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TITLE: Defining a Novel Therapeutic Application of Lactoferrin for Intestinal Inflammation

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**14. ABSTRACT**

Acute intestinal infections can be inciting events for the development of chronic intestinal inflammation, such as occurs in inflammatory bowel diseases (IBD) in susceptible patients. 3.1 million people in the United States suffer from IBD, predominantly Crohn's disease (CD) and ulcerative colitis (UC). The military is not spared from this illness. The peak incidence of IBD occurs between 18-40 years of age and 2/3 of active military personnel are within this age group. IBD is of concern to the military due to the disability it causes and its high costs in terms of lost work hours, chronic health care requirements, and diminished troop readiness. In addition, IBD is on the rise in the military, increasing 2-3 fold over the past 2 decades. Among veteran populations, IBD represents one of the highest hospitalization rates and IBD-associated colorectal cancer is on the rise in this population. These data define an urgent need for identifying novel, inexpensive therapies to treat this disease.

The causes of IBD remain poorly understood yet evidence indicates that there is an imbalance between the anti-inflammatory and pro-inflammatory cells in the intestinal mucosa related to the microbial environment of the intestine. Regulatory T cells are a critical type of immune cell that promotes balance in the intestinal immune response. We have demonstrated that human lactoferrin (hLF), a protein found in breast milk, activates anti-inflammatory, regulatory T cells in intestinal inflammation in mouse models of IBD. We and others have identified that hLF can alter the growth of bacteria in the intestine and that hLF can inhibit or treat infectious intestinal bacteria in the laboratory. These antibacterial effects have not been studied extensively in animals or humans. We know that hLF activates these cells and we will be one of the first to correlate the immune, microbial, and metabolic changes occurring in the intestine in response to this novel agent.

To date this study has identified the optimal route of administration and the optimal iron content for the use of hLF. The significance of this is that oral administration allows for ease of use and the knowledge of the iron content further optimizes this strategy as we move toward its use in patients with intestinal inflammation. In addition, we have determined that the presence of intestinal bacteria is required for optimal efficacy of hLF as an activator of regulatory T cells. This information further informs our understanding of the mechanism by which hLF functions. Finally, the preparatory work for the initiation and completion of all the other aims has been completed.

This study to date is helping us to understand the anti-microbial and anti-inflammatory potential of the naturally occurring protein hLF and better understand the mechanisms by which hLF acts to benefit a well-balanced immune system. Ultimately, this study will inform the development of hLF as a safe, effective, natural therapy for intestinal inflammation in humans.

**15. SUBJECT TERMS**

Inflammatory Bowel Disease, Lactoferrin, Microbiome

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## 1) Introduction:

Anti-inflammatory Foxp3+CD4+ Tregs are critical for the maintenance of immune homeostasis both systemically and in the gastrointestinal tract. Tregs quell an overactive immune response in the intestinal mucosa. The microbiome plays a critical role in this homeostasis, in part through activation of Tregs. Disruption of immune homeostasis is a key factor in development of intestinal inflammation. Many current therapies for inflammatory conditions inhibit the entire immune response without sparing Tregs, thus inhibiting the body's anti-inflammatory response. Selected activation of Tregs, rather than whole-sale suppression of the immune system, could provide a more efficacious and better-tolerated means of restoring immune homeostasis; furthermore, this approach may lend itself to prophylactic measures designed to protect individuals at risk for intestinal inflammation. Our preclinical studies demonstrated that the human iron-binding glycoprotein, LF, ameliorates inflammation and specifically activates the Treg population. The molecular mechanisms through which LF exerts its anti-inflammatory effects and promotes immune homeostasis are not well understood but are likely to occur through multiple pathways. Our ongoing studies effectively demonstrate the therapeutic potential of orally delivered LF in distinct murine models of IBD. Our work to date has focused on the best therapeutic strategy and formulation for LF delivery (e.g., iron free or iron saturated) for enteric infection vs chronic inflammation. Addressing these questions is essential for development of LF as a new treatment option for intestinal inflammation. This work has provided the appropriate quantity, delivery and constituents of LF for use in chronic intestinal inflammation and will be assessed for its similarities in our enteric infection models.

**Clinical Impact:** The results from these studies are helping to define the therapeutic potential and mechanism of action of the natural human colostrum protein, lactoferrin. **Understanding the multimodal effects of hLF will provide essential preclinical data** for a novel method to modulate the microbiome and increase Treg production *in vivo* for the prevention and treatment of intestinal inflammation.

