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High concentrations of atorvastatin reduce *in-vitro* function of conventional T and regulatory T cells

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Summary

Regulatory T cells (T_{regs}) modulate the magnitude of immune responses and possess therapeutic potential in an array of immune diseases. Statins reduce the activation and proliferation of conventional T cells (T_{cons}), and they seem to up-regulate the frequency and function of T_{regs}. However, there is a lack of simultaneous evaluation of the *in-vitro* effect of statins on the functional profile of T_{regs} *versus* T_{cons}. Herein, magnetically purified T_{cons} and T_{regs} were stimulated with CD3/CD28/interleukin (IL)-2 in the presence of atorvastatin (ATV) at 1 or 10 μM. The suppressive function of T_{regs}, the expression of markers associated with T_{reg} function, activation levels, cytokine production and calcium flux in both subpopulations were assessed by flow cytometry. ATV had no cytotoxic effect on T cells at the concentrations used. Interestingly, 10 μM ATV hampered the suppressive capacity of T_{regs}. Moreover, this higher concentration reduced the expression of forkhead box protein 3 (FoxP3), cytotoxic T lymphocyte antigen (CTLA-4) and programmed death 1 (PD-1). In T_{cons}, ATV at 10 μM decreased PD-1 and CD45RO expression. The expression of CD25, CD69, CD95, CD38, CD62L, CCR7 and perforin was not affected in both subpopulations or at any ATV concentrations. Remarkably, 10 μM ATV increased the percentage of tumour necrosis factor (TNF)-α-producing T_{regs}. Although there was a reduction of calcium flux in T_{cons} and T_{regs}, it was only significant in 10 μM ATV-treated T_{cons}. These results suggested that 10 μM ATV affects the cellular functions of both populations; however, this concentration particularly affected several aspects of T_{reg} biology: its suppressive function, cytokine production and expression of T_{reg}-specific markers.

Keywords: activation, Ca^{2+} flux, FoxP3, statin, Regulatory T cell

Introduction

$CD4^+$ regulatory T cells (T_{regs}) that express the forkhead box protein 3 (FoxP3) transcription factor can control the magnitude of immune responses under different clinical conditions, including autoimmune diseases, transplants, atherosclerosis, allergic diseases and colitis [1–5]. Therefore, T_{regs} have become a topic of growing interest due to their therapeutic benefit in the modulation of immune responses. Their suppressive action is largely mediated by cell–cell interactions, soluble factor delivery and functions associated with cytolysis and metabolic disruption [6,7], and these functions can be potentiated or induced by different drugs such as statins. Statins are drugs with potent immunomodulatory actions that inhibit the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) that converts HMGC into mevalonate, a precursor of the biosynthesis of cholesterol and non-steroidal isoprenoid compounds. The immunomodulatory actions of statins have been associated mainly with a reduction of isoprenoid compounds that are essential for the post-translational modifications of proteins participating in several cellular processes, such as survival, proliferation, differentiation and migration [8].

Natural and synthetic statins reduce the level of cellular activation as determined by the expression of CD69 and CD25 molecules, the proliferation of conventional

T cells (T_{cons}) and cell cycle arrest [9–14]. Contradictory results regarding the effect of statins on the production of interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-2, IL-4 and IL-5 by T_{cons} have been associated with the type of statins, incubation time in culture and/or the pre-existing inflammatory condition in which they were evaluated [10–14]. Moreover, it is unclear if statins can modify early events downstream of T cell receptor (TCR) activation, such as calcium (Ca^{2+}) mobilization [9,13]. Interestingly, statins seem to expand the T_{reg} population under both steady-state and inflammatory conditions as well as modulate their phenotypes, suppressive functions and migration patterns both *in vivo* and *in vitro* [15–21]. The statin-induced T_{reg} expansion is primarily attributed to the conversion of forkhead box protein 3 $(FoxP3)^-$ T cells into $FoxP3^+$ T cells through several mechanisms: (i) by negatively modulating the expression of mothers against decapentaplegic homologue 6 (Smad6) and Smad7 proteins responsible for inhibiting the transforming growth factor-β (TGF-β) signalling pathway [22]; (ii) by reducing the phosphorylation of protein kinase B (PKB), mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinases, which are involved in T_{reg} induction [15]; (iii) by increasing the expression of TGF-β and IL-10 [23] and reducing the IL-6/signal transducer and activator of transcription 3 (STAT-3) signalling pathway [24], promoting a higher T_{reg}/T helper type 17 (Th)17 ratio; and (iv) by inducing the transcription factor Krüppel-like factor 2 (KLF2), which promotes T_{reg} accumulation in secondary lymphoid organs [25,26].

Atorvastatin (ATV) is a second-generation statin that reduces blood levels of low-density lipoprotein cholesterol, even at very low therapeutic doses, compared with other available statins [27]. Moreover, because ATV up-regulates the T_{reg} population, we wanted to evaluate whether ATV can differentially modulate several aspects of the biology of T_{regs} and T_{cons}, such as their activation status, suppressive functions, cytokine production and Ca^{2+} mobilization in a dose-dependent manner. Thus, we determined the effector function and Ca^{2+} efflux of T_{cons} and T_{regs} isolated by electromagnetic sorting and activated in the absence or presence of either 1 or 10 μM ATV.

Materials and methods

Isolation of T cell populations and cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA) from healthy individuals recruited at the Universidad de Antioquia, Colombia. Informed consent was obtained from all donors and the research was approved by the Bioethical Board for Human Research from the Universidad de Antioquia. Fresh untouched $CD4^+$ T cells were purified by negative selection with a $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by staining with anti-CD25-phycoerythrin-cyanin 5 (PE-Cy5) (clone M-A251; BD Biosciences, San Jose, CA, USA) and anti-CD127-PE (clone eBioRDR5; eBioscience, San Diego, CA, USA) monoclonal antibodies. $CD25^{high}CD127^{low/-}$ T_{reg} populations and $CD25^-CD127^+$ T_{cons} were isolated in a MOFLO XDP high-speed cell sorter using the single mode (Beckman Coulter, Fort Collins, CO, USA). The purity of the sorted cell populations ranged from 90 to 98%. The efficiency of this procedure was approximately 90%.

The isolated T cells were seed in U-bottomed 96-well plates to a density of $0{\cdot}1–2 \times 10^5$ cells/well and cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cultures were incubated at 37°C in 5% CO_2 and 95% relative humidity. Cells were stimulated for 48 h with 2·5 μg/ml plate-bound anti-CD3 monoclonal antibody (mAb) (clone OKT3; Ancell, Bayport, MN, USA) and 1·5 μg/ml soluble CD28 (clone ANC28.1/5D10; Ancell) in the presence of 20 IU/ml of human recombinant IL-2 (National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD, USA). Stimulus conditions were set at 48 h as T_{reg} cells acquire the highest suppressive capacity at this time, as has been reported by other authors [28].

