Production of induced regulatory T-cells through CRISPR/Cas9-based gene editing

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Dedication

This thesis is dedicated to all those who suffer from autoimmune diseases.

Abstract

Regulatory T-cells (Tregs) are a subset of T-cells essential for maintaining immune tolerance and their dysregulation has been found to have a central role in the progression of various autoimmune diseases. The transplantations of Treg as a form of immune therapy has and continues to be an attractive method for the treatment of such disease based on their immuno-modulatory properties. Despite its potential, Treg adoptive cell transfer therapy is hampered by limited isolation efficiency due to low frequencies in human peripheral blood and poor *in vitro* expansion of a pure population. Herein, a novel CRISPR/Cas9 based technique is described utilizing AAV incorporation of strong transcriptional elements into the promoter region of the Treg master transcription factor, FOXP3, to upregulate expression in isolated primary T-cells and drive them toward a Treg phenotype.

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INTRODUCTION

The human body's ability to fight infection and disease is a complex process which requires the diverse population of cells that make up the immune system. A properly functioning immune system is dependent on the ability of these immune cells to distinguish foreign antigens from self-antigens. The immune system is composed of innate and adaptive networks each mediated by specific cell types. The innate system is primarily made up of granulocytes which phagocytose foreign invaders and secrete granules filled with digestive enzymes to eliminate invading pathogens[1]. It is the first line of defense recognizing molecular structures shared by bacteria and viruses, called pathogen associated molecular patterns (PAMPs), to mount a rapid but non-specific response that lacks long term memory formation of the invader[1]. By comparison, the adaptive immune system has both antigenic specificity and antigenic memory resulting in quicker clearance of a repeated infection[1]. It is comprised of the lymphocytes: NK, B and, T-cells[1].

T-cell is a broad classification that describes the lymphocytes that mature in the thymus[1]. T-cells arise from hematopoietic stem cells (HSCs) in the bone marrow[1]. HSCs differentiate into lymphoid progenitor cells and migrate to the thymus where they differentiate into mature naïve T-cells[1]. All T-cells have a T-cell receptor (TCR) which allows them to recognize antigens presented to them on one of two cell surface receptors known as the major histocompatibility complexes (MHC) class I and II[1]. T-cell receptor antigen specificity is determined during T-cell development in the thymus where the gene recombination event, V(D)J recombination, occurs. V(D)J recombination is a highly regulated process essential for the development of a diverse T-cell repertoire capable of

recognizing nearly two million unique antigens[1]. Autoreactive T-cells are also formed from V(D)J recombination but are deleted from the T-cell repertoire in the thymus in a process called negative selection or central tolerance[1] (**FIGURE 1**).

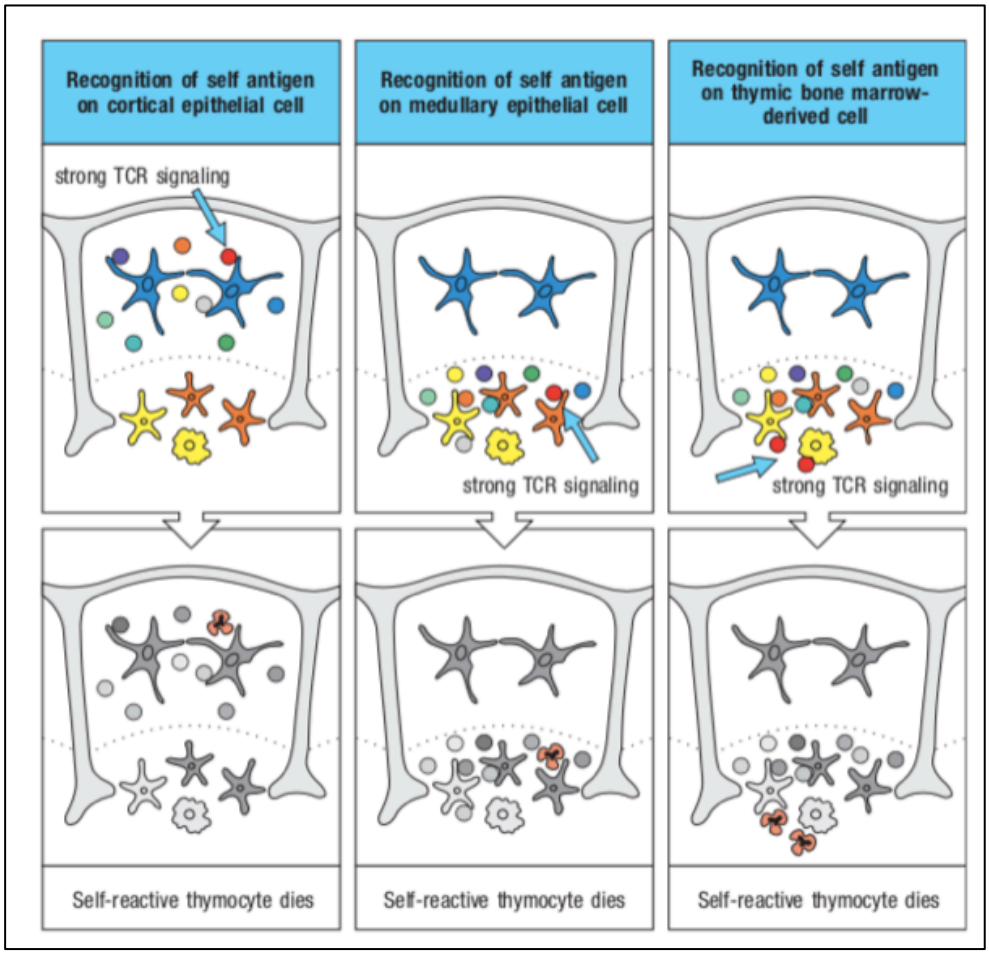


Figure 1: Centeral Tolerance Janeway's Immunobiology, 9th ed. Fig 8.29.

This ability to distinguish between self and non-self-antigens is essential for a functional immune system[1]. Regulatory T-cells (Tregs) are a subtype of T-cell that are important for controlling immune responses to self-antigen[1]. These T-cells can recognize self-antigen but serve to prevent immune responses against them[1]. Following T-cell development in the thymus, T-cells are considered mature but are termed naïve as they have not interacted with their specific antigen[1]. T-cells become activated after encountering their

cognate antigen which is typically displayed by antigen presenting cells (APCs). There are three signals necessary for T-cell activation[1]. The first signal is derived from the interaction between the specific antigen and the TCR[1]. The second is a costimulatory signal promoting survival and proliferation through the CD28 surface protein on the T-cell and the B7 surface protein on the APC[1]. The third signal is conferred by APC cytokine release which can vary depending on the pathogen (**FIGURE 2**). After activation, T-cells are considered mature and can mediate antigen eradication[1].

The first signal necessary for T-cell activation is dependent on the recognition of antigen in the context of self MHC[1]. The first signal is primarily discussed in this thesis as it allows for further classification of T-cells which will be subsequently discussed in

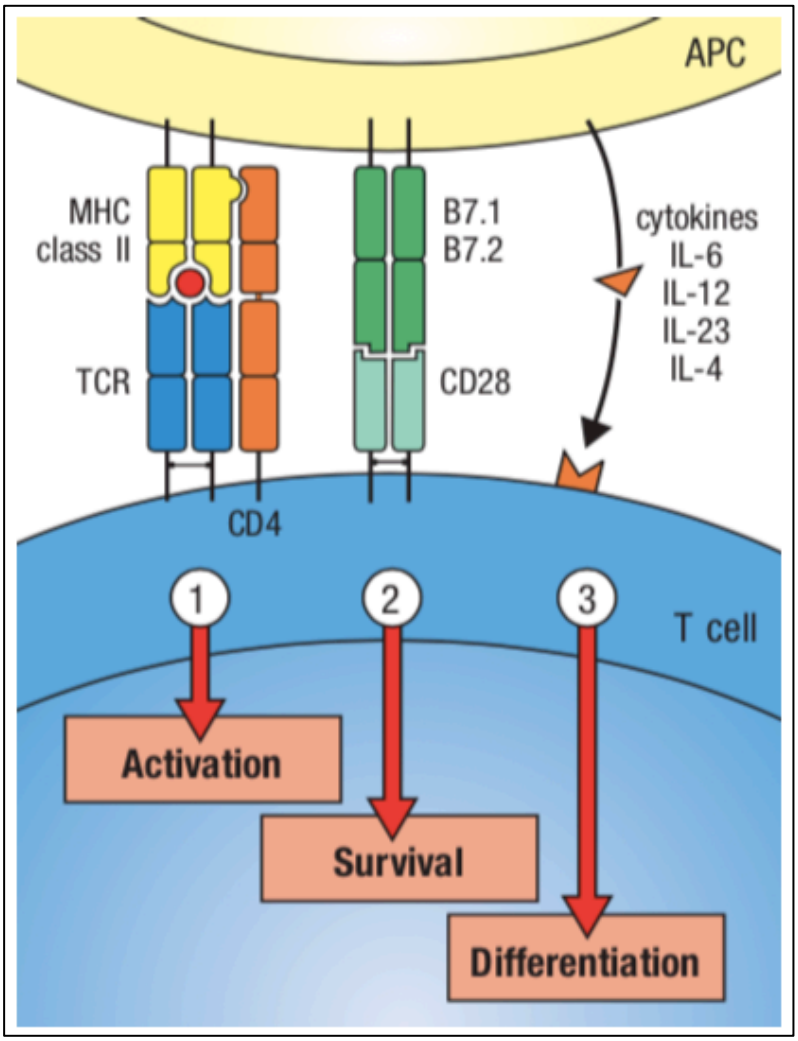


Figure 2: Three signals necessary for T-cell activation. Janeway's Immunobiology, 9th ed. Fig 9.22.

detail. In humans, MHC I and II, also referred to as human leukocyte antigen (HLA), are encoded on chromosome six[1]. There are three main gene classes for MHC I in humans, HLA-A, HLA-B, and HLA-C and three main gene classes for MHC II, HLA-DP, HLA-DQ, and HLA-DR[1]. The specific combination of diverse alleles within each gene class form the highly specific MHC haplotypes for every individiual[1]. MHC matching is especially important during solid organ and allogenic bone marrow transplantation as MHC mismatch can result in graft vs host disease (GVHD)[1]. In GVHD, graft T-cells can recognize host MHC alleles as foreign antigen and mount an immune response directed at all MHC expressing cells[1].

If an alloreactive T-cell recognizes self-MHC through its TCR, the cell surface protein CD3, which is complexed with the TCR, will drive downstream activation and proliferation leading to clonal expansion of alloreactive T-cells[1]. T-cells can be further classified based on their expression of surface markers which help to stabilize this

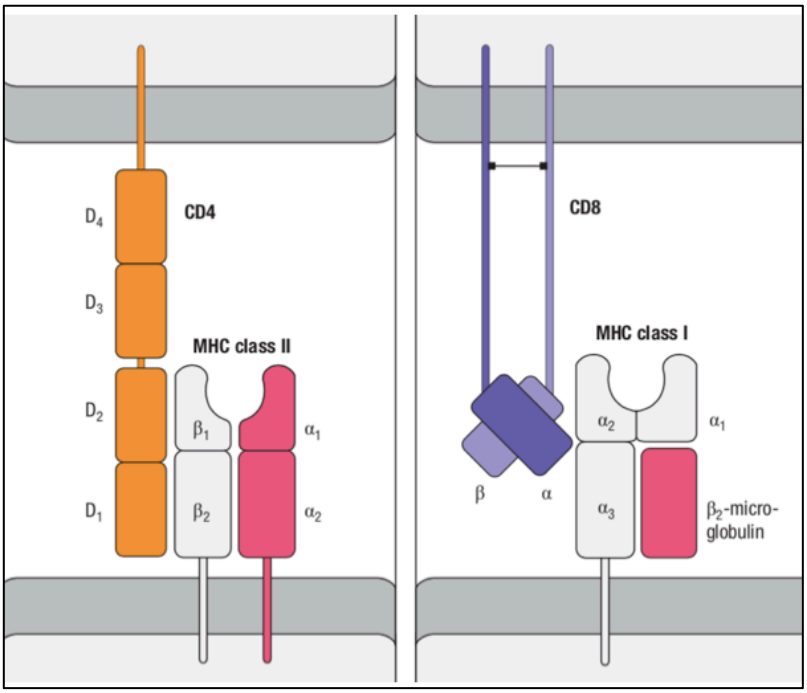


Figure 3: CD8 and CD4 associations with MHC. Janeway's Immunobiology, 9th ed. Fig 4.27.

