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Utilizing cytotoxic lymphocytes for indirect shock-and-kill strategy in HIV-1 treatment

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Abstract

Despite the existence of a treatment, there is still not a cure for HIV-1 infection and there are around 700 000 deaths per year from AIDS-related diseases. A major barrier for a cure is the establishment of latent reservoirs that are impossible to distinguish from healthy cells and thus can escape the immune system. One potential solution is called shock-and-kill strategy, which aims to induce HIV-1 reactivation, exposing latently infected cells to the immune system and making them susceptible to cell death. In our lab, it was seen that when NK cells are stimulated with a pan-caspase inhibitor, they acquire the "shock" ability, but it is still unknown how. In this project, we observed that the supernatant from pan-caspase inhibitor-stimulated NK cells can increase HIV-1 reactivation in two different latency models. Furthermore, the protein levels of three HIV-1 suppressors were found to be increased in the same supernatant. For this reason, their effect in HIV-1 reactivation in latently infected cells was analysed. Although we did not observe an increase in HIV-1 reactivation, the upregulation of these three proteins can be useful in the clinical context. Since they are HIV-1 suppressors, their presence can prevent the infection from spreading after latent cells are reactivated. Altogether, our results show that NK cells stimulated with a pan-caspase inhibitor are secreting a biological product that induces HIV-1 reactivation. This indicates that there is a pathway in NK cells that can potentially be exploited in order for them to be able to induce HIV-1 reactivation.

Key words:
HIV-1, Shock-and-kill strategy, NK cells, latency reservoir, HIV-1 treatment.

Popular science summary

Can we cure HIV using our immune cells?

HIV is a virus that infects specific cells of our immune system and, if the infection is left untreated, leads to acquired immunodeficiency syndrome (AIDS). This syndrome is characterised by a failure of the immune system to control HIV infection, which in turn results in opportunistic infections that can potentially lead to death. Despite the existence of a treatment for HIV infection, there are around 700 000 deaths every year caused by AIDS-related diseases and there have been 35 million deaths since the beginning of the HIV pandemic.

As of today, HIV infection is incurable: the existent life-long treatment prevents the progression of disease, but it does not cure the infection. At some point after the initial infection, the virus hides within the cells, becoming inactive and invisible for the immune system or impossible to target using drugs. These inactive infected cells create the latency reservoirs and are a major barrier to the cure of HIV infection. One potential solution for this problem is called the shock-and-kill strategy. This strategy aims to force the latently infected cells to become active, exposing them to the immune system and making them susceptible to cell death. In our lab, it was observed that when a specific type of immune cells (called NK cells) has specific proteins inhibited, it obtains the ability to induce HIV reactivation. Therefore, our aim with this project was to understand how this can happen.

The proteins being inhibited are called caspases and, to inhibit them, we use a pan-caspase inhibitor. We first wanted to know if NK cells stimulated with a pan-caspase inhibitor are secreting a biological product that induces HIV reactivation and, for that reason, latently infected cells were cultured with the supernatant of stimulated NK cells. This experiment led to an increase in HIV reactivation, showing that NK cells are secreting an important biological product. To identify what that product could be, the production of several proteins was analysed and the protein levels of three HIV suppressors were found to be increased in the supernatant of NK cells stimulated with a pan-caspase inhibitor. Thus, latently infected cells were stimulated with these three proteins, alone or in different combinations. There was no effect in HIV reactivation, which means that the three HIV suppressors do not contribute to viral reactivation.

Our results prove that the inhibition of caspases within NK cells changes the way they behave, making them secrete a biological product that can induce HIV reactivation. Even though higher production of the three HIV suppressors does not increase HIV reactivation, it can be useful in the clinical context to prevent the infection from spreading after latent cells are reactivated. As for future work, it is important to identify what NK cells are secreting, in order to better understand how to use this strategy for a potential HIV cure.

Introduction

HIV

Retroviridae is a family of viruses that are found in all studied vertebrates and that use an RNA reverse transcriptase (RT) during their replication cycle. Their genome consists of two copies of positive sensed ssRNA (+ssRNA) that are contained by an icosahedral capsid and an outer membrane made from their previous host cell lipid membrane and retroviral glycoproteins. Furthermore, all retroviruses have three essential genes: *gag*, which encodes for the matrix, the capsid and the nucleocapsid proteins; *pol*, encoding for the protease, the RT and the integrase proteins; and *env*, which encodes for the surface envelope (SU) and the transmembrane envelope (TM) glycoproteins, which are part of the envelope. Complex retroviruses also encode for additional genes that have a function during replication. Viruses from this family can be transmitted horizontally, by infectious exogenous virions, or vertically, through provirus DNA integrated in germ line cells.[1]

The retroviral replication cycle starts when the virus binds to the host receptor through the SU glycoprotein. This binding leads to conformational changes in the envelope proteins and fusion of membranes, which is mediated by the TM glycoprotein. After internalization of the virus and uncoating of the capsid, the +ssRNA is transcribed into a linear dsDNA by the RT in the cytoplasm. This enzyme lacks proofreading and jumps between the two RNA templates of the retroviruses, which explains their high recombination rate. Most retroviruses can only infect dividing cells because they require the host cell to go into mitoses for the breakdown of the nuclear membrane. At that point, the dsDNA can enter the nucleus, where the proviral integration into the host cell genome takes place. After integration, the provirus replicates with the host cell genome, resulting in daughter cells that have the viral genome integrated. Additionally, the transcription of the provirus by the host RNA polymerase II produces spliced and unspliced RNAs that are transported to the cytoplasm in order to be translated into all the viral proteins. Following translation, there is packaging of the viral RNA genome and assembling of the virion at the host cellular membrane and, finally, budding through the plasma membrane to release the virions. Once the virions are in the extracellular space, the viral polyproteins within the virions are cleaved and the virions become infectious.[1,2]

The human immunodeficiency virus type 1 (HIV-1) belongs to the genus *Lentivirus*. Viruses from this genus infect many vertebrate species and are characterized by having a long incubation period, as well as long-duration infections. However, unlike most retroviruses, HIV-1 does not require its host cells to be in mitoses in order to enter the nucleus, which means it can also infect non-dividing cells. Regarding its tropism, HIV-1 infects CD4-positive (CD4+) cells, using CXCR4 or CCR5 as coreceptors and thus infecting Helper T cells or Macrophages, respectively. The course of an untreated HIV-1 infection can normally be divided into three phases: acute infection, clinically latent phase and, eventually, acquired immunodeficiency syndrome (AIDS). During the acute phase, which typically lasts for around a month, patients can experience mononucleosis or influenza-like symptoms and there is a significant number of CD4+ T cells, both infected and uninfected, that go through apoptosis. Virus titers decrease because of the immune response of cytotoxic lymphocytes and antibodies, leading to the clinically latent phase that has a variable duration. During this period, individuals have no symptoms but there is still viral replication[3], this being the reason why there is a gradual depletion of circulating Helper T cells, eventually leading to AIDS. In this stage, the immune system is failing to control the infection and there is a high viremia, resulting in various opportunistic infections that could potentially lead to death.[1]

Existent treatment and latency reservoir

Despite the existence of a treatment, there have been 35 million deaths since the beginning of the HIV pandemic[4]. In 2019, there were around 38 million people living with HIV infection and 700 000 deaths from AIDS-related diseases[4].

The treatment for HIV-1 infection, which is called antiretroviral therapy (ART), has a life-long duration, several side effects, is expensive, and requires compliance from the patient in order to work properly. ART prevents the progression of disease, but it does not cure HIV-1 infection, as there is viral rebound when the treatment is stopped[5–7]. This happens due to the establishment of latent reservoirs which are, for this reason, a major barrier to the cure of HIV infection[8]. Latently infected cells do not express any different protein from healthy ones, making it impossible for the immune system and for drugs to specifically target infected cells[9].

After HIV-1 integration, the provirus can be silenced through mechanisms that are not completely known or understood. Although it has been proposed that the return of Helper T cells to their resting stage induces latency, there is also evidence that HIV-1 silencing can happen stochastically[10,11]. Breaking HIV-1 latency means reactivating transcription of the provirus, which presumably can happen by activating transcription factors or through epigenetic modulation. In the U3 region of the HIV-1 long terminal repeat (LTR) promoter there are binding sites for several transcription factors: NF-kB, NFAT, Sp1, LEF, Ets-1, and USF[1]. Thus, the stimulation of latently infected cells in order to activate pathways that lead to the activation of these transcription factors could lead to HIV-1 reactivation. Regarding epigenetic modulation, DNA methylation and histone post-translational modifications can regulate HIV-1 latency[12].

