IMMUNOLOGICAL INSIGHTS FROM PRIMATE EOSINOPHIL MAJOR BASIC PROTEIN'S EVOLUTIONARY HISTORY

by

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ABSTRACT

The Red Queen Hypothesis states that hosts must constantly adapt to survive in the face of rapidly evolving pathogens. This cycle of hosts adapting against pathogens and pathogens adapting against host adaptions is referred to as an evolutionary arms race. It is possible to find evidence of these conflicts in the combatants' DNA, where these markers of rapid evolution can help us better understand the role of the proteins involved.

One such protein is Major Basic Protein (MBP). MBP is a cytotoxic protein made by a type of white blood cell called the eosinophil. Eosinophils act as both effectors and regulators of innate immunity, though scientists debate which of these functions is their primary role in host defense. Different studies have found contradicting evidence for the role of eosinophils in both infective and allergic diseases. Some studies identify eosinophils as causative agents of diseases such as asthma, while others find that they are unnecessary for the development of asthma symptoms. Similarly, separate studies have found that eosinophils kill certain parasites, while they are necessary for the survival of others. Therefore, more research needs to be done to elucidate the primary immunological function of eosinophils.

In this study, I show that Major Basic Protein has been involved in an evolutionary arms race throughout the primate lineage. This result provides further evidence that eosinophils' destructive effector functions are important to host health. Additionally, it hints that there may be undiscovered pathogen proteins which antagonize MBP and the other cytotoxic proteins made by eosinophils, opening new avenues for investigation.

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INTRODUCTION

When faced with an invader, the body uses two major systems to defend itself. The slower and more specific of these is the adaptive immune system. This immune response creates antibodies which combat specific pathogens, however, the process takes a significant amount of time. White blood cells search for invaders by checking the antigens present on cells' surfaces. Host cells present "self" antigens, which tell the white blood cells not to attack. When a white blood cell recognizes a non-self antigen from an invading cell, it kills the cell and passes the antigen down a line of immune cells to memory B cells which begin producing antibodies to fight the infection. In this way, the adaptive immune system provides the body with a powerful, yet extremely specific defense against pathogens[1]. The other, more immediate system of immunity is the innate immune system. This nonspecific response acts almost immediately, drawing white blood cells to the site of infection, stimulating the adaptive immune response, and releasing chemicals which hinder the pathogen and potentially damage nearby host cells[2]. If the adaptive immune response represents the highly specific, scalpel-like strategy to host defense, the innate immune response is the hand grenade approach.

While both the adaptive and innate immune systems are integral to host health, both have the potential to seriously damage host tissues. Autoimmune conditions are diseases where the immune system attacks healthy cells, either by mistake or as collateral damage while combatting a pathogen. Scientists have proposed several mechanisms by which the adaptive immune system mistakes self-antigens for pathogenic antigens. In the molecular mimicry model, white blood cells pick up viral antigens which closely resemble self-antigens, leading to antibodies which attack both infected and uninfected

host cells[3]. In the bystander damage model, white blood cells release toxic compounds onto an infected cell, killing uninfected cells around it. The antigens from the uninfected cells are then mistaken as pathogenic antigens, and the adaptive immune system creates antibodies to attack healthy cells[4]. This process is initiated by the innate immune system, whose job is to impede an infection by releasing nonspecific toxic compounds at the site of infection[5]. With these toxic compounds, the innate immune system can cause significant damage on its own[6]. Proteins released during innate immune responses are at least partially responsible for diseases such as asthma.

The symptoms of asthma begin when antibodies recognize allergens inside an asthma patient's airway. This sets off a cascade of events, leading to airway inflammation. The airway walls begin to thicken, and mucus-producing cells grow larger, secreting more, thicker mucus. These symptoms restrict the size of the airway, causing the wheezing and difficulty breathing which are hallmarks of an asthma attack. Inflammation leads to the recruitment of immune cells that react as if the body were being invaded by a pathogen. Granulated immune cells accumulate in the airway sputum where they release cationic proteins in an effort to stop the nonexistent invasion, damaging airway epithelial cells and further exacerbating the symptoms of asthma[7, 8].

One such immune cell is the eosinophil granulocyte. These white blood cells are important effectors in innate immunity, and are found at elevated levels in the sputum of asthma patients, especially during an asthma attack[9]. Eosinophils are filled with protein granules – small compartments in the cell full of toxic proteins[10]. Eosinophils are capable of delivering these proteins via a number of different mechanisms, though recent research indicates that the most common is a process called "piecemeal degranulation."[11] In this

