Increased Levels of Macrophage Inflammatory Proteins Result in Resistance to R5-Tropic HIV-1 in a Subset of Elite Controllers

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ABSTRACT
Elite controllers (ECs) are a rare group of HIV seropositive individuals who are able to control viral replication without antiretroviral therapy. The mechanisms responsible for this phenotype, however, have not been fully elucidated. In this study, we examined CD4+ T cell resistance to HIV in a cohort of elite controllers and explored transcriptional signatures associated with cellular resistance. We demonstrate that a subgroup of elite controllers possess CD4+ T cells that are specifically resistant to R5-tropic HIV while remaining fully susceptible to X4-tropic and vesicular stomatitis virus G (VSV-G)-pseudotyped viruses. Transcriptome analysis revealed 17 genes that were differentially regulated in resistant elite controllers relative to healthy controls. Notably, the genes encoding macrophage inflammatory protein 1α (MIP-1α), CCL3 and CCL3L1, were found to be upregulated. The MIP-1α, MIP-1β, and RANTES chemokines are natural ligands of CCR5 and are known to interfere with HIV replication. For three elite controllers, we observed increased production of MIP-1α and/or MIP-1β at the protein level. The supernatant from resistant EC cells contained MIP-1α and MIP-1β and was sufficient to confer R5-tropic resistance to susceptible CD4+ T cells. Additionally, this effect was reversed by using inhibitory anti-MIP antibodies. These results suggest that the T cells of these particular elite controllers may be naturally resistant to HIV infection by blocking R5-tropic viral entry.

IMPORTANCE
HIV is a pandemic health problem, and the majority of seropositive individuals will eventually progress to AIDS unless antiretroviral therapy (ART) is administered. However, rare patients, termed elite controllers, have a natural ability to control HIV infection in the absence of ART, but the mechanisms by which they achieve this phenotype have not been fully explored. This paper identifies one mechanism that may contribute to this natural resistance: some elite controllers have CD4+ T cells that produce high levels of MIP chemokines, which block R5-tropic HIV entry. This mechanism could potentially be exploited to achieve a therapeutic effect in other HIV-seropositive individuals.

In the absence of antiretroviral therapy (ART), the majority of HIV-seropositive patients have detectable viral loads (VLs) and experience a slow but inevitable decline in the number of CD4+ T cells, eventually culminating in the development of AIDS. However, in rare individuals (<1% of the HIV-seropositive population), viral replication is suppressed to extremely low or undetectable levels. These patients, termed elite controllers (ECs), typically retain relatively high CD4+ T cell counts and do not progress to AIDS, even in the absence of ART (1–4). Sequencing of HIV and in vitro functional assays suggest that most ECs possess replication-competent virus (5). Long-term nonprogressors (LTNPs), representing ~2 to 15% of HIV-seropositive patients, are a second group of individuals with a protective phenotype, in whom the virus continues to replicate but at a reduced level. In LTNPs, the CD4+ T cell count decline and progression to AIDS are significantly delayed (6–9). Genome-wide association studies have revealed several alleles within the human leukocyte antigen B/C (HLA-B/C) block that are enriched in both HIV ECs and LTNPs versus individuals with normal HIV disease progression (termed progressors) (10). Notably, B*57:01, B*27:05, B14/Cw*08:02, B*52, and A*25 alleles are protective, whereas B*35 and Cw*07 alleles confer an elevated risk. However, some ECs lack protective HLA alleles, and other HIV-seropositive patients exhibit viremia and disease progression despite possessing these protective alleles. Collectively, these alleles account for only 20% of the overall variance in HIV control (10). Hence, additional mechanisms must contribute to the virologic suppression characteristic of ECs. Determining how ECs are able to control HIV replication may expand our knowledge of HIV pathogenesis, reveal novel therapeutic targets, and inform vaccine and eradication efforts.

While the mechanisms of viral containment remain largely unknown, a number of key features have been associated with elite control (1, 11, 12). ECs exhibit T cell responses that are qualitatively different from HIV progressors. CD4+ T cells from ECs retain their ability to proliferate and produce interleukin-2 (IL-2) in response to HIV (2, 13–15). ECs do not exhibit some of the...
immune changes that are observed in progressors, such as upregulation of cytotoxic-T lymphocyte-associated antigen 4 (CTLA-4) on CD4+ T cells (16, 17). Interestingly, some ECs have been shown to harbor lower levels of integrated HIV DNA but higher levels of 2-long terminal repeat (2-LTR) circular HIV DNA, suggesting a block at integration post-nuclear entry (18). CD8+ T cells from ECs also exhibit a more multifunctional response to HIV, with greater degranulation and release of multiple cytokines, including gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-2, and macrophage inflammatory protein 1β (MIP-1β) (2, 13, 14, 19–22). Moreover, CD8+ T cells from ECs exhibit a superior ability to control HIV in cocultured CD4+ T cells, through cytotoxic activity (23). Because of the complex interplay of HIV with the host immune system, it is likely that some of these features reflect the primary mechanisms of elite control, whereas others reflect downstream effects or events.

