Technical Advance: Measurement of iNKT cell responses at the single-cell level against rare HIV-1-infected dendritic cells in a mixed culture

Sofia K. Andersson, Dominic Paquin-Proulx, Mirko Kroll, Johan K. Sandberg, and Markus Moll

Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden

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ABSTRACT

iNKT cells recognize lipid antigens, such as α-GalCer, presented in complex with CD1d expressed by DCs. Exposure of DCs to HIV-1 can lead to productive infection, and it was demonstrated recently that HIV-1 inhibits CD1d surface expression in an apparent mode of immune evasion. However, studies of the interaction between T cells, including iNKT cells and HIV-infected DCs in vitro, are hampered by the low frequency of productive infection in DCs. Here, we demonstrate the utility of full-length HIV-1 modified to express eGFP to address this problem. This virus allowed identification of single, rare productively infected cells in a mixed DC population by fluorescence microscopy and enabled detailed studies of the interaction of such cells with individual iNKT cells. iNKT cell responses to α-GalCer presented by HIV-1-positive and -negative DCs were quantified by intracellular IFN-γ staining in iNKT cells forming conjugates with DCs. Whereas complex formation was observed between iNKT cells and uninfected and infected DCs, only iNKT cells in contact with uninfected DCs produced IFN-γ. This microscopy assay, based on full-length HIV-1 modified to express eGFP, thus allows detailed evaluation of HIV-1 immune-evasion mechanisms in rare virus-infected live DCs.

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Introduction

iNKT cells represent a unique population of innate-like lymphocytes combining structural and functional features of NK cells and T cells, including expression of NK cell markers together with an invariant TCR (Vα24-Jα18/Vß11 in humans), rapid activation without priming phase, and quick production of a vast array of cytokines and chemokines [1, 2]. iNKT cells recognize glycolipid antigens presented by the nonclassical MHC molecule CD1d. In humans, CD1d is expressed mainly on professional APCs, such as subsets of DCs, macrophages, and B cells [3, 4], but expression of CD1d has also been reported in epithelial cells of the gastrointestinal tract and the urogenital system in males and females [5, 6], and even T cells may, under certain activation conditions, express CD1d [7]. CD1d surveys endosomal and lysosomal compartments of the cell and presents endogenous and exogenous glycolipid antigens [8]. Importantly, recognition of both types of antigen can lead to iNKT cell activation but has different requirements. Whereas TCR recognition of exogenous glycolipid antigens, e.g., derived from Gram-negative or -positive bacteria or the widely used model antigen α-GalCer, provides a sufficiently strong signal for activation of iNKT cells [9], activation by endogenous glycolipid antigens requires, in addition, an inflammatory cytokine milieu [10–12]. Recently, the endogenous lipid β-D-glucopyranosylceramide has been shown to accumulate in APCs in response to danger signals, such as infection and TLR agonists, and to activate iNKT cells in a CD1d- and cytokine-dependent manner [13]. This activation pathway may be important for pathogens lacking glycolipid structures available for CD1d presentation, including viruses.

The relevance of iNKT cell activation in antiviral immunity is supported by the fact that several viruses have evolved strategies to evade from iNKT cell responses. These strategies comprise depletion and functional impairment of iNKT cells and interference with CD1d expression in APCs. In HIV-1-infected individuals, the iNKT cell compartment in peripheral blood is...
severely depleted [14–16], and even if some patients suffer from only a minimal depletion, their iNKT cells display an exhausted phenotype characterized by elevated programmed cell death protein 1 expression and reduced responsiveness to α-GalCer stimulation [17–19]. Interestingly, similar observations have been made in macaques infected with simian immunodeficiency virus, the precursor of HIV-1 [20]. In addition, HIV-1 uses two of its gene products—the accessory proteins Nef and Vpu—to inhibit the surface expression of CD1d [21–23]. In uninfected cells, CD1d recycles between the cell surface and endosomal compartments to survey the endocytic system and present glycolipid antigens to iNKT cells [8]. This trafficking pattern is blocked in the presence of Nef and Vpu. Whereas Nef interferes with the anterograde transport of CD1d and increases its internalization from the cell surface [21], Vpu decreases the recycling rate of CD1d and retains the protein in early endosomal compartments [23]. Importantly, CD1d down-regulation in HIV-1-infected DCs results in reduced iNKT cell activation and IFN-γ production after α-GalCer stimulation in vitro, indicating the physiological relevance of this immune evasion strategy [23].

