

Scientific Summary

The incidence of Crohn's Disease (CD) is increasing, especially in young children and infants, posing an increasing burden on society.^(1, 2) CD is believed to be the result of a failure of tolerance induction to normal intestinal bacteria in genetically predisposed individuals.⁽³⁾ Tolerance is mediated by regulatory T cells (Tregs). Few treatments seek to augment the suppressive aspects of the immune system such as Tregs. Instead, current treatments rely on modifying the immune system by inhibiting pro-inflammatory responses. These therapies can have serious side effects, especially in children whose growth and development can be permanently affected. There is an urgent need for new therapies that have less potential side effects. Few treatments have sought to utilize Tregs, and none have done so through the skin, as a therapy to suppress inflammation in the gut.

Studies using the neo-antigen keyhole limpet hemocyanin have shown that CD patients have an inherent defect in the ability to form tolerance via the gut.^(4, 5) Thus, attempts at inducing oral tolerance as a treatment for CD have so far not been successful,⁽⁶⁾ and other routes to induce tolerance should be explored. Our initial studies have shown that the skin is a highly active immune organ capable of the induction of effector cells,⁽⁷⁾ as well as immune tolerance. Work utilizing an antigen delivery system through the skin, Viaskin®, has shown the induction of Tregs and suppression of eosinophilic intestinal inflammation.^(8, 9) Our preliminary data demonstrate that tolerance and the induction of Tregs can be achieved by epicutaneous exposure. These Tregs can be found systemically, in the mesenteric lymph nodes and in the intestine and are able to suppress inflammation. *We therefore propose to develop a **novel, innovative approach to treat Crohn's disease** by epicutaneously inducing Tregs to suppress intestinal inflammation via bystander suppression.*

We will perform studies determining the potential of epicutaneous tolerance induction for the treatment of CD. We will begin by examining Tregs generated via epicutaneous exposure to antigen, augmenting their homing to the gut with retinoic acid, and activating them with subsequent feeding of antigen. We will then determine if they are stable during colitis and if they function to suppress intestinal inflammation. Finally, we will determine mechanisms of colitis suppression.

The central hypothesis of this application is that epicutaneous exposure to an innocuous antigen will generate tolerance and an induction of Tregs, which will control inflammation in CD via bystander suppression. Data from these purposed experiments will open a new therapeutic area for the treatment of CD, which will be examined in future clinical studies in humans.

Project Narrative

The proposed research is relevant to the NIH mission because it will develop a novel therapeutic approach for Crohn's disease which would utilize a patient's own immune system to decrease inflammation without the use of potentially dangerous immunosuppressant medications. In addition, such an approach is expected to have an important positive impact because it will likely be applicable to the treatment of other autoimmune disorders.

Facilities and Other Resources

A. Icahn School of Medicine at Mount Sinai

The Icahn School of Medicine at Mount Sinai (ISMMS) was founded in 1968. From its inception the School has been based in and closely joined to the 1,171-bed Mount Sinai Hospital, one of the largest voluntary academic health centers in the United States, established in 1852. Together they form the Mount Sinai Medical Center. Mount Sinai has always been deeply committed to translational research and to the physician scientist as the model of excellence for medical care, research and education. From its inception, ISMMS has housed an NIH-funded General Clinical Research Center, one of the oldest in the country, which has served as a centerpiece for patient-oriented research for over 45 years. The close interrelationship between the clinical and basic sciences permeates the Medical School curriculum.

Mount Sinai Medical Center straddles the border between the Upper East Side of Manhattan, one of the nation's most affluent neighborhoods, and East Harlem, an ethnically diverse community with some of the highest poverty rates and levels of disease in New York City. Mount Sinai is a major provider of medical care and prevention services in East Harlem, as well as a quaternary referral center for patients throughout the tri-state area. Approximately 60,000 people are treated at Mount Sinai as inpatients annually. Outpatient visits exceed 500,000 each year. Members of the Mount Sinai community are thus acutely aware of disparities in health and health care delivery. MSMC devotes approximately \$15 million annually to community activities, while faculty and students donate their time and expertise to hundreds of community-based programs.

In the past three decades, Mount Sinai has become a major research institution. It has a large and diverse grant portfolio spanning the clinical and basic sciences. From 1999 – 2008, Mount Sinai experienced a 97% increase in NIH funding, the strongest growth of any medical school in New York State. With a faculty of more than 3,400 in 54 clinical and basic science departments, institutes, and centers, Mount Sinai ranks among the top 20 medical schools in receipt of NIH grants with an overall medical school ranking of 18 in the *2010 U.S. News and World Report* rankings of America's best graduate schools. In federal fiscal year 2010, total support for ISMMS from NIH and other federal peer-reviewing agencies was \$329.4 million, including 651 federal grants and contracts. Of this, \$275.2 million came from NIH alone and Mount Sinai was ranked 18th among the nation's 125 medical school in 2008 in NIH funding, the last year complete totals for all Schools were released.

A. 1 Academic Departments

Of the 32 clinical and basic science departments and 16 institutes at Mount Sinai, the most directly related to this career development plan are the Department of Pediatrics, Department of Medicine, and the Immunology Institute.

A.1.1 Department of Pediatrics (XXX, MD)

In 2013, the Jack and Lucy Clark Department of Pediatrics of the Icahn School of Medicine at Mount Sinai celebrated its 135th anniversary. A formal pediatric service was established within Mount Sinai Hospital in 1878 and was the first pediatric service in a general hospital in New York City and one of the first in the United States. The Department is organized into 15 clinical and research Divisions. Additional pediatric clinical and research services, specifically surgical subspecialties, are housed within other Departments at the Medical Center.

Previous leaders of the pediatric faculty at Mount Sinai have made major impacts on American pediatrics and child health. XXX, the first chief of the pediatric services at Mount Sinai, is considered by some to be the father of pediatrics in the United States. Subsequent chairs included XXX, XXX, and XXX, all of whom contributed significantly to current understanding of infectious diseases in children. Dr. XXX, a previous chair of the department (and current active faculty member), is one of the founders of human genetics in the United States and has contributed broadly to the areas of biochemical and molecular genetics, as well as cytogenetics. Dr. XXX, immediate past chair, is a leading pediatric hepatologist and physician-scientist. Dr. XXX, the current Chair and a highly notable physician-scientist in pediatric nephrology, was recruited in 2010.

The Mount Sinai Department of Pediatrics has a distinguished tradition of excellence in clinical, translational and basic research, as well as in innovative patient care and professional education. Research activities in the Department continue to expand and encompass a wide range of problems important for child health. The current research budget for the Department is \$18 million, ranking it 12th in research funding nationally among Departments of Pediatrics. Research efforts of particular note include studies focusing on the pathogenesis of parainfluenza and herpes virus infections; the molecular genetics of congenital heart disease; the developmental biology of the liver and biliary tract; the pathophysiology of cholestatic liver disease; the mechanisms of electrolyte transport by the kidney; the noninvasive treatment of congenital heart defects; the immunopathogenic mechanisms of food hypersensitivity; the regulation of the gene responsible for cystic fibrosis; the development of novel treatment strategies for sickle cell anemia; the development of novel therapeutics for solid organ cancers; the pathophysiology of bone marrow failure syndromes; and studies on the immune defense mechanisms of the neonatal intestine. There are extensive ongoing clinical trials as well as clinical studies on environmental toxins. There are also a number of research initiatives in primary care focusing on violence in the home and the use of tympanostomy tubes in children.

The Department has a strong history of supporting Career Development Awardees, through nationally reviewed K23 and K08 mechanisms as well as through KL2 programs and K12 programs.

Clinical resources available within the Department and its clinical arm, the Kravis Children's Hospital at Mount Sinai Medical Center, include both primary and extensive subspecialty pediatric care. The Children's Hospital houses a 75-bed pediatric inpatient unit, a 16-bed pediatric ICU, and an extensive Child Life facility. Pediatric cardiac ICU facilities are located near the operating suites in the adjacent adult hospital. Outpatient pediatric care is provided in a separate ambulatory care center. Comprehensive available pediatric subspecialty care includes pediatric surgery, cardiology, endocrinology, gastroenterology, hepatology, infectious disease, hematology, oncology, genetics, metabolic disease, pulmonary, neurology, immunology, nephrology, and developmental pediatrics.

A.1.1.1 Division of Allergy and Immunology (Chief, XXX, M.D.) and Gastroenterology (Chief, XXX, MD), Department of Pediatrics

The Division of Allergy and Immunology is responsible for the clinical care children with allergic and immunologic disorders at the Mount Sinai Medical Center and is home to a diverse group of researchers. The Division of Gastroenterology is responsible for the care of children with gastrointestinal disorders including IBD, celiac, food allergies, reflux and constipation. A children's IBD center in the Division currently cares for over 500 children with Crohn's disease and ulcerative colitis.

The Division of Allergy and Immunology has laboratories located in the Icahn School of Medicine at Mount Sinai of which Dr. XXX will have access to. The laboratory is well stocked with basic equipment including -20°C and -80°C freezers, CO₂ incubators, a dissecting microscope, and an LSRII flow cytometer. Of particular importance to this career development plan, all reagents and supplies are available in the laboratory. In addition to basic research, the division has multiple ongoing clinical trials including studies on particular relevance to Dr. XXX's research endeavors. These include clinical trials of Chinese herbal therapy for food allergies and asthma and efforts to use epicutaneous therapies to induce tolerance in food allergic patients.

The Division of Gastroenterology has an office cluster located next to the laboratories. Dr. XXX will have an individual office measuring approximately 10 x 15 feet with a phone, fax, copier, printer and 15" Mac book pro with high-speed LAN and wireless access.

In research, the Division of Immunology received \$71 million from the National Institutes of Health in 2008 placing it among the top 20 departments in the country. Many faculty were instrumental in the success of Mount Sinai's application to become a member of the federal Clinical and Translational Science Award Consortium, which resulted in a \$34.6 million grant to the School of Medicine.

Laboratories are located in the Icahn School of Medicine at Mount Sinai of which Dr. XXX will have access to and where his assigned space will be located. The laboratory is well stocked with basic equipment including -20°C and -80°C freezers, CO₂ incubators, laminar flow hoods, multiple centrifuges, a dissecting microscope, and a Fortesa flow cytometer. Of particular importance to this career development plan, all reagents and supplies are available in the laboratory. He is provided with bench space measuring 7.5 feet long as well as

desk space with a phone, fax, copier, printer and 13" Mac book pro computer with high-speed LAN and wireless connections. This space is in close proximity to his mentor's office and lab space.

A.1.2 Division of Gastroenterology (Chief, XXX, MD) and IBD Center (Director, XXX, MD), Department of Medicine

The Division of Gastroenterology has been on the forefront of research, identification, and treatment of gastrointestinal illness since the division's early days. In the first part of the 20th Century, gastric secretion and duodenal contents were studied with the use of Rehfus tubes. An outpatient clinic was founded and devoted solely to GI diseases in 1913. Since the publication of the landmark study on regional enteritis, Crohn's disease, by Mount Sinai physician-researchers XXX, MD, XXX, MD, and XXX, MD, the Hospital has been the leader in the care of inflammatory bowel disease (IBD). Today, Mount Sinai physicians care for the largest population of patients with IBD in the country. In the past year, The

Mount Sinai Medical Center has named XXX, MD, as the Director of The Leona M. and Harry B. Helmsley Charitable Trust Inflammatory Bowel Disease Center, which is scheduled to open in 2014. Dr. XXX is best known for his participation in the identification of NOD2 as a susceptibility gene for Crohn's disease, and the identification of a new subtype of *Escherichia coli* associated with Crohn's disease, as well as the development of the Anti-Saccharomyces Cerevisiae Antibody (ASCA) test, which remains the most sensitive and specific marker for Crohn's disease. He has authored or coauthored more than 500 peer-reviewed articles, books, and book chapters on IBD. Most recently, he was Professor of Hepatogastroenterology at Centre Hospitalier Universitaire de Lille in Lille, France, and President of the European Crohn's and Colitis Organization.