mechanism of protein secretion, uniquely shaped vesicles named “eosinophil sombrero vesicles” retrieve proteins from the eosinophil granules, then transport them to the cytoplasmic membrane to be released in a process similar to exocytosis. This enables eosinophils to release their potent granule proteins in a more controlled manner than simple degranulation would allow. Eosinophils can also release their granule proteins by attaching them to extracellular DNA traps[12]. In one paper, researchers stimulated eosinophils with lipopolysaccharide from Gram-negative bacteria. In response, the eosinophils burst, releasing mitochondrial DNA covered with granule proteins into the environment. The released DNA formed net-like structures, trapping bacteria and promoting their exposure to the cytotoxic proteins. This mechanism likely evolved to hinder invading cells both mechanically, as the DNA nets form a difficult to traverse obstacle for smaller pathogens, as well as a chemically, from the cytotoxic granule proteins. Thus, eosinophil DNA nets help prevent pathogens from spreading beyond the initial site of infection. Finally, eosinophil granules can act completely independently when released from the cell. The surface of eosinophil granules are covered in receptors for various cytokines and interleukins[13]. While researchers initially believed these receptors only served to regulate the movement of granule proteins to the cytoplasmic membrane, additional research has found that the receptors remain functional when released during cell lysis. Upon studying these extracellular granules researchers found that, not only do they continue releasing their contents, but they also become sensitive to signaling molecules in the environment, releasing different proteins in response to different signals[14]. In this way, eosinophils can act as immunological “cluster bombs,”

drastically amplifying their antibiotic effect while preventing the excess tissue damage which would occur through the unregulated release of granule proteins.

Eosinophils' overall function in immunity, however, is still somewhat controversial. Upon discovering the cytotoxic nature of their granule proteins, researchers in the 1980's concluded that Eosinophils had evolved to defend the body against multicellular parasites which are too large for other white blood cells to phagocytize[15]. While eosinophil granule proteins are capable of killing certain helminths *in vitro*, there are a number of issues with this hypothesis. In their review of Eosinophil research, Lee *et al* note that parasitic helminths are an ancient group of organisms, thus Eosinophils should have evolved very early in the development of the Kingdom Animalia. Eosinophils are only present in the vertebrates, however, indicating that they did not arise exclusively to combat helminth parasites[16]. The effect of eosinophils on helminth parasites also seems to vary between different species of worms. In some cases they actively kill the parasite, while in others such as *T. spiralis*, eosinophils are necessary for the parasite's survival[17, 18]. In these cases, eosinophils down-regulate the inflammatory response, allowing the parasite to survive in the host tissue. In some cases, eosinophils may even serve an anti-inflammatory role. One group of researchers created a strain of mice with eosinophils expressing diphtheria toxin receptor protein. By administering diphtheria toxin to the mice, the researchers could kill all of the mice's eosinophils without affecting any other white blood cells. After a long enough period without diphtheria toxin, eosinophil numbers in the mice would recover. The researchers found that, after eliminating eosinophils and challenging the mice with airway allergens, the mice still developed asthma symptoms. However, when they allowed the eosinophils in

the mice to recover, the mice also recovered, no longer showing airway hyperresponsiveness and increased mucus production.

The effect of eosinophils on asthma and allergic diseases is still an open question, but the effect of their granule proteins on bacterial and mammalian cells is much more settled. For both bacteria and host cells, eosinophil proteins are extremely toxic[12]. Major basic protein (MBP) may be one of the most significant of these proteins because it makes up more than half of the granule's contents. MBP is a small (13.8kDa), highly basic protein[19]. It is so basic, in fact, that the eosinophil initially synthesizes it with a highly acidic, 11kDa pro-domain, likely to protect the cell during protein packaging and transport[20]. While MBP's mechanism of toxicity is still unknown, structural and physiological evidence indicates that MBP acts by binding to the cell membrane. Structurally, MBP is similar to a family of proteins called the C-type Lectins. This large family of proteins share a Ca^{2+} dependent carbohydrate binding domain[21]. Interestingly, MBP lacks this Ca^{2+} binding domain, but it is still capable of binding to carbohydrates[22]. In fact, MBP binds to carbohydrates at a site unrelated to the C-type Lectin carbohydrate binding domain[23]. Scientists hypothesize that MBP's ability to bind membrane carbohydrates is a major part of its mechanism of toxicity. They believe that, when MBP binds to a cell, its highly basic nature disrupts the membrane, making it porous and damaging the cell in the process. Additionally, recent research has found that MBP forms amyloid-like oligomeric structures after binding to a cell's membrane[24]. These structures also likely play a part in destabilizing the plasma membrane. In one study supporting the membrane-disruption model of MBP toxicity, researchers added purified MBP to rabbit epithelial cells. They then measured how much current passed through the cells, as a cell

with a damaged membrane would allow more ions to leak through. They found that MBP treated cells conducted much more electricity than a control group, indicating that MBP does increase cell membrane permeability[25].

In the long standing paradigm of eosinophil protein function, MBP is the largest player in asthma of all the eosinophil granule proteins. The literature is conflicting, however, in how much of a role MBP plays in the pathogenesis of asthma. In one study, researchers inserted different eosinophil granule proteins into the airways of crab-eating macaques at varying dosages. When the primates were exposed to asthma-inducing allergens, they found that the individuals who had been dosed with MBP experienced dosage dependent airway hyperresponsiveness, while the other eosinophil granule proteins either had little or no effect on the primates' airways[26]. A related study on human airway epithelial cells showed that MBP initiates airway remodeling similar to the development of asthma[27]. A separate set of studies, however, seem to indicate that MBP is unnecessary for the development of asthma symptoms. In one study, researchers created mice without the MBP gene. When these mice were exposed to asthma-inducing allergens, they developed nearly identical symptoms to mice with the MBP gene. Both types of mice developed airway hyperresponsiveness and both recruited inflammation triggering cells to their airways[28]. Yet another study which produced mice with severely reduced expression of MBP found that the release of MBP is not causatively linked to any pulmonary histopathologies, though it did find a that the release of eosinophil peroxidase (another eosinophil protein) increased the amount of mucin in the airway [29]. Finally, a study comparing the expression levels of MBP, eosinophil cationic protein, and eosinophil peroxidase in the airway with the severity of asthma symptoms found that the

levels of granule proteins were not correlated with symptom severity. They did find, however, that there was higher expression of granule proteins in the airways of patients with asthma than in a healthy control group[30]. Taken together, these papers seem to indicate that, while eosinophil granule proteins including MBP are probably not necessary for the onset of asthma, they can certainly exacerbate the condition.

