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HIV-1 infected human lymphoid tissue remains immune-activated despite ART: soluble and extracellular vesicle-associated cytokines

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ABSTRACT

Background
In the age of efficient antiretroviral therapy (ART), premature aging (that includes the development of various pathologies) of HIV-1-infected patients with suppressed viral replication becomes an important health problem. These pathologies are associated with residual immune activation after successful ART. Cytokines, which sustain immune activation, can be either in a soluble form or can be associated with extracellular vesicles (EVs). Together with classical soluble cytokines, these EV-associated cytokines may play a key role in HIV-1 infection and pathogenesis. These hypotheses should be tested in a biologically-relevant system under controlled laboratory conditions. Human *ex vivo* lymphoid tissues constitute such a system. It was previously used to study HIV-1 pathogenesis, and similar to the situation *in vivo,* soluble cytokines were shown to be upregulated in this system upon HIV-1 infection. Here we investigated the modulation of both soluble and EV-associated cytokines following HIV-1 infection and upon application of ART. We investigated whether the HIV-1-triggered immune activation is decreased when viral replication is suppressed. We considered various hypotheses on the mechanisms of HIV-1-triggered immune activation: persistent immunological and inflammatory response after an initial cytokine storm; presence of defective virions sustaining immune activation; latent herpesvirus infection which may be reactivated during HIV-1 infection and leading to an inflammatory response; or proinflammatory effects of antiretroviral drugs themselves.

Methods
Ex vivo lymphoid tissues were infected with two HIV-1 strains: $X4_{LAI04}$ or $R5_{SF162}$. HIV-1 was either allowed to replicate for 16 days, or tissues were treated with ritonavir (RTV) or AZT-3TC at day 3 post-infection (n=8). *Ex vivo* cultures were also treated with cytokines (n=4), inactivated HIV-1 virions (n=4), or ART in the absence of HIV-1 infection (n=8). HIV-1 replication was analyzed in tissue culture supernatants by measurement of $p24_{gag}$ antigen. EV presence was confirmed by Western Blot and Nanoparticle Tracking Analysis (NTA). 33 cytokines in soluble and EV-associated forms were measured with multiplexed bead-based assays. Herpesvirus DNA copies in tonsillar tissue (n=4) were quantified with droplet digital PCR (ddPCR).

Results
We found that both strains of HIV-1 replicated well in tissues and triggered an upregulation of numerous soluble cytokines as early as day 3 post infection. Many cytokines that were upregulated upon HIV-1 infection in soluble form were increased in EV-associated form as well. However, some cytokines were uniquely upregulated only in the EV form, mainly in response to $X4_{LAI04}$ infection. In early HIV-1 infection, there was a significant increase in the percentage of soluble RANTES and TNF-α compared to EV-associated; additionally, RANTES significantly increased in the percentage of EV surface associated compared to EV internal.
Cytokines which were significantly upregulated throughout culture as evaluated by cumulative totals included: IL-2, IFN-γ, MIP-1α, MIP-1β and RANTES for both virus infections. $X4_{LAI04}$ infection also led to increases in IL-7, IL-18, M-CSF and TNF-α.

An even greater number of cytokines were upregulated in EV-associated form; including many of the same cytokines that were increased in soluble form, but additionally IL-1α, IL-6, IL-13, IL-21, IL-33, GM-CSF and TGF-β were upregulated following $X4_{LAI04}$ infection.
RTV and AZT-3TC treatment of tissues efficiently suppressed viral replication (>99% suppression for both treatments). Despite control of viral replication, cytokines remained upregulated after 13 days of ART treatment, and EV-associated cytokines were less likely to decrease than soluble ones.
$X4_{LAI04}$ induced stronger immune responses as measured by increased soluble and EV-associated cytokines, particularly pro-inflammatory cytokines and the β-chemokines MIP-1α, MIP-1β and RANTES, compared to $R5_{SF162}$ strain. These $X4_{LAI04}$ cytokine upregulations, especially the β-chemokines, were also less likely to be restored after both antiretroviral treatments.
Additional experiments demonstrate that this persistent immune activation was not due to the initial cytokine storm. The emulation of an initial cytokine stimulation was able to boost the production of other cytokines early in culture, but these increases were not maintained over time.
Inactivated $X4_{LAI04}$ was able to trigger a similar, but slightly weaker cytokine release compared to the infectious virus. A single inoculation of inactivated virus elevated many of the same cytokines as live virus; 7 of 13 upregulated cytokines with live virus were increased with inactivated virus. Repeated exposure to inactivated virus generated an even more similar response compared to real infection; almost all the same cytokines were upregulated and some of them were more elevated with inactivated virus (IL-21, MIP-1α, MIP-1β, RANTES and TNF-α).
Herpesvirus (HSV-2, EBV, CMV, HHV-6, and HHV-7) DNA copies were detected at different time points at variable concentrations. However, no obvious pattern of herpesvirus reactivation was observed that would account for the immune activation.
Finally, the antiretroviral treatments themselves were not responsible for immune activation since only small but significant decreases were observed for a few cytokines.

Conclusions
HIV-1 infection of *ex vivo* human lymphoid tissues led to upregulation of various cytokines, in both soluble and EV-associated forms. HIV infection altered distribution of certain cytokines between soluble and EV-associated forms, as well as between EV-surface and EV-encapsulation. Despite viral suppression by ART, the majority of the upregulated cytokines, especially β-chemokines, remained upregulated, similar to the *in vivo* situation. EV-associated cytokines were more likely to remain elevated than soluble ones. The *ex vivo* human tissue model was further employed to test various hypotheses on HIV-triggered immune activation. It was determined that an initial cytokine storm did not change the basal setpoint, there was no correlation with reactivation of latent herpesviruses, and ART by itself did not trigger immune activation; however, the presence of noninfectious viral particles can trigger a response similar to infectious virus.

SOMMARIO

Introduzione
In pazienti HIV-1 infetti sottoposti a terapia antiretrovirale (ART) generalmente si osserva uno stato di persistente attivazione del sistema immunitario nonostante l'inibizione della replicazione virale. Questa condizione è associata all'insorgenza di numerose complicanze. Le citochine possono essere secrete sia come fattori solubili che in associazione a vescicole extracellulari (EVs). Essendo coinvolte nella regolazione del sistema immunitario, le citochine possono giocare un ruolo chiave nell'infezione e patogenesi di HIV-1. Infatti, l'impiego di culture *ex vivo* di tessuti linfoidi ha già permesso di dimostrare che svariate citochine solubili sono upregolate in seguito ad infezione da HIV-1. In questo studio abbiamo valutato la modulazione delle citochine sia in forma solubile che EV-associata in seguito ad infezione e/o trattamento con antiretrovirali, per verificare se lo stato di immuno-attivazione possa essere controllato in seguito all'inibizione della replicazione virale. Inoltre, abbiamo considerato ipotesi alternative potenzialmente responsabili di una persistente risposta infiammatoria: l'iniziale massivo rilascio di citochine (*homeostatic drive effect*); la presenza di virioni difettivi incapaci di replicarsi; la riattivazione di infezioni latenti da parte di herpesvirus; l'effetto tossico e quindi pro-infiammatori esercitato dai composti antiretrovirali stessi.

Metodi
Tessuti linfoidi *ex vivo* sono stati infettati con due ceppi di HIV-1: $X4_{LAI04}$ o $R5_{SF162}$. Le culture infettate sono state mantenute per 16 giorni con o senza l'aggiunta di ritonavir (RTV) o AZT-3TC, a partire dal terzo giorno post-infezione (n=8). Parallelamente, colture non infette *ex vivo* sono state trattate con citochine (n=4), virioni di HIV-1 inattivati (n=4) o ART (n=8). La replicazione di HIV-1 è stata analizzata tramite quantificazione di $p24_{gag}$ rilasciato nei supernanti. La presenza di EVs è stata confermata tramite Western Blot (WB) e Nanoparticle Tracking Analysis (NTA). 33 citochine in forma solubile e EV-associate sono state misurate con "*multiplexed bead-based assays*". Copie di DNA di herpesvirus state quantificate nelle biopsie tonsillari (n=4) tramite "*digital droplet PCR*" (ddPCR).

Risultati
I risultati ottenuti confermano che HIV-1 replica efficacemente in tessuti *ex vivo*, inducendo un incremento di diverse citochine sia in forma solubile che EV-associata già dal terzo giorno post infezione. Tuttavia, alcune di esse sono state upregolate esclusivamente nella forma EV-associate, soprattutto in risposta all'infezione da $X4_{LAI04}$. Inoltre, durante le prime fasi di infezione da HIV-1, è stato osservato un aumento significativo di RANTES e TNF-α in forma solubile rispetto a quella EV-associati e, nel caso di RANTES, una maggiormente concentrazione sulla superficie vescicolare che all'interno di essa. La produzione di numerose citochine (IL-2, IFN-γ, MIP-1α, MIP-1β e RANTES) è risultata elevata lungo tutta la cultura per entrambe le infezioni virali. L'infezione con $X4_{LAI04}$ ha anche indotto un incremento significativo di IL-7, IL-18, M-CSF e TNF-α solubili e di IL-1α, IL-6, IL-13, IL-21, IL-33, GM-CSF and TGF-β nella forma EV-associata.

Il trattamento con RTV o AZT-3TC in tessuti infettati ha inibito la replicazione virale (> 99%) ma non il rilascio di citochine anche dopo 13 giorni di trattamento soprattutto nella forma EV-associata.
L'infezione con $X4_{LAI04}$ ha stimolato il rilascio di concentrazioni più elevate di citochine sia in forma solubile che EV-associata rispetto al ceppo $R5_{SF162}$. In particolare, citochine pro-infiammatorie e β-chemochine (MIP-1α, MIP-1β e RANTES). i cui livelli sono rimasti persistentemente elevati anche in seguito a ART. Ulteriori esperimenti hanno dimostrano che la durevole attivazione immunitaria non è dovuta all'iniziale aumento di citochine. Infatti, la simulazione di una stimolazione iniziale con diverse citochine è stata in grado di influenzare la produzione di altre citochine solamente nelle prime fasi della coltura, ma questi aumenti non sono stati mantenuti nel tempo.
L'inoculazione di una singola dose di virioni di $X4_{LAI04}$ inattivati ha stimolato un rilascio di citochine simile, seppur più lieve, rispetto al virus infettivo (7 su 13 citochine). Tuttavia, ripetute esposizioni al virus inattivato hanno indotto il rilascio di concentrazioni anche più elevate di alcune citochine (IL-21, MIP-1α, MIP-1β, RANTES e TNF-α) rispetto al virus replicante.
Copie di DNA di herpesvirus (HSV-2, EBV, CMV, HHV-6 e HHV-7) sono state rilevate a tempistiche e a concentrazioni diverse. Tuttavia, non è stata osservata alcuna correlazione evidente di riattivazione dell'herpesvirus che possa spiegare un'attivazione immunitaria. Infine, il trattamento dei tessuti bioptici con i farmaci antiretrovirali non ha indotto attivazione immunitaria; al contrario sono state osservate significative, seppur lievi, riduzioni di alcune citochine.

Conclusioni
L'infezione da HIV-1 di tessuti linfoidi umani *ex vivo* correla con un significativo aumento di varie citochine, sia in forma solubile che associata a EV. Nonostante la soppressione virale ART-dipendente, la maggior parte di queste citochine sono rimasta elevate, analogamente a quanto avviene *in vivo*. Pertanto, tale modello *ex vivo* è stato impiegato per testare diverse ipotesi riguardanti la fonte dell'attivazione immunitaria, come: un "*homeostatic drive effect"*, un'attivazione immunitaria innescata da virus difettoso, presenza di co-patogeni, ed un effetto pro-infiammatorio di ART. Futuri studi incentrati sul ruolo esercitato dalle citochine associate ad EV potrebbero contribuire a decifrare il fenomeno di questa persistente immuno attivazione , permettendo lo sviluppo di nuove strategie terapeutiche volte a modulare la produzione di citochine .

LIST OF ABBREVIATIONS

AIDS: Acquired Immunodeficiency Syndrome
APC: Antigen Presenting Cells
APOBEC3H: Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like 3 H
ART: Antiretroviral therapy
AT-2: Aldritrhiol-2
ATP: Adenosine Triphosphate
cART: combined Antiretroviral Therapy
CCR5: C-C Chemokine Receptor Type 5
CDK: Cyclin-Dependent Kinase
CTLs: CD8+ T lymphocytes
CXCR4: C-X-C chemokine receptor type 4
DC: Dendritic Cells
dNTPs: Deoxynucleotide Triphosphates
dsRNA: Double Stranded RNA
ER: Endoplasmic Reticulum
EV: Extracellular Vesicles
FBS: Fetal Bovine Serum
FIs: Fusion Inhibitors
GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
HAART: Highly Active Antiretroviral Therapy
HCMV: Human Cytomegalovirus
HIV: Human Immunodeficiency Virus
HLA: Human Leukocyte Antigen
Homo: Homozygosis
HPV: Human Papilloma Virus
IFN: Interferon
IL: Interleukin
IL-1RII: IL-1 Decoy Receptor
IN: Integrases
INSTI: Integrase Strand Transfer Inhibitors
ISG: Interferon-Stimulated Gene
LTNP: Long Term Non Progressors
LTRs: Long Terminal Repeats
MACS: Multicenter AIDS Cohort Study
MHC: Major Histocompatibility Complex
MIP: Macrophages Inflammatory Protein
MX2: Myxovirus Resistance-2

NIH: National Institute of Health
NK: Natural Killer
NNRTIs: Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs: Nucleotide Reverse Transcriptase Inhibitors
PAMPs: Pathogen-Associated Molecular Patterns
PBMCs: Peripheral Blood Mononuclear Cells
PBS: Phosphate Buffered Saline
PIC: Pre-Integration Complex
PIs: Protease Inhibitors
Pol: Polymerase
PPR: Pattern Recognition Receptors
PR: Protease
RT: Reverse Transcriptase
SIVs: Simian Immunodeficiency Viruses
ss: Single Stranded
ssDNA: Single-Stranded DNA
ssRNA: Single-Stranded RNA
TCR: T Cell Receptor
Th: T Helper CD4+ T
TIM: T Cell Immunoglobulin Domain and Mucim Domain
TLR: Toll-Like Receptors
TNF: Tumor Necrosis Factor
TNFR1: Type I TNF Receptor
VIF: Virion Infectivity Factor
VL: Viral Load
WHO: World Health Organization

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INTRODUCTION

1. Cytokines

Cytokines, a term derived from two greek words "cyto" meaning cell and "kinos" meaning movement, are cell signalling molecules, more often peptides, that facilitate cell-to-cell communication in multicellular organisms [1]. These small proteins can be produced by a large range of cells, including cells of innate and adaptive immunity, like macrophages, B cells, T cells and mast cells but also endothelial and fibroblasts cells.

Commonly, the production of these cell signalling molecules is triggered in response to microorganisms and more largely to antigens [1, 2]. A particular antigen stimulates a specific secretion of cytokines, which, after binding a specific receptor, would activate a precise defense response in the inflammation and/or immunity scope (**Figure 1a**).

However, cytokines play a role not only in pathologic conditions but also in a number of homeostatic and metabolic processes.

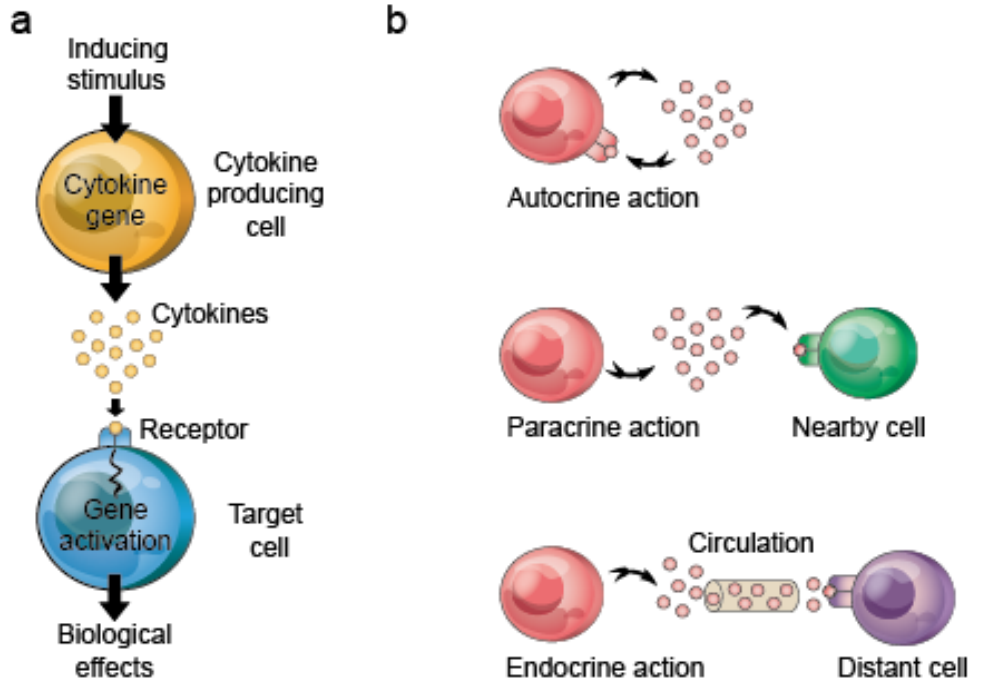

Figure 1: **a**) Example of cytokine release and action after an induced stimulus, **b**) Autocrine, paracrine and endocrine action of cytokines. Image from [3].

These immune regulating proteins may communicate through different strategies: the autocrine mechanism, working on the same cells that secrete them; through paracrine action, activating nearby cells; or the endocrine system, triggering distant cells [4] (**Figure 1b**).

Currently, these molecules play a key role in the clinical practice, indeed they are exploited as both therapeutic targets or potential drugs in different pathological conditions, not only in immunological and inflammatory field, but also macular degeneration, bone loss or even prevention of organ rejection[4].

Cytokine nomenclature has been based on the kind of cell producing them (i.e. monokines and lymphokines produced respectively by monocytes and lymphocytes), their presumed function

or target of action. Although this nomenclature, allowing for exceptions, could be obsolete since cytokines are characterized by considerable redundancy and pleiotropism, this distinction is still largely used by the scientific community.

In detail, cytokines have been categorised as interleukins (ILs), lymphokines, monokines, chemokines (CCs/CXCs), interferons (IFNs), and colony stimulating factors (CSFs) [5]:

- Interleukin (from Latin: “between leukocytes”) is a term initially used by researchers to indicate a protein that is produced by leukocytes and acts on leukocytes. This term today is used for identification of newly discovered cytokines, although it is not directly correlated with their presumed function.
- Chemokines, derived from the ability to induce chemotaxis, are small cytokines that, generally, mediate chemo-attraction between cells.
- Monokines and lymphokines are the cytokines produced respectively by monocytes and lymphocytes.
- Interferons are a group of signalling proteins able to “interfere” with viral replication.
- Colony stimulating factors are glycoproteins that support the growth of cells in semisolid media [5].

1.1 General cytokine characteristics

Although cytokines differ one from each other from a molecular point of view they share some biological properties [6].

- Cytokine secretion is an auto-limited and brief event. These proteins are not accumulated inside the cells; conversely, their synthesis requires a cell signal that induces an *ex-novo* transcription of specific genes. After they are synthetized, they are secreted, creating a “burst” of effects, often necessary to trigger a biological response.
- Biological activity induced by cytokines are frequently pleiotropic and redundant. “Pleiotropic” means that the same cytokine is able to act on different cell types. While “redundancy” refers to the fact that, usually, a particular biological effect could be triggered by different cytokines.
- Cytokines could impact the synthesis and/or activity of other cytokines. In this way a cytokine is able to start a cascade of biological events in which a second or third cytokine could mediate the effect of the first one. In the same way, two cytokines could antagonize each other, or they could work together in synergy.
- Cytokines can operate on the nearby cells, including themselves (paracrine and autocrine action), or on distant cells (endocrine action).
- To execute their function, cytokines bind to specific receptors on the target cell surface. Because of the high affinity, even a low concentration could cause a biological response.
- The receptor expression and in turn the responsiveness of the cell could be regulated by external factors.
- The cellular response to the cytokines is precisely regulated. It undergoes a negative-feedback inhibition mechanism by

influencing the messenger transcription of specific genes, affecting receptor expression and activation.

1.2 Cytokine classification

Cytokines can be classified depending on several factors, such as their structure, the receptor that they bind, or their function.

A functional classification of cytokines includes: hematopoietic cytokines (i.e. growth factors for the blood cell lines, or CSF), primary inflammatory cytokines (i.e., IL-1 and Tumour Necrosis Factor (TNF)), anti-inflammatory or immunosuppressive cytokines (IL-10 and TGF-β), secondary inflammatory cytokines (Chemokines) and acquired immunity cytokines (i.e., IL-2).

1.2.1 Hematopoietic cytokines:

Generally, the hematopoietic cytokines are generated by bone marrow stromal cells, and they can boost the growth and differentiation of immature leukocytes. This family includes the colony-stimulating factors (granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and macrophage colony stimulating factor (M-CSF)) which stimulate the production of colonies of different leukocytes in the bone marrow and are capable to enhance their activity. These factors are produced by several immune cells, such as T-lymphocytes and macrophages [7].

1.2.2 Primary inflammatory cytokines:

The primary inflammatory cytokine family comprises an important “trio” which is representative of the entire family prototype:

IL-1, TNF, IL-6. Commonly, these cytokines are extremely pleiotropic, and they are able to exert their functions in a large variety of tissues. Furthermore, even if these molecules interact with structurally different receptors, their functions are overlapped in an important way [8].

For example, IL-1 and TNF, locally, induce production of adhesive molecules, chemokines, growth factors, and lipidic mediators such as prostaglandins and nitric oxide (NO). These mediators intensify the recruitment and survival of leukocytes in the tissue in which they were recruited. The increase of leukocytes, at a local level, amplifies the innate immune mechanism, which in turn activates the acquired immunity, mainly as a result of molecules such as IL-12. The amplification of the innate immunity is also necessary to address the acquired immunity to polarize to a type I response, characterized by production of IFN-γ, or to a type II response, primarily characterized by the production of IL-4 and IL-13.

At a systemic level these cytokines work through liver stimulation by inducing the production of the “acute phase proteins”. On the other hand, the cascade of inflammatory cytokines is also subject to negative regulation mechanisms, that act both at local and systemic levels [9].

1.2.3 Anti-inflammatory cytokines:

An important negative regulation system is driven by anti-inflammatory cytokines, such as IL-10 and TGF-β, produced by the same cells that synthetize the inflammatory ones, monocytes-macrophages.

Among all the anti-inflammatory cytokines, IL-10 is the most important one as it is able to control the production of inflammatory cytokines such as IL-6, IL-1 or TNF-α. Also, this cytokine can induce the up-regulation of other anti-inflammatory cytokines and down-regulation of pro-inflammatory cytokine receptors. TGF-β has 5 different isoforms (TGF-β1 to -β5). It is known that TGF-β exerts its anti-inflammatory functions by inhibiting macrophage and Th1 cell activity. Furthermore, TGF-β1 is able to block the production of nitric oxide products in macrophages, molecules that usually contribute to cell damage and inflammation [10].

1.2.4 Secondary inflammatory cytokines:

In the secondary inflammatory cytokine family, we consider the chemokines as the major players.

Chemo-attractive stimuli are crucial for the leukocyte recruitment process. These stimuli are important not only to drive the leukocyte migration through the endothelial barrier versus the inflammatory site, but also to increase the adhesiveness at the vascular wall.

The chemokine family is composed of 47 molecules that share some structural characteristics. These molecules are small proteins with a molecular weight between 8-10 kDa and are characterized by the presence of four cysteines in conserved positions that create two disulphide bridges, which are crucial to build their 3-dimensional shape.

The structure is essential for receptor interactions, and the position of the first residues of cysteine allow us to identify four different subfamilies. In the first class, two cysteines are interrupted by

a third amino acid, and they are named Cys-X-Cys (CXC) or α chemokines. The second family is characterized by two consecutive cysteines and it is identified with the name of Cys-Cys (CC) or β chemokines [11].

The third group is particular since it contains only two cysteines, one at the N-terminal and one downstream. These cytokines are named C or γ chemokines. Two cytokines have been included in this family so far, XCL1 (lymphotactin- α) and XCL2 (lymphotactin- β). The fourth and last group have three amino acids between the two cysteines and are termed CX_3C or δ chemokines. To date, the only member of the CX3C family is fractalkine (CX3CL1) which is unique, it is both secreted and tethered to the surface of the cell that expresses it, allowing it to work both as an adhesion and as a chemoattractant molecule. Generally, the α-chemokine genes are located on the 2nd chromosome, while β-chemokines are localized on the 17th chromosome [12].

Although chemokines work in a similar way, different families could attract different kinds of cells. For example, α-chemokines are more active in recruiting neutrophils as well as T and B lymphocytes. While β-chemokines are more functional on mononuclear leukocytes (monocytes, lymphocytes, NK cells, eosinophils and basophils). Lymphotactin and fractalkine are active on T lymphocytes and NK cells. CXCL8 (IL-8) represents an important chemotactic signal for neutrophils. CCL2 (MCP-1) plays an important role in the recruitment of monocytes, while CCL11 (Eotaxin) is active on eosinophilic granulocytes.

Interestingly, in the presence of primary inflammatory molecules, such as TNF-α or IL-1, chemokines are able to induce the activation of other biological responses, as the degranulation and production of reactive oxygen intermediates [13].

Furthermore, chemokines are also able to activate the transcription of genes involved in the migration process, such as chemokine receptors, proteases (used by the leukocyte to degrade the extracellular matrix) and the chemokines themselves. All these functions play an important role in amplifying the inflammatory response.

Over the years, new chemokine functions have been discovered. For instance, it is becoming apparent that chemokines play a key role during viral infections. Indeed, it has been demonstrated that the β chemokines RANTES, MIP (macrophage inflammatory proteins) 1α and 1β (now known as CCL5, CCL3 and CCL4, respectively) can modulate Human Immunodeficiency Virus (HIV)-1 infection [14, 15].

1.3 Adaptive immunity cytokines

These cytokines are involved in response to viral or bacterial infections, supporting and regulating the adaptive immunity. These proteins are produced primarily by T-lymphocytes in response to antigen recognition. Some of these cytokines play a role in regulating the growth and differentiation of different lymphocyte populations, thus playing a fundamental role in the activation phase of the T-dependent

immune response [16]. Other cytokines included in this group recruit, activate and regulate specialized effector cells, such as mononuclear phagocytes, neutrophils and eosinophils, thus contributing to the antigen “killing” on the effector phase of the immune response. The most important cytokines in this family are: IL-2, IL-4, IL-5, and IFN-γ.

IL-2 is produced mainly by CD4+ and CD8+ T-cells. IL-2 is a strong growth factor for T and B lymphocytes, and natural killer (NK) cells. It also increases the activity of these cells, such as the killing ability, the synthesis of cytokines or increased Fas-Fas ligand apoptosis [17].

IL-4 stimulates the production of the IgE antibody isotype and promotes the development of T helper type 2 lymphocytes [18].

IL-5 stimulates the proliferation and differentiation of B-cells and promotes the production of IgA. In concert with IL-5, IL-4 promotes the defence against helminths and arthropods.

Finally, interferons (IFNs) potentially modulate the activity of the entire immune system. Interferons are classified as type I, II and III. Type I interferons include interferon-alpha, interferon-beta, interferon omega, and interferon tau. There is only one type II interferon, interferon-gamma, mainly produced by activated T-cells, which triggers the activity of the cell-mediated immune system, such as cytotoxic-T-lymphocytes (CTLs), NK cells and macrophages [19]. IFN type III, also known as interferon lambda (1-4), is the most recently discovered, and tends to function at anatomical barrier sites. IFN-λ is an epithelial cytokine, which is able to limits viral replication in

epithelial cells and create an extra layer of protection at mucosal sites. [20].

1.4 Cytokine receptors and signal transduction

Cytokines can also be classified according to the receptor they bind. Indeed, to carry out their function, cytokines interact with specific receptors which, despite being structurally diverse, maintain some common characteristics. All the cytokine receptors are composed of one or more transmembrane proteins, whose extracellular portions are designated to cytokine binding, while the cytoplasmatic sides are responsible for the activation of the intracellular signal cascade [21]. The classification of these receptors could be based on the structural homologies of the extracellular domain which are involved in cytokine binding, or it could be based on the mechanism of intracellular transduction eventually shared (**Figure 2**) [21].

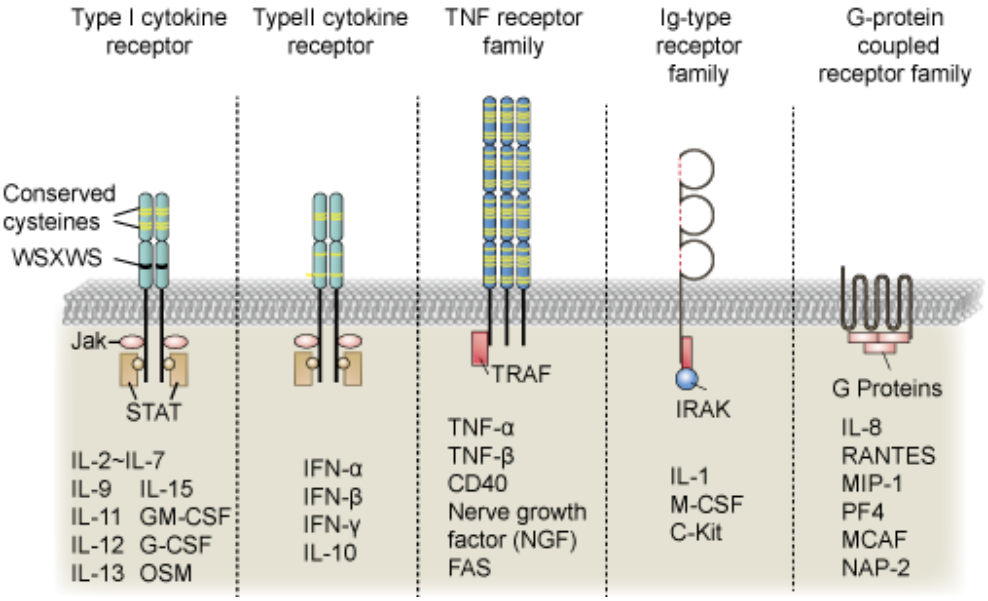

Figure 2: Representation of different cytokine receptor based on the mechanism of intracellular transduction. Image from [3].

According to these criteria we can schematize and distinguish different families of cytokine receptors:

- **Type I cytokine receptor.** Also known as Hemopoietic Growth Factor family, whose members are characterized by conserved motifs in their extracellular amino-acid domain. They have two conserved cysteine residues and, closer to the plasmatic membrane, a tryptophan-serine-X-tryptophan-serine sequence (WSXWS, where X represents any amino acid). Due to this structure, these receptors typically recognize cytokines with a four-α-helix bundle structure (such as IL-2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 15, GM-CSF and G-CSF) and the amino acid residues that differ between the receptors give them the specificity for the different cytokines. These receptors have a multimeric structure, where a peptidic chain is interested in binding the

cytokine, while the others are involved in the signal transduction. The binding with the type I receptor actives the Jak-STAT transduction signal pathway, which is able to modify the gene expression of the targeted cell.

- **Type II cytokine receptor.** At structural level, this family is similar to the type I receptors, as they share the two conserved cysteine residues, but not the WSXWS sequence. Also, as the type I receptors, they are composed by a peptidic chain that binds the cytokine and the other one involved in the Jak-STAT signal transduction pathway. This receptor is also known as Interferon (type 2) family since the main ligands are: IFN-α, β and γ but IL-10,20,24,26 as well.
- **TNF receptor family.** This big family of proteins that share a conserved cysteine-rich extracellular binding domain, and activate pathways triggering a gene expression modification. The most famous pathway is the one that involves the TRAF (TNF Receptor-Associated Factors) proteins, which are able to induce transcription factor activation, such as NF-κB (Nuclear Factor κB) and AP-1 (Activator Protein-1), commonly these transcription factors activate pro-inflammatory and survival genes. In particular, this receptor could bind not only TNF family molecules but also several other non-cytokine ligands like CD40, CD27 and CD30.
- **Immunoglobulin (Ig)-type receptor family**. These receptors are ubiquitously present throughout several cells and tissues. This family is so called due to a structural homology with immunoglobulins (antibodies), cell adhesion molecules, and

even some cytokines. Toll-like/IL-1 receptor (TIR) belongs to this family and similar to other receptors, after the binding with the specific cytokine it is able to active a pathway that modifies the gene transcription according to the signal. Examples of ligand belonging to this family include: IL-1, IL-18 and M-CSF.

- **G-protein coupled receptor family**. Also called Seven transmembrane helix family, or “serpentine” because the peptidic chain crosses the plasma membrane many times like a snake. The name of this group depends on the way they transmit the signal, as it involves triphosphate-guanosine binding proteins.

  The members of this receptor class interact with chemokines and other inflammatory mediators in a rapid and/or transitory way. In this family are the chemokine receptors, which are commonly bond by chemokines, but it has been discovered that class can also be exploited by different viruses as mechanism of entry [22, 23]. For example, HIV-1 can use CXCR4 and CCR5 as co-receptor to facilitate its entrance in the cell [24].

