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The Journal of Undergraduate Research (jur) is dedicated to providing the student body with intellectual perspectives from various academic disciplines. jur serves as a forum for the presentation of original research thereby encouraging the pursuit of significant scholarly endeavors.

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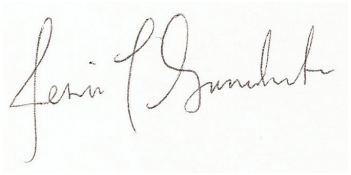
From the Editors

Progress promises a multitude of meanings, and being a decade into the twenty-first century, our society thrives upon advances and improvements. Technology, communication, and information have undergone much change to keep up with the demands of our fast-paced world. In a sense, so too has the extent of undergraduate research evolved.

Over the years of its existence, *jur* has seen an increase in the interest and involvement in scholarly achievement. Our undergraduates demonstrate the limitless bounds of our education and apply our learned knowledge to discover findings of our own. This indicates that progress truly stems at the collegiate level, and our undergraduate research serves as the basis upon which the foundations of scholarly findings and modern advancements are built. We begin to understand the key components, the how and why replication operates. We analyze and draw our own conclusions of political policies. We discern the mechanisms behind signaling pathways. We question the ethics and norms of our society.

jur seeks to produce issues that highlight the extent and importance of undergraduate research. What we provide in the pages of each issue is merely a sampling of laudatory work from our undergraduates who seek to be idealists of all fields.

Sincerely,



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Editors-in-Chief


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table of contents

table of contents

Perspectives on Research

Elizabeth Grayhack, Ph.D.

Assistant Professor, Department of Biochemistry and Biophysics
University of Rochester Medical Center

jur: What are you currently doing in your field of research?

Grayhack: In general, I'm looking at the factors that are inside the protein coding sequence that influence the amount of protein that is made. In particular, I am interested in how the genetic code itself, the synonymous codons that are chosen, affect the amount of protein. I got there in a circuitous way, doing an experiment with Eric Phizicky and Stan Fields in a functional genomics experiment, where we made a library of every yeast gene fused to a purification tag. We did that because we had a set of 6,000 strains, and each one expressed a particular polypeptide. We could use that to assay for a biochemical activity and then know what gene caused it because you could purify every one of those genes by an affinity method, put it in a pool of 100 proteins, assay for a biochemical activity, and get right back to the strain that was responsible for it. So, that was called biochemical genomics. That was where I started making genomic libraries. And then, I made [a genomic library] with Eric and Mark DuMont and Mike Snyder at Yale. The weirdest thing is that you have a thousand-fold difference in expression in this library where you have the same promoter, same terminator, the exact same context, and the only difference is the protein coding sequence. That meant that the protein coding sequence has a lot to do with how much is actually made. We weren't the first people to know that codons affect things. So that's what I study now,; how the actual genetic code that codes the proteins affect how much is made.

jur: Education-wise, how did you become interested in your current field?

Grayhack: I went to an all-female Catholic high school, and at the time that I attended, not that many people went on to college. A few people were going to the University of Illinois, and other people were going on to two-year colleges. My mother had gone off to St. Louis, and she was one of the few women in her generation who had gone to college. She then went to Johns Hopkins to get her bachelor's degree. My dad was a doctor, so my parents were encouraging. I went to a small college in Appleton, Wisconsin, called Lawrence University. From there, I had classes with all of the biochemistry majors. It was amazing. It was great because it was a real confidence builder. I left there, went to Cornell, and worked with Jeff Roberts. Then I worked on bacteriophage *lambda* and the Q protein. I wanted to move on to eukaryotes, but onto something that I can grow quickly. I moved on to work with yeast at the University of California at San Francisco. Because of the combination of the two people I worked for, I was really lucky. Both [mentors] were fantastic and very different. Jeff was extremely smart, very analytical, and really deep. I learned a lot from him. Ira, who died a few years ago of cancer, was very creative and imaginative; so, it was like a study of contrasts. But I just thought I got really lucky that it worked out in both labs I was in. The people there were smart; they were fun. The whole field was also smaller. It was much easier to imagine yourself solving great problems.

jur: Why do you think research is important?

Grayhack: Basic researchers are the prospectors, finding the areas that people who do translational research later on, might in fact find interest in. We contribute [to the fact] that there's information there to be manipulated. It changes the spectrum of drugs available. I think that both types [of research] are very important, but they are fundamentally different. I think there are people whose research is directed at problems that they know impact health, and there are people who are interested in addressing questions that have potential but need to be figured out first. We also, in yeast, develop the tools. An example is the genome sequence–when the genome is sequenced, in a sense, you could say it was a string of As, Gs, Cs, and Ts. You need to figure out how you're going to make use of that information; there's no guidebook. In an organism like yeast (every single functional genomics technique was developed in yeast) and other that are particularly useful have been applied in some way or another to mammalian organisms. The importance of comparing genomes from closely related organisms to find the important signals was first done studying yeast. And then, of course, what are they doing with humans and chimps and everything else? They're using the same techniques to extract information. If you think about my field, which is genomics, last year I think it was, fifty diseases were linked to particular differences and their tendencies to occur in the human genome sequence. Those are not genetic

diseases in the sense that, like cystic fibrosis where a gene causes it; these are really complicated diseases where the tendency to develop the disease is associated with a particular mutation within the genome. I think that's fascinating that in ten or twelve years, we've gone from genome sequencing to being able to do that.

jur: You've mentioned a few changes already, but how else has your field changed over the years, and how has it changed?

Grayhack: A lot. I started out in bacteria and bacteriophage, absolute biochemical, and what I did for my thesis was to purify a protein that had never been purified before to demonstrate its activity in vitro, which would affect gene regulation. I moved out of bacteria and into yeast. I was doing gene regulation in yeast, and then, I moved from there into functional genomics. Now I'm doing work that's between functional genomics and translation. The techniques I've used have changed. I used to do much more biochemistry, and now I do a much stronger component of molecular biology and genetics.

jur: Are there any roadblocks that you have faced?

Grayhack: There are huge roadblocks. There are times when what you're doing doesn't work and you have to rethink yourself. Sometimes you have to go a different way than you might have thought, and find something to do in there that works.

jur: You mentioned earlier that you were part of five chemistry students, was that hard for you?

Grayhack: That was actually easy. The Biology department had a lot more students. I had actually taken college chemistry and never taken any high school chemistry, and when I went into it, the professor said that the last five students who did this failed it. He said that if you get into any trouble and don't understand the lecture, come in and ask me. And I did that. And I understood it all, but that kind of attention was really good. And in graduate school, there was one other woman in my class. So, I wasn't completely alone. I didn't really encounter any sexism in graduate school or as a post doc. I did on the job market—that was the first time—in that somebody said, "Our wives are good biologists, but we husbands have the jobs." I've never quite forgotten that.

jur: Do you have any advice for students who are pursuing something similar to what you are doing?

Grayhack: I think students and all young people should do what they love to do, but the other part to that is that you don't always know what that is. So, in the end, you pick something and try it, and you can't be afraid to pick. And if you pick something that's not right for you, don't be afraid to change. My dad told me that you're going to spend a lot of your life working, so you have to like it.

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Student Review

The master regulator: NF-kappa B

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Nuclear factor kappa B (NF-κB) proteins are a family of transcription factors that have a variety of essential roles in eukaryotic organisms and have been found to play a role in many human diseases. NF-κB was identified as a regulator of expression of the κB light chain in B cells more than two decades ago[1]. The NF-κB family of proteins is composed of transcription factors that have been found to play a part in the control of many normal cellular processes, such as immune and inflammatory response, cellular growth, apoptosis, and developmental processes. Today, research into the function of regulation of the NF-κB family continues with the promise of new insight into human disease. Hyperactivation of these transcription factors has been found in many diseases including cancer, chronic inflammation, asthma and arthritis[2], and mounting research evidence has shown that NF-κB plays a major role in oncogenesis. Immense interest into mechanisms of regulation of NF-κB has arisen of late, which has been further sparked by the continually lengthening list of diseases linked to NF-κB dysfunction.

NF-κB proteins are conserved in a variety of organisms from the fruit fly, to mice, to humans; and have recently been found to occur in simple organisms such as Cnidarians[2]. Various positive and negative regulatory elements exist that are essential for the understanding of NF-κB signaling[1]. Inducing stimuli initiates IKK activation which leads to phosphorylation, ubiquitination, and degradation of IκB proteins[1]. The NF-κB dimers that are released through this process translocate to the nucleus where they bind precise sequences of DNA and upregulate the transcription of target genes[1].

The NF-κB family of closely related transcription factors consists of five genes which give rise to seven proteins that share a Rel Homology Domain (RHD) in their sequence[3]. The RHD contains sequences required for their dimerization and DNA binding and mediates interaction with their specific inhibitors. There are two classes of NF-κB proteins which differ in that one class is synthesized in its mature form and contains a transactivation domain which interacts with the transcriptional apparatus, while the other class is synthesized in its precursor form[4].

NF-κB dimers are found in the cytoplasm of most cells due to their interactions with the inhibitors of NF-κB (IκBs), which prevent nuclear localization and DNA binding. IκB proteins are a family of proteins that contain regions that interact with RHD domains of NF-κB. Through these interactions, IkB proteins primarily regulate the activity of NF-κB[2]. When a cell receives one of many extracellular signals, NF-κB rapidly enters the nucleus and activates targeted gene expression. The NF-κB-IκB interaction that is most well known and studied is that of p50-rel A NF-κB dimer. The interaction of IκB with this dimer prevents NF-κB from binding to DNA and therefore results in NF-κB being maintained in the cytoplasm[2].

Most signals that lead to the activation of NF-κB activate a complex that contains an IκB kinase (IKK), which is serine specific. Once activated, the IKK complex leads to the phosphorylation of two specific serines near the N terminus of

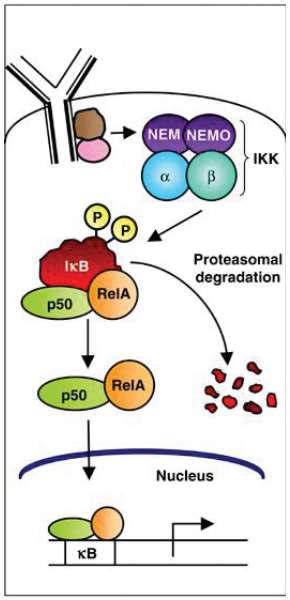

Figure 1. Canonical pathway of NF-κB activation.

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IκB, which makes IκB a target for ubiquitination. Additional pathways have been hypothesized but the shown pathway is by far the most understood. NF-κB that is released as a result of ubiquitination of IκB is now able to enter the nucleus and activate gene expression.

Many exogenous factors lead to the activation of NF-κB. It is a misconception that NF-κB is only activated by "negative" factors. NF-κB regulates many genes important for many biological processes and aging. An example of a human disease that results from NF-κB activation deficiency is incontinentia pigmentosa, an x-linked disorder[5]. The disease is a result of a mutation in NEMO (see Figure 1 to see NEMO's position in the canonical pathway), preventing NF-κB from translocating to the nucleus. The disease is lethal in males (because the mutation prevents activation of NF-kB and males only have one copy of the x-linked gene). In females it may cause skin lesions, alopecia, abnormal teeth and many other symptoms.

A great deal of attention has been given to NF-κB due to its role in regulation of apoptosis and its link to various cancers. The role of NF-κB in apoptosis was first established in 1996 by four independent reports that showed that activation of NF-κB promotes cell survival. These four reports also unanimously showed that downregulation of NF-κB sensitizes the cells to apoptosis[6]. It is speculated that NF-κB may activate genes which suppress cell death by various pathways (mitochondrial and death receptor). NF-κB is also known to induce expression of the Inhibitors of Apoptosis (IAPs) and some members of the Bcl-2 family, which are involved in preventing apoptosis[4]. Other evidence has shown that NF-κB can inhibit or activate apoptotic cell death, depending on various conditions in the cell, such as levels of RelA and c-Rel, which has further complicated the issue[4]. Research has also shown that NF-κB activation also promotes survival of tumor cells and is involved in tumor cell metastasis.

Abnormal activation of the NF-κB pathway has been shown to be a contributing factor to asthma, atherosclerosis, AIDS, muscular dystrophy, heart disease, Alzheimer's disease, and many other human diseases. In addition, studies have also shown that various carcinogens and tumor promoters activate NF-κB. For example, UV radiation has been shown to cause cutaneous inflammation sunburn reactions (characterized by swelling, leukocyte infiltration, and accumulation of proinflammatory cytokines) leading to premature aging, and skin cancer[7]. Suppression of NF-κB has been shown to block the sunburn-induced damage.

Evidence shows that there is sustained, or constitutive activation of NF-κB in tumor cells and cell lines. This contributes to the progression towards malignancy and resistance to therapeutic intervention in multiple human cancers. Several different tumor cell line types have been reported to express constitutively active NF-κB[8]. These include leukemia, lymphoma, myeloma, melanoma, prostate, colon, breast, pancreas, and head and neck squamous cell carcinoma[8]. Moreover this has also been found in samples obtained from cancer patients. Many research studies are being performed to further understand the cause(s) of this constitutive activation[8].

The pharmaceutical industry has been working on developing drugs that are targeted towards the NF-κB pathway and effective against cancer[9]. The importance of NF-κB in tumor progression has been highlighted in several studies that utilized NF-κB inhibitors. As a result, many methods have been developed to prevent the activation of NF-κB. NF-κB inhibitors have already shown promise against tumor growth in xenograft models and have spurred clinical trials in some cases[9]. A wide variety of compounds (e.g. IKK inhibitors, inhibitory peptides, antisense RNA, proteasome inhibitors, chemopreventive agents) are being evaluated for their ability to inhibit NF-κB[10].

