1-1-2014

Baseline natural killer and T cell populations correlation with virologic outcome after regimen simplification to atazanavir/ritonavir alone (ACTG 5201).

John E McKinnon

Robbie B Mailliard

Susan Swindells

Timothy J Wilkin

Luann Borowski

See next page for additional authors

Authors

John E McKinnon, Robbie B Mailliard, Susan Swindells, Timothy J Wilkin, Luann Borowski, Jillian M Roper, and + 5 more

Baseline Natural Killer and T Cell Populations Correlation with Virologic Outcome after Regimen Simplification to Atazanavir/Ritonavir Alone (ACTG 5201)

John E. McKinnon[1]*, Robbie B. Mailliard[2], Susan Swindells[3], Timothy J. Wilkin[4], LuAnn Borowski[2], Jillian M. Roper[5], Barbara Bastow[6], Mary Kearney[7], Ann Wiegand[7], John W. Mellors[2] Charles R. Rinaldo[2] for the A5201 study team[¶]

1 Henry Ford Hospital System, Detroit, Michigan, United States of America, 2 University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 3 University of Nebraska Medical Center, Omaha, Nebraska, United States of America, 4 Weill-Cornell Medical College, New York, New York, United States of America, 5 George Washington University, Washington, DC, United States of America, 6 Social & Scientific Systems, Inc., Silver Spring, Maryland, United States of America, 7 HIV Drug Resistance Program, NCI, Frederick, Maryland, United States of America

Abstract

Objectives: Simplified maintenance therapy with ritonavir-boosted atazanavir (ATV/r) provides an alternative treatment option for HIV-1 infection that spares nucleoside analogs (NRTI) for future use and decreased toxicity. We hypothesized that the level of immune activation (IA) and recovery of lymphocyte populations could influence virologic outcomes after regimen simplification.

Methods: Thirty-four participants with virologic suppression ≥48 weeks on antiretroviral therapy (2 NRTI plus protease inhibitor) were switched to ATV/r alone in the context of the ACTG 5201 clinical trial. Flow cytometric analyses were performed on PBMC isolated from 25 patients with available samples, of which 24 had lymphocyte recovery sufficient for this study. Assessments included enumeration of T-cells (CD4/CD8), natural killer (NK) ($CD3^+CD56^+CD16^+$) cells and cell-associated markers (HLA-DR, CD's 38/69/94/95/158/279).

Results: Eight of the 24 patients had at least one plasma HIV-1 RNA level (VL) >50 copies/mL during the study. NK cell levels below the group median of 7.1% at study entry were associated with development of VL >50 copies/mL following simplification by regression and survival analyses (p = 0.043 and 0.023), with an odds ratio of 10.3 (95% CI: 1.92–55.3). Simplification was associated with transient increases in naïve and $CD25^+$ $CD4^+$ T-cells, and had no impact on IA levels.

Conclusions: Lower NK cell levels prior to regimen simplification were predictive of virologic rebound after discontinuation of nucleoside analogs. Regimen simplification did not have a sustained impact on markers of IA or T lymphocyte populations in 48 weeks of clinical monitoring.

Trial Registration: ClinicalTrials.gov NCT00084019

Introduction

Trials of antiretroviral treatment (ART) simplification to ritonavir-boosted protease inhibitors (PI) alone have shown mixed success, with some trials mirroring the outcome of standard triple therapy and others failing to show equivalence.[1,2] The criteria for selection of patients for treatment simplification trials have varied and include baseline CD4 T-cell counts, duration of prior suppressive antiretroviral therapy and use of specific antiretroviral agents, making it difficult to compare studies in order to identify predictors of virologic outcome. Prior analyses have identified duration of suppressive ART, low hemoglobin and poor adherence as the major predictors of virologic rebound after treatment

simplification.[3] However, these factors were not identified in all trials, suggesting that there are other important determinants of virologic outcomes.[1,2,4–6]

We assessed the immunologic determinants of sustained virologic suppression in the AIDS Clinical Trials Group (ACTG) protocol A5201. This was a prospective, open-label, single-arm pilot trial of simplified maintenance therapy with atazanavir-ritonavir (ATV/r) alone after prolonged virologic suppression.[7,8] At week 48, the Kaplan-Meier estimate of the probability of virologic success was 88%.[8] Poor adherence was only documented in 2 patients in the trial and undetectable atazanavir levels were seen in some of the virologic failures in the A5201 study; however, drug levels did not correlate with treatment outcomes. [8] Studies of regimen simplification assume equal recovery of T and natural killer (NK) cells after the CD4+ T-cell counts have increased following antiretroviral therapy and levels of HIV-1 RNA have been suppressed for a predetermined time period. The A5201 study used an entry criterion of ≥250 CD4+ T cells/mm^3 to minimize risk of insufficient immune recovery prior to maintenance treatment simplification, similar to other maintenance simplification studies.[5,8–19] We hypothesized that the level of immune activation and recovery of lymphocyte populations influence virologic outcomes for patients undergoing induction-maintenance strategies. Indeed, virologic failure of lopinavir/ritonavir (LPV/r) monotherapy was associated with low nadir CD4+ T cell counts and suboptimal medication adherence.[3] In a Swiss study, low nadir CD4+ T cell counts were also associated with virologic failure, but in patients that had only been suppressed for 3 months prior to treatment simplification to LPV/r alone.[20] Additionally, trials of treatment simplification have noted increased number of events of HIV-1 viremia above 50 copies/mL as compared to continued combination therapy, which may lead to an increase risk of virologic failure and emergence of antiretroviral resistance.[23,24]

Our study therefore assessed the immune profile of patients before regimen simplification, the impact of regimen simplification on the T and NK cell populations and immune activation, and whether these immunologic parameters correlated with levels of residual viremia, measured by single copy assay, and detectable viremia above 50 copies/mL.

