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Polymorphisms and Biologic Effects of Acidic Mammalian Chitinase in Asthma

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POLYMORPHISMS AND BIOLOGIC EFFECTS OF ACIDIC MAMMALIAN CHITINASE IN ASTHMA

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Abstract

In this study, we hypothesize that human acidic mammalian chitinase (AMCase) binds and is regulated by the epidermal growth factor receptor (EGFR), and that AMCase interacts with Galectin-3 (Gal-3) to mediate anti-apoptotic functions. We further hypothesize that asthma-associated polymorphisms of AMCase alter chitinase activity and modulate anti-apoptotic effects. We investigated the interactions between AMCase, Gal-3 and EGFR by establishing binding and co-expression *in vitro*; apoptotic effects were evaluated via Annexin V/Propidium Iodide staining. Molecular cloning was performed to generate single nucleotide polymorphisms (SNPs) of AMCase associated with asthma. Our data showed that co-expression of AMCase and EGFR induces chitinase activity; we found that AMCase and Gal-3 bind each other *in vitro*, and that they co-localize in the cytoplasm of cells. Co-transfection of AMCase and Gal-3 demonstrates greater anti-apoptotic effect than Gal-3 alone, while recombinant Gal-3 induces apoptosis, which is not blocked by incubation with recombinant AMCase. From these data, we conclude that AMCase is regulated by EGFR, and that AMCase and Gal-3 physically interact, however contrary to our hypothesis, the anti-apoptotic effects of

AMCase are unlikely to be mediated by Gal-3. Further exploration of this pathway using SNP constructs generated in this study will shed light on the mechanism of AMCase in asthma.

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Introduction

Molecular pathogenesis of asthma

Asthma is a chronic inflammatory airway disease which affects more than twenty million Americans and many millions more worldwide. The increasing prevalence of asthma in developed countries has been correlated with decreases in childhood infections.

Asthma is characterized by reversible airway inflammation, mucus plugging, and airway hyper-responsiveness leading to airflow obstruction. Allergic asthma, which constitutes most cases, is believed to develop as an allergic-type reaction to environmental allergens [1]. Exposure to allergen triggers eosinophil and T cell infiltration into airways, leading to a state of reversible airway inflammation.

Current theories of allergic and asthmatic disease center on the 'hygiene hypothesis' [2]. This hypothesis proposes that childhood exposure to infection is protective against the hyper-excitable inflammatory responses characteristic of allergic and asthmatic disease. The inflammatory response in asthma is believed to be caused by the dysregulation of a subset of T lymphocytes designated as T helper-2 (Th2) cells [3]. Th2 and T helper-1 (Th1) cells are both mutually antagonistic and self-perpetuating: pro-Th2 cytokines simultaneously induce Th2 expansion and suppress Th1 proliferation, and vice versa.

In the early post-natal period, T lymphocyte populations are skewed toward a Th2 predominance, possibly due to selective fetal down-regulation of Th1 cytokines such as IFN-γ, which are toxic to the placenta [4]. High level allergen exposure – to bacteria, viruses, and parasites – during post-natal life leads to redistribution of T cell populations

via anergy and deletion, creating the Th1-dominant adult state. This process of T cell restructuring is known as immune deviation and is believed to fail in atopic patients, leading to allergic and asthmatic disease later in life.

Th2-mediated responses are classically considered to be immunoprotective against parasitic infections. In the absence of previous parasitic exposure, innocuous environmental antigens can elicit an inappropriate Th2 response, leading to asthmatic or allergic disease. Many lines of evidence strongly link Th2 cell predominance to allergic asthma in humans. Th2-stimulatory cytokines are elevated in the airways of asthmatics [5], and high levels of Th2 transcription factors such as STAT6 and GATA3 have been detected on airway biopsy [6, 7]. Th2 cells have been shown to be functionally active in inflammatory asthma, producing pro-inflammatory cytokines including interleukin (IL) -4 and IL-13. The Th2-mediated release of IL-4 and IL-13 has been demonstrated to induce the tissue responses seen in asthma, including recruitment of eosinophils, mucus metaplasia, airway fibrosis and airway hyperresponsiveness [8, 9]. The exact factors which determine immune deviation remain to be fully delineated, however.

The complex interplay between genetics and environmental exposures continues to be the subject of extensive investigation. Chitin is an environmental allergen commonly found in the coats of parasitic nematodes, as well as in fungal cell walls and the exoskeletons and digestive tracts of insects [10]. Chitin is the most common polysaccharide on Earth, after cellulose. Chitin appears to play a role in immune responses, however conflicting studies exist as to the exact function. Protective chitin coats help insulate parasites from host defense mechanisms. As with many pathogen surface markers, chitin has been shown to play a role as a recognition element for hosts,

and to elicit defense responses to pathogens in many species of plants [11]. Interestingly, in a murine model of airway allergy, orally administered chitin was found to down-regulate Th2 mediated IgE production and eosinophilia, inducing a shift toward Th1 dominance [12].

Chitinases, a family of enzymes that cleave chitin, have been identified in many organisms [13]. In plants, chitinases are believed to play a role in resistance to infection by chitin-containing fungi; in fungi, chitinases act as a defense mechanism against parasites [14]. Until recently it was believed that mammals, which lack chitin, also lacked chitinases. Two mammalian chitinases have since been identified and characterized: chitotriosidase [15] and more recently, acidic mammalian chitinase [13].

Chitotriosidase

Chitotriosidase was the first functional mammalian chitinase to be isolated [16]. Exclusively produced by phagocytes, chitotriosidase is enzymatically active *in vitro* against both native chitin and synthetic fluorogenic substrate. The 50 kDa active enzyme secreted by macrophages consists of an N-terminal catalytic domain linked via a hinge region to the C-terminal chitin-binding domain [15].

Chitotriosidase was first identified in the plasma of patients with symptomatic Gaucher's disease [17]. Gaucher's disease is an autosomal recessive lysosomal storage disease characterized by low levels of glucocerebrosidase, which results in accumulation of glucosylceramide inside macrophages. The accumulation of glycolipid results in hepatosplenomegaly, bone lesions and neurological abnormalities. The clinical phenotype of Gaucher's disease is highly variable and difficult to predict.

In addition to accumulation of glycolipids, Gaucher's patients may develop abnormalities in plasma levels of various hydrolases [15]. Patients with symptomatic Gaucher's disease were also observed to have levels of chitotriosidase 100 to 5000-fold (mean, 600-fold) greater than the median level in healthy controls [17]. Levels of chitotriosidase frequently correlate with degree of macrophage activation and hence disease severity; levels decrease with enzyme replacement therapy [18]. As a result, chitotriosidase is currently the most promising biomarker for monitoring of enzyme replacement therapy of Gaucher's disease [19].

Although the exact function of chitotriosidase in mammals has yet to be determined, the available data suggest that chitotriosidase plays a role in innate immunity, similar to the function of plant chitinases in host defenses. GM-CSF has been demonstrated both to increase the synthesis of chitotriosidase in macrophages and to promote its release from polymorphonuclear leukocytes [20]. *In vitro* recombinant human chitotriosidase exhibits anti-fungal activity, and in murine models of systemic aspergillosis and candidiasis, chitotriosidase significantly increases the survival time of neutropenic mice. A common genetic variant consisting of a 24 base pair duplication in the chitotriosidase gene which obviates enzyme activity, has been shown to be associated with susceptibility to *Wucheria bancrofti* filarial infections in India [21], and with bacteremia in children with acute myeloid leukemia [22]. Taken together, these observations point toward a role for chitotriosidase in immune defenses against chitin-containing organisms [20].

Chitotriosidase has also been studied in the context of asthma. Seibold *et al* found that chitotriosidase was upregulated in the lungs of smokers, but not in the lungs of asthmatics [23]. The low level of chitinase activity in the lungs of subjects with asthma was consistent with a protective role for chitinases in the setting of allergic airway disease. Another study found that genetic variants in chitotriosidase have no correlation with asthma. The 24 base pair duplication in chitotriosidase which eliminates enzyme activity, as well as polymorphisms at both the five-prime and the three-prime ends of the gene leading to amino acid exchanges, were found to be evenly distributed between asthmatics and healthy controls [24]. This difference may be due to distinct patterns of tissue expression [25] and functionality of AMCase and chitotriosidase. Given that approximately 6% of the Caucasian and Ashkenazi populations are homozygous for the 24 base pair duplication in chitotriosidase and are therefore deficient in this enzyme [26], it has been proposed that chitotriosidase no longer plays an essential role in host immunity. Instead this function is entirely carried out by AMCase [13].

