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HIV-1 INFECTION INHIBITS CYTOKINE PRODUCTION IN HUMAN THYMIC MACROPHAGES

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Abstract

OBJECTIVE—The thymus serves as a critical site of T lymphocyte ontogeny and selection. Thymic infection by HIV-1 is known to disrupt thymocyte maturation by both direct and indirect means, however; the mechanism behind these effects remains poorly defined. Macrophages represent one of the most important peripheral targets of HIV-1 infection, are resident in the thymic stroma, and play a central role in thymocyte maturation.

MATERIALS AND METHODS—Studies presented here define three primary features and outcomes of thymic macrophages (TM) and HIV-1 infection: (1) The distinctive TM phenotype (surface markers and cytokine production measured by immunofluorescence, FACS and RT-PCR) relative to macrophages from other sources (blood -MDM and bone marrow - BM); (2) Infection of TM by different HIV-1 subtypes (X4, R5 and X4/R5) measured by ELISA and PCR; and (3) The consequences of HIV-1 infection on cytokine production by TM measured by RT-PCR.

RESULTS—The results demonstrate that TM display a distinctive phenotype of HIV-1 receptors (CD4^{lo}, CXCR4^{lo}, CCR5^{med}, CCR3^{hi}), chemokine production (MIP-1α⁺, RANTES⁺, MIP-1b[−], SDF-1[−]), and cytokine production (TNF-α⁺, IL-8⁺, M-CSF⁺, IL-6[−]) relative to either MDM or BM. TM were infected in vitro with R5 and X4/R5-tropic HIV-1 subtypes, and developed syncytia formation during long-term X4/R5 culture. In contrast, TM supported only transient replication of X4-tropic HIV-1. Lastly, infection of TM with HIV-1 abolished the production of all cytokines tested in long-term in vitro cultures.

CONCLUSION—Taken together these results indicate that thymic macrophages are a potential direct target of in situ HIV-1 infection, and that this infection may result in the disruption of macrophage functions that govern normal thymocyte maturation.

Keywords

Thymic macrophages; HIV-1 infection; chemokines; chemokine receptors; cytokines

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INTRODUCTION

The thymus is the primary site of both T-lymphocyte maturation and of orchestration for the coordinate selection process that governs MHC (major histocompatibility complex) restriction within the immune system (1,2). Although the reliance on the ontogenic roles of the thymus for maintenance of a competent immune system decreases with age, there is now ample evidence that developmentally viable remnants of thymic tissue persist in adults. Indeed, pockets of the adult thymic microenvironment may be functionally reactivated following the depletion of peripheral T cells as a consequence of either disease or therapy (3). Maturation and selection of T cells is regulated by a complex and only partially understood interaction between thymocytes and non-lymphoid cells within thymic stroma. The stroma is composed of an amalgam of cell types that includes epithelial cells, macrophages, dendritic cells, endothelial cells and fibroblasts (4,5,6). In view of the exquisite sensitivity of thymocyte maturation and selection to the microenvironment within the thymus, it is perhaps not surprising that thymocyte development can be disrupted, or in some instances ablated, during thymic HIV-1 (human immunodeficiency virus) infection (7,8,9).

There are two primary pathways by which HIV-1 may affect thymocyte development. The first means is by direct infection of thymocytes. Infection of thymocytes by HIV-1 is well documented and can have profound effects on both thymocyte viability and function (6,8,9,10). Although the mechanism for such effects are only partially understood, both thymocytes and mature peripheral T lymphocytes exhibit similar patterns of cytotoxicity to virulent HIV-1 strains/isolates (10,11). The second proposed means of thymocyte alteration is by HIV-1 infection of the stroma, thereby altering developmental capacity through disruption of the thymic microenvironment (12-14). The mechanism and magnitude of these effects, as well as the role of individual components of the stroma are not well defined (15).

