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The effect of fluvastatin on mast cell function: genotype dependence

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The effect of fluvastatin on mast cell function: genotype dependence.

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

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ABSTRACT

THE EFFECT OF FLUVASTATIN ON MAST CELL FUNCTION: GENOTYPE DEPENDENCE.

By

Elizabeth Motunrayo Kolawole

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

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Director: John J. Ryan, Professor, Department of Biology

Fluvastatin, the HMG-CoA reductase inhibitor known for its role in the treatment of hypercholesterolemia and cardiovascular disease, has more recently been shown to play a role in the immune response. Given the critical role that mast cells play in allergy and inflammatory diseases such as asthma, which effects one third of America's population, we assessed the effect of

fluvastatin on mast cell and basophils function. We demonstrate that fluvastatin downregulated IgE-mediated cytokine production. Additionally, in vivo studies showed that fluvastatin suppressed IgE-mediated anaphylaxis. Interestingly, the effects of fluvastatin showed dependence on genetic background, as C57BL/6 mast cells were sensitive, while 129/Sv mast cells were resistant to fluvastatin. Characterizing the role of fluvastatin on mast cells may prove to be therapeutically important.

Part I

The effect of fluvastatin on mast cell function:
genotype dependence

CHAPTER 1 - INTRODUCTION:

Immunology- a brief war and peace

From a distance the human body looks serene, but on closer inspection a battle ensues. Life is an epic fight. The ability to thwart the onslaught of disease-causing agents is a constant struggle that all living organisms face. With increasing complexity of the organism, more elaborate plans of defense are devised and implicated. We call this defense mechanism the immune system. The immune system, beautiful in its complexity, at its best goes unnoticed. Only when an invader breaches our front lines or our defenses turn on us do we see any evidence of its existence. It is in this defective state that we realize how truly intricate and multifaceted our immune system is. Thus, we try to restore the balance, giving rise to the field of immunology.

Throughout history, immunology and the treatment of disease has gone by many names and taken a plethora of forms and iterations. Known as shamanism or divination, healing by supernatural means in ancient times or physician, civilizations have always revered those who possess such capabilities and sometimes feared them. The Smith and Ebers papyrus dating back to 1500 B.C. are ancient Egyptian documents, providing remedies for various illnesses from the use of bark, which contains acetylsalicylic acid, an active ingredient of aspirin, to the treatment of burns and brain injuries (1). While the Egyptians of 1500 B.C. did not know about microorganisms, they knew to treat with copper salts and honey to prevent infection (2) and had healers specializing in different organs.

In 430 B.C Thucydides writes of, “the plaque of Athens” (typhoid fever) and how those who had recovered were then able to treat the sick as they had become unsusceptible. Thucydides might well have been influenced by the great philosophers and historians of his time, such as Hippocrates, Socrates and Galen, who theorized that the body was composed of 4 vital fluids called humors - blood, phlegm, yellow bile and black bile - and that an imbalance between these 4 led to disease. Aristotle was the first to study anatomy in animals, and Herophilus the first to conduct human dissections. Hippocrates believed in starvation for fevers and feeding for colds. Galen however, believed in bloodletting, often using leaches to resolve the humoral imbalance.

Humoralism has been a prevailing theory throughout history, though it has taken many forms, with those in India and China having similar ideas. Indian traditions believed in three humors. Kapna or phlegm was composed of earth and water. Pitta, or bile, being fire and water. Vata was described as being wind or air. Tibetans believed that a blockage of these fluids led to disease and so taking the pulse was important in the identification of such blockages. In the 9th century in the Persian Empire Al-Razi wrote a book of medicine, where he documented and was able to identify allergic reactions, and reasoned that fever was the body fighting disease. Following him, Ibn-Sina’s correctly described blood circulation and recognized that quarantine was required to prevent the spread of infectious disease.

Variolation came before vaccination and dates back to 10th century China where powdered lesions from those with smallpox would be exposed to the

healthy. The numerous delivery methods and vast discrepancies between quantities being used as treatments occasionally resulted in death and disfigurement and therefore were not widely accepted.

From the time of Thucydides, different cultures had recognized that some diseases were contagious. Many believed that these diseases arose spontaneously, an idea that persisted from the time of Aristotle. However, during the renaissance the scientific method of observing, conducting experiments and reaching conclusions moved at a faster pace. In the 18th century James Lind proved that citrus fruits cured scurvy. Subsequently, the English physician Edward Jenner intentionally used cowpox and a vaccine for small pox and the etiology of disease began to be understood.

It was not until the end of the 19th century that the French chemist Louis Pasteur quashed the concept of spontaneous generation. Pasteur's "Germ Theory" (1860) states that microorganisms cause disease and that they are ever present. Pasteur also proposed that microorganisms needed to be prevented from entering the body during surgeries, which led to Joseph Lister developing the antiseptic surgical method. Pasteur also showed that it was microorganisms that are responsible for milk souring and that fermentation is a result of microorganism growth (1858-59). This, along with his development of early vaccines for anthrax, cholera, and tuberculosis earned him the title of the "father" of immunology. Whilst Pasteur is known for early vaccines to these diseases, it was Robert Koch who identified the specific bacteria *Bacillus anthracis*, *Vibrio Cholera* and *Mycobacterium tubercerculosis* being causative for Anthrax, cholera

and tuberculosis respectively, giving rise to bacteriology. Furthermore, “Koch’s postulates” brought about a method of determining if a given microorganism was the causative agent for a particular disease. This vast body of work earned Koch the Nobel Prize in 1905.

The 20th century saw the dawn of modern immunology, with many brilliant scientists in addition to Pasteur and Koch. Behring’s anti-diphtheric serum showed immunity can be transferred, earning him the first Nobel Prize in medicine in 1901. Mechnikov and Ehrlich were awarded for recognizing antibody generation, and Ehrlich for many more wonders, such as chemotherapy and identification of mast cells. He is accredited for being “the father of histology” and conceived the idea of the immune system being able to direct responses against “itself”. Metchnikoff is esteemed for identifying phagocytosis. These great minds, all of which received the Noble Prize in Medicine inside a decade of each other, along with many more paved for way for immunology, unveiling the immune system and its complexities.

This period of history marked the beginning of a new era, a time when scientists would not simply react to some terrible outbreak of disease, but actually begin to unravel the inner working of interactions between host and microorganism. This identification of cause and effect allowed remedies and preventative measures to be outlined. Thus, modern immunology was born.

Immunity –A tale of two theories

The end of the 19th century and 20th century saw a debate between two conflicting theories: the long standing, ever-evolving theory of humoralism, and the immunological wonders that opposed it. Modern humoralism was initiated by Ehrlich's group at the Institute of Infectious Disease in Berlin, with Metchnikoff's group in opposition at the Pasteur Institute in Paris. Humoralism was the prevailing theory of the early 1900's, predominantly led by Ehrlich's proposition that antibodies are responsible for immunity. This inspired subsequent generations, supported by the likes of Von Behring's transferable immunity (1890), Roux's passive immunity (1891), and Bordet's complement and antibody activity in bacteriolysis (1894) to name but a few. Metchnikoff's discovery of Phagocytic cells (1884) inspired the cellularist theory. Phenomenons such as Koch's uncovering delayed type hypersensitivity (1883) and Medawar's hypothesis of allograft rejection (1944) suggested that antibodies were not the definitive immune component, fostering the spread of skepticism regarding the all-encompassing humoral theory and making way for the concept that phagocytic cells were the prominent component of immunity.

The case for Humoralism was strengthened by the immunochemistry work of Heidelberger who demonstrated the precise method for the determination of antibodies, antigen and complement on a weight basis, giving rise to Radioimmunoassays (RIA) and Enzyme Linked Immunosorbent Assays (ELISA). Furthermore, Heidelberger showed that polysaccharides in addition to proteins can act as antigens. Heidelberger in conjunction with Kabat (1953) paved the

way for the understanding of the immunoglobulin molecule, which was elucidated by Porter and Edelman (1959) firmly solidifying the humoral theory. Definitive proof of the Cellularist Theory came from the work of Landsteiner and Chase (1942). They showed that cells transferred from a guinea pig immunized against *Mycobacterium tuberculosis* to naïve mice and then injected with antigen, underwent an immune response, not seen in mice when the serum fraction was transferred. This showed irrefutable proof that antibodies alone did not orchestrate the immune response and that leucocytes play a role, as later shown by Gowen (1962).

Ehrlich v Metchnikoff

Ehrlich's camp and Metchnikoff's camp both sought to prove the other wrong with many scientists believing that it was one theory or the other. But ultimately, both were correct.

Innate immunity

The innate immune system, pioneered by Metchnikoff's phagocytic cells, is the first response to infection. It begins with a physical barrier to the external environment, encompassing the skin and mucosal surfaces. The cellular fraction is composed of circulating cells (basophils, monocytes, eosinophils and neutrophils) and resident cells (mast cells, newly recruited monocytes and macrophages), which migrate to these physical barriers. These cells are activated via receptors such as toll-like receptors (TLR) or immunoglobulin receptors (FcR), and secrete chemokines and cytokines in addition to lipid

mediators, which recruit additional immune cells. The humoral fraction of innate immunity includes activation of the complement cascade.

Adaptive immunity

The adaptive immune system, pioneered by Ehrlich, can be expanded beyond antibodies. Antigen presenting cells (APC) described as “professional” or “non-professional” process and then present antigen fragments to T cells using Major Histocompatibility Complex (MHC) proteins. T cells assist in B cell maturation and differentiation into memory B cells and plasma cells. B cells can undergo isotype switching and somatic hypermutation, producing different antibodies such as IgA, IgD, IgE, and IgG, which bind antigen. Antibodies have many functions, some of which are to neutralize toxins, target infectious organisms, activate complement and promote activation of mast cells, neutrophils, and macrophages.

Mast cells: Importance to innate immunity

Mast cells are classically known for their integral role in IgE-dependent allergic disease such as anaphylaxis and asthma, with anaphylaxis effecting 2% of the American population (3). The prevalence of asthma alone has reached epidemic proportions in westernized countries with an estimated 39.1 million Americans being diagnosed in 2011 and an estimated 6,278 fatalities (4).

In recent years, it has been shown that the mast cell’s role is multifaceted and extends beyond allergic disease, having a key role in inflammatory conditions such as multiple sclerosis (5, 6), rheumatoid arthritis (7), and

atherosclerosis (8). In addition, a mast cell role is not limited to the harmful immune responses, as they participate in host defense against viral (9) and bacterial pathogens (10), as well as immunosuppressive capabilities in connection with allograft tolerance (11) and contact dermatitis (12) (13). Furthermore, mast cells have also been documented at tumor sites and associated with tumor progression (14),(15). The role in tumor biology appears to be linked to promoting angiogenesis, tissue remodeling, and tissue repair (16). These vast amounts of data demonstrate the plethora of complex roles mast cells have. Thus elucidating the mechanisms mast cells employ are of great importance.

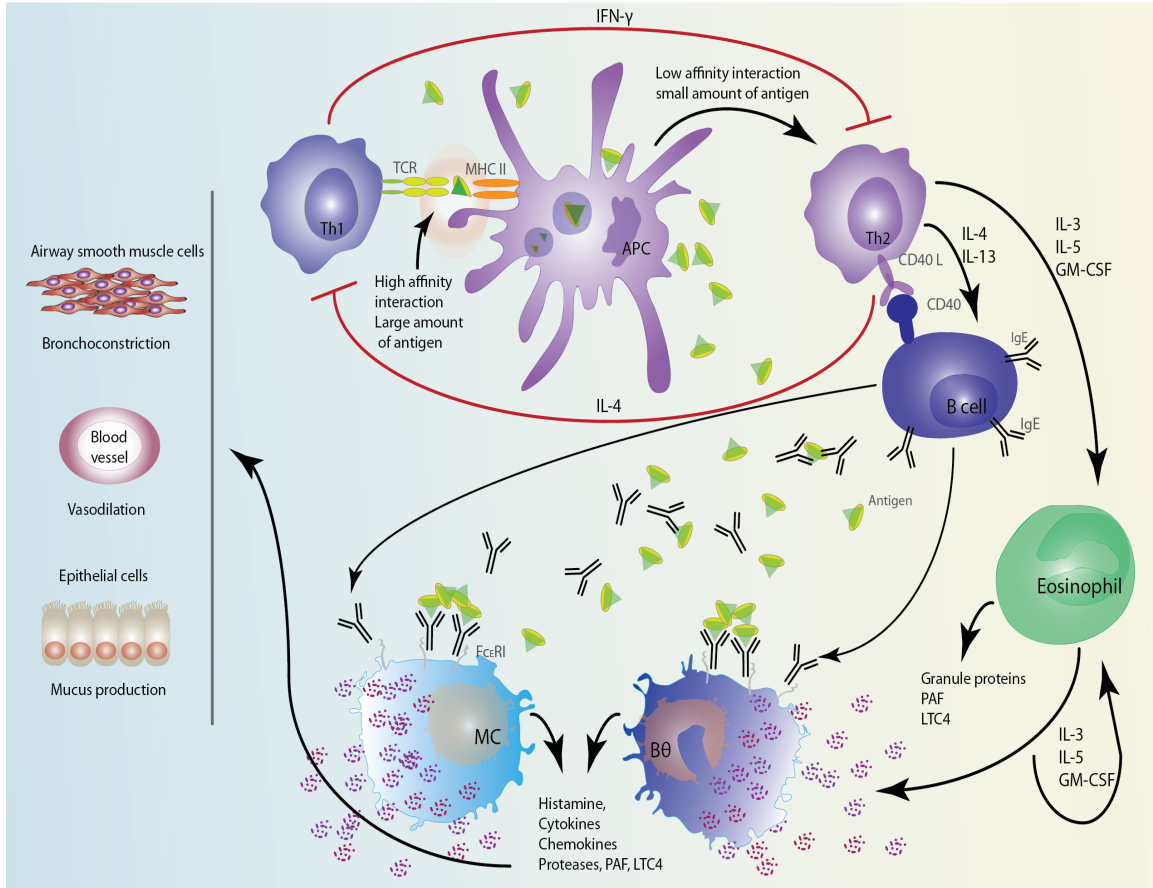


Figure 1

The inflammatory mechanism in allergic inflammation.

Antigen is engulfed by antigen presenting cells (APC). It is then processed by and presented to CD4+ T helper (Th) cells. Th1 production of IFN- γ down regulates the Th2 response, while Th2 production of IL-4 down suppresses Th1 responses. IL-4 is also responsible for class switching of B cell antibody production to IgE. B cells produce IgE that binds to Fc ϵ R1 receptors present in high abundance on mast cells and basophils. Additional Th2 cytokines such as IL-3 and IL-5 stimulate eosinophils and basophils to become active. IgE bound to Fc ϵ R1 is cross-linked by antigen and become activated to release preformed

chemical mediators such as histamine, lipid mediators, such as leucoktriene C4 (LTC4), platelet-activating factor (PAF) and *de novo* cytokines, chemokines and growth factors. These mediators can yield symptoms from mild rhinitis to anaphylactic shock, by inducing vasodilation, contraction of the bronchial smooth muscle and increased mucus secretion in the lungs.

A mast cell rises

Mast cells derive from distinct hematopoietic stem cell (HSC) precursors. Human mast cells arise from pluripotent CD34+ progenitor cells (17), whereas the mouse mast cell progenitors (MCP) are derived from Thy-1^{lo} c-kit^{high} and express c-kit, Fc ϵ RI, ST2 and integrin β (18-20). Whilst development begins in the bone marrow, maturation is completed after immature mast cells migrate through the peripheral blood to vascularized tissues. Interleukin (IL) 3 is important for the development of most hematopoietic lineages, including mast cells. Stem cell factor (SCF) is also important for early development and proliferation of hematopoietic cells (21). However, while other hematopoietic lineages downregulate the SCF receptor c-Kit while immature, mast cells maintain expression. Furthermore, SCF is imperative to mast cell proliferation, survival and activation (22), making the combination of IL-3 and SCF receptors distinct markers of mature mast cells.

Mast cells can be found throughout the body with the exception of the blood. Being largely known for their role in innate immunity, they are strategically placed at the interface to the external world and are found in high numbers in the lungs, skin and mucosal surfaces (20, 23, 24). In connective and mucosal tissues, mast cell maturation is the product of their microenvironment. Mast cells can be activated by an array of different stimuli ranging from immunoglobulin, microbial products such as lipopolysaccharide (LPS) and various cytokines and chemokines (20, 21). Their subsequent activation, resulting in the release of preformed and newly synthesized mediators has a substantial impact on their

microenvironment. The mast cell response can be broadly divided into a pro-inflammatory response leading to cellular migration and infiltration and the anti-inflammatory response, which can result in venom degradation, T-reg migration and immunosuppression (Figures 2 and 3).

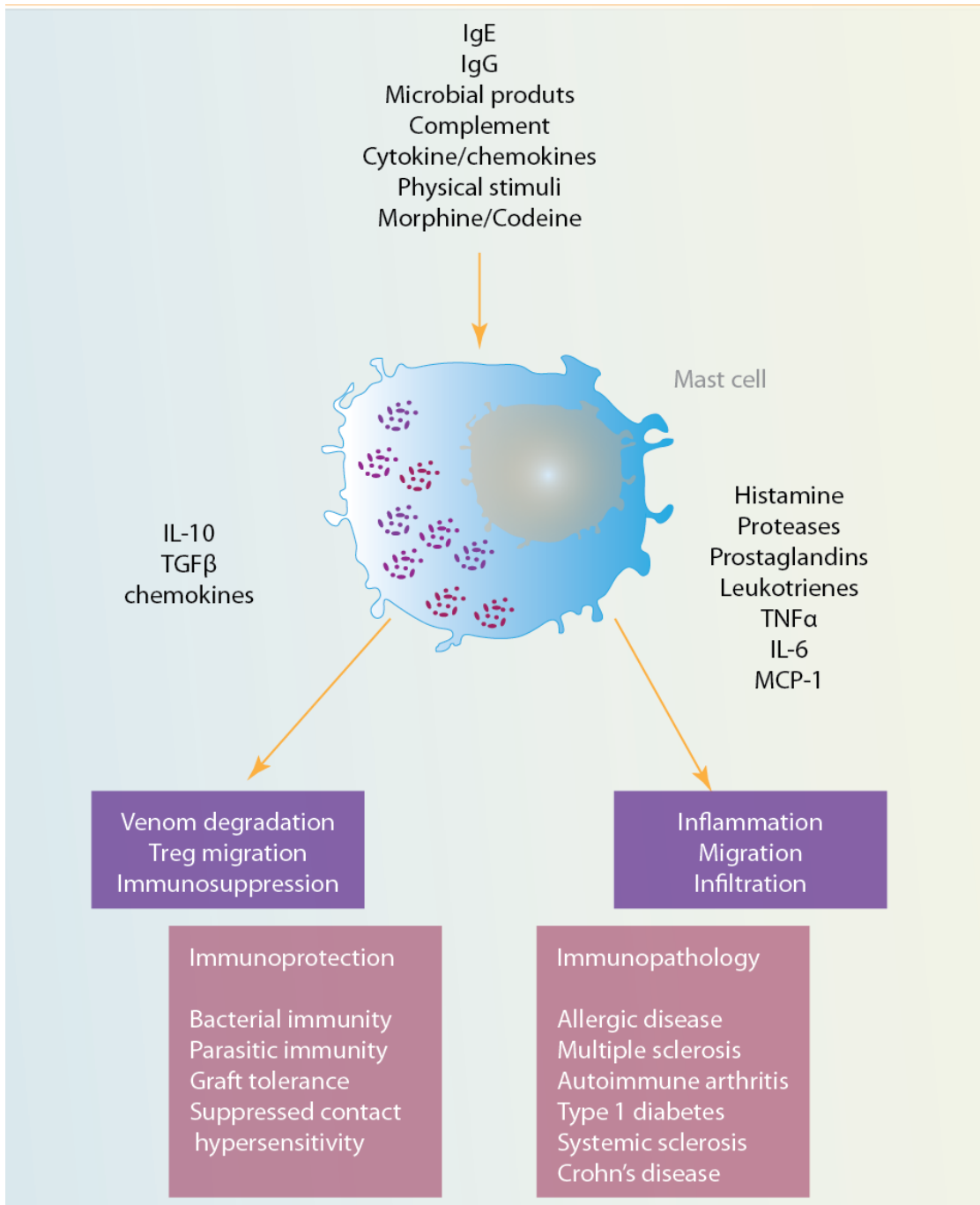


Figure 2

Mast cells: Two arms of activation.

Mast cells can be activated by an array of different stimuli, resulting in pro-inflammatory or anti-inflammatory responses mediated by preformed or newly synthesized factors.

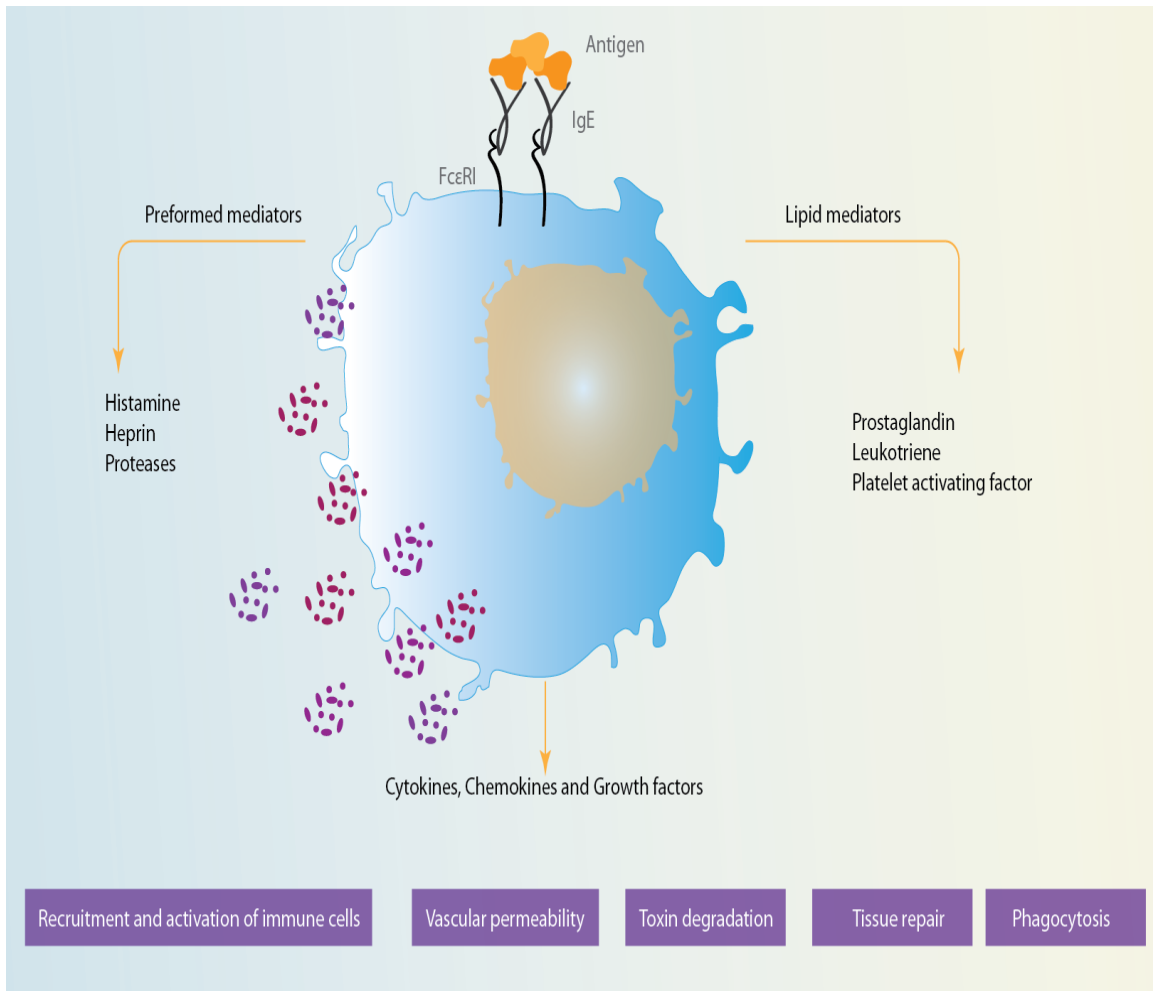


Figure 3

Activation via the FcεRI receptor.

Mast cells are most commonly activated by the high affinity FcεRI receptor. Upon FcεRI aggregation with antigen and IgE, mast cells are activated. Initial stimulation leads to the release of preformed chemical mediators, followed by synthesis of newly-formed chemical mediators such as chemokines and cytokines. These factors result in a myriad of immune responses that act on the surrounding microenvironment.

Statins

Statins are a class of drugs widely used in the treatment of hypercholesterolemia and cardiovascular disease, cardiovascular disease being the leading cause of deaths in the western world (25). Statins act by competitively inhibiting the 3-hydroxy -3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme and subsequently reduces intermediates, downstream of HMG-CoA, resulting in reduced cholesterol (25). Statin chemical structure plays a role in hydrophobicity and consequently, their ability to be absorbed and metabolized, distributed and excreted (26). Recently, it has become apparent that statins exhibit anti-oxidant, anti-atherosclerotic, anti-thrombotic and immunomodulatory functions in addition to lipid lowering {Liao:2005eo}(27). In the rabbit atherosclerosis model, atorvastatin significantly reduced neointimal inflammation and macrophage infiltration (28). Lovastatin has been shown to decrease surface expression of CD11b on monocytes and CD11b-dependent adhesiveness to fixed endothelium (29).

These immunomodulations have been attributed to the fact that the cholesterol biosynthesis pathway also generates a series of vital isoprenoids 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 affect more than just cholesterol formation and exert additional pleiotropic effects.

It has been shown that statins can suppress TNF and IL-1 β production

from macrophages (30) and that statins can suppress mast cell degranulation in rat cell lines (26). In spite of these revelations, the mechanism has yet to be elucidated. Thus, outlining the mechanisms whereby statins alter mast cell responses may prove to be crucial in terms of alternative therapies for allergic disease.

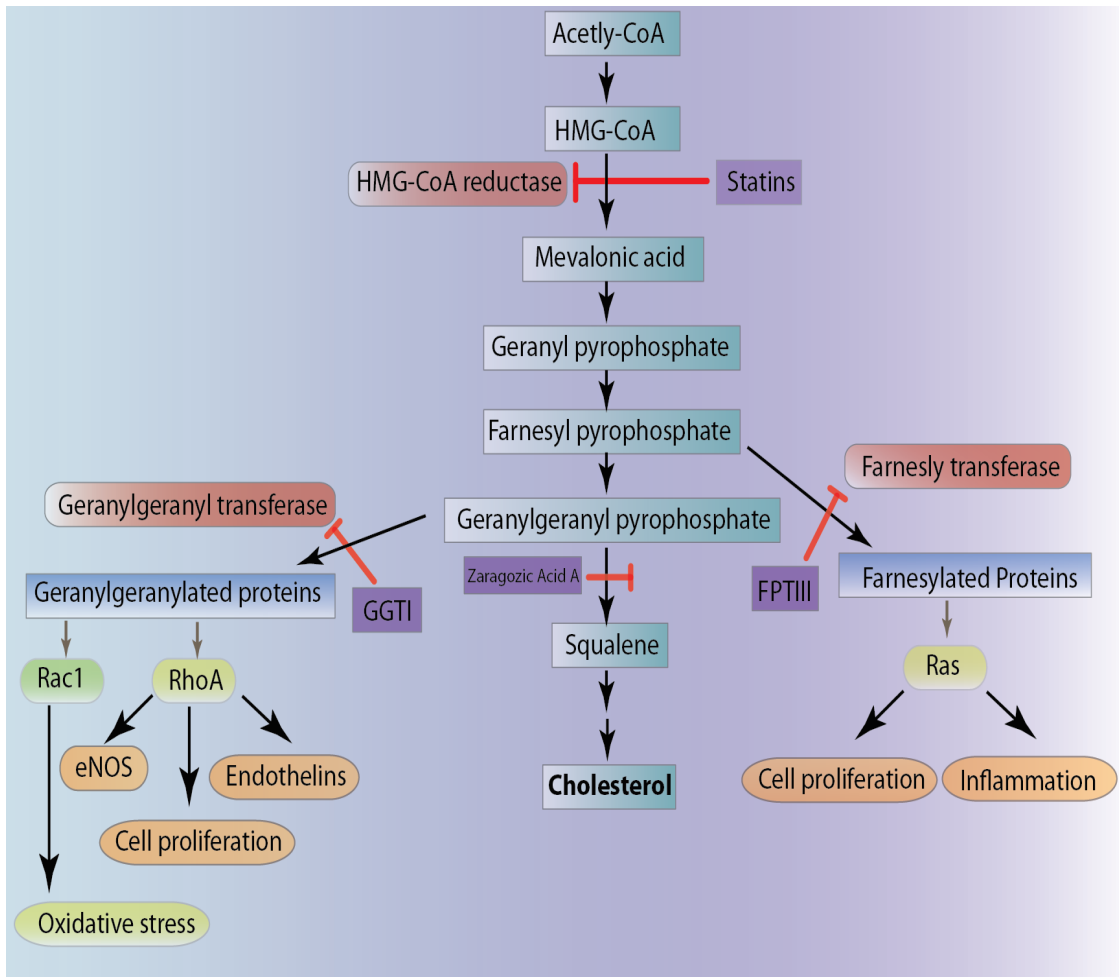


Figure 4

The cholesterol biosynthesis pathway.

The cholesterol biosynthesis pathway or mevalonic acid pathway.

The mevalonic acid pathway is required for the biosynthesis of a range of important molecules, namely cholesterol and the generation of prenylated proteins. Statins are competitive inhibitors of HMGR and decrease intermediates in the mevalonate pathway.

CHAPTER 2 – METHODS & MATERIALS:

Animals

C57BL/6, 129/SvImJ (henceforth referred to as 129/sv) , Balb/c, A/HeJ and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 12 weeks old, with approval from the Virginia Commonwealth University institutional animal care and use committee (IACUC).

Cells

Mouse bone marrow-derived mast cells (BMMCs) were extracted from mice femurs and cultured for 21 days in complete RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM HEPES ((cRPMI); all materials from Biofluids, Rockville, MD), supplemented with IL-3-containing supernatant from WEHI-3 cells and stem cell factor (SCF)-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF was adjusted to 1 or 10 ng/ml, respectively as measured by ELISA. Mouse bone marrow-derived basophils were cultured in cRPMI supplemented with recombinant IL-3 at 20ng/ml (Biolegend, San Diego, CA), for 7-10 days, then sorted by flow cytometry selecting for CD49b-positive cells (Biolegend).

Human mast cell 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 or from the National Disease Research Interchange. Skin MCs were prepared and cultured as described previously (31) and were used after 6–10 week, at which time purity was essentially 100% mast cells, as determined by staining with toluidine blue.

IgE-mediated activation

Human MC or BMMC were sensitized overnight with DNP-specific mouse IgE (1.0 μ g/ml for human MC; 0.5 μ g/ml for BMMC), washed to remove excess unbound IgE and stimulated with DNP-HSA (Ag; 30 or 20 ng/ml for human MC or mouse BMMC, respectively). Ionomycin (1 μ M) was used as positive control.

Passive Systemic Anaphylaxis

Mice were administered 200 μ l of PBS containing 1mg fluvastatin or equivalent dilution of DMSO via intraperitoneal injection, followed by 200 μ l of PBS containing 50ng of mouse anti-IgE. The following day, DNP-HSA 50 μ g/ml was administered via intraperitoneal injection. In some experiments, 5mg of histamine was injected in place of antigen. The core body temperature of each mouse was measured using a rectal microprobe (Physitemp Instruments) at regular intervals. Mice were euthanized using with CO₂ asphyxiation, and blood was collected by cardiac puncture to analyze plasma.

Cytokines and reagents

All cytokines, including murine IL-3, SCF, were purchased from Biolegend. Mouse IgE was generously provided by Dr. Daniel Conrad (VCU). Purified mouse IgE (clone C38-2, κ isotype) was purchased from BD Biosciences (Pharmingen division, San Diego, CA). Antibodies recognizing mouse CD49b, CD107a, CD63, TNF α , IL-4 and IL-6 were purchased from Biolegend. Mouse anti c-Kit, Fc ϵ RI, IL-13, MIP-1 α , MCP-1, p AKT and p-SYK were purchased from eBioscience. Mouse anti-p-ERK was purchased from BD Pharmingen (San Diego, CA). Mouse anti-IL-33 was purchased by R&D systems. Propidium Iodide and DNP-HSA was purchased from Sigma-Aldrich (St. Louis, MO). Caspase 3/7 kit was purchased from Immunochemistry. Cyto ID autophagy detection kit was from Enzo Life Sciences (Farmingdale, NY). The following drugs were used: Fluvastatin (*SML0038*), Simvastatin (S6196), Pravastatin (P4498), Atorvastatin (PZ0001), Lovastatin (PHR1285), Mevalonic acid (44714), Zaragozic acid A (Z2626), all from Sigma-Aldrich. Farnesylation transferase inhibitor III (FPTIII) and geranylgeranyl transferase inhibitor-286 (GGTI-286) were purchased from Calbiochem (Darmstadt, Germany). Farnesyl diphosphate and geranyl geranyl diphosphate were purchased from Echelon (Salt Lake city, UT). Wnt5a and Leptin were purchased from R&D systems (Minneapolis, MN).

Peritoneal mast cell culture

Peritoneal lavage was performed on C57BL/6 and 129/sv mice. Cells were harvested and cultured in cRPMI (as described above) containing IL-3 and SCF

at 10ng/ for 5 days to allow for expansion of mast cells. Mast cells were positively selected and separated using the EasySep Magnet from StemCell Technologies (Vancouver, BC) using c-Kit as a positive marker of mast cells. Flow cytometry was used as confirmation of mast cells.

Cytokine measurement

BMMC treated with fluvastatin or DMSO were cultured in cRPMI 1640 with 10 ng/mL IL-3 and SCF +/- 0.5 µg/mL IgE for 24 hours at a concentration of 1×10^6 cells/mL. BMMC were washed in PBS, resuspended at 1×10^6 cells/mL in cRPMI 1640 with 10 ng/mL IL-3 and SCF +/- 50 ng/mL DNP-HSA for 16 hours, after which supernatants were taken. IL-6, IL-13, TNF- α , and MCP-1 supernatant levels were measured by ELISA kits (Biolegend). ELISAs were developed using BD OptEIA reagents from BD Biosciences.

Flow cytometric analysis

Surface expression of c-Kit and Fc ϵ RI were measured by flow cytometry on a BD FACScalibur. BMMC were cultured in fluvastatin at 10µm/ml or DMSO at varying concentrations for varying times. Cells were then washed in PBS. For directly-labelled antibody staining, cell pellets were incubated in 10µL 2.4G2 rat anti-mouse Fc γ RII/III culture supernatant with PE- anti-c-Kit and FITC- anti-Fc ϵ RI, then incubated for 30 minutes at 4°C, washed in FACS buffer (PBS, 3% FBS, 0.1% Sodium Azide), and analyzed by flow cytometry.

In-cell staining for cytokines

Cells treated with 10µm/ml fluvastatin or DMSO ± IgE at 0.5µg/ml for 24 hours were washed and resuspended in cRPMI containing IL-3 and SCF at 10ng/ml. BMDC were given DNP-HSA for 90 minutes, then treated with 5µM Monensin for 8 hours, fixed in 4% paraformaldehyde, washed twice in PBS and stored overnight at 4°C. Cells were then pelleted and resuspended in saponin buffer (PBS, 0.1% BSA, 0.01M HEPES, 0.5% saponin) for 20 minutes at room temperature. Cell pellets were incubated in 10 µL 2.4G2 rat anti-mouse FcγRII/III culture supernatant with APC- or PE -anti- TNF, IL-6, IL-13, MCP-1, MIP-1, IL-33 or IL-4 in saponin buffer at 4°C for 30 minutes. Basophils were also stained with FITC- anti-CD49b as a marker of basophils prior to fixation.

Degranulation assays

Cells plated at 1×10^6 cells/mL in cRPMI (as described above) and treated with fluvastatin or DMSO for 24 hours ± 0.5 µg/ml of IgE, were washed twice in RPMI and activated ± DNP-HSA for 1 hour and then stained with CD107a or CD63 for 45 minutes at 4°C. cells were then washed twice in FACS buffer and analysed by flow cytometry.

Cell death

Cells were treated with 5µM, 10µM, 20µM or 40µM fluvastatin for 1 to 4 days, then assessed for cell death via caspase-3/7 staining using the methods described in the millipore kit. PI-exclusion yields a live versus dead determination. Prepare Propidium iodide solution to 200 µl/ ml and add 10

microlitres per 200 μ l of cells (resuspended at 1×10^6 cells/mL) and run by flow cytometry.

