Theses and Dissertations   Graduate School

2015

The Effect of Statins on IL-33 Mediated Mast Cell Function

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The Effect of Statins on IL-33 Mediated Mast Cell Function

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

in Biology at Virginia Commonwealth University

By

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Bachelor of Science in Biology

Virginia Commonwealth University, 2015

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Richmond, Virginia

July 31st, 2015

Acknowledgement

First I would like to thank my mentor Dr. John Ryan. His passion and immense knowledge of immunology is truly inspiring, while his support, guidance and patience makes him the ideal mentor for a graduate student. I also would like to thank my committee members Dr. Daniel Conrad, Dr. Jennifer Stewart, Dr. Amanda Dickinson and Dr. David Strauss for helping me complete my thesis and their guidance on my research. Thank you to all of the Ryan lab members for their support, assistance, friendship and acting as a reality check. Additionally, thanks to Dr. Motunrayo Kolawole for helping me throughout this project and serving as a filter for all my ideas. I would also like to thank Dr. William Eggleston and his laboratory members Jennifer Derkits and Robert Lindsey for sparking my interest in research and training me. Thank you to my parents Dayse and Ruben Taruselli, and my sibling Bianca and Felipe for their love and support. I would like to thank my boyfriend Jeffrey Vu, whose love and support is instrumental to my life. Finally, to my cat Sophie Vu, thank you for being by my side, literally.

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List of Abbreviations

Akt- Protein kinase B, serine/threonine-specific protein kinase

BMMC- Bone marrow-derived mast cell

BHK- SCF-producing BHK-MKL cell line

c-Kit- SCF receptor

DMSO- Dimethyl sulfoxide, vehicle for statins

ERK- Extracellular signal regulated kinases

FPTIII- Farnesyl transferase inhibitor

GGTI- inhibitor of geranylgeranyltransferase I

HMGCR- HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase), target of statin drugs

IgE- Immunoglobulin E

IgG- Immunoglobulin G

IL-3- Interleukin 3

IL-6- Interleukin 6

IL-33- Interleukin 33

IL-33Ra- IL-33 receptor, ST2

KLF2- Krüppel-like factor 2, a zinc finger transcription factor

MAP kinases - Mitogen-activated protein kinases

MCP-1- Monocyte chemotactic protein 1

NFκB- Nuclear factor κB

P38- a mitogen-activated protein kinases

P65- a subunit of NFκB

SCF- Stem cell factor, mitogen

TNF- Tumor necrosis factor α

WEHI- IL-3 producing cells

ABSTRACT

THE EFFECT OF STATINS ON IL-33 ACTIVATED MAST CELLS

Marcela Taruselli, Bachelor of Science in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University, 2015.

Director: John J. Ryan, Ph.D., Professor of Biology

This study demonstrates original findings of statin effects on IL-33 stimulated mast cells. Statins are a class of drugs used to lower cholesterol production by targeting HMG CoA reductase. These commonly prescribed drugs have been shown to be immunomodulatory. In this study, we have found that pretreatment with statins has a variety of effects on IL-33 stimulated mast cells. Atorvastatin suppresses TNF and IL-6 production, while fluvastatin significantly enhances release of these proinflammatory cytokines in BMMCs. Although they have differing effects on cytokine production, both statins lowered ST2 expression on the cell surface, decreased cell viability, and enhanced expression of the transcription factor KLF2, a negative regulator of NFκB. Blocking isoprenylation by using geranylgeranyl transferase inhibitor, but not farnesyl transferase, mimicked the effects of atorvastatin, while neither mirrored the effect of fluvastatin. Furthermore, fluvastatin effects were not reversed by mevalonic acid, the product of HMG-CoA reductase. These data indicate that fluvastatin effects are distinct from its activities as

an HMG CoA reductase inhibitor. Fluvastatin effects required the presence of stem cell factor (SCF), and were enhanced by increasing SCF concentrations. Finally, fluvastatin enhanced IL-33-induced cytokine production and neutrophil recruitment in vivo. Collectively, these data suggest that statins can alter the mast cell response, and that drug choice can have divergent effects on outcome.

INTRODUCTION

Mast cells are an integral member of the innate immune system. As a tissue resident cell, they are first responders to skin antigen. Although an important member of innate immunity, they also play an effector role in the adaptive immune system by influencing other cells(1).

Mast cells participate in responses to parasite infection, venom, infectious agents, and autoimmune inflammation (1)(2)(3)(4), although they are more infamously known for their role in allergic disease and asthma. In allergic disease mast cells are activated by antigen interactions with surface-bound IgE. This results in release of early mediators from preformed granules such as histamine, lipid metabolites and proteases, and late mediators requiring new synthesis, such as leukotriene, prostaglandins, cytokines and chemokines (5)(6). Collectively, these elicit itching, immune cell recruitment, vasodilation and edema, and bronchoconstriction,

The lesser investigated ways mast cells can be activated is through other Fc receptors, G-protein coupled receptors, mechanical stimuli, chemokines and cytokines. One newly discovered cytokine that can induce proinflammatory mast cell activation is interleukin 33 (IL-33). IL-33 is an IL-1 family member that acts as an alarmin. Most abundantly expressed by endothelial and epithelial cells, the IL-1 family member is commonly stored in the nucleus of cells and is released upon cell damage or secreted after activation (7). IL-33 activates immune cells by binding to the ST2 receptor, recruiting IL-RacP into the heterodimer, and signaling via MyD88, NFκB, and the MAP kinase (8). Th2, ILC2, basophils, monocytes, and mast cells can be

activated by IL-33 (9). IL-33 has been implicated in a number of diseases and ailments such as cardiac stress, tumor environment, autoimmune disorders, asthma and allergic diseases (8)(10)(11)(12) (13)(14).

IL-33 induces mast cell adhesion, maturation, possibly degranulation, and release of the cytokines IL-13, IL-6, TNF, MIP-1α, MCP-1, IL-1β, IL-4, IL-5 and IL-8 (8)(15)(16)(17)(18). Due to the ability of IL-33 to activate mast cells, it is important to find ways of altering mast cell activation via this alarmin.

Statins are a prevalent class of drug worldwide, used as mainstay therapy for hypercholesterolemia and cardiovascular disease. By targeting HMG-CoA reductase (HMGCR) through competitive inhibition, statins decrease mevalonate production and subsequently cholesterol production(19). Also downstream of HMGCR is the isoprenylation of the small G proteins that are important for cell viability and function, such as Ras, Rac and Rho (20) (Figure 1).

Statins have previously been shown to be immunosuppressive. T cells and monocytes have been shown to have diminished responses to stimuli and decreased migration when treated with statins (21)(22)(23). In mast cells, fluvastatin has been shown to prevent IgE-mediated degranulation (24). Previously in our lab, we have found fluvastatin inhibits IgE-induced proinflammatory cytokine production in BMMCs and decreases the severity systemic anaphylaxis in a mouse model (25). The effects were shown to be due to the blockade of geranylgeranyl transferase. Since statins have been shown to be a potent suppressor of proinflammatory functions of IgE activated mast cells, we decided to investigate the effect of statins on IL-33 activation.

MATERIALS AND METHODS

Animals

Mice of the C57BL/6 strain were used at a minimum of 12 weeks old and purchased from The Jackson Laboratory (Bar Harbor, ME), with approval from the Virginia Commonwealth University institutional animal care and use committee (IACUC).

Mouse Mast Cell Cultures

Mouse bone marrow-derived mast cells (BMMCs) were derived by collecting bone marrow from the C57BL/6 mouse femurs, followed by culturing the stem cells in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing the following purchased from Corning, (Corning, NY): 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1mM sodium pyruvate, and 1mM HEPES. Cultures were supplemented with supernatant from WEHI-3B cells and supernatant from BHK-MKL cells which contain IL-3 and SCF respectively. The final concentration of IL-3 and SCF were adjusted to 1 ng/ml and 10 ng/ml, respectively, as measured by ELISA. BMMCs were used after 21 days of culture.

Cytokines and reagents

The cytokines IL-33, IL-3 and SCF were purchased from Biolegend (San Diego, CA). Mevalonic acid (44714) pravastatin (P4498), lovastatin (PHR1285), simvastatin (S6196), atorvastatin (PZ0001) and fluvastatin (SML0038, were purchased from Sigma-Aldrich. Pitvastatin (147526-32-7) was purchased from Tocris Bioscience (Bristol, UK). Farnesylation transferase inhibitor III (FPTIII) and geranylgeranyl transferase inhibitor-286 (GGTI-286) were purchased from Calbiochem (Darmstadt, Germany). APC-coupled anti-mouse IL-33Ra, PE-coupled anti-mouse CD4, FITC-coupled -coupled anti-mouse CD8, PE-coupled anti-mouse Ly6G, APC-coupled anti-mouse CD49b, PE-coupled anti-mouse CD117, APC-coupled rat IgG1, PE-coupled rat IgG1 and FITC-coupled rat IgG1 isotype control were purchased from Biolegend (San Diego, CA). FITC-coupled rat anti-mouse T1/ST2, and purified rat anti-mouse CD16/CD32 were purchased from BD Biosciences (San Jose, CA). Propidium Iodide was purchased from Sigma-Aldrich (St. Louis, MO).

Cytokine measurement

BMMCs were washed twice (PBS), then resuspended in cRPMI 1640 at a concentration of 1×10^6 cells/ml with IL-3 (10 ng/ml) and indicated concentrations of SCF. Activation of cultures with IL-33 (100 ng/mL) occurred for 16 hours at 37°C. After stimulation, supernatant was collected and measured for IL-6 and TNF levels using ELISA kits from Biolegend (San Diego, CA) and developed using BD OptEIA regeants from BD Biosciences (Franklin Lakes, NJ).

Flow cytometric analysis

For surface staining, cells were cultured at 1×10^6 cells/ml in cRPMI with the indicated treatments prior to staining. Afterwards, cells were washed twice, pelleted and resuspended in PBS containing 3% fetal calf serum and 0.1% sodium azide (FACS Buffer) and the indicated antibodies and anti-CD16/32 clone 2.4G2 (to reduce background staining) (BD Biosciences, Franklin Lakes, NJ). Samples were incubated at 4° C for 45 minutes, washed twice with PBS, and resuspended in 200 µl of PBS. Samples were then analyzed using a on a BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ) to assess the expression of surface molecules.

For cell viability staining, cells were cultured at 1×10^6 cells/ml in cRPMI with the indicated treatments prior to staining. Cells were transferred into FACS tubes at 2×10^5 cells and stained with 10 µl of propidium iodide immediately before analysis on a BD FACSCalibur.

Western Blotting

Cells were cultured at 1×10^6 cells/ml in cRPMI with IL-3 (10ng/ml) and SCF (50ng/ml) with 20µM of fluvastatin or vehicle control for 24 hours. Cultures were then resuspended at 3×10^6 cells/ml and starved for 2 hours of serum and cytokines in cRPMI lacking fetal calf serum. Cultures were then activated with IL-33 (100 ng/ml) and SCF (50 ng/ml) simultaneously, and lysates were collected using Lysis Buffer (Cell Signaling Technology, Danver, MA) containing 1.5x ProteaseArrest (G-Biosciences, Maryland Heights, MO). The Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA) was used to determine protein concentrations. Lysates were separated on 4-20% Mini-Protean TGX Gels (Bio-Rad, Hercules, CA) followed by transfer onto nitrocellulose membrane. Casein Blocking Buffer (LI-COR, Lincoln, NE) was used for 1 hour at room temperature to block the membranes. Membranes were incubated overnight at 4°C in primary antibody used at the manufacturers' protocol (Cell Signaling Technology, Danver, MA)

and 0.1% TWEEN in TBS (TBS-T_ containing 1% casein. Membranes were washed the next

day with TBS-T several times before incubation with secondary goat antibodies against anti-

mouse and anti-rabbit IgG (LI-COR, Lincoln, NE). Membranes were imaged on an Odyssey CLx

infrared scanner (Li-Cor, Lincoln, Nebraska).

Krüppel-like factor 2 qPCR

RNA was harvested from BMMC cultured with fluvastatin (20 µM), atorvastatin

(20µM), or vehicle using TRIzol reagent (Life Technologies, Grand Island, NY). Following

RNA extraction, cDNA was produced using the qScript RNA cDNA Synthesis Kit (Quanta

biosciences, Gaithersburg, MD) following maufactures' protocol. Amplification and qPCR

analysis was conducted using Bio Rad CFX96 Touch™ Real-Time PCR Detection System

(Hercules, CA) and SYBR® Green detection. The reactions performed contained 8 ng of cDNA,

12.5 µl of PerfeCTa SYBR Green SuperMix (Quanta biosciences, Gaithersburg, MD) and KLF2

or β-actin primers (Quanta biosciences, Gaithersburg, MD). Amplification occurred under the

following conditions: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 57 °C for 30 s and

70 °C for 1 min. KLF2 RNA levels in each sample were normalized to the level of β-actin

(housekeeping gene) mRNA.