The importance of these observations is underscored by the potential for profound negative consequences of fetal and/or neonatal HIV-1 infection on functional immune status. Infection of the thymus during critical developmental periods may ablate T cell maturation, the effects of which result in immune deficiency. For example, when left untreated children infected with HIV-1 at birth may acquire a severe immunodeficiency and develop opportunistic infections even though they display higher levels of CD4 positive lymphocytes relative to immunocompromised adults (16). In contrast, thymic infection of adolescents or adults typically has little direct impact on overall immune function. However, this circumstance may become more relevant in severely challenged individuals, or those who have lost peripheral T cell regenerative capacity, for example during immune ablation cancer therapy (17)

Macrophages represent one of the most important peripheral targets of HIV-1 infection, are abundant within the thymic stroma, and are known to play a central role in thymocyte maturation and selection (6,18,19). Accordingly, to determine the mechanism responsible for the inhibitory effects of HIV-1 on the maturation and regeneration of the human immune system, in this work we defined biomarkers for thymic macrophages, established parameters of their infection with subtypes of HIV-1, and investigated the consequences of HIV-1 infection on the production of cytokines.

MATERIALS AND METHODS

Macrophage Isolation and Tissue Culture

Fresh neonatal human thymic tissue, obtained from thoracic surgeries (1 day – 6 month old) on multiple HIV-1 negative individuals, was divided into 3 to 10 mm fragments, then

digested with 0.1% collagenase (Sigma, St.Louis, MO) and DNAase 10 IU/ml (Sigma) solution in Dulbecco's Phosphate-Buffered Saline (PBS) without calcium chloride and magnesium chloride (GibcoBRL, Gaithersburg, MD) for 2 hours at 37°C. The fragments were subsequently passed through a 100 µm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ), and washed with ice cold PBS. Mononuclear cells were isolated from whole blood or bone marrow obtained from healthy adult donors (aged 20 to 55 years) with Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden), and then plated onto 10 cm Biocoat tissue culture plates with collagen cover (BD, Bedford, MA) or directly into Biocoat (collagen cover) 6-well plates. Non-adherent cells were discarded after 12 hours incubation in RPMI medium (GibcoBRL) 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. After 7 days, adherent cells were removed with either Trypsin-EDTA (GibcoBRL) or by use of disposable cell scrapers, and then subjected to experimental analysis. The recovery of the cells was 70%, with a viability of 90% using either technique. The adherent cells were stained with fluorescent-tagged antibodies to determine cell-type: cells were 95% positive for the epitopes CD14 (Sigma, St.Louis, MO) and CD68 (Dako, Carpinteria, CA), which are indicative of macrophages. Hereafter these cells are referred to as thymic macrophages (TM). For comparative purposes macrophages were also isolated, using established procedures (20), from human bone marrow (BM) and peripheral blood (monocyte-derived macrophages - MDM): these cell types were uniformly >98% positive for macrophage CD14 and CD68 epitopes. As a further indicator of enrichment macrophages were incubated in culture with anti-CD14-coated iron polymer beads (Dynal Magnetic Beads, Invitrogen, Carlsbad, CA) for 20 min. at 4°C. Uptake of the beads was then visualized by phase contrast microscopy.

Fluorescent Antibody Analysis of Adherent Cells

Thymic macrophages, isolated as described above, were plated onto Lab-Tec chamber slides (Nunc, Rochester, NY), and maintained in culture 7 days. At the conclusion of this incubation cells were washed with PBS supplemented with 4% BSA (washing buffer/WB). 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% NaN₃. Following fixation, cells were incubated for 30 minutes with 20% goat serum in PBS at 4°C to block non-specific binding. Subsequently, cells were again washed in PBS-BSA and incubated with primary antibody for 1 hour at 4°C. After washing with WB to remove unbound primary antibody, cells were incubated for one hour with secondary antibody conjugated with FITC at 4°C. Macrophage specific epitope staining was evaluated with anti-CD68 Ab (Dako, Carpinteria, CA) at a concentration 1:100, and anti-CD14 Ab (Sigma) at 5 µg/ml. HIV-1 co-receptor staining was evaluated with 12G5 anti-CXCR4 receptor (Dr J. Hoxie, University of Pennsylvania, PA), 7B11 anti-CCR3 receptor and 2D7 anti-CCR5 (Pharmingen, San Diego, CA) at 5 µg/ml. Secondary goat anti-mouse IgG conjugated with FITC (Roche, BMB, Indianapolis, IN), or goat anti-mouse IgG conjugated with Texas Red (Jackson Lab. Inc, West Grove, PA) were both used at a concentration of 1:200. For co-staining with two markers, cells were first incubated with the primary and secondary antibody to one marker, then after intensive washing the procedure was repeated for the second marker using different a fluorochrome for final staining (as described above). At the conclusion of antibody binding reactions cells were washed, mounted with Gel/Mount medium (Biomed, Foster City, CA) and examined under a fluorescent microscope Nikon Optiphot (Tokyo, Japan).

