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*J Immunol* 2014; 193:5294-5305; Prepublished online 13 October 2014; doi: 10.4049/jimmunol.1203436
http://www.jimmunol.org/content/193/10/5294
Inhibition of Protein Geranylgeranylation Specifically Interferes with CD40-Dependent B Cell Activation, Resulting in a Reduced Capacity To Induce T Cell Immunity

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Ab-independent effector functions of B cells, such as Ag presentation and cytokine production, have been shown to play an important role in a variety of immune-mediated conditions such as autoimmune diseases, transplant rejection, and graft-versus-host disease. Most current immunosuppressive treatments target T cells, are relatively unspecific, and result in profound immunosuppression that places patients at an increased risk of developing severe infections and cancer. Therapeutic strategies, which interfere with B cell activation, could therefore be a useful addition to the current immunosuppressive armamentarium. Using a transcriptomic approach, we identified upregulation of genes that belong to the mevalonate pathway as a key molecular event following CD40-mediated activation of B cells. Inhibition of 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting enzyme of the mevalonate pathway, by lipophilic statins such as simvastatin and atorvastatin resulted in a specific inhibition of B cell activation via CD40 and impaired their ability to act as stimulatory APCs for allospecific T cells. Mechanistically, the inhibitory effect resulted from the inhibition of protein geranylgeranylation subsequent to the depletion of mevalonate, the metabolic precursor for geranylgeranyl. Thus, inhibition of geranylgeranylation either directly through geranylgeranyl transferase inhibitors or indirectly through statins represents a promising therapeutic approach for the treatment of diseases in which Ag presentation by B cells plays a role. The Journal of Immunology, 2014, 193: 5294–5305.

In recent years it has been increasingly recognized that apart from Ab production B cells serve a number of other important functions in the immune system. Both Ag presentation and cytokine production by B cells have been shown to play an important role in the physiologic immune response (1). The Ab-independent effector functions of B cells contribute to a wide range of immune-mediated conditions such as autoimmune disease, transplant rejection, and graft-versus-host disease (GVHD) (2–4). It would therefore be desirable to be able to specifically inhibit secretion of inflammatory cytokines and Ag presentation by B cells.

We and others have previously shown that B cells acquire potent Ag-presenting function following crosslinking of CD40 (5, 6). Activation via CD40 is probably the strongest stimulus for the induction of B cell–mediated Ag presentation and cytokine production (7). We therefore sought to identify pathways that are amenable to therapeutic targeting of Ab-independent B cell functions. To identify molecular targets for the modulation of B cell activation and Ag presentation by B cells we conducted a transcriptomic analysis comparing the gene expression profile of human CD40-activated B cells to that of resting B cells and found that CD40 activation resulted in the upregulation of the enzymes in the mevalonate pathway, which is involved in cholesterol biosynthesis (8). This suggested that blockade of this pathway could result in an inhibition of CD40-mediated B cell activation. Statins, which are widely used lipid-lowering drugs, are highly specific inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme of this pathway. Apart from their lipid-lowering effects, statins have been shown to exert a broad range of pleotropic effects. Their immunomodulatory effect is thought to contribute to the atheroprotective activity. Furthermore, recent experiments in animal models and results from clinical studies suggest a beneficial influence of statins on immune-mediated diseases such as rheumatoid arthritis (9). However, the mechanisms underlying the immunomodulatory capacity of statins are still not well understood. We therefore set out to assess the impact of inhibition of the mevalonate pathway by statins on the Ab-independent functions of B cells.
Materials and Methods

Transcriptome analysis

RNA isolation, hybridization to Affymetrix HG-U133A 2.0 chips (Affymetrix, Santa Clara, CA), and array normalization were performed as previously described (10, 11). Briefly, total RNA was isolated from purified human CD19 B cells after lysis in TRIzol (Life Technologies, Darmstadt, Germany) and stored at −80 °C until further processing. Purified RNA was converted to biotin-labeled amplified RNA and hybridized to Affymetrix HG-U133A 2.0 microarrays. The microarray data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE54017. Microarray normalization and analysis were performed using dChip (12) and GenePattern (13). To further characterize the biological processes, which were upregulated, we performed gene ontology (GO) functional annotation using the Database for Annotation, Visualization, and Integrated Discovery (14, 15). The results of the gene enrichment analysis were visualized using the Enrichment Map plugin (version 1.2) (16) for Cytoscape (17) with the default parameters. We used GenMAPP/MAPPFinder and the GO-Elite plugin (18) for GenMAPP-CS (19) to rank and classify the differentially expressed genes based on GO information for each gene. The maps were built using GenMAPP. For each of the existing GO terms the cumulative number of genes which were upregulated, and of all genes represented on the microarray was calculated for every GO term by subtracting the expected number of genes meeting the criterion from the actual number, and division of this value by the SD of the actual number of genes. A positive Z score indicates that there are more genes meeting the criterion in the specific GO term than expected by chance.

