Cytokines Elevated in HIV Elite Controllers Reduce HIV Replication *in vitro* and Modulate HIV Restriction Factor Expression

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Abstract

A subset of HIV infected individuals termed elite controllers (ECs) maintain CD4+ T cell counts and control viral replication in the absence of antiretroviral therapy (ART). Systemic cytokine responses may differentiate ECs from subjects with uncontrolled viral replication or those who require ART to suppress viral replication. We measured 87 cytokines in four groups of women: 73 EC, 42 with pharmacologically suppressed viremia (ART), 42 with uncontrolled viral replication (noncontrollers, NC), and 48 HIV uninfected (NEG) subjects. Four cytokines were elevated in ECs but not NCs or ART subjects: CCL14, CCL21, CCL27, and XCL1. In addition, median SDF-1 levels were 43% higher in ECs than NCs. The combination of the five cytokines suppressed R5 and X4 virus replication in resting CD4+ T cells, and individually SDF-1β, CCL14 and CCL27 suppressed R5 virus replication, while SDF-1β, CCL21, and CCL14 suppressed X4 virus replication. Functional studies revealed that the combination of the five cytokines up-regulated CD69 and CCR5 and down-regulated CXCR4 and CCR7 on CD4+ T cells. The CD69 and CXCR4 effects were driven by SDF-1, while CCL21 down-regulated CCR7. The combination of the EC-associated cytokines induced expression of the anti-HIV host restriction factors IFITM1 and IFITM2 and suppressed expression of RNase L and SAMHD1. These results identify a set of cytokines that are elevated in ECs and define its effects on cellular activation, HIV co-receptor expression, and innate restriction factor expression. This cytokine pattern may be a signature characteristic of HIV-1 elite control, potentially important for HIV therapeutic and curative strategies.

45 **Importance**

46 Approximately 1% of people infected with HIV control virus replication without taking antiviral
47 medications. These subjects, termed elite controllers (ECs), are known to have stronger immune
48 responses targeting HIV than the typical HIV-infected subject, but the exact mechanisms of how
49 their immune responses control infection are not known. In this study we identified five soluble
50 immune signaling molecules (cytokines) in the blood that were higher in ECs than in subjects
51 with typical chronic HIV infection. We demonstrated that these cytokines can activate CD4+ T
52 cells, the target cells for HIV infection. Furthermore, these five EC-associated cytokines could
53 change expression of intrinsic resistance factors, or molecules inside the target cell that fight
54 HIV infection. This study is significant in that it identified cytokines elevated in subjects with a
55 "good" immune response against HIV and defined potential mechanisms as to how these
56 cytokines could induce resistance to the virus in target cells.
57

58 **Introduction**

59 Innate and adaptive immune responses during primary HIV infection are vital in establishing
60 initial host immunologic control of viral replication (1, 2). The extent of HIV replication that
61 persists once host response is mature is a predictor of the subsequent pattern of CD4+ T cell loss
62 over time (3), and cellular immunity is one important factor influencing residual viral replication
63 (4, 5). While it may enable the host to avoid rapid immunologic injury, persistent HIV-associated
64 immune activation is associated with a range of adverse immunologic and clinical outcomes (6).
65 Individuals with rapid disease progression are characterized by high viral load (3), greater T cell
66 activation and turnover (7), and increased levels of inflammatory cytokines including C-reactive
67 protein, IL-6, and tumor necrosis factor (TNF)-α (8). Combination anti-retroviral therapy (ART)
68 significantly inhibits viral replication and dampens immune activation at the level of cellular
69 phenotype and production of inflammatory cytokines (9, 10). Determining the requirements for
70 immune-mediated viral control may support further improvements in HIV therapies and the
71 development of effective vaccines. The study of individuals who demonstrate natural immune-
72 mediated control of HIV replication may provide insight to discriminate effective vs. pathogenic
73 immune responses.
74
75 While HIV has likely been eliminated from one individual (11), generalizable eradication of the
76 virus from host reservoirs is difficult if not impossible with current therapeutic approaches. One
77 alternative to complete eradication of the virus would be functional cure, where replication is
78 controlled to very low levels without the need for continuous ART (12). Elite controllers (ECs)
79 are a subset of individuals who maintain very low levels of viral replication and relatively stable
80 populations of circulating CD4+ T cells without use of ART, and they show reduced immune
81 activation compared to subjects with higher viral load (13–15). The intensive study of ECs has

led to a better understanding of effective host immune responses; however, the exact mechanisms of control have not been elucidated fully. Early studies attributed elite control to infection with less fit or defective viruses (16, 17), while more recent studies describe ECs infected with virus with normal pathogenicity (18, 19). Both CD4+ and CD8+ HIV-specific T cell responses are stronger in ECs compared to subjects with progressive HIV infection (20–22). Some reports note that CD4+ T cells from ECs are less susceptible to HIV infection (23, 24), while others have found decreased virion production from HIV infected cells in ECs (25) as well as decreased viral integration into the cellular genome (26). Moreover, the EC phenotype is associated with specific HLA-B and C genotypes (27). However, genetic polymorphisms only explain 10-15% of the variation in the rate of HIV disease progression in untreated hosts (28, 29). Additionally, the EC phenotype is likely more common in women, who also demonstrate more potent host responses to a variety of pathogens and vaccines (30). As such, it appears that the mechanism(s) of elite control of infection are likely multifactorial.

Considering the varied host response to HIV infection, we sought to study how cytokines contribute to the control of HIV replication. It is known that ECs have elevated measures of inflammation compared to HIV uninfected subjects, which may be associated with HIV co-morbidities such as cardiovascular disease (31), and understanding which components of that inflammatory response are important for viral control would help define effective immunotherapy approaches. To answer this question a panel of soluble mediators selected to include pro-and anti-inflammatory cytokines, chemokines, growth factors, and soluble cytokine receptors was examined in four groups of women with distinctive HIV-related phenotypes: HIV seronegative, EC, ART-treated and aviremic, and off-ART, viremic subjects. We identified five cytokines with elevated expression in the EC compared to the viremic, ART, or HIV negative

groups and we further examined the ability of these cytokines both individually as well as in combination to suppress HIV replication. In addition, cytokine-induced modulation of the expression of multiple surface receptors on CD4+ T cells was measured, including the key HIV co-receptors, CCR5 and CXCR4, as well as the activation marker CD69. Finally, cytokine-induced modulation of innate HIV restriction factors was determined. This study builds on an extensive set of translational research data and sheds important light on which soluble factors of the host immune response can contribute to control of HIV in ECs.

Materials and Methods

Sample selection

Two or more serum samples for each subject were tested, with the samples chosen near the beginning and end of the period of clinical interest (i.e. during the period of elite control for the EC group, during the period of undetectable viremia for the ART group and during a period of the highest level viremia for the NC group). Study participants for each clinical group were drawn from the 1994-95 and 2001-2 enrollment waves of the Women's Interagency HIV Study (WIHS), a multi-site cohort study of HIV among US women. Participants for the current study were chosen from a total of 3,766 WIHS participants to match the three study groups with regards to ethnicity, age, body mass index, HCV antibody status at study entry, and duration of follow-up in the cohort (within one year). HIV non-controllers (NC, n=42; median of 3 time points/ID): antiretroviral therapy naive and had a viral load >2000 RNA copies/ml for at least one of two time points separated by 6 months. Elite Controllers (EC, n=73; median of 7 time points/ID): HIV RNA undetectable (≤80 copies/ml) for at least 2 years, were clinically AIDS-free, minimal prior exposure to ART, and had no more than one viral load blip of 80-1,000 copies/ml allowed per episode, but not at either of the two end visits of the suppression episode.

130 There were five EC who were pregnant during eight time points during the study; there were no
131 differences between the pregnant and non-pregnant time points. ART suppressed (ART, n=42;
132 median of 3 time points/ID): undetectable viral load (<80 RNA copies/ml) for at least 12 months
133 while on a potent combination antiretroviral regimen. HIV uninfected women (NEG, n=48;
134 median of 3 time points/ID) in WIHS undergo the same follow-up procedures as the HIV
135 infected women, and have HIV serology performed every 6 months. Finally, a sample was
136 analyzed from 11 additional female ECs from the SCOPE cohort at UCSF. All participants
137 provided informed consent for study participation per human subject protection protocols
138 approved by all participating institutions.
139
140 **Multiplex cytokine and chemokine analysis**
141 Serum samples were assayed using the High-sensitivity MilliPlex kit (Millipore, Billerica, MA)
142 for interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p70, IL-13, IFN-γ,
143 granulocyte macrophage stimulating factor (GM-CSF) and TNF-α. Standard sensitivity
144 Milliplex Map kit (Millipore) was used to test endothelial growth factor (EGF), fibroblast growth
145 factor (FGF)-2, fractalkine, IL-1α, IL-1 receptor antagonist (Ra), IL-9, IL-12(p40), IL-15, IL-17,
146 interferon induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, MCP-3, monocyte
147 derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β, sIL-2 receptor
148 a (Ra), tumor necrosis factor (TNF)-β, and VEGF. Standard Sensitivity Panel II kit (Millipore)
149 was used to test CCL8/MCP-2, CCL13/MCP-4, CXCL5/ human neutrophil-activating peptide
150 (ENA)-78, CXCL12/stromal derived factor (SDF)-1α/β, CXCL13/ B-cell attracting chemokine
151 (BCA)-1, CCL1/I-309, IL-16, CCL15/MIP-1δ, CCL17/thymus and activation regulated
152 chemokine (TARC), CCL21/6Ckine, CCL24/Eotaxin-2, CCL26/Eotaxin-3, CCL27/cutaneous T-
153 cell attracting chemokine (CTACK), IL-23, leukemia inhibitory factor (LIF), thrombopoiesis

stimulating factor (TPO), TNF-related apoptosis inducing ligand (TRAIL), stromal cell-derived cytokine (SCF), thymic stromal lymphopoietin (TSLP), IL-20, IL-21, IL-28A, and IL-33. Standard Sensitivity Panel III kit (Millipore) was used to test macrophage colony stimulating factor (M-CSF), CXCL9/monokine induced by gamma (MIG), neutrophil attractant protein (NAP), CXCL6/ granulocyte chemotactic protein (GCP)-2, CXCL11/ Interferon-inducible T cell alpha chemoattractant (I-TAC), CCL14/hemofiltrate CC-Chemokine (HCC)-1, CXCL19/MIP-3β, CCL20, MIP-3α, CL1/Lymphotactin, IL-11, and IL-29/IFN-λ1. Soluble Receptors: sCD30, sVEGF-R3, sVEGF-R2, sVEGF-R1, sTNFRII, sTNFRI, sRAGE, sIL-6R, sIL-4R, sIL-2Rα, sIL-1RII, sIL-1RI, sgp130, and sEGFR. Standards and samples were tested in duplicate. Beads were acquired on a Labscan analyzer (Luminex) using Bio-Plex manager 6.1 software (Bio-Rad). Values that were determined to be out of range (OOR) low were assigned ½ the lowest standard. Values that were determined to be OOR high were assigned 2 times the highest standard. Values that were extrapolated beyond the standard curve were assigned the determined value.