To quantify the surface expression of CD4, CCR5, CCR3 and CXCR4 proteins on macrophage populations, antibody binding was evaluated by flow-microfluorimetry (FACS) as described previously (21). Antibodies used for FACS analysis included 12G5 anti-

CXCR4 receptor (Dr J. Hoxie, University of Pennsylvania, PA), 7B11 anti-CCR3 receptor and 2D7 anti-CCR5 (Pharmingen), and anti-CD4 (Immunotech, Marseilles, France). All primary antibodies were used at a concentration of 5 µg/ml. Cells were incubated with antibody as described in the preceding section. Primary antibodies were detected with secondary PE-conjugated goat anti-mouse antibody (Immunotech), at a dilution of 1:100. Macrophages were co-stained with anti-CD14 conjugated with APC (Pharmingen). After the final wash, cells were fixed in 2% paraformaldehyde/PBS prior to analysis, which was performed using a FACSCalibur (Becton Dickinson, San Jose, CA).

HIV Infection and Assay

Macrophages 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 either of the following HIV viral stocks (gift of Dr. F. Gonzalez-Scarano, Univ. of Pennsylvania): Ba-L (R5-tropic), HXB2 or IIIB (X4-tropic), and 89.6 (R5/X4-tropic) at a concentration of 10 ng/ml. 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. P24 ELISA reactions were performed using Alliance HIV-1 p-24 ELISA kit (PerkinElmer, Boston, MA). Briefly, serial dilutions of cell culture supernatant, or in some instances serum or cell suspensions, were placed in a 96-well ELISA plate 5% TritonX-100 and incubated for two hours at 37°C. Following intensive washing, Detector Antibody was added, and the plate was re-incubated for one hour at 37°C. After additional intensive washing samples were incubated with Streptavidin-HRP for 30 minutes at room temperature, treated with OPD Substrate Solution, and analyzed at 492nm in the spectrophotometer - MRX Revelation Plate Reader -(Dynex, Chantilly, VA).

DNA Isolation and PCR Analysis

To harvest DNA, 20 µl of Proteinase K was pipetted into each well of cells (3×10^4 /well) maintained in 24-well plates, then 200 µl of PBS was added followed by 200 µl of lysis buffer. This mixture was heat inactivated for 2 hours at 60°. Samples were subsequently boiled, as described previously (22), for 15 minutes and DNA was purified as per the “blood and body fluid” protocol of the Qiaamp DNA Blood Mini Kit (Qiagen, Valencia, CA). Ten microliters of each purified DNA sample was used as a template for PCR under the following conditions: 1× PCR Buffer, 1.5 mM MgCl₂, 15 mM dNTP, 1× Q reagent, 6 µM LTR-plus primer, 6 µM LTR-minus primer. After denaturing at 97° for 10 minutes, 0.6 µl of Taq Polymerase was added to each sample, and the PCR reaction was carried out under the following cycling parameters –30 seconds at 94°, 2 minutes at 55°, and 1 minute at 72°. This cycle was repeated 42 times, followed by a 10 minute 72° extension, to yield a product of 430 base pairs. All PCR reagents were obtained from Qiagen (Valencia, CA). Primer sequences (LTR: 5'-ACAAGCTAGTACGAGTTGAGCC-3' ; 5'-CACACACTACTTGAAGCACTTCA-3') were obtained as published (23). Ten microliters of product were run on a 1% agarose gel with (10 µg/ml ethidium bromide) for visualization.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Macrophages from each source TM, MDM and BM were analyzed by RT-PCR for mRNA expression of chemokines, cytokines and growth factors, before and after HIV-1 infection. Cells (2×10^5) were lysed in 200 µl of RNazol (Biotech Labs, Houston, TX) containing 22 µl of chloroform as described (24). The aqueous phase was collected and mixed with 1 volume of isopropanol (Sigma, St. Louis, MO). RNA was precipitated overnight at –20°C. The RNA pellet was washed in 75% ethanol and resuspended in 3-times autoclaved H₂O.

