Differential expression of *GRAIL* isoforms in regulatory and effector T cells of mice and human.

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Background and Objectives

GRAIL (RNF128) is an E3 ubiquitin ligase that is expressed in mouse and human CD4 effector T cells (Teffs) and regulatory T cells (Tregs). It plays a role in regulatory T cell function and maintains effector T cell quiescence (**Fig. 1**). We propose that in autoimmune patients, a loss of GRAIL expression may result in reduced Treg function. This may occur though degradation of GRAIL protein or through alternative splicing of the gene. Multiple isoforms of GRAIL have been identified. The canonical isoform of GRAIL (isoform 1) was first discovered and characterized in our lab. This isoform contains a RING finger domain that is essential for ligase activity, a PA domain and transmembrane domain for substrate recognition and localization at the plasma membrane, respectively. Two alternatively spliced isoforms (isoforms 2 and 3) have also been identified (**Fig. 2**), however their expression and function in Teff and Treg cells is unclear. It is possible that one isoform may inhibit or compete with another in mediating various T cell functions. Here, we developed specific QPCR assays to detect and measure the expression of isoforms 1 and isoforms 2/3 of *GRAIL* in human and mouse Teff and Tregs and examined if treatment with various stimuli can alter the expression of these transcripts.

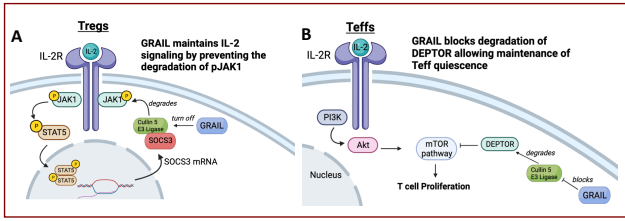

Fig. 1: Role of GRAIL in regulatory (A) and effector (B) T cells. **A:** In Tregs, GRAIL maintains Treg function by blocking the desensitization of the IL-2R. This is accomplished though its inhibition of SOCS3-mediated degradation of pJAK1 on the IL2-R. **B:** In Teffs, Cul5 degrades DEPTOR once GRAIL is diminished by Teff activation

Isoform 1 (428 aa)

MGPPPGAGVSCRGGCGFSRLLAWCFLLALSPQAPG
RGAEAVVWTAYLNVSWRVPHTGVNRTVWELSEEG
VYGQDSPLEPVAGVLVPPDGPGALNACNPHTNFTV
PTVWGSTVQVSWLALICRGGGCTFADKHLAYERG
ASGAVIFNFPGTRNEVIMSHPGAVDIVAIMIGNLKGT
KILQSIQRGIQVTMVIEVGKKHGPWVNHYSIFFVSVS
FFIITAATVGYFIFYSARRLRNARAQSRKQRQLKADAK
KAIGRLQLRTLKQGDKEIGPDGDSCAVCIELYKPNDLV
RILTCNHIFHKTCVDPWLLEHRTCPMCKCDILKAGIE
VDVEDGSVSLQVPVSNEISNSASSHEEDNRSETASSG
YASVQGTDEPPLEEHVQSTNESLQLVNHEANSVAVD
VIPHVDNPTFEEDETPNQETAVREIKS

Isoform 2 (402 aa)

MNQENRSSFFWLLVIFTFLLKITASFSMSAYVTVT
YYNETSNYTAIETCECGVYGLASPVANAMGVVGI
PKNNNYQACDHNTEFSNTKKPWIALIERGNCTFS
EKIQTAGRRNADAVVIYNAPETGNQTIQMANFG
AVDIVAIMIGNLKGTKILQSIQRGIQVTMVIEVGK
KHGPWVNHYSIFFVSVSFFIITAATVGYFIFYSARR
LRNARAQSRKQRQLKADAKKAIGRLQLRTLKQG
DKEIGPDGDSCAVCIELYKPNDLVRILTCNHIFHKT
CVDPWLLEHRTCPMCKCDILKALGIEVDVEDGSV
SLQVPVSNEISNSASSHEEDNRSETASSGYASVQG
TDEPPLEEHVQSTNESLQLVNHEANSVAVDVIPH
VDNPTFEEDETPNQETAVREIKS