Previous studies have yielded conflicting results regarding whether EC CD4+ T cells are resistant to HIV. Several studies demonstrated that phytohemagglutinin (PHA)-activated CD4+ T cells from ECs and LTNP patients are equally susceptible to infection (24–28). Two reports demonstrated that CD3-activated CD4+ T cells from ECs were resistant to HIV infection in culture (29, 30), while a third study using CD3 activation found comparable HIV infection in CD4+ T cells from ECs versus progressors (31). Two studies found that unstimulated CD4+ T cells from ECs and progressors were equally susceptible to HIV infection (32, 33), while a third study found that unstimulated CD4+ T cells from ECs exhibited a block to viral integration (34). These studies used different activation protocols and various viral strains. Thus, it is hard to ascertain if the conflicting and inconsistent results are a consequence of disparate experimental conditions or dissimilar characteristics of the EC cohorts.

The HIV vectors used in laboratory studies can be engineered to incorporate different HIV envelopes, which vary in their coreceptor usage. CCR5 (R5)-tropic envelopes predominate during the early stage of clinical HIV infection, while CXCR4 (X4)-tropic HIV envelopes may evolve as patients progress to AIDS. Additionally, many laboratory studies use HIV vectors pseudotyped with vesicular stomatitis virus G protein (VSV-G), and recent evidence suggests that the ubiquitously expressed low-density lipid receptor (LDL-R) plays a critical role in its viral entry (35). In this study, we examined EC CD4+ T cell resistance to different HIV envelopes. We identified a subset of ECs in our cohort who exhibited CD4+ T cell resistance to R5-tropic HIV but full susceptibility to X4-and VSV-G-pseudotyped viruses. We investigated transcriptional signatures that were associated with cellular resistance and determined that the MIP-1α and/or MIP-1β level was elevated in three out of four individuals with the cellular resistance phenotype. These elevated levels of chemokines were necessary and sufficient to confer this phenotype to other cells in a medium transfer experiment.

MATERIALS AND METHODS

Peripheral blood mononuclear cell collection and CD4+ T cell purification. The Yale Human Investigations Committee and Texas Tech University Health Sciences Center Institutional Review Board approved all studies involving human subjects. Following informed consent, blood was collected from subjects by peripheral venous phlebotomy. Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll-Paque Plus reagent (GE Healthcare Life Sciences, Piscataway, NJ), and CD4+ T cells were purified via magnetic enrichment using CD4 microbeads (Miltenyi Biotech, San Diego, CA). Purity was judged to be between 85% and 97% based upon flow cytometric analysis (the remaining cells were predominantly CD4-low monocytes with <1% contaminating CD8+ T cells and <0.1% contaminating NK cells). Cells were frozen in 10% dimethyl sulfoxide (DMSO)–90% fetal bovine serum (FBS) and stored until use.

CD4+ T cell activation, single-cycle HIV infection assay, and conditioned medium transfer. CD4+ T cell aliquots were thawed and then activated by using plates coated with 1 μg/ml anti-CD3 (clone OKT3; ebioScience, San Diego, CA) in the presence of 2 μg/ml soluble anti-CD28 (clone 28.2; ebioScience) and 100 IU/ml IL-2 for 3 days. They were then infected with a replication-defective HIV vector encoding a fluorescent reporter gene (enhanced yellow fluorescent protein [eYFP] or enhanced green fluorescent protein [eGFP]) and individually pseudotyped with various R5, X4, and VSV-G envelopes, prepared as previously described (36). Three days later, flow cytometry was performed with a FACS Calibur or FACSCanto instrument (Becton Dickinson, Franklin Lakes, NJ) to quantify green fluorescent protein-positive (GFP+)/eYFP+ HIV-infected cells. For the R5-tropic viruses that exhibited a low level of infection, a large number of cells (5 × 10^5 to 2 × 10^6) were analyzed, and gating was set on mock-infected cells such that at most 1 out of 10^5 cells was scored positive.

