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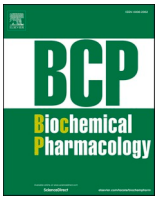

Dasatinib interferes with HIV-1 proviral integration and the inflammatory potential of monocyte-derived macrophages from people with HIV

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ABSTRACT

HIV-1 infection is efficiently controlled by the antiretroviral treatment (ART) but viral persistence in long-lived reservoirs formed by CD4 + T cells and macrophages impedes viral eradication and creates a chronic inflammatory environment. Dasatinib is a tyrosine kinase inhibitor clinically used against chronic myeloid leukemia (CML) that has also showed an anti-inflammatory potential. We previously reported that dasatinib is very efficient at interfering with HIV-1 infection of CD4 + T cells by preserving the antiviral activity of SAMHD1, an innate immune factor that blocks T-cell activation and proliferation and that is inactivated by phosphorylation at T592 (pSAMHD1). We observed that short-term treatment in vitro with dasatinib significantly reduced pSAMHD1 in monocyte-derived macrophages (MDMs) isolated from people with HIV (PWH) and healthy donors, interfering with HIV-1 infection. This inhibition was based on low levels of 2-LTR circles and proviral integration, while viral reverse transcription was not affected. MDMs isolated from people with CML on long-term treatment with dasatinib also showed low levels of pSAMHD1 and were resistant to HIV-1 infection. In addition, dasatinib decreased the inflammatory potential of MDMs by reducing the release of M1-related cytokines like TNFα, IL-1β, IL-6, CXCL8, and CXCL9, but preserving the antiviral activity through normal levels of IL-12 and IFNγ. Due to the production of M2-related anti-inflammatory cytokines like IL-1RA and IL-10 was also impaired, dasatinib appeared to interfere with MDMs differentiation. The use of dasatinib along with ART could be used against HIV-1 reservoir in CD4 and macrophages and to alleviate the chronic inflammation characteristic of PWH.

1. Introduction

The infection caused by the human immunodeficiency virus type 1 (HIV-1) cannot be cured with the current antiretroviral therapy (ART) due to the existence of a stable reservoir comprised of latently infected CD4 + T cells and macrophages [1–3]. Although memory CD4 + T cells are considered the main reservoir for latent HIV-1, macrophages are widely distributed, long-lived, noncycling myeloid cells that may transport the virus to tissues with difficult access for ART, thereby

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Abbreviations

3TC	Lamivudine.
ABC	Abacavir.
ART	Antiretroviral therapy.
BCL-2	B-Cell CLL/Lymphoma 2.
BIC	Bictegravir.
CML	Chronic myeloid leukemia.
CNS	Central nervous system.
COPD	Chronic obstructive pulmonary disease.
CTL	Cytotoxic T lymphocyte.
DMSO	Dimethyl sulfoxide.
dNTPs	Deoxynucleotide triphosphates.
DRV	Darunavir.
DTG	Dolutegravir.
FCS	Fetal calf serum.
FITC	Fluorescein isotiocyanate.
FTC	Emtricitabine.
GFP	Green fluorescent protein.
HC	Heterosexual contact.
HIF-1α	Hypoxia-inducible factor-1 alpha.
HIV-1	Human immunodeficiency virus type 1.
IDU	Injection drug user.
IFNγ	Interferon gamma.
IGF-1	Insulin-like growth factor 1.
IL	Interleukin.
IQR	Interquartile range.
IRB	International review board.
LPS	Lipopolysaccharide.
MCP-4	Monocyte chemotactic protein 4.
MDM	Monocyte-derived macrophage.
MIG	Monokine induced by gamma interferon.
MIP-3α	Macrophage inflammatory protein 3 alpha.
MPIF	Myeloid progenitor inhibitory factor.
MSM	Men who have sex with men.
P	p-value.
PBMCs	Peripheral blood mononuclear cells.
PE	Phycoerythrin.
pSAMHD1	Phosphorylated SAMHD1.
PWH	People with HIV-1.
RPV	Rilpivirine.
RT	Reverse transcripts.
RTV	Ritonavir.
SEM	Standard deviation of the mean.
TAF	Tenofovir alafenamide.
TKI	Tyrosine kinase inhibitor.
TLR	Toll-like receptor.
TNFα	Tumor necrosis factor alpha.

contributing to the viral persistence and to the formation of sanctuaries in tissues that are difficult to access for drugs such as the central nervous system (CNS) [4]. Accordingly, HIV-1 has been detected in macrophages isolated from different tissues such as gut, lungs, liver, urethra, and brain from people with HIV-1 (PWH) [5,6].

Despite the role of macrophages in spreading HIV-1, these cells show low permissiveness to infection, mostly due to the higher expression of the antiviral factor SAMHD1 [7,8]. SAMHD1 is a deoxynucleotide triphosphohydrolase that depletes the pool of intracellular deoxynucleotide triphosphates (dNTPs) necessary for efficient HIV-1 replication in noncycling cells [9,10], but this restriction can be abrogated through its phosphorylation at T592 (pSAMHD1) by cyclin-dependent kinases [11,12] or counteracted by the accessory HIV-2 protein Vpx. It has also been described that most HIV-1 variants cannot enter macrophages as cell-free particles, although infection is greatly improved when viruses are transferred cell-to-cell from infected CD4 + T cells [13–15]. In addition, there is a small fraction of macrophages that is susceptible to infection with a subset of viral variants that show tropism for cells with low expression of CD4 [16].

Infected macrophages may present longer half-life due to HIV-1 promotes resistance to apoptosis in these cells [17], thereby increasing their contribution to viral persistence and pathogenesis. In fact, the elimination of HIV-1-infected macrophages by cytotoxic T lymphocytes (CTLs) is impaired in comparison with that of infected CD4 + T cells [18]. This impaired killing may lead to a higher production of pro-inflammatory cytokines such as IFNγ and TNFα by CTLs [18] that may polarize macrophages into an M1 or pro-inflammatory phenotype [19], contributing to the chronic inflammation state characteristic of PWH [20]. Therefore, in addition to other factors that may contribute to the chronic inflammation characteristic of PWH [21,22], M1 macrophages show an important role in the development of metabolic, cardiovascular, and neurological syndromes associated to HIV-1-related persistent inflammation through the release of pro-inflammatory cytokines [23,24]. Although ART may control proviral reactivation and viral rebound from infected cells, it cannot control the M1-dependent chronic inflammation. Therefore, new strategies are needed to interfere with the viral reservoir in macrophages and to reduce the chronic inflammation that constitutes a hallmark of HIV-1 infection.

The antiviral and immunomodulatory properties of tyrosine kinase inhibitors (TKIs) have sparked interest in their potential use for treating HIV-1 infection [25–27]. TKIs have been safely used in clinic for the treatment of chronic myeloid leukemia (CML) since 2001 [28]. Our group has reported that the TKI dasatinib is a potential candidate as an adjuvant to ART due to its ability to preserve the antiviral activity of SAMHD1 by impeding its phosphorylation [29,30] and because it can interfere with IL-7-mediated homeostatic proliferation of CD4 + T cells [27,31,32]. Consequently, PWH with CML on treatment with ART and dasatinib show lower reservoir size and impaired proviral reactivation [25]. In addition, dasatinib has also shown valuable senolytic properties that help ameliorate immune senescence and inflammation in individuals with pulmonary fibrosis [33] and diabetic kidney disease [34].

In this study, we evaluated the effect and mechanism of action of dasatinib to interfere with HIV-1 infection of monocyte-derived macrophages (MDMs), as well as with the release of M1-related pro-inflammatory cytokines. Due to the essential role of macrophages in HIV-1 persistence, new therapeutic strategies are necessary to interfere with the formation and maintenance of the viral reservoir and to reduce the chronic inflammation characteristic of PWH by simultaneously targeting CD4 + T cells and macrophages.

2. Materials and Methods

2.1. Study Subjects

PWH (n = 15), individuals with CML on treatment with dasatinib (n = 10), and healthy donors (n = 16) were recruited for this study in four different Health Centers in Madrid (Spain): Hospital Universitario Ramón y Cajal, Hospital Universitario Puerta de Hierro Majadahonda, Hospital Universitario Severo Ochoa, and Centro de Salud Doctor Pedro Lain Entralgo. One blood sample was collected by venipuncture in EDTA Vacutainer tubes (Becton Dickinson, Madrid, Spain) and it was immediately processed to isolate peripheral blood mononuclear cells (PBMCs). MDMs were generated directly from fresh PBMCs.

2.2. Ethical statementt

All participants gave informed written consent to participate in this study in accordance with the Helsinki Declaration. Confidentiality and anonymity were protected by current Spanish and European Data Protection Acts. The protocol to perform this study was approved by the Ethics Committee of Instituto de Salud Carlos III (IRB IORG0006384, protocol reference CEI PI 46_2018).

2.3. Dasatinib

Dasatinib was purchased from Selleckchem (Houston, TX). It was used at a concentration of 75 nM in all experiments, according to previous description of IC50 and EC50 in Bermejo et al. [27]. Solvent to this drug was dimethyl sulfoxide (DMSO) that was added to the controls at the same concentration. DMSO had Molecular Biology grade and it was purchased from Sigma-Aldrich (St. Louis, MO, USA).

