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Abstract

Statins are one of the most potent drugs in delaying age-related inflammatory changes in the arterial vessel wall, slowing down the progression of atherosclerosis. Statins have also been shown to abrogate telomere-attributed cardiovascular risk. The goal of our study was to explore a potential effect of atorvastatin on telomerase activity in peripheral blood mononuclear cells (PBMCs) and T-lymphocytes (T cells).

Methods and Results: Treatment with pharmacologically relevant concentrations (0.1-0.3 μM) of atorvastatin resulted in a 6-fold increase of telomerase activity (TA) ($p<0.0001$) in human and mouse PBMCs and CD4 T cells, translating into moderate proliferation of T lymphocytes. In contrast, high doses of atorvastatin (2 - 5 μM) or the addition of LDL cholesterol completely inhibited proliferation, thereby abrogating telomerase activity. The proliferative effect of atorvastatin was ablated by the absense of the catalytic subunit of telomerase, telomerase reverse transcriptase (TERT). Using transgenic GFP-*mTert* reporter mice, we observed a decrease in telomerase-positive lymphocytes from 30% to 15% during the first 5 months of age ($p<0.01$). This suggests that the decrease in immune cell turnover during normal development and maturation is mirrored by a reduction in telomerase activity in lymphocytes in-vivo.

Conclusion: Atorvastatin and cholesterol have opposing effects on telomerase in mononuclear cells and T-lymphocytes. Our study suggests a link between cholesterol metabolism and telomere-related cardiovascular risk.

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Atorvastatin induces T cell proliferation by a telomerase reverse transcriptase (TERT) mediated mechanism

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Introduction

Telomerase is a ribonucleoprotein composed of an RNA subunit (TERC), serving as a template for telomere repeat addition, and a reverse transcriptase (TERT) subunit that facilitates the replication of telomeres, the ends of chromososmes [1,2]. TERT also has additional functions beyond telomere maintenance. These include the control of gene expression, chromatin organisation and mitochondria shuttling [3-5].

Multiple studies so far have provided evidence for an association of short telomeres with conditions of increased oxidative stress, including smoking, obesity and coronary heart disease (CHD) [6-11]. In a cohort of 1500 patients the WOSCOPS substudy identified that the telomere-attributed risk of developing coronary heart disease was attenuated by treatment with pravastatin [12]. Atorvastatin has been demonstrated to reduce oxidative stress in various clinical studies, including patients with atherosclerosis, hypercholesterolemia, rheumathoid arthrits, chronic kidney disease and polycystic ovarian syndrome [13-18]. However, it remains unclear whether short telomere length is only a bystander in atherosclerosis and whether statins can exert a direct effect on telomere length. We previously published that telomere length shortening in CHD patients, when compared to age-matched controls, is more pronounced in T-lymphocytes (T cells) than in myeloid cells, suggesting that T cells could play an important role in ageing- and telomere-mediated atherogenesis [19]. T cell mediated immune responses play important roles at all stages of atherosclerotic lesion development [20] with the majority of T cells in an atherosclerotic lesion being CD4+ T-helper cells (Th1) that produce interferon-γ [21-23].

In vitro, atorvastatin has been shown to inhibit T cell activation and proliferation, thereby exerting an anti-inflammatory effect [24,25]. However, these and similar studies used drug concentrations of up to 10 μM, 30 times higher than in the plasma of atorvastatin-treated patients [26]. In pharmacologically relevant dosages, statins have been shown to induce the Akt pathway and promote proliferation in endothelial cells [27]. We have also shown that statins induce telomere-repeat binding factor TRF2 in endothelial progenitor cells [28]. Telomere length was found to be longer in patients under statin therapy compared to those without [29]. A recent study of 230 subjects showed that statin therapy was associated with higher telomerase activity independently of multiple covariates, including age, gender, cardiovascular risk factors and systemic inflammation [30]. In the same study, subjects on statin treatment also showed significant lower telomere erosion along with aging. The goal

of our study was to investigate the *in-vitro* effect of atorvastatin on telomerase and the mechanistic relation between proliferation and telomerase in this context.

Methods

T-cell receptor ligation and culture of human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-hypaque density gradient (using Biochrom AG Biocoll L 6113/5) and prepared at $5x10^6$/ml in supplemented RPMI 1640. For T-cell activation, 24 well plates (VQR 734-2325) were coated overnight with anti-CD3 (BD 550367) and anti-CD28 (BD 555725) antibodies prepared at 1µl/ml PBS at 4°C, except for unstimulated controls. Cells were then cultured in RPMI 1640 (Gibco 21875-034) supplemented by 3mM L-glutamine, 10% foetal bovine serum (PAA A15-151) and 30µg/ml of pen/strep (Gibco 15070-063). Buffy coats were purchased from the National Blood Service, Newcastle Upon Tyne. Ethical approval was granted by the Newcastle University faculty of medical sciences ethics committee, 000205/2009.

Animals

TERT (Jax strain B6.129S-Tert tm1Yjc/J) [31] and TERC (Jax strain B6.Cg-Terc tm1Rdp/J) [32] animals were purchased from Jackson Laboratory, Maine, USA. *mTert*-GFP transgene reporter mice were previously described [33,34]. To confirm the phenotype of GFP transgenic mice, blood from the mouse tail was taken and mixed with 1% of PBS/EDTA solution before lysis of red blood cells in Red Blood Cell Lysing Buffer (Life Technologies uk) for 20 min at 4°C. Cells were then washed and resuspended in PBS solution and DAPI to confirm cell viability. All the samples were collected using FACS Calibur (BD Biosciences, UK) and were analysed with BD FACSDiva software. All work complied with the guiding principles for the care and use of laboratory animals in the UK. Mice were provided with sawdust and paper bedding and had ad libitum access to food and water. Mice were housed at 20 +/- 2°C under a 12h light/12h dark photoperiod. All mice were held under the UK Home office animal license PPL 60/3864.

Mouse PBMC cell culture

Cells were grown in complete RF10, RPMI 1640 (Gibco 21875-034) supplemented with 0.5mM 2-mercaptoethanol (Sigma M7522), 25mM Hepes Buffer (Gibco 15630-080), 10% FBS (PAA A15 151). 24 well plates were coated overnight with anti CD3 (BD 553238) and CD28 (BD 553295) antibodies prepared at 1µl/ml PBS at 4°C.

PBMCs were isolated by Ficoll-hypaque density gradient and prepared at $2x10^6$/ml in supplemented RPMI 1640.

