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Stains Induce Apoptosis and Autophagy in Primary and Transformed Mast Cells

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STATINS INDUCE APOPTOSIS AND AUTOPHAGY IN PRIMARY AND

TRANSFORMED MAST CELLS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

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Major Advisor:

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Statin drugs are widely employed in the clinic to reduce serum low density lipoproteins (LDLs) in patients with hypocholesteremia. In addition to their cholesterol-lowering effects through HMG CoA reductase antagonism, isoprenyl lipids necessary for membrane anchorage and signaling of small G-proteins are abrogated. We previously found that statins suppress mast cell activation in murine and human cells, suggesting these drugs might be useful in treating allergic disease. While mast cell function is critical to allergic inflammation, mast cell hyperplasia and survival also impact these diseases, and were not studied in our previous work. In this study, we describe Fluvastatin-mediated apoptosis in both primary and transformed mast cells. An IC_{50} was achieved between 1-5μM in both systems, and apoptosis was preceded by mitochondrial dysfunction and caspase release. In addition to apoptosis, our work also uncovered evidence of autophagy, which can serve as a compensatory mechanism during apoptosis. Interestingly, autophagy appeared to be

cyto-protective in the primary cells yet cytotoxic in transformed mast cells. These findings offer insight into the mechanisms of mast cell survival and support the possible utility of statins in mast cell-associated allergic and neoplastic diseases.

Keywords

SCF: Stem cell factor

cKIT: (CD117) receptor for SCF

IL-3: interleukin 3

BMMC: Bone marrow derived mast cells

P815: Mastocytoma cell line

KO: Knock out

IgE: Immunoglobulin E

LDL: Low density lipoprotein

IC_{50}: Inhibitory concentration 50%

PI: Propidium Iodide

TBS: Tris-buffered saline

TBS-T: Tris buffered saline with tween 20

DMSO: Dimethyl sulfoxide

BAF A1: Bafilomycin A1

CQ: Chloroquine

MAPK pathway: Mitogen activated protein kinase pathway

ERK: Mitogen activated protein kinase (MAPK)

AKT/PKB: Protein kinase B, a serine/threonine kinase

Introduction

Statin drugs were released for consumption by the FDA in the early 1990's to combat the rising trend in hypercholesterolemia in the United States. These drugs reduce serum cholesterol and low density lipoproteins (LDLs), which have been linked to increased risk of coronary heart disease (CHD) and stroke (1). However, statins have also been noted to have anti-inflammatory and anti-neoplastic effects that warrant further study. The pharmacological mechanism of statins is HMG-CoA reductase (HMGCR) antagonism. HMGCR is the rate-limiting step in cholesterol synthesis, yielding mevalonic acid. While mevalonic acid is processed to cholesterol, side reactions in this cascade also generate the isoprenoids geranylgeranyl pyrophosphate and farnesyl pyrophosphate. Isoprenylation is required for Ras, Rac and Rho subcellular localization. Since these proteins are critical to cellular proliferation, migration, and cytokine production, there is great interest in determining if isoprenoid blockade is the means by which statins disrupt cell signaling (2, 3). Understanding this process could lead to new drug targets for inflammatory and neoplastic disorders.

Epidemiological studies have noted that asthmatic patients prescribed statins experienced fewer emergency department visits for their allergic conditions as compared to their counterparts (4, 5). These studies have posed the question of what roles statins play in the cellular and molecular signaling of sentinel immune cells that provoke the allergic response. Mast cells play an early role in allergies when activated through the high affinity immunoglobulin E (IgE) receptor (FcεRI). Antigen-mediated crosslinking of IgE bound to FcεR induces the release of preformed granules containing proteases and histamine as well as cytokine secretion. Collectively these mediators induce vasodilation and bronchoconstriction, leading to edema, dyspenia, tissue damage, and even systemic shock. Exploring the importance of isoprenoid

inhibition through statin treatment could reveal fundamental aspects of mast cell biology and offer new therapeutic avenues for allergic disease.

In addition to their role in allergic disease, mast cells can form neoplasms. Mast cell leukemia is a form of acute myelogenous leukemia (AML) that constitutes approximately 3% of leukemias. Diagnosis requires one major or three minor criteria. The major criteria for diagnosis is dense multifocal infiltration of mast cells in the bone marrow or extracutaneous organs. The four prototypical minor criteria are: >25% of mast cell infiltration in biopsies with atypical morphology, mast cells in the bone marrow being >25% immature or atypical, D816V CD117 (cKIT) mutation, expression of CD2 and/or CD25, and total serum tryptase exceeding 20ng/mL consistently (6).

Statins have been shown to induce macroautophagy, hereafter referred to as autophagy (2). Autophagy has several survival adaptation roles, as it degrades internal organelles to create energy while in a catabolic state. However, its adaptation properties are dependent on cellular state and metabolism (7, 8). The autophagic process is often a pro-survival pathway used by cancer cells in response to nutrient deprivation or various toxic insults such as chemotherapeutic agents. But autophagy can also be cytotoxic, as it can enhance intracellular apoptotic signals (9). Interestingly, a recent study has shown that mast cells have a constant autophagic flux necessary for granule maintenance (10). Statins have been shown to alter autophagy and cause mitochondrial dysfunction (11). How this may impact mast cell function and survival is unknown. Statins can also play roles in neoplastic conditions potentially serving both pro- and anti-metastatic functions. The differences observed were dependent on the cancer type, stage, and degree of immunological involvement. In one study, Fluvastatin inhibited metastasis of pancreatic cancer and in another study it was shown to induce apoptosis of transplanted rat

hepatocarcinoma (12, 13). These studies have provided the interest for studying statin induced apoptosis and autophagy.

A recent paper from our group demonstrated that statins are powerful suppressors of IgE-mediated mast cell activation (14, 15) . Of the statins tested, Fluvastatin was most effective in suppressing cytokine production. During these studies, we noted that prolonged treatment caused apoptosis. The current work builds on this initial observation, showing that Fluvastatin induces both apoptosis and autophagy capable of killing primary or transformed mast cells. We provide some mechanistic insights, narrowing the focus of Fluvastatin action and offering support for the possible clinical use of statins in mast cell-associated diseases.

Methods and Materials

Animals:

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained at Virginia Commonwealth University (VCU) facilities in accordance with Institutional Animal Care and Use Committee guidelines (IACUC).

Bone Marrow-Derived Mast cell Cultures (BMMC):

Bone marrow-derived mast cell cultures (BMMC) were extracted from femurs and tibias and placed in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% FBS, 2mM L-glutamate, 10 U/mL penicillin, 10 μg/mL of streptomycin, 1mM sodium pyruvate, and 10mM HEPES (Biofluids, Rockville, MD), supplemented with SCF-containing supernatant from BHK-MKL cells and IL-3 from WEHI-3B cells for 21 days. Final

concentrations of SCF and IL-3 are adjusted to 15 ng/mL and 10 ng/mL, respectively, as measured by ELISA.

Flow Cytometric analysis:

Apoptosis Analysis:

Propidium Iodide-DNA (PI-DNA) staining was performed on cells after 4 hours to 7 days of fixation in PI fixation buffer (36% ethanol, 12.8% fetal calf serum, and 50.8% 1X PBS) . (Thermo Fisher, Waltham, MA). All flow cytometric analysis was done using a BD FACSCaliber (BD Biosciences, San Jose, CA).

Mitochondrial Staining:

$Di(OC_6)_3$ (3,3'-Dihexyloxacarboncyanine iodide), a membrane-permeable dye was used to analyze mitochondrial integrity at a concentration of 1nM (Enzo Life Sciences, Farmingdale, NY). Caspase 7/9 activity DEVD assays were used to assess the presence of active caspases (Thermo Fisher, Waltham, MA). All flow cytometric analysis was done using a BD FACSCaliber (BD Biosciences, San Jose, CA).

Cell Receptor Staining:

Cell surface staining was conducted using antibodies against the following mouse proteins: CD117 (c-KIT), CD25, CD2, CD16/CD32, (eBiosciences, Waltham, MA). All flow cytometric analysis was done using a BD FACSCaliber (BD Biosciences, San Jose, CA).

Autophagy Assessment with Acridine Orange:

 Acridine Orange (AO) staining was used for assessment of acidic vesicles associated with the process of autophagy (Sigma-Aldrich, Saint Louis, MO). Cells were treated with a final concentration of 1µg/mL for 15 minutes. All flow cytometric analysis was done using a BD

FACSCaliber (BD Biosciences, San Jose, CA).

Fluorescent Microscopy:

Cells were stained with anti CD16/CD32, FITC cKIT (CD117), and acridine orange at a concentration of 1µg/mL for 30 minutes. Cells were then washed and spun onto glass slides for imaging using a Cytospin centrifuge (Thermo Fisher, Waltham, MA) and images were captured using a Nikon Eclipse E600/ C1 confocal microscope. Images were created using the EZ-C1 3.80 software.

Western Blotting:

 Western blots were conducted using standard SDS-PAGE protocol on a 4-20% gradient polyacrylamide gel. Nitrocellulose membrane (0.45µM) was purchased from Bio-Rad (Hercules, CA). The following primary antibodies were used: LC3I/II (1:1000), p62 (1:1000), GAPDH (1:1000), ERK1/2 (1:1000), phospho-ERK1/2 (1:1000), AKT (1:1000), phospho-AKTser473 (1:1000), p85 (1:1000), phospho-p85 (1:1000) (Cell Signaling, Danvers, MO). Western blot membranes were blocked with TBS-bovine serum albumin (BSA) and primary antibodies incubated for three days in TBS/0.1% Tween (TBS-T 0.1%) and (0.5% BSA Blocker ™) all purchased from Thermo Fisher, Waltham, MA). Western blots were read using infrared-conjugated secondary antibodies on an Odyssey Imaging System (LI-COR, Lincoln, NE).

Statistics:

Data shown in each figure are the mean ± standard deviations (SD). The number of populations per experiment is depicted as (N). Graphical and statistical analysis were conducted on Graph Pad-Prism 7. For comparisons of two samples, a Student's t-Test was applied unless otherwise stated. Significance was assigned as follows: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

Results:

Fluvastatin induces apoptosis in C57BL/6 BMMCs.