Most recently, NF-κB has been the focus of anti-aging studies. Mechanisms of aging, as well as changes in gene expression during aging are not well understood. However, the NF-κB pathway has been implicated in several ageing studies. In one particular study, NF-κB was blocked for two weeks in the epidermis of aged mice. The skin characteristics of these mice reverted to those of young mice, both in terms of appearance and gene expression. Studies have also linked aging phenotypes to constitutive activation of NF-κB[11]. Resveratrol, a compound synthesized by plants when being invaded by pathogens, has been commercially marketed for the past decade as an anti-aging compound[12]. Though researchers are still investigating the claims made by resveratrol marketers, it is generally agreed that there is some validity that resveratrol could be used to modify the phenotypic symptoms of aging because of its ability to inhibit NF-κB.

Research into the function of regulation of the NF-κB family continues to provide us with greater insight into many human diseases. NF-κB shows much promise in helping us understand aging and cancer in humans, both areas that continue to challenge researchers. With the increasing human life span, it will be important to understand the mechanisms that drive aging and its associated diseases. Learning more about NF-κB will potentially improve quality of life and has the chance to have an impact on reducing health care costs associated with cancer and long-term care.

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Figure 1. Gilmore, T (2006). Introduction to NF-jB: players, pathways, perspectives. Oncogene 25, 6680-6684

The L409 amino acid residue is important for the DNA replication fidelity of thermostable Pfu DNA polymerase

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Effective cell division requires an accurate and efficient mechanism by which genetic information can be replicated and passed on to progeny cells. Excessive errors in genome replication may mutate essential genes and threaten the ability of these daughter cells to survive and proliferate. Organisms that utilize double-stranded deoxyribonucleic acids (dsDNA) as carriers of their genetic code have developed a class of enzymes called DNA polymerases to catalyze this DNA replication process. DNA polymerases incorporate free deoxyribonucleotide triphosphates (dNTPs) into newly synthesized DNA strands in a sequence primarily determined by that of the template DNA strand through Watson-Crick base-pairing rules. However, the wide range of accuracy levels among the known DNA polymerases in faithfully replicating the template strand suggests that simple base-pairing forces are an insufficient explanation for these differences in fidelity. Indeed, the polymerase is actively involved in the dNTP selection, and thus at least partially responsible for replication fidelity.

The DNA polymerase from the thermophillic arhaeon *Pyrococcus furiosus* (Pfu Pol) remains one of the most widely known thermostable high fidelity DNA polymerases. It is commonly used as the replicating enzyme for Polymerase Chain Reactions (PCR) in many laboratory protocols because of its high degree of replication accuracy and inherent thermal stability. While the presence of a 3'→ 5' exonuclease proofreading domain on Pfu significantly contributes to its superior fidelity among often-used enzymes in PCR, exonuclease-deficient Pfu still confers an error rate within an order of magnitude of Taq polymerase, the other most commonly used PCR enzyme lacking this proofreading nuclease activity. This inherent fidelity makes Pfu polymerase a good model for studying the interaction between active site molecular architecture and replication fidelity.

Pfu polymerase belongs to the α-like family of DNA polymerases, a group of closely related enzymes sharing a structure similar to the eukaryotic DNA polymerase α. The crystal structure of another α family member, the RB69 phage polymerase, has been co-crystallized with template-primer and an incoming dNTP (known as the ternary complex) at a resolution of 2.8 Å. Crystallography confirms that the RB69 and Pfu polymerases are extremely similar in structure, with highly conserved topological features. Several functional domains, separated by clefts, radiate from a central cavity. They have been named the "Thumb," "Fingers," "Exo," "Palm," and "N-terminal" domains, with the nomenclature noting its shape's similarity to that of a right hand. The palm domain, thought to be the most highly conserved of the group, has been implicated in the dNTP-binding and nucleotidyl transferase activities central to the accurate polymerization needed for DNA replication. Within this region, the leucine residing at the 409th residue of Pfu Pol has been shown to be highly conserved among α family polymerases and is represented by Leucine-415 in the RB69 polymerase. Conservation of a similarly located amino acid across members of a family of enzymes suggests mechanistic importance and provides a potential target for investigation.

Here, we have created a mutant form of exonuclease-deficient Pfu polymerase in which Leucine-409 was replaced with a methionine residue. This new enzyme (L409M) was assayed for its enzyme fidelity in comparison with the wild-type Pfu Pol. Ongoing work in our laboratory has focused on replacing L409 with bulkier residues, including phenylalanine. Preliminary data has shown that the L409F mutation renders the enzyme unable to complete PCR-based fidelity assays. However, the leucine to methionine substitution presents only approximately four cubic Angstroms in residue volume change and little change in side-chain polarity and this mutant is able to complete a PCR reaction. Therefore, the L409M Pfu polymerase mutant is instructive in determining the role of the 409th residue in maintaining faithful DNA replication in vitro.

Materials and Methods

Site-directed mutagenesis was completed via a two-step overlapping Polymerase Chain Reaction (PCR) method. The first PCR step used a forward primer containing a leucine → methionine substitution (5'-TACCTAGATTTTAGAGCCATGTATCCCTC-GATTATAATTACCCAC-3') and reverse primer that annealed near the C-Terminal domain (5'-GGCTCTAAAATCTAGGTA-3) of the *pfu* gene. A second PCR reaction used the amplicons created in the first step as templates, annealed to a T7 forward primer (5'-TAA TAC GAC TCA CTA TAG GG-3') and the original Pfu reverse primer. This second PCR extended the insert fragment to a size of approximately 2.4 kb, allowing for it to be more easily

used in cloning procedures.

This 2.4 kb amplicon and a pET28a dsDNA plasmid (Novagen, WI) were double-digested with NheI and NcoI restriction endonucleases. The vector and the insert (PCR product) were covalently joined by T4 DNA ligase (Invitrogen, CA) and transformed via electroporation into XL1-Blue *E.coli* cells (Stratagene, CA). Surviving colonies were harvested and the *pfu* clones were isolated from the other cellular components by a Miniprep (Promega, WI) purification procedure. Correct insertion of the PCR amplicon insert into the pET28a backbone in the clones was verified by analytical *Apa*I digest, with successful clones yielding a single linear 7.0 kb band upon agarose electrophoresis. These clones were sequenced, and the results were analyzed for similarity to the *exo*(-) wild type *pfu* gene with the exception of the L→M mutation at amino acid residue 409.

A clone confirmed by sequencing (ACGT Inc., IL) to contain L409M *pfu* was transformed into chemically competent BL-21 (DE3) pLysS *E.coli* cells for over-expression and incubated in 2X-YT rich media under kanamycin/chloramphenicol selection pressure. Kanamycin selects for cells containing the L409M pfu-pET28a clone and cholamphenicol selects for BL-21 cells containing the pLysS plasmid needed for strict transcriptional control over the lacUV5 promoter, which controls expression of the L409M *pfu* gene. Ten milliliters of 100 mM isopropyl β–D-1-thiogalactopyranoside (IPTG) was added to a liter of culture upon an OD reading of 0.1 to induce expression of the mutant protein and the culture was incubated for approximately three more hours. The cells were then pelleted by centrifugation and lysed. Supernatant was isolated from the precipitated impurities in the cell lysate and centrifuged for twenty minutes at 3600 rpm at 4° C.

To isolate and purify the mutant Pfu polymerase protein from the rest of the supernatant, a nickel ion-NTA affinity chromatography method was utilized. This technique involves chelation of hexahistidine tags genetically attached to the L409M Pfu mutant polymerase by embedded nickel ions in a semisolid resin, immobilizing the desired protein while letting all others flow through the column. Subsequent treatment with imidazole, which has a higher affinity for the nickel ions, allows for elution and collection of the purified protein, followed by overnight 4° C dialysis in a Slide-A-Lyzer cassette against 1X-Dialysis Buffer (50 mM Tris-HCl pH 8.01, 1 mM EDTA, 50% glycerol). SDS-PAGE was used to confirm sufficient concentration and purity of Pfu in the eluted fractions.

In order to accurately compare Pfu polymerization activity between L409M and (previously analyzed) wild type, the purified L409M protein preparation was normalized for concentration and activity. Differences in activity among mutants must be based solely on the effects of the introduced substitutions, rather than artifactual differences in the concentration and efficiency levels of protein preparations. To this end, single nucleotide extension assays were performed with varying protein concentrations to ascertain the concentrations at which L409M polymerase extended approximately fifty percent of an oligonucleotide primer.

The L409M Pfu polymerase was used in a primer extension reaction with 40 nM 40mer DNA template (5'-AAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG-3') annealed to 80 nM 23mer Extend A primer (5'-CGCGCCGAATTCCCGCTAGCAAT-3') 5'-radiolabeled with Phorphorus-32. These reactions also included 200 uM oligo-dT, 2.5 mM dATP, and 10X Pfu Buffer (200 mM Tris HCl pH 8.8, 20 mM Mg2SO4, 100 mM KCl, 100 mM (NH4)2SO4, 1% Triton X-100, and 1 mg/mL BSA) with the approximately 40 nM template:primer. The template:primer existed in 250-fold excess over Pfu enzyme in the extension reactions. Other dNTPs were excluded from the reaction mixture so that only an adenine residue could be added onto the primer.

At the commencement of each reaction, the diluted protein was added to the reaction mixture and incubated for five minutes at 55° C. After this incubation, 2x-TC Stop Dye (containing 40 mM EDTA) was added and the reaction was kept at 95° C for five minutes. The EDTA chelates the magnesium ion cofactor from the polymerase enzyme and halts catalysis. For each reaction, a 4 μL sample was electrophoresed through a 16% urea denaturing polyacrylamide gel and visualized by phosphorimaging analysis.

Once the appropriate L409M protein amount was found for comparison to wild type Pfu at fifty percent primer extension, this concentration was used in dATP titration reactions in order to establish the mutant's Michaelis Constant (K_M), or the enzyme concentration at which L409M polymerized at half-maximal velocity. These reactions were performed under identical conditions as those for the Activity Normalization Assays with the exception that polymerase concentration was now fixed at approximately 800 pM and dATP concentration differed between reactions, ranging from final concentrations of 1 μM to 250 μM (See Figure 2). The results were once again electrophoresed in 16% polyacrylamide. Products were then observed via phosphorimaging and quantified by densitometry. The extent of extended primer formation was plotted against dATP concentration and fit to the Michaelis-Menten equation ($V_o = V_{MAX}[S] / K_M + [S]$) to determine the K_M value for L409M Pfu. This process was completed in duplicate to ensure precision. The wild type polymerase K_M had been previously elucidated by similar means.

To measure the extent of mutation conferred by L409M upon a newly synthesized DNA strand, and thus its fidelity, we performed a PCR-based Forward Mutation Assay. This experiment involved the L409M (without the 3'-exo domain) and WT (+/- 3'-exo) Pfu polymerase as the replicating enzymes in PCR reactions upon a template fragment (pUC18 plasmid linearized by restriction endonuclease *AflIII*) containing the lacZα reporter gene fragment. The lacZ gene encodes beta-galactosidase, a hydrolase that can cleave the chromogenic reporter X-GAL to yield a blue product. Primers flanking the lacZ region on the pUC18 fragment (5'-AAAAAAAGATCTTCTTTCCTGCGTTATCCCC-3' and 5'-AAAAAAAGATCTGAGCAA AAGGCCAGC-3') were used with 50 uM dNTPs and 10X-Pfu Buffer to complete PCR in a BioRad Thermal Cycler. Q-PCR had been previously utilized to normalize for template duplications among the mutants in the PCR reactions.

After this PCR was completed and verified by agarose electrophoresis, the amplicon DNA fragment was purified with a Qiagen PCR Purification Kit and double-digested by *DpnI* and *BglII*. *DpnI* specifically degrades dam-methylated adenine bases within a (5'-GA^{m}TC-3') palindrome, thereby destroying any residual plasmid template remaining from the reaction while *BglII* made two cut sites within the PCR products, leaving complementary single-stranded overhangs at each end. This digestion fragment was purified by phenol-ethanol extraction and then subjected to an intramolecular ligation by T4 DNA Ligase (Invitrogen, CA).


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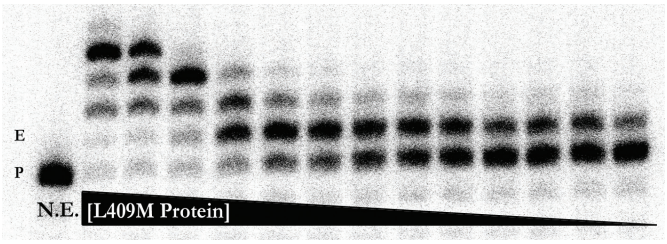


Figure 1. This image is a phosphorimager visualization of a 16% urea denaturing polyacrylamide gel for a L409M Pfu Polymerase dilution series. Single nucleotide extension reactions were performed upon 40 nM 5'-^{32}P-labeled 23mer DNA primer annealed to 80 nM 40mer template combined with 2.5 mM dATP, 200 uM oligodT, and 10X Pfu Buffer. Unextended primers (23mers) are represented in the row labeled with a **P** while single-nucleotide extended primers (24mers) are labeled with an **E**. The reactions were performed for 5 minutes at 55° C, then quenched with 2X-Stop Dye containing 40 mM EDTA. On the left, the N.E. lane contains a no-enzyme negative control reaction, predictably showing no primer extension. The thirteen subsequent treatment groups were treated with a gradually decreasing concentration of L409M Pfu polymerase. The dilution factors (from 2ug/uL protein) for these lanes were: 1:10, 1:256, 1:2000, 1:4000, 1:15000, 1:20000, 1:25000, 1:30000, 1:35000, 1:40000, 1:45000, 1:50000, and 1:100000. The goal of this assay was to determine the protein concentration at which 50% of the primers were singly extended to form 24mers. This would allow for normalization of enzyme activity between L409M and wild type Pfu proteins. The correct concentration of L409M needed for 50/50 was extension was 70 pg/μL or 800 pM, from a 1:28,000 dilution of 2 μg/μL stock protein. Multinucleotide extension beyond the E row illustrates a tendency for L409M Pfu to misincorporate nucleotides onto the primer without respect to template.

