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Effects of Procainamide on CTLA-4 and CD28 Expression in Drug-Induced Lupus: A Research Protocol

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Note: Correction added after original version published on September 19, 2022. We regret any inconvenience caused.

Abstract

Introduction: Lupus erythematosus is a chronic autoimmune disease affecting over 5 million individuals worldwide, characterized by overactivation of B and T lymphocytes. Of these, 10% of patients are diagnosed with drug- induced lupus (DIL), which is caused by high doses of medications such as procainamide, with 20% of procainamide users developing DIL. CTLA-4 and CD28 receptors on T-cell surfaces bind to B7 proteins on antigen-presenting cells, halting and promoting T-cell activation, respectively. Studies indicate that certain lupus symptoms are linked to abnormal *CTLA-4/CD28* expression, resulting in overreactive CD4+ T-cells. However, the exact nature of the relationship between CTLA-4 and lupus remains nonunanimous. Other studies show that procainamide alters DNA methylation[7], while altered *CTLA-4* methylation has been tied to autoimmune disorders. Thus, we hypothesize that procainamide leads to reduced *CTLA-4* expression and/or increased *CD28* expression, causing DIL symptoms.

Methods: We propose an *in vivo* experiment involving time-matched mouse models. CITE-seq analyses would be run on isolated T-cells from the splenic samples to obtain transcriptome and epitope data and to examine the expression of *CTLA-4*, *CD28*, and other potential genes of interest. To corroborate CITE-seq results, the T-cells would undergo immunohistochemical staining with primary and fluorescent antibodies that bind to CTLA-4/CD28.

Expected Results: Results are expected to indicate decreased CTLA-4 gene and protein expression, or increased CD28 gene and protein expression on the surface of procainamide-treated T-cells.

Discussion: If our analyses prove successful, potential next steps involve using gene editing technologies to screen, pinpoint, and correct the molecular mechanisms implicated in procainamide-induced DIL. Further analysis may also implicate additional or other genes of interest involved in the etiology of the disease.

Conclusion: This study is expected to provide insight into the effect of procainamide on molecular mechanisms involved in DIL. Further applications may involve adjunct therapies aimed at mitigating or preventing the development of DIL symptoms.

Keywords: drug-induced lupus (DIL); CTLA-4; CD28; procainamide; overactivation; methylation; transcriptome; immunohistochemistry; CITE-seq

Introduction

DIL is an autoimmune disease in which drug exposure causes the development of a condition resembling systemic lupus erythematosus (SLE) [1]. SLE is a widespread autoimmune disease, with a wide range of symptoms including but not limited to serositis, a butterfly rash, and kidney failure. As these closely resemble the symptoms of other diseases, SLE can be difficult to diagnose initially. SLE is more prominent in women than men and impacted by both genetic and environmental factors. This demographic preference, however, does not extend to DIL [2]. Resulting from the overactivation of B and T lymphocytes [2], DIL represents approximately 10% of all lupus cases [3], with 15000 to 30000 annual new cases in the United States [4]. The pathophysiology of DIL is unclear, with different lupus-inducing drugs causing autoimmunity through different mechanisms. Many agents are believed to cause the inhibition of DNA methylation, which is thought to contribute to the development of DIL. The lupus-like autoimmunity may be the result of improper demethylation in Cluster of Differentiation 4 (CD4+) T-cells, causing them to become autoreactive [1].

T lymphocytes, or T cells, express a specific receptor that recognizes a protein-bound antigen or pathogen [5] and thus play a critical role in regulating immune responses in the body [6]. There are two main types of T cells: cytotoxic T cells

(Cluster of Differentiation 8, or CD8+, T cells) and helper T cells (CD4+ T cells) [5]. Cytotoxic T cells actively kill pathogens, while helper T cells aid in the production of immune response, both cell-mediated and humoral [7]. However, excessive inflammation or overactivation of T cells is a major factor in autoimmune disease [6], such as DIL.

Cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4) is an inhibitory receptor expressed on T cells with high functional relevance to Cluster of Differentiation 28 (CD28), a receptor known to cause T cell activation and proliferation [8,9]. CTLA-4 competes with CD28 for B7 binding, making it essential for T cell regulation and a key factor in maintaining self-antigen immunity [10]. B7 binding to CTLA-4 or CD28 releases ligands that signal T cell activation or suppression, thus regulating an immune response [11]. Improper expression and *CTLA-4* polymorphisms have been linked to many autoimmune diseases, including SLE [10]. A study of CD28's role in T cell proliferation in SLE untreated patients concluded that T cells from the SLE patients had significantly higher responses to CD28 than normal controls [9]. Furthermore, lymphocytes from patients with active SLE showed increased *CD28* gene expression and a heightened response to CD28 when compared to normal subjects, suggesting that CD28/CTLA-4 receptor imbalances may play a role in the autoimmune symptoms seen in lupus cases [9]. Thus, it is hypothesized that, in DIL patients, the effect of certain drugs on the CTLA-4/CD28 pathways bring about temporary SLE-like symptoms which typically disappear within several weeks following cessation of drug use [1].