We recently published that Fluvastatin suppresses IgE-mediated activation of mast cells and basophils. Upon further investigation, it was noted that Fluvastatin also causes apoptosis of bone marrow derived mast cells (BMMC). BMMCs were co-cultured for 72-hours in cRPMI supplemented with SCF and IL-3 at 10 ng/mL, plus either vehicle (DMSO) or Fluvastatin 10 µM. PI-DNA staining was conducted to detect sub-diploid DNA, indicative of apoptosis. It was found that over 50% of the BMMCs were undergoing apoptosis by 48-hours and over 90% by 72-hours in Fluvastatin treatment **(Figure 1A).** A dose response of Fluvastatin was conducted at 72-hours, and an IC_{50} was found between 1-5µM as determined by PI-exclusion via flow cytometry **(Figure 1B).** A 72-hour dose response to Fluvastatin confirmed the presence of active caspase 3/7, as determined by flow cytometry **(Figure 1C).** Interestingly, BMMCs treated with Fluvastatin undergo apoptosis with no discernable cell cycle arrest, even in the 24 hours preceding detectable **(Figure 1D)**. Instead, all phases of the cell cycle were reduced by Fluvastatin, with only the apoptotic fraction increasing**.** These data demonstrate that Fluvastatin induces mast cell apoptosis after a 24-hour delay that is not preceded by cell cycle arrest.

Fluvastatin Decreases SCF Survival Signals in BMMCs.

It has been published that statin-mediated apical antagonism of HMG-CoA reductase in the cholesterol pathway not only lowers plasma cholesterol, but also the production of isoprenyl lipids (16). This effective inhibition of the downstream farnesyl and geranylgeranyl transferases led us to ask how the survival signals provided from cKIT (CD117) binding its ligand stem cell

factor (SCF) are modulated by Fluvastatin. BMMCs were treated for 24-hours in cRPMI supplemented with SCF and IL-3 at 10 ng/mL, plus either vehicle (DMSO) or Fluvastatin 10µM. BMMCs were then starved of IL-3+SCF for four hours in cRPMI prior to SCF (200 ng/mL) re-stimulation. The unstimulated groups (Unstim) were treated with media alone. Whole cell lysates were collected at 5, 15, and 30 minute intervals and Western blots for pERK1/2 and pAKT(Ser473) were performed **(Figure 2A).** A representative blot shows decreased pERK when comparing Fluvastatin treatment to vehicle (DMSO) to groups. The normalization of pERK1/2 to total ERK1/2 was quantified as fold increase from the baseline unstimulated group (Unstim) for each time point **(Figure 2B).** By comparison, AKT phosphorylation trended down at some time points, but did not reach statistical significance. These data support the theory that inhibition of small G-proteins has downstream effects on the cKIT-to-MAPK signaling pathway used for survival.

Apoptosis is dependent on p53 expression and mitochondrial stability

Apoptosis is often mediated through p53 signaling originating from DNA damage, oxidative stress or uncontrolled proliferation (17). Here we examined the role of p53 by using p53-deficient ("knockout" (KO)) BMMCs compared to their background- and age-matched C57BL/6 counterparts. BMMCs were cultured in either vehicle (DMSO) or Fluvastatin (10µM) in cRPMI with IL-3 and SCF at 10 ng/mL over a 72-hour time course. It was found that p53KO BMMCs compared to C57BL/6 wild type (WT) underwent significantly less apoptosis when treated with Fluvastatin **(Figure 3A).** Since p53 activation can induce mitochondrial damage, and statins have been shown to induce mitochondrial dysfunction (11), we sought to determine the effect of overexpressing the anti-apoptotic mitochondrial protein Bcl-2. We cultured age- and strain-matched Bcl-2 transgenic and C57BL/6 WT BMMCs in cRPMI with IL-3 and SCF at 10 ng/mL

over a 72-hour time course and measured apoptosis via PI-DNA. This showed that the Bcl-2 transgenic BMMCs displayed significantly less apoptosis than C57BL/6 WT BMMCs (**Figure 3B).** These results indicate a role for the p53-mitocondrial pathway in Fluvastatin-mediated apoptosis.

Fluvastatin induces cyto-protective autophagy in BMMCs

It has been widely published that statins cause autophagy in a variety of cell types (2). Since autophagy can promote or inhibit apoptosis, we assessed Fluvastatin-induced mast cell autophagy. One measure of autophagy is the accumulation of cleaved light chain associated microtubule (LC3 II), as detected by Western blotting. Chloroquine raises lysosomal pH and inhibits lysosomal fusion, allowing autophagy-induced LC3 II to accumulate. Because mast cells undergo constitutive autophagy (18), we used chloroquine treatment alone to determine the baseline amount of LC3 II present, and compared this to cells receiving the known autophagy inducer Rapamycin as a positive control. **Figure 4A** shows an increase of LC3 II following 24-hour chloroquine treatment, confirming the basal level of autophagy. As expected, Rapamycin+chloroquine increased LC3 II levels. Importantly, Fluvastatin+chloroquine induced more LC3 II than chloroquine alone, suggesting that Fluvastatin increases basal autophagy. Western blot normalization to GAPDH is displayed in **Figure 4B.** These data suggest that Fluvastatin elicits autophagy in primary mast cells at time point preceding apoptosis.

To determine how autophagy affects Fluvastatin-induced apoptosis, we blocked autophagy with chloroquine or bafilomycin A, and measured sub-diploid DNA content. Bafilomycin A1 inhibits the vacuolar H+ pump, preventing degradation of the sequestered components of autophagy. BMMCs were treated for 48-hours in IL-3&SCF plus vehicle (DMSO) or Fluvastatin in combination with chloroquine 10μM (CQ) or bafilomycin A1

(200nM) **(Figure 4C, 4D).** We found that chloroquine or bafilomycin A1 alone slightly

increased apoptosis, and that each significantly enhanced Fluvastatin-mediated apoptosis. These

data suggest that Fluvastatin-induced autophagy is cyto-protective in primary mast cells, since its

blockade increased cell death.

Fluvastatin induces apoptosis in transformed mast cells.

We next determined if Fluvastatin-induced apoptosis extended to neoplastic mast cells. P815

mastocytoma cells were cultured in either vehicle (DMSO) or a dose response of Fluvastatin

prior to collection at 96-hours for analysis with PI-DNA staining **(Figure 5A).** Nearly 80% of

the cells were killed by this treatment, with an IC_{50} between 2.5-5μM, similar to BMMCs. Time

course analysis also revealed a slightly slower apoptotic response than primary mast cells

(**Figure 5B**) and a modest but significant G2 arrest at the 24-hour time point preceding apoptosis

(Figure 5C).

Fluvastatin treatment causes loss of mitochondrial potential and release of caspase 9 in transformed mast cells.

Because Fluvastatin-induced apoptosis was suppressed by Bcl-2 overexpression in BMMC, we

investigated the effect of Fluvastatin on mitochondrial stability in P815 cells. Cells were treated

for 48-hours in vehicle (DMSO) or Fluvastatin and stained with $Di(OC_6)_3$, a dye with high

affinity for the mitochondria. Decreased staining indicates loss of the trapped dye, consistent

with mitochondrial membrane damage. P815 cells treated with Fluvastatin at .625μM or 10μM

exhibited diminished $Di(OC_6)_3$ staining compared to vehicle-treated cells **(Figure 6A).** Given

this indication of mitochondrial damage, we next assessed the presence of active caspase 9,

which results from mitochondrial leakage. P815 cells were treated for 24 and 48-hour with

vehicle (DMSO) and Fluvastatin (10μM), then assessed for active caspase activity via flow cytometry using a fluorescently-labeled, cleavable caspase-9 peptide **(Figure 6B).** There was significant caspase 9 activation at 24- and 48-hours, consistent with the mitochondrial stain data. These data further suggest that Fluvastatin induces apoptotic cell death via a mitochondrial pathway.

P815 cells upregulate cKIT and undergo cytotoxic autophagy in response to Fluvastatin treatment.

P815 cell express a mutant cKIT (D814Y) that is analogous to the common human (D816V) point mutation, and a major contributor to this neoplasm (6, 19, 20). Interestingly, we found that Fluvastatin treatment induced a 2-fold increase in surface cKIT, peaking 24 hours after co-culturing cells, which was just prior to the onset of apoptosis **(Figure 7A)**. We postulate that this may serve to delay apoptosis. To determine if autophagy was also occurring at this time point, P815 cells were treated for 24-hours with vehicle or Fluvastatin (10μM) in the presence or absence of chloroquine. Cells were stained with FITC anti-cKIT for visualization and acridine orange for assessment of acidic vesicles, indicative of autophagic flux. We observed that chloroquine treatment alone increased acridine orange staining, a testament of the basal autophagic flux. Fluvastatin alone appeared to decrease acridine orange staining, perhaps because it promoted autophagic processing to completion. In support of this, Fluvastatin+chloroquine appeared to induce more acridine orange staining than either drug alone **(Figure 7B).** To quantify and substantiate these observations, flow cytometry was used to quantify acridine orange staining. When comparing the basal level of acridine orange staining in the chloroquine (CQ)-treated samples to the combination of Fluvastatin and CQ, a significant increase of MFI was observed **(Figure 7C)**, supporting the fluorescent microscopy findings.

Finally, we tested the functional importance of autophagy in P815 mastocytoma cells undergoing Fluvastatin-mediated apoptosis. In contrast to our findings with primary mast cells, chloroquine decreased Fluvastatin-mediated apoptosis by more than 50% **(Figure 7D).** These data argue that autophagy is cytotoxic in P815 cells, since its blockade reduces apoptosis, and suggest that drugs promoting autophagy might be useful in treating mast cell neoplasia.

Discussion

Mast cells fulfill their primary immunological functions in defense against bacterial and parasitic infections. However in many disease states, environmental antigens become immunogenic and can lead to life threating anaphylaxis. There is an early and a late phase of mast cell activation that collectively cause immediate and long term effects such as vasodilation, capillary leakage, and recruitment and activation of other immune cells. Mast cells can be activated through IgE receptors when antigen aggregates receptor-bound IgE. The early phase of activation occurs when pre-formed granules containing histamine and proteases are released, causing increased vascular permeability and vasodilation. The late phase follows 4-6 hours later, and is elicited by cytokine and chemokine production (21, 22).

