartments after the uptake of viruses and other pathogens or infected cells. By contrast, cellular nucleic acids within the extracellular compartment, are rapidly degraded by nucleases and do not access intracellular vesicles. TLR3, TLR7, TLR8, and TLR9 are sequestered in the ER and are delivered to the endosomes where their N-terminal region is processed by multiple lysosomal proteases, including cathepsins and asparagine endopeptidase, to generate functional receptors that elicit signaling [73, 74].

After the recognition of specific PAMPs, TLRs recruit a set of adaptor molecules that harbor TIR domain and initiate downstream signaling events, leading to the secretion of inflammatory cytokines, type I IFN, and chemokines [75].These responses cause recruitment of neutrophilis, activation of macrophages and induction of IFN-stimulated genes, resulting in direct killing of the infected pathogens. Furthermore, activation of TLR signaling contributed to the induction of adaptative immunity by leads inducing DCs maturation.(**fig. 14**).

The specific response originated by individual TLRs depends on the recruitment of a single, or a specific combination of TIR-domain-containing adaptor protein such as myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adapter protein (TIRAP), TIR domain-containing adapter inducing IFN-β (TRIF or TICAM) or Trif-related adapter molecule (TRAM). All TLRs, except TLR3, activate a common signaling pathway leading to the production of proinflammatory cytokines via myeloid differentiation factor 88 (MyD88), a central adapter protein. The association between TLRs and MyD88 recruits members of the interleukin-1 receptor-associated kinase (IRAK) family. To date, four IRAKs are identified: IRAK1, IRAK2, IRAK4 and IRAK-M [76, 77, 78]. While IRAK1 and IRAK4 possess intrinsic serine/threonine protein kinase activities, IRAK2 and IRAK-M lack this activity, suggesting that they are negative regulator.

In response to stimuli, IRAK4 and IRAK1 are sequentially phosphorylated and dissociated from MyD88, which results in activation of tumor necrosis factor receptor-associated factor 6 (TRAF6) which forms a complex with UBC13 and UEV1A [79]. TRAF6 in turn activates transforming growth factor-β-activated protein kinase 1 (TAK1), a member of the MAP kinase (MAP3K) family, in a ubiquitin-dependent manner [79]. TAK1 forms a complex with TAB1, TAB2 and TAB3 and

activates the IKK complex that leads to NF-κB activation [79]. TAK1 simultaneously phosphorylates two members of the MAP kinase family, MKK3 and MKK6, which successively activate JNK and p38. ERK is also activated in response to TLR ligands through the activation of MEK1 and MEK2, although an upstream kinase activating MEK1 and MEK2 in TLR signaling remains unknown. The signaling pathway from MyD88 to the activation of NF-κB and AP-1 is used by almost all TLRs to control inflammatory responses. However, the activation of the MyD88-dependent pathway requires an additional adapter TIRAP/Mal in terms of TLR2 and TLR4 signaling [80, 81].

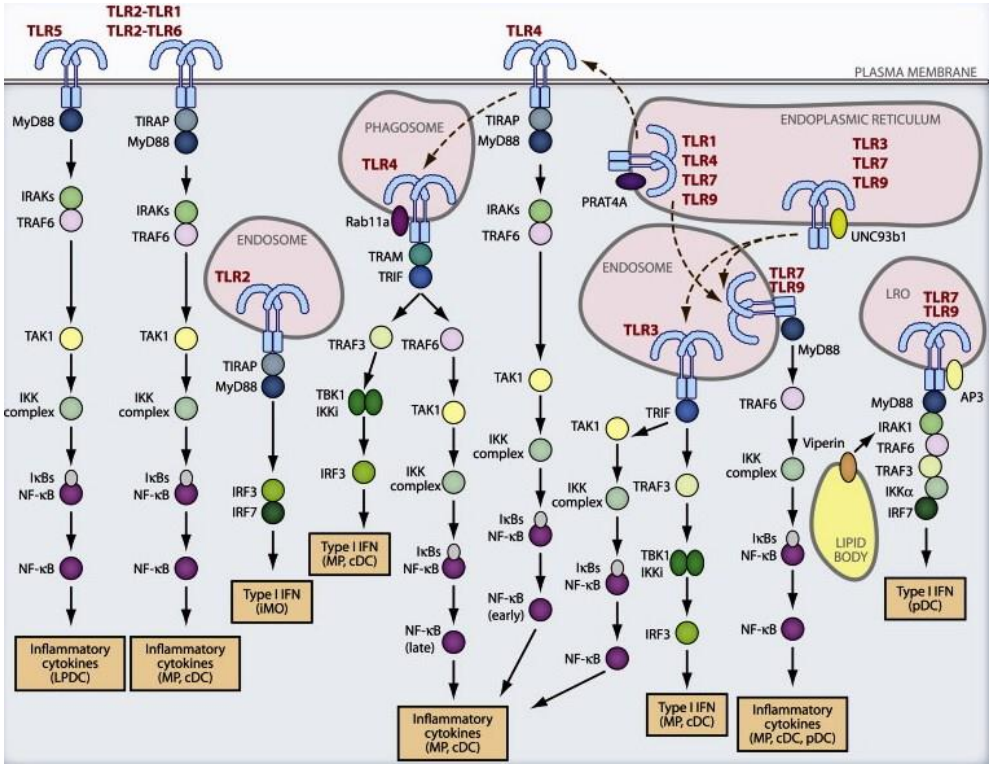


Figure 14 – *TLRs trafficking and signaling* [Kawai T., *Immunity*, 2011]

TLR3 and TLR4 recruits another adaptor protein TIR domain-containing adapter inducing IFNβ (TRIF) (also known as TICAM-1), which leads to induction of type I IFN[82]. TRIF associates with TRAF3 and TRAF6 and subsequently with RIP1 and RIP3 [83, 84]. Recent studies showed that TRADD is also involved in the TRIF-dependent signaling pathway [85]. The downstream signaling molecules for the expression of IFN-inducible genes are shared between the TLR3 and RLR signaling pathways. Simultaneously, TRAF6 and RIP1 are responsible for activating NF-κB through IKKα/β, leading to the expression of proinflammatory cytokines. Transcription of IFN gene is tightly controlled by cooperative activation of several transcription factors, including NF-κB, ATF2/c-Jun, interferon regulatory

factor (IRF)3 and IRF7 [86]. While NF-κB and ATF2/c-Jun are activated by numerous stimuli such as TLR ligands, IL-1β, TNFα and DNA damage, IRF3 and IRF7 are activated when cells are exposed to LPS, poly IC and virus infection, and mainly control type I IFN. IRF3 and IRF7 are structurally related proteins present in the cytoplasm in unstimulated conditions. TLR7 and TLR9 activate distinct signaling pathways in response to viral RNA or DNA in pDC. TLR7 and TLR9 recruit MyD88, which forms a complex with IRAK-1, IRAK-4, and IRF-7 in this cell type [87]. IRAK-1 and IKKα have been identified as potential IRF-7 kinases [88]. Phosphorylated IRF-7 translocates into the nucleus to activate the promoters of type I IFN and IFN-inducible genes. The MyD88-dependent pathway is also critical for NF-κB, leading to the production of cytokines including IL-12 and IL-6.
Localizations of TLR proteins are critical for the recognition of their ligands. An ER protein, UNC93B, was identified as an essential molecule for the translocation of TLR7 and TLR9 from the ER to endosomes [89]. Another ER protein associated with TLR4 (PRAT4A) also control TLR9 trafficking from the ER to endosome/lysosomes [90]. Collectively, recognition of viral nucleotides by TLRs in endosome/lysosomes is controlled by the localizations of TLRs as well as their ligands. This elaborate mechanism may be essential for preventing autoimmune diseases caused by aberrant initiation of TLR signaling.

2.3.1.3. Role of NLRs and inflammasomes

NLRs are a large receptor family that is characterized by the presence of a conserved nucleotide binding oligomerization domain (NOD) motif. Structurally, NLRs consist of an N-terminal protein–protein interaction domains such as the caspase-recruitment domain (CARD), pyrin domain (PYD) or baculovirus inhibitor repeat (BIR) domain; an intermediary NOD domain which is an ATP-binding domain needed for nucleotide binding and self-oligomerization; and an array of C-terminal leucine-rich repeat (LRR) motifs that are presumed to detect conserved microbial patterns and to modulate NLR activity [91] (**fig. 15**).

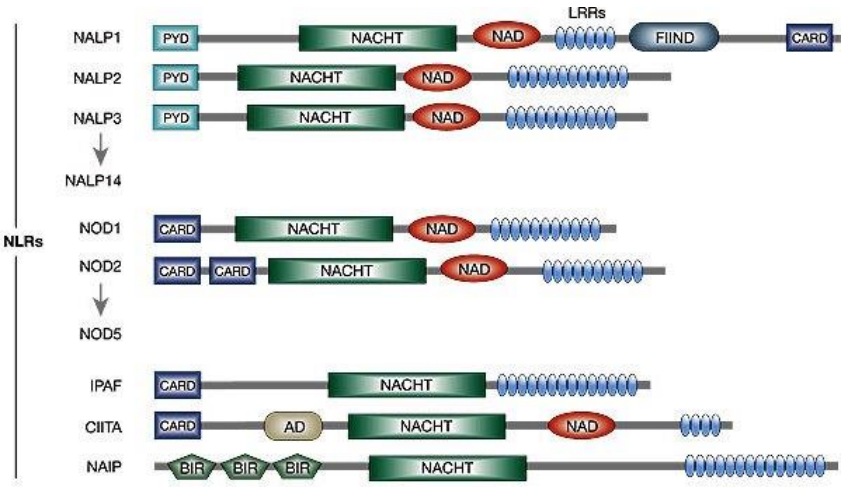


Figure 15 – *NLRs structure* [modified from Meylan E., *Nature*, 2006]

To date, twenty two NLR genes are found in human genome. When a PAMP is sensed by the C-terminal LRR motifs of an NLR, the molecule undergoes conformational changes that trigger oligomerization through the NOD. In turn, NLRs expose the N-terminal effector domains to induce the recruitment and activation of CARD- and/or PYD-containing effector molecules [92]. As a result, NLRs are involved in the activation of multiple signaling pathways. The NLR proteins NOD1 and NOD2 interact with the receptor-interacting serine-threonine protein kinase 2 (RIPK2; also known as RICK or RIP2) to induce NF-κB and MAPK signalling. NOD2 also associates with IPS1 for the induction of type I IFNs in response to viral infection [92].
Moreover, NLRs are involved in the activation of a large protein complex known as inflammasome that results in caspase-1 mediated cleavage of pro-interleukin 1β (IL-1β) and pro-IL-18 into their active form. The activation of caspase-1 is triggered by NLRP3 and ICE-protease-activating factor (IPAF), together with an adapter, ASC (apoptosis-associated speck-like protein containing a CARD) [93] (**fig. 16**).

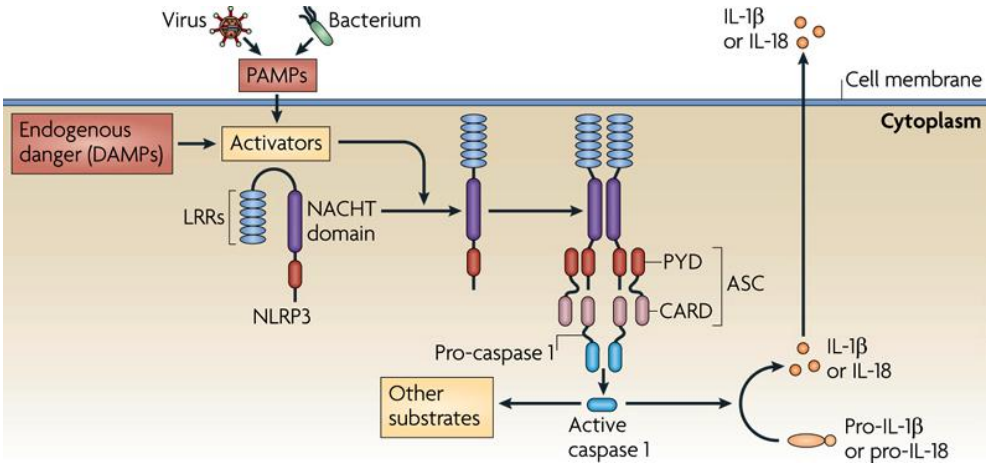


Figure 16 *– NLRP3 inflammasome complex activation* [Tschopp J., *Nat. Rewiev Imm.*2010]

Moreover, NLRP3 is responsible for sensing presence of ATP as well as various crystals by phagocytosis [94, 95]. Although mechanisms of crystal recognition by NALP3 is not fully understood, recent studies suggest that NLRP3 activation is triggered by reaction oxygen species (ROS) produced by a nicotinamide adenine dinucleotide phosphate oxidase or by lysosomal destabilization which may release protease cathepsin B to the cytosol [96-97]. dsRNA and polyI:C were reported to activate the inflammasome via a NLRP3-dependent pathway, but it remains unclear whether NLRP3 directly recognizes dsRNA in the cytoplasm [98]. Moreover, ASC and caspase-1 are essential for dsDNA-induced IL-1β production, suggesting that one of the unknown NALP family members functions as a cytoplasmic DNA sensor. Although a role for NLRP3 in sensing viruses has been proposed, the precise molecular mechanism remains poorly understood. Several lines of evidence indicate that the NLRP3 inflammasome might detect the presence

of viral RNA and DNA in intracellular compartments [99]. The discovery that NLRP3 can detect a wide range of divergent PAMPs, such as bacterial LPS, peptidoglycan and bacterial or viral nucleic acids, as well as endogenous danger signals (DAMPs), such as monosodium urate (MSU), calcium pyrophosphate dihydrate and ATP, has led to the hypothesis that NLRP3 might sense a common downstream effect caused by these molecules, rather than the PAMPs or DAMPs themselves. However, not all NLRs are proinflammatory. NLRP12 and NLRP6 have been shown to downregulate NF-κB signalling subsequent to TLRs activation [100]. NLRP12 has also been reported to regulate alternative NF-κB signaling downstream of TNF family receptors like CD40. Finally, NLR family member X1 (NLRX1) and NLRC5 have been shown to inhibit NF-κB and type I IFN signalling pathways, indicating that these NLRs have an important role as negative regulators [101].

2.3.2. Antiviral mediator and effector molecules

2.3.2.1. Type I IFN-producing cells in response to viral infections

The IFNs are a large family of secreted cytokines involved in antiviral response and immune activation. The IFN family includes two main classes of related proteins characterized by structural homology. Type I IFNs are produced in direct response to virus infection and comprise products of the IFNα family, predominantly synthesized by leukocytes and plasmacytoid dendritic cells (pDC) and products of the IFNβ gene, synthesized by most cell types and in particularly by fibroblasts [102]. Type II IFN consists of the product of the IFNγ gene produced in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells [103]. Both the type I and type II IFN receptors have multichain structures composed of distinct subunits: IFNAR1 and IFNAR2 for the type I IFN receptor, and IFNGR1 and IFNGR2 for the type II IFN receptor. Each subunits interacts with a member of the Janus activated kinase (JAK) family [104]. In particular, the IFNAR1 subunit is constitutively associated with tyrosine kinase 2 (TYK2), IFNAR2 and IFNGR1 are associated with JAK1 and finally IFNGR2 is constitutively associated with JAK2 [105]. The initial phase in both type I and type II IFN mediated response is the rearrangement and dimerization of the receptor subunits followed by autophosphorylation of the associated JAKs. This activation seems to regulate, either directly or indirectly, several other downstream cascades. Multiplicity of signaling is consistent with the pleiotropic biological effects of IFNs on target cells and tissue [105, 106].

2.3.2.2. STATs and Interferon Stimulated Genes (ISGs)

Binding of IFNα or other type I IFNs to their related receptors causes the activation of the receptor-associated JAKs, TYK2 and JAK1, which in turn trigger phosphorylation and consequently activation of signal transducers and activators of transcription (STATs) [107]. The induction of STAT1, STAT2, STAT3 and STAT5 is a common response to different type I IFNs. STAT4 and STAT6 can also be activated by IFNα, but only in endothelial cells or cells of lymphoid origin [108, 109]. After phosphorylation by JAKs, the activated STATs form homodimers or heterodimers that translocate to the nucleus and started transcription by binding specific sites in the promoters of IFN-stimulated genes (ISGs) [107-109]. An important transcriptional complex that is induced by type I IFNs is the ISG Factor 3

(ISGF3) complex, constituted of the phosphorylated forms of STAT1 and STAT2, together with IRF9 [107]. This complex is the only one that binds specific elements known as IFN-stimulated response elements (ISREs) that are present in the promoters of specific ISGs, thereby initiating their transcription. Other STAT complexes bind another type of element, known as an IFN-γ-activated site (GAS) element, that is present in the promoter of different ISGs [110].
Of the hundreds of known ISGs, some have only ISREs or only GAS elements in their promoters, whereas others have both elements; therefore, combinations of different STAT-containing complexes regulates the optimal transcription of a particular gene. Notably, specific responses might be explained by the modulation of distinct STATs functions. The transcription of type II IFN (IFNγ)-dependent genes is regulated by GAS elements, and STAT1 is the most important IFN-γ-activated transcription factor [107-110]. The activation of JAK1 and JAK2 regulate STAT1 phosphorylation, leading to the formation of STAT1-STAT1 homodimers, which translocate to the nucleus and bind GAS elements. In contrast to type I IFNs, IFN-γ does not induce the formation of ISGF3 complexes and thereby cannot induce transcription of genes that have only ISREs in their promoter [111] (**fig. 17**).

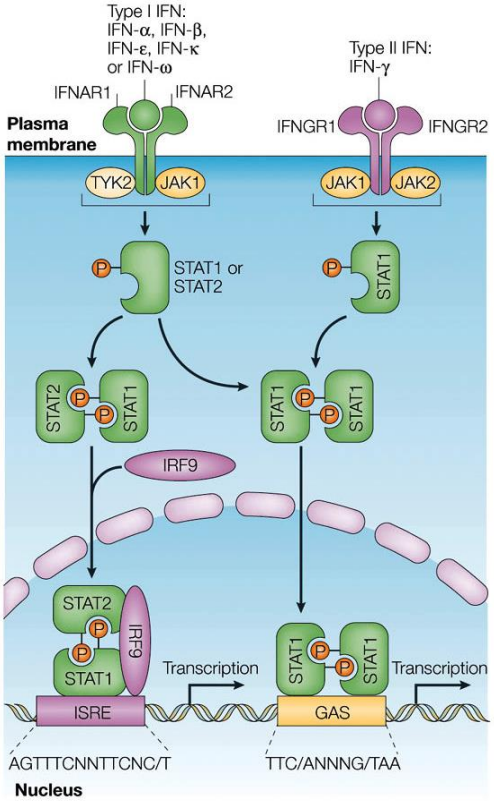


Figure 17 *– IFN receptors and JAK/STAT pathway* [Platanias L.C., *Nat Rev Imm*, 2005]

Importantly, both type I and type II IFNs can induce the recruitment of STAT3. Following its phosphorylation, STAT3 forms homodimers that translocate to the nucleus, where they bind to STAT3-binding elements (SBEs). STAT1 and STAT3 have a high level of sequence similarity but the sets of genes that are activated by these two factors are different and exert opposing effects. In particular, IFN-γ-dependent STAT1 activation usually mediates a pro-inflammatory response that promotes immune cell recruitment through upregulation of adhesion molecule expression (e.g., ICAM-1, VCAM-1), the production of pro-inflammatory mediators (e.g., IP-10, MCP-1, MIG, MIP-1α/β, RANTES) and the enhancing of antigen processing and presentation by MHC class I and II molecules [111]. By contrast, STAT3 is a key mediator of IL-10 signaling [112], which negatively regulates pro-inflammatory responses by directly inhibiting STAT1 activation.