Subsequently, mRNA (0.5 µg) was reverse-transcribed with 500 U of Moloney murine leukemia virus reverse transcriptase (MoMLV-RT) and 50 pmol of an oligodeoxynucleotide primer complementary to the 3' end of the reported gene sequences of the appropriate growth factors and cytokines. The resulting cDNA fragments were amplified using 5 U of Taq polymerase and primers specific for the 5' end of the gene sequences. Amplified products (10 µl) were electrophoresed on a 2% agarose gel and transferred to a nylon filter and further documented photographically. The sequences of primers employed in this study are shown as follow: sense primers - M-CSF (TGT TGT TGG TCT GTC TCC TGG), IL-1b (AGT GAA ATG ATG GCT TAT TAC), IL-6 (AAC TCC TTC TCC ACA AGC GCC TT), G-CSF (GCC AGC TCC CTG CCC CAG AGC TTC), TNF-α (CAG AGG GAA GAG TTC CCC AG), MIP-1a (CCT TGC TGT CCT CCT CTG CAC), MIP-1b (TGT CCT CCT CAT GCT AGT AG), RANTES (TCA TTG CTA CTG CCC TCT GCG), IL-8 (GCT TTC TGA TGG AAG AGA GCT, SDF-1 (AAC GCC AAG GTC GTG GTC GTG CTG) and anti-sense primers: M-CSF (TTC TCC AGC AAC TGG AGA GGT), IL-1b (TAC ACT CTC CAG CTG TAG AGT), IL-6 (CCG AAG AGC CCT CAG GCT GG), G-CSF (GTG TGT CCA AGG TGG GAC CCA ACT), TNF-α (TTG ACC TTG GTC TGG TAG GAG), MIP-1a (CAC TCA GCT CCA GGT CGC TGA), MIP-1b (GTA CTC CTG GCC CAG GAT TC), RANTES (CTC ATC TCC AAA GAG TTG ATG), IL-8 (CTT GGA TAC CAC AGA GAA TGA), SDF-1 (CAC ATG TTG AAC CTC TTG TTT AAA AGC). The specificity of amplified products was further confirmed by Southern blot analysis. Lipopolysaccharide (LPS) activated monocyte extract was used as a positive control and H₂O (instead of mRNA) as a negative control for cytokine production. The control analysis was performed for 21 days of culture in all groups of macrophages. Chemokine expression was also analyzed and confirmed by ELISA analysis (Quantikine immunoassays, R&D Systems Inc., Minneapolis, MN). Cell viability for RNA analysis was verified by amplification of β-actin as previously reported (24).

Statistical Analysis

Arithmetic means, standard deviations and p24 ELISA calculations were performed on a Macintosh OSX computer using Microsoft Excel 2008. Data was analyzed using the Student t-test for unpaired samples.

RESULTS

Co-receptor Expression and Chemokine Production in Thymic Macrophages

Naïve (neither mitogen activated nor HIV-1 infected) thymic macrophages (TM) were first characterized phenotypically by expression of the monocyte surface markers CD14 and CD68 using commercially available antibodies. As shown in Figure 1 (panel A and B), >95% of TM were positive for these markers in comparison to control (panel C). Enrichment of TM to > 95% was verified by ingestion of anti-CD14-coated iron polymer beads, shown in phase contrast (panel D). In light of our interest in understanding HIV-1 infection of TM, staining with CD14 with HIV-1 co-receptors was next determined. A representative immunofluorescence analysis demonstrating CCR5/CD14 co-expression is presented in Figure 1 (panel E).