Isoform 3 (249 aa)

MSAYVTVTYYNETSNYTAIETCECGVY
GLASPVANAMGVVGIPKNNNYQAC
DHNTEFSNTKKPWIALIERGNCTFSE
KIQTAGRRNADAVVIYNAPETGNQTI
QMANFGAVDIVAIMIGNLKGTKILQS
IQRGIQVTMVIEVGKKHGPWVNHYS
IFFVSVSFFIITAATVGYFIFYSARRLRN
ARAQSRKQRQLKADAKKAIGRLQLRT
LKQGDKEIGPDGDSCAVCIELYKPNDL
VRILTCNHIFHKT

Blue: N-terminal unique to isoform 1
Pink: signal peptide
Green: protease-associated (PA domain)
Yellow: transmembrane domain
Red: RING finger motif

Green: N-term unique to Isoform 2
Purple: shared in Isoform 2 and 3
Green: truncated PA domain
Yellow: transmembrane domain
Red: RING finger motif

Purple: shared in isoform 2 and 3
Green: truncated PA domain
Yellow: transmembrane domain
Red: truncated RING finger motif

Fig. 2: Human *GRAIL* transcripts

Methods

Human and Mouse Teff and Tregs: Treg & Teff cells were isolated from lymphocytes of 10-week-old BALB/c, C57/BL6, & NOD (model of Type 1 diabetes) mice using the Dynabeads FlowComp Mouse CD4+CD25+ Treg Kit. Human Teff & Tregs were isolated from PBMCs using the StemCell human CD4+CD127loCD25+ cell isolation kit.

Cell Treatment: Cells were stimulated with anti-CD3/28 beads (1 bead:1 cell), plate bound anti-CD3, IL-2 (1 ng/ml), or a combination of anti-CD3 + IL-2 in a volume of 200 ul of X-Vivo media.

QPCR: RNA was extracted using Trizol and the RNeasy Micro Kit. cDNA was synthesized using Superscript IV, and pre-amplified using the TAQMAN preamplification reagent. QPCR was performed using TaqMan assays and the TaqMan gene expression master mix for *GRAIL* (RNF128) Isoform 1 and *18S* rRNA (house-keeping gene) expression. Custom primers were designed to measure human *GRAIL* encoding isoforms 2/3 and mouse *Grail* isoform 2, using PowerUP SYBR master mix. *GRAIL* transcript expression was normalized using *18S* rRNA expression.

Results

3 isoforms of *GRAIL* have been identified in humans and 2 *Grail* isoforms have been identified in mice. We aligned the mRNA sequences of human *GRAIL* isoforms 1, 2, and 3, and the sequences of mouse *Grail* isoforms 1 and 2 to identify unique regions in each isoform that can be targeted for QPCR analysis. The human and mouse canonical GRAIL isoform 1 express a different exon 1 compared to isoform 2 and/or 3. Commercially available TAQMAN assays targeting the unique exon 1 of isoform 1 were purchased from ThermoFisher. For human *GRAIL* isoforms 2 and 3, we designed a single set of primers that can bind to the region that is shared between these two isoforms (**See Fig. 2**).

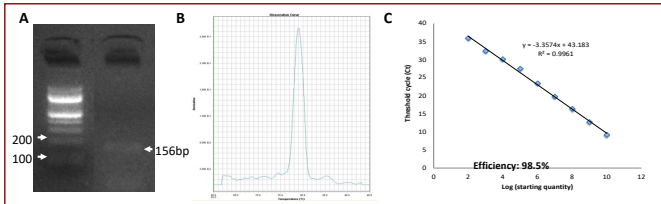

Fig. 3: Testing the specificity and efficiency of the human *GRAIL* isoform 2/3 QPCR primers.
A. RT-PCR results showing the amplification of one product of the expected size using cDNA of PBMCs of a T1D patient.
B. QPCR dissociation curve showing amplification of a single product.
C. Standard curve showing efficient amplification from a Ct range of 9-36.
Note: QPCR primers for the mouse Grail isoform 2 assay were also tested, with similar results.