While it may not play large a role in the development of asthma symptoms, when eosinophils release MBP into the airway in response to an allergen, it undoubtedly damages the host's cells[31]. It seems strange, then, that this kind of double-edged sword would be evolutionarily permissible. Because it is conserved throughout the entire vertebrate lineage, MBP must serve a relatively important function, even though it causes collateral damage to host tissues. The tradeoff between MBP toxicity and host health has been severely under-investigated, however. Especially at the molecular level, there are no studies investigating the ways in which organisms have evolved so that their innate immune system is strong enough to ward off infection, while also protecting themselves from their own cationic proteins.

The tools for such studies certainly exist. Phylogenetic Analysis by Maximum Likelihood (PAML) is a software package developed by Dr. Ziheng Yang at the University of Oxford which allows scientists to investigate large numbers of related genes for interesting evolutionary patterns[32]. One program in the package, CODEML, searches gene sequences for evidence that new, advantageous mutations have recently arisen. This program compares the number of mutations which change the protein's amino acid sequence to those which do not, called the ratio of non-synonymous to synonymous mutations, or the dN/dS ratio, and uses that to determine what kind of

selective pressures the gene is experiencing. A dN/dS ratio less than one indicates that any variants from the gene are dying off, since there are very few mutations which change the chemical character of the protein. This state is called purifying selection, since evolution is selecting organisms with a specific allele. A dN/dS ratio close to one indicates that there are no strong selective pressures acting on the gene. This state is referred to as relaxed selection, since there is little selection acting on the gene. Finally, a dN/dS ratio that is greater than one indicates that natural selection has recently favored novel mutations in the gene in order to combat novel threats from rapidly evolving pathogens. This process is called positive selection.

Positive selection is especially prevalent in immune system proteins. Rapidly breeding pathogens can quickly find new ways to endure, evade, or neutralize host immune processes, so the hosts also begin to evolve rapidly, leading to a molecular arms race. This opens up an interesting method to study immune proteins, as the sites under positive selection are also very likely to have a large effect on the functionality of the protein. In other words, by identifying sites under positive selection, it is possible to identify potentially interesting sites for functional investigations.

In one study using this approach, Dr. Barber of the Elde Lab investigated the evolution of transferrin, a protein which soaks up iron in the blood, preventing pathogens from utilizing it[33]. Because pathogens are under strong selective pressure to pull iron out of transferrin, and hosts are under strong selective pressure to prevent pathogens from doing so, transferrin is evolving rapidly. By examining the DNA sequences of the gene which codes for transferrin in multiple primate species, Dr. Barber was able to identify

which specific sites on the protein are evolving rapidly and test how changes at those sites affected cells' susceptibility to pathogens.

A similar strategy would likely work for a study of MBP. In fact, MBP has even more selective pressures acting on it than transferrin, since it must be toxic enough to kill pathogens and also specific enough to avoid grievously damaging host tissues. These strong selective pressures have likely driven MBP to evolve very rapidly, and seek novel mutations which maximize pathogen toxicity while minimizing damage to host cells. Additionally, because MBP has been influenced by selective pressures from both pathogens and the host, a similar investigation into this protein could uncover exciting new insights into the balance organisms must strike between immune system potency and host health.

METHODS

My goal for this research project is to gain a deeper understanding of the relationship between immune system potency and host tissue damage in MBP. While previous studies of MBP have shown that it is toxic to both host and pathogen cells, few have investigated its role as a double-edged sword of immunity. Most of these studies focus on eosinophils and their role in host tissue damage and only briefly mention MBP, usually in conjunction with the other eosinophil granule proteins. Further, none have studied MBP's positive and negative effects on host health at the amino acid level, likely because they lacked the tools to identify which amino acid changes would have a significant impact on MBP's toxicity without resorting to incredibly time-consuming trial and error. Using techniques from evolutionary biology, I can identify which specific amino acids on the protein are likely to have a large effect on MBP's toxicity to host and/or pathogen cells. I will then be able to test these mutations for their actual impact on MBP's toxicity. With this information, I hope to gain new insights into the ways organisms balance the potency of their innate immune systems against its potential to damage their own tissues.