Shock-and-kill strategy

One potential solution for curing HIV-1 is called shock-and-kill strategy (**Figure 1**, black arrows), which aims to first induce HIV-1 reactivation through a latency reversal agent (LRA) – "shock"[12–14]. When the infection is reactivated, there is viral replication and the infected host cell starts to express viral glycoproteins on its surface, exposing itself to the immune system[12–14]. At this point, the previously latently infected cell becomes susceptible to cell death, which can happen due to the cytopathic effects derived from viral replication, therapeutic strategies and/or cytotoxic lymphocytes detecting and killing these cells – "kill"[12–14]. The goal of this strategy is to gradually kill all the latently infected cells from the patient, thus eliminating the latency reservoirs and curing HIV-1 infection.

Although LRAs have promising *in vitro* results, they have not yet been successful in reducing latent reservoirs *in vivo*[15–19]. Some of the LRAs that have been studied are Protein kinase C agonists (for example, prostratin), PRR Agonists, Immune Checkpoint Inhibitors, Transient CD8+ Lymphocyte depletion, amongst others[12]. These did not work for different reasons which include toxicity for the patient, absent or insufficient reduction in the latency reservoir, inconsistent efficacy and autoimmune-related side effects[12].

Indirect shock-and kill strategy

In our lab, it was seen that Natural Killer (NK) cells could induce HIV-1 reactivation when stimulated with a pan-caspase inhibitor, which led to the idea that cytotoxic immune cells, specifically NK cells, could be used for a modified shock-and-kill strategy. Whereas through the typical strategy an LRA stimulates the reactivation of HIV-1 infection directly in the latently infected cells (**Figure 1**, black arrows), through this indirect approach NK cells stimulated with a pan-caspase inhibitor will be inducing HIV-1 reactivation (**Figure 1**, red arrows). The hypothesis this project is based on is that inhibiting one or several caspases within NK cells modulates their activity, making them acquire the "shock" ability.

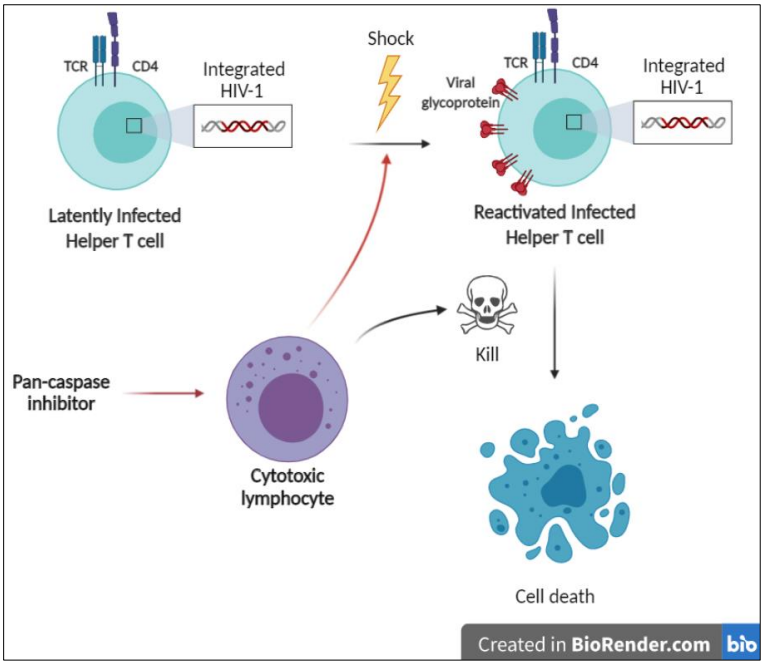

Figure 1: Indirect shock-and-kill strategy. This strategy aims to induce HIV-1 reactivation, which exposes latently infected cells to the immune system and makes them susceptible to cell death. In our lab, it was seen that natural killer cells can acquire the "shock" ability when stimulated with a pan-caspase inhibitor. Black arrows illustrate the typical shock-and-kill strategy. Red arrows illustrate the indirect approach - the principle of this project. CD4, cluster of differentiation 4. TCR, T-cell receptor. This illustration was created with BioRender.com.

As mentioned before, HIV-1 latently infected cells have no different biomarker to discriminate them from healthy cells, meaning that there is no way of specifically targeting these cells. This implies that LRAs must be given systemically to the patient and probably in a higher dose than what would be necessary in order to ensure they reach the latency viral reservoirs. On the other hand, NK cells can be easily targeted and we could administrate only the required concentration of the drug, reducing the side effects and the cost associated to the treatment.

Moreover, NK cells are cytotoxic lymphocytes belonging to the innate immune system. Whereas cells from the adaptive immune system need to detect the major histocompatibility complex (MHC) in the surface of an infected cell in order to kill it, NK cells can kill stressed cells without this need, creating a faster response. Therefore, NK cells could potentially also perform the "kill" step after HIV-1 infection is reactivated.

Aim

The hypothesis of this project is that the inhibition of one or several caspases within NK cells modulates their activity in a way that they acquire the "shock" ability of the shock-and-kill strategy. As it was seen in our lab that NK cells could reactivate HIV-1 infection when stimulated with a pan-caspase inhibitor, the aim of this work was to **map the mechanisms** by which NK cells can induce HIV-1 reactivation.

Materials and methods

Cell culture

Cells were cultured at 37 ºC in a 5 % (v/v) CO_2 humidified atmosphere. The T lymphocyte cell line J-Lat 10.6 (HIV Reagent Program) was maintained in Roswell Park Memorial Institute 1640 (RPMI, Sigma) medium supplemented with 10 % FBS, 20 U/mL penicillin combined with 20 mg/mL streptomycin (Sigma) and 25 mM HEPES. KHYG-1 cells (DSMZ, #ACC725) were maintained in the same conditions and 100 units/mL of recombinant human interleukin-2 (IL-2, PeptroTech). TZM-bl reporter cells (HIV Reagent Program) were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10 % fetal calf serum (Sigma), 2 mM L-glutamine (Sigma), 0.1 mM MEM nonessential amino acids (Gibco), and 20 U/mL penicillin combined with 20 g/mL streptomycin.

In order to use primary NK cells, PBMCs were isolated from buffy coats of anonymous blood donors using Ficoll density centrifugation, aliquoted and stored in liquid nitrogen. A frozen aliquot of PBMCs was thawed and NK cells were negatively selected by incubating the sample in PBS containing 2 % fetal bovine serum (FBS) with 1 mM EDTA and 50 µL/mL of Enrichment Cocktail (Stem Cell Technology, #19055) for 10 min at RT; followed by incubation with 100 µL/mL Magnetic Particles for 5 min at RT and the usage of a magnet to collect the supernatant containing the enriched NK cells. These cells were kept in AIM-V medium (Gibco, #12055091) supplemented with 5 % human AB serum (Sigma) and 500 U/mL of recombinant human IL-2.

Antibodies and Primers

For flow cytometry, LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen; #L34975) was used to analyse cell viability; CellTrace™ Violet Cell Proliferation Kit (Invitrogen; #C34557) was used to label J-lat cells in co-culture assays and BV421 Mouse Anti-Human CD195 (BD Biosciences; #562576) and its isotype (Mouse C57BL/6 IgG2a, κ) were used to analyse CCR5 expression. The primer/probe sets used in this work are listed in **Table I** and were purchased from ThermoFisher (FAM-MGB, TaqMan assay).

Table I: Primer/probe sets used in this work.

Gene	Catalogue number, Assay ID
CCL3	#4331182, Hs00234142_m1
CCL4	#4331182, Hs99999148_m1
CCL5	#4331182, Hs99999048_m1
GAPDH	#4331182, Hs02786624_g1

Stimulation with pan-caspase inhibitor

Seeded KHYG-1 cells, either primary NK or J-lat cells were incubated at 37 ºC for the times indicated with 50 µM of the pan-caspase inhibitor z-VAD-FMK (stock solution in DMSO; Enzo Life Sciences) or only DMSO (0.5 %) as a control.

