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Evaluation of resistance to HIV-1 infection ex vivo of PBMCs isolated from patients with chronic myeloid leukemia treated with different tyrosine kinase inhibitors

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ABSTRACT

Current antiretroviral treatment (ART) may control HIV-1 replication but it cannot cure the infection due to the formation of a reservoir of latently infected cells. CD4+ T cell activation during HIV-1 infection eliminates the antiviral function of the restriction factor SAMHD1, allowing proviral integration and the reservoir establishment. The role of tyrosine kinases during T-cell activation is essential for these processes. Therefore, the inhibition of tyrosine kinases could control HIV-1 infection and restrict the formation of the reservoir. A family of tyrosine kinase inhibitors (TKIs) is successfully used in clinic for treating chronic myeloid leukemia (CML). The safety and efficacy against HIV-1 infection of five TKIs was assayed in PBMCs isolated from CML patients on prolonged treatment with these drugs that were infected ex vivo with HIV-1. We determined that the most potent and safe TKI against HIV-1 infection was dasatinib, which preserved SAMHD1 antiviral function and avoid T-cell activation through TCR engagement and homeostatic cytokines. Imatinib and nilotinib showed lower potency and bosutinib was quite toxic in vitro. Ponatinib presented similar profile to dasatinib but as it has been associated with higher incidence of arterial ischemic events, dasatinib would be the better choice of TKI to be used as adjuvant of ART in order to avoid the establishment and replenishment of HIV-1 reservoir and move forward towards an HIV cure.

1. Introduction

The infection by the human immunodeficiency virus type 1 (HIV-1) is currently incurable. The antiretroviral treatment (ART) is very efficient for controlling the infection and the progression to the acquired immunodeficiency syndrome (AIDS) [1,2], but patients must be taking the medication for life, with the consequent adverse effects and burden on Healthcare Systems [3]. Nowadays, clinical care guidelines recommend universal treatment of HIV infection, regardless the time of infection or CD4 cell count. However, the viral reservoir, which is a major obstacle for eradication, is established very early, well before ART initiation. This reservoir is mainly formed by a small subset of infected memory CD4+ T cells that return to a resting state and persist in the organism for a long time [4,5]. When these latently infected lymphocytes become activated in the context of a normal immune response, a massive viral replication occurs, causing T-cell destruction, peaks of viremia, and the reservoir replenishment [6]. The reseeding of the reservoir may also occur by homeostatic proliferation of the infected CD4+ T cells after low level stimulation induced by cytokines such as interleukine-2 (IL-2) or -7 (IL-7) [7,8].

Several strategies are actively being developed to tackle and destroy the viral reservoir [9,10] such as the use of latency reversal agents (LRAs) to reactivate the latent proviruses without causing global T-cell activation [11]. However, none of them has been fully successful so far and more efforts are necessary to design new strategies that may really facilitate the reservoir destruction. In this context, the lower size of the reservoir, the better disease prognosis and the longer control of viremia after treatment interruption [12,13]. Due to its early development, it is really difficult to completely prevent the reservoir establishment [14,15]. Early treatment has proven quite successful to control the size of the reservoir [15] but it cannot avoid its formation, even when ART is initiated very soon after the infection [16,17]. Moreover, a very small quantity of infected CD4+ T cells is enough to replenish the

reservoir, once the chance is given to the virus [16,18,19]. Therefore, additional strategies aimed at interfering with the formation of the viral reservoir or its replenishment should complement the eradication approaches that are currently being developed.

The idea of using immune-based therapies against HIV-1 infection is lately gaining strength. Whereas ART is only aimed at the control of HIV-1 replication, the concomitant use of immune-based agents could preserve and enhance the immune system of the patient to promote the elimination of the virus. Several attempts are being performed to restore the number and function of specialized CD4+ and CD8+ T cells able to direct a successful response against HIV-1 infection [20–23]. One potential approach would be increasing cellular restriction mechanisms in order to inhibit the reservoir formation or its replenishment. Therefore, it is worth exploring whether cancer immunotherapy could be useful to restore HIV-specific immunity and avoid the formation of the reservoir. In this regard, we described previously that the tyrosine kinase inhibitor (TKI) dasatinib, which is successfully used in clinic for the treatment of chronic myeloid leukemia (CML) and has an immunomodulator profile [24,25], significantly interferes with HIV-1 proviral integration in CD4+ T cells isolated from patients with CML on chronic treatment with it [26]. We determined two mechanisms of action for the inhibition of HIV-1 infection by dasatinib: first, it preserves the antiviral function of the innate immune factor SAMHD1 by impeding its deactivation through the phosphorylation at T592 residue; and second, dasatinib interferes with the activation and proliferation of CD4+ T cells in response to stimulation with PHA and IL-2. As HIV-1 may infect both quiescent and activated CD4+ T cells once SAMHD1 is phosphorylated [27] but only replicates in activated cells [28,29], the use of drugs that interfere with the activation of infected CD4+ T cells in combination with ART could provide an additional mechanism to avoid the establishment of the reservoir. Moreover, preventing the massive replication of HIV-1 that occurs during the acute phase of the disease

would not only avoid the formation of the reservoir, but it would preserve the CD4+ T cell count and the immune response at normal levels, providing an efficient immune system to control the infection.

CML is a neoplastic condition of hematopoietic stem cells characterized by unrestrained growth of myeloid cells in the bone marrow that accumulate in peripheral blood and spleen. It is caused by the formation of a chimeric fusion protein BCR-ABL with uncontrolled tyrosine kinase activity [30,31]. CML treatment is performed with several TKIs. Imatinib was the first TKI against BCR-ABL introduced in clinical practice in 2001 and it highly increased the survival of the patients [32]. The second-generation TKIs nilotinib, dasatinib, and bosutinib were introduced later for CML patients with resistance or intolerance to imatinib [33] and they were all more potent than imatinib against BCR-ABL [34,35]. Recently, the third-generation TKI ponatinib has been approved for CML patients with resistance to the second-generation TKIs, including the T315I mutation of the BCR-ABL kinase domain [36]. Although these drugs target mainly BCR-ABL, they also affect the activity of other kinases, which could be useful in other diseases. Dasatinib and bosutinib display a broader target spectrum than imatinib and nilotinib [37]. Dasatinib and bosutinib inhibit Src family of tyrosine kinases (SFK) such as the non-receptor tyrosine kinase SRC (C-Src proto-oncogene) and the SFK regulator CSK (C-Src tyrosine kinase) –which in turn activates SRC by phosphorylation [38,39]. It is known that dasatinib and bosutinib also target other SFKs such as LYN that is mostly expressed in T and B cells, and LCK that is essential for T-cell development and function [40]. Active LCK induces direct or indirect phosphorylation of many substrates [41], including SAMHD1 [8] and other downstream kinases such as the protein kinase C theta (PKCθ), which leads to full T-cell activation [41,42]. Consequently, in the context of HIV-1 infection, LCK activation enhances viral infection [26] and PKCθ activation is essential for HIV-1 transcription [43,44]. Therefore, using TKIs that could

selectively suppress the activity of SFKs such as LCK and downstream kinases such as PKCθ could be useful to interfere with HIV-1 infection and the reservoir establishment.