There are two phases to this research project. The first is an evolutionary analysis of MBP gene sequences from a panel of species representing each major clade of the primates. This analysis will allow me to identify sites on MBP which are under positive

selection and potentially have a large impact on host or pathogen toxicity. In order to perform this analysis, I will use Polymerase Chain Reaction and Gel Electrophoresis to retrieve MBP genes from primate cDNA. I will then insert these genes into *E. coli* using the TOPO cloning vector and amplify the gene by growing up a large amount of transformed bacteria. After putting the bacteria through a Miniprep plasmid purification procedure, I will use GENEWIZ, a professional DNA sequencing company, to sequence the DNA of the MBP gene for each primate species. With this procedure, I can be more confident about the accuracy of my sequence data because each sequence will come directly from a primate's cells. For those primates which I do not have tissue samples, I will retrieve MBP gene sequences from the NCBI gene database. I will then take all of this sequence data and load it into PAML's CODEML package, which will identify whether or not the gene is under positive selection and which specific amino acid residues on the protein are rapidly evolving.

Assuming MBP is under positive selection, the second phase of this research project will consist of toxicity assays on *E. coli* and HeLa cells to determine the functional effects of mutations at rapidly evolving sites on the protein. Hopefully, these assays will not only show which sites make MBP more toxic to bacterial and/or mammalian cells, but also how organisms balance immune system potency with host health at the molecular level. In order to perform these assays, I will first need to purify each of the proteins whose gene sequences I investigated in the evolutionary analysis stage of this project. To purify the proteins, I will cut each of their genes out of the TOPO vector, then use PCR to re-engineer the sequences of DNA so that the coding region of the gene is flanked on both sides by restriction enzyme cut sites. I will then digest the re-

engineered DNA with restriction enzymes and incubate it with a PGEX plasmid that has been digested by the same restriction enzymes. This will allow the sticky ends on the plasmid and gene to line up, and after DNA Ligase treatment, will result in a useable expression plasmid. PGEX, in addition to containing the necessary promoters for protein expression in bacteria, also attaches a Glutathione S-Transferase (GST) tag to the N-terminus of any protein inserted into it, which makes protein purification possible in later steps. The promoter on pGEX is induced by IPTG, so I will be able to turn on the expression of MBP in cells containing the plasmid by adding IPTG to their growth media. Once I successfully insert an MBP sequence into PGEX, I will use the plasmid to transform BL21 *E. coli* cells, a strain specifically engineered for efficient transformation, expression, and lysis. I will grow up a large culture of the BL21 cells, lyse them, then incubate the lysate with microscopic, glutathione (GSH) covered glass beads. GST has an incredibly high affinity for GSH, so the GST tagged MBP will bind tightly to the beads. Once MBP is bound, I will centrifuge the samples. Since the glass beads are much more massive than anything else in the cell lysate, they will readily spin down into a pellet, and I can remove the remaining bacterial proteins from the supernatant, wash MBP off the beads, and cleave the GST tag[34]. Finally, using these purified proteins, I will perform toxicity assays to collect data on the toxicity of MBP. In these assays, I will add a small amount of MBP to a culture of either *E. coli* cells or Eukaryotic HeLa cells. By comparing the amount of cell death in these test cultures to control cultures in which no MBP was added, I can determine how toxic each species' version of MBP is to both bacterial and host cells. Cross referencing this data with the DNA sequences I retrieved in

the first phase of my project will reveal which amino acid changes have significant impacts on MBP’s functionality.

In addition to the mature form of MBP, we also designed our primers to clone the pro-form of the protein from cDNA. While scientists have long assumed that the highly acidic pro-domain of MBP neutralizes the highly basic mature domain of the protein during packaging and transport, this hypothesis has not been thoroughly tested. After a thorough search of the literature, the only experimental evidence I can find that the pro-domain neutralizes MBP is the 1991 study from Barker et al. which I referenced above. This study did not, however, test the efficacy of MBP’s pro-domain in neutralizing its toxicity. In their experiments, Barker et al. showed that adding acidic peptides to MBP reduced its toxicity, not that pro-MBP is less toxic than the mature form. Since we have both the pro-form and the mature-form of human MBP, we will perform killing assays on HeLa cells with the two forms of MBP to determine which is more toxic to eukaryotic cells. This experiment would provide the first experimental evidence whether or not eosinophils initially synthesize MBP with an acidic pro-domain to protect themselves before they have had the chance to sequester the protein inside a granule.

RESULTS

At present, I have completed the evolutionary analysis phase of the project. I retrieved the gene sequences which code for MBP from nineteen different primate species, representing animals from the Hominoids, Old World monkeys, and New World monkeys. After running these DNA sequences through PAML, I can confidently say that MBP is under positive selection. In all of its tests for positive selection, PAML output a p-value of less than 0.0001. This is much lower than most accepted thresholds for statistical significance, meaning that MBP is almost certainly under positive selection. The analysis found strong signs of positive selection in the hominoid, Old Word, and New World monkey lineages (Fig. 1).

