




Effects of Atorvastatin on T-Cell Activation and Apoptosis in Systemic Lupus Erythematosus and Novel Simulated Interactions With C-Reactive Protein and Interleukin 6

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Objective. We study activation of T helper 17 (Th17) and regulatory T (Treg) cells and induction of apoptosis in cells from patients with systemic lupus erythematosus (SLE) compared with controls and effects of atorvastatin and its simulated interactions with other compounds.

Methods. Mononuclear cells from 10 patients with SLE and 10 controls were cultured in conditions that induce Th17 and/or Treg cell polarization and/or apoptosis and were studied by FACScan. Gene expression was determined by quantitative real-time reverse transcription–polymerase chain reaction. Cytokines in plasma were determined by enzyme-linked immunosorbent assay. The Search Tool for Interactions of Chemicals (STITCH) was used to retrieve information regarding the binding properties of atorvastatin.

Results. Among patients with SLE, the proportion of Th17 (CD4⁺IL17⁺) cells was higher compared with controls after activation, with Th17 or Treg polarizing cytokines, phorbol myristate acetate, and ionomycin. In contrast, Treg cells (CD4⁺CD25⁺CD127^{dim/−}) frequencies were lower. CD95 stimulation induced relatively more apoptosis in Treg cells and less in Th17 cells, as compared with controls. Addition of atorvastatin normalized Th17/Treg cell balance and apoptosis induction. Accordingly, the ratio of RORC/FoxP3 decreased in patients with SLE. Interleukin 17 and interleukin 6 (IL-6) levels were increased in patients with SLE. Atorvastatin interacted strongly with C-reactive protein (CRP) and also significantly with IL-6.

Conclusion. There is a higher proportion of Th17 cells and a lower proportion of Treg cells in patients with SLE after activation. Th17 cells were more resistant than Treg cells to CD95-induced apoptosis in SLE. Atorvastatin normalized these effects. Our findings reveal a novel mechanism behind the imbalance of Th17/Treg cells with implications for treatment in SLE. We determine for the first time simulated interaction between atorvastatin, CRP, and IL-6, implying a novel role of atorvastatin.

INTRODUCTION

Systemic lupus erythematosus (SLE) has various manifestations, including cardiovascular disease (CVD) and increased prevalence of atherosclerotic plaques as well as vulnerable plaques (1,2). Many immunological aberrations have been described, affecting both B-cell (3) and T-cell function (4). Atherosclerosis, the major cause of CVD, is an inflammatory condition characterized by the accumulation of dead cells, oxidized low-density lipoproteins (OxLDL), and activated immune-competent cells,

including T cells, macrophages, and dendritic cells (DCs), in the plaques (5). Studies of SLE could shed light on the role of immunity in atherosclerosis (5,6).

Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is essential for cholesterol synthesis (7). Statins may exert effects additional to low-density lipoprotein (LDL) lowering, as in the Jupiter study, in which effects on CVD risk among individuals with increased C-reactive protein (CRP) levels suggested that inflammation plays a role (8). There is also experimental evidence supporting pleiotropic anti-inflammatory effects of statins (9,10). We

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recently reported that atorvastatin has immunomodulatory effects on OxLDL-induced DC and T-cell activation (11).

In a recent study of incident cases, statin use was associated with reduced mortality in patients with systemic autoimmune disease (12). Still, there is a need for randomized studies of statins in SLE (13). Both in SLE and in atherosclerosis, T-cell subsets may play major roles (3–6). Regulatory T (Treg) cells, a subset of CD4⁺ T cells, suppress autoreactivity in SLE. This is also the case for SLE-related atherosclerosis (4,14,15) and atherosclerosis in general (16). SLE is also characterized by defective clearance of apoptotic cells, like atherosclerosis itself (17).

We here study differences in activation pattern and apoptosis between T helper 17 (Th17) and Treg cells from patients with SLE and controls and the effects of a statin, atorvastatin, which is also analyzed by the Search Tool for Interactions of Chemicals (STITCH) protein-chemical network. The simulation of biological pathways in relation to atorvastatin were done by using STITCH to integrate information about metabolic pathways, crystal structures, binding experiments, and relationships of drug targets (18,19). Moreover, the plethora of atorvastatin interactions with various proteins derived from the chemical-protein network enables us to define a molecular mechanism as well as a mechanistic study of atorvastatin binding to CRP. The implications of these findings are discussed.

MATERIALS AND METHODS

Patients with SLE and controls. Ten patients with SLE and 10 age- and sex-matched population controls (nine were women in each group) for cell experiments and 30 from each group for cytokine analysis in serum were from our previously described SLEVIC study (1). The mean age of patients with SLE and of controls was the same, 55.8 ± 9.45 years. The study was approved by the Karolinska Institutet Research Ethics Committee and is in accordance with the Declaration of Helsinki. All participants gave written informed consent before entering the study.

Isolations and culture of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from patients with SLE and controls according to the manufacturer's protocol (Ficoll-Paque PLUS; GE Healthcare). The cells were added to a tissue culture plate precoated with anti-CD3 antibody (10 $\mu\text{g/ml}$) (eBioscience) together with soluble anti-CD28 antibody (1 $\mu\text{g/ml}$) (eBioscience), interleukin 6 (IL-6) (10 ng/ml), interleukin 1 β (IL-1 β) (10 ng/ml), interleukin 2 (10 ng/ml), transforming growth factor β 1 (TGF- β 1) (10 ng/ml) (Immuno Tools), and interleukin 23 (IL-23) (10 ng/ml) (R&D Systems). Cells were kept in culture medium for 6 days to polarize Th17 and Treg cells, with phorbol myristate acetate (50 ng/ml) and ionomycin (1 $\mu\text{g/ml}$) (Sigma Aldrich) stimulation for 5 hours and with or without anti-CD95 antibody (1 $\mu\text{g/ml}$) (BD Bioscience) stimulation for 3 hours on the day of harvest. Atorvastatin (5 $\mu\text{mol/l}$) (Sigma Aldrich) was added 1 day before harvest for the following experiments.

