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Cytotoxic cell populations developed during treatment with tyrosine kinase inhibitors protect autologous CD4+ T cells from HIV-1 infection

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ABSTRACT

Tyrosine kinase inhibitors (TKIs) are successfully used in clinic to treat chronic myeloid leukemia (CML). Our group previously described that CD4+ T cells from patients with CML on treatment with TKIs such as dasatinib were resistant to HIV-1 infection ex vivo. The main mechanism for this antiviral activity was primarily based on the inhibition of SAMHD1 phosphorylation, which preserves the activity against HIV-1 of this innate immune factor. Approximately 50% CML patients who achieved a deep molecular response (DMR) may safely withdraw TKI treatment without molecular recurrence. Therefore, it has been speculated that TKIs may induce a potent antileukemic response that is maintained in most patients even one year after treatment interruption (TI). Subsequent to in vitro T-cell activation, we observed that SAMHD1 was phosphorylated in CD4+ T cells from CML patients who withdrew TKI treatment more than one year earlier, which indicated that these cells were now susceptible to HIV-1 infection. Importantly, these patients were seronegative for HIV-1 and seropositive for cytomegalovirus (CMV), but without CMV viremia. Although activated CD4+ T cells from CML patients on TI were apparently permissive to HIV-1 infection ex vivo, the frequency of proviral integration was reduced more than 12-fold on average when these cells were infected ex vivo in comparison with cells isolated from untreated, healthy donors. This reduced susceptibility to infection could be related to an enhanced NK-dependent cytotoxic activity, which was increased 8-fold on average when CD4+ T cells were infected ex vivo with HIV-1 in the presence of autologous NK cells. Enhanced cytotoxic activity was also observed in CD8 + T cells from these patients, which showed 8-fold increased expression of TCRγδ and more than 18-fold increased production of IFNγ upon activation with CMV peptides. In conclusion, treatment with TKIs induced a potent antileukemic response that may also have antiviral effects against HIV-1 and CMV, suggesting that transient use of TKIs in HIV-infected patients could develop a sustained antiviral response that would potentially interfere with HIV-1 reservoir dynamics.

Abbreviations: CML, Chronic myeloid leukemia; cART, Combined antiretroviral therapy; CMV, Cytomegalovirus; DMR, Deep molecular response; HIV-1, Human immunodeficiency virus type 1; LGLs, Large granular lymphocytes; LRAs, Latency reversal agents; LUC, Luciferase; Ph+, Philadelphia chromosome positive; PHA, Phytohemagglutinin; RLUs, Relative light units; TI, Treatment interruption; TFR, Treatment-free remission; TKIs, Tyrosine kinase inhibitors

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1. Introduction

The main challenge for the eradication of the infection caused by human immunodeficiency virus type 1 (HIV-1) is represented by its ability to integrate the viral genome into the cellular chromosome, producing long-lived reservoirs in latency. These reservoirs are formed very early after infection [1] and cannot be eliminated with the present combined antiretroviral therapy (cART) or be detected by the immune system [2]. The main components [1] of the viral reservoir are CD4+ T cells and, to a lower extent, macrophages, and is nearly impossible to avoid its formation even with prompt initiation of cART [3]. The reservoir is very stable over time and remains quite unchanged in patients even with tightly controlled viremia [2]. This is due to a large degree to the ability of cells in the reservoir to undergo homeostatic proliferation driven by γc-cytokines such as IL-2, IL-7 and IL-15 that permit low-level proliferation of infected cells without proviral reactivation [4,5]. In this scenario, new strategies are needed not only to avoid the formation of the reservoir but also to interfere with its replenishment and maintenance. Theoretically, this would permit the slow reduction of the reservoir size until it can be controlled by the immune system (functional cure) or is completely eliminated (eradication).

One strategy that is being extensively studied in order to destroy the viral reservoirs and achieve HIV-1 cure is the "kick and kill". This strategy is based on the use of latency reversal agents (LRAs) that would reactivate the latent provirus from the reservoirs so that the infected cells became visible for both cART and the immune system [6]. However, LRAs assayed so far have been quite inefficient to reactivate the provirus and likely LRAs combinations should be used in order to significantly reactivate the latently infected cells [7]. On the other hand, once the provirus has been reactivated and the infected cells begin to produce new viral particles, they should be destroyed by the cytotoxic immune response. Most chronically HIV-infected patients could not elicit a cytotoxic response potent enough to destroy the reactivated cells [8,9]. Therefore, although several LRAs may reactivate latent provirus in cART-treated patients, a significant decrease on the reservoir size could not be demonstrated [10]. For these reasons, new strategies are also needed to increase the cytotoxic activity of CD8 and NK cells in order to efficiently destroy infected cells harboring reactivated proviruses.

Our group previously described that tyrosine kinase inhibitors (TKIs) may interfere with CD4+ T cell activation, which would avoid proviral integration and reduce the reservoir formation in vivo during acute infection [11]. We demonstrated that pretreatment of humanized CD34+ NSG mice with TKI dasatinib reduced plasma viremia and reservoir size in the gut-associated lymphoid tissue (GALT) after intraperitoneal HIV-1 infection. Furthermore, we determined that TKIs could also interfere with the replenishment and maintenance of the reservoir by inhibiting T-cell proliferation induced by homeostatic γc-cytokines [12,13]. TKIs include five FDA-approved compounds that can be used for the treatment of chronic myeloid leukemia (CML): imatinib, nilotinib, dasatinib, bosutinib and ponatinib. These drugs are directed against an aberrant tyrosine kinase termed BCR-ABL that shows constitutive activity and is formed during the reciprocal translocation of chromosomes 9 and 22, rendering a deficient, smaller chromosome 22 which is called Philadelphia (Ph). The introduction of TKIs in the clinic gave rise to an improvement in life expectancy and quality of life of patients with Ph+ CML [14]. TKIs show different selectivity of action against BCR-ABL and other tyrosine kinases. The less selective for BCR-ABL is the TKI, the more efficient seems to be at protecting CD4+ T cells from HIV-1 infection due to off-target effects. Therefore, TKIs that target a wide range of tyrosine kinases, such as dasatinib and ponatinib, have proved to be the most efficient to protect CD4+ T cells from HIV-1 infection [13,15]. The mechanism of action for this direct antiviral effect on CD4+ T cells was related to preserving the antiviral activity of the innate immune factor SAMHD1 both in T-cells [13] as well as in macrophages [16]. SAMHD1 is a deoxynucleotide triphosphate (dNTP) hydrolase that controls the homeostatic balance of cellular dNTPs [17]. When SAMHD1 is active, it degrades dNTPs, and this is concomitant with a resting state of the cell. Phosphorylation of SAMHD1 at T592 by cyclin-dependent kinases deactivates this innate immune factor, resulting in an increase of intracellular dNTPs and entry in S-phase of DNA replication, making HIV-1 infection possible [18]. Therefore, some TKIs may directly protect CD4+ T cells from HIV-1 infection by interfering with SAMHD1 phosphorylation, impeding T-cell activation and proviral integration. However, other mechanisms different from the direct protection of CD4+ T cells might also be triggered by TKIs.