Measurement of neutrophil recruitment and cytokines in vivo

C57BL/6J mice were injected via intraperitoneal (i.p.) with PBS (200 µl) or fluvastatin

(1mg/mouse) 16 hrs and 1 hr prior to i.p. injection with 1 µg of IL-33. Four hours later,

peritoneal lavage and cardiac puncture were performed. Peritoneal cells were stained with

antibodies against CD4, CD8, Ly6G and CD49b prior to analysis by flow cytometry for surface

markers. Plasma cytokines levels were analyzed by ELISA.

Statistics

Data presented are the mean ± SEM. P values were calculated with GraphPad Prism

software by paired or unpaired, two-tailed Student's t Test when comparing 2 samples. For

comparison of 3 or more samples, ANOVA was performed followed by Tukey's or Dunnet's

test. P values of <0.05 were considered statistically significant.

RESULTS

Statins effects on IL-33 activated BMMCs

There have been many studies that demonstrate statins having immunosuppressive effects. Our lab has previously shown a difference in efficacy of statins on suppressing IgE-activated mast cells. Fluvastatin demonstrated the strongest suppression of pro-inflammatory cytokines compared to the other statins, while atorvastatin had no effect on C57BL/6 BMMCs (25). To examine the effect statins had on IL-33 stimulated mast cells, C57BL/6 BMMCs were cultured with a variety of statins (atorvastatin, fluvastatin, simvastatin, pravastatin, lovastatin, pitvastatin) for 24 hours before stimulation (Figure 2). Unlike IgE activation, fluvastatin enhanced TNF and IL-6 production from IL-33-activated BMMCs. Lovastatin and atorvastatin suppressed IL-6 and TNF production, while the remaining statins had no effect on cytokine production.

Atorvastatin suppresses pro-inflammatory cytokine production.

A dose response and time course was conducted to determine the kinetics of atorvastatin effects on IL-33-induced pro-inflammatory cytokine production. BMMCs were treated with atorvastatin with doses ranging from 5-20 μM (Figure 3) or 20 μM for varying times (Figure 4) before activation with IL-33. The data showed that 20 μM was the most effective dose for

suppressing cytokine production, and that longer pretreatments of the drug elicited greater suppression of IL-33 activation.

Atorvastatin effects on receptor expression and cell death.

To examine if the effect of atorvastatin is due to cell death and/or altered receptor expression, we cultured BMMCs for up to three days in atorvastatin or vehicle control and measured receptor expression (Figure 5) and cell death (Figure 6). After two days of culture in atorvastatin, the expression of the IL-33 receptor ST2 and percentage of live cells was slightly but significantly lower than the cells cultured in vehicle. The expression of the SCF ligand, c-Kit, was not affected by atorvastatin. Because the effects on ST2 and cell viability were modest, they appear to be an unlikely explanation for the change in cytokine production.

GGTI but not FPTII suppresses pro-inflammatory cytokines from IL-33 activated mast cells.

HMG CoA reductase is several steps apical to cholesterol production. Along the cholesterol pathway there are reactions generating isoprenoid lipids. Previous studies have found that geranyl geranyl transferase (GGT) inhibition is key to statin immunomodulatory effects (25)(24)(20). To test if the inhibition of the isoprenoid transferases can mimic the effects of atorvastatin, BMMCs were cultured with the GGT inhibitor, GGTI, and/or the farnesyl transferase inhibitor, FPTII, for 24 hours before IL-33 stimulation. GTTI inhibited IL-6 and TNF production, mimicking atorvastatin, while FPTII had no effect on cytokine production (Figure 7). The results suggest that atorvastatin effects may be due to geranyl geranyl transferase inhibition.

The effects of fluvastatin on bone marrow derived mast cells.

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To determine the effects of fluvastatin on different stimuli, cultures pretreated with fluvastatin were activated with IL-33 or IgE crosslinking. Fluvastatin enhanced IL-33-mediated production of the pro-inflammatory cytokines IL-6 and TNF, while suppressing their production in IgE-stimulated BMMCs (Figure 8). The enhancement of cytokines seems to be cytokine specific, as fluvastatin did not exhibit consistent effects on MCP-1 or IL-13 production (data not shown).

To understand fluvastatin kinetics, BMMCs were cultured with varying doses of fluvastatin (5-20 μM) for 24 hours (Figure 9) or pretreated with 20 μM for varying times (Figure 10) before activation with IL-33. Fluvastatin effects were dose- and time-dependent, with the dose of 20 μM and pretreatment for 24 hours eliciting the largest amount of TNF and IL-6 production.

SCF is needed for the enhancement of pro-inflammatory cytokines via fluvastatin.

Previous research has found that statins can stimulate NK cells to produce pro inflammatory cytokines when cultured with the mitogen IL-2 (26). The mast cell mitogen SCF has been shown to enhance IL-33 production of cytokines and phosphorylation events downstream of the IL-33 receptor in HMC1 cells (25, 27). To test if SCF co-stimulation contributes to fluvastatin effects, BMMCs were culture with fluvastatin and varying amounts of SCF (0-100 ng/ml) for 24 hours (Figure 11) or cultured with 50 ng/ml of SCF for varying times (0-24 hrs) before treatment with fluvastatin (Figure 12) followed by activation with IL-33. Fluvastatin-mediated augmentation of IL-6 and TNF plateaued at 50 ng/ml SCF, and did not occur in the absence of SCF. Also, prolonging the exposure to SCF increased the fluvastatin

effects. Therefore the mitogen SCF plays a critical role in fluvastatin's augmenting effects on mast cells.

Cell receptor expression and survival is affected by fluvastatin.

To examine if the effect of fluvastatin is due to increased cell viability and/or altered receptor expression, we cultured BMMCs for up to three days in fluvastatin or vehicle control and measured surface receptor expression (Figure 13) and cell death (Figure 14). After two days of culture in fluvastatin, the expression of ST2 and percentage of live cells was significantly decreased when compared to the vehicle control. The SCF ligand, c-Kit, was decreased by 24 hours of culture in fluvastatin. Diminished ST2 and c-Kit expression should antagonize, not enhance IL-33-induced cytokine secretion, and are thus excluded as the fluvastatin mechanism of action.

Fluvastatin-mediated increase in cytokine production is independent of the HMG-CoA pathway.

To elucidate if fluvastatin effects are related to its known target, HMGCR, we co-cultured mast cells with fluvastatin and the product of HMGCR reaction, mevalonic acid. BMMCs were cultured with fluvastatin (20 μM) for 24 hours and mevalonic acid was added 24 and 4 hours prior to stimulation with IL-33 (Figure 15). The data show that the augmentation of cytokines by fluvastatin was not suppressed by mevalonic acid, suggesting that the ability of fluvastatin to enhance proinflammatory cytokines is an off-target effect of the statin.

Fluvastatin alters phosphorylation events in the IL-33 signaling pathway.

We next sought to investigate the effects fluvastatin has on the IL-33 signaling pathway by examining downstream phosphorylation events. Cell lysates from mast cells cultured with or without fluvastatin 24 hrs prior to activation with IL-33 and SCF were analyzed by western blot (Figure 16). In this experiment, phosphorylation of p65 and Akt were slightly elevated, while phosphorylation of MAP kinase ERK was suppressed in fluvastatin treated cells in comparison to vehicle control. Phosphorylation of p38 was not affected by treatment with fluvastatin. These data show that fluvastatin may alter signaling events in IL-33 stimulated BMMCs, but further testing is needed due to the sample size.

Krüppel-like factor 2 is enhanced by fluvastatin and atorvastatin.

While searching for a mechanism by which statins exert their effects, we noted that previous research found that statins induce Krüppel-like factor 2 (KLF2)(28). To test if statins are enhancing expression of KLF2 in IL-33-stimulated BMMCs, we cultured mast cells in atorvastatin or fluvastatin for 12 hours, then activated with IL-33 for 90 minutes before RNA collection (Figure 17). Our data demonstrate that fluvastatin and atorvastatin both enhance KLF2 message in both unstimulated and IL-33 stimulated BMMCs. Atorvastatin increased expression in stimulated BMMCs ~5 fold while fluvastatin enhanced KLF2 about 10 fold.

Fluvastatin alters IL-33 functions in vivo.

It was previously found that mice injected with IL-33 I.P. have peritoneal neutrophil recruitment. The mouse model found that this recruitment was mast cell dependent via the production of TNFα(29). To test if fluvastatin could alter mast cell function in vivo, we injected mice with fluvastatin 16 and 1 hour prior to IL-33 injection. Cardiac puncture for plasma

cytokines (Figure 18) or peritoneal lavage for cell population (Figure 19) was performed. Our data found that fluvastatin significantly enhanced IL-33-induced IL-6, but not MCP-1 or TNF plasma levels in vivo. Fluvastatin also significantly increased IL-33-mediated recruitment of Ly6G+ neutrophils without altering the percentage of CD4+, CD8+, and CD49b+ cells in the peritoneum (Figure 19).

DISCUSSION

Although statins are only currently approved as a cholesterol lowering drug, several studies have noted their immunomodulatory effects. Statins have been indicated as a treatment for inflammatory diseases such as autoimmune disorders, asthma and cancer (30)(31)(32)(20).

Statins have already been implicated as a treatment for mast cell driven disease. Cervistatin and atorvastatin have been shown to inhibit human mast cell proliferation and IgE degranulation (33). Fluvastatin has also been shown to inhibit IgE-induced degranulation in RBL-2H3 cells, while our lab has previously demonstrated the ability of fluvastatin to inhibit mast cell IgE activation in vitro and in vivo (24)(25). We expanded out our studies to IL-33 stimulated mast cells based on these supportive data. IL-33 is a known stimulator of mast cells and has been implicated in several diseases such as asthma, cardiovascular disease, arthritis, atopic dermatitis and initiating the allergic response (8)(34). Since IL-33 is associated with inflammatory diseases and a known activator of mast cells, we examined if statins can be used as an inhibitor.

In this study we provided novel insight into the effects of statins on IL-33 stimulated mast cells. We found that statins have differing effects on IL-6 and TNF production by IL-33 stimulated mast cells. Atorvastatin and lovastatin suppressed proinflammatory cytokine production while fluvastatin enhanced production. Other statins had no effect on IL-33 stimulated BMMCs. Both fluvastatin and atorvastatin effects on IL-33 stimulated mast cells were

dose and time dependent and independent of receptor expression and cell viability. The effect of fluvastatin appears to be off-target due to the inability of mevalonic acid to reverse the augmentation.

Downstream of the statin target HMGCR, is geranylgeranyl transferase and farnesyl transferase which lead to the isoprenylation of the small G proteins Rac, Ras and Rho. These G proteins have been found to be important for cell function and proliferation (20). The blocking of geranylgeranyl transferase has been shown to be important for fluvastatin's ability to inhibit degranulation and cytokine production and can be reversed using geranylgeranyl pyrophosphate in IgE-stimulated mast cells (24)(25). We determined the effect of these transferases by pretreating cells with GGTI and FPTII. GGTI decreased IL-33-mediated cytokine secretion, while FPTII had no effect. The effect of GGTI mimicked the suppression by atorvastatin, while nether inhibitor imitated the enhancement that fluvastatin causes. These data suggest the ability of atorvastatin to suppress cytokine production is through the inhibition of geranylgeranyl transferase while farnesyl transferase inhibition does not lead to cytokine suppression. Neither of these transferases are implicated in the enhancement via fluvastatin.

The zinc finger transcription factor KLF2 has an significant role in lung development, erythropoiesis, and adipogenesis (35)(36)(37). The transcription factor has also been implicated as an important regulator of T cell maturation and migration, as the overexpression of KLF2 keeps T cells in a naïve state and prevents them from migrating (38). Recently KLF2 has been shown to regulate monocytes as well. KLF2 overexpression inhibits monocyte stimulation by inhibiting NF-κB (39). Simvastatin has been shown to enhance KLF2 in mast cells, while T cells treated with statins had induced KLF2 expression and diminished T cell function (28)(40). Due to the ability of statins to induce KLF2, and KLF2 immunosuppressive effects, we investigated

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the induction of KLF2 by statins. KLF2 expression was increased with atorvastatin pretreatment in IL-33-stimulated and unstimulated cells. Surprisingly, we found that fluvastatin had a greater enhancement of KLF2 mRNA in comparison to vehicle and atorvastatin. Due to both atorvastatin and fluvastatin enhancing KLF2, its role in statin effects on cytokine production remains unclear.

SCF plays an important role in mast cell function. The cytokine drives mast cell maturation, proliferation, migration, and enhances several signaling pathways including IL-33 (1)(41)(27). Due to statins enhancing proinflammatory in NK cells only when co-cultured with a mitogen (26), SCF was explored as a mediator of fluvastatin enhancement of proinflammatory cytokines. We found that SCF was needed for fluvastatin to demonstrate its augmentation of proinflammatory cytokines from IL-33 stimulated BMMCs. Fluvastatin also altered phosphorylation events in the IL-33 signaling pathway in cells co-stimulated with SCF, but further investigation would be needed due to the sample size.