Thus, with an infection rate of 1%, the dynamic range of the flow cytometric assay was ~1,000. In some experiments, cells were incubated in supernatants harvested from CD4+ T cells activated 3 days earlier or supernatants from 293T cells transfected with the following plasmids: pCClL3L1, encoding MIP-1α (Origine, Rockville, MD); pCCl4, encoding MIP-1β (to generate this plasmid, the CCL4 coding sequence was amplified from human cDNA and then ligated into plasmid pCDNA3+1+CAT); and pκ B-arr-HA, encoding β-arrestin (a gift from Bryan Cullen, Duke University). Treatment was performed in the absence or presence of ~70 μg/ml polyclonal goat IgG neutralizing antibodies against MIP-1α and/or MIP-1β or the isotype control (R&D Systems, Minneapolis, MN), followed by the addition of HIV-pseudotyped particles 20 min later.

Quantification of T cell purification, activation, and CCR5 levels. To assess CD4+ T cell purity, activation status, and CCR5 levels, samples were stained with fluorescently labeled antibodies against human CD4-allophycocyanin (APC) (RA-T4), CD3-peridinin chlorophyll protein (PerCP)-cytometric 5.5 (clone OKT3), CD8α-phycocerythrin (PE) (HIT8a), and CD14-fluorescein isothiocyanate (FITC) (61D3) or CCR5-FITC (clone 28.2; eBioscience, San Diego, CA) in the presence of 2 μg/ml soluble anti-CD28 (clone 28.2) and 100 IU/ml IL-2 for 3 days. They were then activated by using plates coated with 1 μg/ml anti-CD3 (clone OKT3; ebioScience, San Diego, CA) in the presence of 2 μg/ml soluble anti-CD28 (clone 28.2; ebioScience) and 100 IU/ml IL-2 for 3 days. They were then infected with a replication-defective HIV vector encoding a fluorescent reporter gene (enhanced yellow fluorescent protein [eYFP] or enhanced green fluorescent protein [eGFP]) and individually pseudotyped with various R5, X4, and VSV-G envelopes, prepared as previously described (36). Three days later, flow cytometry was performed with a FACS Calibur or FACSCanto instrument (Becton Dickinson, Franklin Lakes, NJ) to quantify green fluorescent protein-positive (GFP+)/eYFP+ HIV-infected cells.

RNA sequencing (RNA-Seq). Total RNA was isolated from CD4+ T cells of ECs and healthy donors by using the RNeasy mini kit (Qiagen, Germantown, MD). Isolated RNA was analyzed at the Yale Genome Analysis Center, where cDNA sequencing library preparation and Illumina single-end sequencing were performed. DNA sequence data generated were stored in FASTQC format, and quality control was performed by using FastQC version 0.10.1 (37). Low-quality reads (Q < 20) were removed prior to alignment. Quality-filtered reads were aligned to sequences of the human genome (hg19) downloaded from Illumina’s iGenome resource (Illumina, San Diego, CA), using TopHat version 2.0.5 (38), as previously described (39). Successfully aligned reads were then further analyzed by using Cuffdiff in order to allow the estimation of differential gene expression using functions of the R package “cummeRbund” (40).

Whole-exome sequencing. Genomic DNA was isolated from 10 ml whole blood by using the QIAamp DNA blood maxikit (Qiagen). Targeted capture was performed by using the NimbleGen 2.1 exome reagent.
followed by sequencing on the Illumina Genome Analyzer IIx platform. Sequences were aligned to NCBI build 36 of the human genome by using the ELAND program (Illumina) for single nucleotide variation detection and by BWA software for indel detection. Reads aligned out of the targeted intervals and pairs of reads sharing the same start and end positions were regarded as PCR duplicates and discarded. Single-nucleotide variants (SNVs) and indels were called from the remaining reads by using Samtools software and annotated by using an in-house annotation pipeline. dbSNP (build 135) and 1000 Genomes (May 2011 release) were used to test for novelty of variations.

