Risk Factors for Drug Immunogenicity in a Macaque Model of HIV Infection

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Abstract

HIV-infected patients have a high acquired risk of rash and other delayed hypersensitivity reactions to potentiated sulfonamide antibiotics (trimethoprim/sulfamethoxazole; TMP/SMX), which correlate with low CD4+ counts. Further, this risk is associated with a biomarker, in which patient lymphocytes (especially CD8+ cells) show apoptosis from reactive SMX metabolites in an *in vitro* lymphocyte toxicity assay (LTA). The mechanisms are not understood, but an underlying SMX detoxification defect has been postulated. We hypothesized that acquired metabolic and immunologic alterations were present in retroviral infection that lead to SMX immunogenicity and LTA susceptibility.

SIVmac239-infected rhesus macaques and age-matched controls were administrated TMP/SMX (120 mg/kg/day p.o.) for 14 days, and immune responses were measured. At baseline, blood was assayed for ascorbate, glutathione, IFN-γ, sCD14, and LPS, and liver biopsies and lymphocytes were collected for expression arrays. After treatment, plasma and 24 hour-urinary SMX metabolites were measured, and macaques were tested for SMX-adducts in lymph nodes, anti-drug antibodies, and drug-responsive T cells. Among 9 SIV-infected and 7 non-infected controls, 3 macaques showed drug-responsive T cells and 4 macaques developed anti-SMX antibodies. Two macaques developed transient rash during dosing, and these animals had the highest percentage of lymph node cells with SMX-adducts. However, no association was found between immunologic response and SMX disposition, antioxidants, or inflammatory mediators.

Hepatic expression arrays, along with hepatic activity assays, did not support an effect of SIV infection on SMX detoxification. Instead, genes involved in antigen processing, trafficking, and presentation were over-expressed, and this could contribute to SMX hypersensitivity via promiscuous presentation of SMX-adducted peptides.

Lymphocyte death in the LTA was apparently increased in SIV-infected macaques (29.8% vs. 1.8%, $P = 0.06$), but CD8+ lymphocyte expression arrays from high and low LTA animals did not support impaired detoxification of SMX metabolites. Instead, several pro-apoptotic and lysosomal degradation genes were up-regulated, which could lower the threshold for cell death from reactive SMX metabolites.

In summary, this is the first animal model that demonstrates immunogenicity to SMX at therapeutic dosages; studying more animals with CD4+ counts < 200 cells/µl may better model the risk of SMX immunogenicity and hypersensitivity in HIV-infected human patients.

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List of Abbreviations

AIDS	acquired immunodeficiency syndrome
CD	cluster of differentiation
CO_2	Carbon dioxide
cDNA	complementary deoxyribonucleic acid
cpm	count per minute
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GCL	glutamate-cysteine ligase
GSH	glutathione
GSS	glutathione synthetase
GST	glutathione S-transferase
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC	high performance liquid chromatography
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSA	human serum albumin
IACUC	Institutional Animal Care and Use Committee
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISG	interferon-stimulated genes
KLH	keyhole limpet hemocyanin
LNMC	lymph node mononuclear cell
LPS	lipopolysaccharide
LTA	lymphocyte toxicity assay
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	*N*-acetyltransferase
OVA	ovalbumin

PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
sCD	soluble cluster of differentiation
SEM	standard error of the mean
SI	stimulation index
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus
SMX	sulfamethoxazole
SMX-HA	sulfamethoxazole-hydroxylamine
SMX-NO	sulfamethoxazole-nitroso
TMP	trimethoprim
TNF	tumor necrosis factors
vRNA	viral ribonucleic acid

Chapter 1 – Introduction

1.1 Indications for Sulfonamide Antibiotics, and Adverse Reactions

Sulfamethoxazole (SMX) is a sulfonamide antibiotic that serves as a competitive inhibitor for dihydropteroate synthase, and inhibits synthesis of folate.[1, 2] All organisms require folate for DNA synthesis, repair and methylation.[3, 4] Mammals have an active transport system at the cell membrane to acquire folate,[5] bacteria, certain fungi, and protozoa do not, providing SMX with broad spectrum to fight infections.

Trimethoprim (TMP) is often administrated together with SMX to potentiate the inhibition of bacterial folate synthesis.[2] TMP selectively inhibits bacterial dihydrofolate reductase, another enzyme involves in folate synthesis.[2, 6] TMP/SMX is often administrated in a 1:5 ratio to treat diseases such as bronchitis, urinary tract infections, methicillin-resistant *Staphylococcus aureus*, and *Pneumocystis jiroveci* pneumonia.[7-10]

Pneumocystis pneumonia is caused by a yeast-like fungus, *Pneumocystis jiroveci*. It is an opportunistic pathogen, and mostly infects patients that are immunocompromised, such as those with AIDS, or patients that are on immunosuppressive therapy for autoimmune disease.[11-13] TMP/SMX is the primary treatment recommended by the United States Centers for Disease Control and Prevention to treat and prevent *Pneumocystis jiroveci* pneumonia in HIV-infected patients (www.cdc.gov). Further, the World Health Organization strongly recommends that

AIDS patients (with CD4+ counts ≤ 350 cells/mm^3) start TMP/SMX prophylaxis to prevent opportunistic infections. (www.who.int).

The use of TMP/SMX is essential in AIDS patients but its use is often limited by adverse drug reactions. Adverse drug reactions can be classified into type A and type B reactions. Type A reactions are dose-dependent and associated with actions of the drug, either intended or unintended. For example, TMP/SMX can cause gastrointestinal upset in some patients. However, the most concerning adverse drug reactions of SMX are type B reactions, or idiosyncratic drug reactions.[14, 15] These reactions are dose independent, not associated with the pharmacological effect of the drug, and are often immune-mediated. Therefore, the term drug hypersensitivity is often used interchangeably with idiosyncratic drug toxicity.

Immune-mediated adverse drug reactions are classified by four types of hypersensitivity reactions. Type I hypersensitivity ("immediate") is an allergic reaction mediated by IgE antibodies, and it often occurs within minutes to hours. Type II hypersensitivity is due to cytotoxicity caused by the binding of immunoglobulin. Type III hypersensitivity is caused by immune complex deposition. The major SMX idiosyncratic reaction is type IV, or delayed, hypersensitivity.[14, 15] It is a mediated by drug-responsive T lymphocytes that lead to tissue damage.

SMX hypersensitivity is typically characterized by the delayed onset of fever and skin rash after 5-14 days of treatment.[16] The more severely affected patients may develop hepatic toxicity, thrombocytopenia, or bullous skin eruptions, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, with up to 30% mortality.[17-19] In SMX-hypersensitive patients, anti-SMX antibodies have been detected.[21, 92, 93] In addition, both CD4+ and CD8 T+ cells have been detected by immunohistochemical analyses of skin biopsies at the time of a hypersensitivity reaction.[20, 22- 24]

If TMP/SMX cannot be tolerated, second line treatments such as dapsone, atovaquone, or pyrimethamine can be used, but these treatments are more expensive and less effective when compared to TMP/SMX.[25] Also, they have their own adverse effect profiles. Notably, dapsone, an arylamine that shares structural similarities with sulfamethoxazole, leads to cross-hypersensitivity in 20-39% of sulfonamide-hypersensitive patients.[26-28]

The risk of developing SMX hypersensitivity is 1-3% in the general population.[29-31] However, multiple studies have shown that the risk for developing SMX hypersensitivity in HIV-infected patients increases to 20-57%.[32-35] This increased risk appears to be acquired, and varies with severity of HIV infection. For example, a higher risk is seen in HIV-infected patients with moderately low CD4+ T cell counts, and low CD4/CD8 ratios.[26, 33, 36-38] However, the mechanism of this acquired risk is unknown.

1.2 SMX Biotransformation Pathway and its Associated Risk Factors

SMX is a small, and inactive molecule, but it can act as a prohapten to trigger an immune response.[14, 15] Furthermore, SMX can supplement and stimulate an immune response through pharmacological interaction (p-i concept), by binding directly and reversibly to immune receptors.[136] The development of SMX hypersensitivity depends on SMX bioactivation.[39-41] As shown in **Figure 1.1**, SMX is oxidized to form SMX-hydroxylamine (SMX-HA) by CYP2C8 and CYP2C9 in the liver,[42-44] or by myeloperoxidases in keratinocytes and dendritic cells.[44-46] Nitonavir and nelfinavir, protease inhibitors for treating HIV infection, were shown to induce CYP2C8 and CYP2C9 activity by two-fold in human hepatocytes *in vitro*.[47, 48] This may contribute to an increased risk of hypersensitivity in HIV-infected patients. Variants of CYP2C8 and CYP2C9 have been identified, but none of them have ultra-high expression or activity.[49, 50]

Once SMX is bioactivated to form SMX-HA, this hydroxylamine metabolite can be further oxidized spontaneously to form SMX-nitroso (SMX-NO).[40, 51] The nitroso metabolite can bind to cysteine residues on a protein and form protein adduct,[52] which can be processed and presented in association with MHC-I or MHC-II.[53] This stimulates the activation of T cells and B cells to target SMX-protein adducts,[44, 54] which may lead to the development of hypersensitivity.

The formation of protein adducts can be prevented by several detoxification pathways. *N*-acetylation of SMX in the liver is the primary detoxification pathway. *N*-acetylation of SMX, by *N*-acetyltransferase 1 (NAT1) in leukocytes and *N*-acetyltransferase 2 (NAT2) in the liver, leads to an inactive and non-immunogenic acetylated metabolite for urinary excretion.[55-59] *NAT2* is highly polymorphic with more than 25 alleles that have wide range of expression and activity.[60] Slow acetylation caused by genetic defects in *NAT2* has been reported in about 50% of Caucasians and African Americans.[60-64] In studies that were done in HIV-infected patients, slow acetylation phenotype was over-represented in SMX-hypersensitive patients compared to SMX-tolerant patients, and a discordance was observed between acetylator genotype and phenotype.[65] However, another study found no such discordance between AIDS patients and healthy controls.[66] These observations suggest that HIV infection leads to acquired defects in this drug detoxification pathway, but the mechanism for this is not known.

If SMX is oxidized to SMX-HA, it can be reduced to the parent drug by cytochrome b_5 and NADH cytochrome b_5 reductase.[67] This pathway shows substantial variability in hepatic expression, with some genetic polymorphisms in cytochrome b_5 (encoded by *CYB5A*) and cytochrome b_5 reductase (*CYB5R3*) associated with very low activity.[68] However, no associations were found between these polymorphisms and hypersensitive outcomes in a population of 100 SMX-hypersensitive and 100 SMX-tolerant patients (manuscript submitted). Previous data from our laboratory suggest that much of the wide variability in cytochrome b_5 and b_5 reductase

expression is due to acquired, but not genetic factors,[69] which could be relevant to the risk of acquired hypersensitivity in HIV infection.

If SMX-HA is further oxidized spontaneously to form SMX-NO, it can be reduced to the hydroxylamine metabolite by antioxidants, such as ascorbate and glutathione.[40, 70] Deficiencies in these antioxidants are common in HIV infection, and can lead to decreased capacity for SMX-NO reduction.[40,70-72] This could contribute to increased drug haptenization and risk of SMX hypersensitivity reactions. In support of this, ascorbate and glutathione have been shown to decrease the formation of SMX-NO adducts in lymphoid cells *in vitro*.[22, 73, 74] Furthermore, antioxidant deficiencies may also directly affect cytochrome b_5 expression.[75, 76] However, in one prospective study of AIDS patients,[77] glutathione deficiency was not predictive of sulfonamide hypersensitivity, and supplementation with the glutathione precursor *N*-acetylcysteine did not significantly decrease sulfonamide hypersensitivity incidence.[35] Therefore, glutathione depletion alone is unlikely to contribute to the acquired risk of sulfonamide hypersensitivity in HIV infection.

Due to the impaired immune responses, HIV-infected patients often suffer from opportunistic infections. One common site of infection is the gastrointestinal tract, which can lead to chronic diarrhea.[78-83] HIV itself is also associated with structural and functional abnormalities of the gastrointestinal tract.[84-87] Both opportunistic infections and HIV enteropathy could lead to alteration of SMX bioavailability. However, pharmacokinetic studies in HIV-infected patients

with and without *Pneumocystis jiroveci* pneumonia have shown that SMX bioavailability was not affected by HIV and its complications when compared to healthy individuals.[88, 89]

One interesting finding for *in vivo* SMX biotransformation was that the level of urinary SMX-HA was lower in HIV-infected patients when compared to healthy individuals.[90] This decrease in hydroxylamine metabolites may be due to a reduction in SMX bioactivation by CYP2C8 and CYP2C9, or an increase of SMX-HA reduction by cytochrome b_5 and NADH cytochrome b_5 reductase. Both scenarios could lead to a protective effect against SMX hypersensitivity. Another possibility is that more SMX-HA is oxidized to SMX-NO to form protein adducts (which would not be detected in the urine) because HIV-infected patients are known to suffer from antioxidant deficiencies.[40,70-72] Increased SMX-protein adduct formation could enhance risk of SMX hypersensitivity.

1.3 SMX-Adduct Formation and Cellular Mediated Immune Response in Humans

Serum SMX-protein adducts and anti-SMX antibodies can be detected in both healthy and AIDS patients prescribed TMP/SMX.[21, 91-93] SMX-protein adducts were detected in 6 out of 8 hypersensitive AIDS patients, while adducts were only detected in 1 out of 4 AIDS patients that did not show signs of hypersensitivity.[92] This suggests that SMX-adduct formation could be important in the development of hypersensitivity. In addition to circulating adducts, adducts can

be generated locally in tissues, since SMX may be oxidized by dendritic cells and keratinocytes to form protein adducts.[39, 46, 54, 73, 105]

Dendritic cells are antigen presentation cells that stimulate the activation of adaptive immune responses.[94, 95] Incubation of human dendritic cells with SMX-NO led to instant adduct formation *in vitro*.[44, 54] Some CYP450s and a high level of myeloperoxidase were detected in dendritic cells, but CYP2C8 and CYP2C9 expression were absent.[44, 96] Prolonged incubation of SMX or SMX-HA led to intracellular adduct formation.[39, 54, 73] Furthermore, dendritic cells are capable of the uptake of pre-formed SMX-protein adducts.[39] All these data suggest that dendritic cells are capable of SMX bioactivation and adduct formation.

In addition to SMX adduct formation, SMX and its metabolites also lead to human dendritic cell activation and expression of the co-stimulatory molecule CD40 *in vitro*, which enhances its ability to stimulate the adaptive immune response.[44] In support of this, co-administration of anti-CD40L with SMX-NO has been shown to inhibit T cell responses to the drug *in vivo* in mice.[44] These findings indicate that oxidative SMX metabolites can act as both an antigenic signal and a second "danger" signal, leading to partial maturation of dendritic cells.[54, 97]

Immune dysregulation is clearly present in HIV infection, and despite the immunosuppressed state, there are higher percentages of activated lymphocytes and increases in pro-inflammatory

cytokines in these patients, including higher plasma concentrations of LPS, TNF-α, and IFN-γ.[66, 98-102] These inflammatory mediators have been shown to act on dendritic cells to enhance further presentation of SMX-adducted protein antigens.[73]

In addition to dendritic cells, keratinocytes can also form local SMX-protein adducts. Keratinocyte is the major cell type of skin epidermis, and it is the major target affected by SMX hypersensitivity.[103, 104] Similar to dendritic cells, incubation of keratinocytes with SMX-NO led to immediate surface adduct formation.[20] Keratinocytes also have the ability to oxidize SMX, and generate adducts intracellularly.[46, 105] High concentrations of SMX-HA can lead to cytotoxicity in keratinocytes under glutathione depletion, and SMX-protein adducts that are released can then be taken up by dendritic cells upon keratinocyte cell death.[39, 105] Furthermore, upon incubation of keratinocytes with SMX-HA *in vitro*, co-localization of SMX-protein adducts and MHC-I was observed using confocal microscopy.[46] This result suggests that SMX-protein adducts can be processed and presented by keratinocytes as antigen. This may lead to drug immunogenicity and subsequent cutaneous drug hypersensitivity.

1.4 Lymphocyte Toxicity Assay – A Possible Surrogate Marker for Drug Hypersensitivity

In both immunocompetent and HIV-infected patients, sulfonamide hypersensitivity has been associated with a surrogate biomarker, expressed in the lymphocyte toxicity assay (LTA). Results of this *in vitro* cytotoxicity assay showed that peripheral blood mononuclear cells

(PBMC) from hypersensitive patients are more susceptible to apoptosis in the presence of sulfonamide oxidative metabolites, when compared to cells from sulfonamide-tolerant patients.[106-108] SMX-HA, which converts to SMX-NO, is responsible for the cytotoxicity *in vitro*.[108, 109] Differences in cytotoxicity were not observed with the parent SMX, or with a control compound that generates reactive oxygen species (menadione).[110] These findings, which have been replicated by multiple groups, have been interpreted as a "detoxification defect" in hypersensitive, but not tolerant, patients.[110, 111]

The LTA has been evaluated both retrospectively and prospectively in SMX-hypersensitive patients,[106, 112] and appears to be a durable marker in immunocompetent patients, with increased cytotoxicity still noted years after the hypersensitivity reactions.[113, 114] The LTA has a high specificity (90-99%),[112, 113] with negative results shown to be predictive of uneventful exposure.[106, 107] The sensitivity of the assay, however, varies from 62-100%.[110, 113, 115]

Since SMX is often administrated with TMP, it has been noted that a minority of patients may be hypersensitive to TMP, not SMX. However, TMP metabolites are not routinely tested in the cytotoxicity assay. Notably, rechallenge with TMP alone leads to hypersensitivity in 16-19% of patients with a history of hypersensitivity to TMP/SMX.[116, 117] TMP alone has also been associated with toxic epidermal necrolysis,[118, 119] and TMP-responsive T cells have been found in some patients hypersensitive to TMP/SMX.[120] Human liver microsomes generate reactive TMP metabolites,[121] which could contribute to hapten formation and T cell sensitization. Therefore,

pathways of TMP bioactivation and detoxification, in addition to SMX, may be important in some patients with hypersensitivity reactions to potentiated sulfonamides.

1.5 SMX Immunogenicity in Animal Models

SMX hypersensitivity has been studied in multiple animal models. Upon treating mice with SMX-NO, SMX-protein adducts were detected in serum and multiple lymph nodes.[122] Cells isolated from spleen, inguinal lymph nodes and auricular lymph nodes proliferated and secreted IL-5 when exposed to SMX-NO *in vitro*.[22, 122, 123] Mice treated with SMX did not generate SMX-protein adducts nor drug-responsive T cell-mediated immune responses.[122, 123] In another study, treatment of mice with SMX incubated with S9 fractions (both microsomes and cytosol) from CYP450-induced rat livers led to footpad swelling, a sign of hypersensitivity, but these signs were not seen with SMX treatment alone.[124] These observations suggest that healthy mice, unlike humans, lack the ability to bioactivate SMX to lead to immune responses *in vivo*. Only when SMX metabolite is administrated, can mice develop drug-specific T cell-mediated immune responses with a possible sign of hypersensitivity.

In rats treated with SMX-NO, SMX-protein adducts were detected on the surface of lymphocytes, splenocytes, and epidermal keratinocytes.[20, 22] *Ex vivo* stimulation of these splenocytes with SMX-NO led to proliferation.[20, 22] One study found anti-SMX antibodies in rats sensitized with either SMX-HA or SMX-NO.[125] In another study, incubation of rat splenocytes with SMX-NO

showed that SMX-NO binds preferentially to antigen presenting cells when compared to CD4+ and CD8+ T cells.[126] Further, *ex vivo* proliferation was observed when splenocytes from SMX-NO sensitized rats were again exposed to SMX-NO.[126] However, proliferation was blocked when anti-MHC-I and II antibodies were pre-incubated with the splenocytes.[126] These data suggest that SMX-protein adducts can be processed and presented to T cells by antigen presenting cells, and stimulate T cell activation and proliferation. However, the mechanisms of antigen processing were not examined. Similar to mice, immunization of rats with SMX did not lead to SMX adduct formation nor a drug-responsive cellular-mediated immune response.[20] Therefore rats, like mice, appear to lack the ability to generate enough SMX metabolites for adduct formation *in vivo*.

SMX immunogenicity has also been studied in rabbits. Similar to rodents, treatment of SMX-NO in rabbits led to the activation and proliferation of lymphocytes when exposed to SMX-NO *ex vivo*,[22] but no signs of SMX haptenation and immune responses when rabbits were immunized with SMX itself.[22] This suggested that development of SMX hypersensitivity requires efficient drug bioactivation for protein adduct formation, and both rodent and rabbit models failed to achieve this.

In contrast to rabbits and rodents, pet dogs can show clinical signs of drug hypersensitivity when they are given the parent drug SMX, as TMP/SMX, for clinical infections.[127-129] The dog is therefore the only species, besides humans, that shows clinical signs of SMX hypersensitivity when given with the parent drug SMX. Circulating SMX-protein adducts and anti-SMX

antibodies have also been detected in sera of dogs with the clinical diagnosis of SMX hypersensitivity.[130] However, SMX-specific T cells have not yet been demonstrated. SMX biotransformation, adduct formation, and the immune responses to SMX can therefore be studied in susceptible dogs. However, these cases are sporadic, and client-owned pets cannot be subject to experimentation. Although dogs could be studied in an experimental setting,[135] this is not an appropriate model for the acquired risk of SMX hypersensitivity in HIV infection. A better animal model is necessary.

1.6 SIV as a Model of HIV Infection

Simian immunodeficiency virus (SIV) infection in non-human primates is a useful and widely accepted model of HIV infection.[131-133] SIVmac239, which is pathogenic in rhesus macaques, and HIV-1, are closely related retroviruses that share key similarities in gene organization, receptor recognition, cell tropism, production of Tat, and CD4+ T cell depletion.[131-134] Most importantly, SIVmac239 infection leads to immunosuppression in rhesus macaques, with progression to diarrhea, wasting, and opportunistic infections that models the clinical progression to AIDS in humans.[132, 133] Furthermore, the pharmacokinetics of SMX in macaques are similar to those in humans, suggesting that SMX biotransformation could be similar.[137, 138] The macaque model also allows the use of more invasive procedures, such as collection of lymph nodes and liver tissue, which can be used to assess adduct formation and expression of drug biotransformation pathways.

1.7 Hypothesis and Specific Aims

Based on these findings to date, we hypothesized that a combination of acquired metabolic and immunologic alterations are present in HIV infection that lead to the high risk of sulfonamide hypersensitivity, and that these same alterations are also present in an SIV infection model.

Therefore, the specific aims of this thesis were to:

Aim 1: Determine whether SIV-infected Rhesus macaques show immunologic sensitization to TMP/SMX, and evaluate the associated risk factors (Chapter 2).

Aim 2: Determine the effects of SIV infection on hepatic biotransformation and other pathways, using expression array analyses in livers from infected and control macaques (Chapter 3).

Aim 3: Evaluate the mechanisms for enhanced lymphocyte cytotoxicity from SMX metabolites, observed in HIV-infected patients, using expression array analyses in lymphocytes from drug-naïve SIV-infected macaques (Chapter 4).

1.8 References

1. Brown GM. The biosynthesis of folic acid. II. Inhibition by sulfonamides. *J Biol Chem*. 1962;237:536-40.
2. Bushby SR. Synergy of trimethoprim-sulfamethoxazole. *Can Med Assoc J*. 1975;112:63-6.
3. Kamen B. Folate and antifolate pharmacology. *Semin Oncol*. 1997;24:S18-30-S18-39.
4. Duthie SJ. Folate and cancer: how DNA damage, repair and methylation impact on colon carcinogenesis. *J Inherit Metab Dis*. 2011;34:101-9
5. Henderson GB. Folate-binding proteins. *Annu Rev Nutr*. 1990;10:319-35.
6. Burchall JJ. Mechanism of action of trimethoprim-sulfamethoxazole. II. *J Infect Dis*. 1973;128:Suppl: 437-41.
7. Avgeri SG, Matthaiou DK, Dimopoulos G, et al. Therapeutic options for Burkholderia cepacia infections beyond co-trimoxazole: a systematic review of the clinical evidence. *Int J Antimicrob Agents.* 2009;33:394-404.
8. Drekonja DM, Johnson JR. Urinary tract infections. *Prim Care* 2008;35:345-367, vii.
9. Korbila IP, Manta KG, Siempos, II, et al. Penicillins vs trimethoprim-based regimens for acute bacterial exacerbations of chronic bronchitis: meta-analysis of randomized controlled trials. *Can Fam Physician.* 2009;55:60-67.
10. Drekonja DM, Traynor LM, DeCarolis DD, et al. Treatment of non-life-threatening methicillin-resistant Staphylococcus aureus infections with alternative antimicrobial agents: a 2-year retrospective review. *Diagn Microbiol Infect Dis.* 2009;63:201-207.
11. Gupta D, Zachariah A, Roppelt H, et al. Prophylactic antibiotic usage for Pneumocystis jirovecii pneumonia in patients with systemic lupus erythematosus on cyclophosphamide: a survey of US rheumatologists and the review of literature. *J Clin Rheumatol.* 2008;14:267-272.
12. Rodriguez M, Sifri C, Fishman J. Failure of low-dose atovaquone prophylaxis against Pneumocystis jiroveci infection in transplant recipients. *Clin Infect Dis.* 2004;38:e76-78.
13. Thomas M, Rupali P, Woodhouse A, et al. Good outcome with trimethoprim 10 mg/kg/day-sulfamethoxazole 50 mg/kg/day for Pneumocystis jirovecii pneumonia in HIV infected patients. *Scand J Infect Dis.* 2009;41:862-868.
14. Adam J, Pichler WJ, Yerly D. Delayed drug hypersensitivity: models of T-cell stimulation. *Br J Clin Pharmacol*. 2011;71:701-7.
15. Pichler WJ. Delayed drug hypersensitivity reactions. *Ann Intern Med*. 2003;139:683-93.
16. Cribb AE, Lee BL, Trepanier LA, et al. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. *Adverse Drug React Toxicol Rev.* 1996;15:9-50.
17. Ortiz J, Horn M, Peterson H. Toxic epidermal necrolysis-case report and review of the literature. *Ann Plast Surg.* 1982;9:249-253.
18. Saka B, Kombate K, Mouhari-Toure A, et al. Stevens-Johnson syndrome and toxic epidermal necrolysis in a teaching hospital in Lomé, Togo: retrospective study of 89 cases. *Med Trop (Mars).* 2010;70:255-8.
19. Bell TL, Foster JN, Townsend ML. Trimethoprim-sulfamethoxazole-induced hepatotoxicity in a pediatric patient. *Pharmacotherapy.* 2010;30:539.
20. Naisbitt DJ, Gordon SF, Pirmohamed M, et al. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br J Pharmacol*. 2001;133:295-305.
21. Daftarian MP, Filion LG, Cameron W, et al. Immune response to sulfamethoxazole in patients with AIDS. *Clin Diagn Lab Immunol.* 1995;2:199-204.

22. Farrell J, Naisbitt D, Drummond N, et al. Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *J Pharmacol Exp Ther.* 2003;306:229-237.
23. Miyauchi H, Hosokawa H, Akaeda T, et al. T-cell subsets in drug-induced toxic epidermal necrolysis. Possible pathogenic mechanism induced by CD8-positive T cells. *Arch Dermatol*. 1991;127:851-5.
24. Correia O, Delgado L, Ramos JP, et al. Cutaneous T-cell recruitment in toxic epidermal necrolysis. Further evidence of CD8+ lymphocyte involvement. *Arch Dermatol*. 1993;129:466-8.
25. Helweg-Larsen J, Benfield T, Atzori C, et al. Clinical efficacy of first- and second-line treatments for HIV-associated Pneumocystis jirovecii pneumonia: a tri-centre cohort study. *J Antimicrob Chemother.* 2009;64:1282-90.
26. Carr A, Swanson C, Penny R, et al. Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with Pneumocystis carinii pneumonia and AIDS. *J Infect Dis*. 1993;167:180-185.
27. Holtzer C, Flaherty J, Coleman R. Cross-reactivity in HIV-infected patients switched from trimethoprim-sulfamethoxazole to dapsone. *Pharmacotherapy*. 1998;18:831-835.
28. Jorde U, Horowitz H, Wormser G. Utility of dapsone for prophylaxis of Pneumocystis carinii pneumonia in trimethoprim-sulfamethoxazole-intolerant, HIV-infected individuals. *AIDS*. 1993;7:355-359.
29. Stewart RB, May FE, Cullen SI. Dermatologic adverse drug reactions in hospitalized patients. *Am J Hosp Pharm.* 1979;36:609-12.
30. Bigby M, Jick S, Jick H, et al. Drug-induced cutaneous reactions. A report from the Boston Collaborative Drug Surveillance Program on 15,438 consecutive inpatients, 1975 to 1982. *JAMA*. 1986;256:3358-63.
31. Arndt KA, Jick H. Rates of cutaneous reactions to drugs. A report from the Boston Collaborative Drug Surveillance Program. *JAMA*. 1976;235:918-23.
32. Gordin FM, Simon GL, Wofsy CB, et al. Adverse reactions to trimethoprim-sulfamethoxazole in patients with AIDS. *Ann Intern Med.* 1984;100:495-499.
33. Hennessy S, Strom BL, Berlin JA, et al. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. *J Gen Intern Med.* 1995;10:380-386.
34. Medina I, Mills J, Leoung G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. *N Engl J Med.* 1990;323:776-782.
35. Walmsley SL, Khorasheh S, Singer J, et al. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). *J Acquir Immune Defic Syndr Hum Retrovirol.* 1998;19:498-505.
36. Eliaszewicz M, Flahault A, Roujeau J, et al. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. *J Am Acad Dermatol.* 2002;47:40-46.
37. Rabaud C, Charreau I, Izard S, et al. Adverse reactions to cotrimoxazole in HIV-infected patients: predictive factors and subsequent HIV disease progression. *Scand J Infect Dis.* 2001;33:759-764.
38. Ryan C, Madalon M, Wortham DW, et al. Sulfa hypersensitivity in patients with HIV infection: onset, treatment, critical review of the literature. *WMJ.* 1998;97:23-27.
39. Roychowdhury S, Vyas PM, Svensson CK. Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. *Drug Metab Dispos.* 2007;35:676-681.
40. Cribb AE, Miller M, Leeder JS, et al. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione: implications for idiosyncratic toxicity. *Drug Metab Dispos.* 1991;19:900-906.