TCR/MHC interaction (**FIGURE 3**). CD8+ T-cells express the cell surface protein, CD8, which stabilizes the interaction between the TCR and MHC I[1]. All nucleated cells in the

human body express MHC I[1]. MHC I proteins present intracellular peptides from the cytoplasm. In a healthy cell, these are self-peptides which don't normally elicit an immune response[1]. During a viral infection, a cell can present viral peptides on MHC I which can subsequently activate cytotoxic CD8+ T-cells ultimately leading to the death of the infected cell. Activated CD8+ T-cells or cytotoxic T lymphocytes (CTLs) earn their name due to their ability to directly kill infected cells through the release of soluble factors such as the granzyme and perforin or contact-dependent pathways such as the Fas ligand[1]. Viruses have evolved processes to downregulated MHC I and evade CD8+ T-cell mediated cytotoxicity[1]. In response to this, another type of lymphocyte, called natural killer cells (NK cells), recognize the absence of MHC I on a cell[1]. MHC I interacts with an inhibitory receptor on the surface of NK cells to prevent activation. The absence of this interaction induces NK cell activation and target killing.

CD4+ T-cells express the cell surface protein CD4 which stabilizes the interaction between the TCR and MHC II. Predominately, APCs like macrophages and dendritic cells express MHC II. APCs phagocytose extracellular peptides and present them on MHC II allowing recognition and removal of bacteria, parasites and other pathogens[1]. CD4+ T-cells also require three signals for activation. The first signal necessary for CD4+ T-cell activation is the TCR/MHC II interaction. The second is the costimulatory signal and the third signal is conferred through cytokine signaling release to promote differentiation into the terminal effector subclasses[1] (**FIGURE 2**). Differentiation into these effector subclasses is determined by the context of their activation[1]. In the presence of IFN-γ and IL-12, a mature naïve T-cell will adopt the Th1 phenotype. In the presence of IL-4, the Th2 phenotype will be adopted. In the presence of TGFβ, IL-6 and IL-23 the

Th17 phenotype and, in the presence of just IL-6, the Tfh phenotype will be adopted[1] (**FIGURE 4**).

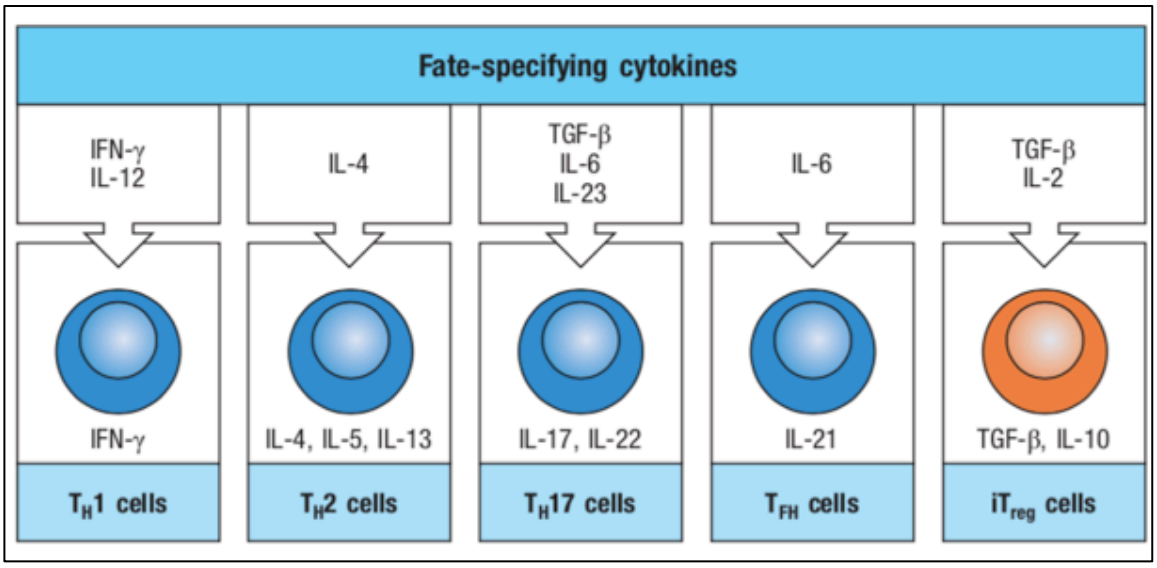


Figure 4: T-cell fate specifying cytokines. Janeway's Immunobiology, 9th ed. Fig 9.31.

Helper T-cells like Th1, Th2 and Th17 help to activate other immune cells by releasing proinflammatory cytokines like IL-4, IL-2 and, IFN-γ[1]. These T-cells are known as the effector T-cells[1]. T-follicular helper cells (Tfh), another type of effector T-cell, help to activate B-cells by driving the humoral response[1] (**FIGURE 5**).

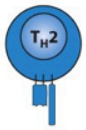
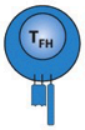
	CD8 cytotoxic T cells	CD4 T_H1 cells	CD4 T_H2 cells	CD4 T_H17 cells	T_{FH} cells	CD4 regulatory T cells (various types)
Types of effector T cell						
Main functions in adaptive immune response	Kill virus-infected cells	Activate infected macrophages Provide help to B cells for antibody production	Provide help to B cells for antibody production, especially switching to IgE	Enhance neutrophil response Promote barrier integrity (skin, intestine)	B-cell help Isotype switching Antibody production	Suppress T-cell responses
Pathogens targeted	Viruses (e.g. influenza, rabies, vaccinia) Some intracellular bacteria	Microbes that persist in macrophage vesicles (e.g. mycobacteria, *Listeria, Leishmania donovani, Pneumocystis carinii*) Extracellular bacteria	Helminth parasites	*Klebsiella pneumoniae* Fungi (*Candida albicans*)	All types	

Figure 5: T-cell classifications. Janeway's Immunobiology, 7th ed. Fig 8-1.

Regulatory T-cells are another important subclass of T-cells due to their immunomodulatory properties. These cells can express either CD4 or CD8, but are mainly considered to be CD4+, and play an important role in self-tolerance in the human immune system[1]. Tregs help control unwanted immune responses to self-antigen that would otherwise lead to autoimmunity. There are two types of regulatory T-cells classified by their location during development[1]. Natural Tregs (nTregs) develop in the thymus and induced Tregs (iTregs) develop in the peripheral lymphoid tissues like the spleen and lymph nodes[1]. nTregs are thought to develop from potentially self-reactive T-cells and strong TCR signaling with self-antigen induces Treg development[35] **(FIGURE 1**). iTregs develop in the periphery from T-cells in the presence of TGFβ (Transforming Growth Factor β) and in the absence of IL-6. IL-6 is a proinflammatory cytokine released by activated macrophages[1]. IL-6 has many functions with one being modulation of body temperature during infection. At higher temperatures, the adaptive immune system

functions more efficiently while bacterial and viral replication is inhibited. IL-6 also drives neutrophils proliferation which limits the infection while the adaptive immune response is developing. TGFβ is an anti-inflammatory cytokine released by Tregs and many other cells in the immune system[1]. It is known to help control unwanted immune responses by preventing the differentiation and proliferation of naïve CD4+ T-cells into effector phenotypes if reactive to self-antigen in the peripherary[26]. This auto-reactive suppression mechanism is also called peripheral tolerance[1]. Without infection and the release of proinflammatory cytokines, peripheral naïve self-reactive T-cells will be activated in high TGFβ environments and can quickly be suppressed or differentiated into Tregs[1] (**FIGURE 6**).

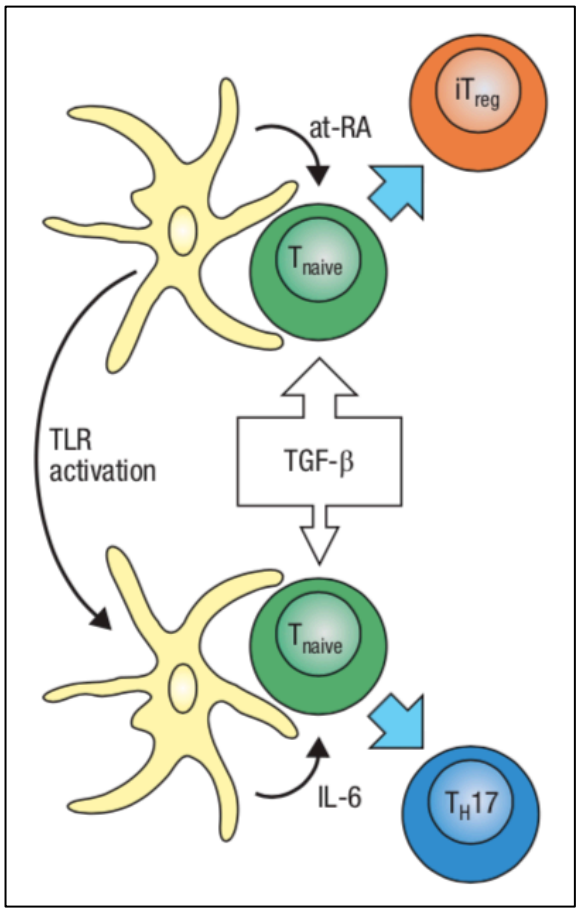


Figure 6: Peripheral tolerance. Janeway's Immunobiology, 9th ed. Fig 9.33.

Tregs function through direct suppression of T-cells and through the release of immunosuppressive cytokines like IL-10 and TGFβ[1]. Tregs directly suppress T-cell activation by preventing traditional T-cell activation. Regulatory T-cells have a TCR that recognizes class II self-peptide. If a self-antigen is presented on an MHC II, the regulatory T-cell can remove the co-stimulatory marker B7 on APCs through the surface protein CTLA-4 and permanently prevent activation of other T-cells by that APC. It has also been shown that regulatory T-cells can be cytotoxic; another mechanism by which they can regulate auto-reactive T-cells[3]. They are thought to kill their target T-cells through both contact dependent and soluble factor pathways[4,5].