Atorvastatin treatment

ATV was provided by Biogen Laboratory, Bogotá, Colombia. It was diluted in dimethyl sulphoxide (DMSO; Carlo Erba, Rome, Italy) at a stock concentration of 100 mM. ATV was added to cultures for 48 h at different concentrations (0·5, 1, 2, 5 or 10 μM) that have been widely used to evaluate statin effects on several cellular subpopulations [12,21]. The highest DMSO concentration used in the experiments was 0·01%, which is below the concentrations causing any toxic effects on the cultures, as has been reported previously [29]. Thus, the T cell subpopulations (T_{con} and T_{reg}) were cultured in the following conditions: unstimulated (or so-called 'basal condition'), stimulated and stimulated and treated with different ATV concentrations.

Viability assay

To assess viability, $CD4^+$ T cells were harvested after 48 h incubation under the previously mentioned conditions and washed twice with phosphate buffered saline (PBS). They were then stained with 700 nM 3,3-dihexyloxacarbocyanine iodide (DIOC-6; Invitrogen Life Technologies, Paisley, UK) and 1 μg/mL 7-aminoactinomycin D (7-AAD; Invitrogen) for 20 min at room temperature in the dark.

The cells were analysed on a LSR Fortessa flow cytometer (BD Biosciences).

Suppression assay

$CD25^{-}CD127^{+}$ T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) at 1·25 μM (Molecular Probes, Eugene, OR, USA). $CD25^{high}CD127^{low/-}$ T_{regs} were treated under the conditions mentioned above for 48 h. Cells were then washed twice with PBS and co-cultured in 96-well round-bottomed plates at a 1:1 ratio in the presence of anti-CD3/CD28 plus IL-2 for 72 h. The proliferation of $CD4^{+}$ T cells was detected as the dilution of CFSE by flow cytometry using FlowJo software version 9.7.6 (TreeStar, Inc., Ashland, OR, USA). The percentage of suppression was calculated as follows: [100 – (% proliferation of T_{cons} in the presence of T_{regs}/% proliferation of T_{cons} alone) × 100].

Flow cytometry

The phenotype, activation status and cytokine production of both T_{cons} and T_{regs} in the presence or absence of ATV was assessed by flow cytometry using anti-human mAbs against surface and intracellular molecules (Supporting information, Table S1). In some cultures, the cells were restimulated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) plus 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) for 5 h to detect cytokines. Antibodies against surface molecules were incubated for 20 min at 4°C. For intracellular staining the cells were fixed/permeabilized according to the manufacturer's instructions using the FoxP3 staining kit (eBiosciences) and incubated with specific antibodies for 30 min. The samples were acquired on an LSR Fortessa flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) with FACSDiva software version 6.1.3 (BD Biosciences) and the data were analysed using FlowJo software version 9.7.6 (TreeStar). In the design of initial experiments, fluorescence minus one controls were used to distinguish positive from negative populations and set the gates. Relevant biological controls, such as unstimulated and stimulated cells, were used to determine the positive populations and the dynamic of antibody fluorescence in further experiments, as previously suggested [30]. Doublets and dead cells were excluded based on the forward/side light-scatter profiles and the absence of fluorescence in the live/dead viability staining (Thermo Fisher Scientific, Karlsruhe, Germany).

Measurement of intracellular Ca^{2+} concentration by flow cytometry

T_{con} and T_{reg} cells were stimulated with (anti-CD3/anti-CD28/IL-2) in the presence or absence of ATV for 48 h. The cells were then washed with PBS and ~0·6 × 10^6 cells were loaded with 4 μg/mL Fluo 3-AM (Invitrogen) and incubated for 60 min at 30°C in the presence of 0·02% pluronic F-127 in Hanks's buffered saline solution (HBSS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1% FBS. The loaded cells were washed and resuspended in PBS. Anti-CD3 at 5 μg/ml was added to the loaded cells and baseline fluorescence was recorded during the first 60 s using a FACSCanto II flow cytometer; cross-linking of CD3 was then performed by adding 10 μg/ml of mouse immunoglobulin (Ig)G and the resulting calcium release was recorded for approximately 15 min. The parameters of the kinetics of calcium fluxes were the areas under the curve (AUC), the maximum values (Max), the values of the slopes and the time required to reach the maximum value (T_{max}) of Ca^{2+} were calculated through FlowJo software version 9.7.6 (TreeStar).

Statistical analysis

The results are expressed as mean ± standard error of the mean (s.e.m.). Normal distribution was assessed by Shapiro–Wilk normality test and comparisons between the groups were performed using one-way analysis of variance (ANOVA) or general linear model (GLM) ANOVA for repeated measurements, followed by Dunnett's *post-hoc* test. Correlations between groups were determined by Spearman's rank correlation. Statistical analyses were performed with Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). *P*-values < 0·05 were considered statistically significant.

Results

High ATV concentration blocks T_{reg} suppressive function

To determine the effect of ATV on the suppressive function of T_{regs}, we evaluated the proliferation of T_{cons} cultured with previously preactivated T_{regs} using anti-CD3/anti-CD28/IL-2 for 48 h in the presence or absence of different concentrations of ATV. While suppressive function was maintained at 0·5–1 μM ATV, 2–10 μM ATV blocked suppressive function (Fig. 1a,b). To rule out the possibility that the loss of suppressive activities was due to increased cell kinetics, DIOC6 or annexin-V staining was performed. We observed that, similarly to lower ATV concentrations, more than 80% of $CD4^{+}$ T cells remained viable at 48 and 72 h of TCR activation in the presence of the highest ATV concentration (10 μM) (Supporting information, Fig. S1a,c). Moreover, both T_{cons} and T_{regs} remained viable at 10 μM ATV as visualized by a live/dead fixable stain, suggesting that ATV concentrations used in this study were non-toxic (Supporting information, Fig. S1b,c).