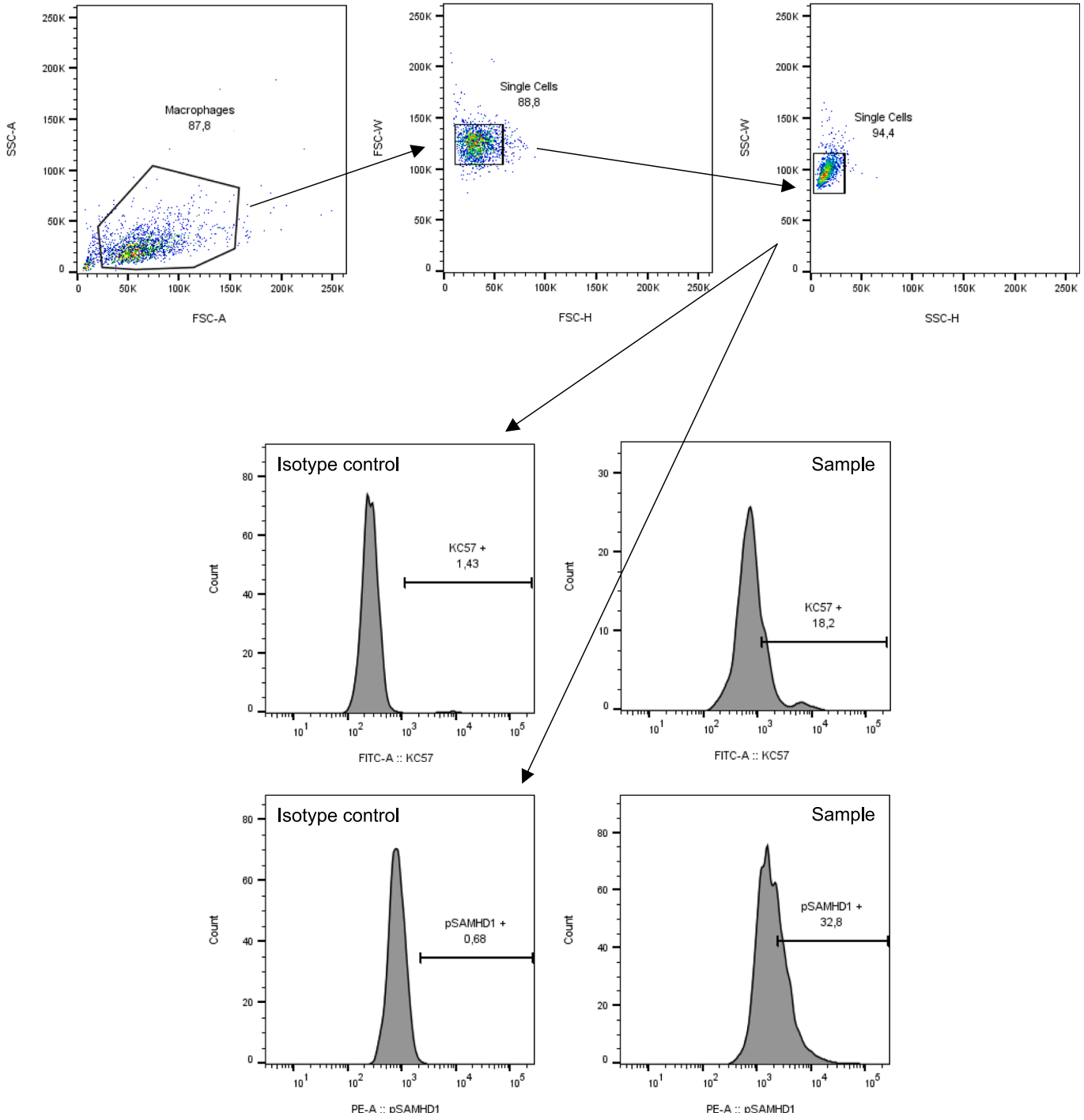

Fig. 1. Gating strategy for the analysis by flow cytometry of the intracellular expression of p24-gag and pSAMHD1. One representative sample of MDMs infected with JR-FL_Renilla stained on the cell surface with specific, conjugated monoclonal antibodies against p24-gag and SAMHD1 phosphorylated at T592. Isotype controls were used to determine the background signal.

2.4. Obtention of monocyte-derived macrophages

PBMCs were isolated by centrifugation through a Ficoll-Hypaque gradient (Pharmacia Corporation, North Peapack, NJ, USA). Monocytes were obtained by positive selection using CD14 MicroBeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufactureŕs instructions. Monocytes were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100μg/ml streptomycin, 100UI/ml penicillin (Biowhittaker, Walkersville, MD) for 2 h without fetal calf serum (FCS) (Gibco, Grand Island, NY) to increase the cell adherence to the plate surface. Once cells were adhered, the culture medium was supplemented with 10 % (v/v) FCS. Monocytes were then cultured for one week until they differentiate and proliferate sufficiently to become confluent [35]. For MDMs treated with dasatinib in vitro, this drug was added to the culture medium at 75 nM after 5 days of culture and incubated 30 min before infection. Cells were then incubated for 48 h more, so the total culture time was 7 days.

2.5. HIV-1 infection of MDMs

HEK293T cells (provided by the existing collection of Intituto de Salud Carlos III, Madrid, Spain) were transfected with plasmid pJR-FL_Renilla [36], (kindly provided by Dr Javier García-Pérez, Instituto de Salud Carlos III, Madrid, Spain), or co-transfected with plasmids pDHIV3_GFP (Green fluorescent protein) and pCMV_VSVG [37] (kindly provided by Dr Vicente Planelles, University of Utah, Salt Lake City, UT) to generate the infectious supernatants by using calcium phosphate method. MDMs were infected with 1 ng p24-gag of JR-FL_Renilla for 30 min at 37 °C. After centrifugation at 1500 rpm (450 rcf) for 30 min at 25 °C, the infected cells were cultured for 48 h or 4 days. Infection was monitored by luminescence using Renilla Luciferase Assay System (Promega, Madison, WI) and by flow cytometry after intracellular staining with anti-HIV-1-core-FITC (Fluorescein isotiocyanate) (anti-p24-gag, clone kc57, Beckman Coulter, Indianapolis, IN) and anti-pSAMHD1-PE (Phycoerythrin) (Thr592, clone D7O2M; Cell Signaling Technology, Danvers, MA). Isotype controls were used to determine the background signal. Data acquisition was performed in a BD LSRFortessa X-20 flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed with FlowJo_V10 software (TreeStar, Ashland, OR). The gating strategy for the analysis is shown in Fig. 1.

Infection with DHIV3_GFP was monitored in living cells by automated time-lapse microscopy with Thunder Imaging System (Leica Microsystems, Wetzlar, Germany) taking images every 30 min for 72 h (40x objective lens, 5 randomly selected fields) per sample. In all cases, MDMs were treated with dasatinib 75 nM or left untreated for 48 h before adding DHIV3_GFP to the culture medium. HIV-1 infection was determined by measuring the mean percentage of GFP+cells in 5 fields per sample.

2.6. Quantification of early and late HIV-1 reverse transcription

Five hours after infection of MDMs with JR-FL_Renilla, total DNA was extracted with QIAamp DNA Blood Mini Kit (Qiagen Iberia, Madrid, Spain) and quantified using Nanodrop 2000C (Thermo Fisher Scientific, Waltham, MA). Early and late reverse transcription (RT) was assessed by qPCR as described previously [38]. qPCR was performed in a StepOne Real-Time PCR system (Thermo Fisher Scientific). Serial dilutions of genomic DNA extracted from 8E5 cell line, which contains a single integrated copy of HIV-1 [39], were used for the standard curve. *ccr5* gene was used as endogenous control.

2.7. Quantification of HIV-1 2-LTR circles and proviral integration

Total DNA was extracted from MDMs 48 h after infection with JR-FL_Renilla, as described above. Analysis of 2-LTR circles was performed by qPCR in a StepOne Real-Time PCR system (Thermo Fisher Scientific) to measure the nuclear import of the viral genome, according to a previous protocol [40]. Proviral integrated DNA was quantified by nested Alu-LTR PCR [41,42] using a QX200 AutoDG Digital Droplet PCR system (Bio-Rad, Hercules, CA). In brief, a first conventional PCR was performed using oligonucleotides against Alu sequence and HIV-1 LTR, with the following conditions: 95 °C, 8 min; 12 cycles: 95 °C, 1 min; 60 °C, 1 min; 72 °C, 10 min; 1 cycle: 72 °C, 15 min. Then, a second qPCR was performed using TaqMan probes with FAM/ZEN/Iowa Black and TaqMan Master Mix (IDT, Integrated DNA Technologies, Coralville, IA). DNA from 8E5 cell line was used for the standard curve. *ccr5* gene was used as housekeeping gene for measuring the input DNA and normalize data.