Viruses, cells, and reagents

Clonal virus stocks were generated by transfection of 4 x 10^6 293T cells with 10 µg of plasmid DNA from HIV molecular clones NL4-3, and 81.A. Transfections were carried out using Fugene 6 (Roche) at a ratio of 1.5 µl Fugene per 1 µg DNA according to the manufacturer's directions. Culture supernatants were harvested 48 hours post infection, centrifuged to remove cell debris, aliquoted, and stored at -80°C until use. The $TCID_{50}$ of each virus stock was determined in MT-2-$CCR5^{hi}$ cells. MT-2-$CCR5^{hi}$ cells were maintained at log phase in RPMI 1640 media (UCSF Cell Culture Facility (CCF)) supplemented with 20% heat-inactivated fetal calf serum (Hyclone), 12 mM HEPES (UCSF-CCF) and penicillin/streptomycin (UCSF-CCF) (R20). Apheresis filters from three donors were purchased from Blood Centers of the Pacific (BCP) and PBMC were

178 isolated, frozen, and maintained in liquid N_2. The cytokines SDF-1β, CCL21, XCL1, CCL27
179 (R&D Systems), and CCL14 (Peprotech) were re-suspended at 100 µg/ml in PBS with carrier
180 protein, aliquoted for single use, and stored at -80°C until use. Cytokines were used in assays at
181 0.5 µg/ml final concentration based on the manufacturer's recommended concentration and/or
182 titration data for suppression of HIV replication.
183
184 **Infection and virus culture assay**
185 PBMC from donors were depleted of CD8+ T cells via CD8 positive selection kits (Stem Cell
186 Technologies) pooled and infected with X4 (pNL4-3) or R5 (81-A) at a multiplicity of infection
187 (MOI) of 10^{-2} for 2hrs. Following infection, cells were washed and seeded into 96-well culture
188 dishes at $1x10^6$ cell/ml, in R20 with 50 IU/ml rhIL-2 and incubated in the presence or absence of
189 indicated cytokines (0.5 µg/ml). On Day 3, cells were washed and replenished with fresh media
190 and indicated cytokines without IL-2 (IL-2 treatment received 200 IU/ml rhIL-2). Following
191 culture, cell viability was determined with acridine orange and propidium iodide labeling using
192 an Auto X4 cell counter (Nexcelom Bioscience). Supernatants were harvested and maintained at
193 -80°C until analysis for HIV p24 by ELISA. Infection supernatants were measured for p24 using
194 the HIV-1 p24 antigen capture ELISA (Applied Bioscience Laboratories) according to the
195 manufacturer's instructions.
196
197 **Immunophenotyping**
198 For immunophenotyping, PBMC were cultured at $2x10^6$ cells/ml with indicated cytokines for 3,
199 6, and 24 hours. Following incubation, cells were washed with PBS and pelleted. Cells were first
200 labeled with Aqua Amine viability dye (Invitrogen) for 30 minutes and then subsequently
201 labeled with CD-3 PE, CD4-AF700, CD8-APC-Cy7, CCR5 AF647, CCR7 PE-Cy7, CXCR4

PerCP Cy5.5, CD38 BV 421, HLA-DR FITC (all from Biolegend) and CD69 PE-CF594 (BD Biosciences) for 20 minutes. Cells were fixed in stabilizing fixative (BD Biosciences) acquired on an LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar). Alternatively, cells were infected as above and cultured for 1 or 6 days prior to immunophenotyping.

Restriction factor qPCR array

CD4+ T cells from cryopreserved donor PBMC were isolated using the EasySep human CD4+ T cell negative selection kits (Stem Cell Technologies) according to manufacturer's instructions. CD4+ T cells were cultured overnight with indicated cytokines. Total RNA was extracted from cells using Qiazol reagent from miRNeasy Mini kit (Qiagen) with the on-column DNAase treatment option using Qiagen RNase-Free DNase Set. DNase-treated clean RNA was transcribe into cDNA using random primers and the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to manufacturer's instructions. A custom made TaqMan® Low Density Array (TLDA) was implemented to measure the mRNA expression of a comprehensive panel of 35 restriction factors with published, direct evidence of inhibitory activity against HIV-1 *in vitro* from Applied Biosystems. Thermal cycling was performed using an ABI ViiA™ 7 Real-Time PCR System. Up to 450 ng cDNA in 200 µl of Applied Biosystems TaqMan® Universal PCR Master Mix, with UNG was loaded onto the designated ports of the TLDA plates. Data was analyzed using the Applied Biosystems ViiA™ 7 software. A panel of six housekeeping genes was included in the TLDA plates (GAPDH, 18S, ACTB, PPIA, RPLP0, and UBC). RPLP0 (60S acidic ribosomal protein P0) was identified as the most stably expressed gene from those six housekeeping genes among all samples using the GeNorm algorithm (32). Therefore, raw cycle threshold numbers of amplified gene products were normalized to the housekeeping gene,

RPLP0, to control for cDNA input amounts. Fold induction was determined using the comparative CT method (32).

Quantitative PCR measurement of IFITM1 and IFITM2 mRNA expression

CD4+ T cells from 12 cryopreserved donor PBMC were isolated as above and cultured for 24 hrs with IFN-α (5 IU/ml, R&D systems), or combined cytokines (0.5 µg/ml). Following culture, cells were lysed and total RNA were extracted from the cells using the Allprep DNA/RNA/miRNA universal kit (Qiagen) with on-column DNAase treatment (Qiagen RNase-Free DNase Set). RNA was transcribed into cDNA using the SuperScript® VILO™ cDNA Synthesis kit (Invitrogen). Quantitative real-time PCR measuring interferon inducible transmembrane protein (IFITM) 1 and 2 using TaqMan® real time PCR was performed using the ABI ViiA™ 7 Real-Time PCR system. Fold induction was determined using the comparative Ct method as indicated above.

Western blot

CD4+ T cells from cryopreserved PBMC were isolated by negative selection (Stem Cell Technologies) and then cultured for 72 hrs with IFN-α, single cytokines or combined cytokines. Following culture, cell lysates were prepared using RIPA buffer (Thermofisher Scientific) supplemented with 'Complete' protease inhibitor (Thermofisher Scientific). Lysates were homogenized by sonication using a Branson Sonfier 150 (Branson Ultrasonics Corporation) and protein was quantified by Pierce BCA protein assay kit (Thermofisher Scientific). In brief, lysates were resolved on Bis-Tris mini gels and transferred overnight at 4°C onto Immobilon-FL PVDF membranes (EMD Millipore) using the X-Cell sure-lock western blot system (Life Technologies). Blocking and antibody incubation steps were performed using Odyssey blocking

buffer (LI-COR). IFITM1 and 2 (Cell Signaling Technologies, Cat. Number 13126 and 13530 respectively) primary antibodies were incubated for 2 hrs at 25°C. After wash steps in PBS-T (PBS plus 0.1% Tween-20), membranes were incubated with LI-COR infra-red secondary antibodies for 45 mins at 25°C. After further wash steps images were obtained using a LI-COR Odyssey scanner and quantified using LI-COR Image Studio software. Where stripping steps were necessary membranes were incubated for 5 mins with LI-COR Newblot PVDF stripping buffer (LI-COR).

Statistical analysis

For participants with data available from two or more time points (all but the three ART subjects noted above), median cytokine levels were calculated for the time points for analysis to minimize the effect of sample-to-sample variation on the association of cytokine levels with clinical group. For analyses of cytokine level correlations with viral load or CD4+ T cell count the observations from each subject were not averaged. Cytokine and viral load values were log-transformed prior to analysis due to non-normal distribution of the data. Differences in subject characteristics between groups were evaluated by ANOVA for continuous variables (Kruskal-Wallis/Mann-Whitney U). P-values were adjusted into FDR (False Discovery Rates) by the Benjamini and Hochberg controlling procedure, a commonly used method for analysis of large sets of biological data (33). Statistical significance was defined as $p < 0.05$ and FDR < 0.1. Linear regression analysis was performed and correlations were determined by Spearman and adjusted by FDR. R/Bioconductor software was utilized for analyses. For cytokines detected in fewer than 50% of subjects, comparisons across clinical groups were performed using the Chi square test using GraphPad Prism software. For viral infectivity and immunophenotyping, significant differences were determined by 2x3 ANOVA vs. media control and unstimulated respectively. For mRNA