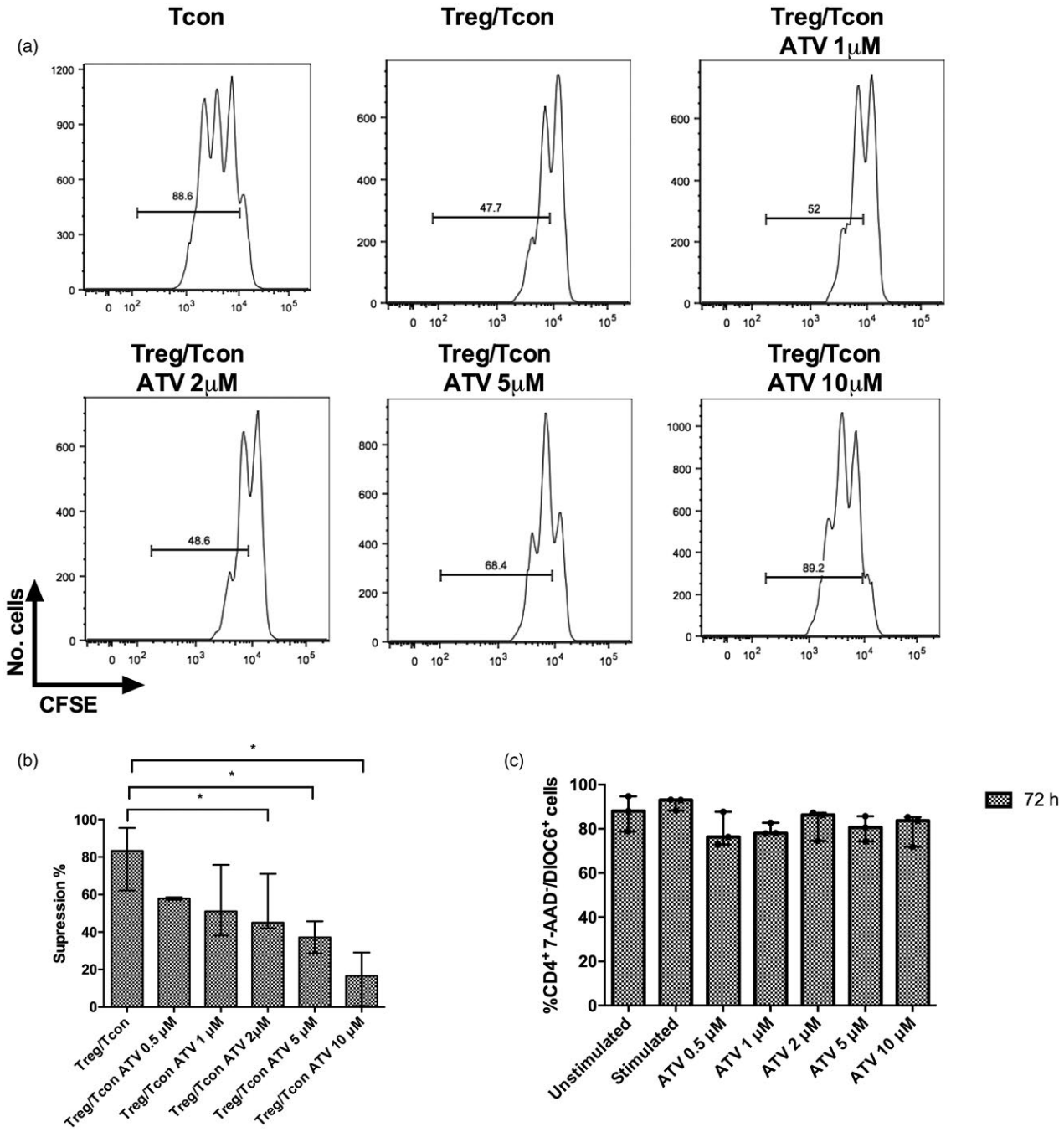


Fig. 1. Ten μM atorvastatin (ATV) reduces the suppressive function of regulatory T cells (T_{regs}). T_{regs} from healthy donors were preactivated with anti-CD3, anti-CD28 and interleukin (IL)-2 in the absence or presence of the indicated ATV concentrations for 48 h. They were then washed and mixed in a 1:1 ratio with conventional T cells (T_{cons}) labelled with carboxyfluorescein succinimidyl ester (CFSE) and activated for 72 h. The CFSE dilution was measured by flow cytometry. Representative histograms of four independent experiments are shown. (a) Percentage suppression of responder cell proliferation is shown for each condition ($n = 4$); (b) untouched total $CD4^+$ T cells were isolated through magnetic separation from the peripheral blood mononuclear cells (PBMCs) of healthy donors and cultured in the conditions mentioned above. Viable cells were determined as $(7\text{-aminoactinomycin D (7-AAD)}^-$ and 3,3-dihexyloxacarbocyanine iodide $(DIOC)\text{-}6^+$ cells ($n = 3$ donors) and (c) statistical significance determined by one-way analysis of variance (ANOVA) and Dunnett's *post-hoc* test (b: co-cultures +2 μm ATV $^*P = 0{\cdot}039$, co-cultures +5 μm ATV $^*P = 0{\cdot}022$ and co-cultures +10 μm ATV $^*P = 0{\cdot}029$ compared with untreated co-cultures).

High ATV concentrations decrease FoxP3, CTLA-4 and programmed death 1 (PD-1) expression on T_{regs}

We then examined if ATV could modulate the expression of molecules associated with T_{reg} function. ATV at the highest concentration reduced the mean fluorescence intensity (MFI) of FoxP3 and CTLA-4 to 42 and 20%, respectively, compared with stimulated cultures without ATV treatment (P = 0·018 and P = 0·033, respectively, Fig. 2a,b). Expression of PD1 was also reduced in the presence of