IFN effectors vary widely in their magnitude of inhibitory activity and display combinatorial antiviral properties [**Tab. 3**]. Collectively, ISGs can target almost any step in a virus life cycle. Some of the most potent antiviral effectors reinforce the system by further inducing IFN or ISGs. Other genes enhance or facilitate viral replication, suggesting that some viruses cooperate with IFN effectors for a survival advantage.

Antiviral interferon-stimulated genes

Gene symbol	Targeted viruses	Viral life cycle	Mechanism related to antiviral activity
ADAR	HCV(r), HDV [enhances CHIKV, HIV-1, MV, VEEV, VSV, WNV, YFV]	replication	viral RNA editing, suppress PKR
APOBEC3	HIV-1, other retroviruses	replication	cytidine deamination of viral genome
BST2 (tetherin)	filovirus, FLUAV, HIV-1, LASV, VSV	egress/budding	block release of nascent virions
C6orf150 (MB21D1)	CHIKV, VEEV, WNV, YFV	translation	unknown
CD74	HIV-1	replication	unknown
DDIT4	HCV	translation	unknown
DDX58 (RIG-I)	numerous RNA and DNA viruses	translation, replication	viral sensing, activation of IRFs
DDX60	HCV, PV, VSV	translation (HCV)	promote RIG-I-like receptor signaling
EIF2AK2 (PKR)	numerous RNA and DNA viruses	translation	targets EIF2A
GBP1, GBP2	EMCV, HCV(r), VSV	replication	unknown
HPSE	CHIKV, VEEV, WNV, YFV	unknown	unknown
IFI44L	HCV	translation	unknown
IFI6/G1P3	HCV(r), YFV	unknown	unknown, possibly antiapoptotic
IFIH1 (MDA5)	numerous RNA and DNA viruses	translation, replication	viral sensing, activation of IRFs
IFIT1/2/3/5	FLUAV, HPV, MHV*, RVFV, SINV, VSV, WNV*	translation, replication	target EIF3 subunits, target HPV helicase, bind 5′-triphosphate RNA
IFITM1/2/3	DENV, filovirus, FLUAV, HIV-1, SARS-CoV, VSV, WNV, YFV	entry	unknown, possibly target endocytic pathway
IRF1	numerous RNA and DNA viruses	similar to IFN	IFN induction, direct ISG induction
IRF7	numerous RNA and DNA viruses	similar to IFN	IFN induction, direct ISG induction
ISG15	FLUAV, HIV-1, HSV-1, JEV, MHV-68, SINV, VVΔE3L	various	modulate protein function by ISGylation
ISG20	BVDV, DENV, EMCV, FLUAV, HCV, SINV, VSV, WNV(v), YFV	viral RNA synthesis	exonuclease activity
MAP3K14 (NIK)	HCV	translation	unknown, possibly NF-κB activation
MOV10	HIV-1, HCV	post-entry (HIV-1)	unknown
MS4A4A	HCV	translation	unknown
MX1 (MxA)	CVB, FLUAV, HCV(r), HPIV3, LACV, MV, SFV, THOV, VSV, others	primary transcription, nucleocapsid shuttling	formation of highly ordered oligomers
MX2 (MxB)	HIV-1, HNTV, LACV, RVFV, VSV	unknown	unknown
NAMPT (PBEF1)	VEEV, WNV	unknown	unknown
NT5C3	HCV	translation	unknown
OAS1/2/3	CHIKV, DENV, EMCV, HCV(r), SFV, SINV, WNV	replication	activate RNaseL to degrade viral genome
OASL	HCV, HCV(r)	translation	unknown
P2RY6	CHIKV	unknown	unknown
PHF15	WNV	unknown	unknown
PML (TRIM19)	numerous RNA and DNA viruses	various	organize multiprotein nuclear bodies
RSAD2 (viperin)	DENV, DENV(v), FLUAV, HCMV, HCV(r), SINV, WNV(v)	egress (FLUAV)	perturb lipid rafts (FLUAV), promote TLR7/9 signaling
RTP4	YFV	unknown	unknown
SLC15A3	CHIKV	unknown	unknown
SLC25A28	CHIKV	unknown	unknown
SSBP3	HCV	translation	unknown
TREX1 (AGS1)	YFV	unknown	unknown
TRIM5	HIV-1, other retroviruses	before reverse transcription	target incoming capsids, promote innate signaling
TRIM25	FLUAV, VSV	similar to IFN	activate RIG-I via ubiquitination
SUN2 (UNC84B)	HIV-1	unknown	unknown
ZC3HAV1 (ZAP)	EBOV, FLUAV, MBGV, NDV, retrovirus, SINV	post-entry, translation	target viral RNA, promote RIG-I signaling

Table 3 - *Antiviral ISGs* [modified from Schoggins J.W., *Elsevier* 2011]

2.3.2.3. Intrinsic immunity to influenza virus

Epithelial cells in the respiratory tract are the primary target of influenza virus. Macrophages and DC can also be infected by FLU virus and play an important role in host innate and adaptive immune responses to the virus. Influenza virus can be identified by various PRRs, including RLR, TLR and NLR (**fig. 19**).

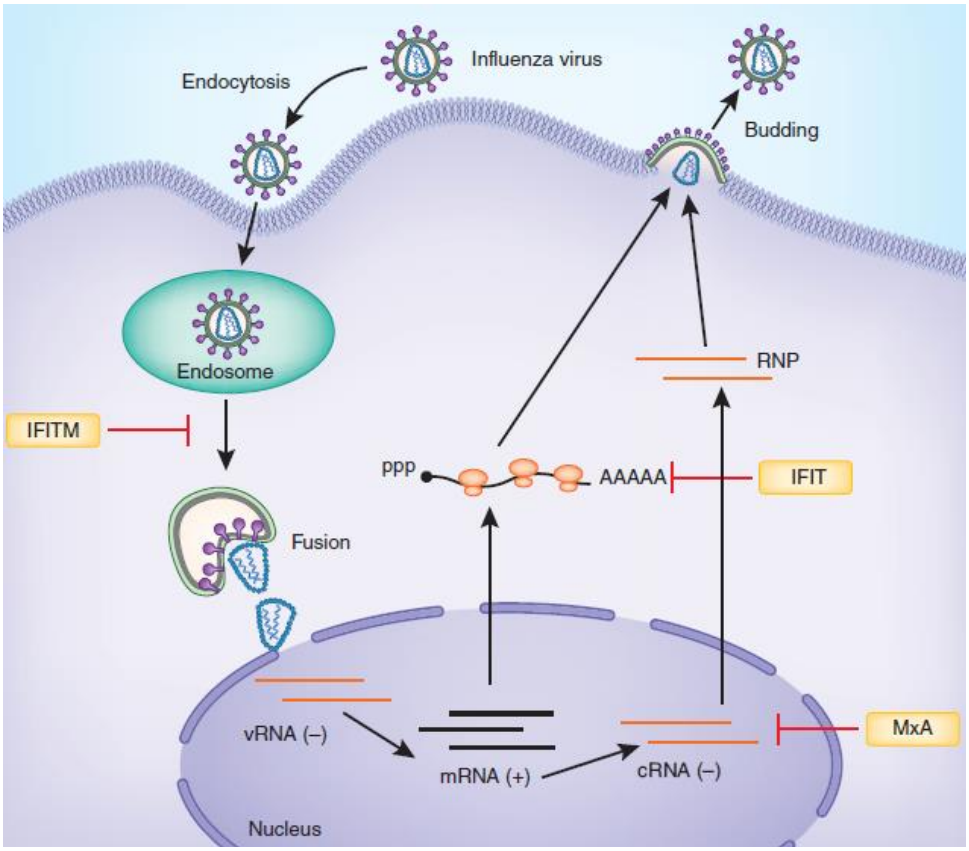


Figure 19 – *Intrinsic antiviral immunity against Influenza Virus* [Yan N., *Nat Imm*, 2012]

In infected fibroblasts, the cytosolic RNA sensor RIG-I recognizes 5'-triphosphate of influenza virus genomic RNA and enhances IFN production through MAVS and IRF3. By contrast, viral protein NS1 helps the virus to evade innate immune detection by sequestering viral RNA or by binding to RIG-I and/or other protein required for RIG-I cascade [113]. In pDC, the endosomal TLR7 recognize influenza virus ssRNA resulting in proinflammatory cytokines and IFN production [114]. Moreover, FLU virus prompts IL-1β production by increasing pro-IL-1β and NLRP3 transcription(signal 1) and by activating NLRP3 inflammasomes (signal 2) [115, 116]. Signal 1 is triggered by the activation of NF-κB as a consequence of viral RNA detection by TLR7. Signal 2 is provided by several sources that all depend on viral M2 protein, including ionic imbalance of trans-Golgi pH,

potassium efflux through the P2X7 receptor ion channel, and elevation of cellular reactive oxygen species (ROS). Studies found that the inflammasome complex is dispensable for viral clearance.[115, 116].

2.3.2.3.1. The IFITM family

The interferon induced transmembrane (IFITM) genes belong to a family of small ISGs containing IFITM1, 2 and 3. IFITM3 has been found in genome-wide screens as a host restriction factor for influenza A virus [117, 118]. Although the mechanism remain to be clarify, the expression of IFITM genes is induced by influenza virus, inhibiting viral entry into target cells.[117]. Moreover, IFITM proteins also obstacle the replication of some flaviviruses, including dengue virus and West Nile virus [117]. Induction of IFITM gene expression has also been observed as a consequence of cytomegalovirus (CMV) and herpes simplex virus (HSV) infection, although there is no established evidence for an antiviral role of these proteins against DNA viruses [119].
IFITM proteins have also been implicated in cancer [120]. Studies have shown that IFITM1 and 3 are associate with cell surface antigen CD81, a protein found to be differently expressed in a variety of cancers. Moreover, all IFITM proteins contain a conserved CD225 domain with antiproliferative activity that can be enhanced by IFNs treatment [121].
Comparison of genes encoding IFITM proteins of different organisms has shown higher than normal sequence variation suggesting that these genes are under positive selection during evolution [120]. Taken together, IFITM proteins represent interesting targets for therapeutical approaches against virus and cancer.

2.3.2.3.2. The IFIT family

The IFN-induced protein with tetratricopeptide repeats (TPRs) (IFIT) family contains four members in humans, namely IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60) and IFIT5 (ISG58). All IFIT proteins are cytoplasmic proteins that contain multiple helix-turn-helix structures (TPRs) involved in protein-protein interactions and protein complexes assemblation [122]. IFIT1 was originally found to inhibit cellular translation by binding the eIF3 initiation factor as a consequence of the non-specific antiviral response mediated by IFN [105].
IFIT1 also prevents cytoplasmic sensing of viral RNA by binding the adaptor protein STING thereby disrupting signaling cascade [124]. This negative feedback of IFN signaling regulated by IFIT1 is important for preserving host cell from toxicity caused by an excessive IFN-production.
Recently, IFIT proteins were found to recognize viral RNA that contains a 5'-triphosphate (5'-ppp) moiety or lacks 2'-O-methylation [125, 126]. Cellular mRNA usually contains a 5'-guanosine cap, necessary requirement for mRNA stabilization and discrimination between self and non-self RNAs. In addition, cellular mRNAs are also methylated at the 2'-O position, whose role remain unclear. Many RNA viruses also encode a methyltransferase that methylates the 2'-O position of viral RNA to mimic host mRNA. This modification is important for these viruses to evade host restriction by IFIT proteins. Viruses defective in methyltransferase exhibited enhanced sensitivity to IFN treatment in an IFIT-dependent manner [125, 126]. Thus, 2'-O-methylation of host mRNAs is critical

for self versus non-self discrimination for at least some RNA viruses.
IFIT1 also binds viral genomic RNA containing 5'-ppp, similar to RIG-I, and exerts a direct antiviral activity [127]. Instead of activating IFN, the binding of IFIT proteins to 5'-ppp viral RNA inhibits viral translation and replication. According to direct antiviral activity, knockdown of IFIT1 results in stronger viral replication without affecting the IFN response. To date, mechanisms of inhibition following recognition by IFIT proteins remain to be clarified.

2.3.2.3.3 Human myxovirus-resistant protein 1 (MX1)

The human myxovirus resistant protein 1 (MX1 or MXA) is a GTPase with wide antiviral activities. Notably, cytoplasmatic MX1 protein has found to exert antiviral activity against influenza virus infection, by blocking virus life cycle subsequently to transcription and viral replication [128]. Different strains of influenza virus vary in their sensitivity to MX proteins, which is determined by specific viral nucleoprotein (NP) [129, 130]. The structure of MXA seems to form an oligomeric ring structure around viral nucleocapsid, thereby inhibiting viral replication [131]. To date, how human MX1 can inhibit a broad spectrum of RNA viruses, some of which replicates in the nucleus, and whether MX1 acts by recognizing a common viral component or structure remains to be clarify

2.3.2.4. Intrinsic immunity to HIV

Mucosal innate immunity is the first line of defense against HIV-1 during early phases of infection, and it also plays a crucial role in the induction of adaptive immune responses. HIV-1 infection results in the activation of both cellular and intracellular innate immunity. Cellular innate immunity includes functions of DCs such as Langerhans cells that are amongst the first group of cells that contact HIV-1 at the site of infection, and that can mediate trans-infection of CD4+ T cells [132]. $\gamma\delta^+$ T cells offer innate responses to HIV through generating antiviral factors, such as RANTES, MIP-1α and MIP-1β [133]. Natural killer (NK) cells also play important roles in cellular innate immunity against HIV by eliminating infected cells and modulating DC functions [134]. Intracellular innate immunity includes several interferon-stimulated host factors that play important roles in restricting HIV-1 replication, such as APOBEC3G, TRIM5α, Tetherin/BST-2 and SAMHD1 [135, 136] (**fig. 20**). HIV-1 neutralizes some of these antiviral factors via its accessory proteins, and avoid upregulation of other antiviral ISGs in infected target cells [137].

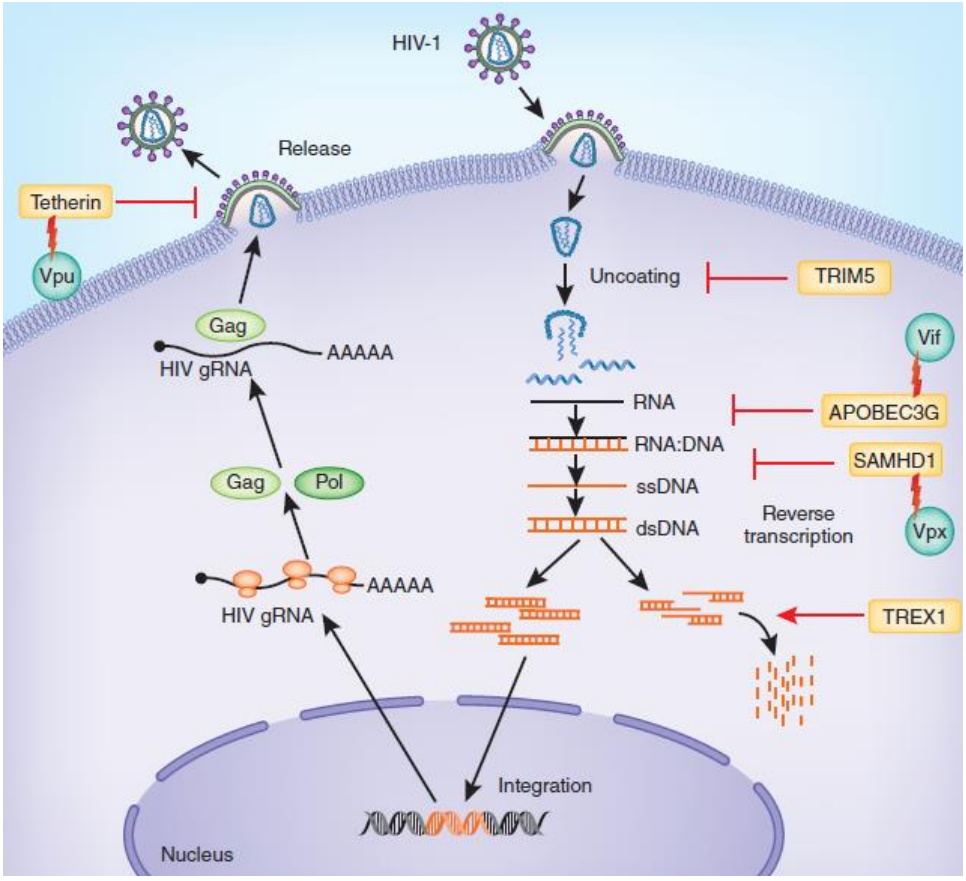


Figure 20 - *Intrinsic antiviral immunity against HIV-1* [Yan N., *Nat Imm*, 2012]

2.3.2.4.1 APOBEC3G

APOBEC3G is one of the first identified intrinsic antiviral factors against HIV-1. It belongs to a family of cytidine deaminases that contains 7 members in primates (APOBEC3A, B, C, DE, F, G, and H). The main restriction factors for HIV-1are represented by APOBEC3G and 3F. These molecules are assembled into HIV-1 virions through interaction with nucleocapsid portion of HIV Gag. Upon infection of target cells and during reverse transcription, APOBEC3G edits C→U in ssDNA (negative strand), which results in G→A mutation in the HIV genome. G→A mutations often results in premature stop codons that partially contributed to the reduced replication. Such G→A mutations are also frequently found in HIV DNA isolated from AIDS patients [138].

APOBEC3G also inhibit reverse transcription and chromosomal integration through undefined mechanisms that are independent of its deaminase activity [139]. HIV-1 Vif counteracts APOBEC3G by promoting its ubiquitination by an E3 ligase complex, thus allowing its degradation by the proteasome in virus producing cells [140].

2.3.2.4.2 Tripartite motif family 5α (TRIM5α)

TRIM5α, is a member of the tripartite motif family (TRIM) that shares a common organization at the N terminus containing a RING domain, a B-box domain and a coiled-coil domain. The RING domain is usually found in E3 ubiquitin ligase and the B-box domain determines substrate specificity. The C terminus of TRIM5α contains a B30.2 domain that binds to the capsid of the incoming virion and is important for restriction. The B30.2 domain of TRIM5α, is also determinant for retrovirus species tropism [141]. The importance of TRIM5α restriction activity was underlined again by the discovery of the TRIM5α-cyclophilin A (TRIMCyp) fusion protein in owl monkey cells [142]. TRIMCyp occurs naturally by in-frame fusion where CypA replaces the B30.2 domain. CypA also binds to the HIV-1 capsid and TRIM-Cyp potently restricts HIV-1 through mechanisms similar to TRIM5α. HIV-1 restriction by these factors occurs early during replication, prior to reverse transcription, and likely during the process of uncoating [141]. TRIM5α promotes the rapid uncoating of HIV-1 capsids *in vitro* [143]. Accelerated disassembly of retroviral capsid may prematurely expose the viral RNA or viral enzymes to degradative processes or disrupt capsid associations with retroviral core that are directly or indirectly critical for reverse transcription.Moreover, TRIM5α was also found to promote innate immune signaling and to act as a PRR for capsid of many retroviruses, including MLV, HIV and SIV [144].

2.3.2.4.3 Theterin

Tetherin was discovered through characterization of HIV-1 accessory protein Vpu [145, 146] which enhances the release of HIV and other retroviral virions, thereby promoting replication. Structurally, tetherin consists of N terminal cytoplasmic domain, a transmembrane domain, extracellular long coiled-coil domain and a C terminal glycosylphosphatidylinositol (GPI) membrane anchor. The short cytoplasmic domain binds to the clathrin adaptors for endocytosis. Tetherin is thought to retain virions at the cell surface, by introducing the GPI membrane anchor into the virion envelope or by dimerization of two tetherins each anchoring at the host cell membrane and the virion envelope, respectively. Thus, tethered virions are internalized by endocytosis and subsequently degraded in the endosomes [147]. Vpu promotes the degradation of tetherin, thereby facilitating HIV infection.
Tetherin targets many other enveloped viruses, such as other retroviruses (MLV, HTLV-1), filoviruses (Ebola virus), and herpesvirus (KSHV) [147]. Each of these viruses encode a viral protein that binds to tetherin and promotes its degradation or inhibits its function through an unknown mechanism. Tetherin also plays a role in immune cell signaling. In fact, tetherin binds to ILT7 (immunoglobulin-like transcript 7), a membrane receptor selectively expressed in pDC leading to the inhibition of TLR-mediated IFN responses in pDCs [148]. Tetherin is also involved in NF- κB activation [149]. The implication of this mechanism in viral infection need further studies.