Flow cytometry

Fluorochrome-labeled Abs against human CD3, CD4, CD8, CD25, CD19, CD20, CD80, CD86, and HLA-DR were purchased from BD Biosciences (Heidelberg, Germany). Annexin V (Life Technologies) staining was performed according to the manufacturer’s instructions. For intracellular cytokine staining, cells were washed after surface marker staining and permeabilized with Fix/Perm buffer solution subsequently washed with permeabilization buffer (both from eBioscience, San Diego, CA). After the cells were washed, the fluorescent conjugate intracellular mAbs against FOXP3 (eBioscience, Frankfurt, Germany) were added and incubated for 30 min in the dark. The cells were washed again and analyzed by FACS. Flow cytometric analysis was done on a FACSCanTo (BD Immunocytometry Systems, Heidelberg, Germany) or Gallios (Beckman Coulter, Krefeld, Germany).

Cell culture

CD40-activated B cells and monocye-derived dendritic cells (DCs) were generated as described previously (20, 21). Briefly, human B cells were purified from buffy coats using the MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. For some experiments naive and memory B cells were purified using the human memory B cell enrichment kit (Miltenyi Biotec). The CD27-negative fraction of the second purification step represented the naive B cell subset. The purified B cells were cultured on irradiated NIH3T3 cells transfected with human CD40L in the presence of reconstituent human IL-4 (R&D Systems, Minneapolis, MN) in IMDM (Invitrogen, Karlsruhe, Germany) supplemented with 10% pooled human serum for 4 d. During the 4-d culture, atorvastatin, simvastatin, or pravastatin (Sigma-Aldrich, Taukirchen, Germany) was added at concentrations of 0.1, 1, and 10 μM. The statin concentrations were chosen to cover a broad range of concentrations, including the lower concentration ranges from 0.1 to 1 μM, which can be achieved with standard doses in humans. In some experiments an excess of mevalonate (Sigma-Aldrich) was added at a concentration of 200 μM to overcome the inhibitory effect of statins on mevalonate synthesis. Vehicle-treated B cells served as control. In each experiment different donors for buffy coats were used to account for genetic variation in the response to statins (22).

T cell proliferation assay

Untouched human CD3+ T cells were purified from buffy coats of healthy donors using the RosetteSep human T cell enrichment mixture (StemCell Technologies, Grenoble, France). Subsequently, the purified T cells were labeled with CFSE (Life Technologies) as described previously (23). The CFSE-labeled T cells were cultured with irradiated stimulator cells, that is, either B cells or DCs, at different ratios. The stimulator cells were washed extensively before use in the assay to ensure that (i) T cells were exposed to the coculture. After 5 d in culture T cell proliferation was assessed by flow cytometric analysis of the percentage of CD25+CFSElow CD4+ or CD8+ T cells. To ensure representation of natural interindividual heterogeneity found in the human population, different donors for both B and T cells were used for each experiment. Unless otherwise indicated, the results of the flow cytometric analysis are expressed as the relative values compared with vehicle-treated controls.

Cytokine production assays

The cytokine production by B cells was analyzed using RayBio human cytokine Ab array 5 (RayBiotech, Norcross, GA) according to the manufacturer’s instructions. In brief, cytokine array membranes, each containing 80 different Abs, were blocked in 2 ml 1× blocking buffer for 30 min. Subsequently, they were incubated with supernatants at room temperature for 1 h. The membranes were then washed and incubated with a mixture of bioin-conjugated Abs against the different proteins. The chemiluminescent signal of membranes was detected using a FLUostar OPTIMA reader (BMG Labtech, Ortenberg, Germany). The relative density of each dot was measured using ImageJ (National Institutes of Health, Bethesda MD). The relative density of each dot was calculated in relationship to the mean densitometric values of the positive controls, which were defined as 10,000 U. Only those proteins with a mean expression of ≥50 U were included in the further analysis. Production of MCP-1 and RANTES was confirmed by ELISA. MCP-1 and RANTES ELISA (Life Technologies) were performed on supernatants of CD40-activated B cell cultures according to the manufacturer’s instructions. The chemiluminescent signal of ELISA plates was detected using a FLUostar OPTIMA reader (BMG Labtech).