DCs are not only important players in iNKT cell activation but also relevant in HIV-1 infection. At the mucosal surface, DCs are among the first cells to encounter HIV-1, and they probably play a crucial role in the establishment of infection. DCs may take up virus or get productively infected and subsequently transport the virus to draining LNs, where it gets access to its main targets, CD4 T cells [24]. It can be speculated that in this initial phase of infection, down-regulation of CD1d from the surface of HIV-1-infected DCs is, in particular, important to avoid recognition by iNKT cells and elimination of infected DCs. Considering the central role of DCs in the activation process of iNKT cells and establishment of HIV-1 infection, it is of high importance to study the interaction of HIV-1-infected DCs and iNKT cells to gain further insight into the relevance both of iNKT cells in HIV-1 infection and strategies to evade iNKT cell responses. However, detailed in vitro studies of these aspects are impeded considerably by the low frequency of productive HIV-1 infection in DCs. Particularly, the consequences of DC HIV-1 infection on iNKT cell activation are hard to assess by standard high-throughput technologies, such as flow cytometry, as the majority of DCs in a mixed culture is HIV-1-exposed but uninfected and displays a healthy phenotype, including normal CD1d expression levels. Therefore, we describe here the establishment of a microcopy-based assay that allows the monitoring of individual iNKT cells in contact with single, rare productively HIV-1-infected DCs in a mixed population.

**MATERIALS AND METHODS**

**Reporter virus construction**

Construction of proviral HIV-1 81A-eGFP was described before [23]. Briefly, an expression cassette consisting of the *egfp* gene, followed by an IRES, was inserted between the *env* and *nef* genes of molecular clone HIV-1 81A using rPCR technology (Fig. 1A). The expression cassette was amplified from the plasmid pEgFP-IRES2-nef-AΔXol [23]. HIV-1 strain 81A is a CCR5-tropic derivative of the CXCR4-tropic virus NL4-3 (group M subtype B) carrying the HIV-1 BaL V3 region [26].

**Cell culture and production of virus stocks**

293T cells were cultured in medium [RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 2 mM L-glutamine and 1% penicillin and streptomycin], containing 10% heat-inactivated FCS. To obtain stocks of 81A and 81A-eGFP viruses, 293T cells were transfected with proviral DNA using Turbofect (MBI Fermentas, Vilnius, Lithuania), according to the manufacturer’s protocol. Forty-eight hours after transfection, cell culture supernatants were harvested and debris removed by centrifugation and filtration. Virus stocks were frozen and stored at −80°C.

**Flow cytometry**

Cell surface and intracellular flow cytometry were performed as described [23]. Antibodies anti-HIV-1 p24-FITC and -PE (Clone KC57), anti-Va24-FITC (Clone C15), and anti-Vβ11-PE (Clone C21) were from Beckman Coulter (Fullerton, CA, USA). Anti-IFN-γ-phycoerythrin (Clone B27), anti-CD3-APC (Clone UCHT1), anti-CD1d-PE (Clone CD1d42), and anti-CD11c-allophycocyanin (Clone B4Ly6) were all from BD Biosciences (San Francisco, CA).
Jose, CA, USA). Data were acquired on a BD LSRFortessa instrument (BD Biosciences) and analyzed using FlowJo Version 9.4.10 software (TreeStar, Ashland, OR, USA).

