# Research Letters

*AIDS* 2014, **28**:2625–2631

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## Long-acting three-drug combination anti-HIV nanoparticles enhance drug exposure in primate plasma and cells within lymph nodes and blood

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**Insufficient HIV drug levels in lymph nodes have been linked to viral persistence. To overcome lymphatic drug insufficiency, we developed and evaluated in primates a lipid-drug nanoparticle containing lopinavir, ritonavir, and tenofovir. These nanoparticles produced over 50-fold higher intracellular lopinavir, ritonavir and tenofovir concentrations in lymph nodes compared to free drug. Plasma and intracellular drug levels in blood were enhanced and sustained for 7 days after a single subcutaneous dose, exceeding that achievable with current oral therapy.**

Combined antiretroviral therapy (cART) can clear HIV from the blood; however, residual virus remains in lymph nodes [1,2]. Oral cART produces lower drug concentrations in lymphoid tissues than in plasma [3–5], which is linked to persistent virus in lymph nodes [3,6,7] and virus rebound upon therapy cessation [8]. Drug nanoparticles have the potential to overcome lymphatic drug insufficiency [9]. We previously developed and demonstrated in primates a lipid nanoparticle (LNP) containing indinavir (IDV) that enhanced drug levels in all analyzed lymph nodes [4,10]. Also, IDV in LNPs enhanced intracellular drug concentrations in peripheral blood mononuclear cells (PBMCs), prolonged plasma residence time, reversed $CD4^+$ T-cell decline, and suppressed viral RNA in both plasma and lymph nodes [10]. Building on findings with IDV-LNPs, we have developed an anti-HIV LNP containing two protease inhibitors, lopinavir (LPV) and ritonavir (RTV), and a reverse transcriptase inhibitor, tenofovir (TFV), for simultaneous, triple drug delivery to HIV host cells in blood and lymph. LPV and RTV were chosen for their stability and strong hydrophobic interactions with LNPs [11]. Ritonavir enhances the efficacy of LPV through metabolic and drug transporter interactions [12,13]. Inclusion of TFV provides a second target of antiviral action to further suppress drug resistance potential, and intracellular retention of phosphorylated TFV prolongs antiviral activity [14].

We evaluated the characteristics of optimized anti-HIV LNPs for primate studies. The aseptically prepared anti-HIV LNPs exhibited 94, 91 and 12% LPV, RTV and TFV incorporation, respectively, with a mean diameter of 52 nm. The well defined unbound drug fractions were included for in-vivo studies. Antiviral potency against HIV-1, evaluated at a fixed LPV : RTV : TFV 1 : 1 : 0.5 mole ratio, revealed at least a three-fold increase in potency with anti-HIV LNP compared to the drugs in soluble form (LPV, RTV, and TFV $EC_{50}$ $30 \pm 0.8$, $30 \pm 0.8$, $15 \pm 0.1$ nmol/l in LNP form, vs. $98 \pm 0.3$, $98 \pm 0.3$, $49 \pm 0.2$ nmol/l in free form).

Primates dosed subcutaneously with LPV, RTV, and TFV (25.0, 14.3, and 17.1 mg/kg) in anti-HIV LNP form exhibited elevated plasma concentrations of LPV and RTV over 7 days (168 h). In contrast, plasma drug levels after administration of free drug in combination subsided to near or below detection limited by 24 h. The increase in total drug exposure [area under the curve (AUC)] provided by the LNP formulation for LPV, RTV, and TFV was 18, 14, and 7-fold, respectively (paired *t* test $P = <0.05$, 0.07, and 0.173) (Table 1). The AUCs for early (0–8 h) versus late drug exposure (8–168 h) were also analyzed. Primates treated with free drug exhibited 92% of total TFV exposure within the first 8 h, whereas those treated with anti-HIV LNPs exhibited only 9.1% in the first 8 h, with the remaining fraction at 8–168 h (Table 1).

