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GRAIL: A unique mediator of CD4 T lymphocyte unresponsiveness

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Abstract

GRAIL (gene related to anergy in lymphocytes, also known as RNF128), an ubiquitin-protein ligase (E3), utilizes a unique single transmembrane protein with a split function motif, and is an important gatekeeper of T cell unresponsiveness. While it may play a role in other CD4 T cell functions including activation, survival, and differentiation, GRAIL is most well characterized as a negative regulator of TCR responsiveness and cytokine production. Here, we review the recent literature on this remarkable E3 in the regulation of human and mouse CD4 T cell unresponsiveness.

Keywords

GRAIL; E3; ubiquitin-protein ligase; anergy; T cell unresponsiveness; ubiquitination; de-ubiquitinating enzymes (DUBs)

Introduction

The ability to distinguish self from non-self is the most important requirement of the mammalian immune system. Central (thymic) and peripheral tolerance mechanisms have evolved to prevent lymphocyte-mediated self-destruction (autoimmunity). Because thymic negative selection is not foolproof, some autoreactive T cells escape negative selection. Peripheral tolerance mechanisms, therefore, need to be in place to maintain CD4 T cell unresponsiveness to self. One important mechanism of peripheral tolerance that maintains CD4 T cell unresponsiveness is anergy [1,2]. Anergic CD4 T cells fail to proliferate or to produce interleukin-2 (IL-2) following immunogenic stimulation. Based on the simplistic two-signal hypothesis, full T cell activation occurs from the simultaneous engagement of the T cell receptor (TCR)(signal one) and CD4 T cell costimulatory molecules such as CD28 (signal two). In the absence of robust activation, (including a variety of extrinsic and intrinsic activation signals), engagement of the CD4 T cell receptor only suboptimally stimulates the T cell (signal one) and, without costimulation, TCR engagement results in a form of CD4 T cell unresponsiveness called anergy [2]. Anergy induction is an active process that is dependent upon tightly controlled biochemical signaling events including up-regulation and degradation of both genes and proteins [3-6]. As demonstrated several years ago, the development of the anergy phenotype in CD4 T cells could be blocked by inhibitors of protein synthesis or by calcineurin, which suggested that the induction of anergy activated a unique genetic program [7]. The induced unresponsive state of anergy was relatively long lived in CD4 T cells and could be reversed by the addition of exogenous IL-2, a distinct

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feature of anergic CD4 T cells. In addition to these molecular events identified earlier, it has recently become evident that the post-translational modification of proteins via ubiquitination plays an essential role in the regulatory mechanisms of CD4 T cell anergy.

The balance between ubiquitination and de-ubiquitination of many cellular proteins is well accepted as an important mechanism for the maintenance of T cell unresponsiveness and prevention of autoimmunity [3,8,9]. Similar to the well studied phosphorylation induced post-translational modification of signaling proteins, ubiquitination is an evolutionarily conserved and reversible process that is also important in signaling and works by covalently attaching monoubiquitin or polyubiquitin chains to target proteins to regulate their stability, activity and localization. Post-translational ubiquitination can result in proteolytic degradation as well as nonproteolytic outcomes that regulate a broad range of critical cellular functions, including gene transcription and protein trafficking. Ubiquitin conjugation of target proteins consists of a sequence of steps that require three classes of modifying enzymes. The initiation step involves an ATP-dependent attachment of ubiquitin to the ubiquitin-activating enzyme (E1). Next, the thiol ester-linked ubiquitin is transferred from the E1 enzyme to a cysteine residue in an ubiquitin-conjugating enzyme (E2). Lastly, the E2 enzyme, together with ubiquitin-protein ligase (E3) transfers ubiquitin to target proteins, where a stable isopeptide bond is formed between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. The E3 determines the specificity in the substrate conjugation process; however, it has been a challenge to uncover specific target lysine sites or consensus ubiquitination motifs on target proteins. This post-translational process is a reversible reaction where the trimming or removal of ubiquitin linkages is mediated by an equally complex process of de-ubiquitination. The diverse family of de-ubiquitinating enzymes (DUBs) can be classified into broad categories based on their enzymatic domains; the most common two are the ubiquitin specific proteases (USP/UBPs) and ubiquitin C-terminal hydrolases (UCHs).

The role of ubiquitin ligases as modulators of central and peripheral tolerance has brought attention to this system as one of the key components of a complex regulatory network designed to maintain an active immune surveillance program [10]. Three ubiquitin-protein ligases, Cbl-b, Itch, and GRAIL have been demonstrated to play a functional role in T cell anergy [1,3,10-13]. Moreover, Itch has been shown to prevent autoimmune activation of peripheral T cells toward a Th2 bias [14], and Cbl-b attenuates T cell hyper-responsive activation absent CD28 costimulation [15-17]. These three E3s function as negative regulators of the immune response and their expression is induced as part of the genetic program tuned by the calcium/calcineurin pathway to help establish and maintain T cell unresponsiveness via setting thresholds for TCR signaling [3,4,14,18-22]. Mechanisms implicated in the development of anergy associated with these E3s include setting the threshold for TCR responsiveness, modulation of TCR-specific signals and repression of cytokine transcription. The induction or function of CD4 regulatory T cells has been suggested for Cbl-b and GRAIL. Moreover, the defective expression of these E3s has been linked to autoimmune or inflammatory diseases, in experimental murine and human models, marking their possible pathogenic roles [11,15,22-24]. While GRAIL is expressed in a variety of tissues including liver and hematopoietic linage cells, only its expression in T cells has been studied extensively. In this review, we will focus our discussion on recent research investigating the biology of GRAIL in T lymphocytes and specifically its role in establishing and maintaining CD4 T cell unresponsiveness. In this issue of Febs Journal, we refer you to excellent discussions on other members of the RING finger E3s including plant RMR [25] and RFN13 [26,27].