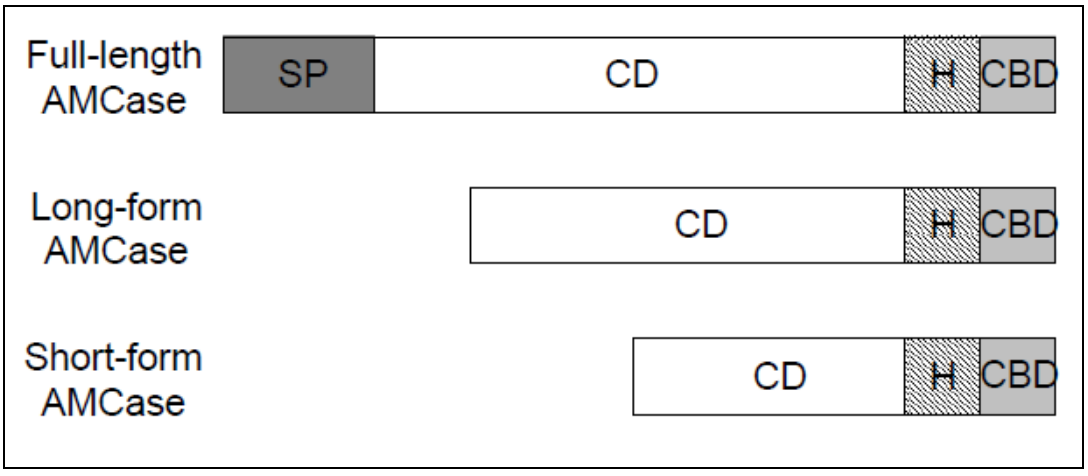
Acidic mammalian chitinase

Acidic mammalian chitinase (AMCase) was the second functional mammalian chitinase to be identified and cloned [13]. AMCase is located on chromosome 1q13 in humans, and is highly conserved between humans and mice. However, unlike murine AMCase which has a major optimum pH at 2 and a secondary optimum pH at 3-6, human AMCase has a single optimum pH of ~4-5 [27]. Chitotriosidase in contrast has a broad optimum pH and is inactivated at acidic pH [13]. Both AMCase and chitotriosidase are members of family 18 of glycosyl hydrolases [27]. In contrast to chitotriosidase which is

expressed in macrophages in humans, AMCase is predominantly expressed in the human lung and gastrointestinal tract. Similar to chitotriosidase, full-length AMCase is secreted as a 50 kDa protein consisting of an N-terminal catalytic domain linked via a hinge region to a C-terminal chitin-binding domain [13]. Unlike AMCase, a small proportion of active chitotriosidase enzyme may be proteolytically cleaved in macrophages to produce a 39 kDa form [28]. This C-terminally truncated chitotriosidase retains hydrolytic activity and accumulates in macrophage lysozymes in humans.

In addition to the full-length enzyme, cloned human AMCase exists in two splice variants (see **Figure 1**) which have been previously described in the literature as TSA 1902-L and TSA 1902-S [29]. These two isoforms arise from sites of alternative translation initiation. They begin at the third and fourth in-frame start codons (see **Figure 7**), and will be referred to in this study as the 'long-form' and 'short-form', respectively.

Figure 1: Isoforms of human AMCase.



The short-form is 315 amino acids long, lacks the signal peptide and almost half of the catalytic domain, and has no chitinase activity. Since it is not biochemically active, the short form was not investigated in this study. The long-form is 368 amino acids long and lacks the signal peptide and the first part of the catalytic domain, but retains the conserved DXXDXDXE catalytic motif that characterizes chitinases although it is chitinolytically inactive. The long-form also contains a highly conserved histidine residue (His207 as numbered from the first start codon) in the active site of the enzyme that has been shown to be responsible for the uniquely acidic optimum pH of AMCase [30].

AMCase and asthma

A growing body of evidence links AMCase with the development of airway inflammation in asthma. When it was initially identified, AMCase was observed to be expressed in the lungs of both humans and rodents [13]. Using a mouse model of allergic airway inflammation, a proteomics approach demonstrated strikingly elevated levels of AMCase and the chitinase family proteins Ym1 and Ym2 in the airways of experimental murine subjects as compared to controls [31]. DNA microarray analysis identified AMCase as upregulated in two murine models of airway inflammation – transgenic IL-13 mice which express IL-13 in the airways, and IL-13/Epi mice, which express IL-13 in the airways, and STAT6 only in nonciliated airway epithelial cells [32].

AMCase has been shown to be integrally involved in Th2-mediated inflammation. In a transgenic mouse model of asthma, our lab has recently shown that AMCase is selectively induced during Th2 inflammatory responses and acts downstream of IL-13

and STAT6 [10]. In this model, inhibition of AMCase activity leads to a markedly diminished Th2 response with decreased eosinophil recruitment, tissue inflammation, and airway hyper-responsiveness. These data implicate AMCase as an important mediator of asthmatic inflammation [33].

Although the role of AMCase in inflammation is still incompletely understood, recent studies have identified some of its functional interacting partners. In a yeast two-hybrid screen, our lab identified epidermal growth factor receptor (EGFR) as a putative binding partner of AMCase. EGFR regulates many functions of epithelial cells, including cell migration, proliferation, differentiation, and survival [34]. EGFR is upregulated or mutated in many human cancers: small molecule EGFR inhibitors such as erlotinib and gefitinib are used as therapy in solid tumors [35].

EGFR has also been implicated in Th2-mediated inflammation in the airways. In a rodent model of asthma, EGFR has been shown to be upregulated in the airways. Pre-treatment with an inhibitor of EGFR tyrosine kinase attenuated the development of inflammation [36]. Bronchial biopsies demonstrated high levels of EGFR mRNA in asthmatics as compared to healthy controls [37]. EGFR has also been shown to act downstream of IL-13 in rodent models of airway inflammation; blocking EGFR signaling prevents IL-13 mediated mucus hypersecretion [38]. Follow-up studies by our lab have shown that AMCase binds EGFR *in vitro*. In addition, AMCase secretion was increased following co-transfection of AMCase and EGFR, but was found to be decreased following inhibition of EGFR, its upstream transactivator ADAM17, or its downstream mediator Ras [39].

Taken together, this evidence has led our group to theorize that airway inflammation in asthma is mediated by Th2 activation. A central theme behind these studies is that AMCase participates in Th2 inflammation by an EGFR-dependant inhibition of apoptosis mechanism. Secretion of IL-13 by activated Th2 cells stimulates the EGFR signaling cascade via ADAM17. Acting through its downstream mediator Ras, EGFR stimulates AMCase secretion, which leads to increased inflammation via an as yet unknown mechanism, possibly involving inhibition of apoptosis via Galectin-3. These effects may be modulated by polymorphisms in the AMCase gene.

Polymorphisms in AMCase in human asthma

In addition to evidence from murine models, expression of AMCase mRNA has been shown to be markedly increased in the lungs of human asthmatics [10]. This association has been explored in population genetic studies. Haplotypes composed of seven single nucleotide polymorphisms (SNPs) of AMCase identified in a German population show a strong correlation ($p<10^{-10}$) with asthma [40].

Polymorphisms in AMCase have also been associated with responses to albuterol treatment in asthmatic patients [41] (see **Appendix**). Polymorphisms correlated with asthma vary with population and genetic background: one recent study identifying polymorphisms in a North Indian population demonstrated minimal overlap with asthma-correlated polymorphisms identified in the German population [40, 42]. Three of the new SNPs identified in the Indian population were found to be correlated with atopic asthma and elevated total serum IgE [42]. One of these SNPs, located in the promoter

region, altered the transcriptional activity of the AMCase promoter; another of the SNPs abrogated binding of the transcription factor Oct-1.

Of the seven SNPs identified in the German population, four are of particular interest to this study (see **Table 1**). The first, leading to the amino acid variant K17R in the catalytic domain within exon 5, is strongly associated with asthma both in adult ($p=0.0031$) and pediatric populations ($p=0.0172$) as compared to controls [40]. Two other polymorphisms in the catalytic domain (Rs2275253 and Rs2275254) were strongly represented in the haplotypes associated with asthma, and may play a role in altered catalytic activity. The fourth polymorphism (Rs2256721) is located within exon 11 in the chitin binding domain (CBD) of AMCase, and as such may modulate chitin binding activity. The Rs2256721 polymorphism has also been strongly correlated with asthma in patients (Elias lab, unpublished data). A fifth SNP (Rs3818822) located in exon 4 was also associated with asthma, particularly in pediatric populations. However, because it does not alter the amino acid sequence of wildtype AMCase this fifth SNP was not included in this study.

