AWARD NUMBER: W81XWH-22-1-0479

TITLE: Identification, Characterization, and Correction of a Defect in Treg Function in SLE

PRINCIPAL INVESTIGATOR: Dr. Charles Fathman, MD

CONTRACTING ORGANIZATION: Stanford University, Stanford, CA

REPORT DATE: July 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2023	Annual	01Jun2022-31May2023

4. TITLE AND SUBTITLE

Identification, Characterization, and Correction of a Defect in Treg Function in SLE

5a. CONTRACT NUMBER
W81XWH-22-1-0479

5b. GRANT NUMBER
LR210017

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Charles G. Fathman

E-Mail: cfathman@stanford.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Board of Trustees of the Leland Stanford Junior University
School of Medicine
Division of Immunology and Rheumatology,
269 Campus Drive West,
Stanford, CA, 94305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of our grant is to develop a protein drug conjugate that can be used to restore function to defective Tregs of autoimmune patients. We previously showed the Tregs of autoimmune patients, including SLE patients, share a common defect in IL-2R signaling. This defect may be restored using neddylation activating enzyme inhibitors (NAEi), however, NAEi are toxic when used systemically. To reduce toxicity, we developed a protein drug conjugate (PDC) consisting of a fusion protein of murine thioredoxin and IL-2 to which three molecules of the NAEi MLN4924 were conjugated through a dipeptide cleavable linker. This mouse PDC (mPDC) was effective in preventing disease in various mouse models of autoimmunity. The purpose of the current grant is to develop a human version of the PDC that can be used to restore function in the defective Tregs of SLE patients, and to develop a diagnostic assay that can be used to identify SLE patients with defective Tregs who may respond to PDC treatment. During this first year of funding we developed 5 different human PDCs and tested the efficacy of these drugs in various assays. Several were selected for additional in vivo and in vitro testing. We identified a single lead candidate. The effectiveness of this drug in restoring or enhancing Treg activity in SLE patients will be tested in the second year of funding. We have also made progress in developing potential diagnostic assays that may be used for identifying individuals who have a defect in IL-2R signaling. The first assay is based on the protein expression of GRAIL and ARF1, a protein that stabilizes GRAIL. Custom antibodies for GRAIL and ARF1 have been made, and will be tested in the second year of funding. The second potential diagnostic assay is based on the mRNA expression of an alternatively spliced variant of GRAIL. We recently found that the Tregs of SLE patients express higher mRNA levels of this non-functional alternatively spliced variant of GRAIL, thus it is possible that this transcript may be used as a biomarker of defective Treg function. Finally, we developed a new lentiviral vector to transduce SLE Tregs to express ARF1 and GRAIL. In the second year of funding, we will test if increased ARF1 and/or GRAIL expression can restore SLE Treg function in vitro.

15. SUBJECT TERMS

Regulatory T cell, Systemic lupus erythematosus (SLE), IL-2 receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	37	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents **Page**

INTRODUCTION:

The goal of our grant is to develop a protein drug conjugate that can be used to restore function to defective Tregs of autoimmune patients. We previously showed the Tregs of autoimmune patients, including SLE patients, share a common defect in IL-2R signaling. This defect may be restored using neddylation activating enzyme inhibitors (NAEi), however, NAEi are toxic when used systemically. To reduce toxicity, we developed a protein drug conjugate (PDC) consisting of a fusion protein of murine thioredoxin and IL-2 to which three molecules of the NAEi MLN4924 were conjugated through a dipeptide cleavable linker. This mouse PDC (mPDC) was effective in preventing disease in various mouse models of autoimmunity. The purpose of the current grant is to develop a human version of the PDC that can be used to restore function in the defective Tregs of SLE patients, and to develop a diagnostic assay that can be used to identify SLE patients with defective Tregs who may respond to PDC treatment. The underlying defect that contributes to reduced Treg function in autoimmune patients may be a loss of functional GRAIL expression in Tregs. GRAIL is required to maintain IL2-R expression on the surface of Tregs, and thus prolong Treg function. To better understand the role of GRAIL in autoimmune disease, we will transduce Tregs to express GRAIL and/or ARF1, a protein that stabilizes GRAIL. We will test if increased expression of GRAIL/ARF1 can boost Treg function in healthy individuals and restore Treg function in autoimmune SLE patients.

KEYWORDS:

1. Regulatory T cells
2. Systemic lupus erythematosus
3. Interleukin-2
4. Autoimmunity
5. GRAIL
6. Ring-finger 128
7. Neddylation inhibitors
8. MLN4924
9. Protein drug conjugate
10. Otubain 1 – Alternative reading frame 1 (ARF1)
11. Alternative splicing
12. Flow cytometry
13. Lentiviral transduction
14. NZBWF1 mice

ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project are listed below:

Specific aim 1: Generate a human IL-2 thioredoxin fusion protein drug conjugate (PDC)

1. We will use a CRO to make the human fusion protein (human thioredoxin/ IL-2) - *Complete*
2. We will have another CRO link drugs to the fusion protein. This will generate 4 human PDCs (2 NAEi's will be used and attached to the fusion protein with dipeptide or glucuronide linker) - *Complete*
3. We will assess the size of the fusion proteins using a non-denaturing gel, and assess the biological activity using a HT-2 assay - *Complete*

Specific Aim 2: Develop an antibody panel for FACS analysis to identify SLE patients who exhibit the correctable Treg IL-2R defect

1. Develop anti-ARF1 mAbs and a flow cytometry panel to identify SLE patients who exhibit the correctable Treg IL-2R defect *(in progress, 50% complete, switched assay).*
2. Validation of the assay on patient Tregs isolated from PBMCs *(in progress, 20% complete, switched assays).*

Specific Aim 3: Test whether PDC treatment repairs the defect in IL-2R signaling in SLE.

1. Assess the ability of the PDCs to correct the IL-2 signaling defect in SLE Tregs *in vitro (in progress, 20% complete)*
2. Test if the human PDCs can block progression of proteinuria in (NZB x NZW) F1 female mice with lupus nephritis *(complete)*

Specific Aim 4: Use of an Otubain1-ARF1 lentiviral vector to restore Treg function in SLE patient samples, and convert CD4 Teffs into CD4 Tregs

1. Produce Otubain1-ARF1 and GRAIL lentiviruses and assess the ability of lentivirus-mediated Otubain1-ARF1 and/or GRAIL expression to repair SLE Tregs *(30% complete)*
2. Transduce SLE Tregs to express ARF1 and check for maintained GRAIL expression and pJAK1 and DEPTOR expression to restore SLE Treg function in vitro *(will perform in the second year of funding)*

Specific Aim 5: Assess ability of forced ARF1 expression in Treg induction

1. Transduce antigen-specific CD4 Teffs with the lentivirus and test conversion to Treg phenotype *(will perform in the second year of funding)*

What was accomplished under these goals?

Major Activities:

1. The four PDCs were synthesized by the CRO. The biological activity of the 2 lead candidates were assessed *in vivo* using an animal model of autoimmunity (cockroach-antigen induced model of acute asthma), and *in vitro* using various Treg assays.

2. Showed that the PDC had little effect on the progression disease in NZBWF1 mice.

3. Established a Treg suppression assay that can be used to assess the Treg activity in healthy controls and patients, and to test if the PDC can boost/restore Treg function in SLE patients.

4. Used a CRO to synthesize antibodies that recognize Otubain1-ARF1 and the functional isoform of GRAIL. The specificity of these antibodies are currently being tested.

5. Designed QPCR assays to measure the expression of different *GRAIL (RNF128)* mRNA transcripts, and showed that the Tregs of SLE patients express higher levels of the non-functional isoforms of *GRAIL*. The higher expression of non-functional *GRAIL* mRNA could potentially serve as a biomarker of the IL-2 signaling defect seen in the Tregs of SLE patients.

6. Designed and synthesized lentiviral constructs for Otubain1-ARF1 and GRAIL, produced lentivirus using these constructs and demonstrated the efficiency of the lentivirus to increase expression of Otubain1-ARF1 and GRAIL in transduced cells.

Significant results/major findings and conclusions:

Evaluation of various human PDCs synthesized by IL-2Rx

Our studies proposed to evaluate the following 4 PDCs generated by the CRO, IL-2Rx:

1) Human IL2-thioredoxin fusion protein conjugated to MLN4924 by dipeptide linkers
2) Human IL2-thioredoxin fusion protein conjugated to TAS4464 by dipeptide linkers
3) Human IL2-thioredoxin fusion protein conjugated to MLN4924 by glucuronide linkers
4) Human IL2-thioredoxin fusion protein conjugated to TAS4464 by glucuronide linkers

We had issues with drugs #3 and #4, and upon further evaluation, IL-2Rx confirmed that the NAEi drugs (MLN4924 or TAS 4464), conjugated to the fusion protein with glucuronide linkers, were not cleaved off after internalization of the drug due to the low glucuronidase activity in Tregs. There was also an issue with the synthesis of drug #2 due to the low solubility of TAS4464 during the dipeptide linkage step. IL-2Rx provided us with drug #1, which we will refer to as the first generation PDC, and also provided us with a modified version of drug #1 that was altered to include a leptin antagonist in the fusion protein. We will refer to this compound as the second generation PDC.

We initially tested the first and second generation PDCs using the cockroach antigen (CRA)-induced model of acute asthma. In previous studies, we showed that the mouse PDC was able to block the development of asthma. The mouse PDC is similar to generation 1 of the human PDC, consisting of a mouse IL2-thioredoxin fusion protein conjugated to MLN4924 by dipeptide linkers. We found that both human PDCs could block the recruitment of leukocytes into the lung air spaces (diapedesis) in the CRA model, but that generation 2 was more effective (**Fig. 1**).

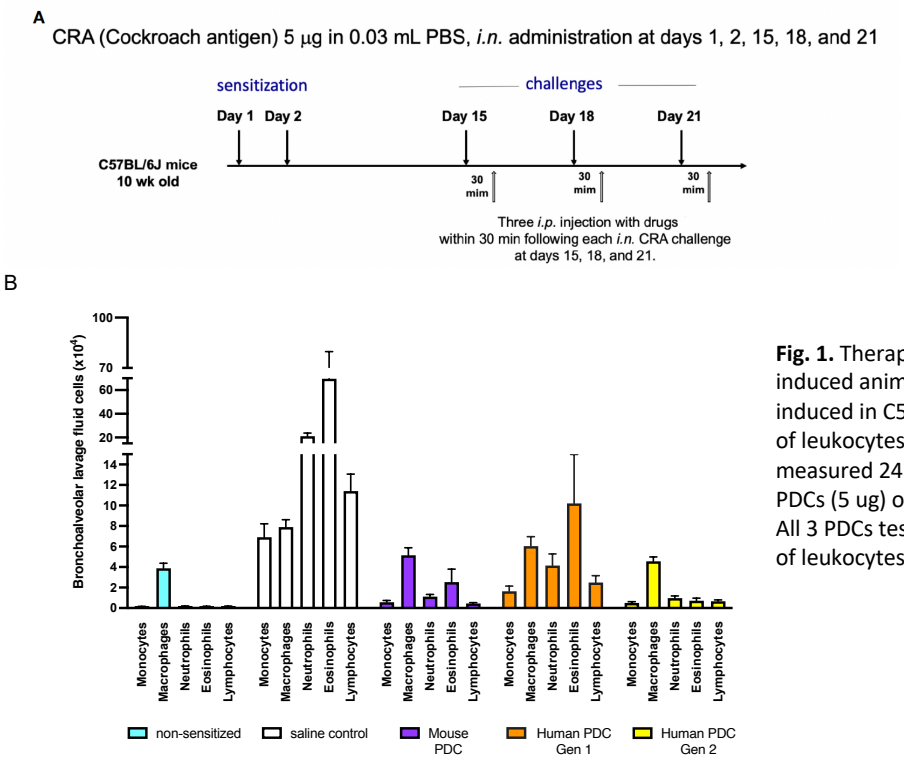


Fig. 1. Therapeutic effect to the PDC in a CRA-induced animal model of asthma. Asthma was induced in C57/BL6J mice (A), and the recruitment of leukocytes into the lung air spaces was measured 24 h after the last treatment with the PDCs (5 ug) or an equal volume of saline (0.1 ml). All 3 PDCs tested were able to reduce the number of leukocytes in the bronchoalveolar lavage fluid.

Effect of PDC on the progression of disease in the NZBWF1 mouse model of SLE

Since the second generation PDC was highly effective in blocking the development of disease in the asthma model (**Fig. 1**), we tested whether the second generation PDC could also block disease progression in the NZBWF1 mouse model of SLE. Mice were injected *(i.p)* with 5 ug of the Generation 2 PDC daily for 4 consecutive days once urine protein levels reached 200-700 mg/dl. Urine was collected every week and protein levels were measured by Bradford assay. No difference in disease progression was observed in the drug-treated animals vs. the saline treated controls. One consideration in these studies, is the timing of drug treatment. A considerable number of mice (~25%) had stunted growth and appeared sick before urine protein levels reached 200 mg/dl, and many of these mice remained below 200 mg/dl. Because SLE is a systemic autoimmune disease with multisystem involvement, it is difficult to determine the optimal time to commence drug treatment, and if the measurement of urine protein levels is the best indication of disease progression.

Effect of PDC on pSTAT5 expression in Tregs

We previously demonstrated that autoimmune patients exhibit a defect in Treg function. In particular, IL-2 induced pSTAT5 expression was lower in the Tregs of autoimmune patients, including SLE patients, compared IL-2 induced pSTAT5 expression in healthy controls. We proposed that this is due to the rapid desensitization of the IL-2 receptor on patient Tregs due to a loss of GRAIL expression. GRAIL is required to maintain IL-2R expression on Tregs. We propose that treatment with the PDC could restore Treg function by blocking the desensitization of the IL2-R. We tested both generation 1 and 2 PDCs in activating pSTAT5 expression in Tregs, and showed that high amounts of the first generation PDC could elicit the same pSTAT5 response as low dose IL-2; however, the generation 2 PDC had little effect on pSTAT5 expression, even at high concentrations (**Fig. 2**). This was surprising as both drugs were able to significantly reduce disease in the CRA asthma model (**Fig. 1**).

