Genomic Response of Human Immune and Non-Immune Cells to Glucocorticoids

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Statement of Compliance

The trial will be carried out in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Good Clinical Practice (GCP) and the following:

- United States Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR Part 312, and/or 21 CFR Part 812)

NIH-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; an IRB determination will be made regarding whether a new consent needs to be obtained from participants who provided consent using a previously approved consent form.

List of Abbreviations

AE	Adverse event/adverse experience
AR	Adverse reaction
BMI	Body Mass Index
CBC	Complete Blood Count
CC	Clinical Center
CFR	Code of Federal Regulations
CRIMSON	Clinical Research Information Management System of the NIAID
CSO	Clinical Safety Office
ECG	Electrocardiogram
GC	Glucocorticoid
GCP	Good Clinical Practice
GRE	Glucocorticoid Response Element
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HRPP	Human Research Protection Program
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IND	Investigational new drug
iPS	Induced Pluripotent Stem Cells
IRB	Institutional Review Board
NIAID	National Institute of Allergy and Infectious Diseases
NIAMS	National Institute of Arthritis and Musculoskeletal and Skin Diseases
NIH	National Institutes of Health
NK	Natural Killer (immune cells)
OCRPRO	Office of Clinical Research Policy and Regulatory Operations
OHRP	Office for Human Research Protections
PBMC	Peripheral Blood Mononuclear Cells
RNAi	RNA interference
SAE	Serious adverse event/serious adverse experience
SAR	Suspected adverse reaction
siRNA	Small Interfering RNA
SUSAR	Serious and unexpected suspected adverse reaction
UP	Unanticipated Problem
UPnonAE	Unanticipated Problem that is not an Adverse Event

Protocol Summary

Full Title:	Genomic Response of Human Immune and Non-Immune Cells to Glucocorticoids
Short Title:	Genomic response to GCs
Conducted by:	Systemic Autoimmunity Branch, NIAMS, NIH
Principal Investigator:	Luis M. Franco, M.D.
Sample Size:	N=50 maximum. The required sample size will be determined by an adaptive study design after an initial recruitment of 20 subjects
Accrual Ceiling:	60
Study Population:	Healthy volunteers ages 18-64 years
Accrual Period:	60 months
Study Design:	This is a study of glucocorticoid action at the genomic level. Healthy volunteers will undergo baseline blood collection, then receive a single intravenous dose of 250 mg of methylprednisolone. Blood will be collected in one of two regimens: 1 and 2 hours or 2 and 4 hours after the start of the infusion. A skin punch biopsy may be obtained before healthy volunteers receive IV methylprednisolone. If so, topical methylprednisolone will be applied to a limited area of skin, contralateral to the site of the initial skin biopsy, and an additional skin biopsy will be obtained (from the area of topical methylprednisolone application) at the 4-hour time point. Individual participation requires 2 visits to the NIH Clinical Center (CC) and 2 follow-up calls. Total participation time is 1-5 weeks. Blood samples will be processed for isolation of hematopoietic cell sub-populations (e.g., neutrophils, B cells, CD4+ T cells, CD8+ T cells, monocytes, and natural killer [NK] cells). Laboratory studies will be performed in the purified cells, with the goal of understanding the human response to glucocorticoids in vivo at the level of RNA (e.g., RNA sequencing, small-RNA-sequencing, real-time PCR), DNA (e.g., ChIP-seq, methylation analysis, DNA sequencing, genotyping), and protein (e.g., flow cytometry, mass spectrometry). At each time point, plasma methylprednisolone levels will be measured and flow cytometry for standard lineage markers will be performed. Skin biopsies will be subjected to RNA

	isolation for RNA sequencing and small-RNA sequencing. A fragment of each skin biopsy will undergo fibroblast isolation and culture for in vitro exposure to glucocorticoids and for the generation of induced pluripotent stem (iPS) cells
Study Duration:	Start Date: October 2016; End Date: October 2022
Primary Objective:	To elucidate fundamental differences in the genomic response of immune and non-immune cells to glucocorticoids
Primary Endpoint:	A list of human genes and non-coding RNAs that are differentially expressed and regulated in response to glucocorticoids between immune and non-immune cells
Secondary Objective:	To identify potential targets for small-molecule or nanoparticle-facilitated RNA interference interventions that reproduce the therapeutic action of glucocorticoids while avoiding harmful effects
Secondary Endpoints:	• A list of protein-coding and non-coding transcripts, their corresponding proteins, and the molecular pathways representing the best candidates for targeted therapeutic alternatives to glucocorticoids • Validation of the targets identified by functional studies

Précis

Glucocorticoids are among the most frequently prescribed immunosuppressive and anti-inflammatory medications worldwide. Long-term use, however, is complicated by serious non-immunologic side effects. Ongoing in vitro experiments with human primary cells in our laboratory suggest that there are indeed fundamental differences in the genomic response of immune and non-immune cells to glucocorticoids. These and other aspects of drug action at the genomic level have not been completely characterized. This study will attempt to generate a list of human genes and non-coding RNAs that are differentially expressed and regulated in response to glucocorticoids between immune and non-immune cells. These data will be used to identify transcripts, their corresponding proteins, and the molecular pathways that are best candidates for targeted intervention. Potential targets could be validated with small interfering RNA (siRNA) libraries, with the long-term goal of developing small-molecule or nanoparticle-facilitated RNA interference (RNAi) interventions that reproduce the therapeutic action of glucocorticoids in immune cells while avoiding their harmful side effects on other tissues.

Healthy volunteers will undergo baseline blood collection prior to receiving a single intravenous dose of 250 milligrams of methylprednisolone sodium succinate. Blood will be collected in one of two regimens: 1 and 2 hours or 2 and 4 hours after the start of the infusion. A skin punch biopsy may be obtained before healthy volunteers receive IV methylprednisolone. If so, topical methylprednisolone will be applied to a limited area of skin, contralateral to the site of the baseline skin biopsy, and an additional skin biopsy will be obtained 4 hours after drug administration, from the area where topical methylprednisolone was applied. Follow-up phone calls 1 and 5 days after discharge will document any adverse effects related to the drug or skin biopsy. Total length of individual study participation is 1-5 weeks.

Blood samples will be processed for isolation of hematopoietic cell sub-populations (e.g. neutrophils, B cells, CD4+ T cells, CD8+ T cells, monocytes, and natural killer [NK] cells). Laboratory studies will be performed in the purified cells, with the goal of understanding the human response to glucocorticoids in vivo at the level of RNA (e.g., RNA sequencing, small-RNA-sequencing, real-time PCR), DNA (e.g., ChIP-seq, methylation analysis, DNA sequencing, genotyping), and protein (e.g., flow cytometry, mass spectrometry). At each time point, serum methylprednisolone levels will be measured and flow cytometry for standard lineage markers will be performed. Skin biopsies will be subjected to RNA isolation for RNA sequencing and small-RNA sequencing. A fragment of each skin biopsy will undergo fibroblast isolation and culture for in vitro exposure to glucocorticoids and for the generation of induced pluripotent stem (iPS) cells.

1 Background Information and Scientific Rationale

1.1 Scientific Background and Preliminary Data

Glucocorticoids are among the most frequently prescribed immunosuppressive and anti-inflammatory medications worldwide (1, 2). Long-term use, however, is complicated by serious non-immunologic side effects. A large gap of knowledge about the differences between the actions of this class of drugs on immune and non-immune cells has prevented the development of more targeted therapies or the use of drug combinations that would preserve the desired immunological effects while avoiding unwanted systemic activities. Glucocorticoids are ligands for a nuclear receptor and their mechanism of action involves broad effects on gene expression via binding to the well-recognized mammalian glucocorticoid response element (GRE). Recent