More comprehensive examination and quantification of the simultaneous presence of the HIV-1 receptor CD4 and co-receptors CXCR4, CCR3 and CCR5 on naïve tissue macrophages is presented in Figure 2. In this analysis we contrasted receptor/co-receptor abundance on purified TM to that seen on macrophages purified from either peripheral blood monocytes (MDM) or bone marrow (BM). All three macrophage types expressed abundant levels of CCR5 relative to control, although CCR5 expression was lower on TM and BM than on MDM. Significant levels of CD4 and CXCR4 were also seen on all

macrophage types, although the expression patterns for both CD4 and CXCR4 were lower on TM than on either BM or MDM. The expression pattern of CCR3 was the most distinctive for TM, as CCR3 was readily observed on TM, but not on either BM or MDM. Thus, the relative HIV-1 receptor/co-receptor expression pattern for TM was CD4^{lo}, CXCR4^{lo}, CCR5^{med}, CCR3^{hi}; the pattern for BM was CD4^{med}, CXCR4^{hi}, CCR5^{med}, CCR3^{neg}; and the pattern for MDM was CD4^{hi}, CXCR4^{med}, CCR5^{hi}, CCR3^{neg}.

Macrophages of different tissue origins may also be distinguished by their pattern of chemokine production. Relative production of chemokines by naïve TM, MDM and BM macrophages was assessed here by RT-PCR on day 7 of culture. As shown in Figure 3, TM (panel A) expressed mRNA for MIP-1 α and RANTES, but not MIP-1 β or SDF-1. In contrast, MDM (panel B) expressed mRNA for all β -chemokines tested (MIP-1 α , MIP-1 β and RANTES), but not the α -chemokine SDF-1. The pattern of chemokine production in BM was identical to that seen in MDM (data not shown). All results obtained in RT-PCR analysis were confirmed by ELISA (data not shown).

In summary, TM are distinguished from BM and MDM derived macrophages by both their expression of surface biomarkers and pattern of chemokine production. These attributes may provide distinctive tools that can be used to decipher the mechanism whereby HIV-1 infection of TM may disrupt thymocyte development.

HIV-1 Infection and Cytopathology of Thymic Macrophages

The capacity of HIV-1 to infect naïve macrophages isolated from different tissue sources was next examined using both ELISA and PCR methodologies. Macrophages isolated from thymus, bone marrow and blood were independently exposed *in vitro* to limiting concentrations of HIV-1 strains with MOI (multiplicity of infection) 0.02, that display known co-receptor utilization selectivity: R5-tropic - Ba-L, X4-tropic - HXB2/IIIB, and R5/X4-tropic - 89.6. The p24 ELISA assay was then employed to follow the kinetics of virus replication in these cultures over time, with the concentration of HIV-1 protein in supernatants collected from cultures at days 0, 7, 14 and 21. As shown in Figure 4, TM (panel A) were susceptible to infection by both R5-tropic and R5/X4-tropic HIV-1. In long-term culture virus replication was highest in TM cells infected with Ba-L, although the kinetics of Ba-L virus replication were slower than seen with the R5/X4 tropic virus 89.6. Early, robust replication of 89.6 virus was followed by decreased p24 concentrations after day 21, which coincided with a high level of cellular cytopathology. As shown in Figure 5 (panel B), syncytia formation in TM was readily observed by day 21 of culture with 89.6 HIV-1 subtypes. Cytopathology was not detected in TM incubated with any other HIV-1 subtype (data not shown). The lowest level of virus replication in TM cultures was obtained with the X4-tropic virus HXB2 where only transient and modest p24 production was observed. In view of this low response, PCR analysis using conserved LTR-primers was conducted on day 7 of TM cultures to verify genome incorporation of HIV-1. Results presented in Figure 6 indicate that integrated viral DNA of R5, X4 and R5/X4-tropic strains were clearly present in TM cultures.

Similar, but not identical results were observed in incubations of MDM with HIV-1. Ba-L and 89.6 viruses displayed an aggressive, early pattern of replication in MDM cultures, ultimately reaching levels 3-4 times that seen with TM (Figure 4). As shown in Figure 5 (panel D) substantial cytopathology was also observed in MDM incubated with 89.6 virus. However, there was no detectable replication of the X4-tropic virus HXB2 in MDM.

The pattern of HIV-1 replication was more distinctive in BM cultures. Sustained replication was only detected with the R5/X4 tropic virus 89.6. Here levels of virus continued to rise throughout 21 days in culture with no sign of cytopathology. In contrast, both HXB2 and

Ba-L displayed a transient burst of replication in BM that was detectable only up to day 7 of culture.

