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Dasatinib protects humanized mice from acute HIV-1 infection

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ABSTRACT

HIV-1 infection remains incurable despite the efficient combined antiretroviral therapy (cART) due to the formation of long-lived viral reservoirs that are mostly settled in CD4+ T cells and maintained by homeostatic proliferation. The use of cytostatic drugs such as tyrosine kinase inhibitors (TKIs) as adjuvants to cART could be helpful to avoid the reservoir establishment and replenishment. We determined previously that TKI dasatinib, which is successfully used for treating chronic myeloid leukemia (CML), shows antiviral effect against HIV-1 infection of CD4+ T cells in vitro. HIV-infected subjects that developed CML may safely combine long-term treatment with TKIs and cART but there is no information about the effect of dasatinib on HIV-1 reservoir in vivo. Therefore, we analyzed the ability of dasatinib to protect NSG mice engrafted with human CD34+ hematopoietic stem cells from HIV-1 infection. Mice were randomly assigned to two groups that received dasatinib or placebo daily by oral gavage. After five days, all mice were infected intraperitoneally with HIV-1 and followed up for 21 days in the absence of cART. Daily administration of dasatinib decreased viral and proviral load in all treated mice, showing in 40% of these mice undetectable viral RNA or DNA in blood. Proviral HIV-1 DNA in gut-associated lymphoid tissue (GALT) was also reduced in all dasatinib-treated mice and under the limit of detection in one of these mice. Finally, treatment with dasatinib modified the distribution of CD4+ and CD8+ T-cell subpopulations, delaying their differentiation into memory T-cell subsets that are a major component of the viral reservoir. In conclusion, dasatinib afforded protection of NSG mice from HIV-1 intraperitoneal infection in the absence of cART.
1. Introduction

Combined antiretroviral treatment (cART) has transformed HIV-1 infection into a chronic disease where the virus remains undetectable. However, treatment interruption leads to an eventual viral rebound in most patients due to the existence of a highly stable HIV-1 reservoir that cannot be eliminated only by cART [1-3]. It has been estimated that more than 80 years would be necessary to produce some significant change in the number of latently infected cells if only cART is used as treatment [4, 5]. Although some chronically infected patients are able to control viral replication after treatment interruption, these individuals have some exceptional characteristics that are not shared by all patients [6-8]. Moreover, such viral control does not imply the clearance of the reservoir during cART.

HIV-1 reservoir is located in higher proportion in central memory (T\textsubscript{CM}), stem cell-like memory (T\textsubscript{SCM}) and effector memory (T\textsubscript{EM}) CD4\textsuperscript{+} T cells [9-11], whereas terminally differentiated effector memory (T\textsubscript{EMRA}) and naïve (T\textsubscript{N}) CD4\textsuperscript{+} T cells show a minor contribution [10, 12]. These latently infected cells remain undetectable for the immune system and cART until they are activated and begin T-cell expansion, proliferation and full viral replication [13]. Therefore, T-cell activation is an essential step for HIV-1 replication and its control may interfere with the formation and replenishment of the reservoir. Src tyrosine kinases (Src-TK) are critical for T-cell activation. We previously demonstrated that p56\textsuperscript{lck}, which is a key signaling Src-TK for T-cell development and maturation, is activated in CD4\textsuperscript{+} T cells during HIV-1 infection [14]. Consequently, some Src-TK inhibitors (TKIs) - currently used in the clinic for the treatment of chronic myeloid leukemia (CML)- are quite efficient to interfere with HIV-1 infection of CD4\textsuperscript{+} T cells [15]. This antiviral effect of TKIs was observed not only after treatment \textit{in vitro} of peripheral blood mononuclear cells (PBMCs) isolated from healthy donors that were
subsequently infected with HIV-1, but also in PBMCs isolated from patients with CML on treatment with TKIs that were infected ex vivo [14, 15]. One of the main mechanism of action of TKIs such as dasatinib against HIV-1 infection was based on preserving the antiviral activity of the innate immune factor SAMHD1 [16], whose phosphorylation and subsequent inactivation is necessary for HIV-1 to initiate the infection of the host cell [17].

