TRANSLATING THE BIOLOGY OF IL-9 INTO THE PATHOGENESIS OF CHRONIC RHINOSINUSITIS

A Thesis

by

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ABSTRACT

Background: Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal sinus mucosa that can have profound effects on patient quality of life and US healthcare costs. Increased IL-9 expression has been identified in CRS patients, particularly those with nasal polyposis (CRSwNP) or eosinophilic CRS (ECRS). *Il-9* gene expression has been found to be uniquely regulated by super enhancer RNA (eRNA).

Aim 1: Perform an in-depth literature review to summarize the current understanding of IL-9 biology, with an emphasis on IL-9's contribution to CRS pathology.

Aim 2: Use antisense treatment of mouse Th9 cells in vitro to suppress IL-9 as initial validation of a possible immunotherapeutic treatment strategy for CRS patients.

Methods: CD4+ T-cells harvested from mouse lymph nodes and spleen were cultured under Th9 polarizing conditions, confirming Th9 differentiation with flow cytometry. Best strategies for robust baseline IL-9 and super enhancer RNA expression were determined by comparing qPCR values with various IL-9 stimulating conditions and RNA extraction methods. Antisense oligonucleotides (ASOs) targeting the *Il-9* gene and super enhancer eRNA were designed using the UCSC In-Silico PCR tool and IDT's OligoAnalyzer tool and were introduced to Th9 cell cultures using electroporation with the Lonza Nucelofector 2b. The fold decrease in gene expression in ASO vs. control conditions were determined using qPCR relative quantification.

Results: Baseline super enhancer expression was 8.9, 7.6, and 3.8 times higher for 3 different super enhancer targets using organic extraction compared to a spin column method for RNA extraction, and 6.8, 7.5, and 6.5 times higher in culture conditions utilizing anti-GITR stimulation compared to OX40L stimulation. Preliminary results of unmodified ASO knockdown

studies showed ~2-fold decrease in IL-9 expression in ASO conditions, but this was not consistent across all conditions.

Conclusions: The addition of anti-GITR to Th9 cultures lead to the greatest baseline super enhancer expression and super enhancer RNA was best isolated using organic extraction methods. Though preliminary ASO studies are promising for IL-9 suppression, additional testing is necessary to determine the ideal concentrations of ASOs and timing of ASO introduction to cell cultures, as well as replicate experiments with modified ASOs.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. David Huston (chair), Dr. Xian Li, and Dr. Wenhao Chen.

All the data was analyzed with assistance from Preston Arnold, a Texas A&M College of Medicine MD/PhD candidate, whose previous work on enhancer RNA was published in *Frontiers in Cell and Developmental Biology* in 2020.

All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

CRS	Chronic Rhinosinusitis
CRSwNP	Chronic Rhinosinusitis with Nasal Polyposis
ECRS	Eosinophilic Chronic Rhinosinusitis
eRNA	Enhancer RNA
ASO	Antisense Oligonucleotide
GITR	Glucocorticoid-induced tumor necrosis factor receptor
APCs	Antigen presenting cells
HPRT	Hypoxanthine-guanine phosophoribosyltransferase

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1. INTRODUCTION

Chronic Rhinosinusitis (CRS) is defined by at least 12 weeks of inflammation of the nasal and paranasal sinus mucosa, manifesting symptoms of facial pressure, nasal obstruction, and dysosmia[1,2,3]. It is a complex disorder that is difficult to classify and hard to differentiate from similar conditions, leading to patients requiring multiple surgeries and medication trials that contribute $10-13 million annually in direct U.S. healthcare costs[1,4]. It is therefore important to better categorize CRS phenotypes and develop individualized treatment plans to reduce inefficiencies in CRS diagnosis and treatment.

Historically, CRS has been broadly divided into two phenotypic subtypes: CRS with nasal polyposis (CRSwNP) and CRS without nasal polyposis (CRSsNP). This dichotomous differentiation is overly simplistic however, given the myriad of various pathologies that contribute to sinonasal inflammation[5]. Diseases such as cystic fibrosis, granulomatosis with polyangitis, and odontogenic sinusitis all contribute to sinonasal inflammation but do not clearly fall into either category[5,6,7,8]. In an effort to delineate CRS phenotypes on a more pathobiological basis, studies have attempted to cluster CRS patients based on cytokine profiles, inflammatory marker patterns, or bacterial colonization[9,10,11]. In such studies, IL-9 elevation has been not only increased in CRS patients generally, but also more associated with CRSwNP and eosinophilic chronic rhinosinusitis (ECRS) patients with atopy and refractory disease[1,9,12,13,14,15,16,17,18]. Understanding this cytokine's regulation and signaling patterns can shed new light on CRS pathogenesis and create new immunotherapeutic opportunities for treatment of specific CRS phenotypic presentations and other IL-9 mediated pathologies.