qPCR and reverse transcription-PCR (RT-PCR). Genomic DNA and total RNA were isolated as described above. cDNA was synthesized with the Promega reverse transcription system using random primers (Promega, Madison, WI). The CCR5 genotype (Δ32 versus WT) was determined by quantitative PCR (qPCR) with primers CCR5-F1 (5′-ACCT CGAGCTCTCATTTTG-3′) and CCR5-664R (5′-CCAGGCCCAAGAT GACTATC-3′), as reported previously (41). To quantify CCR5, STAT-1, CEBPa, and RUNX1 mRNA levels, relative to the EF1α reference gene, qPCR was performed by using the iQ SYBR Green Supermix reagent (Bio-Rad), with primers CCR5-F1 (5′-CAAAAGAAGGTCTTCACTTACAAGG-3′), CCR5-R1 (5′-CCTTGTCCTCTTCTCTTATTTG-3′), hSTAT1F (5′-TGGTTGGAAGAACAGTTCATG-3′), hSTAT1R (5′-TATGCAGTGC CACGGAAGA-3′) (42), CEBPaF (5′-CGGTTGCAAGAAGAAGACGAC-3′), CEBPaR (5′-CGGAATCTCTTCTCTCTGGC-3′) (43), RUNX1F (5′-ACTCTGGCTGACGTTGAA-3′) (44), RUNX1R (5′-GACCTTGCGG TGGTGTGTGTG-3′), EF1αF (5′-TCTGGTGGGAATGTGGTACAACT GC) (44), and EF1αR (AGAGCTTCACTCAAAGCTTCATGG-3′) (44). To measure the copy number of CCL3-like (CCL3L1) and CCL4-like (CCL4L) genes, qPCR was performed on genomic DNA by using the TaqMan no-Amp Erase UNG reagent (Applied Biosystems, Life Technologies, Grand Island, NY), with primers CCL3L1GF (5′-TCTCCACAGCTTCTAAACC AAGA-3′), CCL3L1GR (5′-CTGGACCACTCCTCATCGG-3′), CCL4L1GF (5′-CCTTGCCCTTTCTTTTCAAG-3′), bglbob1(F) (5′-GGCAACCTAAGGT GAAGGC-3′), and bglbob1(R) (5′-GGTGAGCCAGGCCGCTC TACTA-3′), as described elsewhere (46). The nine other patients remained ART naive. HLA typing demonstrated that 7/11 patients in the cohort possess a protective B*57 allele (EC-2, EC-4, EC-5, EC-7, EC-8, EC-9, and EC-11).

A subset of ECs exhibits a novel phenotype of CD4+ T cell resistance to R5-tropic single-cycle HIVs. In order to examine T cell resistance to HIV infection in our cohort, we obtained blood samples from recruited patients, purified CD4+ T cells, and performed a single-cycle HIV infection assay. A post hoc analysis revealed a subset of four ECs (EC-1, -2, -3, and -11) whose CD4+ T cells exhibited relative resistance to YU2-pseudotyped virus, in comparison to the remaining ECs (EC-4 through -10) and healthy controls (Fig. 1A). In contrast, these T cells were fully susceptible to VSV-G-pseudotyped particles (Fig. 1B). For EC-2 and EC-11, we confirmed resistance by using a different HIV backbone and a dose range of YU2 Env-pseudotyped virus (EC-1 and EC-3 were excluded from this analysis because of limited sample availability) (Fig. 1C). The T cells remained fully susceptible to VSV-G-pseudotyped virus under similar conditions (Fig. 1D).

The contrasting results between YU2- and VSV-G-pseudotyped virus led us to question whether the resistance phenotype extended to other R5- and X4-tropic envelopes. We exposed CD4+ T cells to a panel of replication-defective viruses pseudotyped with different envelopes. CD4+ T cells from EC-1, -2, and -11 exhibited significant resistance to the YU2, JRFL, and AD8 R5-tropic envelopes, compared to normal controls (again, EC-3 was excluded from this analysis because of limited sample availability) (Fig. 1E). In contrast, these cells were fully susceptible to viruses containing the HXB2-, NL4.3-, and LAI X4-tropic envelopes as well as VSV-G (Fig. 1E).

Cellular resistance to R5-tropic HIV is not associated with altered CCR5 levels. R5-tropic HIV envelopes mediate entry via the CCR5 coreceptor, whereas X4-tropic HIV’s utilize the CXCR4 coreceptor. To determine if altered CCR5 levels explained this
<table>
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<th>No.</th>
<th>Patient Ethnicity</th>
<th>Race</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>CD4cells</th>
<th>% CD4cells</th>
<th>Viral load(s) (date [mo/yr])</th>
<th>ART (yr)</th>
<th>Yr ofdiagnosis</th>
<th>Risk factor(s)</th>
<th>Comorbidity(ies)</th>
<th>HLA type</th>
</tr>
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</table>

**Legend:**
- HIV: Human Immunodeficiency Virus
- ART: Antiretroviral Therapy
- MIPs: Major Histocompatibility Complex (MHC) Peptide
- HIV Elite Control: Individuals with high viral load suppression and low CD4 cell count

