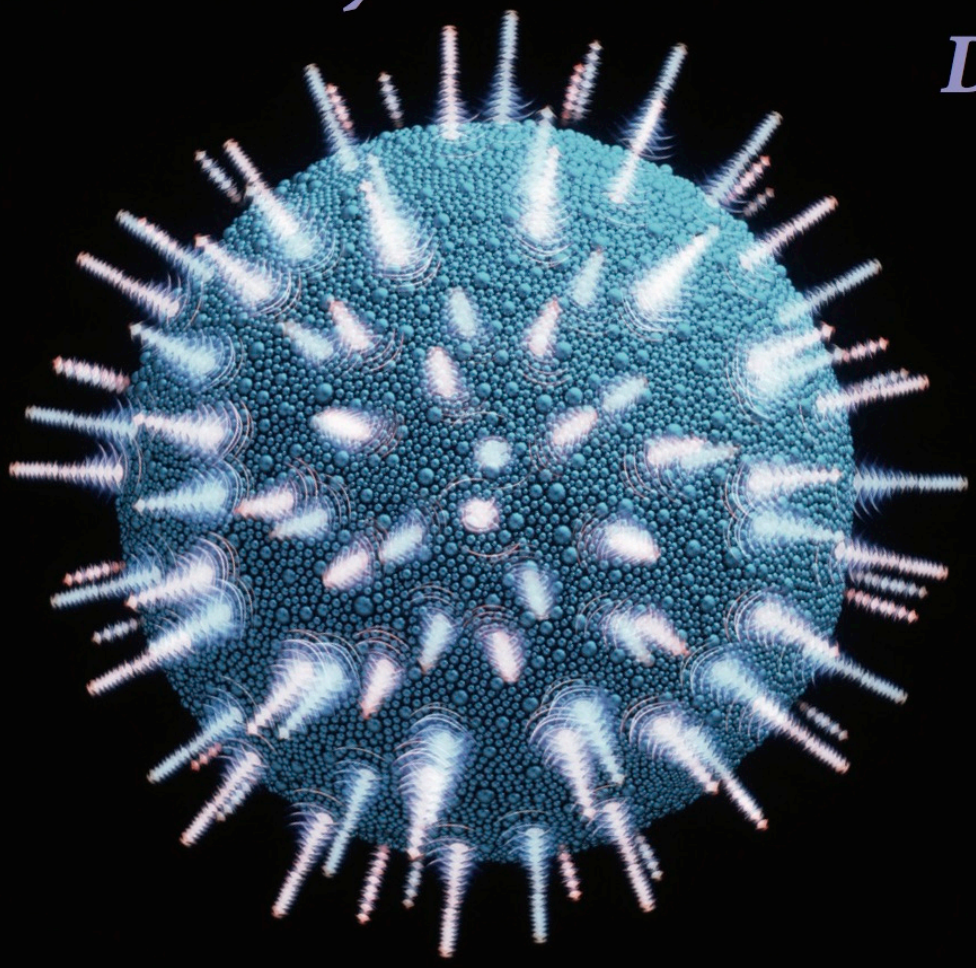
Volume 22 Winter 2012

MURJ

Massachusetts Institute of Technology
Undergraduate Research Journal

Cover Story: Mutated HIV Evades Death

p. 28



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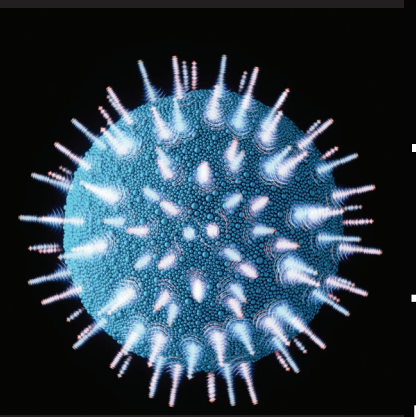

MURJ

Massachusetts Institute of Technology
Undergraduate Research Journal

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February 2012

Dear MIT Community,

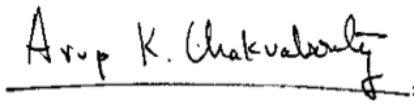
MIT is a perfect example of the American research university, where research and teaching are synergistic functions which together enrich a student's education. In 1969, MIT had the brilliant foresight to initiate the Undergraduate Research Opportunity Program (UROP) to further enable undergraduates to learn by "doing" – it allowed students to work on problems at the forefront of innovation and discovery in the laboratories of MIT faculty. Today, the UROP is an integral part of the MIT culture.

As engineers and scientists, we live in a time of special opportunities. Technological solutions will be key for addressing some of the greatest challenges that humanity faces today. Two examples of grand challenges are: 1] Creating new energy technologies and a sustainable planet. 2] Improving human health by the development of new therapies that can be widely deployed around the globe. MIT is playing a prominent role in addressing these challenges by approaches at the convergence of diverse disciplines, and our undergraduates are at the leading edge of these new frontiers. This is not surprising because, to paraphrase Karl Compton (XXth MIT President), at MIT we develop superb engineering solutions based on excellent science to address major societal problems.

Just this issue of the MIT Undergraduate Research Journal (MURJ) makes vivid what can happen when the ingenuity of our undergraduate students is brought to bear to attack significant problems. Highlighted research projects include reports on education in India and the autoimmune system.

I am honored to introduce this issue of MURJ. Across all departments at MIT, our undergraduates are contributing to solving problems at the frontiers of technology and science. I urge all undergraduate students to learn by doing, and feel the thrill of discovery and innovation, through a UROP experience.

Sincerely,



Arup K. Chakraborty
Robert T. Haslam Professor
Chemical Engineering, Chemistry, & Biological Engineering

Massachusetts Institute of Technology

UNDERGRADUATE RESEARCH JOURNAL
Volume 22, Winter 2012

Printed at Universal Millenium

February 2012,

Dear MIT Community,

We are honored to present to you Issue 22 of the MIT Undergraduate Research Journal (MURJ), a biannual publication highlighting the extraordinary research completed by undergraduates at the Institute. We hope that the work presented in this issue will help exemplify the importance and creativity of work done by undergraduates and showcase their enthusiasm to explore various fields of science and engineering firsthand.

In this issue, we present work spanning from research on the autoimmune system, HIV, and education in India. We also are pleased to present three feature articles on the topics of synthetic biology, senescent cells, and an interview with 2004 Noble prize winning physicist Professor Frank Wilzcek, alongside a collection of several student-written news articles dissecting some of the latest and most interesting happenings within the realms of science.

It is our hope that this journal displays the vibrant intellectuality and curiosity found in undergraduates throughout the Institute. This issue has been made possible by the hard work of many students, departments, and faculty across MIT. We would especially like to thank the MIT Student Activities Office, the Office of Undergraduate Advising and Academic Programming (UAAP), and the MIT Publishing Bureau for all of their help and assistance. Additionally, we must thank all of our executive members and associate editors in reviewing submissions, editing content, and ensuring the utmost quality of work. And lastly, we would like to thank all of our undergraduate authors for sharing their research with us and the larger MIT community. We hope you enjoy this issue!

Sincerely,

Omar Abudayyeh
Co-Editor-in-Chief

Ana Lyons
Co-Editor-in-Chief

Evelyn Wang
Co-Editor-in-Chief

PSB 07-02-0097

Science News In Review

Using E. Coli for Biofuel Production

In the race for an alternative to petrochemicals as a fuel source, a Rice University research group has taken an unconventional route. Conventional methods of making biofuels utilize a cell's fatty acid production capabilities and try to speed up the process to achieve a feasible production of biofuels. However, nature seems to not have evolved an efficient process for long chain fatty acid production. Rice University researchers have taken an unconventional route in that they are using the cell's beta oxidation cycle. The beta oxidation cycle is used by cells to break down fatty acids and provide the organism with energy. The research group, through careful manipulation of genes in E. coli, have managed to reverse the beta oxidation cycle to suit their purposes. They achieved a butanol production rate 10 times higher than anything previously reported. The team also managed to show that through more genetic manipulation, they could get the cells to produce fatty acids of desired lengths.

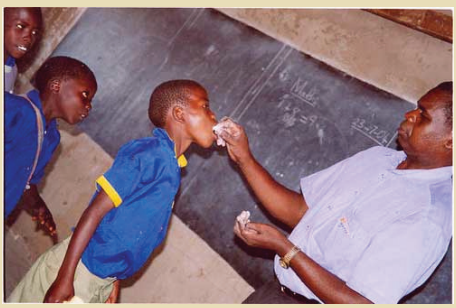
—J. Sanchez

Source: http://www.media.rice.edu/media/NewsBot.asp?MODE=VIEW&ID=16031

Biofuels have increasingly become a point of interest for governments across the world in the search for greener energy sources.
Credit: http://www.our-energy.com/pictures/static_content/biofuels/biofuel_image.jpg

Beyond Aid: From research to action

In the world's largest child deworming program to date, the Indian government announced in late September that more than 17 million children in the northern state of Bihar had been treated for intestinal worm infections. Although other similar programs have been taken up around the world in the past, the bureaucratic and logistical hurdles involved in scaling them up often prevent large-scale introduction of deworming to children, despite strong evidence that these programs are highly effective. For instance, economists Michael Kremer of Harvard and Ted Miguel of UC Berkeley found through a randomized study in Kenya that school absenteeism fell by 25% for children who were offered deworming treatments. Additionally, children who were not treated still benefited from the decrease in transmissible diseases. Similar research has been performed at J-PAL at MIT in Kenya as well as various parts of India, with equally encouraging results. Kremer and Miguel's evidence, along with meetings with politicians and leaders as well as media and advocacy campaigns, that provided the support for large-scale programs such as Deworm the World at UC Berkeley, which has resulted in the deworming of 37 million children around the world.



Deworming in Kenya.
Credit: http://www.povertyactionlab.org/evaluation/primary-school-deworming-kenya

—P. Thaker

Sources: http://blogs.berkeley.edu/2011/10/04/deworming-makes-history-from-research-to-action-in-india/; http://www.povertyactionlab.org/evaluation/primary-school-deworming-kenya

Malaria Vaccine Shows Promise

Malaria is a parasitic disease that results in fever, anemia, and retinal damage, among other symptoms, with severe cases of malaria resulting in coma or death. As a mosquito-borne infectious disease, malaria is widespread, affecting 109 countries. The epidemic nature of the disease is unquestionable, as half of the world population (i.e. 3.3 billion) is at risk.

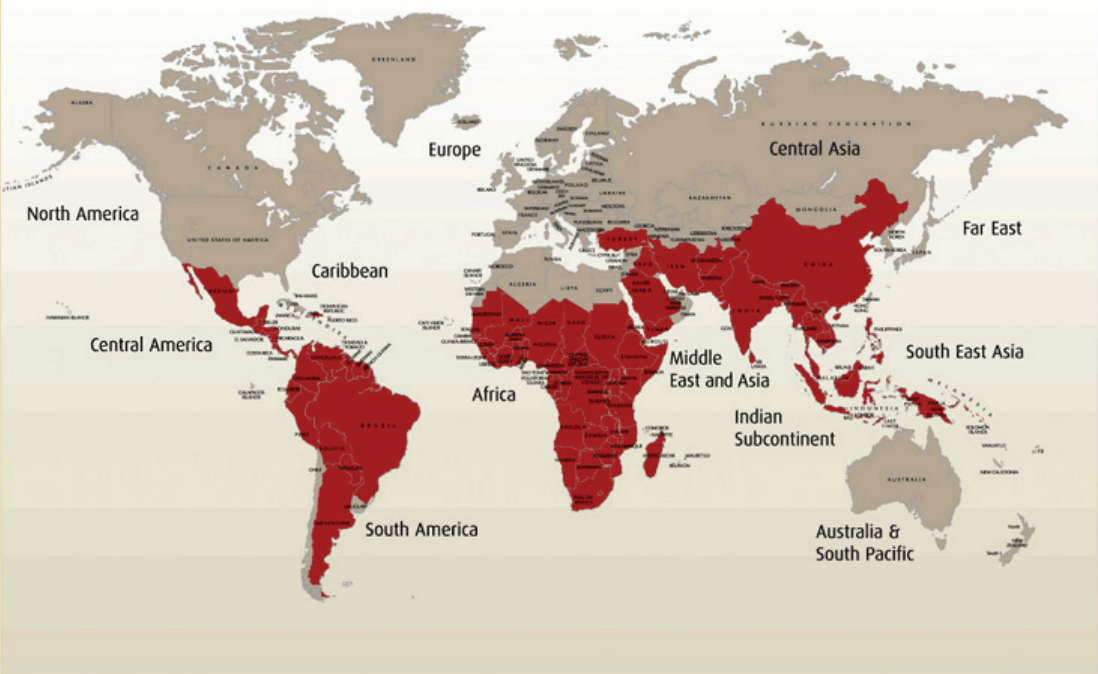
Developed by GlaxoSmithKline Biologicals in Belgium and the PATH Malaria Vaccine Initiative (MVI), a vaccine called RTS,S has been shown to provide some level of protection against malaria. Studies have shown RTS,S successfully reducing malaria episodes in infants by half. In the initial trial performed in 2000 with children from Mozambique, the risk of developing symptoms of malaria decreased by 35. A second, Phase II, trial was conducted in Mozambique, Tanzania, and Kenya, and malaria episodes decreased by 35-53% after the administration of RTS,S. After further improvements, one more trial was required to determine whether the vaccine would perform to GSK Biologicals' and MVI's expectations. Involving more than 15000 infants, the Phase III trial showed that RTS,S cut the risk of malaria episodes by 56% in 6000 children ranging from 5 to 17 months in age. The risk of severe disease was also reduced by 47%.

Although children injected with the vaccine were found to have a slightly higher rate of seizures compared to those injected with the control vaccine (rabies), the independent safety board responsible for overseeing the trials has not made this a point of concern, and the vaccine appears to be safe.

Overall, the researchers and the international community are pleased with the results. While most vaccines nowadays have up to a 90% protection rate, RTS,S is not expected to be a standalone solution to malaria. As Joe Cohen, the leader of the malaria vaccine project at GSK Biologicals, recommends, RTS,S should be used in combination with other malaria-protection methods, such as bed nets. Despite the vaccine's relative success at combating malaria, one major issue with the vaccine that has yet to be addressed is whether it can become more cost-effective for the developing world. Nonetheless, with 881,000 people dying of malaria each year, any advancement that can be made is a welcome one, and RTS,S provides an important step forward towards eradicating malaria.

—C. Wong

Sources: http://news.sciencemag.org/sciencenow/2011/10/malaria-vaccine-meets-modest-expectations.html; http://www.rbm.who.int/keyfacts.html



According to the World Health Organization (WHO), there were 225 million cases of malaria in 2010. In Africa, a child dies every 45 seconds as a result of malaria.

Credit: http://www.smeds.org/7th%20Malaria/Peterson/places%20affected%20by%20malaria.gif

First Successful HIV Vaccine Explained

For the first time since the beginning of the search for a successful vaccine against HIV, scientists finally have begun to find a molecular foundation behind the success of the first successful HIV vaccine trial. In 2009, Thai scientists working on the vaccine trial reported that out of the more than 16,00 people who participated in the study, people who received a drug regimen consisting of two vaccines that failed on their own, were 30 percent less likely to contract HIV. Researchers studying samples from these patients found that subjects with a specific antibody (a Y-shaped immunoglobin antibody) that recognized parts of HIV virus's outer envelope were less susceptible to contraction of HIV whereas patients that with a different IgA antibody were more likely to be infected than subjects who did not produce this specific antibody.

With these results, researchers now are planning new studies to further study these antibodies in both primates and humans. Wayne Koff, leader at the International AIDS Vaccine Initiative, and many other leaders in HIV Vaccine research believe that these results are promising and provide encouraging leads.

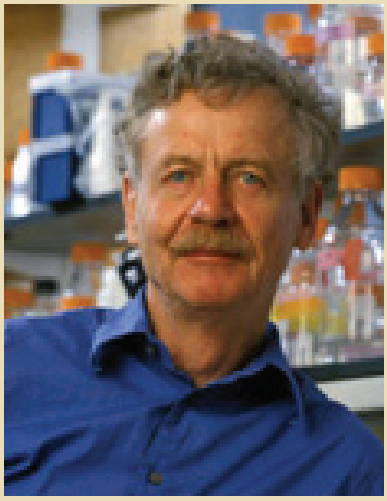
—C. Huang

Source: http://www.nature.com/news/2011/110916/full/news.2011.541.html

In 2009, according to the World Health Organization, 33.3 million people were living with HIV/AIDS.

Credit: http://4.bp.blogspot.com/-XEpu_ivhhv4/TfZKkzsntvI/AAAAAAAAlik/ViSralas9BM/s1600/south-africa-hiv-iac.jpg

MIT Professor Rudolf Jaenisch Awarded National Medal of Science



Dr. Rudolf Jaenisch, MIT Professor of Biology and Whitehead Institute member.

