Research

Corresponding author
Tomasz Rozmyslowicz, MD, PhD
Senior Research Investigator
Department of Pathology and Laboratory Medicine
Perelman School of Medicine
University of Pennsylvania
R. 272 John Morgan Bldg
3620 Hamilton Walk
Philadelphia, PA 19104, USA
Tel. 1-215-898-2874
Fax: 1-215-573-7945
E-mail: rozmyslo@mail.med.upenn.edu

HIV-1 Infection of Human Thymic Stromal Cells and Thymocytes *In Vitro*

Tomasz Rozmyslowicz*, Dareus O. Conover and Glen N. Gaulton

Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

ABSTRACT

We studied *in vitro* Human Immunodeficiency Virus type 1 (HIV-1) infection within human thymic cell cultures as a model to explain the consequences of HIV-1 on the thymic microenvironment. Although HIV-1 inInfection may exert direct thymic cytopathicity, the majority of thymocytes remain uninfected. One hypothesis to explain this effect is that infection of stromal cells, including tissue macrophages, within the thymic microenvironment alters the normal cross-talk between stromal cells and thymocytes thereby disrupting thymocyte maturation. Accordingly, to establish a role for the thymic pathogenicity of HIV-1 we investigated the *in vitro* susceptibility of thymocytes and Thymic Macrophages (TM) to infection with a series of lab adapted HIV-1 that display well defined patterns of tropism: Ba-L(R5), HXB2(X4), and 89.6(R5/X4). We found that thymocytes were most productive in supporting the replication of the R5/X4-tropic virus 89.6 early in culture and displayed more significant replication of the X4-tropic virus HXB2 only after 7-14 days of culture. Replication of the R5 topic virus Ba-L was not detected. In contrast, although HIV-1 replication was delayed overall in cultures of thymic stromal cells enriched for TM, by day 7-21 these cultures supported the replication of both the R5/X4 tropic virus 89.6 and the R5-tropic virus Ba-L, but only transiently HXB2. Thus, while both thymic stromal cells and thymocytes are capable of supporting HIV-1 replication, they display markedly different patterns of susceptibility linked to HIV-1 tropism. Given the exquisite sensitivity of thymocyte development and selection on stromal cell function these results point to new mechanisms for HIV-1 infection in disrupting the maturation of thymocytes.

KEYWORDS: Thymocytes; Thymic macrophages; HIV-1 infection.

ABBREVIATIONS: TM: Thymic Macrophages; DNAase: Deoxyribonuclease; PBS: Phosphate-Buffered Saline; RPMI: Roswell Park Memorial Institute; FBS: Fetal Bovine Serum; BSA: Bovine Serum Albumin; NIH: National Institutes of Health; AIDS: Acquired Immune Deficiency Syndrome; FITC: Fluorescein Isothiocyanate; ELISA: Enzyme Linked Immune Sorbant Assay; OPD: Ortho-Phenylenediamine; HRP: Horseradish Peroxidase; MDM: Monocyte Derived Macrophages; CCR5: C-C chemokine receptor type five; CXCR4: C-X-C chemokine receptor type four.

INTRODUCTION

The thymus functions as the primary site for T-lymphocyte development and selection of histocompatibility recognition linked to antigen presentation.[1,2] Although the dependence on thymic function for maintenance of mature T cells decreases with age, viable thymic tissue persists in adults and may be functionally reactivated following the depletion of peripheral T cells.[3] The development and selection of T cells is tightly regulated by the interaction of thymocytes with the non-lymphoid thymic stroma, which is composed of epithelial cells, dendritic cells, endothelial cells and fibroblasts.[4-6] Thymocyte maturation and selection is a sensitive, multifaceted process that can be disrupted by several infectious agents including HIV-1.[7-9] Although direct infection of thymocytes by HIV-1 may trigger cytopathology, this mechanism alone does not account for the profound loss of thymocytes seen during infection.[10] The capacity of HIV-1

to infect non-lymphoid cells, including thymic stromal cells, has been previously reported.[8-11] However, the magnitude of this infection and consequences on thymic pathology are not clear.[11,12] As is well known, monocytes and macrophages represent one of the most important peripheral targets of HIV-1, yet the infection of TM remains poorly defined.[6,13] In our previous work we investigated HIV-1 infection of TM as a model for the indirect inhibitory effects of HIV-1 on the human immune system and showed that infection of TM triggers alterations in cytokine production.[14] In the present report, we focus attention on the differential susceptibility of thymocytes and thymic stromal TM cells to HIV-1 infection.