**Table Notes:**
- AKA: Above-the-Knee Amputation
- B: Black
- CAD: Coronary Artery Disease
- CKD: Chronic Kidney Disease
- DM: Diabetes Mellitus
- Dyslipid: Dyslipidemia
- GERD: Gastroesophageal Reflux Disease
- GIST: Gastrointestinal Stromal Tumor
- HCV: Hepatitis C Virus
- HTN: Hypertension
- IVDU: Intravenous Drug Use
- LTBI: Latent Tuberculosis
- MI: Myocardial Infarction
- MSM: Men Who Have Sex with Men
- ND: Nondetectable
- NH: Not Hispanic
- W: White

**Additional Information:**
- MHC Peptide Database: HIV Elite Control
- Human Immunodeficiency Virus: Individuals with high viral load suppression and low CD4 cell count
phenotype, we harvested RNA from CD4\(^+\) T cells after activation and measured CCR5 levels by reverse transcription-quantitative PCR (qRT-PCR). We observed similar CCR5 mRNA levels in all subjects (Fig. 2A). Additionally, we quantified CCR5 surface protein levels on CD4\(^+\) T cells by flow cytometry. We observed a substantial upregulation of CCR5 upon activation of CD4\(^+\) T cells, and this occurred to a similar degree in all subjects (Fig. 2B).

Genotyping of CCR5, via qPCR, demonstrated that none of the ECs in this cohort possessed the Δ32 CCR5 mutation, which prevents R5-tropic HIV entry (48). Additionally, whole-exome sequencing demonstrated that none of these subjects had mutations in their CCR5 or CD4 exonic sequences. Hence, altered CCR5 coding sequences or levels of expression did not account for resistance to R5-tropic virus. Upon CD4\(^+\) T cell activation, CD25 was upregulated to a similar degree in all of the ECs and healthy controls, suggesting a comparable activation status (Fig. 2C).

**FIG 1** A subset of ECs shows CD4\(^+\) T cell resistance to R5-tropic HIV. (A and B) CD4\(^+\) T cells from the ECs and healthy controls were activated for 3 days with anti-CD3 and anti-CD28.2 in the presence of IL-2. Cells were then infected with replication-defective HIV-cycT1-internal ribosome entry site (IRES)-eYFP pseudotyped with YU2 (R5 tropic) (A) or VSV-G (pantropic) (B). (C and D) A dose response analysis was performed with replication-defective HIV-ec2GFP pseudotyped with the YU-2 envelope (C) or VSV-G (D). Panels A to D show results of one representative experiment with 3 healthy controls. Data were analyzed by using the Kruskal-Wallis test and Dunn’s multiple-comparison test. (E) Replication-defective HIV-cycT1-IRES-eYFP or HIV-ec2GFP pseudotyped with a panel of envelopes, including VSV-G, YU2, JRFL, and ADA (R5) and HXB2, NL4.3, and Lai (X4). Shown are pooled results from three experimental repeats with 11 healthy control samples for the ADA envelope and 7 healthy control samples for the other envelopes. Data were analyzed separately for each envelope with the Mann-Whitney test. Graphs show individual values and medians. n.s., not significant.
ever, CD69 upregulation was slightly reduced in resistant ECs 1, 2, 3, and 11 (Fig. 2D).

Transcriptional signatures associated with the cellular R5-tropic HIV resistance phenotype. We investigated whether specific transcriptional signatures were associated with the CD4 T-cell resistance phenotype. We activated CD4 T cells from two of the resistant ECs (EC-2 and EC-11) and three healthy controls, prepared RNA, and performed exploratory RNA-Seq transcriptome analyses (EC-1 and EC-3 were again excluded from this analysis because of limited sample availability). The levels of 17 genes were statistically significantly different between the resistant ECs and healthy controls (Table 2). Of the genes on this list, we were intrigued by the presence of CCL3 and CCL3L1, both of which encode MIP-1α. MIP-1α, MIP-1β, and RANTES are natural ligands of CCR5 that inhibit R5-tropic HIV infection by steric hindrance of virus binding (49–52). The CCL3 and CCL3L1 genes were both upregulated in EC-11 versus healthy controls. In contrast, EC-2 had only slightly elevated levels of CCL3 and levels of CCL3L1 similar to those in healthy controls (Table 2).

Cellular resistance to R5-tropic HIV is associated with increased levels of MIP-1α and/or MIP-1β in EC-1, -3, and -11. To determine whether the secreted amounts of MIP-1α and the related chemokines MIP-1β and RANTES were elevated, we harvested supernatants from activated CD4 T cells immediately
prior to the infection assay and measured chemokine levels via an ELISA. T cells from EC-3 and EC-11 exhibited a significant increase in MIP-1α production relative to healthy controls (Fig. 3A), whereas levels for EC-1 and EC-2 were similar to those for healthy controls. The level of MIP-1β production was elevated in EC-1, EC-3, and EC-11 relative to healthy controls (Fig. 3B), whereas EC-2 levels were similar to those in healthy controls. RANTES production did not show a significant difference between the samples (Fig. 3C). Additionally, we measured MIP-1α levels in serum isolated from these patients. In contrast to the results obtained for activated CD4+ T cell media, MIP-1α levels were similar in the sera of both resistant ECs and healthy controls (Fig. 3D).