2. INTERLEUKIN-9

Interleukin-9 (IL-9) is a pleotropic cytokine with diverse cellular sources. Originally known as "P40," IL-9 was first identified by Jacques van Snick's group at the Ludwig Institute for Cancer Research in 1988 as a T-cell growth factor secreted by certain T-helper (Th) cells, allowing for long-term maintenance of Th cell lines without additional antigen stimulation[19]. At around the same time, different groups, including the Institute for Experimental Haematology in Munchen, identified "T-Cell Growth Factor III (TCGF III)" and "Mast Cell Growth Enhancing Activity (MEA)" factor in Th2 cells, which similarly stimulated T-cell and mast cell growth. After comparative sequencing, both were confirmed to be the same 14kDa protein as P40[20]. All three were eventually designated "IL-9" and considered to be a Th2 cytokine. It was not until 2008 that it became clear a subset of IL-9-producing CD4+ T-cells were recognized as a distinct class, known as Th9 cells[21,22].

2.1 Transcription and Regulation

The human *IL-9* gene is found on chromosome 5, within the Th2 cytokine cluster that also includes IL-3, IL-4, IL-5, and GM-CSF genes, while the mouse *IL-9* gene is found on chromosome 13[23,24,25]. In addition to Th9 cells, IL-9 can be secreted by many other cell types (Fig. 1). The most important factors for stimulating IL-9 secretion vary depending on the cell type secreting it. Transcription factors that promote IL-9 expression include Interferon-Regulatory Factor 4 (IRF4), Basic Leucine zipper Transcription Factor ATF-like (BATF), and PU.1, which are critical for Th9 induction by interaction with the *IL-9* gene. TGF-β, which is most commonly associated with Foxp3+ Treg cells, can induce PU.1 interaction with histone acetyltransferases, forming an open chromatin structure at the *Il9* locus[22,26]. Combined with IL-4

and IL-2, TGF-β also activates IRF4, which binds directly to *Il9* to promote its transcription[27]. It is believed that IL-4 operates through the STAT6 pathway to suppress the typical TGF-β-mediated Foxp3+ Treg and Th17 differentiation that would normally suppress Th9 induction[26]. TGF-β and Notch signaling can also activate Smad proteins, which have been shown to both bind the *Il9* promoter directly and form complex with IRF4[26].

Other transcription factors and cytokines implicated in IL-9 production and Th9 polarization include NFκB, NFAT, GATA1 and GATA3, Etv5, thymic stromal lymphopoietin (TSLP), Activin A, Type I Interferons, tumor necrosis factor (TNF) superfamily cytokines like OX40L, GITRL, or TL1A, and interleukins 1, 2, 6, 10, 12, 21, 25, and 33, though the mechanisms by which many of them do so is both poorly understood and cell-type dependent[20,27,28]. Conversely, IFN-γ, IL-21, IL-23, IL-27, STAT1, and cyclosporine A tend to be inhibitory towards IL-9[20,27]. IFN-γ was shown to suppress Th9 differentiation through reduction of sensitivity of T cells to IL-4 as well as activation of STAT1. IL-21 does so through induction of BCL6[26].

2.2. Cellular Sources of IL-9

Many cell types, including both lymphoid and non-lymphoid cells, are known to produce IL-9. Th9 cells, a subset of CD4+ T-cells, act as the primary producers of IL-9. They are well established contributors to anti-helminth protection, anti-tumor activity, autoimmunity, transplant tolerance, and airway hyperresponsiveness[21,26,29]. More recently, they have been investigated in CRS, with Th9 and IL-9 levels appearing in higher concentrations in sinonasal tissue biopsies, blood samples, and nasal polyps of CRS patients, particularly those with CRSwNP and ECRS[1,9,12,13,15,17,18]. Mast cells can also secrete IL-9, particularly in the presence of lipopolysaccharides (LPS) and IL-1. IL-9 then stimulates a number of cell types involved in type

2 immune responses, including mast cells themselves, promoting even further IL-9 secretion in an autocrine fashion[20,27]. Similarly, eosinophils can both produce IL-9 and express IL-9 receptors, which seems to be of particular importance for patients with eosinophilic CRS (ECRS) and comorbid asthma and atopy, given that eosinophils in these patients exhibit higher IL-9 expression[13,18]. Epithelial cells of the airway and nasal mucosa can also produce and respond to IL-9, potentially contributing to CRS pathology. Other cells with the ability to produce IL-9 include Th17 cells, group 2 innate lymphoid cells (ILC2s), natural killer T-cells (NKT), memory CD4+ T-cells, neutrophils, CD8+ T-cells, and osteoblasts. Additional cellular sources of IL-9 can be found in Figure 1.

2.3. The IL-9 Receptor and its Downstream Signaling

The IL-9Rα subunit, first described by the Snick group, is a 64kDa protein and a member of the type 1 hematopoietin receptor superfamily[30,31]. The human *IL9RA* gene is found in one of two unique pseudoautosomal regions on the X and Y chromosomes, specifically pseudoautosomal region 2 (PAR2), which is much shorter than pseudoautosomal region 1 (PAR1) and located on the tips of the long arms[32]. The mouse *IL9RA* gene maps to chromosome 11[33]. Functional and evolutionary implications of this unique gene location have yet to be fully elucidated. The IL-9Rα subunit forms a heterotypic complex with the IL-2Rγ_c subunit, a common subunit of the IL-2R family also present in IL-2, IL-4, IL-7, IL-15, and IL-21 receptor complexes[30,26,34]. The α chain is primarily responsible for ligand binding and exists in both membrane-bound and soluble forms, while the γ_c chain initiates cell signaling[21].

