Relative mRNA Expression Levels of Restriction Factors and Antiviral Genes in Fetal and Adult Human Monocytes and Monocyte-Derived Macrophages

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Abstract

Among untreated HIV-infected pregnant women, the frequency of mother-to-child transmission of HIV is low (5–10%), with most infections occurring at or after birth. Given findings that fetal and adult monocytes are distinct from one another in terms of basal transcriptional profiles, and in phosphorylation of signal transducer and activators of transcription in response to cytokines, we hypothesized that fetal CD14+CD16– monocyte and monocyte-derived macrophages (MDMs) might, compared to their adult counterparts, express higher levels of transcripts for restriction factors and antiviral factors at baseline and/or after stimulation with cytokines that might be induced upon transmission of HIV *in utero*, for example, IFN α , IFN γ , and IL-6. We carried out these experiments and noted that a few genes, including *APOBEC3B*, *APOBEC3C*, and *IFITM2*, were expressed to a greater degree in fetal monocytes compared to adults. Similarly, the expression levels of *APOBEC3F* and *TRIM32* were greater in fetal MDMs. However, most of these differences were not observed after stimulation with cytokines and the vast majority of antiviral genes were more highly expressed in adults. Therefore, the results of this study are not consistent with the hypothesis that increased expression of antiviral genes in fetal myeloid cells confers immune protection to fetuses *in utero*.

Keywords: monocyte, macrophage, restriction factor, interferon

Introduction

A GROWING BODY OF EVIDENCE has emerged in recent years to suggest that cells making up the fetal human immune system are distinct from those found later in life (11). More specifically, it has been found that adult and fetal naïve CD4+ T cells have markedly different propensities to differentiate into Foxp3+ regulatory CD4+ T cells (10) and that fetal CD14+CD16– monocytes have distinct transcriptional signatures at baseline and after stimulation with various inflammatory cytokines, including IFN γ , IL-6, and IL-4 (8). Different transcriptional responses in myeloid cells to inflammatory stimuli might underlie several clinical observations made in the context of infection of the human fetus. Among all fetuses exposed to HIV during the course of a full-term gestation, only 5–10% are infected at the time of birth (9,17). Interestingly, many HIV-exposed uninfected neonates

harbor detectable HIV-specific cytotoxic and helper T cell responses, suggesting direct exposure of the fetal immune system to HIV *in utero* (2). We wondered whether differential myeloid responses, predominant in the fetus and not readily elicited in the adult, might serve to protect the fetus from HIV infection *in utero*.

Intrinsic host-encoded restriction factors and other antiviral genes possess potent antiretroviral activities (4,18) that can interfere with the HIV life cycle, and the expression of these genes is known to vary in cells of different subpopulations and activation states (1,13,15). Previous work by our group led to the development of a quantitative polymerase chain reaction (PCR)-based array capable of examining the expression of a predefined set of antiviral genes in primary cells from adults who were healthy (15), HIV-infected (14), and psoriatic (16). Here, we hypothesized that differential transcriptional patterns found in adult versus fetal monocytes

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and monocyte-derived macrophages (MDMs) might lead to differential expression of restriction factors and other antiviral genes, as detected by this assay. To test this hypothesis, we measured the relative expression of selected viral restriction factors and other antiviral genes in monocytes and MDMs obtained from human fetal bone marrow (FBM) and adult bone marrow (ABM). We also examined the change in expression of these genes after stimulation with IFN α , IFN γ , and IL-6 to determine whether induced antiviral responses would differ between adult and fetal monocytes and MDMs.

Materials and Methods

Isolation of bone marrow monocytes

FBM was obtained from 18 to 22 gestational week specimens provided by the Department of Obstetrics, Gynecology, and Reproductive Sciences, San Francisco General Hospital under the auspices of Committee on Human Research-approved protocols. Fetal samples were excluded in the case of (i) known maternal infection, (ii) intrauterine fetal demise, and/or (iii) known or suspected chromosomal abnormality. Fetal monocytes were isolated from femurs by bisection and mechanical dispersion of marrow in RPMI-1640 (Sigma). ABM samples were obtained from healthy donors (All-Cells, LLC or Lonza, Inc.). Both adult and fetal mononuclear cells were isolated by density centrifugation of a Ficoll-Hypaque gradient (Sigma). All samples, both fetal and adult, were viably cryopreserved before use.