## 2) **Keywords:**

Inflammatory Bowel Disease

Regulatory T cells (Treg)

Microbiome

Lactoferrin

rhLF: recombinant human lactoferrin

CXCR4

### 3) Accomplishments:

Accomplishments are included below based on Major Tasks identified in the SOW of the original application:

#### **SA1- Major Task 1:** *Determine the best route of administration of Lactoferrin (rhLF).*

We have confirmed that Lactoferrin significantly increases FoxP3 expression both *in vivo* and *in vitro* and increases the number of Tregs in the intestine and mesenteric lymph nodes. We have further moved forward to assess the question of whether oral gavage is sufficient, or subcutaneous administration is required to activate regulatory T cells (Tregs) in the murine model of ileitis. We have defined that oral gavage of Lactoferrin is the superior method of treatment with Lactoferrin. Mice treated with oral rhLF have better healing of their inflammation and significantly increased numbers of Tregs. We have reserved the fecal pellets and ceca from these mouse studies to assess by 16S, metagenomics and metabolomics the effect of either oral or subcutaneous rhLF to help determine if this therapy effects the microbiome and how this might relate to its ameliorating effects.

We have further developed the Salmonella model in our laboratory and have initiated these same experiments to assess this question in the Salmonella model to assess the effect of lactoferrin in enteric infection. These experiments are currently in process.

#### **SA1- Major Task 2:** *Identify the best iron content to treat intestinal inflammation.*

This work has just started as we have produced the 3 forms of rhLF. Full iron, half iron and Apo (no) iron containing rhLF have been used to assess the effects of rhLF on increased Treg conversion *in vitro* and development of Tregs *in vivo*. These experiments are just starting but current data is suggesting that full iron containing rhLF is the most effective at activating Tregs. These experiments require more repetition.

We have further developed the Salmonella model in our laboratory and have initiated these same experiments to assess this question in the Salmonella model to assess the effect of lactoferrin in enteric infection. These experiments are currently in process.

#### **SA1- Major Task 3:** *Determine the effect of Lactoferrin on lymphocyte trafficking.*

We have been running the adoptive transfer studies to assess the role of Lactoferrin on Tregs and have noted an early influx of Tregs when treating with rhLF compared to control. One experiment is completed but this requires repetition. In addition, further studies with our CXCR4 deficient mice will be started in the coming 1-2 months.

#### **SA2- Major Task 1:** *Determine if CXCR4 mediates the effect of Lactoferrin on Tregs without inflammation.*

Initial experiments in the CXCR4 conditional knockout mice revolved around immunophenotyping. We noted that there is a significant decrease in the number of CD4 cells in the colon of mice that were deficient in CXCR4 in CD4 T cells. We did not see the similar defect in mesenteric lymph nodes or spleen. This effect seemed to also be seen in B cells as lack of CXCR4 in CD4 T cells decreased the number of CD19 B cells. In the coming 1-2 months we will initiate use of rhLF in the CXCR4 conditional knockout mice compared to WT mice with the expectation that there will be no change in Treg numbers or location.

#### **SA2- Major Task 2:** *Determine the role of LF and CXCR4 on the activation of Tregs during inflammation.*

The Immunophenotyping informed our decision that it will likely be better to use the adoptive transfer model of colitis than the  $TNF^{\Delta ARE} \times CXCR4^{fl} CD4^{cre}$  model of ileitis to assess this question. We will look at the effect of lactoferrin in both of the models, but the cleaner assessment will be in the adoptive transfer model. It will remove the effect on B cells or other cells as a confounder. These experiments have started and are under way.

#### **SA3- Preparatory work for microbiome analyses in Aims 1, 2, and 3**

Murine fecal specimens collected over the course of this project will be subjected to comprehensive microbiome analysis using both 1) 16S rRNA gene sequencing for deep and precise taxonomic assignment of resident bacteria, which permits comparison of results to previous studies conducted in the de Zoeten and Frank labs and 2) Shotgun metagenomic sequencing, which provides broader taxonomic (bacteria, fungi, archaea, bacteriophage) and functional annotations.

Drs. Frank and Robertson have ample experience analyzing 16S rRNA gene sequences, but have more limited experience analyzing metagenomic sequence datasets. Consequently, during the past year they have reviewed best practices in the field, met with local experts, and have begun to install and operate relevant software pipelines/databases on their computer servers.