We previously proposed the use of dasatinib as adjuvant of ART as an alternative to reduce the reservoir size. However, 30% of CML patients on treatment with dasatinib for the first year may develop pleural effusion, which is the most characteristic secondary effect of this TKI and requires dose reduction or even treatment interruption [45]. Therefore, first we analyzed whether a lower dose of dasatinib than the one currently used for treating CML could be effective against HIV-1 infection and second, we determined if other TKIs could also be useful as adjuvant of ART in a clinical setting and with less probability of adverse reactions. The susceptibility to HIV-1 infection ex vivo of PBMCs isolated from patients with CML on chronic treatment with imatinib, nilotinib, bosutinib and ponatinib was then evaluated and compared to dasatinib, and the mechanism of action for this restriction was also analyzed.

2. Materials and methods

2.1. Cells and patients' samples

Thirty seven patients with CML on chronic treatment with one of the assayed TKI or 42 untreated healthy donors were recruited for this study. Peripheral blood lymphocytes (PBMCs) were isolated by centrifugation through a Ficoll-Hypaque gradient (Pharmacia Corporation, North Peapack, NJ). Human CD4+ T lymphocytes were isolated with CD4+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 100μg/ml streptomycin, 100UI/ml penicillin (Biowhittaker, Walkersville, MD). PBMCs were activated with 1μg/ml CD3 (OKT3) antibody (Miltenyi Biotec) and 300 units/ml IL-2 (Chiron, Emeryville, CA) for 72 hours and then, they were maintained in culture only with IL-2.

HIV-negative, CML Phi Chromosome-positive patients receiving treatment with one TKI were obtained from the Hospital Clinic (Barcelona, Spain), Hospital Puerta de Hierro (Majadahonda, Madrid, Spain), Hospital La Princesa (Madrid, Spain) and Hospital Ramón y Cajal (Madrid, Spain). All of them had more than one year of follow-up from CML diagnosis and were taking the current TKI for at least 9 months. All patients were on hematological remission and none of them presented previous or ongoing serious adverse events related to the use of TKI, neither infectious complication related to their hematological disease or to the treatment with the TKI. All of them had normal routine blood and biochemistry test at sampling. Table 1 summarizes the main clinical characteristics of CML patients. Blood samples from the healthy donors were obtained from the Centro Regional de Transfusión from the Complejo Hospitalario de Toledo (Toledo, Spain).

All the procedures followed with human subjects were in accordance with the ethical standards of the Helsinki Declaration. All individuals who participated in this study gave informed written consent. The design of this work has been reviewed and approved by the ethical committees of each participating institution where this study has been carried out, and it conforms to ethical standards currently applied in Spain.

2.2. Reagents, antibodies and vectors

Dasatinib (BMS-354825, Sprycel®; Bristol-Meyers Squibb, New York, NY) was kindly provided by Dr. Stephen Mason (formerly at Bristol-Meyers Squibb) and Dr. Carey Hwang (Discovery Medicine-Virology, Bristol-Myers Squibb). Selleckchem (Deltaclon, Madrid, Spain) provided Imatinib (Gleevec®, Novartis, Basel, Switzerland), nilotinib (Tasigna®, Novartis), bosutinib (Bosulif®, Pfizer, New York, NY), and ponatinib (Iclusig®, Ariad Pharmaceuticals, Cambridge, MA). Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Quimica SL, Madrid, Spain) was used at 25 ng/ml.

Antibodies against SAMHD1 phosphorylated at T592 and total SAMHD1 were purchased from ProSci (Poway, CA) and Abcam (Cambridge, UK), respectively. Monoclonal antibody against the cyclin dependent kinase 1 (CDK1/CDC2) (clone P0H1), CDK2 (clone D-12) and polyclonal antibody against phospho-PKCθ (Thr538) were obtained from Cell Signaling Technology (Danvers, MA). Monoclonal antibody against β-actin (clone AC-15) was obtained from Sigma-Aldrich Quimica SL. Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from GE Healthcare España (Madrid, Spain). Secondary antibody conjugated to Alexa 488 was purchased from Molecular Probes (Eugene, OR). Antibodies against CD4 conjugated to PerCP, chemokine (CXC motif) receptor 4 (CXCR4) conjugated to phycoerythrin (PE), chemokine (C-C Motif) receptor 5 (CCR5) conjugated to

fluorescein isothiocyanate (FITC) and CD25 conjugated to PE were purchased from BD Biosciences (San Diego, CA).

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 [46]. Vector pNL4.3-Renilla was obtained by replacing *nef* gene of HIV-1 proviral clone pNL4.3 with Renilla luciferase (LUC) gene [47]. Vector p3κB-LUC that contains a luciferase gene under the control of three -κB consensus motifs of the immunoglobulin κ-chain promoter was described previously [48]. Plasmid pCMV-Tat that contains full-length *tat* gene (101 aa) was previously described by Dr Arenzana-Seisdedos [48]. Luciferase (LUC) reporter gene under the control of HIV-1 long terminal repeat (LTR) U3 + R region (LAI strain) was also previously described [49]. Mammalian expression vector pcDNA3.1 was purchased from Thermo Fisher Scientific España (Madrid, Spain).