Autophagy detection

For autophagy detection using Cyto ID the detection kit, BMMC were resuspended at 5×10^5 cells/mL and treated with 5 μ M, 10 μ M, 20 μ M and 40 μ M fluvastatin for 1-3 days, then pelleted and stained with the Cyto ID kit using the method described in the kit.

Migration assay

8 μ m polycarbonate 24-well transwell inserts from Corning will be coated in BSA, plates were incubated for 1 hour at 37°C. BMMC were resuspended at 2×10^6 cells/mL in FBS-free cRPMI for 2 hours. Bottom chambers contained 850 μ L of FBS-free cRPMI with IL-3 at 0.5 ng/mL +/- 50 ng/mL SCF in the bottom well and 200 μ L of the previously starved BMMC supplemented with 0.5 ng/mL IL-3 in the upper well. Cells were then incubated for 16 hours at 37°C, then counted using flow cytometry with propidium-iodide exclusion staining. Fold of control was calculated for all groups.

HMG-CoA reductase qPCR

BMMCs were cultured with or without 40 μ M fluvastatin for 6 hours. Then, cells were harvested and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized using the qScript

microRNA cDNA Synthesis Kit (Quantabio, Gaithersburg, MD) following the manufacturer's protocol using oligo dT primers provided in the kit. cDNA was quantified using the Thermo Scientific NanoDrop™ 1000 UV-vis Spectrophotometer (Thermo Scientific, Waltham, MA) according to manufacturer's recommended protocol. qPCR analysis was performed with Bio Rad CFX96 Touch™ Real-Time PCR Detection System (Hercules, CA) and SYBR® Green detection using a relative Livak Method. Each reaction was performed according to the manufacturer's protocol using 8ng of sample cDNA, 12.5 µl of PerfeCTa SYBR Green SuperMix (Quantabio, Gaithersburg, MD) and mmu-miR-155 or SNORD47 (housekeeping gene) primers (Quantabio, Gaithersburg, MD) in a final reaction volume of 10 µM. Amplification conditions for all reactions consisted of a heat-activation step at 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 70 °C for 15 s. Fluorescence data was collected during the extension step of the reaction.

Western blot analysis

Western blotting was performed using 50µg total cellular protein per sample. Protein was loaded and separated over 8–16% or 4–20% gradient SDS polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membranes (Pall Corporation, Ann Arbor, MI), and blocked for 60 minutes in Blotto B buffer (Rockland Immunochemicals, Gilbertsville, PA) plus 0.1% Tween-20. Blots were incubated in a solution of TBS supplemented with 0.1% Tween-20 and 5% BSA (TBST), with the indicated antibodies overnight at

4°C with gentle rocking. Blots were washed six times for 10 minutes each in TBS-T, followed by incubation in Blotto B containing a 1:5,000 dilution of HRP linked anti-IgG matched to the relevant species, from Cell Signaling (Danvers, MA). Size estimates for proteins were obtained using molecular weight standards from Bio-Rad (Hercules, CA).

Statistical Analysis

Data presented are the mean \pm SEM of at least 3 independent experiments. P values were calculated by paired or unpaired, two-tailed Student's *t* test as appropriate. P values of <0.05 were considered statistically significant using GraphPad Prism software.

CHAPTER 3 – RESULTS:

The effect of statins on IgE activated BMMC's

Simvastatin and lovastatin are the most commonly prescribed statin for the treatment of dyslipidemia. It has previously been shown that lovastatin alters isoprenoid generation in rat basophil leukemia cells (RBL-2H3) (32). We panned for a range of statins, in addition to lovastatin; to assess which exert the largest response on IgE activated mast cells. C57BL/6 bone marrow derived mast cells (BMMC's) were pretreated for 24 hours with lipophilic statins (lovastatin, simvastatin, atorvastatin, pitavastatin and fluvastatin) and the hydrophobic statin (pravastatin). BMMC's treated with lipophilic statins significantly suppressed IgE mediated mast cell IL-6, TNF α and IL-13 (Figure 5) production, with the exception of atorvastatin. The hydrophobic pravastatin showed no significant effects on cytokine production but actually slightly enhanced IL-6 and TNF α . Fluvastatin, simvastatin and lovastatin were the most effective at suppressing cytokine production but fluvastatin elicited the strongest response and is the focus of this study.

To determine the kinetics for the effects of fluvastatin on IgE mediated cytokine production, we conducted a time course (Figure 6), and dose response (Figure 7) assays for IL-6, TNF α and IL-13. We established that the effect of fluvastatin on mast cell cytokine production is dose dependent and that 10 μ M fluvastatin suppressed cytokine production on average 50%. Fluvastatin cultured for 24-hours yielded significant suppression of pro-inflammatory cytokines and while suppression is greatest at 72 hours, a large portion of cells are dead at this

point. Interestingly, fluvastatin has no effect on IL-10 production (Figure 8), which in many cases is seen as an anti-inflammatory chemical mediator.

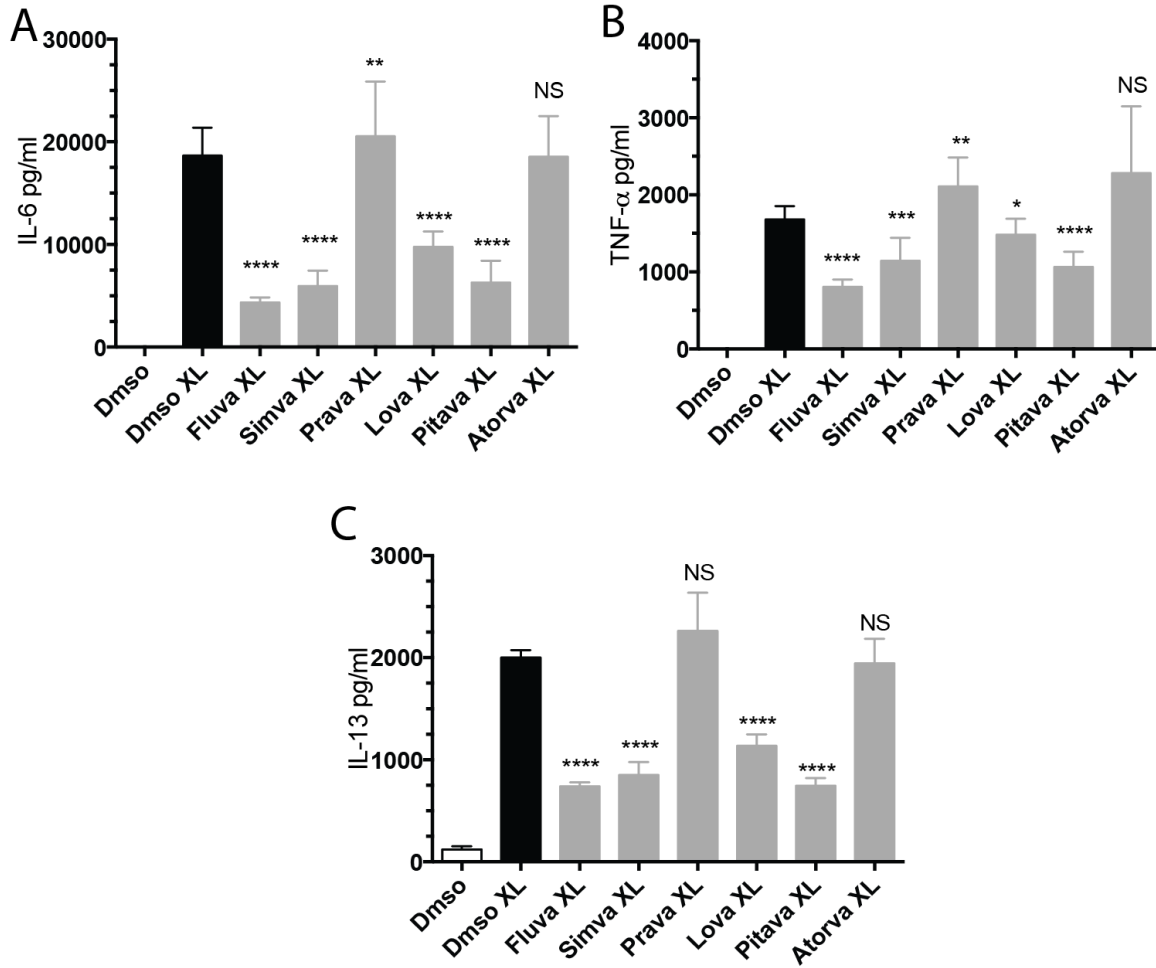


Figure 5

Fluvastatin suppress cytokine production from IgE activated mast cells to a greater extent than other statins.

IgE primed C57BL/6 BMBC's were cultured in IL-3 and SCF, with or without DMSO or 10 μ M statin and then activated with DNP-HSA for 16 hours. Supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine A) IL-6, B) TNF α , C) IL-13 concentrations. The results are expressed as the mean \pm SEM of at least 3 independent experiments conducted in triplicate.

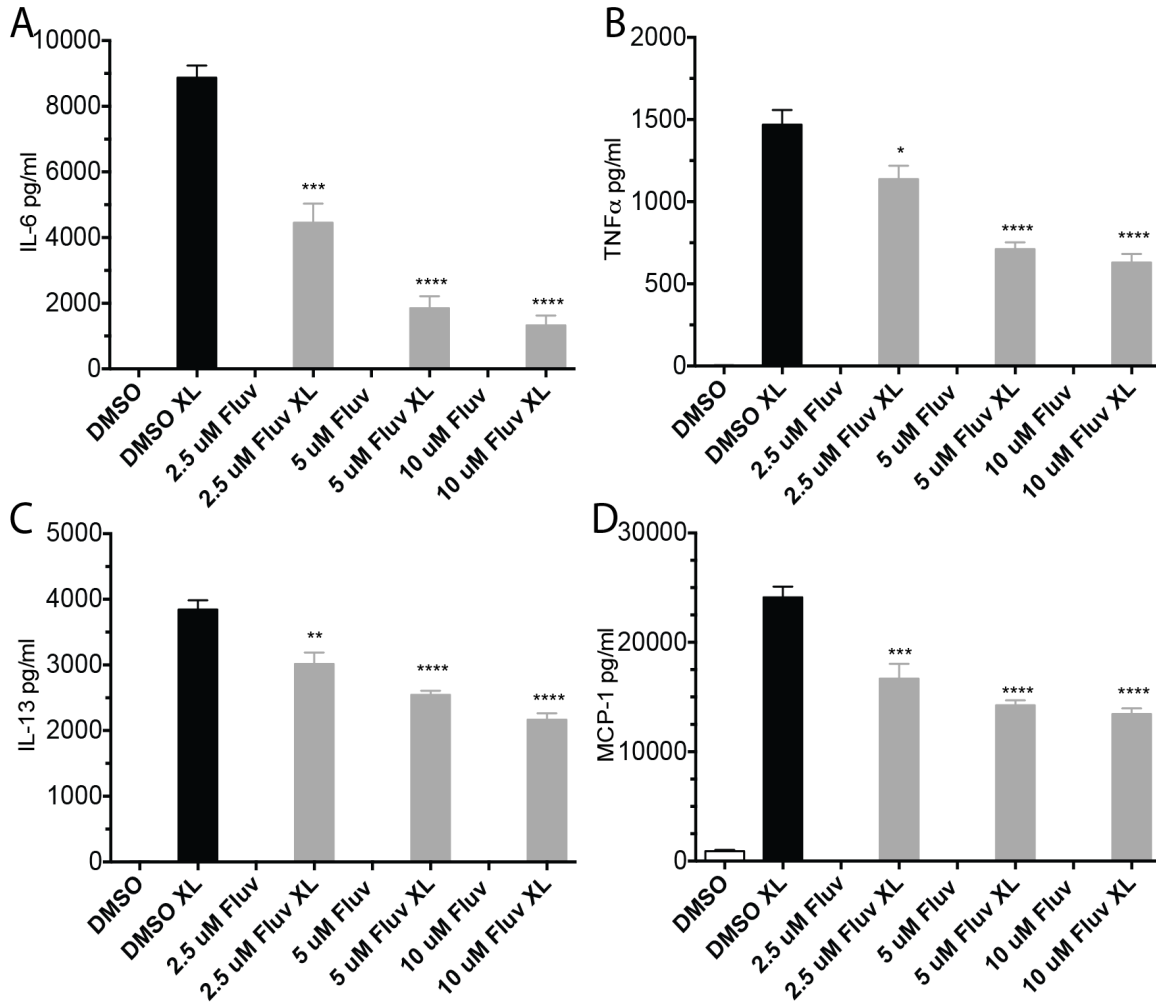


Figure 6

Fluvastatin suppresses IgE activated BMDC's in a dose dependent manner.

IgE primed C57BL/6 BMDC's were cultured in IL-3 and SCF, with or without fluvastatin or DMSO and then activated with DNP-HSA for 16 hours. Supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine A) IL-6, B) TNFα, C) IL-13 concentrations. The results are expressed as the mean ± SEM of at least 3 independent experiments conducted in triplicate.

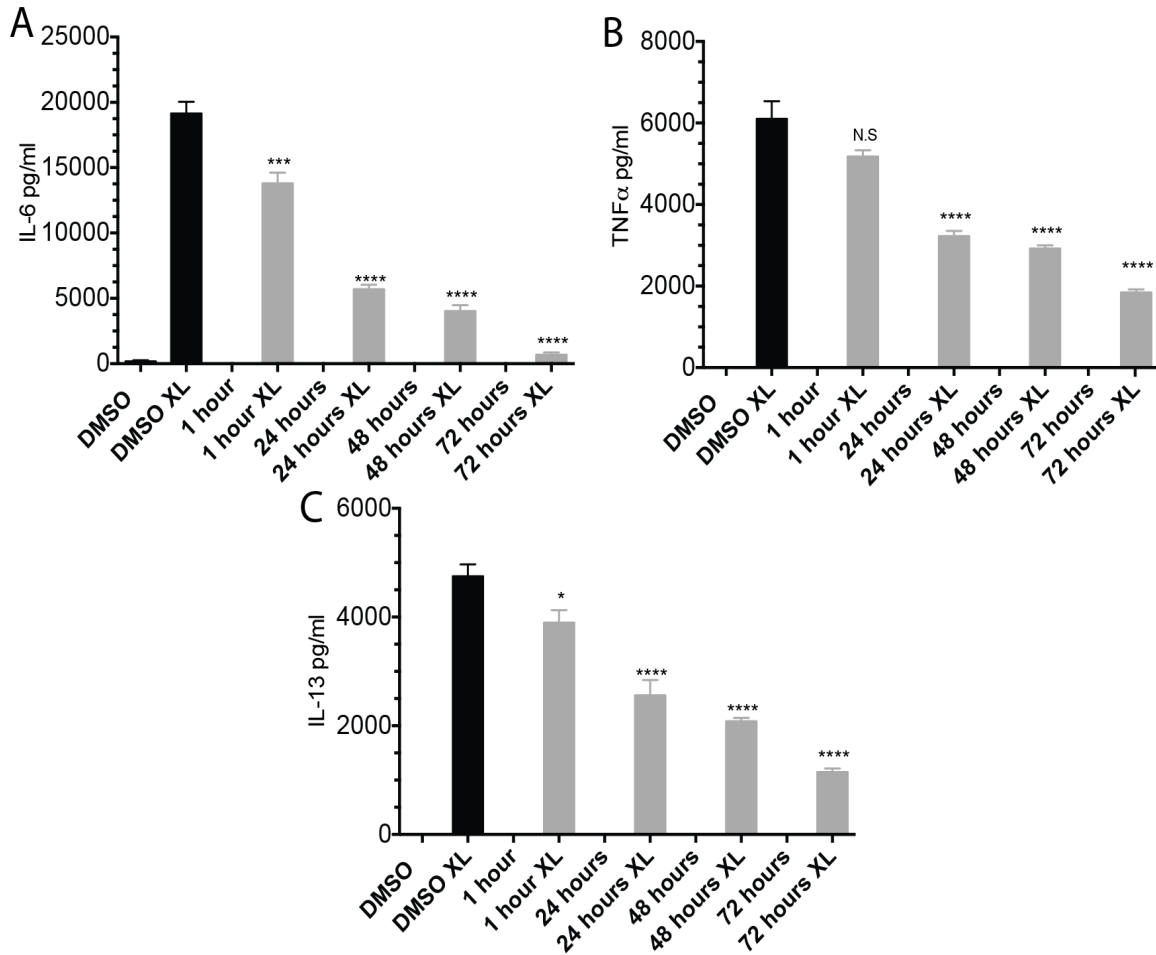


Figure 7

Fluvastatin suppression of IgE activated BMMC's increases with time.

IgE primed BMMC's were cultured in IL-3 and SCF, with or without fluvastatin 10 μ M or DMSO for the stipulated time periods and then activated with DNP-HSA for 16 hours. Supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine A) IL-6, B) TNF α , C) IL-13, D) MCP-1 concentrations. The results are expressed as the mean \pm SEM of at least 3 independent experiments conducted in triplicate.

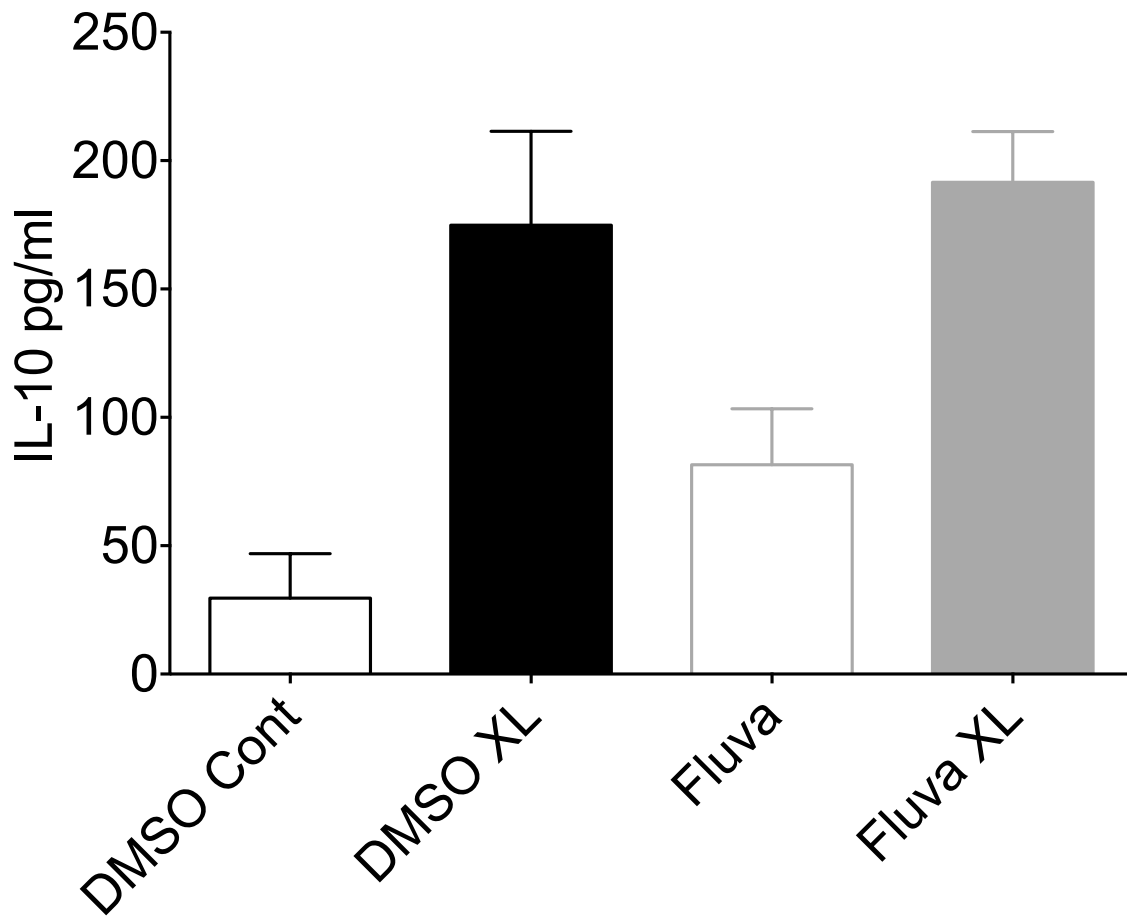


Figure 8

Fluvastatin does not alter IgE mediated IL-10 production in BMMC's

IgE primed C57BL/6 BMMC's were cultured in IL-3 and SCF, with or without a given 10 μ M fluvastatin or DMSO and then activated with DNP-HSA for 16 hours. Supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine IL-10 concentration. The Graph is a representation of 3 independent experiments with an n=9. Data shown are mean \pm SEM.

The effect of fluvastatin on peritoneal mast cells

Thus far, we have showed that fluvastatin suppresses pro inflammatory cytokines on BMMC's that have been cultured in vitro in IL-3 and SCF. To determine if ex vivo mast cells respond in the same way as BMMC's cultured with fluvastatin we assayed peritoneal mast cells. We conducted peritoneal lavage and cultured cells in IL-3 and SCF for 3 days and then isolated and purified the mast cell populations. IgE sensitized mast cells were then cultured in fluvastatin for 24 hours and activated with antigen (DNP-HSA). Here, we show that mast cells cultured ex vivo when treated with fluvastatin suppress cytokine production in the same way as in vitro cultures BMMC's. (Figure 9-10).

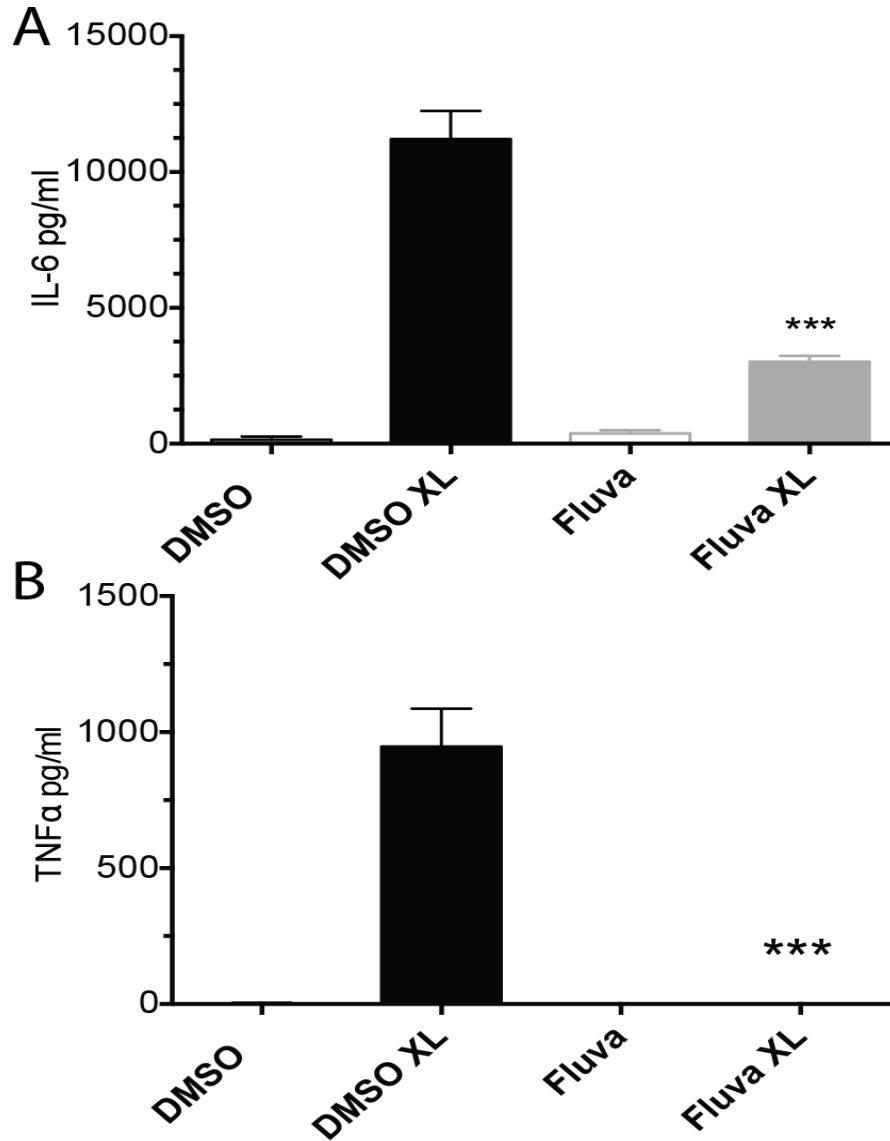


Figure 9

Fluvastatin suppress IL-6 and TNF α production from IgE activated peritoneal mast cells.

Peritoneal mast cells were separated using easySep magnet selecting for c-kit positive cells. Mast cells were then cultured in IL-3 and SCF with or without fluvastatin or DMSO in IgE over night and then activated with DNP-HSA for 16 h

and supernatants were collected as described in the methods and materials. A) IL-6, B) TNF α . The results are expressed as the mean \pm SEM of at least 3 independent experiments conducted in triplicate.

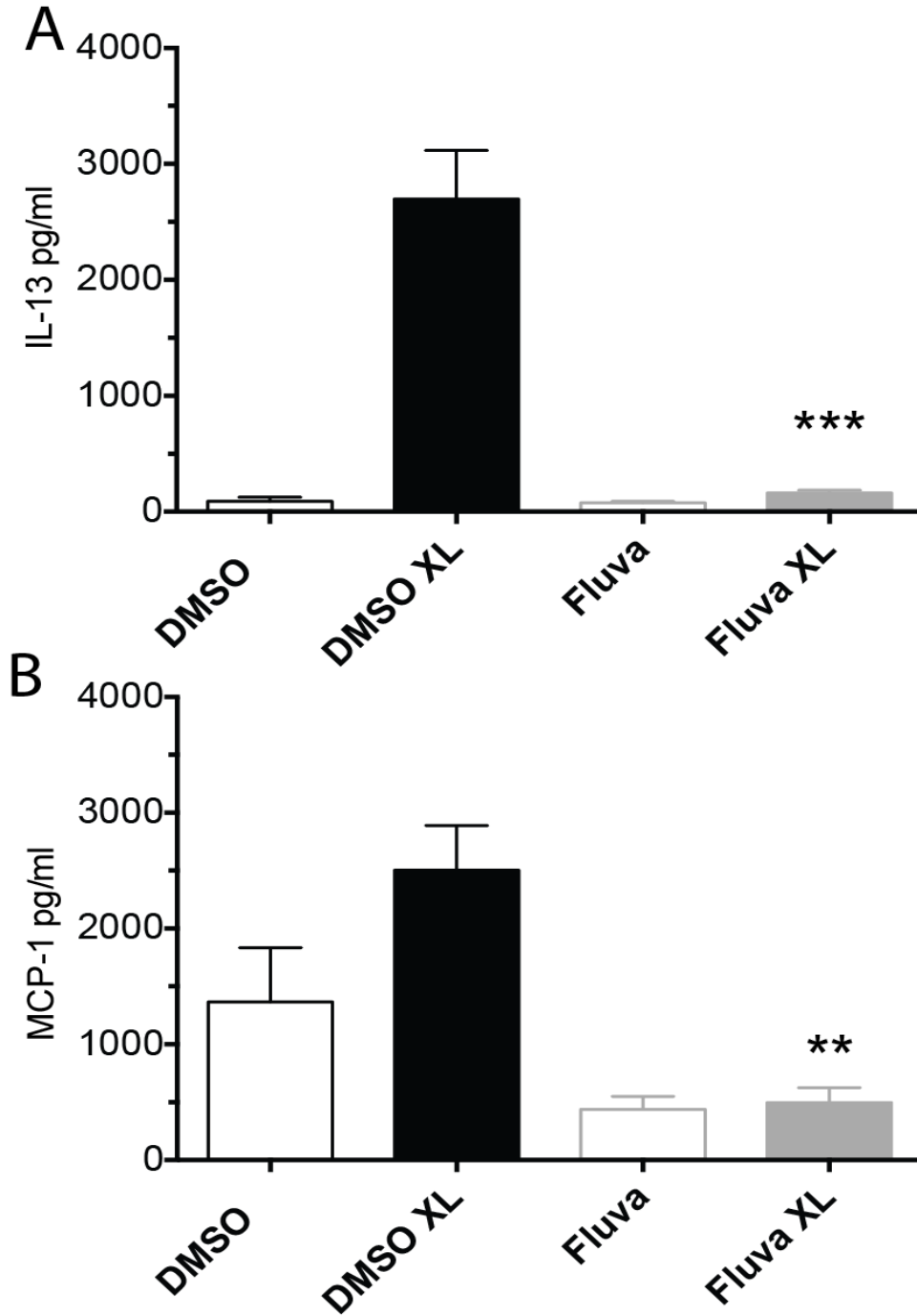


Figure 10

Fluvastatin suppress IL-13 and MCP-1 production from IgE activated peritoneal mast cells.

Peritoneal mast cells were separated using easySep magnet selecting for c-kit positive cells. Mast cells were then cultured in IL-3 and SCF with or without fluvastatin or DMSO in IgE over night and then activated with DNP-HSA for 16 h and supernatants were collected as described in the methods and materials. A) IL-13, B) MCP-1. The results are expressed as the mean \pm SEM of at least 3 independent experiments conducted in triplicate.

The effect of fluvastatin on FcεRI and c-kit receptor expression.

The suppressive effect of fluvastatin on C57BL/6 BMMC's may manifest from altered FcεRI expression. We proceeded by conducting a dose response of fluvastatin for 4 days and assessed FcεRI expression by flow cytometry. Here, we show that fluvastatin treatment does not significantly alter FcεRI expression in C57BL/6 (Figure 11) BMMC's. We also investigated the effect of fluvastatin on c-kit receptor expression since it has been shown that BMMC's cultured with SCF (the c-kit ligand) increase cytokine production. Our data show that fluvastatin does not alter c-kit surface receptor expression on C57BL/6 (Figure 11) BMMC's.

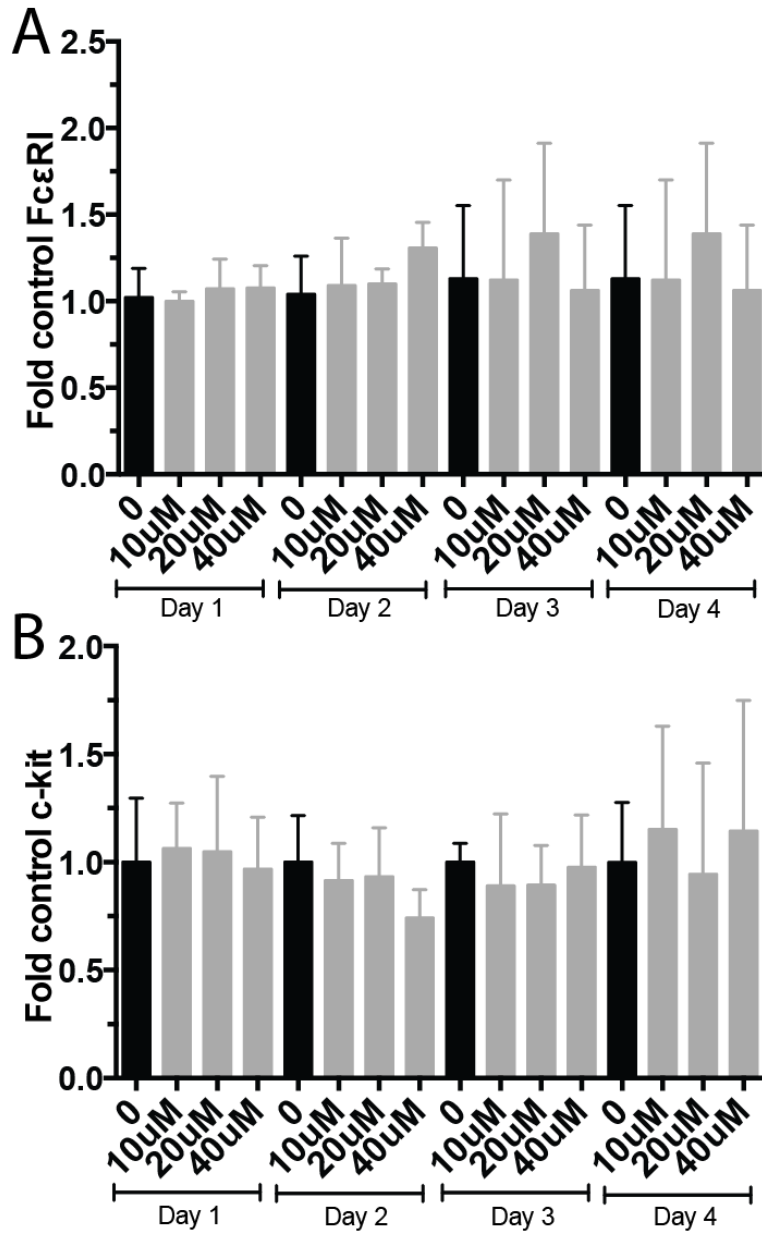


Figure 11

Fluvastatin does not alter Fc ϵ RI or c-kit surface expression on C57BL/6

BMMC's.

C57BL/6 BMMC's were treated with DMSO or fluvastatin at 5 μ M, 10 μ M, 20 μ M and 40 μ M for 1-4 days and surface expression of A) Fc ϵ RI, B) c-kit expression were measured by flow cytometry. The results are expressed as the mean \pm SEM of 3 independent experiments.

The effect of fluvastatin on IgE mediated mast cell cytokine production is counteracted by mevalonic acid (MVA)

Fluvastatin acts by blocking HMG-CoA reductase and inhibiting the production of mevalonic acid (MVA). To assess fluvastatin's target specificity we treated BMMC's with fluvastatin and MVA to see if MVA reversed the effects of fluvastatin. Here, we show that fluvastatin suppresses IgE mediated mast cell production of pro inflammatory cytokines and chemokines and is rescued by mevalonic acid treatment (Figure 12-13).

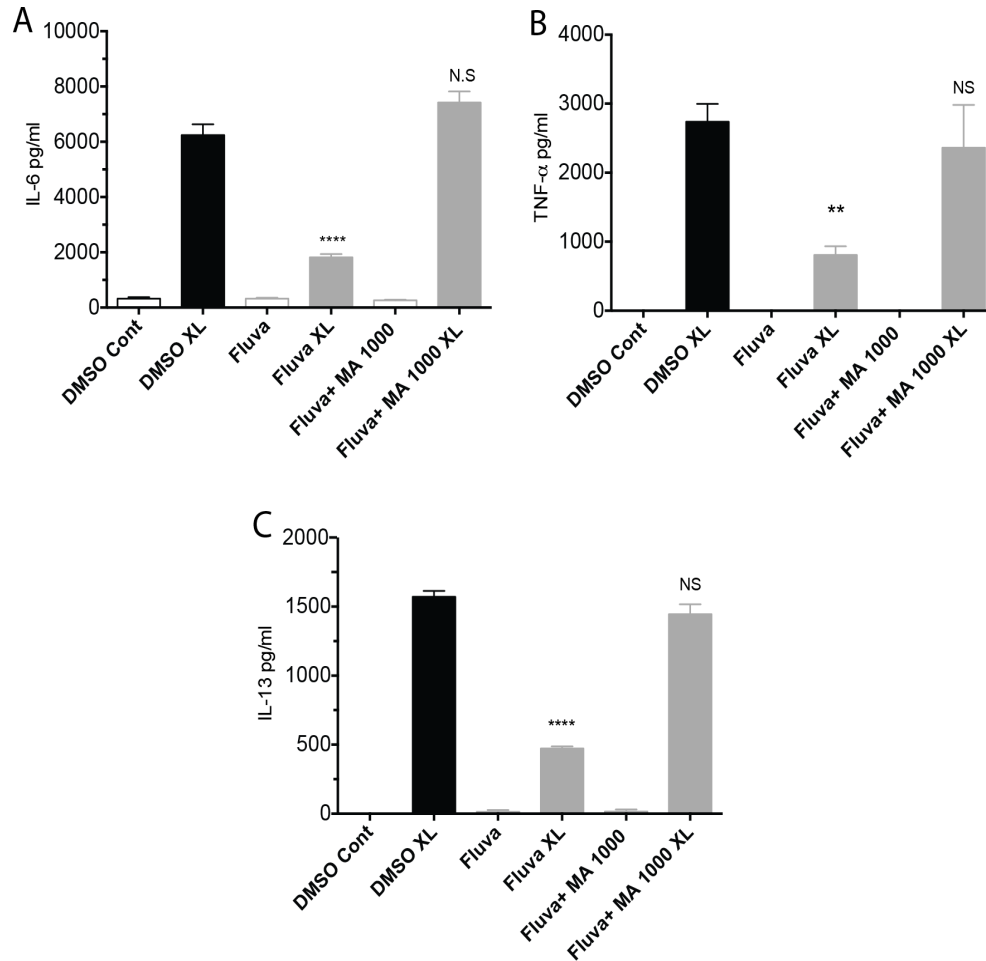


Figure 12

Fluvastatin induced suppression of IgE mediated cytokine production in mast cells is rescued by mevalonic acid.