TKIs have been described as immunomodulatory drugs with the ability to induce the expansion of large granular lymphocytes (LGLs) with cytotoxic activity that have been associated with a better anti-leukemic response mostly in patients with CML who were treated with dasatinib [19–22]. These LGLs show both CD8 and NK cell phenotypes and their expansion appears to be driven by the presence of previous cytomegalovirus (CMV) infection, a ubiquitous herpesvirus with an estimated seroprevalence of 45% to 100% worldwide in the general population [23]. Although TKI treatment against CML is supposedly a life-long treatment, several clinical trials have proven that it is possible to stop treatment under some circumstances and still maintain a durable anti-leukemic response, presumably based on the efficient destruction of cancerous cells by LGLs. This could be due to the generation of a powerful memory cytotoxic response that can be efficiently maintained even after treatment discontinuation. In fact, the expansion of memory NK cells with phenotype CD3-CD56+CD57+ has been described in CMV-seropositive dasatinib-treated patients and also in transplanted patients with CMV reactivation, suggesting that these cytotoxic NK cells could be associated with both anti-leukemic and antiviral responses, even after treatment interruption [24–27]. On the other hand, increased levels of T lymphocytes expressing TCRγδ have also been associated with improved disease-free survival of leukemia in patients who received bone marrow transplant depleted of TCRαβ T cells [28]. These TCRγδ T cells are unconventional cytotoxic cells that recognize antigens without the presence of major histocompatibility complex (MHC) molecules, directly attacking target cells or inducing the activation of other immune cells [29]. The therapeutic potential of these cells is under investigation in the context of hematologic malignances such as CML [30].

Based on the previously exposed, the aim of this study was to determine whether the potent cytotoxic activity against leukemic cells that develops in CML patients during treatment with TKIs may also provide an antiviral activity against HIV-1 that can be maintained even after treatment discontinuation. This finding could be an essential contribution to the "kick and kill" strategy for the elimination of the reactivated viral reservoir in chronically infected patients.

2. Materials and methods

2.1. Patients' samples

Blood samples were obtained from 17 patients with CML Philadelphia Chromosome-positive (Ph+) that were on treatment-free remission (TFR) for an average of 1.1 ± 0.2 years without molecular recurrence of their hematological disease. Patients were recruited at the Hospital Universitario La Princesa, Hospital Universitario Puerta de Hierro and Hospital Universitario Ramón y Cajal (Madrid, Spain). Table 1 summarizes the main clinical characteristics of CML patients. Ten kidney transplant recipients with positive serology for CMV (R+) were also recruited for the study of CMV reactivation. These samples were collected one month postransplantation and all patients were on treatment with valganciclovir to control CMV reactivation at the time of sample collection. Healthy donors with similar age and gender distribution were recruited as controls.

Peripheral blood lymphocytes (PBMCs) were isolated from blood samples by centrifugation through Ficoll-Hypaque gradient (Sigma

Table 1
Clinical characteristics of patients with chronic myeloid leukemia on treatment-free remission that participated in the study.

Patient code	Gender (M/F)	Age at CML diagnosis (years)	Sokal risk score	Molecular response ratio (IS)	Name of previous TKI	Last TKI before discontinuation	Time of treatment with last TKI	Dose of last TKI (mg/day)	Time without TKIs (Off ITK)	Lymphocyte count (10^3/ml)
P1	F	83	–	0.0006	Imatinib	Nilotinib	2y	–	6 m	3.41
P2	M	61	–	0.0004	Imatinib	Nilotinib	2y	–	6 m	2.10
P3	M	48	LOW	0.0051	No previous	Dasatinib	3y 7 m	–	4 m	3.63
P4	F	61	INT	0.0019	No previous	Imatinib	3y 5 m	–	1y 2 m	1.99
P5	M	40	LOW	0.0005	No previous	Nilotinib	5y	–	1y 2 m	1.30
P6	F	63	INT	ND	No previous	Nilotinib	3y 8 m	–	2y 2 m	2.27
P7	M	74	INT	0.0006	No previous	Nilotinib	3y 3 m	–	2y 2 m	1.76
P8	F	38	LOW	0.0000	No previous	Imatinib	14y 4 m	400/300	1y 4 m	2.94
P9	M	31	INT	0.0000	Imatinib	Dasatinib	7y 1 m	140	4y 11 m	1.67
P10	M	53	LOW	0.0020	No previous	Nilotinib	3y 7 m	600	3y 6 m	1.28
P11	F	42	LOW	0.0000	No previous	Imatinib	15y 2 m	400/300	2y	1.54
P12	F	33	LOW	0.0000	Imatinib	Nilotinib	3y 8 m	800/600	3y 5 m	2.66
P13	M	41	LOW	0.0000	No previous	Imatinib	14y 11 m	400/300	2y	1.32
P15	M	46	INT	0.0000	Imatinib, nilotinib	Dasatinib	4y 6 m	50	1y 1 m	4.07
P16	F	71	INT	0.0000	No previous	Nilotinib	4y 4 m	600	3y 8 m	2.21
P17	M	74	INT	0.0000	No previous	Nilotinib	3y 3 m	600	3y 8 m	1.76
P18	M	26	LOW	0.0000	Imatinib	Dasatinib	5y	100	1y 9 m	ND

TKI: tyrosine kinase inhibitor.
INT: intermediate.
IS: International Scale.
ND: not done.
y: years; m: months.