Clinical studies have shown correlations between asthmatic/allergic patients on statin therapy and reduced emergency department visits for their respective ailments (5, 23). The apical antagonism of HMG-CoA reductase by statins in the cholesterol pathway also reduces geranylgeranyl pyrophosphate and farnesyl pyrophosphate production. These isoprenoid lipids are coupled to Ras, Rac and Rho, and are critical to their function (16). The loss of isoprenylation results in production of small G-proteins without membrane anchorage, resulting in a lack of signal transduction following a ligand-receptor interaction. Mechanisms possibly explaining statin anti-

allergic effects include our recent demonstration that Fluvastatin decreases MAPK and AKT signaling pathways activated by IgE crosslinking, with some genotypic constraints (14, 15). In longer time courses, we noted that significant percentages of bone marrow derived mast cells (BMMCs) underwent apoptosis when treated with statins, prompting the current studies.

This work shows that primary mast cells treated with Fluvastatin undergo a p53-dependent apoptosis, but with no discernible cell cycle arrest. Additionally, data from Bcl-2 transgenic BMMCs suggests that stabilizing the mitochondria blocks Fluvastatin-induced death. These findings, coupled with data suggesting a cyto-protective phenotype of autophagy, support the possibility that autophagy results from mitochondria damage, a form of autophagy called mitophagy. In this model, increased apoptosis following autophagy blockade is a result of the cell's inability to dispose of damaged mitochondria through mitophagy. Damaged mitochondria release reactive oxygen species (ROS) and have decreased oxidative phosphorylation, both detrimental to cellular heath and homeostasis (24-26). In addition there are publications showing that Bcl-2 and other anti-apoptotic proteins exit the mitochondria and transit to the endoplasmic reticulum (ER) to become recycled and shuttled to other mitochondria in the cell. Thus autophagy not only protects cells from ROS and metabolic dysfunction, but also controls sensitivity to apoptosis in the cell (27).

The process of mitophagy involves proteins such as PINK1 and Parkin. The presence of these proteins is often used to validate mitophagy (24). The use of confocal microscopy to assess the co-localization of mitochondrial and lysosomal dyes in conjunction with etiology-specific proteins are the next direction necessary in supporting this theory. Signaling data shown in figure 2 also support the known interaction of MAPK and the AKT-mTOR axis in mast cells, which are tasked with the tagging pro-apoptotic (BH3 only) proteins such as Bim for proteasome degradation

in mast cells (28-30). The decreased activity in the MAPK pathway supports this as a plausible mechanism for the increase of Bim or other pro-apoptotic proteins, which in turn antagonize Bcl-2, leading to the release of cytochrome C and caspase activation. In addition, the trend toward a decrease of AKT-PI3K-mTOR axis needs to be further investigated, given studies describing the importance of TORC1 in primary mast cell homeostasis, and TORC2 signaling as a staple in neoplastic states. Additionally, TORC2 activation was found to positively regulate AKT signaling resulting in an amplification of proliferation in neoplastic mast cells (31). This signaling pathway merits further investigation to determine the effect of Fluvastatin on TORC2. These findings have assisted in unraveling mast cell biology and begun to shed light on the molecular mechanism of how statin therapies and autophagy inhibitors may have therapeutic effects on mast cell-associated disease.

The findings our lab made with statin therapy on primary mast cells led us to become interested in the potential efficacy of statins on transformed mast cells. The P815 model used in our study conforms to several aspects of the mast cell leukemia diagnosis, including expression of the mutant cKIT receptor, which causes constitutive growth factor-independent proliferation (32, 33). There has been considerable research employing statin therapy with various cancer types, particularly those with mutant K-Ras or B-Raf. For example, one observational study found that non-small cell lung carcinoma (NSCLC) patients who were on statins had a better survival outcome compared with those on standard treatment (2, 12, 13, 34-39).

The mechanism of Fluvastatin-induced apoptosis in P815 cells was similar to primary cells, with indications of mitchondrial damage. However, autophagy inhibitors decreased Fluvastatin-induced apoptosis, which was the opposite of our observations with primary mast cells. These results suggest a cytotoxic role of autophagy in this system, a difference that can be

potentially explained with observations in this study and the current literature. Mutant cKIT (D816V) leads to strong MAPK, PI3K-mTOR, and STAT5 signaling critical for mast cell survival (40). In Figure 7 it was shown that Fluvastatin treatment caused significant upregulation of the mutant cKIT receptor, possibly as a survival mechanism. These data, coupled with findings in the literature that oncogenic cKIT signals cause endoplasmic reticulum (ER) stress, suggest autophagy might enhance the toxicity of statins (19, 20). The autophagic necessity to sequester and degrade ER components due to stress could abrogate the oncogenic KIT signals upregulated in the P815 cell line. This mechanism may support and provide a biological explanation for the anti-apoptotic phenomenon seen with the use of autophagy inhibitors. Additional research needed to substantiate this theory should include western blots of the SCF pathway and visualization of cKIT signal sequestration in ER autophagosomes (omegasomes) via confocal microscopy or immunoprecipitation of KIT in association with autophagic markers like LC3 or p62.

Understanding the role of statin-mediated autophagy in both primary and transformed mast cells will allow researchers and clinicians to modulate autophagy appropriately as an adjuvant therapy in allergic and neoplastic conditions. A thorough understanding of the biology behind statin-mediated apoptosis could allow the use of small molecular targets to avoid some known side-effects of statin therapy such as rhabdomyolysis (41). For example, knowledge gained in further studies could guide drug selectivity for either geranylgeranyl or farnesyl pyrophosphate branches of the cholesterol synthesis pathway. This knowledge could have the potential to reveal new targets, reduce dangerous side effects, and exploit autophagy correctly in allergic or neoplastic conditions.
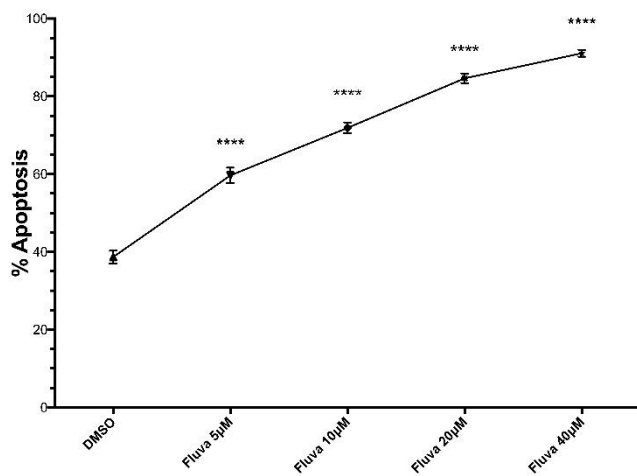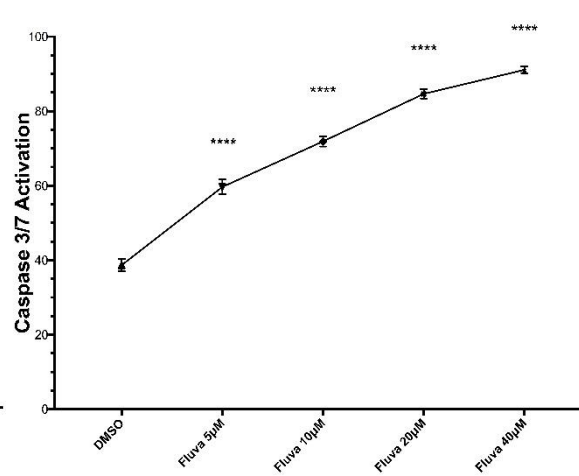
A.

72hr Time Course
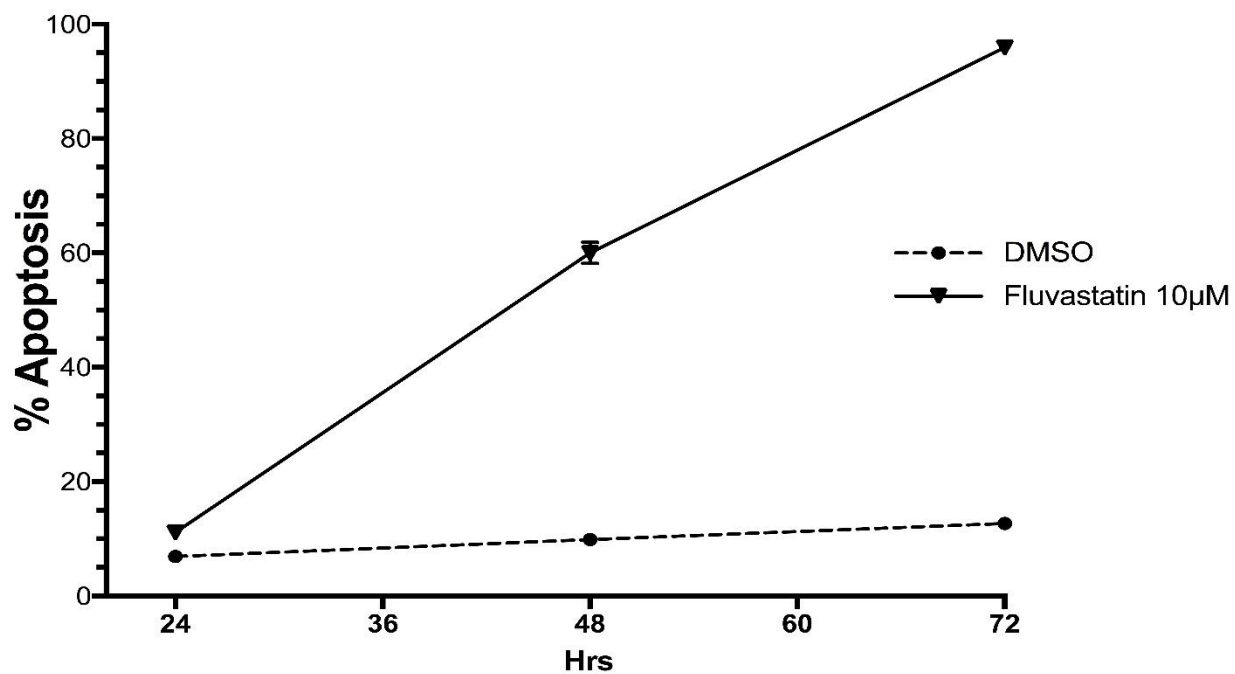
B.