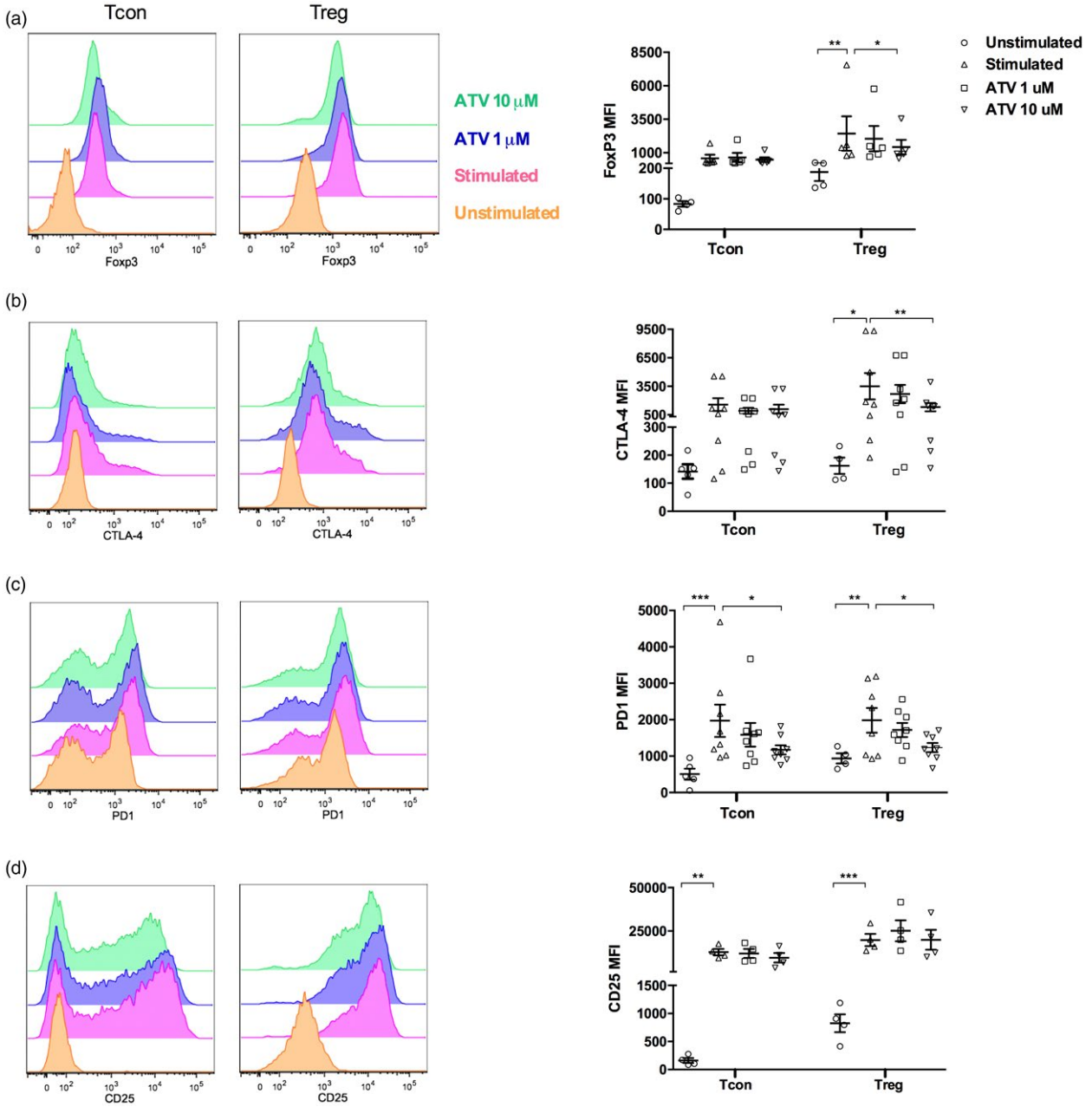


Fig. 2. Ten μM atorvastatin (ATV) reduces the mean fluorescence intensity (MFI) of forkhead box protein 3 (FoxP3), cytotoxic T lymphocyte antigen (CTLA-4) and PD-1 on regulatory T cells (T_{regs}). Histogram of representative flow cytometry analysis and MFI analysis of FoxP3 (n = 5 donors) (a), CTLA-4 (n = 8 donors) (b), programmed death 1 (PD-1) (n = 8 donors) (c) and CD25 (n = 4 donors) (d) on conventional T cells (T_{cons}) and T_{regs} in basal conditions and after activation with anti-CD3, anti-CD28 and interleukin (IL)-2 in the absence or presence of the indicated ATV concentrations for 48 h. Statistical analyses were performed using the general linear model (GLM) analysis of variance (ANOVA) and Dunnett's *post-hoc* tests. Means and standard errors of the mean (s.e.m.) are indicated. *P < 0·05, **P < 0·01 and ***P < 0·001. Each dot represents one individual in the graph.

10 μM ATV by 38% compared with control cultures (P = 0·018, Fig. 2c).

On T_{cons}, ATV treatment did not change FoxP3 expression (Fig. 2a) or CTLA-4 expression patterns compared with stimulated cultures without treatment (Fig. 2b). However, 10 μM ATV significantly reduced expression of PD-1 by 40% compared with cultures without treatment (P = 0·025, Fig. 2c). No significant differences were observed in CD25 and perforin expression in T_{regs} and T_{cons} after ATV treatment (Fig. 2d and Supporting information, Fig. S2).

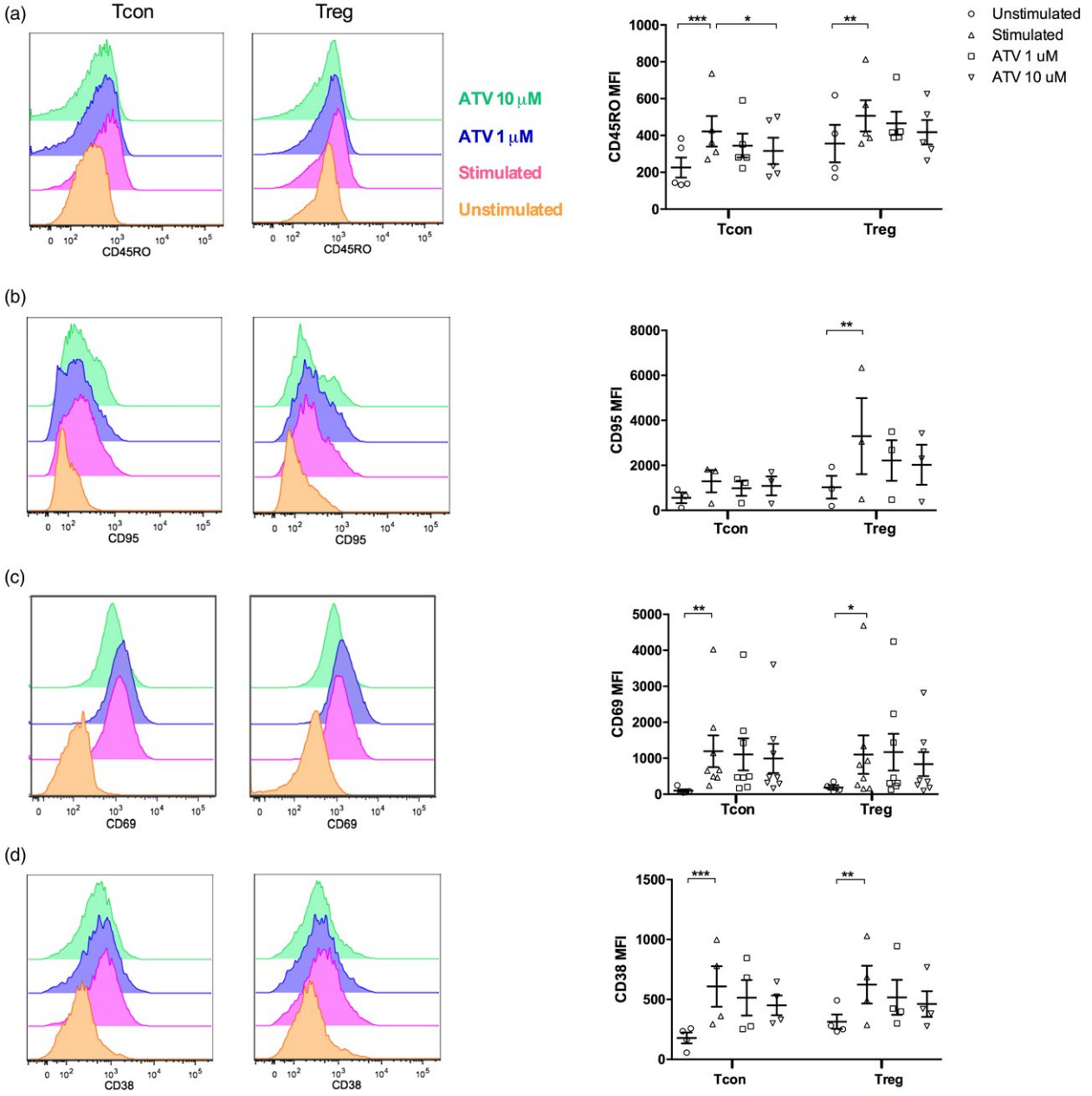


Fig. 3. Ten μM atorvastatin (ATV) reduced CD45RO expression only in conventional T cells (T_{cons}), but did not change activation markers on cell subpopulations. Representative flow cytometry histogram and mean fluorescence intensity (MFI) analysis of CD45RO (n = 5 donors) (a), CD95 (n = 3 donors) (b), CD69 (n = 8 donors) (c) and CD38 (n = 5 donors) molecules (d) in T_{cons} and regulatory T cells (T_{regs}) under basal conditions and after activation with anti-CD3, anti-CD28 and interleukin (IL)-2 in the absence or presence of the indicated ATV concentrations for 48 h. Statistical analyses were performed using general linear model (GLM) analysis of variance (ANOVA) and Dunnett's *post-hoc* tests. Means and standard errors of the mean (s.e.m.) are presented. *P < 0·05, **P < 0·01 and ***P < 0·001. Each dot represents one individual in the graph.