Flow cytometry. PBMCs were resuspended in phosphate-buffered saline, for staining Th17 cells, cells were incubated 30 minutes with FITC-conjugated anti-human CD4 and APC-conjugated anti-human CCR6 (BD Bioscience). For staining Treg cells, cells were incubated with human Treg cocktail (CD4/CD25/CD127) (BD Bioscience). After the surface marker staining, PE-conjugated annexin V and 7-aminoactinomycin D (7-AAD) (BD Bioscience) were added into the samples to quantify the apoptosis. The expression of CD95 and CD95 ligand was detected by PerCP Cy5.5-conjugated anti-human CD95 and PE-conjugated anti-human CD178 (BD Bioscience). Experiments were performed on the BD LSRFortessa cell analyzer (BD Bioscience).

Enzyme-linked immunosorbent assay. We detected the amount of IL-6, interleukin 10 (IL-10), interleukin 17 (IL-17), and tumor necrosis factor α (TNF- α) in conditioned media and human serum with enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems).

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction. Gene expression levels were measured quantitatively by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA of Th17 and Treg cells from polarized cultures were isolated by using the RNeasy Plus macro kit (Qiagen). RNA quality was assessed by the Nanodrop 1000 spectrophotometer (Thermo Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The polymerase chain reaction array layout contained gene-specific primers for Th17 transcription factor RORC and Treg transcription factor FoxP3; GAPDH was included for standardization between samples. The proportion of transcript present in the samples was calculated by using the relative quantification $2^{-\Delta\Delta C_t}$ scheme. A control sample was used as a comparative calibrator. Results represented the relative amount of amplicon in patient samples (fold change) to the mean level of the transcripts in the control samples. Experiments were performed on the Applied Biosystems 7500 Real-Time PCR System (Thermo Scientific).

Chemical-protein network biology for atorvastatin. For large-scale analysis, we fed the query or Medical Subject Headings (MeSH) term "atorvastatin" into the STITCH database to retrieve information regarding the binding of chemicals to the proteins that regulate their activities. The interaction score was set to the highest confidence interval, ie, 0.900, with 20 maximum interactors. The sources of active interactions were referred from text mining, experiments, databases, co-expression, neighborhood, gene fusion, co-occurrence, and predictions. The number of chemicals annotated with each pharmacological action was assessed and measured against a likelihood of sharing a medication goal in the MATADOR database to determine the association's relevance for sharing a pharmacological action. For each

compound, a pattern of activity was calculated on the basis of the anti-cancer drug screens on 60 human tumour cell lines screens, converting the log values growth inhibitory 50% into z scores in each of the cells (a concentration appropriate for 50% inhibition of growth). The use of criteria for the SD of z marks was excluded for compounds with noninformative active patterns. The Pearson correlation of the patterns of activity for all compound pairs was then measured and compared with known action mechanisms. This enabled one to associate the

compounds to well-defined compounds with unknown mechanisms of action. In summary, the thickness of the edge between nodes of the interaction's scales was associated with the protein binding constants (K_i) value. If a K_i was not available, half maximal effective concentration or half-maximal inhibitory concentration was used to determine the depicted strength of the interaction. If there were multiple measurements available, the lowest value (ie, highest reported affinity) was used to determine the thickness of the edge.

