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Characterizing T Cell Phenotype In Patients With Hypersensitivity Reactions To Sulfamethoxazole And Beta-Lactam Antibiotics

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Abstract

Delayed drug hypersensitivity reactions (DHRs) are idiosyncratic, T-cell mediated, and can present days after exposure to the culprit drug, resulting in varying degrees of skin rashes. We hypothesize that differences in activated peripheral T cell subsets and types of mediators released produce different clinical phenotypes of drug hypersensitivity reactions to sulphnamides and beta-lactam antibiotics.

We recruited participants with previous DHRs to sulfamethoxazole or beta-lactams . Peripheral blood mononuclear cells were isolated from participants. T-cell subset proliferation and activation was assessed by T-cell specific surface markers using ^{3}H-thymidine incorporation and flow cytometry, and secreted cytokines were measured using bead-based detection.

There is insufficient evidence to conclude which T-cell subtypes are involved in different DHR clinical presentations. There were no significant differences between DHR participants and controls. More participants should be recruited to increase study power and range of clinical presentations, and consider alternate methods of identifying T-cells and modulators of interest.

Keywords

Drug Hypersensitivity, T cell, sulfamethoxazole, beta-lactam antibiotics

Summary for Lay Audience

Delayed drug hypersensitivity reactions (DHRs) are reactions that are somehow caused by the immune system interacting with the drug. DHRs typically occur days after taking the drug, usually result in a skin rash, and are mediated by T cells. It is still unclear how the T cells cause DHRs, however there are several theories. Two commonly prescribed drugs that cause these reactions are sulfamethoxazole and beta-lactam antibiotics. We believe that reactions mediated by different types of T cells in the blood lead to the various types of skin rashes caused by DHRs.

We recruited participants who had previously visited Dr. Rieder's drug allergy clinic and had previously tested positive for a DHR to either sulfamethoxazole or beta-lactams. We took a sample of blood from each participant, isolated a group of blood cells from the samples called "peripheral blood mononuclear cells" (PBMCs), which contains T cells. We tested these cells in different ways to see how the cells were responding to the drug. We used a technique called scintillation counting to determine if the PBMCs were growing when incubated with the drug, flow cytometry to see which type of T cell was activated in response to the drug, and Luminex to determine which molecules (cytokines) the PBMCs produced after incubation.

We have insufficient evidence to conclude which type of T cell is involved in each skin reaction. Overall, we found no differences between T cell subset activation in response to exposure to the drug treatment, while other studies have previously found differences. Changes in the methods, including increasing number of T cells used in the analysis and expanding the number of T cell subsets analyzed by flow cytometry, and increasing the cytokines analyzed by Luminex, could improve results. We only recruited eight participants, to increase the strength of the conclusions we will need to increase the number of participants. We conclude that some changes in methods could improve and produce more reliable results.

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Chapter 1

1 Introduction

Drugs and medications are an important part of care for many patients, including some of our population's most vulnerable and sick. However, while many individuals can take a drug and benefit from its therapeutic effects, some can experience harmful and potentially fatal reactions. Adverse drug reactions cause a number of problems, which in addition to the effects on the health of the patient can include unexpected effects due to suspected risk of adverse drug reactions including mis-labeling, use of less effective medications and overuse of other types of drugs such as antibiotics leading to resistance.

1.1 Background Information

1.1.1 Definitions

There have been many definitions suggested for adverse drug reactions (ADR) since the World Health Organization (WHO) published their definition in 1972. Specifically, a drug reaction is classified as adverse when, taken at a standard dose, it causes a noxious and unintended reaction.[1] The WHO also defines a drug as a substance or product whose use can benefit the recipient either physiologically or psychologically.[1]

An adverse drug event is an undesired outcome that occurs while a patient is taking a drug, but the adverse outcome is not necessarily attributable to the drug, for example an overdose. This is different from an adverse drug effect, which is an adverse outcome that can be attributed to some action of the drug the patient took, an example being a side effect.[2]

The term "drug allergy", however, is often used out of context to describe conditions that are not mechanistic allergies.[3] This unhelpful labeling has prompted Johansson et al. to suggest revised nomenclature for mechanistic discussions on ADRs.[4] They recommend the term 'hypersensitivity' be used to describe reproducible signs or symptoms due to exposure to a stimulus at a dose that is normally tolerated. They also recommend the term 'allergy' be used only to describe a hypersensitivity reaction that has a specific and

defined immunologic mechanism. When there are other mechanisms that are not immune-mediated, then non-allergic hypersensitivity can be used.

1.1.2 Prevalence

Adverse drug reactions are a burden on the individual and expensive to the health care system. In 1994 it was estimated that in the United States 106 000 patients died of 2,216,000 hospitalized with ADRs as a result of a drug injury.[5] ADRs are responsible for approximately 5% of all hospital admissions and affected 10-20% of all hospital inpatients. In addition to the burden placed on physicians and the healthcare system, ADRs increase the cost of an individual's care and negatively affect their quality of life.[6–10]

1.1.3 Economic Impact

Of all ADRs, approximately 20% are drug hypersensitivity reactions (DHRs).[11,12] Many people in the USA (20-35%) report having at least one prior reaction to medication.[11,13,14] However, not all of these reported reactions are true ADRs: only 1-5% of people with a reported drug allergy actually have one.[11,15] This overreporting can compromise patient care, including the prescribing of less effective treatment options and potentially increasing the cost of care. For example, using alternative antibiotics has been associated with increased treatment failures and higher rates of nosocomial infections (eg. Methicillin resistant *Staphylococcus aureus*, MRSA).[11,16,17] People who report penicillin allergy also spend longer times in hospital than non-allergic patients,[11,18] and have higher costs associated with their prescription medications.[11,19–22] Treating ADRs is expensive, potentially costing thousands of dollars to treat.[11,23] Based on a report from Canada, performing graded oral challenges in children before prescribing antibiotics based on reported allergy is able to save money.[11,24]

1.1.4 Difficulties and Considerations in Diagnosing ADRs

Accurate, reliable, and safe tests are important for both predicting and confirming ADRs. Currently, the risks for many ADRs are overestimated, particularly with reactions to penicillin. In a recent survey,[25] approximately 65% of surveyed patients responded they

would not continue to use a drug even if told by their physician that the reaction was not caused by the drug in question. A penicillin allergy is the most common drug allergy charted during hospital admissions and has been linked to increased time in hospital; increased reliance on broad-spectrum antibiotics; and increased prevalence of *Clostridium difficile*, MRSA, and vancomycin-resistant *Enterococcus* infections.[26] There are consequences of overdiagnosis of drug allergy, including an increase in drug-resistant infections.[3,18,27] However, there is no consensus on the extent of over-diagnosis. Drug allergies are often self-reported, and the frequency can be as high as 39%.[3,28] Sousa-Pinto et al., after completing a meta-analysis, found that 8.3% of their participants had self-reported a drug allergy.[3]

In addition to overcoming personal conviction, accurate diagnoses are also important, as inaccurate diagnoses prevent patients from being treated with potentially beneficial drugs. However, the opposite of this is also true. If a patient is told they are not sensitive to a drug and is then treated with the drug, they could be subjected to a potentially fatal and avoidable reaction.[29,30] Adverse drug reactions can also present similarly to other diseases, so it can be difficult to discriminate between the illnesses.[30] Some drug-induced rashes can be indistinguishable from rashes produced by other agents, such as viruses.[31]

ADRs and DHRs typically affect more children and women than men, for reasons that can either be physiological or sociological. Women are prescribed more antibiotics than men. In a meta-analysis across nine high-income countries, women in general across all ages are 27% more likely to receive an antibiotic, and up to 40% more likely in the 16-54 age group.[32] In the USA specifically, women are prescribed more antibiotics for respiratory tract infections (RTIs) than men are.[32,33] In England in 2019, an analysis by Smith et al. found that adult women receive almost twice as many antibiotic prescriptions than men, while elderly women specifically receive 67.4% more.[34] They also note some drugs are simply more commonly prescribed to women than men, however there is still a disparity of 70% more prescriptions when urinary tract infections (UTIs) are removed from the analysis. In addition to being prescribed more antibiotics alone, women are more likely to consult with their general practitioner (GP) more than men. In the USA, men visit their GP less, but also exhibit more risk-taking and health-compromising

behaviours and conditions than women do, including obesity, smoking, and drinking.[32] Smith et al. describe women seeking primary care 80% more than men across the 12 conditions they examined.[34]

In addition to utilization differences in prescribing, there are potentially pharmacological differences in women compared to men that would lead to an increased incidence of ADRs. For example, Rademaker demonstrated that cytochrome P450 (CYP45) 3A4 is higher in women by up to 40%, which they attribute to different steroid hormones affecting CYP3A4 activity due to competitive inhibition.[35,36] The author suggests that metabolites generated due to drug metabolization by CYP3A4 might be the cause of the ADRs. Rademaker also noted the differences in conjugation of drugs between men and women – specifically referencing temazepam, which is conjugated and cleared faster in men than women.[35,37] Kando observed that women have higher concentrations of drug in plasma than men and receive more drug due to a smaller body mass.[38] In addition, Kando reviewed how hormones can also have an influence on ADRs affecting more women.[38] Different levels of estrogen, progesterone, and testosterone may affect enzyme activity throughout the menstrual cycle, which can affect the metabolization of drugs.[38]

1.2 Classification of ADRs

The most common classification system for ADRs divides ADRs into two main types: Type A and Type B. Type A reactions, the most common, are predictable since the reaction is related to the known pharmacological action of the drug. These reactions are dose-dependent and essentially exhibit amplified effects of the drug. By reducing the dose or eliminating the drug from the patient's therapy, the reaction can often be reversed. Type B reactions, however, are generally more unpredictable due to the potential for delayed onset of signs and symptoms. These often do not exhibit a dose-dependent relationship and the effects are not usually related to the known drug pharmacology.[39] Type B, or "idiosyncratic adverse drug reactions," are further subdivided into two groups: immune-mediated, or hypersensitivity reactions, and non-immune mediated reactions. A breakdown of this classification is outlined in Figure 1-1.

1.2.1 Non-Immune Mediated Reactions

Pseudoallergy is another term for a reaction that is clinically a hypersensitivity but is not immune-mediated. It is classified here because these reactions imitate other reactions and can range from being mild to causing anaphylaxis.[40] While pseudoallergy can be similar to anaphylaxis, unlike anaphylaxis, pseudoallergy results from mast cells and basophils releasing mediators. This process is not mediated by immunoglobulin E (IgE).[41,42] This leads to histamine release, complement activation, among others.[41,43] One example of this is vancomycin-induced redman syndrome. Mast cells and basophils degranulate, releasing histamine, in a manner which is not IgE-dependent,[44] resulting in a flushed face, neck, and ears, and is often accompanied by itching.[45]

1.2.2 Immune-Mediated Reactions

These reactions have been further divided into subtypes. These were originally outlined by Gell and Coombs[46] and have been modified slightly by the addition of further subclassifications.

Type I

Type I reactions are mediated by IgE, bound to mast cells with Fc-IgE receptors. The mast cells release histamine and cause an immediate reaction, including urticaria and fatal anaphylaxis. The reaction can start within seconds of a parentally applied drug, or within minutes of oral intake. Most of these reactions result in pruritis or urticaria, however they can escalate to life-threatening reactions such as anaphylaxis.[40]

Type II

Type II reactions involve the formation of complement-fixing IgG antibodies and occasionally IgM that interact with Fc-IgG receptors on macrophages, natural killer (NK) cells, granulocytes, etc. In these reactions, immune complex activation occurs on the cell surface of erythrocytes, leukocytes, platelets, hematopoietic precursor cells, etc.[40] There are several ways in which this can occur, including opsonization of cells by activated complement components leading to phagocytosis, antibody deposition leading to macrophage and neutrophil recruitment, and anti-body-dependent cell-mediated

cytotoxicity due to eosinophil degranulation.[47] Pichler proposes that, specifically to drugs, the drug can be adsorbed to the membrane, creating a new antigenic complex with the cell membrane.[40] Hemolytic anemia and thrombocytopenia have been listed as side effects to various drugs, including penicillin, cephalosporins, quinidine for the former, and quinine, quinidine, and sulphonamides for the latter.[40]

Type III

In Type III reactions, immune complexes are formed, which is a common event in a normal immune response and does not typically result in symptoms. Efficiency of treatment can decrease if immune complexes are formed by drug-protein interactions. It is not fully understood why an immune complex disease forms, and several reasons can be speculated as to why these occur. An inability to remove complexes can result in inappropriate deposition, leading to recruitment of other immune cells, leading to hypersensitivity, small vessel vasculitis and/or serum sickness.[40]

Type IV

Type IV are T cell mediated hypersensitivity reactions. Originally, Gell and Coombs did not subclassify these reactions, however with new knowledge of the functions of T cells and the types of inflammation they cause, these reactions have been further subclassified from Type IVa to Type IVd.[40]

Type IVa: These are T_h1-type immune reactions, involving the activation of macrophages by IFN-γ and complement-fixing antibody production. This can possibly lead to co-activation of $CD8^+$ T cells, leading to a combination of Type IVa and IVc reactions.[40]

Type IVb: These are T_h2-type immune reactions, with cytokines IL-4, -13, and -5 which promote mast cell and eosinophil responses.[40]

Type IVc: T cells can migrate to the tissue and kill different cells with cytotoxic molecules such as perforin and granzyme B. Cytotoxic T cells ($CD8^+$ T cells) are involved in maculopapular or bullous skin diseases, acute generalized exanthematous pustulosis (AGEP), and contact dermatitis.[40]

Type IVd: This reaction involves T cells coordinating neutrophilic inflammations in the skin, such as in AGEP.[40]

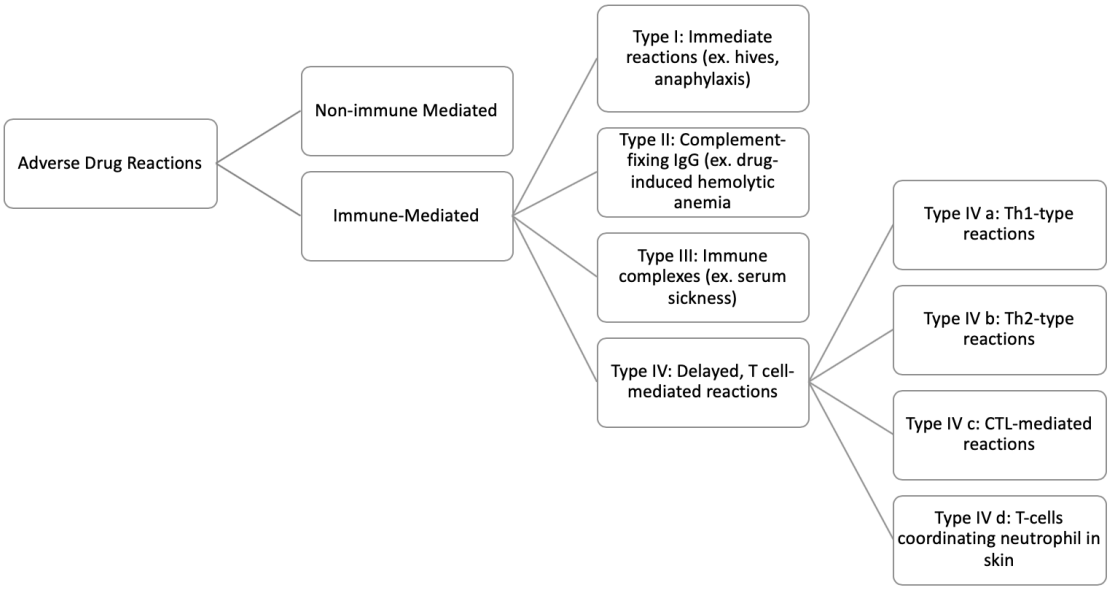


Figure 1-1: Schematic of ADR classification

1.3 Pathophysiology of DHRs

There are several competing hypotheses that attempt to explain the mechanism that drives T cell-mediated drug hypersensitivity reactions. Not all drug reactions support a singular hypothesis, which led to the development of several hypotheses. These hypotheses are modeled in Figure 1-2.

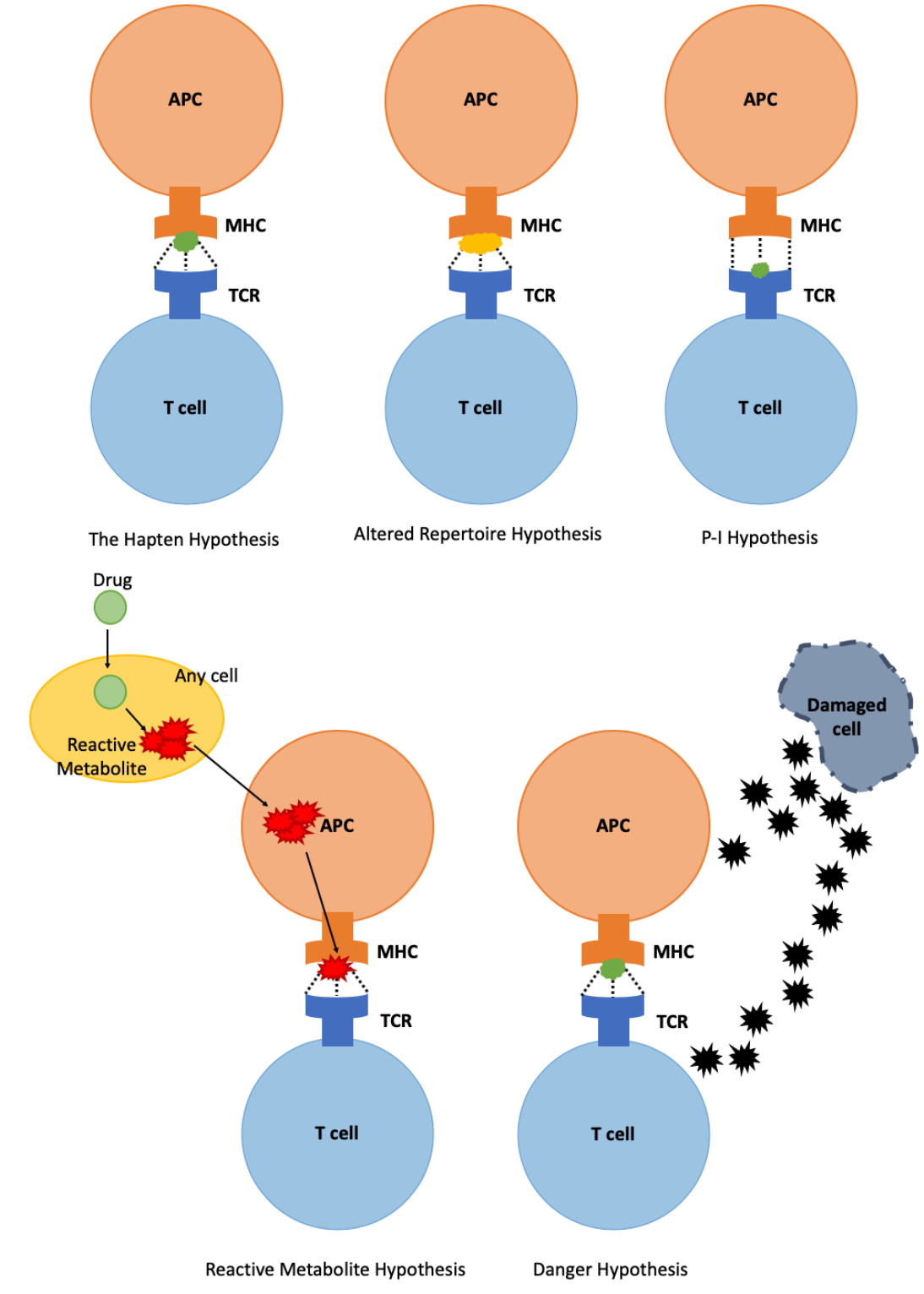


Figure 1-1: Schematic of proposed hypotheses of DHR mechanisms

1.3.1 The Hapten Hypothesis

The hapten/pro-hapten mechanism describes how small, low-molecular weight compounds (<1000 D) are able to elicit an immune response.[40,48–51] The concept of a "hapten" driving an immune response was first published around 1935 by Landsteiner and Jacobs. They theorized that small molecules themselves were not immunogenic, but became immunogenic once bound to a protein.[52] Subsequently the hapten hypothesis was further elucidated. The hypothesis is essentially that a hapten binds to a carrier molecule such as a protein and the modified hapten-carrier molecule can then generate an immune response. Similarly, pro-haptens, which are not chemically reactive, are first metabolized in the liver where they can then bind to a peptide.[40,48,53–55] Beta-lactam antibiotic allergy was felt to be an example of a drug reaction that followed the hapten mechanism. This process occurs due to nucleophilic attack on the drug molecule, opening the beta-lactam ring, resulting in a penicilloyl-protein adduct, which can cause an immune response.[56,57] These drugs often bind to albumin as its carrier protein,[58] since it is the most abundant protein in serum.[57]

1.3.2 The P-I Hypothesis

A competing hypothesis is the p-i (direct pharmacological interaction of drugs with immune receptors) hypothesis, that speculates that chemically inert drugs can activate T cells via specific T cell receptors (TCRs) that interact with the specific drugs.[40,48,49,59] This hypothesis postulates that the drug-TCR interaction is independent from metabolism and processing. The drug binds to the TCR directly, and the major histocompatibility complex (MHC) is not covalently modified. However, MHC binding is still required for full T cell activation.[60] The p-i hypothesis was developed in response to the hapten/prohapten hypothesis stating that a low molecular weight drug must bind to a protein to initiate an immune response, and contradicts the necessity of protein binding. Specifically, Pichler considers how some chemically inert drugs, which cannot become haptenized in the skin, result in positive skin tests.[59–62]

1.3.3 The Danger Hypothesis

The danger hypothesis was proposed as an alternative to the self/non-self concept of immune reactions. Injured cells send danger or alarm signals, which activate antigen-presenting cells (APCs).[63] In this hypothesis, the immune system is triggered by damage signals instead of recognizing non-self antigens.[63–65] It is suggested that the drug or its reactive metabolite must also cause cell damage for a drug reaction to occur.[66] One possibility is that during drug metabolization, accumulation of reactive metabolites can lead to cell death, which generates danger signals.[67] It has been suggested that there are other factors that can act as a danger signal that would increase a patient's chance of having a drug reaction. Administering a patient with mononucleosis ampicillin will often develop a skin rash.[66,68] An example is the 50% chance of developing a drug reaction when sulfamethoxazole administered to patients with AIDS versus 0.5% in uninfected patients.[66,69]

1.3.4 The Altered Repertoire Hypothesis

The altered repertoire hypothesis describes how a drug can interact with human leukocyte antigen (HLA) class I molecules, leading to the presentation of altered peptides that can cause an immune reaction.[70] There has recently been increasing support for this hypothesis. As an example, abacavir is able to activate $CD8^+$ cells in an HLA-B*57:01-restricted manner, leading to a hypersensitivity reaction.[71] After deducing that other hypotheses could not support this observation, Ostrov et al. found that abacavir can bind to the peptide binding groove of HLA-B*57:01 allowing for the presentation of novel peptides that appear as foreign, triggering a response from $CD8^+$ cells.[72]

1.3.5 Reactive Metabolite Hypothesis

The reactive metabolite hypothesis suggests that reactive metabolites of a drug are responsible for drug reactions, instead of the parent drug.[73] This can either be through drug metabolism or if the drug damages a cell and produces danger signals.[73] The reactive metabolite of many drugs is responsible for its activity, and this typically occurs during metabolization by enzymes in cells such as hepatocytes.[74,75] Drug metabolites can accumulate, resulting in an endogenous molecule that can then be taken up by antigen

presenting cells and presented to T cells.[74] One proposed example is the metabolic pathway of sulfamethoxazole, which leads to reactive metabolites.[73] This hypothesis has been linked to the hapten hypothesis, in that reactive metabolite generation may be the initial event triggering an immune response by the creation of a reactive metabolite that, when complexed with a cellular macromolecule, acts as a hapten to generate an immune response.[76]

1.4 Immune-Mediation of DHRs

The innate immune system ("pro-inflammatory" immune response) is responsible for rapid recognition of an antigen. This system consists of neutrophils, macrophages, monocytes, NK cells, dendritic cells (DCs), among others, which direct and inform the adaptive immune system.[77]

The adaptive immune system is responsible for both specific and memory responses, and is composed of B cells, αβ T cells, and γδ T cells. Upon recognizing or being presented with an antigen, these cells expand to perform specific functions: B cells become plasma cells, which make large quantities of immunoglobulins, and T cells (specifically αβ) either directly kill cells or recruit other cell types. While most of these cells die after the culmination of the immune response, some – such as memory T cells – continue to live for many years in order to protect against reinfection after initial contact.[77]

1.4.1 Composition of PBMCs

Peripheral blood mononuclear cells (PBMC) are a cellular population that can be isolated from peripheral blood, and essentially consist of any blood cell featuring a single round nucleus, which include lymphocytes, monocytes, NK cells, and dendritic cells (DCs).[78] These cells are separated from red blood cells, granulocytes (neutrophils, basophils, and eosinophils) by density gradient separation, whereas PBMCs are located in the low-density layer (ie. less dense than 1.077 g/ml). Although there is some variability across individuals, PBMCs are typically composed of approximately 70-90% lymphocytes, 10-20% monocytes, and 1-2% DCs. Of the lymphocytes, approximately 70-80% are $CD3^+$ T cells, 5-10% B cells, and 5-20% NK cells. Typically within $CD3^+$ lymphocytes, $CD4^+$ and $CD8^+$ cells are represented in a 2:1 ratio, respectively.[78–82]

1.4.2 Antigen-Presenting Cells

Antigen-presenting cells (APCs) are cells at the overlap between the innate and acquired immune systems. DCs, B cells, and macrophages are all considered to be professional APCs. What distinguishes these APCs from other cells is the presence of abundant MHC class II receptors. Specifically, DCs and macrophages can detect and phagocytose substances (*e.g.* pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs)), parts of apoptotic cells, process antigen, and move to specific tissues (*e.g.* T cell zone of lymph node). B cells acquire antigen through the B cell receptor (BCR).[83]

Some other cells, such as mast cells, basophils, eosinophils, neutrophils, innate lymphoid cells (ILCs), and $CD4^+$ T cells have also been found to be able to induce MHC class II expression. However, this can only occur in certain immune environments and has not been researched extensively to conclude their role.[83]

1.4.3 Dendritic Cells

DCs are antigen-presenting cells (APCs) that modulate the immune response by stimulating B cells and T cells.[84,85] These cells are located in most tissues throughout the body. They are stellate and have a multiplicity of MHC molecules on their surface.[84,86] There are two stages in the life of a DC: the immature and the mature stage. As an immature DC, these cells have few MHC molecules, capture antigens (with Fcγ and Fcε), but they cannot generate the appropriate stimulatory molecules for T cells (CD40, CD54, CD86). Immature DCs are able to phagocytose particles,[84,87–90] sample antigens in phagocytic vesicles,[84,91] and then express the appropriate receptors to stimulate T cells. Mature DCs, however, are terminally differentiated, and display many MHC class II molecules bound to antigen.[84,92] They upregulate costimulatory molecules, then move to secondary lymphoid tissues to interact with T cells.

1.4.4 B Cells

B cells are “bursal” or “bone marrow” derived cells that date back to the origin of adaptive immunity in jawed vertebrates more than 500 million years ago.[93,94] B cells

originate in the primary lymphoid tissue, which in humans is the bone marrow. In general, they undergo functional maturation in the spleen or lymph nodes, which are the secondary lymphoid organs, and then finally produce antibodies as plasma cells. These cells undergo functional rearrangement of Ig loci in the bone marrow: V, D, and J sequences at the H chain locus and V and J at the L chain loci.[93,95] These allow for a diverse repertoire of B cell receptors in the B cell population.

1.4.5 T Cells

T cells have been previously shown to be involved with DHRs and it is believed that they are the central driver for common and important DHRs.[53,54,96–104] However, the role(s) that different T cell subtypes and their mediators may have in the pathogenesis of Type IV DHRs, if any, have not been fully explored, nor has the impact of different T cells responses to clinical phenotypes of DHRs.

1.4.5.1 Helper T Cells

T helper cells (Th) are involved in the coordination of the adaptive immune response, primarily by the secretion of specific cytokines to recruit other cells toward infected areas. These cells are characterized by $CD4^+$.[105] Specific cytokine environments and master regulators are responsible for the many subtypes of T_h cells that have been discovered.

T_h1 Cells

T helper 1 (T_h1) cells produce the cytokine interferon-γ (IFN-γ). They function to eliminate intracellular pathogens and are involved in two types of hypersensitivity reactions: cell-mediated and delayed.[106,107] Additionally, T_h1 cells are involved in the process of producing opsonins, complement-fixing antibodies, and can lead to macrophage activation.[108]

Both IFN-γ and interleukin-12 (IL-12) are the cytokines in a naïve T cell's environment that promote differentiation to T_h1 subtype. IFN-γ inhibits T_h2 cell differentiation while IL-12 promotes T_h1 growth and does not affect T_h2 cells.[109–112] T-bet is the transcription factor that controls the expression of IFN-γ and is imperative for T_h1 cell differentiation

from naïve $CD4^+$ T cells. T-bet both activates the IFN-γ gene as well as induces exogenous production of IFN-γ.[113] T_h1 cells are found to be involved in organ-specific immune disorders, acute allograft rejection, contact dermatitis, among other immune disorders.[108]

IFN-γ

T_h1 cells are defined by their secretion of IFN- γ, although many cells have been found to secrete this cytokine, including B cells, natural killer T cells (NKT), and professional APCs[114–118] in addition to T_h1, $CD8^+$ T cells, and NK cells.[114,119,120] Multiple studies have found that IFN-y is secreted by PBMCs isolated from study participants with a history of delayed type drug hypersensitivity. IFN-γ is secreted when drug-specific PBMCs are incubated with sulfamethoxazole (SMX)[121] and piperacillin.[104] In addition, Beeler et al. found that PBMCs from AGEP and severe exanthema patients secreted both IFN-γ and interleukin-13 (IL-13).[122]

T_h2 Cells

Another subset of helper T cells are T helper 2 (T_h2) cells. These are associated with extracellular (helminth) responses and stimulating damaged tissue repair. These secrete IL-4, -5, -6, -9, -10, and -13.[106–108]

The cytokine IL-4 promotes the differentiation of a naïve $CD4^+$ T cell to a T_h2 cell.[109,123,124] During T_h2 development, the transcription factor GATA-3 is upregulated. Conversely, it is downregulated during T_h1 development.[125–127]

T_h2 cells are mediators in a variety of different disorders such as Omenn's syndrome, reduced protection against extracellular pathogens, and chronic Graft vs. Host Disease.[108]

IL-4

IL-4 is a potent regulator of immunity,[128] regulating immune functions such as Ig isotype class switching, B cell MHC class II expression, and differentiating certain T_h cell lineages.[129] IL-4 is also secreted by mast cells, eosinophils, and basophils in addition to T_h2 cells.[128] It was first described by Howard and Paul as a comitogen of B cells.[128,130]

IL-4 binds to IL-4R alpha. It can either produce type I signaling when also bound to gamma chain or type II when bound to IL-13Rα1.[128,131,132] This Type I signaling is important for the polarization of naïve $CD4^+$ T cells to T_h2 cells.[128]

IL-13

IL-13 is a cytokine secreted by T_h2 cells.[106–108] It is able to induce the expression of MHC class II in human B cells and phenotypic changes in human monocytes. It also acts on human B cells by inducing proliferation and immunoglobulin production.[133–136] In general, IL-13 has anti-inflammatory properties and plays a regulatory role in the immune response.

IL-13 is secreted by T cells when drug-specific PBMCs are stimulated with either ampicillin (AMP) or SMX.[137] It is also secreted by PBMCs from piperacillin-hypersensitive patients when incubated with the culprit drug.[104] Beeler et al. noted that PBMCs of patients with severe exanthema and AGEP secreted IL-13 as well as IFN-γ.[122]

T_h9 Cells

T_h9 cells are generated by naïve $CD4^+$ cells exposed to TGF-ß and IL-4, and are characterized by their production of IL-9.[138] In contrast, exposing naïve $CD4^+$ cells to IL-4 only leads to the differentiation of T_h2 cells,[138] and $CD4^+$ cells to TGF-ß only leads to induced regulatory T cells (T_{reg}).[138–141] While T_h2 cells also produce IL-9, T_h9 cells produce IL-9 in much greater quantities and have a different role in the immune response than T_h2 cells.[142] These cells also require transcription factors STAT6, PU.1, IRF4, and GATA3.[138,143–146] Although T_h9 cells require such a specific environment to differentiate, they do exist *in vivo*, and can be found in the peripheral blood of allergic patients[147] among CLA^+ (cutaneous leukocyte antigen positive) cells in the blood and skin.[148,149]

IL-9

Many cells are able to produce IL-9, including T_h9 cells, innate lymphoid cells, mast cells, and neutrophils.[138,142,150–153] However, T_h9 cells produce IL-9 in a greater quantity than T_h2.[144,146] When T cells are activated by either PMA (phorbol myristate acetate) or

anti-CD3, IL-9 expression can be induced,[154] with the addition of IL-2 further upregulating production.[155] IL-9 promotes the survival of other cells, such as Th cells, mast cells, among others.[156–158] IL-9 is also able to expand T_h17 and T_{reg} cells.[138,159–162]

IL-9/T_h9 cells have specifically been implicated in allergy and asthma. While IL-9 levels are increased in asthmatic patients,[163] it is unknown if it is involved in other instances of allergy, such as anaphylaxis and atopic dermatitis.

T_h17 Cells

T helper 17 (T_h17) cells are a subset of T_h cells that predominantly secrete IL-17. T_h17 cells were the third T_h subset discovered[164–166] and target extracellular bacteria and fungi. Interestingly, IL-17 was discovered before T_h17 cells.[167–169] When human naïve T cells are exposed to IL-1B and IL-6, T_h17 cells are differentiated.[139,170,171] The transcription factor important in the differentiation of T_h17 cells is orphan nuclear receptor RORγt.[172] IL-17 does not inhibit T_h1 or T_h2 cells,[173] but IL-12, IFN-γ, and IL-4 inhibit T_h17 cells.[165,166,174–177]

While T_h17 cells are characterized by IL-17 production, they also produce IL-17F, IL-22, and some TNF-α (tumour necrosis factor α), but IL-17A is predominant.[164] T_h17 cells produce TNF-α, IL-21, and IL-22, while it is debated whether they produce IL-6.[106,171,178–180]

Both Th17 cells and IL-17 have been implicated in several human diseases, including psoriasis, rheumatoid arthritis, systemic lupus erythematosus, endometriosis, asthma, irritable bowel syndrome, multiple sclerosis, atopic dermatitis, contact hypersensitivity, among others. In atopic dermatitis specifically, acute atopic dermatitis is associated with IL-17, but not chronically.[164,181]

IL-17

T_h17 cells are a major producer of IL-17.[106] IL-17 includes IL-17A, B, D, E, and F.[182] The functions of IL-17A and F include targeting fibroblasts, endothelial and epithelial cells, keratinocytes, macrophages, and stimulating and attracting neutrophils to

inflammation.[178,183] IL-17 is important in extracellular bacteria defense[171] and in the mobilization of neutrophils.[169,171]

Interestingly, a previous study has shown that when PBMCs isolated from piperacillin-hypersensitive patients are incubated with piperacillin, no IL-17 is produced. However, it is produced when incubated with PHA (phytohemagglutinin).[104]

T_h22 Cells

T_h22 cells are a terminally differentiated subtype of helper T cells that secrete IL-22. The cytokine environment required for the differentiation of naïve $CD4^+$ T cells to T_h22 cells is TNF-ß (tumour necrosis factor ß) and IL-6.[184,185] The master regulator of T_h22 cells is aryl hydrocarbon receptor.[185–187]

T_h22 cells were discovered due to their secretion of IL-22, but no signature cytokines of other T cell subtypes, such as IFN-γ, IL-4, and IL-17.[184,185,187,188] However they do secrete small amounts of IL-13 and TNF-ß.[184,188]

IL-22

IL-22 is predominantly secreted by T_h22 cells, but is also secreted in small amounts by T_h1, T_h2, and T_h17 cells[184] and is a member of the IL-10 family of cytokines.[186] IL-22 specifically binds to the IL-22 receptor (IL-22R), a heterodimeric receptor composed of IL-10Rß and IL-22Rα chains, which is abundant on epithelial cells.[184,189] Specifically, IL-22R is expressed in skin, liver, pancreas, intestine, lung, and kidney.[186,190–192] IL-22 is also involved in wound healing.[184,188,193]

IL-22 is implicated in some skin rashes, which can be a symptom of a DHR. For example, IL-22 is upregulated in psoriasis lesions.[186,194–196] IL-22 is also found in greater amounts in atopic[186,197] and contact dermatitis lesions.[186,198]

Regulatory T cells (T_{reg})

The immune system has the dual role of simultaneously protecting the host from pathogens or tumours while also preventing harm from excessive and harmful responses.

If an immune response is misdirected, it can produce an autoimmune disease.[79] Regulatory cells that act to prevent this are characterized as $CD4^+CD25^+FoxP3^+$.[139] T_{reg} cells are a dominant mechanism for preventing excessive response and are specialized for immune suppression. Autoimmune and inflammatory responses can be caused by disruptions in the development or function of T_{regs}. When an adaptive immune response is occurring, both effector cells and T_{regs} are activated and recruited to the site of inflammation to maintain a balanced immune response, in both the quality and magnitude of the adaptive immune reaction.[79]

Previous work has demonstrated the importance of T_{regs} are in controlling the immune response. Manipulating the thymus, as for example a thymectomy in a newborn, results in autoimmune damage and compromised immunity.[79] In addition, removing autoimmune-suppressive T cells from an organism can lead to autoimmune disease, while adding them back inhibits autoimmunity.[199]

Regulatory T cells have many notable functions, but have important roles in inhibiting autoimmunity and protecting against tissue injury.[139,200] The cytokine TGF-ß is important in differentiating naïve $CD4^+$ T cells into T_{reg} cells.[139,201]

1.4.5.2 Cytotoxic T Cells

Broadly, the role of a cytotoxic T lymphocyte (CTL) is to survey all nucleated body cells and destroy any that are a threat.[202] When peptides are formed within the cell due to protein degradation, CTLs can detect the peptides when presented by MHC class I molecules.[203]

A CTL needs to be in close contact to the target cell in order to kill it. The first mechanism is binding of Fas on the target cell to Fas ligand (FasL) on the effector cell, which transfers a death signal.[204–206] The second method is performed through granule exocytosis. In the presence of calcium (Ca^{2+}), granules are exocytosed into the space between the target and effector cells, causing holes to be formed within the target cell membrane.[207] Examples of these “granules” include perforin,[208] and granzymes A and B.[209]

Perforin

Perforin is a protein involved in granule exocytosis in CTLs. Upon polymerization, perforin forms pores in the membrane of a target cell.[210,211] These pores then disrupt ion gradients in a cell and result in osmotic lysis of the target cell.[212,213] Both CTLs and NK cells express perforin.[210,214–216] Perforin has a major role in pathologies such as graft rejection and immune responses against viruses.[205,210,217,218] When Kagi et al. generated perforin-deficient mice, the mice had normal numbers of $CD8^+$ and NK cells, however several immune functions were compromised, including antiviral and transplantation antigen-specific CTL activity.[219] These mice could not clear a lymphocytic choriomeningitis virus infection and also had a diminished ability to control the growth of tumours. However, even in the absence of perforin, there was some lytic activity which was less effective.

Fas

Apoptosis-mediated cell killing by CTLs is able to explain the ability of $CD8^+$ cells in perforin-deficient mice to kill target cells. The cell surface receptor, FasR, can lead to apoptotic cell death.[220–222] When $FasR^-$ or $FasL^-$ mice are generated, they suffer from similar pathologies of $perforin^-$ mice, including autoimmunity and accumulation of large numbers of lymphocytes. $FasL^-$ lymphocytes are able to kill cells, but at a reduced rate of approximately 10-30% compared to wild type CTLs.[220] This residual lysis is probably due to perforin-mediated killing. An important finding was that $perforin^-$ CTLs were inactive against FasR-deficient target cells, meaning that these are the two pathways responsible for CTL-mediated cell death. Overall, Lowin concludes that perforin is likely responsible for approximately two thirds of CTL-mediated killing, while Fas is responsible for the remainder *in vivo*.[220] One downfall of Fas-mediated killing is that FasR expression can be different on cells and can be modulated in certain circumstances, such as when some viruses are able to downregulate FasR expression on cells.[220,221]

Granzyme

Perforin only causes membrane damage and is insufficient in explaining DNA fragmentation and apoptosis shown in CTL target cells.[212,223] Therefore it was hypothesized that another molecule must be responsible for the DNA fragmentation and apoptosis, once it is released from the CTL and is able to enter the target cell via pores made by perforin.[212,224]

1.4.5.3 The T Cell Receptor and Activation Markers

T Cell Receptor

T cell receptors are found on the surfaces of T cells responsible for recognizing antigen from other cells. They are made up of two polypeptides (α and β) linked by disulfide bonds. These polypeptides are associated with other invariant CD3 proteins.[225–228] The α and β polypeptide chains are coded by the rearrangement of germline genes; these random associations allow for the production of thousands of different, mature genes.[225] The TCR recognizes antigens when they are bound to an MHC molecule.[225]

CD69

CD69 is an early membrane receptor, a membrane type II C-type lectin,[121] and is expressed on activated, but not resting, lymphocytes.[229] After activation, an early change that occurs in T cells is the induction of genes encoding cell surface molecules.[230] CD69 has been detected on cell surfaces within 1-2 hours after activation.[230–232] There are other activation markers that exist, for example CD25, CD71, and HLA-DR,[121,233–235] however CD69 is very commonly used for T cells because of how quickly it can be found on cell surfaces. T cells that are stimulated through the TCR (CD3) complex express CD69,[230,232,236–238] therefore mitogens such as PHA and PMA are able to induce the expression of CD69 in lymphocytes.[230,232,238,239] In addition to lymphocytes, CD69 is expressed on all bone-marrow derived cells except RBCs.[229,240] In some instances, CD69 can be expressed on B cells, neutrophils, and freshly isolated monocytes, however the mechanisms by which CD69 is induced are different from T cells.[230]

1.5 Drugs Commonly Associated with DHRs

A number of drugs are associated with ADRs and DHRs, particularly antibiotics (especially beta-lactam antibiotics) and anti-convulsants,[241,242] as well as NSAIDs (non-steroidal anti-inflammatory drugs), anti-retrovirals (eg. Abacavir), sulfonamides, and allopurinol.[242] One issue that is currently lacking in the literature is whether different drugs are linked to DHRs caused by different T cell subsets and whether different DHR phenotypes are associated with different T cell responses.

1.5.1 Sulfamethoxazole

Sulfamethoxazole (SMX) has been previously associated with DHRs. SMX has been previously shown to recognize or activate drug-specific T cell clones.[55,243] Specifically, $CD4^+$ and $CD8^+$ T cells have previously been shown to be involved in reactions to SMX.[103]

Sulfamethoxazole is typically prescribed with trimethoprim (TMP). Bacteria are obligate folic acid synthesizers, as opposed to humans who must obtain folic acid through diet. Both SMX and TMP take advantage of bacteria requiring folic acid to survive by inhibiting the synthesis of tetrahydrofolic acid, a necessary cofactor for bacterial DNA, thymidine, and purines.[244] Typically, it is prescribed as 20 parts SMX to 1 part TMP.[244,245] This is to provide synergistic antibacterial activity as SMX and TMP target the folic acid synthesis pathway at different steps, blockading two separate steps prevents bacterial resistance to either component alone.[244,246]

The primary route of SMX clearance is via metabolism in the liver by *N*-acetyltransferase and *N*-glucoronyl-transferase; these enzymes lead to the production of non-toxic metabolites.[243,247] CYP-450 also metabolizes a small amount of SMX into hydroxylamine (SMX-HA) which under physiology conditions is rapidly converted to nitrososulfonamide (SMX-NO).[247,248] SMX-NO is extremely reactive and may be central to many hypersensitivity reactions.