Ten μM ATV reduced CD45RO expression in T_{cons} but did not change homing and activation markers on cell subpopulations

As ATV can alter the activation status of different cell subpopulations, we then determined how ATV affects the expression of several surface molecules on both subpopulations. Remarkably, only the highest ATV concentration decreased the expression of CD45RO on T_{cons} by 17% compared with untreated stimulated cultures (P = 0·037 Fig. 3a). In contrast, CD95, CD69 and CD38 expression

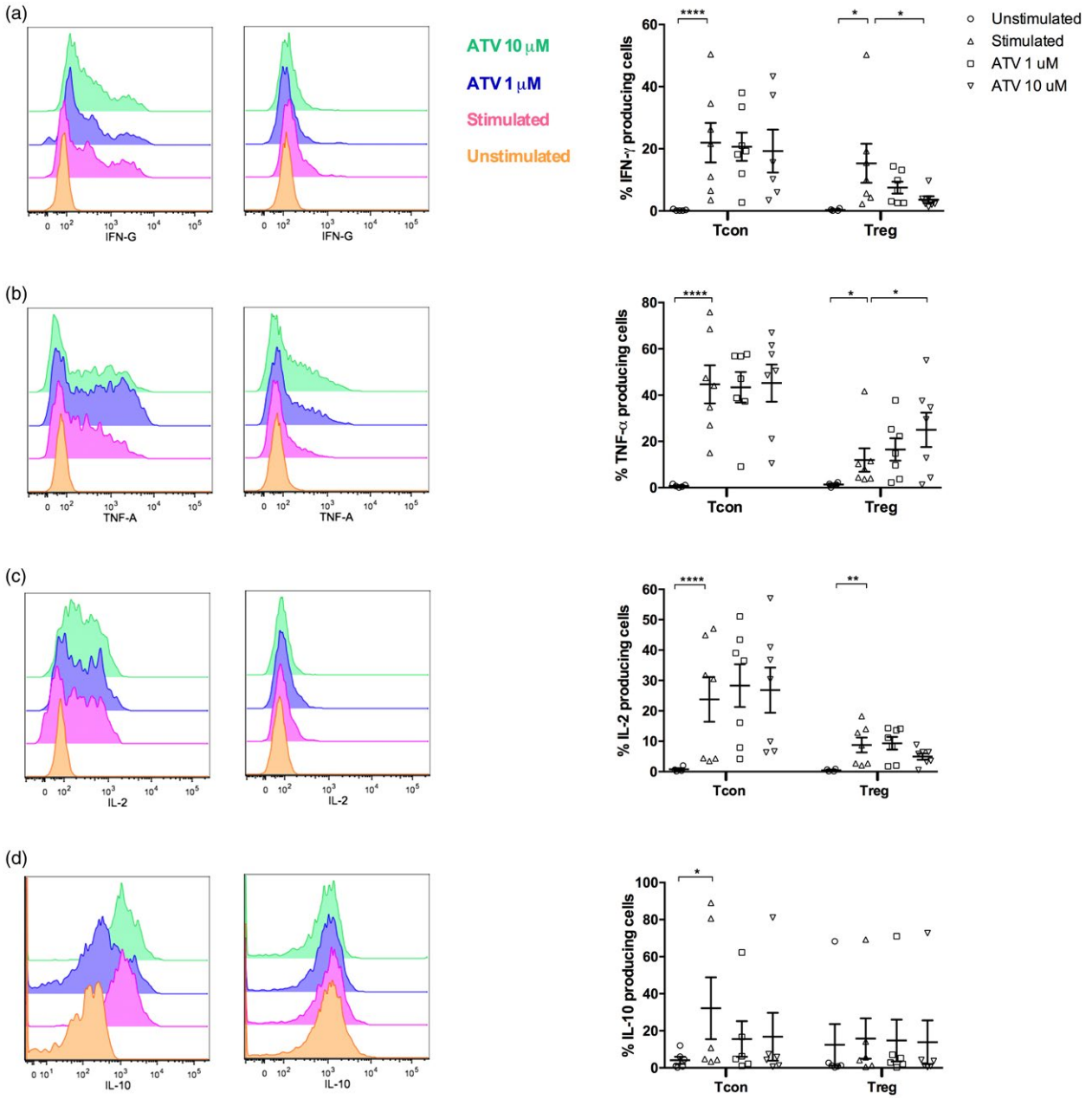


Fig. 4. Regulatory T cells (T_{regs}) treated with 10 μM atorvastatin (ATV) produce more tumour necrosis factor (TNF)-α than untreated-T_{regs}. Conventional T cells (T_{cons}) and T_{regs} were isolated by cell sorting and stimulated with anti-CD3, anti-CD28 and IL-2 in the absence or presence of the indicated ATV concentrations for 48 h. Cells were restimulated with phorbol myristate acetate (PMA)/ionomycin in the presence of brefeldin A for 5 h. Then, they were stained with monoclonal antibodies to detect the percentage of cells expressing interferon (IFN)-γ (n = 7 donors) (a), cells expressing TNF-α (n = 7 donors) (b), cells expressing interleukin (IL)-2 (n = 7 donors) (c) and cells expressing IL-10 (n = 6 donors) (d) by flow cytometry. Statistical analyses of percentages were performed using general linear model (GLM) analysis of variance (ANOVA) and Dunnett's *post-hoc* tests. Means and standard errors of the mean (s.e.m.) are indicated. *P < 0·05, **P < 0·01 and ****P < 0·0001. Left panels show representative flow cytometry histograms, and each dot on the right graphs represents one individual.

was not modified by any ATV concentration on T_{cons} or T_{regs} compared with control cultures (Fig. 3b–d). In addition, we wanted to determine whether ATV changed the circulation pattern of these cell subsets. However, no significant differences in CD62L, CCR7 and CCR5 expression were observed after treatment with statins in both subpopulations (Supporting information, Fig. S3a–c).

T_{regs} treated with 10 μM ATV produce more TNF-α

Functional plasticity is a feature of whole T cell subpopulations, including T_{regs} that can gain the inflammatory functions of Th1, Th2 or Th17 cells influenced by the cytokine environment. Unlike T_{cons}, we observed a lower frequency of T_{regs} producing IL-2, IFN-γ and TNF-α cytokines after anti-CD3/anti-CD28 stimulation. The presence of 10 μM ATV reduced the frequency of IFN-γ-producing T_{regs} to approximately 76% compared with untreated stimulated cultures (P = 0·033, Fig. 4a). Interestingly, the high concentration of ATV increased the percentage of TNF-α-producing T_{regs} from 12 to 25% (P = 0·026, Fig. 4b). Interestingly, we observed a positive correlation between TNF-α-producing T_{regs} and the MFI of CTLA-4 on T_{regs} (r = 0·553, P = 0·007, Supporting information, Fig. S4a) but not with the MFIs of FoxP3 or PD-1 on T_{regs} (Supporting information, Fig. S4b,c). On the contrary, ATV treatment did not modify the production of IL-2, IL-10 and IL-4 in T_{cons} and T_{regs} (Fig. 4c,d and Supporting information, Fig. S5).