273 expression of IFITM 1/2, significant differences were determined by repeated measures ANOVA
274 with Bonferroni's multiple comparison test.
275
276 **Results**
277 **Study subjects**
278 Subjects were classified as elite controllers (EC, n=73), ART suppressed (ART, n=42), non-
279 controllers (NC, n=42), or HIV negative (NEG, n=48); see **Table 1** and Materials and Methods
280 for description of clinical criteria for subject assignment. Eleven of the ECs were drawn from
281 the UCSF SCOPE cohort, and all the remaining samples were drawn from the WIHS cohort.
282 There were no significant differences among groups in terms of median age, race, serologic
283 evidence of HCV infection, or presence of HCV viremia at the time of WIHS enrollment. CD4+
284 T cell counts were significantly lower in the NC group compared to the EC and NEG groups. A
285 small percentage of the EC and NC groups had received ART prior to the period that defined the
286 group status for this present study, which includes treatment provided during pregnancy.
287
288 **Cytokines elevated in HIV infection.**
289 Eighty-seven analytes were chosen to include pro-and anti-inflammatory cytokines, chemokines,
290 growth factors, and soluble cytokine receptors, and these were measured in four HIV disease
291 groups. This was performed using five different multiplex assay kits, and values from all the
292 time points tested for each subject were averaged to give a single value per subject. Twenty-
293 three analytes had fewer than 50% of results detectable above the lowest standard point; these
294 were analyzed categorically using the Chi square test to compare the proportion of subjects with
295 detectable cytokines in each of the clinical groups (CCL26/eotaxin-3, IL-1α, IL-1β, IL-1ra, IL-
296 2Ra, IL-3, IL-4, IL-9, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-20, IL-21, IL-28A, IL-29, IL-

297 33, LIF, M-CSF, SCF, TNF-β, and TSLP). ECs showed a higher percentage of subjects with
298 detectable TSLP compared to ART subjects (p=0.03), ECs had a higher percentage of detectable
299 IL-20 compared to NEG subjects (p=0.03), and EC and NEG subjects had a higher percentage of
300 detectable IL-13 compared to ART and NC subjects (p=0.049). Given that the rate of detection
301 of these factors was less than 50% among the ECs, these cytokines were not further studied. Of
302 the 64 remaining analytes measured, 20 exhibited significant differences between the NEG group
303 and at least one of the HIV infected groups (**Table 2**).
304
305 **Elevated concentration of select cytokines in the plasma of HIV elite controllers**
306 If soluble factors played a role in immune control of HIV, we hypothesized that they would be
307 elevated in ECs compared to HIV-uninfected individuals or women with ART-associated viral
308 suppression. Furthermore, the factors should be elevated in ECs but not in viremic subjects,
309 since cytokines elevated in viremic subjects would likely be HIV antigen driven rather than
310 associated with viral control. We identified four cytokines that were significantly elevated in EC
311 compared to the HIV negative or ART suppressed groups that were not elevated in the viremic
312 subjects ($p<0.05$, FDR<0.1, **Table 3**). Hemofiltrate CC-chemokine-1 (HCC-1, CCL14) and
313 cutaneous T-cell attracting chemokine (CTACK, CCL27) were significantly elevated only in
314 ECs compared to NEG subjects, while 6Ckine (CCL21) was elevated in ECs compared to NEG
315 and NC subjects and in ART vs. NC subjects (**Fig. 1**). Stromal cell-derived factor-1 (SDF-1,
316 CXCL12) was elevated in all HIV infected groups compared to NEG subjects but was included
317 for further study given its known anti-HIV activity and the fact that median levels were 43%
318 higher in ECs than NCs. Finally, lymphotactin (XCL1) was elevated in EC compared to ART
319 subjects (p=0.04), though the difference was not significant after FDR correction. Of note,
320 soluble EGF receptor was also uniquely elevated in EC compared to NEG but was not studied

321 further due to lack of available reagents. Of the five cytokines selected for further *in vitro* study,
322 CCL14 (34), SDF-1 (35, 36), and XCL1 (37) have been previously associated with control of
323 HIV infection.
324
325 **EC-associated cytokines individually and in combination suppress HIV replication**
326 To extend our findings that five cytokines are significantly elevated in the plasma of elite
327 controllers, we measured the effects of these cytokines on HIV infection and replication. To do
328 this, pooled CD8-depleted peripheral blood mononuclear cells (PBMC) from three uninfected
329 healthy donors were infected and cultured in the presence or absence of the indicated cytokines
330 for six days as described in the Materials and Methods. To assess for productive viral infection
331 and replication, viral p24 production was measured by ELISA in the culture supernatant on day
332 6. Individual cytokines were first titrated from 0.1 to 1000 ng/ml to measure suppression of
333 virus, with peak suppression seen for most in the 100-1000ng/ml range (data not shown). Given
334 cost considerations and the manufacturers' recommended concentration range, we tested each of
335 the cytokines at 500 ng/ml. When co-cultured with individual cytokines, SDF-1β, CCL14 and
336 CCL27 all significantly suppressed production of p24 (33%, 76%, and 38% suppression,
337 respectively) when cells were infected with the CCR5-tropic strain 81-A (**Fig. 2A**). As expected,
338 addition of exogenous IL-2 enhanced production of HIV p24. Additionally, SDF-1β, CCL21,
339 and CCL14 significantly suppressed production of p24 (69%, 36%, 54% suppression,
340 respectively) when cells were infected with the CXCR4-tropic strain NL4-3 (**Fig. 2B**). It is
341 interesting to note that SDF-1β and CCL14 each showed some suppression of R5 or X4 virus,
342 respectively, in spite of not targeting these co-receptors. Following these studies, we sought to
343 determine how the five cytokines affected HIV replication in combination. The combined
344 cytokines mediated significant suppression of HIV replication using both 81-A and NL4-3

345 viruses (86% and 93% suppression respectively, **Fig. 2C** and **D**). These results show that several
346 of the individual cytokines found elevated in the serum of elite controllers are capable of
347 inhibiting HIV replication, and this suppression is greatest when the cytokines are combined
348 together.
349
350 It has been previously shown that infection conditions such as exogenous activation or high virus
351 inoculum can influence viral replication (24, 26). Although these cultures were not exogenously
352 activated, there was a possibility that these results were affected by allogeneic stimulation caused
353 by pooling of PBMCs from three different donors. To determine if allogeneic stimulation
354 affected our results, we performed the infectivity assays with three donors individually and
355 pooled (**Fig. 3**). With both 81-A and NL4-3 infectivity assays, the cytokine combinations
356 significantly suppressed HIV replication to similar degrees in all three donors (74-92% and 89-
357 96%, respectively) which was also similar to the results using pooled PBMC (93% and 97%
358 suppression). These results indicate that four of the five cytokines identified as being elevated in
359 ECs have significant anti-HIV activity *in vitro,* and that the combination of these five cytokines
360 yields more potent suppression than any one isolated cytokine.
361
362 **Cytokine-induced modulation of CXCR4, CCR5, CCR7, and CD69 on CD4+ T cells**
363 The role of aberrant immune activation in HIV pathogenesis is well documented and it is clear
364 that elite controllers are distinguished by a relative lack of immune activation and skewed
365 cellular phenotype compared to non-controllers (38). Considering our results that a set of
366 cytokines could suppress HIV replication, we next tested how these cytokines influence the
367 phenotype and function of common targets of HIV infection. PBMC from individual donors

368 were stimulated for 3, 6, and 24 hours with cytokines individually or in combination. No
369 differences in HLA-DR and CD38 expression were observed in cytokine-treated CD4+ T cells
370 (data not shown). CXCR4 surface expression was strongly suppressed (or fluorescent antibody
371 binding was blocked) by SDF-1β or combined cytokine treatment at all time points (**Fig. 4**).
372 There were no significant changes in CCR5 or CCR7 expression at any of the time points,
373 though CCL14 treatment decreased CCR5 expression by 20% compared to untreated cells (**Fig**
374 **4B** and **C**). Interestingly, we observed increased CD69 expression at all three time points in
375 CD4+ T cells stimulated with combined cytokines (**Fig 4D**).
376
377 To further explore the influence of these cytokines on T cell phenotype, similar analyses were
378 performed following infection with HIV. CD8-depleted PBMCs from individual donors were
379 infected with HIV NL4-3 in the presence of the indicated cytokines, and then expression of
380 CCR5/7, CXCR4 and CD69 was measured (**Fig 5**). Following infection for 1 day, we observed
381 significantly increased expression of CD69 in cells incubated with SDF-1β and combined
382 cytokines (**Fig 5A**). CCR5 expression was reduced by CCL14 individually but notably not by
383 the combined cytokines (**Fig 5B**), and CXCR4 expression was significantly reduced when
384 incubated with SDF-1β as well as the combined cytokines (**Fig 5C**). No significant change was
385 seen in CCR7 levels at 24 hours (**Fig 5D**). Next, we performed these analyses with CD8-depleted
386 PBMCs infected for 6 days. As with the single day infections, CXCR4 was significantly reduced
387 when cells were incubated with SDF-1β (**Fig 5E**) and combined cytokines (**Fig 5F**). In addition,
388 combined cytokine incubation resulted in elevated CCR5 expression (**Fig 5G and H**) while
389 CCL21 and combined cytokine incubation resulted in significantly reduced CCR7 expression
390 (**Fig 5I and J**). Consistent with CD69 being an early activation marker (39), no significant
391 changes were seen in CD69 levels at 6 days (data not shown). It is evident from these data that

392 the cytokines we found to be elevated in the plasma of elite controllers can influence the
393 phenotype of CD4+ T cells, especially the markers that are indicative of activation and critical to
394 infection by HIV.
395

396 **Cytokine stimulation induces IFITM1 and IFITM2 expression**

397 Intrinsic immunity is an important mechanism for the immune system to fight viral infections,
398 and there is evidence that host restriction factors play a role in the ability of interferon (IFN)-α to
399 suppress HIV replication (40). We therefore tested whether the cytokines able to suppress HIV
400 replication induced expression of intrinsic restriction factors in target cells. We utilized a
401 customized mRNA profiling array to measure the expression of 31 different innate restriction
402 factors (see Materials and Methods). CD4+ T cells were isolated from three donors and
403 incubated for 24 hours with IL-2, combined cytokines, or media. Following this incubation we
404 measured the expression of all 31 restriction factors (**Fig 6A**). Compared to the unstimulated
405 condition, interferon inducible transmembrane proteins 1 and 2 (IFITM1/2) had significantly
406 elevated relative copy numbers and RNase L and SAMHD1 had significantly decreased relative
407 copy numbers after combined cytokine treatment (**Fig 6B**). To confirm the up-regulation of
408 IFITM1 and IFITM2, we utilized quantitative PCR to measure the relative expression of IFITM1
409 and IFITM2 in the CD4+ T cells of 10 healthy donors. CD4+ T cells were stimulated for 24
410 hours and 72 hours with combined cytokines or with IFN-α as a positive control. We detected a
411 significant elevation in mRNA expression of IFITM1 at 72 hours but not at 24 hours (**Fig 7A**).
412 Additionally, we did not detect a difference in IFITM2 levels at 24 or 72 hours by quantitative
413 PCR (**Fig 7B**). To determine if changes seen at the level of RNA expression translated to
414 differences in protein levels, western blots were performed on parallel samples incubated for 72

hours with IFN-α or combined cytokines. We were able to detect expression of IFITM1 in CD4+ T cells, and this expression was significantly elevated in both the IFN-α stimulated control as well as in the samples incubated with the combined cytokines (**Fig 8A**). We also confirmed basal expression of IFITM2 in the T cells and found a significantly higher level of IFITM2 at the protein level when stimulated with the combined cytokines (**Fig 8B**). While the induction of IFITM1/2 mRNA was modest, at the protein level there was approximately two-fold induction of both these restriction factors by the combined cytokine treatment. It is clear from these data that the combination of cytokines found elevated in ECs is able to modulate expression of innate restriction factors.