Intracellular drug accumulation is pivotal for antiviral effects. Sustained LPV, RTV and TFV in PBMCs were detected for over 7 days in primates treated with anti-HIV LNPs, whereas those on free drug fell near or below the detection limit by 48 h (Table 1). The ratio of anti-HIV LNP to free drug (LNP/free ratio) was used to compare PBMC drug concentrations between the two test groups. This ratio was greater than 1 at all time points beyond 5 h, and greater than 20 at later time points (Table 1). For TFV, with only 12% LNP association, the LNP/free ratio in blood PBMCs was less than 1 at early time points; by 8 h, however, this value increased to over 50. Anti-HIV LNPs also enhanced intracellular drug concentrations in mononuclear cells of lymph nodes (LNMCs). LNMCs isolated from inguinal lymph nodes at 24 h revealed that no LPV and only low levels of RTV were detectable in animals treated with the free drug combination, whereas those treated with anti-HIV LNPs exhibited over 50-fold higher intracellular concentration (Table 1). For TFV, with limited LNP association, the LNP/free ratio was lower and recorded at 0.7; however, the differences between the two groups of macaques were not statistically significant ($P = 1.0$).

While toxicity of anti-HIV LNPs needs further investigation, complete blood count, serum chemistry

**Table 1. The effect of anti-HIV lipid nanoparticle[a] on intracellular levels of lopinavir, ritonavir, and tenofovir in peripheral blood mononuclear cells and inguinal lymph nodes, as well as overall drug exposure in plasma[b].**

| | Drug concentration | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lopinavir | | | Ritonavir | | | Tenofovir (TFV) | | |
| Time (h) | Free drug | LNP | LNP/free ratio[c] | Free drug | LNP | LNP/free ratio[c] | Free drug | LNP | LNP/free ratio[c] |
| Intracellular drug concentration of mononuclear cells in blood (ng/$10^6$ mononuclear cells)[d] | | | | | | | | | |
| 0 | 0.00 ± 0.00 | 0.00 ± 0.00 | **1.0** | 0.00 ± 0.00 | 0.00 ± 0.00 | **1.0** | 0.00 ± 0.00 | 0.00 ± 0.00 | **1.0** |
| 0.5 | 0.67 ± 0.31 | 0.28 ± 0.24 | **0.4** | 0.96 ± 1.67 | 0.41 ± 0.58 | **0.4** | 5.01 ± 5.12 | 1.29 ± 1.83 | **0.3** |
| 1 | 1.93 ± 1.18 | 1.85 ± 1.11 | **1.0** | 2.92 ± 2.62 | 2.57 ± 1.04 | **0.9** | 3.55 ± 4.21 | 1.29 ± 0.93 | **0.4** |
| 3 | 0.91 ± 0.53 | 2.39 ± 1.15 | **2.6** | 2.85 ± 1.50 | 3.05 ± 1.06 | **1.1** | 2.57 ± 2.62 | 1.19 ± 0.92 | **0.5** |
| 5 | 0.32 ± 0.28 | 2.59 ± 1.44 | **8.1** | 0.90 ± 0.60 | 2.20 ± 1.43 | **2.4** | 0.27 ± 0.21 | 1.24 ± 1.16 | **4.6** |
| 8 | 0.70 ± 0.86 | 4.23 ± 2.34 | **6.0** | 0.74 ± 0.99 | 4.57 ± 3.04 | **6.2** | 0.01 ± 0.01 | 0.59 ± 0.27 | **59.0** |
| 24 | 0.16 ± 0.28 | 3.26 ± 2.25 | **20.4** | 0.00 ± 0.00 | 4.09 ± 2.90 | **>409.0** | 0.01 ± 0.01 | 0.70 ± 0.14 | **70.0** |
| 48[c] | 0.01 ± 0.01 | 0.03 ± 0.00 | **3.0** | 0.00 ± 0.00 | 0.00 ± 0.00 | **1.0** | 0.00 ± 0.00 | 2.27 ± 0 | **>227.0** |
| 120[c] | NA | 0.02 ± 0.00 | **NA** | NA | 1.37 ± 0.00 | **NA** | NA | 0.64 ± 0 | **NA** |
| 168 | 0.00 ± 0.00 | 0.08 ± 0.10 | **>8.0** | 0.00 ± 0.00 | 1.32 ± 1.14 | **>132.0** | 0.00 ± 0.00 | 0.25 ± 0.25 | **>25.0** |
| Intracellular drug concentrations in mononuclear cells of inguinal nodes (ng/$10^6$ mononuclear cells)[e] | | | | | | | | | |
| 24 | 0.00 ± 0.00 | 4.85 ± 0.04 | **>485.0** | 0.13 ± 0.13 | 6.57 ± 0.08 | **50.5** | 1.02 ± 0.13 | 0.76 ± 0.09 | **0.7** |
| Plasma drug exposure analysis (AUC in μgh/ml)[f] | | | | | | | | | |
| 0–168 | 3.83 ± 4.04 | 69.6 ± 10.7 | **18.2** | 1.39 ± 1.18 | 19.4 ± 12.2 | **14.0** | 56.6 ± 17.04 | 395.0 ± 344.5 | **7.0** |
| 0–8 (%) | 19.6 | 9.9 | | 30.2 | 11.3 | | 91.8 | 9.1 | |
| 8–168 (%) | 80.4 | 90.1 | | 69.8 | 88.7 | | 8.2 | 90.9 | |