Aldrich, St. Louis, MO). Plasmas were separated and stored at −80 °C until the moment of analysis. Human CD4+ T lymphocytes were isolated from PBMCs by using CD4+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. A wide broad spectrum of NK cell populations was isolated by eliminating CD8+ and CD14+ cells from the CD4-depleted PBMCs by using Human CD8+ T Cell Isolation Kit and Human CD14 MicroBeads (Miltenyi). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100μg/ml streptomycin, 100UI/ml penicillin (Biowhittaker, Walkersville, MD). T cell activation was performed by adding 5μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich) and 300 units/ml IL-2 (Chiron, Emeryville, CA) to the culture medium for 72 h.

2.2. Ethical statement

The individuals participating in this study were recruited in Hospital Universitario La Princesa, Hospital Universitario Puerta de Hierro, Hospital Universitario Ramón y Cajal, and Hospital Universitario 12 de Octubre (Madrid, Spain). All individuals gave informed written consent to participate in the study. Confidentiality and anonymity of all participants was assured by current Spanish and European Data Protection Acts. Protocol for this study was performed in accordance with the Helsinki Declaration and it was previously reviewed and approved by the Ethics Committees of Instituto de Salud Carlos III (CEI PI 46_2018) and all participating hospitals.

2.3. Antibodies and vectors

Antibodies for surface staining CD4-PerCP, CD25-PE, CD69-FITC, CD3-BV510, CD56-FITC, CD16-PercP, and CD8-APC-H7 were purchased from BD Biosciences (San Jose, CA). Antibody for surface staining TCRγδ-PE was obtained from BioLegend (San Diego, CA). Antibodies for intracellular staining of SAMHD1 phosphorylated at Thr592 (pSAMHD1) conjugated with PE and IFNγ conjugated with PE were purchased from Cell Signaling (Cell Signaling Technology Europe, Leiden, The Netherlands) and Beckman Coulter (Indianapolis, IN), respectively.

Vector pNL4-3 wild-type that contains HIV-1 complete genome and induces an infectious progeny after transfection was kindly provided by Dr M.A. Martin [31]. Vector pNL4-3_Renilla was obtained by replacing *nef* gene of HIV-1 proviral clone pNL4-3 with Renilla luciferase (LUC) gene [32].

2.4. HIV-1 infection ex vivo

NL4.3-Renilla infectious supernatants were obtained from calcium phosphate transfection of HEK293T cells (provided by the existing collection of Instituto de Salud Carlos III, Madrid, Spain) with plasmid pNL4-3_Renilla. HIV-1 infection ex vivo by spinoculation was performed in PBMCs and CD4+ T cells isolated from CML patients and controls that were previously activated with PHA and IL-2 for 72 h. Cells were then infected with 1 ng p24 NL4.3-Renilla per 10^6 cells for 30 min at gently rotation, room temperature. After centrifugation at 600xg for 30 min at 25 °C and extensive washing with PBS1X, cells were incubated for 5–7 days in the presence of IL-2. The production of renilla was measured in the cell pellets with Renilla Luciferase Assay System (Promega Biotech Ibérica, Madrid, Spain).

2.5. Quantification of proviral integration by TaqMan qPCR

Whole genomic nucleic acid was extracted from ex vivo infected cells using QIAamp DNA Blood Mini Kit (Qiagen Iberia, Madrid, Spain) and quantified with Nanodrop 2000C (Thermo Fisher Scientific, Waltham, MA). Proviral integrated DNA was quantified with TaqMan probes conjugated with reporter fluorochrome FAM by using a nested Alu-LTR PCR in a StepOne Real-Time PCR System (Thermo Fisher Scientific) as previously described [33,34]. Briefly, 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 PrimeTime TaqMan probes with FAM/ZEN/Iowa Black (Integrated DNA Technologies, Leuven, Belgium) and TaqMan Master Mix (Thermo Fisher Scientific). DNA from 8E5 cell line was used for the standard curve and for quantification of *CCR5* gene as housekeeping gene for measuring the input DNA and normalize data. CCR5 was quantified in the same wells as HIV-1 LTR using TaqMan™ Gene Expression Assay with a Taqman probe conjugated with

reporter fluorochrome VIC (Thermo Fisher Scientific).

2.6. Intracellular staining and flow cytometry analysis

Intracellular staining of pSAMHD1 was performed according to manufacturer's instructions. Briefly, cells were fixed and permeabilized with methanol (Sigma Aldrich). After washing, cells were stained with anti-pSAMHD1-PE and then analyzed by flow cytometry. For intracellular staining of IFNγ, cells were treated for 4 h at 37 °C with PepMix HCMVA pp65 (lower matrix protein 65) and IE1 (immediate-early-1 protein) (JPT Peptide Technologies, Berlin, Germany) to induce specific CD8 response against CMV or with Hsp70 peptide (Abcam, Cambridge, UK) to stimulate cytolytic activity of NK cells, in the presence of brefeldin A and co-stimulator CD28/CD49D (BD Biosciences). Cells were then stained with antibodies against CD3 and CD8 conjugated to PE-Cy7 and APC-H7, respectively, or with CD3, CD56 and CD16 conjugated to APC, FITC and PercP, respectively. After fixation and permeabilization with IntraPrep Permeabilization Reagent (Beckman Coulter), cells were stained with an antibody against IFNγ-PE and then analyzed by flow cytometry.