Sections

What is GRAIL?

GRAIL (gene related to anergy in lymphocytes, also known as RNF128), is a novel ubiquitin-protein ligase (E3), initially identified in a differential display screen of cDNAs obtained from separate aliquots of a T cell clone that had either been rendered anergic, were resting or fully activated. Among the 5 cDNAs that were differentially displayed in the anergic CD4 T cells were two ubiquitin-protein ligases, cbl-b and Rnf 128 [8]. Subsequent structure-function studies characterized Rnf 128 (named GRAIL in our original manuscript) as a 428 amino acids type I transmembrane single subunit E3 with a cytosolic zinc-binding RING finger domain and a luminal or extracellular protease-associated (PA) domain (Figure 1). Unlike other E3s, GRAIL uniquely localizes to the transferrin-recycling endocytic pathway. The RING finger of GRAIL is a C2H2C3 type, and was shown to possess E3 activity. As expected, mutation in the RING finger domain of GRAIL by substitution of asparagine for histidine (H2N2) disrupted ubiquitin ligase activity and enhanced GRAIL's inherent stability [8]. While the cytosolic RING finger domain functioned as an ubiquitin-protein ligase, the extracellular PA domain was subsequently demonstrated to capture transmembrane protein targets for GRAIL mediated ubiquitination [8,28]. This split function motif is unique for a single protein E3, demonstrating initial binding to the cell membrane associated target molecule (including tetraspanins, CD83 and CD40L) through the luminal or extracellular PA domain of GRAIL and subsequent ubiquitination of the cytosolic tail of the transmembrane target (substrate) by the cytosolic RING finger domain of GRAIL [28] (see Table 1). Lastly, the coiled-coil domain was found to interact with Otubain-1, an ubiquitin isopeptidase of the ovarian tumor (OTU) superfamily [29,30]. Thus, GRAIL is a single subunit E3 containing a RING finger and a PA domain that perform dual functions to both recognize and capture GRAIL's substrate (PA domain), and to directly ubiquitinate the captured target protein (RING finger domain).

GRAIL induces and maintains anergy in T cells

Since the cloning of *grail*, numerous studies from our lab and others have clearly demonstrated that GRAIL is necessary for the induction and maintenance of T cell anergy. Earlier studies showed that GRAIL expression correlated with inhibition of cytokine transcription and CD4 T cell proliferation, both anergy “phenotypes.” [8]. Over-expression of GRAIL in T cells was sufficient for the induction of anergy and suppressor function [8,30,31]. Furthermore, ectopic expression of GRAIL was sufficient to abrogate IL-2 transcription after T cell activation in cell lines and primary CD4 T cells [8,30,31]. In agreement, expression of a dominant negative form of GRAIL in naïve CD4+ T cells generated by retroviral transduction of hematopoietic progenitor cells, revealed a block in the development of anergy in an *in vivo* tolerance model, thus demonstrating a necessary role for GRAIL in CD4+ T cell anergy [32]. Accordingly, introduction of epistatic regulators of GRAIL, Otubain-1 (Otub 1) or the alternatively spliced isoform, otubain 1 alternative reading frame 1 (Otub1ARF-1), into ‘naïve’ CD4+ cells *in vitro* and *in vivo*, corresponds to the anergy phenotype of these cells. Otub 1 is a member of deubiquitinating enzymes (DUBs) with the capability to cleave proteins at the Ub-protein bond using its cysteine protease domain [29]. While the Otub 1 expressing cells de-stabilized GRAIL and were resistant to anergy induction, Otub 1-ARF 1 (a catalytically inactive variant) stabilized GRAIL and the T cells expressing Otub 1-ARF 1 were anergic [30]. Two recent studies demonstrated that genetic disruption of the *grail* gene in mice led to a variety of abnormalities in anergic as well as naïve and helper T cells. T cells from *grail*-/- mice are defective in anergy induction *in vitro* and *in vivo* [20,22]. In particular, $grail^{-/-}$ $CD4^{+}$ T cells hyperproliferate [20,33] and produced more cytokines [22] compared to WT cells in response to TCR stimulation alone *in vitro* or with concomitant anti-CD28 costimulation.

Moreover, *in vitro* differentiated CD4 T cells from *grail*$^{-/-}$ mice compared to WT littermates showed significant hypersecretion of IFN-γ in Th1 cells [20,22], lowered IL-4 in Th2 cells [22], and elevated IL-17 and IL-22 in Th17 cells. Consistent with defective anergy *in vitro*, oral tolerance was abolished *in vivo* in *grail*$^{-/-}$ mice using different antigen models. More profound autoimmune symptoms were revealed in aged *grail*$^{-/-}$ mice compared to WT littermates including enlarged spleens and mesenteric lymph nodes, massive infiltration of inflammatory cells in multiple organs, and enhanced susceptibility and severity to experimental autoimmune encephalitis (EAE) [22]. Furthermore, in the EAE model, CD4+ T cell infiltrates from splenocytes and CNS of old *grail*$^{-/-}$ mice produced significantly higher levels of IFN-γ and IL-17 when compared to age-matched littermates [33]. Taken together, results from these studies clearly demonstrate that GRAIL is an important gatekeeper for CD4+ T cell anergy. Its role in other T cell functions will be discussed further below.