1.5 HIV-1 and cytokines

Changes of soluble cytokines in response to HIV infection were widely studied and were reported to play an important role in HIV acquisition and pathogenesis. Following HIV infection there is an altered balance of pro-inflammatory and anti-inflammatory cytokine milieu, leading to different response to the virus and a different HIV disease progression [25] . Recently, it was found that EV-associated cytokines are also altered upon HIV infection [26].

2. Cytokines associated with extracellular vesicles

Cytokines were considered to be classical soluble factors, but recently, it was found that they can be associated with extracellular vesicles (EVs) being either encapsulated or attached to the vesicle surface [27, 28] (**Figure 3**).

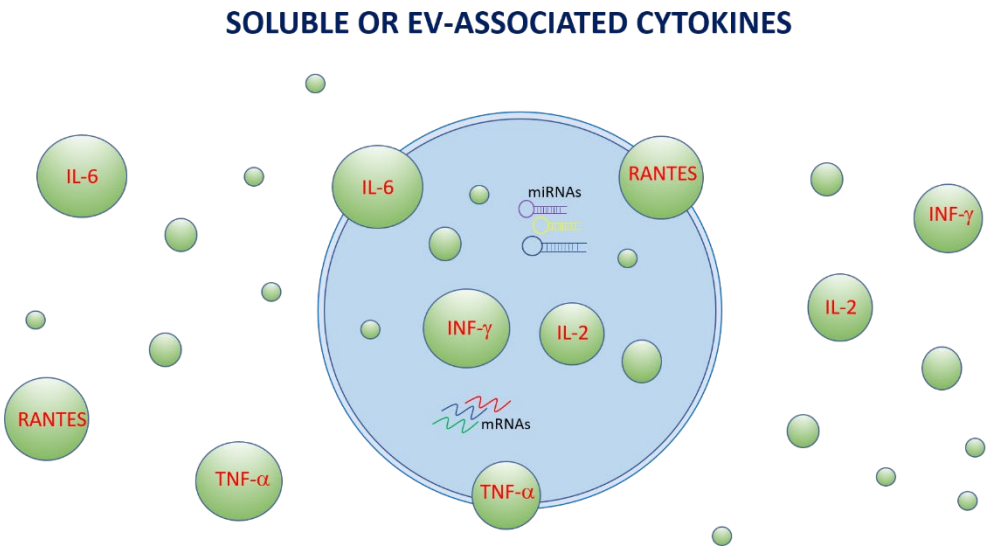

Figure 3: Simplified illustration of soluble and EV-associated cytokines (represented in green) on the extracellular versant.

A recent study from Margolis' Lab showed that in different biological systems, both *ex vivo* and *in vivo*, a particular cytokine could be released both in soluble or EV-associated form. They showed that this different way to release a cytokine is dependent not only by the cell source but also by the kind of stimulus. Thus, bringing up the hypothesis that the association of cytokines with EVs is not just a

casual process, but a finely regulated mechanism that needs to be deepened.

In detail in this study they found that in eight different models, a given cytokine in one system could be produced principally in soluble form, while in another it could be encapsulated in the EVs. For example, some of these cytokines that in placenta explants were mainly in soluble form, were found predominantly associated with EVs in immune cells, such as T cells and monocytes. Furthermore, when associated with the EVs, a cytokine could be found either on the surface or inside as cargo. It has been demonstrated that these vesicles are not just cells debris and waste, but, after interacting with sensitive cells, they are able to trigger a biological activity.

Many questions concerning EVs and their cargo are still unanswered; this is one of the most recent discoveries and several works suggest that these EV-associated cytokines could play an active and important role in health and disease.

Whether the association with the vesicles is a specific property of a particular cytokine or whether it is a precisely regulated physiological mechanism remains to be clarified [28].

3. Extracellular vesicles characterization

The collective term ‘EV’ refers to a heterogeneous population of secreted vesicles that are formed via different pathways [29].

In the last 10 years EVs have begun to be of great interest for investigators. In fact, thanks to the latest discoveries, it was proved

that they are able to mediate cell-cell communication, facilitating the exchange of molecular materials, such as mRNA, miRNA, proteins, lipids, which, consequently, are able to influence the recipient's cells [30].

But, the discovery of these membranous vesicles is not so recent, in fact they were first described in the late 1960s. The first use of the term “extracellular vesicle” in a manuscript title dates back to 1971.

At the beginning, the researchers hypothesized that these vesicles were unnecessary material for the cells, just "trash" that had to be expelled through these lipidic structures [31].

Since that time, it was fully demonstrated that origins, cargo, components, and size are very different, and several classes of EVs have now been described. Throughout this time, different nomenclatures have been used, and the matter is still debated; notwithstanding the scientific community agrees in classifying them depending on size and origins. In general, referring to vesicles that bud-off directly from the plasma membrane, we use the term microvesicles (MVs), commonly they have a size around 150-1000 nm. While, the term exosome is used to indicate a more specific smaller vesicle class (30-150nm) derived from the endosomal system, which are secreted by cells after a fusion process among multivesicular endosomes bodies (MVBs) and the plasma membrane [32, 33] (**Figure 4a**).

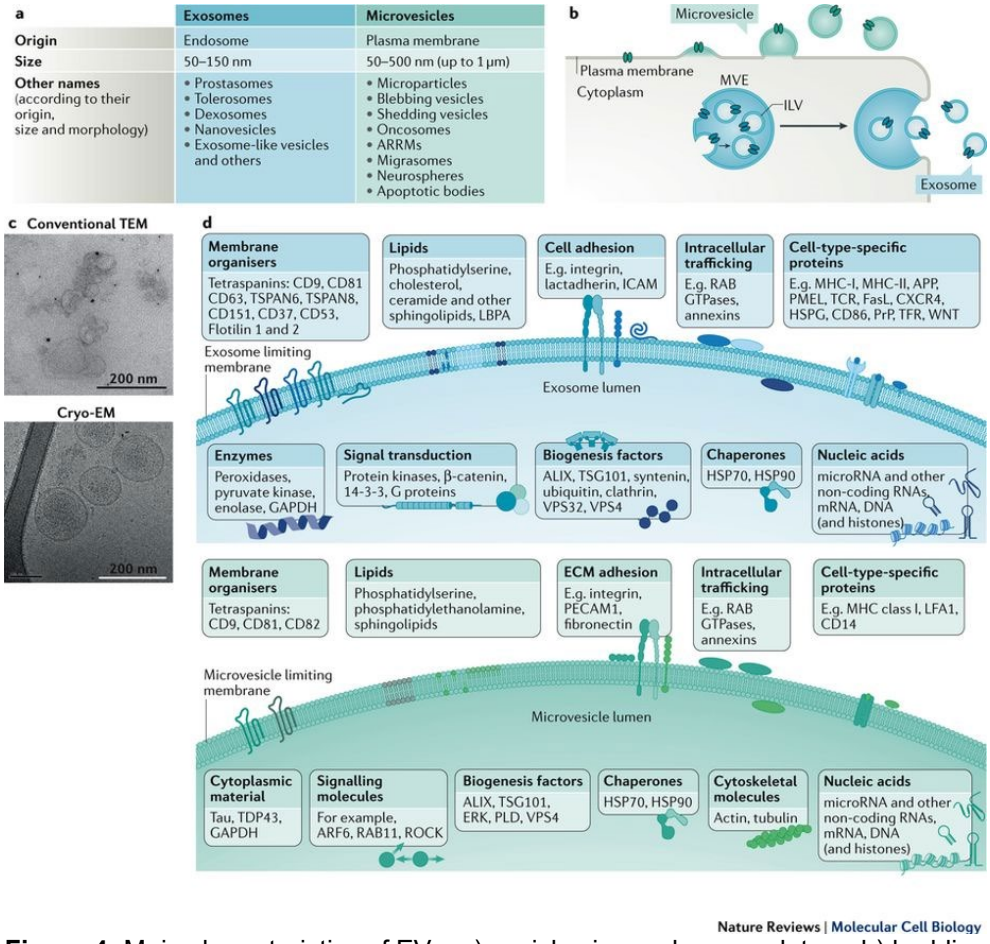

Figure 4: Main characteristics of EVs. a) vesicle size and nomenclature; b) budding pathways: directly from the plasma membrane or as intraluminal vesicles within MVBs; c) TEM or cryo-EM of EVs; d) schematic representation of surface molecules and cargo of exosomes and microvesicles. Image from [33].

Also classified as EVs are other vesicular structures, such as apoptotic bodies, exosome-like vesicles and membrane particles.

Among all, exosomes are the most studied EV population. They are recognized as intraluminal vesicles (ILVs), and they originate by an endosomal route. Exosome membrane formation begins during the early endosome maturation, which leads to the creation of MVBs.

Different ILVs, 30-100nm in diameter, are contained together inside these MVBs, and they may combine and fuse with lysosomes for degradation processes, or they may be released through the plasma membrane, allowing them to be free in the extracellular compartment as exosomes [32, 33] (**Figure 4**).

The precise mechanism has not been completely comprehended so far, but it likely involves the Endosomal Sorting Complex Required for Transport (ESCRT) machinery. This machinery includes four different cytosolic multiprotein complexes (ESCRT-0, I, II, and III), plus other important accessory proteins (TSG-101, Alix, HSC-70, HSP-90β, and VPS-4). These proteins work together, cooperating to facilitate the formation of MVBs, the protein cargo arrangement, and vesicles spread (**Figure 5**).

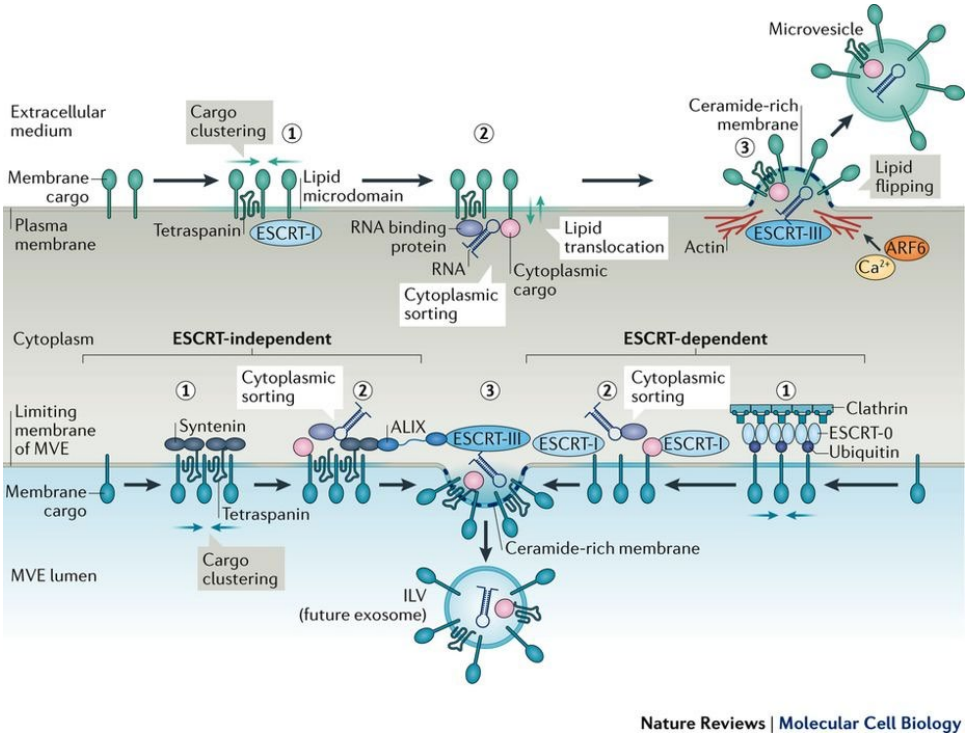

Figure 5: Different mechanisms of EV biogenesis. Image from [33].

ESCRT-0 is the first component triggering the budding; this protein is able to recognize and bind ubiquitinated proteins through precise domains. This interaction leads to a sequential collaboration, first with the ESCRT-I complex and then with ESCRT-II. ESCRT-II attracts and interacts with the last component of this machinery, ESCRT-III. The last protein complex is the most involved in promoting the vesicle release, indeed it is able to induce a flex of the membrane. The interaction of ESCRT-III with VPS-4, a ring necessary for the budding is generated, and following the cleavage to form ILVs, this complex is liberated from the membrane due to the energy released by the ATPase protein VPS-4 (**Figure 5**).

This mechanism is still debated, but several works from different cell sources demonstrated the presence of numerous ESCRT proteins associated with exosomes, thus suggesting the complex to be highly involved [34].

On the other hand, some exosomes could be produced without the need of that complex. Several studies, with the goal of depleting fundamental subunits of the complex, showed that exosomes are produced despite the absence of those unit, suggesting an ESCRT-independent budding way.

This ESCRT-independent manner seems to depend on lipid raft-based domains (mainly ceramides), and tetraspanins proteins.

To confirm this, Trajkovic et al. demonstrated that the inhibition of neutral sphingomyelinases (proteins that permit the transformation of sphingomyelin to ceramide) influenced and decreased the exosomes release [35]. They suggested that ILV formation could begin with the creation of ceramide domains in specific areas of endosome membrane, highly enriched in sphingolipids, that facilitated the membrane curvature and therefore the budding.

Tetraspanins are important transmembrane proteins that can create tetraspanin-enriched microdomains (TEMs) in the plasma membranes, which are involved both in the compartmentalization of protein (such as receptors) and in the proteins communication in the membrane [36].

Several studies have shown that these proteins are involved in the exosome biogenesis, indeed their presence has been detected in the MVBs. They are able to interact with each other, to cluster and

create a group with cytosolic molecules and membrane, facilitating the development of a crater-like structure with an intraluminal hole. The interaction among this domain and formation of this cluster permit the peculiar membrane curvature that induce an inner budding.

3.1 EVs cargo

Vesicles may contain a different cargo composed of proteins, lipids and nucleic acids of different origin. Notably, many studies suggest that the EV content is not just a casual event, but a more finely regulated process. In fact, the cargo of a particular vesicle may change depending on the cell's origin or the specific stimulus it is subjected to. Furthermore, any cell is able to produce and release a very heterogeneous vesicles population (MVs, exosomes, apoptotic bodies), and it may be hard to distinguish between them.

Currently, several studies have shown what may be inside a particular vesicle, but it is still not clear how this process is driven. Some studies strongly suggest that this is a coordinated process which could involve several factors [37, 38]. For example, it was discovered that microRNAs, that are packed inside the exosome, have a specific sequence motif that controls their binding with a nuclear ribonucleoprotein named A2B1 (hnRNPA2B1) [39]. The sumoylation of this nuclear protein seems to be crucial for miRNAs binding, and commonly exosomal hnRNPA2B1 shows this specific modification. Another factor involved in miRNA binding which facilitates their sorting into vesicles is KRAS, an important signal transduction pathway [40]. Indeed, EVs released from cells with a mutation of KRAS gene have

a dissimilar miRNA profile with respect to the cells with a wild type gene.

Ubiquitination represents another protein modification involved in the exosome cargo. In fact, some mass spectrometry analyses showed an increase in ubiquitinated proteins within exosomes.

Not only proteins, but also lipids seem to be necessary for the sorting of specific molecules into vesicles. The most important one include cholesterol and sphingolipids, as they are highly concentrated in the exosomes with respect to their mother cells [41, 42].

The presence of these lipids rafts in the membrane influences its movements and flexibility. Furthermore, it is plausible that some particular protein could interact with these lipid rafts and thus become packed inside the vesicles (such as, flotillin-1 or stomatin).

3.2 EVs trafficking

An important feature that needs further elucidation is the mechanism regulating EV trafficking and how a specific vesicle moves inside the cell. Exosomes are packed inside MVBs and they could be released outside the membrane after their fusion with it, but MVBs could also fuse together with a lysosome. What exactly drives this molecular process is not completely clear yet. A post-translational modification, called ISGylation, has been suggested to be one of the factors able to control the MVBs' destiny [43]. It seems that ISGylated proteins of the MVBs promote their degradation through the fusion with the lysosome, rather than being secreted.

The movement of MVBs for exosome release is reliant on their interaction with microtubules and actin. An important set of proteins implicated in this process is the Rab family, a group of small GTPase proteins. Rab proteins are able to regulate many phases of vesicle transport, including their membrane fusion and budding [44].

Several functional inhibitory studies (drugs inhibition, RNAi) of different Rab GTPase family proteins have demonstrated that this inhibition can modify the exosome cargo and trafficking. In fact, the release of specific EVs, such as the CD63-associated (a tetraspanin member) ones, has been shown to be decreased in exosome cargo, after the silencing of Rab27A or Rab27B messengers [45]. Furthermore, Rab27 proteins control both the MVBs transport and their docking to the plasmatic membrane, promoting and influencing vesicle budding (**Figure 6**).

The Rab family is not the only group of small GTPases involved of vesicle traffic and budding. Indeed, different families such as Rac/cdc42/Rho could have a key role in the EV release. These GTPases could be used also by different viruses; for example it has seen demonstrated that Rho family members are involved in HIV-1 production and release [46].

In summary, exosome budding, and trafficking is regulated by different mechanisms. Several protein and lipid interactions are implicated to facilitate the fusion with the plasma membrane. The most important proteins involved in the membrane fusion are tethering molecules, the small GTPases, and the SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). In

particular, SNARE family members helps the docking and the fusion of EVs with their target plasma or organelles membrane [47].

SNARE proteins are identified as either R or Q SNARE, and they may cluster to create four coiled-coil helices. It is still not completely clear how these complexes work in the membrane fusion, but different knock-down studies have shown their essential implication in this process. They could interact with themselves and with important proteins involved in the exosome pathway (ALIX, CD63, HSC70 and TSG101), also influencing their fate and presence in the vesicle cargo and release.

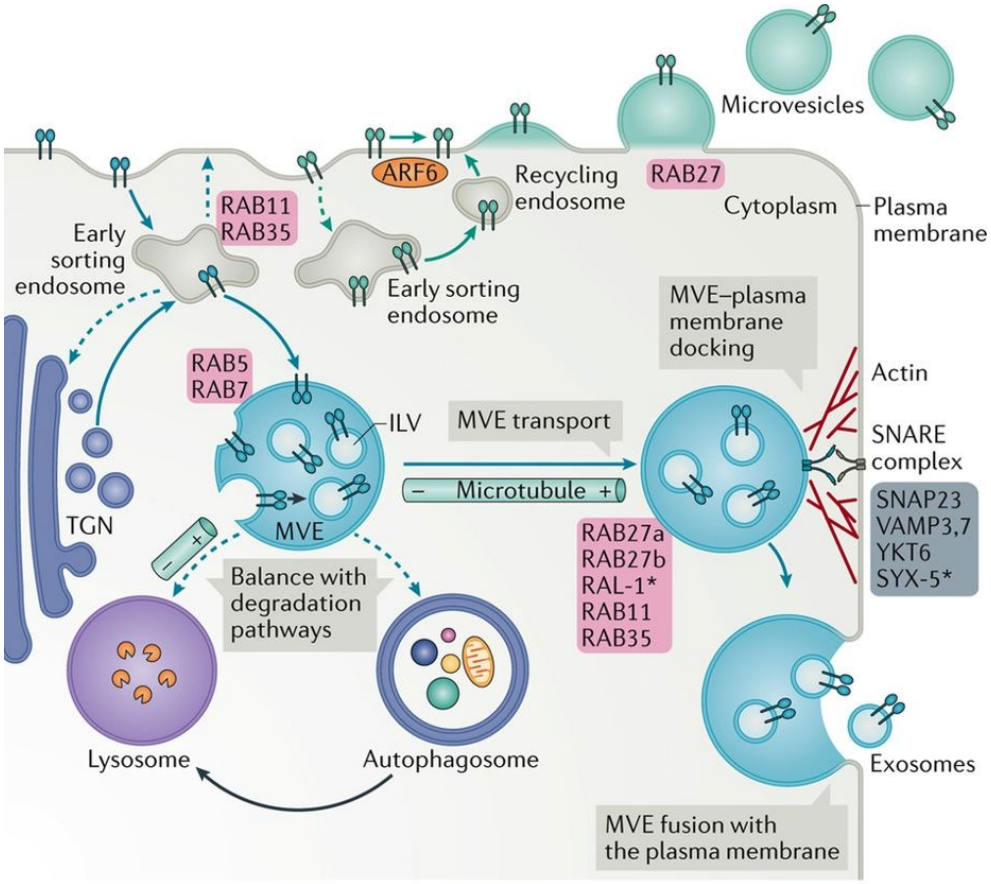

Figure 6: Representation of exosome and microvesicles budding and trafficking, which are regulated by different mechanisms. Image from [33].

The cytoplasmic levels of calcium represent another key factor responsible for exosome release. In fact, some studies have demonstrated that an increase of intracellular calcium facilitates and upregulates the exosome release [48].

In general, there is a large overlapping in terms of cargo, function and release among the different EV types, especially among exosomes and microvesicles. It is therefore extremely hard to exactly

discriminate among them in a given biological material. Indeed, the microvesicles release shares some key point with the exosome pathway.

As with exosomes, the budding of MVs requires their scission from the plasma membrane, depending on use of energy (ATP) and communication with actin and myosin. This implicates also the participation of small GTPases proteins such as ARF6 and ARF1.

Also, TSG101 (an ESCRT subunit) and VPS4, ESCRT-machinery components highly intricated in exosome biogenesis, were described in participating during the fission and budding of arrestin domain-containing protein 1-mediated microvesicles (ARMMs).

Furthermore, microvesicles release is influenced by the cell activity and status. It has been reported that in starvation conditions MVs release is decreased, while an increase of intracellular calcium may induce and increase their budding.

Like exosomes, microvesicles release depends on the lipid raft content as well. The activation of a specific receptor (P2X7), induces the recruitment of SMase (sphingomyelinase) in the plasma membrane, which catalyze the breakdown of sphingomyelin, generating ceramide and phosphorylcholine. As said before, the presence of ceramide is able to promote membrane curvature and then microvesicle spreading.

3.4 Binding and uptake of target cells

After the budding in the extracellular versant, EVs can spread and move towards the target cells, delivering their cargo, which may influence the cellular function and induce a homeostatic or pathological response (**Figure 7**).

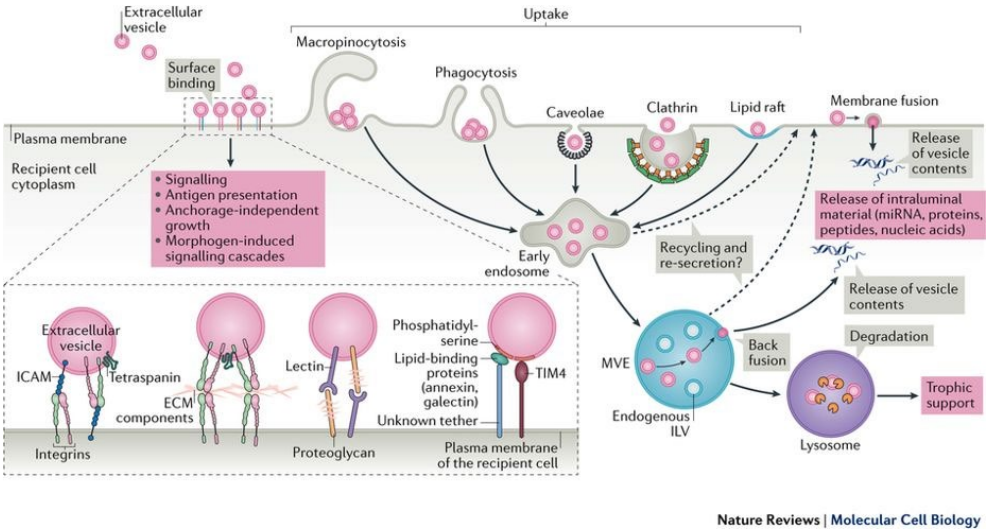

Figure 7: Representation of EV binding and uptake by recipient cells. Image from [33].

The EV uptake requires different processes, which are not full deciphered. First, they have to dock to the plasma membrane through to their interaction with receptors and other proteins. This results in the endocytosis or membrane fusion of the vesicle with the recipient cell. This mechanism depends on the EV source and on the target cell, modulating differently the final effects [49] (**Figure 7**).

Although EVs are a heterogenous group of vesicles, the general principles of their uptake and transport are likely shared among them. Definite proteins present on the EV-surface may give

them a peculiar specificity to proteins expressed on the plasma membrane of the target cells [50-54]. Likewise, cytokines associated with released EVs could have an autocrine action, acting on the same cell that produced them.

The main molecules identified involved in this membrane interaction are: tetraspanins (such as CD63, CD81), lipids (phosphatidylserine), proteoglycans and extracellular matrix proteins (ECM). For instance, ECM proteins, such as intercellular adhesion molecules (ICAMs), could bind integrin units expressed on the EV surface. It has shown that some cancer cells release EVs acting on distant organs through integrin interactions, promoting pre-metastatic development [54].

Integrins can collaborate with the tetraspanins members, facilitating the vesicles docking ad fusion with the target cells [55]. Also, factors other than proteins, such as polysaccharides (i.e. heparan sulfate) and lipids (phosphatidylserine) are involved in these processes and could have an impact on the addressee-cell targeting [56, 57].

After EVs reach their “consignee” cell, their fate could be different, Depending on the cell type, EVs can remain attached to the plasma membrane (i.e. to integrins) and can trigger an intracellular signaling pathway or they can be endocytosed through different mechanisms: Clathrin- dependent or -independent endocytosis, caveolae-mediated endocytosis, membrane fusion or interaction with lipid rafts [58-60].

Subsequently, the uptake addresses the EVs to the canonical endosomal pathway, where they are fused with the MVB leading them to degradation and recycling of the EV cargo [61, 62] . Sometimes, EVs may escape from the degradation fate and they can release their cargos directly in the cytoplasm. This mechanism is poorly comprehended, but it plays an important role in the miRNA delivery and trafficking [63]. Lastly, these vesicles can evade lysosome degradation, and they can be recycled or re-secreted.

Remarkably, EVs can trigger a response just binding and interacting with cellular surface proteins. For example, in the immune response, B lymphocyte are able to produce antigen-presenting exosomes that can interact with T lymphocytes and trigger an antigenic response [64, 65].

In the same way, EV cargo internalization could facilitate the antigen-presentation; in fact, EVs could transport exogenous material (antigens) processed in the endosomes, thus contributing to the acquired immune response. The exact process is not determined yet, but it may be similar to fusion mechanisms exploited by viruses [66].

4. EVs and infectious diseases

EVs are implicated in different cellular pathways. Their intervention in immune system regulation has been demonstrated, but their role in infectious diseases needs further investigation. EVs can be released by infected cells exerting a double effect, facilitating or suppressing the virus spreading.

EVs and viruses (especially retroviruses) are very similar as they share different components. Overall, they have similar size which renders their discrimination and isolation particularly cumbersome. Since both are able to bud from the plasma membrane, they share ECM components (proteins, lipids). Furthermore, their biogenesis takes place in the endosomes, involving the same mechanisms, such as the ESCRT complex. Vesicles and viruses are both released in the extracellular versant to target the recipient cell through plasma membrane binding. Both viruses and EVs have cargo that could influence the status of the targeted cell, stimulating a response. Finally, viruses could exploit EV trafficking to transport their infectious material, rendering the recipient cell easier to be infected, or even worse, as in the case of the hepatitis C virus, they could transport genomic material able to start a new viral infection [67].

In general, during an infection there may be two extremes composed by the infectious virus particle on one hand and the normal host EVs on the other. In the interlude we could find a series of different “hybrid” particles, such as virus-like particles, non-infectious virus, or Virus-induced EVs [68] (**Figure 8**).

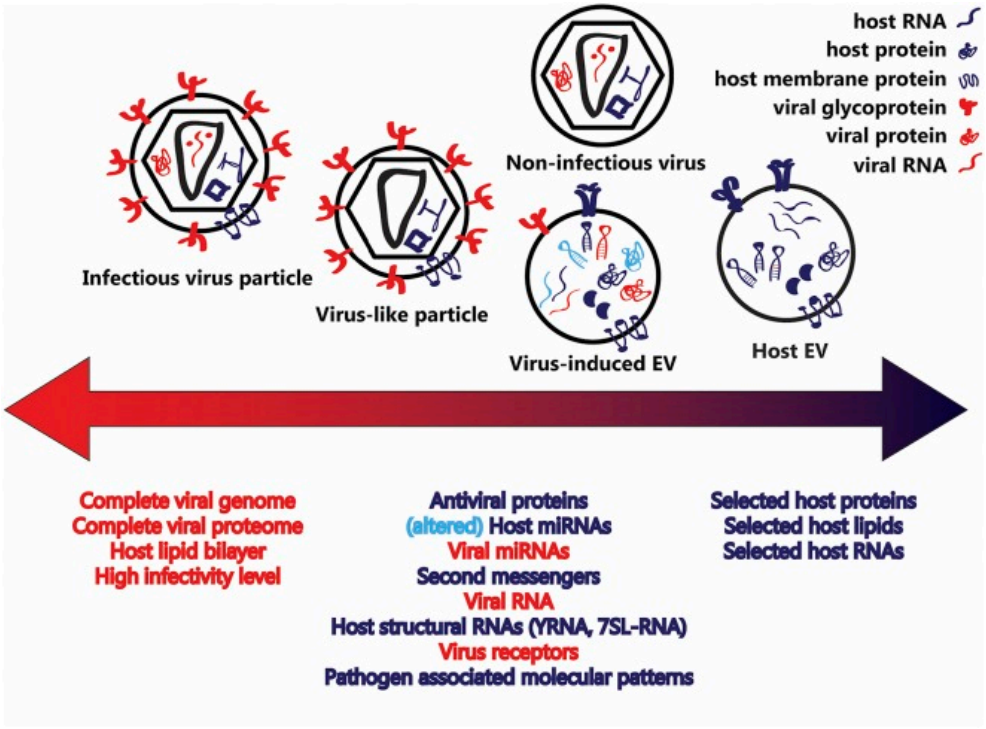

Figure8: Structural similarities among EVs and virus particles. There exists a continuum between infectious virus particles and pure EVs. Image from [68]

Although EVs containing viral components may not be infectious, they could facilitate virus spreading. For instance, Epstein-Barr virus (EBV), a virus belonging to Herpesviridae family, has been shown to transfer oncoproteins and different nucleic acids by exploiting EV transport. Importantly, this transported material has been shown to be functional in the recipient cells [69].

EVs have an ambivalent function during the infection in the case of EVs released from Hepatitis C Virus (HCV) infected cells. HCV viral material can be incorporated inside the vesicles and can stimulate the nearby dendritic cells to produce IFNs for counteracting the viral infection [70]. The transport of viral RNAs may work both as a viral escape strategy to avoid the immune recognition and as a host

strategy to elicitate the immune response in non-infected cells to fight the viral infection.

This way to elude the immune system through the exploiting of exosome release is known as “Trojan exosome hypothesis”. This hypothesis turned out to be important in the fight against HIV and the consequent Acquired Immune Deficiency Syndrome (AIDS), and had important implications in developing of new drugs, controlling of the risk of HIV new infection, and improving the creation of efficacious vaccines [71].

5. Human Immunodeficiency Virus (HIV)

HIV is a retrovirus, an RNA virus which is able to insert DNA copies into the host genome in order to duplicate. This virus belongs to a retroviridae subcategory, genus Lentivirus, that can cause chronic and lethal disease characterized by long a period of incubation. This virus is well known worldwide since it is the major causes of AIDS [72]. The first description is dated to 1981 in the United States of America. In June 1981 in Los Angeles, San Francisco and New York, an increase in unusual pneumonias caused by Pneumocystis carinii (a pathogen rarely found in healthy adults) was reported in some young homosexuals. A rare tumor, Kaposi's sarcoma was reported as well [73]. Although the first report dates back to the beginning of the 1980s, the virus started to propagate earlier. Academics estimated that at some point in the early 1900s, HIV was transmitted in Africa from non-

human primates to humans from a form of simian immunodeficiency virus (SIV) [74].

Initially, this infection was related to bad behaviors (unprotected sex and drug use) and the infection was described by the press as a gay-related immune deficiency (GRID). During this period different names were coined, such as lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type 3 (HTLV-3), or AIDS-related virus (ARV). Only in July 1982 was the term HIV becoming accepted and used [75].

The merit of the first isolation is to be shared between two different scientific teams, one located in the USA led by Robert Gallo [76], and the other one by the French investigators Françoise Barré-Sinoussi and Luc Montagnier [77]. They each called the virus in a different way; Gallo named it HTLV-III and Montagnier LAV, discovering successively that it was the same virus.

At the same time, another group at the University of California run by Jay Levy, independently discovered the AIDS virus and named it ARV. This virus turned out to be dissimilar from the one isolated by the groups of Montagnier and Gallo, indicating the heterogeneity of HIV.

In the 80s and 90s, being infected with HIV was a death sentence, and this awareness brought a lot of alarmism and fear. During these years several antiretroviral drugs were discovered, but they were toxic, or the virus easily gained resistance. The turning point was in 1996 with the introduction of Highly Active Anti-Retroviral

Therapy (HAART), a treatment that combined the effects of different antiretroviral drugs working at different levels [78].

Nevertheless, the grade of new infections, morbidity and deaths due to HIV has remained elevated. The latest data (2018) reports that every year around 1.7 million people are newly-infected, worldwide around 37.9 million people are infected with HIV and only 62% of them can get access to HAART [79]. Finally, yearly 770,000 die from AIDS -related infections [79].