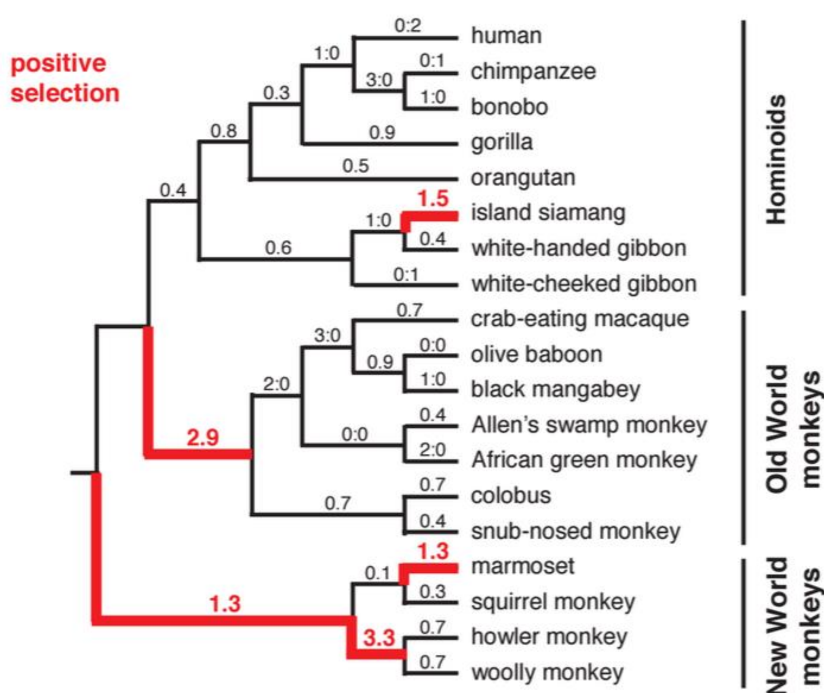

Figure 1. Primate phylogeny showing dN/dS ratios of MBP across each branch. Lineages with a dN/dS ratio greater than 1, suggesting positive selection, are highlighted in red. In cases where there were no non-synonymous or synonymous mutations, ratios of respective substitution numbers are shown.

Additionally, PAML determined that there are nine amino acid residues on MBP which are rapidly evolving. Of these nine, only one is on the pro-domain of the protein. While these sites are spread fairly evenly across MBP's amino acid sequence, when the protein is folded into its 3D conformation, a number of the sites are near its sugar binding domain (Fig. 2). This is a sign that MBP might be engaged in a molecular arms race with pathogens. Scientists theorize that MBP kills cells by binding to their membrane sugars, then using its strong positive charge to destabilize the membrane. Under this model, pathogens with membrane sugars MBP could not bind to would be selected for, and MBP would have to change its sugar binding domain in response. Additionally, one of the sites under positive selection is located in the five amino acid sequence which is predicted to form the spines of amyloid fibers (Fig. 3).

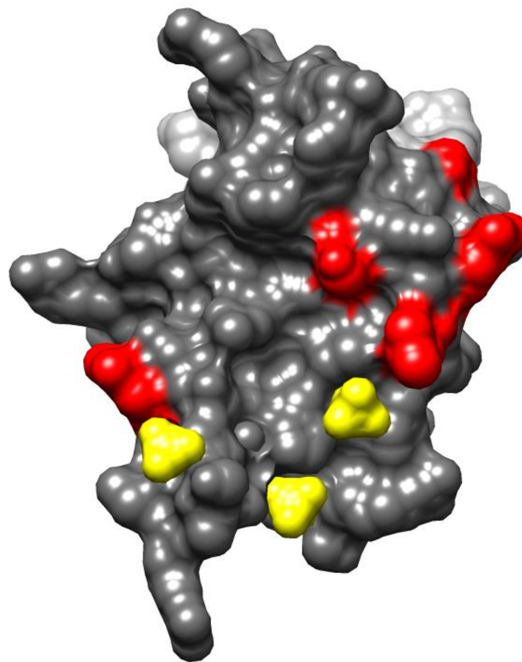

Figure 2. Structure of MBP with sites under positive selection highlighted in red. Bound heparin sulphate, a sugar commonly found on animal cell membranes, is shown in yellow to illustrate that three of the sites under selection are near possible active sites.

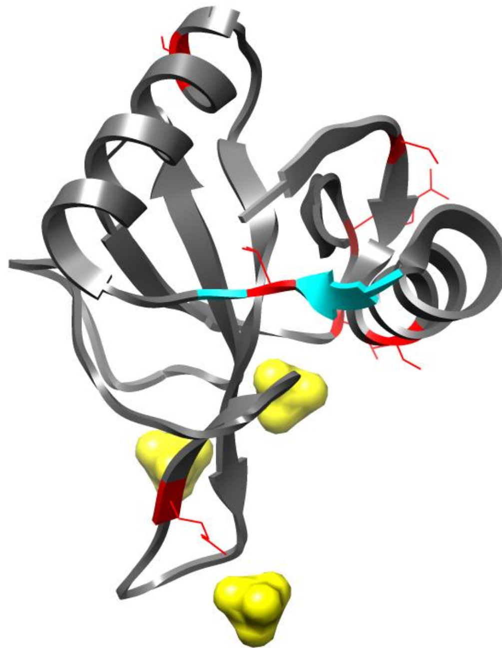

Figure 3. Structure of MBP with sites under positive selection highlighted in red, bound heparin sulphate shown in yellow, and the stretch of amino acids involved in amyloid fiber formation highlighted in blue. One of the residues under positive selection is in the middle of this run of sterically important amino acids.