GRAIL in regulatory T cells (Tregs)

Since the thymically derived Foxp3+CD25+ regulatory T cells as well as adaptive T regulatory cells are special subsets of anergic T cells, we asked whether GRAIL was expressed in Tregs and whether their functions are associated with GRAIL expression. Indeed, GRAIL mRNA expression is increased 10-fold in naturally occurring (thymically derived) CD4(+) CD25(+) T regulatory cells compared to naive CD25(-) T cells [31,34]. Further investigation revealed that CD25(+) Foxp3(+) antigen-specific regulatory T cells were induced after a “tolerizing-administration” of antigen and that GRAIL expression correlated with the CD25(+) Foxp3(+) antigen-specific subset [31]. Using retroviral transduction, forced expression of GRAIL in a T cell line was sufficient for conversion of these cells to a regulatory phenotype even in the absence of detectable Foxp3 [31]. In a well-characterized, Staphylococcal enterotoxin B (SEB)-mediated model of T cell unresponsiveness *in vivo*, GRAIL was shown to be up-regulated in the SEB-exposed CD25(+) and CD25(-)FoxP3(+)Vbeta8(+)CD4(+) T cells and FoxP3(-)CD25(-) Vbeta8(+)CD4(+) T cells [35]. Interestingly, a recent study demonstrated that suppressive and non-proliferative functions of the SEB-expressing FoxP3(+)GRAIL(+) T cells were independent of CD25 expression and glucocorticoid-induced tumour necrosis factor R-related protein. This model system reveals a novel paradigm for chronic non-canonical T cell receptor engagement leading to development of highly suppressive FoxP3(+)GRAIL(+)CD4(+) T cells. While GRAIL is not required for Treg development, it is required for their suppressive function as *grail*$^{-/-}$ Tregs exhibited reduced suppressive activity on the proliferation of naïve responder cells when compared to WT Tregs [20,22]. Interesting, a specific subset of Tregs (CD4+CD62LhighCD25+) do not seem to require GRAIL for suppressive function even though GRAIL mRNA is highly expressed in these cells [20]. On the other hand, Nurieva *et al* demonstrated that *grail*$^{-/-}$ CD4+CD25+ Tregs were not as effective at suppressing WT CD4 T cells compared to WT Tregs [22]. Taken together, these data demonstrate that GRAIL is differentially expressed in naturally occurring and peripherally induced T regulatory cells and that the expression of GRAIL is linked to their functional regulatory activity.

Regulation of GRAIL expression

GRAIL Transcriptional, Translational and Post-translational regulation

In T lymphocytes, GRAIL RNA message and protein expression are both tightly regulated. Originally, GRAIL was found to be highly up-regulated following anergy induction via antigen stimulation in the absence of appropriate costimulation, using ionomycin activation *in vitro*, following peptide stimulation *in vitro* or administration in a tolerizing fashion *in vivo* [8,32,33]. Consistent with the observation that calcium signaling was required for the anergy induction program [4], the activation of NFAT1 homodimers was responsible for

turning on the expression of GRAIL mRNA [36]. Since the transcription factors early growth response 2(Egr2) and 3 (Egr3), known target genes of NFAT, are involved in the induction of the anergy program [37], we were intrigued with the idea that Egr2 and Egr3 (reported 'anergy factors') could regulate GRAIL. Preliminary analysis of the GRAIL 5′ promoter region suggests the presence of Egr binding sites (Su et al, unpublished data), but further investigations are needed to understand and delineate the mechanism(s) that regulate the transcription of GRAIL.

In our search of GRAIL interacting proteins, we have revealed an intricate regulatory network of ubiquitination and deubiquination events that are responsible for controlling the expression of GRAIL protein in anergic T cells (see Figure 2, [28,30] (see table 1)). Specifically, yeast-two hybrid assays identified a GRAIL binding partner, Otubain-1 (Otub1) that mediates the degradation of GRAIL [30]. Subsequently, BacterioMatch genetic interaction assays identified additional control elements including the DUB USP8 [30]. GRAIL was found to exist as a trimolecular complex in cells consisting of GRAIL, Otub1, and USP8(mUBPy); the latter two are deubiquitinating enzymes [DUBs] (see Figure 2) [29,38]. Like most ubiquitin-protein ligases, GRAIL is regulated by auto-ubiquitination linked through Lys48 of ubiquitin, thus yielding to degradation by the proteasome 26S. Therefore, autoubiquitinated GRAIL must be deubiquitinated to be stabilized to maintain CD4 T cell unresponsiveness. While Otub1 is a de-ubiquitinating enzyme or DUB, which binds to autoubiquitinated GRAIL, it does not de-ubiquitinate autoubiquitinated GRAIL [30]. Instead, Otub1 serves an important editing function of GRAIL by mediating the degradation of autoubiquitinated GRAIL through interactions with the DUB, USP8 that prevents GRAIL deubiquitination [30]. Indeed, USP8 functions as a chaperone DUB for auto-ubiquitinated GRAIL, removing the ubiquitin attached to GRAIL but leaving untouched the ubiquitinated target of GRAIL. The DUB function of USP8 is inactivated by Otub1 [30]. Compared to steady-state GRAIL, a dramatic reduction in autoubiquitinated GRAIL was observed in the presence of USP8. An alternative reading frame of Otub1 lacking DUB activity, Otub1ARF-1, can interact with GRAIL and stabilize cellular GRAIL protein levels by stoichiometrically blocking canonical Otub1 binding, thus allowing USP8 to deubiquitinate autoubiquitinated GRAIL. Lastly, Otub1ARF-1, in contrast to Otub1, appears to be expressed only in hematopoietic tissues, suggesting its role is limited to those tissues. Together, these initial studies demonstrate a complex regulation of GRAIL cellular protein levels via the opposing epistatic regulators, Otub1 and its alternative reading frame, Otub1ARF-1, and their differential effects on USP8 activity.