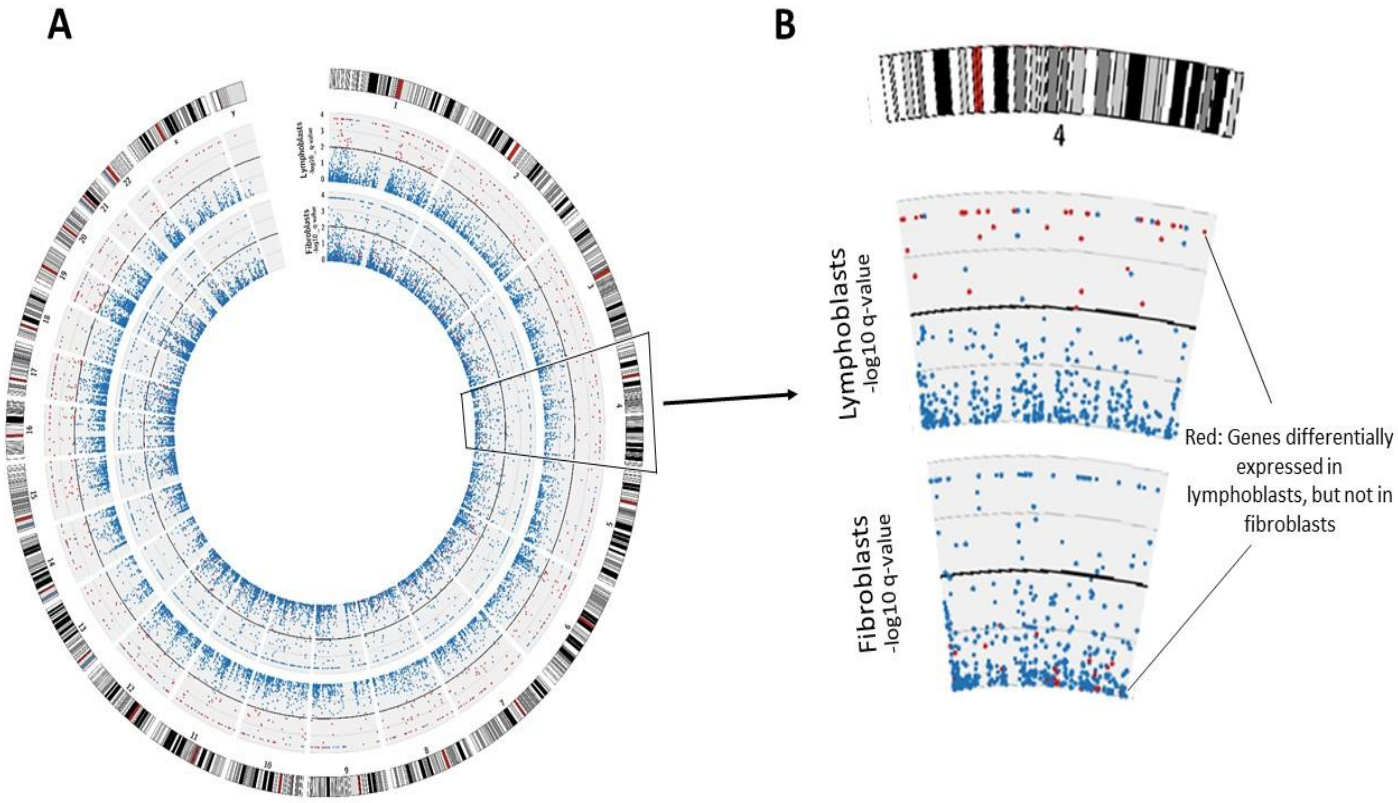
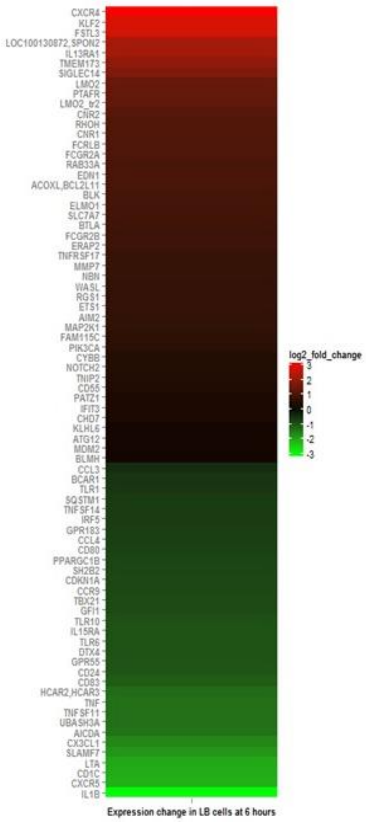


Figure 1. (A) Genome-wide view of the transcriptional response 2 hours after in vitro stimulation with 1 µM dexamethasone in lymphoblasts (outer circle) and fibroblasts (inner circle). Each dot corresponds to a gene. Genes that are differentially expressed (dexamethasone versus vehicle) will appear higher in the plot. The black line represents a differential expression q-value of 0.01 (-log10 q-value = 2). Genes marked in red are differentially expressed in lymphoblasts but not in fibroblasts, as seen in the close-up of chromosome 4 (B).

discoveries, including that of a negative GRE (3, 4) and of a glucocorticoid-responsive long non-coding RNAs (5) suggest that other important aspects of drug action at the genome level remain incompletely characterized. Ongoing in vitro experiments with human cells in our laboratory suggest that there are indeed fundamental differences in the genomic response of immune and non-immune cells to glucocorticoids **(**Figure 1**)**.

We have identified genes that are known to be involved in immune function and are differentially expressed in immune but not in non-immune cells after in vitro exposure to glucocorticoids. Within this group, we have observed genes that increase and genes that decrease in expression after glucocorticoid stimulus **(**Figure 2**)**.



This includes genes that had been previously observed to respond to glucocorticoids (e.g., decreased expression of the cytokines *IL1B* and *TNF*), as well as genes that were previously not known to be affected by glucocorticoids (e.g., increased expression of *KLF2*, a transcription factor involved in T cell quiescence, regulation of T cell migration(6), negative regulation of B cell clonal expansion (7), and regulation of acute and chronic inflammation (8)). In addition, the genome-wide view of the transcriptional response provided by RNA sequencing has given us a more complete picture of the effects of glucocorticoids on hematopoietic and non-hematopoietic cell types than was previously available. Among genes that are differentially expressed in hematopoietic but not in non-hematopoietic cells after a glucocorticoid stimulus, we have observed a strong representation of TNF signaling molecules (decreased expression of *TNFSF11*, *TNFSF14*, *TNF*, *LTA*, and *LTB*), TLR signaling molecules (decreased expression of *TLR1*, *TLR6*, *CCL3*, *CD80*, and *CCL4*), and genes known to be involved in leukocyte migration and margination (*CXCR4*, *CXCR5*, *KLF2*).

Figure 2. Immune genes that are differentially expressed in lymphoblasts but not in fibroblasts include upregulated and downregulated genes.

Importantly, a number of genes known to be involved in immune function and differentially expressed in lypmhoblastoid cells are not expressed at all in fibroblasts **(**Figure 3**)**, providing support for the hypothesis that there are potential targets for mimicking the immunologic effects of glucocorticoids without direct adverse consequences on non-immune cells.

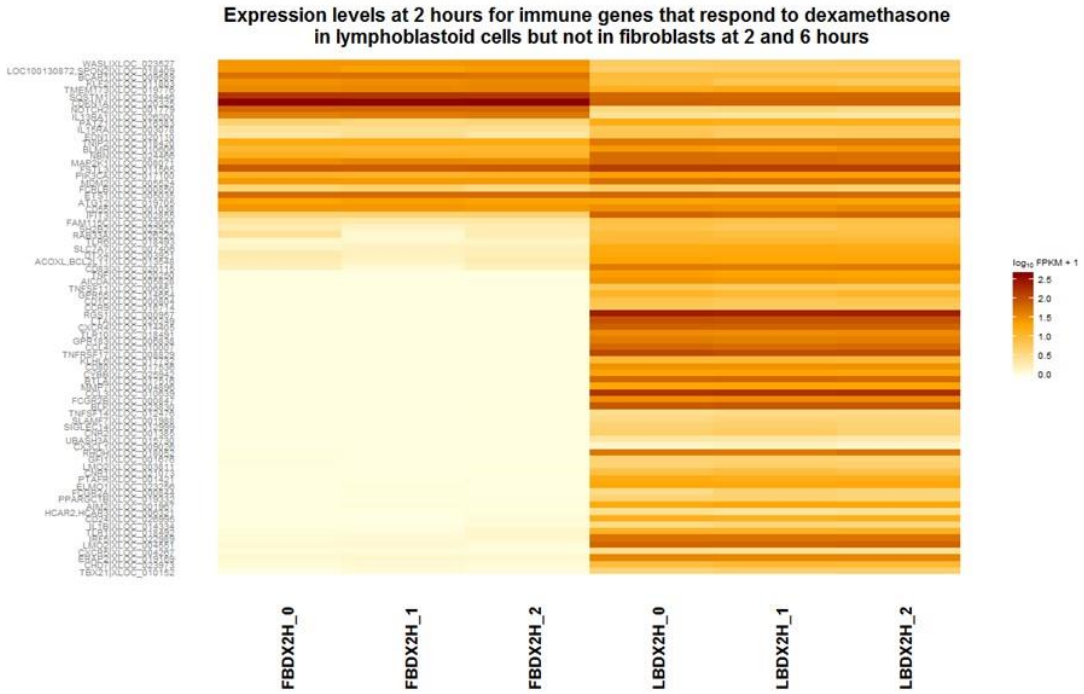


Figure 3. Many of the immune genes that are differentially expressed in lymphoblasts at 2 and 6 hours are not expressed at all in fibroblasts. Expression levels are displayed in fragments per kilobase of transcript per million mapped reads (FPKM) in an RNA sequencing experiment 2 hours after stimulation with 1 μM dexamethasone. The 3 columns on the left are technical triplicates of a fibroblast culture, and the 3 columns on the right are technical triplicates of a lymphoblast culture.

Finally, our in vitro data suggests that there is a significant and differential response of the non-coding genome to the glucocorticoid stimulus: Approximately 16% of the transcripts that are differentially expressed in lymphoblasts but not in fibroblasts after a glucocorticoid stimulus correspond to long-non-coding RNAs (Table 1).

