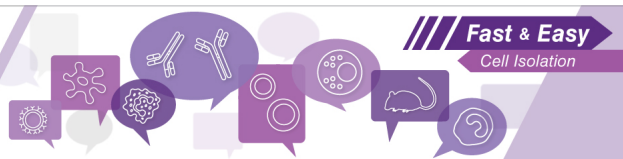


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Fluvastatin Suppresses Mast Cell and Basophil IgE Responses: Genotype-Dependent Effects

Elizabeth Motunrayo Kolawole,* Jamie Josephine Avila McLeod,* Victor Ndaw,* Daniel Abebayehu,* Brian O. Barnstein,* Travis Faber,* Andrew J. Spence,* Marcela Taruselli,* Anuya Paranjape,* Tamara T. Haque,* Amina A. Qayum,* Qasim A. Kazmi,* Dayanjan S. Wijesinghe,[†] Jamie L. Sturgill,[‡] Charles E. Chalfant,^{§,¶,||,#} David B. Straus,* Carole A. Oskeritzian,** and John J. Ryan*

Mast cell (MC)– and basophil-associated inflammatory diseases are a considerable burden to society. A significant portion of patients have symptoms despite standard-of-care therapy. Statins, used to lower serum cholesterol, have immune-modulating activities. We tested the in vitro and in vivo effects of statins on IgE-mediated MC and basophil activation. Fluvastatin showed the most significant inhibitory effects of the six statins tested, suppressing IgE-induced cytokine secretion among mouse MCs and basophils. The effects of fluvastatin were reversed by mevalonic acid or geranylgeranyl pyrophosphatase, and mimicked by geranylgeranyl transferase inhibition. Fluvastatin selectively suppressed key FcεRI signaling pathways, including Akt and ERK. Although MCs and basophils from the C57BL/6J mouse strain were responsive to fluvastatin, those from 129/SvImJ mice were completely resistant. Resistance correlated with fluvastatin-induced upregulation of the statin target HMG-CoA reductase. Human MC cultures from eight donors showed a wide range of fluvastatin responsiveness. These data demonstrate that fluvastatin is a potent suppressor of IgE-mediated MC activation, acting at least partly via blockade of geranyl lipid production downstream of HMG-CoA reductase. Importantly, consideration of statin use for treating MC-associated disease needs to incorporate genetic background effects, which can yield drug resistance. *The Journal of Immunology*, 2016, 196: 1461–1470.

Statins are widely used in the treatment of hypercholesterolemia and cardiovascular disease (CVD) (1). These drugs act by competitively inhibiting the 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) enzyme, resulting in reduced cholesterol (1). Statin structures influence their ability to be absorbed, metabolized, distributed, and excreted (2). Statins also exhibit antioxidant, antiatherosclerotic, antithrombotic, and immunomodulatory functions (3, 4). In the rabbit atherosclerosis model, atorvastatin significantly reduced neointimal inflammation and macrophage infiltration (5). Similarly, lovastatin has been shown to decrease CD11b surface expression on monocytes and CD11b-dependent adhesiveness to fixed endothelium (6).

The anti-inflammatory effects of statins are thought to be critical to protection from CVD. In fact, several clinical trials have demonstrated many non-lipid-lowering statin benefits on CVD (reviewed in Ref. 7). These effects have been attributed to

reducing the production of isoprenoids that are part of the cholesterol synthesis pathway and are involved in modifying cell signaling proteins. In particular, the geranylated and farnesylated proteins, which include the small GTPase family such as Ras, Rac, and Rho, are responsible for controlling multiple cell signaling pathways. It is therefore not surprising that statins exert pleiotropic effects.

It has been shown that statins can suppress TNF and IL-1β production from macrophages (8). Fluvastatin, a synthetic lipophilic family member, inhibits mast cell (MC) degranulation in rat cell lines (2), but a mechanism has yet to be elucidated. Moreover, human and in vivo models have not been thoroughly assessed. Our data show that statins suppress a full range of FcεRI-mediated MC signaling in vitro and in vivo, with consistent effects on mouse basophils and primary human MCs. Suppression appears to be exerted at the level of geranylgeranyl lipid

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Abbreviations used in this article: BMMC, bone marrow–derived MC; cRPMI, RPMI 1640 media supplemented with 10% FCS, sodium pyruvate, penicillin, and streptomycin; CVD, cardiovascular disease; DNP-HSA, DNP-coupled human serum albumin; FPTIII, farnesyl transferase inhibitor III; GGTI-286, geranylgeranyl transferase inhibitor-286; HMGCR, 3-hydroxy-3-methylglutaryl CoA reductase; MC, mast cell; MVA, mevalonic acid; PSA, passive systemic anaphylaxis; SCF, stem cell factor; ZA, zaragozic acid A.

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generation. Interestingly, statins are strikingly susceptible to genetic background, likely because of drug-induced control of HMGCR expression. Our data support consideration of statins as a means of suppressing inflammatory disease, with the caveat that genetic background may determine drug efficacy.

Materials and Methods

Cytokines and reagents

Cytokines were purchased from BioLegend. Mouse IgE for in vivo studies was generously provided by Dr. Daniel Conrad (Virginia Commonwealth University). Purified mouse IgE (clone C38-2, κ isotype) was purchased from BD Biosciences (San Diego, CA). Abs recognizing mouse CD49b, CD63, TNF, IL-4, and IL-6 were from BioLegend. Abs against c-Kit, Fc ϵ R1, Syk, IL-13, MIP-1 α (CCL-3), MCP-1 (CCL-2), phosphoserine-473-AKT, and phosphothreonine 202/phosphotyrosine 204-ERK-1/2 were from Cell Signaling Technology (Danvers, MA). Propidium iodide and DNP-coupled human serum albumin (DNP-HSA) were from Sigma-Aldrich (St. Louis, MO). Zaragozic acid A (ZA) and all statin drugs except pitavastatin were from Sigma-Aldrich. Pitavastatin (ab141958) was from Abcam (Cambridge, MA). Farnesylation transferase inhibitor III (FPTIII) and geranylgeranyl transferase inhibitor-286 (GGTI-286) were from Calbiochem (Darmstadt, Germany). Farnesyl diphosphate and geranylgeranyl diphosphate were from Echelon (Salt Lake city, UT).

Animals

C57BL/6J and 129/SvImJ mice were from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 10 wk old, with approval

from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Mouse MC and basophil culture

Mouse bone marrow-derived MCs (BMMCs) were cultured as described previously (9). Mouse bone marrow-derived basophils were cultured in RPMI 1640 media supplemented with 10% FCS, sodium pyruvate, penicillin, and streptomycin (cRPMI), which was supplemented with rIL-3 at 20 ng/ml (BioLegend, San Diego, CA) for 7–10 d, then sorted by flow cytometry selecting for CD49b⁺ cells (BioLegend). Peritoneal lavage cells were cultured in cRPMI containing IL-3 (5 ng/ml) and stem cell factor (SCF) (10 ng/ml) for 5 d. MCs were positively selected using the EasySep Magnet from STEMCELL Technologies (Vancouver, BC), with c-Kit as a positive marker. Flow cytometry confirmed purity, which was essentially 100%.

Human skin MC culture

All protocols involving human tissues were approved by the human studies Internal Review Board at the University of South Carolina. Surgical skin samples were obtained from the Cooperative Human Tissue Network of the National Cancer Institute. Skin MCs were prepared and cultured as described previously (10) and were used after 8–16 wk, at which time purity was essentially 100% MCs, as determined by staining with toluidine blue.

IgE-mediated activation

Human MCs or BMMCs were sensitized overnight with DNP-specific mouse IgE (1.0 μ g/ml for human MC; 0.5 μ g/ml for BMMCs), then washed and resuspended 1×10^6 cells/ml in complete media with cytokines. Cells were stimulated with DNP-HSA (Ag; 30 or 20 ng/ml for human MC or mouse BMMCs, respectively) for the indicated times.