The computational infrastructure of the Frank lab has now been significantly upgraded to accommodate analysis of the large sequence datasets generated through next-generation metagenomic sequencing. This

work includes: 1) Installation and testing of metagenomic data analysis pipelines (e.g. SqueezeMeta<sup>1</sup> [*broad functional/phylogenetic classification*] and additional databases such as CARD/RGI [*antibiotic resistance module detection*]; 2) Adaptation and testing of our microbiome statistical software (written in R) to accommodate metagenomics data; and 3) Design and construction, by Dr. Robertson, of a high-performance computer server consisting of 40 Xeon cores, 384GB ECC RAM, 9TB PCIe SSD, 80TB RAID6 (the hardware for this system was *not* funded by our DOD grant but will be instrumental in forthcoming data analyses). The SqueezeMeta software package<sup>1</sup> is a modern, open-source, fully automated pipeline for metagenomic data analysis that can be installed locally on our computer servers. It links together a robust set of tools for co-assembling metagenomes, discovering and annotating open reading frames (ORF), mapping reads to ORFs, and summarizing sequence hits against several databases (COG, KEGG, PFAM). However, our initial tests of this pipeline using test datasets revealed limitations in scaling up analyses to the size of metagenomic sequence datasets that we expect to generate in this project. Over the previous year we have performed detailed analyses of the SqueezeMeta pipeline and both modified the code to provide significantly better performance and reported bugs to the developers. Finally, Dr. Frank's lab has completed an evaluation of commercial kits used for generation of shotgun metagenomic libraries and will use the plexWell™ kits from seqWell Inc. for upcoming experiments.

Overall, we now have a robust and reliable set of benchtop protocols and software tools that are capable of generating and efficiently processing the metagenomic datasets we require to accomplish project goals.

**Major Task 1:** *Evaluate 16S profiling profiles providing lactoferrin at different iron concentrations and via differing routes of Aim 1 and Aim 2 intestinal samples.*

Samples have been collected from SA1 for assessment. We are continuing to collect these samples to complete the analysis on only a few chips to assess in a controlled fashion and maintain consistency. These initial evaluations will be assessed from SA1 by the end of 2022

**SA3- Major Task 2:** *Evaluate the metagenomic profiles of Aim 1 and Aim 2 cecal samples*  
See above

**SA3- Major Task 3:** *Assess the Evaluate cecal and serum metabolomic profiles in Aim 1 and Aim 2 samples after differing iron concentrations.*  
*Ongoing*

**SA3- Major Task 4:** *Evaluate the effect of Lactoferrin on a germ-free model of intestinal inflammation.*  
We have further assessed for the requirement for bacteria to attain the effect of increased regulatory T cells after treatment with rhLF. This set of experiments have been performed in germ free mice. In this model we assessed the effect of rhLF on mice without microbiome. We have completed 2 replicates of this experiment and have noted that there is no change in Treg numbers without the presence of the microbiome. This work will need to be repeated at least twice more to meet statistical significance. We have saved stool from these mice to assure there is no bacterial contamination and will assess for metabolomic changes in the fecal pellets.

**SA3- Major Task 5:** *Data Integration*  
This work is ongoing throughout the entire time of this grant

#### 4) Impact

Inflammatory bowel diseases (IBD) including ulcerative colitis and Crohn's disease are increasing in prevalence. A study in 2016 determined that over 3.1 million people in the United States are affected. IBD represents not only a major economic burden to the nation but also a major economic and suffering burden to patients. Within the military, IBD represents a disease with one of the highest hospitalization rates in veterans. This disease is on the rise in the general population but there has been a 2-3 fold increase in the military and the sequelae of this disease, including colorectal cancers, are on the rise. The results from these studies will define the therapeutic potential and mechanism of action of the natural human colostrum protein, lactoferrin. **Understanding the multimodal effects of hLF will provide essential preclinical data** for a novel method to modulate the microbiome and increase Treg production *in vivo* for the prevention and treatment of intestinal inflammation.

Many of the medications currently used to treat IBD broadly suppress the immune system, including Tregs, and have severe and even deadly side effects. In contrast, lactoferrin (LF), a naturally occurring, iron-binding human glycoprotein could be used to activate Tregs and reinstate the balance of the immune response in the intestine. This unique proposal is the first comprehensive evaluation of the use of human recombinant lactoferrin (rhLF) produced in rice, for the treatment of IBD. We will clarify the mechanism by which LF provides its influence on the immune response by combining immunologic, microbiome, and metabolome data. Our work will advance the fundamental understanding of methods to activate Tregs in T-cell mediated diseases. This proposal will build on multiple original observations to further define the mechanism by which LF influences the immune response.

Completion of the Aims will delineate the mechanisms by which lactoferrin ameliorates intestinal inflammation occurring in both acute infections and chronic IBD. We will specifically target the interplay between lactoferrin, gut microbiota, and Treg activity in the setting of experimental models of IBD. *The clinical- translational contribution of this work is significant* as it provides novel data regarding lactoferrin's potential as a safe, naturally occurring, easily administered, evolutionarily conserved agent to treat IBD and other inflammatory conditions. *The proposed research is innovative* as it evaluates the anti-inflammatory effects of a ubiquitous protein as a more cost effective and safe therapy than present options for inflammatory diseases. This work will establish a critical framework for developing and testing compounds that promote immunological and physiological balance within the immune system. The mechanistic insights from mouse studies will inform clinical studies of IBD, and other inflammatory conditions (e.g., enteric infections, autoimmune disease) in humans and stimulate rational development of new therapeutic strategies for its treatment by evaluating the intestinal environment, the microbiome and the immune response. If successful this work will very likely lead to evaluation of this compound first in patients with IBD, then in a wider array of inflammatory conditions.