Long-term culture and growth curves

Long-term culture of human PBMCs was prepared at $5x10^6$ cells per 2ml RPMI 1640, and supplemented with MACSibead human T cell activation beads at a ratio of 1 bead to every 4 cells (Militenyi 130-091-441) in flat bottom 24 well plates. Medium was changed as cells were counted by Neubauer chamber, and re-stimulated at day 14 with additional beads as per manufacturer's instructions. Mouse cultures were established from $2x10^6$ cells per ml RPMI 1640 supplemented by MACSibead mouse T cell activation beads (Militenyi 130-093-627) at a ratio of 1 bead to every 2 cells. Medium was changed every 2-3 days with addition of IL-2 and atorvastatin (cells split 1:2 if necessary) and cells re-stimulated every 7 days as per manufacturer's instructions. Individual wells were compiled after a total cell number of $25x10^6$ was reached, the resultant aspirated pellet resuspended in 50ml complete mouse medium to achieve the required $2x10^6$ /ml in 50ml flasks.

Hypoxia and normoxia culture conditions

Hypoxia (3%) and normoxia (atmospheric 20%) culture conditions were maintained throughout experiments in Heraeus Hera Cell 150 incubators. Conditions of 5% CO_2 and 37°C were constant.

Pharmacological activators and inhibitors

Atorvastatin was kindly donated by Pfizer and prepared in dimethyl sulfoxide (DMSO, Sigma 472301) to working dilutions of 5µM, 2µM, 0.3µM, 0.1µM and 0.02µM. Interleukin-2 (IL-2, R&D 202-IL) was prepared from frozen stocks (-20°C) of 100µg/ml and added in a working concentration of 1.5µg/ml. Ly294002 (Cell Signaling 9901) was prepared at working doses of 10µM, 2µM and 0.4µM in DMSO. Akt kinase 1/2 inhibitor (Sigma A6730) was prepared at working doses of 10µM, 2µM and 0.4µM in DMSO. For all experiments and conditions, DMSO concentrations in culture medium never exceeded 0.1% and 0.1% DMSO was added to control wells.

Addition of LDL cholesterol in cholesterol-free media

Human plasma LDL cholesterol (Sigma L7914) was diluted in 150 mM NaCl and 0.01% EDTA to working stocks of 200, 100 and 50mg/dl added to RPMI supplemented by 10% lipoprotein deficient serum (Sigma S5394) 30µg/ml of pen/strep (Gibco 15070-063). Cholesterol was added serially at each media change

every 2-3 days.

Immunomagnetic sorting of human CD4 and CD8 T-cells

Single cell suspensions of PBMCs were centrifuged in a Ficoll-Hypaque gradient, and re-suspended in 90µl ice-cold MACS buffer (2mM EDTA, 0.5ml BSA per 100ml PBS) per 10^7 cells. PBMC suspensions were then incubated with 10µl per 10^7 cells anti-CD8 mAb labelled magnetic beads (Militenyi Biotec 130-045-201) under refrigerated conditions to prevent antibody capping and non-specific binding. Cells were then passed through a pre-cooled magnetic LS column (Militenyi 130-042-401) apparatus and the column was repeatedly washed with buffer. Immediately collected cells constituted a fraction depleted of CD8 T cells, which were retained in the column and forced into a separate collection tube by replacement of the column plunger. This fraction was passed down the column a second time to improve its purity. The depleted fraction was identically treated with anti-CD14 beads (Militenyi 71-5775-40), and the depleted fraction with anti CD4 beads (Militenyi 71-5775-40). A multi conjugated anti CD3 (PE), CD8 (FITC), CD4 (PerCP) [BD Tritest 342445] antibody was added at 10µl per $5x10^5$ cells and incubated for 20 min at 4°C to measure purity of CD4+ and CD8+ populations post isolation (above 80% was acceptable). Analysis was performed using the FACSCanto apparatus (BD Biosciences) with the use of FACSDiva software (BD Biosciences).

Proliferation assay with CSFE

Human PBMCs were isolated by Ficoll-Hypaque density gradients and cultured in RPMI 1640 (Gibco 21875-034), supplemented by 3mM L-glutamine, 10% foetal bovine serum (PAA A15-151) and 30µg/ml of pen/strep (Gibco 15070-063). Cells at $5x10^6$/ml in serum free RPMI 1640 were incubated at 37°C for 3 min with 0.25 µM carboxyfluorescein diacetate (CFSE, Invitrogen CellTrace C34554). Staining was terminated by addition of 10% foetal bovine serum (PAA A15-151) at 37°C for 10 mins. Cells were cultured at $5x10^6$/well in 100 µl of culture medium in 24 flat-bottom plates in a standard 37°C CO_2 incubator for 3 to 5 days. Cultures were stimulated with 1.5µg/µl IL-2 and atorvastatin (Pfizer US). Dry cell pellets ($1x10^6$) were stained with anti-CD4 (BD PE Cy-7560644), CD8 (APC-H7 560273) and CD14 (Invitrogen Pacific Blue MHCD1428) antibodies for 20 mins in the dark at RT, washed in 1xPBS before aspirated pellets were suspended in 500µl FACS buffer (1xPBS supplemented with 1mM EDTA, 25mM HEPES and 1% FBS) and measured on FACSCanto II (BD) using FacsDiva. Murine PBMCs from $TERT^{+/+}$ and $TERT^{-/-}$ mice were isolated by Ficoll density gradient centrifugation, and freshly Isolated PBMCs

were resuspended in PBS (0.1% BSA) at $1x10^6$ cells/ml before incubation with CFSE (final concentration: 0.25 µM) for 10 min at 37°C. Cells were washed and resuspended in culture medium for 15 min to stabilize CFSE staining and then cultured in a 48-well plate at $1x10^6$ per well with 10% foetal bovine serum RPMI 1640 (Gibco 21875-034). Cultures were stimulated with 1.5µg/µl IL-2 and atorvastatin (Pfizer US) for 5 days in a standard 37°C CO_2 incubator. For T-cell activation of human and murine cells, wells were coated overnight with anti-CD3 and anti-CD28 antibodies as described above.