72hr Dose Response

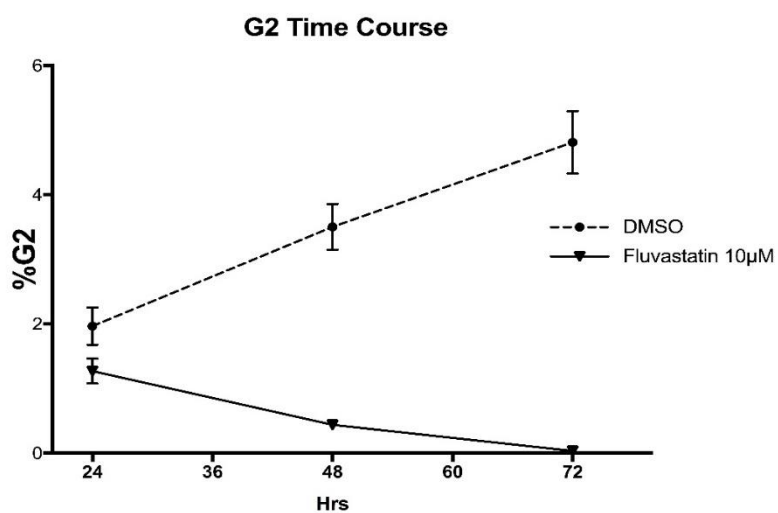

C.

72hr Caspase 3/7 Activation

D.

G1 Time Course
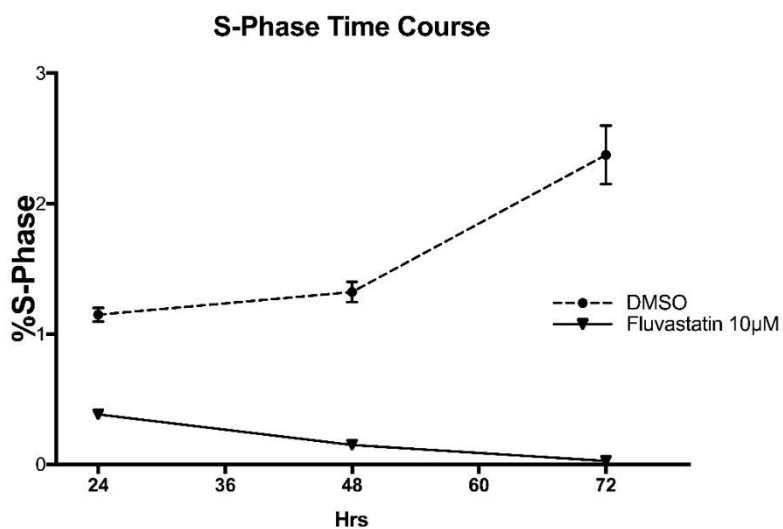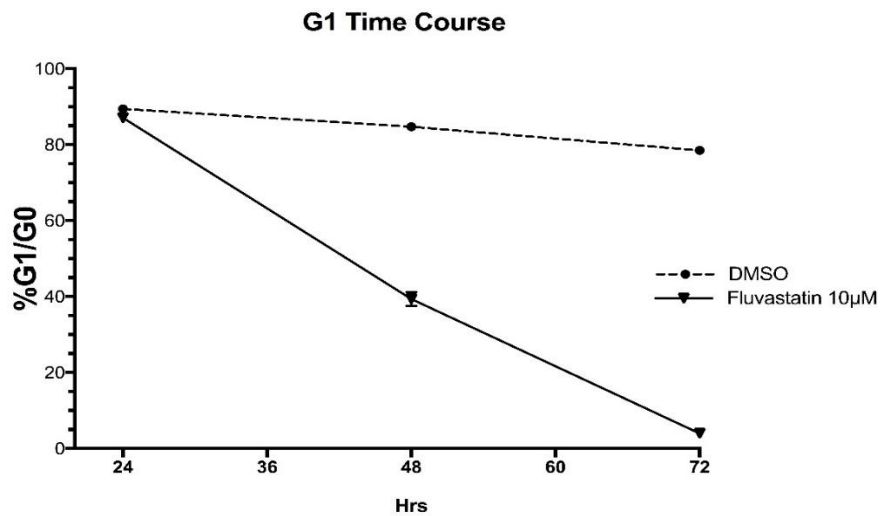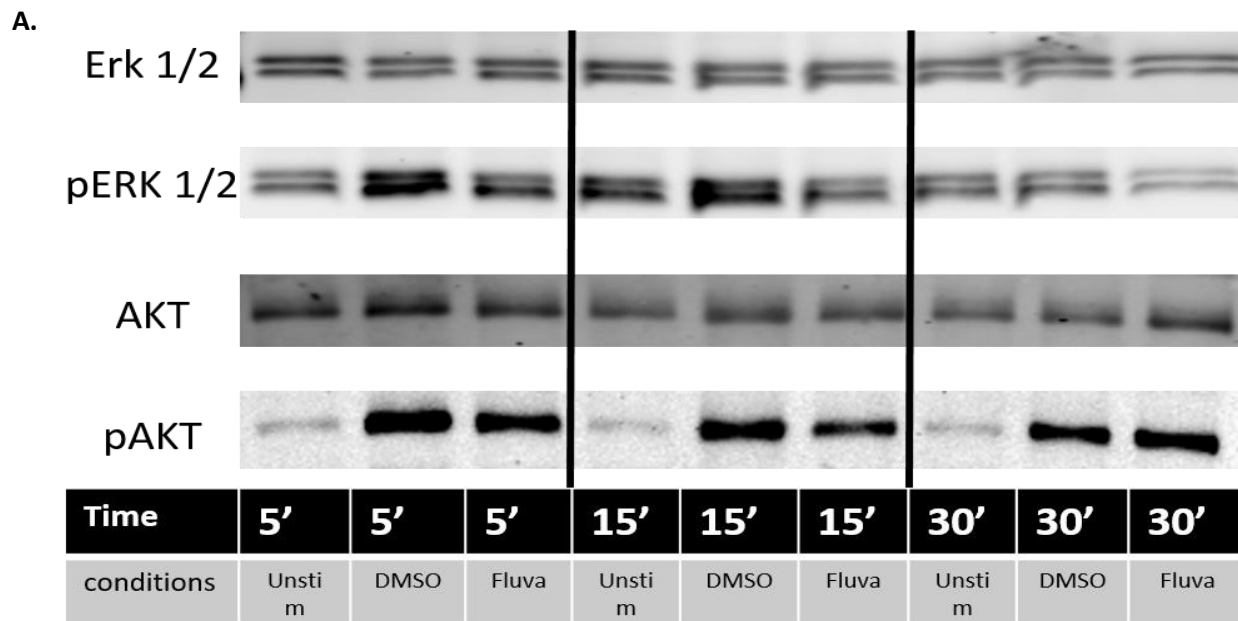

S-Phase Time Course

G2 Time Course

Figure 1
Fluvastatin induces apoptosis in C57BL/6 BMMCs.

BMMCs were co-cultured with Fluvastatin at a concentration of 10μM in a 72hr time course. Apoptosis was evidenced via flow cytometry PI-DNA staining through the presence of for sub-diploid DNA. (**Figure 1A)** A dose response of Fluvastatin was conducted at the 72hr time point on the BMMCs and apoptosis was measured via flow cytometry via PI-exclusion. **(Figure 1B)** BMMCs were co-cultured with Fluvastatin in a dose response and analyzed via flow cytometry for the presence of caspase 3/7 at 72hrs. (**Figure 1C)** BMMCs were co-cultured with Fluvastatin at 10μM for 72hrs and analyzed for cell cycle progression via PI-DNA staining. **(Figure 1D)** This figure is representative of three independent experiments (N=3). A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

A.

Time	5'	5'	5'	15'	15'	15'	30'	30'	30'
conditions	Unstim	DMSO	Fluva	Unstim	DMSO	Fluva	Unstim	DMSO	Fluva

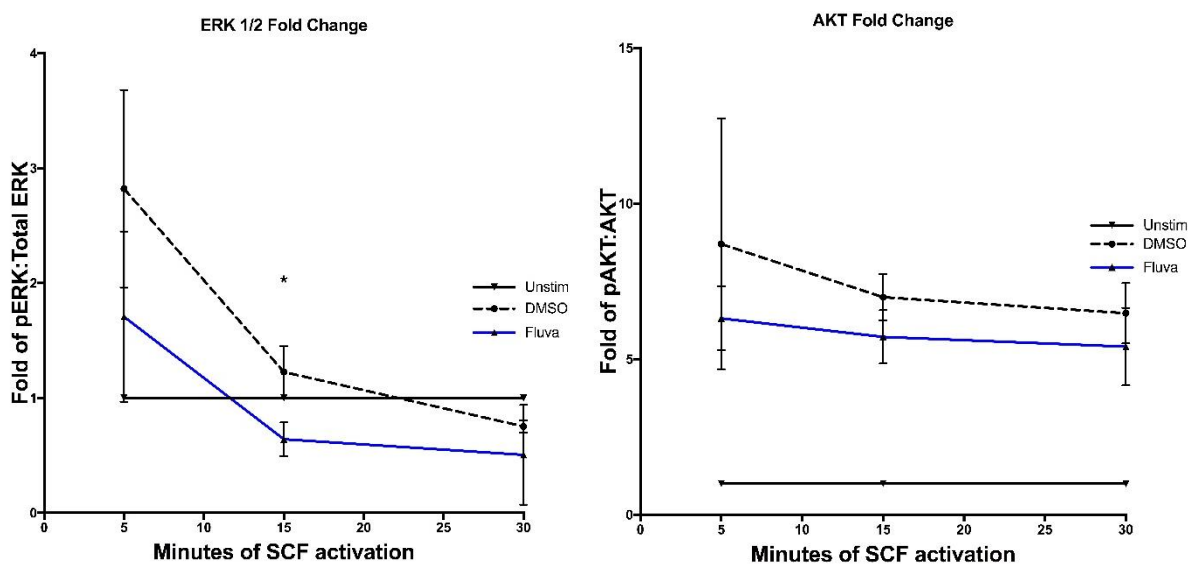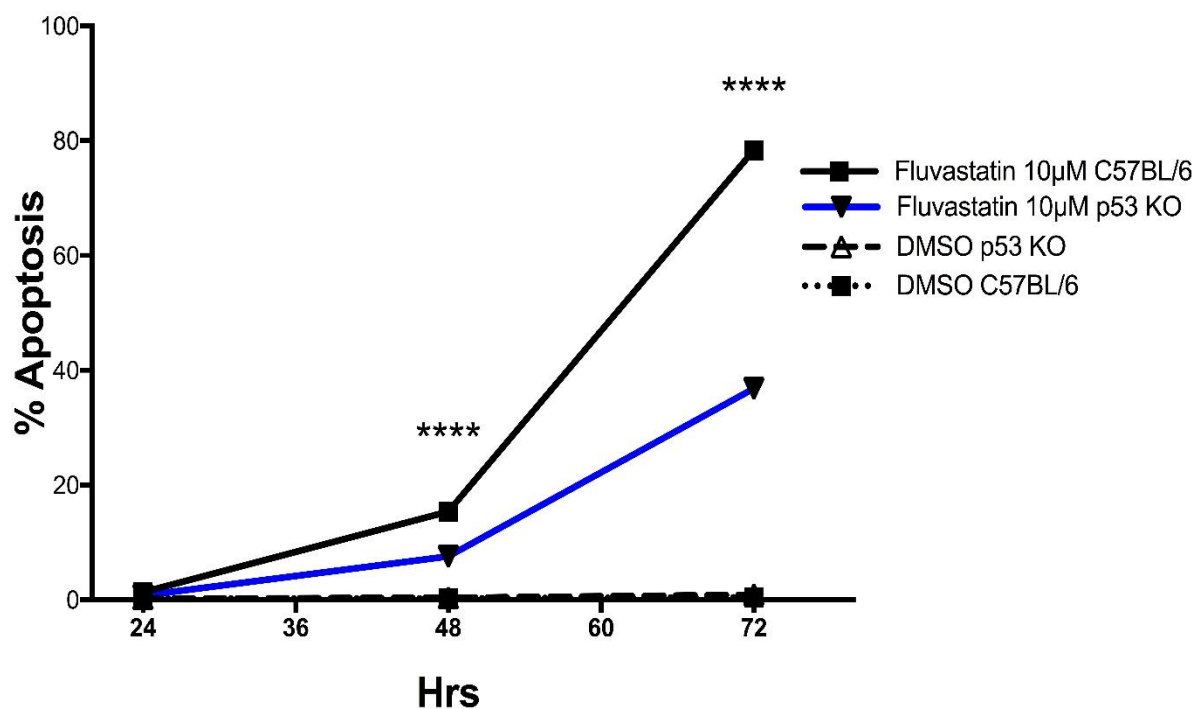**B.**