Upon binding of IL-9 to IL-9Rα, a conformational change in the heterocomplex allows Janus kinase 1 (JAK1) interaction with the BOX1 motif on IL-9Rα. JAK3 interacts with the γ_c

chain in the same fashion. This BOX1 motif has been found to be essential for phosphorylation of JAK1 and JAK3 tyrosine residues[35]. Downstream signaling is then carried out through Src homology 2 (SH2) signaling molecules like insulin receptor substrates (IRS), utilizing phosphatidylinositol-3 kinases (PI3-K), Mitogen-Activated Protein Kinase (MAPK), or Signal Transducer and Activator of Transcription (STAT) pathways, with STAT1, STAT3, and STAT5 being of particular importance[21,35,36] (Fig. 2).

Numerous cell types express the IL-9 receptor, with some of the earliest recognized being mast cells and hematopoietic stem cells (HSCs). In mast cells, IL-9 amplifies mastocytosis and mast cell production of proteases and proallergic cytokines like IL-5, IL-6, and IL-13[20]. In HSCs, IL-9 binding promotes phosphorylation of the BCL2-associated agonist of cell death (BAD) protein, preventing caspase-mediated apoptosis[21]. Eosinophils have also been found to express the IL-9R and studies have shown that IL-9 enhances eosinophil survival, though this may be indirectly through IL-5R upregulation[18,37]. In the airway, IL-9 binds to smooth muscle cells and epithelial cells. Effects on smooth muscle cells include their release of eotaxin, IL-13, and IL-8, all of which recruit neutrophils and eosinophils to the area that can cause inflammatory damage[26]. Effects on epithelial cells include goblet cell metaplasia, mucus gene induction, and alteration of epithelial barriers through changes to membrane bound e-cadherin and claudin-1[38]. Additional cells expressing the IL-9 receptor can be found in Figure 1.