Human studies

The protocol was approved by the Ethics Committee and written informed consent was obtained from all participants. To be eligible for inclusion, all healthy volunteers had to be free of morbidities. All healthy controls received 10 mg atorvastatin once daily for 14 consecutive days. Blood samples were obtained before treatment, on days 3, 10, and 14 after the start of therapy and then again 7 d after discontinuation of therapy, that is, on day 21. PBMCs from these blood samples were prepared by Ficoll gradient centrifugation. Subsequently, samples were frozen for later use in FBS containing 10% DMSO in liquid nitrogen.

Statistical analysis

Were appropriate a Student t test or ANOVA was used to compare groups. To correct for multiple comparisons, we used a Bonferroni post hoc test. A p value < 0.05 was considered statistically significant.

Results

Increased expression of the genes involved in the mevalonate pathway upon signaling via the CD40 signalosome

To identify potential targets for the inhibition of B cell activation, we compared the gene expression profiles of resting B cells with those of CD40-activated B cells. To determine biological pathways, which were overrepresented among the upregulated genes, we used several bioinformatic enrichment analysis tools, including the Database for Annotation, Visualization, and Integrated Discovery, Elite-GO, GenMAPP, and MAPPFinder. With the help of these different bioinformatic enrichment tools we performed a comprehensive functional analysis to identify overrepresented biological processes and pathways. The global analysis revealed several functional clusters that contained significantly enriched differentially expressed genes. Besides the expected upregulation of genes associated with cell division and cell cycle, we observed an enrichment of genes associated with lipid metabolism such as cholesterol biosynthesis, statin pathway, fatty acid β oxidation, and fatty acid biosynthesis (Fig. 1, Supplemental Table 1). Among the list of genes that were most strongly upregulated following CD40 ligation was the gene encoding HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway. Close inspection of gene expression changes showed that essentially all enzymes involved in the cholesterol biosynthetic pathway were coordinately upregulated in B cells following activation via CD40 (Fig. 1B, 1C). The consistent upregulation of the genes belonging to the mevalonate pathway suggested that this metabolic pathway is important for the activation of B cells.
FIGURE 1. Coordinated upregulation of genes belonging to the mevalonate pathway following CD40 activation. (A) Enrichment map of cellular processes overrepresented in upregulated genes. Nodes represent enriched GO terms. Node size correlates with the total number of genes associated with each GO term. GO terms of related functions are grouped in clusters. (B) Schematic depiction of the cholesterol biosynthetic pathway. Red color designates genes that were significantly upregulated >2-fold. (C) Heat map showing the expression of genes encoding for enzymes of the mevalonate/cholesterol biosynthetic pathway. Red indicates high expression and blue indicates low expression. For each gene, the probe set with the highest dynamic range across all profiled samples was selected from the probe sets that mapped to a given gene symbol. FDFT1, farnesyl-diphosphate farnesyltransferase 1; FDPS, farnesyl diphosphate synthase; FNTA, farnesyl transferase; GGPS, geranylgeranyl pyrophosphate synthase; HMGCS1, HMG-CoA synthase; HMGCR, HMG-CoA reductase; IDI, isopentenyl diphosphate isomerase; MVD, mevalonate pyrophosphate decarboxylase; MVK, mevalonate kinase; PMVK, phosphomevalonate kinase; SQUE, squalene epoxidase.
High sensitivity of Ag presentation to inhibition of the mevalonate pathway by lipophilic but not hydrophilic statins