Co-culture assay and incubation with supernatant of NK cells

For the co-culture assay, J-lat cells were labelled with 2 µM CellTrace Violet dye on day one. On day two, $10x10^6$ J-lat were collected and half was stimulated with 6 µM of prostratin (Sigma-Aldrich) for 4 h at 37 ºC, while the other half was left unstimulated as a control. Cells were resuspended to a concentration of $0.5x10^6$ J-lat cells/mL and incubated with z-VAD-FMK for 1 h at 37 ºC. After this incubation period, a cell suspension of $0.5x10^6$ KHYG-1 cells or only medium were added to the J-lat cells in a 1:1 ratio of J-lat:KHYG-1. z-VAD-FMK or

DMSO were added to adjust the concentration and cells were incubated overnight (ON) in the same conditions as KHYG-1 are maintained.

For the incubation of J-lat cells with supernatant of KHYG-1 cells experiment, a cell suspension of $2x10^6$ KHYG-1 cells/mL, or only RPMI medium, was incubated with z-VAD-FMK ON at 37 ºC. On day two, a cell suspension of $1x10^6$ J-lat cells was incubated with 6 µM of prostratin for 4 h at 37 ºC or left unstimulated as a control. J-lat cells were then washed twice with PBS and resuspended to a concentration of $5x10^5$ J-lat cells/mL using supernatant from KHYG-1 cells undiluted, diluted 2x, diluted 4x or only medium and incubated ON at 37 ºC.

For the incubation of TZM-bl cells with supernatant of NK cells experiment, cell suspensions of $1x10^6$ KHYG-1 or primary NK cells cells/mL were stimulated with z-VAD-FMK and incubated ON at 37 ºC. TZM-bl cells were seeded in a concentration of $2x10^5$ cells/mL and incubated ON at 37 ºC. On day two, TZM-bl cells were challenged with 200 µL of the supernatant from KHYG-1 or primary NK cells in triplicates. As a negative control, the cells were left unstimulated and, as a positive control, they were stimulated with 6 µM of prostratin, also in triplicates. TZM-bl cells were incubated in these conditions ON at 37 ºC and on the following day luminescence was measured.

Flow cytometry

For the co-culture assay and the incubation with supernatant from NK cells experiment, J-lat cells were washed in ice-cold PBS and incubated with Near IR viability dye for 30 min at 4 ºC in the dark. The cells were then washed with ice-cold PBS and fixed with 2 % paraformaldehyde for 20 minutes at 4 ºC in the dark. They were washed with ice-cold PBS one last time and finally resuspended in ice-cold PBS and stored at 4 ºC until they were analysed.

For the CCR5 expression experiment, both "new" and "old" J-lat cells were kept in five different conditions and TZM-bl cells were used as a positive control. "New" J-lat cells had been in culture for less than 5 passages, whereas "old" cells had been in culture for around 25-30 passages. On day one, a cell suspension of $1x10^6$ KHYG-1 cells/mL was stimulated with z-VAD-FMK ON at 37 ºC. On day two, "new" and "old" J-lat cells were resuspended to a concentration of $0.5x10^6$ cells/mL in: (1) only RPMI 1640 medium, (2) 100 U/mL IL-2, (3) z-VAD-FMK, (4) 3 mL of supernatant of KHYG-1 cells or (5) 3 mL of supernatant of KHYG-1 cells that were stimulated with z-VAD-FMK and were incubated ON at 37 ºC. On day three, cells were washed twice in FACS buffer (PBS with 1 % BSA and 2 mM EDTA) and incubated with no antibody, BV421 isotype or CCR5-BV421 antibody diluted in FACS buffer with Near IR viability dye for 30 min at 4 ºC in the dark. The cells were then washed with FACS buffer and fixed with 2 % paraformaldehyde for 20 minutes at 4 °C in the dark. They were washed twice with ice-cold PBS and finally resuspended in ice-cold PBS and stored at 4 ºC until they were analysed.

Acquisition was performed on BD FACSVerse (BD Bioscience), using lasers and filter/bandwidth setting as indicated: CellTrace Violet (405, 448/45), GFP (488, 527/32), Near IR (640, 783/56) and CCR5 (405, 448/45).

Flow cytometry analysis was performed using FlowJo 10.7.1 (TreeStar, Inc, Ashland, OR). The gating strategy used for flow cytometry analysis is provided in Figure 2A. Data was analysed and plotted using GraphPad Prism v8.4.2 (GraphPad, Inc., USA).

Cytokine array

A cell suspension of $1x10^6$ KHYG-1 cells/mL was incubated with z-VAD-FMK ON at 37 ºC. The following day, 700 µL of supernatant was used to perform a Proteome Profiler™, Human Cytokine Array (R&D Systems; #ARY005B) according to the instructions of the manufacturer. This array allows the determination of the relative expression levels of 36 human cytokines.

RT-qPCR
A cell suspension of $1x10^6$ KHYG-1 or primary NK cells/mL were stimulated with z-VAD-FMK and incubated ON at 37 ºC. Total RNA was extracted with Quick-RNA™ Miniprep Plus Kit (ZYMO RESEARCH, R1057) according to the instructions of the manufacturer. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, #4368814), with RNasin® Ribonuclease Inhibitor (Promega, #N2511). qPCR reactions were done using Premix Ex Taq™ (Probe qPCR) (Takara, #RR390W) with the primer/probe sets listed in **Table I.** The qPCR was performed on an ABI Fast 7500 system (Applied Biosystems) according to manufacturer's protocol. Gene expression analysis was performed using the ΔΔCT method and data was analysed and plotted using GraphPad Prism v8.4.2. RNA samples were stored at -20 ºC and cDNA samples at 4 ºC.

Incubation with CCL3, CCL4 and CCL5
TZM-bl cells were seeded in a concentration of $2x10^5$ cells/mL and incubated ON at 37 ºC. On day two, cells were stimulated, in triplicates, with Recombinant Human MIP-1α (CCL3; PeproTech, #300-08), Recombinant Human MIP-1β (CCL4; PeproTech, #300-09), Recombinant Human RANTES (CCL5; PeproTech, #300-06), CCL3 + CCL4, CCL3 + CCL5, CCL4 + CCL5 and CCL3 + CCL4 + CCL5 in different concentrations: 10 ng/mL, 20 ng/mL or 50 ng/mL. As a negative control, the cells were left unstimulated and as a positive control, they were stimulated with 6 µM of prostratin, also in triplicates. TZM-bl cells were incubated in these conditions ON at 37 ºC. On the following day, luminescence was measured.

Luciferase assay
Cells were washed with PBS and incubated with 50 µL of 1X Passive Lysis Buffer (Promega, #E1941) for 30 min at 4 ºC. The cells were centrifuged at maximum speed for 5 min at 4 ºC and 20 µL of lysate was added to a white 96-wells plate. Then, 100 µL of luciferase reagent (Promega, #E1483) were added and luminescence was read using Tecan microplate reader (Tecan Infinite 200 Pro). Data was analysed and plotted using GraphPad Prism v8.4.2.

HMGB1 ELISA
Cell suspensions of $1x10^6$ cells/mL of KHYG-1 cells or primary NK cells were stimulated with z-VAD-FMK and incubated ON at 37 ºC. On day two, 100 µL of their supernatant was used to perform an ELISA to detect Human HMGB-1 (Novus Biologicals; #NBP2-62766): undiluted, diluted 3x and diluted 10x. There were three biological replicates, as well as three technical replicates for each sample and dilution. Data was analysed and plotted using GraphPad Prism v8.4.2.

Ethical consideration
This study used immune cells obtained from PBMCs of anonymous healthy individuals. This has been approved by regional ethics committees of Stockholm (2013/1944–31/4). All participants have given informed consent. The patient information was anonymized and de-identified prior to analysis.

Results

KHYG-1 cells stimulated with a pan-caspase inhibitor increase HIV-1 reactivation

Firstly, we wanted to confirm that when NK cells are stimulated with a pan-caspase inhibitor, they are able to increase HIV-1 reactivation. In order to do this, we did a co-culture experiment using J-lat and KHYG-1 cells stimulated with the pan-caspase inhibitor z-VAD-FMK, which is a broad-spectrum caspase-inhibitor[20,21] (**Figure 2**). KHYG-1 is an NK leukaemia cell line that was established from a patient with aggressive NK cell leukaemia[22]. The J-lat cell line is a model for HIV-1 latent infection, having an integrated copy of the HIV-1 genome. This genome was edited so that the non-essential *nef* gene was replaced by a gene encoding for GFP. For this reason, HIV-1 replication and thus reactivation can be measured by analysing the percentage of GFP-positive cells through flow cytometry. J-lat cells were cultured either unstimulated or activated with the latency reversal agent prostratin that induces HIV-1 reactivation. They were cultured only with medium or with pan-caspase inhibitor-stimulated or unstimulated KHYG-1 cells.