LNP, lipid nanoparticle. NA, not available.

[a]Anti-HIV LNPs composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), *N*-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (MPEG-2000-DSPE), lopinavir, ritonavir, and tenofovir (TFV) were prepared by thin film hydration method, as previously published [4]. All drugs were dried with the lipid film, rehydrated in bicarbonate-buffered saline, and size-reduced by high-pressure homogenization under aseptic conditions to produce drug-lipid nanoparticles with a mean 52 nm diameter. Free drug suspension dosages were prepared in bicarbonate-buffered saline using biocompatible solvent and surfactant to suspend the highly hydrophobic drugs.

[b]Four primates (*Macaca nemestrina*) were administered anti-HIV LNPs and free drug in a cross-over study at a normalized dose of 25 mg/kg LPV, 14.3 mg/kg RTV, and 17.1 mg/kg tenofovir (TFV) subcutaneously. All experiments were done under an approved Institutional Animal Care and Use Committee (IACUC) protocol. Blood and lymph nodes were collected over 7 days at indicated time points and drug concentrations were determined with a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) published assay [11]. Data are expressed as mean ± SD. Ratios for comparative analysis are shown in bold.

[c]LNP/free ratio is the mean anti-HIV LNP drug concentration divided by mean free drug concentration. In cases where the drug level was below detectable limits, the number was taken as '0.01' to calculate ratio.

[d]PBMCs were isolated from whole blood by density gradient method [15], and cell pellets of 2 million PBMCs each were analyzed. ($n = 4$/group; at 48 h and 120 h, $n = 2$/group).

[e]An inguinal lymph node was collected at 24 h ($n = 2$/group) and mononuclear cells were isolated by pressing tissue through a 200 μm cell strainer. Pellets of 2 million cells each were analyzed.

[f]Area under the curve (AUC) was calculated from plasma drug concentrations using the trapezoidal rule. The fractional percentage of total AUC in the early phase (0–8 h) and late phase (8–168 h) was calculated from the mean of the total AUC and the mean AUC in the indicated time range. Values are expressed as a percentage of the total AUC ($n = 4$/group).

panel, C-reactive protein, and complement levels revealed no treatment impact. No anti-HIV LNP-treated animals demonstrated an elevation in C-reactive protein, white blood cell count, blood urea nitrogen (BUN), creatinine, or liver enzymes. Total complement levels were highly variable, but variations were not significant. Also, no significant increase in cholesterol levels was noted with anti-HIV LNP administration (162 ± 16.4 vs. 191 ± 14.8 mg/dl). On physical examination of the injection site, naïve animals receiving free drug demonstrated a local reaction consisting of firm, nonerythematous swellings that resolved over the following weeks. Animals treated with anti-HIV LNP demonstrated no local reaction and their platelet counts remained within normal range.