We hypothesized that inhibition of mevalonate biosynthesis using competitive HMG-CoA reductase inhibitors such as simvastatin would result in a disruption of CD40-mediated B cell activation. To test this hypothesis we studied the effect of the lipophilic statins simvastatin and atorvastatin or the hydrophilic statin pravastatin on purified human B cells, which were activated via CD40. Presence of either simvastatin or atorvastatin led to a decreased activation, which was reflected by a change in morphology and immunophenotype. Upon activation, B cells typically form dense round clusters through homotypic adhesion. Even at low concentrations of ≤1 μM, simvastatin and atorvastatin resulted in a dose-dependent loss of the typical cluster formation (Fig. 2A). Treatment with statins primarily led to growth arrest but not increased cell death. As shown in Fig. 2B and 2C, treatment with simvastatin and atorvastatin inhibited the proliferation of B lymphocytes after stimulation via CD40. On the contrary, the rate of apoptosis was increased only slightly by treatment with atorvastatin (Fig. 2D). Following CD40-mediated activation, B cells typically upregulate the expression of MHC and costimulatory molecules. Flow cytometric analysis demonstrated that, compared with controls, both

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surface expression of MHC class II and the important costimulatory molecules CD80 and CD86 was decreased on statin-treated B lymphocytes (Fig. 2E). HLA-DR expression was slightly more sensitive to inhibition by lipophilic statins than was expression of CD80 or CD86. The inhibitory effect of statins was reversible, as renewed CD40 stimulation after removal of statins led to an increase in the cell surface expression of CD80, CD86, and MHC class II (data not shown). Importantly, as demonstrated previously the concentrations of statins needed to inhibit B cell activation were significantly lower than those needed to inhibit T cell activation (Supplemental Fig. 1) (8). Because naive and memory B cells have been reported to respond differently to CD40 signaling, we examined whether there was a difference in the effect of statins on these two B cell populations (24, 25). As shown in Fig. 3 expression of CD80, CD86, and HLA-DR by both naive and memory B cells was inhibited to a similar degree by atorvastatins.

As a consequence of the decreased activation, statin-treated B cells also displayed a diminished immunostimulatory activity. CD40-activated B cells treated with 1 μM simvastatin or atorvastatin had a lower capacity to activate alloreactive T cells, as shown by the decreased activation and proliferation of CFSE-labeled allogeneic T cells (Fig. 4A, 4B). Additionally, statin-treated B cells induced a preferential expansion of Foxp3+CD25high cells (Fig. 4C, 4D). The effect of statins on the APC function of human B cells was entirely dependent on the inhibition of mevalonate synthesis (Figs. 2B, 2E, 4A, 4B). Addition of an excess amount of mevalonate completely abolished the inhibitory activity of lipophilic statins. This indicates that mevalonate-independent mechanisms of statins, such as the allosteric interference with LFA-1 signaling or disruption of lipid rafts, play a negligible role (26).

To determine the optimal statin for further clinical evaluation, we compared the activity of three of the most commonly prescribed statins, that is, simvastatin, atorvastatin, and pravastatin. Surprisingly, we observed striking differences in the inhibitory activity of distinct statins. Unlike the lipophilic statins, the hydrophilic statin pravastatin had no inhibitory effects. The morphology of pravastatin-treated B cells did not differ from untreated controls (Fig. 2A). Only at very high doses of 10 μM could we observe a small inhibitory effect of pravastatin on B cell clustering. Likewise, high concentrations of pravastatin were required for inhibition of CD40-mediated upregulation of MHC class II, CD80, and CD86 (Fig. 2E). These results are in line with observations made by others with regard to the differential effects of lipophilic and hydrophilic statins on immune cell function (27, 28). One possible explanation for the inability of pravastatin to inhibit B cell activation could be a lack of expression of solute carrier organic anion transporter family member 1B1 (SLCO1B1), which encodes a transporter protein responsible for the cellular uptake of statins.

Analysis of the gene expression microarray data revealed that SLCO1B1 was not expressed in either resting or activated B lymphocytes (data not shown). The SLCO1B1 gene encodes the organic anion transporter polypeptide 1B1, which is a member of the solute carrier family and mediates the cellular uptake of statins (29). Liver cells do express this receptor, and thereby hydrophilic statins can enter into liver cells (30). The absence of this transporter prevents hydrophilic statins from entering the cell whereas lipophilic statins can still cross the cell membrane (31). Thus, the lack of organic anion transporter polypeptide 1B1 on B cells could explain the inability of pravastatin to inhibit B cells.