Discussion

The current study examined what cytokines from a broad panel of potentially important mediators were elevated in women who control HIV in the absence of ART. From a panel of 87 cytokines, 4 were found to be elevated in ECs and not elevated in NCs or ART subjects. In addition SDF-1 was included for further analysis given modestly higher levels in ECs compared to NCs and its known anti-HIV properties. Identified cytokines included factors previously associated with HIV control, including CCL14, SDF-1, and XCL1. In addition, we noted that CCL21 and CCL27 levels were elevated in ECs, and to our knowledge this is the first report demonstrating that CCL27 is elevated in elite controllers and can suppress virus replication *in vitro*. We further showed that the combination of the five cytokines resulted in up-regulation of CD4+ T cell CD69 surface expression and down-regulation of CXCR4 at 24 hours, and down-regulation of CCR5 and CCR7 expression at six days. The CD69 and CXCR4 effects were driven by SDF-1, while CCR7 down-regulation was induced by CCL21. We also demonstrated that the combination of the five EC-associated cytokines induced expression of IFITM1 mRNA

439 and induced expression of both IFITM1 and 2 at the protein level. These results identify a set of
440 cytokines that are elevated in EC subjects and define its effects on cellular activation, HIV co-
441 receptor expression, and innate restriction.
442
443 Multiple studies have measured levels of circulating cytokines in HIV infected subjects
444 (reviewed in (41)), with many measuring a correlation between HIV virus load and cytokines. In
445 prior work we found that CXCL10 correlated with HIV viral load, while IL-17, CCL22, CXCL9,
446 and fractalkine (CX3CL1) all showed an inverse correlation with viral load (10). IL-21 has also
447 been shown to inversely correlate with viral load (42). IL-10, IL-18, and sCD30 correlated
448 positively with viral load in a cohort of subjects with low viral load (<1,000 RNA copies/ml) and
449 non-controllers (43). Very few studies have examined systemic cytokine levels in ECs. A recent
450 study by Platten et al. comparing ECs to viremic controllers (with viral load between 50 and
451 6,000 RNA copies/ml) found that of 25 cytokines measured, 3 showed lower levels in ECs:
452 CXCL10, CXCL9, and CCL4 (44). In our current study the median level of CXCL10 was 40%
453 lower in ECs, CXCL9 was 57% lower in ECs, and CCL4 was 13% higher in ECs compared to
454 NCs. The results of our study recapitulated those of Platten et al. with the exception of CCL4. It
455 has been shown that a subset of ECs contain CD4+ T cells that produce high levels of CCL4,
456 rendering these cells resistant to R5 virus infection (45). In total, our identification of cytokines
457 elevated in ECs but not NCs or ART subjects revealed cytokines not previously associated with
458 control of HIV infection, and our findings were largely consistent with the few prior comparative
459 observations made in ECs.
460
461 Advantages of the current study include the large panel of analytes measured in a large enough
462 cohort to observe the population distribution of cytokines. There are also limitations of the study

463 worth mentioning. By including CD4 count in the propensity matching score with ECs, the
464 resulting NC group of women had relatively low viral loads (median 1,100 RNA copies/ml), and
465 including women with a higher viral load may have allowed better differentiation between the
466 EC and NC women. In addition, XCL1 was included in the *in vitro* analyses in spite of failing
467 the FDR correction for significant elevation in ECs compared to the ART group. In an original
468 analysis XCL1 was significantly elevated so it was included in *in vitro* studies. It was later
469 discovered that a subject had been misclassified, and on re-analysis of the data XCL1 failed the
470 FDR test for significance. It should also be noted that the concentration of cytokines used in the
471 virus culture assays (500 ng/ml) is approximately 2 logs higher than that found in the plasma of
472 elite controllers. We assume but have not proven that cytokine concentrations would be higher
473 in the local environment of the lymph node or other close intercellular signaling environments.
474 Finally, two well-characterized laboratory-adapted X4 and R5 HIV viruses were examined and
475 yielded reproducible results, but multiple X4 or R5 strains or primary isolate viruses were not
476 tested.
477
478 To the best of our knowledge there has been very little reported linking CCL27 to HIV infection,
479 just one report of elevated CCL27 in HIV infected subjects with eosinophilic dermatitis
480 compared to HIV infected subjects without eosinophilic dermatitis (46). CCL27 binds to T cells
481 expressing CCR10 and is thought to be important in attracting these cells to the skin during
482 inflammatory responses (47). It is possible that CCL27 is associated with more vigorous HIV-
483 specific immune responses in ECs (15), as vaccination studies using CCL27 plasmid DNA as an
484 adjuvant demonstrated enhanced T cell and antibody responses, including at mucosal sites (48,
485 49).
486

Our data showed higher levels of CCL21 in EC and ART compared to NC subjects, which is in contrast to a prior report showing decreased levels of CCL21 after initiation of ART, and a correlation of CCL21 with viral load in a cross-sectional analysis (50). In viremic subjects, stimulation of PBMCs with CCL21 increased TNF-α secretion, while the same effect was not seen in HIV uninfected subjects or those with low viral load (51). Incubation of resting CD4+ T cells with CCL21 does not cause activation of the cells (52), consistent with our data. However, CCL21-treated cells incorporated more HIV DNA after *in vitro* infection, showing a potential role for the chemokine in promoting HIV latency (52). In contrast to our data showing inhibition of X4 virus replication by CCL21 in resting, CD8-depleted CD4+ T cells, a prior report showed enhanced HIV replication of R5 and X4 viruses in previously activated PBMCs (53). One limitation of our study is that we measured cytokine levels in peripheral blood, but these factors likely exert much of their effects in the microenvironment of lymph node and gut lymphoid tissues. It was recently shown that CCL21 expression is decreased in the lymph nodes in both acute and chronic HIV infection (54). Finally, the kinetics of CCL21 interaction with CD4+ T cells might be important, as CCR7 was down-regulated on the surface of CCL21-treated cells after six days but not by 24 hours (**Fig 5D and I**).

In spite of being elevated in ECs, XCL1 did not show HIV suppressive activity, irrespective of virus co-receptor usage in our assays. These data are consistent with findings published after our experiments were performed showing that an alternative all-β conformation of XCL1 forms a dimer, binds glycosaminoglycans, and suppresses HIV replication via blockade of attachment and entry into cells, and that this form of the protein lacks chemotactic activity (37). The protein supplied by R&D Systems is tested to demonstrate chemotactic activity, so we conclude that the version of XCL1 used in our experiments was in the classic XCL1 conformation and would not

511 be expected to suppress HIV replication. In fact, the R&D Systems XCL1 was tested recently
512 and was shown to have only modest suppressive activity compared to other preparations (55). If
513 XCL1 were to be utilized for anti-HIV therapy it would need to be formulated in the β-dimer
514 form, and a variant with a disulfide bond that stabilizes the all-β conformation has been
515 engineered (56).
516
517 CCL14 has been reported to suppress R5 but not X4 virus replication (34), while the current
518 study showed significant suppression of both R5 and X4 virus. In addition, the ability of CCL14
519 to suppress CCR5 expression was modest at day 1 and not detected at day 6 **(Fig. 5)**, implying
520 that the ligand may have had relatively low affinity for CCR5. The commercial preparation of
521 CCL14 used for the current study contained amino acids 2-74 of the complete peptide, while
522 peak suppression of R5 virus was found to reside in a truncated protein spanning residues 9-74 of
523 the sequence, with a peptide spanning residues 6-74 of CCL14 unable to suppress HIV
524 replication (57). It is unclear whether or not the longer peptide used in the current study
525 underwent proteolytic processing *in vitro* to gain activity or whether the commercial peptide
526 acted in a fashion independently of CCR5 blocking activity.
527
528 Interestingly, in the current study SDF-1β, which blocks HIV entry via the CXCR4 receptor,
529 caused activation of CD4+ T cells *in vitro*. The anti-HIV effects of this molecule may in fact
530 drive some of the residual immune activation seen in EC subjects. SDF-1 exists in two
531 predominant forms in humans, SDF-1α, and SDF-1β, which is identical to SDF-1α but possesses
532 four additional C-terminal residues (58). The original articles describing suppression of HIV
533 replication of X4 but not R5 virus used SDF-1α (35, 36). SDF-1β is about twice as potent as
534 SDF-1α in suppressing HIV replication, consistent with our results in Figure 2A-B, in spite of

the fact that the CXCR4 binding domain resides in the N-terminus of the protein (59). Subsequent work showed that the peptides from the C-terminus of SDF-1β but not SDF-1α can suppress X4 HIV replication, independently of binding CXCR4. Our results showing suppression of R5 virus by SDF-1β but not by SDF-1α are consistent with the concept that the C-terminal portion of SDF-1β possesses HIV suppressive activity independent of CXCR4 blockade. The differential ability of the SDF-1 isoforms to suppress HIV does not appear to be linked to activation of the target cells, as SDF-1α and β induced a similar degree of CD69 up-regulation on CD4+ T cells at 24 hours (data not shown). Taken together, our results support the notion that SDF-1β has the ability to suppress HIV replication through CXCR4-dependent and independent mechanisms.