ERK 1/2 Fold Change

AKT Fold Change

Figure 2
Fluvastatin Decreases SCF signaling pathway in BMMCs.

C57BL/6 BMMCs were treated for 24hrs with Fluvastatin (Fluva) or vehicle (DMSO) and then starved for 4 hours prior to SCF stimulation (200ng/mL). Whole cell lysates were collected after 5, 10, or 15 minutes of SCF activation with the exception of the unstimulated group (Unstim). ERK 1/2 and AKT were probed by Western blotting. (**Figure 2A).** Normalization of phosphorylated: total protein quantified as fold induction over minutes of SCF stimulation (200ng/mL) **(Figure 2B).** This figure is representative one experiment (N=3). A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

A.

Apoptosis C57BL/6 vs. p53KO

Legend:
- Fluvastatin 10µM C57BL/6
- Fluvastatin 10µM p53 KO
- DMSO p53 KO
- DMSO C57BL/6

B.

Apoptosis C57/BL/6 vs. Bcl-2

Legend:
- Fluvastatin 10µM C57BL/6
- DMSO C57BL/6
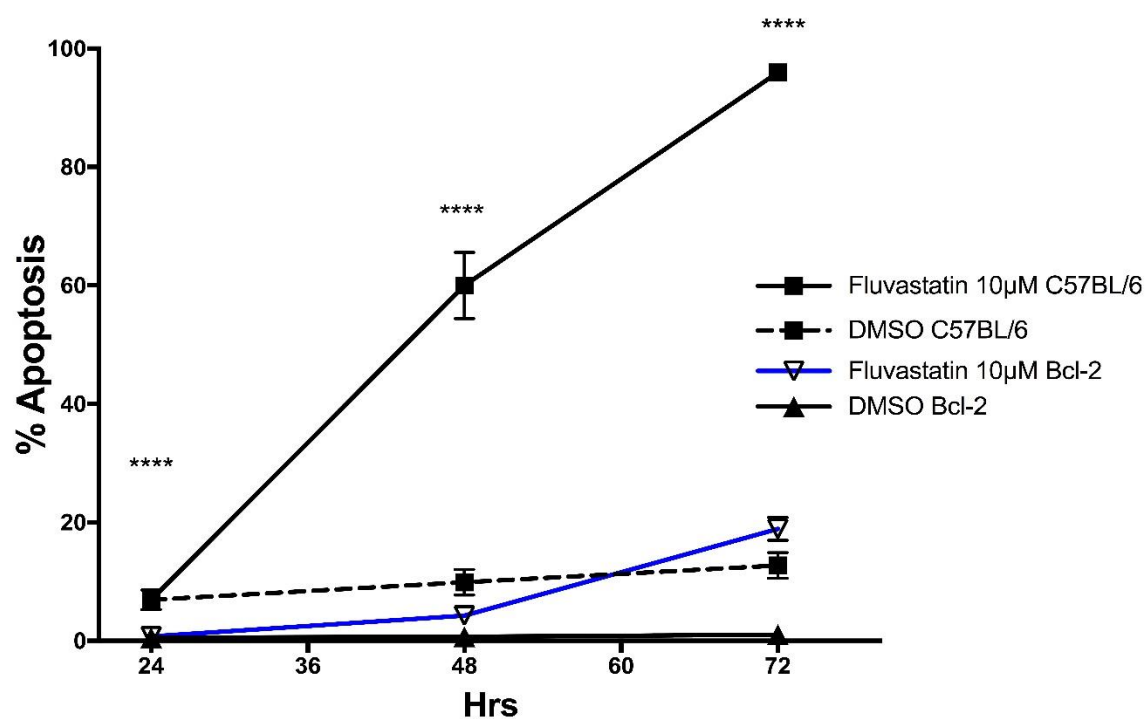- Fluvastatin 10µM Bcl-2
- DMSO Bcl-2

Figure 3
 Apoptosis is dependent on p53 expression and mitochondrial stability.

C57BL/6 background p53KO with age-matched WT C57BL/6 BMMCs were co-cultured with Fluvastatin 10μM over a 72-hour time course (**Figure 3A).** (Representative N=1) C57BL/6 background Bcl-2 transgenic with age matched C57BL/6 BMMCs were co-cultured with Fluvastatin 10μM over a 72-hour time course. Apoptosis was evidenced by the presence of sub-diploid DNA identified via flow cytometry and PI-DNA staining. **(Figure 3B)**. Figure3A is representative of two independent experiments (N=3). Figure 3B is representative of three independent experiments (N=3). A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

A.

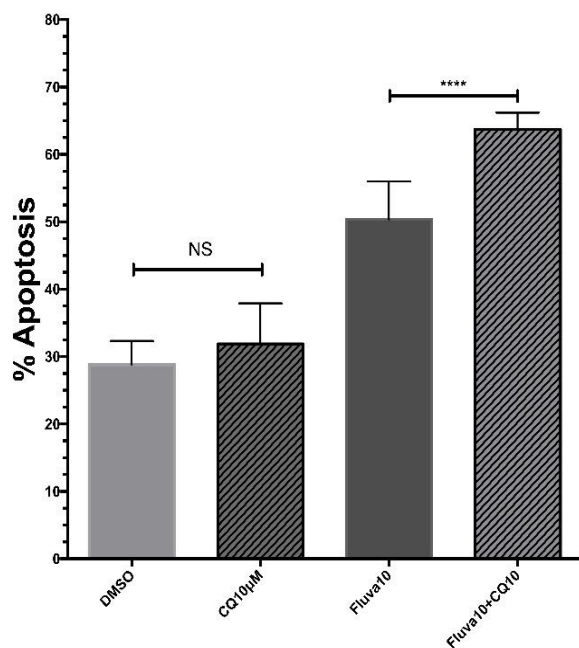|	DMSO	CQ	Rapamycin	Rapamycin	Fluvastatin	Fluvastatin
CQ 10µM	-	+	-	+	-	+
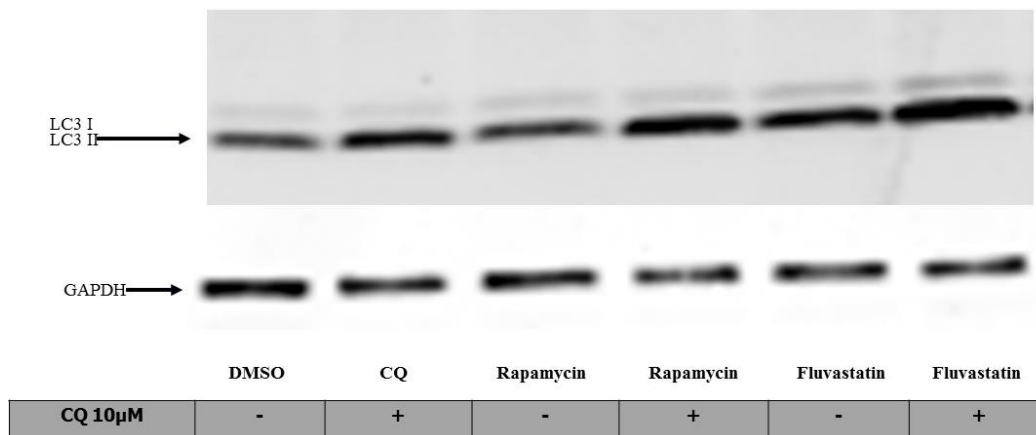
B.
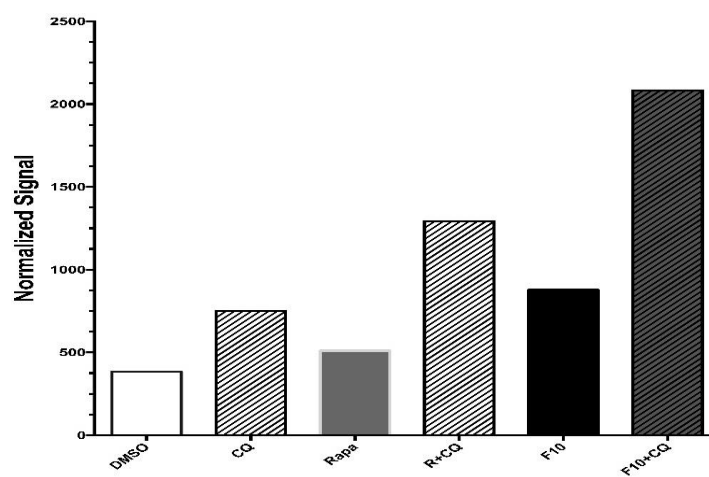
C.