Culture and stimulation of monocytes and MDMs

For cytokine stimulation experiments, sorted cells were incubated for 4 h in sterile R10 media (RPMI medium 1640 [GIBCO], 10% fetal bovine serum [FBS; Gemini], 100 U/mL penicillin [Mediatech], 100 μ g/mL streptavidin [Mediatech], 2 mM L-glutamine [Mediatech], 20 mM HEPES [Mediatech]), and appropriate amounts of cytokine: 150 IU/mL IFN α (PBL), 40 ng/mL IFN γ (Biolegend), or 70 ng/mL IL-6 (Biolegend). MDMs were produced by culturing purified monocytes in sterile R10 with 50 ng/mL of M-CSF (Gibco), changing the media every 3 days. After 6 days of culture, MDMs were stimulated with cytokines and subsequently lysed in 1 mL of TRIzol reagent (Invitrogen).

Flow cytometry

Mononuclear cell preparations were incubated in fluorescence activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 2% FBS and 2 mM EDTA) with fluoro-chrome-conjugated antibodies against human surface antigens, including CD3-Alexa700 (UCHT1; BD Biosciences), CD14-qDot605 (TüK4; Invitrogen), CD16-PacificBlue (3G8; BD Biosciences), and HLA-DR-PE-Cy7 (L234, BD Biosciences). All cells were stained with a live/dead marker (Amine-Aqua/AmCyan; Invitrogen) to exclude dead cells from the analysis, filtered through 70 μ m mesh filters (Falcon), and sorted by FACS (FACS Aria; BD Biosciences) directly into PBS with 2% FBS and 2 mM EDTA. The purity of sorted CD3-HLA-DR+CD14+CD16- classical monocytes was assessed by reanalyzing a small fraction of sorted cells and consistently found to be 88.7% $\pm$ 5.2% for fetal samples and 92.3% $\pm$ 4.1% for adult samples.

Gene expression

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), followed by a 15 min spin at 12,000 g at 4°C. RNA was extracted from the aqueous layer using the RNeasy kit (Qiagen) with in-solution DNAase treatment (Qiagen RNase-Free DNase Set) and eluted in 20 μ L of RNase-free water. DNase-treated RNA was transcribed into cDNA using random primers and the SuperScript VILO cDNA Synthesis Kit (Invitrogen), according to the manufacturer's instructions. Quantitative real-time PCR assays utilized custom-made TaqMan low density arrays (TLDA) from Applied Biosystems, following the manufacturer's instructions. All gene cards used for quantification were first described in reference (13). Thermal cycling was performed using an ABI ViiA7 Real-Time PCR System. cDNA (in 100 μ L of Applied Biosystems TaqMan Universal PCR Master Mix with UNG) was loaded onto the designated ports of the TLDA plates. Data were analyzed using ABI ViiA7 software. A panel of six housekeeping genes (*GAPDH*, *18S*, *ACTB*, *PPIA*, *RPLP0*, and *UBC*) was included in the TLDA plates and the gene, *PPIA*, was identified as the most stably expressed using the GeNorm algorithm. Raw cycle threshold numbers of amplified gene products were accordingly normalized to the levels of *PPIA* to control for cDNA input amounts. Fold induction was determined using the comparative Ct method.

Data analysis

All analyses were performed using a two-tailed unpaired nonparametric Mann-Whitney *t*-test and plots were generated using median and interquartile range. Antiviral factors with differential expression between adult and fetal samples with a statistical significance of $p < 0.05$ were highlighted.

Results and Discussion

Live classical (CD3-HLA-DR+CD14+CD16-) monocytes were sort-purified from seven adult and seven fetal donor bone marrow tissues (with representative examples in Figs. 1A, B, respectively). Sorted and plated adult monocytes showed the size and morphology of monocytes when examined microscopically (Fig. 1C). Upon M-CSF stimulation, adult MDMs were found to be adherent, flattened, and elongated, consistent with the expected morphology of MDMs (Fig. 1D). Fetal monocytes and M-CSF-stimulated MDMs demonstrated the same morphologies as their adult counterparts (data not shown).