41. Reilly TP, Bellevue FH, Woster PM, et al. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. *Biochem Pharmacol.* 1998;55:803-810.
42. Cribb AE, Spielberg SP, Griffin GP. N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab Dispos.* 1995;23:406-414.
43. Miller JL, Trepanier LA. Inhibition by atovaquone of CYP2C9-mediated sulphamethoxazole hydroxylamine formation. *Eur J Clin Pharmacol*. 2002;58:69-72.
44. Sanderson JP, Naisbitt DJ, Farrell J, et al. Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J Immunol.* 2007;178:5533-42.
45. Vyas PM, Roychowdhury S, Koukouritaki SB, et al. Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes: II. Expression and role of flavin-containing monooxygenases and peroxidases. *J Pharmacol Exp Ther*. 2006;319:497-505.
46. Roychowdhury S, Vyas PM, Reilly TP, et al. Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. *J Pharmacol Exp Ther*. 2005;314:43-52.
47. Dixit V, Hariparsad N, Li F, et al. Cytochrome P450 enzymes and transporters induced by anti-human immunodeficiency virus protease inhibitors in human hepatocytes: implications for predicting clinical drug interactions. *Drug Metab Dispos*. 2007;35:1853-9.
48. Kirby BJ, Collier AC, Kharasch ED, et al. Complex drug interactions of HIV protease inhibitors 2: in vivo induction and in vitro to in vivo correlation of induction of cytochrome P450 1A2, 2B6, and 2C9 by ritonavir or nelfinavir. *Drug Metab Dispos*. 2011;39:2329-37.
49. Daily EB, Aquilante CL. Cytochrome P450 2C8 pharmacogenetics: a review of clinical studies. *Pharmacogenomics*. 2009;10:1489-510.
50. Zhou SF, Zhou ZW, Huang M. Polymorphisms of human cytochrome P450 2C9 and the functional relevance. *Toxicology*. 2010;278:165-88.
51. Naisbitt DJ, O'Neill PM, Pirmohamed M, et al. Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: implications for immune mediated toxicity. *Bioorg Med Chem Lett.* 1996;6:1511-1516.
52. Callan HE, Jenkins RE, Maggs JL, et al. Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation. *Chem Res Toxicol.* 2009;22:937-48.
53. Schnyder B, Mauri-Hellweg D, Zanni M, et al. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest*. 1997;100:136-41.
54. Elsheikh A, Lavergne SN, Castrejon JL, et al. Drug antigenicity, immunogenicity, and costimulatory signaling: evidence for formation of a functional antigen through immune cell metabolism. *J Immunol.* 2010;185:6448-6460.
55. Cribb AE, Nakamura H, Grant DM, et al. Role of polymorphic and monomorphic human arylamine N-acetyltransferases in determining sulfamethoxazole metabolism. *Biochem Pharmacol*. 1993;45:1277-82.
56. Dierolf D, Scheitza S, Bonifas J, et al. Cyanamide-mediated Inhibition of N-acetyltransferase 1. *Toxicology*. 2012;302:1-10.
57. Wang D, Para MF, Koletar SL, et al. Human N-acetyltransferase 1 *10 and *11 alleles increase protein expression through distinct mechanisms and associate with sulfamethoxazole-induced hypersensitivity. *Pharmacogenet Genomics*. 2011;21:652-64.
58. Cribb AE, Grant DM, Miller MA, et al. Expression of monomorphic arylamine N-acetyltransferase (NAT1) in human leukocytes. *J Pharmacol Exp Ther*. 1991;259:1241-6.

59. Nuss CE, Grant DM, Spielberg SP, et al. Further investigations of the role of acetylation in sulphonamide hypersensitivity reactions. *Biomarkers*. 1996;1:267-72.
60. Fretland AJ, Leff MA, Doll MA, et al. Functional characterization of human N-acetyltransferase 2 (NAT2) single nucleotide polymorphisms. *Pharmacogenetics*. 2001;11:207-15.
61. Weber WW, Hein DW. N-acetylation pharmacogenetics. *Pharmacol Rev*. 1985;37:25-79.
62. Cascorbi I, Drakoulis N, Brockmöller J, et al. Arylamine N-acetyltransferase (NAT2) mutations and their allelic linkage in unrelated Caucasian individuals: correlation with phenotypic activity. *Am J Hum Genet*. 1995;57:581-92.
63. Grant DM, Hughes NC, Janezic SA, et al. Human acetyltransferase polymorphisms. *Mutat Res*. 1997;376:61-70.
64. Hein DW, Doll MA, Fretland AJ, et al. Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. *Cancer Epidemiol Biomarkers Prev*. 2000;9:29-42.
65. Carr A, Gross AS, Hoskins JM, et al. Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulphamethoxazole in HIV-infected patients. *AIDS*. 1994;8:333-7.
66. O'Neil WM, Drobitch RK, MacArthur RD, et al. Acetylator phenotype and genotype in patients infected with HIV: discordance between methods for phenotype determination and genotype. *Pharmacogenetics*. 2000;10:171-82.
67. Kurian JR, Bajad SU, Miller JL, et al. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther*. 2004;311:1171-1178.
68. Sacco JC, Trepanier LA. Cytochrome b5 and NADH cytochrome b5 reductase: genotype-phenotype correlations for hydroxylamine reduction. *Pharmacogenet Genomics*. 2010;20:26-37.
69. Rhoads K, Sacco JC, Drescher N, et al. Individual variability in the detoxification of carcinogenic arylhydroxylamines in human breast. *Toxicol Sci*. 2011;121:245-56.
70. Trepanier LA, Yoder AR, Bajad S, et al. Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J Acquir Immune Defic Syndr*. 2004;36:1041-50.
71. Naisbitt DJ, Vilar FJ, Stalford AC, et al. Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. *AIDS Res Hum Retroviruses*. 2000;16:1929-38.
72. Walmsley SL, Winn LM, Harrison ML, et al. Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: toxicological and pathological implications. *AIDS*. 1997;11:1689-97.
73. Lavergne SN, Wang H, Callan HE, et al. "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. *J Pharmacol Exp Ther* 2009;331:372-381.
74. Manchanda T, Hess D, Dale L, et al. Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol Pharmacol*. 2002;62:1011-26.
75. Degkwitz E, Walsch S, Dubberstein M, et al. Influence of L-ascorbate on the contents of cytochromes P-450 and b5 in several organs of guinea pigs. *Enzyme*. 1973;16(1):237-45.
76. Degkwitz E, Kim KS. Comparative studies on the influence of L-ascorbate, D-arabino-ascorbate and 5-oxo-D-gluconate on the amounts of cytochromes P-450 and b5 in liver microsomes of guinea pigs. *Hoppe Seylers Z Physiol Chem*. 1973;354:555-61.
77. Wolkenstein P, Loriot MA, Aractingi S, et al. Prospective evaluation fo detoxification pathways as markers of cutaneous adverse reactions to sulphonamides in AIDS. *Pharmacogenetics*. 2000;10:821-828.
78. Smith PD, Quinn TC, Strober W, et al. NIH conference. Gastrointestinal infections in AIDS. *Ann Intern Med*. 1992;116:63-77.

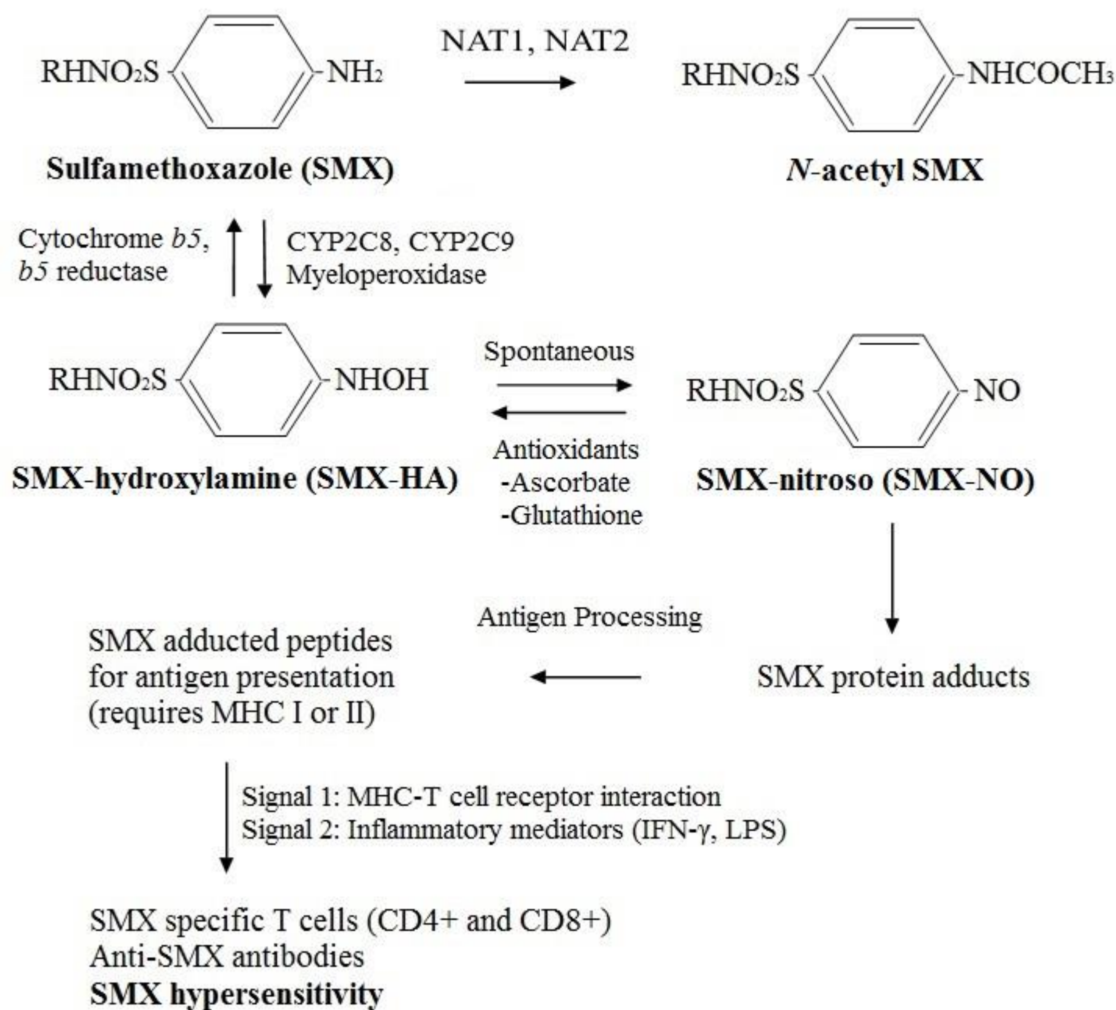
79. Jacobson MA, Mills J. Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS). Clinical findings, diagnosis, and treatment. *Ann Intern Med*. 1988;108:585-94.
80. Desportes I, Le Charpentier Y, Galian A, et al. Occurrence of a new microsporidan: Enterocytozoon bieneusi n.g., n. sp., in the enterocytes of a human patient with AIDS. *J Protozool*. 1985;32:250-4.
81. Gray JR, Rabeneck L. Atypical mycobacterial infection of the gastrointestinal tract in AIDS patients. *Am J Gastroenterol*. 1989;84:1521-4.
82. Velásquez JN, Risio CA, Etchart CB, et al. First report of Cystoisospora belli parasitemia in a patient with acquired immunodeficiency syndrome. *Acta Parasitol*. 2016;61:172-7.
83. Nsagha DS, Njunda AL, Assob NJ, et al. Intestinal parasitic infections in relation to CD4(+) T cell counts and diarrhea in HIV/AIDS patients with or without antiretroviral therapy in Cameroon. *BMC Infect Dis*. 2016;16:9.
84. Ullrich R, Zeitz M, Heise W, et al. Small intestinal structure and function in patients infected with human immunodeficiency virus (HIV): evidence for HIV-induced enteropathy. *Ann Intern Med*. 1989;111:15-21.
85. Wang H, Kotler DP. HIV enteropathy and aging: gastrointestinal immunity, mucosal epithelial barrier, and microbial translocation. *Curr Opin HIV AIDS*. 2014;9:309-16.
86. Reka S, Kotler DP. Correlation of intestinal structure and function in HIV infection. *Gastrointest Endosc Clin N Am*. 1998;8:841-56.
87. Bjarnason I, Sharpstone DR, Francis N, et al. Intestinal inflammation, ileal structure and function in HIV. *AIDS*. 1996;10:1385-91.
88. Chin TW, Vandenbroucke A, Fong IW. Pharmacokinetics of trimethoprim-sulfamethoxazole in critically ill and non-critically ill AIDS patients. *Antimicrob Agents Chemother*. 1995;39:28-33.
89. Klepser ME, Zhu Z, Nicolau DP, et al. Oral absorption of trimethoprim-sulfamethoxazole in patients with AIDS. *Pharmacotherapy*. 1996;16:656-62.
90. van der Ven AJ, Vree TB, van Ewijk-Beneken Kolmer EW, et al. Urinary recovery and kinetics of sulphamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulphamethoxazole. *Br J Clin Pharmacol*. 1995;39:621-5.
91. Meekins CV, Sullivan TJ, Gruchalla RS. Immunochemical analysis of sulfonamide drug allergy: identification of sulfamethoxazole-substituted human serum proteins. *J Allergy Clin Immunol*. 1994;94:1017-24.
92. Gruchalla RS, Pesenko RD, Do TT, et al. Sulfonamide-induced reactions in desensitized patients with AIDS--the role of covalent protein haptenation by sulfamethoxazole. *J Allergy Clin Immunol*. 1998;101:371-8.
93. O'Neil CE, Reed MA, Lopez M, et al. Evaluation of immune parameters in HIV+ subjects reporting adverse reactions to sulfamethoxazole. *Int Arch Allergy Appl Immunol*. 1991;94:246-7.
94. Howard CJ, Charleston B, Stephens SA, et al. The role of dendritic cells in shaping the immune response. *Anim Health Res Rev*. 2004;5:191-5.
95. Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immuno*l. 2006;311:17-58.
96. Sieben S, Baron JM, Blömeke B, et al. Multiple cytochrome P450-isoenzymes mRNA are expressed in dendritic cells. *Int Arch Allergy Immunol*. 1999;118:358-61.
97. Pichler WJ, Naisbitt DJ, Park BK. Immune pathomechanism of drug hypersensitivity reactions. *J Allergy Clin Immunol*. 2011;127:S74-81.
98. Sandler NG, Wand H, Roque A, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis*. 2011;203:780-90.

99. Aukrust P, Svardal AM, Müller F, et al. Increased levels of oxidized glutathione in CD4+ lymphocytes associated with disturbed intracellular redox balance in human immunodeficiency virus type 1 infection. *Blood*. 1995;86:258-67.
100. Watanabe D, Uehira T, Yonemoto H, et al. Sustained high levels of serum interferon-gamma during HIV-1 infection: a specific trend different from other cytokines. *Viral Immunol* 2010;23:619-625.
101. Aukrust P, Liabakk NB, Müller F, et al. Serum levels of tumor necrosis factor-alpha (TNF alpha) and soluble TNF receptors in human immunodeficiency virus type 1 infection--correlations to clinical, immunologic, and virologic parameters. *J Infect Dis.* 1994;169:420-4.
102. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med.* 2006;12:1365-71.
103. Khalili B, Bahna SL. Pathogenesis and recent therapeutic trends in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Ann Allergy Asthma Immunol*. 2006;97:272-80
104. Schwartz RA, McDonough PH, Lee BW. Toxic epidermal necrolysis: Part I. Introduction, history, classification, clinical features, systemic manifestations, etiology, and immunopathogenesis. *J Am Acad Dermatol*. 2013;69:173.e1-13
105. Reilly TP, Lash LH, Doll MA, et al. A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J Invest Dermatol*. 2000;114:1164-73.
106. Carr A, Tindall B, Penny R, et al. In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin Exp Immunol.* 1993;94:21-25.
107. Rieder MJ, Uetrecht J, Shear NH, et al. Diagnosis of sulfonamide hypersensitivity reactions by in-vitro "rechallenge" with hydroxylamine metabolites. *Ann Intern Med*. 1989 Feb 15;110(4):286-9.
108. Rieder MJ, Uetrecht J, Shear NH, et al. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. *J Pharmacol Exp Ther*. 1988;244:724-8.
109. Rieder MJ, Krause R, Bird IA. Time-course of toxicity of reactive sulfonamide metabolites. Toxicology. 1995;95:141-6.
110. Wolkenstein P, Charue D, Laurent P, et al. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. *Arch Dermatol.* 1995;131:544-51.
111. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. *Clin Biochem*. 2000;33:517-24.
112. Elzagallaai AA, Jahedmotlagh Z, Del Pozzo-Magaña BR, et al. Predictive value of the lymphocyte toxicity assay in the diagnosis of drug hypersensitivity syndrome. *Mol Diagn Ther*. 2010;14:317-22.
113. Neuman MG, Shear NH, Malkiewicz IM, et al. Immunopathogenesis of hypersensitivity syndrome reactions to sulfonamides. *Transl Res*. 2007;149:243-53.
114. Zawodniak A, Lochmatter P, Beeler A, et al. Cross-reactivity in drug hypersensitivity reactions to sulfasalazine and sulfamethoxazole. *Int Arch Allergy Immunol*. 2010;153:152-6.
115. Riley RJ, Cribb AE, Spielberg SP. Glutathione transferase mu deficiency is not a marker for predisposition to sulphonamide toxicity. *Biochem Pharmacol*. 1991;42:696-8.
116. Bonfanti P, Pusterla L, Parazzini F, et al. The effectiveness of desensitization versus rechallenge treatment in HIV-positive patients with previous hypersensitivity to TMP-SMX: a randomized multicentric study. C.I.S.A.I. Group. *Biomed Pharmacother*. 2000;54:45-9.
117. Carr A, Penny R, Cooper DA. Efficacy and safety of rechallenge with low-dose trimethoprim-sulphamethoxazole in previously hypersensitive HIV-infected patients. *AIDS*. 1993;7:65-71.

118. Das G, Bailey MJ, Wickham JE. Toxic epidermal necrolysis and trimethoprim. *Br Med J (Clin Res Ed)*. 1988;296:1604-5.
119. Nwokolo C, Byrne L, Misch KJ. Toxic epidermal necrolysis occurring during treatment with trimethoprim alone. *Br Med J (Clin Res Ed)*. 1988;296:970.
120. Mauri-Hellweg D, Bettens F, Mauri D, et al. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol*. 1995;155:462-72.
121. Damsten MC, de Vlieger JS, Niessen WM, et al. Trimethoprim: novel reactive intermediates and bioactivation pathways by cytochrome p450s. *Chem Res Toxicol*. 2008;21:2181-7.
122. Cheng L, Stewart BJ, You Q, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol Pharmacol*. 2008;73:1769-75.
123. Hopkins JE, Naisbitt DJ, Humphreys N, et al. Exposure of mice to the nitroso metabolite of sulfamethoxazole stimulates interleukin 5 production by CD4+ T-cells. *Toxicology*. 2005;206:221-31.
124. Choquet-Kastylevsky G, Santolaria N, Tedone R, et al. Induction of delayed-type hypersensitivity to sulfamethoxazole in mice: role of metabolites. *Toxicol Lett*. 2001;119:183-92.
125. Gill HJ, Hough SJ, Naisbitt DJ, et al. The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. *J Pharmacol Exp Ther*. 1997;282:795-801.
126. Naisbitt DJ, Farrell J, Gordon SF, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol*. 2002;62:628-37.
127. Cribb AE, Spielberg SP. An in vitro investigation of predisposition to sulphonamide idiosyncratic toxicity in dogs. *Vet Res Commun*. 1990;14:241-52.
128. Trepanier LA, Danhof R, Toll J, Watrous D. Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. *J Vet Intern Med*. 2003;17:647-52.
129. Trepanier LA. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. *J Vet Pharmacol Ther*. 2004;27:129-38.
130. Lavergne SN, Danhof RS, Volkman EM, et al. Association of drug-serum protein adducts and anti-drug antibodies in dogs with sulphonamide hypersensitivity: a naturally occurring model of idiosyncratic drug toxicity. *Clin Exp Allergy*. 2006;36:907-15.
131. Ambrose Z, KewalRamani VN, Bieniasz PD, et al. HIV/AIDS: in search of an animal model. *Trends Biotechnol*. 2007;25:333-7.
132. Lackner AA, Veazey RS. Current concepts in AIDS pathogenesis: insights from the SIV/macaque model. *Annu Rev Med*. 2007;58:461-76.
133. Valentine LE, Watkins DI. Relevance of studying T cell responses in SIV-infected rhesus macaques. *Trends Microbiol*. 2008;16:605-11.
134. Cullen BR, Garrett ED. A comparison of regulatory features in primate lentiviruses. *AIDS Res Hum Retroviruses*. 1992;8:387-93.
135. Lavergne SN, Volkman EM, Maki JE, et al. Evaluation of the clinical, immunologic, and biochemical effects of nitroso sulfamethoxazole administration to dogs: a pilot study. *Toxicology*. 2005;208:63-72.
136. Pichler WJ. The p-i Concept: Pharmacological Interaction of Drugs With Immune Receptors. *World Allergy Organ J*. 2008;1:96-102.
137. Craig WA, Kunin CM. Distribution of trimethoprim-sulfamethoxazole in tissues of rhesus monkeys. *J Infect Dis*. 1973;128:Suppl:575-9 p.

138. Craig WA, Kunin CM. Trimethoprim-sulfamethoxazole: pharmacodynamic effects of urinary pH and impaired renal function. Studies in humans. *Ann Intern Med*. 1973;78:491-7.

Figure 1.1: Sulfamethoxazole (SMX) biotransformation with generation of reactive metabolites that lead to hapten formation, immunogenicity, and clinical signs of delayed sulfoanmide hypersensitivity in immunocompetent and HIV-infected patients.



Chapter 2 – Immunogenicity of Trimethoprim/Sulfamethoxazole in a Macaque Model of HIV Infection

2.1 Introduction

The sulfonamide antibiotic sulfamethoxazole (SMX), in combination with trimethoprim (TMP), is the standard of care treatment for opportunistic infections such as *Pneumocystis jiroveci* pneumonia in patients with HIV/AIDS (cdc.gov). One major adverse effect of TMP/SMX is the development of drug hypersensitivity, which is characterized by the delayed onset of fever and skin rash after 5-14 days of treatment.[1] Sulfonamide hypersensitivity occurs in only about 3% of the general population, but the incidence increases to 20-57% in HIV-infected patients.[2-5] This risk varies with severity of illness, with higher risk seen at low CD4+ counts (< 200 but greater than 30 cells/µl).[3,6-10] However, the mechanisms for this increased risk are not well understood.

The development of sulfonamide hypersensitivity requires the bioactivation of SMX to form SMX-hydroxylamine (SMX-HA; **Figure 2.1**).[11-13] The hydroxylamine molecule spontaneously oxidizes to SMX-nitroso (SMX-NO), which generates protein adducts that can act as tissue haptens.[14-16] This leads to the generation of SMX-responsive T cells and anti-drug antibodies that appear to mediate the clinical signs SMX hypersensitivity.[15-19]

There are several detoxification pathways that prevent the accumulation of the proximate immunogen SMX-NO. A primary pathway is *N*-acetylation of SMX to form a non-immunogenic metabolite (*N*-acetyl SMX). In some studies of HIV-infected patients, impaired *N*-acetylation

was over-represented in sulfonamide-hypersensitive patients compared to sulfonamide-tolerant patients,[20-22] with a discordance between a normal *N*-acetyltransferase genotype and a slow acetylator phenotype,[23, 24] especially in patients with lower CD4+ counts.[25] A second detoxification pathway is the reduction of SMX-NO back to SMX-HA by antioxidants such as ascorbate and glutathione (GSH).[26, 27] Blood ascorbate and GSH concentrations can be depleted in HIV-infected patients, causing impaired ability to reduce SMX-NO.[26, 27] SMX-HA can be further reduced back to SMX by the cytochrome b_5 reductase pathway (**Figure 2.1**).[28]

In addition to metabolic factors, inflammatory mediators may also contribute to the development of SMX hypersensitivity in HIV infection. For example, IFN-γ and LPS enhance the presentation of SMX-adducted protein antigens by human dendritic cells *in vitro*,[29] and IFN-γ increases the cytotoxicity of SMX-responsive T cells toward human keratinocytes.[30] High concentrations of these inflammatory mediators have been detected in untreated HIV-infected patients,[31, 32] but they have not been evaluated in relationship to the risk of SMX immunogenicity or hypersensitivity.

Despite evidence that acquired metabolic and inflammatory changes may contribute to the risk of SMX hypersensitivity in HIV infection, this adverse drug reaction remains difficult to predict. One barrier to understanding mechanisms of risk is the lack of an appropriate animal model that expresses the immunologic and toxicologic aspects of SMX hypersensitivity. In rodents and rabbits, administration of SMX itself does not lead to an immune response, and the SMX-NO metabolite must be administered directly to stimulate drug-responsive T cells; however, rash or

other signs of hypersensitivity do not develop.[15, 18, 19, 33] Pet dogs can develop sulfonamide hypersensitivity with clinical signs similar to humans,[34] but the reaction cannot be reliably reproduced in the experimental setting.[35] Further, these models do not address the risk of SMX hypersensitivity in HIV infection.

Simian immunodeficiency virus (SIV) infection in non-human primates is a well-accepted model of HIV infection.[36-38] HIV-1 and SIVmac239 are closely related retroviruses that share key similarities in receptor recognition, cell tropism, and CD4+ depletion.[36-39] Importantly, SIVmac239 infection leads to immunosuppression in rhesus macaques, with progression to diarrhea, wasting, and opportunistic infections that models the clinical progression to AIDS in humans.[36-38]

We hypothesized that SIV-infected macaques would be a useful model of SMX immunogenicity and hypersensitivity in the setting of HIV infection. The primary aim of this study was to determine whether TMP/SMX at therapeutic dosages was sufficient to lead to SMX adduct formation, SMX immunogenicity, or signs of drug hypersensitivity in SIVmac239-infected rhesus macaques. Secondary aim was to determine whether differences in antioxidant concentrations, pro-inflammatory mediators, or SMX disposition were observed in HIV infection, and whether any of these biomarkers were predictive of SMX immunogenicity in macaques.

2.2 Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich, except SMX-HA and SMX-NO, which were purchased from Dalton Chemicals, and N-acetyl SMX, which was obtained from Frinton Laboratories.

Animals

Rhesus macaques (*Macaca mulatta*) chronically infected with SIVmac239, along with age- and sex-matched non-infected controls, were selected from the Wisconsin National Primate Research Center. Animals were studied after reaching set-point viremia, at least 10 weeks after inoculation, and no animals had a history of prior to TMP/SMX exposure. MHC genotyping data were available for Mamu-A1 (6 alleles), Mamu-B (2 alleles), Mamu-DRB (3 alleles) and Mamu-DRB1 (6 alleles), obtained using PCR with sequence-specific priming.[61]

All macaques were evaluated with a physical exam, complete blood count, and serum biochemical panel prior to TMP/SMX dosing. Infected animals also had a CD4+ count and viral load performed. Macaques were fed a fixed formula global primate diet (Teklad, Harlan Laboratories) throughout the study, except for fasting overnight before sample collection under sedation prior to dosing and on day 14. The Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison approved all procedures.

Baseline measurements and dosing

At least 24 hours prior to dosing, animals were sedated with ketamine and dexmedetomidine for baseline sample collection. Whole blood was collected in heparinized tubes for antioxidants,

IFN-γ and sCD14 measurements, and in serum tubes for LPS assays, and was transported on ice. All macaques were then dosed orally for 14 days with TMP/SMX at 120 mg/kg/day, which is the daily dosage used to treat *Pneumocystis jiroveci* pneumonia in humans.[4, 6, 7] The drug was formulated in a banana-flavored paste (Banana crème, Professional Compounding Centers of America) and mixed in food for administration. Animals were observed to confirm that the full dose was consumed each day.

Post-dosing sample collection

Twenty-four hour voided urine was collected after the 13th dose of TMP/SMX, for measurement of SMX metabolites. Total urine volume was measured, 500 mg ascorbate was added to prevent oxidation of SMX-HA,[40] and aliquots were frozen at -80°C. Animals were sedated 2-3 hours after the 14th dose of TMP/SMX for another complete blood count, biochemical panel. In addition, whole blood was collected in heparinized tubes, and was kept at room temperature to harvest plasma for drug metabolite and anti-SMX antibody assays, and for peripheral blood mononuclear cell (PBMC) isolation for drug-responsive T cell assays. One to two inguinal lymph nodes were harvested using sterile surgical technique and placed on ice for lymph node mononuclear cell (LNMC) isolation. Animals were recovered from sedation, except for some SIV-infected macaques that were scheduled for necropsy as part of their original study protocols.

Quantification of plasma ascorbate

Plasma ascorbate was measured from chilled baseline heparinized blood by HPLC. Briefly, equal volume of 0.33% perchloric acid was added to plasma samples to precipitate proteins by

centrifugation at 13,000 × *g* for 5 min. The supernatant were filtered for HPLC analysis. Ascorbate was quantified by HPLC using a C18 Ultrasphere ODS column (4.6 mm × 25 cm; Beckman Coulter) and ultraviolet detection at 254 nm. Mobile phase A was 0.05% triethylamine and 1.0% glacial acetic acid in water, with acetonitrile as mobile phase B. Gradient elution was performed from 0% to 20% mobile phase B over 5 min, isocratic elution of 20% mobile phase B for 10 min, a gradient over 5 min to 0% mobile phase B, followed by isocratic elution of 0% for 2 min at the end. The flow rate was 0.75 ml/min.

Quantification of erythrocyte reduced GSH

Erythrocyte reduced GSH were measured from chilled baseline heparinized blood by HPLC, as previously described with slight modification.[27] Briefly, 125 µl red blood cell (RBC) was incubated with 150 µl of 9 mM monobromobimane in PBS in dark for 10 min. 25 ul of 60% perchloric acid was added to RBC samples to precipitate proteins by centrifugation at 13,000 × *g* for 15 min. The supernatant was incubated with 5-sulfosalicylic acid, dithiothreitol, *N*-ethylmorpholine, and acetonitrile in dark at 37℃ for 5 min. Trichloroacetic acid (3% final volume) was added to RBC lysates to precipitate proteins by centrifugation at 13,000 × *g* for 5 min. The supernatant was filtered for HPLC analysis. GSH was quantified by HPLC using a C18 Ultrasphere ODS column (4.6 mm × 25 cm; Beckman Coulter) and fluorescence detection with excitation at 394 nm and emission at 480 nm. Mobile phase A was 0.05% triethylamine and 1.0% glacial acetic acid in water, with acetonitrile as mobile phase B. Gradient elution was performed from 10% to 40% mobile phase B over 10 min, isocratic elution of 40% mobile phase

B for 5 min, a gradient over 5 min to 10% mobile phase B, followed by isocratic elution of 10% for 2 min at the end. The flow rate was 2 ml/min.

Erythrocyte reduction activity assay

In addition, to assess the capacity for reduction of SMX-HA, activity of the cytochrome b_5/b_5 reductase pathway, which reduces SMX-HA to SMX,[28] was determined in chilled pre-dosing blood using cytochrome *c* as a prototype substrate, as previously described with slight modification.[59] Briefly, 5 ul erythrocyte was lysed by adding 20X volume of deionized water. 50 µl lysed erythrocyte was added to 80 µM cytochrome *c* in 1 ml PBS. 10 µM dicoumarol was added to inhibit competing NAD(P)H:quinone oxidoreductase activity.[60] Human serum albumin instead of lysed erythrocyte was added as negative control. The reaction mix was transferred to flat-bottom, 96-well plates in duplicate, and incubated at 37°C for 5 min. A final concentration of 5 mM NADH was added to the warmed reaction mix, and reduced cytochrome *c* was read immediately at 550 nm every 60s for 7 min.

Measurement of IFN-γ, LPS, and sCD14

Pre-treatment plasma and sera were thawed at room temperature. Plasma IFN-γ was quantified with a commercially available Monkey IFN-γ ELISA kit (U-CyTech biosciences) according to the manufacturer's protocol. For determination of LPS, sera were diluted with endotoxin-free water and heated at 70°C for 15 min to inactivate serum proteins. LPS was quantified with a commercially available QCL-1000™ Endpoint Chromogenic Limulus Amebocyte Lysate Assay

(Lonza; limit of quantitation 0.1 EU/ml) according to the manufacturer's directions. Plasma sCD14 was quantified with a commercially available sCD14 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's protocol.