The Center will offer patients a collaborative team of experts in gastroenterology, clinical immunology, nutrition, pathology, psychology, radiology, colorectal and laparoscopic surgery, and genetics and genomics. Its Young Adult IBD Transitional Program will assure patients a seamless transition from pediatric to adult gastroenterologists. The Center's scientists will conduct genomic, proteomic, metabolomic, and microbiomic research to create disease models that would identify biomarkers for disease type and pathways, with the ultimate goal of developing approaches to disrupt the pathogenesis of the disease and to treat at-risk patients before IBD develops. Mount Sinai's IBD Registry and Biobank, which analyzes complex data at every stage of a patient's life, will collaborate to drive this research and expand Mount Sinai's clinical trial capabilities.

A. 2 Research

Infrastructure

The Icahn School of Medicine at Mount Sinai has made a strong commitment to expanding its biomedical research infrastructure. This well funded base has enabled Mount Sinai to become the fastest growing medical school in New York State and to attain its current status in NIH grant funding. Over the past five years, Mount Sinai has recruited new research faculty members in areas such as neural aging; gene therapy; human genetics; cancer biology; cardiovascular biology; bioinformatics; infectious diseases; immunobiology; stem cell biology; epidemiology; pediatrics; and developmental biology. The School has also established a number of innovative and interdisciplinary research institutes. These representative and innovative programs and departments provide a translational and basic science infrastructure that reflects the commitment of the institution, and fosters an interdisciplinary approach with strong collaboration between basic scientists and clinical colleagues.

A.2.1 Translational Research Institutes

Since 2007, the Icahn School of Medicine at Mount Sinai has developed 16 translational research institutes. These institutes are interdisciplinary and comprise the work of multiple departments in a given area of research. Seven of the 16 are disease-oriented: the Brain Institute; the Tisch Cancer Institute; the Child Health and Development Institute; the Cardiovascular Research Institute; the Immunology Institute; the Metabolism Institute; and the Emerging Pathogens Institute. Six of the 16 complement and advance the work of the disease-oriented institutes and research efforts within multiple clinical and basic science departments: the Experimental Therapeutics Institute; the Institute of Translational Epidemiology; the Charles R. Bronfman Institute for Personalized Medicine; the Black Family Stem Cell Institute; Conduits (the Institutes for Translational Sciences); and the Translational and Molecular Imaging Institute. The institutes that most directly relate to this career development plan are Conduits, the Institute of Translational Epidemiology, and the Immunology Institute.

A.2.1.2 Conduits – the Institutes for Translational Sciences (XXX, MD)

Conduits, as its name implies, provides a pipeline to rapidly move scientific discoveries from the bench to the bedside and out to the community. Funded in 2009 by a \$34.6 million 5-year Clinical and Translational Science Award (CTSA) from the National Center for Research Resources, Conduits enables Mount Sinai to provide the infrastructure and services to improve the research experience for investigators and their collaborators. It also prepares future research scientists through innovative educational and training programs that stress translational medicine, establishes an integrated academic home for translational science, and taps the rich resources of Mount Sinai's diverse patient population. Critical to the success of Conduits is an institutional commitment to connect community members and research scientists so that the values and culture of the community are respected throughout the research process.

The mission of Conduits is to expand the institutional research enterprise, streamline and centralize institutional infrastructure for clinical and translational research, foster interdisciplinary research, and support innovative educational and training programs that stress translational medicine. Conduits provide consultation, oversight, and facilities for clinical and translational research; engages the community and its affiliates to translate health benefits to the public; and develops new methodologies to improve trial design and reduce participant burden. In summary, Conduits establishes an integrated academic home for translational science.

Multiple key programs are housed within Conduits for easy access by Mount Sinai faculty and trainees. These separate but integrated divisions include Biomedical Informatics, Community Engagement and Research programs, resources for translational discoveries (including the Clinical Research Centers (CRC), the Office of Clinical Research (OCR), and the Biostatistics, Ethics, and Research Design (BERD) Program), resources for experimental therapeutics and technology development, and extensive resources for research education, training, and career development.

A.2.1.3 Immunology Institute (XXX, MD, PhD)

The Immunology Institute has maximized the translational aspect of its research by focusing on major immune system functions and diseases. We have designed this strategy to promote interdisciplinary collaboration and the efficient transfer of results into new treatments. The role of immunity in disease processes can be grouped into four general areas: Autoimmunity, Host-pathogen interactions, Inflammation and immunoregulation, and Immunocompetence. Immunology Institute researchers focused on immunoregulation and immunocompetence, based on Mount Sinai's expertise in the fields of inflammation, immune deficiency, and immunoregulation. Other determining factors include the existence of a robust patient population, the lack of strong competition within New York City for immunocompromised patients, and substantial potential for extramural funding in these areas, both from the National Institutes of Health and from disease-oriented foundations.

The Immunology Institute is also home to transplantation research. Developing better immunosuppressant medication for use in organ transplantation and increasing the viability of pancreatic islet cells for transplantation are particularly important areas within the transplant program. Advances in the understanding of immunoregulation and immunocompetence will have a strong impact on treatment of immune mediated disorders.

Dr. XXX will have access to the faculty and resources of the Institute. As such he will also participate in course work, journal clubs and seminars given by the Institute.

A.2.1.4 Mindich Child Health and Development Institute (Director, Bruce Gelb, M.D.)

The Mount Sinai Child Health and Development Institute builds on the research programs of the Department of Pediatrics, but also includes scientists from the Departments of Developmental Biology, Preventive Medicine, Human Genetics, and Psychiatry. The mission of the Institute is to conduct groundbreaking research that ensures all children will have the opportunity to lead healthy and productive lives free from disease and disability.

Research in the Institute investigates the cellular, molecular, and genetic mechanisms underlying common, yet complex diseases of children including neuro-developmental and neuropsychiatric disorders, diabetes, obesity, asthma, and food and environmental allergies. The Institute searches for environmental causes and for the associations between genes and the environment that make some children especially susceptible. The Institute studies contextual and psychosocial factors that modulate children's vulnerability as well as epigenetic influences embedded in the protein constituents of chromatin that promise to serve as an important adjunct to DNA sequence in the analysis of biologic response to environmental exposures. The goal of research in the Institute is to discover the preventable causes of these diseases, the molecular mechanisms that can be treated, and the ways in which access to care and health promotion for children can be improved.

As a faculty member in this institute, Dr. XXX will have access to all shared resources.

A.2.2 Shared Research Facilities

In response to the relentless and fast-paced evolution of the biomedical sciences and the growing research needs of faculty, the Icahn School of Medicine at Mount Sinai has made a major investment in the creation of state-of-the-art Shared Research Facilities (SRF). The SRF bring state-of-the-art instrumentation and methodologies crucial to modern biomedical research within the reach of all Mount Sinai investigators, fellows and graduate students. The experts who staff the SRF not only provide research services, but are also sources of instruction and training and thus constitute a major intellectual resource for the entire institution. The SRF are administered through the Office of the Dean and subsidized by institutional funds of approximately 50 percent.

Five initial SRF were created in 1998 with a total capital investment by Mount Sinai of approximately \$1.5 million and an annual operating budget of over \$500,000. These SRF complement the activities of 10 Department-based Cores that provide additional specialized services to the community. Support for these Departmental Cores comes from earmarked allocations in the departmental budgets, extramural grant support and below-cost fees for service. Ongoing support for the SRF, Cores and specialized Centers increased substantially over the last few years. Support for the SRF by the Icahn School of Medicine at Mount Sinai totals approximately \$3 million per year, of which user fee charges to investigators from their grants and other resources recoups approximately \$1.3 million. These facilities are available to all researchers within the institution. Shared Research Facilities are open to all faculty, with equal access regardless of departmental or center affiliation; priority is given to ISMMS faculty over external users

There are 12 freestanding SRF at Mount Sinai. The SRF and Departmental cores currently available to investigators at Mount Sinai and relevant to this career development plan include:

Shared Research Facility	Director
Biorepository Cooperative Histology	XXX, MD
Flow Cytometry	XXX
Microarray, PCR, Bioinformatics	XXX XXX
Microscopy	XXX
Mouse Genetics	XXX
Scientific Computing	xxx

A.2.3 General Clinical Research Center (XXX, MD)

The Mount Sinai General Clinical Research Center (GCRC) is a combined adult and pediatric NIH-funded GCRC. The GCRC serves as the Institutional hub for clinical research at Mount Sinai and a major resource for all Mount Sinai investigators. The GCRC is located at 1184 Fifth Avenue, where it occupies an entire 7500 square foot floor. The GCRC sits in the Pediatric Hospital. Extensive renovations were undertaken and completed in December 2006. The renovated space gives increased privacy and improved functionality. There are eight private inpatient bedrooms and two large outpatient rooms, each divided into four cubicles with privacy walls. A Core Processing Laboratory was relocated to another site nearby. The new space has a waiting room with internet access for participants, a nursing station, medication room, kitchen, secure storage for research records, conference room, and administrative offices. The GCRC is staffed by advance practice nurses, research nurses, and research bio-nutritional staff who can assist investigators conduct their clinical research projects. In addition to patient care, the GCRC can provide full dietary and nutrition support and can provide funding for pilot projects for young investigators. Other GCRC services include a fully equipped molecular laboratory for DNA isolation and extraction, an imaging core, and a statistical/database management core.

A. 3 Mount Sinai Graduate School of Biological Sciences

The Mount Sinai Graduate School of Biological Sciences is located on the Mount Sinai campus with the School of Medicine and the Mount Sinai Hospital. The mission of the Graduate School is to provide a rigorous educational experience in biomedical sciences to excellent, research-oriented students.

The Graduate School of Biological Sciences sponsors PhD training in five traditional basic science departments and also is home of Mount Sinai's Medical Scientist Training Program (MD/PhD Program). There are currently 217 students matriculated in the Graduate School: 60 students in the MD/PhD and 152 in the PhD Program. Mount Sinai's research faculty is in a period of active growth in numerous interactive and important areas of research. Currently, there are more than 200 members of the Graduate Faculty, most of whom are anxious to have the opportunity to work with students and fellows who will choose to pursue projects in their laboratories. Thus, students and fellows enjoy interactions with new faculty and the new programs and viewpoints they bring to the Graduate School throughout their stay at Mount Sinai. The Graduate School also hosts two masters degree programs – the Master of Public Health (MPH) program and the Master of Science (MS) in Clinical Research program.

A.3.1 Clinical Research Education

The Graduate School of Biologic Sciences provides formal training in patient-oriented clinical and translational research (didactic and experiential) to fellows, junior faculty, and other allied health professionals who hold a professional doctoral level degree in medicine, dentistry, nursing, pharmacy or other relevant professional fields. Two degree-granting programs and one certificate program are offered. In addition, course may be taken outside of these programs.

The Masters of Science in Clinical Research (MSCR) is a 2-year, part-time program that provides an exceptional educational experience in the knowledge, skills, and experience to successfully launch clinical and/or translational research-intensive careers. The MSCR has two main components: (1) graduate coursework, and (2) a mentored clinical research experience. Graduate courses include biostatistics, epidemiology, research design, data analysis, and grant writing. The mentored clinical research project leading to a Masters thesis is designed to be completed in 2 years. The PhD program is designed for those who desire a more intense educational experience to help to prepare them for a career in clinical or translational research. The program provides a strong didactic foundation combined with a mentored clinical research experience leading to a doctoral degree in Clinical Research. The Clinical Research Training Program (CRTP) is the more modest, 1 year, part-time certificate version of the MSCR program, which includes the core of class work without the Masters thesis requirement and 2nd year research seminars.

A. 4 Computer Support

The Mount Sinai Levy Library has a Computer Help Desk that provides extensive technical support for email, desktops, and network file storage requests and problems. In addition, the library distributes a number of site-licensed software products for both business productivity and statistical analysis. Some of the statistical packages that they offer are SAS, SPSS and MatLab. Mount Sinai provides network connectivity to the Internet with both wired and wireless networks.

