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Determining Polymorphisms In Tnf Production As They Relate To Infection With Ebov

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Determining polymorphisms in TNF production as they relate to infection with EBOV

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DISCLAIMER: This is a theoretical proposal done as an intellectual exercise.

Abstract

This is a theoretical proposal done as an intellectual exercise. The purpose of this project is to practice and familiarize oneself with the process of applying for a grant from the National Institute of Health to conduct research on human subjects. This proposed study plans to sequence blood samples from infected individuals, exposed and uninfected individuals, and unexposed individuals from the 2018 outbreak in the Democratic Republic of Congo to determine the presence of polymorphisms in TNF production as they relate to Ebolavirus infection. It is hypothesized that certain polymorphisms in the regulation and production of TNF that cause a decreased production thereof have a protective effect against Ebola disease. This hypothesis is based on a synthesis of other's published work. Completion of this study will identify key polymorphic targets for developing interventions and allocating healthcare resources during a potential future Ebola epidemic in the Democratic Republic of Congo. The work proposed here will also develop experimental techniques and understanding of infectious disease immunology and genetics that together will constitute a widely-applicable platform for assessing risk during other disease outbreaks.

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Project Title:

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Project Summary/Abstract:

Ebola virus has surfaced several times since its discovery in 1976 to cause devastating outbreaks in the continent of Africa. The most recent outbreak took place in the Democratic Republic of Congo (DRC) from 2018-2020. This was the largest Ebola outbreak in the DRC, and the second largest Ebola outbreak in the world. Better methods for diagnosis and intervention are necessary to prevent future outbreaks from becoming worse. This proposed study plans to sequence blood samples from infected individuals, exposed and uninfected individuals, and unexposed individuals from the 2018 outbreak in the North Kivu province of the Democratic Republic of Congo to determine the presence of polymorphisms in TNF production as they relate to Ebolavirus infection. It is hypothesized that certain polymorphisms in the regulation and production of TNF that cause a decreased production thereof have a protective effect against severe Ebola disease. This hypothesis is based on a synthesis of other's published work. Completion of this study will identify key polymorphic targets for developing interventions and allocating healthcare resources during a potential future Ebola epidemic in the Democratic Republic of Congo or elsewhere. The work proposed here will also develop experimental techniques and understanding of infectious disease immunology and genetics that together will constitute a widely-applicable platform for assessing risk during other disease outbreaks.

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Project Narrative:

The proposed research is *relevant to public health* because it will allow physicians and healthcare workers to determine whether a patient is more or less at risk of succumbing to Ebola before infection even occurs. This could determine when and where interventions to stop disease course and spread should occur. This proposal is *relevant to the part of NIH's mission* that pertains to fostering fundamental creative discoveries and innovative research strategies as a basis for ultimately protecting health.

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Specific Aims:

This proposal's *objective* is to determine the impact that polymorphisms in TNF production have on Ebola disease progression.

Infection with Ebolavirus is characterized by an abnormal host immune response known as a cytokine storm. A cytokine storm is when vast quantities of different inflammatory cytokines are produced in a short period of time. One of these cytokines is the tumor necrosis factor, or TNF. This cytokine is responsible for signaling cells to apoptosis. Therefore, a sudden influx of TNF would result in mass cell death, leading to organ failure and death in Ebola patients. A polymorphism preventing increased TNF production would theoretically aid in preventing increased cell death and organ damage in an individual. The work we propose to conduct will determine whether this is true, as well as open new possibilities for study in TNF polymorphisms and their relation to Ebolavirus.

Our *long-term goal* is to explore genetic risk factors as they relate to Ebola disease by examining blood samples. Here, our *central hypothesis* is that certain polymorphisms in the regulation and production of TNF that cause a decreased production have a protective effect against Ebola disease. This hypothesis is based on a synthesis of other's published work. The *rationale* is that completion will identify key polymorphic targets for developing interventions and allocating healthcare resources during a potential future Ebola epidemic in the Democratic Republic of Congo. The work we propose here will also develop experimental techniques and understanding of infectious disease immunology and genetics that together will constitute a widely-applicable platform for assessing risk during other disease outbreaks.