2.3.2.4.4 SAMHD1 and TREX

SAMHD1 restricts the infection of dendritic and other myeloid cells by human immunodeficiency virus type 1 (HIV-1), but in lentiviruses of the simian immunodeficiency virus of sooty mangabey (SIVsm)–HIV-2 lineage, SAMHD1 is counteracted by the virion-packaged accessory protein Vpx. HIV-1 does not encode Vpx, and Vpx-deficient SIVmac or HIV-2 fails to replicate in DC.
SAMHD1 appears to inhibit HIV-1 reverse transcription and innate immune responses to HIV [150]. SAMHD1 consists of a SAM domain responsible for protein-protein interaction and a HD domain with nucleotide phosphohydrolase activity. Vpx binds SAMHD1 and brings it to an ubiquitin ligase complex for ubiquitination and subsequent degradation. Restriction of SAMHD1 is related to the hydrolysis of intracellular deoxynucleoside triphosphates (dNTPs), thus lowering their concentration to below those required for a functional synthesis of viral DNA. [151].
SAMHD1 may play an additional role in limiting innate immune signaling to HIV replication, especially in DCs. Indeed, DCs rendered permissive to HIV-1 infection through the expression of Vpx, which causes SAMHD1 degradation, induces type-I IFNs[152]. Thus, HIV-1 seems to avoid infecting DCs as they do not induce IFNs, but stealthily passes through DCs to facilitate its infection of helper T cells. Consistent with an inhibitory effect of SAMHD1 on IFN induction, mutations in the SAMHD1 gene are associated with Aicardi-Goutières Syndrome (AGS) [153], an autoimmune disease characterized by elevated levels of IFNα.
Interestingly, another AGS-associated gene, TREX1, has been shown to be important for HIV replication, specifically innate immune responses to HIV DNA [137]. TREX1 is a 3' exonuclease that contains conserved exonuclease N-terminal motifs and a C-terminal hydrophobic region important for its localization to the cytoplasm and endoplasmic reticulum (ER). TREX1 bound to cytosolic HIV DNA and digested excess non-productive HIV DNA that would otherwise activate interferon expression via a pathway dependent on the kinase TBK1, the adaptor STING and the transcription factor IRF3 [137]. TREX1 also prevents autoimmunity induced by DNA derived from endogenous retroelements [154], which may explain the AGS disease in patients carrying loss of function mutations of TREX1.
Although both SAMHD1 and TREX1 are related to the same autoimmune disease, they show opposite effects on HIV-1 replication. SAMHD1 is antiviral whereas TREX1 is proviral for HIV-1, through distinct mechanisms. Interestingly, both proteins seems to target the reverse transcription step, by limiting the dNTP supply (SAMHD1) or by inhibiting immune recognition of non-productive RT products (TREX1). Taken together, HIV reverse transcription is becoming an increasingly important process in the HIV life cycle that is carefully regulated by a concerted effort of viral and host factors.

2.3.2.4.5 IFN-induced myxovirus resistance 2 (MX2)

Human dynamin-like IFN-induced myxovirus resistance 2 (MX2, also known as MXB) is a member of the IFN-inducible guanosine triphosphatase (GTPase) superfamily that includes proteins involved in several cellular processas well as in resistance to intracellular pathogens [155]. To date, relatively little informations about MX2 role and function are available. Recently, a group of researchers has described MX2 protein as a strong inhibitor of HIV-1 infection and as a key effector

of IFN-α-mediated resistance to HIV-1 infection [156]. In this study, the ability of MX2 to suppress infection have been confirmed in presence of different strains of HIV-1 and, similarly, on divergent simian immunodeficiency viruses. In contrast, no effect was shown on other retrovirusessuch as murine leukaemia virus. Thus, susceptibility to MX2 protein is dictated by the capsid region of viral Gag protein, and the block to infection occurs at a late post-entry step causing the suppression of nuclear accumulation and chromosomal integration of nascent viral complementary DNA.. Interesting, human MX1, closely related protein that has long been recognized as a broadly acting inhibitor of RNA and DNA viruses, including the orthomyxovirus influenza A virus [157, 158], does not affect HIV-1, whereas MX2 is ineffective against influenza virus. Thus, MX2 anti-HIV-1 resistance factor may represent a new therapeutic approach for the treatment of HIV-1 infection.

2.3.2.4.6 Antiviral role of 25-hydroxycholesterol (25-HC)

Cholesterol is an essential component of cellular membranes and its biosynthetic pathway must be tightly regulated. The sterol regulatory element–binding protein (SREBP) family members provide a feedback regulatory system involved in biosynthesis and uptake of cholesterol preferentially by SREBP2 [159]. The liver X receptor (LXR) family members, instead, are involved in the elimination of excess cholesterol [160]. Additionally, SREBP1 and LXR transcription factors also work together to integrate cholesterol homeostasis with fatty-acid metabolism [161, 162].
LXR family consists of two members LXRα, and LXRβ, that regulate, as heterodimers with retinoid X receptors (RXRs), the expression of target gene by binding to LXR-response elements (LRE) on their promoters [163]. In the absence of activating ligands, LXR-RXR heterodimers interact with corepressor complexes that actively repress gene expression. LXRs positively regulate the expression of genes encoding lipid-transport proteins, such as ATP-binding cassette A1 (ABCA1), ATP-binding cassette G1 (ABCG1), apolipoprotein E (ApoE) and several enzymes involved in fatty-acid remodeling [164-166]. In contrast, LXRs negatively regulate gene expression of NF-κB and AP-1 [167, 168].
Sensing cellular cholesterol content is a key element for the regulation of intracellular cholesterol homeostasis. Once SREBP-cleavage activating protein (SCAP) senses low cholesterol levels in the ER, dissociates from ER-retention protein INSIG1 and INSIG2, and permits the proteolytically cleavage of SREBP releasing the active N-terminal transcription factor [162]. This is then delivered to the nucleus where it binds to sterol-regulatory element (SREs) in the promoter of target genes. By contrast, in presence of high cholesterol levels, SREBP precursors are sequestered in the ER. The activities of LXR and SREBP pathway are finely regulated by cholesterol precursors and oxysterols [162] (**fig. 18**).
The connection between virus and cholesterol has been widely established in the last few years. Many viral pathogens indeed exploit cellular lipid metabolism to ensure a correct amount of cholesterol for the release of functional virions [169].
According to this observation, studies have provided evidence that activation of LXRs and/or inhibition of SREBP pathways results in the inhibition of viruses replication, including HIV and HCV [169-174]. Moreover, increased level of the ER-associated enzyme cholesterol-25-hydroxylase (CH25H), responsible for the production of 25-hydroxycolesterol (25-HC), has been related to TLR stimulation

and IFNs. [175, 176]. Notably, distinct from known IFN-mediated antiviral mechanism, CH25H was recently found to inhibits growth of a wide range of enveloped viruses by production of a soluble oxysterols, 25-HC [177, 178]. Independent of its known regulatory effect on metabolism, 25-HC impairs virus-cell fusion by inducing cellular membrane changes. In particular, HIV induce a switch of cholesterol trafficking from physiological efflux to virus-controlled transport, thus reducing the ability of a cell to export excessive cholesterol. Intracellular cholesterol metabolism control is essential for the correct assembly and release of functional virions.

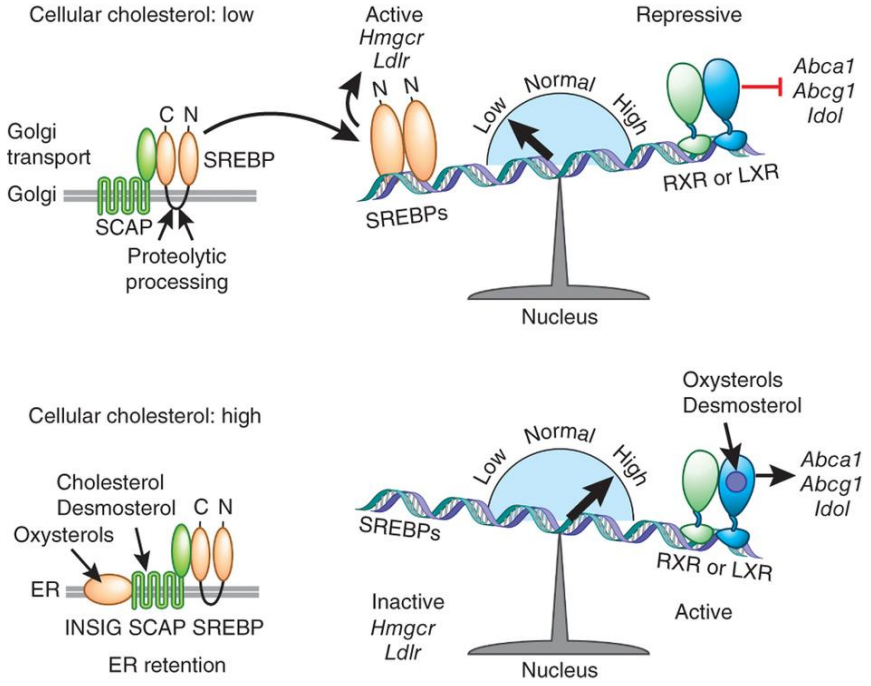


Figure 18 – *Regulation of cellular cholesterol homeostasis* [Spann N.J. *Nat Imm.*, 2013]

AIM

The amplitude of the spectrum of pathogens targeted by thiazolides makes it very unlikely that the action of these compounds is mediated by a pathogen-specific mechanism(s), suggesting instead that thiazolides act as immunomodulants. The potential effect of these compounds on immune responses has nevertheless not been investigated so far. In particular, as innate immunity and type 1 IFNs are pivotal in early and potent antiviral immune responses not antigen-restricted, it is plausible to hypothesize that thiazolides could potentiate this arm of the immune response. To verify this possibility, we performed extensive *in vitro* analyses on the immunomodulatory effects of two generations of thiazolides, TIZ and RM4848 a second generation non-nitro thiazolide, using two different viral models: Influenza and HIV-1. In particular, the aim of this study was to evaluate thiazolide effects on TLR and type I IFNs expression key protein involved in viral recognition and innate immune responses against viruses.

MATERIALS AND METHODS

1. SAMPLE COLLECTION AND DRUGS PREPARATION

1.1. Thiazolides

TIZ and the second generation thiazolides RM4848 were provided by Romark Laboratories (Tampa, Florida). Powders were resuspended in dimethyilsulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) to obtain a 40 μg/ml solution and stored at 2-8°C until use.

1.2. Blood sample collection and PBMCs separation

Whole blood was collected from 20 healthy blood donors by venipuncture in Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA, Becton Dickinson, Rutherford, NJ, USA) following informed consent. Samples were centrifuged at 1400 rpm for 10 minutes and plasma was collected and stored at -20 for subsequent analysis. The remaining sample, composed by erythrocytes, peripheral blood mononuclear cells (PBMCs), granulocytes and platelets, was diluted in phosphate buffered saline (PBS, PBI International, Milano, Italy) and separated on a lymphocytes separation medium (Lympholyte-H, Cederlane Laboratories, Burlington, NC, USA) for 25 minutes at 2300 rpm. PBMCs layer were carefully removed and washed twice in PBS (PBI, Milan, Italy) and cell number and cellular vitality were determined.

1.3. Cell count

Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). ADAM-MC automatic cell counter measures total cell numbers and cell viabilities by cutting edge detection technologies. Instead of tryphan blue staining, which can lead to inaccurate data, ADAM-MC utilizes two sensitive fluorescence dye staining solutions, AccuStain Solution T (Propidium Iodide/lysis solution) and AccuStain Solution N (Propidium IodideI/PBS). AccuStain Solution T allows plasma membrane disruption and nucleus staining for measurement of total cell concentration. AccuStain Solution N allows staining of non-viable cells, thus leaving viable cells completely intact. A 532 nm optic laser is automatically focused onto the cell solution inserted into a disposable microchip and cell analysis is made by a CCD detection technology.

1.4. MTT assay

Toxicity of thiazolides was determined by a MTT based assay (Sigma Aldrich, St. Louis, MO, USA). The MTT system is a method to measure the activity of living cells via mitochondrial dehydrogenases activity. Viable cells reduce the yellow tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5diphenyl formazan (MTT) to purple formazan insoluble crystals.
Cells were cultured in RPMI medium (Euroclone, Milan, Italy) supplemented with 20% FBS (Euroclone, Milan, Italy), penicillin and streptomycin, L-glutamine and gentamycin (Euroclone, Milan, Italy) alone or in presence of increasing concentration (0.5 μg/ml, 1 μg/ml, 10 μg/ml, 20 μg/ml and 200 μg/ml) of drugs, diluted in medium culture for 1, 3, 5, 7 and 10 days. Every two days the 50% of medium was removed and replaced with fresh medium containing drugs at the

corresponding concentrations. After culturing, cells were washed five times in RPMI without phenol red (Euroclone, Milan, Italy). PBMCs were then incubated in medium RPMI without phenol red plus 10% FBS containing MTT (500 µg/ml) for a period of 4 hours. Insoluble formazan product was dissolved by incubation with an amount of an acidified Solubilisation Solution (Sigma Aldrich, St. Louis, MO, USA) equal to original culture media volume. Finally formazan absorbance were measured at 595 nm with IMark microplate reader (Biorad, Hercules, CA, USA). PBMCs viability were established by dividing the absorbance reading of the formazan by the dry weight of cell cultures. Toxicity were determined by dividing viability of the drug- treated cells by viability of untreated cell control.

2. INFLUENZA VIRUS ANALYSIS

2.1. Influenza Virus (FLU) strains

The influenza virus (FLU) used is live UV-inactivated influenza virus (A/Bangkok/RX73 and A/Puerto Rico/8/34 strains; 1:800) and the 1998–1999 formula of influenza virus vaccine (1:5000; Wyeth Laboratories Inc., Marietta, PA, USA). The influenza virus vaccine is an inactivated trivalent subunit formulation that contains the hemagglutinin antigens of influenza A H1N1, influenza A H1N3, and influenza B virus strains (each at 30 mg/ml). Viruses were kindly provided by Dr. Shearer, National Institutes of Health (NIH, Bethesda, Washington, USA).

2.2. PBMCs stimulation with thiazolides in presence of FLU antigens

PBMC re-suspended at $4x10^6$/ml in RPMI 1640 (Euroclone, Milan, Italy) were incubated for 4 hours with two different doses of TIZ or RM4848 (1 or 10 µg/ml) (Romark Laboratories, L.C. Tampa, Florida) in presence/absence of FLU antigens (final dilution 1:400, National Institutes of Health, Bethesda, MD, USA). Anti-CD28 mAb (R&D Systems, Minneapolis, MN, USA) was added during incubation (1mg/well) to facilitate co-stimulation. For cytokine analyses, 10 µg/ml Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) was added to cell cultures during the last 18 hours to block protein secretion.

2.3. Comparison between TIZ or IFNα treatment in FLU-stimulated and unstimulated PBMCs

PBMCs stimulated with FLU antigens were re-suspended at $4x10^6$/ml in complete medium and incubated for 4 hours with TIZ (10 µg/ml) (Romark Laboratories, L.C. Tampa, Florida) or IFNα (final diluition 1:400 IU/ml) (Sigma Aldrich, St. Louis, MO, USA). PBMCs not stimulated with FLU in the same conditions were used as control. Anti-CD28 mAb (R&D Systems, Minneapolis, MN, USA) was added during incubation (1mg/well) to facilitate co-stimulation. For cytokine analyses, 10 µg/ml Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) was added to cell cultures during the last 18 hours to block protein secretion.

2.4. Flow cytometry analysis

All flow cytometry analysis were performed using a CYTOMICS FC-500 flow cytometer (Beckman-Coulter, Miami, FL, USA) equipped with a double 15-mV argon ion laser operating at 456 and 488, interfaced with CXP 21 software (Beckman-Coulter, Miami, FL, USA). For each analysis 20000 events will be acquired and gated on CD4, CD8 or CD14 expression and side scatter properties. Green fluorescence from FITC was collected through a 525 nm band pass filter (FL1), orange-red fluorescence from PE was collected through a 575 nm band pass filter (FL2), red fluorescence from 7-AAD was collected through a 620 nm band pass (FL-3), red fluorescence from PE- Cy5 was collected through a 670 nm band pass filter (FL4), deep red fluorescence from PE- Cy7 was collected through a 755 nm band pass filter (FL-5). Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL-1, FL-2, FL-3, FL-4 and FL-5.

2.4.1. TLRs expression on monocytes

After stimulation, PBMCs were resuspended in PBS and stained for surface mAb CD14PECY5 (Beckman-Coulter, Fullerton, CA, USA). After a 15-minute incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldehyde in PBS. Cells were then permeabilizated with saponin 0,5% (Sigma-Aldrich, St. Louis, MO, USA) and monoclonal antibodies for TLR3 PE (eBioscience, San Diego, CA, USA) and TLR8 FITC (Imgenex Corporation, San Diego, CA, USA) or TLR7 FITC (R&D Systems, Minneapolis, MN, USA) were added. Cells were incubated for 45 minutes at 4°C in the dark, washed and fixed in 1% paraformaldehyde in PBS.

2.4.2. Percentage of IFNα-secreting pDC

PBMCs, stimulated with TIZ or IFNα and resuspended in PBS were stained for surface mAb CD123 PECy7, LIN^- PECy5 (CD8, CD4, CD19, CD14) and HLA-DRII PE (Beckman-Coulter, Fullerton, CA, USA). After a 15-minute incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldehyde in PBS. Cells were then permeabilizated with saponin 0,5% (Sigma-Aldrich, St. Louis, MO, USA) and monoclonal antibodies for IFNα FITC (eBioscience, San Diego, CA, USA) were added. Cells were incubated for 45 minutes at 4°C in the dark, washed and fixed in 1% paraformaldehyde in PBS.

2.4.3. Intracellular cytokines expression

After stimulation, PBMC were washed in PBS and stained for CD4 PECy5, CD8 PECy7 or CD14PECy5 (Beckman-coulter, Fullerton, CA, USA) for 15 min at RT in the dark. PBMCs will be fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) 15 min at 4°C and then washed. Cells were then re-suspended in 0,5% Saponin (Sigma-Aldrich, St. Louis, MO, USA) and stained for IL2 PE and IFNγ FITC (Beckman-Coulter, Fullerton, CA, USA). After a 45 minutes incubation at 4°C in the dark, cells will be washed and fixed in 1% paraformaldehyde in PBS.

2.4.4. Statistical analysis

Comparisons between groups were analyzed to evaluate immunological differences. Kruskal & Wallis analysis of variance was performed for each variable; Bonferroni correction was applied to the results. Two-sided p-values were considered. Data analysis was performed using the SPSS statistical package (SPSS Inc. Chicago, Illinois, USA).

2.5. RNA extraction and Real Time PCR

RNA was extracted from cultured PBMCs by using the acid guanidium thiocyanate–phenol–chloroform method. RNAzol B reagent (Duotech, Milan, Italy), a monophase solution containing phenol and guanidine thiocyanate, was used. PBMCs were lysed in RNAzol B and the lysate were separated into aqueous and organic phase by the addition of chloroform (20% of RNAzol B initial volume used). Samples were centrifuged (at 12,000g 15 minutes at 4°C) to efficiently remove DNA and proteins from the aqueous phase containing RNA. The pure, not degraded, RNA is obtained from the aqueous phase by the isopropanol precipitation and washing with 75% ethanol.