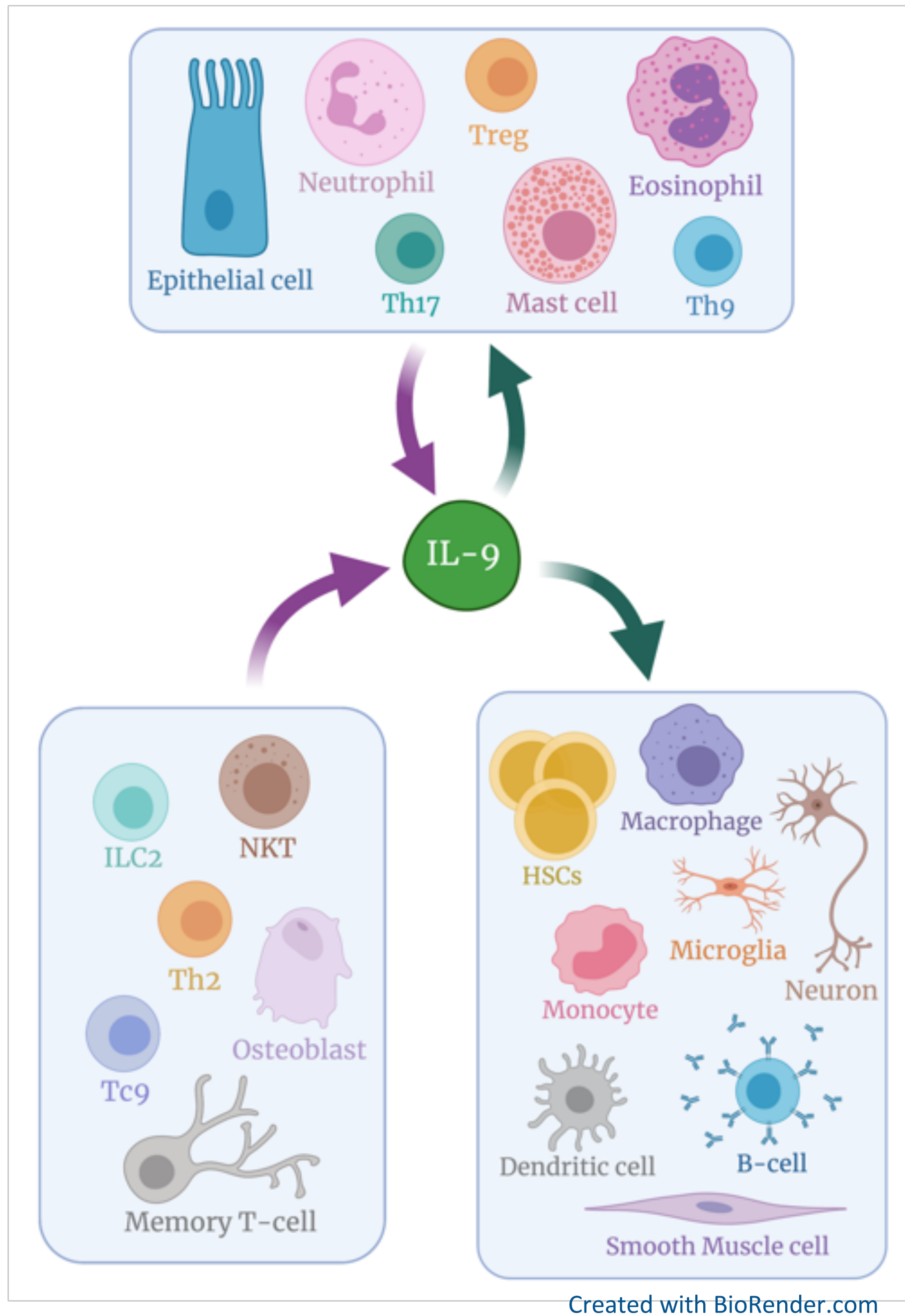

Figure 1. Cellular interactions with IL-9. Boxes with purple arrows pointing away from them contain cells that produce IL-9. Boxes with green arrows pointing toward them contain cells that express the IL-9 receptor and are affected by IL-9 binding to this receptor.

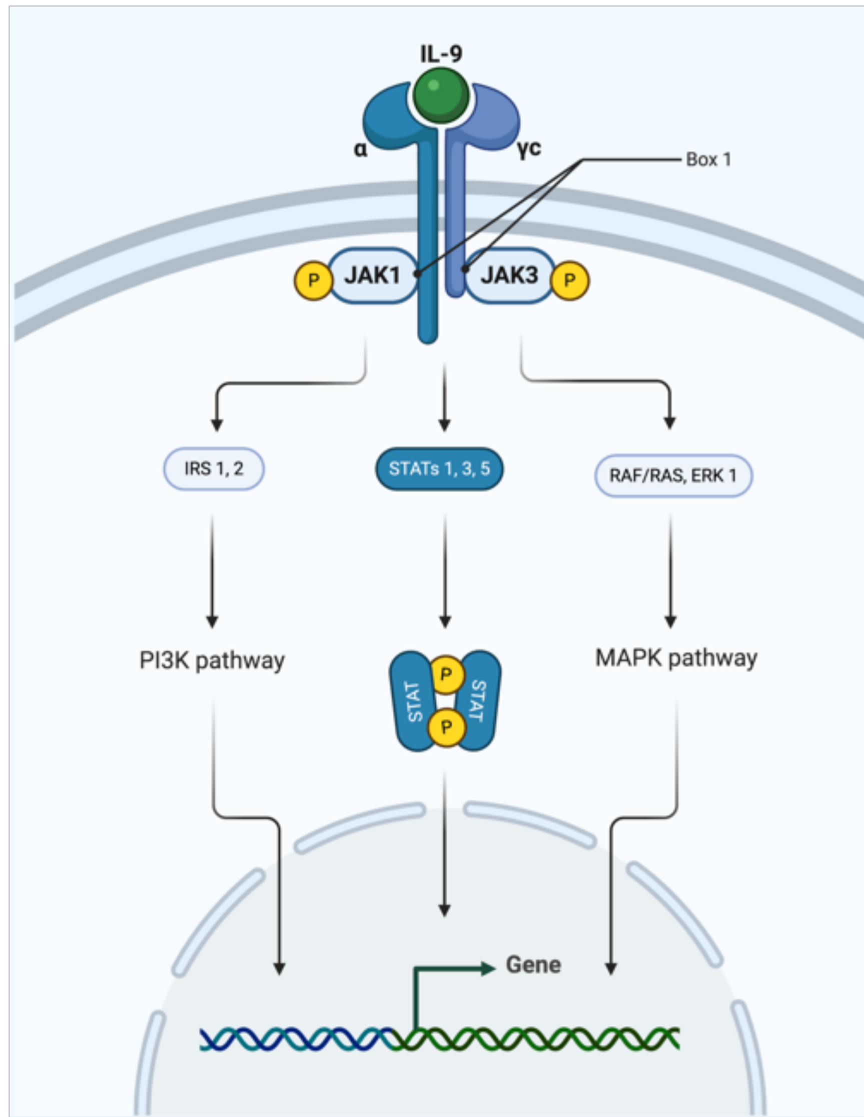

Figure 2. IL9-R and downstream intracellular signaling. The IL-9 receptor is a heterodimer comprised of an IL-9Rα subunit and a common γ_c subunit that is shared amongst other receptors of the IL-2 receptor family. IL-9 binding promotes JAK1 and JAK3 phosphorylation, which initiates either PI3K, MAPK, or JAK-STAT signaling pathways.

3. THE EMERGING ROLE OF SUPER ENHANCERS

Though a broad range of regulatory factors act upon IL-9-producing cells throughout the body, none are specific to IL-9 transcription compared to other cytokines. In fact, lineage-defining transcription factors for Th9 cells have yet to be identified, sparking debate over whether they are truly a distinct T-cell class versus a mere Th2 subtype[29]. In 2017, however, one unique aspect of the IL-9 regulatory unit was established. Xiao et al. utilized chromatin immunoprecipitation sequencing (ChIP-seq) to localize 3 super enhancer clusters that are crucial for IL-9 transcription[28].