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We will test our central hypothesis and attain our objective via the following *specific aims:*

1: To determine whether there is a polymorphism in TNF production. For this, we will use blood samples from uninfected and unexposed individuals and infect them with either activated or inactivated Ebola virus. We will also use ELISA assays to examine the levels of TNF produced upon infection. A DNA sequencer will be used to determine whether samples that produced less TNF contained a polymorphism that led to this diminished production. *Working hypothesis*: Individuals who have lower TNF production upon infection with Ebola have a polymorphism in the TNF-alpha gene.

2: To determine the role of TNF in Ebola disease progression. For this, we will use blood serums of recently infected individuals, as well as serums throughout their disease progression. ELISA assays will be used to examine the levels of TNF, and when they are varying. We will simultaneously measure the levels of cytokines related to TNF. This aim is not completely dependent on the first aim, as there may or may not be a genetic difference between samples. *Working hypothesis:* Mortality will be dependent on high levels of TNF, with low levels being protective against death from Ebola.

The *expected outcome* of this work is a comprehensive understanding of how genetic capabilities of TNF production may be a risk factor for succumbing to Ebola disease. The results will have an important *positive impact* because they lay the groundwork to develop further studies on understanding the role of TNF in Ebola.

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Research Strategy:

A) Significance:

Ebola virus was discovered in the 1970s in what is now the Democratic Republic of Congo (Feldmann et al 2011). The virus causes a hemorrhagic fever that currently has a 60% mortality rate (Feldmann et al 2011). Transmission between humans occurs through contact with bodily fluids including saliva and blood.

Research into how exactly the virus works in the body is limited, since outbreaks usually occur in remote areas where data collection is difficult. However, it is known that Ebola infection is characterized by a cytokine storm immune response in the host (Feldmann et al 2011). A cytokine storm is when different inflammatory cytokines are produced at a significantly higher rate than what is considered normal.

Infection with Ebolavirus triggers the expression of several inflammatory cytokines including interferons, interleukins 2, 6, 8, and 10, interferon-inducible protein 10, monocyte chemoattractant protein 1, TNFα, and reactive oxygen and nitrogen species (Wauquier et al, 2010). Although monocytes or macrophages seem to produce many of these cytokines, as shown in vitro, other cell types could produce inflammatory cytokines in the intact animal or person. Overall, virus-induced expression of these cytokines seems to result in an immunological imbalance that partly contributes to the progression of disease. Proinflammatory responses recorded in fatal cases of Ebola hemorrhagic fever are dysregulated, whereas early and well-regulated inflammatory responses have been associated with recovery (Wauquier et al, 2010).

Inhibition of the type I interferon response is a key feature of filovirus pathogenesis. The Ebola virus viral protein (VP) 35 functions as a type I interferon antagonist, by

blocking activation of interferon regulatory factor 3 and possibly by preventing transcription of interferon β (Wauquier et al, 2010). Additionally, other studies suggest that expression of VP24 of the Ebola virus interferes with type I interferon signaling; mutations in VP24 have been linked to adaptation of Zaire Ebola virus to produce lethal disease in mice and Guinea pigs (Feldmann et al, 1999).

Results from several studies show an important role for reactive oxygen and nitrogen species in pathogenesis of Ebola hemorrhagic fever (Hensley et al, 2002). Increased concentrations of nitric oxide in blood were reported in non-human primates experimentally infected with Zaire Ebola virus and were noted in patients infected with Zaire Ebola virus and Sudan Ebola virus. Increased blood concentrations of nitric oxide in patients were associated with mortality. Abnormal production of nitric oxide has been associated with several pathological disorders including apoptosis of bystander lymphocytes, tissue damage, and loss of vascular integrity, which might contribute to virus-induced shock. Nitric oxide is an important mediator of hypotension, and hypotension is a prominent finding in most of the viral hemorrhagic fevers including those caused by Ebola virus (Hensley et al, 2002).

Lymphocytes are depleted by apoptosis even though they are not susceptible to Ebola infection, and the apoptosis is likely to be due to impairment of dendritic cells, proapoptotic factors from activated monocytes/macrophages and direct effect of viral glycoproteins (Morikawa et al, 2007).

In summation, an impaired and ineffective host innate immune response leads to high concentrations of virus and proinflammatory mediators in the late stages of disease, which is important in the pathogenesis of hemorrhage and shock. The

prevailing data suggest that infection and activation of antigen-presenting cells is fundamental to the development of Ebola hemorrhagic fever. The release of proinflammatory cytokines, chemokines, and other mediators from antigen presenting cells, and perhaps other cells, causes impairment of the vascular and coagulation systems leading to multiorgan failure and a syndrome that in some ways resembles septic shock. The goal of this proposed study is to determine whether the genetic variation in cytokines released during the cytokine storm (TNF specifically) determines the outcome of disease.