A. 5 Office

Mount Sinai's faculty members have private offices in the Mount Sinai Medical Center with adjacent office support staff and office equipment. The close proximity of the staff facilitates interactions and cross-fertilization of research ideas among the faculty members. In addition, conference facilities are available in all departments, as well as elsewhere in the Medical Center. The Division of Gastroenterology, Department of Pediatrics provides administrative and secretarial support for faculty and has scanners, fax and photocopy machines.

A. 6 Other

Faculty have access to the Mount Sinai Medical Center's other resources, including libraries, researchers, and medical practitioners. The Gustave L. and Janet W. Levy Library provides access to a range of biomedical information resources in both digital and paper formats, including on-site and off-campus access to over 17,000 electronic journals and 70 licensed databases. The library is open approximately 100 hours per week and makes available to the Mount Sinai community one hundred computers with a variety of installed software

as well as Internet access. It also serves as a resource on communications, web, and computing issues for the school.

Major Equipment

Laboratory

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6,000 sq. ft. of open bench space with 36 total benches. There are nine cell culture facilities (200 sq ft each) with CO₂ incubators, centrifuges and laminar flow hoods, and alcoves that are utilized as common space for chemicals, refrigerator/freezers, pH meters, table top and floor centrifuges, FPLC (Pharmacia), and gamma and beta counter (Packard). There are six chemical/fume hoods used for radioisotopes (one hood), PCR or chemicals. There are two common equipment rooms (4,000 sq. ft.) which house shared equipment and freezers.

Animal

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There is a barrier facility, which is housed in the Icahn Research Building where the Immunobiology Center exists. This is for rodents only. This is in addition to the facility in the Annenberg Building that houses small and large animals. There is a transgenic core and a knockout core run by Dr.XXX.

Computer

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A 13" Mac Book Pro computer is provided for use in the laboratory and a 15" Mac Book Pro is provided in the office space. The Immunobiology Center has a graphics station with scanner, slide maker and Tektronic color printer, which is available to all faculty and students.

Office

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Offices are provided for all faculty in separate office suites adjacent to the laboratories. Offices are supported by a central reception area, which houses the administrator and administrative secretaries, Xerox and Faxing facilities, and online access to recent journals (e.g. Nature, Immunity, Cell, Science, J Exp Med., J Immunology, J Biologic Chemistry, Immunology Today).

Shared Core Facilities

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Mount Sinai has several institutional core facilities including the DNA microarray core, a peptide synthesis core, a DNA sequencing core, a protein sequencing core, a confocal and imaging core, a transgenic core, and a molecular biology "store" for generation of DNA probes core. Each floor has 2 darkrooms with X-O-Mats, 3 walk-in cold rooms, a walk-in freezer (-20°C), a dishwasher and autoclave facility, and conference rooms (2)

Major Equipment

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The flow cytometry core is in the Icahn Building. It has a MoFlo cell sorter, an LSRII, a FACScaliber, FACSvantage and a BD FACScan. There are 3 full time operators. There are 2 ultracentrifuges (Beckman), 2 superspeeds (Sorvall and Beckman), a Wallac Microbeta scintillation counter and a Tomtec 96 well harvester, a Packard gamma and beta counter. There are 4 MJ Research PCR machines, and X-O-Mat, Beckman bacterial shaker, a Vacuum oven, 4 liquid nitrogen (Cryomed) repositories, a Queue -135 freezer, a cryomed programmed cell freezer, Shandon cytospin (2), 2 Speed-Vacs, 2 FPLCs (Pharmacia), fraction collector and gel apparatus, a minigel apparatus (Pharmacia), multiple -70 freezers, and multiple power supplies for SDS-PAGE and DNA sequencing. We also have 2 transfer apparatus for Western blots.

INTRODUCTION:

	Candidate	Career Development	Research	Mentors/ Collaborators	Environment/ Institutional Commitment
Reviewer 1	2	3	4	2	1
Reviewer 2	2	2	4	2	2
Reviewer 3	2	2	3	2	2

The candidate, mentors, and environment were scored as outstanding to exceptional with minor weaknesses noted in the career development and research plan, which we addressed below. **Reviewers' comments are in bold** with replies following them. Changes in the proposal have a typeface change including Aim 2 and Aim 3, which were extensively changed and reorganized given reviewers' comments with guidance from my mentors and advisors.

(1) Candidate's productivity. While productivity declined while my mentor, XXX, was ill and ultimately passed away, this has increased in the past year including one co-first author paper and multiple co-authored papers. In addition, a first author paper with my preliminary data is soon to be submitted for publication.

(2) Career development plan diffuse with mix of clinically oriented and basic science elements. The career development plan has been revised to put more focus on the basic immunology of tolerance formation and Treg mechanisms during epicutaneous tolerance induction during the first 3 years and to transition to translational research training during years 4 and 5. The basic aspects of immunology will focus more closely on mucosal inflammation and tolerance induction. The addition of translational/clinical research training during the last two years of the grant will enable me to participate in clinical trials that result from this project. As such the training focus will transition in parallel with that of my research endeavors. (Career Development Plan)

(3) Concerns regarding the research plan included:

T cell trafficking: The goal of the first subaim is to optimize migration of immunosuppressant Tregs to the gut rather than discover more about the mechanisms involved since much is already known in this field. This has been more clearly stated (Aim 1 introduction).

Immune status in the OTII transfer: The transfer experiment involves transferring OTII cells from OTII mice on a RAG background to immune-competent mice. This has been clarified in the methods (Aim 1.1). In this immuno-competent model there are data showing effective induction of epicutaneous tolerance.

Use of Salmonella-OVA infectious model: There is precedent for these experiments in the literature. However given the complex immunology involved in this model, the more straight-forward transfer model has been chosen instead. Use of an antigen specific model is done initially since this may have broader applicability to other diseases of the GI tract. Significantly more detail and rationale has been added as requested by the reviewer (Aim 2.1).

Use of IL-10^{-/-} model of colitis: In Aim 2.2, the IL-10 model has been replaced with the input of Dr. XXX and Dr. XXX. IL-10 may in fact play an important role in bystander suppression in the gut given data from multiple studies implicating Tr1 cells in suppressing colitis. Additionally, IL-10 and TGF- β will be better examined throughout my experiments. Consequently, additional mechanistic experiments have been added. (Aim 3)

Longevity of Tregs, if they can be rapidly re-induced upon oral antigen administration, and stability: In response to these excellent points, the proposal examines delayed oral antigen exposure (Aim 1.3) and addresses the stability of Tregs in an inflammatory milieu (Aim 1.3).

Insufficient justification for the chosen experiments and the need for more robust investigation of the underlying mechanisms: Additional details explaining the previous studies done to induce tolerance in colitis models have been added along with relevant citations. (Innovation, Background and Preliminary Data) Studies here are unique as they induce tolerance via a different route than oral and in addition investigate the immunological mechanisms, which we may alter to optimize the ability of this therapy to be effective. In humans, early oral tolerance has failed to treat autoimmune diseases. Additionally, Crohn's patients have been shown to have defects in oral tolerance. Thus, this proposal avoids the oral route and utilizes a new approach to induce Tregs epicutaneously based on promising preliminary data. Additional in depth studies to examine the mechanism have been introduced throughout the grant including examining the need for various subsets of Treg cells (Aims 3).

(4) Advisor with expertise in experimental IBD models would be beneficial. We note that Dr. XXX (co-mentor) has recently published work, which focuses on the intestinal immune system in murine models in *Science*. The expertise of Dr. XXX has also been clarified emphasizing his IBD experience (Career Development Plan and Biosketch). Finally, we have added Dr. XXX in an advisory capacity. He has extensive experience in

murine models of colitis and has given considerable input into the revision of this grant.

Candidate Background

My passion for a career as a physician scientist started as an undergraduate at Cornell University where I performed research in an entomology laboratory researching genetic factors underlying pesticide resistance. Once I decided to pursue a career in medicine, I transitioned my interest in research towards that end. I conducted research at New York Hospital on the immunologic responses to alternative therapies for pediatric patients with AIDS and the effectiveness of intra-aortic balloon pumps. After completing my undergraduate degree, I attained a position as a research assistant at Stony Brook Health Science Center in an immunology laboratory. My project focused on examining outer surface proteins for use as diagnostic tests for *Borrelia burgdorferi* infection. With each research experience I found it stimulating to discuss scientific theories and develop methods to prove or disprove them. During medical school I continued to cultivate my interest in research. I was awarded the prestigious Washington Health Policy Fellowship from the American Medical Student Association. This allowed me to work in the Department of Health and Human Services on Healthy People 2010 and resulted in a first-authored paper in *JAMA Online*. This public health research included the assessment of leading health indicators and evaluation of preventive health education. This was a valuable opportunity as I obtained insight into the world of epidemiology and clinical research.

I then completed a rigorous clinical residency in Pediatrics at Yale Children's Hospital. While concentrating on my clinical training, I maintained my enthusiasm for research and decided to pursue subspecialty training that would allow me to gain in-depth research experience. As a pediatric gastroenterology fellow at The Icahn School of Medicine at Mount Sinai I had the opportunity to perform research under the guidance of Dr. XXX in the Immunology Institute. Upon completion of my fellowship, I was selected to continue under my mentor's guidance as a Child Health Research Career Development Award Scholar (CHRCDA K12) and faculty member. Through this work we showed that many physiologic routes of antigen exposure are able to induce sensitization but the epicutaneous route was optimal. I presented these data at multiple national conferences and published a first-authored paper in the *Journal of Allergy and Clinical Immunology*. Towards the end of this funding I was awarded a grant from the Crohn's and Colitis Foundation of America to examine the efficacy and mechanism of a Chinese herbal therapy for the treatment of colitis. I presented this work at numerous national conferences and published a first-authored manuscript in *Inflammatory Bowel Diseases*. My research goals evolved through ongoing discussions with my then mentor, Dr. XXX, to work towards bridging these seemingly disparate research experiences with a focus on informing and developing a novel therapy for the treatment of Crohn's disease. However, over the two years, Dr. XXX was battling a progressive illness and unfortunately passed away. In this context and with his continued support, I assembled a group of mentors and advisors who could assure my success. The pre-clinical research proposed in this grant is a necessary next step in my career path prior to performing translational studies that include clinical trials in IBD subjects.

I am committed to pursuing a career as a physician-scientist. I value academic medicine for its intellectually stimulating environment and the opportunities it provides to practice medicine and science on the cutting-edge. My long-term goal is to become an independent investigator, as a full-time academic faculty member, performing translational research and clinical trials on tolerance induction and IBD. Along with my devotion to research, I continue to be committed to direct patient care, which gives me access to select patient populations and allows me to keep my science geared towards helping children and their families. Finally, work in an academic center will allow me to mentor other health professionals in both clinical and laboratory settings.

This Career Development Award will support both the needed directed-guidance on extending the basic pre-clinical studies aimed toward informing interventional studies for IBD as well as training on the conduct of clinical trials, which is a significant gap in my training thus far and will be need for future research endeavors. Specifically, this award would provide me with continued protected time for research to gain proficiency in selected techniques and enhance my understanding of immunology as well as protected time to gain experience and expertise in the conduct of clinical trials specific to IBD populations through symposia, graduate level courses, and mentorship. To accomplish my goal of becoming an independent investigator, I will devote 80% of my time to the laboratory and 20% to clinical duties. My Division Chief and Department Chair are extremely supportive of this plan. My clinical duties are restricted to attending on the inpatient pediatric gastroenterology service for 6 weeks per year and 1 office-based practice session each week. I now have the opportunity to realize my goal of becoming a physician investigator. Together with my experience and commitment to research, the scientific infrastructure, the graduate level courses available, institutional support,

protected time and outstanding mentoring provide the perfect combination to optimize my chances for success as an independent investigator. I am committed to making full use of this priceless opportunity.