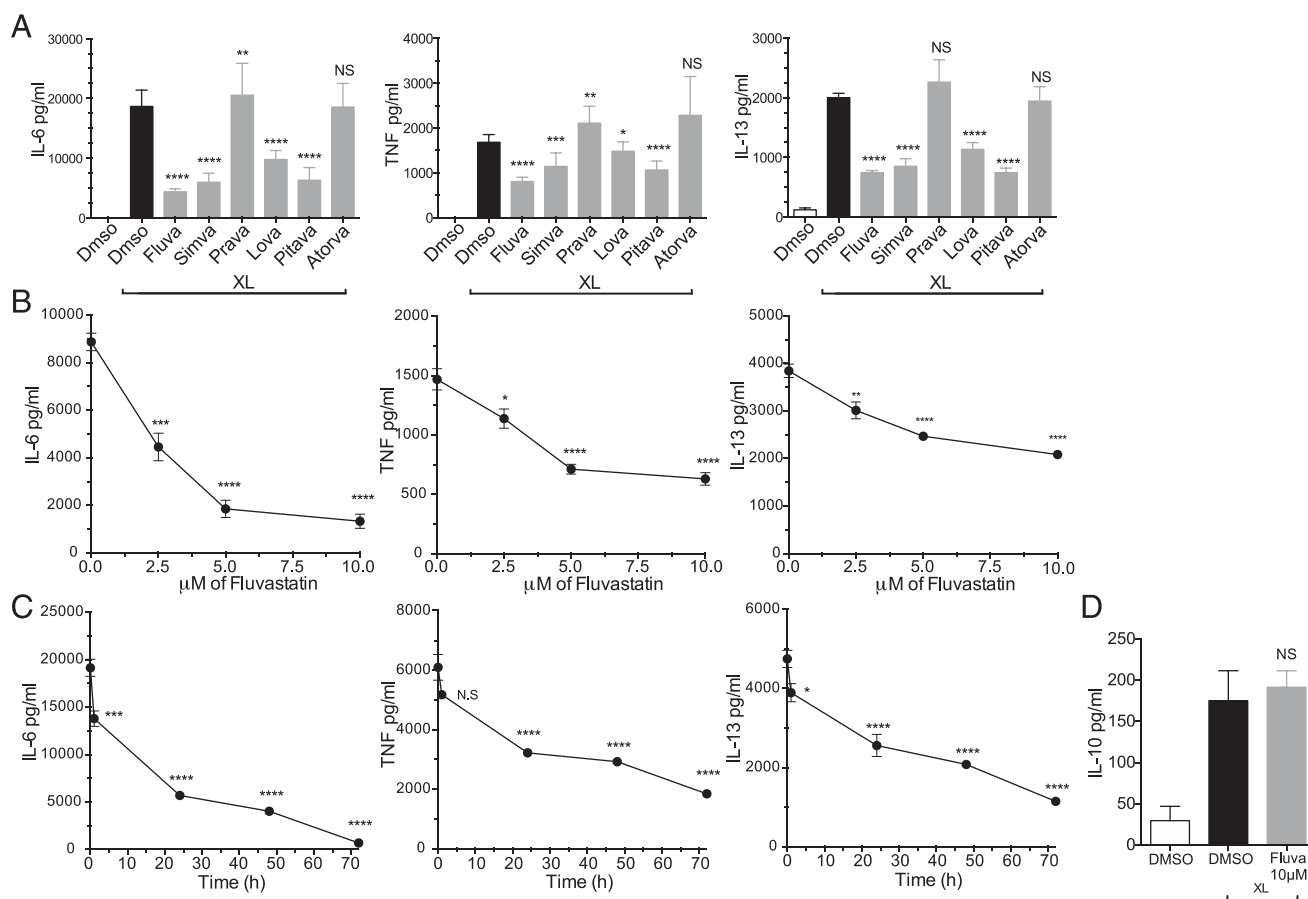


FIGURE 1. Statins suppress IgE-mediated cytokine production in mouse MCs. (A) C57BL/6J BMMCs were cultured with DMSO or 10 μ M of the indicated statin for 24 h, then activated as described. IL-6, TNF, and IL-13 levels in culture supernatants were determined by ELISA. (B) BMMCs were cultured with the indicated concentrations of fluvastatin before activation as in (A). (C) BMMCs were treated with 10 μ M fluvastatin as in (A) for the indicated times before Ag-mediated activation for 16 h. (D) BMMCs were cultured with 10 μ M fluvastatin as in (A), and culture supernatants were tested for the presence of IL-10 by ELISA. Data are means $\pm$ SEM of five (A) and three (B–D) independent experiments done in triplicate and analyzed by unpaired *t* test, comparing fluvastatin- and DMSO (control)-treated groups. For each independent experiment, *n* = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Cytokines were measured by standard ELISA kits from BioLegend (San Diego, CA) or BD Biosciences (San Diego, CA).

Flow cytometric analysis

Surface c-Kit and FcεRI expression were measured by standard flow cytometry on a BD FACSCalibur. To detect intracellular cytokines, we stained cells as described previously (9). Basophils were also stained with FITC-anti-CD49b before fixation.

Degranulation assays

Cells were plated at 1×10^6 /ml in cRPMI, as described earlier, and treated with fluvastatin or DMSO (vehicle, diluted 1:5000) for 24 h $\pm$ 0.5 μ g/ml IgE, were washed twice in RPMI 1640, and were activated $\pm$ DNP-HSA for 1 h before staining with CD63, followed by flow cytometry analysis.

Migration assay

Migration was assayed as described previously (9), using 8 μ m polycarbonate 24-well transwell inserts from Corning.

HMG-CoA reductase quantitative PCR

BMMCs were cultured $\pm$ 40 μ M fluvastatin for 5 h. RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized using the SuperScript III Reverse Transcriptase (Invitrogen by Life Technologies, Grand Island, NY) using oligo deoxythymidine primers. Quantitative PCR analysis was performed with BioRad CFX96 Touch Real-Time PCR Detection System (Hercules, CA) and SYBR Green detection using a relative Livak method. Primers included: HMGCR pri-

mers q.HMGCR Forward: 5'CCTGTAAGTCAAGGGTCAAGATGAT-3' and q.HMGCR Reverse: 5'CCAGCGACTGTGAGCATGAA-3' or β -actin Mouse β -Actin Forward: 5'GATGACGATATCGTGC-3', Mouse β -Actin Reverse: 5'CTCGTCACCCACATAGGAGTC-3' (housekeeping gene) primers (Invitrogen by Life Technologies, Grand Island, NY). Melting curve analysis was performed between 50°C and 95°C.

Quantitative measurement of cholesterol

Cholesterol levels were analyzed via liquid chromatography tandem mass spectrometry using a Shimadzu Nexera UPLC coupled to a QTRAP 6500 mass analyzer (AB SCIEX, Framingham, MA). Lipids were extracted from the cell pellet using a modified Bligh and Dyer method as previously described (11, 12).

Western blot analysis

Western blotting was performed as described previously (9). Blots were visualized and quantified using a LiCor Odyssey CLx Infrared imaging system (Lincoln, NE).

Passive systemic anaphylaxis

Mice were administered 200 μ l PBS containing 1 mg fluvastatin or equivalent dilution of DMSO via i.p. injection, followed by 200 μ l PBS containing 50 μ g mouse IgE anti-DNP. The following day, mice were again administered 200 μ l PBS containing 1 mg fluvastatin, or DMSO 1 h before DNP-HSA (50 μ g) was administered via i.p. injection. In some experiments, 8 mg histamine was injected in place of Ag. The core body temperature of each mouse was measured using a rectal microprobe

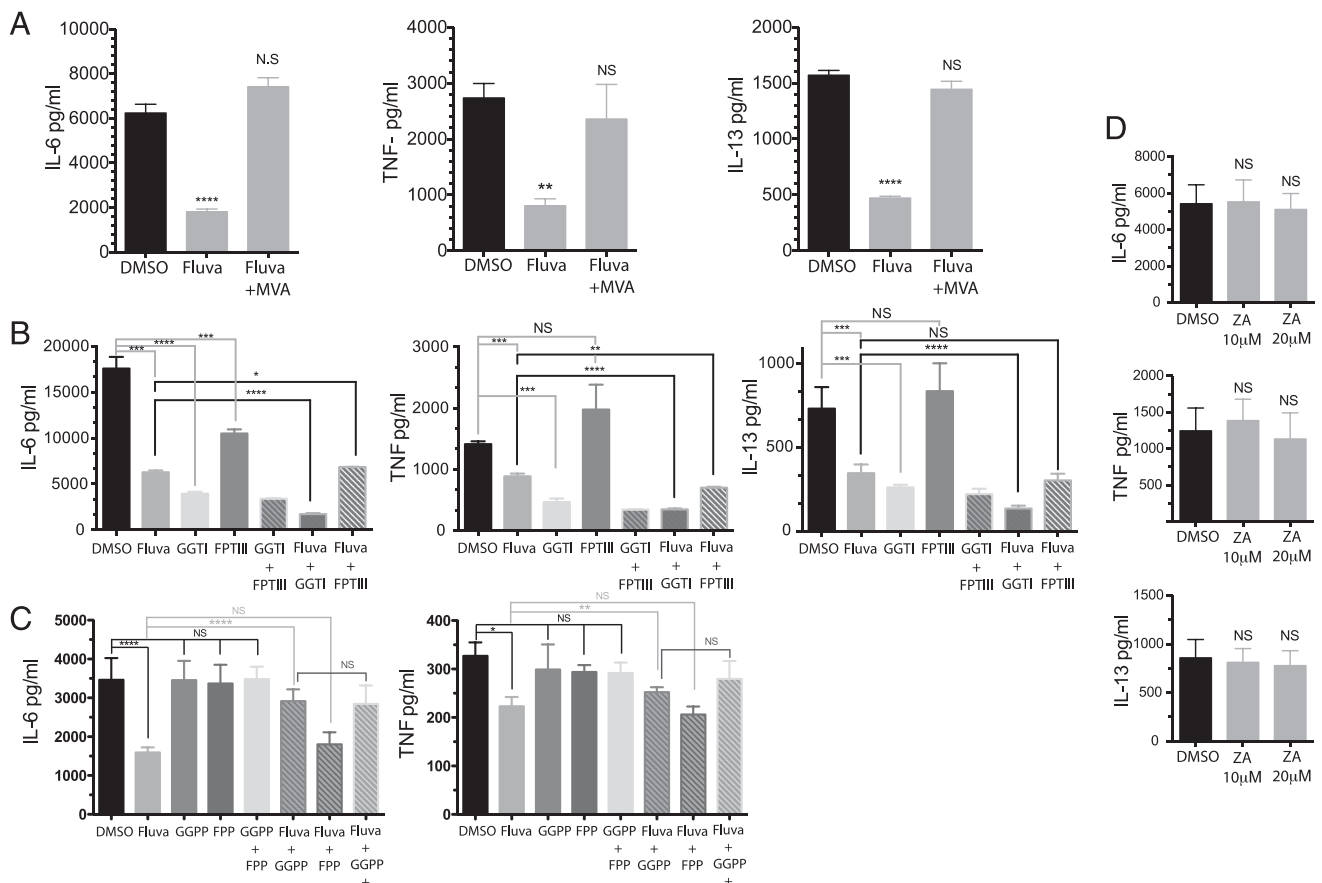


FIGURE 2. Fluvastatin-mediated suppression is linked to isoprenoid biosynthesis. **(A)** IgE-primed C57BL/6J BMMCs were cultured for 24 h in the presence of either DMSO or 10 μ M fluvastatin $\pm$ 1000 mM MVA as indicated. Cells were then activated with Ag for 16 h and culture supernatants were analyzed by ELISA. MVA alone did not alter cytokine production (data not shown). **(B)** BMMCs were cultured for 24 h with IgE plus the indicated inhibitors (20 μ M) or fluvastatin (10 μ M), then activated for 16 h with Ag. Culture supernatants were analyzed by ELISA. **(C)** BMMCs were cultured for 24 h with IgE in the presence of DMSO, fluvastatin (10 μ M) $\pm$ the indicated farnesyl, or geranyl pyrophosphate (20 μ M) before Ag activation for 16 h. Culture supernatants were assayed by ELISA. **(D)** BMMCs were cultured for 24 h with IgE plus DMSO or the indicated concentration of ZA, before Ag activation for 16 h. Culture supernatants were analyzed by ELISA. Data are means $\pm$ SEM of three (A, B, and D) or four (C) independent experiments done in triplicate and analyzed by unpaired *t* test comparing fluvastatin- and DMSO (control)-treated groups. For each independent experiment, *n* = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

(Physitemp Instruments). Mice were euthanized and blood was collected by cardiac puncture to analyze plasma.