Flow cytometry data acquisition was performed using BD LSR Fortessa X-20 flow cytometer (BD Biosciences) with BD FACSDiva software. Data analyses were carried out with FlowJo v10 software (TreeStar, Ashland, OR).

2.7. NK cytotoxicity assays

CD4+ T cells were purified from PBMCs of CML patients on TFR and controls and the rest of cells were depleted of CD8+ T cells and CD14+ cells, as described above. Flow cytometry of the remaining cells proved they contained 75% on average of mixed populations of cells with NK phenotype (CD56+) (data not shown). CD4+ T cells were activated with PHA and IL-2 for 72 h and then infected ex vivo with NL4-3_Renilla in the presence or absence of NK-enriched cells (ratio 1:1). After measuring the production of renilla in the cell pellets (relative light units, RLUs), cytotoxic activity was calculated according to the following equation:

$$\textit{Fold cytotoxicity} = \frac{\text{average renilla (RLUs) in isolated CD4 T cells}}{\text{average renilla (RLUs) in coculture CD4: NK enriched cells (1: 1)}}$$

2.8. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8.0 (Graph Pad Software Inc., San Diego, CA). Statistical significance in the differences between groups was calculated using unpaired, nonparametric Mann-Whitney *t*-test to compare ranks. Two-tailed P values (p) < 0.05 were considered statistically significant in all comparisons and were represented as *, **, ***, or **** for $p < 0.05$, $p < 0.01$, $p < 0.001$, or $p < 0.0001$, respectively.

3. Results

3.1. Study population

Seventeen CML Philadelphia Chromosome-positive (Ph+) patients were recruited for this study (57% male, 43% female) (Table 1). CML diagnosis mean age was 61 ± 5.5 years and all of them were on treatment with imatinib, nilotinib and/or dasatinib for 5.3 ± 0.4 years on average. Due to sustained DMR, these patients discontinued TKI treatment and were on TFR for an average of 1.1 ± 0.2 years without molecular recurrence of their hematological disease. Eleven patients (65%) were treated with only one TKI before discontinuation, 5 patients (29%) were treated with two TKIs and one patient (6%) was treated with all three TKIs before discontinuation. The most frequent last TKI was nilotinib (54%), followed by 23% with dasatinib and 23% with imatinib. All patients were HIV-1 seronegative and CMV seropositive without viremia, they had normal routine blood and biochemistry tests, and they were in good health at the time of sampling, without any concomitant complication. Mean lymphocyte count was 2.4 ± 0.3 × 10^3/ml.

Ten patients that received a kidney allograft were also recruited for the study (60% male, 40% female). Blood samples were taken one month after transplantation. Patients mean age at transplantation was 66 ± 12.7 years and all of them were on treatment with valganciclovir as prophylaxis for CMV infection.

A total of 30 healthy donors with similar age and gender distribution were recruited as controls for the different analyses.

3.2. CD4+ T cell activation in PBMCs from CML patients on TFR

PBMCs isolated from blood samples of CML patients on TFR (n = 4; P2-P5, Table 1) and from healthy donors (n = 9) were stained for surface activation markers and analyzed by flow cytometry. CD4+ T cells from CML patients on TFR showed 2.7-fold lower expression of CD25+/CD69+ than cells from healthy donors ($p < 0.05$) in non-activated conditions (Fig. 1A, left graph) (graph scale was maintained

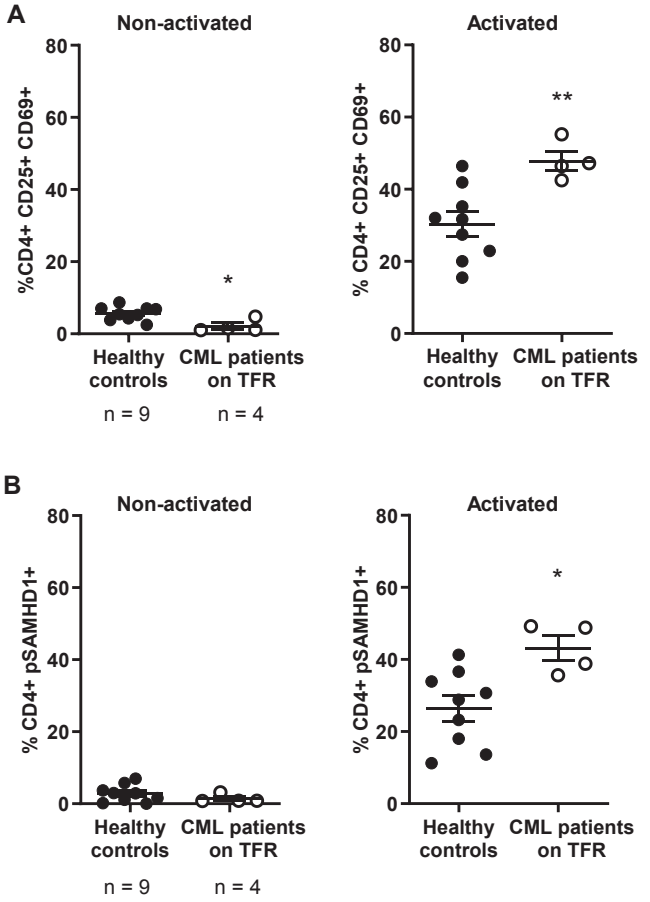

Fig. 1. Expression of activation markers in CD4+ T cells from patients with CML on TFR. (A) Analysis by flow cytometry of the expression of activation markers CD25 and CD69 in resting and activated CD4+ T cells from CML patients on TFR in comparison with cells from healthy donors. T cell activation was performed with PHA and IL-2 for 3 days. (B) SAMHD1 phosphorylation was analyzed in the same cells by flow cytometry after intracellular staining. Each dot corresponds to one sample and lines represent mean ± standard error of the mean (SEM). Statistical significance was calculated using unpaired, non-parametric Mann-Whitney *t* test. *p < 0.05; *p < 0.01.

at 80% for comparison with activated cells). Ex vivo activation with PHA and IL-2 for 3 days showed increased expression of activation markers in CD4+ T cells from both patients and controls, with expression of CD25/CD69 in cells isolated from CML patients being 0.6-fold higher on average than that of controls ($p < 0.01$) (Fig. 1A, right graph). Intracellular staining of pSAMHD1 in non-activated PBMCs showed 2.0-fold lower phosphorylation in CD4+ T cells from CML patients on TFR than in those from healthy donors (Fig. 1B, left graph) (graph scale was maintained at 80% for comparison with activated cells). Treatment ex vivo with PHA and IL-2 for 3 days increased the levels of pSAMHD1 in both groups, with pSAMHD1 being 0.6-fold higher on average in CD4+ T cells from CML patients than in control cells ($p < 0.05$) (Fig. 1B, right graph).