**CCL3-like and CCL4-like gene copy numbers are elevated in EC-1, EC-2, and EC-3, and the STAT-1 mRNA expression level is elevated in EC-11.** The CCL genes encoding MIP-1α, MIP-1β, and RANTES lie in a cluster at chromosome 17q12 (53). The original genes, CCL3 and CCL4, are each present as a single copy and encode MIP-1α and MIP-1β, respectively. However, segmental duplication of this region has resulted in additional genes that exhibit copy number variation (CNV) (47). This CNV locus includes the full-length CCL3-like (CCL3L) genes CCL3L1 and CCL3L3 as well as a truncated gene, CCL3L2. These genes exhibit a high degree of homology to each other and the CCL3, with CCL3L1 being identical to CCL3L3 at the nucleotide level. Likewise, there are multiple CCL4-like (CCL4L) genes in this region, including CCL4L1 and CCL4L2, which are identical to each other at the amino acid level and highly homologous to CCL4. Moreover, this CNV region exhibits a complex architecture, with variations in breakpoints and CNVs inserted into larger CNVs (47).

The relative CCL3L gene copy number varies among different ethnic and racial backgrounds. The CCL-like gene copy number regulates chemokine production levels (45, 46). Some studies suggest that individuals with a high gene copy number (n) in relation to their racial background are less likely to seroconvert and have a lower rate of HIV progression to AIDS if seropositive (46).

**CCL3-like and CCL4-like gene copy numbers were therefore quantified by qPCR.** Both EC-1 and EC-3 exhibited high relative copy numbers of CCL3-like genes (n = 17.0 and 11.8, respectively) (Fig. 4A) and CCL4-like genes (n = 8.4 and 8.1, respectively) (Fig. 4B) for individuals of European descent. It is therefore likely that the higher relative copy numbers of CCL3-like genes and CCL4-like genes explain the increased levels of production of MIP-1α in EC-3 and MIP-1β in both individuals. Additionally, EC-2 exhibited a somewhat elevated relative gene copy number, higher than the copy number in EC-3 and EC-2 in both individuals. Additionally, EC-2 exhibited a somewhat elevated relative gene copy number for an individual of African descent (CCL3-like genes, n = 13.3 [Fig. 4A]; CCL4-like genes, n = 6.1 [Fig. 4B]), although we detected a normal level of chemokine production by ELISA and transcriptome analyses (Fig. 3 and Table 2). In contrast, EC-11 exhibited an intermediate relative gene copy number for an individual of African ancestry (CCL3-like genes, n = 6.7 [Fig. 4A]; CCL4-like genes, n = 3.2 [Fig. 4B]). It is therefore unlikely that the gene copy number is responsible for the increased levels of chemokines in EC-11. Whole-exome sequencing demonstrated that none of the patients in our cohort had mutations in CCL3 or CCL4 exonic sequences.

In order to further explore the mechanism of the increase in MIP levels in EC-11, we utilized qPCR to quantify the levels of three transcription factors that bind to the promoters of the CCL3, CCL3-like, CCL4, and CCL4-like genes. STAT-1 mRNA levels were elevated in EC-11 T cells versus healthy control T cells (Fig. 4C). In contrast, RUNX1 and C/EBPα mRNA levels were similar between EC-11 and control T cells (data not shown).

**MIP-1α and MIP-1β produced by EC-3 and EC-11 CD4+ T cells are necessary and sufficient to confer the R5-tropic HIV cellular resistance phenotype in a medium transfer experiment.** We employed a medium transfer experiment to determine whether the high levels of MIP-1α and MIP-1β were responsible

### Table 2: Transcriptome analysis of activated CD4+ T cells from EC-2 and EC-11 versus healthy controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcriptome expression value (no. of fragments/kb of exon/million fragments mapped)</th>
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<tr>
<td></td>
<td>EC-2</td>
<td>EC-11</td>
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<tr>
<td>IFIT2</td>
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</table>