Career Goals and Objectives

This award will be critical to my development as a translational investigator. My current goals seek to merge the knowledge I have gained from both of my research endeavors into an innovative project, which utilizes epicutaneous tolerance induction for the treatment of colitis. While my projects have exposed me to a wide variety of models and techniques, I must acquire further experiences and new skills in order to gain independence. I will expand beyond my experiences to more in depth studies on the mechanisms of tolerance induction through the skin, the function of Tregs and their ability to suppress immune responses in the gut. Importantly, I will also focus on acquiring the knowledge to transition my research towards human application and clinical trials. My career development plan builds on my strong foundation in immunology and clinical gastroenterology and is an extension of my previous experiences. I have developed this plan in consultation with my mentors and advisors. It is designed to address my long-term goals. The Icahn School of Medicine at Mount Sinai has extensive core facilities to support my work, and a rich educational environment with a wide range of lectures, workshops and graduate level courses.

By providing substantial salary support, this Career Development Award will allow me to continue focusing on laboratory investigations and acquiring new skills and knowledge to advance this novel scientific project towards clinical trials. Eighty percent of my time will continue to be protected from clinical responsibilities during the award period. The guidance from my renowned and successful mentors, Dr. XXX and Dr. XXX, as well as an outstanding group of advisors, and the protected time will assure that I emerge as an independent investigator who can compete successfully for extramural funding. Moreover, I believe my scientific niche provides a realistic and unique career focus with direct clinical relevance and the strong potential for continued scientific growth. I plan to make meaningful contributions towards our understanding of epicutaneous tolerance induction as a treatment for colitis in preclinical studies in murine models with the intent of directly applying this knowledge towards clinical trials in humans with Crohn's disease.

To prepare for more long-term studies in humans I have established a collaborative agreement with DBV Technologies along with Dr. XXX to utilize Viaskin[®], a patch that can deliver antigen epicutaneously. This technology is fully integrated into the current grant in anticipation of using it in future human studies. In the final two years of this grant, I will seek funding from the Immune Tolerance Network to begin preliminary studies in patients with Crohn's Disease to determine if they can be tolerized through the skin. Currently, it is unknown if they have systemic defects in tolerance in addition to the defects in oral tolerance discovered by my former mentor, Dr. XXX. This will be followed by the development of an R01 and clinical trials in Crohn's disease. In addition, discoveries made during this granting period have the potential to be applied towards other inflammatory gastrointestinal disorders such as celiac disease.

My progress towards these goals will be addressed informally by my mentors and advisors. Formal Faculty Advisory Committee meetings led by Drs. XXX and attended by my mentors and myself will continue to review my advancement and progress towards independence twice a year. Faculty Advisory Committee meetings review my progress over the prior six months and include: a rating of progress, identification of problems affecting research progress and solutions to them, assuring protected research time, a detailed description of goals for the coming six months, reviewing presentations and publications since the last meeting, and reviewing course work and conference plans. Discussions of these topics occur as a group and a formal write up of recommendations is done after each meeting.

I ultimately expect to become an independent investigator, as a full-time academic faculty member, continuing research related to tolerance formation and IBD. I plan to focus on translation studies and performing clinical trials and see this career development award as a critical step toward that end. Along with my devotion to research, I continue to be dedicated to direct patient care, which gives me access to select patient populations and allows me to keep my science geared towards helping children and their families.

Career Development/Training Activities During Award Period

A Career Development Award would supply me with the continued protected time needed to complete training in advanced immunology, learn new lab techniques and gain expertise in methods needed to transition my work to human studies. My previous research experience in immunology research under the mentorship of Dr. XXX began to provide many of these, however over the past two years, my development slowed while my mentor struggled to fight a severe illness that ultimately claimed his life. This award will allow me to undertake concentrated research training in advanced immunologic approaches and learn skills needed to perform clinical trials. The major focus of my research training is to define the mechanisms of tolerance development during epicutaneous antigen exposure and utilize this as a treatment for inflammatory bowel disease. My principle mentors will be Dr. XXX who is an expert in immunology, cytokine biology, and tolerance and Dr. XXX who is an expert in monocyte physiology and intestinal immunology.

The laboratory where I have designated space is fully equipped to perform studies of immunobiology. My preliminary work has been and will continue to be supported through funds from The Department of Pediatrics as well as grants provided to my mentors. Within the laboratory, I interact with post-doctoral fellows, junior faculty members, PhD students and lab technicians. Both of my mentors have an extensive track record of mentoring junior faculty and are part of the Immunology Institute with 54 faculty and close to 100 post-docs and students. This is a dynamic environment with constant interactions between members. Formal meetings occur on a weekly basis where on-going research is discussed. As part of the Immunology Institute, formal training is provided in immunology including lectures, journal clubs and a course series. Appropriate supplies, equipment and computer access are all readily available. Bench and desk space are designated to me adjacent to Dr. XXX's laboratory. A technician is currently being hired. My career development plan integrates formal coursework, laboratory exposure, institutional and national conferences, and mentored research, with the goal of applying for independent funding during the final 2 years of the award.

Formal coursework (Immunology: Years 1-3. Translational/Clinical: Years 4-5): I plan to expand my scientific knowledge base in immunology and gain new knowledge in translational research and clinical trials. I will take the following courses at the Mount Sinai Graduate School of Biological Sciences: **1. *Advanced Topics In Immunology (BSR6502)***; **2. *Immunology Seminar Series***; **3. *Systems Biomedicine (BSR1800)***: This course integrates knowledge of core biochemical, cell biological and molecular mechanisms together with basic bioinformatics and systems biology concepts and applications in the context of human biomedical research; **4. *Clinical Investigation for the Translational Scientist (G791)***: This course will improve my understanding of translating basic data into the clinical arena; **5. *Clinical Research Training Courses***: These courses will be taken towards the end of the grant period and will give me the knowledge necessary to apply my work to patients and perform clinical trials: Biostatistics (MPH0300, 0800 and CLR0320), Methods in Clinical Research (CLR0006, 0016, 0007), Applied Linear Models, Advanced Topics in Clinical Trials Research (CLR1020) and Clinical Trials Management (CLR8010).

Basic Laboratory Work (Years 1-5): I have become proficient in the use of mouse models of anaphylaxis and tolerance induction, isolation and characterization of dendritic cell sets/subsets and T cell subsets, and the use of mouse models of colitis/ileitis. During the training period, I will expand my expertise to study the molecular mechanisms underlying Treg cell function, and will acquire more advanced laboratory techniques to elucidate the molecular basis of their interactions, cytokine production and cell signaling. I will also expand my expertise with mouse models including functional models to study Tregs and new models of colitis and ileitis. This will be accomplished by working directly with members of my mentors' groups, as well as members of my Research Advisory Committee, who have expertise in mucosal immunology, regulatory T cells, and IBD both in murine models and in humans. Through mastery of these new skills, I will have a more comprehensive knowledge of the strengths and limitations of specific approaches.

Institutional Conferences (Years 1-5): Throughout my training period, I will attend the following conferences to continually expand my knowledge and help me attain independence: **1. Immunology Institute:** Work in Progress Conference (weekly) **2. Young Faculty Development Conference and Lecture Series:** Institution wide conference to help young faculty further their careers including workshops on grant writing, manuscript writing, and promotion (monthly). In addition, I will maintain my clinical competence by attendance at relevant Departmental and Divisional Conferences.

National and International Scientific Meetings (Years 1-5): I plan to attend the following meetings as a forum for presenting my work, and as an opportunity to stay abreast of recent developments. In addition, these meetings offer the opportunity to identify potential collaborators for future projects:

1. Annual CCFA Advances in Inflammatory Bowel Diseases Conference 2. Annual Meeting of The *North American Society of Pediatric Gastroenterology, Hepatology and Nutrition*; 3. Keystone Symposia that focus inflammatory diseases: Recent Advances in Basic and Translational Research and Therapeutic Treatments (J4); 4. Society for Mucosal Immunology Conference.

Mentored Research (Years 1-5): The cornerstone of my career development plan is my mentorship structure, which is based on the expertise of outstanding immunobiologists with skills that complement each other and who collaborate often. I have also established a research advisory committee to help monitor and foster my scientific and professional progress from different scientific perspectives. Office space is provided to me by the Department of Pediatrics and laboratory space is provided in the Department of Immunology. These are located in close proximity thus allowing the sharing of common facilities, encouraging collaboration and giving me easy access to my mentors and advisors.

1. Primary Mentors

XXXX

2. Research Advisory Committee

XXXX

Career Advancement and Transition to Independence (Years 1-5):

The Department of Pediatrics is committed to advancing the careers of its physician-scientists and has instituted formal physician-scientist discussion groups and formal career advisory meetings. I will attend the bimonthly meeting of the group, led by Drs. XXX and XXX. Discussion focuses on issues relevant to career advancement: developing a mentor network, establishing a research niche, funding grants, time management, protecting writing time, and understanding the appointments and promotions process. A program within this group assists in the transition to independent research and includes: a grant writing course, internal R submission and mock study section reviews.

Pediatric Faculty Advisory Committee (FAC): As mandated for junior physician-scientists at Mount Sinai, Drs. XXX and XXX will meet with me and my mentors on a formal basis biannually to assure my professional progress, career development and transition to independent research.

Meetings include: a rating of progress, identification of problems affecting research progress and solutions to them, assuring protected research time, a detailed description of goals for the coming six months, reviewing presentations and publications since the last meeting, and reviewing course work and conference plans. Discussions of these topics occur as a group and a formal write up of recommendations is done after each meeting

Career Development Goals	Career Development Activities	Year 1	Year 2	Year 3	Year 4	Year 5
1. Improved practical skills in the conduct of basic research	1. Mentored research 2. Research conferences 3. Work in Progress meetings 4. RCR Training	+	+	+	+	+
2. Expand knowledge and experience in molecular immunology, IBD, Treg and DC interaction	1. Formal coursework in immunology 2. Scientific meetings A. CCFA and NASPGHAN B. Keystone and SMI Meetings	+	+	+	+	+
3. Further experience in relevant lab techniques	1. Exposure to molecular biology techniques 2. Immune response assessment	+	+	+	+	
4. Develop expertise in the presentation of findings	1. Abstract for CCFA, SMI and/or NASPGHAN 2. Original manuscript submission	+	+	+	+	+
5. Translation of mouse studies into human IBD studies	1. Formal coursework in clinical research 2. Exposure to human research work 3. Development of translational pilot project				+	+
6. Apply for independent funding	1. Development and submission of ITN grant 2. Development of R01 plan 3. Submission of R01				+	+

Training in the Responsible Conduct of Research

The plan for RCR instruction is based on the philosophy that trainees and faculty at The Icahn School of Medicine at Mount Sinai (ISMMS) receive formal training in bioethics, scientific integrity and the responsible conduct of all aspects of research: animal, laboratory-based and patient-oriented. Individual research mentors are expected to serve as role models and provide one-on-one practical instruction as well as fulfill the RCR training described below.

The Office of Postdoctoral Affairs at ISMMS offers a mandatory two-hour long annual **RCR Workshop** for all postdoctoral research fellows and faculty members. My mentors and I have already participated in this course and will attend the required course refresher annually.