**Inhibition of the mevalonate pathway modulates cytokine production**

B cells serve as an important source of cytokines and chemokines. B cell–derived cytokines and chemokines play a key role in regulating immune responses (32). Activated B cells produce a large number of cytokines and chemokines (32, 33). Using cytokine/chemokine Ab arrays we examined whether inhibition of the mevalonate pathway affected the secretome of CD40-activated B cells. Fig. 5A shows representative cytokine arrays of supernatants from CD40-activated B cell cultures treated with vehicle control (top) or 1 μM atorvastatin (bottom). As expected, CD40-activated B cells produced cytokines such as MDC, TARC, and IL-8, which are known to be upregulated in human B cells after crosslinking of CD40 (5, 34). To our surprise, despite the pronounced effects on B cell APC function, statins had a comparatively small impact on cytokine and chemokine production by CD40-activated B cells. Most cytokines and chemokines, which are known to be produced in response to CD40 activation, remained unaffected by inhibition of the mevalonate pathway. However, treatment with atorvastatin resulted in a characteristic

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**FIGURE 3.** Inhibition of naive and memory B cells. Purified human naive and memory B cells were activated via CD40 in the presence of the indicated concentrations of atorvastatin. Results are expressed as the relative cell surface expression of CD80, CD86, and HLA-DR of atorvastatin-treated B cells compared with vehicle-treated controls. Bar charts represent the means ± SD of three independent experiments (n = 3). *p < 0.05 compared with vehicle-treated controls.
change in the pattern of cytokine and chemokine secretion (Fig. 5B). The expression of the proinflammatory cytokines MCP-1/CCL2 and RANTES/CCL5 was inhibited by treatment with statins. Using ELISA, we confirmed the statin-mediated downregulation of RANTES/CCL5 and MCP-1/CCL2 (Fig. 5C).

Specific dependence of B cells and signaling through CD40 but not TLR on the mevalonate pathway

We next assessed whether the effects of statins are exclusive for B cells or also apply to other APCs such as DCs. As shown in Fig. 6A, maturation of immature DCs by CD40L was much less affected than B cell activation. This indicates that the dependence on the mevalonate pathway is cell type–specific and that other APCs such as DCs are less vulnerable to depletion of mevalonate. Furthermore, we investigated whether the effects of HMG-CoA reductase inhibition are specific for the CD40 signaling pathway. Using publicly available gene expression data, we assessed whether the enzymes involved in the mevalonate pathway were also upregulated following B cell activation via other signaling pathways. Our analysis showed that B cell activation through the BCR or pattern recognition receptors such as TLR9 did not result in an equal upregulation of enzymes involved in cholesterol biosynthesis, suggesting that these modes of B cell activation are less dependent on this pathway (data not shown).

FIGURE 4. The APC function of B cells is attenuated by lipophilic but not hydrophilic statins. Assessment of the effect of statins on the APC function of CD40-activated B cells is shown. Purified B cells were activated via CD40 in the presence of simvastatin, atorvastatin, pravastatin, or vehicle control for 4 d. After extensive washing the B cells were used as APCs in an allogeneic MLRs with purified CFSE-labeled human CD4+ and CD8+ T cells as responders. (A) FACS plots showing the percentage of activated, proliferating allogeneic CD4+ (upper panel) or CD8+ (lower panel) T lymphocytes after 5 d of stimulation. (B) Bar charts represent the means ± SD of three independent experiments (n = 3). Results are expressed as the percentage of proliferating T cells relative to vehicle-treated controls. (C) Representative FACS plots showing the percentage of Foxp3+CD25high T cells after stimulation of allogeneic CD4+ T cells with CD40-activated B cells treated either with 1 μM atorvastatin or vehicle. Plots are gated on proliferating CD3+CD4+CFSElow T cells. (D) Bar charts showing the mean percentage of FOXP3+CD25high T cells of three independent experiments ± SD (n = 3). *p < 0.05, **p < 0.01 compared with vehicle-treated controls.
In line with these results, B cell activation by CpGs, which act through TLR9, was not inhibited to the same extent by statins as was activation via CD40 (Fig. 6B). The concentration of atorvastatin and simvastatin that was required to inhibit B cell activation by CpG was much higher than that needed to inhibit activation via CD40 (Fig. 2E).