2.5.1. DNAse treatment and retrotranscription (RT)

RNA was dissolved in RNase-free water, and purified from genomic DNA with TURBO DNAse (Applied Biosystems/Ambion, Austin, TX, USA), a genetically engineered form of bovine DNase I with greater catalytic efficiency than conventional DNase I at higher salt concentrations and lower DNA concentrations. A reaction mixture, containing 1µg of RNA, Turbo DNasi 1U and TURBO DNasi Buffer, were incubated 30 minutes at 37 °C. Then DNase was inactivated by DNase inactivation reagent (Applied Biosystems/Ambion, Austin, TX, USA), that binds and removes the divalent cations from DNase. 1 µg of RNA was reverse transcribed into first-strand cDNA in a 20-µl final volume. A reaction mixture, containing 1 µM random hexanucleotide primers, 1 µM oligo dT and the RNA, was heated at 70 °C for 5 minutes to melt secondary structure within the template. The mixture was immediately cooled on ice to prevent secondary structure from reforming. A dNTPs mix, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20 U Recombinant RNasi inhibitor and M-MLV 5X reaction buffer were added (Promega, Fitchburg, WI, USA). The reaction mix were incubated 60 minutes at 42 °C and then heated 5 minutes at 95 °C to inactivate the RT.

2.5.2. Real time PCR

cDNA quantification of genes involved in this study was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Ramsey, MN). Reactions were performed using a SYBR Green PCR mix (Promega, Fitchburg, WI, USA). SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. Reactions were performed according with the following thermal profile: an initial denaturation (95 °C ,15 minutes) followed by 40 cycles of 15 sec at 95 °C (denaturation) and 1 min at 60 °C (annealing) and 20 seconds at 72 °C (extension). By recording the amount of fluorescence emission at each cycle, the PCR reaction was monitored during exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Melting point or dissociation curve analysis for amplicon identification, was performed.
The threshold line is the level of the detection or the point at which a reaction the reaction reaches a fluorescent intensity above background (the mean of fluorescence values detected from to third to tenth cycle, when target amplification it is no appreciable yet). The threshold was set placed above baseline activity and in the exponential increase phase of the amplification for the most accurate reading. The parameter Ct (Threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the Ct value. A Ct value of 40 or higher means no amplification and this value was not included in the calculations.
Results were expressed as ΔΔCt and presented as ratios between the target gene and the GAPDH housekeeping mRNA. All the samples were analyzed in triplicate.

2.5.3. Real Time PCR Arrays

2.5.3.1. Human Toll-like Receptors signaling pathway

TLR signalling pathways were analysed in a PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SABiosciences Corporation, Frederick, MD, USA). This approach permits the monitoring of mRNA expression of 84 genes (**tab. 4**) related to the TLR pathway, plus five housekeeping genes, following the procedures suggested by the manufacturer. Controls were also included on each array for genomic DNA contamination, RNA quality, and general PCR performance. The results were analysed by SABioscences online software. Only targets showing at least a 2-fold modulation were considered significative.
The experiments have been run on all of the subjects included in the study pooled into distinct groups on the basis of the treatment (no drugs, TIZ or RM4848). Thus, results represent the mean value of the different targets analysed in each group. Furthermore, those targets showing marked differences between groups have been confirmed by Real time PCR on each individual member of the sample confirming the data obtained in the array.

Toll-Like Receptors: CD180 (LY64), SIGIRR, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10.
Pathogen-Specific Responses:
Bacterial: CCL2 (MCP-1), CD14, CD180 (LY64), FOS, HRAS, IL10, IL12A, IL1B, IL6, IL8, IRAK1, HMGB1, HSPA1A (HSP70 1A), JUN, LTA (TNFB), LY86 (MD-1), LY96, NFKBIA (IKBA/MAD3), PTGS2 (COX2), RELA, RIPK2, TLR2, TLR4, TLR6, TNFRSF1A, TICAM1 (TRIF).
Viral: EIF2AK2 (PRKR), IFNB1, IFNG, IL12A, IL6, IRF3, PRKRA, RELA, TBK1, TLR3, TLR7, TLR8, TNF, TICAM1 (TRIF).
Fungal/Parasitic: CLEC4E, HRAS, HSPA1A (HSP70 1A), IL8, TLR2, TIRAP.
TLR Signaling:
Negative Regulation: SARM1, SIGIRR, TOLLIP.
TICAM1 (TRIF)-Dependent (MYD88-Independent): IRF3, MAP3K7 (TAK1), TAB1, NR2C2, PELI1, TBK1, TICAM2, TLR3, TLR4, TRAF6, TICAM1 (TRIF).
MYD88-Dependent: IRAK1, IRAK2, MAP3K7 (TAK1), TAB1, MYD88, NR2C2, TIRAP, TLR1, TLR10, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TRAF6.
Downstream Pathways and Target Genes:
NFκB Pathway: BTK, CASP8, CHUK (IKKa), ECSIT (SITPEC), FADD, IKBKB, IL10, IL1B, IRAK1, IRAK2, IRF3, LY96, MAP3K1 (MEKK), MAP3K7, MAP4K4, NFKB1, NFKB2, NFKBIA (IKBA/MAD3), NFKBIL1, NFRKB, PPARA, REL, RELA, TNF, TNFRSF1A, UBE2N, UBE2V1.
JNK/p38 Pathway: ELK1, FOS, IL1B, JUN, MAP2K3 (MEK3), MAP2K4 (JNKK1), MAP3K1 (MEKK), MAP3K7, MAPK8 (JNK1), MAPK8IP3, TNF.
JAK/STAT Pathway: CCL2 (MCP-1), CSF2 (GM-CSF), IFNG, IL12A, IL2, IL6.
Interferon Regulatory Factor (IRF) Pathway: CXCL10 (INP10), IFNA1, IFNB1, IFNG, IRF1, IRF3, TBK1.
Cytokine-Mediated Signaling Pathway: CCL2 (MCP-1), CSF3 (GCSF), IL1A, IL1B, IL6, IRAK1, IRAK2, RELA, SIGIRR, TNF, TNFRSF1A.
Regulation of Adaptive Immunity: CD80, CD86, HSPD1, IFNG, IL10, IL12A, IL1B, IL2, MAP3K7, TRAF6.
Adaptors & TLR Interacting Proteins: BTK, CD14, HMGB1, HRAS, HSPA1A (HSP70 1A), HSPD1, LY86 (MD-1), LY96 (MD-2), MAPK8IP3, MYD88, PELI1, RIPK2, SARM1, TICAM1 (TRIF), TICAM2 (TRAM), TIRAP, TOLLIP.
Effectors: CASP8 (FLICE), EIF2AK2 (PRKR), FADD, IRAK1, IRAK2, MAP3K7 (TAK1), TAB1, NR2C2, PPARA, PRKRA, ECSIT (SITPEC), TRAF6, UBE2N, UBE2V1.

Table 4 *– Complete list of TLR pathway genes analyzed (SABiosciences)*

2.5.3.2. Human Type I IFN response

Type I IFNs signalling pathways were analysed in a PCR array (SABiosciences Corporation, Frederick, MD, USA) (see Human TLRs signaling for details) (**tab. 5**).

Interferons & Receptors: IFNA1, IFNA2, IFNA4, IFNAR1, IFNAR2, IFNB1, IFNE, IFNW1.

Interferon-Responsive Genes:

Innate Immunity: ADAR, BST2, CASP1 (ICE), CCL2 (MCP-1), CCL5 (RANTES), DDX58, GBP1, HLA-E, HLA-G, IFI27, IFI30, IFI6 (G1P3), IFIH1, IFIT1, IFIT2, IFIT3, IFITM1 (9-27), IFITM2, IFITM3, IRF1, IRF2, IRF7, IRF9 (ISGF3G), ISG15 (G1P2), ISG20, JAK2, MX1, MX2, MYD88, NMI, OAS1, OAS2, PML, SOCS1, STAT1, STAT2, TAP1 (ABCB2), TLR3, TLR7.

Adaptive Immunity: CD80, CRP, MYD88, TAP1 (ABCB2).

Cytokines: CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (INP10), IL15, TNFSF10 (TRAIL).

Apoptosis: ADAR, CASP1 (ICE), CCL2 (MCP-1), CCL5 (RANTES), EIF2AK2 (PRKR), IFI16, IFI27, IFI6 (G1P3), IFIH1, JAK2, MX1, MYD88, PML, STAT1, TIMP1, TLR3, TNFSF10 (TRAIL).

Other Genes Involved in Type I Interferon Response:

Innate Immunity: CD86, CIITA, HLA-A, HLA-B, IFNA2, IFNA4, IFNAR1, IFNAR2, IFNB1, IRF3, IRF5, JAK1, NOS2 (iNOS), SH2D1A, STAT3, TICAM1 (TRIF), TLR8, TLR9, TMEM173, TRAF3, TYK2.

Adaptive Immunity: IFNA2, IFNB1, IL10, TLR8, VEGFA.

Cytokines: CD70 (TNFSF7), IL10, IL6, VEGFA.

Apoptosis: BAG3, CAV1, CD70 (TNFSF7), CDKN1B (p27KIP1), IFNA2, IFNB1, IL10, IL6, MAL, MET, MNDA, PRKCZ, PSME2, SHB, TICAM1 (TRIF), TMEM173, TRAF3, VEGFA.

Table 5 *– Complete list of Type I IFN pathway genes analyzed (SABiosciences)*

2.6. Clinical trial design

Six hundred and twenty four (624) patients were enrolled at 74 outpatient study centers throughout the United States. Eligibility criteria include age 12-65 years, oral temperature ≥ 100.4º F, at least one respiratory symptom and one constitutional symptom, symptom onset ≤48 hours of enrolment, laboratory confirmed influenza in the community, not at risk of influenza complications based on CDC criteria. Patients were randomized to receive NTZ: 600 mg, NTZ: 300 mg or Placebo twice daily for 5 days and were followed for 28 days. Symptoms were graded (0 to 3, absent to severe) and recorded twice daily in a patient diary. Nasopharyngeal swabs collected at baseline and day 7 for all patients and on days 2, 3, 4 and 5 for a subset of patients subjected to PCR and culture to identify and quantify viruses. The primary endpoint was time to alleviation of symptoms (all symptoms absent or mild). The study protocol was approved by a central Ethical Committee and the study was carried out under the Food and Drug Administration IND No 107.316.

3. HUMAN IMMUNODEFICIENCY VIRUS 1

3.1. HIV-1 strains

The laboratory-adapted HIV-1 strains used in the experiments was the R5 tropic HIV-1_{BaL} (contributed by Drs. S. Gartner, M. Popovic and R. Gallo, courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program). The virus were provided through the EU programme EVA centre for AIDS Reagents (The National Institute for Biological Standards and Control NIBSC, Potter Bars, UK).

3.1.1. HIV-1_{BaL} expansion in CCR5$^+$ JURKAT T cells

The HIV-1_{BaL} was grown to high titer in CCR5$^+$ Jurkat T cells, stably expressing CCR5. Cells were maintained in a logarithmic phase of growth for 2 to 3 days before infection, at the concentration between 0.1 to 0.5x10^6 cells/ml in complete medium. Cells were seeded at density of 2×10^6 cells/ml and were incubated with HIV-1_{Bal} for 3 hours at 37°C. After infection cells were washed and maintained in culture at density of 1x10^6 cells/ml in complete RPMI medium for 21 days. Two times per week the 50% of medium and of the cells was removed and replaced with fresh RPMI medium. Supernatants were collected, aliquoted and stored in liquid nitrogen. Viral concentration in supernatants was monitored by p24 ELISA (PerkinElmer, Waltham, MA, USA). HIV-1_{BaL} concentration in supernatants 21 days after infection was 617ng/ml.

3.2. HIV-1_{BaL} infection assay

Whole blood was collected from 20 healthy volunteers by venupuncture in Vacutainer tubes containing EDTA (Becton Dickinson, NJ, USA), and PBMC were separated on lymphocyte separation medium (Organon Teknica, Malvern, PA, USA). PBMCs (10×106 cells/mL) were resuspended in medium containing 1ng/1x10^6 cells of HIV-1_{BaL} p24 viral input and incubated for 24 hours at 37°C and 5% Co_2. Cells were then washed and resuspended in complete medium with interleukin-2 (IL-2,15 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA), phytohemagglutinin (PHA, 7.5 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and TIZ (10 µg/mL) or RM4848 (10µg/ml) or TO901317 (LXR agonist, 1 µM) (Sigma-Aldrich, St. Louis, MO, USA) for 3 days. After viability assessment cells were washed and resuspended in RPMI complete medium added with IL2 and TIZ or RM4848 or TO901317 at the same concentrations. Cells were then plated in non-treated 24-well tissue culture plates and incubated at 37°C and 5% CO2. After other 4 days (7 days post infection), 1 x 10^6 PBMC were collected for gene expression analyses whereas 2.5 x 10^5 cells for each condition for protein expression analysis. Supernatants were collected and stored at -20°C for subsequent analysis. After 7 days (day 10 post infection) supernatants were collected for batched ELISA of p24 antigen. Absolute levels of p24 were measured using the Alliance HIV-1 p24 ELISA Kit (PerkinElmer, Waltham, MA, USA). 1 x 10^6 PBMC were collected for gene expression analyses whereas 2.5 x 10^5 cells for each condition for protein expression analysis. Every maximum 3 days half medium was changed with fresh one.

The RM4848 action and kinetic were evaluated during the HIV-1_{BaL} infection assay by adding the compound to cell cultures at different time-points. In particular, PBMCs were treated with RM4848 for two days before infection, during infection, for 10 days post infection, or for all the experiment. Untreated condition was used as control. Supernatants were collected and stored at -20°C for subsequent analysis.

3.3. Flow cytometry analysis

All flow cytometric analyses were performed using a CYTOMICS FC-500 flow cytometer (Beckman-Coulter, Miami, FL, USA). See 2.4 paragraph for details about the method.

3.3.1. Identification of apoptosis by 7-amino actinomycin D (7AAD)

To evaluate the toxicity of thiazolides cells were incubated with different concentrations of drugs for 1, 4 or 7 days. After the incubation cells were stained with DNA incorporating dye 7-aminoactinomicyn D (7-AAD, Beckman-coulter, Fullerton, CA, USA) for 15 minutes at room temperature in the dark. After, 500µl of PBS were added and samples were analyzed by flow cytometry.
Scattergrams were generated by combining forward light scatter with 7AAD fluorescence, and regions were drawn around clear-cut populations having negative, dim, and bright fluorescence.

3.3.2. Intracellular cytokines expression

After stimulation, PBMC were washed in PBS and stained for CD4PC5, CD8PC7 or CD14PC5 (Beckman-coulter, Fullerton, CA, USA) for 15 min at RT in the dark. PBMCs were fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) 15 min at 4°C and then washed. Cells will be then re-suspended in 0,5% Saponin (Sigma-Aldrich, St. Louis, MO, USA) and stained for IL2 PE, IFNγ FITC (Beckman Coulter). After a 45 minutes incubation at 4°C in the dark, cells will be washed and fixed in 1% paraformaldehyde in PBS.

3.4. ELISA assay

3.4.1. p24 ELISA

Culture supernatants were collected at day 7 and day 10 after HIV-1_{Bal} infection assay. p24 concentration, as a measure of HIV infection, was assayed using the Alliance HIV-1 p24 Antigen kit (Perkin Elmer, Boston, USA) following manufacturer's instructions. Microplate wells are already pre-coated with a monoclonal antibody endowed with a high specificity and affinity to HIV-1 p24. Standards and samples (when needed) were diluted in inoculated culture medium. A five point standard curve using two-fold serial dilution and an high standard of 100 pg/ml were prepared. A linear standard curve was generated. Firstly Triton X-100 was added to all microplate wells (except substrate blank) to disaggregate HIV-1 virion, then the standards and samples were added to designated wells and the plate were incubated 2 hours at 37 °C. Plate were washed with wash buffer (phosphate buffer plus 1% Tween-20). Biotinylated polyclonal antibody to HIV-1 p24 was added to all wells except substrate blank at 37 °C for 1 hours. After

washes streptavidine conjugated with the horseradish peroxidase was added and the microplate was incubated 30 minutes at room temperature (RT). After washing the chromogenic substrate ortho-phenilenediamine-HCL (OPD) were added 30 minutes to all wells at RT. Following incubation with OPD, yellow color directly proportional to amount of p24 captured was generated. Reaction was stopped by adding stop solution (4N sulfuric acid) to all wells. Plates were immediately read at 490 nm, with the correction wavelength set at 655 nm, using the IMark microplate reader equipped with Microplate Manager® 6 software (both from Biorad, Hercules, CA, USA).

3.4.2. Multiplex Immunoassay

3.4.2.1. Measurement of Cytokine Concentrations by Multiplex Immunoassay and ELISA Immunoassay

Cytokines concentrations were determined using multiplex sandwich immunoassays with the Fluorokine Multi Analyte Profiling Kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol. The following cytokines were included in the kit: IL2, IFNγ, MCP-1, MIP1α, MIP1β and RANTES. Cytokines concentrations were calculated using a standard curve derived from the reference cytokine concentration standards supplied by the manufacturer.

3.4.2.2. Statistical analysis

Wilcoxon Rank Sum tests were used to evaluate differences in p24 levels, cytokine expression and cell counts. All tests were two-tailed and were performed in R *(*http://cran.r-project.org*).*

3.5. RNA extraction, retrotranscription (RT) and Real Time PCR

See paragraph 2.5. for details about the methods.

3.5.3. Real Time PCR Arrays

3.5.3.1. Human Toll-like Receptors signaling pathway

TLR signalling pathways were analysed in a PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SABiosciences Corporation, Frederick, MD, USA). See paragraph 2.5.3.1 for details about the methods.

3.5.3.2. Human Type I IFN response

Type I IFNs signalling pathways were analysed in a PCR array (SABiosciences Corporation, Frederick, MD, USA). See paragraph 2.5.3.2 for details about the methods.

3.5.3.3. HIV-1 human response

Type I IFNs signalling pathways were analysed in a PCR array (SABiosciences Corporation, Frederick, MD, USA) (**tab. 6**). See paragraph 2.5.3.1 for details about the methods.

HIV Receptors & Natural Ligands: CCL2 (MCP-1), CCL4(MIP-1B), CCL5 (RANTES), CCR5, CD4, CXCL12 (SDF1), CXCR4.
Cellular Cofactors Involved in HIV Infection:
Viral Genome Replication: APOBEC3G, CD209, HTATSF1.
Inflammatory Response: CCR2, CCR3, CCR4.
Antimicrobial Humoral Response: CCR2, YY1.
Apoptosis: EP300, LTBR, PTK2B.
Cell Cycle: CCNT1, CDK7, CDK9, EP300, RBL2, SMARCB1.
Cell Proliferation: CDK7, CDK9, PTK2B.
Cell Adhesion: CCR3, CD209, PTK2B.
Transcription Factors & Regulators: APEX1, BCL11B, CCNT1, CREBBP, EP300, HMGA1, HTATSF1, NFATC1, RBL2, SMARCB1, TFCP2, TSG101, YY1.
Other Genes: BANF1, BTRC, CBX5, CD247 (CD3Z), COPS6, ELANE, LTBR , PPIA, TRIM5, VPS4A, XPO1.
Innate Immune Response:
Humoral Response: IL12B, IL1B, KLRD1, XCL1.
Response to Virus: CCL4 (MIP-1B), CCL5 (RANTES), CCL8 (MCP-2), CXCL12 (CSF1), IFNB1, TNF.
Inflammatory Response: CCL2 (MCP-1), CCL3 (MIP-1A), CCL4 (MIP-1B), CCL5 (RANTES), CCL8 (MCP-2), CXCL12 (SDF1), IL10, IL1B, IL8, SERPINA1, TNF.
Natural Killer Cell Activation: IFNB1, IL12B, IL2.
Defense Response Against Pathogens: CD69, CX3CL1, IFNA1, IFNB1, IFNG.
Cell Adhesion: CCL2 (MCP-1), CCL4 (MIP-1B), CCL5 (RANTES), CD44, CX3CL1, CXCL12 (CSF1), IL8, SELL (LECAM1), TNF.
Other Genes: CD74, CR2, IFNG, IL16, MBL2, PRDX1, SERPINC1 (ATIII), SLPI, TGFB1, TNFRSF1B.
Immune Evasion: CD4, FCAR, MAP3K5.
Cellular Proteins Induced or Activated by HIV Infection:
Apoptosis: BAD, BAX, BCL2, CASP3, CASP8 (FLICE), CDKN1A (p21CIP1/WAF1), GADD45A, NFKBIA (I?Ba/MAD3), STAT1, TNFSF10 (TRAIL).
Cell Cycle: BAX, BCL2, CDK9, CDKN1A (p21CIP1/WAF1), GADD45A, IRF1, STAT1.
Cell Proliferation: BCL2, CDK9, CDKN1A (p21CIP1/WAF1), IRF2.
Transcription Factors & Regulators: CDK9, CEBPB, FOS, IRF1, IRF2, NFATC1, NFKBIA, STAT1, STAT3.