In summary, taking advantage of the high LNP incorporation efficiency of two lipophilic protease inhibitors, LPV and RTV, and the ability to encapsulate hydrophilic TFV, we constructed a combination anti-HIV LNP and analyzed intracellular drug concentrations and plasma kinetics in macaques (*Macaca nemestrina*). Primates administered subcutaneously with anti-HIV LNPs exhibited elevated and extended intracellular drug levels in mononuclear cells of both blood and lymph nodes, indicating the potential for this approach to overcome lymph node drug insufficiency and associated viral persistence in patients on oral cART therapy [3–5]. The ability of anti-HIV LNP to extend plasma drug levels for over 1 week with higher total drug exposure supports consideration as a long-acting agent to improve patient compliance. Importantly, three drugs packaged together in anti-HIV LNPs could reduce drug resistance potential by consistently and simultaneously delivering all three drugs above therapeutic levels in the same cell. This reduces the likelihood of varying intracellular

concentrations for each drug, as achieved when delivered in free form or in separate particles. Therapeutic efficacy of anti-HIV LNPs could be further enhanced by organelle targeting with pH-responsive drug release [16,17] and expressing $CD4^+$-binding peptide [18] to target HIV host cells; for a review, see Gunaseelan *et al.* [19]. In summary, anti-HIV LNPs show promise for overcoming drug insufficiency in lymphoid tissues and improving patient compliance in search of a cure for AIDS.

## Acknowledgements

J.P.F. – Contributed to study design and execution, analyzed data, and drafted the manuscript. J.K. – Analyzed plasma and cell samples and provided critical review of the manuscript. C.S. – Processed blood samples. J.S. – Contributed to data analysis. R.J.Y.H. – Contributed to conception and study design, provided critical review of the manuscript.

We thank Michael Gough, Jason Ogle, and Chris English of the WaNPRC Research Support Group for their contributions in conducting the primate study.

Source of support: Supported by NIH grants, AI-077390 (S1, S2, S3), RR00166, RR025014, and UL1-TR000423. RJYH is also supported by the Milo Gibaldi endowment.

### Conflicts of interest

There are no conflicts of interest.

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*Received: 7 May 2014; revised: 16 July 2014; accepted: 21 July 2014.*

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DOI:10.1097/QAD.0000000000000421

## Effects of atorvastatin and pravastatin on immune activation and T-cell function in antiretroviral therapy-suppressed HIV-1-infected patients

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**This retrospective study was designed to assess statin effects on T-cell activation from HIV-infected**

**individuals. Peripheral blood mononuclear cells from antiretroviral therapy suppressed HIV-infected individuals receiving atorvastatin or pravastatin were evaluated for T-cell activation, exhaustion and function. Atorvastatin was associated with a significant reduction in CD8 T-cell activation (HLA-DR, CD38/HLA-DR) and exhaustion (TIM-3, TIM-3/PD-1) whereas pravastatin had no effect. In contrast, pravastatin increased antigen specific interferon γ production. These results suggest a differential effect of statins on immune activation and function.**

Immune activation contributes to HIV pathogenesis [1–10]. Even with suppressive antiretroviral therapy (ART), immune activation persists and remains elevated compared with HIV-uninfected individuals [11–15]. Although statins primarily serve as lipid-lowering therapy [16,17], they also reduce T-cell surface signaling molecules and decrease T-cell activation [18–21]. Recent studies demonstrate that statins reduce T-cell activation and are associated with decreased morbidity and mortality among HIV-infected persons [22–24]. Specifically, atorvastatin significantly reduced T-cell activation in viremic HIV-infected individuals [25]. Here, we evaluated the effects of pravastatin and atorvastatin on T-cell activation and exhaustion in ART-suppressed individuals.

We used cryopreserved samples from 21 HIV-infected persons who were virologically suppressed (<50 copies/ml) with ART for more than 12 months (range, 20–137) and remained suppressed throughout follow-up [26]. Seventeen received adjunctive statin therapy for more than 6 months (range, 3–32): seven individuals received 10 mg atorvastatin; 10 received pravastatin (five with 20 mg; five with 40 mg). A median viability of 97% and recovery at 87% was observed for peripheral blood mononuclear cell (PBMC) samples used in this study. As a control group, we identified four ART-suppressed individuals (median $CD4^+$ T-cell count: 498 cells/$mm^3$; median duration of ART: 119 weeks) not receiving statins. The study was approved by the Institutional Review Board at University of Alabama at Birmingham, Alabama, USA.