1.5.2 Beta-lactam antibiotics

Beta-lactam antibiotics have been previously implicated in T cell-mediated DHRs. It has been previously shown that $CD4^+$ and $CD8^+$ T cell clones could be activated by piperacillin in patients with cystic fibrosis and beta-lactam antibiotic hypersensitivity.[100] Brander et al. also found a heterogeneous T cell response (of $CD4^+$ and $CD8^+$ T cells) to penicillin-hypersensitive individuals.[249]

Beta-lactam antibiotics are widely used for treating bacterial infections in humans, but can also cause many immune-mediated allergic reactions.[250] β-lactam antibiotics inhibit cell wall synthesis in bacteria by inhibiting transpeptidases, inducing lysis and cell death.[34,251] Bacteria have developed a resistance to beta-lactam antibiotics, making many drugs ineffective.[27,252–254]

The basic structure of a penicillin consists of a beta lactam ring, which is condensed to a thiazolidine ring. At position 6 of the structural backbone, there is an amine-bonded side chain, which is different depending on the type of penicillin.[250] Specifically, amoxicillin (AMX) is a type of penicillin. It is prescribed either alone or combined to clavulanic acid (CLV).[255]

Beta-lactam antibiotics have a ring structure that is responsible for the reactive and antibiotic activity.[256] Once opening, penicillin is rearranged to metabolic by-products, including penicillic acid, penilloic acid, and penicilloic acid. In addition, 6-aminopenicillanic acid may be formed. Beta-lactamases may produce other metabolites, including penicilloic acid. While not anti-bacterial, these metabolites may form immunogenic conjugates with proteins.[257]

Beta-lactams can cause both immediate reactions (typically occurring one hour after drug intake), and non-immediate reactions.[258–260] In terms of the non-immediate DHRs, maculopapular or morbilliform exanthemas (MPE) are the most common.[258,261] It has been previously shown that AMX may be mediated via the hapten hypothesis.[57]

1.6 Clinical Phenotype of DHRs

DHRs can result in a variety of clinical phenotypes, which range in severity. It is not always known how some individuals experience a more severe rash than another when taking the same drug. Typically, with delayed type DHRs, exanthemas (widespread rash that expands quickly) are the most common.[262] Several types of lesions also occur, including pustular, vesicular, and bullar, however maculopapular is the most common.[262] Examples of skin reactions that can occur include erythema multiforme, drug reactions with eosinophilia and systemic symptoms (DRESS), Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis (SJS/TEN), maculopapular exanthema, and fixed drug eruption.[262,263] Some drugs have been previously associated with different eruptions, including AMX with bullous skin disease, MPE, and AGEP,[49] and NSAIDs, phenytoin, SMX, and AMX have been observed to cause FDE. Some of these skin reactions have other accompanied symptoms, such as fever (can be seen with DRESS and SJS/TEN) headaches, malaise, and fatigue. In some cases, the reaction can induce an autoimmune response, with one example occurring with prolonged DRESS potentially leading to drug-induced lupus erythematosus. DHRs can also lead to systemic organ involvement. Eosinophilia is common, while drug-induced hepatitis,[262,264] drug-induced nephritis, and serum sickness are more rare.[262]

1.6.1 Skin Rashes

There are many kinds of rashes, and many different diseases or sensitivities can result in very similar-looking rashes. Allergies can lead to a set of specific types of rashes. Schlossberg outlines several of these distinctive types of reactions well.[265] A rash that is pruritic is characterized as severe itching.[266] Maculopapular rash is a type of eruption characterized by macules (spots) and papules (bumps). There are several causes of maculopapular rash, including Lyme disease, Rickettsiosis, rubella, EBV (Epstein-barr virus), SLE (systemic lupus erythematosus), and allergy, among many more.[265] Another type of rash are vesico-bullous rashes. Vesicles are small blisters, while bullae are large fluid-filled blisters. There are also several causes of these types of rashes, including herpes simplex virus, staphylococcemia, HIV (human immunodeficiency virus), and

allergy.[265] Several cytokines that can be secreted by different T cell subsets have been described in association with certain skin rashes (described below).

1.6.1.1 Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis

A very serious type of rash associated with ADRs are seen in Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis (SJS/TEN), which are acute life-threatening illnesses that affect the mucous membranes and cause acute epidermal detachment.[267–269] SJS and TEN are two conditions on a spectrum with varying degrees of skin necrosis defined by the percentage of body surface area (BSA) affected. SJS involves <10% BSA, SJS/TEN overlap is 10-30% BSA, and TEN is >30% BSA. SJS is fatal in approximately 10% of all patients, while TEN is fatal in 30-50%. The vast majority (80-95%) of TEN cases are due to ADRs. Other, less common, causes include recent immunization, acute graft-vs-host disease, and contrast medium. Early symptoms include fever, malaise, and sore throat followed by blistering, target lesions, and various degrees of skin detachment.[267,270] The skin lesions typically start on the body's trunk.[267,270,271]

Murata et al. determined that increased soluble Fas ligand (sFasL) is associated with SJS-TEN, by demonstrating that there was increased sFasL in five of seven cases before disease onset.[272] This difference would set early SJS/TEN apart from other drug eruptions. The authors note that since sFasL can be due to cell apoptosis in other diseases, it is important that this test is used only on people experiencing a cutaneous adverse reaction with high risk for SJS/TEN. Nomura et al. obtained serum samples from people at the onset of SJS/TEN and compared the cytokine levels to those of people with other delayed-type ADRs.[273] They found that patients with TEN had higher levels of TNF-α, IL-10 IL-1Rα, IL-6, and GM-CSF. Patients with drug-induced hypersensitivity syndrome (DIHS) had increased IL-5 when compared to erythema multiforme (EM) and maculopapular (MP) type rashes. DIHS and TEN had high IL-13 levels, while SJS and TEN had slightly elevated IFN-γ levels. Pro-inflammatory cytokines and anti-inflammatory cytokines are especially increased in TEN. Once SJS/TEN symptoms improved, IFN-gamma, IL-10, IL-1Rα, and IL-6 levels decreased.[273]

CCL27 is production is augmented by TNF-α in keratinocytes by the NFκB pathway.[274] Wang et al wanted to know if CCL27 influences the course of SJS/TEN. In serum CCL27 was elevated during SJS/TEN, but low in blister fluid.[275] In contrast, they found serum TNF-α to be low, while blister TNF-α was high. They concluded that while both contribute to progression of SJS/TEN, their effects were different.[275] Granulysin has also been found to be responsible for widespread keratinocyte death.[98,270] Chung and Hung found granulysin RNA in the blister cells of SJS/TEN, and granulysin was found to be in greater concentrations than other cytotoxic molecules. Upon injecting the skin of mice with granulysin, the mice developed an SJS/TEN-like reaction.[98,270]

1.6.1.2 Acute Generalized Exanthematous Pustulosis

Acute generalized exanthematous pustulosis (AGEP) was previously classified as psoriasis as it looked similar and was very rare.[276] However, Baker and Ryan observed that some people presenting with AGEP had no previous history of psoriasis, and their illness was acute, short, and did not recur. They therefore attributed it to an infection or drugs.[277]

AGEP is characterized by a pustular rash with fever over 38 °C[96,278] on edematous erythema with a high neutrophil count.[96] AGEP will spontaneously resolve within 15 days of onset.[96,279]

Approximately 90% of all AGEP cases have been associated with drugs, specifically aminopenicillins.[96,279] AGEP has been previously associated with IL-8 secretion by T cells.[96]

1.6.1.3 Drug Reaction with Eosinophilia and Systemic Symptoms

Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS, also referred to as DIHS, Drug Induced Hypersensitivity Syndrome) is a drug reaction that has been found to result after treatment with sulfanimide, phenytoin, dapsone, allopurinol, etc.[280–284] DRESS has a late onset (typically 2-8 weeks after use), and also involves fever, eosinophilia, skin eruption, and lymphocyte activation.[280,285] Chen et al. examined DRESS records from 60 former patients in a Taiwan medical centre.[280] They found that

allopurinol was the most common cause, and the average latency period was 20.7 days. Some had other symptoms, including exfoliative dermatitis or blistering/purpuric eruption, in addition to the diffuse exanthematous eruption that all patients had. The authors also noted some differences in DRESS between patients, therefore questioned whether it is one disease or a spectrum. DRESS has been previously associated with IL-5 and eotaxin secretion.[286,287]

1.7 Diagnostic and Predictive Tests for DHRs

1.7.1 In vivo

In vivo tests that currently exist have been used to investigate DHRs are provocation tests and skin tests. Provocation tests work best for immediate reactions, since delayed reaction tests are not standardized enough for dose and symptom duration. There is also some difficulty with defining a positive result. Skin tests have been previously documented[121,259,260,288–290] to have a low sensitivity and are only of utility in immediate hypersensitivity. They can be performed using a patch, a skin-prick, or an intracutaneous test.[121]

1.7.2 In vitro

1.7.2.1 Lymphocyte Toxicity Assay

The lymphocyte toxicity assay (LTA) was developed from assays used to study metabolic differences between cells of patients and cells of controls. This assay uses isolated PBMCs to determine if there is greater cell death of drug hypersensitive patient cells compared to the cells of healthy control when incubated with the drug.[291] Dr Rieder's lab has had a long experience using these assays as a predictive and mechanistic tool for studying DHRs.[29]

1.7.2.2 Lymphocyte Transformation Test

The lymphocyte transformation test (LTT) is a widely used method in studying drug hypersensitivity.[291] This test has been shown to be useful in exploring hypersensitivity reactions.[96,102,103,121,292] The basic principle of this test involves measuring the incorporation of radio-labelled thymidine (^{3}H-thymidine) in PBMCs to measure cell

division.[243,291,293,294] The result in counts per minute (cpm) is expressed as a stimulation index (SI). Increased proliferation results in a greater SI value, however high SI values are not correlated with clinical severity.[121]

1.7.2.3 Flow Cytometry to Study Activated T Cell Subsets

As previously mentioned, CD69 is a marker present soon after T cell stimulation. However, there are others including CD25, CD71, and HLA-DR.[121,233–235] Two relevant papers use flow cytometry to detect CD69 upregulation after drug stimulation include one by Nishio[101] and another by Beeler and colleagues.[295] Beeler et al. observed that CD69 is upregulated more in drug-allergic individuals than in non-allergic individuals and suggest CD69 as a promising tool for detecting drug-reactive T cells in peripheral blood.[295] In addition, CD69 measurement by flow cytometry has been used in other allergies as well, including delayed hypersensitivity reactions to iodine contrast media.[296]

1.8 Rationale

My research will attempt to fill the gap that exists in the literature concerning the T cell phenotypes of drug hypersensitivity reactions. Currently, there are limited numbers of papers that link T cell subsets with different reactions. There are some papers that link individual cytokines, chemokines, enzymes, or proteins (see section *Immune Mediation of DHRs*) however there is not a full understanding of the physiology of these reactions. There are several “hypotheses” that attempt to explain the pathophysiology of these reactions, however there are many unknowns and there is no one mechanism more supported than the others.

Flow cytometry has been used previously to study these reactions, however it has been limited in favour of other methods, including ELISA/ELISpot, the LTT, and cloning drug-specific T cell subsets. Flow cytometry is beneficial because it allows the detection of multiple fluorochromes bound to multiple proteins on the cell surface, which is beneficial in staining for both cell type and activation. The overall idea would be to confirm that the immune cells are dividing in response to drug stimulation, determine which immune cells are activated during this stimulation, and which cytokines are secreted as a result. Since certain cytokines are associated with specific T cell subsets,

these secreted cytokines can be used to determine which T cells could be involved in the reactions. Consenting participants with confirmed reactions to sulfamethoxazole and beta-lactam antibiotics will be asked to participate in this study. SMX and AMX were chosen due how they are both commonly associated with DHRs. The ultimate goal is to conduct a comprehensive study of the phenotypes of T cell involvement in specific clinical presentations of hypersensitivity reactions to various medications.

1.8.1 Hypothesis

I hypothesize that differences in activated peripheral T cell subsets and types of mediators released produce different clinical phenotypes of drug hypersensitivity reactions to sulphnamides and beta-lactam antibiotics.

1.8.2 Aims

In this study, my aim was to study and characterize lymphocytes isolated from peripheral blood samples from patients with clinical presentations suggestive of delayed-type drug hypersensitivity reactions and compare with drug tolerant controls. To do this, I will use different techniques such as scintillation counting, flow cytometry, and multiplex bead-based assays to measure in vitro PBMC and T cell proliferation. I will use radiolabeled thymidine incorporation to measure proliferation in response to treatment, flow cytometry to determine activated T cell subsets, and Luminex, a bead-based detection assay, to analyze cytokine release of T cell subsets.

Chapter 2

2 Experimental Design and Methods

This research project is part of a larger drug safety project being conducted in the Rieder laboratory. The first stage of the project, being performed by another lab member, is to learn about the symptoms that patients with drug hypersensitivity have by completing a detailed survey to determine the extent and nature of the reaction. Another component of the project is to extract DNA from the isolated PBMCs and study markers in each subgroup of patients exhibiting different clinical presentations of drug hypersensitivity. My portion of the project was to characterize the T cell phenotypes of the different clinical presentations of drug hypersensitivity reactions. To date, while there has been some research into the different types of T cells involved in certain DHRs, much is still unknown. To do this, I obtained venous blood samples from patients and controls and isolated and characterized isolated PBMCs/T cells. A methods schematic is detailed in Figure 2-1. Research ethics had been previously obtained by Dr. Rieder's laboratory for this project (REB # 1118833E).

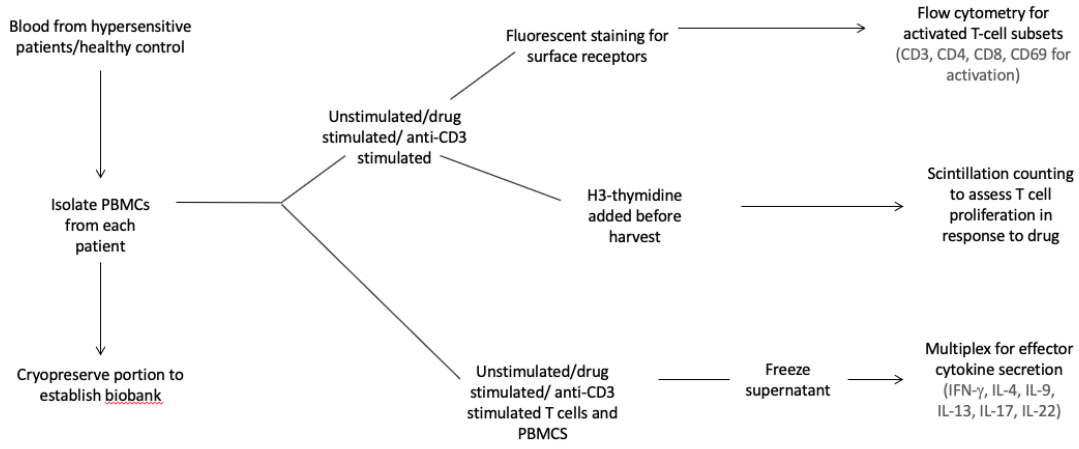


Figure 2-2-1: Schematic of methods used

2.1 General Methods

2.1.1 PBMC Isolation

We isolated PBMCs from whole blood using density gradient separation. First, approximately 15 ml of blood was diluted 1:1 in 1× phosphate buffered saline (PBS, protein-free, pH 7.2, Gibco by Life Technologies, catalogue no. 20012-027). Then, 30 ml of the blood + PBS 1:1 mixture was carefully layered over 15 ml of Ficoll Histopaque-1077 (Sigma, catalogue no. 10771), and centrifuged for 20 minutes (increased to 30 minutes if blood was drawn the day before) at 1500 rpm. The grey PBMC layer was transferred to new 50ml tubes by serological pipette, and washed twice, each time with a full tube (50 ml) of PBS and centrifuged at 1600 rpm for 10 minutes. The PBMCs were diluted to 5×10^6 cells/ml in Roswell Park Memorial Institute (RPMI) medium (+ L-glutamine, 1640 1×, catalogue no. 11875-093) supplemented with 10% human AB serum (Sigma, catalogue no. H3667) by manual counting with a hemocytometer.

2.1.2 Preparing Universal Mitogen Stimulation Plates

To prepare for stimulation, anti-CD3 antibody (Ultra-LEAF purified antihuman CD3 antibody, clone OKT3, Biolegend, catalogue no. 317325) was coated on the bottom of a U-bottom 96-well plate. To the required number of labelled wells, 2 µl anti-CD3 antibody (at 1mg/ml) and 98 µl of 1× protein-free PBS are added. The plate was wrapped in parafilm and left in the fridge (4 °C) overnight. The following day, the plates were centrifuged at 1600 rpm for 10 minutes and the supernatant decanted before PBMCs or T cells were added into coated wells.

2.1.3 Assessing Proliferation by Scintillation Counting

For scintillation counting, the stimulants included anti-CD3, the drug the patient was sensitive to, or culture media (RPMI + 10% human AB serum) as an unstimulated control. The PBMCs were incubated with the stimulant, drug, or media for 54 hrs and 4 days in an incubator at 37 °C and 5% CO_2. Sixteen hours before harvesting the plates, 1 µCi of tritiated thymidine (Thymidine, [methyl-^{3}H], Perkin Elmer, catalogue no. NET027005MC, lot no. 201510) was diluted in culture media 1:10, then 10 µl of the

tritiated thymidine/media mixture was added to both stimulated (by anti-CD3 and drug) and unstimulated PBMCs (alternatively, the 3H-thymidine is diluted 1:25 in media, and added to all wells with a repeater pipettor if number of wells is very high). The PBMCs were then washed and harvested using a cell harvester (Tomtec, Hamden, CT), and the thymidine incorporation is assessed using a MicroBeta counter (Micro Beta Jet, Perkin Elmer1450 LSC & Luminescence Counter). Proliferation is assessed by calculating the stimulation index (SI), by subtracting average background counts per minute (cpm) from the average cpm of each type of sample, then dividing by the average of the unstimulated wells.

2.1.4 Assessing Proliferation of T Cell Subsets by Flow Cytometry

2.1.4.1 Flow Cytometry Protocol

PBMCs were seeded into a U-bottom 96-well plate, then stimulated *in vitro* with the drug the patient had a reaction to (ie. AMX or SMX) at the test concentrations, or stimulated with anti-CD3 (see § *Preparing Universal Mitogen and Stimulation Plates* section), a T cell mitogen, as a controlled comparison. The cells were incubated for 54 hrs in an incubator at 37 °C and 5% CO_2.

The PBMCs were stained with eBioscience Fixable Viability Dye eFluor 506 (ThermoFisher Scientific, catalogue no. 65-0866-14) according to manufacturer's instructions to identify dead cells in the sample. The cells were also stained with allophycocyanin (APC) mouse antihuman CD3 (BD Pharmingen, clone UCHT1, catalogue no. 561810) to identify all T cells, brilliant violet 421 (BV421) mouse antihuman CD8 (BD Horizon, clone RPA-T8, catalogue no. 562429) to identify cytotoxic T cells, phycoerythrin (PE) mouse antihuman CD4 (BD Pharmingen, clone RPA-T4, catalogue no. 561843) to identify helper T cells, phycoerythrin-cyanine 5 (PE-Cy5) antihuman CD69 (eBioscience, clone FN50, catalogue no. 25-0699-41), all according to manufacturer's instructions, to identify activated T cells. Single stain controls were performed with Invitrogen UltraComp eBeads (ThermoFisher Scientific, catalogue no. 01-2222-42). All cells were fixed by resuspension in 100 µl of 4% paraformaldehyde

solution and 200 µl of 1× PBS + 3% FBS (foetal bovine serum) prior to flow cytometry analysis. Samples were analyzed within 4 days post-fixing.

2.1.4.2 Flow Cytometry Specifications

All flow cytometry experiments were performed at the London Regional Flow Cytometry Facility at Robarts Research Institute, Western University. The PBMCs were analyzed on a Becton Dickinson LSR II analysis cytometer (BD Biosciences), using FACSDiVa software (version 8.0.1). The flow cytometer is equipped with a 50 mW Coherent Cube 402 nm violet diode laser, a 2 mW Coherent Sapphire state 488 nm blue laser, a 50 mW Coherent Compass 561 nm solid-state yellow-green laser, and a 40 mW Coherent Cube 640 nm red diode laser.

The violet laser trigon was used to detect Brilliant Violet 421 (detector C, with a 450/50 bandpass (bp) filter and no longpass (lp) mirror), and to detect the fixable viability dye (detector B, 525/50 bp, 505 lp). The yellow-green laser octagon was used to detect PE (detector E, 582/15 bp), and PE-Cy7 (detector A, 78/60 bp, 755 lp). The red laser trigon was used to detect APC (detector C, 670/30 bp).

2.1.4.3 FlowJo Gating Procedure

Data was analyzed using FlowJo software (FlowJo, LLC). On FlowJo, the gates were set using FMO (fluorescence minus one) controls on the fully stained samples. First, forward scatter area (FSC-A) was gated against the aqua viability dye to gate only live cells. Side scatter height (SSC-H) was gated against side scatter width (SSC-W), and forward scatter height against forward scatter width to gate single cells. Then, CD3 APC was compared against SSC-A to gate $CD3^+$ and $CD3^-$ stained cells. Within the $CD3^-$ population, CD69 PE-Cy7 was compared against SSC-A to identify with $CD3^-CD69^+$ population. Within the $CD3^+$ lymphocyte population, CD4 PE was compared against SSC-A to determine the $CD4^+$ population. Within this gate, CD69 was compared against SSC to determine the $CD4^+CD69^+$ population. From the $CD3^+$ population, CD8 was compared against SSC-A to determine the $CD8^+$ population. Within this population, CD69 was compared against SSC-A to determine the $CD8^+CD69^+$ population.

2.1.5 Cryopreservation of PBMCs and T Cells

This method has been adapted from the Canadian Healthy Infant Longitudinal Development (CHILD) Study, from their 8-Year Biological Samples Collection and Processing SOP, 23 July 2018 edition.[297] Once all appropriate experiments were prepared with the patient PBMCs and T cells, aliquots of cells were frozen under liquid nitrogen for future analysis.

The 2× freezing medium was prepared by combining 15 ml of heat-inactivated fetal bovine serum (FBS), 15 ml of RPMI 1640, and 10 ml of dimethyl sulfoxide (DMSO), aliquoted in 3 ml volumes and remainder stored at -20 °C.

Both T cells and PBMCs are resuspended at 1×10^7 cells/ml in RPMI 1640 + FBS 1:1. Labelled cryovials were placed on ice, then 500 µl of PBMC or T cell suspension were added to each tube. Then we added 500 µl of 2× freezing medium to each cryovial while on ice.

The Mr Frosty (Nalgene, Thermo Scientific, USA) was prepared by removing the insert and adding 250 ml of isopropanol to the container. The vial holder was replaced into the container. The isopropanol was changed after every fifth use. The Mr. Frosty was placed into the fridge at 4 C more than four hours before it is required. The PBMC and T cell aliquots were placed into the Mr. Frosty and then into the -80 °C freezer for 4 to 24 hours (typically overnight), and then the samples were placed into the liquid nitrogen long term storage tank into a known and recorded location.

2.2 Specific Experimental Procedures and Methods

2.2.1 Assessing Highest CD69 Expression on T Cells

I explored the length of the incubation for incubating isolated PBMCs with the culprit drug or universal mitogen (anti-CD3). This experiment was based off of a study by Beeler et al.[295] The evening before isolating the PBMCs and plating the cells, I coated 21 wells (three wells per timepoint for five timepoints, five FMO wells, one viability dye compensation control well) of a U-bottom 96-well plate with anti-CD3 and PBS, as previously described (see § *Preparing Universal Mitogen and Stimulation Plates*) and

allowed the plate to rest in the fridge. I started with 15 ml healthy volunteer blood and LEAF anti-CD3 as the universal mitogen. I did not have access to hypersensitive patient samples, so a drug stimulant was not used. I isolated the PBMCs from the 15 ml of blood (as described in § *PBMC Isolation*), suspended the PBMCs in 10 ml of RPMI with 1× penicillin/streptomycin and 10% human AB serum, counted the cells in a hemacytometer, and adjusted the concentration to 5×10^6 cells/ml. The plate with anti-CD3 coated wells was centrifuged at 1600 rpm for 10 minutes, then I decanted the supernatant and added 200 ul of PBMC suspension to 21 coated wells and 12 uncoated wells. Every 18 hours I harvested three wells of anti-CD3 stimulated and three unstimulated samples for flow cytometry and stained with Aqua Viability Dye, CD3 APC, CD4 PE, CD8 BV421, and CD69 PE-Cy7 (as described in § *Assessing Proliferation of T Cell Subsets by Flow Cytometry* subsection A, *Flow Cytometry Protocol*) for a total of five timepoints. After staining, the PBMCs were suspended in 100 µl 1 × PBS + 3% FBS and 100 µl 4% paraformaldehyde for flow cytometer acquisition. Single stain compensation controls for CD3 APC, CD4 PE, CD8 BV421, and CD69 PE-Cy7 were made fresh using comp beads. In total, 15 stimulated timepoint samples, 15 unstimulated timepoint samples, five FMO samples, and five single stain compensation controls were analyzed. To analyze the data, the unstimulated values were subtracted from the stimulated values in an attempt to normalize.

2.2.2 Testing Healthy Cells with Concentrations of SMX-HA and SMX Parent Drug

The purpose of this experiment was to determine the magnitude of cell death caused by different concentrations of either sulfamethoxazole hydroxylamine reactive metabolite (SMX-HA) or sulfamethoxazole parent drug (SMX). As SMX-HA is more reactive than SMX and can cause cell death, we used both drugs to determine the best one to use in this experiment. The evening before the experiment, I coated nine wells of a U-bottom 96-well plate with anti-CD3 (see § *Preparing Universal Mitogen and Stimulation Plates*) for flow cytometry.

I isolated PBMCs from approximately 15 ml of healthy volunteer blood and suspended the PBMCs in 10 ml of RPMI 1640 supplemented with 10% human AB serum. Using a

hemacytometer, the PBMCs were counted and the concentration was adjusted to 5×10^6 cells/ml. The 96-well plate was centrifuged at 1600 rpm for 10 minutes, and the supernatant decanted. I added 100 µl of healthy PBMCs to each of the anti-CD3 coated wells and to 21 non-coated wells. Of the nine anti-CD3 coated wells, five of the stimulated wells were for FMOs, one of the stimulated wells was for the viability dye single stain control, three stimulated wells were for control wells – to these wells I added 100 µl RPMI 1640 + 10% human AB serum. I also added 100 µl of media to three unstimulated wells to be stained with the full panel of fluorescent antibodies. For unstimulated wells, media was added because no drugs were required, but the final volume required was 200 µl. Sulfamethoxazole parent drug was added to nine unstimulated wells in 200 µM, 100 µM, and 50 µM concentrations, three wells each. Sulfamethoxazole hydroxylamine was added to nine unstimulated wells in 100 µM, 50 µM, and 25 µM concentrations, three wells each. The plate was incubated for 54 hrs. The cells were harvested into flow cytometry tubes, stained with viability dye, CD3 APC, CD4 PE, CD8 BV421, and CD69 PE-Cy7, acquired on the LSR II flow cytometer with FACS Diva and analyzed using FlowJo (see § *Assessing Proliferation of T Cell Subsets by Flow Cytometry*). Once a concentration of SMX-HA was found to potentially cause cell death, two more healthy control participants were tested with anti-CD3 and the concentration previously found to reduce cell viability, to ensure results were consistent.

2.2.3 Processing Patient Samples

2.2.3.1 Recruiting Participants

Subjects were recruited from patients seen at Dr. Rieder's Drug Safety Clinic at London Health Science Centre, Victoria Hospital in London, Ontario. Prospective patients were determined from a database of Dr. Rieder's DHR patients, on the basis of having previously been tested by LTA (Lymphocyte Toxicity Assay) for a T-cell mediated drug hypersensitivity reaction to either sulfamethoxazole or beta-lactam antibiotics. The LTA testing was either being conducted simultaneously for patients with a high likelihood based on clinical history, or one to eight years previously. For these participants, blood was drawn either at Robarts Research Institute, London, Ontario by Dr. Rieder, or at University Hospital, in London, Ontario by phlebotomy staff. Inclusion criteria were that

they had a positive LTA test, or they were in the process of being tested and their clinical history strongly suggested DHR, and had a recorded rash or skin condition attributed to drug exposure. Patients who were very likely to have had a DHR based on history alone who were referred to LTA testing. For these participants, blood was drawn at the same time as the blood for the LTA testing. I called previous or current patients and asked if they would be willing to provide a sample for the study, or to add one 1-2 tubes of blood to their LTA testing. The details of the experiment and their privacy were discussed over the phone, and they were sent a copy of the Letter of Information (LOI) with the consent forms either in advance or in person before the appointment. Participants were given a $10 Tim Horton's gift card for their time and their sample.

2.2.3.2 Receipt and Preparation of Participant Samples

After patient samples were received, a control sample (~15 ml) of blood was drawn from a healthy, consenting volunteer. Typically one control was used per one DHR participant. The control was ideally someone who has taken the drug the patient participant has a hypersensitivity to but did not have an adverse response, or someone who has served as a control in previous LTA tests but consistently tests negatively. One control sample and one patient sample were analyzed at the same time. Depending on availability of samples, the samples were processed and PBMCs were isolated either the same day or the next day (see § *PBMC Isolation*). If PBMCs were isolated the day after blood was collected, an extra 10 minutes was added to the centrifugation at the Ficoll separation step for those samples only. PBMCs were re-suspended after isolation to 5×10^6 cells/ml in RPMI + 10% human AB serum. If testing only one drug, 15 ml was prepared, or 20ml for two drugs, to accommodate additional wells.

2.2.3.3 Preparing SMX and AMX to study Activation

To stimulate PBMCs, four different concentrations were made from analytical grade powder for each drug, AMX and SMX. Two serial dilution charts (see Figure 2-2 and Figure 2-3) were used for each patient to make the appropriate dilutions. Drugs were diluted 1:1 with 100 µL of PBMC suspension to achieve final concentrations of AMX 1000 µM, 500 µM, 100 µM, and 10 µM; SMX 800 µM, 400 µM, 200 µM, and 100 µM.

All drug preparations were made fresh the day of plating, and either used immediately or kept in the dark until required (usually only maximum of 1 hr).

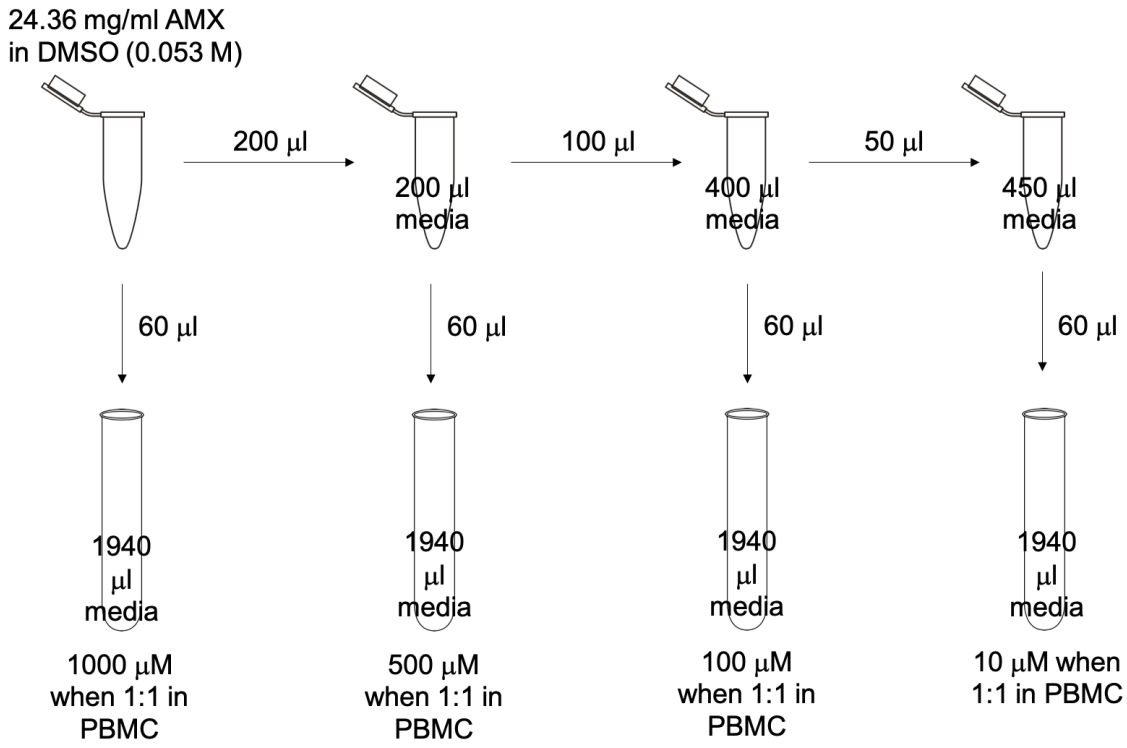


Figure 2-2: Serial dilution chart for amoxicillin

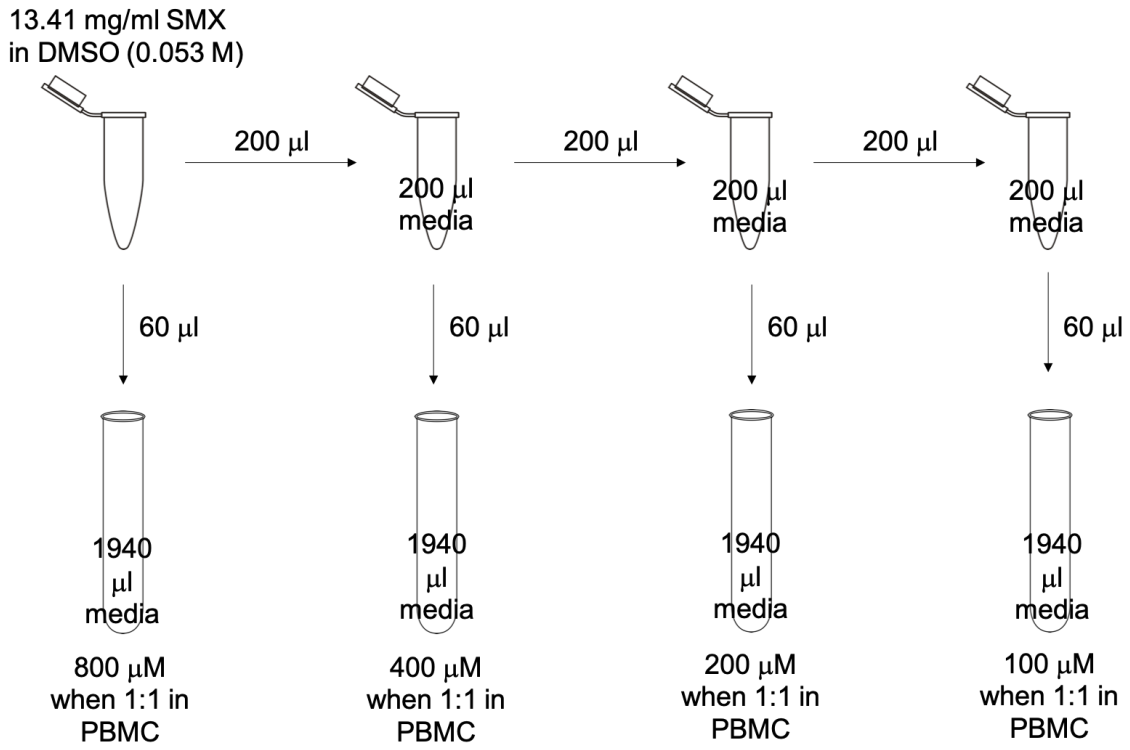


Figure 2-3: Serial dilution chart for sulfamethoxazole

2.2.3.4 Assessing Proliferation by Scintillation Counting

The evening before the PBMCs were plated onto 96-well plates, I made the appropriate number of anti-CD3 coated plates. Which plates were made depended on which experiments would be conducted the next day – this was important because cell harvesting and scintillation counting for proliferation testing could not be performed on a weekend due to equipment availability, so the 54 hrs and 4 days scintillation counting plates were coated strategically to avoid this. For each control and participant plate at both time points, I coated four wells. The following day, 100 ul patient and control PBMCs at 5×10^6 cells/ml in RPMI + 10% human AB serum were plated with either 100 ul of media (RPMI + 10% human AB serum as negative control), anti-CD3 with added media to bring volume up to 200 µl, or the 100 µl of the four different concentrations of the appropriate drug. Four wells of each condition were plated. At 16 hours before harvesting, 1 µCi of 3H-thymidine was added to each well (see § *Assessing Proliferation by Scintillation Counting* for details). See Figure 2-4 for a sample plate layout.

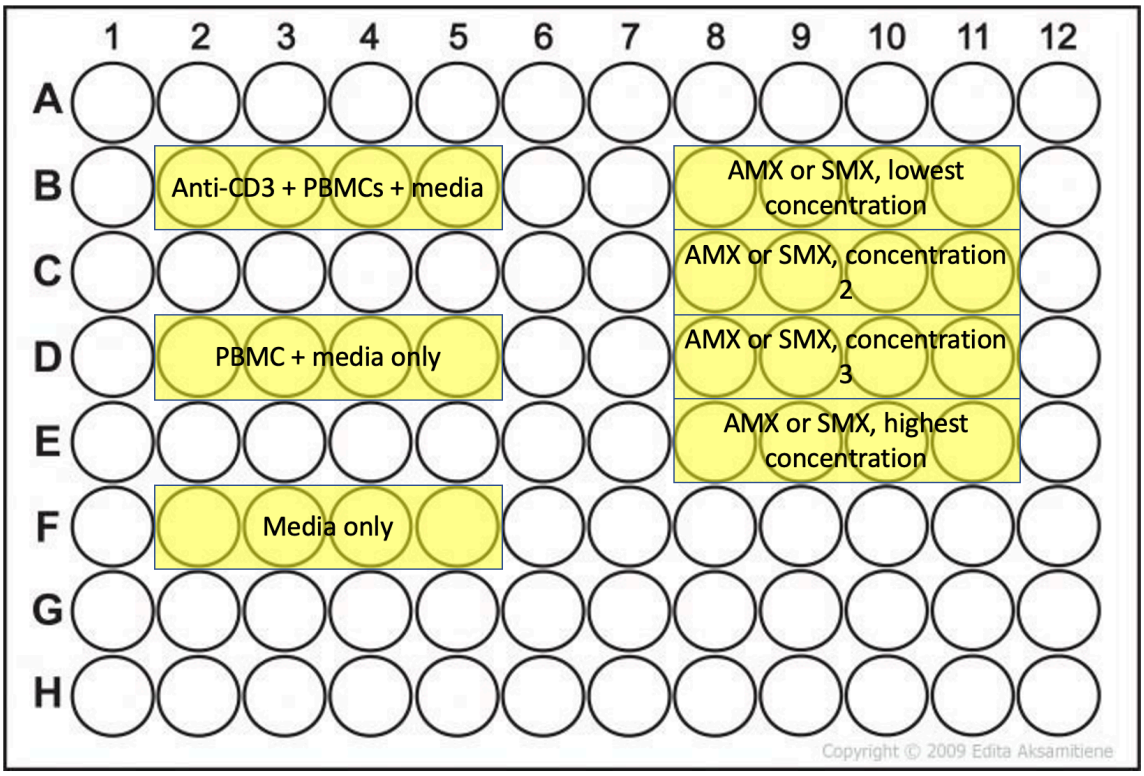


Figure 2-4: Sample scintillation counting plate layout

If two drugs were plated, the wells occupied rows A-H columns 8-11. One plate is made for control PBMCs, a second plate is made for patient PBMCs, and two plates are processed for each 54 hrs and 4 days incubation period.

2.2.3.5 Flow Cytometry for Activated CD4 and CD8 T Cells

The flow cytometry plate cells were harvested into flow cytometry tubes using a plastic transfer pipette, and PBS to rinse the wells to ensure all cells were transferred into the tubes. The cells were washed with PBS and centrifuged at 1600 rpm for 10 minutes. The supernatant was removed by pipette and the cell pellet resuspended in 1 ml of PBS. To all tubes except the viability dye FMO, I added 1 µl of viability dye. The anti-CD3 stimulated PBMCs were stained with one test each of CD3 APC, CD4 PE, CD8 BV421, and CD69 PE-Cy7 (see § Assessing Proliferation of T Cell Subsets by Flow Cytometry subsection A, Flow Cytometry Protocol, for details) while the FMO tubes were stained appropriately (see Figure 2-5 for a sample flow cytometry staining panel for patient samples). PBMCs were washed twice with 1 × PBS + 3% FBS, and resuspended in 100

ul 4% paraformaldehyde, to fix the cells, and 200 µl of 1× PBS + 3% FBS. The experiment was acquired on the LSR II as soon as possible after staining (within 2-3 days, depending on availability of the cytometer for a 3-hour time slot).

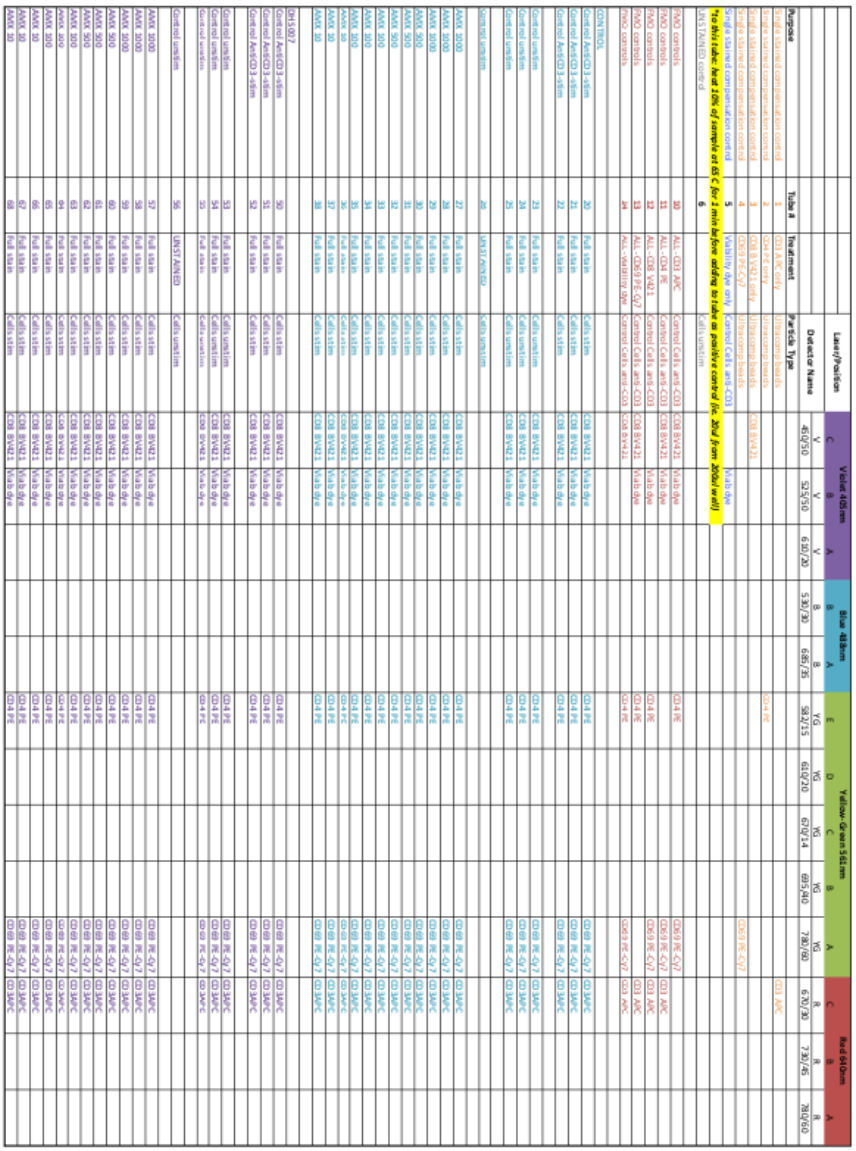


Figure 2-5: Sample flow cytometry staining panel for patient and control samples

2.2.3.6 Assessing Cytokine Release

Isolated PBMCs and T cells from both control and patient samples were stimulated with anti-CD3, the drug concentrations, and culture media (unstimulated) in duplicate. The evening before plating, two anti-CD3 plates are prepared (see § *Preparing Universal*

Mitogen Stimulation Plates). T cells were only isolated and plated if there were enough cells remaining from the patient and control samples to justify isolating them without compromising cell numbers for other experiments. The plates were divided into two (one half for PBMCs, one half for T cells), and two wells are coated in each half (4 wells total per plate). PBMCs were plated in duplicate at 5×10^6 cells/ml in RPMI + 10% human AB serum with either media (control), anti-CD3, or the four concentrations of either drug (AMX or SMX). After a 3-day incubation at 37 °C and 5% CO_2, the supernatant was removed from samples, stored in labelled Eppendorf 2 ml tubes, and frozen at -80 C until analysis could be completed.

Stimulated DHS and control participant PBMCs were subjected to Luminex analysis. Since the AMX data was more promising, only AMX participants and controls were included in the Luminex analysis. Control and DHR 006 participants were not analyzed due to heavy RBC contamination in DHS006 samples.

A custom Luminex kit was ordered from Bio-Rad (Hercules, CA), specific for analytes IFN-y, IL-9, IL-22, IL-17A, and IL-13 (PO # 0222850). The analysis was performed according to manufacturer's instructions. The beads were diluted as instructed by the manufacturer. Since it was a custom kit, some beads were 2× as concentrated as others, so they were diluted appropriately. A plate layout was generated (see Figure 2-6), with all blanks, standards, and samples analyzed in duplicate. A standard curve was generated for the beads. Bio-Rad suggests neat to 1:10 dilution, but since some of the standards had such a high starting concentration, we chose neat to 1:12 dilution to accommodate this, as well as to fill up the plate.

Frozen supernatants were thawed over ice and centrifuged for 3 minutes at 12000 rpm to pellet any debris. Since the supernatants were composed of culture media (with added protein), no BSA was added to the samples. Then 50 µl of the diluted beads were added to each well and washed two times with wash buffer and the magnetic plate. Then, 50 µl of samples, standards, and blank were added to each well, covered, and incubated with shaking for 1 hr. With 10 mins remaining in the incubation, the antibodies were diluted to 1× according to instructions. The plate was washed three times with wash buffer and the

magnetic plate, then 25 µl of detection antibodies was added to each well. The plate was covered and incubated with shaking for 30 mins. With 10 mins remaining in the incubation, the streptavidin-PE (SA-PE) was prepared. The plate was washed three times with wash buffer and the magnetic plate, then 50 µl of diluted SA-PE was added to each well. The plate was covered and incubated for 10 mins. A final round of three washes was performed, and each well was resuspended in 125 µl of assay buffer. The plate was covered and placed on the shaker until analysis.