Credit: http://www.wi.mit.edu/research/faculty/jaenisch.html

This past October, Whitehead professor Rudolf Jaenisch was awarded the National Medal of Science in a White House ceremony by current president Barack Obama. Joining eight other current faculty members at MIT, Jaenisch was honored for his pioneering research in "improving [the] understanding of epigenetic regulation of gene expression: the biological mechanisms that affect how genetic information is variably expressed, [leading] to major advances in our understanding of mammalian cloning and embryonic stem cells".

Jaenisch's current research goals include the desire to understand epigenetic regulation and its role in both normal mammalian development and deviations that lead to diseases. His lab's work focuses on understanding the mechanisms that allow somatic cells to revert to a state more like pluripotent stem cells and then use that understanding to study human diseases. Jaenisch's research achievements thus far include the first to generate transgenic mice and later showing that therapeutic cloning can treat genetic diseases. He was also able to successfully take reprogram somatic cells into pluripotent stem cells and use these cells to treat genetic diseases like sickle-cell anemia and Parkinson's disease.

—C. Huang

Source: http://web.mit.edu/newsoffice/2011/medal-of-science-jaenisch.html

The Connection Between Infertility and Miscarriage

According to the Mayo Clinic, 10-15% of American couples are infertile.
Credit: http://www.onmedica.com/GetResource.aspx?resourceId=a96c9e57-e2ad-4010-b0a1-a89e858917bd&width=210

Researchers from Imperial College London have linked an enzyme to infertility and miscarriage. The research team collected data from over 100 women. Women who were infertile had high levels of the enzyme SGK1. To further the discovery from only a statistical linkage, researchers collected data from the womb lining of mice. The data pointed to a decrease in SGK1 levels during the time that the mice can become pregnant. Insertion of more SGK1 into the mice rendered them unable to become pregnant. The researchers believe that a decrease in SGK1 levels is key to embryo acceptance by the uterus. Unfortunately, the problem is more complex. While low levels of SGK1 help combat infertility, constant low levels of SGK1 after impregnation may be linked to miscarriages. The mice whose SGK1 genes were blocked became pregnant but showed signs of bleeding. As a result of this discovery, the research team will narrow their focus with the hope that this knowledge will eventually be used to treat human infertility and recurring miscarriages.

—J. Sanchez

Source: http://www.bbc.co.uk/news/health-15305064

Language and the Brain

Cognitive scientists have long been interested in the area of functional specificity, a field that studies whether certain parts of the brain control certain actions, movements, or other cognitive tasks. Some areas of cognition, such as divisions of motor control, have been localized to specific sets of neurons. Evelina Fedorenko, a research scientist in the Department of Brain and Cognitive Scientists at MIT, has extended this list of exclusive sets of neurons to include certain parts of the brain that are specialized to language through a new use of functional magnetic resonance imaging, or fMRI, techniques. Where previous studies have been inconclusive, or suggested that these areas overlap with other cognitive tasks such as music and arithmetic, Fedorenko's methodology allowed her, along with Walter A. Rosenblith Professor of Cognitive Neuroscience Nancy Kanwisher, and undergraduate Michael Behr, to analyze nine regions of the brain and show that eight of them had no significant activation for seven tasks other than language. The researchers' next steps will be to rule out further non-linguistic tasks from these areas of the brain and determine which particular linguistic functions each of the eight regions has.

—P. Thaker

Source: http://web.mit.edu/newsoffice/2011/language-brain-0830.html

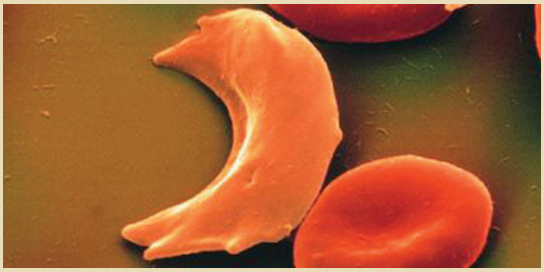
Direct correlations have been found between particular bodily functions or cognitive tasks and specific areas of the brain.
Credit: http://medicmagic.net/wp-content/uploads/2010/04/brain1.jpg

Transforming Sickle Cells to Normal Red Blood Cells

Sickle cell disease is characterized by abnormal hemoglobin that is unable to carry the normal amount of oxygen as a result of its sickle-like shape, and occurs only after birth. Dr. Stuart Orkin of Harvard Medical School, Children's Hospital, and the Howard Hughes Medical Institute and his team had previously identified a protein (BCL11A) that was responsible for the conversion from fetal hemoglobin to adult hemoglobin - a transition that occurs in all humans after birth. In a recent study, the Orkin group blocked the BCL11A protein from being produced in mice affected by sickle cell disease, and observed that the mice reverted to producing fetal hemoglobin. The mice with sickle cell disease also showed improved health (reduced symptoms). The results of Orkin's study may have implications on future treatments for the disease for humans. At present, there is no widely available cure to sickle cell disease, an illness that affects 3- to 5-million people across the globe.

—C. Wong

Source: http://news.sciencemag.org/sciencenow/2011/10/young-blood-to-the-rescue.html



(Left) "Sickle cell" - mutant hemoglobin, (Right) a normal red blood cell.
Credit: http://sicklecellcurefoundation.org/wp-content/uploads/2009/09/sickle-cells-586x284.jpg

MURJ Features

Forever Young?: *Slowing Down Aging*

By: Elliot Akama-Garren

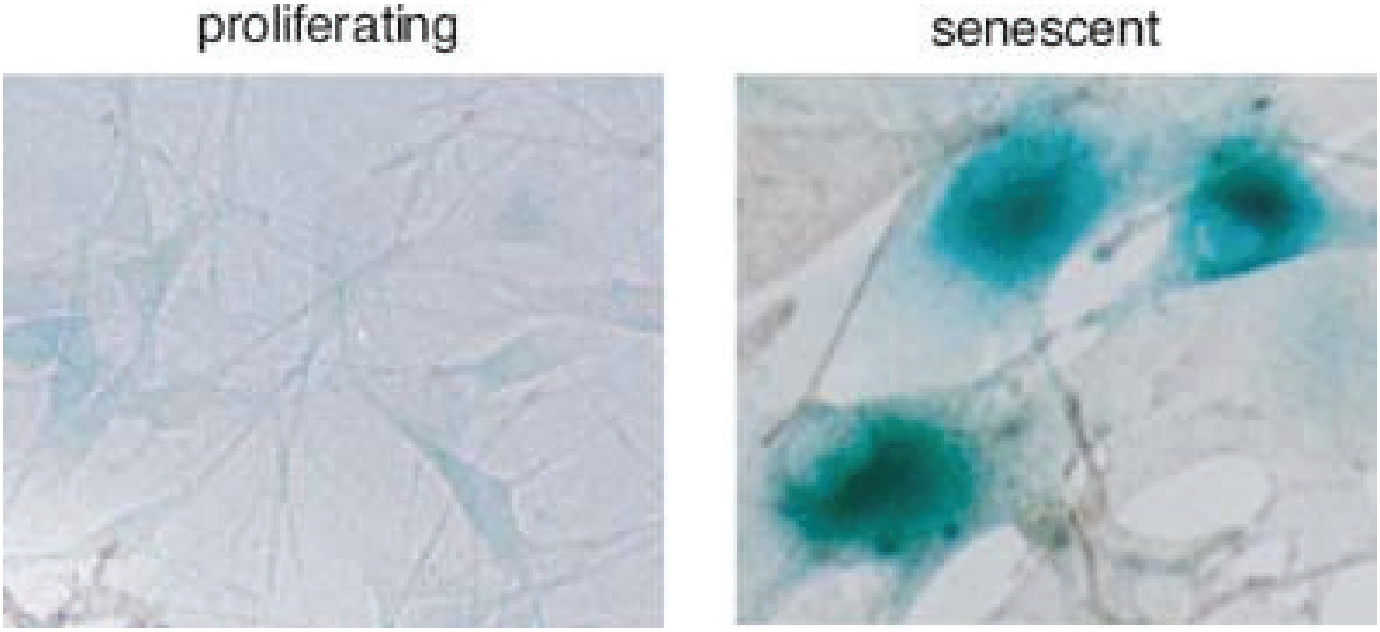
The possibility of a fountain of youth has taken one step closer to reality. Researchers at the Mayo Clinic have delayed the onset of wrinkles, muscle wasting, and cataracts in mice [1]. French scientists also announced that they have successfully reprogrammed cells from 100 year old humans into stem cells, capable of restoring youth [2]. These two discoveries suggest the possibility of slowing the aging process.

Both of these studies involve eliminating "senescent cells," cells that have stopped dividing and contribute to aging [3]. Normally, the immune system gets rid of these senescent cells; however, as people age the number of these cells increases. The recent interest in senescent cells has brought life to the field of aging research. Over the past 20 years, researchers have identified new genes that contribute to aging, but have produced few applicable results. Experiments done with senescent cells "suggest therapies that might work in real patients," according to Dr. Norman Sharpless of the University of North Carolina.

In the Mayo Clinic study [4], researchers gave mice a drug that killed all senescent cells. To measure aging, researchers investigated the formation of cataracts, muscle wasting, and loss of fat (which contributes to wrinkles). As a result of clearing out senescent cells, the onset of these symptoms was "dramatically delayed" in mice.

The Mayo Clinic study is the first to show that eliminating senescent cells might be beneficial, let alone possible. Dr. Sharpless says this finding is "a fundamental advance by our field." Before this study, researchers knew that senescent cells could be damaging, but had no way of killing the cells themselves.

Senescent cells cannot simply be killed in humans. Although senescent cells contribute to aging, they also prevent tumors from



Senescent cells, shown on the left, are larger and flatter than the proliferating cells on the right. One marker of cellular senescence is expression of beta-galactosidase, which is responsible for its blue color.

Credit: http://media.wiley.com/CurrentProtocols/CB/cb1809/cb1809-fig-0001-1-full.jpg

progressing. In response, Dr. Jan van Deursen from the Mayo Clinic study suggests the possibility of simply, "[priming] the immune system, [boosting] it a little bit, to make sure senescent cells are removed." Daniel Peeper from the Netherlands Cancer Institute proposes that it may be more effective to remove the factors produced by senescent cells, not the cells themselves.

French scientists from INSERM have a different approach: reprogramming senescent cells into stem cells. Dr. Jean-Marc Lemaitre and his team have reprogrammed senescent cells from 100 year old humans into embryonic stem cells, which can differentiate into many cell types like neurons and heart cells, potentially rejuvenating the body.

"By reprogramming cells from elderly patients, the INSERM study opens the possibility of repairing damaged organs using cells that would be tolerated by a patient's immune system."

Dr. Lemaitre was able to reprogram senescent cells by using six genetic factors, allowing him to reprogram 100 year old cells for the first time. By reprogramming cells from elderly patients, the INSERM study opens the possibility of repairing damaged organs using cells that would be tolerated by a patient's immune system.

Both of these studies introduce the possibility of one day reversing aging. However, Dr. Jesus Gil from the Medical Research Council's Clinical Sciences Center says that handling senescent cells must be "taken with a bit of caution." Senescent cells are not yet completely understood, but have been found to play a role in preventing cancer. It is important to note that although eliminating senescent cells might slow aging, it cannot prevent it. Dr. van Deursen says, "If you remove the senescent cells you improve things considerably, but you can't reverse the process or completely stop the aging because it has other causes."

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Synthetic Biology: *Exploring Mother Nature and the Final Frontier*

By: Ebaa Al-Obeidi

In essence, synthetic biology serves as a way to harness biology in reliable, robust, engineered systems. This engineered biology has found applications in diverse fields like genetics and even space exploration. Taking a closer look reveals how this intriguing field isn't just the stuff of science fiction, and how MIT is making an indelible mark on its advancement.

Synthetic biology emerged from the Human Genome Project, and has developed as a new discipline which applies the project's breakthroughs – namely, the knowledge of DNA sequences and the chemistry of cells – to tackle diverse challenges like new fuel sources, medicine, and agriculture. At the Institute, there have been a number of recent advances in synthetic biology. For example, investigators in the Analog Circuits and Biological Systems Group at the Research Laboratory of Electronics have made forays in cell-inspired electronics by mimicking cell functions with transistors. By recognizing that the chemical reactions that lead to protein production in cells are reminiscent of electronic circuits, analog circuits can enable accurate modeling of biological systems. These models can be used to study the interactions of genes and protein regulation.

Using a different type of circuit, the Weiss Laboratory for Synthetic Biology has developed a way of effectively killing cancer cells from the inside out. The circuit is made of genes that detect molecules specific to a type of cervical cancer cell. If the molecules are present, the genes will be signaled to produce a protein that induces programmed cell death. Such a circuitry system could be used to detect many other diseases by allowing scientists to build a library of cell-death-inducing programs specific to various types of cancer.

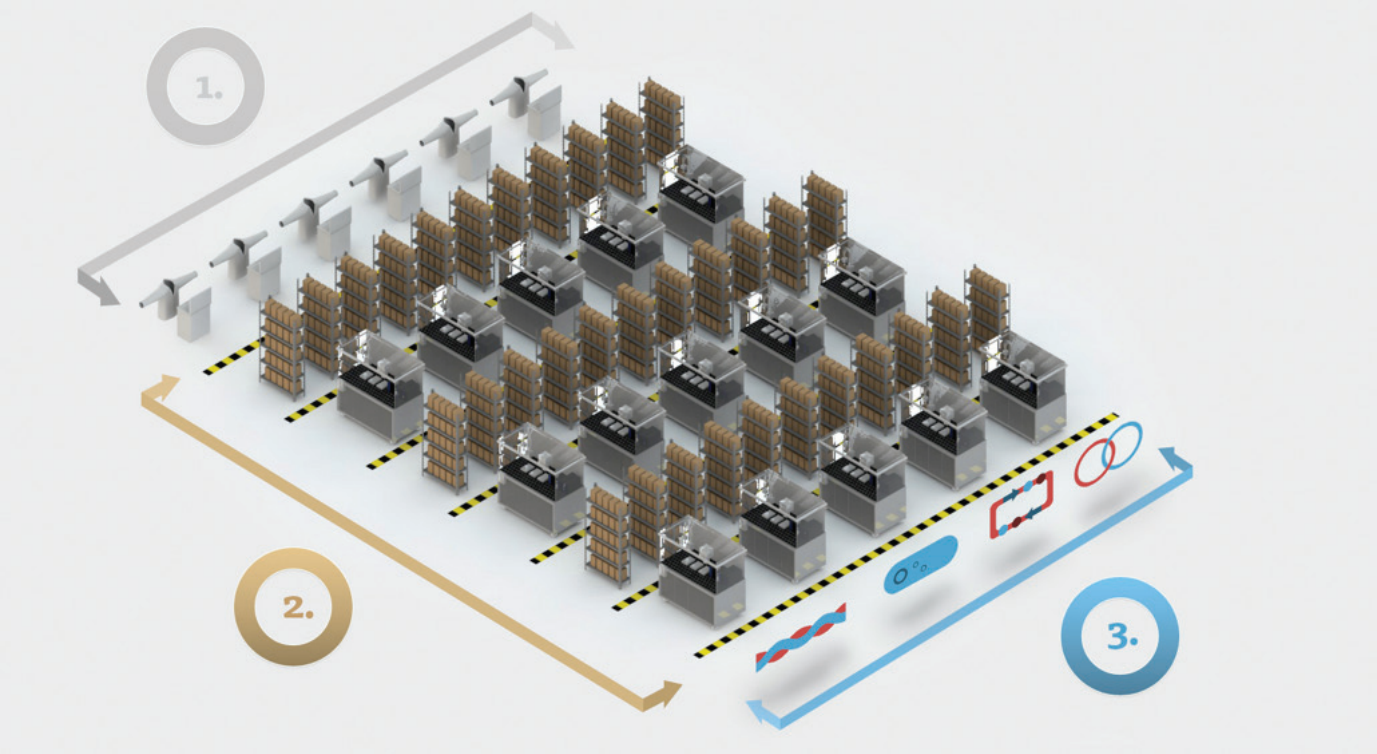
"…virus-resistant bacteria could be engineered and cells could be redesigned to produce new proteins."

Pursuing the theme of programming, new technology has been unveiled by MIT and Harvard that can edit DNA at the genetic scale. This novel tool allows researchers to rewrite genetic code and give cells new functions. For instance, virus-resistant bacteria could be engineered and cells could be redesigned to produce new proteins.

To foster more of this creative thinking, the Institute hosts the International Genetically Engineered Machine Competition (iGEM), which is a weekend-long synthetic-biology showdown where teams from around the world strut their scientific stuff and present genetic machines constructed from toolkits given to all teams. The beauty of the competition is that it instills a sense of wonder at nature, an awe which drives teams to invent unique solutions to diverse problems.