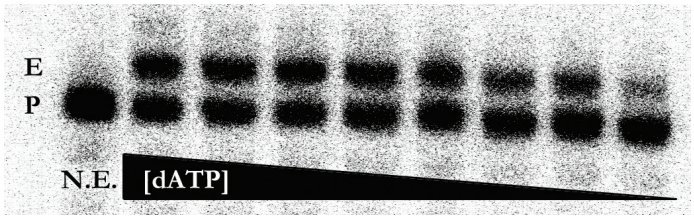


Figure 2. This image is a phosphorimager visualization of a 16% urea denaturing polyacrylamide gel for a L409M Pfu Polymerase dATP Titration Assay. Reactions were completed similarly to those in the Protein Dilution Series (Figure 1) except that protein concentration was fixed at 71pg/μL and dATP concentration was gradually decreased. Single nucleotide extension reactions were performed upon 40 nM 23mer DNA primer annealed to 80 nM 40mer template combined with varying dATP concentrations, 200 uM oligodT, and 10X Pfu Buffer. Unextended primers (23mers) are represented in the row labeled with a **P** while single-nucleotide extended primers (24mers) are labeled with an **E**. The reactions were performed for 5 minutes at 55° C, then quenched with 2X-Stop Dye containing 40 mM EDTA. On the left, the N.E. lane contains a no-enzyme negative control reaction, expectedly showing no primer extension. The eight subsequent treatment groups were treated with a gradually decreasing concentration of dATP. The final dATP concentrations represented in these lanes are: 250 μM, 100 μM, 50 μM, 25 μM, 10 μM, 5 μM, 2.5 μM, and 1.0 μM. The fraction of extended primer over the total primer added per lane was quantified by densitometry and the observed product concentrations were fit to Michaelis-Menten kinetics.

This newly ligated pUC18 construct was then electroporated into XL1-Blue *E. coli* cells (Stratagene, CA). These were spread onto plates under carbenicillin selection pressure with IPTG as an expression inducer and X-GAL as the chromogenic substrate of beta-galactosidase action. When cells expressing this lacZα-containing pUC18 fragment were plated onto dishes containing X-GAL, colonies with fully functional lacZ gene expression were blue in color. Mutations introduced by L409M Pfu Polymerase during the PCR led to decreased beta-galactosidase activity, yielding white bacterial colonies. The number of blue and white colonies for the L409M (exo-), WT (exo-), WT (exo+), and a no-enzyme control assays were counted and the ratios of white:blue colonies were calculated for each treatment group. Relative differences in the frequency of white colonies in a given sample size per plate (~300 colonies) speak to the extent of mutations conferred by each Pfu Polymerase onto the lacZα gene in the initial PCR reaction, and therefore provide a method by which relative fidelity can be quantified. The Forward Mutation Assay was performed in triplicate for all treatment groups to ensure precision.

Results and Discussion

Normalization of L409M and Wild Type DNA polymerase Activities

As described in the Methods section, the single nucleotide extension reactions were performed with varying L409M Pfu protein dilution factors to normalize for any concentration and efficiency differences between the L409M and wild type proteins brought about by preparation procedures. The goal of this assay was to find the concentration of polymerase displaying 50% extension of 32P-labeled 23mer at the maximal dATP concentration used in our single nucleotide extension reactions (250 uM final). This protein concentration will be used for the following enzyme characterizations in comparison to wild type Pfu. After electrophoresis, through 16% urea denaturing polyacrylamide, the phosphor-image of 50% extension should show two similarly radioactive bands representing the small unextended primer (P) and extended primer (E). A scan of these reactions with increasing L409M dilutions from 1:10 to 1:100,000 is depicted in Figure 1.

While the wild type exo(-) enzyme used for comparison was found to reach 50% extension at the 1:180,000 dilution factor mark, Figure 1 demonstrates that the fifty percent extension point for L409M Pfu lied between 1:25,000 and 1:30,000. This point was later found to reside at 1:28,000 by a more refined set of protein dilution reactions, yielding a concentration of 70 pg/uL. This would be the dilution factor used for this mutant in subsequent kinetics assays. The multi-nucleotide extension seen in Figure 1 is unexpected as the reactions contained only dATP nucleotides for incorporation. Also, the template:primer anneals such that the template thymidine used to base-pair with incoming dATP is followed by two adenine residues to further discourage multi-nucleotide extension. The presence of multiple incorporations in a single nucleotide extension assay reveals the presence of nucleotide misincorporation, which had been previously seen in assays for wild type (*exo-*).

dATP Titration and Michaelis-Menten Kinetics Results

The dATP Titration Assay was performed with L409M Pfu polymerase at a concentration of 800 pM (a dilution of 1:28,000 from an initial protein concentration of 2 ug/uL) in order to determine how the mutant enzyme incorporated a single nucleotide over a range of dATP concentrations. The resultant 16% poly-

acrylamide gel loaded with these reaction products is depicted in Figure 2. The radioactivity levels of the quantified bands in Figure 2 represent the amount of extended primer in relation to unextended, thereby providing data for extended primer product formation over a range of substrate (dATP) concentrations. These are the parameters necessary to fit the L409M extension profile to fundamental Michaelis-Menten kinetics ($V_o = V_{MAX}[S] / K_M + [S]$). Figure 3A graphically displays L409M extended product formation as a function of dATP concentration.

Fitting L409M extension levels at varying dATP concentrations provides a value for the the Michaelis Constant K_M, which describes a component of catalytic efficiency at substrate concentrations below the enzyme's saturation point by revealing the concentration of dATP that must be added to the reaction for the polymerase to reach half-maximal velocity (1/2Vmax) in extending upon the DNA primer in the steady state. Combining the K_M finding displayed in Figure 3A for L409M with the value previously found for wild type (exo-) Pfu, Figure 3B shows that the mean K_M values (reactions duplicated) for these two enzymes differ by less than one micromole per liter, being 1.6 uM and 2.5 uM, respectively. All K_M values found via nonlinear regression analysis of Michaelis-Menten plots were confirmed by double-reciprocal Lineweaver-Burk plot analysis (data not shown).

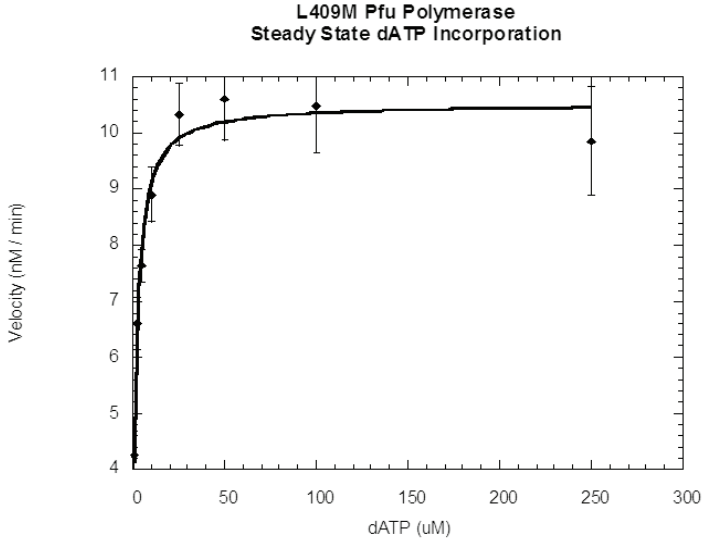
The difference in K_M values suggests that the substitution of Leucine-409 with methionine in Pfu polymerase has little effect on its relative ability to incorporate incoming dATP nucleotides in the steady state, although comprehensive pre-steady state analysis is needed to directly compare dNTP binding affinity. Also, while the normalization assays for wild type and L409M ensure equal extension ability among the enzyme samples added to the reactions, pre-steady state kinetic analysis would be required to arrive at a quantitative percent activity figure for each polymerase. This will be a topic of future research on the L409M mutant, as fractional activity data is needed to accurately assess L409M turnover number (k_{cat}). However, the present kinetics data shows that any observed fidelity differences between L409M and wild type Pfu are not coupled with drastic difference in K_M value.

Fidelity Assay Results

A Forward Mutation Assay was performed to measure the mutation rate of L409M, and thus its fidelity, within a ~600 bp lacZα gene fragment. The first step of this procedure was to use the mutant L409M polymerase in a PCR reaction on a pUC18 fragment containing the lacZα gene. The subsequent steps are detailed in the Methods section. Successful completion of the PCR and corresponding Q-PCR experiments (not shown) show that L409M is functional enough to extend primers and allows for enough processivity to extend them many thousands of bases. However, this result speaks only to the effectiveness of the enzyme at completing the reaction, not the extent to which its products are mutated.

As described in the Methods section, the PCR product was digested and ligated to itself to form a circular pUC18 fragment (containing the lacZα gene region) that was transformed via electroporation into XL1-Blue E.coli. These cells were spread on plates containing the chromogenic beta-galactosidase substrate X-GAL, allowing a blue-white assay to be completed in which blue colonies represented functional lacZα expression while white

A.



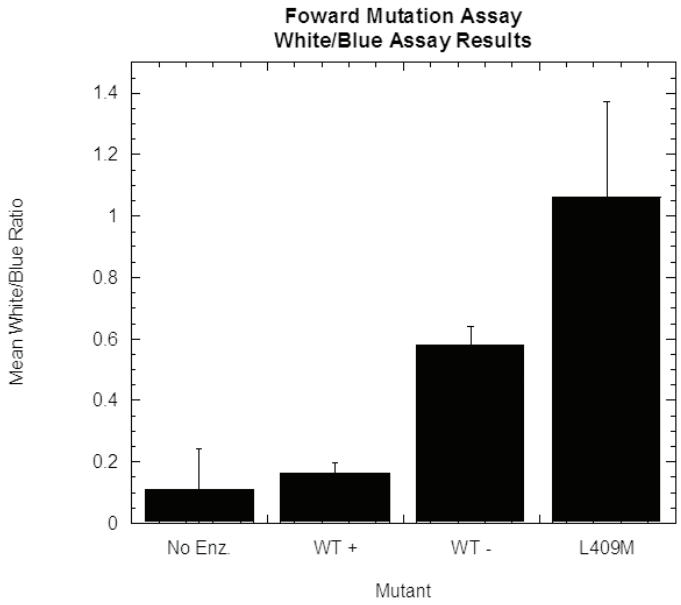
B.

Mean Km Values for L409M and WT (exo-) Pfu Polymerase

Mutant	Km (uM)	S.D. (uM)
L409M	1.6	0.38
WT (*exo* -)	2.5	0.25

Figure 3. (**A**) After quantification of the extended and unextended bands from the dATP Titration Experiment for L409M Pfu polymerase (duplicated), the concentration of extended product (over five minute reactions) was graphed as a function of dATP concentration and fit to the Michaelis-Menten equation. The nonlinear fit provides the Michaelis Constant (K_M) by calculation of the dATP concentration needed for half-maximal velocity, or that needed to reach half of the maximal product formed in the fixed five minute reactions. **(B)** This chart compiles the mean K_M value for L409M Pfu calculated from Figure 4A with the previously determined K_M for wild type (exo-) Pfu polymerase. At 1.6 μM for L409M and 2.5 for wild type (exo-), these two values differ by less than 1 μM. This provides initial data suggesting similar dATP incorporation kinetics in the steady state (to be confirmed by pre-steady state analysis). All K_M values shown have been confirmed by Lineweaver-Burk linear regression analysis.

A.



B.

Mean White/Blue Assay Results

Mutant	Mean White:Blue Ratio	S.D.
N.E. CONTROL	0.11	0.13
WT +	0.16	0.035
WT -	0.58	0.060
L409M	1.1	0.31

Figure 4. (A) This is a graphic representation of differences in the number of white *E. coli* colonies per blue colony between forms of Pfu polymerase. The ratio of white:blue colonies represents the frequency with which lacZ reporter genes are mutated and made defective by mutant Pfu polymerase enzymes in the initial Forward Mutation Assay PCR, thereby keeping X-GAL-treated colonies from hosting beta-galactosidase-mediated production of a blue dye. **(B)** This chart details the data presented in Figure 4A from the triplicated Forward Mutation Assay. As expected, the No Enzyme control had the lowest appearance of white colonies, as no Pfu polymerase performed PCR upon these pUC18 templates. WT+, harboring a 3'→ 5' proofreading ability, had a mutation rate barely above that seen with no enzyme at all. This supports wild type Pfu's reputation as a high-fidelity polymerase. Upon loss of the exonuclease domain, however, mutation rate increases 3.6-fold, confirming the proofreading activity's role in maintaining Pfu's fidelity. Lastly, for the exonuclease-deficient L409M mutant, the mutation rate rises 1.9-fold above that for WT-. This supports the hypothesis that even a small change in active site architecture near the dNTP-binding motif could have notable effects on Pfu replication fidelity.

colonies represented mutation. This conferred to the extent that beta-galactosidase was functionally impaired. These procedures were completed in triplicate for the WT (exo+), WT (exo-), and L409M Pfu proteins, along with a no-enzyme negative control to assess background white colony frequency, in order to elucidate fidelity changes brought about by the L409M mutation. The results of these blue-white assays are depicted in chart and graph form in Figures 4A and 4B, respectively.