TRAP telomerase activity assay

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) assay as previously established using an end-labeled telomerase substrate (TS)[5'-AatorvastatinCCGTCGAGCAGAGTT-3'] primer and ACX [5'-GCGCGG$(CTTACC)_3$CTAACC-3'] reverse primer. The PCR mastermix volume was 25µl per sample and contained 1xSYBR Green (Applied Biosystems Mastermix 4309155), ds.H_2O and 1mM EGTA supplemented by 0.1µg of each primer. PCR was performed in MicroAmp Fast-tubes (Applied Biosystems 4358293) each sample in triplicate. The reaction mixture was first incubated at 25 °C for 30 min to allow the telomerase in the protein extracts to elongate the TS primer by adding TTAGGG repeat sequences. The PCR was then started at 95 °C for 10 min, followed by a 40-cycle amplification (95 °C for 15 s, 60 °C for 1min) and 60 minute melting curve stage (95°C for 15s, 60°C for 1min, 95°C for 15s). The fluorescent dye SYBR Green (Applied Biosystems 4309155) was used to bind to ds DNA as new amplicons were produced and generate fluorescent signals. These were collected and analysed with detection software (Applied Biosystems). Telomerase activity in cell lines or samples was calculated based on the threshold cycle (C_T).

Telomere length (Flow-FISH)

Telomere length measurements were carried out as previously described [28,35,36]. In brief, each sample, containing 100,000 bovine thymocytes as an internal standard and 200,000 cells from the patient, was resuspended in a hybridization mixture containing telomere-specific N-terminal FITC-conjugated $(C_3TA_2)_3$ peptide nucleic acid probe, washed and counterstained with propidium iodide before analysing by FACS analysis.

Statistics

Significance between data was compared by one-way ANOVA followed by Tukey's post hoc analysis, or 2 way ANOVA followed by Bonferroni's post-hoc analysis. $P<0.05$ was considered statistically significant.

Results

Atorvastatin induces telomerase at pharmacological dosages

PBMCs were isolated from healthy volunteers and co-stimulated with α-CD3 and α-CD28 in culture. The addition of atorvastatin lead to an 6-fold increase in telomerase activity, peaking at 0.3 μM concentration after 5 days (Figure 1A). A time course study confirmed that telomerase activity (TA) was only temporarily induced and reached its maximum after 5 days, then receding back to baseline levels at day 10 (Figure 1B). Statins have been shown to exert some of their protective effects on vascular cells through the Akt/PI3 kinase pathway [27]. Induction of telomerase by atorvastatin was prevented by inhibitors of the Akt and PI3 kinase pathway, respectively (Figures 1B and 1C). To determine whether specific subsets of T cells were affected by atorvastatin, we examined populations of purified human CD4 and CD8 T cells seperately. Here we found that induction of TA was most pronounced in CD4 compared to CD8 T cells (650% vs 256%, Figure 1E). Finally, we analysed mononuclear cells from patients with acute ST elevation myocardial infarction (STEMI), a state of high inflammation. We found TA in unstimulated PBMCs to be 5-fold reduced compared to controls ($p<0.05$ vs healthy controls, $p<0.01$ vs post-infarction; Figure 1F).

The effect of atorvastatin on T-lymphocyte proliferation is dose-dependent

In order to test the effect of statins on T-cell proliferation, mononuclear cells from healthy volunteers were co-stimulated and treated with a wide dose range of atorvastatin. Physiologically relevant dosages of up to 0.3 μM led to a significant increase in cell number after 5 days in culture (Figure 2A) as well as a moderate increase after 28 days (Figure 2B). Proliferation assays using CSFE and co-staining for CD4 T cells demonstrated an increase in the rate of proliferating CD4 T cells from 42±4% up to 73±1% under 0.3 μM atorvastatin ($p<0.001$, Figures 2C-D). As previously published, higher concentrations (2 - 5 μM) of atorvastatin inhibited T-cell proliferation (Figure 2D) without inducing cell death (data not shown). Baseline as well as statin-induced proliferation was completely abrogated by co-treatment with an Akt-kinase inhibitor (Figure 2E).

LDL cholesterol suppresses telomerase

We next sought to investigate whether the statin effect on telomerase was co-dependent on cholesterol metabolism. For this we used cholesterol free media and

added increasing concentrations of LDL cholesterol. At 100 mg/dl TA was completely suppressed and no statin effect visible (Figure 3A). To exclude that the reduction in telomerase activity was not an artificial result of the PCR-based assay, we performed growth curves in human (Figure 3B) and murine (Figure 3C) T-cells. For both species, LDL cholesterol significantly inhibited T cell proliferation.

Telomerase activation in lymphocytes declines with age

We have previously generated an *mTert*-GFP transgenic mouse model in which GFP expression indicates endogenous *mTert* expression and telomerase activity. *mTert*-GFP^+ cells among peripheral blood lymphocytes decreased from 30% to 15% continuously between the 2nd and the 5th month ($p<0.01$, Figure 3D and 3E), suggesting a correlation of telomerase-positive lymphocytes with cell turnover in-vivo.

Telomerase mediates the proliferative effect of atorvastatin

Atorvastatin induced TA under 3% ($p<0.001$, Figure 4A) and to a lesser degree under 20% (Figure 4B) oxygen in murine T cells. The linearity of the TRAP assay was confirmed for murine cells (Figure 4C). So far we have shown that the induction of TA is paralleled by a moderate increase in T cell proliferation. To prove a causal relationship, we isolated murine splenocytes with a genetic knockdown of TERT [31], the catalytic subunit of telomerase, and monitored T cell proliferation over 4 weeks in culture. As expected, T cells from heterozygous $TERT^{+/-}$ mice already displayed reduced proliferation ($p<0.05$, Figure 4D). Knock-out of TERT further reduced T cell growth over 4 weeks ($p<0.0001$ vs wild type, Figure 4D). Atorvastatin only enhanced proliferation in $TERT^{+/+}$ splenocytes with preserved telomerase activity at a dose of 0.3 μM, but not in $TERT^{-/-}$ cells (Figure 4E). At the highest dose (5 μM), atorvastatin completely inhibited ^{3}H-thymidine incorporation at later timepoints, independent of telomerase (data not shown).

To exclude the possibility that differences in proliferation were secondary to telomere length (TL) shortening in the telomerase-deficient animals, we measured mean telomere length of splenocytes. As expected, splenocytes from first generation (F1) $mTert^{-/-}$ mice did not reveal any TL shortening (Figure 5A), while heterozygous $mTerc^{+/-}$ mice already displayed shorter TL ($p<0.001$). Finally, we attempted to explain the role of telomerase for statin-induced T cell proliferation. The statin-induced increase in proliferation was completely dependent on functional mTert (Figure 5B) with a residual effect in $TERC^{-/-}$ T cells (Figure 5C). Again, shorter TL in

cells from second generation TERC mice (F2) had no impact on the statin effect (Figure 5B and 5C).