Super enhancers are clusters of multiple enhancer regions that coordinate to drive unusually high levels of gene expression[39,40,41]. Comparison of typical enhancers to super enhancers is represented in a 2019 review article by Wu and Shen. It displays a super enhancer unit that is more densely packed with transcription factors, mediator complexes, polymerases, and enhancer RNA (eRNA) than a typical enhancer[42]. Enhancer RNA (eRNA) is RNA that has been transcribed from the enhancer regions of DNA. The exact function of eRNA is still under investigation, but studies have suggested that individual eRNAs can actually contribute directly to regulation of their target genes, through processes such as chromatin modification, maintenance of enhancer-promoter architecture, and assistance with transcription factor binding[43]. Standardized protocol for super enhancer identification involves the use of ChIP-seq to locate enhancer regions with significantly greater histone acetylation than others, indicating more frequent chromatin remodeling for gene transcription[40]. They are often associated with genes that are key to defining tissue or cell identity[40,39,41]. According to the Super Enhancer

database (SEdb), there are currently 542 super enhancers recognized in the human genome from 240 different tissues and cell types[44].

In the case of IL-9, super enhancer induction is facilitated by OX40, which was previously known to stimulate Th9 differentiation, but through unknown mechanisms. OX40 typically signals through the NF-κB pathway, and in Th9 cells specifically, stimulates RelB, a transcription factor that recruits histone acetyltransferase p300 to the *IL-9* gene locus, promoting an open chromatin structure that allows for Brd4 binding[28]. Brd4-bound regions are recognized "hot spots" for super enhancer formation[39]. Enhanced expression of eRNA from the IL-9 super enhancer region, an indicator of robust super enhancer formation, was strongly associated with increased IL-9 expression and Th9 cell induction. OX40, RelB, and histone acetylase p300 have proved vital not only for in vitro IL-9 expression but also for in vivo Th9 cell-mediated allergic airway inflammation[28]. There is much more to learn about the IL-9 super enhancer and IL-9 regulation in general, but these are promising discoveries that can contribute to the development of targeted IL-9 therapeutics.

4. METHODS

The goals of this research project were to increase understanding of IL-9 biology, particularly regarding CRS pathogenesis, and test the hypothesis that **antisense oligonucelotides targeting IL-9 super enhancer eRNA could suppress IL-9 expression**. This was approached with 1) a comprehensive literature review summarizing updated findings related to IL-9 functionality at a cellular and molecular level, with emphasis on IL-9 contribution to pro-inflammatory processes that would affect the nasal mucosa, and 2) an in vitro experiment in which mouse Th9 cell cultures were treated via electroporation with ASOs targeting IL-9 super enhancer eRNA and assessed for decreased IL-9 expression.

4.1. Experimental Design and Protocol Optimization

An overview of the experimental design is provided by Figure 3. Naïve CD4+ T-cells were harvested from the spleen and lymph nodes of C57BL/6 (B6) mice and cultured in Th9 inducing conditions (TGF-β, IL-4). Th9 cell differentiation was confirmed using flow cytometry on day 3 of incubation, identifying cells that stained positive for IL-9 and negative for IFN-γ. These cell cultures were then treated with ASOs via electroporation with the Lonza Nucelofector 2b device. IL-9 and super enhancer RNA levels were measured using qPCR, comparing their expression in ASO treated vs. control (cells treated with a scrambled ASO of the same length, ordered from IDT Technologies) conditions, using relative quantification standardized to the β-actin housekeeping gene. The ΔΔCT method was used to determine relative gene expression of IL-9 and super enhancer RNA in ASO treated cells compared to their controls (Fig. 4).

In order to optimize the protocol for this experiment, it was necessary to first identify the most ideal baseline conditions for robust Th9 differentiation and IL-9/super enhancer RNA activity. Two strategies previously demonstrated in the literature to stimulate Th9 differentiation were compared: 1) adding OX40L-expressing antigen presenting cells (APCs) to cell cultures, and 2) adding anti-GITR antibodies to cell cultures, using flow cytometry to determine the percentage of Th9 differentiated cells and qPCR relative quantification to evaluate IL-9 and super enhancer RNA expression in each condition. Two RNA extraction methods were also compared: 1) a standard spin column method, and 2) organic extraction, which has the potential to more effectively isolate smaller RNA segments like super enhancer eRNA. qPCR was used to assess IL-9 and super enhancer RNA levels using each extraction method.

4.2. ASO Design

With the UCSC In-Silico PCR tool, forward and reverse qPCR primer sequences (provided in the supplemental material of Xiao et al.[28]) were used to identify the IL-9 and super enhancer sequences between them. Reverse complements of these sequences were then manually scanned to identify the most stable for building ASOs 20 nucleotides in lengths. Stability was assessed using IDT Technology's OligoAnalyzer tool, selecting ASOs with the most stable melting points and least potential for self-dimerization or formation of hairpin structures.

4.3. Nucleofection

Based on previous literature regarding ASO transfection methods, electroporation was determined to be the best approach, given its efficacy in T-cells, which are historically very difficult to transfect, and its better ability to penetrate the nucleus where super enhancer eRNA is

located[45,46,47,48]. A Lonza Nucleofector 2b device was used to transfect cells with unmodified ASOs targeting IL-9, IL-9 super enhancer, and positive control (targeting the mouse HPRT gene) regions after 24 hours of incubation, with the aim of identifying the most consistently effective ASOs to order in a modified, more stable form. Cells were collected for qPCR analysis at least 24 hours after nucleofection, giving them time to recovery from the harsh electroporation process.