Although cancer has become a leading cause of morbidity and mortality in HIV-infected patients [18], CML is not commonly associated to HIV-1 [19]. Nevertheless, some HIV-infected patients have developed CML while on cART and they have started a long-term treatment with TKIs, including dasatinib [20-22]. Once drug interactions between cART and TKIs have been discarded, it has been described that these patients normally showed good tolerance to TKIs and excellent clinical evolution in their hematological disease. However, no information about the effect of TKIs on the progression of the viral reservoir has been described for these patients.

In the present study, we analyzed the ability of dasatinib to interfere with HIV-1 infection in vivo using highly immunodeficient NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>m1Wjl</sup>/SzJ (NSG) mice engrafted with human CD34<sup>+</sup> hematopoietic stem cells in order to give rise to a functional human immune system [23], and we evaluated whether the antiviral effect of TKIs against HIV-1 infection that was observed in vitro and ex vivo may be reproduced in vivo. The results obtained could support the strategy of using TKIs as adjuvants of cART in order to restrain the formation and maintenance of the reservoir in HIV-infected patients.
2. Materials and methods

2.1. Animal model

NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice were selected for this study since it is an extremely immunodeficient mouse strain that permits the humanization by engraftment of a wide range of primary human cells such as human CD34⁺ hematopoietic stem cells. Therefore, CD4⁺ T cells, the main target for HIV-1 infection, are preserved in this model. Mice engrafted with human CD34⁺ hematopoietic stem cell were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were engrafted with cells from the same human donor in order to reduce the experimental variability. Mice were included in the experiments four months after CD34⁺ engraftment. To avoid the possibility of developing a graft versus host disease (GVHD) that could interfere with the results, mice were closely monitored for hair changes and weight loss, main markers of the disease. Significant hair changes or weight reduction > 15% were not observed during the time of the study. Regarding to immune reconstitution, mean human CD45⁺ cells was 63.7%, of which 20.4% on average were CD3⁺ T cells (Table 1). Mice that were not guaranteed a minimum of 20% human CD3⁺ T cells were not included in the study.

2.2. Ethical statement

All animal procedures were performed according to protocol #10242 that was reviewed by the Animal Experimentation Ethics Committees of the University Hospital Germans Trias i Pujol (Barcelona, Spain) and Instituto de Salud Carlos III (Madrid, Spain), and approved by Generalitat de Catalunya (Barcelona, Spain) and Comunidad de Madrid (Madrid, Spain) (PROEX 253/15), according to current Spanish and European Union legislation regarding the protection of experimental animals. Mice were supervised daily following a strict protocol in order to ensure animal welfare.
2.3. Sample Size and Experimental Design

Sample size was determined to compare plasma viral load and total HIV-1 DNA between two groups of mice - untreated or treated with dasatinib - through power analysis using G-POWER software (Universität Düsseldorf, Germany). Assuming normality of the variables in log scale and non-inferiority of treatment effect over untreated mice, we considered a one-tailed T-test for the difference between two independent means and a signification level (alpha) of 0.05. We determined a sample size of n=5 mice per group to observe an effect size of 1.7 (means differences over 1.7 times the standard deviation) reaching a power of 80%. We used then 5 mice per group in order to accomplish the Three Rs principles for more ethical use of experimental animals.

Ten NSG mice were randomly assigned to group 1 (n=5) or group 2 (n=5) (Fig 1). Mice in group 1 were treated daily with 100µl of placebo (citrate buffer 80mM pH 3.1) by oral gavage. Mice in group 2 were treated daily with 100µl of dasatinib 20mg/kg (0.5mg/day) by oral gavage. Dasatinib (BMS-354825) for in vivo use was kindly provided by Bristol-Myers Squibb (BMS) (New York, NY) and previously prepared as oral solution for multiple dose in citrate buffer 80mM pH 3.1. Citrate buffer was prepared using citric acid monohydrate and sodium citrate dehydrate purchased from Sigma-Aldrich Merck (Sigma Aldrich Química, Madrid, Spain), according to the protocol provided by BMS (New York, NY). In order to ensure a high drug bioavailability, mice did not have access to food or water at least 12 and 3 hours before oral gavage, respectively. Direct delivery of the drug into the stomach by oral gavage ensured precise and accurate dosing of animals [24]. Since dasatinib bioavailability is highly dependent on gastric pH [25], it was administered in acid solution to facilitate fast absorption kinetics due to its ability to permeate the gastric mucosa [26]. An appropriate volume (lower than 5 ml/kg) was used to avoid stress or discomfort, slowly delivered to ensure better tolerance [27]. All mice
were treated for 5 days before administering a single intraperitoneal injection of HIV-1_{NL4-3} (17,500 TCID₅₀ per dose). X4-tropic HIV-1 was used due to the ability of dasatinib to downregulate the expression of CCR5 on the cellular surface in vitro [15] and the subsequent potential interference with the viral entry of R5-tropic viruses. Mice were maintained for 21 days after infection and whole blood samples were collected every 7 days. Samples of GALT were collected post-mortem at day 21.