To assess statin effects on T-cell proliferation, we performed in-vitro assays as previously described [27] using cryopreserved PBMC from four statin-naive individuals. We compared in-vitro effects of three statins on T-cell proliferation in response to Staphylococcal enterotoxin B (SEB) using cells from four ART-suppressed controls (no statin). Compared with pravastatin, both atorvastatin and rosuvastatin significantly suppressed SEB-mediated $CD4^+$ and $CD8^+$ T-cell proliferation which was partially restored with exogenous mevalonate (Fig. 1a,b).

Ex-vivo analyses of expression levels of activation (CD38, HLA-DR), exhaustion (PD-1, TIM-3), and memory (CD27, CD45RO) markers were performed on cryopreserved PBMC from the 17 individuals receiving adjuvant statins before and during statin therapy and the four controls using surface staining with fluorochrome conjugated antibodies for specific cell surface markers [27]. Clinical and demographic features were not significantly different between groups (data not shown). Hyperlipidemia was the indication for statin use in all individuals; none were diabetic patients. In addition, baseline levels of immune activation and exhaustion were similar between the two groups. $CD8^+$ T-cell activation (HLA-DR and HLA-DR/CD38) and exhaustion (TIM-3) markers were significantly reduced following atorvastatin but not pravastatin treatment (Fig. 1c,d). A significant reduction of $CD4^+$ T-cell exhaustion markers (TIM-3 and PD-1) was also observed with atorvastatin ($P < 0.05$). We did not identify any sex-specific differences in activation or exhaustion marker expression. To evaluate whether ART alone explained these changes, we measured changes in expression levels on PBMC from four virologically suppressed and statin naive individuals. We identified no changes in activation (CD38, HLA-DR) or exhaustion markers (TIM-3, PD-1), suggesting that ART therapy alone did not dictate our findings.

We measured CD27 and CD45RO expression to assess changes in memory T-cell subsets: naive (CD27+ CD45RO+), intermediate (CD27+CD45 RO−), late (CD27−CD45RO+) and terminally differentiated (CD27−CD45RO−) phenotypes [28]. A shift to an earlier stage of differentiation and a reduction in terminally differentiated subset was noted with atorvastatin but no change with pravastatin (Fig. 1e,f). Atorvastatin-related reduction of activation marker expression was restricted to terminally differentiated (CD27−CD45RO−) subset of $CD8^+$ T-cells (HLA-DR [44% decline, $P = 0.063$] and CD38/HLA-DR [55% decline, $P = 0.031$]); data not shown. These differences were not explained by different proportions of terminally differentiated $CD8^+$ T-cells between the two groups before or during statin time points ($P = 0.13$, Fig. 1e, f). By contrast, $CD4^+$ T-cell activation (HLA-DR and CD38/HLA-DR) increased with pravastatin in the early differentiated subset (CD27+CD45RO+; $P \leq 0.0312$), but this change was not reflected in the total $CD4^+$ T-cell population (data not shown).

As atorvastatin was able to reduce markers associated with chronic T-cell activation and exhaustion, we next evaluated whether statin treatments improved T-cell functionality. When PBMC were antigenically stimulated with HIV (gag) or cytomegalovirus (CMV) (pp65) peptide pools, increased proliferation and interferon-γ production by CMV-specific $CD4^+$ and $CD8^+$ T-cell subsets was observed only for the pravastatin group (data not shown). No functional changes for HIV were observed in either statin group.