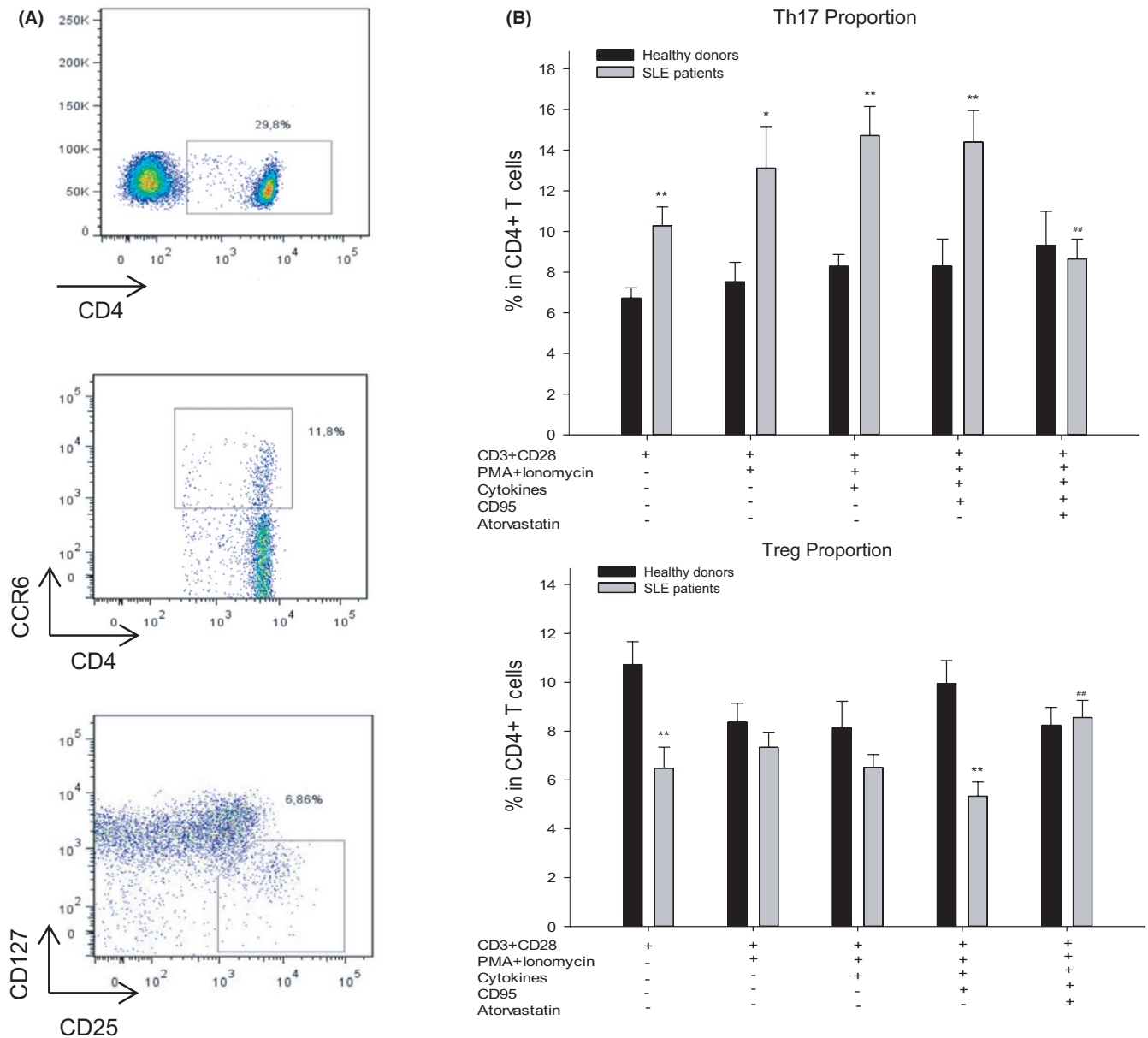


Figure 1. The proportion of T helper 17 (Th17) (CD4⁺CCR6⁺) and regulatory T (Treg) (CD4⁺CD25⁺CD127^{dim/-}) cells in CD4⁺ T cells from patients with systemic lupus erythematosus (SLE) and healthy donors. CCR6, CD25, and CD127 expression was determined by flow cytometry analysis in CD4⁺ T cells after 6-day culture with Th17 or Treg polarizing cytokines, with PMA and ionomycin stimulation with CD95. Atorvastatin was added 1 day before harvest. **A**, Representative dot plot analysis shows CCR6 and CD25/CD127 expression in CD4⁺ T-cell population. **B**, The percentage \pm SEM of 10 different cultures performed. Differences between Th17 and Treg cells from healthy donors and patients with SLE were statistically significant. * $P \leq 0.05$; ** $P \leq 0.01$. Differences between Th17 and Treg cells from patients with SLE with or without atorvastatin were statistically significant. ## $P \leq 0.01$. PMA, phorbol 12-myristate 13-acetate.

Molecular docking analysis. The CRP protein structure bound to phosphorylcholine (PC) was retrieved from the Protein Data Bank (PDB) database bearing PDB identifier 1B09 (20). The ligand atorvastatin was retrieved from PubChem database bearing identifier 60823. To confer the binding orientation and efficacy of the atorvastatin binding to CRP, we performed molecular docking analysis using Autodock Vina. The protein (CRP) preparation was performed by using Autodock Wizard by adding hydrogens and Kollman charges. The ligand preparation was done in a similar way by merging 31 nonpolar hydrogens and adding Gasteiger charges. Twenty-two aromatic carbons were detected with 16 rotatable bonds.

Statistical analysis. Results are expressed as mean \pm SEM. Relative messenger RNA expression levels of the genes in patients' PBMCs versus controls' were evaluated by using the nonparametric Wilcoxon signed-rank test and comparing the medians against a hypothetical median. Determinations of significant differences between groups were compared by using Student's *t*-test. A *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Data on patients with SLE. Patients for cytokine determination and for experimental studies were chosen randomly. In the SLE group ($n = 30$) in which cytokines were measured, the median value for SLAM was 5, and 16 individuals were on glucocorticoids, 16 were on disease-modifying antirheumatic drugs (DMARDs), and six were on statins. In the group in which experimental studies were performed ($n = 10$), the median value for SLAM was 6, and six individuals were on glucocorticoids, seven were on DMARDs, and two were on statins.

Polarization of Th17 and Treg cells among patients with SLE and controls and effects of atorvastatin. To generate the Th17 and Treg cells from PBMCs in vitro, Th17 cells were polarized by adding IL-6, IL-1 β , TGF- β 1, and IL-23, whereas Treg cells were generated by TGF- β 1. The proportion of Th17 and Treg cells was measured by FACScan (Figure 1). We observed significantly more Th17 cells and less Treg cells in patients with SLE compared with controls. After atorvastatin treatment, the proportion of Th17 cells was decreased significantly, whereas the proportion of Treg cells was increased significantly (Figure 1).

Apoptosis of Th17 and Treg cells induced by CD95 was influenced by atorvastatin in patients with SLE. To investigate the apoptosis of Th17 and Treg cells in response to CD95, early, late, and total stages of apoptosis were quantified by annexin V and 7-AAD. CD95-induced apoptosis of the Th17 cells from patients with SLE showed lower frequency, but the frequency of Treg cells was higher. After treatment with atorvastatin, there

was an increase of the apoptosis in Th17 cells but a decrease in Treg cells responding to CD95 (Figure 2), indicating that Th17 cells were more resistant to CD95-induced apoptosis compared with Treg cells in patients with SLE and that atorvastatin reversed the response of Th17 and Treg cells to CD95-induced apoptosis.

Expression of CD95 ligand in Th17 cells was induced by atorvastatin in patients with SLE. To further investigate underlying mechanisms, we detected the expression of CD95 and CD95 ligand in Th17 and Treg cells with or without atorvastatin. We found after the atorvastatin treatment, the expression of CD95 ligand in the Th17 cells of patients with SLE was upregulated, whereas this effect was not seen in Treg cells (Figure 3).

RORC/FOXP3 ratio in SLE was decreased in patients with SLE treated with atorvastatin. Next, we measure the gene expression of specifying transcriptional factors for Th17 and Treg cells by a real-time polymerase chain reaction. The gene levels of ROR γ t/RORC and FOXP3 in controls was used as a comparative calibrator. The ratio of ROR γ t/RORC and FOXP3 expression in patients with SLE versus controls was shown in Figure 4; the ratio was significantly decreased by atorvastatin treatment but showed no significant difference when CD95 was added.

Levels of TNF- α , IL-6, IL-10, and IL-17 in plasma of patients with SLE. TNF- α , IL-6, IL-10, and IL-17 in the plasma from 30 patients with SLE and 30 controls were detected by ELISA. Compared with controls, we observed a significant increase in IL-6 and IL-17 levels and a tendency for TNF- α levels to increase in patients with SLE (Figure 5).