Classically, regulatory T-cells are identified as being CD4+ or CD8+, CD25+ and, FOXP3+. FOXP3 (forkhead box P3) is the master transcription factor of the Treg phenotype[1]. FOXP3 is essential for Treg development and induces the expression of Treg related genes like the high affinity IL-2 receptor (CD25)[1]. IL-2 is a cytokine released in low quantities by naïve T-cells that induces proliferation of neighboring and distant naïve T-cells as well as itself. Upon activation, T-cells release much higher concentrations of

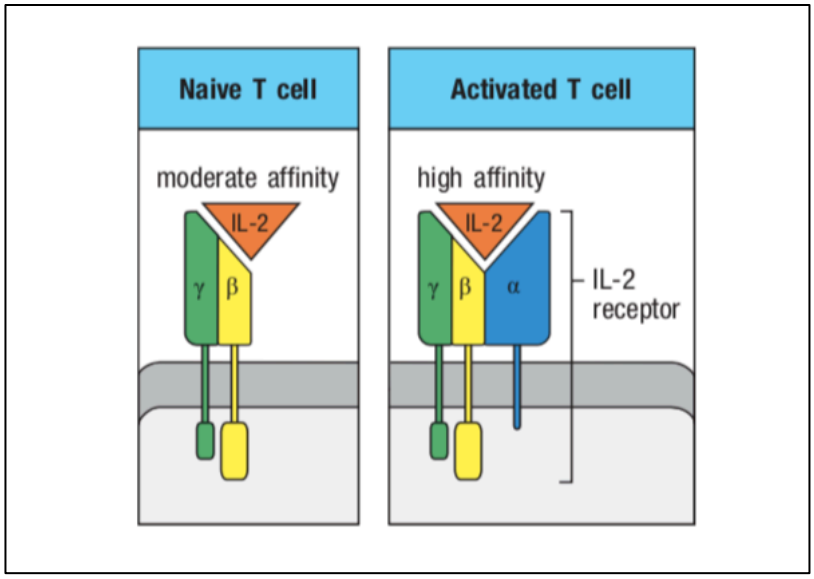


Figure 7: Moderate and high affinity IL-2 receptor i.e. CD25. Janeway's Immunobiology, 9th ed. Fig 9.23.

IL-2 to induce an immune response[1]. The IL-2 receptor has both a moderate affinity and high affinity isoform[1] (**FIGURE 7**). All T-cells express the moderate affinity receptor and only express the high affinity receptor upon activation[1]. Because Tregs constitutively express the high affinity receptor, they preferentially bind IL-2 present in the milieu of various organs, preventing the activation of other T-cells[1]. If IL-2 is released at high concentrations, such as during times of infection and inflammation, Tregs will be unable to sequester all the IL-2 and T-cell activation will occur. FOXP3 also decreases Treg ability to release IL-2 preventing additional IL-2 accumulation and unwanted immune responses[1]. Tregs are also characterized by their low expression of the IL-7 receptor, CD127, which is associated with naïve and memory T-cells[7,8.] IL-7 is a cytokine secreted by stromal cells in the thymus and the bone marrow and is necessary for T-cell development and homeostasis[9]. Mice with knock-out mutations in the FOXP3 locus have increased fatal auto-immunities[10]. Tregs also have high expression of CTLA-4 which is a negative costimulatory receptor[1]. CTLA-4 binds and removes B7 on APCs preventing T-cell activation through that APC[1]. One final indication of Treg phenotype is the expression of Helios. Helios is upregulated in response to FOXP3 and is involved with the epigenetic silencing of IL-2 release[11] (**TABLE 1**).

Treg associated marker[1, 8,11]	**Treg marker role[1, 8, 11]**
FOXP3	Master transcription factor of Treg fate.
CD25	High affinity IL-2 receptor
CD127	IL-7 receptor, present on memory T-cells
Helios	Treg transcription factor
CTLA-4	Negative co-stimulatory to B7

Table 1: Regulatory T-cell associated markers.

The dysfunction of regulatory T-cells have been implicated in a variety of autoimmune diseases such as irritable bowel syndrome (IBS). It is thought that Tregs play an important role in suppressing the immune response against gut microbiota by releasing IL-10 and that the loss of these cells induces IBS[1]. Another autoimmune disease, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), is caused by a missense mutation in the FOXP3 gene resulting in severe allergic reactions, anemia, polyendocrinopathy, thrombocytopenia and, usually leads to early death[1]. Recently, regulatory T-cells have also been shown to play a role in the tumor microenvironment (TME) by suppressing the immune response and in doing so limiting antitumor immunity[12]. Current research aims to remove the Tregs from the TME in the hope that it will allow reactivation of the immune system and tumor clearance[12]. A summary of Treg related diseases is shown in **TABLE 2**. These phenotypes demonstrate the crucial nature of functional Tregs for immune system homeostasis and highlight their potential as a therapeutic agent for treating autoimmune disease and preventing transplantation rejection[13, 14]. However, a barrier to the development of such treatments is the low endogenous frequency of Tregs. Tregs make up only 5-10% of all the CD4+ T-cells and only 0.1-1% of all CD8+ cells in the human body making isolation very expensive and inefficient[1, 15]. Culturing CD4+ T-cells with TGF-β is known to give rise to *in vitro* Tregs through increased FOXP3 expression[16]. However, these Tregs, termed induced Tregs (iTregs), a separate population from the *in vivo* iTregs discussed earlier, have unstable FOXP3 expression and under inflammatory conditions such as in the presence of certain inflammatory cytokines such as IL-4, IL-6 and IL-12, they lose their FOXP3 expression altogether and can revert back to other subtypes of T-cells such as

Th1, Th2 and, Th17[16]. This is problematic for transplantation as inducing effector phenotypes could worsen autoimmunity or graft rejection.

Treg associated disease[28]	Treg role in disease progression[28]
Immune Dysregulation, Polyendocrinopathy, enteropathy, X-linked (IPEX)	Loss of function mutation of FOXP3. Autoimmunty against skin, intestines, endocrine glands, blood cells, liver and, kidney. Often fatal within first two years of life.
Irritable Bowel Syndrome (IBS)	Decreased Treg suppressive function and increased autoimmunity against commensal bacteria
Multiple Sclerosis (MS)	Decreased Treg suppressive function and increased autoimmunity against myelin
Rhumatoid Arthritis (RA)	Decreased Treg suppressive function and increased autoimmunity against joint tissues
Cancer	Suppress immune mediated tumor clearance

Table 2: Regulatory T-cell associated diseases.

Access to a biobank of Tregs would be beneficial when treating any number of autoimmune diseases. This is especially true given Tregs constitute such a small percentage of T-cells in the human body. *In vitro* production of regulatory T-cells would be the ideal method for generating the sizable numbers necessary for transplantation. Currently, *in vitro* production of regulatory T-cells is being studied by culturing naïve CD4+ T-cells and isolated Tregs with TGFβ, retinoic acid (RA) and, rapamycin but the resulting FOXP3+ populations have either unstable FOXP3 expression or secrete the proinflammatory cytokine IL-4[29, 37]. Alternatively, differentiation of induced pluripotent stem cells (iPSCs) into Tregs is also under investigation but the costs of such treatments are estimated to be nearly $45,000 per treatment and resulting cells tend to skew toward a CD8+ T-cell phenotype[29, 39]. Due to the importance of the master transcription factor

FOXP3 in controlling the expression of regulatory T-cell related genes, upregulation of FOXP3 in naïve CD8+ and CD4+ T-cells could result in the conversion of these T-cells into regulatory T-cells. This thesis discusses an approach for the targeted upregulation of FOXP3 in isolated T-cells.

There are a number of ways in which targeted gene upregulation can be achieved, for example, the introduction of exogenous plasmids and gene modification using viruses[7]. Lentivirus and retrovirus are often utilized for their large packaging capacity and ability to integrate a gene of interest into the genome[17, 19]. However, plasmids are inefficient and do not integrate into the genome resulting in only transient gene expression and certain viral gene editing techniques are often difficult and can result in random integration and mutagenesis[18]. To prevent these gene editing induced risks while also maintaining stable expression of the FOXP3 gene, we hypothesize that the targeted insertion of a strong promoter into the endogenous promoter region of the FOXP3 locus using an adeno-associated viral vector would allow controlled insertion into the T-cell genome to cause both stable and permanent FOXP3 gene expression. Adeno-associated viral vectors (AAV) are non-pathogenic single-stranded DNA viruses which have also been used as a gene delivery system[20]. AAV vectors show promise as successful integration doesn't require host cell proliferation, and the wild type virus integration is targeted to the AAVS1 locus in the human genome[20]. Removing the endogenous proteins packaged in the AAV and inserting a template of interest can remove the ability of the AAV to integrate into the AAVS1 locus and allows it to act as a donor delivery system[34]. This donor system could be used to repair CRISPR/Cas9 induced double stranded breaks which will be described in the next section.

CRISPR/Cas9 is a novel gene editing technique which has quickly become a useful tool in gene therapy research due to cost, availability, and generation time compared to other gene editing techniques[38] (**TABLE 3**). CRISPR/Cas9 is a component

Platform	Cost	Availability	Generation Time
Meganuclease	$50,000	low	Months/year
ZFN	$5,000	low/medium	Months/year
TALEN	$500	high	Week(s)
CRISPR/Cas9	$5	highest	days

Table 3: Costs associated with various gene editing techniques.

of the adaptive immune system present in bacteria against bacteriophage infection[21]. The bacteria integrate fragments of bacteriophage genome into the CRISPR, or clustered regulatory interspace short palindromic sequence, locus[21]. These fragments, termed spacers, are separated by repetitive regions in the CRISPR locus. Upon secondary infection, RNA complementary to the bacteriophage sequence is generated from these spacers, called CRISPR RNA (crRNA). Upstream of the CRISPR locus, a second region is transcribed creating the trans-activating CRISPR RNA (tracrRNA)[21]. The tracrRNA binds to the crRNA creating a partial double-stranded RNA which is then loaded onto a CRISPR associated (Cas) protein[21] (**FIGURE 8**). Complexation of the Cas protein with the RNA activates the nuclease activity which can target, cleave, and destroy the invading nucleic acid sequences recognized by the crRNA[21]. The Cas9/RNA complex scans the invading genome for sequence complementary to the crRNA. Identification of a complementary sequence must occur near a proto-spacer adjacent motif (PAM) which are short sequences present in invading viral genomes[21]. Recognition of a PAM site by the Cas9 indicates foreign viral DNA is present and Cas9 should be activated. Different Cas9

proteins recognize different PAM sequences. One example of a PAM sequence used by the *S. pyogenes* Cas9 is NGG where N is any nucleotide that is followed by two guanines[21].

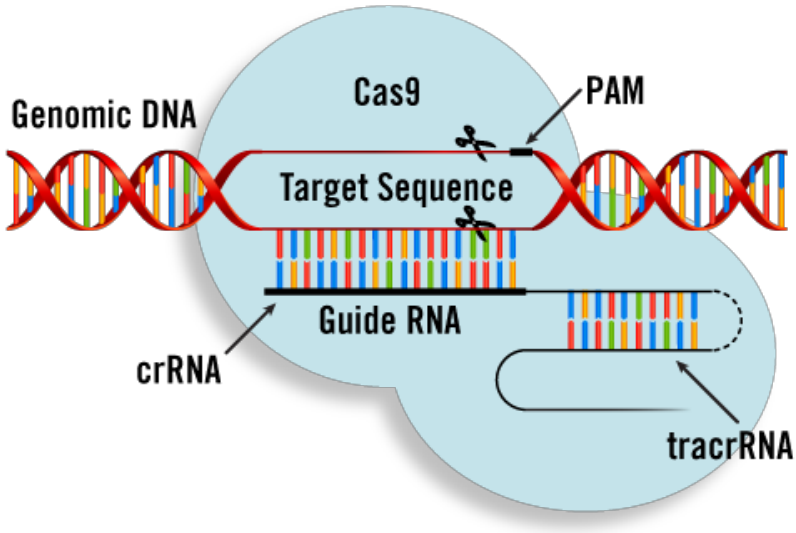


Figure 8: CRISPR/Cas9 complex. NJSTA.