I have made some progress on the functional assay phase of this project, but I have also run into significant roadblocks. Initially, to make sure our procedure would work, I moved forward with the pro-form and mature form of the human and woolly monkey MBP orthologs. I successfully used PCR to add restriction enzyme cut sites to either end of the MBP genes, then inserted the modified genes into PGEX expression vectors. Next, I used the resulting plasmid to transform *E. coli* cells and begin expressing MBP. Western blots of the transformed cell cultures were promising, indicating that the cells were successfully expressing all four forms of MBP (Fig. 4). Unfortunately, I had significant difficulties in actually purifying MBP from the transformed cell culture. MBP

naturally aggregates *in vivo*, and expressing MBP in bacteria likely exacerbated the problem. After I lysed the bacteria and centrifuged them in order to separate the larger cell components from their soluble proteins, most of the MBP centrifuged down into the pellet with the other, larger cell detritus. MBP monomers would have remained dissolved in the supernatant, but because the MBP molecules bound together into massive aggregates of protein, they centrifuged down and became unavailable to later steps of the purification procedure.

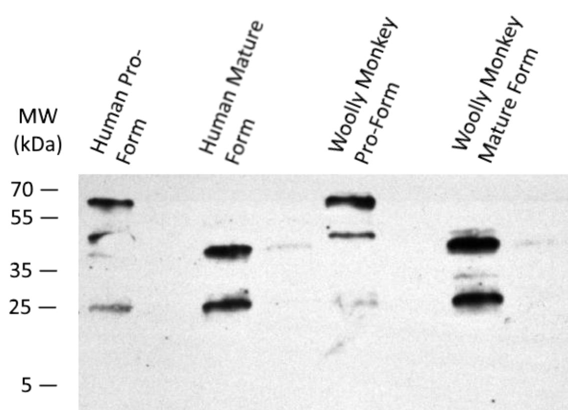

Figure 4. Western blot from cell cultures of bacteria transformed with MBP pGEX vectors. With the 26kDa GST tag, we expected that the pro-form of MBP would be approximately 51kDa, and the mature form would be approximately 40kDa.

To test whether or not the MBP generated by the transformed cells was functional, I performed an assay comparing the growth of cells expressing the pro-form of MBP versus cells expressing the mature form of MBP. I hypothesized that, since the pro-domain should neutralize the cytotoxic effects of MBP, cells expressing mature MBP would grow relatively slowly. I inoculated six cultures of cells with IPTG, three expressing pro-MBP and three expressing mature MBP, then grew them over 22 hours, periodically measuring their OD600 (Fig. 5). Finally, I performed a one-way ANOVA on the OD600 values for each group after 22 hours to determine if the mean OD600's were

not equal. This analysis produced a p-value of 0.282, indicating that there was no statistically significant difference between the OD600's of the two groups.

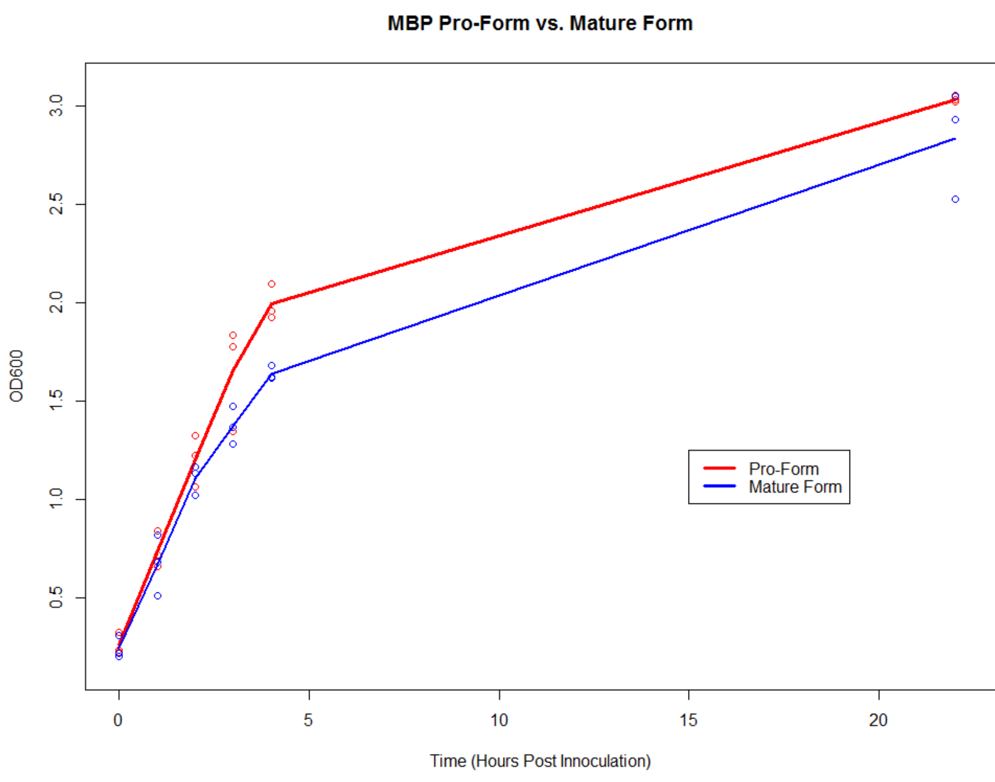

Figure 5. Assay comparing the growth of bacterial cells expressing Human pro-MBP and Human mature MBP. An ANOVA test to determine whether or not the OD600 of the cultures were significantly different after 22 hours returned a p-value of 0.282.