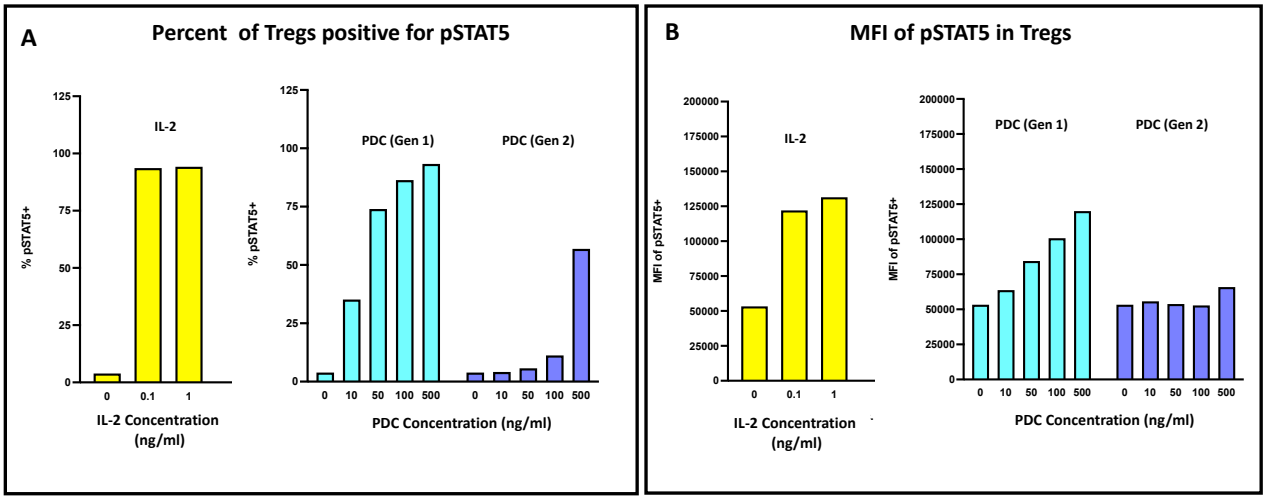


Fig. 2. Expression of pSTAT5 (percent positive and MFI) in Tregs treated with IL-2 or PDC (generation 1 and 2)

It is possible that the PDCs modulate Treg function via other pathways. To examine if the PDCs can restore Treg function in SLE patients, we have started Treg suppression assays. In these assays, CD4 Teff and Tregs are isolated from the PBMCs of SLE patients or healthy controls. The Teffs are labelled with a proliferation dye (Cell Tracer violet; CTV) and activated with CD3/28 beads. The labelled Teffs are co-cultured with Treg at various ratios, and treated with or without IL-2 or the PDCs. Teff proliferation is assessed 5 days later by FACS analysis of CTV intensity in the Teffs. The proliferation modeling function on the Flowjo FACS analysis software will be used to evaluate the suppressive activity of the Tregs of SLE patients vs. healthy individuals, in the presence or absence of IL2 or PDC.

We have already optimized the assay to use 50,000 Teff cells, stimulated with 1 CD3/28 bead per 32 cells, in the presence or absence of Tregs. Our preliminary data show that the Generation 1 and 2 PDC appears to boost the suppressive activity of Tregs of an autoimmune type 1 diabetes (T1D) patient. **(Fig. 3)**.

Effect of PDCs on Teff proliferation (T1D patient)	Division index	Suppression
Teff only (Avg of triplicates)	0.503	
Teff only + Gen 1 PDC (5ng/ml)	0.53	no
Teff only + Gen 2 PDC (7.5ng/ml)	0.54	no

Effect of PDCs on Treg suppression of Teff Proliferation (T1D patient)	Division index	Suppression (%)
Teff only (Avg of duplicates)	0.60	
Treg + Teff	0.54	10.0
Treg+ Teff + Gen 1 PDC (5ng/ml)	0.48	20.0
Treg + Teff + Gen 2 (7.5ng/ml)	0.49	18.3

$$\% \text{ suppression} = 100 - \frac{(DI, \text{Teff+Treg})}{(DI, \text{Teff only})} 100$$

Fig. 3: Preliminary data showing that Generation 1 and 2 PDCs can boost the suppressive function of Tregs in an established autoimmune (T1D) patient.

In the next year of funding, we will test whether treatment with Generation 1 or 2 PDCs can restore function to the Tregs of SLE patients. In collaboration with Dr. Matt Baker, we have collected blood, and isolated approximately 10 million peripheral blood mononuclear cells (PBMCs) from 20 active SLE patients during this first year of funding. Based on previous experiments, we expect to isolate approximately 100,000 Tregs from each PBMC sample. This is a sufficient number of Tregs to test the suppressive effect of several PDCs using our Treg suppression assay. However, we will also attempt to expand the Tregs in culture for 2 weeks, and test whether the expanded Tregs function similar to freshly isolated Tregs. If they do, then expansion of the Tregs will allow us to perform additional assays to test different culture conditions (i.e. various PDC concentrations, different amounts and types of stimulant, and culture time).

Custom antibodies for Otubain1-ARF1 and the functional isoform of GRAIL

The loss of GRAIL expression underly the Treg defect observed in SLE patients (and in patients suffering from other autoimmune diseases; see attached manuscript). Thus, the expression of GRAIL and proteins such as Otubain1-ARF1 (ARF1), that help maintain the expression of GRAIL, may potentially be used to identify individuals with a defect in Treg function. The expression of GRAIL and Otubain1-ARF1 can be measured in Tregs by Western blot or ELISA assays or by flow cytometric analysis. However, all these techniques require specific antibodies that detect the correct isoform of GRAIL or Otubain1-ARF1. There are 3 known alternatively spliced protein-coding isoforms of GRAIL (**Fig. 4**). Only one isoform, isoform 1, contains the PA and RING finger domains. The PA domain is required for identifying targets of GRAIL, while the RING finger domain is required for transferring ubiquitin to these targets. Isoform 1 has a unique N-terminal that can be used to differentiate it from isoform 2, which lacks the PA domain, and isoform 3 which lacks both the PA and RING finger domains.

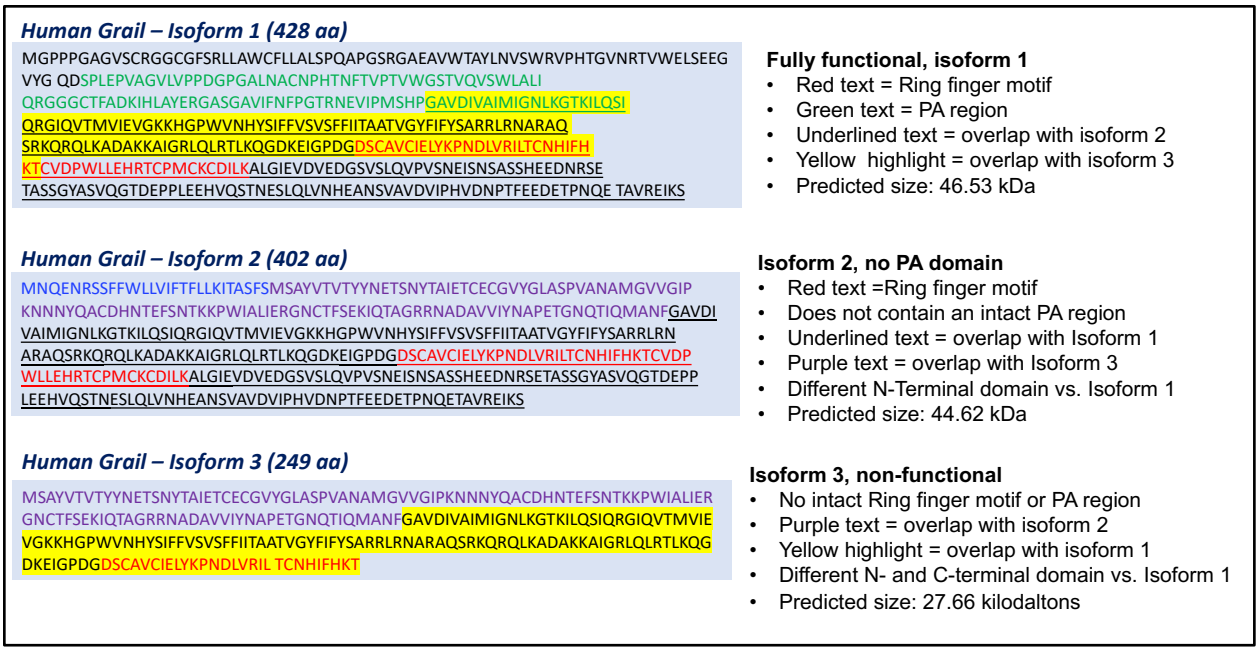


Fig. 4: Three different isoforms of GRAIL (RNF128)

Most commercially available antibodies for GRAIL are synthesized using whole recombinant GRAIL protein, or using a proprietary immunogen. Thus, the specificity of these antibodies against each isoform of GRAIL is unknown. Using lysate of cells overexpressing GRAIL, we screened anti-GRAIL antibodies produced by BD Pharmigen and Abcam, and an anti-GRAIL antibody against the C-terminus, previously synthesized by our laboratory, and showed that the proteins identified by these 3 antibodies were different (**Fig. 5**). To evaluate the specificity of these antibodies we performed deglycosylation experiments. Since Isoform 1 of GRAIL contains 3 N-linked glycosylation sites, and Isoforms 2 and 3 do not, we tested whether deglycosylation could reduce the size of the bands identified by these antibodies. The bands detected by the Abcam and BD GRAIL antibodies did not change after N-linked deglycosylation of the sample. However, the bands detected by our C-terminal anti-GRAIL antibody were reduced (**Fig. 5**). We showed reduced expression of the ~75 kDa band and increased expression of the ~66 kDa (possibly the O-linked glycosylated form of Isoform 1), and ~46 kDa (likely the non-glycosylated form of Isoform 1) bands, demonstrating that the C-terminal anti-GRAIL antibody can bind to Isoform 1 of GRAIL. This C-terminal antibody also bound to a band that was slightly smaller than the ~46 kDa band, that may represent Isoform 2 of GRAIL that is ~2 kDa smaller than Isoform 1. This is a possibility since isoform 1 and 2 share the same C-terminal domain. The N-terminal of Isoform 1 is unique. Thus, we selected the following immunogen within the N-terminal domain for the synthesis of custom antibodies against Isoform 1 of GRAIL: SPQAPGSRGAEAVWTAYLNVSWRVPHTGVNRTVWELSEEGVYGQDSPLEPVA GVLVPPDGPGALNACNPHTNFTVPTVWGSTVQVSWLALIQRGGGCTFADKIHLAYERGA (**see Fig. 4**). Various hybridoma clones were created and screened. We are currently evaluating the specificity of the purified N-terminal antibodies.

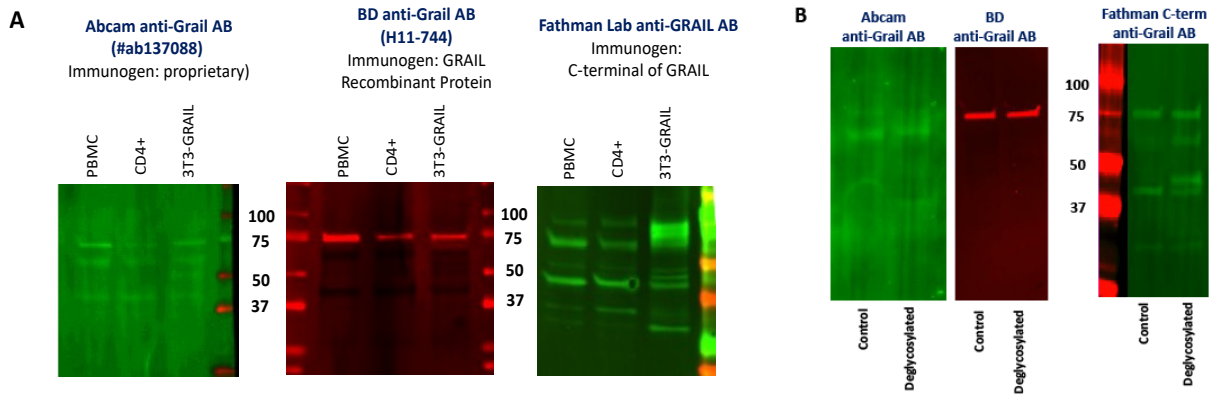


Fig. 5: Immunoblotting analysis to compare the specificity of different anti-GRAIL antibodies. A) Western blot analysis was performed to compare commercially available anti-GRAIL antibodies from Abcam and BD, and the C-terminal anti-GRAIL antibody synthesized in our lab using lysate of human PBMCs, human CD4+ T cells, and NIH3T3 cells overexpressing GRAIL. The C-terminal anti-GRAIL antibody was the only that could show increased GRAIL expression in the transduced cell line. B) Deglycosylation experiments were performed using the PBMC lysate. Only the C-terminal anti-GRAIL antibody showed increased expression of the ~66 and ~ 46 kDa bands.

Evaluation of different *GRAIL (RNF128)* transcripts in Tregs

It is possible that the loss of GRAIL and the defect in Treg function seen in SLE patients may be due to the alternative splicing of *GRAIL*. To examine this, we designed and optimized a QPCR assay that is specific for isoforms 2/3 of GRAIL (**Fig. 6**). Using this assay, we found that the expression of the non-functional isoforms of GRAIL appear to be higher in the Tregs of SLE patients compared to that of healthy controls (n=5/group; **Fig. 6**). In the second year of funding, we plan to assess *GRAIL* splicing in the Tregs of additional SLE patients and controls to determine if increased mRNA expression of the non-functional isoforms of *GRAIL* correlate with disease and if increased expression of *GRAIL isoforms 2/3* could serve as a potential biomarker of reduced Treg function.

Previous studies performed in our lab and others have shown that inflammation can drive the alternative splicing of multiple genes involved in the pathogenesis of various autoimmune diseases. In the next year of funding, we will examine if inflammation can drive the splicing of *GRAIL*. We will measure the gene expression of the functional and non-functional isoforms of *GRAIL in vitro* in Tregs treated with various inflammatory cytokines vs. PBS-treated Tregs, and *in vivo* by comparing *GRAIL* transcript expression in the inflamed tissues of various autoimmune-prone strains of mice vs. those of congenic healthy control mice.