Statistical analysis

The *p* values were calculated using GraphPad Prism software, by paired or unpaired two-tailed Student *t* test as appropriate. The *p* values <0.05 were considered statistically significant. Unless otherwise stated, results are expressed as the mean $\pm$ SEM of at least three independent experiments conducted in triplicate. In all figures, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Results

Statins inhibit IgE-mediated cytokine production by mouse MCs

Statins alter isoprenoid generation and degranulation in RBL-2H3 cells (13). We tested several statin family members to determine their effects on IgE-activated (XL) primary MCs. C57BL/6J BMMCs treated for 24 h before Ag-induced activation generally showed decreased IL-6, TNF, and IL-13 production, with the exception of atorvastatin and pravastatin treatment (Fig. 1A). Fluvastatin responses tended to be of the greatest magnitude, hence we focused our studies on this drug.

Time and dose dependence for fluvastatin-mediated suppression were established by assessing IgE-induced IL-6, TNF, and IL-13 secretion. A total of 10 μ M fluvastatin yielded maximal inhibition, suppressing cytokine production $\geq$ 50%. The IC₅₀ for this effect was $\sim$ 2.5–5 μ M (Fig. 1B). A 24-h incubation yielded

significant suppression with no change in cell viability (Fig. 1C). By contrast, 72-h treatment provided the greatest suppression but decreased cell viability (data not shown); hence we used a 24-h time point for further study. Finally, it was interesting to note that fluvastatin had no effect on production of IL-10, a cytokine that is generally anti-inflammatory (Fig. 1D). This divergence from the effects on proinflammatory cytokines suggested some specificity in how FcεRI signaling was altered.

Fluvastatin effects are mediated via blockade of isoprenoid lipids

Fluvastatin acts by blocking HMGCR, inhibiting the production of mevalonic acid (MVA) (3, 14–16). To assess target specificity, we attempted to reverse the effects of fluvastatin with MVA. As shown in Fig. 2A, MVA restored IgE-mediated cytokine production in the presence of fluvastatin, indicating that drug effects were in fact due to HMGCR blockade.

MVA metabolism leads to cholesterol production, but also yields farnesyl pyrophosphate and geranylgeranyl pyrophosphate, precursors of isoprenoid lipids. These are critical for protein prenylation, including modifications to small GTPases such as Ras, Rac, and Rho. To determine the importance of prenylation, we used the compounds GGTI-286 and FPTIII, selective inhibitors of geranylgeranylation and farnesylation, respectively. GGTI-286 treatment for 24 h mimicked the effects of fluvastatin (Fig. 2B). Treatment with fluvastatin plus GGTI-286 suppressed cytokine

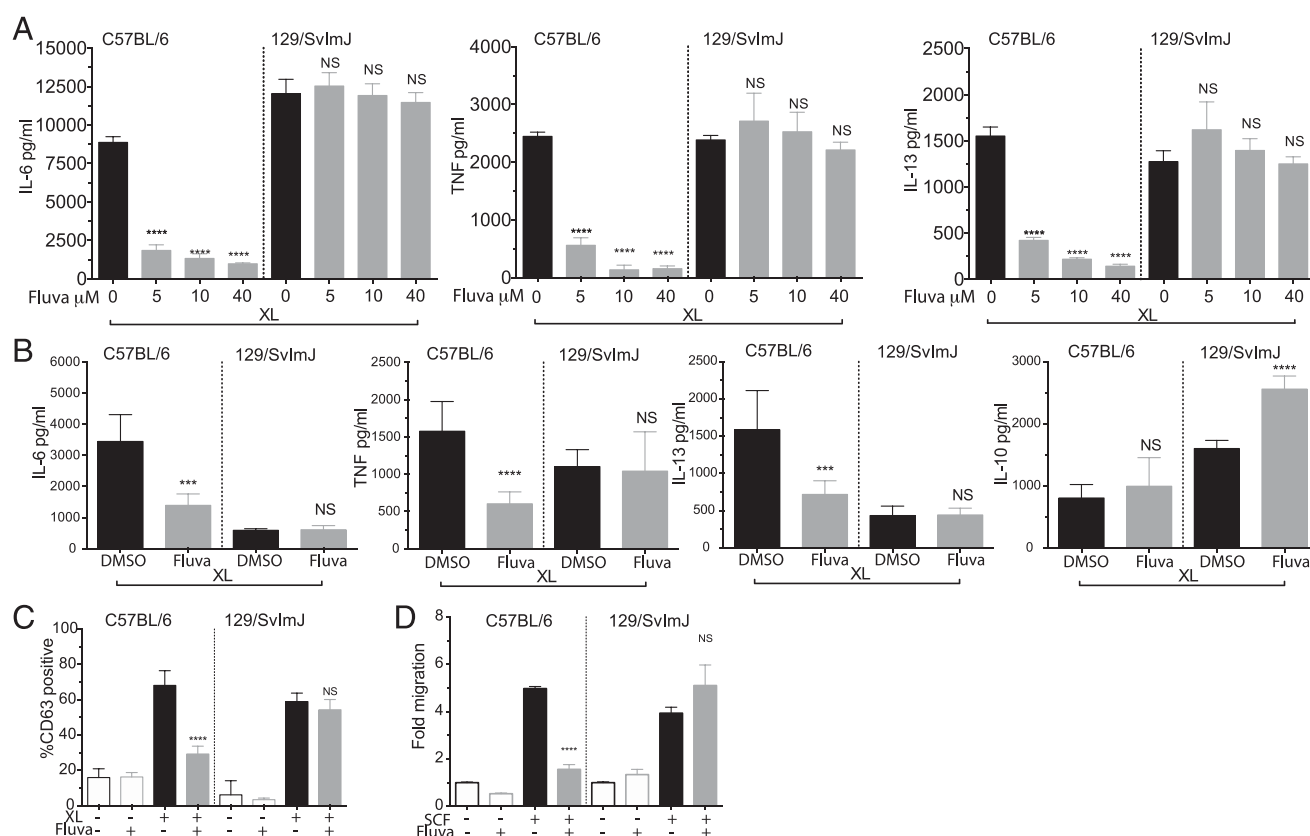


FIGURE 3. 129/SvImJ mouse MCs are resistant to fluvastatin. **(A)** C57BL/6J or 129/SvImJ BMMCs were cultured in the indicated concentrations of fluvastatin and activated as described in Fig. 1A. Culture supernatants were analyzed by ELISA. **(B)** Purified peritoneal MCs from the indicated mouse strains were cultured for 24 h with DMSO or 10 μ M fluvastatin, then activated with Ag. Culture supernatants were assessed by ELISA. **(C)** BMMCs from the indicated strains were cultured as described in Fig. 1A and activated with Ag for 1 h before staining to detect surface CD63 by flow cytometry. **(D)** BMMCs from the indicated strains were cultured with DMSO (–) or fluvastatin (10 μ M) for 24 h, then tested for migration toward an SCF gradient as described in *Materials and Methods*. All data shown are from a minimum of three experiments. Data are means $\pm$ SEM of three independent experiments done in triplicate and analyzed by unpaired *t* test comparing fluvastatin- and DMSO (control)-treated groups. For each independent experiment, *n* = 3. **(B)** A representative of one of three independent experiments done in triplicate is shown. ****p* < 0.001, *****p* < 0.0001.

production more than either compound alone. In contrast, FPTIII treatment provided little or no inhibition (Fig. 2B).

To confirm our findings with these inhibitors, we attempted to reverse fluvastatin-mediated inhibition by restoring isoprenoid lipids. BMMCs were treated with fluvastatin in the presence or absence of either geranylgeranyl pyrophosphate or farnesyl pyrophosphate. As shown in Fig. 2C, geranylgeranyl pyrophosphate partially rescued IgE-mediated cytokine production when compared with fluvastatin-treated cells. By contrast, farnesyl pyrophosphate had little or no effect.

Although isoprenoid blockade appeared to be important for fluvastatin effects, cholesterol depletion could also be critical. To test this, we used the squalene synthase inhibitor ZA, which blocks cholesterol synthesis downstream of isoprenoid generation. ZA treatment reduced BMMC cholesterol levels 59%, similar to the effects of fluvastatin (data not shown), but had no impact on IgE-mediated cytokine production (Fig. 2D). Collectively, these data suggest that fluvastatin effects on Ag-induced cytokine production are predominantly due to reduced geranylgeranylation events involved in FcεRI signaling.