MATERIAL AND METHODS

Thymic Cell Isolation and Tissue Culture

Fresh neonatal human thymic tissue was obtained from elective thoracic surgeries of HIV-1 negative individuals aged 1 day-6 months. Tissue was diced into 3-10 mm fragments, and incubated with 0.1% collagenase (Sigma, St. Louis, MO, USA) and Deoxyribonuclease (DNAase) 10 IU/ml (Sigma) solution in Dulbecco's Phosphate-Buffered Saline (PBS) without calcium chloride and magnesium chloride (GibcoBRL, Gaithersburg, MD, USA) for 2 hours at 37 °C. The fragments were subsequently passed through a 100 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA), and washed with ice cold PBS. Mononuclear cells were isolated from whole blood by Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden), and plated onto 10 cm Biocoat tissue culture plates with collagen cover (BD, Bedford, MA, USA) or directly into Biocoat (collagen cover) 6-well plates. Non-adherent cells (thymocytes) were placed in separate dishes (Falcon 24 well plate) for further culture. After 12 hours incubation in Roswell Park Memorial Institute (RPMI) medium (GibcoBRL) adherent cells were supplemented with 10-20% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. Cells were removed for analysis with either Trypsin-EDTA (GibcoBRL) or by use of disposable cell scrapers, and utilized for experimental analysis. Adherent cells were stained with fluorescent-tagged antibodies to determine cell-type: cells were 95% positive for the epitopes CD14 (Sigma, St. Louis, MO, USA) and CD68 (Dako, Carpenteria, CA, USA), which are indicative of TM.

Fluorescent Antibody Analysis of Thymic Cells

Thymic stromal cells, isolated as described above, were plated onto Lab-Tec chamber slides (Nunc, Rochester, NY, USA), and maintained in culture 7 days. At the conclusion of incubation cells were washed with PBS supplemented with 4% Bovine Serum Albumin (BSA). When necessary to visualize intracellular antigens, cells were first permeabilized by treatment with -20 °C methanol for 5-10 minutes, and then rinsed with PBS. All other incubations were conducted at 4 °C in PBS with 0.1% NaN$_3$. Following fixation, cells were incubated for 30 min-

utes with 20% goat serum in PBS at 4 °C to block non-specific binding. Subsequently, cells were washed in PBS-BSA and incubated with primary antibody for 1 hour at 4 °C. After washing with buffer to remove unbound primary antibody, cells were incubated for one hour with secondary antibody conjugated with FITC at 4 °C. Cell epitope specific staining was evaluated with anti-CD68 Ab (Dako, Carpinteria, CA, USA) at a concentration 1:100, and anti-CD14 Ab (Sigma) at 5 µg/ml. HIV-1 staining was evaluated with HIV-1 gp41 monoclonal antibody (2F5) at 5 µg/ml, obtained from the National Institutes of Health (NIH) Acquired Immune Deficiency Syndrome (AIDS) Research and Reference Reagent Program. HIV-1 co-receptor staining was evaluated with the anti-CCR5 monoclonal 2D7 (Pharmingen, San Diego, CA, USA) at 5 µg/ml. Secondary goat anti-mouse IgG conjugated with Fluorescein Isothiocyanate (FITC) (Roche, BMB, Indianapolis, IN, USA), or goat anti-mouse IgG conjugated with Texas Red (Jackson Lab. Inc, West Grove, PA, USA) were both used at a concentration of 1:200. At the conclusion of antibody binding cells were washed, mounted with Gel/Mount medium (Biomeda, Foster City, CA, USA) and examined under a fluorescent microscope Nikon Optiphot (Tokyo, Japan).