Table 1: AMCase SNPs associated with asthma [40]

SNP	Exon	BP#	WT Base	SNP Base	Amino Acid exchange
--	5	477	A	G	K17R
Rs2275253	9	1118	A	G	I231V
Rs2275254	10	1164	T	C	F269S
Rs2256721	11	1398	T	G	V324G

None of these five non-synonymous SNPs have been fully functionally characterized with regard to their effect on chitinase activity or chitin binding. Two of these SNPs (K17R and Rs2256721) have been associated with asthma independent of haplotype; two others (Rs2275253 and Rs2275254) are prominently represented in multiple haplotypes associated with asthma. Although it remains to be established what impact an isolated SNP may have on functional activity, this strong association data coupled with plausible biochemical mechanisms seemed to support the investigation of these SNPs both individually and as part of haplotype constructs.

Summary

These observations show that AMCase is upregulated in both humans and mice with airway inflammation is involved Th2 cell-mediated inflammation, acts downstream of IL-13 and EGFR, and that genetic polymorphisms associated with clinical asthma are present in human populations. Taken together, these data suggest that AMCase plays a role in the pathobiology of asthma, and that polymorphisms in human populations have significant implications for understanding mechanisms of asthmatic disease and treatment. This study will seek to investigate the functional role of AMCase by developing the molecular tools to further explore the impact of non-synonymous single nucleotide polymorphisms of AMCase on chitinase activity and apoptotic effects.

Hypothesis

We hypothesize that human AMCase participates in Th2 inflammation by an EGFR-dependant inhibition of apoptosis mechanism. We hypothesize that AMCase interacts with Galectin-3 to mediate downstream anti-apoptotic functions of AMCase, and that asthma-associated polymorphisms of human AMCase alter chitinase activity and modulate downstream anti-apoptotic functions of AMCase, increasing Th2-mediated inflammation in human asthma.

Specific Aims

In this study, we seek to characterize the effects of human AMCase on apoptosis, and to develop the biochemical tools to explore the effect of polymorphisms of AMCase associated with asthma. We approach this problem in two stages:

1. Investigate the interactions between wildtype AMCase and Galectin-3 and their effect on apoptosis.

In a yeast two-hybrid screen our lab has determined that Galectin-3 binds to human AMCase. Galectin-3 is a known regulator of apoptosis that is upregulated during cellular proliferation [43]. Galectin-3 has been shown to have anti-apoptotic effects when over-expressed in cell lines, conferring resistance to anti-Fas antibody and staurosporine [44]. Paradoxically, exogenous Galectin-3 has been shown to induce apoptosis in Jurkat cells, a human T cell line [45]. We believe that the inflammatory actions of AMCase may be mediated via protein-protein interactions with Galectin-3, inhibiting apoptosis and thereby increasing Th2 cell-mediated inflammation and the severity of asthmatic

disease. In this study, we will seek to establish the effects of interactions between AMCase and Galectin-3 on apoptosis. We will over-express full-length AMCase and Galectin-3 in Jurkat T cells and induce apoptosis via staurosporine. We will also study the effects of exogenous AMCase and Galectin-3 by stimulating un-transfected Jurkat T cells with recombinant Galectin-3 to induce apoptosis; we will then incubate with recombinant AMCase to determine whether AMCase blocks Galectin-3 mediated apoptosis, as detected by Annexin V/PI staining.

2. Develop mammalian expression constructs of AMCase SNPs associated with asthma.

Polymorphisms of AMCase demonstrating a strong association with human asthma have been identified by population genetics in multiple studies [40, 42]. Given that the exact function of AMCase has yet to be fully elucidated, investigation of genetic polymorphisms with known clinical implications has significant potential to aid in understandings of the biochemical role of AMCase. In this study, we propose to generate constructs expressing SNPs of AMCase that have been associated with asthma. Specifically, we will seek to introduce SNPs identified in the literature [40] and by our lab (Elias lab, unpublished data) using site-specific mutagenesis. We plan to generate these constructs both for long-form (Open Biosystems clone) and full-length (MedImmune clone) for comparison. The SNPs to be investigated in this study are listed in **Table 1**.

Methods

Cloning. Long-form AMCase was purchased from Open Biosystems (Huntsville, AL), clone ID #5185486. Full-length AMCase was obtained as a gift from MedImmune (Gaithersburg, MD). Site-directed mutagenesis was conducted with Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers were designed using Stratagene's Quik Change Mutagenesis Program. Clones were sequenced to confirm accuracy, and amplified in host bacteria DH5α. DNA was prepared using QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD) or using PowerPrep HP Plasmid Maxiprep System (Marligen Biosciences, Ijamsville, MD) according to the manufacturer's instructions.

A549 cell transfection. Cultured A549 cells were grown in 6-well culture plates (BD Biosciences, San Jose, CA) to 80% confluence. Transfection of 2 μg of plasmid DNA per well was performed using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At designated time points the cells were harvested to assay for chitinase activity or FACS analysis.

Jurkat T cell transfection. Jurkat T cells were cultured in IMDM (Invitrogen) with 10% FBS at a maximum of $1x10^6$ cells per mL. Cells were harvested by centrifugation, washed once in IMDM without serum, and resuspended at a final concentration of $2.5x10^6$ cells/mL in a final volume of 100 μl. Maxiprep DNA was added at a concentration of 100 ug/mL, and samples were electroporated at 250 volts/960 μF.

Samples were transferred to 10 mL culture flasks (BD Biosciences, San Jose, CA) containing IMDM with 10% FBS, and incubated at 37˚C. At designated time points the cells were harvested for FACS analysis.

Chitinase bioactivity assay. A549 cells were cultured in DMEM (Invitrogen) with 10% FBS to 80% confluence. Cells were washed twice in serum-free media, transfected as above, and cultured in serum-free Opti-MEM. Supernatants from transfected cells were harvested at designated time points. Aliquots (50 µl) of each sample were mixed with 30 µl of buffer (0.1M citrate, 0.2M phosphate, pH 5.2) and 20 µl of 0.5 mg/ml 4-methylumbelliferyl-D-N,N'-diacetylchitobioside (Sigma, St. Louis, MO) at a final concentration of 0.17 mM. The samples were incubated at 37˚C for 15 minutes, and the reactions were stopped by adding 1 mL of stop solution (0.3 M glycine/NaOH buffer, pH 10.6). The fluorescence intensity of released 4-methylumbelliferone was measured using a fluorometer (excitation 350 nm, emission 450 nm). Chitinase extracted from *Serratia marcescens* (Sigma) was used as a positive control.

T cell apoptosis assay. Electroporated Jurkat T cells ($1x10^6$/ml) were harvested by centrifugation, resuspended in RPMI 1640 (Invitrogen) to a final concentration of $2.5x10^4$ cells/mL, and incubated with 0.5 µM of staurosporine in RPMI containing 10% FBS. Samples were incubated for 6 hours at 37˚C. Apoptosis was detected by FACS staining for Annexin V/PI, as described below.

Recombinant protein stimulation assay. Untransfected Jurkat T cells were harvested by centrifugation, resuspended in RPMI 1640 (Invitrogen) to a final concentration of $2.5x10^4$ cells/mL, and incubated with either 10 μg/mL recombinant human Galectin-3 (R&D Systems Inc, Minneapolis, MN.), 10 μg/mL recombinant human AMCase, or PBS negative control in RPMI containing 10% FBS. Samples were pre-incubated for 30 minutes on ice then incubated for 6 hours at 37˚C. Apoptosis was detected by FACS staining for Annexin V/PI, as described below.

Annexin V/PI staining. Samples were stained with Annexin V/Propidium Iodide kit (BD Pharmingen, San Jose, CA) according to the manufacturer's instructions. Briefly, cells were harvested by centrifugation at indicated time points, washed twice in cold PBS, and resuspended in 250 μl of 1X binding buffer. Samples were incubated with 5 μl each of Annexin V and Propidium Iodide for 15 minutes in the dark. Staining was stopped by the addition of 400 μl of 1X binding buffer, and the cells were analyzed by FACS.

Immunoprecipitation. Cell monolayers were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM sodium fluoride, and lysed with lysis buffer (15 mM HEPES [pH 7.9], 10% glycerol, 0.5% NP-40, 250 mM NaCl, 0.1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM DTT, and 1 tablet of Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Pleasanton, CA) per 10 ml lysis buffer). The lysates were clarified by centrifugation at 10,000 *g* for 15 minutes, and supernatant protein concentrations determined. Lysates were incubated 1 hour at 4°C

with appropriately diluted antibody-bead conjugates (TBD) to tagged vector, resolved by centrifugation at 10,000 *g* for 1 minute and stored at -20°C prior to Western blot analysis.