Impact of HIV-1 Infection on Cytokine Production by Thymic Macrophages

As noted above, peripheral blood and tissue macrophages produce a number of chemokines and cytokines that are crucial to normal biological function: for example, several cytokines produced by TM are proposed to play key roles in thymocyte maturation and/or selection (25,26). To evaluate the potential impact of HIV-1 infection on TM function we utilized RT-PCR methodology to examine the production of cytokines IL-6, IL-8, TNF- α and M-CSF in TM cultures incubated with or without HIV-1 89.6. Each of these cytokines was previously shown to be produced in short-term (7 day) MDM cultures (27-29). Typically, the maintenance of macrophages in culture is supported by the addition of M-CSF and GM-CSF to regulate their growth and differentiation (27,30); however, because the addition of these cytokines might of themselves alter the levels of HIV-1 infection and cytokine production, TM were cultured here in cytokine free media as described above (31). The viability of these cells was >80% for up to 21 days in all cultures. As shown in Figure 7 (lane 1), TM produced TNF- α , and to a lesser degree IL-8 and M-CSF, but not IL-6 in short-term 7 day cultures. The impact of longer-term culture (21 days) on cytokine production is shown in lane 2. TM displayed augmented production of IL-8, but the production of TNF- α and M-CSF was absent. The impact of HIV-1 infection on cytokine production was next evaluated in these cultures. As shown in lane 3, infection of TM cultures with the R5/X4 virus 89.6 eliminated the production of all cell cytokines tested by day 21.

DISCUSSION

Macrophages constitute an important subset of cells within the thymic stroma and are susceptible to HIV-1 infection (8,32-34). Our working hypothesis is that unique features of thymic macrophages may regulate not only their susceptibility to subtypes of HIV-1 and subsequent physiological response to this infection, but also the consequences of this infection on thymocyte maturation. Accordingly, studies presented here focused on defining three primary features and outcomes of thymic macrophages: the distinctive TM phenotype (surface markers and cytokine production) relative to macrophages resident in blood or other tissues (MDM and BM); infection of TM by different HIV-1 subtypes (X4, R5 and X4/R5); and the consequences of HIV-1 infection on cytokine production by TM.

Although TM comprise only a small percentage of the total thymic cellular population they play a central role in thymocyte maturation, selection and the generation of immune tolerance to many self-antigens (5,6). Physical interaction between thymocytes and TM is key to processes such as MHC presentation and apoptosis. Similarly, macrophages produce panoply of chemokines and cytokines, the distribution of which is often unique to the specific tissue location and activation status. The cytokines IL-1, IL-4, IL-6, IL-7, TNF- α , and INF- γ among others have all been implicated in thymocyte maturation (25,26,34,35). Nevertheless, we lack precise knowledge of the unique properties and roles of TM relative to other thymic stromal cells, and indeed, of the distinctive biological features of TM relative to other tissue and circulating macrophages. Knowledge of HIV-1 infection in non-lymphoid cells is primarily restricted to circulating macrophages and monocytes (22). This can be attributed in large measure to the important role that these cells play in establishing and maintaining normal immune function, and their ease of isolation for study. Circulating MDM are also well-established targets of R5-tropic HIV-1 species, and the consequences of these infections on altered macrophage and immune function are comprehensively described (36). However, tissue specific macrophages frequently display markedly different functions and phenotypes than their circulating counterparts (30). Given the extraordinary tissue

burden of HIV-1 infection, an increased knowledge of these distinctive cells is essential if we are to fully understand HIV-1 biology and pathology.

