Application for

Pew Research Grant

Project title:

Regulation of the thyroid hormone activator deiodinase-2 in macrophages during inflammation

Submitted by

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October 19, 2020

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End product: presentation in poster form at a conference of peers, such as the Association of Southeastern Biologists, held every March. A refereed paper is possible but could require additional data.

Research question: Is NFκB required for induction of DIO2 by LPS in macrophages?

The project plan is to use CRISPR gene editing to knock out the transcription factor NFκB in a mouse macrophage cell line, and to measure deiodinase-2 RNA expression after treatment with lipopolysaccharide (LPS). The project will be carried out by Dr. Thierfelder, working with undergraduate students. The ethics of gene editing will be deliberately discussed with these students.

The **goals** of the project are as follows:

1. To determine definitively whether the NFκB transcription factor is required for transcriptional activation of DIO2 in macrophages after stimulation with LPS
2. To establish CRISPR as a practical tool for use in mammalian cells at Union
3. To demonstrate to students and Christian observers that CRISPR can be used for ethical and morally laudable purposes in support of healthcare

Importance:

- Contributes to understanding of how the inflammatory response is regulated in cells of the immune system
- Connecting thyroid hormone (TH) regulation to inflammation is a step toward linking thyroid metabolism to diseases that involve chronic inflammation (obesity, atherosclerosis, diabetes)
- Understanding thyroid regulation is a step toward better regulating TH levels in patients with diseases involving thyroid hormone (Grave's, Hashimoto's, non-thyroidal illness syndrome)
- Potential implications for management of metabolic syndrome, cancer metastasis, and pre-term pregnancy loss
- Supports training Union students in a powerful, current technique that equips them for work in frontline biomedical research, and will help Union labs explore a wide spectrum of molecular genetic questions
- Equips students to consider the ethics of CRISPR applications from a vantage point of knowledge and experience, and to participate in decisions about biomedical ethics when they take up positions in science, medicine, government, or the church

Background and literature:

<u>Thyroid hormones, deiodinases, and inflammation</u>

Thyroid hormone (TH) functions as one of the body's primary regulators of metabolism, acting on most cell types to increase the extraction of energy from proteins, carbohydrates, and fats, and serving as a regulator of growth. The thyroid gland releases thyroxine, or T_4, a low-activity form of the hormone that circulates to the tissues, where it is converted in cells as needed to its active form, called triiodothyronine (T_3).

One of the processes that can alter the levels of thyroid hormones in tissues is inflammation. Inflammation is the body's first, rapid response to infection by bacteria, viruses, and other microbes, resulting in suppression or elimination of the pathogen, as well as stimulation of longer-term responses such as the production of antibodies. Chronic inflammation is also associated with metabolic syndrome diseases like obesity and diabetes, as well as with cancer. During inflammation, cells called macrophages recognize molecular patterns on the surfaces of invading microbes, consume those microbes, and secrete chemicals that boost the inflammatory response. Gram-negative bacteria like *Salmonella* exhibit a

characteristic molecule called lipopolysaccharide, or LPS, which binds to the macrophages. This binding in turn triggers a cascade of protein activation and gene expression inside the macrophage, resulting in a variety of actions by the macrophage that support inflammation. Current data suggest that one of those actions is the conversion of T_4 to T_3, which results in the production of energy needed to support local and systemic inflammation (Kwakkel et al., 2014). Macrophages also play critical roles in maintaining the inflammatory state in obese tissue (Herrero et al. 2010), and in controlling the growth of tumor cells (Genin et al., 2015).