Western blotting. Samples were mixed with an equal volume of 2x SDS-PAGE sample buffer (100 mM Tris-Cl [pH 6.8], 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and heated in a boiling water bath, and equal amounts loaded onto 10% or 12% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to Immun-Blot PVDF membranes (Bio-Rad). After transfer, the membranes were blocked for 1 hour in 2% nonfat dried milk in TBST, rinsed, incubated with the appropriate primary antibodies for 1.5 hours at room temperature or overnight at 4°C, washed, incubated with secondary antibody for 1.5 hours at room temperature, and washed in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. Immunoreactive proteins were visualized using the 20x LumiGLO reagent and 20x Peroxide according to the manufacturer's instructions (Cell Signaling Technology Inc., Beverly, MA). The membranes were exposed to BioMax MR film (Eastman Kodak Company, Rochester, NY).

Results

Characterization of the AMCase and Galectin-3 interaction

In a yeast two-hybrid screen, our lab identified Galectin-3 as a direct binding partner of AMCase. Binding was confirmed *in vitro* by immunoprecipitation in A549 cell lysates cotransfected with Galectin-3 and AMCase constructs, as shown in **Figure 2** (data courtesy of Chun Hua He). A549 cells were transfected with myc-tagged Galectin-3 (Gal3), full-length AMCase, Galectin-3 and AMCase, or empty vector. 24 hours post-transfection, samples were harvested and immunoprecipitated with anti-AMCase or anti-myc antibody, then analyzed by Western blot using anti-AMCase or anti-myc antibody for detection. Co-transfected samples contained both AMCase and myc-tagged Galectin-3, while single transfected and empty vector samples failed to contain both proteins.

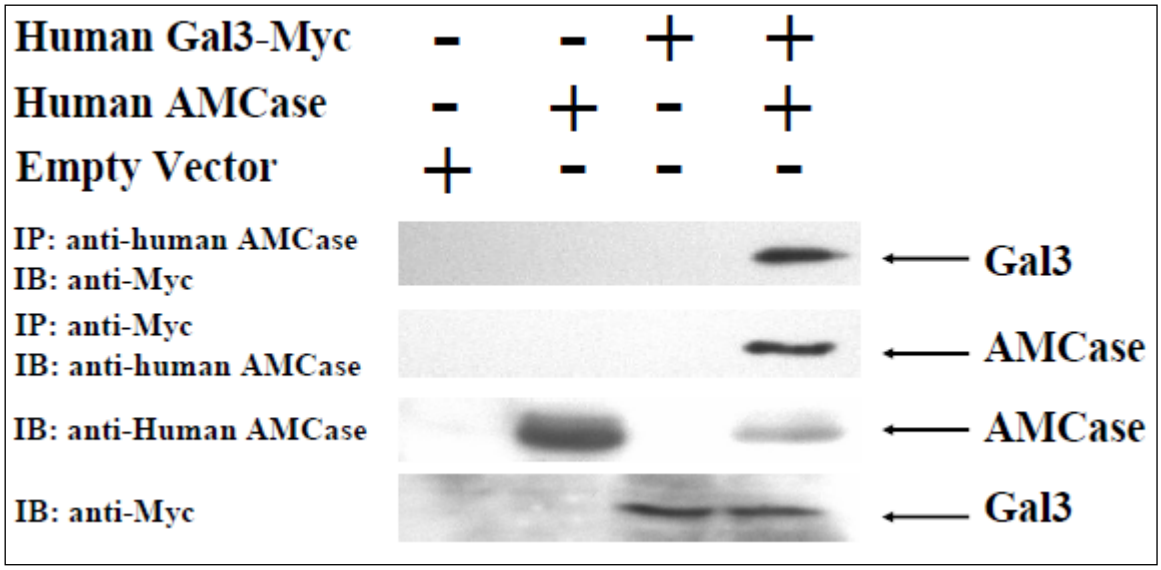
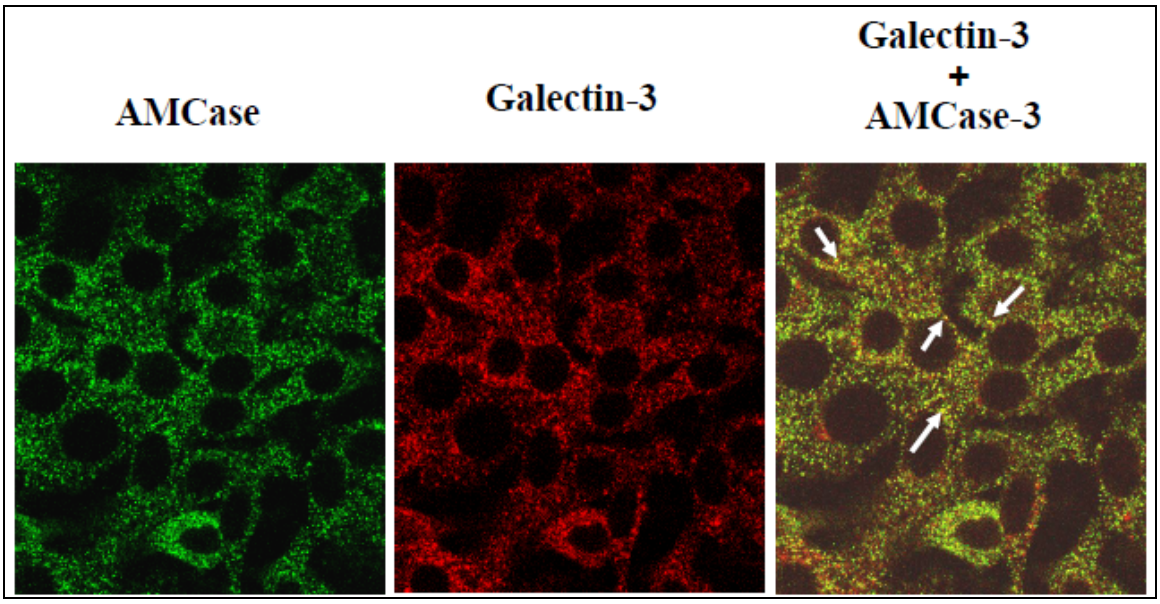


Figure 2: AMCase binds Galectin-3 *in vitro*.

Our lab has also demonstrated that AMCase and Galectin-3 co-localize in the cytoplasm of A549 cells. A549 cells were co-transfected with AMCase and Galectin-3. 24 hours post-transfection, cells were stained with anti-AMCase and anti-Galectin-3 antibodies, and incubated with secondary antibody tagged with GFP (AMCase) or RFP (Galectin-3). As show in **Figure 3** (data courtesy of Chun Hua He), AMCase and Galectin-3 were observed to co-localize in the cytoplasm, as indicated by arrows in the merged panel.

Figure 3: AMCase and Galectin-3 co-localize in A549 cells.

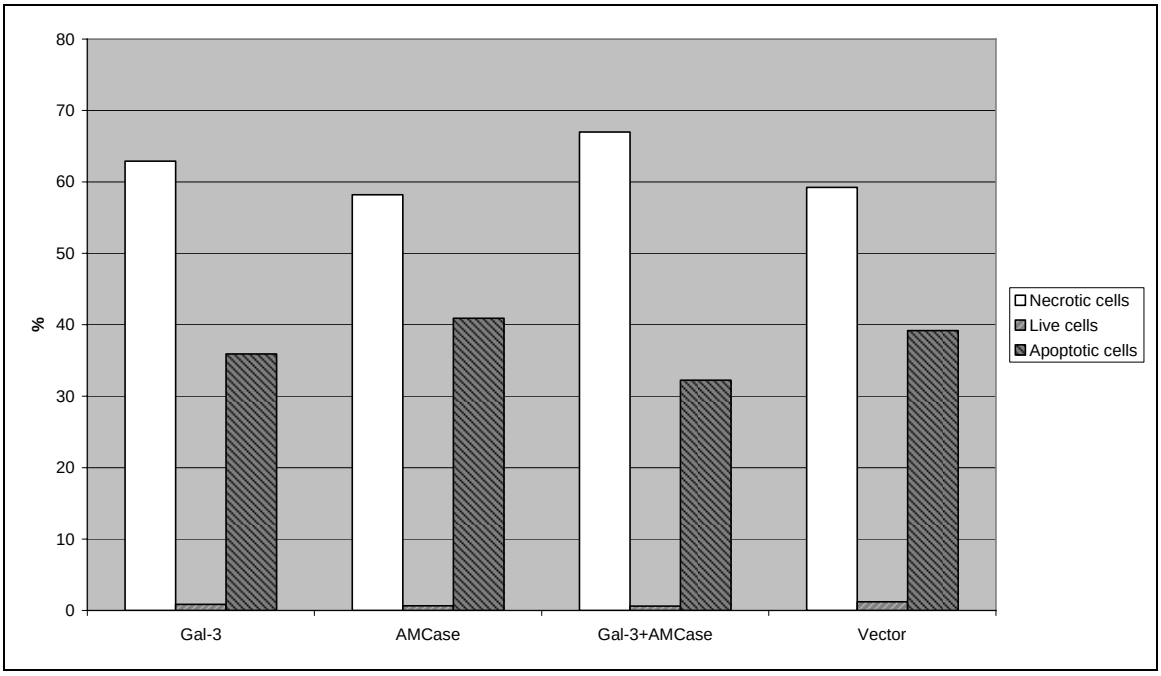


The effect of AMCase on Galectin-3 in staurosporine induced apoptosis of Jurkat T cells