One would expect a wild type Pfu polymerase with proofreading ability to effectively replicate the genome of its host organism with a limited amount of error. This is supported by Figure 4, in which wild type *exo*(+) plates showed only slightly higher mutation rate than plates with no enzyme at all. When the proofreading exonuclease function is disabled, mutation rate should increase. Figure 4 shows that indeed it does, with wild type *exo*(-) presenting a 3.6-fold increase in white colonies per blue colony over wild type *exo*(+). For L409M, the white:blue colony ratio averaged at 1.1. Its white:blue ratio lies 1.8-fold above wild type *exo*(-), 6.6-fold above *exo*(+), and 9.6-fold above the no-enzyme control. Therefore, the leucine → methionine substitution at residue 409 decreases the fidelity of the polymerase approximately two-fold, as its 1.1 white:blue ratio is 1.9 times higher than 0.56, the ratio for wild type (exo-).

The L409M mutation reduces the ability of Pfu polymerase to faithfully replicate the lacZ reporter gene in PCR without damaging the function of the enzyme expressed from it, beta-galactosidase. Unpublished work from this laboratory suggests that replacing L409 with isoleucine produces similar fidelity detriments. These observations suggest that L409 is an important component in maintaining the replication fidelity of Pfu polymerase, as even slight modification to this residue yields significant loss of fidelity.

Conclusion

Faithful replication of DNA requires polymerase enzymes with sufficient fidelity to minimize mutations that may hinder the fitness of progeny cells. Pfu DNA polymerase ranks among the most accurate enzymes commonly used in PCR reactions and therefore represents an appropriate target for investigation into the role of the active site architecture in determining this fidelity. We introduced a leucine-to-methionine substitution at residue 409, within the highly conserved dNTP-binding motif, to investigate whether subtly changing the geometry of this region would affect the Pfu polymerase's ability to correctly replicate a lacZα reporter gene in a PCR-based Forward Mutation Assay. The results of these experiments show that the L409M (exo-) mutant presents replication fidelity nearly two-fold below exonuclease-deficient wild type Pfu polymerase, as *E. coli* colonies containing the damaged beta-galactosidase are 1.83 times more common in L409M than WT (exo-). Furthermore, steady state kinetic analysis of L409M yielded a Michaelis Constant (K_M) of 1.6 uM versus 2.5 uM for wild type (exo -). This means L409M Pfu functions at half-maximal catalytic velocity at a comparable, if not lower, dATP concentration than the exonuclease-deficient wild type enzyme while still severely hindering the fidelity with which the polymerase pairs the incoming nucleotide with the correct template base. Future work will focus on a mechanistic understanding of this altered state through pre-steady state kinetic analysis and elucidation of L409M Pfu dNTP-binding parameters. In any case, the correlation between this subtle change in the Pfu polymerase active site and its drastic

effects on the mutant's ability to faithfully replicate DNA speaks to the refined nature of this enzyme as a product of evolution.

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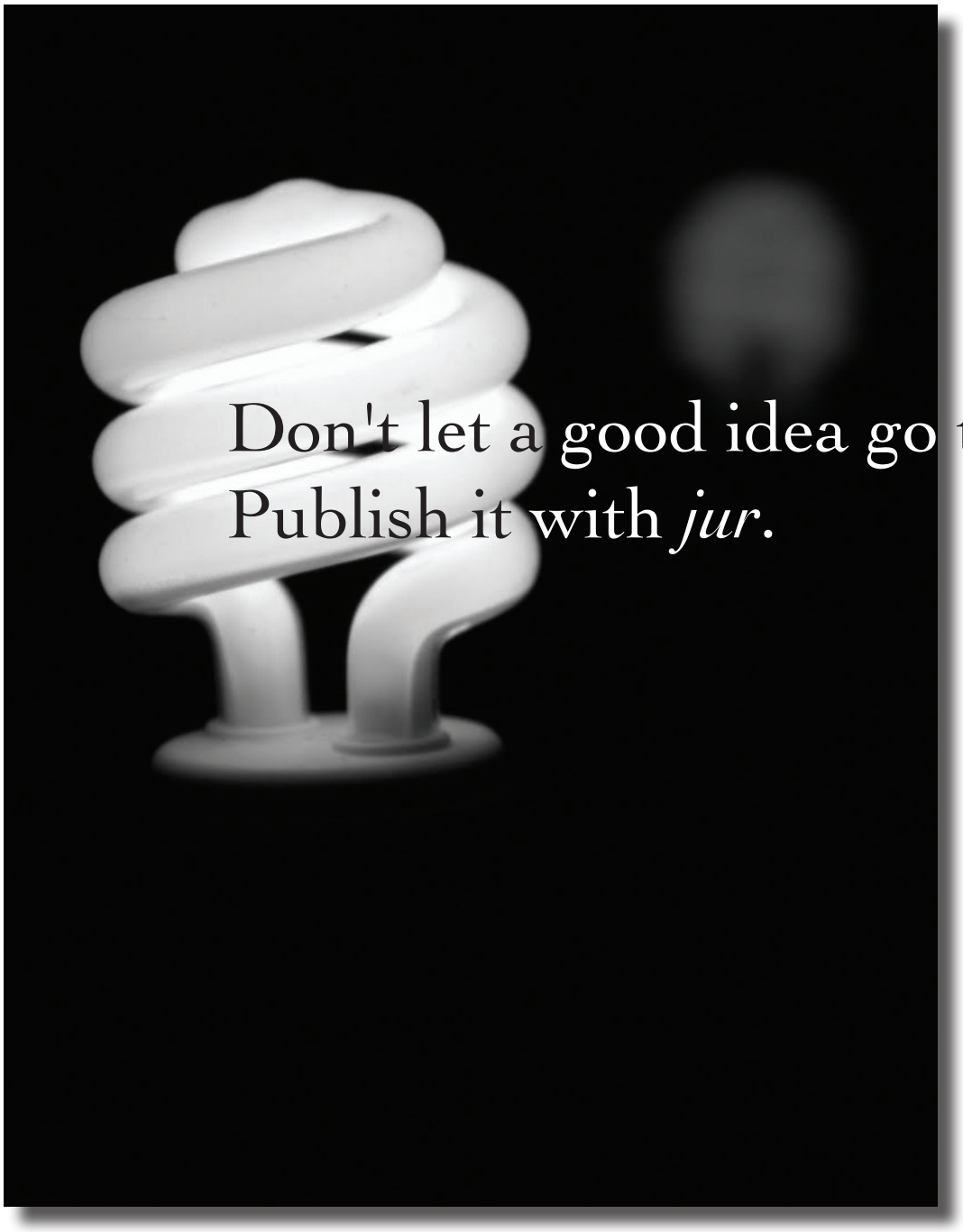

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Student Interview

Nate Lindsey, 2010

jur: How did you get started in research?

Lindsey: I heard about the project over Meliora Weekend. A woman named M.J. Ebenhack, who is the president of the group AHEAD energy, talked about small-scale energy transitions during a panel discussion on alternative energy. The group was starting a project at a Peace School in Uganda. The school burned firewood and charcoal, which are both a bad source of energy. M.J. asked me to be the engineer/technical person on the project.

jur: What kind of research have you been involved in over the course of your career?

Lindsey: During sophomore year, I worked with the Center of Visual Science. They were creating a retinal scanner and they needed someone to code a manual. I also did an REU in Chemistry, Research Experience for Undergraduates through the National Science Foundation, working with the Krauss group. We did work with quantum dots and solar energy.

jur: What kind of research are you involved in currently?

Lindsey: This project is an independent research study. This project looks at how to engineer small-scale alternative energy. We did a desk study researching different possibilities of alternative energy before going to Uganda. When I was on the ground I realized there was no wind and thus all of that research that I did on wind was useless. But I did realize that solar energy was a good prospect.

jur: What are the kinds of techniques or approaches you use with your research that makes it different from other people who are conducting the same research in your field?

Lindsey: We are working in the developing world while most research on alternative energy is done here in America. When you take it to the developing world, there's a difference in coding that has to be performed. Some coding isn't even available there. Also, we are looking at hybrid systems instead of looking at just one alternative source such as solar energy. Thus we are coming in with an open mind and were saying that we can look at solar, wind, biodigestion, and efficient cooking.

jur: Is there any research that opposes your findings, or believes in a different conclusion?

Lindsey: On the panel discussion on alternative energy, M.J. was in support of small scale solutions. On that panel discussion, there was also Steven Chu, a University of Rochester alumnus and United States Secretary of Energy for Obama. He was in support of taking all of the minds of the world and developing one technology for alternative energy, so it was a more global view.

jur: What kind of broader application could your research have?

Lindsey: Across the developing world, plenty of people are still living below the energy poverty level. They are consuming two kilowatts of energy and we are consuming six. This research can be applied across the developing world to decrease this discrepancy between the energy used by different groups.

JUR: Is this a project you plan to pursue into the future, or is there something else?

Lindsey: This is going to be my senior design thesis. I made my own major, which requires a capstone project. This project requires a full year research project which you design. This project is the beginning of my final project. I'll continue this into the summer and maybe next year.

jur: How does research develop and challenge the intellect, especially when it comes to students?

Lindsey: Research culminates and synthesizes everything you learn. You have to look at a question and figure out what it is asking by making an assessment. This could be applied to working in companies answering the question of what it takes for them to go green, for example.

jur: Do you have any advice for undergraduates that plan to pursue research in your field?

Lindsey: Don't be afraid to go up to someone to express your interest, and tell them that you are ready to take on the responsibilities. Ask professors and other figureheads to be a part of their projects.

Perspectives on Research

Elizabeth Colantoni, Ph.D.

Assistant Professor of Classics

jur: Can you describe your academic and professional background?

Colantoni: Sure. As an undergraduate I was a French major and I also took some archeology classes. It always happens that people ask me how I got into what I am currently doing, and the answer is: nepotism. My mother is an archaeologist. So sometimes when I was a kid, I would go and see the work that she was doing. When I was in college, I took archeology classes for fun, and since it was enjoyable, I realized maybe that's what I should do for a living. And after that, I did some slightly different things than what most people who study classical archeology would do: I got a Masters degree in anthropology and new-world archaeology. I worked for two years for the National Park Service doing archaeological work, writing reports, doing excavations at sites in Florida, South Carolina, the US Virgin Islands, and then after that, I spent the year as a student at the University of Rome. That was a great experience, and I really enjoyed being in Italy and actually studying the ancient monuments in the context where you can read about it in your textbook and then go and actually see what you were reading about. After this, I went on to get a PhD in classical archaeology at the University of Michigan, where I wrote a dissertation on early Roman religion, looking at the physical evidence for Roman religious practices in a time period for which we don't have written sources. There are later written sources, like Livy and various other authors who write about the history of early Rome but are removed by about six or seven centuries. So what I did was to try to look at the material remains from that time period and to talk about what was happening then based on contemporary evidence, rather than trying to use the later tales of the beginning of Rome.

jur: Okay, so what kind of research are you doing now, and what kind of research have you been involved in, in the past?

Colantoni: Okay, well, I'm working on looking at the physical remains from Rome in the 7th or 8th centuries B.C., and using those physical remains to create a narrative about what was happening in Rome at that time, particularly focusing on religion, but also other things. For example, there is a site in Rome that is the earliest evidence for a temple there. And at this site, the people who excavated this site studied the bones found there and were able to establish that a lot of the animals that were sacrificed there were newborn. For me, when thinking about working on religious life in early Rome, one of the things that I am interested in is the religious calendar. Ancient Rome had a religious calendar that, in a lot of ways is similar to our own calendar, where the year was divided up into months, with certain festivals falling at the same time each year. These festivals helped create a rhythm to the year in the same way that today we have Valentine's Day and the holiday season in December. However, we don't know how far back that calendar goes. In studying this site where they have evidence of offering of animals, baby animals are born at roughly the same time every year. And so, I make the argument that because they are sacrificing baby animals, at the site, long before we have evidence for the Roman calendar, there was already some kind of annual festival that was taking place when Rome wasn't really a big city. So, from the beginning, Rome had a religious calendar. That's the kind of thing that I've been working on. The other thing that I've been doing is running an excavation in Italy at a site an hour outside of Rome. It has remains from the prehistoric period all the way up to the present. Most of the remains, at this point, are a part of a Roman villa, but we still need to do some more excavation to find out about that. I'm actually working right now on selecting students for a group to go this summer, taking a class for credit and excavate the site. This will be a part of my research also.

jur: So, what drew you to researching about ancient Rome in particular?

Colantoni: I went to Italy off and on as a kid because of the work that my mom does as an archaeologist. I just like Italy; it's a fascinating place. The modern culture is interesting as some of the ways in which modern and ancient culture connect, but there are clear differences between the ancient Romans and Italians today; it would be wrong to think that people in Italy are just quaint modern versions of antiquity. But it's a place where for thousands of years, civilization has really thrived and produced incredible artwork and literature. I really just enjoy learning more about it. When I went

as a student, when I went to the University of Rome for a year, that experience for me was really defining in that it made me realize that this was what I wanted to focus on. I actually had studied other things, like when I was an undergraduate studying French, I had actually focused on Roman France, because the Romans had conquered most of the Mediterranean world including Gaul, which is now France. There are a lot of really interesting Roman remains in France. That really tied together my two majors and is actually something that I hope to get back to at some point. But having spent the year in Rome was, for me, something I found to be very inspiring.

jur: Why do you think it is important to research this and what do you think are the impacts?