A

>NM_024539.3 Homo sapiens ring finger protein 128 (RNF128), transcript variant 2, mRNA

Region that overlaps between the 2 non-functional GRAIL isoforms: 247bp-570bp

ATAGAGCTGATGTATCCAGAGGTTATGTTGCTAGAGGTGAGATCAGTTACCTACGTGCAACTGAAATTTC
AAACTTCTGTTCAGCAGGGACGTGAGTGGACAATGGTGACTGATAGTTGGAAATATCAGCAAACATCTTA
AATTTTATACTCAAATGAATGAGCAATGAACCAGGAGAATAGGTCCAGTTTTTTTTGGCTCCTTGTAATT
TTTACCTTTTTACTTAAAATTACAGCATCTTTTTCAATGAGTGCCTATGTGACTGTGACTTATTACAATG
AAACCAGCAACTACACTGCAATAGAGACATGTGAATGTGGCGTTTATGGATTAGCTTCACCAGTGGCTAA
TGCTATGGGAGTGGTAGGCATCCCTAAGAACAATAACTACCAAGCTTGTGACCACAACACTGAGTTTAGT
AATACTAAGAAGCCCTGGATTGCGCTGATAGAAAGAGGTAATTGTACATTTTCAGAAAAAATTCAAACAG
CGGGCAGAAGAAATGCTGATGCTGTTGTGATTTACAATGCTCCAGAGACTGGCAATCAGACGATACAGAT
GGCAAATTTTGGTGCAGTAGACATTGTTGCAATCATGATCGGCAATCTGAAAGGCACAAAAATTCTGCAA
TCTATTCAAAGAGGCATACAAGTGACAATGGTCATAGAAGTAGGGAAAAAACATGGCCCTTGGGTGAATC
ACTATTCAATTTTTTTCGTTTCTGTGTCCTTTTTTATTATTACGGCGGCAACTGTGGGCTATTTTATCTT
TTATTCTGCTCGAAGGCTACGGAATGCAAGAGCTCAAAGCAGGAAGCAGAGGCAATTAAAGGCAGATGCT

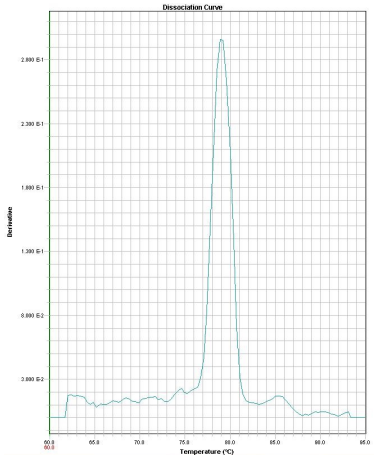
OLIGO	start	len	tm	gc%	any_th	3'_th	hairpin	seq
LEFT PRIMER	352	20	58.94	55.00	0.00	0.00	0.00	GCTATGGGAGTGGTAGGCAT
RIGHT PRIMER	505	20	59.12	50.00	0.00	0.00	0.00	CATTTCTTCTGCCCGCTGTT

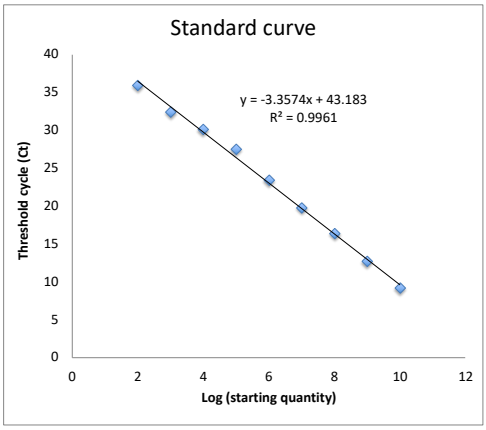
SEQUENCE SIZE: 700
INCLUDED REGION SIZE: 700

PRODUCT SIZE: 154, PAIR ANY_TH COMPL: 0.00, PAIR 3'_TH COMPL: 0.00

B

Specificity and efficiency of QPCR Assay for Human GRAIL isoform 2/3 (non-functional)





Efficiency: 98.5%

Fig. 6 Development of a QPCR assay to measure non-functional isoforms 2/3 of *GRAIL.* **A**) QPCR primers were selected from the region of the human *RNF128 (GRAIL), variant 2 tra*nscript highlighted in yellow. This region is shared between isoforms 2 and 3 of *GRAIL* (**See Fig. 4**). **B**) This primer set was able to amplify a single product of the expected size using cDNA template from peripheral blood cells of a T1D patient. The primers were also 98.5% efficient in amplifying product between Cts 10-35 (over a 8-log range of starting material).

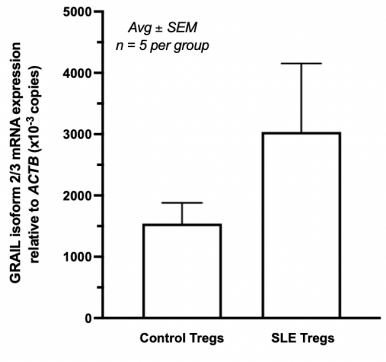


Fig. 7: Increased expression of *GRAIL isoform 2/3* mRNA in the Tregs of SLE patients

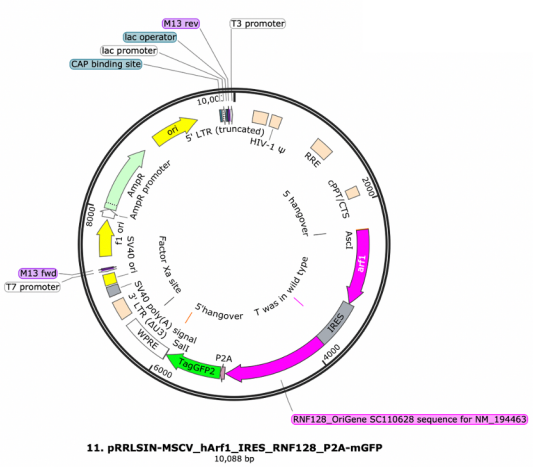


Fig. 8. The lentiviral plasmid for the combined expression of GRAIL and Otubain1-ARF1 was created using the pRRLSIN-MSCV backbone plasmid. Positions of the Otubain1-ARF1 (arf1) and GRAIL (RNF128) transcripts are shown in pink. The Otubain1-ARF1 transcript was placed before the GRAIL transcript, separated by an IRES. A P2A site was added after the GRAIL transcript, followed by a GFP tag.

Overexpression of Otubain1-ARF1 and GRAIL by lentiviral transduction

Since the loss of GRAIL expression may lead to the Treg defect seen in SLE patients, we proposed to test if overexpression of GRAIL, and/or the stabilizer of GRAIL, Otubain1-ARF1, could restore Treg function, or convert CD4 T cells to CD4 Tregs. We previously showed that transduction of murine CD4 Teffs with GRAIL converted T effector cells into T regulatory cells, suggesting that the expression of GRAIL is linked to the regulatory phenotype of Tregs. This indicates that conventional antigen-specific CD4 Teffs may be converted into antigen-specific CD4 Tregs by transduction of CD4 Teffs with *GRAIL* or *Otubain1-ARF1*.

We have designed and synthesized several lentiviral constructs that can be used to transduce cells to express GRAIL, Otubain1-ARF1, or both GRAIL and Otubain1-ARF1. To produce the GRAIL lentivirus and Otubain1-ARF1 lentivirus, the coding region of each transcript was inserted into the pRRLSIN-MSCV backbone plasmid, along with a P2A site and mGFP tag. The same backbone plasmid was used to make the lentiviral plasmid containing both GRAIL and Otubain1-ARF1. For this construct, Otubain1-ARF1 expression is driven by the MSCV promotor, an ideal promotor for the overexpression of genes in T-cells. The Otubain1-ARF1 transcript was placed before the GRAIL transcript, separated by an IRES. In this arrangement, we expect slightly higher Otubain1-ARF1 expression (~10% higher) than GRAIL. This can help stabilize the overexpression of GRAIL. A P2A site was added after the GRAIL transcript, followed by mGFP (**Fig. 8**), allowing for the separate (non-fused) expression of mGFP and GRAIL.

The lentiviral plasmids, along with the envelope and packaging helper plasmids, were transfected into HEK293T cells using Transit-LT1 transfection reagent (Mirus). Lentivirus was harvested from the supernatant 48 h later and concentrated using Lenti-X reagent (Takara), according to manufacturer's instructions. HEK293T and/or primary Teff cells were transduced with various amounts of lentivirus. GRAIL and/or ARF1 expression was evaluated 48 or 72 h later by measuring GFP expression using FACS, mRNA expression by QPCR, or protein

expression by immunoblotting. Our preliminary experiments showed that the GRAIL lentivirus was effective in increasing GRAIL expression in HEK293T and T effector cells (**Fig. 9**), and the GRAIL+ Otubain1-ARF1 lentivirus was effective in overexpressing GRAIL and Otubain1-ARF1 expression in HEK293T cells (**Fig. 10**). Studies are currently in progress to evaluate the Otubain1-ARF1 lentivirus.

In the second year of funding, we will transduce Tregs of SLE patients with these lentiviruses to examine if increased GRAIL and/or Otubain1-ARF1 expression could restore SLE Treg function *in vitro*. Treg function will be assessed using the Treg suppression assay, and by measuring pSTAT5 expression in response to low dose IL-2. In addition, we will test if forced expression of GRAIL and/or Otubain1-ARF1 could convert antigen-specific CD4 T effector cells to "regulatory" cells.

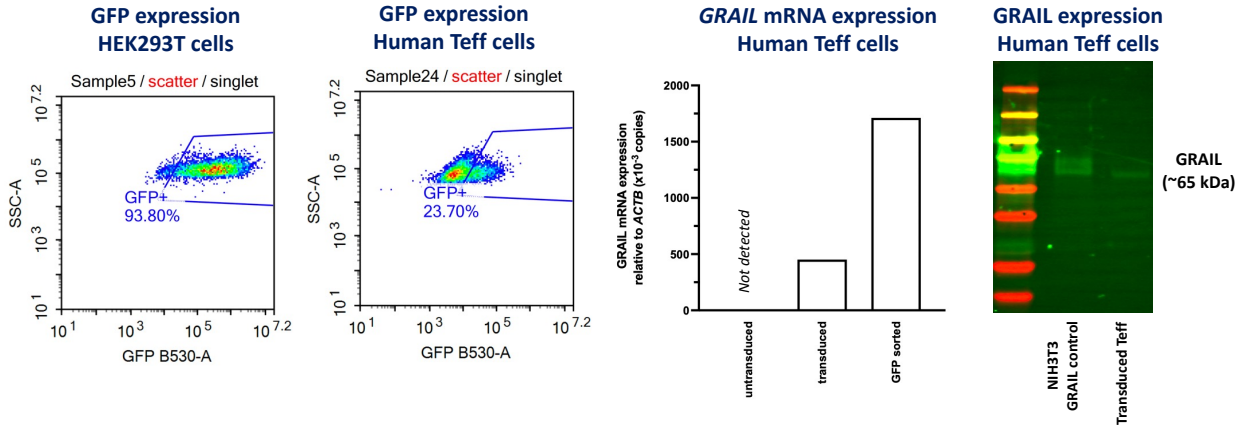


Fig. 9. Testing the efficacy of the GRAIL lentivirus. HEK293T and T effector cells were transduced with the GRAIL lentivirus. Expression of GFP, *GRAIL* mRNA, and GRAIL protein was measured by flow cytometry, QPCR, and immunoblotting, respectively, 48 h after transduction.

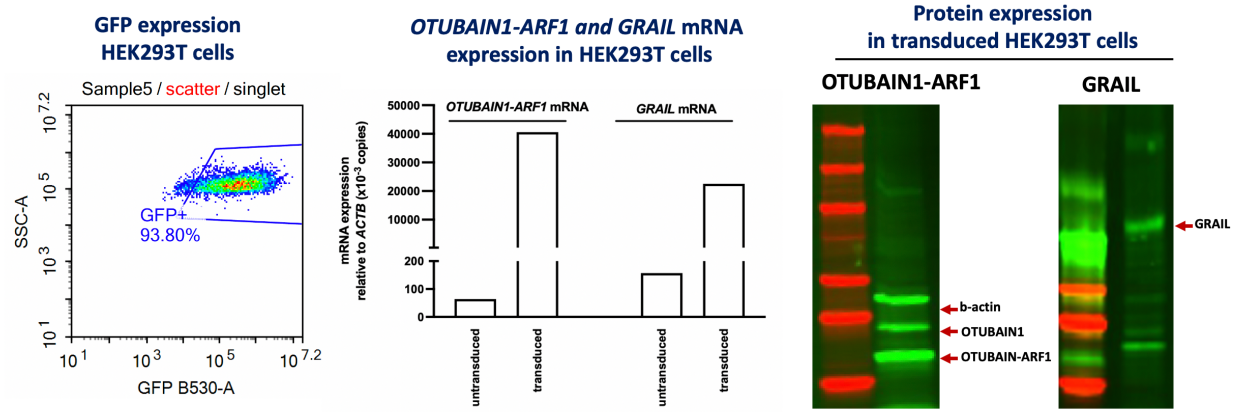


Fig. 10. Evaluating the efficacy of the GRAIL+OTUBAIN1-ARF1 lentivirus. HEK293T cells were transduced with the GRAIL+OTUBAIN1-ARF1 lentivirus. Expression of GFP, *GRAIL* and *OTUBAIN1-ARF1* mRNA and protein was measured by flow cytometry, QPCR, and immunoblotting, respectively, 48 h after transduction.

What opportunities for training and professional development has the project provided?
1:1 Training/mentoring of undergraduate students in the area of immunology, autoimmunity, and biomedical research. Training them to become proficient in the following technical skills: immunoblotting, flow cytometry, ELISA assays, QPCR, cell culture, data analysis, animal research.

How were the results disseminated to communities of interest?
Some of our data was published in the following article (See Appendix): C.G. Fathman, L. Yip, D. Gomez-Martin, M. Yu, CM Seroogy, C.R Hurt, J. Lin, J. Jenks, K. Nadeau, L. Soares. How GRAIL controls Treg function to maintain self-tolerance. *Frontiers in Immunology.* 2022; 8; 13:1046631 (doi: 10.3389/fimmu.2022.1046631). 2022.

What do you plan to do during the next reporting period to accomplish the goals?

1. Using the Treg suppression assay, test if Treg function is reduced in SLE patients and whether treatment with the PDCs can restore function.

2. Evaluate the gene and protein expression of GRAIL and/or Otubain1-ARF1 in SLE patients and healthy controls by FACS, ELISA, immunoblotting and/or QPCR to identify a biomarker/diagnostic assay that can of show reduced Treg function in SLE patients.

3. Examine if inflammation can drive the splicing of *GRAIL*. We will measure the gene expression of the functional and non-functional isoforms of *GRAIL in vitro* in Tregs treated with various inflammatory cytokines vs. PBS-treated Tregs, and *in vivo* by comparing *GRAIL* transcript expression in the inflamed tissues of various autoimmune-prone strains of mice vs. those of congenic healthy control mice.

4. Determine if over expression of GRAIL and/or Otubain1-ARF1 can restore function to the Tregs of SLE patients

5. Determine if overexpression of GRAIL and/or Otubain1-ARF1 in antigen-specific CD4 Teffs can convert them to a Treg phenotype.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

1. We have added an additional Treg suppression assay to assess the ability of Tregs to suppress activated Teffs in healthy controls and SLE patients and to test if treatment with the can correct this defect.
2. In the first year of funding, we showed that the non-functional isoforms of *GRAIL* (isoforms 2 and 3) are upregulated in the Tregs of SLE patients. In the second year we plan to test whether inflammation may drive the alternative splicing of GRAIL in the Tregs of SLE patients.

Actual or anticipated problems or delays and actions or plans to resolve them:
None.

Changes that had a significant impact on expenditures

- Part of the budget from year 1 was not spent due to some of the following issues listed below. We anticipate that we can complete most of the aims proposed for year 2, but may need a no-cost extension at the end of year 2.
- There was a delay in the production of the PDCs by the CRO because the glucuronide linkage of the drug to the fusion protein failed. The company replaced these PDCs with Gen 2 drug.
- We initially proposed to evaluate the efficacy of the PDCs in restoring Treg function by measuring IL-2 induced pSTAT5 expression. However, our data suggests that the PDCs act via a different pathway. Thus, we had to establish a Treg suppression assay to evaluate Treg function and to test the efficacy of PDCs. This includes testing whether in vitro expanded Tregs can be used for these assays.
- We have completed collection of the SLE patient samples in year 1. Any salary/funds allocated to patient sample collection will be diverted to establishing the Treg suppression assay.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

PRODUCTS:

Journal publication:

C.G. Fathman, L. Yip, D. Gomez-Martin, M. Yu, CM Seroogy, C.R Hurt, J. Lin, J. Jenks, K. Nadeau, L. Soares. How GRAIL controls Treg function to maintain self-tolerance. *Frontiers in Immunology.* 2022; 8; 13:1046631 (doi: 10.3389/fimmu.2022.1046631). 2022.