We have also demonstrated that fluvastatin effects are consistent in vivo. Fluvastatin induced IL-6 production and enhanced the number of Ly6G+ neutrophils in our IL-33-stimulated mouse model. Previous models found that IL-33 recruitment of neutrophils is mast cell driven and TNF-dependent (29). Interestingly, we found that fluvastatin increased systemic IL-6 but not TNF, although local effects on the peritoneum were not ruled out.

These data could yield novel insights into allergic disease and cancer biology. IL-33 has recently been shown to exacerbate inflammation associated with allergic diseases such as asthma (14)(42)(13). Our data suggest that atorvastatin may help reduce allergic inflammation by antagonizing IL-33 effects. Additionally, fluvastatin has been shown to decrease tumor size and reduce proliferation in breast cancer patients (43). Also mast cells have been shown to be vital to the tumor microenvironment, as several tumors have been found to secrete SCF leading to mast

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cells being associated with them (44). IL-33 has also been found at the site of the tumor and has been implicated as useful in cancer therapy by enhancing CD8+T and NK cells responses (45)(46). The ability of fluvastatin to enhance proinflammatory cytokines in the presence of SCF may contribute to tumor inhibition, and warrants further study.

In summary, our data demonstrate novel insights into statin effects on IL-33-mediated mast cell function. These effects are completely distinct based on the statin used, ranging from no effect to either inhibitory or stimulatory. Atorvastatin may be suppressive by preventing GGT function. Fluvastatin requires SCF for its enhancing effects, and is functional in vivo. These data suggest further investigation into the on- and off- target effects of statins and their possibilities as therapeutics in a variety of diseases.
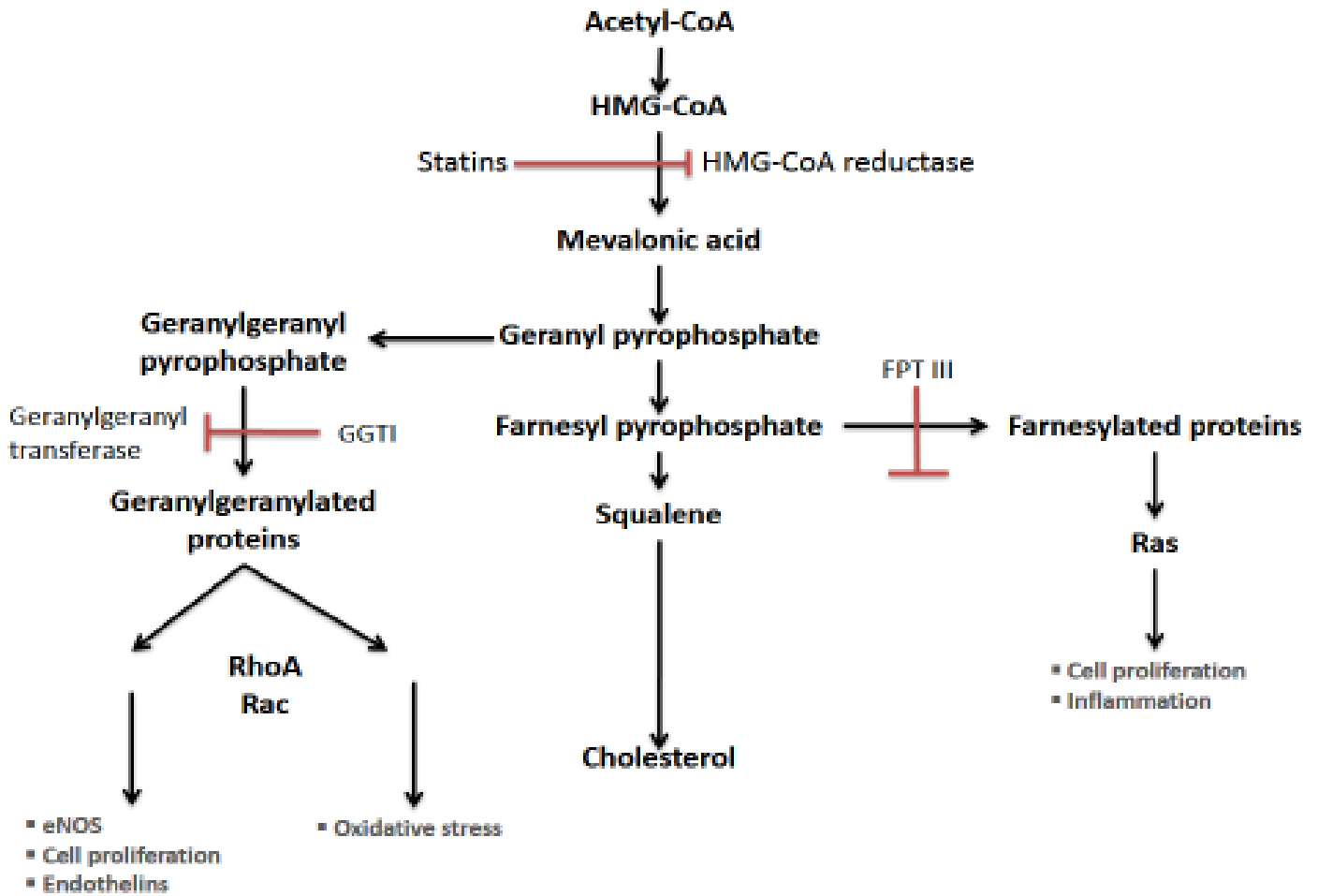
Figure 1. Statins inhibit HMG CoA reductase and downstream events in the cholesterol synthesis pathway. Statins are HMGCR inhibitors which prevent the formation of mevalonate. This subsequently inhibits the isoprenylation of the small g proteins. The inhibitors GGTI and FPT III can be used to block isoprenylation without decreasing cholesterol production.
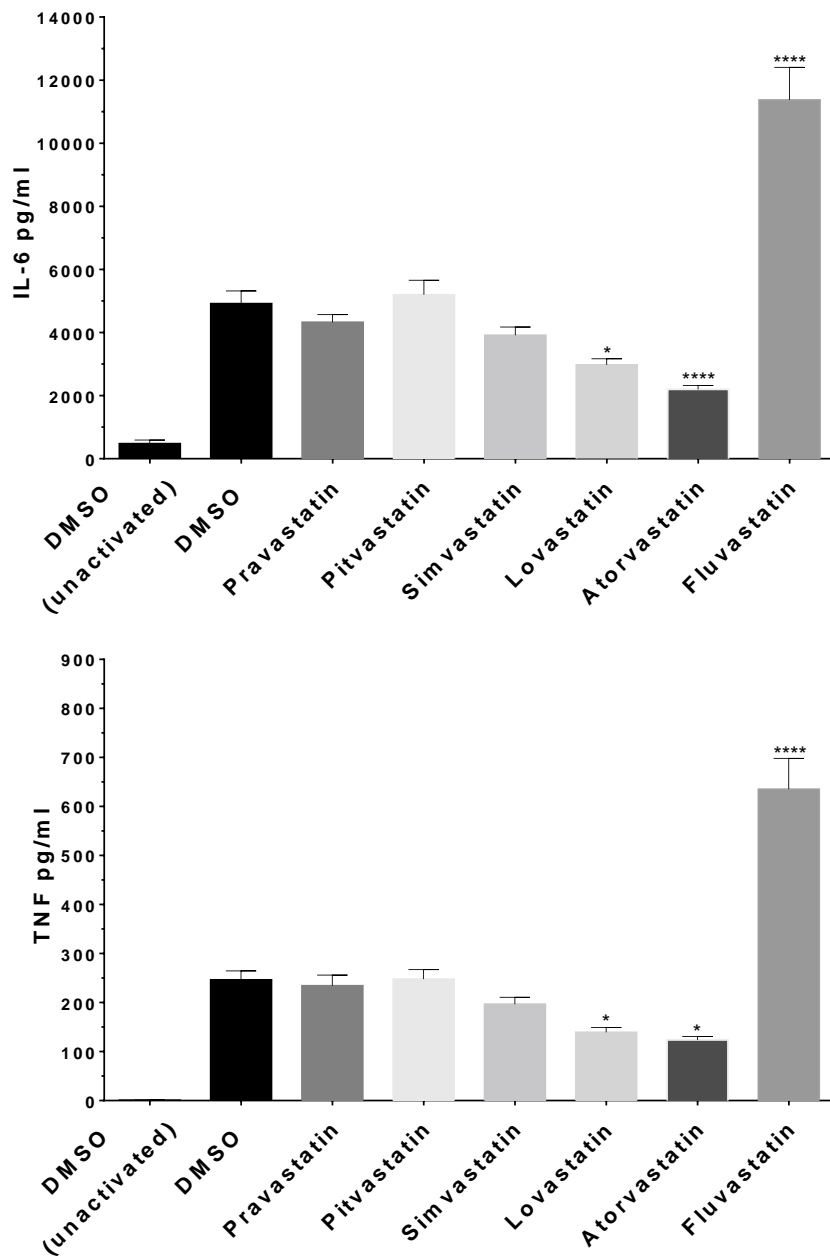
Figure 2. Statins alter pro-inflammatory cytokine production in IL-33-stimulated mast cells. C57BL/6J BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (50ng/ml) in the presence or absence of statins (20 μM), then activated with IL-33 (100 ng/ml) for 16 hrs. Cytokines were measured by ELISA. Samples were compared by ANOVA followed by Dunnet's test. Data shown are means ± SEM from 3 samples and representative of 3 separate experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001