2.4. HIV quantification in blood and GALT

Plasma was used for HIV-RNA quantification with Abbott m2000 RealTime System (Abbott Laboratories, Chicago, IL). We also lysed blood cells and quantified HIV-DNA as previously described [28]. Peyer patches from gut samples were collected at the end time point, mechanically disaggregated and used to quantify HIV-DNA by qPCR. DNA was isolated from tissues using DNeasy Blood & Tissue kit (Qiagen Iberia, Madrid, Spain) and HIV-1 proviral integration was quantified by using nested Alu-LTR PCR as described previously [29, 30] with modifications [31], using StepOne Real-Time PCR System (Applied Biosystems).

2.5. Cell population in blood

Whole blood was stained to characterize cell populations in CD4⁺ and CD8⁺ T cells (hCD45⁺CD3⁺CD8⁺/-CD45RA⁺/-CCR7⁺/⁻). All conjugated antibodies were purchased from BD Biosciences Europe (Eysins, Switzerland) and analyses were performed using a BD LSRSFortessa X-20 flow cytometer (BD Biosciences) and FlowJo v10 software (TreeStar, Ashland, OR).

2.6. Statistical analysis

Statistical analysis of the results was performed using R v.3.4 and Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA). Comparisons were analyzed using non-
parametric rank based tests. For variables affected by detection limits, Peto-Prentice test and paired Prentice-Wilcoxon test for censored data were used. P values ($p$) < 0.05 were considered statistically significant in all comparisons and were represented as *. 
3. Results

3.1. Study design

Ten NSG mice of identical genetic background (Table 1) were randomly divided into Group 1 (n=5), treated daily with oral placebo, and Group 2 (n=5), treated daily with dasatinib (Fig. 1). Treatment was administered by oral gavage. After five days of treatment, all mice were intraperitoneally injected with HIV-1 and then maintained until day 21 post-infection. Blood samples were taken at day 7, 15 and 21 days post-infection (dpi). Mice were sacrificed at day 21 and samples of GALT were taken.

3.2. Effect of treatment with dasatinib on HIV-1 viremia and proviral integration

Plasma viremia increased over time in all animals (Fig. 2a). However, an overall reduction of 3.1-fold was observed in dasatinib-treated mice at 21 dpi. Two mice from the dasatinib-treated group (#2 and #5) showed persistent undetectable plasma viremia. Proviral HIV-DNA in peripheral blood cells also increased over time in all mice (Fig. 2b), showing an overall decrease of 3.1-fold in all dasatinib-treated mice at 21 dpi, in agreement with plasma viral load, with two mice (#2 and #5) showing no detectable proviral HIV DNA with our assay. Integrated provirus was also quantified in GALT at 21 dpi, and it was reduced 1.4-fold in dasatinib-treated group (Fig. 2c). One mouse from this group (#5) showed no proviral HIV DNA in GALT. The other mouse with no detectable viral RNA and proviral HIV DNA in blood (#2) had, however, detectable provirus in GALT.

3.3. Changes in T-cell subpopulations associated with dasatinib treatment

The effect of dasatinib on the distribution of CD4+ and CD8+ T-cell populations was analyzed by flow cytometry after 21 days of infection. We observed an increase of 25% and 29% on average in naïve CD4+ and CD8+ T cells (p<0.05), respectively, in the group
of NSG mice treated with dasatinib for 26 days, in comparison with the control group (Fig. 3a). These data indicated that treatment with dasatinib *in vivo* impeded the differentiation of both CD4$^+$ and CD8$^+$ naïve to memory T cells in humanized mice (Fig. 3b). In fact, the percentage of CD4$^+$ and CD8$^+$ T$_{CM}$ cells was reduced 15% and 7%, respectively ($p<0.05$), in dasatinib-treated mice, whereas both effector memory T-cell types (T$_{EM}$ and T$_{EMRA}$) were reduced by 8% ($p<0.05$) and 2% in CD4$^+$ T cells, and by 16% and 6% in CD8$^+$ T cells ($p<0.05$), respectively.
4. Discussion