48hr Treatment of Fluvastatin with Autophagy Modulation

D.

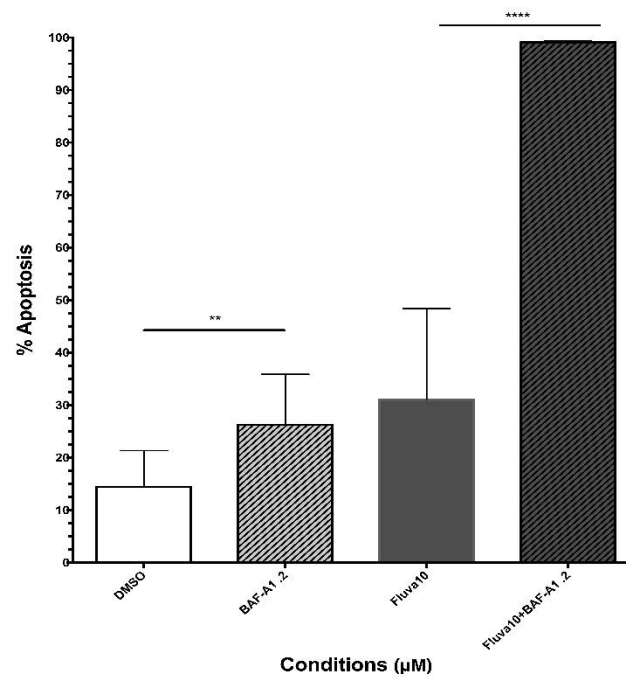48hr Treatment of Fluvastatin with Autophagy Modulation

Figure 4
 Fluvastatin induces cyto-protective autophagy in BMMCs

C57BL/6 BMMCs were treated for 24-hours with vehicle (DMSO), chloroquine 10μM (CQ), rapamycin 200nM (rapa), or Fluvastatin 10μM alone or in combination. Whole cell lysates were collected and western blots were conducted for LC3I/II **(Figure 4A).** LC3 II protein levels normalized to GAPDH quantified in **(Figure 4B).** C57BL/6 BMMCs were co-cultured with vehicle (DMSO), chloroquine 10μM (CQ), bafilomycin A1 (BAF A1), or Fluvastatin 10μM (Fluva) alone or in combination for 48-hours. **(Figure 1C,D)** Figure 4A and 4B are representative of one experiment with N=3. Figure 4C is representative of three independent experiments (N=3). Figure 4C is representative of two independent experiments (N=3). A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

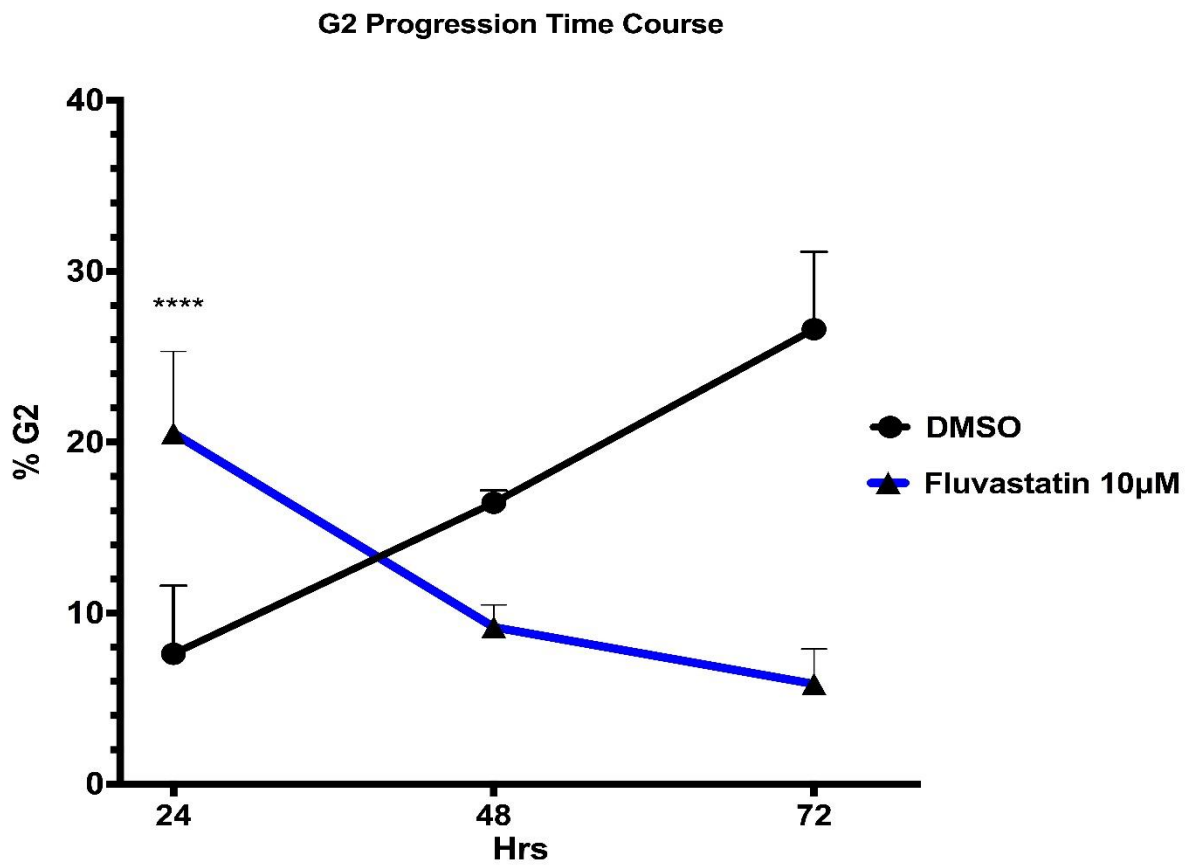**A.**

B.

C.

G2 Progression Time Course
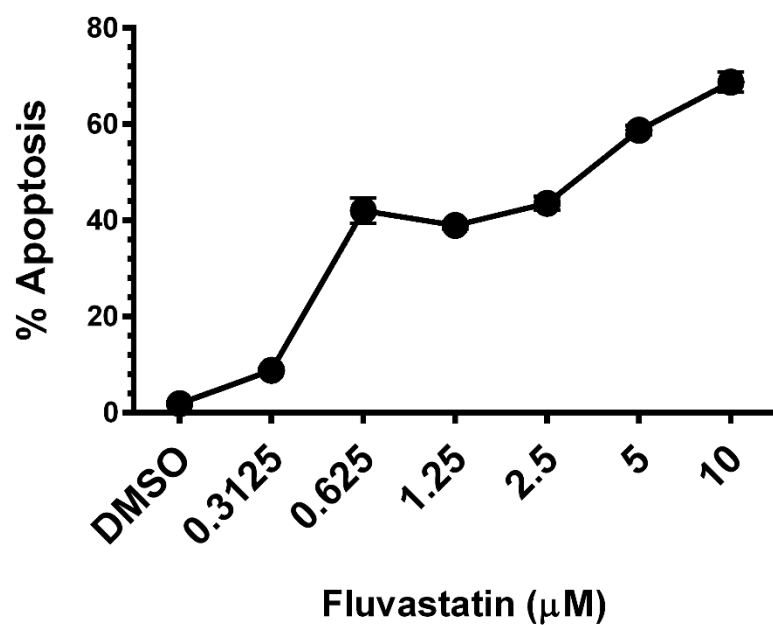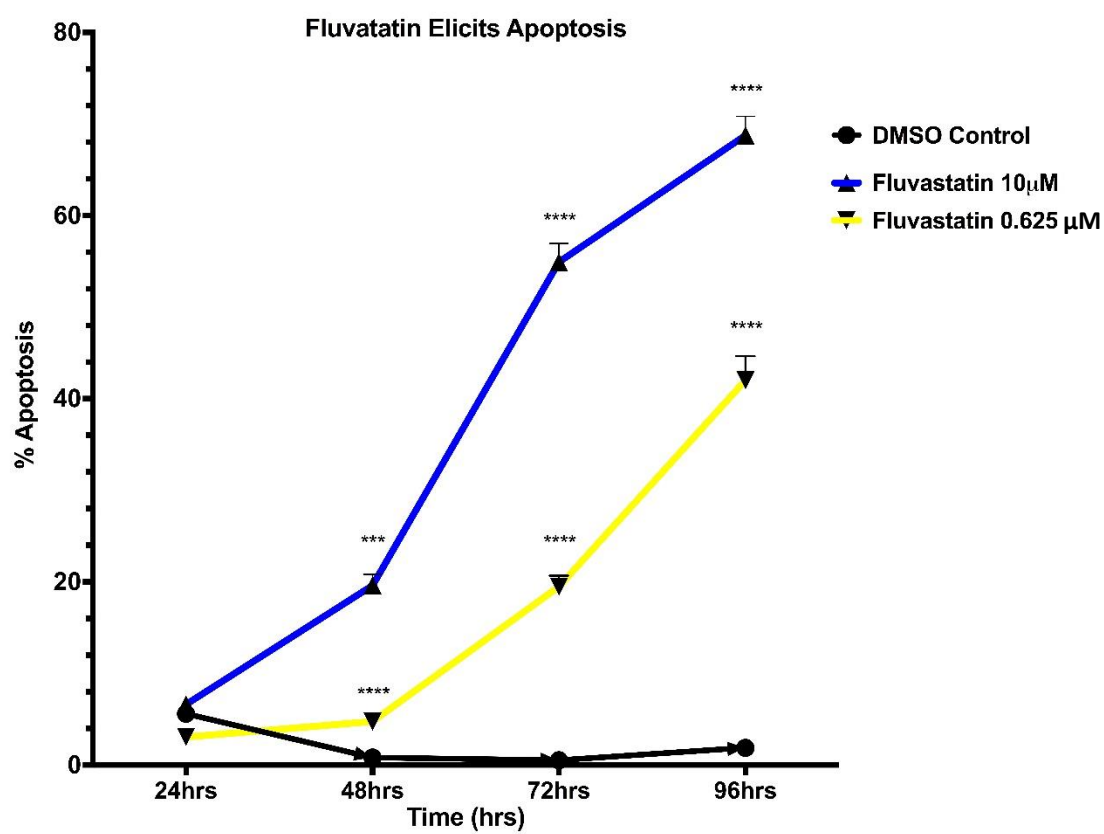
Figure 5
Fluvastatin induces apoptosis in transformed mast cell lines.