B) Innovation:

The common approaches to studying disease progression of Ebolavirus is to infect primates, mice, or other model organisms and observe the outcome. This study will build upon what is already known by using human blood samples in two different and novel ways. First, unexposed samples will be infected with Ebolavirus to determine the difference in TNF production. Second, samples of infected individuals will be used to observe TNF and cytokine production in relation to disease progression. Our approach will allow for clear results that will either support or deny our hypotheses.

C) Approach:

Research Personnel:

This will be a joint effort between the Ebola Treatment Center at the Beni General Hospital in Beni, DRC, and the National Institute of Allergy and Infectious Disease Integrated Research Facility in Frederick, Maryland and both their staff.

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Specific Aim 1: To determine whether there is a polymorphism in TNF production.

***Introduction*:**

The *objective* of this aim is to determine what polymorphisms occur in the TNF-alpha gene and how those affect TNF production upon infection with Ebolavirus. At this point, there is no specific polymorphisms in mind, any and all will be sequenced and vetted equally against the regular TNF-alpha gene. The *working hypothesis* of this aim is that a polymorphism in the TNF-alpha gene is seen in samples that produce lower levels of TNF. Our *approach* to testing this working hypothesis will be to use ELISA assays to measure TNF levels and to use DNA sequencers to sequence the TNF-alpha genes.

***Justification and Feasibility*:**

Several TNF-alpha polymorphisms associated with TNF production have been identified. There have studies where lowered TNF production due to a polymorphism have been beneficial, such as with tuberculosis where reduced production of TNF due to polymorphisms leads to the inability of the tuberculosis to maintain its granuloma, garnering protection (Correa et al, 2005). Other studies have observed that lowered TNF promotes infection, such as with chronic hepatitis B, where low TNF production is linked to defective viral clearance (Zum et al, 1998). Most studies, however, describe TNF's relationship with autoimmune disease rather than infectious diseases. Since there is a lot unknown about TNF polymorphisms reacting to foreign infections, this proposed study will only add to the limited resources in the field.

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***Research Design*:**

This aim will be completed in three parts. The first part will be accomplished by obtaining, storing, and infecting blood samples from individuals who were uninfected and unexposed to Ebolavirus in the areas surrounding Beni. These surrounding areas will be from the Tshopo and Maniema provinces, as they were less affected by the outbreak, and are more likely to have unexposed individuals (Mbala-Kingebeni et al, 2021). The second part will be accomplished by using commercially available ELISA kits according to manufacturer's directions to measure the levels of TNF produced upon infection. The assays will be performed 5 days post infection, as this is considered late stage disease and will have been sufficient time to allow for TNF production (Hensley et al, 2002). The third part will be accomplished by using a DNA sequencer to determine whether samples that produced less TNF contained a polymorphism that led to this diminished production. All polymorphisms found will be considered and ranked against samples containing regular TNF during analysis.

***Expected Outcomes*:**

The overall outcome of this aim will be comprehensive knowledge of structure and makeup of TNF and its respective polymorphisms upon Ebola infection. This knowledge will be used to identity individuals with key polymorphisms that lower TNF production.

***Potential Pitfalls and Possible Alternatives*:**

It will be difficult to ensure samples from unexposed individuals in surrounding areas of Beni are actually unexposed. If we are unable to obtain enough samples to

conduct a thorough analysis, we will use samples from individuals in the US who have not traveled to the DRC, so as to ensure complete unexposure.

Specific Aim 2: To determine the role of TNF in Ebola disease progression.

***Introduction*:**

The *objective* of this aim is to determine how production of TNF in an Ebola-infected individual affects disease progression. The *working hypothesis* of this aim is that low levels of TNF production will be protected against death from Ebola. Conversely, our hypothesis also includes that high levels of TNF will be associated with greater mortality from Ebola disease. Our *approach* to testing this working hypothesis will be to use ELISA assays to examine levels of TNF at different intervals upon infection.