The gating strategy used in flow cytometry analysis is shown in **Figure 2A**. As expected, there was an increase in the percentage of GFP-positive cells, and thus HIV-1 reactivation, when J-lat cells were activated with prostratin, regardless of the remaining conditions (**Figure 2B;** e, f, g, h). Comparing with J-lat cells cultured with only medium with or without the pan-caspase inhibitor (**Figure 2B;** a, b, e, f), HIV-1 reactivation did not increase when cells were co-cultured with unstimulated KHYG-1 cells (**Figure 2B;** c, g), but it increased when they were co-cultured with KHYG-1 cells stimulated with a pan-caspase inhibitor (**Figure 2B;** d, h). This increase was observed both when J-lat cells were left unstimulated and when activated with prostratin (**Figure 2B;** d, h). Thus, when KHYG-1 cells are stimulated with a pan-caspase inhibitor, they can increase HIV-1 reactivation. The percentage of live cells was close to 100 % in all conditions (**Figure 2C**).

Supernatant of KHYG-1 stimulated with a pan-caspase inhibitor increases HIV-1 reactivation in two latency models

We then wanted to understand whether NK cells are secreting a biological product that is responsible for the increase in HIV-1 reactivation, for example a cytokine, or if it is due to cell-to-cell contact. For this purpose, KHYG-1 cells and primary NK cells were either left unstimulated or were stimulated with the pan-caspase inhibitor z-VAD-FMK and their supernatant was collected. The J-lat cells were unstimulated or activated with prostratin and they were cultured only with medium or with supernatant from unstimulated or pan-caspase inhibitor-stimulated KHYG-1 cells (**Figure 3**).

There was an increase in HIV-1 reactivation when J-lat cells were cultured with the supernatant of KHYG-1 cells stimulated with a pan-caspase inhibitor, but only when J-lat cells were previous activated with prostratin (**Figure 3A;** h). HIV-1 reactivation increased when J-lat cells were activated with prostratin, as expected and seen in the co-culture experiment (**Figure 3A;** e, f, g, h). The percentage of live cells was close to 100 % under all conditions (**Figure 3B**).

To know if this result was reproducible in another latency model, we used TZM-bl cells. These cells have a luciferase gene controlled by the HIV-1 LTR promoter, allowing the determination of HIV-1 reactivation by measuring the production of luciferase through a luminescence readout. TZM-bl cells were either left unstimulated, as a negative control, or were stimulated with prostratin, as a positive control. TZM-bl cells were cultured with supernatant from KHYG-1 cells or primary NK cells unstimulated or stimulated with the pan-caspase inhibitor z-VAD-FMK. The supernatant was used undiluted, diluted 2x and diluted 4x (**Figure 3C**).

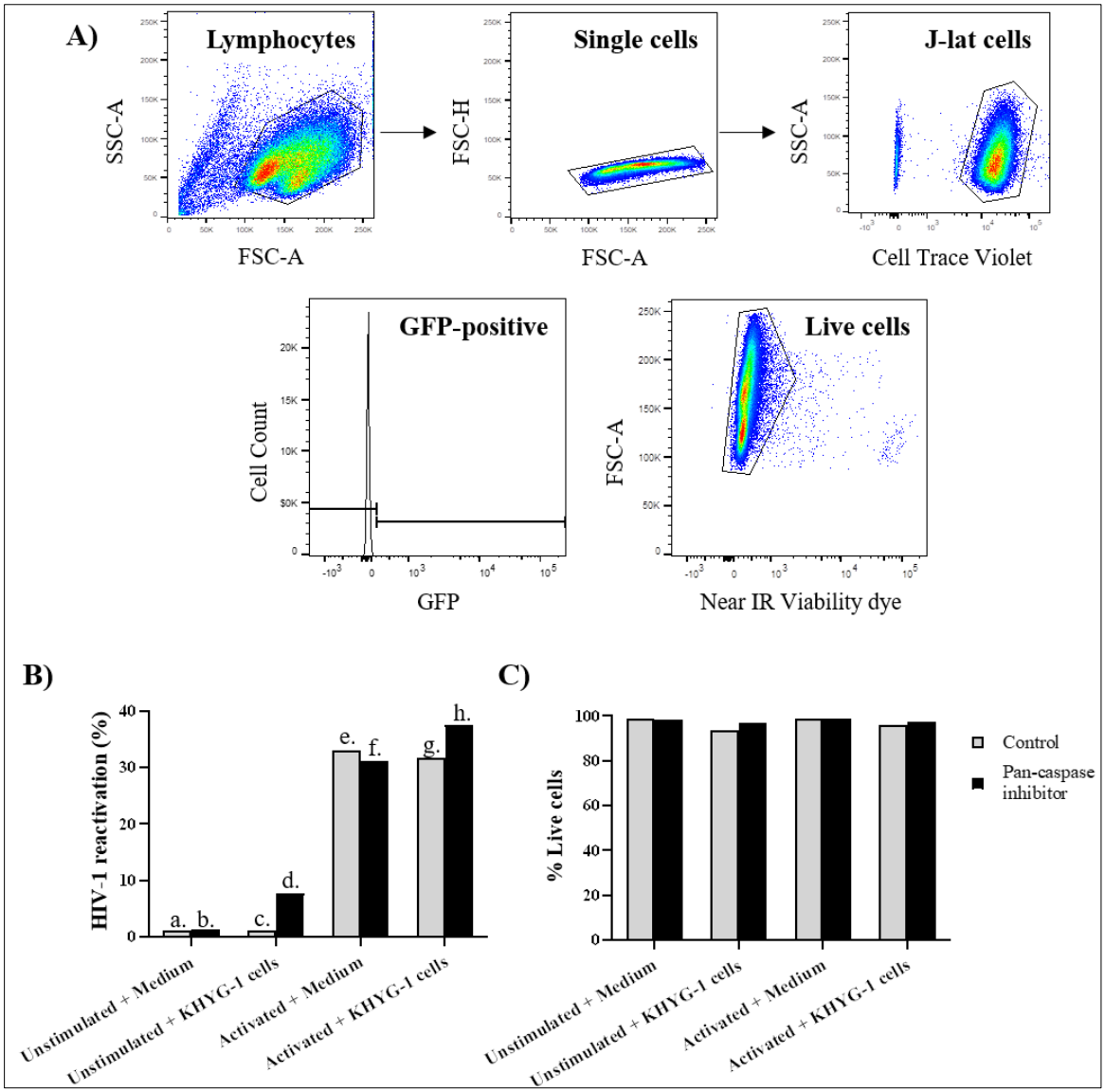

Figure 2: KHYG-1 cells stimulated with a pan-caspase inhibitor increase HIV-1 reactivation. **(A)** Gating strategy used for flow cytometry analysis. J-lat cells have an integrated copy of the HIV-1 genome, which was altered to include a gene encoding for GFP. These cells were either left unstimulated or were activated with prostratin (an LRA) and were cultured with only medium or with KHYG-1 cells that were unstimulated or stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK. Through flow cytometry, HIV-1 reactivation was determined by the percentage of GFP-positive cells **(B)** and the percentage of live J-lat cells was determined by the percentage of Near IR viability dye-negative cells **(C)**. FSC-A, forward scatter area. FSC-H, forward scatter height. SSC-A, side scatter area.

HIV-1 reactivation increased when TZM-bl cells were cultured with supernatant from KHYG-1 cells that were stimulated with a pan-caspase inhibitor, compared to the negative control as well as to when cells were cultured with the supernatant from unstimulated KHYG-1 cells. Additionally, HIV-1 reactivation increased more when the supernatant was more concentrated (**Figure 3C**). HIV-1 reactivation did not increase significantly when TZM-bl cells were cultured with supernatant from primary NK cells, neither unstimulated nor stimulated with a pan-caspase inhibitor (**Figure 3C**). The experiment with the supernatant from KHYG-1 cells was repeated two more times and results were similar. From these results, we can affirm that the supernatant of pan-caspase inhibitor-stimulated KHYG-1 cells increases HIV-1 reactivation in two latency models.