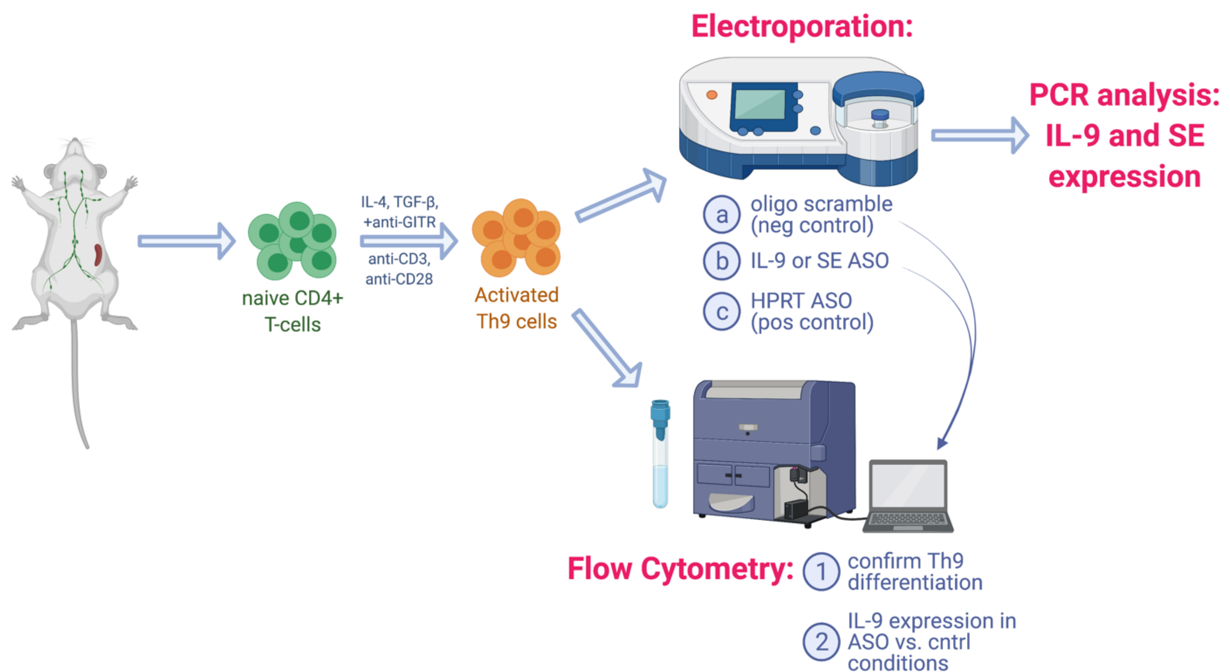

Figure 3. Experimental design. Naïve CD4+ T-cells harvested from mouse spleen and lymph nodes were cultured in Th9 activating conditions, confirming differentiation by flow cytometry. Cells were then treated with IL-9, IL-9 super enhancer, positive control, and negative control ASOs via electroporation and analyzed for IL-9 and super enhancer expression using qPCR relative quantification.

$\Delta\Delta CT$ Method:

$$\Delta CT_{ASO} = CT_{gene} - CT_{\beta\text{-actin}}$$

$$\Delta CT_{cntrl} = CT_{gene} - CT_{\beta\text{-actin}}$$

$$\Delta\Delta CT = \Delta CT_{ASO} - \Delta CT_{cntrl}$$

$$\text{Fold change} = (1/2)^{\Delta\Delta CT}$$

*CT = cycle threshold

Created with BioRender.com

Figure 4. Methods for qPCR relative quantification calculation. This shows the calculation steps utilized to find the fold change in gene expression in ASO conditions compared to controls, using the ΔΔCT method.

5. RESULTS

5.1. Th9 Differentiation

The addition of anti-GITR stimulating antibodies to T-cell cultures had the strongest effect on Th9 differentiation and IL-9/super enhancer expression. Cultures with Th9 conditions (IL-4 and TGF-β) plus anti-GITR displayed 44% Th9 cells, which was greater than the 30% observed with IL-4 and TGF-β alone and the 0.1% with only T-cell activation (anti-CD3 and anti-CD28) (Fig. 5). Cells cultured with anti-GITR also expressed 11.6-fold higher levels of IL-9 and up to 7.5-fold higher levels of super enhancer eRNA compared to those cultured with OX40L APCs. Three different super enhancer regions exhibited 5.9-, 5.3- and 5.4-fold increases in eRNA levels when cultured with anti-GITR, as opposed to IL-4 and TGF-β alone (Fig. 6).

5.2. RNA Extraction

Though the use of spin columns and organic extraction were comparable in terms of isolating IL-9 RNA, organic extraction was more effective for isolating super enhancer eRNA. Three super enhancer regions displayed 8.9-, 7.6-, and 3.8-fold greater eRNA expression after utilizing organic extraction compared to a standard spin column method for RNA isolation (Fig. 7).

5.3. Nucleofection

Cells were treated with IL-9, super enhancer, positive control (HPRT), and negative control (ASO scramble) ASOs after 24 hours and collected >24 after nucleofection for qPCR analysis. Using a fluorescent positive control vector (pmaxGFP Control Vector) provided in the Lonza Nucleofector kit, it was confirmed that this method of electroporation was successfully

allowing entrance into cells, as GFP fluorescence was visible intracellularly in ~90% of cells treated with the positive control vector.