The procedures used to isolate macrophages as well as the age of donors can have a considerable impact on experimental outcomes (32,37). As consistent methods for the isolation of tissue macrophages are not uniformly utilized, we initially established both isolation and culture maintenance procedures that were uniformly successful in preparing macrophages regardless of tissue source. Importantly, these procedures minimize activation of macrophages during isolation and culture, as activation has profound consequences on macrophage function, including altered co-receptor expression and susceptibility to HIV-1 (27,30). Using these procedures, macrophages were isolated to a high level of enrichment and successfully cultured for up to 21 days from thymus, bone marrow and blood without the addition of exogenous growth factors. As we were interested in understanding HIV-1 infection of macrophages, we next characterized the pattern of HIV-1 receptor and co-receptor expression, and found that macrophages isolated and enriched from each tissue source had a unique profile: the relative HIV-1 receptor/co-receptor expression pattern for TM was CD4^{lo}, CXCR4^{lo}, CCR5^{med}, CCR3^{hi}; the pattern for BM was CD4^{med}, CXCR4^{hi}, CCR5^{med}, CCR3^{neg}; and the pattern for MDM was CD4^{hi}, CXCR4^{med}, CCR5^{hi}, CCR3^{neg}. The most distinctive marker for TM was CCR3. The chemokine receptor CCR3 plays an important role in the regulation of eosinophil migration, which is involved in many inflammatory pathologies including asthma (38). The presence of CCR3 also facilitates infection by primary R5-tropic HIV-1 isolates along with CCR5 (39), and may be used by a subset of primary HIV-1 isolates as a co-receptor for infecting microglia (40).

As demonstrated in Figures 4 and 7, TM are susceptible to infection with R5 and X4/R5-tropic HIV-1 subtypes. TM as well BM, but not MDM, also showed transient replication of the X4-tropic virus HXB2. It was reported previously that monocytes and MDM are generally resistant to infection with X4-tropic strains of HIV-1, and that this phenomenon does not depend on CXCR4 expression or function (21). Although the detection of HXB2 antigens was not persistent in long-term culture, the presence of provirus in TM at day 7 of culture was verified by PCR analysis. Multiple factors undoubtedly govern the susceptibility of tissue macrophages to HIV-1 infection. For example, there is growing evidence that the pattern and relative amounts of chemokine receptors differentially influence HIV-1 infection depending on cell type (21,41), whereas, even low levels of CD4 appear capable of consistently mediating HIV-1 infection (30). Whether the distribution of HIV-1 receptors and co-receptors, such as the high levels of CCR3 seen here, regulate HIV-1 susceptibility in TM or otherwise influence thymic pathology is currently under investigation.

Results presented here also demonstrate that the chemokines and cytokines produced by TM were distinctive relative to other tissue macrophages, that this pattern was altered during extended time in culture, and lastly, that infection with HIV-1 abolished cytokine production by TM. The production and secretion of chemokines and cytokines plays an important role in infection, replication and spreading of viruses within individual tissue and throughout the body (42). For example, by regulating the adhesive and homing properties of lymphocytes chemokines influence cell migration to both infected tissues and lympho-hematopoietic organs such as the thymus (8,24). Both TM and MDM produced the β -chemokines MIP-1a and RANTES, and failed to produce the α -chemokine SDF-1. SDF-1 is known to interfere in some cell populations with infection caused by X4-tropic viruses (41,43,44). This may in part explain infection of TM and MDM seen here with the X4 virus HXB2. However, unlike MDM, TM also failed to produce the β -chemokine MIP-1 β . The MIP-1 β protein is a chemoattractant for monocytes, and plays an important role in differential attraction of lymphocytes and induction of adhesive properties in T cells (45). The b-chemokines are also produced by CD8⁺ T cells (46), and inhibit R5-tropic infection of T cells through

competition for chemokine receptors and/or receptor down-regulation (47). Lastly, although not shown, the total level of β -chemokines secreted by TM was twenty times lower than seen for MDM in our in vitro cultures. Collectively, these observations are consistent with in vitro susceptibility of TM to both R5 and X4/R5-tropic viruses seen here, and suggest that TM, like other tissue macrophages, may be more differentiated than monocytes or freshly isolated MDM (8,30).