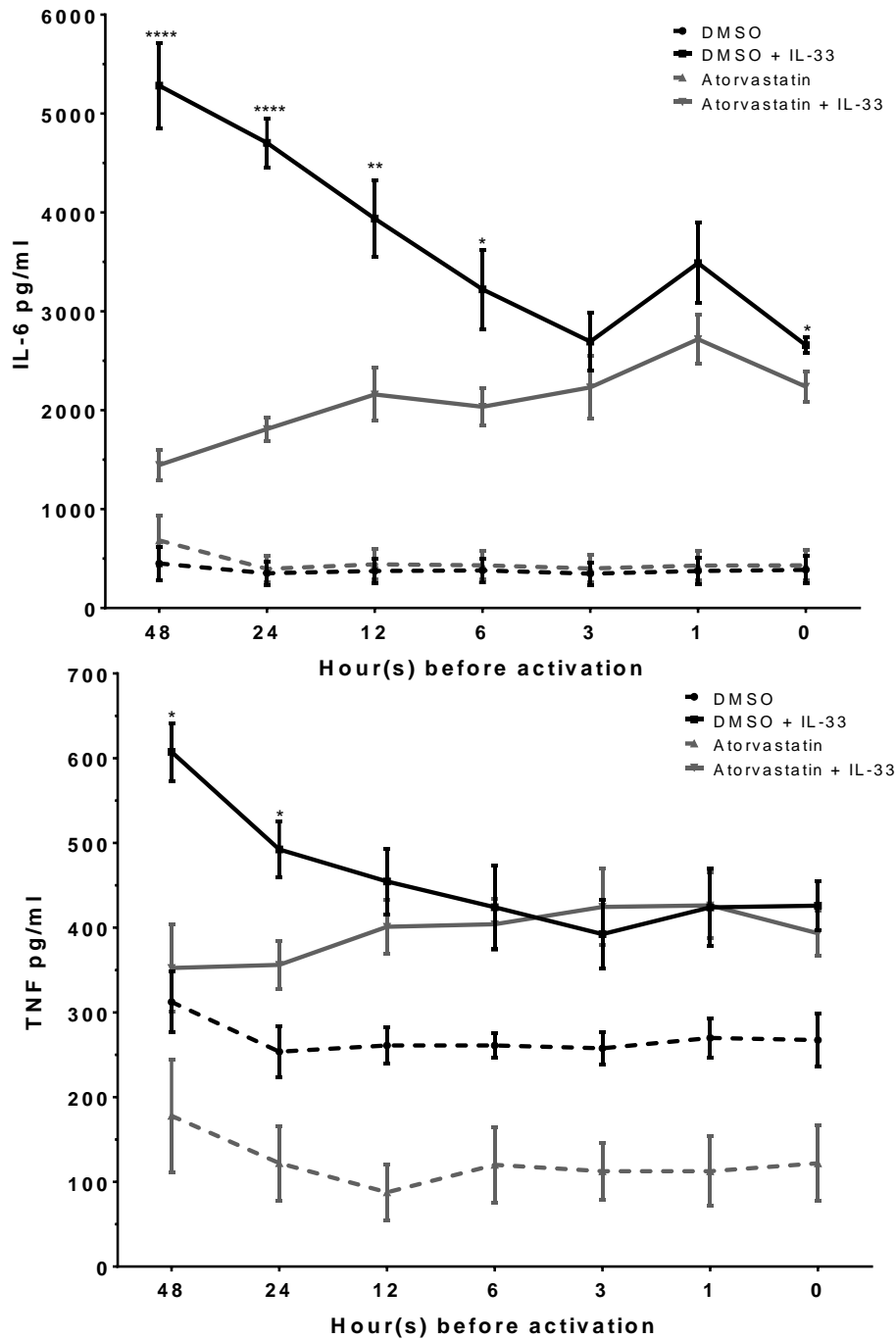
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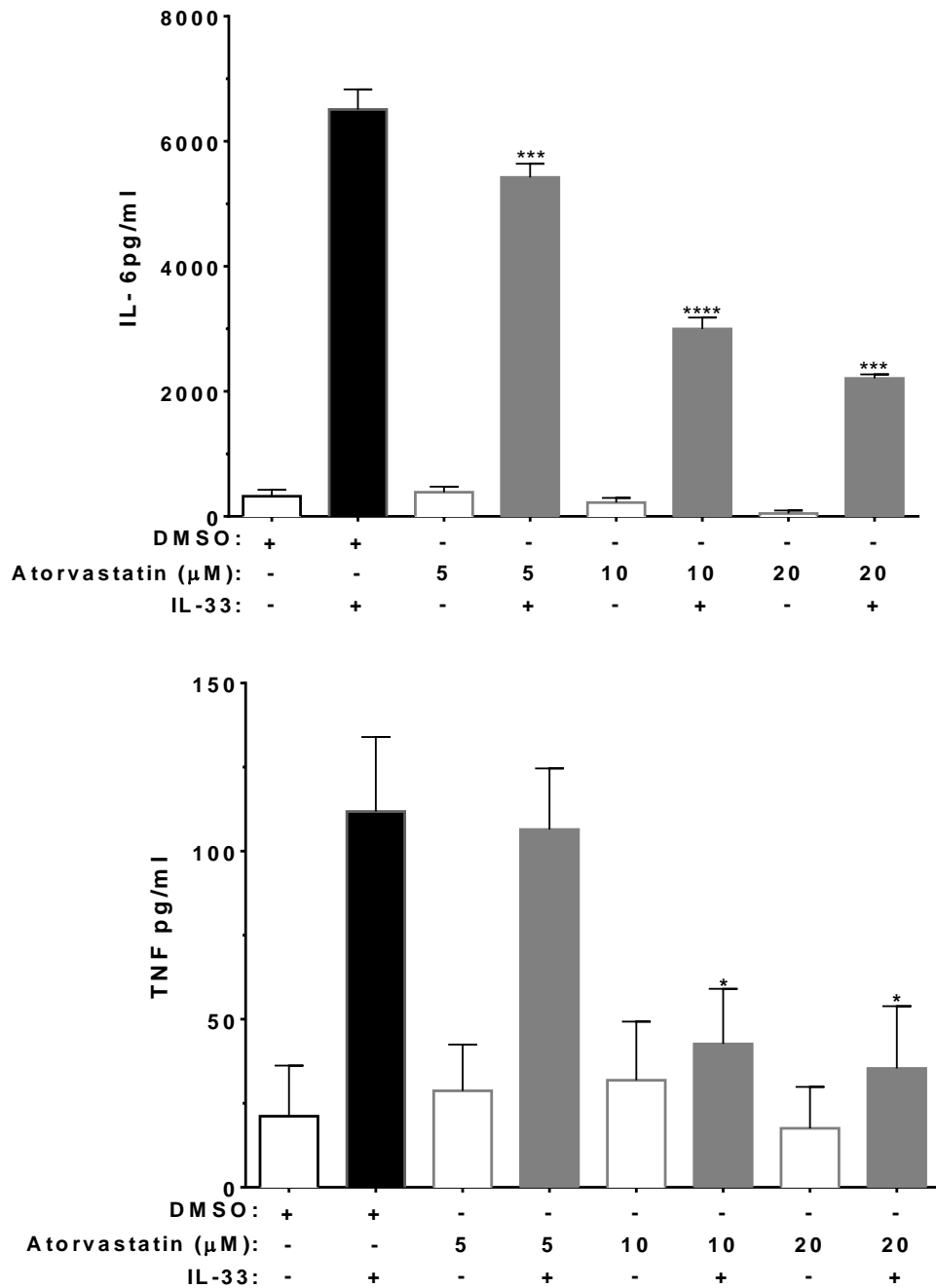
Figure 3. Atorvastatin alters pro inflammatory cytokine production in a dose- dependent manner. C57BL/6 BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (10ng/ml) ± atorvastatin at the indicated concentrations. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours, and cytokines were measured by ELISA. Samples were compared by ANOVA followed by Dunnet's test. Data shown are means ± SEM from 6 samples and 2 separate experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Figure 4. Atorvastatin suppresses pro inflammatory cytokine production in a time-dependent manner. C57BL/6 BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (10ng/ml) in the presence or absence of atorvastatin at 20 μM for varying time before activation. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours, and cytokines were measured by ELISA. Samples were compared by ANOVA followed by Tukey's test at each time point. Data shown are means ± SEM from 3 samples and representative of at least 2 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
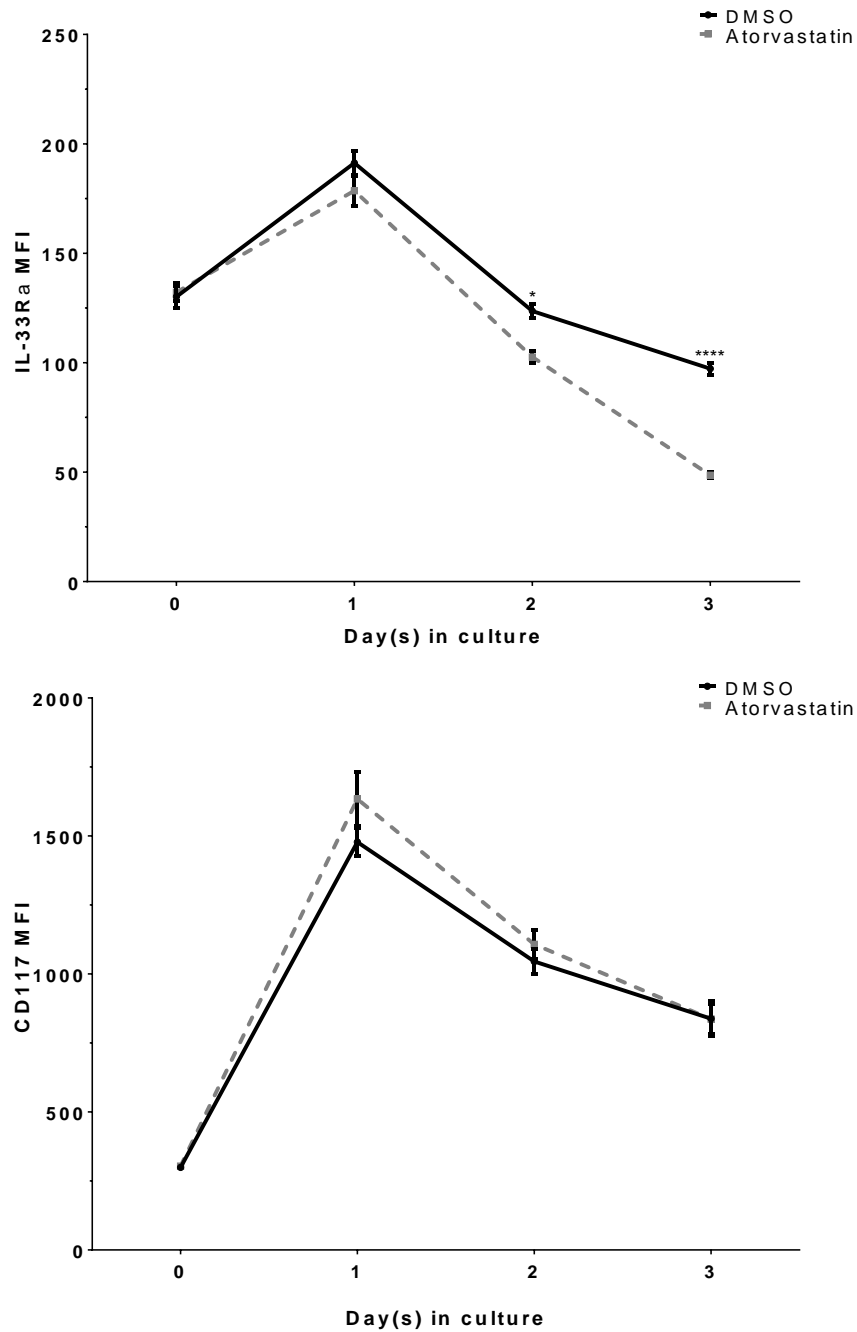
Figure 5. Atorvastatin alters surface receptor expression of mast cells. C57BL/6J BMMCs were cultured in IL-3 (10ng/ml) + SCF (10ng/ml) for 0- 3 days ± atorvastatin at 20 μM. IL-33Ra and CD117 surface expression was measured by flow cytometry. Data shown are means ± SEM from 3 samples and representative of at least 2 separate experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
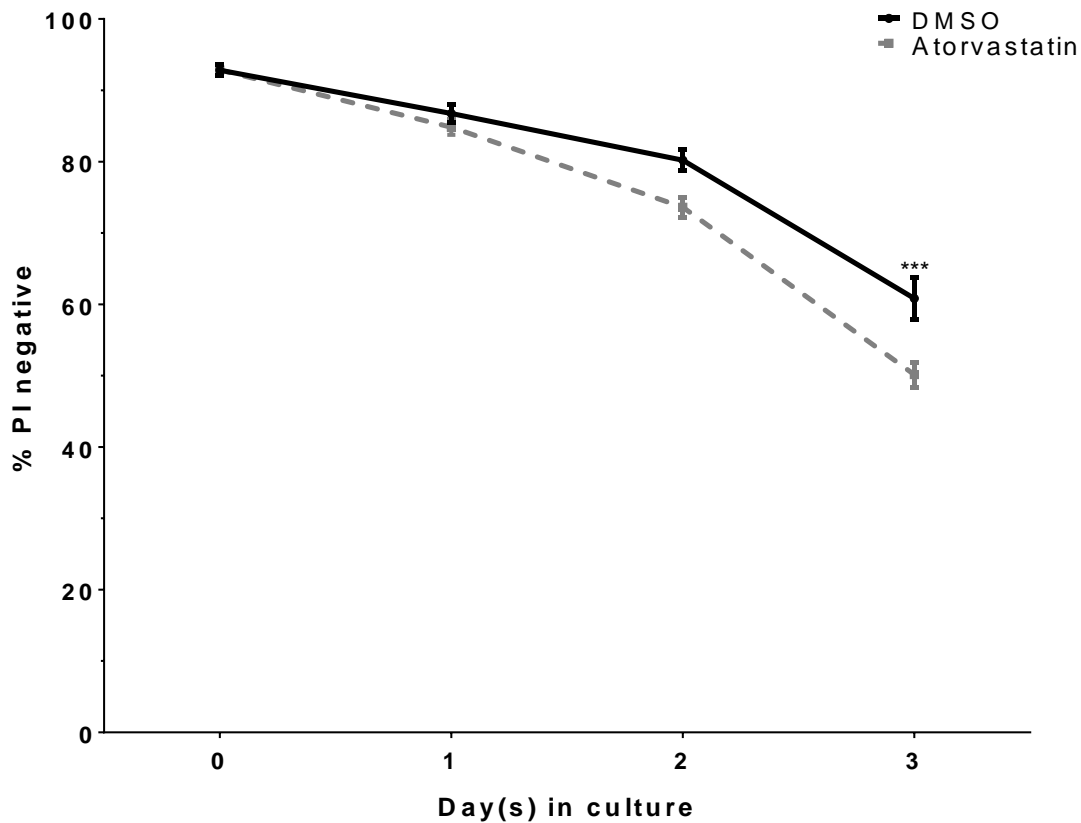
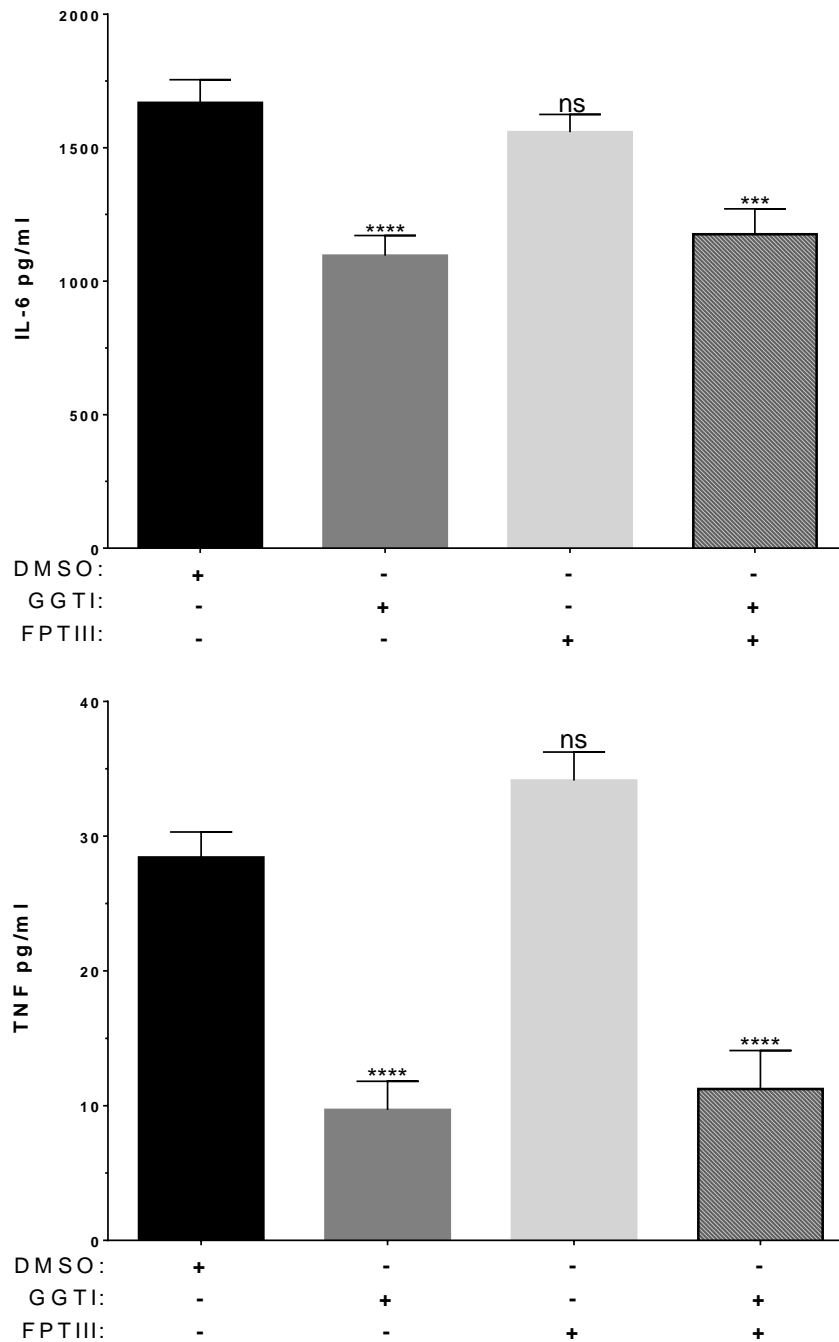**Figure 6. Survival of mast cells is altered by atorvastatin**. C57BL/6J BMMCs were cultured in IL-3 (10ng/ml) + SCF (10ng/ml) for 0- 3 days ± atorvastatin at 20 μM. Cell survival was measured by flow cytometry via propidium iodide (PI) exclusion. Data shown are means ± SEM from 3 samples and representative of at least 2 separate experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Figure 7. GGTI suppresses pro-inflammatory cytokine production in IL-33 stimulated BMMCs. C57BL/6J BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (50 ng/ml) in the presence or absence of GGTI, ± FPTIII at 20 μM. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours and cytokines were measured by ELISA. Samples were compared by ANOVA followed by Tukey's test. Data shown are means ± SEM from 9 samples and 3 independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.