Status of publication: published

Acknowledgement of federal support: Yes

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project? *(at least one person month of effort)*

Name:	C. Garrison Fathman
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Oversaw the progress of project, provided direction and feedback
Funding Support:	

Name:	*Linda Yip*
Project Role:	*Senior Research Scientist*
Researcher Identifier	0000-0002-6459-2063 (ORCID ID)
Nearest person month worked:	6
Contribution to Project:	Performed experiments using the mouse model of SLE, performed various assays on the Tregs and Teff cells of SLE patients and healthy controls, screening of custom GRAIL and ARF1 antibodies, oversee lab expenses to maintain costs within allocated expenses.
Funding Support:	

Name:	Fangyuan Wang
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Performed lentivirus experiments and Treg suppression assays
Funding Support:	*Myra Reinhard Foundation Funds*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Previously active grant that has closed:
Funding Agency: NIH
Funding Amount:
Funding date: 4/10/18-3/31/2023
PI: C.Garrison Fathman (10% effort)
Title: Whole blood gene expression to identify biomarkers of disease risk, progression and response to therapy in Type 1 diabetes

New active grant:
Funding Agency: JDRF (Juvenile Diabetes Research Foundation)
Funding Amount:
Funding date: 6/1/22-5/31/2024
PI: C.Garrison Fathman (30% effort)
Title: Restoration of Treg function to prevent progression to hyperglycemia

What other organizations were involved as partners?

Organization Name: IL-2Rx is a Delaware corporation whose registered office is at 1209 Orange Street, Wilmington, DE 19801. It was co-founded by Drs C G Fathman MD and Luis Soares PhD.

Location of Organization: IL-2Rx, a Delaware corporation whose registered office is at 1209 Orange Street, Wilmington, DE 19801. The current IL-2Rx lab is in Florida where Dr. Soares and his team made the prototype protein drug conjugates (PDCs). Currently, the PDCs are being manufactured by a CRO (Wu Xi) under GMP conditions for potential use in future clinical trials.

Partner's contribution to the project; IL-2Rx made the initial PDCs that we used in the animal studies and murine and human Treg assays performed at Stanford.

Financial support: There is no financial support from IL-2Rx to the project.

In-kind support: IL-2Rx provided the Fathman lab with 10 mg of the initial PDC [a fusion protein of human thioredoxin and IL-2 to which three molecules of MLN 4924 (TK924) were attached by dipeptide linkage for enzymatic cleavage inside the target cell (Tregs)]. This PDC has been used in the studies reported within this document. Subsequently additional prototypic PDCs incorporating a leptin antagonist into the fusion protein have been constructed and are being studied in the Fathman lab.

Facilities: None of the IL-2Rx facilities are used outside of the preparation and analysis of the PDC constructs.

Collaboration: Dr. Soares provides data on the PDC structure and data on his analysis of the biological activities in studies using indicator cell lines for IL-2 activity of the constructs.

Personnel exchanges: There is no exchange of personnel.

Other: Nothing additional to report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *not relevant*

QUAD CHARTS: *none*

APPENDICES:

Journal publication: C.G. Fathman, L. Yip, D. Gomez-Martin, M. Yu, CM Seroogy, C.R Hurt, J. Lin, J. Jenks, K. Nadeau, L. Soares. How GRAIL controls Treg function to maintain self-tolerance. *Frontiers in Immunology.* 2022; 8; 13:1046631 (doi: 10.3389/fimmu.2022.1046631). 2022.

Please see attached manuscript.

frontiers | Frontiers in Immunology

TYPE Hypothesis and Theory
PUBLISHED 08 December 2022
DOI 10.3389/fimmu.2022.1046631

OPEN ACCESS

EDITED BY
Dawn Elaine Smilek,
UCSF, United States

REVIEWED BY
George C. Tsokos,
Harvard Medical School, United States
Amy Rosenberg,
EpiVax (United States), United States

*CORRESPONDENCE
C. Garrison Fathman
cfathman@stanford.edu

SPECIALTY SECTION
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

RECEIVED 16 September 2022
ACCEPTED 21 November 2022
PUBLISHED 08 December 2022

CITATION
Fathman CG, Yip L, Gómez-Martín D, Yu M, Seroogy CM, Hurt CR, Lin JT, Jenks JA, Nadeau KC and Soares L (2022) How GRAIL controls Treg function to maintain self-tolerance.
Front. Immunol. 13:1046631.
doi: 10.3389/fimmu.2022.1046631

How GRAIL controls Treg function to maintain self-tolerance

C. Garrison Fathman[1*], Linda Yip[1], Diana Gómez-Martín[2], Mang Yu[3], Christine M. Seroogy[4], Clarence R. Hurt[5], Jack T. Lin[1], Jennifer A. Jenks[3], Kari C. Nadeau[3,6] and Luis Soares[1,5]

[1]Department of Medicine, School of Medicine, Stanford University, Palo Alto, CA, United States, [2]Departamento de Inmunología y Reumatología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico City, Mexico, [3]Department of Pediatrics, School of Medicine, Stanford University, Palo Alto, CA, United States, [4]Department of Pediatrics, Division of Allergy, Immunology and Rheumatology, University of Wisconsin, Madison, WI, United States, [5]IL-2Rx, San Jose, CA, United States, [6]Sean N. Parker Center for Allergy & Asthma Research, School of Medicine, Stanford University, Palo Alto, CA, United States

Regulatory T cells (T_{regs}) normally maintain self-tolerance. T_{regs} recognize "self" such that when they are not working properly, such as in autoimmunity, the immune system can attack and destroy one's own tissues. Current therapies for autoimmunity rely on relatively ineffective and too often toxic therapies to "treat" the destructive inflammation. Restoring defective endogenous immune regulation (self-tolerance) would represent a paradigm shift in the therapy of these diseases. One recent approach to restore self-tolerance is to use "low dose IL-2" as a therapy to increase the number of circulating T_{regs}. However, studies to-date have not demonstrated that low-dose IL-2 therapy can restore concomitant T_{reg} function, and phase 2 studies in low dose IL-2 treated patients with autoimmune diseases have failed to demonstrate significant clinical benefit. We hypothesize that the defect in self-tolerance seen in autoimmunity is not due to an insufficient number of available T_{regs}, but rather, due to defects in second messengers downstream of the IL-2R that normally control T_{reg} function and stability. Previous studies from our lab and others have demonstrated that GRAIL (a ubiquitin E3 ligase) is important in T_{reg} function. GRAIL expression is markedly diminished in T_{regs} from patients with autoimmune diseases and allergic asthma and is also diminished in T_{regs} of mice that are considered autoimmune prone. In the relevant pathway in T_{regs}, GRAIL normally blocks cullin ring ligase activity, which inhibits IL-2R desensitization in T_{regs} and consequently promotes T_{reg} function. As a result of this defect in GRAIL expression, the T_{regs} of patients with autoimmune diseases and allergic asthma degrade IL-2R-associated pJAK1 following activation with low dose IL-2, and thus cannot maintain pSTAT5 expression. pSTAT5 controls the transcription of genes required for T_{reg} function. Additionally, the GRAIL-mediated defect may also allow the degradation of the mTOR inhibitor, DEP domain-containing mTOR interacting protein (Deptor). This can lead to IL-2R activation of mTOR and loss of T_{reg} stability

in autoimmune patients. Using a monoclonal antibody to the remnant di-glycine tag on ubiquitinated proteins after trypsin digestion, we identified a protein that was ubiquitinated by GRAIL that is important in T_{reg} function, cullin5. Our data demonstrate that GRAIL acts a negative regulator of IL-2R desensitization by ubiquitinating a lysine on cullin5 that must be neddylated to allow cullin5 cullin ring ligase activity. We hypothesize that a neddylation inhibitor in combination with low dose IL-2 activation could be used to substitute for GRAIL and restore T_{reg} function and stability in the T_{regs} of autoimmune and allergic asthma patients. However, the neddylation activating enzyme inhibitors (NAEi) are toxic when given systemically. By generating a protein drug conjugate (PDC) consisting of a NAEi bound, *via* cleavable linkers, to a fusion protein of murine IL-2 (to target the drug to T_{regs}), we were able to use 1000-fold less of the neddylation inhibitor drug than the amount required for therapeutically effective systemic delivery. The PDC was effective in blocking the onset or the progression of disease in several mouse models of autoimmunity (type 1 diabetes, systemic lupus erythematosus, and multiple sclerosis) and a mouse model of allergic asthma in the absence of detectable toxicity. This PDC strategy represents targeted drug delivery at its best where the defect causing the disease was identified, a drug was designed and developed to correct the defect, and the drug was targeted and delivered only to cells that needed it, maximizing safety and efficacy.

KEYWORDS

GRAIL, regulatory T cell, neddylation, cullin RING ligase, immune regulation, low dose IL-2, protein drug conjugates

Introduction

Autoimmune diseases can develop due to a defect in peripheral regulatory T cells (T_{regs}) (1–4). There have been several anecdotal reports of the successful use of low dose interleukin 2 (IL-2) to treat autoimmune diseases (5, 6). However, as recently reported, placebo-controlled phase 2 clinical trials using low dose IL-2, muteins of IL-2, or pegylated IL-2 as a potential therapy to treat autoimmune diseases have not reported statistically significant clinical responses, despite a significant increase in the level of circulating T_{regs} in the treated patients (5). Although there are data showing that the defect in Tregs in autoimmunity and allergy may be due to insufficient numbers of Tregs, here we present data to suggest that a defect in IL-2 receptor (IL-2R) signaling in Tregs leads to diminished Treg function and underlies both autoimmune diseases and allergic asthma. In this study, we compared the responses to low dose IL-2 *in vitro*, of equal numbers of Tregs isolated from patients with autoimmune diseases or allergic asthma to Tregs from healthy controls, to ask if the defect might be in the number of Tregs. Rather than the defect being in the number of Tregs (as studied *in vitro*) our studies identified a common druggable defect in IL-2 receptor second messenger signaling that was shared among patients with autoimmune diseases and patients with severe food allergy. We found that T_{regs} from these patients lost inhibition of IL-2R desensitization (Figure 1A), which equates to a loss of regulatory function. When activated with low dose IL-2 *in vitro*, T_{regs} from healthy subjects inhibit IL-2R desensitization to prolong pSTAT5 and Deptor expression for ~4 hours, allowing the genes for T_{reg} function to be transcribed *via* pSTAT5 to maintain the T_{reg} phenotype (7), and inhibiting mTOR activation to maintain T_{reg} stability *via* Deptor (8, 9). Deptor is a mTOR inhibitor and functions similarly to the immunosuppressant Rapamycin, although by a distinct mechanism of action (9). In patients with autoimmune diseases or allergic asthma, there is a defect in the inhibition of IL-2R desensitization. Following activation with low dose IL-2, cullin ring ligase (CRL) degradation of IL-2Rβ chain-associated pJAK1 and Deptor occur, leading to rapid reduction in both pSTAT5 and Deptor expression. This diminishes transcription of the genes required for regulation and allows activation of mTOR that leads to a loss of T_{reg} stability.

T_{regs} from healthy controls constitutively express a ubiquitin E3 ligase called GRAIL (Gene Related to Anergy In Lymphocytes) (10) that we found to ubiquitinate the exact lysine (K724) on Cul5

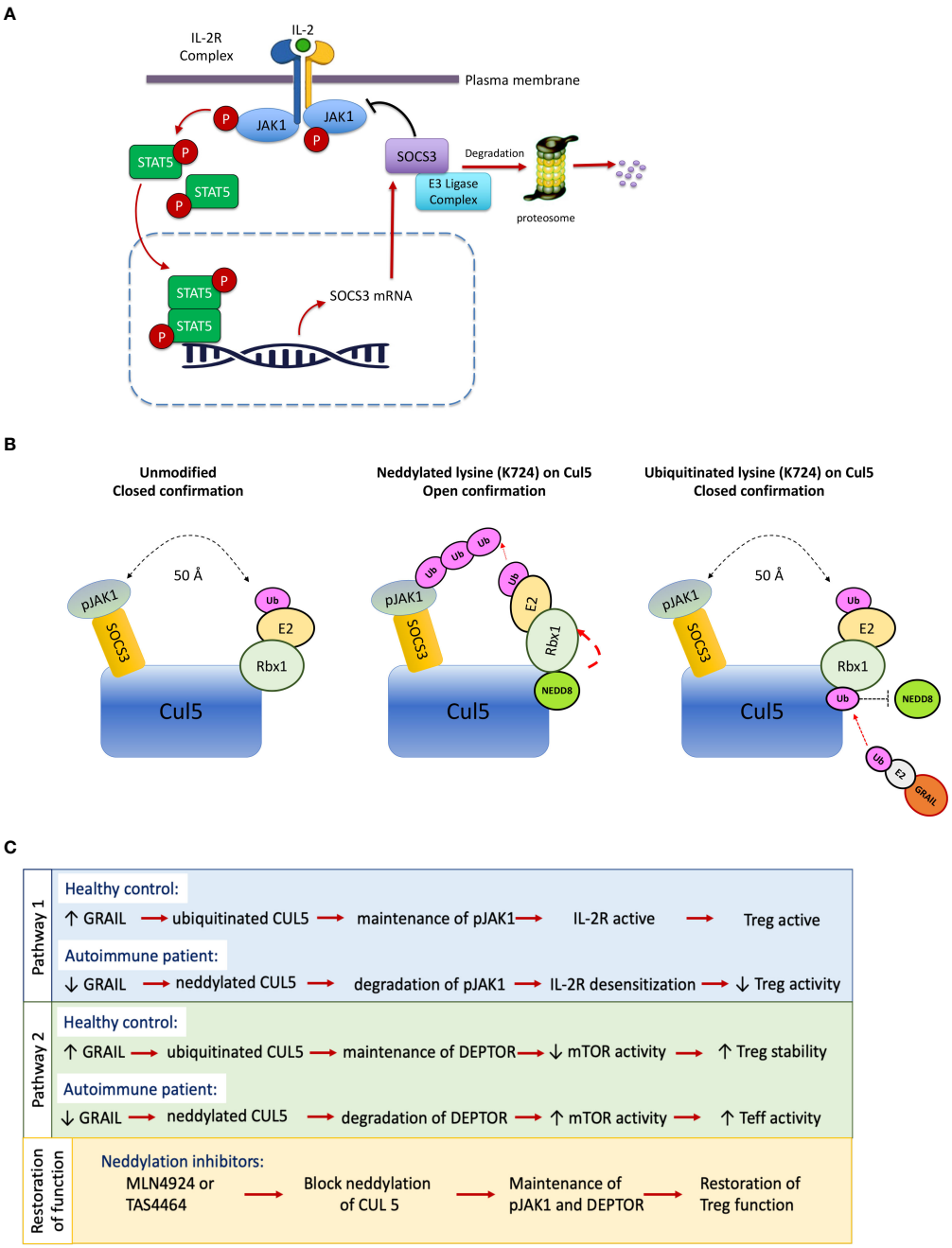


FIGURE 1
IL-2 receptor desensitization, neddylation of Cullin5 and restoration of T_{reg} function. Desensitization of the IL-2R means turning it off. SOCS3 is a negative regulator of IL-2Rβ chain–associated pJAK1 and forms a cullin ring ligase to ubiquitinate pJAK1. **(A)** Healthy individuals inhibit IL-2R desensitization in their T_{regs} by a protein, GRAIL, that is constitutively expressed in normal T_{regs}. Patients with autoimmunity have a defect in this pathway of inhibition of desensitization. **(B)** Upon neddylation of Cullin5 (Cul5) at lysine 724, one end of the Rbx1 protein is untethered and undergoes a 50A shift, bringing the ubiquitin transferase (E2) in proximity of the target of the SOCS3, pJAK1, to transfer ubiquitin for degradation. GRAIL, constitutively expressed in T_{regs}, is a competitive inhibitor of this process, as it ubiquitinates lysine 724 and does not allow neddylation and release of the E2 bound to the Rbx1 protein, thus inhibiting IL-2R desensitization. **(C)** Reduced GRAIL expression in autoimmune patients leads to diminished T_{reg} function *via* two distinct pathways that favor the neddylation of CUL5. Treatment with neddylation inhibitors such as MLN9424 may restore T_{reg} activity.

proteins that needs to be neddylated as a condition for CRL activation. Once neddylated, the ubiquitin transferase (E2) attached to the Rbx1 protein on the cullin5 backbone undergoes a 50A shift to bring the E2 in proximity of the target of the CRL to allow transfer of ubiquitin. The SOCS3/Cullin5 CRL ubiquitinates pJAK1 (9, 10). Deptor is also degraded by an activated cullin5 CRL, the F box protein βTrCP of the Cul5/Elongin B complex (11). Following IL-2R activation, poly-ubiquitination by the Cullin 5 CRLs leads to proteasomal degradation of pJAK1 and Deptor, desensitizing (turning off) the IL-2R signaling (Figure 1B). In T_{regs} from healthy controls, GRAIL acts as a competitive inhibitor of neddylation by ubiquitinating the exact lysine on cullin5 that needs to be neddylated to activate the CRL. Thus, in Tregs, GRAIL acts as a competitive inhibitor of neddylation and blocks activation of the cullin5 CRLs that normally degrade pJAK1 and Deptor in IL-2 activated CD4 T effector cells (11–13). GRAIL protein but not *GRAIL* mRNA expression is reduced in T_{regs} from SLE patients. The competitive inhibition of neddylation by GRAIL in the T_{regs} from the patients with SLE is thus diminished with a resultant loss of inhibition of IL-2R desensitization and prolongation of IL-2R signaling (Figure 1C). These defects can be corrected by the application of a small molecule drug called a neddylation activating enzyme inhibitor (NAEi) that replaces the function of GRAIL to block neddylation and inactivate the cullin5 ring ligases (14).