The effects of statins are dependent on the inhibition of geranylgeranylation

Mevalonate serves as the precursor for cholesterol and isoprenoids. Inhibition of mevalonate production thus not only interferes with the production of cholesterol but indirectly also with that of other important intermediates such as isoprenoids, which are required for the...
FIGURE 6. Inhibitory activity of statins is selective for B cells and CD40 signaling. (A) Human immature DCs were matured with anti-CD40 Abs in the presence of IL-4 and G-CSF and the indicated concentration of atorvastatin. After maturation, the expression of CD80, CD86, and HLA-DR was determined using flow cytometry. Shown are the relative expression values compared with untreated controls. Bar charts represent the means ± SD of three independent experiments (n = 3). (B) Human B cells were activated with CpG in the presence of vehicle or the indicated concentration (0.01, 0.1, 1, and 10 µM) of atorvastatin, simvastatin, or pravastatin. After 3 d of stimulation the cell surface expression of CD80, CD86, and HLA-DR was determined by flow cytometry. The results are shown as relative cell surface expression compared with vehicle-treated controls. Bar charts represent the means ± SD of three independent experiments (n = 3). *p < 0.05 compared with vehicle-treated controls.

Posttranslational modification by protein geranylgeranylation or farnesylation is important for the function of several proteins such as the members of the Ras family of small GTPases such as Rac, Rab, and RhOa. To identify the specific mechanism by which inhibition of mevalonate synthesis leads to inhibition of the Ag-presenting function we performed experiments in which we substituted different downstream substrates of the mevalonate pathway. To this end we added either geranylgeranylated, the metabolite required for protein isoprenylation, or squalene, the precursor for cholesterol synthesis to B cells, which were activated in the presence of atorvastatin. The inhibitory effect of atorvastatin was fully reversible by addition of geranylgeranyl pyrophosphate, which does not restore cholesterol biosynthesis, but not by addition of squalene, the precursor for cholesterol (Fig. 7A).

To verify these findings and to further explore the precise mechanism of inhibition, we added either an inhibitor or geranylgeranylated or farnesylated to the cultures. We found that inhibition of geranylgeranyl transferase but not of farnesyltransferase mimicked the effects of statins on B cell activation (Fig. 7B). These data suggest that the inhibitory activity of statins is due to interference with protein isoprenylation, more specifically geranylgeranylation, but not farnesylation or cholesterol biosynthesis. Furthermore, these results excluded the possibility that direct interference with LFA-1 signaling plays a role in mediating the effects of statins (26).

**Atorvastatin demonstrates inhibitory effects on Ag-presenting B cells in humans in vivo**

Because there are already many approved lipophilic statins available, we had the opportunity to test in healthy human volunteers whether the effects we observed in vitro could also be achieved in vivo. We chose atorvastatin for these experiments because it has fewer interactions with calcineurin inhibitors such as cyclosporin A or tacrolimus than does simvastatin and thus will be the most likely candidate for future clinical studies in transplant recipients. Five healthy subjects received atorvastatin once daily for 14 d. Statin treatment resulted in a reduction of activated B cells, that is, CD86⁺ B cells (Fig. 8). After the treatment with atorvastatin was stopped, the number of activated B cells returned to pretreatment levels within a week. The number of CD86⁺ B cells thus could serve as a sensitive surrogate biomarker for the effects of immunomodulatory drugs on APCs. The extent of the immunomodulatory effect on APCs in vivo seemed to correlate with the degree of cholesterol lowering (data not shown). This correlation could reflect differences in drug levels due to differences in the individual pharmacodynamics and pharmacokinetics. A higher drop in serum cholesterol would thus be an indicator for higher blood levels of atorvastatin, which would be associated with a stronger APC inhibitory effect.

**Discussion**

T cell responses play a crucial role in the pathogenesis of many autoimmune diseases and in alloreactive tissue damage in GVHD and transplant rejection (36). Consequently, most current immunosuppressants primarily target T lymphocytes. Unfortunately, these drugs have many side effects and lead to broad and unspecific immunosuppression. Long-term immunosuppression increases the patient’s risk of secondary cancers and severe life-threatening infections (37, 38). Moreover, despite the profound immunosuppression induced by these agents, many patients still suffer from active disease. In addition to T cells, APCs, such as DCs and B cells, are also important for the induction and maintenance of autoimmune and alloreactive T cell responses (39). Inhibition of APCs could therefore show synergistic effects with conventional immunosuppressants such as cyclosporine A.