In general, the main problem in HIV infection is the recipient cells; because of particular surface proteins, HIV is able to infect CD4+ T cells, leading to their death. CD4+ T cells are very important in the regulation of the adaptive immune system and their loss is characterized by a severe immune deficiency leading to opportunistic infections, development of cancer, and dementia. Therefore, if not correctly treated, HIV infection can lead to AIDS and consequently to death [80].

5.1 HIV characteristics

HIV is a lentivirus, a subtype of viral Retroviridae family, and two main types have been described: HIV-1 and HIV-2. HIV-1 is the most diffused and virulent strain and it was probably transmitted from Central African chimpanzees (SIVcpz). HIV-2 is less virulent and aggressive and thus more confined to occidental African areas; it presumably derived from the West African sooty mangabey strain of SIV (SIVsm).

5.2 HIV-1 genome and structure

The genomic material of HIV is composed of two identical single-strand RNAs that are surrounded by the core proteins that permit the formation of the viral particle. In general, a HIV virion is a sphere of around 120nm. Through a special enzyme, reverse transcriptase, HIV is able to convert its genome from RNA to DNA which may be integrated into the human genome via an enzyme called integrase and is defined as provirus [81].

The full genome is 9749 nucleotides long, it is bordered by long terminal repeats (LTRs) and it is composed of coding and non-coding regions involved in the production of regulatory and accessory proteins and in the regulation of viral expression. On the 5'-LTR region there is the "start", better known as gene promoter, to encode all the HIV proteins. In order, in 5' to 3' direction there are three main genes, GAG, POL, ENV [82] (**Figure 9**).

1. **GAG**, an acronym standing for group-specific antigen, is a gene shared among different retroviruses, which encodes viral capsid proteins. In detail, the gene codes for a polyprotein which through cleavage by the viral protease leads to the formation of matrix protein (MA or p17), capsid protein (CA or p24), spacer peptide 1 (SP1 or p2), nucleocapsid protein (NC or p7) and P6 protein. All these proteins together are essential for the correct packaging of HIV virions and to vehicle them into the plasma membrane, thus facilitating the budding. The protein p17 favors the anchorage of GAG polyprotein to lipid

rafts in the plasma membrane. The capsid protein is often used in diagnostics and research as an index of viral replication [83].

2. **POL** is a retroviral gene necessary for the synthesis of DNA-polymerase, more precisely for reverse transcriptase (RT), Ribonuclease H (RNase H), integrase (IN), and HIV protease (PR). RT has an RNA-dependent DNA polymerase activity, which is able to convert RNA into complementary DNA (cDNA), this proviral DNA can be inserted into the host DNA via IN. Importantly, the PR is required for the correct cleavage of GAG polyprotein [84].
3. **ENV**, the envelop gene, codes for a big protein named p160, which is a homotrimer which is cut by furin, a host PR, resulting in the production of gp120 and gp41. These proteins work together: gp120 is important for targeting and entry into the recipient cell since it is a glycoprotein able to interact with the CD4 receptor (present in T helper cells); after this binding a conformation change occurs in gp41 which can then assist the fusion and entry into the target cell [85].

5.2.1 Crucial regulatory components:

- **TAT** (Trans-Activator of Transcription) is a regulatory protein that considerably increases HIV transcription. Through the interaction with host proteins Tat is able to mediate their phosphorylation, massively enhancing the level of transcription of the HIV dsDNA and its genes. Tat seems also to play an active part in the HIV-1 disease processes. Furthermore, Tat

can bind the 5' RNA ends of new viral transcripts, a stem-loop conformation known as trans-activating response element (TAR). This interaction can boost the full-length viral RNA synthesis through the recruitment of the positive transcription elongation factor (P-TEFb) complex (CDK9/cyclin T) [86].

- **REV** (regulator of expression of virion proteins) is a protein that binds to the viral genome via an arginine-rich RNA-binding motif that also acts as an NLS (nuclear localization signal), required for the transport of Rev to the nucleus from the cytosol during viral replication. Rev recognizes a complex stem-loop structure of the env mRNA located in the intron separating the coding exon of Tat and Rev, known as the HIV Rev response element. Rev is important for the synthesis of major viral proteins and is hence essential for viral replication [87].

5.2.2 Accessory regulatory proteins:

- **vpr** (lentivirus protein R): Vpr is a virion-associated, nucleocytoplasmic shuttling regulatory protein. It is believed to play an important role in replication of the virus, specifically, nuclear import of the preintegration complex. Vpr also appears to cause its host cells to arrest their cell cycle in the G2 phase. This arrest activates the host DNA repair machinery which may enable integration of the viral DNA. HIV-2 and SIV encode an additional Vpr related protein called Vpx which functions in association with Vpr [88].
- **vif** (Viral Infectivity Factor): Vif is a highly conserved, 23 kDa phosphoprotein important for the infectivity of HIV-1 virions

depending on the cell type. HIV-1 has been found to require Vif to synthesize infectious viruses in lymphocytes, macrophages, and certain human cell lines. Vif expression is not required for the same process in HeLa cells or COS cells, among others [89].

- **nef** (negative factor): NEF is a N-terminal myristoylated membrane-associated phosphoprotein. It is involved in multiple functions during the replication cycle of the virus. It is believed to play an important role in cell apoptosis and in increasing virus infectivity [89].
- **vpu** (Virus protein U): Vpu is specific to HIV-1. It is a class I oligomeric integral membrane phosphoprotein with numerous biological functions. Vpu is involved in CD4+ cell degradation, through the ubiquitin proteasome pathway activation, as well as in the successful release of virions from infected cells [90] .
- **tev (T**at, **E**nv, re**V):** This gene is only present in a few HIV-1 isolates. It results from the fusion of tat, env, and rev genes, and codes for a protein with some of the properties of tat, but little or none of the properties of rev [91].

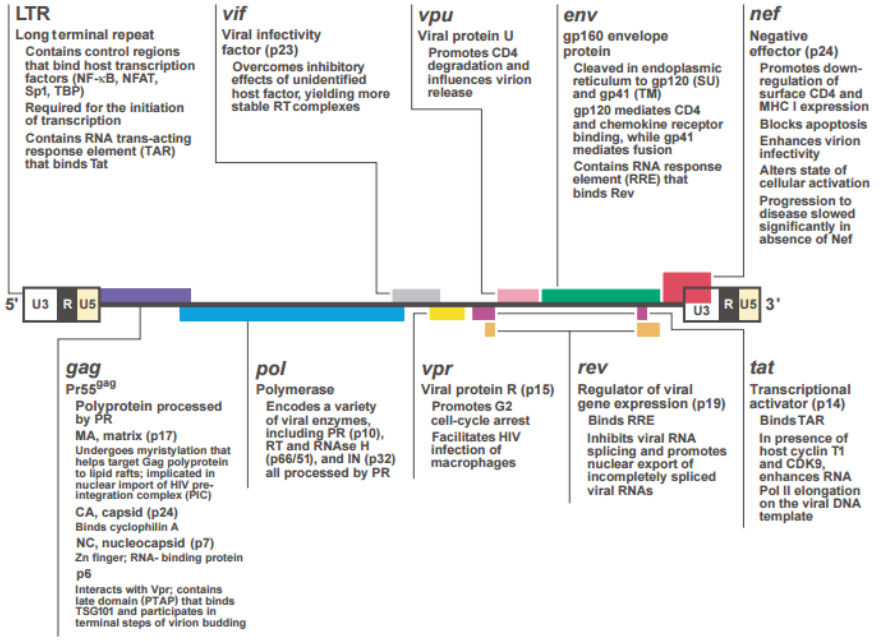

Figure 9: Schematic HIV-1 genome illustration. Each gene is marked with a colour block. Genes using the same sequences of other genes are shown by overlapping blocks. The blocks which are separated by lines indicate gene coding sequences which are separated in the genome and require RNA splicing to produce the respective messenger. Image from [92]

5.3 Groups and subtypes

HIV-1 is the most usual and virulent strain of HIV. This virus is very variable and has a lot of different subtypes that are spread worldwide in different percentages. Scientists have identified four different groups: M, N, O, and P. Presumably each group derives from a different and independent non-human primate [93].

The major group is M, and around 90% of infections are due to this family. This group has nine different subtypes, named with an alphabetic letter as well: A, B, C, D, E, F, G, H, I (or CRF04_cpx), J, K. The most studied, although covering only 12% of infections, is the

B subtype, since this is more widespread in the developed countries such as Europe, North and South America, Australia, and Japan. Furthermore, around 89 subtypes could be found since the possibility of a genetic material combination among subtypes, recognized as circulating recombinant forms (CRFs).

The N group stands for non-M and non-O, and since 2015 less than 20 infection cases were reported.

The O group is tagged as “outlier”, as it was found exclusively in a small population in West-Central Africa (around Cameroon).

Lastly, the P group is a “pending” group, since in 2009 a new HIV strain was isolated from a Cameroonian woman, and it was proven to be genetically closer to a SIV infecting wild gorillas (SIVgor) than SIVcpz (chimpanzees) [94].

5.4 HIV-1 replication cycle

HIV-1 replication can be divided into different steps: viral entry, uncoating, viral DNA synthesis and integration, messengers’ transcription, viral maturation and spreading.

Initially, HIV-1 makes contact with the target cells through its surface glycoprotein gp120, which is able to recognize CD4 receptors, making virtually all CD4+ cells infectable (T helper lymphocytes, macrophages, monocytes and dendritic cells). The interaction between gp120 and CD4 molecules induces a small conformational change revealing a binding “pocket” for an auxiliary receptor (co-receptor), commonly the chemokine receptor CCR5, although CXCR4

could be used as well depending on the virus tropism. This interaction induces a gp41 conformational change leading to the fusion of viral and cellular membranes and in turn the entry of viral components inside the cell [95].

Once the virion is inside, the CA proteins covering the virus must be removed to release the inner material containing all the necessary elements to drive viral DNA synthesis, integration and future replication. The RT converts the single RNA into double strand DNA. This proviral DNA interacts with other host and viral proteins thus forming the Pre-Integration Complex (PIC) which facilitates the transport of viral components through nuclear pores. The RT enzyme does not have proof-reading activity, thus favoring the rise of diversity in viral genomes [96].

In the nucleus the virus takes a “one way” route, because the IN permits the integration of the viral DNA into the host DNA, which cannot be reverted.

This last step is the “bridge” from early to late stages of the viral cycle, which includes the transcription of viral RNA, the transduction of viral proteins, the assembly and budding of new virions.

The integrated virus can remain latent in a silent phase for an undetermined and extensive period. In the 5’ LTR regions there is an important non-coding DNA sequence enriched in thymine and adenine bases called TATA box, a consensus box for different host transcription factors, such as NF-kB, SP1, AP-1 and STAT-family, which are highly expressed and functional in stimulated cells of the immune system. In fact, different sets of cytokines could stimulate HIV gene expression in different immune cell populations. For example, IL-

2 and TNFα induce HIV-1 activation in infected T cells, while IL-1, IL-3, IL-6, IFNγ and GM-CSF activate viral expression in infected macrophages [97-99]. This is a crucial step in HIV replication, since the role played by different cytokines may pilot the HIV fate, helping or counteracting AIDS development.

Transcription is also regulated by two important viral proteins, tat and rev, which drive the transcription and synthesis of new viral proteins.

Once HIV-1 has all its material scattered in the cytosol, gag intervenes recruiting and facilitating the packaging of new virions. Myristic acid modifications of MA also facilitate gag association with the plasma membrane instead of the intracellular membranes. HIV-1 release is controlled by many viral and host factors, as such the interaction of gag with vacuolar protein sorting (VPS) proteins, important for creating new particles and releasing them extracellularly. These VPS proteins are the same as those involved in the MVB formation and other trafficking and budding processes. In the assembly stage, the ESCRT machinery and its accessory proteins (such as ALIX) are involved [100]. In the last stage the protease plays a key role by permitting the correct maturation of viral proteins, essential to produce infectious virions (**Figure 10**).

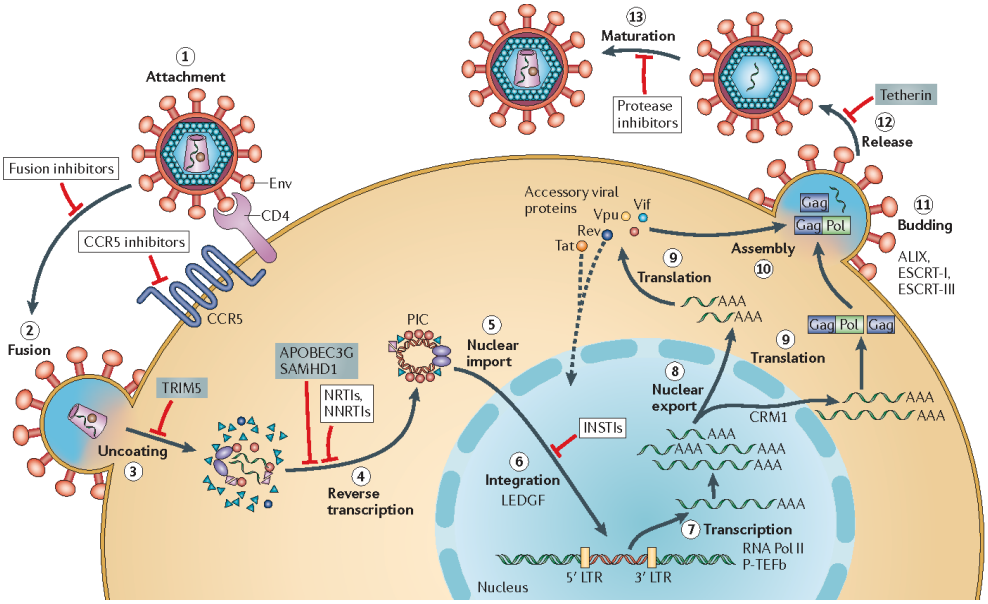

Figure 10: Schematic picture of HIV-1 replication cycle. Image from [101].

5.5 HIV-1 pathogenesis

Commonly HIV is transmitted through sexual intercourse (horizontal transmission), or through the sharing of infected needles among drug users.

Vertical transmission may also occur, when a mother transmits the virus to her children, but this is becoming more uncommon due to successful antiretroviral treatments. HIV pathogenesis, in the absence of antiretroviral treatment, is characterized by an acute phase, a chronic phase and AIDS development [82].

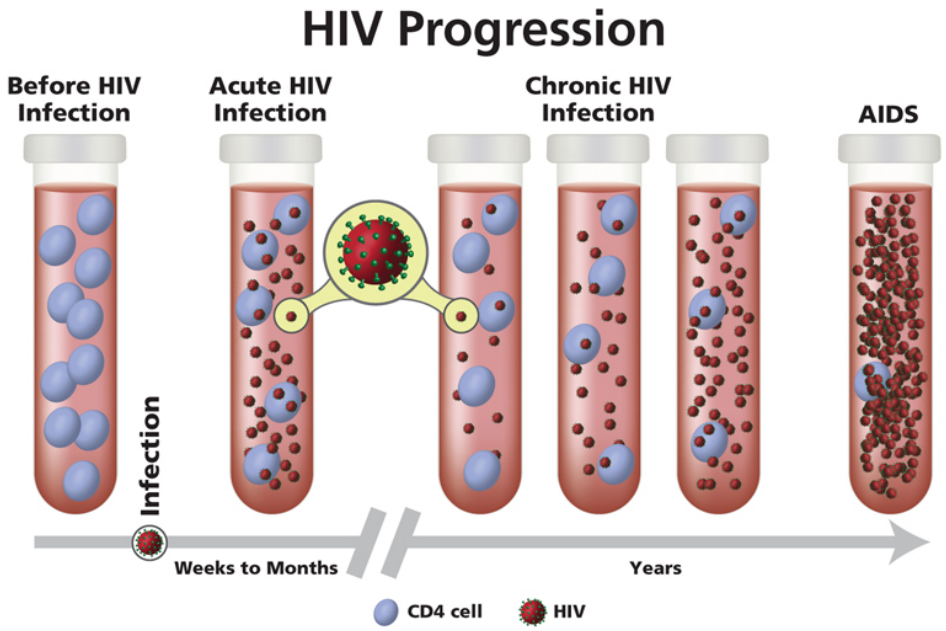

Figure 11: Simplified illustration of HIV-1 progression in infected individuals. Image from [79].

Acute phase is generally fast and brief (around two or four weeks), and during that time some infected individuals have a flu-like syndrome, characterized by fever and headaches. In this phase the virus is very active and infective, targeting as many CD4+ cells as possible. HIV-1 can penetrate the mucosal epithelia where it can interact with CD4+ cells in the mucosal lymphoid tissues, resulting in an important lymphocyte exhaustion (**Figure 11**).

In a second phase, HIV-1 slows down its replication, is less aggressive, and the infection enters an asymptomatic phase. Although the virus is less destructive, it continues to slowly kill immune cells, and if not treated, the infection may progress to AIDS in an average of ten years [102].

The last phase is known as full-blown AIDS. The immune system is highly compromised and can no longer control opportunistic infections of pathogens that usually are not a human threat. The diagnosis is CD4+ cell count based; once the number of cells is lower than 200 cells/mm^3 the patients enter AIDS stage. Of note, the response to HIV-1 infection as well as the progression of the disease are highly variable depending on different host (genetic, immune response), viral (replication ability) and environmental factors [82].

5.6 Immune response to HIV-1

The first response to HIV-1 infection is driven by the innate immune system, a mechanism common to other viruses. Pathogen-associated molecular patterns (PAMPs), well-conserved components shared among pathogens, are recognized by pattern recognition receptors (PRR). PRRs are able to identify bacterial carbohydrates, nucleic acids (both RNA and DNA of pathogens), specific peptides shared among microorganisms, lipids, glucans, and peptidoglycans. These receptors have different qualities and can be subdivided into different groups. In HIV infection, the most important are Toll-Like Receptors (TLRs) and Retinoic Acid-Inducible gene I (RIG-I) [103].

HIV-1 single strand RNA (ssRNA) is recognized by TLR-7 and -8 expressed on the surface of monocytes/macrophages and DCs, which trigger a cytokine storm. The main cytokine released soon after HIV-infection is IFNα, followed by the momentary release of IL-15, IL-18, IL-22, IL-10, IFNγ, TNFα, and CXCL10. IFNα is highly important because it is able to activate a cascade of signals and a set of genes

called “interferon stimulated genes” (ISG), which are involved in anti-HIV mechanisms. Furthermore, viral DNA could be identified by interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS), inducing a signal pathway ending with the production of restriction factors such as Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), Tripartite motif-containing protein 5 (TRIM5a), SAM domain and HD domain-containing protein 1 (SAMHD1) and Tetherin [104].

APOBEC3G is a restriction factor for HIV genome, resulting in replication inhibition, due to its cytidine deaminase activity. Vif can influence APOBEC3G fate, leading to its ubiquitination and then degradation [105].

TRIM5a is involved in the first stages of infection. After the virion entry, this protein facilitates the viral uncoating and proteasomal degradation of HIV-1 components [106].

SAMHD1, is a phospohydrolase able to transform nucleotides into nucleosides and inorganic phosphate, then depriving the RT of its “blocks” to convert viral RNA into cDNA thus preventing viral replication [107].

Tetherin, also known as CD317, is a transmembrane lipid raft-associated protein, mostly expressed in response to stimuli from the IFN pathway. This protein is able to tie virions, through the viral protein vpu, and inhibit their release [108].

5.7 HIV-1 acquired immune responses

HIV-1 triggers humoral and cell-mediated immune responses, although these are not enough to counteract and stop the infection. CD8+ T cytotoxic cells (CTLs) are the main protagonists to control HIV-1 infection, indeed specific CTLs for viral-peptides (mainly nef and env), after the interaction with these components, go through an expansion [109, 110].

These cells interact with the Major Histocompatibility Complex-1 (MHC-1) expressed on the surface of infected cells, which leads to production, release and activation of the perforin/granzyme axis. CTLs can also induce the activation of the Fas/Fas ligand pathway, leading to cell apoptosis [111, 112]. However, this response is typically ineffectual, since HIV-1 is highly mutable, allowing it to escape from the immune system response.

Also, the targeted cells (CD4+ T cells) may contribute to the fight against HIV-1; indeed, CD4+ T cells are specialized T helper cells able to increase CTL activity. Helper T cells can differentiate into different subsets, Th1, Th2, regulatory T cells (Tregs) and Th17, displaying different functions mediated by the production and release of different cytokines. Th1 cells help and sustain the CTL activation and differentiation into memory or differentiated cells, through the production of IFNγ, TNF α and IL-1. While, the humoral immune response is regulated by Th2 through IL-4, IL-5 and IL-13 secretion [113].

Treg cells, as suggested by their name, are regulatory lymphocytes that prevent exaggerated immune reactions that could

lead to autoimmune diseases or persistent inflammation, then losing immune system control. Their function is ambiguous, they could attenuate the immune responses, thus suppressing an immune activation state, or on the other hand they may inhibit antiviral responses, facilitating HIV-1 progression and spreading [114].

Humoral response intervenes within 6 to 9 weeks after HIV contact. Usually, the most immunogenic epitopes are from gp120 and gp41, the two envelope proteins which are most accessible for B cell recognition and expansion [115]. Other viral proteins such as p24, RT, gag and proteins of pol gene can trigger a humoral response, but by virtue of the high mutagenesis of the HIV-1 genome, these antibodies fail in controlling viral infection [116].

6. Antiretroviral therapy (ART)

Currently, a combination of antiretroviral therapy (cART) has proven to be efficient in governing viral replication, significantly dropping the risk of transmission and prolonging life expectancy in infected individuals, thus decreasing morbidity and mortality [117-119]. These antiretroviral compounds are able to exert their action by inhibiting different steps of HIV replication.

Zidovudine, also known as AZT, was the first anti-HIV drug approved by the FDA, commercialized and used since 1987 [120]. Since then, more than 25 antiviral drugs have been approved and differentially used. Because of the high variability of HIV genome and

its capacity to “change” and gain resistance to different drugs, a combination of drugs started to be used since 1996.

With the introduction of new compounds, their combination and interchangeability, HIV-1 infected people can live a “normal” life; what was a sentence of death, now is a “controlled” non-fatal chronic disease [121].

6.1 Antiretroviral classification

The HIV-1 cycle can be influenced and inhibited at different levels of its life cycle, and depending on where the drug acts, they can be divided into 7 different categories (**Figure 12**):

- **Non-nucleoside RT inhibitors (NNRTIs):** Molecules belonging to this group can bind and block the RT subunit of HIV, inhibiting its replication and spreading. They are divided into first and second-generation drugs and those currently approved and used include: Efavirez (EFV), Nevirapine (NVP), Etravirine (TMC-125) and Rilpivirine (TMC-278) [122].
- **Nucleotide RT inhibitors (NRTIs):** Similar to NNRTIs, NRTIs act on the HIV RT, but exploiting a different mechanism. They are able to compete with the host's nucleosides, influencing the nucleotide addition by RT during the DNA polymerization and therefore blocking the formation of new cDNA molecules. Since these drugs are modified nucleosides, they are very toxic as the host DNA polymerase can use them to synthetize host molecules. Some were first discovered for their anti-

cancerogenic activity, for instance AZT. Other current commercial drugs include: Lamivudine (3TC), Tenofovir (TDF), Abacavir (ABC), and Emtricitabine (FTC) [123].

- **Protease inhibitors (PIs):** PIs are able to block HIV-1 PR enzyme, essential for the correct maturation of gag polyprotein, resulting in the production of defective and un-infectious viral particles. Some of the FDA approved drugs are: Ritonavir (RTV), Darunavir (DRV), Fosamprenavir (FPV), Tipranavir (TPV), Indinavir (IDV), Nelfinavir (NFV), and Lopinavir (LPV) [124].
- **Fusion Inhibitors (FIs):** These drugs are able to contrast the HIV-1 fusion end viral entry to the recipient cell, by blocking gp120 and gp41. The most famous drug in this group is Enfuvirtide (T20) [125].
- **Chemokine receptor 5 (CCR5) antagonists:** Molecules belonging to this group are able to compete with HIV-1 entrance through the binding of one of its co-receptors. Drugs in this category are not extensively used, since the role of RANTES in HIV-1 pathogenesis needs further investigation and its indiscriminate use could lead to mutual selection of CXCR4 tropic strains. The most famous compound in this class is Maraviroc (MVC) [126].
- **Integrase inhibitors (INIs):** These compounds are very promising since they act directly on a viral protein, thus reducing the possible side effects. The drug is able to inhibit the integrase enzyme and consequently the viral integration in the host DNA, blocking the development of cells harboring virus. In

2019, a drug belonging to this group, Dolutegravir (DTG), was recommended as the first- and second-line treatment for all persons with HIV [127]. This drug is becoming a preferred treatment since no resistance by HIV has been recorded so far. Other important and well tolerated drugs are: Raltegravir (RAL) and Elvitegravir (EVG) [128].

- **Post-attachment Inhibitors:** This is a very recent group of drugs able to block the interaction of gp120 and the host co-receptor. In 2018 the first inhibitor was approved, Ibalizumab, a humanized monoclonal antibody (mAb) rather than a drug, which is able to block the post-binding conformation of gp120-CD4, resulting in a defective interaction with CCR5 or CXCR4 and thus preventing the viral entry and future replication. Ibalizumab, also known as Trogarzo, may be used when all the other drugs fail in controlling viral replication, due to HIV-1 multidrug resistance [129].

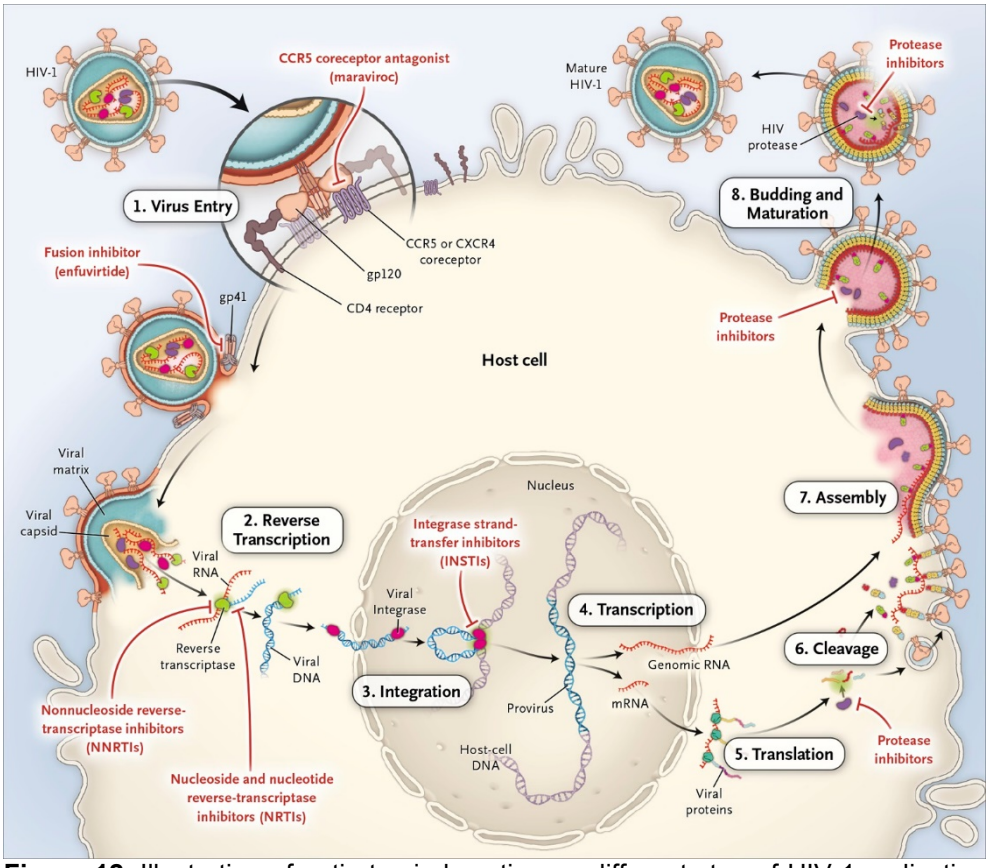

Figure 12: Illustration of antiretrovirals action on different step of HIV-1 replication cycle. Reproduced with permission from (Single-Pill Combination Regimens for Treatment of HIV-1 Infection. Monica Gandhi, Rajesh T. Gandhi. The New England Journal of Medicine), Copyright Massachusetts Medical Society. [130]

In clinical practice these drugs are not used as a standalone but rather in combination. This approach, also defined as Highly Active ART (HAART), often employs a mixture of compounds able to block the viral replication at different stages. This treatment allows a synergic effect of the drugs resulting in a more efficient and faster effect, and importantly reduced resistance formation. The most used combinations are: one NNRTI and two NRTIs compounds, or one PI and two NRTI [131].

The action of HAART is quite immediate lowering plasma viral copies to less than 50 copies per mL (in four to six months) and facilitating a slow recovery of CD4+ T cell numbers (around 100-150 cells/mm3 per year) [132]. Notwithstanding, the achievement of ART is not only drug and viral related, but it is also conditioned by environmental and individuals' genetic factors. Furthermore, the prolonged use of these drugs may entail severe side effects, which require interruption of the cure.

Finally, we have to take into consideration that although the viral load is almost undetectable following ART treatment, the virus is never completely eliminated from the body, and the so-called reservoirs can be reactivated following drug suspension or particular triggering conditions. Because of all these reasons the requirement of better regimens, less toxic molecules and possibly a vaccine able to prevent HIV-1-infection are still mandatory for the scientific community.

7. Immune activation despite ART

Immune activation is characterized by a persistent inflammation status, both in early and late phases of HIV-1 infection, resulting in upregulated serum levels of pro-inflammatory cytokines (IL, chemokines, IFNs), inflammatory mediators, coagulation components, and acute phase proteins. In the acute phase there is a so-called cytokine storm, thus the high secretion and production of IFNα, IL-15, IL-10, TNFα, IP-10, IFNγ, and IL-6 [133].

These cytokines are able to influence the T cell fate, activating them or facilitating their apoptosis, increasing the immune system deficiency. For instance, during these phases, activated macrophages and monocytes produce more IL-6 compared to a control group, which, in the SMART study, was associated with high mortality [134]. Also, several studies showed increased ISGs production during the acute phase, helping the counteraction against HIV-1.

Cytokines may play an important role in this residual immune activation [135]. In fact, in 2015 a study reported that in a big cohort of HIV-1-infected individuals, the Multicenter AIDS Cohort Study (MACS), after the first year under HAART, several inflammation biomarkers were not recovered, particularly markers of monocyte and macrophage activation were persistent [136]. These elements contribute to maintain a persistent state of immune activation and inflammation, representing an important therapeutic goal to be targeted in order to improve the state of infected individuals under ART.

The exact origin of this event is still not fully understood and different theories have been supposed and partially confirmed. Probably this activation state is not due to a single factor, but rather to a combination of different events that facilitate an inflammation status, such as an immune dysregulation (referred as homeostatic drive), residual non-detected viral replication, bacterial gut translocation, and the appearance of opportunistic co-infections [137] [138].

Surprisingly, even if the viral replication is inhibited by ART (undetectable viremia), the inflammatory environment persists; it may

decrease but it never recovers to a normal condition, suggesting that factors other than viral products are involved. Therefore, investigators started to look for other reasons that could explain the characteristic T cell depletion that occurs in HIV-1 infected ART-controlled patients. The main events considered include thymus depletion, non-efficacious hematopoiesis, an increase of fibrosis in lymphoid tissues, and immune activation. This was highly suggested from the Sousa study, showing a connection between CD4+ T cell decrease and immune activation in HIV-2 infection, whose disease is characterized by a controlled immune system activity for a long period and low viral replication [134]. A similar picture is also peculiar of a particular HIV-1-infected population, who naturally control viral replication without any ART auxilium, the so called long-term non-progressors (LTNP) and elite controllers [139, 140].

Increased immune activation could also be secondary to the presence of co-infections, such as herpes and hepatitis viral infections. For example, it has been reported that cytomegalovirus (CMV), or Human betaherpesvirus 5 (HHV-5), a virus that usually is not pathogenic in healthy controls, is reactivated following epithelial barrier dysfunction, thus inducing an IL-6 release, which, as previously stated, contributes to the chronic immune activation [141]. HHV-5 belongs to the herpesvirus family, and in immune compromised patients it could shed to different organs leading to Th cell proliferation and activation (**Figure 13**). Likewise, CMV shedding has been connected with an increased number of proviral HIV-1 DNA copies in the blood, and higher CD4+ T cell activation and proliferation [142].