Our studies propose a paradigm shift in immune therapy away from immunosuppression to restoration of self-tolerance. These studies demonstrate that repairing the defect in T_{reg} function can restore normal inhibition of desensitization of the T_{reg} IL-2R to low dose IL-2 and allow transcription of the genes required for regulatory function and block the activation of mTOR to maintain the T_{reg} phenotype (Figure 1C). As described below, we developed a novel protein drug conjugate (PDC) of a fusion protein of murine IL-2 and thioredoxin to which we attached three molecules of a neddylation activating enzyme inhibitor (MLN4924) (14) by cleavable linkers (15). The PDC blocks the neddylation dependent activation step of the cullin5 ring ligases that controls pJAK1 and Deptor degradation. As shown below, the PDCs were effective in blocking progression of disease in animal models of autoimmunity and asthma.

Identification of a defect in inhibition of T_{reg} IL-2R desensitization in autoimmunity

We hypothesize that restoration of peripheral tolerance in T_{regs} of autoimmune/allergy patients requires enhancement of T_{reg} function (restoration of downstream messenger effects) rather than simply an increase in the number of poorly functioning circulating T_{regs}. To address this hypothesis, we asked if the same number of T_{regs} isolated from patients with systemic lupus erythematosus (SLE, one of the diseases that has been treated with low dose IL-2 (16), would have a similar, or less robust response to low dose IL-2 *in vitro* when compared to T_{regs} from healthy controls. If their response to IL-2 activation was lower despite using the same number of Tregs from the SLE patients, this would suggest that there was a defect in their

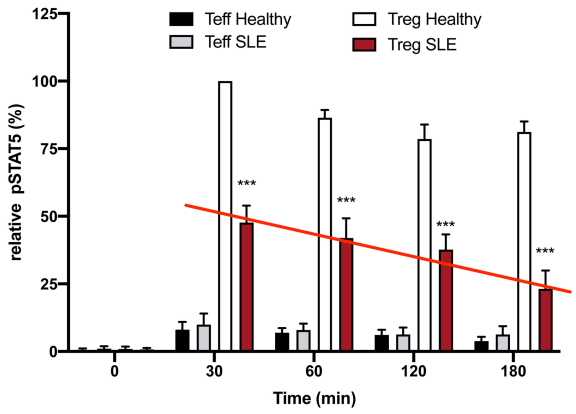


FIGURE 2
Defective inhibition of IL-2 desensitization in the T_{regs} of SLE patients. The 25 SLE patients studied were selected from a clinic in Mexico City irrespective of current disease status. These graphs represent Western blot data of pSTAT5 phosphorylation in T_{effs} and T_{regs} of the SLE patients and healthy sex and age-matched controls after stimulation with low dose IL-2 (1 ng/ml; 25 IU/ml) for the indicated amounts of time. Expression is shown as a percent of pSTAT5 measured from 30 min-stimulated control T_{regs}. A defect in the inhibition of IL-2R desensitization is observed in the T_{regs} of SLE patients (red line) n=6 per group ***p < 0.001 by 2-way ANOVA.

function. Dr. Diana Gomez-Martin, a former post-doc in the Fathman lab, isolated and then studied T_{regs} from patients with SLE seen in her rheumatology clinic in Mexico City. Interestingly, equal numbers of T_{regs} from the SLE patients studied, regardless of disease activity status, expressed similar amounts of cell surface CD25 as the controls when measured by FACS (data not shown) but had less pSTAT5 30 minutes following low dose IL-2 activation *in vitro* and had a more rapid loss of pSTAT5 expression than that seen in T_{regs} from healthy controls when stimulated with low dose IL-2 (Figure 2). This diminished pSTAT5 expression represents a defect in the normal inhibition of IL-2R desensitization seen in T_{regs} from healthy controls that maintain pSTAT5 expression for ≥4 hours. We hypothesize that the lower initial expression and subsequent loss of pSTAT5 expression in the SLE Tregs leads to ineffective transcription of the genes required for Treg function.

The role of ubiquitin ligases in autoimmunity

As described by Amy Lin and Tak Mak in 2007, ubiquitin E3 ligases play an important role in controlling immune regulation (17). Ubiquitin E3 ligases are important in maintenance of self-tolerance and in the suppression of autoreactive T cells (18, 19). These authors suggested the possibility that there could be ways to exploit the therapeutic potential of manipulating ubiquitination, particularly for autoimmune disorders. Although traditionally, the addition of a ubiquitin chain targets a protein for degradation by the proteasome, mono- or pauci-ubiquitination of the protein can specify a nonproteolytic fate. The studies described below support their suggestion.

Could defective desensitization of the T_{reg} IL-2R be related to GRAIL expression?

The IL-2R is sensitized (turned on) by ligand engagement to activate the JAK1/STAT5 pathway. Prolonged IL-2R signaling in activated CD25+ CD4 T effector cells (T_{eff}) could result in proliferation and potentially chronic inflammation. However, when the IL-2R on CD4 T_{regs} is activated, pSTAT5 dimerizes and translocates into the nucleus where it initiates the transcription of multiple genes, including negative regulators such as SOCS3. In turn, SOCS3 feeds back into the signaling cascade desensitizing the IL-2R by inactivating pJAK1 (20) (Figure 1A). SOCS3 desensitizes (turns off) IL-2R signaling, thereby creating a negative feedback loop (21). SOCS3 is a member of a family of SOCS proteins. This multi-member family of proteins is important in creating non-redundant feedback inhibition of tyrosine kinase cytokine receptor activation (22). The SOCS family consists of a group of eight intracellular proteins: SOCS 1–7 and CIS, all possessing an SH2 domain, C-terminal SOCS box, N-terminal extended SH2 subdomain, and a variable N-terminal region (23). The SOCS box can recruit factors to form an E3 ligase complex (a cullin ring ligase, CRL) that ubiquitinates the target protein, leading to its proteasomal degradation. The IL-2β chain–associated pJAK1 is degraded by the SOCS3/Cul5 CRL. T_{regs} from patients with SLE had a defect in inhibition of IL-2R desensitization, pSTAT5 levels were initially lower in the T_{regs} from the patients, and STAT5 phosphorylation in response to low-dose IL-2 and was lost more rapidly in the T_{regs} from the disease patients compared to the T_{regs} from healthy controls (Figure 2).

Studies from the Seroogy lab demonstrated that GRAIL, a single-protein ubiquitin ligase (8), was constitutively expressed in T_{regs} (24). These studies revealed that both GRAIL mRNA and protein expression were increased in naturally occurring thymically derived T_{regs} (mRNA levels were 10-fold higher compared to CD25- T cells). These studies also demonstrated that CD25+ Foxp3+ antigen- specific T cells were induced after a "tolerizing-administration" of antigen, and that GRAIL mRNA expression was upregulated in the CD25+ Foxp3+ antigen-specific subset. Collectively, these data demonstrated that GRAIL was differentially expressed in naturally occurring and peripherally induced CD25+ T_{regs} and that the expression of GRAIL was linked to their functional regulatory activity. By studying GRAIL expression in the T_{regs} isolated from patients with SLE, Dr. Gomez-Martin demonstrated a statistically significant reduction in the amount of GRAIL protein expressed in the T_{regs} from the SLE patients compared to the healthy controls, regardless of disease activity (Figure 3). Diminished GRAIL expression was also seen in T_{regs} from mice that are autoimmune prone (25).

How could GRAIL expression influence IL-2R desensitization?

To ask how GRAIL expression might play a role in IL-2R desensitization, we looked for proteins ubiquitinated by GRAIL that were involved in IL-2R signaling. To identify a potentially relevant target of GRAIL mediated ubiquitination, we made use of a technique called "global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling" (25). This

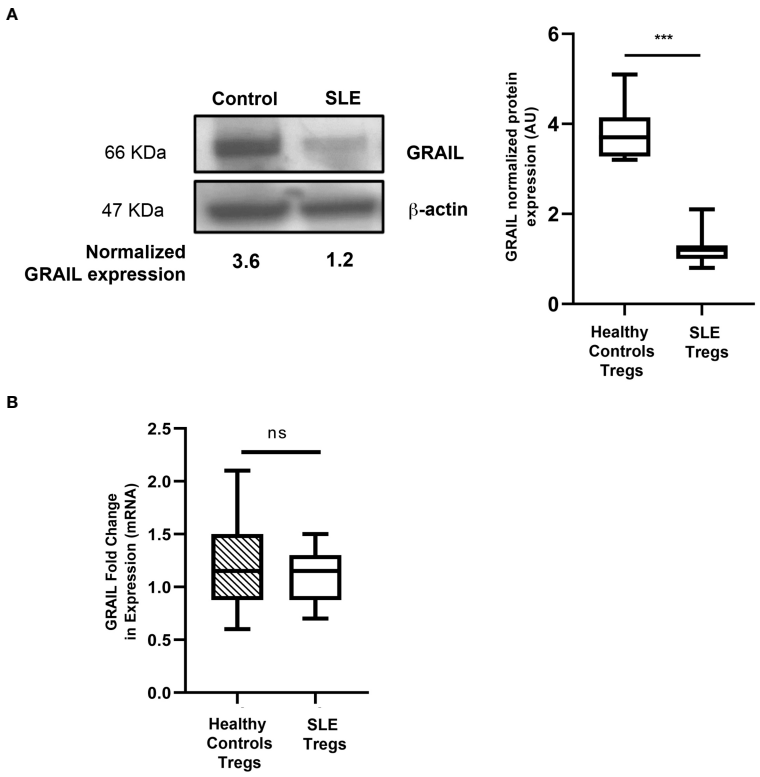


FIGURE 3
GRAIL expression in the Tregs of patients with SLE. **(A)** Immunoblotting data showing loss of GRAIL expression in the T_{regs} of a representative SLE patient vs. healthy control; the graph shows the pooled data in which SLE T_{regs} are deficient in GRAIL protein expression vs. healthy controls (HC=10, SLE=25 ***$p < 0.0001$ by unpaired T test). The T_{regs} from SLE patients were found to be deficient in GRAIL expression and had diminished T_{reg} function irrespective of disease activity (data not shown). **(B)** No differences were found in GRAIL mRNA levels in T_{regs} from SLE patients compared to healthy controls (HC=10, SLE=10 $p=0.4715$ by unpaired T-test). ns, not significant.

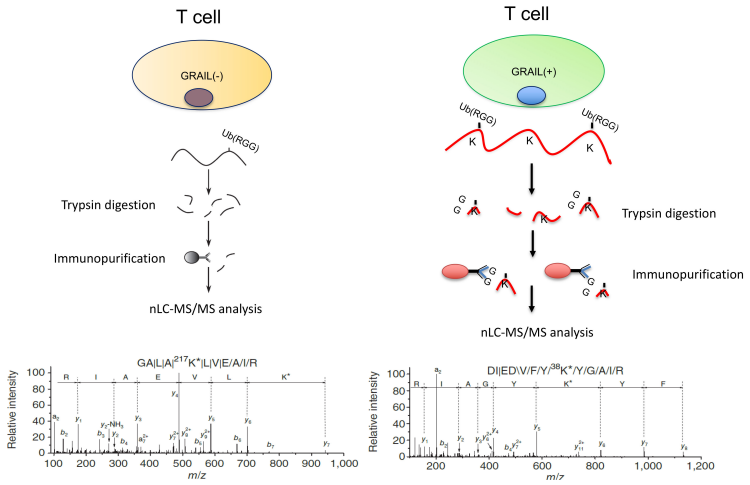


FIGURE 4
Proteins ubiquitinated by GRAIL. We identified proteins ubiquitinated by GRAIL using a new E3 target identification system from Cell Signaling Technologies. A monoclonal antibody recognizes the di-glycine tag on ubiquitinated lysines on proteins that can then be identified by differential mass spectrometry and sequencing. GRAIL mono-ubiquitinates lysine 724 on Cul5. This is the exact lysine that must be neddylated to allow the Cul5 ring ligase to function. Thus, GRAIL acts as a competitive inhibitor of neddylation to inhibit IL-2R desensitization in Tregs.

technology utilizes a monoclonal antibody that recognizes a di-glycine (K-ϵ-GG) tag that remains on lysines of cytosolic proteins following their ubiquitination (Figure 4). Ubiquitinated proteins were then identified by differential mass spectrometry following affinity purification using this monoclonal antibody. We used this technology to compare trypsin digests of Jurkat cells that expressed an inducible form of GRAIL vs. non-induced control Jurkat cells. Cullin5 (Cul5) was identified as a target of GRAIL-mediated ubiquitination. Importantly, the target of GRAIL ubiquitination on Cul5 was K724, the exact lysine that needs to be neddylated to allow the SOCS3/Cul5 CRL to function (Figure 1B). Ubiquitination of K724 by GRAIL acts as a competitive inhibitor of neddylation. This results in inhibition of IL-2R desensitization (prolonged IL-2R signaling through pJAK1), allowing the T_{reg} transcriptome for "function" to be stably transcribed.