Characterization of viral constructs

293T cells were transfected with proviral DNAs 81A or 81A-eGFP or mock-transfected, respectively. Twenty-four hours after transfection, cell lysates were prepared and samples run on a 12% polyacrylamide gel, transferred onto a nitrocellulose membrane. Membranes were probed with pooled anti-HIV-1 sera from five patients and HRP-conjugated anti-human IgG (GE Healthcare, Buckinghamshire, UK). After stripping, membranes were probed with anti-β-actin mAb (MP Biomedicals, Solon, OH, USA) and HRP-conjugated anti-mouse IgG (GE Healthcare). For microscopy analysis, 293T cells were fixed with 4% paraformaldehyde, cytopsion onto glass microscope slides, and analyzed for expression of HIV-1 p24 and eGFP. Images were obtained on a Nikon A1R confocal system with a 60×/1.49 oil objective using NIS-Elements AR software (Version 3.2; Nikon Instruments Europe B.V., Amstelveen, The Netherlands).

HIV-1 infection of DCs

DCs were generated from human monocytes and infected as described [23]. Briefly, buffy coats were obtained from healthy blood donors as approved by the Ethics Committee at Karolinska Institutet. To obtain immature DCs, monocytes were enriched from PBMCs using RosetteSep human monocyte enrichment cocktail (StemCell Technologies, Vancouver, BC, Canada) and cultured for 6 days in medium supplemented with 5% heat-inactivated human serum (Cambrex Bio Science, Verviers, Belgium), 6.5 ng/mL human rIL-4 (R&D Systems, Minneapolis, MN, USA), and 250 ng/mL human rGM-CSF (PeproTech, Rocky Hill, NJ, USA). DCs were infected with viral stocks or mock-infected in the presence of cytokines and serum. After 4–7 days of culture, cells were analyzed for expression of HIV-1 p24, eGFP, and CD1d by flow cytometry or microscopy.

iNKT cell activation assays

CD1d-restricted iNKT cell lines were established as described [27]. Briefly, iNKT cells were expanded from PBMCs by culture in medium supplemented with 10% heat-inactivated FCS, 10 ng/mL IL-2 (PeproTech), and 100 ng/mL α-GalCer (KRN7000; Enzo Life Sciences, Farmingdale, NY, USA). After 10–14 days, iNKT cells were isolated based on their uniform expression of the TCR V24 chain. Isolated cells were stimulated with irradiated α-GalCer-pulsed monocytes in the presence of IL-2 and then restimulated every 2nd week. The purity of iNKT cell lines was analyzed by flow cytometry using Vα24 and Vβ11 antibodies.

Activation of iNKT cells after coculture with DCs from 81A, 81A-eGFP, or mock-infected cultures was assessed by flow cytometry and microscopy. For the flow cytometry assay, DCs were preincubated with different concentrations of α-GalCer (0.1-100 ng/mL) overnight at 37°C before adding iNKT cells at a ratio of 1:2. After 6 h of coculture in the presence of brefeldin A (2 mg/mL; GolgiPlug; BD Biosciences), cells were surface-stained with anti-CD3 and anti-CD11c mAb to discriminate between DCs and iNKT cells. Following permeabilization, cells were stained with anti-IFN-γ mAb and analyzed on a BD LSRFortessa instrument. To assess iNKT cell activation by microscopy, iNKT cells were cocultured with DCs preloaded with α-GalCer (100 ng/mL) at a 1:2 ratio in the presence of brefeldin A. Minimal coinoculation times for complex formation and cytokine detection were 30 min for TNF-α and 2 h for IFN-γ. After fixation with 4% paraformaldehyde, cell complexes were cytopsion onto glass microscope slides using a Cytospin 4 cytoentrifuge (800 rpm, 5 min; Thermo Shandon, Cheshire, UK) and permeabilized with 0.1% saponin (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained with anti-IFN-γ mAb (Clone 25,718; 1:50; R&D Systems) or anti-TNF-α mAb (Clone 28,401; 1:50; R&D Systems), blocked with 1% normal goat serum (Dako, Glostrup, Denmark), and finally, incubated with anti-mouse Alexa-594 secondary antibody (1:500; Molecular Probes, Eugene, OR, USA) and DAPI (Sigma-Aldrich). All incubations with primary and secondary antibodies and serum were at room temperature for 30 min. Images were obtained on a Nikon A1R confocal system with a 60×/1.49 oil objective using NIS-Elements AR software (Version 3.2). IFN-γ production by iNKT cells was analyzed by visual inspection of randomly taken images of DC-iNKT cell complexes. To avoid operator bias, image acquisition and analysis were performed independently by different investigators.