Simulation of atorvastatin interactions. To link the derived chemical-chemical associations to the protein binding, a variety of databases of chemical-protein interactions mapped to MeSH terms aforementioned in the Methods were depicted (Figure 6A and B). The numbers of nodes and edges obtained from the atorvastatin pathway were 37 and 70 and protein-protein interaction (PPI) enrichment *P* values. The clustering coefficient was predicted to be 0.703. Moreover, the PPI enrichment *P* value was predicted to be 0.0285. This signifies that the atorvastatin interaction pathway reveals some significant insights in chemical-protein binding regulating several biological processes and functions. In the atorvastatin pathway, we identified a good binding affinity toward proteins (eg, CRP with a score of 0.900 [curated database] and a combined score of 0.991 and IL-6 with a score of 0.881) and lipid-related proteins (eg, HMGCR with a score of 0.865 and apolipoprotein E with a score of 0.856). There was no direct interaction between atorvastatin and components of OxLDL as malondialdehyde and PC (data not shown). In addition to the CRP association with atorvastatin, we further extended the network to CRP subcomponents to study the pattern of direct and indirect interactions of atorvastatin with CRP to activate CRP

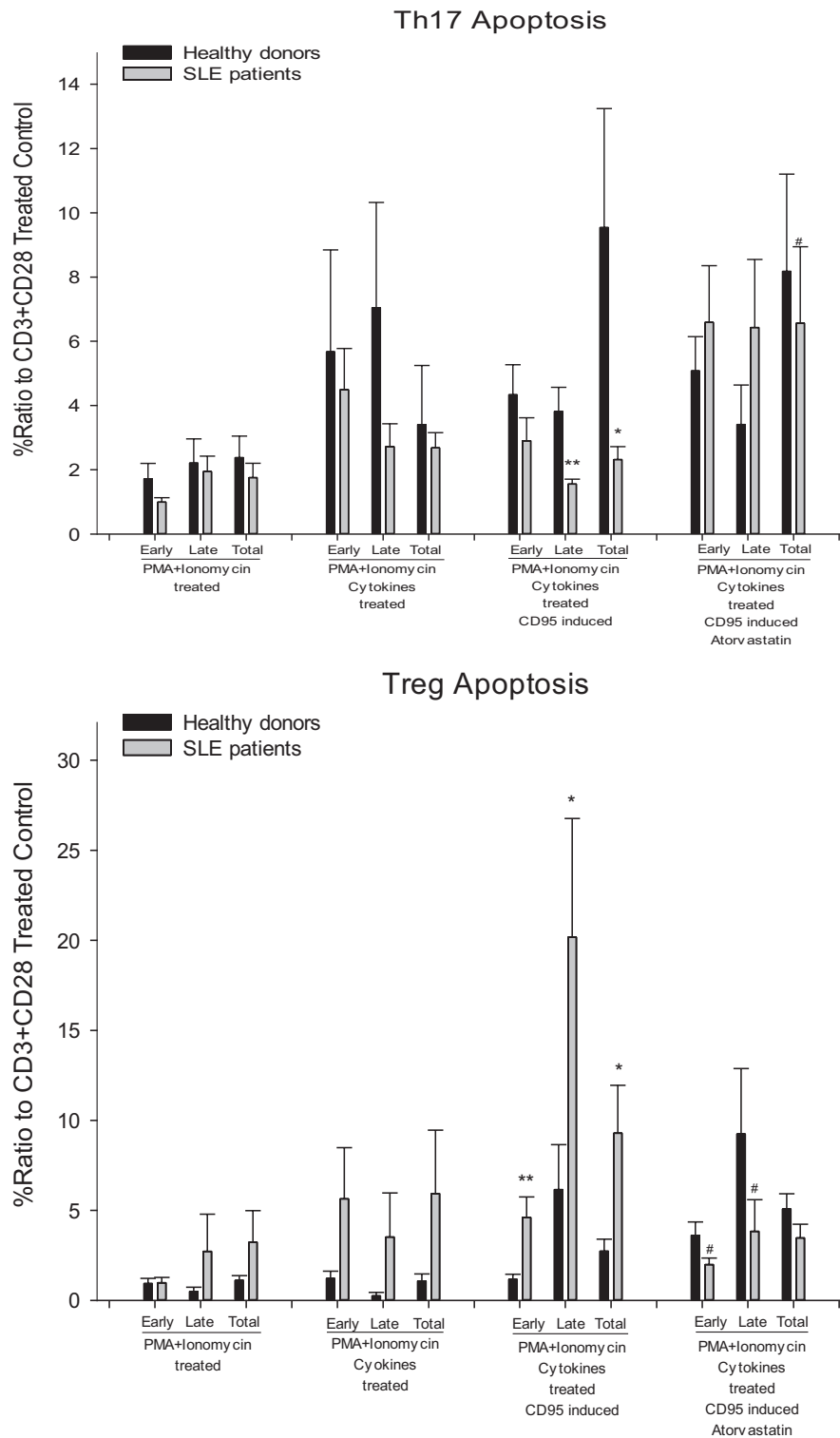


Figure 2. Effects of atorvastatin on the apoptosis of T helper 17 (Th17) ($CD4^+CCR6^+$) and regulatory T (Treg) ($CD4^+CD25^+CD127^{dim/-}$) cells in healthy donors and patients with systemic lupus erythematosus (SLE). Th17 and Treg cells were cultured alone or in the presence of Fas ligating antibody anti-CD95 for 3 hours. Atorvastatin was added 1 day before harvest. Apoptosis was quantified by annexin V and 7-aminoactinomycin D (7-AAD) staining by using flow cytometry. Early stage of apoptosis: annexin V⁺ 7-AAD⁻; late stage of apoptosis: annexin V⁺ 7-AAD⁺; total apoptosis was calculated as the sum of early and late apoptosis cells. Data show the percentage \pm SEM of 10 different cultures performed. Differences between Th17 and Treg cells from healthy donors and patients with SLE were statistically significant. * $P \leq 0.05$; ** $P \leq 0.01$. Differences between Th17 and Treg cells from patients with SLE with or without atorvastatin were statistically significant. # $P \leq 0.05$. PMA, phorbol 12-myristate 13-acetate.