2.3. Cell viability and proliferation

Cell viability was determined with CellTiter-Glo Luminescent Cell Viability Assay (Promega Ibérica, Madrid, Spain) using an Orion Microplate Luminometer and Simplicity software (Berthold Detection Systems, Oak Ridge, TN). Cell proliferation was measured by flow cytometry after labeling with 2μM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, Carlsbad, CA) [50]. Briefly, cells were labeled with CFSE 2μM and then washed and cultured in 96-well plates in the absence or presence of ITKs and antiCD3/CD28/IL-2 for 3 days or IL-7 (R&D Systems, Minneapolis, MN) for 10 days. Samples were analyzed with FACS Calibur flow cytometer (Becton Dickinson, San José, CA) and FlowJo software v7.2.5 (TreeStar, Ashland, OR) using non-linear curve-fitting techniques. The number of generations (Gn) was fixed at eight peaks, each including the corresponding number of events for each generation.

2.4. HIV-1 infection

Infectious supernatants were obtained from calcium phosphate transfection of HEK293T cells (provided by the existing collection of the Instituto de Salud Carlos III, Madrid, Spain) with plasmid pNL4-3_Renilla. PBMCs previously activated with PHA/IL-2 for two days were infected with 0.5 ng of NL4-3_Renilla in 96-well plates. After incubation for 48 hours in the presence of serial dilutions of ITKs, Renilla activity was quantified as relative light units (RLUs) in the cell lysates using Renilla Luciferase Assay (Promega) in Orion Microplate Luminometer (Berthold Detection Systems). IC_{50} was calculated using sigmoidal dose–response formula in Graph Pad Prism Software (Graph Pad Software Inc., San Diego, CA). Cell viability was evaluated in parallel as described above. Half-maximal cytotoxic concentration (CC_{50}) was calculated using GraphPad Prism Software (sigmoidal dose–response formula). Results were expressed using SI (CC_{50}/IC_{50}).

PBMCs isolated from CML patients and activated previously with antiCD3/CD28/IL-2 for 72 hours were infected by spinoculation with 1 ng p24/10^6 cells for 30 minutes at gently rotation, room temperature. After centrifugation at 600xg for 30min at 25°C and extensive washing with PBS1X, cells were left in culture for 5-7 days only with IL-2.

2.5. Flow cytometry analysis of SAMHD1

PBMCs from CML patients on treatment with ITKs and untreated healthy donors were activated with PHA/IL-2 for 5 days. Cells were then fixed with 1% paraformaldehyde and permeabilized with methanol. After washing, cells were blocked with PBS/BSA 1% and stained with anti-phospho-T592 SAMHD1 and a secondary antibody conjugated to FITC (Dako, Glostrup, Denmark). After staining with fluorescent labelled antibodies against CD3 and CD4 (BD Biosciences), flow cytometry analysis was performed using FACS Calibur cytometer (Becton Dickinson) and FlowJo software.

2.6. Quantification of early and late retrotranscription by TaqMan qPCR

Five hours after infection, DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen Iberia SL, Madrid, Spain) and quantified using Nanodrop 2000C (Thermo Fisher Scientific). Early and late retrotranscription (RT) were assessed by qPCR as previously described by Konig et al [51]. Briefly, 100ng DNA were mixed with 1μM forward and reverse primers for early RT and late RT, 0.2μM FAM-TAMRA probe, and 1xTaqMan Universal Master Mix II (Thermo Fisher Scientific). qPCR was performed in triplicate using standard cycling conditions in a StepOne Real-Time PCR system (Thermo Fisher Scientific). A standard curve was performed using serial dilutions of genomic DNA from 8E5 cell line, which contains a single integrated copy of HIV-1 [52]. We used *ccr5* gene as endogenous control.

2.7. Quantification of proviral integration and 2-LTR circles by TaqMan qPCR

Purified CD4+ T cells were subjected to whole genomic nucleic acid extraction as described above. Analysis of 2-LTR circles was performed as described previously [53]. Using a StepOne Real-Time PCR System (Thermo Fisher Scientific), proviral integrated DNA was quantified by nested Alu-LTR PCR as described previously [54,55]. Briefly, we performed a conventional PCR using two oligonucleotides against Alu sequences and one oligonucleotide against HIV-1 LTR, using the following conditions: 95ºC, 8 min; 12 cycles: 95ºC, 1 min; 60ºC, 1 min; 72ºC, 10 min; 1 cycle: 72ºC, 15 min. A second qPCR was performed with FAM/ZEN/Iowa Black TaqMan probes and TaqMan Master Mix (Thermo Fisher Scientific). DNA standard curve was performed by using genomic DNA from 8E5 cell line and *ccr5* gene was used as housekeeping gene for measuring the input DNA and normalize data.

2.8. Immunoblotting assays

Whole protein extracts were obtained as described [53] and protein concentration was determined by using Bradford method [54]. Forty micrograms of protein extracts were analyzed by fractionation in sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto Hybond-ECL nitrocellulose paper (GE Healthcare España), blocked and incubated with primary and secondary antibodies. The proteins of interest were detected with SuperSignal West Pico/Femto Chemiluminescent Substrate (Pierce, Rockford, IL) and a BioRad Geldoc 2000 (BioRad Laboratories, Madrid, Spain). Densitometry was performed in a Gel Doc 2000 System (BioRad) by using Quantity One software. Gel bands were quantified and background noise was subtracted from the images using Image J software (NIH, USA). The relative ratio of the optical density units corresponding to each sample was calculated per lane using β-actin as internal control.

2.9. Transfection of PBMCs by electroporation

PBMCs were transfected with a Gene Pulser Electroporation System (BioRad). In brief, 10^7 PBMCs isolated from healthy donors were collected in 350μl of RPMI without supplement and mixed with 1μg/10^6 cells of plasmid DNA. Cells were transfected in a cuvette with 4mm electrode gap (MBP Molecular BioProducts, Thermo Fisher Scientific), at 280V, 1500μF and maximum resistance. After transfection, cells were divided in wells, treated with each TKI and activated with PHA and IL-2. After incubation for 72 hours, Luciferase or Renilla activity were assayed with Luciferase or Renilla Assay Systems (Promega), according to manufacturer's instructions. RLUs were measured in cell lysates with a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN). In all transfections, data were normalized according to cell viability that was determined by flow cytometry and

by measuring protein concentration [56] in a microplate reader Sunrise (Tecan Group Ltd., Männedorf, Switzerland).