The CRISPR/Cas9 system is a powerful tool which can be used to induce double stranded breaks (DSB) and is especially helpful in eukaryotic cells. For use in human cells, the crRNA and the tracrRNA can be fused via a linker to create a single guide RNA (sgRNA) allowing for an easier delivery of a single gRNA species[21]. Upon delivery of the Cas9 and the gRNA, a ribonucleoprotein (RNP) forms and will be directed by the gRNA to the complementary DNA sequence in the host genome[21]. The formation of double stranded breaks can destabilize chromosomes and can often be fatal to the cell[22]. There are two methods that eukaryotic cells use to repair double stranded breaks: non-homologous end joining (NHEJ) and homology directed repair (HDR)[21] (**FIGURE 9**). NHEJ is the simpler pathway and fuses the broken ends of DNA together and often results in insertions or deletions (indels) in an error prone fashion[21]. HDR requires a homologous template often in the form of the homologous chromosome *in vivo*[21].

Exogenous DNA with homology to the DSB can also be used to incorporate DNA into the host genome by using the HDR repair machinery[21]. DSB recruit proteins such as BRCA1, BARD51 and, Rad51 which induce strand invasion into the homologous chromosome[33]. The break can then be resynthesized complementary to the template with DNA polymerase and repaired with DNA ligase[33]. These donor templates are often plasmids, PCR products or linearized plasmids and result in HDR efficiencies of 0.2-2.1%[23]. These poor HDR efficiencies are due to many factors ranging from the template to the stage of the cell cycle when the double stranded break occurs[24]. In order to address low HDR rates, adeno-associated virus has been shown to be an efficient donor template with 12-fold increased HDR efficiencies compared to plasmids[24]. This might be due to

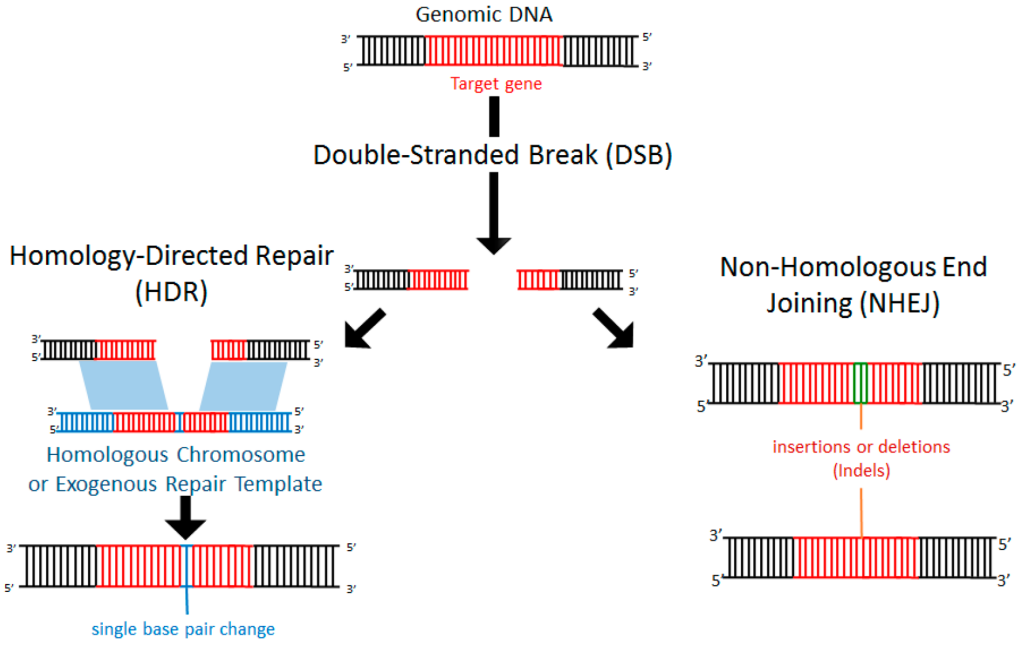


Figure 9: Homology-directed repair vs non-homologous end joining. MDPI.

the large quantities of donor template AAV can deliver to the cell. This essentially 'floods' the cell with the donor template in order to increase the rate at which HDR occurs.

Due to the increased efficiency of AAV-based donor delivery, an *in vitro* protocol for the production of regulatory T-cells was developed using CRISPR/Cas9 coupled with AAV delivery of donor DNA to primary T-cells obtained from peripheral blood. Increasing the expression of the Treg master transcription factor, FOXP3, is hypothesized to induce the development of regulatory T-cells from naïve T-cells. To increase FOXP3 expression, CRISPR/Cas9 was targeted to the promoter region of the FOXP3 locus to induce a DSB followed by AAV donor-mediated HDR delivering transcriptional activating elements. Inclusion of homology arms in the donor matching the regions flanking the cut site generated by CRISPR/Cas9 in the FOXP3 promoter region could allow AAV delivery of the strong polymerase II promoter, MND, and a ubiquitous chromatin opening element (UCOE) [30, 31] to induce the regulatory T-cell phenotype (**FIGURE 10**). The UCOE sequences drives further gene upregulation by preventing gene silencing and methylation[25]. Herein, the donor carrying AAV is termed UMET for the UCOE, MND promoter, tEGFR, and T2A sequences present. Inclusion of tEGFR simplified selection of successfully transfected and transduced cells as FOXP3 expression was brought under the control of the starting methionine of the tEGFR. tEGFR also serves as a safety mechanism as a monoclonal antibody against tEGFR can be used for targeted depletion[25, 36]. In this thesis, Isolated T-cells were transfected via electroporation with spCas9 peptide and a sgRNA targeting the FOXP3 promoter region. The cells were then transduced with the UMET AAV inserting the UCOE element, an MND promoter, and the selectable marker tEGFR. Validation of the genetic modifications was conducted by Sanger sequencing of the locus to verify in-frame insertion of the UMET without indel formation. The modified cells were then characterized for the regulatory T-cell

expression patterns through qPCR of FOXP3 expression and flow cytometry for various markers including CD25, CD127- and, Helios.

Efficient production of regulatory T-cells is essential for treatment of various autoimmune diseases. The use of the gene editing technique CRISPR/Cas9 to drive regulatory T-cells from naïve T-cells by insertion of strong transcriptional elements into the FOXP3 locus is a novel technique to address the unmet need of stable, large scale, Treg production.

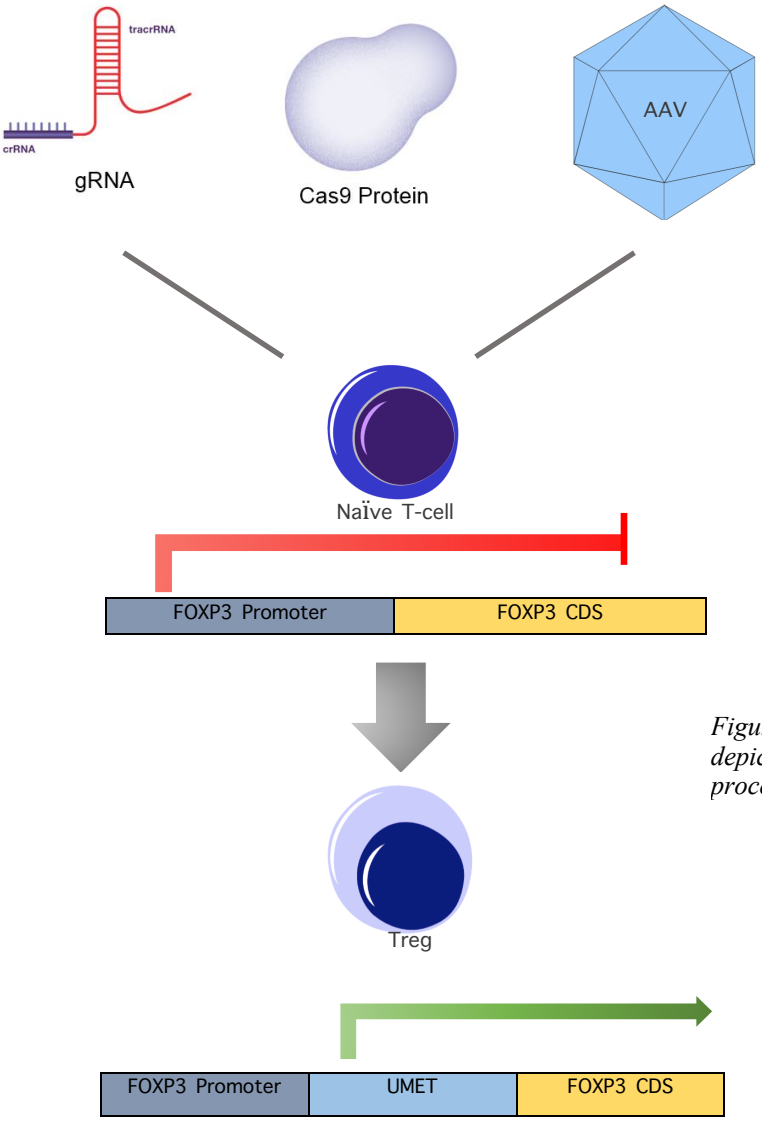


Figure 10: Illustrative depiction of experimental process. ThermoFisher.

METHODS

Cell Culture

Human embryonic kidney 293T cells (293Ts) were purchased from ThermoFisher (Waltham, MA, USA). 293Ts were cultured in Dulbecco's Modification of Eagle's Medium with glutamax, non-essential amino acids, penicillin/streptomycin and 10% fetal bovine serum (ThermoFisher Waltham, MA, USA). Cells were grown at 37 ◦C and 5% CO2.

Primary human T-cells were isolated from fresh blood using the RosetteSep Human T Cell Enrichment Cocktail (STEMCELL Technologies, Cambridge, MA, USA). The Enrichment Cocktail targeted all cells excepted T-cells with an antibody. The targeted cells are pelleted using a centrifuge and the T-cells isolated. All T-cells were obtained in accordance with the Declaration of Helsinki requirements for research on human subjects. T-cells were cultured in X-VIVO-20 (Lonza, Allendale, NJ, USA) with 10% AB serum (Valley Biomedical, Winchester, VA, USA), 300 IU of IL-2 (PeproTech, Rocky Hill, NJ, USA), N-Acetyl-L-Cysteine, penicillin/streptomycin, and Gluta-MAX-I (ThermoFisher Waltham, MA, USA). T-cells were stimulated with CD3/CD28 Dynabeads (ThermoFisher, Waltham, MA, USA) at a 3:1 bead to cell ratio. Stimulation of CD3 and CD28 is necessary for T-cell proliferation. CD4+ T-cells were isolated using EasySep Human CD4 Positive Selection Kit (STEMCELL Technologies, Cambridge, MA, USA) and CD8+ T-cells were isolated using EasySep Human CD8 Positive Selection Kit II (STEMCELL Technologies, Cambridge, MA, USA). Cells were grown at 37 ◦C and 5% CO2.

tEGFR+ cells were isolated through addition of 3.0 µg/mL of monoclonal phycoerythrin (PE) labeled anti-human EGFR antibody (Biolegend, San Diego, CA,

USA; Clone AY13) and the EasySep PE positive selection kit (STEMCELL Technologies, Cambridge, MA, USA)

Cells were counted using a hemocytometer and 0.4% Trypan blue staining (ThermoFisher, Waltham, MA, USA).

Regulatory T-cells were a generous gift of Dr. Keli Hippen at the University of Minnesota, Department of Pediatric Blood and Marrow Transplant. Tregs were cultured in the same manner as primary T-cells.