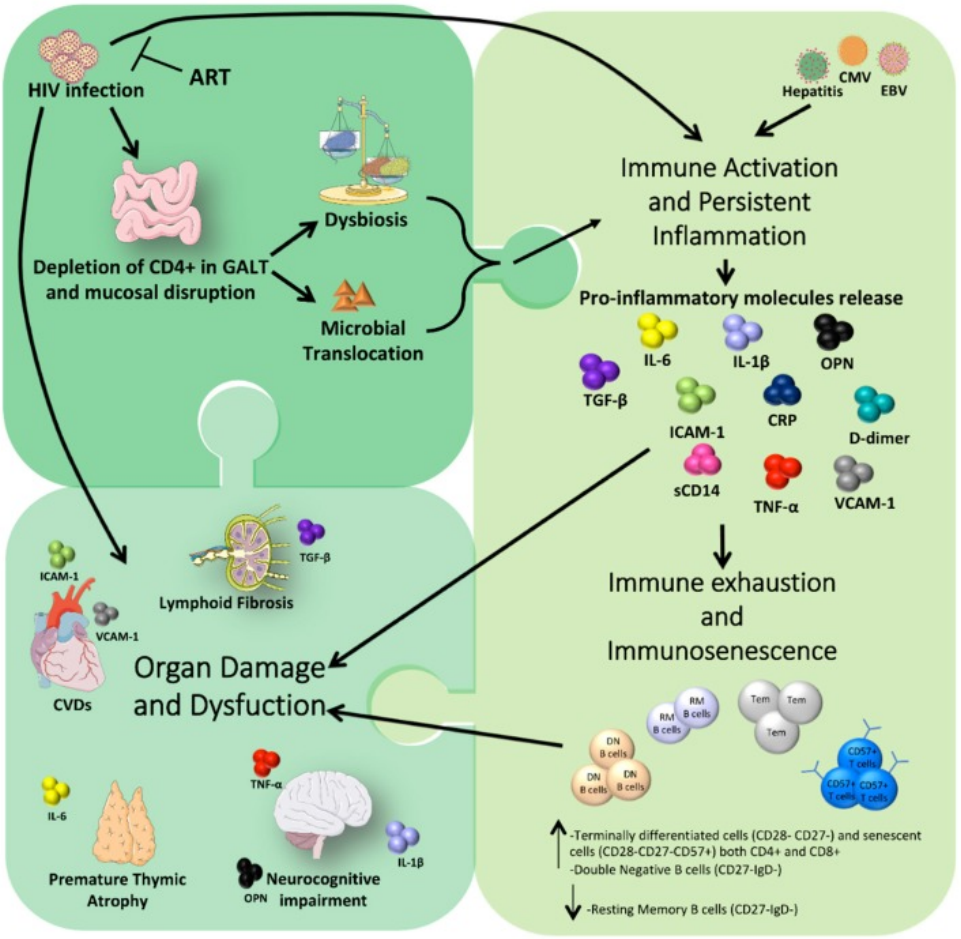

Figure 13: Different factors which may be involved in the immune activation triggered by HIV-1. Image from [143]

Immune activation is critical and needs to be controlled, since this high inflammation status is associated with an increase of age-related disease development, such as cardiovascular disease, neurocognitive dementia, kidney failure, but also autoimmune and cancer diseases [119, 144, 145]. In fact, the connection among inflammation and aging has been exhaustively reported in the

literature, as the production and secretion of pro inflammatory cytokines or coagulation factors were described to be highly implicated in these processes [146, 147].

In this context, it is mandatory to mention a particular pathological state called the immune reconstitution inflammatory syndrome (IRIS), which is characterized by an aberrant inflammatory response and a paradoxical worsening of previous viral infection immediately after the start of ART [148].

The recovery of CD4+ T cell count and functionality leads also to recovery in the immune system capacity to fight pathogens which have been hidden, increasing the inflammatory status. This peculiar condition may create an exaggerated reaction resulting in a tissue-destructive inflammation status. Furthermore, residual non-detected HIV replication due to HIV-1 reservoirs may be highly involved in the persistent inflammation status, since it also keeps the immune system activated [148, 149]. HIV-1 reservoir is the major reason for treatment failure and incapability to eradicate it. The current therapy is able to target only specific stages of HIV-1 replication but is not able to selectively kill the infected cells and is not able to inactivate the viral integrated genome.

Importantly, Gianella described a positive correlation between proviral DNA copies and EBV replication. This may suggest that herpesviruses are implicated in the activation of latent HIV-1 reservoirs. Future therapeutic approaches against herpesviruses could be helpful in fighting the residual viral replication [142]. The presence of viral reservoirs and the residual replication of HIV-1 have

been reported to mostly affect the body areas with sub-optimal drug access or a high dysregulation of the immune system, such as lymphoid tissues, central nervous system (CNS), gastrointestinal (GI) tracts, and genital tracts [150-153]. In detail, a higher HIV-1 residual replication has been described to be mostly present in lymph nodes, where there is a higher presence of HIV-1 replication per cell and lower drug access [154]. In summary, HIV-1 sub-optimal replication and inflammation status are inter-dependent processes. On one side, a persistent state of inflammation favors CD4+ cell activation and proliferation, which could provide new recipient cells for HIV-1 replication maintenance. On the other hand, the presence of HIV-1 reservoir and low replication maintains an “alert” state in the immune system, leading to persistent immune activation.

The lymph node is the perfect organ for HIV-1 infection and replication as its architecture facilitates cell-to-cell interaction (among infected cells and new target cells), because of the high number of resident and circulating CD4+ cells, a pro-inflammatory setting and also low drug infiltration [155, 156]. Recent data demonstrated that an early start of ART is the best procedure in counteracting HIV-1 replication and the diffusion of viral reservoir [157]. Nevertheless, ART itself is not enough to eradicate viral replication and immune activation. The investigators should focus more attention on finding the connection between viral latency and this persistent immune activation status, to provide new strategies to defeat HIV-1 infection in the future.

8. Human *ex vivo* lymphoid tissue model

To better understand HIV-1 pathogenesis, its latency, and its involvement in immune activation, conventional in vitro cultures are very limited models [158]. These include experiments on cell line cultures (such as THP-1 and U-937, monocyte cell lines, or Jurkat and MT4, T cell lines), or peripheral blood mononuclear cells (PBMCs), which are very practical and convenient models for HIV-1 exploration, but show some limitations, since they are not able to reproduce the complexity of an organ, the involvement of several cell types, the original tissue architecture and finally the cell-to-cell interactions [159].

Grivel and Margolis advanced and adjusted new human *ex vivo* tissue models to study HIV-1 infection phenomena as well as other infectious diseases [160-164]. Briefly, this system consists of culturing dissected fresh human tissue blocks ($2mm^3$) on a collagen foam structure (Gelfoam sponges) at the air-liquid interface, allowing them to stay immobilized in a specific zone and also to acquire all the nutrients necessary to survive from the culture media. These *ex vivo* tissues maintain important cell to cell interactions and support viral infection without artificial stimulation [165]. They established *ex vivo* models of HIV-1 infection in different human tissues, including lymphoid tonsillar tissues, cervico-vaginal tissues (endo and ecto-cervix), and rectosigmoid tissues.

In comparison to *in vitro* models that need an exogenous stimulus such as IL-2 and phytohemagglutinin (PHA), which may change cellular surface receptors and functionality, the *ex vivo* models are able to support productive HIV-1 infection and replication without

any external activator. Furthermore, and most important, *ex vivo* system is able to maintain the tissue cytoarchitecture, imperative not only for the cell-to-cell interaction but also for maintaining the characteristics of the *in vivo* cell network among lymphocyte subtypes and follicular-dendritic cells. Tissue functionality is still preserved after such cultivation including the ability to release cytokines similar to the ones released *in vivo*, and the expression patterns of key cell-surface molecules relevant to HIV-1 infection such as HIV-1 co-receptors, for over two weeks.

This *ex vivo* lymphoid tissue model has been widely used for studies on HIV-1 pathogenesis, including studies on HIV-1 immune activation, co-pathogen infections and preclinical evaluations of potential antivirals [160-164, 166-168]. Importantly, the *ex vivo* system has been reported to be successfully infected by different herpesviruses such as Human simplex virus-2 (HSV-2), HHV-6, HHV-7, HCMV, and also by vaccinia and measles viruses [166, 168-170].

Conversely, it is also true that the system has some limits in the study of infectious pathogenesis, as the tissues decline after two to three weeks in culture, and *ex vivo* cultures cannot reflect the complexity of *in vivo* systemic aspects. The model also is affected by donor-to-donor variability, and the handling skills of the operators. The procedure itself is laborious and needs experienced staff, and many blocks of tissues are essential to overcome tissue heterogeneity.

In summary, human *ex vivo* cultured tissue systems are important and essential tools in the study of normal and pathological conditions, above all in the case of infectious processes including but

not limited to HIV-1 pathogenesis, under controlled laboratory conditions.

AIM OF THE STUDY

My Ph.D. project was focused on the depiction of the immune response triggered by HIV-1 infection in human tissues. In particular, we investigated the cellular and molecular mechanisms responsible for persistent immune activation in HIV-1-infected ART-treated patients, despite HIV-1 viral suppression.

To this end we cultured human lymphoid tissues *ex vivo*, to analyse the spectrum of cytokines released by these tissues upon infection with various HIV-1 strains. Such analyses are basically important to clarify the failure of human immune system in controlling HIV-infection.

Furthermore, we focused on the identification of extracellular vesicles (EVs) that carry various cytokines and are released by the infected tissues. We investigated whether suppression of HIV-1 replication, by commonly used antivirals, is able to restore cytokines, which are upregulated due to infection, back to normal levels. Indeed, such alteration is significantly correlated to the onset of early diseases in treated patients with undetectable viremia.

Specific goals:

- To explore the role played by cytokines, both in soluble and EV-associated form, during HIV-1 infection in an *ex vivo* model of infection.
- To elucidate and characterize the difference between early and late infection in cytokine production.

- To decipher the role played by EV-associated cytokines, and understand if their release and compartmentalization is influenced by an infectious disease as HIV-1 infection.
- To test the efficiency of different antiretroviral drugs in viral suppression, and determine if suppression leads to restored cytokine levels, both in soluble and in EV-associated form.
- To elucidate if an initial cytokine storm or immune stimulus is involved in the cytokine balance setpoint, that could trigger a prolonged inflammatory status.
- To understand if the HIV-1 virion itself, without actually infecting and starting a replication cycle, is enough to trigger immune activation.
- To evaluate the presence of co-infections such as human cytomegalovirus or other herpesviruses, which may contribute to long-term immune activation and sustained high levels of pro-inflammatory cytokines.

MATERIALS AND METHODS

1. Sample preparation, treatment and storage

Tonsillar tissues were obtained from routine tonsillectomies performed at the Children's National Medical Center in Washington DC. All tissues were anonymous pathological samples obtained according to an Institutional Review Board approved protocol. Healthy tonsil tissues were dissected as previously described [165]. Briefly, tissues were separated from damaged, cauterized and bloody parts, then they were dissected into small strips, which were finally dissected into 2 × 2 × 2 mm blocks and cultured on collagen sponges (GelFoam) at the air-liquid interface (**Figure 14**).

Nine 2-mm^3 blocks per gel foam in 3ml medium were used, with a minimum of 18 blocks per condition, in RPMI 1640 (Thermo Fisher) supplemented with 15% FBS (Gemini Bioproducts), 100 µM Modified Eagle's medium (MEM)-nonessential amino acids, 1 mM sodium pyruvate, 50 µg/ml gentamicin, 2.5 µg/ml Amphotericin B (all from Thermo Fisher). Medium was collected and changed at day 3, 6, 9, 12 and 16. Collected medium was centrifuged at 2000 × g for 10 minutes to remove cells and aliquots were frozen at −80 °C.

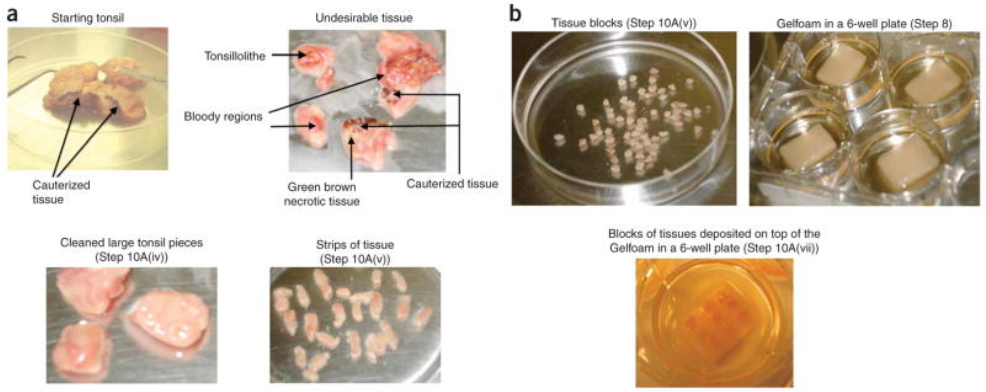

Figure 14: Tonsil tissue dissection. a) tissues were cleaned of cauterized and undesirable parts and then dissected into small strips; b) small strips were then dissected into 2 × 2 mm blocks and 9 blocks per well were cultured on collagen sponges at the air-liquid interface. Image from [160].

1.1 HIV-1 infection and antiretroviral treatment

Tonsils were infected with two HIV-1 strains, $X4_{LAI04}$ [$3.5x10^5$pg/mL] or $R5_{SF162}$ [$1.6x10^5$pg/mL]. Immediately after thawing the HIV-1 viral stock at 37 °C, the inoculum was pipetted directly on each tonsillar tissue block, 5μL for the $X4_{LAI04}$, and 7.5μL for the $R5_{SF162}$.

In the first set of experiments, HIV-1 was allowed to replicate in tissue culture for 16 days, or tissues were treated with ritonavir, a protease inhibitor, at a final concentration of 5μM (n=8). Ritonavir treatment was performed at day 3 following HIV inoculation and subsequently at every medium change (day 3, 6, 10, 13 and 16) until the end of culture.

In a second set of experiments, HIV-1 was allowed to replicate for 16 days, or tissues were treated with two nucleotide retro transcriptase inhibitors, AZT (zidovudine) and 3TC (lamivudine), both

at a final concentration of 5μM (n=8). The AZT-3TC treatment was started 2 days post HIV inoculation and subsequently at every medium change (day 2, 5, 8, 12 and 15) until the end of culture.

1.2 Simulation of cytokine storm

To further test if the initial immune stimulation could persist throughout the culture length, we treated lymphoid tissue with cytokines either singly or in combination.

Tonsils were treated at day 0 with cytokines which were upregulated after viral infection: IFNγ (1000pg/mL), RANTES (1000pg/mL), TNFα (100pg/mL), or with a mixture of these cytokines plus IL-2 [25pg/mL], IL-7 [100pg/mL], MIP-1a [500pg/mL] and MIP-1b [500pg/mL] (n=4). In all the experimental conditions, molecules were added only at day 0 and medium was collected and replaced every 3 days (day 3, 6, 9, 12).

1.3 Inactivated virus treatment

To test if the virion itself, without replicating and infecting any cell, is able to trigger an immunoactivated status that may persist throughout the culture length, we treated tonsil tissues with inactivated HIV-1 virions. In detail, the virus is the same $X4_{LAI04}$ strain treated with aldrithiol-2 (AT-2), which is able to change the zinc finger nucleocapsid proteins of HIV-1, therefore deactivating the viral infectivity. After modification, virions retain their surface conformation and functionality, indeed these non-active viral particles are able to bind

CD4+ T cells and mediate a viral stimulation similar to native virions. However, several studies demonstrated that the viral life cycle is interrupted immediately before reverse transcription activation [171].

Tonsils were infected with either 5µL HIV-1 $X4_{LAI04}$ or $X4_{LAI04}$ AT-2 inactivated virions and HIV was allowed to replicate for 12 days. In separate experiments, tonsils were treated with $X4_{LAI04}$ AT-2 inactivated virions at day 0 and subsequently at every medium change (day 3, 6, 10, and 13) until the end of culture (n=2).

2. Evaluation of HIV-1 replication

We evaluated HIV-1 replication in tissue culture supernatants by measurement of HIV-1 $p24_{gag}$ antigen, using a dynamic immunofluorescent cytometric bead assay, as described previously [172].

In detail, MagPlex carboxymethylated microspheres (Luminex Corporation, Austin, TX) were coupled with anti-p24 monoclonal antibody (ImmunoDiagnostics, Inc., Woburn, MA). Microspheres were pelleted at 8000×g for 2 min (12.5×10^6) and resuspended in 160µl of 0.1M monobasic sodium phosphate pH 6.2, and after were activated by adding 20µl of freshly prepared 50 mg ml^{-1} solution of sulfo-N-hydroxysuccinimide (Pierce, Rockford, IL) in dH2O, and 20µl of 50 mg ml−1 solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) in water, and incubated for 20 min. Beads were washed two times in PBS and at the third centrifugation they were resuspended in 250µl of PBS. The beads were mixed with capture

antibody to a final 1mL volume, next the mixture was incubated for 2h at RT with gentle mixing. After the incubation beads were washed with PBS 1ml of PBS, 0.1% BSA, 0.02% Tween-20, 0.05% azide, pH 7.4 (PBS–TBN) and resuspended in a volume of 1 ml of PBS–TBN. Beads count was performed by measuring on an automatic hemacytometer (Cellometer AutoM10, Nexcelom Bioscience, Lawrence, MA). The beads were stored at 4°C until their use.

For the assay, all dilutions were made in a buffer of PBS–TBN supplemented with 0.1% goat serum and 0.1% mouse serum. 45µl of sample were lysed by the addition of 5µl of 10% TritonX100 and incubated at 37°C for 1h. Next, 50µl of coupled beads were added in each well in 96 well plates at the concentration of $1x10^3$/mL and then the plates were incubated for 2h at RT. Then, plates were washed with 200µl of PBS–0.02% Tween-20 on a magnetic plate washer (Biotek). 50µl of the RD1-labeled anti-p24 KC57 antibody (Beckman Coulter, Miami, FL) at final concentration of 0.5µg ml^{-1} were added to plates and incubated 1h at RT. Plates were washed and 85µl buffer were added to all wells. Analysis was performed on a Luminex 200 (Bio-Rad, Hercules, CA) acquiring 100 microspheres/well.

3. EVs isolation and characterization

3.1 Preparation of EV fractions

Culture supernatant samples were split into multiple fractions. One aliquot was untreated while another one was treated with ExoQuick TC (SBI) according to manufacturer's protocols. Briefly,

ExoQuick was added to supernatant at a ratio of 40µl of ExoQuick to 200µl supernatant and refrigerated overnight at 4°C. ExoQuick/sample mixtures were centrifuged at 1500 × g for 30 minutes to pellet EVs. Supernatant was collected and saved for cytokine measurement of EV-free supernatant. The pellet was centrifuged again at 1500 × g for 5 minutes and all traces of fluid were removed resulting in an EV enriched preparation. The pellet was resuspended in 180µl 1X PBS and used for cytokine measurement on intact and lysed EVs. Fractions were stored at 4°C and run within 24 hours.

3.2 NanoSight measurement of EVs

Untreated aliquots of samples were diluted and characterized with Nanoparticle Tracking Analysis software using a NanoSight NS300 (Malvern), which uses light scattering and Brownian motion to obtain particle size distributions and concentrations. Measurements were collected from all culture conditions at day 10 from 5 experiments. Briefly, samples were diluted 1:200 in particle-free PBS and analyzed using the following setting: screen gain 10.0, detection threshold 7, infusion rate speed 25. Samples were analyzed by 2 video captures of 60 seconds each to generate averaged concentrations of EV/ml ± SEM and mean ± SEM particle size (nm).

3.3 EV markers characterization.

3.3.1 Protein Extraction

1mL of supernatant for each condition was used to isolate the EVs ExoQuick TC (SBI) procedure and the final pellet was resuspended directly in 200µL of RIPA buffer with a protease inhibitor cocktail (Thermo Scientific Halt Protease Inhibitor Cocktail). The tubes were vortexed and placed in ice for 30 minutes, with cycles of vortex every 5 minutes. Tubes were centrifuged at 16'000g for 20 minutes at 4°C to pellet the unwanted cell debris. The supernatant was recovered and stored at -20°C until required.

3.3.2 BCA protein quantification

To quantify extracted proteins, 10µl for each condition were loaded into a 96 well plate. 200µl of working reagent, prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B, were added to each well. The plate was incubated at 37 °C for 30 min before performing the absorbance readings at 562 nm on a plate reader (Tecan).

3.3.3 Western Blot

EV markers were detected by Western Blot, to test the effective presence of EVs after ExoQuick isolation. We tested culture supernatants at day 3 of pooled control (CTRL), $X4_{LAI04}$ and $R5_{SF162}$ conditions. 10 µg of proteins were loaded on a 4–20% precast polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and

separated by SDS-PAGE, then transferred to PVDF membranes. The membranes were blocked with 5% milk and then washed and probed for 2h with anti-CD63 (2 µg/ml), anti-Rab27A (0.2 µg/ml), anti-TSG101 (0.2 µg/ml), and anti-Calnexin (0.2 µg/ml) (Thermo Fisher Scientific, Waltham, MA). The membranes were washed and incubated for 45min with goat peroxidase-conjugated anti-mouse IgG secondary antibody at 1:3000 dilutions (Bio-Rad). Peroxidase activity and digital images were detected by using V3 Western Workflow™ (Bio-Rad).

4. Cytokine measurement

We previously developed an in-house multiplexed bead-based assay for measurement of the following 33 cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-18, IL-21, IL-22, IL-33, Calgranulin A (Calg A or S100A8), Eotaxin (CCL11), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulated alpha (GRO-α or CXCL1), interferon-γ (IFN-γ), interferon-γ-induced protein (IP-10 or CXCL10), interferon-inducible T-cell alpha chemoattractant (ITAC or CXCL11), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1 or CCL2), monokine induced by IFN-γ (MIG or CXCL9), macrophage inflammatory protein-1α (MIP-1α or CCL3), MIP-1β (CCL4), MIP-3α (CCL20), regulated on activation normally T-cell expressed and secreted (RANTES or CCL5), transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) as described previously [28, 173].

All antibody pairs and cytokine standards were purchased from R&D Systems except those for IL-4 (Biolegend) and IL-21 (eBioscience). Individual magnetic Luminex bead sets with 33 distinct spectral signatures (regions) were coupled to cytokine specific capture antibodies according to manufacturer's recommendations and stored at 4 °C (as described in p24 microsphere coupling). All cytokine pairs were verified to be free of cross reactivity. Standards and samples were diluted in assay buffer (1X PBS with 20 mM Tris-HCl, 1% each normal mouse and goat serum (Gemini Bioproducts) and 0.05% Tween 20) and combined with bead mixtures and incubated overnight at 4°C. Intact EV samples and lysed EV samples, to which Triton X was added at final concentration of 1%, were run in separate wells, and separate standard curves were also set up in 1% Triton X. Plates were washed 2 times and incubated with mixtures of polyclonal biotinylated anti-cytokine antibodies (R&D Systems) in assay buffer for 1 hour at room temperature. Plates were washed three times and incubated for 30 minutes with 16 µg/ml streptavidin-phycoerythrin (Thermo Fisher) in PBS. Plates were washed 2 times and beads were resuspended in PBS. Plates were read on a Luminex 200 analyzer with acquisition of a minimum of 100 beads for each region and analyzed using Bioplex Manager software (BioRad). Cytokine concentrations were determined using 5P regression algorithms. Concentrations of analytes in EV free supernatants were adjusted for dilution by ExoQuick reagent.

4.1 Statistical analysis of cytokine measurements

Results from all timepoints were analyzed by pairwise comparison with Wilcoxon signed-rank test with Benjamini-Hochberg correction for multiple comparison between different pairs of treatments in a day-to-day manner. Values of $p < 0.05$ were considered statistically significant; graphs are presented with p-values in log10-scale. Results analyzed for single timepoints are represented as means ± SEM, and statistical significances were determined by two-tailed paired Student's t-test using Microsoft Excel version 16.15.

5. Evaluation of endogenous herpesviruses replication

To evaluate the presence of endogenous co-infections and to verify their involvement in immune activation during HIV-1 infection [167], we extracted DNA from uninfected or HIV-1 infected tonsillar tissues treated or not with ritonavir.

5.1 Qiagen DNA extraction

For each condition and for 3 different time points (day 0, 6 and 12), 2 tissue blocks were collected and stored with RNAlater (Invitrogen), an aqueous stabilization solution that rapidly permeates tissues to stabilize and protect cellular nucleic acids. The tissues in RNAlater were stored at -20°C until required.

DNA isolation from tissue was performed by QIAamp DNA Mini kit (Qiagen). To disrupt the blocks tissue, 2 blocks for each condition were mixed with 180µl of Buffer ATL and 20uL of proteinase K, and then incubated for 3h at 56°C (vortexing every 30 minutes). Next, 200µl of Buffer AL were added to the samples and incubated for 10 min at 70°C. After the incubation 200µl of absolute ethanol were added to each tube. Then, these mixtures were transferred to QIAamp Mini spin columns (in a 2 ml collection tube) and centrifuged at 6000g for 1 min. The spin columns were then transferred to new 2 ml collection tubes and 500µl of Buffer AW1 were added to each tube and centrifuged at 6000g for 1 min. The spin columns were placed again in new 2 ml collection tubes and 500µl of Buffer AW2 were added and the tubes were centrifuged at 20'000g for 3 min. Finally the spin columns were placed in sterile DNase/RNase free 1.5 ml microcentrifuge tube, and to each column 100µl of Buffer AE were added, then they were incubated at room temperature for 2 min and centrifuged at 6000g for 1 min (this step was repeated twice for optimize the DNA elution, resulting in a final volume of 200µl).

5.2 Droplet digital PCR (ddPCR)

To quantify the DNA copies of different Herpesviruses we took advantage of the new droplet digital PCR system (BioRad), which allowed us to detect precisely very low copies of DNA inside a sample. Annealing and extension set up were optimized for each primer/probe. We quantified DNA copies of different Herpesviruses: HSV-2, HHV-3, HHV-4 (or EBV), HHV-5 (or CMV), HHV-6 and HHV-7. Ribonuclease

P protein subunit p30 (RPP30), was used as Housekeeping DNA to normalize for cell distribution (**Table 1**). The primer-probes were conjugated with HEX (6-carboxy-2,4,4,5,7,7 hexachlorofluorescein succinimidyl ester) and black hole quencher 1 (BHQ1) or with 6-carboxyfluorescein (FAM) and Tetramethylrhodamine quencher (TAM) (Sigma-Aldrich, Saint Louis, MO).

The ddPCR reaction mixture consisted of 11µl of a 2x ddPCR supermix for probes (Bio-Rad), 1.10 µl of each 20× primer-probe mix, and 5µl of template DNA and 5µl of DEPC water in a final volume of 22.1µl for each well. The plate was then loaded with the samples and placed in the droplet generator (Bio-Rad), which mixed 20µl of the reaction with 20µl of droplet generation oil (Bio-Rad), allowing the creation of droplets. The plate was then transferred into a 2720 Thermal Cycler (Applied Biosystems) for PCR amplification, which was performed with the following settings: 94°C for 10 min (polymerase activation), 40 cycles of 94°C for 10 min and an annealing/extension T of 57.1°C for 1 min (for HHV-3, HHV-4, CMB UL-55, and HHV-6 primers/probes) or 59°C (for RPP-30, HSV-2, AND HHV-7 primers/probes), followed by 98°C for 10 min (deactivation step) and ending at 4°C. A droplet reader (Bio-Rad) was used to count every single generated droplet and to discriminate them into negative (droplets without any DNA copy amplified) and positive (droplets with the target DNA amplified and emanating fluorescence) ones. Data obtained were analyzed by QuantaSoft™ Analysis Pro (version 1.0.596) software and quantitation of target molecules was presented as copies/µl of PCR, which was then calculated to reflect copies per 10^6 cells.

PRIMER/PROBE SEQUENCES.

	Forward sequence	Reverse sequence	Probe Sequence
RPP30-HEX	GATTTGGACCTGCGAGCG	GCGGCTGTCTCCACAAGT	TTCTGACCTGAAGGCTCTGCGC
HSV-2 UL27-FAM	CGCATCAAGACCACCTCCTC	GCTCGCACCACGCGA	CGGCGATGCGCCCCAG
HHV-3 ORF62-FAM	CGGCATGGCCCGTCTAT	TCGCGTGCTGCGGC	ATTCAGCAATGGAAACACACGACGC
HHV-4 LMP2A-FAM	AACGATGAGGAACGTGAAT	AGTCATCCCGTGGAGAGTA	AGAGCCCCCACCGCCTTA
CMV UL55-HEX	TGGGCGAGGACAACGAA	TGAGGCTGGGAAGCTGACAT	TGGGCAACCACCGCACTGAGG
HHV-6 U67-FAM	CGCTAGGTTGAGGATGATCGA	CAAAGCCAAATTATCCAGAGCG	CCCGAAGGAATAACGCTC
HHV -7 U67-FAM	AGCGGTACCTGTAAAATCATCCA	AACAGAAACGCCACCTCGAT	GAGAACATCGCTCTAACTGGATCA

Table 1: Forward and reverse primers, and probes for detection by ddPCR of RPP30, HSV-2, HHV-3, HHV-4, CMV (HHV-5), HHV-6, HHV-7. RPP-30 and CMV probes were conjugated with HEX, all the other probes with FAM.

RESULTS

1. HIV-1 replication

Tonsillar explants (n=8) were infected with HIV-1 strains, $X4_{LAI04}$ or $R5_{SF162}$, at the beginning of culture as previously described [161, 163]. Between days 6 to 16 of culture, median p24 concentration in culture supernatants from $X4_{LAI04}$-infected tissues was 416.92 ± 168.55 ng/ml and 340.57 ± 92.64 ng/ml in $R5_{SF162}$-infected tissues. Following ritonavir treatment p24 concentration significantly decreased in both $X4_{LAI04}$ infected tissues (1.43 ± 0.92 ng/ml; 99.66% inhibition) and in $R5_{SF162}$ infected tissues (0.87 ± 0.32 ng/ml; 99.74% inhibition). These results demonstrate that tonsillar explants are permissive to HIV-1 infection, and treatment with ritonavir is able to inhibit HIV replication ($p<0.05$) (**Figure 15**).

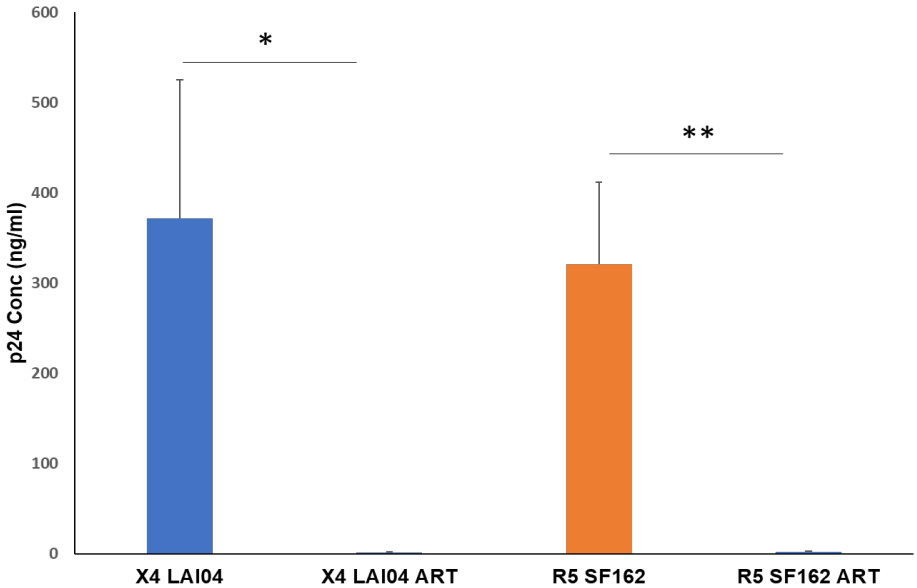

Figure 15: Cumulative p24 production by HIV-1-infected human lymphoid tissues *ex vivo.* Tissue blocks were inoculated with $X4_{LAI04}$ or $R5_{SF162}$ and incubated for 16 days. Matched tissue blocks were treated with ritonavir (5μM) at day 3 and ritonavir was added with every medium change through day 16. HIV-1 replication as evaluated by p24 measurement with a Luminex assay for each timepoint and cumulative total is shown (Mean ± SEM, n=8).

HIV-1-infected tissues (n=8) were treated with AZT-3TC resulting in almost complete suppression of infection as well: infected AZT-3TC treated tissues released 0.28 ± 0.05 ng/ml with $X4_{LAI04}$ infection, and 0.23 ± 0.06 ng/ml in $R5_{SF162}$ infection, resulting in an inhibition of 99.77% and 99.65% respectively.

2. EV characterization

2.1 NanoSight analysis of EVs in *ex vivo* lymphoid tissues

We next quantified EVs released by *ex vivo* infected tissues with/without ritonavir to determine if tissues respond to any of these conditions with fluctuations in EV release. EVs were quantified at day 9 to allow time either for HIV to replicate well or for ART to be effective. Tonsils released on average 3.14 ± 0.35 x 10^{10} EVs/ml with an average size of 160 ± 5.75 nm. We found no significant differences between the six conditions, suggesting that HIV-1 infection and treatment do not affect EV release and size (**Table 2, Figure 16**).

NanoSight determination of vesicle concentration and size

	Concentration (vesicles/ml)	**Particle Size (nm)**
Control	2.7 ± 0.25 x 10^{10}	160.8 ± 6.78
Control + RTV	3.51 ± 0.56 x 10^{10}	159.2 ± 7.66
$X4_{LAI04}$	3.11 ± 0.34 x 10^{10}	163.0 ± 5.35
$X4_{LAI04}$ + RTV	3.48 ± 0.47 x 10^{10}	160.4 ± 5.45
$R5_{SF162}$	2.87 ± 0.07 x 10^{10}	155.6 ± 6.43
$R5_{SF162}$ + RTV	3.17 ± 0.31 x 10^{10}	163.4 ± 5.38

Table 2: Culture supernatants were analyzed by NanoSight NS300. Presented are data from five representative tissues at day 9 (accumulation of EVs over days 6-9) for each condition. Mean ± SEM. No significant differences were observed between various conditions.