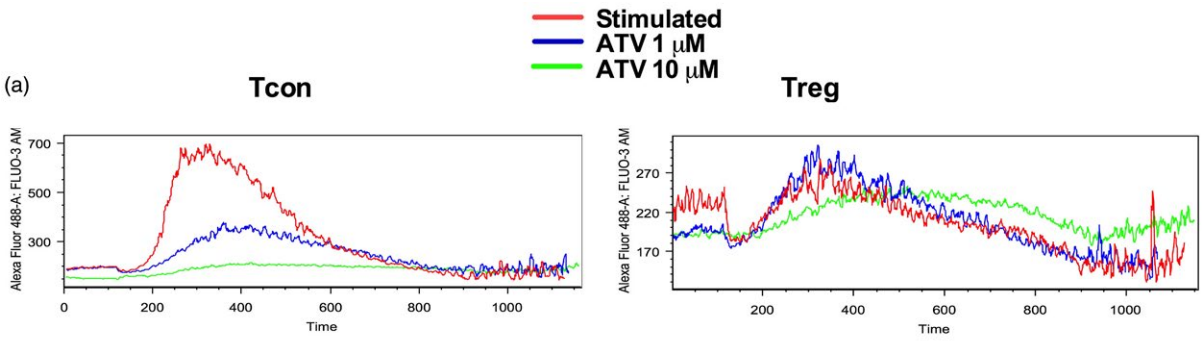
Higher ATV concentrations impaired Ca^{2+} flux in T_{cons}

To determine the influence of ATV on early events downstream of TCR activation, we evaluated Ca^{2+} mobilization elicited by CD3 on both cell populations by flow cytometry (Fig. 5a). In general, in response to CD3, we observed a higher Ca^{2+} mobilization in T_{cons} compared with T_{regs}, corroborating previous work [31]. When the T_{cons} were treated with ATV, there was a marked reduction in the AUC from 161 133 (without ATV) to 135 581 (P = 0·606) and to 105 447 (P = 0·020) with 1 μM and 10 μM ATV, respectively. In the case of T_{regs}, the effect of ATV was less evident. ATV induced a negligible increase in the AUC from 121 108 to 124 604 and 126 099 at 1 and 10 μM ATV, respectively, but there was also a delay in the times to achieve the maximal peaks (Fig. 5b). The effect of ATV on T_{cons} was also evident, probably being dose-dependent regarding the maximum values (Max), as ATV decreased these values from 381 to 260 (P = 0·750) and 175 (P = 0·011) at 1 and 10 μM ATV, respectively (Fig. 5a,b).

Discussion

In recent years, statins have been proposed as positive regulators of T_{regs}. Here we found that pretreatment of T_{regs} with 0·5–1 μM ATV did not modify suppressive function, and while at the highest ATV concentrations (2–10 μM), T_{regs} lost their suppressive function and became TNF-α-producing T_{regs}.

(a)



(b)

	Condition	AUC	Max	Slope	Tmax(s)
Tcon	Stimulated	161133 (60067)	381 (173)	1.417 (0.948)	291 (32)
	Stimulated+ 1.0 μM ATV	135581 (29808)	260 (67)	0.5287 (0.1698)	408 (64)
	Stimulated+ 10 μM ATV	105497 (19640)	175 (33)	0.1 (0.063)*	401 (35)*
Treg	Stimulated	121108 (14850)	235 (28)	0.4113(0.1429)	352 (28)
	Stimulated+ 1.0 μM ATV	124604 (17119)	232 (38)	0.3893 (0.1676)	335 (35)
	Stimulated+ 10 μM ATV	126099 (18055)	211 (30)	0.1403 (0.044)*	502 (28)*

Fig. 5. Ten μM atorvastatin (ATV) impairs Ca^{2+} flux in conventional T cells (T_{cons}). Representative histograms of Ca^{2+} flux in T_{cons} and regulatory T cells (T_{regs}) isolated and activated with anti-CD3, anti-CD28 and interleukin (IL)-2 in the presence or absence of different ATV concentrations for 48 h. Cross-linking of the CD3 monoclonal antibody (mAb) was performed through immunoglobulin (Ig)G (a). Area under the curve (AUC), Max value, slope and T_{max} (b). Statistical analyses were performed using the general linear model (GLM) analysis of variance (ANOVA) and Dunnett's *post-hoc* tests. Means and standard errors of the mean (s.e.m.) are indicated (n = 3 donors); *P < 0·05.

Early reports suggested that statins up-regulate T_{reg} suppressive function [15,23,32]; however, subsequent studies demonstrated contradictory results that could be due to differences in assays or other present technical issues. For instance, in several *in-vitro* experiments, regardless of the concentration used, statins were added directly into T_{reg}/T_{con} co-cultures [16]; this could lead to an over-estimation of the T_{reg} suppressive function because of statin-induced anti-proliferative effects on T_{cons} [14], an event that we also witnessed (Supporting information, Fig. S6). Although statins can also affect the proliferative response of T_{regs} [17,33], further studies are necessary to determine if T_{regs} are less susceptible to statin-induced anti-proliferative activities. In other studies, human T_{regs} were obtained as $CD4^+CD25^+$ T cells, which might not represent true T_{regs}; therefore, activated T_{cons} could proliferate in the presence of strong stimulation, masking the suppression activity of T_{regs} [15,34]. This could be problematic, especially when the proliferative response is evaluated through $[^3H]$-thymidine incorporation, which does not particularly distinguish the proliferating cell population [35]. Similarly, contradictory results could be derived from the type of statin used, considering the lipophilic or hydrophilic nature of these drugs, that could facilitate or block its spread across the cell membrane [36]. Moreover, others have demonstrated higher T_{reg} suppressive capacity in the presence of high concentrations of statins *in vitro* (25 μM) [32]; however, these concentrations could affect also cell viability [37]. Although we used supratherapeutic ATV concentrations (1 μM), this did not affect T_{reg} suppressive function, which was similar to the effect of 0·001 μM ATV [37], a similar concentration to that found in individuals taking low-dose statins [38]. Similar to our findings, pretreatment with simvastatin at 2 μM or ATV or lovastatin at 5 μM impaired T_{reg} suppressive capability in mice, as evaluated by the $[^3H]$-thymidine assay [33], highlighting the relevance of the mevalonate pathway in co-ordinating T_{reg} functions.