Table 6 *– Complete list of HIV-1 human response pathway genes (SABiosciences)*

RESULTS AND DISCUSSION

1. THIAZOLIDES

1.1. Evaluation of thiazolide toxicity

The colorimetric tetrazolium salt (MTT) assay, that monitors metabolic activity of cultured cells, was adapted to analyse the viability of cells exposed to thiazolides. After having established a suitable cell seeding density, PBMC isolated from 5 healthy controls were treated with different doses of TIZ or RM4848 (1, 5, 10, 20 and 100 µg/ml) at different time-points (1, 3, 5 and 10 days) to test the dose-dependence and the time-course of PBMC viability reduction (**fig. 19**). The higher dose (100 µg/ml) of thiazolides reduced cell viability after 3 or more days of drug exposure. The most severe effect was observed 10 days after treatment with MTT reduction of 35% for TIZ and 86% for RM4848 compared to drug-untreated cells. Results obtained demonstrated that concentrations lower than 20 µg/ml were sufficient to maintain pharmacological properties without affecting cell viability.

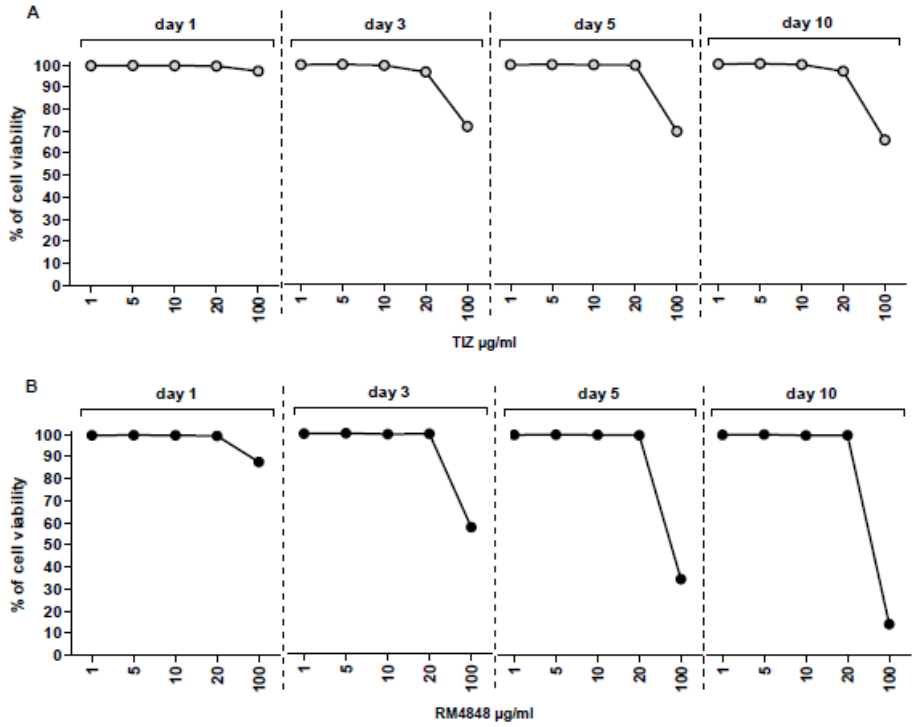


Figure 19 *– Percentage of PBMC viability after culture with different doses of TIZ (1, 5, 10, 20 and 100 µg/ml) (**A**) or RM4848 (1, 5, 10, 20 and 100 µg/ml) (**B**) at different time-points (1, 3, 5 and 10 days) assessed by MTT assay.*

2. INFLUENZA VIRUS MODEL

2.1. Thiazolides enhance TLR7 and TLR8 expression on immune cells

Early antiviral response requires TLR activation induced by ligation with pathogen-associated molecular patterns (PAMPs). In particular TLR3, TLR7 and TLR8 recognize nucleic acids produced during viral replication. We examined whether TLR expression could be modulated by two different doses of TIZ or RM4848 (1 and 10 µg/ml). To this purpose, TLR3, TLR7 and TLR8 expression was evaluated on $CD14^+$ cells (monocytes) both in unstimulated conditions and after FLU-stimulation. Results showed that, whereas thiazolides did not influence TLR3 expression (data not shown), TLR7 and TLR8 were up-regulated in a dose dependent manner on the surface of FLU-stimulated $CD14^+$ cells, achieving statistical significance only in the presence of the higher dose of drugs (**fig. 20**). A similar trend was observed in unstimulated conditions in the presence of thiazolides but without reaching statistical significance.

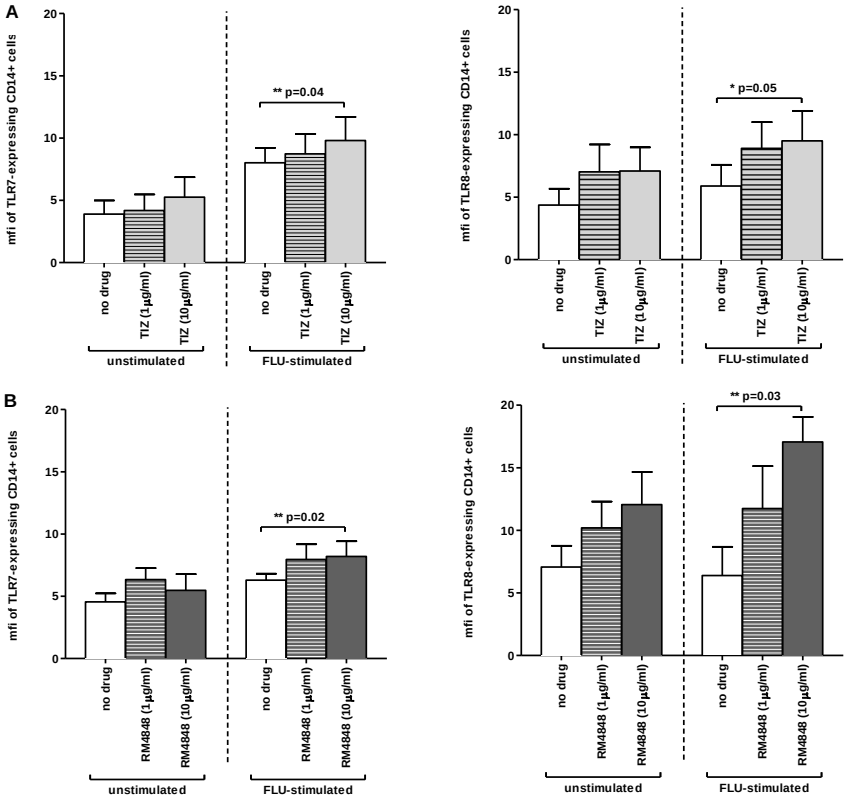


Figure 20 *- Mean fluorescence intensity (mfi) of TLR7- and TLR8- expressing CD14+ cells in unstimulated and FLU-stimulated conditions and in the absence or in the presence of two different doses of TIZ (1.0 µg/ml; 10 µg/ml) (**A**) or RM4848 (1.0 µg/ml; 10 µg/ml) (**B**). Mean values ± S.D. and p values are indicated.*

Activation of TLR7 and TLR8 triggers inflammatory responses characterized by type I IFN production in virus-infected cells via activation of downstream NF-κB and type I IFN promoters. Thus, modulation of TLRs expression by thiazolides seems to be the first key element for activation of innate immune response against viruses.

2.2. Thiazolides upregulates gene expression of molecules involved in TLR pathway

As thiazolides upregulate TLR7 and TLR8 expression, we next evaluated the possible effects of these compounds on genes involved in the TLR-associated transduction pathway using a real-time PCR array.
4 hour-TIZ incubation in unstimulated condition resulted in the upregulation of TLR3, TLR7, TLR8, TLR9 expression, along with the increase of downstream molecules such as the adaptor protein TICAM2, the interferon regulatory factor TBK1 and the effectors NR2C2 and UBE2V1. Moreover, as a consequence of TLR activation, an augmented mRNA expression of IFNα1, IFNβ1, CSF3 and IL6 (genes involved in cytokine-mediated signaling), and IL12A and IL2 (key regulators of adaptive immunity) was observed as well (**fig. 21A**).

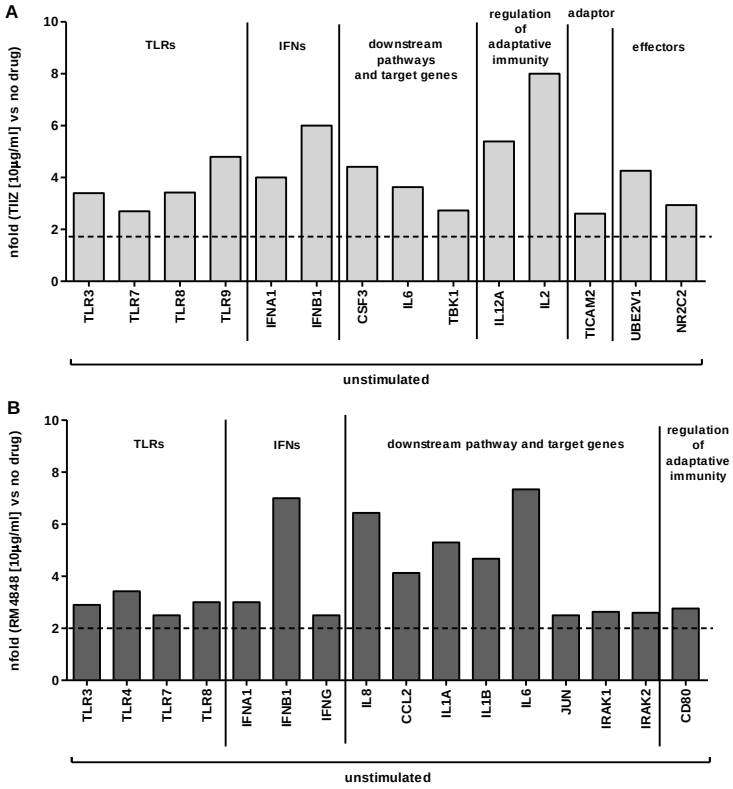


Figure 21 *– mRNA expression of genes involved in TLR signaling after TIZ (10µg/ml) (**A**) or RM4848 (10µg/ml) (**B**) treatment in unstimulated condition.*

In unstimulated cells, incubation with RM4848 (10 µg/ml) significantly upregulated: TLR3, TLR4, TLR7, TLR8, IFNα1, IFNβ1, and IFNγ, different downstream and target genes (IL6, IL1A, IL1B, CCL2, IL8, JUN, IRAK1, IRAK2) and a regulator of adaptative immunity (CD80) compared to the untreated condition (**fig. 21B**).
Upon FLU-stimulation, thiazolide effect on TLR pathway resulted in a more evident gene expression modulation. In particular, TIZ treatment was associated with the upregulation of TLR7, TLR8, and TLR9, different downstream and target genes (CSF3, CXCL10, IL6, MAP3K1, NFKB2, NFKBIL1, NR2C2, SIGIRR, TBK1), some regulators of adaptative immunity (IL12A, IL2, CD80), specific adaptors (HRAS, MAPK8IP3, MyD88, TICAM2, TIRAP), and the effector molecule EIF2AK2 mRNA expression which in turn inhibits protein synthesis. As a consequence of TLR pathway activation, a consistent upregulation of IFNs (IFNα1, IFNβ1, IFNγ) was reported as well (**fig. 22A**).
RM4848 (10 µg/ml) treatment in FLU-stimulated cells showed an increased mRNA expression of TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10, downstream pathway genes (IL6, JUN, NFKB2), regulators of adaptative immunity (CD80, IL12A, IL2), effectors (UBE2V1, TRAF6), and IFNs (IFNα1, IFNβ1, IFNγ) (**fig. 22B**).

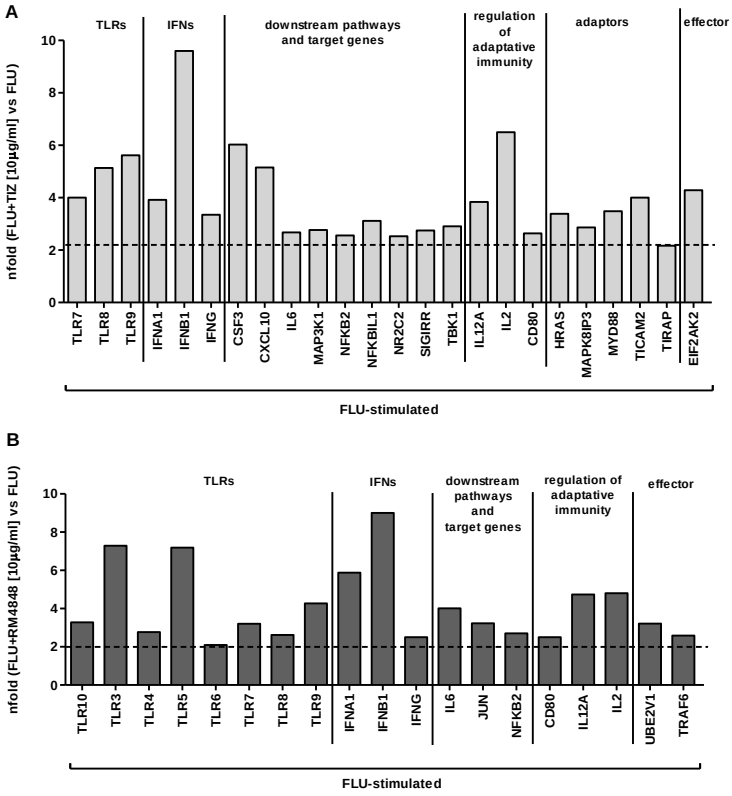


***Figure 22** – mRNA expression of genes involved in TLR signalling after TIZ (10 µg/ml) (**A**) or RM4848 (10 µg/ml) (**B**) treatment in FLU-stimulated condition.*

2.3. Thiazolides increase IFNα-secreting plasmacytoid Dendritic Cells

Dendritic cells (DCs) are specialized sentinels of the immune system that detect invading pathogens and play a crucial role in orchestrating immune responses. In particular, in response to viral infection, a specialized DC subset, plasmacytoid dendritic cells (pDC), produces high quantities of type I IFNs. Thus, IFN production is considered as a hallmark response underlying cellular antiviral immune responses.

Since thiazolide treatment of FLU-stimulated PBMCs resulted in an increased expression of type I IFNs, we verified the effects of these compounds on pDCs activation. Although no significant effect was detected in unstimulated conditions, the percentage of IFNα-secreting pDC was significantly increased in the presence of the highest dose of TIZ (p-value=0.05) (**fig. 23A**) or RM4848 (p-value=0.05) (**fig. 23B**) in FLU-stimulated cell cultures.

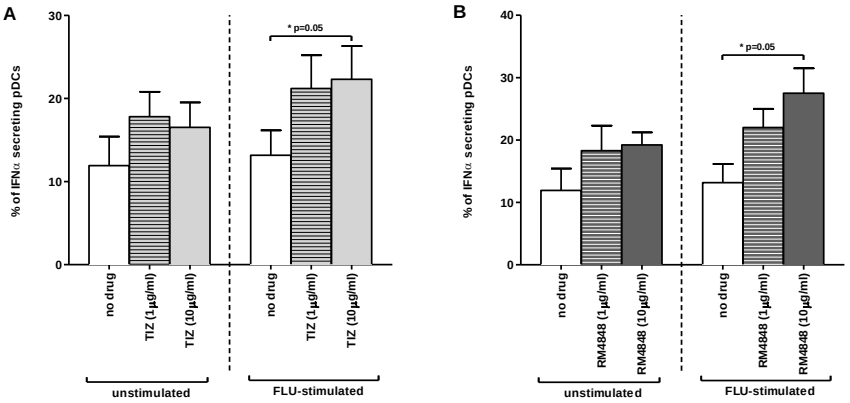


Figure 23 *- IFNα-secreting pDC (%) evaluated on total pDC in unstimulated and FLU-stimulated conditions. Data obtained in the absence or in the presence of two different doses of TIZ (1.0 µg/ml; 10 µg/ml) (**A**) or RM4848 (1.0 µg/ml; 10 µg/ml) (**B**) are indicated. Mean values, S.E. and p values are shown.*

2.4. Induction of the type I IFN pathway in response to thiazolides

Activation of TLRs results in a variety of cellular responses including production of IFNs, pro-inflammatory cytokines and effector cytokines that direct adaptive immune responses. We, therefore, performed a Real-time PCR array to determine whether induction of type I IFNs observed in the presence of thiazolides could influence the expression of ISGs.

Results obtained in unstimulated PBMCs treated with TIZ (10 µg/ml) showed an increase of IFNα1, IFNα2, IFNα4, and IFNβ1, and of different ISGs expression (ADAR, CXCL10, GBP1, IFI27, IFI16, MX1, OAS1, OAS2) (**fig. 24A**). Similarly, incubation with RM4848 (10 µg/ml) resulted in the upregulation of IFNα1, IFNα2, IFNα4, IFNβ1, and IFNγ, ISGs (IFI30, IRF5, IRF7, ISG15, SHB, STAT2), antigen presentation genes (HLA-A, HLA-B, HLA-DQA1) and genes involved in type I IFN pathway (VEGFA) (**fig. 24B**).