	2 hours	6 hours
Total transcripts DE in lymphoblasts but not in fibroblasts	586	2047
lncRNA transcripts DE in lymphoblasts but not in fibroblasts	96	315
Proportion of DE transcripts that are lncRNA	16.4%	15.4%

FDR for differential expression: 0.01

Table 1. Approximately 16% of the transcripts that are differentially expressed in immune but not in non-immune cells after a glucocorticoid stimulus correspond to long non-coding RNAs. FDR=False Discovery Rate

At the completion of this study, we expect to generate a list of human genes and non-coding RNAs that are differentially expressed and regulated in response to glucocorticoids between immune and non-immune cells in vivo. These data will be used to identify transcripts, their corresponding proteins, and the molecular pathways that are best candidates for targeted intervention. Potential targets could then be validated with siRNA libraries, with the long-term goal of developing small-molecule (9, 10) or nanoparticle-facilitated-RNAi (11, 12) interventions that reproduce the therapeutic action of glucocorticoids in immune cells while avoiding the harmful side effects of this class of drugs on other tissues.

1.2 Rationale for Performing the Study in Healthy Subjects

While the long-term goal of this line of research is to allow the development of targeted therapeutic interventions for people with inflammatory and autoimmune disorders, the immediate obstacle to accomplishing that goal is a lack of understanding of the human genomic response to glucocorticoids in immune and non-immune cells. The immediate goal of this study is to understand this response. For that purpose, one could start with either patients or healthy volunteers. However, patients who require glucocorticoids, even with a common clinical indication, show great variability in terms of disease state, co-morbidities, and the use of other immunosuppressive and anti-inflammatory drugs. For an initial study, these strong confounders would likely obscure the identification of the specific effects of glucocorticoids, and would likely require a large number of subjects to reach an answer that could potentially be obtained by studying a smaller number of less clinically variable subjects, such as healthy volunteers. Similarly, the risk of side effects from an experimental administration of glucocorticoids would

likely be higher in patients with significant co-morbidities and additional immunosuppressive drugs than in a well-selected cohort of healthy volunteers. Therefore, the safest and most scientifically rational approach to the question of how the human genome responds to glucocorticoids is to perform an initial study in a cohort of healthy volunteers, and to use the results obtained in this cohort as the basis for designing and interpreting future studies in patients with inflammatory and autoimmune disorders.

1.3 Choice of a Glucocorticoid and Routes of Administration

Chemical modifications to the 21-carbon corticosteroid backbone (e.g. introduction of a carbon 1-2 double bond, methylation at carbon 6, fluorination at carbon 9), have led to the diverse set of glucocorticoids that is currently available for clinical use. These modifications have mostly altered the pharmacokinetic properties of the drugs, and the relative proportion of mineralocorticoid and glucocorticoid activity, but they do not appear to have significantly altered the mechanism of action or the side effect profile of glucocorticoids. Therefore, the choice of a specific glucocorticoid for our study was based on the frequency of its use in clinical practice, the availability of reports of safe use in studies of healthy human volunteers, and the availability of an intravenous formulation (to reduce potential inter-individual variation in rates of absorption). Methylprednisolone is widely used in clinical practice, with indications in the management of diverse conditions including dermatomyositis (13), lupus nephritis (14), acute asthma exacerbations (15), and relapses of multiple sclerosis (16). Intravenous methylprednisolone has been used in multiple studies of healthy volunteers since the early 1970s (17-19), without reports of serious adverse events (SAEs) in those studies. Sodium succinate and phosphate esters are available for intravenous use. Therefore, we have identified methylprednisolone sodium succinate as the glucocorticoid of choice for our study. Because our study involves a comparison of the effect of glucocorticoids in immune and non-immune cells, in addition to blood we will serially sample a non-hematopoietic tissue. Skin is composed mostly of non-hematopoietic cells and is easily accessible, so we have selected the skin as a non-hematopoietic tissue for sampling. It is unclear from existing literature whether the concentrations of methylprednisolone in skin after intravenous administration are sufficient to induce gene expression changes after short-term administration. However, high concentrations of methylprednisolone in the skin can be achieved with topical administration. Although topical methylprednisolone is widely used in clinical practice around the world, an IND application will be required for topical methylprednisolone use in our study given that topical methylprednisolone is not currently marketed in the United States.

1.4 Choice of Dose of Glucocorticoid

Clinically, the dosing of glucocorticoids has been described in categorical terms based on prednisone equivalents per day (20). These categories are low dose (prednisone-equivalent dose > 0 and ≤ 7.5 mg/day), medium dose (prednisone-equivalent dose >7.5 and ≤ 30 mg/day), high dose (prednisone-equivalent dose > 30 and ≤ 100 mg/day), very high dose (prednisone-equivalent dose > 100 and ≤ 250 mg/day), and pulse dose (prednisone-equivalent dose >250 mg/day). Complete saturation of the cytosolic glucocorticoid receptor is achieved with very high and pulse doses of glucocorticoids (21). Importantly, however, some of the pharmacologic effects of glucocorticoids that are not mediated directly through their interaction with the glucocorticoid receptor appear to be higher at the pulse dose range when compared to the very high dose range (20). Although the effects that are not mediated directly through binding of the glucocorticoid molecule to its cytosolic receptor have been termed "non-genomic effects", it is clear that some of those effects ultimately lead to changes in gene expression, via direct or indirect interactions with other transcription factors (including NFκB, AP-1, and STAT5) or competition for nuclear co-activators. Therefore, in order to examine the full range of the human transcriptional response to glucocorticoids, the pulse dose range is the most scientifically sound. From a clinical point of view, pulse doses of methylprednisolone sodium succinate are routinely employed when a maximum anti-inflammatory effect is desired, as is the case in dermatomyositis (13), lupus nephritis (14), and relapses of multiple sclerosis (16). Although the most significant clinically observable effects on inflammation are obtained at pulse doses of glucocorticoids, long-term administration of these doses is not possible without severe, dose-limiting side effects. Therefore, most of the clinical use of pulse doses of glucocorticoids involves short courses of 3 to 10 days. Our study involves a single intravenous dose. It is important, in this context, to make an objective assessment of the potential risks of short-term administration of pulse doses of glucocorticoids, based on the available literature. There have been isolated case reports of serious cardiovascular events following the administration of methylprednisolone at a dose of 1 gram (22), but a causal relationship is difficult to establish. A retrospective study of adverse events (AEs) in 84 patients with rheumatic diseases who received a combined total of 275 treatments with 1 gram IV methylprednisolone daily for 3 consecutive days found a low rate of AEs. Again, the events that were reported were difficult to attribute to the methylprednisolone infusion due to the concomitant presence of medical illness and the use of other drugs (23). We were unable to identify any studies that directly assessed the potential long-term effects of a single dose of methylprednisolone, likely because the drug is clinically used in courses of 3 to 10 days. The use of doses of methylprednisolone up to 30 mg/kg IV in healthy volunteers was initially reported in 1970 (17). In this small study (6 subjects received the highest dose and 6 subjects received placebo), no short-term toxic effects were identified even at a high rate of infusion (30 mg/kg infused over 10 minutes). Subsequent studies have administered methylprednisolone phosphate at doses up to 1 gram IV to small groups (n = 6 and n = 12) of healthy volunteers (18, 19), again without reports of serious short-term AEs.

While a dose of 1 gram of methylprednisolone IV is likely to be safe based on the existing data, we have selected a lower dose, near the lower end of the pulse-dose range. The dosage forms of methylprednisolone sodium succinate that are clinically available include vials of 40, 125, 500, and 1000 mg. Therefore, we have selected a dose of 250 mg, equivalent to 312.5 mg of prednisone.

2 Study Objectives and Endpoints

Primary Objective: To elucidate fundamental differences in the genomic response of immune and non-immune cells to glucocorticoids

Primary Endpoint: A list of human genes and non-coding RNAs that are differentially expressed and regulated in response to glucocorticoids between immune and non-immune cells

Secondary Objective: To identify potential targets for small-molecule or nanoparticle-facilitated-RNAi interventions that reproduce the therapeutic action of glucocorticoids while avoiding harmful effects

Secondary Endpoints:

- A list of transcripts and molecular pathways representing the best candidates for targeted therapeutic alternatives to glucocorticoids
- Validation of such targets by functional studies

3 Study Design

Healthy volunteers will present to the NIH CC for a brief screening visit. A baseline whole-blood sample will be obtained on the screening day from volunteers who are eligible for the study and choose to enroll. Enrolled volunteers will return for a day-long study visit ("infusion visit"): following a pre-infusion whole-blood sample, volunteers will receive a single intravenous dose of 250 milligrams (mg) of methylprednisolone sodium succinate infused over 30 minutes. Whole-blood samples will then be obtained serially, in one of two regimens: 1 and 2 hours or 2 and 4 hours after the start of drug administration. A 3-mm skin punch biopsy sample may be obtained prior to IV methylprednisolone administration. If so, a single application of topical methylprednisolone 0.1% will be applied to a small area (2 x 2 cm) of the skin contralateral to the baseline biopsy site post IV methylprednisolone administration, and a second skin punch biopsy will be performed 4 hours after the start of drug administration, in the area of skin where topical methylprednisolone was applied. Samples will be processed as follows:

Table 2. Processing and use of samples from healthy volunteers

Visit	Volunteer sample	Initial processing	Downstream use
Screening visit	Baseline whole-blood sample (103 mL)	10 mL: Complete blood count, acute care panel, prothrombin time (PT) & partial thromboplastin time (PTT), interferon gamma release assay (IGRA), and serum pregnancy test	NA
		10 mL: HIV and hepatitis A, B and C testing	NA
		5 mL: DNA purification	Genome-wide genotyping and/or DNA sequencing
		8 mL: Plasma purification	Plasma banking
		60 mL: Isolation of hematopoietic cell sub-types (e.g. neutrophils, B cells, CD4+ T cells, CD8+ T cells, monocytes, NK cells)[a]	RNA-level, DNA-level, protein-level, and/or functional assays
		10 mL: PBMC isolation	PBMC banking
Infusion visit	Pre-infusion and 4-hour skin biopsies (up to 3 mm x 2)	Fibroblast isolation and establishment of a fibroblast cell line	In vitro exposure of fibroblasts to glucocorticoids iPS generation for in vitro study of other non-hematopoietic cell types
		RNA isolation	RNA sequencing Small-RNA sequencing
	Pre-infusion whole-blood sample (88 mL)	60 mL: Isolation of hematopoietic cell sub-types (e.g. neutrophils, B cells, CD4+ T cells, CD8+ T cells, monocytes, NK cells)	RNA-level, DNA-level, protein-level, and/or functional assays
		10 mL: PBMC isolation	PBMC banking
		8 mL: Plasma purification	Plasma banking
		5 mL: Complete blood count, acute care panel and serum pregnancy test	NA
		5 mL: Serum	Concentration of methylprednisolone
	Serial post-infusion whole-blood samples (88 mL x 2)	60 mL: Isolation of hematopoietic cell sub-types (e.g. neutrophils, B cells, CD4+ T cells, CD8+ T cells, monocytes, NK cells)	RNA-level, DNA-level, protein-level, and/or functional assays
		10 mL: PBMC isolation	PBMC banking
		8 mL: Plasma purification	Plasma banking
		5 mL: Complete blood count and acute care panel	NA
		5 mL: Serum	Concentration of methylprednisolone

[a]The purpose of this initial sample will be to assess the level of variation in the baseline.

Abbreviations: iPS induced pluripotent stem (cells), NA not applicable, NK natural killer, PBMC: Peripheral blood mononuclear cell, TBNK: Flow cytometry panel for T lymphocytes, B lymphocytes, and NK cells

Volunteers will be discharged after collection of the 2-hour or 4-hour blood and biopsy samples (depending on the regimen of blood collection), measurement of vital signs, and documentation of any AEs. Follow-up phone calls 1 day and 5 days after discharge will document any adverse effects related to the drug or skin biopsy. If any AEs that may require treatment are documented on the follow-up phone calls, volunteers will be asked to come to the NIH CC's outpatient clinic for evaluation by the Principal Investigator.

We propose an adaptive study design, in which we will recruit an initial cohort of 20 subjects. After processing the samples from these subjects, we will perform differential expression analysis to determine if recruitment of additional subjects (up to a maximum of 50) would impact the biological interpretation of the data. If additional data would be unlikely to change the study outcome, then no further recruitment will be pursued.

4 Study Population

4.1 Recruitment Plan

Healthy volunteers (N=50 maximum) may be recruited through the NIH Clinical Research Volunteer Program, the Patient Recruitment and Public Liaison Office, or through the posting of advertisements. All flyers and advertisements will be submitted to the NIH IRB for approval.

4.2 Inclusion Criteria

1. Age 18 to 64 years
2. Willingness to have samples stored for future research
3. Willingness to undergo genetic testing

4.3 Exclusion Criteria

1. Body Mass Index < 18 or > 35
2. Difficult peripheral venous access (as determined by study staff at screening)
3. History of severe allergic reaction to glucocorticoids
4. History of autoimmune or autoinflammatory disease
5. Active solid or hematologic malignancy
6. History of a skin condition (such as psoriasis, pemphigus, or atopic dermatitis) that could affect the results of the transcriptional analysis of the skin biopsy samples
7. Diabetes mellitus
8. Cancer chemotherapy within the past 5 years
9. Surgery within the past 8 weeks
10. History of recent (within the past 30 days) infection

11. A positive test for human immunodeficiency virus, or hepatitis A, B or C virus infection (viral markers hepatitis screen, which includes HBsAg, anti-HCV IgG, anti-HAV IgM)
12. A positive or indeterminate test for latent tuberculosis (interferon gamma release assay)
13. History of parasitic, amebic, fungal or mycobacterial infections, or other possible latent infections
14. Coagulation test (PT and PTT) results outside of normal range
15. History of a bleeding disorder
16. Use of a glucocorticoid (including topical or inhaled), a nonsteroidal anti-inflammatory drug (including aspirin and selective COX-2 inhibitors), an anti-epileptic drug, an anticoagulant, a statin, a selective serotonin reuptake inhibitor, a macrolide, an azole, diltiazem, troglitazone, rifabutin, ranitidine, rifampin, quinine, quinidine, cyclosporine, amiodarone, or St. John's wort, within the past 30 days. A list of prohibited medications, including specific examples for each class, is provided as an attachment.
17. Vaccination within the past 30 days
18. Receipt of an immunosuppressant or immunomodulatory drug within the past 30 days
19. Pregnancy, current or within the past 90 days, or trying to become pregnant during the study
20. Current breastfeeding
21. Complete blood count (CBC) and/or acute care panel values are both outside of the NIH Department of Laboratory Medicine normal reference range and deemed clinically significant by the principal investigator
22. Any electrocardiogram (ECG) abnormality that is clinically significant
23. Any condition that, in the investigator's opinion, may put the participant at undue risk or compromise the study's scientific objectives
24. Participation in a clinical protocol which includes an intervention that, in the opinion of the investigator, may affect the results of the current study

4.4 Justification for Exclusion of Special Populations

Children: Minors will be excluded because there is the potential for greater than minimal risk and no prospect for direct benefit.

Adults who lack capacity to consent: Because this study has no prospect for direct benefit, adults who lack decision-making capacity to provide informed consent will be excluded at screening. Enrolled participants who permanently lose capacity to consent during the study will be withdrawn (section 11.7), in accordance with NIH Human Research Protection Program (HRPP) Policy 403.

Pregnant and breastfeeding individuals: Pregnant volunteers will be excluded because there are no adequate and well-controlled studies of glucocorticoids in pregnant women. Glucocorticoids should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Breastfeeding volunteers will be excluded due to the potential for serious adverse reactions in nursing infants from glucocorticoids.

NIH staff: NIH staff and family members of study team members may be enrolled in this study as this population meets the study entry criteria. Neither participation nor refusal to participate as a subject in the research will have an effect, either beneficial or adverse, on the participant's employment or position at NIH. Every effort will be made to protect participant information, but such information may be available in medical records and may be available to authorized users outside of the study team in both an identifiable an unidentifiable manner.

The NIH investigator will provide and request that the NIH staff member review the Frequently Asked Questions (FAQs) for Staff Who are Considering Participation in NIH Research and the Leave Policy for NIH Employees Participating in NIH Medical Research Studies (NIH Policy Manual 2300-630-3). Please see section 15.1.1 for consent of NIH staff.

5 Study Agents

5.1 Methylprednisolone sodium succinate for injection

5.1.1 Disposition and Dispensation

Study agent will be distributed via the NIH CC Pharmacy according to standard pharmacy procedures.

5.1.2 Formulation

Methylprednisolone sodium succinate for injection, USP (SOLU-MEDROL sterile powder, Pfizer, Inc.) is an anti-inflammatory glucocorticoid which occurs as a white, or nearly white, odorless hygroscopic, amorphous solid. It is very soluble in water and in alcohol; it is insoluble in chloroform and is very slightly soluble in acetone.

Methylprednisolone sodium succinate has the same metabolic and anti-inflammatory actions as methylprednisolone. When given parenterally and in equimolar quantities, the two compounds are equivalent in biologic activity.

125-milligram Act-O-Vial System: Each 2 mL (when mixed) contains methylprednisolone sodium succinate equivalent to 125 milligrams methylprednisolone; also 1.6 mg monobasic sodium phosphate anhydrous; and 17.4 mg dibasic sodium phosphate dried.

5.1.3 Dosage and Administration

Study volunteers will receive 250 milligrams administered intravenously over 30 minutes.

5.1.4 Storage

Protect from light. Store unreconstituted product and solution at a controlled room temperature of 20° to 25°C (68° to 77°F). Use solution within 48 hours after mixing.

5.2 Topical methylprednisolone

5.2.1 Disposition and Dispensation

Study agent will be distributed via the NIH CC Pharmacy according to standard pharmacy procedures.

5.2.2 Formulation

1g Advantan emulsion 0.1% (Bayer) contains methylprednisolone aceponate (21-acetoxy-11beta-hydroxy-6alpha-methyl-17-propionyloxy-1,4-pregnadiene-3,20-dione) 1 mg, as the active ingredient. It is an oil-in-water emulsion containing medium chain triglycerides, caprylic-capric-stearic triglyceride, polyoxyethylene alcohol 2-stearylether, polyoxyethylene alcohol-21-stearylether, benzyl alcohol, disodium edetate, glycerol, and purified water.

5.2.3 Dosage and Administration

Advantan emulsion 0.1% will be applied thinly to the skin in a 2 x 2 cm area. This is a one-time application.

5.2.4 Storage

Store below 25°C (77°F).

6 Study Schedule

This study will involve 2 study visits at the NIH CC and 2 follow-up phone calls. Only those volunteers found to be eligible after the screening procedures will return to continue the study. Volunteers will be referred to their Primary Care Physician if any screening tests need further follow-up.