Colantoni: I will say, maybe not specifically the things that I'm researching on but the methodologies that I use and the things that I teach, deal with the contrast and relationship between texts and physical remains, whether it's art or archaeological remains. The ability to analyze physical evidence and visual evidence and to create a narrative using that, in contrast with textual sources and words and ideas that are expressed in that way is a skill that I use in my research and is something that I try to teach in my classes. It is also something that is really relevant to the modern world. So much of what we deal with is not just text anymore, but very visual image-based, and it's really important for someone to be able to assess images and propaganda in the modern world to be able to understand what's happening around them. So while I'm dealing with the ancient world, it's very much the same kinds of skills that are relevant to the modern world. I think that for me, and for students, it's a good exercise to be able to sharpen those skills while dealing with something that's a little bit removed from you, but is also interesting. It's important to be able to use those things in your everyday life and know your position in the everyday world, and make the most of things around you.

jur: How can students at the University of Rochester get involved in this sort of research?

Colantoni: Well, they can come see me. This is an area that the University has a new program in, the program of archaeology and architecture. There are a couple of different directions in which that goes, depending on if the student is more interested in the engineering side of ancient structures—you may know Professor Renato Profuccio who is in the engineering department and looks at ancient structures from a very technical standpoint. Then there's also David Walsh in the art history department who focuses more on architecture, and I focus more on the archaeology side of things. So if a student is interested in these areas, coursework for the new program would definitely be a way to get involved. Also, I am doing that summer program with excavations in Italy, and that would be a way for someone to get a really firsthand experience excavating and seeing ancient objects firsthand. They can learn to interpret ancient objects as they come out of the ground, not just read a textbook and have somebody tell you that this is what is significant about something, and rather, experience these things for themselves. So I think that if a student is interested in that then he or she should look into this new program. Obviously there are other ways beyond that that someone could get involved depending on their level of interest, but that would be a starting place.

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The Chinese conundrum

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China is an interesting exception to Huntington's theory of political democracy which states that the higher the level of economic development of a given country, the greater its chance of political democracy. In its history, China has gone through a civil war, invasion and occupation, a revolutionary modernization, a radical reversal from communism to a version of capitalism, and recently, extraordinary rates of economic growth. Huntington in *The Third Wave – Democratization In The Late Twentieth Century* claims that economic development is the basis of political democracy. China's political democracy has been ambiguous so far, and somewhat of a dichotomy. The Chinese are not explicitly confrontational against their leaders or the establishment, but there has been growing evidence of a slow but steadily rising opposition against the powers in rule. While this is not definitively indicative of a democracy, it is certainly suggestive of one. There are three factors that justify such thinking. The first is the increasing influence of the private sector on the Chinese economy; the second is the rising political participation by the people of China; the third is the willingness of the CCP (Chinese Communist Party) to change. All of these factors point to a deep rooted change in traditional thinking that suggest that democracy in China within the near future is not an impossibility.

Many experts claim that political democracy and economic growth are two sides of the same coin. The emerging dominance of the private sector is a crucial explanation for why one would expect China to transition towards a more democratic state of affairs. It is an important indicator underlying the impending change towards a more democratic China. The private sector has long been a passive participant in the Chinese economy. Shackled by government bureaucracy, Chinese businesses were never able to fully reach their true potential. In recent times however, their impact on the Chinese economy has been noteworthy, which is a testament to the decreasing restrictions and influence of the government on the private sector. This is a significant development as it indicates increasing autonomy and independence of the private sector–a characteristic typical of more open political structures.

Let us first examine all of the economic policies that have taken place in China. Then we shall analyze if any of these economic policies have had a bearing on the political democracy in the region. The rapid rate of economic growth has liberalized the economy to a certain extent. Rapid development has in turn forced the Chinese government to enact major reforms in the areas of housing, business and government. One of the key economic reforms introduced by the central government was the rapid decentralization of local governments and businesses. Previously, the Chinese government invested public funds in private businesses in order to have controlling power over key decisions. However, this trend has decreased significantly, as depicted in the China's industrial output by the Type of Enterprise graph (China Industry Economy Statistical Yearbook; China Statistical Yearbook).

Between 1997 and 2007, the private sector accounted for nearly all of China's net employment growth (Gilboy and Read, 3). This highlights a major shift in the attitude of the Chinese people. Once puppets in the hands of the government, smaller, privately owned firms are now becoming self-organized and proactive. Gilboy and Read give an example of a business association in the eastern city of Wenzhou that worked closely with the local government in order to develop and enforce quality standards after hearing complaints of Wenzhou-produced products. Some of the most effective lobbyists are state firms, including tobacco and energy companies (Gilboy & Read, 4). Another interesting and similar decentralization reform has also occurred in housing. In the 1960's, most of China's urban population lived in state owned homes. Between 1960 and 2000, most of these homes were sold at market rates. Today, over 460 million people own their own homes. From organizing condominium resident boards, to protesting against shoddy construction, homeowners are quickly becoming a potent force to reckon with, due to privatization and the increasingly market driven demand for these homes. (Gilboy & Read, 5). Agricultural de-collectivization, another major reform initiated by the government, geared the agricultural sector towards a more competitive and market-based environment. The combination of a greater decentralization in areas such as housing, private businesses and local governments along with higher employment and education rates have made the Chinese society more aggressive in demanding their rights.

Increasing participation by the people is a suggestive second indicator of political awareness in a country. The Chinese are in-

dulging in more demonstrative behaviors to voice their opinions. There has been a change in attitudes, put more simply, a role reversal where the government serves the people instead of the people serving the government. This shift in perspective by itself justifies the claim made by those who believe China's political future lies in democracy.

In his book *Political Participation in Beijing*, Tianjin Shi is in agreement with the above perception that the servile attitude with which the Chinese people once looked upon their government exists no more. Shi paints a picture of a dynamic, feisty, and aggressive population determined to pursue its interests—even if it has to resist the government in doing so. While Shi stays clear of making strong claims about the current political state of China, he is quick to emphasize that contrary to popular belief, dramatic displays of opposition and resistance are not a prerequisite for meaningful mass involvement in politics.

Shi disagrees with the notion that the Chinese are politically apathetic. His survey of 757 Beijing inhabitants, conducted in 1988, reveals that there is a level of political awareness. Most of the people Shi interviewed were genuinely interested in politics—with only a tenth of the people deemed to be apathetic. O'Brien supports this point when he concludes that "a full three quarters of the respondents had undertaken at least one voluntary political act other than voting, between 1983 and 1988." (O'Brien, 160). As previously mentioned, Shi does not make any judgments about the state of political democracy in China. Rather, he concentrates on how local attitudes are changing, which enables the Chinese to participate in politics.

Gilboy and Read provide two examples that explicitly highlight the newfound political activism and awareness of the Chinese people. In 2003, Lu Jun set up a website informing people of how they could protect themselves from employment discrimination against people with Hepatitis B. Lu Jun founded *Yirenping Zhongxin* a website for Hepatitis B carriers. The group developed a savvy strategy for lobbying representatives to the National People Congress (NPC) and Chinese People's Political Consultative Conference. *Yirenping Zhongxin*, secured support for legal protection from some of China's official alternative political parties. The new employment protection act took effect on January 1, 2008, forbidding employers from discriminating against Hep-B carriers.

Gilboy and Read provide another example of political activism in the Chinese people involving the public outcry over a white collar Chinese migrant who was beaten to death for not carrying the right identification papers. The case received media attention and public condemnation and caused the government to issue a new administrative regulation titled, "Relief Methods for Vagrants and Beggars." These examples are instances of greater resistance and expression of dissatisfaction by the people. This opposition has revealed itself in the form of individuals increasingly opposing the establishment they work in, which is shown in the Political Participation in Beijing diagram (Shi, 155).

O'Brien argues that Shi's book seems more an analysis of what people are willing to risk in order to attain democracy rather than a description of the Chinese attitudes towards democracy. He contends that Shi shies away from making any strong claims that there is, of yet, no substantial strategic path to democracy. O'Brien criticizes the idea underlying the term "political opportunity structure" that Shi coins to describe the notion that given the opportunity to resist, the Chinese will retaliate. The "political opportunity structure" clarifies what the leadership will allow and prevent; the popular response to cycles of opening (*fang*) and closing (*shou*) of the political system, and the realm in which the Chinese can operate to achieve their aims. It is up to savvy individuals to exploit the changing authoritarian system to their minimum disadvantage. This, O'Brien argues, suggests that the government has a ceiling on the amount of opposition it will tolerate, and citizens are expected to operate within that space. This does not fit Shi's representation of a dynamic and aggressive China.

O'Brien's second criticism of Shi's study is his sample selection. According to O'Brien, Shi's study is flawed because his sample is unrepresentative of China's beliefs. Beijing residents differ from the rest of the Chinese population in their attitudes and ideology. Shi's sample does not take into account the beliefs of people living in villages or in less developed cities. O'Brien states, "[Beijing's] citizens are considered the most political, sophisticated and outspoken people in the country… a small survey conducted during a liberal interregnum is hardly definitive" (O'Brien, 163).

The third criticism leveled against Shi's analysis can be explained, using the diagram of increasing opposition (Shi, 155). The increase in percentages of people creating resistance, are mostly individualized cases. These are less reactionary, cautious forms of resistance. When it comes to more explicit acts of defiance against an establishment, such as organizing people to fight against leaders or persuading others to attend campaign or briefing meetings at the workplace, the opposition seems to decrease, the diagram shows a decrease in opposition (Shi, 155).This brings us to O'Brien's last criticism. He argues that regime challenging actions are rare and that protest activities are usually individualized and "directed against work unit leaders rather than the political system itself" (O'Brien, Pg.165). Gilboy and Read echo similar sentiments: "The Xioman and Shanghai [protest] walks illustrate how new social groups … continue to adapt, and experiment with ways to act on new interests, while avoiding or preventing direct challenges to CCP rule." (Gilboy and Read, 6). O'Brien's last argument is that, because political resistance is uneven, weak and fragile, it cannot be taken seriously enough to make the claim that China is becoming more politically democratic. There needs to be a systematic and organized approach to democracy, which currently does not exist. These viewpoints are affirmed by the trend of decreasing support of democracy in PRC regions as opposed to other regions (Chu and Chang, 332-33).

Yun Feiyang from the Epoch Times injects a gloomy dose of economic reality, "… the standard of living in China is one of the lowest in the world…in big cities in China, there are cars everywhere, however 90% of them are made in the US, Korea and Japan. The financial gap between town and country is unbelievably large, and half of the farmers in China live in poverty". It seems as though the flickering flames of political democracy in China have only promoted the popular view that some get rich first.

A personal opinion is that the trend shown in the PRC diagram is not an accurate depiction of the attitudes of the Chinese people towards democracy today (Chu and Chang, 332-33). While O'Brien and Gilboy & Read criticize that no substantial developments towards a legitimate democracy have taken place, they fail to acknowledge that the change that has taken place is by itself a substantial improvement. The reverent attitude, with which the

Chinese once looked upon their government, has all but disappeared. Where does this stand with regards to Huntington's theory of economic development? While Huntington's theory cannot be proved in entirety, it is applicable to a certain extent. The reason why the theory may be only partially accurate is that we may have caught China in a transition mode. This transition mode probably explains the reason why the attitudes held by the Chinese towards political democracy are dichotomous in nature, almost a passive form of resistance.

With regards to Feiyang's commentary on the economic situation of his country, one could argue that these issues are, unfortunately, not unique to China alone. The OECD (Organization of Economic Cooperation Countries) states, "Income inequality rose twice as fast in Japan as in other rich countries between the mid 80s and 2000." China is still on a lower rung of the economic ladder in comparison with nations such as US, Korea and Japan; it is unfair to level criticisms of economic disparity against China when it has not yet realized its full potential economically, and even more so politically.

Reform is inevitable in any country. The Chinese leadership realizes that the fundamental thinking used to underlie the people's subservient attitude has all but disappeared. The recognition (by the CCP) that there is a need to reform is, by itself, a significant indication of greater flexibility in the political structure – a feature typified by democracies. This is the third indicator that not only highlights a shift in traditional Chinese views on what role a government should play, but more crucially emphasizes CCP's willingness to change.

The CCP is realizing that it needs to change with its citizens if it wants to continue remaining in power. In the words of Gilboy and Read, "…the CCP must change in order to survive, with a renewed focus on improving legitimacy, transparency and governing capacity in response to a changing society" (5). The authors make the argument that the CCP has already changed quite a bit since it took a turn away from totalitarianism in 1978. CCP now has an intra-party promotion mechanism, where education and competence rather than personal loyalty are the important criteria for promotion. Gilboy and Read state that "[the] government has become more institutionalized, ensuring that power and policies are linked to specific offices and government positions rather than personalities." Gilboy and Read predict that in the future, the reforms that the Chinese government decides to implement will be determined by its citizens, and that the citizens' reactions to these policies, will determine future reforms. This will, in essence, create a two way policy arrangement.

The argument that CCP's change is inevitable is a logical one. The party has to change in order to survive because there are pressures pulling at the government from many different sides. As stated by Gilboy and Read, "Demands on the leadership are emerging from new proprietors such as private businesses, homeowners, environmentalists…migrant laborers and poor farmers who feel that they too share a personal stake in China's future" (Gilboy and Read, 6). These different pressures tugging at the CCP from different sides are coercing it into changing in order to accommodate people's demands. However, for this change to occur, the nation will have to be exposed even more to the dividing conflicts of interest between the party and its people, making the existing schisms more obvious. The CCP is attempting greater legitimacy and transparency by having intra-party elections, but to come up to Western standards of democracy is a "long (and) potentially tumultuous path" (Golboy and Read). It is unclear what Gilboy and Read see as a tangible future for China. They do not make any generalizations, but merely express their opinion that the path to democracy for China, while beneficial, will not be an easy transition.