The ubiquitin E3 ligase GRAIL is crucial to the maintenance of the T_{reg} phenotype. Diminished GRAIL expression seen in the T_{regs} from SLE patients (Figure 3) was accompanied by a loss of inhibition of IL-2R desensitization (Figure 2) and defective regulatory function when CD4+CD25- effector and CD4+CD25+ regulatory T cells from the SLE patients in Figure 3 were studied in autologous co-cultures (effector:regulatory T cells ratio 1:1) in 24-well plates and were either left unstimulated (RPMI), or were activated by means of the combination of plate-bound anti-CD3 antibody (5 μg/ml) and soluble anti-CD28 antibody (2.5 μg/ml) for 48 hours. Following this period, cells were harvested for proliferation assays. Cell proliferation was evaluated by FACS (BD LSRII Fortessa; BD Biosciences) according to the CFSE dilution protocol. SLE % average suppression of autologous Teffs was 35.2%; Healthy donors 78.4%, p=0.002.

The Fathman lab had previously shown that GRAIL is associated with and regulated by two isoforms of the ubiquitin-specific protease Otubain; Otub1 and Otub1 alternative reading frame 1, (Otub1-ARF1) (26). In these studies, they reconstituted bone marrow (BM) cells in lethally irradiated OVA reactive T cell receptor-transgenic mice (DO11.10) with DO11.10 BM cells retrovirally transduced to express one of the two isoforms of Otubain. They showed that following antigenic stimulation, Otub1-expressing cells contained negligible amounts of endogenous GRAIL, proliferated well and produced large amounts of IL-2. In contrast, cells expressing Otub1-ARF1 contained large amounts of endogenous GRAIL, demonstrated diminished functional responses; they proliferated poorly and produced undetectable amounts of IL-2 following antigenic stimulation. Thus, these two proteins have opposing functions in controlling the stability of GRAIL and the resultant phenotype of CD4 T cells.

Additional studies from the Fathman lab (27) demonstrated that *Otub1* translation in CD4 T_{effs} was under the control of mTOR. IL-2R driven AKT activation of mTOR, in response to CD4 T_{eff} stimulation, leads to GRAIL degradation in anti-OVA transgenic DO11.10 CD4 T_{eff} cells. In this study it was demonstrated that GRAIL was expressed in quiescent naive mouse and human CD4 T cells and has a functional role in inhibiting T cell proliferation. Following TCR engagement and CD28 co-stimulation, the resultant expression of IL-2 activates an Akt-pathway to activate mTOR leading to selective mRNA translation. Pre-existing mRNA for *Otub1* is translated, resulting in the degradation of GRAIL in CD4 T_{eff} cells, and the proliferation of these cells. Akt activation of mTOR appears to be a critical component of IL-2R signaling regulating GRAIL expression in CD4 T_{effs}. Maintenance of GRAIL expression in T_{regs} is crucial for T_{reg} stability and function GRAIL expression is regulated by the two isoforms of Otubain1 (26). Our unpublished data have demonstrated a marked increase in Otubain1-ARF1 mRNA in Tregs. This catalytically inactive isoform blocks GRAIL degradation by competitive inhibition of Otub1to maintain USP8 activity (26). USP8 is a deubiquitinating enzyme that de-ubiquitinates auto-ubiquitinated GRAIL. In Tregs, GRAIL ubiquitinates lysine 724 on Cul5 and blocks its neddylation. When ubiquitinated, lysine 724 can no longer be neddylated. Ubiquitinated K724 on cullin 5 does not allow the ubiquitin transferase on the Cul5 protein E2 to undergo the 50A shift required to be able to transfer ubiquitin to its target pJAK1 (Figure 1B) to maintain pJAK1 expression and thus pSTAT5 transcriptional activity (26) (Figure 1B).

How did we identify a drug strategy?

Our studies demonstrated that GRAIL-mediated ubiquitination of Cul5 serves as a negative regulator of the SOCS3/Cul5 CRL and is used by T_{regs} to inhibit IL-2R desensitization (26). Thus, GRAIL mediated ubiquitination of Cul5 in T_{regs} inhibits IL-2R desensitization and allows prolonged IL-2R signaling and prolonged pSTAT5 expression in response to low-dose IL-2. We reasoned that if this is true, it should be possible to restore T_{reg} function in the "defective" T_{regs} from patients, or experimental animals with autoimmune diseases that have diminished GRAIL expression, by blocking neddylation with a small molecule inhibitor of neddylation. This drug would substitute for GRAIL activity. Several small-molecule drugs are inhibitors of the neddylation activating enzyme [*neddylation activating enzyme inhibitors* (*NAEi*)]. We selected one such NAEi, called MLN4924, for our initial studies (14).

There is no previous unifying hypothesis of a common defect leading to the immune dysregulation seen in autoimmune and inflammatory diseases. We reasoned that if diminished GRAIL expression leading to loss of inhibition of IL-2R desensitization was a common defect in mouse models of autoimmunity, where a defect in GRAIL expression had been

identified (28), it might be possible to restore T_{reg} function using low-dose IL-2 in combination with an NAEi in these mouse models of disease. Our initial studies in restoring T_{reg} function were in the NOD mouse model of type one diabetes (T1D). T_{regs} from NOD mice had been previously demonstrated to have diminished GRAIL expression (28). We asked if low dose IL-2 in combination with a neddylation activating enzyme inhibitor (NAEi) would have a better effect in preventing disease progression than low dose IL-2 alone. We were able to inhibit progression to hyperglycemia in female NOD mice when we treated them with combination therapy (low-dose IL-2 and the NAEi, MLN) for 21 days, beginning at 12 weeks of age (Figure 5). At this age, these mice have pronounced islet inflammation but are normoglycemic. Although the data suggested that the combination was more effective than low-dose IL-2, the systemic administration of the NAEi was toxic at therapeutically effective doses (data not presented).

Why did we develop a protein drug conjugate approach?

In order to decrease the amount of the NAEi given systemically to the mice to restore T_{reg} function, we reasoned that we could develop a protein drug conjugate (PDC) similar to an antibody drug conjugate using the same type of cleavable linker strategy (15) (Figure 6). The PDC would specifically target the high affinity IL-2R constitutively expressed on T_{regs} and, following activation-induced receptor mediated endocytosis, deliver the attached drugs directly to signaling endosome of the T_{regs}. This is a Trojan Horse model for targeted drug delivery to diminish off target toxicity by using the high affinity receptor (IL-2R) on the target cell (T_{reg}) and the ligand (IL-2) to activate the receptor (IL-2R). Once engulfed by receptor mediated endocytosis, the drugs attached by cleavable linkers to the IL-2 fusion protein are specifically released inside the target cell (the T_{reg}).

Recombinant IL-2 produced in *E. coli*, was generated by a biotech company, IL-2Rx, by a refolding process from denatured protein recovered from inclusion bodies. To decrease disulfide scrambling during refolding, cysteine 160 in the mouse IL-2 sequence was mutated to serine, leaving only the native disulfide-forming cysteine pair at positions 68 and 115. As documented by antibody-drug conjugate technology development, cysteine-based conjugation vs. lysine conjugation is preferred, due to the randomness of the geographic reactivity of amino-reactive moieties in proteins that contain large numbers of lysines. To increase drug-conjugation potential, IL-2Rx developed a fusion protein consisting of murine IL-2 and murine thioredoxin, with a small, four-amino-acid spacer peptide separating them (Figure 6B). This resulted in the production of a largely soluble, small fusion protein, and, after re-bridging the two cysteines on IL-2, allowed three sites on the thioredoxin portion of the PDC for

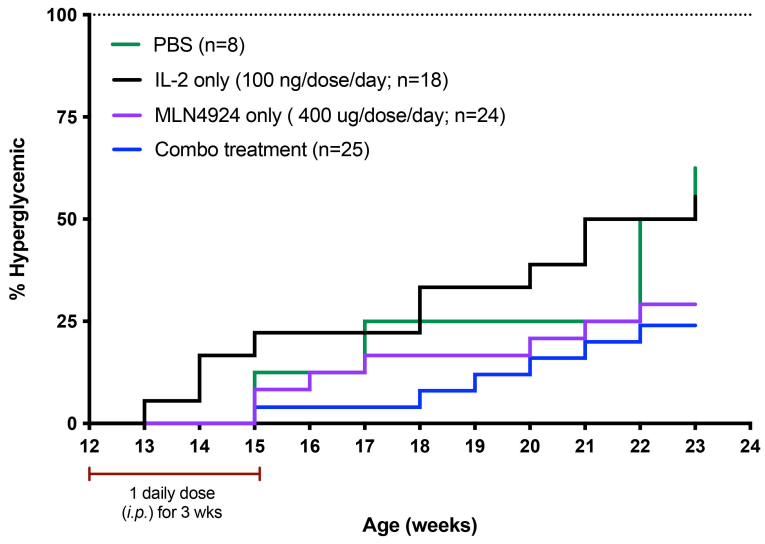


FIGURE 5
Use of the NAEi MLN4924 to treat NOD disease and undergoes receptor mediated endocytosis. IL-2 binding to the IL-2R triggers cellular pathways leading to second messenger signaling activity. Defective second messenger signal pathways identified in T_{regs} from patients with autoimmune diseases that require inhibition of CRL activity, can be corrected by NAEi's attached to the PDC by stable linkers that are enzymatically cleaved only inside the target cell to deliver the drug payload to the appropriate cell. B) PDC (ATA-003) consisting of 3 molecules of an NAEi (MLN4924) bound by di-peptide linkers to the thioredoxin portion of the murine IL-2 fusion protein.

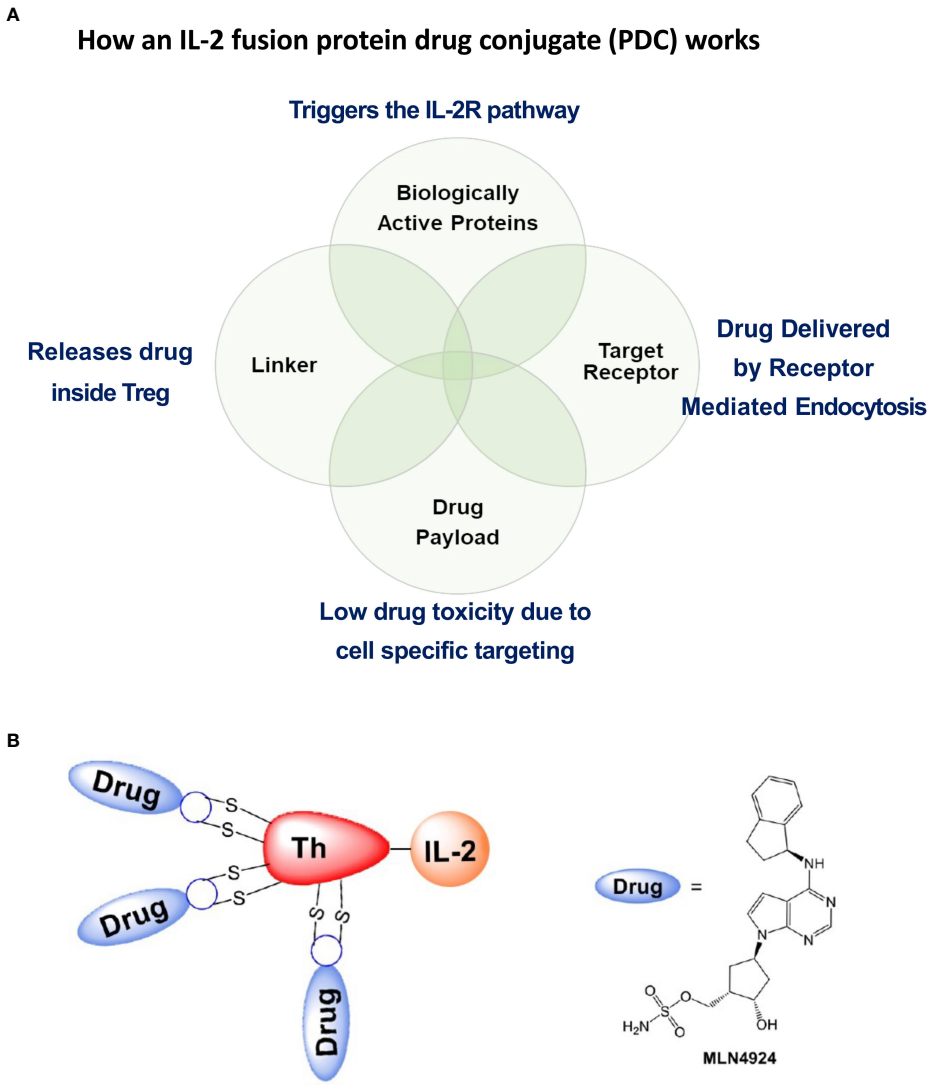


FIGURE 6
Salient features of the IL-2 fusion PDC. **(A)** The PDC binds to Treg cell surface IL-2R's that, following PDC binding, undergo receptor mediated endocytosis. IL-2 binding to the IL-2R triggers cellular pathways leading to second messenger signaling activity. Defective second messenger signal pathways identified in Tregs from patients with autoimmune diseases that require inhibition of CRL activity, can be corrected by NAEi's attached to the PDC by stable linkers that are enzymatically cleaved only inside the target cell to deliver the drug payload to the appropriate cell. **(B)** PDC (ATA-003) consisting of 3 molecules of an NAEi (MLN4924) bound by di-peptide linkers to the thioredoxin portion of the murine IL-2 fusion protein.

cysteine-based drug conjugation. Using conventional dipeptide linkage, IL-2Rx produced a small molecule–linked PDC, called ATA-003, consisting of an NAEi (MLN4924) with an optimized dipeptide linker and a maleimide moiety attached to the murine IL-2 fusion protein (Figure 6B). Analytical methods showed that the fusion protein had three molecules of MLN linked to cysteines by dipeptide cathepsin cleavable bonds to the fusion protein. After passing tests *in vitro* to quality assess the compound, we used ATA-003 to treat animal models of autoimmunity.

Treatment of NOD mice with the PDC to block progression to hyperglycemia

Although the NOD model is a spontaneous model of an autoimmune disease, the pathophysiology is of immune mediated organ destruction leading to loss of the insulin-secreting beta cells of the islets of Langerhans. Since restoring

T_{reg} function (tolerance) will not regenerate islet cells, we chose to try and stop progression to hyperglycemia by treating normoglycemic NOD mice at a time consistent with late-stage islet infiltration with immune cells (insulitis). In this study, we treated 12-week-old female NOD mice with the PDC ATA-003 (2 µg per injection equivalent to 25,000 units of IL-2 as the PDC weighs two times as much as IL-2), low dose IL-2 alone (1 µg or 25,000 units per injection), or saline for 5 days and followed the mice for three months following the 5-day treatment. In a second cohort of animals, we administered one single dose of ATA-003 (2 µg) or IL-2 (1 µg 25.000 IU) every 2 weeks or every month after the initial 5-day dose. We found that the PDC treated mice had a much more pronounced response than the mice treated with low dose IL-2 alone, as the PDC treated mice did not progress to hyperglycemia for over 2 months after therapy started, and the administration of a bi-weekly or monthly booster dose delayed disease onset by an additional 4 weeks (Figure 7). The PDC was also much more effective in delaying disease onset and progression than systemic treatment with the combination of IL-2 and MLN4924 (Figure 5), and there was no detectable toxicity.