Fluvastatin effects can be altered by genetic background

To determine whether genetic background alters fluvastatin responsiveness, we compared BMMCs from C57BL/6J and 129/SvImJ mice, which possess many polymorphic variations. As shown in Fig. 3A, 129/SvImJ BMMCs were strikingly resistant to fluvastatin at concentrations up to 40 μM. In fact, 129/SvImJ BMMCs were resistant to simvastatin, pravastatin, and partially to atorvastatin (Supplemental Fig. 1). To establish that these differences were not due to their *in vitro* differentiation, we compared fluvastatin responses using peritoneal MCs, which yielded a similar outcome (Fig. 3B). We also tested BMMCs cultured from BALB/c and A/J mice, both of which were also resistant to fluvastatin effects (Supplemental Fig. 2). These data support the hypothesis that genetic background can yield drug resistance.

We further showed that fluvastatin inhibits other MC functions, effects that are also absent among 129/SvImJ MCs. FcεRI-

induced degranulation, as judged by membrane expression of the intragranular protein CD63, was selectively inhibited by fluvastatin in C57BL/6J BMMCs (Fig. 3C). Similarly, fluvastatin diminished SCF-mediated migration of C57BL/6J, but not 129/SvImJ BMMCs (Fig. 3D).

Fluvastatin suppresses Ag-stimulated basophils

We also investigated the effects of fluvastatin on C57BL/6J and 129/SvImJ basophils. Culture for 24 h with fluvastatin before IgE+Ag-mediated activation reduced IL-4, IL-6, IL-13, TNF, and MCP-1 production, and inhibited degranulation among C57BL/6J, but had no significant effect on 129/SvImJ basophils (Fig. 4).

Fluvastatin selectively alters FcεRI signaling pathway

The suppressive effects of fluvastatin could be related to reduced FcεRI or c-Kit expression. However, fluvastatin treatment with concentrations reaching 40 μM for up to 4 d had no significant effect on FcεRI or c-Kit surface expression as measured by flow cytometry (data not shown). Hence loss of receptor expression does not appear to explain fluvastatin effects. As such, we investigated how fluvastatin impacts FcεRI signaling.

After Ag cross-linking, the Src family tyrosine kinases Fyn and Lyn, as well as the tyrosine kinase Syk, are recruited to FcεRI (17). Fluvastatin had no effect on Fyn, Lyn, or Syk expression in C57BL/6J BMMCs (Fig. 5A).

Downstream of FcεRI apical tyrosine kinases, a number of signaling pathways are activated. Fluvastatin inhibited Akt and ERK phosphorylation (Fig. 5B) in C57BL/6J, but not 129/SvImJ BMMCs. In contrast, JNK or p38 phosphorylation was unaffected (Fig. 5B). Taken together, these data demonstrate selective inhibitory effects of fluvastatin treatment, acting both apically and distally from FcεRI.

129/SvImJ resistance correlates with fluvastatin-mediated induction of HMGCR

Although the C57BL/6J and 129/SvImJ strains have significant genetic variability, the fluvastatin target HMGCR is reported on the

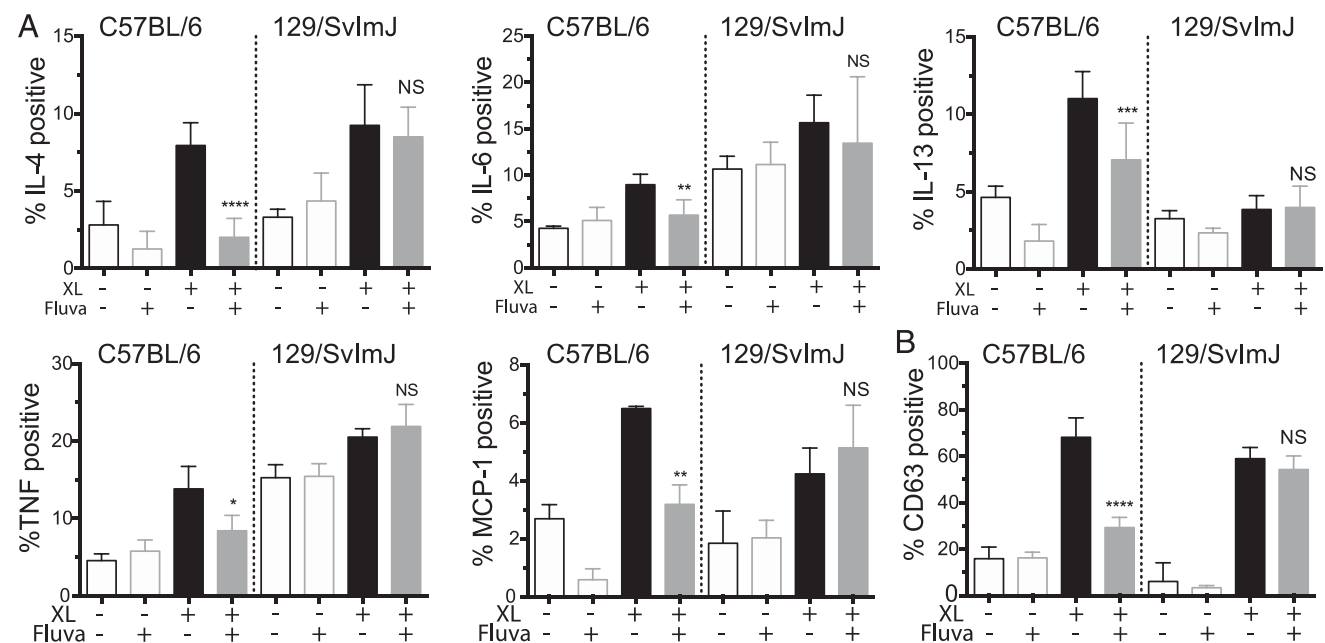


FIGURE 4. Fluvastatin suppresses IgE responses in C57BL/6J but not 129/SvImJ basophils. Basophils from the indicated mouse strains were cultured in fluvastatin as described in Fig. 1, then activated with Ag before detecting (A) intracellular cytokines or (B) surface CD63 as described. Data are means $\pm$ SEM of three independent experiments done in triplicate and analyzed by unpaired *t* test comparing fluvastatin- and DMSO (control)-treated groups. For each independent experiment, *n* = 3. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

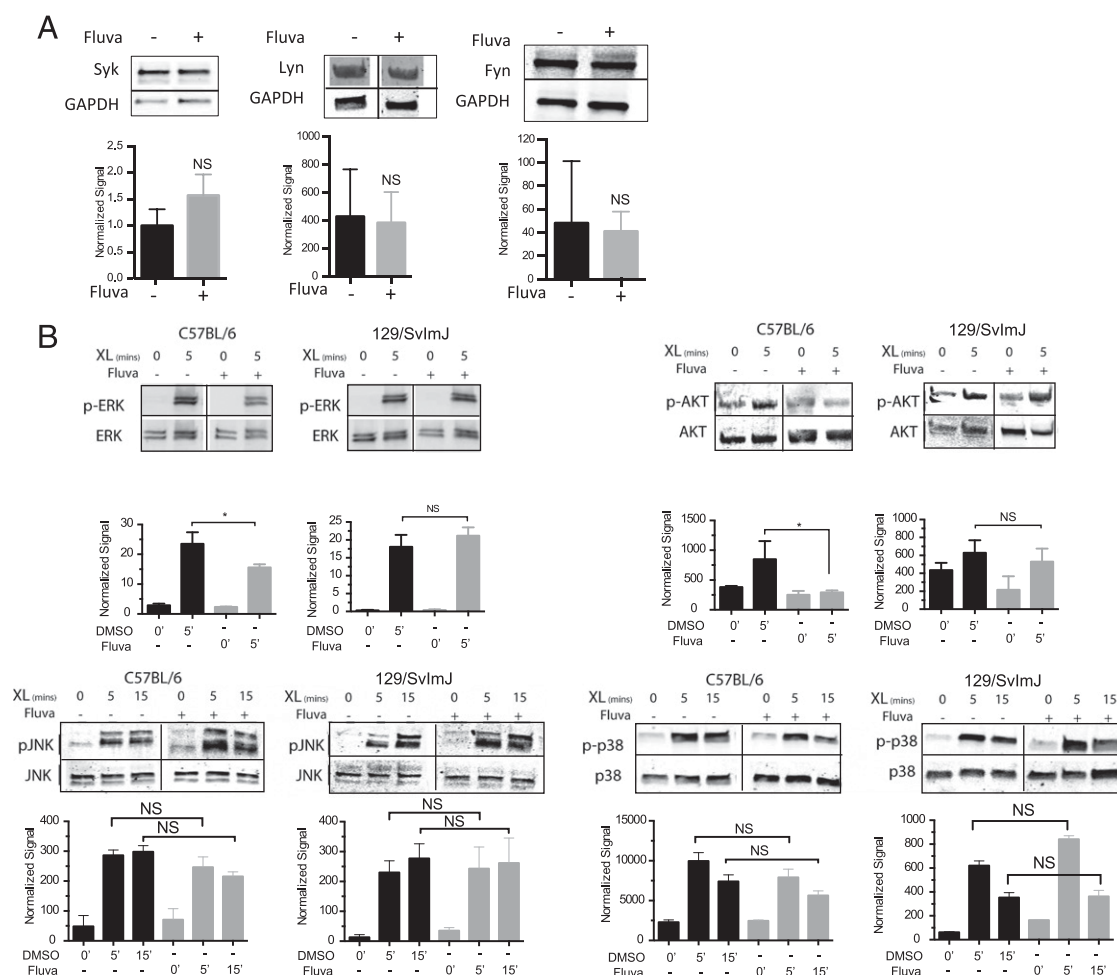


FIGURE 5. Fluvastatin suppresses FcεRI signaling. **(A)** C57BL/6 BMMCs were cultured in DMSO or fluvastatin as described in Fig. 1, then subjected to Western blotting to detect Syk, Lyn, or Fyn. **(B)** BMMCs from the indicated strains were cultured for 24 h with IgE plus DMSO or fluvastatin (10 μM) before Ag-mediated activation for 5 min, then analyzed by Western blotting to detect the indicated total and phosphorylated proteins. Black lines indicate where parts of the images are joined. Data are means ± SEM analyzed by unpaired *t* test of **(A)** nine samples and **(B)** three independent experiments. **p* < 0.05.