C57BL/6 BMMC's were cultured in IL-3 and SCF, with or without fluvastatin 10 μ M or DMSO for 24 h with or without mevalonic acid 1000 μ M and IgE sensitized. Mast cell cultures were washed in PBS and then treated again with fluvastatin 10 μ M or DMSO for 24 h with or without mevalonic acid and then given DNP-HSA for 16 h and supernatant taken for ELISA. A) IL-6, B) TNF α , C) IL-13. The Graph is a representation of 3 independent experiments with an n=9. Data shown are mean \pm SEM.

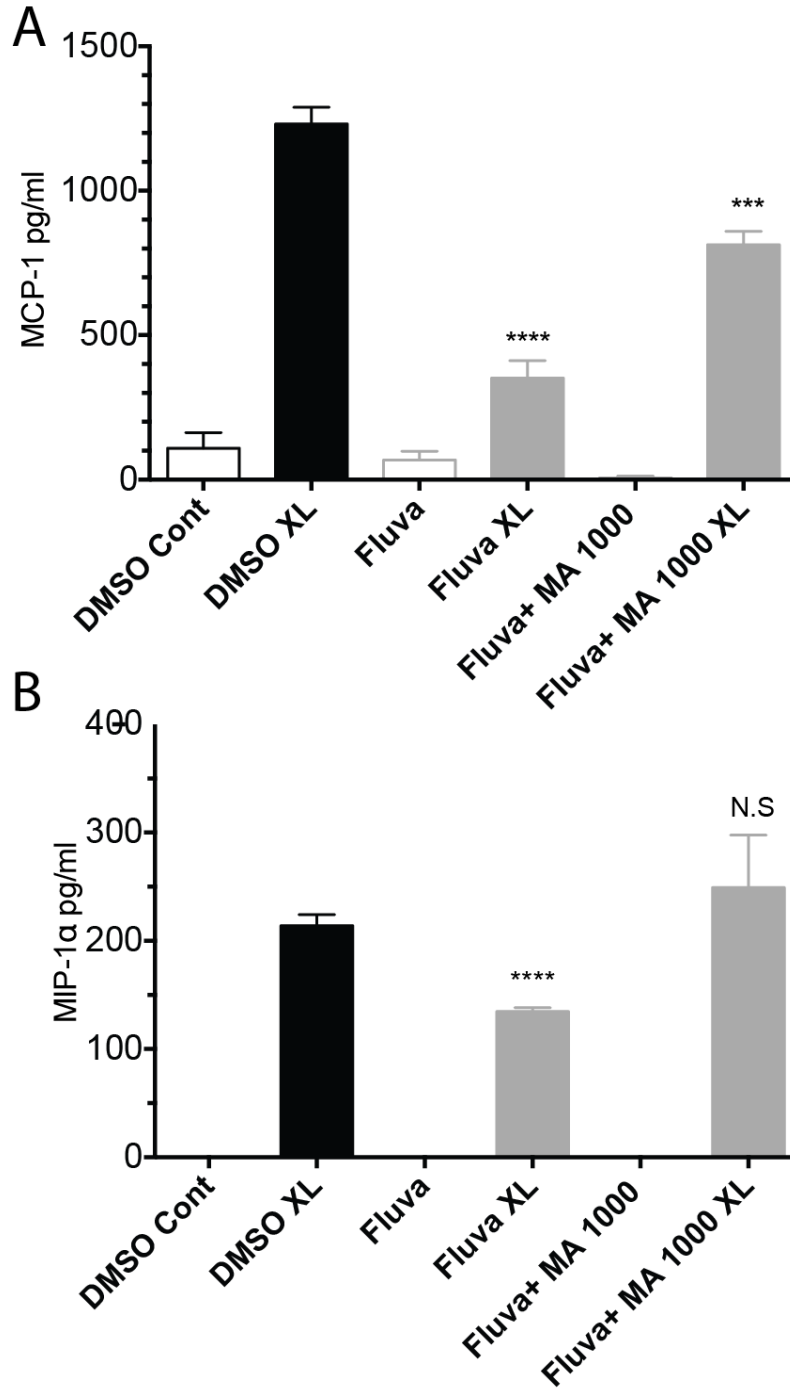


Figure 13

Fluvastatin induced suppression of IgE mediated chemokine production in mast cells is blunted by mevalonic acid.

C57BL/6 BMMC's were cultured in IL-3 and SCF, with or without fluvastatin 10 μ M or DMSO for 24h with or without mevalonic acid 1000 μ M and IgE sensitized. Mast cell cultures were washed in PBS and then treated again with fluvastatin 10 μ M or DMSO for 24 h with or without mevalonic acid and then given DNP-HSA for 16 hours and supernatant taken for ELISA. A) MCP-1, B) MIP-1 α . The Graph is a representation of 3 independent experiments with an n=9. Data shown are mean \pm SEM.

The effect geranylgeranyl pyrophosphate inhibitor and farnesyl pyrophosphate inhibitor on IgE mediated mast cell cytokine production.

MVA continues to be metabolized to the farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which leads to the production of prenylated proteins, which include the small GTPases such as Ras, Rac, Rho and CDC42. Thus, we investigated the effects of GGTI-286 and FPTIII, which selectively inhibit geranylgeranylation and farnesylation respectively. Here, we show that IgE sensitized mast cells pretreated with GGTI-286 for 24 hours significantly inhibit IgE induced cytokine production to a similar degree as fluvastatin treated mast cells (Figure 14-15). Treatment with fluvastatin and GGTI-286 suppress cytokine production more significantly than either drug alone. FPTIII treated mast cells show cytokine suppression to a lesser extent than fluvastatin for IL-6 and MCP-1 (Figures 14, and 15 respectively). For TNF α , IL-13 and MIP-1 α FPTIII shows no significant suppression (Figures 14-15 respectively). Additionally, BMMC's treated with both fluvastatin and FPTIII show suppressed cytokine levels to the same extent as fluvastatin alone, suggesting that the FPTIII arm of the cholesterol biosynthesis pathway does not significantly contribute to cytokine suppression. These data suggest that fluvastatin suppression of antigen induced cytokine production alters geranylgeranylation more so than farnesylation.

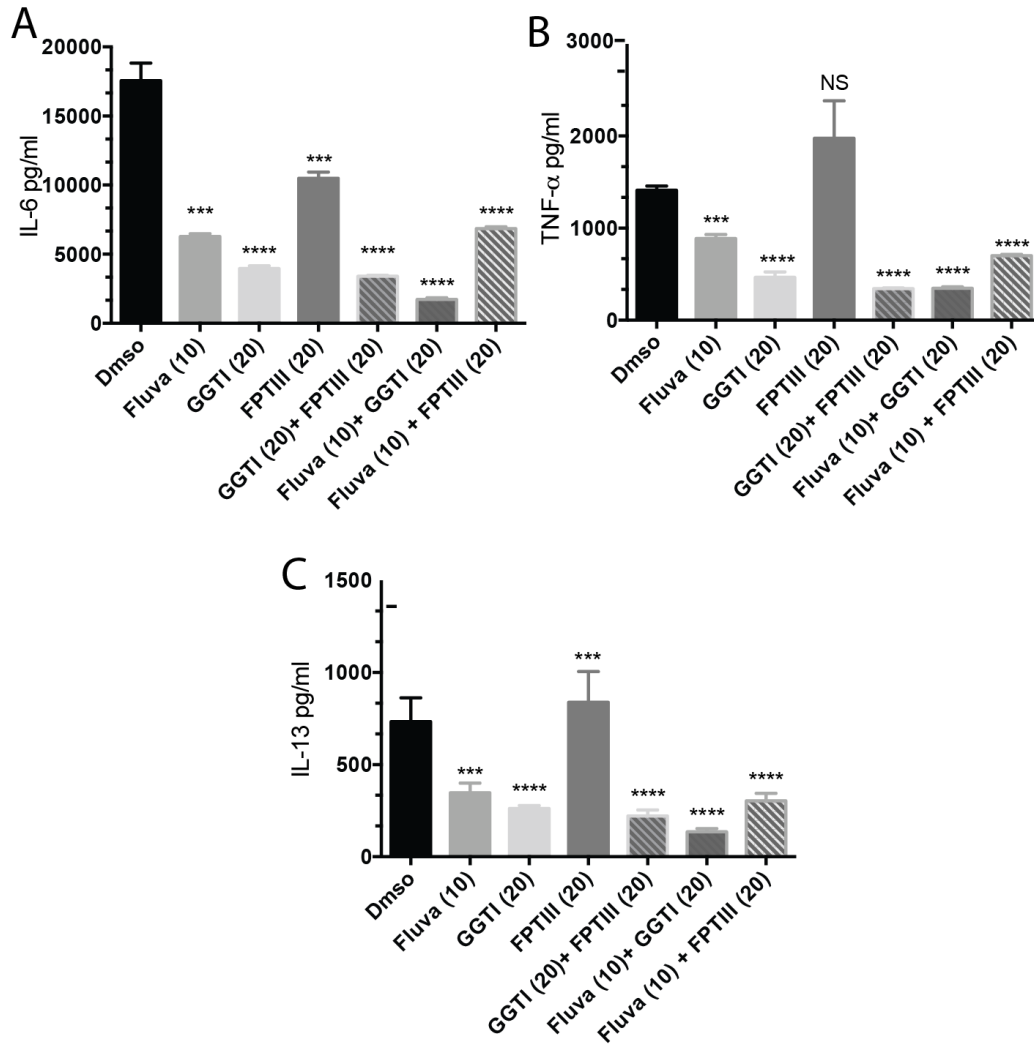


Figure 14

GGTI-286 significantly suppresses Ag induced cytokine production on BMDC and FPTIII to a lesser extent.

C57BL/6 BMDC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO, fluvastatin 10 μ M, GGTI-266 20 μ M or FPTIII 20 μ M or combinations for 24 hours and then stimulated with DNP-HSA (50ng/ml) for 16 hours. Supernatants were harvested and ELISA's were performed. A) IL-6, B) TNF α , C) IL-13. The Graph is a representation of at least 4 independent experiments with an n=12. Data shown are mean \pm SEM.

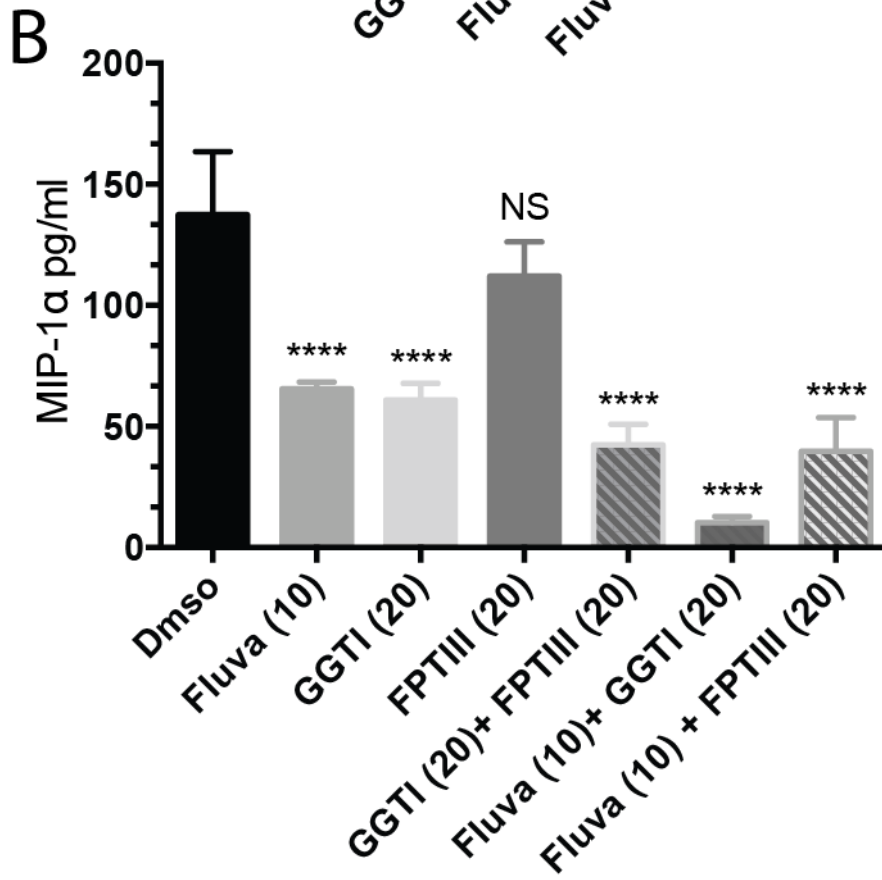
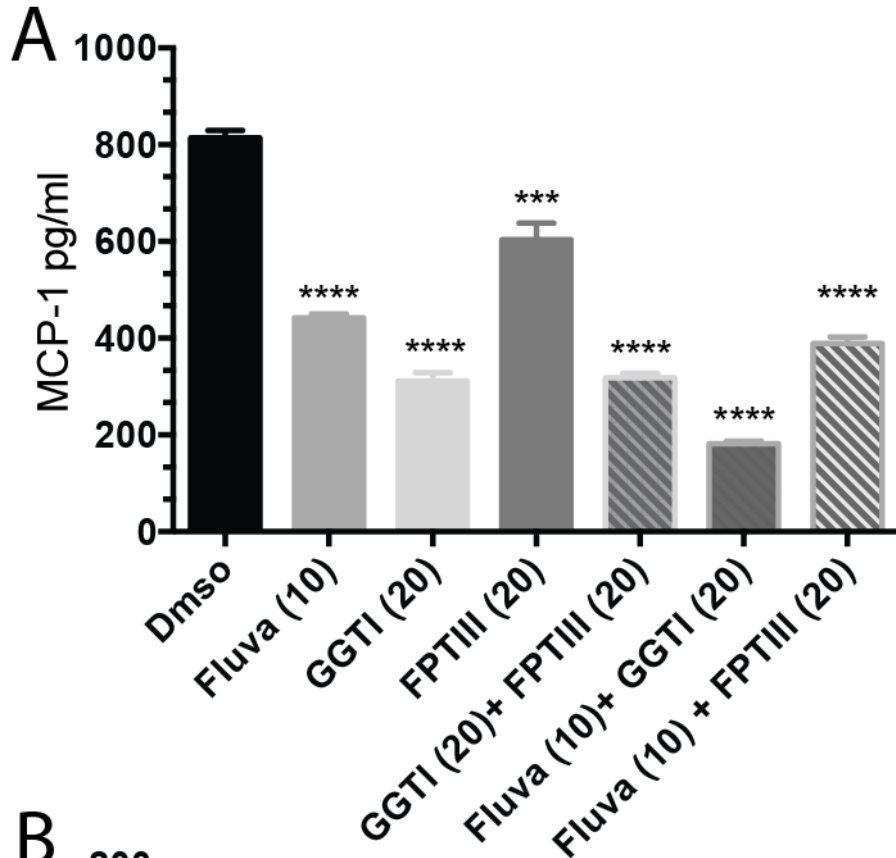


Figure 15

GGTI-286 and FPTIII suppress Ag induced chemokine production on BMMC.

C57BL/6 BMMC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO, fluvastatin 10 μ M, GGTI-266 20 μ M or FPTIII 20 μ M or combinations for 24 hours and then stimulated with DNP-HSA (50ng/ml) for 16 hours. Supernatants were harvested and ELISA's were preformed. A) MCP-1, B) MIP-1 α . The Graph is a representation of at least 4 independent experiments with an n=12. Data shown are mean \pm SEM.

The effect of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) on IgE mediated mast cell cytokine production in response to Fluvastatin treatment

Inhibition of MA also alters production of the intermediate isoprenoids GGPP and FPP. GGPP and FPP have been shown to affect posttranslational modification of signaling molecules including small G-proteins(25). GGPP is known solely for the production of prenylated proteins such as Rac Rho and Cdc42. FPP on the other hand, leads to the production of sterol and non-sterol products in addition to prenylation such as Ras. We therefore, sought to investigate which branch of the isoprenoid pathway was responsible for cytokine production suppression in mast cells. Following treatment with GGTI-286 and PTIII we shows that GGTI-286 treatment mimicked fluvastatin treated mast cells, suggesting fluvastatin acts by blocking production of geranylgeranylated proteins more so than farnesylated proteins. We then wanted to treat mast cells with exogenous GGPP and FPP and see if these treatments restored cytokine production in fluvastatin treated cells. Here, we show that treatment of IgE sensitized mast cells activated with antigen and pretreated with either GGPP or FPP has little effect to no effect on cytokine production (Figure 16-17). Treating mast cells with GGPP and fluvastatin shows a partial rescue of cytokine production in relation to fluvastatin alone treated mast cells, suggesting that fluvastatin acts predominately by blocking geranylgeranylated proteins. Treatment of fluvastatin with FPP shows little to no rescue of cytokine production in relation to fluvastatin alone treated mast cells (Figure 16-17). In addition

treatment of fluvastatin, GGPP and FPP are not statistical difference that fluvastatin and GGPP treated cells for IL-6, TNF α (Figure 16), IL-13 and MCP-1 (Figure 17).

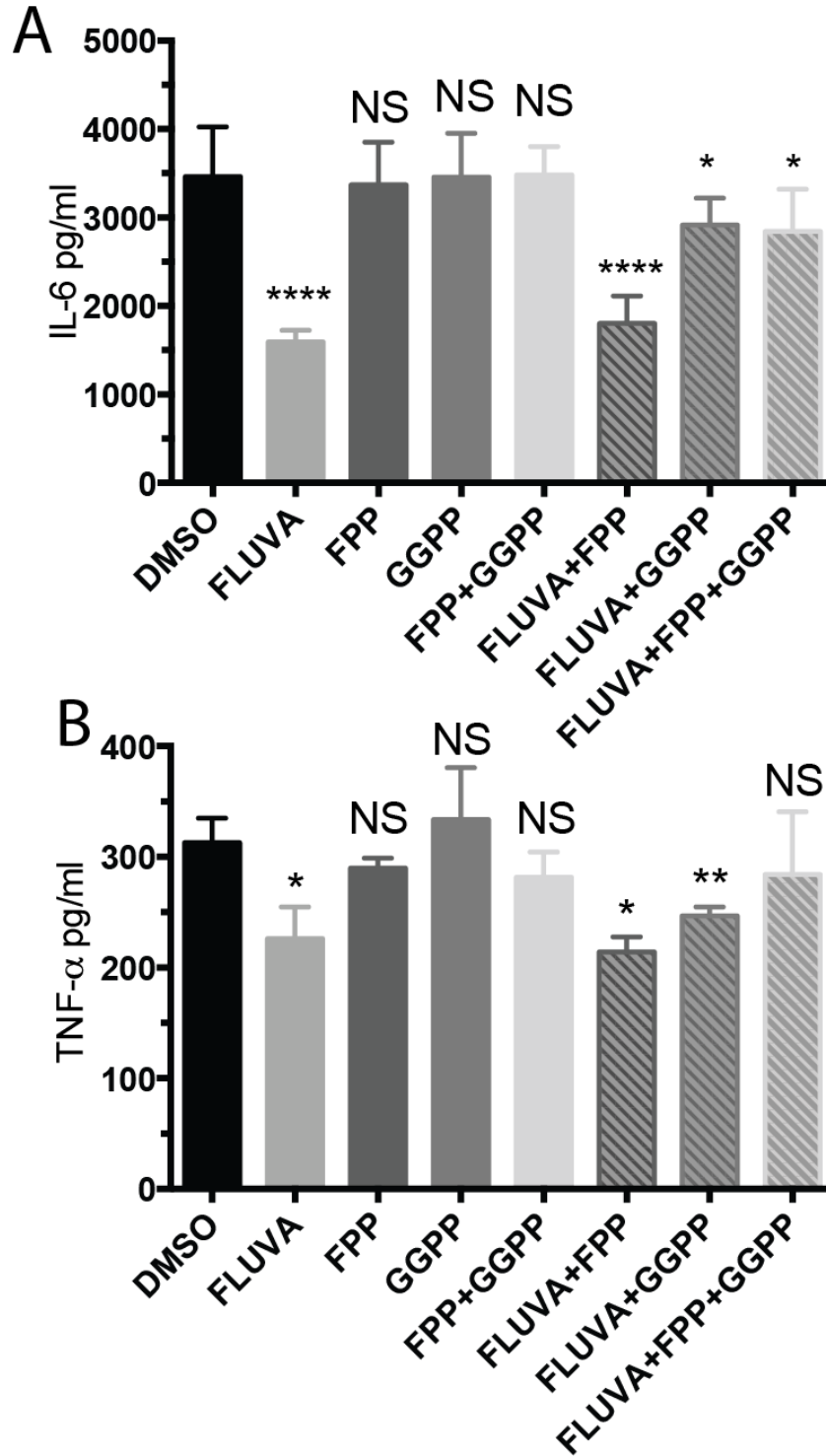


Figure 16

Geranylgeranyl pyrophosphate (GGPP) partially rescues Antigen induced IL-6 and TNF α production from fluvastatin treated BMMC's. C57BL/6

BMMC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO, fluvastatin 10 μ M, GGPP 20 μ M or FPP 20 μ M or combinations for 24 hours and then stimulated with DNP-HSA (50ng/ml) for 16 h. Supernatants were harvested and ELISA's were preformed. A) IL-6, B) TNF α . The Graph is a representation of 3 independent experiments with an n=9. Data shown are mean \pm SEM.

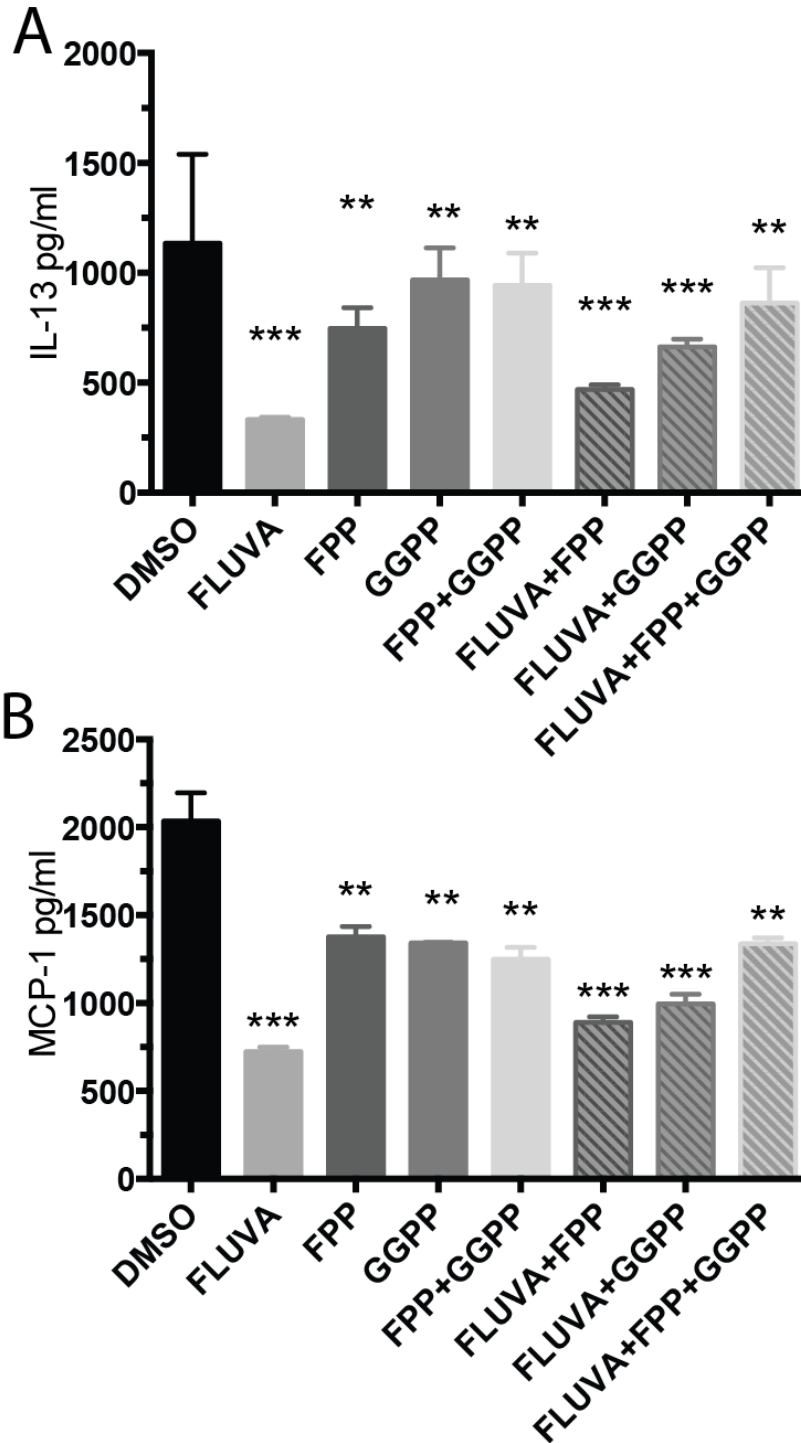


Figure 17

Geranylgeranyl pyrophosphate (GGPP) partially rescues Antigen induced IL-13 and MCP-1 production from fluvastatin treated BMMC's. C57BL/6

BMMC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO, fluvastatin 10 μ M, GGPP 20 μ M or FPP 20 μ M or combinations for 24 h and then stimulated with DNP-HSA (50ng/ml) for 16 h. Supernatants were harvested and ELISA's were performed. A) IL-13, B) MCP-1. The Graph is a representation of 3 independent experiments with an n=9. Data shown are mean \pm SEM.

The effect of zaragozic acid A on BMMC's cytokine production.

Thus far, we have demonstrated that isoprenoid generation is critical for IgE Induced cytokine production. We next investigated the potential role of cholesterol in the suppression of cytokines without altering isoprenoid production. To achieve this, we used the squalene synthase inhibitor zaragozic acid A (ZA), which blocks cholesterol synthesis downstream of isoprenoid synthesis. Here, we show that BMMC's treated with ZA does not alter cytokine production (Figure 18) and propose that fluvastatin's method of suppressing cytokines is independent of cholesterol.

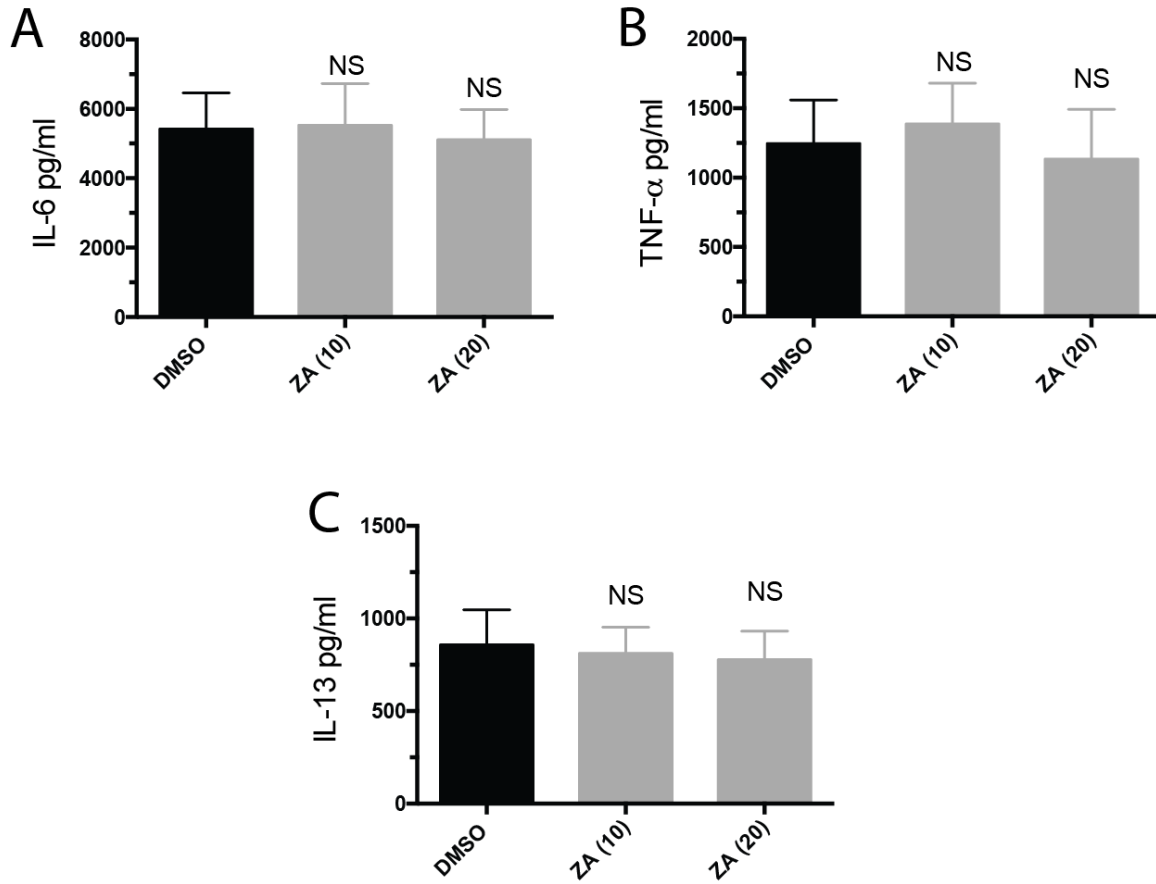


Figure 18

Zaragozic acid A does not alter cytokine production of IgE sensitized and Antigen activated BMBC's.

C57BL/6 BMBC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO or zaragozic acid A (ZA) for 24 h and then stimulated with DNP-HSA (50 ng/ml) for 16 h. Supernatants were harvested and ELISA's were preformed. A) IL-6, B) TNF α , C) IL-13. The Graph is a representation of 3 independent experiments with an n=9. Data shown are mean \pm SEM.

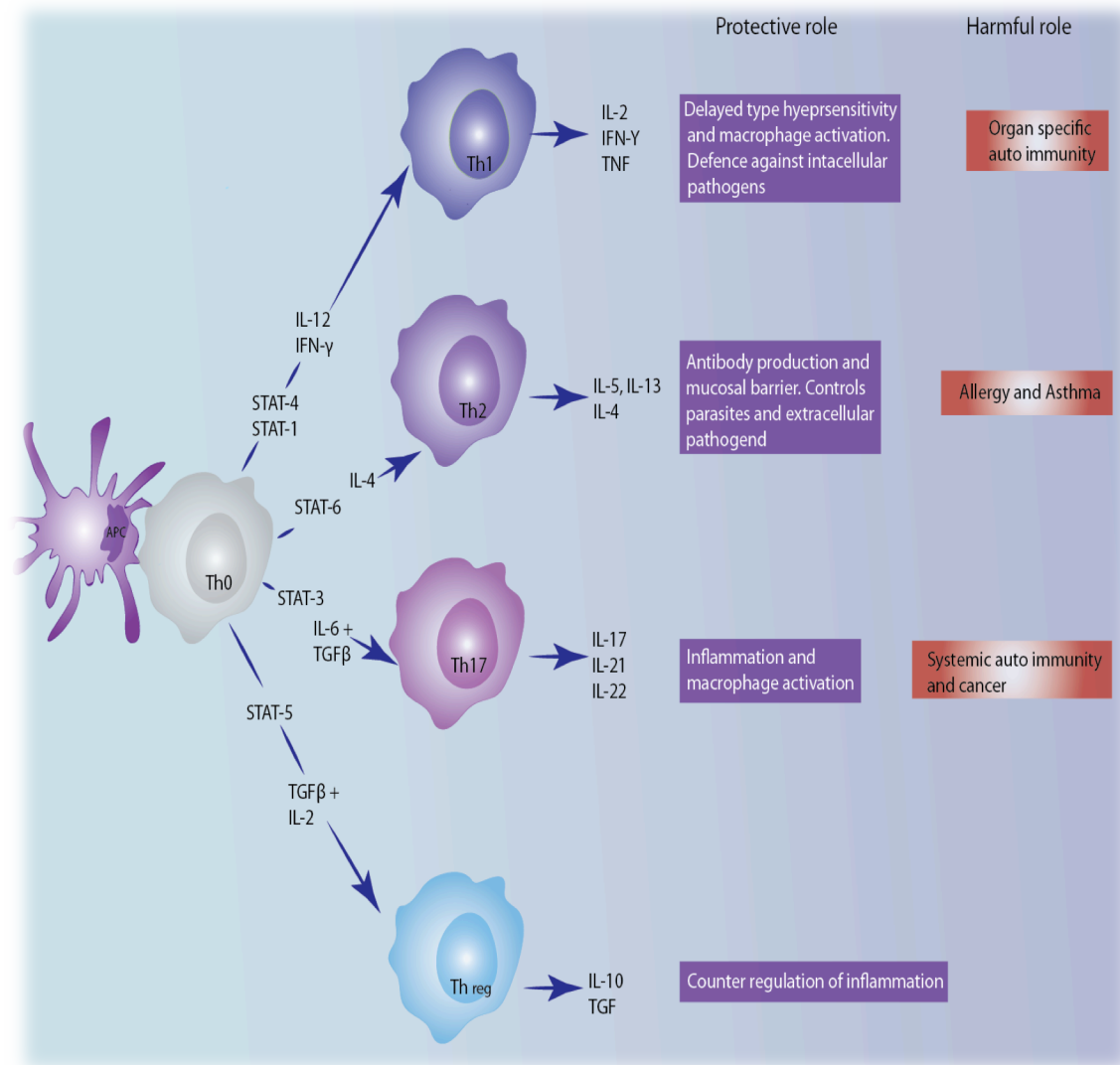


Figure 19

T helper cell differentiation.

T helper cells are characterized by distinct cellular function and cytokine secretion. Their subsets also account for different immune pathologies and atopy. Whilst there are many more T cell subsets than shown, we will focus on genetic strains that are predisposed to generating a Th1 prone (C57BL/6)

immune response or a Th2 prone (129/sv) immune response, Th2 prone mice being more susceptible to asthma and allergy.

The effect of fluvastatin on IgE activated mast cells: genetic influence

Thus far, we have demonstrated that fluvastatin suppresses antigen-induced cytokine production on C57BL/6 mice, which are Th1 prone. To determine if genetic background alters fluvastatin responsiveness we looked at 129/sv mice, which are Th2 prone in comparison to C57BL/6 mice. As shown in Figure 20, (TNF α , IL-13 and MCP-1 respectively) the Th1 prone C57BL/6 mice show suppression of cytokines. Interestingly, the Th2 prone 129/sv mice show resistance to fluvastatin treatment when assessing cytokine production. We further demonstrate that C57BL/6 mice treated with fluvastatin or ZA suppress cholesterol levels, whereas 129/sv mice treated with fluvastatin or ZA do not alter cholesterol levels (Figure 21).

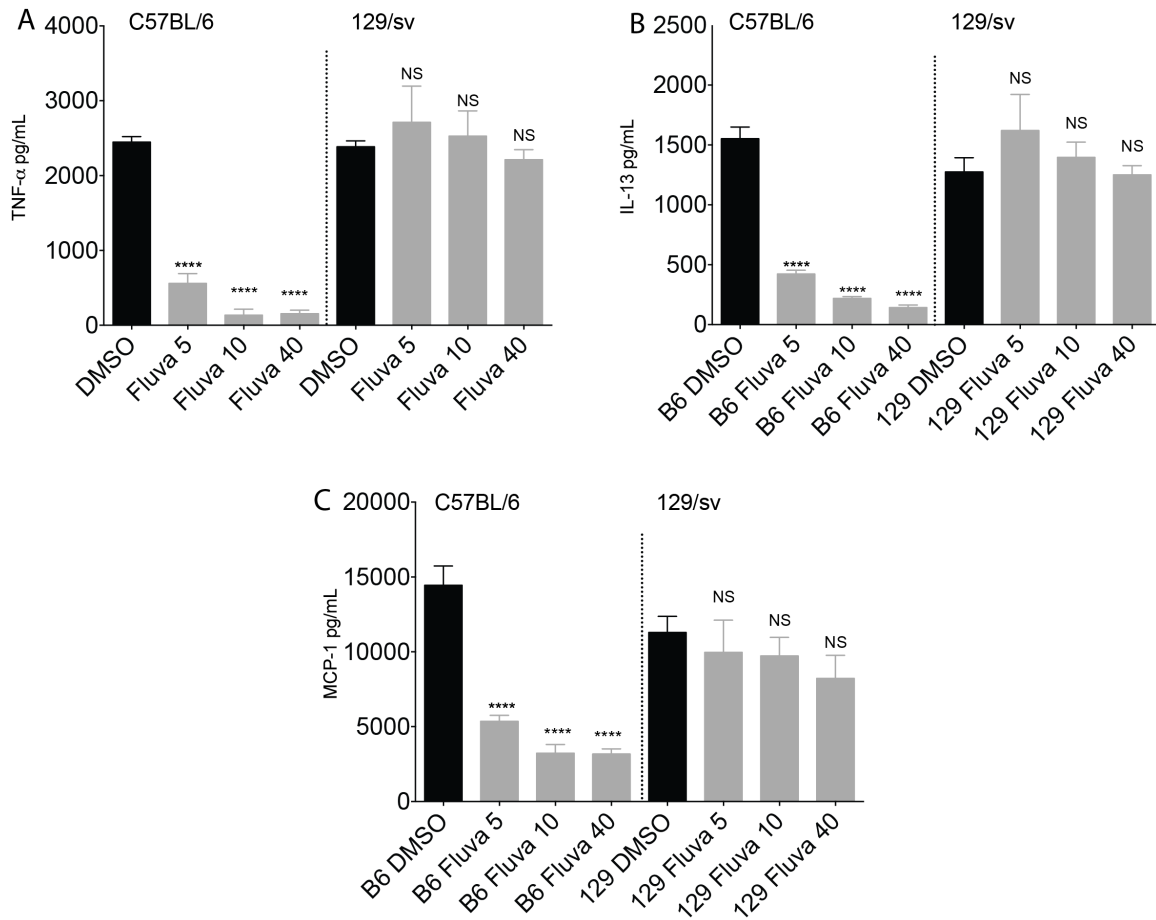


Figure 20

Fluvastatin suppress TNF α , IL-13 and MCP-1 production from IgE activated BMMC's from 129/sv IgE sensitized BMMC's were cultured in IL-3 and SCF with or without fluvastatin at 5 μ M, 10 μ M and 40 μ M for 24 h and then activated with antigen for 16 h. Supernatants were collected as described in the methods and materials. A) TNF α , B) IL-13, C) MCP-1. The results are expressed as the mean \pm SEM of 3 independent experiments.