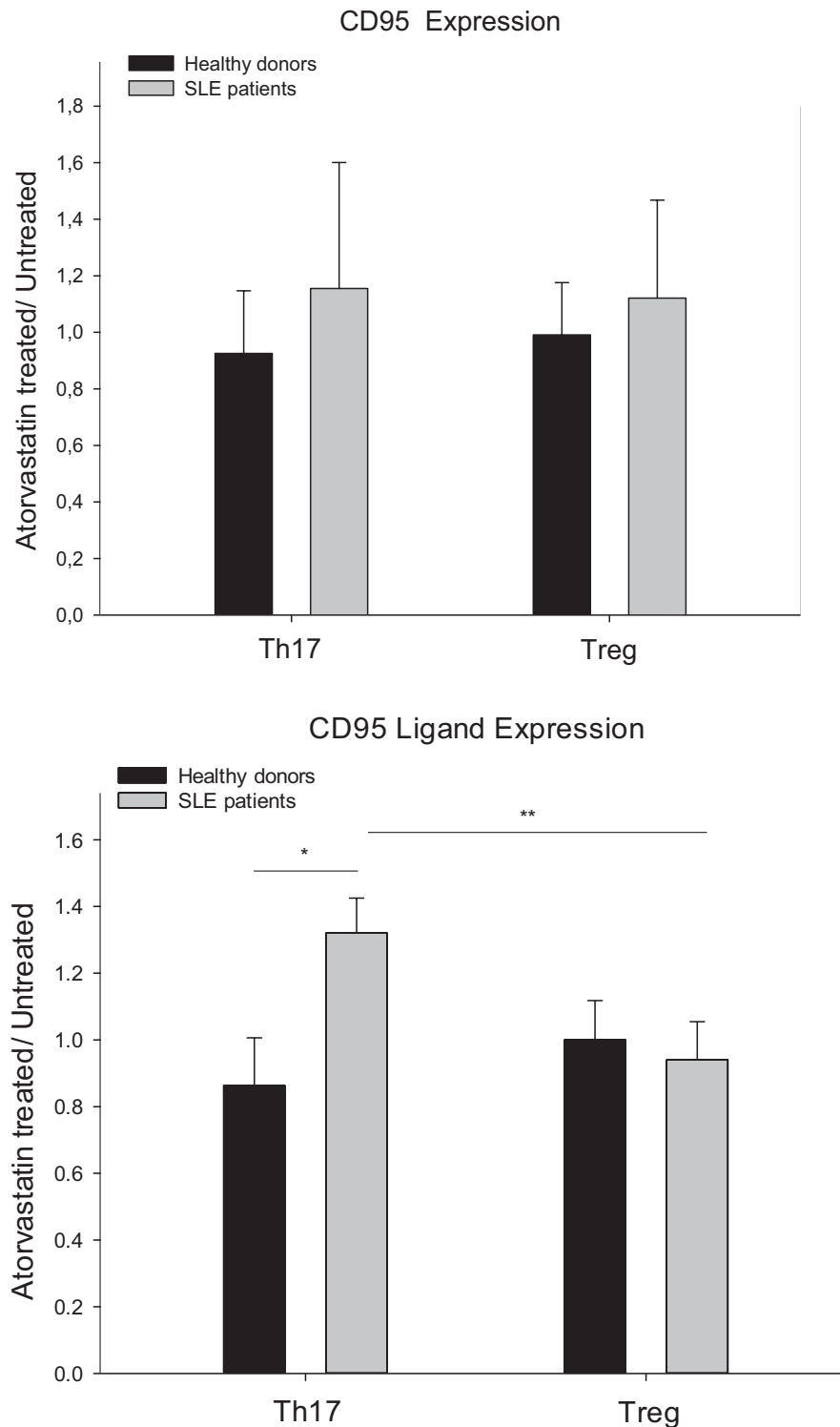


Figure 3. The expression of CD95 and CD95 ligand of T helper 17 (Th17) ($CD4^+CCR6^+$) and regulatory T (Treg) ($CD4^+CD25^+CD127^{dim/-}$) cells in $CD4^+$ T cells from patients with systemic lupus erythematosus (SLE) and healthy donors. CCR6, CD25, and CD127 expression was determined by flow cytometry analysis in $CD4^+$ T cells after 6-day culture with Th17 or Treg polarizing cytokines with PMA and ionomycin stimulation. Atorvastatin was added 1 day before harvest. The expression of CD95 and CD95 ligand of Th17 ($CD4^+CCR6^+$) and Treg ($CD4^+CD25^+CD127^{dim/-}$) cells in $CD4^+$ T cells from patients with SLE and healthy donors was analyzed by flow cytometry according to the MFI values. The percentage \pm SEM of 10 different cultures performed is shown. Differences of the expression of CD95 ligand between Th17 cells from healthy donors and patients with SLE with or without atorvastatin were statistically significant. * $P \leq 0.05$; ** $P \leq 0.01$. MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate.