Our recent studies add further complexity to GRAIL-USP8 reciprocal regulation. We showed that the stabilization effect of USP8 on GRAIL was directly dependent on USP8 DUB activity, as GRAIL was completely degraded in the presence of an enzymatically inactive mutant, C748S USP8 (Su et al, unpublished observations). Furthermore, the presence of wild-type GRAIL along with USP8 increased the amount of ubiquitinated USP8, which was further enhanced when the DUB activity of USP8 was abolished. This increased ubiquitination was dependent on the E3 activity of GRAIL, as no enhanced ubiquitination of USP8 was observed in the presence of the H2N2 ligase defective mutant of GRAIL. These data suggest a reciprocal E3-DUB relationship in which GRAIL can ubiquitinate USP8, and ubiquitinated USP8 can de-ubiquitinate GRAIL. Since Otub1was previously shown to interact with USP8, we asked whether it had any effect on USP8 modulation of GRAIL stability. Interestingly, Otub1 expression completely abolished USP8-mediated stabilization of GRAIL when all 3 proteins were co-expressed. Moreover, the catalytic inactive C748S USP8 mutant made no difference on Otub1-mediated GRAIL stability. Indeed, when the catalytically inactive C748S USP8 mutant was co-expressed with Otub1, a dramatic reduction in USP8 ubiquitination levels was seen, which possibly affects USP8 activity on GRAIL stability. Thus, our current working model is that Otub 1 promotes

GRAIL degradation by de-ubiquitination of ubiquitinated USP8, thereby diminishing USP8 activity (Figure 2).

How then is the regulator of GRAIL, Otub1 controlled? Recent results from our laboratory demonstrate that GRAIL is expressed in resting CD4 T cells, whereas Otub1 is not. Upon CD4 T cell activation, Otub1 protein translation is enhanced and GRAIL is degraded, allowing for proliferation and cytokine production of the CD4 T cells [24]. Specifically, in naïve CD4 T cells, the loss of GRAIL is mechanistically controlled through a pathway involving CD28 costimulation, IL-2 production and IL-2R signaling, and ultimately, mTOR-dependent translation of select mRNA ([24] and unpublished data) (Figure 3). In particular, IL-2R signaling leads to Akt and mTOR activation, Otub1 translation, de-ubiquitination of ubiquitinated USP8, and subsequent degradation of GRAIL that permits T cell proliferation. In the absence of costimulation (CTLA4-Ig), IL-2R blockade (anti-IL-2), or rapamycin treatment, Otub1 is not translated, and GRAIL expression is maintained. Thus, all three small molecule treatments function through the same final common pathway via blockade of mTOR phosphorylation of S6 with resultant block of Otub1 translation, maintenance of ub-USP8 and resultant GRAIL stability and CD4 T cell unresponsiveness. Accordingly, interference of this pathway using CTLA4-Ig, anti-IL-2, or rapamycin prevents Otub1 protein expression, and thus maintains GRAIL expression, which inhibits T cell proliferation [24]. Thus, there is a common mechanism in the maintenance of unresponsiveness: CTLA4-Ig blocks IL-2 production, anti-IL-2 removes IL-2, and rapamycin blocks mTOR activation downstream of IL-2R signaling; they all inhibit Otub1 translation and maintain functional ub-USP8 and stabilize GRAIL.

Molecular basis of GRAIL mediated T cell unresponsiveness

Major progress was made in the past few years in characterizing the molecular basis by which GRAIL regulates functional unresponsiveness in CD4+ T cells. Data from our lab suggest that GRAIL may maintain cells in the unresponsive/anergic state by modulating the expression of a number of costimulatory molecules including CD40L [39], a critical costimulatory molecule required for T cell activation, and a previously unrealized costimulator, CD83 (previously described as a cell surface marker for mature dendritic cells) [40]. GRAIL binds to the extracellular portion of CD40L or CD83 via its protease-associated (PA) domain, and facilitates transfer of ubiquitin molecules from the intracellular GRAIL RING finger to the cytoplasmic portion of CD40L or CD83. CD40L and CD83 degradation is dependent on the PA domain and a functional RING finger. Downregulation of CD40L occurred following ectopic expression of GRAIL in naïve T cells from $CD40^{-/-}$ mice, and expression of GRAIL in bone-marrow chimeric mice was associated with diminished lymphoid follicle formation. Similarly, GRAIL-mediated down-modulation of CD83 proceeds via the ubiquitin-dependent 26S proteosome pathway. Ubiquitin modification of lysine residues K168 and K183, but not K192, in the cytoplasmic domain of CD83 was shown to be necessary for GRAIL-mediated degradation of CD83. Reduced CD83 surface expression levels were seen both on anergized CD4 T cells and following GRAIL expression by retroviral transduction, whereas GRAIL knock-down by RNA interference in CD4 T cells resulted in elevated CD83 levels. Furthermore, CD83 expression on CD4 T cells contributes to T cell activation as a costimulatory molecule. This study supports the novel mechanism of ubiquitination by GRAIL, identifies CD83 as a substrate of GRAIL, and ascribes a role for CD83 in CD4 T cell activation. Taken together, these data provide a model for intrinsic T cell regulation of co-stimulatory molecules and a molecular framework for the initiation of CD4 T cell anergy.