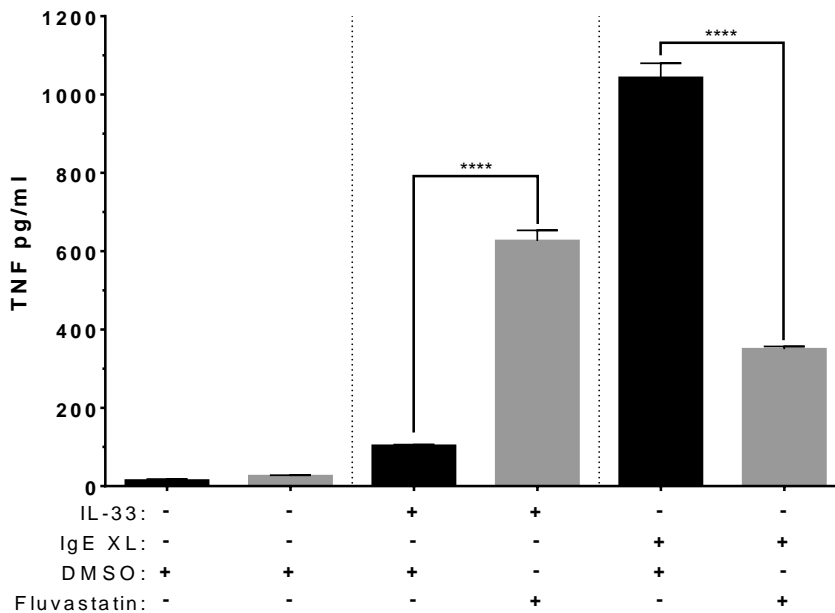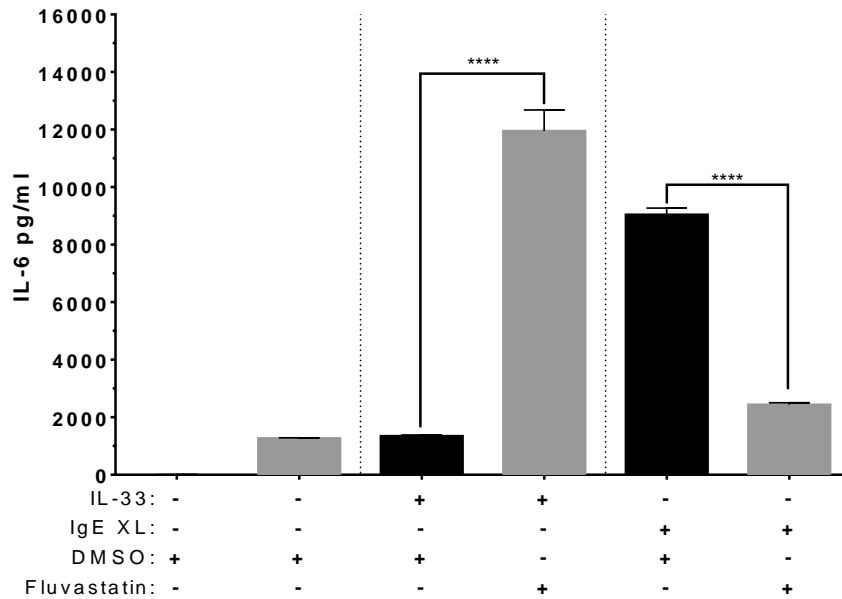
Figure 8. **Fluvastatin has differing effects on pro-inflammatory cytokine production in IL-33-stimulated vs. IgE-stimulated mast cells.** C57BL/6J BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (50ng/ml) +/- 0.5 µg/ml IgE in the presence or absence of Fluvastatin (20 µM). Cells were then activated with IL-33 (100 ng/ml) or 50ng/ml DNP-HSA for 16 hours. Cytokines were measured by ELISA. Stimulated samples were compared by ANOVA followed by Tukey's test. Data shown are means ± SEM from 3 samples. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
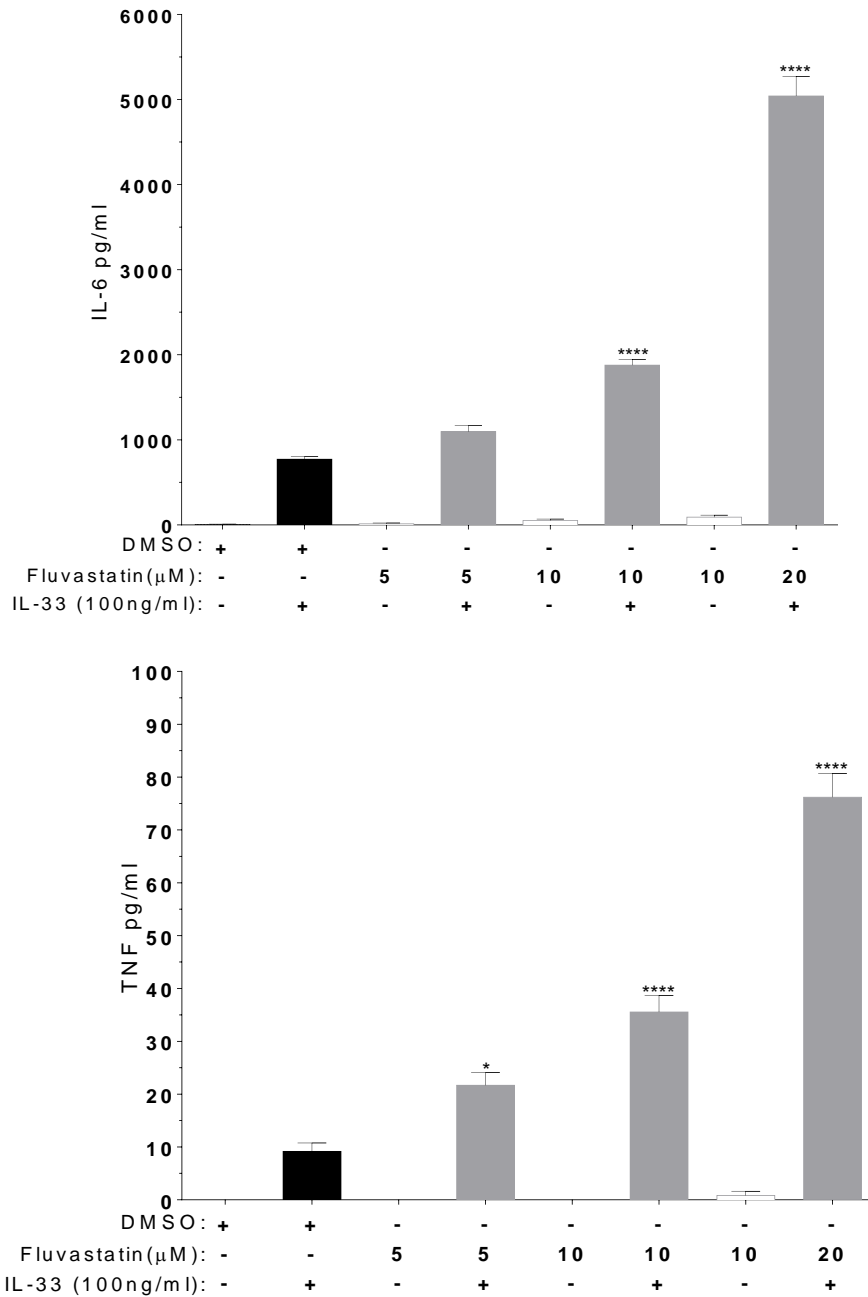
Figure 9: Fluvastatin enhances pro inflammatory cytokine production in a dose- dependent manner. C57BL/6 BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (50ng/ml) ± fluvastatin at the indicated concentrations. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours, and cytokines were measured by ELISA. Stimulated samples were compared by ANOVA followed by Dunnet's test. Data shown are means ± SEM from 3 samples and representative of at least 3 separate experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
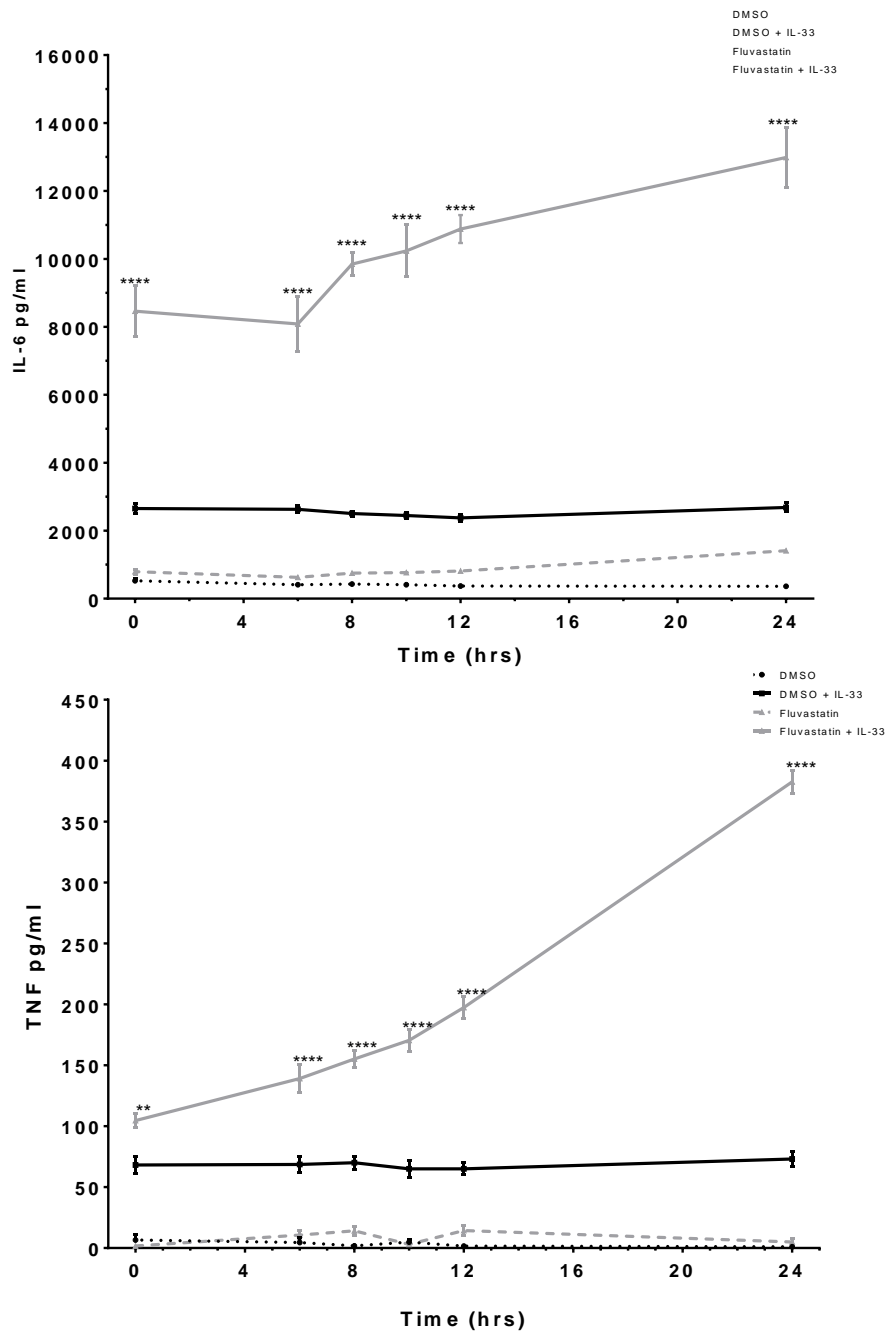
Figure 10. Fluvastatin enhances pro inflammatory cytokine production in a time-dependent manner. C57BL/6 BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) and SCF (50ng/ml) in the presence or absence of Fluvastatin at 20 μM for the indicated time before activation. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours, and cytokines were measured by ELISA. Stimulated samples were compared by Student's t-Test. Data shown are means ± SEM from 3 samples and representative of at least 3 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
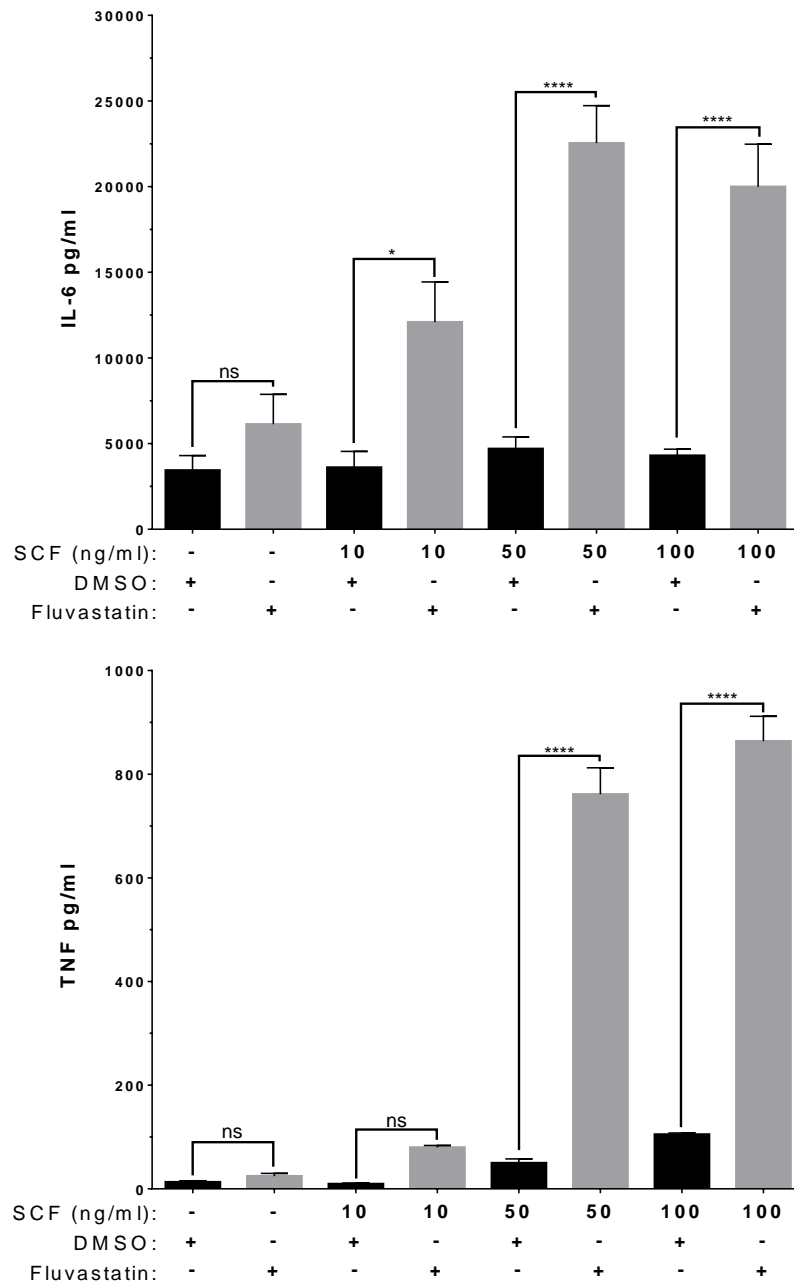
Figure 11: SCF is required for Fluvastatin-mediated increase in cytokine production.
C57BL/6J BMMCs were cultured for 24 hrs in IL-3 (10ng/ml) in the presence or absence of
fluvastatin (20 μM) ± SCF at 10, 50, or 100 ng/ml. Cultures were then activated with IL-33 (100
ng/ml) for 16 hours and cytokines were measured by ELISA. Samples were compared by
ANOVA followed by Tukey's test. Data shown are means ± SEM from 6 samples and 2 separate
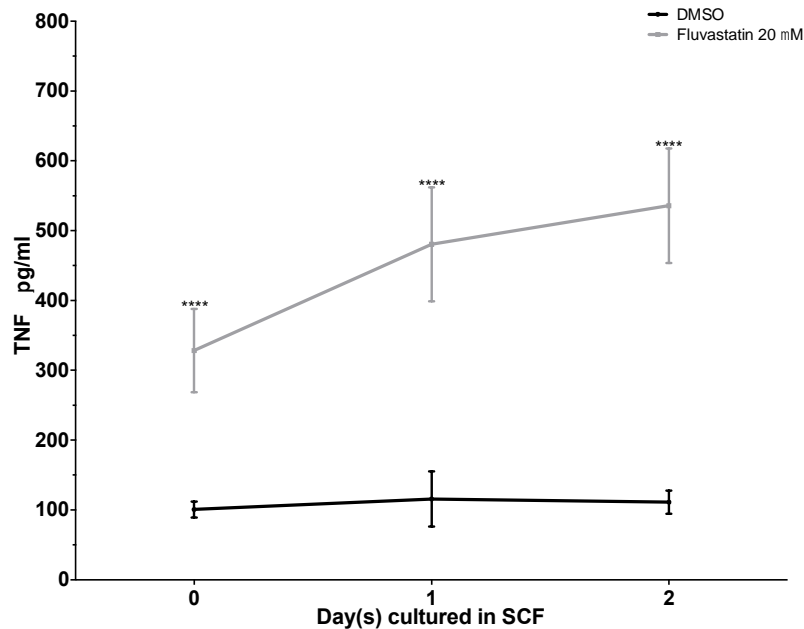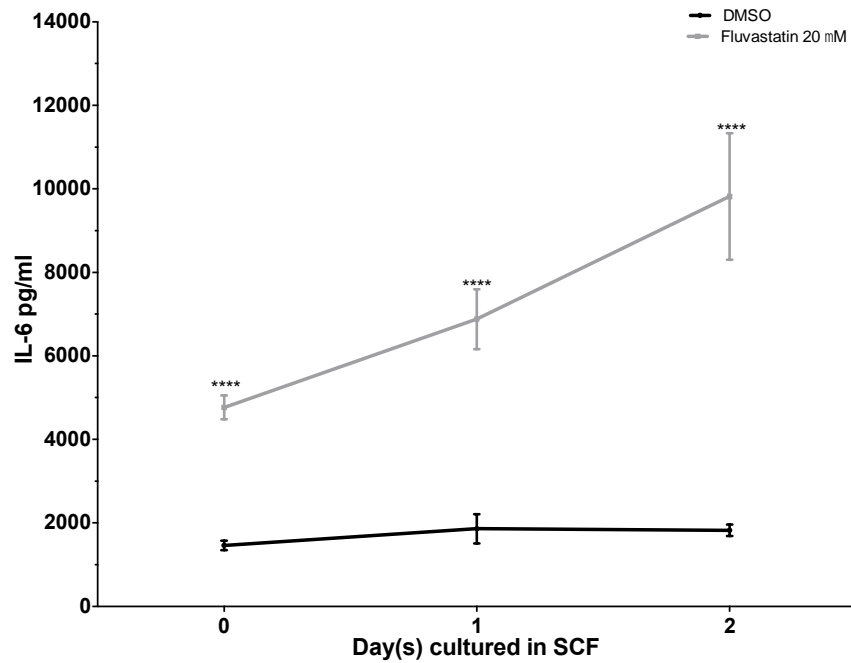experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Figure 12: SCF enhances Fluvastatin-mediated increase in cytokine production in a time dependent manner. C57BL/6 BMMCs were cultured in IL-3 (10ng/ml) and SCF (50 ng/ml) for 0-2 days before the addition of Fluvastatin (20 μM) or vehicle. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours and cytokines were measured by ELISA. Samples were compared by Student's t-Test. Data shown are means ± SEM from 3 samples and representative of at least 2 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