Materials and Methods

Study population

The Institutional Review Boards of all the participating and enrolling institutions listed in the acknowledgements approved the A5201 study and each participant provided written informed consent, these include: the University of Colorado Health Sciences Center, Duke University, Stanford University, the University of Nebraska Medical Center, Weill-Cornell Medical College, the University of Pittsburgh, the University of Cincinnati, the University of Hawaii–Manoa, the University of Iowa, the University of North Carolina–Chapel Hill, the University of Texas–Southwestern Medical Center, and the University of Puerto Rico. Thirty-four participants underwent regimen simplification to ATV/r during the A5201 study. Participants included in the study were receiving a protease inhibitor plus at least 2 NRTIs with plasma HIV-1 RNA suppression below 50 copies/mL for at least 48 weeks immediately prior to study entry.[7] Four participants experienced virologic failure by protocol definition (defined as 2 consecutive plasma HIV-1 RNA levels ≥ 200 copies/mL) at weeks 12, 14, 20 and 28 after simplification. None of these virologic failures developed PI resistance either by standard genotyping or by single genome sequencing.[8] The virologic

failures were restarted on triple combination therapy and remained on study until week 54. Of the 34 patients, 25 participants had available cryopreserved PBMC samples for flow cytometry testing, which were collected at entry, and weeks 6, 18, 30 and 54. The 25 participants with available PBMCs did not differ in age, race, nadir or baseline CD4 T cell count, and had been virologically suppressed on average for the same duration as the full study cohort (data not shown). At each time point, 20×10^6 peripheral blood mononuclear cells (PBMC) were obtained and stored at −140°C. Of the 25 patients with available PBMC samples, one was excluded from the analyses due to poor sample quality (<10% viable PBMC from expected). Most of the PBMC samples were collected at the same timepoints as the HIV-1 viral load samples. All virologic failures as defined in the A5201 clinical trial had available PBMCs and underwent flow cytometry analyses. Clinical trial data including virologic outcomes, CD4 nadir and HIV-1 RNA levels were included in our analyses to assess for potential impact on outcomes or in immune parameters. In all participants, HIV-1 RNA levels were measured with the Roche Amplicor Cobas v1.5 assay. To assess the impact of treatment simplification on HIV-1 residual viremia in patients with long standing HIV-1 suppression, the single copy assay (SCA), having a limit of detection of 1.1 copies/mL as reported by Palmer et al, was used in a subset of 13 participants, which included all 4 participants with study defined virologic failure and 9 participants who had been previously amplified efficiently by SCA in a prior study.[21,22] In this analysis, we will examine the correlation of persistent HIV-1 residual viremia with immune activation and cell population parameters.

Prior studies of PI monotherapy/simplification using lopinavir/ritonavir have shown an increased prevalence of detectable HIV-1 RNA above 50 copies/mL; persistently detectable HIV-1 RNA levels may potentially lead to virologic failure and emergence of HIV resistance.[23,24] Therefore, we have used as criteria for
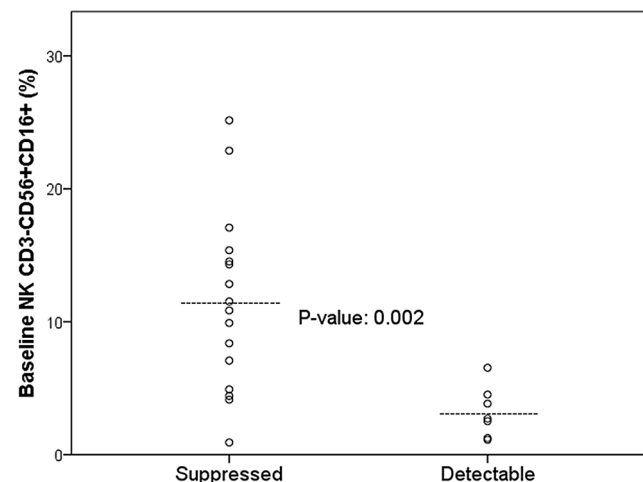
Figure 1. Baseline NK Cell percentages by HIV-1 RNA Outcome following Regimen Simplification. Figure 1 compares the baseline NK cell levels, defined by CD3-CD56+CD16+ cells, and virologic outcome (HIV-1 RNA below or above 50 copies/mL, throughout the study). The circles represent each study participant with either sustained HIV-1 RNA suppression during the trial and those participants who developed detectable viremia following treatment simplification. The difference in the median NK cell levels between the groups with detectable and suppressed viremia was statistically significant with a p-value of 0.002. The median level is noted by the line in the scatterplot for each group.

doi:10.1371/journal.pone.0095524.g001

Table 1. Univariate analysis of Baseline T and NK Cell Populations and Immune Activation Markers with in Participants with and without detectable HIV-1 RNA after Regimen Simplification.