2.10. Immunofluorescence assay

Cells were adhered on PolyPrep slides (Sigma-Aldrich), fixed with 2% paraformaldehyde in PBS1X and permeabilized as previously described [57]. Cells were then stained with specific antibody against LCK phosphorylated at Y394 and a secondary antibody conjugated to Alexa 555. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Dapi) (Sigma-Aldrich). Images were obtained with a Leica DMI 4000B Inverted Microscope (Leica Microsystems, Barcelona, Spain). The intensity mean per pixel was calculated in all images using LAS AF Lite software (Leica Microsystems) and values were represented in bar diagrams showing statistical significance.

2.11. Kinase activity assay

CD4+ T cells isolated from PBMCs of healthy donors were treated for 3 days with antiCD3/CD28/IL-2 in the presence or absence of ITKs. The enzymatic activity of Src-family tyrosine kinases Src, Lck, Fyn, Lyn and Hck was measured jointly in 5 μg of protein lysates from these cells using ProFluor Src-Family Kinase Assay kit (Promega), according to manufacturer's instructions. Data provided by the Src-Family Kinase rhodamine 110 substrate were obtained with a GloMax Multi Detection System (Promega) and then normalized according to the fluorogenic control substrate Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-AMC), which was used as negative control.

2.12. Statistical analysis

Graph Pad Prism 7.0 (Graph Pad Software Inc.) was used to perform the statistical analysis of the results. Two-way analysis of variance (ANOVA) with Bonferroni post-test

analysis was made to describe the statistical differences among groups. P values (p) < 0.05 were considered statistically significant in all comparisons between groups. p was represented as *, **, ***, or **** for $p<0.05$, $p<0.01$, $p<0.001$, or $p<0.0001$, respectively.

3. Results

3.1. Study population

Blood samples from 37 CML Phi Chromosome-positive (Phi+) patients receiving treatment with TKIs were obtained from the Hospital Clinic (Barcelona, Spain), Hospital Puerta de Hierro (Majadahonda, Madrid, Spain), Hospital La Princesa (Madrid, Spain) and Hospital Ramón y Cajal (Madrid, Spain). All patients had more than one year of follow-up from CML diagnosis and were on treatment with imatinib (n = 11), nilotinib (n = 9), dasatinib (n = 13), bosutinib (n = 3) or ponatinib (n = 1). Some patients had previous exposition to different TKIs but they were on treatment with the current TKI for at least 9 months. All patients were on hematological remission and none of them presented previous or ongoing serious adverse events related to TKI use, neither infectious complication related to their hematological disease or to the treatment with TKIs. All of them were HIV-negative and had normal routine blood and biochemistry test at sampling. The clinical characteristics of these patients are summarized in Table 1. Forty two healthy patients were also recruited for the study to provide a basal, untreated control. All individuals gave informed written consent to participate in the study.

3.2. PBMCs isolated from CML patients showed different susceptibility to HIV-1 infection

PBMCs isolated from blood samples of CML patients on chronic treatment with different TKIs (n = 37) or from healthy donors (n = 42) were activated with anti-CD3/CD28 and IL-2 for three days and then infected ex vivo by spinoculation with HIV-1 X4-tropic strain NL4.3_Renilla. After extensive washing, cells were incubated for 5 days with IL-2. HIV-1 infection was analyzed in triplicates by measuring the production of Renilla, which is proportional to the synthesis of p24-gag [47]. PBMCs from all patients, except those who were on treatment with imatinib, showed lower production of Renilla (p<0.0001 for nilotinib

and dasatinib; $p<0.05$ for bosutinib) (Fig. 1A). Similar results were obtained with PBMCs from the only patient on treatment with ponatinib who was recruited for this study. No TKI was added to the culture medium; the infection results were only related to the susceptibility of the cells to be infected after treatment with TKIs in vivo.

Integration of the provirus was also analyzed by Alu-qPCR of total DNA. All TKIs, except imatinib, were very efficient to interfere with the proviral integration, significantly decreasing the formation of the reservoir ($p<0.001$ for dasatinib; $p<0.01$ for nilotinib; $p<0.05$ for bosutinib) (Fig. 1B).

3.3. Susceptibility of SAMHD1 to be phosphorylated upon activation decreased in PBMCs isolated from CML patients

T-cell activation eliminates the antiviral function of SAMHD1 through its phosphorylation at T592 by cyclin A2/CDK1 [27], rendering the cells susceptible to HIV-1 infection. The phosphorylation of SAMHD1 at T592 was analyzed by flow cytometry in PBMCs from CML patients on chronic treatment with imatinib (n = 5), nilotinib (n = 5), dasatinib (n = 6), bosutinib (n = 2) or ponatinib (n = 1), as well as from healthy, untreated donors (n = 6), after stimulation with PHA and IL-2 for 5 days. The phosphorylation of SAMHD1 was delayed in PBMCs from all CML patients, except in those treated with imatinib (Fig. 2A). Dasatinib ($p<0.0001$), bosutinib ($p<0.05$), nilotinib ($p<0.01$) and ponatinib were the most potent to preserve SAMHD1 function in vivo. A similar pattern was observed when only CD4+ T cells were analyzed, although it was only significant in cells from patients on treatment with dasatinib ($p<0.01$) (Fig. 2B).

One of the mechanism of action described for SAMHD1 to restrict HIV-1 infection is by depleting the intracellular pool of dNTPs [58], which affects the early viral reverse transcription. The formation of viral transcripts was evaluated by qPCR 5 hours after the

infection ex vivo with HIV-1 in PBMCs from CML patients and healthy donors (Fig. 2C). Imatinib was very effective to block both early and late transcription (p<0.0001), as well as dasatinib (p<0.0001), bosutinib (p<0.0001) and ponatinib. Nilotinib slightly interfered with late retrotranscription (p<0.01) but showed no significant effect on early reverse transcription.