A Bio-Plex 200 readout system (Bio-Rad) was used to analyze the cytokines. This system uses Luminex ® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/ml) were automatically calculated from standard curves, generated by the Bio-Plex Manager software (v. 6.1, Bio-Rad, Hercules, CA).

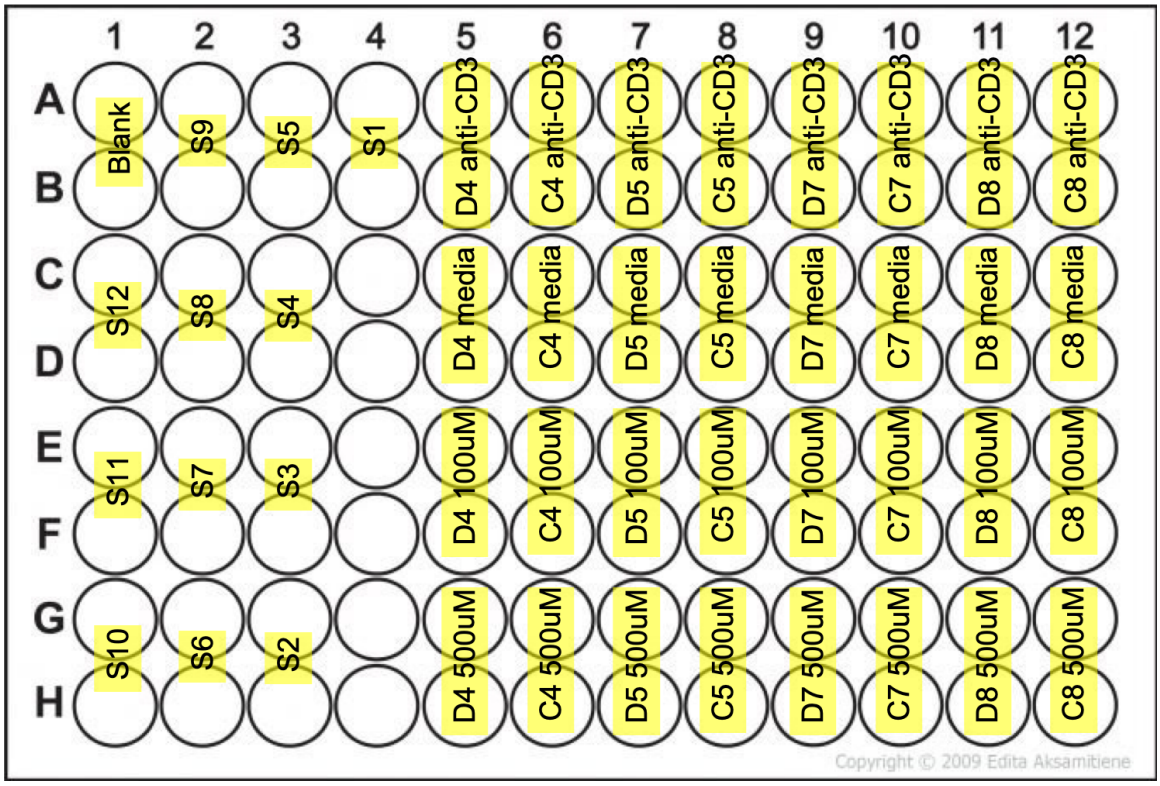


Figure 2-6: Luminex sample plate layout

S1-S12: standard bead dilutions, D4-D8: DHS participant, C4-C8: control participant. All samples were supernatants of PBMCs incubated with AMX.

2.3 Statistical Methods

An ANOVA can be used to test three or more groups without inflating the alpha value, which would be the case with multiple comparisons.[298] Repeated measures ANOVA (rANOVA) was used to perform many of the statistical analyses as the same participants were tested repeatedly across the dependent variable. This is beneficial as it reduces the error.

However, one important aspect of rANOVA is sphericity. When sphericity is violated, the test becomes too liberal (increases Type I error rate) and therefore might indicate a significant result when it should actually be not significant. As a result, there are some tests that correct the rANOVA by increasing *p* to make the test more conservative. We chose to analyze sphericity-violated rANOVAs with the Greenhouse-Geisser correction. A previous analysis by Muller and Barton (1989) supports the use of the Greenhouse-Geisser correction (over Huynh-Feldt) due to acceptably controlling for Type I error while also maximizing power.[299]

While we did use statistical methods when appropriate, it is important to note that the N of the study was relatively small for certain tests, it would be prudent to increase the number of participants in the study to increase the power.

Chapter 3

3 Experimental Set 1

It has been previously shown that T cells appear to be key mediators of DHRs.[53,54,96–104] While it is not fully understood how they are involved, it is thought that they, as well as their mediators, are primarily responsible for the immunological pathogenesis of these reactions. In addition, CD69 has been previously shown to be an activation marker that is expressed on the T cell surface rapidly after activation.[295] However, CD69 does not remain on the cell surface indefinitely, and its quantity can change after activation. For our own purposes, it was important to do our own tests to observe what methods would work for us. This includes which metabolites to use, length of incubation time, percent of CD69 expression.

3.1 Using SMX Yields more Live Cells than SMX-HA

In addition to sulfamethoxazole (SMX) parent drug, there are two intermediates that can also cause a reaction, however they differed in their toxicity. To decide which form of SMX we would use, I tested concentrations of SMX and sulfamethoxazole hydroxylamine (SMX-HA). PBMCs from one volunteer donor were incubated with culture media (unstimulated), 500μM, 100μM, and 200μM SMX, and 25μM, 50μM, and 100μM SMX-HA, and analyzed by flow cytometry for the percentage of live cells remaining after incubation (Figure 3-1).

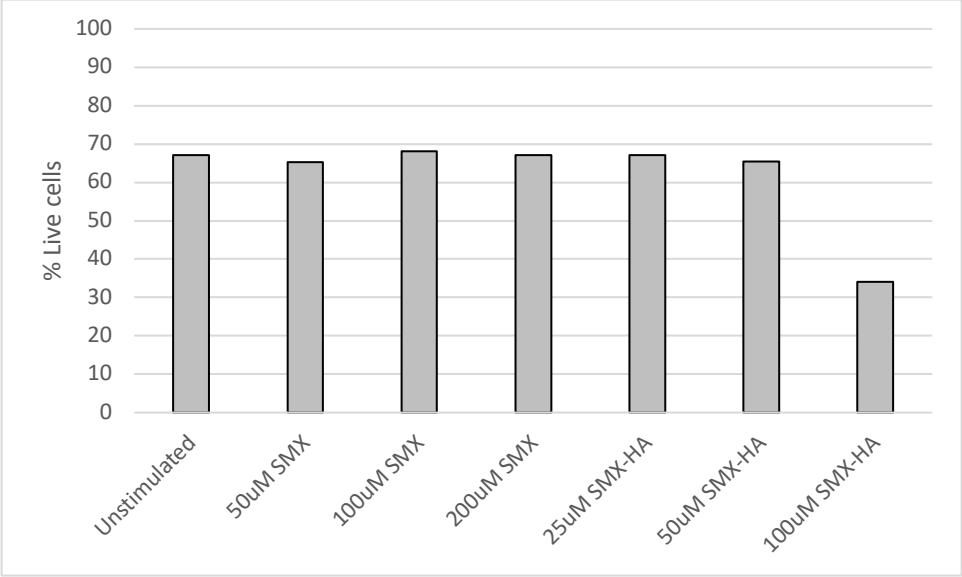


Figure 3-1: Percentage of live cells after PBMCs from a healthy control were incubated with either culture media, SMX, or SMX-HA for 54 hours
Concentrations of 50µM, 100µM, and 200µM SMX; 25µM, 50µM, and 100µM SMX-HA for 54 hrs. N = 1.

Without statistical analysis, given the small sample size (N=1), there do not seem to be any differences between the SMX-stimulated samples. However, there was a decrease in cell viability when the PBMCs were incubated with 100µM SMX-HA. To confirm, this concentration was repeated with two additional healthy controls, increasing the sample size to 3 (Figure 3-2). A Student's t test confirmed that 100µM SMX-HA significantly reduces cell viability ($p = 0.0017$).

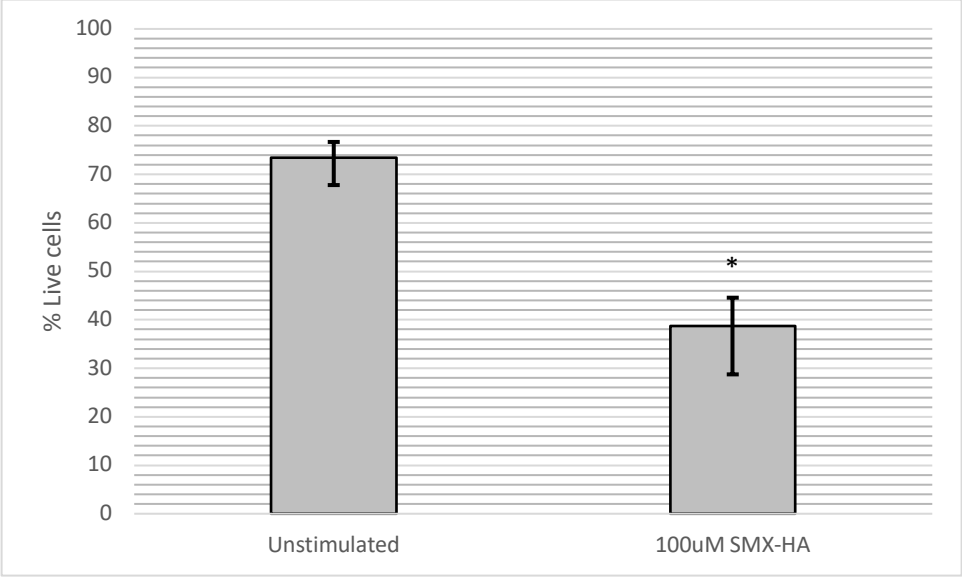


Figure 3-2: Percentage of live cells remaining after PBMCs from three controls were incubated with 100uM SMX-HA for 54 hrs

The PBMCs incubated with 100µM SMX-HA have a significantly lower number of live cells remaining after incubation compared with unstimulated PBMCs with media alone (student's t test, one-way, $p = 0.0017$). N = 3, ± SEM.

3.2 Length of Incubation affects Viability of PBMCs

To determine the best length of time for incubating PBMCs with a stimulant, PBMCs were incubated with plate-bound anti-CD3 for five intervals of 18 hours, in an experiment similar to Beeler et al.[295] First, the percent of live PBMCs remaining after incubation was analyzed every 18 hours by flow cytometry (Figure 3-3). There was a gradual decrease in viability as the timepoints increased. At 90 hrs, viability decreased to an average of approximately 50% viability. Our chosen timepoint of 54 hrs did not differ significantly from 18hrs.

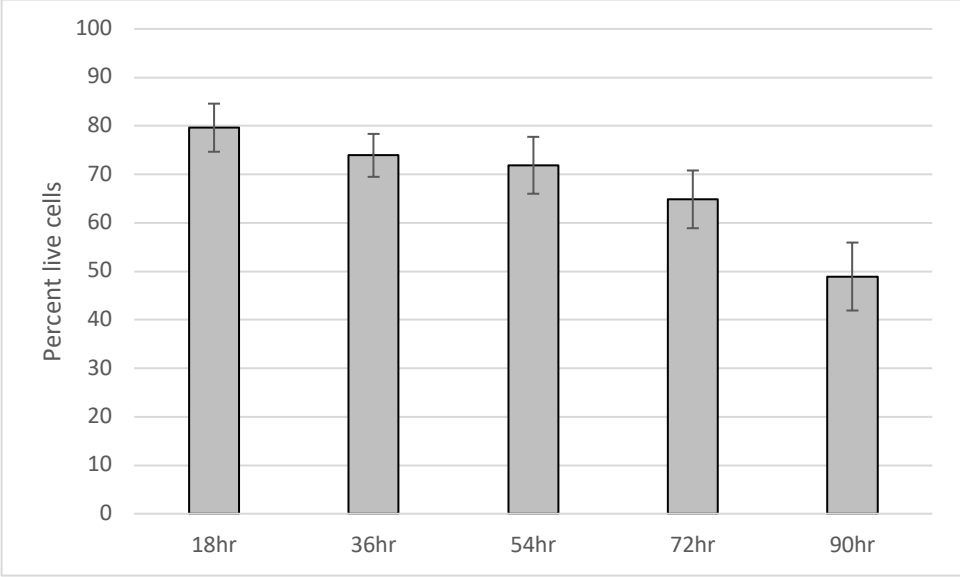


Figure 3-3: The percent of live cells remaining after PBMCs were incubated with plate-bound anti-CD3 at 18 hr intervals for 90 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was a significant effect of time on number of live PBMCs remaining. $F(1.164, 2.328) = 25.351$, $p = 0.027$. N = 3, ± SEM.

3.3 Percent of T Cells expressing CD69 during Incubation

PBMCs were also analyzed for percent CD69 expression and MFI of CD69 across CD3+, CD3+CD4+, and CD3+CD8+ cell types. Unstimulated percent of CD69 expression values were subtracted from anti-CD3 stimulated values. With the CD3+ percent CD69 and MFI (Figures 3-4 and 3-5), there were no significant timepoints, however the overall appearance of the data can be described. With both the percent CD69 and the MFI of CD69, 18, 54, and 72 hr timepoints appeared to be the highest. With CD3+CD4+ percent CD69 and MFI (Figures 3-6 and 3-7), the trends were different, but again not significant. With the percent CD69 (Figure 3-6), there seemed to be an overall decrease in percent CD69 expression from the 18 hr to the 90 hr timepoint. With the MFI of CD69 (Figure 3-7), the 18 hr, 54 hr, and 72 hr timepoints were the highest, however the values were more

variable. With CD3+CD8+ percent CD69 and MFI (Figures 3-8 and 3-9), while also providing no significant timepoints.

While there were no significant differences between timepoints (that either caused a significantly higher or lower cell viability), the timepoint 54 hrs was chosen for the participant experiments.

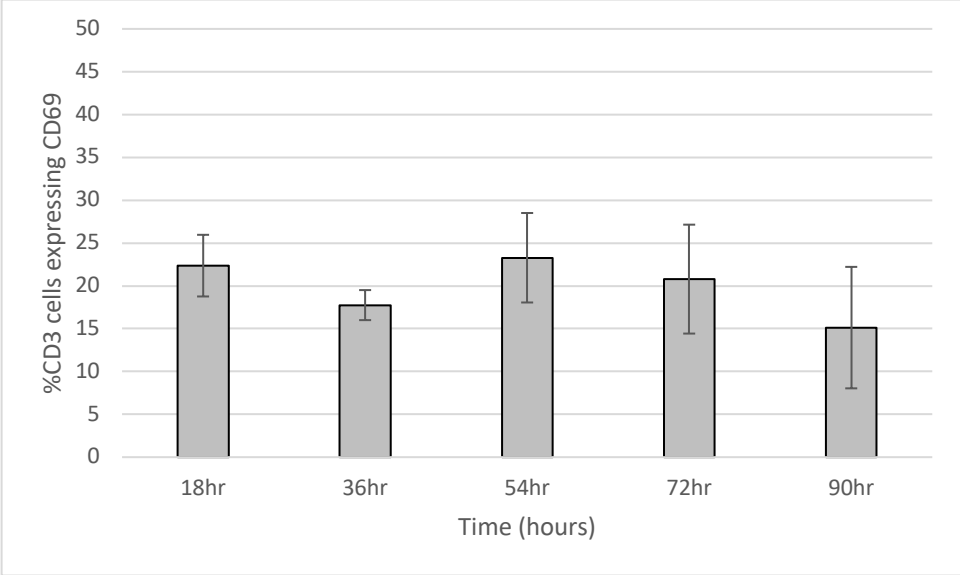


Figure 3-4: Percentage of CD3+ T cells expressing CD69, assessed by flow cytometry, after PBMCs from three healthy control participants were incubated with anti-CD3 for 90 hrs for intervals of 18 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was no significant effect of time on percent CD69 expression on $CD3^+$ T cells. $F(1.041, 2.081) = 1.494$, $p = 0.346$. N = 3, ± SEM.

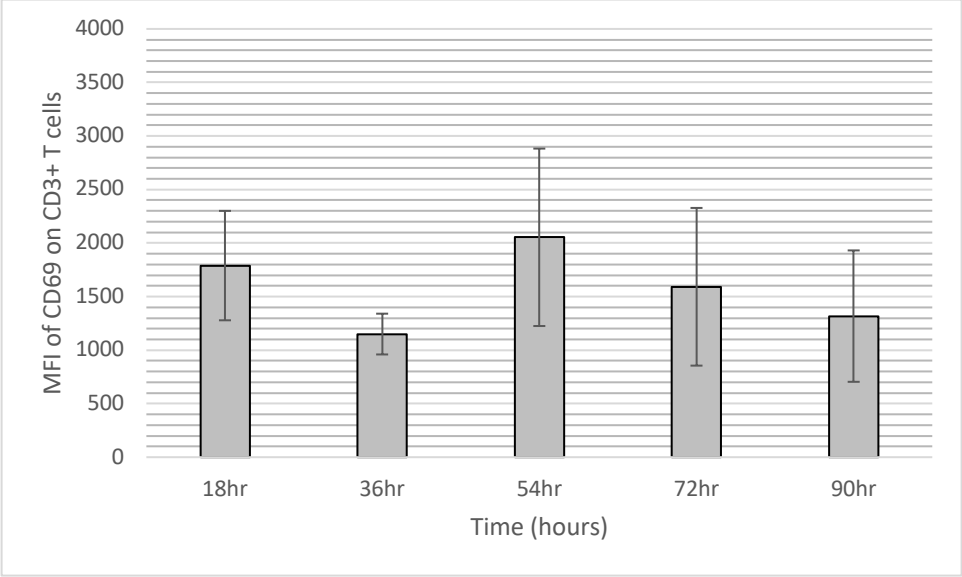


Figure 3-5: MFI of CD69 expressing CD3+ T cells, assessed by flow cytometry, after PBMCs from three control participants were incubated with anti-CD3 for 90 hrs for intervals of 18 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was no significant effect of time on the MFI of CD69 expression on $CD3^+$ T cells. $F(1.072, 2.145) = 0.495$, $p = 0.563$. N = 3, ± SEM.

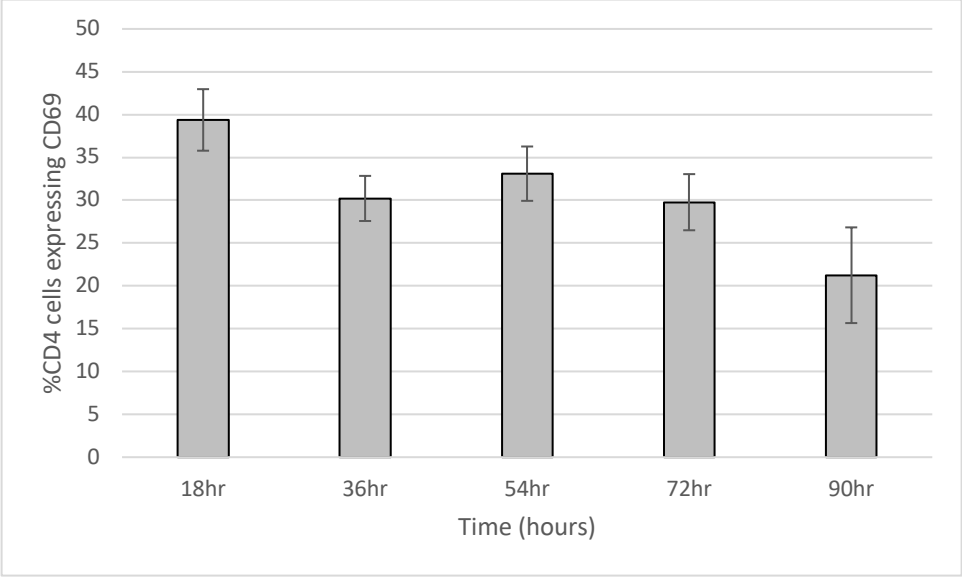


Figure 3-6: Percentage of CD3+CD4+ T cells expressing CD69, assessed by flow cytometry, after PBMCs from three healthy control participants were incubated with anti-CD3 for 90 hrs for intervals of 18 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was no significant effect of time on % CD69 expression on $CD3^{+}CD4^{+}$ T cells. $F(1.087, 2.173) = 3.660$, $p = 0.188$. N = 3, ± SEM.

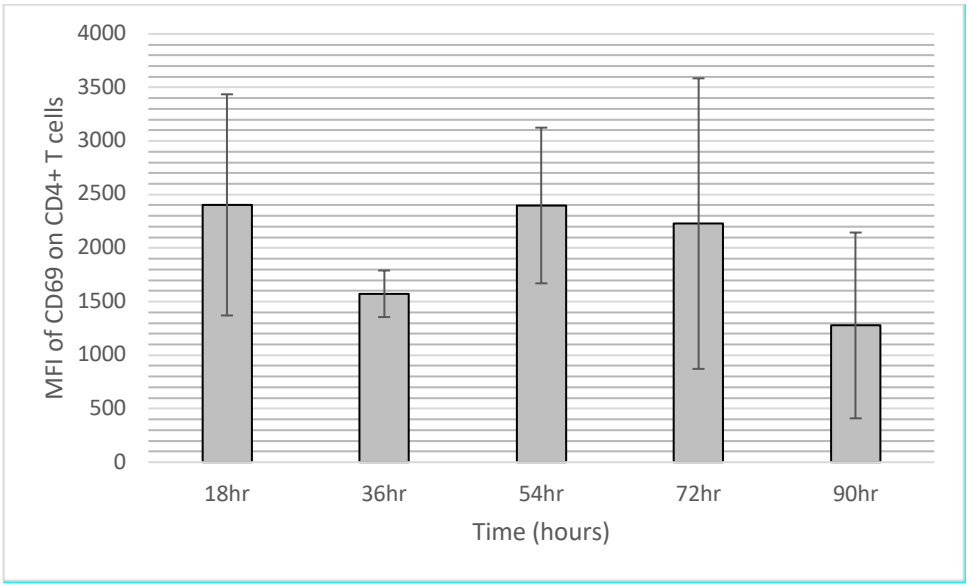


Figure 3-7: The MFI of CD69 expressing CD3+CD4+ T cells, assessed by flow cytometry, after PBMCs from three healthy control participants were incubated with anti-CD3 for 90 hrs for intervals of 18 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was no significant effect of time on the MFI of CD69 expression on $CD3^{+}CD4^{+}$ T cells. $F(1.072, 2.144) = 0.362$, $p = 0.619$. N = 3, ± SEM.

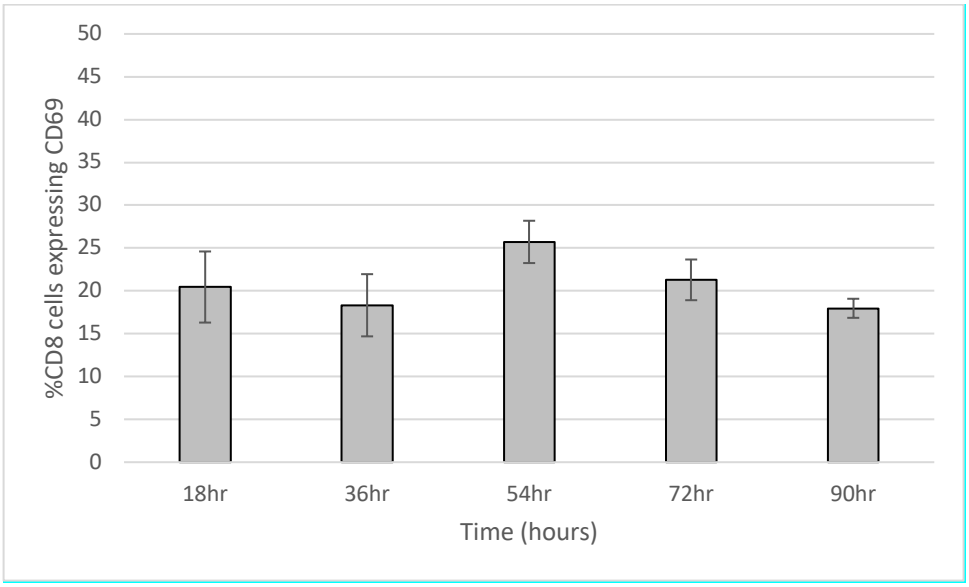


Figure 3-8: Percentage of CD3+CD8+ T cells expressing CD69, assessed by flow cytometry, after PBMCs were incubated with anti-CD3 for 90 hrs for intervals of 18 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was no significant effect of time on percent CD69 expression on $CD3^{+}CD8^{+}$ T cells. $F(1.689, 3.378) = 1.735$, $p = 0.296$. N = 3, ± SEM.

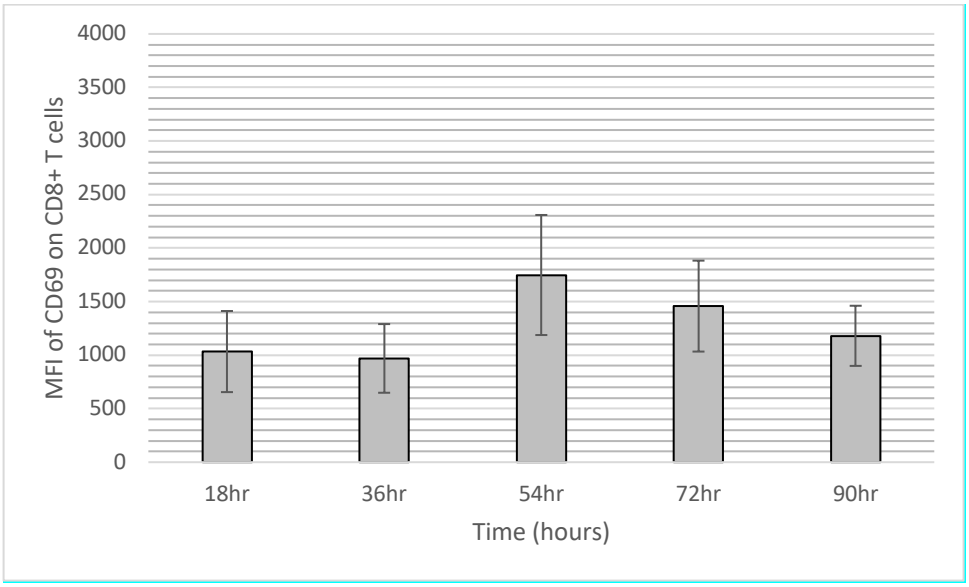


Figure 3-9: The MFI of CD69 expressing CD3+CD8+ T cells, assessed by flow cytometry, after PBMCs from three control participants were incubated with anti-CD3 for 90 hrs for intervals of 18 hrs

A repeated measures ANOVA was conducted to determine if any time intervals were significant. Mauchly's Test of Sphericity indicated that the assumption of sphericity was violated, therefore the Greenhouse-Geisser correction was used. There was no significant effect of time on the MFI of CD69 expression on $CD3^{+}CD8^{+}$ T cells. $F(1.216, 2.432) = 0.744$, $p = 0.493$s. N = 3, ± SEM.

Chapter 4

4 Experimental Set 2

Both sulfamethoxazole (SMX) and beta-lactam antibiotics (for example, penicillin) have been previously shown to elicit DHRs.[137,258,259,261] In addition, both are commonly prescribed antibiotics. The lymphocyte transformation test (LTT) is an effective diagnostic test for delayed type DHRs.[96,102,103,121,292] Flow cytometry has been previously used to detect CD69 upregulation after drug stimulation.[101,295,296] While its use in delayed type DHRs has been limited, it is a promising way to detect cellular response using surface proteins. In addition, differences in cytokine secretion, which can be analyzed in several different ways, are an effective way to study DHRs.[137,186,300] The goal was to determine if there were patterns among the clinical presentations of people previously diagnosed with DHRs to SMX and AMX.

4.1 Description of Participant Population

The mean age of DHR participants (Table 4-1) was 50.5 years, with a standard deviation of approximately 19.1 years, and the ratio of female to male participants was 7:1. The mean age of the control participants (Table 4-2) was 31.125 years, with a standard deviation of 13.3, and a female to male participant ratio of 4:4. Student's t-test confirmed that there was a significant difference in average age between the two participant populations (Student's t-test, one-tailed, $p = 0.017$). All control AMX participants had previously taken the drug, while the control participants either had never taken sulfamethoxazole or had no recollection.

It is important to note that within the SMX participants, of the four oldest participants, three were DHS participants. Within the AMX participants, of the four oldest, again three were DHS.

Table 4-1: List of participants with drug allergies that took part in the study, recruited from Dr. Michael Rieder's drug allergy clinic at London Health Sciences Centre, in London, Ontario, Canada

Participant identifier	Age as of collection date	Listed sex	Drug tested	History/symptoms with drug
DHS001	52	F	SMX	Rash (unspecified)
DHS002	72	F	SMX	No recollection
DHS003	48	F	SMX	Oral blistering (SJS?)
DHS004	51	F	AMX	Serum-sickness like
DHS005	22	F	AMX	Morbilliform rash
DHS006	63	M	AMX	SJS
DHS007	24	F	AMX	Rash (unspecified)
DHS008	68	F	AMX and SMX	Pruritic rash, requiring hospitalization

Table 4-2: List of control participants that participated in the study, recruited from Western University, in London, Ontario, Canada

Participant identifier	Age as of collection date	Listed sex	Drug tested	History/symptoms with drug
C001	27	F	SMX	No recollection
C002	21	F	SMX	Never taken sulfa
C003	60	F	SMX	Never taken sulfa
C004	41	M	AMX	Taken before, no adverse reactions
C005	23	M	AMX	Taken before, no adverse reactions

C006	24	F	AMX	Taken before, no adverse reactions
C007	24	M	AMX	Taken before, no adverse reactions
C008	26	M	AMX and SMX	Taken AMX before, unsure about SMX

4.2 Scintillation Counting Results

Control and SMX/AMX participant PBMCs were also incubated with the drug and ^{3}H-thymidine to determine if the drug stimulated DHS participant PBMCs, resulting in the proliferation of cells and a high stimulation index (SI). SI is a ratio of unstimulated PBMCs to drug stimulated PBMCs. Typically, an SI of 2 is required to say that the PBMCs are responding to the drug *in vitro*.

4.2.1 Scintillation Counting Results from SMX-Hypersensitive Participants

When control and DHR participant PBMCs were incubated with SMX for 54 hrs, there was no significant change in SI (Figure 4-1), and there was no trend observed for either control or DHR participants (ie. no overall increase or decrease). There were no SIs at or greater than 2, therefore none shows a positive proliferative response to the drug treatment.

Control and DHR participant PBMCs were also incubated with SMX for 4 days (Figure 4-2). Again, none of the concentrations of SMX were found to be significant. None of the participants had a SI greater than 1.2.

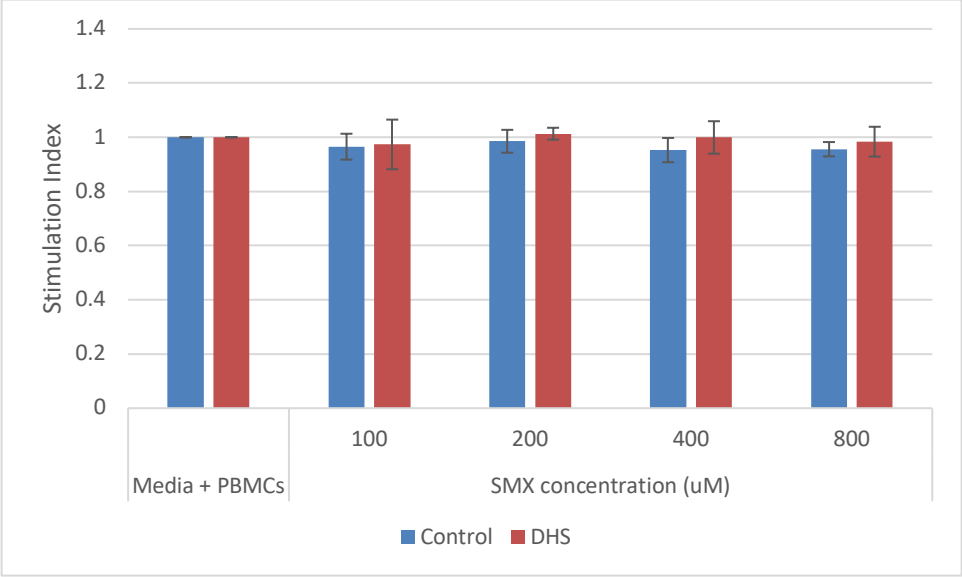


Figure 4-1: SI of control and DHS participants after incubation with SMX for 54 hrs

A repeated measures ANOVA was conducted to determine if the concentration of SMX had an effect on the proliferation of control and DHS PBMCs. Mauchly's test of sphericity was not significant ($p = 0.263$), therefore the assumption was met and a Greenhouse-Geisser correction was not used. $F(4, 24) = 0.443$, $p = 0.776$. N(control) = 4 and N(DHR) = 4, ± SEM.

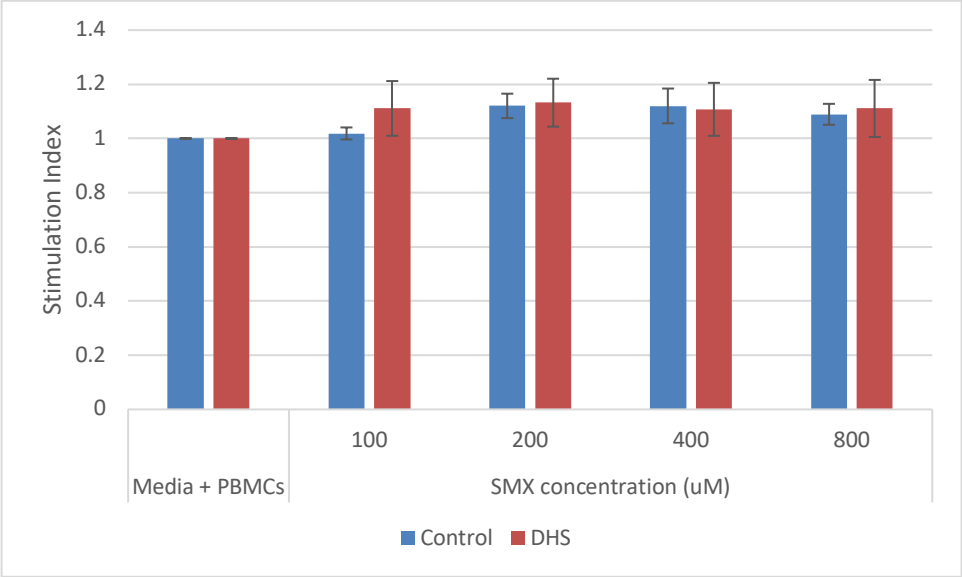


Figure 4-2: SI of control and DHS participants after incubation with SMX for 4 days

A repeated measures ANOVA was conducted to determine if the concentration of SMX had an effect on the proliferation of control and DHS PBMCs. Mauchly's test of sphericity was not significant ($p = 0.468$), therefore the assumption was met and a Greenhouse-Geisser correction was not used. $F(4, 24) = 3.234$, $p = 0.029$. N(control) = 4 and N(DHR) = 4, ± SEM.

4.2.2 Scintillation Counting Results from AMX-Hypersensitive Participants

In addition to SMX, AMX participants were also recruited. Participant PBMCs were incubated with AMX for 54 hrs (Figure 4-3). No participant had a SI greater than 1.2, and no significant differences between control and DHR participant or unstimulated and drug concentration were found. When incubated with AMX for four days, there were no significant differences between control and DHR participant, or between unstimulated and drug stimulated (Figure 4-4).

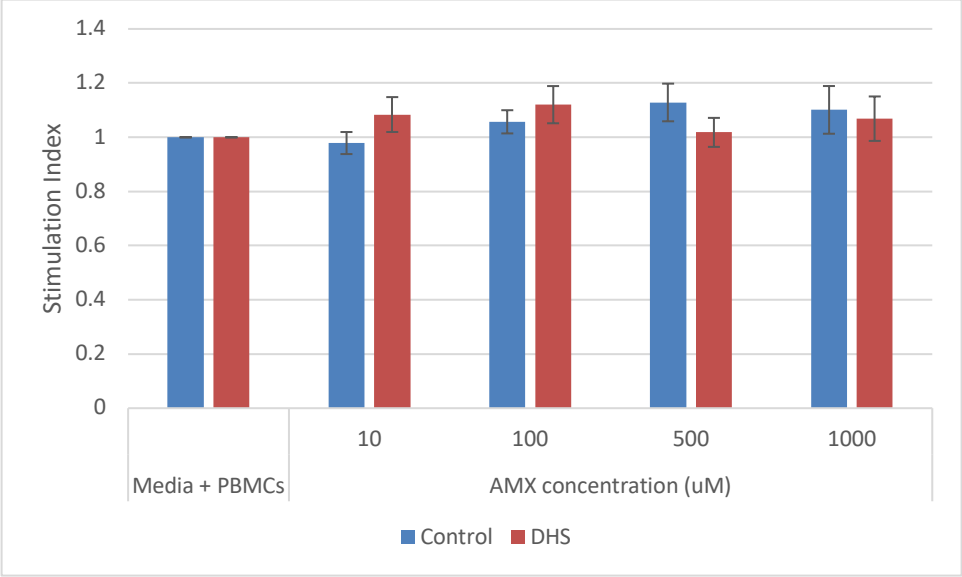


Figure 4-3: SI of control and DHS participants after incubation with AMX for 54 hrs

A repeated measures ANOVA was conducted to determine if the concentration of SMX had an effect on the proliferation of control and DHS PBMCs. Mauchly's test of sphericity was not significant ($p = 0.606$), therefore the assumption was met and a Greenhouse-Geisser correction was not used. $F(4, 32) = 1.457$, $p = 0.238$. N(control) = 4 and N(DHR) = 4, ± SEM.

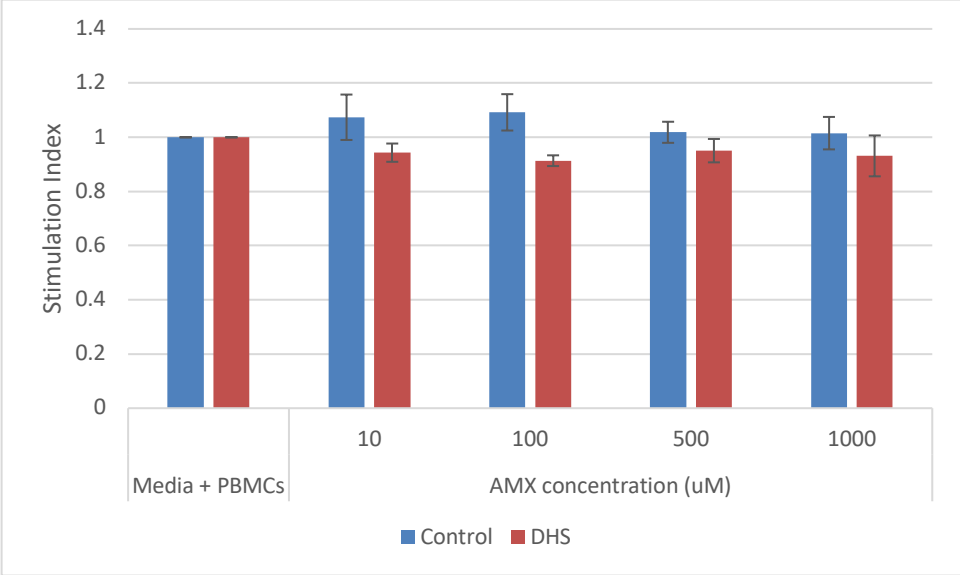


Figure 4-4: SI of control and DHS participants after incubation with AMX for 4 days

A repeated measures ANOVA was conducted to determine if the concentration of SMX had an effect on the proliferation of control and DHS PBMCs. Mauchly's test of sphericity was not significant ($p = 0.681$), therefore the assumption was met and a Greenhouse-Geisser correction was not used. $F(4, 32) = 0.296$, $p = 0.878$. N(control) = 4 and N(DHR) = 4, ± SEM.

4.3 Flow Cytometry Results

4.3.1 Representative Flow Cytometry Charts

To gate the PBMCs analyzed by flow cytometry, first live cells were gated according to viability dye (Figure 4-5-i). Then, single cells were gated until the cells could be gated as $CD3^+$ and $CD3^-$ (Figure 4-5-v). $CD3^+$ cells were further gated for being $CD69^+$ (4-5-vi), $CD4^+$ (4-5-vii), and $CD8^+$ (4-5-viii). Both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were gated for CD69 (4-5-x and 4-5-xi, respectively). In addition, CD3- cells were also examined for CD69 expression (Figure 4-5-ix). Just as CD69 expression can be represented as dot plots, it can also be presented as MFI of CD69 histograms (Figure 4-10). All gating and analyses were performed on FlowJo. All participants (both control and DHR) were gated in the same way.

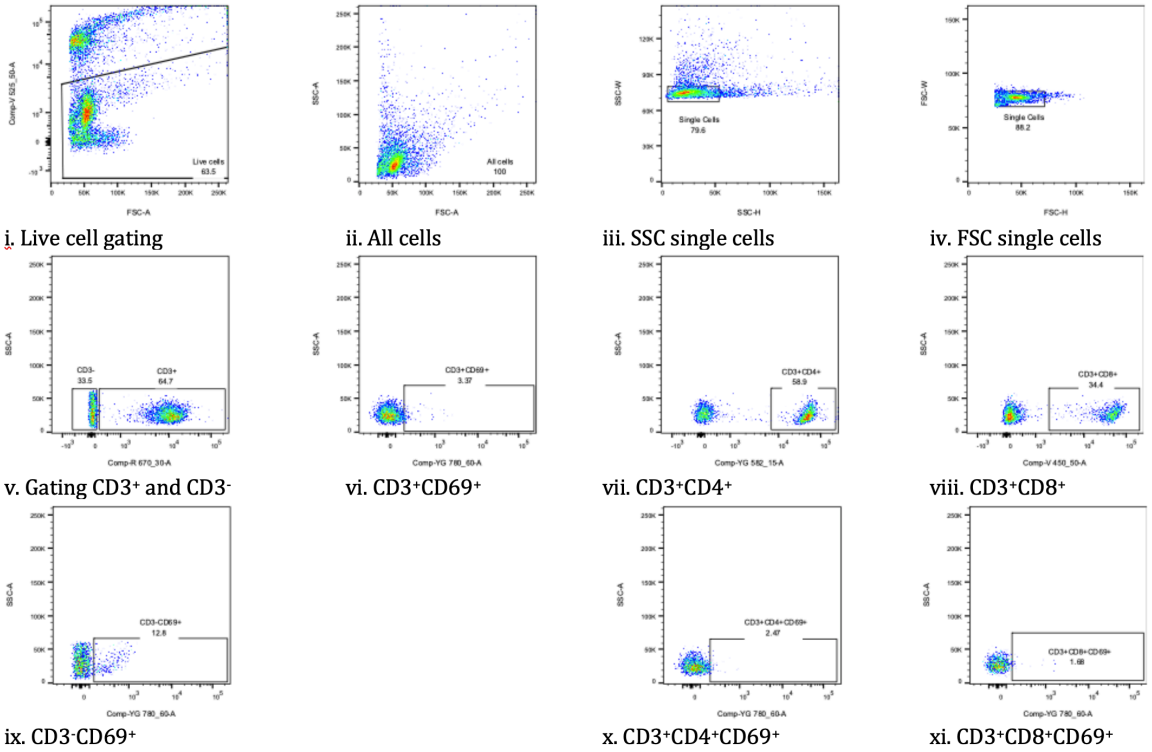
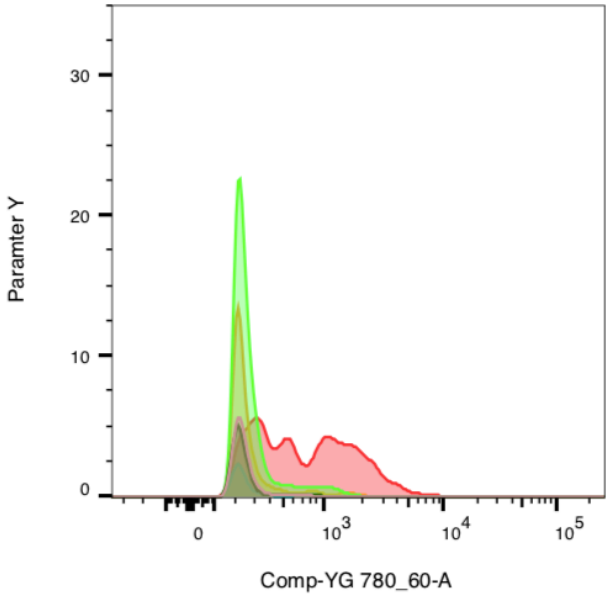


Figure 4-5: Representative flow cytometry dot plot and gating procedure



	Sample Name	Subset Name	Count
	DHS005_66_DHS005_10uM AMX_046.fcs	CD3+CD69+	66.0
	DHS005_63_DHS005_100uM AMX_043.fcs	CD3+CD69+	58.0
	DHS005_60_DHS005_500uM AMX_040.fcs	CD3+CD69+	271
	DHS005_57_DHS005_1000uM AMX_037.fcs	CD3+CD69+	146
	DHS005_53_DHS005_control unstim_033.fcs	CD3+CD69+	30.0
	DHS005_50_DHS005_antiCD3_030.fcs	CD3+CD69+	326

Figure 4-6: Representative MFI of CD69 expression histogram

4.3.2 Participants were Eliminated According to Cell Viability and Percent of $CD3^+$ Cells

The percent of live cells remaining after incubation with both SMX and AMX was assessed for each control and DHR participant. In addition to CD69 expression, cell viability and the percent of T cells ($CD3^+$) of total cells acquired were looked at. Cell viability was measured using a viability stain that enters cells with compromised cellular membranes, and binds to proteins within the cell. Cells positively stained with the dye can be gated as dying or dead cells on the flow cytometer and can be excluded from subsequent analysis.