RESULTS AND DISCUSSION

Reporter virus HIV-1 81A-eGFP productively infects DCs

To identify productively, HIV-1-infected cells in a mixed culture, easily and without need of further manipulation, we constructed the CCR5-tropic reporter virus HIV-1 81A-eGFP [23]. The 81A-eGFP proviral construct is based on the molecular clone HIV-1 81A and contains an expression cassette consisting of the egfp gene, followed by an IRES between the env and nef genes (Fig. 1A). Microscopic analysis of 293T cells transfected with the 81A-eGFP-encoding plasmid showed coexpression of the viral capsid protein p24 and eGFP, demonstrating functionality of the inserted expression cassette (Fig. 1B). To ascertain fully functional viral gene expression after insertion of the expression cassette, transfected 293T cells were analyzed by Western blot using human anti-HIV-1 serum. Similar protein expression profiles were detected in cells transfected with 81A-eGFP and the parental construct 81A, indicating full functionality of the engineered viral genome (Fig. 1C). Next, virus stocks were produced in 293T cells and used for DC infection experiments. Productively infected DCs coexpressing p24 and eGFP could first be detected at Day 4 postinfection, and the infection peaks at approximately Day 6 (Fig. 2A). The rate of productive infection in monocyte-derived DCs varied greatly between donors and rarely exceeded 10% in our hands (data not shown). As expected, virus infection and eGFP expression in DCs were monitored easily by microscopy without further manipulation of the cells (Fig. 2B). Insertion of the eGFP-IRES cassette directly upstream of the nef gene implicates IRES-dependent translation of the viral Nef protein. This mode of translation did not significantly impair expression of the Nef protein in 81A-eGFP-infected DCs, as indicated by the efficient down-regulation of HLA-A2, which is Nef-dependent (data not shown) [28]. In summary, the reporter virus HIV-1 81A-eGFP infects DCs and gives rise to the expression of eGFP without interfering with normal viral gene expression and therefore, represents a tool to study the interaction of HIV-1-infected DCs with other types of immune cells without further need of methodology to visualize productive virus infection.

Low frequency of HIV-1 infection hampers detection of differences in IFN-γ production in iNKT cells cocultured with uninfected and infected DC cultures by flow cytometry

DCs are important APCs initiating T cell responses. Moreover, DCs are relevant in HIV-1 infection, as they are among the first cells in contact with the virus at mucosal sites and transporting the virus to draining LNs, where it is transmitted to T
In vitro infection of DC cultures with HIV-1 generally results in low frequencies of productively infected cells (Fig. 2A and B). Clearly, the virus interferes with a number of functional features of the single infected DC. The functional consequences of this may, however, not be experimentally accessible when mixed DC cultures with high numbers of virus-exposed but uninfected cells are assessed using assays with low resolution, unable to zoom in on the specific interaction between an infected DC and a target cell of interest. To illustrate the problem, we cocultured iNKT cells with α-GalCer-pulsed DC cultures infected with 81A, 81A-eGFP, or mock, respectively, and assessed iNKT cell IFN-γ production by intracellular cytokine staining and flow cytometry (Fig. 3). We have shown recently that HIV-1 down-regulates CD1d in productively infected DCs, and it is expected that CD1d down-regulation translates into impaired activation of CD1d-restricted iNKT cells [23]. Stimulation with 81A-, 81A-eGFP-, and mock-infected DC cultures resulted in similar frequencies of IFN-γ-positive iNKT cells over the whole range of tested α-GalCer concentrations (Fig. 3). Although CD1d was down-regulated in the rare virus-positive DC population, this result is not surprising, as the majority of uninfected DCs in the cultures (81.1–98.9%) showed normal CD1d expression levels (data not shown) and was therefore able to activate iNKT cells efficiently.