Having demonstrated that AMCase and Galectin-3 physically interact and co-localize *in vitro*, the effect of AMCase on Galectin-3 modulation of apoptosis was then investigated in the Jurkat T cell apoptosis assay. Staurosporine, a broad-spectrum protein kinase inhibitor induces apoptosis through the mitochondrial apoptosis pathway [46], was

incubated with Jurkat T cells which had been electroporated with AMCase, Galectin-3, both or empty vector; DNA content was equalized with empty vector. Cells were incubated with staurosporine for 6 hours, and stained with Annexin V/PI. Apoptosis was assessed by FACS. The results of the pilot experiment are demonstrated in **Figure 4.**

Figure 4: The effect of AMCase and Galectin-3 on staurosporine induced apoptosis in Jurkat T cells.

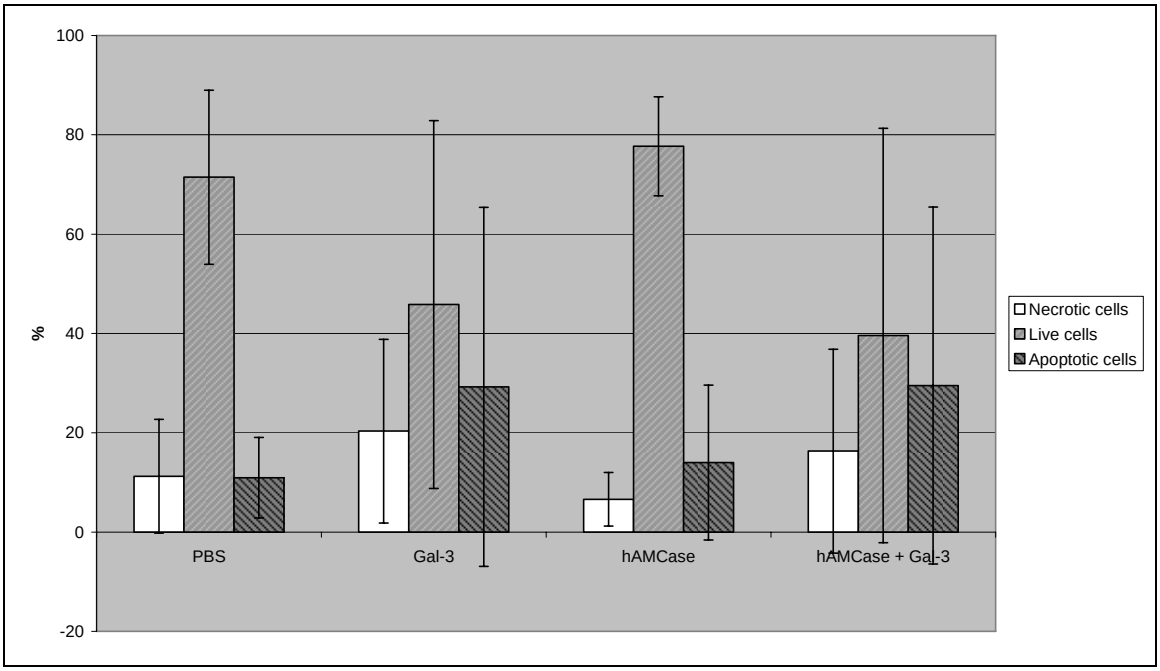


Galectin-3 was observed to be mildly protective against staurosporine-induced apoptosis, consistent with the known anti-apoptotic effect of over-expressed Galectin-3 [44]. AMCase alone showed a greater degree of apoptosis than empty vector alone, however AMCase co-transfected with Galectin-3 demonstrated less apoptosis than either Galectin-3 alone or the empty vector control. These data support our hypothesis that Galectin-3 and AMCase interact and Galectin-3 mediates anti-apoptotic effects of AMCase.

However, the high level of cell death, indicated by the number of necrotic cells (greater than 50% in all samples) is a known effect of electroporation we concluded that a different experimental approach was required to accurately characterize the effect of AMCase on apoptosis.

As an alternative means to evaluate apoptosis, we next studied the induction of apoptosis by recombinant Galectin-3. Un-transfected Jurkat T cells were incubated with recombinant Galectin-3, recombinant AMCase, both, or negative control (PBS) for 6 hours as described in ***Methods***. Samples were stained with Annexin V/PI to detect apoptosis, and analyzed by FACS. All assays were performed in triplicate. **Figure 5** presents the average data and standard deviation for three independent experiments.

Figure 5: Annexin V/PI staining of Jurkat T cells incubated with recombinant protein.



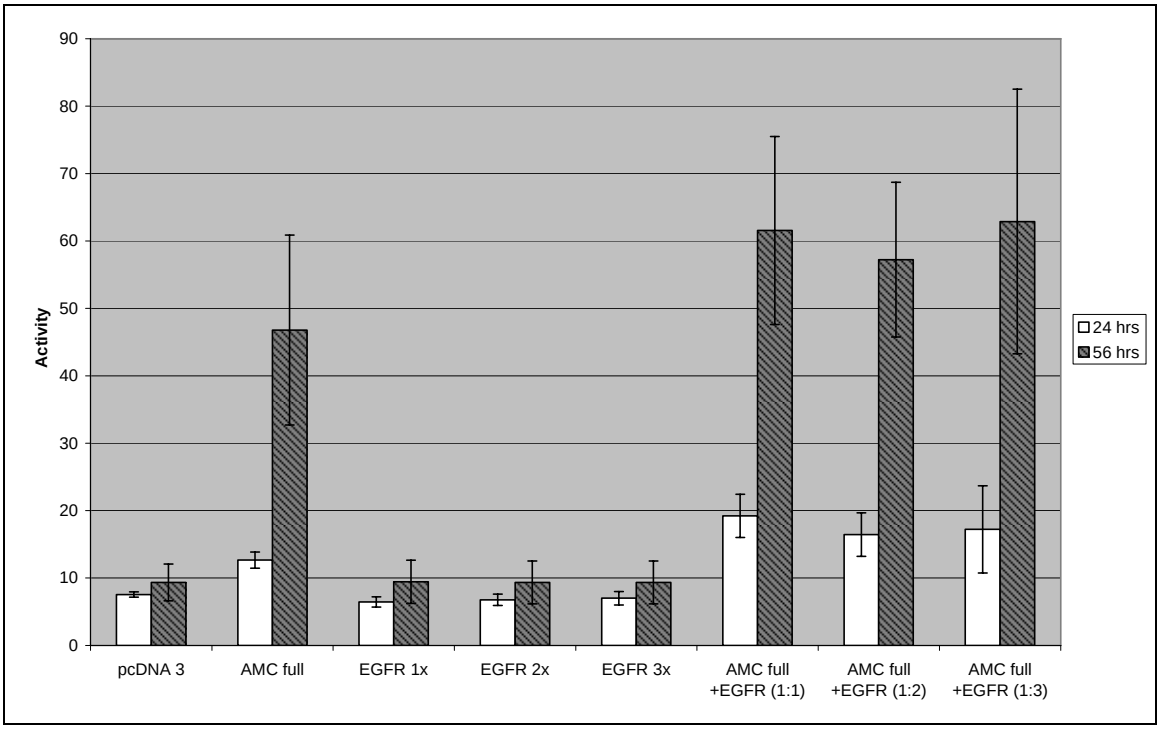
As seen in **Figure 5**, recombinant Galectin-3 induced apoptosis in Jurkat T cells, consistent with previous studies [45]. Incubation with recombinant AMCase produced levels of apoptosis similar to the negative control. However, co-incubation with exogenous AMCase and Galectin-3 did not block Galectin-3 mediated apoptosis in this system.