Four DHR participants and three control participants were tested in culture media (unstimulated), four concentrations of SMX (100, 200, 400, 800 μM), and anti-CD3 (data not shown for anti-CD3). Within the DHR participants (DHS001-003, 008) tested (see Figure 4-7), two participants had cell viability under 50% across all conditions (DHS001, average cell viability of 38.12%, and 002, average cell viability of 9.18%). Two SMX concentrations could not be acquired in DHS008 (100μM and 200μM). Within the three control participants (Control001-003) tested (see Figure 4-8), Control002 had less than 50% viability across all conditions (average cell viability of 14.43%). In Control001, only the SMX 800μM condition had viability at slightly under 50%, all other conditioners were higher. Because of these low viabilities, DHS001, DHS002, and Control002 were excluded from subsequent analyses.

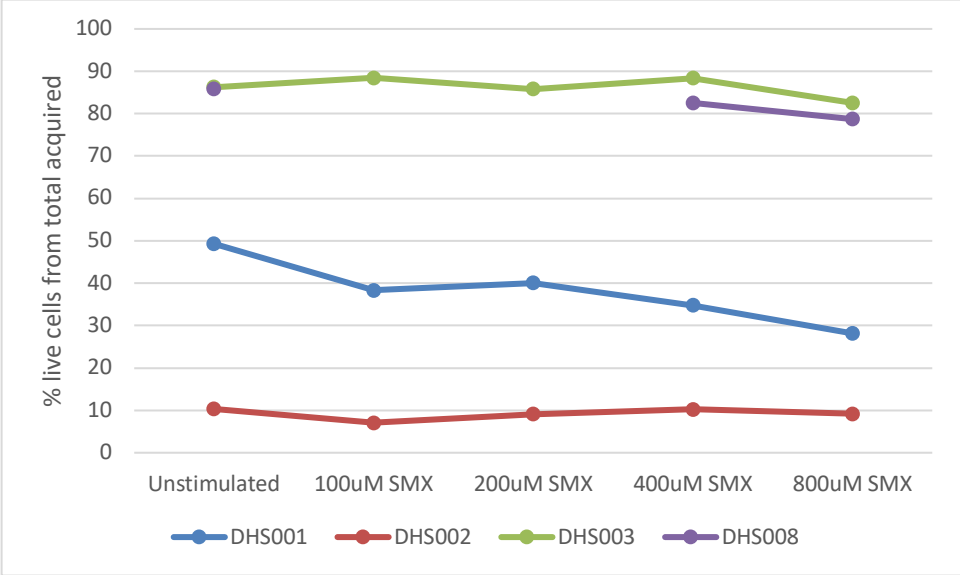


Figure 4-7: Percent of live PBMCs analyzed from total number of DHR participant PBMCs after incubation with concentrations of SMX, acquired by flow cytometry, as a measure of cell viability

PBMCs were incubated with culture media (unstimulated), anti-CD3, and SMX at concentrations 100µM, 200µM, 400µM, and 800µM.

Figure 4-8: Percent of live PBMCs analyzed from total number of control participant PBMCs after incubation with concentrations of SMX, acquired by flow cytometry, as a measure of cell viability

PBMCs were incubated with culture media (unstimulated), anti-CD3, and SMX at concentrations 100µM, 200µM, 400µM, and 800µM.

In addition to SMX, five DHR participants and four control participants were tested with culture media (unstimulated), four concentrations of AMX (10µM, 100µM, 500µM, 1000µM), and anti-CD3. All participants, both DHR (see Figure 4-9) and control (see Figure 4-10), had cell viability greater than 50% (lowest viability was 70.77% and greatest was 90.39%), therefore all were included in subsequent analyses.

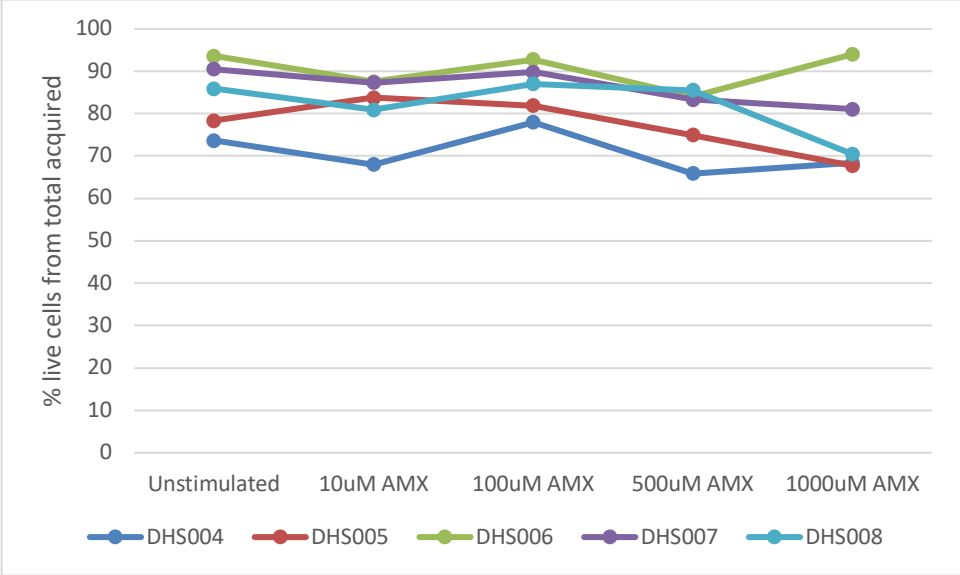


Figure 4-9: Percent of live PBMCs analyzed from total number of DHR participant PBMCs after incubation with AMX, acquired by flow cytometry, as a measure of cell viability

PBMCs were incubated with culture media (unstimulated), anti-CD3, and AMX at concentrations 10µM, 100µM, 500µM, and 1000µM.

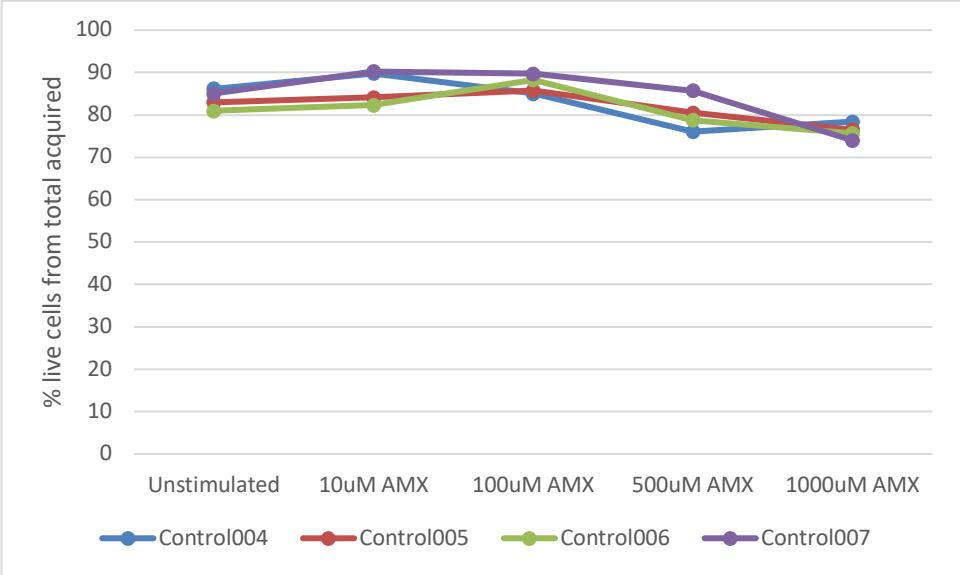


Figure 4-10: Percent of live PBMCs analyzed from total number of control participant PBMCs after incubation with AMX, acquired by flow cytometry, as a measure of cell viability

PBMCs were incubated with culture media (unstimulated), anti-CD3, and AMX at concentrations 10µM, 100µM, 500µM, and 1000µM.

The percent of $CD3^+$ T cells was also analyzed by flow cytometry. Within the SMX DHR participants (see Figure 4-11), the percentages of $CD3^+$ at each concentration were very different. DHS001 and DHS002 both had low percent of $CD3^+$ T cells (averages of 14.85% and 0.54%, respectively). In the SMX control participants (see Figure 4-12), Control002 also had a very low percentage of $CD3^+$ T cells acquired (approximately 1.31% across all conditions).

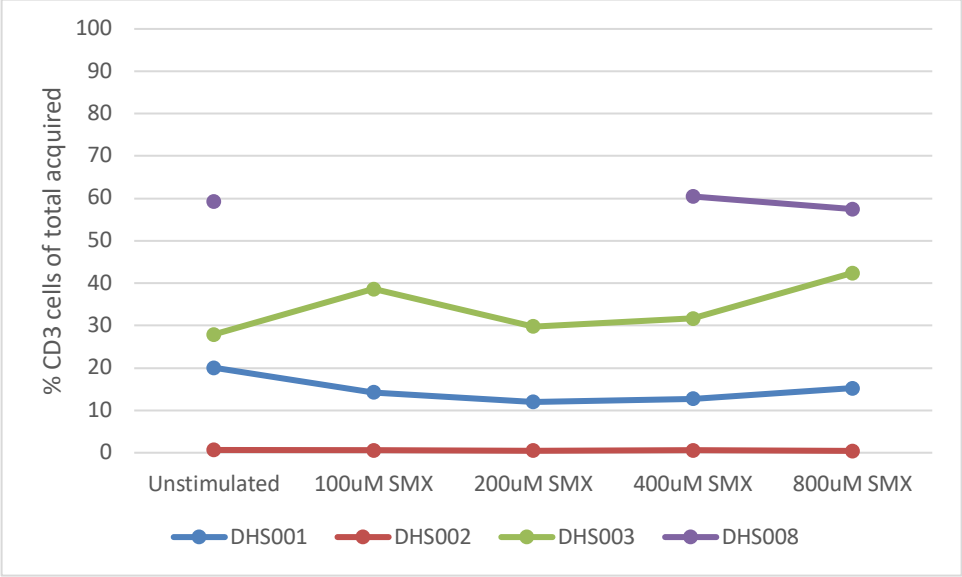


Figure 4-11: Percent of CD3+ cells analyzed from total number of DHR participant PBMCs after incubation with SMX, acquired by flow cytometry

PBMCs were incubated with culture media (unstimulated), anti-CD3, and SMX at concentrations 100µM, 200µM, 400µM, and 800µM.

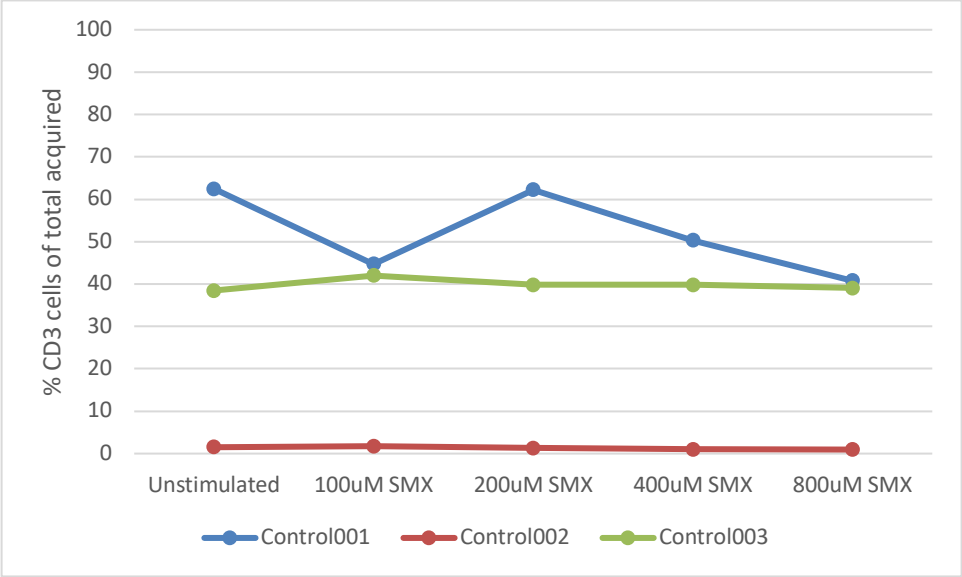


Figure 4-12: Percent of CD3+ cells analyzed from total number of control participant PBMCs after incubation with SMX, acquired by flow cytometry

PBMCs were incubated with culture media (unstimulated), anti-CD3, and SMX at concentrations 100µM, 200µM, 400µM, and 800µM.

As for AMX control (Figure 4-13) and DHR participants (Figure 4-14), most percentages of $CD3^+$ T cells were high, except for DHS006, which was very low (average of approximately 0.74% across all conditions). Because of this, DHS006 was removed from all subsequent analyses.

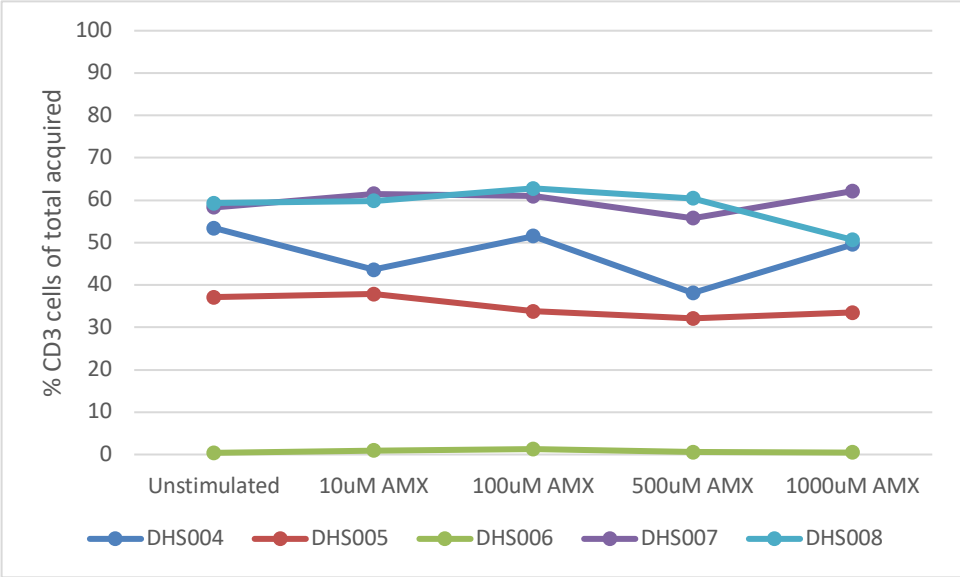


Figure 4-13: Percent of CD3+ cells analyzed from total number of DHR participant PBMCs after incubation with AMX, acquired by flow cytometry

PBMCs were incubated with culture media (unstimulated), anti-CD3, and AMX at concentrations 10µM, 100µM, 500µM, and 1000µM.

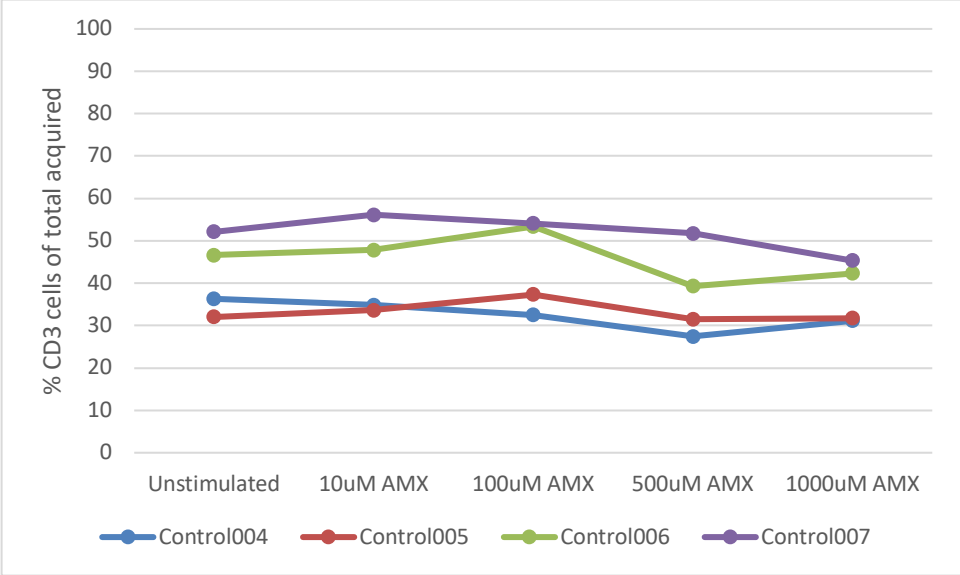


Figure 4-14: Percent of CD3+ cells analyzed from total number of control participant PBMCs after incubation with AMX, acquired by flow cytometry
PBMCs were incubated with culture media (unstimulated), anti-CD3, and AMX at concentrations 10µM, 100µM, 500µM, and 1000µM.

4.3.3 Control and DHR Participant Results with SMX Incubation

Two control and DHR participant results were analyzed after participants had been removed, and graphed according to percent of CD69 expression and MFI of CD69 across $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^-$ cell types as a result of incubation with increasing concentrations of SMX (Figures 4-15 to 4-30).

With $CD3^+$ cells (Figure 4-15 to 4-18), there was no increase in proliferation across concentrations, and no detectable difference between control and DHR participants. With percent CD69 (Figure 4-15), the only participant that had some increase beyond unstimulated was DHS008 at 800µM SMX. However, when averaged with DHS003 (Figure 4-16), the average did not appear to be different from unstimulated. As for the individual MFI values (Figure 4-17), all values of control and DHR participant were at or below that of unstimulated regardless of concentration, which is also reflected in the averaged values (Figure 4-18).

It is important to note that as sample size of SMX participants is small, no statistics were calculated.

With helper ($CD3^+CD4^+$) T cells, similarly to $CD3^+$, there were no noticeable increases. With percent of CD69 expression (Figure 4-19 and 4-20), all normalized values were below normalized unstimulated levels. Only DHS008 had an increase between 400µM and 800µM SMX, however both values were not greater than unstimulated. MFI values were also lower than unstimulated for all concentrations (Figure 4-21 and 4-22). Control001 showed an odd increase compared to unstimulated in MFI with 100µM SMX, but was below unstimulated for all other concentrations (seen in Figure 4-21).

As for $CD3^+CD8^+$, overall there were no noticeable differences between the concentrations of SMX. For percent CD69 (Figure 4-23), again DHS008 had a slight increase between 400uM and 800uM SMX, but not greater than unstimulated. Control001 had a slight increase over unstimulated for 100µM and 200µM SMX, but returned to at or below unstimulated in the higher concentrations. On average (Figure 4-24), no concentrations showed an effect. With MFI (Figures 4-25 and 4-26), Control001, 003 and DHS003 all had similar values, with none of the MFI values greater than unstimulated, however DHS008 MFI values at 400 and 800µM were very close to 0 when normalized to unstimulated (Figure 4-25). On average, no overall differences were seen between the groups.

Similar patterns were seen again with $CD3^-$ cell types (Figures 4-27 to 4-30). With percent CD69 (Figures 4-27 and 4-28), DHS008 showed again an increase between 400µM and 800µM, which was slightly above unstimulated. Control001 showed an increase above normalized unstimulated for all concentrations, while Control003 and DHS003 values were all below normalized unstimulated. Overall, none of the average values differed from unstimulated (Figure 4-28). With MFI of CD69, Control001, Control003, DHS003 all were very similar to unstimulated values across all concentrations. Only DHS008 showed a low MFI at 400µM and 800µM concentrations. Overall the MFI of control values did not differ from unstimulated (Figure 4-29 and 4-30), with DHS only slightly lower due to DHS008.

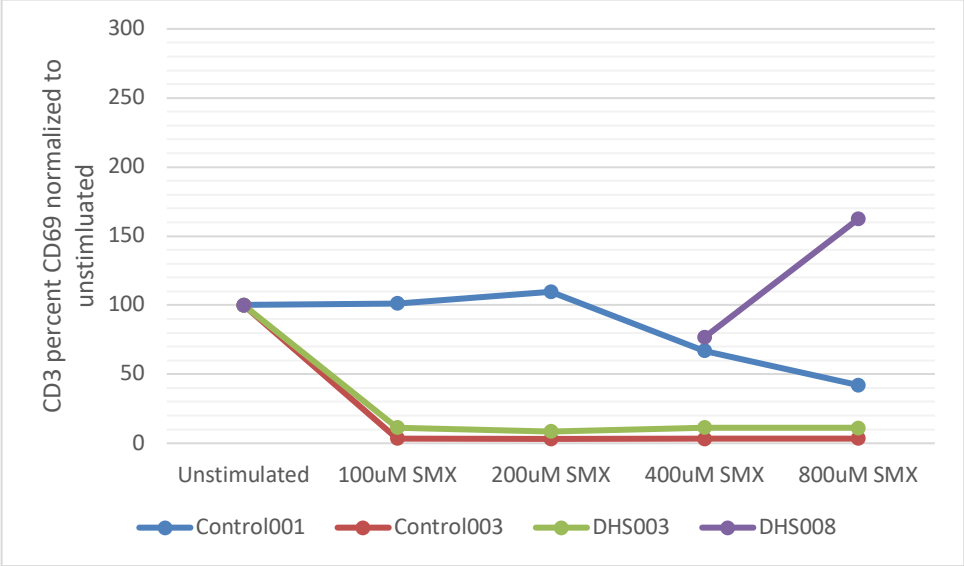


Figure 4-15: Percent of CD3+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

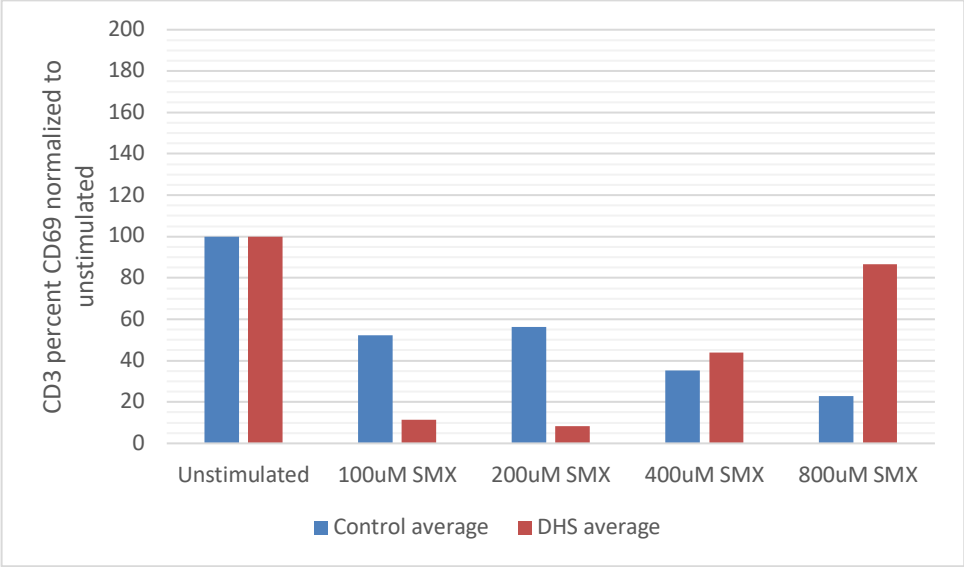


Figure 4-16: Average percent of CD3+ T cells expressing CD69 normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

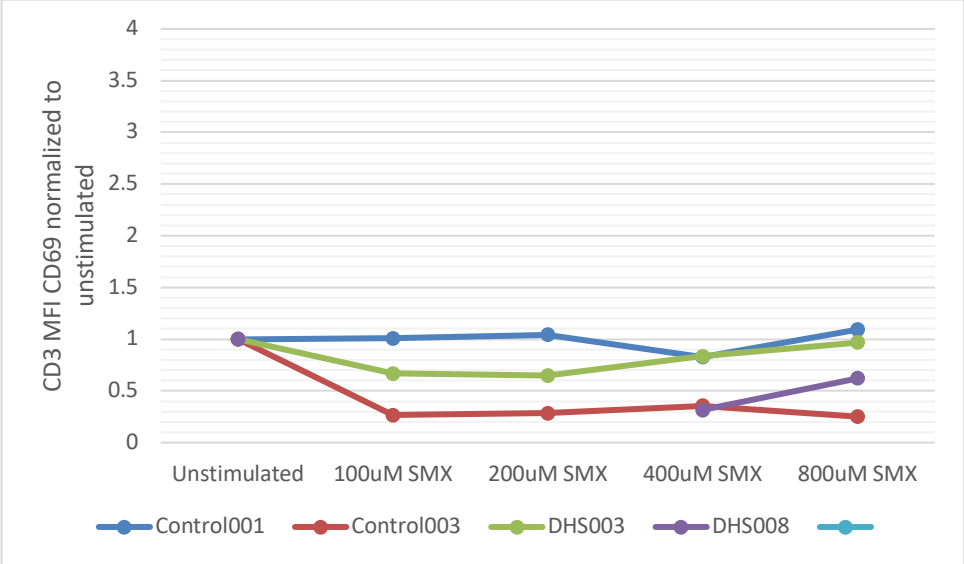


Figure 4-17: MFI of CD3+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

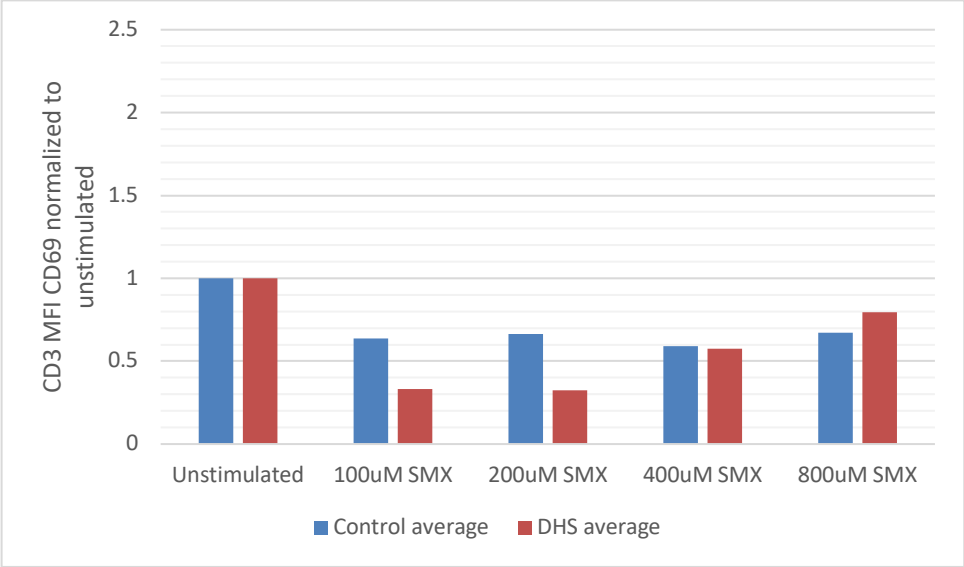


Figure 4-18: Average MFI of CD69 on CD3+ T cells normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

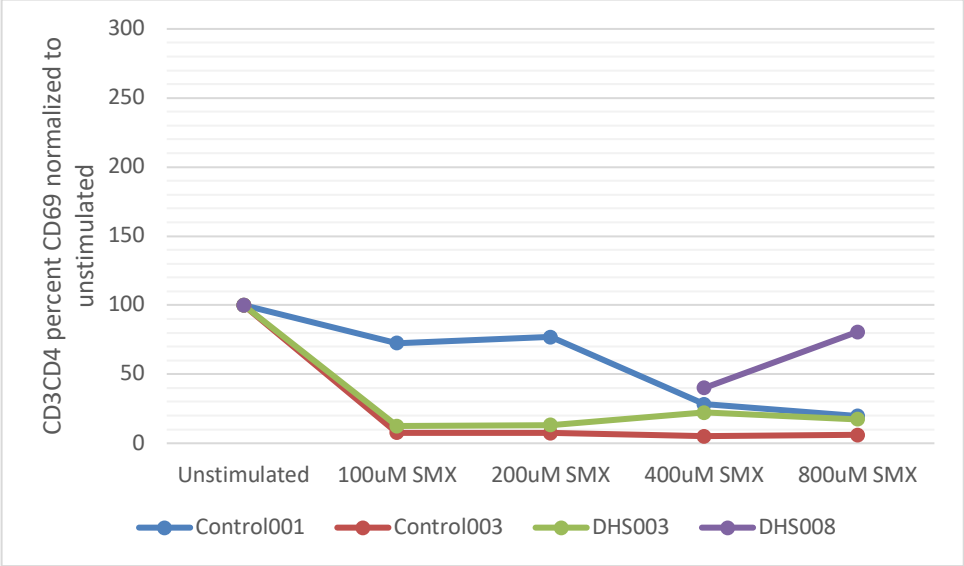


Figure 4-19: Percent of CD3+CD4+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

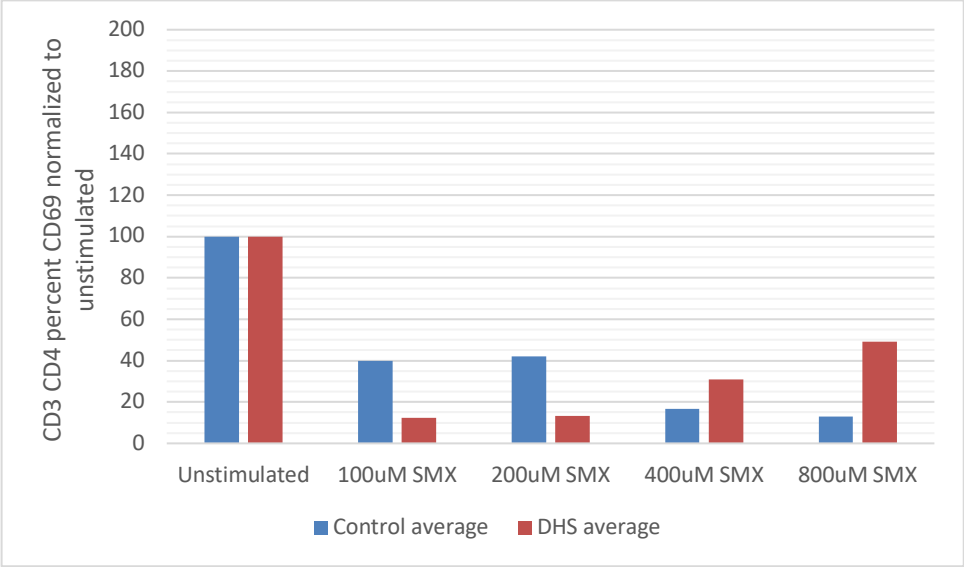


Figure 4-20: Average percent of CD3+CD4+ T cells expressing CD69 normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

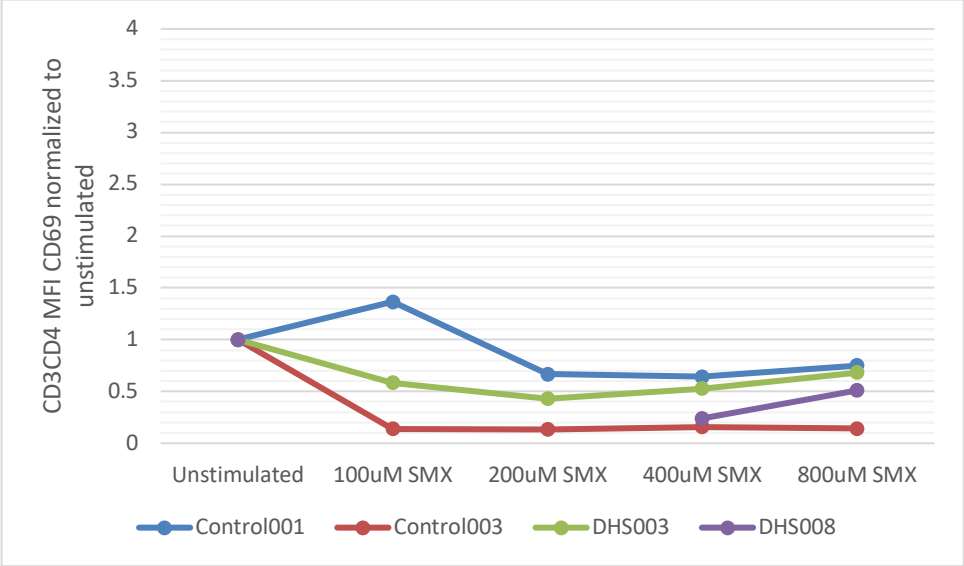


Figure 4-21: MFI of CD3+CD4+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

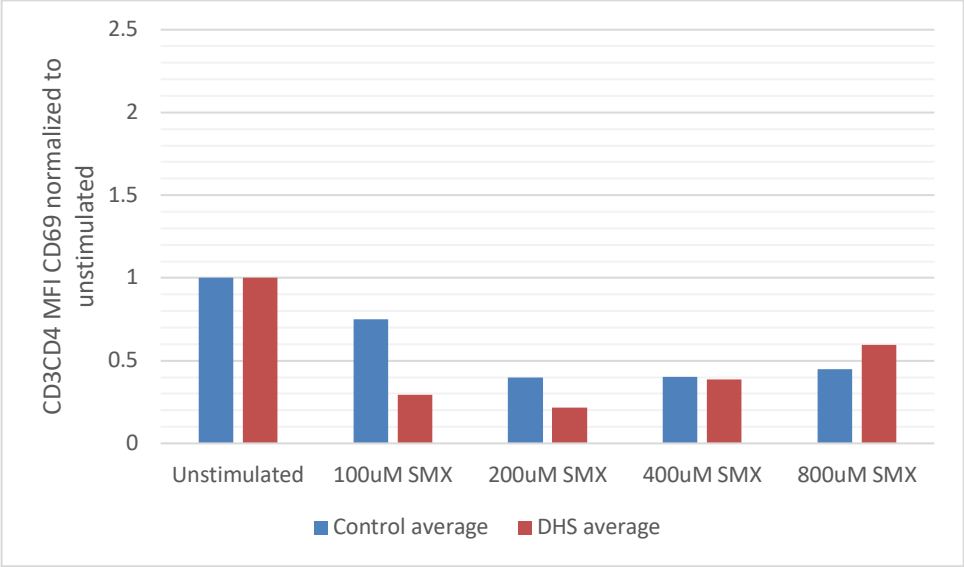


Figure 4-22: Average MFI of CD69 on CD3+CD4+ T cells normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

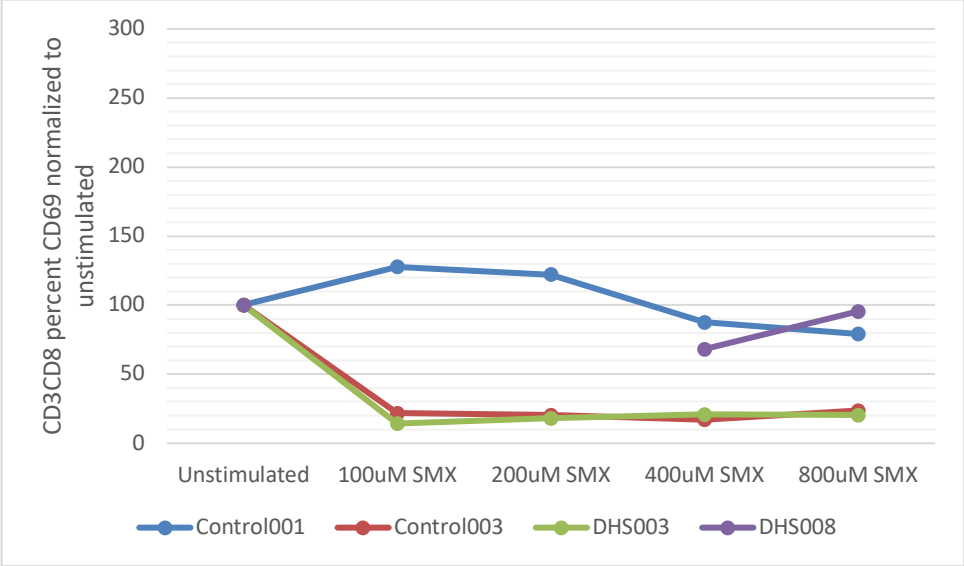


Figure 4-23: Percent of CD3+CD8+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

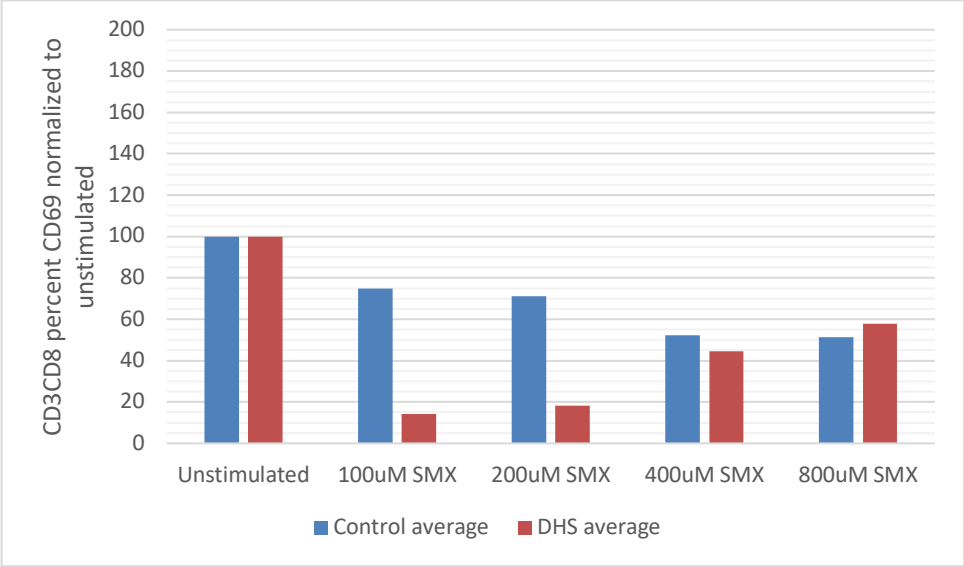


Figure 4-24: Average percent of CD3+CD8+ T cells expressing CD69 normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

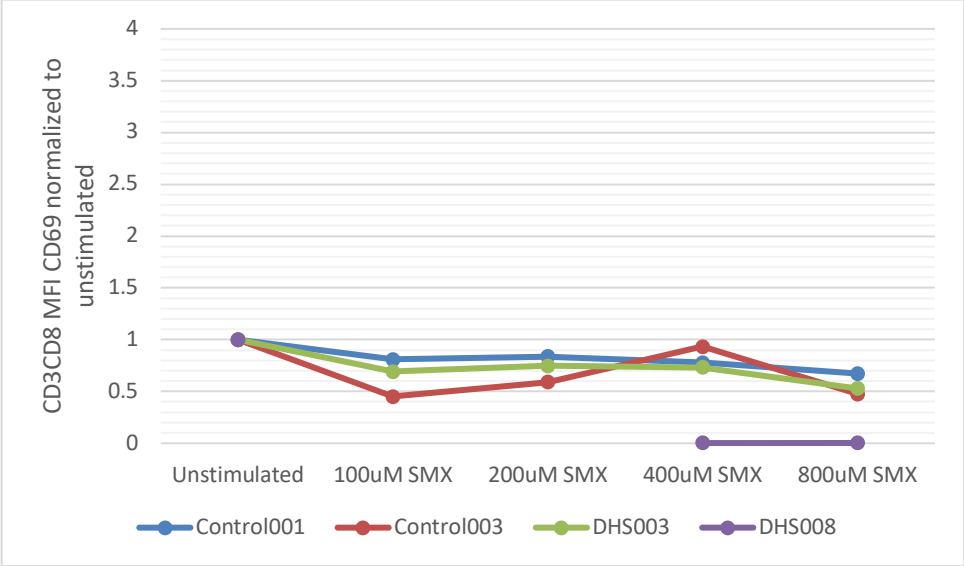


Figure 4-25: MFI of CD3+CD8+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

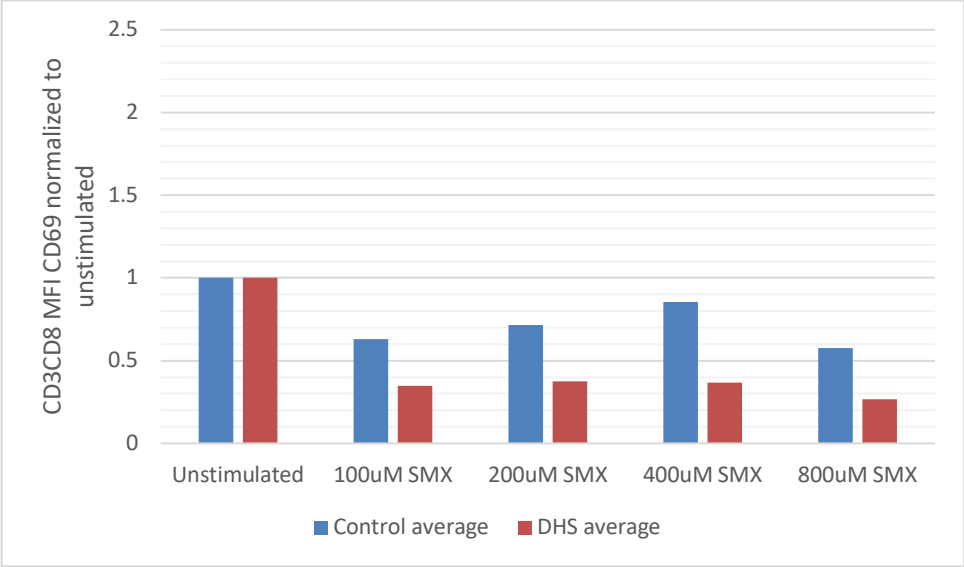


Figure 4-26: Average MFI of CD69 on CD3+CD8+ T cells normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

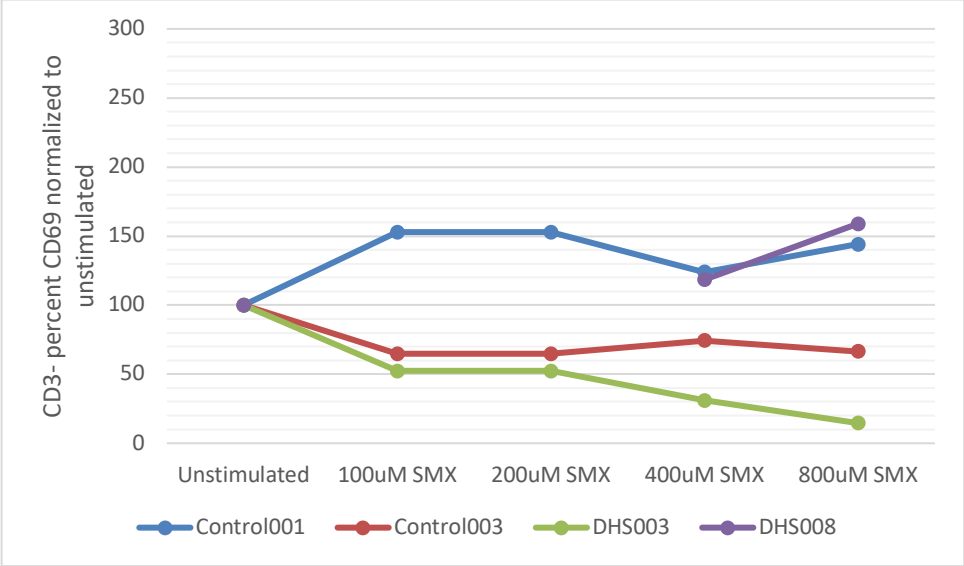


Figure 4-27: Percent of CD3- PBMCs expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

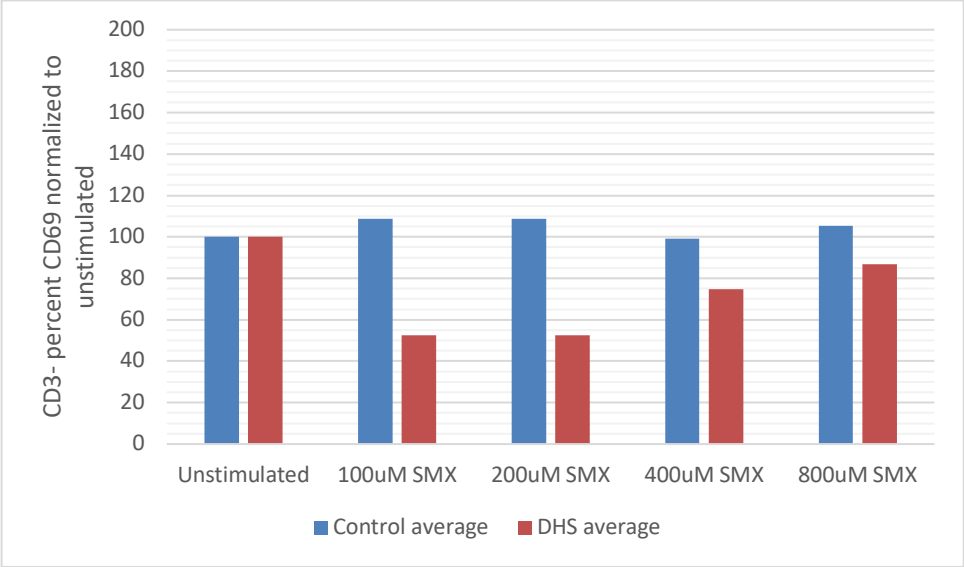


Figure 4-28: Average percent of CD3- PBMCs expressing CD69 normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

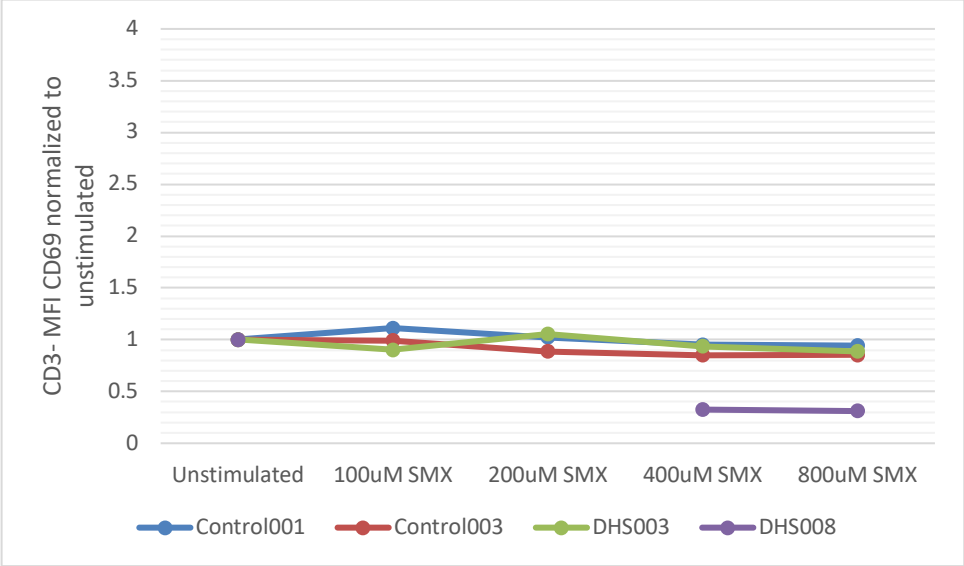


Figure 4-29: MFI of CD3- PBMCs expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

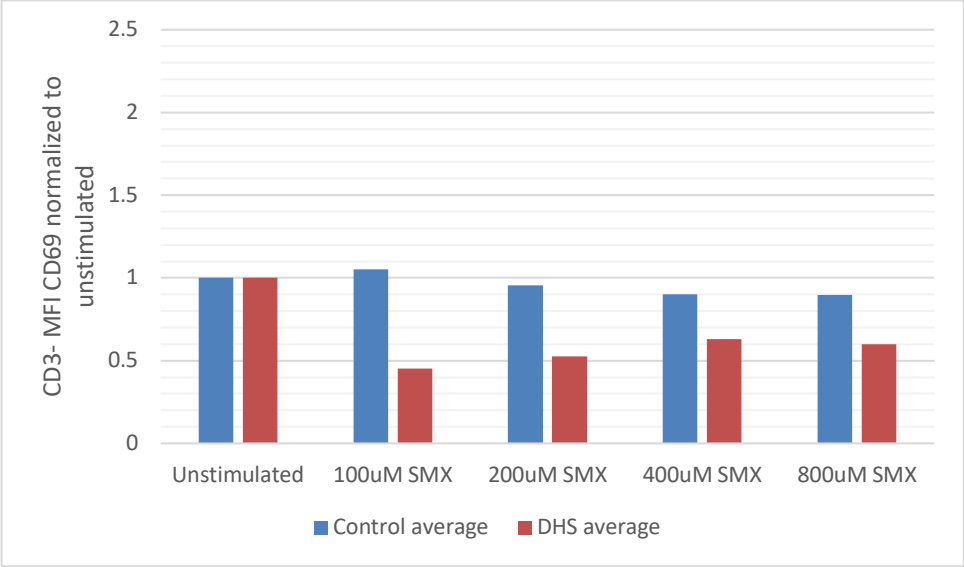


Figure 4-30: Average MFI of CD69 on CD3- PBMCs normalized to unstimulated control, as a result of control and DHS participant PBMCs incubated with culture media (unstimulated control) and four concentrations of SMX

4.3.4 Control and DHR Participant Results with AMX Incubation

A total of four control participants and four DHR participants for AMX were recruited for this study, which allowed statistical analyses to be performed, unlike with SMX.

