Virology 510 (2017) 9–21

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The histone deacetylase inhibitor SAHA simultaneously reactivates HIV-1 from latency and up-regulates NKG2D ligands sensitizing for natural killer cell cytotoxicity

Maria Giovanna Desimio, Erica Giuliani, Margherita Doria*

Laboratory of Immunoinfectivology, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

ARTICLE INFO

Keywords:
HIV-1
Viral latency
LRA
HDACi
SAHA
NK cell
NKG2D
MICA/B
ULBP2

ABSTRACT

In pilot HIV-1 eradication studies, patients' immune responses were ineffective at killing viral reservoirs reactivated through latency reversing agents (LRAs) like suberoylanilide hydroxamic acid (SAHA). We hypothesized that T cells harboring reactivated HIV-1 express MIC and ULBP ligands for the activating NKG2D receptor of natural killer (NK) cells. Here, we demonstrated that MICA/B and ULBP2 are induced by SAHA on primary T cells harboring reactivated virus. Using latently HIV-1-infected J-Lat 6.3/8.4/9.2 and J1.1 cell lines, we showed that SAHA reverts latency and, simultaneously, up-regulates MICA/B and ULBP2 acting at the transcriptional level and through ATR activation, thus sensitizing T cells with reactivated virus to NKG2D-mediated killing by NK cells. Moreover, IL-2 and IL-15 potently boosted NKG2D expression and cytotoxicity of NK cells against SAHA-reactivated $p24^+$ target cells. Therefore, immunotherapy with cytokines enhancing NKG2D-mediated NK-cell cytotoxicity combined with administration of LRAs up-modulating NKG2D ligands, represents a promising approach towards HIV-1 eradication.

1. Introduction

The HIV-1 pandemic still represents a major challenge for public health and biomedical research. An efficacious antiretroviral therapy (ART) has been developed, which effectively suppresses HIV-1 replication and greatly decreases the morbidity of HIV-1-infected patients. However, HIV-1 is not eradicated by ART and persists in a replication-competent form in reservoirs, mainly latently infected resting $CD4^+$ T cells, where is rapidly reactivated upon therapy interruption. At present, HIV-1-infected patients must be chronically treated with ART, which causes resistance and long-term toxicity problems, and are exposed to a higher risk of developing immune-related pathologies, thus a cure for HIV-1 is clearly a priority.

One of the most promising approaches to finding a cure for HIV-1 infection is elimination of viral reservoirs via the so-called 'shock-and-kill' strategy (Deeks, 2012). The 'shock' consists in using latency reversing agents (LRAs) that reactivate latent reservoirs by inducing transcription of quiescent provirus, then followed by the 'killing' of reactivated infected cells by the host immune system and by viral cytopathic effects (CPEs), while exposing *de novo* produced virus to the effect of ART. The histone deacetylase inhibitors (HDACis), such as suberoylanilide hydroxamic acid (SAHA or Vorinostat), panobinostat, and romidepsin, are among the most prominent LRA candidates. Indeed, consistent with the role histone deacetylases play in repressing transcription, HDACis were shown to disrupt HIV-1 latency in both cell lines and primary T cells, in resting $CD4^+$ T cells derived from patients, and, more importantly, *in vivo* (Spivak and Planelles, 2016; Wightman et al., 2012). However, SAHA-induced HIV-1 reactivation in an *in vitro* model did not result in the death of infected cells by CPE or by killing via patient's $CD8^+$ T lymphocytes, unless these latter cells were previously stimulated (Shan et al., 2012). Moreover, in pilot clinical trials the individual administration of SAHA, panobinostat, and romidepsin resulted in increased cell-associated HIV-1 RNA but failed to decrease viremia and reservoir size (Archin et al., 2012, 2014; Elliott et al., 2014; Rasmussen et al., 2014; Søgaard et al., 2015). These results suggests that combining pharmacological reactivation of latent HIV-1 with therapies that stimulate the antiviral immune response may be essential for viral eradication.