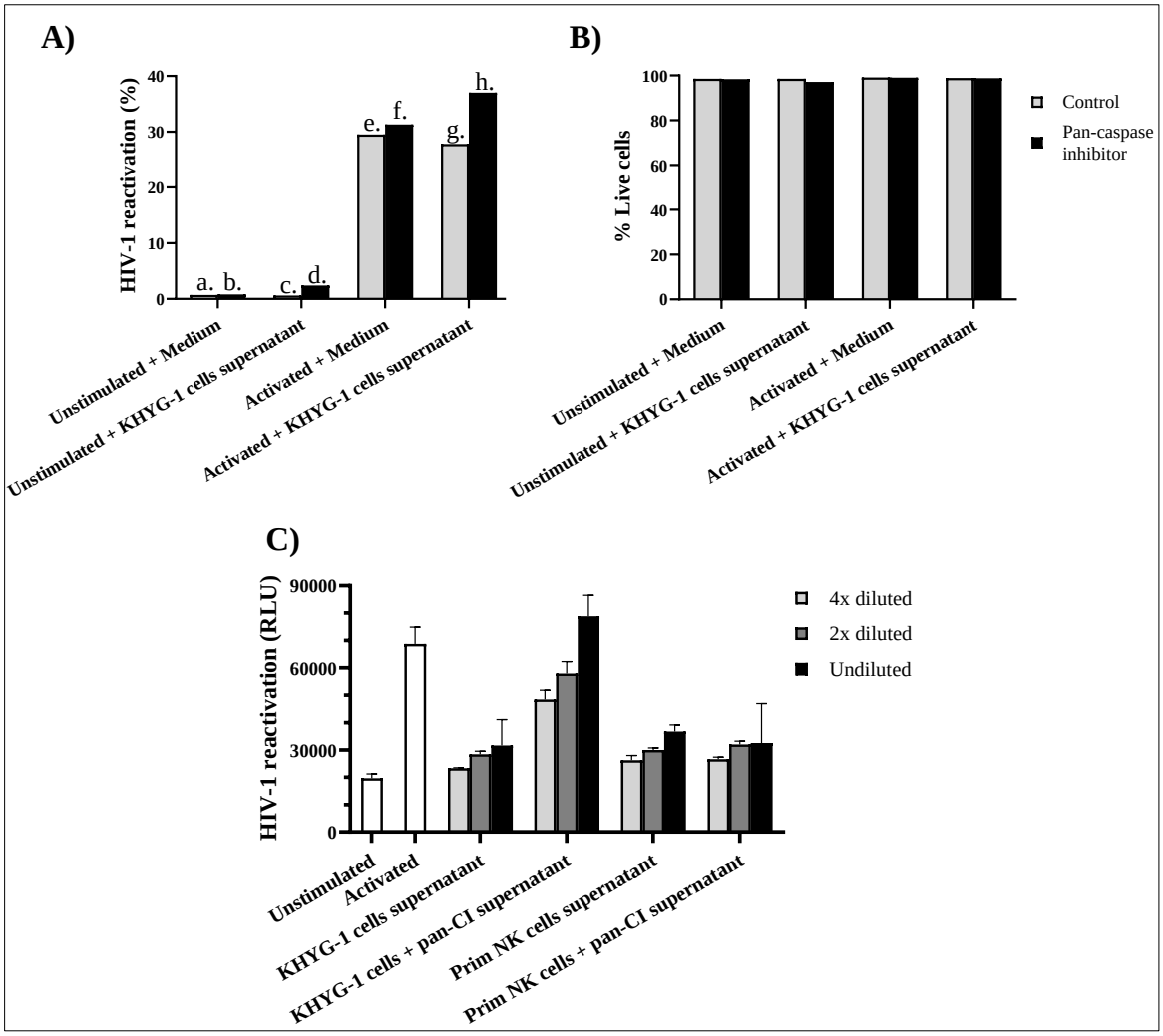

Figure 3: Supernatant of KHYG-1 stimulated with a pan-caspase inhibitor increases HIV-1 reactivation in two latency models. KHYG-1 cells and primary NK cells were left unstimulated or were stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK. Their supernatant was collected and then used to culture latently infected cells. J-lat cells have an integrated copy of the HIV-1 genome, which was altered to include a gene encoding for GFP. These cells were left unstimulated or were activated with prostratin (an LRA) and were cultured with only medium or with the supernatant of KHYG-1 cells that were unstimulated or stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK. Through flow cytometry, HIV-1 reactivation was determined by the percentage of GFP-positive cells **(A)** and the percentage of live J-lat cells was determined by the percentage of Near IR viability dye-negative cells **(B)**. TZM-bl cells have a luciferase gene controlled by the HIV-1 long terminal repeat (LTR) promoter, allowing the determination of HIV-1 reactivation by measuring the production of luciferase through a luminescence readout. TZM-bl cells were either left unstimulated, as a negative control, or were stimulated with prostratin, as a positive control. They were cultured with supernatant from KHYG-1 cells or primary NK cells that were unstimulated or stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK. The supernatant was used undiluted, diluted 2x and diluted 4x **(C)**. pan-CI, pan-caspase inhibitor. Prim NK cells, primary NK cells. RLU, Relative Luminescence Units.

CCL3, CCL4 and CCL5 protein expression is increased by KHYG-1 cells when stimulated with a pan-caspase inhibitor

Since the supernatant from KHYG-1 cells stimulated with the pan-caspase inhibitor increased HIV-1 reactivation, cytokines produced by these cells were analysed using a Human Cytokine Array. For this purpose, KHYG-1 cells were left unstimulated or were stimulated with the pan-caspase inhibitor z-VAD-FMK. Their supernatant was collected and used to perform the Cytokine Array, which allows the determination of the relative expression levels of 36 human

cytokines (**Figure 4**). CCL3, CCL4 and CCL5 protein levels were found to be increased in the supernatant of KHYG-1 cells that were stimulated with a pan-caspase inhibitor (**Figure 4A**) when compared to the supernatant of unstimulated KHYG-1 cells (**Figure 4B**). IL-2, MIF and Serpin E1 were found to be approximately equally present in both supernatants, and no other cytokine was detected (**Figure 4**). This experiment was repeated one more time and the results were similar.

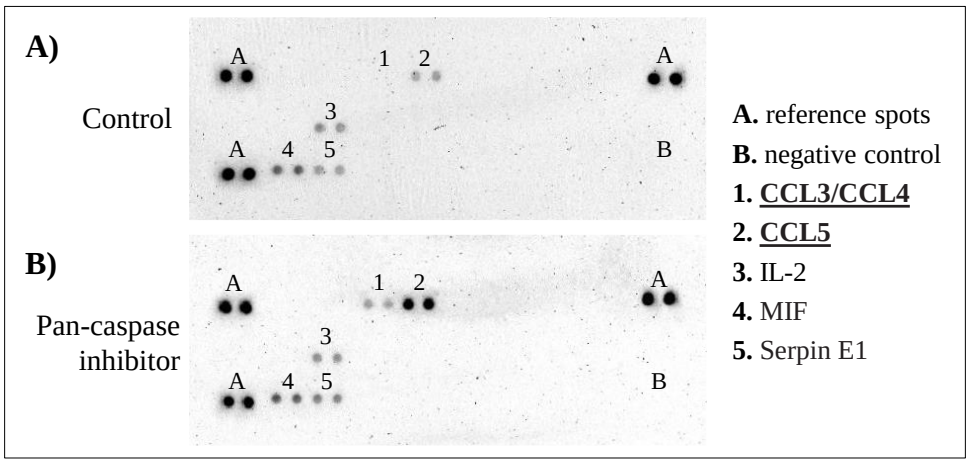

Figure 4: CCL3, CCL4 and CCL5 protein expression is increased by KHYG-1 cells when stimulated with a pan-caspase inhibitor. KHYG-1 cells were left unstimulated **(A)** or stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK **(B)** and their supernatant was used to perform a Human Cytokine Array, in order to determine the relative expression levels of 36 human cytokines.