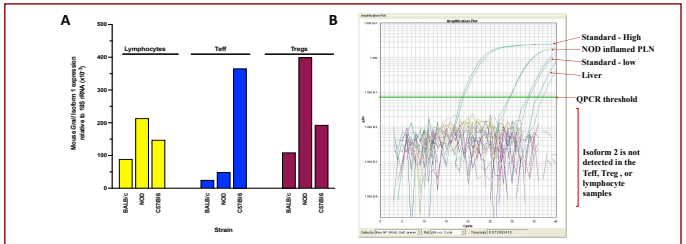

Fig. 3. The canonical isoform (isoform 1) is the only *Grail* transcript expressed in lymphocytes, Teffs and Tregs of mice. QPCR was performed to measure isoform 1 and 2 in lymphocytes, Teff, and Tregs of control BALB/c mice, autoimmune-prone C57/BL6, and NOD mice (model of T1D). Only isoform 1 was detected.

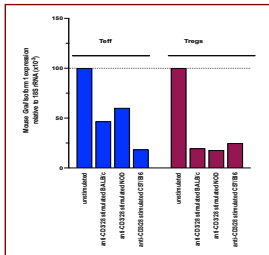

Fig. 4. Anti-CD3/28 activation of Teffs and Tregs result in reduced expression of the canonical isoform 1 of *Grail*.

Table 1: QPCR Primers for *GRAIL* isoform 2/3 and *Grail isoform 2*

Species	Primer	Sequence	Amplicon
Human *GRAIL* isoform 2/3	Forward	5' GCTATGGGAGTGGTAGGCAT 3'	154 bp
	Reverse	5' CATTTCTTCTGCCCGCTGTT 3'	
Mouse *Grail* Isoform 2	Forward	5' GCCTTGGATTGCGCTGATAG 3'	155 bp
	Reverse	5' CAATGTCCCCAGCACCAAAA 3'	

Table 2: *GRAIL* transcript expression in human cells

	Tregs		Teffs	
	Isoform 1	Isoform 2/3	Isoform 1	Isoform 2/3
Control LY18	689.07	2393.69	931.85	1301410.41
Control LY25	39.44	825.86	738.14	5826.37
Control FW2	376.00	1476.43	821.41	2180.45
SLE	5.66	1405.38	0.21	476.08
Allergy	22.35	28.54	971448	37520847

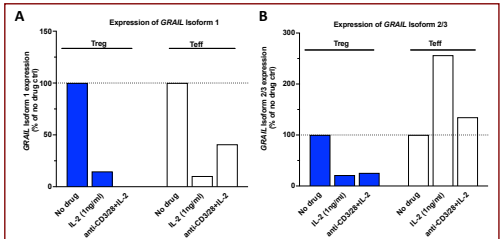

Fig. 5: Expression *of GRAIL* isoform 1 (**A**) and isoform 2/3 (**B**) after 24h treatment with low dose IL-2 (1ng/ml) with and without anti-CD3/28 stimulation.

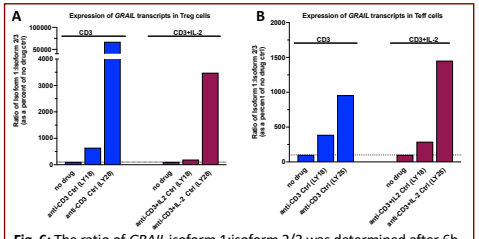

Fig. 6: The ratio of *GRAIL* isoform 1:isoform 2/3 was determined after 6h stimulation with anti-CD3 alone or with IL-2 (1 ng/ml) in Tregs (**A**) and Teffs (**B**) of healthy controls. Treatment increased the relative amount of isoform 1.

Major Findings

- Teff and Tregs of mice express only the canonical isoform 1 of *Grail* (**Fig. 3**), while human Teffs and Treg express multiple isoforms of *GRAIL* **(Table 2)**
- Activation with IL-2 and anti-CD3/28 beads generally resulted in a loss of *GRAIL* transcript expression **(Fig. 4 & 5)**
- Stimulation with anti-CD3 or CD3+IL-2, generally upregulated the expression of *GRAIL* transcripts **(Fig. 6)**

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