With hope to broaden that appreciation for synthetic biology, Natalie Kuldell, an instructor in MIT's Department of Biological Engineering, created biobuilder.org. The site is intended for all ages and was designed as a resource for students and teachers to learn about engineering in the domain of biology. It teaches about the fascinations of biology through cartoons that illustrate the importance of engineering in pursuit of answers.

MIT associate professor of



Gingko BioWorks, a synthetic biology startup founded by MIT alumni, break down their technology into three steps.
Credit: http://ginkgobioworks.com/tech.html

Biological Engineering Christopher Voigt is Editor-In-Chief of a new journal from the American Chemical Society titled ACS Synthetic Biology, a publication forum for cutting edge research in the field. The emergence of the journal is a testament to synthetic biology's place in engineering the future. The field tackles some of society's most pressing challenges, from energy to health, and strives to understand and improve the human condition.

Synthetic biology has also been the motivation for engineering on the macroscopic scale by stimulating the construction of new start-ups and research centers. Ginkgo BioWorks in Boston is a new synthetic biology startup founded by five MIT scientists that aims to push the field to the factory level. They specialize in rapid prototyping and synthesis of pieces of DNA to create functional genetic pathways. They describe their technology as a three-fold approach to creating synthetic biological organisms: first, their engineers use CAD/CAM tools to design new organisms. One current project involves work on an organism that can convert electricity and carbon dioxide to fuel. Second, a robotic automation process in Kendall Square produces the organisms. Third, the organisms are customized with reusable strains of bacteria and genetic parts to better fit its purposes.

MIT is a part of the Synthetic Biology Engineering Research Center (SynBERC), a coalition of biology labs here and at four other top universities. A major project of the center has been to create a registry for the standardization of biological parts called BioBricks, which are pieces of DNA that can be put together like LEGOs to create unique microbes. Other topics of research include the development of fuels, organ tissue and tumor-destroying bacteria. Another stronghold of synthetic biology is MIT's Synthetic Biology Center, which focuses on establishing the field as an engineering discipline.

In addition to its broad applications in genetics and agriculture, synthetic biology has begun to carve out a unique niche in outer space exploration. The NASA Ames Research Center declared in May 2011 its mission to define the field of Space Synthetic Biology as it began construction of a new center focused on research in the field. The interdisciplinary research effort will utilize synthetic biology as an enabling technology to explore our solar system.

Synthetic biology is a precocious field at the interface between science, engineering, and Mother Nature. It has engineered an impressive repertoire of cutting edge scientific achievements, and pioneers investigation into novel solutions to

society's most daunting challenges. By putting MIT's motto of Mens et Manus to task, breakthroughs in synthetic biology begin to change the world, and enable the exploration of new ones.

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Nobel Spotlight: *Professor Frank Wilczek MIT Physics Department*

By: Paige Finkelstein

This issue's Nobel Spotlight feature covers Professor Frank Wilczek, the 2004 Nobel Prize laureate for physics. Unlike a typical interview you may have read about this Nobel laureate, this article also focuses on his non-academic interests and personal life details. You can see what you have in common with Professor Schrock under "Quick Facts" or read up on some of his other research interest.

MURJ: Do you have any hidden talents?

Frank Wilczek: Well, there are some that will remain hidden – and the talent is limited – but I do play the piano, the accordion, and the drums. The drums were mostly in the past when I was a teenager.

MURJ: If you could meet anyone in the world – dead or alive – who would it be?

FW: I think the person I would like to meet is James Clerk Maxwell – from Maxwell's equations. Maxwell is quite an interesting character.

MURJ: What is your favorite class to teach?

FW: The kind of class I'm teaching almost exclusively now is more of a seminar. I talk about very advanced subjects and theoretical physics so it is kind of integrated with my research work. Next semester I've been drafted to do a recitation of 8.04 – quantum mechanics – so I will be doing that, which should be fun. In the past I taught 8.012 and 8.022, both the lectures and the recitation… and that was a less enjoyable teaching because it's a lot more work – grading a lot is not very fun.

MURJ: The coolest thing about MIT is….

FW: I think MIT is a whole universe. It's a vague answer, but I really enjoy the engineering spirit and the down-to-earthiness. It's not natural to me; I'm more of a dream theorist. It's refreshing to get dragged back down sometimes.

MURJ: If I wasn't a Nobel Prize researcher I would be a….

FW: I don't really know actually. I initially struggled with it [physics as a career path] and I certainly did not come to physics as my first inclination when I was thinking about what to do in college. I was very interested in questions about how the mind works, so I probably would have gravitated towards something along those lines, though I'm not sure what. My taste is also very mathematical, so I would have been looking for something quantitative too.

MURJ: Can you give us a summary about the research that won you the Nobel Prize?

FW: I do research in theoretical physics across a broad spectrum of things, but the common thread is quantum mechanics quantum theory. I've dealt with things all the way from the properties of elementary particles to properties of materials at low temperature. The Nobel Prize work, specifically, was about fundamental interactions. We know there are four [fundamental interactions] in the present state of physics. There is gravity and electromagnetism – sort of the classic forces – then there are things called the strong and weak forces that govern how atomic nuclei are held together and

how they decay. It was discovered in the 20th century that we needed new forces, and what I did was figure out what the basic equations for the strong forces are. They turn out to be kind of like Maxwell equations on steroids, where instead of one electromagnetic field – one photon – it turns out you have eight, and instead of one type of electric charge, you have three. I found what those equations are, and just as important, how to tell that those are the right equations.

MURJ: How did you get involved with that research?

FW: I did this work when I was about twenty-two years old. I arrived at Princeton as a graduate student in mathematics but I wasn't sure of exactly what I wanted to do. However, what I discovered at Princeton was that a revolution in physics was underway. New techniques of analyzing behavior of quantum systems and promising ideas about fundamental forces were being developed, so I just sort of jumped in.

MURJ: So was winning the Prize always a goal for you?

FW: Well, it is and it isn't a goal in some sense. I had no doubt that if experiments conformed to my theories then this work would be Prize worthy. When I was a little kid learning about what it was like to be a scientist, the Nobel Prize is sort of a glittering thing out in the distance, so just saying, "I'm going to win the Nobel Prize" is not an algorithm to get one. You know you have to actually solve some sort of problem.

MURJ: How did you find out you won?

FW: Well, I knew when the announcement was going to be made, and I didn't sleep out of anticipation. At five AM, I looked at my clock and thought the announcement wasn't going to be made until six o'clock, so I thought that since I wasn't sleeping, I might as well get up and take a shower. But I was wrong – what I didn't know was that they didn't wait until the official announcement was made to call the winners; they actually call them first. So I was in the shower when my wife came in with the phone, and told me that there was a woman with a Swedish accent on the line. I was completely soaking wet, but took the call anyway. I thought the call would be in the form of short congratulations, and I'd be able to get dressed, but it wasn't like that either. They wanted to explain how to deal with the press, what the practical arrangements were, and then at least half a dozen people wanted to congratulate me. So I basically had to sit there wet on the phone for at least twenty minutes.

"When I was a little kid learning about what it was like to be a scientist, the Nobel Prize is sort of a glittering thing out in the distance, so just saying, "I'm going to win the Nobel Prize" is not an algorithm to get one."

MURJ: What was your first reaction?

FW: I definitely was shocked – that is for sure. Of course, any year it is a surprise because there are many candidates that are more or less plausible. I mean, usually you don't win a Nobel Prize. It was sort of like the Spanish Inquisition, where nobody really expects it, but it happens.

MURJ: The coolest thing about winning a Nobel Prize is…

FW: Everything is pretty cool. The thing that surprised me was actually the ceremony itself. It was a great party. It's not just a presentation, it's a whole week of events in Sweden – one after another, where you think, "Nothing could top this" but somehow the next event would. The actual moment of getting the thing [the Nobel Prize] and facing the audience and the trumpet fanfare that played when they announced the winners was really electric. I could feel my hair standing up; there is nothing else quite like that. The whole atmosphere is like a fairytale. Everything is taken care of and you have an attaché – a Swedish diplomat – with a schedule and limousine to take you all around. Everyone in Stockholm knows about it [the ceremony] and the whole country is excited.

MURJ: So where is your prize right now?

FW: Well, for security reasons, it [the actual medal] stays hidden. We have a duplicate medal that I keep on display at home though.

MURJ: What is your current research about?

FW: My current is similar in the sense that it is theoretical physics and uses quantum mechanics and mathematical ingenuity to do new things. The details are different though, because I have always had this style of moving on; I don't like to keep doing the same thing. Recently,

what I got most excited about is basically the closest one can get to a perpetual motion machine. It sounds ridiculous, but we know of something close to perpetual motion – when you have a super conductor, the current under the right circumstances will go around forever. So that in a sense is perpetual motion; on the other hand, since the current is constant, nothing is actually moving. So I've been cooking up models that are variants of conventional super conductors where something actually does move.

MURJ: For our readers out there – do you accept UROPs?

FW: I mostly work with graduate students, but I do from time to time accept some undergraduates.

MURJ: In regards to the current neutrino debate, what are you thoughts on that?

FW: [Professor Wilczek laughs] I think it's crap. Things might be faster than the speed of light, but I don't think this particular experiment is it. First, the measurement they are trying to do is very, very difficult; there are many ways in which there could be error. Second, there are other experiments closely related to this one – some that are much more accurate – that don't see similar results. It's hard to interpret these results in a manner that is consistent with those other experiments and everything else we know.

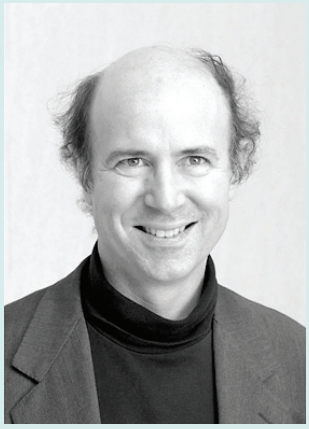
MURJ: Anything last thoughts you want to share with our MURJ readers? Maybe some advice for winning their own Nobel Prize someday?

FW: I think it is very important to think not only about what you are doing in the moment, but what you want to do in the long run. Consider your options and don't put yourself in a box. See what's out there and constantly be exploring and questioning and finding your passion. What you find yourself spontaneously gravitating to is they key. At least for me, I could try to impose discipline on myself by telling myself what I should be interested in or what I should try to work on, but there are some things that I just want to do and find myself doing spontaneously. If you have impulses leading you in a direction within reason, you should go with that. For example, if your passion is checkers or World of Warcraft, you might want to reconsider….

For more information on Professor, visit http://web.mit.edu/physics/people/faculty/wilczek_frank.html.

Quick Facts:

Birthplace: Long Island, New York
High School: MartinVan Burren High
Alma Matter: University of Chicago
Favorite Sports Team: New York Knicks
Favorite Restaurant: Sidney's Grille or Bertucci's
Favorite Band: The Beatles, Eric Clapton, the Rolling Stones
Length of Time at MIT: A little over 10 years
Favorite Spot at MIT: His office or the Stata Center



Professor Frank Wilczek, winner of the Nobel Prize for Physics 2004

MURJ Reports

Epidermal Growth Factor Receptor Inhibition Treats Experimental Autoimmune Encephalomyelitis[1]

Elliot Akama-Garren[2], Christina Swanson[3], William H Robinson[3]

1. Department of Medicine, Division of Rheumatology, Stanford University School of Medicine and the VA Palo Alto Health Care System
2. Student Contributor, Class of 2015, MIT
3. Mentors, Department of Economics, MIT

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system. Tyrosine kinases are critical in inflammatory regulation and are promising targets for the treatment of autoimmune diseases, as shown in animal models of rheumatoid arthritis. Here we show that inhibition of epidermal growth factor receptor (EGFR) with lapatinib, erlotinib, and gefitinib decreases the severity of experimental autoimmune encephalomyelitis (EAE), a murine model of MS. To further understand the mechanism of EGFR inhibition, we used qPCR to investigate the role of EGFR in EAE, showing that EGFR is differentially transcribed in peritoneal macrophages and brain endothelial cells (bEnd.3), as compared to splenoctyes. We show that lapatinib did not affect classical macrophage activation or cytokine production, confirming that EGFR inhibition does not indiscriminately suppress the immune system, making it an appealing therapeutic candidate. We also show that lapatinib reduces bEnd.3 proliferation, cytokine and chemokine production, and adhesion molecule expression, suggesting that EGFR inhibition reduces inflammatory cell infiltration across the BBB. In conclusion, EGFR inhibition is a novel and potentially promising treatment for MS that warrants further investigation because of the specific nature of tyrosine kinase expression and the selectivity of pharmacological inhibitors such as lapatinib.

Introduction

Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease of the central nervous system (CNS) that affects approximately one million individuals worldwide. MS is characterized by inflammation in the white matter of the brain and by paralysis, lack of coordination, and visual impairment [1]. Currently, treatment of MS is limited to immunosuppressive or immunomodulatory therapy that often lead to adverse effects [2].

Recent research has shown the capability of tyrosine kinase inhibitors to treat autoimmune disease by selectively targeting certain cell types. Imatinib, an inhibitor of the tyrosine kinase enzyme ABL, has the capability to treat animal models of rheumatoid arthritis [3]. Given these findings, we investigated the therapeutic potential of various tyrosine kinase inhibitors in experimental autoimmune encephalomyelitis (EAE), a common mouse model of MS.

Epidermal growth factor receptor (EGFR) is a member of the ErbB receptor family, a group of four tyrosine kinase receptors responsible for cell survival, proliferation, invasion, metastasis, and angiogenesis [4]. Recent research has shown that expression of EGFR in peripheral blood mononuclear cells is increased during relapse compared to remission [5]. EGFR has also been shown to promote astrocyte proliferation and development [6]. However, the role of EGFR in MS has never been investigated.

Here we studied the effects of EGFR inhibition on EAE severity. We also investigated possible mechanisms of action.

Methods

Animals and EAE induction

A total of 50 C57/BL6 female mice were obtained from Jackson Laboratories (Sacramento, CA) and housed at VA Palo Alto Hospital. Experiments were performed under protocols approved by the Stanford University Committee of Animal Research and in accordance with NIH guidelines. All efforts were made to minimize the numbers of animals used and to ensure minimal suffering. We induced EAE by subcutaneous immunization with 100 mg of MOG_{35-55} emulsified in complete Freund's adjuvant (CFA) containing 4 mg/mL heat-killed Myobacterium tuberculuosis H37Ra (Difco). Pertussis toxin was given at 400 ng per mouse on the day of immunization and 48 hr later. EAE symptoms were monitored daily using a clinical score of: 1) Tail paralysis, 2) hind limb weakness, 3) hind limb paralysis, 4) forelimb paralysis, and 5) death.

Splenocyte isolation and stimulation

Primary splenocyte cultures were established from naive DBA/J mice or treatment mice and red blood cells were lysed using ACK lysis buffer. Cells were cultured in RPMI 1640 supplemented with 10%

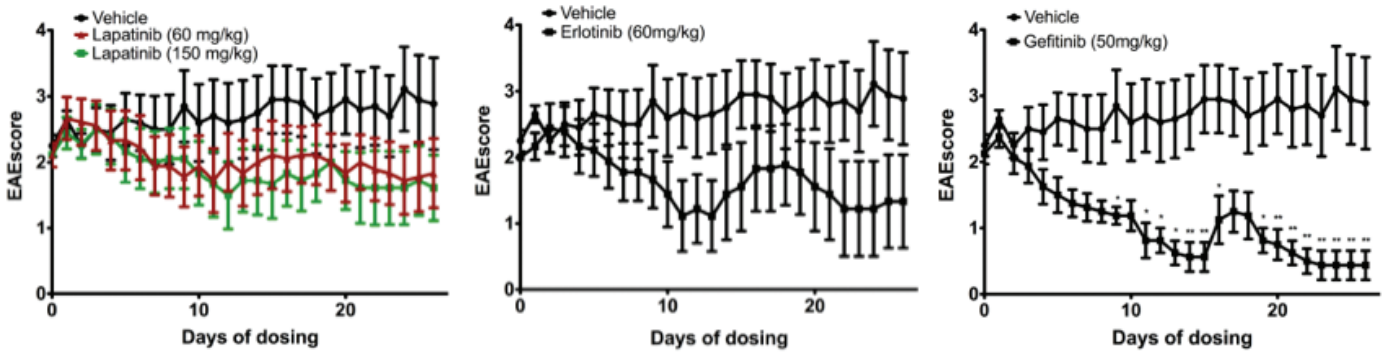


Figure 1. EGFR inhibition treats EAE. EAE was induced in C57/BL6 mice by injection of MOG peptide emulsified in CFA. Mice received daily treatment with lapatinib (60 mg/kg or 150 mg/kg), erlotinib (60 mg/kg), gefitinib (50 mg/kg), or vehicle by oral gavage. All three EGFR inhibitors reduced EAE severity compared to vehicle. *P < 0.05, **P < 0.01 compared with vehicle treated mice.