Quantification of SMX metabolites in plasma and urine after dosing

SMX, SMX-HA, and *N*-acetyl SMX concentrations were quantified in pooled 24-hour urine after 13 days of dosing, using HPLC with ultraviolet detection as previously described with slight modifications,[41] and were normalized for urine volume. SMX metabolites were also quantified in plasma 2-3 hours after the 14th dose of TMP/SMX, to approximate the timing of peak SMX-HA concentrations.[42] Briefly, trichloroacetic acid (1% final volume) was added to plasma or urine samples to precipitate proteins by centrifugation at 13,000 × *g* for 5 min. The supernatant was filtered for HPLC analysis. SMX, SMX-HA and *N*-acetyl SMX were quantified by HPLC using a C18 Ultrasphere ODS column (4.6 mm × 25 cm; Beckman Coulter) and ultraviolet detection at 274 nm. Mobile phase A was 0.05% triethylamine and 1.0% glacial acetic acid in water, with acetonitrile as mobile phase B. Elution was performed with 20% mobile phase B for 12 min, followed by a gradient over 3 min to 80% mobile phase B, isocratic elution with 80% mobile phase B for 4 min, a gradient over 2 min to 20% mobile phase B, followed by isocratic elution of 20% for 3 min at the end. The flow rate was 2 ml/min.

Synthesis of SMX-conjugated proteins

SMX was conjugated to human serum albumin (HSA), ovalbumin (OVA), or keyhole limpet hemocyanin (KLH) by a diazotization reaction. SMX was dissolved in 1 N hydrochloric acid. Ice-cold sodium nitrite was then added dropwise while the reaction was kept on ice with stirring. The mixture was added dropwise to HSA, OVA, or KLH in 0.13 M sodium chloric and 0.16 M boric acid. pH was held at 9-9.5 with sodium hydroxide. After the last addition, the mixture was kept on ice for 1 hour with stirring. The pH was adjusted to 7.4 with hydrochloric acid. The mixture was dialyzed with 300X volume of PBS. The dialyzed SMX-conjugated protein was lyophilized.

Production of anti-SMX antibodies

A specific pathogen free, barrier raised New Zealand white rabbit was immunized with SMX-KLH in Freund's complete adjuvant on day 0 (Panigen Inc, Blanchardville, WI), and SMX-KLH in Freund's incomplete adjuvant on day 21 and 42. Sera were collected for anti-SMX antibodies two weeks after the final immunization.

Detection of surface SMX adducts in lymph node cells

Inguinal lymph nodes were teased apart and used to isolate LNMC. LNMC were washed with ice-cold PBS containing 10% heat-inactivated FBS, and centrifuged at 300 × *g* for 10 min at 4°C. ACK lysing buffer (Lonza) was added to pelleted cells to remove erythrocytes. Isolated LNMC were placed in heat-inactivated FBS with 10% DMSO, placed in a Mr. Frosty™ freezing

container (Thermo Scientific) at -80°C for 24 hours, and then transferred to a liquid nitrogen dewar for storage.

For determination of SMX-protein adducts by flow cytometry, LNMC were thawed at 37°C and washed with PBS containing 10% heat-inactivated FBS; 500,000 cells were stained with polyclonal rabbit anti-SMX sera (1:30,000), along with polyclonal goat anti-rabbit IgG linked with FITC (1:20 dilution; BD Biosciences). Validation experiments for detecting B cells revealed excessive background without the primary antibody, so subsequent experiments were focused on T cell populations. T cells were identified with specific antibodies for macaque CD3 (linked to Alexa Fluor 700, BD Biosciences) clone SP34-2, CD4 (linked to APC, Miltenyi Biotec Inc) clone M-T466, and CD8 (linked to Pacific Blue; BD Biosciences) clone RPA-T8. Reactions were analyzed for surface SMX adducts in lymphocyte subpopulations on an LSR II analytical flow cytometer (BD Biosciences), and data were analyzed using FlowJo software.

PBMC isolation

After 14 days of dosing, PBMC were isolated from room temperature heparinized blood as previously described with slight modification.[44] Briefly, whole blood was diluted with an equal volume of 37°C HBSS buffer, then layered on Lymphocyte Separation Media (Lonza), and centrifuged at 400 × *g* for 30 min at room temperature. The interface containing PBMC was washed and resuspended with 37°C PBS with 10% heat inactivated FBS. RBC were lysed by

addition of ACK lysing buffer (Lonza). Isolated PBMC were immediately processed on the same day for detection of drug-responsive T cells.

Detection of drug-responsive T cells

The lymphocyte transformation test was used to detect drug-responsive T cells in PBMC (or in LNMC if PBMC yield was poor), using previously described techniques.[45] Briefly, cells (200,000 cells/well) were resuspended in RPMI media with 10% heat-inactivated FBS, 1% L-glutatamine, 1% antibiotic/antimycotic, and incubated in triplicate with SMX (1000 μM), SMX-NO (75 and 100 μM), TMP (75 and 100 μM), media with drug vehicle (1% DMSO, 1.5% NaOH), or concanavalin A (2 μg/ml) as a positive control, in flat-bottom, 96-well plates for 72 hours at 37°C with 5% CO_2. SMX-NO and TMP were also incubated with concanavalin A to ensure that these drug concentrations were not inhibiting cell proliferation. Lymphocyte proliferation was measured using incorporation of [^{3}H]-thymidine,[45] and proliferative responses were calculated as stimulation indices (SI; cpm in drug-treated cultures divided by cpm in cultures containing media plus vehicle alone). A positive result for T cell proliferation was defined as a stimulation index in response to TMP, SMX or SMX-NO *in vitro* that was two-fold or greater than drug vehicle controls.[15, 46, 47]

Detection of anti-SMX antibodies

Post-dosing plasma was assayed for anti-SMX antibodies using an ELISA protocol and BD OptEIA™ reagents (Reagent Set B, BD Biosciences). Flat-bottom, 96-well microtiter plates were coated in triplicate overnight at 4°C with 10 mg/ml OVA or 10 mg/ml SMX-OVA. Plates

were blocked for 2 hours with assay diluent at room temperature. Macaque plasma was centrifuged 4℃, diluted up to 1:512 with coating buffer, and incubated in plates at 4℃ overnight. HRP-linked anti-macaque IgG secondary antibodies (1:30,000; Bethyl Laboratories, Inc.) were added to plates and incubated for 2 hours at room temperature. The presence of macaque anti-SMX antibodies was detected using the OptEIA™ kit substrate solution per the manufacturer's instructions, with ultraviolet detection at 450 nm. Absorbance results for SMX-OVA were blanked for absorbance in OVA-coated wells; plasma samples were considered positive for anti-SMX antibodies if the blanked absorbance was 2 standard deviations above the mean blanked absorbance for plasma from SMX-naïve rhesus macaques. These latter samples were obtained from 6 SIV-negative individual animals from the Wisconsin National Primate Research Center colony.

Statistical analyses

Outcomes were compared between groups (SIV positive versus negative; immune responders versus non-responders) with Mann Whitney tests, using commercial software (Prism 6.0, Graphpad Software, Inc.), with $P < 0.05$. The prevalence of Mamu genotypes was compared between immune responders and non-responders using a Fisher's exact test. Data are reported as medians and observed ranges.

2.3 Results

Animals

Sixteen rhesus macaques were studied: 9 (8 male, 1 female) chronically infected with SIVmac239, and 7 non-infected controls (6 male, 1 female). For the SIV-infected group, the median age (8.6 years, range 7.2-18.3) and weight (10.3 kg, range 9.0-16.5) were comparable to those of the control animals (10.2 years, range 7.3-20.5, and 11.8 kg, range 8.9-18.5). The SIV group had been infected for a median of 13 months (range 9-68), with a median pre-dosing CD4+ count of 351 cells/µl (range 49-1149 cells/µl) and a median viral load of 614,000 copies/ml (range 453,000-792,000 vRNA copies/ml, with one outlier macaque having a viral load of 10,868,000 copies/ml; **Table 2.1**).

No macaques developed hematologic or serum biochemical abnormalities after 2 weeks of TMP/SMX dosing. One non-infected macaque developed transient facial erythema and eyelid swelling with decreased feed intake on day 6 of dosing, and one SIV-infected macaque developed lethargy and a transient, non-pruritic rash on the face, chest and axilla on day 7. This latter animal had the highest viral load (10,868,000 vRNA copies/ml) and lowest CD4+ counts (49 cells/µl) of all the macaques. However, in both cases, skin eruptions resolved despite continued dosing.

Surface SMX adducts on T lymphocytes

We next evaluated SMX adducts on the surface of T lymphocytes from the peripheral lymph nodes in both groups of macaques. The percent of adducted CD4+ and CD8+ lymphocytes was variable, with no significant differences between SIV-infected and non-infected groups (**Figure**

2.2). However, in each case the highest percentage of adducted cells was found in the two macaques with transient rashes.

Immunologic responses to TMP/SMX

Three of 16 macaques (1 SIV-infected and 2 controls) dosed with TMP/SMX had detectable T cells (using an SI threshold of 2-fold)[15,46,47] that proliferated in response to SMX-NO (SI 2.0-3.2; vehicle cpm 160-333). Two of these animals also had T cell responses to TMP (SI 2.0-2.4; vehicle cpm 333-760), but no macaques had detectable T cells that responded to the parent drug SMX (median SI, 0.83; **Table 2.2**).

Four macaques (3 SIV-infected and 1 control) had detectable circulating antibodies to SMX, including the SIV-positive macaque with the facial, chest, and axillary rash. Titers ranged from 1:32 to 1:128 (**Table 2.1**). Overall, 4 of 9 of SIV-positive (44%), and 3 of 7 SIV-negative (43%) macaques had at least one positive immune marker to SMX (drug-responsive T cells or anti-SMX antibodies), and this was not significantly different between groups.

Antioxidants, IFN-γ, LPS, and sCD14

Plasma ascorbate concentrations were very similar between groups (**Figure 2.3A**). Although erythrocyte reduced GSH concentrations did not differ statistically (median GSH 2.0 mM in each group), a subset of SIV-infected, but not control, macaques had very low GSH concentrations ($<$ 1 mM; **Figure 2.3B**), including the SIV-infected macaque with the rash on the face, chest and axilla (GSH 0.59 mM). Circulating plasma IFN-γ concentrations did not differ significantly

between infection groups (control macaques, median 0.0 pg/ml, range 0.0-5.5; SIV group, median 1.8 pg/ml, range 0.0-7.0; *P* = 0.21). Only one macaque had detectable serum LPS (15.4 EU/ml); this SIV-infected animal had pneumonitis at necropsy and also had evidence of drug-responsive T cells to SMX-NO and TMP. Circulating plasma sCD14 concentrations also did not differ between groups (control macaques, median 0.97 µg/ml, range 0.56-1.50; SIV group, median 1.11 µg/ml, range 1.03-1.67; *P* = 0.39).

Plasma and urine SMX metabolites

Plasma and urinary concentrations of *N*-acetyl SMX were not significantly different between SIV-infected and control animals (**Table 2.3**). In contrast, SMX-HA concentrations in plasma and urine were significantly lower in the SIV group compared to controls (*P* = 0.036 for both; **Figure 2.4**). In addition, SMX-HA comprised a lower percentage of total measured plasma drug in SIV infection compared to controls (*P* = 0.049; **Table 2.3**). To determine whether capacity for SMX-HA reduction differed in SIV infection, we also measured the activity of the cytochrome b_5 reductase pathway in erythrocytes in both groups. There was a trend for lower activities in SIV-infected macaques, but this difference did not reach significance (*P* = 0.08; **Figure 2.5**).

Predictors of immunogenicity

Finally, we grouped the macaques by whether they had a detectable immune response to TMP/SMX and compared potential predictors of immunogenicity. Seven of the 16 macaques had either anti-SMX antibodies or drug-responsive T cells (with no macaque having both; **Table 2.1**).

There were no discernable differences in blood antioxidants, LPS, IFN-γ, sCD14, SMX metabolites, or cytochrome b_5 reductase activities between "immune responder" and non-responder macaques (**Table 2.4**). However, the Mamu-DRB1_0401 allele was significantly over-represented in immune responder macaques (4 of 7) compared to non-responder animals (0 of 9; $P = 0.019$).

2.4 Discussion

Sulfonamide hypersensitivity develops in 20-57% of critically ill HIV-infected patients,[2-5] but the mechanisms for increased risk are not well understood. The risk varies with severity of illness, with higher incidence seen at lower CD4+ counts.[3, 6, 8-10] One study found an increased relative risk for SMX hypersensitivity of 1.28 with each decrement in CD4+ count of 50 cells/µl,[9] although extremely low CD4+ counts (< 27/µl) appear to be protective.[7] The association of viral load with SMX hypersensitivity is less well documented, since many older reports lack information on viral loads.[2, 4-6, 17, 20, 22-25, 59] However, one study did report a relative risk of 1.42 for adverse events from TMP/SMX for each log increment in viral load.[9]

Animal models of SMX hypersensitivity have been disappointing. Rodents and rabbits do not develop immune responses when dosed with the parent SMX, and require parenteral dosing with SMX-HA or SMX-NO metabolites in order to develop drug-specific immune responses.[15, 16, 18, 33] In addition, these species show no evidence of cutaneous rash or systemic illness even when given relatively high dosages of the reactive SMX-NO metabolite. Pet dogs can develop sulfonamide hypersensitivity when treated with TMP/SMX therapeutically, with clinical signs

similar to those seen in humans.[34] However, none of these animal models are appropriate to address the mechanisms of risk for SMX hypersensitivity in the setting of retroviral infection.

In the present study, we found that like humans,[4, 6, 7] macaques can generate drug-tissue adducts, drug-responsive T cells and anti-SMX antibodies when given oral TMP/SMX at therapeutically relevant dosages. Three of 16 animals had detectable drug-responsive T cells and 4 of 16 animals developed anti-SMX antibodies after 2 weeks of treatment with the parent drug, SMX. This is in contrast to rodents and rabbits,[15, 16, 18, 33] and may be due to differences between these species and primates in SMX bioactivation, adduct formation, peptide processing, or antigen presentation.

Two macaques also developed transient facial or truncal rashes during dosing, which were similar in appearance and time of onset to human patients with SMX hypersensitivity.[2, 5, 48] These same two macaques also had the greatest number of SMX-adducted CD4+ and CD8+ lymphocytes in lymph nodes. However, we did not detect drug-responsive T cells in either animal, and only one of the two had anti-SMX antibodies. In addition, in both cases, the rash resolved over 24-48 hours despite continued administration of TMP/SMX. Sulfonamide-induced rashes can sometimes resolve despite continued drug administration in human patients,[2] and can occasionally be seen without detectable anti-SMX antibodies or drug-responsive T cells.[49-52] However, it is uncertain whether the observed events in our macaques were induced by TMP/SMX administration.

Neither drug-protein adducts nor anti-SMX antibodies were sufficient for the development of drug hypersensitivity in macaques, since we observed both markers in animals without clinical or biochemical evidence of an adverse reaction. This is consistent with previous findings in human patients with incomplete concordance between these immune markers and clinical hypersensitivity.[17, 50, 53] We also observed drug-responsive T cells in macaques without clinical or biochemical evidence of SMX hypersensitivity. Further, we re-dosed two of the SIV-negative animals that had drug-responsive T cells for an additional 14 days with the TMP/SMX, 4-8 months after initial exposure, without adverse event (data not shown). Therefore, even with dosing of the parent drug in non-human primates, T cell or antibody responses against SMX were not sufficient to lead to drug hypersensitivity. It is unclear whether detectable drug-responsive T cells are sufficient for SMX hypersensitivity in humans, since most studies have evaluated a relatively small number of patients or have not included SMX-tolerant patients.[18, 46, 54-56]

We next evaluated antioxidant status, pro-inflammatory mediators, and SMX disposition in SIV infection. For antioxidants, we focused on ascorbate and GSH, which can mediate the reduction of immunogenic SMX-NO back to SMX-HA.[11, 27] Plasma ascorbate concentrations did not differ between SIV-infected and non-infected animals. In our previous study in HIV-infected patients, plasma ascorbate concentrations were significantly decreased in un-supplemented individual; however, ascorbate concentrations were normal in those patients that self-reported taking a vitamin C supplements.[27] The macaques in our study were fed a uniform diet containing stabilized vitamin C, and were offered fruits and vegetables daily. Therefore, if ascorbate

disposition were altered in SIV infection as it is in HIV infection, high levels of vitamin C intake could have masked this. Since ascorbate appears to be important in reducing, and thus detoxifying, plasma SMX-NO,[27, 44] additional studies are indicated in macaques with moderate ascorbate restriction to model the nutritional status of un-supplemented HIV-infected patients.

Median GSH concentrations were also not significantly different between groups. However, 3 of 9 SIV-infected macaques had very low erythrocyte GSH concentrations (≤ 1 mM), including the macaque with the highest viral load and axillary rash. We previously found erythrocyte GSH concentrations ≤ 1 mM in about 57% of HIV-infected human patients using the same methodology.[27] Others have also found significantly lower blood GSH concentrations in untreated HIV infection,[23] which correlate inversely with viral load.[58] However, low blood GSH alone was not a risk factor for sulfonamide hypersensitivity in one study in AIDS patients.[23] Because ascorbate and glutathione are interdependent pathways,[62] and either antioxidant can reduce SMX-NO,[26, 27] studying additional animals with combined GSH and ascorbate deficiencies may help to address the role of antioxidant status in the pathogenesis of SMX hypersensitivity.

We next evaluated several pro-inflammatory mediators in SIV infection. Both IFN-γ and LPS have been shown to enhance SMX adduct formation within dendritic cells *in vitro*,[29] and serum IFN-γ, plasma LPS, and plasma sCD14, which is secreted in response to circulating LPS,[57] are reportedly increased in HIV-infected patients.[32, 57, 63] In our study, median circulating IFN-γ concentrations were not significantly different in SIV-infected macaques (1.8 pg/ml) compared

to non-infected controls (0.0 pg/ml). Serum IFN-γ concentrations can vary from undetectable to > 10 pg/ml in HIV-infected patients, and do not appear to correlate with clinical status, viral load, or CD4+ count,[32] so our findings are not unexpected. Serum LPS was detected in only one SIV-infected macaque, and median plasma sCD14 concentrations (in the range of 1.0 μg/ml) did not differ significantly between groups. The detection of LPS is also variable in HIV infection, and while median plasma sCD14 concentrations are higher in HIV-infected patients than in non-infected controls, concentrations can vary between 1.0 to 3.0 μg/ml in either group.[63]

We next examined SMX disposition in SIV-infection. Plasma and urinary SMX-HA concentrations were significantly lower in the SIV-infected group, which is similar to what has been found in HIV-infected patients.[42] This may result from further oxidation of SMX-HA to SMX-NO, which binds to tissue proteins and is not filtered into the urine. This finding in our population cannot be attributed to enhanced reduction of SMX-HA back to SMX, since cytochrome b_5 reductase activities were not increased in SIV infection.

In contrast to SMX-HA, we did not find differences in plasma or urinary *N*-acetyl SMX concentrations between SIV-infected and control animals. However, there was considerable individual variability among animals, and we may have had inadequate power to show an effect of SIV infection, particularly without information on *N*-acetyltransferase 2 (*NAT2*) genotype. Studies of *N*-acetylation in HIV infection have been conflicting, and associations with SMX hypersensitivity have been mixed.[7, 20, 24, 59] Some groups of HIV-infected patients show discordance between normal *NAT2* genotypes and a slow acetylator phenotype.[23, 24] In particular, impaired *N*-acetylation has been shown in clinically ill AIDS patients,[20-22] and has been

correlated with lower CD4+ counts < 200 cells/µl.[25] This acquired metabolic defect could be due to down-regulation of *NAT2* expression or decreased availability of acetyl-CoA donors, but the mechanism has not been characterized. An effect of SIV infection on *N*-acetylation might have been detected had we included more animals with advanced disease, as only 3 of the SIV-infected macaques had CD4+ counts < 200/µl. Slow acetylation appears to be a risk factor for SMX hypersensitivity in HIV infection,[23] and screening macaques for CD4+ counts < 200/µl before enrollment may provide a stronger model for studying both the mechanisms of impaired *N*-acetylation as well as hypersensitivity risk.

Overall, we did not see an association between SMX immunogenicity and antioxidant concentrations, selected pro-inflammatory mediators, or SMX disposition in our study. However, we did observe an association between SMX immunogenicity and Mamu-DRB1 genotype. This is intriguing, since allelic variants in the orthologous gene in humans (HLA-DRB1) increase the risk of idiosyncratic drug reactions to nevirapine in HIV-infected patients, as well as to asparaginase, lapatinib, amoxicillin/clavulanate and aromatic antiepileptics in immunocompetent populations.[64-68] Screening macaques for this genotype might strengthen the power of this model to detect SMX hypersensitivity.

Our study was limited by the availability of chronically infected rhesus macaques, particularly those with low CD4+ counts. Perhaps due to our small study population, along with intrinsic biological variability among animals, made it difficult to demonstrate significant differences between SIV-infected and control macaques for several outcomes.

We can conclude, however, that macaques provide a model of SMX immunogenicity that resembles the response seen in human patients with SMX hypersensitivity. Studying more animals with CD4+ counts < 200/µl, along with moderately restricted ascorbate intake to match deficiencies seen in humans, may better model the risk of SMX hypersensitivity in HIV-infected human patients. In addition, the role of Mamu-DRB1 genotype in modeling hypersensitivity to SMX and other drugs in retroviral infection deserves further study.

2.5 References

1. Cribb AE, Lee BL, Trepanier LA, et al. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. *Adverse Drug React Toxicol Rev.* 1996;15:9-50.
2. Gordin FM, Simon GL, Wofsy CB, et al. Adverse reactions to trimethoprim-sulfamethoxazole in patients with AIDS. *Ann Intern Med.* 1984;100:495-499.
3. Hennessy S, Strom BL, Berlin JA, et al. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. *J Gen Intern Med.* 1995;10:380-386.
4. Medina I, Mills J, Leoung G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. *N Engl J Med.* 1990;323:776-782.
5. Walmsley SL, Khorasheh S, Singer J, et al. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). *J Acquir Immune Defic Syndr Hum Retrovirol.* 1998;19:498-505.
6. Carr A, Swanson C, Penny R, et al. Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with Pneumocystis carinii pneumonia and AIDS. *J Infect Dis.* 1993;167:180-185.
7. Eliaszewicz M, Flahault A, Roujeau J, et al. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. *J Am Acad Dermatol.* 2002;47:40-46.
8. Kennedy C, Pimentel J, Lewis D, et al. Crossover of HIV-infected patients from aerosolizd pentamidine to trimethoprim-sulfamethoxazole: lack of hematologic toxicity and relationship of side effects to CD4+ lymphocyte count. *J Infect Dis.* 1993;168:314-317.
9. Rabaud C, Charreau I, Izard S, et al. Adverse reactions to cotrimoxazole in HIV-infected patients: predictive factors and subsequent HIV disease progression. *Scand J Infect Dis.* 2001;33:759-764.
10. Ryan C, Madalon M, Wortham DW, et al. Sulfa hypersensitivity in patients with HIV infection: onset, treatment, critical review of the literature. *WMJ.* 1998;97:23-27.
11. Cribb AE, Miller M, Leeder JS, et al. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione: implications for idiosyncratic toxicity. *Drug Metab Dispos.* 1991;19:900-906.
12. Reilly TP, Bellevue FH, Woster PM, et al. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. *Biochem Pharmacol.* 1998;55:803-810.
13. Roychowdhury S, Vyas PM, Svensson CK. Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. *Drug Metab Dispos.* 2007;35:676-681.
14. Manchanda T, Hess D, Dale L, et al. Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol Pharmacol.* 2002;62:1011-1026.
15. Naisbitt D, Gordon S, Pirmohamed M, et al. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br J Pharmacol.* 2001;133:295-305.
16. Naisbitt D, Farrell J, Gordon S, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol.* 2002;62:628-637.
17. Daftarian MP, Filion LG, Cameron W, et al. Immune response to sulfamethoxazole in patients with AIDS. *Clin Diagn Lab Immunol.* 1995;2:199-204.
18. Farrell J, Naisbitt D, Drummond N, et al. Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *J Pharmacol Exp Ther.* 2003;306:229-237.

19. Gill HJ, Hough SJ, Naisbitt DJ, et al. The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. *J Pharmacol Exp Ther.* 1997;282:795-801.
20. Carr A, Gross AS, Hoskins JM, et al. Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulphamethoxazole in HIV-infected patients. *AIDS* 1994;8:333-337.
21. Kaufmann GR, Wenk M, Taeschner W, et al. N-aceyltransferase 2 polymorphism in patients infected with human immunodeficiency virus. *Clin Pharmacol Ther.* 1996;60:62-67.
22. Lee Bl, Wong D, Benowitz NL, et al. Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. *Clin Pharmacol Ther.* 1993;53:529-535.
23. Wolkenstein P, Loriot M-A, Aractingi S, et al. Prospective evaluation fo detoxification pathways as markers of cutaneous adverse reactions to sulphonamides in AIDS. *Pharmacogenetics.* 2000;10:821-828.
24. O'Neil W, MacArthur RD, Farrough MJ, et al. Acetylator phenotype and genotype in HIV-infected patietnts with and without sulfonamide hypersensitivity. *J Clin Pharmacol.* 2002;42:613-619.
25. Quirino T, Bonfanti P, Arcidiacono M, et al. Acetylator phenotype prevalance in HIV-infected patients without previous trimethoprim-sulfamethoxazole hypersensitivity. *Biomed Pharmacother.* 1999;53:286-287.
26. Naisbitt D, Vilar F, Stalford AC, et al. Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. *AIDS Res Hum Retroviruses.* 2000;16:1929-1938.
27. Trepanier LA, Yoder AR, Bajad S, et al. Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J Acquir Immune Defic Syndr.* 2004;36:1041-1050.
28. Kurian J, Bajad S, Miller J, et al. NADH cytochrome b_5 reductase and cytochrome b_5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther.* 2004;311:1171-1178.
29. Lavergne SN, Wang H, Callan HE, et al. "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. *J Pharmacol Exp Ther.* 2009;331:372-381.
30. Schnyder B, Frutig K, Mauri-Hellweg D, et al. T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazol-induced skin reaction. *Clin Exp Allergy.* 1998;28:1412-1417.
31. Sandler NG, Wand H, Roque A, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis.* 2011;203:780-790.
32. Watanabe D, Uehira T, Yonemoto H, et al. Sustained high levels of serum interferon-gamma during HIV-1 infection: a specific trend different from other cytokines. *Viral immunol.* 2010;23:619-625.
33. Cheng L, Stewart BJ, You Q, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol Pharmacol.* 2008;73:1769-1775.
34. Trepanier L, Danhof R, Toll J, et al. Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. *J Vet Intern Med.* 2003;17:647-652.
35. Lavergne S, Volkman E, Maki J, et al. Evaluation of the clinical, immunologic, and biochemical effects of sulfamethoxazole-nitroso administration to dogs: a pilot study. *Toxicology.* 2005;208:63-72.
36. Ambrose Z, KewalRamani VN, Bieniasz PD, et al. HIV/AIDS: in search of an animal model. *Trends Biotechnol.* 2007;25:333-337.
37. Lackner AA, Veazey RS. Current concepts in AIDS pathogenesis: insights from the SIV/macaque model. *Annu Rev Med.* 2007;58:461-476.
38. Valentine LE, Watkins DI. Relevance of studying T cell responses in SIV-infected rhesus macaques. *Trends Microbiol.* 2008;16:605-611.
39. Cullen BR, Garrett ED. A comparison of regulatory features in primate lentiviruses. *AIDS Res Hum Retroviruses.* 1992;8:387-393.

40. Lee B, Delahunty T, Safrin S. The hydroxylamine of sulfamethoxazole and adverse reactions in patients with acquired immunodeficiency syndrome. *Clin Pharmacol Ther.* 1994;56:184-189.
41. Trepanier L, Miller J. NADH-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. *Xenobiotica.* 2000;30:1111-1121.
42. VanderVen AJAM, Mantel MA, Vree TB, et al. Formation and elimination of sulphamethoxazole hydroxylamine after oral administration of sulphamethoxazole. *Br J Clin Pharmacol.* 1994;38:147-150.
43. Abouraya M, Sacco JC, Hayes K, et al. Dapsone-associated methemoglobinemia in a patient with slow NAT2*5B haplotype and impaired cytochrome b5 reductase activity. *J Clin Pharmacol.* 2012;52:272-278.
44. Lavergne SN, Kurian JR, Bajad SU, et al. Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. *Toxicology.* 2006;222:25-36.
45. Abouraya M, Scacco J, Kahl B, et al. Evaluation of sulfonamide detoxification pathways in haematologic malignancy patients prior to intermittent trimethoprim-sulfamethoxazole prophylaxis. *Br J Clin Pharmacol.* 2011;71:566-574.
46. Kalish RS, LaPorte A, Wood JA, et al. Sulfonamide-reactive lymphocytes detected at very low frequency in the peripheral blood of patients with drug-induced eruptions. *J Allergy Clin Immunol.* 1994;94:465-472.
47. Maria V, Victorino R. Diagnostic value of specific T cell reactivity to drugs in 95 cases of drug induced liver injury. *Gut.* 1997;41:534-540.
48. Caumes E, Roudier C, Rogeaux O, et al. Effect of corticosteroids on the incidence of adverse cutaneous reactions to trimethoprim-sulfamethoxazole during treatment of AIDS-associated Pneumocystis carinii pneumonia. *Clin Infect Dis.* 1994;18:319-323.
49. Nyfeler B, Pichler W. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Immunol.* 1997;27:175-181.
50. Gruchalla R, Pesenko R, Do T, et al. Sulfonamide-induced reactions in desensitized patients with AIDS - the role of covalent protein haptenation by sulfamethoxazole. *J Allergy Clin Immunol.* 1998;101:371-378.
51. Meekins CV, Sullivan TJ, Gruchalla RS. Immunochemical analysis of sulfonamide drug allergy: identification of sulfamethoxazole-substituted human serum proteins. *J Allergy Clin Immunol.* 1994;94:1017-1024.
52. Lavergne SN, Danhof RS, Volkman EM, et al. Association of drug-serum protein adducts and anti-drug antibodies in dogs with sulphonamide hypersensitivity: a naturally occurring model of idiosyncratic drug toxicity. *Clin Exp Allergy.* 2006;36:907-915.
53. O'Neil CE, Reed MA, Lopez M, et al. Evaluation of immune parameters in HIV+ subjects reporting adverse reactions to sulfamethoxazole. *Int Arch Allergy Appl Immunol.* 1991;94:246-247.
54. Burkhart C, vonGreyerz S, Depta J, et al. Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. *Br J Clin Pharmacol.* 2001;132:623-630.
55. Mauri-Hellweg, Bettens F, Mauri D, et al. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol.* 1995;155:462-472.
56. Schnyder B, Burkhart C, Schnyder-Frutig K, et al. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J Immunol.* 2000;164:6647-6654.
57. Wilson NL, Vance DE, Moneyham LD, et al. Connecting the dots: could microbial translocation explain commonly reported symptoms in HIV disease? *J Assoc Nurses AIDS Care.* 2014;25:483-495.
58. Sbrana E, Paladini A, Bramanti E, et al. Quantitation of reduced glutathione and cysteine in human immunodeficiency virus-infected patients. *Electrophoresis.* 2004;25:1522-1529.