HIV Infection and Assay

Thymocytes and stromal cultures enriched for TM were isolated as described above and then plated onto either Falcon 24, 48 or 96 well plates as required for subsequent assay. Cells were maintained for one week in RPMI medium supplemented with 10% FBS and subsequently incubated with one of the following HIV viral stocks at a concentration of 10 ng/ml: Ba-L (R5-tropic), HXB2 (X4-tropic), and 89.6 (R5/X4-tropic). After a 12-hour incubation, cells were washed 5 times in PBS solution and zero time samples were collected. Cells were then re-cultured in the presence of RPMI medium supplemented with 10% FBS for the times indicated in Results. After collection of all required time points, p24 ELISA was used to determine infection status. Enzyme Linked Immune Sorbant Assay (ELISA) reactions were performed using the Alliance HIV-1 p-24 ELISA kit (PerkinElmer, Boston, MA, USA). Briefly, serial dilutions of cell culture supernatant, or in some instances cell suspensions, were placed in a 96-well ELISA plate 5% TritonX-100 and incubated for two hours at 37 °C. Following washing, detector antibody was added, and the plate was re-incubated for one hour at 37 °C. After additional washing samples were incubated with Streptavidin-Horseradish Peroxidase (HRP) for 30 minutes at room temperature, treated with Ortho-Phenylenediamine (OPD) substrate solution, and analyzed at 492 nm in a MRX Revelation Plate Reader (Dynex, Chantilly, VA, USA).

Statistical Analysis

Arithmetic means, standard deviations and p24 ELISA calculations were performed, using Microsoft Excel 2011. Data was analyzed using the Student t-test for paired and unpaired samples. Statistical significance was defined as p<0.05.

RESULTS

Receptor Expression on Thymic Stromal Cells

Naïve, uninfected thymic stromal cells were first examined for expression of the classical monocyte surface markers CD14 and CD68 which are characteristic for macrophages and dendritic cells.[15,16] As shown in Figure 1, these cells stained positively with both CD68 (Panel A) and CD14 (Panel B) antibodies relative to control (Panel D). Staining for the CCR5 chemokine receptor (HIV-1 co-receptor) was shown in parallel (Panel C).
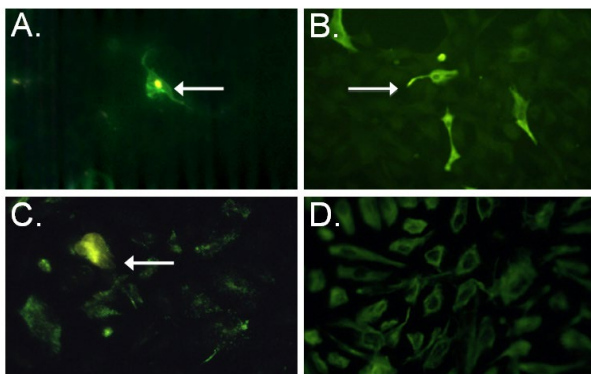

Figure 1: Biomarker Expression on Thymic Stromal Cells (Macrophages). Freshly isolated TM was analyzed for biomarker expression by fluorescence microscopy following incubation with antibodies to CD68, CD14, and CCR5. Panel A: anti-CD68 (FITC), Panel B: anti-CD14 (FITC), Panel C: anti-CCR5 (FITC), Panel D: negative control antibody (FITC).

Cultures of thymic stromal cells enriched for TM were then incubated with the R5/X4-tropic virus 89.6, and after 7 days cells were stained with gp41-Texas Red to confirm the presence of HIV-1 infection (Figure 2).
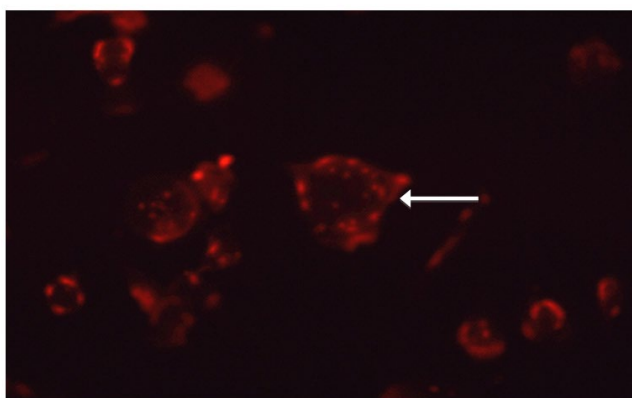