3.3. PBMCs from CML patients on TFR were resistant to HIV-1 infection

PBMCs were isolated from blood samples of CML patients on TFR (n = 7; P1-P7, Table 1) and healthy donors (n = 28) and then activated with PHA and IL-2 for 3 days. Cells were then infected with HIV-1NL4-3_Renilla recombinant clone and incubated for 7 days. Proviral integration was analyzed by Alu-qPCR and it was reduced 12.5-fold on average in cells isolated from CML patients on TFR in comparison with cells from healthy donors ($p < 0.001$) (Fig. 2A). Provirus-driven gene expression was analyzed by quantifying the production of Renilla luciferase (RLUs) and it was reduced 5.2-fold on average in PBMCs from CML patients on TFR in comparison with healthy donors ($p < 0.0001$) (Fig. 2B). Therefore, although SAMHD1 in CD4+ T cells from these patients was phosphorylated and so less active, cells were still less permissive to HIV-1 infection than those from untreated donors. We speculated that an increase cytotoxic activity in NK or CD8 cells could be responsible for the unexpected inhibition of infection (see next section).

3.4. NK activity was enhanced in PBMCs from CML patients in TFR

Expression of NK activation marker CD56 and of the receptor for triggering cell lysis CD16 were analyzed by flow cytometry in PBMCs from patients with CML on TFR (n = 11; P8-P18, Table 1), in comparison with healthy donors. Expression of CD56 showed a wide variability in PBMCs from CML patients on TFR compared with healthy donors, and it was increased 1.4-fold on average (Fig. 3A, left dot plot). PBMCs from CML patients showed a subpopulation of CD3-CD56 + CD16 + NK cells that was increased 2.2-fold on average compared with cells from healthy donors ($p < 0.05$) (Fig. 3A, middle dot plot). Conversely, the population of less cytotoxic cells CD3-CD56 + CD16- was reduced 2.0-fold on average in CML patients ($p < 0.01$) (Fig. 3A, right dot plot). Interestingly, CML patients whose PBMCs showed higher CD56 + expression were the same patients that also expressed the highest proportion of CD3-CD56+CD16+ cytotoxic population.

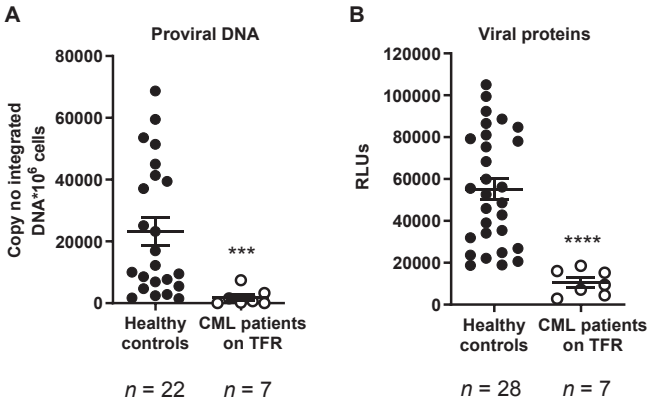

Fig. 2. PBMCs from CML patients on TFR showed resistance to HIV-1 proviral integration and transcription. (A) Analysis by qPCR of proviral integration in PBMCs infected in vitro with NL4-3_Renilla for 7 days and (B) quantification of the synthesis of renilla by chemiluminescence in the same cells. Each dot corresponds to one sample and lines represent mean ± SEM. Statistical significance was calculated using unpaired, non-parametric Mann-Whitney *t* test. *** $p < 0.001$; **** $p < 0.0001$.

In order to determine whether these NK cell populations with higher expression of activation markers were also more effective, we analyzed the ability to produce IFNγ from both CD16 ± NK cell populations in response to Hsp70 peptide which may induce the proliferation and cytotoxic activity of human NK cells [35]. Synthesis of IFNγ was increased 1.7-fold on average in CD3-CD56+CD16+ cells from CML patients on TFR (n = 5; P8-P12, Table 1) (Fig. 3B, left graph), whereas the synthesis of IFNγ from CD3-CD56+CD16− cells remained unchanged compared with control cells (Fig. 3B, right graph). Besides, the population of NK cells with memory phenotype CD3-CD56+CD57+ was increased 1.4-fold on average in CML patients on TFR compared with healthy donors ($p < 0.01$) (Fig. 3C).

3.5. Antiviral activity of CD8+ T cells from CML patients on TFR

Total levels of CD8+ T cells in PBMCs from CML patients on TFR were not different than those in PBMCs from healthy donors (data not shown). However, analysis by flow cytometry of the expression of TCRγδ receptor in PBMCs from these patients (n = 11; P8-P18, Table 1) showed that the proportion of CD3+ T cells expressing TCRγδ was higher than in PBMCs from healthy donors, both CD8+ and CD8−, as they were increased 6.3-fold ($p < 0.0001$) (Fig. 4A) and 2.6-fold ($p < 0.01$) (Fig. 4B), respectively.