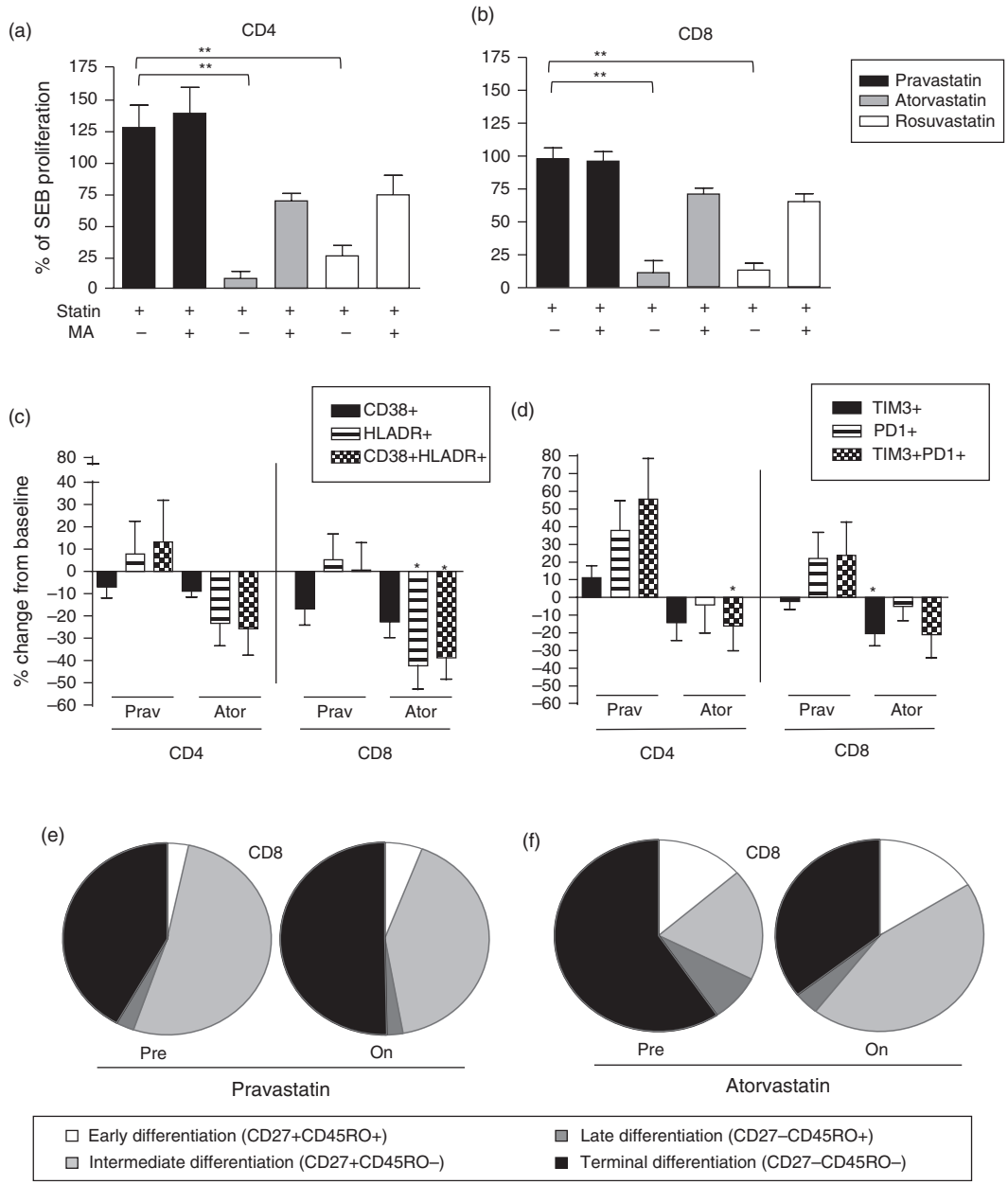


**Fig. 1. (a, b) In-vitro effects of statins on T-cell proliferation.** For in-vitro experiments, PBMC from four HIV-infected individuals with undetectable viral load on antiretroviral therapy were labelled with carboxyfluorescein succinidyl ester and stimulated with Staphylococcal enterotoxin B (SEB) for 5 days in the presence of the indicated statins, with or without mevalonic acid (MA, +/−). Experiments were performed in triplicate and data are expressed as a % of SEB only stimulated proliferation in $CD4^+$ (a) and CD8 (b) T-cell subsets. Proliferation in the presence of atorvastatin and rosuvastatin (without mevalonic acid) was significantly different from the pravastatin group in a Wilcoxon rank-sum test ($^{**}P < 0.01$). (c–f) In-vivo effects of statins on markers of immune activation and exhaustion in T cells. The in-vivo effect of atorvastatin and pravastatin on markers of T-cell activation and immune exhaustion are shown in panels c and d. The expression of CD38 and HLA-DR (c) and TIM-3 and PD-1 (d) is shown as percentage change in marker expression relative to prestatin, that is, baseline (mean + SEM). The two statin groups were compared by the Mann–Whitney *U* test ($^{*}P \leq 0.05$). The proportion of the four CD8 T-cell subsets, that is, naive (CD27+CD45RO+), intermediate (CD27+CD45RO−), late (CD27−CD45RO+), and terminally (CD27−CD45RO−) differentiated subsets before (pre) and during (on) pravastatin and atorvastatin treatment are shown as pie charts (e and f, respectively).