FBS and penicillin-streptomycin-L-glutamine. For experimentation, adherent splenocytes were cultured in RPMI 1640 containing 1% FBS and penicillin-streptomycin-L-glutamine and stimulated with MOG (10 ng/mL), anti-IGM (5 mg/mL), dynabeads, ConA (5 mg/mL), or LPS (10 ng/mL).

Peritoneal macrophage isolation and stimulation

Resident peritoneal macrophages were isolated from naive DBA/J mice by i.p. injection and withdrawal of 5–7 ml of complete DMEM media and adherent macrophages were cultured overnight in complete DMEM media supplemented with 10% FBS and penicillin-streptomycin. For experimentation, peritoneal macrophages were cultured in complete DMEM media supplemented with 1% FBS and penicillin-streptomycin and stimulated with MCSF (10 ng/mL) or LPS (3 ng/mL, 10 ng/mL, 50 ng/mL, or 100 ng/mL).

bEnd.3 cultures and stimulation

An immortalized mouse brain microvascular endothelial cell line (bEnd.3) was grown in advanced DMEM supplemented with 10% FBS and penicillin-streptomycin-L-glutamine. For experimentation, bEnd.3 cells were cultured in advanced DMEM containing 5% FBS and penicillin-streptomycin-L-glutamine and stimulated with TNF-α (10 ng/mL), IFN-γ (10 ng/mL), IL-1β (5 ng/mL), or LPS (100 ng/mL).

RNA isolation and qPCR

Real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR) analysis was performed on mouse spinal cords and splenocyte, peritoneal macrophage, and bEnd.3 lysates. Mice were anesthetized with CO_2 and lumbar spinal cords were excised and homogenized in TRIzol reagent. Splenocytes, peritoneal macrophages, and bEnd.3 cells were lysed using TRIzol reagent. Total RNA was extracted from spinal cords and cell lysates using RNeasy Mini Kit. For quality control, RNA purity was verified using an OD260/280 ratio between 1.8 and 2.0. cDNA was synthesized using the qScript cDNA synthesis kit. qPCR reactions were performed in triplicate using SYBR Green Master Mix. Each primer set was validated by calculating qPCR efficiencies of serial dilutions from 0 to 10 ng of original sample, and constructing a relative efficiency plot comparing target and reference ΔCp values to ensure that the absolute slope of fit line was less than 0.1. Samples were analyzed using SDS 7700 software and normalized to the expression of internal control genes Ywhaz or Hprt1.

Cytokine analysis

Enzyme-linked immunosorbent assay (ELISA) was used to measure cytokine concentrations in serum and supernatant samples. Cytokine concentrations were measured using commercial kits (Peprotech), according to the manufacturers' recommendations. Each cytokine concentration was determined by its optical density, measured using a microplate spectrophotometer at 450 nm and corrected at 540 nm.

Proliferation analysis

Bromodeoxyuridine (BrdU) was diluted 1:100 and added to cells 24 hr before stopping the experiment. Cells were then stained with a mAb specific to BrdU and BrdU incorporation was measured using a BrdU flow kit (Calbiochem) according to the manufacturers's recommendations. Samples were analyzed using a microplate spectrophotometer at 450 nm and corrected at 540 nm.

Histology and immunohistochemistry

Mice were anesthetized with CO_2 followed by perfusion through the left ventricle with PBS. Brains were removed and stored in OCT compound at -80°C. Frozen sections of mouse brain (5 µm) were then fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes. Sections were then permeabilized with 0.5% Triton X100 for 6 min. Endogenous peroxidase was inactivated with 0.03% H_2O_2. Nonspecific binding was blocked with 1.5% normal goat serum, and sections were then incubated with rabbit anti-EGFR (1:200) or rabbit anti-VWF (1:200, Millipore). Sections were washed in PBS and incubated with a biotinylated goat anti-rabbit antibody (1:250), which was developed with diaminobenzedine tetrahydrochloride using a VECTASTAIN avidin-biotin kit (Vector Laboratories) according to the manufacturers's recommendations. Sections were

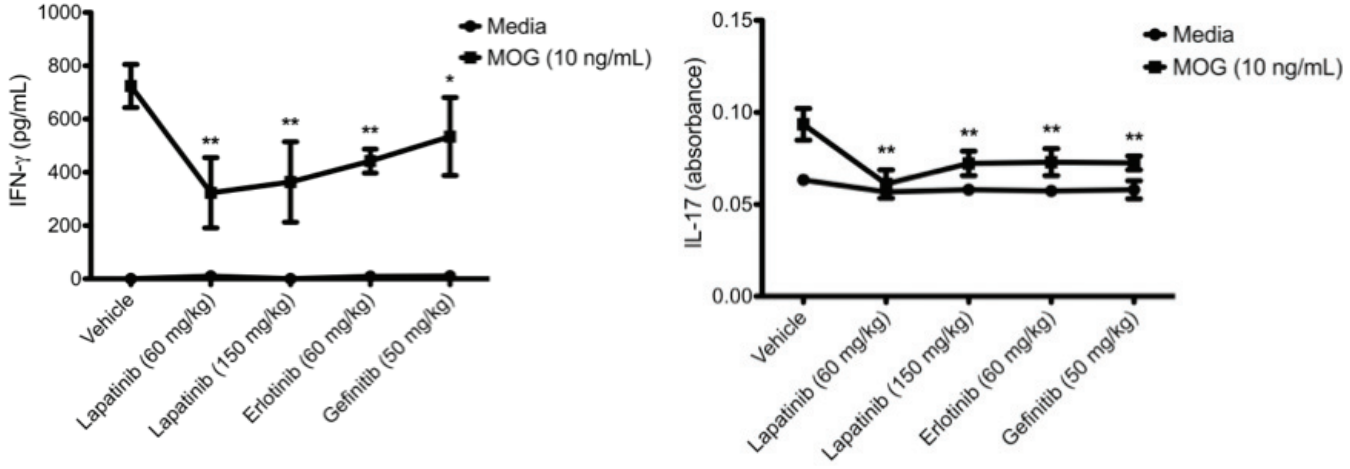


Figure 2. EGFR inhibition reduces IFN-γ and IL-17 production by MOG stimulated splenocytes. Splenocytes were isolated from each treatment group and treated with media or 10 ng/mL of MOG peptide. After 24 hr supernatants were collected and IFN-γ and IL-17 production was measured. MOG peptide significantly stimulated IFN-γ and IL-17 production compared to media, which was significantly reduced in treatment groups. *P < 0.05, **P < 0.01 compared to vehicle.

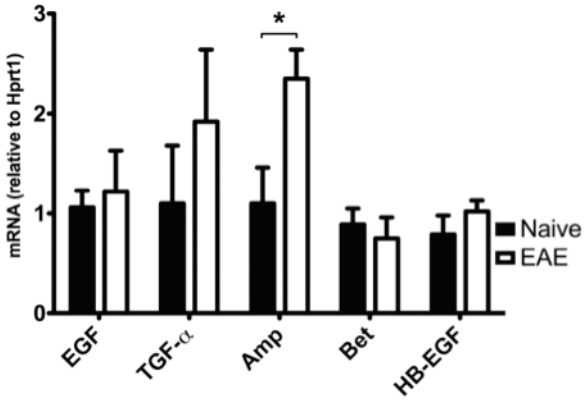


Figure 3. Amphiregulin expression is increased in EAE spinal cord. Spinal cords were isolated from mice with EAE scores of 3 or higher and naive controls. Tissue was then homogenized and EGF, TGF-α, amphiregulin (Amp), betaceullin (Bet), and HB-EGF expression was analyzed by qPCR. Amphiregulin is the only EGFR ligand found to be significantly increased in EAE. *P < 0.05.

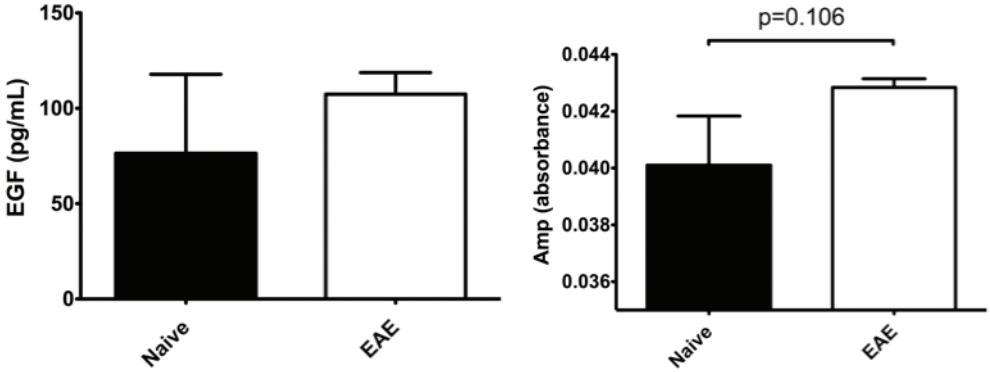


Figure 4. Amphiregulin expression is increased in EAE serum. Serum was isolated from mice with EAE scores of 3 or higher and naive controls. EGF and amphiregulin concentrations were measured by ELISA. Significantly higher concentrations of amphiregulin was found in EAE. *P < 0.05.

then stained with hematoxylin, dehydrated, and mounted using Entallen mounting medium.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego CA). Statistical significance was analyzed by two-tailed Student's t-test.

Data are represented as mean±S.D, except EAE studies which are shown as mean±S.E.M. $P < 0.05$ was considered significant. The symbol * indicates $P<0.05$; ** indicates $P<0.01$; and *** indicates $P<0.001$.

Results

EGFR inhibition reduces EAE severity

To investigate the effects of EGFR inhibition on EAE, we treated mice with EGFR inhibitors lapatinib ditosylate (laptinib), erlotinib, and gefitinib. Lapatinib is a tyrosine kinase inhibitor approved for the treatment of Her-2 expressing breast cancers [7]. Lapatinib specifically inhibits EGFR with IC_{50} of 10.2 nM and downstream Erk1/2 and Akt signaling [8]. Erlotinib is approved to treat non-small cell lung cancer and inhibits EGFR with IC_{50} of 2 nM [9]. Gefitinib is also approved to treat non-small cell lung cancer and inhibits EGFR with IC_{50} of 31 nM [10].

EAE was induced by injection of C57/BL6 mice with MOG emulsified in CFA. Mice were evaluated daily for clinical symptoms of EAE and once mice reached a score of 2 were allocated to treatment with lapatinib (150 mg/kg or 60 mg/kg), erlotinib (60 mg/kg), gefitinib (50 mg/kg), or vehicle. Mice were treated daily by oral gavage. All three inhibitors significantly reduced disease severity compared to vehicle (Figure 1).

To examine the effects treatment had on autoreactive lymphocytes, splenocytes were isolated from each treatment group and stimulated with 10 ng/mL of MOG peptide. After 24 hr, supernatants were collected and IFN-γ and IL-17 production was measured by ELISA. MOG peptide significantly stimulated IFN-γ and IL-17 production compared to media. Splenocytes from treatment groups produced significantly less IFN-γ and IL-17 after MOG stimulation compared to vehicle splenocytes (Figure 2). Together these results demonstrate that inhibition of EGFR significantly reduces EAE severity.

Amphiregulin expression is increased in EAE

To examine the role EGFR plays in MS pathogenesis, we examined levels of EGFR ligands in EAE compared to naive mice. Spinal cords were isolated from mice with EAE scores of 3 or higher and naive controls. The tissue was then homogenized and EGF, TGF-α, amphiregulin, betaceullin, and HB-EGF expression was analyzed by quantitative PCR (Figure 3). Also, serum was isolated from mice with EAE scores of 3 or higher and naive controls. EGF and amphiregulin concentrations were measured by ELISA (Figure 4). We found that amphiregulin expression is significantly increased in EAE while EGF, TGF-α, betaceullin, and HB-EGF expression is not significantly altered.

EGFR is expressed on macrophages and brain endothelial cells in EAE

To examine the mechanism by which EGFR inhibition decreases EAE severity, we investigated EGFR expression in cell types commonly implicated in MS. Splenocytes from naive DBA/J mice were treated with media, 5 mg/mL anti-IGM to stimulate B-cells, dynabeads to stimulate T-cells, 5 mg/mL ConA to stimulate T-cells, and LPS to stimulate monocytes. Peritoneal macrophages from naive DBA/J mice were treated with 10 ng/mL MCSF for 48 hr or 3 ng/mL LPS for 72hr. Immortalized mouse brain endothelial cells (bEnd.3) were treated with media or 10ng/mL TNF-α for 48 hr. EGFR mRNA expression in each of these cell cultures was measured using quantitative PCR (Figure 5). We found that EGFR is differentially expressed in peritoneal macrophages and brain endothelial cells compared to lymphocytes.

To investigate EGFR expression *in vivo*, brains from mice with EAE scores of 3 or higher were isolated and sectioned. Immunohistochemistry was performed on serial sections for EGFR and endothelial cell marker von-Willebrand Factor (VWF) (Figure 6). Endothelial cells expressed EGFR, confirming that EGFR is expressed on brain endothelial cells in EAE.

EGFR inhibition does not affect macrophage activation or cytokine production

Upon finding high expression of EGFR in peritoneal macrophages, we examined the effects of EGFR inhibition on macropohage activation and cytokine production. To investigate the effects of EGFR inhibition on classical macrophage activation, peritoneal macrophages from naive DBA/J mice were pretreated with media or 10 μM lapatinib for 1 hr. Cells were then stimulated with media or LPS (10 ng/mL, 50 ng/mL, or 100 ng/mL) in order to activate classical macrophages. After 24 hr, supernatants were collected and TNF-α and IL-12p40 concentrations were measured by ELISA (Figure 7). LPS stimulated both TNF-α and IL-12p40 production in a dose dependent matter compared to media control, but treatment with lapatinib failed to reduce TNF-α and IL-12p40 production.

To investigate the effects of EGFR inhibition on macrophage cytokine production, peritoneal macrophages from naive DBA/J mice were incubated with increasing concentrations of lapatinib. After 24 hr, supernatants were collected and TNF- α, IL-6, and IL-10 concentrations was measured by ELISA (Figure 8). Treatment with lapatinib did not significantly alter TNF- α, IL-6, and IL-10 production.

EGFR inhibition reduces brain endothelial cell proliferation, cytokine and chemokine production, and adhesion molecule expression

Upon finding high expression of EGFR in brain endothelial cells, we examined the effects of EGFR inhibition on brain endothelial cell angiogenesis and inflammation. To investigate the effect of EGFR inhibition on brain endothelial cell proliferation, bEnd.3 cells were pretreated with media, 5 μM lapatinib, or 10 μM lapatinib for 1 hr. Cells were then stimulated with media, 10 ng/mL of TNF-α, 10 ng/

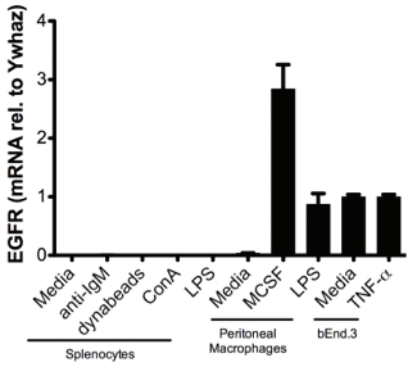


Figure 5. EGFR is expressed on macrophages and brain endothelial cells. Splenocytes from naive DBA/J mice were treated with media, 5 mg/mL anti-IGM, dynabeads, 5 mg/mL ConA, and LPS. Peritoneal macrophages from naive DBA/J mice were treated with media, 10 ng/mL MCSF for 48 hr or 3 ng/mL LPS for 72hr. bEnd.3 cells were treated with media or 10ng/mL TNF- for 48 hr. EGFR mRNA expression in each of these cell cultures was measured using qPCR. Peritoneal macrophages and bEnd.3 cells differentially express EGFR compared to lymphocytes.

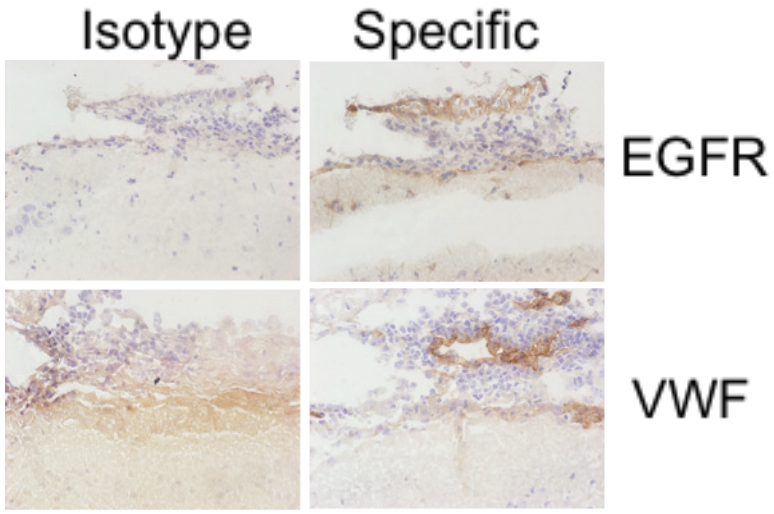


Figure 6. EGFR is expressed on endothelial cells in the EAE brain. Immunohistochemistry was performed on serial sections of brains from mice with EAE scores 3 or higher for EGFR and VWF. EGFR staining correlated with VWF staining, confirming that brain endothelial cells express EGFR.