Over 100 drugs are known to induce DIL, with some only having a small association with DIL, and others with risks as high as 20% in the first year of therapy [3]. The drug with the highest incidence of causing DIL is procainamide, a medication used to treat arrhythmia [2]. Procainamide is a class 1A antiarrhythmic which reduces myocardial excitability and contractility by binding to fast sodium channels, thus inhibiting recovery after repolarization [12]. Despite an increase in procainamide use in recent years, notable side effects resulting from long-term use are, primarily: DIL, gastrointestinal disturbances, and autoimmune blood dyscrasias [12,13]. Interestingly, procainamide has been implicated in improper DNA methylation [14], which has been tied to autoimmune disorders, including lupus [15].

Research on procainamide's effect on the immune system and its correlation with the pathology of DIL is limited. However, as procainamide has the highest known incidence of causing DIL, studying its interactions with T cell function may prove essential for better understanding both the etiology and pathophysiology of DIL. We hypothesize that procainamide decreases to reduced *CTLA-4* expression and/or increased *CD28* expression, leading to the T-cell overactivation seen in DIL. Thus, we aim to investigate the impact of procainamide on DNA methylation related to CTLA-4/CD28 and, in turn, DIL.

Methods

Induced Disease Model

The proposed methodology involves an *in vivo* experiment using mouse models: a control group (n=40), and an experimental group (n=50). The strain of mice would be A/J mice, based on literature demonstrating that A/J mice produced the highest spike in antinuclear antibodies (ANA) following long-term procainamide treatment [16,17]. As DIL is expected to occur in around 20% of the experimental group, a larger sample size will be used for the experimental group so that approximately 10 positive samples may be compared with 10 of each control group. Two groups of lupus-free mice would be procured, both subject to identical living conditions, including daily exercise; a natural diet; and habitat. Following the induced disease model, the experimental group of mice would take 6g/L of drinking water of procainamide [16], orally, for a length of time sufficient to mimic long-term treatment in humans, which may span several weeks to lifelong procainamide use. Similarly, in mice, this period is also expected to span a minimum of three weeks [17]. For this study, the procainamide treatment will span 37 weeks. The dosage and timeframe are determined based on literature confirming the development of ANA in A/J mice given the same dosage for 37 weeks [16]. Half the control group (n=40) would orally be given a fluid-based solution, such as drinking water, that does not contain the active drug in order to demonstrate that drug administration does not influence experimental outcomes. Lupus symptoms in mice vary depending on strain, and literature on specific lupus symptoms for A/J mice is limited; however, the most common symptoms for mice are autoantibody production, glomerulonephritis, lymphoid activation and hyperplasia, and arthritis [18,19]. An accurate and reproducible marker for detecting positive murine cases of lupus in ANA testing [17,20], which will be used to identify DIL-positive cases in the experimental group.

T-Cell Isolation

Following disease induction, T-cells would be isolated from the spleens of mice from both the control and experimental groups by centrifugation using the technique described by Grosjean et al. (2021) for examination [21]. This would be done immediately following the 37 weeks of treatment, as it is expected based on literature that DIL, if it develops, would have done so to a significant extent [16,17]. First, the collected spleen would be torn apart and placed in a centrifuge with a buffer of 1 X Dulbecco′s Phosphate Buffered Saline and 2% Fetal Bovine Serum [21]. The sample would be centrifuged once at 450 x g for 7 minutes at room temperature, then again after the addition of Red Blood Cells lysis buffer to the cell pellet [21]. The cells would be gently resuspended by pipetting and incubated for 10 minutes at room temperature before the second round of centrifuging [21]. 5 µL of cells from the resulting material would be mixed with 45 µL of trypan

blue then loaded into a hemocytometer to identify and count viable cells [21].

ANA Testing

Due to the wide range of symptoms that overlap with other diseases, testing for ANA antibodies will take place as part of the experimental procedure to determine whether DIL has indeed been induced in the mice. ANA testing may be done using well plates covered in human Hep-2 cells blocked with PBS containing 5% goat serum [22,23]. The plates would be incubated with mouse serum, then washed with PBS. FITC-labeled anti-mouse IgG would be added for 15 minutes and subsequently washed [22,23]. ANA production would be scored by comparing staining of wells; bright staining would indicate positive cases, while no staining or faint staining would reveal low to no antinuclear antibody production [22,23].