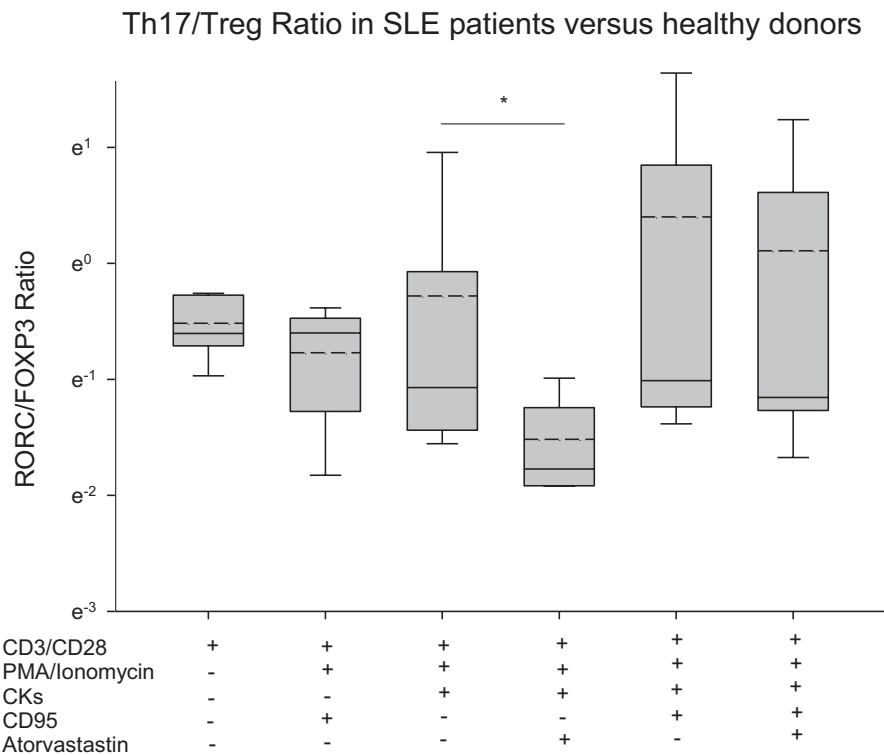


Figure 4. RORC/FOXP3 ratio in patients with patients with systemic lupus erythematosus (SLE) versus healthy donors. RNAs extracted from peripheral blood mononuclear cells of patients with SLE ($n = 6$) and healthy donors ($n = 6$) were reverse transcribed, and the gene expression levels were determined by quantitative real-time reverse transcription–polymerase chain reaction using gene-specific primers for indicated genes and glyceraldehyde-3-phosphate dehydrogenase to normalize the messenger RNA. The proportion of transcript present in the samples was calculated using the relative quantification $2^{-\Delta\Delta C_t}$ scheme. The relative amount of RORC and FOXP3 transcription in patients' samples (fold change) was calculated by using the level of the transcripts in the healthy donors' samples as a comparative calibrator. Final results represent the RORC/FOXP3 ratio in patients with SLE versus healthy donors. In the figure, results are represented in box plots given the median (horizontal bars within boxes) and the mean (cross points); box limits correspond to quartiles, and vertical lines indicate the 5th to 95th percentile range. Results are represented in a log 10 scale, and the indicated numbers are the antilog values. *Significance levels set at $P < 0.05$. CK, creatine kinase; PMA, phorbol 12-myristate 13-acetate.

subcomponents (Supplementary Figure 1). Furthermore, the biological processes, molecular functions, and pathways associated with the aforementioned pathways have been depicted in Supplementary Table 1.

Molecular docking of atorvastatin with CRP to show the efficacy and binding orientation. Previous findings suggest that phosphate oxygen of PC binds to CRP and projects away from the surface of the protein, leaving an adjacent hydrophobic pocket. The residues involved in PC binding to CRP are GLN150, GLU81, PHE66, SER74, ASP140, and two calcium ions (Figure 7A). From the molecular docking analysis, atorvastatin binds to CRP with a binding affinity of -7.1 kcal/mol. The binding orientation of the atorvastatin was observed adjacent to PC binding in a hydrophobic pocket, which is far away from the surface of the protein. The amino acid residues that are involved in atorvastatin binding revealed that ARG116, PRO93, ALA92, THR90, LYS114, VAL86, and VAL111 form conventional hydrogen bonds as well as alkyl bonds with oxygen atoms of atorvastatin (Figure 7B).

DISCUSSION

We here report that in patients with SLE, stimulation of mononuclear leukocytes led to a significantly higher proportion of Th17 cells ($CD4^+IL17^+$) than among age- and sex-matched controls, as determined by FACScan expression of $CD4^+IL17^+$. The stimulation of cells was done in different ways and in combinations in which all cell cultures were treated with CD3/CD28, and then we added other stimuli: phorbol myristate acetate and ionomycin and Th17 polarizing cytokines and CD95. All experiments with cell stimulation showed a significantly higher Th17 cell proportion in patients with SLE than among controls. The addition of atorvastatin abolished these differences and normalized Th17 cell proportion among patients with SLE to the same levels as healthy individuals.

Furthermore, after using the same stimuli (except that the cytokines added were Treg stimulatory), the proportion of Treg cells ($CD4^+CD25^+CD127^{dim/-}$) was significantly lower among patients with SLE than among controls for CD3/CD28, CD3/CD28 with CD95, and when a combination of all stimuli was used

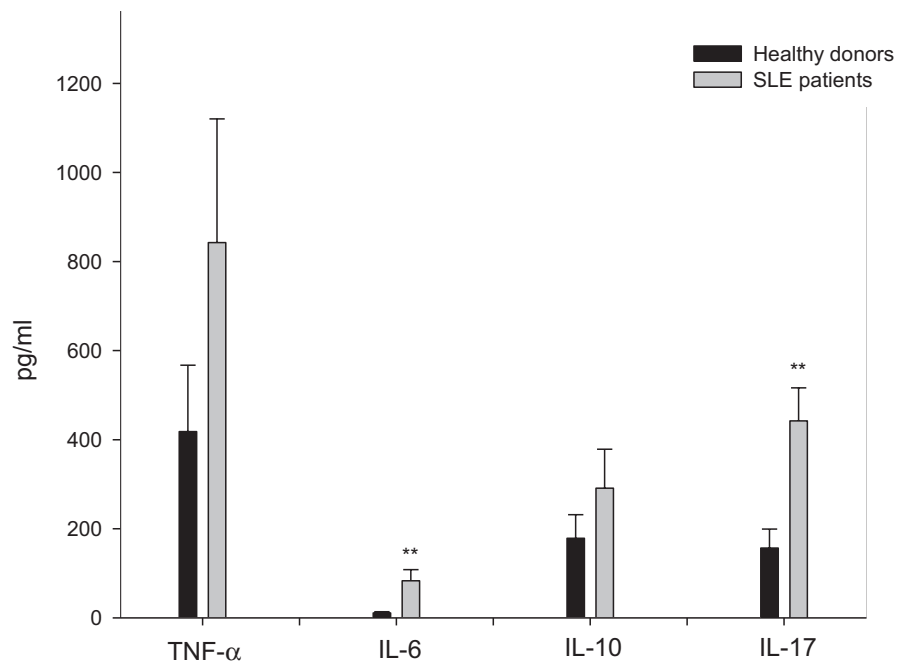


Figure 5. Plasma levels of tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 17 (IL-17) in patients with systemic lupus erythematosus (SLE). Results are expressed as mean \pm SE. Differences between IL-6 and IL-17 expression from healthy donors (n = 30) and patients with SLE (n = 30) were statistically significant. ** $P < 0.01$.

and nonsignificantly lower for other stimuli. The addition of atorvastatin abolished the difference in Treg cells between patients and controls, and the difference in T cells without atorvastatin in patients with SLE was significant.