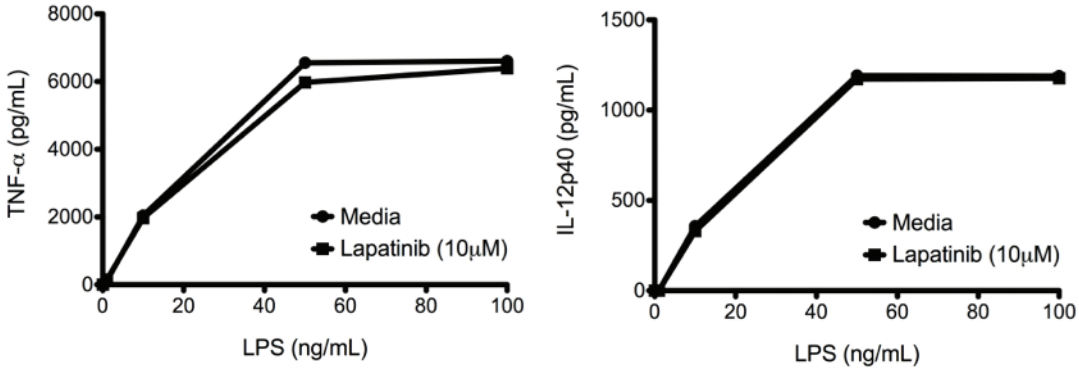


Figure 7. Lapatinib does not reduce classical macrophage activation. Peritoneal macrophages from naive DBA/J mice were pretreated with media or 10 μM lapatinib for 1 hr. Cells were then stimulated with media or LPS (10 ng/mL, 50 ng/mL, or 100 ng/mL) in order to activate classical macrophages. After 24 hr, supernatants were collected and TNF-α and IL-12p40 concentrations were measured by ELISA. Treatment with lapatinib did not significantly reduce classical macrophage activation as measured by TNF-α and IL-12p40 production.

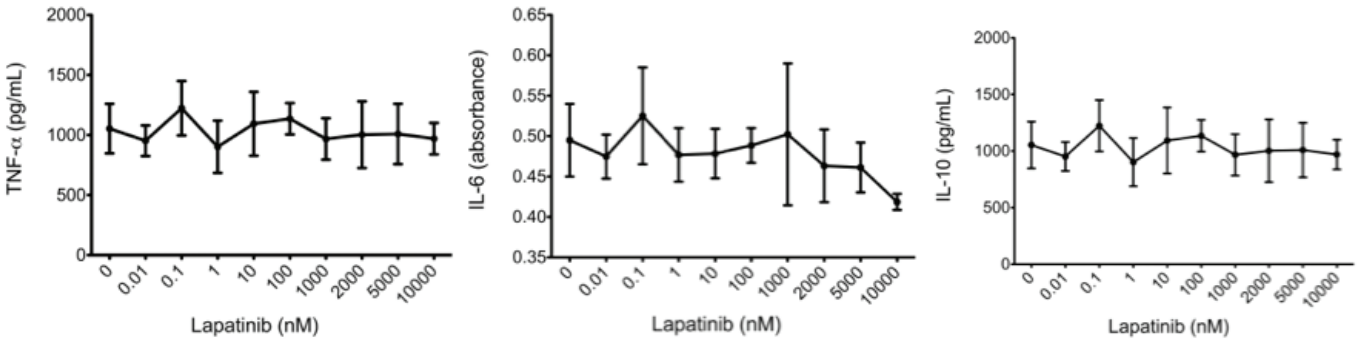


Figure 8. Lapatinib does not alter macrophage TNF-α, IL-6, or IL-10 production. Peritoneal macrophages from naive DBA/J mice were incubated with increasing concentrations of lapatinib. After 24 hr, supernatants were collected and TNF- α, IL-6, and IL-10 concentrations were measured by ELISA. Treatment with lapatinib did not significantly alter TNF- α, IL-6, or IL-10 production.

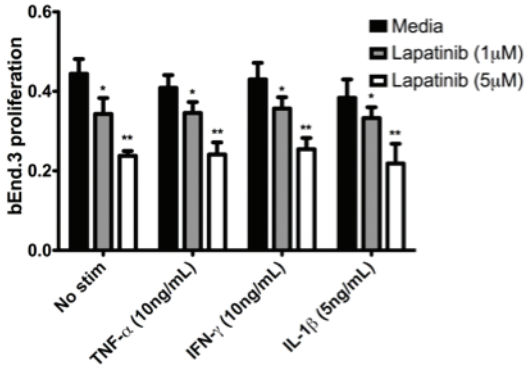


Figure 9. Lapatinib reduces bEnd.3 proliferation. bEnd.3 cells were pretreated with media, 5 μM lapatinib, or 10 μM lapatinib for 1 hr, then stimulated with media, 10 ng/mL of TNF-α, 10 ng/mL of IFN-γ, or 5 ng/mL of IL-1β. After 24 hr, cells were collected and BrdU incorporation was measured. Lapatinib significantly reduced bEnd.3 proliferation in a dose dependent manner. *P < 0.05, **P < 0.01.

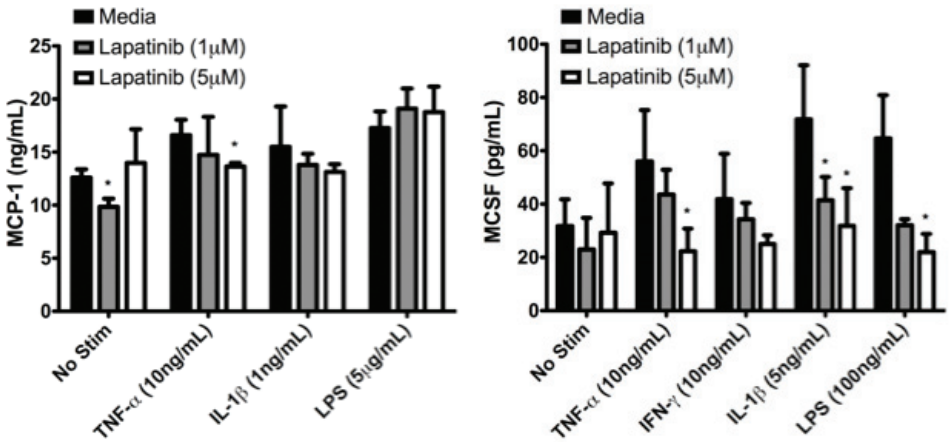


Figure 10. Lapatinib reduces bEnd.3 MCP-1 and MCSF production. bEnd.3 cells were pretreated with media, 1 μM lapatinib, or 5 μM lapatinib for 1 hr, then stimulated with media, 10 ng/mL of TNF-α, 10 ng/mL of IFN-γ, 5 ng/mL of IL-1β, or 100 ng/mL of LPS. After 24 hr, supernatants were collected and MCP-1 and MCSF production was measured by ELISA. Lapatinib significantly reduced MCP-1 production in TNF-α stimulated bEnd.3 cells and MCSF production in TNF-α, IFN-γ, IL-1β, and LPS stimulated bEnd.3 cells. *P < 0.05.

mL of IFN-γ, or 5 ng/mL of IL-1β. After 24 hr, cells were collected and BrdU incorporation was measured (Figure 9). Lapatinib significantly reduced bEnd.3 proliferation in a dose dependent matter.

To investigate the effect of EGFR inhibition on brain endothelial cell chemokine and cytokine production, bEnd.3 cells were pretreated with media, 1 μM lapatinib, or 5 μM lapatinib for 1 hr. Cells were then stimulated with media, 10 ng/mL of TNF-α, 10 ng/mL of IFN-γ, 5 ng/mL of IL-1β, or 100 ng/mL of LPS. After 24 hr, supernatants were collected and MCP-1 and MCSF production was measured by ELISA (Figure 10). Treatment with lapatinib significantly reduced MCP-1 production in TNF-α stimulated bEnd.3 cells. TNF-α, IFN-γ, IL-1β, and LPS increased MCSF production, which was significantly reduced with lapatinib treatment.

To further investigate the mechanism by which EGFR inhibition affects brain endothelial cell chemokine production and adhesion molecule expression, bEnd.3 cells were pretreated with media or 5 μM lapatinib for 15 min. Cells were then stimulated with media, 10 ng/mL of TNF-α, or 100 ng/mL of LPS. After 45 min, mRNA was collected from the cells and analyzed by quantitative PCR (Figure 11). Treatment with lapatinib significantly reduced MCP-1, RANTES, MMP-3, and osteopontin expression. In addition, treatment with lapatinib reduced the expression of pro-inflammatory adhesion molecules ICAM-1 and VCAM-1. Treatment with lapatinib also significantly increased the expression of the tight junction molecule occludin and adherens junction molecule VE-Cadherin in LPS stimulated bEnd.3 cells.

Discussion

In this study we demonstrate that EGFR inhibition can decrease the severity of EAE by selectively targeting endothelial cell mediated inflammation. In MS, endothelial cells are responsible for releasing pro-inflammatory cytokines and chemokines as well as recruiting lymphocytes into the CNS [11].

In vivo, our data indicate that EGFR inhibition with lapatinib, erlotinib, and gefitinib decreases EAE severity. We also show that splenocytes from treatment groups had decreased IFN-γ and IL-17 production upon MOG stimulation. In addition, we show that expression of amphiregulin, an EGFR ligand, is increased in EAE serum and spinal cord section when compared to naive mice. These results suggest that EGFR signaling contributes to MS pathogenesis, and its inhibition provides a promising potential therapy for MS.

To determine why EGFR inhibition decreased the severity of EAE, we examined the levels of EGFR expression in cell types commonly implicated in MS pathogenesis, including splenocytes, macrophages, and endothelial cells. We show that EGFR is differentially transcribed in macrophages and bEnd.3 cells as compared to essential immune cells, such as T cells, B cells, and monocytes. These data support our conclusion that EGFR inhibition selectively targets brain endothelial cells, which play a central role in MS pathogenesis.

To further investigate macrophages and brain endothelial cells, we first examined the effects of lapatinib on macrophage activation and cytokine production. We show that EGFR inhibition did not affect classical macrophage activation as measured by TNF-α and IL-12p40 production. In addition, lapatinib treatment did not

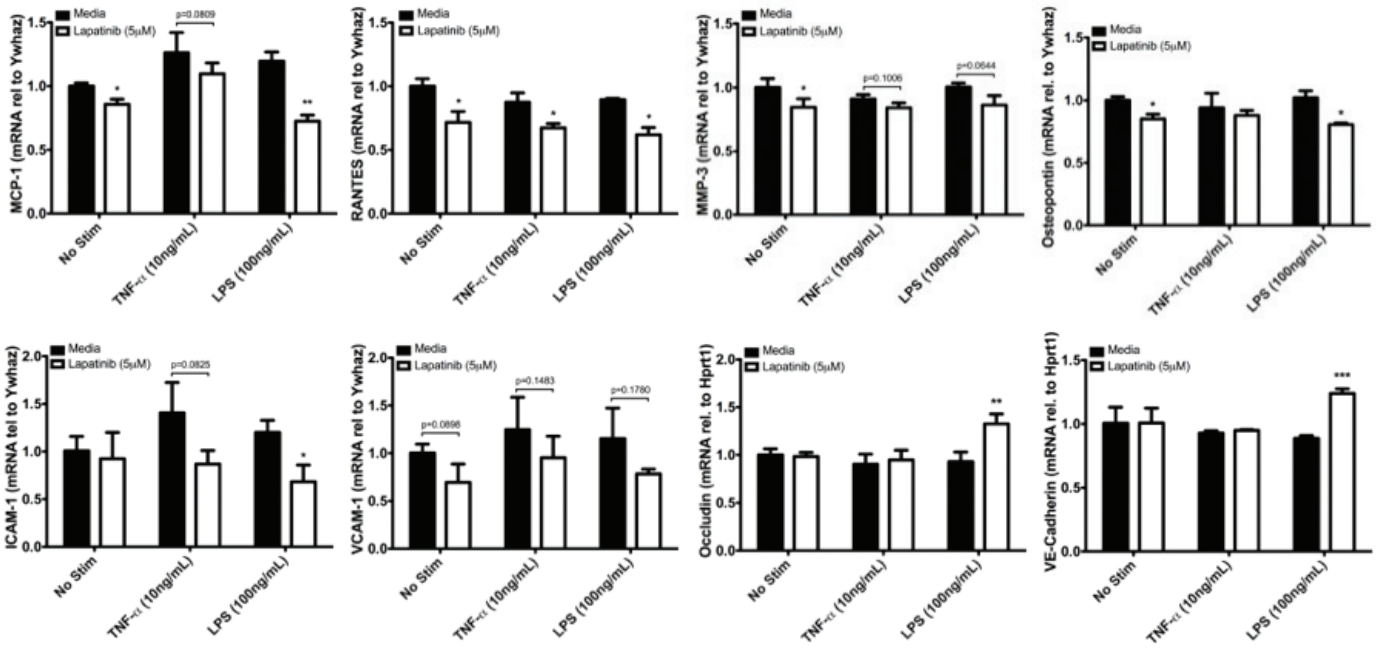


Figure 11. Lapatinib reduces bEnd.3 cytokine, chemokine, and adhesion molecule expression and increases tight and adherens junction molecule expression. bEnd.3 cells were pretreated with media or 5 μM lapatinib for 15 min, then stimulated with media, 10 ng/mL of TNF-α, or 100 ng/mL of LPS. After 45 min, mRNA was collected from the cells and analyzed by qPCR. Lapatinib significantly reduced MCP-1, RANTES, MMP-3, osteopontin, ICAM-1, and VCAM-1 expression. Lapatinib also significantly increased occluding and VE-Cadherin expression. *P < 0.05.

significantly alter TNF- α, IL-6, or IL-10 production. These results suggest that EGFR inhibition does not treat EAE by inhibiting macrophage driven inflammation.

Next, we examined the effects of EGFR inhibition on brain endothelial cells. We show that lapatinib reduced bEnd.3 proliferation. This suggests that EGFR inhibition decreases angiogenesis in the brain, a component of inflammation in MS [12]. We also found that lapatinib reduces MCP-1 and MCSF production in bEnd.3 cells. Lapatinib treatment also reduced MCP-1, RANTES, MMP-3, and osteopontin expression in bEnd.3 cells. In MS, MCP-1 and RANTES are chemokines released by brain endothelial cells that attract monocytes and macrophages to sites of demyelination [13]. Endothelial cells also release MMP-3 into the surrounding extracellular matrix, forming a perivascular cuff around vessels that facilitates infiltration by lymphocytes into the CNS [14]. Expression of osteopontin, a molecule that binds to α4β1 integrin on T Cells to activate the NF-κB pathway, is also increased in MS [1]. These results suggest that EGFR inhibition decreases endothelial cell driven inflammation in the brain by reducing cytokine, chemokine, and MMP expression.

We further demonstrate that lapatinib treatment reduced VCAM-1 and ICAM-1 expression in bEnd.3 cells. In MS, expression of VCAM-1 is increased, which binds to α4β1 integrin, allowing T-Cells to cross the blood brain barrier (BBB) and into the CNS. Similarly, ICAM-1 is an adhesion molecule that recruits lymphocytes, monocytes, and macrophages across the BBB [15]. Thus, these results suggest that EGFR inhibition reduces immune cell trafficking across the BBB by decreasing expression of adhesion molecules VCAM-1 and ICAM-1 on brain endothelial cells.

We also found that lapatinib treatment increased occludin and VE-cahderin expression in bEnd.3 cells. In MS, occludin expression is decreased in endothelial cells, contributing to loss of integrity of tight junctions in the BBB. VE-cahderin expression is also decreased, leading to loss of integrity of adherens junctions in the BBB [14]. Due to weaker tight and adherens junctions, the BBB is made more penetrable by infiltrating cells in MS. Thus, these results suggest that EGFR inhibition increases the integrity of tight and adherens junctions in the BBB by contributing to increased expression of occludin and VE-cadherin.