2.8. Analysis of the intracytosolic expression of IFNγ and TNFα

MDMs treated or not with dasatinib that were infected in vitro with JR-FL_Renilla for 48 h were detached with StemPro Accutase Cell Dissociation Reagent (Thermo Fisher Scientific). After washing, cells were treated with 2 μg/ml lipopolysaccharide (LPS) from Escherichia coli serotype R515 (Enzo Life Sciences, New York, NY) for 4 h at 37 °C. Cells were then fixed and permeabilized with IntraPrep Reagent (Beckman Coulter) and the intracytosolic expression of IFNγ (Interferon gamma) and TNFα (Tumor necrosis factor alpha) was analyzed by flow cytometry after staining with specific conjugated antibodies (Beckman Coulter). Isotype controls were used to determine the background signal. Data acquisition was performed in a BD LSRFortessa X-20 flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed with FlowJo_V10 software (TreeStar, Ashland, OR). The gating strategy for the analysis is shown in Fig. 2.

2.9. Quantification of cytokines released by MDMs

Supernatants obtained from the culture of MDMs treated or not with dasatinib for 48 h and then infected in vitro with JR-FL_Renilla for 48 h were analyzed to determine the levels of cytokines released by M1-like MDMs (Interleukin (IL)-1β, IL-6, IL-8/CXCL8, Monokine induced by gamma interferon (MIG)/CXCL9, IL-12P70) and M2-like MDMs (Interleukin-1 receptor antagonist protein (IL-1 RA), IL-10, Monocyte chemotactic protein 4 (MCP-4)/CCL13, Myeloid progenitor inhibitory factor (MPIF)/CCL23, and Macrophage inflammatory protein 3 alpha (MIP-3α)/CCL20). Customized ProcartaPlex Immunoassay (Thermo Fisher Scientific) was used according to the manufactureŕs instructions and analyzed with Luminex 200 system (Thermo Fisher Scientific) and xPONENT 4.3 software.

2.10. Statistical analysis

Software GraphPad Prism 10.1.2. (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. Quantitative variables were represented by the mean and the standard deviation of the mean (SEM). Normal distribution of variables was determined with Kolmogorov-Smirnov normality test. According to the results, comparisons within groups were calculated using paired *t*-test and Wilcoxon signed-rank test; comparisons between groups were calculated using Kruskal-Wallis one-way, ordinary one-way ANOVA analysis, and Mann-Whitney U of variance by ranks, as appropriate. P values (p) < 0.05 were considered statistically significant in all comparisons.

3. Results

3.1. Clinical characteristics of study subjects

Fifteen PWH were recruited for this study at Hospital Universitario Severo Ochoa (Madrid, Spain). Sociodemographic and clinical data of these participants are summarized in Table 1. Most participants (80 %) were male, and the median age was 36 years old (Interquartile range

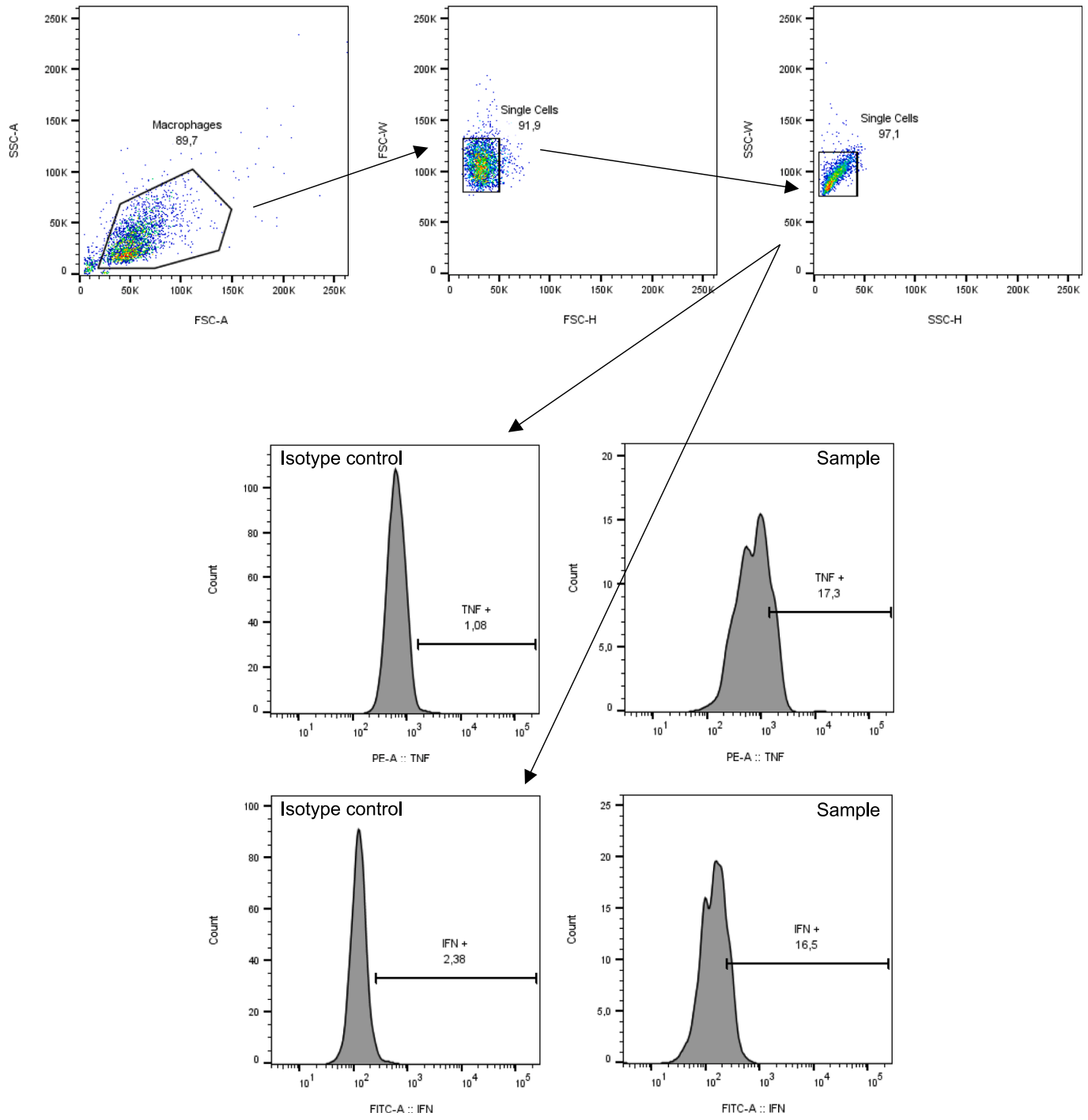

Fig. 2. Gating strategy for the analysis by flow cytometry of the intracellular expression of TNFα and IFNγ. One representative sample of MDMs infected with JR-FL_Renilla strain and stimulated with lipopolysaccharide stained in the cytoplasm with specific, conjugated monoclonal antibodies against TNFα and IFNγ. Isotype controls were used to determine the background signal.

(IQR) 33–45). Sexual transmission between men who have sex with men (MSM) was the most prevalent transmission route (66.7 %). Time of infection with HIV-1 until sampling was 8.6 years (IQR 3.2–13.0), median viral load log value at diagnosis was 4.8 (IQR 3.7–5.4), and the viral load at sampling was undetectable. Median nadir CD4 was 441 cells/ml (IQR 168–524), and median CD4 and CD8 counts were 640 cells/ml (IQR 544–998) and 990 cells/ml (IQR 613–1,122), respectively. Median CD4/CD8 ratio was 0.9 (IQR 0.5–1.2). All participants were on treatment with ART at the moment of sampling and most of them were receiving dolutegravir/lamivudine (DTC/3TC, 40 %) or bictegravir/emtricitabine/tenofovir alafenamide (BIC/FTC/TAF, 40 %).

Ten individuals with CML on treatment with dasatinib were recruited at the Hospital Universitario Ramón y Cajal and Hospital Universitario Puerta de Hierro Majadahonda (Madrid, Spain). Sociodemographic and clinical data of these participants are summarized in Table 2. Most participants were male (80 %), and the median age was 50 years old (IQR 31–57.8). Most individuals (90 %) were in chronic phase CML, and 5 individuals (50 %) were at low Sokal risk. All participants (100 %) showed hematological and cytogenetic remission of CML, with a median molecular response of 4.5 log (IQR 3–5). Current TKI for all

Table 1
Sociodemographic and clinical data of PWH who participated in the study.

Participants, n	15
Age (years), median (IQR)	36 (33–45)
Gender, n (%)	Male, 12 (80)
	Female, 3 (20)
Transmission route, n (%)	MSM, 10 (66.7)
	HC, 2 (13.3)
	IDU, 3 (20)
Time of infection with HIV (years), median (IQR)	8.6 (3.2–13.0)
Viral load at diagnosis (log), median (IQR)	4.8 (3.7–5.4)
Nadir CD4 (cells/ml), median (IQR)	441 (168–524)
CD4 count (cells/ml), median (IQR)	640 (544–998)
CD8 count (cells/ml), median (IQR)	990 (613–1,122)
CD4/CD8 ratio, median (IQR)	0.9 (0.5–1.2)
ART, n (%)	DTG/3TC, 6 (40)
	BIC/FTC/TAF, 6 (40)
	ABC/3TC/DTG, 1 (6.7)
	RPV/FTC/TAF, 1 (6.7)
	DRV/RTV/3TC, 1 (6.7)

3TC, lamivudine; ABC, Abacavir; ART, antiretroviral therapy; BIC, bictegravir; DRV, darunavir; DTG, dolutegravir; FTC, emtricitabine; HC, heterosexual contact; IDU, injection drug user; MSM, men who have sex with men; RPV, rilpivirine; RTV, ritonavir; TAF, tenofovir alafenamide.