The enzymes that convert T_4 to T_3 are called deiodinases, because they act by removing an iodine atom. There are two of these: deiodinase type I (Dio1) acts in the liver, while type II (Dio2) acts in other tissues. A third deiodinase, Dio3, inactivates both T_4 and T_3 in all tissues. The levels of the deiodinase enzymes in tissues are carefully regulated to ensure that active thyroid hormone is neither locally deficient (a hypothyroid condition) nor in excess (hyperthyroid). Like all proteins, the deiodinase enzymes are encoded by DNA genes (designated *Dio* in mice), which are copied (transcribed) into RNA; the genetic code in the RNA is then read (translated) to synthesize the deiodinase proteins. Gene regulation—that is, controlling the amount of protein that is expressed and active at a particular time in a cell—can in theory occur at any stage of this process. With respect to TH activation, a cell that needs more active TH would be expected to raise the level of Dio2 enzyme compared to Dio3; if less TH activity is needed, the reverse would occur.

This study focuses on the regulation of deiodinase-2, the enzyme that activates TH in most tissues. The literature suggests that Dio2 expression is regulated primarily at two levels: transcription, that is, the amount of RNA being copied from the *Dio2* gene; and protein stability, the amount of time the Dio2 enzyme is allowed to persist in the cell before it is degraded (Arrojo e Drigo and Bianco, 2011; Gereben et al., 2000). RNA synthesis is controlled in cells by proteins called transcription factors. One of the primary transcription factors activated by the binding of LPS is nuclear factor kappa B, abbreviated NFκB. NFκB is known to regulate the transcription of many pro-inflammatory genes (Sharif et al., 2007). A study by Lamirand et al. (2011) demonstrated that in astrocytes, a type of brain cell that is an important target of thyroid hormones, *Dio2* transcription was altered in response to LPS, and that the regulation depended on activation of NFκB. A subsequent study by Kwakkel et al. (2014) considered whether NFκB was also required for LPS-induced regulation of Dio2 in macrophages. These authors used a technology called gene knockdown to reduce the level of NFκB in the cells, then stimulated them with LPS. Since *Dio2* RNA production still increased in these cells upon exposure to LPS, they concluded that NFκB was not required. However, a close examination of their data shows that their experiment was only partially effective, resulting in a reduction of NFκB expression by 50%, but not in its elimination. In a genetic context, expression of a gene at 50% of its normal level can be sufficient for normal function; therefore, we contend that their conclusion was only weakly supported. We propose, instead, to completely eliminate NFκB expression in the same macrophage cell line using CRISPR technology, which has arisen since that paper was published (Zhang et al. 2013), and to determine using a technique called RT-qPCR whether *Dio2* RNA induction is negated. This should provide a definitive answer to whether LPS operates on *Dio2* via NFκB in macrophages, as it does in astrocytes, supporting the hypothesis that this is a global mechanism (as we predict it will); or if it does not, suggesting that a different biochemical pathway supports *Dio2* transcription in macrophages.

This question was addressed previously by Union student Jonathan Bowman, who received the Biology Department's research award for his work. Jonathan and Dr. Jennifer Gruenke modified macrophages to express activated NFκB constitutively, that is, even without LPS treatment. After Dr. Gruenke's retirement, Jonathan and I examined levels of Dio2 protein, rather than RNA, and his data suggested that activation of NFκB might result in permanent expression of Dio2. However, the protein experiments were difficult and the results inconclusive. Therefore, they need to be confirmed.

Should this experimental approach work as we hope for deiodinase-2, we could also use it to examine transcriptional regulation of the other two deiodinases (*Dio1* and *Dio3*), which is only partly understood (Dentice and Salvatore, 2011). *Dio3* is particularly interesting, since it is known to be essential for safe development of the fetus (Huang et al., 2005; Charalambous and Hernandez, 2013; Deng et al., 2014), and is also involved in metastasis of a uterine cancer called choriocarcinoma (Huang et al., 2017).