Discussion

Statins are established as a first-line therapy for secondary prevention in patients with documented coronary heart disease, improving morbidity and mortality. While the preponderance of evidence strongly suggests that a lower serum concentration of LDL cholesterol is the principal mechanism responsible for improved outcome, the benefits of statin therapy, particularly in primary prevention, may extend beyond those attributable to lipid-lowering (termed pleiotropic effects) [37]. Taking into account that atherosclerosis is an inflammatory disease characterized by intense immunological activity, these data suggest the possibility that statins are, in addition to their lipid-lowering capacity, potential "anti-inflammatory" drugs targeting monocytes, macrophages and lymphocytes. By contrast, however, a recent meta-analysis of data from 170,000 patients showed that each 1 mmol/L LDL cholesterol reduction reduces the risk of occlusive vascular events by about 20%, irrespective of baseline cholesterol concentration [38]. Thus, although an attractive concept, the existence of pleiotropic effects of statins remains highly controversial. Convincing mechanistic support for such effects has yet to be demonstrated. Many of the hypothesised pleiotropic effects have been thought to occur through inhibition of isoprenoid synthesis with subsequent inhibition of isoprenoid-mediated activation of small GTP-binding proteins, such as Rho family members, Rac1 and RhoA, observed *in vitro* [24-26]. Nevertheless, the dosages of statins commonly used in those in-vitro studies by far exceed therapeutic concentrations. The inhibitory concentration IC_{50} for cholesterol biosynthesis is 9.8 nM, while IC_{50} for protein prenylation is 2.6 µM (lovastatin). Given that therapeutic plasma levels of statins do not exceed 0.3 µM, it seems unlikely that inhibition of protein prenylation will play a role in vivo [26]. Our results clearly show though, that the anti-proliferative effect of atorvastatin only occurs at suprapharmacological doses (2-5 µM) as a result of blocked protein prenylation. Therefore it is highly unlikely to expect that statin therapy exerts an anti-proliferative effect on lymphocytes in-vivo. On the other side, inhibition of cholesterol synthesis seems to parallel the pro-proliferative effect of atorvastatin and activation of telomerase. Although the principal target cell responsible for the effect of statins on plasma cholesterol is the hepatocyte, statins lead to inhibition of cholesterol synthesis in all extra-hepatic tissues [39]. A recent study suggests that therapeutic concentrations of statins modulate key cell signaling pathways in diverse

cell types as a direct consequence of cholesterol depletion [40]. The authors show that lovastatin induces Ras signaling in a cholesterol-dependent fashion. Depletion of cholesterol from kidney and endothelial cells had the same effect as lovastatin treatment. The PI3-kinase/Akt-kinase pathway is a downstream target of Ras signalling, and a positive regulator of telomerase [41]. Thus, telomere and telomerase dependent effects on cell metabolism could very well be a consequence of lower intracellular cholesterol concentrations due to statin therapy. Our study shows that pro-telomeric effects of atorvastatin are downstream of Akt and counteracted by direct addition of LDL cholesterol. Should statins prove to exert telomerase-dependent effects on atheroprotective lymphocyte populations, telomerase could become a direct target for other forms of age-decelerating therapy. Finally, we provide further evidence that the proliferative capacity of T-lymphocytes is not dependent on, but can be significantly augmented by the activation of telomerase. Using the TERT-GFP reporter mouse, we also show that high turnover in the lymphocyte compartment at an early age is paralleled by a high number of telomerase-positive cells *in-vivo*. Together, our results indicate a bidirectional relationship between proliferation and telomerase – inhibition of one will negatively affect the other.

Given the importance of T-lymphocytes in atherogenesis and our findings that in patients with CHD telomeres from peripheral blood lymphocytes shorten faster than in myeloid cells,[19,36] our data suggest that telomere length might be a downstream target in T cells. However, this does not exclude that critically short telomeres in subsets of human T cells will affect their proliferation by driving a senescent phenotype of these cells. Restoring telomerase activity in T-cells, e.g. through small molecule activators such as TA-65 [42,43], could prove to be a powerful therapeutic intervention in age-related diseases such as atherosclerosis. Future studies have to identify the cell-specific role of telomerase in atherogenesis. In the mouse model, this can be accomplished by studying cholesterol-fed Apo E null x mTert-GFP reporter mice. We could not find identify telomerase-independent functional differences in murine T cells. However, mouse telomeres are several-fold longer than human telomeres. In our opinion, decreased telomerase activity in human T cells might accelerate telomere shortening and cellular senescence over a long period of time. Our unpublished data from the Newcastle 85+ study (Spyridopoulos et al., manuscript in preparation) suggests that immunosenescence can be an independent predictor of outcome in the elderly, possible via accelerated atherosclerosis.

Disclosures

None.

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Figure Legends

Figure 1: Atorvastatin induces telomerase in T-lymphocytes. A. Telomerase activity in human PBMCs, activated by T-cell receptor ligation with α-CD3 and α-CD28 antibodies coated to wells (n=5 different donors). Atorvastatin was added at the appropriate concentrations and cells cultured for 5 days under normoxia. **B.** Telomerase activity over a 10-day time course of human PBMCs (n=3 different donors). **C-D.** Telomerase activity in activated human PBMCs. An Akt kinase-specific inhibitor (**C**) and a PI3-kinase specific inhibitor (Ly294002, **D**) were added at day 0 at the indicated concentrations (n=3 different donors). **E.** Comparitive telomerase activity in MACS isolated CD4 and CD8 human T cells (n=3 different donors). **F.** Telomerase activity of human PBMCs isolated from patient blood samples. Samples from healthy controls (n=13), patients with acute STEMI (n=8) and 3 months post STEMI (n=17) were collected and measured by TRAP qPCR. ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2: Atorvastatin induces proliferation in CD4 T-lymphocytes by an Akt-dependent pathway. A. Cell counts of human PBMCs measured after 5 days in culture, activated by T-cell receptor ligation with α-CD3 and α-CD28 antibodies. **B.** Long-term growth curves of isolated human CD4 T-cells grown over 28 days using expansion kit beads. **C.** Representative histograms of gated CD4 T cells from a single CSFE experiment. **D.** Proliferation of human CD4 T cells with atorvastatin, quantified as $CD4^{hi}CSFE^{lo}$ cells among all PBMCs. **E.** Proliferation of human CD4 T cells under 0.3 μM atorvastatin treated with an Akt kinase-specific inhibitor that was added at day 0 at the indicated concentrations. AT = atorvastatin, IL-2 = interleukin-2, un=unstimulated cells, co=activated cells without statin, ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All p-values are against control, calculated by 1-way ANOVA and Tukey post-hoc tests. All experiments n=3 different donors.