Table 2
Sociodemographic and clinical data of individuals with CML who participated in the study.

Participants, n	10
Age at CML diagnosis (years), median (IQR)	50 (31–58)
Gender, n (%)	Male, 8 (80)
	Female, 2 (20)
CML phase, n (%)	Chronic, 9 (90)
	Accelerated, 1 (10)
	Blast, 0 (0)
Sokal risk score, n (%)	Low, 5 (50)
	Intermediate, 2 (20)
	High, 3 (30)
Molecular response (log), median (IQR)	4.5 (3–5)
Time of treatment with dasatinib (years), median (IQR)	5 (3.9–7.2)
Dose of dasatinib (mg/day), median (IQR)	70 (50–100)
Lymphocyte count (cells/ml), median (IQR)	1,550 (975.9–2,025)

CML, Chronic myeloid leukemia; IQR, Interquartile range; TKIs, Tyrosine kinase inhibitors.

participants was dasatinib with a median time of treatment of 5 years (IQR 3.9–7.2). Median daily dose of dasatinib was 70 mg (IQR 50–100). Median lymphocyte count was 1,550 cells/ml (IQR 975.9–2,025).

Sixteen healthy donors were recruited at the Primary Healthcare Center Doctor Pedro Lain Entralgo (Madrid, Spain). They had a median age of 45 years old (IQR 31.5–63) and 65 % were male.

3.2. Dasatinib interfered with HIV-1 infection in MDMs

MDMs from all participants were incubated with dasatinib 48 h before being infected with HIV-1 strain JR-FL-Renilla [36]. After 48 h of infection, the intracytoplasmic expression of p24-gag and SAMHD1 phosphorylation were assessed by flow cytometry and virus-driven expression of renilla was analyzed by luminescence. Infection was followed-up for additional 48 h (4 days in total) to determine if the effect of dasatinib may be maintained over time without further addition to the culture medium. The expression of p24-gag in MDMs from healthy donors was 3.2- ($p = 0.0216$) and 2.2-fold ($p = 0.0326$) lower after 48 h and 4 days of incubation in the presence of dasatinib, respectively, in comparison with untreated cells (Fig. 3A). Similarly, the expression of p24-gag in MDMs from PWH was 2.2- ($p = 0.0002$) and 2.6-fold ($p = 0.0350$) lower after 48 h and 4 days of incubation in the presence of dasatinib, respectively. These levels of infection were similar in MDMs isolated from CML individuals on treatment with dasatinib. Analogous pattern was observed when the expression of renilla was analyzed as a measure of proviral transcription, which was 4.0-fold ($p = 0.0397$) lower in MDMs from healthy donors after 4 days of incubation (Fig. 3B). This decrease was also observed in MDMs from PWH treated with dasatinib, although there was no significance in the comparisons with untreated cells. In accordance with the expression of p24-gag and renilla, the levels of pSAMHD1 were 5.3- ($p = 0.0162$) and 2.5-fold ($p = 0.0159$) lower in MDMs from healthy donors after treatment with dasatinib for 48 h and 4 days, respectively. In MDMs from PWH, similar decrease was observed after treatment with dasatinib for 48 h and 4 days of treatment (3.2-fold; $p = 0.0109$) (Fig. 3C). MDMs from CML individuals on treatment with dasatinib showed levels of pSAMHD1 that were 8.2- ($p = 0.0364$) and 8.7-fold ($p = 0.0116$) lower in comparison with MDMs from healthy donors and PWH, respectively. In all parameters, uninfected cells were used as mock controls to determine the response to dasatinib in the absence of infection.

HIV-1 infection was also monitored by time-lapse microscopy in living cells. MDMs from healthy donors and CML individuals on treatment with dasatinib were seeded in 6-well plates. MDMs from healthy donors were treated with dasatinib for 48 h or left untreated. All MDMs were then infected with DHIV3-GFP virus [37] and monitored for 72 h by confocal microscopy. GFP+ (infected) cells were visible 42 h after infection in untreated MDMs from healthy donors (Fig. 4A; Video file S1), while GFP expression was undetectable in 5 fields randomly selected in the well with dasatinib-treated MDMs ($p = 0.0032$) (Fig. 4A and B; Video file S2). The GFP expression in MDMs isolated from CML individuals on treatment with dasatinib was 12-fold ($p = 0.0369$) lower in comparison with untreated MDMs from healthy donors and the scarce GFP+-infected cells were visible after 64 h of infection in one of five fields randomly selected (Fig. 4B; Video file S3).

3.3. Dasatinib reduced HIV-1 proviral integration in MDMs

To evaluate the step in HIV-1 life cycle that was blocked by dasatinib in macrophages, MDMs from healthy donors were treated with dasatinib for 48 h and then infected with JR-FL_Renilla for 48 h. Viral reverse transcripts (RT) were analyzed by qPCR 5 h after infection. There were no differences between MDMs pre-treated or not with dasatinib in the formation of early and late RT (Fig. 5A). However, pre-treatment with dasatinib significantly interfered with the formation of 2-LTR circles in MDMs during the first 48 h of infection as it was 3.9-fold ($p = 0.0259$) lower than in untreated MDMs (Fig. 5B). The integration of the provirus was 4.0-fold ($p = 0.0391$) lower in MDMs pre-treated with dasatinib (Fig. 5C).

3.4. Dasatinib reduced the intracytosolic expression of pro-inflammatory cytokines in MDMs

The level of TNFα that was expressed in response to LPS in the cytosol of MDMs from PWH infected in vitro with JR-FL_Renilla for 48 h under basal conditions was similar to that in cells isolated from healthy donors (Fig. 6A). When cells were pre-treated in vitro with dasatinib for 48 h before infection with JR-FL_Renilla and LPS stimulation, the expression of TNFα in MDMs isolated from healthy donors and PWH was 1.2- ($p = 0.0244$) and 1.2-fold ($p = 0.0215$) lower, respectively. MDMs isolated from CML individuals on treatment with dasatinib showed very low levels of TNFα expression in comparison with MDMs from healthy donors (−4.4-fold, $p = 0.0380$) and PWH (−5.3-fold, $p = 0.0051$) pre-treated with dasatinib for 48 h.

The intracytosolic expression of IFNγ in response to LPS in MDMs isolated from healthy donors and PWH was lower after pre-treatment with dasatinib for 48 h (−2.1-fold, $p = 0.0098$ and −1.8-fold, $p = 0.0637$, respectively) (Fig. 6B). MDMs isolated from CML individuals on treatment with dasatinib expressed higher levels of IFNγ than MDMs isolated from PWH treated in vitro (3.1-fold, $p = 0.0224$).