With $CD3^+$ percent CD69 (Figure 4-31), Control004 was similar or lower to unstimulated across all concentrations, Control005 was highest at 10µM AMX but decreased to unstimulated levels at 1000µM. Control006 was similar to unstimulated but lowered a lot by 1000µM, and Control007 was lower than unstimulated through 500µM, but increased slightly past unstimulated at 1000µM. DHS004 was mostly similar to unstimulated except at 100µM AMX, and DHS005, while initially lower at than unstimulated at 10µM, increased above unstimulated values at 500µM and 1000µM. While DHS007 was consistent or lower than unstimulated, DHS008 had a spike at 10µM before returning around unstimulated levels. When comparing the average control and DHR participants across AMX concentrations (Figure 4-32), there were no significant differences observed. There was a slight but not significant increase in DHS averages at 10µM, 500µM, and 1000µM AMX, and a slight decrease at 100µM, but none were significantly different from unstimulated when analyzed by repeated measures ANOVA. Control participant averages were very similar to unstimulated values. When a repeated measures ANOVA was conducted on the control averages alone, the *p* value was not significant (> 0.05). MFI for each individual participant (Figure 4-33) did not reveal any differences, other than DHS007 slightly increasing as concentration increased, and DHS008 was very low in comparison to the unstimulated values and the other participants (both control and DHS). With the average MFI values (Figure 4-34), other than a very slight but not significant increase at 500µM for the control participants, everything was very similar to unstimulated values and there were no significant differences between group or concentration. When analyzed by repeated measures ANOVA, neither control participants or DHS participant MFI levels showed no significant differences between treatment concentrations and unstimulated.

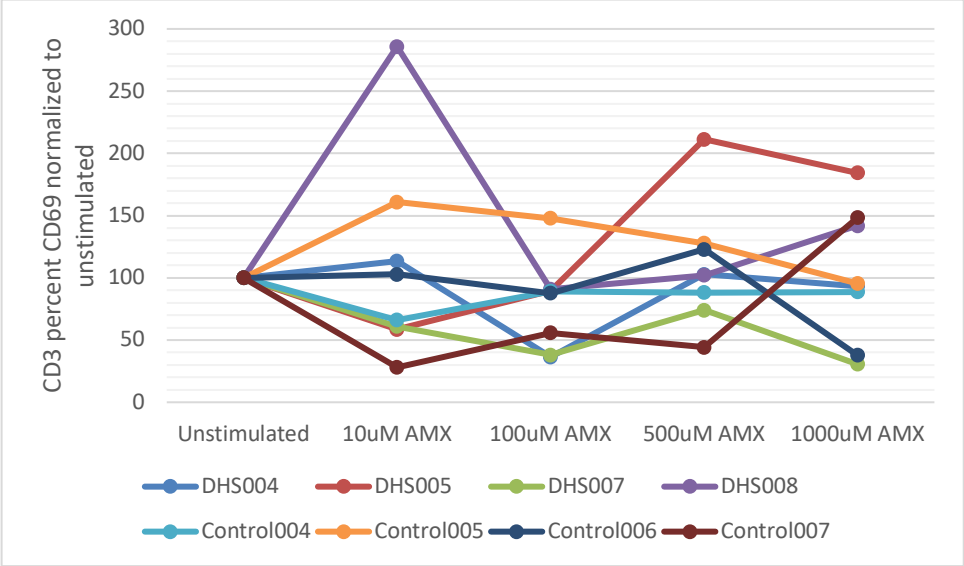


Figure 4-31: Percent of CD3+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

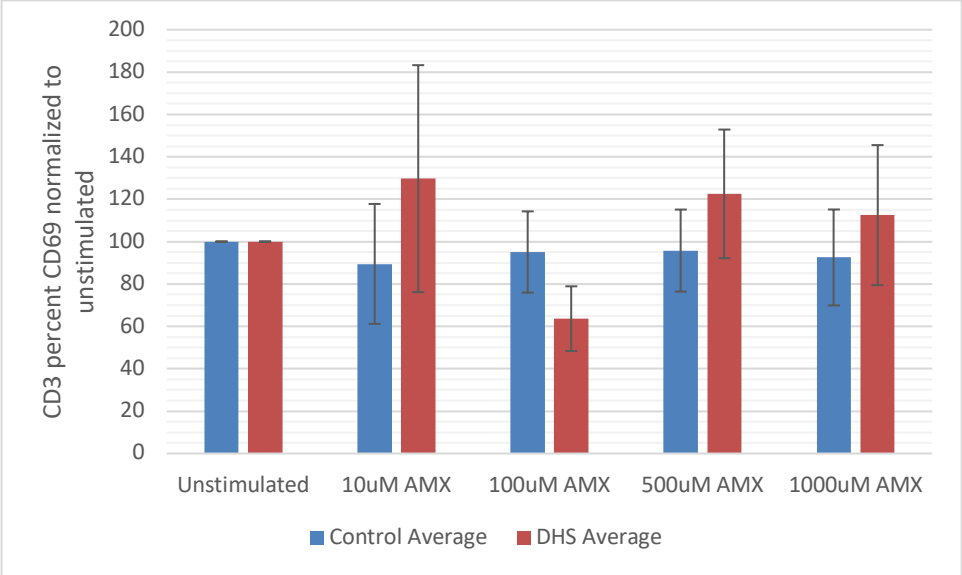


Figure 4-32: Percent of CD3+ T cells expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^+$ T cells. Mauchly's Test of Sphericity was insignificant ($p = 0.277$) and therefore the assumption of sphericity was met, and

therefore a Greenhouse-Geisser correction was not used. F(4,24) = 0.510, p = 0.729. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, therefore a Greenhouse-Geisser correction was used. F(1.281,3.842) = 0.043, *p* = 0.897. For DHS participants, Mauchly's Test of Sphericity was violated, therefore a Greenhouse-Geisser correction was used. F(1.443,4.329) = 0.815, *p* = 0.461.

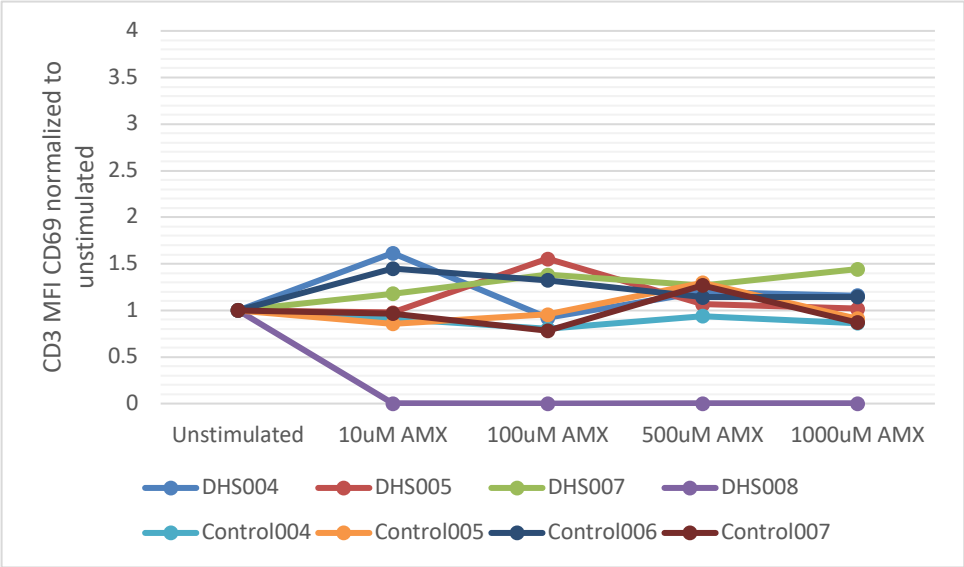


Figure 4-33: MFI of CD3+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

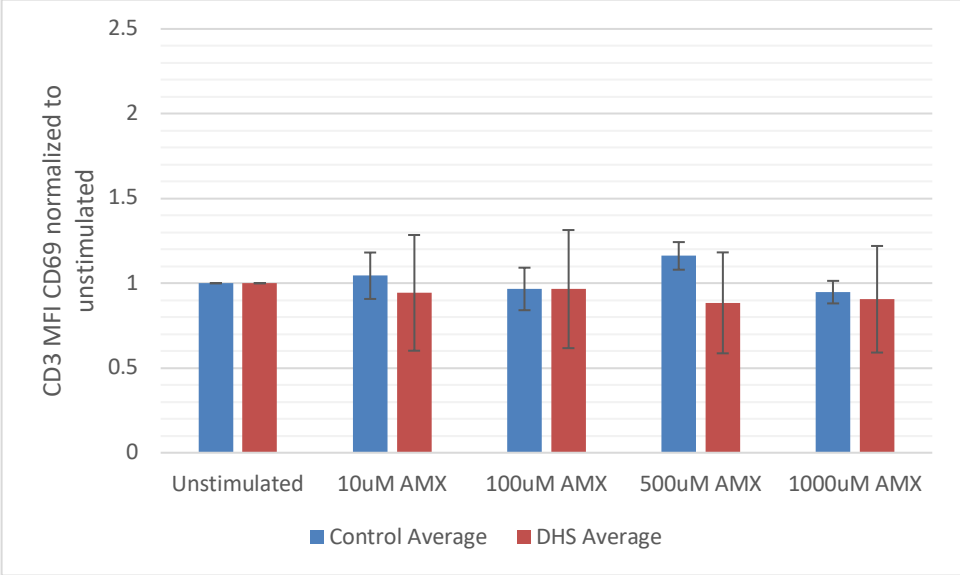


Figure 4-34: MFI of CD3+ T cells expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^+$ T cells. Mauchly's Test of Sphericity was insignificant ($p = 0.491$), the assumption of sphericity was met, and therefore a Greenhouse-Geisser correction was not used. $F(4,24) = 0.155$, $p = 0.959$. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.531,4.593) = 1.232$, $p = 0.355$. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.916,5.748) = 0.073$, $p = 0.925$.

With $CD3^+CD4^+$, in the individual percent (Figure 4-35), most participants were very similar to unstimulated. With the control participants, Control005 increased above unstimulated at 10µM but decreased back down to unstimulated by 1000µM AMX. Control006 decreased at 1000µM, while Control007 increased at 1000µM. For the DHR participants, DHS004 increased at 10µM, decreased below unstimulated at 100µM, then returned to unstimulated values at 500 and 1000µM. DHS005 increased from below

unstimulated at 10µM and 100µM to increase above unstimulated at 500µM and 1000µM AMX. DHS007 was consistently lower than unstimulated across all concentrations, and DHS008 was close to unstimulated. When comparing average values at the different concentrations (Figure 4-36), there were no noticeable differences between the groups and unstimulated. A repeated measures ANOVA indicated no significant differences between any treatment groups and unstimulated for both control participants and DHS participants. Control006 was slightly increased at 100µM, and DHS008 was low across all concentrations similar to the other cell types. Individual MFI values did not reveal any obvious differences between participants (Figure 4-37). Comparing average MFI values (Figure 4-38) did not reveal any significant differences between control and unstimulated or differences between any of the AMX concentrations.

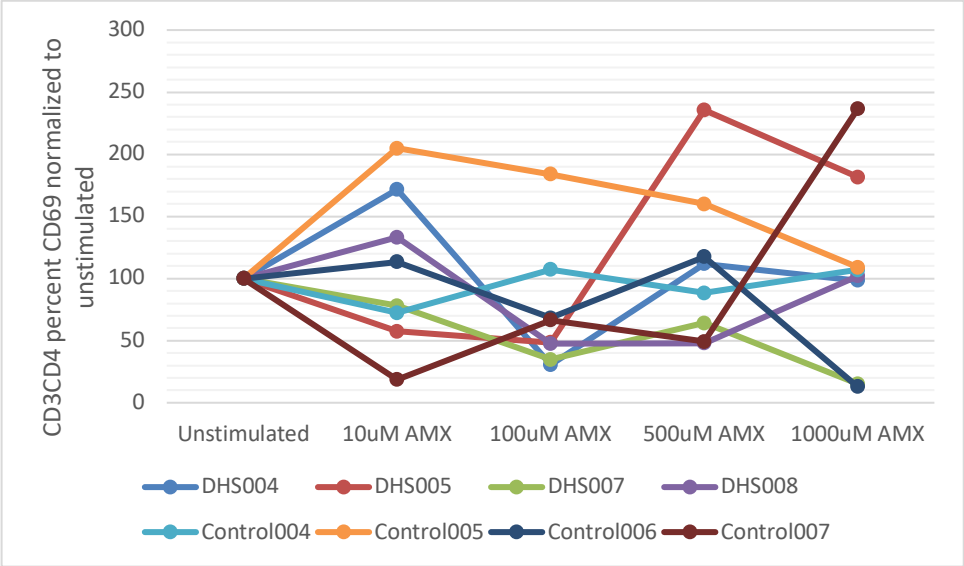


Figure 4-35: Percent of CD3+CD4+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

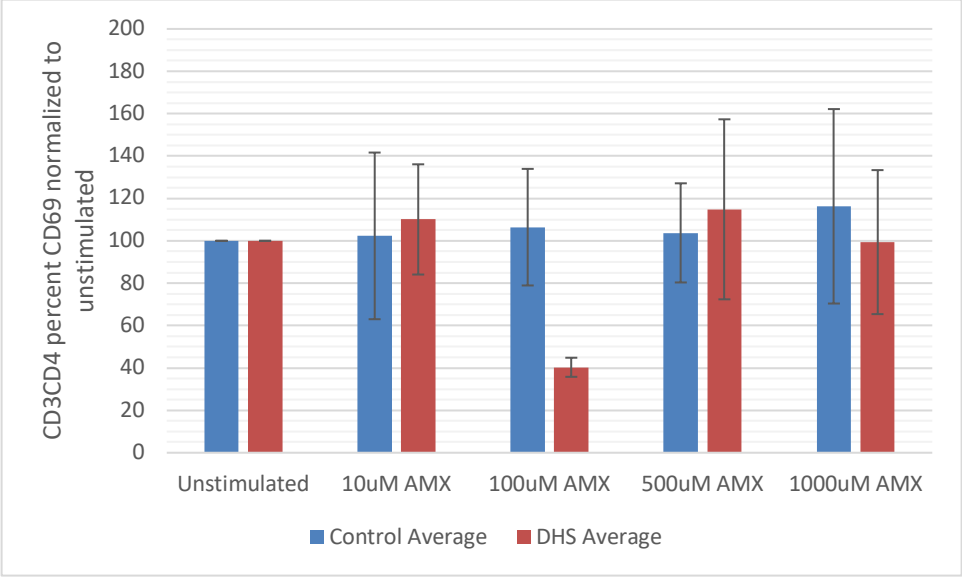


Figure 4-36: Percent of CD3+CD4+ T cells expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^{+}CD4^{+}$ T cells. Mauchly's Test of Sphericity was insignificant ($p = 0.471$), the assumption of sphericity was met, and therefore a Greenhouse-Geisser correction was not used. $F(4,24) = 0.555$, $p = 0.697$. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on $CD3^{+}CD69^{+}$ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.257,3.772) = 0.043$, $p = 0.893$. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.508,4.524) = 1.354$, $p = 0.330$.

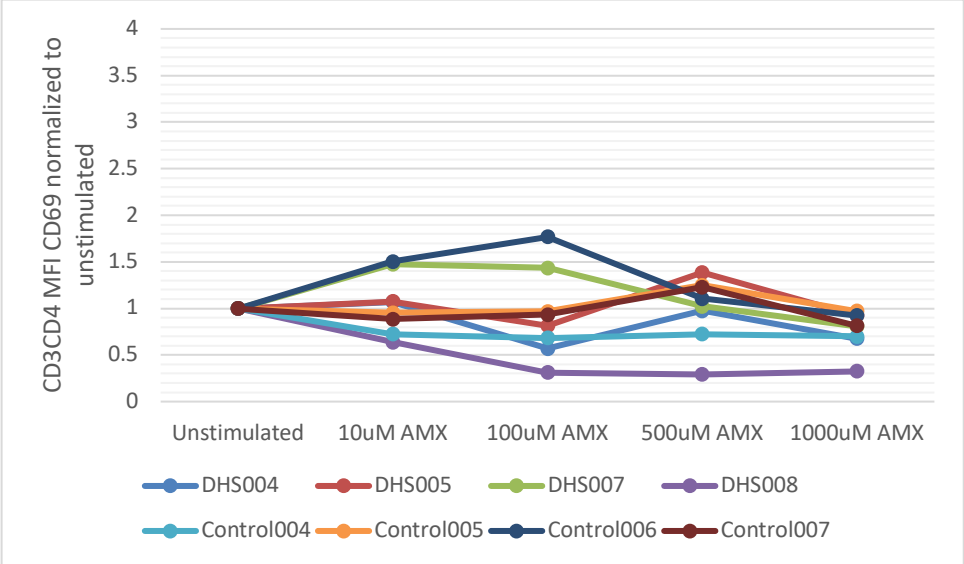


Figure 4-37: MFI of CD3+CD4+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

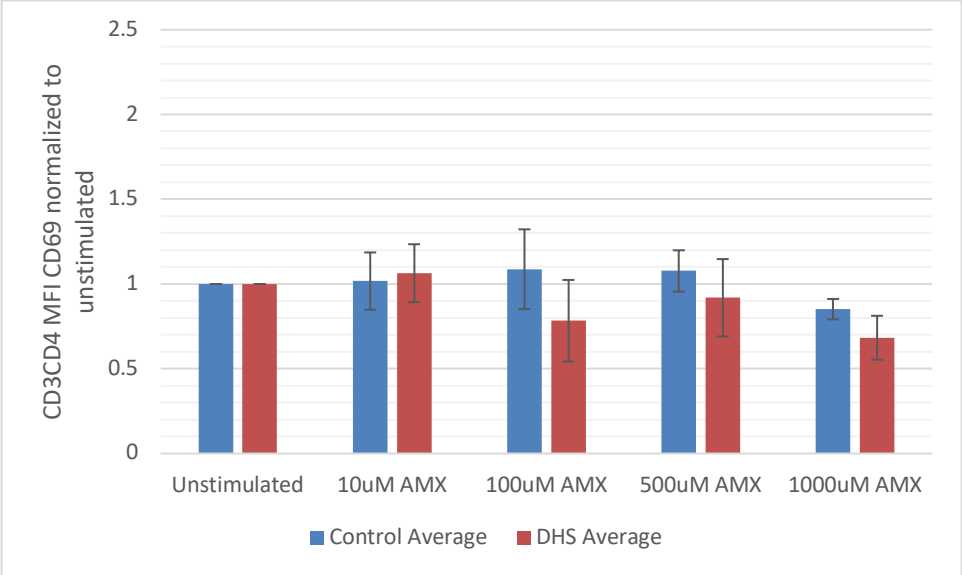


Figure 4-38: MFI of CD3+CD4+ T cells expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^{+}CD4^{+}$ T cells. Mauchly's Test of Sphericity was significant ($p = 0.012$), the assumption of sphericity was not met, and

therefore a Greenhouse-Geisser correction was used. F(1.832,10.995) = 1.685, $p = 0.230$. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on $CD3^+CD69^+$ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. F(1.384,4.151) = 0.699, $p = 0.496$. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. F(2.073,6.219) = 1.638, $p = 0.269$.

$CD3^+CD8^+$ cell types did not show many individual differences with CD69 expression (Figure 4-39); only control004 showed a slight increase as with increasing concentrations with a slight decrease from 500µM to 1000µM, and DHS005 showed an increase as concentration increased. No other participants visibly differed from the control. With the average percent CD69 values (Figure 4-40), there were no significant differences found with the repeated measures ANOVA, either when the data for control and DHS participants was combined or when analyzed separately. There was a noticeable difference at 100µM AMX between control and DHS participants, where the control participant average was slightly higher, but this difference was not significant overall with the repeated measures ANOVA. Student's t-test conducted between the control and DHS participants at 100uM had a p value of 0.069. With individual MFI values (Figure 4-41), all values were similar to unstimulated. When the average MFI values were compared (Figure 4-42), no significant differences were observed with a repeated measures ANOVA. However, there is a difference seen at 1000µM, where the DHS participants mean MFI was slightly higher than control. While this difference was not significant with the ANOVA, a one-tailed student's t-test revealed this comparison to have a p value of 0.0034.

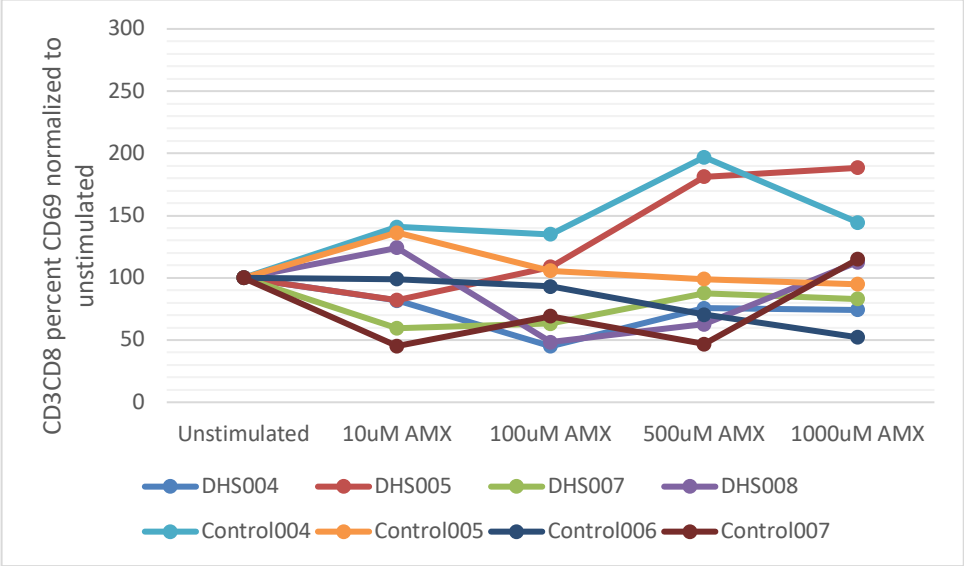


Figure 4-39: Percent of CD3+CD8+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

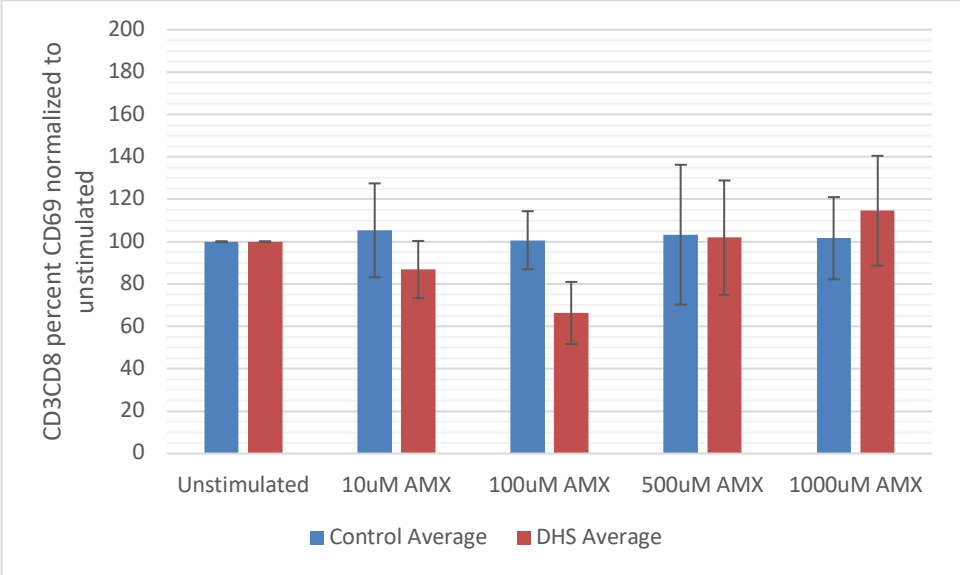


Figure 4-40: Percent of CD3+CD8+ T cells expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^{+}CD8^{+}$ T cells. Mauchly's Test of Sphericity was insignificant ($p = 0.168$), the assumption of sphericity was met, and

therefore a Greenhouse-Geisser correction was not used. F(4,24) = 0.714, p = 0.590. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. F(2.026,6.079) = 0.019, p = 0.982. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. F (1.439,4.318) = 1.420, p = 0.318.

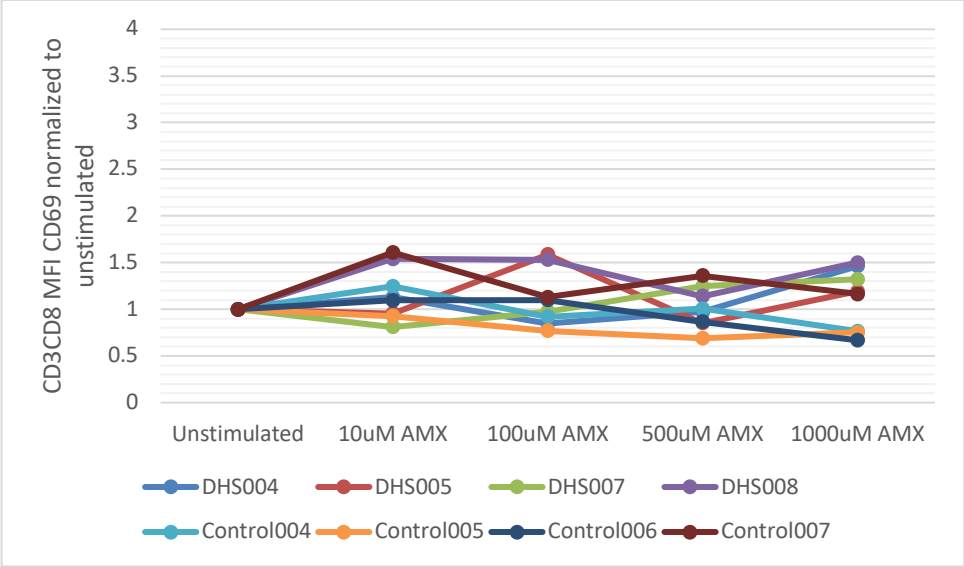


Figure 4-41: MFI of CD3+CD8+ T cells expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

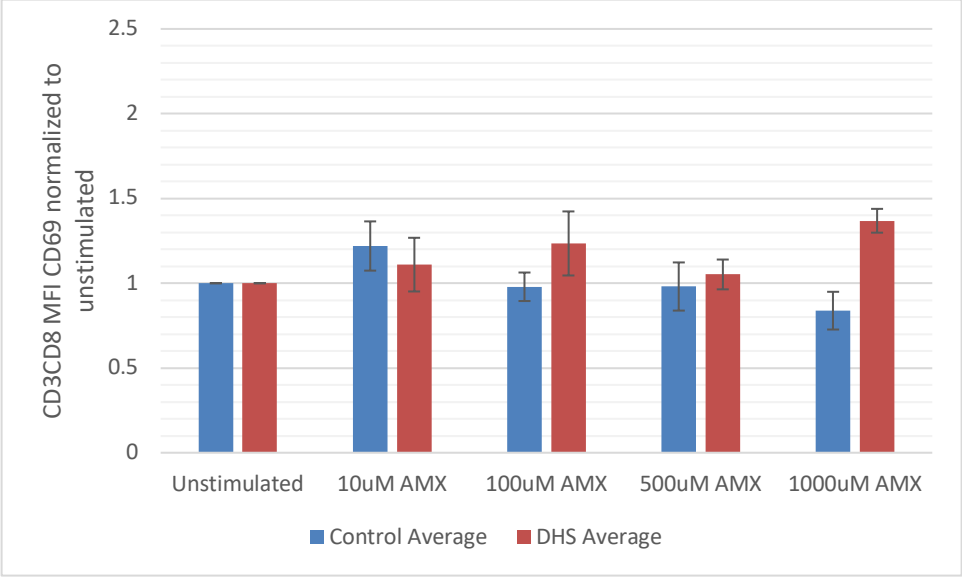


Figure 4-42: MFI of CD3+CD8+ T cells expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^{+}CD8^{+}$ T cells. Mauchly's Test of Sphericity was insignificant ($p = 0.407$), the assumption of sphericity was met, and therefore a Greenhouse-Geisser correction was not used. $F(4,24) = 1.028$, $p = 0.412$. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.669,5.007) = 3.677$, $p = 0.107$. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.979,5.936) = 1.672$, $p = 0.265$.

Similar patterns were seen with $CD3^{-}$ cells. With the individual participants (Figure 4-43), most were very similar to unstimulated. Control004 was slightly lower across all concentrations than unstimulated, while Control007 decreased from unstimulated as concentration of AMX increased. DHS007 was consistently slightly higher than unstimulated at all concentrations. Overall, when the average values were compared (Figure 4-44), no significant differences were observed with a repeated measures

ANOVA. One small noticeable difference was seen again at 1000µM, where the DHR percent CD69 value was slightly higher than control. While not significant in the ANOVA, a student's t-test at this concentration had a p value of 0.003. With individual MFI values, a few differences are seen (Figure 4-45). For the most part, values were consistent around unstimulated. DHS004 however increased from unstimulated as concentration increased, and there was a sharp increase in the MFI of CD69 with DHS005 at 100µM. DHS007 and DHS008 were consistently lower than unstimulated. With the average MFI values (Figure 4-46), there were no significant differences seen. The sharp increase of DHS008 is responsible for variability and an increase (but not significant) in average MFI values at 100µM.

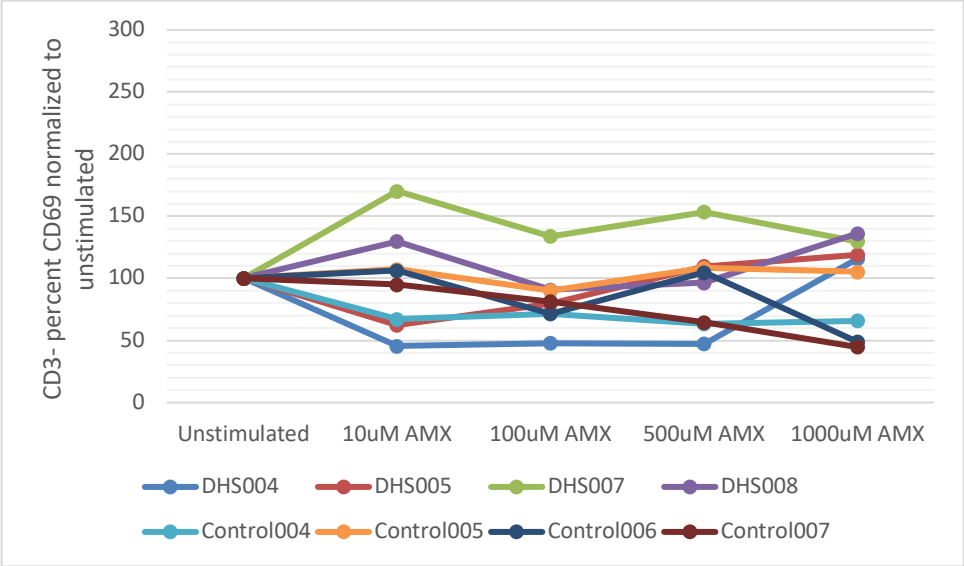


Figure 4-43: Percent of CD3- PBMCs expressing CD69 in individual control and DHS participant PBMCs incubated with AMX

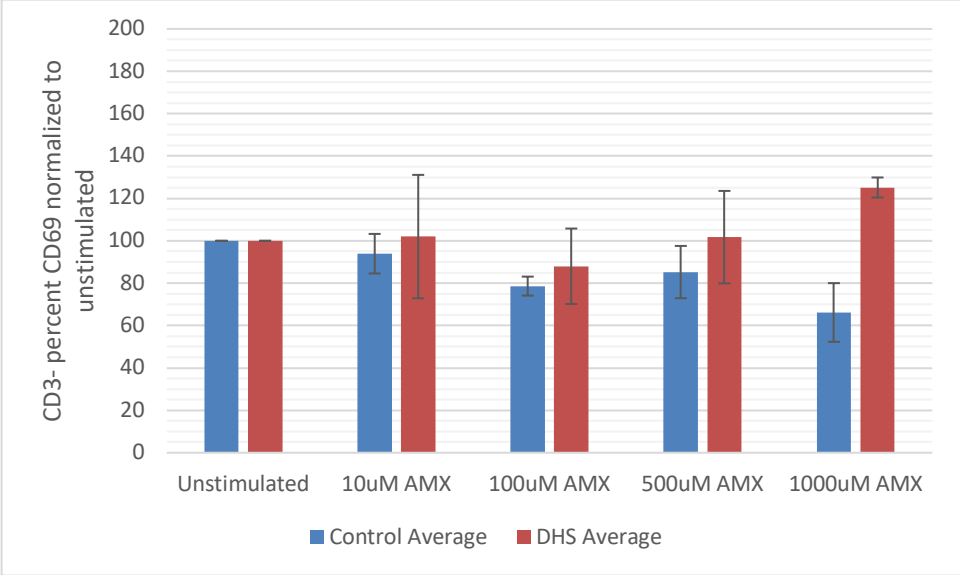


Figure 4-44: Percent of CD3- PBMCs expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^-$ T cells. Mauchly's Test of Sphericity was insignificant ($p = 0.437$), the assumption of sphericity was met, and therefore a Greenhouse-Geisser correction was not used. $F(4,24) = 0.719$, $p = 0.587$. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(2.233,6.700) = 2.833$, $p = 0.126$. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. $F(1.449,4.348) = 1.050$, $p = 0.396$.

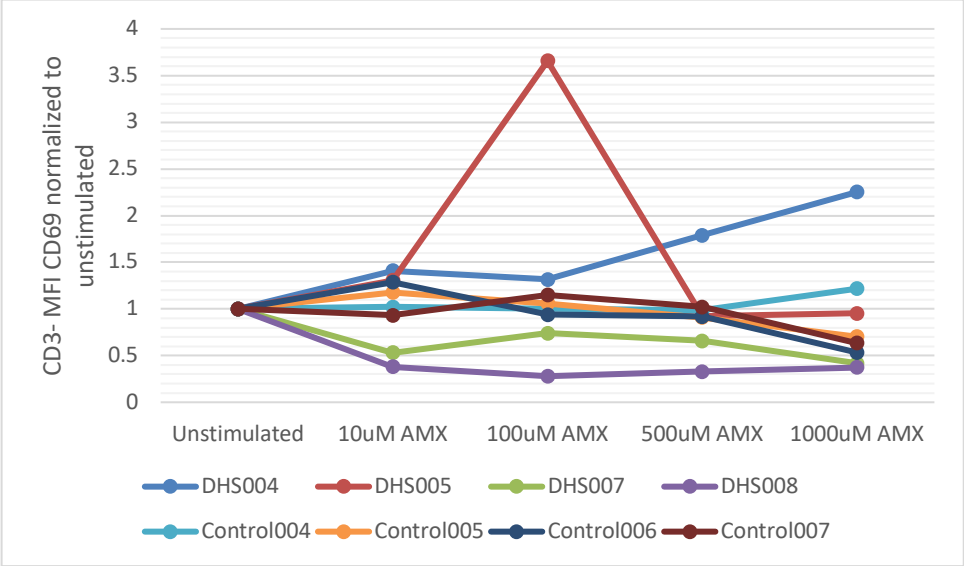


Figure 4-45: MFI of CD3- PBMCs expressing CD69 in individual control and DHS participant PBMCs incubated with SMX

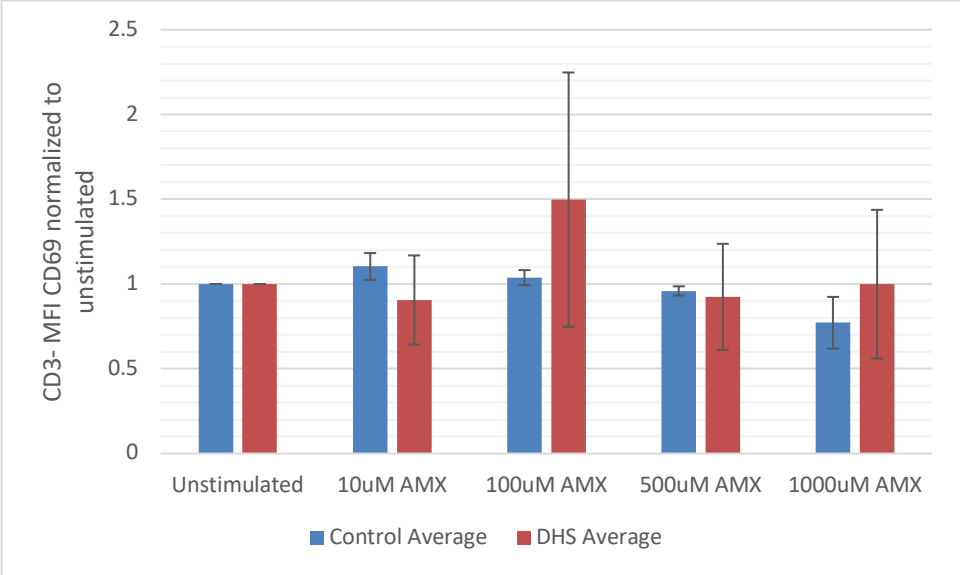


Figure 4-46: MFI of CD3- PBMCs expressing CD69 normalized to unstimulated control, as a result of PBMCs incubated with culture media (unstimulated control) and four concentrations of AMX

A repeated measures ANOVA was conducted to determine if any concentration had a significant effect on CD69 expression by $CD3^-$ T cells. Mauchly's Test of Sphericity was significant ($p = 0.022$), the assumption of sphericity was not met, and therefore a

Greenhouse-Geisser correction was used. F(1.603,9.621) = 0.646, p = 0.513. N(control participants) = 4 and N(DHR participants) = 4, ± SEM. Repeated measures ANOVAs were also conducted on CD3+ CD69+ control participants and DHS participants separately. For control participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. F(1.611,4.834) = 2.157, p = 0.212. For DHS participants, Mauchly's Test of Sphericity was violated, so a Greenhouse-Geisser correction was used. F(1.481,4.443) = 0.477, p = 0.597.

4.3.5 Comparison of SMX and AMX Grouped Concentrations

Since four concentrations each of AMX and SMX were used, they were combined in increasing concentrations to see if there was any effect on increasing concentration on the isolated PBMCs from hypersensitive individuals. Control participants were not included in this analysis. Percent CD69 expression and MFI of CD69 values were normalized to unstimulated values. See Figures 4-47 to 4-54.

Across all cell types, there were no significant differences in percent of CD69 expression or MFI of CD69 when drug concentration was increased. Overall, there did not seem to be any trends that exceeded unstimulated values. Within CD3+ cell types, the second concentration (C2) was consistently low in percent CD69 compared to unstimulated and other concentrations, but nothing was found by repeated measures ANOVA to be significant (Figure 4-47, 4-49, 4-51). There was a slight decrease from unstimulated in the MFI of CD69 on CD3+CD4+ cells (Figure 4-50). Within the CD3- cell types, the second concentration (C2) appeared to be very high, but also had high variability (Figure 4-53).

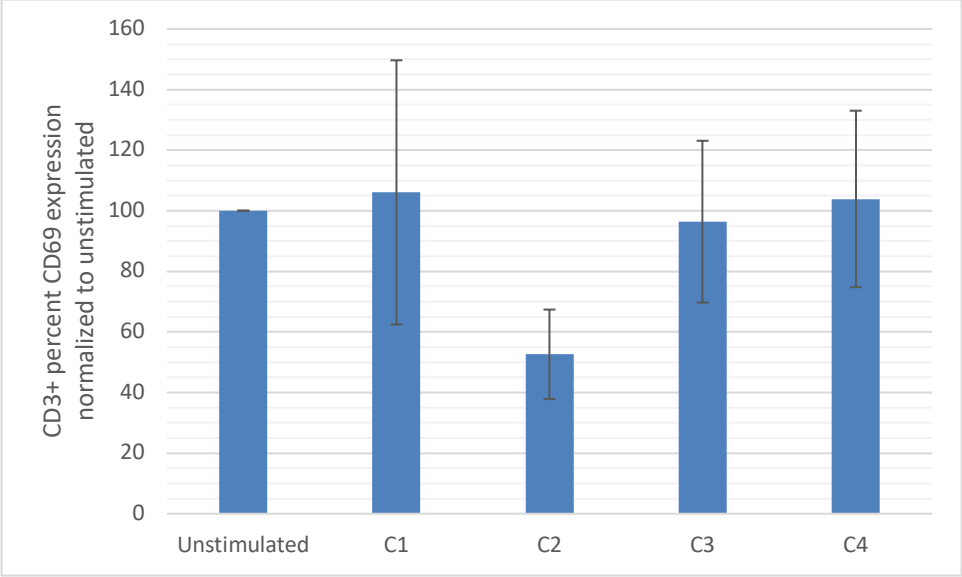


Figure 4-47: Average percent of CD69 expression on CD3+ PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. $F(4,16) = 0.782$, $p = 0.553$.