Figure 3. LDL cholesterol suppresses telomerase and proliferation in T-lymphocytes. A. Telomerase activity measured by qPCR in activated human lymphocytes grown in lipoprotein free medium (n=5 different donors). un = unstimulated cells, co = control, atorvastatin concentrations in μM. **B.** Cell counts of human lymphocytes after 5 day culture with cholesterol free medium with the addition of LDL-cholesterol (n=2 different donors, AT = atorvastatin 0.3 μg/ml). **C.** Growth

curve of mouse splenocytes with cholesterol free medium with the addition of LDL-cholesterol (n=3 different donors). **D.** Gating strategy for *mTert*-GFP^{+} lymphocytes. Peripheral blood was obtained from the tail vein and counterstained with DAPI. **E.** Quantification of GFP-positive lymphocytes in the peripheral blood at different ages. ns=not significant, **p<0.01.

Figure 4. Atorvastatin-induced T-cell proliferation depends on functional TERT. **A-B.** Telomerase activity measured by qPCR of splenocytes from TERT$^{+/-}$ (n=6 experiments, all from different mice) and TERT$^{-/-}$ (n=6) after 5 day culture under 3% and 20% oxygen conditions. **C.** Telomerase activity measured by qPCR of a titration of TerT$^{+/+}$ splenocytes after 5 day culture under 3% oxygen. **D.** Long-term growth curve of splenocytes from TERT$^{+/+}$ (n=4), TERT$^{+/-}$ (n=4) and TERT$^{-/-}$ (n=3) under 3% oxygen conditions. **E.** Proliferation of splenocytes from TERT$^{+/+}$ (n=3) and TERT$^{-/-}$ F1 (n=3), measured at 72 hours under 20% oxygen by gating on CSFE^{lo} cells.'-' indicates unstimulated cells, 'co' indicates α-CD3 and α-CD28 stimulated cells without atorvastatin. ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5. Atorvastatin-induced T-cell proliferation depends on functional TERT. **A.** Mean telomere length of T lymphocytes from TERT$^{+/+}$ (n=6 experiments, all from different mice), F1$^{-/-}$ (n=3), F4$^{-/-}$ (n=6), TERC$^{+/+}$ (n=6), TERC$^{+/-}$ (n=3) and TERC$^{-/-}$ F2 (n=6) mice determined by Flow-FISH. Human PBMCs and bovine thymocytes served as a control. Long-term growth curve of splenocytes from TERT$^{+/+}$ (n=4) and TerT$^{-/-}$ (n=3) under 3% oxygen conditions. AT = atorvastatin 0.3 µg/ml. All comparisons against TERT wt using 1-way ANOVA with Tukey's post-hoc tests. **B.** Cell counts of splenocytes from TERT$^{+/-}$ (n=3), TERT$^{-/-}$ F2 (n=3) and TERT$^{-/-}$ F3 mice after 5 day culture under 3% oxygen conditions. **C.** Cell counts of splenocytes from TERC$^{+/-}$ (n=3) and TERC$^{-/-}$ F2 (n=3) mice after 5 day culture under 3% and 20% oxygen conditions. For dose response curves, 2-way ANOVA with Bonferoni's multiple comparison test was used. p-values were calculated of each statin dose against control (co). ns=not significant, *p<0.05, ***p<0.001, ****p<0.0001.