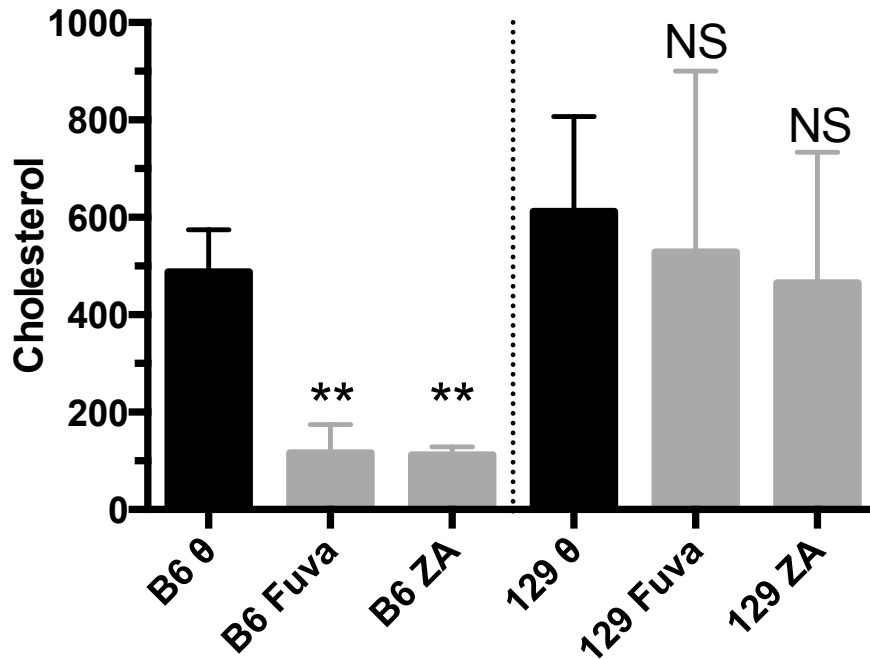


Figure 21

Fluvastatin and zaragozic acid suppress cholesterol production on the C57BL/6 background but not the 129/sv BMMC's.

C57BL/6 and 129/sv BMMC's were cultured in IL-3 and SCF with or without fluvastatin or ZA for 24 h spun down and resuspended in PBS. Cholesterol levels were measured using mass spectrometry.

The effect of fluvastatin on intracellular cytokine versus extracellular cytokine secretion.

We have shown that fluvastatin suppresses cytokine secretion. We next wanted to evaluate fluvastatin's ability to alter intracellular cytokine production via in cell staining. It may be that mast cells treated with fluvastatin make the same levels of cytokines, but have defects in pathways that transport these chemical mediators. We therefore compared intracellular cytokine production, to secreted cytokines. Our data show that fluvastatin suppresses intracellular IL-6 (Figure 22) and TNF α (Figure 23) production in addition to secreted cytokines on the C57BL/6 Th1 prone genetic background but not the 129/sv Th2 prone background. These data suggest that fluvastatin does not just impede secretion, but also generation of these cytokines.

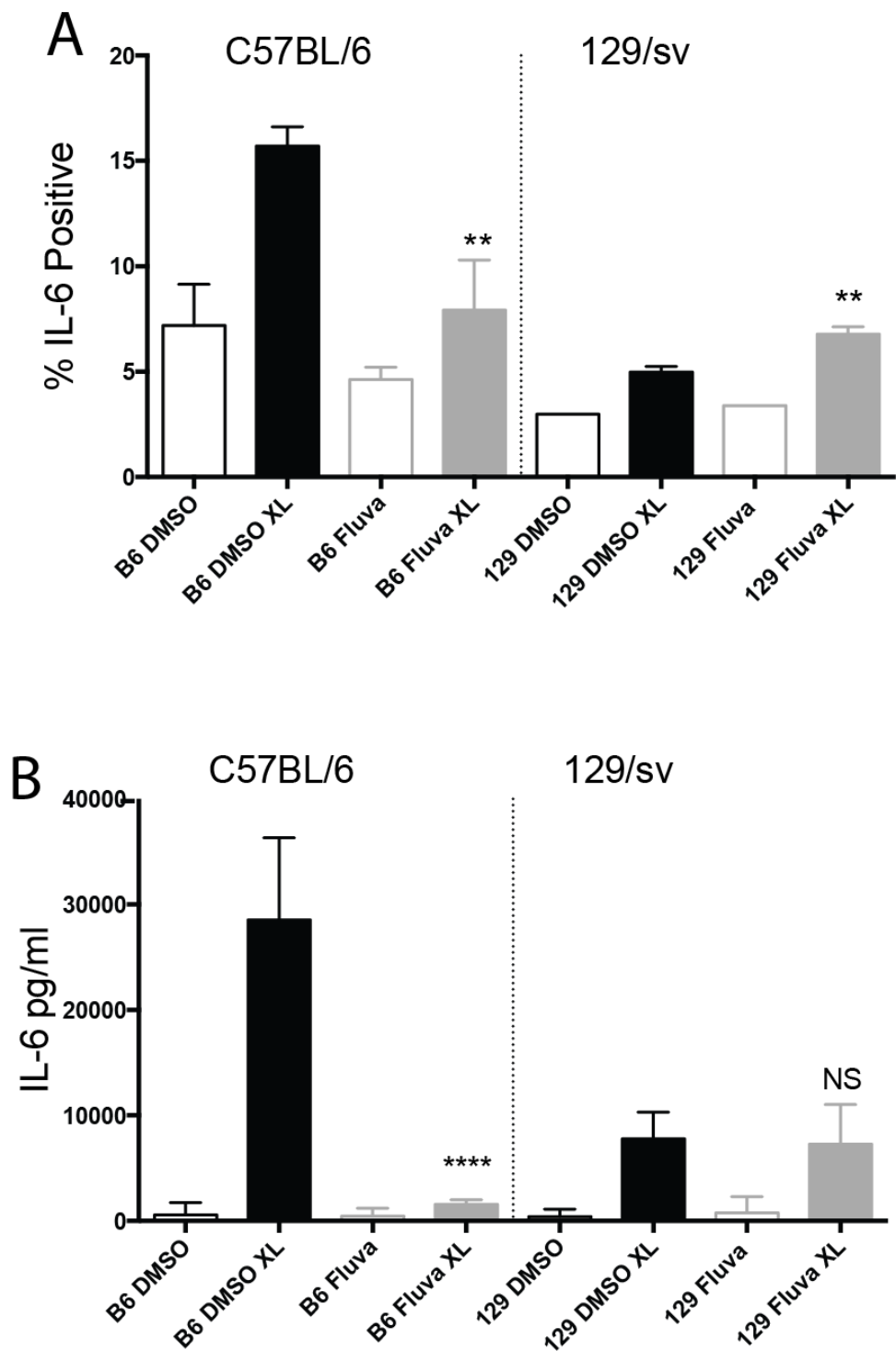


Figure 22

Fluvastatin suppresses IL-6 on C57BL/6 BMDC'S intracellularly and extracellularly but 129/sv BMDC'S are resistant.

IgE sensitized C57BL/6 and 129/sv BMMC's were cultured in IL-3 and SCF with or without fluvastatin or DMSO A) cells were then activated with antigen for 90 minutes and then cultured with monensin for 6 hours and fixed in 4% paraformaldehyde. Cells were then permeabilized in saponin buffer and intracellularly stained with PE-anti IL-6. B) Cells were then activated with antigen for 16 hours, supernatants were harvested and ELISA's run. The results are expressed as the mean \pm SEM of 2 independent experiments n=6.

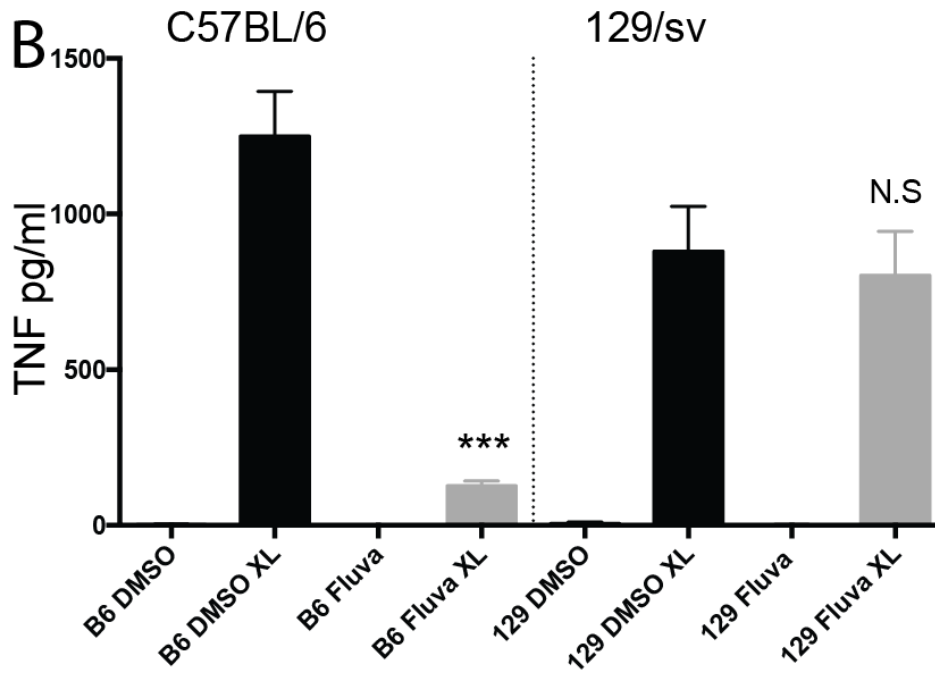
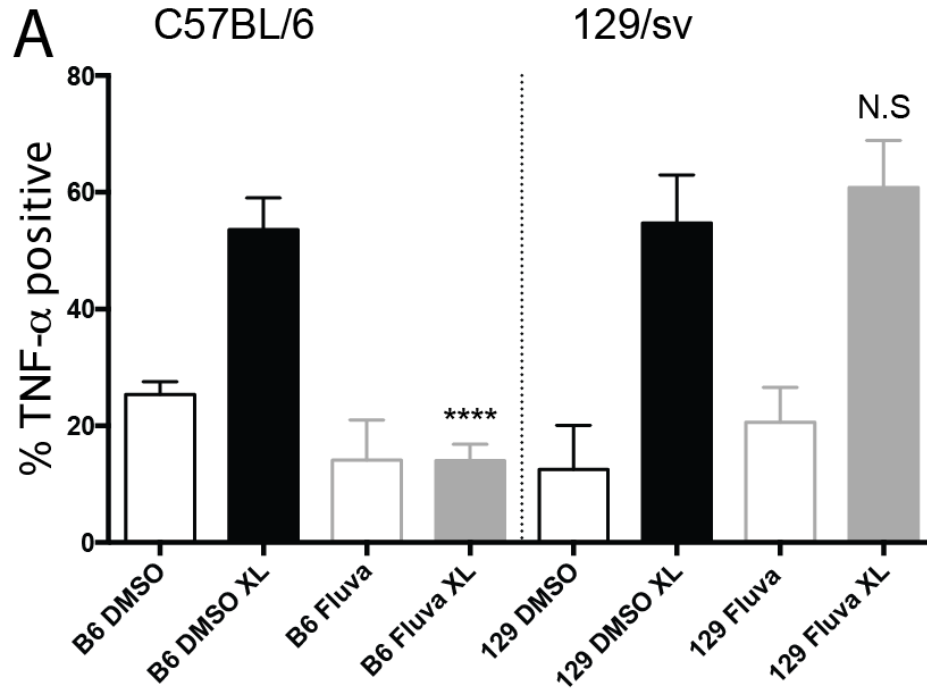


Figure 23

Fluvastatin suppresses TNF α on C57BL/6 BMMC'S intracellularly and extracellularly but 129/sv BMMC'S are resistant.

IgE sensitized C57BL/6 and 129/sv BMMC's were cultured in IL-3 and SCF with or without fluvastatin or DMSO A) cells were then activated with antigen for 90 minutes and then cultured with monensin for 6 hours and fixed in 4% paraformaldehyde. Cells were then permeabilized in saponin buffer and intracellularly stained with PE-anti TNF α . B) Cells were then activated with antigen for 16 h, supernatants were harvested and ELISA's run.

Additional Th1 and Th2 prone genetic strains

In addition to the Th2 prone 129/sv mice we looked at the Th2 prone Balb/c mice. Here, we show that Balb/c BMMC's are also resistant to fluvastatin, when assessing IgE induced cytokine production (Figure 24) but 129/sv and Balb/c mice did not show resistance to GGTI (Figure 25-26). Another Th2 prone strain the A/J mice. When looking at A/J's ability to produce cytokine in response to antigen, we see a variation. For IL-6 production, overall we see a slight suppression at 5 μ M but for higher fluvastatin concentrations of 20 μ M we see a slight enhancement (Figure 27). For TNF α (Figure 27 B) we see suppression with increasing concentration of fluvastatin. When looking at C3H/HeJ mice whose genetic background is Th1 prone, we show that IL-6 (Figure 28) and TNF α (Figure 28B) production is suppressed.

We next compared peritoneal mast cells of A/J, and C3H/HeJ in comparison to C57BL/6 and 129/sv. Here, we show that whilst the peritoneal mast cells for C57BL/6 show suppression, 129/sv show resistance to fluvastatin treatment where as the A/J, and C3H/HeJ peritoneal mast cells do not show the same Th1 and Th2 prone responses (Figure 29). Previously, we have shown that fluvastatin does not suppress IL-10 production on C57BL/6 BMMC's. We next looked at IL-10 production on peritoneal mast cells from C57BL/6 and 129/sv interestingly show enhancement (Figure 30). These data suggest that fluvastatin resistance that is evident on some Th2 genetic backgrounds is not all-inclusive and is more complex than simply a Th1 versus a Th2 genetic background.

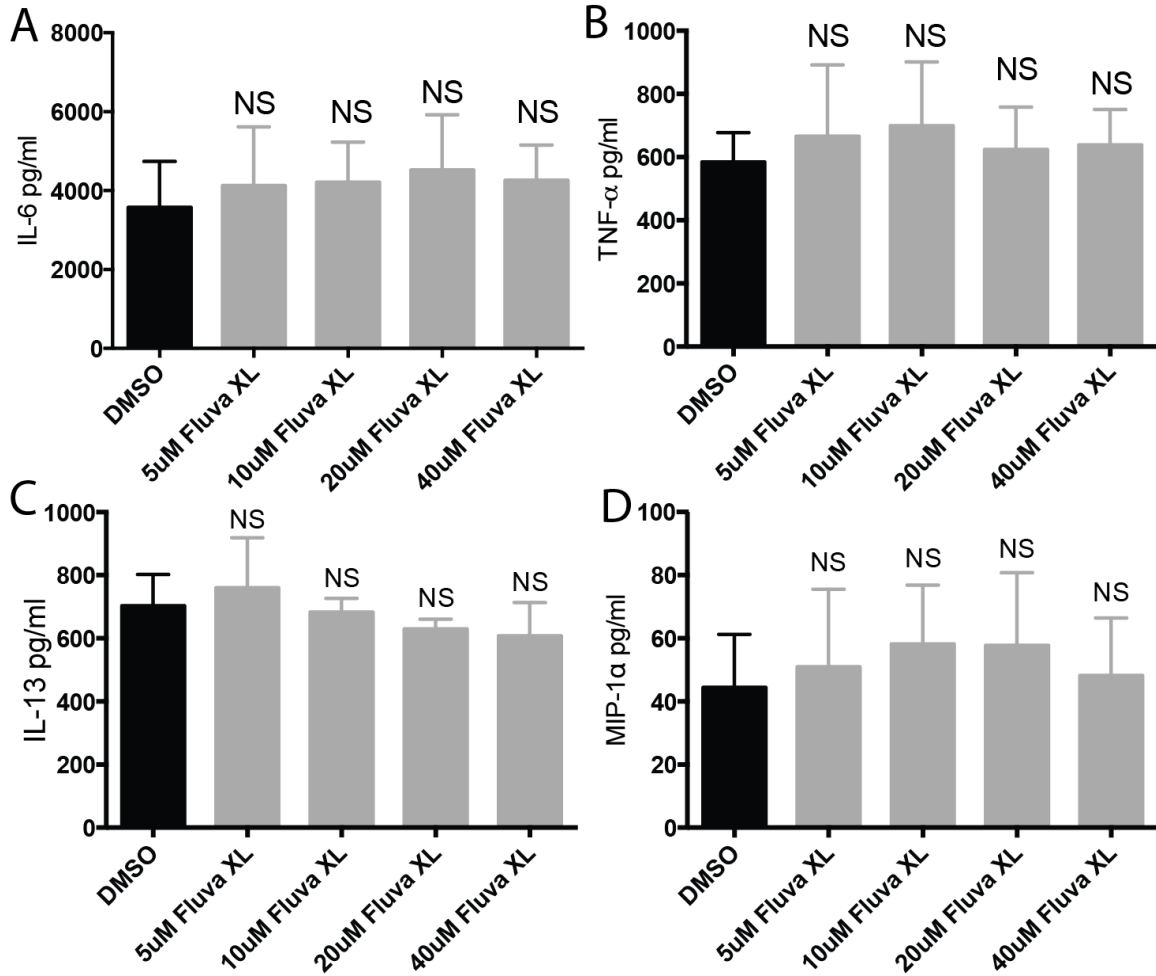


Figure 24

Fluvastatin does not suppress IgE activated cytokine or chemokine production from Balb/c BMMC's.

Balb/c IgE sensitized BMMC's were cultured in IL-3 and SCF, with or without fluvastatin or DMSO and then activated with DNP-HSA for 16 h and supernatants were collected as described in the methods and materials. A) IL-6, B) TNF α , C) IL-13, D) MCP-1. The results are expressed as the mean \pm SEM of 3 independent experiments.

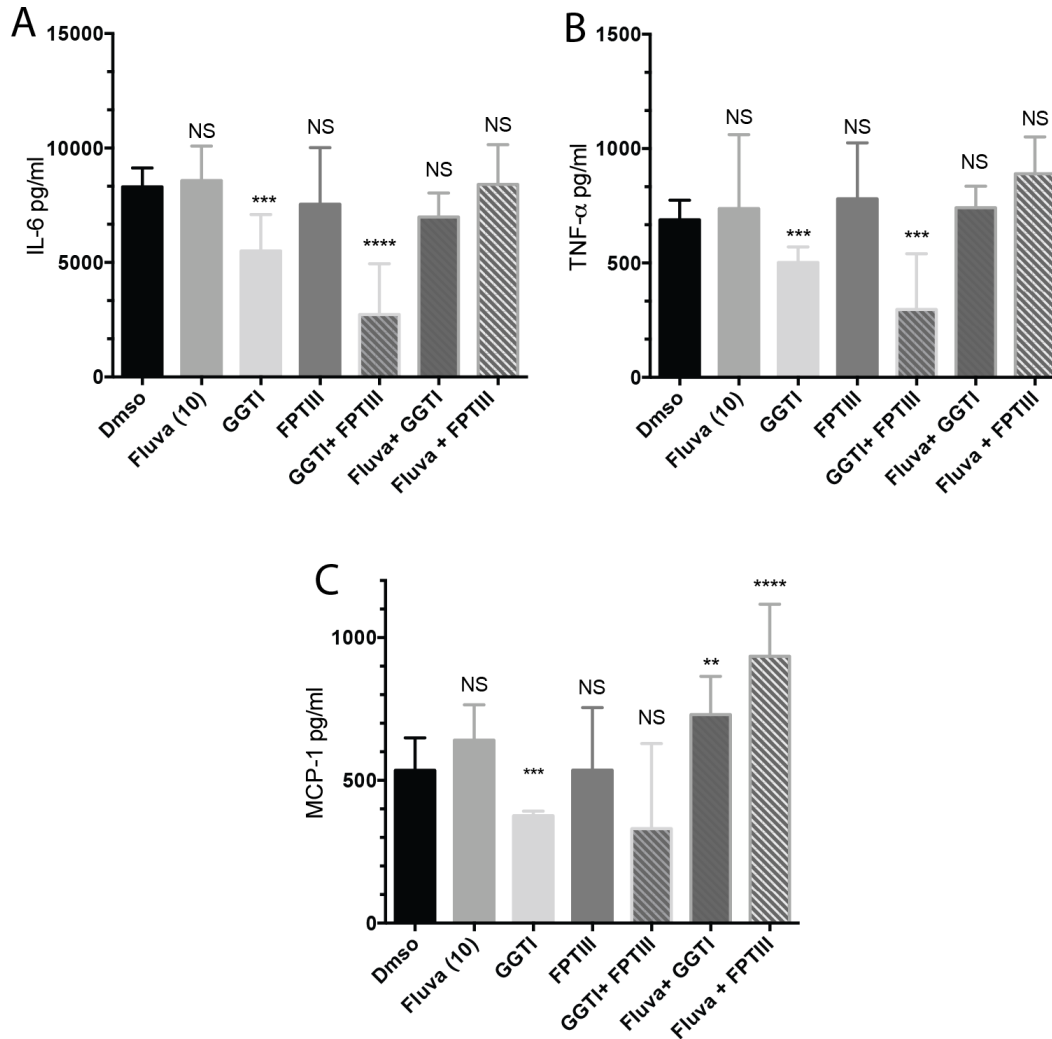


Figure 25

GGTI-286 significantly suppresses Ag induced cytokine production on 129/sv BMMC's but fluvastatin does not.

129/sv BMMC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO, Fluvastatin 10 μ M, GGTI-266 20 μ M or FPTIII 20 μ M or combinations for 24 h and then stimulated with DNP-HSA (50 ng/ml) for 16 h. Supernatants were harvested and ELISA's were preformed. A) IL-6, B) TNF α , C) MCP-1. The Graph is a representation of 4 independent experiments with an n=12. Data shown are mean \pm SEM.

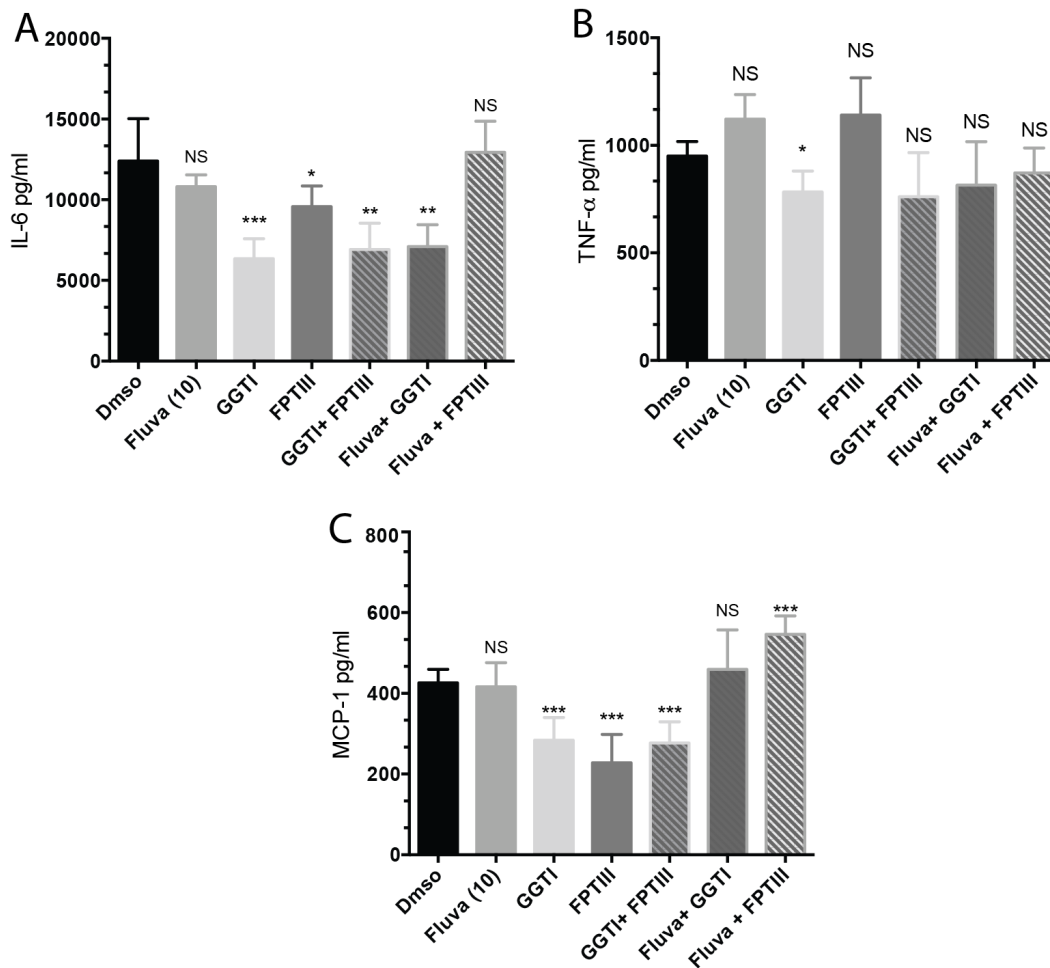


Figure 26

GGTI-286 and FPTIII significantly suppresses Ag induced cytokine production on Balb/c BMMC's but fluvastatin does not.

Balb/c BMMC's were cultured in IL-3 and SCF and IgE sensitized and pretreated with or without DMSO, fluvastatin 10 μ M, GGTI-266 20 μ M or FPTIII 20 μ M or combinations for 24 h and then stimulated with DNP-HSA (50 ng/ml) for 16 hours. Supernatants were harvested and ELISA's were performed. A) IL-6, B) TNF α , C) MCP-1. The Graph is a representation of 4 independent experiments with an n=12. Data shown are mean \pm SEM.

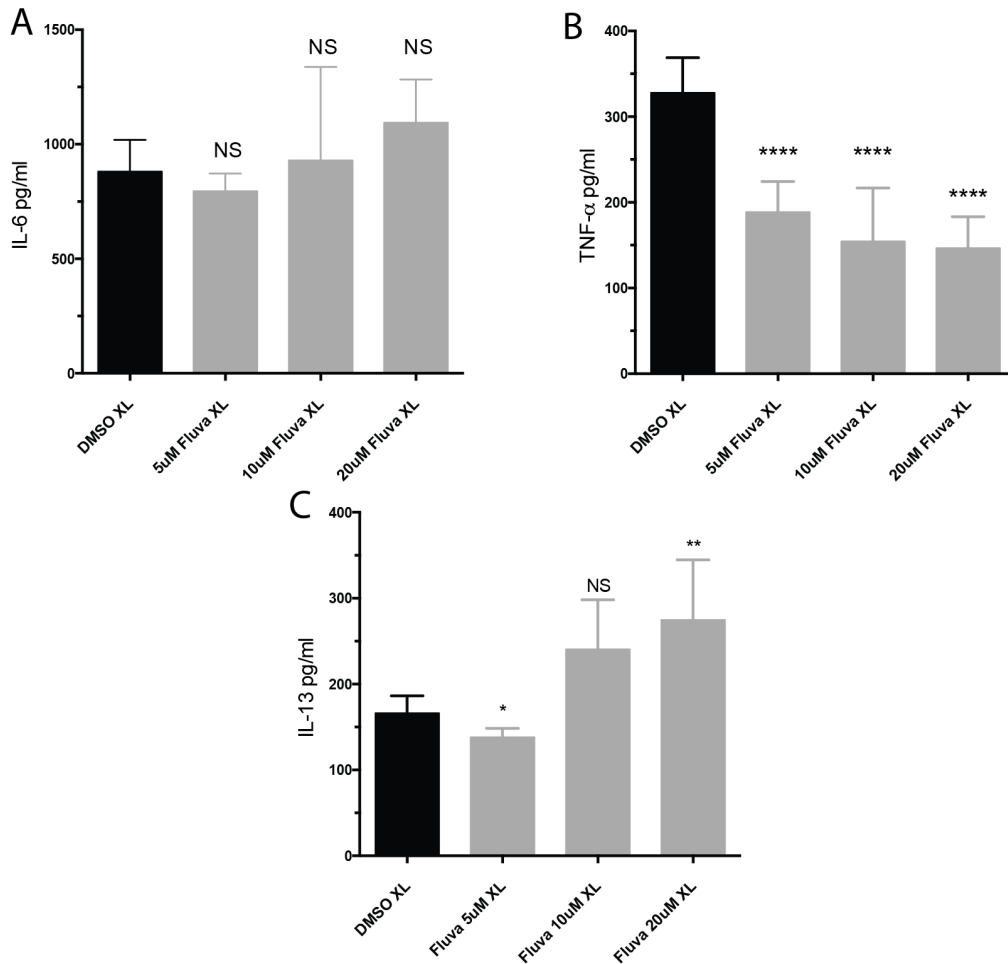


Figure 27

Fluvastatin does not suppress IgE activated IL-6 production But does suppress TNF α , additionally Fluvastatin does not suppress consistently suppress IgE activated IL-13 production from A/J BMMC's.

IgE sensitized A/J BMMC's were cultured in IL-3 and SCF, with or without fluvastatin or DMSO and then activated with DNP-HSA for 16 h and supernatants were collected as described in the methods and materials. A) IL-6, B) TNF α , C) IL-13. The results are expressed as the mean \pm SEM of 2 independent experiments.

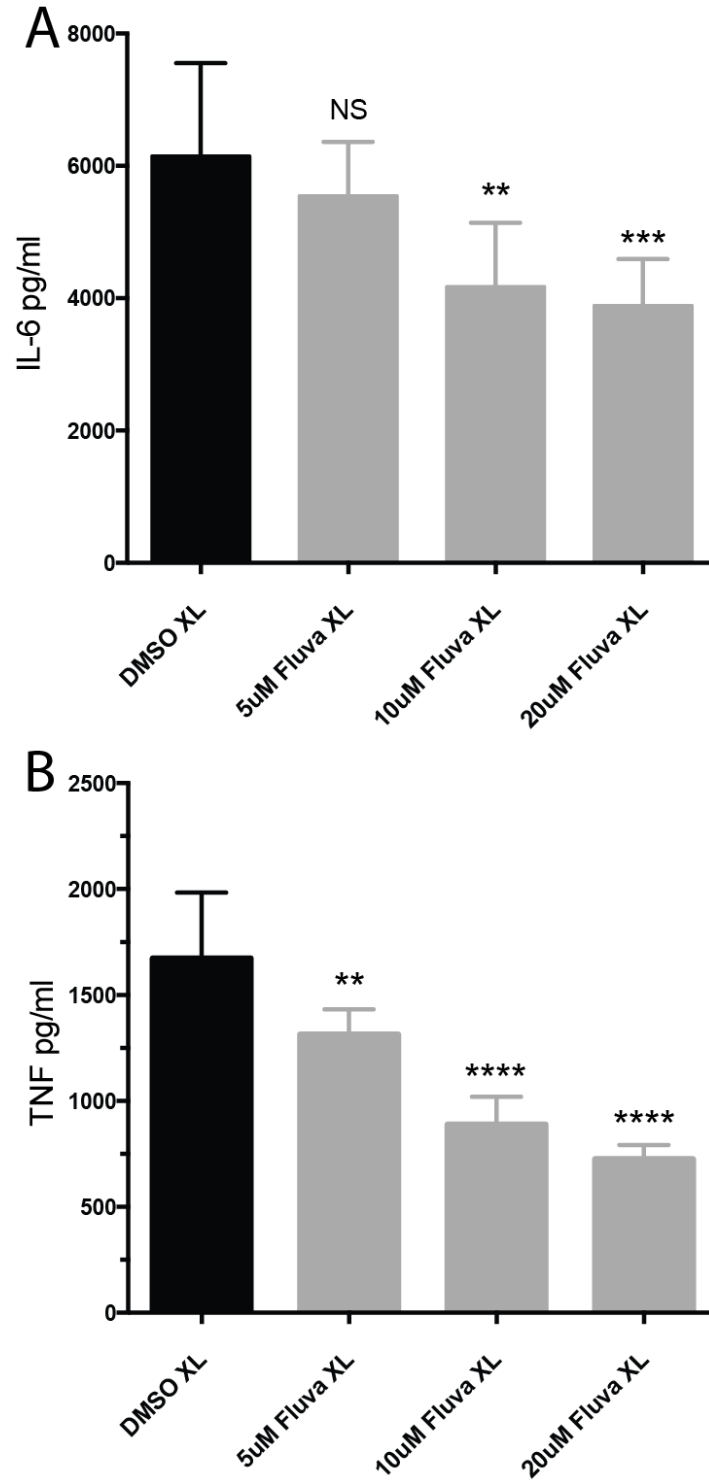


Figure 28

Fluvastatin mediated suppression of IL-6 and TNF α by IgE activated mast cells increases with dose on C3H/HeJ.

IgE sensitized C3H/HeJ BMMC's were cultured in IL-3 and SCF, with or without fluvastatin or DMSO and then activated with DNP-HSA for 16 h and supernatants were collected as described in the methods and materials. A) IL-6, B) TNF α . The results are expressed as the mean \pm SEM of 2 independent experiments.

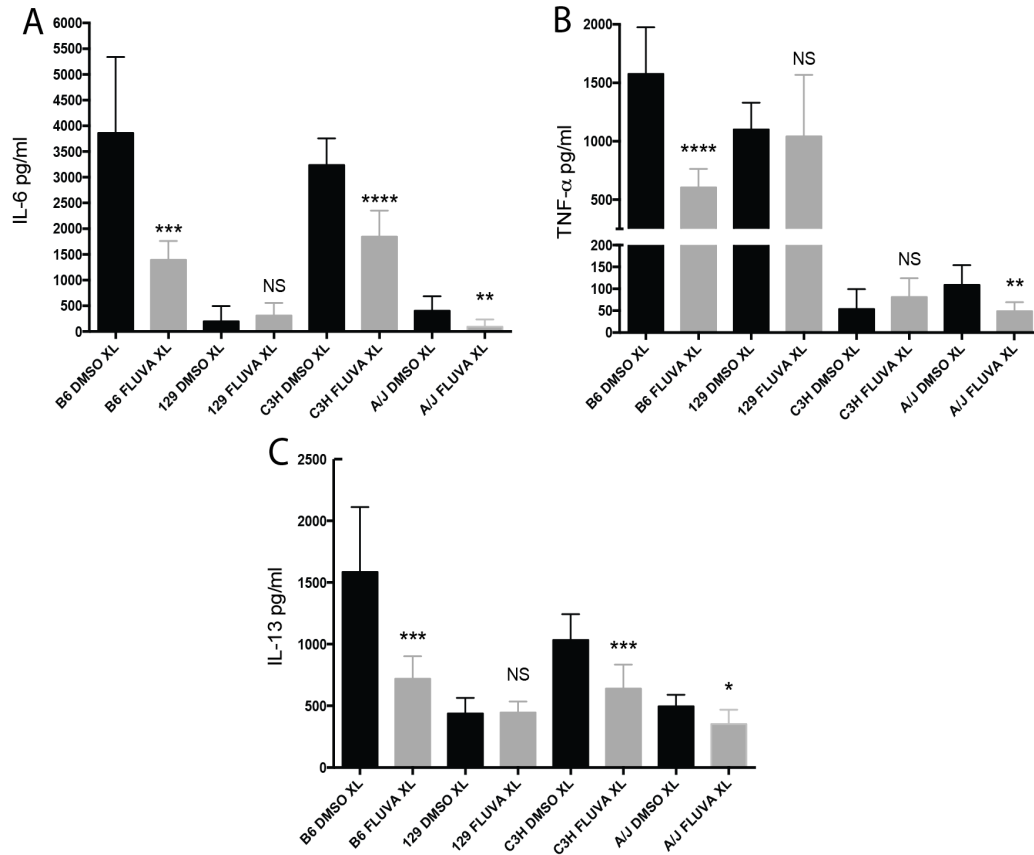


Figure 29

The effect of fluvastatin from IgE activated peritoneal mast from C57BL/6, 129/sv, C3H/HeJ and A/J.