Jackson Lab Phenome site (<http://phenome.jax.org>) to lack coding polymorphisms between these strains. We confirmed this by sequencing MC HMGCR mRNA from these strains (data not shown). However, coding variability is only one means of achieving drug resistance. We demonstrate that 129/SvImJ BMMCs increased HMGCR mRNA expression 2.5-fold after fluvastatin treatment, whereas C57BL/6J BMMCs showed no change in expression (Fig. 6A). Furthermore, this increased expression appeared to be functionally significant. As shown in Fig. 6B, fluvastatin treatment reduced cellular cholesterol levels ~60% in C57BL/6J BMMCs, whereas 129/SvImJ BMMCs were unaffected. Finally, we found that FcεRI-mediated IL-6 production by 129/SvImJ BMMCs could be suppressed by blocking isoprenylation events downstream of HMGCR with GGTI-286 ± FPTIII (Fig. 6C). Thus, the 129/SvImJ background is responsive to isoprenoid blockade, indicating that drug resistance lies above this step. Collectively these data support the hypothesis that HMGCR induction yields drug resistance in 129/SvImJ MCs.

Fluvastatin suppresses passive systemic anaphylaxis in C57BL/6J but not 129/SvImJ mice

We next used an MC-dependent passive systemic anaphylaxis (PSA) model to assess the functional relevance of fluvastatin sensitivity on C57BL/6J and 129/SvImJ mice. In this article, we show that fluvastatin pretreatment significantly mitigated the loss of

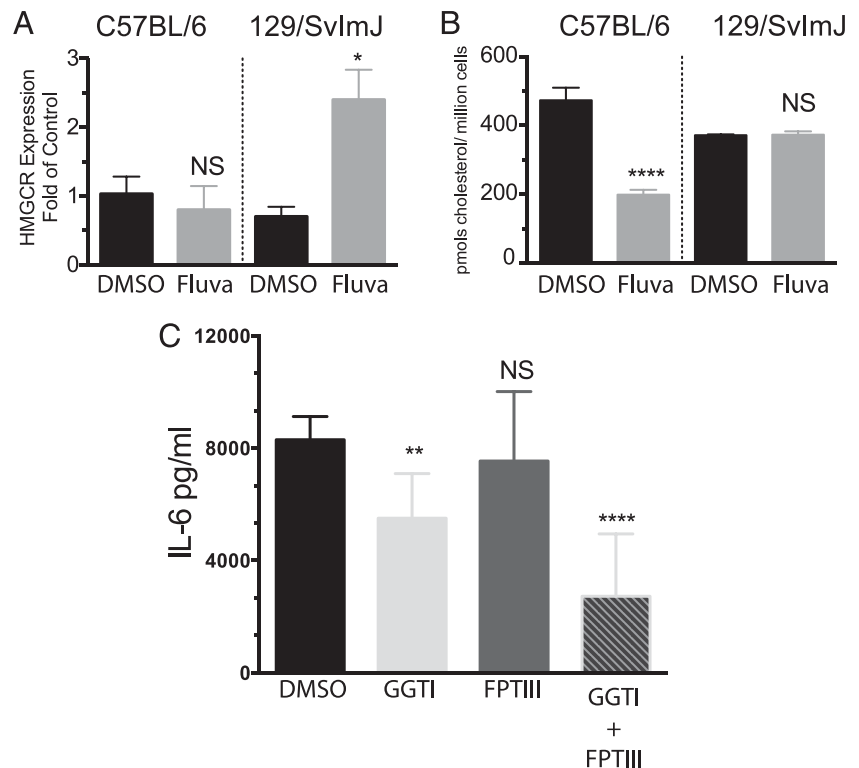
core body temperature in C57BL/6J mice but had no effect on the 129/SvImJ background (Fig. 7A). Plasma MIP-1α levels from these mice showed the same trend, with fluvastatin-mediated suppression observed in C57BL/6J but not 129/SvImJ mice (Fig. 7B). These data show that fluvastatin dampens the early and late phases of MC-dependent anaphylaxis, and that genetic background can greatly alter the drug response.

In addition to inhibiting MC degranulation, fluvastatin could be suppressing the vascular response to histamine. However, we show that fluvastatin pretreatment before histamine administration did not suppress anaphylaxis (Fig. 7C). We therefore conclude that fluvastatin effects are largely due to MC suppression, not effects on the vasculature.

Variable responsiveness to fluvastatin is consistent among primary human MCs

Given the stark variation in fluvastatin responses among mouse strains, we assessed primary human skin MCs from eight healthy subjects. Interestingly, we found a large variation in fluvastatin responses (Fig. 8). When investigating TNF and MCP-1 production, we noted relative decreases in cytokine production from <20% to >80%. These data suggest that fluvastatin can significantly blunt IgE-mediated activation in human MCs, but that genetic variability may greatly impact the extent of drug responsiveness.

FIGURE 6. 129/SvImJ MCs exhibit fluvastatin-induced HMGCR upregulation correlating with drug resistance. **(A)** BMMCs were cultured for 5 h with DMSO or fluvastatin (40 μ M). Total RNA was analyzed for HMGCR expression by quantitative real-time PCR. Data shown are from one of two independent experiments using three populations each analyzed by unpaired *t* test comparing fluvastatin- and DMSO (control)-treated groups. **(B)** BMMCs were cultured with DMSO or fluvastatin (10 μ M) for 24 h. Lysates were analyzed for the presence of cholesterol by mass spectroscopy. Data shown are means and SEM of three samples analyzed by paired *t* test, comparing the samples with vehicle with fluvastatin. **(C)** 129/SvImJ BMMCs were cultured for 24 h with IgE plus the indicated inhibitors (20 μ M) or fluvastatin (10 μ M), then activated for 16 h with Ag before ELISA analysis of supernatants. Data are from three independent experiments done in triplicate and analyzed by unpaired *t* test comparing fluvastatin- and DMSO (control)-treated groups. **p* < 0.05, ***p* < 0.01, ****p* < 0.0001.



Discussion

Statins are best known for their lipid-lowering effects and are widely prescribed to prevent CVD. However, several clinical trials have suggested that statin anti-inflammatory benefits are important, separate from their effects on lipids (reviewed in Ref. 7). These drugs have pleiotropic effects on MCs. Lovastatin and fluvastatin have been reported to inhibit IgE-mediated degranulation in the rat MC line RBL-2H3 (2). Furthermore, cerivastatin and atorvastatin have been shown to suppress growth and IgE-mediated histamine release in human basophils (18). Our work demonstrates that a range of statins inhibit IgE-induced cytokine production from MCs and basophils with varying degrees of efficacy. There were some exceptions: pravastatin slightly enhanced cytokine production, and atorvastatin had no significant effect. Pravastatin is more hydrophilic than the “lipophilic” simvastatin, lovastatin, and fluvastatin (19). However, the most lipophilic statins, lovastatin and simvastatin (20), were not the most suppressive in our assays. Interestingly, the suppressive capabilities of fluvastatin did not extend to IL-10, widely regarded as an anti-inflammatory cytokine (21, 22).

Fluvastatin effects were reversed with MVA pretreatment, confirming targeted specificity to the mevalonate pathway. Our data showed that the geranylgeranyl transfer step is critical. These data were supported by studies using the squalene synthase inhibitor ZA (23), which did not alter IgE-induced cytokine production, despite suppressing cholesterol synthesis. These latter results suggest that fluvastatin effects are not due to large-scale changes in cholesterol-containing lipid rafts. Taken together, these data argue that geranylgeranylation has the largest effect on IgE-mediated cytokine production, and suggest this pathway as a potential target for controlling the MC response.

MCs are best known for their role in IgE-mediated inflammation. Asthma and allergy studies support broad effects of statins in MC-associated disease. PBMC proliferation and inflammatory responses were suppressed by fluvastatin in patients with allergic asthma (24). Furthermore, atorvastatin in conjunction with inhaled

corticosteroids improved lung function and airway inflammation in atopic asthmatics (25). These and other studies have led to the conclusion that statins are beneficial for asthma management (26). Cellular and molecular mechanisms are suggested from human and animal studies. Statins may suppress T cell activation by decreasing IFN- γ -induced MHC II expression on monocytes (27). T cell activation, proliferation, and migration are also suppressed by statin treatment (24). In addition to their effects on immune cells, statins have been shown to suppress bronchial wall remodeling (28).

Mouse models also support the rationale for statin therapy. Simvastatin inhibits airway hyperresponsiveness in a murine model (29). This is partly due to suppression of T cell-produced IL-4 and IL-5 (30). More recently, Kim et al. (31) showed that simvastatin reduced OVA-specific IgE levels, and the number of macrophages, neutrophils, and eosinophils in bronchoalveolar lavage fluid in an MC-independent airway hyperresponsiveness model. Moreover, simvastatin also reduced thioglycolate-induced peritoneal inflammation (32) in which the predominate infiltrate is neutrophils.