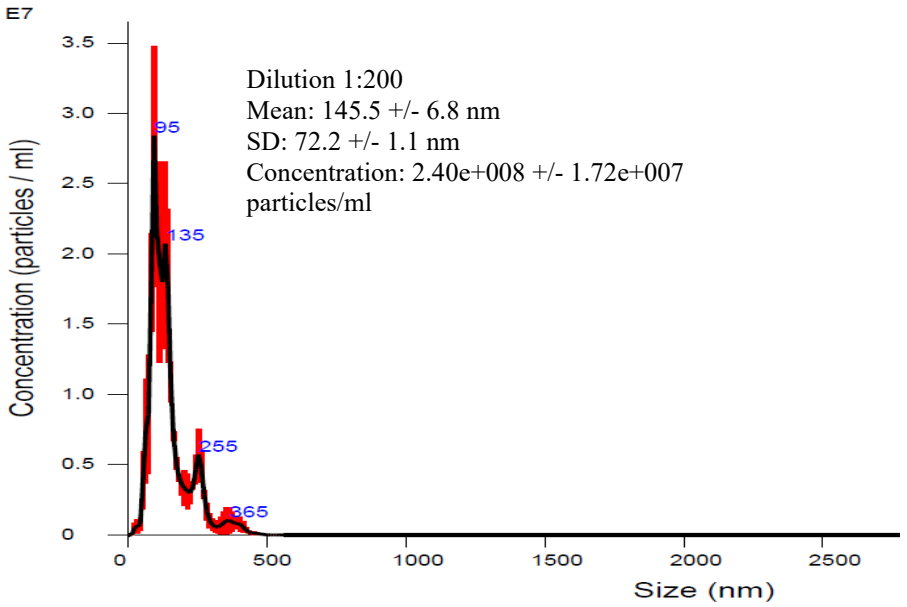

Figure 16: NanoSight histogram of EVs isolated from culture supernatants. EVs from all conditions at day 9 were characterized by NanoSight. Shown is representative histogram of sizing and concentration of EVs isolated from culture supernatants.

2.2 Detection of EV markers by western blot

To further confirm the presence of vesicles after ExoQuick isolation, we tested three different positive EV markers.

In all the conditions, the proteins extracted from ExoQuick isolated EVs showed the presence of CD63, Rab27A and TSG101. These findings confirm the efficient isolation of EVs in our procedures. Calnexin, a negative marker for exosomes, was also identified, but it could be associated to other microvesicles/apoptotic bodies in our preparations (**Figure 17**).

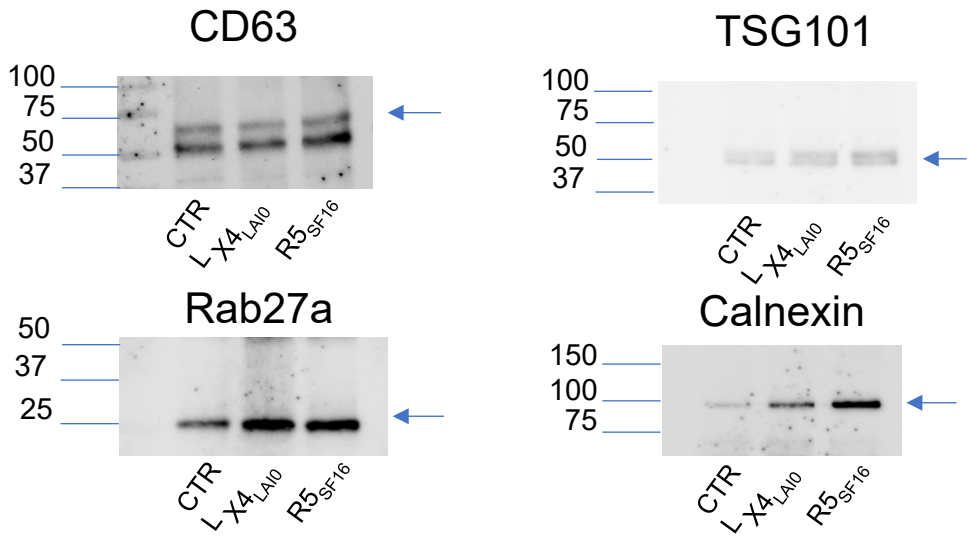

Figure 17: EVs isolated from culture supernatants express typical EV markers. EVs from control (CTRL), $X4_{LAI04}$ - and $R5_{SF162}$ -infected matched tissues at day 3 post-infection were characterized by Western blot. Analysis confirmed the presence of CD63, Rab27A, and TSG101. Calnexin expression, a marker negative for exosomes, was observed as it can be associated with microvesicles/apoptotic bodies.

3. Cytokine release by *ex vivo* lymphoid tissues

In order to evaluate immune activation induced by *ex vivo* HIV-1 infection, we measured the concentration of 33 cytokines that were released into the culture media of HIV-1-infected and control tonsils.

In order to distinguish cytokines that were soluble or EV-associated, we treated tissue culture supernatants with ExoQuick TC to sediment EVs. Cytokine measurements were made on the supernatant (without EVs), and on the EV fraction both intact (surface cytokine) and lysed (surface cytokines + internal cytokine).

Cytokines were analysed at different time points: 1) at the first time point and medium change (day 3: early infection); 2) at the last

time point (day 16: late infection) and 3) the cumulative cytokine production (the sum of cytokines released throughout the culture length: day 3, 6, 9, 12, 16). Results are based on data from the experiments using ritonavir treatment, unless otherwise noted. AZT-3TC treatment was used to confirm these initial results with ritonavir.

Both soluble (**Table 3**) and EV-associated cytokines (**Table 4**) were released in different concentrations. The following tables represent the cumulative production for each cytokine in the six different conditions.

	Control	Control + RTV	$X4_{LAI}$	$X4_{LAI}$ + RTV	$R5_{SF162}$	$R5_{SF162}$ + RTV
IL-1α	1361.82±198.12	1093.99±145.49	1492.19±194.7	1335.47±145.73	1170.54±177.11	1191.77±131.66
IL-1β	76.32±11.72	68.09±9.54	86.91±15.38	80.51±13.98	86.29±14.71	74.67±12.13
IL-2	20.96±3.25	18.62±3.5	39.95±6.5	36.56±6.05	26.68±4.57	22.94±6.02
IL-4	10.63±8.58	7.89±6.69	8.45±5.18	6.25±1.57	1.22±0.64	1.51±0.85
IL-6	463754.29±76438.6	443009.23±73731.4	525812.97±65573.63	566358.67±89171.92	379324.68±68350.78	412441.53±78761.23
IL-7	69.8±16.9	88.1±26.79	149.26±40.45	143.12±44	116.64±34.74	103.36±40.08
IL-8	1211878.66±196423.68	1082906.46±182926.87	1214343.01±127776.39	1208241.36±131351.8	961275.51±125634.61	956923.76±107703.39
IL-10	356.11±107.54	337.27±83.02	447.95±133.66	409.72±109.82	376.9±112.95	340.57±131.77
IL-12 p70	3.44±1.71	3.57±1.64	4.64±1.83	2.75±1.37	3.32±1.61	4.04±1.54
IL-13	1520.2±352.22	1592.21±396.43	2556.06±568.71	2722.16±654.88	2352.42±469.97	3146.73±1376.08
IL-15	16.61±5.18	12.18±3.88	18.48±5.42	15.19±4.45	16.05±4.35	15±4.36
IL-16	34664.66±5688.6	32220.78±5713.05	35114.47±6305.56	38215.82±7119.55	33169.6±6571.74	31297.08±6065.36
IL-17	2051.56±494.46	1818.29±441.13	1902.73±453.4	1991.14±468.07	2273.06±643.29	1900.77±486.8
IL-18	42.34±8.33	38.46±8.65	53.42±9.56	44.86±9.7	48.29±10.62	38.02±7.99
IL-21	192.48±44.34	213.16±49.71	350.46±105.97	416.57±129.08	241.97±70.22	192.33±94.8
IL-22	136.51±65.75	99.94±46.87	235.23±169.84	250.97±121.7	85.22±48.06	90.11±40.85
IL-33	772.66±304.56	703.97±231	971.26±216.31	869.46±233.64	892.55±254.38	942.92±300.3
S100A8	963.85±185.07	914.01±197.06	1039.44±253.26	1118.6±291.22	1011.35±253.61	1001.61±248.81
Eotaxin	485.72±188.93	394.26±149.86	298.14±75.07	290.62±90.69	255.09±55.96	248.92±76.17
GM-CSF	3025.1±338.55	3000.37±323.4	3281.61±452.95	3384.62±430.78	3042.33±392.85	2868.09±360.56
Gro-α	1119890.78±375968.09	1042337.78±352505.62	994598.68±332434.97	1057801.72±377359.73	886166.98±351223.09	853858.25±347261.09
IFN-γ	1110.06±160.5	1176.74±226.98	1799.4±224.29	1715.99±225.39	1370.28±202.78	1201.43±228.89
IP-10	410055.31±116200.18	329887.04±71846.11	421303.61±107194.84	285815.21±67303.17	408711.72±106273.36	289197.3±44097.62
I-TAC	19669.33±6843.53	12843.28±4647.17	16191.46±3925.22	12992.08±3429.75	17904.81±5720.31	14047.93±5108.07
M-CSF	287.27±41.15	266.68±43.53	344.47±42.4	322.01±43.58	285.75±50.33	270.94±55.86
MCP-1	88644.54±18242.71	76959.03±17611.32	79819.94±15834.58	89653.46±22528.44	67544.15±17687.99	67428.52±14217.77
MIG	548518.34±345533.55	398674.78±273624.88	454192.71±315432.71	384689.61±267246.67	459916.09±314880.66	432605.9±307405.39
MIP-1α	271.46±88.48	252.44±68.01	824.09±280.41	727.72±220.33	386.58±125.27	291.1±80.94
MIP-1β	566.3±201.52	578.74±156.28	2203.2±432.85	2170.16±427.84	755.63±212.83	617.87±164.26
MIP-3α	16371.61±5697.23	16171.5±6003.7	19820.19±7268.79	18075.28±5981.02	18176.96±6576.33	15428.4±5886.41
RANTES	756.26±227.44	859.97±237.64	1753.17±513.41	1833.8±560.6	1004.5±300.35	974.97±269.3
TGF-β	363.18±141.8	339.17±93.21	461.98±116.39	412.94±128.84	283.98±79.47	254.34±92.9
TNF-α	43.98±5.12	42.12±5.83	95.69±19.82	78.19±17.07	49.49±7.34	43.11±7.01

Table 3: Sum of cytokine release in soluble form from day 0-16. Mean concentration ± SEM (pg/ml) of 33 cytokines as measured by in-house multiplexed bead-based assay (n=8). Culture supernatants were treated with ExoQuick to sediment EVs. Measurements were made on EV-free supernatants.

	Control	Control + RTV	$X4_{LAI}$	$X4_{LAI}$ + RTV	$R5_{SF162}$	$R5_{SF162}$ + RTV
IL-1α	28.67±5.58	23.3±3.84	39.47±6.23	37.4±5.43	28.42±6.79	27±4.08
IL-1β	20.03±5.01	20.31±6	24.41±5.33	22.73±6.41	21.66±6.93	15.22±5.23
IL-2	2.8±0.88	2.99±1.05	24.69±2.89	24.99±4.35	2.4±0.7	3.08±1.21
IL-4	1.1±0.39	3.12±2.38	7.45±2.24	9.28±3.12	0.74±0.4	2.34±1.07
IL-6	3832.67±357.37	4033.84±489.42	5408.33±633.94	5977.76±708.59	3673.53±555.58	4007.6±691.41
IL-7	7.63±2.26	5.99±2.01	61.26±18.9	58.81±16.23	8.22±2.8	12.23±4.46
IL-8	11400.2±1010.01	11025.12±1070.75	11916.63±968.47	12706.18±1261.05	10297.49±776.94	10090.38±692.91
IL-10	391.84±64.44	330.3±54.17	441.66±75.16	421.25±53.75	348.25±77.58	396.88±78.64
IL-12 p70	0.12±0.08	1.19±1.14	0.37±0.25	1.51±1.06	0.29±0.21	0.09±0.09
IL-13	888.26±259.7	462.59±160.65	2166.67±654.01	2137.89±682.59	1958.05±1096.29	1902.55±912.64
IL-15	27.34±18.63	17.59±12.19	26.52±18.77	7.31±3.76	4.39±2.4	6.9±5.28
IL-16	1071.69±153.35	1018.83±161.82	1224.46±258.19	1249.21±230.72	952.94±206.23	1048.68±211.55
IL-17	125.57±33.36	108.07±20.23	125.54±31.73	112.1±22.54	132.85±33.43	106.58±21.39
IL-18	1.95±0.36	1.98±0.53	2.52±0.72	2.63±0.69	2.45±0.6	3.25±0.68
IL-21	53.98±18.14	31.49±13.03	135.14±43.06	153.44±55.23	70.55±34.25	59.34±27.2
IL-22	0±0	0±0	139.2±70.94	57.36±22.12	0±0	0±0
IL-33	481.14±281.18	662.4±305.62	1218.31±500.05	1123.03±496.07	657.95±262.99	577.92±233.67
S100A8	56.93±24.49	66.51±24.84	44.28±18	76.55±24.9	66.93±17.6	116.52±44.02
Eotaxin	84.8±31.21	131.66±68.8	74.55±34.34	157.78±78.99	125.08±46.41	91.52±38.22
GM-CSF	79.02±9.51	80.99±13.77	154.32±14.52	155.19±21.07	97.99±10.71	76.29±10.89
Gro-α	42670.35±10826.79	33143.93±6403.26	55126.38±18878.26	62670.98±22781.47	32952.45±6743.49	35358.71±8483.2
IFN-γ	282.8±62	291.39±70.57	844.8±146.65	834.73±130.75	332.48±81.97	340.83±88.81
IP-10	121067.33±62897.97	56284.52±16873.01	45463.66±12962.08	35554.12±11541.41	79929.9±26385.77	56627.75±16303.63
I-TAC	3815.34±1174.7	2716.96±793.25	2706.27±452.22	2213.52±381.73	3532.26±907.04	2859.77±816.84
M-CSF	50.09±9.16	55.78±9.85	79.89±11.38	93.62±22.12	42.62±4.96	42.1±7.91
MCP-1	3578.06±947.8	2535.07±366.09	1857.17±261.84	1857.84±206.5	2254.82±286.39	2668.93±352.25
MIG	14028.48±4789.14	11789.89±3993.77	9829.69±3349.92	8248.86±2879.56	13002.53±4080.36	11571.56±4030.19
MIP-1α	10.47±2.3	7.54±2.34	71.87±22.77	58.22±13.66	14.19±3.04	8.45±2.19
MIP-1β	37.08±14.56	34.66±7.66	203.19±52.62	179.41±38.68	47.17±12.32	36.49±8.18
MIP-3α	1025.76±360.11	1030.36±454.67	1224.06±526.24	946.77±338.02	1017.42±409.72	1166.04±460.14
RANTES	236.34±54.74	262.42±58.3	410.07±117.05	414.74±114.08	285.46±66	307.16±78.06
TGF-β	554.62±172.93	532.42±194.17	734.33±241.95	682.96±246.76	597.57±210.3	528.05±180.06
TNF-α	26.51±4.15	30.19±9.18	41.76±7	36.16±7.28	27.48±7.04	23.96±4.79

Table 4: Sum of cytokines in EV-associated form from day 0-16. Mean concentration ± SEM (pg/ml) of 33 cytokines was measured by in-house multiplexed bead-based assay (n=8). Culture supernatants were treated with ExoQuick to sediment EVs. Measurements were made on EV fractions (intact and lysed). Presented are total amounts of EV-associated cytokines (encapsulated + surface-associated).

Cytokine concentrations ranged from high (IL-6, IL-8, GRO-α, IP-10, MCP-1, and MIG) to low (IL-2, IL-4, IL-7, IL-12, IL-15, TGF-β, and TNF-α). Most cytokines were predominantly released in soluble form, but many cytokines including IL-4, IL-10, IL-13, IL-15, IL-33, Eotaxin, TGF-β, and TNF-α were released in at least 25% in EV-associated form. Most EV-associated cytokines were distributed more on the surface rather than internal; only IL-4, IL-10, IL-13, IL-15, IL-18, IL-33, Calg A and RANTES were at least 50%) inside of EVs compared to on the surface.

3.1 Cytokine production in early HIV-1 infection

To evaluate early responses to HIV-1 infection we measured cytokines released by uninfected and infected tissues at day 3 post-infection. We observed a significant increase in soluble IL-1β, IL-2, IL6, IL-8, IFN-γ, IP-10, ITAC, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, RANTES, and TNF-α from $X4_{LAI04}$ infected tissues compared to uninfected ones. Soluble cytokine analyses in culture supernatants from $R5_{SF162}$ infected tonsils showed a significant increase in IL-1β, GM-CSF, IFN-γ, IP-10, M-CSF, MIG, MIP-1α, MIP-1β, RANTES, and TNF-α ($p<0.05$, n=8) (**Figure 17**).

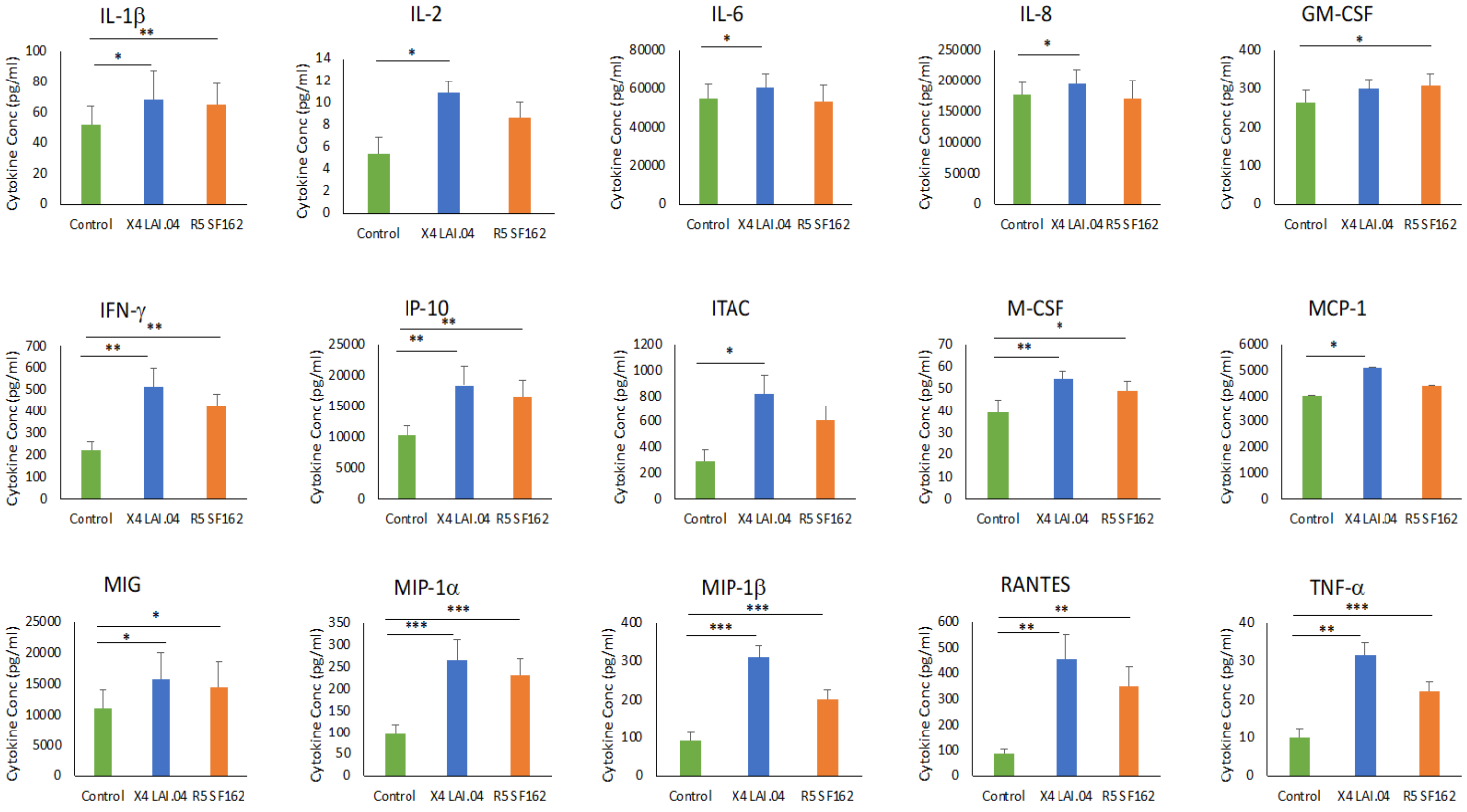

Figure 17: Concentrations of soluble cytokines that were significantly upregulated at day 3 by HIV-1 infection (Mean ± SEM; n=8; * p<0.05, **p<0.01, ***p<0.001).

Increases of cytokines occurred not only in the soluble fractions of cytokines but also in the EV-associated form. Indeed, significant increases were observed for IP-10, MIP-1α, MIP-1β, RANTES, and TNF-α in $X4_{LAI04}$ infected tissues, and in IL-1β, IL-18, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in $R5_{SF162}$ infected tissues ($p<0.05$, $n=8$) (**Figure 18**).

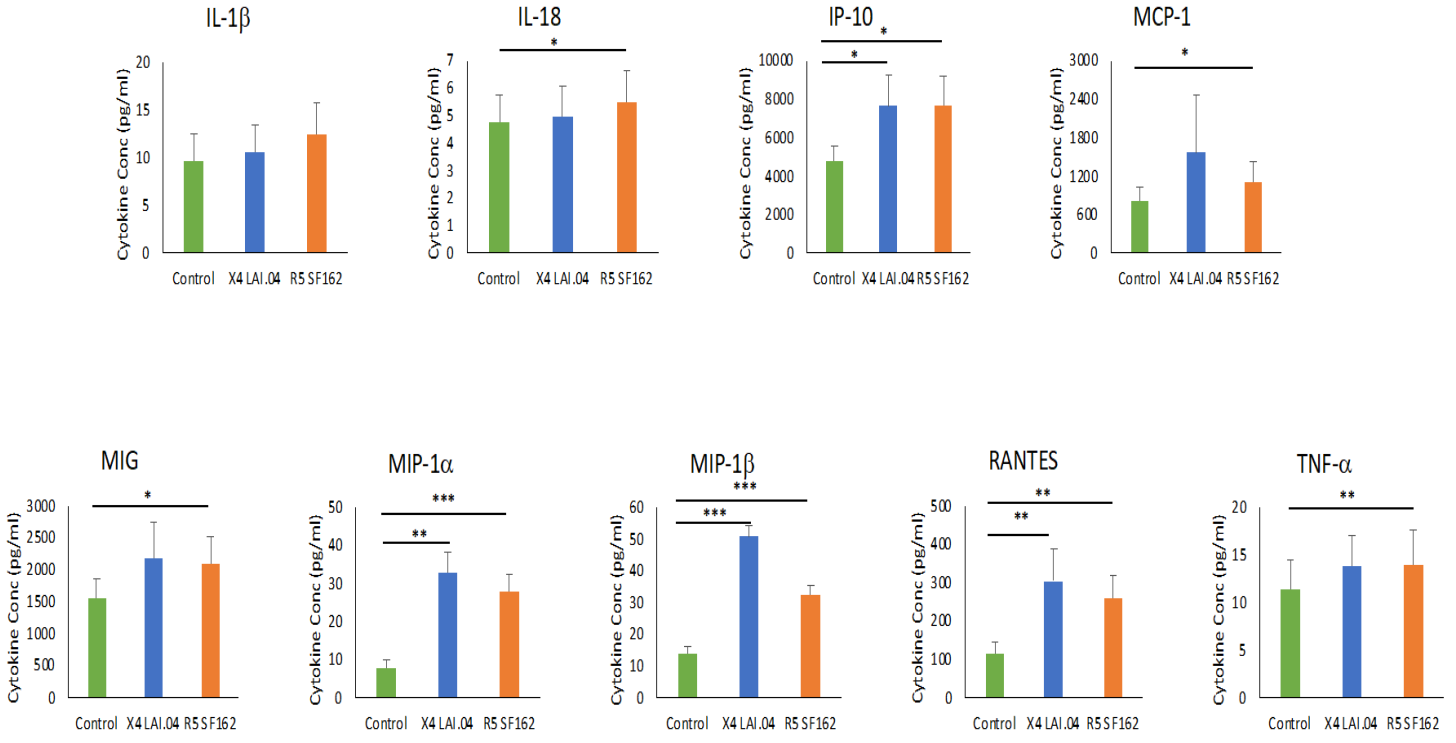

Figure 18: Concentrations of EV-associated (EV Total) cytokines upregulated significantly at day 3 (Mean ± SEM; n=8; *p<0.05, **p<0.01, ***p<0.001). Except for IL-18, these were also upregulated in soluble form.

3.2 Cytokine production throughout HIV-1 infection

Next, we evaluated cytokine release by uninfected and infected tissues as cumulative production of cytokines from day 3 to day 16. We observed significant increase in the soluble form of the cytokines IL-2, IL-7, IL-18, IFN-γ, M-CSF, MIP-1α, MIP-1β, RANTES and TNF-α following $X4_{LAI04}$ tissue-infection compared to uninfected tissues. $R5_{SF162}$ infection resulted in a significant increase in IL-2, IFN-γ, MIP-1α, MIP-1β and RANTES ($p<0.05$, $n=8$) (**Figure 19**).

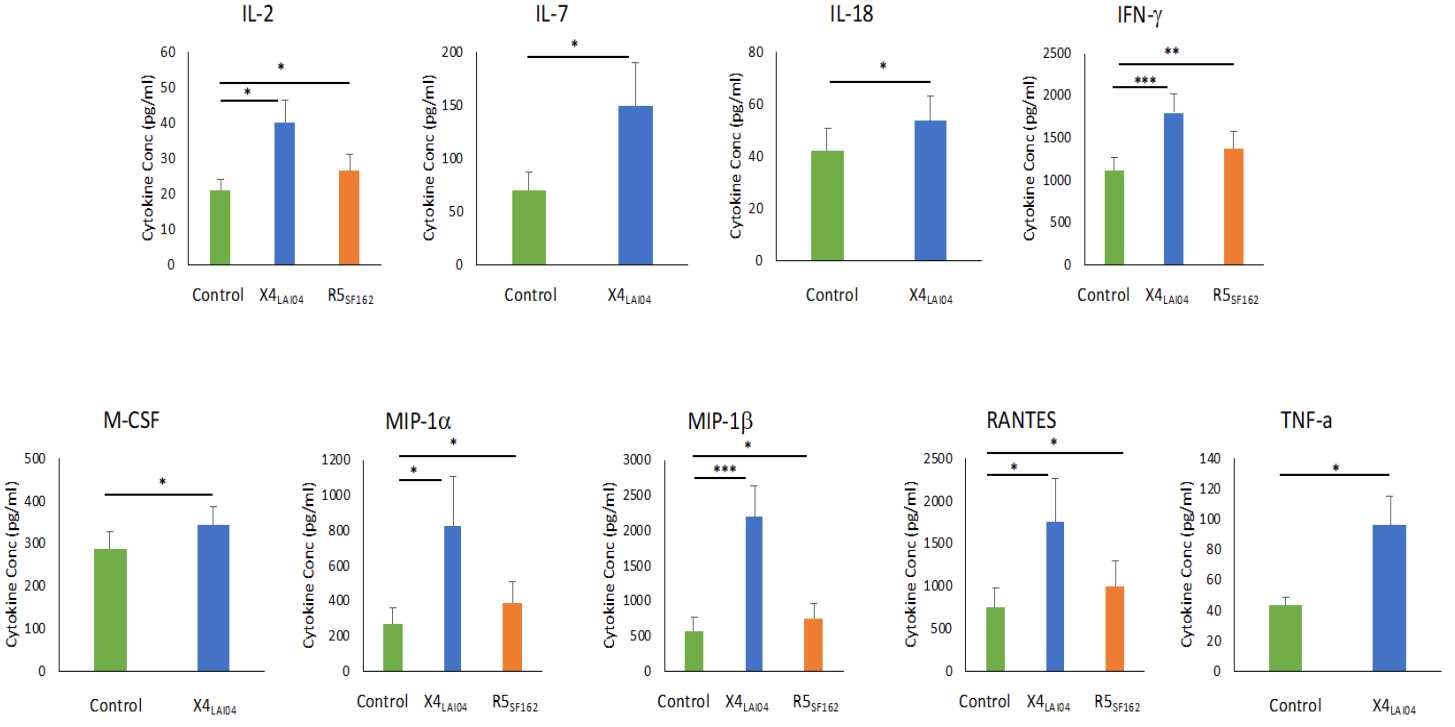

Figure 19: Soluble cytokine increased in infected tissues $X4_{LAI04}$ and $R5_{SF162}$ as cumulative production. Sum of soluble cytokines (day 3-16) upregulated with HIV infection (Mean ± SEM; n=8; *p<0.05, **p<0.01, ***p<0.001).

Cytokine increases occurred in EV-associated form as well. Even though the cytokine profile roughly mirrored the one observed for the soluble cytokines, a greater number of cytokines were augmented in EV-associated form in $X4_{LAI04}$ infected tissues. Significant increases were observed in cumulative data for: IL-1α, IL-2, IL-4, IL-6, IL-7, IL-13, IL-21, IL-33, GM-CSF, IFN-γ, M-CSF, MIP-1α, MIP-1β, RANTES, TGF-β and TNF-α following $X4_{LAI04}$ infection, and RANTES upon $R5_{SF162}$ infection ($p<0.05$, $n=8$) (**Figure 20**).

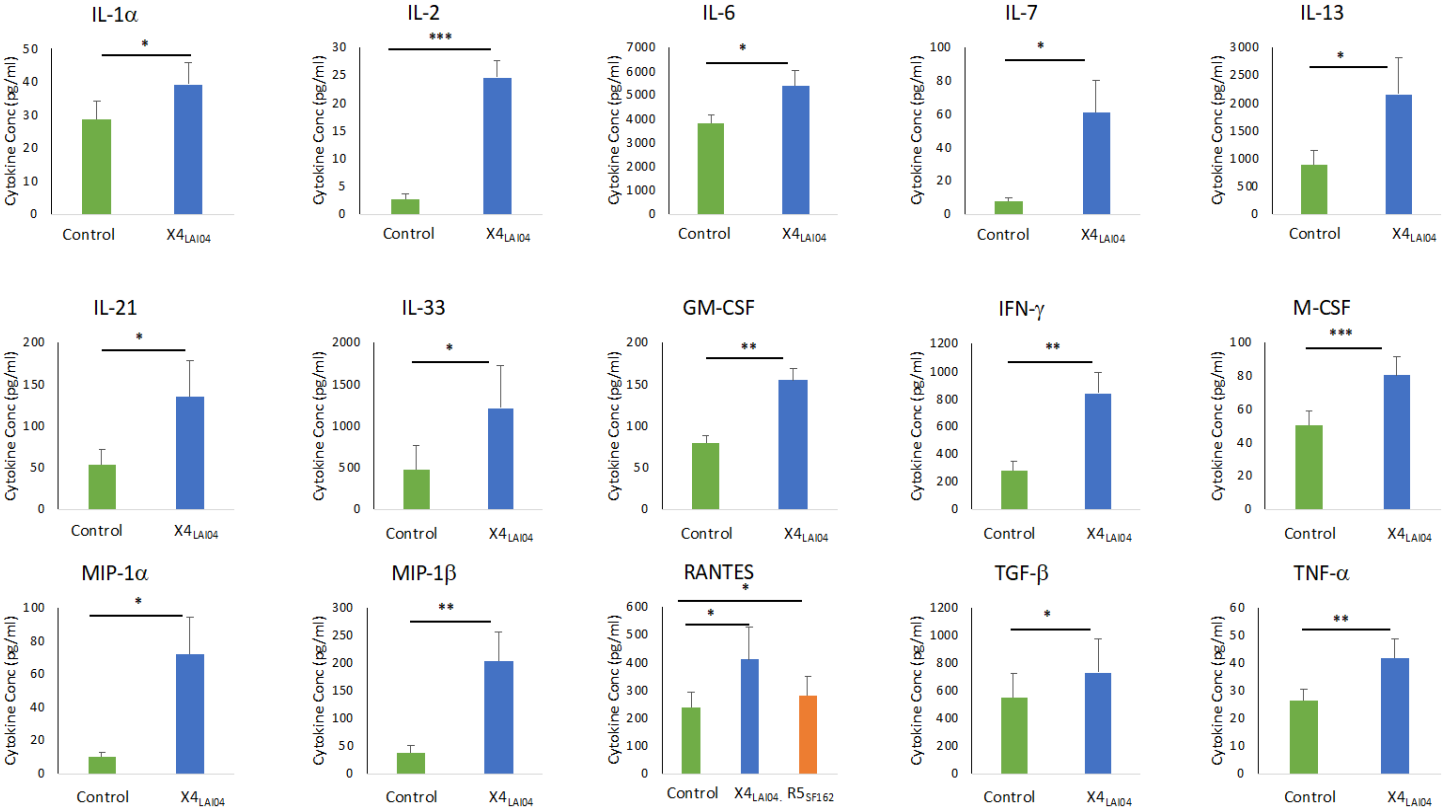

Figure 20: EV-associated cytokine upregulated in infected tissues $X4_{LAI04}$ and $R5_{SF162}$ as cumulative production (sum) of cytokines from day 3 to day 16, compared to uninfected tissues (Control) (Mean ± SEM, n=8; *p<0.05, **p<0.01, ***p<0.001).