6.1 Screening visit (Day -30 to Day -1)

The following tests will be conducted after informed consent is obtained:

- Physical exam (including BMI assessment) with vital signs
- Medical history and medication review
- ECG

- Blood draw for the following:
    - CBC with differential
    - Acute care panel
    - Serum pregnancy test (for volunteers capable of becoming pregnant)
    - PT and PTT
    - Screening for HIV and hepatitis A, B and C. Test results obtained within the past 30 days will be accepted.
    - Screening for latent tuberculosis with interferon gamma release assay (IGRA)
    - Baseline purification of six hematopoietic cell sub-types (following confirmation of volunteer eligibility)
    - Baseline flow cytometry
    - Banking of baseline plasma, peripheral blood mononuclear cells (PBMCs), and DNA

6.2 Infusion visit (Day 0)

- Targeted physical exam
- Vital signs on first encounter, every 30 minutes from the time of infusion until the time of collection of the four-hour blood sample, and discharge.
- Serum pregnancy test (for volunteers capable of becoming pregnant). For volunteers screened within the past 24 hours, the test will not be repeated.
- Interim medical history and medication review
- Pre-infusion blood draw (for analyses listed in Table 2)
- Optional: Pre-infusion skin biopsy from one arm
- Optional: Application of topical methylprednisolone to the contralateral arm
- Intravenous infusion of methylprednisolone sodium succinate (30 minutes)
- Nursing staff will monitor study subjects for the presence of behavioral abnormalities (altered mood, hyperactivity, disorientation, psychosis)
- Blood draws at 1 and 2 hours or 2 and 4 hours (+/-10 minutes) after the start of infusion (for analyses listed in Table 2)
- Optional: Skin biopsy (at the site where topical methylprednisolone was applied) 4 hours after the start of infusion

6.3 Follow-up Phone Calls (Day 1 and Day 5)

Follow-up phone calls will be made one day (+/- 0 days) and five days (+/- 2 days) after discharge to document the presence or absence of side effects related to the skin biopsy or study drug.

7 Study Procedures/Evaluations

Methylprednisolone sodium succinate infusion: Volunteers will receive a single intravenous dose of methylprednisolone sodium succinate administered through an intravenous catheter over a period of 30 minutes.

Blood draw: Blood will be drawn at screening, immediately prior to the drug infusion, and in one of two regimens: 1 hour and 2 hours (+/- 10 minutes) after the infusion, or 2 hours and 4 hours (+/-10 minutes) after the start of the infusion. Blood will be used for laboratory evaluations and storage as indicated in Table 2. The amount of blood drawn for research purposes will be within the limits allowed for adult research subjects by the NIH CC (Medical Administrative Policy 95-9, Guidelines for Limits of Blood Drawn for Research Purposes in the Clinical Center: http://cc-internal.cc.nih.gov/policies/PDF/M95-9.pdf).

Punch biopsy of the skin, with application of methylprednisolone emulsion: A full-thickness skin sample will be taken from the posterior aspect of the upper third of one arm at baseline for some volunteers. The sample will be obtained with a 3-mm skin biopsy punch, following intradermal anesthesia with 2% lidocaine injected with a 30-gauge needle. Prior to insertion of the biopsy punch, the skin will be stretched along the long axis of the arm, perpendicular to the lines of least tension. After collection of the sample, the elliptical wound will be closed with gel foam or steri-strips and covered with a dressing. Methylprednisolone emulsion will be applied to the same location (upper third of the posterior aspect) on the contralateral arm, which will be biopsied 4 hours after the start of infusion, following the same procedure. The skin biopsy sample will be divided into two fragments: The most superficial fragment (which is expected to contain the epidermis and dermis) will be used for RNA purification, and the deeper fragment (which is expected to contain sub-dermal connective tissue) will be employed to generate a fibroblast culture for future testing of in vitro exposure to glucocorticoids and for future generation of iPS cells to study the response of other cell types to glucocorticoids.

ECG (12-lead) will be performed to rule out electrical cardiac abnormalities and ensure eligibility to receive the study agent.

8 Potential Risks and Benefits

8.1 Risks

Methylprednisolone sodium succinate infusion: Side effects from a one-time, intravenous dose of methylprednisolone may include metallic or bitter taste in the mouth during and possibly after infusion, upset stomach, sleep disturbance (insomnia, sleepiness/fatigue), increased appetite, water retention, swelling in hands/ankles/feet, weight gain, temporary increase in blood glucose, temporary increase in blood pressure, mood disturbance or unusual behavior, electrolyte

imbalance, muscle cramps, back pain, headaches, and frequent urination lasting 1-2 weeks after infusion.

Individuals who received methylprednisolone to treat illness have had skin problems such as rash or dry skin, easy bruising, growth of body hair, irregular menstrual cycles, abnormal results of liver enzyme tests, enlargement of the liver, inflammation of the pancreas, numbness in the feet and hands, eye problems, and bone problems such as osteoporosis and damage to the hip bone. Many of these effects were dependent on the length of treatment and we do not expect that they will occur with a single dose.

Corticosteroids may decrease resistance to infection and the ability to localize infection. Infections with any pathogen in any location of the body may be associated with the use of corticosteroids alone or in combination with other immunosuppressive agents. Corticosteroids may reactivate or exacerbate latent fungal, bacterial, or viral infections. Individuals with known or suspected active or latent infections, as well as those using immunosuppressive agents, will be excluded from study participation. In addition, subjects will be advised to limit travel for 4 weeks following the methylprednisolone infusion.

Allergic reactions including anaphylactoid reaction, anaphylaxis and angioedema have been reported in patients receiving methylprednisolone.

Serious cardiac, musculoskeletal, neurologic/psychiatric and other side effects of intravenous methylprednisolone have been reported in patients with underlying disease. Such effects are not expected in this study involving administration of a single dose in healthy volunteers.

There are reports of cardiac arrhythmias and/or cardiac arrest following the rapid administration of large IV doses of methylprednisolone (greater than 0.5 gram administered over a period of less than 10 minutes). Bradycardia has been reported during or after the administration of large doses of methylprednisolone sodium succinate, and may be unrelated to the speed or duration of infusion.

Methylprednisolone emulsion: The most common side effects are itching, burning and redness of skin. Allergic skin reactions are rare. The following side effects may occur: vesiculation folliculitis, hypertrichosis, skin thinning, telangiectasia, and perioral dermatitis. Systemic absorption of topically applied corticosteroids may occur, particularly under the following conditions: when large quantities are used, or when application is made to wide areas of the body, or to damaged skin, when potent topical corticosteroids are used, and when the occlusive dressing technique is applied. Depression of the hypothalamic-pituitary-adrenal axis with consequent suppression of the adrenal gland may occur. Benign increased intracranial pressure

has been rarely reported. Methylprednisolone emulsion must not be applied to the face or to skin crease areas, and should not come in contact with the eyes.

Blood draw: The risks of drawing blood include pain, bruising, bleeding, and, rarely, fainting or infection.

Skin biopsy: The risks of skin biopsy include local pain, bleeding, infection, and potential scar and keloid formation. The use of local and systemic glucocorticoids can increase the risk of infection and poor healing of the biopsy site. A local anesthetic administered prior to biopsy can cause a mild burning sensation upon injection, skin irritation and rarely allergic reaction. Oral analgesics will be used to manage pain.

ECG: An ECG is of minimal risk to the subjects and causes no significant side effects. Unexpected or incidental medical findings unrelated to the study may occur and may require the subject to follow up with an appropriate physician for standard medical care or follow up. The electrodes may feel cold when applied; in rare cases some people will develop a rash or skin irritations where the patches are placed. This type of irritation usually resolves by itself or occasionally with topical medication.

RNA sequencing, DNA sequencing, and genome-wide genotyping: Studies of RNA molecules (RNA sequencing) will be performed to identify changes in gene expression in response to the study drug. Studies of DNA molecules (genome-wide genotyping) will be performed to identify variants in the genome that may influence gene expression. These studies will involve assays of specific regions of the genome. Depending on the findings, sequencing of DNA (either specific regions or the entire DNA molecule) may be performed in the future. These studies are not designed or intended to produce data regarding health risks, incurable conditions, or information contradictory to stated biological relationships between individuals. Therefore, it is not expected that the results from these studies will be returned to volunteers. In the rare event of detecting an incidental DNA finding related to a volunteer's health, we will share the information with the volunteer, after confirming the result on an additional sample (collected prospectively or previously stored) in a laboratory certified by the Clinical Laboratory Improvement Amendments program. The confirmed result would be placed in the NIH medical record. Such information may have psychological implications; genetic counseling and advice is available from the NIH to help volunteers understand the implications of incidental findings, if necessary. Following genetic testing, data will be shared in a controlled-access public database in accordance with NIH guidelines, for other investigators to benefit from the information (e.g., dbGaP, the Database of Genotypes and Phenotypes). However, no personal, identifiable information will be shared in this process, as the results will only be shared with a code.

8.2 Benefits

This research is not designed to benefit study volunteers. Knowledge gained from this research may inform the development of future anti-inflammatory and immunosuppressive medications.