Treatment of (NZB x NZW) F1 lupus nephritis with the PDC

We next asked if our PDC would be better than low dose IL-2 in arresting progression of proteinuria in the (NZB x NZW) F1 (BWF1) mouse model of lupus nephritis. In this study, we asked if our novel PDC could prevent the progression of proteinuria in BWF1 female mice that already had significant kidney disease. Three cohorts of BWF1 female mice were initially studied. At an age between 20 and 24 weeks, the mice developed increasing levels of proteinuria. Once a mouse developed 500 mg/dl of proteinuria, they were randomly assigned to receive either the PDC (2 µg per day), low-dose IL-2 (1 µg per day 25,000 IU), or saline as control, by intraperitoneal injections. The amount of IL-2 in the PDC is the same as in the low-dose IL-2, as the PDC weighs about twice as much as IL-2 alone. The mice were given five, daily *i.p.* injections of the PDC, IL-2, or saline, starting when they had achieved 500 mg/dl proteinuria. Our data demonstrate that the PDC was far superior in blocking progression in proteinuria than the low-dose IL-2 alone (Figure 8). Saline treated controls rapidly reached 3 gm/dl and were sacrificed (data not shown).

Using the PDC to treat a mouse model of asthma

There is strong evidence in animals that Tregs act as key regulators of allergic diseases. One example is that the adoptive transfer of ovalbumin (OVA) peptide-specific Tregs into the OVA-sensitized mice attenuated airway hyper-responsiveness along with reduced number of eosinophils and production of Th2 cytokines in the lung following allergen challenge (29). A recent review suggested that Tregs are a viable target to attenuate allergic inflammation in asthma (30). It has already been demonstrated that patients with severe food allergy have a defect in T_{reg} function (31). Additionally, low dose IL-2 has been used to treat experimental food allergy (32). We initially asked if T_{regs} from patients with severe food allergy had the same defect in inhibition of IL-2R desensitization as was seen in the T_{regs} from patients with SLE (Figure 2). Using Tregs isolated from PBMC's from patients with severe food allergy, Jennifer Jenks from Kari Nadeau's lab was able to demonstrate a similar defect in inhibition of the T_{reg} IL-2R response to activation by low dose IL-2 (Figure 9). These data again demonstrate that the defect in allergic asthma does not seem to be due to the number of Tregs, but rather to a defect in their function; ineffective second messenger signaling following IL-2R activation.

To address the potential of the PDC to block an allergen induced allergic response in an animal model, we turned to Mang Yu in Steve Galli's lab to study the effect of the PDC compared to low dose IL-2 in a mouse model of cockroach antigen (CRA) induced asthma (Figure 10A). In the initial study, 1 cohort (n = 5) of 10-week-old female C57BL/l6J (B6) mice with diminished GRAIL expression in their T_{regs} (28), was left untreated as a control for the studies on drug effect. Three cohorts (n = 5/group) were sensitized to the CRA by intranasal administration of 5 µg of CRA in 0.03 mL PBS at days 1 and 2. They were then challenged with the same dose of CRA intranasally at days 15, 18 and 21. Thirty minutes following each of the three challenges, the 3 cohorts of mice received either 0.1 mL of saline *i.p.*, or 0.1 mL of saline containing 1 µg (25,000 IU) of IL-2 *i.p.*, or 0.1 mL of saline containing 2 µg of the PDC *i.p.* The mice were then sacrificed 24 hours following the last antigen challenge and treatment, and bronchoalveolar lavage fluid was collected for evaluating the recruitment of leukocytes into the lung air spaces (diapedesis). The results of the study are presented in Figure 10B. Remarkably, the number of leukocytes in the alveoli of PDC treated mice was almost the same as the control unsensitized mice when the various leukocyte subsets were examined compared to the extensive diapedesis into the lungs of the saline treated mice. The low dose IL-2 treated mice had a modest decrease in leukocyte recruitment compared to that seen in the PDC treated mice.

A separate cohort of mice (same strain, gender, and age) sensitized, challenged, and treated in an identical manner as above, were assessed for their pulmonary function (Figure 10C) using the following methodology: Invasive measurements of airway reactivity in anesthetized, tracheostomized, mechanically ventilated mice were performed 24 hours after the last intranasal exposure (CRA or PBS) by using the BUXCO FivePoint Mouse RC System (Data Sciences International, New

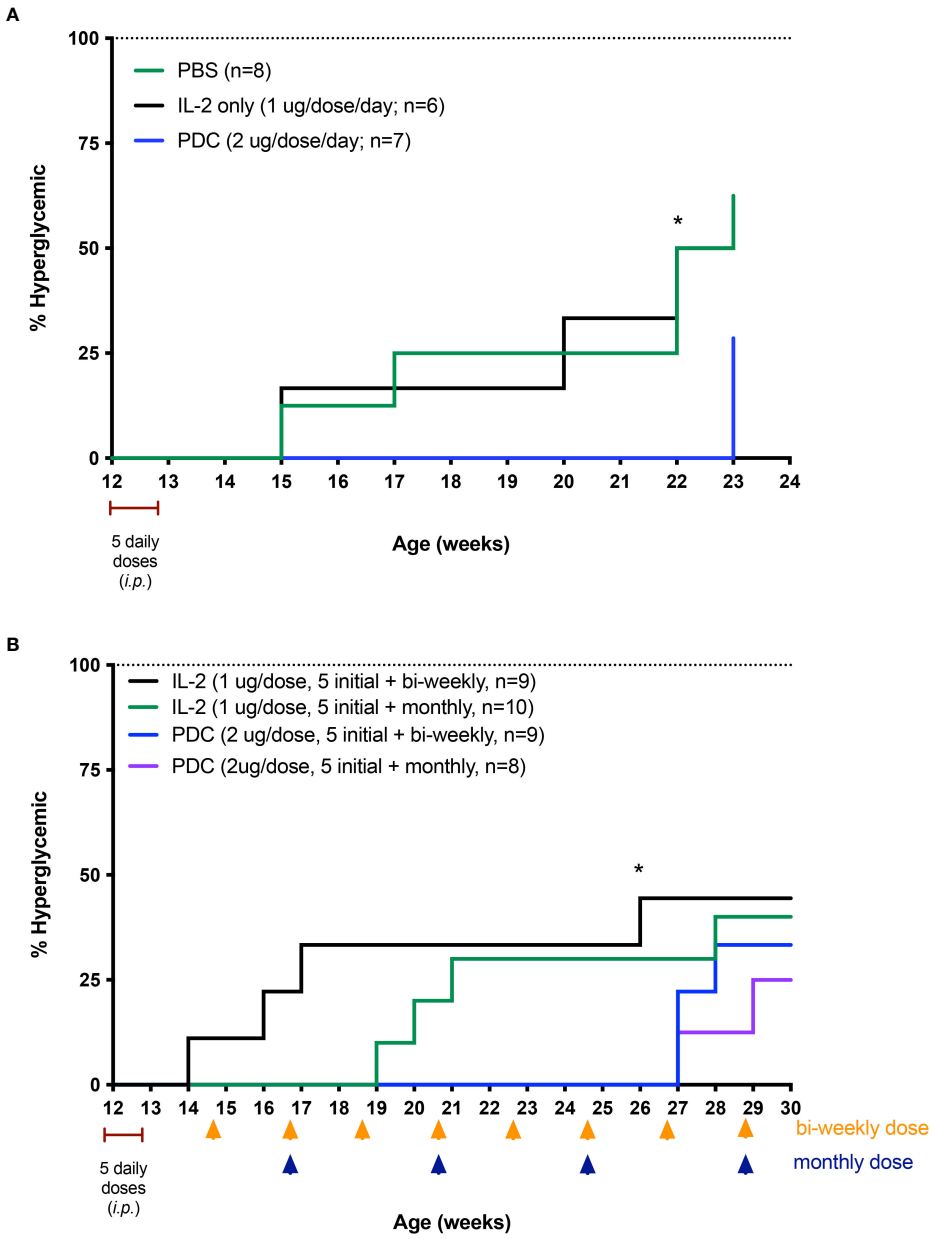


FIGURE 7
Therapeutic effect of the PDC, protein drug conjugate (ATA-003) in preventing disease in the NOD mouse model of T1D. **(A)** NOD mice were treated *i.p.* with low-dose IL-2 (1 µg; 25,000 IU/day), PDC (2 µg/day), or an equal volume of PBS, phosphate buffered saline (0.1 ml) for 5 consecutive days starting at 12 weeks of age. Disease incidence was significantly different between PDC vs. PBS-treated groups ($P < 0.04$), and between PDC vs. IL-2 treated groups ($P < 0.04$; Log rank Mantel-Cox test) at 22 weeks of age. Separate cohorts of NOD mice were treated similarly but with an additional dose of PDC or IL-2 administered every 2 weeks (yellow arrows) or every month (blue arrows) after the initial 5 daily doses **(B)**. Blood sugar was measured weekly until mice became hyperglycemic. Disease incidence was significantly different between biweekly treated PDC vs. biweekly treated IL-2 groups at 26 weeks of age (* $P < 0.03$; Log rank Mantel-Cox test).

Brighton, NM.). Aerosolized, methacholine was administered in increasing concentrations (0, 10, 20, and 40 mg/mL), with individual doses separated by 2 minutes. Lung resistance (R_L) was continuously computed by fitting flow, volume, and pressure to an equation of motion for each aerosol challenge period, which consisted of a 0.5-minute aerosol exposure and a 1.5-minute period after exposure, as described previously (28).

Tregs have been identified as a good candidate for therapy in allergic asthma. Current studies have used two approaches to attempt to increase Treg numbers in a variety of inflammatory

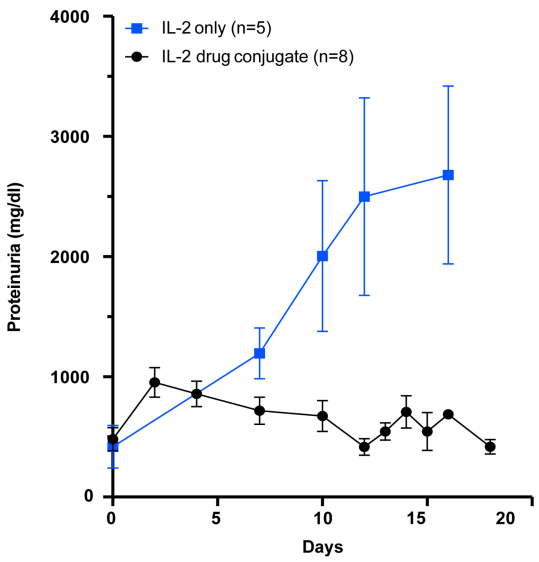


FIGURE 8
Therapeutic effect of the PDC (ATA-003) in preventing nephritis in an animal model of SLE. Groups of female (NZB × NZW) F1 mice aged more than 24 weeks with a proteinuria score of greater than 500 mg/dl were either given the PDC ATA-003 (2 µg/day; n = 8) or a molar equivalent of low-dose IL-2 (1 µg; 25,000 IU/day; n = 5) for 5 days and followed for 3 weeks. Mice that received saline rapidly progressed to severe proteinuria in a manner similar to the low dose IL-2 treated mice (data not included).

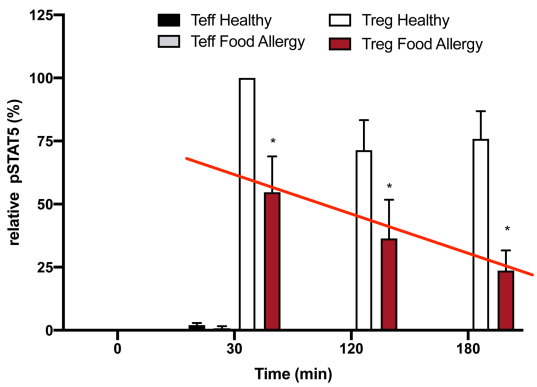


FIGURE 9
Loss of inhibition of T_{reg} IL-2R desensitization in food allergy patients. Expression of pSTAT5 in human T_{effs} vs. human T_{regs} in healthy and food allergy participants (n=3/group). Expression of pSTAT5 is shown relative to levels measured in T_{regs} of healthy controls stimulated with low dose IL-2 (1 ng; 25 IU/ml) for 30 mins. Cultures were harvested at the indicated times after stimulation and pSTAT5 levels were measured by immunoblotting. Data are normalized to levels measured in 30 min IL-2 stimulated control T_{regs}. (mean ± S.D.; $p < 0.05$ for * with two-way ANOVA).

disorders, either using low dose IL-2 or by adoptive transfer of autologous Tregs expanded *ex vivo* back into patients (33). To date there are no convincing studies that the approach of increasing Treg numbers in allergic asthma will work as therapy. Our approach using the PDC described above to restore function to the defective Tregs seen in allergen induced asthma represents a revolutionary form of therapy to treat allergic asthma in man.

Discussion

Our hypothesis is that the defect in T_{regs} cells seen in autoimmunity and allergic asthma is not due to a deficiency in the number of T_{regs}, but rather due to a defect in T_{reg} IL-2R signaling that leads to defective second messenger activity leading to a loss of inhibition of IL-2R desensitization. This defect leads to diminished pSTAT5 and Deptor expression following low dose IL-2 activation of the T_{reg} IL-2R in patients with autoimmune and inflammatory diseases (Figure 1C). We hypothesized that this leads to incomplete pSTAT5 transcription of the genes required for T_{reg} function (34). Deptor is an endogenous inhibitor of mTOR activation and is recognized by the F box protein βTrCP of the Cul5/Elongin B complex for polyubiquitination and consequent proteasomal degradation following IL-2 activation of conventional CD25+CD4 T_{effs} (11). mTOR regulates multiple biological processes including those involved in cell growth and proliferation (34). Cell proliferation and differentiation show a remarkable inverse relationship. In conditions where T_{reg} numbers are sufficient, restoration of function in the absence of proliferation may actually be advantageous given the remarkable inverse relationship between cell proliferation and differentiation (35). GRAIL mediated maintenance of Deptor to inhibit mTOR kinase activation in non-autoimmune T_{regs}, suggests an important role for this protein in the maintenance of T_{reg} homeostasis (36).