A customized RNA expression array (1,13,14) was used to measure the baseline expression levels of antiviral genes and restriction factors in fetal and adult monocytes and MDMs. Compared with fetal monocytes, adult monocytes had significantly higher levels of expression of the following genes: *APOBEC3A* ($p < 0.05$), *CDKN1A* ($p < 0.01$), *EIF2AK2* ($p < 0.01$), *HERC5* ($p < 0.05$), *ISG15* ($p < 0.01$), *MX2* ($p < 0.001$), *PML/TRIM19* ($p < 0.01$), *RSAD2* ($p < 0.001$), and *TRIM22* ($p < 0.01$) (Mann-Whitney *t*-test) (Fig. 2A). In contrast, *APOBEC3B*, *APOBEC3C*, and *IFITM2* were significantly overexpressed in fetal monocytes ($p < 0.05$). Interestingly, most of these genes were expressed at equivalent levels in fetal and adult cells upon maturation *in vitro* into MDMs, the exceptions being *APOBEC3F* and

FIG. 1. Phenotypic and morphologic characterization of human fetal and ABM-derived monocytes and MDMs. **(A)** FACS plots and gating strategy of a representative ABM sample from which single, live cells with the phenotype of CD3-HLADR+CD14+CD16- classical monocytes were sorted. **(B)** FACS plots and gating strategy of a representative FBM sample from which single, live cells with the phenotype of CD3-HLADR+CD14+CD16- classical monocytes were sorted. **(C)** ABM-derived monocytes were viewed by light microscopy 4 h after sort purification. **(D)** ABM-derived adherent MDMs were viewed by light microscopy 6 days after stimulation of sort-purified monocytes with 50 ng/mL M-CSF. ABM, adult bone marrow; FACS, fluorescence activated cell sorting; FBM, fetal bone marrow; MDMs, monocyte-derived macrophages.

TRIM32, which were more highly expressed in fetal MDMs ($p < 0.05$), and *RNASEL*, which was more highly expressed in adult MDMs ($p < 0.01$) (Fig. 2A). Since fetal and adult monocytes were cultured *in vitro* as they matured into MDMs, it is possible that more differences would have been observed if it were possible to study macrophages directly isolated from fetal and adult tissues.

Since many restriction factors and antiviral genes relevant to HIV infection are induced by IFN α (4) and since IFN α has been observed to decrease viral replication in adult MDMs (7), we investigated whether there might be differential expression of these genes in fetal versus adult monocytes and MDMs. Fetal and adult monocytes and MDMs were stimulated with IFN α and, as expected, IFN α induced the expression of most antiviral genes in all four populations with the exception of *BRD4*, *CPSF6*, *CTR9*, and *TRIM28* (not shown). After stimulation, fetal monocytes continued to express *APOBEC3B* ($p < 0.01$) to a greater degree than adult monocytes. On the other hand, *CH25H* ($p < 0.05$), *CDKN1A* ($p < 0.01$), *BRD4* ($p < 0.05$), and *LGALS3BP* ($p < 0.05$) were expressed to a significantly greater degree by adult monocytes relative to fetal monocytes (Fig. 2B). Comparing adult and fetal MDMs, there

was no significant difference in antiviral gene expression after IFN α stimulation (Fig. 2B).

Previously, it had been shown that IFN γ stimulation of adult and fetal monocytes leads to differential signal transducer and activators of transcription 1 and 5 (STAT1 and STAT5) activation and to differential expression of genes associated with antigen presentation and innate pathogen responses (8) and, in adult MDMs, to decreased HIV replication (7). We hypothesized that these effects may be associated with the expression of specific antiviral genes in adult and fetal monocytes and MDMs after stimulation with IFN γ . As shown in Figure 3A, IFN γ treatment of adult monocytes induced a significantly higher expression of *CDKN1A* ($p < 0.05$), *MX2* ($p < 0.05$), *MOV10* ($p < 0.05$), *RSAD2* ($p < 0.05$), *ISG15* ($p < 0.05$), *BST2* ($p < 0.05$), *PML/TRIM19* ($p < 0.05$), *TRIM26* ($p < 0.05$), and *TREX1* ($p < 0.05$) compared to that found in fetal monocytes. When MDMs were stimulated with IFN γ , adult cells had greater expression of *SAMHD1* ($p < 0.05$), *RTF1* ($p < 0.05$), and *SLFN11* ($p < 0.05$) (Fig. 3A).