9 Research Use of Stored Human Samples, Specimens, or Data

Intended Use: Samples and data collected under this protocol may be used to study the body's response to glucocorticoids. Genetic testing will be performed on some of these samples. A separate signed informed consent document will be obtained for any other research not described in this protocol.

Storage: Samples will be stored in a secure location with limited access. Samples and data will be coded prior to storage using barcodes assigned by the investigators. The database linking a barcode with a specific individual will be kept in password-protected computers. Only investigators will have access to the samples and data.

Tracking: Data acquired under this protocol will be tracked using the Clinical Research Information Management System of the NIAID (CRIMSON). After coding, laboratory specimens will be tracked using the Biological Specimen Inventory (BSI) system, under a NIAID-wide contract for laboratory specimen banking and tracking.

Disposition at the completion of the protocol:

- In the future, other investigators (both at NIH and outside) may wish to use these samples and/or data for research purposes. If the planned research falls within the category of "human subjects research" on the part of the NIH researchers, NIH IRB review and approval will be obtained. This includes the NIH researchers sending out coded and linked samples or data and getting results that they can link back to their subjects.

Reporting the loss or destruction of samples/specimens/data to the IRB:

- Any loss or unanticipated destruction of samples or data (for example, due to freezer malfunction) that meets the definition of a reportable event will be reported to the NIH IRB according to NIH HRPP Policy 801.
- Subjects may decide at any point not to have their samples stored. In this case, the principal investigator will destroy all known remaining samples and report what was done to both the subject and to the IRB.

Handling of specimens/data from subjects who must be removed from the study: Subjects who are enrolled may need to be removed from the study after the screening visit, if the laboratory and/or ECG results obtained on that visit show an abnormality that meets the study's exclusion criteria. Subjects may also need to be removed from the study if they develop a

condition or side effect that makes them ineligible to continue participation, if the study is cancelled or stopped, or if they fail to comply with the study requirements. In any of these cases, the subject will be notified by the Study Coordinator. Any blood or skin samples collected prior to removal of a subject will be processed in the same way as those obtained from subjects who are not excluded from the study. Data obtained from these samples may be used for the purpose of establishing the level of variation of the baseline measurements under different circumstances, which can be informative. At the time of notification of removal from the study, subjects will be reminded of their right, as detailed in the consent form, to request for their samples to be destroyed. The purpose of this provision is to ensure that research samples of potential use are not unnecessarily destroyed, while effectively respecting the research subjects' right to request destruction of their samples.

10 Remuneration Plan for Volunteers

Volunteers will be compensated for their time and inconvenience according to the following schedule:

Screening visit: \$20 for the 1st hour plus \$10 for each additional hour (20+10[3]) = \$50
Screening blood draw: \$20
Infusion visit: \$20 for the 1st hour plus \$10 for each additional hour (20+10[6]) = \$80
Pre- and post-infusion blood draws: \$20 (x3) = \$60
Pre- and post-infusion punch biopsies of the skin: \$50 (x2) = \$100
Total with biopsy = \$310
Total without biopsy = \$210

In the event that follow-up blood draws need to be scheduled after the infusion visit, as detailed in Section 11.2.1 below, each additional visit will be compensated at \$20.

Volunteers will receive compensation by check, direct deposit, or Automated Clearing House (ACH) payment after their participation in the study is complete.

Travel-related expenses (e.g., transportation, hotel, meals) will not be paid for or reimbursed under this protocol.

11 Assessment of Safety

11.1 Definitions

The NIAID Clinical Safety Office (CSO) is responsible for sponsor safety oversight of this study, and the definitions below comply with CSO requirements.

Adverse Event (AE): An AE is any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (eg, abnormal physical exam or laboratory finding),

symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to the research.

Adverse Reaction (AR): An AE that is caused by an investigational agent (drug or biologic).

Suspected Adverse Reaction (SAR): An AE for which there is a reasonable possibility that the investigational agent caused the AE. 'Reasonable possibility' means that there is evidence to suggest a causal relationship between the drug and the AE. An SAR implies a lesser degree of certainty about causality than AR, which implies a high degree of certainty.

Serious Adverse Event (SAE): An SAE is an AE that results in one or more of the following outcomes:

- death
- a life-threatening event (places the subject at immediate risk of death from the event as it occurred)
- an inpatient hospitalization or prolongation of an existing hospitalization
- a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- a congenital anomaly/birth defect
- a medically important event*

*Medical and scientific judgment should be exercised in deciding events that may not be immediately life threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed above.

Unexpected Adverse Event: An AE is unexpected if it is not listed in the Investigator's Brochure or Package Insert (for marketed products) or is not listed at the specificity or severity that has been observed. It is the responsibility of the IND sponsor to make this determination.

Serious and Unexpected Suspected Adverse Reaction (SUSAR): A SUSAR is an SAR that is both serious and unexpected.

Unanticipated Problem (UP): A UP is any event, incident, experience, or outcome that is

1. unexpected in terms of nature, severity, or frequency in relation to
    a. the research risks that are described in the IRB-approved research protocol and informed consent document, Investigator's Brochure, or other study documents; and
    b. the characteristics of the subject population being studied; and

2. possibly, probably, or definitely related to participation in the research; and
3. places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized. (Per the IND sponsor, an AE with a serious outcome will be considered increased risk.)

Serious Unanticipated Problem (UP): A UP that meets the definition of a Serious Adverse Event or compromises the safety, welfare or rights of subjects or others.

Unanticipated Problem that is not an Adverse Event (UPnonAE): A UP that does not fit the definition of an AE, but which may, in the opinion of the investigator, involve risk to the subject, affect others in the research study, or significantly impact the integrity of research data. Such events would be considered non-serious UPs. For example, we will report occurrences of breaches of confidentiality, accidental destruction of study records, or unaccounted-for study drug.

11.2 Documenting, Recording, and Reporting Adverse Events

AEs occurring from the time the informed consent is signed through study day 5 will be documented, recorded, and reported to the CSO.

The following signs or symptoms are induced by or associated with Methylprednisolone sodium succinate, and any combination of these is expected to occur. These events will not be recorded or reported to the CSO unless they reach a Grade of 3 or higher, are deemed a risk to the participants' rights or well-being, or are deemed appropriate to report under specific circumstances by the Principal Investigator:

- Hyperglycemia
- Leukocytosis
- Neutrophilia
- Monocytopenia
- Eosinopenia
- Flushing
- Increased appetite
- Bitter taste in mouth
- Metallic taste in mouth
- Anxiety

The following signs or symptoms are induced by or associated with Methylprednisolone sodium succinate. These events will not be recorded or reported to the CSO unless they reach a Grade of

4 or higher, are deemed a risk to the participants' rights or well-being, or are deemed appropriate to report under specific circumstances by the Principal Investigator:

- Lymphopenia (Lymphocyte Count Decreased)

The following signs or symptoms are induced by or associated with Methylprednisolone emulsion 1%, and any combination of these is expected to occur. These events will not be recorded or reported to the CSO, unless they reach a Grade of 3 or higher, are deemed a risk to the participants' rights or well-being, or are deemed appropriate to report under specific circumstances by the Principal Investigator:

- Application site burning
- Application site itching
- Application site redness

At each contact with the subject, information regarding AEs will be elicited by appropriate questioning and examinations and will be:

- immediately documented in the subject's medical record/source document,
- recorded in CRIMSON, and
- reported as outlined below (eg, IND sponsor, IRB, and Food and Drug Administration [FDA]).

If a diagnosis is clinically evident (or subsequently determined), the diagnosis rather than the individual signs and symptoms or lab abnormalities will be recorded as the AE.

All abnormal laboratory findings will be reviewed on a routine basis by the principal investigator to identify potential safety signals. An abnormal lab not included on the toxicity table should be assessed in a similar fashion to the criteria above.

11.2.1 Follow-up of reportable abnormal laboratory values

Abnormal laboratory values noted during the infusion visit that require immediate medical intervention will be managed by the study team at the NIH CC, and followed until resolution.

Abnormal laboratory values that do not require immediate medical intervention, but that will be recorded and reported based on the criteria listed in Section 11.2, will be followed until resolution. Laboratory values that remain within the reportable range at the final (4-hour) time point on the day of the infusion visit, will be repeated on an outpatient basis at the NIH CC, until they have returned to the non-reportable range. The first outpatient follow-up blood draw will be scheduled within 72 hours of discharge from the Day Hospital. In the unlikely event that

subsequent follow-up blood draws are necessary, the follow-up intervals will be at the discretion of the Principal Investigator.

11.3 Investigator Assessment of Adverse Events

The investigator will assess all AEs with respect to seriousness (criteria listed above), severity (intensity or grade), and causality (relationship to study agent and relationship to research) according to the following guidelines.

11.3.1 Severity

The Investigator will grade the severity of each AE according to the "Common Terminology Criteria for Adverse Events (CTCAE)" (v 4.0) which can be found at: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm

11.3.2 Causality

Causality (likelihood that the event is caused by the study agent(s)) will be assessed considering the factors listed under the following categories:

Definitely Related

- reasonable temporal relationship
- follows a known response pattern
- clear evidence to suggest a causal relationship
- there is no alternative etiology

Probably Related

- reasonable temporal relationship
- follows a suspected response pattern (based on similar agents)
- no evidence of a more likely alternative etiology

Possibly Related

- reasonable temporal relationship
- little evidence for a more likely alternative etiology

Unlikely Related

- does not have a reasonable temporal relationship

  OR

- good evidence for a more likely alternative etiology

Not Related

- does not have a temporal relationship

OR

- definitely due to an alternative etiology

Note: Other factors should also be considered for each causality category when appropriate. Causality assessment is based on available information at the time of the assessment of the AE. The investigator may revise the causality assessment as additional information becomes available.