In the fall of 2013 I completed the **Responsible Conduct in Research Course** offered by the Graduate School of Biological Sciences at Mount Sinai Medical School (BSR 1003). This 16-hour course, directed by Charles Mobbs, PhD, Professor of Neurosciences and Geriatrics, encompasses a series of presentations in different formats that include presentations by guest speakers, panels of faculty members and videos. Each session also has an hour devoted to small group discussion of specific pre-assigned case studies with a faculty mentor. These discussions are followed by a full class wrap-up in which the groups compare notes on their conclusions, insights and remaining questions. Each student is required to submit a report discussing the assigned case study each week, and at the end of the course is required to submit a real-life case study of an ethical research dilemma encountered, and an analysis of the resolution of the dilemma. The main resource is the website created by the Office of Research Integrity (ORI) which presents official NIH positions on most of the issues described in the course. This material is supplemented by additional assigned reading, with use of selected videos and other special materials. The primary textbook used is "Scientific Integrity, An Introductory Text with Cases" by Francis L. Macrina. In Fall 2012, the specific topics for the eight two hour sessions were: (i) research misconduct; (ii) experimental design and data management practices; (iii) mentor and trainee responsibilities; collaborative research; (iv) conflicts of interest; intellectual property; (v) the protection of human subjects; (vi) the welfare of laboratory animals; (vii) publication, responsible authorship, and peer review; (viii) peer review, the grant process, and fiduciary responsibility. Guest lecturers included the directors of the IRB, IUCAC and the Office of Industrial Liaison. Students are required to take advantage of the available instruction on radiation safety, animal use or human subjects issues if these are part of their research work. In particular, Reginald W. Miller, D.V.M., DACLAM, who has had a major role in administration of Mount Sinai's Animal Care facilities and been a long-standing participant in RCR education (and was one of the pre-doctoral RCR small group leaders), was appointed to the position of Research Integrity Officer in the Office of the Dean three years ago. He is overseeing a coordinated increase in RCR education at all levels of the institution that will add even greater vitality to a program that has been a long-standing institutional commitment. At the end of the course students are required to take and pass (>85% correct answers) an online test produced by the Collaborative Institutional Training Initiative (CITI), founded in March 2000 through a collaboration between the University of Miami and the Fred Hutchinson Cancer Research Center, to ensure that the students meet broad standards for RCR. This RCR course must be retaken every four years.

This formal RCR course work is supplemented by a series of practical seminars that discuss real world situations. Specifically, the monthly *Center-Wide Ethics Luncheons*, convened monthly by Rosamond Rhodes, PhD, Professor of Medical Education and Director of Bioethics Education, brings together physicians, scientists, medical students, nurses, social workers, and patient representatives to discuss the ethical issues surrounding a case at the medical center.

Finally, as part of the bimonthly **Pediatric Physician-Scientist Career Development Discussion Group**, Drs. XXX will review and discuss specific ISMMS policies and procedures on ethical practices in research, all available on the website (<http://www.mssm.edu/about-us/services-and-resources/faculty-resources/handbooks-and-policies/faculty-handbook/research-environment/research-integrity>): responsibilities of authors and data retention, press relations, manuscript policy, policy and procedures on ethical practices in research (including procedures for handling allegations of misconduct in research and policy and procedures on protecting whistleblowers), use of Mount Sinai's name, conflict of interest in research, policies on intellectual property (ownership and commercial development) and policy on harassment.

Specific Aims:

Crohn's disease (CD) is increasing in incidence in both adults and children.^(1, 2) CD is a multi-factorial, inflammatory disease of the gastrointestinal tract that is driven by disturbances in the innate and adaptive immune responses.⁽¹⁰⁾ Its pathophysiology is believed to be the result of aberrant immune responses or the failure to form tolerance to bacterial antigens in a genetically predisposed individual.⁽³⁾ CD is associated with increased Th1/Th17 effector T cell responses.⁽¹¹⁾ This results in loss of the steady-state balance between pro-inflammatory effector cells and suppressive regulatory cells.⁽³⁾ CD also leads to loss of intestinal mucosal immune tolerance^(4, 5) and promotion of local immune responses that result in intestinal injury.⁽¹²⁾ While there are numerous CD treatment options, all suppress the pro-inflammatory component of the immune system, many have serious potential side effects and none utilize patients' immune systems to decrease inflammation. The use of oral tolerance as therapy for CD has met with limited success and Treg based therapies are just beginning to be studied. Oral tolerance is the suppression of immune responses to antigens that have been administered previously by the oral route. The inciting antigen in CD is unknown and thus Treg therapies must suppress inflammation in an antigen non-specific manner (bystander suppression). Tregs expanded with the egg protein ovalbumin ex-vivo and infused back into patients with CD suppressed disease activity after one oral exposure to egg.⁽¹³⁾ In-vivo approaches to generate Tregs would have a lower risk of adverse effects, be easier to implement, and be more cost effective than ex-vivo methods. Our initial studies found that the skin is highly active immunologically and can induce both effector⁽⁷⁾ and regulatory responses. Similarly, studies utilizing an antigen delivery device, Viaskin®, show induction of Tregs and efficacy for the treatment of gastrointestinal eosinophilic inflammation.⁽⁸⁾ The skin would be an ideal in vivo route by which to induce Tregs that could treat CD. There has been no application of this concept towards the treatment of CD. Our preliminary data show that epicutaneous antigen exposure induces immune tolerance, the generation of gut-homing Tregs, and can be utilized to decrease gastrointestinal inflammatory cytokine production.

Our long-term goal is to translate our pre-clinical research on epicutaneous tolerance induction into a treatment for CD. The central hypothesis of this application is that epicutaneous exposure to an innocuous antigen will generate Tregs that will control inflammation in CD via bystander suppression. Our preliminary results support the hypothesis that epicutaneous exposure is a highly relevant route of Treg induction and may be utilized to treat CD.

We plan to objectively test our central hypothesis by pursuing the following specific aims:

1. Generate Tregs by epicutaneous antigen exposure and augment gastrointestinal homing. Based on our preliminary data, epicutaneous exposure to antigens induces antigen specific Tregs that home to the intestines. We hypothesize that we can optimize their migration, proliferation and stability in the intestines with the addition of retinoic acid during skin exposure and by subsequent oral feeding of antigen.

1.1 Can we augment generation and migration of Tregs to the intestines by utilizing retinoic acid?

1.2 Can we augment migration and activation of Tregs by oral feeding of antigen?

1.3 Are Tregs stable in the inflammatory milieu of colitis?

2. Determine if epicutaneous Treg induction can treat colitis. Our preliminary data strongly suggest that bystander suppression will be able to abrogate colitis. We hypothesize that epicutaneously induced Tregs will suppress colitis in two different murine models of intestinal inflammation.

2.1 Can TCR-transgenic Tregs suppress T cell-mediated colitis?

2.2 Can endogenous Tregs suppress intestinal inflammation in a spontaneous model of ileitis?

3. Determine the mechanism of colitis suppression. We hypothesize that Foxp3⁺ T cells suppress colitis through TGF- β and IL-10 dependent mechanisms.

3.1 Are Foxp3⁺ Tregs necessary for bystander suppression of colitis?

3.2 Does elimination of TGF- β receptor on effector T cells prevent bystander suppression of colitis?

3.3 Does elimination of IL-10 receptor on effector T cells prevent bystander suppression of colitis?

Our studies will develop a novel method for treating CD. Our results are expected to have an important positive impact on the health, nutrition and growth of children afflicted with CD and other inflammatory gastrointestinal diseases. The investigation of epicutaneous tolerance induction as a treatment for colitis will lead to further investigations in humans. Our studies in colitis models will lead to clinical trials utilizing a targeted therapy, which could spare the use of potentially toxic immuno-modulating medications.

Research Strategy

Significance:

Crohn's disease (CD) affects 241 per 100,000 adults and 58 per 100,000 children in the United States. The incidence in both adults and children has increased in the past 60 years,^(1, 2) especially in children under 10 years old.⁽¹⁴⁾ CD is believed to be the result of failure of tolerance to normal gut bacteria in genetically predisposed individuals.⁽³⁾ Under normal conditions, tolerance to food and microbiota is mediated by regulatory T cells (Tregs). Studies using the neo-antigen keyhole limpet hemocyanin (KLH) have shown that CD patients have an inherent defect in the ability to form tolerance via the gut.^(4, 5) The course of CD is characterized by remission and exacerbation. Abdominal pain, growth failure, nausea, diarrhea, vomiting, anorexia, rectal bleeding and weight loss are common symptoms of the disease. Extra-intestinal manifestations include arthritis and arthralgias, inflammation of the liver and sclerosing cholangitis, specific inflammation of the eyes (episcleritis and uveitis) and specific skin lesions (pyoderma gangrenosum and erythema nodosum). Current treatments rely on modifying the immune response by inhibiting pro-inflammatory responses but not by correcting or augmenting aspects of the immune system that could suppress inflammation. Current immunosuppressant medications have significant toxic adverse effects, including myelosuppression, hepatitis and increased risk of malignancies. Additionally, resistance or intolerance to treatment is common, with up to 18% of children requiring surgery within 5 years from disease onset.⁽¹⁵⁾ There is an urgent need to develop new therapies in light of these potentially serious side effects, especially in children whose growth and development can be permanently affected. An effective treatment would offer a life-altering option for patients affected by this disease.

Innovation:

Immune tolerance has been investigated as a treatment for autoimmune diseases such as rheumatoid arthritis, diabetes and demyelinating neuropathies by inducing antigen-specific Tregs to control inflammation.⁽¹⁶⁻¹⁸⁾ Tregs specific to bystander antigens can also suppress inflammation.^(19, 20) This may be especially useful when the triggering antigen is unknown, as is the case with CD. Studies utilizing oral tolerance induction in rodent models of colitis have shown some success in ameliorating disease.⁽²¹⁻²⁴⁾ However, CD patients have an inherent defect in the ability to form oral tolerance and thus alternative routes of tolerance induction must be investigated.^(4, 5) Tregs generated in vitro to a dietary antigen (ovalbumin from egg) could suppress inflammation in patients with CD.⁽¹³⁾ A significant improvement in disease activity was observed after transfer of Tregs followed by dietary exposure to egg. Our initial work has shown that the skin is a highly active immune organ capable of the induction of effector cells,⁽⁷⁾ as well as immune tolerance. In unpublished work, we show that tolerance and the generation of Tregs can be achieved by epicutaneous antigen exposure to the same extent as that which is induced orally and these Tregs migrate to the mesenteric lymph nodes (MLN) and gastrointestinal tract. Immunization by topical (skin) application has only recently been investigated as vaccines and immunotherapy for food allergens.⁽²⁵⁻²⁹⁾ Epicutaneous immunotherapy using Viaskin® patches induces Tregs⁽⁸⁾ and suppresses eosinophilic gastrointestinal inflammation in mice, demonstrating the potential for skin-induced Tregs to treat intestinal inflammation.⁽⁹⁾ Our studies are innovative because they utilize a novel method of Treg generation to treat colitis. We will account for the defect in oral tolerance found in CD patients by inducing immunomodulation through the skin. In contrast to current immunosuppressive therapies, we propose to use patient's own immune regulatory mechanisms to dampen inflammation. To date there have been no investigations utilizing epicutaneous tolerance induction as a treatment for CD nor have there been studies examining the ability to enhance proliferation of Tregs in the gut in vivo as a means to treat intestinal inflammation. Our preliminary data demonstrates the novelty of our approach.

Background and Preliminary Data:

Oral tolerance for the treatment of murine models of colitis have utilized foreign antigens like ovalbumin, as well as intrinsic antigens like murine cecal antigen-1 and colonic extracted protein to suppress inflammation.⁽²¹⁻²³⁾ However, one rodent study failed to show induction of oral tolerance with antigen alone and showed that nasal tolerance induction was more efficacious for treating colitis.⁽²⁴⁾ IBD patients have an inherent defect in the ability to form tolerance via the gut and thus alternative routes of tolerance induction must be investigated.^(4, 5) Epicutaneous immunotherapy has been shown to induce CD25⁺Foxp3⁺ CD4 T cells systemically and has shown efficacy for the treatment of gastrointestinal eosinophilic inflammation in mice.^(8, 9) Our preliminary data are evidence of the feasibility of the proposed experiments:

Immune tolerance is induced by epicutaneous antigen exposure:

Epicutaneous antigen exposure inhibits Th1-polarized cytokine and antibody responses. We exposed BALB/c mice to ovalbumin (OVA) or KLH by the oral or epicutaneous route for 5 days followed by immunization. Control mice were not exposed to antigen prior to immunization. Antigen-specific IgG2a production was significantly suppressed by prior epicutaneous exposure to antigen (Fig 1A and B), as was IFN- γ production

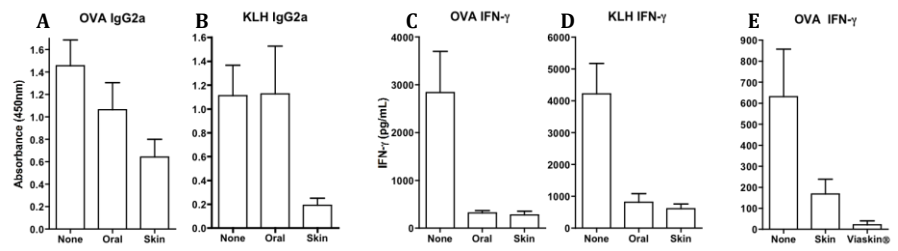


Fig 1: Induction of Tolerance by Epicutaneous Antigen Exposure: A&B: OVA and KLH specific IgG2a after oral and epicutaneous (skin) exposure and immunization. C&D: Antigen specific IFN- γ levels after oral and epicutaneous exposure and immunization. E: OVA-specific IFN- γ after epicutaneous (skin) or Viaskin® exposure with OVA and immunization. All epicutaneous (skin and Viaskin®) groups with $p < 0.05$ as compared to control (none) groups.