CRISPR/Cas9

Chemically modified guide RNAs were purchased from Synthego (Redwood City, CA, USA). spCas9 mRNA was obtained through Aldevron (Fargo, ND, USA). Both were used at a concentration of 1 μg per reaction. FOXP3 guide RNA target sequences (5'-3') were as follows:

FOXP3 guide 1: AGAGGTATCAATGAGATAAT

FOXP3 guide 2: ATTGATACCTCTCACCTCTG

FOXP3 guide 3: CCCCTCACCACAGAGGTGAG

Gene Transfer

Guide RNA testing plasmids were delivered to 293Ts through Lipofectamine 2000 (ThermoFisher, Waltham, MA, USA) in cis using the Guide-IT CRISPR/Cas9 system (Clontech, Mountain View, CA, USA). T cells were electroporated using the Neon Electroporation System (ThermoFisher, Waltham, MA, USA) in 10 μL reactions using buffer T. T cell Neon settings: 1400 volts, 10 ms, 3 pulses. CD3/CD28 Dynabeads were removed from the T-cells 1 hour before electroporation.

Control GFP mRNA was obtained through TriLink BioTechnologies (San Diego, CA, USA). AAV-6 particles were produced by Vigene (Rockville, MD, USA) and were

added at the indicated MOI. FOXP3 upregulation testing was performed using gRNA 2 in T-cells.

Molecular Analysis

Surveyor assay: T-cells were transfected with the gRNA and Cas9 mRNA. Cells were plated at 100,000 cells/ well in a 96 well plate for 18 hours. Genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (ThermoFisher, Waltham, MA, USA). The FOXP3 locus was amplified from genomic DNA using AccuPrime *Taq* DNA Polymerase, High Fidelity (ThermoFisher, Waltham, MA, USA) using primers indicated in **APPENDIX 1** at 94C 2 min followed by 40 rounds of 94C 40 s, 59C 40 s, 68C 1 min, and a final extension of 68C for 10 min. Surveyor assay was performed using the Surveyor Mutation Detection Kit (Integrated DNA Technologies, Coralville, IA, USA) with resolution on a 10% polyacrylamide gel. The surveyor assay is a mismatch cleavage assay that uses a nuclease that can detect single base pair mutations. Without incorporation of a homologous template, indels will form at the edited locus. Incubating PCR product from edited and non-edited cells creates duplexes that have base mismatches from the NHEJ in the edited cells. The nuclease can cleave the mismatches and the fragments can be run on an agarose gel to visualize.

Inside/out HDR PCR: Genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (ThermoFisher, Waltham, MA, USA). Genomic DNA was amplified using primers indicated in **APPENDIX 1** using Phusion High-Fidelity DNA Polymerase (New England BioLabs, MA, USA) with the following conditions: 98C × 30 s and 40 cycles of 98C × 5 s, 58C × 10 s, and 72C × 2 s. Amplicons were TA cloned (ThermoFisher, Waltham, MA, USA) and sequenced after plating colonies (Sequetech, Mountain View, CA, USA).

Inference of CRISPR Edits (ICE): Sanger sequencing files were analyzed for insertions and deletions using the ICE algorithm (https://ice.synthego.com/#/; Synthego, Redwood City, CA, USA) after being transfected with the gRNA and Cas9 RNP (Aldervron, Fargo ND) followed by transduction with AAV. Genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (ThermoFisher, Waltham, MA, USA). The FOXP3 locus was amplified from the gDNA using primers indicated in **APPENDIX 1** using Phusion High-Fidelity DNA Polymerase (New England BioLabs, MA, USA) with the following conditions: at 94C 2 min and 40 rounds of 94C 40 s, 59C 40 s, 68C 1 min, and a final extension of 68C for 10 min. Amplicons were TA cloned (ThermoFisher, Waltham, MA, USA) and sequenced prior to plating colonies (Sequetech, Mountain View, CA, USA).

Quantitative real time PCR: Total RNA was isolated using RNeasy Mini Kit (Qiagen, Vinlo, Netherlands) and then reverse transcribed with SuperScript IV VILO MasterMix (ThermoFisher, Waltham, MA, USA) and analyzed by TaqMan gene expression assay using the $2^{-\Delta\Delta CT}$ method. All final values were multiplied by 10. FOXP3 probe was Hs01085834_m1 and GAPDH probe was Hs99999905_m1 (ThermoFisher, Waltham, MA, USA). Unedited T-cells were used as the negative control.

Flow Cytometry

Flow cytometry data was acquired on a BD LSRII Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data was analyzed with FlowJo 10.5.2 (FlowJo, LLC, Ashland, OR, USA). The following antibodies were used: CD4: PerCP-Cy5.5 clone OKT4; EGFR: PE-Dazzle clone AY13; CD8: FITC clone SK1; FOXP3: PE clone 259D; CTLA-4: PE-Cy7 clone BNI; CD25: BV711 clone BC96; Helios: AF647 clone 22F6;

CD127: PE-Cy7 clone A019D5; EGFR: APC clone AY13; Zombie Violet™ Fixable Viability Kit. All obtained from BioLegend (San Diego, CA, USA). Unedited T-cells were used as the negative control.

RESULTS

Determining FOXP3 gRNA

Three guide RNA sequences were chosen in the promoter region of the FOXP3 locus (**FIGURE 11A**). To first determine which guide would yield the highest upregulation of FOXP3, HEK-293T cells were lipofected with the gRNA, spdCas9 fused to the transcriptional activator VP64, and lambda activator. To determine FOXP3 expression, qPCR was performed. The results demonstrated gRNA 2 gave the most upregulation of FOXP3 under these conditions (**FIGURE 11B**) with expression increased as much as 10-fold. Based on these results, gRNA 2 identified as the lead candidate and used in all additional experimentation.

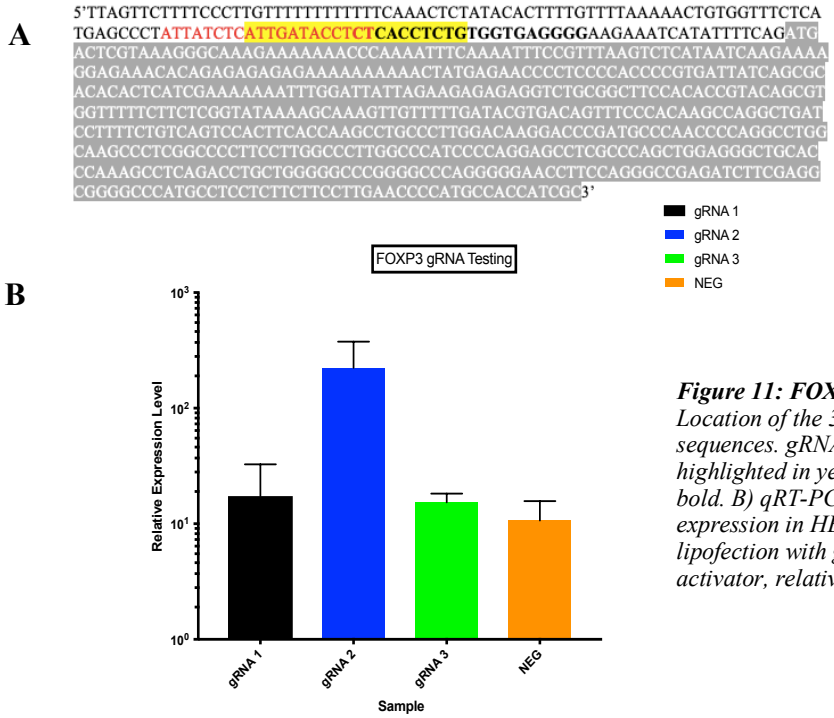


Figure 11: FOXP3 gRNA testing. *A) Location of the 3 FOXP3 gRNA sequences. gRNA 1 in red, gRNA 2 highlighted in yellow and gRNA 3 in bold. B) qRT-PCR of FOXP3 expression in HEK-293T cells after lipofection with gRNA, Cas9 and, V(64 activator, relative to NEG, n=3*

FOXP3 gRNA 2 Surveyor Assay and ICE

To test the activity of gRNA 2/Cas9 in the FOXP3 promoter region, a surveyor assay was performed. A surveyor assay is a mismatch cleavage assay used to determine the nuclease activity of the Cas9 by studying indel formations at the locus of interest. Primary human T-cells were isolated and electroporated with spCas9 RNP and chemically modified sgRNA 2 obtained through Synthego. Chemically modified gRNAs have been shown to increase HDR rates and are more resistance to exonuclease activity[40]. The FOXP3 locus was amplified through PCR and the FOXP3 locus from untransfected cells was used as a negative control (**APPENDIX 1**). After the surveyor assay was performed, the resulting PCR products were run on an agarose gel (**FIGURE 12A**). The results show fragmented final product consistent with effective Cas9 cutting. Edited FOXP3 PCR product was also analyzed using Interference of CRISPR Edits (ICE) program by Synthego to determine editing frequency[41] (**FIGURE 12B**). ICE analysis is similar to TIDE (Tracking Indels by Decomposition) and determines the major indels formed and editing frequency. Similar to the gene activation data, these results showed that gRNA 2 drives effective nuclease activity and shows indel formation that is consistent with CRISPR/Cas9 cutting[32].

A

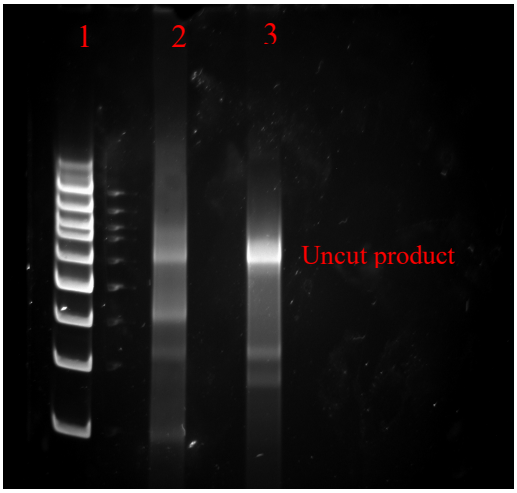


Figure 12: FOXP3 gRNA 2 Surveyor and ICE. *A) Surveyor results after electroporation with gRNA 2 and Cas9. Lane 1 – ladder, lane 2 – T-cells with electroporation of gRNA and Cas9, lane 3- T-cells with no treatment. B) ICE results showing editing frequencies of gRNA 2.*

B

FOXP3 AAV6 Donor Production and Editing Frequency

After observing the ability of gRNA 2 to effectively target the FOXP3 locus and the modest increase in FOXP3 expression in HEK-293T cells, an AAV6 donor was designed in order to insert a UCOE and MND promoter into the endogenous promoter

region of the FOXP3 locus in isolated primary peripheral blood T-cells. Donor arms were derived targeting 400-500 bp regions on either side of the gRNA 2 cut site (**FIGURE 13A, APPENDIX 2**). The right and left homology arms flanked the UCOE, MND promoter and, a 2A sequence to allow co-expression of the following tEGFR (UMET). AAV serotype 6 particles bearing the donor template were obtained through Vigene. Donor template was designed to mediate in frame insertion with the starting methionine of the FOXP3 locus.

To determine the optimal AAV donor dose, various MOIs (Multiplicity of Infection) were tested (**FIGURE 13B**). The chemically modified gRNA 2 was electroporated with Cas9 mRNA to isolated T-cells. Immediately after electroporation, the AAV donor was added at the various doses. qRT-PCR of the FOXP3 expression showed that all doses resulted in similar levels of expression. MOI of 2.5 x 10^6 was used for the rest of experimentation.

Efficiency of AAV transduction was determined by tEGFR expression through flow cytometry. Efficiency was tested for the bulk (pan) population of T-cells isolated from peripheral blood, CD4+ T-cells isolated from peripheral blood and, CD8+ T-cell isolated from peripheral blood. Results showed similar HDR efficiencies by tEGFR expression for all three populations (**FIGURE 13C, D, E**).

The accuracy of homology directed repair was determined using homologous recombination PCR (HR-PCR) (**FIGURE 13E**). PCR was performed to amplify the regions of insertion between both the left and right arms. Sequencing results showed HR occurred without indel formation (**FIGURE 13F, G**). Collectively, this data shows that the use of AAV donor to insert strong transcriptional elements into the FOXP3 promoter

did insert without unintended indel formation into the targeted locus and gave increased expression of FOXP3.