These findings were largely confirmed by qRT-PCR, in which we demonstrated an increased RORC/FOXP3 transcription ratio, which was significantly decreased after the addition of atorvastatin, significantly more so in SLE-derived T cells. These findings are also in line with and extend our previous report in which the proportion of Th17 T cells was higher among patients with SLE but lower in relation to Treg cells at baseline, with no added stimuli (21). Some of these T-cell activating compounds are implicated in autoimmune disease, and cytokines are also well known to promote T-cell polarization (22). CD95 was previously thought to play a role in apoptosis regulation, but recent studies indicate that it is also proinflammatory, induces nonapoptotic signals, and is overexpressed in SLE (23).

Because a Th17/Treg cell imbalance is a known feature of SLE, our findings thus imply that irrespective of activating agents, this skewed induced T-cell response could contribute to SLE. We also, for the first time, present evidence that this type of skewed T-cell response in SLE is abolished by a statin, atorvastatin. This opens the possibility that statins may be an effective treatment against SLE. Although results vary, there are promising results from statin studies in rheumatoid arthritis, SLE, and multiple sclerosis (24). Still, in SLE, results are not clear-cut. Also concentration may play an important role; for example, in one study, the activated

phenotype of Treg cells was lost when cultured in a higher concentration of atorvastatin than we used in our studies herein (25).

There are other aspects in the T17/Treg cell imbalance in SLE, such as metabolic pathways involving differences as compared with controls in relation to glycolysis, lipid synthesis, glutaminolysis, and highly activated mammalian/mechanistic target of rapamycin, which promote Th17 cell differentiation and function. It is possible that influencing cholesterol metabolism with statins decreases Th17 cells through a mechanism related to lipid levels (26). Furthermore, DCs are directly implicated in cholesterol homeostasis, and the immune response and expansion of the DC population led to decreased cholesterol levels, whereas depletion of DCs instead resulted in raised cholesterol levels (27).

Lipid rafts could also be implicated because T-cell rafts from patients with SLE exhibit differences as compared with controls, and statins may normalize raft-associated T cell receptor signaling in T cells from patients with SLE (28,29).

Specific antigens could play a direct role in SLE-related T-cell activation. OxLDL, abundant in atherosclerotic plaques and also raised in SLE and associated with CVD and disease complications (30), induces DC and T-cell activation, an effect reversed by atorvastatin (11). Small hairpin RNA knockdown of heat shock protein 60 (HSP60) attenuated this effect, and HSP60 is induced by OxLDL. HSP60 in itself induces DC activation and an major histocompatibility complex class II-dependent activation of blood- and plaque-derived T cells (31). HSP60 has been implicated as an important antigen in SLE, which could play a role in

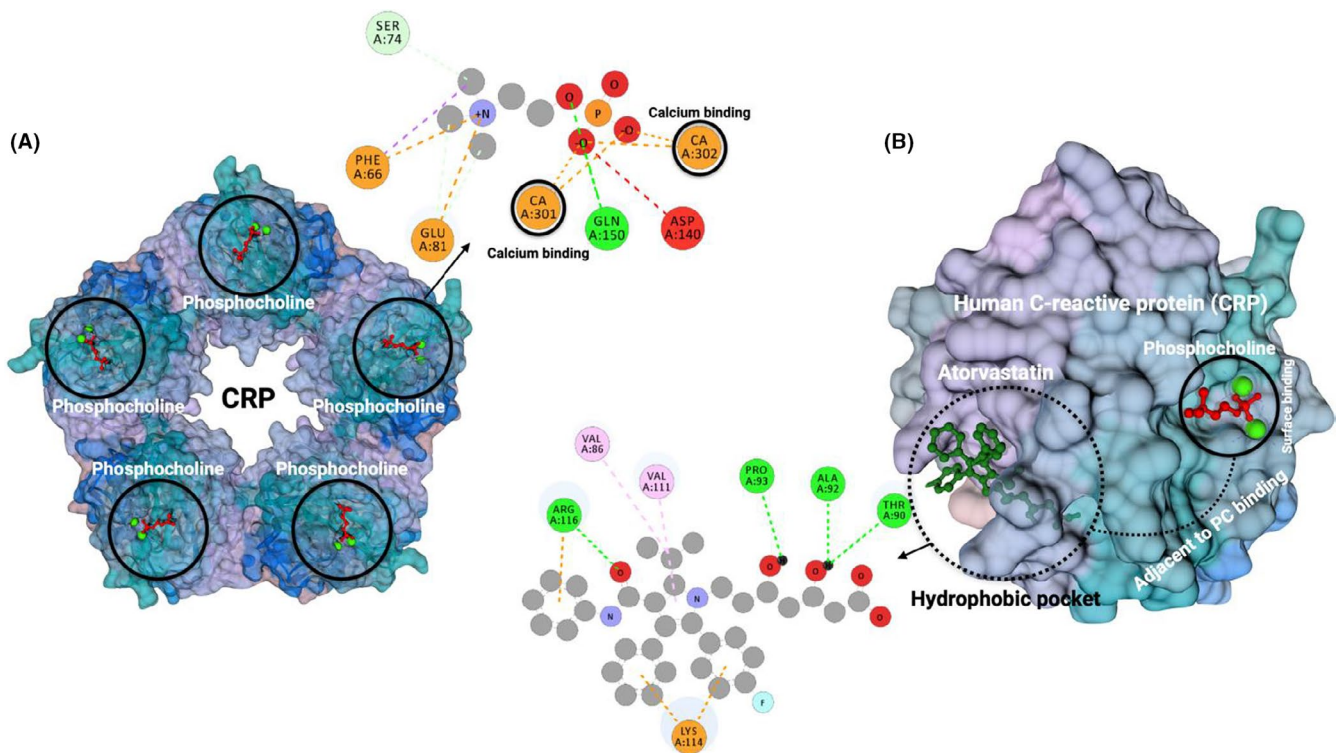


Figure 7. Molecular docking analysis of atorvastatin with C-reactive protein (CRP). **A**, phosphorylcholine (PC) binding to pentameric CRP (Protein Data Bank identifier: 1B09) on the surface of the protein with 2D interaction **B**, Binding orientation of atorvastatin with a single CRP chain in the hydrophobic pocket. 2D, two-dimensional.