The paradigm of antibody-drug conjugate (ADC) therapy is that delivery of a drug is restricted to the target cell population by means of the specificity of the antibody and the conditional release of the drug warhead in the vicinity of the cell. Our protein-drug conjugate (PDC) model improves on that concept by making use of the high affinity IL-2 receptor in conjunction with low-dose IL-2 activation (Figure 6). Our PDC has the advantage of targeting almost exclusively T_{regs} that constitutively express the high affinity IL-2R. Importantly, IL-2R activation by the PDC triggers internalization of the PDC by receptor mediated endocytosis and the drug is delivered to the late endosomes of the T_{reg} where the drug is released by cathepsin cleavage (of the di-peptide bond) to repair the defective signaling pathway activated by IL-2. This can be considered a "Trojan Horse" model for drug delivery. The IL-2 portion of the PDC activates the IL-2R receptor and, following receptor mediated

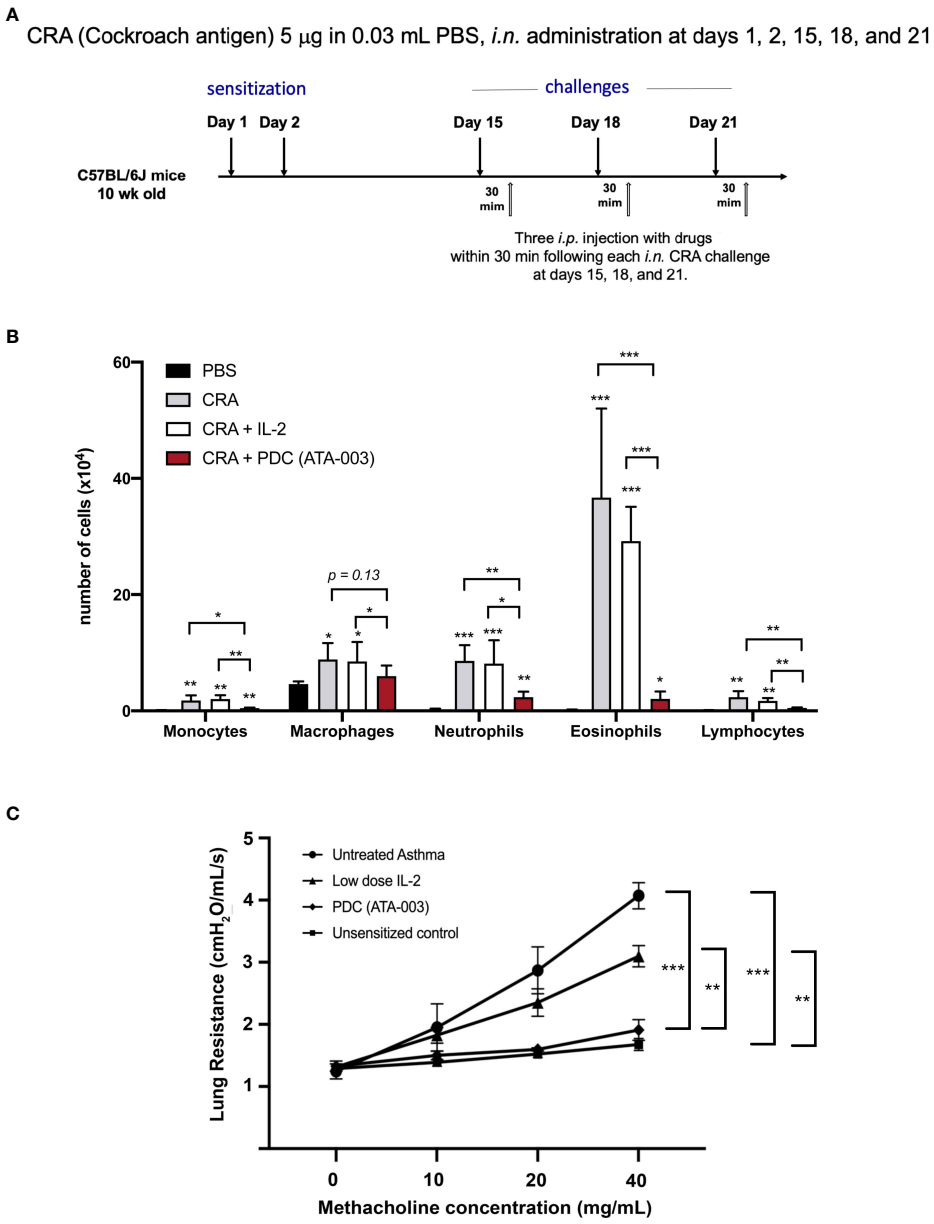


FIGURE 10
Therapeutic effect to the PDC in a CRA-induced animal model of asthma. **(A)** Asthma was induced in C57/BL6J mice. **(B)** In one cohort of mice, the recruitment of leukocytes into the lung air spaces (diapedesis) was measured 24 h after the last treatment with PDC (2 μg), IL-2 (1 μg 25,000 IU) or an equal volume of saline (0.1 ml). *p < 0.05, ** p < 0.01, *** p < 0.001 compared to PBS -treated group or groups indicated. **(C)** In a second cohort of mice, pulmonary function was evaluated. Lung resistance for each group was compared using two-way ANOVA (Tukey's multiple comparisons test; ***p < 0.001, **p < 0.01).

endocytosis (opening the gate of the T_{reg} membrane for targeted drug delivery), delivers the drug directly to the signaling endosome where it corrects the defect in IL-2R signaling. Several publications have now described signaling defects downstream of the IL-2 receptors in T cells from autoimmune subjects both in humans and in animal models (28, 37). This restoration of immune regulation (blockade of disease progression) relies on the fact that a Cullin5-dependent CRL ubiquitin ligase complex is responsible for pJAK1 degradation in the proteasome (35). The Nedd8 activating enzyme inhibitor MLN-4924 blocks the ability of the CRL E2 transferase to deliver ubiquitin to the target of the E3 ligase, pJAK1 (Figure 1B).

Neddylation of lysine 724 on the cullin backbone of the CRL5 is an absolute requirement for CRL5 function. Neddylation will come to a halt as the inactivated nedd8, bound by the NAEi, accumulates in the targeted T_{reg}. In addition to presumably restoring pSTAT5 transcription of the genes required for T_{reg} function, the PDC also blocks Deptor degradation to maintain mTOR in an inactive state and thus stabilizes the T_{reg} phenotype. mTOR activation is required for generation of effector T cells from naïve cells, and its absence or inhibition causes activated naïve T cells to default to a regulatory lineage (38). We are currently evaluating the homologous human fusion protein of the mouse thioredoxin/IL-2 fusion protein PDC as a potential drug candidate to treat several autoimmune diseases and, potentially, allergic asthma. Preliminary studies of isolated activated CD25+ human CD4+T effectors (Teffs) demonstrated that their response to the PDC *in vitro* was similar to their response to a molar equivalent of hIL-2 in proliferation measured by dye dilution 48 hours after stimulation with anti-CD3/28 beads.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Stanford School of Medicine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Stanford School of Medicine.

Author contributions

CF, LS and LY were the main authors of this article. The text was reviewed by CS, KN, DG-M, JL, JJ and MY and CRH, all of whom participated in deriving the data discussed in the manuscript. All authors contributed to the article and approved the submitted version.

Funding

CF was funded by grants from the Juvenile Diabetes Research foundation (JDRF), the Lupus Research Alliance (ALR), the Department of Defense (DoD: LR210017) and the National Institutes of Health (RO1 AI-49903, RO1 CA065237, U19 AI 082719, and U19 AI70352). KN was funded by grants from NIH (NIAID: R01/U01/U19).

Acknowledgments

We thank Dr. Steve Galli for his helpful discussions, and acknowledge the Naddisy Foundation, Sean N. Parker Center for Allergy and Asthma Research, and the Reinhart Foundation for their support.

Conflict of interest

CF is on the Board of IL-2Rx, and LS and CRH are part-time contract employees of IL-2Rx. KN is on the SAB of IL-2Rx.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

References

1. Hatzioannou A, Boumpas A, Papadopoulou M, Papafragkos I, Varveri A, Alissafi T, et al. Regulatory T cells in autoimmunity and cancer: A duplicitous lifestyle. *Front Immunol* (2021) 12:731947. doi: 10.3389/fimmu.2021.731947

2. Long SA, Buckner JH. CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game. *J Immunol* (2011) 187(5):2061–6. doi: 10.4049/jimmunol.1003224

3. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N. Regulatory T cells and human disease. *Annu Rev Immunol* (2020) 38:541–66. doi: 10.1146/annurev-immunol-042718-041717

4. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* (2006) 212:8–27. doi: 10.1111/j.0105-2896.2006.00427.x

5. Grasshoff H, Comduhr S, Monne LR, Muller A, Lamprecht P, Riemekasten G, et al. Low-dose IL-2 therapy in autoimmune and rheumatic diseases. *Front Immunol* (2021) 12:648408. doi: 10.3389/fimmu.2021.648408

6. Klatzmann D, Abbas AK. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol* (2015) 15(5):283–94. doi: 10.1038/nri3823

7. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in treg cell function. *Nat Immunol* (2016) 17 (11):1322–33. doi: 10.1038/ni.3540

8. Catena V, Fanciulli M. Deptor: not only a mTOR inhibitor. *J Exp Clin Cancer Res* (2017) 36(1):12. doi: 10.1186/s13046-016-0484-y

9. Wedel J, Bruneau S, Liu K, Kong SW, Sage PT, Sabatini DM, et al. DEPTOR modulates activation responses in CD4(+) T cells and enhances immunoregulation following transplantation. *Am J Transplant* (2019) 19(1):77–88. doi: 10.1111/ajt.14995

10. Anandasabapathy N, Ford GS, Bloom D, Holness C, Paragas V, Seroogy C, et al. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity* (2003) 18(4):535–47. doi: 10.1016/s1074-7613(03)00084-0

11. Okumura F, Joo-Okumura A, Nakatsukasa K, Kamura T. The role of cullin 5-containing ubiquitin ligases. *Cell Div* (2016) 11:1. doi: 10.1186/s13008-016-0016-3

12. Kumar B, Field NS, Kim DD, Dar AA, Chen Y, Suresh A, et al. The ubiquitin ligase Cul5 regulates CD4(+) T cell fate choice and allergic inflammation. *Nat Commun* (2022) 13(1):2786. doi: 10.1038/s41467-022-30437-x

13. Gao F, Fan Y, Zhou B, Guo W, Jiang X, Shi J, et al. The functions and properties of cullin-5, a potential therapeutic target for cancers. *Am J Transl Res* (2020) 12(2):618–32.

14. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* (2009) 458(7239):732–6. doi: 10.1038/nature07884

15. Bargh JD, Isidro-Llobet A, Parker JS, Spring DR. Cleavable linkers in antibody-drug conjugates. *Chem Soc Rev* (2019) 48(16):4361–74. doi: 10.1039/c8cs00676h

16. He J, Zhang R, Shao M, Zhao X, Miao M, Chen J, et al. Efficacy and safety of low-dose IL-2 in the treatment of systemic lupus erythematosus: A randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis* (2020) 79(1):141–49. doi: 10.1136/annrheumdis-2019-215396

17. Lin AE, Mak TW. The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells. *Curr Opin Immunol* (2007) 19(6):665–73. doi: 10.1016/j.coi.2007.10.002

18. Fang N, Fang D, Wang HY, Altman A, Liu YC. Regulation of immune responses by E3 ubiquitin-protein ligases. *Curr Dir Autoimmun* (2002) 5:161–75. doi: 10.1159/000060552

19. Wang J, Maldonado MA. The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Mol Immunol* (2006) 3(4):255–61.

20. Cohney SJ, Sanden D, Cacalano NA, Yoshimura A, Mui A, Migone TS, et al. SOCS-3 is tyrosine phosphorylated in response to interleukin-2 and suppresses STAT5 phosphorylation and lymphocyte proliferation. *Mol Cell Biol* (1999) 19 (7):4980–8. doi: 10.1128/mcb.19.7.4980

21. Kershaw NJ, Murphy JM, Liau NP, Varghese LN, Laktyushin A, Whitlock EL, et al. SOCS3 binds specific receptor-JAK complexes to control cytokine signaling by direct kinase inhibition. *Nat Struct Mol Biol* (2013) 20(4):469–76. doi: 10.1038/nsmb.2519

22. Kershaw NJ, Murphy JM, Lucet IS, Nicola NA, Babon JJ. Regulation of janus kinases by SOCS proteins. *Biochem Soc Trans* (2013) 41(4):1042–7. doi: 10.1042/BST20130077

23. Babon JJ, Sabo JK, Zhang JG, Nicola NA, Norton RS. The SOCS box encodes a hierarchy of affinities for Cullin5: Implications for ubiquitin ligase formation and cytokine signalling suppression. *J Mol Biol* (2009) 387(1):162–74. doi: 10.1016/j.jmb.2009.01.024

24. MacKenzie DA, Schartner J, Lin J, Timmel A, Jennens-Clough M, Fathman CG, et al. GRAIL is up-regulated in CD4+ CD25+ T regulatory cells and is sufficient for conversion of T cells to a regulatory phenotype. *J Biol Chem* (2007) 282(13):9696–702. doi: 10.1074/jbc.M604192200

25. Xu G, Paige JS, Jaffrey SR. Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat Biotechnol* (2010) 28(8):868–73. doi: 10.1038/nbt.1654

26. Soares L, Seroogy C, Skrenta H, Anandasabapathy N, Lovelace P, Chung CD, et al. Two isoforms of otubain 1 regulate T cell anergy *via* GRAIL. *Nat Immunol* (2004) 5(1):45–54. doi: 10.1038/ni1017

27. Lin JT, Lineberry NB, Kattah MG, Su LL, Utz PJ, Fathman CG, et al. Naive CD4 t cell proliferation is controlled by mammalian target of rapamycin regulation of GRAIL expression. *J Immunol* (2009) 182(10):5919–28. doi: 10.4049/jimmunol.0803986

28. Godoy GJ, Olivera C, Paira DA, Salazar FC, Ana Y, Stempin CC, et al. T Regulatory cells from non-obese diabetic mice show low responsiveness to IL-2 stimulation and exhibit differential expression of anergy-related and ubiquitination factors. *Front Immunol* (2019) 10:2665. doi: 10.3389/fimmu.2019.02665

29. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after *in vivo* transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* (2005) 202(11):1539–47. doi: 10.1084/jem.20051166

30. Zhang J, Zou Y, Chen L, Xu Q, Wang Y, Xie M, et al. Regulatory T cells, a viable target against airway allergic inflammatory responses in asthma. *Front Immunol* (2022) 13:902318. doi: 10.3389/fimmu.2022.902318

31. Martin-Orozco E, Norte-Munoz M, Martinez-Garcia J. Regulatory T cells in allergy and asthma. *Front Pediatr* (2017) 5:117. doi: 10.3389/fped.2017.00117

32. Bonnet B, Vigneron J, Levacher B, Vazquez T, Pitoiset F, Brimaud F, et al. Low-dose IL-2 induces regulatory T cell-mediated control of experimental food allergy. *J Immunol* (2016) 197(1):188–98. doi: 10.4049/jimmunol.1501271

33. Roth-Walter F, Adcock IM, Benito-Villalvilla C, Bianchini R, Bjermer L, Boyman O, et al. Immune modulation *via* T regulatory cell enhancement: Disease-modifying therapies for autoimmunity and their potential for chronic allergic and inflammatory diseases-an EAACI position paper of the task force on immunopharmacology (TIPCO). *Allergy* (2021) 76(1):90–113. doi: 10.1111/all.14478

34. Laplante M, Sabatini DM. mTOR signaling. *Cold Spring Harb Perspect Biol* (2012) 4(2):a011593. doi: 10.1101/cshperspect.a011593

35. Ruijtenberg S, van den Heuvel S. Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. *Cell Cycle* (2016) 15(2):196–212. doi: 10.1080/15384101.2015.1120925

36. Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* (2009) 137(5):873–86. doi: 10.1016/j.cell.2009.03.046

37. Long SA, Cerosaletti K, Bollyky PL, Tatum M, Shilling H, Zhang S, et al. Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4(+)CD25(+) regulatory T-cells of type 1 diabetic subjects. *Diabetes* (2010) 59(2):407–15. doi: 10.2337/db09-0694

38. Powell JD, Delgoffe GM. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* (2010) 33(3):301–11. doi: 10.1016/j.immuni.2010.09.002