T & NK cells	HIV-1 Viremia						Median Test	T-test
	UNDETECTABLE (n = 16)			DETECTABLE (n = 8)				
	Mean (%)	Std. Deviation	Median (%)	Mean (%)	Std. Deviation	Median (%)	Sig. (2-tailed)	Sig. (2-tailed)
T cell populations								
CD4+	48.07	13.48	48.71	43.81	16.22	50.91	0.52	0.56
CD8+	41.13	14.61	42.29	47.25	13.50	44.00	0.36	0.35
CD4+CD45RO−CCR7+	13.26	16.28	8.60	9.61	7.29	6.70	0.58	0.47
CD4+CD45RO+CCR7+CD27+	0.23	0.31	0.05	0.91	1.94	0.20	0.17	0.39
CD4+CD45RO+CCR7+CD27−	1.70	3.81	0.25	1.49	2.13	0.30	0.89	0.87
CD4+CD25+	8.75	9.47	4.70	6.16	5.25	4.60	0.51	0.41
CD8+CD45RO−CCR7+	8.08	9.40	5.50	9.73	5.15	10.30	0.67	0.60
CD8+CD45RO+CCR7+CD27+	0.04	0.07	0.00	0.04	0.08	0.00	0.90	0.91
CD8+CD45RO+CCR7+CD27−	0.31	0.53	0.10	0.24	0.24	0.20	0.76	0.69
CD8+CD25+	1.41	2.20	0.55	2.00	2.93	0.60	0.60	0.64
NK cell populations								
CD3−CD56+	30.55	17.52	27.37	13.62	9.53	9.58	**0.03**	**0.01**
CD3−CD56+CD16+	11.52	6.70	11.19	3.21	1.92	2.71	**0.00**	**0.00**
CD3+CD56+	11.52	11.71	7.18	3.76	1.92	4.31	**0.10**	**0.02**
IA Markers: CD4+ T cells								
CD4+CD38+	31.59	14.26	33.50	29.01	12.48	28.60	0.68	0.67
CD4+HLADR+	23.93	26.52	13.55	4.99	2.07	4.20	0.08	**0.01**
CD4+CD69+	21.25	22.47	12.15	6.67	1.54	6.30	0.11	**0.02**
CD4+CD95+	45.58	13.76	45.95	39.21	15.43	42.50	0.34	0.37
CD4+CD279+	1.80	5.79	0.30	1.47	2.59	0.70	0.89	0.85
CD4+CD38+HLADR+	5.61	7.95	3.55	1.57	1.27	1.20	0.20	0.07
IA Markers: CD8+ T cells								
CD8+CD38+	10.80	7.82	8.05	9.69	5.31	8.60	0.74	0.70
CD8+HLADR+	20.29	16.05	16.80	6.74	2.66	6.10	**0.04**	**0.00**
CD8+CD69+	15.49	19.13	8.35	3.99	2.44	3.70	0.13	**0.03**
CD8+CD95+	37.88	17.26	35.95	48.83	15.34	52.10	0.16	0.15
CD8+CD279+	0.21	0.23	0.10	0.77	0.89	0.40	**0.02**	0.15
CD8+CD38+HLADR+	2.96	3.21	1.90	1.81	0.91	1.60	0.37	0.21
IA Markers: NK cells								
CD3−CD56+CD69+	31.44	20.38	27.04	17.06	10.36	15.86	0.09	**0.04**
CD3−CD56+CD94+	55.85	18.92	55.49	54.19	23.43	62.73	0.86	0.87

Table 1. Cont.

T & NK cells	HIV-1 Viremia							Median Test	T-test
	UNDETECTABLE (n=16)			DETECTABLE (n=8)				Sig. (2-tailed)	Sig. (2-tailed)
	Mean (%)	Std. Deviation	Median (%)	Mean (%)	Std. Deviation	Median (%)			
CD3⁻CD56⁺CD158a⁺	30.11	19.75	33.11	21.78	15.41	22.42		0.33	0.29
CD3⁻CD56⁺CD158b⁺	25.71	19.13	30.51	37.05	23.45	45.51		0.23	0.29

The significant p-values for univariate comparisons are in bold.
doi:10.1371/journal.pone.0095524.t001

detectable HIV-1 RNA any value above 50 copies/mL, which is different from the protocol defined virologic failure of confirmed HIV-1 RNA levels above 200 copies/mL. Eight of the 24 participants tested by flow cytometry had at least one timepoint with HIV-1 RNA >50 copies/mL during the trial, 3 with single, 2 with two consecutive and 3 with multiple detectable timepoints. Seventeen participants had no HIV-1 RNA >50 copies/mL during the trial. We compared immune parameters between the participants with undetectable versus detectable HIV-1 RNA viremia levels during the study.

Flow cytometric analyses

PBMC samples collected as part of the A5201 protocol at entry, weeks 6, 18, 30 and 54 were used for the analyses. We expanded the flow cytometry testing to include both T cell and NK cell populations, as the latter is associated with early viremic control and may contribute to the control of HIV-1 viremia in patients on ART. The impact of the NK cell population on HIV-1 residual viremia has not been previously evaluated. Cryopreserved PBMC samples were thawed and cell viability was determined using a Vi-CELL analyzer (Beckman Coulter, California) with a target of ≥ 75% viability for each sample to be used for testing. For each timepoint, 1.5×10^6 cells were incubated in FACS buffer containing 2%BSA and 0.1% NaN3 along with appropriate combinations of antibodies for 30 minutes following manufacturer's recommendations. The cells were then washed and resuspended in FACS buffer. Flow cytometry data was immediately acquired by using the LSR II flow cytometer (BD Biosciences), in accordance to the manufacturer's instructions. The antibodies used were purchased from BD Biosciences (CD3-PB, anti-CD4-AF700, anti-CD45RO-PE, anti-CD27-FITC, anti-CD69-PC-7, anti-CCR7-APC-AF700, anti-CD38-PERCY5,5, anti-HLADR-APC-H7, anti-CD25-APC, anti-CD95-FITC, anti-PD1-PE, and anti-CD69-PC7, anti-CD16-PERCY5,5, anti-CD158a-FITC, anti-CD158b-PE, anti-CD94-APC, and anti-CD69-PC7), Beckman-Coulter (anti-CD8-ECD), eBioscience (anti-CCR7-APC-AF700) and BioLegend (anti-CD56-AF700). Additional negative controls were included for each sample during staining and acquisition steps. The parent cells were the CD3⁺ T cell lymphocytes for our analyses and all other T and NK cell percentages are based from this parent population. A gate was set on the population of singlet events as determined by the linear relationship between forward scatter height and area. This was followed by a gate on the live population of lymphocytes based on forward and side scatter light properties. The CD56 and CD3 parameters were plotted, and a gate was set on the NK cells based on their classical definition of being CD56+/CD3- lymphocytes. All flow cytometry gating and data were reviewed by both flow cytometry technicians (two) and by the principal investigator to confirm the validity of the samples, the gating strategy and the final numbers, prior to inclusion into the final data set.

Statistics

All statistical analyses were performed using SPSS statistics 20 (IBM). Sample size was limited to patients participating in the A5201 single arm study with available PBMC samples for analyses. We used both parametric analyses including means test, t-test, and non-parametric analyses including median test, the Mann-Whitney-U, mixed model analyses, and Cox multivariate regression analysis and cumulative survival analyses. Both parametric and non-parametric test were used as appropriate. Univariate analyses were used to examine potential predictors of sustained virologic suppression with p-values ≤ 0.1 which were subsequently analyzed using both forward and backward multivariate regression analysis. Multivariate analyses was used to

Table 2. Univariate and Multivariate Regression Analysis of Baseline T and NK Cell Populations and Immune Activation Markers and the Risk of Detectable Plasma HIV-1 RNA.