CCL3 and CCL4 mRNA levels are upregulated in KHYG-1 cells when stimulated with a pan-caspase inhibitor

In order to confirm if CCL3, CCL4 and CCL5 genes were being upregulated, their relative mRNA expression was analysed. KHYG-1 cells (**Figure 5A**) and primary NK cells (**Figure 5B**) were left unstimulated or were stimulated with the pan-caspase inhibitor z-VAD-FMK and their total RNA was extracted in order to perform an RT-qPCR. Unstimulated and stimulated cells were compared, using the mRNA levels of the GAPDH gene to normalise the values. It was observed that CCL3 and CCL4 were upregulated in KHYG-1 cells when stimulated with a pan-caspase inhibitor, whereas CCL5 had no significant increase or decrease in expression (**Figure 5A**). This experiment was repeated two more times and the results were similar. On the other hand, the relative mRNA levels of CCL3, CCL4 and CCL5 were not significantly different between unstimulated and stimulated primary NK cells (**Figure 5B**).

J-lat cells do not express CCR5

CCL3, CCL4 and CCL5 are C-C chemokine receptor type 5 (CCR5) ligands[23] and, for this reason, CCR5 expression by J-lat cells was analysed through flow cytometry with an anti-CCR5 antibody (**Figure 6**). As negative controls of the antibody, there were samples with no antibody or with an isotype. "New" and "old" J-lat cells were cultured with medium or stimulated with: IL-2, the pan-caspase inhibitor z-VAD-FMK, supernatant of KHYG-1 cells or supernatant of KHYG-1 cells that were stimulated with z-VAD-FMK. "New" J-lat cells had been in culture for less than 5 passages, whereas "old" cells had been in culture for around 25-30 passages. TZM-bl cells were used as a positive control since these cells were genetically engineered to overexpress CCR5, among other characteristics[24] (**Figure 6A**). Unstimulated "old" J-lat cells did not express CCR5 (**Figure 6B**), as well as unstimulated "new" J-lat cells and both cells in the remaining four different conditions (data not shown).

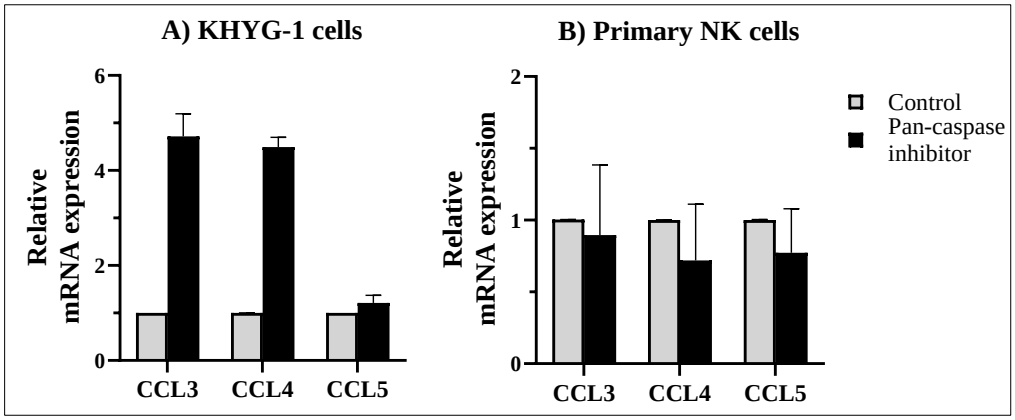

Figure 5: CCL3 and CCL4 mRNA levels are upregulated in KHYG-1 cells when stimulated with a pan-caspase inhibitor. KHYG-1 cells **(A)** and primary NK cells **(B)** were left unstimulated or were stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK. Relative mRNA expression of CCL3, CCL4 and CCL5 was acquired with RT-qPCR of total RNA using the primer/probe sets listed in **Table I**. The values were normalised to the relative expression of GAPDH.

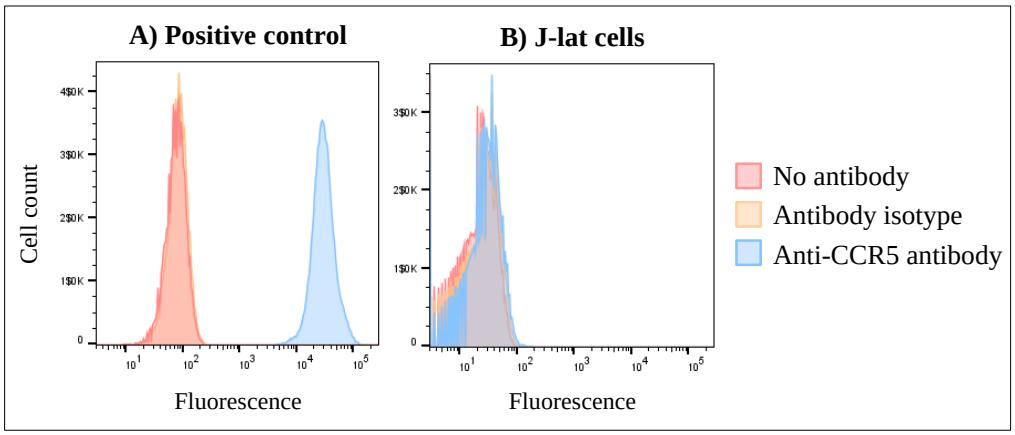

Figure 6: J-lat cells do not express CCR5. "New" and "old" J-lat cells were cultured in five different conditions: unstimulated or stimulated with: IL-2, 50 µM of the pan-caspase inhibitor z-VAD-FMK, supernatant of KHYG-1 cells or supernatant of KHYG-1 cells that were stimulated with 50 µM of z-VAD-FMK. "New" J-lat cells had been in culture for less than 5 passages, whereas "old" cells had been in culture for around 25-30 passages. CCR5 expression was measured through flow cytometry, using an antibody anti-CCR5 and, as controls, no antibody and an isotype. TZM-bl cells were used as a positive control **(A)** and all conditions in "new" and "old" cells had the same result **(B)**.

CCL3, CCL4 and CCL5 do not increase HIV-1 reactivation

In order to analyse if the cytokines CCL3, CCL4 and CCL5 are responsible for the increase in HIV-1 reactivation, TZM-bl cells were stimulated with these cytokines in different concentrations (10, 20 or 50 µg/mL), either each cytokine alone or in different combinations (**Figure 7**). TZM-bl cells were left unstimulated, as a negative control, or were stimulated with prostratin, as a positive control. There was no significant increase in HIV-1 reactivation with any of the cytokines or the combinations (**Figure 7**).

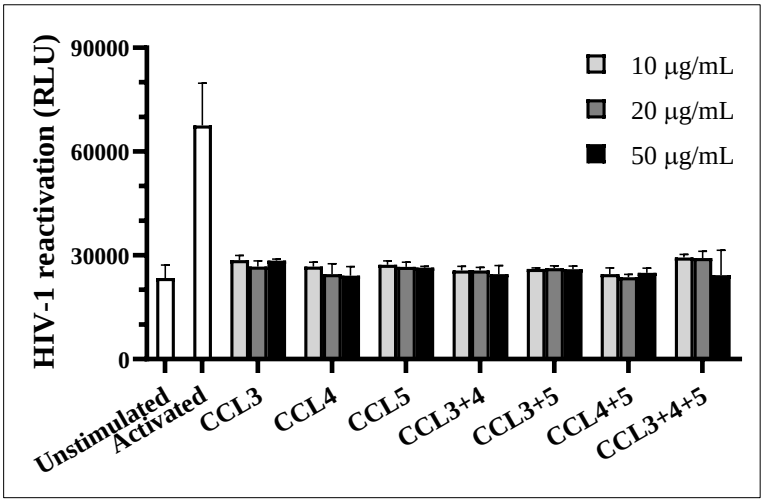

Figure 7: CCL3, CCL4 and CCL5 do not increase HIV-1 reactivation. TZM-bl cells have a luciferase gene controlled by the HIV-1 long terminal repeat (LTR) promoter, allowing the determination of HIV-1 reactivation by measuring the production of luciferase through a luminescence readout. TZM-bl cells were left unstimulated, as a negative control, or were stimulated with prostratin, as a positive control. They were cultured with CCL3, CCL4, CCL5, CCL3 + CCL4, CCL3 + CCL5, CCL4 + CCL5 or CCL3 + CCL4 + CCL5 in different concentrations: 10 μg/mL, 20 μg/mL and 50 μg/mL. RLU, Relative Luminescence Units

HMGB1 is not produced by KHYG-1 cells, neither unstimulated nor stimulated with a pan-caspase inhibitor

In a study published by Nowak *et al.*[25], it was shown that the High mobility group box protein 1 (HMGB1) upregulates HIV-1 replication in latently infected cells and concomitantly upregulates the expression of CCL3, CCL4 and CCL5. For this reason, we hypothesised that HMGB1 could be upregulated in KHYG-1 cells stimulated with the pan-caspase inhibitor, and thereby could be responsible for the increase in HIV-1 reactivation, as well as for the upregulation of the three cytokines. In order to analyse HMGB1 production by KHYG-1 cells and primary NK cells, these cells were left unstimulated or stimulated with the pan-caspase inhibitor z-VAD-FMK and their supernatants were used undiluted, diluted 3x and diluted 10x to perform an ELISA (**Figure 8**). There was no HMGB1 present in the supernatant of KHYG-1 cells, regardless of stimulation with the pan-caspase inhibitor (**Figure 8**). Furthermore, there was around 1000 pg/mL of HMGB1 present in the supernatant of primary NK cells, both the unstimulated and the stimulated with the pan-caspase inhibitor (**Figure 8**). Thus, pan-caspase inhibitor treatment does not affect the production of HMGB1.