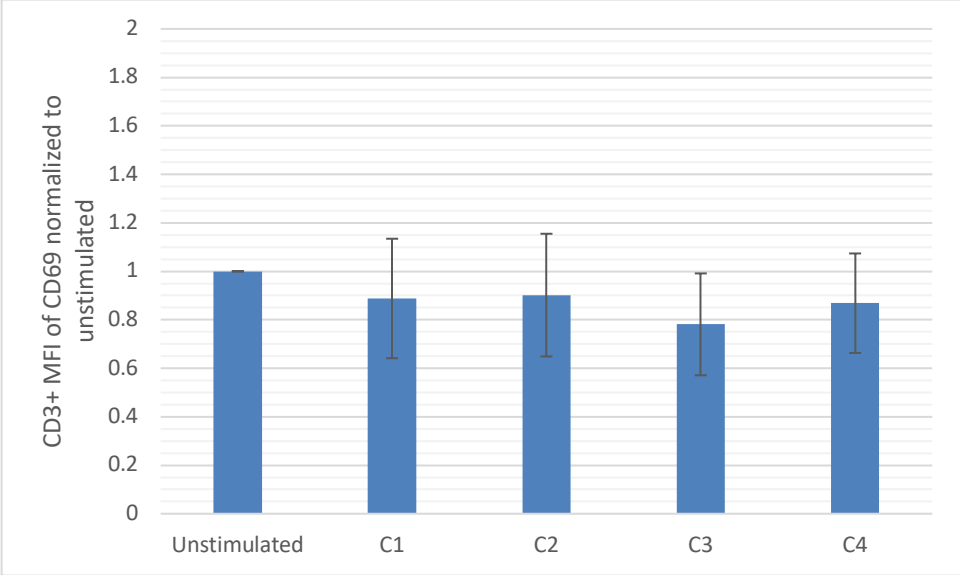


Figure 4-48: Average MFI of CD69 on CD3+ PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. $F(4,16) = 0.130$, $p = 0.969$.

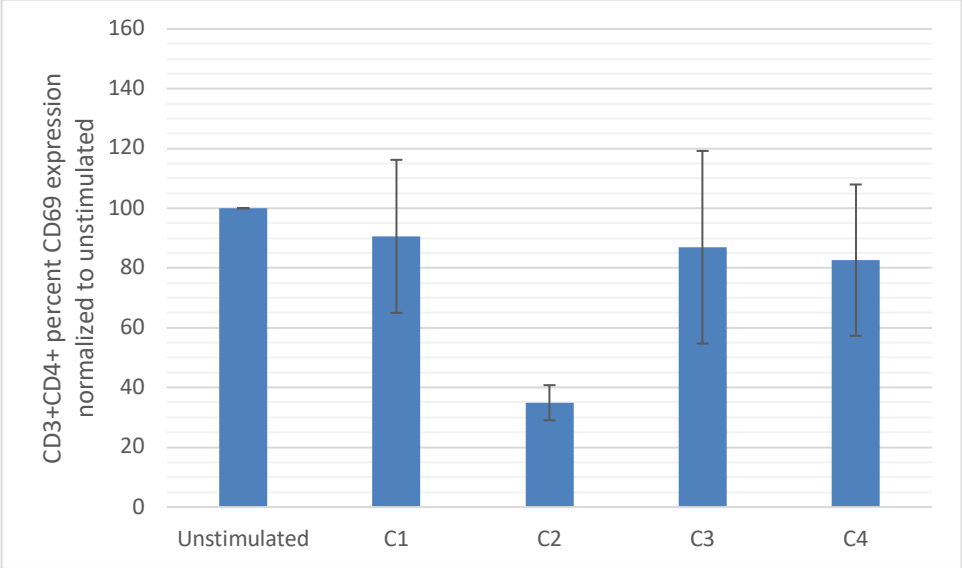


Figure 4-49: Average percent of CD69 expression on CD3+CD4+ PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. $F(4,16) = 1.468$, $p = 0.258$.

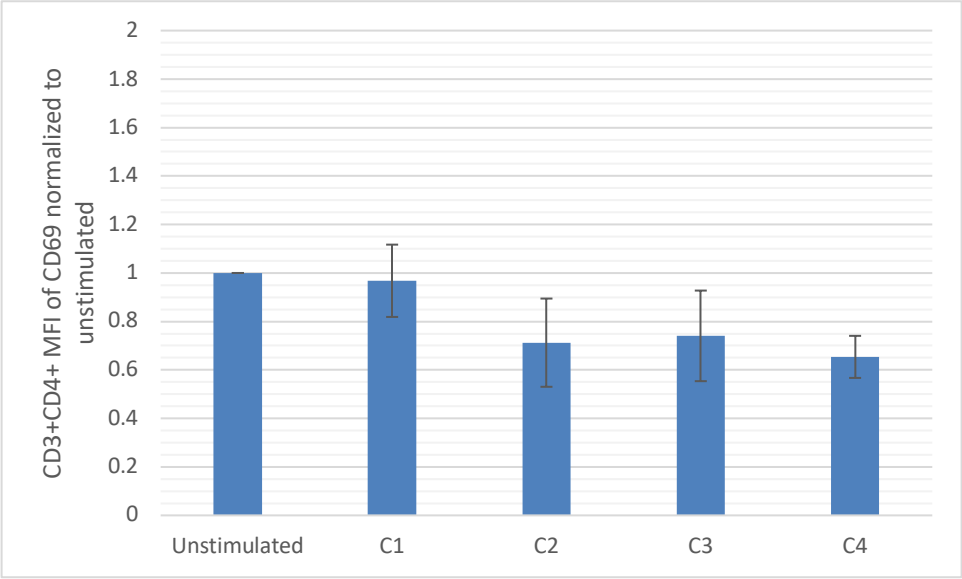


Figure 4-50: Average MFI of CD69 expression on CD3+CD4+ PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. F(4.16) = 1.891, p = 0.161.

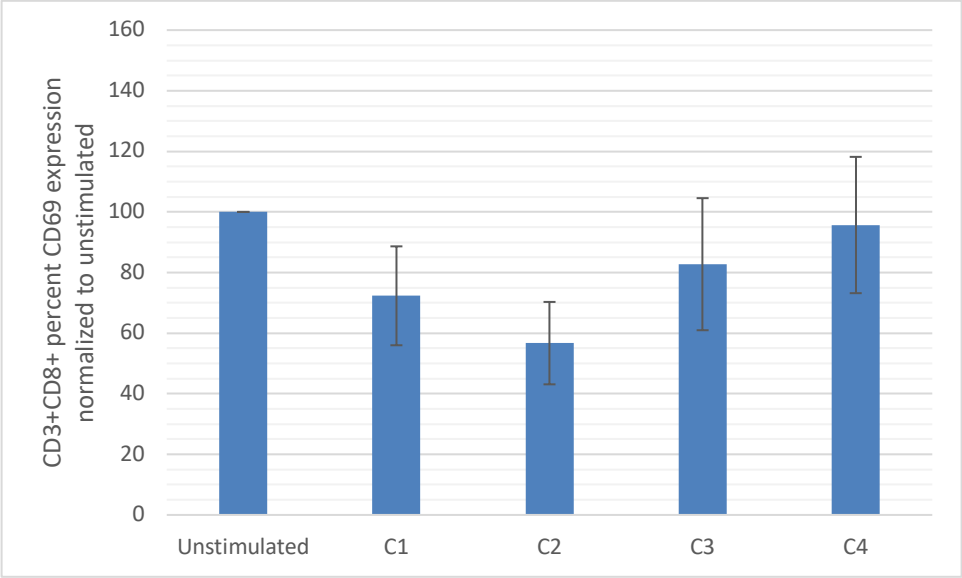


Figure 4-51: Average percent of CD69 expression on CD3+CD8+ PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. F(4,16) = 1.604, p = 0.222.

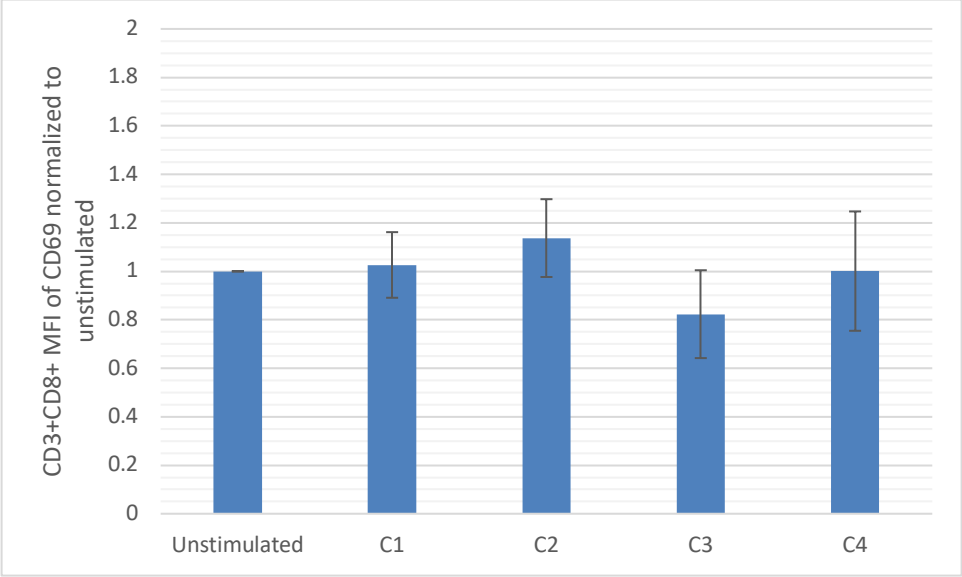


Figure 4-52: Average MFI of CD69 of CD69 expression on CD3+CD8+ PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. $F(4,16) = 0.758$, $p = 0.568$.

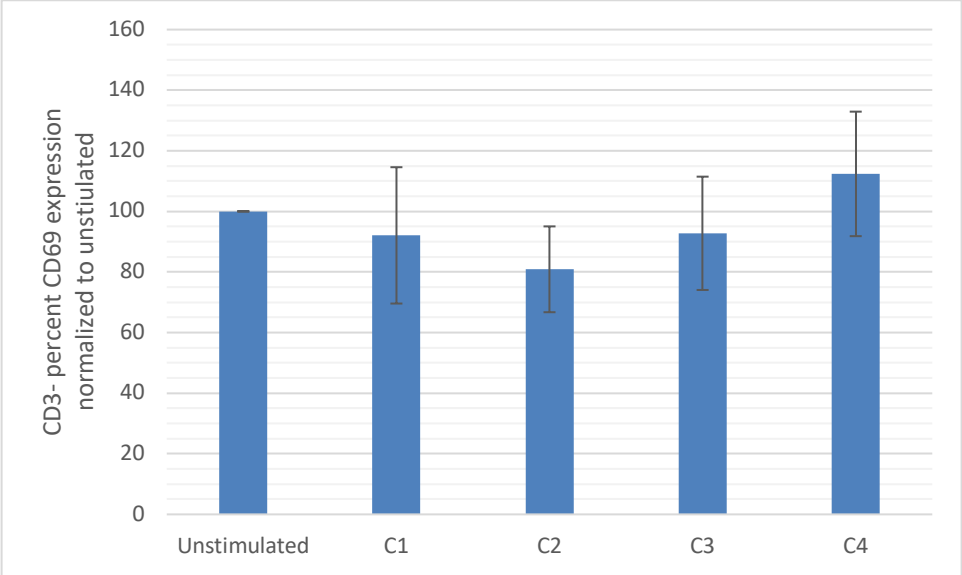


Figure 4-53: Average percent of CD69 expression on CD3- PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was not violated. F(4,16) = 0.475, p = 0.753.

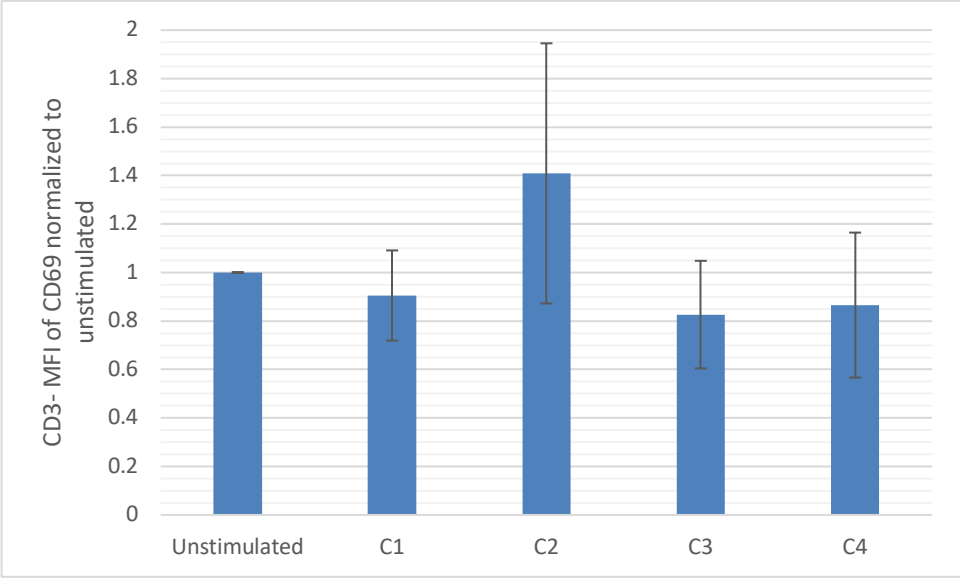


Figure 4-54: Average MFI of CD69 expression on CD3- PBMCs isolated from hypersensitive participants, combining SMX and AMX concentrations

N = 6, ± SEM. A repeated measures ANOVA was used to determine if there were any differences among the unstimulated and the increasing drug concentrations of drugs. Mauchly's Test of Sphericity was violated (p = 0.41), therefore a Greenhouse-Geisser correction was used. F(1.480,5.921) = 0.559, p = 0.550.

4.3.6 Control and DHS Participant Response to Anti-CD3

In addition to analyzing the different concentrations of SMX and AMX compared to unstimulated, I also analyzed whether there were any differences between control and DHR participant response to anti-CD3 stimulation (Figures 4-55 to 4-62). To do this, all participants were combined, because anti-CD3 was the positive control for all participants regardless of drug type. Student's t-tests were used to compare the percent CD69 expression and MFI of CD69 on $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^-$ cells.

Overall, $CD3^-$ cells had a significantly lower percent expression of CD69 compared to the $CD3^+$ cells. When activated with anti-CD3, all participant $CD3^+$ cells had an average

percent CD69 expression of 33.10%, while $CD3^-$ had 1.27% (student's t-test, one-tailed, p <0.005). This was similar for MFI of CD69 – $CD3^+$ cells had an average MFI of CD69 of 2.36, while $CD3^-$ had 1.27 (student's t-test, one-tailed, paired, $p < 0.01$).

The only instance where there was a significant difference between control and DHS participant response to stimulation with anti-CD3 was with the percent of CD3+ cells expressing CD69 (Figure 4-57). In this case, 27.68% of CD3+CD4+ cells were expressing CD69, which is significantly lower than the control participants, where 65.42% of CD3+CD4+ cells were expressing CD69 (student's t-test, one-tailed, p = 0.043). Meanwhile, there were no significant differences in MFI of CD69 by CD3+CD4+ T cells in response to stimulation with anti-CD3 (student's t-test, one-tailed, p = 0.38), see Figure 4-58. There were no other significant differences between any other responses to anti-CD3 in the other cell types analyzed ($p > 0.05$), see Figures 4-55 and 4-56, 4-58, 4-59 and 4-60, 4-61 and 4-62.

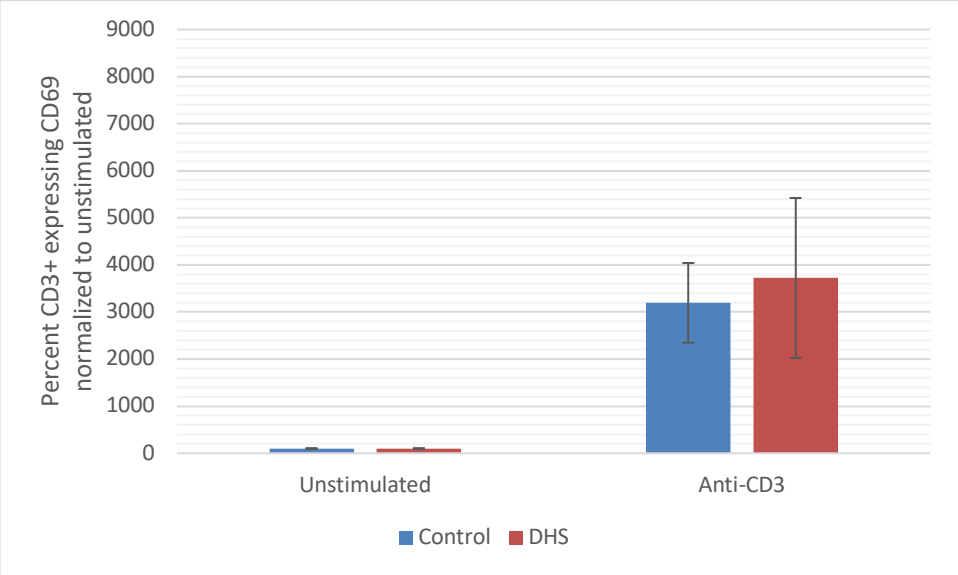


Figure 4-55: Average percent of CD69 expression on CD3+ PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.39.

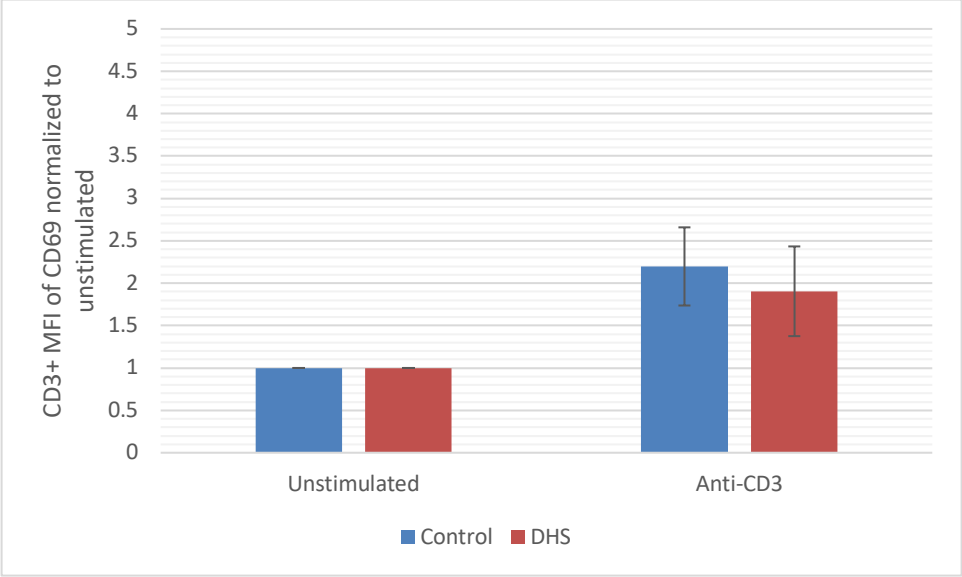


Figure 4-56: Average MFI of CD69 expression on CD3+ PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.34.

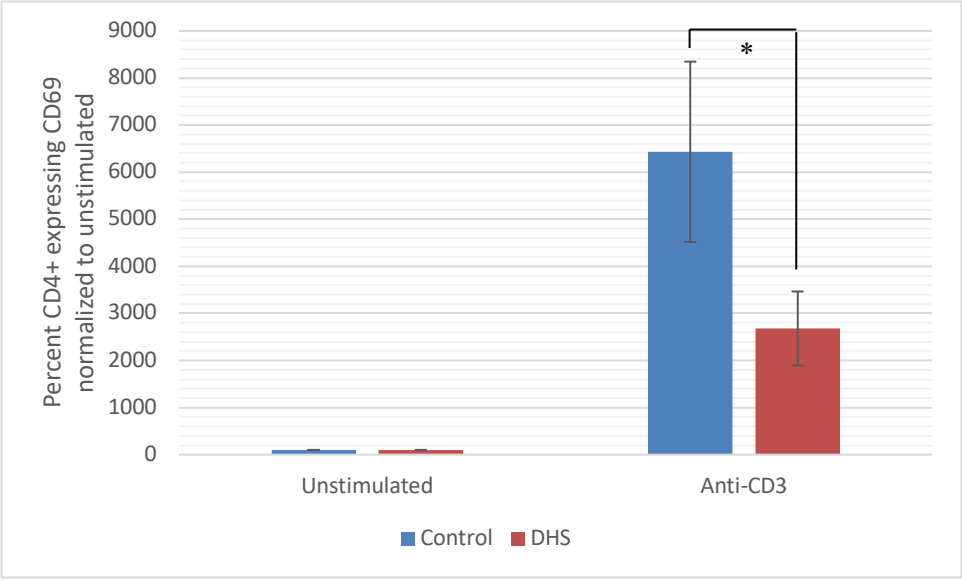


Figure 4-57: Average percent of CD69 expression on CD3+CD4+ PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.049.

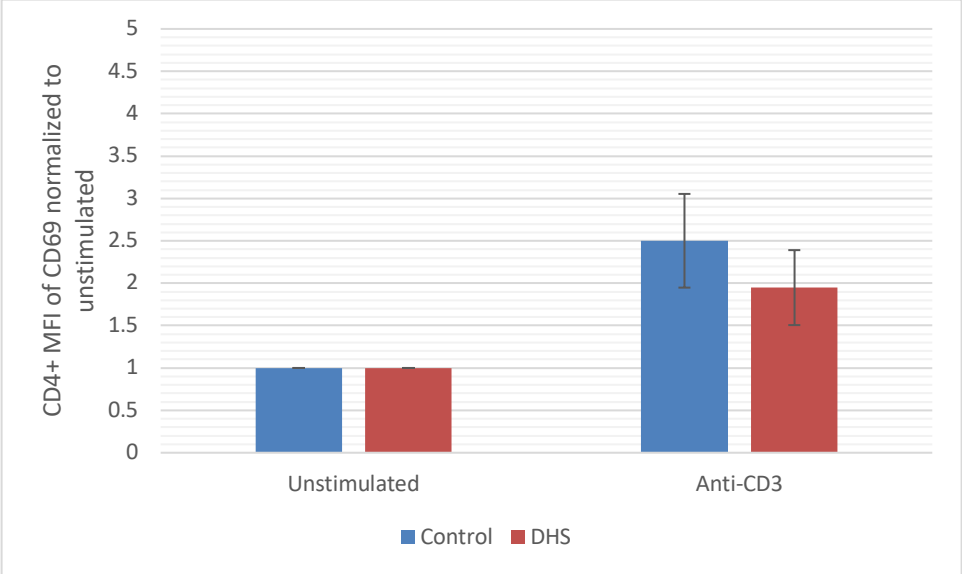


Figure 4-58: Average MFI of CD69 expression on CD3+CD4+ PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.22.

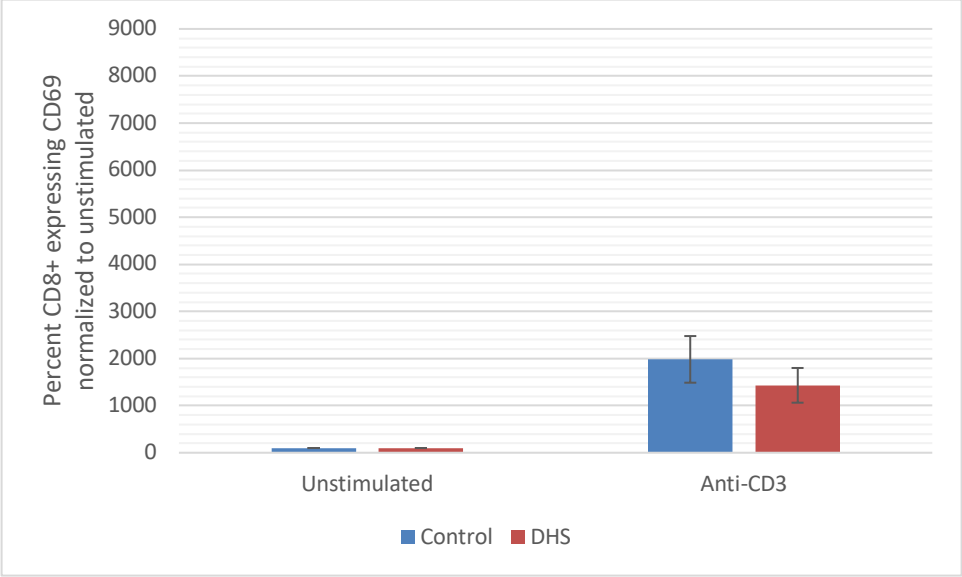


Figure 4-59: Average percent of CD69 expression on CD3+CD8+ PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.20.

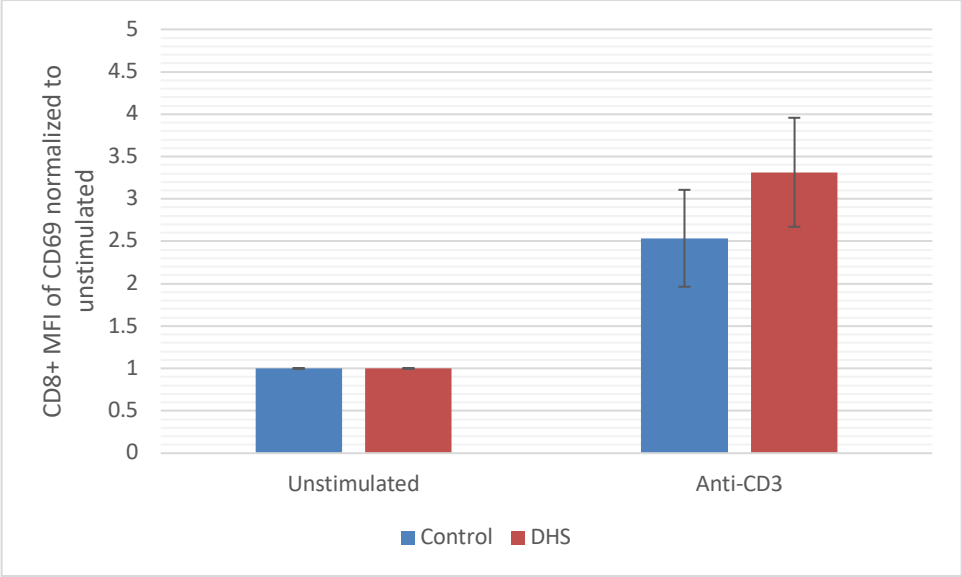


Figure 4-60: Average MFI of CD69 expression on CD3+CD8+ PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.19.

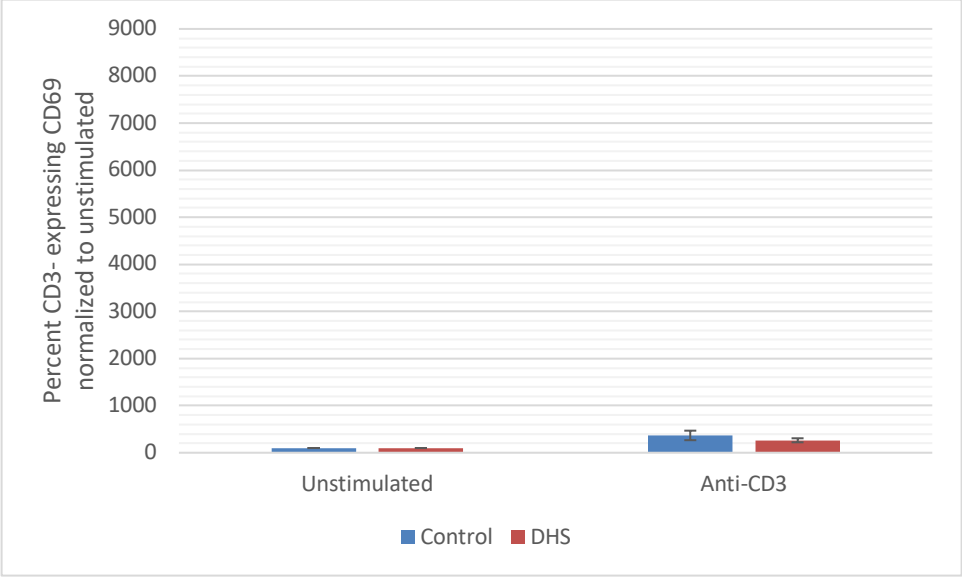


Figure 4-61: Average percent of CD69 expression on CD3- PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.18.

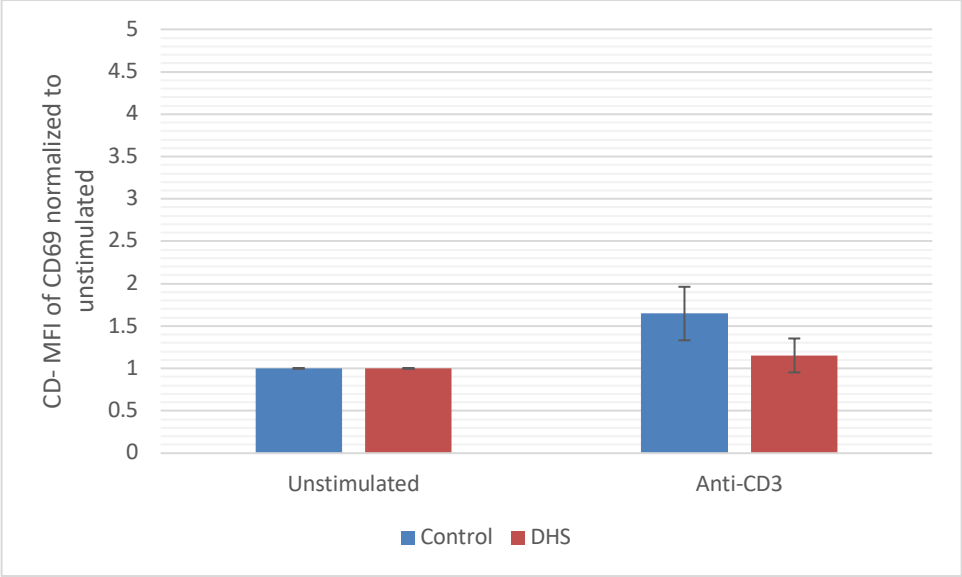


Figure 4-62: Average MFI of CD69 expression on CD3- PBMCs isolated from control and hypersensitive (DHS) participants stimulated with anti-CD3

N = 6, ± SEM. Student's t-test was used to compare control and DHS anti-CD3-stimulated values. Student's t-test, one-tailed, p = 0.12.

4.3.7 Combining SMX Concentrations in Control and DHS Participants

In addition to comparing the effect of individual concentrations of SMX to unstimulated, the concentrations of SMX were combined by taking the average of percent CD69 expression and MFI of CD69 and compared to unstimulated (Figures 4-63 to 4-70). Drug-stimulated values were normalized to unstimulated values. No significant differences were found between control and hypersensitive participants stimulated with SMX. However, in three instances, the drug-stimulated percent CD69 or MFI of CD69 values were observed to be lower than unstimulated: $CD3^+CD4^+$ percent CD69 (Figure 4-65), $CD3^+CD4^+$ MFI of CD69 (Figure 4-66), and $CD3^+CD8^+$ percent CD69 (Figure 4-67). All others appeared to be similar to unstimulated.

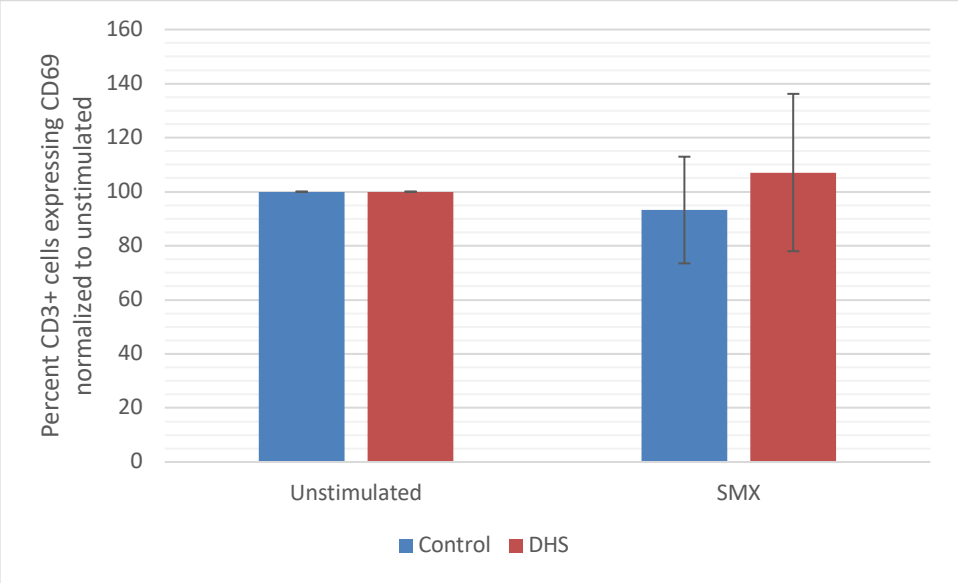


Figure 4-63: Average percent of CD69 expression of CD3+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, p = 0.38.

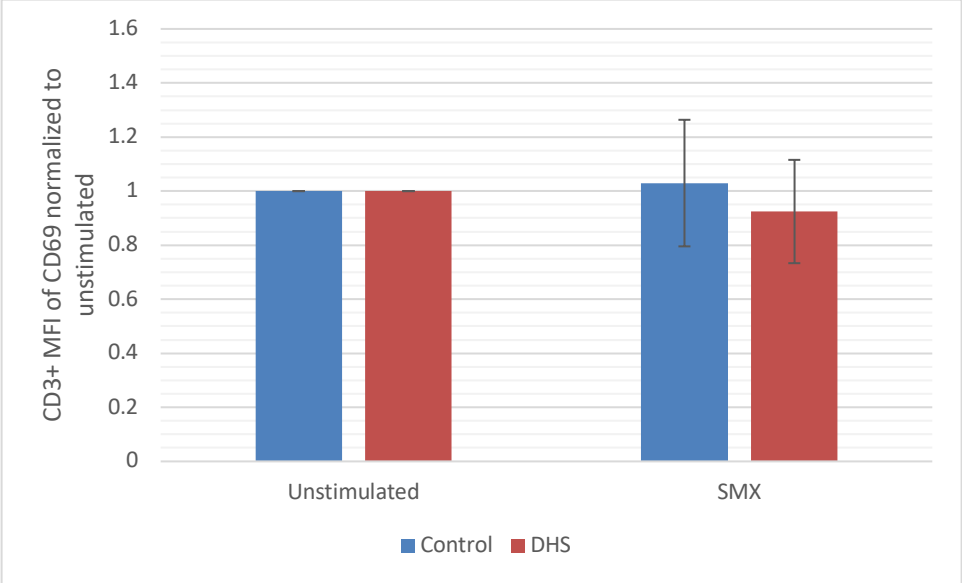


Figure 4-64: Average MFI of CD69 expression of CD3+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, $p = 0.49$.

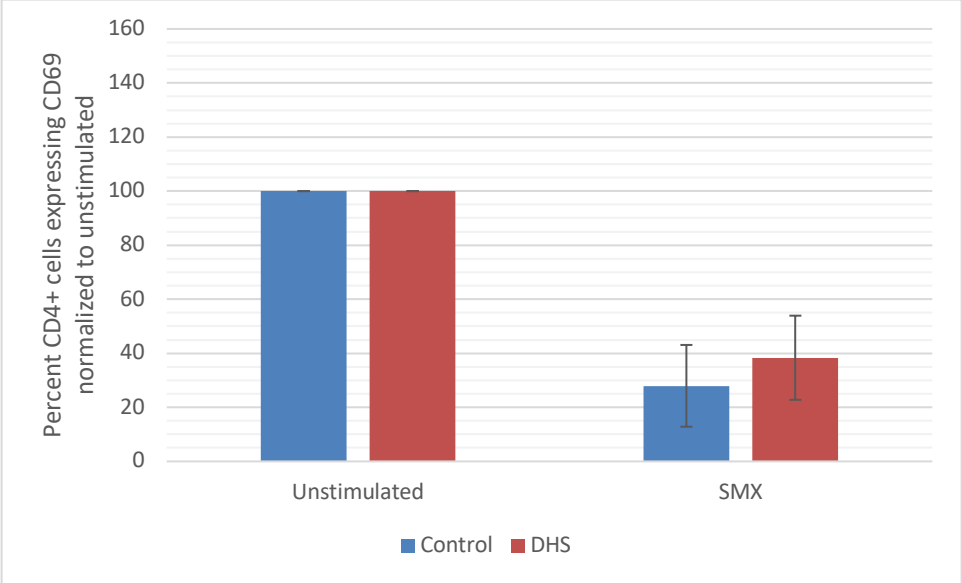


Figure 4-65: Average percent of CD69 expression of CD3+CD4+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, p = 0.38.

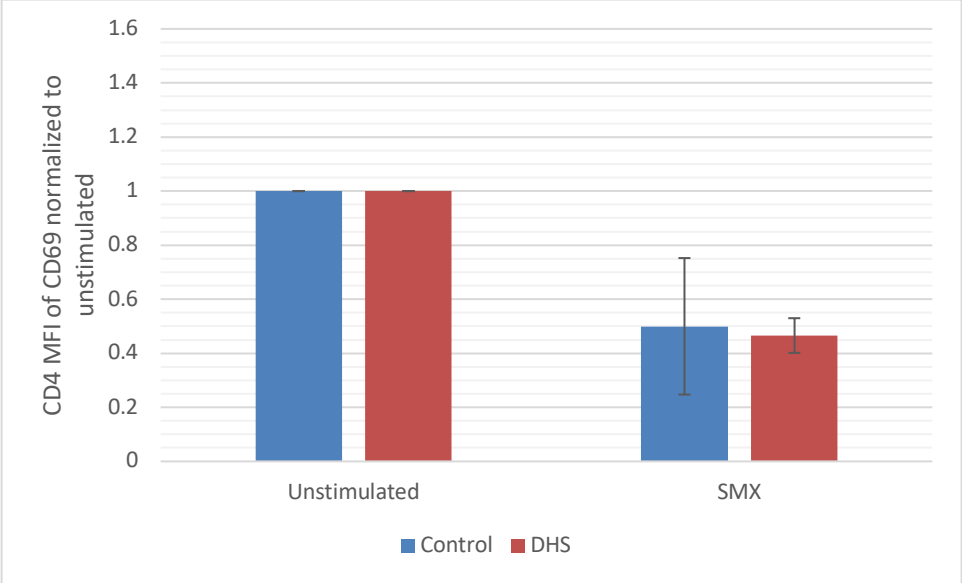


Figure 4-66: Average MFI of CD69 expression of CD3+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, $p = 0.47$.

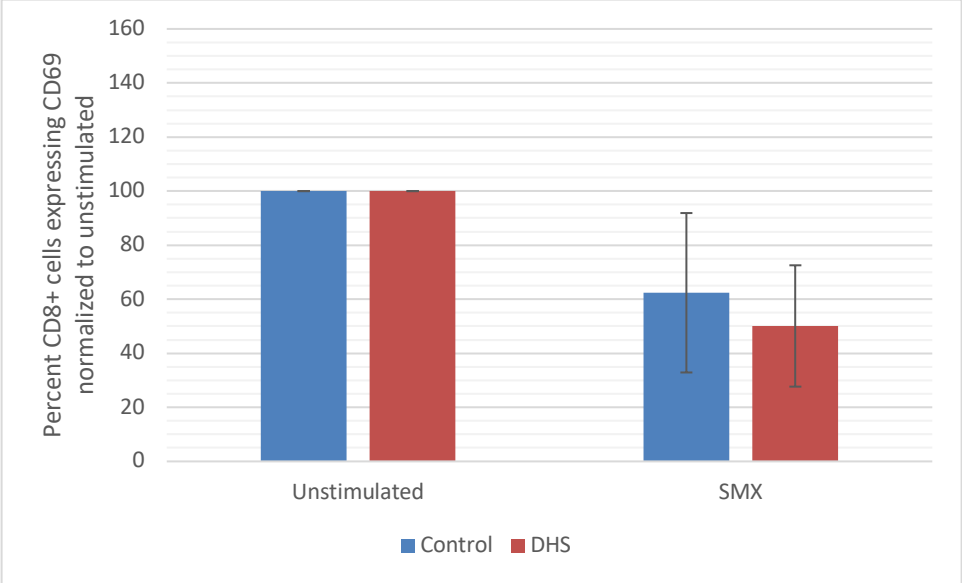


Figure 4-67: Average percent of CD69 expression of CD3+CD8+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, p = 0.42.

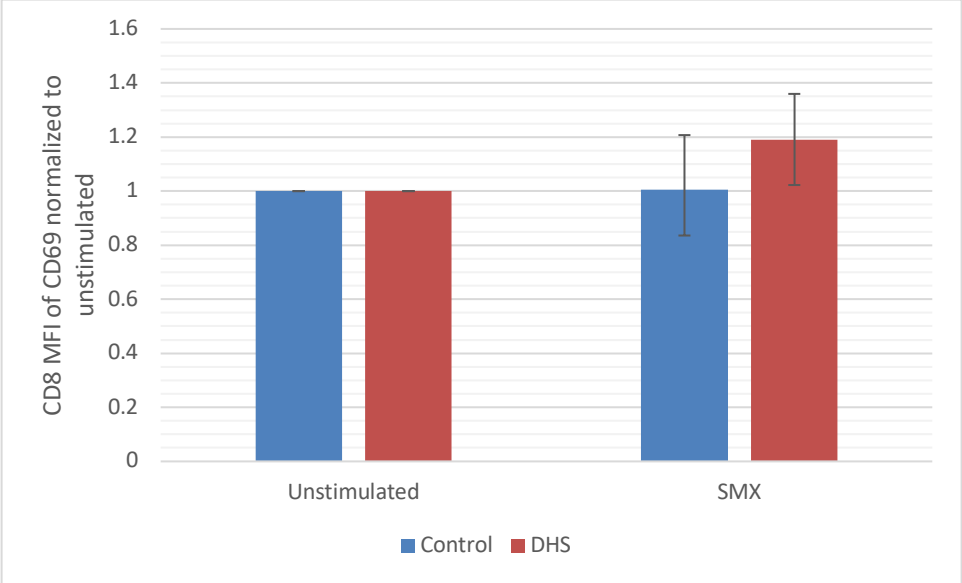


Figure 4-68: Average MFI of CD69 expression of CD3+CD8+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM.Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, $p = 0.26$.

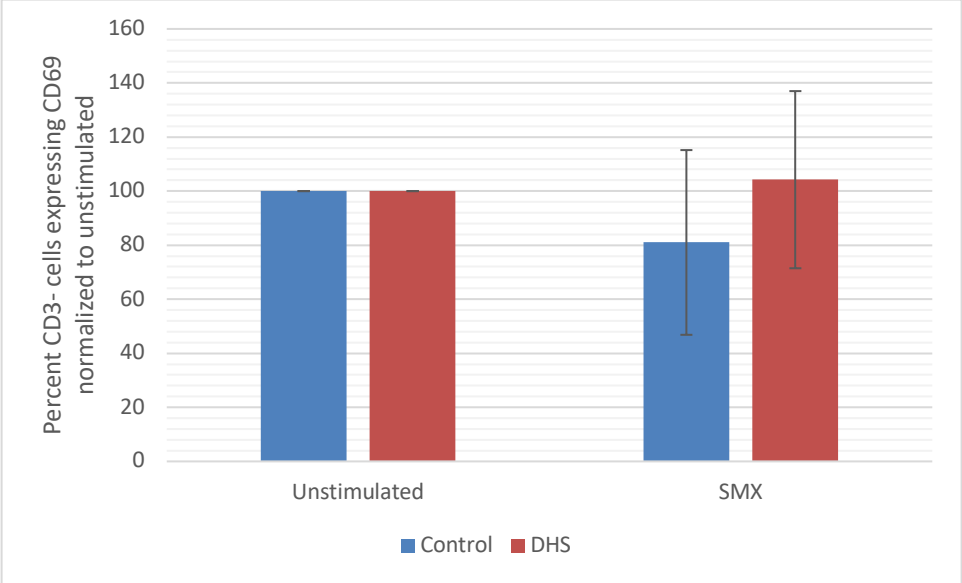


Figure 4-69: Average percent of CD69 expression of CD3- PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, p = 0.43.