Together these data indicate that EGFR signaling promotes brain endothelial cell trafficking of immune cells into the CNS, which is reduced with EGFR inhibition. We show that EGFR inhibition decreases brain endothelial cell proliferation, cytokine, and chemokine release, preventing the recruitment of immune cells to the CNS. In addition, EGFR inhibition reduces immune cell penetration of the BBB by decreasing the expression of VCAM-1 and ICAM-1. We also show that EGFR inhibition increases the expression of tight and adherens junction molecules, further reducing the penetrability of the BBB.

Conclusion

We show that EGFR is a novel target for the treatment for MS. In contrast to existing globally immunosuppressive treatments, EGFR inhibition selectively targets brain endothelial cells and does not have significant effects on T Cells, B Cells, monocytes, or macrophages. We show that the mechanism by which EGFR inhibition decreases EAE severity is the reduction lymphocyte trafficking across the BBB. Treatment with lapatinib decreased brain endothelial cell proliferation, cytokine, and chemokine production.

EGFR inhibition still warrants further investigation as a potential therapy for MS. Future experiments will be to examine the effects of amphiregulin, an EGFR ligand that is increased in EAE. We must also examine the expression of EGFR and its ligands in human brain endothelial cells during the course of MS.

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Fitness Costs of HIV-1 Mutational Escape from Cytotoxic T Lymphocytes in Acute Infection[1]

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Cytotoxic T lymphocytes (CTLs) are an important mechanism for suppression of HIV-1 viremia in acute infection, because they recognize and lyse infected cells by binding viral epitopes presented by host major histocompatibility complex class I (MHC-I) on infected cells. Viral persistence in infected persons occurs despite CTL suppression for many reasons, including HIV-1 mutational escape from CTLs. When these mutations decrease epitope binding by CTLs, the virus can escape the immune response. While such mutations allow the virus to escape lysis from CTLs, many mutations confer a fitness cost. This is shown by the tendency of HIV-1 to revert back escape mutations from the prior host when it is transmitted to a MHC-unmatched host. It is unclear whether these fitness costs arise during the rapid viremia of acute infection. One person identified in acute infection was studied to determine the fitness costs of potential escape mutations using an assay that competes viruses to measure their relative fitness. An unmodified virus representing virions circulating before developing escape mutations was competed against a virus containing potential escape mutations in the gag-pol region that developed within several weeks after infection. The two mutated viruses analyzed both indicated increased fitness as compared to the unmodified virus, showing that these two mutations actually increase the fitness of the virus.

Introduction

Understanding the structure and mechanism of the HIV-1 virus is critical to developing an effective vaccine. HIV-1 is a spherical retrovirus that measures approximately 100-120 nm in diameter. The cone-shaped viral capsid encloses two identical single-stranded RNA molecules and viral enzymes, including reverse transcriptase, integrase, protease, and regulatory proteins such as Nef and Vpr. HIV-1 infects host cells by binding to cell receptors with the gp120 protein [13]. These viruses predominantly infect CD4 T cells, which are responsible for supporting humoral and cell-mediated immunity [14]. After binding, the viral and cellular membranes fuse, and the viral RNA is released into the cell with other viral enzymes. The virus reverse-transcribes the viral RNA into DNA, which is integrated into the host genome by the viral integrase [13]. Activation of cellular transcription thus induces transcription of viral DNA into mRNA, which can then enter the cytoplasm and generate viral proteins on host ribosomes. Infected CD4 T cell levels are gradually depleted, both through the direct effect of the virus and through activation of cytotoxic T lymphocytes (CTLs), which recognize and target virally infected cells [9].

Attempts have been made to exploit the virus-depleting capabilities of the CTLs [9]. The T cell receptors of CTLs recognize cells displaying viral fragments bound to the Major Histocompatibility Complex Class I (MHC-I) glycoproteins, which are found on every nucleated cell, including the infected CD4 cells. These CTLs also express the cell-surface protein CD8, which binds to the invariant portions of the MHC-I molecule, facilitating and stabilizing binding [8]. Once the CTLs have recognized and bound to the CD4 cells that display viral fragments, they release granules containing perforin, granzymes, and other proteins into the infected cell, causing cell lysis and death [3]. Viremia occurs early in HIV-1 infection and peaks three weeks after exposure; viral loads then diminish, remaining at a low level until they rise in the rampant viremia associated with the onset of AIDS. These diminished viral loads are associated with the emergence of CTLs targeted for HIV-1 epitopes [9]. CTLs are thus an important mechanism for suppressing HIV-1 and limiting its spread; however, suppression of HIV-1 is incomplete [15].

One reason for the incomplete suppression of HIV-1 is its high mutation rate. Viral replication is rapid [6] and error-prone [4]. The errors in viral reverse transcription lead to mutations, known as epitope escape mutations, which decrease the ability of CTLs to recognize and bind to HIV-infected cells. These mutations have prevented the development of an effective CTL-targeted vaccine. Epitope escape mutations can decrease binding between the epitope and MHC Class I molecules; they can also decrease T cell receptor (TCR) recognition, or change epitope processing. These escape mutations can arise in either acute or chronic infection. During acute infection, the viral replication rate is so high that every possible point mutation could be generated in a single day. The potential for escape mutation development is therefore particularly high during this period [5].

	GWR1.3.3M3	GWR1.3.3M9	GWR1.3.3M3+9	GWR1.3.3M4	GWR1.3.3M8	GWR1.3.3M10	GWR1.3.3M8+10
AA Position	Gag465	Gag481	Gag465 + 481	Pol241	Pol616	Pol623	Pol616 + 623
GWR1.3.3 Sequence	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Mutated sequence	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Amino acid change	F → L	K → Q	F → L K → Q	D → E	K → R	S → P	K → R S → P
Nucleotide change	TTT → CTT	AAG → CAG	TTT → CTT AAG → CAG	GAC → GAA	AGA → AAA	TCC → CCC	AGA → AAA TCC → CCC

Table 1. *Summary of Amino Acid Mutations*
Beginning with the wildtype sequence, point mutations were induced in GWR1.3.3. For each mutation, the amino acid position is given in the table. The amino acid sequence of the wildtype GWR1.3.3 in the indicated location is given, along with the sequence of the mutated virus. The amino acid change is given, along with the change in the single nucleotide to make the mutation.
.

GWR	Date	Viral Load	CD4	HLA Type		
				A	B	C
Symptom Onset	5/19/2004	N/A	N/A	A01, A02	B08, B18	Cw7, Cw7
v1	6/2/2004	749000	445			
v2	6/14/2004	83900	534			
v3	6/23/2004	1150000	538			
v4	8/2/2004	1420000	419			
V5	8/30/2004	1250000	441			

Table 2. *Summary of Patient Information*
Symptoms arose on 5/19/2004 for patient GWR. Visit 1 (6/2/2004) cells had a viral load of 749000. Visit 2 (6/14/2004) cells had a decreased viral load of 83900. Visit 3 (6/23/2004) cells had an increased viral load of 1150000. Visit 4 (8/2/2004) cells had a viral load of 1420000. Visit 5 (8/30/2004) cells had a viral load of 1250000. Patient GWR has a HLA type of A01/A01, B08/B18, and Cw7/Cw7.

	GWR1.3.3M3 Gag455-472	GWR1.3.3M9 Gag474-488	GWR1.3.3M4 Pol234-248	GWR1.3.3M8 Pol609-622	GWR1.3.3M10 Pol618-631
Consensus	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Consensus t=1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
t=1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
t = 2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
t = 3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
t = 4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
t=5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Pattern	Diverge from consensus sequence	Diverge from consensus sequence	Diverge from consensus and then revert back	Revert back to consensus sequence	Diverge from consensus sequence

Table 3. *Summary of all mutations selected*
Patient samples from five time-points during acute infection were evaluated for mutations. Top row shows the names of all the mutations, along with their positions in the gag or the pol protein. The consensus sequence illustrates the consensus sequence as determined by the HIV World Database for all HIV-1 Clade B infected individuals. Consensus t=1 shows the sequence that is used as the wildtype sequence in the experiment. t=1, t=2, and t=5 show the proportion of samples with each sequence during visits 1, 2, and 5 respectively. The pattern shows whether the mutation is a divergence from the consensus, a reversion back to the consensus, or, in the example of GWR1.3.3M4, a divergence followed by a rapid reversion.

There is, however, a tension between the selective pressure that causes an escape mutation and the fitness costs of that mutation. Mutations have fitness costs when the mutation decreases the survival of the virus. This tension is reflected in the tendency of HIV-1 viruses that have developed escape mutations to rapidly revert back to their original sequence when transferred to a MHC-unmatched individual [10]. The fitness costs of many escape mutations are so significant that the mutations only arise in the presence of compensatory mutations [11]. The exact factors allowing viral escape in early infection are unknown, which hinders the development of an effective CTL vaccine [5]. Mutations in the viral sequence are generally deleterious, incurring a fitness cost, but the severity of the fitness costs varies for each mutation. It is still unclear whether escape mutations that arise in acute infection incur a fitness cost, for fitness costs typically arise when the virus targets constant regions of the virus in chronic infection. Some escape mutations in early infection may even increase the fitness of the virus. The virus can undergo positive or purifying selection by the CTLs. Positive selection occurs to favor a particular mutation that increases the fitness of the virus. Negative, or purifying, selection selects against deleterious mutations. Recently, evidence has appeared that indicates that viruses in acute infection have high viral fitness [2]. By isolating escape mutations from an individual and replicating them in an *in vitro* system with no immune pressure, the fitness costs of each mutation in early infection can be investigated.

To examine the effect of fitness cost in acute infection in HIV-1 infected individuals, samples from patient GWR were isolated and examined in an ex vivo system. Escape mutations were isolated and then engineered into a wildtype virus. The fitness, or growth rate, of the mutated virus was compared to the fitness of the wildtype virus. In both mutations studied, the fitness of the mutated virus was

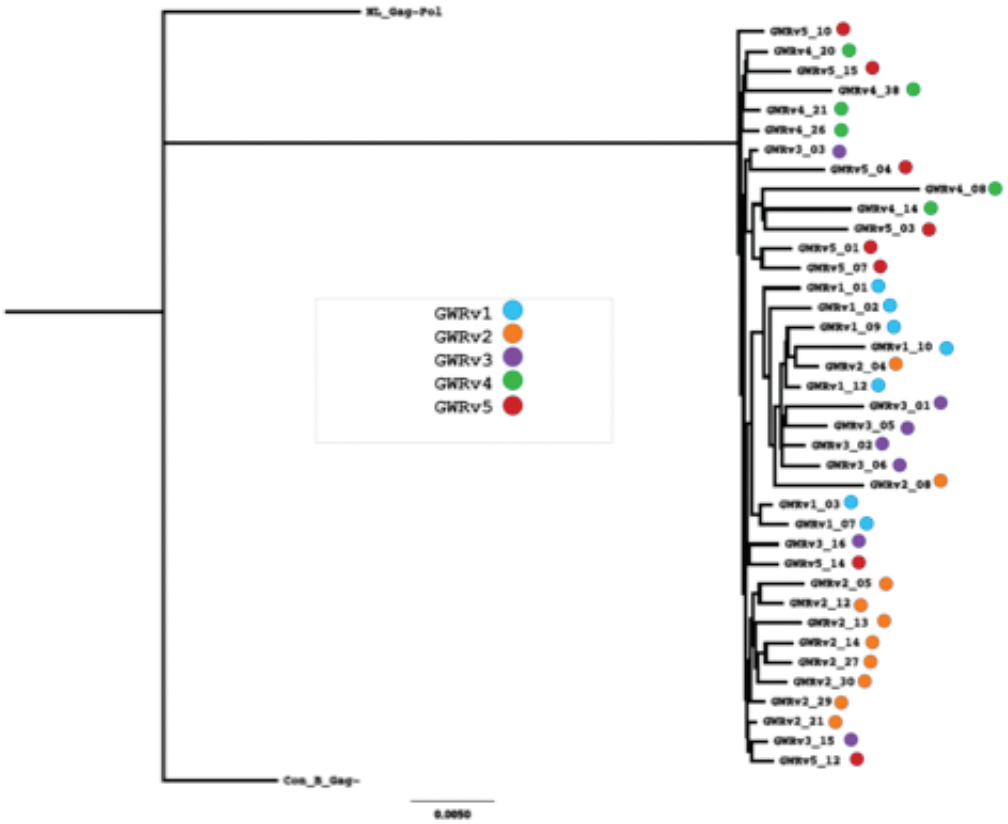


Figure 1. *Phylogenetic Tree of GWR from visits 1-5*
A neighbor-joining tree rooted on Clade B consensus and evaluated with 100 bootstrap replicates was generated for full-length gag-pol. Shorter branches indicate that the virus is more closely related to the consensus sequence, while longer sequences indicate that the virus is further from consensus. Two viruses that branch from the same node are closely related.

better than the fitness of the wildtype virus, suggesting that these particular escape mutations do not confer a significant fitness cost, or adversely affect the survival, to the virus.

Materials and Methods

Patient GWR

Peripheral blood mononuclear cells (PBMC) and plasma samples were collected from an antiretroviral-untreated patient who was identified during early infection by the Los Angeles acute infection cohort (Primary Infection Network; Dr. Erin Daar).

Evolutionary analysis

To examine the evolutionary pattern of mutations that occurred during acute infection, a neighbor-joining tree was created for full-length gag-pol using a 100 replicate bootstrap analysis and SENDBS Software.

Plasmid Modification

Plasmid p83-2 contained an extraneous EcoRI site. PCR-based point mutagenesis of the EcoRI site was used to mutate the plasmid sequence. The mutation was confirmed with sequencing on site by a Biosystems 3130 Genetic Analyzer.

HIV-1 Point Mutations

The sequence from the initial visit (v1) of GWR was used as a wildtype, referred to hereafter as GWR1.3.3. Using PCR-based point mutagenesis of the wildtype sequence, five distinct gag-pol point mutations were induced in amino acid positions GagF465L, GagK481Q, PolD241E, PolK616R, and PolS623P of the full-length

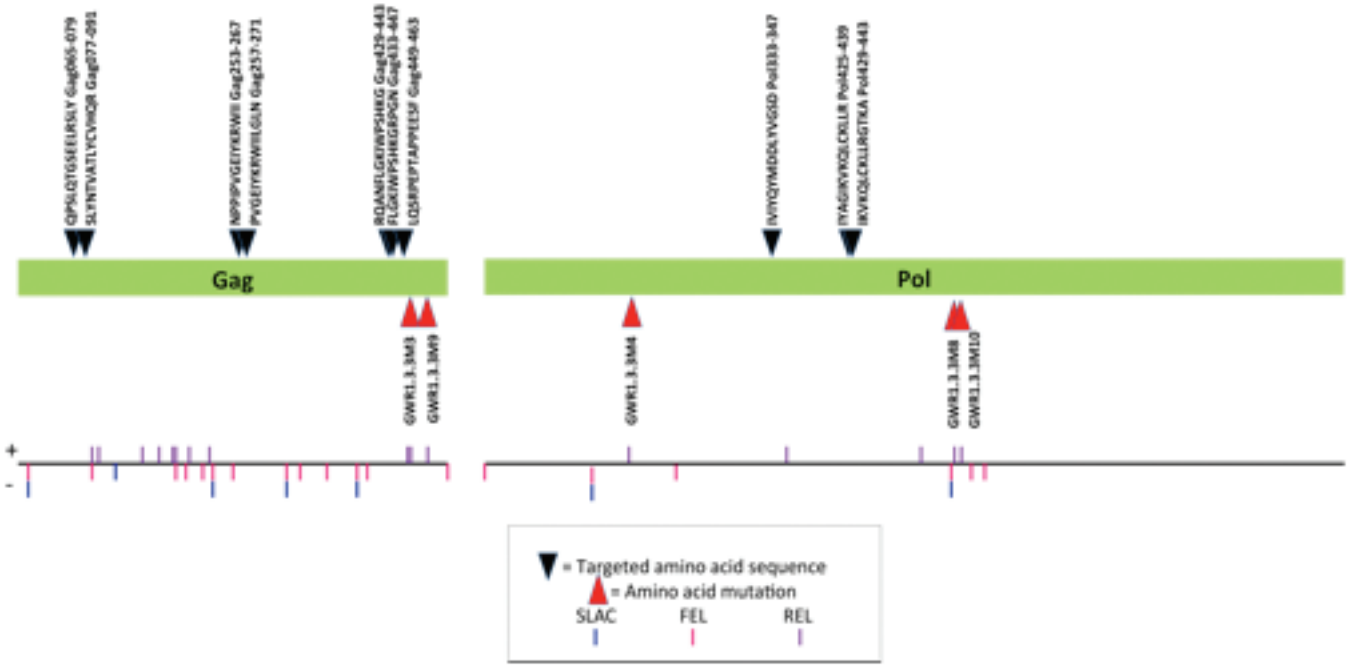


Figure 2. *All selected mutations indicate positive selection*
CTL-targeted regions of the Gag-Pol sequence are shown. The red arrows indicate locations of GWR1.3.3 M3, 9, 4, 8, and 10. Below are the results of SLAC (Single Ancestor Counting), FEL (Fixed Effects Likelihood), and REL (Random Effects Likelihood) analysis for positive or purifying selection. These statistical tests evaluate single site codon selection. The purple lines up show positive selection according to REL analysis. The pink and blue lines show negative selection according to FEL and SLAC analysis, respectively. All the selected mutations show positive selection.