CITE-seq and Transcriptome and Epitope Sequencing

Using single-cell RNA sequencing (scRNA-seq), a cellular profile may be obtained that allows us to examine the entire transcriptome of a given cell [24]. This experiment involves the use of Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq). A unique molecular identifier (UMI) would be assigned to the mRNA molecule corresponding to *CTLA-4*, *CD28*, and other genes of interest, such as cluster of differentiation 70 (*CD70*) [25,26]. These would then be matched, using a cell barcode, to a specific $CD4^+$ T-cell [25,26]. Beads covered in poly(dT) would be used to conjugate antibodies by way of a poly(A) sequence and polymerase chain reaction (PCR) handle known as an antibody-derived tag (ADT) [25,26]. This would be followed by the lysis of antibody-stained cells, resulting in the capture of mRNA and ADTs [25,26]. By repeating this process and scaffolding the data, the entire antibody-based transcriptome and epitope data of the cell may be obtained [27].

Immunohistochemistry

Immunohistochemistry will be used to detect and analyze protein expression in T-cells, providing both qualitative and semiquantitative data in one test. IHC shows protein localization and surface distribution, giving us further insight into procainamide's effect on CTLA-4 and CD28. T-cells from the same mouse spleens used in the CITE-seq procedure would undergo immunofluorescence staining, as per the method outlined by Im et al. (2020) [28]. The cells would be prepared in a cell culture dish and covered with 4% formaldehyde in PBS to a depth of 2-3mm [28,29]. The cell line would be fixed for 10 minutes and then washed with a PBS Wash Buffer [28,29]. The sample would then be blocked with 200 µL of blocking buffer, streptavidin (0.1%), and biotin (0.01%) [28,29]. Small volumes (200µL) of prepared primary polyclonal antibodies (Cat # CTLA4-FITC from Fabgennix for CTLA-4 and Cat # MA1-10172 from Invitrogen for CD28) would be added at a dilution of 1:100 to cover the cell line [28,29]. This would be incubated overnight at 22°C for 90 minutes [28,29]. The sample would be rinsed with PBS Wash Buffer and then washed with 100mL distilled water for 2 minutes [28,29]. Biotinylated anti-Hamster IgG secondary antibodies (Cat # 31750 from Invitrogen) would be added to the sample and incubated for 90 minutes at 22°C then rinsed and fixed [28,29]. Finally, the sample would be incubated for 30 minutes with 200 µL of fluorophore-conjugated Streptavidin dilution in the dark and rinsed again [28,29]. After the preparation of slides with 200 µL ProLong Gold Antifade Reagent with DAPI, the samples would be examined immediately at 495 nm to excite CTLA-4 bound fluorophores, and at the appropriate wavelength of the chosen fluorophore-conjugated Streptavidin [28,29]. The data may be quantified through the use of image analysis softwares such as the Aperio Area Quantification FL Algorithm, which would measure intensity and area of each fluorescent dye [28,29]. This may be used to corroborate the results of, or to further examine genes of interest following the CITE-seq analysis.

Anticipated Results

We predict that the development of autoimmune symptoms of procainamide-induced lupus are the result of overactivation of $CD4^+$ T-cells caused by improper *CTLA-4/CD28* expression. Running CITE-seq on isolated splenic $CD4^+$ T-cells are expected to show a decrease in CTLA-4 expression, an increase in CD28 expression, or both. The immunohistochemistry data is expected to reflect the CITE-seq data, with low fluorescence of antibodies bound to CTLA-4 and/or high fluorescence of antibodies bound to CD28, compared to the control group of mice.

Further analysis of the transcriptome would allow us to investigate the possibility of mutations or protein misfolding, or dysfunction in both or either CTLA-4/CD28 proteins despite normal amounts of protein present in the cell. As around 20% of procainamide users develop DIL [2], symptoms in the experimental group are expected to appear in approximately 20% of experimental mice. In this case, results may highlight genetic differences between symptomatic and non-symptomatic mice. Results may be obtained as symptoms appear, if they do; results are expected between three weeks to four months following the onset of treatment [17]. Note that DIL-positive T cells are ANA positive cells.