DISCUSSION

The growth assay experiment showed that the growth of bacteria expressing the pro-form of MBP and bacteria expressing the mature form of MBP was not significantly different. This result indicates that the bacteria are not producing functional protein, though there are a few problems with my experimental design that make it difficult to conclude the MBP is non-functional. When eosinophils arrive at the site of an infection, they release MBP out into the environment, where it attaches to the outer surface of the cell membrane. The MBP produced by the bacteria in this experiment stayed inside of the cells, where it may have lacked membrane carbohydrates to bind. This could have prevented otherwise functional mature MBP from inhibiting cell growth by preventing it from localizing to the cell membrane. Intracellular mature MBP would still likely have some effect on cell growth, however. When eosinophils initially synthesize MBP, they attach an acidic pro-domain to the protein even though it is on the inside of the cell membrane. This seems to indicate that mature MBP has the potential to damage cells even if it does not have access to the outer surface of the cellular membrane. The 26kDa GST tag attached to the protein may have further hampered this experiment. Mature MBP is only 13kDa in size, so it is possible that the much larger GST tag sterically inhibited MBP, preventing it from binding to carbohydrates on the inside of the cell membrane. This would also prevent MBP from accumulating on the bacterial membrane, potentially reducing the killing power of the protein. Finally, my experiment lacked a control group of bacteria. It is possible that both the pro-form and mature form of MBP are similarly toxic, so without a control group to compare to the MBP expressing bacteria, it is

impossible to tell whether the MBP the bacteria are producing negatively impacts their growth.

I have likely had so much difficulty purifying MBP because I have been using a Prokaryote to express a volatile Eukaryotic protein. Bacteria face two major hurdles when trying to properly synthesize and fold proteins originating from Eukaryotes. First, bacteria sometimes lack the necessary post-translational machinery to ensure that eukaryotic proteins fold properly[34]. When a protein folds improperly, the cell simply throws it into a pile of other misfolded proteins. This wad of misshapen proteins is called an inclusion body, and was likely the final stop for many molecules of MBP. The second difficulty for bacteria expressing Eukaryotic proteins is simply an overload of the protein. Many expression plasmids, PGEX included, are designed to maximize protein expression. In these cases, the bacteria's post-translational machinery is overloaded by the amount of protein the bacteria are synthesizing. When all of the bacteria's chaperone proteins are in use, but peptides are still being synthesized, much of that protein simply remains unfolded and aggregates into inclusion bodies[35].

Since it is unlikely I will be able to purify MBP from bacteria, future research expanding upon this work with functional studies will need to take a different approach to purify orthologs of MBP. In most papers which use purified MBP for their experiments, the researchers extract the protein from the blood of patients suffering from eosinophilia, a condition where the number of eosinophils in the blood is much higher than normal. Using this method, researchers would be able to retrieve substantial amount of human MBP relatively easily. Since the functional portion of this study relies on comparing the

toxicity of MBP from multiple primates with the human ortholog of MBP, however, a different method of retrieving MBP would be necessary.

Luckily, in a paper from 1995, Popken-Harris et al. successfully purified a recombinant form of MBP using Chinese hamster ovary (CHO) cells[36]. For this project, Popken-Harris and her team inserted the gene encoding MBP into an expression plasmid and transformed this plasmid into the Eukaryotic CHO cells. Unlike a procedure which retrieves MBP from the blood of patients, this procedure could be modified to produce MBP from different primate species. Researchers would simply need to insert the different genes into the expression plasmid during the initial step, which would allow them to express and purify any ortholog of MBP in the CHO cells.

Data from toxicity assays on the various orthologs of MBP would likely reveal one of three results. First, it is possible that there are sites under selection which affect MBP's toxicity to host and pathogen cells, but these sites are mutually exclusive. This possibility would be exciting because of its implications for immunology. Knowing which mutations in MBP could increase its antibacterial activity without increasing host toxicity could lead to future studies of other immune proteins, and possibly inform future drug research. While MBP is most likely not the single causative agent of asthma, it does appear to exacerbate symptoms in people who suffer from asthma. Knowing which sites on MBP most contribute to its host toxicity would allow future researchers to create compounds that inhibit these sites, potentially alleviating some of the more severe asthma phenotypes. This is probably the least likely outcome, however, since natural selection would presumably have already selected for mutations which decrease self-toxicity if they had no ramifications on pathogen toxicity.

Second, it is possible that mutations at the sites under selection in MBP will have a range of effects. Some of the mutations might have a slightly larger effect on pathogen toxicity than host cell toxicity and vice versa. Additionally, many of the mutations would probably have a significant impact on toxicity towards both pathogens and the host, since MBP binds similar molecules on all cell surfaces. These sites, while not necessarily useful for medicine, would nonetheless shed light on how organisms cope with an immunological double-edged sword such as MBP. It would be interesting to see which species have sacrificed the integrity of their own tissues in order to combat pathogens, and which were pushed down a more defensive evolutionary path, i.e. utilizing a less potent ortholog of MBP for the sake of their own cells. This information could give a hint to the kinds of pathogens different species have encountered over the course of their evolutionary histories and the geographic distributions of those pathogens.