Previous studies showed that some TKIs such as dasatinib could be an efficient tool against HIV-1 as they may protect CD4\(^+\) T cells from infection and proviral integration both \textit{in vitro} and \textit{ex vivo} [14, 15]. Dasatinib may also interfere with CD4\(^+\) T cell proliferation induced by homeostatic cytokines such as IL-2 and IL-7 \textit{in vitro} [32], which could be useful to hinder the replenishment of HIV-1 reservoir. These effects have been mostly related to the ability of dasatinib to interfere with SAMHD1 phosphorylation and subsequent inactivation, which would lead to CD4\(^+\) T cell activation and proliferation [32]. Although these results seemed very promising, we had no warranty of the effect of dasatinib during HIV-1 acute infection \textit{in vivo}, whether it could really impede HIV-1 infection and reservoir establishment. Therefore, we treated NSG mice with dasatinib for 5 days and then they were infected with HIV-1 through intraperitoneal injection. The presence of viral RNA and DNA in blood was analyzed every 7 days after the infection and the size of the reservoir in GALT was analyzed after 21 days of infection. We chose the NSG mice model since this strain preserves functional T cells with the ability to mature and differentiate into subpopulations and it has been validated for long-term studies of infectious diseases [33]. Dasatinib was administered before HIV-1 infection in order to ensure that we were preserving SAMHD1 from phosphorylation and to be able to reproduce the protective effects against HIV-1 infection that we observed \textit{in vitro} [14]. A 2.4-fold decrease on average in the viral load of the dasatinib-treated group was observed at day 7. This restraint was observed throughout the duration of the experiment, being 3.1-fold reduced in the dasatinib-treated group at day 21. This restrictive effect caused by dasatinib was variable but it was observed in all dasatinib-treated mice, which would discard that this observation was due to a general failure of infection in dasatinib-treated group. The fact that we obtained significant differences among groups proved that we had
enough statistical power for the significance of the results. Accordingly, we concluded that pre-treatment of mice with dasatinib afforded protection against HIV-1 acute infection, regarding the placebo group and in the absence of cART. Moreover, two out of five mice showed no detectable viral load or total DNA in blood and one of these mice showed undetectable proviral DNA in GALT. Besides, we isolated 3.2-fold more total DNA from the gut tissue of dasatinib-treated mice than from the placebo group at 21 dpi (data not shown). As HIV-1 acute infection is characterized by massive depletion of activated CD4$^+$ T cells mainly in GALT [34], dasatinib could be able to protect the mucosal cells from viral damage. The dosage of dasatinib used for this study (20 mg/kg/day) was selected according to previous in vivo experiments [35] and pharmacokinetic data obtained from the supplier BMS [36]. In fact, near maximal absorption of dasatinib occurs when it is given at this dosage [37], without causing toxicity as the animals did not show changes in weight, behavior or general appearance during the time of treatment.

The effect of dasatinib on the distribution of CD4$^+$ T-cell subpopulations was also analyzed since differentiation of latent memory T cells is essential for the establishment, expansion and maintenance of HIV-1 reservoir. CD4$^+$ T$_{CM}$ cells are the largest fraction of memory population and a predominant site for persistent HIV-1 infection even in patients on cART [9]. The calculated half-life for T$_{CM}$ cells reaches 4.8 years [10, 11] and they proliferate under homeostatic stimuli such as IL-7, ensuring a highly stable HIV-1 reservoir that is replenished most likely by clonal expansion of infected memory CD4$^+$ T cells [38], rather than de novo infection [39]. Moreover, memory stem cells, a slightly earlier developmental stage of T$_{CM}$ cells, also contribute to the long-term viral reservoir, showing an estimated half-life of 9.2 years and higher susceptibility to homeostatic proliferation [40]. We observed that treatment with dasatinib in vivo modified the distribution of T-cell subpopulations in the humanized mice, favoring the population of
naïve CD4⁺ and CD8⁺ T cells over the differentiation to memory or effector T cells. This could imply that dasatinib was interfering with the generation of CD4⁺ T_CM subpopulation, which is the most important component of the viral reservoir. As Lck is essential for T-cell development, differentiation and activation [41], and dasatinib inhibits Src-TKs such as Lck [35], it could be expected that these modifications in the pattern of T-cell subpopulations may also be observed in patients with CML on treatment with dasatinib. However, to our knowledge, this study has not yet been performed.