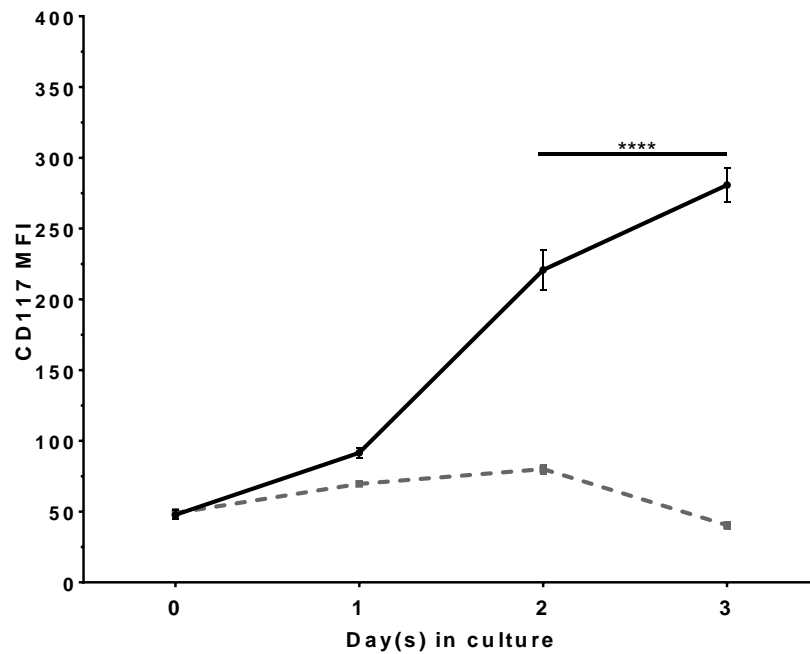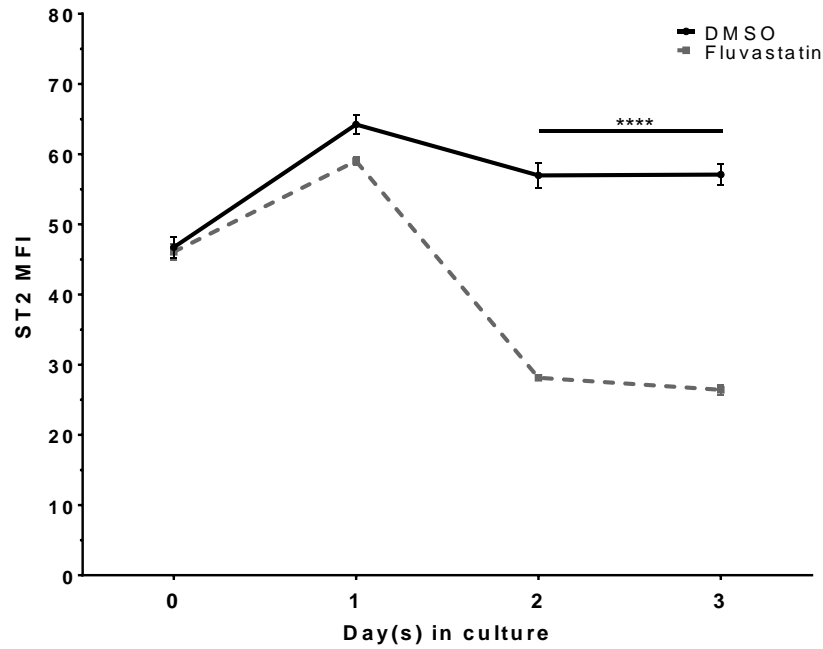
Figure 13: Fluvastatin alters surface receptor expression of mast cells. C57BL/6J BMMCs were cultured in IL-3 (10ng/ml) + SCF (50ng/ml) for 0- 3 days ± fluvastatin at 20 μM. ST2 and CD117 surface expression was measured by flow cytometry. Data shown are means ± SEM from 6 samples and 2 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001
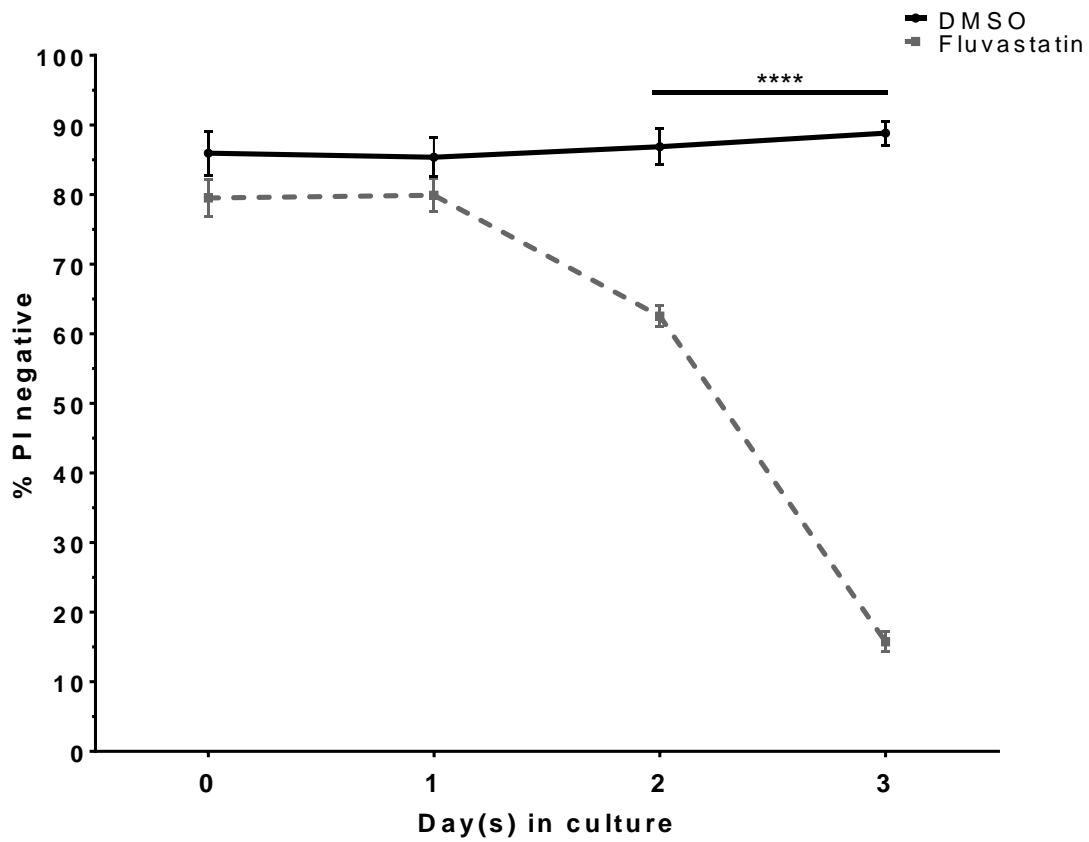
Figure 14: Fluvastatin alters survival of mast cells. C57BL/6J BMMCs were cultured in IL-3 (10ng/ml) + SCF (50ng/ml) for 0- 3 days ± atorvastatin at 20 μM. Cell survival was measured by flow cytometry via propidium iodide (PI) exclusion. Data shown are means ± SEM from 6 samples and 2 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001
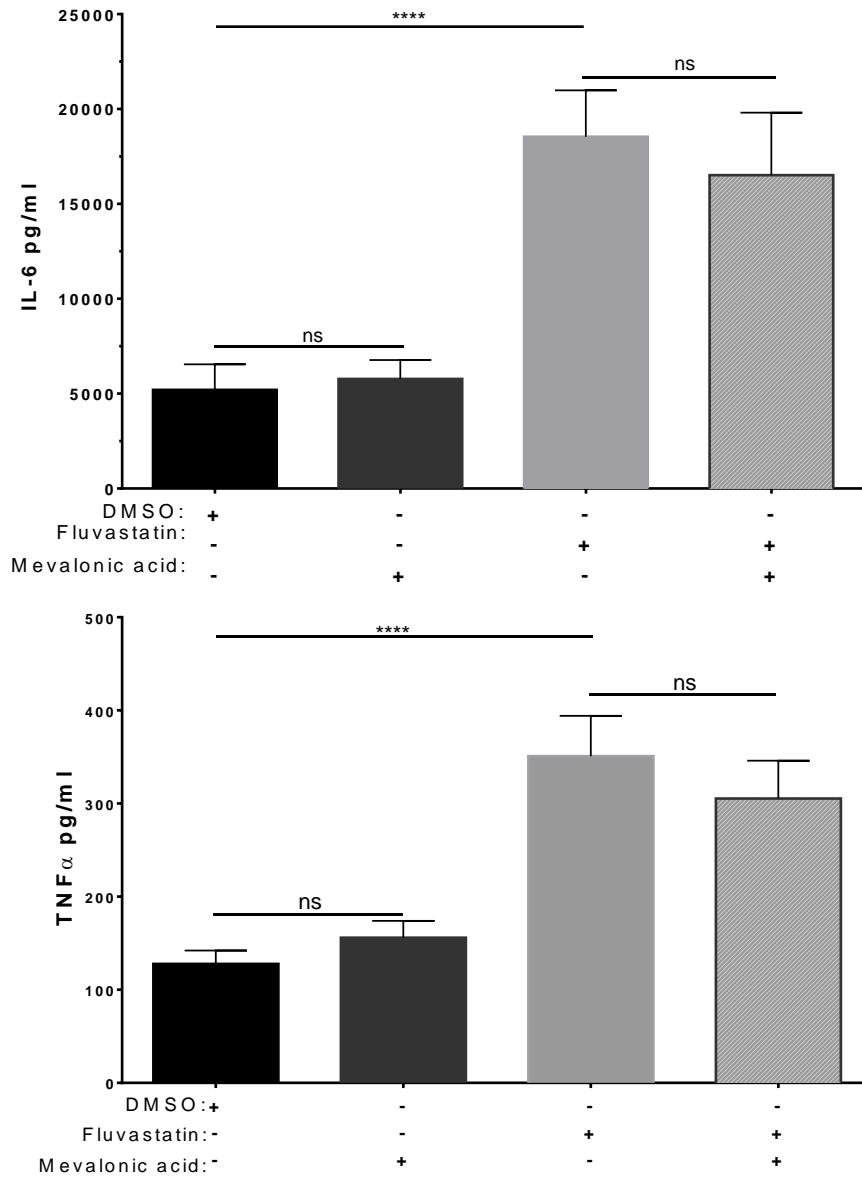
Figure 15: Fluvastatin-mediated increase in cytokine production is independent of the HMG-CoA pathway. C57BL/6J BMMCs were cultured in IL-3 (10ng/ml) and SCF (50 ng/ml) in the presence of vehicle or fluvastatin (20 μM) for 24 hours ± mevalonic acid (1000 μM) for24 and 4 hours before stimulation. Cultures were then activated with IL-33 (100 ng/ml) for 16 hours and cytokines were measured by ELISA. Samples were compared by ANOVA followed by Tukey's test. Data shown are means ± SEM from 3 samples and representative of 3 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
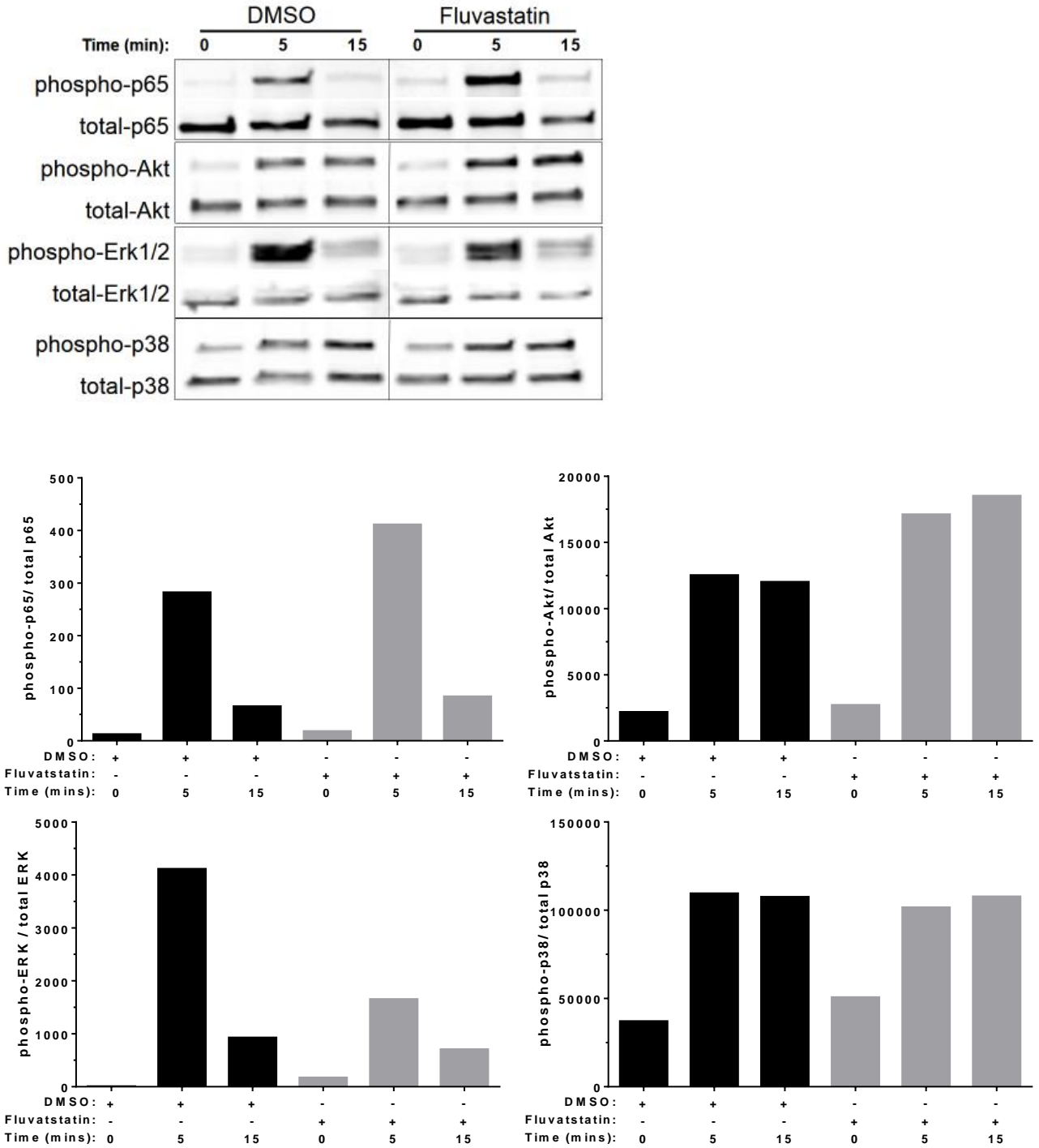
Figure 16. Fluvastatin alters phosphorylation of p65, Akt, and ERK in the IL-33 signaling pathway. C57BL/6J BMMCs were cultured in IL-3 (10ng/ml) and SCF (50 ng/ml) in the presence of vehicle or fluvastatin (20 μM) for 24 hours. Cultures were then activated with IL-33 (100 ng/ml) and SCF (50 ng/ml) for 0-15 mins prior to collection of cell lystates. Lysates were separated by western blot and imaged via Odyssey CLx infrared scanner. Quantification shown are the phospho /total ratio of p65, Akt, ERK1/2, and p38 from 1 sample.
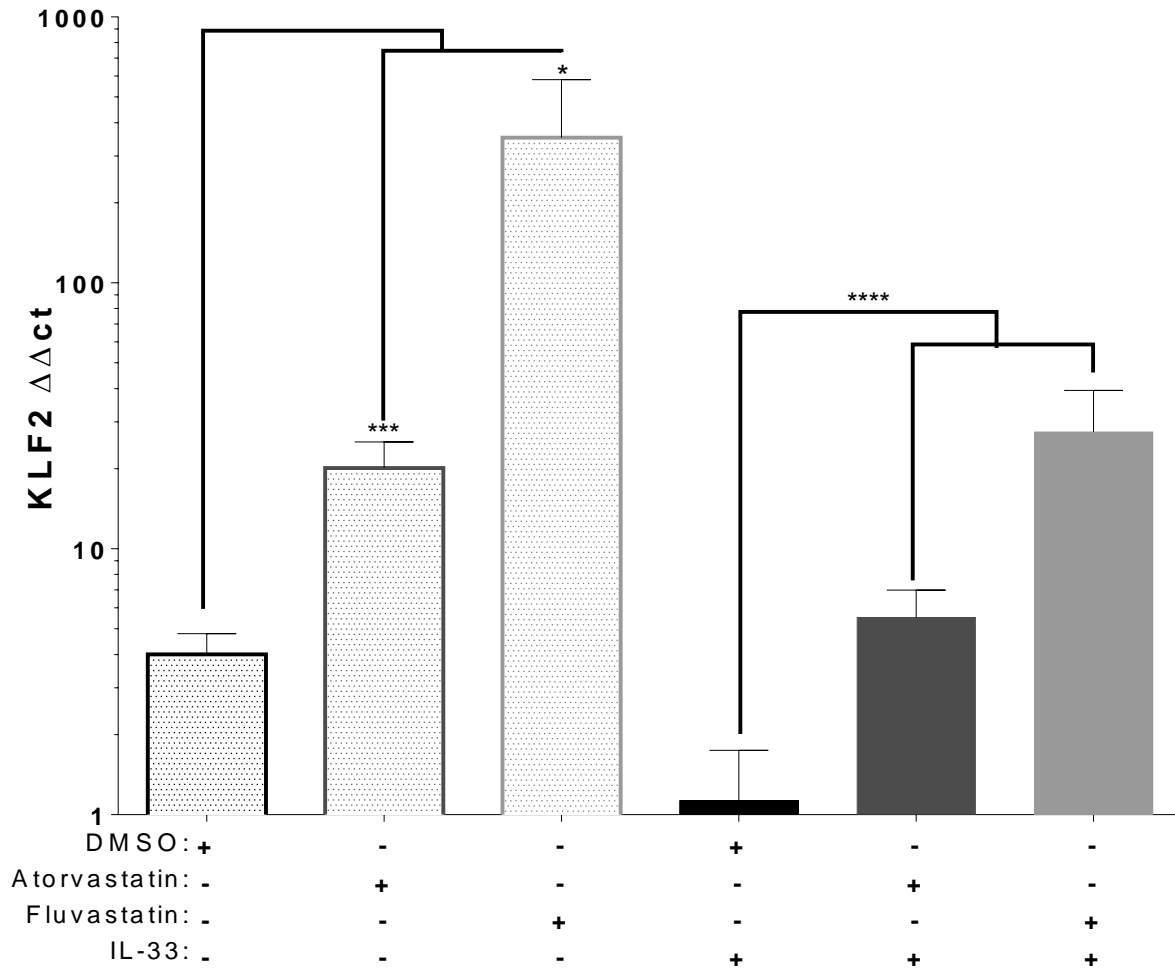
Figure 17. Statins induce mRNA expression of Krüppel-like factor 2. C57BL/6 BMMCs were cultured for 12 hrs in IL-3 (10ng/ml) SCF (50 ng/ml) in the presence of vehicle or the indicated statin (20 μM). Cultures were then activated with IL-33 (100 ng/ml) for 90 mins and total RNA analyzed for KLF2 expression by quantitative real time PCR. Samples were compared by Student's t-Test. Data shown are means ± SD from 3 samples. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