AMCase chitinase activity and EGFR

AMCase has been shown to demonstrate functional chitinase activity *in vitro* [13]. Previous studies in our lab demonstrated elevation of chitinase activity with co-expression of AMCase and EGFR. In order to confirm this finding and to validate the assay method, A549 cells were transfected with either full-length AMCase (2 μg), EGFR (2 μg, 4 μg or 6 μg) plus empty vector to equalize amounts of DNA, or co-transfected with full-length AMCase (2 μg) and EGFR (2 μg, 4 μg or 6 μg). Empty pcDNA3 vector was used as a negative control. All transfections were done in triplicate. Chitinase activity was measured at 24 and 56 hours. **Figure 6** presents average data and standard deviation from three independent experiments.

As shown in **Figure 6**, co-transfection of AMCase and EGFR caused a marked increase in chitinase activity when compared to AMCase alone ($p=0.03$ at 24 hour time point by Student's T test); this effect did not show a dose-dependent response to the amount of EGFR transfected.

Figure 6: Chitinase activity in transfected A549 cells at 24 and 56 hours.



Cloning of polymorphisms of AMCase.

Human AMCase exists endogenously as both full-length and C-terminally truncated forms. Numerous genetic variants of AMCase including multiple SNPs have been identified in the literature. The SNPs of interest in this study are shown in **Figure 7**. Human AMCase consists of a catalytic domain (amino acids 1-391) which includes the N-terminal signal peptide (amino acids 1-21, double underline), hinge domain (amino acids 392-428, dotted underline) and chitin binding domain (amino acids 429-476, single underline). Start codons for full-length (amino acid 1), long-form (amino acid 109) and short-form AMCase (amino acid 162) are indicated by bold text. SNPs of interest are

highlighted in gray (Exon 5, base pair 477, A to G; Exon 9, base pair 1118, A to G; Exon 10, base pair 1164, T to C; Exon 11, base pair 1398, T to G).

Figure 7: Sequence of human AMCase cDNA (AF290004).

```
1    gctttccagtctggtggtgaatcctccatagtctgaagcctttgtgataaccacagaatca 61

62   gaacatataaaaagctctgcgggactggtgctgactgcaaccatgacaaagcttattctc  121
 1                                              M  T  K  L  I  L    6

122  ctcacaggtcttgtccttatactgaatttgcagctcggctctgcctaccagctgacatgc  181
7     L  T  G  L  V  L  I  L  N  L  Q  L  G  S  A  Y  Q  L  T  C   26

182  tacttcaccaactgggcccagtaccggccaggcctggggcgcttcatgcctgacaacatc  241
27    Y  F  T  N  W  A  Q  Y  R  P  G  L  G  R  F  M  P  D  N  I   46

242  gacccctgcctctgtacccacctgatctacgcctttgctgggaggcagaacaacgagatc  301
47    D  P  C  L  C  T  H  L  I  Y  A  F  A  G  R  Q  N  N  E  I   66

302  accaccatcgaatggaacgatgtgactctctaccaagctttcaatggcctgaaaaataag  361
67    T  T  I  E  W  N  D  V  T  L  Y  Q  A  F  N  G  L  K  N  K   86

362  aacagccagctgaaaactctcctggccattggaggctggaacttcgggactgcccctttc  421
87    N  S  Q  L  K  T  L  L  A  I  G  G  W  N  F  G  T  A  P  F  106

422  actgcc**atg**gtttctactcctgagaaccgccagactttcatcacctcagtcatcaaattc  481
107   T  A  **M**  V  S  T  P  E  N  R  Q  T  F  I  T  S  V  I  K  F  126

482  ctgcgccagtatgagtttgacgggctggactttgactgggagtaccctggctctcgtggg  541
127   L  R  Q  Y  E  F  D  G  L  D  F  D  W  E  Y  P  G  S  R  G  146

542  agccctcctcaggacaagcatctcttcactgtcctggtgcaggaaatgcgtgaagctttt  601
147   S  P  P  Q  D  K  H  L  F  T  V  L  V  Q  E  **M**  R  E  A  F  166

602  gagcaggaggccaagcagatcaacaagcccaggctgatggtcactgctgcagtagctgct  661
167   E  Q  E  A  K  Q  I  N  K  P  R  L  M  V  T  A  A  V  A  A  186

662  ggcatctccaatatccagtctggctatgagatcccccaactgtcacagtacctggactac  721
187   G  I  S  N  I  Q  S  G  Y  E  I  P  Q  L  S  Q  Y  L  D  Y  206

722  atccatgtcatgacctacgacctccatggctcctgggagggctacactggagagaacagc  781
207   I  H  V  M  T  Y  D  L  H  G  S  W  E  G  Y  T  G  E  N  S  226

782  cccctctacaaatacccgactgacaccggcagcaacgcctacctcaatgtggattatgtc  841
227   P  L  Y  K  Y  P  T  D  T  G  S  N  A  Y  L  N  V  D  Y  V  246

842  atgaactactggaaggacaatggagcaccagctgagaagctcatcgttggattccctacc  901
247   M  N  Y  W  K  D  N  G  A  P  A  E  K  L  I  V  G  F  P  T  266

902  tatggacacaacttcatcctgagcaacccctccaacactggaattggtgcccccacctct  961
267   Y  G  H  N  F  I  L  S  N  P  S  N  T  G  I  G  A  P  T  S  286
```

```
962  ggtgctggtcctgctgggccctatgccaaggagtctgggatctgggcttactacgagatc  1021
287   G  A  G  P  A  G  P  Y  A  K  E  S  G  I  W  A  Y  Y  E  I   306

1022 tgtaccttcctgaaaaatggagccactcagggatgggatgcccctcaggaagtgccttat  1081
307   C  T  F  L  K  N  G  A  T  Q  G  W  D  A  P  Q  E  V  P  Y   326

1082 gcctatcagggcaatgtgtgggttggctatgacaacatcaagagcttcgatattaaggct  1141
327   A  Y  Q  G  N  V  W  V  G  Y  D  N  I  K  S  F  D  I  K  A   346

1142 caatggcttaagcacaacaaattggaggcgccatggtctgggccattgatctggatgac  1201
347   Q  W  L  K  H  N  K  F  G  G  A  M  V  W  A  I  D  L  D  D   366

1202 ttcactggcactttctgcaaccagggcaagtttcccctaatctccaccctgaagaaggcc  1261
367   F  T  G  T  F  C  N  Q  G  K  F  P  L  I  S  T  L  K  K  A   386

1262 ctcggcctgcagagtgcaagttgcacggctccagctcagcccattgagccaataactgct  1321
387   L  G  L  Q  S  A  S  C  T  A  P  A  Q  P  I  E  P  I  T  A   406

1322 gctcccagtggcagcgggaacgggagcgggagtagcagctctggaggcagctcgggaggc  1381
407   A  P  S  G  S  G  N  G  S  G  S  S  S  S  G  G  S  S  G  G   426

1382 agtggattctgtgctgtcagagccaacggcctctaccccgtggcaaataacagaaatgcc  1441
427   S  G  F  C  A  V  R  A  N  G  L  Y  P  V  A  N  N  R  N  A   446

1442 ttctggcactgcgtgaatggagtcacgtaccagcagaactgccaggccgggcttgtcttc  1501
447   F  W  H  C  V  N  G  V  T  Y  Q  Q  N  C  Q  A  G  L  V  F   466

1502 gacaccagctgtgattgctgcaactgggcataaacctgacctggtctatattccctagag  1561
467   D  T  S  C  D  C  C  N  W  A                                  476

1562 ttccagtctcttttgcttaggacatgttgcccctacctaaagtcctgcaataaaatcagc  1621

1622 agtc                                                          1625
```

Long-form AMCase with sequence identical to wildtype AMCase (AF290004) was obtained from Open Biosystems. SNPs associated with asthma were identified in the primary literature [40] (see **Table 1**) and by our group (Elias lab, unpublished data), as noted above. Primers were designed to introduce four of these SNPs in exon 5, 9, 10 and 11 (see **Table 2**). Site-directed mutagenesis was performed to generate these constructs.

Table 2: Primers used in site-directed mutagenesis of long-form AMCase (Open Biosystems).