3.4. Correlation of TKIs between IC50 in vitro and Cmax in vivo

IC50 and CC50 of each TKI to inhibit HIV-1 replication in vitro was calculated in PBMCs from healthy donors that were treated with serial dilutions of TKIs and simultaneously activated with PHA and IL-2 for 48 hours. Cells were then infected with NL4.3_Renilla and incubated for 2 days more before measuring Renilla to assess the progression of the infection. A correlation of IC50 and CC50 calculated in vitro with the maximum serum concentration (Cmax) that each TKI may achieve in vivo was established as shown in Table 2. Imatinib and nilotinib were the least potent in vitro to inhibit HIV-1 replication, with IC50 of 8.25 μM (4.07 μg/ml) and 9.28 μM (4.91 μg/ml), respectively (Fig. 3A and B). Imatinib reaches Cmax of 2.35 μg/ml after a single dose of 400 mg in healthy adults [59,60], which means that an increase of 1.7 times in Cmax would be needed to attain an antiviral activity against HIV-1 for this inhibitor. Nilotinib reaches Cmax of 1.59 μg/ml after a steady dose of 400 mg twice a day in CML patients [61,62], which means that 3.0 times more concentration in serum would likely be needed to attain an effective antiviral activity. The selectivity index (SI = CC50/IC50) was also calculated to determine the therapeutic index in vitro, attaining maximum antiviral activity with minimal cytotoxicity. SI was at least 2.42 and 3.23 for imatinib and nilotinib, respectively. Bosutinib and ponatinib were more potent, with IC50 of 618.4 nM (327.28 ng/ml) and 145.6 nM (77.46 ng/ml), respectively (Fig. 3C and D). Bosutinib showed a low therapeutic range in vitro for the inhibition of HIV-1 as SI was < 2

(1.66). Bosutinib attains a Cmax of 120 ng/ml in CML patients [63] and 141 ng/ml in healthy adults [64] after a single dose of 600 mg. Therefore, an increase of at least 2.3 times in Cmax would be needed to exert an antiviral effect on HIV-1 replication. Ponatinib reaches a Cmax of 54.7 ng/ml after a single dose of 45 mg in healthy adults [65], which nearly correlates with the IC50 calculated in vitro to inhibit HIV-1 replication. Ponatinib showed an SI of 68.68. Finally, we previously determined that dasatinib is very potent and safe to inhibit HIV-1 replication, with SI > 612 and IC50 of 16.34 nM (8.26 ng/ml) [66], which is 5 times lower than the Cmax of 41.52 ng/ml that is attained in healthy adults taking 50 mg once daily [25].

As there was a great difference between the IC50 of dasatinib for HIV-1 inhibition and the Cmax in vivo, we decided to continue the study using it at 37.5 nM that was closer to IC90 and equally safe and effective. Besides, for the in vitro study nilotinib and imatinib were used at 10 μM, bosutinib at 0.5 μM and ponatinib at 150 nM. In order to evaluate if TKIs were able to interfere with HIV-1 replication at these concentrations in vitro, PBMCs isolated from healthy donors were treated with each TKI and then activated with anti-CD3/CD28/IL-2 for three days. Cells were infected by spinoculation with NL4-3_Renilla and the synthesis of Renilla (Fig. 4A) and proviral integration (Fig. 4B) were analyzed. Dasatinib (p<0.001) and ponatinib (p<0.001) were the most potent, which correlated with the results obtained with PBMCs from CML patients. In contrast, bosutinib showed a similar effect to the cells treated with imatinib and nilotinib, although it appears to be more potent in vivo. In these conditions, dasatinib, bosutinib and ponatinib effectively interfered with SAMHD1 phosphorylation induced by anti-CD3/CD28/IL-2, but PBMCs treated with imatinib and nilotinib showed similar levels to the control cells (Fig. 4C). The expression level of CDK1 and CDK2 correlated with the phosphorylation of SAMHD1. All TKIs except imatinib were effective to block SAMHD1 phosphorylation induced by IL-7 (Fig. 4D).

3.5. Effect of TKIs on viral transcription

In order to evaluate if the inhibition of HIV-1 replication was also at transcriptional level, the specific effect of TKIs on NF-κB transcriptional activity was analyzed by transfecting PBMCs with vector p3κB-LUC. Immediately after transfection, cells were treated with each TKI and activated with PHA and IL-2 for three days. Production of luciferase was measured by chemiluminescence. All TKIs interfered with NF-κB-dependent transcriptional activity (p<0.0001), being imatinib the least potent (Fig. 5A). Although NF-κB is essential for HIV-1 transcription [67], other important transcription factors also bind to LTR promoter such as NF-AT or SP1 [68]. In order to determine the effect of TKIs on all these transcription factors including NF-κB, PBMCs isolated from healthy donors were transfected with pLTR-LUC vector in the presence or absence of the main HIV-1 transcriptional regulator Tat, which is also responsible for an efficient viral transcript elongation and splicing [69]. In the absence of Tat, all TKIs showed similar inhibitory effect on the early phase of LTR-mediated transcription, which is mostly cellular dependent (Fig. 5B, co-transfection of pLTR-LUC with empty pcDNA3). When Tat was overexpressed by co-transfection of pCMV-Tat vector, TKIs conserved the ability to interfere with the late phase of LTR-mediated transcription, which is mostly dependent on Tat (p<0.001), although some seemed more effective such as nilotinib and bosutinib (Fig. 5B, co-transfection of pLTR-LUC with pCMV-Tat). The effect of TKIs on viral transcription in the presence of all viral proteins was evaluated by transfection of PBMCs with infectious pNL4-3_Renilla expression vector in the same conditions as described before. We observed that the restraining effect on HIV-1 transcription persisted with all TKIs, (p<0.0001) (Fig. 5C), being imatinib the least potent.

3.6. Effect of treatment with TKIs on the expression of HIV-1 co-receptors CCR5 and CXCR4.

We already described that simultaneous treatment of PBMCs with dasatinib and PHA/IL-2 for 48 hours did not significantly modify the expression levels of CXCR4 and CCR5 on the cell surface [26]. Now we analyzed by flow cytometry if the expression levels of co-receptors were altered in PBMCs after treatment with anti-CD3/CD28/IL-2 for 5 days, in the presence or absence of each TKI. In cells not treated with TKIs, the addition of anti-CD3/CD28/IL-2 to the culture medium down-regulated an average of 25% the expression of CXCR4 ($p<0.001$), as was described before [70], but none of the assayed TKIs reduced significantly further the expression of this co-receptor (Fig. 6A). Expression of CCR5 was 10% up-regulated on average upon activation with anti-CD3/CD28/IL-2 [71] in cells not treated with TKIs (Fig. 6B). Treatment with imatinib and nilotinib did not significantly affect the expression of CCR5, but dasatinib, bosutinib and ponatinib ($p<0.05$) impeded the up-regulation of CCR5 upon activation, which remained the same as in resting cells. This effect was most likely related to the status of activation in the T cells.