All patients recruited for this study were seropositive for CMV. Analysis of levels of IgG against CMV showed that they were increased 2.1-fold on average in plasma from these patients in comparison with healthy donors that were also seropositive for CMV ($p < 0.05$) (n = 11; P8-P18, Table 1) (Fig. 4C). These antibodies were not wide-spectrum, neutralizing antibodies, as we found no significant differences in the potency of neutralization in comparison with those from plasma of healthy donors or patients who went through renal transplant and afterwards suffered CMV reactivation (data not shown). The antiviral activity against CMV of memory CD8 + T cells that could be developed during TKI treatment was evaluated in PBMCs isolated from CML patients on TFR previously stimulated with CMV peptides. The production of IFNγ from CD3 + CD8 + T cells was increased 18.7-fold on average in PBMCs from these patients compared with CMV seropositive controls ($p < 0.05$) and was very similar to patients with renal transplant who suffered CMV reactivation and needed treatment with valganciclovir ($p < 0.05$) (Fig. 4D). CMV was not reactivated in the patients with CML on TFR that were recruited for this study.

3.6. NK cytotoxic response in PBMCs isolated from CML patients in TFR interfered with HIV-1 infection of autologous CD4+ T cells

We analyzed whether the antiviral memory response developed against CMV during TKI treatment was also efficient to impede HIV-1 infection of autologous CD4+ T cells. Therefore, CD4+ T cells purified from PBMCs of CML patients on TFR (n = 9; P8-P13, P16-P18, Table 1) and healthy donors were infected ex vivo with NL4-3_Renilla in the presence or absence of CD56 + NK cells (ratio 1:1), which were isolated from the autologous PBMCs. CD4+ T cells from CML patients that were cocultured with autologous NK cells showed a reduction of 2.2-fold in the integration of HIV-1 provirus, compared with cells from healthy donors ($p < 0.001$) (Fig. 5A). NK cytotoxic activity was calculated by making a ratio between the copies of integrated proviral DNA per million of CD4+ T cells infected alone and the copies of provirus integrated in CD4+ T cells that were infected in the presence of NK cells. According to this calculation, the sustained NK cell response

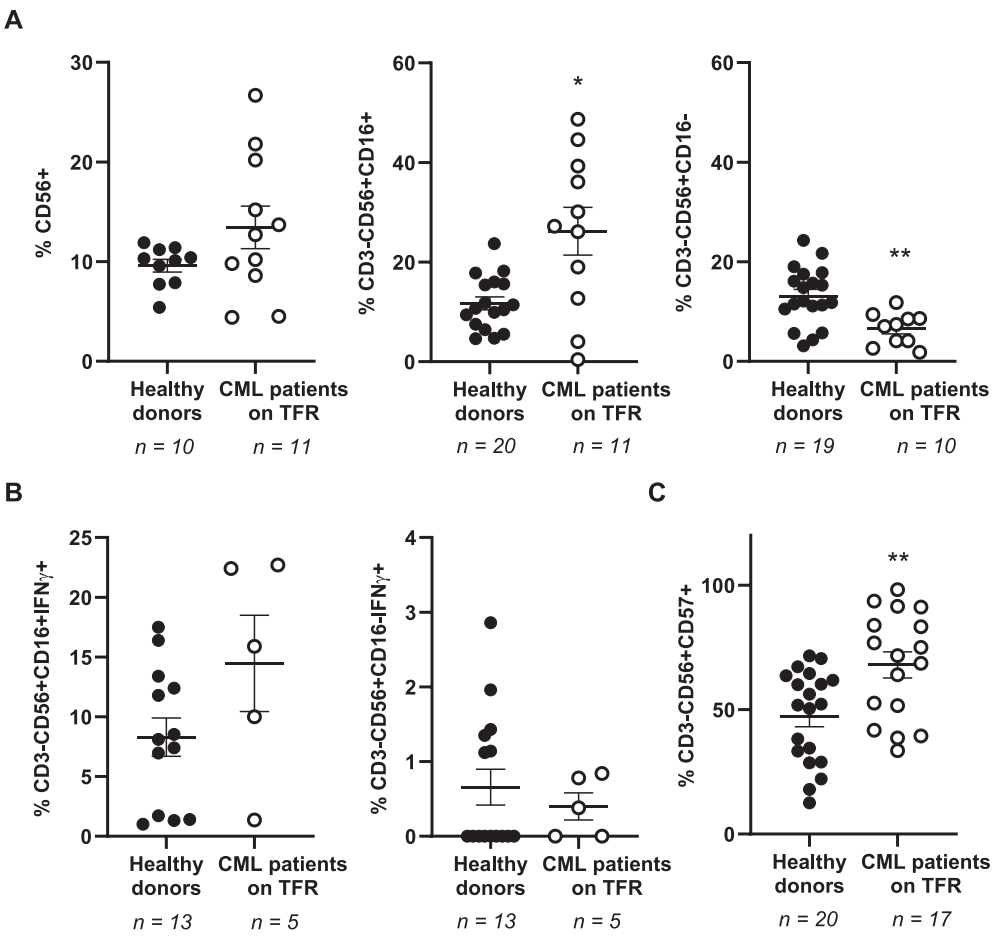

Fig. 3. Phenotype and cytotoxic activity of NK cell populations in patients with CML on TFR. (A) Analysis by flow cytometry of the expression levels of CD56 and CD16 in NK cells from PBMCs of patients compared with healthy donors. (B) Percentage of intracellular levels of IFNγ produced by NK cells with CD3-CD56 + CD16 ± phenotype from PBMCs of CML patients on TFR compared with healthy donors. (C) Expression levels of NK cells with memory phenotype CD3-CD56 + CD57 + in PBMCs from CML patients on TFR compared with healthy donors. Each dot corresponds to one sample and lines represent mean ± SEM. Statistical significance was calculated using unpaired, non-parametric Mann-Whitney *t* test. * $p < 0.05$; ** $p < 0.01$.

that was developed during TKI treatment allowed that cells from patients with CML on TFR showed 8.0-fold more cytotoxic activity against HIV-infected CD4 + T cells, in comparison with healthy donors ($p < 0.0001$) (Fig. 5B).