Figure 1

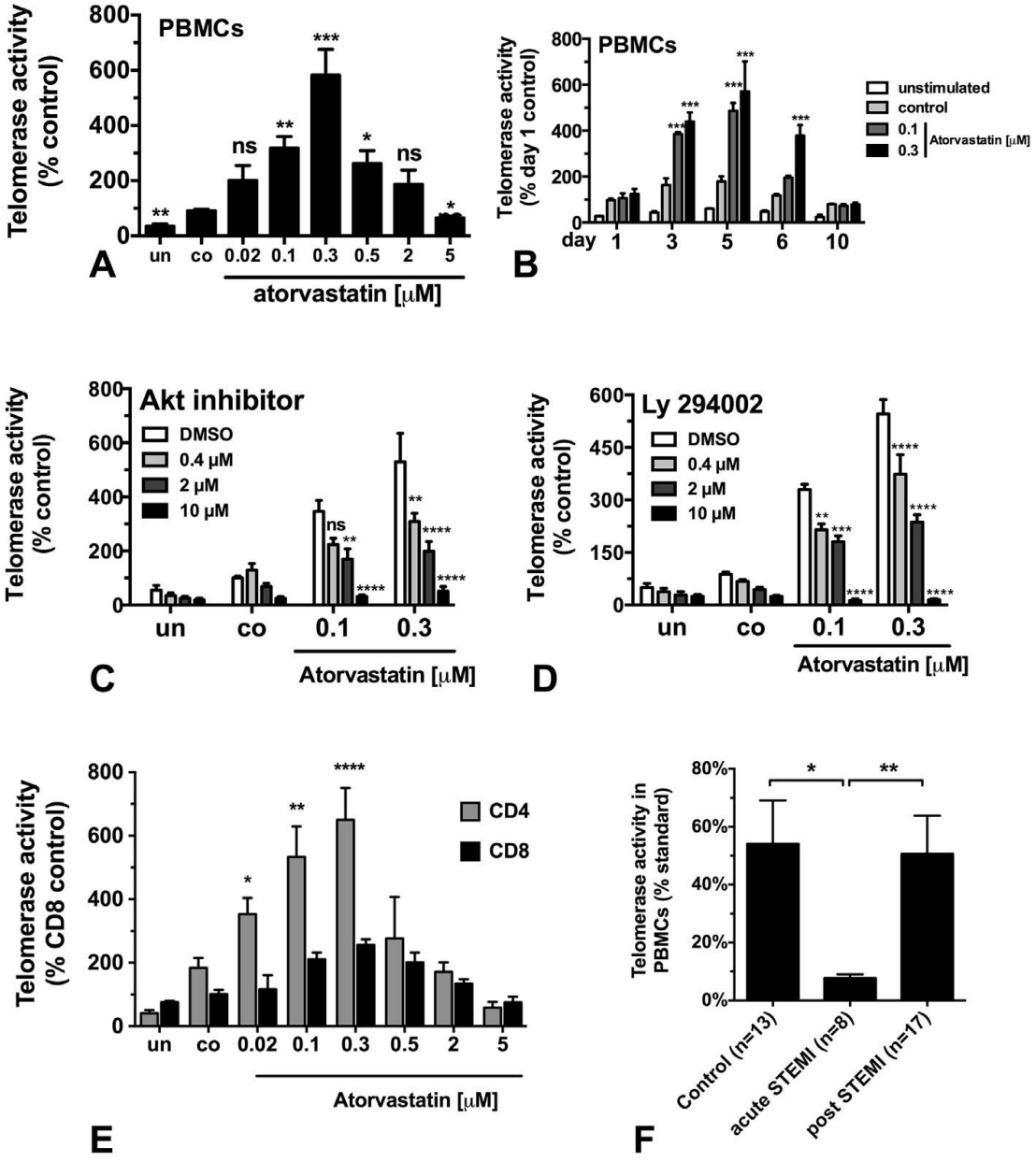

PBMCs
Telomerase activity (% control)
un
co
0.02
0.1
0.3
0.5
2
5
atorvastatin [μM]
A
PBMCs
Telomerase activity (% day 1 control)
day 1
3
5
6
10
unstimulated
control
0.1
0.3
Atorvastatin [μM]
B
Akt inhibitor
DMSO
0.4 μM
2 μM
10 μM
C
Ly 294002
D
CD4
CD8
E
Telomerase activity in PBMCs (% standard)
Control (n=13)
acute STEMI (n=8)
post STEMI (n=17)
F

Figure 2

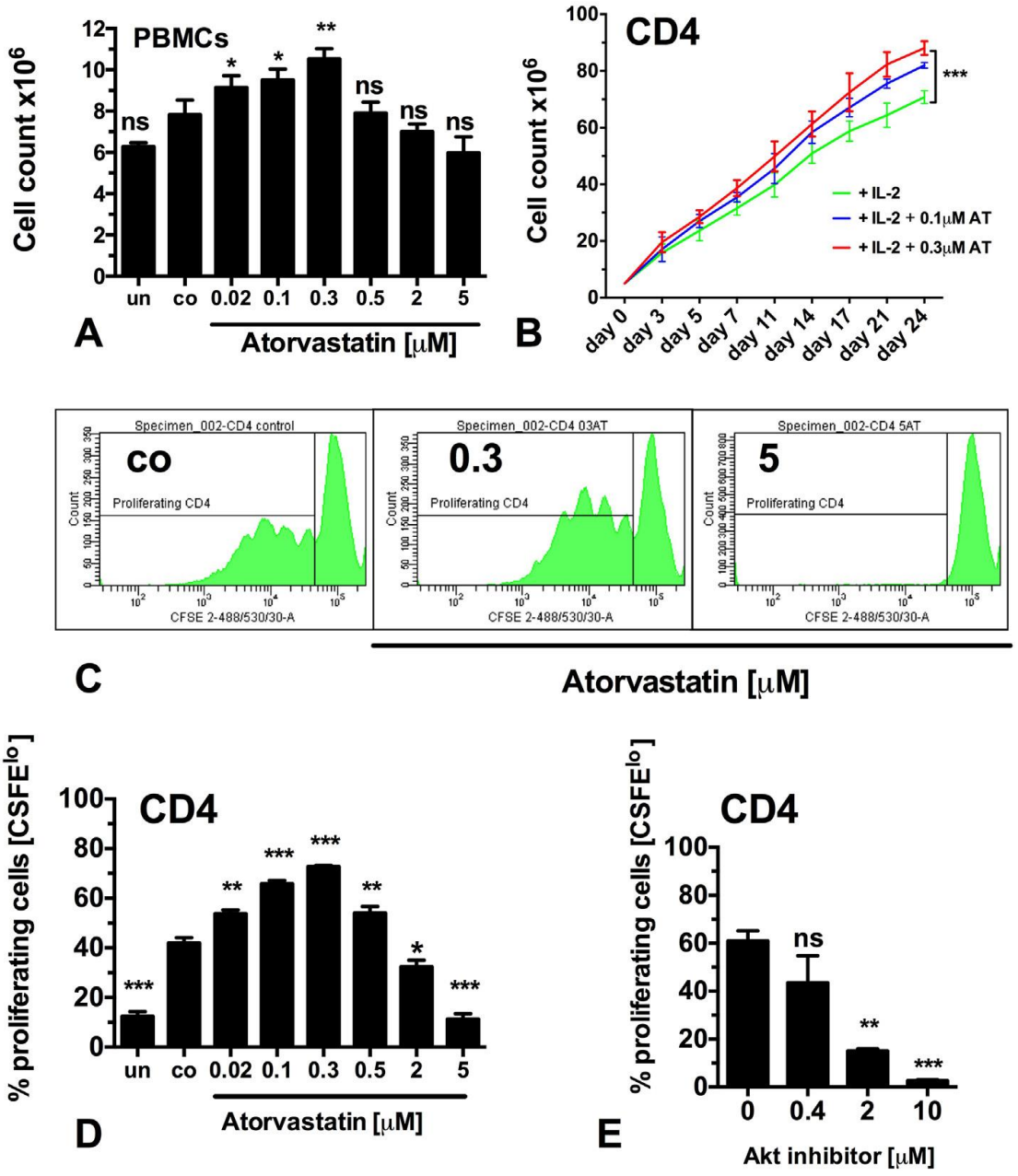

PBMCs
Cell count x10^6
ns
*
*
**
ns
ns
ns
un
co
0.02
0.1
0.3
0.5
2
5
A
Atorvastatin [μM]
CD4
Cell count x10^6
***
+ IL-2
+ IL-2 + 0.1μM AT
+ IL-2 + 0.3μM AT
day 0
day 3
day 5
day 7
day 11
day 14
day 17
day 21
day 24
B
Specimen_002-CD4 control
co
Proliferating CD4
Count
CFSE 2-488/530/30-A
Specimen_002-CD4 03AT
0.3
Proliferating CD4
Count
CFSE 2-488/530/30-A
Specimen_002-CD4 5AT
5
Proliferating CD4
Count
CFSE 2-488/530/30-A
C
Atorvastatin [μM]
% proliferating cells [CSFE^lo]
CD4
***
**
***
***
**
*
***
un
co
0.02
0.1
0.3
0.5
2
5
D
Atorvastatin [μM]
% proliferating cells [CSFE^lo]
CD4
ns
**
***
0
0.4
2
10
E
Akt inhibitor [μM]