Peritoneal mast cells were separated using easySep magnet selecting for c-kit positive cells. IgE sensitized mast cells were then cultured in IL-3 and SCF with or without fluvastatin or DMSO and then activated with DNP-HSA for 16 h and supernatants were collected as described in the methods and materials. A) IL-6, B) TNF α and C) shows IL-13 production. The results are expressed as the mean \pm SEM of 3 independent experiments.

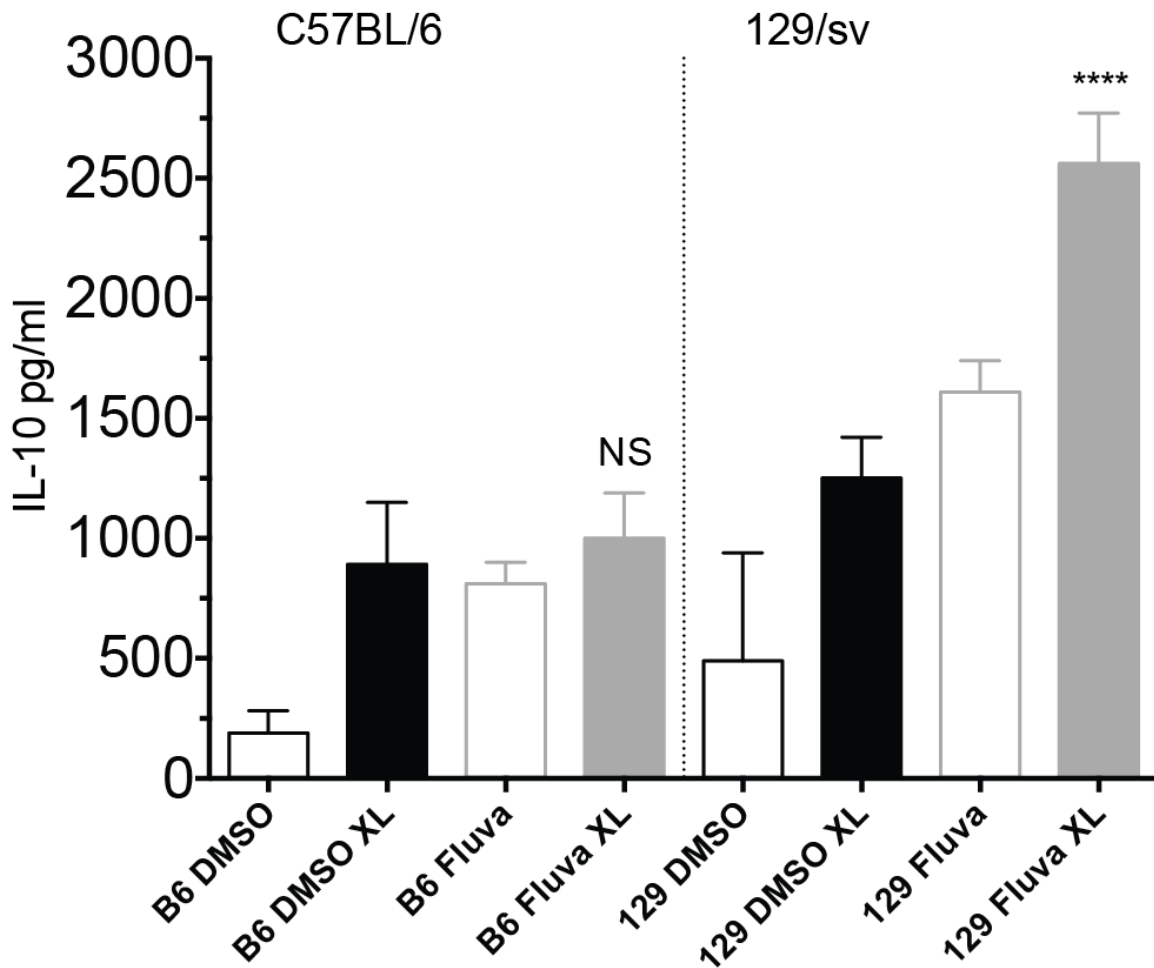


Figure 30

Fluvastatin shows no suppression of IL-10 in C57BL/6 peritoneal mast cells but actually enhances IL-10 production in 129/sv peritoneal mast cells.

Peritoneal mast cells from C57BL/6 and 129/sv mice were separated using easySep magnet selecting for c-kit positive cells. IgE sensitized mast cells were then cultured in IL-3 and SCF with or without fluvastatin or DMSO and then activated with DNP-HSA for 16 h and supernatants were collected as described in the methods and materials. The results are expressed as the mean \pm SEM of 3 populations.

Fluvastatin effect on mast cell degranulation

It has previously been shown that fluvastatin suppresses mast cell degranulation (26) in RBL-2H3 cells. Here, we show that fluvastatin suppresses mast cell degranulation on C57BL/6 BMMC's but not Th2 prone 129/sv BMMC's (Figure 31) demonstrating that fluvastatin resistance on 129/sv BMMC's extends beyond cytokine production.

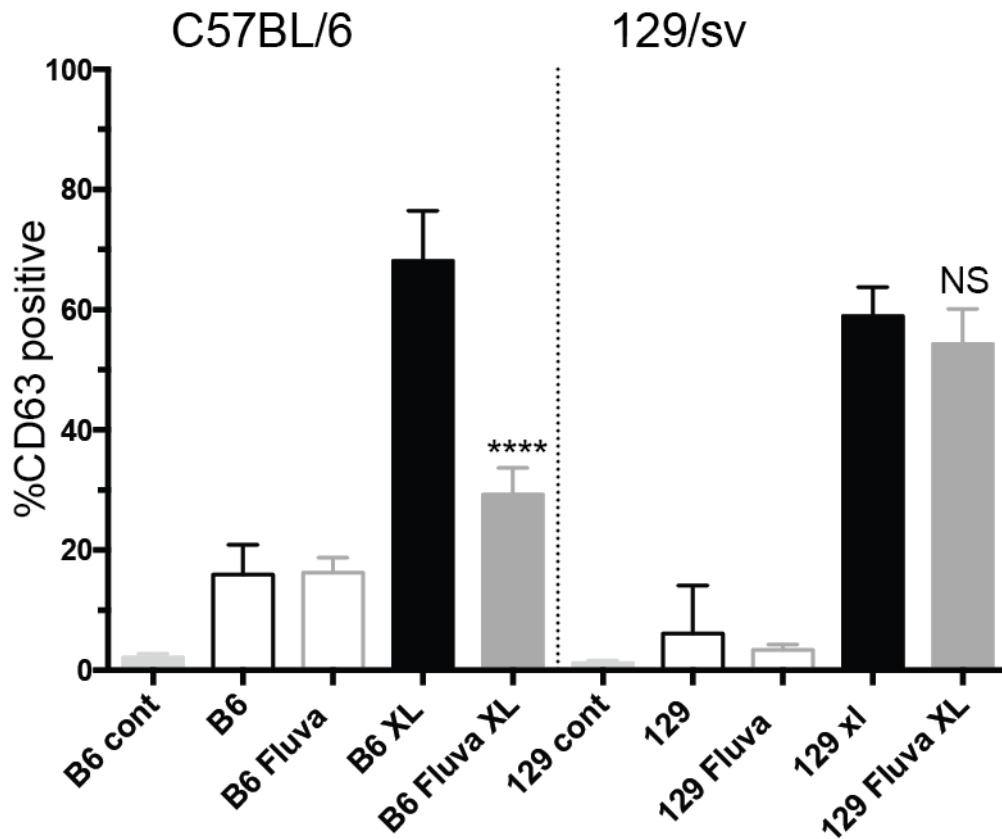


Figure 31

Fluvastatin suppresses mast cell degranulation from C57BL/6 but not 129/sv BMMC's. C57BL/6 and 129/sv IgE sensitized BMMC's were cultured in IL-3 +SCF with fluvastatin or DMSO for 24 h and then activated for 1 h with antigen. Cells were then stained for CD63 for 45 minutes and then run by flow cytometry.

Fluvastatin and migration

SCF induces chemotactic migration of mast cells and is important for survival and proliferation. We wanted to assess the potential for fluvastatin to suppress mast cell migration by setting up a transwell assay. It has previously been shown that C57BL/6 BMMC's migrate to SCF for 24 hours suppresses BMMC migratory capabilities. Here, we compare C57BL/6 and 129/sv BMMCs ability to migrate towards SCF and show that the Th2 prone 129/sv BMMC's are resistant to the suppressive capabilities of fluvastatin (Figure 32).

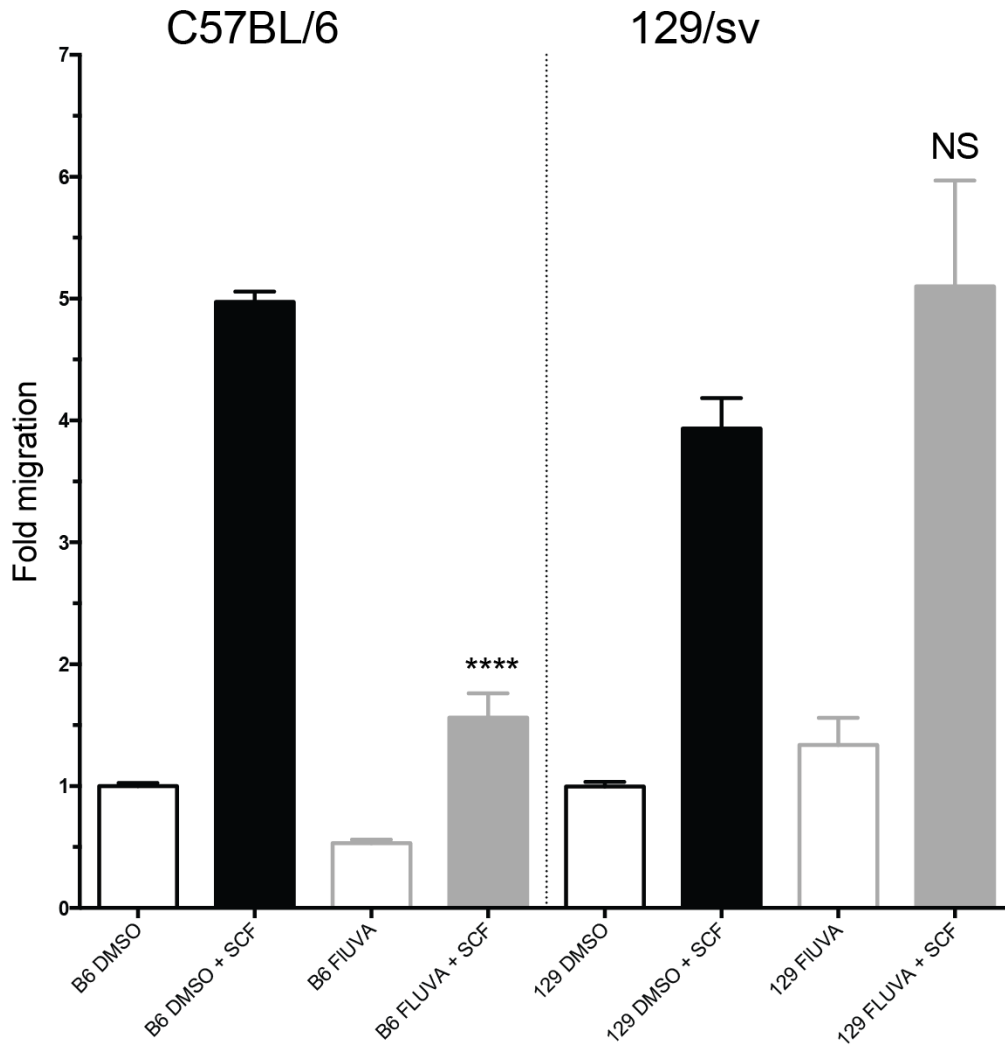


Figure 32

Fluvastatin suppresses mast cell migration of C57BL/6 BMMC's but not 129/sv BMMC's.

C57BL/6 and 129/sv BMMC's were tested through 8 μ m transwell membranes for migration in response to SCF or fluvastatin as describes in methods and materials. Fold migration is based on comparison to media alone samples. The results are expressed as the mean \pm SEM of 2 independent experiments.

Fluvastatin elicits cell death in C57BL/6 BMMC's more so than 129/Sv BMMC's.

We next wanted to assess the effect of fluvastatin on mast cell death by looking at dead cells (Figure 33), apoptosis (Figure 34) and autophagy (Figure 35) on C57BL/6 and 129/sv mice (Figure 36), apoptosis (Figure 37) and autophagy (Figure 38) by flow cytometry as a time course assay. Here, we show that C57BL/6 mice show increased cell death, apoptosis and autophagy in a dose dependent manner after 3 days of fluvastatin treatment whereas 129/sv BMMC's show resistance.

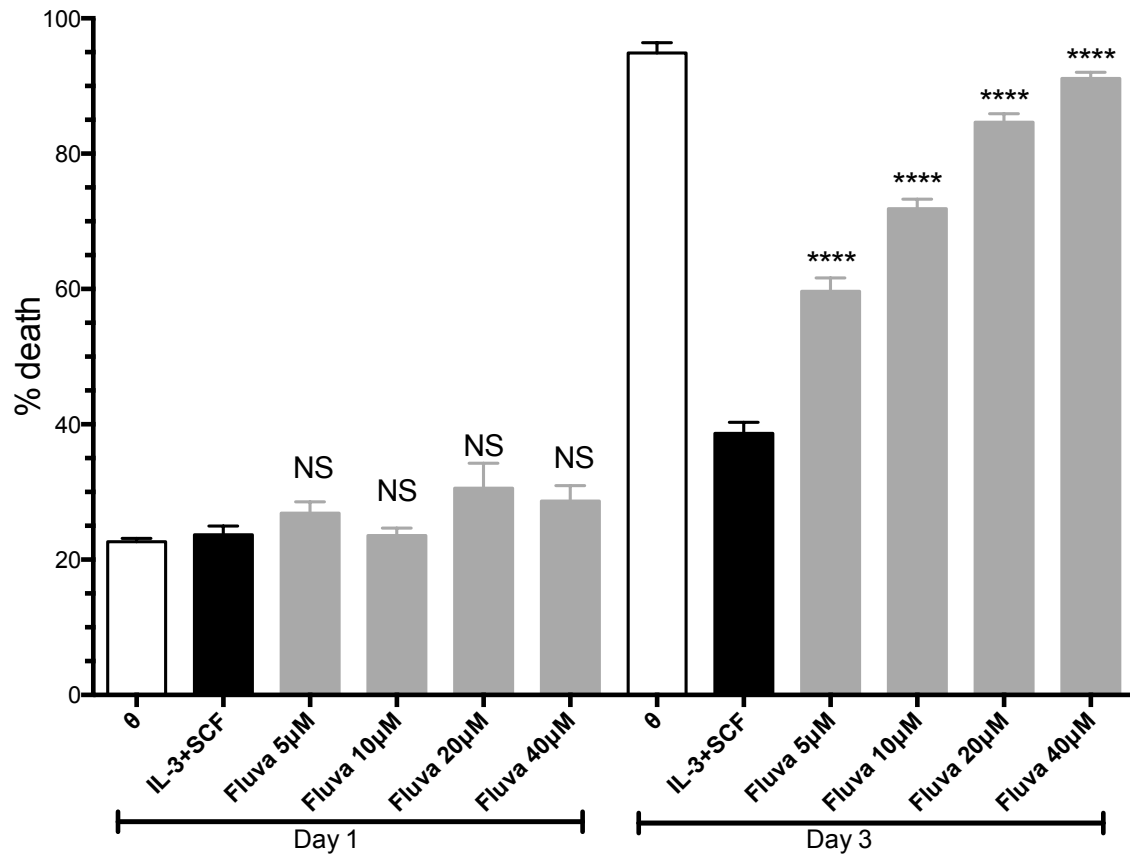


Figure 33

Fluvastatin enhances mast cell death in C57BL/6 mice in a dose dependent way over 3 days.

C57BL/6 BMMC's were cultured with 5 μM, 10 μM, 20 μM or 40 μM fluvastatin for 1 through 3 days and then stained with propidium iodide 200 μL (PI exclusion) a few seconds before running by flow cytometry. The results are expressed as the mean ± SEM of n=6.

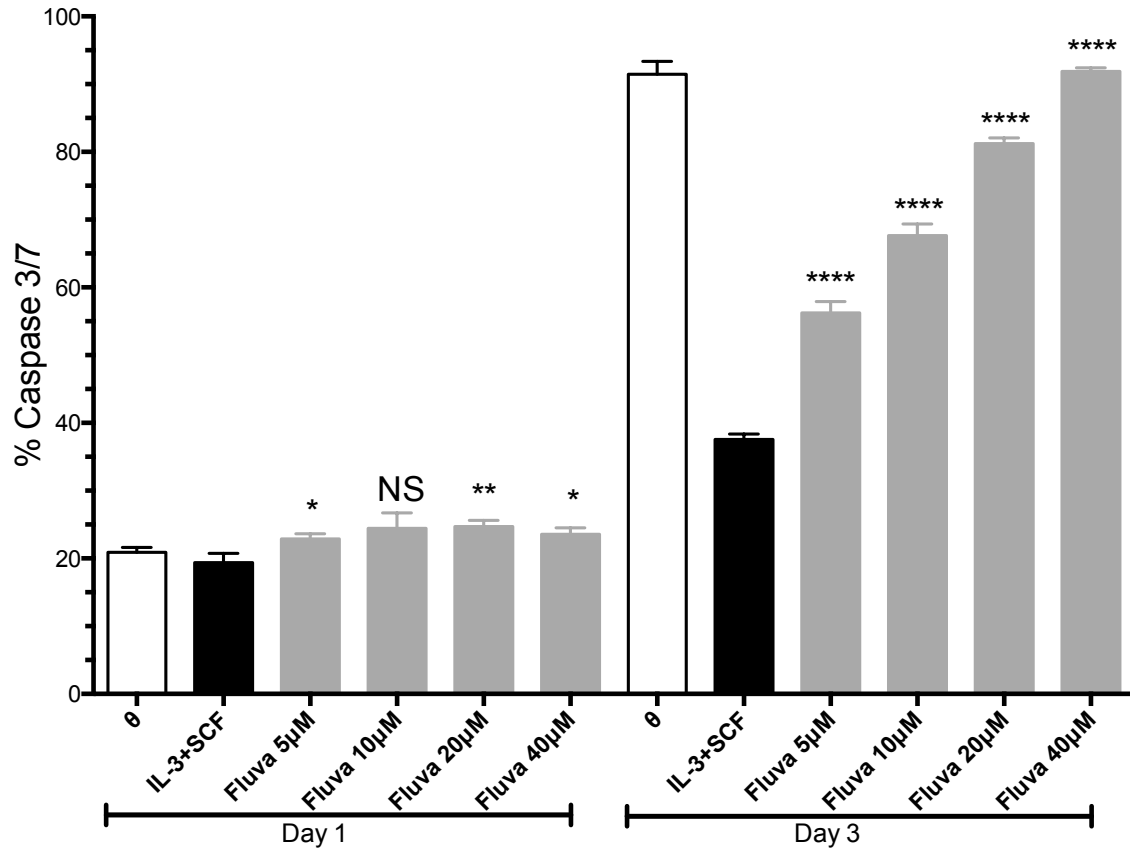


Figure 34

Fluvastatin enhances mast cell apoptosis in C57BL/6 mice in a dose dependent way over 3 days.

C57BL/6 BMMC's were cultured with 5 µM, 10 µM, 20 µM or 40 µM fluvastatin for 1 through 3 days and then stained with caspase 3/7 green detection reagent for 45 minutes and then run by flow cytometry. The results are expressed as the mean ± SEM of n=3.

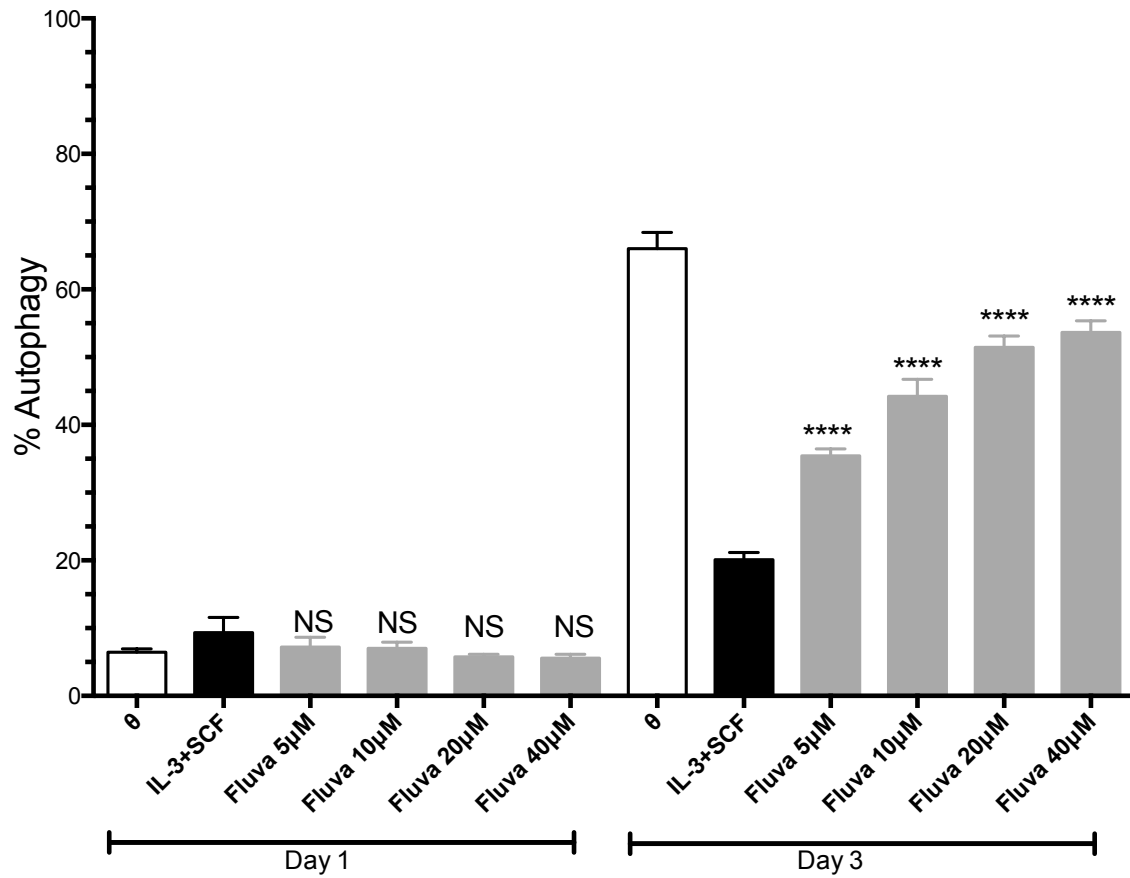


Figure 35

Fluvastatin enhances mast cell autophagy in C57BL/6 mice in a dose dependent way over 3 days.

C57BL/6 BMBC's were cultured with 5 µM, 10 µM, 20 µM or 40 µM fluvastatin for 1 through 3 days and then stained with Cytold autophagy kit as describes in methods and materials. Samples were run by flow cytometry. The results are expressed as the mean ± SEM of n=6.

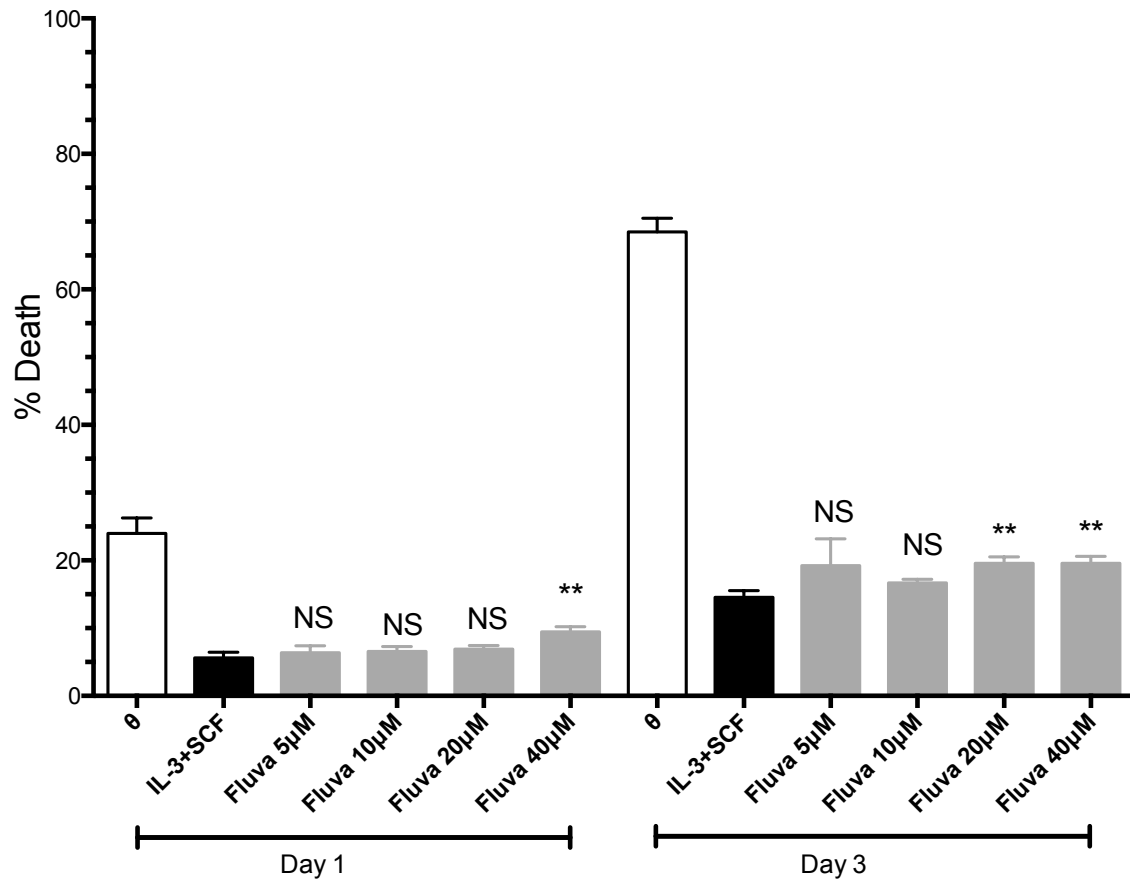


Figure 36

Fluvastatin enhances mast cell death in 129/Sv mice in a dose dependent way over 3 days.

129/sv BMMC's were cultured with 5 µM, 10 µM, 20 µM or 40 µM fluvastatin for 1 through 3 days and then stained with propidium iodide 200 µL (PI exclusion) a few seconds before running by flow cytometry. The results are expressed as the mean ± SEM of n=6.

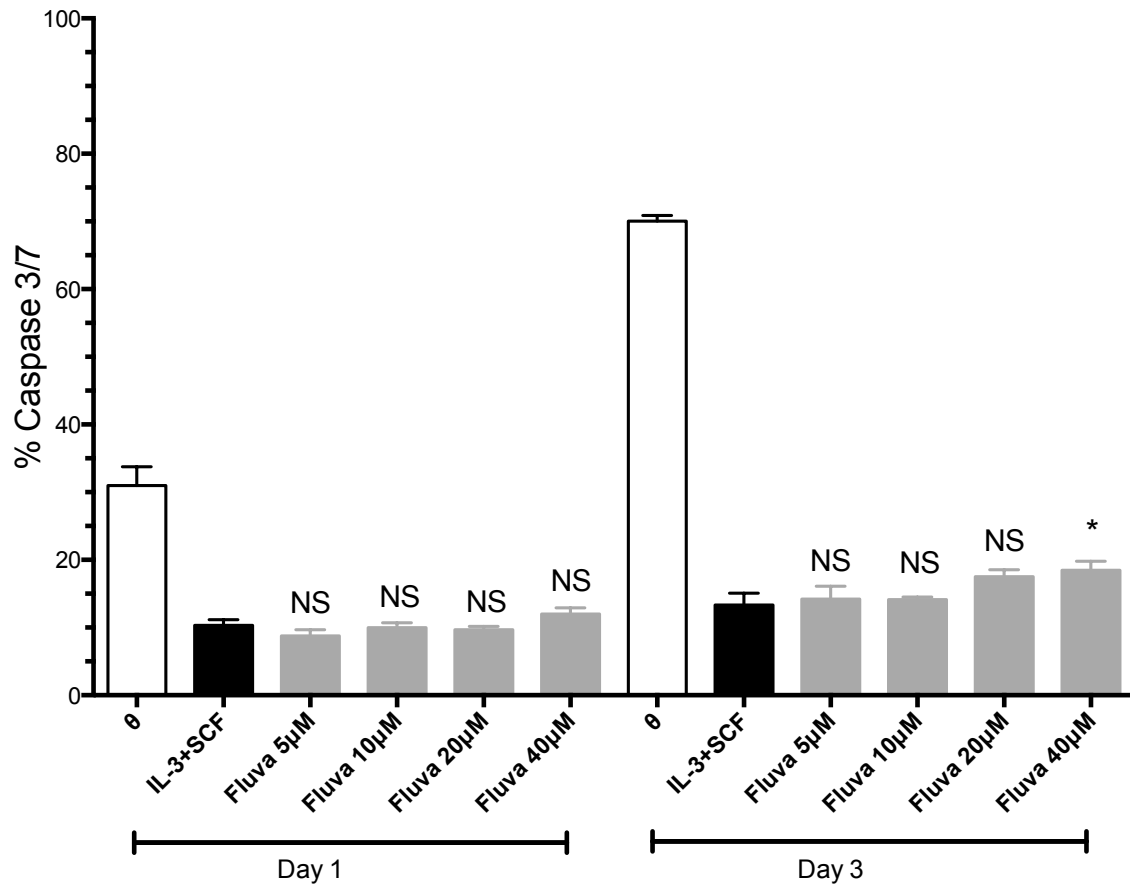


Figure 37

Fluvastatin enhances mast cell apoptosis in 129/SvImJ mice in a dose dependent way over 3 days.

129/sv BMMC's were cultured with 5 μM, 10 μM, 20 μM or 40 μM fluvastatin for 1 through 3 days and then stained with caspase 3/7 green detection reagent for 45 minutes and then run by flow cytometry. The results are expressed as the mean

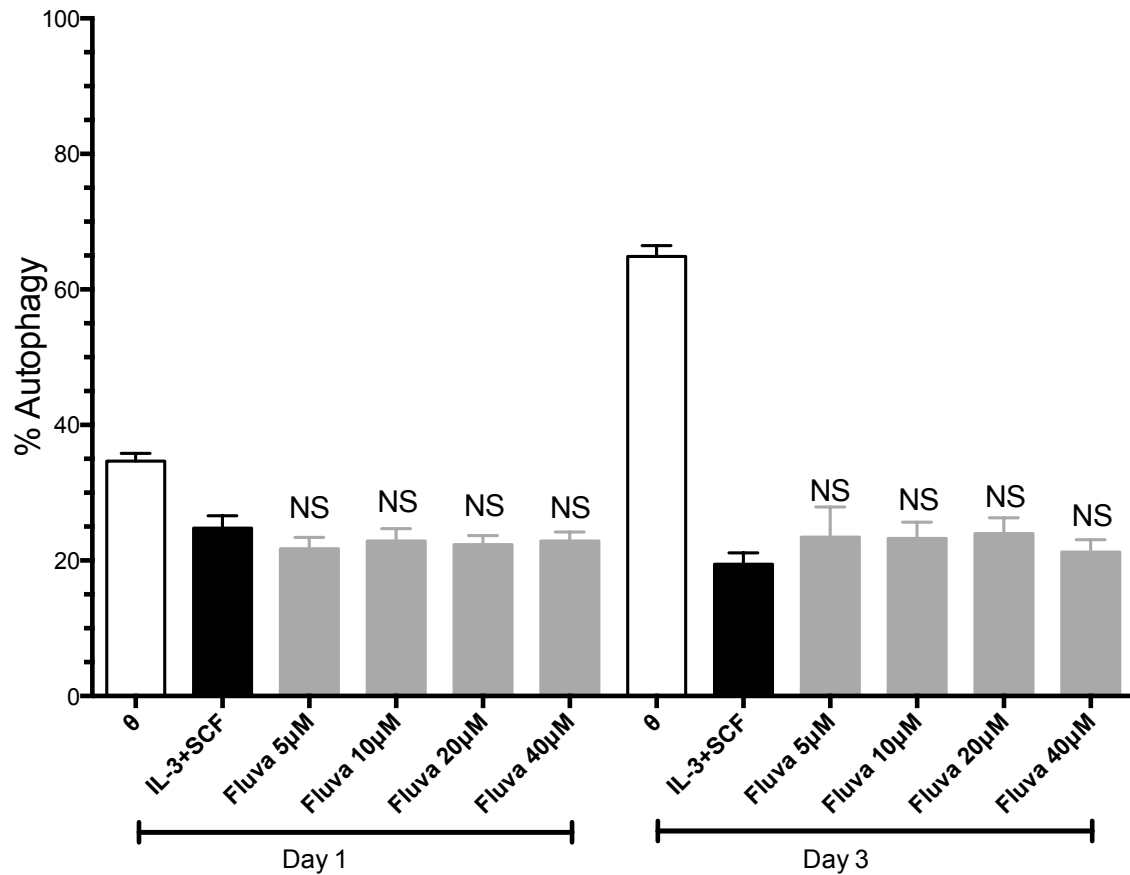


Figure 38

Fluvastatin enhances mast cell autophagy in 129/sv mice in a dose dependent way over 3 days.

129/sv BMMC's were cultured with 5 µM, 10 µM, 20 µM or 40 µM fluvastatin for 1 through 3 days and then stained with CytolD autophagy kit as describes in methods and materials. Samples were run by flow cytometry. The results are expressed as the mean ± SEM of n=6.

The effect of fluvastatin on antigen stimulated basophils

To this point, we have investigated the suppressive capabilities of fluvastatin on mast cell function with particular emphasis on IgE signaling and cytokine production. Basophils express the Fc ϵ RI and upon aggregation of this receptor are capable of producing a similar range of chemokine's and cytokines. We therefore decided to investigate the potential suppressive abilities of fluvastatin on basophils from a C57BL/6 and 129/sv genetic backgrounds. Here, we show that IgE sensitized basophils activated with antigen from a C57BL/6 background pretreated with fluvastatin for 24 h show suppression of IL-4, IL-6, IL-13 (Figure 39). The suppression of IL-6 (Figure 39 B) and TNF α (Figure 40 A) on the C57BL/6 and the cytokine production in general is less so, but still evident. MCP-1 (Figure 40 B) and MIP-1 α (Figure 40 C) show similar trends. However, no significant suppression of cytokines or chemokines is evident in the Th2 prone 129/sv treated with fluvastatin.