11.4 Investigator Reporting Responsibilities to the Sponsor

11.4.1 Adverse Events

AE data will be submitted to the IND sponsor when requested for periodic safety assessments, review of IND annual reports, review of IND safety reports, and preparation of final study reports.

11.4.2 Serious Adverse Events

All SAEs (regardless of relationship and whether or not they are also UPs) must be reported on the Safety Expedited Report Form (SERF) and sent to the Clinical Safety Office (CSO) by fax or e-mail attachment. Deaths and immediately life threatening SAEs must be reported to the CSO within 1 business day after the site becomes aware of the event. All other SAEs must be reported within 3 business days of site awareness.

CSO CONTACT INFORMATION:
Clinical Safety Office
5705 Industry Lane
Frederick, MD 21704
Phone: 301-846-5301
Fax: 301-846-6224
E-mail: rchspsafety@mail.nih.gov

SAEs that have not resolved by the end of the follow-up period are followed until final outcome is known. If it is not possible to obtain a final outcome for an SAE (eg, the subject is lost to follow-up), the reason a final outcome could not be obtained will be recorded by the investigator in CRIMSON and on the SERF.

SAEs that occur after study day 5 that are reported to and are assessed by the investigator to be possibly, probably, or definitely related to study drug must be reported to the CSO.

11.4.3 Unanticipated Problems

UPs that are also AEs must be reported to the CSO by fax or e-mail attachment using the NIH Problem Report Form no later than 7 calendar days of site awareness of the event. UPs that are not AEs are not reported to the CSO.

11.5 Sponsor's Reporting Responsibilities

SUSARs, as defined in 21 CFR 312.32 and determined by the IND sponsor, will be reported to FDA and all participating investigators as IND Safety Reports.

The IND sponsor will also submit an IND Annual Report of the progress of the investigation to the FDA as defined in 21 CFR 312.33.

11.6 Halting Rules for the Protocol

Halting the study requires immediate discontinuation of study agent administered for all subjects and suspension of enrollment until a decision is made whether or not to continue enrollment and study agent administration.

The halting rules are:

- 2 or more subjects experience the same or similar SAEs that are possibly, probably, or definitely related to the study agent;

OR

- 2 or more of the same or similar AE in different subjects that are grade 3 or above and are possibly, probably, or definitely related to the study agent;

OR

- any safety issue that the principal investigator and/or the CSO determines should halt the study.

The principal investigator and/or CSO will determine if the study should be halted. In addition, the FDA may halt the study at any time following review of any safety concerns.

11.6.1 Reporting a Study Halt

If a halting rule is met, a description of the AE(s) or safety issue must be reported by the principal investigator within 1 business day to the CSO and IRB by fax or email.

11.6.2 Resumption of a Halted Study

The IND sponsor, in collaboration with the principal investigator, will determine if it is safe to resume the study. The principal investigator will notify the IRB of the decision on resumption of the study.

11.7 Withdrawal Criteria for an Individual Subject

An individual subject will be withdrawn for any of the following:

- An individual subject's decision. The investigator will attempt to determine the reason for the subject's decision.
- The subject permanently loses decision-making capacity to provide informed consent.
- The investigator determines that continued participation in the study would not be in the best interest of the subject.

Subjects who withdraw or are withdrawn from the study at any point after the pre-infusion skin biopsy will, if willing, continue to receive follow-up phone calls for safety assessments.

11.7.1 Replacement of Withdrawn Subjects or Subjects Who Discontinue Study Agent

Subjects who withdraw or are withdrawn prior to the pre-infusion skin biopsy will be replaced. If a subject is replaced, all the data collected from that subject will still be included for the safety assessment.

11.8 Safety Oversight

11.8.1 Safety Review and Communications Plan

A Safety Review and Communications Plan (SRCP) has been developed for the protocol. The SRCP is an internal communications document between the principal investigator and the CSO, which delineates the safety oversight responsibilities of the principal investigator, the CSO, and other stakeholders. The SRCP also includes the overall plan for conducting periodic safety surveillance assessments.

11.8.2 Sponsor Medical Monitor

A medical monitor, representing the IND sponsor (OCRPRO), has been appointed for oversight of safety in this clinical study. The Sponsor Medical Monitor will be responsible for performing safety assessments as outlined in a Safety Review and Communications Plan (SRCP).

12 Reporting Procedures

12.1 Reporting to the NIH IRB

Reportable events will be tracked and submitted to the IRB as outlined in Policy 801.

12.1.1 NIH Definitions of Protocol Deviation and Non-compliance

Protocol Deviation: Any change, divergence, or departure from the IRB-approved research protocol.

1. Major Deviation – Deviation from the IRB-approved protocol that have, or may have the potential to, negatively impact, the rights, welfare or safety of the subject, or to substantially negatively impact the scientific integrity or validity of the study.
2. Minor Deviation – A Deviation that does not have the potential to negatively impact the rights, safety, or welfare of subjects or others, or the scientific integrity or validity of the study.

Non-compliance: Failure of an investigator to follow the applicable laws, regulations, or institutional policies governing the protection of human subjects in research, or the requirements or determinations of the IRB, whether the failure is intentional or not.

- Continuing Non-compliance – A pattern of recurring non-compliance that either has resulted, or, if continued, may result in harm to subjects or otherwise materially compromise the rights, welfare and/or safety of subjects, affect the scientific integrity of the study or validity of the results. The pattern may comprise repetition of the same non-compliant action(s), or different non-compliant events. Such non-compliance may be unintentional (e.g. due to lack of understanding, knowledge, or commitment), or intentional (e.g. due to deliberate choice to ignore or compromise the requirements of any applicable regulation, organizational policy, or determination of the IRB).
- Serious Non-compliance – Non-compliance, whether intentional or not, that results in harm or otherwise materially compromises the rights, welfare and/or safety of the subject. Non-compliance that materially affects the scientific integrity or validity of the research may be considered serious non-compliance, even if it does not result in direct harm to research subjects.

12.2 Reporting to the NIAID Clinical Director

The principal investigator will report UPs, major protocol deviations, and deaths to the NIAID clinical director according to institutional timelines.

13 Site Monitoring Plan

According to the ICH GCP guidelines, section 5.18, and FDA 21 CFR 312.50, clinical protocols are required to be adequately monitored by the study sponsor. This study monitoring will be conducted according to the "NIAID Intramural Clinical Monitoring Guidelines." Monitors under contract to the NIAID/OCRPRO will visit the clinical research site to monitor aspects of the study in accordance with the appropriate regulations and the approved protocol. The objectives of a monitoring visit will be: 1) to verify the existence of signed informed consent documents

and documentation of the consent process for each monitored subject; 2) to verify the prompt and accurate recording of all monitored data points, and prompt reporting of all SAEs; 3) to compare CRIMSON data abstracts with individual subjects' records and source documents (subjects' charts, laboratory analyses and test results, physicians' progress notes, nurses' notes, and any other relevant original subject information); and 4) to help ensure investigators are in compliance with the protocol. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements (Office for Human Research Protections [OHRP]), FDA, and applicable guidelines (ICH GCP) are being followed. During the monitoring visits, the investigator (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit.

The investigator (and/or designee) will make study documents (eg, consent forms, CRIMSON data abstracts) and pertinent hospital or clinical records readily available for inspection by the local IRB, the FDA, the site monitors, and the NIAID staff for confirmation of the study data.

A specific protocol monitoring plan will be discussed with the principal investigator and study staff prior to enrollment. The plan will outline the frequency of monitoring visits based on such factors as study enrollment, data collection status, and regulatory obligations.

14 Statistical Considerations

Definition of the Study Endpoints

- The primary endpoint of this study is a set of lists of human genes and non-coding RNAs (small RNAs and lncRNAs) that are differentially expressed in vivo after a glucocorticoid stimulus in each of six immune cell types (B cells, CD4+ T cells CD8+ T cells, NK cells, neutrophils, and monocytes) and one non-immune tissue (skin).
- The secondary endpoint is a list of the genes and molecular pathways that represent the best candidates for targeted therapeutic alternatives to glucocorticoids.

Bioinformatics Pipeline and Data Handling

The output of the RNA and small-RNA sequencing runs (bcl files) will be converted to FASTQ format with the software program bcl2fastq. Quality control of the resulting FASTQ files will be performed with the software program FASTQC.

RNA sequencing data will be aligned to the most recent human genome assembly using the software program TopHat (24). The resulting BAM files, the most recent version of the GENCODE human genome annotation, and a mask file of ribosomal and mitochondrial RNA annotations will be used to generate a transcriptome assembly with the software program Cufflinks (25), which models expression at the level of spliced transcripts, and not only at the level of genes or exons. A merged transcriptome file with data from all subjects in an analysis will then be generated with the software program Cuffmerge (26). Transcript abundance will be

estimated with the software program Cuffquant (26), which outputs expression values for each transcript normalized by the length of the transcript and by the number of mapped reads for a particular sample (fragments per kilobase of transcript per million mapped reads, or FPKM).