4. Discussion

Infection with HIV-1 is currently incurable due to the formation and maintenance of long-lived reservoirs that harbor replication competent proviruses. Early cART allows to preserve the integrity of the immune response but is unable to impede the formation of the reservoir that occurs very early after the infection [1]. New strategies are being developed by laboratories all over the world to tackle and destroy the viral reservoir, but success has been incomplete so far. The strategy of "kick and kill" seems very promising but without an adequate stimulation of the immune response it is not useful [36,37]. Therefore, new strategies are needed to enhance the cytotoxic immune response that may destroy cells with reactivated provirus and eliminate the viral reservoir. In this study, we analyzed whether the potent antileukemic response that is induced by treatment with TKIs during CML may also have an antiviral activity against HIV-1. In fact, it has already been described that this cytotoxic response may be active against other viruses such as CMV [19,38].

PBMCs from CML patients on treatment with TKIs are highly resistant to HIV-1 infection ex vivo [12,15]. This resistance is, to a large degree, derived from the ability of TKIs to interfere with phosphorylation of SAMHD1 in CD4 + T cells [15,16]. TKIs may also interfere with activation and proliferation of other essential immune cells such as dendritic cells, NK and B cells [39]. Therefore, CD4 + T cells isolated from CML patients on treatment with TKIs are unable to proliferate in response to γc-cytokines such as IL-2 or IL-7 [13]. Accordingly, dasatinib may be useful to avoid the formation of HIV-1 reservoir when administered prior to infection, as a pre-exposure prophylactic drug [40]. But it is unclear whether TKIs would have an effect on the reservoir that is already formed in chronically infected patients despite its negative effect on T-cell proliferation, which may affect the maintenance and replenishment of the viral reservoir. In this regard, it has been described that TKIs such as imatinib and dasatinib may also act as immunomodulators to induce a potent cytotoxic response against cancerous cells [41–43]. In some patients with CML that have been on treatment with TKIs and maintained a DMR for at least 2 years is possible to safely interrupt treatment. However, in most cases TKI treatment is discontinued even if *BCR-ABL* is still detected as it is not so frequent that CML patients become completely negative for the cancerous clone. Due to unknown reasons, only 50% of patients who stop treatment maintain molecular recurrence-free survival [44], pointing at the possibility that the memory immune response that is developed during TKI treatment in these patients may control the disease recurrence after treatment interruption. This raises the possibility that, at least in some patients, CML might be cured after transient treatment with TKIs [45]. As unspecific antileukemic responses could also be directed against cells infected with viruses, the possibility of applying this sustained cytotoxic response against chronic viral infection should be analyzed.

In this work, we determined that CD4 + T cells isolated from patients with CML who withdrew from treatment with TKIs more than one year earlier recovered the capacity of being activated and proliferate in response to PHA and the γc-cytokine IL-2. This recovery of CD4 + T cell activity indicated that the apparently immunosuppressive effect induced by TKIs on these cells could be restored after treatment interruption. Surprisingly, these CD4 + T cells showed continued resistance to HIV-1 infection, which now could not be related to the interference

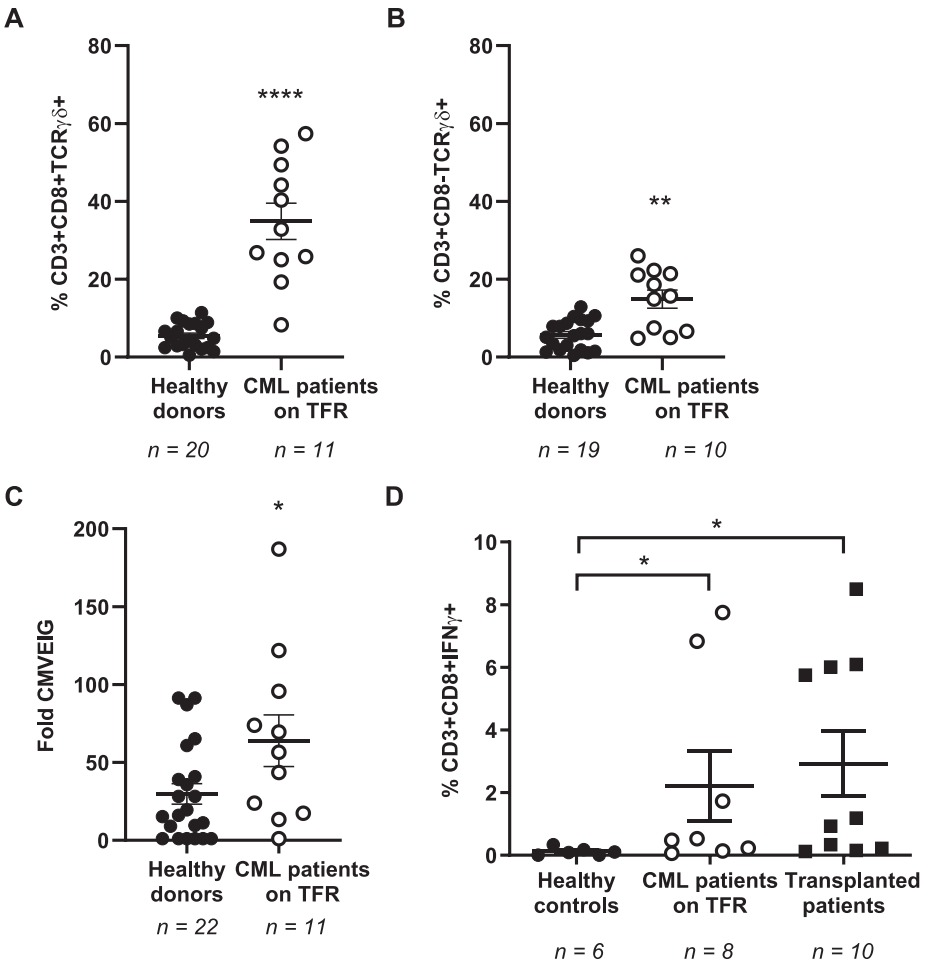

Fig. 4. Phenotype and cytotoxic activity of CD8+ T cell populations in patients with CML on TFR. Analysis of cell populations expressing TCRγδ that were CD3+CD8+ (A) or CD8- (B) in patients with CML on TFR compared with healthy donors. (C) Fold levels of IgG against CMV in plasma from CML patients on TFR, compared with healthy donors. (D) Production of IFNγ in CD3+CD8+ T cells from CML patients on TFR was analyzed by flow cytometry after stimulation with CMV peptides, compared with PBMCs from patients who went under renal transplant and suffered afterwards CMV reactivation and from healthy donors. Each dot corresponds to one sample and lines represent mean ± SEM. Statistical significance was calculated using unpaired, non-parametric Mann-Whitney *t* test. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