59. Smith C, Brown I, Torraca B. Acetylator status and tolerance of high-dose trimethoprim-sulfamethoxazole therapy among patients infected with human immunodeficiency virus. *Clin Infect Dis.* 1997;25:1477-1478.
60. Abouraya M, Sacco JC, Hayes K, et al. Dapsone-associated methemoglobinemia in a patient with slow NAT2*5B haplotype and impaired cytochrome b5 reductase activity. *J Clin Pharmacol*. 2012;52:272-8.
61. Kaizu M, Borchardt GJ, Glidden CE, et al. Molecular typing of major histocompatibility complex class I alleles in the Indian rhesus macaque which restrict SIV CD8+ T cell epitopes. *Immunogenetics*. 2007;59(9):693-703.
62. Martensson J, Meister A. Glutathione deficiency decreases tissue ascorbate levels in newborn rats: ascorbate spares glutathione and protects. *Proc Natl Acad Sci U S A*.1991;88(11):4656-4660.
63. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006;12(12):1365-1371.
64. Wang W, Hu FY, Wu XT, An DM, Yan B, Zhou D. Genetic predictors of Stevens- Johnson syndrome and toxic epidermal necrolysis induced by aromatic antiepileptic drugs among the Chinese Han population. *Epilepsy Behav*. 2014;37:16-19.
65. Fernandez CA, Smith C, Yang W, et al. HLA-DRB1*07:01 is associated with a higher risk of asparaginase allergies. *Blood*. 2014;124(8):1266-1276.
66. Yip VL, Alfirevic A, Pirmohamed M. Genetics of immune-mediated adverse drug reactions: a comprehensive and clinical review. *Clin Rev Allergy Immunol*. 2015;48(2- 3):165-175.
67. Parham LR, Briley LP, Li L, et al. Comprehensive genome-wide evaluation of lapatinibinduced liver injury yields a single genetic signal centered on known risk allele HLADRB1* 07:01. Pharmacogenomics J. 2016;16:180-5.
68. Cornejo Castro EM, Carr DF, Jorgensen AL, Alfirevic A, Pirmohamed M. HLAallelotype associations with nevirapine-induced hypersensitivity reactions and hepatotoxicity: a systematic review of the literature and meta-analysis. *Pharmacogenet Genomics*. 2015;25(4):186-198.

Figure 2.1: Sulfamethoxazole (SMX) biotransformation with generation of reactive metabolites that lead to hapten formation, immunogenicity, and clinical signs of delayed sulfoanmide hypersensitivity in immunocompetent and HIV-infected patients.

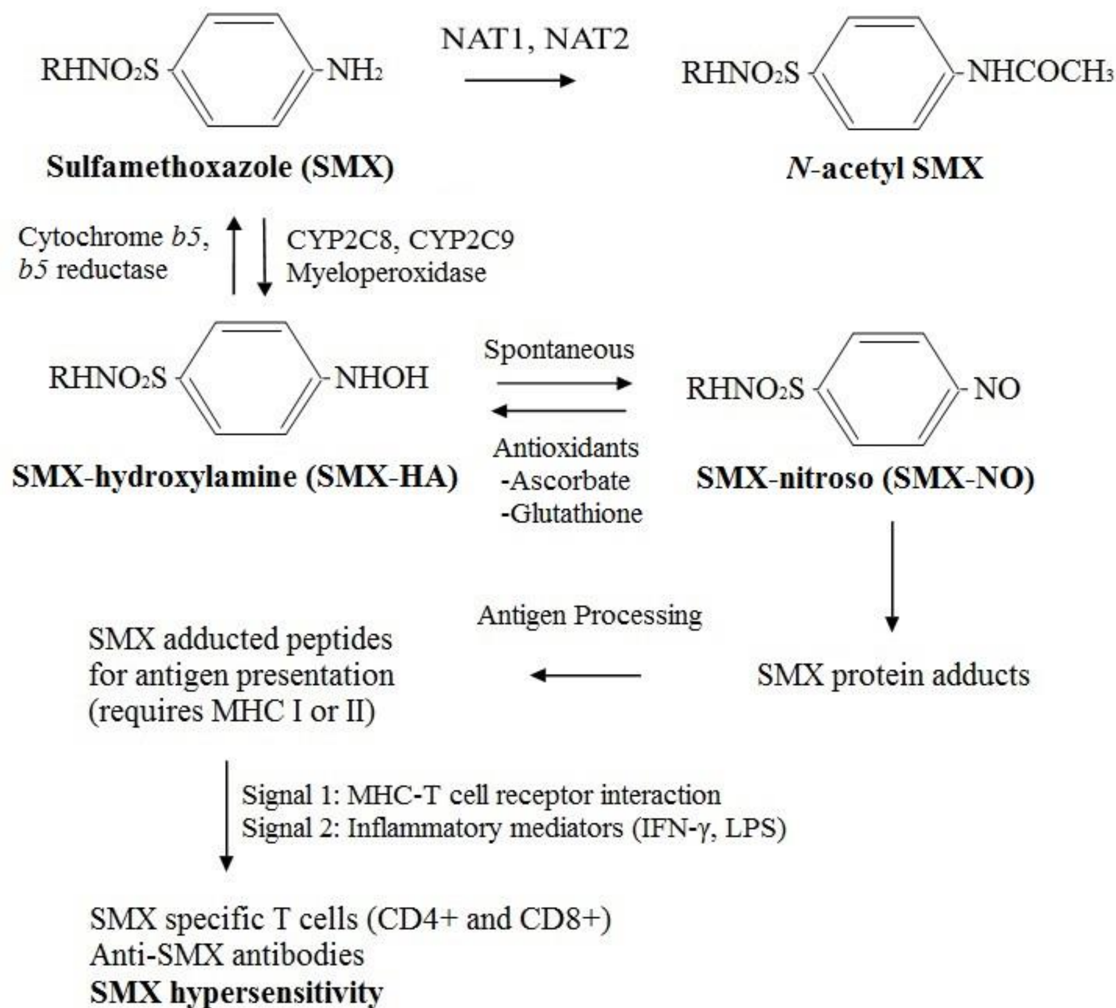


Table 2.1 Immune outcomes in 17 macaques dosed orally with TMP/SMX at 120 mg/kg/day for 14 days.

Animal ID	Condition	CD4 (cells/µl)	Viral load (copies/ml)	DRB1*0401/ 0406/ 0411	Rash	T cell response	Anti-SMX antibodies
r2267	SIV	49	10,868,000	Pos	Yes		Pos, 1:64
r98016	SIV	355	453,200				
r2353	SIV	351	792,000				
r2366	SIV	116	614,000				
r2360	SIV	175	780,000	Pos		Pos	
r2355	SIV	1149	470,000				
r2367	SIV	573	786,667	Pos			Pos, 1:128
r1049	SIV	MD	315,667				
r4087	SIV-female	MD	520,000	Pos			Pos, 1:32
r2252	Control	ND	ND		Yes		
r4024	Control	ND	ND			Pos	
r5090	Control	ND	ND			Pos	
r99070	Control	ND	ND				Pos, 1:128
r4050	Control	ND	ND				
r3015	Control	ND	ND				
r4031	Control-female	ND	ND				

MD: missing data.

ND: not determined

Figure 2.2: Surface drug (SMX; sulfamethoxazole) adducts detected in inguinal lymph node lymphocytes from SIV-infected macaques and non-infected controls, after dosing with TMP/SMX at 120 mg/kg/day per os for 14 days. Highlighted data points indicate outlier high adduct formation in 2 macaques that developed transient erythematous rashes during treatment.

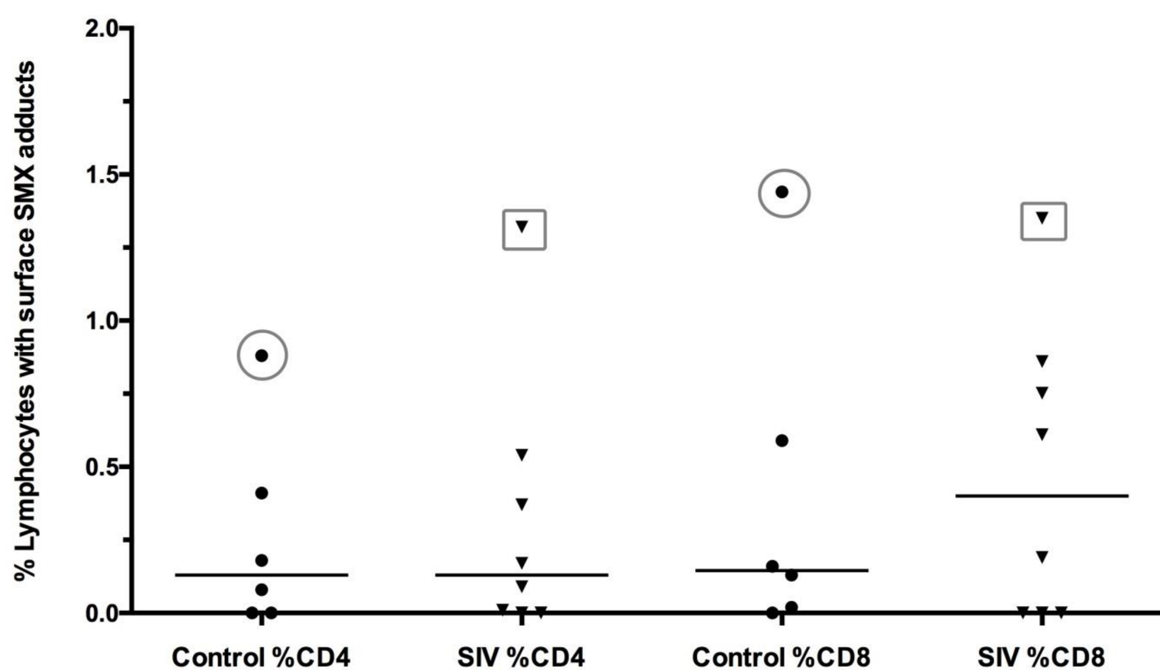
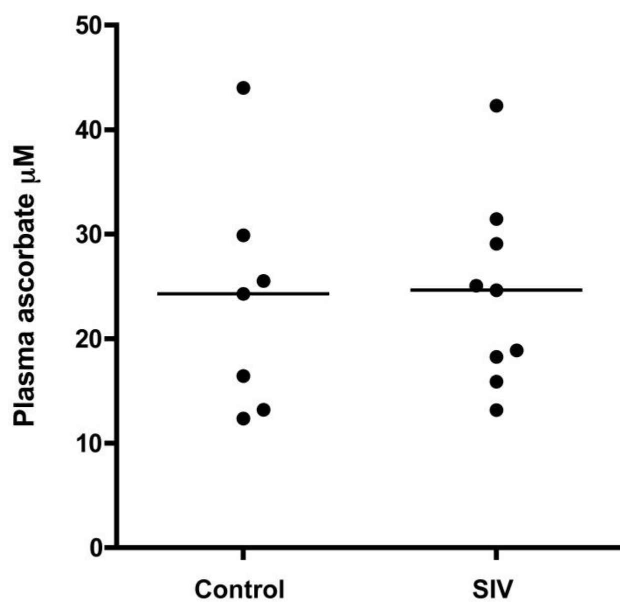


Table 2.2: Detailed lymphocyte transformation test data showing counts per minute (cpm) and stimulation indices (SI) after incubating macaque PBMC with drug vehicle, 1000 µM SMX, 75-100 µM SMX-NO, or 75-100 µM TMP.

Animal ID	**Condition**	**Vehicle**	**SMX 1000 µM**		**SMX-NO 100 µM**		**SMX-NO 75 µM**		**TMP 100 µM**		**TMP 75 µM**	
		cpm	**cpm**	**SI**	**cpm**	**SI**	**cpm**	**SI**	**cpm**	**SI**	**cpm**	**SI**
r2267	SIV	167.83	160.00	0.95	93.50	0.56	not determined		171.97	1.02	not determined	
r98016	SIV	91.77	85.30	0.93	71.43	0.78	not determined		119.47	1.30	not determined	
r2353	SIV	1627.10	746.03	0.46	1001.60	0.62	887.37	0.55	581.33	0.36	744.23	0.46
r2366	SIV	253.53	438.30	1.73	463.17	1.83	268.83	1.06	197.97	0.78	233.73	0.92
r2360	SIV	333.50	198.17	0.59	955.03	2.86	278.93	0.84	794.23	2.38	257.57	0.77
r2355	SIV	226.43	133.27	0.59	171.37	0.76	183.70	0.81	138.20	0.61	185.00	0.82
r2367	SIV	118.47	108.20	0.91	103.47	0.87	106.20	0.90	79.47	0.67	98.17	0.83
r1049	SIV	103.60	101.67	0.98	89.50	0.86	105.47	1.02	54.03	0.52	171.80	1.66
r4087	SIV- female	95.10	90.33	0.95	95.10	1.00	100.33	1.06	63.87	0.67	133.50	1.40
r2252	Control	223.23	105.53	0.47	173.80	0.78	298.27	1.34	137.77	0.62	236.40	1.06
r4024	Control	760.13	374.93	0.49	1552.07	2.04	1467.53	1.93	624.20	0.82	1527.77	2.01
r5090	Control	160.17	174.37	1.09	146.57	0.92	525.97	3.28	131.67	0.82	244.20	1.52
r99070	Control	289.13	140.40	0.49	116.10	0.40	204.07	0.71	105.60	0.37	138.87	0.48
r4050	Control	150.33	112.00	0.75	121.00	0.80	149.00	0.99	106.67	0.71	124.33	0.83
r3015	Control	393.80	177.00	0.45	208.40	0.53	317.27	0.81	168.13	0.43	289.23	0.73
r4031	Control-female	118.40	182.53	1.54	123.00	1.04	138.50	1.17	112.07	0.95	146.47	1.24

Figure 2.3: Plasma ascorbate (panel A) and erythrocyte reduced GSH (panel B) concentrations in SIV-infected macaques and non-infected controls prior to dosing with TMP/SMX.

A.



B.

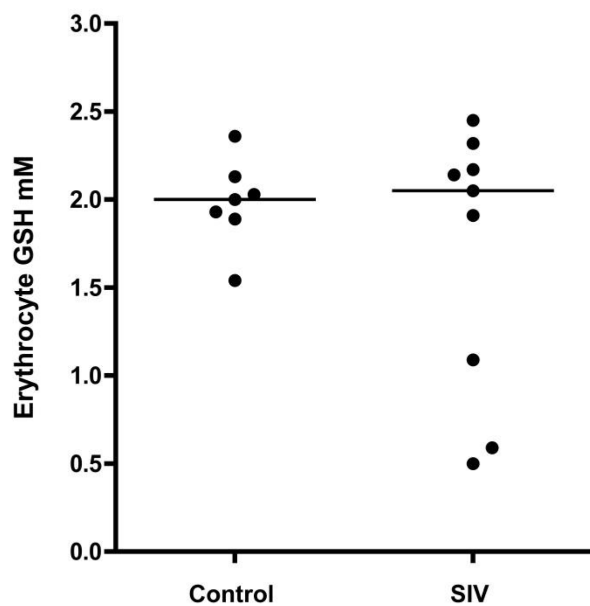


Table 2.3: Steady state plasma concentrations and 24-hour urinary excretion of sulfamethoxazole-hydroxylamine (SMX-HA) and *N*-acetyl SMX metabolites, along with baseline cytochrome b_5 reductase activities (a surrogate for SMX-HA reduction), in rhesus macaques given 120 mg/kg/day of trimethoprim/SMX per os for 14 days. Data are reported as medians and observed ranges. Data are reported as medians and observed ranges.

	Control	**SIV** [1]	**P value**
Plasma SMX-HA (µM)	1.04 (0.0-1.25)	0.0 (0.0-0.30)	0.027*
Plasma SMX-HA (% of total measured drug) [2]	0.6 (0.0-5.4)	0.0 (0.0-0.6)	0.045*
Urinary SMX-HA (µmoles excreted in 24 h.)	2.2 (0.0-5.6)	0.0 (0.0-3.7)	0.032*
Urinary SMX-HA (% of total measured drug) [2]	0.5 (0-2.3)	0.0 (0-1.4)	0.098
Plasma *N*-acetyl SMX (µM)	9.7 (2.4-43.0)	6.9 (0.8-24.9)	0.62
Plasma *N*-acetyl SMX (% of total measured drug) [2]	11.0 (5.3-32.8)	10.1 (5.4-50.2)	0.31
Urinary *N*-acetyl SMX (µmoles excreted in 24 h.)	238 (83-841)	135 (17-2021)	0.27
Urinary *N*-acetyl SMX (% of total measured drug) [2]	55.1 (32.7-61.4)	54.4 (12.0-74.9)	0.45
Cytochrome b_5 reductase activity (nmole/µl RBC/min)	1267.0 (714.3-2381)	738.1 (276.2-2429)	0.080

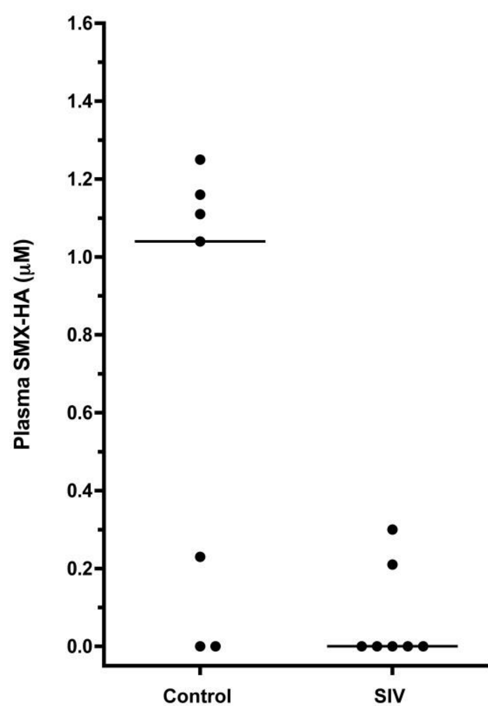
[1] Metabolite concentrations censored for 2 SIV-infected macaques with poor TMP/SMX ingestion on days 13 and 14.

[2] Calculated using a denominator of measured SMX, SMX-HA, and *N*-acetyl SMX.

* Significantly lower than non-infected control macaques, by one tailed Mann Whitney test.

Figure 2.4: Approximate peak plasma (panel A) and 24-hour urinary (panel B) concentrations of SMX-hydroxylamine (SMX-HA) in SIV-infected and control macaques after two weeks of TMP/SMX dosing.

A.



B.

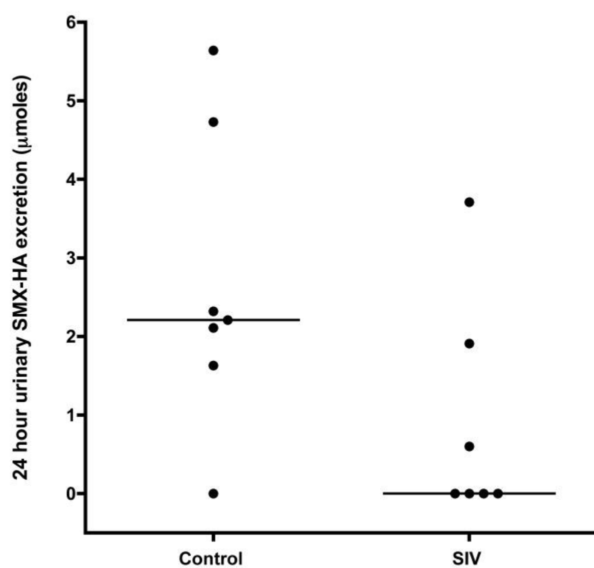


Figure 2.5: Cytochrome b_5 reductase activities (a surrogate for SMX-HA reduction), measured in erythrocytes of control and SIV-infected macaques prior to TMP/SMX dosing. *P* = 0.08 between groups.

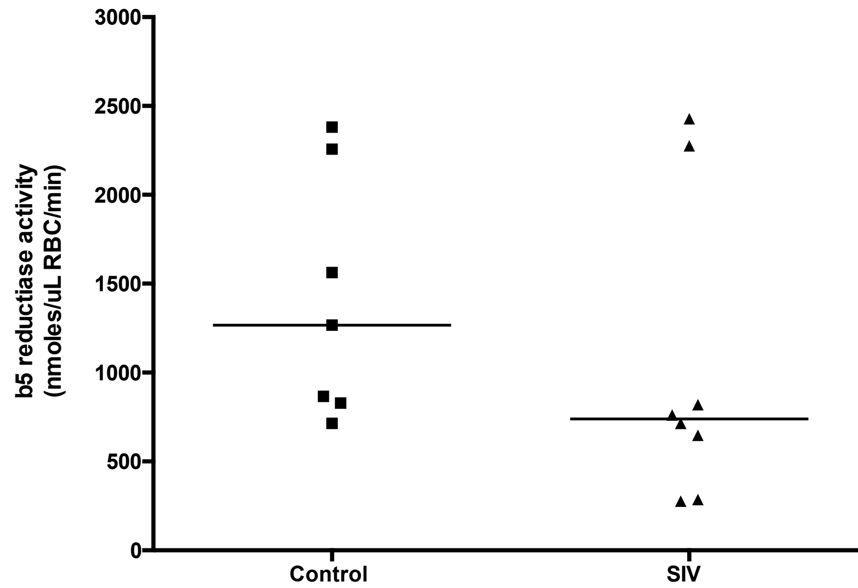


Table 2.4: Potential markers of a detectable immune response to TMP/SMX (defined as the presence anti-SMX antibodies or drug-responsive T cells) in macaques after oral administration of 120 mg/kg/day for 14 days. Data are reported as medians and observed ranges.

	Detectable immune response	**No detectable immune response**	**P value**
SIV status	Infected n = 4 Control n = 3	Infected n = 5 Control n = 4	1.00
Plasma IFN-γ (pg/ml)	1.21 (0.0-5.46)	1.71 (0.0-7.01)	0.86
Plasma sCD14 (µg/ml)	1.11 (0.69-1.50)	1.05 (0.56-1.67)	0.60
Plasma ascorbate (µM)	18.3 (12.4 - 44.0)	24.6 (13.2 - 42.3)	0.30
Erythrocyte reduced GSH (mM)	2.05 (0.59-2.17)	2.00 (0.50-2.45)	0.84
Plasma peak SMX-HA (µM)	86.0 (3.1-352.7)	64.8 (8.0-503.0)	1.00
Plasma peak *N*-acetyl SMX (µM)	12.0 (0.8-33.5)	5.8 (2.4-43.0)	0.74
Urinary SMX-HA (µmoles)	1.8 (0.0-4.7)	1.1 (0.0-5.6)	0.75
Urinary *N*-acetyl SMX (µmoles)	186.2 (16.6-556.5)	153.9 (40.7-2021.0)	1.00
Cytochrome *b*5 reductase activity (nmole/µl/min)	842.9 (285.7-2429)	828.6 (285.7-2429)	0.98

Not shown: LPS (only detected in one SIV-infected animal without a detectable immune response).

Chapter 3 – Effects of SIV Infection on Hepatic Expression

3.1 Introduction

An increased risk of sulfamethoxazole (SMX) hypersensitivity is observed in HIV-infected patients.[1-4] 20-57% HIV-infected patients suffer from SMX hypersensitivity while it only affects 3% of the general population.[1-7] Skin rash occurs in most affected patients, and liver toxicity, thrombocytopenia, and bullous skin eruptions, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, are observed in the more severely affected patients.[8-11] The risk factors associated with an unusually high incidence of sulfonamide hypersensitivity are still not clear, but the increased risk is thought to be due to metabolic alternations in HIV infection.

The development of SMX hypersensitivity depends on SMX bioactivation.[12-14] As shown in **Figure 3.1**, SMX is oxidized to form SMX-hydroxylamine (SMX-HA) by CYP2C8 and CYP2C9 in the liver.[15, 16] This hydroxylamine metabolite can be further oxidized spontaneously to form SMX-nitroso (SMX-NO).[16, 17] The nitroso metabolite can bind to cysteine residues on a protein and form protein adduct,[18] which can be processed and presented in association with MHC-I or MHC-II.[125, 126] This stimulates the activation of T cells and B cells to target SMX-protein adducts,[16, 19] which may lead to the development of drug hypersensitivity.

The formation of protein adducts can be prevented by several detoxification pathway. *N*-acetylation of SMX by *N*-acetyltransferase 2 (NAT2) in the liver leads to an inactive and non-immunogenic acetylated metabolite (*N*-acetyl SMX) for urinary excretion.[20-22] If SMX is oxidized to SMX-HA, it can be reduced to the parent drug by cytochrome b_5 and NADH cytochrome b_5 reductase.[23] If SMX-HA is further oxidized to form SMX-NO, it can be reduced to the hydroxylamine metabolite by antioxidants such as ascorbate, and glutathione (GSH). [13, 24]

Trimethoprim (TMP) is often administrated with SMX.[104-107] Rechallenge with TMP alone leads to hypersensitivity in 16-19% of patients with a history of hypersensitivity to TMP/SMX.[108, 109] TMP alone has also been associated with toxic epidermal necrolysis,[110, 111] and TMP-responsive T cells have been found in some patients allergic to TMP/SMX.[112] Incubation of TMP with human liver microsomes generate reactive TMP metabolites,[113] which could contribute to hapten formation and T cell sensitization. One study showed that TMP has 50% inhibitory effect on CYP2D6,[114] which could suggest that TMP is a substrate for CYP2D6.

N-acetylation of SMX in the liver is the primary detoxification pathway. [20-22] *NAT2* is highly polymorphic with a wide range of expression and activity.[22, 25-29, 37] It has been shown that the slow *N*-acetylation phenotype was over-represented in HIV-infected patients compared to the general population in several studies, with the slow phenotype correlated with low CD4+ T cell counts.[30-35, 48] These observations suggest that HIV infection leads to acquired defects in this

drug detoxification pathway, but its role in the individual risk of sulfonamide hypersensitivity in HIV infection has not been established.[33, 36]

HIV-infected patients also have deficiencies in blood antioxidants, including GSH and ascorbate, and this decreases the capacity for SMX-NO reduction.[13, 24, 38, 39] This could contribute to the increased risk of hypersensitivity. It has been shown that glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS), involved in GSH synthesis, are down-regulated in macrophages of HIV-infected patients, and this could also occur in the liver.[40] Furthermore, antioxidant deficiencies may also directly affect hepatic cytochrome b_5 expression.[41, 42] A lower cytochrome b_5 expression could increase the risk of sulfonamide hypersensitivity by decreasing the reduction of SMX-HA back to the parent compound, SMX.

Studies on urinary metabolites have suggested that HIV-infected SMX-hypersensitive patients may have altered function of some CYP450s compared to HIV-infected SMX-tolerant patients, and may lead to increased SMX bioactivation. [43] Although SMX-HA is the immediate precursor of the immunogenic SMX-NO, urinary SMX-HA concentrations were actually lower in HIV-infected patients, and in two seropositive patients that developed liver toxicity from TMP/SMX.[44, 45, 72] While this seems counter-intuitive, it could reflect enhanced oxidation of SMX-HA to SMX-NO, which binds rapidly to plasma proteins and would not be detected in urine. These findings demonstrate the need for a better understanding of hepatic biotransformation pathways in HIV infection.

Because obtaining liver biopsy samples from HIV-infected patients has ethical and practical constraints, we used an SIV-infected rhesus macaque model of HIV infection to examine the effects of retroviral infection on expression of drug biotransformation pathways. We hypothesized that retroviral infection would lead to enhanced SMX bioactivation and decreased detoxification pathways in the liver.

3.2 Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich, except SMX-HA which was purchased from Dalton Chemicals, and *N*-acetyl SMX, which was obtained from Frinton Laboratories.

Animals

Male rhesus macaques (*Macaca mulatta*) chronically infected with SIVmac239, along with age- and sex-matched non-infected controls, were selected from the Wisconsin National Primate Research Center. Animals were studied after reaching set-point viremia, at least 10 weeks after inoculation, and no animals had a history of prior TMP/SMX exposure. All macaques were evaluated with a physical exam, complete blood count, and serum biochemical panel prior to liver biopsy. Infected animals also had a CD4+ count and viral load performed. Macaques were

fed a fixed formula global primate diet (Teklad, Harlan Laboratories). The Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison approved all procedures.

Liver samples collection and storage

Macaques were sedated with ketamine and dexmedetomidine, and 2-3 Tru-cut needle biopsies were obtained from the liver transcutaneously with ultrasound guidance. Liver samples were put in RNAlater for 24 hours at 4℃. Excess RNAlater was removed, and the samples were stored at -80℃ until RNA extraction.

RNA extraction

Total RNA was extracted by homogenizing liver samples in TRIzol (Ambion) according to manufacturer's protocol, with slight modifications. Briefly, 200 µl chloroform was added to 1 ml of lysate, vortexed and centrifuged at 16,000 × *g* for 5 min at 4℃. The aqueous phase at the top was added to 500 µl ice-cold isopropanol in a new tube, and allowed to precipitate at -20℃ for 1 hour. RNA was pelleted by centrifuging at 16,000 × *g* for 15 min at 4℃. The supernatant was removed, and the RNA pellet was washed with 75% ethanol. Ethanol was removed by centrifuging at 16,000 × *g* for 1 min with the centrifuge lid open. The ethanol-free RNA pellet was resolubilized in RNase free water (Ambion). RNA samples were treated with 2 U DNase I

(Ambion) for 30 min at 37℃ according to the manufacturer's protocol to remove DNA contamination. DNase I was inactivated by adding EDTA to a final concentration of 5 mM, followed by heating at 75℃ for 10 min. RNase inhibitor (Applied Biosystems) was added to a final concentration of 1 U/µl. RNA integrity was accessed by Agilent 2100 BioAnalyzer, and quantified by NanoDrop ND-1000 (ThermoFisher). RNA was stored at -80℃ until preparation for arrays.

Microarray processing

High quality RNA was selected from 3 SIV-infected male macaques (ages 7.2-18.3) and 3 age-matched controls (ages 7.3-20.5) for expression arrays. 100 ng of total RNA was used to generate sense-strand cDNA with the Ambion WT Expression Kit. cDNA was fragmented and labeled with biotin by the GeneChip WT Terminal Labeling Kit (Affymetrix). The fragmented and labeled cDNA was hybridized with the GeneChip Rhesus Gene 1.0 ST Array (Affymetrix) using the Hybridization Oven 640 (Affymetrix). The microarray chip was washed and stained using the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) with the automated GeneChip Fluidics Station 450 (Affymetrix). The stained microarray chip was scanned by the GeneChip Scanner 3000 7G (Affymetrix). Both fluidics station and scanner were controlled by the Affymetrix GeneChip Command Console Software v4.0.0.1567G.

Microarray data analysis

Raw data exported from the Affymetrix platform was analyzed by GeneSpring GX (Agilent). Data were normalized by the Robust Multi-chip Average (RMA) method. Gene expression was compared between SIV-infected and non-infected macaques using a moderated t-test,[102] with $P \leq 0.005$ and ≥ 2-fold difference in expression as thresholds for significance. Microarray probe sets that had signal intensity lower than 100 in both SIV and control groups were excluded. Genes with significant differences in expression between groups at the level of $P \leq 0.005$ were further exported to DAVID Bioinformatics Resources 6.7 (david.ncifcrf.gov) for pathway analysis using the KEGG PATHWAY Database. Interferon (IFN)-inducible genes were further identified using INTERFEROME v2.01.[59]

Quantitative real-time PCR (qPCR) confirmation of transcript expression

Hepatic RNA from individual macaques was also used for qPCR to confirm altered expression of select transcripts. cDNA was generated from 2.5 µg RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). cDNA was diluted 1:100, and incubated with gene-specific probe (0.125 µM), and forward and reverse primers (0.67 mM) for qPCR, using the FastStart Essential DNA Probes Master kit (Roche) and the LightCycler 96 Instrument (Roche). Primers and probes for recognizing sequence-specific DNA were designed using the Universal ProbeLibrary (Roche). qPCR cycles were as follows: 95°C for 10 min pre-incubation, then 95°C for 10s, 60°C for 20s, for 55 cycles amplification. β-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were used as reference genes.[101] Samples were run in triplicate, and all data were normalized to the reference genes.