Discussion

Analysis and Interpretation of Results

Analysis of the results of CITE-seq and immunofluorescent staining is expected to yield information on the count of CTLA-4/CD28 proteins as well as any genetic alterations, such as methylation or demethylation of these and other genes of interest. A potential gene of interest is the *CD70* gene, which has shown to have increased expression as a result of

demethylation of a certain promoter sequence in DNA [30]. Procainamide competitively inhibits DNA methyltransferase (Dnmt); in lupus CD4+ T cells, Dnmt is inhibited via inhibition of the extracellular-signal-related kinase (ERK) pathway that affects the same promoter sequence [30]. Thus, our results may implicate *CD70* instead of or in addition to *CTLA-4/CD28*. The transcriptome and epitope data obtained from CITE-seq should be analyzed initially and these analyses corroborated with the relative abundance of CTLA-4/CD28 proteins as shown through immunohistochemistry.

Through gene expression and surface protein counts, the results are expected to reveal whether the regulation of T-cell activation is impaired by way of defects in the *CTLA-4/CD28* pathways [8,9]. Such results would verify the presented hypothesis and provide insight into the pathophysiology of procainamide-induced lupus by revealing an area of interest through which procainamide could influence T-cell function and induce DIL. A deficiency in CTLA-4 or an excess of CD28 on the surface of procainamide-treated T-cells is expected. The results would be interpreted such that the lack of CTLA-4, which inhibits T-cell activation, caused the overactivation of the T-cell due to an inability to prevent B7 docking. On the other hand, the excess of CD28, which promotes T-cell activation, did so excessively in procainamide-treated mice. In either case, the results would be interpreted to suggest that the balance between CTLA-4 and CD28, inhibitory and promoter proteins respectively, caused T-cell overactivation in mice exhibiting symptoms of DIL. If improper methylation of the *CTLA-4/CD28* genes is observed through the transcriptome data, this would suggest that defects in one or both of these pathways is implicated in the development of DIL. However, as lupus is a multifactorial disease, it is important to note that alternative interpretations are possible. For example, B7 docking sends primary activation signals to CD4+ T cells. In one alternative interpretation, overactivation could be attributed to increased sensitivity to or errors in pathways that produce secondary activation signals, such as those from CD70 molecules [31,32]. Alternatively, the implication of other genes of interest – such as improper methylation targeting genes other than *CTLA-4/CD28*, or altered gene expression – would be interpreted such that the CTLA-4/CD28 pathways are not directly involved in DIL. This would be the case if the results implicate the aforementioned *CD70* gene, in which case the results may be interpreted such that DIL is the result of procainamide-induced overexpression of CD70. Contrarily, analysis of the transcriptome may not implicate genetic changes at all. Such results would lead the researcher to reject the original hypothesis in favor of an alternative hypothesis regarding the molecular and/or genetic nature of procainamide-induced DIL. A potential alternative hypothesis would be mutations in DNA transcription or deficient or damaged mRNA molecules directly related to the development of DIL.

The anticipated results are in line with previous research that broaches the subject of procainamide-induced DIL. Current research links DIL and CTLA-4/CD28 pathways, while other studies show that procainamide alters DNA methylation [14]. Further, altered methylation of the CTLA-4 gene is tied to autoimmune disorders [33]. While the exact nature of the link between procainamide, DNA methylation, and CTLA-4/CD28 pathways is unknown, the anticipated results of this study would solidify a direct correlation between defects in these pathways and DIL. Genetic alterations, such as improper methylation, are expected to become evident in the CITE-seq data, granting further insight into the cellular and molecular basis of procainamide-induced DIL. Knocking out the implicated gene would also establish a causal relationship between the drug, defects in the respective pathway, and DIL.

Moreover, data obtained from symptomatic and non-symptomatic procainamide-treated mice are expected to provide insight into potential individual risk factors for DIL. As around 20% of procainamide users develop DIL [2], a comparable ratio is expected to be present in the experimental group in the drug-induced model. This allows for a sub-study of two subgroups of drug-treated mice: those that did and those that did not show symptoms following long-term procainamide treatment. Any potential risk factors may be extrapolated to define human risk factors. Symptoms, most of which are not unique to DIL, will be attributed either to DIL or other factors, such as adverse effects or faulty administration, based on presence or absence of ANA antibodies in the isolated T cells.

Strengths and Limitations of Proposed Methodology

The proposed study allows for examination of the entire transcriptome/epitope to identify which areas, if any, are impacted by procainamide. This in turn allows the researcher to generate alternative hypotheses easily, should the original be disproved; these hypotheses can then be further tested using immunohistochemistry or other methods. However, important limitations of this study include the inability to fully replicate environmental stressors and biological factors that may influence gene expression in DIL patients. For example, human patients who develop DIL due to procainamide use take procainamide to relieve arrhythmia; however, this will not be replicated in most of our samples. Thus, this study focuses on the genetics of DIL induced in a laboratory setting and does not, to any great extent, account for epigenetic factors that complicate the disease model. Further, the great heterogeneity of lupus symptoms may make it difficult to pinpoint DIL if rare or unexpected symptoms arise; ANA testing must thus be conducted to differentiate between adverse effects and symptoms of DIL. Additionally, it is uncertain precisely when DIL symptoms are expected to appear, as this differs between individuals; this, despite close monitoring, may prove problematic in carrying out the study under the proposed methodology.