(Fig 1C and D). Similarly, mice exposed to OVA utilizing Viaskin® (applied for 48 hours weekly for 2 weeks) and subsequently immunized had suppressed IFN- γ production (Fig 1E). Thus, we have shown that epicutaneous antigen exposure generates systemic immune tolerance.

Epicutaneous antigen exposure generates gut-homing Tregs:

Tregs include both Foxp3⁺ Tregs and Foxp3⁻ Tregs, which include Tr1 cells that secrete IL-10 and Th3 cells, which are LAP⁺ indicating that they express TGF- β . We transferred CD4⁺ T cells from DO11.10 or OT-II mice into BALB/c or C57BL/6 mice respectively. Mice were exposed daily on the skin to OVA for 5 days or via use of Viaskin® applied for 48 hours.

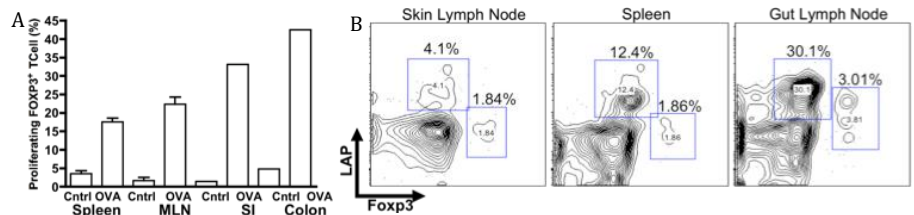


Fig 2: Epicutaneous Exposure to Antigen Induces Tregs: A: Bar graph demonstrating OVA-specific FOXP3⁺ cells in the spleen, MLN, small intestine (SI) and colon after 5 days of daily epicutaneous exposure to OVA as compared to non-exposed controls (Ctrl). B: Flow cytometry plots gated on proliferating OVA-specific CD4⁺ cells from skin draining LNs, spleen and MLN (gut draining LNs) after exposure to Viaskin® with OVA identifying Foxp3⁺ and Foxp3⁺LAP⁺ Tregs.

T cells were then isolated and assessed by flow cytometry. There was induction and proliferation of OVA-specific Tregs (both Foxp3⁺ and LAP⁺) in the skin draining lymph node (SLN), spleen, MLN and intestines after epicutaneous exposures to OVA (Fig 2A and B). We have not yet assessed IL-10 production. Importantly, we have shown that Tregs are induced by epicutaneous exposure and can be found in the spleen, MLN and intestines indicating that they may be able to suppress colitis.

Topical retinoic acid enhances generation of gastrointestinal homing

Tregs: Subcutaneous injections of the vitamin A derivative, retinoic acid (RA), given during vaccination have been shown to redirect immune responses from skin to the gut by increasing gut-homing receptors on T cells.⁽³⁰⁾ Topical RA given at the time of epicutaneous OVA exposure increased the migration of antigen-specific Foxp3⁺ T cells to the MLN, and more strikingly to the small and large intestine (Fig 3). This indicates that RA may increase the efficacy of tolerance induction through the skin.

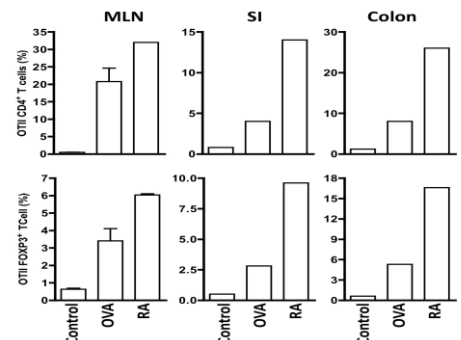


Fig 3: Migration of OT-II/CD4⁺ T cells increases with RA: epicutaneous exposure to OVA without (OVA) and with RA (RA) induced increased migration of OVA-specific T cells and Foxp3⁺ cells to the mesenteric lymph node (MLN), small intestine (SI) and colon.

Skin-derived Tregs prevent colitis:

We next determined whether Tregs from the SLN are as effective as Tregs from the MLN in the suppression of colitis. When RAG^{-/-} mice are transferred with CD45RB^{hi} (naïve T cells) cells from C57BL/6 mice they develop colitis. If both CD45RB^{hi} and CD45RB^{low}CD25⁺ cells from spleens are transferred into RAG^{-/-} mice they do not develop colitis due to the presence of Tregs in the CD45RB^{low} population.^(31, 32) We tested the capacity of CD25⁺ cells

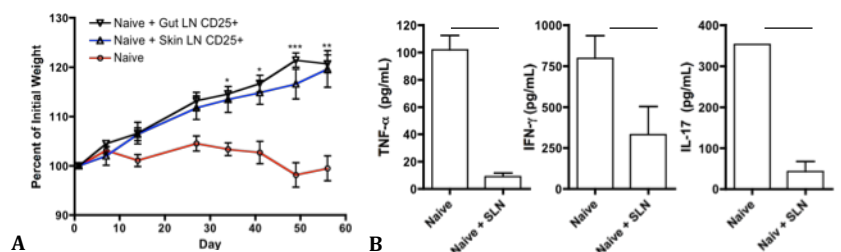


Figure 4: Tregs from SLN are able to Maintain Weight Gain and Prevent Colonic Inflammation: A: Weight as a percent of initial weight for mice co-transferred with CD25⁺ T cells from MLN (Gut LN) or SLN (Skin LN) and naïve T cells as compared to transfer of naïve T cells alone (Naïve). B: TNF- α , IFN- γ and IL-17 production from colonic tissue from mice transferred with naïve T cells alone as compared to those co-transferred with CD25⁺ T cells from SLN. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

derived from SLN or MLN to suppress colitis induced by CD45RB^{hi} cells. Mice receiving Tregs from either SLN or MLN maintained growth curves and had suppressed inflammatory cytokine production in the colon as compared to mice given naïve T cells alone (Fig 4A and B). These data indicate that Tregs derived from the skin can effectively suppress the development of colitis.

Intestinal inflammatory cytokine production is abrogated via bystander suppression:

Since the inciting antigen in CD is unknown, inflammation must be suppressed via bystander suppression.

Tregs expanded with OVA in vitro and infused back into patients with CD are able to decrease disease activity after dietary exposure to egg.⁽¹³⁾ This shows the potential of bystander tolerance in the treatment of CD. We exposed C57BL/6 mice on the skin to OVA or PBS for 5 days. Three days later all mice were gavaged with OVA to induce Treg homing to the gastrointestinal tract, along with streptomycin that was given to increase susceptibility to *Salmonella typhimurium*, which was given by gavage the following day. Four days later mice were sacrificed due to significant weight loss. Minced colon tissue was cultured and supernatants analyzed for cytokine secretion. IFN- γ , IL-17 and TNF- α were all significantly decreased in the mice tolerized and fed OVA (Table 1). Thus, we have demonstrated in an infectious model of colitis that inflammatory cytokines can be inhibited via bystander suppression.

	Untreated (\pm SEM)	OVA Exposed (\pm SEM)
IFN- γ (pg/mL)	107.7 \pm 16.4	57.1 \pm 10.5 *
IL-17A (pg/mL)	275.4 \pm 108.1	33.1 \pm 9.6 *
TNF- α (pg/mL)	229.6 \pm 27.3	139.1 \pm 26.6 *
IL-10 (pg/mL)	84.9 \pm 52.1	107.6 \pm 8.4

Table 1: Suppression of Inflammatory Cytokines in the Colon after Epicutaneous OVA Exposure and Subsequent Oral Exposure: IFN- γ , IL-17A, TNF- α , and IL-10 production from colons of mice exposed to OVA epicutaneously (OVA Exposed) or not (Untreated) and subsequently fed OVA and infected with *S. typhimurium*. *p<0.05

Our preliminary data demonstrate the potential of epicutaneous tolerance to inhibit immune responses in the gastrointestinal tract via bystander suppression and the feasibility of our experimental design.

Approach:

Aim 1: Generate Tregs by epicutaneous antigen exposure and augment gastrointestinal homing.

We hypothesize that epicutaneous exposure to antigen induces Tregs with gastrointestinal homing capacity that will have the ability to suppress T effector responses. To date this skin-intestinal immune communication has not been explored as a treatment for colitis. The addition of RA to the skin and the subsequent feeding of oral antigen are likely to augment gastrointestinal homing and the stability of Tregs.

Justification and Feasibility: Our preliminary data shows that epicutaneous exposure induces tolerance and gut-homing Tregs (Fig 1-2). Our goal is to optimize the conditions conducive to homing from the skin in order to get maximal suppression of inflammation in the gastrointestinal tract. Viaskin® patches loaded with OVA, with or without RA, have been obtained by formation of a collaborative agreement with DBV Technologies. All methods are well established and all mouse strains are readily available.

Experimental Design:

1.1 Can we augment generation and migration of Tregs induced by epicutaneous exposure to the intestines by utilizing retinoic acid?

We have shown that the addition of RA during epicutaneous exposure increases migration of antigen-specific T cells to the intestines (Fig 3). Additionally, RA treated Tregs have been shown to be resistant to Th17 and other Th cell conversion that is found in an inflammatory milieu.⁽³³⁾ We will examine dose-dependent effects of RA on Treg generation and gastrointestinal homing. We will transfer OVA specific T cells from OT-II (CD45.2⁺) mice on a RAG^{-/-} background to reduce any contribution from endogenous non-transgenic TCR into immunocompetent C57BL/6 (CD45.1⁺) mice. Subsequently we will expose mice for 48 hours once a week for two weeks to Viaskin-OVA on the skin in the presence or absence of various doses of RA (5, 10, 25, 50 μ g). Three days later, SLN, MLN, and small and large intestine will be examined by flow cytometry immediately after 4 hours of stimulation with PMA/Ionomycin/BFA for OVA specific T cells (CD4, CD45.2, Foxp3, CD25, IL-10 and LAP). We will detect effector T cell populations by examining IFN- γ and IL-17. IL-6R will be examined as well since down-regulation of expression and signaling of IL6R is associated with increased stability of Tregs when they are treated with RA.⁽³³⁾ Finally, we will also identify gut homing markers (CCR9 (small bowel and thymus homing)⁽³⁴⁾, CCR6, CCR10 and α 4 β 7) on T cells in the SLN.

Anticipated Results: As shown in our preliminary data, we expect that epicutaneous exposure will induce Tregs that migrate to the MLN and intestines. The addition of RA is expected to increase the generation of Tregs and thus their frequency in the responder population and increase gastrointestinal homing.

1.2 Can we augment migration and activation of Tregs induced by epicutaneous exposure to the intestines by oral feeding of antigen?

We hypothesize that subsequent oral feeding of antigen after epicutaneous exposures will increase homing and activation of Tregs in the MLN and intestines. We will determine the optimal oral dose of OVA needed to induce gastrointestinal-homing of Tregs. The same experimental design will be used as above. Groups of mice in these experiments will or will not be exposed to OVA with RA at the optimal dose determined in Aim 1.1 and 3 days later will be gavaged fed or not with various doses (300ug, 1mg, 3mg, and 10mg) of OVA. After 24, 48 and 72 hours we will examine MLN and lamina propria of small and large intestine by flow cytometry as described above. In addition, Ki67 will be examined by flow cytometry to determine if feeding of OVA induces proliferation of Tregs in the MLN and intestines.