3.3 Cytokine production in late HIV-1 infection

Analyses performed on culture supernatants from the final day of cultures (16 days post infection) showed that some cytokines maintained a 1.5-fold increase compared to the matched control tissues. As for soluble cytokines these included: IL-2, IL-18, IL-21, MIP-1α, MIP-1β, and RANTES for both viruses; Calg A in $X4_{LAI04}$ infected tissues; and IL-7, IL-13, and IL-33 and TNF-α in $R5_{SF162}$ infected tonsils. Those which were significant included IL-2, IL-18, MIP-1α, MIP-1β, and RANTES for $X4_{LAI04}$ infection, and MIP-1α and RANTES for $R5_{SF162}$ infection ($p<0.05$, $n=8$) (**Figure 22**).

EV-associated cytokines showing at least an 1.5-fold increase included: IL-1α, IL-7, IL-13, IL-18, IL-21, GM-CSF, IFN-γ, MIP-1α, MIP-1β, and RANTES for both viruses; IL-2, IL-10, IL-16, IL-33, M-CSF, and TNF-α in $X4_{LAI04}$ infected tissues only. IL-6 was elevated 1.4-fold in $X4_{LAI04}$ infected tissues. Those which were significantly increased compared to control tissues were: IL-1α, IL-2, IL-7, GM-CSF, IFN-γ, M-CSF, MIP-1α, MIP-1β, RANTES and TNF-α in $X4_{LAI04}$ infection only, and RANTES in $R5_{SF162}$ infection ($p<0.05$, $n=8$) (**Figure 23**). Overall, a greater number of cytokines remained elevated in EV-associated form compared to soluble (16 cytokines versus 11, respectively).

A few significant decreases were observed at late timepoints only in $X4_{LAI04}$ infection, including soluble ITAC and MCP-1, and EV-associated IP-10, ITAC, MCP-1, and MIG ($p<0.05$, $n=8$) (**Figure 21**).

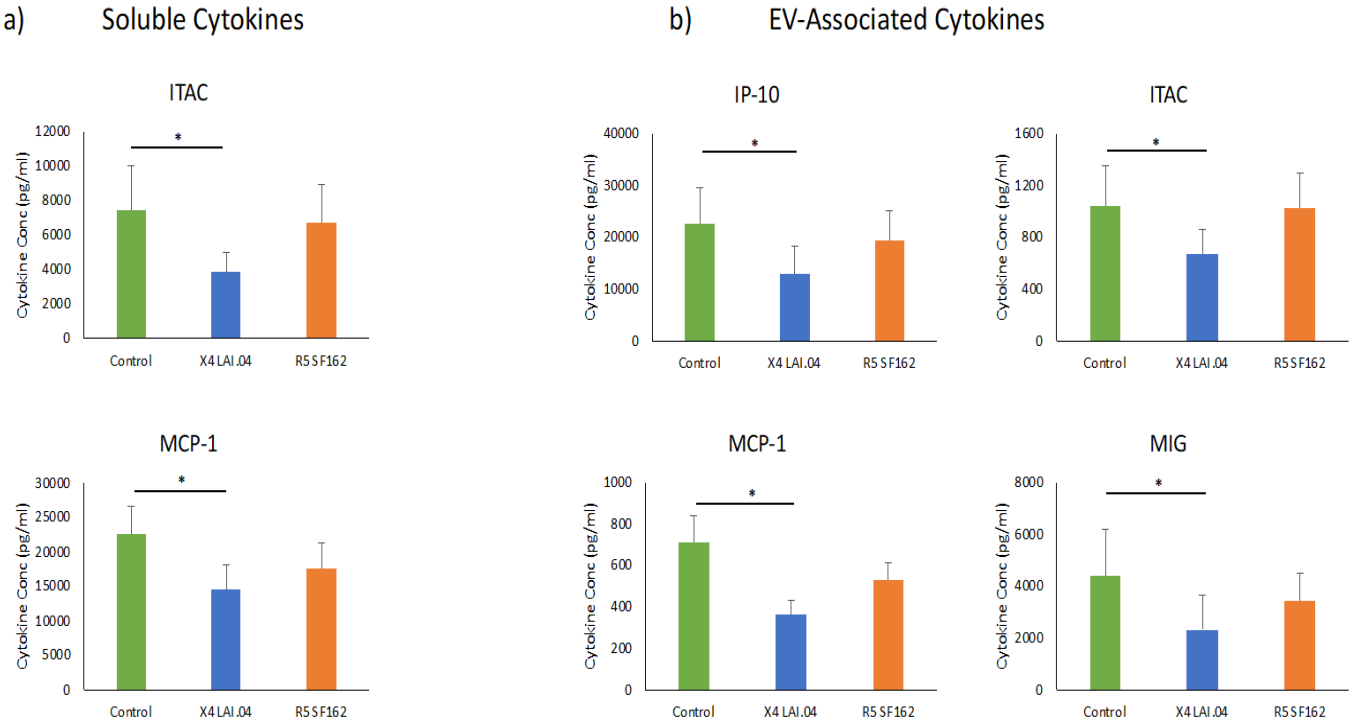

Figure 21: Soluble and EV-associated cytokines decreased at day 16 post-infection with both $X4_{LAI04}$ and $R5_{SF162}$ compared to uninfected tissue culture (Control) (Mean ± SEM, n=8; *p<0.05).

3.4 Cytokine production in *ex vivo* HIV-1 infected tissues treated with ritonavir

We then investigated whether HIV-1-replication suppression by ritonavir lead to control of immune activation. To this end we analysed cytokine production, in ritonavir treated/untreated tissues infected with different HIV-1 viral strains.

In particular, we focused our attention on those cytokines expression of which remained upregulated 16 days after HIV-1-infection and compared them to matched infected tissues treated with ritonavir (after 13 of treatment). As shown in **Figure 22,** with a few exceptions, despite controlling HIV-replication ritonavir was not able to reduce cytokine expression.

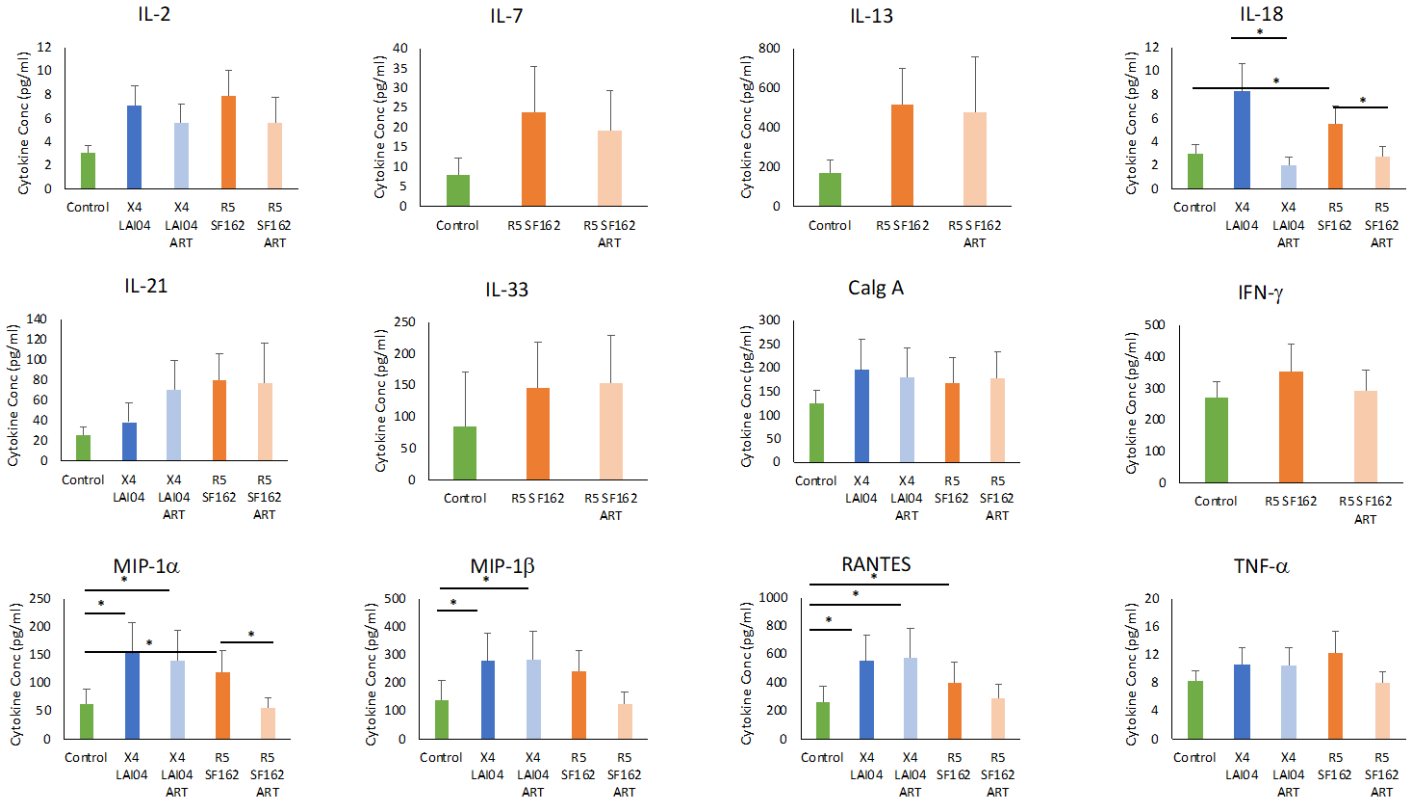

Figure 22: Soluble cytokines at day 16 in infected tissues $X4_{LAI04}$ and $R5_{SF162}$ which maintained a 1.5-fold increase compared to the matched control tissues. Ritonavir treatment (ART), was not sufficient to lower most of elevated cytokines after 13 days of therapy (Mean ± SEM; n=8; * $p<0.05$).

Only soluble IL-18 significantly decreased after 13 days of ritonavir treatment for both viruses, and soluble MIP-1α significantly decreased in $R5_{SF162}$ infection ($n=8$, $p<0.05$) Decreases were also observed for soluble MIP-1β, RANTES, and TNF-α in $R5_{SF162}$ ART but they did not reach significance.

In the EV-associated cytokines none of the upregulated cytokines were significantly decreased by ritonavir treatment (**Figure 23**), but decreases were observed for IL-10 and IL-13 production in $X4_{LAI04}$ infection and IL-1α, IL-7, GM-CSF, MIP-1α and MIP-1β in $R5_{SF162}$ ART condition, though they did not reach significance.

To summarize, most of the cytokines upregulated by HIV-1 infection remained elevated even after 13 days of ritonavir. EV-associated cytokines were less likely to decrease compared to soluble ones.

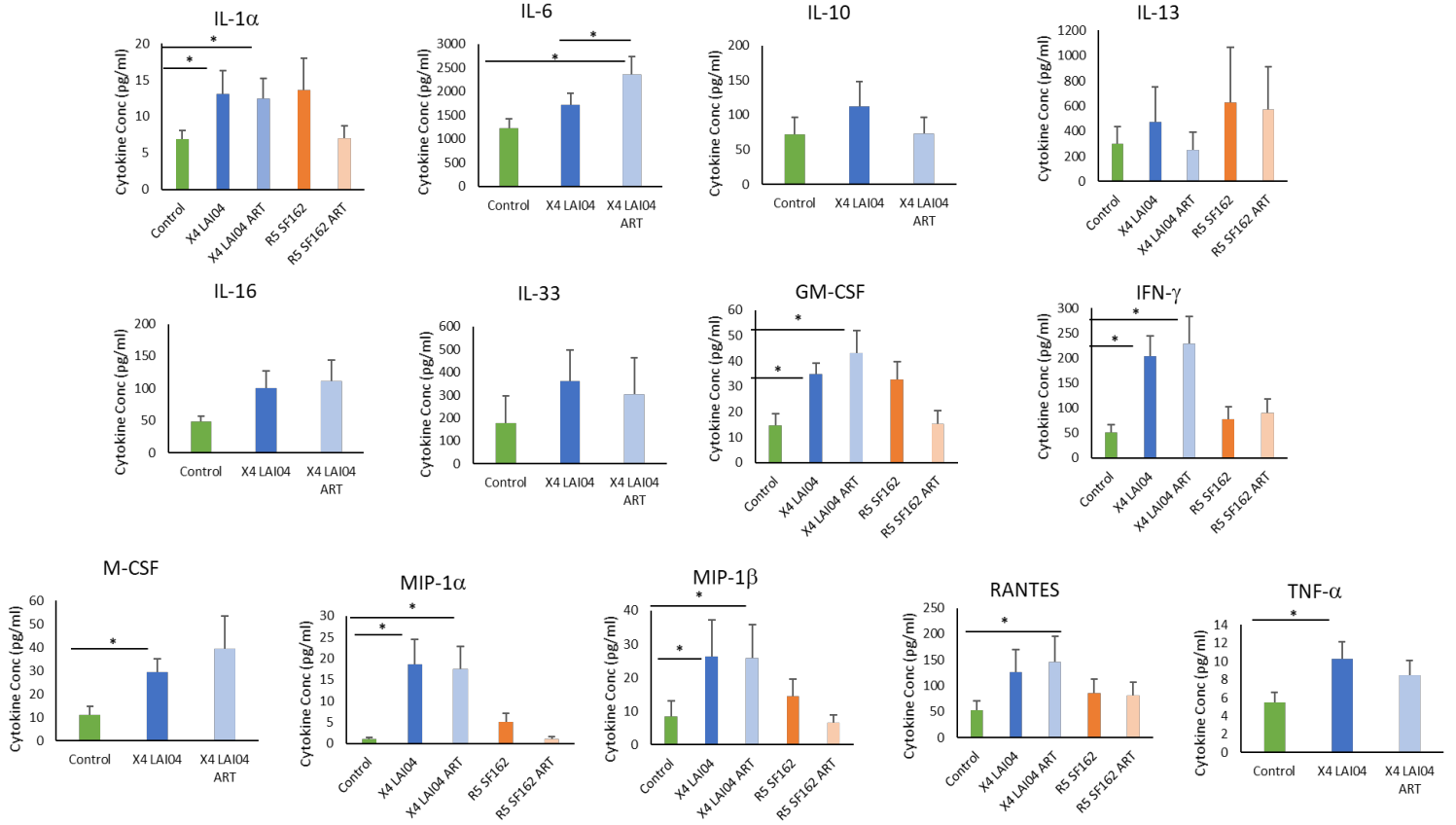

Figure 23: EV-associated cytokine at day 16 in infected tissues $X4_{LAI04}$ and $R5_{SF162}$ which maintained a 1.5-fold increase compared to the matched control tissues. Ritonavir treatment (ART) was not sufficient to lower most of elevated cytokines after 13 days of therapy (Mean ± SEM; n=8; *p<0.05).

3.5 Cytokine production in *ex-vivo* HIV-1 infected tissues treated with AZT-3TC

The above results showed that despite antiretroviral therapy, using a protease inhibitor, tissues remain persistently immune activated. To further confirm our analyses, we tested an alternate antiretroviral therapy using a combination of AZT and 3TC, which both belong to the NRTI antiretroviral group, and are largely used in combination in the hodiern regimens [174].

Similar to ritonavir treatment (and described above in HIV-1 Replication section), the treatment with AZT-3TC efficiently stopped the replication of HIV-1 in our *ex vivo* model.

Evaluation of cytokine production upon AZT-3TC treatment demonstrates that tissues remain immune-activated despite ART treatment, though the cytokine profile varied presumably as a consequence of tissue variability. Four out of six soluble cytokines (IL-12, MIP-1α, MIP-1β, RANTES) which were elevated (1.5-fold increase over control tissues) upon $X4_{LAI04}$ infection were elevated in AZT-3TC treated tissues as well. Two out of ten cytokines (IL-12, IL-22) which were elevated in $R5_{SF162}$ infected tonsils were also elevated with AZT-3TC treated cultures (**Figure 24**).

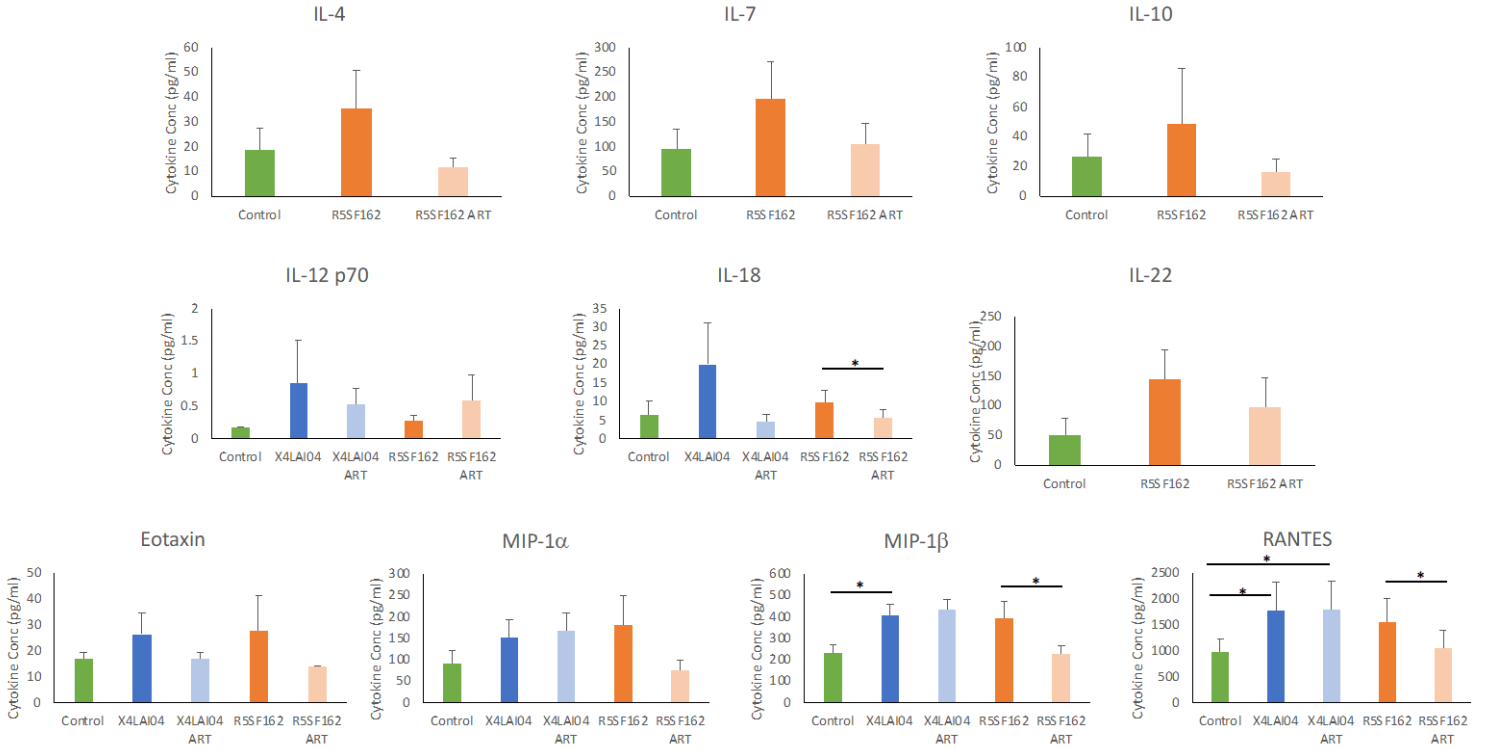

Figure 24: Soluble cytokines that were elevated by at least a 1.5-fold increase late in infection with $X4_{LAI04}$ and $R5_{SF162}$. Most of these cytokines were not restored to control levels after 13 days of AZT-3TC treatment (ART) compared to the matched control tissues (Mean ± SEM; n=8; *p<0.05).

EV-associated cytokines were also elevated after AZT-3TC treatment. Twelve out of the 14 cytokines (IL-2, IL-4, IL-7, IL-13, IL-15, IL-21, IL-33, IFNγ, M-CSF, MIP-1α, MIP-1β, RANTES) which were increased in $X4_{LAI04}$ infected tissues were persistently higher despite ART treatment compared to control tissue. Likewise, two out of the six cytokines previously described, were still elevated in $R5_{SF162}$ infected AZT-3TC treated biopsies (**Figure 25**).

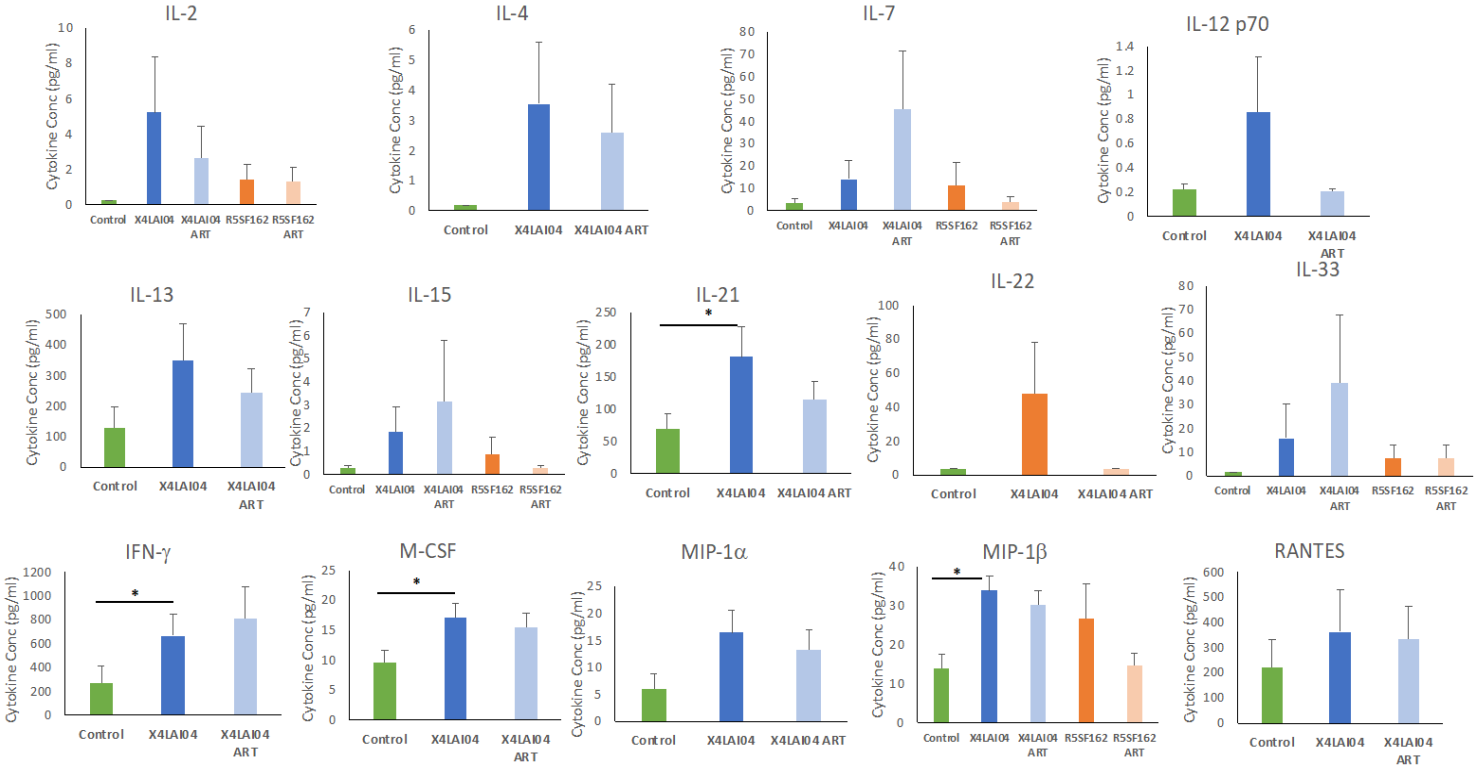

Figure 25: EV-associated cytokines that were elevated by at least a 1.5-fold increase late in infection with $X4_{LAI04}$ and $R5_{SF162}$. Most of these cytokines were not restored to control levels after 13 days of AZT-3TC treatment (ART) compared to the matched control tissues (Mean ± SEM; n=8; *p<0.05).

Notably, AZT-3TC treatment resulted in a significant soluble IL-6 increase compared to that measured in $X4_{LAI04}$ or $R5_{SF162}$ infected tissues ($p<0.05$, $n=8$). EV-associated IL-6 production was also elevated, and reached statistical significance ($p<0.05$, $n=8$) in $X4_{LAI04}$ infected biopsies. Increased level of soluble and EV-associated IL-6 was also observed in $X4_{LAI04}$ ritonavir treatment, although did not reach significance.

Compared to HIV-1 infection alone, AZT-3TC treatment also resulted in a significant decrease of soluble IP-10, ITAC, and MIG for both viral strains ($p<0.05$, $n=8$). EV-associated levels of these chemokines were also decreased with both viral strains, though only IP-10 reached significance ($p<0.05$, $n=8$). A similar trend (except for EV-associated levels with $R5_{SF162}$) was observed following ritonavir treatment, though none of these were statistically significant.

3.6 Difference in cytokine production between X4 and R5 virus infection.

We then investigated whether $X4_{LAI04}$ and $R5_{SF162}$ infections differently upregulate cytokine release. When evaluating those soluble cytokines which were elevated by at least 1.5-fold in HIV-1 infection compared to control cultures, these cytokines were almost always more elevated in $X4_{LAI04}$ infection. Cumulative soluble cytokine concentrations that were significantly higher in $X4_{LAI04}$ infection compared to $R5_{SF162}$ ones included IL-2, IL-7, IFN-γ, M-CSF, MIP-1α, MIP-1β, RANTES and TNF-α ($p<0.05$, $n=8$), increases were also seen in IL-1α, IL-6, IL-8, and IL-21, but these were not significant (**Figure 26**).

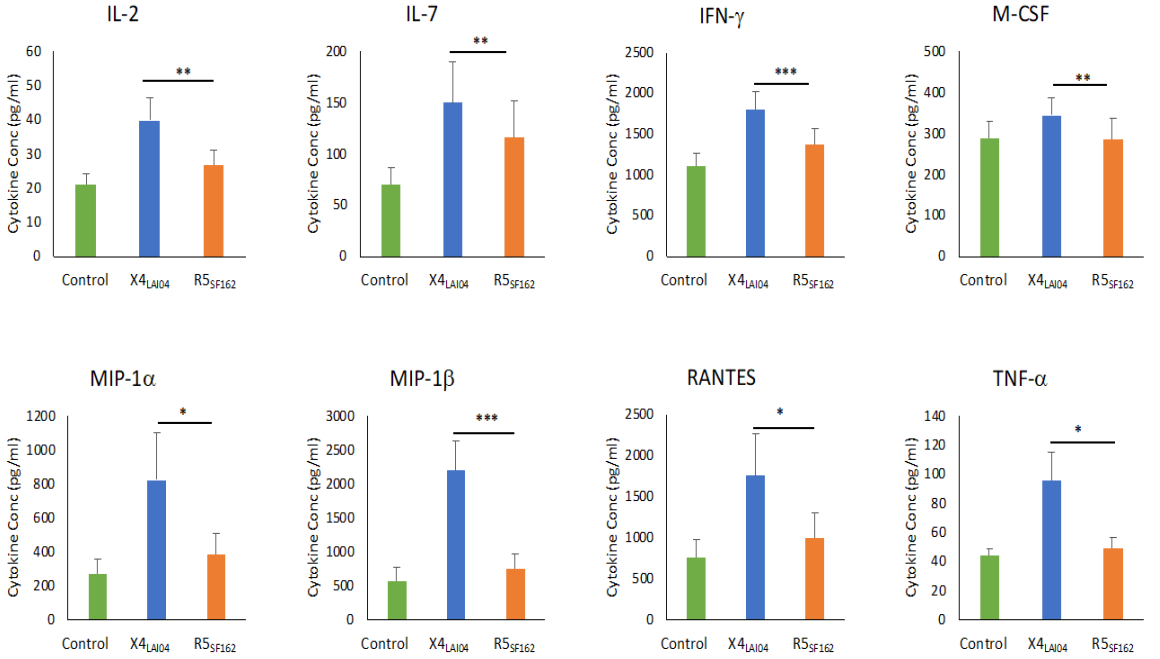

Figure 26: Significant differences between $X4_{LAI04}$ and $R5_{SF162}$ in soluble cytokines, which were increased in cumulative production (sum) from day 3 to day 16. $X4_{LAI04}$ triggered a stronger immune response compared to $R5_{SF162}$ (Mean ± SEM; n=8; *p<0.05, **p<0.01, ***p<0.001).

When looking at the EV-associated cytokines that are upregulated in HIV-1 infection by at least 1.5 fold over control cultures, amounts of IL-2, IL-6, IL-7, IL-21, IFN-γ, M-CSF, MIP-1α, MIP-1β, RANTES and TNF-α were significantly upregulated in culture media from $X4_{LAI04}$ infected biopsies compared to $R5_{SF162}$ infection ($p<0.05$, n=8) (**Figure 27**).

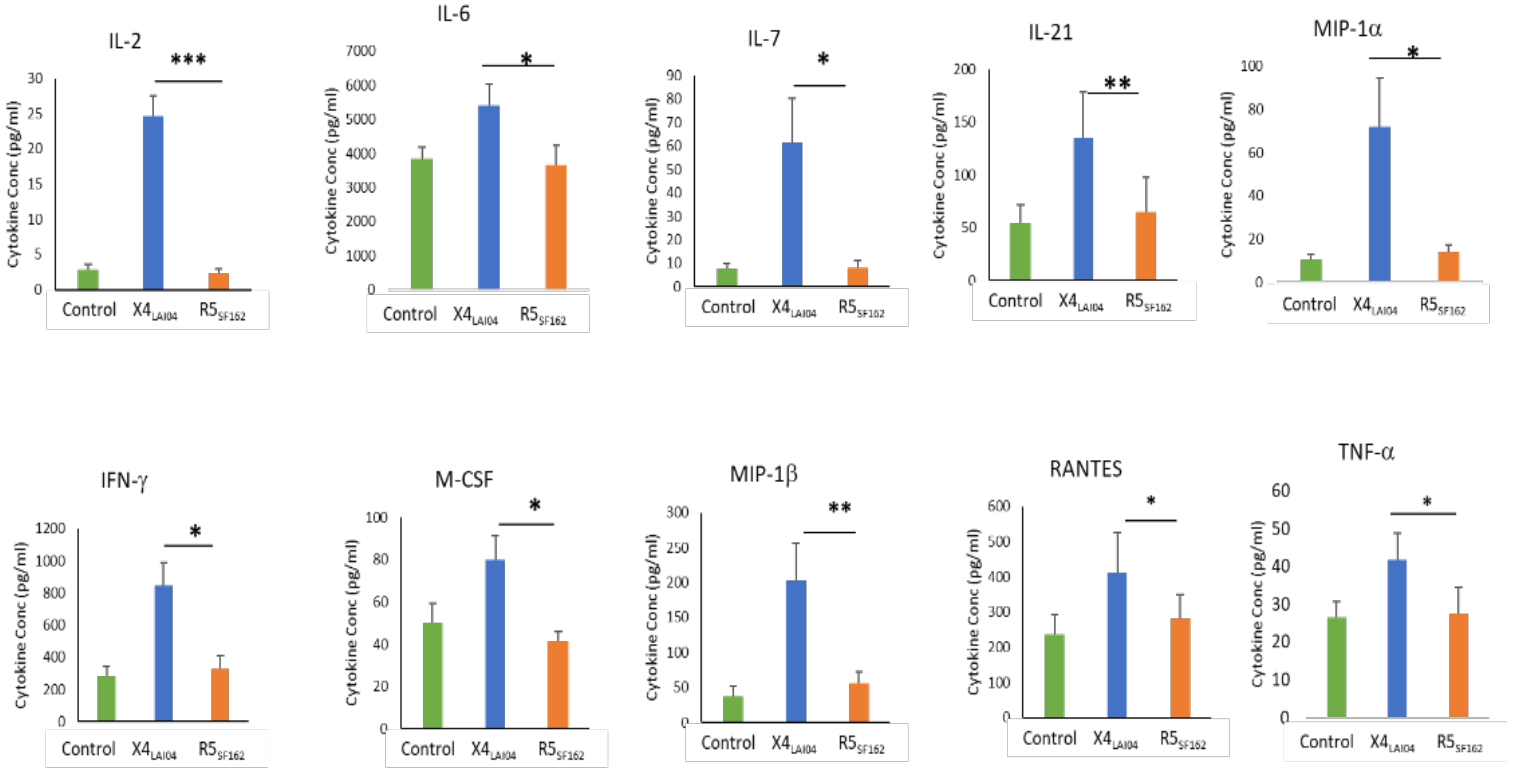

Figure 27: Significant differences between $X4_{LAI04}$ and $R5_{SF162}$ in EV-associated cytokines, which were increased in cumulative production (sum) from day 3 to day 16. $X4_{LAI04}$ triggered a stronger immune response compared to $R5_{SF162}$ (Mean ± SEM; n=8; *p<0.05, **p<0.01, ***p<0.001).