We found that sorted T_{regs} showed an activated phenotype, as has been reported previously, with higher expression of FoxP3, CD25, and PD-1 compared with their T_{con} counterparts. However, this feature was lost when they were cultured with the highest ATV concentration as a reduction of these molecules was observed. In line with these findings, previous reports have observed a down-regulation of functional markers such as CTLA-4, the inducible co-stimulator (ICOS) and the glucocorticoid-induced TNFR-related protein (GITR) on murine T_{regs} pretreated with ATV, lovastatin or simvastatin at concentrations ≥ 2 μM [33,39] as well as FoxP3 expression on $CD4^+CD25^+$ cells treated *in vitro* with 10 μM ATV [37], highlighting the relevance of the mevalonate pathway co-ordinating T_{reg} functions. Accordingly, the loss of FoxP3 in response to 10 μM ATV could be associated with reduced expression of CTLA-4 and PD-1 and with the inhibition of T_{reg} suppressive function, as it has been previously suggested [40,41]. Interestingly, these effects were partially observed on T_{cons}, as only the expression of PD-1 along with CD45RO was reduced by 10 μM ATV. Moreover, activation and homing markers such as CD25, CD69, CD95, CD38, CD62L and CCR7 were not modified by ATV on T_{cons} or T_{regs}. Our results are partially supported by others; for instance, CD45RO expression has been previously shown to be reduced after ATV treatment [11]; however, several authors [9,11,12] have demonstrated a reduction in CD25 expression after ATV treatment, whereas it remained unchanged in our experiments. Similarly, the ATV effects on CD69 expression has been controversial [9,11,12], possibly because to differences in stimuli, activation time and experimental conditions. In general, these findings contrasted with our previous results that demonstrated higher expression levels of functional markers on T_{regs} in individuals on temporary treatment with statins [17]. Such a difference could be explained because therapeutic doses are not equivalent to the doses used *in vitro* or is probably because of transitory regulation. As our study was limited to *in-vitro* assays, these results could not be directly extrapolated to individuals on statin therapy. Thus, more studies are necessary to understand the dynamics of T_{con} and T_{reg} activation through clinical trials that allow evaluating the effects of long-term statin use on these cell subsets.

In addition to phenotypical changes, ATV induced alterations on the Ca^{2+} influx, as 10 μM ATV reduced maximal peak (Max) and total mobilized Ca^{2+} (AUC) elicited by CD3 on T_{cons}. Although both ATV concentrations delayed the time required to reach the peak Ca^{2+} concentration (slope and T_{max}) on T_{cons}, there was no statistical significance. Altogether, these results suggested that T_{cons} are more susceptible than T_{regs} to the effect of ATV. Probably, this lower effect on T_{regs} could be due to their TCR-induced attenuated Ca^{2+} mobilization compared with T_{cons} [31]. Furthermore, this lower Ca^{2+} mobilization on T_{cons} could be related to lower proliferation. It is noteworthy to mention that although 10 μM ATV reduced Ca^{2+} mobilization, it did not affect cytokine production by T_{cons} as Ca^{2+} activates nuclear factor of activated T cells (NFAT), which is essential to cytokine production [42]. In contrast, ATV-treated T_{regs} become TNF-α-producing cells, which could be attributed to lower FoxP3 expression that, in turn, down-regulated CTLA-4 expression, allowing for inflammatory phenotypes by producing Th1 cytokines [43,44].

Taken together, our results suggested that 10 μM ATV disturbs the function of T_{regs}, diminishing their 'regulator'

phenotype via decreasing the expression of surface molecules and suppressive function and inducing the acquisition of an inflammatory profile (TNF-α-producing T_{regs}). This concentration also affected T_{con} function by reducing their proliferation, delaying Ca^{2+} mobilization and loss of PD-1 expression, suggesting that ATV differentially affected the function of both cell populations. Moreover, our findings may explain why some individuals taking statins over a prolonged time at high doses are at higher risk to develop autoimmune diseases [45]. Although our results are interesting, the low sample size in our study does not allow us to generalize our conclusions, and thus more studies are needed to determine the effect of low concentrations of ATV that simulate the plasmatic concentrations reached in individuals taking low therapeutic doses of statins on T_{reg} function and the role of the mevalonate pathway on the biology of T_{regs}.

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Disclosures

The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

1 Mor A, Planer D, Luboshits G *et al.* Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. Arterioscler Thromb Vasc Biol 2007;**27**:893–900.

2 Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 2002;**168**:1080–6.

3 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;**155**:1151–64.

4 Ling EM, Smith T, Nguyen XD *et al.* Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 2004;**363**:608–15.

5 Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol 2003;**170**:3939–43.

6 Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;**101**:455–8.

7 Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;**133**:775–87.

8 Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. Nat Rev Immunol 2006;**6**:358–70.

9 Ghittoni R, Patrussi L, Pirozzi K *et al.* Simvastatin inhibits T-cell activation by selectively impairing the function of Ras superfamily GTPases. FASEB J 2005;**19**:605–7.

10 Azor MH, dos Santos JC, Futata EA *et al.* Statin effects on regulatory and proinflammatory factors in chronic idiopathic urticaria. Clin Exp Immunol 2011;**166**:291–8.

11 Brinkkoetter PT, Gottmann U, Schulte J, van der Woude FJ, Braun C, Yard BA. Atorvastatin interferes with activation of human CD4(+) T cells via inhibition of small guanosine triphosphatase (GTPase) activity and caspase-independent apoptosis. Clin Exp Immunol 2006;**146**:524–32.

12 Blank N, Schiller M, Krienke S *et al.* Atorvastatin inhibits T cell activation through 3-hydroxy-3-methylglutaryl coenzyme A reductase without decreasing cholesterol synthesis. J Immunol 2007;**179**:3613–21.

13 Zhao N, Dong Q, Qian C *et al.* Lovastatin blocks Kv1.3 channel in human T cells: a new mechanism to explain its immunomodulatory properties. Sci Rep 2015;**5**:17381.

14 Dunn SE, Youssef S, Goldstein MJ *et al.* Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. J Exp Med 2006;**203**:401–12.

15 Tang TT, Song Y, Ding YJ *et al.* Atorvastatin upregulates regulatory T cells and reduces clinical disease activity in patients with rheumatoid arthritis. J Lipid Res 2011;**52**:1023–32.

16 Mausner-Fainberg K, Luboshits G, Mor A *et al.* The effect of HMG-CoA reductase inhibitors on naturally occurring CD4+CD25+ T cells. Atherosclerosis 2008;**197**:829–39.

17 Rodríguez-Perea AL, Montoya CJ, Olek S, Chougnet CA, Velilla PA. Statins increase the frequency of circulating CD4+ FOXP3+ regulatory T cells in healthy individuals. J Immunol Res 2015;**2015**:762506.

18 Mira E, Leon B, Barber DF *et al.* Statins induce regulatory T cell recruitment via a CCL1 dependent pathway. J Immunol 2008;**181**:3524–34.

19 Kagami S, Owada T, Kanari H *et al.* Protein geranylgeranylation regulates the balance between Th17 cells and Foxp3+ regulatory T cells. Int Immunol 2009;**21**:679–89.

20 Zhang X, Jin J, Peng X, Ramgolam VS, Markovic-Plese S. Simvastatin inhibits IL-17 secretion by targeting multiple IL-17-regulatory cytokines and by inhibiting the expression of IL-17 transcription factor RORC in CD4+ lymphocytes. J Immunol 2008;**180**:6988–96.

21 Jameel A, Ooi KG, Jeffs NR, Galatowicz G, Lightman SL, Calder VL. Statin modulation of human T-cell proliferation, IL-1β and IL-17 production, and IFN-γ T cell expression: synergy with conventional immunosuppressive agents. Int J Inflam 2013;**2013**:434586.