5.4. ASO Knockdown: Preliminary Data

Unmodified IL-9 and super enhancer ASOs were used for preliminary evaluation of ASO knockdown potential. Unmodified HPRT ASOs and scrambled ASOs were used as positive and negative controls, respectively, in these experiments. A modified HPRT ASO was also utilized to prove that the nucleofection protocol was broadly effective for gene suppression prior to ordering any modified IL-9 and super enhancer ASOs.

Cells treated with HPRT ASOs, as compared to those treated with an ASO scramble, at 3 different doses (100nM, 250nM, and 500nM) exhibited a 3.1-, 2.1-, and 30.0-fold reduction in gene expression. Similarly, treatment with modified HPRT ASOs displayed a 20.0-fold reduction in gene expression at the 500nM concentration. Treating cells with a 500nM concentration of 3 different unmodified IL-9 ASOs resulted in a 1.5-, 1.8-, and 1.7-fold reduction in IL-9 expression, with no reduction at lower concentrations. Initial studies treating cells with various concentrations of unmodified super enhancer ASOs showed up to 1.8-fold decrease in super enhancer eRNA expression, however, results were inconsistent across different concentrations without a dose-dependent pattern, and effects on IL-9 expression were variable. For this reason, it is likely necessary to conduct further studies with more stable modified ASOs and determine the ideal concentrations of super enhancer ASOs to use.

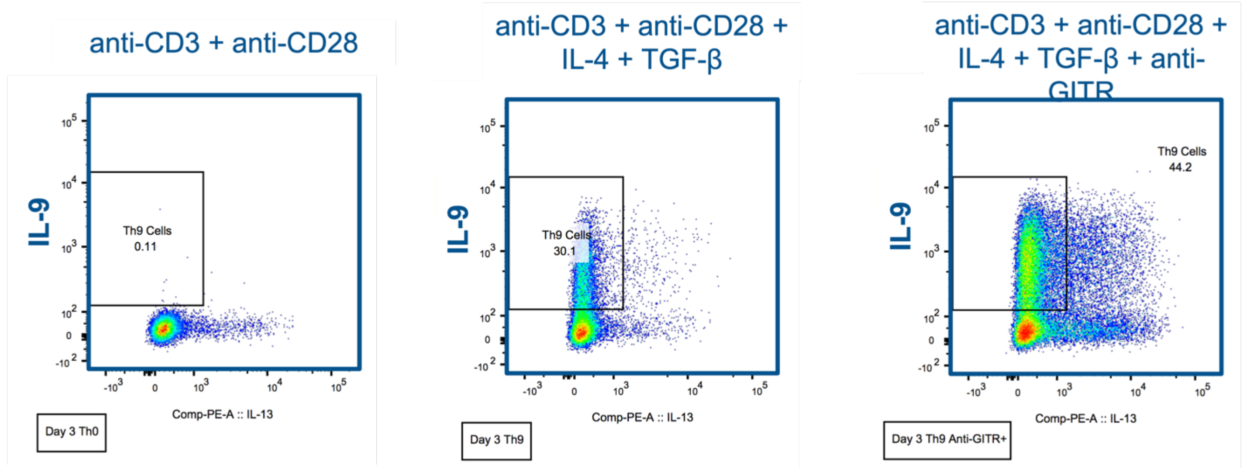

Figure 5. Th9 differentiation in anti-GITR conditions. (Left) Flow cytometry analysis of CD4+ T-cells cultured in basic T-cell activating conditions without any Th9 differentiating factors. Only 0.11% of cells were Th9 differentiated, as measured by the percentage that stained positive for IL-9 production. (Middle) Flow cytometry analysis of CD4+ T-cells cultured in Th9 conditions (IL-4 and TGF-β), displaying 30.1% Th9 differentiation. (Right) Flow cytometry analysis of CD4+ T-cells cultured in Th9 conditions (IL-4 and TGF-β) with the addition of anti-GITR stimulating antibodies, showing 44.2% Th9 differentiation.