The passive anaphylaxis model does not require Ag processing/presentation or a role for T and B cells. While focusing on the MC and vasculature, this assay demonstrated that fluvastatin treatment before Ag challenge dramatically reduced the severity of anaphylaxis in C57BL/6J mice. To rule out fluvastatin primarily suppressing the vascular response to histamine, we pretreated mice with fluvastatin and bypassed the MC response by injecting histamine, conditions under which fluvastatin had no effect. These data support the theory that fluvastatin directly suppresses MCs in vivo and support the use of statins as a therapy for MC-associated disease.

Downstream of the IgE receptor, Syk, Fyn, and Lyn are recruited and activated. Lyn is predominantly a negative regulator, as noted in Lyn-deficient mice (33). We further show fluvastatin suppressed Akt and Erk phosphorylation in C57BL/6J but not 129/SvImJ BMMCs. These are critical regulators of MC responses to IgE, hence their collective suppression could logically explain reduced cytokine production and anaphylaxis.

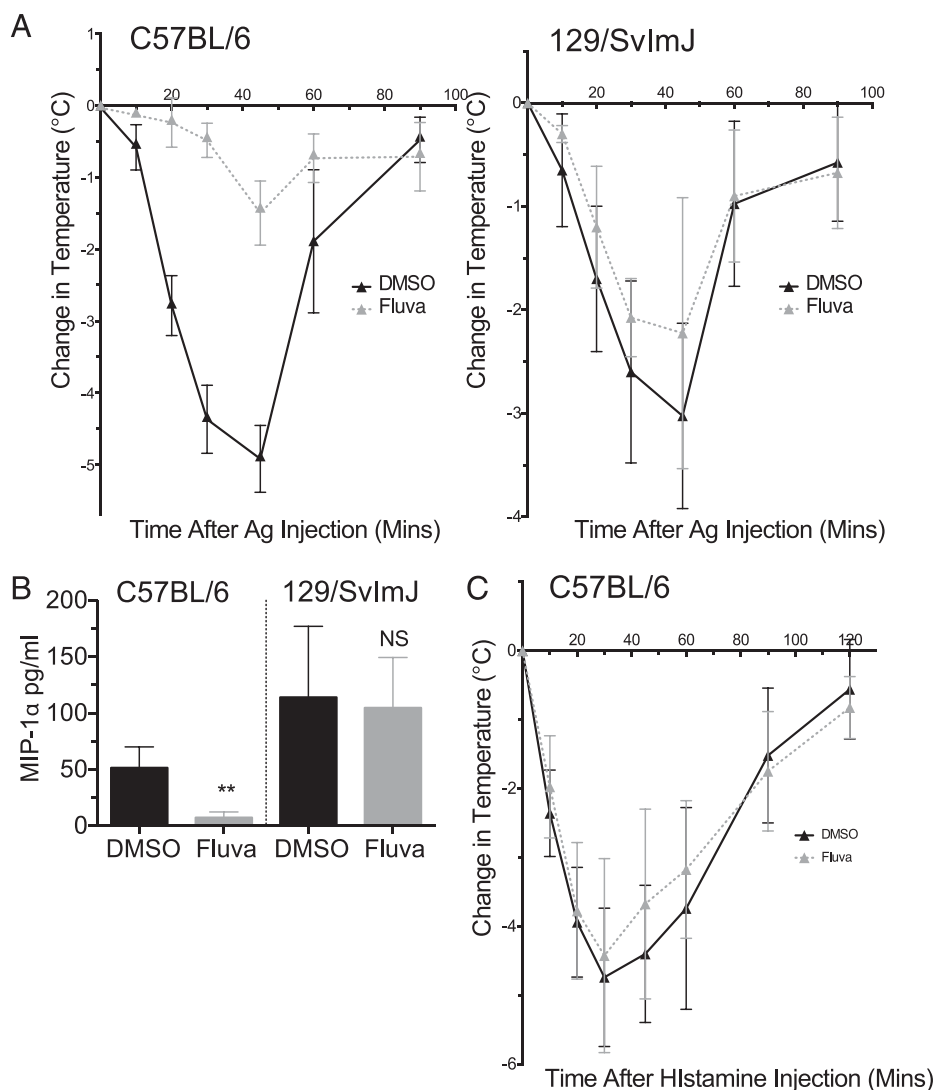


FIGURE 7. Fluvastatin suppresses PSA in C57BL/6J but not 129/SvImJ mice. **(A)** and **(B)** Mice from the indicated strains were subjected to PSA as described. Plasma was analyzed for MIP-1 α levels by ELISA. **(C)** Mice were injected with DMSO or fluvastatin as described in (A), then challenged with 8 mg histamine. Change in core body temperature was recorded. (A and C) $n = 5$ from two independent experiments. (B) Data are means $\pm$ SEM from two independent experiments. All data were analyzed by unpaired t test comparing fluvastatin- and DMSO (control)-treated groups. ** $p < 0.01$, **** $p < 0.0001$.

Variable responses to statins among C57BL/6J and 129/SvImJ MCs are consistent with previous studies from our group. We found that MC precursors from the BALB/cJ background are resistant to IL-10 (34) and that 129/SvImJ BMMCs are resistant to TGF β I-mediated suppression (9). Thus, it was intriguing to note that 129/SvImJ, BALB/cJ, and A/J were all fluvastatin resistant. Because C57BL/6J is Th1-prone and 129/SvImJ, BALB/c, and A/J are Th2-prone mice, a simple conclusion would be that genetic variations predisposing to strong Th1 or Th2 development have related polymorphisms yielding drug resistance. However, it is important to note that these mice have many genetic variations unrelated to T cell or inflammatory phenotypes. These observations support a deeper investigation into the mechanisms explaining this pharmacogenomic effect.

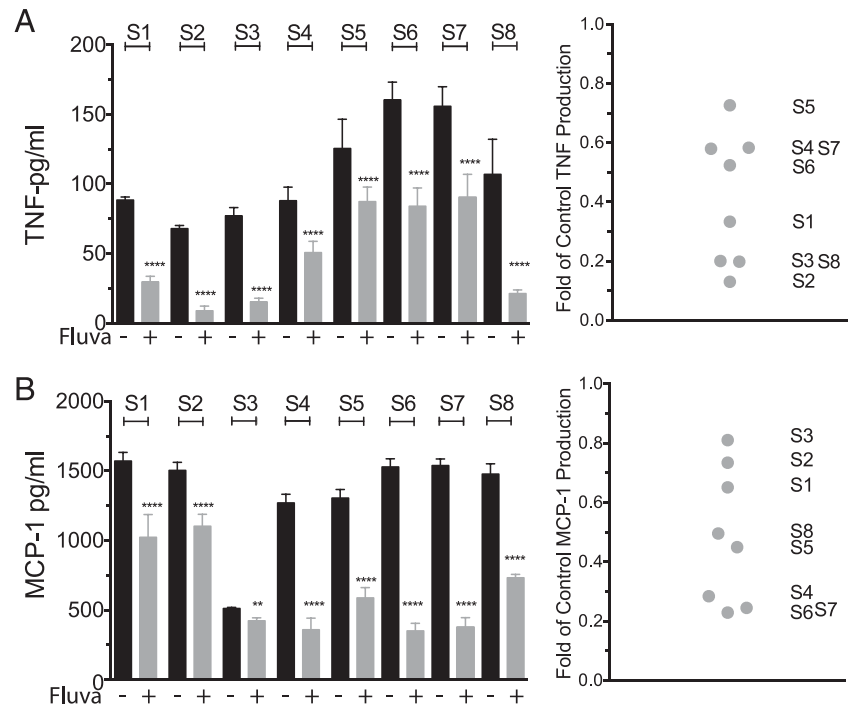
Because statins target HMGCR, we initially postulated that the genetic variation between the C57BL/6J and 129/SvImJ strains was a polymorphism or steady-state alteration in HMGCR expression between strains. Instead, we found that 129/SvImJ MCs increase HMGCR expression after fluvastatin treatment, which correlated with resistance to fluvastatin-mediated decrease in cellular cholesterol. Importantly, statin-induced HMGCR upregulation has been noted by others. For example, Ness and coworkers (35) found that HMGCR levels are significantly increased in rats fed atorvastatin. Lastly, GGTI suppressed Fc ϵ RI-mediated IL-6 production in 129/SvImJ MCs, demonstrating that these cells can be

suppressed by targeting enzymatic steps subsequent to HMGCR. We therefore propose that increased HMGCR is at least partly responsible for fluvastatin resistance in 129/SvImJ mice.

It is important to state that other explanations for fluvastatin resistance are possible and warrant further study. There is wide variation in statin responsiveness among patients, possibly because of several genetic differences (36). Clinical studies have largely focused on cytochrome P450 enzyme polymorphisms, the lipid metabolism genes apolipoprotein E and B, cholesteryl ester transfer protein, and the LDL receptor (37). For example, the P450 subunit Cyp2c9 is the primary pathway for fluvastatin metabolism (38). It might be that variants of the Cyp2c9 gene also contribute to fluvastatin resistance. However, the mouse Cyp2c9 orthologs Cyp2c65/66 have no coding region polymorphisms between C57BL/6J and 129/SvImJ strains on Jackson Lab's Phenome database. Although Cyp2c9 polymorphisms may not explain our murine data, variations among human donors are not known, not mutually exclusive with altered HMGCR induction, and should be further examined.