Infection by $X4_{LAI04}$ and $R5_{SF162}$ viral strains also showed differences in cytokine upregulation (notably MIP-1α, MIP-1β, and RANTES) when infection was suppressed by both RTV and AZT-3TC treatments. Although both soluble and EV-associated cytokine levels remained upregulated in ART-treated HIV-1-infected tissues their concentrations were significantly higher in $X4_{LAI04}$ -infected biopsies compared to $R5_{SF162}$ - ones.

4. Cytokine distribution

4.1 Fraction of cytokines shifting between soluble and EV-associated compartments

We examined the distribution of cytokines between soluble and EV-associated forms. We observed few significant changes: On day 3 post $X4_{LAI04}$ infection there was a slight shift of IP-10 to the soluble form and a more significant shift in RANTES and TNF-α towards the soluble forms upon infection with both $X4_{LAI04}$ and $R5_{SF162}$ infection (**Figure 28a**).

The shift towards soluble RANTES (both $X4_{LAI04}$ and $R5_{SF162}$ infection), and soluble TNFα (only for $X4_{LAI04}$ infection) continued through day 6.

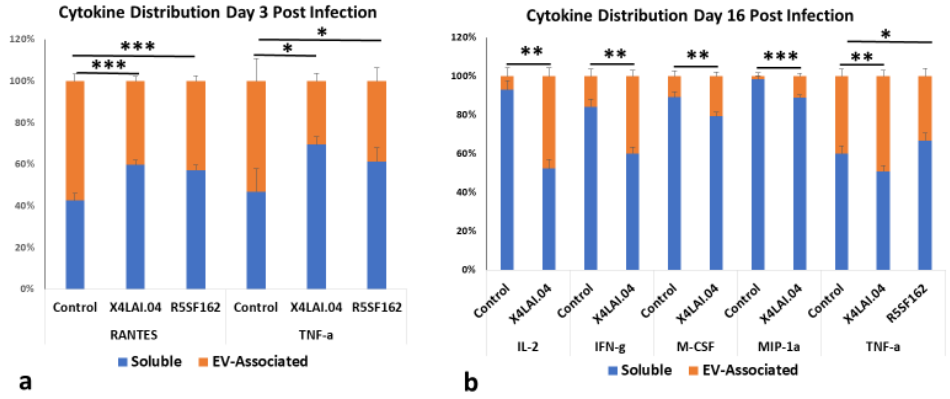

Figure 28: Cytokines were measured by Luminex in soluble and EV-associated forms at various timepoints and ratios are determined between the cytokine concentrations in these two forms. (a) day 3; (b) day 16 (Mean ± SEM, n=8, *p<0.05, **p<0.01, ***p<0.001).

On day 16, $X4_{LAI04}$ infection resulted in a significant increase in EV-associated IL-2, IFN-γ, M-CSF, MIP-1α, and, in particular, TNF-α. Conversely, we observed a shift towards the soluble TNF-α in $R5_{SF162}$ infected tonsils (**Figure 28b**). No significant difference in the relative release of cytokines in soluble and EV-associated forms were observed between tissues following both ART-treatment.

4.2 Fractions of cytokines shifting between EV-surface and EV-internal compartments

Finally, we evaluated cytokine distribution between EV surface and inner EV space. The only significant shift early in HIV infection was in RANTES concentration, that was more associated with the EV surface in both $X4_{LAI04}$ (day 3 and 6) and $R5_{SF162}$ (day 3) infections compared to uninfected tissues (**Figure 29**). At day 16, $X4_{LAI04}$

infection resulted in a significant decrease in EV-surface compared to EV internalization for IL-1α, IL-17 and GM-CSF. Neither antiviral treatments significantly affected cytokine distribution between EV surface and EV encapsulation.

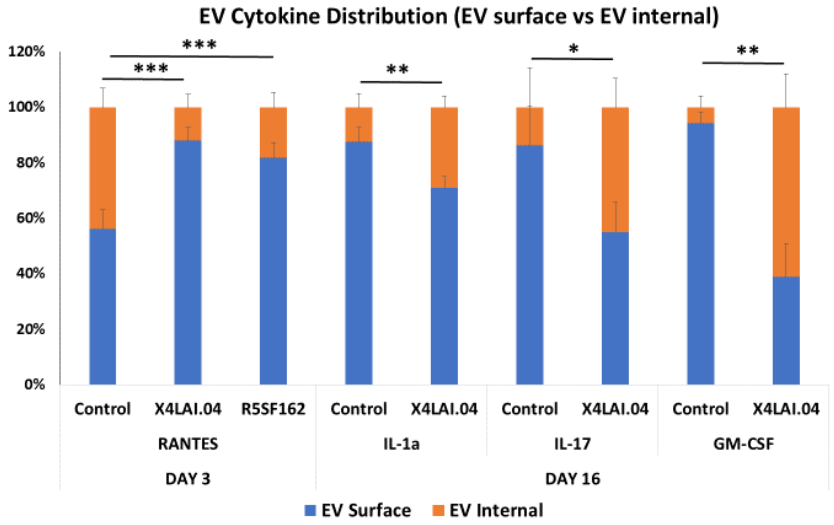

Figure 29: Cytokines encapsulated in EVs and associated with EV surface were measured by Luminex at various timepoints and ratios between these amounts were determined (Mean ± SEM, n=8, *p<0.05, **p<0.01, ***p<0.001).

5. Statistical evaluation of cytokine production

We performed principal component analysis of the data that demonstrated that responses of tissues were similar and none of the tissues were outliers. Wilcoxon ranked sign tests were used as another method to evaluate pairwise cytokine production over the entire culture length between all culture conditions. All timepoints were used for all donors for each condition and the following comparisons were made: uninfected vs uninfected ART (ritonavir treatment), uninfected vs $X4_{LAI04}$, $X4_{LAI04}$ vs $X4_{LAI04}$ ART, uninfected vs $X4_{LAI04}$

ART, uninfected vs $R5_{SF162}$, $R5_{SF162}$ vs $R5_{SF162}$ ART, uninfected vs $R5_{SF162}$ ART, and $X4_{LAI04}$ vs $R5_{SF162}$. These comparisons were made for both soluble and EV-associated cytokines.

Heatmaps generated for these comparisons show similar results (**Figure 30**) to those already stated above and highlight aforementioned conclusions: HIV infection leads to many increases in cytokines in both soluble and EV-associated form with $X4_{LAI04}$ resulting into higher increases in more cytokines compared to R5; ART therapy did not restore cytokines back to basal levels, and there are few changes in cytokine release due to ART itself.

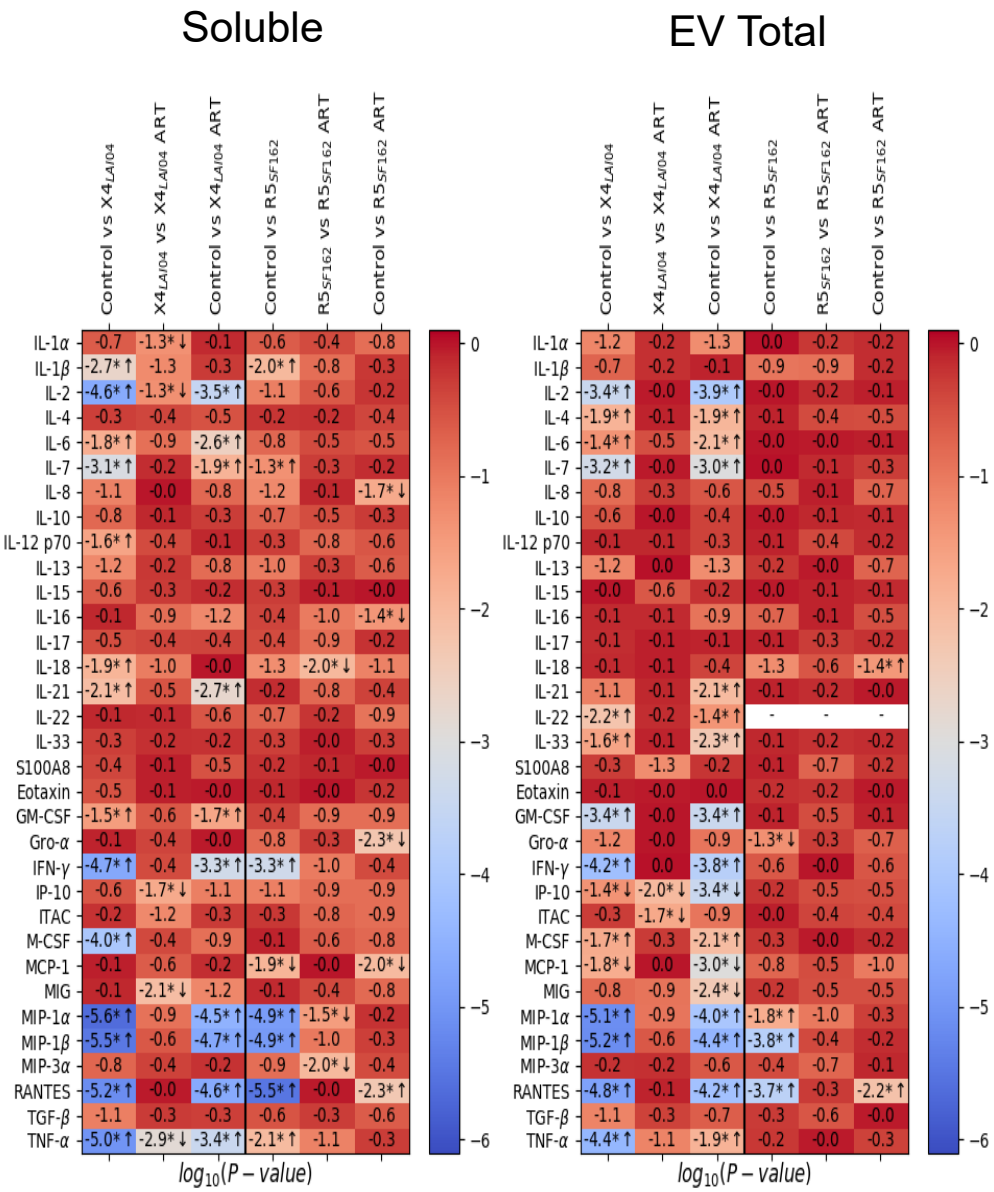

Figure 30: Comparison of cytokine levels between uninfected, HIV-1 infected, and antiretroviral treated human lymphoid tissues *ex vivo*. Data were analyzed with Wilcoxon signed-rank test for paired nonparametric data with Benjamini-Hochberg correction for multiple comparison between different pairs of treatments in a day-to-day manner. Graphs are presented with p-values in $\log_{10}$-scale, presented are comparison of the levels of soluble and EV-associated cytokines between the uninfected (Control) group, HIV-1 infected (16 days, $X4_{LAI04}$ and $R5_{SF162}$), and HIV-infected and treated for 13 days with 5μM ritonavir ($X4_{LAI04}$ ART and $R5_{SF162}$ ART); (* indicates significance, n=8, arrows indication direction of change up or down).

6. Investigation of possible mechanisms of immune activation despite viral suppression.

The above results confirmed that our *ex vivo* model replicates what is seen in the *in vivo* situation: despite halting HIV-1 infection there is a persistent state of inflammation. The next step was to investigate possible mechanisms of this immunoactivation. Several hypotheses have been proposed for this phenomenon and the *ex vivo* model proved useful in evaluating some of them.

6.1 Initial cytokine release as a potential cause for the cytokine storm

First, I hypothesized that an initial upregulation of certain cytokines could activate the immune cells and stimulate them to produce a cascade of other cytokines. To test this, we treated tonsillar *ex vivo* tissues with exogenous cytokines which were shown in the above experiments to be upregulated after the infection (IFNγ, RANTES, TNFα, and a combination of IFNγ, IL-2, IL-7, MIP-1α, MIP-1β, RANTES and TNFα).

Evaluation of cytokines three days after addition of exogenous cytokines demonstrated an upregulation of Eotaxin and TGF-β upon RANTES stimulation, and of Gro-α with combined cytokine stimulation.

Cumulative production of cytokines throughout the culture length also revealed only a few upregulated cytokines: IL-10, IL-15

and TGF-β following the exogenous TNF-α stimulation, and TGF-β with RANTES stimulation. Furthermore, no significant increase was found at the last time point (day 12) for all the exogenous cytokine stimulations.

These results suggest that the persistent increase of cytokines in our *ex vivo* model is not due to an initial stimulus of cytokine storm.

6.2 The presence of virions as a potential cause for the immune activation

Another hypothesis that we tested was that the presence of virus alone, either in very low levels, or in defective form, is enough to trigger immune activation. To test if the virion itself, without actually infecting and starting a replication cycle, is able to trigger such a response, we inoculated *ex vivo* tissues with equivalent amounts of HIV-1 ($X4_{LAI04}$) or $X4_{LAI04}$ inactivated with Aldrithiol (AT-2) and quantified those cytokines that in our experimental model were at least 1.5-fold higher than in matched control tissues.

Early after infection (day 3), $X4_{LAI04}$ infected tissues showed an upregulation of IL-2, IL-4, IL-15, IL-21, IFN-γ, IP-10, ITAC, MIG, MIP-1α, MIP-1β, RANTES, and TNF-α, while the inactivated $X4_{LAI04}$ triggered a similar though slightly dampened response, upregulating IL-4, IL-21, ITAC, MIP-1α, MIP-1β, RANTES, and TNF-α.

As in previous experiments, at the final timepoint many cytokines remained at least 1.5-fold upregulated with $X4_{LAI04}$ (**Figure 31**). With a single dose of inactivated $X4_{LAI04}$, 7 cytokines were still

elevated. Although we saw a similar pattern between infectious and inactivated virus, the increases with inactivated virus were usually lower than with replicating virus.

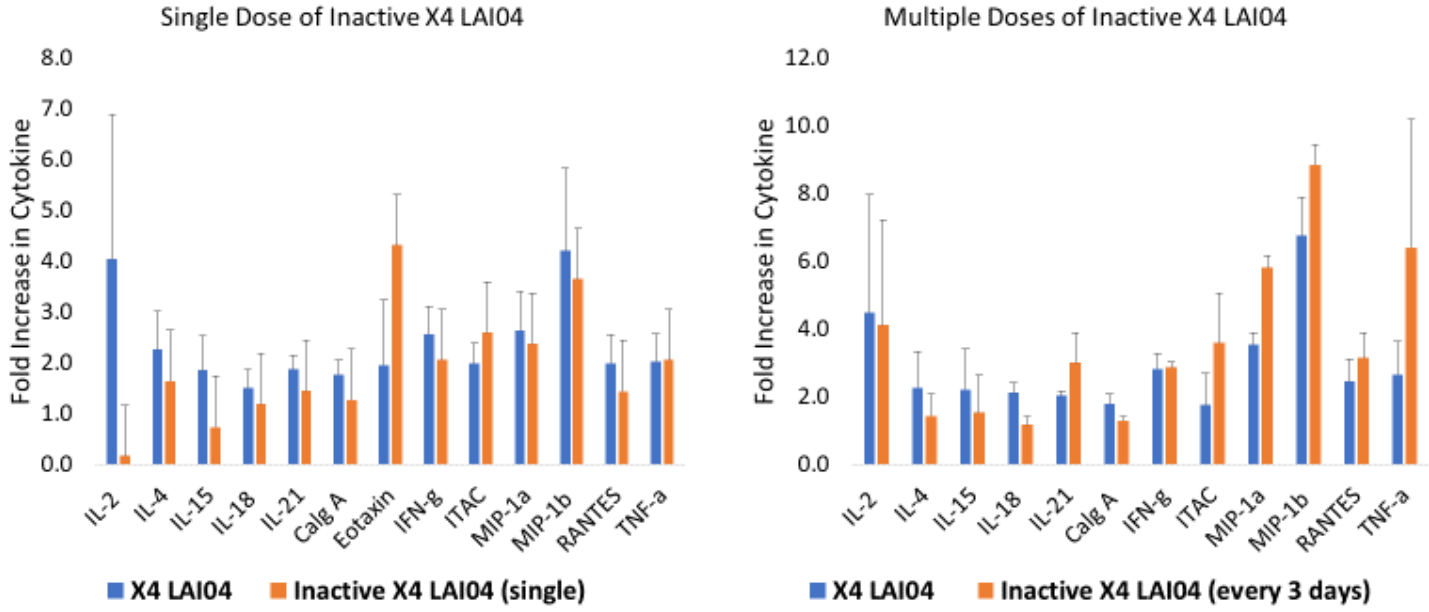

Figure 31: Cytokines which at day 12 were at least 1.5-fold higher than in matched control tissues after inoculation with HIV-1 ($X4_{LAI04}$) or $X4_{LAI04}$ inactivated with aldrithiol-2 (AT-2) in *ex vivo* lymphoid tissue. Left graph demonstrates results with single dose at day 0 of inactivated AT-2 HIV-1; right graph reflects changes with multiple doses of inactivated AT-2 HIV-1 at day 0 and every change of medium (Fold increase ± SEM, n=2-4).

The inactivated virus could mimic the residual viral replication or production of defective viruses that are not detected by routine viral measurements. To further address this possibility, in separate tissues we tested a repeated exposure to the inactivated virus, adding inactive virus every three days. Again we saw similar upregulation of cytokines between infectious $X4_{LAI04}$, and with multiple doses of inactive $X4_{LAI04}$; we observed 9 of the same 12 cytokines to be still upregulated; 5 of these cytokines were higher with the inactive virus (IL-21, MIP-1α, MIP-1β, RANTES, and TNF-α) (**Figure 31**).

6.3 HIV-1 co-pathogens as a potential cause for immune activation

Another possibility for the immune activation observed in HIV-1 suppressed individuals is that latent co-infecting viruses are reactivated thus leading to cytokine upregulation.

To test this theory, we evaluated the presence of multiple herpesviruses in uninfected and $X4_{LAI04}$ infected of *ex vivo* tissues with and without ART to look for reactivation of any latent viruses. Herpesviruses (HSV-2, EBV, CMV, HHV-6, and HHV-7) were quantified by digital droplet PCR in tissue blocks at the time of infection and 6 and 12 days post-infection.

No consistent patterns of virus replication were observed for any condition or any virus (**Figure 32, 33, 34, 35, 36**). These results, while preliminary, suggest that immune activation in our *ex vivo* tissues is not secondary to herpesviruses reactivation.

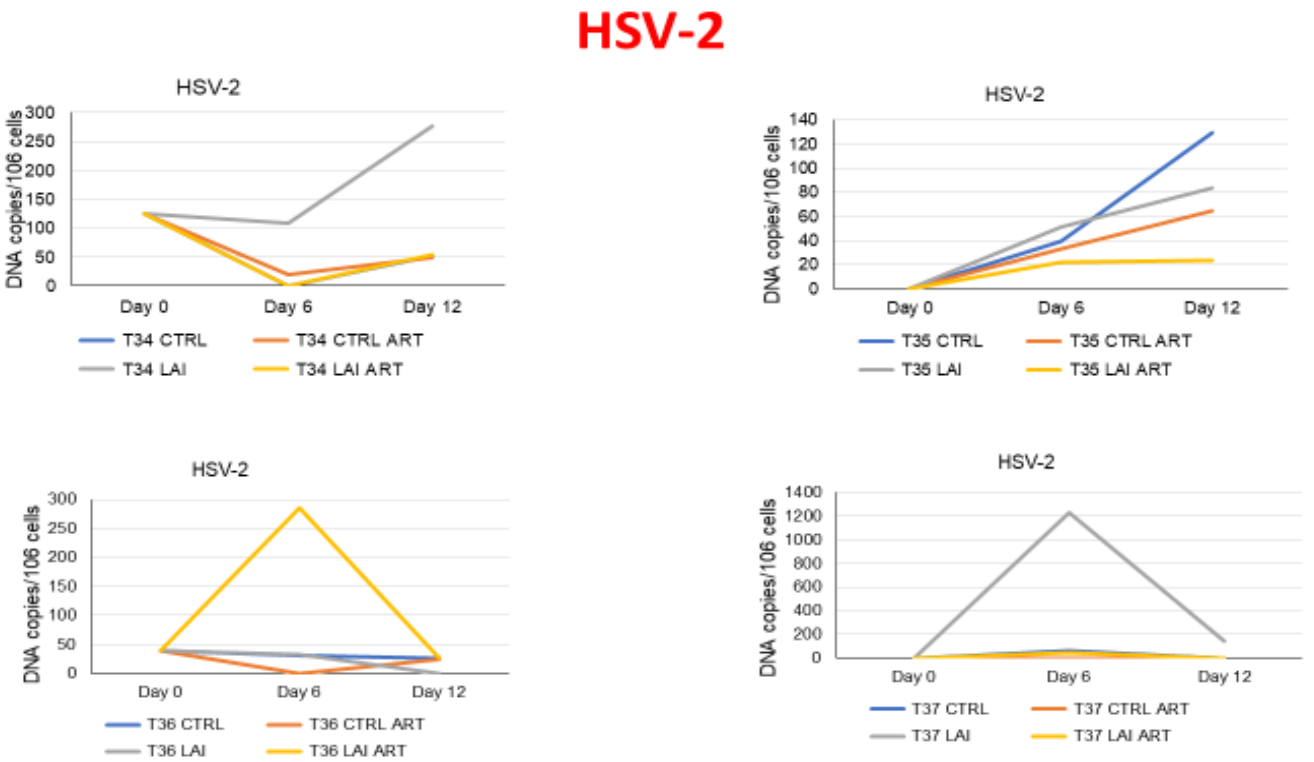

Figure 32: DNA copies were detected by ddPCR of endogenous **HSV-2** in 4 independent tissue blocks (T34-T35-T36-T37), at day 0, 6 and 12, in tissue uninfected (CTRL) and infected with $X4_{LAI04}$ (LAI) and treated or not with RTV (ART). DNA copies per number of cells was calculated using RPP30 as a reference.

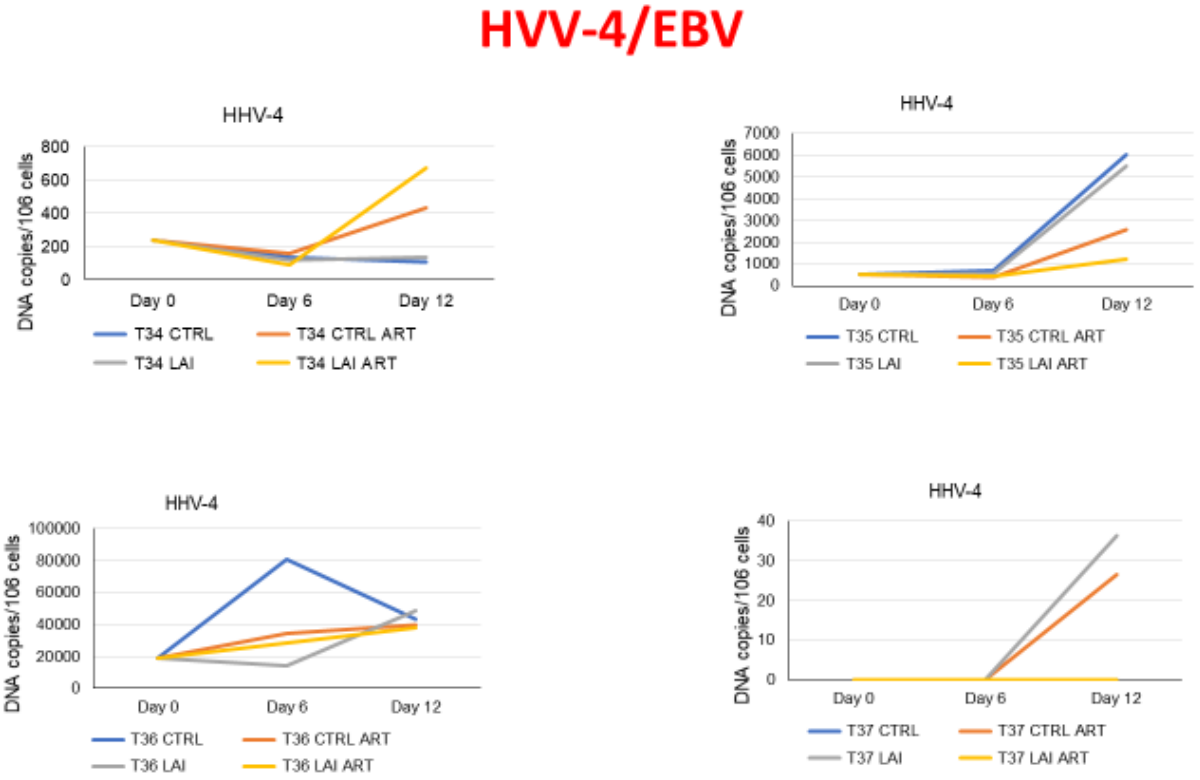

Figure 33: DNA copies detected by ddPCR of endogenous HVV-4 (also known as EBV) in 4 independent tissue blocks (T34-T35-T36-T37), at day 0, 6 and 12, in tissue uninfected (CTRL) and infected with $X4_{LAI04}$ (LAI) and treated or not with RTV (ART). DNA copies per number of cells were calculated using RPP30 as a reference.

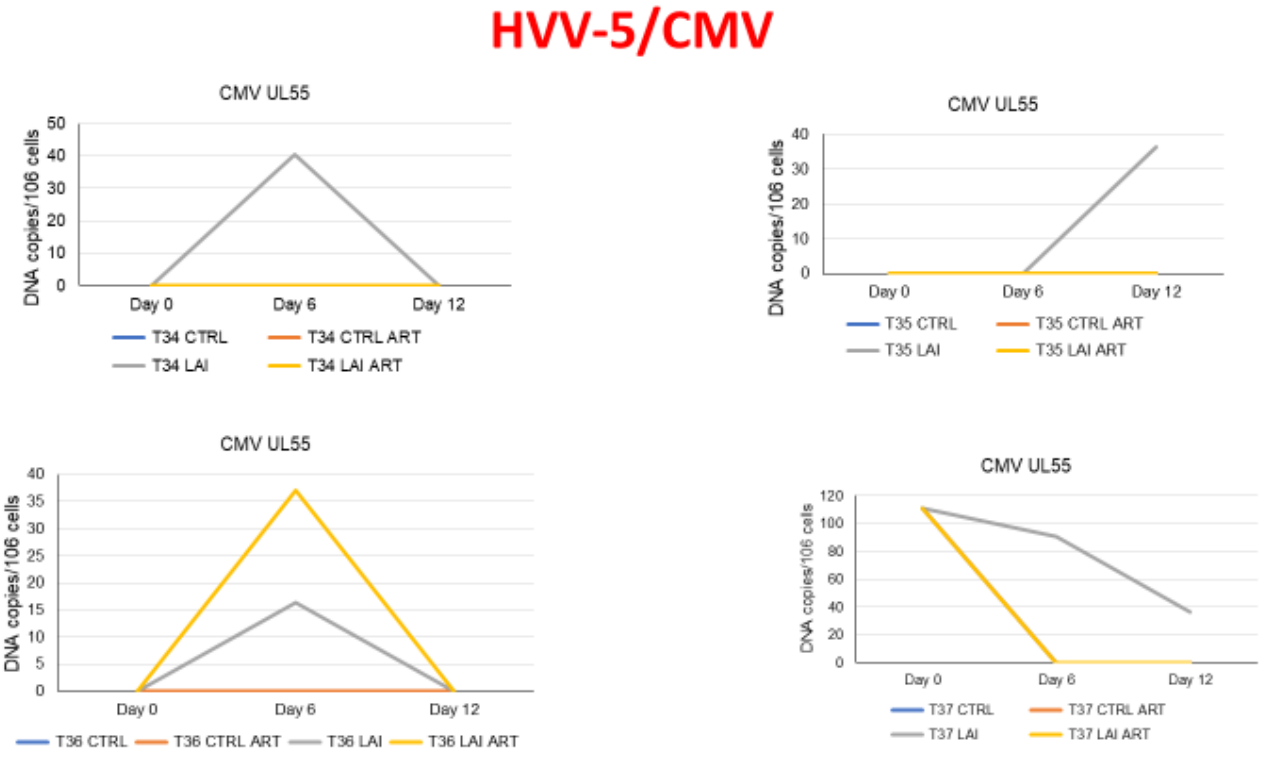

Figure 34: DNA copies were detected by ddPCR of endogenous HVV-5 (also known as hCMV) in 4 independent tissue blocks (T34-T35-T36-T37), at day 0, 6 and 12, in tissue uninfected (CTRL) and infected with $X4_{LAI04}$ (LAI) and treated or not with RTV (ART). DNA copies per number of cells were calculated using RPP30 as a reference.

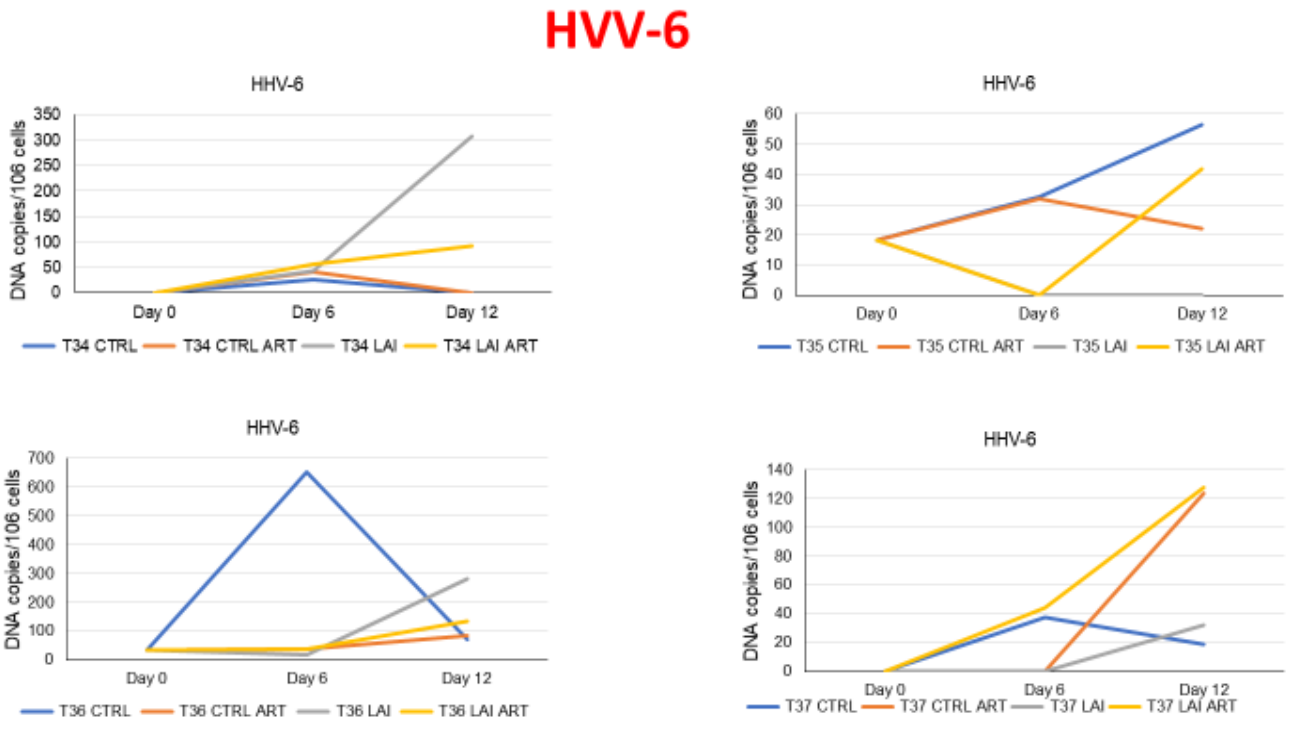

Figure 35: DNA copies were detected by ddPCR of endogenous HVV-6 in 4 independent tissue blocks (T34-T35-T36-T37), at day 0, 6 and 12, in tissue uninfected (CTRL) and infected with $X4_{LAI04}$ (LAI) and treated or not with RTV (ART). DNA copies per number of cells were calculated using RPP30 as a reference.