Gag-Pol sequence. These mutations will hereby be referred to as GWR1.3.3M3, GWR1.3.3M9, GWR1.3.3M4, GWR1.3.3M8, and GWR1.3.3M10, respectively (Table 1). In addition, mutations in position GWR1.3.3M3+9 and in position GWR1.3.3M8+10 were induced together to examine the effect of potential compensatory mutations. All mutations were confirmed by full-length Gag-Pol sequencing by a Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Virus Stocks

Each gag-pol mutant was produced by coelectroporation of 7x10^6 T1 cells, an HIV-1 permissive cell line, with p83-2 derivatives and p83-10 plasmid DNA linearized with EcoR1, followed by culture in a 37°C humidified incubator for one week. The presence of virus was tested using a p24 ELISA assay (Perkin Elmer, Waltham, MA). A second round of infection was performed by infecting 10x10^6 T1 cells with viruses generated by electroporation.

Assay of Fitness Costs

An Applied Biosystems StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA) was used to assess the fitness costs of the mutated viruses. Wild-type viruses were tagged with a reporter in the wild-type murine CD24 gene in the nef locus [7]. Mutated viruses were tagged with a slightly modified version of the same gene, containing an antibody epitope from influenza hemagglutinin [1]. Genomic DNA was isolated from wildtype and mutated HIV-1 viruses, and the amplification of this DNA was monitored via real-time PCR over a period of < 5 days. The growth rates of the viruses were plotted, with a bigger increase in viral growth indicating an increased viral fitness.

Selection

Selection at each amino acid was calculated using three different codon-based selection techniques using HYPHY Software Package. Random Effects Likelihood (REL) tests were used to evaluate positive selection, while the Single Ancestor Counting (SLAC) and Fixed Effects Likelihood (FEL) tests were used to evaluate negative selection.

Results

HIV-1 easily escapes from CTL pressure

Samples were taken from a single patient in the first three months after HIV infection (Table 2). Symptoms arose in patient GWR on 5/19/2004. The first blood samples were taken on 6/2/2004, containing a viral load of 749000 and a CD4 count of 445. The second blood samples were taken on 6/14/2004, showing a viral load of 83900 and a CD4 count of 534. Visit 3 samples were taken on 6/23/2004, with an increased viral load of 1150000 and a CD4 count of 538. Visit 4 samples were taken on 8/2/2004, with a viral load of 1420000 and a CD4 count of 419. Visit 5 samples were taken on 8/30/2004 with a viral load of 1250000 and a CD4 count of 441.The HLA type of patient GWR was identified as A01/A02, B08/B18, Cw7/Cw7.

A number of sites were observed evolving at the protein level within the five samples collected in the acute stage of infection, demonstrating remarkable HIV sequence plasticity. Five mutations were identified in patient GWR during acute infection (Table 3). The consensus sequence is the most common amino acid sequence in Clade B HIV-1 infected individuals as determined by the HIV Sequence Database [12]. For each mutation selected, the amino acid change was identified and engineered within the context of the visit 1 clone. Each mutation was then identified as either a divergence from this consensus sequence or a reversion from a mutated sequence back to the consensus sequence.

The virus was seen to evolve across the five time points of acute infection (Fig. 1). In the phylogenetic tree, shorter branches are closer to consensus than are longer branches; two viral sequences that branch from the same node are thus closely related to each other. Phylogenetic analysis (Fig. 1) revealed that the viral sequences began to branch out more during the later time points than they had across earlier visits. The mutations identified in the sample from visit 2, for example, are closely grouped together, while the mutations from visit 5 spread out across the tree. This phylogenetic analysis suggests that the virus is undergoing a form of divergent evolution, although further samples would be needed to confirm the observed trend.

CTL-targeted regions of the Gag-Pol protein of the virus were identified using IFN-γ ELISpot (Fig 2). CTL-targeted regions are the sites of pressure from the immune system on the virus. The selected sites of sequence evolution were identified, showing the Gag-Pol locations of GWR1.3.3M3, 4, 8, 9, and 10. Additionally, sites of positive or purifying selection were identified. Positive and purifying selection sites were identified by SLAC (Single Ancestor Counting) analysis, FEL (Fixed Effects Likelihood) analysis, and REL (Random Effects Likelihood) analysis to evaluate single site codon selection. REL analysis indicates positive selection, while FEL and SLAC analysis indicate purifying selection. All the selected mutations show positive selection.

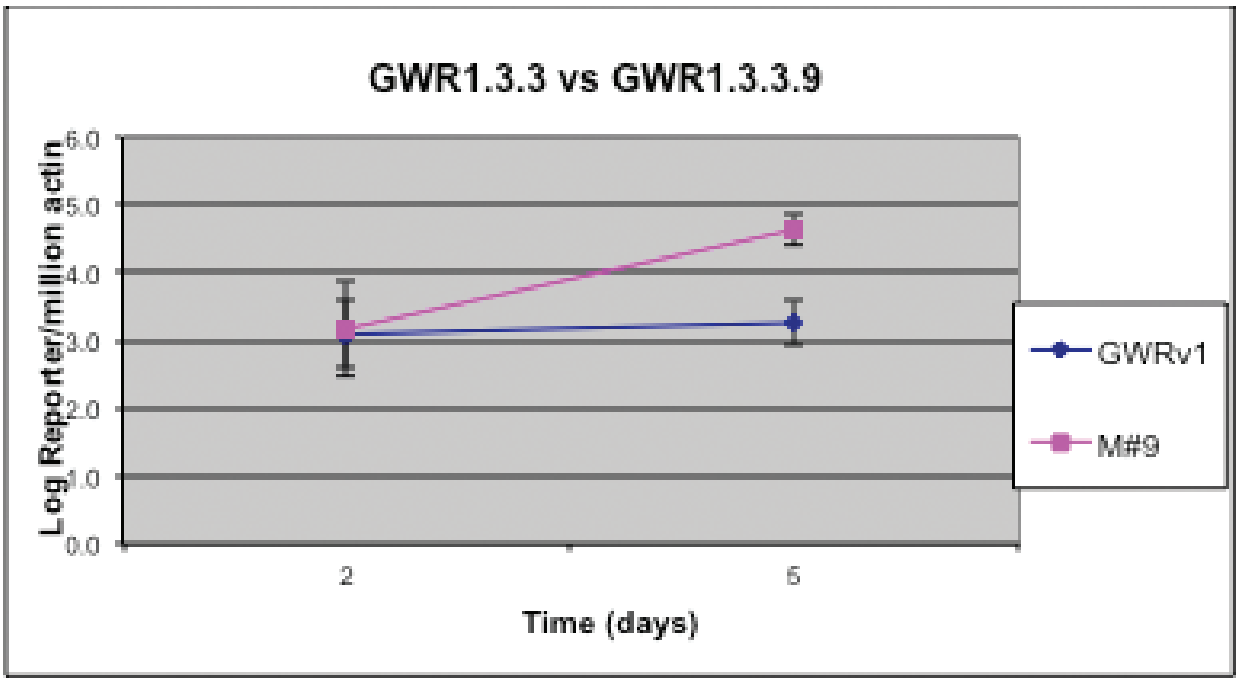
Real-time PCR analysis was performed comparing the growth rate of GWR1.3.3M9 and GWR1.3.3M3+9 compared to the virus from visit 1 (Figure 3). Genomic DNA (gDNA) was isolated on day 2 and day 5 post-infection. To determine which virus grew faster, and was thus the most fit and able to survive, the growth rates of each virus were obtained.

Both GWR1.3.3M9 and GWR1.3.3M3+9 had higher growth rates than the wildtype GWR1.3.3. Wildtype GWR1.3.3 in the M9 experiment had a growth rate of 0.054, while GWR1.3.3M9 had a growth rate of 0.488 (Fig. 3A). Wildtype GWR1.3.3 in the M3+9 experiment had a growth rate of 0.082 while GWR1.3.3M3+9 had a growth rate of 0.600 (Fig. 3B). The increased growth rate relative to wildtype indicates that the mutations in GWR1.3.3M9 and GWR1.3.3M3+9 increased the fitness, or survival, of the virus, confirming the REL analysis that indicated positive selection.

Discussion

Real time analysis of samples from an HIV-infected individual in the acute stages of infection supports the hypothesis that HIV

A



B

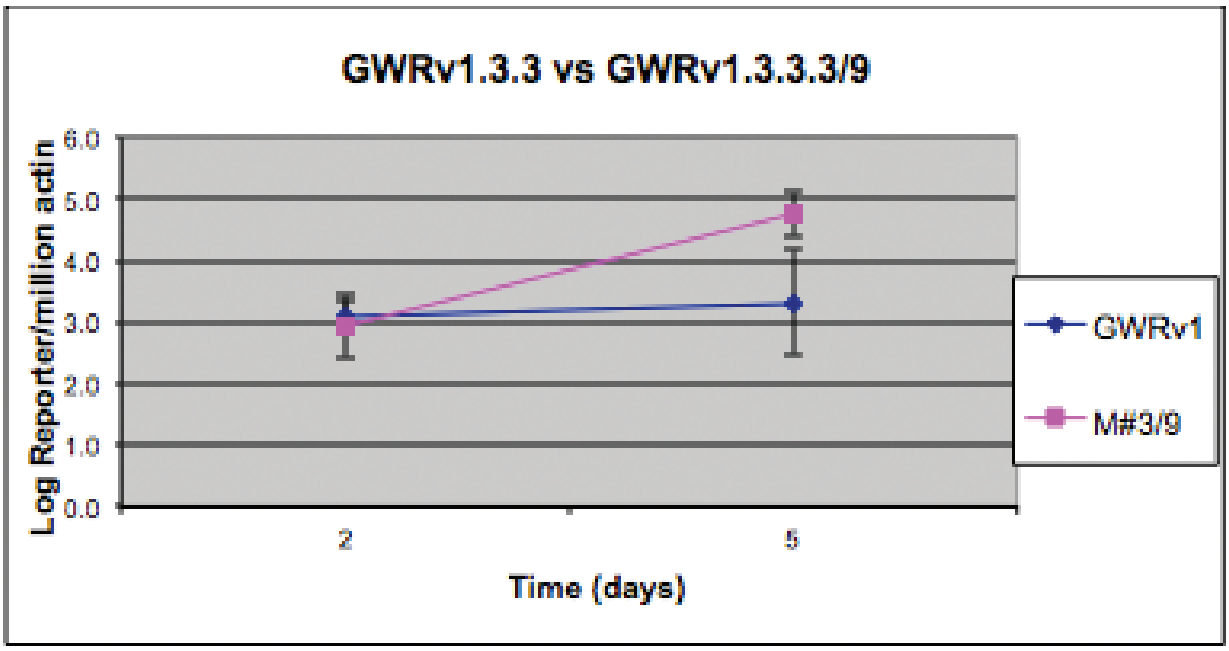


Figure 3. *Fitness Cost Assay of GWR1.3.3M9 and GWR1.3.3M3+9*
Both GWR1.3.3M9 and GWR1.3.3M3+9 had higher growth rates than GWR1.3.3. GWR1.3.3M9 had a growth rate of 0.488 compared to the wildtype growth rate, which was 0.054. GWR1.3.3M3+9 had a growth rate of 0.600 compared to the wildtype growth rate of 0.082.

mutates rapidly to escape CTL pressure. The mutated viruses GWR1.3.3M9 and GWR1.3.3M3+9 have an increased fitness, or survival rate, as compared to wildtype GWR1.3.3, as indicated by the higher replication rate and the positive selection at these sites. Although both mutations studied increased viral fitness, further analysis is required before this result can be generalized to all mutations that occur during the acute stages of infection. Real time PCR analysis of the other mutations is currently underway, and will help to determine whether these escape mutations also confer increased fitness. All of the selected mutations underwent positive selection, and all but one (GWR1.3.3M8) demonstrated a divergence from the consensus sequence. Because the GWR1.3.3M8 mutation represents a reversion back to the consensus sequence, real-time PCR analysis may yield give insight into the relationship between fitness and divergence from the consensus sequence. The reversion in GWR1.3.3M8 could indicate that the wildtype virus is more fit. This could indicate that the earlier mutation had incurred a fitness cost, which decreased the survival of the mutated virus.

Little is known about the presence of fitness costs in acute infection. There is evidence that escape mutations during acute infection in HIV-1 do not confer significant fitness costs and therefore do not adversely affect the survival of the virus because of the rapid evolution of the virus during this time. The virus replicates and mutates so rapidly that any deleterious mutation is quickly changed, while during the slower evolution process in chronic infection, these deleterious mutations cannot be changed as rapidly. The viruses isolated during early infection, in fact, are shown to have high fitness compared to viruses from chronic infection, as measured by real time PCR analysis of the viral replication rate [2]. Escape mutation is complex, with a delicate balance existing between selective pressure exerted by CTLs and fitness cost incurred by the mutations [10]. The information gained from patient GWR may ultimately help to elucidate the role of fitness cost in viral mutational escape from CTLs in HIV-1 infected individuals.

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Returns to Education in India[1]

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Education is a major investment for both ordinary citizens and governments. Therefore, the returns to education, that is, the increase in wages associated with additional schooling, is significant for people making career decisions as well as national education policy-makers. Previous studies of the United States have shown that additional education, especially at the college level, translates to substantially higher wages. This paper analyzes the returns to education for India, a developing economy, for the years 1983-2004. It also investigates the differences in returns for men and women and dynamics in the returns over the twenty-year period. These results paint a helpful picture of the recent Indian labor market. As expected, higher education leads to higher wages. Women have higher returns to education at the college and secondary levels, and returns have decreased over time for the college and secondary levels. Lastly, compared to the United States, India has higher returns to education.

Introduction

Education is an important investment made by students and governments. As with all investments, the returns to education, or the increase in wages associated with additional schooling, are of interest to the investors, who include individuals making career plans and government officials allocating funds to schools.

Returns to education in India in particular are of interest for two reasons. First, numerous papers, including Angrist and Krueger (1992) [1], have investigated returns to education in the United States and other developed, Western countries. On the other hand, India is still considered to be developing. Furthermore, many Eastern cultures, including India, place greater value on education than do Western cultures. It would be curious to see if this translates to higher returns in the labor market.

Second, India's economy possesses several interesting characteristics. The rate of economic growth surpasses 8 percent [9]. Yet, the mean years of schooling is 4.4 years and the literacy rate is only 74 percent, far behind the rest of the world [9]. However, the level of unemployment among educated people is high [3]. Low education attainment levels provide a reason for increasing government spending on education, but high unemployment rates for the educated do not. Therefore, accurate estimates of returns to education could have extremely important implications for government policy and provide information about the economy responsible for such phenomenal growth. Previous papers by Duraisamy (2000) [3] and Dutta (2006) [4] have investigated the returns to education in India in the 1980's and early 90's and for men respectively. In this paper, we expand their analysis to investigate the returns to education from 1983-2004, for both men and women, throughout India.

This paper analyzes the returns to education in India using data from national employment surveys provided by the Indian Ministry of Statistics and the Minnesota Population Center at the University of Minnesota. We estimate the average returns to education, examine any differences in the returns for men and women, and investigate any changes in the returns over the years 1983 to 2004. Section 2 describes the Indian education system and the data set, and Section 3 states our methods of analysis. Section 4 reports our findings and addresses some objections to the analysis, and Section 5 concludes.