The variability in fluvastatin responsiveness seen in murine MCs was corroborated by 18–87% suppression among human MC cultures, when measuring Fc ϵ RI-mediated cytokine secretion. Even though larger numbers of donors are needed to corroborate these findings, these data demonstrate the possible utility of statins for treating MC-associated disease, but also the inherent variability

FIGURE 8. Human MCs show varied responses to fluvastatin. Human skin MCs from eight subjects were cultured with DMSO or fluvastatin as described in Fig. 1A. Sixteen hours after Ag stimulation, culture supernatants were analyzed for the presence of TNF (A) or MCP-1 (B) by ELISA. Scatterplot shows the normalized fold of DMSO control for each subject. Each supernatant was analyzed in sextuplicate for each subject. Data are means $\pm$ SEM, analyzed by paired *t* test comparing vehicle with fluvastatin treatment. ***p* < 0.01, *****p* < 0.0001.



that should be anticipated. It is possible that statin-induced HMGCR expression could be used to screen for statin responsiveness. Understanding how statins alter IgE-mediated signaling, and the significance of genetic background, could prove to be clinically useful. Presently, a simplistic interpretation of our data would be that statins may be less efficacious among Th2-prone individuals. Our data also suggest that geranylgeranyl transfer inhibitors might be therapeutically useful, especially among statin-resistant populations. However, given the complexity of the cholesterol synthesis cascade, further study of correlations between statin-mediated MC suppression and polymorphisms is warranted and may provide insight for novel therapies.

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Disclosures

The authors have no financial conflicts of interest.

References

- Liao, J. K. 2002. Isoprenoids as mediators of the biological effects of statins. *J. Clin. Invest.* 110: 285–288.
- Fujimoto, M., T. Oka, T. Murata, M. Hori, and H. Ozaki. 2009. Fluvastatin inhibits mast cell degranulation without changing the cytoplasmic Ca²⁺ level. *Eur. J. Pharmacol.* 602: 432–438.
- Liao, J. K., and U. Laufs. 2005. Pleiotropic effects of statins. *Annu. Rev. Pharmacol. Toxicol.* 45: 89–118.
- Vaughan, C. J., M. B. Murphy, and B. M. Buckley. 1996. Statins do more than just lower cholesterol. *Lancet* 348: 1079–1082.
- Bustos, C., M. A. Hernández-Presa, M. Ortego, J. Tuñón, L. Ortega, F. Pérez, C. Díaz, G. Hernández, and J. Egido. 1998. HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in a rabbit model of atherosclerosis. *J. Am. Coll. Cardiol.* 32: 2057–2064.
- Weber, C., W. Erl, K. S. C. Weber, and P. C. Weber. 1997. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. *J. Am. Coll. Cardiol.* 30: 1212–1217.
- Tousoulis, D., C. Psarros, M. Demosthenous, R. Patel, C. Antoniadis, and C. Stefanadis. 2014. Innate and adaptive inflammation as a therapeutic target in vascular disease: the emerging role of statins. *J. Am. Coll. Cardiol.* 63: 2491–2502.
- Pahan, K., F. G. Sheikh, A. M. Namboodiri, and I. Singh. 1997. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J. Clin. Invest.* 100: 2671–2679.
- Fernando, J., T. W. Faber, N. A. Pullen, Y. T. Falanga, E. M. Kolawole, C. A. Oskertizian, B. O. Barnstein, G. Bandara, G. Li, L. B. Schwartz, et al. 2013. Genotype-dependent effects of TGF- β 1 on mast cell function: targeting the Stat5 pathway. *J. Immunol.* 191: 4505–4513.
- Kambe, N., M. Kambe, J. P. Kochan, and L. B. Schwartz. 2001. Human skin-derived mast cells can proliferate while retaining their characteristic functional and protease phenotypes. *Blood* 97: 2045–2052.
- Wijesinghe, D. S., J. C. Allegood, L. B. Gentile, T. E. Fox, M. Kester, and C. E. Chalfant. 2010. Lipidomics and Bioactive Lipids: Mass-Spectrometry-Based Lipid Analysis. *J. Lipid Res.* 51: 641–651.
- McDonald, J. G., B. M. Thompson, E. C. McCrum, and D. W. Russell. 2007. Extraction and analysis of sterols in biological matrices by high performance liquid chromatography electrospray ionization mass spectrometry. In *Lipidomics and Bioactive Lipids: Mass-Spectrometry Based Lipid Analysis*, Vol. 432. H. A. Brown, ed. Academic Press, New York, p. 145–170.
- Deanin, G. G., J. L. Cutts, J. R. Pfeiffer, and J. M. Oliver. 1991. Role of isoprenoid metabolism in IgE receptor-mediated signal transduction. *J. Immunol.* 146: 3528–3535.
- Endo, A., M. Kuroda, and Y. Tsujita. 1976. ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterol synthesis produced by *Penicillium citrinum*. *J. Antibiot.* 29: 1346–1348.
- Endo, A., Y. Tsujita, M. Kuroda, and K. Tanzawa. 1977. Inhibition of cholesterol synthesis in vitro and in vivo by ML-236A and ML-236B, competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Eur. J. Biochem.* 77: 31–36.
- Tobert, J. A. 2003. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. *Nat. Rev. Drug Discov.* 2: 517–526.
- Kambayashi, T., and G. A. Koretzky. 2007. Proximal signaling events in Fc epsilon RI-mediated mast cell activation. *J. Allergy Clin. Immunol.* 119: 544–552, quiz 553–554.
- Majlesi, Y., P. Samorapoompichit, A. W. Hauswirth, G.-H. Scherthaner, M. Ghannadan, M. Baghestanian, A. Rezaie-Majd, R. Valenta, W. R. Sperr, H.-J. Bühring, and P. Valent. 2003. Cerivastatin and atorvastatin inhibit IL-3-dependent differentiation and IgE-mediated histamine release in human basophils and downmodulate expression of the basophil-activation antigen CD203c/E-NPP3. *J. Leukoc. Biol.* 73: 107–117.
- Hamelin, B. A., and J. Turgeon. 1998. Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. *Trends Pharmacol. Sci.* 19: 26–37.
- Serajuddin, A. T. M., S. A. Ranadive, and E. M. Mahoney. 1991. Relative lipophilicities, solubilities, and structure-pharmacological considerations of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors pravastatin, lovastatin, mevastatin, and simvastatin. *J. Pharm. Sci.* 80: 830–834.
- Ryan, J. J., M. Kashyap, D. Bailey, S. Kennedy, K. Speiran, J. Brenzovich, B. Barnstein, C. Oskertizian, and G. Gomez. 2007. Mast cell homeostasis: a fundamental aspect of allergic disease. *Crit. Rev. Immunol.* 27: 15–32.
- Couper, K. N., D. G. Blount, and E. M. Riley. 2008. IL-10: the master regulator of immunity to infection. *J. Immunol.* 180: 5771–5777.
- Ness, G. C., Z. Zhao, and R. K. Keller. 1994. Effect of squalene synthase inhibition on the expression of hepatic cholesterol biosynthetic enzymes, LDL

- receptor, and cholesterol 7 α hydroxylase. *Arch. Biochem. Biophys.* 311: 277–285.
24. Samson, K. T. R., K. Minoguchi, A. Tanaka, N. Oda, T. Yokoe, Y. Yamamoto, M. Yamamoto, S. Ohta, and M. Adachi. 2006. Inhibitory effects of fluvastatin on cytokine and chemokine production by peripheral blood mononuclear cells in patients with allergic asthma. *Clin. Exp. Allergy* 36: 475–482.
 25. Hothersall, E. J., R. Chaudhuri, C. McSharry, I. Donnelly, J. Lafferty, A. D. McMahon, C. J. Weir, J. Meiklejohn, N. Sattar, I. McInnes, et al. 2008. Effects of atorvastatin added to inhaled corticosteroids on lung function and sputum cell counts in atopic asthma. *Thorax* 63: 1070–1075.
 26. Lokhandwala, T., D. West-Strum, B. F. Banahan, J. P. Bentley, and Y. Yang. 2012. Do statins improve outcomes in patients with asthma on inhaled corticosteroid therapy? A retrospective cohort analysis. *BMJ Open* 2: e001279.
 27. Kwak, B., F. Mulhaupt, S. Myit, and F. Mach. 2000. Statins as a newly recognized type of immunomodulator. *Nat. Med.* 6: 1399–1402.
 28. Michalik, M., E. Soczek, M. Kosińska, M. Rak, K. A. Wójcik, S. Lasota, M. Pierzchalska, J. Czyż, and Z. Madeja. 2013. Lovastatin-induced decrease of intracellular cholesterol level attenuates fibroblast-to-myofibroblast transition in bronchial fibroblasts derived from asthmatic patients. *Eur. J. Pharmacol.* 704: 23–32.
 29. Xu, L., X.-W. Dong, L.-L. Shen, F.-F. Li, J.-X. Jiang, R. Cao, H.-Y. Yao, H.-J. Shen, Y. Sun, and Q.-M. Xie. 2012. Simvastatin delivery via inhalation attenuates airway inflammation in a murine model of asthma. *Int. Immunopharmacol.* 12: 556–564.
 30. McKay, A., B. P. Leung, I. B. McInnes, N. C. Thomson, and F. Y. Liew. 2004. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J. Immunol.* 172: 2903–2908.
 31. Kim, D. Y., S. Y. Ryu, J. E. Lim, Y. S. Lee, and J. Y. Ro. 2007. Anti-inflammatory mechanism of simvastatin in mouse allergic asthma model. *Eur. J. Pharmacol.* 557: 76–86.
 32. Weitz-Schmidt, G., K. Welzenbach, V. Brinkmann, T. Kamata, J. Kallen, C. Bruns, S. Cottens, Y. Takada, and U. Hommel. 2001. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat. Med.* 7: 687–692.
 33. Falanga, Y. T., N. S. Chaimowitz, N. Charles, F. D. Finkelman, N. A. Pullen, S. Barbour, K. Dholaria, T. Faber, M. Kolawole, B. Huang, et al. 2012. Lyn but not Fyn kinase controls IgG-mediated systemic anaphylaxis. *J. Immunol.* 188: 4360–4368.
 34. Speiran, K., D. P. Bailey, J. Fernando, M. Macey, B. Barnstein, M. Kolawole, D. Curley, S. S. Watowich, P. J. Murray, C. Oskeritzian, and J. J. Ryan. 2009. Endogenous suppression of mast cell development and survival by IL-4 and IL-10. *J. Leukoc. Biol.* 85: 826–836.
 35. Ness, G. C., C. M. Chambers, and D. Lopez. 1998. Atorvastatin action involves diminished recovery of hepatic HMG-CoA reductase activity. *J. Lipid Res.* 39: 75–84.
 36. Chasman, D. I., D. Posada, L. Subrahmanyam, N. R. Cook, V. P. Stanton, Jr., and P. M. Ridker. 2004. Pharmacogenetic study of statin therapy and cholesterol reduction. *JAMA* 291: 2821–2827.
 37. Schmitz, G., and W. Drobnik. 2003. Pharmacogenomics and pharmacogenetics of cholesterol-lowering therapy. *Clin. Chem. Lab. Med.* 41: 581–589.
 38. Kajinami, K., M. E. Brousseau, J. M. Ordovas, and E. J. Schaefer. 2004. CYP3A4 genotypes and plasma lipoprotein levels before and after treatment with atorvastatin in primary hypercholesterolemia. *Am. J. Cardiol.* 93: 104–107.