IL-6 is an important cytokine in the context of HIV infection (3), increasing HIV replication in adult MDMs (7). It is also a contributor to inflammatory environments in the

FIG. 2. Relative expression of antiviral genes in adult versus fetal monocytes and MDMs, before and after stimulation with IFN α . (A) Relative expression of select antiviral genes in ABM- versus FBM-derived monocytes and MDMs without cytokine stimulation ($n=7$). (B) Relative expression of select antiviral genes in ABM- versus FBM-derived monocytes and MDMs after 4 h of stimulation with 150 IU/mL of IFN α ($n=7$).

FIG. 3. Relative expression of antiviral genes in adult and fetal monocytes and MDMs, following stimulation with IFN γ or IL-6. (A) Relative expression of selected antiviral genes in ABM- versus FBM-derived monocytes and MDMs after 4 h of stimulation with 40 ng/mL of IFN γ ($n=4$). (B) Relative expression of selected antiviral genes in ABM- versus FBM-derived monocytes after 4 h of stimulation with 70 ng/mL of IL-6 ($n=4$). (MDM data not shown as there were no significant differences.)

fetus that can result in spontaneous abortion (5) and a cytokine previously studied in the context of transcriptional profiles in fetal and adult myeloid cells (8). We accordingly investigated the effects of IL-6 on the induction of antiviral genes in fetal and adult myeloid cells. After stimulation with IL-6, adult monocytes showed greater expression of *TRIM5* ($p < 0.05$), *APOBEC3A* ($p < 0.05$), *CDKN1A* ($p < 0.05$), *MX2* ($p < 0.05$), *TRIM22* ($p < 0.05$), *RSAD2* ($p < 0.05$), *CNP* ($p < 0.05$), *ISG15* ($p < 0.05$), *PML/TRIM19* ($p < 0.05$), *TRIM21* ($p < 0.05$), *TRIM26* ($p < 0.05$), *ELF2AK2* ($p < 0.05$), and *TREX1* ($p < 0.05$). However, when differentiated into MDMs before stimulation, adult and fetal cells did not demonstrate differential expression of any antiviral genes (Fig. 3B and Table 1).

To our knowledge, this is the first comparative study of the expression of transcripts for restriction factors and antiviral genes in fetal and adult human monocytes and MDMs. Given our previous observations that fetal monocytes phosphorylate

canonical and noncanonical STATs and respond more strongly to IFN γ , IL-6, and IL-4 than do adult monocytes (8), we hypothesized that fetal monocytes and, in particular, fetal MDMs might more readily upregulate restriction factors and other antiviral factors than their adult counterparts and, accordingly, be less permissive to HIV infection. If so, this might provide some degree of protection to the fetus against mother-to-child transmission of HIV and explain, at least in part, why the frequency of HIV infection of the fetus *in utero* is low (9,17).

Our findings are not consistent with this hypothesis. First, transcripts for most restriction factors and antiviral factors are expressed at higher levels in resting adult compared to resting fetal monocytes. Second, differences in the expression of such factors largely disappear when monocytes matured to macrophages *in vitro*. Indeed, since macrophages are generally much more permissive to HIV infection than are resting monocytes (12), this is the stage of myeloid differentiation that is likely most relevant in the context of HIV.

TABLE 1. SUMMARY TABLE OF DIFFERENTIAL GENE EXPRESSION IN UNSTIMULATED AND CYTOKINE-STIMULATED FETAL VERSUS ADULT MONOCYTES AND MONOCYTE-DERIVED MACROPHAGES