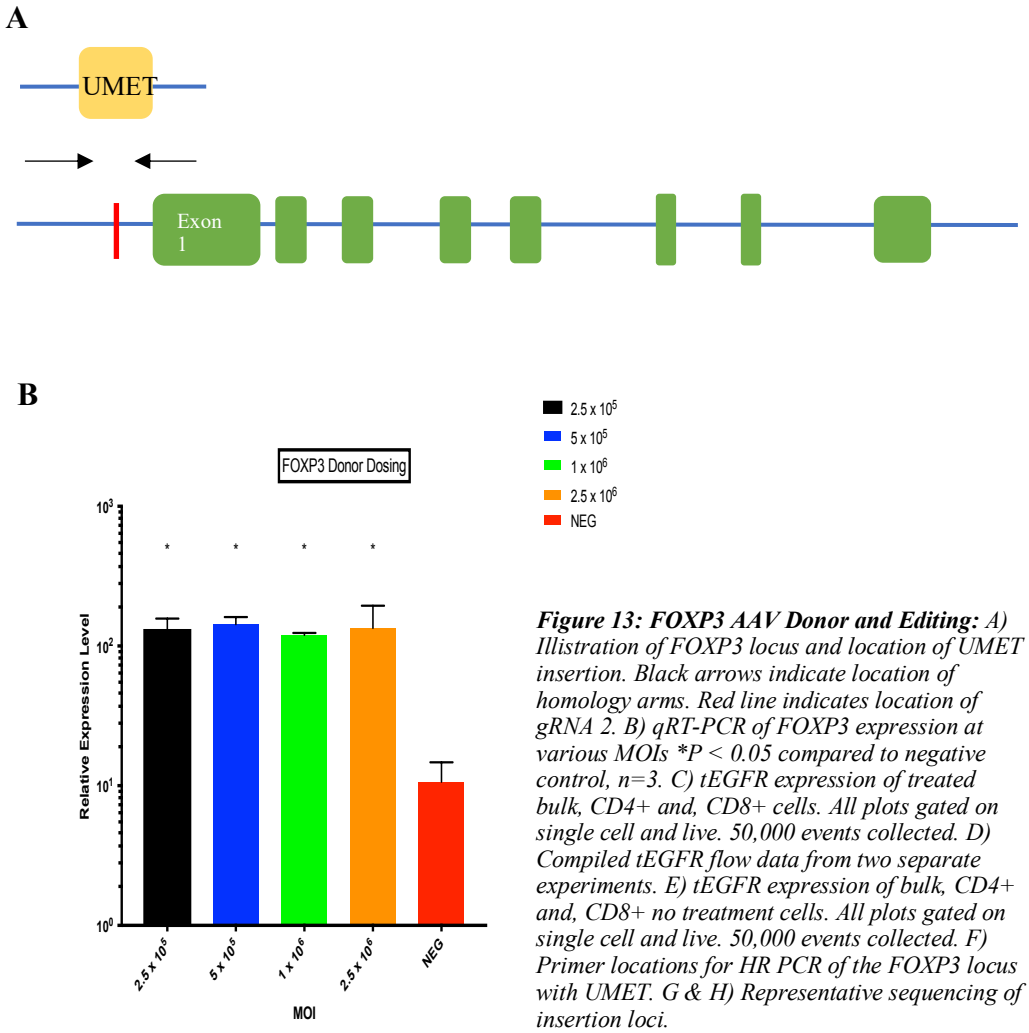
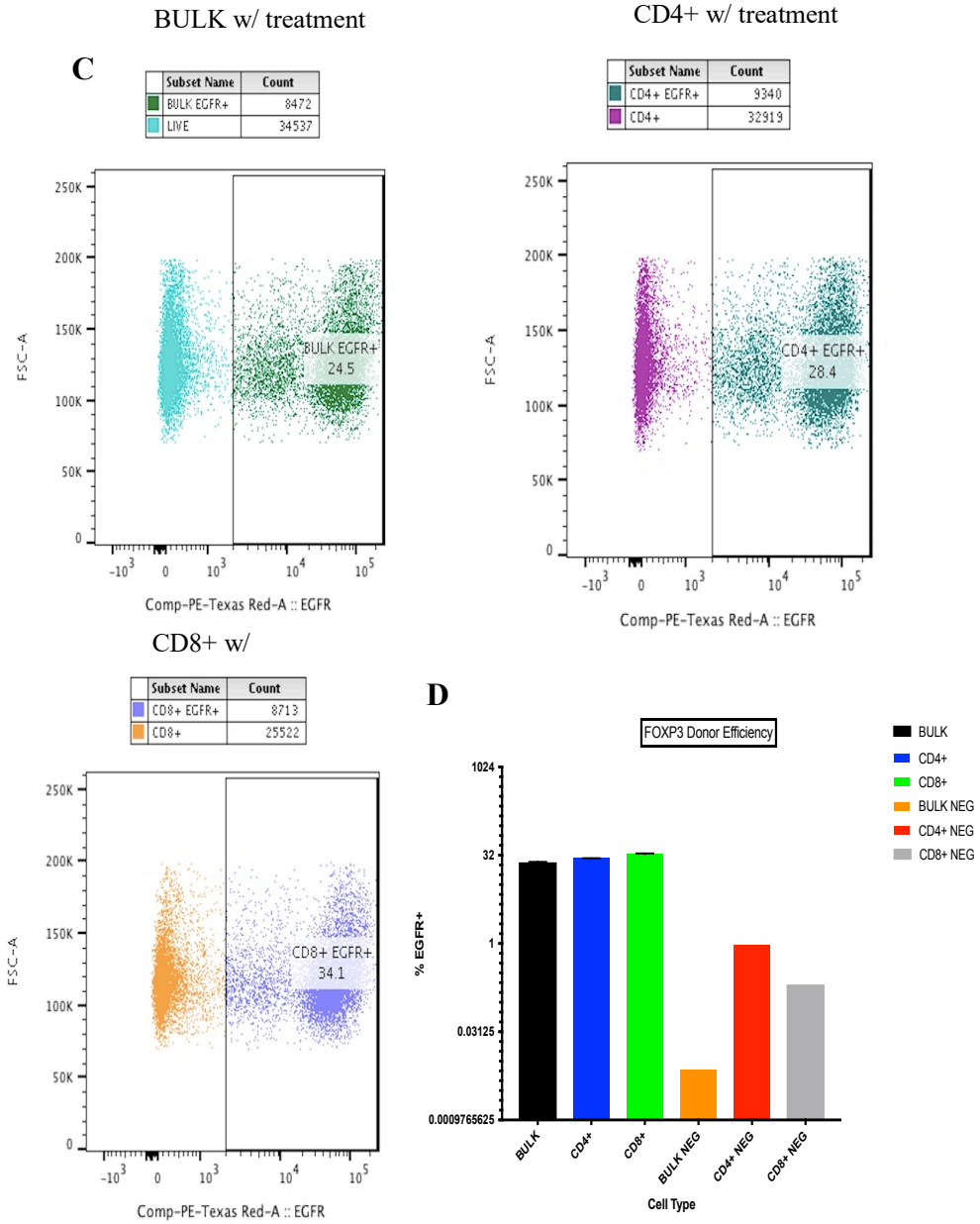
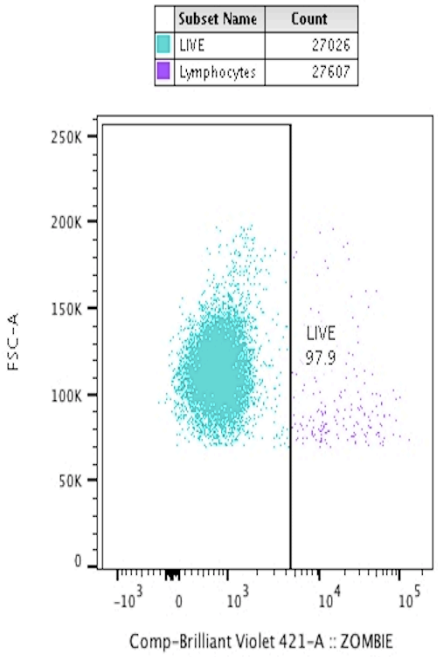


Figure 13: FOXP3 AAV Donor and Editing: *A) Illistration of FOXP3 locus and location of UMET insertion. Black arrows indicate location of homology arms. Red line indicates location of gRNA 2. B) qRT-PCR of FOXP3 expression at various MOIs *P < 0.05 compared to negative control, n=3. C) tEGFR expression of treated bulk, CD4+ and, CD8+ cells. All plots gated on single cell and live. 50,000 events collected. D) Compiled tEGFR flow data from two separate experiments. E) tEGFR expression of bulk, CD4+ and, CD8+ no treatment cells. All plots gated on single cell and live. 50,000 events collected. F) Primer locations for HR PCR of the FOXP3 locus with UMET. G & H) Representative sequencing of insertion loci.*

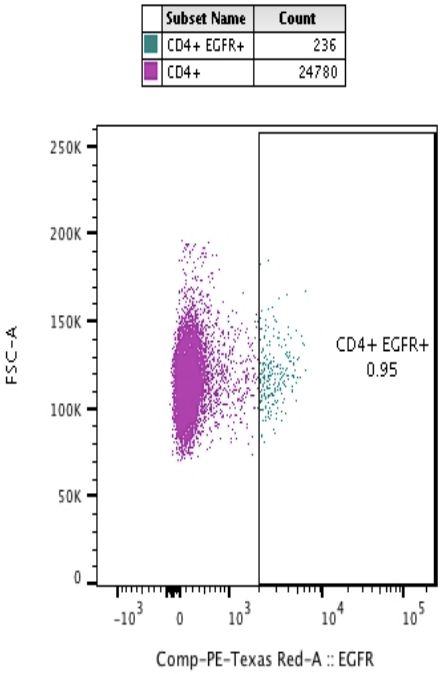

BULK w/ treatment
CD4+ w/ treatment
C
Subset Name
Count
BULK EGFR+
8472
LIVE
34537
CD4+ EGFR+
9340
CD4+
32919
BULK EGFR+
24.5
CD4+ EGFR+
28.4
FSC-A
Comp-PE-Texas Red-A :: EGFR
CD8+ w/
CD8+ EGFR+
8713
CD8+
25522
CD8+ EGFR+
34.1
D
FOXP3 Donor Efficiency
BULK
CD4+
CD8+
BULK NEG
CD4+ NEG
CD8+ NEG
% EGFR+
Cell Type