Background and Data

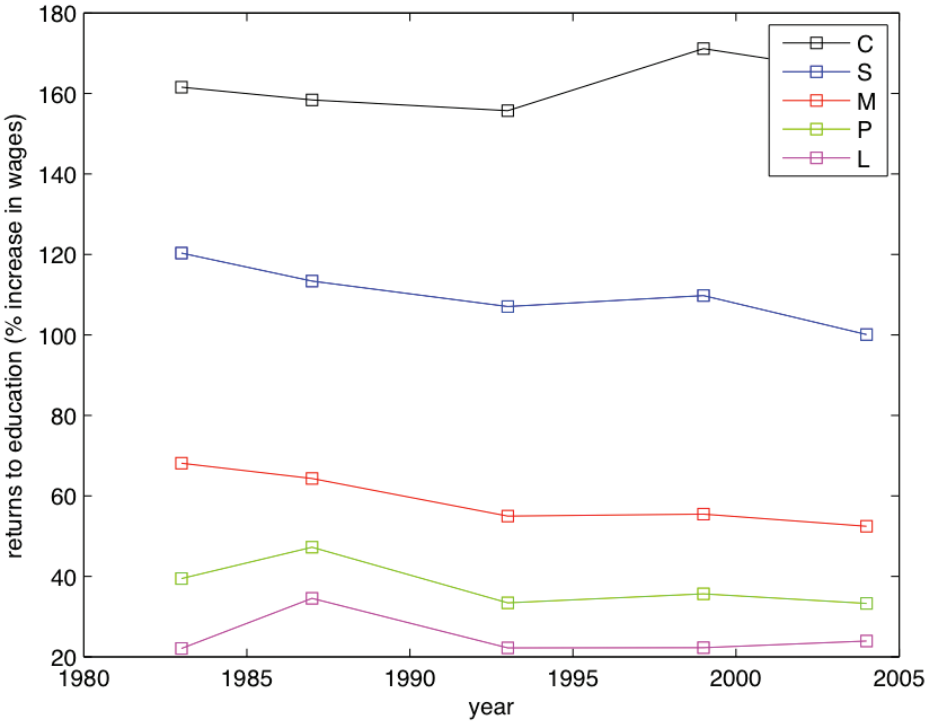
The education system in India consists of five levels: preprimary, primary, middle, secondary, and higher levels, which encompasses technical school, college, and university [6]. Primary education lasts five years, middle three years, and secondary four years [6]. Since 1976, education has been controlled by the central government [6]. In 1986, the National Policy on Education was enacted in order to improve the Indian education system. It sought to increase enrollment across the board and ensure equal access to primary education for all children, and also expanded the university system [6]. Furthermore, in 1993 the government launched a nation-wide campaign to increase literacy, especially among women [6]. These policies have served to increase the literary rate from 43.7 percent in 1981 to 74 percent in 2011, a great improvement [9].

For our analysis we use cross-sectional data from five national Socio-Economic Surveys conducted by India's National Sample Survey Organization on a household basis in 1983, 1987, 1993, 1999, and 2004 [7]. Each survey contains data on approximately 600,000 people, but the sampling methods differ [7]. The data is weighted, which means that every person in the sample represents some percentage of the population [7].

We preprocessed the data so that only employed persons were kept in the data set. For ease of analysis, persons with zero income or unknown age, sex, marital status, or education level were deleted. Due to inconsistencies in reporting, various categories in certain variables were also combined.

In our analysis we utilize the following variables:

- Lnwage: natural logarithm of the weekly wage income in rupees
- Age: age, between 0 and 99
- Age2: Age squared



- Married: indicator variable for married
- Male: indicator variable for male
- C: indicator variable for undergraduate or graduate as the highest education level attained
- S: indicator variable for secondary as the highest education level attained
- M: indicator variable for middle as the highest education level attained
- P: indicator variable for primary as the highest education level attained
- L: indicator variable for literate and less than primary as the highest education level attained
- Eightyseven, Ninetythree, Ninetynine, Ohfour: indicator variables for the sample year (We include indicator variables for four years, not five, to prevent multi-collinearity)
- State1, State2, . . . , State30: indicator variables for the state of residence
- MaleC, MaleS, MaleM, MaleP, MaleL: interaction variables between Male and C, S, M, P, L
- C87, C93, C99, C04, S87, S93, S99, S04, M87, M93, M99, M04, P87, P93, P99, P04, L87, L93, L99, L04: interaction variables between C, S, M, P, L, and Eightyseven, Ninetythree, Ninetynine, Ohfour

Methods of Analysis

Aggregate Returns to Education

We first estimate the aggregate, or average, increase in income associated with certain education levels.

Throughout this paper, we follow the usual Mincerian earnings function framework, with age as a proxy for experience [3]. We first propose the following basic Ordinary Least Squares (OLS) model:

$$(1)\ Lnwage_i = \beta_0 + \beta_1 Age_i + \beta_2 Age2_i + \beta_3 Married_i + \beta_4 Male_i + \beta_5 C_i + \beta_6 S_i + \beta_7 M_i + \beta_8 P_i + \beta_9 L_i + \beta_{10} Eightyseven_i + \ldots + \beta_{13} Ohfour_i + \beta_{14} State1_i + \ldots + \beta_{43} State30_i + \varepsilon_i$$

Because we do not have data on the exact number of years of schooling, we use the dummies C, S, M, P, and L. We include the variables Married, Male, Eightyseven, Ninetythree, Ninetynine, Ohfour, and State1 to State30 to control for inflation and differences between married and single people, between males and females, and in regional labor markets.

However, problems arise when simply using OLS. First, there may be omitted variable bias (OVB) due to factors, such as innate ability, that are correlated with both wages and education level. Unfortunately, with the data available, we cannot correct for OVB. This is discussed further in Section 4.5. Second, there are unobserved fixed effects that are correlated with certain explanatory variables, making OLS inconsistent. For example, the place of residence is a determinant of education - the quality and availability of education varies, especially between urban and rural areas. To correct for this problem, we convert our data to panel data with the

Table 1: Returns to Education, US vs. India (Percents)

Education Level	US	India
College	105.6	163.5
Secondary	79.2	109.6
Middle	52.8	58.4
Primary	33	37.4
Literacy	13.2	24.3

state of residence as the panel variable and use fixed effects (FE) regression. We also modify our model to the following:

$$(2)\ Lnwage_i = \beta_0 + \beta_1 Age_i + \beta_2 Age2_i + \beta_3 Married_i + \beta_4 Male_i + \beta_5 C_i + \beta_6 S_i + \beta_7 M_i + \beta_8 P_i + \beta_9 L_i + \beta_{10} Eightyseven_i + \ldots + \beta_{13} Ohfour_i + \varepsilon_i$$

To confirm the validity of this FE model versus a random effects (RE) model, we carried out a Hausman test [5]. We rejected the null hypothesis that the unobserved fixed effects discussed above are uncorrelated with explanatory variables. Therefore, we use the coefficients from a FE regression of specification (2), with robust standard errors (RSE) to account for heteroskedasticity as estimates of the aggregate returns to education. The results are given in Table 1 of the Appendix.

Differences for Males and Females

India, like many other countries, has made strides in women's rights in the last thirty to forty years. We next investigate differences in the returns to education for males and females.

To capture the differences between the returns to education for males and females, we add the interaction variables MaleC, MaleS, MaleM, MaleP, and MaleL to specification (2). Therefore, we use the following model:

$$(3)\ Lnwage_i = \beta_0 + \beta_1 Age_i + \beta_2 Age2_i + \beta_3 Married_i + \beta_4 Male_i + \beta_5 C_i + \beta_6 S_i + \beta_7 M_i + \beta_8 P_i + \beta_9 L_i + \beta_{10} Eightyseven_i + \ldots + \beta_{13} Ohfour_i + \beta_{14} MaleC_i + \beta_{15} MaleS_i + \beta_{16} MaleM_i + \beta_{17} MaleP_i + \beta_{18} MaleL_i + \varepsilon_i$$

Once again, to confirm the validity of this FE model versus a random effects (RE) model in this situation, we carried out a Hausman test [5]. We rejected the null hypothesis that unobserved fixed effects are uncorrelated with explanatory variables. Therefore, we use the coefficients from a FE regression of specification (3) with RSE as estimates of the differences in returns to education for men and women. The results are given in Table 2 of the Appendix.

Movements in Returns to Education over Time

Lastly, we investigate changes in the returns to education in India from 1983 to 2004. This analysis could shed light on the effects of the educational policies of the 1980's on the labor market.

To capture changes over time in the returns to education, we add the interaction variables C87, C93, C99, C04, S87, S93, S99, S04, M87, M93, M99, M04, P87, P93, P99, P04, L87, L93, L99, and L04 to specification (2). Thus, we use the following model:

$$(4)\ Lnwage_i = \beta_0 + \beta_1 Age_i + \beta_2 Age2_i + \beta_3 Married_i + \beta_4 Male_i + \beta_5 C_i + \beta_6 S_i + \beta_7 M_i + \beta_8 P_i + \beta_9 L_i + \beta_{10} Eightyseven_i + \ldots + \beta_{13} Ohfour_i + \beta_{14} C87_i + \beta_{15} C93_i + \beta_{16} C99_i + \beta_{17} C04_i + \beta_{18} S87_i + \beta_{19} S93_i + \beta_{20} S99_i + \beta_{21} S04_i + \beta_{22} M87_i + \beta_{23} M93_i + \beta_{24} M99_i + \beta_{25} M04_i + \beta_{26} P87_i + \beta_{27} P93_i + \beta_{28} P99_i + \beta_{29} P04_i + \beta_{30} L87_i + \beta_{31} L93_i + \beta_{32} L99_i + \beta_{33} L04_i + \varepsilon_i$$

Again, to confirm the validity of this FE model versus a random effects (RE) model in this situation, we carried out a Hausman test [5]. We rejected the null hypothesis that unobserved fixed effects

are uncorrelated with explanatory variables. Therefore, we use the coefficients from a FE regression of specification (4) with RSE as estimates of the variations in returns to education in various years. The results are given in Table 3 of the Appendix.

Results

Aggregate Returns to Education

From Table 1, we may conclude that, compared to being illiterate and not having a primary education, being literate and not having a primary education is associated with a 24.3 percent increase in wages, having a primary education is associated with a 37.4 percent increase, having a middle school education is associated with a 58.4 percent increase, having a secondary education is associated with a 109.6 percent increase, and having a college education is associated with a 163.5 percent increase. As expected, higher education is associated with higher financial returns. It is worth noting that these estimates are quite precise; the t-statistics range from 18.1 to 32.0, much higher than our cutoff for statistical significance, which is 3.0.

Differences for Males and Females

For specification (3), the difference in the returns to a college education for men and women is equal to the coefficient of MaleC. Similarly, the difference for a secondary education is the coefficient of MaleS, the difference for a middle school education is the coefficient of MaleM, the difference for a primary education is the coefficient of MaleP, and the difference for literacy and no primary education is MaleL.

By computing the t-statistics for those five coefficients in the regression results given in Table 2, we conclude that the difference in returns to education between men and women is significant only for the college, secondary, and primary levels. The economic returns to college or secondary education are higher for women, while the return to primary education is higher for women.

These observations may be explained by cultural norms. In India, much fewer women than men possess secondary or college educations. Those who do likely are the most intelligent and able, and thus are expected to have higher earnings and returns to education. At the primary level, the numbers of men and women are more equal, so the higher returns for men may merely be a reflection of cultural bias against women. These results are similar to those of Duraisamy (2000) [3] for the years 1983 to 1994.

Movements in Returns over Time

For specification (4), the percent increase in wages associated with a college education is equal to the coefficient of C in 1983, the sums of the coefficients of C and C87 in 1987, the sums of the coefficients of C and C93 in 1993, the sums of the coefficients of C and C99 in 1999, and the sums of the coefficients of C and C04 in 2004. The returns to the other levels of education in each of the five years are defined similarly.

Figure 1 is a plot of the percent increases in wages associated with college, secondary, middle, primary, and literate and no primary education levels in 1983, 1987, 1993, 1999, and 2004, calculated from the estimates in Table 3.

According to Psacharopoulos (1994) [8], globally the returns to education tend to decrease over time in a country. India somewhat follows this pattern. From hypothesis testing, we conclude that the returns to education change significantly over these five years for the college, secondary, middle, and literate and no primary

Table 2: Panel Estimates of the Aggregate Returns to Education

Variables	FE with RSE
C	1.635 (0.0511)
S	1.096 (0.0399)
M	0.584 (0.0225)
P	0.374 (0.0187)
L	0.243 (0.0134)
Constant	2.171
Number of Observations	410653
R^2	0.666

Notes: Individual level observations. Fixed effects panel regression with robust (clustered) standard errors. The dependent variable is the natural logarithm of wages. See Section 2 for variable descriptions.

Table 3: Panel Estimates of the Differences in Returns for Men and Women

Variables	FE with RSE
C	1.868 (0.0787)
S	1.381 (0.0559)
M	0.522 (0.0310)
P	0.274 (0.0134)
L	0.234 (0.0180)
MaleC	-0.306 (0.0577)
MaleS	-0.351 (0.0469)
MaleM	0.049 (0.0395)
MaleP	0.097 (0.0304)
MaleL	-0.007 (0.0247)
Constant	2.131
Number of Observations	410653
R^2	0.668

Notes: Individual level observations. Fixed effects panel regression with robust (clustered) standard errors. The dependent variable is the natural logarithm of wages. See Section 2 for variable descriptions.

Table 4: Panel Estimates of the Dynamics in Returns to Education

Variables	FE with RSE	Variables	FE with RSE
C	1.616 (0.0635)	M87	-0.0381 (0.0411)
S	1.204 (0.0532)	M93	-0.1318 (0.0370)
M	0.681 (0.0327)	M99	-0.1266 (0.0334)
P	0.394 (0.0235)	M04	-0.1572 (0.0343)
L	0.221 (0.0148)	P87	0.0781 (0.0454)
C87	-0.0317 (0.0349)	P93	-0.061 (0.0293)
C93	-0.0585 (0.0383)	P99	-0.038 (0.0314)
C99	0.0961 (0.0450)	P04	-0.062 (0.0279)
C04	0.0338 (0.0403)	L87	0.125 (0.0299)
S87	-0.0698 (0.0351)	L93	0.001 (0.0205)
S93	-0.1329 (0.0307)	L99	0.002 (0.0205)
S99	-0.1060 (0.0424)	L04	0.018 (0.0126)
S04	-0.2027 (0.0518)	Constant	2.159
Number of Observations	410653	R^2	0.666

Notes: Individual level observations. Fixed effects panel regression with robust (clustered) standard errors. The dependent variable is the natural logarithm of wages. See Section 2 for variable descriptions.

education levels. From Figure 1, it appears that for the college, secondary, and middle education levels, returns generally decreased, while at the literate and no primary level, returns increased.

The decreasing returns at the college and secondary levels can be explained by the government education reforms in the 1980's discussed in Section 2. These reforms increased the number of universities and the availability of education throughout India. Hence, the percentages of individuals at higher education levels increased [6], thereby lowering the wages for those jobs requiring more education and leading to smaller returns in the labor market.

Comparison to the United States

In Section 1, we cited differences in cultural values of education between Eastern and Western countries as a motivation for our research. Therefore, to discover if Indians' greater emphasis on education does indeed translate to higher returns to education, we take the returns to education for the United States given in a paper by Angrist and Krueger [1], and compare them to our results. According to Angrist and Krueger, in the United States from 1979-85, an extra year of schooling was associated with a 6.6 percent increase in weekly earnings.

For ease of comparison, we convert these yearly returns to returns of education levels. Assuming that college takes 16 years, secondary 12, middle 8, primary 5, and literacy 2, we estimate that for the United States the return to k years of schooling equates to 6.6k percent increase in earnings over those of an illiterate person. Table 1 shows the percent increase in wages over those of an illiterate person associated with each education level for the US and India.

It is apparent that the returns to education in India are higher than those in the United States. Whether this is due to differences in cultural views of education or the fact that India is a developing country while the US is not is unclear.

Weaknesses

As mentioned previously, our specifications are subject to sources of omitted variable bias such as natural ability. Usually an instrumental variable is used to correct this, but none are available in the data; there is no one variable that is correlated with the level of education but is uncorrelated with wages. However, we may make conjectures about the direction of the bias. Natural ability is positively correlated with education level, and it tends to increase earnings. Therefore, the OVB creates an upward bias in our estimates of the returns to education - this also accounts for the extremely high t-statistics we have seen.

In addition, there may be errors in variables, such as misrepresentations of age or education level. This may create downward bias in our estimates of the returns to education. For the United States, Card (2001) [2] estimates the downward bias to be about 10 to 15 percent, "enough to offset a modest upward ability bias". Therefore, the overall bias is ambiguous.

Another weakness of this analysis is possibility of sample selection bias. We do not know the method of sampling for all five surveys, and each survey includes less than 1 percent of the total population. In addition, only approximately half of the Indian population is in the labor force [3]. Since we restricted our data to those who are employed, there may be selection bias against various groups such as women and the elderly. Generally, there is no good way to correct for sample selection bias. Heckman developed a method to correct for selection bias arising from low labor force participation, but such a method requires data non non-wage income, which we do not have [3]. Therefore, we cannot correct for sample selection bias with the data available.

Conclusion and Future Work

From our analysis, we may conclude that higher education leads to higher economic returns. In addition, the returns to college or secondary education are much higher for women and generally decreased in the years 1983 to 2004. Lastly, India has greater returns to education than the United States, which may be attributed to cultural and economic differences.

Therefore, it is apparent that, from an economic perspective, in India it is beneficial in the long run to receive higher education. Whether this implies that the government should spend more on education depends on the cost of education and needs further investigation.

One direction for further research would be to investigate where the differences between the returns to education for men and women changed from 1983 to 2004. Another would be to examine the effect of outsourcing on wages and returns to education in India, as any foreign companies have moved operations there in the last ten years. Yet another would be to compare the returns to education for India and China, currently the two fastest growing economies in the world [9].

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