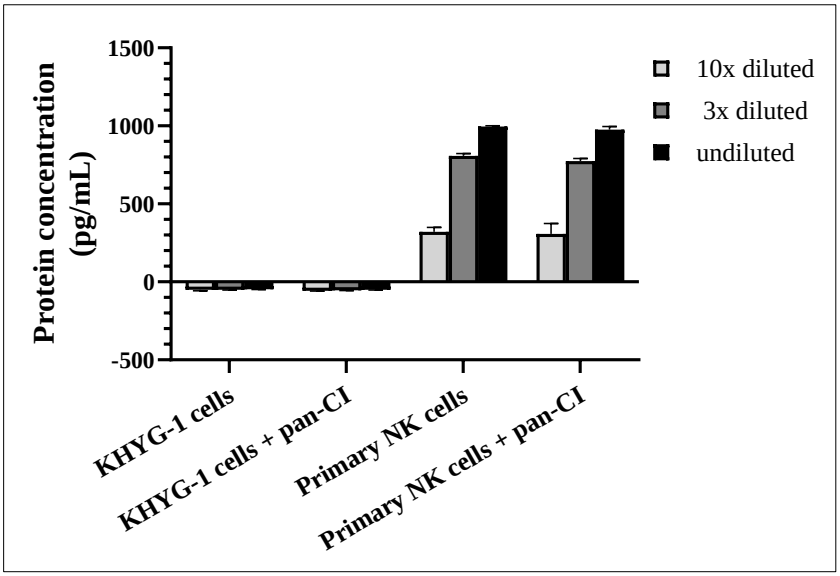

Figure 8: HMGB1 is not produced by KHYG-1 cells, neither unstimulated nor stimulated with a pan-caspase inhibitor. KHYG-1 cells and primary NK cells were either left unstimulated or stimulated with 50 µM of the pan-caspase inhibitor z-VAD-FMK and their supernatants were used to perform an ELISA to detect Human HMGB-1. The supernatants were used undiluted, diluted 3x and diluted 10x. pan-CI, pan-caspase inhibitor.

Discussion

Latency reservoirs in HIV-1 infection are a major barrier to its cure[8] since latently infected cells are impossible to distinguish from healthy ones[9]. For this reason, both the immune system and drugs cannot identify and specifically eliminate these cells. The "shock-and-kill" strategy is a potential solution that aims to induce HIV-1 reactivation, which exposes latently infected cells to the immune system and makes them susceptible to cell death[12–14]. To break HIV-1 latency, there should be epigenetic modulation of the provirus or activation of specific transcription factors that can bind the HIV-1 LTR promoter[1,12]. Although a very promising strategy, there has not been a successful reduction of latent reservoirs *in vivo* yet[15–19]. In our lab, it was observed that NK cells, specifically KHYG-1 cells, can acquire the "shock" ability when stimulated with the pan-caspase inhibitor z-VAD-FMK. Therefore, the main aim of this project was to map the mechanisms by which NK cells can induce HIV-1 reactivation. Some options for mechanisms are the secretion of a biological product or the expression of ligands on the NK cells surface that can induce activation of a pathway in latently infected cells that ultimately leads to transcription of the provirus. Specifically, it was investigated whether NK cells stimulated with a pan-caspase inhibitor secrete one or several cytokines that can induce HIV-1 reactivation. If so, our goal would be to identify these cytokines.

Firstly, a co-culture experiment was performed in order to confirm that KHYG-1 cells stimulated with a pan-caspase inhibitor are able to increase HIV-1 reactivation. There was an increase in HIV-1 reactivation when J-lat cells, which are latently infected cells, were co-cultured with KHYG-1 cells that had been stimulated with a pan-caspase inhibitor. This increase was observed both when the target cells were left unstimulated and when they were activated with an LRA. On the other hand, HIV-1 reactivation did not increase when KHYG-1 cells were left unstimulated. These results indicate that the inhibition of one or several caspases in KHYG-1 cells modulates their activity in a way that they obtain the ability to induce HIV-1 reactivation in latently infected cells. Thus, it is likely that there is a pathway in NK cells that can potentially be exploited in order for these cells to be able to induce HIV-1 reactivation and therefore perform the "shock" step in the shock-and-kill strategy.

The percentage of live J-lat cells was close to 100 % in every condition, which was not expected since KHYG-1 cells are cytotoxic lymphocytes and HIV-1 reactivation should expose J-lat cells. This might be happening because KHYG-1 cells are less successful in killing cells, as reported previously in our lab as well as in other groups (personal communication). In literature, KHYG-1 cells are usually described as highly cytotoxic towards K562 cells, but lower against other cell lines[22,26,27]. In these papers, they often used a high Effector:Target (E:T) ratio, while we used a 1:1 ratio. Therefore, J-lat cells could be less susceptible than K562 cells to KHYG-1 cell killing due to different receptors or ligands that mediate NK cell activation and subsequent killing. Moreover, a higher E:T ratio could result in more killing of J-lat cells.

Secondly, we wanted to understand whether the increase in HIV-1 reactivation is due to cell-to-cell contact or due to a biological product, for example a cytokine, that is being released by pan-caspase inhibitor-stimulated KHYG-1 cells. To test this, J-lat cells were cultured with supernatant from KHYG-1 cells. HIV-1 reactivation increased when J-lat cells were cultured with the supernatant of KHYG-1 cells that had been stimulated with the pan-caspase inhibitor. However, unlike in the co-culture experiment, this increase was only observed when J-lat cells had been reactivated with an LRA. This result indicates that the supernatant of KHYG-1 cells stimulated with a pan-caspase inhibitor does have an effect in HIV-1 reactivation, even though it is not the same as in the co-culture experiment. Thus, stimulated KHYG-1 cells are producing and releasing a biological product to the extracellular space that can induce HIV-1 reactivation. This product could potentially be a cytokine, since NK cells are immune cells, which normally act through cytokines. The fact that the effect in HIV-1 reactivation was not as strong as in the co-culture experiment might mean that cell-to-cell contact is important, or it might be because

these cells tend to be close together when they are co-cultured. For this reason, a small amount of a biological product is enough to induce reactivation in J-lat cells co-cultured with NK cells, while it might be too diluted when J-lat cells are cultured only with the supernatant.

In order to know if the effect in HIV-1 reactivation was reproducible, TZM-bl cells, which are another latency model, were used and cultured with supernatant from KHYG-1 cells and primary NK cells. There was an increase in HIV-1 reactivation when TZM-bl cells were cultured with supernatant from KHYG-1 cells that were stimulated with a pan-caspase inhibitor. Importantly, the increase was bigger when the supernatant was more concentrated, proving causation. This result, together with the previous one which utilised J-lat cells, shows that the supernatant of pan-caspase inhibitor-stimulated KHYG-1 cells can increase HIV-1 reactivation in two different latency models. Therefore, when KHYG-1 cells are stimulated with a pan-caspase inhibitor, they secrete a biological product that contributes to this effect. Regarding primary NK cells, their supernatant did not cause a significant increase in HIV-1 reactivation. However, we believe this result is not representative since the primary NK cells were dying over time during the experiment (based on manual cell counting, data not shown), meaning that their culture conditions were not optimal for their growth. For this reason, one of the future goals is to try to optimize the protocol of isolation of primary NK cells from PBMCs, as well as the conditions in which they are cultured, in order to have healthy primary NK cells that will behave more naturally.