3.7. Some TKIs showed a strong cytostatic effect on CD4+ T cells

SAMHD1 phosphorylation and CCR5 up-regulation are linked to T-cell activation. Therefore, we first analyzed the effect of TKIs on activation markers such as CD69 and CD25, which is expressed early upon activation [72]. CD4+ T cells isolated from healthy donors that were treated with anti-CD3/CD28/IL-2 for 3 days showed an average increase of 80% in CD25/CD69 expression, which was maintained after treatment with imatinib or nilotinib (Fig. 7A). The effect of bosutinib was modest but dasatinib and ponatinib were very efficient to avoid the expression of CD25 and CD69, keeping them at a basal level. The expression of CD25/CD69 was also analyzed in CD4+ T cells isolated from CML patients treated with TKIs and a similar pattern was observed (Fig. 7B). Treatment with dasatinib in vivo reduced 1.5-fold the expression of CD25/CD69 on CD4+ T cells surface, upon TCR-

stimulation (p<0.05). The same erratic behavior of bosutinib in vitro was observed after in vivo treatment.

The effect of TKIs on T cell proliferation was analyzed by flow cytometry after labeling with CFSE CD4+ T cells isolated from healthy donors that were simultaneously treated with TKIs and anti-CD3/CD28/IL-2 for 3 days (Fig. 7C) or with IL-7 for 10 days (Fig. 7D). Dasatinib, bosutinib and ponatinib impeded T-cell proliferation mediated by anti-CD3/CD28/IL-2. All TKIs interfered with T-cell proliferation induced by IL-7, being dasatinib and ponatinib the most potent.

3.8. Activity of Src-kinases essential for T cell activity is partially suppressed by TKIs

LCK induces direct or indirect phosphorylation of PKCθ, which is essential for most T cell activation pathways [41]. PKCθ is phosphorylated and translocated to the plasma membrane upon T-cell receptor (TCR)/CD28 engagement at the immunological synapse, initiating a cascade of events that culminates in the activation of transcription factors such as NF-κB, NF-AT, and AP-1 [73]. Therefore, LCK/PKCθ/CD28 interaction is essential for T-cell activation. After TCR engagement, LCK is phosphorylated at tyrosine 394 (Y394) in the catalytic domain to induce its kinase activity [74]. LCK phosphorylation at Y394 was analyzed by fluorescence microscopy in PBMCs isolated from healthy donors that were treated with each TKIs and then activated with anti-CD3/CD28/IL-2 for 3 days. Phosphorylation of LCK was observed in activated, untreated PBMCs and in those also treated with imatinib, nilotinib and bosutinib (Fig. 8A). Cells treated with dasatinib or ponatinib showed a level of Lck phosphorylation similar to untreated, non-activated cells (p<0.01). The intensity mean per pixel was calculated and values represented in a bar diagram.

The kinase activity of the Src-family tyrosine kinases SRC, LCK, FYN, LYN and HCK was measured jointly by fluorometry in CD4+ T cells isolated from healthy donors and treated with antiCD3/CD28/IL-2 in the presence or absence of different TKIs. All TKIs reduced the activity of these Src kinases ($p < 0.0001$), being bosutinib, nilotinib and dasatinib the most potent (Fig. 8B).

4. Discussion

HIV-1 reservoir is formed immediately after the infection [14–17,19] and although prompt initiation of ART may limit its size [75–77], it is not enough to ensure its elimination. Even in patients with long-term undetectable viremia, the eradication of a reservoir of 10^6 cells would take more than 50 years of ART [78]. This is mostly due to the reservoir is not static but the latently infected cells may proliferate at a low rate, even clonally expand [79], continuously replenishing the reservoir [80]. Although productively infected cells die quickly, the reservoir is formed mostly by long-lived, memory CD4+ T cells that ensure the viral persistence even with ART [5,81]. These cells remain invisible to the immune system and ART unless they are activated, being then able to produce full viral replication. However, the strategies directed to reactivate and destroy the latently infected cells using LRAs have not been completely successful so far [82,83]. Therefore, new approaches to tackle and eliminate the reservoir should be developed.

During primary or acute infection there is a massive activation of CD4+ T cells that is responsible not only for the reservoir formation and establishment, but also for the viral spread and destruction of infected cells [68]. Once the reservoir is settled, several mechanisms such as the homeostatic proliferation induced by IL-7 maintain and replenish the reservoir [8]. The phosphorylation and subsequent deactivation of the restriction factor SAMHD1 that occurs during T-cell activation [27], induced either by TCR engagement or homeostatic cytokines [26], is essential for all these events. SAMHD1 phosphorylation permits the synthesis of viral cDNA by reverse transcription [84], which is subsequently integrated as a provirus in the cell genome. TCR engagement induces SAMHD1 phosphorylation in CD4+ T cells and releases a cascade of events that ends in the activation of essential transcription factors such as NF-κB, necessary for T-cell proliferation and proviral transcription. LCK recruitment to the immunological synapse is required for

initiating this activation pathway [85,86]. Therefore, the inhibition of tyrosine kinases such as LCK in CD4+ T cells could be valuable to interfere with T-cell activation and simultaneously, with HIV-1 replication [26]. In this regard, the family of TKIs that is currently used for the treatment of CML could be valid candidates, as they are potent and safely used in these patients. TKIs may control CML but so far there is no evidence that they can cure it through the complete elimination of the BCR-ABL+ clone. Therefore, although there are multiple clinical trials demonstrating the possibility of treatment-free remission in many patients [87], usually these drugs need to be taken for life. Nevertheless, TKIs have definitely improved the clinical course of CML and the survival of the patients [88,89]. Although imatinib was the first approved, the rest of TKIs produce faster and deeper responses, and they are approved in resistance or intolerance to imatinib (nilotinib, dasatinib, bosutinib, ponatinib), or in first line (all but ponatinib). The reason why ponatinib was not approved in first line was the high incidence of arterial ischemic events [88].