Figure 18. Fluvastatin enhances Il-33-mediated IL-6 production in vivo. C57BL/6J mice were injected i.p. with vehicle or Fluvastatin (1mg/mouse) 16 and 1 hour prior to IL-33 injections. Mice were then treated with IL-33 (1 µg/mouse) or PBS and cardiac puncture was performed 4 hours after injection. Plasma was collected and assessed for TNF, IL-6, and MCP-1 via ELISA. Samples were compared by ANOVA followed by Tukey's test. Data shown are means ± SEM from 4 samples. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
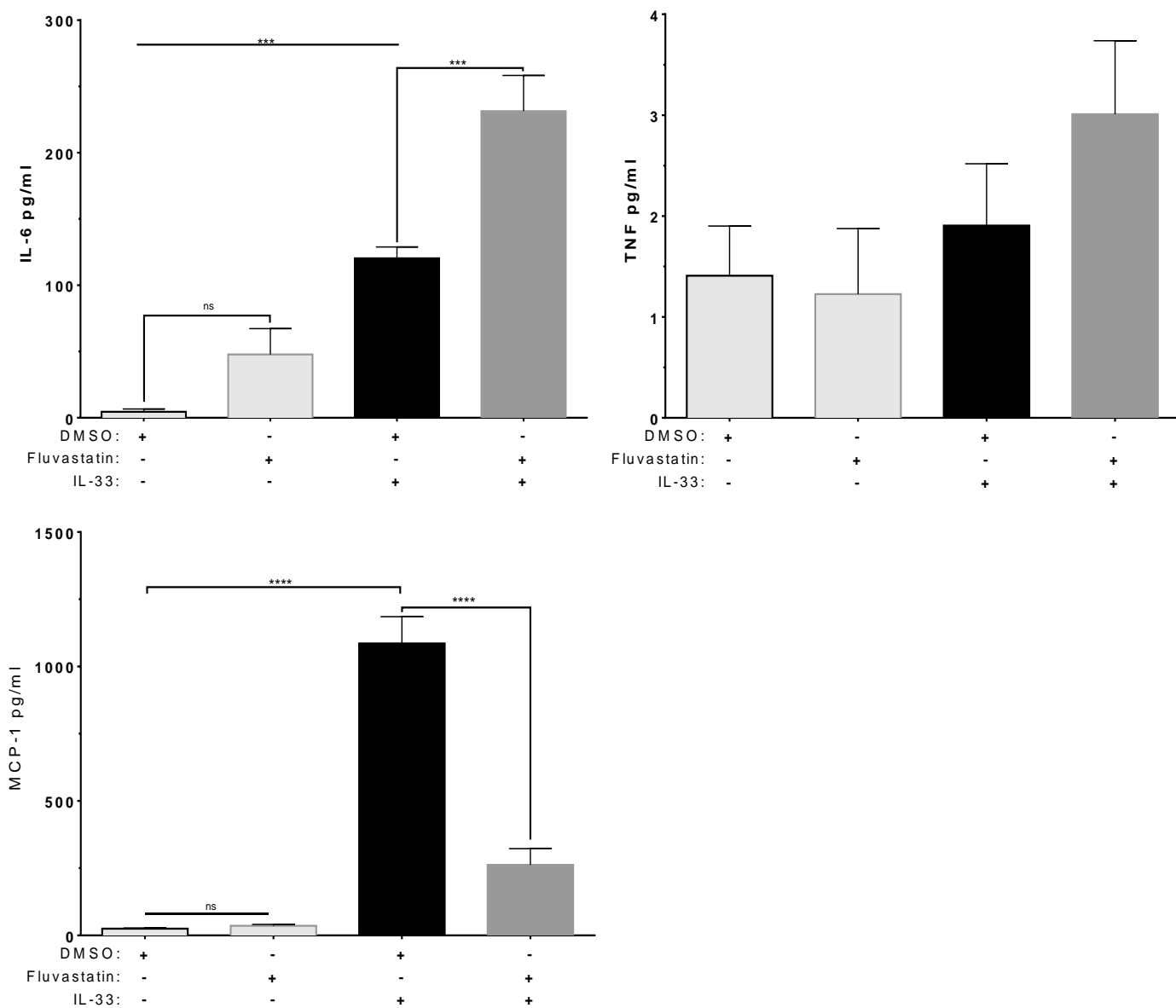
Figure 19. Fluvastatin enhances IL-33-mediated neutrophil recruitment. C57BL/6J mice were injected i.p. with vehicle or Fluvastatin (1mg/mouse) 16 and 1 hour prior to IL-33. Mice were injected with or without 1 μg of IL-33 and 4 hours after injection. Cells were stained with Ly6G, CD49b, CD8 and CD4 antibodies. Samples were analyzed using flow cytometry. Samples were compared by ANOVA followed by Tukey's test. Data shown are means ± SEM from 4 samples. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