The correlative data showing higher levels of a subset of cytokines in ECs but not in NCs could point to cytokines that contribute to the EC phenotype or alternatively are merely markers for the phenotype. Our *in vitro* data suggest that these cytokines play a role in suppression of HIV replication. The mechanism for some cytokines such as CCL14 and SDF-1 is at least partially mediated through blocking of HIV co-receptors. We also found that the combination of cytokines we identified increased CD69 expression and decreased CXCR4 expression at 24 hours post-HIV infection, and increased CCR5 and decreased CXCR4 and CCR7 expression 6 days post-infection *in vitro*. Decreased CXCR4 expression would protect against infection with X4 virus, consistent with an anti-HIV effect of the combined cytokines. The early up-regulation of CD69 and later up-regulation of CCR5 implies activation of host CD4+ T cells in response to the cytokines, which is typically thought to make cells more susceptible to HIV infection (60). However, if this activation were associated with enhanced cell-intrinsic immunity, the deleterious effects of cellular activation could be balanced by intracellular blockade of viral

559 replication. Finally, the later down-regulation of CCR7 could affect T cell migration to lymph
560 nodes. Given the fact that residual viral replication in ECs appears to be concentrated in lymph
561 node CD4+ T follicular helper and memory cells (61), the interaction between CCL21 and CCR7
562 could be important in maintaining the EC phenotype.
563
564 We found that the combination of cytokines studied increased expression of the restriction
565 factors IFITM1 and IFITM2, while it decreased expression of RNase L and SAMHD1. Our *in*
566 *vitro* infection data point to increased resistance of cells to HIV infection after exposure to the
567 combination of cytokines studied, though it is not clear what the relative effects of increasing
568 some restriction factors and decreasing others would have *in vivo*. The interferon-induced
569 transmembrane proteins were recently shown to suppress HIV replication (62). IFITM2 but not
570 IFITM1 impedes HIV entry into cells, and neither protein affects cell proliferation or CD4 cell
571 surface expression, though the intracellular moiety of IFITM1 is required for anti-HIV activity
572 (62). More recently it has been shown that IFITMs, particularly IFITM2 and IFITM3, co-
573 localize with Env and Gag proteins and can be incorporated into nascent virions, which can
574 impair fusion to target cells (63, 64). IFITMs have relatively modest HIV suppressive activity,
575 and it is hypothesized that these proteins act in part by interfering with viral protein production
576 (65). The NL4-3 strain of HIV has been reported to be resistant to inhibition by full-length but
577 not C-terminal truncated IFITM1, potentially due to differential cellular localization of the two
578 IFITM1 protein species (66). HIV can mutate Vpu and Env genes to increase cell-to-cell
579 transmission and avoid IFITM1 restriction (67). Finally, IFITM1 expression has been shown to
580 be elevated in CD4+ T cells from HIV infected subjects, and the percentage of activated
581 CD4+CD38+HLA-DR+ T cells from elite controllers correlates strongly with IFITM1
582 expression (68). How IFITMs mediate HIV suppression is an area of active research, and the

583 combination of cytokines elevated in ECs provides a second mechanism in addition to IFN-α for
584 induction of these HIV restriction factors.
585
586 In conclusion, the current study identified four cytokines elevated in ECs but not NCs, in
587 addition to SDF-1 which was relatively elevated in ECs compared to NCs. These cytokines can
588 modulate CD4+ T cell activation, HIV co-receptor expression, and expression of the HIV
589 restriction factors IFITM1, IFITM2, RNase L, and SAMHD1. Of note, incubation of target cells
590 with the combination of cytokines studied resulted in more potent suppression of HIV replication
591 than individual cytokines at the same doses. The data presented here provide rationale for pre-
592 clinical testing of these cytokines in animal models of HIV, particularly for studying
593 combinations of cytokine therapies. Understanding the cytokine profile associated with control
594 of HIV could be critical to establishing post-ART suppression of viral replication in designing a
595 functional cure for HIV. Furthermore, the cytokine profile we identified has implications for
596 evaluation of responses induced by preventive and therapeutic HIV vaccines.
597
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603
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878 **Figure Legends**

879 **Figure 1. Correlation of cytokines with elite control of HIV**

880 Multiplex assays were used to measure 87 cytokines in samples from patients in the WIHS and
881 SCOPE cohorts. Sample groups included: 74 EC, 42 ART, 42 Viremic, and 49 HIV negative
882 patients. Bars represent median values. Asterisks denote statistical significance as determined by
883 Mann-Whitney test. Corrections for multiple comparisons were made using the method of
884 Benjamini and Hochberg, with false discovery rate (FDR) <0.1 and $p<0.05$ considered
885 significant *$p<0.05$, **$p<0.01$.

886

887 **Fig 2. *In vitro* suppression of HIV by individual and combined cytokines**

888 Pooled CD8-depleted PBMC from three donors (MLR stimulated) were infected with (**A**) 81-A
889 or (**B**) NL4-3 virus at an MOI of 10^{-2} and co-cultured with individual indicated cytokines. **(C, D)**
890 Infections were co-cultured with SDF-1α/β, CCL21, XCL1, CCL14, and CCL27 (Combo), IL-2
891 or media alone. Supernatants were measured for p24 by ELISA on day 6. Data from 4
892 experiments were combined for analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$, 2x3 ANOVA vs.
893 media control.

894

895 **Fig 3. *In vitro* suppression of HIV in individual and pooled donor PBMC**

896 Infections with (**A**) 81-A and (**B**) NL4-3 virus were performed as previously described in pooled
897 (MLR-stimulated) or non-pooled (resting) PBMC and co-cultured with combined SDF-1α/β,
898 CCL21, XCL1, CCL14, and CCL27 (Combo), IL-2 alone, or media alone. Culture supernatants
899 were measured for p24 on day 6. Data were combined for analysis from two experiments.

900

Fig 4. Cytokine stimulation of PBMC increases CD69 and decreases CCR7 and CXCR4 expression

PBMCs from three donors were separately stimulated with individual cytokines or combined SDF-1α/β, CCL21, XCL1, CCL14, and CCL27 (Combo) for the indicated times. Following incubation, surface expression of (**A**) CXCR4, (**B**), CCR5, (**C**) CCR7, and (**D**) CD69 was measured by flow cytometry. Mean and SEM for three donors are shown. *p<0.05, **p<0.01, 2x3 ANOVA vs. unstimulated condition.

Fig 5. HIV infected cultures co-cultured with cytokines increases CD69 and decreases CXCR4, and CCR7 expression

Resting CD8-depleted PBMCs from three donors were infected with HIV NL4-3 and co-cultured with indicated cytokines. Combo indicates co-culture with SDF-1α/β, CCL21, XCL1, CCL14, and CCL27. Following infection for 1 day cells were measured by flow cytometry for expression of (**A**) CD69, (**B**) CCR5, (**C**) CXCR4, and (**D**) CCR7. Cells from 6-day infections were measured by flow cytometry for expression of (**E-F**) CXCR4, (**G-H**) CCR5 and (**I-J**) CCR7. Individual and combined cytokine experiments were performed separately. Mean and SEM for three donors are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, 2x3 ANOVA vs. media control.

Fig 6. Gene expression profile of HIV restriction factors

CD4+ T cells from three donors were negatively selected and stimulated overnight with indicated cytokines. Combo indicates SDF-1α/β, CCL21, XCL1, CCL14, and CCL27. **(A)** 31 HIV innate restriction factors were measured on a custom qPCR array and normalized to housekeeping genes. **(B)** Elevated expression of IFITM1 and IFITM2 mRNA (top), and

925 decreased expression of RNase L and SAMHDI mRNA (bottom). Fold induction was
926 determined using the Ct method. Mean and SEM of three donors are shown, and conditions were
927 compared using repeated measures ANOVA. *p<0.05, **p<0. 01.
928
929 **Fig 7. Elevated mRNA expression of IFITM1 and IFITM2**
930 CD4+ T cells from 10 donors were negatively selected and stimulated for 24 or 72 hrs with IFN-
931 α, combined cytokines (SDF-1α/β, CCL21, XCL1, CCL14, and CCL27), or media alone. Lysates
932 were then measured by qPCR for **(A)** IFITM1 and **(B)** IFITM2. Mean and SEM are shown, with
933 comparisons between groups made using repeated measures ANOVA. *p<0.05, ****p<0.0001
934
935 **Fig 8. Cytokine induced protein expression of IFITM1 and IFITM2**
936 CD4+ T cells were negatively selected and stimulated with IFN-α, combined cytokines (SDF-
937 1α/β, CCL21, XCL1, CCL14, and CCL27), or media alone for 72 hrs. **(A)** Total cell lysates were
938 transferred to PVDF membranes and probed for expression of IFITM1 (left) or IFITM2 (right).
939 GAPDH was used as a loading control. Images from two representative experiments are shown.
940 **(B)** Plots summarize the expression ratio as a percentage of IFITM1 or IFITM2 compared to
941 GAPDH expression after each treatment. Mean and SEM from 10 donors are shown;
942 comparisons were made using repeated measures ANOVA of log-transformed data, *p<0.05,
943 **p<0.01.
944
945

946 **Table 1. Demographics of study cohort**

	EC	ART	NEG	NC
Number	73	42	48	42
Median age (years) (IQR)	42 (34-47)	40 (35-46)	39 (32-47)	36 (32-42)
CD4 count (cells/µl) (IQR)	720 (562-929)	729 (528-934)	824 (682-1094)	625 (453-796)
HIV VL (RNA copies/ml) (IQR)	<80 (<80-<80)	<80 (<80-<80)	- -	1100 (448-3475)
Race				
Black	73%	76%	73%	79%
Hispanic	12%	17%	19%	12%
White	10%	7%	6%	7%
Other	3%	-	-	2%
Asian/Pacific Islander	1%	-	-	-
Native American	1%	-	2%	-
HCV antibody+	40%	33%	35%	26%
HCV RNA+	25%	19%	29%	14%
History of ART	7%	100%	-	5%