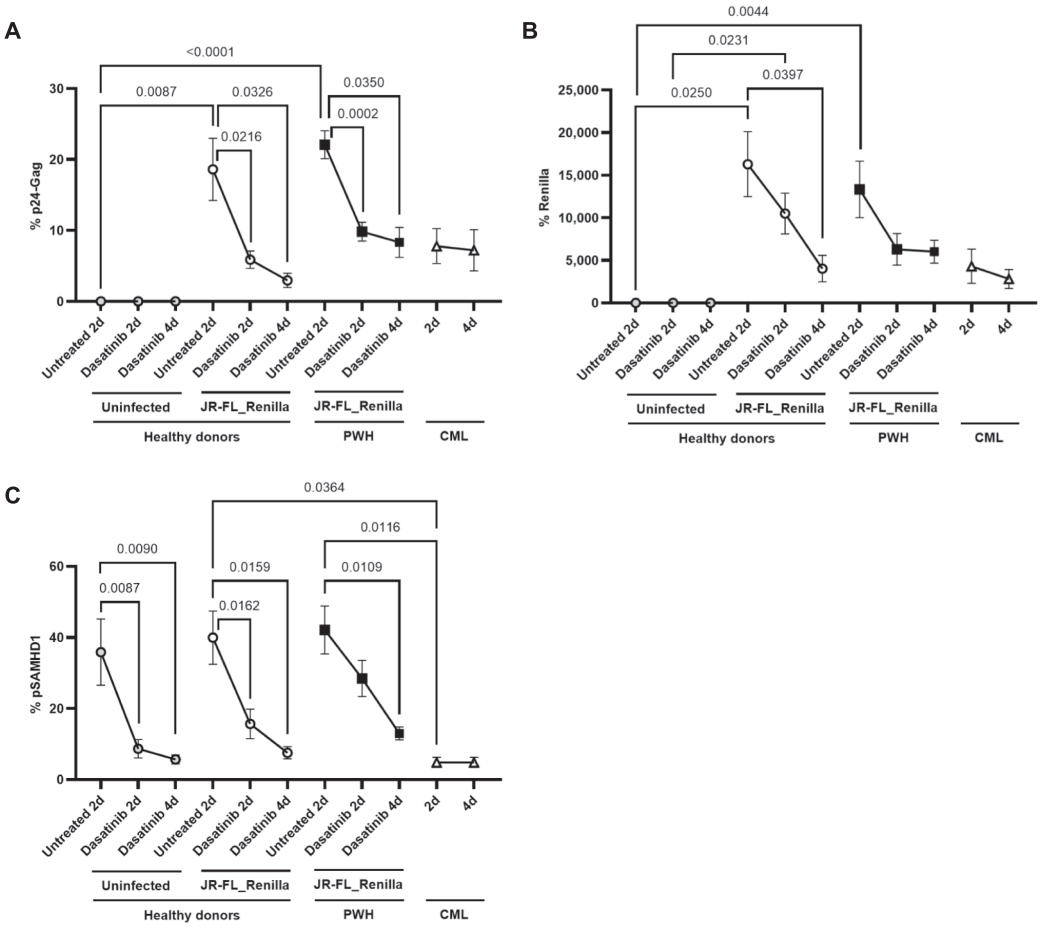

Fig. 3. Susceptibility to infection with JR-FL_Renilla of MDMs isolated from healthy donors, PWH, and people with CML on treatment with dasatinib for 48 h and 4 days. Quantification by flow cytometry of the intracytosolic expression of p24-gag (A), renilla (B), and pSAMHD1 (C) in MDMs isolated from healthy donors, PWH, and CML individuals on treatment with dasatinib that were infected in vitro with pJR-FL_Renilla. Each dot corresponds to the mean ± standard error of the mean (SEM). Each symbol represented a different cohort: healthy donors (open circles), PWH (closed squares), and people with CML on treatment with dasatinib in vivo (open triangle). Paired *t*-test and Wilcoxon signed-rank test were applied to calculate the statistical significance within groups and ordinary one-way ANOVA test, Kruskal-Wallis one-way test, and Mann-Whitney U of variance by rank was applied to calculate the statistical significance between groups, as appropriate.

3.5. Dasatinib interfered with the release of M1- and M2-related cytokines

The release of M1- and M2-related cytokines was analyzed in the supernantants of MDMs infected in vitro with JR-FL_Renilla for 48 h. Dasatinib was added to the culture medium 48 h before the infection. The release of pro-inflammatory cytokines mainly produced by M1-like macrophages such as IL-1β and IL-12 was 2.4- ($p = 0.0048$) and 1.4-fold ($p = 0.0024$) lower, respectively, from MDMs of PWH after treatment with dasatinib in vitro for 48 h (Fig. 7A). Treatment with dasatinib did not modify the release of IL-6 by MDMs from PWH and healthy donors, but this cytokine was undetectable in the supernatants of MDMs isolated from CML individuals on treatment with dasatinib in vivo ($p = 0.0004$), as occurred with IL-1β ($p = 0.0039$), in comparison with MDMs from PWH. The capacity to release IL-12 by MDMs from people with CML remained unchanged in comparison with MDMs from PWH treated with dasatinib in vitro, and it was higher than MDMs from healthy donors (1.7-fold; $p = 0.0149$). The release of chemokine MIG/CXCL9 to the culture medium by MDMs from healthy donors and PWH was 2.0- ($p = 0.0273$) and 1.6-fold ($p = 0.0313$) lower, respectively, after treatment in vitro with dasatinib, while it was nearly undetectable by MDMs from people with CML (Fig. 7B, right graph). No significant differences were observed in the levels of chemokine IL-8/CXCL8 released by MDMs of PWH and healthy donors after treatment with dasatinib in vitro, but this chemokine was undetectable in the supernatants of MDMs from CML individuals ($p < 0.0001$ in comparison with healthy donors; $p = 0.0010$ in comparison with PWH) (Fig. 7B, left graph).

The levels of anti-inflammatory cytokines primarily released by M2-like macrophages such as IL-1RA and IL-10 were also lower in the supernatants of MDMs from healthy donors and PWH infected with JR-FL_renilla for 48 h if they were previously treated with dasatinib in vitro (−2.7-fold, $p = 0.0020$ and −2.1-fold, $p = 0.0002$, respectively, for IL-1RA; −2.2-fold, $p = 0.0012$ and −2.2-fold, $p = 0.0011$, respectively, for IL-10) (Fig. 8A). IL-1 RA and IL-10 were undetectable in the supernatants of MDMs isolated from CML individuals on treatment with dasatinib in vivo ($p < 0.0001$ and $p = 0.0031$, respectively, in comparison with PWH). No significant changes were observed in the levels of chemokines MIP-3α/CCL20 and MPIF/CCL23 released by MDMs from healthy donors and PWH after short treatment in vitro with dasatinib, but they were undetectable in the supernatants of MDMs from CML individuals on treatment with dasatinib in vivo ($p = 0.0201$ and $p = 0.0073$, respectively, in comparison with PWH) (Fig. 8B). The release of MCP-4/CCL13 was lower by MDMs from healthy donors (−2.2-fold, $p = 0.0313$) after treatment with dasatinib in vitro but it remained unchanged in MDMs from PWH (Fig. 8C). However, the level of this chemokine was higher in the supernatants of MDMs from CML individuals on treatment with dasatinib in vivo, in comparison with MDMs from healthy donors and PWH (11.4-fold, $p < 0.0001$ and 6.1-fold, $p =$

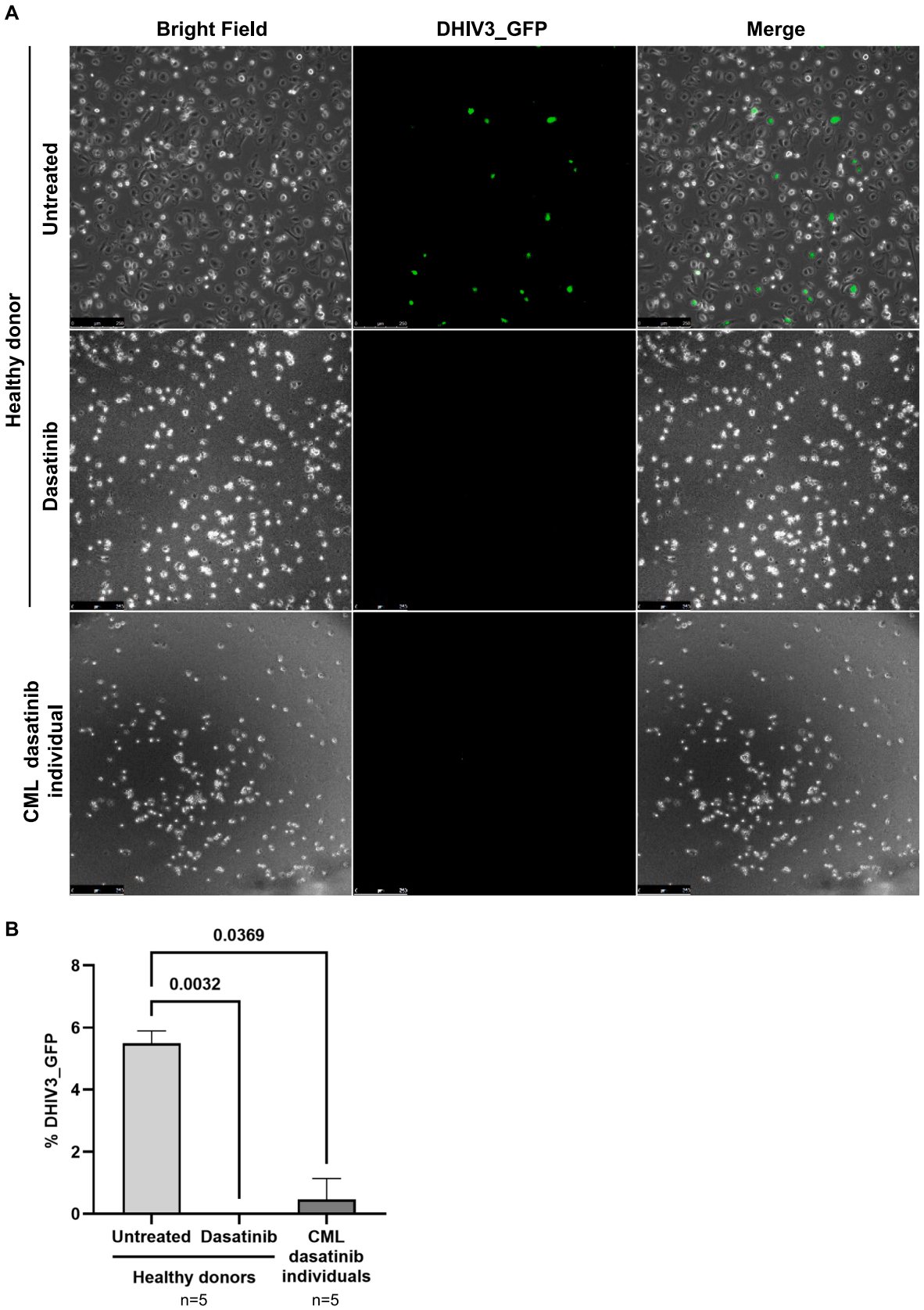

Fig. 4. Analysis by time-lapse confocal microscopy of HIV-1 infection in living MDMs. (A) Maximum image projection of representative fields of MDMs isolated from healthy donors untreated or treated with dasatinib in vitro for 48 h before being infected with DHIV3_GFP for 72 h. MDMs isolated from CML individuals on treatment with dasatinib in vivo were infected in the same conditions. (B) Statistical significance in the comparison of the number of infected GFP+cells per field between groups was analyzed by Kruskal-Wallis one-way test.