Due to viral control is achieved at an early stage of HIV-1 infection mainly by CD8⁺ T-cells [42, 43], the influence of dasatinib on effector CD8⁺ T-cell populations might prove counterproductive. Development from naïve to T_CM cells is quite different in CD4⁺ and CD8⁺ T cells, and it has been described that CD4⁺ T cells are more sensitive to the inhibitory effect of dasatinib in vitro [44]. However, 94% of total CD8⁺ T cells were naïve in mice treated with dasatinib, as compared to 78% naïve CD4⁺ T cells. Because dasatinib-treated mice showed lower viral replication and smaller reservoir size than placebo-treated mice, even with a lower proportion of CD8⁺ T_EM and T_EMRA cells, this suggested that low CD8 differentiation could also be consequence of low viral replication. Intriguingly, although mice from dasatinib-treated group showed plasma viremia and proviral HIV DNA under the limit of detection (#2 and #5), all mice from this group showed the same percentage of CD4⁺ and CD8⁺ T-cell subpopulations, even with percentages of effector CD8⁺ T cells under the average in placebo-treated mice. This may indicate that the antiviral effect of dasatinib was not dependent on a higher number of cytotoxic cells but likely more related to the protection of CD4⁺ T cells from acute infection and to a low viral reservoir of memory cells. In fact, it has been described that patients able to maintain undetectable levels of HIV-1 replication in the absence of cART also have very low levels of infected CD4⁺ T_CM cells [45], which is unusual in most patients on cART [46].
Our study presents several limitations. First, we used the intraperitoneal route of infection; it would be important to test if the protection could be higher with a different route of infection such as intravaginal. Second, in this study we analyzed the ability of dasatinib to interfere with the reservoir formation as a preventive drug but we should determine if dasatinib will also exert its antiviral effect in individuals already infected with HIV-1 and on treatment with cART. Finally, it has been described that dasatinib may have the ability to enhance the cytotoxic activity of Natural Killer (NK) cells [47] but NK cell expansion is very poor in NSG model. Therefore, other animals expressing human NK cells such as Hu-Rag2<sup>−/−</sup>;γ<sup>−/−</sup> mice should be used to test this potential anti-HIV-1 effect of dasatinib.

In conclusion, we provided the proof of concept that dasatinib is a valid option to safely treat HIV-1 infection in order to interfere with the reservoir formation and replenishment, thereby supporting the initiation of a pilot clinical trial with HIV-infected patients.
Author contributions

M.C. and J.A conceived the study. M.C., M.S., C.G., B.R.S., E.M performed the experiments, data acquisition and curation. M.C., M.S., S.R.M., V.U analyzed data and applied the statistical analysis. M.C., M.S., J.A., and J.M.P. wrote the manuscript. All authors revised and approved the final manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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References


Figure legends

**Fig. 1.** Experimental design. NSG mice were divided into two groups treated daily with placebo (group 1, n=5) or dasatinib (group 2, n=5) from 5 days before HIV-1 intraperitoneal injection. All mice were then treated daily with either placebo (group 1) or dasatinib (group 2) for 21 days. Blood samples were taken every 7 days and GALT samples were taken at 21st day, post-mortem.

**Fig. 2.** Analysis of viral and proviral load in blood and tissue. (a) Viral load was analyzed in plasma of NSG mice in both groups treated with placebo and dasatinib 7, 15 and 21 days after HIV-1 infection. Total HIV-1 DNA was analyzed in blood cells (b) and integrated HIV-1 DNA was analyzed in GALT (c). Each symbol represents data of an individual mouse. Open symbols indicate undetectable values. Bars represent the median with interquartile range; non-parametric test, p<0.05 (*).

**Fig. 3.** Quantification of T cell populations. (a) Blood samples of each NSG mice from both placebo- and dasatinib-treated groups were analyzed by flow cytometry after staining with specific antibodies to quantify the different CD4+ and CD8+ T-cell subpopulations. Each symbol represents data of an individual mouse. Error bars represent the median with interquartile range; non-parametric test, p<0.05 (*). (b) Representation of previous data from CD4+ and CD8+ T-cell subpopulations in pie plots showing the percentage of each subpopulation.