Anticipated Results: We expect that oral OVA exposure will lead to enhanced numbers of proliferating Foxp3⁺ and LAP⁺ Tregs and increased IL-10⁺ T cells in the intestine compared to mice exposed on the skin but unfed and also compared to mice fed but not initially exposed on the skin.

1.3 How does the inflammatory milieu of colitis affect Tregs and augmentation of gastrointestinal-homing?

The therapeutic use of Tregs may be limited by data showing that an inflammatory environment may alter Treg function from suppressive to pro-inflammatory through their acquisition of a Th-17 phenotype.⁽³⁵⁾ However, the question of Treg stability remains contentious along with the origin of this “unstable” population. RA may prevent this conversion since Tregs cultured with RA maintained their phenotype when placed into an inflammatory milieu.⁽³⁵⁾ The optimal doses of RA and oral feeding from Aims 1.1 and 1.2 will be evaluated in the setting of colitis. We will utilize the CD45RB^{hi} → RAG transfer model of colitis. In experimental groups, naïve CD4⁺ T cells from C57BL/6 mice will be transferred into RAG1^{-/-} mice. These mice develop colitis at 4-6 weeks in our facility. Control groups will receive total CD4⁺ T cells and thus will not develop colitis due to the presence of Tregs in the CD45RB^{low} population. At 6 weeks, naïve CD4⁺ T cells will be isolated from OT-II/RAG^{-/-} mice and transferred into all mice. The next day, groups of mice will be epicutaneously exposed to OVA or Viaskin® alone with or without RA and then gavaged fed OVA or water 3 days later. Subsequently, mice will be sacrificed and MLN and lamina propria from intestines examined for OT-II Tregs by flow cytometry (CD4, CD45.1, Foxp3, CD25, LAP, IL-10). This will be quantitatively compared to the OT-II effector T cell population (IFN-γ, IL-17, TNF-α, IL-6).

Since CD is a chronic disease, we will also examine the life span and reactivation of these cells. Mice will be gavaged fed OVA as above and then weekly. Mice will be sacrificed 2 and 4 weeks after the first feed. MLN and lamina propria of intestines will be assessed by flow cytometry as above.

Anticipated Results: We anticipate that Tregs will be stable during colitis and expect to find similar proportions of Tregs as compared to effector T cells, especially with the addition of RA.

Potential Problems and Alternatives: This methodology has been extensively utilized by our lab and will pose no technical difficulties. Our preliminary results demonstrate that Tregs migrate to the MLN and intestines, thus we do not anticipate any problems with this aim. If Tregs are unstable during colitis we will verify this by utilizing fate mapping with OT-II TCR transgenic Foxp3GFP^{Cre}ROSA26^{RFP} mice in the transfer model of colitis. If confirmed, possible interventions include co-administering an adjuvant like cholera toxin B, which has been shown to promote Foxp3⁺ Tregs, or cytokines IL-10 and TGF-β.⁽³⁶⁾

Aim 2: Determine the ability of Tregs induced by epicutaneous exposure to suppress colitis.

In aim 1 we will optimize the generation and gastrointestinal homing of Tregs induced via epicutaneous antigen exposure. The next step will be to determine efficacy of Tregs to suppress colitis. We will start by maximizing potential efficacy by providing a large number of antigen-specific Tregs, which is only possible with a transgenic system. We will then test efficacy in a more physiologic system. *We hypothesize that the induction and proliferation of Tregs will serve to decrease intestinal inflammation via bystander suppression.*

Justification and Feasibility: Our preliminary data shows that Tregs isolated from SLN are able to prevent colitis (Fig 4) and that bystander suppression is able to decrease intestinal inflammation (Table 1). Here we will determine the ability to treat rather than prevent colitis in two models with many similarities to human CD. We will do so with the optimization of Treg migration determined in Aim 1. All methods are well established and all mouse strains are readily available.

Experimental Design:

2.1 Can TCR-transgenic Tregs induced by epicutaneous exposure suppress T cell-mediated colitis?

Oral tolerance induction to OVA suppressed colitis in a CD45RB^{hi} transfer model that was induced by co-transfer of OVA-specific and wild type naïve T cells.⁽²¹⁾ Suppression of colitis was partially due to the expression of non-Tg TCR that responded to gut flora. However, there was also an increase in TGF-β and IL-10 secretion and thus the possibility of suppression due to bystander responses. We will utilize a similar transfer model given that it is widely accepted and has many similarities to human CD.⁽³⁷⁾ We will isolate naïve T cells

from C57BL/6 (CD45.1⁺) mice and transfer them into RAG1^{-/-} mice. After the onset of colitis, at 6 weeks, we will transfer naïve T cells from OT-II (CD45.2⁺) mice into the same RAG1^{-/-} mice. A control group will not receive OT-II cells. Unlike the published model,⁽²¹⁾ we will use OT-II mice on a RAG^{-/-} background to eliminate the role of non-Tg TCR. The next day we will expose mice on the skin to OVA with RA or Viaskin® alone. After 72 hours, mice will be gavage fed with OVA or water. Exposures and feedings will be done with the optimal doses determined in Aim 1. Mice will be monitored for weight loss and sacrificed 2 weeks later. The extent of inflammation will be scored based on microscopic examination of the large intestine by a pathologist blinded to the treatment group. MLN and large intestines will be taken, stimulated for 4 hours with PMA/Ionomycin/BFA, and T cells examined for the formation of T effector cells (Th17, IFN- γ , TNF- α) as compared to the formation of Tregs (CD45.2, Foxp3, CD25, IL-10, LAP) by flow cytometry. The immune milieu will be examined by looking at secretion of cytokines (IL-6, IL-10, IL-17, TNF- α , IFN- γ , TGF- β) from MLN cultured for 72 hours with anti-CD3/CD28 antibodies and minced colonic tissue cultured overnight without any stimulus. MLN and large intestine will also be examined by rtPCR for chemokines and cytokines.

Anticipated Results: In this model, we expect that epicutaneous exposure will result in tolerance to OVA, an induction and migration of OVA-specific Tregs to the colon and attenuated Th1 responses.

2.2 Can endogenous Tregs induced by epicutaneous exposure suppress intestinal inflammation in a spontaneous model?

We will determine if we can suppress ileitis and colitis via bystander suppression using SAMP1/YITFc mice, which develop spontaneous ileitis and cecal inflammation mediated by monocytes and T cells by 6 weeks of age in our facility.⁽³⁸⁾ This model is similar to human disease clinically and histologically. It has particular relevance to this proposal since it is Th1-mediated and high IFN- γ is found even prior to the onset of ileitis.⁽³⁹⁾ At 6 weeks of age, we will expose mice on the skin to OVA with RA or Viaskin® alone. After 72 hours, mice will be gavage fed with OVA or water. Mice will be sacrificed one week later. Inflammation in the ileum and cecum will be scored based on microscopic examination by a pathologist blinded to the treatment group. MLN, ileum and cecum will be taken, and T cells stimulated for 4 hours with PMA/Ionomycin/BFA and examined for the formation of T effector cells (Th17, IFN- γ , TNF- α) as compared to the formation of Tregs (Foxp3, CD25, IL-10, LAP) by flow cytometry. The immune milieu will be examined by looking at secretion of cytokines (IL-6, IL-10, IL-17, TNF- α , IFN- γ , TGF- β) from MLN cultured for 72 hours with anti-CD3/CD28 antibodies and minced intestinal tissue cultured overnight without any stimulus. MLN and intestines will also be examined by rtPCR for chemokines and cytokines.

Anticipated Results: We expect that Tregs will migrate to the intestines and abrogate ileitis and colitis via bystander suppression. Microscopic inflammation will be decreased and Th1 effector cells and cytokine production will be suppressed.

Potential Problems and Alternatives: We expect that inflammation will be abrogated in the first model given our strong preliminary results. However, in the SAMP1 mice we may not see suppressed inflammation due to possible defects of Tregs in this model.⁽⁴⁰⁾ Similarly, CD patients may also have a defect in Tregs. Thus, we may consider inducing tolerance prior to 6 weeks. These experiments can be validated with other antigens such as KLH, since this neo-antigen can be safely used in humans. Alternatively other antigens may be more ideal such flagellin or CEA which are already present in the intestines, obviating the need for subsequent oral feedings. Murine models will be performed under the guidance of Drs. XXX.

Aim 3: Determine the mechanism of colitis suppression.

In Aim 2 we will determine the efficacy of Tregs induced by epicutaneous antigen exposure to abrogate inflammation in two models of CD. Foxp3⁺ Tregs and TGF- β are important for the induction of oral tolerance⁽⁴¹⁾ and IL-10 producing Tr1 cells are implicated in suppression of colitis.⁽²⁰⁾ In experimental colitis models, Tregs were protective by producing IL-10 and TGF- β .⁽⁴²⁻⁴⁴⁾ We, therefore, hypothesize that suppression of inflammation in the intestines will require a combination of Treg populations.

Justification and Feasibility: Our preliminary data shows the generation and migration of both Foxp3⁺ and LAP⁺ Tregs. Therefore, both populations are likely involved in suppression of inflammation in the intestines. However, we did not assess the role of IL-10 producing Tregs, which are likely to play an important role. We will utilize transgenic models given the ability to tract OVA-specific T cells. All methods are well established in our laboratory and all mouse strains are readily available.

Experimental Design:

3.1 Are Foxp3⁺ Tregs necessary for bystander suppression of colitis?

We will first determine the necessity of Foxp3⁺ Tregs for suppression of colitis. We will again utilize the CD45RB^{hi} transfer model described in Aim 2.1. However, after the onset of colitis at 6 weeks, mice will be transferred with naïve T cells from DREG/OT-II (Depletion of REGulatory T cells) mice, which carry a DTR

transgene under the control of a Foxp3 promoter and thus allow for the specific depletion of Foxp3⁺ Tregs by injection of diphtheria toxin (DT). The next day mice will be exposed on the skin to OVA with RA or Viaskin® alone. DT will be given 5 days after skin exposure via intraperitoneal injection to deplete any OVA-specific Foxp3⁺ Tregs. DT will not affect wild-type T cells. Control mice will not be given DT. The next day OVA will be gavage fed. Mice will be sacrificed 2 weeks later and outcomes measured as in Aim 2.1.

Anticipated Results: We anticipate that tolerance will be mediated by Foxp3⁺ Tregs given literature supporting this concept,⁽⁴⁵⁾ and thus we expect to see more severe colitis in mice depleted of Foxp3⁺ cells.

3.2 Does elimination of TGF- β receptor on effector T cells prevent bystander suppression of colitis?

Next we will determine the need for TGF- β to suppress colitis. We will use the CD45RB^{hi} transfer model described in Aim 2.1. However, the initial transfer will be done with naïve T cells isolated from TGF- β receptor knockout mice. Thus, any TGF- β produced by Tregs will have no effect on the suppression of effector T cell responses. TGF- β receptor knockout mice develop colitis by 3-4 months of age. Thus naïve T cells will be isolated in these mice at 2 months of age. Control mice will be transferred with naïve T cells from C57BL/6 mice. At 6 weeks, we will transfer naïve T cells from OT-II mice into the same RAG1^{-/-} mice. The next day mice will be exposed on the skin to OVA with RA or Viaskin® alone. The following day mice will be gavage fed with OVA. Mice will be sacrificed 2 weeks later and outcomes measured as in Aim 2.1.

Anticipated Results: We anticipate that TGF- β will play a significant role in decreasing inflammation given its role in tolerance and suppression of colitis,^(20, 41) and thus we expect to see more severe colitis in mice transferred with cells from TGF- β r^{-/-} mice.