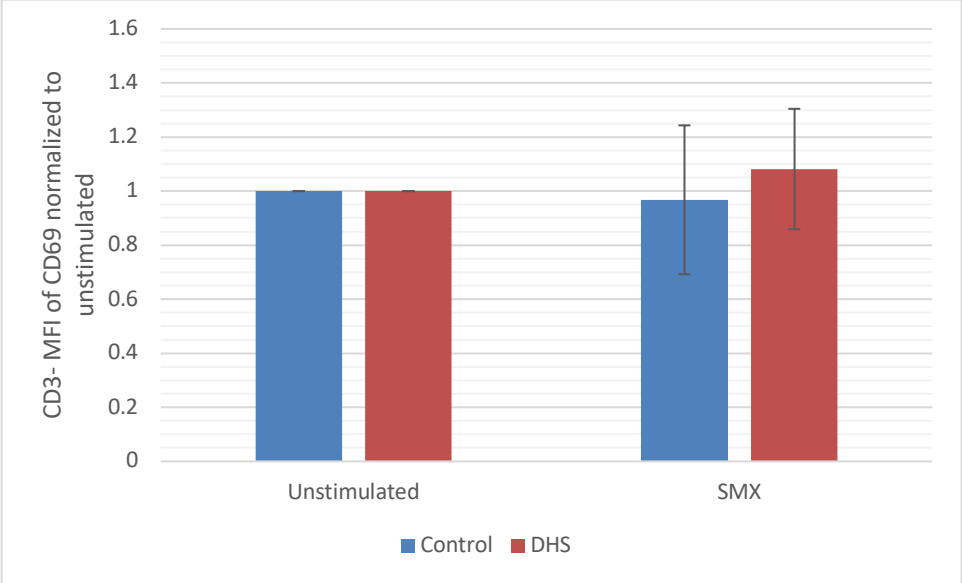


Figure 4-70: Average MFI of CD69 expression of CD3- PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of SMX

N(control) = 2, N(DHS) = 2, ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to SMX. Student's t-test, p = 0.33.

4.3.8 Combining AMX Concentrations in Control and DHS Participants

All of the concentrations of AMX were combined to see if there was any effect between unstimulated and AMX in general. Student's t-tests were performed between control AMX and DHS AMX values. No significant differences were found between these values with any cell type (Figures 4-71 to 4-78).

All of the concentrations of AMX were combined to see if there was any effect between unstimulated and AMX in general. Student's t-tests were performed between control AMX and DHS AMX values. No significant differences were found between these values with any cell type (Figures 4-71 to 4-78). Similar to SMX results, there were no significant differences found between control and hypersensitive participants stimulated

with AMX. There were no instances where AMX-stimulated values appeared lower than unstimulated.

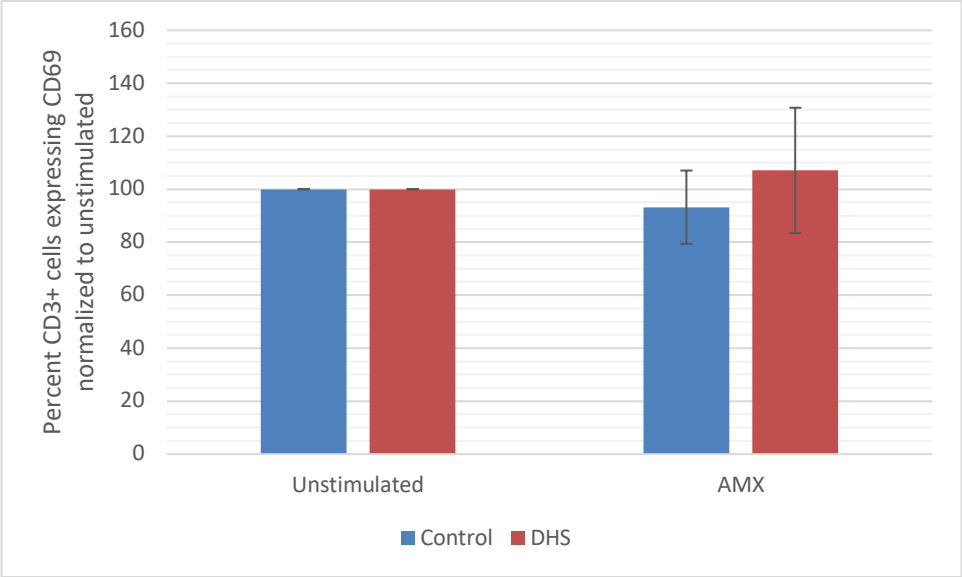


Figure 4-71: Average percent of CD69 expression of CD3+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, p = 0.32.

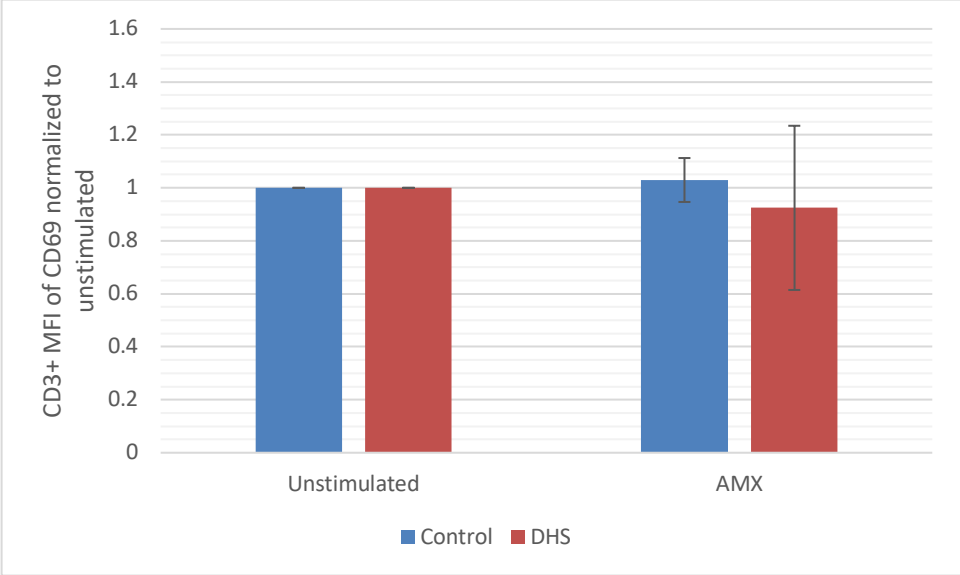


Figure 4-72: Average MFI of CD69 of CD3+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4; ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, $p = 0.38$.

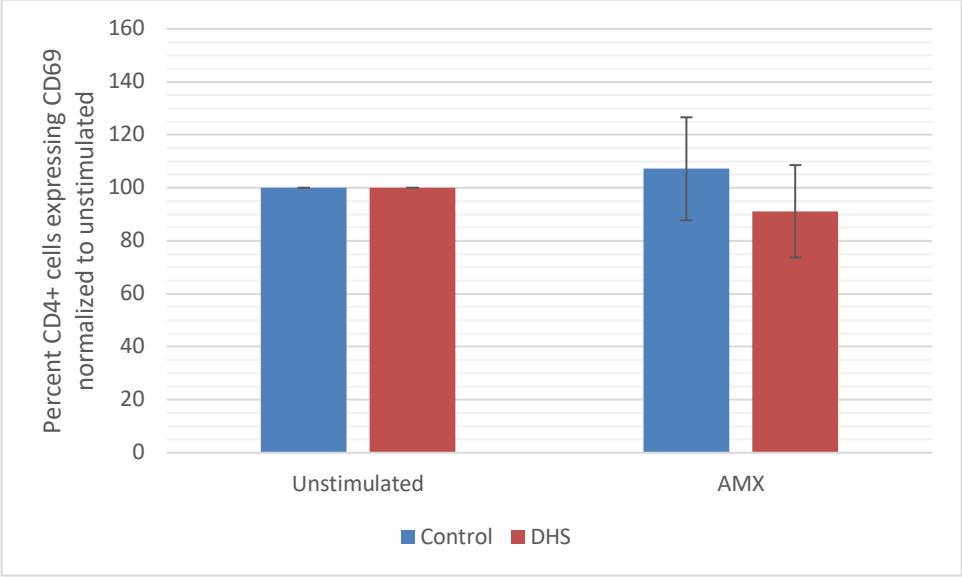


Figure 4-73: Average percent of CD69 expression of CD3+CD4+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, $p = 0.28$.

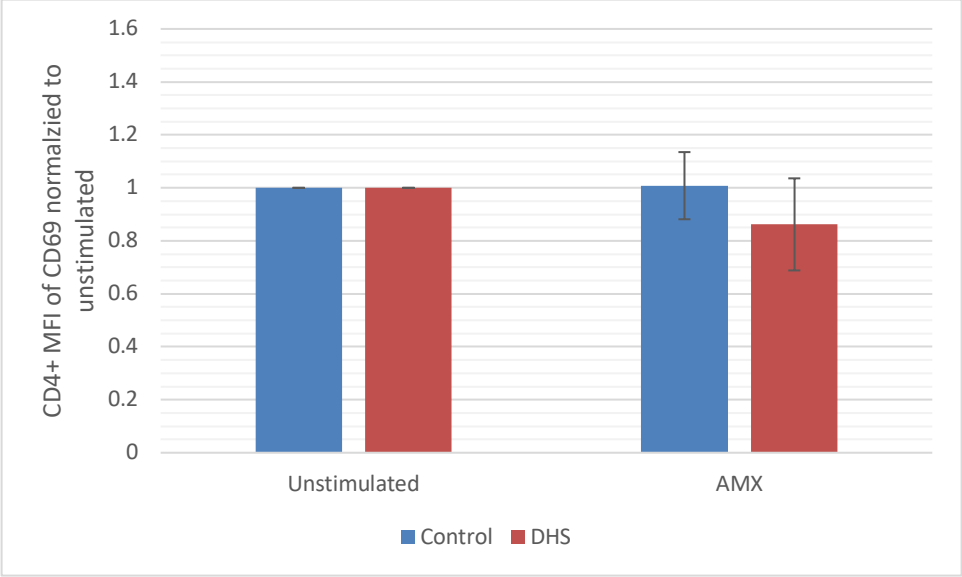


Figure 4-74: Average MFI of CD69 of CD3+CD4+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4; ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, $p = 0.26$.

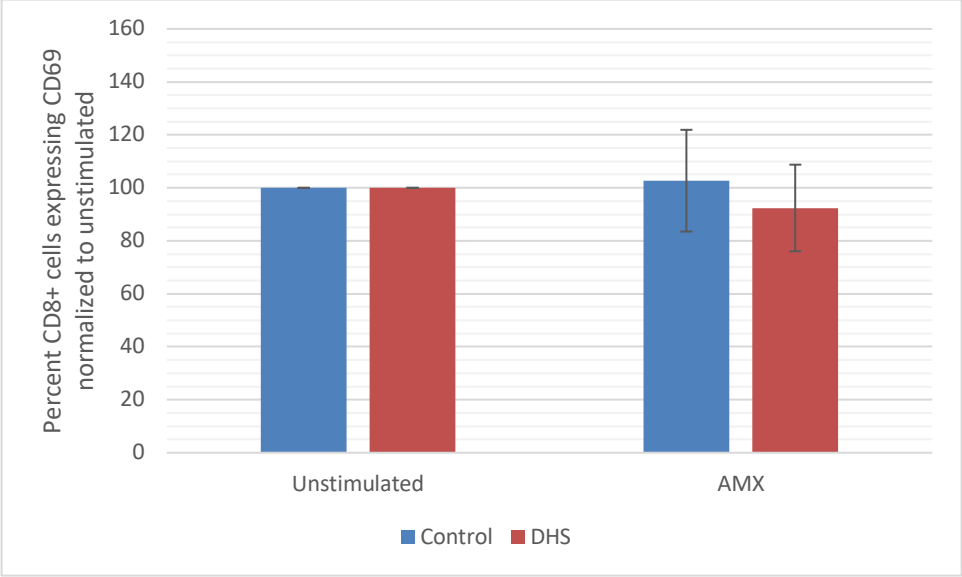


Figure 4-75: Average percent of CD69 expression of CD3+CD8+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, p = 0.35.

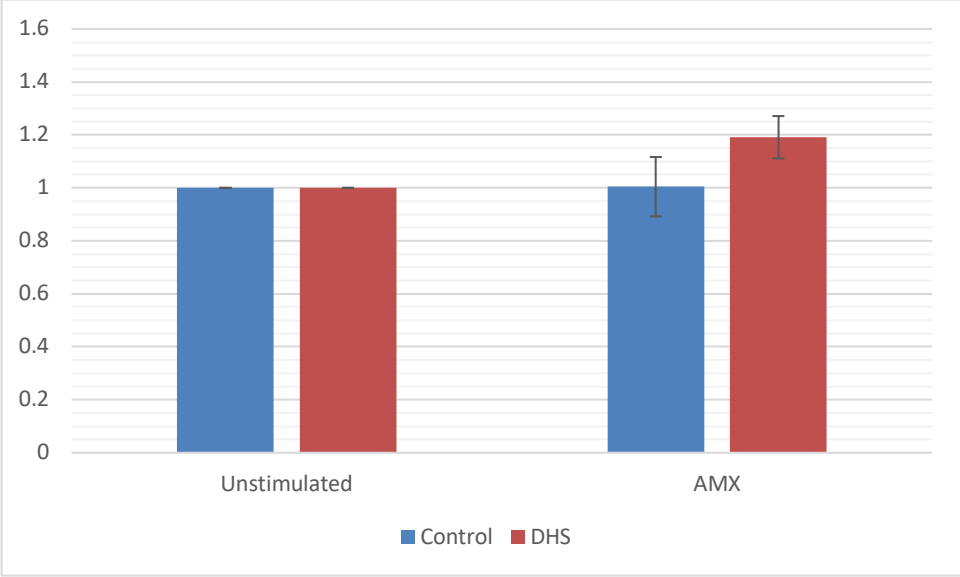


Figure 4-76: Average MFI of CD69 of CD3+CD8+ PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4; ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, $p = 0.11$.

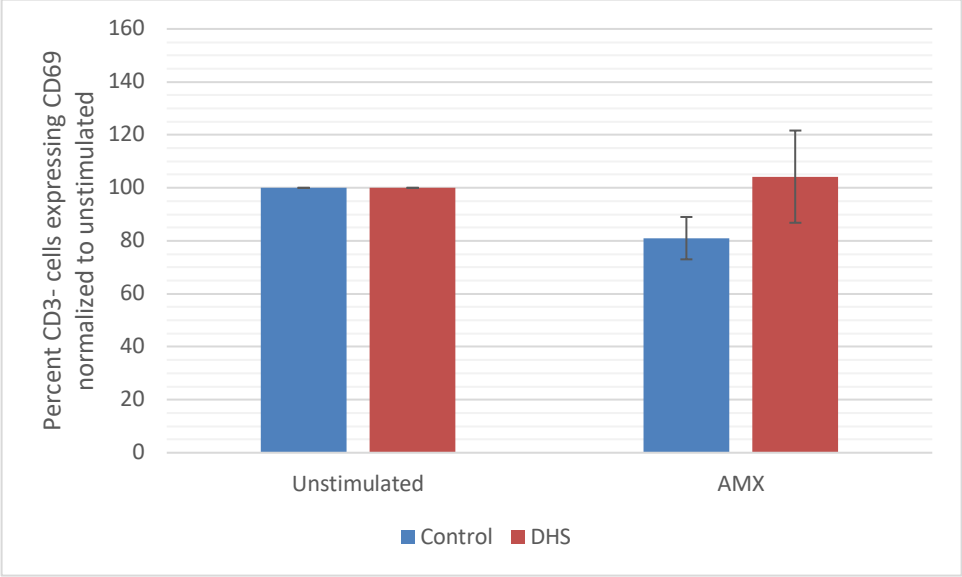


Figure 4-77: Average percent of CD69 expression of CD3- PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4, ± SEM. Student's t-test was used to compare percent of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, p = 0.14.

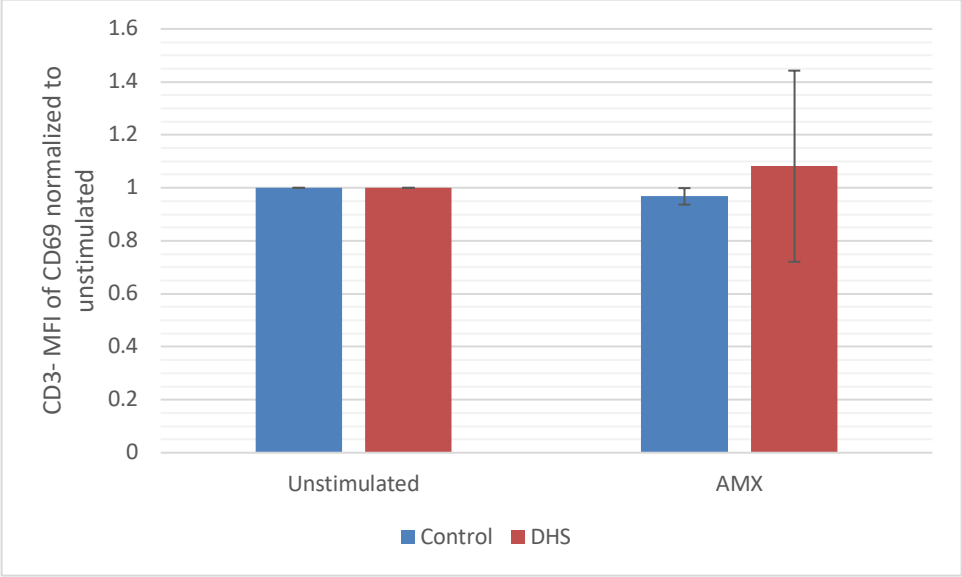


Figure 4-78: Average MFI of CD69 of CD3- PBMCs isolated from control and hypersensitive (DHS) participants as a result of exposure to combined concentrations of AMX

N(control) = 4, N(DHS) = 4; ± SEM. Student's t-test was used to compare MFI of CD69 expression between control and DHS participants after exposure to AMX. Student's t-test, p = 0.38.

4.4 Cytokine Analysis Results

Cytokines IFN-y, IL-4, IL-9, IL-13, IL-17A, and IL-22 were analyzed by a custom Luminex kit by Bio-Rad. These cytokines were analyzed from the cell culture supernatant. Any cytokine secretion, if seen at all, occurred in anti-CD3 stimulated samples. Most results were highly variable due to only one sample of four having any detectable level of cytokine.

IFN-7 was the only cytokine that had values in all conditions, however it is likely that most of these were extrapolations (specifically values under approximately 19 pg/ml). Anti-CD3 stimulation of both control and DHS PBMCs resulted in IFN-y secretion (Figure 4-79). Control004 had some secretion of IL-4, and IL-17A in the media (Figure 4-80, 4-83). Anti-CD4 stimulation resulted in secretion of IL-4 and IL-9 in one DHS

participant only, and not in the controls (Figure 4-80, 4-81). IL-22 was also secreted when control and DHS participants were stimulated with anti-CD3 (Figure 4-84).

It is important to note that if a bar is missing from the bar graph, it signifies that the result was 0, or that there were no detectable levels of cytokine.

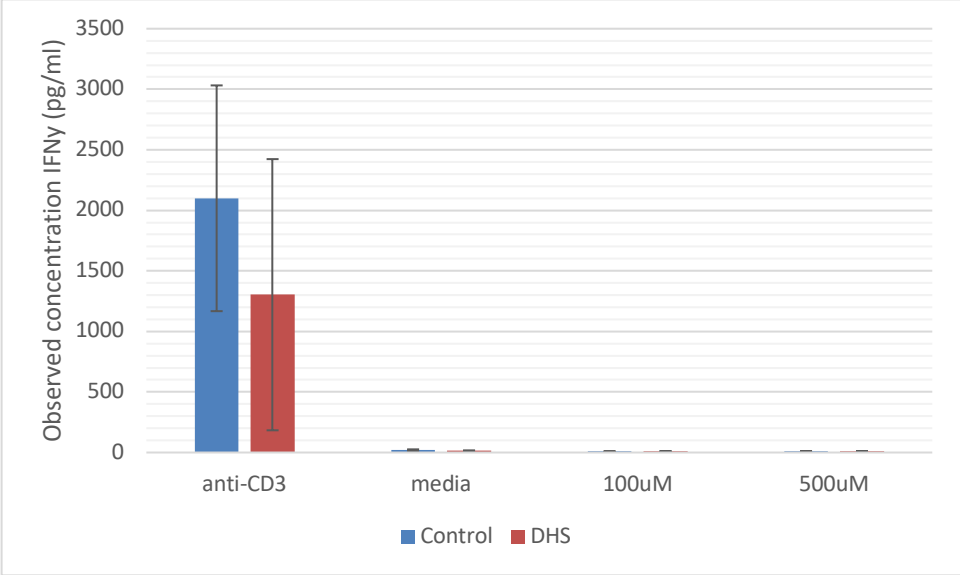


Figure 4-79: Observed concentration of IFN-y in pg/ml after incubation of PBMCs isolated from control and AMX-hypersensitive participants in anti-CD3, media (negative control), 100uM AMX, and 500uM AMX

N(control) = 4, N(DHS) = 4.

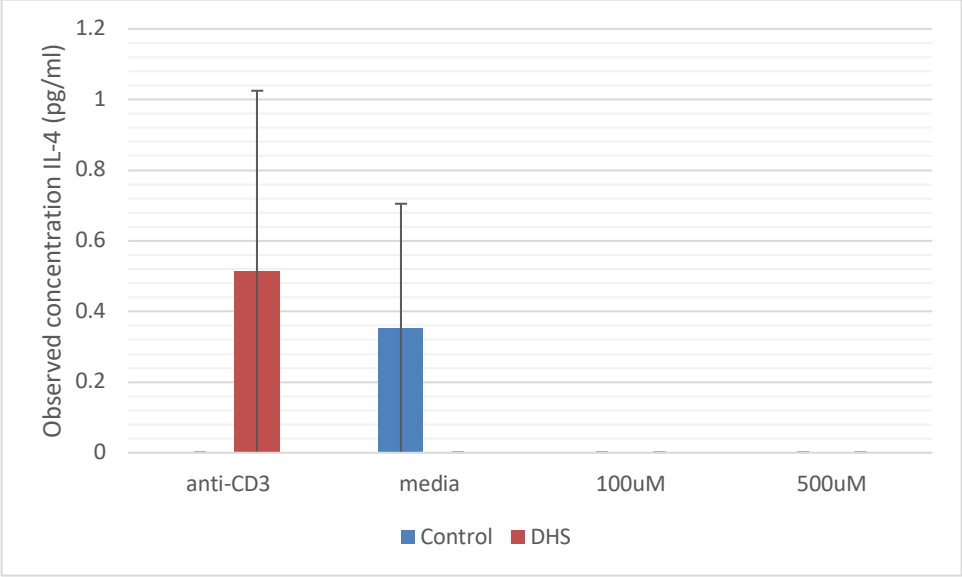


Figure 4-80: Observed concentration of IL-4 in pg/ml after incubation of PBMCs isolated from control and AMX-hypersensitive participants in anti-CD3, media (negative control), 100uM AMX, and 500uM AMX

N(control) = 4, N(DHS) = 4.

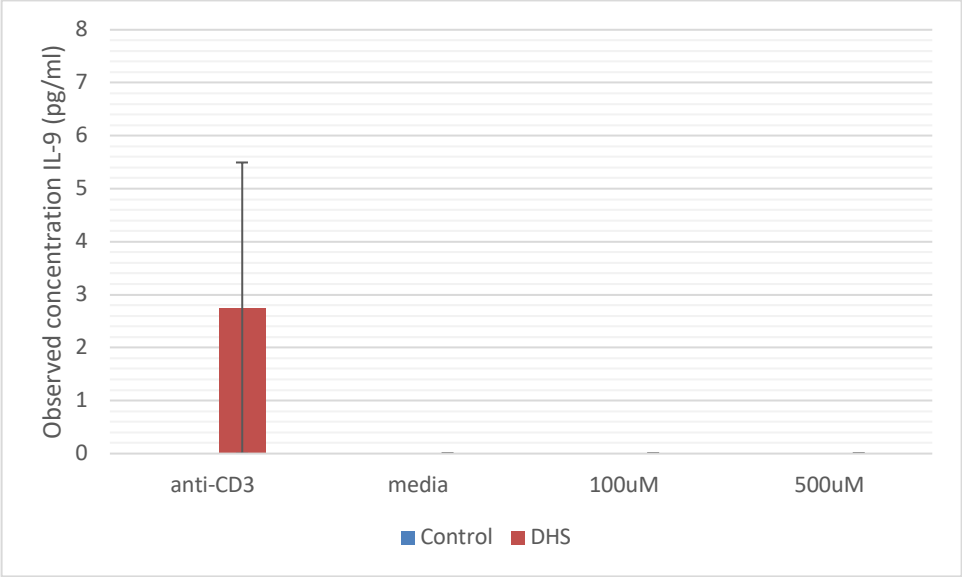


Figure 4-81: Observed concentration of IL-9 in pg/ml after incubation of PBMCs isolated from control and AMX-hypersensitive participants in anti-CD3, media (negative control), 100uM AMX, and 500uM AMX

N(control) = 4, N(DHS) = 4.

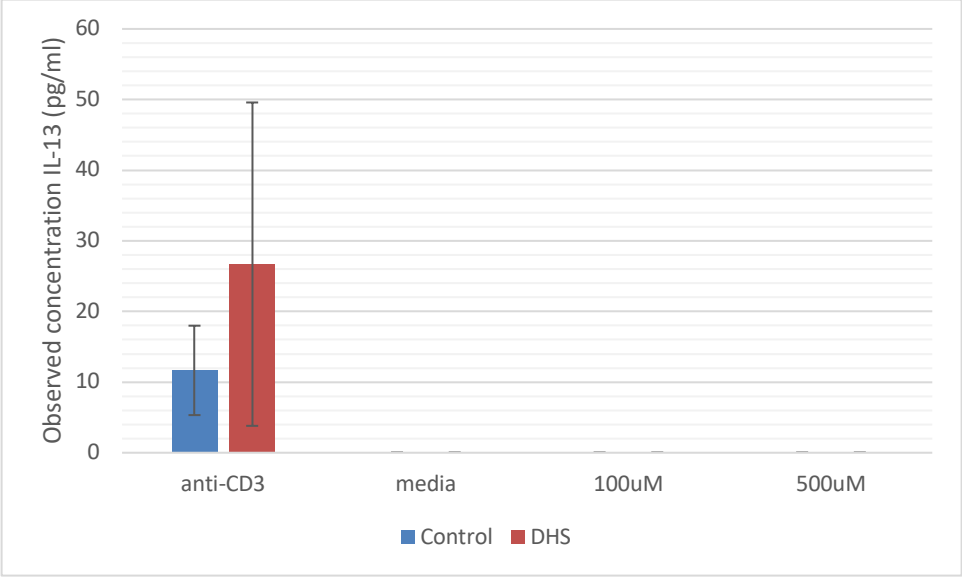


Figure 4-82: Observed concentration of IL-13 in pg/ml after incubation of PBMCs isolated from control and AMX-hypersensitive participants in anti-CD3, media (negative control), 100uM AMX, and 500uM AMX

N(control) = 4, N(DHS) = 4.

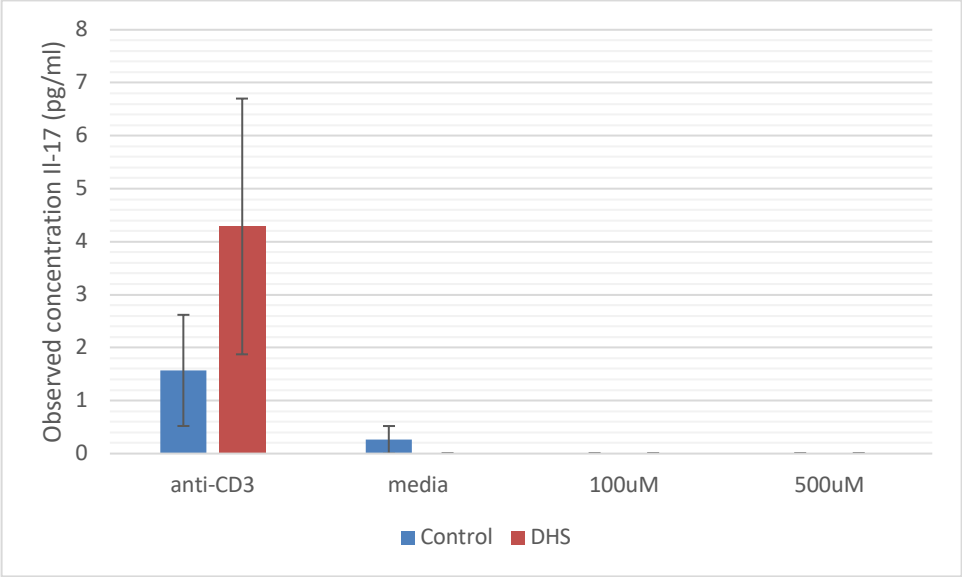


Figure 4-83: Observed concentration of IL-17A in pg/ml after incubation of PBMCs isolated from control and AMX-hypersensitive participants in anti-CD3, media (negative control), 100uM AMX, and 500uM AMX

N(control) = 4, N(DHS) = 4.

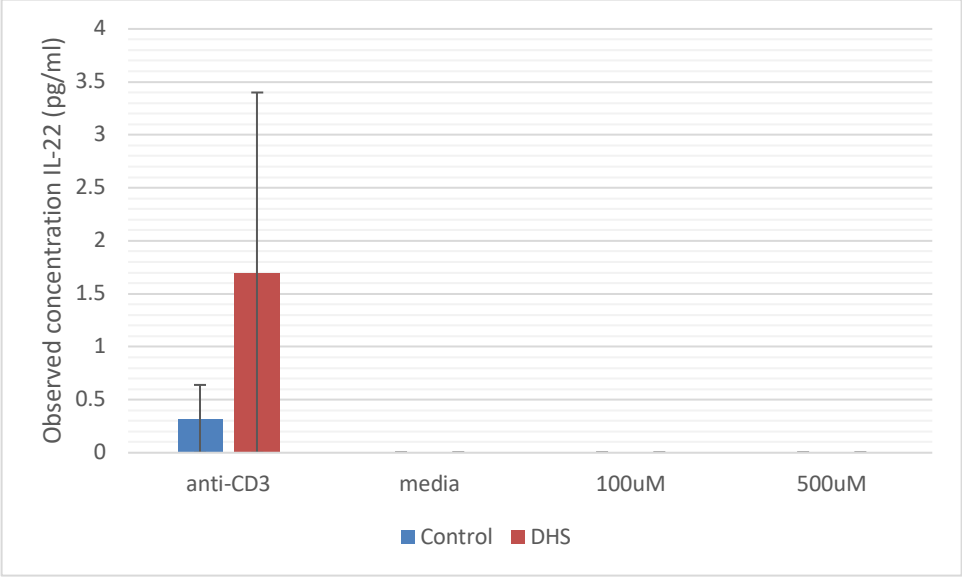


Figure 4-84: Observed concentration of IL-22 in pg/ml after incubation of PBMCs isolated from control and AMX-hypersensitive participants in anti-CD3, media (negative control), 100uM AMX, and 500uM AMX

N(control) = 4, N(DHS) = 4.

The cytokine concentrations of IL-4, IL-9, IL-13, IL-17A, and IL-22 were secreted in much lower concentrations than IFN-γ in response in anti-CD3 stimulation (Figure 4-85). However, not all participants had cytokines secreted when stimulated with anti-CD3. All participants secreted some amount of IFN-γ upon stimulation (Figure 4-86). For example, DHS005 was the only participant secreting IL-4 (Figure 4-87) and IL-9 (Figure 4-88), no other participants secreted detectable amounts of these cytokines. DHS005 also secreted greater amounts of cytokines when secreted with anti-CD3 compared to the other participants. Most participants secreted some small amount of IL-13 and IL-17A (Figures 4-89 and 4-90).

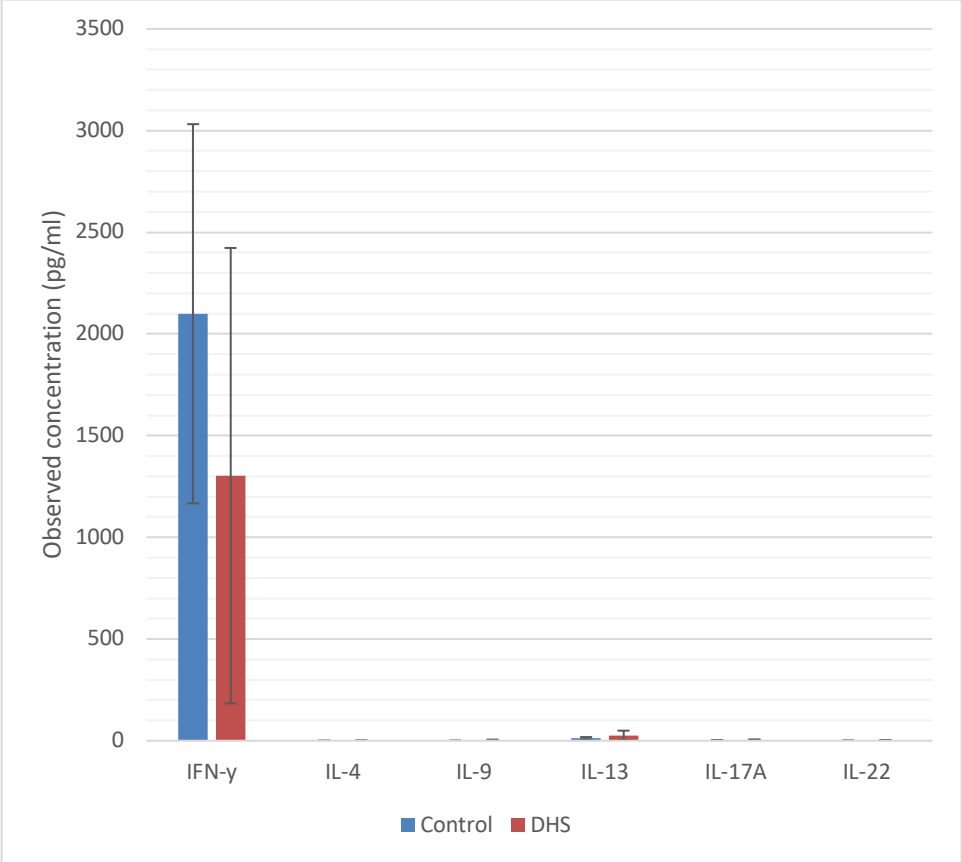


Figure 4-85: Average observed concentration of each cytokine analyzed in result to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3

N(control) = 4, N(DHS) = 4.

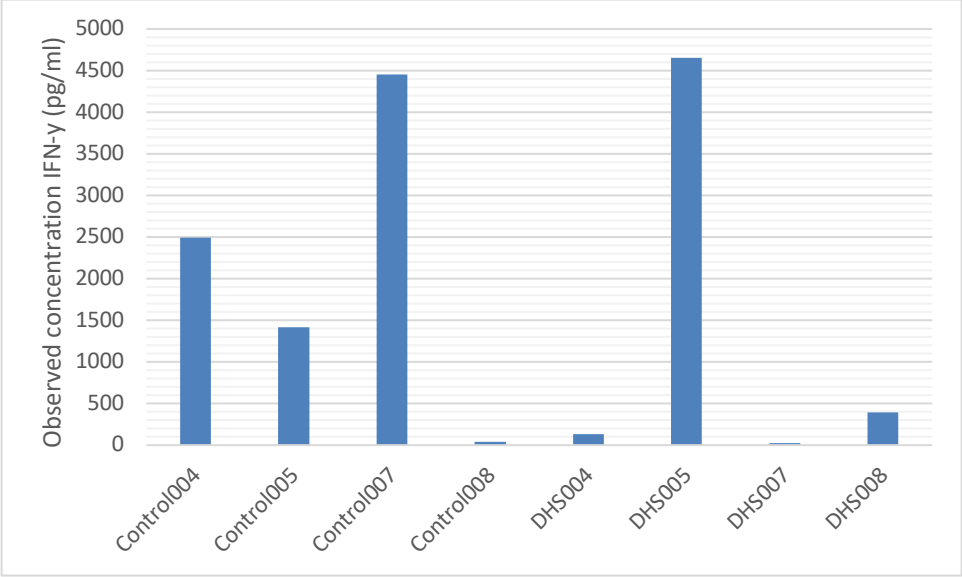


Figure 4-86: Observed concentration of IFN-y (pg/ml) in response to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3
N(control) = 4, N(DHS) = 4.

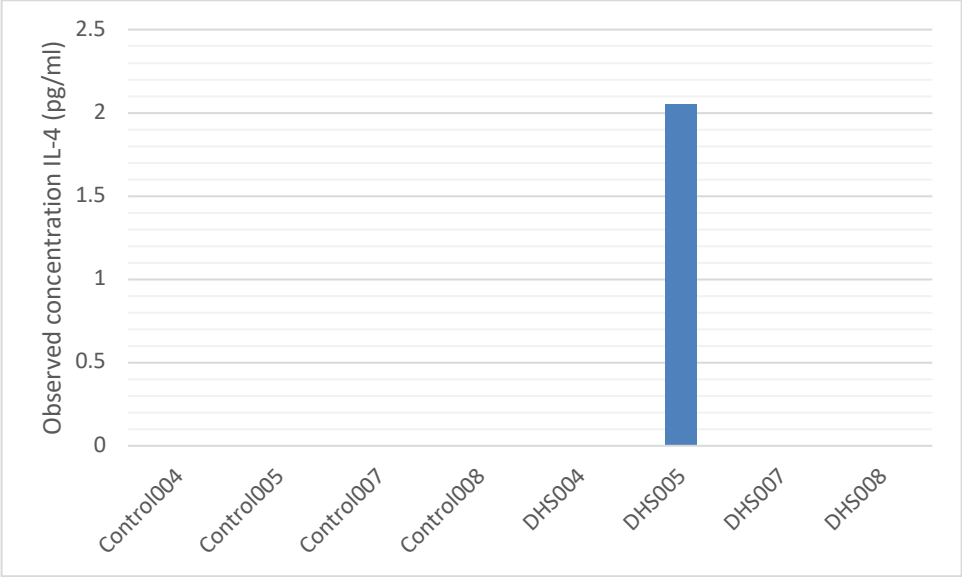


Figure 4-87: Observed concentration of IL-4 (pg/ml) in response to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3
N(control) = 4, N(DHS) = 4.

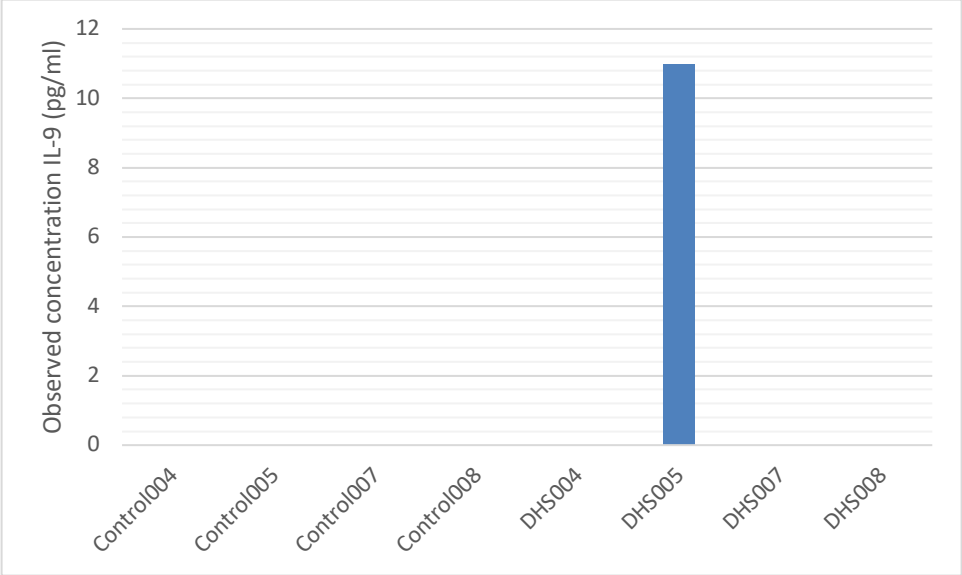


Figure 4-88: Observed concentration of IL-9 (pg/ml) in response to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3
N(control) = 4, N(DHS) = 4.

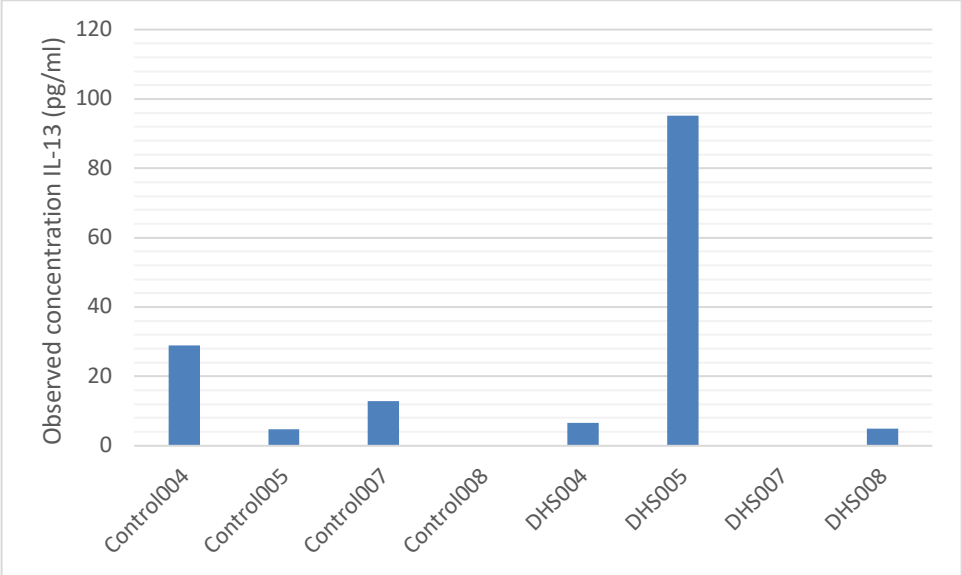


Figure 4-89: Observed concentration of IL-13 (pg/ml) in response to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3
N(control) = 4, N(DHS) = 4.

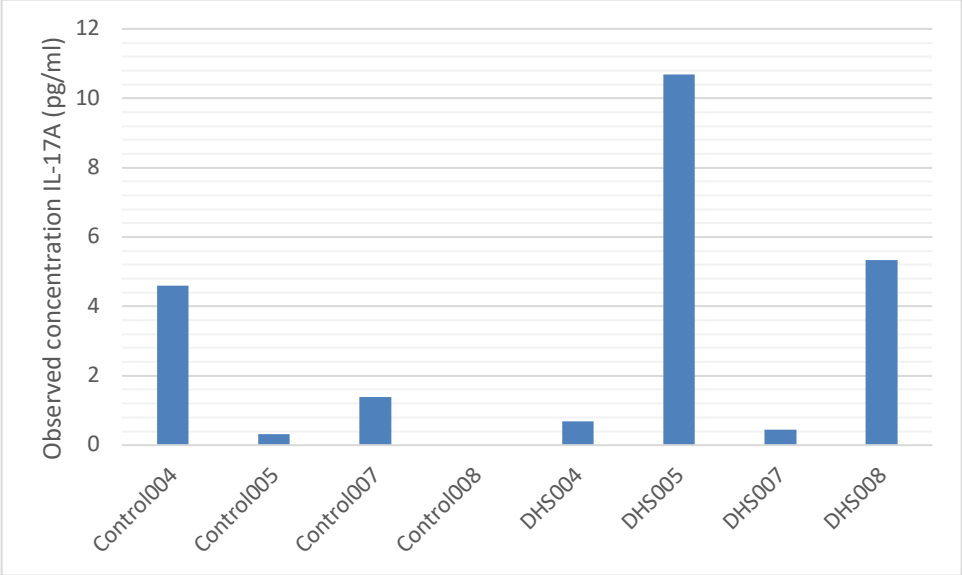


Figure 4-90: Observed concentration of IL-17A (pg/ml) in response to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3
N(control) = 4, N(DHS) = 4.

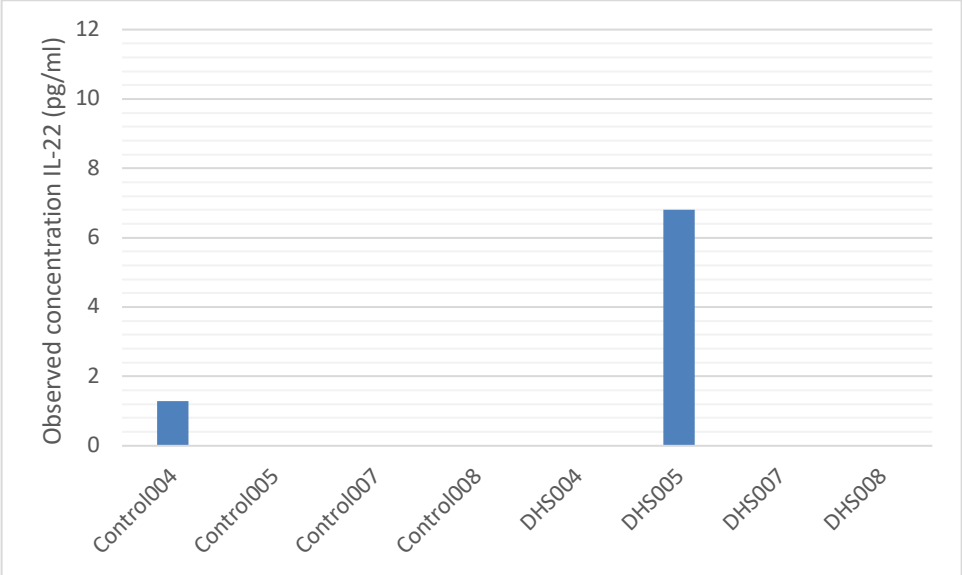


Figure 4-91: Observed concentration of IL-22 (pg/ml) in response to incubation of PBMCs isolated from control and AMX-hypersensitive participants with anti-CD3
N(control) = 4, N(DHS) = 4.