**Microscopic assessment of cytokine production in iNKT cells cocultured with DCs from HIV-1 81A-eGFP-infected cultures**

Possible solutions to the problem caused by high numbers of uninfected DCs include enrichment of the rare HIV-1-positive DC population and alternative methods to assess iNKT cell responses. Enrichment of live HIV-1-positive DCs using magnetic-bead sorting relies on cell surface markers and antibodies allowing discrimination between virus-positive and -negative cells. We used magnetic-bead sorting based on the surface expression of the viral glycoprotein gp120 (positive selection) as well as virus-induced down-regulation of CD4 or HLA-A2 (negative selection), but neither positive- nor negative-selection approaches resulted in sufficiently pure populations of HIV-1-positive DCs (data not shown). Moreover, such selection procedures have the general disadvantage of surface molecule cross-linking and possible subsequent alterations in the respective cell populations. Reporter viruses, such as 81A-eGFP, open the possibility for flow cytometric cell sorting based on the intracellular expression of reporter genes. This would, however,
require equipment in an environment suitable for handling of live, HIV-1-infected material, and such arrangements are not readily available in most laboratories interested in this type of study.

An alternative way to circumvent the background problem is to study specifically the interaction of an infected DC with one or more iNKT cells by microscopy. To establish such an assay, we first studied the conditions for complex formation between DCs and iNKT cells and subsequent iNKT cell IFN-γ production in the absence of infection. Co-culture for 30 min, PFA fixation, and cytopsin onto glass microscopy slides resulted in the formation and preservation of DC-iNKT cell complexes, as detected by DAPI staining and microscopy (Fig. 4A). To study IFN-γ production, iNKT cells were co-cultured with α-GalCer-pulsed DCs in the presence of brefeldin A for different times. After fixation and cytopsin, cells were permeabilized with saponin and intracellularly stained for IFN-γ. As expected, iNKT cell cytokine production, but not iNKT cell–DC complex formation, required loading of DCs with lipid antigen (Fig. 4B, upper panel). Robust complex formation and IFN-γ production coincided and were detectable already after 2 h of coculture (Fig. 4B, lower panel). Longer periods of coculture did not result in a more pronounced complex formation or higher degree of IFN-γ production. Therefore, we decided to use a coincubation time of 2 h for all experiments with IFN-γ production as readout.

Finally, iNKT cells were cocultured with DC cultures infected with the reporter virus 81A-eGFP for 6 days, and IFN-γ production was assessed as described above. Interestingly, robust complex formation was observed between iNKT cells and uninfected and infected DCs, suggesting that mechanisms of cell adhesion may not be affected significantly by HIV-1 infection. Moreover, as CD1d is known to be down-regulated in HIV-1 infected cells [21–23], these data may indicate that CD1d–TCR interaction may not be absolutely required for efficient complex formation. However, further investigations using live-cell imaging technology will be required to determine if the average contact time between iNKT cells and DCs is affected by HIV-1 infection and CD1d down-regulation. In contrast to complex formation, IFN-γ production was largely con-
staining with anti-TNF-α, circles; IFN-γ, eGFP, green; DAPI, blue; DCs, white. (A) Schematic representation of the assay. (B) α-GalCer-loaded DCs from a DC culture infected with HIV-1 81A-eGFP for 6 days were cocultured with iNKT cells for 2 h in the presence of brefeldin A. Following cytopsining, cells were fixed, permeabilized, and stained with anti-IFN-γ mAb and DAPI. IFN-γ, red; eGFP, green; DAPI, blue; DCs, white circles; IFN-γ-positive iNKT cells in DC contact, *; original scale bar, 20 μm. (C) Quantification of iNKT cell IFN-γ production after coculture with HIV-1 81A-eGFP or mock-infected DC cultures. Data represent the average of three independent experiments ± srm; *P < 0.05 (Student’s t test). (D) Staining with anti-TNF-α mAb was performed after coculture of iNKT cells with α-GalCer-loaded DCs from an infected culture for 30 min. TNF-α, red; eGFP, green; DAPI, blue; original scale bar, 20 μm.