Additionally, the family of Rho guanine dissociation inhibitors (RhoGDI) has been identified as a GRAIL substrate [41] and thus GRAIL, like Cbl-b, can regulate T cell activation via modulation of the actin cytoskeleton. We demonstrated in Jurkat T cells, that

GRAIL polyubiquinated (via non-K48 on ubiquitin) and stabilized RhoGDI; thus, allowing it to inhibit RhoA GTPase activity, resulting in impaired IL-2 production and proliferation. Since signal transduction of Rho family proteins is critical in regulation of actin cytoskeleton reorganization, these data suggest that one mechanism of action for GRAIL's biological activity is mediated by alterations in the actin cytoskeleton. Indeed, recent reports show GRAIL expression resulted in reduced T/APC conjugation efficiency as assessed by flow cytometry [42]. Moreover, the T/APC conjugates revealed altered polarization of polymerized actin and LFA-1 to the T/APC interface that can be restored by knocking down GRAIL expression. These data support the notion that GRAIL is involved in the alteration of actin cytoskeletal rearrangement under anergizing conditions and thus modulates TCR signaling events in anergic T cells. This is consistent with other published work that anergic T cells demonstrate profound impairment in signaling events upon engagement of their T cell receptors [43-45]. In contrast to naïve T cells, TCR signaling in anergic T cells exhibits lowered influx of calcium, diminished Ras activation, defective LAT palmitoylation resulting in impairment of PLC-γ phosphorylation and PI3K recruitment to the TCR, diminished ERK and JNK phosphorylation, and impaired translocation of the transcription factor AP-1 to the nucleus. Interestingly, while GRAIL had little impact on proximal TCR signaling such as calcium flux and Vav phosphorylation, distal signaling events demonstrated significantly decreased JNK phosphorylation [42]. Genetically, naïve *grail*$^{-/-}$ T cells show no significant differences of total and phosphorylated levels of ZAP70, phospholipase Cγ1, and MAP kinases p38 and JNK, but elevated baseline levels of MAP kinase ERK1/2 [20,22]. Nurieva *et al* suggested recently that GRAIL targets endocytosed TCR-CD3 complex via ubiquitination and proteosome-mediated degradation [22]. Thus, unlike Cbl-b that plays a critical role in modulating proximal TCR signal transducers including PKCθ, PI3K, PLCγ [46,47], GRAIL appears to affect distal TCR signaling protein expression and functions. Clearly, more detailed analysis of GRAIL-mediated TCR signaling events is still needed.

Other targets of GRAIL thus far identified include tetraspanins CD151 and CD81 [28] (see Table 1). While the functional relevance of these molecular interactions is currently under investigation, the cytosolic NH2 terminal domain of all tetraspanins tested is the target of GRAIL mediated ubiquitination. These preliminary data supported the possibility that this function allows ubiquitination of other transmembrane proteins with short cytosolic tails. It is also highly possible that, like RhoGDI, tetraspanins are involved in the regulation of actin cytoskeleton reorganization during TCR signaling. This is supported by a report which showed that CD81 redistributed to the central zone of the immunological synapse (IS) on the T cell [48] and interestingly, CD81 is also shown to be redistributed in toward the contact area on the APC. In addition, CD81 interfaces between the plasma membrane and the actin cytoskeleton by activating Syk leading to the phosphorylation and mobilization of ezrin, and thus, recruiting F-actin to facilitate cytoskeletal reorganization [49]. Similarly, CD151 function has also been linked to cytoskeletal reorganization [50-52]. Consistent with how GRAIL modulates the T/APC interactions and TCR signals as discussed above, it is tempting to propose that GRAIL does this by downregulating the expression of tetraspanins and thus, limits the reorganization of the IS and TCR signaling in anergic T cells.

GRAIL may control the cell cycle

We recently showed that GRAIL may maintain CD4 T cell unresponsiveness by blocking entry into the cell cycle. Specifically, we have shown that GRAIL holds “all” CD4 T cells (SP thymocytes, naïve, memory, and Tregs) in cell cycle arrest at the G1-S inter-phase [24]. As discussed above, activation of mTOR via IL-2R signaling allows selective mRNA translation, including the epistatic regulator of GRAIL, Otub1, whose expression results in the degradation of GRAIL and allows T cell proliferation. Indeed, blocking the mTOR

pathway via CTLA-4 Ig, anti-IL-2 or rapamycin results in blockade of Otub1 expression, maintenance of GRAIL stability, and inhibition of CD4 T cell proliferation. These observations provide a mechanistic pathway sequentially linking CD28 costimulation, IL-2R signaling, and mTOR activation as important requirements for naive CD4 T cell proliferation through the regulation of Otub1 and GRAIL expression. Our findings also extend the role of GRAIL beyond anergy induction and maintenance, suggesting that endogenous GRAIL regulates entry into cell cycle and proliferation of primary naive CD4 T cells. Consistent with this proposal is the demonstration that naïve CD4+ *grail*$^{-/-}$ T cells are hyperproliferative to TCR stimulation *in vitro* and *in vivo*. Clearly, the expression of GRAIL in T cells significantly alters proliferative capacity, likely by holding the cells in the G_1/S transitional phase, as our earlier studies suggest. Our laboratory is currently conducting various screens to search for GRAIL interacting proteins, with focus on candidate substrates that mediate cell cycle progression in order to provide a mechanistic link between GRAIL function and T cell unresponsiveness.