Our study demonstrates a differential effect of atorvastatin and pravastatin on T-cell activation, exhaustion and function. Atorvastatin significantly reduced markers of activation and exhaustion on T-cell populations; an effect most pronounced on terminally differentiated effector memory $CD8^+$ T cells. Given that this T-cell subset contributes to the detrimental inflammatory state of chronic viral infections, these changes may be very

important in the context of HIV infection, even in persons with suppressed viremia [29,30]. We focused on virologically suppressed individuals with persistent immune activation. Similar to data from viremic individuals, we found reduced $CD8^+$ T-cell activation markers with atorvastatin [25]. This appears to be statin-specific, as pravastatin had no effect on these markers.

Atorvastatin, but not pravastatin, reduced markers of exhaustion (TIM-3 and TIM-3/PD-1) on $CD8^+$ T cells. PD-1is upregulated on both $CD4^+$ and $CD8^+$ T cells during chronic HIV infection even after ART administration and has been correlated with functional T-cell exhaustion in chronic viral infections [31]. Reducing PD-1 and TIM-3 expression may restore T-cell function [32,33]. Whether the reduction of these inhibitory pathways by statins will yield improved immune responses needs further evaluation.

Limitations of our study include its small sample, non-randomized approach, and baseline $CD4^+$ cell count differences. Speculatively, as the pravastatin group had higher $CD4^+$ before and during statin $CD4^+$ count, this group might exhibit lower levels of immune activation prior to statin initiation. However, baseline expression of markers was not different between groups (data not shown). In addition, a comparison of activation markers in patients ($CD4^+ < 500$ cells/mm$^3$) showed similar patterns as shown in Fig. 1c and d, which suggests that a higher $CD4^+$ cell count in pravastatin was not responsible for a lack of detectable impact on the activation/exhaustion markers.

In conclusion, atorvastatin reduced markers of T-cell activation and exhaustion in virologically suppressed HIV-infected individuals. Given the anti-inflammatory effects of statins and the recent American College of Cardiology/American Heart Association guidelines suggesting the benefits of statins extend beyond lipid-lowering, future research will assess which statin provides the greatest benefit for HIV-infected persons.

## Acknowledgements

The authors are indebted to the participants enrolled in the Center For AIDS Research Network of Integrated Clinical Systems (CNICS) studies at UAB. The authors are also very grateful to Marion Spell and the UAB Center For AIDS Research Flow core for technical support and also to Tiffanie Mann for excellent technical assistance.

Author contributions: E.T.O., A.J.Z., P.A.G. and A.B. contributed to the conception and design of the experiments; S.S. and S.M.K. performed the experiments; A.B. and S.S. analyzed the data; A.O.W. performed the statistical analysis of the data; E.T.O., G.B. and P.A.G. provided clinical care for the patients; G.B. helped identify patients for the study. E.T.O. and A.B. wrote the manuscript. A.B. supervised the entire project. E.T.O. and S.S. contributed equally to the writing of the article.

Source of funding: This work was supported by the NIH-funded CNICS (R24 AI067039). Flow cytometry was performed, in part, in the UAB Center for AIDS Research Flow Cytometry Core, which is funded by NIH grant P30 AI027767. A.Z. was supported by AI099867 and S.K. was supported in part by training grant AI007051.

Presentation: This work was presented at the Immune Activation in HIV Infection: Basic Mechanisms and Clinical Implications meeting (poster # 1008), 3–8 April 2013, Breckenridge, Colorado.

### Conflicts of interest

There are no conflicts of interest.

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*Received: 25 March 2014; revised: 28 August 2014; accepted: 29 August 2014.*

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DOI:10.1097/QAD.0000000000000475