3.3 Does elimination of IL-10 receptor on effector T cells prevent bystander suppression of colitis?

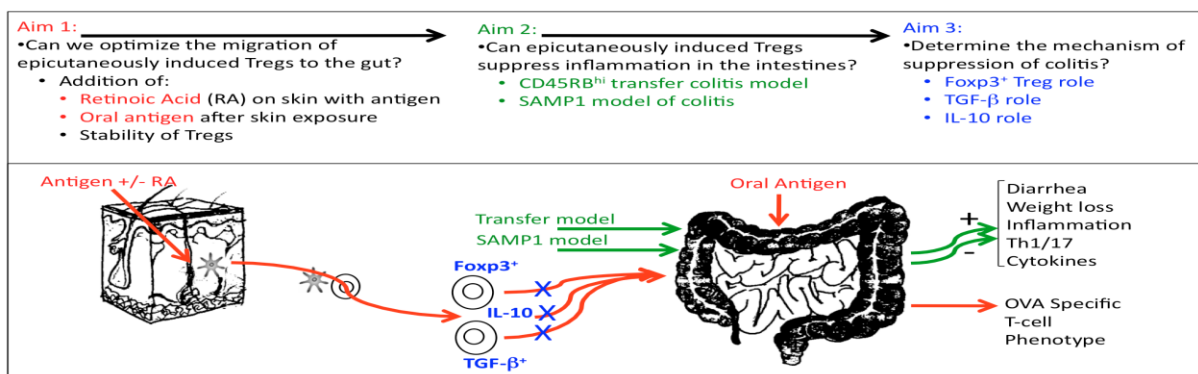
Finally, we will determine the need for IL-10 to suppress colitis. We will use the CD45RB^{hi} transfer model described in Aim 2.1. However, the initial transfer will be done with naïve T cells from IL-10 receptor knockout mice. Thus, any IL-10 produced by Tr1 Tregs will have no effect on the suppression of effector T cell responses. IL-10 receptor knockout mice develop colitis by 3-4 months of age. Thus naïve T cells will be isolated in these mice at 2 months of age. Control mice will be transferred with naïve T cells from C57BL/6 mice. At 6 weeks, we will transfer naïve T cells from OT-II mice into the same RAG1^{-/-} mice. The next day mice will be exposed on the skin to OVA with RA or Viaskin® alone. The following day mice will be gavage fed with OVA. Mice will be sacrificed 2 weeks later and outcomes measured as in Aim 2.1.

Anticipated Results: We anticipate that IL-10 will play a role in decreasing inflammation given its role in tolerance and suppression of colitis,^(20, 41) and thus we expect to see more severe colitis.

Potential Problems and Alternatives: We do not anticipate any technical difficulties with these experiments given our experience with the transfer model. We will consider confirming our results with use of neutralizing antibodies to TGF- β and IL-10 given during epicutaneous and oral exposures in the models in Aim 2. This may cause worse colitis even in non-tolerized mice making it important to have non-tolerized control groups.

Statistics: Differences between groups will be analyzed using the Mann-Whitney U test or Kruskal-Wallis ANOVA assuming non-parametric distribution and depending upon the number of groups being compared.

Research Plan Overview:



Future Studies: These studies are the necessary pre-clinical studies before we can perform future clinical trials and develop an R01. This is the first step towards transitioning to human studies, which will be facilitated by Dr. XXX, and by Dr. XXX. As such, my educational plan initially focuses on advanced immunology and towards the end of the granting period focuses on acquiring the necessary knowledge to design and perform clinical trials.

Vertebrate Animals

We have shown that tolerance and antigen-specific regulatory T cells (Tregs) can be induced via epicutaneous application of antigens. Our results support the hypothesis that exposure through the skin may be an effective route of tolerance induction that may be utilized to treat gastrointestinal diseases including colitis. (Full details of preliminary work and experiments are available in the Research Strategy Section.)

1. Detailed Description of Proposed Use of Animals

In Aim 1, we seek to optimize the generation and migration of Tregs induced by epicutaneous exposure to the gastrointestinal tract.

1.1 and 1.2. In order to assess this, our initial experiments will first seek to optimize migration of Tregs from the skin to the MLN and intestine after epicutaneous exposure to OVA. We will assess if oral antigen feeding and the application of retinoic acid to the skin will increase migration of Tregs. We will isolate CD4⁺ T cells from female OTII/RAG^{-/-} mice and transfer them into C57BL/6 (CD45.1⁺) female mice. We will expose them on the skin to OVA with or without retinoic acid utilizing Viaskin®. Mice will be anesthetized with ketamine/xylazine in standard doses. A depilatory cream, which has been shown to cause no skin irritation or barrier disruption, will be applied to the skin for 30-60 sec prior to skin exposures. Viaskin® has also been shown not to cause any skin irritation and will be applied for 48 hours. Three days later mice will be fed or not fed with OVA in various doses by gavage. Each experimental and control group will consist of 5 mice each. We will need 8 experimental groups and 2 control groups plus 20 OTII/RAG^{-/-} mice (Subtotal 70 mice).

1.3. In this portion of Aim 1 we will determine the stability of Tregs and our ability to optimize their intestinal homing in the inflammatory milieu of colitis. We will utilize a transfer model of colitis where CD45Rb^{hi} or total CD4⁺ T cells are isolated from female C57BL/6 and transferred into RAG1^{-/-} mice. Mice receiving CD45Rb^{hi} T cells will develop colitis 4-6 weeks later in our facility. Mice will lose weight and have diarrhea. At 6 weeks mice will be transferred with CD4⁺ T cells isolated from OTII/RAG^{-/-} mice. The following day mice will be tolerized on the skin with Viaskin® with OVA with or without RA (as described above) and then gavage fed OVA or water 3 days later. Mice will be sacrificed 1 week later or if any mice lose more than 20% of their initial weight or have BCS equal to or less than 2 (6 groups of 5 RAG1^{-/-} mice each plus 20 C57BL/6 mice and 20 OTII/RAG^{-/-} mice = subtotal 70 mice).

(Total for Aim 1 = 140 mice)

In Aim 2, we will determine the ability of Tregs induced by epicutaneous exposure to suppress colitis in two different models.

2.1 We will utilize a transfer model of colitis where CD45Rb^{hi} (naïve) cells are isolated from female C57BL/6 and transferred into RAG^{-/-} mice. Mice develop colitis between 4 and 6 weeks. At 6 weeks mice will be transferred with naïve OTII/RAG^{-/-} cells. The next day they will be exposed on the skin to OVA as described in Aim 1 with and without retinoic acid. They will subsequently be gavage fed OVA at the optimal dose determined in Aim 1. Mice will lose weight and have diarrhea. Mice will be monitored daily for weight loss and BCS. Mice will be sacrificed if mice lose more than 20% of their initial weight or they have BCS equal to or less than 2 (15 C57BL/6 mice, 15 OTII/RAG^{-/-} mice and 4 groups of 5 RAG^{-/-} mice = Subtotal of 50 mice).

2.2. Here female SAMP1/YITFc (SAMP1) mice will be utilized which spontaneously develop ileitis and cecal inflammation at 6 weeks of age in our facility. Mice will slowly lose weight soon after inflammation starts. Mice will be monitored twice a week for weight loss and BCS. Mice will be sacrificed if mice lose more than 20% of their initial weight or they have BCS equal to or less than 2. At the start of weight loss, mice will be exposed on the skin to OVA as described in Aim 1. Three days later mice will then be gavage fed or not with OVA. Mice will continue to be assessed for weight loss and BCS until being sacrificed 2 weeks later (4 groups of mice and 5 mice per group = Subtotal 20 mice)

(Total for Aim 2 = 70 mice)

In Aim 3, we seek to determine the mechanism of colitis suppression. We will determine which Treg population is necessary for suppression of immune responses in the gut.

3.1. We will first determine the need for Foxp3⁺ Tregs. We will utilize a transfer model of colitis where CD45Rb^{hi} cells are isolated from female C57BL/6 and transferred into RAG1^{-/-} mice. 6 weeks later mice will again be transferred but now with naïve T cells from DREG/OTII mice. The next day mice will be exposed to OVA with/without retinoic acid as described in Aim 1. Three days later mice will be given diphtheria toxin IP to deplete Foxp3⁺ Tregs. The next day they will be gavage fed with OVA. Mice will be weighed daily and

sacrificed 2 weeks later or if mice lose more than 20% of their initial weight or they have BCS equal to or less than 2 (4 groups of 5 RAG1^{-/-} mice plus 15 C57BL/6 mice and 15 DREG/OTII mice = Subtotal 50 mice)

3.2. We will then determine the need for TGF- β . We will utilize a transfer model of colitis where CD45Rb^{hi} cells are isolated from female TGF- β receptor knockout mice and transferred into RAG1^{-/-} mice. 6 weeks later mice will again be transferred but now with naïve T cells from OTII/RAG^{-/-} mice. The next day mice will be exposed to OVA with/without retinoic acid as described in Aim 1. Three days later mice will be gavaged with OVA. Mice will be weighed daily and sacrificed 2 weeks later or if mice lose more than 20% of their initial weight or they have BCS equal to or less than 2 (4 groups of 5 RAG1^{-/-} mice plus 15 TGF- β r^{-/-} mice and 15 OTII/RAG^{-/-} mice = Subtotal 50 mice)

3.3. In the final subaim we will determine the need for IL-10. We will utilize a transfer model of colitis where CD45Rb^{hi} cells are isolated from female IL-10 receptor knockout mice and transferred into RAG1^{-/-} mice. 6 weeks later mice will again be transferred but now with naïve T cells from OTII/RAG^{-/-} mice. The next day mice will be exposed to OVA with/without retinoic acid as described in Aim 1. Three days later mice will be gavaged with OVA. Mice will be weighed daily and sacrificed 2 weeks later or if mice lose more than 20% of their initial weight or they have BCS equal to or less than 2 (4 groups of 5 RAG1^{-/-} mice plus 15 IL-10r^{-/-} mice and 15 OTII/RAG^{-/-} mice = Subtotal 50 mice)

(Total for Aim 3 = 150 mice)

2. Justification for the Use of Animals

Murine models are ideal because many of the cells involved in tolerance induction in mice are similar to those in humans. The mechanisms of colitis/ileitis also overlap with those in human IBD. Additionally, many of the mechanisms we will study are unable to be studied in humans or examined by computer modeling. Numbers per group were determined from our previous experiments showing statistical significance, similar protocols and previous reports in the literature. None of the strains of mice used are in short supply and all special strains (OTII/RAG^{-/-}, RAG^{-/-}, DREG/OTII, TGF- β r^{-/-}, IL-10r^{-/-}, SAMP1/YITFc) are readily available at Mount Sinai in my colony or those of my mentors, members of my advisory committee or consultants. Standard strains of mice (C57BL/6 and BALB/C) are readily available from vendors.

3. Veterinary Care

The Icahn School of Medicine at Mount Sinai has an IACUC, which will verify all protocols and a rodent barrier facility, which is housed in the Icahn Research Building. This facility has a full staff of veterinary technicians and doctors. Mice are cared for daily and evaluated if ill appearing or at the request of the investigator. IACUC approval will be obtained and verified for all experiments prior to funding of this grant

4. Procedures for Ensuring Limited Discomfort, Distress, Pain and Injury

In most experiments, mice are expected to be healthy without any distress after the minor procedure of fur removal using a depilatory cream and application of innocuous proteins to the skin. This has been shown to not cause any irritation.

Mice with colitis have the potential to lose weight, have diarrhea and become ill. We will monitor these animals on a daily basis for signs of distress including mobility, observation of their physical condition and body weight. To assess physical condition, the body condition scoring (BCS) will be used.

During all stressful procedures, Ketamine/Xylazine will be used.

5. Method of Euthanasia

If mice have a BCS of equal to or less than 2 they will be euthanized. If animals appear moribund or lose more than 20% of their baseline body weight they will be euthanized using Ketamine/Xylazine and cervical dislocation. Euthanasia methods used are consistent with the recommendations of the American Veterinary Medical Association Guidelines.