Figure 3

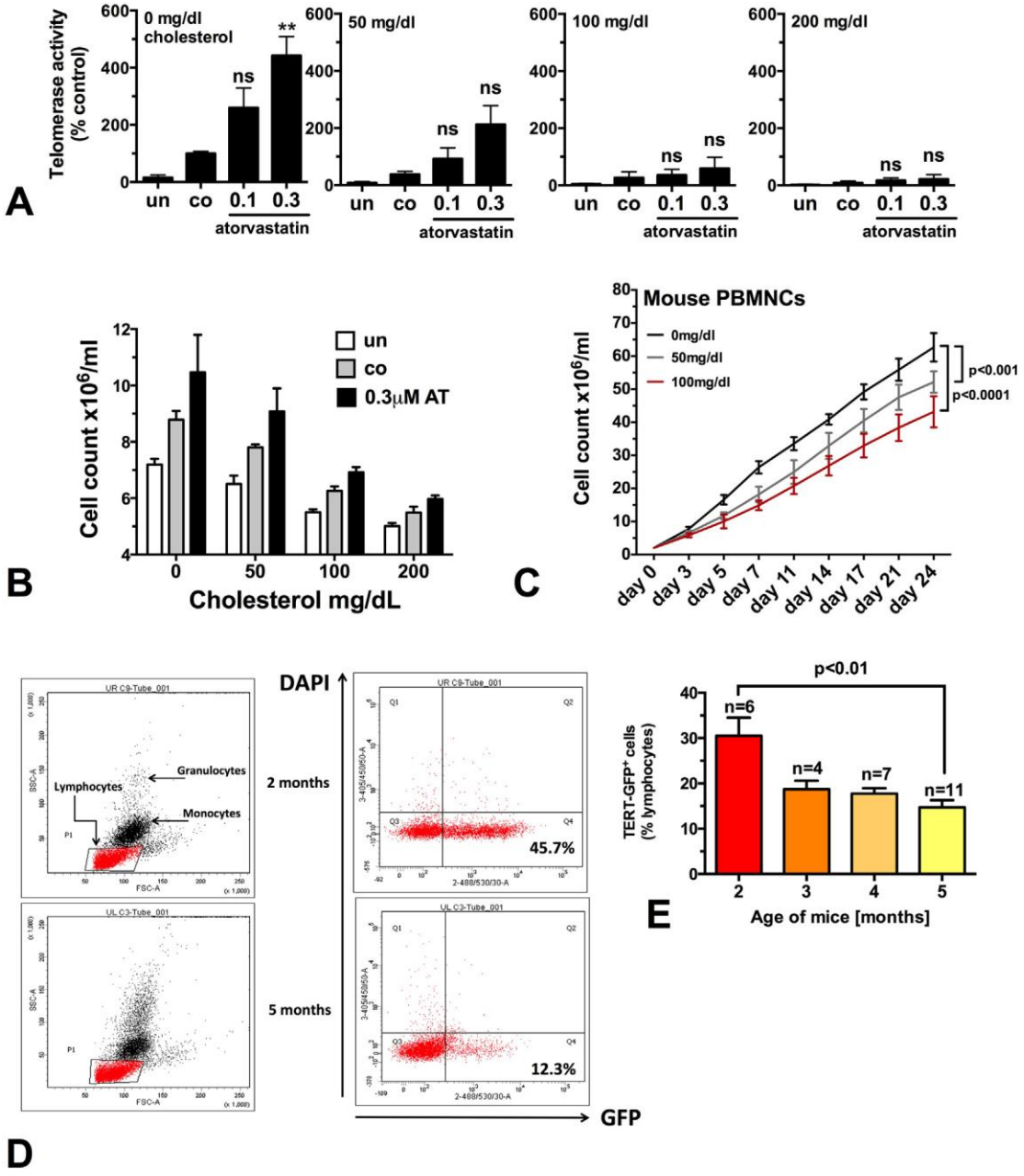

A
Telomerase activity (% control)
0 mg/dl cholesterol
50 mg/dl
100 mg/dl
200 mg/dl
ns
**
un
co
0.1
0.3
atorvastatin
B
Cell count x10^6/ml
un
co
0.3μM AT
Cholesterol mg/dL
C
Mouse PBMNCs
0mg/dl
50mg/dl
100mg/dl
p<0.001
p<0.0001
day 0
day 3
day 5
day 7
day 11
day 14
day 17
day 21
day 24
D
DAPI
Granulocytes
Lymphocytes
Monocytes
2 months
5 months
45.7%
12.3%
GFP
E
p<0.01
n=6
n=4
n=7
n=11
TERT-GFP+ cells (% lymphocytes)
Age of mice [months]