Figure 2: Biomarker Expression on Thymic Macrophages Infected with HIV-1. Cells enriched for TM were incubated with HIV-1 (89.6) and analyzed for gp41 expression at day 7 by fluorescence microscopy following incubation with anti-gp41 (Texas Red).

HIV-1 Infection of Thymocyte and Thymic Stromal Cell Cultures

To examine potential differences in susceptibility of subpopulations of thymic cells to various HIV-1 strains, we first incubated thymocytes with each of three laboratory derived HIV-1 strains: X4-tropic HXB2, R5-tropic Ba-L and R5/X4-tropic 89.6 at a concentration of 10 ng/ml (m.o.i. 0.02) as de-

scribed above. We then utilized p24 ELISA to follow the kinetics of virus replication in these cultures over time from 1-14 days. As shown in the left side of Figure 3, thymocytes in short-term culture (day 1-5) supported the replication of both X4-tropic and R5/X4-tropic HIV-1 to relatively equal levels, but not the R5-tropic Ba-L virus. On longer-term incubation, shown on the right of the figure, virus replication at day 14 of culture was greatly enhanced for HXB2 relative to 89.6 virus.
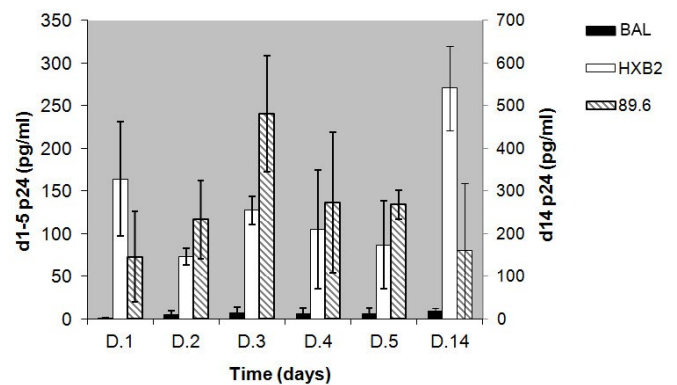

Figure 3: Thymocytes Infection with HIV-1 Strains. Thymocytes were incubated in culture with HIV-1 subtypes as described in Materials and Methods, and then aliquots of culture supernatant were analyzed for HIV-1 production at the times indicated using p24 ELISA. Infection with Ba-L (black bars), HXB2 (white bars) 89.6 and (striped bars) are presented. The data are shown as the mean p24 antigen production±SE of triplicate experiments.

Thymic stromal cells enriched for TM were similarly inoculated with the three different HIV-1 strains (Ba-L, HXB2 and 89.6). Both the kinetics and tropism of virus replication was different in TM cultures as compared to thymocytes. As shown in Figure 4, virus replication in TM cultures was slower overall then seen with thymocyte cultures, with virus not readily detected until day 7. With regard to tropism, cultures enriched for TM showed evidence of early replication of 89.6 virus, which peaked at day 7-14, whereas replication of the R5-tropic virus Ba-L continued to rise throughout culture from days 7-21 of culture. In contrast to thymocyte cultures, replication of the X4-tropic virus HXB2 was detected only transiently on day 7.
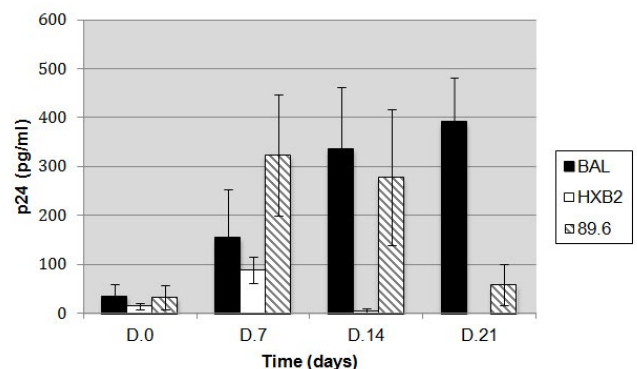