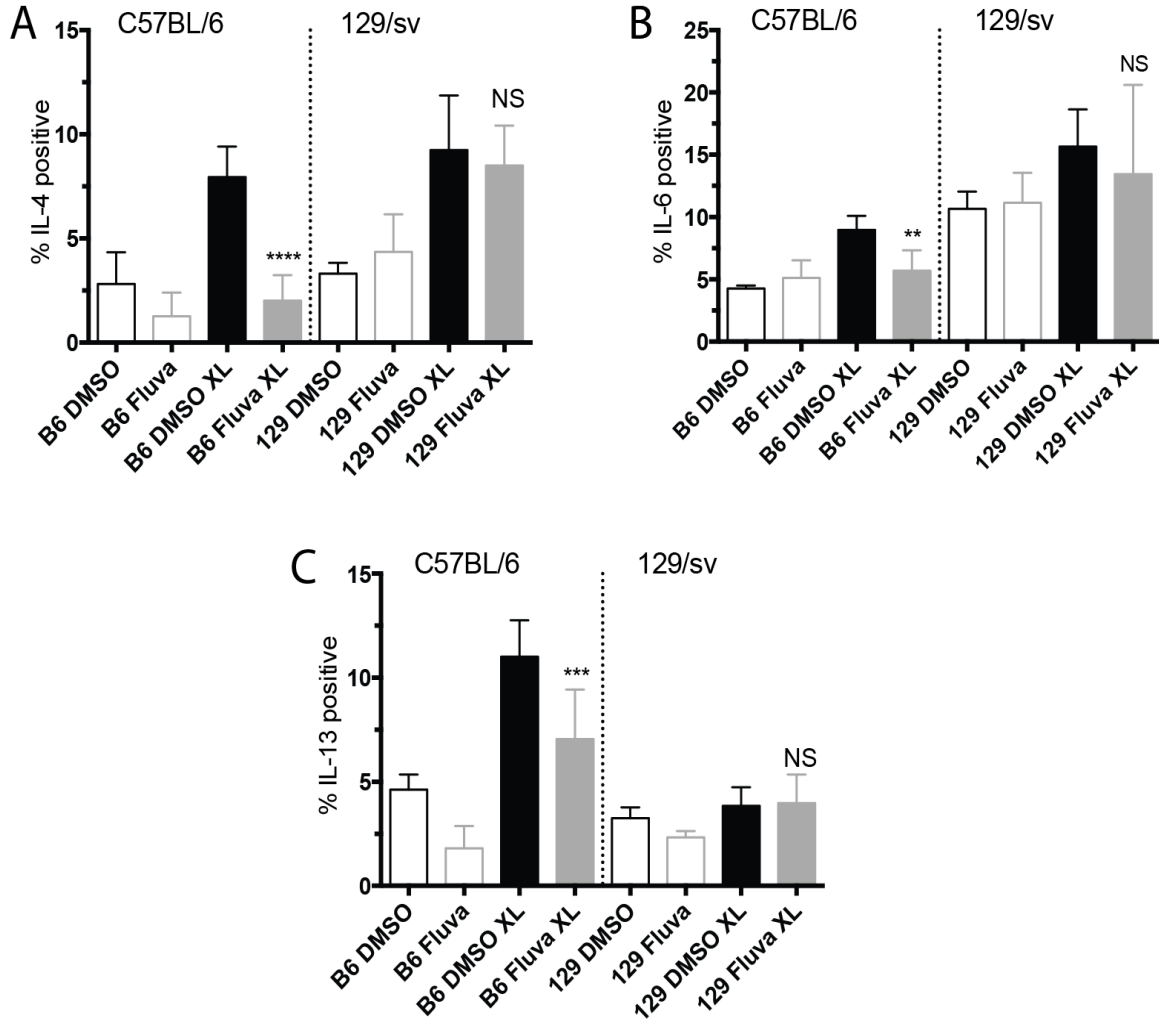


Figure 39

Fluvastatin suppress IL-4, IL-6 and IL-13 production from IgE activated basophils on a C57BL/6 but not a 129/sv genetic background.

C57BL/6 and 129/sv IgE bone marrow were cultured in IL-3 20 ng/ml for 8 days and then cultured with or without IgE at 0.5 μ g/ml, in fluvastatin 10 μ m or DMSO for 24 h. in cell staining was conducted as described in methods and materials with CD49b as a basophil marker. A) Shows IL-4, B) shows IL-13 and C) shows IL-6 production from basophils on a C57BL/6 and 129/sv genetic background.

The results are expressed as the mean \pm SEM of n=6.

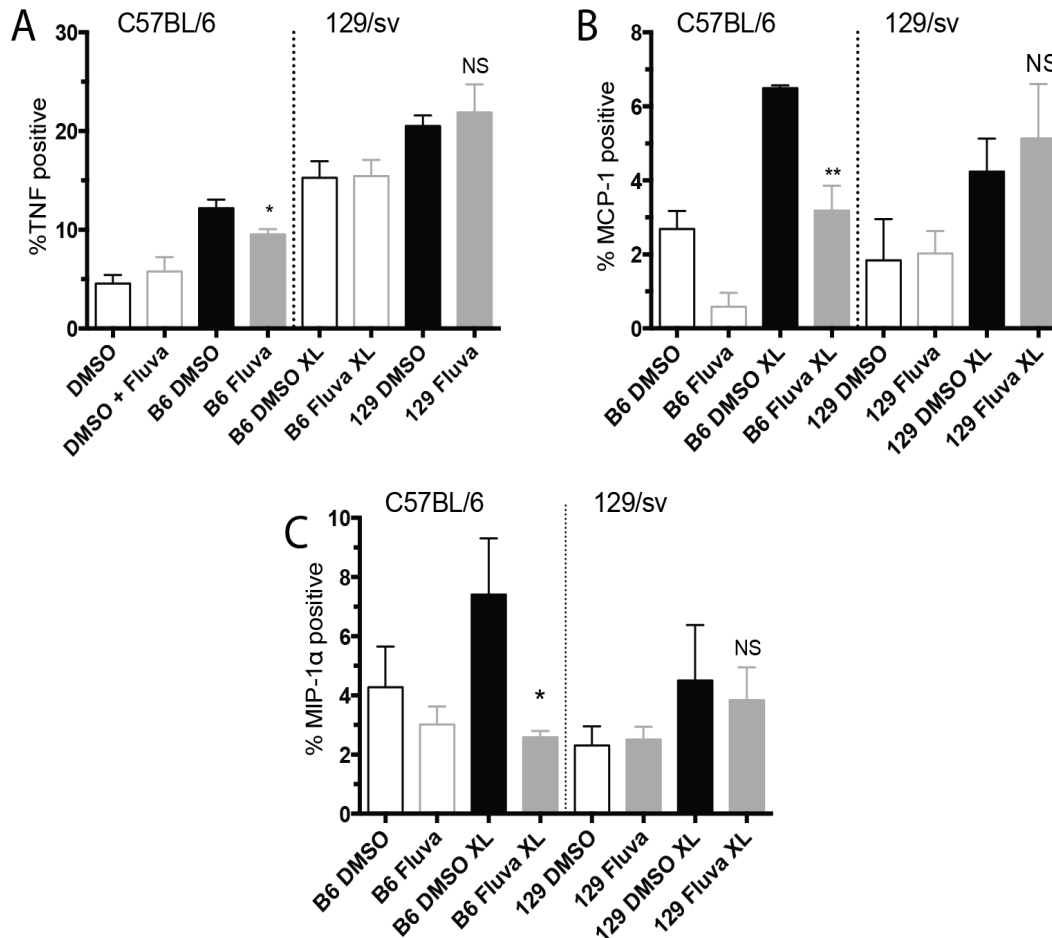


Figure 40

Fluvastatin suppress $TNF\alpha$, MCP-1 and MIP- α from IgE activated basophils on a C57BL/6 but not a 129/sv genetic background.

C57BL/6 and 129/sv IgE bone marrow were cultured in IL-3 20 ng/ml for 8 days and then cultured with or without IgE at 0.5 μ g/ml, in fluvastatin 10 μ m or DMSO for 24 h. in cell staining was conducted as described in methods and materials with CD49b as a basophil marker. A) Shows $TNF\alpha$, B) shows MCP-1 and C) shows MIP- α production. The results are expressed as the mean \pm SEM of n=6.

Fluvastatin effect on basophil degranulation

We, and others have shown that fluvastatin suppresses mast cell degranulation (26) in RBL-2H3 cells, and we have shown that the suppression is evident on C57BL/6 mice but not Th2 prone 129/sv mice. Here, we show that fluvastatin suppresses basophil degranulation in C57BL/6 mice but not Th2 prone 129/sv mice (Figure 41).

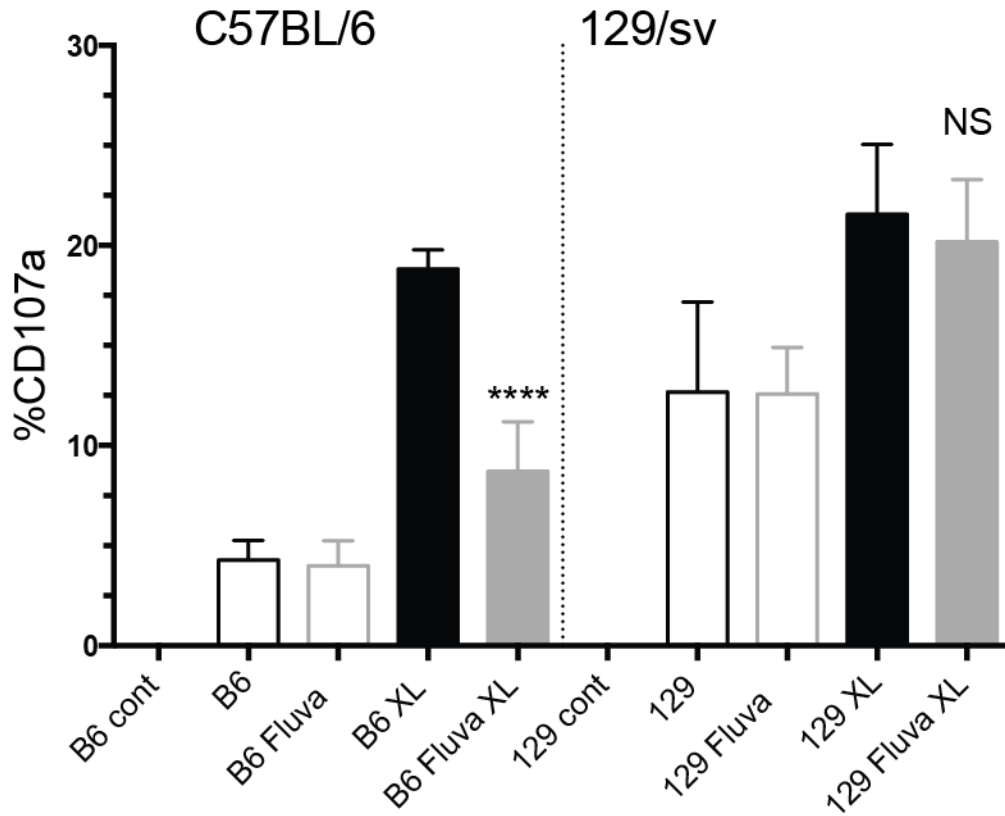


Figure 41

Fluvastatin suppresses basophil degranulation from C57BL/6 but not 129/sv mice. C57BL/6 and 129/sv IgE sensitized basophils were cultured in IL-3 20 ng/ml with fluvastatin or DMSO for 24 h and then activated for 1 h with antigen. Cells were then stained for CD107a for 45 minutes and then run by flow cytometry. The results are expressed as the mean \pm SEM of n=6.

Fluvastatin alters membrane bound Ras on C57BL/6 mice

As previously states, fluvastatin treatment does not suppress Fc ϵ RI expression. Our data suggests that fluvastatin suppresses geranylgeraylation more so that farnesylation. We therefore wanted to investigate the effect fluvastatin may have on membrane bound Ras, a prenylated protein involved in IgE mediated signaling. Here, we show suppression of membrane bound Ras on the C57BL/6 but not 129/sv BMMC's.

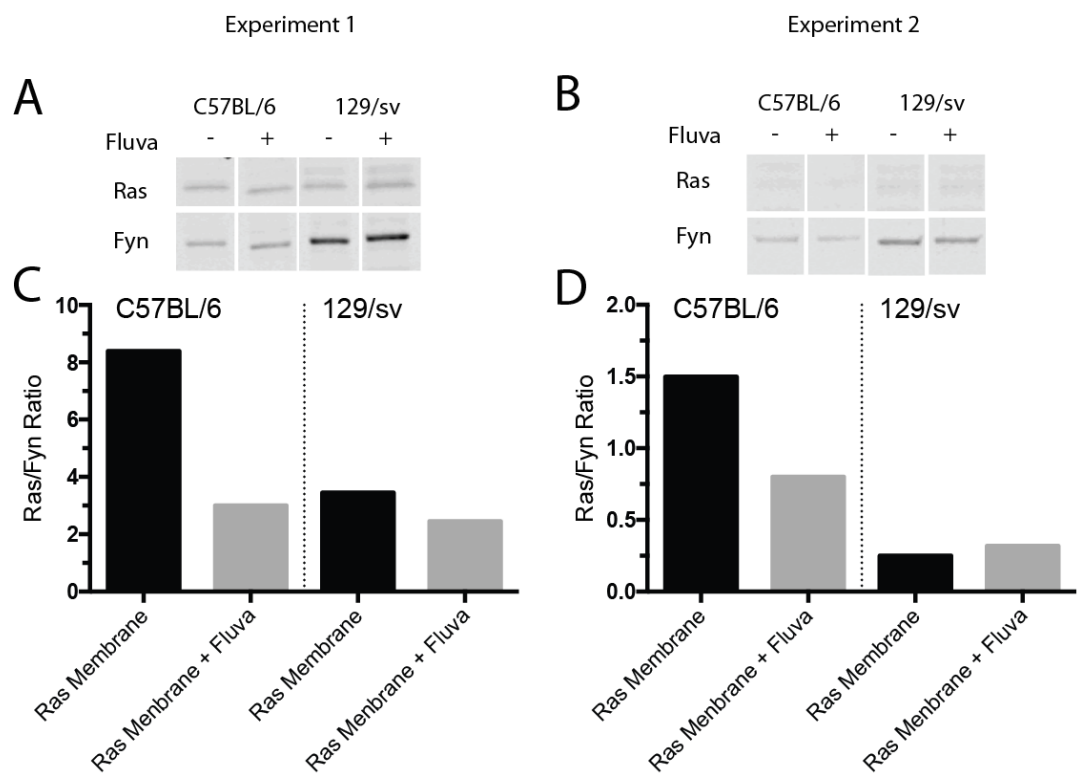


Figure 42

Fluvastatin treatment dramatically suppresses membrane bound Ras in C57BL/6 BMMC's, whereas there is little difference in 129/sv BMMC's. BMMC's treated with or without fluvastatin for 24 h. Cells were lysed and cytospin. Cell membranes were collected and probed for Ras. Fyn was used as a control. Data represents 2 Independent experiments after normalizing to Fyn.

Fluvastatin selectively suppresses Fyn but upregulates Lyn in C57BL/6 BMMC's

Down stream of the FcεRI, Fyn and Lyn are recruited and activated. Both Fyn and Lyn can act as positive regulator, recruiting LAT. Additionally, Lyn can act as a negative regulator. Here, we demonstrate that fluvastatin treatment suppresses Fyn activation and augments Lyn activation on the C57BL/6 BMMC's.

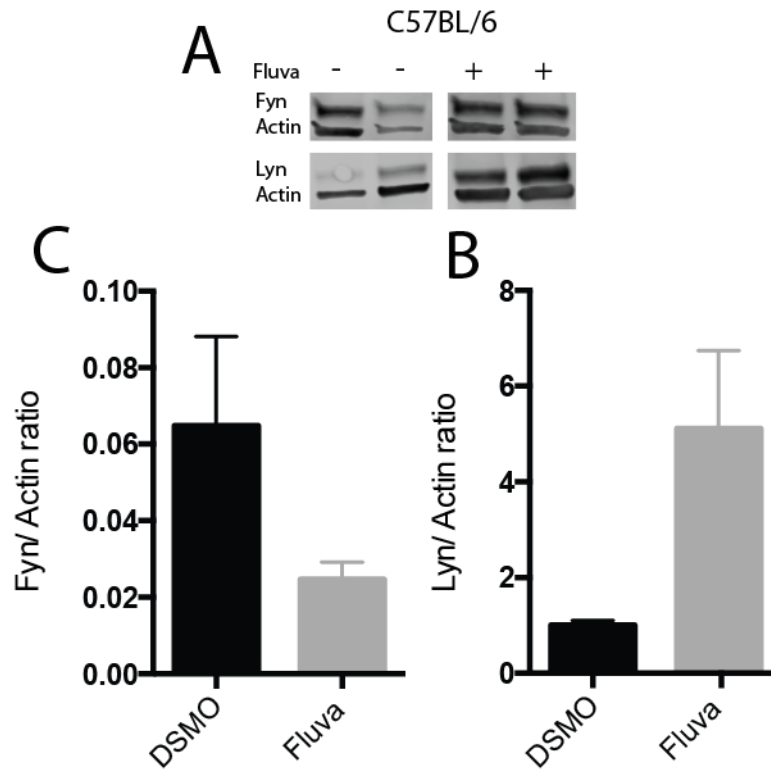


Figure 43

Fluvastatin selectively suppresses Fyn and upregulate Lyn.

C57BL/6 BMMC's were IgE primed and cultured in IL-3 and SCF, with or without fluvastatin overnight and activated with antigen for 5 minutes, followed by cell lysing. Lysates were blotted for Fyn and Lyn. A) Shows a representative blot for Fyn and Lyn from two separate experiments that yielded similar results. B) Shows quantification of Fyn analysis form two independent experiments. C) Shows quantification of Lyn analysis form two independent experiments.

Fluvastatin selectively suppresses ERK and STAT5 pathway

The suppressive effects of fluvastatin are evident through the suppression of membrane Ras on C57BL/6 BMMC's. Down stream of Fyn and Ras, we show that fluvastatin suppresses ERK (Figure 44) a member of the MAP kinases. Greatest suppression in fluvastatin treated C57BL/6 BMMC's, is evident at 5 minutes of antigen activation. Additionally, it has been shown that Stat5 is critical for IgE mediated signaling (33-35). We therefore investigated fluvastatin suppression of Stat5. Here, we show that fluvastatin treatment also suppresses Stat5 expression (Figure 45) in C57BL/6 BMMC's but has little effect on 129/sv BMMC's. Taken together, these data suggest a role for protein prenylation being important for IgE induced signaling in primary BMMC's.

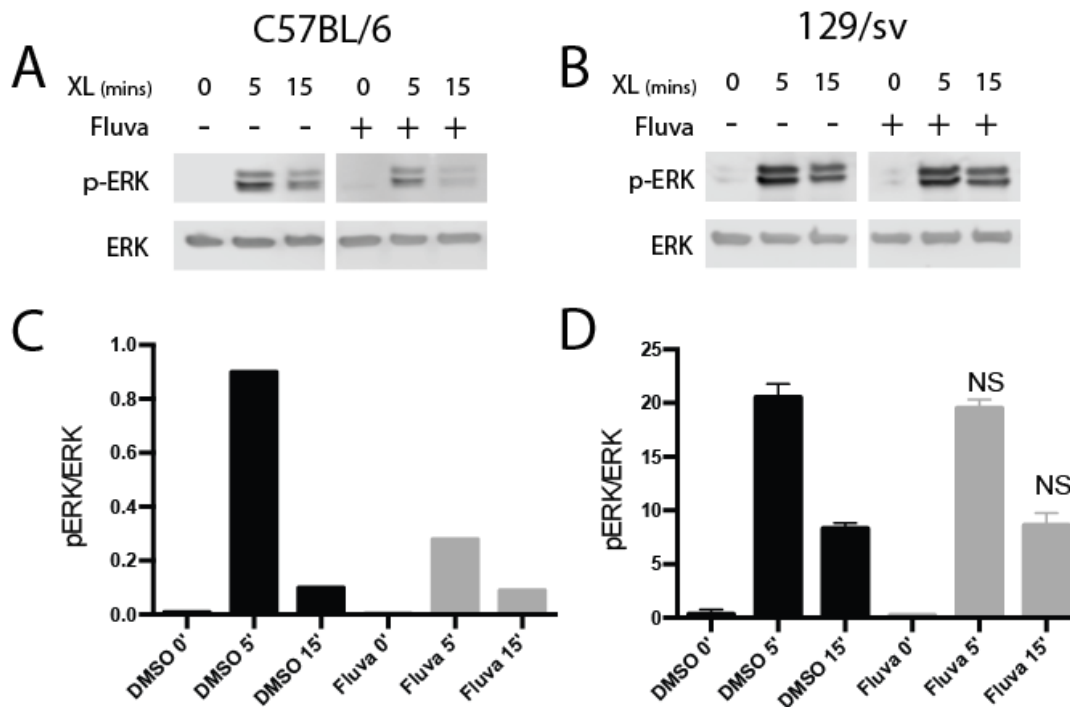


Figure 44

Fluvastatin selectively suppresses ERK on C57BL/6 BMMC's.

C57BL/6 and 129/sv BMMC's were IgE primed and cultured with or without fluvastatin in IL-3 and SCF overnight and activated with or without antigen for 0, 5 and 15 minutes, followed by cell lysing. Lysates were blotted ERK and p-ERK. A) Shows a representative blot for ERK and pERK for C57BL/6 BMMC's from two separate experiments that yielded similar results. B) Shows quantification of the p-ERK/ERK ratio for C57BL/6 BMMC's form two independent experiments. C) Shows a representative blot for ERK and pERK for 129/sv BMMC's from two separate experiments that yielded similar results. D) Shows quantification of the p-ERK/ERK ratio for 129/sv BMMC's form two independent experiments.

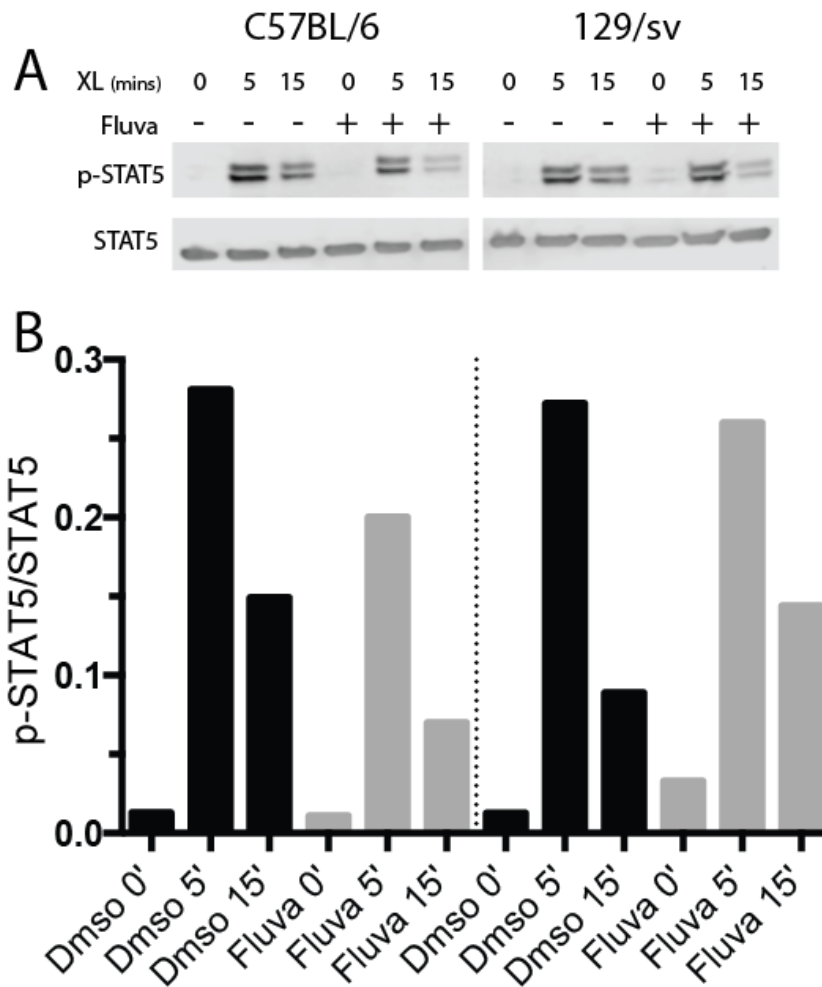


Figure 45

Fluvastatin selectively suppresses Stat5 on C57BL/6 but not 129/sv BMMC's.

C57BL/6 and 129/sv BMMC's were IgE primed and cultured with or without fluvastatin in IL-3 and SCF overnight and activated with or without antigen for 0, 5 and 15 minutes, followed by cell lysing. Lysates were blotted Stat5 and p-Stat5.

A) Shows a representative blot for Stat5 and p-Stat5 for C57BL/6 and 129/sv BMMC's from one experiment. B) Shows quantification of the p-Stat5/ Stat5 ratio for C57BL/6 and 129/sv BMMC's from one experiment.

Fluvastatin selectively augments HmG-CoA reductase expression on 129/SvImJ but not C57BL/6 BMMC's.

Thus far, we have demonstrated that C57BL/6 mice are sensitive to fluvastatin, and that 129/sv are resistant. We initially hypothesized that it might be that 129/sv mice have an increase in MVA or that they have increased expression of HMGCoA reductase (HMGCR). Here, we demonstrate that 129/sv mice have increased HMG-CoA reductase levels upon treatment with fluvastatin, in comparison to the C57BL/6 BMMC's (Figure 46). These data suggest that the increase in HMG-CoA reductase levels in 129/sv BMMC's, is in part responsible for 129/sv mice being resistant to fluvastatin.

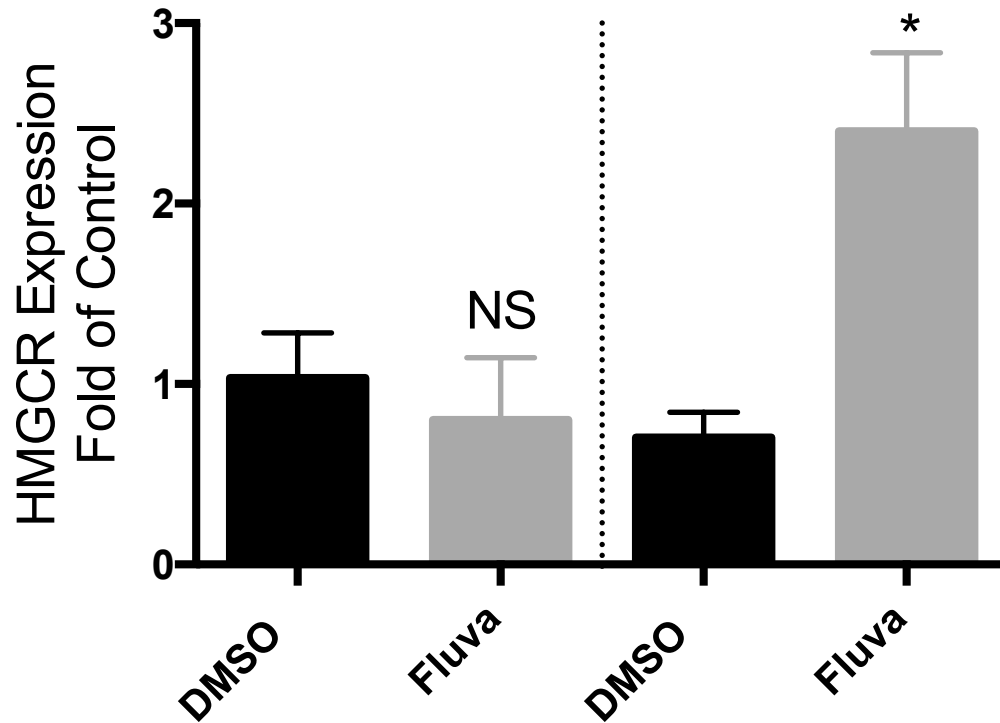


Figure 46

Fluvastatin selectively augments HMGCR reductase expression on 129/Sv but not C57BL/6 BMMC's.

Expression of HMGCR mRNA in C57BL/6 and 129/sv BMMC's. BMMC's were plated at 1×10^6 cell ml and treated with DMSO or Fluvastatin for 6 h. mRNA expression was determined by comparative qPCR. Data are expressed as the mean \pm SD of triplicate measurements from 2 independent experiments n=6.

Fluvastatin suppresses passive systemic anaphylaxis on C57BL/6 mice but not 129/sv

Thus far, we have demonstrated the suppressive effects of fluvastatin in vitro and ex vivo and that fluvastatin's suppressive capabilities are exhibited on the Th1 C57/BL/6 but not the Th2 129/sv mice. We next wanted to extend our study the functional relevance of fluvastatin sensitivity on both the C57BL/6 and 129/sv mice. IgE dependent passive systemic anaphylaxis (PSA) is a mast cell dependent process and thus, we investigated the effects of fluvastatin on mast cells within this in vivo system. Here, we show that fluvastatin dampens PSA on C57BL/6 mice (Figure 48 A), in a dose dependent manner (Figure 47) but, mice treated with fluvastatin on 129/sv background continued to be fluvastatin resistant (Figure 48 B). Plasma chemokine levels from C57BL/6 mice also show suppression of serum MIP-1 α (Figure 49) on C57BL/6 but not 129/sv mice. These data, taken together, suggest that fluvastatin dampens the early and late phase of mast cell dependent anaphylaxis. We were unable to fully investigate additional cytokines and chemokines due to low serum levels.

Histamine causes bronchoconstriction, vasodilation and vascular leakage, features observed during systemic anaphylaxis. We wanted to investigate whether fluvastatin is affecting the vasculature in conjunction with mast cells of the C57BL/6 mice. Here, we show that fluvastatin pretreatment 16 h before administration of histamine does not significantly alter PSA (Figure 50). We therefore conclude, that it is the effects fluvastatin exerts on mast cells that causes a reduction of PSA on C57BL/6 mice.

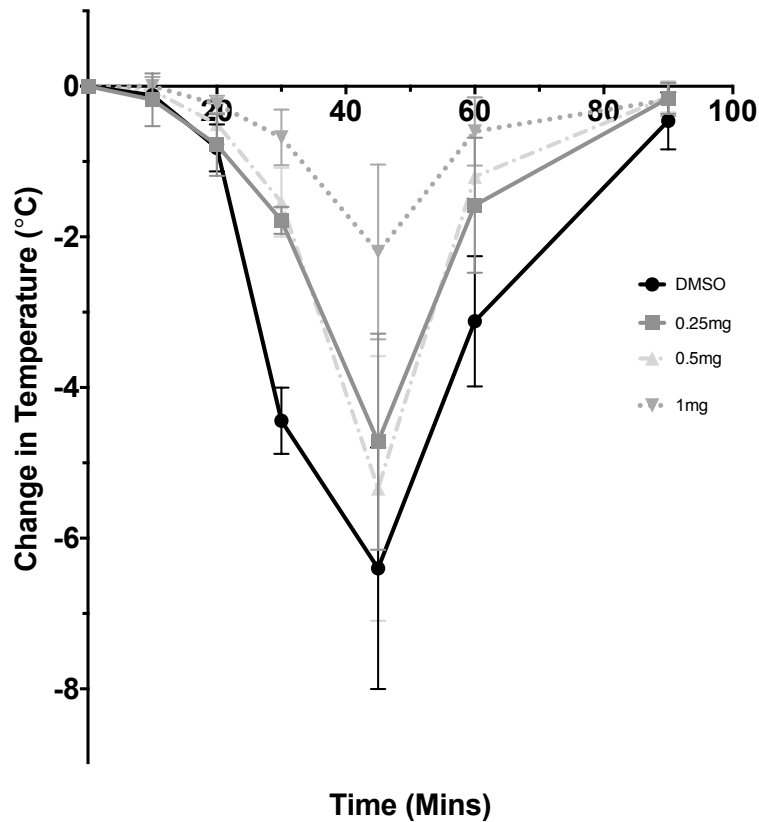


Figure 47

Fluvastatin dampens IgE induced PSA in C57BL/6 mice in a dose dependent manner.

C57BL/6 mice were sensitized with 50 μ g of IgE by intraperitoneal injection overnight with or without fluvastatin at 1 mg, 0.5 mg or 0.25 mg or DMSO. Mice were then injected with fluvastatin or DMSO the next morning and left for 90 minutes to rest. PSA was induced by intraperitoneal administration of DNP-HSA antigen. Changes in core body temperature were measured by rectal probe at regular intervals. (DMSO n=5), (fluvastatin 1 mg n=5), (fluvastatin 0.5 mg n=5) and (fluvastatin 0.25 mg n=5).

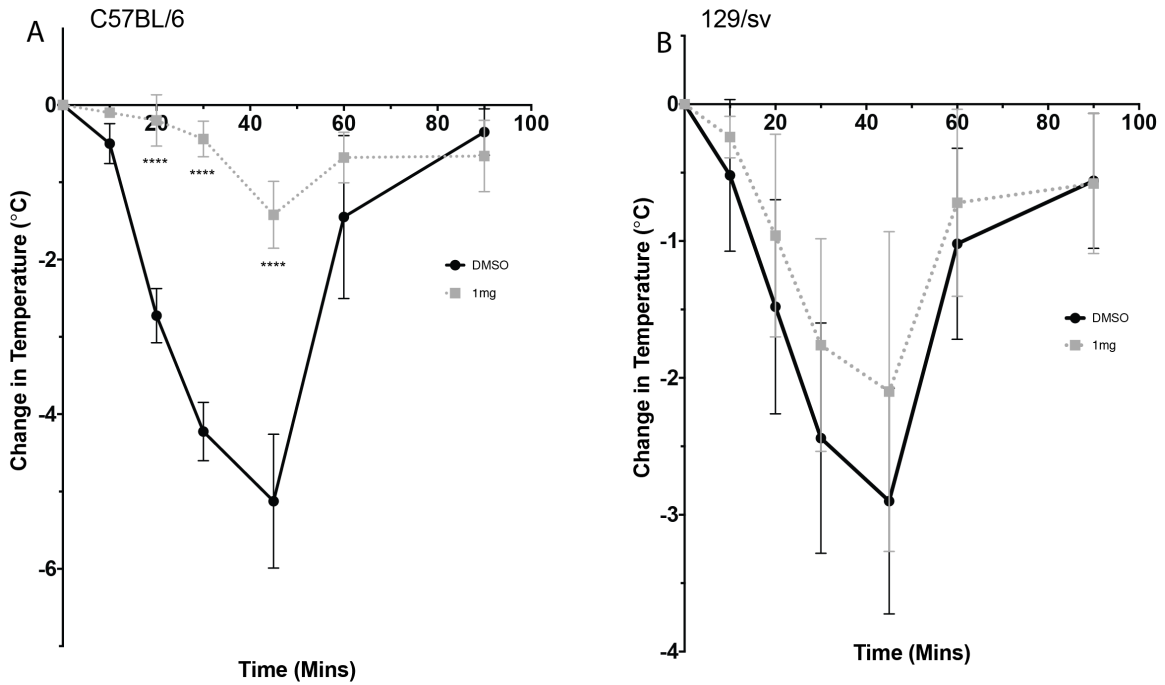


Figure 48

The effect of fluvastatin on PSA is strain dependent.

C57BL/6 mice were sensitized with 50 μ g of IgE by intraperitoneal injection overnight with or without fluvastatin at 1mg, or DMSO. Mice were then injected with fluvastatin or DMSO the next morning and left for 90 minutes to rest. PSA was induced by intraperitoneal administration of DNP-HSA antigen. A) Fluvastatin suppresses IgE induced PSA in C57BL/6 mice. B) Fluvastatin does not alter IgE induced PSA in 129/sv mice. Changes in core body temperature were measured by rectal probe at regular intervals.

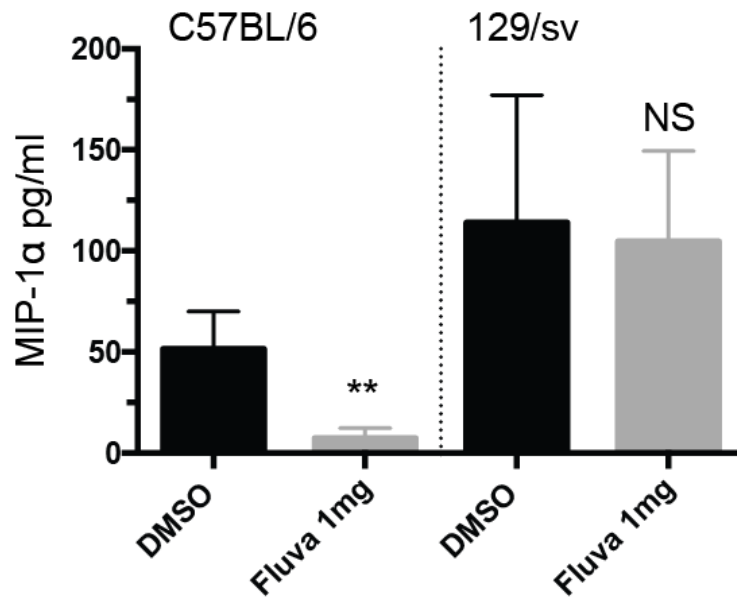


Figure 49

Plasma levels of circulating MIP-1 α following IgE- induced PSA on C57BL/6 and 129/SvImJ mice.

Mice were administered fluvastatin and sensitized to IgE 16 h prior to the induction of PSA and then again 1 h before Mice were injected i.p with antigen. Mice were then sacrificed 4 h after the induction of PSA and cardiac puncture was performed to assess circulating plasma MIP-1 α levels. Data shown represents the mean \pm SEM. C57BL/6 DMSO mice (n=2), C57BL/6 fluvastatin mice (n=2) 129/sv DMSO mice (n=5), 129/sv fluvastatin mice (n=5).