CRISPR

Beyond the immediate goal of understanding how *Dio2* is regulated in macrophages, this project aims to introduce CRISPR as a working technique to labs at Union. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a gene editing technology that was discovered in the mid-2000s by several scientists, two of whom were awarded the Nobel prize in chemistry for it this year (Jinek et al., 2012). Although biologists have been modifying the genomes of cells and some animals since the late 1980s, this technology, which is adapted from natural bacterial defenses against viruses, is faster, cheaper, easier, and more versatile than all preceding methods. CRISPR can be used to edit genes in almost any cell or organism and can even be introduced to breeding populations that will propagate the mutations to their offspring in the wild. It is also technically versatile: every gene in a cell or organism can be modified simultaneously, in a single experiment (multiplexing), rather than one at a time; and many different kinds of modifications can be introduced, as required for the experiment being performed (Addgene, 2020). CRISPR has already been used to modify immune system cells (T cells) taken from cancer patients, boosting their ability to attack the patient's specific tumor when re-introduced into their circulation (Miliotou and Papadopoulou, 2018).

The power of this technique is greatly accelerating the pace of discovery in biomedical laboratories. But some applications of CRISPR, especially heritable (germline) modifications of human embryos generated by in vitro fertilization, are ethically problematic for a variety of reasons, and I intend to discuss these applications with students and require them to consider them in their reports. I will comment on this further in the section below on the relationship of this project to Christian faith.

In our experiment, we will be introducing deletions of DNA bases into the NFκB gene that will render it non-functional (called a "knockout"). Cells with this mutation will not produce any NFκB at all. As explained above, this kind of mutation will definitively indicate whether NFκB is essential for LPS induction of the *Dio2* gene, as opposed to the uncertain conclusion produced by gene knockdown. It will also serve to confirm the preliminary data obtained by Jonathan Bowman. It is important to note that we are modifying a gene in a cultured mouse cell line, not in an embryo or other cell that can pass the modification on to future generations. Some of the first steps in this procedure have already been carried out in Union's molecular biology course lab and are being continued by Union student Luke Spivey. In addition, Union student Leigh Walker is beginning similar experiments targeting genes in a breast cancer cell line that may contribute to metastasis.

Christian faith in relation to the practice of biology and to this project

Faith and the practice of biology

My approach to nature and to teaching and practicing biology begins with the belief that God created the natural world for himself, and that "in him (Christ) all things hold together (Colossians 1:17)." My primary goals for students are that they see God's nature and character reflected in his creation, and that science becomes for them an inducement to worship. God intends that creation leave us in awe of him (Psalm 19:1, Job 38 and 39), and declares that everyone can see his eternal power and divine nature in it (Romans 1:20). Furthermore, God has charged us to "work" and "take care of" his creation (Genesis 2:15), and I believe he will hold us responsible for our treatment and use of it. Our attitude toward it

ought therefore to be one of stewardship, rather than either veneration or exploitation, and our use of it ought to be directed toward obeying and glorifying God, and serving others.

Although we are told to do everything, including our work, for the glory of God (1 Corinthians 10:31), it can be difficult for a Christian scientist to see how his or her labor is any different from that of an unbeliever; for all scientists are fundamentally driven by the thrill of discovery and the beauty of nature. In my experience, the difference is not in the physical or intellectual practice of science, but in acknowledgment, gratitude, and submission toward its author. Psalm 136 says that he made the heavens by his "understanding." When God allows us to comprehend something marvelous in the created order—such as the regulatory networks that are the subject of this project—it is this divine wisdom that he is permitting us to see. That is the connection I try to help students grasp, and that I try to keep in mind myself in the lab.

Of course, biomedical research generally has built into it a component of promoting health, which is an appropriate avenue of labor for Christians. Beyond that, striving for excellence, service to and love of colleagues, humility, contentment rather than greed for praise or money, faith toward God in times of discouragement or frustration, and especially in the case of modern molecular genetics, ethical use, are all appropriate ways to glorify God as a biologist. In addition, Christians should see the world of science as a true mission field. The scientific community is unusually diverse culturally, ethnically, and philosophically, which provides a built-in opportunity for international and intercultural ministry.