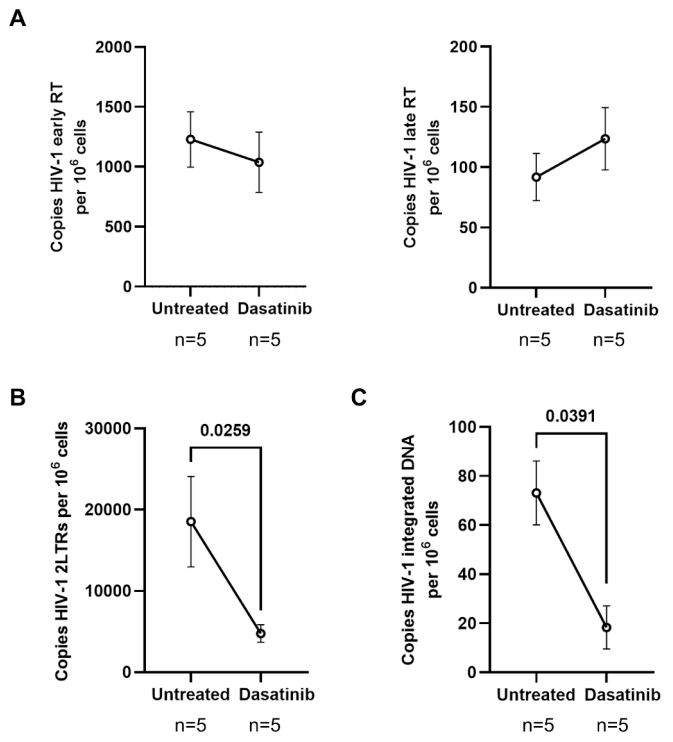

Fig. 5. Dasatinib interfered with HIV-1 proviral integration and nuclear import. A) Analysis by qPCR of the early and late reverse transcription of pJR-FL_Renilla in MDMs isolated from healthy donors in response to treatment in vitro with dasatinib for 48h. The formation of 2-LTR circles (B) and the integration of the provirus (C) were also analyzed in these cells by qPCR and ddPCR, respectively. Each dot corresponds to the mean ± SEM. Paired *t*-test was applied to calculate the statistical significance between conditions.

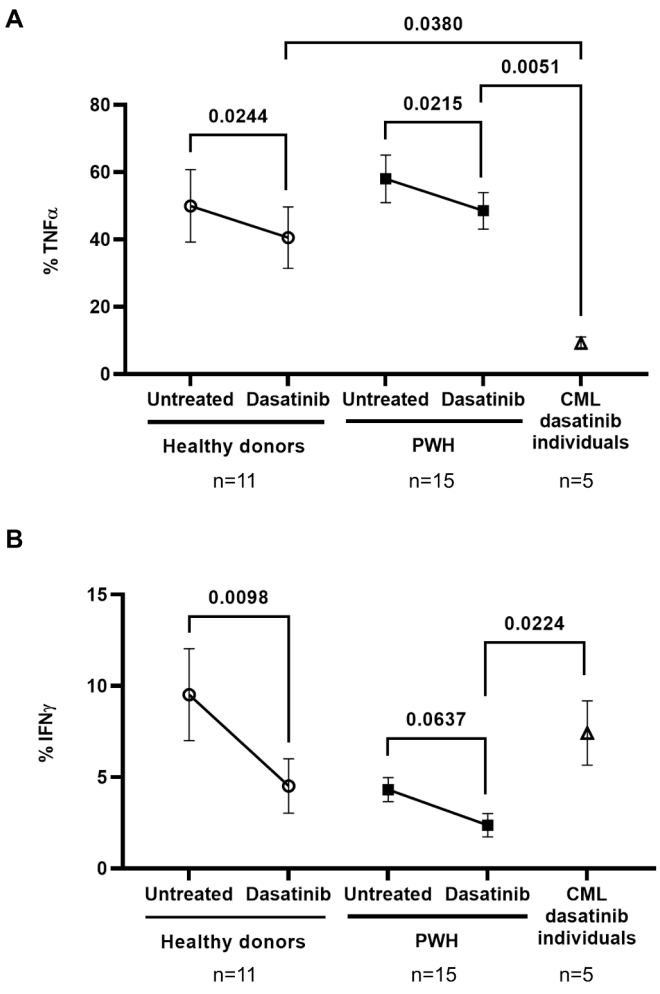

Fig. 6. Influence of dasatinib on the intracytosolic expression of pro-inflammatory cytokines. The intracellular expression of TNFα (A) and IFNγ (B) was determined by flow cytometry in MDMs cells isolated from healthy donors, PWH, and individuals with CML on treatment with dasatinib in vivo that were previously infected with pJR-FL_Renilla for 48 h and stimulated with LPS for 4 h. Dasatinib in vitro was added to MDMs from healthy donors and PWH 48 h before infection with pJR-FL_Renilla. Each dot corresponds to the mean ± SEM. Each symbol represented a different cohort: healthy donors (open circles), PWH (closed squares), and people with CML on treatment with dasatinib in vivo (open triangle). Wilcoxon signed-rank test was applied to calculate the statistical significance within groups and Kruskal-Wallis one-way and Mann-Whitney U of variance by rank were applied to calculate the statistical significance between groups.

0.0009, respectively).

4. Discussion

Both CD4 + T cells and macrophages should be considered as targets in the strategies aimed at eradicating HIV-1, long-lived viral reservoirs. These two types of cells are very different in morphology and function, but they share the common receptor CD4 that constitutes the main receptor for HIV-1 infection [43]. The innate immune antiviral factor SAMHD1 efficiently protects CD4 + T cells from HIV-1 infection while not phosphorylated, i.e., in resting cells [44]. However, T-cell activation mediated by the engagement of TCR or interleukin receptors induces SAMHD1 phosphorylation and inactivation, rendering the cell susceptible to infection [27,31]. The expression of SAMHD1 is higher in macrophages, which should induce less permissiveness to HIV-1 infection in these cells than in CD4 + T cells [7,8]. However, in our study, SAMHD1 was highly phosphorylated in MDMs isolated from PWH and healthy donors, in agreement with previous reports [12], which would abrogate its antiviral activity. This indicated that HIV-1 may be less sensitive to SAMHD1 antiviral activity in MDMs than in CD4 + T cells and that other mechanisms are likely involved in the lower susceptibility to infection by HIV-1 in macrophages, such as the necessity for a cell–cell interaction that may facilitate the infection [45].

Dasatinib is very effective to inhibit HIV-1 infection of CD4 + T cells and this inhibition mostly relies on the interference with SAMHD1 phosphorylation [29]. In fact, we described previously that dasatinib was not able to interfere with the replication of HIV-1 in CD4 + T cells when the virions were carrying SIVsm Vpx [29] which targets SAMHD1 for proteasomal degradation [7,46]. The antiviral effect of dasatinib has also been described in MDMs by Szaniawski et al. who demonstrated not only a positive correlation between the level of pSAMHD1-T592 and HIV-1 infectivity for MDMs treated with dasatinib, but also that the presence of Vpx abrogated the inhibitory potential of dasatinib on HIV-1 infection of MDMs [30], due to Vpx induces SAMHD1 ubiquitination and nuclear degradation [7,8]. We observed that MDMs from PWH showed higher susceptibility to HIV-1 infection in vitro than those from healthy donors, but dasatinib had a similar antiviral effect in all MDMs, independently of the origin. However, this protective effect exerted in MDMs by treatment with dasatinib in vitro was not as complete as in resting CD4 + T cells, likely because MDMs showed a high level of SAMHD1 phosphorylation that was not totally counteracted after 48 h of treatment. This could be due to the chronic inflammation characteristic of PWH that may be influencing the activation status of the macrophages. Therefore, more time of treatment with dasatinib would be necessary to decrease the levels of pSAMHD1 and produce a full protective effect against HIV-1 infection in MDMs, as was shown in the experiments in which cells were treated with dasatinib for 4 days. In fact, MDMs from CML individuals on long-term treatment in vivo with