Interestingly, all HDACis with reported LRA properties, including SAHA, valproic acid, trichostatin A, sodium butyrate, romidepsin, and panobinostat, can induce the expression of NKG2DLs, and thus have been extensively used in tumor immunology studies and clinical trials for non HIV-1-related diseases (Chretien et al., 2014). NKG2D is expressed by all natural killer (NK) cells and $CD8^+$ T cells, as well as by

* Correspondence to: Bambino Gesù Children's Hospital, Piazza S. Onofrio 4, 00165 Rome, Italy.
E-mail address: doria@uniroma2.it (M. Doria).

http://dx.doi.org/10.1016/j.virol.2017.06.033
Received 8 February 2017; Received in revised form 14 June 2017; Accepted 29 June 2017
Available online 06 July 2017
0042-6822/

subsets of γδ T cells and NKT cells, and interacts with MHC-class-I-related sequence A (MICA) and B (MICB) proteins and six different cytomegalovirus UL16-binding proteins (ULBP1-6), cell-surface molecules normally absent on healthy cells but up-regulated on virus-infected or transformed cells (Raulet et al., 2013). Ligand engagement by NKG2D delivers a potent activating signal to NK cells and a co-stimulus to $CD8^+$ T cells resulting in cytotoxicity and release of cytokines, thus functioning as a key activating pathway for immune-mediated elimination of infected cells and tumors (Barber and Sentman, 2011; Bryceson et al., 2006; Groh et al., 2001; Maasho et al., 2005). In addition, in non-pathologic conditions, activated T cells transiently express NKG2DLs and are exposed to NKG2D-mediated clearance by NK cells that is relevant to restrict immune responses and maintain T cell homeostasis (Zingoni et al., 2013). Work performed by various groups including our own, has demonstrated that the NKG2D/NKG2DLs pathway plays an important role in anti-HIV-1 cytotoxic responses. In fact, upon productive HIV-1 infection of activated $CD4^+$ T lymphocytes, expression of *NKG2DL* genes (*i.e. MICA*, *MICB*, and *ULBP1-2*) is induced via activation of the DNA damage response (DDR) pathway due to viral DNA integration and to the activity of the viral Vpr protein, resulting in cell-surface ligand up-regulation (Cerboni et al., 2007a; Matusali et al., 2013; Richard et al., 2013; Ward et al., 2007, 2009). On the other hand, HIV-1 has evolved the capacity to reduce the NKG2DL levels via the activity of the viral proteins Nef, that impairs ligand cell-surface expression, and Vif, that limits activation of the DDR pathway, thus reducing the NKG2D-mediated killing of infected T cells by NK cells (Cerboni et al., 2007a; Norman et al., 2011). Despite virus countermeasures, *ex-vivo* expanded HIV-1-infected $CD4^+$ T cells derived from $HIV\text{-}1^+$ patients up-regulate NKG2DLs, especially ULBP2, and are killed by autologous NK cells mainly through NKG2D triggering (Fogli et al., 2008). Based on this evidence, we thought to investigate the expression of NKG2DLs in the context of latent HIV-1 reactivation. Here we show that SAHA, one of the best characterized HDACi with LRA property, simultaneously reactivates latent HIV-1 and up-regulates cell-surface MICA, MICB, and ULBP2 hence exposing T cells that exit latent infection to NK-cell lysis through NKG2D-mediated recognition. These results have implications for an adjuvant NK cell-based immunotherapy to achieve the ultimate goal of a cure for HIV-1.

2. Materials and methods

2.1. Cells, antibodies, and reagents

Jurkat E6-1, J-Lat full length clones 6.3, 8.4, and 9.2, J-Lat GFP clone A72, and J1.1 cells (all from NIH AIDS Reagent Program) were maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.2 mM L-glutamine, and 100 units/ml penicillin-streptomycin (all from Euroclone). PBMCs were obtained by Ficoll separation of buffy coats from a donor bank. Primary NK cells were isolated from PBMCs by negative selection with Dynabeads Untouched Human NK Cells Kit (Invitrogen-Life Technologies) and stimulated with 500 IU/ml of IL-2, 50 ng/ml of IL-7, or 10 ng/ml of IL-15 (all from Peprotech) for 18 h before cytotoxicity assays. Primary $CD4^+$ T cells were isolated from PBMCs by negative selection with the EasySep $CD4^+$ T-cell Enrichment Kit (Stem Cell Technologies) according to manufacturer's protocol. (Sigma-Aldrich) for 18 h before cytotoxicity assays.

The purity (~95%) of isolated NK ($CD3^-CD56^+CD16^{-/+}$) and $CD4^+$ T cells ($CD3^+CD4^+$) was assessed by immunolabeling with specific BD Biosciences antibodies and FACS analysis.