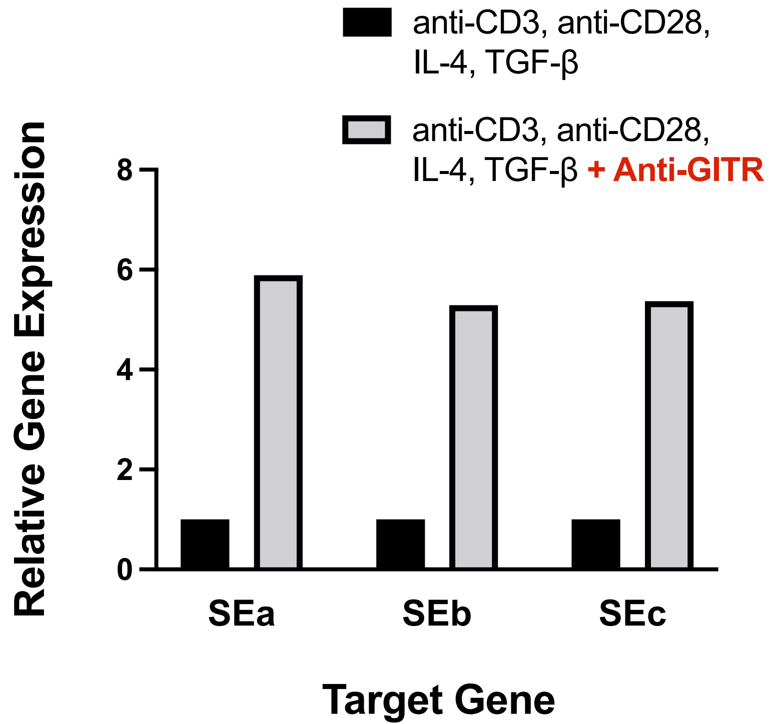

Figure 6. IL-9 super enhancer gene expression in the presence of anti-GITR. Three super enhancer regions (SEa, SEb, SEc) exhibit 5.9-, 5.3- and 5.4-fold increases in eRNA levels respectively when cultured with anti-GITR, as opposed to IL-4 and TGF-β alone.

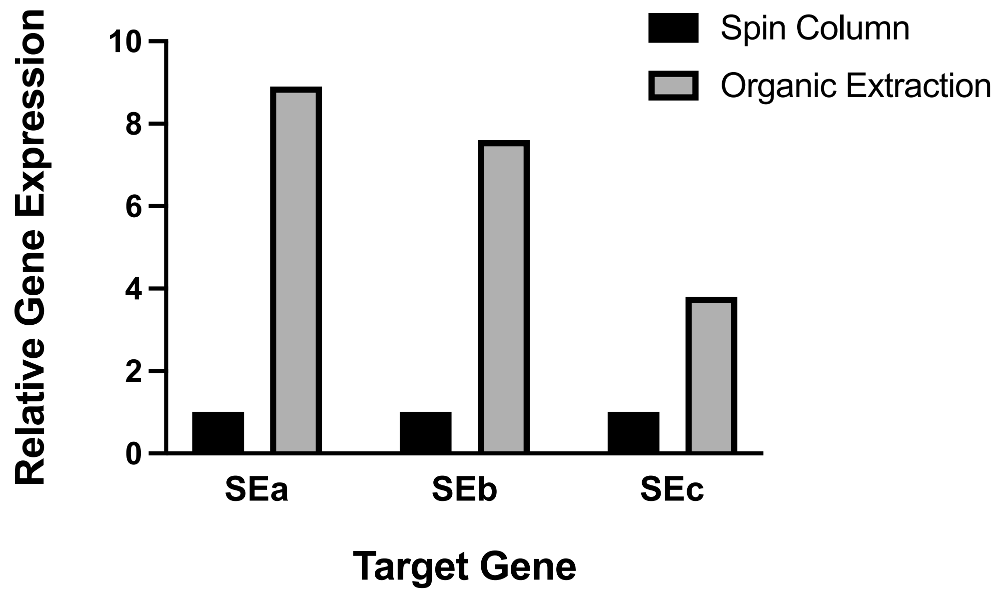

Figure 7. RNA extraction methods. Three super enhancer regions (SEa, SEb, SEc) show 8.9-, 7.6-, and 3.8-fold greater eRNA expression respectively, after utilizing organic extraction compared to a standard spin column method for RNA isolation.

6. SUMMARY AND CONCLUSIONS

Chronic Rhinosinusitis (CRS) is a debilitating disease, particularly for patients with refractory cases. As the study of CRS pathogenesis evolves, endotyping and cytokine profiling become increasingly important for identification of immunotherapeutic targets. Due to recent findings that IL-9 expression is increased in some CRS patients, particularly those with nasal polyps, comorbid atopy, and refractory disease, it is valuable to better understand the IL-9 biology, especially in terms of pro-inflammatory processes of the nasal mucosa. The unique discovery of super enhancer regulation of IL-9 has created an opportunity for in-depth analysis of the potential suppression of IL-9 by inhibiting super enhancer activity. This research sought to do so through antisense treatment targeting super enhancer regions and the IL-9 gene directly.

It was found that the best approach to an experiment testing ASO knockdown of IL-9 super enhancer eRNA would be through the in vitro culture of mouse Th9 cells and subsequent treatment with ASOs via nucleofection. Experiments for protocol optimization showed that strategies for the most robust baseline Th9 differentiation and IL-9/super enhancer production would include the addition of anti-GITR to traditional Th9 stimulation cell culture conditions and RNA isolation with an organic extraction method that better targets eRNA. Nucleofection with the Lonza Nucleofector 2b resulted in effective transfection of cells and considerable knockdown of both unmodified and modified HPRT positive controls. Because preliminary knockdown studies with unmodified IL-9 and super enhancer ASOs did not produce clear results, the best next steps would be to test more stable modified IL-9 and super enhancer ASOs at various concentrations to determine if there is a consistent dose-dependent knockdown effect. If successful, future directions include replicating studies in human cells and creating both

animal and human CRS models to determine the biological effects of ASO-mediated IL-9 suppression, in the hopes for progression to clinical trials and production of biologic therapies for future patients suffering with Chronic Rhinosinusitis.

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