P815 cells were treated with vehicle (DMSO) or increasing doses of Fluvastatin during a 96-hour dose response prior to PI-DNA staining for apoptosis (sub-diploid DNA) **(Figure 5A).** P815 cells were co-cultured in vehicle (DMSO) or Fluvastatin at 10µM for a 96-hour time course and analyzed via PI-DNA staining for apoptosis and cell cycle arrest **(Figure 5B,C).** This figure is representative of three independent experiments (N=3). A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001
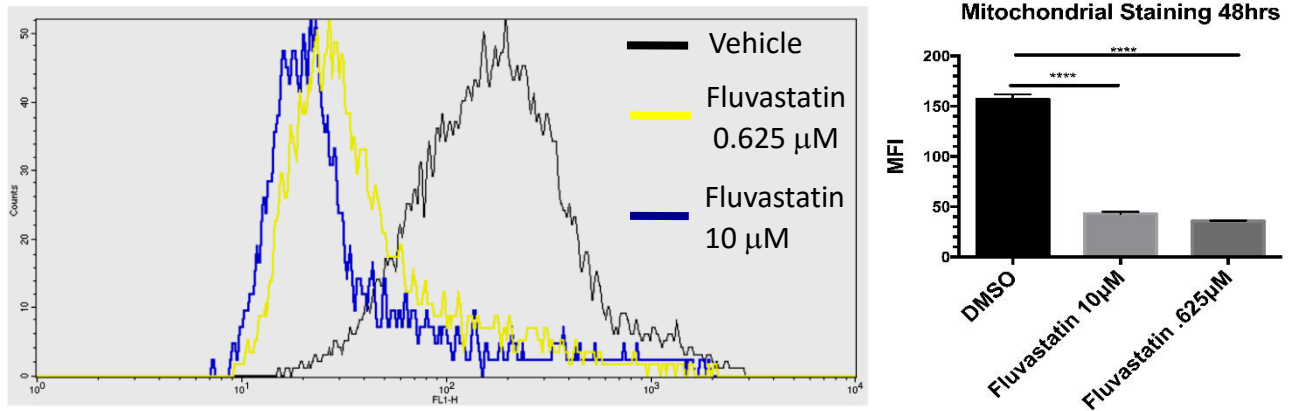
A.

B.
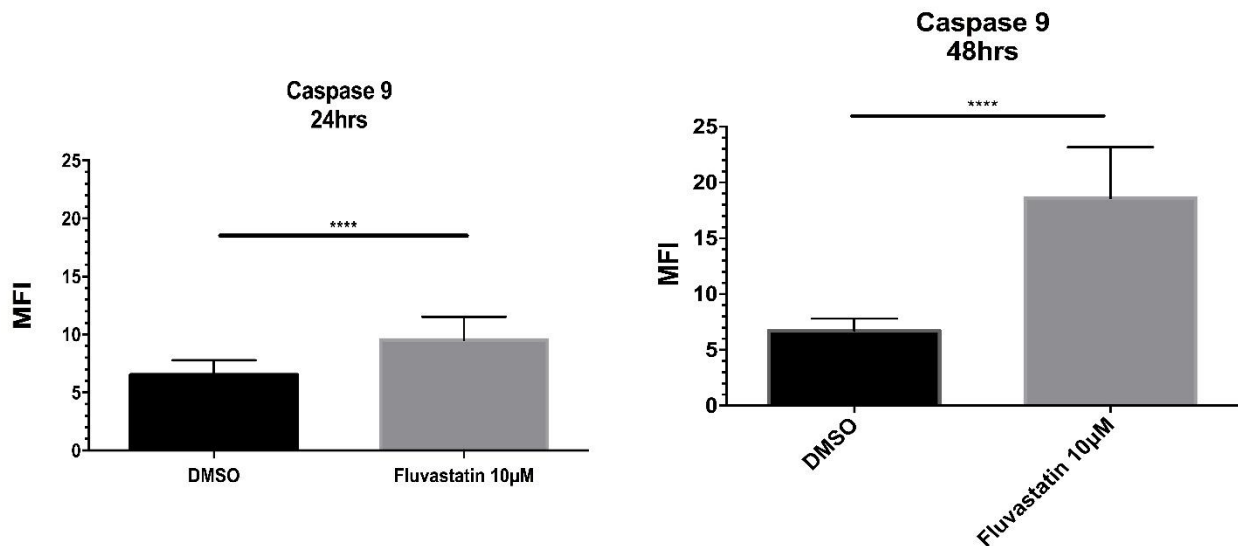
Figure 6

Fluvastatin treatment causes loss of mitochondrial potential and release of caspase 9 in transformed mast cells.

P815 cells were treated for 48-hours with vehicle (DMSO) or Fluvastatin at 10µM or .625µM and then stained with $Di(OC_6)_3$ for analysis of mitochondrial potential. A histogram representation and a graph displaying the mean florescence intensity (MFI) **(Figure 6A).** P815 cells were treated with vehicle (DMSO) or Fluvastatin 10µM for 24 and 48-hours and then assessed for caspase 9 presence via flow cytometry **(Figure 6B).** This figure is representative of three independent experiments. A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

A.

P815 Upregulation of cKIT
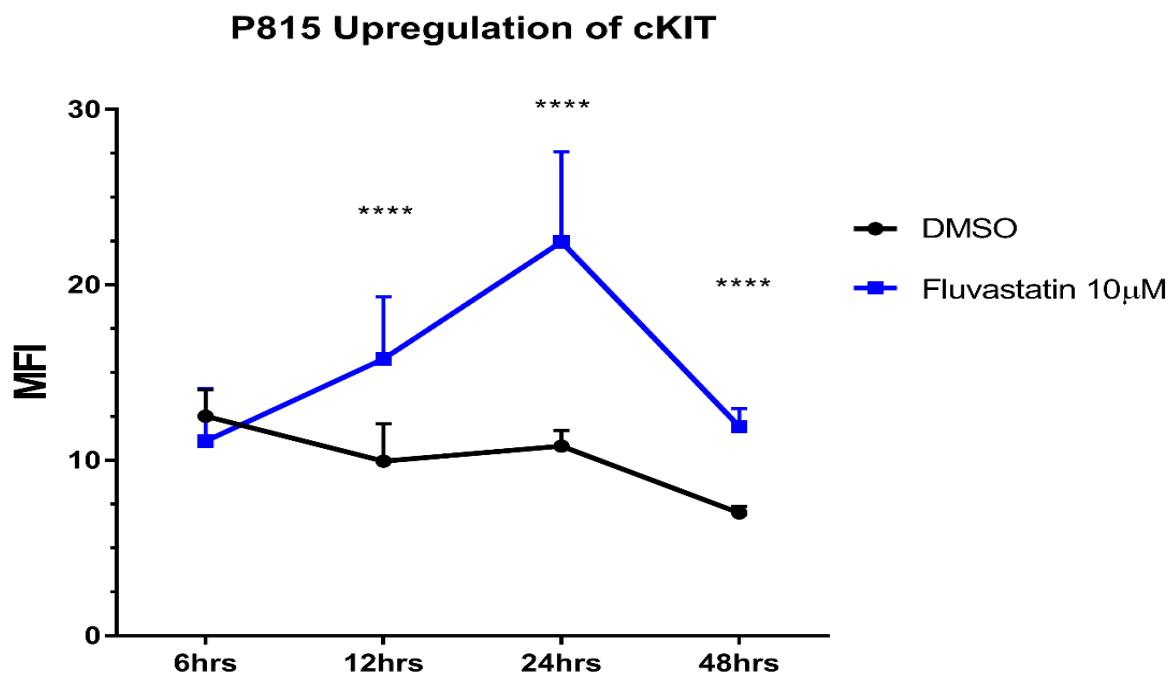
B.

C.

D.