Figure 5. Microscopic assessment of cytokine production in iNKT cells cocultured with DCs from HIV-1 81A-eGFP-infected cultures. (A) Schematic representation of the assay. (B) α-GalCer-loaded DCs from a DC culture infected with HIV-1 81A-eGFP for 6 days were cocultured with iNKT cells for 2 h in the presence of brefeldin A. Following cytopsining, cells were fixed, permeabilized, and stained with anti-IFN-γ mAb and DAPI. IFN-γ, red; eGFP, green; DAPI, blue; DCs, white circles; IFN-γ-positive iNKT cells in DC contact, *; original scale bar, 20 μm. (C) Quantification of iNKT cell IFN-γ production after coculture with HIV-1 81A-eGFP or mock-infected DC cultures. Data represent the average of three independent experiments ± srm; *P < 0.05 (Student’s t test). (D) Staining with anti-TNF-α mAb was performed after coculture of iNKT cells with α-GalCer-loaded DCs from an infected culture for 30 min. TNF-α, red; eGFP, green; DAPI, blue; original scale bar, 20 μm.

Staining with anti-TNF-α, circles; IFN-γ, eGFP, green; DAPI, blue; DCs, white. (A) Schematic representation of the assay. (B) α-GalCer-loaded DCs from a DC culture infected with HIV-1 81A-eGFP for 6 days were cocultured with iNKT cells for 2 h in the presence of brefeldin A. Following cytopsining, cells were fixed, permeabilized, and stained with anti-IFN-γ mAb and DAPI. IFN-γ, red; eGFP, green; DAPI, blue; DCs, white circles; IFN-γ-positive iNKT cells in DC contact, *; original scale bar, 20 μm. (C) Quantification of iNKT cell IFN-γ production after coculture with HIV-1 81A-eGFP or mock-infected DC cultures. Data represent the average of three independent experiments ± srm; *P < 0.05 (Student’s t test). (D) Staining with anti-TNF-α mAb was performed after coculture of iNKT cells with α-GalCer-loaded DCs from an infected culture for 30 min. TNF-α, red; eGFP, green; DAPI, blue; original scale bar, 20 μm.

Single, rare, productively infected DCs and individual iNKT cells and was clearly superior to a flow cytometry approach that suffered from background problems caused by high frequencies of uninfected DCs in the cultures. Whereas no effect of HIV-1 infection and CD1d down-regulation on iNKT cell IFN-γ production was detectable when using bulk DC cultures for stimulation and flow cytometry, microscopy revealed that infected, eGFP-positive DCs were unable to activate iNKT cells efficiently (Figs. 3 vs. 5C). Microscopy has the disadvantage of low throughput and challenges in quantification of results. Computerized analysis tools for standardized quantification would help alleviate these challenges. Importantly, the assay can be easily modified to evaluate other outcomes of the DC–iNKT cell interaction, including early (e.g., synapse formation, vesicle polarization) and late events (e.g., cytokine production, degranulation) of iNKT cell activation. We have studied TNF production and degranulation and found that infected, eGFP-positive DCs are inferior in activating iNKT cells as compared with uninfected DCs in the same culture (Fig. 5D, and data not shown). Moreover, the eGFP-expressing HIV-1 opens up the possibility to compare the interaction of uninfected and infected DCs in the same culture with iNKT cells using live-cell imaging techniques. For example, temporal aspects of the interaction between iNKT cells and uninfected and infected DCs could be investigated after suitable modification of the described assay. It should also be noted that the proviral construct 81A-eGFP can be further manipulated to knock out viral genes of interest, and we have already constructed a mutant lacking expression of the Vpu protein (data not shown). Finally, the described assay is not restricted to the study of DC–iNKT cell interactions, as any type of T cell can be used in the cocultures. Therefore, the methodology described here, including the eGFP-expressing reporter virus, is likely to facili-
tate detailed studies of HIV-1 infection in DCs and many as-
pects of T cell-mediated immunity.

AUTHORSHIP
S.K.A., D.P.P., and M.K. designed research, performed experi-
ments, and analyzed data. J.K.S. and M.M. conceived of and
designed the research and supervised the work. S.K.A. and
M.M. wrote the paper.

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