Primer efficiency was determined by running qPCR for each gene with 1:10, 1:100, 1:1,000 and 1:10,000 dilutions of cDNA. Primer sequences, efficiency, and probe numbers are shown in **Table 3.1**. Statistical analyses for relative mRNA expression between groups were performed using relative expression software tool (REST) 2009 version1, which utilized the method described by Pfaffl and Vandesompele.[62-64, 103]

Liver microsome preparation

Additional banked liver samples were obtained from the Wisconsin National Primate Research Center for hepatic biotransformation activity assays. Liver samples were homogenized on ice in PBS, pH 7.4, and centrifuged at 9000 × *g* for 20 min at 4°C. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C to obtain microsomal and cytosolic fractions, which were stored at -80°C. Protein concentration was determined by the Bradford protein assay (Bio-rad).

Hepatic SMX oxidation activity assay

Activity of SMX oxidation to SMX-HA was assayed as previous described, with slight modification. [115] Briefly, 1 mM SMX was incubated with 250 μg microsomal protein and 1 mM NADPH in PBS for 30 min at 37°C. Both ascorbate and reduced GSH were added to final concentrations of 1 mM to prevent SMX-HA oxidation. 1 mM final concentration of α-Lipoic acid and 6-Propyl-2-thiouracil were added to prevent SMX-HA reduction.[116] Inhibition of SMX-HA reduction was evaluated by adding 100 μM SMX-HA instead of 1 mM SMX to the reaction

mix. Both negative control and SMX-HA standards were incubated with human serum albumin instead of microsomal protein. Reactions were terminated by adding half volume of ice-cold methanol. The reaction mix was centrifuged at 16,000 × *g* for 10 min at 4°C, and the supernatant was filtered and subject to HPLC analysis.

Hepatic SMX-HA reduction activity assay

SMX-HA reduction was assayed as previously described, with slight modification.[60] Briefly, 1 mM SMX-HA was incubated with 125 μg microsomal protein and 1 mM NADH in PBS for 30 min at 37°C. Both ascorbate and reduced GSH were added to final concentrations of 1 mM to prevent SMX-HA oxidation. Both negative control and SMX standards were incubated with human serum albumin instead of microsomal protein. Reactions were terminated by adding half volume of ice-cold methanol. The reaction mix was centrifuged at 16,000 × *g* for 10 min at 4°C, and the supernatant was filtered and subject to HPLC analysis.

Hepatic SMX *N*-acetylation activity assay

N-acetylation of SMX was determined as previously described.[61] Briefly, 300 μM SMX was incubated with 250 μg cytosolic protein and 3 mM acetyl CoA for 20 min at 37°C. Both negative control and *N*-acetyl SMX standards were incubated with human serum albumin instead of cytosolic protein. Reactions were terminated by adding 10% volume of ice-cold 15% perchloric

acid. The reaction mix was centrifuged and filtered as the SMX-HA reduction assay prior to HPLC analysis.

HPLC analysis of SMX metabolites

SMX, SMX-HA and *N*-acetyl SMX were quantified by HPLC using a C18 Spherisorb ODS2 Column (4.6 mm × 25 cm, 5 µm particle size; Waters) and a Shimadze LC-20AD solvent delivery system with ultraviolet detection at 274 nm. Mobile phase A was 0.05% triethylamine and 1.0% glacial acetic acid in water, with acetonitrile as mobile phase B. Elution was performed with 20% mobile phase B for 12 min, followed by a gradient over 3 min to 80% mobile phase B, isocratic elution with 80% mobile phase B for 4 min, a gradient over 2 min to 20% mobile phase B, followed by isocratic elution of 20% for 3 min at the end. The flow rate was 2 ml/min, yielding retention times for SMX, SMX-HA, and *N*-acetyl SMX of 8.269 min ± 0.005 SEM, 6.381 min ± 0.001 SEM and 10.406 min ± 0.030 SEM, respectively.

Statistical analyses

Unless described otherwise, outcomes were compared between groups (SIV-positive versus negative) with Mann Whitney tests, using commercial software (Prism 6.0, Graphpad Software, Inc.), with $P < 0.05$. Data are reported as medians and observed ranges.

3.3 Results

Microarray analysis – SMX biotransformation pathways

Gene expression of SMX biotransformation pathways is shown in **Table 3.2**. Genes involved in SMX oxidation (*CYP2C8* and *CYP2C9*) and TMP bioactivation (*CYP2D6*), were not differentially expressed in livers between SIV-infected and non-infected macaque. Genes involved in SMX detoxification, including *NAT2* and *CYB5R3*, were not differentially expressed; *CYB5A* was not included in the microarray. Genes involved in GSH synthesis and conjugation, included *GSS*, *GCL*, *GSTM1* and *GSTP1*, were also not differentially expressed; *GSTT1* was not included in the microarray.

Hepatic bioactivation activities of SMX

In order to confirm a lack of effect of retroviral infection on hepatic SMX bioactivation, oxidation of SMX was evaluated between 5 SIV-infected and 5 non-infected male macaques. No oxidation activities were detected in both SIV-infected and non-infected macaques. Incubation of SMX-HA in the present of cytochrome b_5 reductase inhibitors, α-Lipoic acid and 6-Propyl-2-thiouracil, did not prevent SMX-HA reduction activities.

Hepatic detoxification activities of SMX and its metabolites

In order to confirm a lack of effect of retroviral infection on hepatic SMX detoxification pathways, reduction of SMX-HA and *N*-acetylation of SMX were evaluated between 5 SIV-infected and 5 non-infected male macaques. Reduction activates between the two groups were similar, with a median of 0.54 nmol/min/mg (range 0.38-0.88) in the SIV-infected group, and 0.43 nmol/min/mg (range 0.36-0.96) in the control group (**Figure 3.2**). *N*-acetylation activates between the two groups were similar, with a median of 0.11 nmol/min/mg (range 0.08-0.20) in the SIV-infected group, and 0.09 nmol/min/mg (range 0.03-0.18) in the control group (**Figure 3.3**).

Microarray analysis – non-biotransformation genes

Although key SMX biotransformation genes were not altered, 154 microarray probe sets were differentially expressed between the SIV-infected and non-infected control macaques at $P \leq 0.005$. Of those, 138 of the probe sets were associated with an identified gene, and 59 genes showed a two-fold or greater difference in expression. Among the 59 genes, four were down-regulated and 55 were up-regulated (**Table 3.3**). 33 of these 59 genes were IFN-inducible, with 7 induced only by type I IFN, one only by type II IFN, and 25 by either type I or type II IFN.

Pathway analyses

Pathway analyses were performed with the 138 identified differentially expressed genes, without requirement for a 2-fold change. Analyses revealed five pathways that were altered by SIV

infection, including RIG-I-like receptor signaling, ISGylation, systemic lupus erythematosus (SLE) development, immunoproteasomal degradation, and antigen presentation (**Table 3.4**).

qPCR confirmation of transcript expression

Based on the results of the expression array, up-regulated genes involved in ISGylation (*ISG15*, 3.6 fold; *HERC5*, 5.8 fold; and *HERC6*, 10 fold), and immunoproteasomal degradation (*PSMB10*, 1.3 fold; *PSME1*, 1.5 fold; and *PSME2*, 1.5 fold) were selected for validation by qPCR. Target gene expression was normalized to the reference genes, *ACTB*, *GAPDH*, and *HPRT1*. All of the genes were confirmed to be up-regulated in the SIV-infected macaque livers compared to controls, with $P \leq 0.001$ (**Table 3.5**).

3.4 Discussion

An increased risk of sulfonamide hypersensitivity is observed in HIV-infected patients,[1-7] but the mechanisms for this are not well understood. The development of hypersensitivity depends on SMX bioactivation to form the reactive SMX-NO metabolite, which binds to protein.[12-18] These SMX-protein adducts can then act as haptens to stimulate an immune response, which can ultimately lead to drug hypersensitivity.[16, 19]

In order to understand the increased risk, we first determined whether retroviral infection affected the gene expression of SMX biotransformation pathways, using liver biopsy samples from SIV-infected macaques as model for HIV infection. However, we found no evidence for differential expression of genes involved in SMX oxidation (*CYP2C8* and *CYP2C9*). We were unable to assess this *in vitro* because forward oxidation was undetectable in both SIV-infected and control liver microsomes, due to rapid reverse reduction. The cytochrome b_5 reductase inhibitors, α-Lipoic acid, and 6-Propyl-2-thiouracil, only inhibited NADH cytochrome b_5 reductase activity up to 40%.[65] At this rate, SMX-HA reduction activity is faster than SMX oxidation in the hepatic microsomal proteins. This may explain why SMX induced hepatotoxicity is relatively rare; rapid reduction of SMX-HA may prevent the generation of SMX-NO and formation of protein adducts in the liver. However, SMX as a parent drug can still escape hepatic transformation and be oxidized locally by keratinocytes and dendritic cells, and lead to the development of cutaneous drug hypersensitivity.[12, 16, 19, 66-71]

There were also no detectable differences in the hepatic reverse reduction pathway, either in gene expression of *CYB5R3* or in SMX-HA reduction. In **Chapter 2**, we found that levels of plasma and urinary SMX-HA were significantly lower in the SIV-infected macaques after dosing with TMP/SMX for 14 days. This is similar to what has been found in HIV-infected patients, who show decreased excretion of SMX-HA compared to healthy subjects.[44, 45, 72] These observations together with the *CYB5R3* expression and hepatic SMX-HA reduction activity data suggest that the decreased SMX-HA concentrations are not a result of increased hepatic

reduction. Instead, decreased measurable SMX-HA in SIV infection may be due to increased oxidation to SMX-NO.

We also found no detectable differences in hepatic *N*-acetylation of SMX, either in gene expression of *NAT2* or enzyme activities. In **Chapter 2**, we found that the levels of plasma and urinary *N*-acetyl SMX were not different between the SIV-infected and non-infected macaques after dosing with TMP/SMX for 14 days. This is in contrast to previous studies suggesting HIV infection acquired slow acetylation.[30-36, 48] However, these studies involved acutely ill AIDS patients,[30-32] with lower CD4+ counts < 200 cells/µl,[35] while the SIV-infected macaques in this study had a median CD4+ counts of 351 cells/µl (range 49-1149 cells/µl; **Chapter 2**). It is possible that an effect of SIV infection on *N*-acetylation might have been detected had we included more animals with advanced disease (CD4+ counts < 200 cells/µl).

Finally, we found no differences in genes involved in GSH synthesis and conjugation (*GCL*, *GSS*, *GSTM1*, *GSTP1*, and *GSTT1*). This is contradictory to other studies that show decreased *GCL* and *GSS* gene expression in a model of Tat transfection or in macrophages of HIV-infected patients.[40, 116] Similar to our expression array, we found no differences in erythrocyte GSH levels between SIV-infected and non-infected controls (**Chapter 2**). The inability to show alterations in GSH level and genes involve in GSH regulation could due to the lack of SIV-infected macaques with high viral loads.

Although SIV infection did not affect hepatic SMX biotransformation, it could still lead to acquired risks for drug hypersensitivity through other pathways. HIV can infect multiple hepatic cells *in vivo*, including Kupffer cells, sinusoidal cells and hepatocytes.[53-55] *In vitro* studies of HIV-infected hepatocytes also showed that HIV infection can lead to apoptosis and fibrogenesis.[56, 57] These observations suggest that HIV infection could alter hepatic gene expression in other pathways.

Immune dysregulation is clearly present in HIV infection, and despite the immunosuppressed state, there are higher percentages of activated lymphocytes and increases in pro-inflammatory cytokines in these patients.[46-49] Microarray studies in retroviral infection have shown an increased expression of IFN-inducible genes in lung tissue and macrophages.[50, 51] These changes might lead to alterations in antigen processing and presentation[58] that could promote drug hypersensitivity. For example, multiple genes that were up-regulated in our expression array are inducible by type I (IFN-α and IFN-β) or type II (IFN-γ) IFN. Up-regulation of IFN-inducible genes including the genes involved in RIG-I-like signaling pathway in the SIV-infected macaques are responsible for fighting against viral infections, and this has been shown in multiple studies of viral infection.[58, 73,75, 76] Up-regulation of IFN-γ can lead to drug hypersensitivity through enhancing presentation of SMX-adducted protein antigens by human dendritic cells *in vitro*,[69] and increasing cytotoxicity of SMX-responsive T cells toward human keratinocytes.[117] However, we did not see a significant increase of serum IFN-γ in SIV-infected

macaques (**Chapter 2**), and this could suggest that the up-regulated IFN-inducible genes were induced by type I IFN.

One pathway that was up-regulated in SIV infection was ISGylation. It involves a group of IFN-inducible genes that mediate the conjugation of ISG15 protein on a target protein.[79] ISG15 has a strong anti-viral activity,[84-86] and it has been shown to conjugate to more than 150 protein targets with a wide spectrum of functions. [87] The up-regulation of this pathway during SIV infection could be expected due to its anti-viral properties. Furthermore, ISGylation may be involved in altering proteasomal degradation indirectly, and this could promote drug hypersensitivity by generating SMX-adducted peptides for antigen presentation.[79, 118, 119]

Another group of genes that was up-regulated in SIV infection included those related to SLE, which is an autoimmune disease caused by autoantibodies targeting cell nuclei.[88, 89] While it seems improbable to associate autoimmunity with a disease of immunodeficiency, multiple reports have shown that HIV-infected patients can be diagnosed with SLE,[90, 91] and up-regulation of IFN-inducible genes have been detected in SLE patients.[92, 93] Type I IFN has been associated with the pathogenesis of SLE via the activation of autoreactive T cells and B cells.[120-122] These observations suggest that HIV-infected patients are capable of developing autoimmune disease, and the up-regulation of IFN-inducible genes may lower the threshold for drug hypersensitivity.

Additional up-regulated genes in SIV infection were those involved in immunoproteasomal degradation. *PSMB10* (β2i), *PSME1* (PA28α), and *PSME2* (PA28β) encode immunoproteasome subunits that can be up-regulated by type I IFN, and they enhance production of selective set of antigenic peptides for MHC-I presentation.[94-98] Up-regulation of these genes may increase the chance of processing SMX-adducted proteins, and generating SMX-adducted antigenic peptides for MHC-I presentation. Multiple studies report the up-regulation of immunoproteasome in autoimmune diseases.[94] It is possible that up-regulation of the immunoproteasome contributes to the risk of SMX hypersensitivity by increasing peptide processing even without enhanced drug metabolite generation or adduct formation.

Consistent with this hypothesis, genes involved in antigen presentation were also up-regulated in SIV infection. Short peptides produced by immunoproteasomal degradation are transported into the endoplasmic reticulum,[98,100] where tight binding of the peptide and MHC-I allows the release of MHC-I to the cell surface for antigen presentation.[98, 100] *B2M* (β2 microglobulin), *MAMU-A*, *MAMU-E*, and *MAMU-F* encode are macaque MHC-I receptor components, and their expressions were up-regulated in our SIV-infected macaques. This suggests that SIV infection may increase antigen presentation in a global manner, and this may have a role in SMX as well as other drug hypersensitivities in HIV infection, including the antituberculosis drug thiacetazone, and the antiretroviral drugs, efavirenz and nevirapine.[123-125]

Overall, these data do not support a role for enhanced bioactivation or impaired detoxification of SMX in retroviral infection. Instead, these results raise the possibility that the increased risk of SMX hypersensitivity could be due to non-specific up-regulation of antigen processing and antigen presentation. This hypothesis would be consistent with the increased risk of hypersensitivity to other structurally unrelated drugs. Additional studies are needed to assess antigen processing and presentation for immunogenic and non-immunogenic drugs in the setting of HIV infection.

3.5 References

1. Gordin FM, Simon GL, Wofsy CB, et al. Adverse reactions to trimethoprim-sulfamethoxazole in patients with AIDS. *Ann Intern Med*. 1984;100:495-9.
2. Hennessy S, Strom BL, Berlin JA, et al. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. *J Gen Intern Med.* 1995;10:380-6.
3. Medina I, Mills J, Leoung G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. *N Engl J Med*. 1990;323:776-82.
4. Walmsley SL, Khorasheh S, Singer J, et al. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). *J Acquir Immune Defic Syndr Hum Retrovirol*. 1998;19:498-505.
5. Stewart RB, May FE, Cullen SI. Dermatologic adverse drug reactions in hospitalized patients. *Am J Hosp Pharm*. 1979;36:609-12.
6. Bigby M, Jick S, Jick H, Arndt K. Drug-induced cutaneous reactions. A report from the Boston Collaborative Drug Surveillance Program on 15,438 consecutive inpatients, 1975 to 1982. *JAMA*. 1986;256:3358-63.
7. Arndt KA, Jick H. Rates of cutaneous reactions to drugs. A report from the Boston Collaborative Drug Surveillance Program. *JAMA*. 1976;235:918-23
8. Cribb AE, Lee BL, Trepanier LA, et al. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. *Adverse Drug React Toxicol Rev*. 1996;15:9-50
9. Bell TL, Foster JN, Townsend ML. Trimethoprim-sulfamethoxazole-induced hepatotoxicity in a pediatric patient. *Pharmacotherapy*. 2010;30:539.
10. Ortiz J, Horn M, Peterson H. Toxic epidermal necrolysis-case report and review of the literature. *Ann Plast Surg*. 1982;9:249-53.
11. Saka B, Kombate K, Mouhari-Toure A, et al. Stevens-Johnson syndrome and toxic epidermal necrolysis in a teaching hospital in Lome, Togo: retrospective study of 89 cases. *Med Trop (Mars)*. 2010;70:255-8.
12. Roychowdhury S, Vyas PM, Svensson CK. Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. *Drug Metab Dispos*. 2007;35:676-81.
13. Cribb AE, Miller M, Leeder JS, et al. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione: implications for idiosyncratic toxicity. *Drug Metab Dispos*. 1991;19:900-6.
14. Reilly TP, Bellevue FH 3rd, Woster PM, et al. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. *Biochem Pharmacol*. 1998;55:803-10.
15. Cribb AE, Spielberg SP, Griffin GP. N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab Dispos.* 1995;23:406-14.

16. Sanderson JP, Naisbitt DJ, Farrell J, st al. Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J Immunol*. 2007;178:5533-42.
17. Naisbitt DJ, O'Neill PM, Pirmohamed M, et al. Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: implications for immune mediated toxicity. Bioorg. *Med Chem Lett*. 1996;6:1511-6.
18. Callan HE, Jenkins RE, Maggs JL, et al. Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation. *Chem Res Toxicol*. 2009;22:937-48.
19. Elsheikh A, Lavergne SN, Castrejon JL, et al. Drug antigenicity, immunogenicity, and costimulatory signaling: evidence for formation of a functional antigen through immune cell metabolism. *J Immunol*. 2010;185:6448-60.
20. Nuss CE, Grant DM, Spielberg SP, et al. Further investigations of the role of acetylation in sulphonamide hypersensitivity reactions. *Biomarkers*. 1996;1:267-72.
21. Mengelers MJ, Kleter GA, Hoogenboom LA, et al. The biotransformation of sulfadimethoxine, sulfadimidine, sulfamethoxazole, trimethoprim and aditoprim by primary cultures of pig hepatocytes. *J Vet Pharmacol Ther*. 1997;20:24-32.
22. Cribb AE, Nakamura H, Grant DM, et al. Role of polymorphic and monomorphic human arylamine N-acetyltransferases in determining sulfamethoxazole metabolism. *Biochem Pharmacol*. 1993;45:1277-82.
23. Kurian JR, Bajad SU, Miller JL, et al. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther*. 2004;311:1171-8.
24. Trepanier LA, Yoder AR, Bajad S, et al. Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J Acquir Immune Defic Syndr*. 2004;36:1041-50.
25. Fretland AJ, Leff MA, Doll MA, et al. Functional characterization of human N-acetyltransferase 2 (NAT2) single nucleotide polymorphisms. *Pharmacogenetics*. 2001;11:207-15.
26. Weber WW, Hein DW. N-acetylation pharmacogenetics. *Pharmacol Rev*. 1985;37:25-79.
27. Cascorbi I, Drakoulis N, Brockmöller J, et al. Arylamine N-acetyltransferase (NAT2) mutations and their allelic linkage in unrelated Caucasian individuals: correlation with phenotypic activity. *Am J Hum Genet*. 1995;57:581-92.
28. Grant DM, Hughes NC, Janezic SA, et al. Human acetyltransferase polymorphisms. *Mutat Res*. 1997;376:61-70.
29. Hein DW, Doll MA, Fretland AJ, et al. Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. *Cancer Epidemiol Biomarkers Prev*. 2000;9:29-42.
30. Carr A, Gross AS, Hoskins JM, et al. Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulphamethoxazole in HIV-infected patients. *AIDS*. 1994;8:333-7.

31. Kaufmann GR, Wenk M, Taeschner W, et al. N-aceyltransferase 2 polymorphism in patients infected with human immunodeficiency virus. *Clin Pharmacol Ther*. 1996;60:62-7.
32. Lee Bl, Wong D, Benowitz NL, et al. Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. *Clin Pharmacol Ther.* 1993;53:529-35.
33. Wolkenstein P, Loriot M-A, Aractingi S, et al. Prospective evaluation fo detoxification pathways as markers of cutaneous adverse reactions to sulphonamides in AIDS. *Pharmacogenetics.* 2000;10:821-8.
34. O'Neil W, MacArthur RD, Farrough MJ, et al. Acetylator phenotype and genotype in HIV-infected patietnts with and without sulfonamide hypersensitivity. *J Clin Pharmacol*. 2002;42:613-9.
35. Quirino T, Bonfanti P, Arcidiacono M, et al. Acetylator phenotype prevalance in HIV-infected patients without previous trimethoprim-sulfamethoxazole hypersensitivity. *Biomed Pharmacother*. 1999;53:286-7.
36. Eliaszewicz M, Flahault A, Roujeau J, et al. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. *J Am Acad Dermatol*. 2002;47:40-6.
37. Jones AE, Brown KC, Werner RE, et al. Variability in drug metabolizing enzyme activity in HIV-infected patients. *Eur J Clin Pharmacol*. 2010;66:475-85.
38. Naisbitt DJ, Vilar FJ, Stalford AC, et al. Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. *AIDS Res Hum Retroviruses*. 2000;16:1929-38.
39. Walmsley SL, Winn LM, Harrison ML, et al. Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: toxicological and pathological implications. *AIDS*. 1997;11:1689-97.
40. Morris D, Guerra C, Donohue C, et al. Unveiling the mechanisms for decreased glutathione in individuals with HIV infection. *Clin Dev Immunol*. 2012;2012:734125.
41. Degkwitz E, Walsch S, Dubberstein M, et al. Influence of L-ascorbate on the contents of cytochromes P-450 and b5 in several organs of guinea pigs. *Enzyme*. 1973;16(1):237-45.
42. Degkwitz E, Kim KS. Comparative studies on the influence of L-ascorbate, D-arabino-ascorbate and 5-oxo-D-gluconate on the amounts of cytochromes P-450 and b5 in liver microsomes of guinea pigs. *Hoppe Seylers Z Physiol Chem*. 1973;354:555-61.
43. Lehmann D, Holohan P, Blair D. Comparisons of oxidative metabolism and reductive capacity in sulfonamide-tolerant and -intolerant patients with human immunodeficiency virus. *J Clin Pharmacol*. 1996;36:1149-53.
44. van der Ven A, Vree T, van Ewijk-Beneken Kolmer EW, et al. Urinary recovery and kinetics of sulphamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulphamethoxazole. *Br J Clin Pharmacol*. 1995;39:621-5.
45. Lee B, Delahunty T, Safrin S. The hydroxylamine of sulfamethoxazole and adverse reactions in patients with acquired immunodeficiency syndrome. *Clin Pharmacol Ther*. 1994;56:184-9.

46. Sandler NG, Wand H, Roque A, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J Infect Dis*. 2011;203:780-90.
47. Aukrust P, Svardel A, Muller F, et al. Increased levels of oxidized glutathione in CD4+ lymphocytes associated with disturbed intracellular redox balance in HIV virus type 1 infection. *Blood*. 1995;86:258-67.
48. O'Neil WM, Drobitch RK, MacArthur RD, et al. Acetylator phenotype and genotype in patients infected with HIV: discordance between methods for phenotype determination and genotype. *Pharmacogenetics.* 2000;10:171-82.
49. Watanabe D, Uehira T, Yonemoto H, et al. Sustained high levels of serum interferon-gamma during HIV-1 infection: a specific trend different from other cytokines. *Viral Immunol*. 2010;23:619-25.
50. Schaefer TM, Fuller CL, Basu S, et al. Increased expression of interferon-inducible genes in macaque lung tissues during simian immunodeficiency virus infection. *Microbes Infect*. 2006;8:1839-50.
51. Zahoor MA, Xue G, Sato H, et al. HIV-1 Vpr induces interferon-stimulated genes in human monocyte-derived macrophages. *PLoS One*. 2014;9:e106418.
52. Cao YZ, Dieterich D, Thomas PA, et al. Identification and quantitation of HIV-1 in the liver of patients with AIDS. *AIDS*. 1992;6:65–70.
53. Housset C, Boucher O, Girard PM, et al. Immunohistochemical evidence for human immunodeficiency virus-1 infection of liver Kupffer cells. *Hum Pathol*. 1990;21:404–408.
54. Hufert FT, Schmitz J, Schreiber M, et al. Human Kupffer cells infected with HIV-1 in vivo. *J Acquir Immune Defic Syndr*. 1993;6:772–777.
55. Crane M, Iser D, Lewin SR. Human immunodeficiency virus infection and the liver. *World J Hepatol*. 2012;4:91-8.
56. Vlahakis SR, Villasis-Keever A, Gomez TS, et al. Human immunodeficiency virus-induced apoptosis of human hepatocytes via CXCR4. *J Infect Dis*. 2003;188:1455-60.
57. Tuyama AC, Hong F, Saiman Y, et al. Human immunodeficiency virus (HIV)-1 infects human hepatic stellate cells and promotes collagen I and monocyte chemoattractant protein-1 expression: implications for the pathogenesis of HIV/hepatitis C virus-induced liver fibrosis. *Hepatology*. 2010;52:612-22.
58. de Veer MJ, Holko M, Frevel M, et al. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol*. 2001;69:912-20.
59. Rusinova I, Forster S, Yu S, et al. Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res*. 2013;41 (Database issue):D1040-6.
60. Sacco JC, Trepanier LA. Cytochrome b5 and NADH cytochrome b5 reductase: genotype-phenotype correlations for hydroxylamine reduction. *Pharmacogenet Genomics*. 2010;20:26-37.
61. Trepanier LA, Ray K, Winand NJ, et al. Cytosolic arylamine *N*-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. *Biochem Pharmacol*. 1997;54:73-80.
62. Michael Pfaffl. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res*. 2001;29:e45.

63. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:RESEARCH0034.
64. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002;30:e36.
65. Gan L, von Moltke LL, Trepanier LA, et al. Role of NADPH-cytochrome P450 reductase and cytochrome-*b*5/NADH-*b*5 reductase in variability of CYP3A activity in human liver microsomes. *Drug Metab Dispos*. 2009;37:90-6.
66. Naisbitt DJ, Gordon SF, Pirmohamed M, et al. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br J Pharmacol*. 2001;133:295-305.
67. Roychowdhury S, Vyas PM, Reilly TP, et al. Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. *J Pharmacol Exp Ther*. 2005;314:43-52.
68. Reilly TP, Lash LH, Doll MA, et al. A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J Invest Dermatol*. 2000;114:1164-73.
69. Lavergne SN, Wang H, Callan HE, et al. "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. *J Pharmacol Exp Ther* 2009;331:372-381.
70. Sieben S, Baron JM, Blömeke B, et al. Multiple cytochrome P450-isoenzymes mRNA are expressed in dendritic cells. *Int Arch Allergy Immunol*. 1999;118:358-61.
71. Pichler WJ, Naisbitt DJ, Park BK. Immune pathomechanism of drug hypersensitivity reactions. *J Allergy Clin Immunol*. 2011;127:S74-81.
72. van der Ven AJ, Mantel MA, Vree TB, et al. Formation and elimination of sulphamethoxazole hydroxylamine after oral administration of sulphamethoxazole. *Br J Clin Pharmacol*. 1994;38:147-50.
73. Nasr N, Maddocks S, Turville SG, et al. HIV-1 infection of human macrophages directly induces viperin which inhibits viral production. *Blood*. 2012;120:778-88.
74. Anderson SL, Carton JM, Lou J, et al. Interferon-induced guanylate binding protein-1 (GBP-1) mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus. *Virology*. 1999;256:8-14.
75. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol*. 2014;14:36-49.
76. Perry AK, Chen G, Zheng D, et al. The host type I interferon response to viral and bacterial infections. *Cell Res*. 2005;15:407-22.
77. Satoh T, Kato H, Kumagai Y, et al. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A*. 2010;107:1512-7.
78. Wang MQ, Huang YL, Huang J, et al. RIG-I detects HIV-1 infection and mediates type I interferon response in human macrophages from patients with HIV-1-associated neurocognitive disorders. *Genet Mol Res*. 2015;14:13799-811.

79. Zhang D, Zhang DE. Interferon-stimulated gene 15 and the protein ISGylation system. *J Interferon Cytokine Res*. 2011;31:119-30.
80. Haas AL, Ahrens P, Bright PM, et al. Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. *J Biol Chem*. 1987;262:11315-23.
81. Dastur A. Beaudenon S. Kelley M. Krug RM. Huibregtse JM. Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. *J Biol Chem*. 2006;281(7):4334–8.
82. Versteeg GA, Hale BG, van Boheemen S, et al. Species-specific antagonism of host ISGylation by the influenza B virus NS1 protein. *J Virol*. 2010;84:5423-30.
83. Malakhov MP, Malakhova OA, Kim KI, et al. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J Biol Chem*. 2002;277:9976-81.
84. Künzi MS, Pitha PM. Role of interferon-stimulated gene ISG-15 in the interferon-omega-mediated inhibition of human immunodeficiency virus replication. *J Interferon Cytokine Res*. 1996;16:919-27.
85. Langevin C, van der Aa LM, Houel A, et al. Zebrafish ISG15 exerts a strong antiviral activity against RNA and DNA viruses and regulates the interferon response. *J Virol*. 2013;87:10025-36.
86. Okumura A, Pitha PM, Harty RN. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. *Proc Natl Acad Sci U S A*. 2008;105:3974-9.
87. Zhao C, Denison C, Huibregtse JM, et al. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc Natl Acad Sci U S A*. 2005;102:10200-5.
88. Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv Immunol*. 1989;44:93-151.
89. Arbuckle MR, McClain MT, Rubertone MV, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med*. 2003;349:1526-33.
90. Clutterbuck DJ, Watson J, De Ruiter A, et al. Presence of HIV infection in patients diagnosed with systemic lupus erythematosus. *Rheumatology (Oxford)*. 2000;39:1047-8.
91. Mody GM, Patel N, Budhoo A, et al. Concomitant systemic lupus erythematosus and HIV: case series and literature review. *Semin Arthritis Rheum*. 2014;44:186-94.
92. Han GM, Chen SL, Shen N, et al. Analysis of gene expression profiles in human systemic lupus erythematosus using oligonucleotide microarray. *Genes Immun*. 2003;4:177-86.
93. Obermoser G, Pascual V. The interferon-alpha signature of systemic lupus erythematosus. *Lupus*. 2010;19:1012-9.
94. Vasuri F, Capizzi E, Bellavista E, et al. Studies on immunoproteasome in human liver. Part I: absence in fetuses, presence in normal subjects, and increased levels in chronic active hepatitis and cirrhosis. *Biochem Biophys Res Commun*. 2010;397:301-6.
95. de Graaf N, van Helden MJ, Textoris-Taube K, et al. PA28 and the proteasome immunosubunits play a central and independent role in the production of MHC class I-binding peptides in vivo. *Eur J Immunol*. 2011;41:926-35.