72hr Treatment of P815's
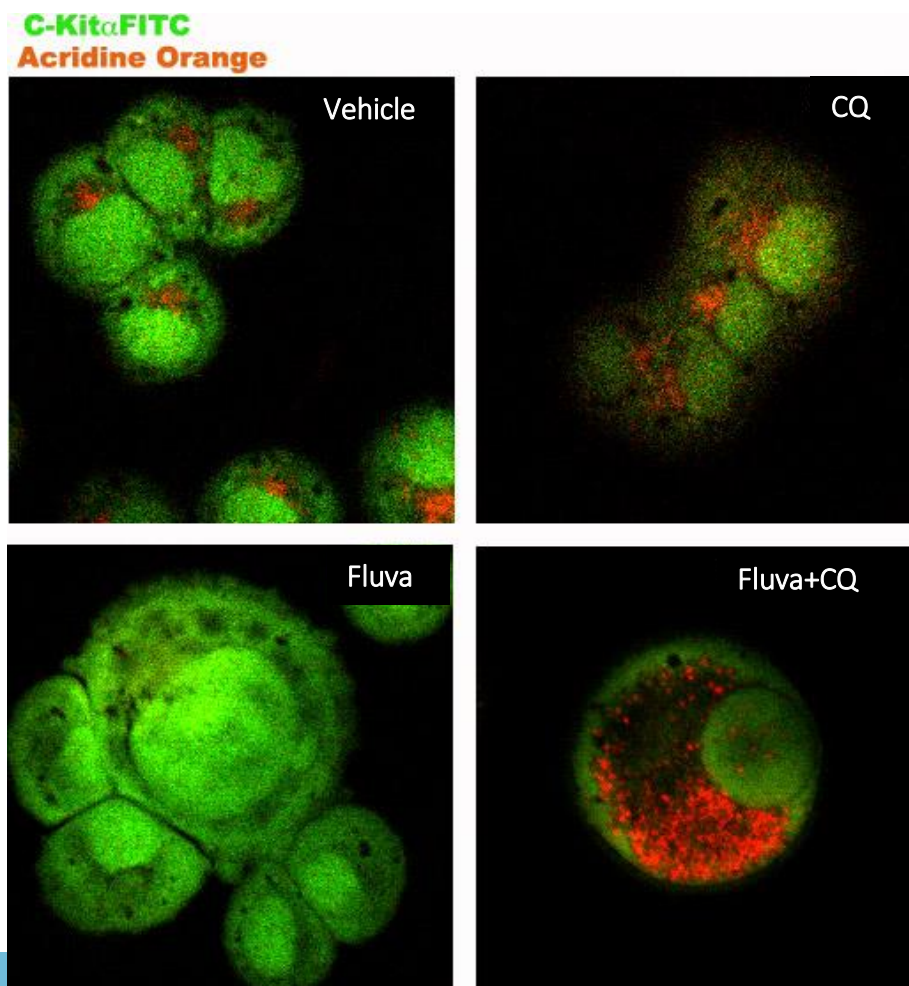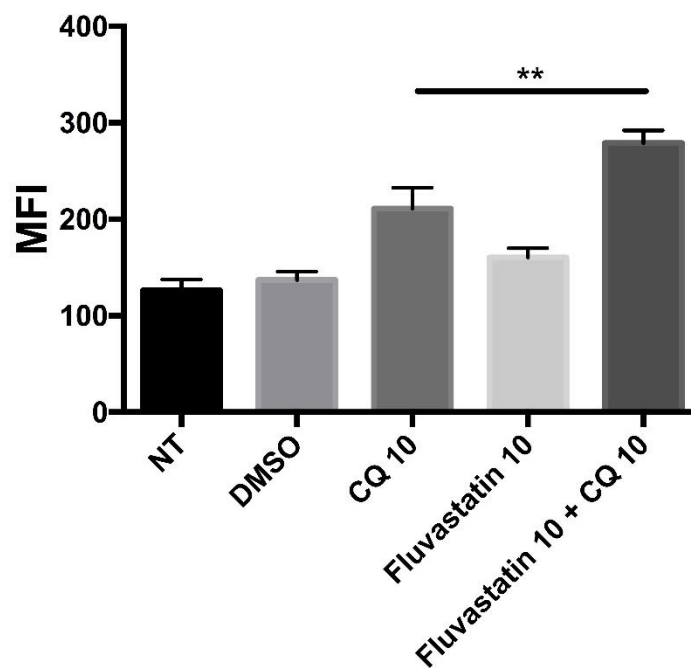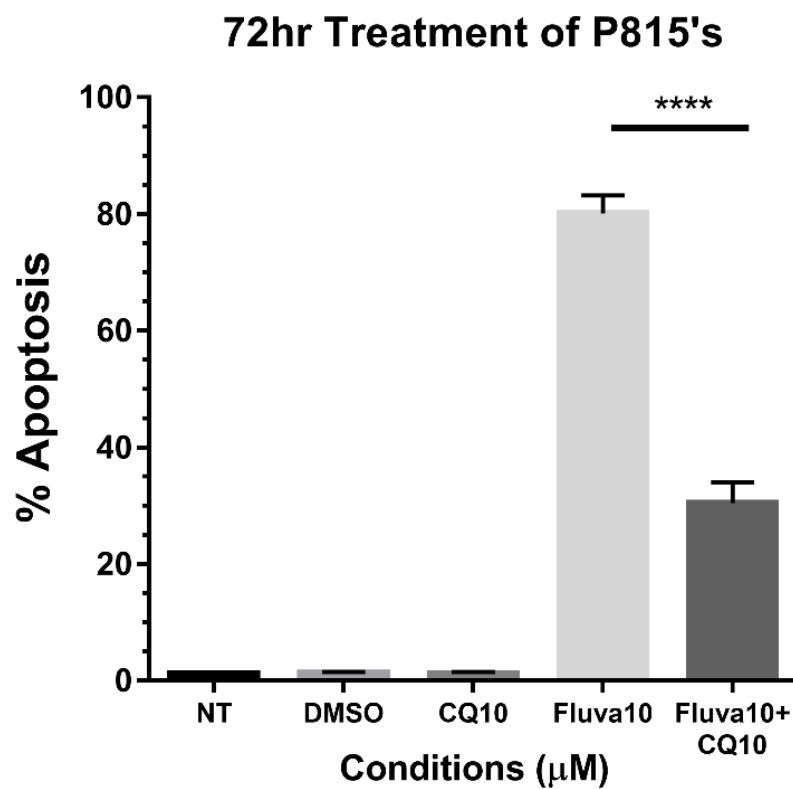
Figure 7

P815 cells exhibit pro-survival upregulation of D814V cKIT and cyto-toxic autophagy.

P815 cells were treated with either vehicle (DMSO) or Fluvastatin 10µM for 6, 12, 24, and 48-hours before cKIT receptor staining **(Figure 7A).** P815 cells were treated with (DMSO), chloroquine 10µM (CQ), Fluvastatin 10µM (Fluva), or Fluvastatin 10µM with chloroquine 10µM for 24-hours. Prior to fluorescent microscopy, P815 cells were stained with FITC anti-cKIT, and acridine orange **(Figure 7B)** and mean florescence intensity (MFI) from flow cytometry of acridine orange staining conditions displayed in fluorescent imaging **(Figure 7C).** P815 cells were treated for 72-hours with vehicle (DMSO), chloroquine 10µM (CQ), Fluvastatin 10µM (Fluva), and the combination treatment of chloroquine 10µM with Fluvastatin 10µM **(Figure 7D).** All figure 7A, 7B, 7C, and 7D are representative of three independent experiment (N=3). A student's t-Test was conducted and statistical significance is as depicted: *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

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Vita

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Born November 24, 1989 in Westchester, New York.

Education

Northern Virginia Community College, Woodbridge, Virginia

- Emergency Medical Technician (2007)

Virginia Commonwealth University, Richmond, Virginia
(Undergraduate Degrees)

- Pre-Medicine
- Bachelors of Science in Psychology (2012)

Georgetown University Medical Center and **George Mason University G²**, Manassas, Virginia
 (Graduate Degree)

- Advanced Biomedical Sciences (2013)

Virginia Commonwealth University, Richmond, Virginia

- Masters in Biology (current)

Certifications and Qualifications

- Emergency Medical Technician

- Advanced Cardiac Life Support from American Heart (ACLS)

- American Heart Association: CPR for the Healthcare Provider

- Health Care Insurance Portability and Accountability Act (HIPPA)

- **Bilingual**: Spanish (Proficiently read ,write and speak)

- Dedicated, creative professional with excellent interpersonal and communication skills.

- Able to rapidly and efficiently adjust to new and unpredictable situations.

- Phlebotomy technician

- Proficient with Microsoft programs.

- Proficient with Cerner and Connect Care electronic medical record programs. (EMR)

- Red Cross Lifeguard Certified

- Certified Pool Operator

Awards

- American Association of Immunology (AAI) trainee abstract award (2014)

- American Association of Immunology (AAI) trainee abstract award (2015)

- American Red Cross Life Saver Recipient for actions taken on 1/16/2014

- Fairfax County Fire and Rescue Citizen Life Saver Award

- Fairfax County Glen McCarthy Award (2008)

Work Experience

Virginia Commonwealth University, Richmond, Virginia   August 2014-Current

- Research assistant
- Biology laboratory teaching assistant.

Lifetime Athletic, Centreville, Virginia   (April 2013-August 2014)

- Aquatics Department Head
  o Manage lifeguards in respect to budgeting and scheduling.
  o Train staff for healthcare related emergencies.
  o Run swim lesson program with monthly financial goals critical in budgeting payroll.
  o Create and grow new programs within the department budget to create revenue.
  o Hiring skilled staff for coaching and other competitive swimming related tasks.
  o Training of staff in regards to customer service and retention with clients.
  o Maintenance of licensure and mechanical function of filters.
  o Daily testing with DPD-FAS reagents.
  o Maintaining current knowledge of county regulations and requirements.
  o Fostering business relationships with maintenance and monthly chemical delivery companies.

Bon Secours Richmond Health System, Richmond, Virginia

- Emergency Medical Technician, Emergency Department   (July 2011-August 2012)

  o Trained on psychiatric and cardiac arrest codes.
  o Performed vitals.
  o Phlebotomy.
  o Insertion of IV lines.
  o Casting and splinting of fractures and sprains.
  o Communicated with patients and documented procedures using Connect Care (EMR) services.

Molecular Medicine Research Building (MMRB), Richmond, Virginia   (December 2010-August 2012)

- Research Lab Assistant
  o Worked with laboratory animals.
  o Fluent in research, and Microsoft programs.
  o Performed various biotechniques.
  o Competent with various immunological assays and tests. (ELISA's, flow cytometry, preparing vaccines with various adjuvants in sterile conditions and isolating serum from patients and mice.)
  o Worked under Dr. Dan Conrad.

  MCV Hospital, Richmond, Virginia
- Emergency Medical Technician, Radiology Department   (September 2010-August 2010)
  o Transported and provided care for patients from cardiac procedure areas to back to ICU or floors.

- o Gained experience with in hospital terminology and treatments for critical care patients.

- Care Partner, Acute Care Medicine (Critical Care Hospital, MCV)   (May 2010-August 2010)
    - o Performed all phlebotomy, vitals every six hours or PRN and assisted patients with medical care.
    - o Emergency department rotations as a medic occurred as needed to supplement staff.

Tuckahoe Volunteer Rescue Squad, Richmond, Virginia

- Emergency Medical Technician   (August 2009-April 2010)
    - o Provided emergency medicine
    - o Attended the Emergency Vehicle Operator Course (EVOC)

Northern Virginia Pools, Chantilly, Virginia

- Pool Manager   (May 2008-August 2008)
    - o Managed employees in regards to scheduling and training.
    - o Pool systems operator: Regulated pool chemical levels to state regulations and performed maintenance of filters and pumps.

Life Time Fitness, Centreville, Virginia

- Aquatics Supervisor   (September 2005-May 2008)
    - o Managed employees in regards to scheduling along with providing work related medical instruction.
    - o Pool systems operator: Regulated pool chemical levels to state regulations and performed maintenance of filters and pumps.
    - o Provided customer service for patrons in and was in charge of any and all issues on deck.