It is important to study how democracy would affect China in different scenarios. In his article in Asia Policy, Cheng Li presents three different future political scenarios for China in the year 2020. The first scenario is that of a new emerging and democratic China; the second scenario is that of a nation plagued by prolonged chaos due to the persisting problem of economic disparity. The third scenario is a resilient and more authoritarian China. Li predicts that the first scenario will come about by the increasing number of checks and balances within the ruling party. He separates the ruling party into the elitist group, terming them as China's "red states", which represent the interests of the coastal region and the populist coalition or China's "blue states", which voice the concerns of the inland region. Li argues that these two groups are equally powerful, and that the politics of China dividing itself over these two main political groups in the future is not hard to envision. He notes: "it is not difficult to imagine that the CCP will split along the lines of an elitist coalition and a populist coalition…largely because of the incremental nature of this institutional development, this split can be achieved in a non violent way." In 2020, the elections and competition within the CCP may extend to general elections in the country, which consequently, Li argues, will increase intra-party democracy to give birth to a constitutional democracy.

The second scenario highlighted is that of prolonged and continued violence. Li cites several factors for what could set off such a scenario. The tension between local and central governments, global financial crises, conflicting interests between civilian and military groups, ethnic factions and the military confrontation across the Taiwan strait are just some of the triggering factors that could cause a dip in economic and political stability in China. Li predicts a dire situation where "the central government loses its control over provincial administrations, the CCP no longer functions, the military splits, civil war breaks out hoodlums who cause looting all over the country and a massive Chinese exodus leads migrants to every corner of the world." The third situation predicts a more resilient and authoritarian China. The CCP's ability to change so easily might actually make them more sustainable in the future. Their flexibility is a reason why China could become a resilient authoritarianism.

All of these scenarios are unlikely in my opinion. The CCP can only adapt to a certain extent. There will come a point when the fast changing attitudes and beliefs of people will be so beyond CCP's basic ability to change that there will be a clash between the core and fundamental values of the CCP, and the new ideas held by the people. The third scenario is too optimistic in predicting that a transition from a traditional party to a party that changes at the whim and mercy of its people will occur easily. In contrast, the second situation is too dire a prediction and reaches no resolute end. It is simply a prediction of mass violence and threat to people's sovereignty. That is likely to happen for some time if there were any triggering factors such as war or a clash in two extreme values (like the one between a party and the people), however it is unlikely that this chaos will go unresolved. The second seems more of a means in achieving some greater political outcome, rather than be-

ing an end in itself. Scenario one assumes too easy and smooth of a transition from communism to constitutional democracy. The mere presence of two equally powerful parties does not imply democracy. There are likely to be conflicts of interest as the country decides to transform its very inveterate beliefs in communism to a constitution it has always opposed, democracy. One thing that is clear, however, is that there will be division and opposition to whatever decisions China decides to undertake in the future. It is therefore important to understand that the three scenarios can only be simplified versions of what China could become in the year 2020.

China does not disprove Huntington's theory. It does not entirely prove it either. What it does affirm, however, is that it is somewhere in the middle. The burgeoning passive resistance of its people, coupled with CCP's willingness to change, is by itself a remarkable deviation from the Chinese people's traditional views towards democracy. It is only a matter of time before the global community waits and watches to see whether Huntington's theory disproves or unravels the mystery of the Chinese conundrum.

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LPA2 Receptor as an important component of the IL-12 negative feedback mechanism

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Sepsis and toxic shock syndrome are serious medical conditions that result from unregulated inflammation. Inflammation is normally controlled by the body's innate immune system. Toll-like receptors are a key part of the innate immune system and help regulate inflammation. This study was on the Toll-like receptor (TLR) 2 and 4 pathways and how they are affected by lysophosphatidic acid receptor 2 (LPA2). We conducted experiments with bone marrow derived dendritic cells (BMDC) from wild type and LPA2 knock out mice that showed phosphorylation of Akt in wild type cells, but not in LPA2 knock out cells. Phosphorylated protein kinase B (pAkt) is known to control a feedback mechanism in the TLR pathway that prevents inflammation from occurring[2]. Ongoing work has shown that mice deficient in LPA2 develop enhanced inflammation in response to TLR2 and TLR4 exposure. This indicates that LPA2 plays an important role as a negative feedback receptor, which prevents inflammation when a TLR is activated.

The innate immune system is the body's first line of defense against pathogens and their products. Innate responses recognize common structural features found on foreign molecules and pathogens. Phagocytes, cells that can engulf, digest, and kill most pathogens, play a key role in the innate response. Interactions with pathogens stimulate large numbers of phagocytes to activate genes that lead to the expression of proteins that destroy pathogens[4].

The sentinels of the innate immune system are the dendritic cells (DCs). They are usually present in the skin and mucous membranes of the nose, lungs, stomach, and intestines[5]. Also known as dendrocytes, they have a dual function of phagocytosis and antigen presentation. Immature DCs are found throughout the body as active phagocytes. Once an antigen is ingested, the DC matures and migrates to the lymph nodes where they present the antigen to T cells[4]. After T cells are presented with an antigen by DCs, it becomes activated. The activated T cells then secrete various cytokines that mediate immune responses to pathogens. Cytokines are critical to the development and function of the innate immune response. These soluble signaling proteins can recruit and activate further immune cells like macrophages, neutrophils, and lymphocytes, to intensify the body's response to a pathogen[4]. They can also signal T-cells to hunt down infected cells and mediate T-cell proliferation.

Toll-like receptors play an important role in the innate immune system. They are a class of pattern recognition receptors that are used by the innate immune system to identify pathogen-associated molecular patterns[4]. TLRs span the membrane of the cell surface and are non-catalytic receptors that recognize molecules that are broadly shared by pathogens, but distinguishable from host molecules[3]. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS) and TLR2 recognizes bacterial cell wall components including peptidogylcan. We can use LPS and Pam3Cys (a synthetic TLR2 ligand) to activate TLR4 and TLR2 signaling, respectively.

TLR activation results in a signaling cascade that allows a protein complex called nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to travel into the nucleus and promote the transcription of genes that cause inflammation. NF-κB plays a key role in regulating the immune response to infection because it is a transcription factor for many pro-inflammatory genes such as interleukin-12 (IL-12)2. IL-12 is a cytokine that is naturally produced in dendritic cells in response to antigen stimulation. It was already known that phosphorylated protein kinase B (pAkt) and Phosphoinositide 3-kinase (PI3K) are important for the negative regulation of IL-12. For example, the PI3K-Akt pathway can inhibit NF-κB and the expression of inflammatory genes[2]. However it was not clear how the PI3K-Akt pathway itself was controlled.

There is increasing evidence that lysophospholipids including acid (LPA) regulate immune responses, but relatively little is known about the effects of LPA on DCs. LPA is a bioactive lipid that can induce cell proliferation, migration, and cytokine release. It can be generated intracellularly and extracellularly by different metabolic pathways, and is now known to bind a family of G-protein coupled receptors including LPA2[1, 5]. The lack of data about the effects of LPA on dendritic cells prompted our research into the effects of a LPA2 knock out in dendritic cells.

To activate the LPA2 we used Pam3Cys and LPS. Pam3Cys is a synthetic analog of the immunologically active N-terminal

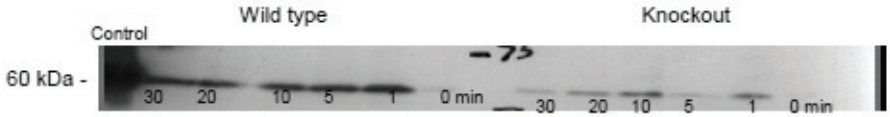

Figure 1. pAkt difference between wild type and knock out BMDC treated with Pam3Cys. Time points at 0, 1, 5, 10, 20, 30 minutes. pAkt band at 60kDa. Control is Jurkat Extract + Calyculin A (Cell Signaling). It clearly shows that there was strong phosphorylation of the wild type and very little in the knockout. The zero time point for the Pam3Cys samples had no phosphorylation. This shows that phosphorylation occurred after the samples were exposed to Pam3Cys.

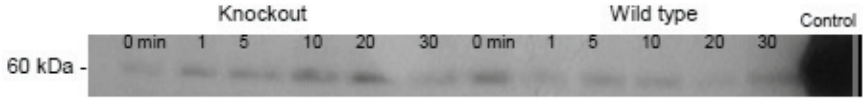

Figure 2. pAkt difference between wild type and knock out BMDC treated with LPS. Time points at 0, 1, 5, 10, 20, 30 minutes. pAkt Band at 60kDa. Control is Jurkat Extract + Calyculin A (Cell Signaling). The samples show similar amounts of weak phosphorylation with no differences between the wild type and knock outs. The amount of phosphorylation is the same as the zero time point, which means LPS had no effect on Akt phosphorylation in the cells.

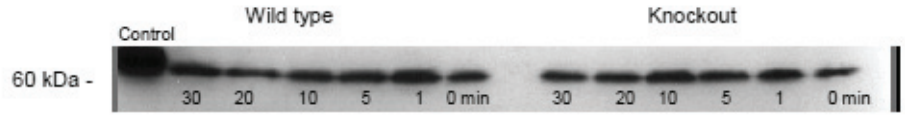

Figure 3. Total Akt difference between wild type and knock out BMDC treated with Pam3Cys band at 60kDa. Actin difference between wild type and knock out BMDC treated with Pam3Cys band at 42kDa. Time points at 0, 1, 5, 10, 20, 30 minutes. Control is Jurkat Extract + Calyculin A (Cell Signaling). This figure shows the same amount of Akt is present in both types of cells, but only in the wild type is Akt phosphorylated.

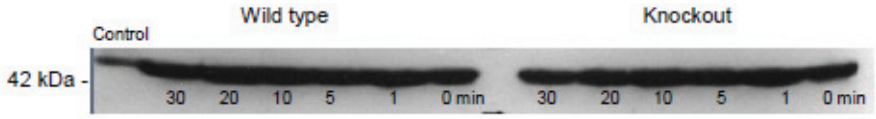

Figure 4. Actin difference between wild type and knock out BMDC treated with Pam3Cys band at 42kDa. Time points at 0, 1, 5, 10, 20, 30 minutes. Control is Jurkat Extract + Calyculin A (Cell Signaling). The amount of actin present in each of the samples is equal. Because the amount of actin is equal in all the samples, the amount of protein loaded in each lane is equal

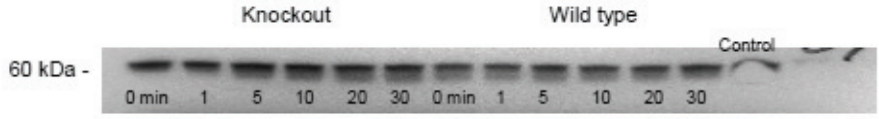

Figure 5. Total Akt difference between wild type and knock out BMDC treated with LPS band at 60kDa. Actin difference between wild type and knock out BMDC treated with Pam3Cys band at 42kDa. Time points at 0, 1, 5, 10, 20, 30 minutes. Control is Jurkat Extract + Calyculin A (Cell Signaling). This figure shows that there was Akt present in these samples, but it was not phosphorylated when the cells were treated with LPS.

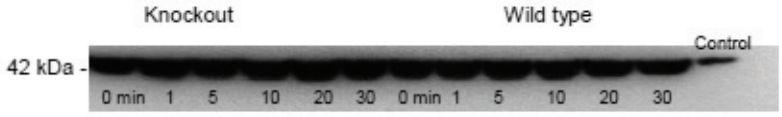

Figure 6. Actin difference between wild type and knock out BMDC treated with LPS band at 42kDa. Time points at 0, 1, 5, 10, 20, 30 minutes. Control is Jurkat Extract + Calyculin A (Cell Signaling). The amount of actin present in each of the samples is equal, which means that each well was loaded correctly.

portion of bacterial lipoprotein that activates TLR2 in monocytes and macrophages. LPS is a major component of Gram-negative bacterial outer cell membrane and acts as an endotoxin. LPS activates TLR4 in monocytes and macrophages. By stimulating the cells with these ligands, we could see the effect the LPA2 receptor had on each TLR pathway.

Methods

For this experiment we used lysates from LPA2 knock out and wild type bone marrow derived dendritic cells. They were collected from the bone marrow of wild type and LPA2 knock out C57BL/6 mice. The cells were then cultured using IMDM-10 complete media. These cells were treated with either synthetic lipopeptide Pam3Cys-SKKKK (Pam3-CSK4) or LPS from *E.coli* serotype O55:B5. Whole cell lysates were collected at time points of 0, 1, 5, 10, 20, and 30 minutes. They were stored at -80ºC until used in the experiment.

The samples were probed with pAkt antibody through western blotting. This was done by loading 30µg of sample per lane along with one lane of 5µg of Jurkat Extract + Calyculin A

(Cell Signaling) on a 10% SDS gel. The gel was run for 3 hours at 100V and then transferred to a nitrocellulose membrane using 80V for 90 minutes. The membrane was then blocked with 5% BSA/Tris-Buffered Saline tween (0.1%) (TBSt) for 45 minutes to reduce nonspecific binding of the primary antibody. After two washes for five minutes in TBSt (0.1%), anti-phospho-Akt (Cell Signaling) antibody was applied in a 1:200 dilution overnight at 4ºC. The next day the membrane was washed three times for five minutes with TBSt (0.1%) each time. Then α-rabbit IgG-HRP (Cell Signaling) antibody was applied for 1 hour at room temperature in 5% milk/TBSt (0.1%). The membrane was then washed three times for five minutes each. ECL Plus chemiluminescent (Santa Cruz luminal) was then applied to visualize the pAkt bands.