947

948

949 **Table 2. Cytokine levels by study group**

Cytokine	NEG	EC	ART	NC
CCL1/I-309	2 (1 - 3.4)	2.4 (1 - 3.9)	2.9 (1.6 - 4.1)	2.4 (1.4 - 3.7)
CCL2/MCP-1	400 (300 - 600)	400 (290 - 550)	460 (340 - 640)	430 (300 - 650)
CCL3/MIP-1α	90 (39 - 190)	60 (25 - 160)	60 (23 - 150)	**60 (34 - 110)**
CCL4/MIP-1β	70 (46 - 110)	80 (50 - 140)	80 (48 - 140)	70 (40 - 100)
CCL7/MCP-3	6.8 (1.6 - 24)	3.1 (1.6 - 25)	1.6 (1.6 - 13)	1.6 (1.6 - 12)
CCL8/MCP-2	46 (34 - 57)	**56 (41 - 77)**	**59 (42 - 80)**	**65 (46 - 97)**
CCL11/Eotaxin	85 (49 - 150)	94 (61 - 140)	85 (68 - 150)	82 (56 - 120)
CCL13/MCP-4	80 (50 - 140)	**105 (65 - 170)**	**140 (80 - 230)**	**110 (70 - 150)**
CCL14a/HCC-1	3400 (1700 - 5200)	**5200 (2600 - 16300)**	5500 (3100 - 20000)	3700 (2100 - 11700)
CCL15/MIP-1δ	2800 (1500 - 4100)	3800 (2300 - 5600)	3600 (2100 - 5700)	2500 (1700 - 5100)
CCL17/TARC	120 (77 - 160)	110 (65 - 190)	160 (100 - 230)	140 (97 - 210)
CCL19/MIP-3β	110 (75 - 170)	110 (76 - 150)	110 (76 - 150)	**160 (116 - 220)**
CCL20/MIP-3α	13 (8.5 - 22)	12 (8.7 - 21)	13 (9.3 - 22)	13 (7.6 - 23)
CCL21/6Ckine	520 (340 - 720)	**730 (470 - 1000)**	600 (450 - 770)	530 (400 - 690)
CCL22/MDC	2500 (1900 - 3400)	2700 (1900 - 3500)	3000 (2300 - 4000)	2200 (1800 - 3100)
CCL24/Eotaxin-2	510 (310 - 790)	650 (400 - 1070)	580 (350 - 950)	490 (270 - 800)
CCL27/CTACK	530 (400 - 750)	**710 (520 - 930)**	640 (510 - 840)	630 (450 - 870)
CXCL5/ENA-78	2800 (1300 - 4200)	2800 (1200 - 4800)	2800 (1300 - 4900)	3100 (1800 - 4600)
CXCL6/GCP-2	260 (160 - 350)	220 (140 - 350)	290 (160 - 370)	290 (190 - 420)
CXCL7/NAP-2	7100 (5800 - 8900)	9500 (6900 - 12300)	8800 (6800 - 11100)	8000 (5600 - 9300)
CXCL8/IL-8	16 (6.2 - 48)	13 (6.2 - 32)	14 (5.2 - 40)	13 (6.5 - 26)
CXCL9/MIG	800 (480 - 1400)	**1300 (800 - 2700)**	**1100 (800 - 1900)**	**3000 (1700 - 5300)**
CXCL10/IP-10	250 (130 - 480)	230 (150 - 450)	300 (180 - 830)	**380 (260 - 640)**
CXCL11/I-TAC	88 (53 - 160)	91 (56 - 150)	116 (64 - 240)	**170 (110 - 260)**
CXCL12/SDF-1α+β	1800 (1100 - 3500)	**3300 (1600 - 4800)**	**3200 (1900 - 4800)**	**2300 (1400 - 3500)**
CXCL13/BCA-1	20 (13 - 30)	**29 (17 - 42)**	27 (17 - 48)	**59 (37 - 88)**
EGF	160 (90 - 260)	150 (70 - 280)	180 (80 - 330)	210 (90 - 320)
FGF-2	22 (9.6 - 53)	13 (1.6 - 31)	9 (1.6 - 25)	14 (1.6 - 30)
Flt3-Ligand	1.6 (1.6 - 34)	1.6 (1.6 - 36)	1.6 (1.6 - 42)	1.6 (1.6 - 20)
Fractalkine	25 (1.6 - 85)	11 (1.6 - 128)	28 (1.6 - 230)	1.6 (1.6 - 67)
G-CSF	30 (19 - 52)	28 (13 - 45)	29 (18 - 44)	26 (13 - 41)
GM-CSF	0.49 (0.07 - 2)	0.43 (0.07 - 2.3)	0.07 (0.07 - 0.7)	0.19 (0.07 - 1.1)
GRO	2300 (1400 - 3900)	2900 (1700 - 5900)	2600 (1700 - 3900)	3300 (1800 - 5100)
IFN-α2	1.6 (1.6 - 25)	1.6 (1.6 - 19)	1.6 (1.6 - 23)	1.6 (1.6 - 5.1)
IFN-γ	0.62 (0.07 - 2.2)	0.07 (0.07 - 1.8)	0.07 (0.07 - 1)	0.08 (0.07 - 1.4)
IL-2	0.18 (0.07 - 1.5)	0.07 (0.07 - 3.3)	0.07 (0.07 - 0.4)	0.16 (0.07 - 0.8)
IL-5	0.14 (0.07 - 0.4)	0.14 (0.07 - 0.5)	0.18 (0.07 - 0.4)	0.14 (0.07 - 0.3)
IL-6	6.5 (2.3 - 25)	3.6 (1.7 - 9.1)	5 (2.6 - 13)	4.7 (2.2 - 13)
IL-7	8.2 (4.9 - 13)	7.6 (4.2 - 13)	8.7 (5.6 - 12)	9.9 (5.4 - 14)
IL-10	9.6 (5.3 - 21)	8.3 (4.6 - 15)	9 (4.8 - 19)	11.3 (5.6 - 20)
IL-11	91 (39 - 130)	95 (52 - 150)	120 (52 - 200)	**130 (60 - 200)**
IL-16	30 (5 - 55)	40 (15 - 76)	46 (25 - 69)	38 (17 - 63)
IL-23	66 (24 - 660)	84 (24 - 950)	65 (24 - 410)	77 (24 - 320)

Cytokine	NEG	EC	ART	NC
RAGE	57 (30 - 94)	52 (33 - 85)	78 (46 - 140)	53 (36 - 87)
sCD30	36 (21 - 64)	26 (15 - 46)	29 (21 - 56)	34 (21 - 53)
SCD40L/CD154	20000 (20000 - 20700)	20000 (20000 - 20000)	20000 (19300 - 20000)	20000 (20000 - 20000)
sEGFR	43000 (29000 - 53000)	**53000 (39000 - 64000)**	52000 (41000 - 61000)	50000 (40000 - 59000)
sgp130	85000 (62000 - 110000)	94000 (64000 - 110000)	93000 (77000 - 120000)	65000 (45000 - 100000)
sIL-1RI	31 (24 - 44)	29 (22 - 42)	**39 (28 - 52)**	33 (25 - 43)
sIL-1RII	4800 (2700 - 5900)	4900 (2900 - 7000)	**7700 (5300 - 10200)**	4200 (2900 - 6300)
sIL-2Ra	650 (450 - 840)	640 (400 - 1000)	750 (450 - 1000)	**800 (600 - 1100)**
sIL-4Ra	400 (280 - 570)	340 (250 - 460)	380 (270 - 540)	390 (280 - 480)
sIL-6	8400 (6400 - 12000)	**11000 (7600 - 15000)**	**12000 (7900 - 15000)**	**10000 (7600 - 12000)**
TGF-α	11.7 (6.1 - 18)	7.2 (3.2 - 14)	7.4 (3.9 - 12)	8.8 (4.8 - 12)
TNF-α	6.8 (4.1 - 10)	**8.9 (6 - 14)**	**10 (7.4 - 14)**	**10.9 (6.5 - 16)**
TNFR1	890 (540 - 1200)	830 (590 - 1200)	1100 (800 - 1400)	780 (580 - 1080)
TNFR2	3400 (2500 - 4800)	**4100 (2900 - 5900)**	**4400 (3400 - 5900)**	**4200 (2800 - 5300)**
TPO	350 (170 - 610)	340 (170 - 620)	410 (280 - 590)	410 (290 - 660)
TRAIL	36 (20 - 52)	**54 (30 - 75)**	**53 (32 - 70)**	**60 (42 - 82)**
VEGF	240 (140 - 490)	130 (56 - 290)	270 (102 - 540)	210 (126 - 370)
VEGFR1	120 (86 - 200)	160 (85 - 260)	**190 (110 - 260)**	130 (93 - 190)
VEGFR2	13000 (9000 - 17400)	15000 (10000 - 21000)	15000 (12000 - 18900)	16000 (12000 - 20300)
VEGFR3	1800 (1100 - 3300)	1700 (910 - 2700)	**2600 (1600 - 3900)**	2000 (1200 - 2800)
XCL1-lymphotactin	82 (42 - 130)	99 (62 - 160)	83 (48 - 120)	86 (44 - 130)

950

951 Median (range) cytokine levels are shown for each group (pg/ml). Cytokine values significantly
952 different from the NEG group are shown in bold ($p<0.05$, FDR<0.1).