96. Dick TP, Ruppert T, Groettrup M, et al. Coordinated dual cleavages induced by the proteasome regulator PA28 lead to dominant MHC ligands. *Cell*. 1996;86:253-62.
97. Vigneron N, Van den Eynde BJ. Proteasome subtypes and regulators in the processing of antigenic peptides presented by class I molecules of the major histocompatibility complex. *Biomolecules*. 2014;4:994-1025.
98. Basler M, Kirk CJ, Groettrup M. The immunoproteasome in antigen processing and other immunological functions. *Curr Opin Immunol*. 2013;25:74-80.
99. Wang J, Maldonado MA. The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Mol Immunol*. 2006;3:255-61.
100. Hulpke S, Tampé R. The MHC I loading complex: a multitasking machinery in adaptive immunity. *Trends Biochem Sci*. 2013;38:412-20.
101. Huggett J, Dheda K, Bustin S, et al. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun*. 2005;6:279-84.
102. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.
103. Pabingera S, Rödigerb S, Kriegnera A, et al. A survey of tools for the analysis of quantitative PCR (qPCR) data. *Biomol Detect Quantif*. 2014;1:23-33.
104. Avgeri SG, Matthaiou DK, Dimopoulos G, et al. Therapeutic options for Burkholderia cepacia infections beyond co-trimoxazole: a systematic review of the clinical evidence. *Int J Antimicrob Agents* 2009;33:394-404.
105. Drekonja DM, Johnson JR. Urinary tract infections. *Prim Care.* 2008;35:345-367, vii.
106. Korbila IP, Manta KG, Siempos, II, et al. Penicillins vs trimethoprim-based regimens for acute bacterial exacerbations of chronic bronchitis: meta-analysis of randomized controlled trials. *Can Fam Physician.* 2009;55:60-67.
107. Drekonja DM, Traynor LM, DeCarolis DD, et al. Treatment of non-life-threatening methicillin-resistant Staphylococcus aureus infections with alternative antimicrobial agents: a 2-year retrospective review. *Diagn Microbiol Infect Dis.* 2009;63:201-207.
108. Bonfanti P, Pusterla L, Parazzini F, et al. The effectiveness of desensitization versus rechallenge treatment in HIV-positive patients with previous hypersensitivity to TMP-SMX: a randomized multicentric study. C.I.S.A.I. Group. *Biomed Pharmacother.* 2000;54:45-9.
109. Carr A, Penny R, Cooper DA. Efficacy and safety of rechallenge with low-dose trimethoprim-sulphamethoxazole in previously hypersensitive HIV-infected patients. *AIDS.* 1993;7:65-71.
110. Das G, Bailey MJ, Wickham JE. Toxic epidermal necrolysis and trimethoprim. *Br Med J (Clin Res Ed).* 1988;296:1604-1605.
111. Nwokolo C, Byrne L, Misch KJ. Toxic epidermal necrolysis occurring during treatment with trimethoprim alone. *Br Med J (Clin Res Ed).* 1988;296:970.
112. Mauri-Hellweg, Bettens F, Mauri D, et al. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol.* 1995;155:462-472.

113. Damsten MC, de Vlieger JS, Niessen WM, et al. Trimethoprim: novel reactive intermediates and bioactivation pathways by cytochrome p450s. *Chem Res Toxicol.* 2008;21:2181-2187.
114. Wen X, Wang JS, Backman JT, et al. Trimethoprim and sulfamethoxazole are selective inhibitors of CYP2C8 and CYP2C9, respectively. *Drug Metab Dispos*. 2002;30:631-5.
115. Cribb AE, Spielberg SP. Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine. *Drug Metab Dispos*. 1990;18:784-7.
116. Choi J, Liu RM, Kundu RK, et al. Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. *J Biol Chem*. 2000;275:3693-8.
117. Schnyder B, Frutig K, Mauri-Hellweg D, et al. T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazol-induced skin reaction. *Clin Exp Allergy.* 1998;28:1412-1417.
118. Liu M, Li XL, Hassel BA. Proteasomes modulate conjugation to the ubiquitin-like protein, ISG15. *J Biol Chem*. 2003;278:1594-602.
119. Desai SD, Haas AL, Wood LM, et al. Elevated expression of ISG15 in tumor cells interferes with the ubiquitin/26S proteasome pathway. *Cancer Res*. Jan 15;66:921-8.
120. Le Bon A, Thompson C, Kamphuis E, et al. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J Immunol.* 2006;176:2074–8.
121. Kiefer K, Oropallo MA, Cancro MP, et al. Role of type I interferons in the activation of autoreactive B cells. *Immunol Cell Biol*. 2012;90:498–504.
122. Lauwerys BR, Ducreux J, Houssiau FA. Type I interferon blockade in systemic lupus erythematosus: where do we stand? *Rheumatology (Oxford)*. 2014;53:1369-76.
123. Nunn P, Kibuga D, Gathua S, et al. Cutaneous hypersensitivity reactions due to thiacetazone in HIV-1 seropositive patients treated for tuberculosis. *Lancet*. 1991;337:627-30.
124. Chintu C, Luo C, Bhat G, et al. Cutaneous hypersensitivity reactions due to thiacetazone in the treatment of tuberculosis in Zambian children infected with HIV-I. *Arch Dis Child.* 1993;68:665-8.
125. Yunihastuti E, Widhani A, Karjadi TH. Drug hypersensitivity in human immunodeficiency virus-infected patient: challenging diagnosis and management. *Asia Pac Allergy*. 2014;4:54-67.
126. Schnyder B, Mauri-Hellweg D, Zanni M, et al. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest*. 1997;100:136-41.

Figure 3.1: Sulfamethoxazole (SMX) biotransformation with generation of reactive metabolites that lead to hapten formation, immunogenicity, and clinical signs of delayed sulfoanmide hypersensitivity in immunocompetent and HIV-infected patients.

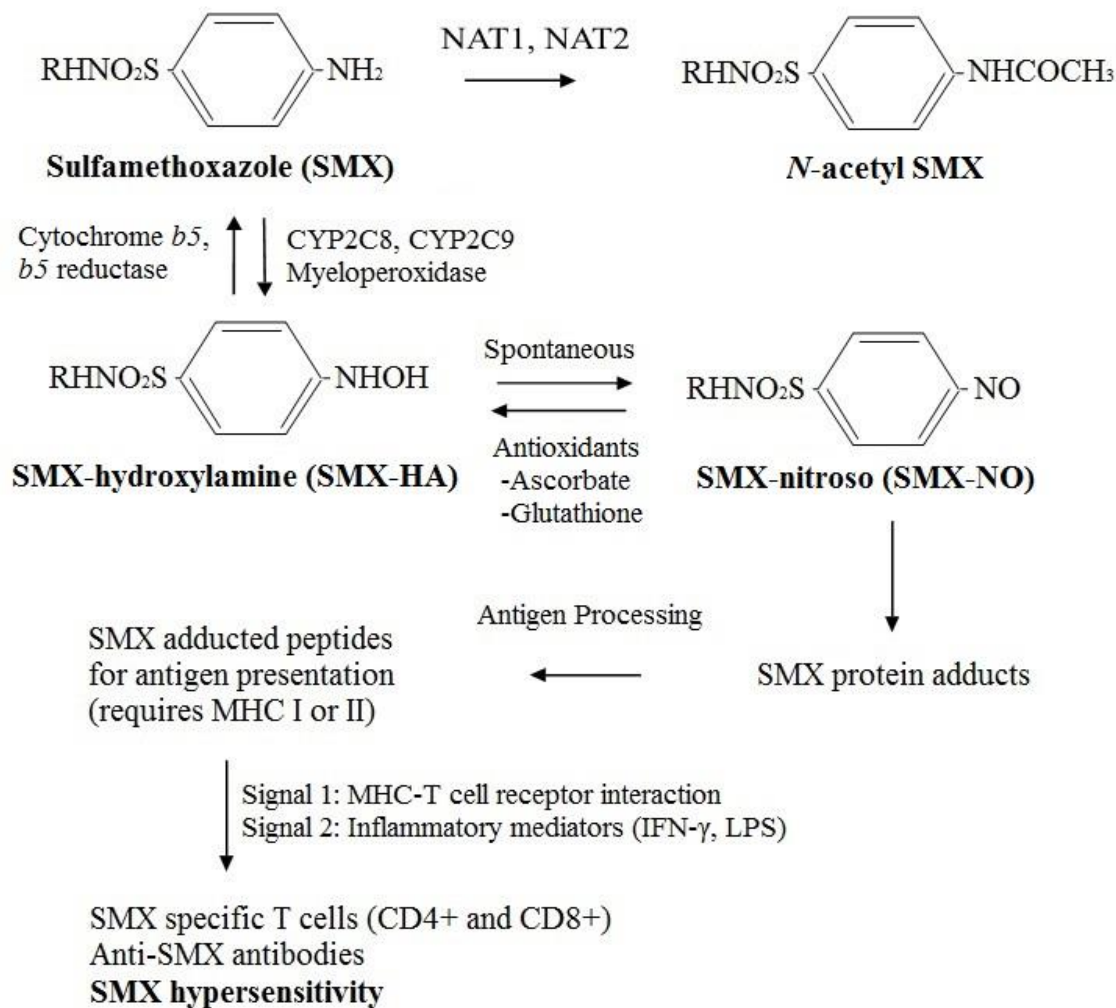


Table 3.1: Primers and gene-specific probes were designed using the Universal ProbeLibrary (Roche). Listed are the primer sequences, probe number, and amplification efficiency for each gene.

Genes	Primer Sequences	Probe Number	Amplification Efficiency
ACTB	Forward: aggtcatcaccattggcaac Reverse: cgtggatgccacaggact	122	1.98
GAPDH	Forward: tcagccgcattttctcttg Reverse: gcccaatacgaccaaatcc	60	1.98
HPRT1	Forward: tgaccttgatttattctgcatacc Reverse: cgagcaagacgttcagtcc	73	2.02
ISG15	Forward: cggcagttgagaggcagt Reverse: accttcaggtcccagctca	140	2.00
HERC5	Forward: tgggccacactgagagtaaa Reverse: tcaactttctgattgtctagtgcttc	12	1.92
HERC6	Forward: ccaaaagccatcattggaag Reverse: ccaatgtcatcagcatcatca	60	2.00
PSMB10	Forward: ggttccagccgaacatga Reverse: cccaggtcacccaagattc	135	1.91
PSME1	Forward: agaacctgctcgggagctat Reverse: attgctcaggttggcttcat	42	1.93
PSME2	Forward: gaaagtcctgtccctgcttg Reverse: aatcttggggatcaggtgct	129	2.08

Table 3.2: Genes that may affect the SMX biotransformation pathway in the microarray. None of the genes involved were differentially expressed in the microarray.

Pathway	Gene	Fold Change	P value
SMX oxidation	CYP2C8	1.0	0.94
	CYP2C9	-1.1	0.32
TMP oxidation	CYP2D6	-1.1	0.53
Detoxification	NAT2	1.2	0.05
	CYB5A	not in array	
	CYB5R3	-1.3	0.19
Glutathione synthesis and conjugation	GCL	1.0	0.88
	GSS	1.0	0.95
	GSTM1	1.1	0.74
	GSTP1	1.4	0.28
	GSTT1	not in array	

Figure 3.2: SMX-HA reduction activities between SIV-infected and non-infected control macaques were similar. Activities range from 0.38 to 0.88 nmol/min/mg with a median 0.54 nmol/min/mg in the SIV-infected group, and activities range from 0.36 to 0.96 nmol/min/mg with a median 0.43 nmol/min/mg in the control group.

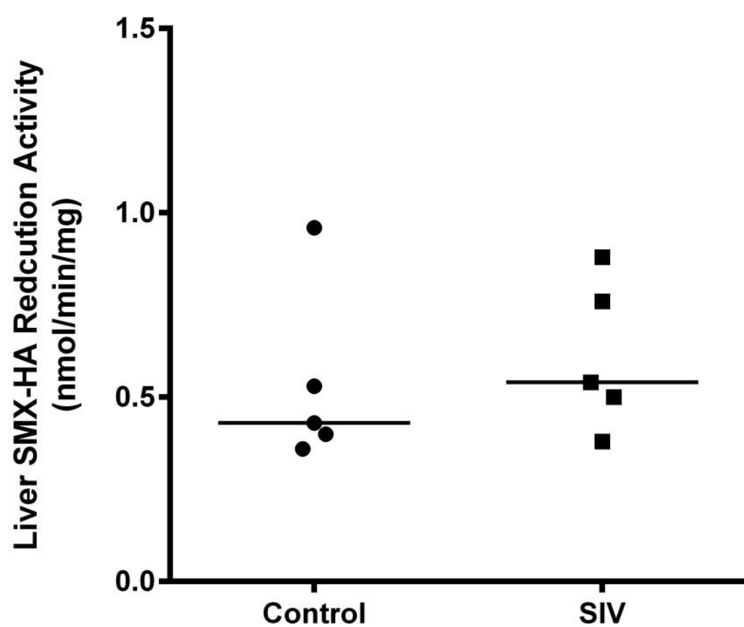


Figure 3.3: *N*-acetylaion activities between SIV-infected and non-infected control macaques were similar. Activities range from 0.08 to 0.20 nmol/min/mg with a median 0.11 nmol/min/mg in the SIV-infected group, and activities range from 0.03 to 0.18 nmol/min/mg with a median 0.09 nmol/min/mg in the control group.

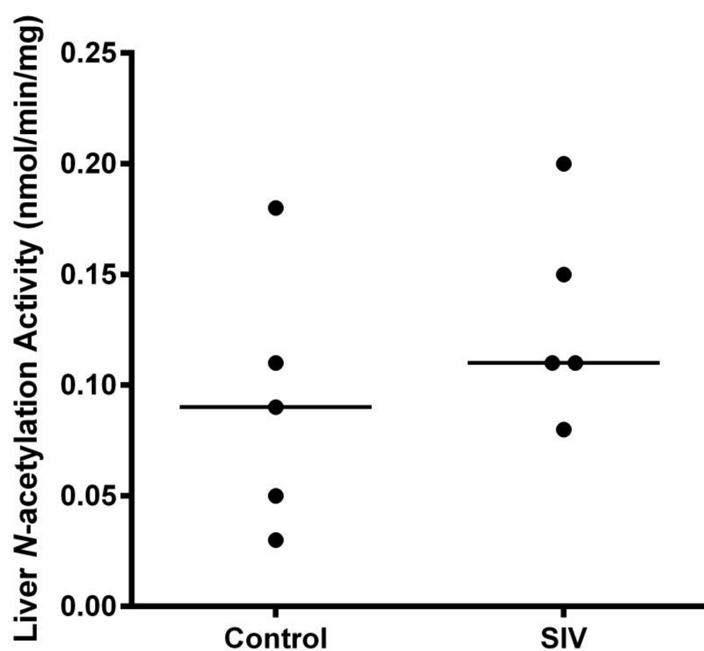


Table 3.3: 59 genes had a two-fold or higher expression ($P \leq 0.005$) in the microarray. 33 of these genes were IFN-inducible.

Gene Symbol	Fold Change	P value	IFN-Inducible (Type I, Type II, or both)
ACE2	-2.96	0.003	Type I
ADAR	2.16	0.001	
ALPL	2.83	0.001	
AOAH	2.08	0.001	
B2M	3.11	0.002	
BST2	2.38	<0.001	Both
CCDC69	2.03	0.004	
CMPK2	4.23	<0.001	Type I
DDX58	5.03	<0.001	Both
DDX60	6.06	<0.001	Both
DDX60L	2.26	0.001	Type I
DHX58	3.06	<0.001	Type I
DNHD1	2.55	0.001	
FAM162A|DTX3L	2.29	<0.001	
FAM205B|CCL21	2.31	<0.001	
GBP1	3.67	<0.001	Both
HEPACAM2	2.39	<0.001	
HERC5	5.80	<0.001	Type I
HERC6	9.97	<0.001	Type I
HIPK2|PARP12	2.13	0.003	
HIST1H2AC	2.00	0.002	
IFI27	2.34	0.003	Both
IFI44	6.68	<0.001	Both
IFI44L	7.78	<0.001	Both
IFI6	12.25	<0.001	Both
IFIH1	3.59	<0.001	Both
IFIT1	4.78	<0.001	Both
IFIT2	3.27	0.004	Both
IFIT3	5.38	0.001	Both

Table 3.3 (continue): 59 genes had a two-fold or higher expression ($P \leq 0.005$) in the microarray. 33 of these genes were IFN-inducible.

Gene Symbol	Fold Change	P value	IFN-Inducible (Type I, Type II, or both)
ISG15	3.64	<0.001	Both
LHFP	-2.37	0.001	
LOC699022	-2.17	<0.001	
LOC700208	4.28	<0.001	
LOC705561	3.38	<0.001	Both
LOC719747	2.16	0.002	
MAMU-A	2.04	<0.001	
MAMU-DQA1	2.82	0.001	Type II
MAMU-E	2.30	0.002	
MAPK6	2.34	0.005	
MX1	14.21	<0.001	Both
MX2	5.04	0.001	Both
OAS2	10.08	0.001	Both
PARP14	3.37	<0.001	Both
PARP15	2.66	0.001	
PARP9	2.57	<0.001	Both
PIK3AP1	2.20	0.005	
PLAC8	2.97	<0.001	
PM20D1	-2.73	0.005	
RNF213	2.73	0.001	
RSAD2	2.54	<0.001	Both
SECTM1	2.08	0.005	Both
SLC26A11	2.91	0.001	
STAT1	5.21	<0.001	Both
TAP1	2.00	0.001	Both
TRIM14	3.64	0.001	Type I
UBQLNL	3.82	0.002	
UNC45B	3.57	0.001	
USP18	3.51	0.001	Both
XAF1	3.09	<0.001	Both

Table 3.4: Genes up-regulated in RIG-I-like receptor signaling, ISGylation, SLE development, immunoproteasomal degradation, and antigen processing and presentation in the microarray.

Function	Pathway	Gene	Fold Change	P value
Anti-viral	RIG-I-like receptor signaling	DDX58	5.0	< 0.001
		DHX58	3.1	< 0.001
		IFIH1	3.6	< 0.001
	ISGylation	UBQLNL	3.8	0.002
		ISG15	3.6	< 0.001
		HERC5	5.8	< 0.001
		HERC6	10.0	< 0.001
		USP18	3.5	< 0.001
Autoimmune	Systemic lupus erythematosus (SLE)	FCGR3	1.9	0.001
		C1QA	1.7	0.002
		C1QC	1.8	< 0.001
		HIST1H2AC	2.0	0.002
		HIST1H3D	1.9	< 0.001
		MAMU-DQA1	2.8	0.001
Antigen processing	Immunoproteasomal degradation	PSMB10	1.3	0.002
		PSME1	1.5	0.001
		PSME2	1.5	0.003
Antigen presentation	Peptide transporter	TAP1	2.0	< 0.001
	MHC-I	MAMU-A	2.0	< 0.001
		MAMU-E	2.3	0.002
		MAMU-F	1.7	0.003
		B2M	3.1	0.002

Table 3.5: qPCR confirmation of transcript expression from expression array. All of the genes were confirmed to be up-regulated in the SIV-infected macaque livers compared to controls, with $P \leq 0.001$.

Gene	Fold Change	95% Confidence Interval
ISG15	30.8	6.4-163.2
HERC5	17.6	4.6-94.8
HERC6	91.9	7.8-947.6
PSMB10	3.5	1.3-7.6
PSME1	4.6	1.4-16
PSME2	3.2	0.9-13.1

Chapter 4 – Effects of SIV Infection on SMX Biotransformation Pathways in CD8+ Lymphocytes

4.1 Introduction

An increased risk of sulfamethoxazole (SMX) hypersensitivity is observed in HIV-infected patients.[1-4] 20-57% HIV-infected patients suffer from SMX hypersensitivity while it only affects 3% of the general population.[1-7] Skin rash occurs in most affected patients, and liver toxicity, thrombocytopenia, and bullous skin eruptions, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, are observed in the more severely affected patients.[8-11] The risk factors associated with an unusually high incidence of sulfonamide hypersensitivity are still not clear, but the increased risk is thought to be due to metabolic alternations in HIV infection.

The development of SMX hypersensitivity depends on SMX bioactivation.[12-14] As shown in **Figure 4.1**, SMX is oxidized to form SMX-hydroxylamine (SMX-HA) by CYP2C8 and CYP2C9 in the liver.[15, 16] This hydroxylamine metabolite can be further oxidized spontaneously to form SMX-nitroso (SMX-NO).[16, 17] The nitroso metabolite can bind to protein and form protein adduct,[18] which can be processed and presented in association with MHC-I or MHC-II. This stimulates the activation of T cells and B cells to target SMX-protein adducts,[16, 19] which may lead to the development of hypersensitivity.

The formation of protein adducts can be prevented by several detoxification pathways. *N*-acetylation of SMX leads to an inactive and non-immunogenic acetylated metabolite (*N*-acetyl SMX) for urinary excretion.[20-22] However, associations between SIV infection and urinary *N*-acetyl SMX or hepatic gene expression of *N*-acetyltransferase 2 (*NAT2*) were not observed in our rhesus macaque model (**Chapter 2** and **Chapter 3**). If SMX is oxidized to SMX-HA, it can be reduced to the parent drug by cytochrome b_5 and NADH cytochrome b_5 reductase,[23] but differences in hepatic gene expression and activities for this pathway between SIV-infected macaques and non-infected controls were not seen (**Chapter 3**). If SMX-HA is further oxidized to form SMX-NO, it can be reduced to the hydroxylamine metabolite by antioxidants, such as ascorbate, and glutathione (GSH).[13, 24] However, both plasma ascorbate and erythrocyte GSH level were not different between SIV-infected macaques and non-infected controls (**Chapter 3**). Although hepatic gene expression and activities showed no differences in SMX biotransformation between the two groups of macaques, metabolic alterations could still occur in dendritic cells, keratinocytes and lymphocytes, and lead to acquired risk of drug hypersensitivity.[12, 19, 25-27]

Development of sulfonamide hypersensitivity has been associated with a surrogate marker, the lymphocyte toxicity assay (LTA). Results of this *in vitro* cytotoxicity assay showed that peripheral blood mononuclear cells (PBMC) from SMX-hypersensitive patients are more susceptible to apoptosis in the presence of sulfonamide oxidative metabolites, when compared to cells from sulfonamide-tolerant patients.[28-30] SMX-HA is often used in the assay, but it oxidizes

spontaneously to SMX-NO, which is responsible for the cytotoxicity *in vitro*.[30, 31] Addition of ascorbate or GSH, which reduce SMX-NO, decrease the cytotoxicity.[32] Differences in cytotoxicity were not seen with SMX, or with control compounds that generate reactive oxygen species (menadione).[33] These findings, which have been replicated by multiple groups, have been interpreted as a "detoxification defect" in hypersensitive, but not tolerant, patients.[33, 34]

In addition, this apparent defect can be demonstrated in family members of hypersensitive patients who have never been administered potentiated sulfonamides, with either normal, intermediate, or abnormal cytotoxicity profiles,[33-35] which suggests a heritable defect. However, it is not clear whether this marker is an epiphenomenon or is directly related to the pathogenesis of systemic hypersensitivity.

Another study was done to determine the cell type(s) among PBMC that is/are responsible for the enhanced susceptibility to SMX-HA. PBMC were isolated into different cell populations, and the enhanced SMX metabolite susceptibility was seen only when the cell populations included CD8+ T cells, while CD4+ T cells and B cells alone were minimally affected.[36] This suggests that CD8+ T cells may be either more susceptible to SMX-HA or they are responsible for causing cell death to other cell types *in vitro*.

The LTA has also been studied in Jurkat cells and MOLT3 cells (both immortalized line of human T lymphocytes) experimentally infected with HIV, and these cells also showed increased cytotoxicity compared to non-infected cells in the presence of sulfonamide metabolites, while parent SMX was no more toxic than vehicle.[37, 38] This susceptibility to sulfonamide metabolites can be replicated by transfecting lymphoid cells with the HIV-transactivator protein, Tat,[39] which alters a large number of host cellular genes in HIV infection.[40-42] Specifically, transfection of Jurkat cells with Tat leads to enhanced cytotoxicity in the presence of SMX metabolites, but not the parent SMX, that parallels that of HIV-infected cells.[39] Furthermore, cells from HIV-infected patients that developed SMX hypersensitivity show significantly greater cytotoxicity from SMX metabolites than HIV-infected tolerant patients,[28, 35, 43] which suggests that the cytotoxicity phenotype may be useful to understand individual risk, even among patients with HIV infection. However, the mechanisms for this predictive phenotype are not well understood, and the assay is time consuming.

We hypothesized that the increased cytotoxicity seen in HIV infection, and in hypersensitive individuals in particular, may be due to acquired alterations in SMX biotransformation or apoptotic pathways. We tested this hypothesis in an SIV model of HIV infection in rhesus macaques. The specific objective of this study was to determine whether retroviral infection altered expression pathways in CD8+ T cells that would explain the susceptibility to SMX metabolite cytotoxicity *in vitro*.

4.2 Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich, except SMX-HA, which was purchased from Dalton Chemicals, and *N*-acetyl SMX, which was obtained from Frinton Laboratories.

Animals

Male rhesus macaques (*Macaca mulatta*) chronically infected with SIVmac239, along with age- and sex-matched non-infected controls, were selected from the Wisconsin National Primate Research Center. Animals were studied after reaching set-point viremia, at least 10 weeks after inoculation, and no animals had a history of prior TMP/SMX exposure. All macaques were evaluated with a physical exam, complete blood count, and serum biochemical panel prior to sample collection and TMP/SMX exposure. Infected animals also had CD4+ counts and viral load performed. Macaques were fed a fixed formula global primate diet (Teklad, Harlan Laboratories). The Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison approved all procedures.

Samples collection and PBMC isolation

Blood samples were obtained prior to dosing macaques with TMP/SMX at 120 mg/kg/day per os for 14 days for a related study on SMX immunogenicity in SIV infection **(Chapter 2**). Macaques

were sedated with ketamine and dexmedetomidine for whole blood collection in heparinized tubes. Whole blood collected was diluted with an equal volume of 37°C HBSS buffer, then layered on Lymphocyte Separation Media (Lonza), and centrifuged at 400 × *g* for 30 min at room temperature. The interface containing PBMC was washed and resuspended with 37°C PBS with 10% heat-inactivated FBS. Red blood cells were lysed by addition of ACK lysing buffer (Lonza). Isolated PBMC were immediately processed on the same day for the LTA and for CD8+ cell isolation.

<u>Lymphocyte toxicity assay (LTA)</u>

PBMC were resuspended in HBSS media with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 15 mM HEPES at a density of 80,000 cells/well. Cells were plated in quadruplicate with 1000 μM SMX, 25-800 μM SMX-HA, or vehicle (1% DMSO) control in flat-bottom, 96-well plates for two hours at 37°C, 5% CO_2. SMX and SMX-HA were dissolved in 1% DMSO final well concentration. After incubation, cells were washed three times with PBS with 10% heat-inactivated FBS by centrifugation. Cells were then resuspended in HBSS media with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 15 mM HEPES at 37°C, 5% CO_2 for 19 hours. Dead cells were stained with YO-PRO-1 Iodide dye,[27, 44] and measured by a fluorescence reader with excitation at 485 nm and emission at 530 nm. To normalize cell death with the total cell number in each well at the end of the assay, all cells were then permeabilized with 2% Triton-X for second (total cell) florescence reading. Percent cell death was calculated

by dividing the fluorescence of dead cells after drug exposure by the fluorescence of all cells after Triton-X permeabilization.

CD8+ cell isolation

CD8+ cells were isolated from fresh PBMC using the mini magnetic-activated cell separation system, MiniMACS (Miltenyi Biotec Inc), according to manufacturer's protocol. Briefly, CD8+ cells were isolated through positive selection using antibodies targeting CD8 surface receptors and conjugated to magnetic beads. The labeled cells were bound in separation column under a magnetic field, and elution of the separation column in the absence of the magnetic field recovered a purified CD8+ cell population. The purity of isolated cells was confirmed by flow cytometry on an LSR II analytical flow cytometer (BD Biosciences). Isolated CD8+ cells were put in 200 µl RNAlater for 24 hours at 4°C and stored at -80°C until RNA extraction.

RNA extraction

Total RNA was extracted by lysing CD8+ cells with TRIzol (Ambion) according to the manufacturer's protocol with slight modifications. Briefly, CD8+ cells stored in RNAlater were thawed and 200 ul ice-cold PBS was added, followed by centrifugation at 3,000 × *g*. The supernatant was removed, and 1 ml TRIzol was added. Cells were lysed by pipetting up and down for 5 times, followed by addition of 200 µg glycogen to help precipitating RNA. 200 µl chloroform was added and vortexed, followed by centrifugation at 16,000 × *g* for 5 min at 4°C.

The aqueous phase at the top was added to 500 μl ice-cold isopropanol in a new tube, and precipitated at -20℃ for 1 hour. RNA was pelleted by centrifuging at 16,000 × *g* for 15 min at 4℃. The supernatant was removed, and RNA pellet was washed with 75% ethanol. Ethanol was removed by centrifuging at 16,000 × *g* for 1 min with the lid open. The ethanol-free RNA pellet was resolubilized in RNase free water (Ambion). RNA samples were treated with 2 U DNase I (Ambion) for 30 min at 37℃ according to the manufacturer's protocol to remove DNA contamination. DNase I was inactivated by adding EDTA to a final concentration of 5 mM, followed by heating at 75℃ for 10 min. RNase inhibitor (Applied Biosystems) was added to a final concentration of 1 U/μl. RNA integrity was accessed by Agilent 2100 BioAnalyzer, and quantified by NanoDrop ND-1000 (ThermoFisher). RNA was stored at -80℃ until preparation for arrays.

Microarray processing

High quality RNA was selected for expression array analyses from 3 SIV-infected macaques with high cytotoxicity from 800 μM SMX-HA (> 22%) and 3 control macaques with low cytotoxicity (< 2%). For each animal, 10 ng of total RNA was used to generate sense strand cDNA with the GeneChip WT Pico Reagent Kit (Affmetrix), followed by fragmentation and labeling with biotin. The fragmented and labeled cDNA was hybridized with the GeneChip Rhesus Gene 1.0 ST Array (Affymetrix) using the Hybridization Oven 640 (Affymetrix). The microarray chip was washed and stained using the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) with the automated GeneChip Fluidics Station 450 (Affymetrix). The stained

microarray chip was scanned by the GeneChip Scanner 3000 7G (Affymetrix). Both fluidics station and scanner were controlled by the Affymetrix GeneChip Command Console Software v4.0.0.1567G.