To gain an insight about the differences between cytokine production of unstimulated and pan-caspase inhibitor-stimulated KHYG-1 cells, a Human Cytokine Array was performed. Out of 36 human cytokines analysed, IL-2, MIF and Serpin E1 were found to be approximately equally present in supernatants from unstimulated and stimulated cells, while CCL3, CCL4 and CCL5 protein levels were found to be increased in the supernatant of KHYG-1 cells that were stimulated with a pan-caspase inhibitor. The fact that we detected IL-2 in the supernatant of both unstimulated and stimulated KHYG-1 cells proves that the cytokine array system works, since IL-2 was added to culture these cells. Because of this result, it was hypothesised that the cytokines CCL3, CCL4 and CCL5 - either one, two, or all three of them -, could be responsible for the increase in HIV-1 reactivation. In order to first investigate if their genes were being upregulated, their relative mRNA expression was analysed in KHYG-1 cells and primary NK cells, comparing unstimulated cells with pan-caspase inhibitor-stimulated cells. Relative mRNA levels of CCL3, CCL4 and CCL5 in primary NK cells were not significantly different when these cells were stimulated with a pan-caspase inhibitor. As mentioned above, however, this result might not be representative and new experiments need to be performed in order to draw conclusions. In KHYG-1 cells stimulated with a pan-caspase inhibitor, CCL3 and CCL4 were found to be upregulated, whereas CCL5 was not downregulated nor upregulated. The reason for the latter result could be either that upregulation of CCL5 production happens during translation or that in fact this cytokine is not upregulated. Based on the cytokine array result, it is likely that CCL5 is being upregulated during translation, for examples through mRNA stabilization or increased export from the cytoplasm. To our knowledge, the production of CCL3, CCL4 and CCL5 in human NK cells after stimulation with a pan-caspase inhibitor has not been studied. There are, however, studies on stimulated human PBMCs[28], infected mice[29], infected human bronchiolar cells[30] and murine macrophages[31]. Teresa Krakauer[28] has observed that staphylococcal enterotoxin B (SEB)-stimulated and staphylococcal toxic shock syndrome toxin 1 (TSST-1)-activated PBMCs produce less CCL3 and CCL4 when they are stimulated with a pan-caspase inhibitor. Furthermore, in a paper published by Breyne *et al.*[29], CCL5 production is shown to decrease when *Escherichia coli*-infected mice are treated with a pan-caspase inhibitor. In both studies, pan-caspase inhibitor treatment seems to be counteracting the highly activated inflammatory responses induced by SEB/TSST-1 or bacteria. Nonetheless, the production of these cytokines was not analysed in inactivated, unstimulated, and non-infected

cells or mice, which would be important controls. Therefore, it is possible that the stimulation of PBMCs or mice with a pan-caspase inhibitor would upregulate the expression of CCL3, CCL4 and/or CCL5. In a study done by Edward Brydon *et al.*[30], the stimulation of influenza virus-infected bronchiolar cells with a pan-caspase inhibitor increased expression of CCL5. In this paper, the authors assume that the upregulation happens due to the increased cell survival caused by the inhibition of caspases, which could be an explanation. However, it would again be important to analyse CCL5 expression in non-infected cells treated with or without pan-caspase inhibitor to know if the stimulation with a pan-caspase inhibitor has an effect regardless of the influenza infection. Finally, in a paper published by Wim Martinet *et al*[31], stimulation of a murine macrophage cell line with a pan-caspase inhibitor is shown to induce cell death through the induction of autophagy, whilst upregulating the expression of CCL3 and CCL4. This upregulation could be due to the stimulation with a pan-caspase inhibitor or could be cell death-induced. These four studies did not focus on the effect of pan-caspase inhibitor-stimulation on the expression of CCL3, CCL4 and/or CCL5 since it was not their aim, which explains the absence of controls in three of the studies. Furthermore, the upregulation we see in NK cells could be a cell type-specific response and it can be misleading to compare with PBMCs, a mice model, bronchiolar cells or macrophages.

CCL3, CCL4 and CCL5 are cytokines that, in spite of having different functions, have one characteristic in common: they are CCR5 ligands[23]. CCR5 is one of the most commonly used receptors by HIV-1 to enter cells[32] and, for this reason, the three cytokines normally act as HIV-1 suppressors[33] by blocking its entry into the host cell. In the context of latency reservoirs, however, the HIV-1 genome is already integrated in the host cell genome and there is no active infection, meaning the cytokines would not be inhibiting infection in our model. Moreover, a recent study has shown that maraviroc, a CCR5 antagonist used for HIV-1 treatment, can also act as a latency reversal agent[34], which reinforced our idea that CCL3, CCL4 and CCL5 could be inducing HIV-1 reactivation. Since these cytokines are CCR5 ligands and there is some controversy in literature regarding the J-lat cell line expressing CCR5[35–39], its expression was analysed in several conditions. TZM-bl cells were used as positive control, as it is known that they express CCR5[24], and it was observed that J-lat cells do not express this receptor. Nonetheless, we wanted to analyse if CCL3, CCL4 and CCL5 increased HIV-1 reactivation, since they could be acting through another receptor and, besides that, physiologically latently infected cells do express CCR5. This was tested by stimulating TZM-bl cells with each cytokine or with different combinations of them. There was no significant increase in HIV-1 reactivation with any of the cytokines or the combinations, meaning that although these cytokines are being upregulated, they are not responsible for the increase in HIV-1 reactivation, at least not in the analysed concentrations and in this model. As future work, we will test different concentrations of CCL3, CCL4 and CCL5, and analyse the stimulation of J-lat cells with these three cytokines. Even though these cytokines might not be responsible for the HIV-1 reactivation, their upregulation can be useful in the clinical context. Since they are HIV-1 suppressors, their presence can prevent the infection from spreading after latent cells are reactivated.

In a study published by Nowak *et al.*[25], HMGB1 was reported to cause an increase of HIV-1 replication in latently infected cells and an upregulation of expression of CCL3, CCL4 and CCL5. Therefore, we hypothesised that the stimulation of NK cells with a pan-caspase inhibitor could be upregulating the production of HMGB1 by these cells. HMGB1 could then be the protein responsible for the increase in HIV-1 reactivation and for the overexpression of CCL3, CCL4 and CCL5. HMGB1 was not found in the supernatant of KHYG-1, whereas there was around 1000 pg/mL of HMGB1 present in the supernatant of primary NK cells. More importantly, there was no difference between unstimulated and pan-caspase inhibitor-stimulated NK cells, indicating that HMGB1 is not the protein responsible for HIV-1

reactivation. Regarding the difference between KHYG-1 cells and primary NK cells, we believe it is due to the way primary NK cells were cultured. The medium we used to culture primary NK cells contains human serum and we suspect that either the serum has HMGB1, or it has some protein that induces the production of HMGB1 by the primary NK cells.

There are some strengths and weaknesses in this project. In flow cytometry experiments, we could have used more replicates per sample, in order for results to be more significant. Nonetheless, we did use two different latency models, which strengthens our findings. Another weakness is the fact that we used latency models, but no primary infected cells. However, there is currently no way of isolating these cells from donors since it is impossible to distinguish them from healthy cells. Furthermore, some of the experiments were repeated, which shows that the results are reproducible.

As future work, we plan to: perform co-culture experiments using primary NK cells cultured in a different way, as well as other effector cells, for example, PBMCs; stimulate J-lat cells and TZM-bl cells with different concentrations of CCL3, CCL4 and CCL5; perform a Stable isotope labelling by amino acids in cell culture (SILAC) experiment in order to identify proteins present in the supernatant; and we also aim to identify which caspase(s) within KHYG-1 cells is/are mediating the “shock” ability.

In summary, the results obtained in this project prove that the inhibition of one or several caspases in the KHYG-1 cells modulates their activity. Since their supernatant can increase HIV-1 reactivation in two different latency models, it is possible to conclude that KHYG-1 cells stimulated with a pan-caspase inhibitor secrete a biological product, yet to be identified, that contributes to this effect. Finally, even though increased secretion of CCL3, CCL4 and CCL5 does not induce HIV-1 reactivation, it can be very useful in the clinical context to prevent the infection from spreading after latent cells are reactivated.

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