Searching for the best option of TKI to interfere with the formation of HIV-1 reservoir, we determined that all assayed TKIs except imatinib were able to avoid proviral integration in PBMCs from CML patients treated with TKIs that were infected ex vivo with HIV-1. These PBMCs also showed resistance to SAMHD1 phosphorylation induced ex vivo, which correlated with a low reverse transcription of HIV-1 in PBMCs from patients treated with dasatinib, bosutinib and ponatinib. Although imatinib was the least efficient to avoid SAMHD1 phosphorylation and proviral integration, it was able to impede HIV-1 reverse transcription. Something similar could occur with nilotinib that was quite effective against HIV-1 infection when administered in vivo but not when used in vitro. This could be due to a low half-life of imatinib and nilotinib in cell culture but we also have to consider that all patients recruited for this study were treated for at least 9 months with these TKIs, whereas PBMCs from healthy donors used for in vitro experiments were only treated once with each

TKI at the beginning of the experiment. Another discrepancy was that although ponatinib was the most efficient both in vivo and in vitro to avoid HIV-1 infection and inhibited the phosphorylation of LCK at Y394, it was not the most active against the kinase activity of SFKs such as SRC, LCK, FYN, LYN and HCK. In view of the potent cytostatic effect of ponatinib on PBMCs, likely other kinases different than the ones assayed are affected by this TKI. In fact, it is known that ponatinib potently inhibits FLT3, KIT, and RET [91] and we have preliminary results showing that ponatinib induces a great deregulation of the transcriptome in PBMCs from healthy donors treated in vitro (data not shown).

The ability of TKIs to impede HIV-1 infection ex vivo was related to their antiviral effect in vitro, comparing IC50 in vitro with Cmax in vivo. Although PBMCs from CML patients treated with imatinib could not avoid later stages of viral replication such as proviral integration or synthesis of viral proteins, when imatinib was used in vitro at the calculated IC50 (~ 10 μM; SI > 2.42), it showed more antiviral activity. As this IC50 was nearly twice the Cmax achieved in vivo, a higher dose of imatinib in vivo than the one currently used for CML treatment would be necessary for HIV-infected patients. Nilotinib was not as efficient as imatinib at early stages of the infection, as it could not avoid reverse transcription despite the low phosphorylation of SAMHD1 observed in PBMCs from CML patients in response to PHA and IL-2. However, although nilotinib interfered with proviral integration and synthesis of viral proteins both in vitro and ex vivo, according to IC50 (~ 10 μM; SI > 3.23) it would be necessary to administer three times the current dose of nilotinib to HIV-infected patients. Bosutinib was very potent in vitro (IC50 = 618 nM) and interfered with SAMHD1 phosphorylation, but it showed the lowest therapeutic range against HIV-1 infection (SI = 1.66). These results did not correlate with data of bosutinib in vivo, because even being one of the most toxic TKI, SI is not so low in vivo [35]. PBMCs from CML patients treated with bosutinib were resistant to both early and late stages of infection but twice the lowest dose of

bosutinib used in vivo would be necessary for HIV-infected patients. Finally, TKIs with the lowest IC50 for interfering with HIV-1 replication and to preserve SAMHD1 antiviral function were dasatinib (IC50 = 16.34 nM; SI > 612) and ponatinib (IC50 = 145 nM; SI > 68), which impeded HIV-1 reverse transcription, SAMHD1 phosphorylation, proviral integration and synthesis of viral proteins both ex vivo and in vitro. When IC50 was compared to Cmax in vivo, less than one-fifth of the regular dosage of dasatinib and nearly the same dosage of ponatinib would be necessary for HIV-infected patients. Therefore, treatment of HIV-infected patients with dasatinib would be safer than in CML patients as quite lower dose would be needed to interfere with HIV-1 replication.

SAMHD1 phosphorylation is linked to T-cell activation and cell cycle progression [92]. All TKIs able to interfere with TCR-mediated phosphorylation of SAMHD1 also impeded T-cell proliferation and activation, as well as an increase in CCR5 surface expression, except bosutinib that did not affect the expression of activation markers such as CD25 and CD69. Due to dasatinib, bosutinib and ponatinib maintained CCR5 expression on the cell surface at a resting level, all analyses were performed with X4-tropic HIV-1 strain as the expression of CXCR4 remained unchanged after TKI treatment. Besides, all TKIs showed similar restrictive effect on HIV-1 transcription, mostly at the level of NF-κB, being imatinib the least potent. Although this restriction was partially overcome by the presence of other cellular transcription factors that bind to LTR in PBMCs or by the overexpression of the viral regulator Tat, all TKIs significantly interfered with viral transcription when fully infectious HIV-1 genome was transfected. This would mean that a low reactivation of the provirus might be produced during treatment with TKIs.

We may conclude that the preferred TKIs to be evaluated in clinic as adjuvant of ART would be dasatinib or ponatinib as they showed at least four effective mechanisms of action against HIV-1 infection. First, they interfered with SAMHD1 phosphorylation in vivo,

making CD4+ T cells refractory to HIV-1 infection and proviral integration. Second, they induced a cytostatic effect even under TCR-mediated stimulation, which would avoid the massive CD4+ T cell activation that occurs during the acute infection and would reduce the size of the reservoir. Third, by interfering with NF-κB activity, these TKIs would avoid the proviral reactivation, impeding reservoir reseeding. Finally, dasatinib and ponatinib impeded IL-7-mediated T-cell proliferation, which theoretically would avoid the homeostatic proliferation of the reservoir in chronically infected patients on ART. It is intriguing that, despite their myelosuppressive and inmunomodulating effects, the rate of infections with the BCR/ABL inhibitors is rather low, and the occurrence of viral and opportunistic infections is anecdotal [93]. This indicates the possibility that other immune mechanisms of compensation not completely understood might have been developed in these patients [94]. However, the high risk of opportunistic infections that may occur in HIV-infected patients makes recommendable that only patients with CD4 counts above 350-500 cells/ml should be included in clinical studies with dasatinib, as this is the TKI with highest potential for infectious complications [95]. Traditionally, HIV-infected patients are excluded from clinical trials, which supposes a challenge for applying the results of safety to these patients. However, although the incidence of CML in HIV-infected patients is low and coincidental [96,97], some cases of HIV-infected patients with CML have been described and they were safely treated with the same dose of imatinib usually used for uninfected individuals, achieving remission and tolerance no differently than non-HIV patients [97,98]. The main problem that may arise then is the possibility of interaction between TKIs and ART. Because dasatinib is metabolized though CYP3A4 pathway [99], ART regimens containing dolutegravir or raltegravir should be considered if co-administered with dasatinib as they would have no expected pharmacokinetic interactions [100]. However, combinations of ritonavir, cobicistat or any other potent CYP3A4 inhibitors should be avoided as they might

become toxic if administered simultaneously with dasatinib [66,101]. Ponatinib is an interesting choice as it had a similar ex vivo profile to dasatinib but it should be used cautiously in the clinical setting as it has been related to ischemic adverse events [91].