E

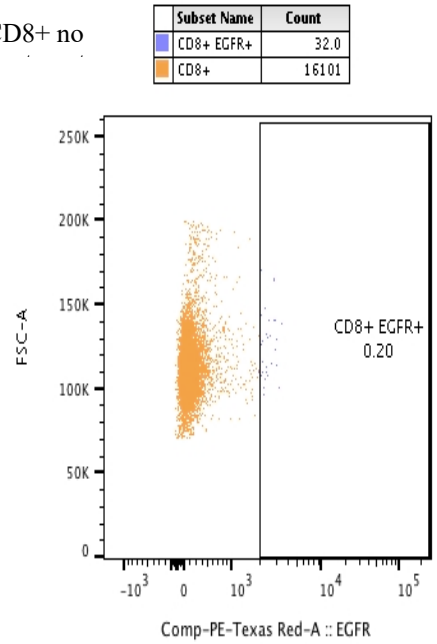
BULK no treatment



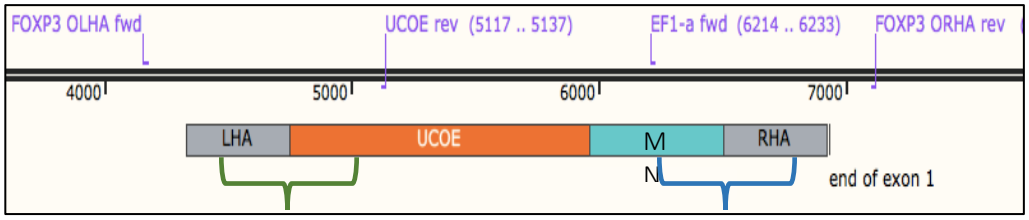
CD4+ no treatment



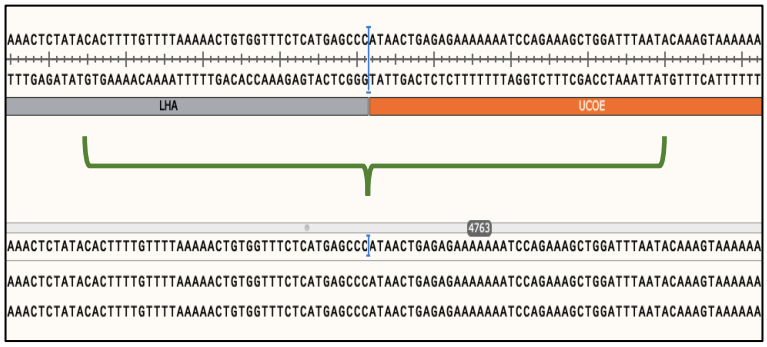
CD8+ no



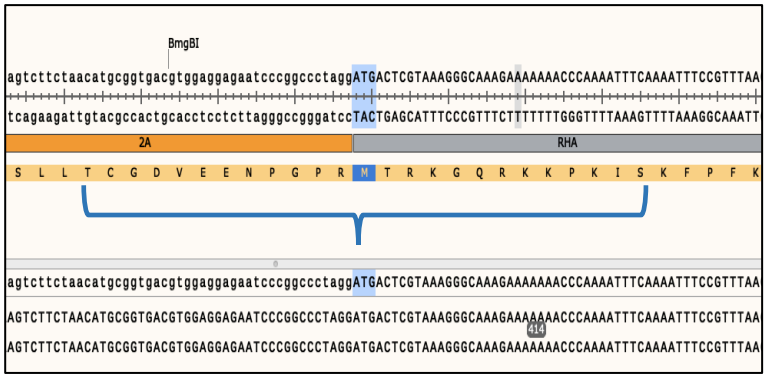
F



G



H



FOXP3 Expression and Treg Panel

EGFR selection was performed to isolate cells that had been successfully modified. qRT-PCR was performed on these cells to determine FOXP3 expression on bulk, CD4+ and, CD8+ EGFR+ T-cells (**FIGURE 14A**). FOXP3 expression was increased in each population and up to 100-fold in the pan population.

After successful upregulation of FOXP3 expression was determined, the expression of other regulatory T-cell related markers was investigated using flow cytometry. Tregs are known to be high in the surface marker CD25 and low in the surface marker CD127. Tregs also have expression of the transcription factor Helios. The results showed high expression of CD25 and the transcription factor Helios and low expression of CD127 (**FIGURE 14B)**. The results suggest that the upregulation of FOXP3 through the insertion of a UCOE and strong promoter leads to expression of Treg-specific proteins. Isolated regulatory T-cell cells were compared to the edited T-cells levels as a positive control (**FIGURE 14C**). 6.68% of treated CD4+ T-cells were CD25+, CD127-, FOXP3+ and, Helios+ and 31.6% of treated CD8+ T-cells were CD25+, CD127-, FOXP3+ and, Helios+. The results suggest a preference for edited CD8+ T-cells but further experimentation is necessary to elucidate this trend.

The results presented in this thesis show that the use of an AAV donor combined with CRISPR/Cas9 for the upregulation of FOXP3 gives targeted homology directed repair without indel formation and significantly increased FOXP3 expression. The increased FOXP3 expression drives the cells to express Treg related proteins potentially leading to an efficient protocol for the production of Tregs.

A

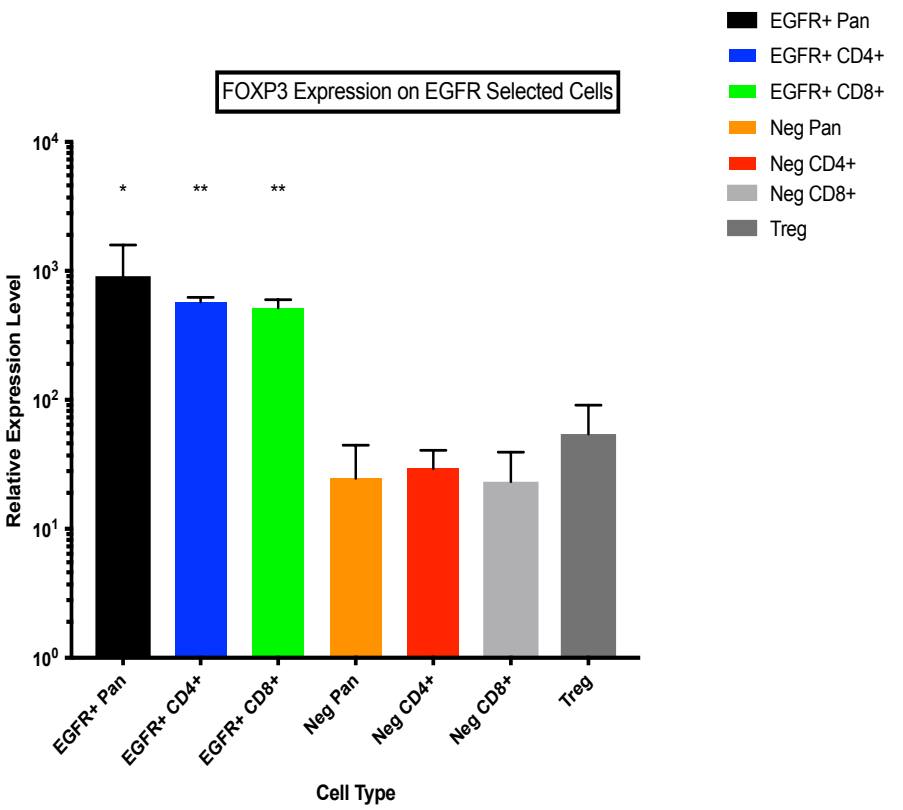
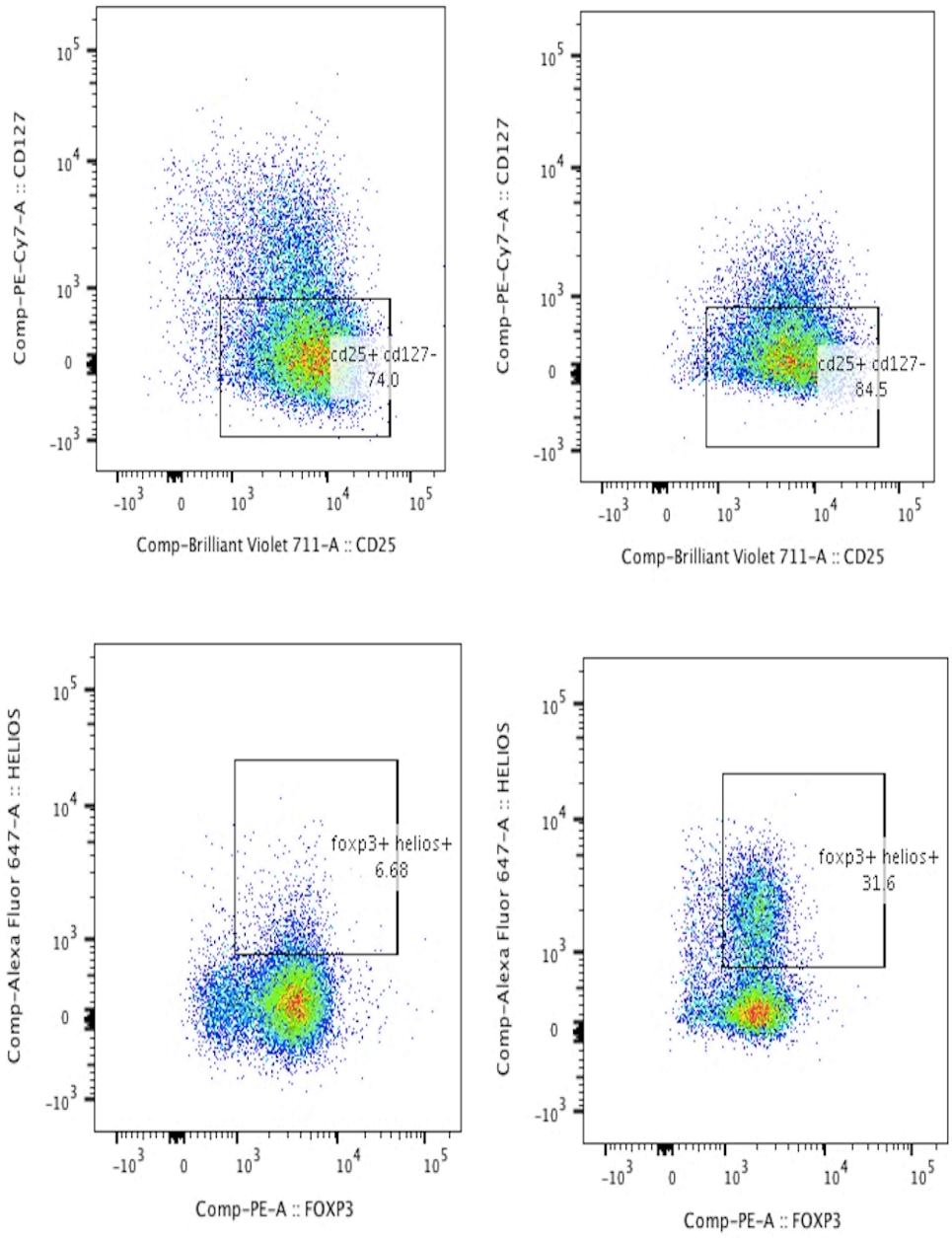


Figure 14: FOXP3 Expression and Treg Panel: *A) qRT-PCR of FOXP3 expression of tEGFR selected bulk, CD4+ and, CD8+ cells. *P<0.01, **P<0.001 compared to corresponding negative control. n=5. B) Flow cytometry data of treated CD4+ and CD8+ cells. All plots were gated on single cell and live. FOXP3 Helios+ subgates were performed on the CD25+ CD127- enriched population. 50,000 events collected. C) Flow cytometry data of isolated regulatory T-cells. All plots were gated on single cell and live. 50,000 events collected. FOXP3 Helios+ subgates were performed on the CD25+ CD127- enriched population D) Flow cytometry data of CD4+ and CD8+ no treatment cells. All plots were gated on single cell and live. FOXP3 Helios+ subgates were performed on the CD25+ CD127- enriched population. 50,000 events collected*