Future Steps and Significance of Findings

The expected findings present critical implications for future research in the field of immunology. Potential next steps include the use of emerging biotechnology and further transcriptome and epitope analysis and replication studies to further understand and probe the effects of procainamide on the CTLA-4/CD28 pathways and other potential genes of interest. One example of such an application is the use of gene editing, such as CRISPR-Cas9, to screen and knock-out the top upregulated genes from isolated splenic T-cells based on previously obtained transcriptome and epitope data. This would pinpoint the exact molecular mechanisms involved in DIL. Then, gene editing could be used to counter or balance the effects of procainamide on *CTLA-4/CD28*, offering tremendous benefits for patients undergoing long-term or lifelong treatment with procainamide or other problematic drugs. Alternatively, drug research could be conducted to modify or develop adjuvant therapies for drugs known to induce DIL. These drugs would aim to balance or counteract the genetic and autoimmune effects of procainamide, such as by regulating T-cell activation in the presence of the drug. Thus, conclusions drawn from this research, including those concerning potential risk factors in individuals, may be used to improve the quality and efficiency of care for DIL patients and patients undergoing long-term treatments with drugs linked to DIL.

Conclusions

Drug-induced lupus is an under-researched autoimmune disease comprising 10% of all lupus cases [3]. The autoimmune symptoms of DIL are hypothesized to be due to drugs interfering with methylation in $CD4^+$ T-cells; however, there is insufficient experimental evidence to back these claims, and the genes involved are unknown. Our study aims to understand the interactions between procainamide, the drug with the highest case incidence, and T cell protein expression to further understand the pathophysiology of DIL. We predict CTLA-4, a receptor that binds to B7 antigen-presenting cells and inhibits T-cell activation, will be under expressed in the procainamide-treated mice. We also expect to see increased expression of CD28, a receptor that binds to B7 antigen-presenting cells and promotes T-cell activation. Understanding the pathophysiology of DIL would allow for the development of novel treatments for DIL patients as well as preventative adjuvant therapies for high-risk patients undergoing long term treatments with drugs linked to DIL. Further research with CTLA-4 and CD28 could reveal new approaches to treating autoimmune diseases, similar to immune checkpoint inhibitors, by shifting the balance between T cell activation and inhibition.

List of Abbreviations Used

DIL: drug-induced lupus
SLE: systemic lupus erythematosus
CD4+: cluster of differentiation 4
CD8+: cluster of differentiation 8
CTLA-4: cytotoxic T-lymphocyte-associated protein 4
CD28: cluster of differentiation 28
ANA: antinuclear antibody
scRNA-seq: single-cell RNA sequencing
CITE-seq: Cellular Indexing of Transcriptomes and Epitopes by Sequencing
UMI: unique molecular identifier
CD70: cluster of differentiation 70
PCR: polymerase chain reaction
ADT: antibody-derived tag
PBS: Phosphate-buffered saline
Dnmt: DNA methyltransferase
ERK: extracellular-signal-related kinase

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study is a research protocol proposal only. No participants were recruited for the study and no ethics approval was obtained; animal experimentation guidelines and ethics should be adhered to when completing the study.

Authors' Contributions

RK: made contributions to the design of the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.
NCS: contributed to study design and planning, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.

Acknowledgements

We extend our gratitude to the Undergraduate Research in Natural and Clinical Science and Technology Journal and the sponsors; executives; volunteers; and partners of the IgNITE Medical Case Competition for this opportunity to explore the field of immunology. We also thank Nicolas Castonguay (University of Ottawa) for providing invaluable guidance, feedback, and support throughout the ideation and production of this research and for cultivating growth.

Funding

This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Alita Gideon, Pierre Lemieux
Article Dates: Received Jul 12 21; Accepted Sep 09 22; Published Sep 19 22

Citation
Please cite this article as follows:
Kim R, Sheth NC. Effects of procainamide on CTLA-4 and CD28 expression in drug-induced lupus: A research protocol. URNCST Journal. 2022 Sep 19: 6(9). https://urncst.com/index.php/urncst/article/view/395
DOI Link: https://doi.org/10.26685/urncst.395