Role of GRAIL in controlling T cell activation and proliferation in primary T cells

While the role for GRAIL in regulating CD4 T cell proliferation has been demonstrated in clones and in transgenic expression systems, the expression, regulation, and function of endogenous GRAIL or Otub1 in naive CD4 T cell activation is only at its infancy. In a recent study, we asked how the expression of GRAIL and Otub1 was regulated during mouse and human naive CD4 T cell activation. We demonstrated that Otub1 was expressed and GRAIL was degraded when naive CD4 T cells were productively activated to undergo proliferation [24]. Our studies revealed that the loss of GRAIL was mechanistically controlled through a pathway involving CD28 costimulation, IL-2 production and IL-2R signaling, and ultimately, mTOR-dependent translation of select mRNAs. Blocking mTOR by using CTLA4-Ig, anti-IL-2, or rapamycin prevented Otub1 protein expression and maintained GRAIL expression that inhibits T cell proliferation. This study was the first demonstration that endogenous GRAIL protein regulation in primary human and mouse naive CD4 T cells plays an important role in controlling T cell activation and proliferation. A recent study showed that Notch signaling via Jagged-1 during TCR activation in primary human T cells upregulates GRAIL mRNA and induces a novel form of T cell hyporesponsiveness that differs from anergy [53]. While this interesting form of hyporesponsiveness is not anergy, this study in primary human T cells suggested that expression of GRAIL mRNA was associated with hypoproliferation and T cell activation, and not necessarily just anergy. In mice, GRAIL expression can be traced to Qa-2$^+$ CD4 single-positive thymocytes poised for export to the periphery [24]; thus, GRAIL expression may be an important component of peripheral tolerance in naive CD4 T cells, in addition to its role in CD4 T cell anergy. Qa-2$^+$ CD4 single-positive thymocytes, but not earlier stage thymocytes, respond to TCR ligation in a manner similar to peripheral CD4 T cells [54]. The observations of GRAIL expression in Qa-2$^+$ CD4 single-positive thymocytes and expression in peripheral naive CD4 T cells suggest a possible role for GRAIL in CD4 T cell tolerance to TCR self-peptide/MHC encountered during the transition from the thymus to the peripheral environment. For the naïve CD4 T cell, TCR engagement of self selecting-peptide/MHC needs to remain a nonresponsive event, and yet TCR engagement is necessary for maintaining their survival and keeping them poised for potential activation by non-self. When foreign Ag is presented as non-self-peptide in the context of MHC class II, the increased affinity/avidity of the TCR engagement, as well as the presence of danger-induced APC costimulatory signals following B7-CD28 ligation, breaks the GRAIL-maintained quiescent state of the naive CD4 T. Subsequently, IL-2 signals through the IL-2R on CD4 T cells via mTOR to ensure GRAIL degradation to allow proliferation. Interestingly, *grail*$^{-/-}$ mice do not display abnormalities in thymic T cell development; however, their naïve peripheral CD4+ T cells are hyperproliferative upon TCR stimulation *in vitro* and *in vivo*

[20,22]. Thus, maintenance of GRAIL serves to preserve quiescence of naive CD4 T cells and its down-regulation is required to allow activation and proliferation.

Since GRAIL may be a key factor for maintenance of cellular quiescent, it is tempting to hypothesize its involvement in genetic imprinting and mechanisms of epigenetic regulation. In fact, it is well documented that the *il-2* locus is methylated in anergic cells (and Tregs) [55-57]. It is entirely possible that an E3 may regulate chromatin structure or histone deacetylation. How chromatin (nucleosome remodeling), histone deacetylation, DNA hypermethylation all contribute to maintaining T cell quiescence (or 'anergy') is still unclear, but it would not be surprising that these diverse mechanisms are interconnected and that E3s including GRAIL may somehow have a part in this regulatory process.

Association of GRAIL with autoimmune disorders and other functions

The significance of GRAIL's role in disease comes from data associating aberrant expression of GRAIL to a number of autoimmune and infection models. The Non-obese diabetic (NOD) mouse serves as a murine model of human type 1 diabetes that develops increasing incidence of hyperglycemia with age [58]. The disease process is thought to occur initially through autoimmune T cell activation, possibly in the pancreatic lymph nodes, followed by inflammation of the islets of Langerhans (insulitis) that, at ~12 wk of age, leads to islet β-cell destruction and resultant hyperglycemia [59]. In search of genes differentially expressed during disease initiation and progression, we conducted genome-wide analyses of gene expression in pancreatic lymph nodes (PLNs) from NOD and disease-resistant NOD.B10 ($H\text{-}2^b$) congenic mice [60]. At certain ages, including 12 wk, *grail* mRNA was decreased in PLNs of NOD mice compared with NOD.B10 mice. This differential *grail* expression was verified by quantitative PCR of pancreatic lymph node RNA samples from multiple 12-wk-old NOD and NOD.B10 mice [24]. Our findings suggest a potential peripheral tolerance role for GRAIL on naive CD4 T cells *in vivo*, which might be lost during NOD disease pathogenesis. Consistent with this hypothesis, oral tolerance is abolished *in vivo* using 2 different models: in OT-II TCR transgenic $grail^{-/-}$ mice fed with ovalbumin and in experimental allergic encephalitis, a model of organ-specific autoimmunity, oral tolerization with myelin basic protein [20,22]. Moreover, Nurieva et al recently reported that $grail^{-/-}$ mice are more prone to develop autoimmune symptoms compared to wild-type mice and exhibit exacerbated EAE [22]. In a study of primate HIV infection, GRAIL was up-regulated in anergic CD4 T cells isolated from disease-susceptible SIV-infected rhesus macaques, whereas SIV-resistant sooty mangabey primates showed no increase in GRAIL [61]. Hyporesponsiveness of Th2 cells in the late phase of *Schistosoma mansoni* infection in mice or chronic antigen restimulation of Th2 cells in vitro correlated with elevated GRAIL mRNA expression and the knock down of GRAIL via siRNA blocked repeated antigen induced hyporesponsiveness [62]. A role for GRAIL in human disease was recently demonstrated in patients successfully treated for ulcerative colitis: patients in remission expressed higher levels of GRAIL in CD4 T cells versus patients with ongoing disease or normal controls [23]. All these findings suggest that regulation of GRAIL plays an important role in peripheral tolerance and its dysregulation contributes to human immune disorders.