For flow cytometric analysis, isotype control IgG_1 and IgG_{2a} (EuroBioScience) and the following mouse monoclonal antibodies (MAbs) were used: anti-MICA (AMO1; BamOmaB); anti-MICA/B (MAB13001), anti-MICB (MAB1599), anti-ULBP1 (MAB1380), and anti-ULBP2/5/6 (MAB1298) from R & D Systems; anti-phospho S139-H2AX (JBW301; Upstate Biotechnology); anti-p24-fluorescein isothiocyanate (FITC) (KC57; Beckman Coulter); anti-NKG2D-phycoerythrin (PE) (1D11, eBioscience). As a secondary antibody, Alexa647-coniugated goat anti-mouse IgG (GAM) (Invitrogen) was used. The antibodies employed in Western blotting were as follows: anti-HIV-1 human serum derived from an HIV^+ patient, anti-Nef polyclonal sheep serum (ARP444; kind gift of Mark Harris, University of Leeds, Leeds, UK), MAbs against MICA (AB150355; Abcam), MICB (MAB1599; R & D Systems), ULBP2 (AF1298; R & D Systems), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (MAB374; Millipore). As secondary reagents, horseradish peroxidase-conjugated GAM (Cell Signaling) and protein G (Bio-Rad) were used.

The anti-NKG2D (149810; R & D Systems) MAb or isotype control IgG_1 were used in cytotoxicity assays.

Where indicated, cells were treated with 10 μM KU55933 (Selleckchem), 5 mM Caffeine (Sigma-Aldrich), 10 ng/ml tumor necrosis factor alpha (TNF-α) (R & D Systems), 2.5 μM 5-aza-2'deoxycytidine (AZA-CdR), 10 μM suberoylanilide hydroxamic acid (SAHA), 20 nM Romidepsin (ROM), 3 mM Sodium Butyrate (NaBut), or with equivalent amounts of dimethyl sulfoxide (DMSO) when used as a solvent (all from Sigma-Aldrich). Other reagents used were: phorbol-12-myristate-13-acetate (PMA), Ionomycin (Iono), and 7-aminoactinomycin D (7-AAD) from Sigma-Aldrich); HIV-1 protease inhibitor Atazanavir (ATV; Reyataz, Bristol-Myers Squibb) and HIV-1 integrase inhibitor Raltegravir (RAL; NIH AIDS Reagent Program).

2.2. Flow cytometry

The following procedures were performed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3 (staining buffer, SB). To label cell-surface molecules, cells were incubated for 20 min at 4 °C first with specific MAbs then, after a wash, with GAM-Alexa647. For simultaneous detection of intracellular p24, cells were fixed and permeabilized with BD Biosciences reagents, then incubated at room temperature for 30 min with anti-p24-FITC MAb. The latter procedure was also employed to label NKG2D ligands intracellularly. For γ-H2AX staining, cells were fixed in 1% paraformaldehyde (PFA) for 20 min at 4 °C and then in 70% ethanol at −20 °C for 1 h, permeabilized with 0.2% Triton X-100, stained with JBW301 MAb and then with Alexa647-GAM. All immunolabeled cells were finally washed, resuspended in 1% PFA, and acquired on a FACSCanto II (BD Biosciences) collecting at least 2×10^5 cells in the life gate population. Data analyses were performed using FlowJo software. The geometric mean fluorescence intensity (MFI) value obtained with isotype control staining was subtracted to the ligand-specific MFI value. The extent of ligand up-regulation (*n*-fold) was calculated by dividing the MFI obtained for each cell condition by the MFI of control DMSO-treated cells.

2.3. Establishment and reactivation of latently infected $CD4^+$ T cells

Primary $CD4^+$ T cell cultures latently infected with HIV-1 were established and then reactivated with PMA (100 ng/ml) and ionomycin (0.5 mg/ml) or SAHA (335 nM) as previously described (Lassen et al., 2012) with the following modifications: spinoculation of freshly isolated $CD4^+$ T lymphocytes was performed with 300 ng $p24/10^6$ cells of NL4-3 HIV-1, either wild-type (NIH AIDS Reagent Program) or unable to express Nef (Nef^-, kindly provided by F. Kirchhoff) pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G), then cells were cultivated in the presence of 20 μM ATV for 3 days before stimulation with PMA/Iono or SAHA and addition of 30 μM RAL, and finally analyzed by FACS for intracellular p24 accumulation and cell-surface NKG2DLs expression at 72 h post-stimulation.