22 Kim YC, Kim KK, Shevach EM. Simvastatin induces Foxp3 T regulatory cells by modulation of transforming growth factor-beta signal transduction. Immunology 2010;**130**:484–93.

23 Meng X, Zhang K, Li J *et al*. Statins induce the accumulation of regulatory T cells in atherosclerotic plaque. Mol Med 2012;**18**:598–605.

24 Jougasaki M, Ichiki T, Takenoshita Y, Setoguchi M. Statins suppress interleukin-6-induced monocyte chemo-attractant protein-1 by inhibiting Janus kinase/signal transducers and activators of transcription pathways in human vascular endothelial cells. Br J Pharmacol 2010;**159**:1294–303.

25 Pabbisetty SK, Rabacal W, Maseda D *et al*. KLF2 is a rate-limiting transcription factor that can be targeted to enhance regulatory T-cell production. Proc Natl Acad Sci USA 2014;**111**:9579–84.

26 Pabbisetty SK, Rabacal W, Volanakis EJ *et al*. Peripheral tolerance can be modified by altering KLF2-regulated Treg migration. Proc Natl Acad Sci USA 2016;**113**:E4662–E4670.

27 Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. BMJ 2003;**326**:1423.

28 Chellappa S, Lieske NV, Hagness M, Line PD, Taskén K, Aandahl EM. Human regulatory T cells control TCR signaling and susceptibility to suppression in CD4+ T cells. J Leukoc Biol 2016; **100**:5–16.

29 de Abreu Costa L, Henrique Fernandes Ottoni M, Dos Santos MG *et al*. Dimethyl sulfoxide (DMSO) decreases cell proliferation and TNF-α, IFN-γ, and IL-2 cytokines production in cultures of peripheral blood lymphocytes. Molecules 2017;**22**:1789.

30 Mahnke YD, Roederer M. Optimizing a multicolor immunophenotyping assay. Clin Lab Med 2007;**27**: 469–85, v.

31 Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells *in vivo*. Nat Immunol 2002;**3**:33–41.

32 Zhao X, Liu Y, Zhong Y *et al*. Atorvastatin improves inflammatory response in atherosclerosis by upregulating the expression of GARP. Mediators Inflamm 2015;**2015**:841472.

33 Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. Nature 2013;**499**:485–90.

34 Moon BI, Kim TH, Seoh JY. Functional modulation of regulatory T cells by IL-2. PLOS ONE 2015;**10**:e0141864.

35 Zhang L, Manirarora JN, Wei CH. Evaluation of immunosuppressive function of regulatory T cells using a novel *in vitro* cytotoxicity assay. Cell Biosci 2014;**4**:51.

36 Sirtori CR. The pharmacology of statins. Pharmacol Res. 2014;**88**:3–11.

37 Guasti L, Maresca AM, Schembri L *et al*. Relationship between regulatory T cells subsets and lipid profile in dyslipidemic patients: a longitudinal study during atorvastatin treatment. BMC Cardiovasc Disord 2016;**16**:26.

38 Lins RL, Matthys KE, Verpooten GA *et al*. Pharmacokinetics of atorvastatin and its metabolites after single and multiple dosing in hypercholesterolaemic haemodialysis patients. Nephrol Dial Transplant 2003;**18**:967–76.

39 Mao R, Xiao W, Liu H *et al*. Systematic evaluation of 640 FDA drugs for their effect on $CD4^+Foxp3^+$ regulatory T cells using a novel cell-based high throughput screening assay. Biochem Pharmacol 2013;**85**:1513–24.

40 Sharma MD, Shinde R, McGaha TL *et al*. The PTEN pathway in Tregs is a critical driver of the suppressive tumor microenvironment. Sci Adv 2015;**1**:e1500845.

41 Le Mercier I, Lines JL, Noelle RJ. Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators. Front Immunol 2015;**6**:418.

42 Feske S. Calcium signalling in lymphocyte activation and disease. Nat Rev Immunol 2007;**7**:690–702.

43 Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol 2007;**8**:277–84.

44 Fessler J, Raicht A, Husic R *et al*. Novel senescent regulatory T-cell subset with impaired suppressive function in rheumatoid arthritis. Front Immunol 2017;**8**:300.

45 de Jong HJ, Klungel OH, van Dijk L *et al*. Use of statins is associated with an increased risk of rheumatoid arthritis. Ann Rheum Dis 2012;**71**:648–54.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Untouched total $CD4^+$ T cells were isolated through magnetic separation from PBMCs of healthy donors and cultured during 48 h under TCR activation as it was mentioned on material and methods. Viable cells were determined as 7-AAD- and DIOC-6+ cells ($n = 3$ donors) (a). Representative flow cytometry plot showing live and dead isolated T cells (Tcon and Treg) by using Live/dead fixable cell stain (b). Percentage analysis of live cells in subsets of T con and Treg (c). No statistical significant differences were found ($n = 4$).

Fig. S2. Mean fluorescence intensity (MFI) analysis of perforin ($n = 3$ donors) on Tcons and Tregs in basal conditions and after activation with anti-CD3, anti-CD28 and IL-2 in the absence or presence of the indicated concentrations of ATV for 48 h. No significant differences were found between groups.

Fig. S3. Representative flow cytometry histogram and MFI analysis of CD62L ($n = 8$ donors) (a), CCR7 molecules ($n = 3$

donors) (b), and CCR5 ($n = 4$ donors) in Tcons and Tregs in basal conditions and after activation with anti-CD3, anti-CD28 and IL-2 in the absence or presence of the indicated concentrations of ATV for 48 h. Statistical analyses were performed using the GLM ANOVA, Dunnett's post-hoc tests. Mean and SEM. $^{****}P < 0{\cdot}0001$, $^{***}P < 0{\cdot}0002$. Each dot represents one individual in the graph.

Fig. S4. Correlation of TNF-a-producing Tregs percentage with CTLA-4 MFI (a), FoxP3 MFI (b), and PD1 MFI (c) in Treg cells. Spearman's rank correlation coefficients (r) and p values (*P*) are indicated.

Fig. S5. Tcons and Tregs were isolated by cell sorting and stimulated with anti-CD3, anti-CD28 and IL-2 in the absence or presence of the indicated concentrations of ATV for 48 h. Cells were re-stimulated with PMA ionomycin in the presence of brefeldin A for 5 h. Then, they were stained with monoclonal antibodies to detect the percentage of cells expressing IL-4 ($n = 7$ donors). No significant differences were found between groups.

Fig. S6. Untouched total CD4+ T cells were isolated through magnetic separation fromÐBMCs of healthy donors and they were labeled with CFSE at 1·25 μM. Cells were washed twice with PBS and put in co-cultured in 96-well round-bottom plates at a ratio 1:1 in the presence of CD3/CD28/IL-2 for 72 hours and different atorvastatin (ATV) concentrations. Proliferation of $CD4^+$ T cells was detected as the dilution of CFSE on flow cytometry and Index division (ID) was calculated using FlowJo software. ($n = 3$ donors). Statistical analyses were performed using One-Way ANOVA, Dunnett's post hoc test. Mean and SEM. $^{*}P = 0{\cdot}011$, $^{**}P = 0{\cdot}001$.

Table S1. Antibodies used for flow cytometry.