Two recent studies implicate GRAIL's role in other functions besides anergy/tolerance regulation. The first study investigated the role of GRAIL in non-lymphoid development [63]; specifically, the role of GRAIL during hematopoiesis since GRAIL was known to be expressed in the bone marrow [8]. Their data demonstrated that GRAIL was expressed during hematopoietic development in the bone marrow and appeared to be differentially regulated at the common myeloid progenitor (CMP) developmental branch point. In the second study, the potential function of GRAIL in nutrient metabolism was investigated by generating mice in which the expression of GRAIL was reduced specifically in the liver

[64], another tissue where GRAIL is abundantly expressed [8]. Adenovirus-mediated transfer of a short hairpin RNA specific for GRAIL mRNA markedly reduced the amounts of GRAIL mRNA and protein in the liver. The results of this study demonstrated that GRAIL in the liver is essential for maintenance of normal glucose and lipid metabolism in living animals [64]. These studies, together with our data on GRAIL's role in regulating cell cycle, suggest broader functions of GRAIL besides regulation of the immune system.

Conclusions

Since the cloning of GRAIL several years ago, we have seen important advances in our understanding of its molecular basis in the induction and maintenance of CD4 T cell anergy or functional unresponsiveness. The study of GRAIL especially highlights ubiquitination and de-ubiquitination mechanisms in the regulation of CD4 T cell anergy and proliferation. GRAIL is associated with the CD4 T cell anergy phenotype *in vitro* and *in vivo*, and its expression in CD4 naïve T cells creates an anergy phenotype. This function of GRAIL is tightly regulated by Otub1 and its differentially spliced isoform, Otub-ARF 1, which stabilizes or destabilizes GRAIL, respectively, by either allowing or preventing auto-ubiquitination and proteasomal degradation of GRAIL protein. To date, GRAIL substrates include the family of tetraspanins, RhoGDI proteins, CD83, and CD40L, suggesting the modulation of actin-cytoskeleton and expression of costimulatory molecules and other cell surface receptors might be critical for the anergy. Our work on primary mouse and human naïve CD4 T cells revealed that the loss of GRAIL is mechanistically controlled through a pathway involving CD28 costimulation, IL-2 production and IL-2R signaling, and ultimately, mTOR-dependent translation of select mRNAs including Otub1. Blocking mTOR prevents Otub1 protein expression and maintains GRAIL expression, which inhibits T cell proliferation. These data suggest that endogenous GRAIL protein regulation in primary human and mouse naive CD4 T cells plays an important role in controlling T cell activation and proliferation. The essential contribution of GRAIL to tolerance induction and maintenance is demonstrated in *grail*$^{-/-}$ mice and more significantly, GRAIL is linked to a number of immune dyregulations including autoimmunity (T1D, EAE, IBD) and SIV infection. One mechanism whereby GRAIL maintains CD4 T cell unresponsiveness may be through holding cells in cell cycle arrest. Additionally, GRAIL may play a broader role, as demonstrated in HSC and glucose and lipid metabolism models as well as other forms of T cell unresponsiveness such as demonstrated in Jagged-1 mediated Notch signaling during TCR activation in human T cells. These discoveries hint at the exciting possibility that GRAIL may be an attractive therapeutic target for a number of different autoimmune and infectious disease models, and may be involved in proliferative disorders such as cancer.

While these advances have provided a better understanding of GRAIL biology, further work is clearly needed to fully unravel the complex regulation of GRAIL function and to understand how GRAIL mediates the unresponsive phenotype in T cells and how it functions in other non-immune models. For example, more work is required to characterize the distribution of the varied isoforms of Otub1 in CD4+ T cell subsets and activation conditions that lead to alterations in the balance between otub1 and Otub1 ARF-1. Additionally, the mechanism by which Otub1 regulates GRAIL expression, in particular identifying the substrate of its DUB activity still needs to be investigated. Other important questions include how Otub1 uses its cysteine protease activity to regulate GRAIL. Clearly there are other substrates of GRAIL required to help mediate the establishment and maintenance of anergy. What are these? In addition to the translational and post-translational regulation of GRAIL protein expression, what are the transcriptional regulators of GRAIL besides NFAT? Current studies in our laboratory include analyzing the molecular pathway(s) in CD4 T cells expressing GRAIL, and in particular, how GRAIL may be modulating the TCR signaling pathway in anergic T cells. The potential function of GRAIL

in CD4 Tregs is an exciting area to pursue due to the important role of Tregs in immune modulation. Since GRAIL is widely expressed in non-lymphoid tissues, what's the role of GRAIL in these tissues? In light of GRAIL's possible function in the regulation of the cell cycle, what are other disease models where GRAIL may play a role (i.e. cancer)? Many of these questions are currently under investigation in our laboratory and others. We anticipate exciting discoveries about this remarkable E3 in the near future, and hope that this information will enable us to manipulate the GRAIL pathway for the treatment of various immune-and non-immune related disorders.

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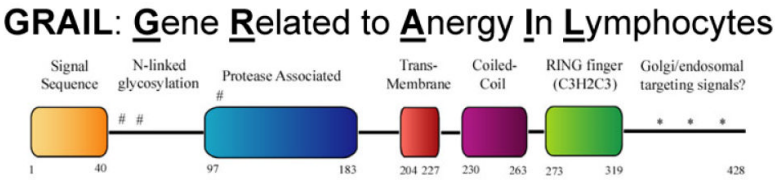

Figure 1.