We also determined levels of TNF- α , IL-6, IL-10, and IL-17 in plasma of patients with SLE compared with controls. The levels of all these cytokines were higher among patients with SLE, but only IL-6 and IL-17 differed significantly. This finding is thus in line with the observations that T-cell activation in SLE predominantly leads to a T17 cell response, with the production of the proinflammatory cytokine IL-17. In line with this, treatment with IL-17 inhibitors is an option for drug development in SLE (38).

We also here report the simulation of biological pathways in relation to atorvastatin. In drug research, the role of the interaction network is even more prevalent because diseases are often caused by multiple changes in the same route or protein complex (39). Considering the target protein neighborhood and the network topology itself, a better understanding of the drug's cellular effects can be achieved (40).

The efficacy of the drug on the organism depends on its involvement by target proteins and the degree to which it interrupts the protein-protein and protein-chemical interaction networks. This is linked to the drug's dosage, the intensity with which it modulates target activity, and the distribution within different tissues of target proteins (41).

The major novel finding using STITCH herein is that atorvastatin interacts strongly with CRP. The properties of CRP appear to be complex. In general, CRP is an acute inflammatory protein that play a role in host defense, including agglutination and capsular

swelling, and CRP binds to PC (exposed on dead cells, oxidized LDL, and some microorganisms and bacteria), which promotes phagocytosis and complement activation. The main producer of CRP is believed to be liver cells, but several other cell types also have this property, such as smooth muscle cells, macrophages, and others (42,43).

In a chronic inflammatory setting, with low-grade inflammation and a small increase in CRP levels, the risk of CVD is raised (44). In the Jupiter study, atorvastatin was protective in relation to CVD when CRP levels were raised (8). Although CRP in acute situations thus plays a protective role, potentiating the response to pathogens and damage, a low-grade inflammation with only marginally raised CRP levels may instead be detrimental, even though the role of CRP in this context still is only partially known. For example, CRP increases the production of proinflammatory cytokines, such as IL-6, TNF- α , and IL-8, and in atherosclerotic lesions, CRP binds to damaged cell membranes, promoting complement activation and inflammation and damage to heart tissue, and recruits monocytes (42,45,46).

In its native form, CRP consists of five identical subunits not covalently bound to form a symmetrical disk of approximately 115 kDa and is a member of the protein pentraxin complex. On one side, the pentamer is connected to ligands, such as PC, and on the other side, the effector molecules are linked, such as C1q. Previous studies on CRP binding to PC demonstrated that PC

binds to CRP via phosphate oxygen that projects away from the surface of the protein. Multipoint clamps to a PC-bearing surface of one planar face of the CRP molecule will allow recognition sites for C1q, detected by mutagenesis on the other exposed face. This allows CRP to affect essential supplements physiologically and/or pathologically. The hydrophobic pocket next to the binding PC is an opportunity to develop CRP binding inhibitors that may be therapeutic in SLE (20).

In our current study, we found a binding orientation of atorvastatin in the hydrophobic pocket of single-chain CRP (20). Under a variety of conditions, including low pH, absence of calcium, increased temperatures, and urea chelation, native CRP can disassociate from its pentameric form to its monomeric substrates. We have not established a direct association of the five subunits with atorvastatin. However, indirect effects may occur through native CRP.

Whether atorvastatin could play a role in this interaction remains to be seen. If atorvastatin through interaction with CRP could inhibit proinflammatory properties of CRP in chronic inflammation as in both SLE and atherosclerosis, this could contribute to its beneficial effects, a possibility that deserves further studies. However, the possible interaction of atorvastatin reported here is not supported by experiments, to the best of our knowledge, but currently, we predict the binding of atorvastatin with CRP (ligand recognition and binding by CRP) might thus contribute to a range of metabolic, scavenging, and host-defense functions, potentially with increased concentration if CRP is accumulated locally.

Interestingly, atorvastatin also interacts with IL-6, which is a cytokine with a wide range of biological activities and a potent inducer of CRP with a central role as a mainly proinflammatory cytokine, which may play a role in atherosclerosis and CVD (47). If this interaction is functional, atorvastatin may inhibit IL-6 effects. Other interactions of atorvastatin include with lipid-related compounds (which is expected), such as HMGCR (a transmembrane glycoprotein) and the rate-limiting enzyme in cholesterol biosynthesis as well as in the biosynthesis of nonsterol isoprenoids essential for normal cell function, including ubiquinone and geranylgeranyl proteins (48). Another is apolipoprotein E, mediating the binding, internalization, and catabolism of lipoprotein particles in accordance with the LDL (apolipoprotein B/E) receptor.

There are limitations to this study. One is that we only used an *ex vivo* system with PBMCs from patients with SLE and matched controls. It would be of interest to study statins in mouse models and to expand the number of included individuals, as we here limited this to 10 patients with SLE and 10 matched control individuals. In addition, randomized studies of statins in patients with SLE are warranted. The simulations of interactions also need to be studied experimentally.

Taken together, we here demonstrate that different stimuli promote Th17 cells more than Treg cells in patients with SLE compared with controls. Th17 cells were more resistant than Treg cells to CD95-induced apoptosis in patients with SLE compared

with controls, which could reveal a novel mechanism behind the imbalance of Th17/Treg cells documented in SLE. Atorvastatin reversed these effects and normalized T-cell apoptosis, suggesting a potential role of statins in the treatment of SLE, a possibility deserving further treatment studies. We also determine direct and strong potential interaction between atorvastatin and CRP and, to a lesser extent, IL-6. These findings could imply a novel role of atorvastatin in the regulation of inflammation.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Frostegård had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Liu, Frostegård.

Acquisition of data. Sun, PandaAjeganova, Hafström.

Analysis and interpretation of data. All authors.

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