*Transcriptome expression values (fragments per kilobase of exon per million fragments mapped) for EC-2, EC-11, and three healthy controls (HCs) (HC-1, HC-2, and HC-3) are shown. For the 17 genes shown, expression values were significantly different between ECs and healthy controls. Please note that this table omits a duplicate entry for CCL3L1, two processed pseudogenes, PSFHP1 and RP11-1082B1.1; and two transcripts with no database entry, which were also significant. P values were calculated from the difference in the distribution of observed log fold changes (i.e., Student’s t test on the log2 fold change) by using Cuflinks.*
We treated activated CD4\(^+\) T cells from a healthy donor with cell culture supernatants from EC-3, EC-11, or healthy control CD4\(^+\) T cells and then infected these cells with YU2-pseudotyped HIV. EC-3 and EC-11 supernatants suppressed YU2-pseudotyped HIV infection relative to healthy control supernatants (Fig. 5A). Furthermore, the addition of anti-MIP-1\(\alpha\) and anti-MIP-1\(\beta\) neutralizing antibodies reversed this effect (Fig. 5A). These data suggest that a secreted factor(s) from EC-3 and EC-11 T cells confers cellular resistance to R5-tropic HIV and that MIP-1\(\alpha\) and MIP-1\(\beta\) are necessary for this effect. In contrast, supernatants from EC-3 and EC-11 T cells did not alter infection by HXB2-pseudotyped HIV (Fig. 5B).

In a separate experiment, we treated CD4\(^+\) T cells from healthy controls with culture supernatants from cells transfected with pCCL3L1 (encoding MIP-1\(\alpha\)), pCCL4 (encoding MIP-1\(\beta\)), a control plasmid encoding \(\beta\)-arrestin, or complete RPMI. These cells were then infected with YU2-pseudotyped virus. Supernatants containing MIP-1\(\alpha\) or MIP-1\(\beta\) suppressed YU2-pseudotyped HIV infection relative to that of the \(\beta\)-arrestin control (Fig. 5C). The addition of the appropriate neutralizing antibody reversed this effect (Fig. 5C). These data indicate that MIP1-\(\alpha\) and MIP-1\(\beta\) are indeed sufficient to inhibit R5-tropic HIV infection.

**DISCUSSION**

This study demonstrates a novel HIV cellular resistance phenotype in a subset of ECs: CD4\(^+\) T cells were intrinsically resistant to R5-tropic HIV but fully susceptible to X4-tropic and VSV-G-pseudotyped HIV for 4 of 11 patients in our cohort (EC-1, -2, -3, and -11). The remaining patients in the cohort likely accomplish HIV control via other mechanisms that may be CD4\(^+\) T cell extrinsic. These and previous studies support the notion that ECs are a diverse group of patients who accomplish HIV control via multiple distinct and unknown mechanisms.

Previous studies investigated whether ECs harbor CD4\(^+\) T cells that are intrinsically resistant to HIV infection but obtained mixed results (24–34). We speculate that these conflicting results are a consequence of the different experimental conditions among these studies. Viral infection rates may be influenced by the CD4\(^+\) T cell activation protocol, the physiological state of the cells, the viral strains used, viral titers, cell density, the frequency of medium change, and the use of spinoculation or lack thereof. It is possible that the specific experimental conditions used in our study are critical to observe the phenotype of R5-tropic resistance in a subset of ECs. Alternatively, the patient characteristics of our cohort may be unique. Although it would be of interest to deter-
mine HIV tropism in these ECs, the low levels of plasma viremia make it extremely challenging to do so.

For three patients in our cohort, EC-1, EC-3, and EC-11, we determined that increased levels of MIP-1\(^+\)H9251\) and/or MIP-1\(^+\)H9252\) secretion were associated with cellular resistance. Activated CD4\(^+\) T cells from these patients produced significantly more MIP-1\(^+\)H9251\) (EC-3 and EC-11) and more MIP-1\(^+\)H9252\) (EC-1, EC-3, and EC-11) than did those from healthy controls. The level of RANTES production was not elevated in these patients. We did not observe an elevation in chemokine levels in the sera of these patients. It is likely that CD4\(^+\) T cells must be activated to induce chemokine production. Alternatively, it is possible that even unactivated CD4\(^+\) T cells produce more chemokines in these patients, but this is not sufficient to elevate serum levels. Even in the absence of activated serum chemokine levels, MIP-1\(^+\) T cells produce more chemokines in these patients, but this is insufficient to elevate serum levels. Even in the absence of activated serum chemokine levels, MIP-1\(^+\) locally produced by activated CD4\(^+\) T cells could bind cell surface CCR5 in an autocrine or paracrine fashion, inhibiting the entry of R5-tropic HIV.