	Gene expression in fetus > adult		Gene expression in adult > fetus	
	Monocytes	MDMs	Monocytes	MDMs
Baseline	<i>APOBEC3B</i> * (4.433 $\pm$ 5.302), <i>APOBEC3C</i> * (6.388 $\pm$ 5.952), <i>IFITM2</i> * (19.20 $\pm$ 13.73)	<i>APOBEC3F</i> * (0.3137 $\pm$ 0.5090), <i>TRIM32</i> * (3.043 $\pm$ 2.777)	<i>APOBEC3A</i> * (11.46 $\pm$ 15.62), <i>CDKN1A</i> ** (74.41 $\pm$ 29.26), <i>ELF2AK2</i> ** (32.08 $\pm$ 45.32), <i>HERC5</i> * (10.88 $\pm$ 14.75), <i>ISG15</i> ** (1.96 $\pm$ 1.90), <i>MX2</i> *** (26.40 $\pm$ 35.52), <i>PML</i> ** (16.08 $\pm$ 26.30), <i>RSAD2</i> *** (9.814 $\pm$ 9.056), <i>TRIM22</i> ** (22.23 $\pm$ 16.63)	<i>RNASEL</i> ** (1.69 $\pm$ 1.49)
IFN α stim	<i>APOBEC3B</i> ** (8.098 $\pm$ 14.34)	None	<i>CH25H</i> * (0.5308 $\pm$ 0.8212), <i>CDKN1A</i> ** (113.6 $\pm$ 68.03), <i>BRD4</i> * (88.30 $\pm$ 80.51), <i>LGALS3BP</i> * (1.603 $\pm$ 2.199)	None
IL-6 stim	None	None	<i>TRIM5</i> * (13.38 $\pm$ 3.84), <i>APOBEC3A</i> * (24.42 $\pm$ 51.61), <i>CDKN1A</i> * (63.14 $\pm$ 11.39), <i>MX2</i> * (35.3 $\pm$ 62.3), <i>TRIM22</i> * (35.69 $\pm$ 20.59), <i>RSAD2</i> * (11.67 $\pm$ 29.16), <i>CNP</i> * (7.467 $\pm$ 4.531), <i>ISG15</i> * (1.666 $\pm$ 6.363), <i>PML/TRIM19</i> * (31.68 $\pm$ 39.47), <i>TRIM21</i> * (12.41 $\pm$ 8.79), <i>TRIM26</i> * (17.72 $\pm$ 6.86), <i>ELF2AK2</i> * (29.04 $\pm$ 38.25), <i>TREX1</i> * (13.05 $\pm$ 4.977)	None
IFN γ stim	None	None	<i>CDKN1A</i> * (118.1 $\pm$ 36.5), <i>MX2</i> * (56.47 $\pm$ 53.72), <i>MOV10</i> * (20.28 $\pm$ 6.42), <i>RSAD2</i> * (410.1 $\pm$ 307.8), <i>ISG15</i> * (3.129 $\pm$ 6.127), <i>BST2</i> * (19.98 $\pm$ 13.67), <i>PML/TRIM19</i> * (134.9 $\pm$ 110.2), <i>TRIM26</i> * (28.86 $\pm$ 16.24), <i>TREX1</i> * (17.89 $\pm$ 7.30)	<i>SAMHD1</i> * (134.7 $\pm$ 50.0), <i>RTF1</i> * (8.553 $\pm$ 2.919), and <i>SLFN11</i> * (12.04 $\pm$ 5.801)

Median and interquartile range are reported in this table. All statistical analyses were performed using a two-tailed unpaired nonparametric Mann–Whitney *t*-test. Antiviral factors with differential expression between adult and fetal samples with a statistical significance of $p < 0.05$ are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MDMs, monocyte-derived macrophages.

There are, of course, important caveats to these findings that must be taken into account. First, we have only measured the relative transcript levels of given restriction factors and antiviral factors, and there is not always a positive correlation between mRNA and protein expression levels (6). Second, the transcripts assayed in this study represent but a subset of the total family of factors that might play a protective role *in utero*. Third, and given limitations in the cell yields obtained from each of the bone marrow specimens, it was not possible to show directly that HIV infection levels are (or are not) similar in fetal and adult monocytes and MDMs. Finally, and not least, it is possible that other mediators, alone or in combination with certain restriction factors and antiviral factors, might play a role in protecting the fetus from HIV infection *in utero*. That being said, we believe that the current data sets address important and novel aspects of myeloid cell biology *in utero*, and may help to guide the design of future studies in this area.

In sum, notwithstanding the fact that fetal myeloid cells demonstrate unique transcriptional and immunologic properties (8), our data are not consistent with the hypothesis that increased expression of transcripts for restriction factors and antiviral genes within them confers fetal resistance to HIV. Future studies might extend these observations to address the above caveats and to more completely explore the apparent resistance of the human fetus to HIV infection *in utero*.

Authors' Contributions

K.T., R.A.S.R., M.M.L., W.Y., and J.M.M. designed the experiment. K.T., E.R.K-L., W.Y., and D.S. performed the sample preparation and *in vitro* assays. R.A.S.R. and V.D.C. carried out the quantitative PCR-based arrays. K.T., R.A.S.R., D.S., and J.M.M. interpreted the data and wrote the article.

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Author Disclosure Statement

No competing financial interests exist.

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