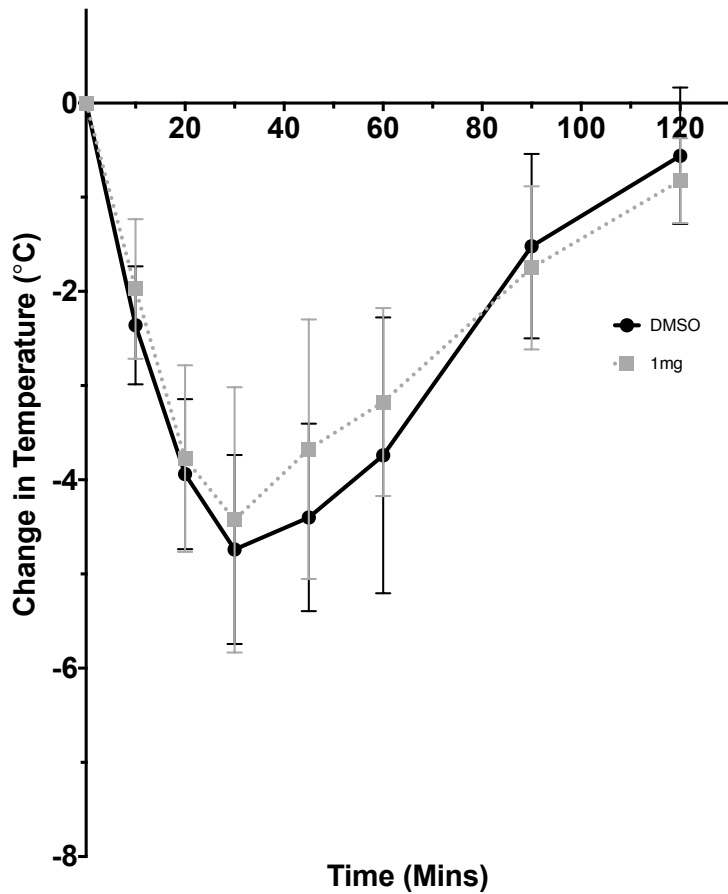


Figure 50

Fluvastatin does not alter histamine induced PSA in C57BL/6 mice.

C57BL/6 mice were injected by intraperitoneal injection overnight with or without fluvastatin at 1 mg, or DMSO. Mice were then injected with fluvastatin or DMSO the next morning and left for 90 minutes to rest. PSA was induced by intraperitoneal administration of histamine. Changes in core body temperature were measured by rectal probe at regular intervals. Mice were sacrificed 4 hours after the induction of PSA and cardiac puncture was performed to assess serum cytokine levels.

The variable responsiveness of fluvastatin is consistent with primary human skin mast cells.

We extended our study of the fluvastatin induced differential production of IgE mediated cytokines and chemokine production, to primary human skin mast cells from five donors. Interestingly, we show a large variation in fluvastatin responsiveness between the five donors (Figure 51). When investigating MCP-1 production we show we show suppression from as little as 18% to as much as 71% as seen using cluster analysis. With $\text{TNF}\alpha$ we see a variation in sensitivity ranging from 30% to 87% suppression. These data suggest that the variability in cytokine and chemokine suppression induced by fluvastatin may have a human correlate.

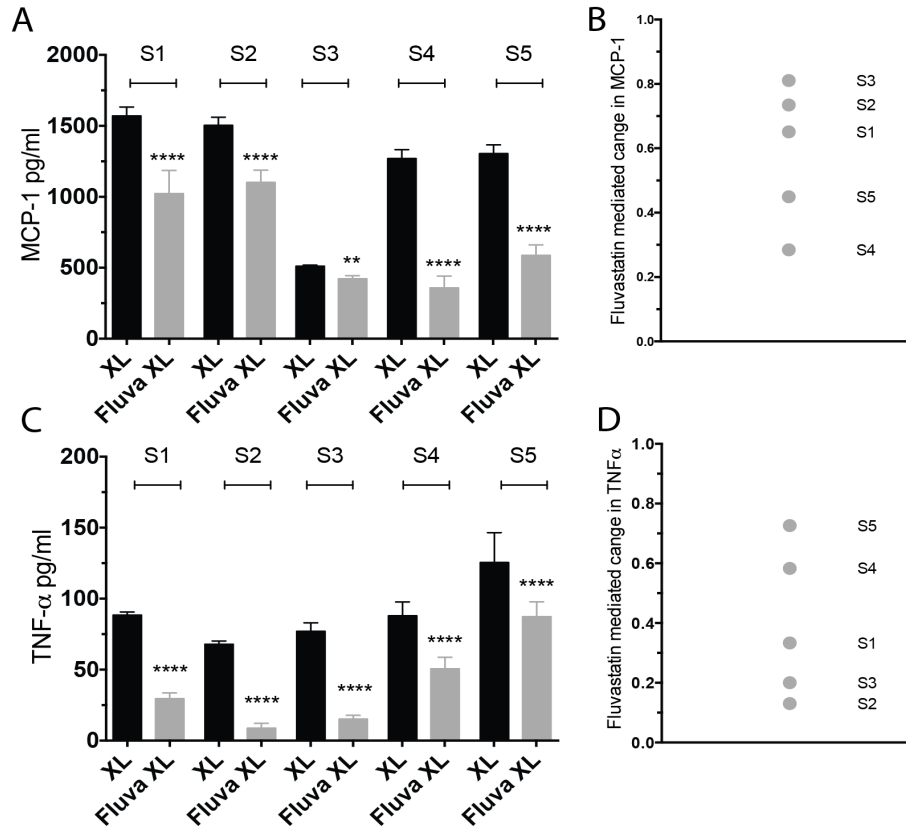


Figure 51

Variation in human mast cell responsiveness to fluvastatin

Mast cells cultured from human skin donors were sensitized to IgE and treated with or without fluvastatin for 24 h. Human skin donors were then activated with antigen for 16 hours as described in methods and materials. Supernatants were then collected and then analyzed for the production of MCP-1 and TNF- α by ELISA. Total of 5 donors labels sample 1-5, each in quintuplicate. A) MCP-1 chemokine levels. B) Fold of control MCP-1 levels S1=34% suppression, S2= 26% suppression, S3= 18% suppression, S4=71% suppression, S5= 54% suppression. C) TNF- α cytokine levels. D) Fold of control TNF- α levels. S1=66% suppression, S2= 87% suppression, S3= 80% suppression, S4=42%

CHAPTER 4– DISCUSSION:

Statins, as HMG-CoA reductase inhibitors (36) display effects in addition to lipid lowering and exhibit pleiotropic effects on immune cells. Given that statins alter protein prenylation in addition to the lipid modification that occurs on many proteins including those of the immune system, it is not surprising that statins alter immune function. Statins display comparable biochemical properties and effects on mevalonate, but have different structures and exhibit different potencies. It has been shown that statins, in particular lovastatin, can inhibit function of the RBL-2H3 cells (37) in vitro, and that fluvastatin inhibits degranulation of RBL-2H3 cells (26) in vitro. Furthermore, cerivastatin and atorvastatin have been shown to suppress growth and IgE-mediated histamine release in human basophils (38).

This study establishes that a range of statins inhibit IgE-induced cytokine production from Th1-prone C57BL/6 BMMC's with varying degrees. There were some exceptions to this: pravastatin slightly enhanced cytokine production, and atorvastatin produced no significant effect. While atorvastatin has been shown to strongly suppress human mast cell degranulation in vitro (39), pravastatin's effects may be due to its hydrophilic properties preventing it from permeating the cell surface membrane to the extent of more lipophilic statins such as simvastatin, lovastatin and fluvastatin (40). This being said, the most lipophilic statins, lovastatin and simvastatin (41), whilst demonstrating significant suppression, do not display the greatest suppression. Additionally, we demonstrate that fluvastatin; a moderately lipophilic statin, suppresses cytokine

production with the strongest effect and in a dose dependent manner on primary murine mast cells. Furthermore, fluvastatin's suppression is shown in vitro and ex vivo. Interestingly, fluvastatin's suppressive capabilities did not extend to IL-10, widely known as an anti-inflammatory cytokine (42-44).

This study also demonstrates that fluvastatin effects on IgE-induced cytokine production on primary murine mast cells were reversed with MVA pretreatment, confirming targeted specificity to the mevalonate pathway. These data support the findings that suppressed degranulation mediated by fluvastatin was also rescued by MVA in RBL-2H3 cells (26). As previously stated, MVA inhibition by statins suppresses the generation of prenylated proteins. Prenylation is a posttranslational modification, with covalent binding of isoprenoid lipids to conserved cysteine residues near the C terminus of target proteins (45, 46). These include the Ras superfamily of small GTP binding proteins, such as Ras, Rho and Rac.

Prenylated proteins can be further divided into farnesylated proteins and geranylgeranylated proteins. Since farnesylated and geranylgeranylated proteins play a critical role in maintaining cell function (47), we investigated the effect of geranylgeranylation and farnesylation by using GGTI and FPTIII respectively. Here, we show that GGTI pretreatment significantly inhibited cytokine production and mimicked the action of fluvastatin, whereas FPTIII only slightly suppressed IL-6 production. These data suggest that fluvastatin's ability to suppress IgE-induced cytokine production might be mediated by geranylgeranylation as opposed to farnesylation. Farnesylation might be more important for the

arachidonic acid pathway in mast cells (26, 48) (49). It has been reported that dolichol and cholesterol, which are also synthesized from the mevalonate pathway, did not restore inhibition of degranulation in RBL-2H3 cells (37) and were not tested here.

To further corroborate the involvement of geranylgeranylation for IgE induced cytokine production, we attempted to reverse fluvastatin suppression with exogenous GGPP and FPP to restore geranylgeranylation and farnesylation, respectively. We found that GGPP partially rescued cytokine production, whereas FPP has no significant effect. These data further support the theory that fluvastatin blockade of geranylgeranylation, more so than farnesylation, suppresses IgE-induced cytokine production. The importance of upstream lipids such as isoprenoids was further supported by studies using the squalene synthase inhibitor zaragozic acid A (ZA) that blocks the formation of squalene from transfarnesyl pyrophosphate (50). ZA pretreatment did not alter IgE-induced cytokine production despite suppressing cholesterol synthesis, as measured by mass spectrometry. 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 mast cell response.

Allergic asthma is characterized by reversible obstruction of airway hyper responsiveness, infiltration of inflammatory immune cells into the lungs, coupled with Th2-mediated cytokines. The mechanism has yet to be fully elucidated, but

the involvement of inflammatory and structural cells are critical. Current therapies can be improved upon, especially for patients who respond poorly to traditional treatments. These include those who remain symptomatic despite high dose corticosteroid treatment, and account for approximately 10% of asthmatics (51). Given the time, expense, and risks associated with new drug developments, exploring previously-approved drugs for alternative disease states may prove to be beneficial. In the case of asthma, statins are a prime candidate.

There are studies to support statin use in asthma and allergic disease. Peripheral blood mononuclear cell (PBMC) proliferation and inflammatory responses were suppressed by fluvastatin in patients with allergic asthma (52). Furthermore, Atorvastatin in conjunction to inhaled corticosteroids improved lung function and airway inflammation in atopic asthmatics (53). These and other studies have led to the conclusion and statins are beneficial for asthma management (54). Cellular and molecular mechanisms supporting these clinical benefits are forthcoming from human and animal studies. Statins may suppress T cell activation by decreasing MHC II expression on monocytes induced by IFN- γ (55). T cell activation, proliferation, and migration are also suppressed by statin treatment (52). This includes Th2 lymphocytes, which play a key role in the initiation and prolongation of airway inflammation. In addition to their effects on immune cells, statins have been shown to suppress bronchial wall remodeling (56).

Among the statins, Simvastatin has been studied in some detail for its suppressive effects. It has anti-inflammatory capabilities in the murine model of

allergic asthma (57). This is in partly due to suppression of T lymphocytes and T cell-produced IL-4 and IL-5 (58). More recently, Kim et al showed that simvastatin reduced ovalbumin- specific IgE level, number and total of macrophages, neutrophils and eosinophils into bronchoalveolar lavage fluid (59) in a mast cell-independent model. Moreover, Simvastatin also reduced thioglycolate-induced peritoneal inflammation (60) in which the predominate infiltrate is neutrophils. This suggests that statins might be beneficial in “non-allergic” asthma, which is associated with strong Th17 responses and neutrophilic infiltration (61-63).

Taken together, many studies have investigated immune modulating cells and the effect statins have on allergic lung function. However, these in vivo studies have overlooked mast cells, and its fundamental role in allergic asthma and anaphylaxis. In the airways of asthmatics, mast cells are not only present (64-66), but fundamental for initiating and maintaining allergic inflammation by interactions with resident and infiltrating cells in the airway.

As many treatments have shown, targeting and suppressing specific aspects of mast cell function is beneficial to allergic disease. Using the mast cell-dependent passive systemic anaphylaxis (PSA) model, we assessed the functional validity of our in vitro data. Anaphylaxis is a life threatening acute systemic reaction caused largely by IgE-mediated release of mediators from mast cells and basophils to allergen triggers, such as venom, food and medication (67). Estimated occurrences of anaphylaxis vary greatly, with the majority of studies agreeing that its prevalence is increasing (68-74). Estimates

predict a frequency of approximately 50 to 2000 episodes per 100,000 people (67). Anaphylaxis can also be IgE-independent, caused by agents such as serum complement activation or direct mast cell degranulation. These IgE-independent pathways are often directed by non-immunological factors such as exercise, cold air, or medications such as vancomycin or opioids, and are not fully understood (69, 75). Regardless of the activating pathway, mast cells and to a lesser extent, basophils are central effector cells in anaphylaxis. Degranulation and the immediate release of preformed mediators such as histamine, leukotrienes, and platelet activating factor (PAF) occur between 5 and 30 minutes after cell activation (76). This gives rise to the hypersensitivity response (69). The delayed response arises 2-6 hours after initiation and involves the production of cytokines and chemokines (77).

This study demonstrates that pretreatment with fluvastatin prior to induction of IgE-mediated PSA dramatically reduced the severity of anaphylaxis in C57BL/6 mice. These data clearly show the importance of mast cells as a target and fluvastatin as a therapy for allergic disease. To rule out fluvastatin primarily provoking a response on the vasculature, we pretreated mice with fluvastatin and then bypassed the mast cell response by injecting histamine i.p. Our results showed no significant drop in core body temperature. These data demonstrate that the dramatic suppression of anaphylaxis is a result of fluvastatin acting on the mast cells and not the vasculature. Strikingly, mice of the Th2-prone 129/SvImJ background demonstrated no alteration in anaphylaxis following pretreatment with fluvastatin. These in vivo data support our in vitro

findings that 129/SvImJ mast cells are resistant to fluvastatin-mediated suppression. We postulate that genetic variation could be the cause of these altered responses in inbred mice.

In addition to the classical anaphylactic pathway, numerous studies have shown that anaphylactic responses can occur in mice deficient in mast cells (78), IgE (79) or Fc ϵ RI α chain {Dombrowicz:1997wy}. These data provide an argument for an alternative pathway, whereby IgG1- dependent activation of the Fc γ RIII receptor is required (73). Studies show that although mast cells are involved, the major cells involved in this pathway are basophils, macrophages and neutrophils (80). This is a mechanism that can be explored in future work.

Down stream of the IgE receptor, Fyn and Lyn are recruited and activated. Lyn acts both as a positive and negative regulator, the predominant phenotype being a negative regulator, as seen in Lyn Knockout mice that demonstrate increased PSA (81, 82). Our data suggest that statins might be useful in inflammatory disorders not typically thought of as mast cell-driven, such as lupus.

Along with Fyn down regulation, we further show fluvastatin suppressed Erk phosphorylation in C57BL/6 but not 129/SvImJ BMMC's. Additionally, Stat5 phosphorylation was suppressed on C57BL/6 but not 129/SvImJ BMMC. We previously found that Fyn and Stat5 physically interact, and that Stat5 is crucial for mast cell function and survival (33, 83, 84). Given the importance of Fyn and

Stat5 for IgE-mediated cytokine release, suppressing this pathway could explain the reduced cytokine secretion. Additionally, the Ras–MAPK cascade is important for differentiation and survival. reduced activation of Stat5 and ERK could partially explain the increased cell death observed among C57BL/6 mast cells treated with fluvastatin for prolonged periods.

Variable responses to fluvastatin among C57BL/6 and 129/SvImJ mast cells are consistent with previous studies from our group. Previously, we demonstrated that mast cell precursors from the Th2-prone 129/SvImJ background are resistant to IL-10 (85), and BMDC are resistant to the TGF- β -mediated suppression (35). Additionally, 129/SvImJ mice display increased circulating levels of IgE and an increase in Fc ϵ RI expression (86). Therefore we were intrigued by the lack of fluvastatin effects on 129/SvImJ mice. We further found that the commonly used Th2-prone Balb/c strain also demonstrated fluvastatin resistance when assessing IgE-mediated mast cell activation. A simple conclusion would be that genetic variations predisposing to strong Th1 or Th2 development show linkage to genetic effects yielding drug resistance. However, it is important to note that while the C57BL/6 mice are Th1 prone and 129/SvImJ are Th2 prone, this is not their only variation. Moreover, fluvastatin responses are not simply a Th1-prone versus a Th2-prone phenomenon. Mast cells from Th2-prone A/J mice did not show complete fluvastatin resistance in vitro and ex vivo. These observations prompted a deeper investigation into the mechanisms explaining this pharmacogenomic effect.

There is a wide variation in inter-individual responses to statin therapy, and many have hypothesized that genetic differences may contribute to this variation (87). Clinical data for pharmacogenetic interactions with statins have largely focused on polymorphisms in the cytochrome P450 enzyme, which assists in statin export; the lipid metabolism genes apolipoprotein E and B (APOE,APOB), which control cholesterol transport across the plasma membrane; and cloesteryl ester transfer protein (CETP) and the LDL receptor (LDLR) which regulate LDL/VLD protein binding (88-90). CYP2C9 is the primary pathway for fluvastatin metabolism (91). It might be that functional variants of the CYP2C9 gene are genetic determinants for the lipid response to fluvastatin therapy. However, this hypothesis while important for in vivo study, would not explain our in vitro findings.

In addition to these plausible mechanisms yielding statin resistance, there could be pharmacodynamic gene-drug interactions involving gene products expressed as receptors after the drug enters circulation (92). A third possibility is polymorphisms in genes that are in the causal pathway of disease and are subsequently able to modify the effects of drugs (93). For example, in one study, a subgroup of coronary heart disease patients did not respond to simvastatin treatment. This study demonstrated that patients with high baseline synthesis of cholesterol showed simvastatin responsiveness, whereas those with low cholesterol synthesis were resistant (94).

Our data show that cholesterol levels are not significantly different between C57BL/6 and 129/SvImJ BMMC. While, 129/SvImJ BMMC are resistant

to fluvastatin, they are suppressed by GGTI. We therefore propose that the variation is above the step of geranyl pyrophosphate in the mevalonic acid pathway. Thus, it is entirely plausible that the variation is at the level of HMG-CoA, being either a polymorphism or alteration in expression between strains. We therefore sequenced HMGCR (HMG-CoA reductase) from C57BL/6 and 129/SvImJ cDNAs, but found no variations between the strains (data not shown). We did however demonstrate that 129/SvImJ mast cells have greater than two-fold increase in HMGCR following fluvastatin treatment. We therefore propose that this increase in HMGCR is at least in part responsible for fluvastatin resistance in 129/SvImJ mice.

The alteration in fluvastatin responsiveness seen in murine mast cells correlates with variable responsiveness among primary human skin mast cells. Comparing various donors, we found 18% to 87% suppression by fluvastatin, when measuring IgE-mediated cytokine secretion. These data demonstrate both the possible utility of inhibiting mast cells responses in patients, and the inherent variability that should be anticipated. Larger numbers of donors are needed to fully corroborate these findings. Understanding how fluvastatin and statins in general alter IgE-mediated mast cell signaling and the significance of genetic background could prove to be important for treating allergic disease.

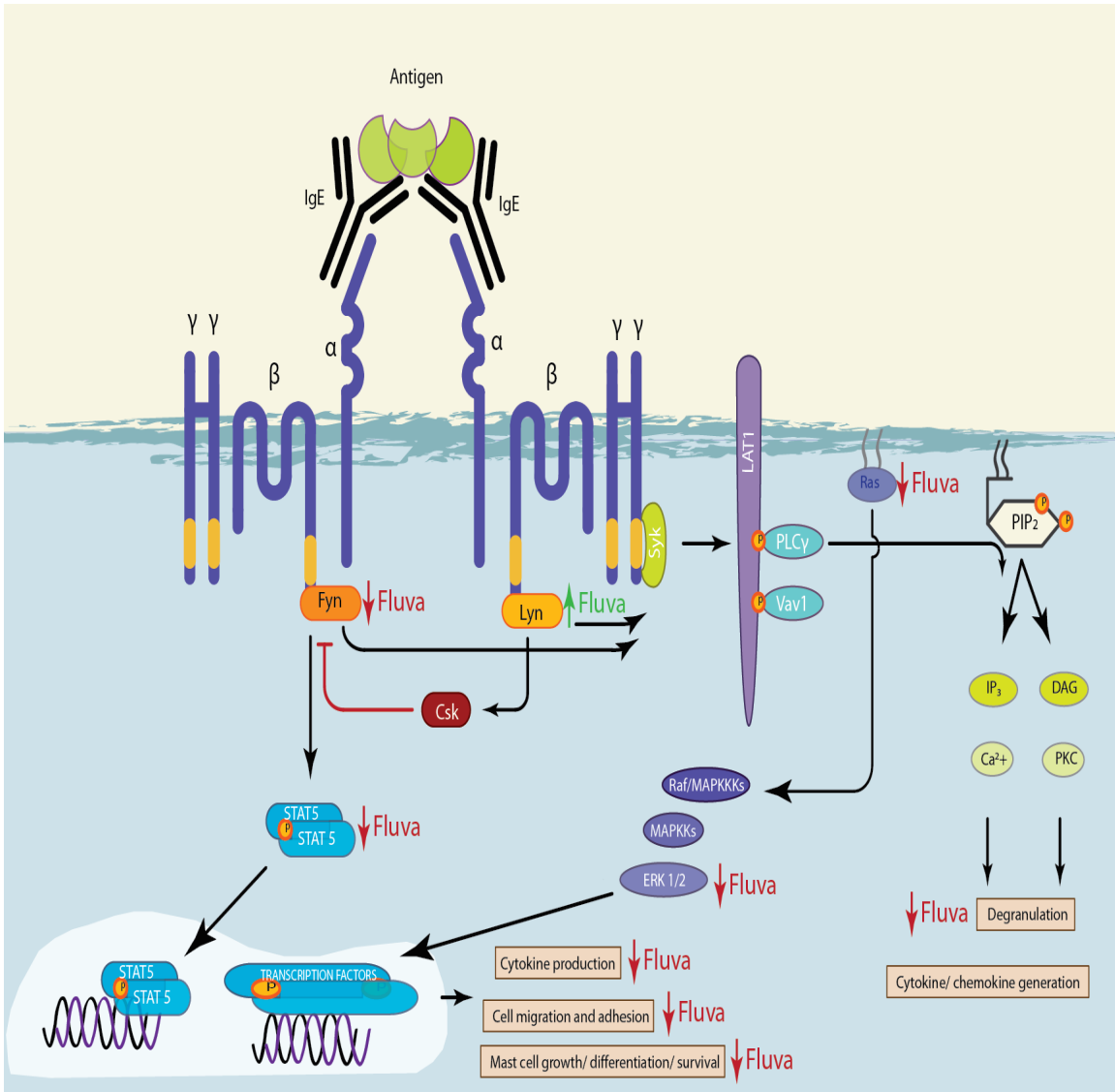


Figure 52

The proposed mechanism whereby fluvastatin suppresses the FcεRI signaling cascade.

Following cross-linking of the FcεRI receptor, the Src family kinases Fyn and Lyn become activated. Fyn and Lyn both activate Syk and subsequently LAT. Upon phosphorylation, this scaffold protein serves as a multimolecular signaling complex allowing for positive signaling downstream of the FcεRI receptor. Lyn can also act as a negative regulator by recruiting Csk, which inhibits Fyn. This

figure illustrates the observed signaling molecules inhibited and enhanced by fluvastatin treatment.

Part II

The divergent roles of Wnt5a and Leptin on
mast cell function

Abstract

The prevalence of obesity has reached epidemic proportions in western countries. Increased levels of adipose tissue are strongly associated with disease states such as chronic heart disease, atherosclerosis, insulin resistance and type II diabetes (95). Obesity is also associated with increased risk of inflammatory conditions such as allergic asthma and allergic rhinitis (96) (97). The obese state has been described as chronic low-grade inflammation (95, 98). Thus, obesity and inflammation are inextricably linked. The role of mast cells in inflammatory disease is unequivocal. Mast cells are instrumental effector cells in IgE-mediated allergic diseases such as asthma (97, 99) and rhinitis (99) (96). The receptor for IgE is the high affinity multimeric Fc receptor FcεRI, which when activated through a IgE-antigen aggregation results in signal transduction. This yields degranulation and the production of inflammatory and chemotactic mediators. We hypothesize that the adipokine Wnt5a enhances IgE-mediated mast cell function and migration. We further hypothesize that the adipokine leptin suppresses IgE-mediated mast cell cytokine production. These effects may vary on different genetic backgrounds.

Chapter 1 - INTRODUCTION:

The prevalence of obesity is on the rise and reaching epidemic proportions, with 75 million Americans considered obese (95, 100, 101). The implications of obesity are extensive, altering metabolic processes and immune responses associated with metabolic syndrome, chronic heart disease, atherosclerosis, insulin resistance and type II diabetes (95, 101, 102). Recent studies have explored the relationship between obesity and asthma, indicating that obesity antedates asthma (102) (103) (104). While the link between obesity and airway hyper-responsiveness (AHR) is now known, the relationship of obesity to other inflammatory diseases such as bronchitis, sinusitis, and rhinitis, the latter of which affects 40% of the American population, is less explored.

Mast cells are instrumental in inflammatory disease. It has been demonstrated that in obese individuals mast cell numbers are significantly increased in white adipose tissue (WAT) compared to non-obese (105). Furthermore, since obesity had recently been described as chronic low-grade inflammation, it seems logical to explore the relationship between inflammatory cells and obesity (98, 106). While the role of adipocytes and their adipokines are well characterized in relation to obesity, their links to allergic inflammation are not well-understood (95, 107).

The resistance or susceptibility of mice strains to various diseases such as asthma has been linked to differences in the transcription rates of several immunologically important molecules such as cytokines, chemokines and their receptors associated with Th1 and Th2 type immune responses. Using Th1-

prone C57BL/6 and Th2-prone and 129/sv mice, we show that the adipokine leptin suppresses mast cells from C57BL/6 mice, but that mast cells from Th2-prone mice are resistant to the effects of leptin (108, 109). We propose that like Th2-prone mice, there are Th2-prone individuals that are predisposed to allergic disease. And while this predilection is by no means the only contributing factor, we hypothesize that there is a change in the response of obese Th2-prone individuals that leads to increased incidence of allergic disease when compared to Th1-prone individuals. Outlining the role of the mast cell and its response to adipokines may prove to be crucial for the development of new therapies.

Wnt5a has potential to augment mast cell function

Obesity elicits a dysregulation of adipokines released from adipocytes and an infiltration of immune cells into adipose tissue. Many adipokines have been characterized. Our first focus is the effects of Wnt5a on mast cell function. Wnt proteins are fundamental for basic developmental processes¹⁶. Wnt5a has been shown to signal via Frizzled receptors (Fz) with co-receptors LRP5/6 in the canonical pathway, and through Fz and Ror2/PTK7/RYK receptors in the non-canonical pathway(110). However, Wnt5a has recently been implicated as having inflammatory activities, and is elevated in obese individuals (111-113) (114). Furthermore, the evolutionarily conserved Wnt5a has been implicated as being a link between the innate and adaptive immune system for microbial infection(115). It has also been suggested that the inflammatory Wnt5a signaling pathway is Beta catenin-independent, suggesting that other pathways are

involved (114). In addition, there is evidence to suggest that Wnt5a and Ror2 activate JNK, contributing to the inflammatory response. For example, these factors can activate pro-inflammatory cytokine production in endothelial cells in individuals with Rheumatoid arthritis (114) (116-118). Taken together, these findings serve as a basis to investigate the inflammatory capabilities of Wnt5a on mast cell signaling.

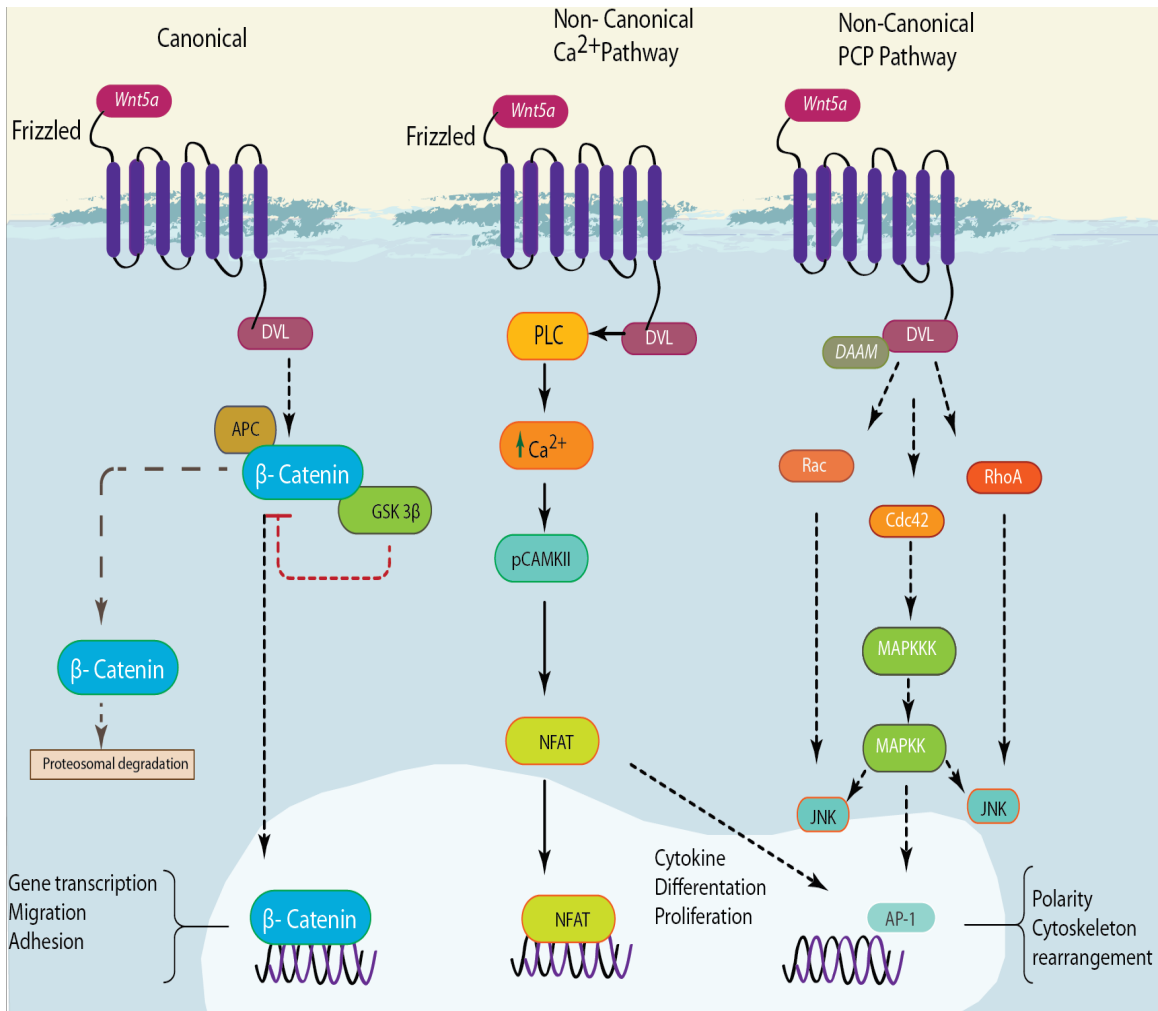


Figure 53

The 3 proposed Wnt signaling pathways.

A) Canonical Wnt/ β -catenin pathway. B) non- canonical Wnt/ Ca^{2+} pathway and the C) non-canonical Wnt/PCP pathway. A) Canonical Wnt β -catenin pathway regulates cell fate decisions during development of vertebrates and invertebrates. Wnt binds to Frizzled receptors that recruit disheveled which, in turn results in a down stream signaling cascade that displaces GSK-3 β from the APC/Axin/GSK-3 β complex allowing β -catenin to move to the nucleus. In the absence of Wnt molecules binding frizzled, β -catenin is targeted for proteosomal degradation. B) In the non-canonical Wnt/ Ca^{2+} pathway, Wnt binds to frizzled and increases intracellular calcium, which activates CAMKII and transcription

factors like NFAT and AP-1. C) the non-canonical Wnt/PCP pathway is characterized by asymmetric distribution of frizzled. This results in cell polarization. Additionally this pathway activates Rho GTPases such as Rac1, Cdc42 and RhoA, resulting in cytoskeleton rearrangement and can also activate JNK.

The complexities of leptin extend into mast cell homeostasis.

Leptin is primarily produced and synthesized by adipocytes, but its receptors are found on many cell types, including monocytes and mast cells - and leptin effects are felt in many systems (119) (120). Leptin is most commonly known for its role in energy utilization and storage by regulating energy intake and expenditure. Leptin levels correlate with adipose tissue mass. Leptin also regulates metabolic, endocrine, bone metabolism and immune function (120, 121). The leptin receptor knockout (KO) mice db/db, and ob/ob mice lacking leptin secretion, are not only obese but exhibit endocrine/immune deficiency (119). Of the 6 leptin isoforms, (Ob-Ra to Ob-Rf) only the long isoform Ob-Rb is fully functional and capable of downstream signaling through Jak2/Stat3, PI3K MAPK cascade and SOCS3 (119, 120, 122-124).

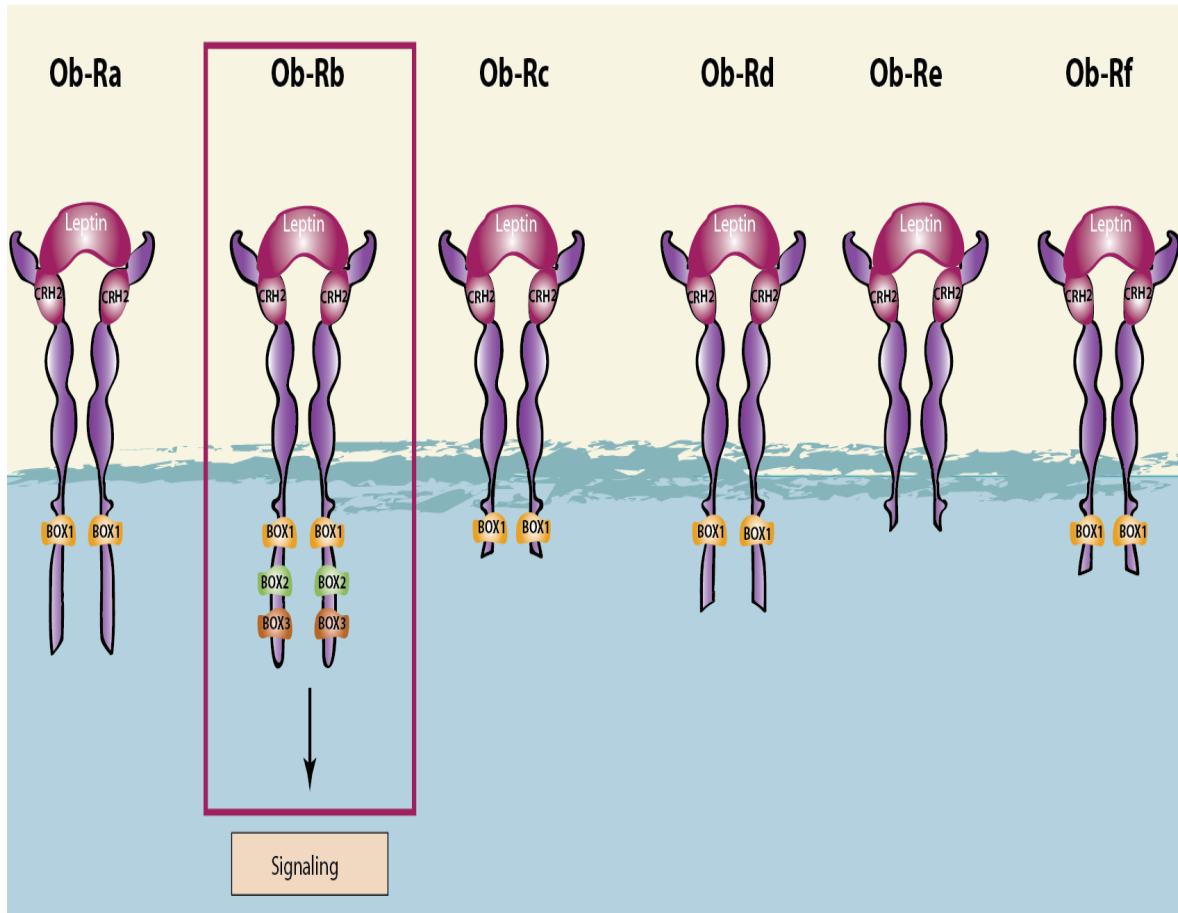


Figure 54

The isoforms of the leptin receptor.