B

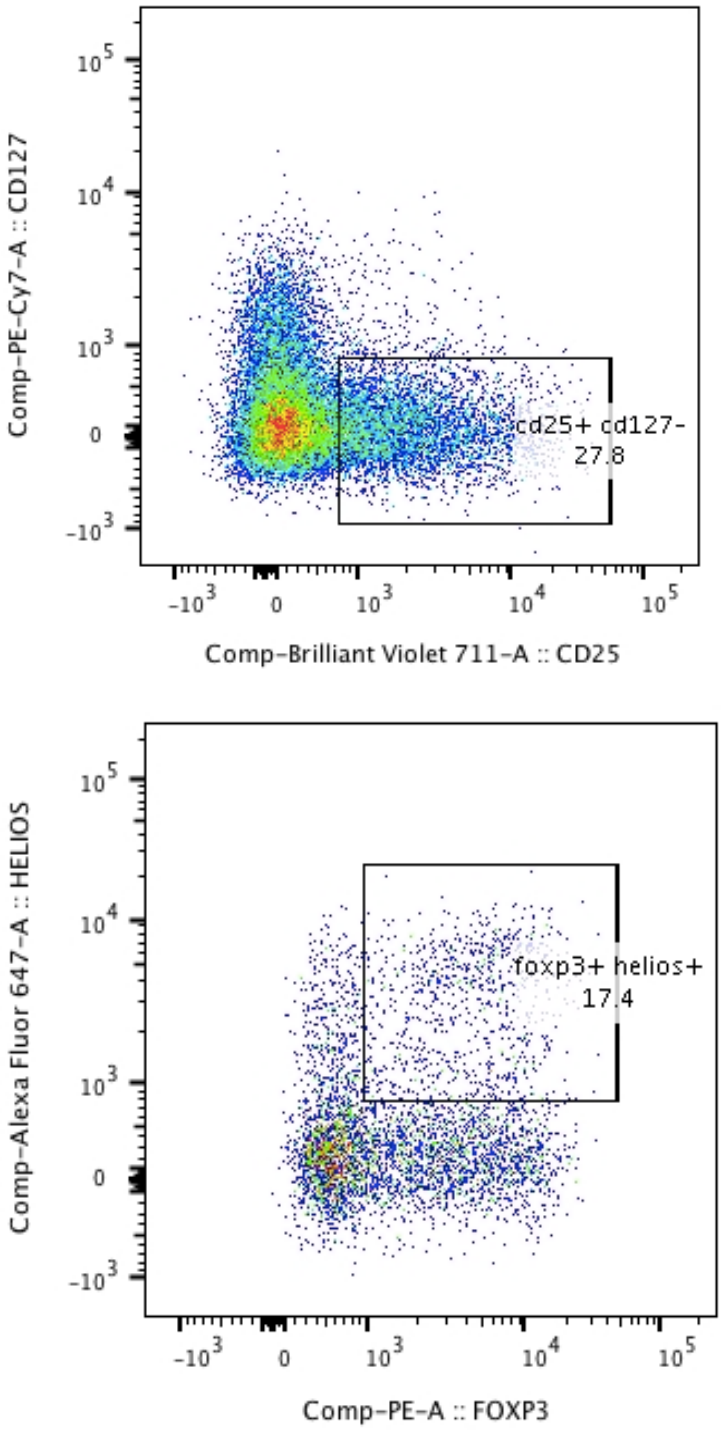
CD4+ T-cells

CD8+ T-cells



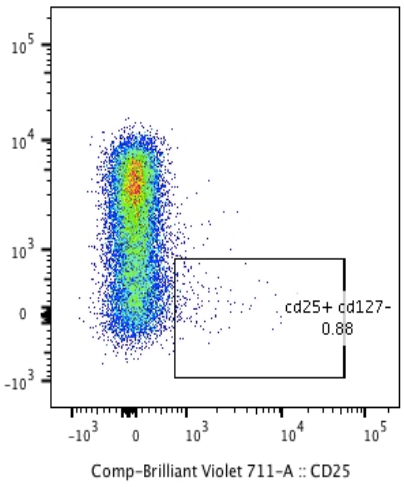
C

Isolated Treg

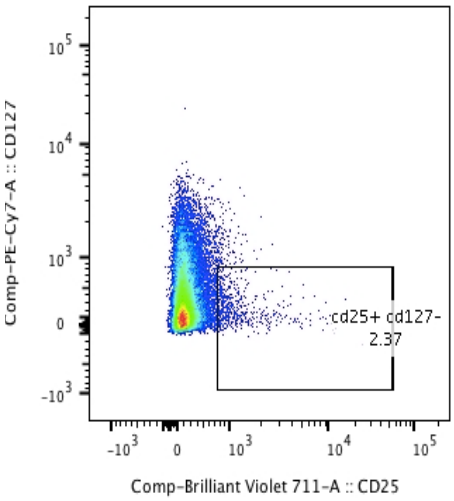


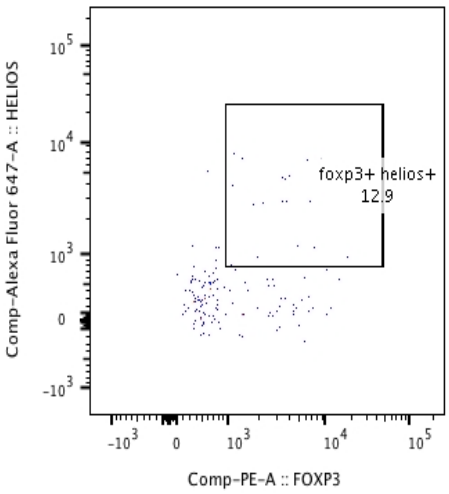
D

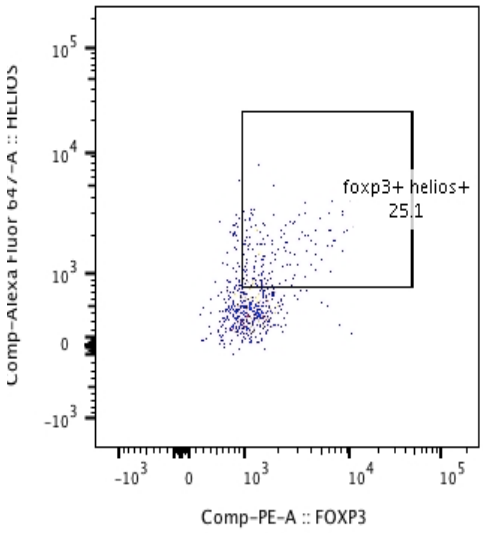
CD4+ no treatment



CD8+ no treatment







DISCUSSION

Regulatory T-cells constitute an important subclass of T-cells necessary for the regulation of the immune system[1]. Tregs are known to have roles in various autoimmune diseases like IBS and MS[28] along with determination of allogenic transplantation success[28]. Adoptive cell transfer therapy of regulatory T-cells has long been researched in the hopes of treating autoimmunity and helping to mitigate the side effects of allogenic bone marrow and organ transplantation[13]. However, Tregs constitute only 5-10% of all T-cells in the human body which makes isolating the number of cells needed for transplantation difficult[1]. Current research into the production of regulatory T-cells results in regulatory T-cells with unstable Treg characteristics that are often lost if cultured with proinflammatory cytokines or Tregs that express T-effector related markers[16, 29]. Induced pluripotent stem cells are another field of research into the production of regulatory T-cells, however the differentiation and transplantation of these types of Tregs is very expensive and their differentiation often leads to a CD8+ phenotype[29, 39].

A new approach to develop regulatory T-cells from both CD4+ and CD8+ cells has been described in this thesis. This approach uses the novel gene editing technique CRISPR/Cas9 with adeno-associated virus transduction to upregulate FOXP3 in T-cells. FOXP3 is considered the master transcription factor necessary for the regulatory T-cell phenotype[1]. We used CRISPR/Cas9 targeted to the promoter region of the FOXP3 locus to insert strong transcriptional elements to increase its expression. CRISPR/Cas9 can induce double stranded breaks (DSB) in mammalian cells. The repair of this DSB can be through non-homologous end joining (NHEJ) or homology directed repair (HDR) if an appropriate homologous template is available[21]. Adeno-associated virus has been shown

to be an efficient template for HDR[24]. Using CRISPR/Cas9 and AAV6 delivery of a donor template, a ubiquitous chromatin opening element (UCOE) and a strong promoter, MND, was delivered to induce the expression of FOXP3 in naïve isolated T-cells[30,31].

The results indicate that the use of AAV as a donor template gives HDR at rates of near 30%. These cells have up to 100-fold increase in FOXP3 expression. These cells are also CD25+, CD127- and, Helios+. The results suggest that there may be a preference for CD8+ regulatory T-cells but further investigation must be done to further elucidate this trend.

To further expand the research described in this thesis, additional regulatory T-cell markers should be looked at like CTLA-4, and PD-1[1]. After additional characterization, a repression assay should be performed in order to test the functional ability of these cells. Finally, the use of CRISPR/Cas9 and AAV donor delivery of a UCOE and MND promoter should be expanded in other cell types such as induced pluripotent T-cells to determine if this approach offers another pathway for the production of regulatory T-cells from stem cells.

In conclusion, a method for the production of regulatory T-cells has been discussed. This method uses a CRISPR/Cas9 induced double stranded break paired with the AAV delivery of strong transcriptional activating elements into the FOXP3 locus of isolated peripheral T-cells. Increased expression of FOXP3 was observed along with increased expression of other Treg related markers. While further research is still necessary, the results reported thus far suggest a Treg-like-cell has been developed.

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APPENDIX (1)

Primer Sequences

Primer:	Sequence (5'-3'):
FOXP3 FWD	AACTTACATGCCCCTCACCCTC
FOXP3 REV	TCGATGAGTGTGTGCGCTGAT
FOXP3 Left Arm FWD	CATCGTGAGGATGGATGCATTA
UCOE REV	TCAAGCAACTCTCCTACCTCT
EGFR FWD	CCAGTGTGCCCACTACATT
FOXP3 Right Arm REV	CTGGACAGCATTTCAAGTTGTT

Table A1: Primer names and sequences

APPENDIX (2)

FOXP3 left homology arm (LHA) and right homology arm (RHA) Sequences

LHA: 418 bp

TCCATGAATTTTAGGGGATGACAGTGGGCTCTCCGCTTTCTCCTCCATGAAGT

AACTTACATGCCCCTCACCCTCTGTGGGAGGGGTGTTGCAGGGGGTGCAGAA

CTCCCCTCGCCGGGTAGTTCAAGCAATGGGGACCATATCAATTCCATCTATAG

GGAAACTGAGGCCTGGAGTAGGGCGAGGCCTCTGGGAACCCAGCCCTATTCT

GTCTCTTTCCCTGGCATTTCCCATCCACACATAGAGCTTCAGATTCTCTTTCTT

TCCCCAGAGACCCTCAAATATCCTCTCACTCACAGAATGGTGTCTCTGCCTGC

CTCGGGTTGGCCCTGTGATTTATTTTAGTTCTTTTCCCTTGTTTTTTTTTTTTCA

AACTCTATACACTTTTGTTTTAAAAACTGTGGTTTCTCATGAGCCC

RHA: 500 bp

TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTAGGATGACTCGTAAA

GGGCAAAGAAAAAAACCCAAAATTTCAAAATTTCCGTTTAAGTCTCATAATC

AAGAAAAGGAGAAACACAGAGAGAGAGAAAAAAAAAACTATGAGAACCCC

TCCCCACCCCGTGATTATCAGCGCACACACTCATCGAAAAAAATTTGGATTAT

TAGAAGAGAGAGGTCTGCGGCTTCCACACCGTACAGCGTGGTTTTTCTTCTCG

GTATAAAAGCAAAGTTGTTTTTGATACGTGACAGTTTCCCACAAGCCAGGCT

GATCCTTTTCTGTCAGTCCACTTCACCAAGGTGAGTGTCCCTGCTCTCCCCTA

CCAGATGTGGGCCCCATTGGAGGAGATGGCAGGGAGGTAGGCACGGCGGGG

GGGTCAGGGGCCCTCTGGTACAGTGGGATGTACCCAGCTACCGTACTGGCCG

TCGTTTTACTTGTAGGTAACCACGTGCGGACC