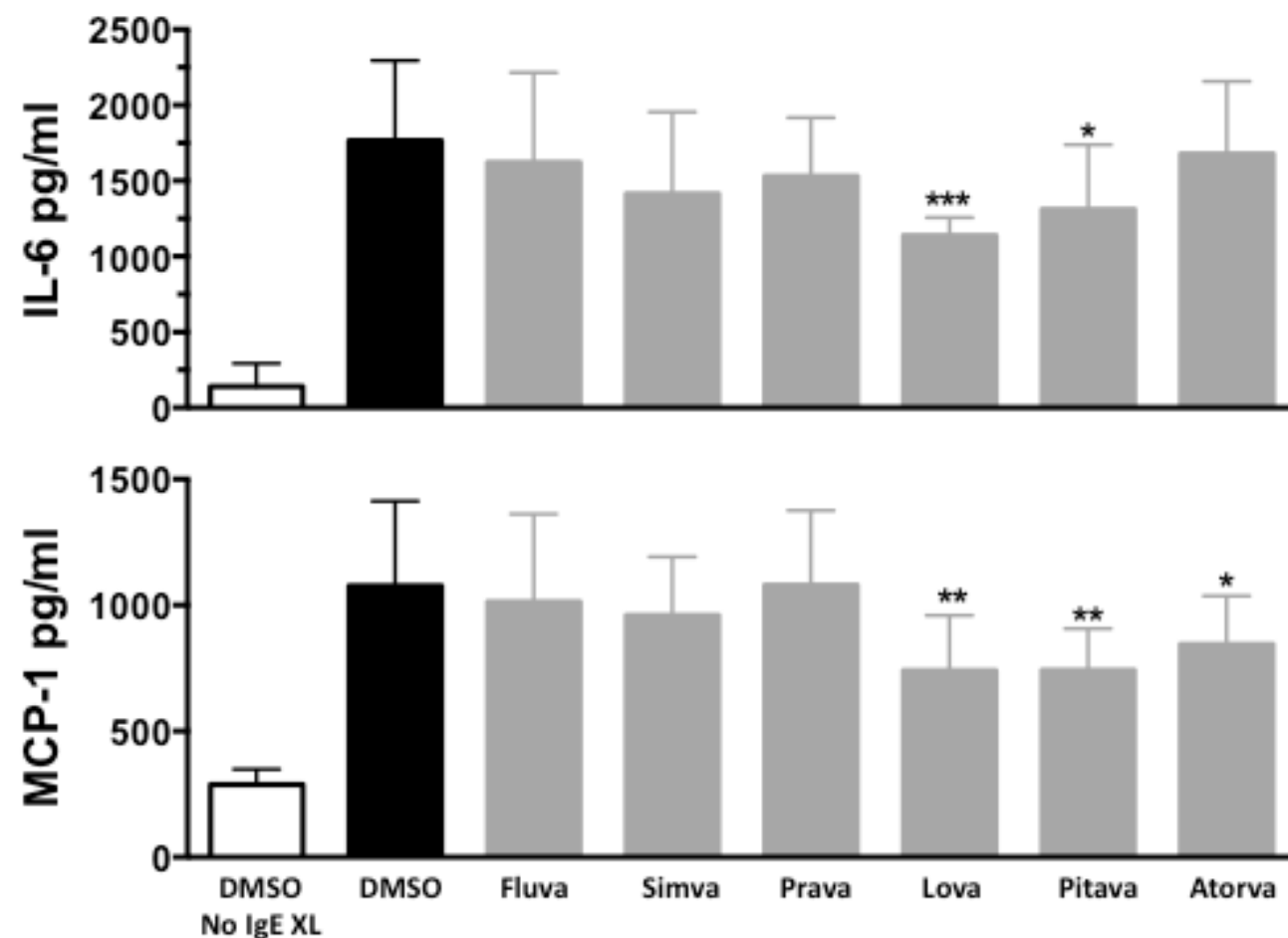


Figure S1. 129/SvImJ BMMC are resistant to several statin drugs.

129/SvImJ BMMC were cultured for 24 hours in the indicated statins (10 μ M) and activated with IgE+antigen (IgE XL) as described in Figure 1. Data are means and SD from 5 populations analyzed in triplicate, from two independent experiments.

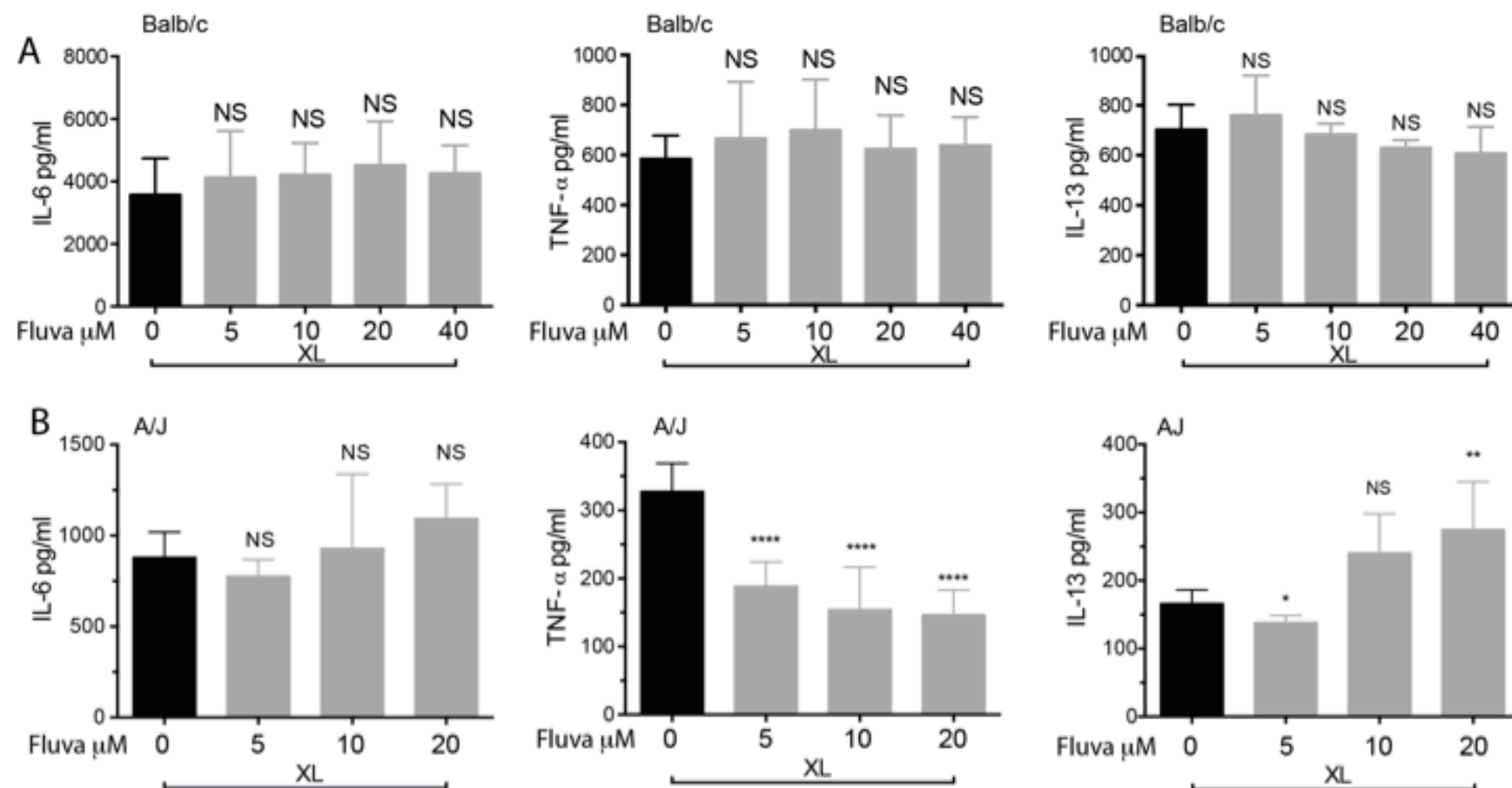


Figure S2. Varied responses to fluvastatin among mouse strains.

BMMC from the indicated mouse strains were cultured and activated as described in Figure 1. Culture supernatants were assayed by ELISA. Data are means and SEM of (A) three independent experiments and (B) three sample.