In recent years it has been increasingly recognized that in addition to Ab production, Ab-independent functions of B cells play a crucial role in the physiologic immune response and many immunemediated diseases. It has been shown that B cell effector mechanisms contribute to autoimmunity and alloreactive immune responses and that targeting of B cells represents a promising therapeutic approach (40–46). The molecular and cellular mechanisms by which B cells regulate the immune response are currently
not well understood. Apart from the well-known Ab-mediated mechanisms, Ab-independent functions such as Ag presentation and cytokine production play a crucial role in B cell–mediated immunopathology (47–51). Alternatively, regulatory B lymphocytes can foster tolerance induction through a number of mechanisms, including the production of immunosuppressive cytokines and expansion of regulatory T cells (52–55). Thus, the contribution of B cells to immunopathology is complex, and B cells seem able to both enhance and inhibit immune responses (56). Ideally, B cell–targeted therapies for autoimmune diseases, transplant rejection, or GVHD would therefore inhibit the immunostimulatory B cell subsets while sparing B cells with immunoregulatory function.

To identify pathways that are important for the activation of B cells and the development of APC function, we performed a global transcriptomic analysis comparing resting B cells and CD40-activated B cells. We chose CD40 as the activation stimulus because CD40L–CD40 interaction is one of the most important signaling pathways for the activation of APCs (57–60). Furthermore, CD40 is crucial for the regulation of autoimmunity and alloreactive immune responses (61–63). Ligation of CD40 on B lymphocytes by its ligand CD154 results in the activation of several intracellular signaling pathways, which mediate B cell activation, B cell proliferation, upregulation of the Ag presentation machinery, isotype switching, and differentiation into Ab-producing cells (64, 65). A GO and pathway-based analysis demonstrated that the list of genes that were upregulated following B cell activation via CD40 was enriched for genes related to cholesterol biosynthesis. We found that virtually all genes of the mevalonate pathway were coordinately upregulated in human B cells following CD40 activation (Fig. 1). This observation prompted us to further investigate the involvement of this metabolic pathway in the activation and function of APCs. The most strongly upregulated gene was the gene for HMG-CoA reductase, the rate-limiting enzyme of the mevalonate biosynthetic pathway. Thus, the blockade of this metabolic pathway with HMG-CoA reductase inhibitors, such as statins, seemed to be a promising strategy for the inhibition of B cell activation.

Indeed, we found that statins inhibited the activation and Ag-presenting function of CD40-activated B cells in a dose-dependent fashion. Statins interfered with CD40-mediated B cell activation, resulting in a reduced expression of costimulatory molecules such as CD80 and CD86 (Fig. 2A, 2B). As a consequence, the Ag-presenting
function of statin-treated B cells was reduced (Fig. 4). Treatment with statins resulted not only in quantitative but also in qualitative differences. Whereas statin-treated B cells demonstrated a decreased ability to stimulate allogeneic CD4+ and CD8+ T cells, their ability to expand Foxp3+CD25highCD4+ T cells seemed to be enhanced (Fig. 4C, 4D). FOXP3 is generally considered to be a marker for regulatory T cells but can also be transiently expressed in activated but hyporesponsive effector T cells (66). Therefore, we cannot determine with certainty whether the FOXP3+ T cells in our experiment represent genuine regulatory T cells. However, irrespective of whether the FOXP3+ T cells represent regulatory or hyporesponsive T cells, the increased percentage of CD25highFOXP3+ T cells demonstrates that atorvastatin-treated B cells possess a decreased ability to induce effective T cell responses. Comparative secretome analysis revealed that treatment with statins also altered cytokine and chemokine secretion by CD40-activated B cells. We observed a strong downregulation of the production of the proinflammatory cytokines RANTES/CCL5 and MCP-1/CCL2 (Fig. 5). Interestingly, both cytokines have previously been shown to be critically involved in allograft rejection, GVHD, and autoimmunity (67–74). Statins affect a variety of immune cells, including T cells, macrophages, and APCs such as DCs (75). However, most of these effects have only been observed in vitro and required high concentrations of statins. Most previous reports, which studied the mechanisms of the immunomodulatory effects of statins on immune cells, employed very high concentrations, most often ≥10 μM. It is therefore not clear whether these effects can also be obtained at concentrations that can be achieved in vivo. Of note, even though T cells are thought to be a major target of the immunomodulatory action of statins, we confirmed our previous result that the concentrations of simvastatin and atorvastatin required for inhibition of B cell activation are considerably lower than those required for inhibition of T cell activation (8). Additionally, although it has previously been shown that the differentiation of DCs can be suppressed by statins (76, 77), our findings indicate that the DC maturation is less dependent on mevalonate biosynthesis (Fig. 6A). The effects of statins appear to be particularly pronounced for the CD40-mediated activation of B lymphocytes. We show that statins preferentially inhibit APC activation via CD40 because signaling via CD40 seems to depend more strongly on the mevalonate pathway than do other B cell–activating pathways such as TLR9 signaling induced by CpGs (Fig. 6B). Importantly, the concentrations of statins at which we observed a significant inhibition of B cell activation can be readily attained in human subjects (78). Our finding that administration of atorvastatin to healthy individuals leads to a detectable decrease of activated CD86+ B cells in the peripheral blood suggests that B activation can be affected by standard doses of statins in vivo (Fig. 8).