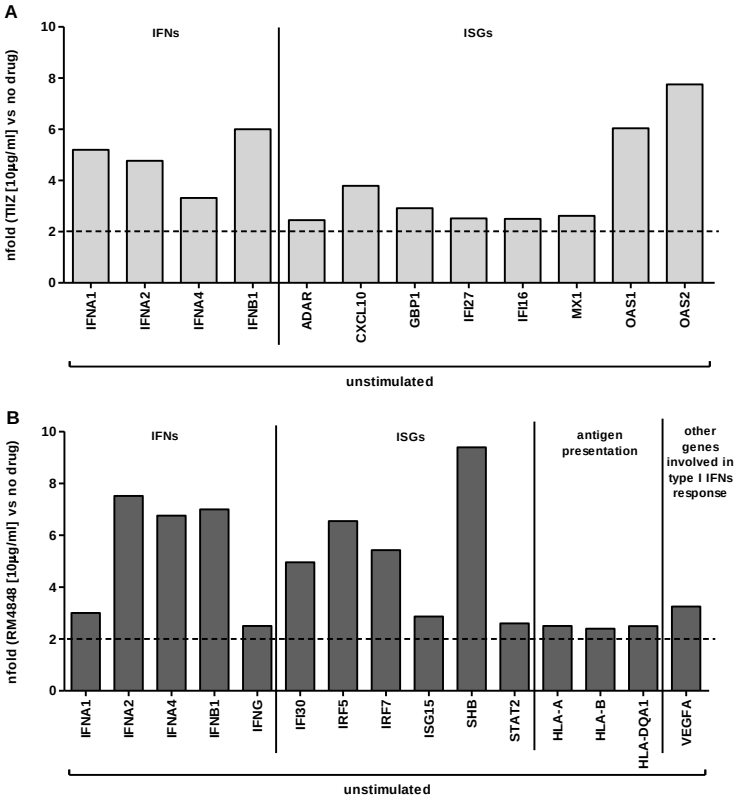


Figure 24 *- mRNA expression of genes involved in type I IFN signaling after TIZ (10 µg/ml) (**A**) or RM4848 (10 µg/ml) (**B**) treatment in unstimulated condition.*

Data obtained in FLU-stimulated PBMCs following 3 hour-incubation with TIZ showed an increase of both type I and type II IFNs (INFα1, IFNAR2, IFNβ1, IFNγ). Furthermore, the compound upregulated specific ISGs (IFI6, IFI27, IRF1, MX1), antigen presentation genes (TAP1, HLA-A, HLA-B), and other genes involved in type I IFN pathway (CD70, PRKCZ, PRKRA, TRAF3) (**fig. 25A**).
Upregulation of type I and type II IFNs (INFα1, INFα2, INFα4, IFNβ1, IFNγ), ISGs (CXCL10, IFI27, IFIT1, NRG1, OAS1, OAS2, SHB), antigen presentation genes (HLA-A, HLA-B), and of a gene involved in this pathway (CD70) was observed after RM4848 stimulation as well (**fig. 25B**).
Notably, the increase of restricted HLA-class I antigen gene expression would suggest an indirect effect of thiazolides on the onset of antiviral acquired immune responses.

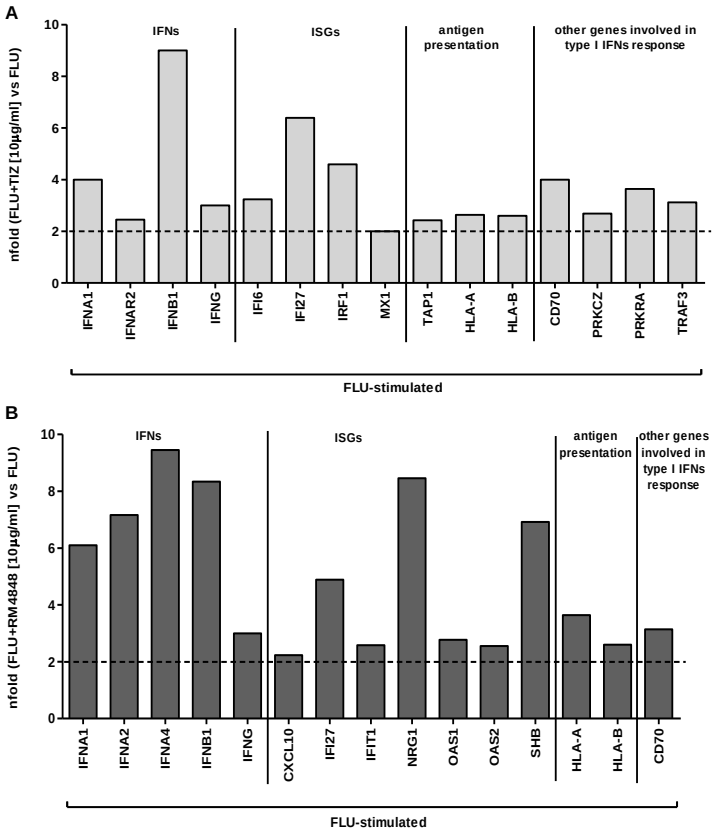


Figure 25 *- mRNA expression of genes involved in type I IFN pathway following TIZ (10 µg/ml) (**A**) or RM4848 (10 µg/ml) (**B**) treatment in FLU-stimulated condition*

2.5. Modulation of intracellular cytokine expression

An efficient antiviral immune response, including immunity to FLU virus, requires activation of both innate and acquired immune responses. Thus, we verified whether thiazolides would increase production of cytokines involved in the elicitation of acquired immune responses by antigen-stimulated lymphocytes.

IFNγ and IL2 production by CD4+ T cells was evaluated both in unstimulated condition or upon stimulation with FLU-specific antigens. Only in FLU-stimulated cells, results showed a statistically significant increase in IFNγ production in the presence of the highest dose of TIZ (p-value=0.03) and of both doses of RM4848 (1 µg/ml: p-value=0.04; 10 µg/ml: p-value=0.05). A similar trend was observed for IL2 synthesis (TIZ 10 µg/ml p-value=0.03; RM4848 10µg/ml p-value=0.04), thus confirming an important role of these compounds in the induction of an effective antigen-specific adaptive immunity (**fig. 26**).

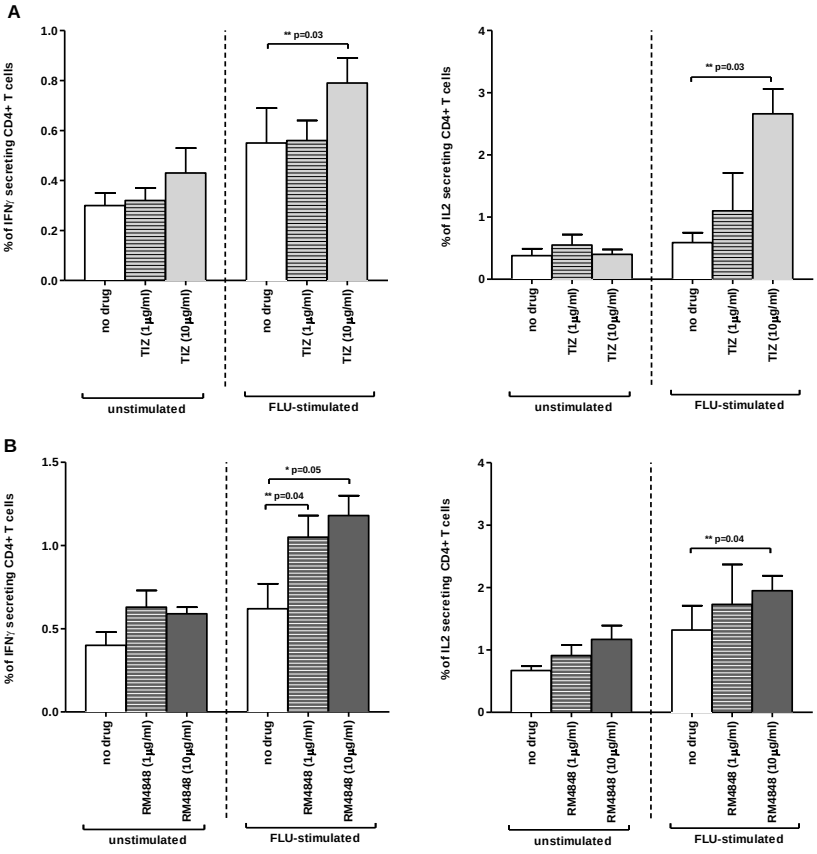


Figure 26 *- Percentage of IFNγ- and IL2- secreting CD4+ T cells in unstimulated and FLU-stimulated conditions. Data obtained in the absence or in the presence of two different doses of TIZ (1.0 µg/ml; 10 µg/ml) (**A**) or RM4848 (1.0 µg/ml; 10 µg/ml) (**B**) are indicated. Mean values, S.E. and p values are shown.*

2.6. Thiazolide efficacy compared to IFNα stimulation

IFNα effects on immune system were extensively explored and nowadays IFNα is used as a standard treatment in several pathologic conditions. Unfortunately IFNα use is accompanied by a wide variety of possible side effects that may hamper reaching and maintaining the dose needed for maximal therapeutic effect while their occurrence can outweigh clinical benefit of IFNα treatment. Since we previously demonstrated that thiazolides positively affected a wide range of genes involved in type I IFN pathway, we thus compared the immunomodulatory effects of TIZ to those exerted by IFNα in FLU-stimulated PBMCs.

Following 4 hour-IFNα stimulation, we analysed genes involved in both TLR and type I IFN pathway. Results indeed showed an upregulation of a large number of genes (**fig. 27**) involved in the generation of both innate and acquired immune responses.

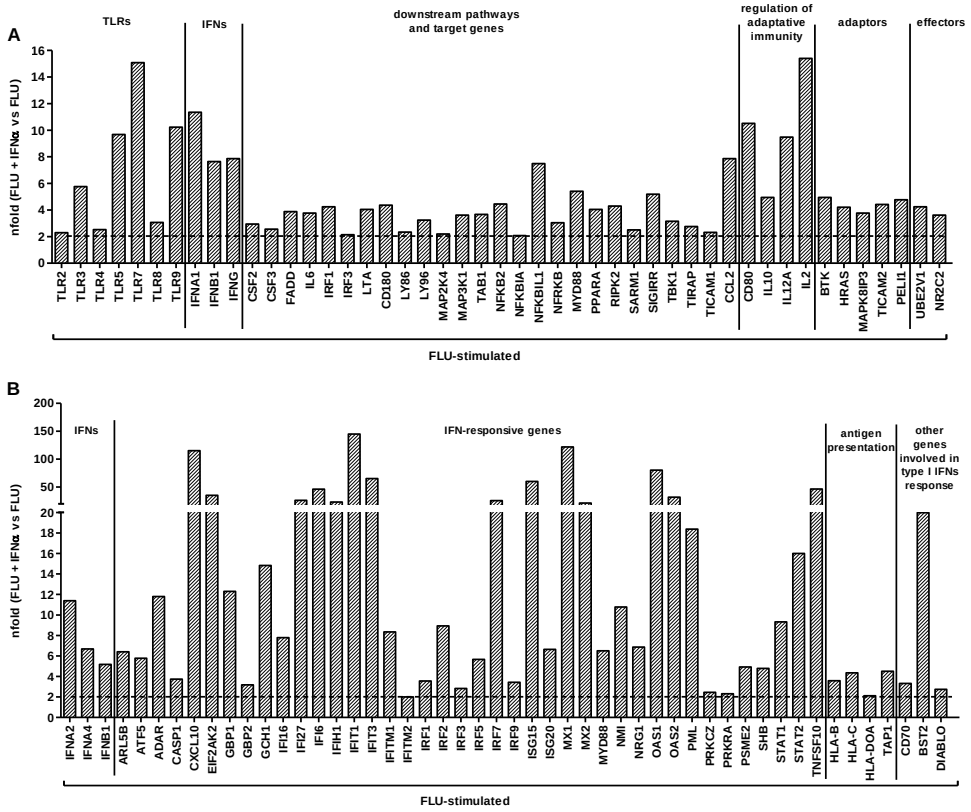


Figure 27 *– mRNA expression of genes involved in TLR (**A**) and in type I IFN (**B**) pathway following IFNα treatment in FLU-stimulated cells.*

Although IFNα immunomodulation was greater than that observed following TIZ treatment, in terms of either number of upregulated genes or relative level of mRNA expression, the less intense effects of thiazolides (**fig. 28**) could be notwithstanding considered positive in an effort to reduce side effects that characterized IFNα treatment.

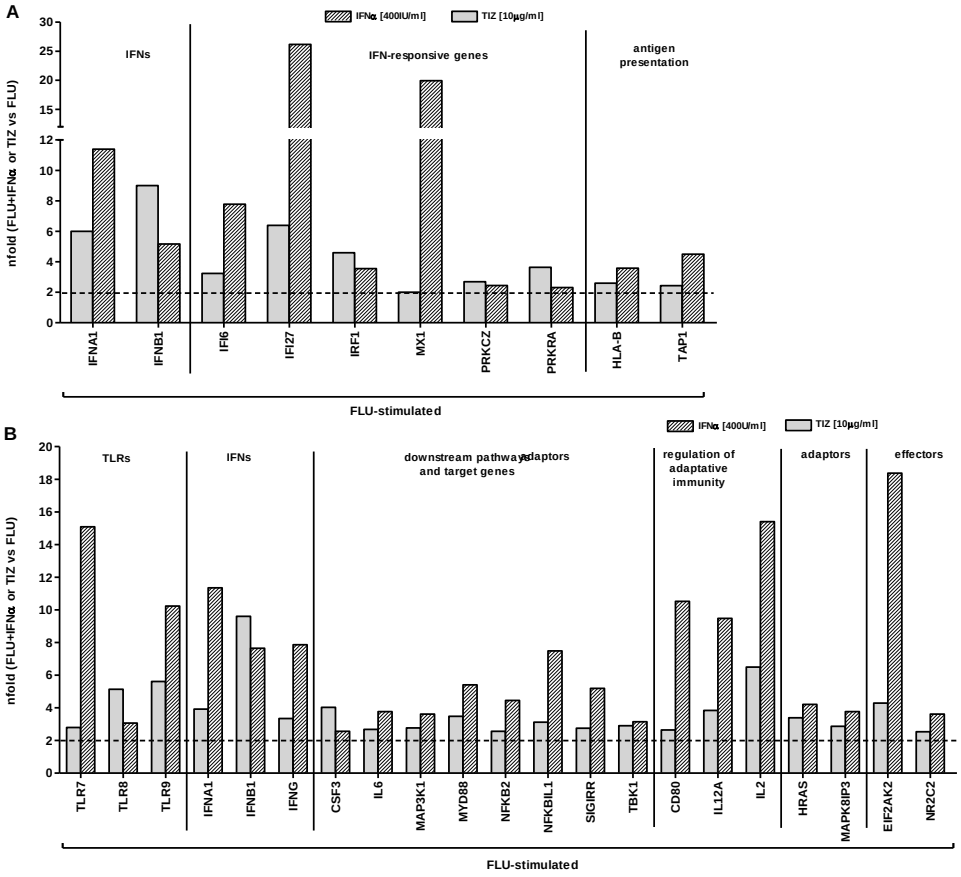
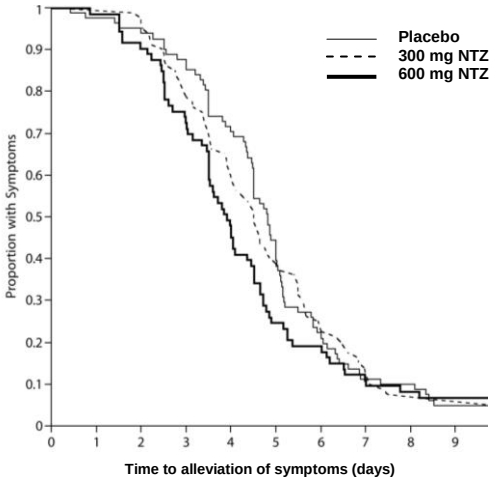


Figure 28 *– Comparison between mRNA expression of genes involved in TLR (**A**) and type I IFN (**B**) pathway following IFNα or TIZ treatment in FLU-stimulated cells.*

2.7. Clinical trial for the treatment of uncomplicated influenza

The results obtained *in vitro,* together with recent data showing that TIZ blocks Influenza virus replication at a post-entry level, resulting in impairment of the transport of influenza hemagglutinin to cell surface, justified a human clinical trial in uncomplicated influenza in adults and adolescents. The choice of testing NTZ, the first of thiazolides, was mainly due to the fact that this molecule has been used in clinical trials against a number of other types of infections caused by protozoa, anaerobic bacteria and viral infections such as rotavirus and HCV. Six hundred and twenty-four adults and adolescents with FLU symptoms were enrolled in the study within 48 hours of symptom onset. 257 of the 624 patients (41%) had laboratory confirmed influenza infection by RT-PCR or viral culture at baseline. Approximately half of the influenza-infected patients enrolled in the US clinical trial were infected with influenza A subtype H1N1 (“swine flu”) with approximately 30% being infected with influenza B and 20% with influenza A subtype H3N2. Patients were blindly divided into three groups: placebo (N=89); NTZ 300mg (N=89); NTZ 600mg

(N=79). Patients treated with NTZ 600mg for five days experienced statistically significant reduction in time from beginning treatment to alleviation of FLU symptoms compared to patients receiving the placebo (95 hours vs. 117 hours, *P*=0.01, **fig. 29**) (P=0.008).



Median time to alleviation of symptoms

	Median (IQR)	P value (vs. Placebo)
Placebo	116.7 (91-144)	
300 mg NTZ	109.1 (82-152)	0.5208
600 mg NTZ	95.5 (71-126)	0.0084

p values from Prentice-Wilcoxon test interpreted at 0.025 significance level to adjust for multiplicity

Figure 29 *– Time to alleviation of symptoms following treatment with placebo, 300 or 600 mg of NTZ in a cohort of FLU-infected patients (**A**). Median time to alleviation and pvalues vs placebo are shown (**B**).*

Time to resolution of symptoms for the low dose group were less than for the placebo, but the difference was not statistically significant (P=0.521). Patients in the 600 mg NTZ treatment group also experienced statistically significant reductions in quantitative viral shedding compared to patients receiving the placebo (P=0.0006, **fig. 30**).

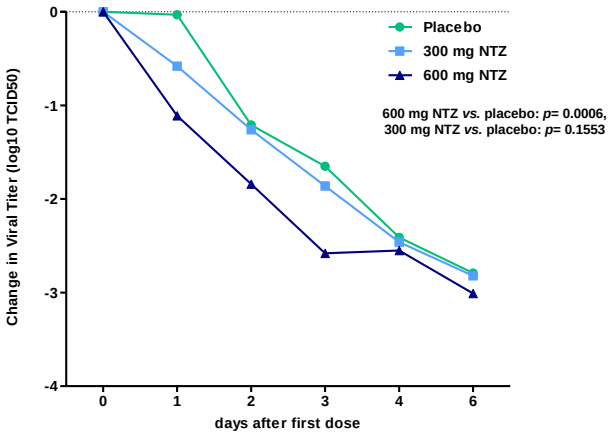


Figure 30 *– Change over time in viral titer following treatment with placebo, 300 or 600 mg of NTZ in a cohort of FLU-infected patients*

Additional results showed that seroprotection and seroconversion rates (Influenza B and H3N2 strains) were higher in both groups of NTZ patients compared to the placebo control group (**fig. 31**).

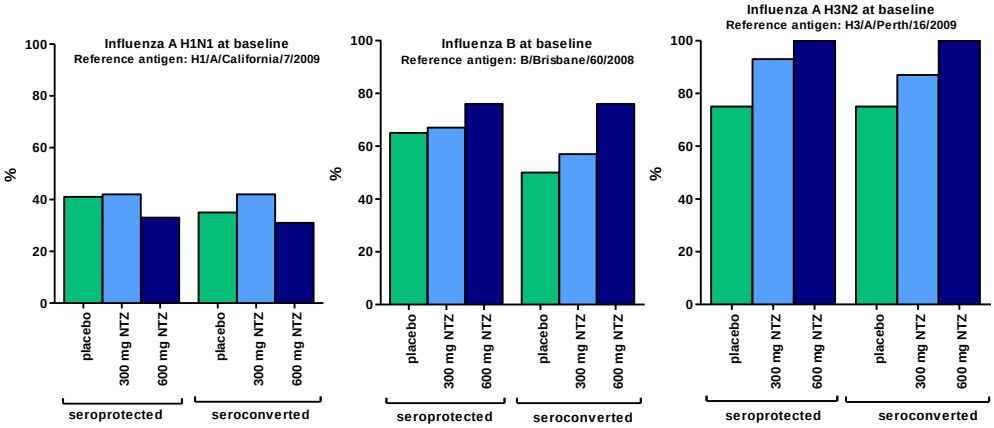


Fig. 31 *– Seroprotection and seroconversion rates in patients infected with different influenza strains, after treatment with placebo, 300 or 600 mg of NTZ*

Notably, no differences in the severity or the frequency of adverse events were reported in the NTZ compared to placebo group of patients (**tab. 7**). These data indicate that immunostimulatory effects associated with NTZ *in vitro* and *in vivo* are translated into significant clinical benefits.