SNP	Exon	BP#	WT Base	SNP Base	Primers
--	5	477	A	G	Forward: 5’-catcacctcagtcatcagattcctgcgccagtatg-3’ Reverse: 5’-catactggcgcaggaatctgatgactgaggtgatg-3’
Rs2275253	9	1118	A	G	Forward: 5'-gtgggttggctatgacaacgtcaagagcttcgatattaa-3' Reverse: 5'-ttaatatcgaagctcttgacgttgtcatagccaacccac-3'
Rs2275254	10	1164	T	C	Forward: 5'-tcaatggcttaagcacaacaaatctggaggcgccat-3' Reverse: 5'-atggcgcctccagatttgttgtgcttaagccattga-3'
Rs2256721	11	1398	T	G	Forward: 5'-tggattctgtgctggcagagccaacggcc-3' Reverse: 5'-ggccgttggctctgccagcacagaatcca-3'

Constructs were sequenced after each round of mutagenesis to identify clones containing the desired sequence of base pairs. Constructs developed included the single-nucleotide polymorphisms identified as significantly correlated with asthma [40] as well as various combinations of SNPs (**Table 3**).

Table 3: Long-form AMCase constructs containing combinations of SNPs correlated with asthma.

Construct	Exon 5 SNP	Exon 9 SNP	Exon 10 SNP	Exon 11 SNP
Wildtype				
1	+			
2		+		
3			+	
4				+
5		+	+	
6		+		+
7			+	+

Full-length AMCase acquired from MedImmune was sequenced and found to contain nine SNPs (**Table 4**). Three of these SNPs (numbered 6, 7 and 9 in **Table 4**) were previously identified as associated with asthma [40]. Two of these SNPs (numbered 5 and 8 in **Table 4**) consisted of silent mutations in the catalytic domain, though neither of these two synonymous mutations have been identified in the current literature as clinically associated with asthma. The remaining four SNPs (numbered 1, 2, 3, and 4 in

Table 4) were located in the signal peptide domain of AMCase, and were of undetermined clinical significance.

Table 4: Single-nucleotide polymorphisms identified in full-length AMCase clone (MedImmune) by sequencing.

	SNP	**Domain**	**Exon**	**BP#**	**WT Base**	**SNP Base**	**WT AA**	**New AA**
1	--	SP	--		A	G	AAC (N)	GAC (D)
2	--	SP	--		G	A	GAC (D)	AAC (N)
3	--	SP	--		G	T	AGG (R)	ATG (M)
4	--	SP	--		C	T	AAC (N)	AAT (N)
5	--	Catalytic	9	1099	G	A	GTG (V)	GTA (V)
6	Rs2275253	Catalytic	9*	1118	A	G	ATC (I)	GTC (V)
7	Rs2275254	Catalytic	10*	1164	T	C	TTT (F)	TCT (S)
8	--	Catalytic	10	1268	C	T	GGC (G)	GGT (G)

9	Rs2256721	CBD	11*	1398	T	G	GTC (V)	GGC (G)

SNPs in the signal peptide domain of full-length AMCase were corrected in a stepwise manner to generate wildtype sequence using point mutagenesis as described in the ***Methods*** section above. Subsequently, constructs containing the desired clinically-relevant SNPs (designated by an asterix in **Table 4**) were generated by sequential correction via point mutagenesis. Primers used for mutagenesis are listed in **Table 5**; SNPs associated with asthma are designated with an asterix.

Table 5: Primers used in site-directed mutagenesis of full-length AMCase (MedImmune).

	SNP	**Exon**	**BP#**	**WT Base**	**SNP Base**	**Primers**
1	--	--		A	G	Forward: 5'-cttcatgcctgacaacatcgacccctgcctctg-3' Reverse: 5'-cagaggcaggggtcgatgttgtcaggcatgaag-3'
2	--	--		G	A	
3	--	--		G	T	Forward: 5'-cgcctttgctgggaggcagaacaacgag-3' Reverse: 5'-ctcgttgttctgcctcccagcaaaggcg-3'
4	--	--		C	T	Forward: 5'-ccaccatcgaatggaacgatgtgactctctacc-3' Reverse:

						5’-ggtagagagtcacatcgttccattcgatggtgg-3’
5		5*	477	A	G	Forward: 5’-catcacctcagtcatcagattcctgcgccagtatg-3’ Reverse: 5’-catactggcgcaggaatctgatgactgaggtgatg-3’
6	--	9	1099	G	A	Forward: 5'-ccttatgcctatcagggcaatgtgtgggttggctat-3' Reverse: 5'-atagccaacccacacattgccctgataggcataagg-3'
7	Rs2275253	9*	1118	A	G	Forward: 5’-tatgggttggctatgacaacatcaagagcttcgatattaag-3’ Reverse: 5’-cttaatatcgaagctcttgatgttgtcatagccaacccata-3’
8	Rs2275254	10*	1164	T	C	Forward: 5'-tcaatggcttaagcacaacaaatttggaggcgccat-3' Reverse: 5'-atggcgcctccaaatttgttgtgcttaagccattga-3'
9	--	10	1268	C	T	Forward: 5'-gaaggccctcggcctgcagagtgcaag-3' Reverse: 5'-cttgcactctgcaggccgagggccttc-3'
10	Rs2256721	11*	1398	T	G	Forward: 5'-tggattctgtgctgtcagagccaacggcc-3' Reverse: 5'-ggccgttggctctgacagcacagaatcca-3'

Constructs were sequenced after each round of mutagenesis to identify clones containing the desired sequence of base pairs. The two silent mutations in the catalytic domain were

not altered due to low potential for changes in enzyme activity or expression; all constructs generated contained these two point mutations, making the backgrounds of all constructs comparable.

Discussion

AMCase has been implicated in asthma and other immune-mediated allergic disease based upon studies from animal models of asthma that demonstrate it is involved in the development of Th2 inflammatory responses, as well as population-based association studies that have demonstrated that genetic variation in AMCase is associated with asthma. The effect of genetic mutations in AMCase on its biologic function remains completely unknown.

We hypothesized that human AMCase participates in Th2 inflammation by an EGFR-dependant inhibition of apoptosis mechanism. We theorized that AMCase interacts with Galectin-3 to mediate downstream anti-apoptotic functions of AMCase, and that asthma-associated polymorphisms of human AMCase alter chitinase activity and modulate downstream anti-apoptotic functions of AMCase, increasing Th2-mediated inflammation in human asthma.

In these studies, we have begun to elucidate the biologic effects of AMCase. We have demonstrated that AMCase binds to and co-localizes with Galectin-3 *in vitro*. In an over-expression system, Galectin-3 demonstrated known anti-apoptotic effects [44], while co-transfection of AMCase and Galectin-3 was more protective against apoptosis than either Galectin-3 or empty vector alone. In an exogenous protein assay, recombinant Galectin-3 induced apoptosis consistent with published data [45], while recombinant AMCase stimulation resulted in similar levels of apoptosis as observed in negative controls. These data seem to suggest that while AMCase and Galectin-3 bind and interact, and co-expression appears to increase anti-apoptotic effects, contrary to our hypothesis the anti-apoptotic effects of AMCase may be independent of a direct

interaction with Galectin-3. It is also possible that the over-expression assay is a better model of an endogenous system, while the experimental system in which recombinant protein is added exogenously does not sufficiently resemble *in vivo* conditions. Alternatively, the interaction may be a complex one, mediated by different factors intracellularly as compared with extracellularly.

In this investigation, we also demonstrated that co-transfection of AMCase and EGFR augments chitinase activity in transfected A549 cells. This data is consistent with our hypothesis that AMCase and EGFR interact, and with published data from our lab establishing EGFR as an upstream regulator of AMCase [39]. This raises the intriguing possibility that the downstream functions of EGFR may be mediated by AMCase.

Lastly, in order to further explore the function of AMCase and particularly the role that genetic variants of AMCase play in human asthma, in this study we have developed expression constructs of SNPs associated with clinical asthma. These constructs have great potential as tools with which to study molecular functionality of AMCase, and are further discussed in ***Future directions***, below.

Although it is clear that AMCase plays a significant role in Th2-mediated inflammation in the lung, its exact function is yet to be determined, and contrasting data exists. In a murine model, chitin has been shown to induce eosinophil and basophil recruitment to the lung [47], an effect which seemed to be mediated by leukotriene B4 produced by macrophages. BLT1, the high-affinity receptor for leukotriene B4, has an essential role in CD8+ T-cell recruitment to the lung in allergic asthma and airway hyper-responsiveness [48, 49]. Eosinophil recruitment to the lung was blocked both by pre-treatment with recombinant AMCase, and by depletion of macrophages, contrary to

previous data which seemed to implicate AMCase as an inducer of Th2-mediated inflammation [10]. Taken together, these data suggest a role for chitin as a molecular-recognition element implicated in the innate immune response and recruitment of eosinophils and basophils to the lung. In this context, AMCase seems to act in an anti-inflammatory manner to decrease eosinophil influx, in contrast to findings by our lab that AMCase has a pro-inflammatory function mediated by IL-13 [10]. The causes of this apparent paradox have yet to be delineated, however, some STAT pathways have been shown to have both pro- and anti-inflammatory actions [50].