Microarray data analysis

Raw data exported from the Affymetrix platform were analyzed by GeneSpring GX (Agilent). Data were normalized by Robust Multi-chip Average (RMA) method. Gene expression was compared between groups (SIV-infected/ high cytotoxicity and non-infected/low cytotoxicity) using a moderated t-test,[45] Microarray probe sets that had signal intensity lower than 100 in both SIV and control groups were excluded. Initially, genes that had differential expression at the level of $P \leq 0.005$ were exported to DAVID Bioinformatics Resources 6.7 (david.ncifcrf.gov) for pathway analysis using the KEGG PATHWAY Database. Due to the small number of genes that were significant at this threshold, $P \leq 0.010$ was used instead to select genes to be exported for pathway analysis. Interferon-inducible genes were identified using INTERFEROME v2.01.[46]

Statistical analyses

Unless described otherwise, outcomes were compared between groups (SIV-positive versus negative) with Mann Whitney tests, using commercial software (Prism 6.0, Graphpad Software, Inc.), with $P < 0.05$. Data are reported as medians and observed ranges.

4.3 Results

Lymphocyte toxicity assay (LTA)

Cytotoxicity in the presence of 800 μM SMX-HA was used for all statistical analyses. The background PBMC cytotoxicity (from media with drug vehicle) was higher in the SIV-infected macaques compared to the non-infected controls (SIV-infected macaques, median 39.3%, range 23.3-48.8; control macaques, median 26.6 %, range 14.1-37.3, $P = 0.01$; **Figure 4.2A**). Drug cytotoxicity was calculated by subtracting the % cytotoxicity of vehicle from that seen with SMX or SMX-HA.

Increased drug cytotoxicity was observed in PBMC incubated with SMX-HA (800 μM) when compared to SMX in the SIV-infected macaques (SMX-HA cytotoxicity, median 29.8%, range 0.0-44.3%; SMX cytotoxicity, median 0.6 %, range 0.0-4.8; $P = 0.005$). However, this differential metabolite susceptibility was not seen in the control macaques (SMX-HA cytotoxicity, median 1.8%, range 0.0-24.0; SMX cytotoxicity, median 3.8 %, range 0.0-28.9; $P = 0.50$; **Figure 4.2B**).

Overall, SMX-HA cytotoxicity was apparently higher in the SIV-infected macaques comparing to the control group, but this did not reach statistical significance due to individual variability

(SIV group, median 29.8%, range 0.0-44.3; control macaques, median 1.8 %, range 0.0-24.0; *P* = 0.06).

All macaques were dosed with TMP/SMX for 14 days for the immunogenicity study (**Chapter 2**). Therefore, cytotoxicity data were also analyzed with animals grouped depending on whether they developed a detectable immune response after dosing (either drug-responsive T cells or anti-SMX antibodies; **Chapter 2**) The background vehicle cytotoxicity was not different between macaques that had any immunologic response (n = 6) and those without (n = 8) (median 32.7%, range 14.1-43.3, and 34.2 %, range 18.6-48.8, respectively; *P* = 0.63; **Figure 4.3A**).

Interestingly, increased SMX-HA cytotoxicity was observed when compared to SMX in macaques *without* immunologic responses (SMX-HA cytotoxicity, median 14.2%, range 0.0-44.3; SMX cytotoxicity, median 0.7 %, range 0.0-14.1; *P* = 0.001; **Figure 4.3B**), but metabolite cytotoxicity was not statistically different from SMX in macaques *with* immunologic responses (SMX-HA cytotoxicity, median 12.1%, range 0.0-42.4; SMX cytotoxicity, median 2.7 %, range 0.0-28.9; *P* = 0.25).

Further, when macaques were categorized by immune response rather than SIV status, there was no difference in SMX-HA cytotoxicity between groups (negative immune macaques, median 14.2 %, range 0.0-44.3; positive immune group, median 12.1%, range 0.0-42.4; *P* = 0.36).

Microarray analysis – SMX biotransformation and apoptotic pathways

Three SIV-infected macaques with high SMX-HA cytotoxicity ($>$ 22% for 800 μM SMX-HA) and three control macaques with low SMX-HA cytotoxicity ($<$ 2% for 800 μM SMX-HA) were selected for expression array analyses in CD8+ cells.

Gene expression of SMX biotransformation pathways is shown in **Table 4.1**. Genes involve in SMX oxidation (*CYP2C8* and *CYP2C9*) were not differentially expressed in CD8+ cells between the SIV-infected high cytotoxicity and non-infected low cytotoxicity group. Genes involved in SMX detoxification, including *NAT1* and *CYB5R3*, were also not differentially expressed, and *CYB5A* was not included in the microarray. Genes involved in GSH synthesis and conjugation, including *GSS*, *GCL*, *GSTM1* and *GSTP1*, were not differentially expressed; *GSTT1* was not included in the microarray. However, *IFI27* (*ISG12*), was up-regulated 15.8 fold in the SIV-infected high cytotoxicity group (**Table 4.2**). This gene product has been shown to sensitize cells to apoptotic stimuli through mitochondrial membrane destabilization in HT1080 cells (fibrosarcoma cell line).[47, 48]

Microarray analysis – other genes

Transcripts detected by 170 microarray probe sets were differentially expressed between the high cytotoxicity/SIV-infected macaques and the low cytotoxicity/non-infected macaques at $P \leq 0.01$. Of these, 142 were identified to be associated with a known gene, and 22 showed a two-fold or greater difference in expression. Among the 22 genes, seven were down-regulated and 15 were up-regulated (**Table 4.2**). Twelve of these 22 genes were interferon (IFN)-inducible, with 1 induced only by type I IFN, and 11 by either type I or type II IFN.

Pathway analysis

Pathway analyses were performed with the 142 identified differentially expressed genes, without requirement for a 2-fold change. Analyses revealed two pathways that were altered: RIG-I-like receptor signaling and lysosomal degradation (**Table 4.3**).

4.4 Discussion

In vitro cytotoxicity from SMX metabolites toward PBMC has been used as a prospective and retrospective marker of sulfonamide hypersensitivity in both HIV-infected and immunocompetent patients.[28-33] Further, cell death in the LTA is dependent on the presence of CD8+ T cells in the PBMC.[36] However, the mechanism for this enhanced cytotoxicity in SMX-hypersensitive patients is still unclear. In this study, we examined the LTA in SIV-infected macaques and non-infected controls prior to 14 days of TMP/SMX dosing, a protocol that led to immune responses to SMX in some macaques (**Chapter 2**).

As shown previously in HIV-infected and Tat-transfected lymphoid cells,[39] the background cytotoxicity from drug vehicle (1% DMSO in 100 µl media) was higher in PBMC from the SIV-infected macaques when compared to non-infected controls. DMSO (> 2% final concentration) has been shown to induce apoptosis in EL-4 lymphoma cells (T lymphocyte cell line),[49] and Burkitt's lymphoma cells (B lymphocyte cell line).[50] These observations suggest that retroviral infection may lower the threshold for apoptosis in PBMC.

Beyond vehicle cytotoxicity, net cytotoxicity from SMX-HA incubation (800 µM) was higher than SMX alone in the SIV-infected macaques, but not in the control group. Further, there was a trend toward higher cytotoxicity of SMX-HA in SIV-infected PBMC compared to non-infected PBMC. More samples may be required to show statistical significance because of a wide range in cytotoxicity observed in SIV-infected macaques.

These observations are similar to those reported in PBMC from HIV-infected patients by other groups,[28, 35, 43] suggesting a possible acquired alternation in SMX-HA detoxification in PBMC during retroviral infection. It is possible that ascorbate and GSH deficiencies seen in HIV-infected patients,[13, 24] lead to increased SMX-HA oxidation to SMX-NO, which enhances cytotoxicity.[32] Future experiments in retroviral-infected PBMC with the addition of ascorbate

and GSH during SMX-HA exposure could suggest whether redox stress caused by intracellular ascorbate or GSH deficiencies contributes to enhanced cytotoxicity.

The phenotype of increased *in vitro* SMX-HA cytotoxicity has been associated with subsequent SMX hypersensitivity in HIV-infected patients.[28-30, 35, 43] Therefore, we also categorized macaques by whether they had a detectable drug-specific immune response following two weeks of oral treatment with TMP/SMX at therapeutic dosages, and compared *in vitro* cytotoxicity results between groups.

As expected, background vehicle cytotoxicity was not different between macaques that had a subsequent detectable immune response to TMP/SMX, compared to those that did not. In addition, as found in human patients (both immunocompetent and HIV-positive, SMX-hypersensitive or not),[28-33] SMX-HA was more cytotoxic than SMX. However, in this model, SMX-HA cytotoxicity did not distinguish between macaques that later developed immunologic responses to TMP/SMX and those that did not. Since only two macaques developed a skin rash during dosing (and this could not be confirmed to be caused by TMP/SMX), we cannot draw conclusions about the use of *in vitro* drug cytotoxicity to predict *in vivo* drug hypersensitivity in this model.

We next determined whether SIV-infected PBMC with high *in vitro* SMX-HA cytotoxicity showed differences in expression of SMX biotransformation or apoptotic pathways. We focused on CD8+ cells since a previous study showed that human CD8+ cells were much more susceptible to SMX-HA cytotoxicity than CD4+ T cells or B cells.[36] As found in the macaque liver (**Chapter 3**), SMX biotransformation pathways were not differentially expressed in CD8+ cells in SIV infection, including *CYP2C8*, *CYP2C9*, *CYB5R3*, *NAT1* (the latter present in leukocytes but not in liver),[58] and genes involved in GSH synthesis and conjugation (*GCL*, *GSS*, *GSTM1*, *GSTP1*, and *GSTT1*). This suggests that increased lymphocyte cytotoxicity from SMX-HA seen in retroviral infection is not due to altered gene expression of SMX biotransformation pathways.

Although SIV infection did not appear to affect SMX biotransformation in CD8+ lymphocytes, other pathways were of course affected, particularly those induced by IFN. Pathway analysis of our array data showed that SIV infection up-regulated anti-viral pathways of RIG-I-like receptor signaling, including *DDX58* (RIG-1), *IFIH1* (MDA5), *IRF7*, and *ISG15* that encode proteins that sense viral infection and lead to the release of type I IFN.[52-56] The up-regulation of this pathway during SIV infection could be expected due to its anti-viral properties.

In addition, *IFI27* (ISG12), an IFN-inducible gene, has been shown to enhance apoptosis in HT1080 cells,[47, 48] and this could further explain the enhanced vehicle and SMX-HA cytotoxicity in retroviral infection. Other than *IFI27*, multiple IFN-inducible genes have been shown to

enhance apoptosis in PBMC,[57] and could potentiate apoptotic effect of retroviral infection.[58] Therefore, it is possible that the up-regulated IFN-inducible genes could lower the threshold for apoptosis when lymphocytes are exposed to reactive drug metabolites such as SMX-HA.

Another pathway that was altered in SIV infection was lysosomal degradation, included *ABCA2* (-1.3 fold), *GALC* (-1.5 fold), and *PPT1* (1.3 fold). Deficiencies in ABCA2, GALC, or PPT1 activity have been associated with lysosome malfunction, and can lead to enhanced cell death in macrophages, myelinating cells, and neurons respectively.[59-63] Lysosomal degradation activities have not been evaluated specifically in lymphocytes during retroviral infection, but deserve further study.

Overall, these data do not support an association between increased lymphocyte cytotoxicity from SMX-HA in SIV infection and altered SMX biotransformation pathways. However, as expected, SIV infection up-regulated multiple IFN-inducible genes. Specifically, *IFI27* up-regulation may contribute to the enhanced SMX-HA susceptibility. Further investigation in *IFI27* and other IFN-inducible genes may provide new insights for enhanced PBMC susceptibility to SMX-HA, and the acquired risk in drug hypersensitivity during retroviral infection.

4.5 References

1. Gordin FM, Simon GL, Wofsy CB, et al. Adverse reactions to trimethoprim-sulfamethoxazole in patients with AIDS. *Ann Intern Med*. 1984;100:495-9.
2. Hennessy S, Strom BL, Berlin JA, et al. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. *J Gen Intern Med.* 1995;10:380-6.
3. Medina I, Mills J, Leoung G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. *N Engl J Med*. 1990;323:776-82.
4. Walmsley SL, Khorasheh S, Singer J, et al. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). *J Acquir Immune Defic Syndr Hum Retrovirol*. 1998;19:498-505.
5. Stewart RB, May FE, Cullen SI. Dermatologic adverse drug reactions in hospitalized patients. *Am J Hosp Pharm*. 1979;36:609-12.
6. Bigby M, Jick S, Jick H, Arndt K. Drug-induced cutaneous reactions. A report from the Boston Collaborative Drug Surveillance Program on 15,438 consecutive inpatients, 1975 to 1982. *JAMA*. 1986;256:3358-63.
7. Arndt KA, Jick H. Rates of cutaneous reactions to drugs. A report from the Boston Collaborative Drug Surveillance Program. *JAMA*. 1976;235:918-23
8. Cribb AE, Lee BL, Trepanier LA, et al. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. *Adverse Drug React Toxicol Rev*. 1996;15:9-50
9. Bell TL, Foster JN, Townsend ML. Trimethoprim-sulfamethoxazole-induced hepatotoxicity in a pediatric patient. *Pharmacotherapy*. 2010;30:539.
10. Ortiz J, Horn M, Peterson H. Toxic epidermal necrolysis-case report and review of the literature. *Ann Plast Surg*. 1982;9:249-53.
11. Saka B, Kombate K, Mouhari-Toure A, et al. Stevens-Johnson syndrome and toxic epidermal necrolysis in a teaching hospital in Lome, Togo: retrospective study of 89 cases. *Med Trop (Mars)*. 2010;70:255-8.
12. Roychowdhury S, Vyas PM, Svensson CK. Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. *Drug Metab Dispos*. 2007;35:676-81.
13. Cribb AE, Miller M, Leeder JS, et al. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione: implications for idiosyncratic toxicity. *Drug Metab Dispos*. 1991;19:900-6.
14. Reilly TP, Bellevue FH 3rd, Woster PM, et al. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. *Biochem Pharmacol*. 1998;55:803-10.
15. Cribb AE, Spielberg SP, Griffin GP. N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab Dispos.* 1995;23:406-14.

16. Sanderson JP, Naisbitt DJ, Farrell J, st al. Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J Immunol*. 2007;178:5533-42.
17. Naisbitt DJ, O'Neill PM, Pirmohamed M, et al. Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: implications for immune mediated toxicity. Bioorg. *Med Chem Lett*. 1996;6:1511-6.
18. Callan HE, Jenkins RE, Maggs JL, et al. Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation. *Chem Res Toxicol*. 2009;22:937-48.
19. Elsheikh A, Lavergne SN, Castrejon JL, et al. Drug antigenicity, immunogenicity, and costimulatory signaling: evidence for formation of a functional antigen through immune cell metabolism. *J Immunol*. 2010;185:6448-60.
20. Nuss CE, Grant DM, Spielberg SP, et al. Further investigations of the role of acetylation in sulphonamide hypersensitivity reactions. *Biomarkers*. 1996;1:267-72.
21. Mengelers MJ, Kleter GA, Hoogenboom LA, et al. The biotransformation of sulfadimethoxine, sulfadimidine, sulfamethoxazole, trimethoprim and aditoprim by primary cultures of pig hepatocytes. *J Vet Pharmacol Ther*. 1997;20:24-32.
22. Cribb AE, Nakamura H, Grant DM, et al. Role of polymorphic and monomorphic human arylamine N-acetyltransferases in determining sulfamethoxazole metabolism. *Biochem Pharmacol*. 1993;45:1277-82.
23. Kurian JR, Bajad SU, Miller JL, et al. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther*. 2004;311:1171-8.
24. Trepanier LA, Yoder AR, Bajad S, et al. Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J Acquir Immune Defic Syndr*. 2004;36:1041-50.
25. Roychowdhury S, Vyas PM, Reilly TP, et al. Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. *J Pharmacol Exp Ther*. 2005;314:43-52.
26. Lavergne SN, Wang H, Callan HE, et al. "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. *J Pharmacol Exp Ther*. 2009;331:372-381.
27. Reilly TP, Lash LH, Doll MA, et al. A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J Invest Dermatol*. 2000;114:1164-73.
28. Carr A, Tindall B, Penny R, et al. In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin Exp Immunol.* 1993;94:21-25.
29. Rieder MJ, Uetrecht J, Shear NH, et al. Diagnosis of sulfonamide hypersensitivity reactions by in-vitro "rechallenge" with hydroxylamine metabolites. *Ann Intern Med*. 1989 Feb 15;110(4):286-9.
30. Rieder MJ, Uetrecht J, Shear NH, et al. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. *J Pharmacol Exp Ther*. 1988;244:724-8.

31. Rieder MJ, Krause R, Bird IA. Time-course of toxicity of reactive sulfonamide metabolites. *Toxicology*. 1995;95:141-6.
32. Lavergne SN, Kurian JR, Bajad SU, et al. Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. *Toxicology*. 2006;222:25-36.
33. Wolkenstein P, Charue D, Laurent P, et al. Metabolic predisposition to cutaneous adverse drug reactions. Role in toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. *Arch Dermatol.* 1995;131:544-51.
34. Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. *Clin Biochem*. 2000;33:517-24.
35. Shear NH, Spielberg SP, Grant DM, et al. Differences in metabolism of sulfonamides predisposes to idiosyncratic toxicity. *Ann Intern Med.* 1986;105:179-184.
36. Hess DA, Sisson ME, Suria H, et al. Cytotoxicity of sulfonamide reactive metabolites: apoptosis and selective toxicity of CD8(+) cells by the hydroxylamine of sulfamethoxazole. *FASEB J*. 1999;13:1688-98.
37. Rieder MJ, Krause R, Bird IA, et al. Toxicity of sulfonamide reactive metabolites in HIV-infected, HTLV-infected, and noninfected cells. *J Acquir Immune Defic Syndr Hum Retrovirol.* 1995;8:134-140.
38. Wijsman J, Dekabam G, Rieder M. Differential toxicity of reactive metabolites of clindamycin and sulfonamides in HIV-infected cells: influence of HIV infection on clindamycin toxicity in vitro. *J Clin Pharmacol.* 2005;45:346-351.
39. Arp J, Rieder MJ, Urquhart B, et al. Hypersensitivity of HIV-1-infected cells to reactive sulfonamide metabolites correlated to expression of the HIV-1 viral protein tat. *J Pharmacol Exp Ther.* 2005;314:1218-1225.
40. Choi J, Liu R-M, Kundu R, et al. Molecular mechanism of decreased glutathione content in HIV type 1 Tat-transgenic mice. *J Biol Chem.* 2000;275:3693-3698.
41. Ambrosino C, Palmieri C, Puca A, et al. Physical and functional interaction of HIV-1 Tat with E2F-4, a transcriptional regulator of mammalian cell cycle. *J Biol Chem.* 2002;277:31448-31458.
42. Toborek M, Lee YW, Pu H, et al. HIV-Tat protein induces oxidative and inflammatory pathways in brain endothelium. *J Neurochem.* 2003;84:169-179.
43. Neuman M, Malkiewicz I, Phillips E, et al. Monitoring adverse drug reactions to sulfonamide antibiotics in human immunodeficiency virus-infected individuals. *Ther Drug Monit.* 2002;24:728-736.
44. Reilly TP, MacArthur RD, Farrough MJ, et al. Is hydroxylamine-induced cytotoxicity a valid marker for hypersensitivity reactions to sulfamethoxazole in human immunodeficiency virus-infected individuals? *J Pharmacol Exp Ther*. 1999;291:1356-64.
45. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.
46. Rusinova I, Forster S, Yu S, et al. Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res*. 2013;41 (Database issue):D1040-6.

47. Cheriyath V, Leaman DW, Borden EC. Emerging roles of FAM14 family members (G1P3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. *J Interferon Cytokine Res*. 2011;31:173-81.
48. Rosebeck S, Leaman DW. Mitochondrial localization and pro-apoptotic effects of the interferon-inducible protein ISG12a. *Apoptosis*. 2008;13:562-72.
49. Liu J, Yoshikawa H, Nakajima Y, et al. Involvement of mitochondrial permeability transition and caspase-9 activation in dimethyl sulfoxide-induced apoptosis of EL-4 lymphoma cells. *Int Immunopharmacol*. 2001;1:63-74.
50. Lin CK, Kalunta CI, Chen FS, et al. Dimethyl sulfoxide suppresses apoptosis in Burkitt's lymphoma cells. *Exp Cell Res*. 1995;216:403-10.
51. Cribb AE, Grant DM, Miller MA, et al. Expression of monomorphic arylamine N-acetyltransferase (NAT1) in human leukocytes. *J Pharmacol Exp Ther*. 1991;259:1241-6.
52. Satoh T, Kato H, Kumagai Y, et al. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A*. 2010;107:1512-7.
53. Wang MQ, Huang YL, Huang J, et al. RIG-I detects HIV-1 infection and mediates type I interferon response in human macrophages from patients with HIV-1-associated neurocognitive disorders. *Genet Mol Res*. 2015;14:13799-811.
54. Künzi MS, Pitha PM. Role of interferon-stimulated gene ISG-15 in the interferon-omega-mediated inhibition of human immunodeficiency virus replication. *J Interferon Cytokine Res*. 1996;16:919-27.
55. Langevin C, van der Aa LM, Houel A, et al. Zebrafish ISG15 exerts a strong antiviral activity against RNA and DNA viruses and regulates the interferon response. *J Virol*. 2013;87:10025-36.
56. Okumura A, Pitha PM, Harty RN. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. *Proc Natl Acad Sci U S A*. 2008;105:3974-9.
57. Waddell SJ, Popper SJ, Rubins KH, et al. Dissecting interferon-induced transcriptional programs in human peripheral blood cells. *PLoS One*. 2010;5:e9753.
58. Balachandran S, Roberts PC, Kipperman T, et al. Alpha/beta interferons potentiate virus-induced apoptosis through activation of the FADD/Caspase-8 death signaling pathway. *J Virol*. 2000;74:1513-23.
59. Calpe-Berdiel L, Zhao Y, de Graauw M, et al. Macrophage ABCA2 deletion modulates intracellular cholesterol deposition, affects macrophage apoptosis, and decreases early atherosclerosis in LDL receptor knockout mice. *Atherosclerosis*. 2012;223:332-41.
60. Reddy AS, Patel JR, Vogler C, et al. Central nervous system pathology progresses independently of KC and CXCR2 in globoid-cell leukodystrophy. *PLoS One*. 2013;8:e64647.
61. Voccoli V, Tonazzini I, Signore G, et al. Role of extracellular calcium and mitochondrial oxygen species in psychosine-induced oligodendrocyte cell death. *Cell Death Dis*. 2014;5:e1529.
62. Cho S, Dawson PE, Dawson G. Role of palmitoyl-protein thioesterase in cell death: implications for infantile neuronal ceroid lipofuscinosis. *Eur J Paediatr Neurol*. 2001;5 Suppl A:53-5.

63. Cho S, Dawson PE, Dawson G. Antisense palmitoyl protein thioesterase 1 (PPT1) treatment inhibits PPT1 activity and increases cell death in LA-N-5 neuroblastoma cells. *J Neurosci Res*. 2000;62:234-40.

Figure 4.1: Sulfamethoxazole (SMX) biotransformation with generation of reactive metabolites that lead to hapten formation, immunogenicity, and clinical signs of delayed sulfoanmide hypersensitivity in immunocompetent and HIV-infected patients.

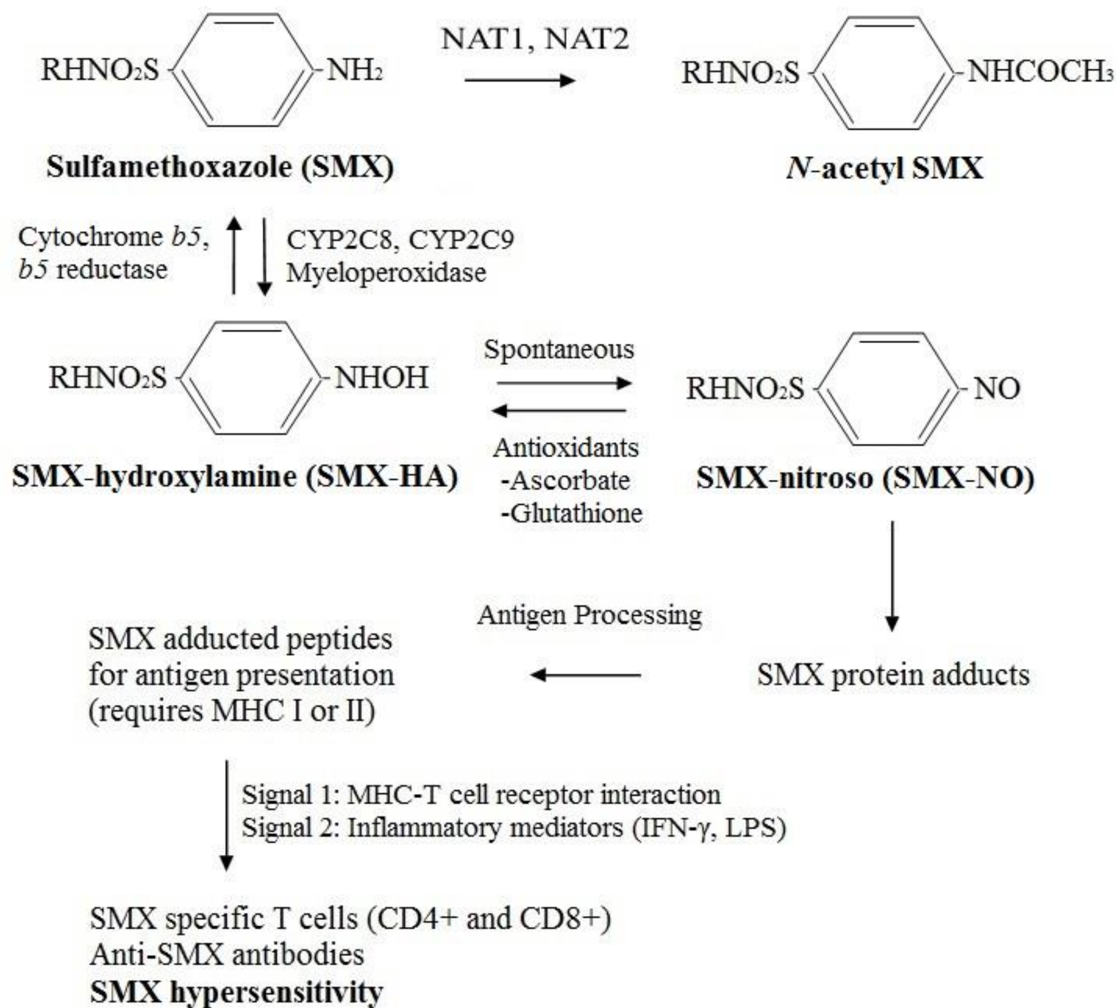


Figure 4.2A: Enhanced background vehicle cytotoxicity in SIV-infected macaques. Control macaques, median 26.6 %, range 14.1-37.3, versus SIV group, median 39.3%, range 23.3-48.8; *P* = 0.01.

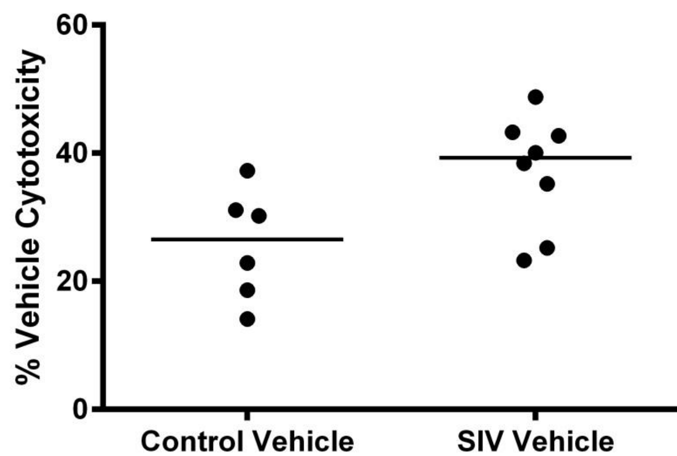


Figure 4.2B: Higher *in vitro* cytotoxicity of SMX-HA compared to SMX alone in PBMC from SIV-infected macaques (SMX-HA, median 29.8%; SMX, median 0.6 %; *P* = 0.005), but not in non-infected control macaque PBMC (SMX-HA, median 1.8%; SMX, median 3.8%; *P* = 0.50). Cytotxicity results were normalized by subtracting cytotoxicity seen from drug vehicle alone. There was also a trend toward higher SMX-HA cytotoxicity in the SIV-infected PBMC (29.8%) compared to the control group (1.8%); *P* = 0.06.

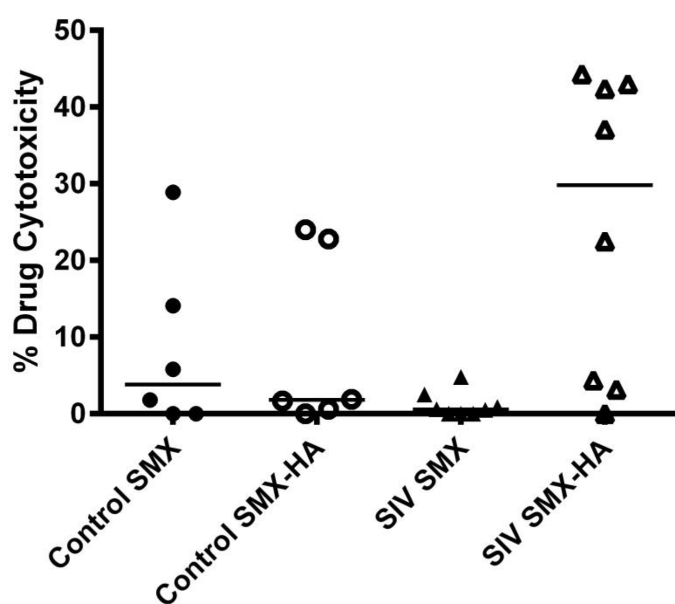


Figure 4.3A: The background vehicle cytotoxicity was not different between macaques that had detectable immunologic responses after oral TMP-SMX dosing (Pos immune; median 32.7%, range 14.1-43.3), and those without (Neg immune, median 34.2 %, range 18.6-48.8, $P = 0.63$).

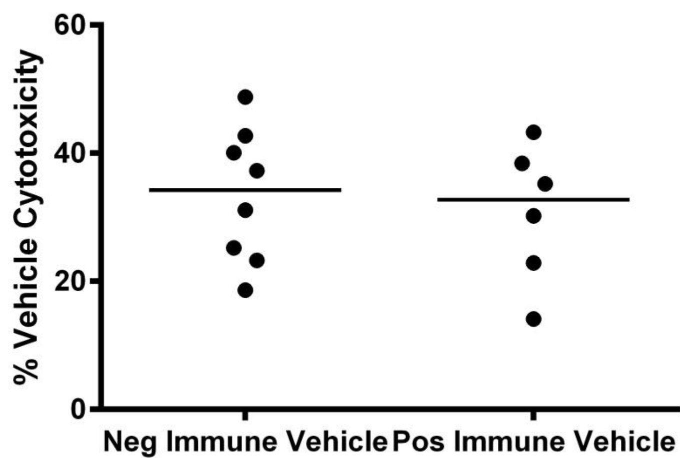


Figure 4.3B: In macaques without a subsequent immune response to TMP/SMX, the SMX metabolite SMX-HA was more toxic to PBMC than the parent drug SMX, as expected (P = 0.001). The same pattern was seen in macaques that did later develop a detectable immune response to TMP/SMX, but the difference did not reach significance (P = 0.25). Cytotoxicity results were normalized by subtracting cytotoxicity seen from drug vehicle alone. When comparing the two groups of macaques directly, SMX-HA cytotoxicity was not different between macaques with immune responses and those without (P = 0.36).