***Justification and Feasibility*:**

There is previous literature indicating that infection with Ebolavirus promotes production of TNF. Hensley et al conducted a study in 2002 where they collected sera and plasma from nonhuman primates and assayed the samples to determine levels production of several different cytokines (Hensley et al, 2002). Samples were taken pre-exposure (before they were purposefully infected), at the early stage of disease (2-3 days post infection), late stage of disease (5-6 days post infection), and the terminal stage of disease (6-7 days post infection). There were no detectable cytokines observed in the early disease stages. By day 5, there was increased IFN and TNF-alpha in 75% of the samples. Infection was lethal to all nonhuman primates infected. This indicates that there is an initial release of TNF, followed by a drop in production and then a gradual increase.

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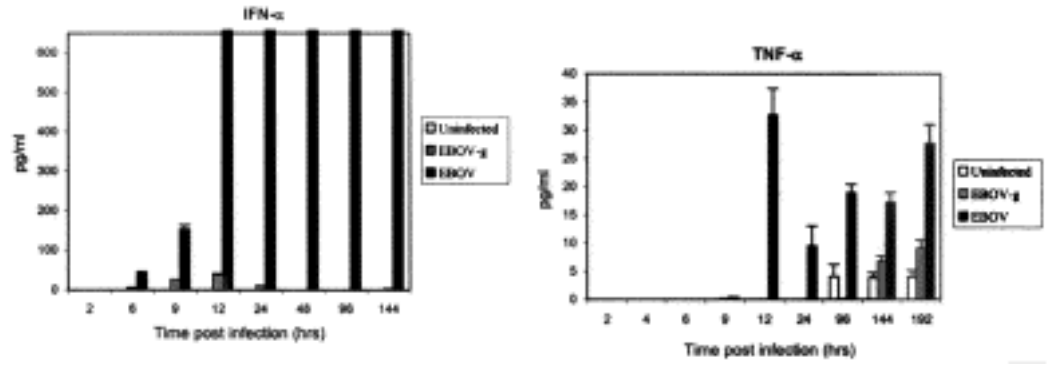

Fig. 1. Analysis of cytokine accumulation in EBOV-infected nonhuman primates. EBOV represents infectious Ebolavirus, EBOV-g represents inactivated ebolavirus. (Hensley et al, 2002)

***Research Design*:**

This aim will involve several extractions of blood samples from recently infected individuals. First, individuals who were recently infected must be identified in the Beni General Hospital. Determining how recent the infection was will be the responsibility of the Hospital staff to inform us when diagnosis occurred. The earlier the diagnosis, the more accurate our results will be. Once infected individuals are identified, blood samples will be taken at predetermined intervals throughout their disease progression. These intervals are at diagnosis, at the early stage of disease (2-3 days post infection), late stage of disease (5-6 days post infection), and the terminal stage of disease (6-7 days post infection). Each sample will be stored properly until the ELISA assay to ensure that they were not corrupted or damaged, affecting our results. Once samples are determined to be intact, they are to be assayed by using commercially available ELISA kits according to manufacturer's directions to measure TNF levels. The different levels will be observed over time, allowing us to determine any trends in TNF production upon Ebolavirus infection.

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***Expected Outcomes*:**

The overall outcome of this aim will be an understanding of the role of TNF in Ebola disease progression. This knowledge will be used to determine which individuals have a higher risk of mortality upon infection.

***Potential Pitfalls and Possible Alternatives*:**

Our working hypothesis is that the higher levels of TNF produced will increase the chance of mortality in an individual. This requires that we perform ELISA assays at predetermined checkpoints before and after infection with Ebolavirus. Missing a checkpoint or incorrectly determining that checkpoint will result in inconsistent data. It can be difficult to determine exact checkpoints since it will be difficult to determine when exactly individuals were infected. To ameliorate this potential mishap, we will ensure that there are multiple reminders put in place to remind the staff of when to take measurements, as well as alerting staff at the Beni Treatment Center to record exactly when infection was diagnosed.

Use of Human Subjects:

This study involves the analysis of human blood samples to be collected at the Beni Ebola Treatment Center and surrounding areas. Although usually painless, there is a small risk of a cut to the skin from the needle used for extraction, or lightheadedness from extracting too much blood. The Beni General Hospital (where the Ebola Treatment Center is found) has been collecting blood samples since its inception, and therefore the staff is well trained in how to obtain blood samples with minimal to no injury to the individual.

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