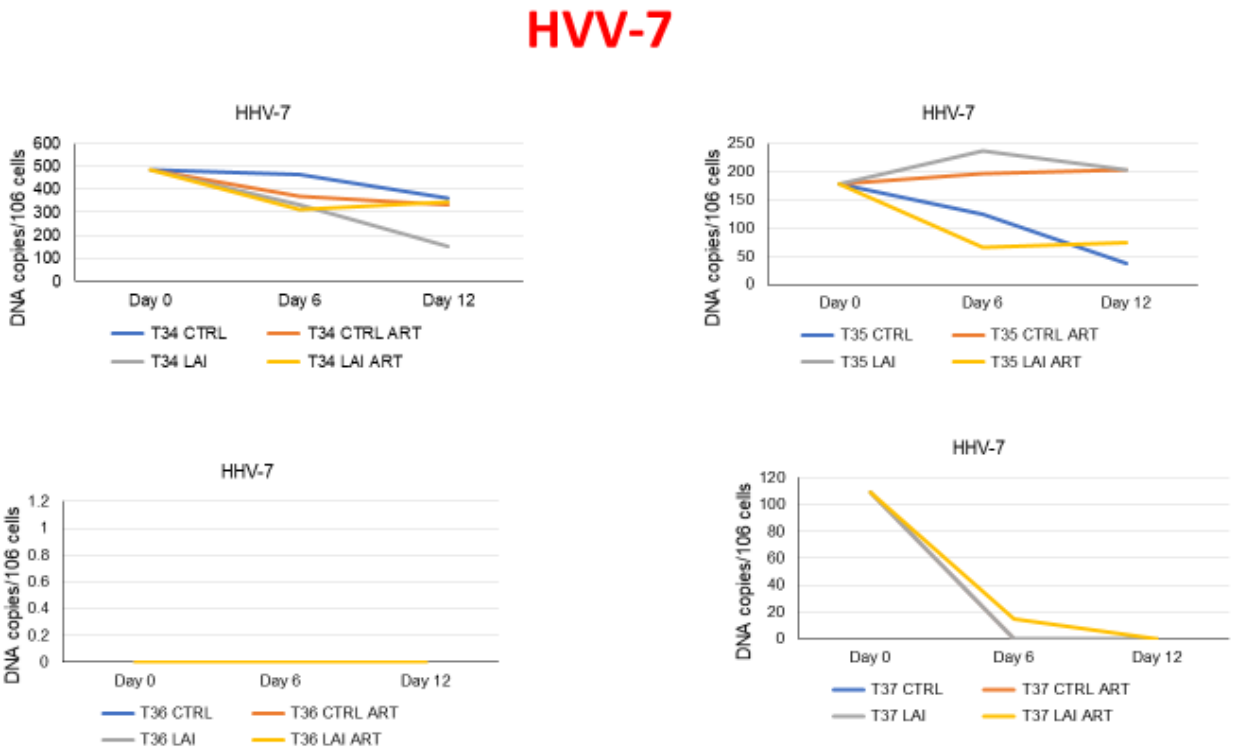

Figure 36: DNA copies were detected by ddPCR of endogenous HVV-7 in 4 independent tissue blocks (T34-T35-T36-T37), at day 0, 6 and 12, in tissue uninfected (CTRL) and infected with $X4_{LAI04}$ (LAI) and treated or not with RTV (ART). DNA copies per number of cells were calculated using RPP30 as a reference.

6.4 ART treatment as a potential cause for immune activation

It has also been hypothesized that ART itself may lead to this immunoactivation, and this *ex vivo* model allows for easy testing of this premise. As previously reported no significant increases in cytokine levels were observed either with ritonavir or with AZT-3TC after maximal exposure to the drugs.

6.4.1 Ritonavir

To rule out the possibility that ART itself, in the absence of HIV-1 infection, affects cytokine levels, we treated uninfected tissues with ritonavir. We compared cytokines released by uninfected tissues treated with ART to those by matched controls.

At the final time point there was a significant decrease only for IL-1α and ITAC (10.1 and 30.8% decreases, respectively, $p<0.05$, $n=8$) (**Figure 37**).

None of these cytokines were among those upregulated following HIV-1-infection. Few cytokines were induced in EV-associated form as well. At day 16, after maximum exposure to ritonavir, we observed significant decreases in IL-16, IP-10 and MCP-1 (21.5 – 39.2% decreases, $n=8$, $p<0.05$) (**Figure 37**).

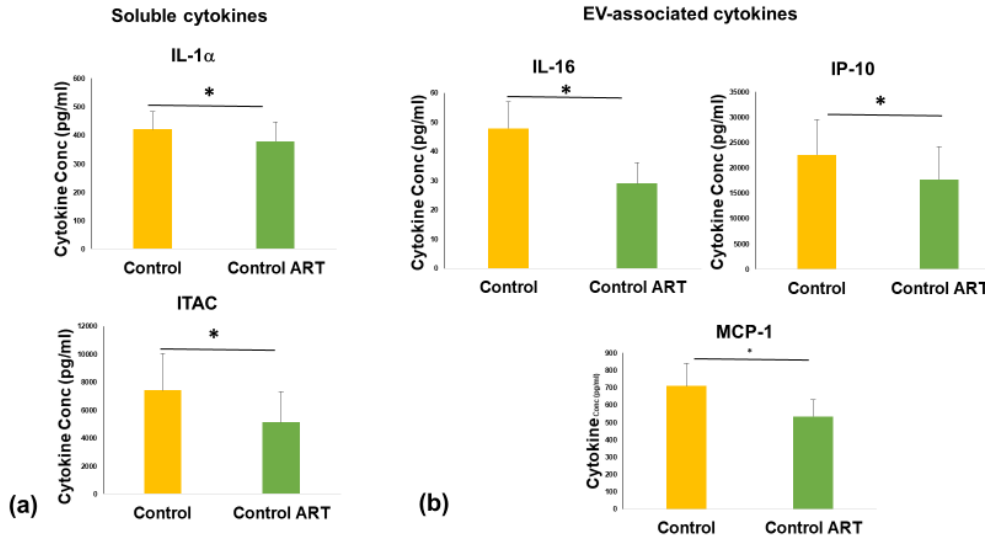

Figure 37: Cytokines in uninfected tissues treated with ritonavir were not upregulated. After 13 days of ART, cytokines were measured by Luminex and their amounts in untreated (control) and matched ritonavir treated (control ART) tissues were compared. **(a)** soluble cytokines **(b)** EV-associated cytokines **(**Mean ± SEM, n=8, *p<0.05).

6.4.2 AZT-3TC

We also evaluated whether AZT-3TC alone could contribute to increase cytokine levels, especially because high concentrations of these compounds may interact with the DNA polymerase used by human cells, in particular by mitochondria [175], resulting in cellular damage and immune activation. As in the ritonavir treatment, we compared cytokines produced by uninfected tissues treated with AZT-3TC to those by matched controls.

At day 15 of culture, after 13 days of ART, no significant increases were observed for any cytokine, in fact the only significant changes were decreases in soluble IL-1β and IL-2 (24.0 and 33.2% decreases, respectively, p<0.05, n=8) (**Figure 38**). In EV-associated

cytokines, the only significant change was a decrease in IL-10 (29.7%, $p<0.05$, n=8) (**Figure 38**).

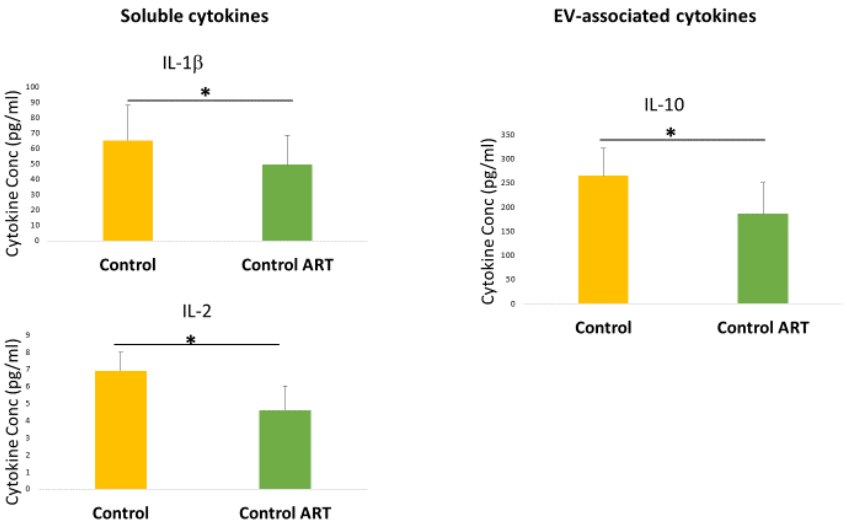

Figure 38: Cytokines in uninfected tissues treated with AZT-3TC were not upregulated. After 13 days of treatment, cytokines were measured by Luminex and their amounts in untreated (control) and matched AZT-3TC treated (control ART) tissues were compared. On the left soluble cytokines (**a**), and on the right EV-associated cytokines (**b**) (Mean ± SEM, n=8, *$p<0.05$).

Thus, in both ART regimens, the treatment itself doesn't show a notable involvement in the persistent immune activation; the most common pro-inflammatory cytokines were maintained to the same level as in control uninfected tissues.

DISCUSSION

Immune activation is now considered as a driving force of HIV-1 disease [176]; however, the mechanisms responsible are not fully understood. Moreover, residual immune activation persists even in those patients in which HIV-1 replication was successfully suppressed by ART for many years. This improper immune activation is associated with the development of various pathologies approximately 15 years earlier than in the control population [177]. Numerous studies have characterized the cytokine modulation observed in HIV-1 infected individuals, from initial infection through chronic disease [178]. These studies demonstrated that HIV-1-triggered immune activation is associated with the upregulation of many cytokines and with the changes in the entire cytokine network [25, 149]. These cytokines that remain upregulated in HIV-1 infected individuals despite viral suppression by ART may contribute to the development of several inflammatory-related disease such as osteoarthritis, heart disease, kidney and liver diseases, metabolic syndrome, dementia, cancer [137]. Understanding the mechanisms prompting immune activation in HIV-1-suppressed patients is paramount to develop efficient therapies able to fully restore the immune systems of patients with treated HIV-1 infection.

Evaluation of cytokines in immune activation has relied on measurement of cytokines in soluble form; however, recently it was demonstrated that they can be associated with EVs, which are considered a new form of cell-cell communication [28]. Furthermore, cytokines can be either on the surface of EVs or carried as internal cargo [28]. The system of EV-encapsulated cytokines, which is not

revealed by traditional assays, may play a significant role in health and disease.

It has become clear that EVs are involved in the progression of pathogenesis of many diseases including cancer, autoimmune disorders, and viral infections. Indeed, in virally-infected cells, especially infected with HIV-1, not only cellular but also viral proteins and RNAs are packaged into EVs and affect the recipient cells [179-183]. A recent study showed that exosome associated cytokines were increased in plasma of HIV-1-positive individuals [184]. It has also been demonstrated that EVs containing viral products are continually released and may cause neurocognitive and immunological dysfunction in HIV-1 infected individuals being treated with several FDA-approved drugs [185]. Furthermore, EVs in ART-treated HIV-1 patients carry proteins related to immune activation and oxidative stress, have immunomodulatory effects on myeloid cells, and may have pro-inflammatory and redox effects during pathogenesis [186].

Further research aimed at elucidating the physiological role of EVs in intercellular communication, immune modulation, and immune surveillance in HIV-1 infection and treatment is necessary to better counteract the viral replication and efficiently treat infected individuals. A laboratory-controlled experimental model is needed to study these mechanisms.

In the present work we used a system of human lymphoid tissues *ex vivo* to study the changes of free and EV-associated cytokine network in HIV-1 infection and to investigate the effects of ART on these processes. *Ex vivo* tissues reproduce the *in vivo*

situation more faithfully than isolated cell cultures, as they maintain tissue cytoarchitecture, which is important for cell-cell interactions. *Ex vivo* lymphoid cultures support productive HIV-1 infection without exogenous cell activation and retain expression patterns of key cell-surface molecules relevant to HIV-1 infection [163, 164]. This tissue model has previously been used to study pathogenesis of HIV-1 and other pathogens [161, 163, 165], HIV-1 immune activation [162, 166], co-pathogen infections [187] and preclinical evaluations of potential antivirals [167, 168, 170].

Ex vivo human lymphoid tissues were productively infected with a prototypic X4 (LAI04) or a prototypic R5 (SF162) HIV-1 over 16 days of infection and treated by ART. Immune activation was evaluated by measurement of 33 cytokines not only soluble cytokines, but also “insoluble” cytokines, namely those associated with EVs. HIV-1 infection, with both $X4_{LAI04}$ and $R5_{SF162}$, triggered a significant upregulation of many soluble cytokines early in infection: IL-1β, IFN-γ, IP-10, M-CSF, MIG, MIP-1α, MIP-1β, RANTES, and TNF-α. Other cytokines were increased for only one strain of the viruses: IL-2, IL-6, IL-8, ITAC and MCP-1 for $X4_{LAI04}$ infection, and GM-CSF with $R5_{SF162}$ infection.

Analysis of cumulative cytokine production over the entire culture length revealed that IL-2, IFN-γ, MIP-1α, MIP-1β and RANTES were increased with both viral strains. Many of these cytokines are typical of the acute TH1 response which protects against intracellular pathogens. In $X4_{LAI04}$ infection, IL-7, IL-18, M-CSF and TNF-α were also significantly increased. In general, these results validate the use

of this model for studying cytokine responses of lymphoid tissues to HIV-1 infection.

Evaluation of EV-associated cytokines demonstrated that some of the same cytokines upregulated in soluble form were also upregulated in EV-associated form. The β-chemokines (MIP-1α, MIP-1β, and RANTES), in particular, were consistently upregulated throughout infection with both virus strains in soluble and EV-associated forms. However, several cytokines (IL-1α, IL-6, IL-13, IL-21, IL-33, GM-CSF and TGF-β) were uniquely upregulated in the EV form, particularly upon $X4_{LAI04}$ infection.

The majority of cytokines that are EV-associated are located on the EV surface, but certain cytokines are also found inside EVs, including IL-4, IL-10, IL-13, IL-15, IL-18, IL-33, Calg A and RANTES. Several cytokines were observed to shift distribution early during HIV-1 infection toward increased soluble compared to EV associated (RANTES and TNF-α for both virus strains), as well as toward increased EV surface compared to EV internal (RANTES). During late days of infection, significant increases were noted in EV-associated IL-2, IFN-γ, M-CSF, MIP-1α and TNF-α cytokines in $X4_{LAI04}$ infection, whereas we observed a shift to more soluble TNF-α in $R5_{SF162}$ infection.

These different forms of cytokines may exert different actions, for example they may act on different cells or activate different pathways. EV surface cytokines may address particular EVs with a definite cargo to specific target cells [28, 188]. Different forms of cytokine may contribute either to host defence or viral spread. For

example, EVs released by HIV-infected cells may contain viral envelope proteins (Env) [189] and these EVs facilitate HIV-1 infection [190], or could influence non-infected and uninfectable bystander cells through the release of negative regulatory factor (Nef) inside vesicles [191].

After establishing that the *ex vivo* human lymphoid tissue model successfully recapitulates immune activation during HIV-1 disease *in vivo*, we next moved to studying antiretroviral treatment of these infected tissues. It has already been established *in vivo* that low-level immune activation persists in patients successfully treated with antivirals. Various hypotheses were formulated about the causes supporting continuous immune activation; however, none were convincingly validated [137, 192] in part because of the complexity of the system and limitations of experimental approaches *in vivo*.

Here, the phenomenon of residual immune activation after successful ART is reproduced in our *ex vivo* human lymphoid tissues, opening a new strategy to study this phenomenon under controlled laboratory conditions. ART (RTV) suppressed productive HIV-1 infection in this model, yet many soluble cytokines remained upregulated after 13 days of ART: soluble IL-2, IL-18, IL-21, MIP-1α, MIP-1β, and RANTES (for both $X4_{LAI04}$ and $R5_{SF162}$ infections), Calg A (exclusively for $X4_{LAI04}$ infection), and IL-7, IL-13, and IL-33 and TNF-α (only for $R5_{SF162}$ infection). Only soluble IL-18 significantly decreased after 13 days of ART treatment for both $X4_{LAI04}$ and $R5_{SF162}$ infections, and MIP-1α for $R5_{SF162}$ infection. IL-18 is produced by monocytes and macrophages and is able to enhance IFNγ production and T cell

immune responses. It has been described as a defensive cytokine capable of inhibiting intracellular infections [193]. Notwithstanding, in HIV-1 infection IL-18 may play an ambivalent function. IL-18 is able to counteract HIV-1 infection in PBMCs [193], but it is also able to facilitate HIV-1 replication by upregulating CXCR4 HIV-1 coreceptor expression and the pro-apoptotic mediator TRAIL. For these reasons, IL-18 was pointed to one of the factors responsible for the immunodeficiency and immune activation processes in HIV-1-infected patients. While successful HAART is accompanied by a decrease in IL-18 levels, virologic treatment failure is associated with persistently raised IL-18 serum levels [194].

EV-associated cytokines tended to be more increased late in HIV infection compared to soluble cytokines, and this pattern was seen with RTV treatment as well. None of the EV-associated cytokines that remained upregulated in HIV-1 infected tissues were significantly decreased by ritonavir treatment. Fourteen of 16 EV-associated cytokines elevated in HIV-1 infection remained elevated after 13 days of ritonavir for $X4_{LAI04}$ infection, and 6 of 10 remain elevated for $R5_{SF162}$.

Ritonavir, a protease inhibitor, is one of several antiretroviral drugs already in use in the HAART regimens. It has been reported that despite the use of reverse transcriptase inhibitors, HIV-1 is able to evade ART and spread by exploiting cell-to-cell interactions [195]. An *in vitro* study suggested that different drugs may have a different impact on HIV-1 cell-to-cell spread, which could influence its replication [196]. For example, protease inhibitors successfully arrested the cell-to-cell spread of HIV-1 among T lymphocytes [196].

Another study demonstrated that despite inhibition with protease inhibitors, HIV-1 products are constantly released in association with EVs [185]. Release of these viral products may contribute to immune/cytokine responses. Taking into account these published results, to further confirm our analysis, we explored the effects of alternate antiretroviral compounds, in particular a combination of AZT and 3TC. Both these compounds belong to the NRTIs antiretroviral group and are used in combination in the current HAART [174].

Evaluation of cytokine production with AZT-3TC treatment of HIV infected lymphoid tissues confirmed that tissues remain immune-activated with this second ART regimen also. In particular, in the context of $X4_{LAI04}$ infection 4 of the 6 increased soluble cytokines remained elevated after AZT-3TC treatment. In $R5_{SF162}$ infection, 2 of 10 soluble cytokines which were elevated in infection were still raised after AZT-3TC treatment.

As with ritonavir, EV-associated cytokine release was only marginally modified AZT-3TC usage. In particular, in $X4_{LAI04}$ infection 12 out of 14 EV-associated cytokines remained upregulated after 13 days of AZT-3TC therapy. Of the six cytokines elevated in $R5_{SF162}$ infection, 2 remained upregulated with AZT-3TC. These data again demonstrate a pattern of EV-associated cytokines being less likely to be restored by ART than the soluble ones.

Another finding was that AZT-3TC treatment of $X4_{LAI04}$ and $R5_{SF162}$ infected tissues resulted in a significant increase in soluble IL-6 compared to untreated infected tissues. EV-associated IL-6 was also

increased in $X4_{LAI04}$ infected tissue treated with AZT-3TC. Soluble and EV-associated IL-6 upregulations were observed in ritonavir treated $X4_{LAI04}$ infected tissues, though these differences did not reach significance. These results are in line with those reported in *in vivo* studies where high IL-6 levels were observed in patients treated with NNRTI [197].

AZT-3TC treatment led to significant decreases of soluble α-chemokines (IP-10, ITAC, and MIG), and EV-associated IP-10 (other α-chemokines were decreased but did not reach significance) compared to infection alone. Similar trends were observed with ritonavir treatment as well. It has been reported that in HIV-1 infected individuals receiving AZT-3TC therapy, IP-10 is reduced after successful treatment, while it increases in those individuals failing in therapy [198]. This information together with the IL-18 decrease in ritonavir treatment, demonstrate the proficiency of different drugs in *ex vivo* model [199].

Cytokine upregulation and the ability of ART to restore cytokine levels to baseline was strain dependent in these experiments. While $X4_{LAI04}$ infects a wide population of CD4+ T cells, $R5_{SF162}$ infects only a subpopulation of these cells that express CCR5 [163]. Commonly, R5 is responsible for and dominates early infection while X4 emerges later, usually during the chronic infection [200]. The reason why one strain appears most in late infection and the other is more a “gatekeeper” in the early stage of infection is still not well deciphered; disclosing this “mystery” may be important to properly drive the treatment of HIV-1 infected individuals at different phases of disease.

In our *ex vivo* model, infection with $X4_{LAI04}$ resulted in significantly higher levels of many cytokines compared to the $R5_{SF162}$, most notably pro-inflammatory cytokines like IL-2, IL-7, IFNγ, M-CSF, TNF-α, and β-chemokines like MIP-1α, MIP-1β and RANTES. This pattern was more evident with EV-associated cytokines. IL-2, IL-6, IL-7, IL-21, IFN-γ, M-CSF, MIP-1α, MIP-1β, RANTES and TNF-α were significantly upregulated in $X4_{LAI04}$ infection compared to $R5_{SF162}$ infection. The β-chemokines (MIP-1α, MIP-1β, and RANTES) were also less likely to be restored when infection was suppressed by ART in $X4_{LAI04}$ compared to $R5_{SF162}$. These results confirmed that the X4 tropic virus is more "aggressive" and for this reason triggers a stronger inflammation status, upregulating a higher number of cytokines compared to R5 tropic virus.

To my knowledge this is the first study describing higher levels of specific EV-associated chemokines upon X4 virus infection, not only compared to the uninfected condition, but also compared to R5 virus infection, both in treated and untreated conditions. These data show that increased levels of these EV-associated chemokines are correlated with higher immune activation and inflammation that could impact HIV-1 infection as well. Therefore, designing new drugs that control their levels could be advantageous to prevent immune activation in HIV-1 positive patients.

While the *ex vivo* model of human lymphoid tissue replicates many aspects of the *in vivo* situation, it also has obvious limitations. For example, unlike *in vivo,* where immune activation can be traced for years in individuals with suppressed HIV-1 infection, these *ex vivo*

experiments are limited to two to three weeks for preservation of tissue cytoarchitecture. Despite this limitation, the *ex vivo* lymphoid tissue model does mimic aspects of immune activation that persist despite viral control.

This *ex vivo* system allows the investigation of other determinants that can be associated with immune activation which are difficult to investigate *in vivo*. These possible factors include: cytokine storm leading to changes in normal setpoints; undetectable viremia in tissues, not reflected in blood; other viral infections (i.e. HCV, CMV), which can be easily reactivated in the HIV-1 infected population [201-204]; and side effects induced by ART itself [205].

It has been hypothesized that an initial cytokine storm may influence and stimulate an immune-inflammatory response, changing the normal setpoint, which despite ART may persist and may not be reversed [137]. To test this possibility, cytokine storms were simulated by treating tonsillar *ex vivo* tissues with exogenous cytokines (those which were shown in the previous experiments to be upregulated after HIV-1 infection). Several cytokines were tested singly or in combination for their ability to initiate a cycle of upregulation of other cytokines. This addition of exogenous cytokines resulted in temporary increases of a few cytokines, but they were not sustained over the length of culture; by day 12 all cytokine levels were similar to those in control tissues.

Therefore, these data do not confirm the hypothesis that an initial cytokine increase may affect future and persistent release of cytokines. Analogous to what happens *in vivo*, where cells

immediately use the cytokines or these are cleared from the extracellular versant, the *ex vivo* model allows clearance of these extra metabolites every change of medium (every 3 days), and cell activation does not persist.

Next, we tested the hypothesis that the presence of virions without viral replication is enough to trigger sustained cytokine upregulation using a replication incompetent virus, AT-2 inactivated $X4_{LAI04}$. AT-2 is a mild oxidizing reagent, which eliminates the infectivity of HIV while maintaining its structure and ability to be processed for presentation to T cells.

In the *ex vivo* lymphoid tissue, defective virions were able to trigger an initial cytokine release similar to that observed with infectious virus. In detail, a single inoculation of AT-2 virions increased the levels of IL-4, IL-21, ITAC, MIP-1α, MIP-1β, RANTES, and TNF-α early in virion exposure. And many of the cytokines (7 of the 13) that were upregulated late in HIV infection remained elevated with inactivated virus.

To further address this possibility, repeated exposures to AT-2 inactivated virus were also tested. This recurring exposure to inactivated virus triggered a cytokine response even more similar to actual HIV-1 infection. In these experiments, 9 of 12 cytokines increased with infectious virus also remained elevated with inactivated virus. Five of these were even higher with inactivated virus (IL-21, MIP-1α, MIP-1β, RANTES, and TNF-α).

These results suggest that the presence of viral particles (albeit large amounts of virus in these experiments), without productive

infection, may be sufficient to trigger a response similar to the infectious virus. Furthermore, it has already been shown that AT-2 inactivated virus does not kill cells, therefore the surviving CD4+ T cells may be persistently immune stimulated due to the presence of virions. Therefore, these defective virions may support a consistent and perpetual cytokine release.

Another potential mechanism involved in the persistent state of immune activation is the presence co-pathogens due to immunodeficiency as a result of HIV-1 infection. It has been described that latent co-infecting viruses, in particular herpesviruses which in healthy individuals are silent, may be reactivated and lead to cytokine upregulation. This hypothesis was tested by measuring the endogenous presence of several herpesviruses (HSV-2, EBV, CMV, HHV-6, and HHV-7). Using a ddPCR technique, DNA copies of different herpesviruses were detected in tonsillar tissue blocks. Data showed donor to donor tissue variability, but overall no strong pattern of reactivation was observed. This suggests that immune activation in *ex vivo* lymphoid tissues is not directly correlated with herpesvirus re-activation and replication.

Finally, we also hypothesized that ART itself may trigger immunoactivation. To test the possibility that ART itself, in the absence of HIV-1 infection, affects cytokine levels, we treated uninfected tissues with either ritonavir or AZT-3TC. No significant cytokine increases were observed for either compound, only a few decreases. Ritonavir was able to induce a minor decrease of soluble IL-1α and ITAC, and EV-associated IL-16, IP-10 and MCP-1. AZT-3TC

decreased soluble IL-1β and IL-2 and EV-associated IL-10. Thus, in both ART regimens, the drugs themselves did not trigger persistent immune activation.

CONCLUSION

In conclusion, our analyses showed that HIV-1 infected *ex vivo* lymphoid tissues upregulate the production of numerous cytokines, both in soluble and EV-associated forms, which in general, follow the same upregulation pattern. The levels of these cytokines remain upregulated despite suppression of viral replication by two different antiretroviral regimens. EV-associated cytokines were less likely to be restored back to levels of control tissues, compared to soluble cytokines, and this was most striking with the β-chemokines. Different strains of HIV-1 induced similar patterns of cytokine responses; however, the X4 tropic strain induced stronger cytokine increases than the R5 tropic strain, which were less likely to decrease with ART.

We observed a few significant shifts between soluble and EV-associated cytokine compartments with HIV-1 infection, as well as between the EV surface or internal space. RANTES, in particular, showed a specific pattern in early infection; it shifted to a more soluble form, and in association with EVs, its presence shifted to more on the surface.

In general, these results are consistent with the findings *in vivo* [206] and demonstrate that HIV-1 infected *ex vivo* lymphoid tissues provide an adequate *in vivo*-relevant model to study immune activation in ART-treated HIV infection.

The *ex vivo* lymphoid tissue system allowed the testing of several hypotheses on the possible mechanisms involved in persistent inflammation. These results established that in this *ex vivo* tissue system the HIV-1-triggered immune activation is:

I. independent from an initial cytokine storm which may change the basal setpoint.

II. influenced by the presence of viral particles, which in absence of productive infection, may trigger a response similar to infectious virus.

III. not directly correlated with the presence of endogenous herpesviruses (HSV-2, EBV, CMV, HHV-6, and HHV-7).

IV. not affected by ART by itself (ritonavir or AZT-3TC), which in the absence of HIV-1 infection, does not increase cytokine levels.

Future investigations into the role played by EV-associated cytokines and the mechanisms of the residual immune activation in the described *ex vivo* system under controlled laboratory conditions may help to decipher the phenomenon of the persistent immune activation and lead to the development of new therapeutic strategies aimed at controlling or modulating cytokine production.

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SCIENTIFIC PRODUCTS

List of scientific products from January 2017 to September 2019

Publication:

- Persistent immune activation in HIV-1 infected *ex vivo* model tissues subjected to antiretroviral therapy: Soluble and extracellular vesicle-associated cytokines.
  Mercurio V*, Fitzgerald W*, Molodtsov I, Margolis L (***accepted for publication in JAIDS***)

- Evaluation of salivary and plasma microRNA expression in patients with Sjögren's syndrome, and correlations with clinical and ultrasonographic outcomes.
  Talotta R*, **Mercurio V*,** Bongiovanni S, Vittori C, Boccassini L, Rigamonti F, Batticciotto A, Atzeni F, Trabattoni D, Sarzi-Puttini P, Biasin M.
  Clin Exp Rheumatol. 2019 May-Jun;37 Suppl 118(3):70-77. Epub 2019 Jan 10.

- Interleukin 21 (IL-21)/microRNA-29 (miR-29) axis is associated with natural resistance to HIV-1 infection.
  Ortega PAS, Saulle I, **Mercurio V**, Ibba SV, Lori EM, Fenizia C, Masetti M, Trabattoni D, Caputo SL, Vichi F, Mazzotta F, Clerici M, Biasin M.
  AIDS. 2018 Nov 13;32(17):2453-2461.

- Analysing the role of STAT3 in HIV-1 infection.
  S.V. Ibba, C. Fenizia, P. Serna Ortega, **V. Mercurio**, I. Saulle, E.M. Lori, D. Trabattoni, M. Clerici, M. Biasin. JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS. - ISSN 0393-974X. - 33:5(2019 Sep 06).

Under submission:

- Evaluation of the Effect of Protective Genetic Variants on cART Success in HIV-1-infected Patients.
  E. Lori, A. Cozzi-Lepri, **V. Mercurio**, S. V. Ibba, S. Lo Caputo, F. Castelli, A. Castagna, A. Gori, G. Marchetti, C. Venditti, M. Clerici, A. D'Arminio Monforte, M. Biasin (***In submission***)

- Dual-targeted anti-CMV/anti-HIV heterodimers
  C. Vanpouille, **V. Mercurio**, Meghan Delaney, R. Palomino, L. Margolis. (***In submission***)

Congresses:

- IAS 2019, 21-24 July 2019, Mexico City (Mexico). "*Extracellular vesicle-associated cytokines in HIV-infected human ex vivo tonsils*". Poster **presenter**.
- IAS 2019, 21-24 July 2019, Mexico City (Mexico). "*Sterol metabolism modulates susceptibility to HIV-1 Infection in HESNs*". Poster **presenter**.
- IAS 2019, 21-24 July 2019, Mexico City (Mexico). *"Endoplasmic reticulum associated aminopeptidases 2 (ERAP2) is released in the secretome of activated MDM and reduces in vitro HIV-1 infection*". Poster.
- Exosomes, Microvesicles, and Infectious Disease 2019, May 31-June 1, 2019, POTOMAC (Maryland, USA). "*Extracellular vesicle-associated cytokines in HIV infected human ex vivo tonsils*". **Speech presenter.**
- ISEV 2019, 24-28 April 2019, Kyoto (Japan). "*Extracellular vesicle-associated cytokines in HIV infected human lymphoid tissue ex vivo*". Poster **presenter.**
- CROI 2019, 4-7 March 2019, Seattle (USA)."*EXTRACELLULAR VESICLE–ASSOCIATED CYTOKINES IN HIVINFECTED HUMAN EX VIVO TONSILS*". Poster **presenter**.
- ICAR 2018, 22 May 2018, Rome (Italy). "*ANALYSES OF PROTECTIVE GENETIC POLYMORPHISMS IN HIVINFECTED CART TREATED PATIENTS*." Poster.

- HIVR4P 2018, 21-25 October 2018, Madrid (Spain). *“Evaluation of Protective Genetic Variants in HIV-1-Infected cART Treated Patients*”. Poster.
- 9th IAS Conference on HIV Science, 23-26 July 2017, Paris (FRANCE). "*INTERLEUKIN-21/microRNA-29 AXIS IN NATURAL RESISTANCE TO HIV-1 INFECTION*". Poster.
- 9th ICAR Congress, 12-14 June 2017, Siena. "*INTERLEUKIN - 21/microRNA-29 AXIS IN NATURAL RESISTANCE TO HIV-1 INFECTION*". Poster.

AWARDS

- ✓ CROI-ICAR Awards 2019 for Young Italian Investigators. Seattle, Washington, U.S.A., March 6, 2019 and Milan, Italy, June 5, 2019.

- ✓ Young Investigator Scholarship to attend the 2019 Conference on Retroviruses and Opportunistic Infections (CROI 2019), Seattle, Washington, U.S.A., 4-7 March 2019. Awarded by the International Antiviral Society (IAS)-USA.

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RESEARCH INTEGRITY

I, Vincenzo Mercurio, declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

I confirm that this thesis presented for the doctoral course in Molecular and Translation Medicine at University of Milan, has:

- been composed entirely by myself
- been solely the result of my own work
- not been submitted for any other degree or professional qualification

Parts of this work on 10th September 2019 were submitted and are currently under reviews in Journal of AIDS.
Results reported in this work comply with the four fundamental principles of research integrity of The European Code of Conduct for Research Integrity (ALLEA, Berlin, 2018):

- Reliability in ensuring the quality of research, reflected in the design, the methodology, the analysis and the use of resources;
- Honesty in developing, undertaking, reviewing, reporting and communicating research in a transparent, fair, full and unbiased way;
- Respect for colleagues, research participants, society, ecosystems, cultural heritage and the environment;
- Accountability for the research from idea to publication, for its management and organization, for training, supervision and mentoring, and for its wider impacts.

I have no financial disclosure or conflict of interest to declare.