Cox Regression Analysis

Cell Markers	Univariate (p-value)	Univariate OR (95% CI)	Multivariate (p-value)	Final OR (95% CI)
CD4+ CD69+	0.071	13.2 (1.24–140.69)	NS	
CD4+ DR+	0.093	4.2 (0.60–28.62)	NS	
CD4+ CD38+ DR+	0.039	13.2 (1.24–140.69)	NS	
CD8+ CD69+	0.033	4.2 (0.60–28.62)	NS	
CD8+ DR+	0.045	13.2 (1.24–140.69)	NS	
CD8+ CD279+	0.1	0.13 (0.01–1.34)	NS	
CD3+ CD56+	0.012	13.2 (1.24–140.69)	NS	
CD3− CD56+	0.033	13.2 (1.24–140.69)	NS	
CD3− CD56+ CD16+	0.007	13.2 (1.24–140.69)	**0.043**	10.3 (1.92–55.3)
CD3− CD56+ CD69+	0.053	4.2 (0.60–28.62)	0.055	
CD3− CD56+ CD158a+	0.093	1.71 (0.29–10.30)	NS	

Cox regression analyses included all variables with p-values ≤0.1 in the univariate analyses, and age, race and CD4 nadir prior to starting ART. Odds ratio above 1.0 are associated with increased risk of virologic failure.
doi:10.1371/journal.pone.0095524.t002

determine if any of the factors were true predictors of virologic outcome and to eliminate any potential bias or confounding of multiple comparisons/repeated testing producing a false positive finding.[25] We reported the highest significant p-value for the significant variables when examining both the multivariate forward and backward regressions analyses. Statistical significance was defined as P-values <0.05, using a 2-tailed test for multivariate analyses.

Results

Baseline immunologic parameters

Participants entering the study had a median CD4+ T cell count of 616 (range: 443–756) cells/mm³. We compared the baseline T and NK cell parameters between participants maintaining HIV-1 RNA suppression and those who did not (HIV-1 RNA > 50 copies/mL). At baseline, the CD4+ and CD8+ T cell populations were similar for naïve, central memory, effector memory and CD25+ T cells between participants who maintained suppression of HIV-1 RNA and those with detectable HIV-1 RNA (all p-values were non-significant, Table 1). Nadir CD4 cell counts did not correlate with virologic outcomes of detectable HIV-1 RNA above 50 copies/mL or with the assessed immune parameters in multivariable analyses. However, the percentage of CD3− cells that expressed CD56+ was significantly higher in participants with undetectable viremia versus those with detectable levels with a mean of 30.6% versus 13.6% (p = 0.03), and for co-expression of CD56+CD16+ with mean value of 11.5% versus 3.2% (p<0.01). T cells expressing NK cell associated marker CD56+ cell levels were also higher in participants with sustained viral load (VL) suppression as compared to participants with detectable VL, with median values of 7.2% versus 4.3% (p<0.01) (Table 1, Figure 1). NK cell baseline differences were statistically significant in both univariate and multivariate analyses including Cox regression (Table 2). Higher NK cell population levels correlated in our analyses with sustained HIV suppression following treatment simplification.

Significant differences in T cell immune activation at baseline were seen between the two groups. CD4+CD38+HLADR+, CD8+CD69+, and CD3−CD56+CD69+ percentages were higher in the participants whose HIV-1 RNA levels remained suppressed below 50 copies/mL during the study. In comparison of participants with undetectable versus detectable viremia, the median percentages for CD4+HLADR were 13.6% vs. 4.2% (p = 0.01), CD4+CD69+ were 12.2% versus 6.3% (p = 0.02),
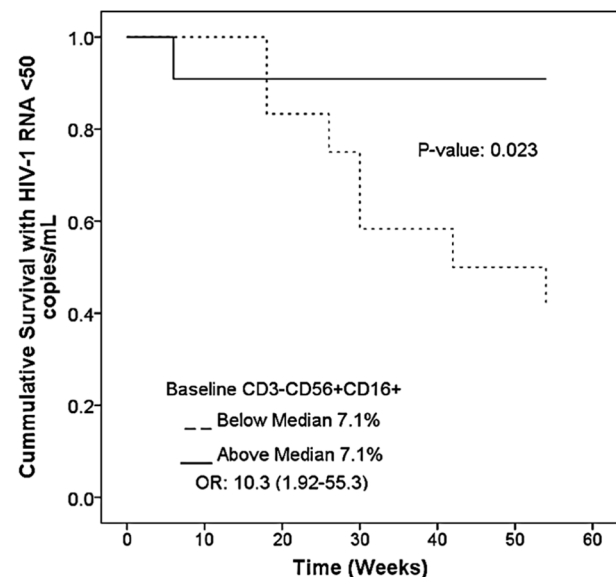

Figure 2. Proportions with HIV-1 RNA < 50 copies/ml after regimen simplification by baseline NK cell levels. Figure 2 shows a Kaplan-Meier plot for the survival with HIV-1 RNA level above 50 copies/mL during the A5201 study based on the participant's NK cell level at study entry. The p-value is 0.023 by Kaplan-Meier analyses for the difference at 54 weeks between the groups above and below the median NK cell level.
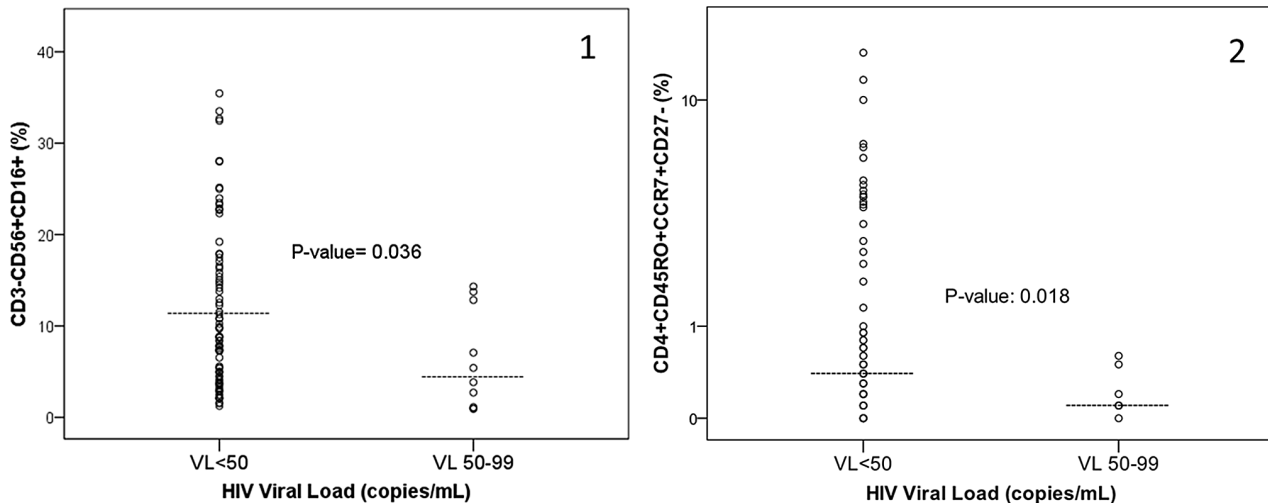doi:10.1371/journal.pone.0095524.g002