Figure 4

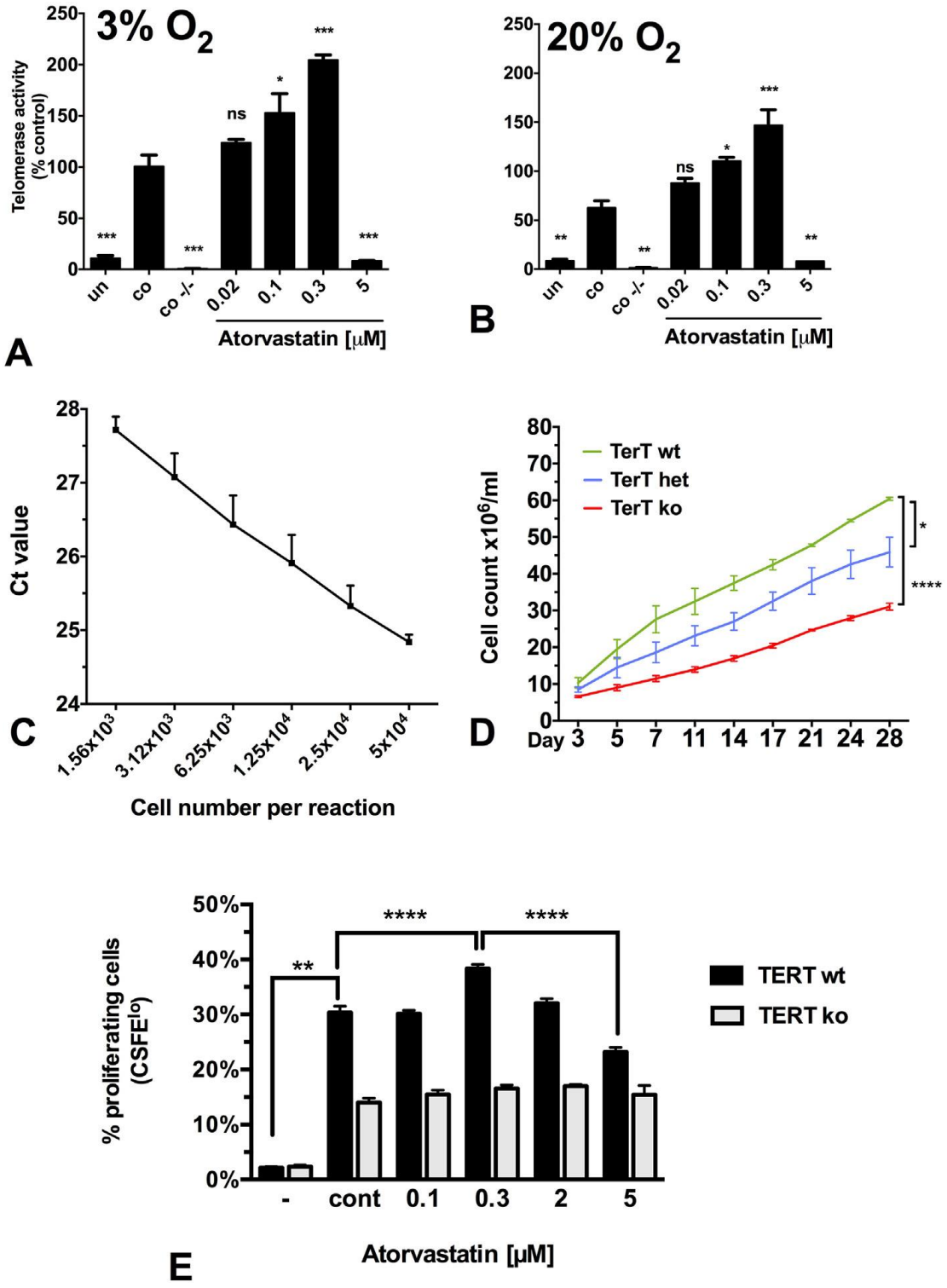
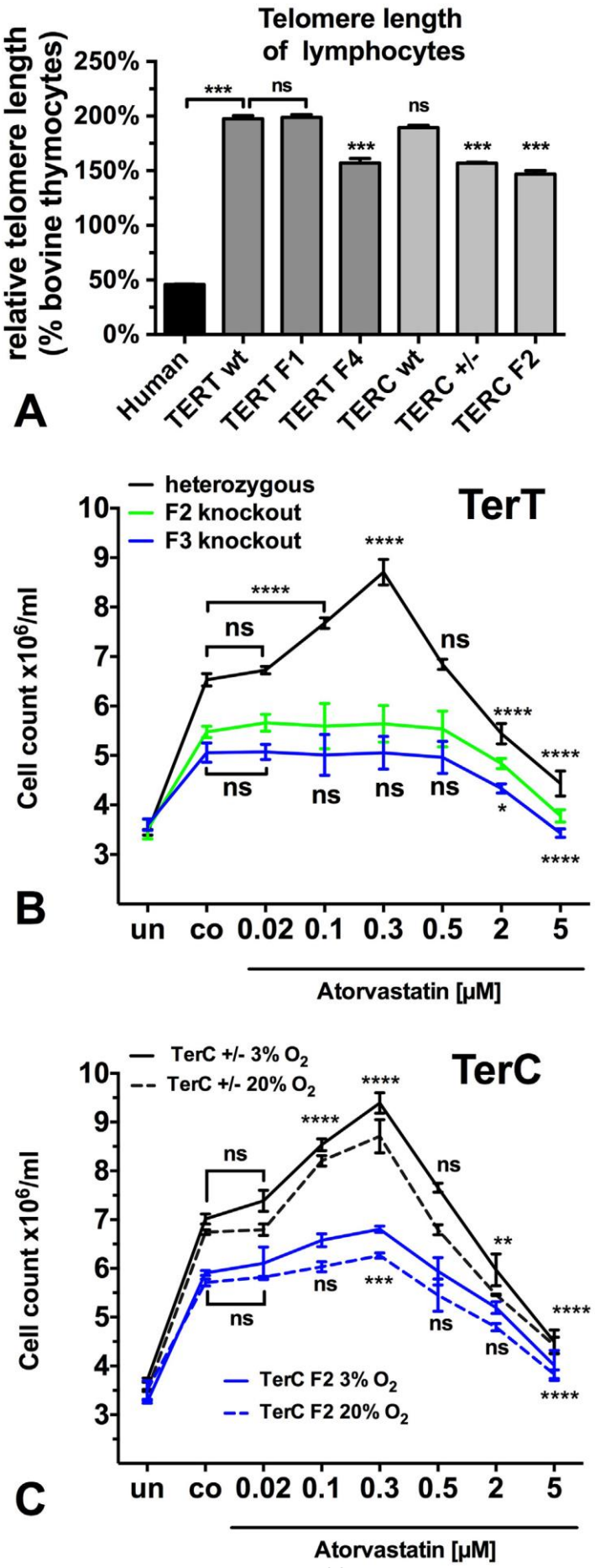

3% O2
Telomerase activity (% control)
250
200
150
100
50
0
***
ns
*
***
***
un
co
co -/-
0.02
0.1
0.3
5
Atorvastatin [µM]
A
20% O2
**
**
ns
*
***
**
B
Ct value
28
27
26
25
24
1.56x10^3
3.12x10^3
6.25x10^3
1.25x10^4
2.5x10^4
5x10^4
Cell number per reaction
C
Cell count x10^6/ml
80
70
60
50
40
30
20
10
0
TerT wt
TerT het
TerT ko
*
****
Day 3 5 7 11 14 17 21 24 28
D
% proliferating cells (CSFE^lo)
50%
40%
30%
20%
10%
0%
**
****
****
-
cont
0.1
0.3
2
5
Atorvastatin [µM]
TERT wt
TERT ko
E

Figure 5


Telomere length
of lymphocytes
relative telomere length
(% bovine thymocytes)
250%
200%
150%
100%
50%
0%
***
ns
ns
***
***
***
Human
TERT wt
TERT F1
TERT F4
TERC wt
TERC +/-
TERC F2
A
heterozygous
F2 knockout
F3 knockout
TerT
Cell count x10^6/ml
10
9
8
7
6
5
4
3
****
****
ns
ns
ns
ns
ns
ns
ns
****
****
*
****
un
co
0.02
0.1
0.3
0.5
2
5
Atorvastatin [µM]
B
TerC +/- 3% O2
TerC +/- 20% O2
TerC
Cell count x10^6/ml
10
9
8
7
6
5
4
3
****
****
ns
ns
ns
***
ns
ns
ns
**
****
****
TerC F2 3% O2
TerC F2 20% O2
un
co
0.02
0.1
0.3
0.5
2
5
Atorvastatin [µM]
C