Adverse Events

Most common adverse events (1%) No. (%) of Patients Reporting	Placebo (n=212)	300 mg NTZ (n=201)	600 mg NTZ (n=211)
Diarrhea	7 (3.3%)	4 (2%)	17 (8.1%)
Headache	24 (11.3%)	12 (6%)	17 (8.1%)
Bronchitis	3 (1.4%)	10 (5%)	7 (3.3%)
Oropharyngeal pain	7 (3.3%)	5 (2.5%)	10 (4.7%)
Abdominal pain	7 (3.4%)	4 (2%)	8 (3.8%)
Vomiting	2 (0.9%)	3 (1.5%)	8 (3.8%)
Chromaturia	-	6 (3%)	8 (3.8%)
Cough	8 (3.8%)	5 (2.5%)	8 (3.8%)
Sinusitis	8 (3.8%)	6 (3%)	3 (1.4%)
Nausea	6 (2.8%)	1 (0.5%)	6 (2.8%)
Pyrexia	5 (2.4%)	4 (2.0%)	6 (2.8%)
Rhinorrhea	7 (3.3%)	5 (2.5%)	4 (1.9%)
LFT abnormal	4 (1.9%)	5 (2.5%)	5 (2.4%)
Wheezing	3 (1.4%)	2 (1%)	5 (2.4%)
Nasal congestion	5 (2.4%)	3 (1.5%)	5 (2.4%)
Insomnia	4 (1.9%)	-	5 (2.4%)

Table 7 *– Common adverse events observed in 1% of patients included in the clinical trial*

3. HIV-1_{BAL} INFECTION

3.1. Strong antiviral activity of thiazolides against HIV-1_{BaL} infection in vitro

Since thiazolides were shown to be effective in triggering a protective antiviral response against a wide variety of viruses, we verified suitability of these compounds in the treatment of another viral infection, in particular HIV-1. In this regard, thiazolide antiviral activity was investigated in human PBMCs post HIV-1_{BaL} infection. p24 viral antigen release from infected cells was significantly reduced after TIZ or RM4848 treatment compared to untreated conditions (p-value< 0.01 for both compounds). Percentages of HIV-1 infection inhibition were 87.8% and 96.5% after TIZ- and RM4848-treatment, respectively (**fig. 32**).

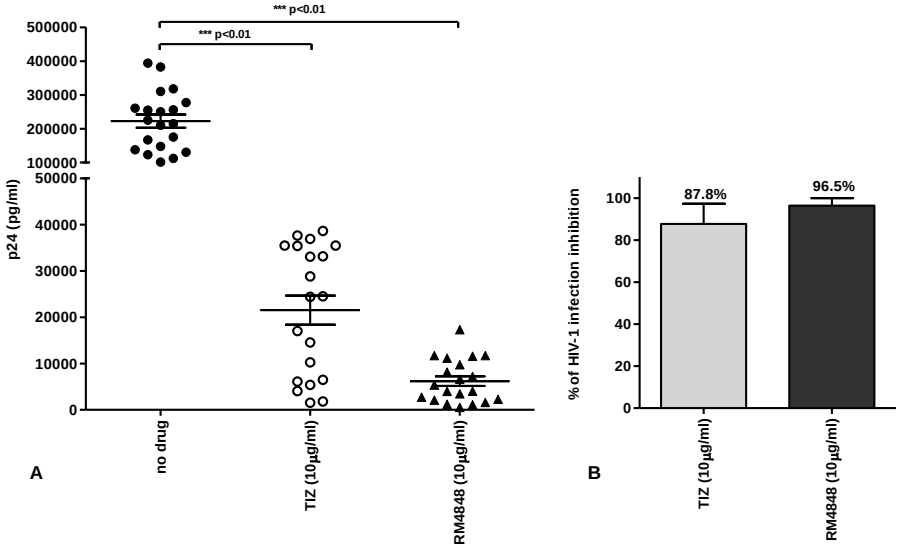


Figure 32 *– p24 viral antigen levels in the supernatant of HIV-1_{BaL} infected PBMCs treated with TIZ (10 µg/ml) or RM4848 (10 µg/ml) 10 days post-infection (**A**). Percentage of viral replication inhibition following TIZ (10 µg/ml) or RM4848 (10 µg/ml) treatment in HIV-1_{BaL} infected PBMCs 10 days post-infection (**B**). Mean values ± standard errors are shown.*

As we observed a stronger effect of RM4848 on viral replication compared to TIZ, we decided to further deepen its action and kinetic by adding RM4848 to cell cultures at different time-points. In this regard, the experiment was set up as shown in **Fig. 33.**

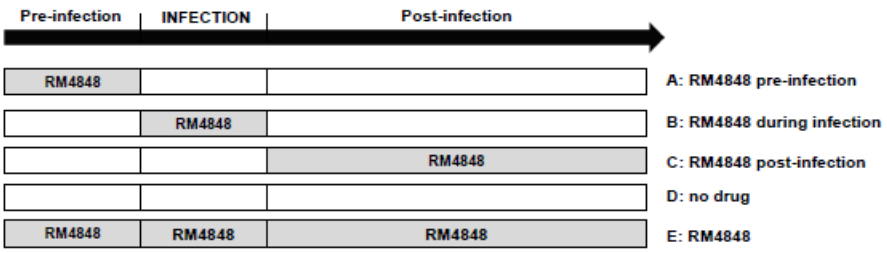


Figure 33 *– Schematic representation of the experiment performed to test RM4848 action and kinetics.*

A reduction of p24 levels was observed in all RM4848 treated-samples compared to untreated controls, suggesting that second generation thiazolides exert an immunomodulatory effect rather than directly interfering with HIV-1 replicative life cycle. However, inhibition of viral replication reaches statistical significance only when RM4848 treatment is performed post infection or maintained over time in cell culture until 10 days post infection; otherwise it's effect is progressively weakened presumably because of a gradual attenuation of the immunomodulatory properties of the compound in all the other conditions (**fig. 33**).

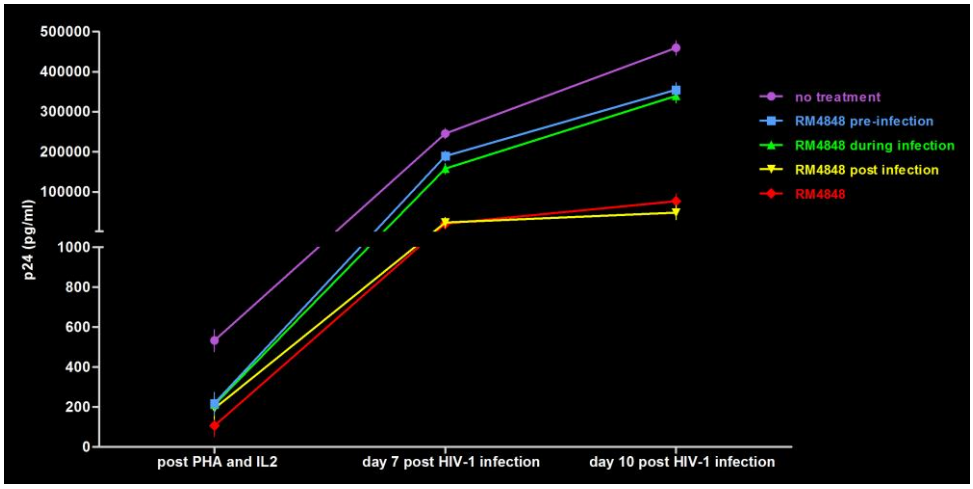


Figure 33 *– p24 viral antigen levels of HIV-1_{BaL} infected PBMCs treated with RM4848 (10 µg/ml): pre-infection (blue), during infection (green), post infection (yellow), during all the experiment course (red), at different time points. Mean ± standard errors are shown.*

3.2. Evaluation of thiazolide cytotoxicity in HIV-1-infected cells

Viability of cells is a crucial factor influencing the quality of HIV-1 replication assay. Viability of HIV-1-infected cells was monitored during treatment with thiazolides by 7-AAD staining, in order to evaluate the percentage of cell death as a result of thiazolide cytotoxicity. Results indicated that TIZ or RM4848 exposure did not alter the viability of PBMCs at the concentrations assayed at different time-points (**fig. 34**).

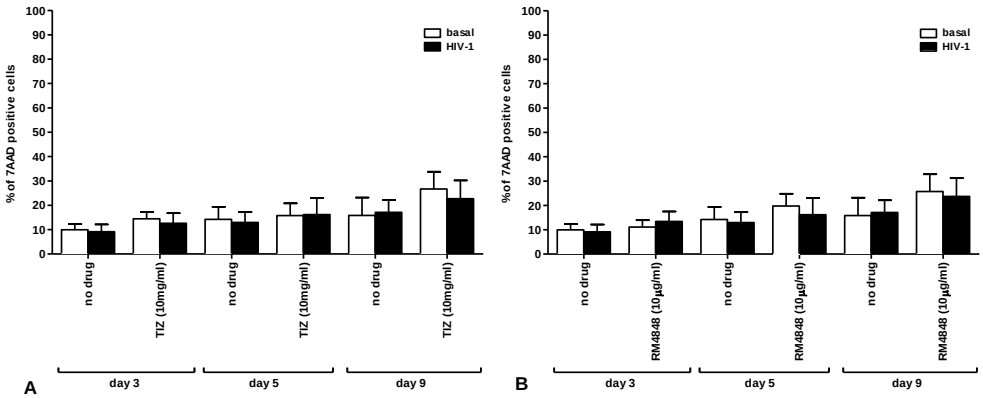


***Figure 34** – Percentage of 7-AAD positive (non viable) PBMCs treated with TIZ (10 µg/ml) (**A**) or RM4848 (10 µg/ml) (**B**) after 3, 5 or 7 days of incubation, in basal condition or after HIV-1_{BaL} infection. Mean values ± standard errors are shown.*

3.3. Modulation of type I IFN pathway after thiazolide treatment

To determine whether thiazolide treatment could increase type I IFN and ISG expression levels also in the *in vitro* model of HIV-1-infection, we performed a Real-time PCR array that screens simultaneously 84 gene involved in type I IFN response.

Results from uninfected cells incubated with TIZ showed an increased expression of IFNα1, IFNα2, IFNα4, IFNβ1, and IFNγ, ISGs (ARL5B, IRF7, MET, OAS2, PRKRA, SHB, STAT2, TRAF3), and HLA-C a gene involved in antigen presentation (**fig. 35A**). RM4848 treatment resulted in the upregulation of IFNα2, IFNα4, IFNAR1, IFNAR2, IFNβ1, and IFNγ, ISGs (CAV1, IFI30, IRF5, IRF7, ISG15, ISG20, ITIH2, MX2, SHB, STAT2), antigen presentation genes (HLA-C, HLA-DQA1), and a downstream effector gene involved in this pathway (VEGFA) (**fig. 35B**).

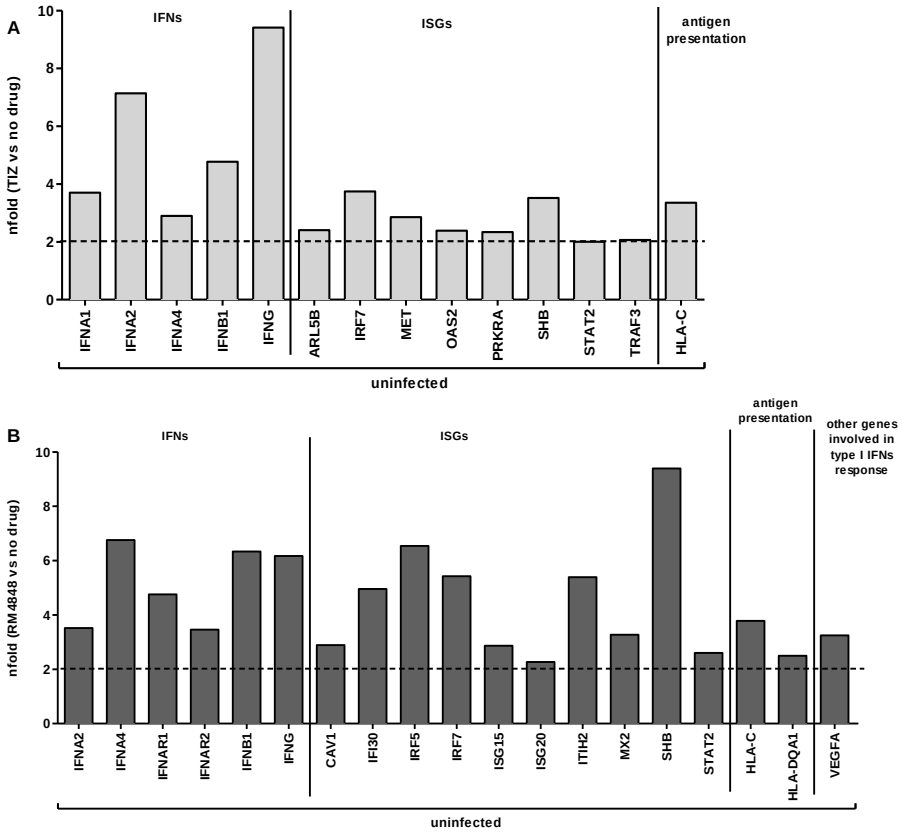


Figure 35 *– mRNA expression of genes involved in type I IFN pathway following TIZ (10 μg/ml) (**A**) or RM4848 (10 μg/ml) (**B**) treatment in uninfected condition*

Results from HIV-1-infected cells incubated with TIZ showed an upregulation of 18 genes. Data showed an increase in IFNα1, IFNα2, IFNα4, IFNβ1 and in the HLA-C gene. Moreover, TIZ moderately upregulated different ISGs (IRF1, IRF2, IRF7, MX1, MyD88, OAS2, SHB, TRAF3) and a gene involved in the pathway (CD70). Again, RM4848 effect was shown to be stronger than TIZ (**fig. 36**) in this action.

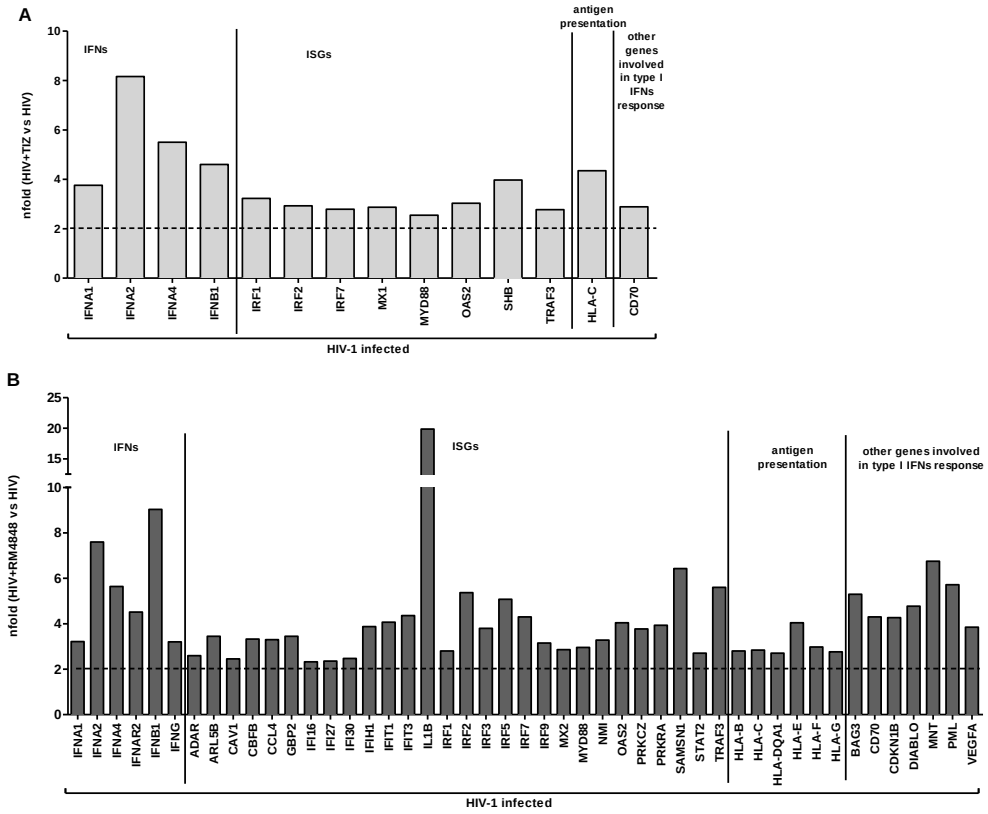


Figure 36 *- mRNA expression of genes involved in type I IFN pathway following TIZ (10 μg/ml) (**A**) or RM4848 (10 μg/ml) (**B**) treatment in HIV-1-infected condition*

3.4. Analysis of anti-HIV-1 human response pathway

To determine whether the anti-HIV effects of thiazolides result in a specific modulation of cellular factors directly involved in HIV-1 life cycle, we used an anti-HIV-1 human response pathway array that screens for the expression of different genes known to directly interfere with HIV.

Data showed that thiazolides result in the reinforcement of innate immune responses rather than affecting cellular mechanisms used by HIV-1 for replication and survival. These data confirmed the hypothesis of an effective immune stimulation exerted by these compounds. Notably, RM4848 seems to be endowed with a stronger immunomodulatory activity compared to TIZ (**fig. 37**).

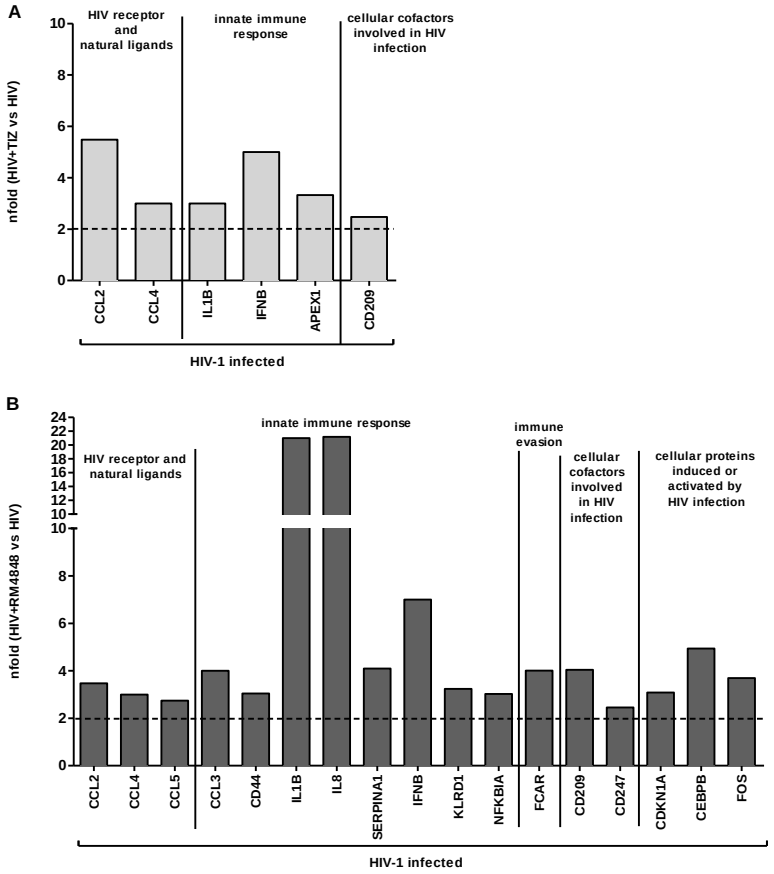


Figure 37 *– mRNA expression of genes involved in HIV-1 human response pathway following TIZ (10 μg/ml) (**A**) or RM4848 (10 μg/ml) (**B**) treatment in HIV-1-infected condition*

3.5. Thiazolides modulate cytokine and chemokine production in HIV-1 infected PBMCs

Cytokine and chemokine production by *in vitro* HIV-infected thiazolide-treated PBMCs was measured in supernatants 3 days after HIV-1_{BaL} infection by Multiplex Detection Immunoassay. Results showed that IFNγ and IL2 (Th1-cytokines) production was significantly increased in HIV-1 infected cells in the presence of TIZ (p-value=0.05) or RM4848 (p-value=0.05). Interestingly, chemokine production (MCP-1, MIP1-α, MIP1-β, RANTES), known to be endowed with anti-HIV properties, was strongly modulated by thiazolides as well.
In particular, HIV-infected cells treated with TIZ (**fig. 38**) or RM4848 (**fig. 39**) showed an increased release of MIP-1α (TIZ: p-value=0.03, RM4848: p-value=0.05), MIP-1β (TIZ: p-value=0.03, RM4848: p-value=0.05), MCP-1 (TIZ: p-value=0.05, RM4848: p-value=0.05) and RANTES (TIZ: p-value=0.05, RM4848: p-value=0.03).