Chapter 5

5 Discussion

5.1 Aims and Objectives

Adverse drug reactions account for approximately 5% of all hospital admissions and affect millions annually.[5] Of these ADRs, DHRs account for approximately 20%.[11,12] Despite this, diagnosing DHRs is still difficult, resulting in overdiagnosis, reliance on substitute antibiotics, and limited understanding of the underlying mechanisms.[3,18,27] There are several competing hypotheses that attempt to explain how these DHRs occur (reviewed in § *Pathophysiology of DHRs*), however there is no consensus.

The aim of this thesis is to study and characterize lymphocytes from peripheral blood samples from participants with clinical presentations suggestive of delayed-type drug hypersensitivity reactions by comparing them to healthy controls. Since the underlying cause and pathophysiology of T cell-mediated hypersensitivity reactions is still not fully understood, the goal was to use a combination of techniques to study drug-specific lymphocytes. Previous findings suggest that there are different T cells involved with different physical presentations of drug allergy. We hypothesized that differences in activated peripheral T cell subsets and types of mediators released have a direct impact on the clinical presentations of DHRs. We wanted to determine if there are any cell types or cytokines involved in the different clinical presentations we observed and recruited. We specifically chose sulfamethoxazole (SMX) and penicillin due to the high number of cases typically referred to Dr. Rieder's clinic. Due to less than optimal cell count for the ^{3}H-thymidine and flow cytometry experiments, and RBC contamination in PBMCs leading to inconsistent cell counts in cytokine analysis, the results are inconclusive. However, this work has led to discovery of better methods and insights for improvements, optimization, and future studies.

5.2 SMX Parent Drug was Best Form of Sulfamethoxazole

Sulfamethoxazole, which inhibits the synthesis of tetrahydrofolic acid in bacteria,[244] is metabolized in the liver by *N*-acetyltransferases and *N*-glucoronul-transferase, resulting

in the production of non-toxic metabolites.[243] CYP-450 can also metabolize small amounts of SMX into two metabolites, SMX-NO and SMX-HA.[247,248] Since SMX, SMX-NO, and SMX-HA can be involved in the immune response,[60,122,243] it was important to determine if any of these will cause damage to the PBMCs they would be incubated with. We know that SMX-HA is a reactive metabolite that can lead to greater cell death, so we needed to see if it would cause greater cell death than it would show cell activation. Our preliminary experiments used SMX and SMX-HA because SMX-NO was too unstable for use under the experiment conditions.

First, I used PBMCs isolated from one volunteer and incubated these PBMCs with either unstimulated media, three concentrations of SMX, or three concentrations of SMX-HA. In the highest concentration of SMX-HA (100μM) used, there was a decrease in percent cell viability (Figure 3-1), but since sample size was only 1, I repeated the highest concentration with PBMCs isolated from two additional volunteers and found a significant decrease in percent cell viability after incubation (Figure 3-2). Therefore, we chose SMX since it did not negatively affect cell viability like SMX-HA.

5.3 54 Hour Incubation was the Optimal Length of Time

We needed to determine what the optimal length of time for incubating PBMCs with universal mitogen to minimize cell death and to show maximum activation. Since we used CD69 as an activation marker, this would show how CD69 is expressed over a time course and allow us to decide how long to incubate the PBMCs with drugs for. If the incubation time is not optimal, we would not see the maximum number of CD69-activated cells, and it would be more difficult to see an effect since dividing cells would closely resemble non-dividing cells. This experiment was modeled after a similar experiment by Beeler et al.,[295] however we added an extra timepoint and used control participants only.

I isolated PBMCs from three volunteers. After incubating the isolated PBMCs for intervals of 18 hours for a total of 90 hrs with anti-CD3, and staining for viability and CD69, we found a decrease in live cells (Figure 3-3). A rANOVA revealed that there was a significant decrease in cell viability as incubation time increased.

For determining the optimum length of incubation time for best CD69 expression, no significant timepoints were found for any of the cell types ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) examined (Figures 3-4 to 3-9). There was considerable variability across all timepoints, specifically in the MFI values. There were some noticeable but not significant increases at 54 hours for $CD3^+$ and $CD8^+$ cells (Figures 3-3, 3-5), while $CD4^+$ cells had the highest CD69 expression at the 18-hour timepoint.

Overall an incubation time of approximately 54 hours was chosen as a compromise between cell viability and CD69 expression. If closer to 90 hours was chosen, that would have left only approximately 50% of cells in the sample viable, even if there is no significant difference between 54 hours and 90 hours for percent CD69 expression.

For this experiment, both anti-CD3-stimulated and unstimulated samples were prepared for flow cytometry analysis, and the unstimulated values were subtracted from the stimulated values. However, it would be wise to repeat this experiment with a 0-hour timepoint to better normalize the timepoints in addition to stimulated and unstimulated samples at each timepoint.

5.4 Participant Recruitment

For this study, patients were recruited from Dr. Michael Rieder's drug safety clinic at London Health Sciences Centre, Victoria Hospital in London, Ontario. This research would not be possible without this direct access to ADR patients.

While we are fortunate to have potential access to a wide number of people of all ages seeking an *in vitro* toxicity assay for potential ADRs, there are some pitfalls. Typically, these participants are referred to the clinic by their primary care physician due to a suspected previous ADR to a drug their primary care physician is interested in prescribing them. However, many times these participants have no real recollection of when the reaction occurred, let alone the specifics including type of resulting skin reaction or how many days after ingestion of the drug that the reaction occurred. This is the primary reason why some cases are described as "rash, unspecified." These patients are included due to a positive LTA. The LTA test typically occurred sometime in the

previous 10 years before this study. It has been previously reported that drug-specific T cells can be re-activated after 12 years since previous exposure[122] or over 20 years since previous exposure.[301] Recruitment was challenging, as the participants often would not answer phone calls, phone number was out of service and not updated with hospital, no interest, no availability, or difficulty accessing RRI due to location of home or work.

We opted to test adult former patients (>18 years of age at the time of study) due to more easily obtained consent and a larger volume of blood drawn. Children and adolescents would be more complicated due to parental consent, for example parents might not be inclined to have blood drawn from their children for research. Children would also have to be taken out of school, or their parents would have to drive them before or after, while adults are usually more flexible with schedules and transportation.

Out of all of Dr. Rieder's former patients, many were eliminated immediately if the result of their LTA was negative. From all of the patients with positive LTAs, we excluded all those not positive to sulfamethoxazole and penicillin. I verified the final selected patients' LTA results, confirmed their eligibility to participate with Dr. Rieder, called former patients for consent to participate, and explained the contents of the letter of information and consent package over the phone (see *Appendix § Letter of Information*).

In addition, participants were also recruited directly from Dr. Rieder's clinic at the same time as LTA testing. These participants were asked to participate either because they had a strong history indicative of a delayed type ADR to either sulfamethoxazole or penicillin, or because they already had testing and were doing a re-test for a different drug.

The participant pool consisted of middle-aged women with the exception of one man. Many of the participants eligible to participate were women; I did call prospective male participants, however many did not return my phone call. In contrast, the women I spoke to seemed very eager to participate in the study. It is also worth noting that while my study is biased towards women, ADRs are also more prevalent in women. There are a few possible explanations to why more women were recruited. The first being that more women tend to access health care services and have more consultations with their GP

than men do, and in effect are prescribed more antibiotics. The second reason is women are more often diagnosed with an ADR than men. Overall, women are prescribed more antibiotics than men,[32] especially for conditions such as RTIs,[32,33] and UTIs.[32] Women also seek primary care much more than men do.[34,302,303] Women also have physiological differences, such as higher levels of CYP450, different levels of hormones, different rates of drug conjugation, and differences in body mass.[35–38]

Recruitment of participants also depends on the location of the patient themselves. The clinic often acquires samples from referred patients who live outside of London, often around Toronto or elsewhere throughout Southwestern Ontario. Many of these patients have a positive LTA and a history of ADR to the drugs that we are interested in, however often it is not possible to acquire a sample due to the participant residing outside of London. This can be due to a variety of reasons, some of which were experienced over the course of the recruitment of patients. Often, private clinics (ie. Life Labs or community health services) are inconsistent with their ability to draw blood for this lab/clinic. In addition, patients are often not motivated enough to participate, since it often involves driving to University Hospital or Robarts, which is inconvenient for many in addition to not wanting an extra blood test. Many of the patients were able to participate in this study by simply happening to be in the area at the time.

It would open up many more opportunities for patient recruitment if we could access these samples outside of the London area, or arrange with other clinics around London to be able to draw the blood to make it easier for participants who work during the day. Currently, University Hospital can do draws from about 8 am until 4 pm daily, but other private clinics might be open on the weekends or in the evenings. In the future it would be ideal to make arrangements with other hospitals or blood-drawing clinics in other cities to access a greater number of participants.

5.5 Defense of Drug Choice

Sulfamethoxazole and penicillin were chosen as our culprit drugs of choice, rather than a survey of all drugs tested for in the clinic, because these two drugs are some of the most

commonly tested for. We were able to obtain a large list of potentially eligible participants, some of which had consented to be a part of Dr. Rieder's database.

However, there are some of the downsides of these drug choices, one example being sulfamethoxazole. SMX is a frequently prescribed antibiotic for UTIs,[304] which are mostly seen in women. While they can and do occur in men, typically men do not suffer from as many UTIs due to increased length of urethra,[305–307] among other reasons including urethral opening proximity to vaginal mucosa compared to the dry epithelium of the glans penis.[305,306]

Choosing the appropriate reactive metabolite was important (see section *Determining Best Form of Sulfamethoxazole*). As previously mentioned, SMX-NO, and SMX-HA are reactive metabolites of SMX. SMX-NO has previously been used in studies.[243] SMX-HA is used in Dr. Rider's LTA analyses. Currently in the lab, we have stock of SMX-HA and SMX, however SMX-NO is unstable, so it would not be as useful. We found SMX-HA to negatively affect cell viability (Figures 3-1 and 3-2). In addition, it would be interesting to use liver microsomal metabolites rather than the AMX parent drug, to allow for reactive metabolites to be used in the experiment.[102,257]

5.6 Scintillation Counting

Beeler et al. suggest that a SI of at least 2 is a moderately positive result for DHR when using the LTT, while a SI of 3 is a strong positive result.[295]

Scintillation counting was performed on both SMX and AMX groups of participants, after incubation of 54 hrs (established as incubation length for flow cytometry) and 4 days. I found none of the concentrations of SMX or AMX caused a significant increase in SI at either timepoint (Figure 4-1, 4-3, Figure 4-5, 4-7). In addition, the age of the participant did not correlate with any noticeable increase in SI.

In some of the raw data for the scintillation counting, the cpm were low for anti-CD3-stimulated PBMCs and some for drug-stimulated samples were indistinguishable from unstimulated wells. I learned later on that there was an issue with cell counting and there was increased RBC contamination after the experiment was complete. I did not know I

was making this error and it was not corrected in time. We had previously attempted this when testing different mitogens and T cell stimulants (ex. anti-CD3, PHA, etc) and the results were better. In order to improve on this and obtain the results shown in other successful LTT studies, the incubation time should be extended to six days and the cell counting optimized to decrease RBC contamination.

Another possibility in the future would be to use CFSE (carboxy fluorescein succinimidyl ester) instead of ^{3}H-thymidine incorporation. CFSE is a common flow cytometry-based assay to assess cell proliferation, and it works by staining desired cells with CFSE before incubation, and quantifying the labelled cells by number of divisions. There is a progressive halving of fluorescence with each cell division.[308] There are several benefits to using CFSE over ^{3}H-thymidine incorporation. First, there is no radiation required and therefore no radiation permits or special training would be required. In addition, since it's a flow cytometry-based assay, it can discriminate between cell types, for example between $CD4^{+}$ vs $CD8^{+}$ cell types dividing, if those surface markers are also stained for.[308] CFSE is also stably and uniformly incorporated into cells, meaning cells can be cultured for weeks and still have measurable levels of CFSE remain in the cells.[309–313] Previous studies have used CFSE to stain T cells for cell proliferation in response to drug stimulation after six-[122,295,314] or seven-[315] day incubations.

One potential downside of this method would be cost – the flow cytometer is rented with a set rate. One bottle of ^{3}H-thymidine can last a long time, one well only requires 1ul, and use of the scintillation counter is free to the lab. For the CFSE analysis by flow cytometry, a new experiment would have to be set up with CFSE, in addition to all other desired fluors (for example, to differentiate T cells from PBMCs, and different T cell subsets), with our own FMO and isotype controls. A separate flow cytometry appointment would need to be set up aside from CD69 experiments, since staining for CD69 is done after only 54 hours of incubation, instead of six days.

5.7 Flow Cytometry

Flow cytometry was used with the stimulated PBMCs to determine which type of cell ($CD3^{+}$, $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$, $CD3^{-}$) was expressing CD69. These PBMCs were gated

according to cell surface marker expression as dot plots (Figure 4-5) and as histograms (Figure 4-6).

First, flow cytometry results were used to screen participants. Both cell viability and percent CD3 cells of total cells acquired were examined. To include participants, it was decided that the majority of PBMCs analyzed had to be alive (>50% cell viability after staining with fixable viability dye). With SMX participants (consisting of four DHR participants and three control participants), two DHS participants had viability less than 50% (Figure 4-7), and one control participant (Figure 4-8). It is not clear why these particular participants (DHS001, DHS002, and Control002) had low cell viability. For AMX testing, there were five DHR participants and four control participants. All participants had cell viability greater than 50% (Figures 4-9 and 4-10) so no AMX participants were excluded on this basis.

In addition to cell viability criteria, participants were also excluded based on percent of $CD3^+$ T cells acquired. If this percentage was too low, there would not be enough to see cells in gates further in the flow cytometry analysis. Typically, $CD3^+$ T cells are approximately 70% of the composition of PBMCs.[78–82,316,317] However, since there is individual variation between individuals, we removed participants with less than 30% of CD3+ cells acquired. Because of this, for SMX participants, DHS001, DHS002, and Control002 were removed – these were previously removed due to low cell viability. Out of the AMX participants, DHS006 was removed for a very low percentage of $CD3^+$ T cells. This is likely because once the blood was drawn, the laboratory that drew the blood refrigerated the sample, causing red blood cell contamination in the PBMC layer during Ficoll separation. This tube was centrifuged for longer to attempt to remove more RBCs, however the RBC count was still very high which was especially noticeable in the flow cytometry results.

For SMX participants, there was no significant increase in percent CD69 or MFI of CD69 across any of the cell types ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-$, see Figures 4-15 to 4-18, 4-19 to 4-22, 4-23 to 4-26, 4-27 to 4-30, respectfully). There was some variability in the groups at each concentration, however there were not enough participants to draw

conclusions. At the 100µM and 200µM SMX concentrations for DHS participants, one of the participants had very low cell numbers at these concentrations so these samples had to be removed.

There were few participants (N < 3 in each control and DHS) in this portion of the study, therefore no statistics could be performed. Ideally more participants would be recruited with hypersensitivities to SMX. To improve collection and results, red blood cell contamination would be reduced at the counting stage, and more cells would be acquired by flow cytometry (upwards of 50 000 cells).

Similar to SMX results, there was no significant increase in percent CD69 or MFI of CD69 across any of the cell types ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-$), see Figures 4-31 to 4-34, 4-35 to 4-38, 4-39 to 4-42, 4-43 to 4-46. There were some noticeable increases in a few instances in each cell type, however none of these increases were significant. There were two instances in this analysis where there was a noticeable difference between control and DHS. At 100µM AMX, the percent of CD69 on control $CD3^+CD4^+$ cells was higher than the DHS cells. This is unexplained and could be attributed to several things, including too few participants and low cell count. In addition, with $CD3^-$ cells, there was greater CD69 expression on DHS cells compared to control cells at 1000µM. There were no other noticeable differences at the other concentrations. While this difference could also be attributed to error, this concentration is also high and could have had an effect on CD69 expression. Again, there should be a greater number of participants, more cells acquired, and use of an isotype control to confirm this.

In addition to looking at each concentration individually, the increasing concentrations were grouped as concentration 1 (C1, 10µM AMX and 100µM SMX), concentration 2 (C2, 100µM AMX and 200µM SMX), etc (Figures 4-47 to 4-54). This analysis was done with results from the DHS participant group across both SMX and AMX participants. The goal was to see if there are any noticeable increases across increasing concentrations. These were separated according to cell type, percent CD69 expression and MFI of CD69, similar to previous analyses. Similar to other results, there were no significant differences between unstimulated and increasing concentrations across any cell types.

There were only a few noticeable, but not significant, differences. First with $CD3^+CD4^+$ percent CD69, at C2 (200µM SMX, 100µM AMX) there was an observable decrease below unstimulated and the other concentrations, while the other concentrations (1, 3, 4) showed no difference from unstimulated. $CD3^+CD4^+$ MFI of CD69 had a slight decrease across concentrations, however none of these concentrations differed from unstimulated. With $CD3^-$ MFI of CD69, C2 had large variability, which was different from the other concentrations, which did not differ from unstimulated. It is not fully known why these small differences occurred, however as with the previous analyses, a larger participant group, improved methods, the results would be more accurate.

Another analysis that was done was to look at how the control and DHS participants responded to anti-CD3 stimulation (Figures 4-55 to 4-62). Since anti-CD3 is a positive control for all of the participants across both drug types, all participants were combined regardless of which drug they had a hypersensitivity to. First, $CD3^-$ had a lower percent CD69 compared to $CD3^+$ cells, which is still consistent considering while some cells in the $CD3^-$ population might express CD69, they may not express this protein in the same capacity as T cells, or some may not express it at all.

There was one instance in this analysis where there was a difference between the percent of CD69 being expressed between control and DHS cells. $CD3^+CD4^+$ control PBMCs expressed a significantly higher percent of CD69 when compared to DHS participants when stimulated with anti-CD3 (Figure 4-57), however this difference was not reflected in the MFI of CD69 (Figure 4-58). This means that more cells expressed similar numbers of CD69. The reason for this is unknown so far.

AMX concentrations were combined to see if control or DHS participant PBMCs reacted differently to incubation to amoxicillin in general, regardless of concentration (Figures 4-71, 4-72 to 4-77, 4-78). There were no significant differences between control and DHS participants in either percent of CD69 or MFI of CD69 across all cell types. While this analysis does not take into account how the PBMCs would respond in different concentrations, it is reasonable to expect some differences across the concentrations that would affect CD69 expression.

Beeler et al. (2008) demonstrated that 1.9% of $CD4^+$ T cells from drug-allergic participants expressed CD69 in response to sulfapyridine, 1.1% in response to tetanus toxoid, and 0.1% to media. In our flow cytometry analysis, we did not see this. However, when looking at very few PBMCs, our analysis could be only seeing a few stimulated T cells. Increasing the number of PBMCs analyzed would increase the number of $CD4^+CD69^+$ T cells, which would make the difference between $CD69^-$ and $CD69^+$ cells more distinct. Deciding on where to place the gate for the $CD69^-$ T cells was decided by FMOs, however isotype controls should be used to place the gate more accurately. This is because isotype controls reduce non-specific binding of fluorescent-labeled antibodies. Even if we are seeing this 1-2% activation in T cells, it could be lost in the murky area between $CD69^+$ and $CD69^-$ due to lack of isotype controls.

With flow cytometry, starting cell count does not matter as much as cells are counted during analysis, and analysis is stopped once a certain threshold is reached. In the future, a greater number of cells should be analyzed, instead of 10000, closer to 100 000 PBMCs. This, coupled with decreasing RBC contamination, could show more $CD69^+$ cells if they exist in the sample, making a positive response more noticeable.

There are also some improvements that can be made to the protocol, which can be established with improvements in the number of fluorescent channels on a flow cytometer, or the availability of such a flow cytometer at Western University. The fluorescent antibodies that were used for this experiment included CD3-APC, CD4-PE, CD8-BV421, and CD69-PE-Cy7. CD3 is a pan-T cell marker, CD4 is present on Th cells, CD8 is present on CTLs, and CD69 is a T cell activation marker.[105,225,229,318] These four markers are for broad classes of T cells and activation, and we could get more specific than this. In the future, we could look at activation of more specific types of Th cells, including T_h1, T_h2, T_h9, T_h17, T_h22, and T_{reg} cells by flow cytometry by introducing other markers to the flow panel.

This study is very underpowered. For the flow cytometry work alone, if looking at drug and control, we would need N=28, or N=14 each for patient participants and controls, for each drug, for 80% power. This power calculation was performed by using the

differences in percent of CD69 T cells in response to drug stimulation observed in the study conducted by Beeler et al.[295] Adding in different clinical presentations would increase the number of participants. With six conditions, drugs, and participant/control groups, the required participant number increases to 72, with N = 12 per condition. The work completed so far has only 8 participants in total and is therefore incredibly underpowered. In addition to making adjustments to the methods, many more participants would need to be recruited. One benefit though is that this experiment could be considered a pilot project, to further provide insight into future studies.

5.8 Luminex

A custom Luminex assay purchased from Bio-Rad was used to assess the concentrations of IFN-y, IL-4, IL-9, IL-13, IL-17A, and IL-22 in the cell culture supernatant. Four DHS participants and their corresponding controls were selected from the AMX participants. DHS006 and Control006 were not chosen due to high RBC contamination and low percentage of $CD3^+$ cells observed in flow cytometry results.

The majority of the samples that expressed any level of cytokine were those stimulated with anti-CD3. Anti-CD3 promotes cell proliferation which could lead to greater amounts of cytokines released. Few cells in tested cultures would produce undetectable amounts in control and drug treatments. However, these results were highly variable because usually only one or two participants of the four had a measurable release of cytokines. None of the samples incubated with 100µM and 500µM AMX secreted detectable levels of cytokines, and most of the control (media) did not secrete any detectable levels of any of the cytokines. Only DHS005 secreted low but detectable levels across all six cytokines evaluated.

Given that there are likely inconsistent numbers of cells across samples, a protein quantification would be required to compare samples to each other. The total protein acquired would be divided by the amount of cytokine secretion to normalize to protein content. However, only the supernatant was used and it was frozen to accumulate enough samples for Luminex. The cells were centrifuged and the supernatant was collected off the top, and the plate and cells were discarded. In addition, the media used was

supplemented with 10% human serum, which contains protein. Our original concept was to conduct a total protein quantification on the remaining supernatant used for Luminex, and use RPMI 1640 + 10% human AB serum as background. This background protein quantification would be subtracted from the protein content of the samples. We attempted to do this and tested the controls and standard curve with the protein quantification kit. It was recommended to use a buffer without protein, such as 1% PBS. After analyzing the standard curve prepared in both PBS and RPMI 1640 + 10% human AB serum, the media curve was very warped, and it was suggested by BioRad that the media and serum were not compatible with the kit. It is also a possibility that the proteins in the kit increased the total protein content higher than the upper limit of the kit. In the future, it would be beneficial to ensure the PBMC concentration is the same among all participants with more accurate cell counting. In addition, conduct protein quantification alongside isolating and freezing the supernatant, so that cells could be lysed and a total protein quantification could be done on lysed cells. Alternatively it could be done as it was, and the supernatant could be frozen and analyzed later. It would also be beneficial to leave out the serum if we will be doing a protein quantification.

Other methods we had previously considered included ELISAs (enzyme-linked immunosorbent assay) and ELISpot (enzyme-linked immunospot) assays. An ELISpot is similar to an ELISA, however the cells are bound to the bottom of a 96-well plate, and the cytokines being released are also bound to the bottom of the plate, near the secreting cell. When the dye is applied, the cell secreting the cytokine being analyzed turns blue. The number of cells secreting the cytokine can be counted. This method would be useful if we tried cloning drug-specific T cells.[104] ELISAs could be used, however each kit for a single cytokine was approximately $600. For this initial attempt, Luminex was less expensive. However, if this experiment continues in the future, the ELISAs may be a more economical solution. In addition, the ELISA kit has the ability to measure additional proteins, including granzyme B, which is secreted by cytotoxic T cells. Granzyme B, for example, is not measurable by the Bio-Rad customizable Luminex kit we used. This would be relevant due to the involvement of cytotoxic T cells in Stevens-Johnson Syndrome, for example Chung and Hung 2010, Murata et al. 2008, among others.[270,272]

In addition to surface phenotyping greater populations of T cells by flow cytometry, we can also look at cytokine secretion using flow cytometry. While Luminex is a viable solution, it could be beneficial to try intracellular cytokine staining. One downside to this is that the flow cytometer currently available is able to examine 14 colours. We would have to carefully choose which cytokines to analyze in order to accommodate the viability dye, cell surface markers, and intracellular dyes. However, to accomplish all of this at once by flow cytometry, the staining would take more time and the flow cytometer would require more fluorescent channels, depending on how many T_h cell types are being analyzed and how many cytokines would be looked at.

To correctly power this study for Luminex, a survey of the literature for the difference between baseline and stimulated cytokine concentrations for each cytokine would need to be done. A smaller difference would require additional participants to support a conclusion.

5.9 Future Studies

There are many future directions that can be taken with this project. Regarding participant recruitment, it would be prudent to focus more on recruiting more age- and sex-matched controls. In this current study, the gender profiles of the DHR participants differed from those of the controls, as well as the average age was significantly older in the DHR compared to the controls. Another interesting avenue to explore would be matching the reason the drug was taken. For example, if one participant had a DHR to SMX taken for a UTI, then the control would have taken SMX for a UTI but did not have a reaction.

An interesting direction this research could take is to genotype HLA-type participants in addition to the *in vitro* testing done. There are many GWAS (Genome-Wide Association Studies) from throughout the globe,[319–323] including some in Canada.[324] It would be beneficial to genotype adult patients recruited, and also extend this to paediatric patients as well. These GWAS help to identify culprit genes that contribute to the development of ADRs, and are often specific to origin/ethnicity. Genotyping is a huge component of personalized medicine. By phenotyping people who have or have had ADRs, we can look

for patterns between genes (specifically HLA genes), drug type and clinical presentation. This would be extremely beneficial to people who are looking to take a specific drug.

With so few participants, this is a low-powered study, especially considering the vast numbers of different skin reactions that exist and recruiting more people over time to increase the number of hypersensitive participants as well as controls to increase the power would reveal more promising results.

Children are some of the most common users of prescription antimicrobials.[325] It would be very beneficial to include children into this analysis. Children are not just smaller versions of adults: the physiology of a child and their ability to metabolize drugs is different than that of an adult, in addition to several other differences.[326,327] While there could be a genetic component to penicillin hypersensitivity in children, it could be another underlying pathway resulting in adverse reactions occurring.[328] One of the main reasons children were excluded from this study is because of the volume of blood required for the study. With adults, we drew approximately 15 ml of peripheral blood, while in children typically we must draw less because they are smaller. Improving the methods, for example by combining the current methods into one large flow cytometry panel, could decrease the total number of isolated PBMCs required to acquire the same information. This would decrease the amount of blood required to be drawn, which could open up this experiment to consenting and assenting children and teenagers.

In addition to collecting and plating PBMCs for Luminex, I originally isolated $CD3^+$ cells for plating for cytokine release. However due to the cost of reagents for one 96-well plate and prioritizing PBMCs over isolated T cells, the supernatant for these T cells is still frozen. This is important since there would be no RBC contamination unlike in the PBMC experiments. The cell count is consistent across all samples and these samples could be tested reliably without protein quantification. While limited by not including $CD3^-$ derived cytokines, it would be interesting to see T cell only derived cytokines profiles. Ideally this analysis would be run alongside PBMCs.

Our current study asked participants who had a DHR diagnosed in previous years, similar to previous reports (for example Beeler et al. 2007).[295] It would be interesting to continue

this research in DHR patients who are currently undergoing a DHR, and also get leftover skin biopsy and blister fluid samples from active lesions. Some previous studies have done this, and have examined activated T cells and cytokines present in both peripheral blood and skin/blister fluid with promising results.[275] They also analyzed peripheral blood during an active DHR and after resolution. This is important since it can demonstrate important diagnostic markers.

5.10 Conclusions

Overall, we cannot draw conclusions due to too few subjects and the need for different experimental approaches. However, there are several improvements that can be made that have been proposed. In the future it would be prudent to adjust the methods, including using flow cytometry to measure proliferation instead of scintillation counting. We could also expand the number of T cell subsets we analyze, by both increasing the surface markers by flow cytometry and the cytokines tested using a bead-based detection assay. It would be interesting to look at different drugs in addition to sulfamethoxazole and beta lactam antibiotics, to learn more about the role drug type has with DHRs. This study could span years to accumulate enough participants and increase power. There is much work to be done in the field of drug hypersensitivity; efforts put towards faster identification and detection of DHRs can contribute to decreased healthcare burden and increased quality of life.

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Appendices

List of Abbreviations

ADR – adverse drug reaction

AGEP – acute generalized exanthematous pustulosis

AMX – amoxicillin

APC – antigen presenting cell

BCR – B cell receptor

BSA – body surface area

CBZ – carbamazepine

CCR – chemokine receptor

CD – cluster of differentiation

CLA – cutenaous leukocyte antigen

Cpm – counts per minute

CTL – cytotoxic T cell

DAMP – danger-associated molecular patterns

DC – dendritic cell

DHR – drug hypersensitivity reaction

DIHS – drug-induced hypersensitivity syndrome

DNA – deoxyribonucleic acid

DRESS – drug rash with eosinophilia and systemic symptoms

DRESS – drug reaction with eosinophilia and systemic symptoms

EM – erythema multiforme

FACS – fluorescence-activated cell sorter

FasL – Fas ligand

GP – general practitioner

GVHD – Graft vs Host Disease

GWAS – genome wide association study

HLA – human leukocyte antigen

IFN-y – interferon-gamma

Ig – immunoglobulin

IL – interleukin

ILC – innate lymphoid cell

LTA – lymphocyte toxicity assay

LTE – lymphocyte transformation test

MHC – major histocompatibility complex

MPE – maculopapular exanthemas

MRSA – methicillin resistant staphyllocaucus aureus

NK – natural killer

NKT – natural killer T cells

NSAID – non-steroidal anti-inflammatory drugs

p-i – direct pharmacological interaction of drugs with immune receptors

PAMP – pathogen-associated molecular patterns

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline, pH = 7.2

PE – phycoerythrin

PE-Cy7 – phycoerythrin cyanine-7

Pen – penicillin

PMA – phorbol myristate acetate

R – receptor (ie. IL-4R)

rANOVA – repeated measures analysis of variance

RBC – red blood cell

RNA – ribonucleic acid

RPMI – Roswell Park Memorial Institute (media)

RTI – respiratory tract infection

SI – stimulation index

SJS – Stevens-Johnson Syndrome

SJS – Stevens-Johnson Syndrome

SLE – systemic lupus erythematosus

SMX – sulfamethoxazole

SMX-HA – sulfamethoxazole hydroxylamine

SMX-NO – nitrososulfonamide

Strep – streptomycin

T_c – cytotoxic T cell (also see CTL)

TCR – T cell receptor

TEN – Toxic Epidermal Necrolysis

TGF-b – tumour growth factor

T_H – helper T cell

TMP – trimethoprim

TNF – tumor necrosis factor

TNF-b – tumour necrosis factor

T_{reg} – regulatory T cells

UTI – urinary tract infection

ViD – viability dye

WHO – World Health Organization

Ethics Approval

Western Research

Date: 18 January 2018

To: Michael Rieder

Project ID: 3091

Study Title: Canadian Pharmocogenomic Network for Drug Saftey - 11883E

Application Type: Continuing Ethics Review (CER) Form

Review Type: Delegated

Date Approval Issued: 18/Jan/2018

REB Approval Expiry Date: 24/Jan/2019

Dear Principal Investigator Full Name ,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Erika Basile, Director, Office of Human Research Ethics, on bahalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Date: 4 January 2019

To: Michael Rieder

Project ID: 3091

Study Title: Canadian Pharmocogenomic Network for Drug Saftey - 11883E

Application Type: Continuing Ethics Review (CER) Form

Review Type: Delegated

REB Meeting Date: 29/Jan/2019

Date Approval Issued: 04/Jan/2019

REB Approval Expiry Date: 24/Jan/2020

Dear Michael Rieder,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Daniel Wyzynski, Research Ethics Coordinator, on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Letter of Information

Letter of Information

This Letter of Information and the Informed Consent form are directed to the participants in this study. The pronouns 'you' and 'your' should be read as referring to the participant.

Principle Investigator: Michael J. Rieder MD, PhD, FRCPC, FAAP, FRCP (Glasgow)
Professor, Departments of Paediatrics, Physiology & Pharmacology and Medicine, Schulich School of Medicine and Dentistry, Western University
Department of Paediatrics
London Health Sciences Centre
[redacted]

Study Coordinator: Abdelbaset Elzagallaai, BSc Pharm, MSc, PhD
Research Associate and Laboratory Manager, Department of Pediatrics, Schulich School of Medicine and Dentistry, Western University
Robarts Research Institute [redacted]

Graduate Student: Christine Caron, BSc Biology, MSc candidate
Graduate Student, Department of Pathology and Laboratory Medicine, Western University
Robarts Research Institute [redacted]

Sponsor: Canadian Institutes for Health Research (CIHR)

Place of Research: Robarts Research Institute, Western University

Title of Study: Characterizing T-cell phenotype in patients with hypersensitivity reactions to sulfonamides and beta-lactam antibiotics

Background
Adverse drug reactions are a serious problem and are among the top five causes of all deaths in Canada and the United States. We know that some people who take a drug suffer an adverse reaction while others can take the same drug without any adverse effects. Some of these adverse reactions are mediated by cells in the immune system that circulate through the blood. We would like to learn more about how these cells differ between patients with a history of drug reaction and those without.

Purpose of Study
The purpose of our research is to prevent adverse drug reactions and the subsequent skin rashes through improved understanding of the physical features of involved immune cells and their secretions. These studies will help to find out what role different cell types and their respective secretions have in predicting who will have adverse drug reactions and what type of adverse skin reaction will result.

Since not all of the immune cells and cell secretions involved in different responses to drugs have been discovered yet, we would like to bank your biological sample to study in the future. This way, we can go back and test different types of cells present in your sample, or look at different secretions that those cells produce when stimulated with the drug.

The exact plan for these future studies is not known at this time since it will depend on other discoveries being made in the area of pharmacological and immunological research.

Invitation
You are being invited to participate in a study looking at immune cells in your peripheral blood (specifically, blood from a vein in your arm), and then have the unused sample of your cells isolated from blood stored for future use in research studies. This process is referred to as biobanking.

Participation
Before you decide to consent, it is important for you know why we wish to collect and bank your peripheral immune cells and what will be done with them. This consent form will tell you what will be collected and stored, where it will be stored, who will have access to it, how it may be used in the future, and the possible benefits, risks, and discomforts associated with providing a sample.

If you wish to participate in this study, you are invited to sign this form. Participation in this study is voluntary, therefore you have the right to refuse to allow us to obtain a blood sample and/or have your isolated immune cells banked. Refusing to participate or have your cells banked will not affect your participation in this study nor will refusing to participate in the study as a whole affect your present or future medical care.

Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide.

Procedures
As part of this study, a small amount of your blood (approximately 15-20 millilitres, or 3-4 teaspoons) will be drawn using a needle from a vein in your arm.

Once the blood is collected, the peripheral blood mononuclear cells (PBMCs; immune cells that are in circulation through arteries and veins) will be isolated. The cells will be examined for how they respond to stimulation with the drug that caused the adverse reaction. Then, we would like to take a portion of the isolated PBMCs and freeze them for future analysis. Upon consent, your samples will be labelled with a unique numeric code so that your name will not be attached to the sample. Only the study's Principal Investigator and his designates will hold the key containing your unique numeric code with your identifying information. This sample will be held frozen at Robarts Research Institute, Western University.

We are also asking your permission to keep some information obtained from your medical records. This information is only pertinent to the adverse drug reaction you experienced to sulfamethoxazole or beta-lactam antibiotics. Only the relevant information obtained from these records will be stored in our secure study databases located at Robarts Research Institute. This information will be identified using the same study identity number as the biological sample. No information that could identify you personally will be included. Only the study investigators will have access to the database.

Every measure will be taken to ensure your privacy. The cells you provide will only be used for research described in this consent form. You will not receive the results of this or any future tests. Your participation in this study will not become part of your medical record. The analyses conducted by our investigators are focused on the response of cells upon stimulation by the specific drug you have an adverse reaction to and a control stimulant. Therefore it is extremely unlikely that we will uncover any new information relevant to your health or overall wellbeing. However, in the rare event that this occurs, the biobank will be requested to re-identify you so that we can notify your physician of our findings.

Responsibilities
After your blood sample has been taken you do not need to do anything else to participate in this study.

Risks and Discomforts
Blood withdrawing is identical to any blood sample taking for routine blood tests at a hospital or a medical lab. The possible harms and discomforts of the study mostly involve the collection of the blood sample. There may be some slight pain and discomfort when the needle is inserted into the vein for blood collection, and some minor bleeding, bruising, swelling, or feeling faint or dizzy after it is removed.

Benefits
The research that may be done with your biological sample is not expected to benefit you or your family members directly. However, we hope that the information gained from these studies can be used in the future to improve the safety and efficacy of beta-lactam antibiotics and sulfamethoxazole.

Withdrawal
It is possible to withdraw from the study at any stage upon your request. All your samples will be properly destroyed and data will be deleted.

Confidentiality
Protecting your privacy is our number one priority. Your rights to privacy are legally protected by federal and provincial laws that require safeguards to ensure that your privacy and personal data are respected. These laws also give you the right of access to the information that has been provided and, if need be, an opportunity to correct any errors in this information. In most cases, your personal information or information that could identify you will not be revealed to any third party, including your family members and your physician, without your expressed consent.

You will be assigned a unique study code number as a participant in this study. This number will not include any personal information that could identify you (for example it will not include your Ontario Health Insurance Card number, or Social Insurance Number, your initials, etc.). Only this number will be used on any research-related information collected about you during the course of this study, so that your identity will be kept confidential. Only the Principal Investigator, the study coordinator, and the person collecting your blood sample will have access to the names of participants in this study. Lists of participants will be kept locked in the office of the study coordinators. All computer files will be kept encrypted and locked with restricted access passwords known only to the investigators. Information about the drugs you take and your blood sample will be sent to our analysis center, at the Robarts Research Institute, for analysis.

No information or records that disclose your identity will be published, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

Name, address, and phone number are collected in the rare instance that we need to contact you regarding any unclear information. This extra contact will be minimal, and we do not expect to contact every patient enrolled.

Since your sample and information will be kept for many years or until it is used entirely or withdrawn, we will update our security measures for protecting your data and for preserving your sample as they become available.

Contact Person(s) for Participants
If you have any questions about the study and/or treatment and care, you may contact Dr. Abdelbaset (Baset) Elzagallaai, the study coordinator [redacted] or Christine Caron [redacted]

No Waiver of Rights
You do not waive any legal rights by signing this consent form.

Consent Form – Research Copy

Title of Study: Characterizing T-cell phenotype in patients with hypersensitivity reactions to sulfonamides and beta-lactam antibiotics

I have read the accompanying letter of information and have had the nature of the study explained to me and I agree to participate in the study. All questions have been answered to my satisfaction.

Participant's Date of Birth

Participant's Name (please print) Name of Person Obtaining Informed Consent (please print)

Participant's (or Guardian's) Signature Signature of Person Obtaining Informed Consent

Date Date

Consent Form – Participant Copy

Title of Study: Characterizing T-cell phenotype in patients with hypersensitivity reactions to sulfonamides and beta-lactam antibiotics

I have read the accompanying letter of information and have had the nature of the study explained to me and I agree to participate in the study. All questions have been answered to my satisfaction.

Participant's Date of Birth

Participant's Name (please print) Name of Person Obtaining Informed Consent (please print)

Participant's (or Guardian's) Signature Signature of Person Obtaining Informed Consent

Date Date

Curriculum Vitae

Academic Background

September 2017 – August 2020	Western University Master of Science in Pathology and Laboratory Medicine student under the supervision of Dr. Michael Rieder
January 2014 – April 2017	Algoma University Undergraduate student in Honours Bachelor of Science in Biology, minor in Psychology

Awards and Scholarships Received

2018	Dr. Frederick Winnett Luney Graduate Research Award
2017	NSERC-USRA Scholarship Algoma University Dean's Honour List
2016	Algoma University Academic Achievement Scholarship NSERC-USRA Scholarship Algoma University Dean's Honour List
2015	Algoma University Academic Achievement Scholarship NSERC-USRA Scholarship Algoma University Dean's Honour List
2014	Lake Superior State University Dean's List
2013	Lake Superior State University Canadian Student Entrance Scholarship Sault Ste. Marie Zonta Scholarship Kewadin Club Scholarship

Work Experience

September 2018 – present	Graduate Teaching Assistant (full time, 140 hours per semester) – Western University, Department of Biology: Introductory Biology (BIOL1001/1201A, BIOL1002/1202A) skills sessions B teaching assistant, involves co-leading approximately 40 students through a wet lab environment, grading assignments, proctoring midterms and final exams, entering grades
May 2017 – August 2017; May 2016 – August 2016; May 2015 – August 2015,	NSERC Undergraduate Student Research Assistant – Algoma University: Received NSERC-USRA grant to work in Algoma University's plant biochemistry lab under the supervision of Dr. Isabel Molina on projects involving the study of suberin deposition in poplar trees
September 2015 – April 2016	Research Assistant – Algoma University, Department of Biology: Worked in plant biochemistry lab at Algoma University under the supervision of Dr. Isabel Molina, studying suberin deposition in poplar tree studies

January 2015 – December 2015	Teaching Assistant – Algoma University, Department of Biology: Introductory Chemistry (CHMI1006) course teaching assistant; duties involved helping students, grading tests, quizzes, assignments, and holding tutorial sessions for the classes
September 2014 – December 2014	Teaching Assistant – Algoma University, Department of Biology: Introductory chemistry (CHMI1006) lab teaching assistant; duties included supervising the students during lab experiments, answering questions, and helping the lab instructor
September 2014 – April 2016	Tutor – Algoma University: Tutor for a variety of subjects, including general chemistry and organic chemistry

Research Experience

September 2017 – present	Western University – London, Ontario: MSc student • Supervisor Dr. Michael Rieder • Project involves studying pathophysiology of drug hypersensitivity reactions, specifically culturing, stimulating, and staining immune cells, and assessing phenotype and cytokines
2015 – 2017	Algoma University – Sault Ste. Marie, Ontario: NSERC-USRA Scholarship • Supervisor Dr. Isabel Molina • Involved in continual research experiments surrounding *Populus* sp. wounding experiments and mutant analysis
August 2016, August 2017	University of California San Diego – San Diego, California • Part of NSERC-USRA scholarship • Supervisor Dr. Laurie Smith, collaboration with Dr. Isabel Molina • Collecting and phenotyping corn leaves as part of National Science Foundation project on drought-resistant maize
2016 – 2017	Algoma University – Sault Ste. Marie, Ontario: Thesis student • Supervisor Dr. Isabel Molina • Involved in determining gene responsible for unique bark phenotype in *Populus* 717 mutant using forward genetics and to determine whether mutation is related to suberin deposition
2015 – 2016	Algoma University – Sault Ste. Marie, Ontario • Supervisor Dr. Isabel Molina • Plant Biochemistry • Hired part-time to begin growing cuttings of mutant *Populus* ssp. 717 mutant and performing lipid analyses on suberin and associated waxes, while also performing general lab duties such as TLC on other lab projects

Conference Experience

June 2019	Canadian Society of Pharmacology and Therapeutics Joint Annual Conference, "From Base to Summit: Pharmacology at its Peak", Calgary, Alberta Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions*
April 2019	London Health Research Day, London, Ontario • Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions*
March 2019	Western University Department of Pathology Research Day, London, Ontario • Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions*
June 2018	Robart's 5th Annual Research Retreat, London, Ontario • Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions*
May 2018	Canadian Society of Pharmacology and Therapeutics Joint Annual Conference, "Translating Innovative Technology to Patient Care", Toronto, Ontario • Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions* London Health Research Day, London, Ontario • Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions*
April 2018	Western University Department of Pathology Research Day, London, Ontario • Presented poster *Investigating the Pathophysiology of T-Cell Mediated Drug Hypersensitivity Reactions*
March 2017	Ontario Biology Day 2017 Undergraduate Conference at Laurentian University, Sudbury, Ontario • Presented undergraduate thesis poster *Characterization of a Mutant Poplar Hybrid (Populus sp.) with an Abnormal Bark Phenotype* •

Leadership, Outreach, and Volunteer Experiences

October 2018—present	Vice President of Social Events with Western Pathology Association • Planning social events for undergraduate and graduate students as well as staff and faculty in the Pathology department to help with teambuilding and morale
May 2018	Volunteer at Science Rendezvous event with the Western Pathology Association's "Crime Scene Investigation" booth at Western University, London, Ontario

April 2017	Volunteer at Sault Ste. Marie's Science Festival "Hangar After Dark" event at the Canadian Bushplane Heritage Centre, Sault Ste, Marie, Ontario
2015—2016	President of the Algoma University Biology Club
April 2016	Science Fair at Algoma University • Presented overview of ongoing research conducted in Dr. Isabel Molina's laboratory
January 2015—April 2016	General Chemistry Teaching Assistant • Created and conducted tutorial sessions for first year students
September 2014—April 2015	Community Ecology Lab Volunteer • Duties included sorting and counting seeds of various species after collection from field for future analysis and experimentation
September 2014—December 2014	General Chemistry Lab Teaching Assistant • Aided students in performing lab experiments for first year general chemistry