with SAMHD1 phosphorylation. On the other hand, this apparently immunosuppressive effect does not cause significant clinical consequences as patients with CML that are on treatment with TKIs are rarely susceptible to opportunistic infections, although some reports describe that continued treatment with dasatinib may increase the risk of bacterial infections [46–48]. Conversely, this cytostatic effect on CD4+ T cells occurs simultaneously to an expansion of LGLs with CD8 and NK phenotypes that are developed during treatment with TKIs, mostly with dasatinib but also with other TKIs such as imatinib [20]. In fact, 30% of patients receiving dasatinib develop absolute lymphocytosis with clonal expansion of cytotoxic T lymphocytes and NK cells that rapidly control the leukemic cells, making treatment very successful in these patients [19,21,22]. Interestingly, the expansion of LGLs with powerful memory response during treatment with TKIs appears to be driven by previous infection with CMV even in the absence of viremia [23,26,38]. We found that all patients with CML recruited for this study that had a sustained antileukemic response even after more than one year on TFR were seropositive for CMV, showing an enhanced humoral

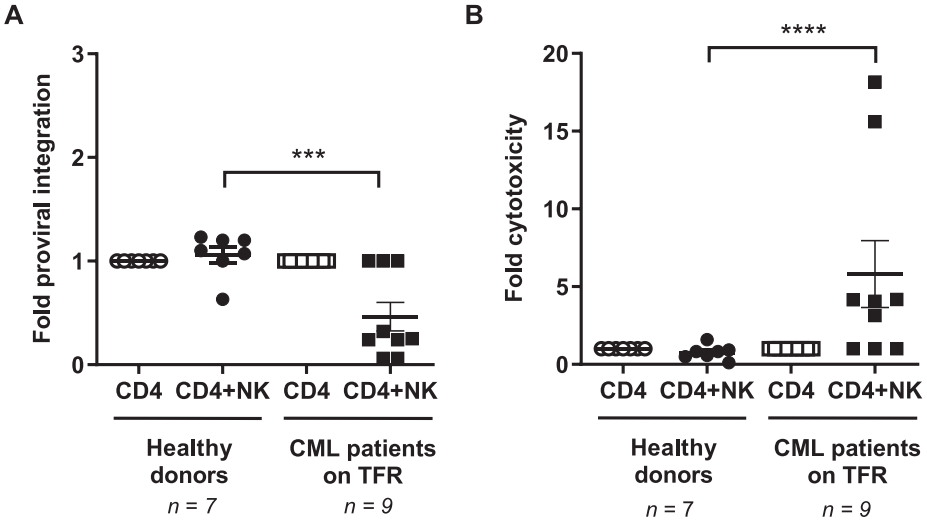

Fig. 5. Antiviral effect of NK cells from CML patients on TFR against HIV-1 infection ex vivo of autologous CD4+ T cells. (A) Fold of HIV-1 proviral integration in CD4+ T cells isolated from patients with CML on TFR compared with cells from healthy donors, in the presence or absence of autologous NK cells, and (B) fold of cytotoxic activity of these NK cells. Each dot corresponds to one sample and lines represent mean ± SEM. Statistical significance was calculated using unpaired, non-parametric Mann-Whitney *t* test. *** $p < 0.001$; **** $p < 0.0001$.

response against this virus and the presence of CMV-specific CD8 + T cells. In fact, this cellular response was very similar to the one observed in patients with renal transplant who suffered CMV reactivation and viremia and needed treatment with valganciclovir. However, patients with CML on TFR did not show CMV viremia. This could indicate that the presence of CMV along with leukemic cells and treatment with TKIs may trigger a potent immune response against both leukemic and virus infected cells. This immune response could be maintained after TFR due to the generation of both humoral and cellular memory responses, being the latter based on cytotoxic cells such as CD3- CD56 + CD57 + NK cells and TCRγδ + CD8 ± lymphocytes. Both cell population types were significantly increased in PBMCs from patients with CML on TFR recruited to our study. Moreover, these cells were able to trigger an unspecific memory antiviral response that is effective not only against endogenous CMV but also against CD4+ T cells infected ex vivo with HIV-1. These results may portend a great advance towards the development of strategies based on the stimulation of a potent cytotoxic response against HIV-1 reservoir to assist LRAs during the reactivation and destruction of latently infected cells with competent proviruses.

In conclusion, the results presented here, along with previous reports that demonstrate the antiviral effect of TKIs, suggest that TKIs could be used transiently in HIV-infected patients to modulate the immune response and generate a potent, sustained antiviral activity in order to interfere with HIV-1 reservoir replenishment and to destroy the infected cells reactivated from latency.

Author contributions

MC, MRH, VP and JA conceived the study. VGG, JLS, GB and AL selected the patients and obtained the blood samples. LV, SRM, NC and MP performed the cytometry analyses. EM, SRM and MRLH performed the HIV-1 infections in vitro. VS, PPR and FO performed the analysis of CMV antibodies and production of IFNγ. MC, LV and MRLH wrote the manuscript and performed the statistical analyses. All authors contributed to data collection and analysis and approved the final manuscript.

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.

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