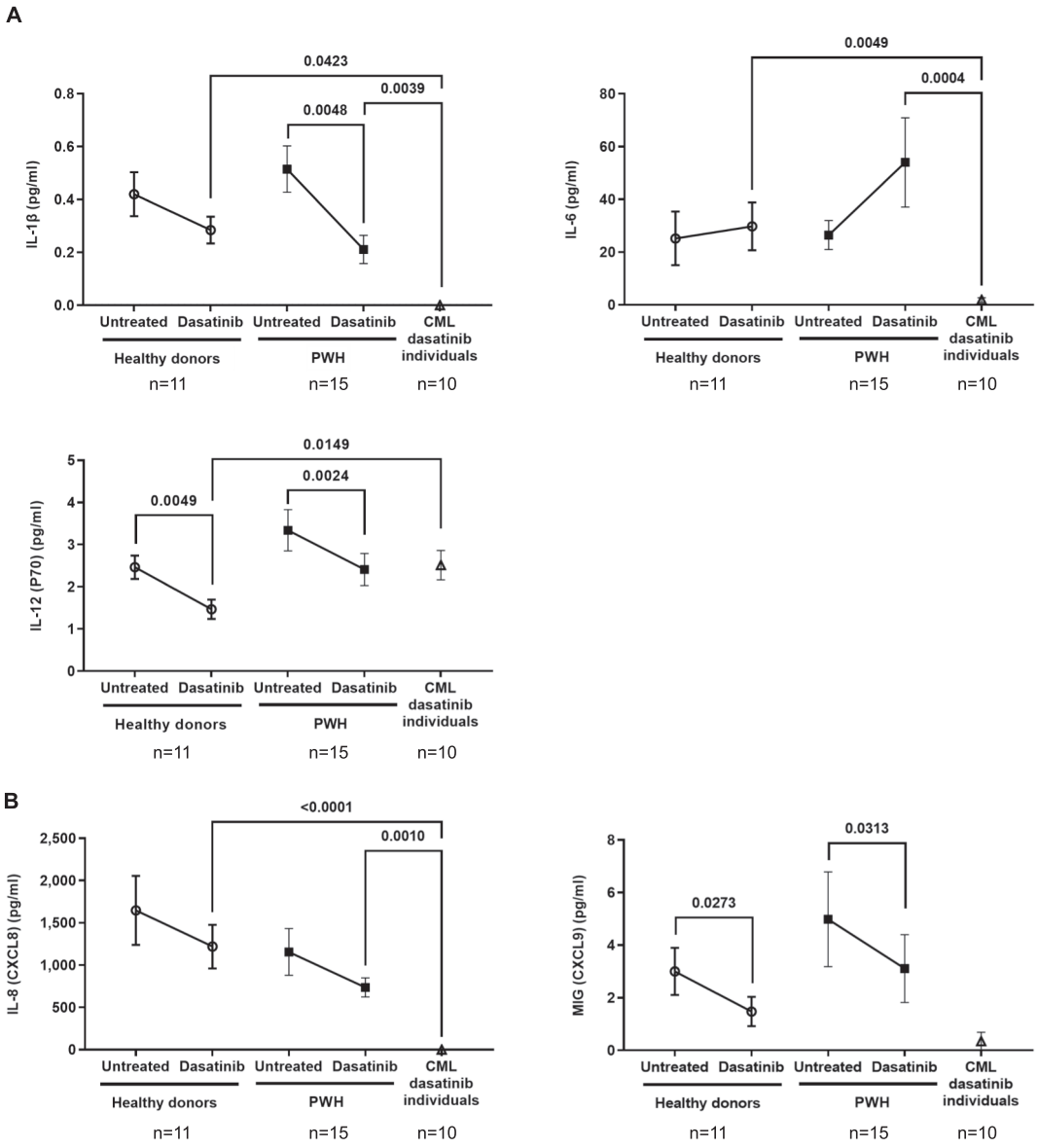

Fig. 7. Quantification of M1-related pro-inflammatory cytokines in the culture supernatants on MDMs. Cytokines IL-1β, IL-6, and IL-12 (A) and chemokines IL-8/CXCL8 and MIG/CXCL9 (B) were quantified by Luminex assay in the supernatants of MDMs isolated from healthy donors, PWH, and individuals with CML on treatment with dasatinib in vivo that were previously infected with pJR-FL_Renilla for 48 h. Dasatinib in vitro was added to MDMs from healthy donors and PWH 48 h before infection with pJR-FL_Renilla. Each dot corresponds to the mean ± SEM. Each symbol represented a different cohort: healthy donors (open circles), PWH (closed squares), and people with CML on treatment with dasatinib in vivo (open triangle). Wilcoxon signed-rank test was applied to calculate the statistical significance within groups and Kruskal-Wallis one-way and Mann-Whitney U of variance by rank were applied to calculate the statistical significance between groups.

dasatinib showed nearly undetectable levels of pSAMHD1 under basal conditions. Monitorization by time-lapse microscopy of infection with DHIV3_GFP strain in living MDMs treated with dasatinib or isolated from people with CML showed nearly undetectable expression of GFP, which was also delayed in comparison with healthy donors. Surprisingly, using a different readout such as the expression of renilla and p24-gag in MDMs infected with JR-FL_Renilla strain, we observed that MDMs from CML individuals were still susceptible to HIV-1 infection. This difference could be due to luminescent assays are more sensitive than fluorescent assays [47], but also to the existence of additional mechanisms that may permit HIV-1 infection in macrophages and that could be independent on SAMHD1. In addition, we observed a discordance between the levels of p24-gag and renilla produced by the infected MDMs, which has already been described and may be due to the detection of renilla by chemiluminescence is more sensitive than detection of p24-gag by ELISA [48]. Moreover, as *renilla* has been inserted into the location of *nef* inside the viral genome in HIV-1 JR-FL_Renilla strain [36], we cannot rule out that dasatinib was also acting at transcriptional and/or splicing level to interfere with a complete, efficient proviral reactivation, as was observed in CD4 + T cells isolated from PWH and CML treated with ART and dasatinib [25]. In fact, dasatinib completely inhibits HIV-1 reverse transcription in CD4 + T cells, mostly due to the inhibition of pSAMHD1 and dNTPs depletion, thereby impeding the formation of 2-LTR circles and the integration of the provirus [29]. Dasatinib may also have a direct or indirect effect on the proviral integration likely based on the interference with the chromatin opening that occurs during T-cell activation [49] and that would also impede the proviral integration and reactivation from latency. In MDMs, due to the inhibition of SAMHD1 phosphorylation by dasatinib was not complete and dNTPs may still be available, HIV-1 reverse transcription was not totally inhibited in these cells, contrary to what was observed in CD4 + T cells. However, we observed a low quantity of 2-LTR circles and proviral