Figure 3. Comparison of NK cells and T cell populations based on HIV-1 viremia below and above 50 copies/mL. Figure 3 demonstrates that the population percentage of CD3-CD56+CD16+ cells (panel 1) and CD4-CD45RO+CCR7+CD27- cells (panel 2) were decreased in samples with HIV-1 RNA levels of 50–99 copies/mL as compared to samples obtained with HIV-1 RNA below 50 copies/mL with p-values of 0.036 and 0.018, respectively. The median level for each cell population is indicated by the line in the scatterplot for each group.
doi:10.1371/journal.pone.0095524.g003

$CD4^+CD38^+HLADR^+$ were 3.6% versus 1.20% (p = 0.07), $CD8^+HLADR^+$ were 16.8% versus 6.1% (p<0.01), $CD8^+CD69^+$ were 8.4% versus 3.7% (p = 0.03) and for $CD56^+CD69^+$ were 27.0% versus 15.9% (p = 0.04) (Table 1). The lower T cell immune markers may represent early migration of activated T cells from peripheral circulation to the tissues where active HIV replication is ongoing in participants with detectable viremia.

Association of baseline immunologic status with virological outcome

Cox univariate regression analyses of baseline immunologic parameters as continuous variables were performed to assess for any associations with the development of detectable HIV-1 RNA levels above 50 copies/mL. Only 9 of the T or NK cell populations or immune activation parameters analyzed had a p-value of ≤0.1 in univariate regression, including $CD4^+CD69^+$, $CD4^+CD38^+HLA-DR^+$, $CD8^+HLA-DR^+$, $CD8^+CD69^+$, $CD8^+CD279^+$, $CD3^+CD56^+$, $CD3^-CD56^+$, $CD3^-CD56^+CD16^+$, $CD3^-CD56^+CD69^+$ and $CD3^-CD56^+CD158a^+$. These immunologic variables were included in a multivariable regression analysis with forward and backward regression. We also included participant demographic characteristics, including age and race, and nadir $CD4^+$ T cell count prior to start of antiretroviral treatment. Only one parameter-population remained in the model, $CD3^-CD56^+CD16^+$, with a p-value of 0.043 in forward regression and 0.034 in backwards regression analyses. NK cells expressing the $CD69^+$ cell-associated marker of early activation did not remain in the model but the p-value neared statistical significance at 0.055 (Table 2). Cumulative survival analyses of the impact of NK cells levels, as a dichotomous variable, determined that participants with NK cell levels above the median of 7.1% had reduced risk of developing detectable HIV-1 viremia above 50 copies/mL as compared to those with NK cell levels below the median (p = 0.023) (Figure 2). The odds ratio of detectable HIV-1 RNA viremia in participants with low NK cell levels was 10.3 (95% CI: 1.92 to 55.3). Independent of baseline levels of immune activation and other T cell populations, the baseline NK cell level was the only predictive marker of detectable HIV-1 viremia in multivariate regression following treatment simplification. Inter-

estingly, $CD8^+CD38^+HLA-DR^+$ levels were statistically significantly different at week 30 of the study in participants with and without detectable viremia (p = 0.034) but the difference was not present by week 54 (p = 0.67).

Correlation of T and NK cell populations and Immune Activation (IA) Markers with HIV-1 RNA levels

Levels of T and NK cell populations and immune activation markers were analyzed with HIV-1 RNA levels using both the Single Copy Assay (SCA) and/or the Roche Amplicor v1.5 assay. During the trial, blood samples with detectable HIV-1 RNA levels below 50 copies/mL by SCA correlated with higher NK cells expressing $CD158b^+$ and $CD94^+$ markers (r = 0.455 & 0.375, p-values = 0.006 & 0.027, respectively). Other parameters were not significantly correlated (data not shown).

When comparing samples with HIV-1 RNA levels 50–99 copies/mL to those below 50 copies/mL by Roche Amplicor v1.5, we determined that the higher viral load group had lower median levels of $CD4^+CD45RO^+CCR7^+CD27^-$ (0.1% vs. 0.4%, p = 0.018), $CD8^+CD38^+HLA-DR^+$ (1.3% vs. 1.8%, p = 0.013), NK cells $CD3^-CD56^+CD16^+$ (4.6% vs. 11.5%, p = 0.036) and CD56+CD94+ (38.3% vs. 59%, p = 0.005), in univariate analyses (Figure 3). Other T and NK cell populations and IA markers were not significantly different when compared at different HIV viral load levels. In multivariate analyses, only NK cell levels remained significant with detectable viremia (Table 2).