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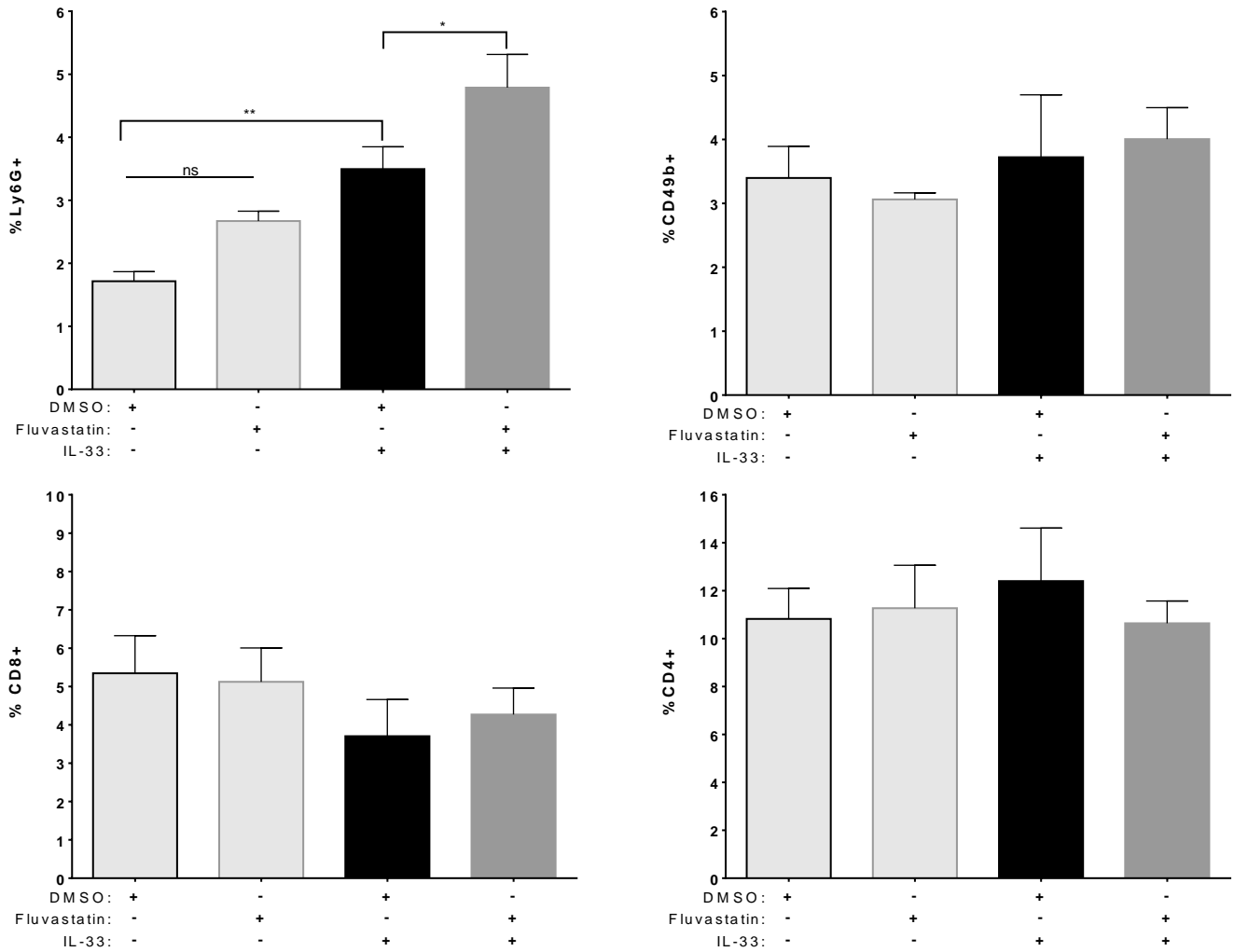1. Galli, S. J., M. Grimbaldeston, and M. Tsai. 2008. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nature Reviews Immunology*.

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