Table 4.1: Gene expression of genes that may affect the SMX biotransformation pathway in the microarray. None of the genes involved were differentially expressed in the microarray.

Pathway	Gene	Fold Change	P value
SMX oxidation	CYP2C8	1.0	0.16
	CYP2C9	-1.1	0.40
Detoxification	NAT1	1.2	0.05
	CYB5A	not in array	
	CYB5R3	-1.2	0.70
Glutathione synthesis and conjugation	GCL	1.0	0.88
	GSS	1.2	0.29
	GSTM1	1.2	0.41
	GSTP1	1.1	0.84
	GSTT1	not in array	

Table 4.2: Twenty-two genes with two-fold or higher difference in expression by microarray analysis (at $P \leq 0.01$) in CD8+ cells from 3 SIV-infected macaques with high SMX-HA cytotoxicity and 3 non-infected controls with low SMX-HA cytotoxicity. Twelve of the genes were IFN-inducible.

Gene	Fold Change	P value	IFN-inducible (Type I, Type II, or Both)
CR1	-2.3	0.005	
DDX58	2.2	0.002	Both
DDX60	2.6	0.005	Both
GBP1	2.3	0.003	Both
IFI27	15.8	0.000	Both
IFI6	4.8	0.002	Both
IFIT3	2.9	0.001	Both
INADL	-2.9	0.005	
ISG15	6.6	0.003	Both
LOC696781	-2.1	0.003	
LOC700208	2.0	0.001	
LPIN1	-2.1	0.001	
MICALCL	-2.0	0.006	
MX1	6.1	0.004	Both
MX2	5.3	0.001	Both
OAS2	3.5	0.001	Both
PRPF4|SLC31A1	2.1	0.002	
SPATS2L	2.2	0.005	Type I
TCF7L2	2.0	0.003	
XAF1	2.5	<0.001	Both
ZDBF2	-2.3	0.002	
ZDBF2	-2.7	0.004	

Table 4.3: CD8+ lymphocyte gene expression was altered in RIG-I-like receptor signaling and lysosomal degradation in the microarray.

Function	Pathway	Gene	Fold Change	P value
Anti-viral	RIG-I-like receptor signaling	DDX58	2.2	0.002
		IFIH1	1.4	0.008
		IRF7	1.5	0.006
		ISG15	6.6	0.003
Antigen processing	Lysosomal degradation	GALC	-1.5	0.005
		PPT1	1.3	0.008
		ABCA2	-1.3	0.008

Chapter 5 – Summary and Conclusions

TMP/SMX is an important antibiotic for treating multiple infections, especially in treating and preventing *Pneumocystis jirovecii* pneumonia in HIV-infected patients.[1-4] However, administration of TMP/SMX has been limited by drug hypersensitivity reactions. Most SMX-hypersensitive patients suffer from fever and skin rash,[5] and the more severely affected patients may develop liver toxicity, thrombocytopenia, or bullous skin eruptions, which can cause up to 30% mortality.[6-8] In SMX-hypersensitive patients, anti-SMX antibodies and drug-responsive T cells have been detected.[9-15]

The risk of developing SMX hypersensitivity is 1-3% in the general population.[16-18] However, multiple studies have shown that the risk for SMX hypersensitivity in HIV-infected patients increases to 20-57%.[19-22] This increased risk appears to be acquired, and varies with severity of HIV infection.[20, 23-26] However, the mechanism of this acquired risk is unknown.

Animal models including mice, rats, rabbits, and dogs have been used to investigate the risk of SMX hypersensitivity.[9,11, 27-36] However, these models lack similar SMX biotransformation profiles as humans, or a retroviral infection status that models HIV infection.

The lymphocyte toxicity assay (LTA) has been used as a surrogate biomarker to investigate the risk of SMX hypersensitivity. In this *in vitro* cytotoxicity assay, peripheral blood mononuclear cells (PBMC) from hypersensitive patients are more susceptible to apoptosis in the presence of SMX-hydroxylamine (SMX-HA), when compared to PBMC from sulfonamide-tolerant patients.[37-39] However, the mechanism for the increased cell susceptibility to SMX-HA is unknown.

In order to understand the acquired risk factors for SMX hypersensitivity in HIV infection, SIV-infected rhesus macaques were used. We hypothesized that a combination of acquired metabolic and immunologic alterations are present in HIV infection that lead to the high risk of sulfonamide hypersensitivity, and that these same alterations are also present in an SIV infection model. In Aim 1 (**Chapter 2**), we determined whether SIV-infected macaques can show immunologic sensitization to TMP/SMX, and evaluated the associated risk factors. In Aim 2 (**Chapter 3**), we determined the effects of SIV infection on hepatic biotransformation and other pathways, using expression array analyses in infected and control livers. In Aim 3 (**Chapter 4**), we evaluated the mechanisms for enhanced lymphocyte cytotoxicity from SMX metabolites, observed in HIV-infected patients, using expression array analyses in SIV-infected macaque lymphocytes.

To determine whether SIV-infected rhesus macaques can be a model suitable for investigating SMX hypersensitivity, SIV-infected and non-infected macaques were dosed with TMP/SMX.

Drug-responsive T cells and anti-SMX antibodies were detected in both SIV-infected and non-infected macaques. One SIV-infected and one non-infected macaque developed transient facial and truncal rash during dosing, which was similar in appearance and time of onset to that seen in human patients with SMX hypersensitivity.[19, 22, 40] These observations suggest that rhesus macaques, like humans, are capable of SMX bioactivation, that could lead to the development of SMX hypersensitivity.

Although immunologic responses were seen in macaques, we did not see an association between SMX immunogenicity and antioxidant status, cytokine concentrations, or SMX disposition in our study. This could be due to the lack of SIV-infected macaques with high viral loads and abnormal CD4+ counts, because a higher risk of hypersensitivity is observed in HIV-infected patients with moderately low CD4+ counts.[20, 23-26] Furthermore, the formula global primate diet fed to macaques in our studies was healthily supplemented with ascorbate, and animals did not develop the low plasma ascorbate concentrations seen in HIV-infected patients, which has been shown to impair SMX-nitroso (SMX-NO) reduction capacity. It is possible that studying animals with higher viral loads and lower CD4+ counts, and with moderately restricted ascorbate intake, could increase the sensitivity of the model for SMX hypersensitivity by affecting SMX disposition and antioxidant levels in SIV-infected macaques. Also, immunohistochemical analyses of skin biopsies at the time of a hypersensitivity reaction could be done to confirm the event of SMX hypersensitivity, by documenting drug adducts and drug-responsive T cells in affected tissues. In addition, intravenous (IV) or intraperitoneal (IP) administration of SMX-NO

to a limited number of animals might trigger an overt cutaneous hypersensitivity reaction in the macaque model.

To further investigate whether SMX biotransformation is affected by retroviral infection, liver biopsies were obtained from SIV-infected macaques to evaluate expression of genes involved in SMX biotransformation pathways. Using an expression array, we found that genes involved in SMX biotransformation was not affected by SIV infection, and hepatic activity assays further confirmed that SMX biotransformation activities were not affected. This is in contradiction to some studies that have shown impaired *N*-acetylation and glutathione (GSH) production in HIV infection.[20, 41-48, 50, 51] However, other studies have not found changes in *N*-acetylation or plasma reduced GSH concentrations in HIV infection,[49, 64, 65] and alterations may depend on stage and severity of retroviral infection. Therefore, hepatic biotransformation genes should also be evaluated in more macaques with advanced disease (CD4+ counts < 200 cells/μl).

Although we did not find an effect of SIV infection on hepatic SMX biotransformation genes, SIV significantly altered genes involved in immunologic pathways, as expected. Multiple IFN-inducible genes, including antiviral genes, were up-regulated to fight against SIV infection, as has been shown by other studies.[59-62] Among these IFN-inducible genes, some are involved in ISGylation, immunoproteasomal degradation, and antigen presentation. Up-regulation of these genes could increase processing of SMX-protein adducts into SMX-adducted peptides. Together with up-regulated MHC-I, presentation of SMX-adducted protein antigen could be more efficient,

and contribute to the acquired risk of SMX hypersensitivity. To test this, type I and type II IFN could be administered to healthy macaques during TMP/SMX administration to determine whether IFN-inducible genes lead to increased drug-antigen presentation in lymph node cells, development of drug-responsive T cells, or even clinical signs of drug hypersensitivity.

Interestingly, a few genes that are involved in systemic lupus erythematosus (SLE), an autoimmune disease, were also up-regulated in SIV infection. While it may seem improbable to associate autoimmunity pathways with a disease of immunodeficiency, HIV-infected patients, although rare, can actually be diagnosed with SLE,[52, 53] and up-regulation of IFN-inducible genes have been detected in SLE patients.[54, 55] The association between type I IFN and the activation of autoreactive T cells and B cells in SLE suggests that autoimmunity in SMX hypersensitivity may be partially associated with up-regulated IFN pathways.[56-58] We did not see a difference in plasma IFN-γ (type II IFN) levels between SIV-infected and non-infected macaques, and between macaques that showed immunologic responses (drug-responsive T cells, anti-SMX antibodies) and those did not. Therefore, the levels of type I IFN (IFN-α and IFN-β) should also be evaluated in future studies. It is possible that treatments to lower the up-regulated IFN response in retroviral infection could lower the threshold for drug-antigen processing and presentation, and reduce the pathogenesis of SMX and other drug hypersensitivity, but this theory would require further investigation. In addition, the possible adverse effects of such interventions on viral load would need to be considered, because this could suppress immune responses against active retroviral infection.

In **Chapter 4**, we evaluated whether findings of the lymphocyte toxicity assay (LTA) in HIV-infected patients could be repeated in SIV-infected macaques, and further explored the mechanism of enhanced PBMC cytotoxicity. We found that PBMC from SIV-infected macaques incubated with vehicle have higher background cytotoxicity when compared to the controls. DMSO ($> 2\%$ final concentration) has been shown to induce apoptosis in lymphocytes cell line,[66, 67] and this suggests that retroviral infection may lower threshold for apoptosis in PBMC.

An apparently increased SMX-HA cytotoxicity in PBMC from SIV-infected macaques was observed when compared to PBMC from the controls, but it just missed statistical significance at $P = 0.06$. Although a larger sample size is needed to explore this trend, this result is similar to findings that HIV-infected patients have higher SMX-HA-induced *in vitro* cytotoxicity.[37-39] This enhanced cytotoxicity was not seen with the parent drug SMX, and could suggest an acquired lymphocyte susceptibility in retroviral infection due to oxidative stress from SMX-HA, which oxidizes to SMX-NO.

In HIV-infected patients, despite higher *in vitro* SMX-HA cytotoxicity overall, the LTA can still distinguish between patients with and without SMX hypersensitivity.[37, 68, 69] However, only one SIV-infected and one non-infected macaque developed rash during TMP/SMX dosing in our study, and it was inconclusive whether it was sign of hypersensitivity caused by SMX. Therefore,

we could not evaluate the LTA as a predictor of drug hypersensitivity in this macaque model under the conditions of the study. However, we did not see an association between the presence of detectable immunologic makers (drug-responsive T cells, anti-SMX antibodies) in TMP/SMX dosed macaques and enhanced SMX-HA cytotoxicity. This may be due to the limitation of the lymphocyte transformation test for drug-responsive T cell detection. This assay may have false negatives in some macaques, including those that developed rash, because only a finite number of PBMC are present in a given blood sample to be plated in the assay, while a small number of undetected circulating drug-responsive T cells may be sufficient for a hypersensitivity reaction. Furthermore, hypersensitivity reactions to TMP, but not SMX, could lead to false negatives in the LTA.[70, 71] Limitations in both the LTA and the lymphocyte transformation test, together with an apparently low incidence of rash in macaques with CD4+ counts below 200/µl, may have lowered the statistical power to evaluate LTA as a surrogate marker for SMX hypersensitivity.

In the LTA, CD8+ T cells appear to be most susceptible to apoptosis upon exposure to SMX-HA *in vitro,* compared to CD4+ T cells and B cells.[63] A defect in SMX-HA detoxification in CD8+ T cells has been hypothesized.[63] In order to determine whether metabolic alterations are present in CD8+ cells from high cytotoxicity LTA assays, expression arrays were carried out in PBMC from SIV-infected macaques with high cytotoxicity, and from control macaques with low cytotoxicity. Similar to the liver expression array, we found that SMX biotransformation pathways were not altered, and multiple IFN-inducible genes, including antiviral genes, were up-regulated in SIV infection, which is a common response in retroviral infection.[59-62] Multiple IFN-

inducible genes have been shown to enhance apoptosis in PBMC.[74, 75] Specifically, IFI27, which was up-regulated in the expression array, have been shown to enhance apoptosis in PBMC,[72] and could potentiate apoptotic effect of retroviral infection.[73] Future experiments of LTA incubated with type I and type II IFN, or IFI27 specifically, could confirm whether increased PBMC susceptibility in LTA is IFN related.

Pathway analyses also revealed that genes involved in lysosomal degradation were altered in SIV infection. It has been shown that deficiency in lysosomal enzyme expression lead to increase cell death in neurons and macrophages,[76-80] and this could also occur in CD8+ T cells. Further experiments could be focused in this direction by transfecting lymphocyte cell lines with siRNA targeting lysosomal degradation and performing the LTA with SMX-HA.

Overall, SIV-infected macaques have the potential to be a useful animal model for investigating the risk of SMX hypersensitivity in HIV infection. Encouraging results include demonstrated immunogenicity to SMX when dosed in a manner similar to human patients, possible drug-associated rash in two animals, and similar effects of retroviral infection on SMX-HA concentrations, and in some animals, blood GSH concentrations. Drawbacks of the model are its expense, and difficulty in accruing enough animals with low CD4+ counts to recapitulate the "risk window" seen in HIV infection. However, the lack of differences in SMX biotransformation pathways and measurable drug adducts in SIV infection, even in animals with immune responses to SMX, suggests that risk for **SMX hypersensitivity could involve**

pathways that are downstream from adduct formation. Although it has been hypothesized that HIV-infected patients have defects in SMX drug disposition,[20, 41-48, 50, 51] our observations did not support this. Instead, the up-regulation of antigen processing, antigen presentation, and "autoimmune" (SLE) genes in retroviral infection could be related to the risk of drug hypersensitivity reactions to many structurally unrelated drugs in HIV infection. For example, HIV- infected patients also have a higher incidence of drug hypersensitivity reactions to the antituberculosis drug thiacetazone, and the antiretroviral drugs, efavirenz and nevirapine.[81-83] Further studies on drug hypersensitivity in retroviral infection should address mechanisms of drug immunogenicity downstream from adduct formation. These investigations can be focused on the role of IFN inducible genes in drug hypersensitivity, opening up a new possibility to prevent drug hypersensitivity in HIV-infected patients.

References

1. Avgeri SG, Matthaiou DK, Dimopoulos G, et al. Therapeutic options for Burkholderia cepacia infections beyond co-trimoxazole: a systematic review of the clinical evidence. *Int J Antimicrob Agents.* 2009;33:394-404.
2. Drekonja DM, Johnson JR. Urinary tract infections. *Prim Care* 2008;35:345-367, vii.
3. Korbila IP, Manta KG, Siempos, II, et al. Penicillins vs trimethoprim-based regimens for acute bacterial exacerbations of chronic bronchitis: meta-analysis of randomized controlled trials. *Can Fam Physician.* 2009;55:60-67.
4. Drekonja DM, Traynor LM, DeCarolis DD, et al. Treatment of non-life-threatening methicillin-resistant Staphylococcus aureus infections with alternative antimicrobial agents: a 2-year retrospective review. *Diagn Microbiol Infect Dis.* 2009;63:201-207.
5. Cribb AE, Lee BL, Trepanier LA, et al. Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: clinical syndromes and pathogenesis. *Adverse Drug React Toxicol Rev.* 1996;15:9-50.
6. Ortiz J, Horn M, Peterson H. Toxic epidermal necrolysis-case report and review of the literature. *Ann Plast Surg.* 1982;9:249-253.
7. Saka B, Kombate K, Mouhari-Toure A, et al. Stevens-Johnson syndrome and toxic epidermal necrolysis in a teaching hospital in Lomé, Togo: retrospective study of 89 cases. *Med Trop (Mars).* 2010;70:255-8.
8. Bell TL, Foster JN, Townsend ML. Trimethoprim-sulfamethoxazole-induced hepatotoxicity in a pediatric patient. *Pharmacotherapy.* 2010;30:539.
9. Naisbitt DJ, Gordon SF, Pirmohamed M, et al. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br J Pharmacol*. 2001;133:295-305.
10. Daftarian MP, Filion LG, Cameron W, et al. Immune response to sulfamethoxazole in patients with AIDS. *Clin Diagn Lab Immunol.* 1995;2:199-204.
11. Farrell J, Naisbitt D, Drummond N, et al. Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *J Pharmacol Exp Ther.* 2003;306:229-237.
12. Miyauchi H, Hosokawa H, Akaeda T, et al. T-cell subsets in drug-induced toxic epidermal necrolysis. Possible pathogenic mechanism induced by CD8-positive T cells. *Arch Dermatol*. 1991;127:851-5.
13. Correia O, Delgado L, Ramos JP, et al. Cutaneous T-cell recruitment in toxic epidermal necrolysis. Further evidence of CD8+ lymphocyte involvement. *Arch Dermatol*. 1993;129:466-8.
14. Gruchalla RS, Pesenko RD, Do TT, et al. Sulfonamide-induced reactions in desensitized patients with AIDS--the role of covalent protein haptenation by sulfamethoxazole. *J Allergy Clin Immunol*. 1998;101:371-8.
15. O'Neil CE, Reed MA, Lopez M, et al. Evaluation of immune parameters in HIV+ subjects reporting adverse reactions to sulfamethoxazole. *Int Arch Allergy Appl Immunol*. 1991;94:246-7.
16. Stewart RB, May FE, Cullen SI. Dermatologic adverse drug reactions in hospitalized patients. *Am J Hosp Pharm.* 1979;36:609-12.
17. Bigby M, Jick S, Jick H, et al. Drug-induced cutaneous reactions. A report from the Boston Collaborative Drug Surveillance Program on 15,438 consecutive inpatients, 1975 to 1982. *JAMA*. 1986;256:3358-63.
18. Arndt KA, Jick H. Rates of cutaneous reactions to drugs. A report from the Boston Collaborative Drug Surveillance Program. *JAMA.* 1976;235:918-23.

19. Gordin FM, Simon GL, Wofsy CB, et al. Adverse reactions to trimethoprim-sulfamethoxazole in patients with AIDS. *Ann Intern Med.* 1984;100:495-499.
20. Hennessy S, Strom BL, Berlin JA, et al. Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pneumonia prophylaxis. *J Gen Intern Med.* 1995;10:380-386.
21. Medina I, Mills J, Leoung G, et al. Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. *N Engl J Med.* 1990;323:776-782.
22. Walmsley SL, Khorasheh S, Singer J, et al. A randomized trial of N-acetylcysteine for prevention of trimethoprim-sulfamethoxazole hypersensitivity reactions in Pneumocystis carinii pneumonia prophylaxis (CTN 057). *J Acquir Immune Defic Syndr Hum Retrovirol.* 1998;19:498-505.
23. Carr A, Swanson C, Penny R, et al. Clinical and laboratory markers of hypersensitivity to trimethoprim-sulfamethoxazole in patients with Pneumocystis carinii pneumonia and AIDS. *J Infect Dis*. 1993;167:180-185.
24. Eliaszewicz M, Flahault A, Roujeau J, et al. Prospective evaluation of risk factors of cutaneous drug reactions to sulfonamides in patients with AIDS. *J Am Acad Dermatol.* 2002;47:40-46.
25. Rabaud C, Charreau I, Izard S, et al. Adverse reactions to cotrimoxazole in HIV-infected patients: predictive factors and subsequent HIV disease progression. *Scand J Infect Dis.* 2001;33:759-764.
26. Ryan C, Madalon M, Wortham DW, et al. Sulfa hypersensitivity in patients with HIV infection: onset, treatment, critical review of the literature. *WMJ.* 1998;97:23-27.
27. Cheng L, Stewart BJ, You Q, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol Pharmacol*. 2008;73:1769-75.
28. Hopkins JE, Naisbitt DJ, Humphreys N, et al. Exposure of mice to the nitroso metabolite of sulfamethoxazole stimulates interleukin 5 production by CD4+ T-cells. *Toxicology*. 2005;206:221-31.
29. Choquet-Kastylevsky G, Santolaria N, Tedone R, et al. Induction of delayed-type hypersensitivity to sulfamethoxazole in mice: role of metabolites. *Toxicol Lett*. 2001;119:183-92.
30. Gill HJ, Hough SJ, Naisbitt DJ, et al. The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. *J Pharmacol Exp Ther*. 1997;282:795-801.
31. Naisbitt DJ, Farrell J, Gordon SF, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol*. 2002;62:628-37.
32. Cribb AE, Spielberg SP. An in vitro investigation of predisposition to sulphonamide idiosyncratic toxicity in dogs. *Vet Res Commun*. 1990;14:241-52.
33. Trepanier LA, Danhof R, Toll J, Watrous D. Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. *J Vet Intern Med*. 2003;17:647-52.
34. Trepanier LA. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. *J Vet Pharmacol Ther*. 2004;27:129-38.
35. Lavergne SN, Danhof RS, Volkman EM, et al. Association of drug-serum protein adducts and anti-drug antibodies in dogs with sulphonamide hypersensitivity: a naturally occurring model of idiosyncratic drug toxicity. *Clin Exp Allergy*. 2006;36:907-15.
36. Lavergne SN, Volkman EM, Maki JE, et al. Evaluation of the clinical, immunologic, and biochemical effects of nitroso sulfamethoxazole administration to dogs: a pilot study. *Toxicology*. 2005;208:63-72.
37. Carr A, Tindall B, Penny R, et al. In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin Exp Immunol.* 1993;94:21-25.

38. Rieder MJ, Uetrecht J, Shear NH, et al. Diagnosis of sulfonamide hypersensitivity reactions by in-vitro "rechallenge" with hydroxylamine metabolites. *Ann Intern Med*. 1989 Feb 15;110(4):286-9.
39. Rieder MJ, Uetrecht J, Shear NH, et al. Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. *J Pharmacol Exp Ther*. 1988;244:724-8.
40. Caumes E, Roudier C, Rogeaux O, et al. Effect of corticosteroids on the incidence of adverse cutaneous reactions to trimethoprim-sulfamethoxazole during treatment of AIDS-associated Pneumocystis carinii pneumonia. *Clin Infect Dis.* 1994;18:319-323.
41. Mengelers MJ, Kleter GA, Hoogenboom LA, et al. The biotransformation of sulfadimethoxine, sulfadimidine, sulfamethoxazole, trimethoprim and aditoprim by primary cultures of pig hepatocytes. *J Vet Pharmacol Ther*. 1997;20:24-32.
42. Cribb AE, Nakamura H, Grant DM, et al. Role of polymorphic and monomorphic human arylamine N-acetyltransferases in determining sulfamethoxazole metabolism. *Biochem Pharmacol*. 1993;45:1277-82.
43. Carr A, Gross AS, Hoskins JM, et al. Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulphamethoxazole in HIV-infected patients. *AIDS*. 1994;8:333-7.
44. Kaufmann GR, Wenk M, Taeschner W, et al. N-aceyltransferase 2 polymorphism in patients infected with human immunodeficiency virus. *Clin Pharmacol Ther*. 1996;60:62-7.
45. Lee Bl, Wong D, Benowitz NL, et al. Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. *Clin Pharmacol Ther.* 1993;53:529-35.
46. Wolkenstein P, Loriot M-A, Aractingi S, et al. Prospective evaluation fo detoxification pathways as markers of cutaneous adverse reactions to sulphonamides in AIDS. *Pharmacogenetics.* 2000;10:821-8.
47. O'Neil W, MacArthur RD, Farrough MJ, et al. Acetylator phenotype and genotype in HIV-infected patietnts with and without sulfonamide hypersensitivity. *J Clin Pharmacol*. 2002;42:613-9.
48. Quirino T, Bonfanti P, Arcidiacono M, et al. Acetylator phenotype prevalance in HIV-infected patients without previous trimethoprim-sulfamethoxazole hypersensitivity. *Biomed Pharmacother*. 1999;53:286-7.
49. O'Neil WM, Drobitch RK, MacArthur RD, et al. Acetylator phenotype and genotype in patients infected with HIV: discordance between methods for phenotype determination and genotype. *Pharmacogenetics.* 2000;10:171-82.
50. Morris D, Guerra C, Donohue C, et al. Unveiling the mechanisms for decreased glutathione in individuals with HIV infection. *Clin Dev Immunol*. 2012;2012:734125.
51. Choi J, Liu RM, Kundu RK, et al. Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. *J Biol Chem*. 2000;275:3693-8.
52. Clutterbuck DJ, Watson J, De Ruiter A, et al. Presence of HIV infection in patients diagnosed with systemic lupus erythematosus. *Rheumatology (Oxford)*. 2000;39:1047-8.
53. Mody GM, Patel N, Budhoo A, et al. Concomitant systemic lupus erythematosus and HIV: case series and literature review. *Semin Arthritis Rheum*. 2014;44:186-94.
54. Han GM, Chen SL, Shen N, et al. Analysis of gene expression profiles in human systemic lupus erythematosus using oligonucleotide microarray. *Genes Immun*. 2003;4:177-86.
55. Obermoser G, Pascual V. The interferon-alpha signature of systemic lupus erythematosus. *Lupus*. 2010;19:1012-9.
56. Le Bon A, Thompson C, Kamphuis E, et al. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J Immunol.* 2006;176:2074–8.

57. Kiefer K, Oropallo MA, Cancro MP, et al. Role of type I interferons in the activation of autoreactive B cells. *Immunol Cell Biol*. 2012;90:498–504.
58. Lauwerys BR, Ducreux J, Houssiau FA. Type I interferon blockade in systemic lupus erythematosus: where do we stand? *Rheumatology (Oxford)*. 2014;53:1369-76.
59. de Veer MJ, Holko M, Frevel M, et al. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol*. 2001;69:912-20.
60. Nasr N, Maddocks S, Turville SG, et al. HIV-1 infection of human macrophages directly induces viperin which inhibits viral production. *Blood*. 2012;120:778-88.
61. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol*. 2014;14:36-49.
62. Perry AK, Chen G, Zheng D, et al. The host type I interferon response to viral and bacterial infections. *Cell Res*. 2005;15:407-22.
63. Hess DA, Sisson ME, Suria H, et al. Cytotoxicity of sulfonamide reactive metabolites: apoptosis and selective toxicity of CD8(+) cells by the hydroxylamine of sulfamethoxazole. *FASEB J*. 1999;13:1688-98.
64. van der Ven AJ, Vree TB, van Ewijk-Beneken Kolmer EW, et al. Urinary recovery and kinetics of sulphamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulphamethoxazole. *Br J Clin Pharmacol*. 1995;39:621-5.
65. van der Ven AJ, Blom HJ, Peters W, et al. Glutathione homeostasis is disturbed in CD4-positive lymphocytes of HIV-seropositive individuals. *Eur J Clin Invest*. 1998;28:187-93.
66. Liu J, Yoshikawa H, Nakajima Y, et al. Involvement of mitochondrial permeability transition and caspase-9 activation in dimethyl sulfoxide-induced apoptosis of EL-4 lymphoma cells. *Int Immunopharmacol*. 2001;1:63-74.
67. Lin CK, Kalunta CI, Chen FS, et al. Dimethyl sulfoxide suppresses apoptosis in Burkitt's lymphoma cells. *Exp Cell Res*. 1995;216:403-10.
68. Neuman M, Malkiewicz I, Shear N. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. *Clin Biochem.* 2000;33:517-524.
69. Neuman M, Malkiewicz I, Phillips E, et al. Monitoring adverse drug reactions to sulfonamide antibiotics in human immunodeficiency virus-infected individuals. *Ther Drug Monit.* 2002;24:728-736.
70. Bonfanti P, Pusterla L, Parazzini F, et al. The effectiveness of desensitization versus rechallenge treatment in HIV-positive patients with previous hypersensitivity to TMP-SMX: a randomized multicentric study. C.I.S.A.I. Group. *Biomed Pharmacother.* 2000;54:45-49.
71. Carr A, Penny R, Cooper DA. Efficacy and safety of rechallenge with low-dose trimethoprim-sulphamethoxazole in previously hypersensitive HIV-infected patients. *AIDS.* 1993;7:65-71.
72. Cheriyath V, Leaman DW, Borden EC. Emerging roles of FAM14 family members (G1P3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. *J Interferon Cytokine Res*. 2011;31:173-81.
73. Rosebeck S, Leaman DW. Mitochondrial localization and pro-apoptotic effects of the interferon-inducible protein ISG12a. *Apoptosis*. 2008;13:562-72.
74. Waddell SJ, Popper SJ, Rubins KH, et al. Dissecting interferon-induced transcriptional programs in human peripheral blood cells. *PLoS One*. 2010;5:e9753.
75. Balachandran S, Roberts PC, Kipperman T, et al. Alpha/beta interferons potentiate virus-induced apoptosis through activation of the FADD/Caspase-8 death signaling pathway. *J Virol*. 2000;74:1513-23.
76. Calpe-Berdiel L, Zhao Y, de Graauw M, et al. Macrophage ABCA2 deletion modulates intracellular cholesterol deposition, affects macrophage apoptosis, and decreases early atherosclerosis in LDL receptor knockout mice. *Atherosclerosis*. 2012;223:332-41.

77. Reddy AS, Patel JR, Vogler C, et al. Central nervous system pathology progresses independently of KC and CXCR2 in globoid-cell leukodystrophy. *PLoS One*. 2013;8:e64647.
78. Voccoli V, Tonazzini I, Signore G, et al. Role of extracellular calcium and mitochondrial oxygen species in psychosine-induced oligodendrocyte cell death. *Cell Death Dis*. 2014;5:e1529.
79. Cho S, Dawson PE, Dawson G. Role of palmitoyl-protein thioesterase in cell death: implications for infantile neuronal ceroid lipofuscinosis. *Eur J Paediatr Neurol*. 2001;5 Suppl A:53-5.
80. Cho S, Dawson PE, Dawson G. Antisense palmitoyl protein thioesterase 1 (PPT1) treatment inhibits PPT1 activity and increases cell death in LA-N-5 neuroblastoma cells. *J Neurosci Res*. 2000;62:234-40.
81. Nunn P, Kibuga D, Gathua S, et al. Cutaneous hypersensitivity reactions due to thiacetazone in HIV-1 seropositive patients treated for tuberculosis. *Lancet*. 1991;337:627-30.
82. Chintu C, Luo C, Bhat G, et al. Cutaneous hypersensitivity reactions due to thiacetazone in the treatment of tuberculosis in Zambian children infected with HIV-I. *Arch Dis Child*. 1993;68:665-8.
83. Yunihastuti E, Widhani A, Karjadi TH. Drug hypersensitivity in human immunodeficiency virus-infected patient: challenging diagnosis and management. *Asia Pac Allergy*. 2014;4:54-67.