The studies completed to date suggest that lactoferrin that is fully bound with iron is the best product to use to ameliorate inflammation in the intestine. In addition, the best method of administration appears to be oral administration. These studies together suggest that oral lactoferrin may be a viable therapy for intestinal inflammation. Additionally, it appears that the presence of bacteria in the intestine is required for the complete and direct activity of lactoferrin in the gut. Yet further studies will analyze how this gut microbiome will change in the presence of lactoferrin and whether these mechanisms are similar for treatment of enteric infections.

Through a multi-disciplinary, experimental approach, we propose to comprehensively evaluate the natural human protein lactoferrin as a novel activator of regulatory T-cells and potential therapeutic agent for human IBD and other acute and chronic inflammatory diseases. Completion of the Specific Aims will identify the mechanism(s) by which LF relieves inflammatory conditions. Our work has identified a novel means of modulating anti-inflammatory function through administration of a human protein that potentially represents the first viable, non-toxic method to activate Tregs to treat IBD. Identifying the most effective methods to administer LF and fully characterizing its anti-inflammatory mechanisms will set the stage for clinical evaluation of LF as a novel treatment regimen for IBD.

## **5) Changes and Problems**

Currently our original proposals specific aims have not changed. It appears that lactoferrin affects the development and activity of Tregs in the intestine and therefore there has been no need to alter our hypotheses or the focus of our studies. We are currently evaluating the role of CXCR4 in this response. Due to the recent construction of a new animal facility some of our work on the Salmonella Colitis model was paused but this work is now complete, and we are able to proceed with the enteric infection model and will do so immediately. This did not delay progress but diverted our attention to other components of the proposal so that there was no lost time.

The major issues that we have come up against in our work has been the hiring of a post-doctoral fellow to work on the project. As has been seen across the academic spectrum of research post-doctoral fellows are scarce. We have a new job posting out and await responses, with the hope of hiring a post-doctoral fellow by January of this coming year. Despite the lack of a complete workforce in the laboratory we are able to continue and remain on time based on the SOW.

## 6) Products

There are currently no products completed but development of the 1<sup>st</sup> manuscript is under way

## 7) Participants & Other Collaborating Organizations

Name: Edwin F. de Zoeten  
Project Role: PI  
Researcher Identifier (e.g. ORCID ID): 0000-0002-0946-9759  
Nearest person month worked: 5

Contribution to Project: Dr. de Zoeten has co-led this project and overseen the work of Tom Nguyen. He has been involved in experimental design, problem solving, writing up results and organizing support.  
Funding Support: NA

Name: Tom Nguyen B.S.  
Project Role: Professional Research Assistant  
Researcher Identifier (e.g. ORCID ID): NA  
Nearest person month worked: 9

Contribution to Project: Dr. de Zoeten has co-led this project and overseen the work of Tom Nguyen. He has been involved in experimental design, problem solving, writing up results and organizing support.  
Funding Support: NA

Name: Daniel N. Frank  
Project Role: PI  
Researcher Identifier (e.g. ORCID ID): [0000-0001-6669-228X](https://orcid.org/0000-0001-6669-228X)  
Nearest person month worked: 3  
Contribution to Project: Dr. Frank has co-led this project and overseen the work of Dr. Robertson and Ms. Kofonow.  
Funding Support: na

Name: Charles E. Robertson, PhD.  
Project Role: Research Associate  
Researcher Identifier (e.g. ORCID ID): na  
Nearest person month worked: 3  
Contribution to Project: Dr. Robertson has worked to install, test, and improve software required for metagenomic analysis of study specimens.  
Funding Support: na

Name: Jennifer Kofonow, MS  
Project Role: Professional Research Assistant  
Researcher Identifier (e.g. ORCID ID): na  
Nearest person month worked: 3  
Contribution to Project: Ms. Kofonow has established benchwork protocols and tested reagent kits for metagenomic analysis of study specimens.  
Funding Support: na

Dr de Zoeten and Dr. Frank continue to partner on this project. We have regularly scheduled meetings to discuss progress, timelines, and concerns. These meetings involve team members and focus on meeting our SOW timelines and future plans. This partnership continues to work well and there are currently no concerns.

8) Special Reporting Requirements None

9) Appendices None