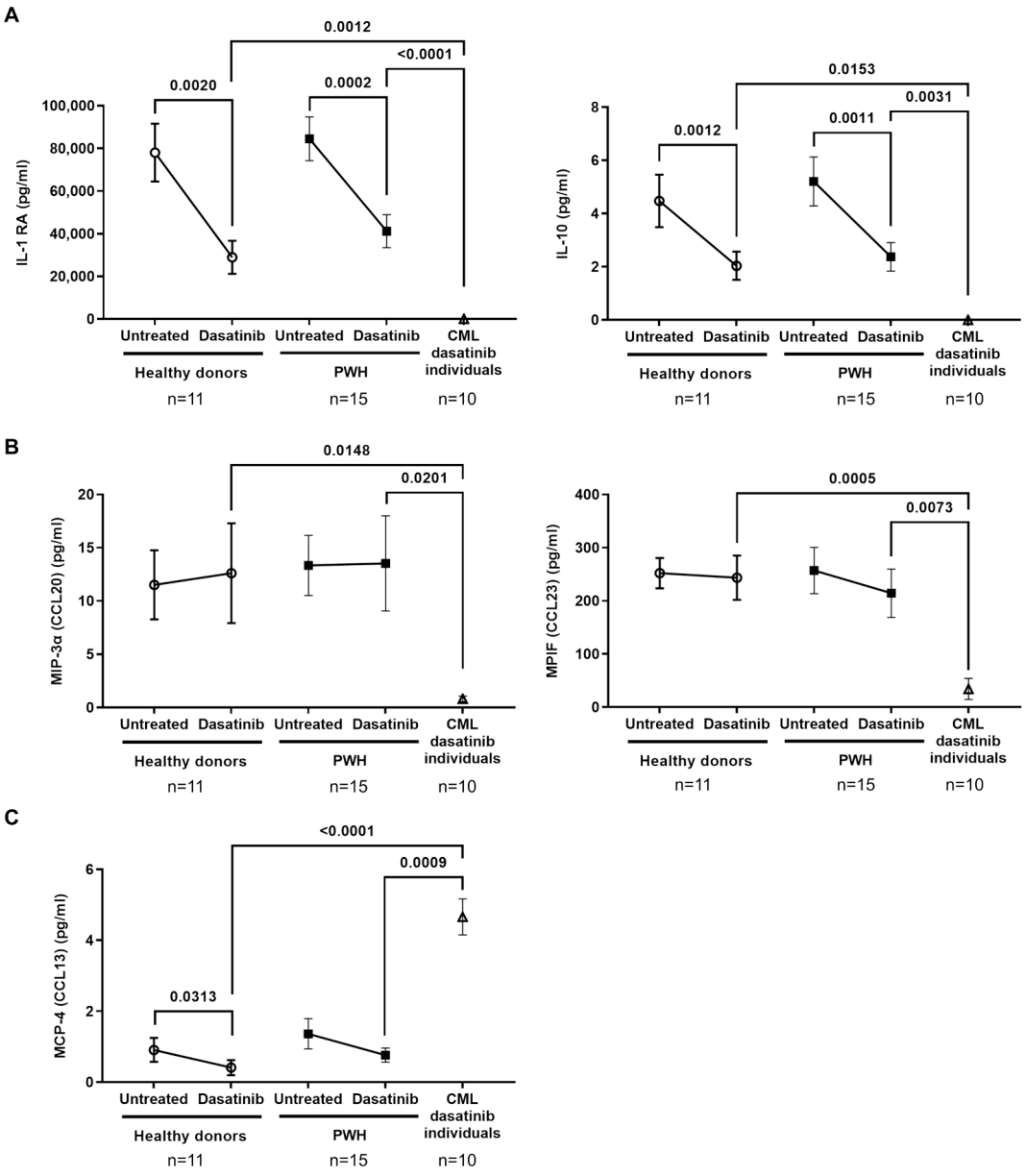

Fig. 8. Quantification of M2-related anti-inflammatory cytokines in the culture supernatants on MDMs. Cytokines IL1-RA and IL-10 (A) and chemokines MIP-3α/CCL20 and MPIF/CCL23 (B), and MCP-4/CCL13 (C) were analyzed by Luminex assay in the supernatants of MDMs isolated from healthy donors, PWH, and individuals with CML on treatment with dasatinib in vivo that were previously infected with pJR-FL_Renilla for 48 h. Dasatinib in vitro was added to MDMs from healthy donors and PWH 48 h before infection with pJR-FL_Renilla. Each dot corresponds to the mean ± SEM. Each symbol represented a different cohort: healthy donors (open circles), PWH (closed squares), and people with CML on treatment with dasatinib in vivo (open triangle). Paired *t*-test and Wilcoxon signed-rank test were applied to calculate the statistical significance within groups and ordinary one-way ANOVA test, Kruskal-Wallis one-way test and Mann-Whitney U of variance by rank was applied to calculate the statistical significance between groups, as appropriate.

integration in the presence of dasatinib that would indicate that the interference with HIV-1 infection in macrophages probably occurs in a different step such as the nuclear import.

Dasatinib has also been tested as senotherapeutic in several clinical trials to improve cellular senescence. The first human trial with dasatinib for this indication was performed in combination with the flavonol quercetin that targets the apoptosis regulator B-Cell CLL/Lymphoma 2 (BCL-2), insulin-like growth factor 1 (IGF-1), and hypoxia-inducible factor-1 alpha (HIF-1α), demonstrating that this combination was safe, well-tolerated, and effective to improve the physical function of individuals with idiopathic pulmonary fibrosis [33]. The combination of dasatinib and quercetin has also been effective to reduce intestinal inflammation in aged mice [50] and dasatinib also improved pancreatic fibrosis and interfered with the infiltration of macrophages in a mouse model [51]. Although several factors may contribute to the chronic inflammation characteristic of PWH, such as persistent viral replication, microbial translocation, and co-infections with other pathogens [21,22], macrophages may also have a central role due to PWH usually present high levels of these cells with pro-inflammatory phenotype [6,52,53]. Therefore, we evaluated the potential effects of dasatinib toward controlling the production of pro-inflammatory cytokines characteristically released by M1-like macrophages. In vitro treatment with dasatinib for 48 h significantly reduced the release from MDMs of potent inflammatory cytokines such as IL-1β or TNFα in response to HIV-1 infection, although there was no effect on the production of IL-6. However, MDMs isolated from CML individuals did not produce detectable levels of TNFα, IL-1β, IL-6, as well as chemokines IL-8/CXCL8 and CXCL9, thereby supporting the role of dasatinib as an anti-inflammatory drug. In

addition, dasatinib slightly modified the capacity of MDMs to release IL-12 and IFNγ that are essential for the polarization of T helper cells Th0 to Th1 with antiviral activity [54]. Nevertheless, although dasatinib may interfere with the release of IFNγ from CTLs [55], it did not completely inhibit the intracytosolic expression of IFNγ in MDMs from people with CML on long-term treatment, in comparison with untreated MDMs from healthy donors. In view of these results, it appeared that dasatinib was able to control inflammation by impeding the differentiation of macrophages into the pro-inflammatory M1 phenotype. It has been described that treatment of primary murine macrophages with dasatinib suppresses the production of IL-6, IL-12, and TNFα after Toll-like receptors (TLRs) stimulation, but that it also increases the release of IL-10 [56], suggesting that the anti-inflammatory potential of dasatinib is also based on favoring differentiation to M2-like MDMs. However, we observed that treatment of MDMs with dasatinib in vitro for 48 h also decreased the release of M2-related anti-inflammatory cytokines such as IL-1RA and IL-10, which would indicate that dasatinib is more likely hindering the polarization of both M1 and M2 macrophages, as was described previously in mice with chronic pancreatitis [51]. In fact, in the supernatants of MDMs isolated from individuals with CML on long-term treatment with dasatinib in vivo there were undetectable levels of all assayed M2-related cytokines with the notable exception of CCL13 that was significantly higher. CCL13 is a chemokine also known as MCP-4 (monocyte chemoattractant protein 4) that has been related to multiple anti-microbial activities such as the migration of T cells, dendritic cells, monocytes, and eosinophils, degranulation of eosinophils and basophils, expression of adhesion molecules, and antibacterial activity against Gram-negative bacteria [57–59]. Therefore, higher levels of CCL13 in individuals with CML could be associated with protection against infections as has been observed in individuals with cirrhosis and chronic obstructive pulmonary disease (COPD) [60,61]. This likely contributes to the low incidence of opportunistic infections and herpesvirus reactivation reported in CML individuals on long-term treatment with dasatinib [62,63], despite the interference with T-cell activity and proliferation [55] and the inhibitory effect on the M1-like macrophages reported here. One potential limitation of our study is that MDMs from individuals with CML may be affected by the disease, which would prevent confirming if all the changes observed in MDMs isolated from these individuals were completely dependent on the treatment with dasatinib. Therefore, these results will be confirmed in the clinical trial NCT05780073 promoted by our group in which chronically infected PWH on ART will take dasatinib for 24 weeks.

In conclusion, dasatinib interfered with HIV-1 proviral integration in macrophages, but it was not as efficient as in CD4 + T cells after short-treatment, likely due to MDMs presented higher levels of pSAMHD1 in basal condition than resting CD4 + T cells [29,33,59]. Therefore, longer time would be necessary for dasatinib to completely impede HIV-1 infection of one of the cell contributors to the viral reservoir, as was observed in people with CML on long-term treatment. Dasatinib was also able to decrease the inflammatory potential of MDMs without interfering with its antiviral activity, as the production of IL-12 and IFNγ, which is essential for Th1 polarization, although it was reduced after short-term treatment in vitro, it was preserved in MDMs from people with CML on long-term treatment with dasatinib. The anti-inflammatory effect appeared to be based on a decrease in the release of M1-related pro-inflammatory cytokines. But the release of M2-related anti-inflammatory cytokines was also diminished, which pointed towards an interference with the differentiation of MDMs to M1- and M2-like phenotypes, similarly to the effect produced by dasatinib on the differentiation of CD4 + T cells memory subpopulations [25,64]. However, it is important to consider that these experiments were performed in the context of an acute HIV-1 infection and that further analyses will be necessary to evaluate if dasatinib would have the same effect in latent infection. Consequently, more studies are required to determine the validity of using dasatinib in combination with ART to impede the reservoir formation and maintenance in both CD4 + T cells and macrophages, as well as to ameliorate the chronic inflammation characteristic of PWH by interfering with the inflammatory potential of the macrophages.

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CRediT authorship contribution statement

Sara Rodríguez-Mora: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Clara Sánchez-Menéndez:** Resources, Methodology, Investigation, Formal analysis. **Guiomar Bautista-Carrascosa:** Writing – original draft, Resources, Methodology, Investigation. **Elena Mateos:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Lucia Moreno-Serna:** Resources, Methodology, Investigation. **Diego Megías:** Methodology, Investigation, Formal analysis, Data curation. **Juan Cantón:** Resources, Methodology, Investigation, Formal analysis. **Valentín García-Gutiérrez:** Resources, Methodology, Investigation. **María Aránzazu Murciano-Antón:** Resources, Methodology, Investigation. **Miguel Cervero:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Adam Spivak:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vicente Planelles:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mayte Coiras:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We greatly appreciate all the participants for their contribution to this study.

AUTHOR CONTRIBUTIONS

MCoi, SRM, AS, and VP conceptualized the project and wrote the manuscript. GBC, MAMA, MCer, JC, and VGG selected and recruited the participants and collected the blood samples. SRM, EM and LMS processed and stored all blood samples. SRM, EM, CSM, and LMS performed the analytical experiments. SRM, LMS, and DM performed the microscopy analyses. SRM, GBC, LMS, DM, AS, VP, and MCoi collected and analyzed the clinical data and laboratory results. All co-authors read and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcp.2024.116512.

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