For probing using Akt total and actin antibody the western blot membrane was first stripped using 1X stripping buffer. It was then washed three times; five minutes each, with TBSt (0.1%). 1:1000 anti-Akt (Cell Signaling) and 1:3000 anti-actin (Cell Signaling) antibodies were applied for 1.5 hours at room temperature. The membrane was then washed three times; five minutes each, with TBSt (0.1%). 1:4000 α-rabbit IgG-HRP (Cell Signaling) antibody was applied for 1 hour at room temperature in 5% milk/TBSt (0.1%). The membrane was then washed three times for five minutes each, ECL Plus chemiluminescent (Santa Cruz luminal) was then applied to visualize the Akt total and actin bands.

Results

First, we tried to determine what affect Pam3Cys had on LPA2 and the TLR2 pathway. To do this, we probed with anti-pAkt used western blotting to see the rate of Akt phosphorylation in LPA2 knockout and wild type BMDCs treated with Pam3Cys. The samples collected at time points of 1, 5, 10, 20, and 30 minutes all showed strong Akt phosphorylation in the wild type cells and very little phosphorylation in the knock out (Figure 1). The zero time point for the Pam3Cys samples had no phosphorylation. This shows that phosphorylation occurred after the samples were exposed to Pam3Cys.

Next, we looked at how LPS affected LPA2 and the TLR4 pathway. This was done by probing with anti-pAkt on a western blot. We used LPA2 knockout and wild type BMDCs treated with LPS to determine the rate of Akt phosphorylation. The samples show similar amounts of weak phosphorylation with no differences between the wild type and knock outs (Figure 2). The amount of phosphorylation is the same as the zero time point, which means LPS had no effect on Akt phosphorylation in the cells.

Finally, we looked at the amount of total Akt and actin in the samples. Total Akt serves as a control to make sure each sample has the same overall amount of Akt in it. Probing with anti-Akt measured the amount of phosphorylated and non-phosphorylated Akt present. Actin is a protein used in the cytoskeleton of cells and is a control in this experiment to prove that each lane was loaded with the same amount of protein. To determine the amount of total Akt and actin present in the Pam3Cys samples, the membranes were stripped and re-probed with anti-Akt and anti-actin at the same time. The total Akt bands for Pam3Cys treated cells are even and consistent between the wild type and knock out cells (Figure 3). This shows the same amount of Akt is present in both types of cells but only in the wild type is Akt phosphorylated. The amount of actin present in each of the samples is also equal (Figure 4). Because the amount of actin is equal in all the samples, the amount of protein loaded in each lane is equal.

To determine the amount of total Akt and actin present in the LPS samples, the membranes were stripped and re-probed with anti-Akt and anti-actin at the same time. The total Akt bands for the LPS treated cells are also even and consistent between the wild type and knockout cells (Figure 5). This shows that there was Akt present in these samples but it was not phosphorylated when the cells were treated with LPS. The amount of actin present in each of the samples is equal, which means that each well was loaded correctly (Figure 6).

Discussion

These results suggest that the LPA2 receptor is an important component of the negative feedback mechanism that controls IL-12 production. It shows that when LPA2 is present, more Akt is phosphoralyted and because pAkt is known as a negative regulator for IL12 production, LPA2 counteracts antigens that cause inflammation. However, it seems that LPA2 only helps regulate certain TLR pathways. When the cells were treated with Pam3Cys which is an antigen for TLR2, there was a great difference in phoshporalytion between the wild type and knock out cells. But when cells were treated with LPS, which is an antigen for TLR4, there was no difference in phosphoralytion between the wild type and knock out. Previous research has shown that pAkt and PI3K are responsible for the negative regulation of IL-12[1], but this suggests that they are only intermediaries and LPA2 is a main component needed for negative regulation of IL-12. To confirm these results, we are planning to do further experiments, such as a gel shift assay to confirm that more NF-κB is bound to DNA in the knock out cells compared to the wild type. We are also planning to see if LPA alone can phosphorylate Akt in the absence of any other cell stimulation. Ultimately, if selective ligands of LPA2 can be discovered, they may potentially be used as anti-inflammatory therapies.

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Student Interview

Brian Palmisano, 2009

jur: First, tell us a bit about yourself.

Palmisano: I grew up in a small town called Quakertown in southeast Pennsylvania with two sisters, Amy and Megan, and my parents, Bill and Kate. I'm a senior biochemistry and chemistry double major. I'm interested in disease-oriented research related to cardiovascular disease and diabetes. I'm especially interested in how lipid metabolism and insulin signaling play roles in these diseases.

jur: Can you tell us a bit about the research you do?

Palmisano: At the moment, I'm not involved in any specific laboratory experiments. I'm working with one of my research advisors, Dr. Charlie Sparks, on preparing data from an ongoing project into a paper for publication. This involves designing additional experiements and piecing a bunch of data into a coherent body of evidence.

I've had other research experiences here at Rochester and the University of Pennsylvania. I worked for Dr. John Jaenike and Dr. John Werren during my freshman and sophomore years. Both professors conduct evolutionary biology and genetics research related to the parasitic bacteria *Wolbachia*. Over the last two summers, I worked at the University of Pennsylvania as part of an internship in molecular biology. There, I worked with Dr. Dan Rader and got interested in cholesterol and lipid metabolism research as it relates to cardiovascular disease. He actually knew Drs. Janet and Charles Sparks here at the Medical Center, and I worked with them over the last two years. Last year, I worked with Dr. Janet Sparks on a project related to insulin signaling.

jur: Could you provide more information about your cholesterol research or any experiments?

Palmisano: Well, it's an exciting time for biomedical research because scientists can identify potential genes contributing to a particular disease in genome-wide association studies. When I worked at the University of Pennsylvania, I was able to work on trying to determine the function of one such gene candidate, which was predicted to be involved in lowering LDL, the "bad" cholesterol. These kinds of studies have been conducted in many fields and now the challenge is to figure out what these genes do. It was really exciting to be a part of this cutting edge area of genetics.

jur: What got you interested in biology before this? Was there a particular topic or subject in which you were interested?

Palmisano: I actually didn't have any research or much science experience coming into college. I took a handful of science classes in high school, but that was it. From those basic courses, I realized that I liked science. The more biology classes I took, the more I liked it, and that continued in college. I like biochemistry because it reveals how living things work all the way down to the molecular level.

jur: What are your plans after school? Are you considering grad school?

Palmisano: I've been remodeling the answer to that question over the last year. Originally, I wanted to go to graduate school for a PhD to conduct research related to cardiovascular disease or diabetes. As I thought more about my interests, I realized I was interested in the medical aspects of diseases as well. So, I'm going to pursue an MD/PhD in the future. It's what I'm interested in, so if the extra degree adds a few extra years, then it's well worth it. The dual degree will let me pursue both the scientific and medical aspects aspect of the diseases I was already interested in studying. Pursuing an MD or a PhD alone would be fine, but I feel that I'll have more opportunities with the MD/PhD.

jur: How do you feel about research and science education in general? Is there any advice you would like to share with the future undergraduates?

Palmisano: I think research is a great extension of and supplement to science education. It's one thing to learn something in a book or to do a pre-designed experiment in a lab course, but it's much more exciting to use those methods and tools

you learn about in courses to discover something new.

As for advice, I would encourage anybody interested in science to try research in a lab. U of R is a great place to try research because there are so many different opportunities available here. I didn't have any experience before I came to U of R, but I've worked in a few labs since coming here and now I've got a lot of great experience. People might get discouraged or shy away from research because things don't always work out, but that's all part of it. That little bit of frustration pays off dividends when things finally work. Plus, when you're doing research, you're discovering something that nobody ever knew before.

jur: Is there anything else you'd like to add, a comment, perhaps?

Palmisano: I'd encourage people from all backgrounds to get involved in research. Science and medicine can benefit a lot from a diverse group of doctors and researchers. I came from a very modest background, and my parents may not know a lot about biomedical research, but I can apply what I learned growing up to different situations in the lab, and that helps me to be successful.

Brian Palmisano was one of several undergraduate students from the University of Rochester to be named a NIH Undergraduate Scholar. In 2008, Brian was selected as one of 14 recipients of the National Institutes of Health (NIH) Undergraduate Scholarship. In addition to a scholarship worth up to $20,000, Brian was also awarded a paid, 10-week summer research internship and a full-time, one year research position following graduation. Brian is currently completing his one year research position at the National Heart, Lung and Blood Institute (NHLBI) in Dr. Alan Remaley's laboratory at the main NIH campus in Bethesda, MD. In the fall of 2010, Brian will attend medical school to complete an MD/PhD dual degree.

The war on rape

Vanessa Rooney, 2009
Adviser: Kathryn Van Wert
Department of English

Rape is a cruel expression of dominance, a method of destruction, a product of inequality and a form of irreversible identity theft that has damaged the lives of hundreds of thousands of victims around the world. Though it is so common, it is difficult to categorize rape and to accurately understand why it happens. Individual rapists can be analyzed psychologically and biologically, but without taking into account the effects of culture, religion, political stability and social standing, it is nearly impossible to portray the crime accurately. Because rape is not something a society wants to deal with, this concept has not been studied until recent years when mass rapes of women during times of war forced it onto the public agenda. These rapes are examples of some of the most brutal acts of humanity that, during times of war, take on a different form than those attacks during times of peace. In this paper, I will examine episodes of rape and argue that a society's view on women, rape and soldiers during times of peace affects, and even encourages, the atrocities of mass rapes during times of war.

The main goal of rape during war is to completely destroy a society by terrorizing one family at a time. By destroying and humiliating women, the dynamics of the family crumble. This tactic is only effective because it acknowledges women as people who play a central role in the construction of a family, whether it is by rearing children or providing support for all family members. One would think that if men viewed women in this way then there would be no rape, especially during war when the emphasis is on protecting women and children. But the fact that mass rapes are so frequent shows that the value of women in families is overshadowed by deep-rooted feelings about gender roles. The problem of women being harmed is exploited to gain support for war, but it is barely addressed during peacetime. Does society truly care about the hurt women? In many ways, these women are nothing other than pawns of combat similar to land and homes.

This is a product of the perception in society that women are the property of men, which is a perception that has been maintained over hundreds of years. As Sharon Frederick points out in her book *Rape; Terror of War*, the first laws against rape stated that rape was actually a crime against the husband or father of the female victim (12). Without legal capacity, a woman was the property of her father or husband whose only roles of worth were to cook, clean and raise children (which were deemed to be easy tasks). As women finally began to gain certain rights, such as the right to own property and the right to vote, the issue of sexism was brought to the forefront of the public agenda. Issues of gender and sexuality finally became topics that people discussed openly, whereas in the past they were hidden because of society's more conservative nature.

However, this perception of women as property has not yet disappeared. It is suppressed by the rise of feminism and gained respect for women, but by looking at rape during times of war one can see that the instinct to think this way is still there, "the subordination of women during times of war is an extension of the power relations between the sexes within society generally" (Colombini 4). Many soldiers believe that enemy women are their spoils of war that are owed to them as a reward for winning a battle, a prize they have gained. The fact that the vast majority of women raped during war are 'enemy women' shows that the more inferior the women, the more property-like they become. Inferior women are more likely to be thought of as "booty" because the soldiers cannot relate to them at all, whether it is because of their ethnicity or their religion or simply because they are the enemy. This de-emphasizes sex in rape because rape becomes a pure expression of victory over everything the enemy possesses.

Because women are portrayed by the media as sexual objects who are both catty and overly emotional, rape seems like a justified, purely sexual crime. In her book, *Aftermath,* Susan Brison quotes a joke made by Howard Stern after the Columbine shootings which, in an extreme form, portrays the idea that women, regardless of the situation, can be thought of in a sexual light:

> "there were some really good looking girls running out with their hands over their heads. Did those kids [the suspects] try to have sex with any of the good looking girls? They didn't even do that? At least if you're going to kill yourself and kill all the kids, why wouldn't you have some sex?" (92)

Howard Stern, intentionally or not, encouraged rape as a last resort to dominate the school. This comment completely undermined the seriousness of the situation by finding a way to sexualize

women fearing for their lives. This provokes the idea that, during combat of any kind, women should be sexual companions for men.

Sharon Frederick argues that many people believe that rape is an inevitable product of armed combat because the soldiers fear for their lives and are in the most need of companionship (especially sexual) at a time when none is available. However, Frederick also points out that rape of enemy women is a completely separate issue from the dearth of "willing women" (7). The violent nature of the rapes themselves is evidence enough that rape during wartime is not about sexuality or a man's need for intimacy. If the rapes are not provoked by a need for sexual intimacy then they must be provoked by a different, more complex need that could have originated before combat began.

There are many different theories about how culture affects criminal behavior, but the overarching agreement among social scientists is that it does. Culture is, according to Parviz Saney in her book *Crime and Culture in America*, a "set of ideas, traditions, appropriate emotions and symbols" (31). Individuals are expected to obey certain social norms based on the specific ideals put forth by the culture of their birth. According to Saney, people are motivated to act by their desire to obtain what society thinks they should have and thus "every type of learned behavior is accompanied by a value judgment" (37). Evidence of this comes in the form of an individual's actions, specifically interactions with others. Human beings learn through their interactions with the people around them what behaviors are deemed acceptable and unacceptable in their culture. Because breaking these rules will have serious social consequences for the offender, individuals' behaviors are generally kept in check by their culture.