953

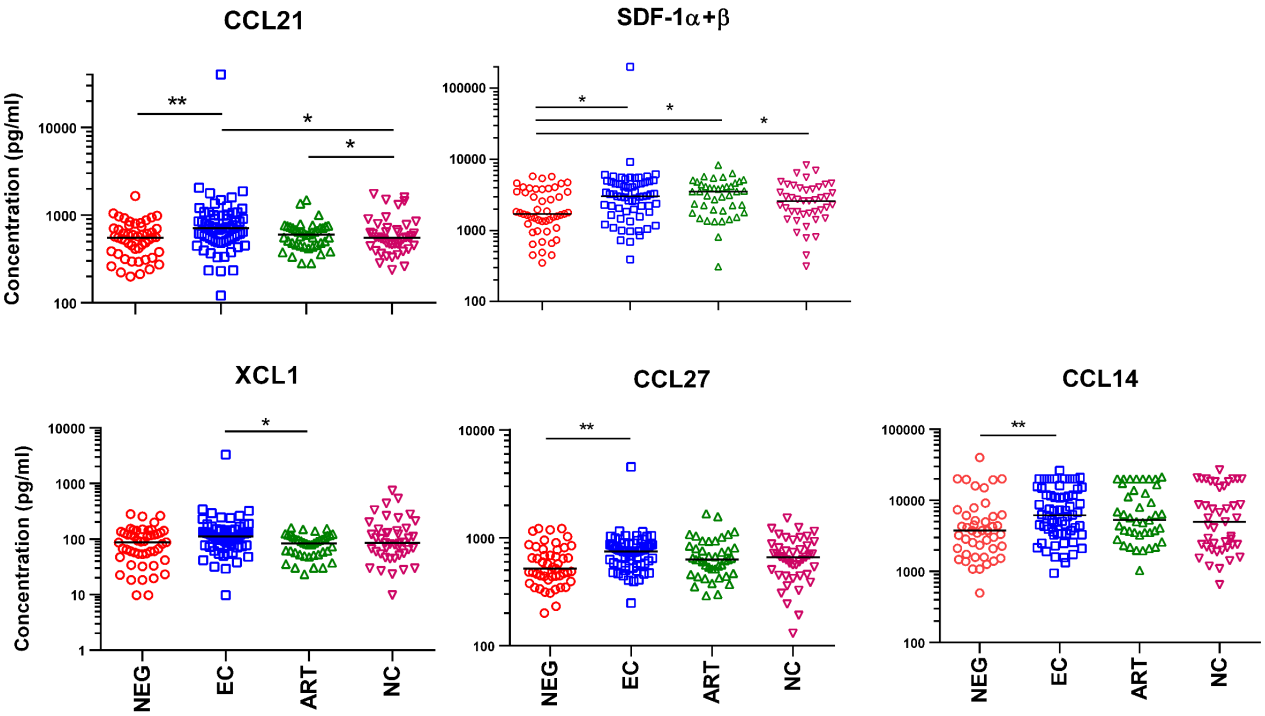
954 (**Table 3**). **Table 3. Cytokines showing significant differences between clinical groups**

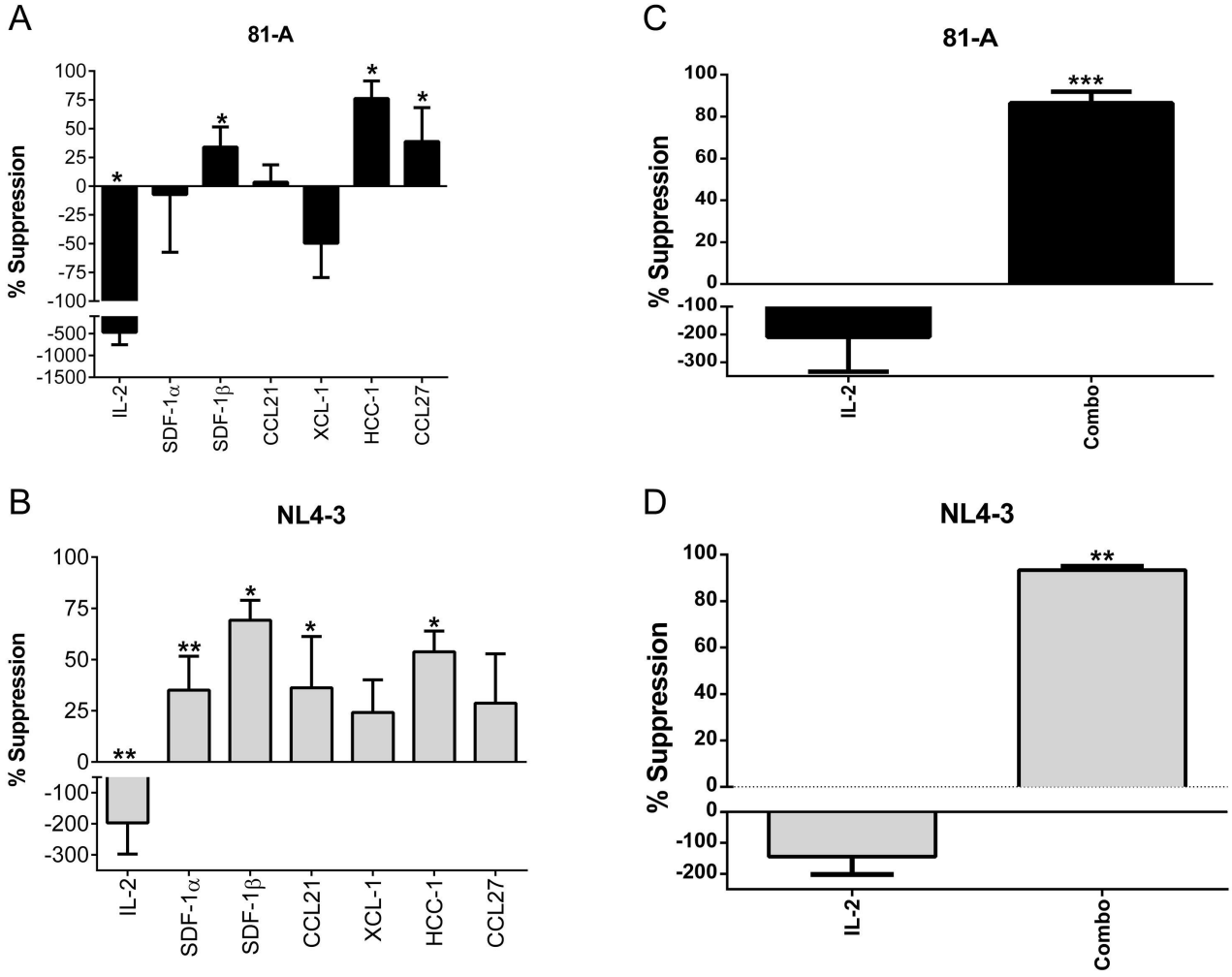
Cytokine	NEG vs EC	NEG vs ART	NEG vs NC	EC vs ART	EC vs NC	ART vs NC
CCL14a/HCC-1	0.007 (0.04)					
CCL21/6Ckine	0.006 (0.04)				0.02 (0.05)	0.04 (0.08)
CCL27/CTACK	0.006 (0.04)					
CXCL12/SDF-1α+β	0.046 (0.09)	0.02 (0.09)	0.03 (0.09)			
XCL1/lymphotactin				0.04 (0.26)		
sEGFR	0.005 (0.03)					
CCL8/MCP-2	0.006 (0.02)		0.002 (0.02)		0.048 (0.07)	
CCL13/MCP-4	0.05 (0.09)		0.03 (0.09)			
CXCL13/BCA-1	0.046 (0.07)		<0.001 (<0.001)		<0.001 (<00.01)	<0.001 (<0.001)
CXCL9/MIG	0.001 (0.002)		<0.001 (<0.001)		<0.001 (<0.001)	<0.001 (<0.001)
sIL-6	0.02 (0.04)		0.02 (0.04)			
TNF-α	0.006 (0.01)	<0.001 (0.002)	<0.001 (0.002)		0.047 (0.07)	
TNFR2	0.02 (0.04)		0.007 (0.02)			
TRAIL	0.006 (0.01)		<0.001 (0.003)			0.006 (0.01)
CCL19/MIP-3β			0.007 (0.01)		<0.001 (<0.001)	<0.001 (<0.001)
CXCL10/IP-10			<0.001 (<0.001)		0.002 (0.004)	0.01 (0.01)
CXCL11/I-TAC			<0.005 (<0.01)		<0.005 (<0.01)	0.006 (0.01)
sIL-1RII		<0.001 (<0.001)		<0.001 (<0.001)		<0.001 (<0.001)
sIL-2Ra			0.007 (0.04)			
VEGFR1						0.005 (0.02)
VEGFR3		0.02 (0.07)		0.002 (0.02)		