All 6 leptin receptor isoforms share the same ligand binding domain, Cytokine Receptor Homolog (CRH). With the exception of the OB-Re, which is the secreted form, all other isoforms are membrane anchored. JAK's associate with the conserved Box 1 motif, which is required for JAK2 activation. The OB-Rb isoform has the longest intracellular domain, which is vital for leptin signaling.

Although SOCS3 is a feedback inhibitor of leptin and is activated by the Jak2/Stat3 pathway, structurally, the leptin receptor is similar to the class I cytokine receptor (gp130) superfamily that include the inflammatory cytokine receptor IL-6. It also signals much like the IL-6 receptor, and has been demonstrated to act as a pro inflammatory mediator in macrophages (119, 125). Furthermore, leptin production is increased in inflammation (126), promoting a Th1 response. However, it has been shown that the loss of the leptin or its receptor results in exacerbated airway hyperresponsiveness, suggesting an inhibitory role for leptin also (127).

RESULTS:

Wnt5a enhances IgE induced cytokine production in primary BMMC's.

We initially investigated the adipokines Wnt5a, leptin, adiponectin and resistin and found wnt5a and leptin to yield the most interesting results. Here, we show that IgE sensitized BMMC's pretreated with Wnt5a for 3 show a significant increase in IL- and $\text{TNF}\alpha$ (Figure 55) production on the Th1 prone C57BL/6 mast cells. Interestingly Wnt5a did not significantly alter IL-10 production (Figure 56) on C57BL/6 mast cells. To determine the potential of genotype dependence, we treated BMMC's from the Th2 prone 129/SvImJ with Wnt5a. Here, we show that 129/SvImJ exhibit an increase in IL-6, $\text{TNF}\alpha$, IL-13 and MCP-1 (Figure 57). We demonstrate that the effect of Wnt5a on murine mast cell is not genotype restricted.

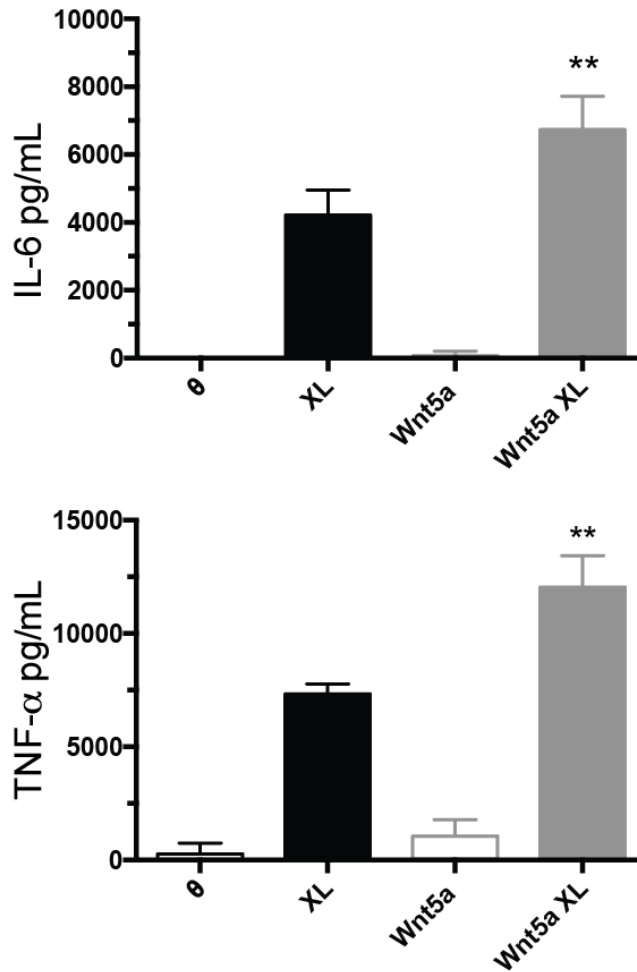


Figure 55

Wnt5a Enhances cytokine production from IgE activated BMMC's.

C57BL/6 were cultured in IL-3 and SCF, with or without Wnt5a for 3 days. BMMC's were then sensitized with IgE over night and then activated with DNP-HSA for 16 hours and supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine IL-6 and TNF α concentrations. The results are expressed as the mean \pm SEM of 3 independent experiments.

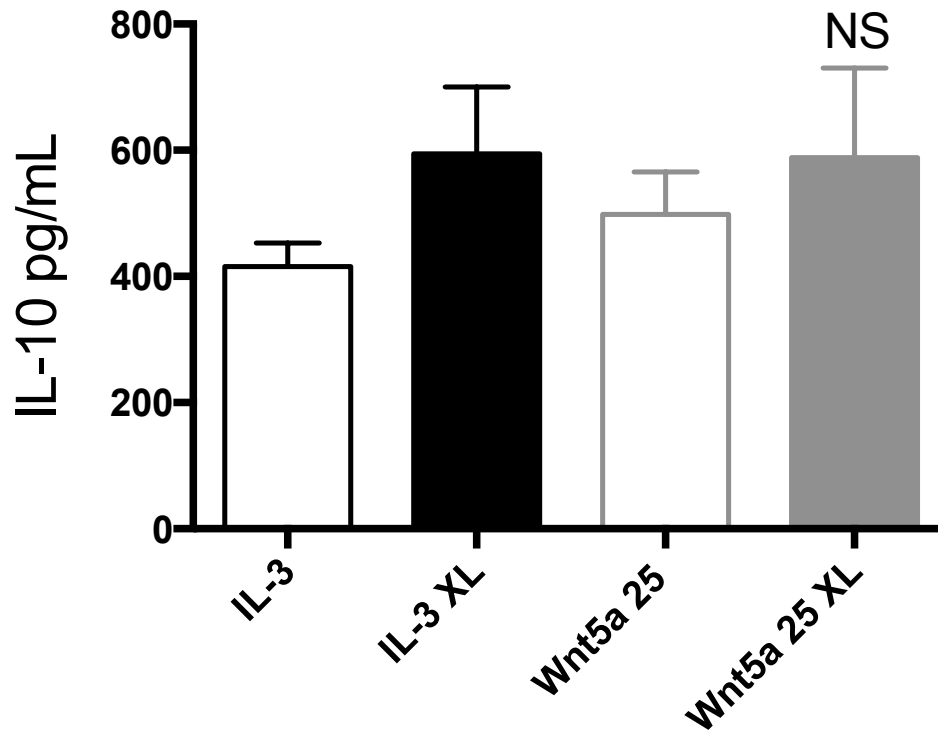


Figure 56

Wnt5a does not alter IL-10 production from IgE activated mast cells.

C57BL/6 were cultured in IL-3 and SCF, with or without Wnt5a for 3 days. BMMC's were then sensitized with IgE over night and then activated with DNP-HSA for 16 hours and supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine IL-10 concentrations. The results are expressed as the mean \pm SEM of 2 independent experiments.

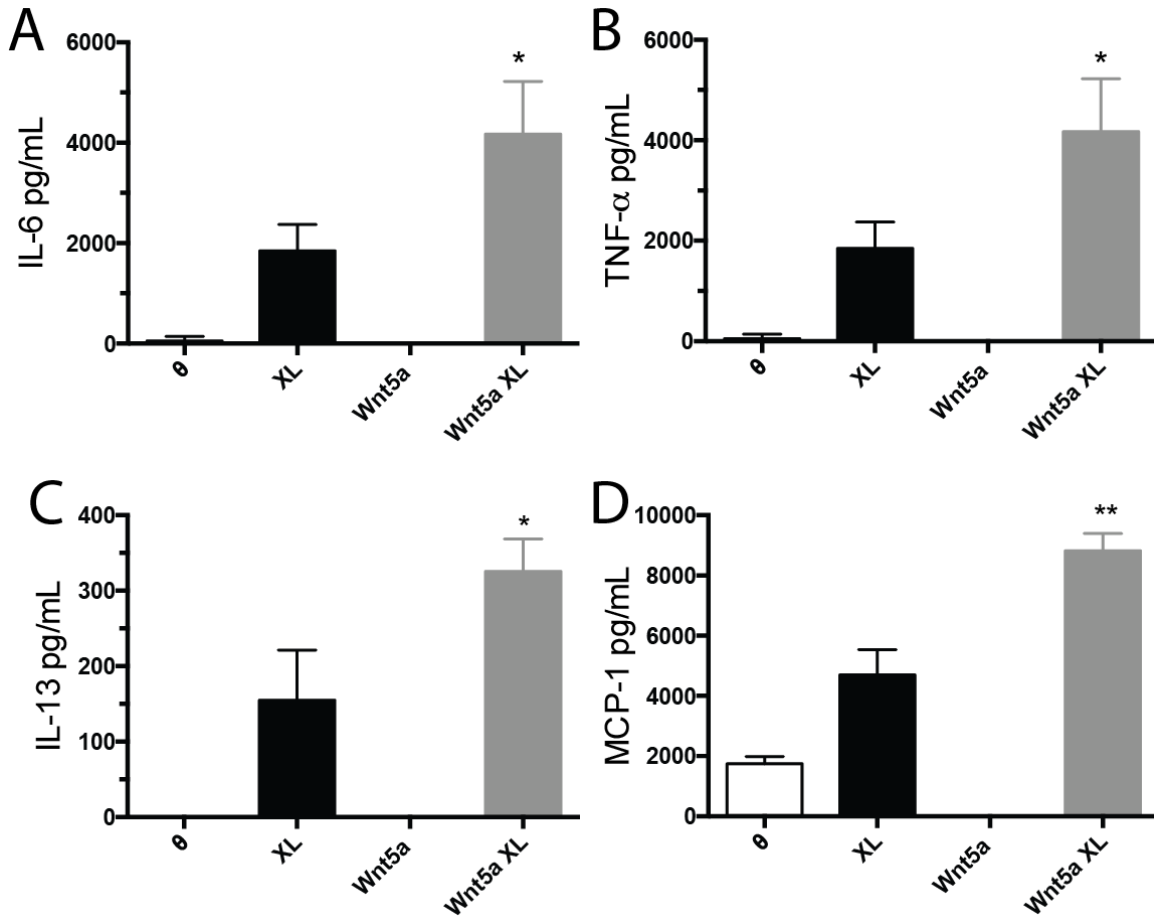


Figure 57

Wnt5a Enhances cytokines and chemokine production from IgE activated mast cells.

129/SvImJ BMMC's were cultured in IL-3 and SCF, with or without Wnt5a for 3 days. BMMC's were then sensitized with IgE over night and then activated with DNP-HSA for 16 hours and supernatants were collected as described in the methods and materials and assessed by standard sandwich ELISA to determine A) IL-6, B) TNF α , C) IL-13 D) MCP-1 concentrations. The results are expressed as the mean \pm SEM of 3 independent experiments.

Wnt5a enhances mast cell migration

Following Wnt5a's ability to increase IgE mediated pro-inflammatory cytokines; and given that mast cells migrate to sites of infection, we wanted to investigate the ability of Wnt5a to alter mast cell migration and mast cell migration towards antigen. Here, we show that mast cells from C57BL/6 mice migrate towards Wnt5a (Figure 58 A), and more so in the presence of antigen (Figure 59 B).

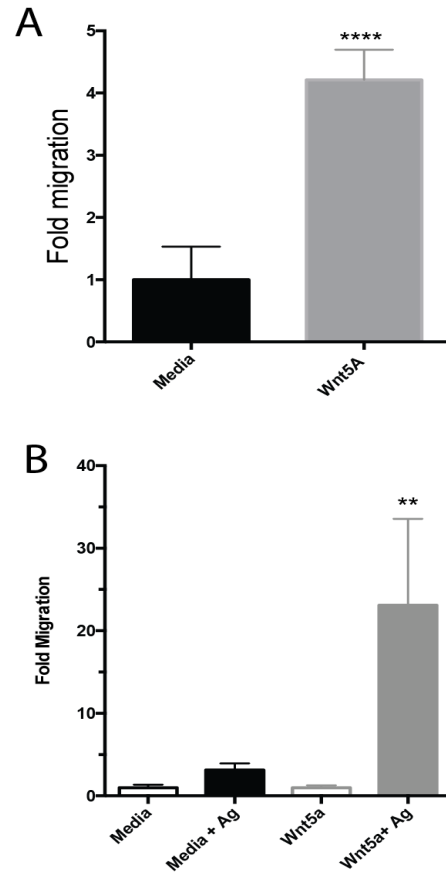


Figure 58

Wnt5a enhances mast cell migration.

A) C57BL/6 BMDC's were tested through 8µm transwell membrane for migration in response to media alone or Wnt5a (100ng/ml). Fold migration is in response to samples containing media alone. The results are expressed as the mean ± SEM of 2 independent experiments. B) C57BL/6 BMDC's were cultured for 3 days IL-3 (10ng/ml) and SCF (10ng/ml) ± Wnt5a (100ng/ml) and sensitized with IgE. BMDC's were then tested through 8µm transwell membrane for antigen-induced migration. Fold migration is in response to samples containing media alone. The results are expressed as the mean ± SEM of 2 independent experiments.

Wnt5a enhances IgE induced cytokine production in basophils

We next wanted to see if Wnt5a and leptin altered basophil cytokine production. Here, we show that Wnt5a increases IL-6 (Figure 59) and TNF α (Figure 60) production but pretreatment with leptin does not significantly alter basophil cytokine production.

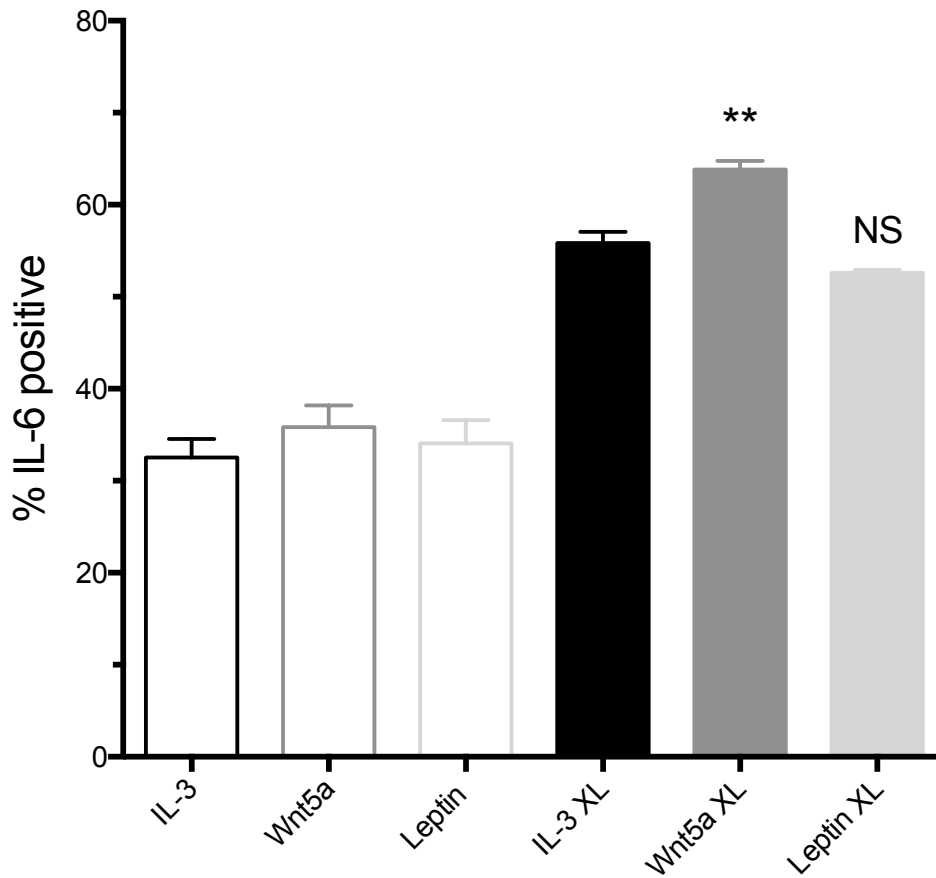


Figure 59

Wnt5a increases IgE mediated IL-6 production in basophils.

Bone marrow derived basophils from C57/BL6 mice were cultured for 3 days in IL-3 (10ng/ml). On day 4 they were treated with \pm Wnt5A (25ng/ml) or \pm leptin (250ng/ml) and then cross-linked with IgE (0.5ug/ml) and DNP-HSA antigen (IgE XL) on day 7. Cytokines levels were measured intracellularly by flow cytometry.

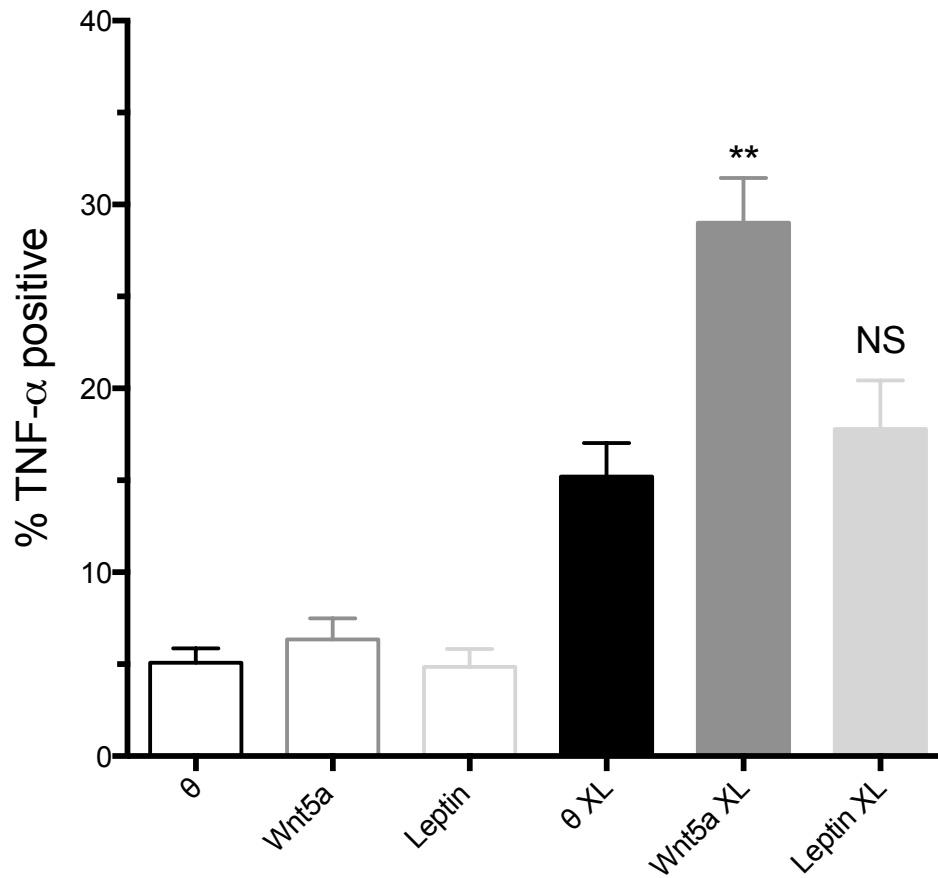


Figure 60

Wnt5a increases IgE mediated TNF α production in basophils.

Bone marrow derived basophils from C57/BL6 mice were cultured for 3 days in IL-3 (10ng/ml). On day 4 they were treated with \pm Wnt5A (25ng/ml) or \pm Leptin (250ng/ml) and then cross-linked with IgE (0.5ug/ml) and DNP-HSA antigen (IgE XL) on day 7. Cytokines levels were measured intracellularly by flow cytometry.

Leptin suppresses IgE induced cytokine production on mast cells

As mentioned previously, leptin plays a role in inflammation and thus we wanted to assess the ability of leptin to alter mast cell cytokine production whilst looking at any potential genotype dependence. Interestingly, we show that BMMC's pretreated with or without leptin for 3 days that were then IgE sensitized and activated with antigen actually suppress cytokine production on C57BL/6 mice but on the Th2 prone 129/SvImJ mice we see that BMMC's are resistant to the suppressive capabilities of leptin.

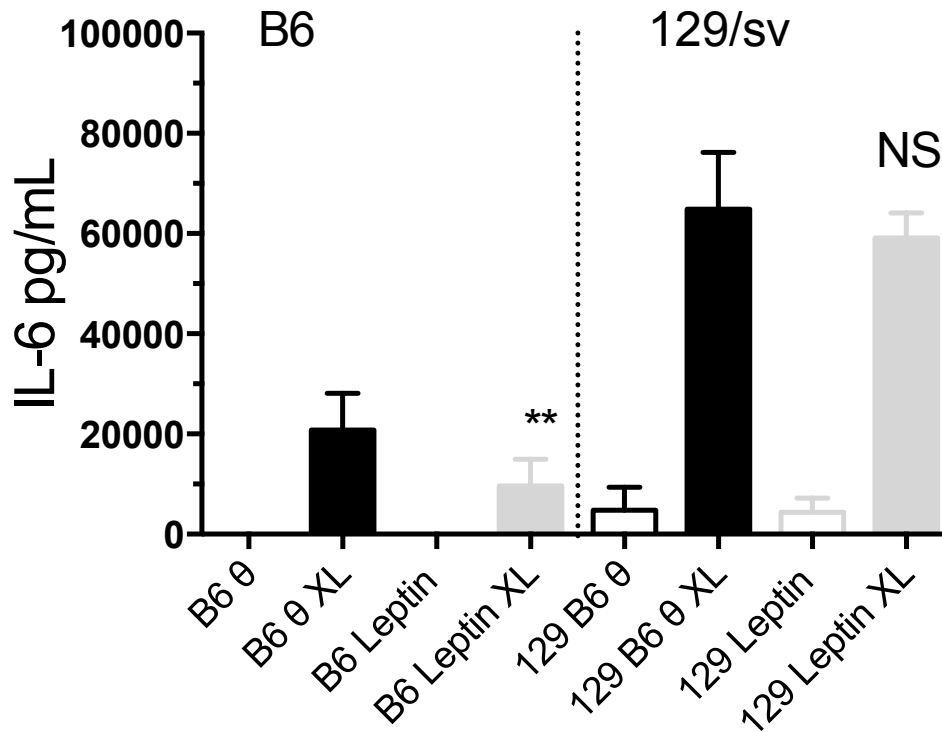


Figure 61

Leptin suppresses IL-6 production from IgE activated BMBC's on the Th1 prone C57BL/6 background but not the TH2 prone 129/SvImJ BMBC's.

C57BL/6 and 129/SvImJ IgE sensitized BMBC's were cultured in IL-3 and SCF with or without Leptin for 3 days, then sensitized with IgE and then activated with antigen for 16 hours. Supernatants were collected as described in the methods and materials. The results are expressed as the mean \pm SEM of 3 independent experiments.

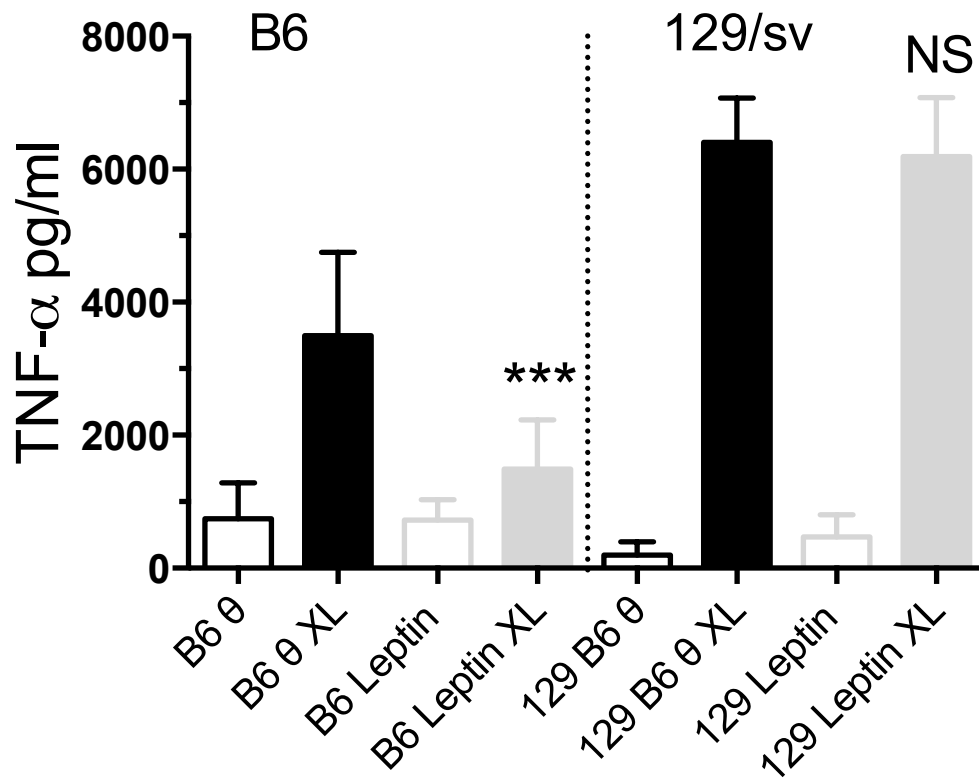


Figure 62

Leptin suppresses TNF α production from IgE activated BMMC's on the Th1 prone C57BL/6 background but not the TH2 prone 129/SvImJ BMMC's.

C57BL/6 and 129/SvImJ IgE sensitized BMMC's were cultured in IL-3 and SCF with or without Leptin for 3 days, then sensitized with IgE and then activated with antigen for 16 hours. Supernatants were collected as described in the methods and materials. The results are expressed as the mean \pm SEM of 3 independent experiments.

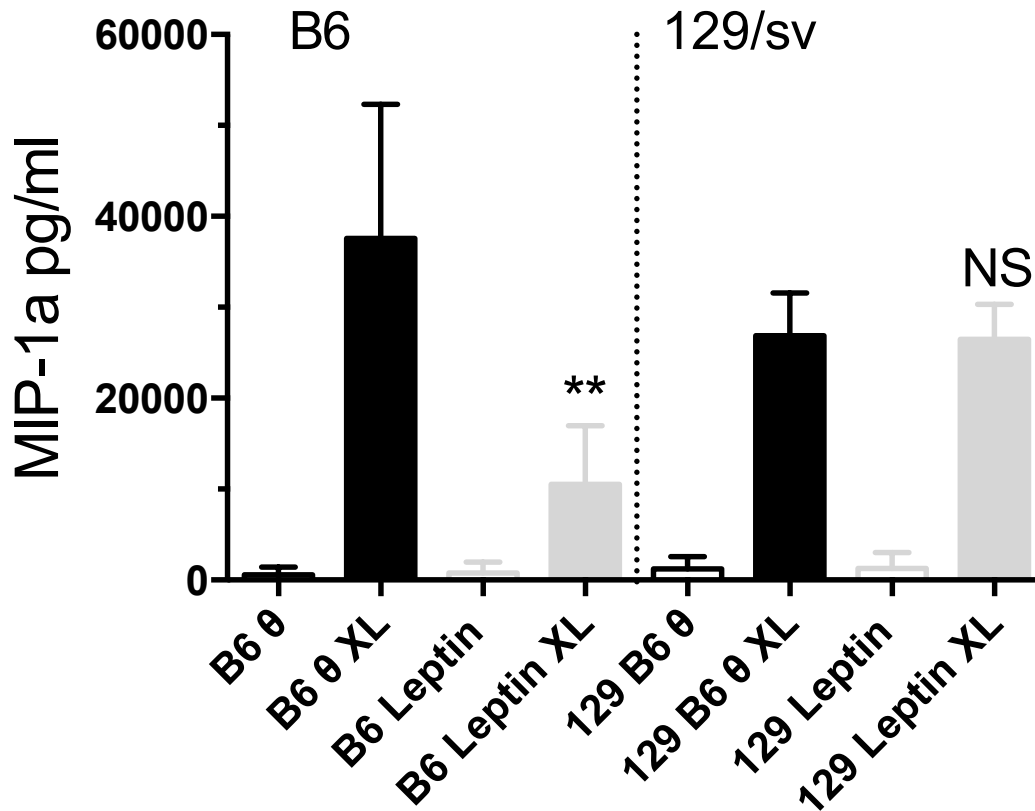


Figure 63

Leptin suppresses MIP-1 production from IgE activated BMMC's on the Th1 prone C57BL/6 background but not the Th2 prone 129/SvImJ BMMC's.

C57BL/6 and 129/SvImJ IgE sensitized BMMC's were cultured in IL-3 and SCF with or without Leptin for 3 days, then sensitized with IgE and then activated with antigen for 16 hours. Supernatants were collected as described in the methods and materials. The results are expressed as the mean \pm SEM of 3 independent experiments.

Leptin receptor deficiency exacerbates anaphylaxis, but reduces histamine release

We next wanted to investigate the effect leptin deficiency has on passive systemic anaphylaxis, which is dependent upon mast cell IgE mediated activation. We therefore assessed this response on leptin receptor knockout mice (db/db mice) and their Wild type (WT) counterparts. While the difference between the KO and wild type is small, the KO mice take a significantly longer time to recover (Figure 64). The db/db mice also demonstrated a reduced responsiveness to histamine (Figure 65).

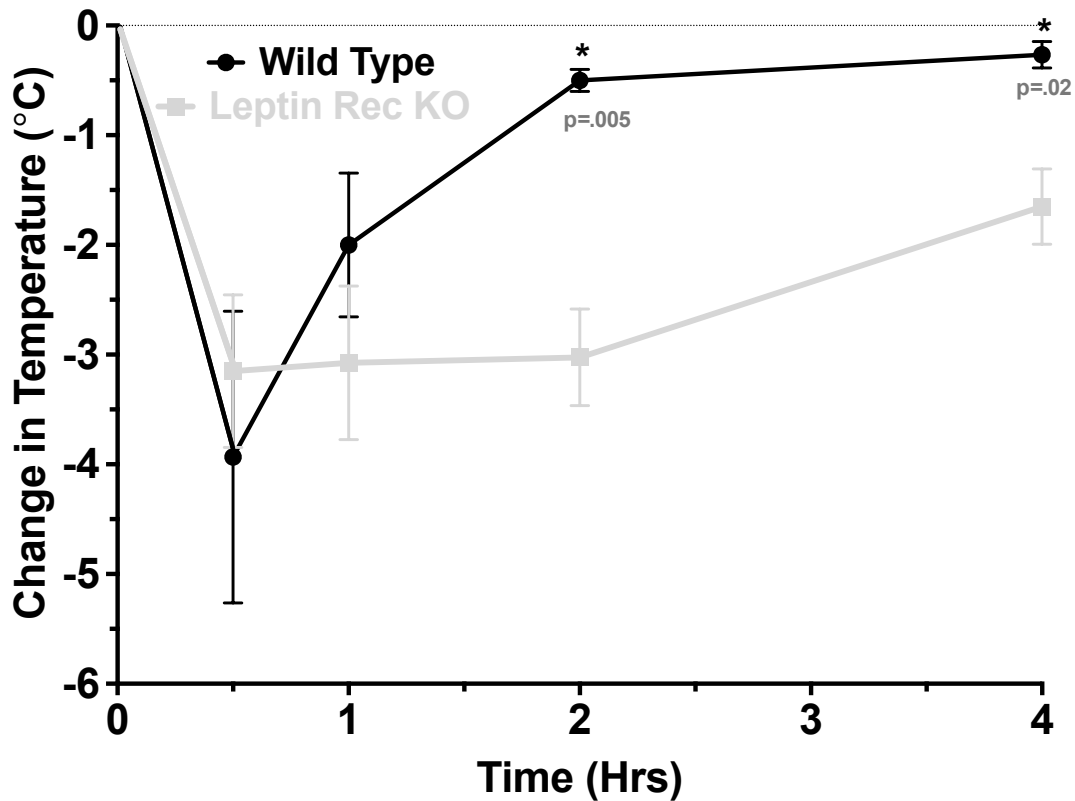


Figure 64

Leptin receptor deficiency worsens anaphylaxis, but reduces histamine release.

WT (C57BL/6) or Leptin receptor knockout mice (n=4) A) were sensitized with IV injection of IgE anti-DNP, and then injected with DNP-BSA the following day. Change in body temperature was measured by rectal probe. B) Mice were injected with 5mg histamine, i.v. Change in core body temperature was measured by rectal probe at regular intervals.

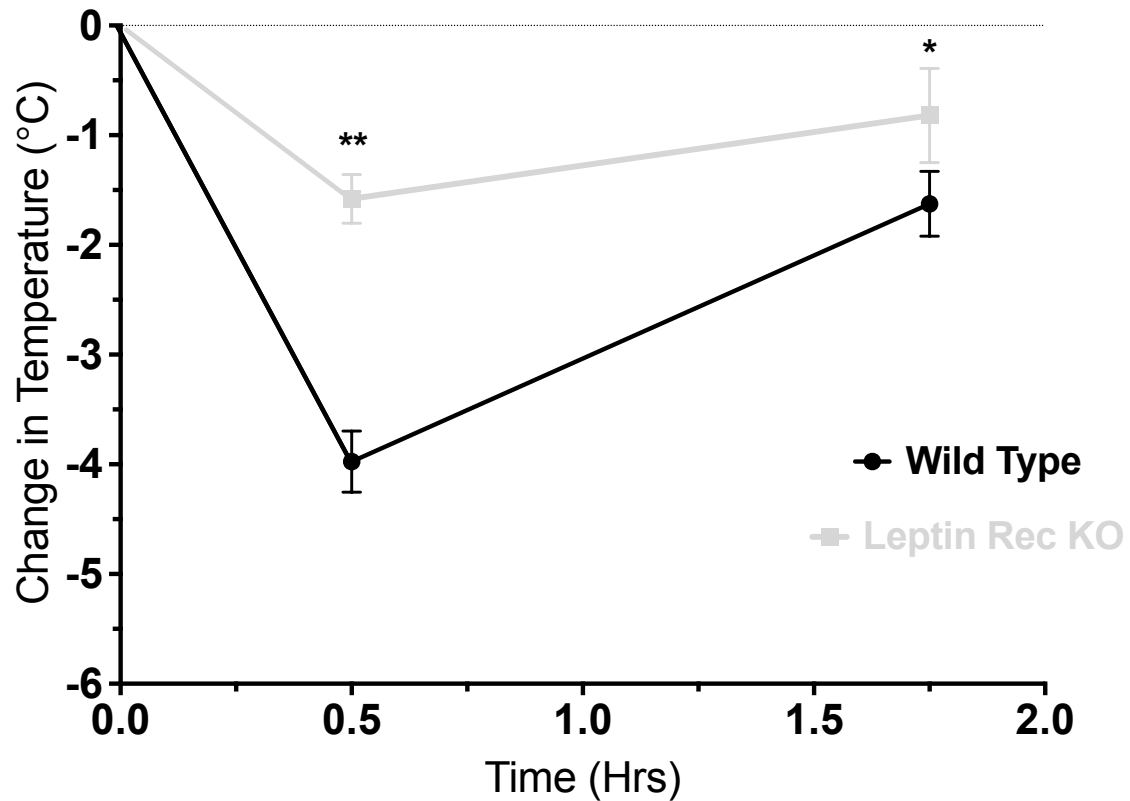


Figure 65

Leptin receptor deficiency reduces histamine release.

WT (C57BL/6) or Leptin receptor knockout mice (n=4) A) were sensitized with IV injection of IgE anti-DNP, then injected with DNP-BSA the following day. Change in body temperature was measured by rectal probe. B) Mice were injected with 5mg histamine, IV, and change in temperature was measured.

DISCUSSION:

The effects of Wnt5a and leptin on mast cells

Recently it has been shown that adipokines play a role in immunomodulation, in particular that Wnt5a has inflammatory capabilities (95, 111, 112) and that Wnt5a is implicated as being a link between the adaptive and innate immune system. Here, we show that Wnt5a significantly enhances the release of inflammatory mediators from mast cells. Furthermore, these data suggest that Wnt5a also enhance mast cell migration. Additionally, our preliminary data show that pretreatment with leptin causes suppression of cytokine production on the Th1 prone genetic background but not the Th2 prone 129/SvImJ background. Unfortunately, although interesting out data did not consistently repeat leading us to halt this investigation.

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