Impact of treatment simplification on T and NK cell populations and IA

Comparisons of the T and NK cell populations and levels of IA markers before and after treatment simplification revealed no impact of treatment simplification on the immune parameters examined. Participants with suppressed viremia during the study tended to have an increase in the percentage of $CD4^+CD25^+$ cells with treatment simplification (p = 0.08), but by study week 54 the levels were similar to baseline (p = ns). The $CD4^+CD45RO^+CCR7^+CD27^-$ cell population also showed a trend to increase following simplification in the participants with

suppressed viremia during the study but did not reach statistical significance (p = 0.073). No statistically significant difference was seen in immune activation levels following treatment simplification in either outcome group. All other T and NK cell markers were not statistically associated with treatment simplification (all p-values >0.08 (data not shown).

Discussion

We examined the impact of baseline T and NK cell populations and markers of immune activation on virologic outcomes following regimen simplification to atazanavir/ritonavir alone. In addition, the impact of regimen simplification on immunologic parameters was investigated. Our study is the first to measure a possible predictive value of baseline NK cell levels for virologic outcome. Participants with NK cell levels below the median had 10 times higher risk of developing HIV-1 RNA levels above 50 copies/mL during the study. The difference in NK cell levels was seen in our study group while patients were still on their original combination antiretroviral therapy and before any intervention had occurred. Increased incidence of detectable viremia above 50 copies/mL has been reported in other trials of treatment simplification strategies and has led to a concern for increased risk of virologic failure and possible development of drug resistance mutations. [16,23,24,26] Therefore, understanding the immunologic control of low level viremia in participants undergoing treatment simplification and selecting a stricter 50 copies/mL threshold was considered to be more informative than using the study defined confirmed virologic failure above 200 copies/mL for our analyses.

The NK cell levels and immune activation marker differences at baseline demonstrate that despite prolonged HIV-1 RNA suppression on antiretroviral therapy, participants had significant differences in immunologic parameters at entry into the study. Therefore the immune status of patients entering HIV clinical trials as currently assessed by CD4$^+$ T cell levels prior to study entry does not provide a complete assessment of functional immune status reconstitution, and duration of HIV-1 RNA suppression may not be sufficient as an entry criterion for trials of maintenance therapy. Other parameters that were associated with detectable HIV-1 viremia at some point during the study included markers of immune activation HLA-DR and CD38, which could be indicators of undetected viral replication and/or ongoing inflammation, and in turn impact treatment outcomes. These markers reached statistical significance in univariate analyses but did not remain in the model as significant factors in multivariate analyses, possibly due to the limited sample size of our study. Larger studies are needed to further evaluate if the cell markers not retained in the regression models were affected by the limited sample size and have a significant impact on ART treatment responses.

Regimen simplification to atazanavir/ritonavir was not found to be an independent predictor of changes in either T or NK cell populations or on the levels of immune activation markers. We observed initial gains in CD4$^+$ naïve and CD4$^+$CD25$^+$ T cells in participants with virologic suppression during the trial but the differences were not sustained after 48 weeks of simplification. In the OK04 trial regimen simplification to LPV/r was associated with a modest increase in CD4$^+$ T cells at 48 weeks.[6] CD4$^+$ T cells increases were not detected in our study as previously reported.[8]

HIV-1 residual viremia below 50 copies/mL and low level viremia between 50–100 copies/mL were associated with distinct changes in immune cell populations and activation markers. NK cell CD158b$^+$ and CD94$^+$ surface cell markers tended to increase

with rising residual viremia by SCA. Once the HIV-1 RNA levels were between 50–99 copies/mL, then CD4$^+$ central memory T cells, NK cells and markers of inflammation tended to decrease as compared to samples from participants with suppressed HIV-1 RNA levels. These paradoxical decreases in markers of inflammation could be related to migration of activated cells from the circulation to the periphery as HIV-1 viral replication expands. These findings may be relevant as we monitor patients with low level detectable viremia or viral blips, as these could signify that the low level increase in plasma HIV-1 RNA is due to actual replication instead of assay variability.

Both the innate and adaptive immune systems have a role in the host response against HIV-1 viral replication. Initial viremia control in acute infection is achieved by cytotoxic activity of CD8$^+$ T and NK cell responses.[27,28] NK cells are known to be involved in both the early antiviral response to HIV-1 infection and represent an important part of innate and possibly adaptive immune response to control HIV-1 viral replication.[27–32] Concomitant CD4$^+$ and CD8$^+$ T cell responses to Gag peptides and NK cell responses to Env and Reg peptides have been correlated with improved viremia control and higher CD4$^+$ T cell counts.[33] NK cell responses to HIV-1 peptides have been demonstrated to persist even in absence of CD4$^+$ and CD8$^+$ T cell responses.[33] NK cell population levels and the phenotypic changes identified in our study suggest a more robust role of these cells in functional and cytotoxic activities related to control of HIV-1 replication in patients with detectable viremia below 50 copies/mL. Prior studies have shown that either changes in NK cell phenotype or functionality can impact HIV-1 viral control when assessed in long-term non-progressors and HIV-1 controllers.[34,35] Decreases in CD56^{bright} subsets, NKp30 and NKp46 expression in NK cells have been associated with reduced activation and cytotoxic activity with progressive HIV-1 infection.[36–39] NK cell exhaustion may also be associated with decreased viremic control, as HIV controllers expressed normal Siglec-7 levels as compared to HIV-1 progressors.[40] Similarly, PBMCs obtained from of long term non-progressors show normal to low expression of different inhibitory natural killer receptors (iNKR) in CD3+CD8+ CTL cells and lack of inhibition of HIV-1 specific cytotoxic activity by iNKR in vitro suggesting that normalized receptor expression may be needed for on-going anti-HIV cytotoxic activity.[41] Virologic failure may be as dependent on the host innate immune responses beyond just CD4$^+$ T cell recovery as it is on regimen potency.