Lastly, there is the possibility that none of the sites under selection have any significant effect on MBP’s toxicity to either Prokaryotic or Eukaryotic cells. Even though it is the most negative result, this possibility would potentially be the most interesting. The selective pressure on MBP to bind rapidly evolving pathogen antigens is the simplest explanation for its rapid evolution. A completely negative result, however, would show that changing pathogen carbohydrates are not driving MBP’s evolution. This result would imply that pathogens have other ways to evade the cytotoxic effects of MBP, beyond simply avoiding the protein binding to their membranes.

In fact, the evolutionary data from this study provides some evidence that this may be the case. Often, the molecular arms races which leave signs of positive selection in the genomes of extant species are the result of interactions between immune proteins

made by a host and inhibitory proteins made by a pathogen to neutralize those same immune proteins[37]. If MBP kills pathogens by binding to the carbohydrates on their cell membrane, those pathogens would be under strong selective pressure to block MBP's carbohydrate binding domain, thus preventing the protein from accumulating on the pathogen and killing it. The presence of strong signs of positive selection throughout the primate orthologs of MBP imply that some pathogens may be synthesizing compounds which mimic their carbohydrate motifs. These compounds would occupy MBP's carbohydrate binding domain, effectively neutralizing it in the extracellular space. In turn, this would place strong selective pressure on MBP to change its carbohydrate binding domain so that it could no longer be blocked by inhibitory pathogen compounds. The evolutionary conflict between MBP and these inhibitory proteins would lead to rapid evolution, leaving traces of positive selection in the genome of primates similar to the signals found in this study.

Similarly, the evidence of positive selection in the region of MBP which forms amyloid fibers suggests this region of the protein might also be a target for antagonism. The researchers who identified this region on the protein note that MBP's highly non-specific toxicity resembles amyloid toxins such as Aβ oligomers which kill cells through aggregation. Whether aggregation disrupts the membrane, or helps to localize more MBP to the cell surface, increasing the effect of their strong charge is unclear. A pathogen which could prevent MBP from forming amyloid fibers on its membrane, however, would significantly reduce the protein's ability to disrupt the membrane.

After a thorough review of the literature, however, I can find no known proteins made by pathogens which inhibit MBP. This is not surprising, since most organisms

contain thousands of genes with unknown functions. Nonetheless, these results provide evidence that there may be additional virulence factors in certain pathogens which help them avoid destruction during an inflammatory response. These results hint at an interesting area of research in identifying and describing undiscovered inhibitors of MBP. In addition to deepening our understanding of the mechanisms by which pathogens establish an infection in the body, finding these inhibitory proteins of MBP could reveal new drug targets against pathogens. Drugs which prevent inhibitors of MBP from attaching to the protein could increase the efficiency of the innate immune response, helping the immune system to clear infections more quickly, or keep infections localized more efficiently.

This study also has implications for MBP's role in asthma. The literature indicates that MBP most likely is not necessary for the development of asthma symptoms, however, because of its ability to damage airway epithelial cells, MBP likely exacerbates the symptoms of asthma. The evolutionary data from this study shows that MBP has a very high dN/dS ratio, meaning that selection is favoring new alleles in the protein. This variability in MBP may explain some of the phenotypic variability in asthma patients. The severity of asthma can vary widely in different patients with asthma. If some of the sites on MBP which are variable in human populations lead to an increase in the protein's toxicity, these different alleles may explain a small part of the variability of asthma symptoms.

Finally, this study has implications for the function of eosinophils in the innate immune system. Recent gene knockout studies in mice have contradicted the old paradigm that eosinophils are a causative agent of allergic disease, and that their primary

function is to release toxic proteins at the site of infections, hampering pathogens and causing collateral damage to host tissues. Instead, these newer studies have shown that eosinophils and their proteins are not necessary for the development of allergic diseases such as asthma, and in some cases, seem to be anti-inflammatory. They note that eosinophils reduce the inflammatory response to certain parasites, allowing the parasite to survive in the host tissue, but saving that tissue from damage by the innate immune response. One especially provocative study showed that eosinophils are necessary for the cessation of airway hyperresponsiveness in certain mouse models of asthma. These studies have led scientists to the Local Immune and Remodeling/Repair (LIAR) hypothesis of eosinophil function. In this hypothesis, eosinophils are not nonspecific, destructive end-stage effector cells. Instead, they are regulators of local immunity, promoting tissue homeostasis and repair after inflammation. While this hypothesis does not completely discount eosinophils' role as cytotoxic cells, it does downplay this function. The strong signatures of positive selection in MBP, however, indicate that eosinophils' ability to kill pathogens with their granule proteins is very important to their function. If eosinophils were primarily anti-inflammatory, then we would expect to see relaxed selection in eosinophil granule proteins, since these non-specific, cytotoxic functions are also filled by other granulated cells such as neutrophils. Instead, there are signs that eosinophil granule proteins like MBP have recently been in evolutionary conflict with pathogens. The evolutionary data from this study show that the ability of MBP to kill pathogens has significantly impacted the fitness of its host, leading to rapid evolution of the protein. Thus, while eosinophils almost certainly have anti-inflammatory functions, their role as destructive immune cells should not be neglected.

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