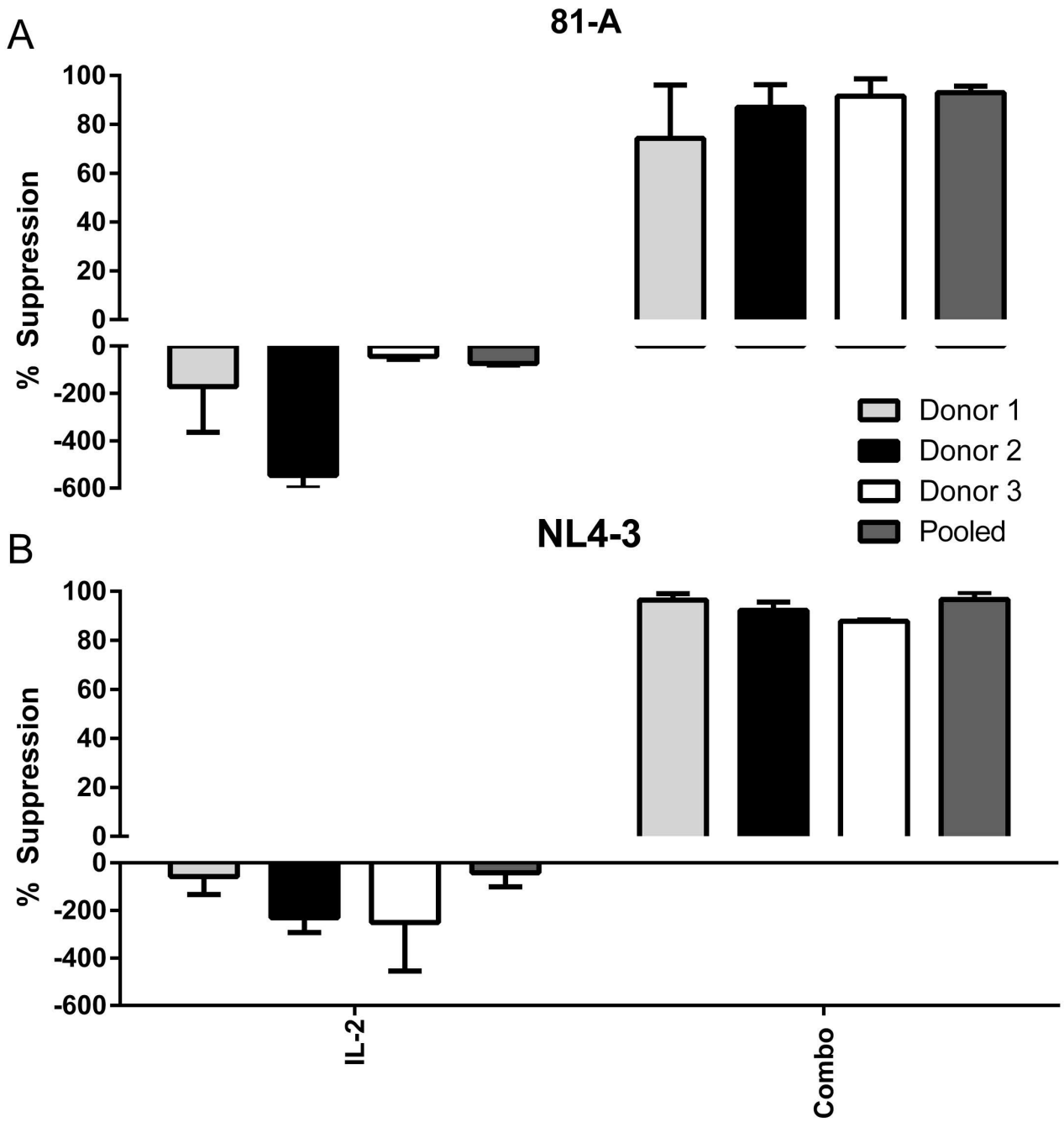
955

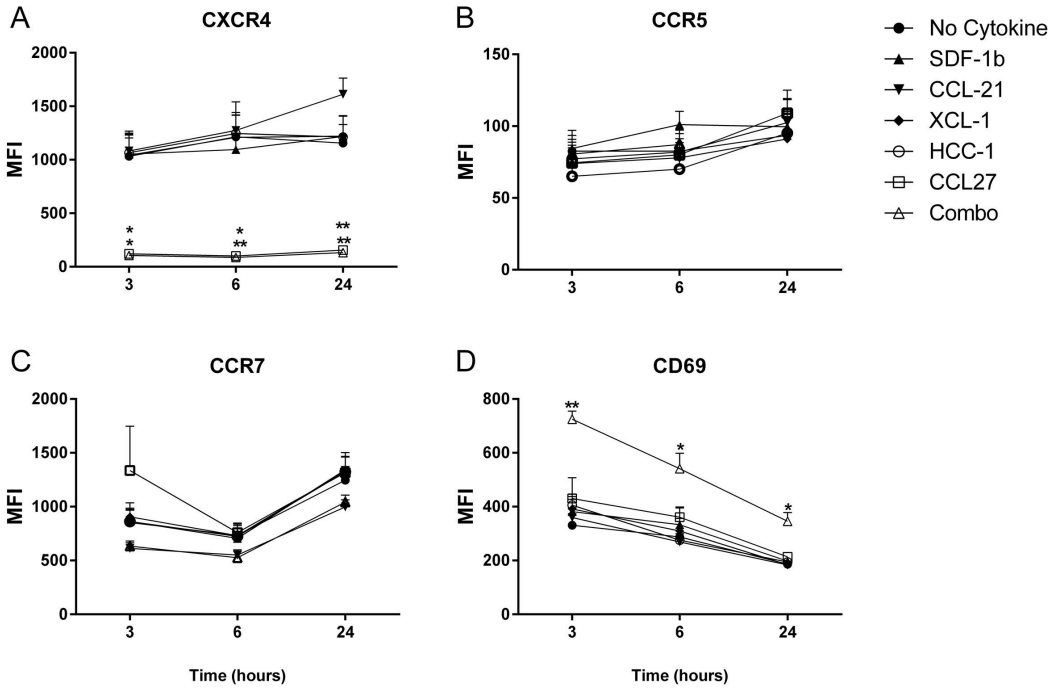
956 Values shown are p value and (FDR), with p value <0.05 and FDR <0.1 considered significant.
957 The overall p value and FDR statistic for comparison between groups were significant for each
958 analyte shown. Bold cytokines were selected for further *in vitro* analysis of ability to suppress
959 HIV replication.

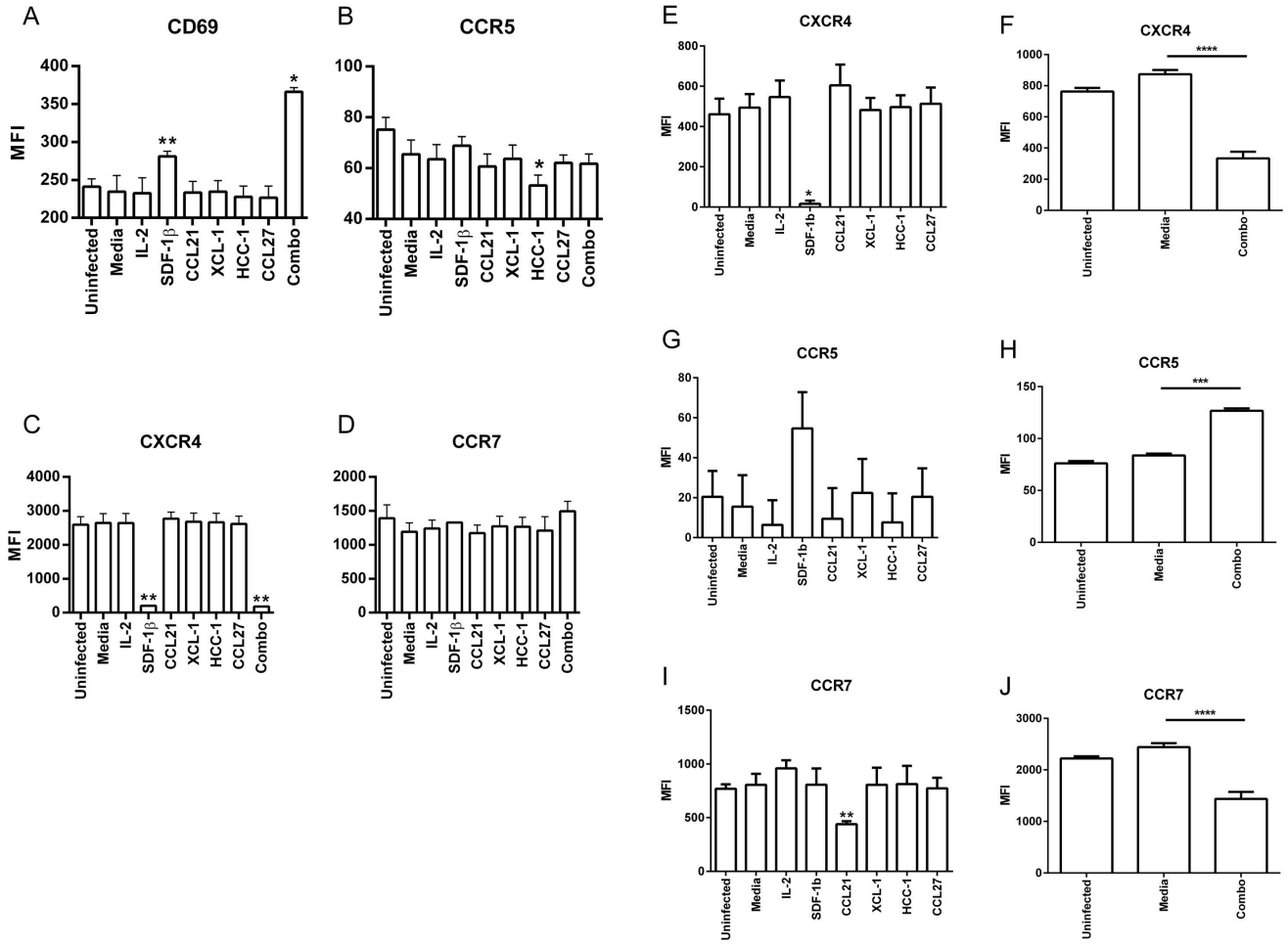
960











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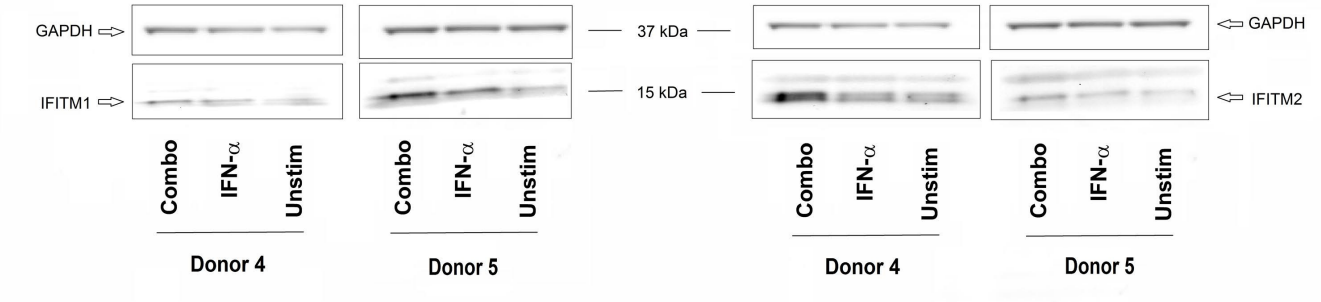

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