Does the continuing existence of criminal behavior suggest that most cultures do not do a good enough job of teaching people that violence is unacceptable? If people are truly motivated to act by how culture tells them they should, then this suggests that there is a part of our culture that promotes violence or certain types of violence. While many scientists would say that violent behavior is part of human nature, culture usually makes us hide our primal instincts in the hope of assimilation. There are by nature going to be some people who seek attention by breaking social rules or, for biological reasons, are unaware of social cues; however, the average citizen is not a rapist because our culture tells people not to act on certain instincts. This unfortunately suggests that if those social boundaries were not in existence, rape would not be condemned and would thus happen far more frequently. This can only be verified by studying how the variation between societies in their emphasis on cultural values affects the rate of crime.

Cultural variation is a product of differences in the expectations of the citizens in a society. In many cultures around the world, people are taught to accept their social status as their fate given to them by some form of higher power. For example, Indian culture follows a hierarchical caste system based on specific guidelines found in Hindu scripture. According to Saney, this sets a more rigid code of expectations and thus there is less of a chance of individuals breaking it. American culture, on the other hand, is based upon the fact that people can change their social status through hard work. Because this makes American culture more individualistic there is a greater chance that individuals will transgress the social norm (12). This is in accordance with the statistics given by Saney (15) that the United States has the highest rate of homicide, murder, assault and rape but the lowest rate of group violence and assassination.

The idea of group violence ranges from gang fights to civil or world wars, but there are fundamental differences regarding who fights them and how they are received by society. The most important distinction is that the members of a gang are considered criminals whereas soldiers are not to be associated with criminal behavior. Thus far I have discussed criminal behavior during times of peace, but during war the concept of a criminal is not well defined since soldiers are considered heroes to some and criminals to others. During peacetime, rape and murder are clearly criminal acts, but during war, they are at times accepted and even encouraged. Because of this, a soldier's idea of criminal behavior is tainted; the enemy's activities are criminal while their own activities are somehow for the greater good of the people they are protecting.

During war, soldiers transgress the social norm of not partaking in criminal behavior because it is deemed acceptable for them to do so. This causes a conjuring of enormous moral dilemmas for soldiers. It is accepted that during times of war, soldiers have to choose which morals of their normal lives they want to uphold and which ones to put aside and decide that 'desperate times call for desperate measures.' This could suggest that the soldiers who rape during war had suppressed tendencies towards criminal behavior during times of peace. While this may be true for some, war changes soldiers once they are in combat or go through something traumatic. Soldiers have an incredibly hard time dealing with their actions and being so far away from family and normal life that sometimes their normal, peacetime judgment is clouded.

The feeling of admiration towards soldiers partially comes from our recognition that they give up normal life to fight for the better of our country. U.S soldiers, for example, are portrayed as heroes and ideal all-American gentlemen that demand respect. We honor our veterans because they risked their lives to protect us and our country. Stories about war tell of its atrocities, so we admire the courage a soldier must invest in combat. If we have nothing but respect for soldiers without turning a critical eye to how victory is won, then the issue of mass rape is lost amongst the exciting stories of adventure, escape and terror. Somehow, we as citizens, justify this by feeling personally benefited when our soldiers destroy the enemy because the media tells us that the world will become a safer and more just place without them.

The media does this by reminding us that the world today is a far more dangerous place for civilians. Soldiers no longer fight other soldiers on a battlefield; they fight unmarked civilian soldiers in cities and villages. It is the civilians that are the most impacted by mass rape. This makes the issue of where fighting takes place more prevalent. America, for example, has not truly been a battle ground since the Civil War and thus its women have not experienced firsthand the atrocities of mass rape during war. War has also become more impersonal and cruel with the invention of new weapons and technology that distance the soldiers from their targets. When war becomes impersonal, total domination becomes the emphasis. Even though rape is an incredibly personal act with the intent of destroying certain people, the tone of war has made it easier to commit the crime of dehumanization (Frederick 7).

American culture explains the increasing amount of dehumanization during war by medicalizing all the unexplained problems of its soldiers and its citizens. Soldiers often suffer from various psychological disorders such as Posttraumatic Stress Disorder which,

according to some, explains the instances of grotesque rapes. A dominant theory among American psychologists as stated in the book, *Men Who Rape,* by Nicholas Groth is that rapists have a form of "psychological dysfunction and sexual deviation" (4) which separates them from the average human being. Groth believes that one of the fundamental reasons for why rape still continues on such a large scale is because it has not fully been accepted into the realm of psychological disorders. The assumption that this theory makes is that a rapist has a clinically diagnosable distorted view of reality and, more specifically, a distorted view of how others will react to their actions.

The major problem with labeling rapists as "ill" is that it takes the emphasis away from the victim and places it on the attacker. This is not to say that American culture promotes empathy towards rapists but it does, in a way excuse them. When emphasis is taken away from the intense pain and suffering caused to the victim, it downplays the immensity of the crime and allows it to continue. When rape becomes 'not such a big deal', the media will not pay any attention to the issue and thus, the cycle continues; rape is kept out of the public agenda and the victim becomes unimportant. According to Brison, evidence of this can be found in most crime films and TV shows when the victim (of a crime other than murder) is only a part of the plot for initial few minutes while the rest is about the cop chasing the bad guy. Brison argues that this is an example of why the criminal justice system in the U.S. has little concern for victims of rape--after all, victims are not even the ones with the right to a speedy trial (10).

Women go through a ludicrous amount of strife to get their cases heard. The sheer amount of paperwork alone would discourage any woman from testifying against her opponent. Even though many psychologists agree that personal narrative in a court or outside is an extremely effective method of healing (Brison 14), it is nonetheless difficult to testify in a rape case where the burden of proof lies solely upon the woman's capability to prove her validity. This is an important explanation for why rape still occurs and why so many rapes go unreported or unpunished. The fact that women have to prove that their rape was not somehow their fault shows the underlying inequality between men and women that influences culture. Women are taught at a very early age not to be alone at night (especially in a city) and that strangers are all harmful. They are also taught that if they are perfect, young women, nothing bad can happen to them. There is a commonly believed myth that if a girl is wearing a skirt that is too short or a top that is too low, she shouldn't be surprised if a man takes advantage of her because she is giving off the impression that she is looking for sex. If men start to believe these myths then rape becomes less of an offense.

This issue is of crucial importance in places such as Afghanistan where women are already restricted in everyday life by the laws of their country. There are places where women are not allowed to go outside without being accompanied by male relative, to work outside of the house, to attend school and are certainly not allowed to go anywhere without every inch of their bodies being covered up. Islam puts extreme importance on the virginal woman and the faithful woman, so if a woman is raped, she is considered impure and even an adulteress. Because the Quran does not sanction rape specifically and it is, in fact, considered an extremely offensive religious crime, culture finds an excuse for why it must be happening. There is a common belief that women who are raped must have been acting in a promiscuous manner by wearing jewelry or showing skin, in which case they are showing no respect for the Quran. This infers that every single rape is entirely a woman's fault. Therefore a woman's case is very hard to make and it is incredibly hard to indict someone for rape (Frederick 53).

In cases of rape during war, soldiers know that it is almost impossible for them to get in trouble for their actions. For one, there are so many victims and so many attackers that it would be impossible to try them all. Additionally, during war, when there are so many atrocities, soldiers can be assured that rape is on the bottom of the list of things to be charged for (if they are charged at all). Besides, would a court truly believe an enemy woman over a soldier who was willing to die for his country? This makes rape an effective tactic of war because it involves domination without killing and little chance of penalty.

This is many a time used as evidence in feminist theory that rape is not an act of pure sexual force, but an act of dominance fueled by the need for power. Feminist literature seems to agree that rape is an act of aggression, violence and dominance by sexual means but is not a product of uncontainable lust. This would adequately describe the reason for the excessive vaginal mutilation that occurs during times of war. If sexual pleasure is the goal then soldiers would not, for their own purposes, destroy vaginas in such dehumanizing and demoralizing ways. Vaginal mutilation is purely an act of humiliation and that makes a woman feel "useless" and her attacker feel empowered.

Many scientists such as Thornhill in his novel, *The Natural History of Rape*, argue that this theory completely undermines the theory of evolution of species. Thornhill argues that this is feminist propaganda to make men seem more violent and completely undermine the "fact" that "biologically driven sexual motives play a role in the commitment of sexual assault" (125). Connel agrees when he says that "rape and combat- however regrettable- are part of the unchanging order of nature" (215). But even though Connel sites testosterone as a factor of aggression, he acknowledges that is not the "answer to rape."

Because testosterone is not the answer, Connel and Thornhill agree that the problem is other men. According to Connel "most violence is not a matter of individual pathology" (215), so rape must be a collective idea. Though this does explain why rape during war is almost exclusively committed by groups of soldiers, it does not account for rapes during times of peace which are (for the most part) committed by individuals. Additionally, it does not provide an explanation for why a group would collectively decide to commit mass rape during war. It is possible that the cultural and political climate could explain both of these problems. Connel and Thornhill are suggesting that it is a violent society or community that makes rape a "collective idea" even if the rape is committed by an individual. During war, when violence is inevitable, it is the specific character of the war (which is greatly influenced by the specific cultural and political climate at the time) that dictates what kind of violence will be used.

Fighting begins in many countries because a large group initiates the overthrowing of a corrupt government or a forced removal of another ethnic group. In Haiti, for example, the goal of mass rape was to drive families out of the country who supposedly supported Aristide. Rape was specifically used to show the men of the community that their political activities would be punished

in the form of rape of their wives and daughters (Frederick 27). Political instability, in general, causes large groups of people to rally together for a cause in a violent way. If the government emphasizes freedom of speech and peaceful dissent, people are more likely to break rules privately and alone because they fear being labeled an outcast. Most rapes that occurred during World War I (WWI), World War II (WWII) and civil wars in almost every country were rapes of 10,000 to 600,000 women and girls. This cannot possibly be the act of individuals acting alone.

There is some evidence that the rapes which occurred during WWII were an official tactic of the German army. There are some cases in which every single woman and girl in a village was documented to have been raped; this can only arise through planning and organization. Eventually, this worked against the German army when the war turned in favor of the Russians. The Russian soldiers decided to use the same tactic and rape German women as an act of revenge. This proves that the Russian soldiers had no real empathy towards the women who were raped since they were willing to commit the very same act against other women; to take 'an eye for an eye' was far more important than upholding the honor of women (17).

The media however, portrayed the soldiers as people fighting solely for that honor. During WWI, the enemies of the Germans used the fact that the German army was mass raping women in France and Belgium as propaganda to gain support for the war by making people realize that the enemy was a danger that needed to be destroyed. Even though this was effective because the media successfully played off of society's empathy for victims of the enemy forces, there was not enough empathy to punish the offenders in a way other than attempting to kill them or rape their women. The fact that the rapes went unpunished during WWI proved that society chose to forget about them thus paving the way for even more brutal and blatant rapes (14).

The rapes during WWII had slightly different motives. The major difference was that the German army was attempting to create a "master race" by biologically cleansing the "dirty" people in Europe. This does not mean that the soldiers wanted to procreate with Russian women; they wanted to humiliate and destroy them enough so that they would never have children again. The evidence of this tactic of humiliation was that rape was usually done in front of family members (usually fathers and husbands) so that the soldiers could prove their dominance over them (16). This idea could not possibly have been concocted during times of war alone. Anti-Semitism and sexism were common in German society and war allowed the German army to act on the hatred which had been brewing since WWI.

Also during WWII in Nanking, the Japanese raped far over 40,000 Chinese women, children, pregnant women and even elderly women. These were some of the first documented vaginal mutilations. Rape was not enough; the vaginas of these women were ripped apart and mutilated until they were useless. When news of these rapes reached the West, the Japanese decided to save face by creating a movement called the "comfort system" which was, in essence, a legal form of forced prostitution for soldiers. In Manila, 500 women were imprisoned in a hotel for 8 days and raped repeatedly by soldiers rotating through.

It was because of this that General Tomoyuki Yamashita became the first general in history to be "held criminally liable for acts committed by his troops" (20). This was an enormous accomplishment in relation to the Nuremberg trials where the "Tribunal essentially ignored crimes against women…there was no prosecutions, nor was there any effort to at least publicly document sexual atrocities" (17). Considering the history of women attempting to win court battles against their attackers, it was not surprising that it took until the late part of the 1900's to finally gain some justice for rapes during war. But the fact that it took 40,000 women to be raped in order to gain public acknowledgement of the issue speaks volumes to the importance (or unimportance) of women in society.

Today, one of the most prevalent cases of mass rape is going on in the Democratic Republic of the Congo. In an article by Eve Ensler, she describes a hospital for women who are survivors of the atrocious rapes. These rapes are, like all the others, characterized by brutal vaginal mutilation. One case Ensler describes is of a woman whose attacker "unloaded his entire cartridge into her vagina." She surprisingly survived and walked all the way to the Panzi hospital for treatment. She walked because she could not ask for help since she was afraid of what her culture would think of her if they knew she was raped.

In an interview, a doctor of the Panzi hospital mentions that many people come to the hospital, cry, and send some money, but do nothing further. Even though it is idealistic to expect that everyone who cares should drop what they are doing and go work at the Panzi hospital, it is surprising that so few alternatives to giving money have been exercised. Women may be able to convince themselves that it would never happen to them if they live in a 'safe place' but the fact that very few of them are willing to go out of their way to help those women in need just further proves society's hushing influence on women. Without raising awareness about the subject, it will never reach the level of publicity that it needs for real help to be given. Women can't be so afraid of disturbing the notion of a perfect soldier or bringing attention to themselves by speaking out against such atrocities as rape even if it have been deemed a "woman's issue" that men supposedly won't want to talk about. Even the fact that rape is considered a woman's issue and not a national problem proves the underlying existence of sexism in many societies. While acknowledging that there are matters in the world (especially during war) which deserve equal if not greater attention, when the number of women raped reaches 80-100,000, it is time to act.

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