Finally, as a Christian scientist I am deeply dismayed by the strained relationship that has existed between evangelicals and science for more than a century. There are many reasons for this, but I sincerely hope and pray that as I interact with students in class and in the lab and with my fellow believers elsewhere, I can serve to ameliorate this state of affairs, by educating Christians about how science is really practiced and about the kinds of knowledge it can be trusted to provide; by equipping Christian students to practice science that is good both qualitatively and ethically; and by demonstrating to all that a person can be a thoughtful, competent scientist as well as a committed Christian.

Faith in relation to this project

This project serves to encourage worship in that it will provide new insight into the intricate organic networks that underlie the immune and physiological responses to infection and disease. It supports healthcare as a form of Christian service in that it addresses thyroid, inflammatory, and metabolic diseases, and cancer. It serves students by training them in rigorous, current, practical science; and it serves Union in providing an important tool for further education and exploration of what God has made.

Importantly, this project seeks to train students in the *ethical* use of a powerful new technique that has already generated worldwide controversy due to its reported use by a scientist in China to modify human embryos and implant them, producing children carrying genetic alterations that all their descendants will inherit (Wee 2019). Most scientists consider this unethical, not because embryos were modified, but because they were implanted and brought to term, propagating both the intentional mutation and any that were accidental (Reyes and Lanner, 2017). They are also worried about backlash against even legitimate uses of CRISPR (Cyranoski 2019). This project is offered at precisely the time when CRISPR is spreading across scientific labs worldwide facilitating a dramatic increase in genomic editing, most of which will be ethically uncontroversial, but some of which will not be. Dr. Jennifer Doudna, one of the Nobel winners mentioned previously, discussed the ethics of CRISPR use in embryos in her book *A Crack in Creation* (Doudna and Sternberg, 2017). Although her evaluation is thoughtful and considerate, she clearly does not believe that personhood begins at conception, and she is cautiously optimistic about its use in the future to correct diseases in *in vitro*-conceived embryos. Although some Christians have written about this development (Carter 2020), more ought to be involved in this kind of discussion where

it matters. But the only way they will be is if they are knowledgeable enough to rise to her level of influence.

Sadly, it is often the case that new developments in science appear and become entrenched before Christians are aware of them, due in part to disengagement from and suspicion of science within our community. As a result, we have little input into ethical decisions when they are being made by those in power, and when we do respond, it is often out of ignorance and fear, leading either to undiscerning rejection or uncritical acceptance of ideas and technologies. This project provides one small way to teach biology students how to use genetic engineering techniques thoughtfully and ethically, rather than either complying with the demands of their profession uncritically, or reacting to pressures from the evangelical community irrationally.

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Time frame: the specific question should be answerable by the end of 2021. Dissemination in some form may occur as early as spring of 2021, or may take longer depending on both progress in the project and on conference and seminar schedules. In support of this assertion, some preliminary work has been done in molecular biology course labs and is being continued in my work with undergraduates. Receipt of the Pew grant would allow us to finish this experiment, establish CRISPR in our labs, and employ it to answer other questions.

Budget:

<u>CRISPR knockout of NFκB</u>	
Synthetic DNAs:	$350
Competent bacteria:	$125
Plasmid isolation kit:	$100
Promega DNA cleanup kit:	$130
Enzymes:	$250
DNA markers:	$50
Plasmid Safe kit:	$162
QuickExtract DNA isolation solution:	$150
Phusion PCR kit:	$150
Surveyor kit:	$320
Electroporation cuvettes:	$175
<u>Cell culture and treatment</u>	
Media, trypsin:	$100
Plasticware:	$150
Fetal bovine serum:	$350
Puromycin:	$95

Lipopolysaccharide (LPS):	$50
Measurement of gene expression	
Trizol for RNA extraction:	$100
NEB Luna RT-qPCR kit:	$263
Stipends	
Students	$600
Dr. Thierfelder	$845
Total	$4500