As noted above, HIV-1 may alter thymocyte development by either direct cytopathic effect on infected thymocytes or by altering the milieu established by the thymic stroma, of which macrophages are a key component (6,8-14). Although it is well known that the transcriptional analysis have many limitations our results demonstrate that the cytokines IL-8, TNF- α and M-CSF are produced in short-term TM cultures established without the addition of exogenous growth factors. After 21 days in culture only IL-8 production was detected even though cell viability remained above 80%. In contrast, when HIV-1 infected TM cultures were maintained for 21 days all cytokine production was eliminated and extensive syncytia formation was observed. The loss of cytokine production by TM could impact thymic function in several ways. As the release of TNF- α and IL-8 are known to work synergistically with INF- γ to inhibit viral replication (48-50), decreased cytokines might result in enhanced HIV-1 infection and cytotoxicity towards thymocytes. Additionally, thymocyte maturation could be disrupted through the loss of the supporting thymic cytokine microenvironment (25,26,34,35). Similar results were reported in the inhibition of myelopoiesis during HIV-1 bone marrow infection (28). Whether these or other mechanisms govern thymic HIV-1 infection awaits further investigation.

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Figure 1. Biomarker Expression on Thymic Macrophages

Freshly isolated TM were 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: negative control irrelevant Ab (FITC), Panel D: TM stained with CD14 linked to anti-CD14-coated iron polymer beads, Panel E: CCR5 and CD14 (FITC/Texas Red).

Figure 2. Comparative Distribution of Chemokine Receptors and CD4 on Macrophage Types Macrophages were isolated from thymus (TM), peripheral blood (MDM) and bone marrow (BM), and then analyzed for expression of CD4 and the chemokine/co-receptors CXCR4, CCR3, and CCR5 by flow microfluorimetry. Results are presented as the mean channel fluorescence + SE obtained in three experiments using cells isolated from three different donors.

Figure 3. RT-PCR Analysis of Chemokine Production in Macrophage Types

Macrophages isolated from thymus and peripheral blood were examined for chemokine production by RTPCR on day 7 of culture using primers specific to either MIP-1α, MIP-1β, RANTES or SDF-1. Reaction products were separated on agarose gels and visualized by ethidium bromide staining. Panel A: TM; Panel B: MDM. The data are representative of three separate analyses.

Figure 4. Macrophage Infection with HIV-1 Strains

Macrophages were isolated from thymus, peripheral blood and bone marrow, incubated in culture with HIV-1 subtypes as described in Methods, and then aliquots of culture supernatant were analyzed for HIV-1 production at the times indicated using p24 ELISA. Panel A: TM, Panel B: MDM, Panel C: BM. Infection with Ba-L (closed squares), 89.6 (open squares) and HXB2 (open triangles) are presented. The data are shown as the mean p24 antigen production $\pm$ SE of triplicate experiments.

Figure 5. Morphological Analysis of HIV-1 Infected Macrophage Cultures

Macrophages were isolated from thymus and peripheral blood, incubated in culture with or without HIV-1 subtypes as described in Methods, and cell morphology was assessed on day 21 by phase contrast microscopy. Panel A: non-infected TM, Panel B: TM infected with 89.6 virus, Panel C: non-infected MDM, Panel D: MDM infected with 89.6 virus. Areas of syncytia formation (SF) are noted with arrows.

Figure 6. PCR analysis of HIV-1 DNA integration into TM

Integration of HIV-1 DNA into TM cells at 7 days of culture was examined by PCR using a set of conserved HIV-1 primers within the LTR region. Results shown are: Lane 1- uninfected TM controls, Lanes 2-4, TM cells incubated with either Ba-L, HXB2 or 89.6, and Lane 5 - SupT1 cells chronically infected with IIIB virus. The conditions for infection are described in Methods. Results shown are the PCR reaction products (474bp) analyzed on agarose gels and visualized by ethidium bromide staining. The data are representative of three separate analyses.

Figure 7. RT-PCR analysis of HIV-1 effect on cytokine expression in TM

Thymic macrophages were established in culture and then incubated without or with HIV-1 89.6 as described in Methods. At selected times, cultures were examined for cytokine production by RT-PCR using primers specific to either IL-8 (A), IL-6 (B), TNF- α (C) or M-CSF (D). Reaction products were separated on agarose gels and visualized by ethidium bromide staining. Lane 1 - uninfected TM cells at culture day 7, Lane 2 - uninfected TM cells at culture day 21, Lane 3 - TM infected with 89.6 HIV-1 at culture day 21, Lane 4 - positive cytokine production control, Lane 5 - negative cytokine production control. The data are representative of three separately conducted experiments.