In conclusion, the use of one TKI as dasatinib or ponatinib as adjuvant of ART during a short period could limit the formation and replenishment of the reservoir, favoring its depletion by interfering with proviral reactivation and homeostatic proliferation. Besides, these TKIs could also help ART in controlling viremia, eventually allowing lower dosage of ART, or during controlled interruptions of treatment. The possible development of adverse effect induced by dasatinib would be less frequent because a lower dosage could be used for HIV-infected patients. This strategy merits further investigation in clinic as it could induce a better disease prognosis, creating a more adequate scenario for eradication strategies and longer controlled treatment interruptions.

Author contributions

MC and JAl conceived the study. GB, JAm, JMM, VG, JLS, RD and FC selected the patients and obtained the blood samples. MB, NC, CR and MP performed the immunoblotting and cytometry analyses. EM, SRM and MRLH performed the HIV-1 infections in vitro and PBMCs transfections and immunofluorescences. MC, JAl and MB wrote the manuscript and performed the statistical analyses. All authors contributed to data collection and analysis and approved the final manuscript.

Conflict of interest statement

JMM has received consulting honoraria and/or research grants from AbbVie, Bristol-Myers Squibb, Cubist, Genentech, Medtronic, Novartis, Gilead Sciences, and ViiV Healthcare outside the submitted work. The other authors have declared that no conflict of interest exists.

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Figure legends

Figure 1. Susceptibility to HIV-1 infection of PBMCs from CML patients on long-term treatment with TKIs. (A) Analysis of the synthesis of viral proteins in PBMCs from CML patients (n = 37) or healthy donors (n = 42) infected ex vivo with HIV-1 X4-tropic strain NL4.3_Renilla for 5 days. (B) Proviral integration was also analyzed in these cells by Alu-qPCR with TaqMan probes. All measurements were done in triplicate and are represented as mean ± the standard error of the mean (SEM). Statistical significance was calculated using one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 2. Phosphorylation of SAMHD1 and HIV-1 reverse transcription was hindered in CD4+ T cells from CML patients treated with TKIs. Measurement by flow cytometry of SAMHD1 phosphorylation in PBMCs (A) and CD4+ T cells (B) from CML patients, compared to healthy controls. (C) Analysis of early and late reverse transcription (RT) in PBMCs from CML patients and healthy donors infected ex vivo with HIV-1 X4-tropic strain NL4.3_Renilla for 5 hours. All measurements were done in triplicate and are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. *p < 0.05; **p < 0.01; ****p < 0.0001.

Figure 3. Measurement of IC_{50} and CC_{50} of several TKIs in PBMCs activated with PHA/IL-2 and infected with NL4-3_Renilla strain. (A) PBMCs activated with PHA and IL-2 for 48 hours were incubated in 96-well plates with increasing concentrations of imatinib (A), nilotinib (B), bosutinib (C) and ponatinib (D) and then infected with NL4-3_Renilla strain. IC_{50} and CC_{50} were calculated by measuring the production of Renilla (RLUs). All measurements were done in triplicate and are represented as mean ± SEM.

Figure 4. Analysis of in vitro effect of TKIs in HIV-1 infection and SAMHD1 phosphorylation. The synthesis of renilla (A) and the proviral integration (B) was analyzed in PBMCs from healthy donors incubated for three days with each TKI and antiCD3/CD28/IL-2

and then infected with NL4-3_Renilla. All measurements were done in triplicate and are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001. SAMHD1 phosphorylation and its correlation with the expression of CDK1 and CDK2 was evaluated by immunoblotting in PBMCs from healthy donors after treatment with each TKI and activation with antiCD3/CD28/IL-2 (C) or IL-7 (D). The relative ratio of the optical density units corresponding to each sample was calculated by densitometry regarding the internal loading control (β-actin) per each lane.

Figure 5. Effect of TKIs on viral transcription. Analysis of chemiluminescence in PBMCs transfected by electroporation with p3κB-LUC (A), pLTR-LUC along with pcDNA3 or pCMV-Tat (B), or pNL4-3_Renilla (C) and then incubated for 72 hours in the presence or absence of each TKI and PHA/IL-2. All measurements were done in triplicate and are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. ****p < 0.0001.

Figure 6. TKIs interfered with the expression of HIV-1 co-receptors. Analysis by flow cytometry of the expression of CXCR4 (A) and CCR5 (B) in PBMCs isolated from healthy donors and treated with antiCD3/CD28/IL-2 in the presence or absence of each TKI. All measurements were done in triplicate and are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. *p < 0.05; ***p < 0.001.

Figure 7. Cytostatic effect of TKIs. Expression of CD25/CD69 in CD4+ T cells isolated from healthy donors and treated for 3 days with TKIs and/or antiCD3/CD28/IL-2 (A) or in CD4+ T cells isolated from CML patients treated with different TKIs, activated with antiCD3/CD28/IL-2 for 3 days (B). All measurements were done in triplicate and are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. *p < 0.05; ***p < 0.001; ****p < 0.0001. In CD4+ T cells from healthy donors, T cells

proliferation was analyzed after CFSE staining and treatment with TKIs and antiCD3/CD28/IL-2 for 3 days (C) or IL-7 for 10 days (D).

Figure 8. Effect of TKIs on kinases essential for T cell activation. (A) Phosphorylation of LCK at Y394 was analyzed by fluorescence microscopy in PBMCs from healthy donors treated with TKIs and/or antiCD3/CD28/IL-2 for 3 days. Nuclei were stained with Dapi. Intensity mean per pixel was calculated and values were represented in a bar diagram showing statistical significance. (B) Quantification of Src-kinase activity (SRC, LCK, FYN, LYN and HCK) was performed by fluorimetry in CD4+ T cells isolated from healthy donors, treated with with TKIs and/or antiCD3/CD28/IL-2 for 3 days. The fluorogenic peptide AAF-AMC was used as negative control. All measurements were done in triplicate and are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. *$p < 0.05$; ****$p < 0.0001$.