The A5201 study was designed as a single arm study, which limits our ability to compare our results to control participants not undergoing treatment simplification. SCA HIV-1 viral load data are also limited to 13 participants who were known to have amplifiable HIV-1 gag sequences, limiting the depth of the analyses below 50 copies/mL. Low level detectable HIV-1 viremia above 50 copies/mL seen in this study may represent either new full rounds of replications due to incomplete suppression or viral shedding from previously infected cells. In the A5201 study, no PI resistance mutations were detected by either standard or single genome sequencing to support possible viral evolution and therefore new rounds of replication. Poor medication adherence will impact virologic rebound and was documented in at least two A5201 study participants. However, most study participants indicated good medication adherence during the trial and had been suppressed for longer to 12 months prior to study entry.[8] Atazanavir drug levels were evaluated in the original study but were not correlated with virologic outcomes, except if no level was detected (data not shown). Study medication adherence is unlikely to have impacted baseline T and NK populations prior to study

intervention, unless inconsistent medication adherence had been unrecognized prior to study entry and had not resulted in virologic rebound. Due to the original study sample size and sample availability, our final sample size is relatively small and some of the variables tested may not have reached statistical significance due to a type B error. Larger studies will be needed to further assess our study results and elucidate the impact of other immunologic parameters on HIV-1 viremic control. The strength of our study, however, lies in the depth and robustness of the immunologic flow cytometric analyses which included T and NK cells, together with correlation with clinical outcomes and HIV-1 RNA levels from both single copy assay and standard viral load testing.

In summary, this study demonstrates that patients on suppressive ART for similar timeframes have distinct immune cell populations and levels of cellular activation, despite $CD4^+$ T cell counts on average above 600 cells/mm^3, and even after adjusting for nadir $CD4^+$ T cell counts. Different levels of immune reconstitution and activation may explain the differences in treatment outcomes in trials of maintenance regimen simplification, and could explain why some patients may not require triple combination therapy to maintain HIV-1 suppression. Future trials of maintenance therapy strategies should include more in-depth immune assessments to further confirm these findings and better understand the relationship between the NK cell populations and other immune markers and treatment outcomes. Baseline assessment of NK cell levels and immune activation markers could provide information for early detection of patients at increased risk of virologic failure.

Acknowledgments

Author Contributions

References

1. McKinnon JE, Mellors JW, Swindells S (2009) Simplification strategies to reduce antiretroviral drug exposure: progress and prospects. Antivir Ther 14: 1–12.

2. Bierman WF, van Agtmael MA, Nijhuis M, Danner SA, Boucher CA, et al. (2009) HIV monotherapy with ritonavir-boosted protease inhibitors: a systematic review. AIDS 23: 279–291.

3. Pulido F, Perez-Valero I, Delgado R, Arranz A, Pasquau J, et al. (2009) Risk factors for loss of virological suppression in patients receiving lopinavir/ritonavir monotherapy for maintenance of HIV suppression. Antiviral Therapy 14: 195–201.

4. Perez-Valero I, Arribas JR, Perez-Valero I, Arribas JR (2011) Protease inhibitor monotherapy. Current Opinion in Infectious Diseases 24: 7–11.

5. Arribas JR, Horban A, Gerstoft J, Fatkenheuer G, Nelson M, et al. (2010) The MONET trial: darunavir/ritonavir with or without nucleoside analogues, for patients with HIV RNA below 50 copies/ml. AIDS 24: 223–230.

6. Arribas JR, Delgado R, Arranz A, Munoz R, Portilla J, et al. (2009) Lopinavir-ritonavir monotherapy versus lopinavir-ritonavir and 2 nucleosides for maintenance therapy of HIV: 96-week analysis. Journal of Acquired Immune Deficiency Syndromes: JAIDS 51: 147–152.

7. Swindells S, DiRienzo AG, Wilkin T, Fletcher CV, Margolis DM, et al. (2006) Regimen simplification to atazanavir-ritonavir alone as maintenance antiretroviral therapy after sustained virologic suppression. JAMA 296: 806–814.

8. Wilkin TJ, McKinnon JE, DiRienzo AG, Mollan K, Fletcher CV, et al. (2009) Regimen simplification to atazanavir-ritonavir alone as maintenance antiretroviral therapy: final 48-week clinical and virologic outcomes. J Infect Dis 199: 866–871.

9. Wong AH, Williams K, Reddy S, Wilson D, Giddy J, et al. (2010) Alterations in natural killer cell receptor profiles during HIV type 1 disease progression among chronically infected South African adults. AIDS Res Hum Retroviruses 26: 459–469.

10. Pialoux G, Raffi F, Brun-Vezinet F, Meiffredy V, Flandre P, et al. (1998) A randomized trial of three maintenance regimens given after three months of induction therapy with zidovudine, lamivudine, and indinavir in previously untreated HIV-1-infected patients. Trilège (Agence Nationale de Recherches sur le SIDA 072) Study Team. New England Journal of Medicine 339: 1269–1276.

11. Reijers MH, Weverling GJ, Jurriaans S, Wit FW, Weigel HM, et al. (1998) Maintenance therapy after quadruple induction therapy in HIV-1 infected individuals: Amsterdam Duration of Antiretroviral Medication (ADAM) study. Lancet 352: 185–190.

12. Reijers MH, Weverling GJ, Jurriaans S, Wit FW, Weigel HM, et al. (2001) The ADAM study continued: maintenance therapy after 50 weeks of induction therapy. AIDS 15: 129–131.

13. Katlama C, Fenske S, Gazzard B, Lazzarin A, Clumeck N, et al. (2003) TRIZAL study: switching from successful HAART to Trizivir (abacavir-lamivudine-zidovudine combination tablet): 48 weeks efficacy, safety and adherence results. HIV Medicine 4: 79–86.

14. Gathe JC Jr, Yeh RF, Mayberry C, Nemecek J, Miguel B, et al. (2007) Single-agent Therapy with Lopinavir/ritonavir Suppresses Plasma HIV-1 Viral Replication in HIV-1 Naive Subjects: IMANI-2 48-Week Results; July 22–25.

15. Arribas JR, Pulido F, Delgado R, Lorenzo A, Miralles P, et al. (2005) Lopinavir/ritonavir as single-drug therapy for maintenance of HIV-1 viral suppression: 48-week results of a randomized, controlled, open-label, proof-of-concept pilot clinical trial (OK Study). Journal of Acquired Immune Deficiency Syndromes: JAIDS 40: 280–287.

16. Pulido F, Arribas JR, Delgado R, Cabrero E, González-García J, et al. (2008) Lopinavir-ritonavir monotherapy versus lopinavir-ritonavir and two nucleosides for maintenance therapy of HIV. AIDS 22: F1–F9.