Small RNA sequencing data will be aligned to the most recent human genome assembly using the software program STAR (27). The resulting BAM files and the most recent version of the GENCODE human genome annotation will be used to annotate and quantify small RNAs with the software program ShortStack (28, 29).

Visualization and gating for the flow cytometry data will be performed with the software program FlowJo. Analysis of flow cytometry and high-throughput cytokine measurement data will be performed in R using custom scripts.

Differential Expression Analysis:

Differential expression analysis for the RNA-seq data will be performed with the software program Cuffdiff (25). In short, the Cuffdiff algorithm models how variability in the measured fragment counts for a transcript depends on its expression level as well as its splicing structure. The algorithm captures uncertainty in a transcript's fragment count as a beta distribution and the overdispersion in this count (determined by globally fitting the observed variance in fragment counts as a function of the mean across replicates) with a negative binomial distribution. Under the resulting beta negative binomial model of fragment count variability, it estimates count variances for each transcript in each sample. These variance estimates are used for statistical testing to report significantly differentially expressed genes and transcripts. Raw p-values and False Discover Rate (FDR)-based q-values (30) are reported.

Differential expression analysis for the small-RNA-seq data will be performed in R, with the package DESeq (31). In short, the DESeq algorithm models fragment count data with a negative binomial distribution and a shrinkage estimator for the distribution’s variance. Our choice of this algorithm for the small-RNA-seq data is based on the fact that most human small RNA molecules are not spliced, and DESeq uses raw fragment counts, without the assumption of spliced transcripts employed by the Cufflinks/Cuffdiff algorithms.

For each cell type, we will assess the level of variation in the baseline measurements by comparing the expression values on the day of the screening visit to the 0-hour (pre-infusion) values on the day of the glucocorticoid infusion. We will then perform differential analysis comparing each of the two post-infusion time points (2 hours and 4 hours), independently, against the baseline. We will employ a cutoff value of < 5% FDR to select the transcripts that will be considered differentially expressed in each cell type at each time point. The resulting gene lists will be contrasted to determine, for example, which genes are uniquely differentially expressed in a specific cell type, or common to a specific hematopoietic lineage (myeloid or

lymphoid). They will also be used for pathway enrichment analysis, to determine the molecular pathways and biological functions that are most responsive to the glucocorticoid stimulus in each cell type. The genes and pathways that are found to be modified by the glucocorticoid stimulus in immune cells but not in non-immune cells (skin cells from this in vivo study and the primary human cells we have studied in vitro), will be selected as the top candidates for targeted therapeutic intervention, which we expect will form the basis of future studies.

Sequencing library preparation and sequencing batches will be carefully designed such that samples from the same subject and cell type are run in the same batch and samples from different subjects are evenly distributed across batches. This will keep batch effects to a minimum in the identification of differentially expressed genes. For analyses across different subjects, such as comparisons among different cell types, linear regression will be adopted with batch as regression variable to adjust for batch difference. While prior experience suggests that library preparation is the greatest source of batch-to-batch variation in RNA-seq experiments, if the source of batch effects is unclear or cannot be adequately modeled, the R package SVA will be used.

Summary Statistics for Demographic and Clinical Data

Demographic characteristics and medical history will be summarized, e.g., proportion, mean, median, standard deviation, and minimum and maximum values. Means and standard deviations will be reported for all continuous variables; percent will be reported for categorical variables.

Sample Size Justification

Given the high-throughput data generation strategy of next-generation sequencing, the use of different starting materials for sequencing (mRNA, small RNA, DNA), and the use of serial sampling, standard sample-size calculation methods are not appropriate in this case and an adaptive study design is a more realistic approach. Sample size consideration will be based primarily on the power to identify differentially expressed genes. A recent pilot study of glucocorticoid action performed by the NIH Center for Human Immunology (CHI) (32) administered intravenous hydrocortisone at doses of 50 or 250 mg to a group of 20 healthy volunteers, where 10 subjects received each dose. They performed transcriptional analysis by expression microarrays in PBMCs before and at six time points after the infusion. They used the R package limma for analysis, which implements linear models and empirical Bayes methods for assessing differential expression in microarray experiments (33). P-values underwent multiple-testing correction using the method of Benjamini and Hochberg to estimate the false-discovery rate (FDR). Using cut-off values of < 5% FDR-adjusted p-value and > 0.5 absolute average log2-fold-change, they found evidence of differential expression in over 400 genes at the lower dose and over 600 genes at the higher dose, with a maximum number of differentially expressed genes 4 hours after the infusion for both doses. This was accomplished with only 10 subjects per dose,

despite technical difficulties (notably hemolysis), and the use of hydrocortisone (which is approximately five times less potent than methylprednisolone, the glucocorticoid we plan to use in our study). In addition, the data from the study by Olnes et al. used bulk PBMCs, which are likely to give a more variable expression signal than the purified cell sub-types that we plan to employ in our study. Based on this information, we estimate that an initial recruitment of at least 20 subjects is likely to reveal the changes in gene expression over time that our study aims to define in individual cell sub-types. We propose an adaptive study design, in which we will recruit an initial cohort of 20 subjects. Based on the observed data from 20 subjects, we will then assess the conditional power that can be obtained with up to 30 additional subjects. We will also perform a bootstrap procedure, sampling with replacement up to 50 units from the 20 observed subjects, to evaluate the improvement in gene detection. For example, if the number of genes that can be detected reaches a plateau after 30 subjects, we will stop at that number. However, if the number of detected genes is seen to rise with a sample size of 30, but the pathways and the biological interpretation of the data remain unchanged, additional subjects will be unlikely to change the outcome of the study and no further recruitment will be pursued. Due to the adaptive sample size determination, we will use a nominal significance level $\alpha < 0.05$.

15 Ethics/Protection of Human Subjects

15.1 Informed Consent Process

Informed consent is a process where information is presented to enable a person to voluntarily decide whether or not to participate as a research subject. It is an ongoing conversation between the human research subject and the researchers which begins before consent is given and continues until the end of the subject's involvement in the research. Discussions about the research will provide essential information about the study and include: purpose, duration, experimental procedures, alternatives, risks, and benefits. Subjects will be given the opportunity to ask questions and have them answered.

Informed consent will be obtained in person in a private setting or remotely via telephone or video conference, by a study team member authorized to obtain consent. The subjects will sign the informed consent document prior to undergoing any research procedures. The signature may be obtained in ink or digitally using a mouse, stylus, or finger. The subjects may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the subjects for their records. The researcher will document the signing of the consent form in the subject’s medical record. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

Remote Consent Process: The informed consent document will be sent via secure email or file transfer to the potential participant. An explanation of the study will be provided over the

telephone or an NIH-approved videoconferencing platform (e.g., Microsoft Teams) after the participant has had the opportunity to read the consent form. The participant can print the appropriate form to sign and date in ink, or they can sign and date electronically with a finger, stylus, or mouse.

The participant will return the signed and dated consent form to the consenting investigator, who will sign and date it with the date it was received. The consent form can either be printed and signed and dated in ink, or signed and dated digitally with a finger, stylus, or mouse. A fully executed copy will be sent to the participant for their records.

The informed consent process will be documented on a progress note by the consenting investigator. The investigator will confirm that written consent has been obtained prior to initiating any study interventions.

15.1.1 Considerations for Consent of NIH Staff

Consent for NIH staff will be obtained as detailed above and will comply with the requirements of NIH HRPP Policy 404 Research Involving NIH Staff as Subjects.

Consent from NIH staff for whom this research is taking place within their own work unit or is conducted by any of their supervisors will, when possible, be obtained by an individual in a non-supervisory relationship with that staff member. When consent of that staff member is conducted, a third party will be present to observe the consent process in order to minimize the risk of undue pressure on the staff member.

15.2 Subject Confidentiality

All records will be kept confidential to the extent provided by federal, state, and local law. The study monitors and other authorized representatives of the sponsor may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records. Records will be kept locked and all computer entry and networking programs will be done with coded numbers only. Clinical information will not be released without written permission of the subject, except as necessary for monitoring by the IRB, NIAID, or the OHRP.

To further protect the privacy of study participants, a Certificate of Confidentiality has been issued by the NIH. This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

16 Data Handling and Record Keeping

16.1 Data Capture and Management

Study data will be maintained in CRIMSON and collected directly from subjects during study visits and telephone calls, or will be abstracted from subjects' medical records. Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary to confirm the data abstracted for this study. Data entry into CRIMSON will be performed by authorized individuals. The investigator is responsible for assuring that the data collected are complete, accurate, and recorded in a timely manner.

16.2 Record Retention

The investigator is responsible for retaining all essential documents listed in the ICH GCP guideline. Study records will be maintained by the principal investigator for a minimum of 5 to 7 years and in compliance with institutional, IRB, state, and federal medical records retention requirements, whichever is longest. All stored records will be kept confidential to the extent required by federal, state, and local law.

Should the investigator wish to assign the study records to another party and/or move them to another location, the investigator will provide written notification of such intent to OCRPRO/NIAID with the name of the person who will accept responsibility for the transferred records and/or their new location. Destruction or relocation of research records will not proceed without written permission from OCRPRO/NIAID.

Appendix A: References

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