High relative copy numbers of CCL3-like and CCL4-like genes were detected in EC-1 and EC-3 and likely account for the increased chemokine production that we observed in CD4\(^+\) T cells from these patients. Interestingly, the relative copy numbers of CCL3-like and CCL4-like genes were also somewhat elevated in EC-2. However, this individual displayed normal CCL3L1 gene expression levels and normal MIP-1\(^+\)\(1\alpha/\beta\) levels in stimulated T cell culture supernatants. It remains plausible that an elevated CCL copy number is somehow mechanistically responsible for cellular resistance in EC-2 despite not exhibiting an obvious elevation at the RNA or protein level in CD4\(^+\) T cells. In contrast, EC-11 had intermediate relative copy numbers of CCL3-like and CCL4-like genes for an individual of African ancestry. Hence, the copy number does not explain the elevated levels of chemokine production that we observed for this individual. EC-11 exhibited elevated mRNA levels of STAT-1, a transcription factor with a binding site in the CCL gene promoters. Elevated STAT-1 levels may thus play a role in cellular resistance in EC-11. However, the mechanism for the elevation of STAT-1 mRNA levels in this individual remains to be fully explored.

In a medium transfer experiment, secreted products from EC-3 and EC-11 CD4\(^+\) T cells were sufficient to confer cellular resistance to R5-tropic HIV. MIP-1\(^+\) and MIP-1\(^-\beta\) were necessary for this effect, since chemokine-neutralizing antibodies fully reversed the phenotype. Additionally, transfected medium containing the MIP-1\(^+\) \(1\alpha\) or MIP-1\(^-\beta\) chemokine suppressed R5-tropic HIV infection, indicating that the chemokines themselves were also sufficient for these effects, consistent with data from previous reports (30, 54).

Previous studies obtained mixed results regarding the association between the CCL3L1 and CCL4L1 copy numbers and HIV/
AIDS susceptibility and disease progression (reviewed by Hollox and Hoh [55]). For example, some reports demonstrated an association between high CCL3L1 copy numbers and a reduced prevalence of HIV seropositivity, slower progression to AIDS, and improved recovery of CD4+ T cells (45, 56–58), whereas other studies and follow-up correspondences failed to detect an association even within the same data set (59–61). Some researchers have suggested that the CCL3L1 copy number estimation method used in those association studies could be flawed, for example, due to DNA degradation, inconsistent quality among DNA samples, the use of inappropriate DNA amounts in the real-time PCR assay, inappropriate comparison of threshold cycle (CT) values from samples run in different real-time PCR experimental repeats, or inappropriate rounding of copy numbers to the nearest integer value. Of note, every DNA sample used in our study was isolated by the same investigator, using the same purification method. The DNA obtained was found to be of high quality, with a 260/280-nm light absorbance ratio of ~1.8 and no obvious degradation, as evidenced by agarose gel electrophoresis of the samples. The amount of DNA used for the real-time PCR assay was 6.6 ng for every sample, and all samples were included in every experimental replicate, with consistent values being observed between replicates. A serial dilution of the CCL3L1 plasmid DNA and genomic DNA suggested that the CT of the samples was within the range in which the 2ΔΔCT method yielded an appropriate estimation of the gene copy number. We did not round our copy number values to an integer but instead reported them to two or three significant digits. Finally, previous reports criticized the fact that multiple reports that found a positive association used duplicated patient cohorts. In contrast, the patient cohort in this report is unique and was not used in any previous studies.

Protective HLA alleles were previously implicated as a determining factor in HIV control versus progression (10). Most notably, the B*57 alleles are associated with HIV control. Seven of 11
patients in our cohort possessed B*57 alleles (EC-2, EC-4, EC-5, EC7, EC-8, EC-9, and EC-11), including two of the elite controllers who exhibited the pattern of R5-tropic cellular resistance described in this report (EC-2 and EC-11). It is plausible that CD4+ T cell resistance synergizes with an HLA-based mechanism (i.e., enhanced CD8+ T cell response to HIV antigens) to control HIV replication in these patients. We also observed reduced CD69 upregulation upon CD4+ T cell activation in the resistant ECs versus healthy controls. The significance of this finding is unclear.

In summary, the factors contributing to the natural control of HIV are likely multifactorial. Increased production of MIP-1α and MIP-1β can now be added as a mechanism that drives cellular resistance to R5-tropic virus in some of these individuals, likely contributing to their EC phenotype. Future studies will be required to determine precisely how the levels of these chemokines are elevated in EC T cells and their relative importance among all ECs and LTNPs. While limited by a small sample size, our findings support the continued investigation of coreceptors as a target for drug development and functional eradication of HIV. Whether the expression levels of the natural ligands for CCR5 or CXCR4 can be increased for therapeutic benefit with minimal risk remains to be explored.

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