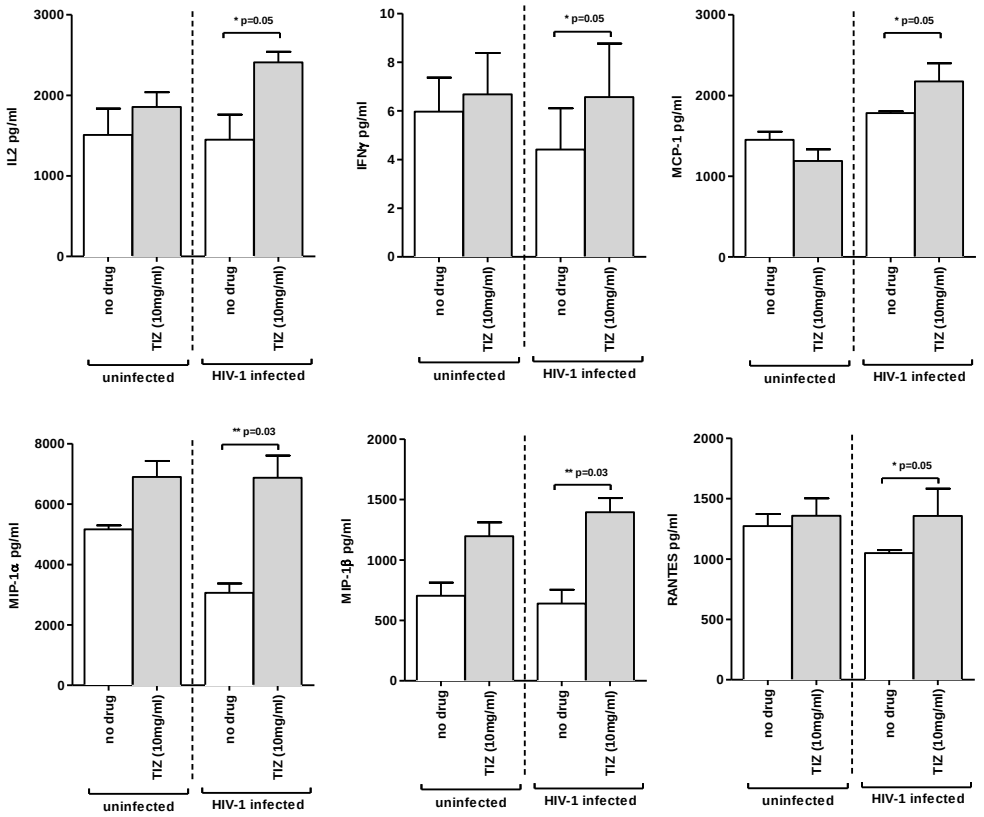


Figure 38 *– Cytokines and chemokines supernatant concentration of uninfected and HIV-1 infected cells untreated (white bars) ore treated (grey bars) with TIZ (10 µg/ml) 3 days post-infection . Mean values ± standard errors are shown.*

Recently, it was suggested that NTZ acts after viral entry and before or during reverse transcription step. The increased production of pro-inflammatory cytokines and chemokines reported in the *in vitro* assays, nevertheless indicate that TIZ or RM4848 could either obstacle viral entry or reduce the infection process of target cells once the virus has replicated.

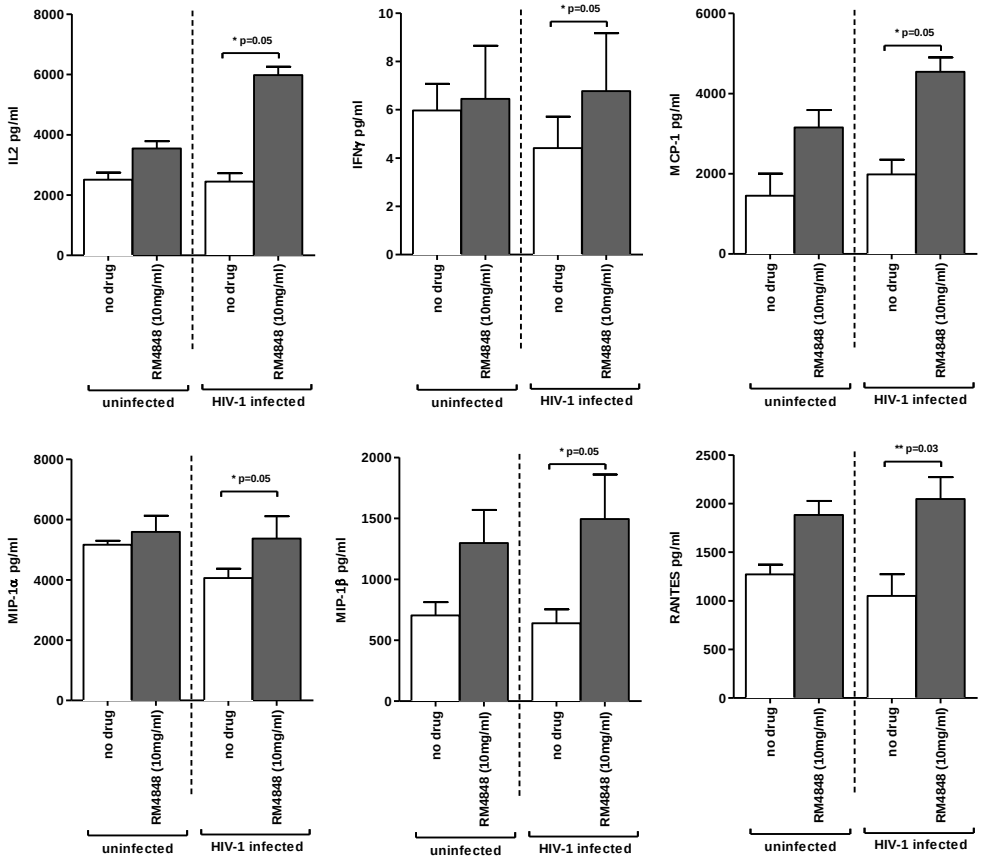


Figure 39 *- Cytokines and chemokines supernatant concentration in uninfected and HIV-infected cells in presence of RM4848 (10 µg/ml) at day 3 post-infection. Mean and p values are shown*

3.6. Interferon-inducible cholesterol-25-hydroxylase (CH25H) broadly inhibits viral entry

Since we previously demonstrated that thiazolide treatment results in IFN and ISG upregulation, we thus verified whether pharmacological immunomodulation may also affect cholesterol metabolism, and in particular CH25H expression. In this regard, we designed custom PCR arrays to analyse a wide range of genes involved in intracellular cholesterol trafficking and metabolism. Results obtained in uninfected PBMCs confirmed our hypothesis, showing an upregulation of genes involved in cholesterol metabolism and efflux (CH25H, NR1H3, RXRα, SREBF1, SREBF2) and of different membrane receptors and cholesterol transporters (MSR1, ABCA1) in the presence of TIZ (10 µg/ml). RM4848 stimulation showed a similar trend with a higher effect compared to TIZ (**fig. 40**).

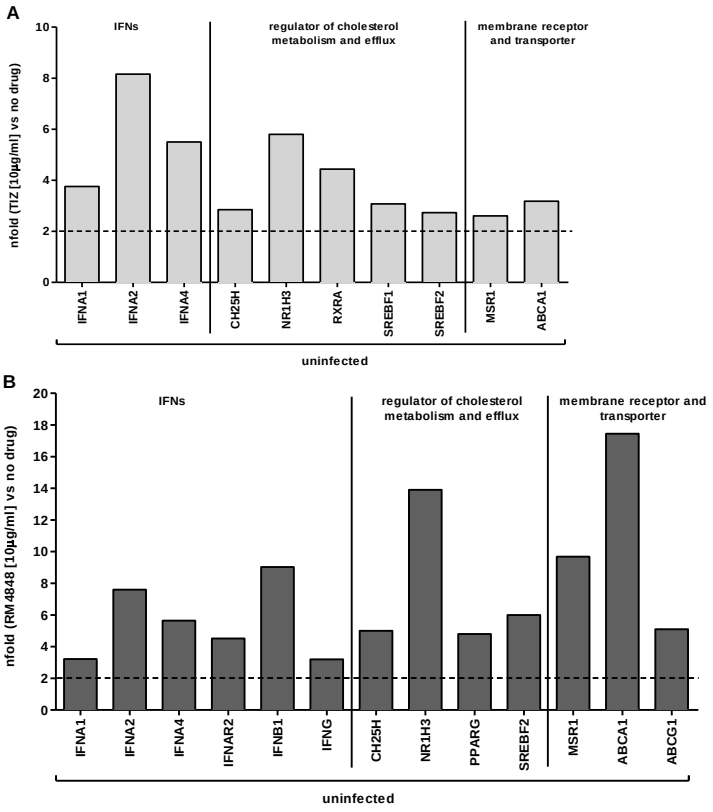


Figure 40 *– Cholesterol metabolism and efflux gene expression following stimulation with TIZ (10 µg/ml) (**A**) or RM4848 (10 µg/ml) (**B**) in uninfected conditions.*

Data obtained on HIV-1-infected TIZ-stimulated PBMCs confirmed upregulation of CH25H, NR1H3, PPARγ, SREBF1, and SREBF2 expression, which represent the main regulators of cholesterol metabolism and efflux. Moreover, MSR1, LDLR, ABCG1 and ABCA1, membrane receptors involved in cholesterol metabolism and efflux were also increased by TIZ. RM4848 stimulation resulted in the upregulation of CH25H, NR1H2, NR1H3, PPARγ, RXRα, RXRβ, SREBF1, SREBF2 and of MSR1, LDLR, ABCA1, and ABCG1 as well (**fig. 41**).

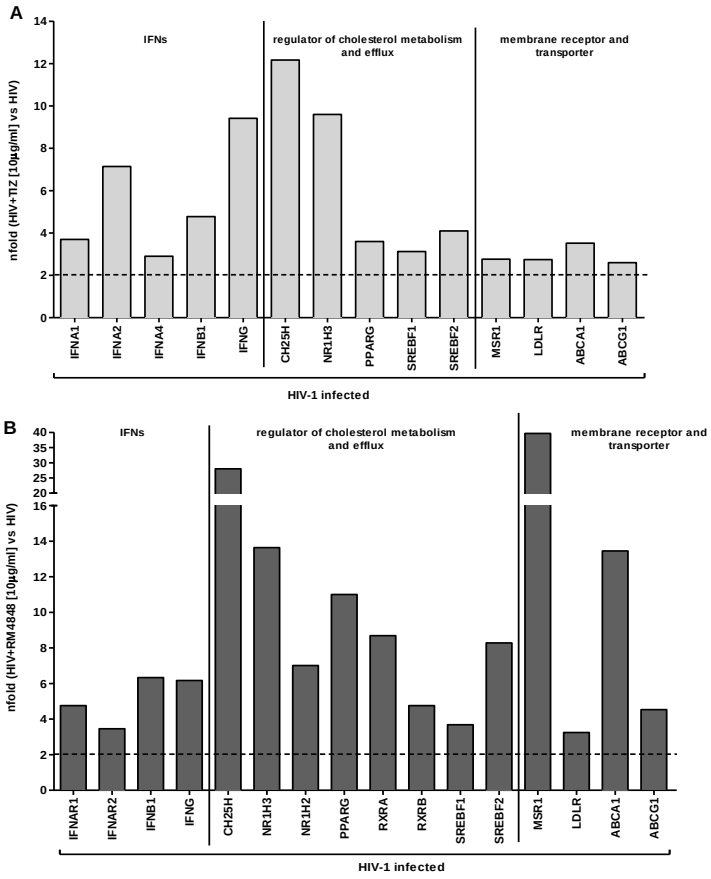


Figure 41 *- Cholesterol metabolism and efflux gene expression following stimulation with TIZ (10 µg/ml) (**A**) or RM4848 (10 µg/ml) (**B**) in HIV-1 infected cells.*

Results herein presented showed that thiazolides potently induce antiviral responses by enhancing type I IFN production and the expression of a wide range of genes involved in cholesterol metabolism. Among these, CH25H and 25-HC, a natural oxysterol that is produced through a CH25H-mediated enzymatic reaction, play an important role in preventing viral spread to target cells. It is well known that most natural oxysterols act as ligands for LXR, which has been extensively shown to mediate cholesterol efflux throughout the cells. Although we did not quantify 25-HC levels in the supernatants of HIV-1_{BaL} infected cells, we verified a possible correlation between upregulation of genes involved in cholesterol efflux and *in vitro* inhibition of HIV-1 infection. In this context, we stimulated HIV-1_{BaL} infected cells with a synthetic agonist of LXR (TO-901317) and assessed viral infectivity by p24 dosage. Data showed a statistically significant decrease of cell infection by 47% in TO-901317-treated samples compared to controls.

These results suggest that reduction of viral replication is partially achievable through modulation of cholesterol efflux mechanisms (**fig. 42**). This hypothesis should be nevertheless confirmed by further studies.

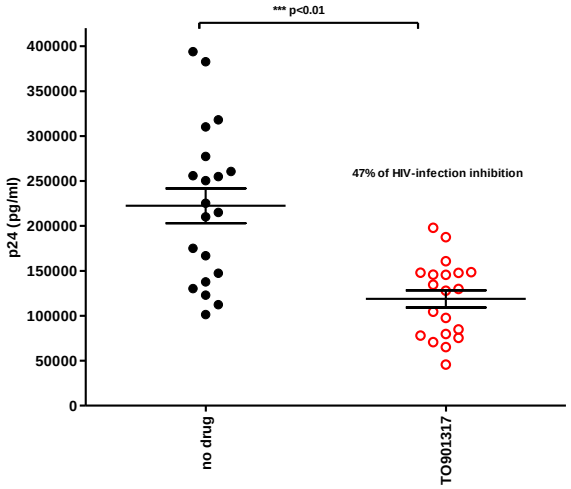


Figure 42 *- p24 viral antigen levels of HIV-1_{BaL} infected PBMCs in presence or absence of TO-901317 (1 µM) 10 days post-infection. Mean ± standard errors are shown.*

CONCLUSIONS

The ability of thiazolides to interfere with pathogens that are radically different in their structure and in the pathogenic mechanisms of action suggested that these compounds are endowed with a potent and non pathogen-specific activity.
In this regard, we initially investigated the possibility that thiazolides effects could be associated with the elicitation of both innate and acquired immunity in influenza virus model. Our results indicate that first and second generation thiazolides were able to upregulate gene expression of multiple components of the TLR- and type I IFN-signal transduction pathways, of multiple chemokines and cytokines, as well as the production of IFNα by pDC, and that of IL2 and IFNγ by CD4+ T lymphocytes.
The fact that NTZ had been extensively used in clinical setting, and recent data indicating that TIZ inhibits viral replication of several strains of influenza A H1N1, H3N2 and influenza B viruses at a post-entry level [37], prompted us to further verify the possible effect of NTZ in the *in vivo* condition. Results obtained in a double-blinded, placebo-controlled clinical trial in patients with Influenza infection showed a clear effect of NTZ in reducing the severity of FLU symptoms and viremia, and in increasing seroprotection and seroconversion rates. These data indicate that the ability of NTZ/TIZ to upregulate innate immune responses *in vitro* are reflected in a clear clinical advantage. Because of the study design we could not perform *ex-vivo* analyses on cells of patients enrolled in the trial. It is nevertheless reasonable to believe that the immunomodulatory effect of NTZ we observed *in vitro* are responsible for the activation of a multifaceted immune response *in vivo,* resulting in the observed clinical benefit of the drug.
Moreover, immunomodulatory effects of thiazolides were stronger in FLU-stimulated cells compared to unstimulated control, suggesting that thiazolides result in the reinforcement of immune responses. This remarkable selectivity seems to justify the lack of adverse clinical side effects that characterize the clinical use of NTZ. Indeed, data obtained both in clinical trials as well as data presented here, did not evidence any drug-related toxicity. This feature clearly sets apart NTZ in respect to other immune based therapies (e.g. IFN, IL-2, IL-12, etc) that are associated with common and severe side effects resulting from a generalized and extensive immune activation (i.e. cytokine storm).
Considering the overall results obtained in influenza virus model, we further reasoned on the possible role of thiazolides in elicitation of effective innate immune responses against HIV-1. In this context, induction of robust innate immune responses is correlated with a reduced susceptibility to HIV-1 infection *in vivo* a condition distinctive of subjects who despite repeated exposure to the virus do not seroconvert (HIV exposed seronegative individuals, HESN). Indeed, recent data showed that *in vitro* HIV replication is significantly reduced in PBMCs of HESN and that this effect is secondary to the elicitation of a prompter and more robust innate immune response [179].
Thiazolide treatment resulted in the reduction of *in vitro* HIV-1 replication in cells isolated from healthy individuals, conferring to these cells an “HESN-like” status. As our results demonstrated that thiazolides induce a modulation of the whole innate immune compartment we could reasonably speculated that this effect is responsible for HIV-1-infection reduction. Actually, in the presence of both thiazolides cytokine and chemokine secretion well as that of type I IFN genes and interferon-stimulated genes (ISGs) were significantly augmented in HIV-1_{BaL}

infected human PBMCs. Among ISGs, the increased expression of OAS1, OAS2, PRKRA, MX1 and MX2 is particularly intriguing as their activity is strongly associated with the induction of antiviral resistance mechanisms. Furthermore, experimental observations support the hypothesis that IFN effectors work in cooperation to achieve a fully functional antiviral state. Combination of different ISGs expressed together results in a greater magnitude of antiviral activity than either gene alone. Thus, the greater efficacy of RM4848 in reducing HIV-1-infection could be correlated to a broadly range of ISGs upregulated compared to TIZ.

To better unravel the mechanism of action exerted by thiazolides and given the high number of ISGs induced by these drugs, we finally focused our attention on CH25H, an oxysterols producing enzyme. CH25H inhibits growth of a wide range of enveloped viruses by producing 25HC, which in turn impairs viral entry at the virus-cell fusion step by inducing cellular membrane changes. Interestingly, our results confirmed the involvement of CH25H in thiazolide-mediated effects. This fascinating perspective led us to deepen the intriguing connection between modulation of cholesterol metabolism and reduction of viral infection mediated by thiazolides. Consequently, we found an increase of cholesterol metabolism (LXRα, PPARγ) and influx or efflux (ABCA1, ABCG1, MSRI, LDLR) genes. RM4848 results in a stronger effects compared to TIZ. Further, we reported that cholesterol efflux induction by an LXR agonist (TO-901317) is associated with a reduction of HIV-1-infectivity.

Results herein presented allow us to speculate that mechanisms of antiviral activity of thiazolides are entirely new, and are mediated by a robust activation of innate immune system and a modulation of genes involved in cholesterol efflux and metabolism. However, to date, an explanation for the mechanism of action of thiazolides in various disease conditions remains theoretical but clearly deserving further studies. Given the complexity of IFN regulation pathway, major information could arise from animal studies in which the immune system can be manipulated. Additional disease models and future clinical trials will be needed to elucidate the role of thiazolides in immune-system regulation. In conclusion, thiazolides could be considered by the medical community for the design of novel preventive and therapeutical approaches against HIV-1 infection.

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