17. Cameron W, da Silva B, Arribas J, Myers R, Bellos N, et al. (2006) A two-year randomized controlled clinical trial in antiretroviral-naïve subjects using lopinavir/ritonavir (LPV/r) monotherapy after initial induction treatment compared to an efavirenz (EFV) 3-drug regimen (Study M03-613). 13-18 August; Toronto, Canada.

18. Kahlert C, Hupfer M, Wagels T, Bueche D, Fierz W, et al. (2004) Ritonavir boosted indinavir treatment as a simplified maintenance "mono"-therapy for HIV infection. AIDS 18: 955–957.

19. Pulido F, Serrano O, Rivero A (2009) Atazanavir/ritonavir for maintenance of virologic suppression:48 week primary analysis of the 96 week multicenter, open-label, single-arm, pilot OREY Study. 12th EACS Conference; Cologne, 11–14 November. pp. Oral Abstract PS 4/6.

20. Gutmann C, Cusini A, Gunthard HF, Fux C, Hirschel B, et al. (2010) Randomized controlled study demonstrating failure of LPV/r monotherapy in HIV: the role of compartment and CD4-nadir. AIDS 24: 2347–2354.

21. Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, et al. (2003) New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. Journal of Clinical Microbiology 41: 4531–4536.

22. Palmer S, Maldarelli F, Wiegand A, Bernstein B, Hanna GJ, et al. (2008) Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. Proceedings of the National Academy of Sciences of the United States of America 105: 3879–3884.

23. Delaugerre C, Flandre P, Chaix ML, Ghosn J, Raffi F, et al. (2009) Protease inhibitor resistance analysis in the MONARK trial comparing first-line lopinavir-ritonavir monotherapy to lopinavir-ritonavir plus zidovudine and lamivudine triple therapy. Antimicrobial Agents & Chemotherapy 53: 2934–2939.

24. Cameron DW, da Silva BA, Arribas JR, Myers RA, Bellos NC, et al. (2008) A 96-week comparison of lopinavir-ritonavir combination therapy followed by lopinavir-ritonavir monotherapy versus efavirenz combination therapy. Journal of Infectious Diseases 198: 234–240.

25. LaValley MP (2008) Logistic regression. Circulation 117: 2395–2399.

26. Doyle T, Smith C, Vitiello P, Cambiano V, Johnson M, et al. (2012) Plasma HIV-1 RNA detection below 50 copies/ml and risk of virologic rebound in patients receiving highly active antiretroviral therapy. Clinical Infectious Diseases 54: 724–732.

27. Berger CT, Alter G, Berger CT, Alter G (2011) Natural killer cells in spontaneous control of HIV infection. Current Opinion in HIV & AIDS 6: 208–213.

28. Alter G, Teigen N, Ahern R, Streeck H, Meier A, et al. (2007) Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. Journal of Infectious Diseases 195: 1452–1460.

29. Alter G, Altfeld M (2009) NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. Journal of Internal Medicine 265: 29–42.

30. Sun JC, Beilke JN, Lanier LL, Sun JC, Beilke JN, et al. (2009) Adaptive immune features of natural killer cells. Nature 457: 557–561.

31. Tiemessen CT, Shalekoff S, Meddows-Taylor S, Schramm DB, Papathanaso-poulos MA, et al. (2009) Cutting Edge: Unusual NK cell responses to HIV-1 peptides are associated with protection against maternal-infant transmission of HIV-1. Journal of Immunology 182: 5914–5918.

32. Chakrabarti LA, Simon V, Chakrabarti LA, Simon V (2010) Immune mechanisms of HIV control. Current Opinion in Immunology 22: 488–496.

33. Tiemessen CT, Shalekoff S, Meddows-Taylor S, Schramm DB, Papathanaso-poulos MA, et al. (2010) Natural Killer Cells That Respond to Human Immunodeficiency Virus Type 1 (HIV-1) Peptides Are Associated with Control of HIV-1 Infection. Journal of Infectious Diseases 202: 1444–1453.

34. Vieillard V, Fausther-Bovendo H, Samri A, Debre P, and French Asymptoma-tiques a Long Terme A-COSG, et al. (2010) Specific phenotypic and functional features of natural killer cells from HIV-infected long-term nonprogressors and HIV controllers. Journal of Acquired Immune Deficiency Syndromes: JAIDS 53: 564–573.

35. Johansson SE, Hejdeman B, Hinkula J, Johansson MH, Romagne F, et al. (2010) NK cell activation by KIR-binding antibody 1-7F9 and response to HIV-infected autologous cells in viremic and controller HIV-infected patients. Clinical Immunology 134: 158–168.

36. Bjorkstrom NK, Ljunggren HG, Sandberg JK, Bjorkstrom NK, Ljunggren H-G, et al. (2010) CD56 negative NK cells: origin, function, and role in chronic viral disease. Trends in Immunology 31: 401–406.

37. Barker E, Martinson J, Brooks C, Landay A, Deeks S, et al. (2007) Dysfunctional natural killer cells, in vivo, are governed by HIV viremia regardless of whether the infected individual is on antiretroviral therapy. AIDS 21: 2363–2365.

38. Mavilio D, Benjamin J, Daucher M, Lombardo G, Kottilil S, et al. (2003) Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates.[Erratum appears in Proc Natl Acad Sci U S A. 2004 Apr 20;101(16):6326]. Proceedings of the National Academy of Sciences of the United States of America 100: 15011–15016.

39. De Maria A, Fogli M, Costa P, Murdaca G, Puppo F, et al. (2003) The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). European Journal of Immunology 33: 2410–2418.

40. Brunetta E, Fogli M, Varchetta S, Bozzo L, Hudspeth KL, et al. (2009) The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia. Blood 114: 3822–3830.

41. Costa P, Rusconi S, Mavilio D, Fogli M, Murdaca G, et al. (2001) Differential disappearance of inhibitory natural killer cell receptors during HAART and possible impairment of HIV-1-specific CD8 cytotoxic T lymphocytes. AIDS 15: 965–974.