A variety of molecular mechanisms for the immunomodulatory effects of statins, including cholesterol depletion, lipid raft disruption, or inhibition of protein isoprenylation, have been described (79, 80). Mechanistically, we demonstrate that the effects of statins on B lymphocytes rely entirely on the inhibition of protein geranylgeranylation and not on inhibition of farnesylation or cholesterol depletion. In addition to the mechanistic understanding, our study provides instructive insights for the clinical application of statins as immunomodulators. One important conclusion from our study is that even though they all target HMG-CoA reductase and have demonstrated similar efficacy for lowering cholesterol, statins are not interchangeable with respect to their immunomodulatory effects. This should be taken into account for the interpretation of the results of clinical trials that study the use of statins as an immunomodulatory drug. Especially in the setting of transplantation, hydrophilic statins have been preferentially studied because they do carry a lower risk of serious drug interactions with other immunosuppressants such as cyclosporine A or tacrolimus (81). However, several studies indicate that lipophilic statins can be safely administered concomitantly with cyclosporine A or tacrolimus when appropriate safety precautions are applied and the patients are monitored closely (82, 83).

Together with data from other groups, the present study underscores the importance of lipid metabolism for activated and malignant human B cells (84). Given that inhibitors of geranylgeranylation are still in the early phases of clinical development and that there are numerous statins that are already on the market, lipophilic statins such as simvastatin and atorvastatin appear to be ideal candidates for the rapid translation into clinical application. Experimental and clinical evidence support the idea that statins could be a useful adjunct conventional immunosuppressant (85–88). Using a bioinformatic approach, Khatri et al. (89) lately identified statins as potential drug candidates for the prevention of transplant rejection. They furthermore demonstrated that atorvastatin significantly extended allograft survival in mice and humans. Interestingly, the same study found that atorvastatin reduced allograft infiltration by B cells in a murine model of cardiac transplant rejection. Additionally, several clinical trials demonstrate the safety of statins and suggest their effectiveness in the prevention of GVHD (90–94). Statins will certainly not replace conventional immunosuppressants. However, immunomodulation of Ag presentation by statins or inhibitors of geranylgeranylation could have an immunosuppressant-sparing effect. In addition to their immunomodulatory properties, statins may offer several additional benefits, which make their use in the setting of transplantation particularly appealing (95). Theoretically, the cardioprotective effects (96, 97) of statins together with their potential antimicrobial (98, 99) and antineoplastic effects (100–103) might confer additional benefits that could lead to better clinical outcomes.

In summary, our data demonstrate that the mevalonate pathway is crucial for CD40-mediated B cell activation and that targeting lipid metabolism of B cells represents a promising therapeutic strategy. We show that statins inhibit the immunostimulatory capacity of B cells by indirectly preventing geranylgeranylation of proteins through depletion of geranylgeranyl. Our study provides a firm rationale for the investigation of inhibitors of geranylgeranylation such as lipophilic statins or more specific inhibitors of geranylgeranyl transferase for the suppression of B cell activation in immunologic conditions where activated B cells play a pathogenic role. The use of statins as immunomodulators could thus become another example for the promise of drug repurposing as a means of accelerated drug discovery (104).

Acknowledgments
We thank Anne Fiedler for expert technical assistance. Furthermore, we are indebted to Bastian von Tresckow, Geothy Chakupukral, and the members of Cologne Interventional Immunology for helpful advice and discussions.

Disclosures
The authors have no financial conflicts of interest.

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