Future directions

In this study, we have developed the biochemical tools to investigate genetic polymorphisms in AMCase that have been clinically associated with asthma. Our initial data shows that the anti-apoptotic effects of AMCase are not mediated by a simple interaction with Galectin-3. Rather, it is likely that AMCase acts through a complex activation cascade which includes Galectin-3 and other as yet unidentified mediators. In order to further explore the biologic function of AMCase, we have developed constructs expressing SNPs of AMCase associated with asthma in human populations. Our goal is to use these constructs in future experiments to delineate the effects of SNPs and haplotypes of SNPs on chitinase activity and apoptosis.

Chitinase activity is an important characteristic of AMCase. Functional chitinases are enzymatically active against chitin. Their chitinase activity can be measured by using a fluorogenic substrate such as 4-methylumbelliferyl-D-N,N'-diacetylchitobioside (4-MU). Both chitotriosidase and AMCase have been demonstrated to have functional chitinase activity against 4-MU [13, 16]. Our lab has replicated chitinase activity in wildtype AMCase.

Using the AMCase constructs generated in this study, future experiments will seek to quantify chitinase activity in constructs containing polymorphisms associated with asthma as compared to wildtype AMCase. AMCase constructs will be over-expressed in A549 cells, and chitinase activity against 4-MU measured. Cells will be harvested and lysates probed by Western blot to monitor protein expression levels.

In order to further explore the impact of polymorphisms in AMCase, future experiments would investigate whether polymorphic AMCase constructs demonstrate

altered levels of apoptosis as compared to wildtype. Full-length AMCase constructs generated in this study are currently in a bacterial expression vector containing RFP (pDSRed, Clontech), which fluoresces in the same range of wavelengths (excitation 557 nm, emission 579 nm) as the tagged Propidium Iodide (BD Pharmingen; emission spectrum 562-588 nm) which is currently available, precluding the use of Annexin V/PI staining as an assay for apoptosis. To facilitate future experiments using PI, the existing panel of AMCase constructs can be cloned into an alternative expression vector such as pcDNA3 in order to eliminate confounding signal between the RFP and PI. These constructs can then be over-expressed by transfection in A549 cells, and apoptosis induced by incubation with Fas ligand as compared to negative control. Transfected samples can then be evaluated for apoptosis by Annexin V/PI staining.

In order to further investigate the role that polymorphisms in AMCase play in asthma, future experiments should be aimed at generating and biochemically testing haplotypes of AMCase which have been identified as clinically significant in asthma. In their study of German asthmatics, Bierbaum et al identified sixteen haplotypes with a frequency greater than 1% in either the asthmatic or the control population [40]. The five haplotypes with the greatest difference in frequency between asthmatics and healthy controls are shown in **Table 6** below.

Table 6: Haplotyes of AMCase identified in a German population [40]

			Haplotype*				
Polymorphism	**Position**	**Amino Acid exchange**	**1**	**2**	**3**	**4**	**5**

Rs12033184	Promoter	None	wt	wt	**M**	**M**	**M**
Rs3818822	Exon 4	None	**M**	**M**	wt	**M**	**M**
	Exon 5	K17R	wt	wt	wt	Wt	**M**
Rs2275253	Exon 9	I231V	wt	**M**	**M**	Wt	**M**
Rs2275254	Exon 10	F246S	**M**	wt	**M**	**M**	Wt
	Exon 10	F269S	wt	wt	wt	Wt	Wt
Rs2256721	Exon 11	V324G	**M**	wt	wt	**M**	Wt

*wt= wildtype allele, **M**=mutant allele

Of these five haplotypes, two (Haplotypes 2 and 4) are positively associated with asthma, occurring with greater frequency in asthmatic populations as compared with healthy controls. This difference is particularly marked for pediatric asthmatics as compared with healthy children, as shown in **Table 7.** The remaining three haplotypes are negatively associated with asthma, occurring with greater frequency in control subjects.

Table 7: AMCase haplotype frequency in asthmatics compared with controls [40]

Haplotype	**Asthma**	**Control Subjects**		**Frequency Difference**	
		Adults	**Children**	**Adults**	**Children**
1	0.105921	0.109709	0.166181	-0.00379	-0.06026
2*	0.232742	0.194970	0.182000	0.037772	0.050742
3	0.073852	0.085775	0.102604	-0.011920	-0.02875
4*	0.146976	0.102581	0.076349	0.044395	0.070627
5	0.099457	0.139256	0.142457	-0.039800	-0.043000

*Haplotype with greater frequency in asthmatics as compared with healthy controls

Our goal is to generate the haplotypes of AMCase that appear with greater frequency in the asthmatic population, and to investigate these haplotypes *in vitro* for biochemical and functional activity, including chitinase activity and evaluation of apoptosis.

Summary

Chitin is a ubiquitous environmental allergen known to have a role as a recognition element for some host responses. Plant and fungal chitinases serve as defense mechanisms against parasitic infection. AMCase and chitotriosidase, two recently discovered mammalian chitinases, are likewise hypothesized to function in innate immunity. AMCase has been implicated in Th2 cell-mediated inflammation, which is classically considered to be the branch of the immune system involved in both parasitic infection and the development of atopic and asthmatic disease. Several lines of evidence have linked AMCase to clinical asthma, and SNPs of AMCase have been associated with development of asthmatic disease in human populations. In this study, we have shown that AMCase is stimulated by co-transfection EGFR, that it inhibits apoptosis, and that it binds and co-localizes with Galectin-3 *in vitro*. However, contrary to our hypothesis, the anti-apoptotic effects of AMCase do not seem to be mediated by a direct interaction with Galectin-3. We have also developed the molecular tools to explore the effect of genetic polymorphisms have on the function of AMCase. Future experiments will be focused on delineating the biochemical activity of polymorphic AMCase, to help shed light on this important mediator of inflammatory asthma.

Appendix: SNPs in AMCase in African American populations [41]

Table 1. Non-synonymous AMCase SNPs

Variants	Alleles	AA Change	Position	Allele	Frequency of Noted Allele		
					MX	PR	AA
SNP1+236	A/G	Asn/Asp	Exon 4	G	0.09	0.135	0.26
SNP2+242	G/A	Asp/Asn	Exon 4	A	0.09	0.135	0.26
SNP3+285	G/T	Arg/Met	Exon 4	T	0.09	0.135	0.26
SNP4+407	G/A	Gly/Arg	Exon 5	A	0.035	0.1	0.15
SNP5+477	G/A	Arg/Lys	Exon 6	G	0.06	0.1	0.05
SNP6+1118	A/G	Ile/Val	Exon 10	G	0.36	0.55	0.79
SNP7+1164	C/T	Ser/Phe	Exon 11	C	0.30	0.39	0.39
SNP8+1398	T/G	Val/Gly	Exon 12	G	0.35	0.54	0.78

Table 2. Results of African American Case-Control Analysis for Asthma and Atopic Asthma Disease Status

SNP#	Allele	Frequency (%)			Odds Ratio (95% CI)		χ^2 p Value	
		Cases (n=352)	Atopic Cases (n=202)	Controls (n=352)	Asthma	Atopic Asthma	Asthma	Atopic Asthma
SNP3	T	17.0	16.2	25.9	.59/(.41,.85)	.55/(.35,.87)	0.004	0.009
SNP4	A	16.1	17.8	14.5	1.05	1.27	0.83	0.30
SNP5	G	4.9	4.6	4.5	1.07	0.99	0.85	0.98
SNP6	A	22.5	20.6	20.7	0.90	0.99	0.56	0.95
SNP7	C	32.6	30.4	39.2	0.75	.67/(.47,.98)	0.08	0.04
SNP8	T	23.4	21.5	21.7	1.10	0.93	0.59	0.95

Table 3. Allelic and Genotypic Association test for AMCase SNPs with Bronchodilator Response (Delta-FEV_1) in African Americans

Allelic Regression			Genotypic Regression	
SNP	Cases (n=352)	Atopic Cases (n=202)	Cases (n=176)	Atopic Cases (n=101)
	p value	p value	p value	p value
SNP3	0.887	0.718	0.863	0.902
SNP4	0.852	0.385	0.879	0.417
SNP5	0.052	0.503	0.051	0.491
SNP6	0.018	0.012	0.025	0.013
SNP7	0.403	0.342	0.316	0.343
SNP8	0.009	0.014	0.017	0.016

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