Figure 4: Thymic Macrophages Infection with HIV-1 Strains. Thymic Macrophages were incubated in culture with HIV-1 subtypes, and then aliquots of culture supernatant were analyzed for HIV-1 production at the times indicated using p24 ELISA. Infection with Ba-L (black bars), HXB2 (white bars) and 89.6 (striped bars) are presented. The data are shown as the mean p24 antigen production±SE of triplicate experiments.

DISCUSSION

Thymic macrophages represent an important subset of cells within the thymic stroma.[17] Although, TM comprise only a small percent of the total thymic cell population they play a central role in thymocye development and selection.[5,6] In view of this, disruption of TM function by infection with viruses such as HIV-1 might have a profound negative effect on thymocyte maturation.

Thymic macrophages are difficult to maintain in cell culture for study.[17,18] Therefore, we focused the first steps of our analysis on establishing an appropriate TM isolation procedure, and subsequently on the maintenance of cultures enriched for TM for up to 21 days *in vitro* with greater than 90% viability. We then compared thymocytes to stromal TM cultures with regard to their susceptibility to HIV-1 infection. Here we detected significant differences in the strain dependent tropism of infection and replication as measured by p24 ELISA analysis. Thymocytes supported the replication of R5/X4-tropic (early) and X4-tropic (early-late) HIV-1 strains, but not the R5-tropic virus Ba-L at any time point. In contrast, stromal cells enriched for TM supported the replication of R5/X4-tropic (mid) and R5-tropic (late) HIV-1 strains, but only transiently the X4-tropic virus HXB2.

It was reported previously that thymic macrophages are more mature and differentiated *in vivo* than monocytes and Monocyte Derived Macrophages (MDM).[17] However, similar to monocyte and MDM cultures, the production of endogenous chemokines decreased with TM maturation. The differentiation of tissue macrophages was also correlated with an increase in C-C chemokine receptor type five (CCR5) expression.[8,14,19] This may explain the data we obtained, as in our study cultures enriched for TM were preferentially susceptible to viruses that utilize CCR5 as a co-receptor, and replication of the R5 virus Ba-L rose continually throughout 21 days in culture.

It was also reported previously that monocytes and MDM are generally resistant to infection with X4-tropic strains of HIV-1 and that this phenomenon is not dependent on C-X-C chemokine receptor type four (CXCR4) expression or function.[19] Nonetheless we observed here, in replicate assays, transient replication of the X4-tropic virus HXB2 in TM cultures. The validity of this observation is reinforced by work from a prior study in which we also observed integration of X4 virus HXB2 DNA in TM cultures.[14] Collectively these results support the hypothesis that the ability of chemokine receptors to mediate viral entry may differ not only among different cell lineages but also within a single lineage depending upon the tissue origin.[20,21] Lastly, although not described above, infection with R5-tropic virus did not induce morphological changes to TM cells such as syncytia formation; whereas, syncytia formation and cytoxicity was observed in TM cultures incubated with R5/X4-tropic virus. This may explain the observed decrease in 89.6 virus replication after day 14 of culture.

In summary, HIV-1 infection of the thymus can have profound consequences on thymocyte maturation. These effects may result from direct infection of thymocytes but may also occur *via* infection of TM stromal cells, thereby disrupting the cell cross talk that is so crucial to thymocyte maturation. This hypothesis is consistent with previous reports that changes in cytokine production of HIV-1 infected bone marrow cells induced the disregulation of normal myelopoietic development.[22]

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

CONSENT

Regarding the consent statement, please be advised that the conduct of this research, including the acquisition of human subject materials was reviewed and approved by the University of Pennsylvania Institutional Review Board (IRB). As the research utilized only tissue samples that were provided to authors in an anonymized manner, direct patient consent was not required. Thus, this is also not a publication that reports on a patient based study and consent is not required for the publication of the manuscript.

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