Schematic representation of the structural domains of GRAIL (**G**ene **R**elated to **A**nergy **I**n **L**ymphocytes). GRAIL is a 428 amino acids type I transmembrane single subunit ubiquitin E3 ligase protein with a cytosolic zinc-binding RING finger domain and a luminal or extracellular protease-associated (PA) domain. The RING finger domain is C2H2C3 type and functions as an ubiquitin E3 ligase, the PA domain captures transmembrane protein targets for ubiquitination. This split function motif is unique for a single protein E3 ligase. Unlike other E3 ligases, GRAIL is uniquely localized to the transferrin-recycling endocytic pathway.

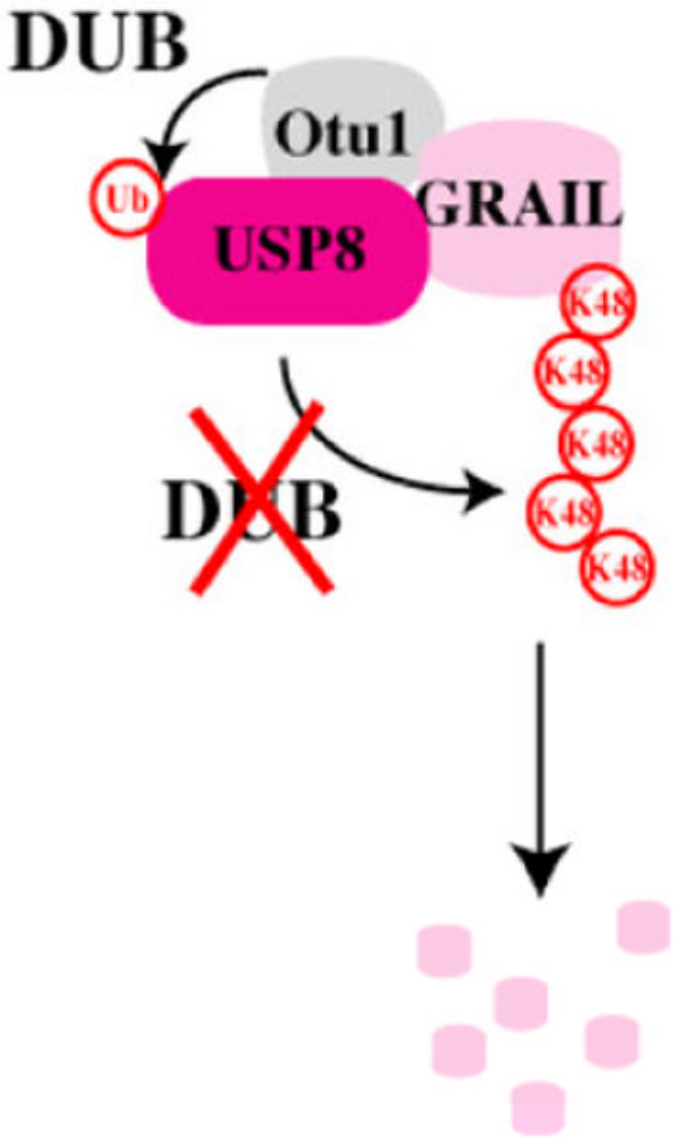

Figure 2.

Molecular basis of GRAIL regulation. GRAIL is associated with and regulated by two isoforms of the ubiquitin-specific protease otubain 1 (Otub1). Otub1, a deubiquitinating enzyme or DUB, binds to ub-GRAIL but does not deubiquitinate it. USP8, is a DUB that binds to GRAIL and to Otub1 in a tri-molecular complex. USP8 can function as a DUB for auto-ubiquitinated GRAIL, however USP8's DUB function is blocked by Otub1, but not catalytic mutants of Otub1 or it's alternatively spliced isoform, Otub1-ARF1. USP8 must be ubiquitinated to function as a DUB for auto-ubiquitinated GRAIL. Otub1, (but not catalytic mutants), de-ubiquitinates ubiquitinated USP8, inactivating it and allowing auto-ubiquitinated. GRAIL to be degraded by the 26S proteosome.

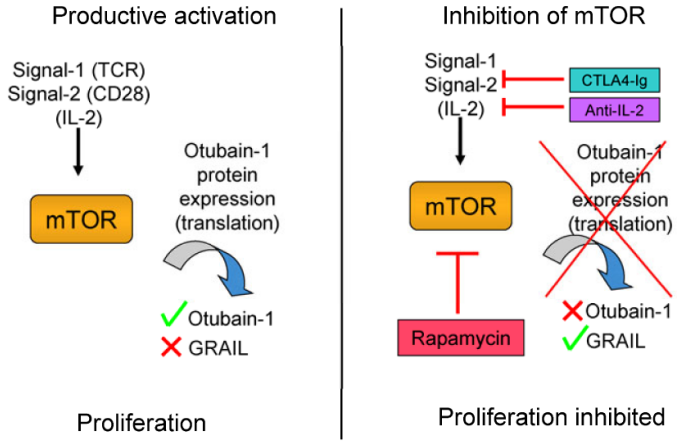

Figure 3.

GRAIL and Otub1 regulation by the mTOR pathway controls naïve CD4 T cell proliferation. (left panel) Productive activation of naive CD4 T cells leading to proliferation comes about through TCR engagement (signal 1) and CD28 costimulation (signal 2); IL-2 production, signaling through the IL-2R leading to phosphorylation of Akt and activation of mTOR, expression of Otub1 protein, and subsequent GRAIL degradation, allowing proliferation to occur. (Right panel) Three independent mechanisms that block mTOR activation result in inhibition of naïve T cell proliferation. CTLA4-Ig blocks CD28 costimulation, does not allow IL-2 production, thus prevents Akt phosphorylation, mTOR is inactive, and Otub1 protein is absent, leading to the maintenance of GRAIL, inhibiting proliferation. Anti-IL-2 blocks IL-2R engagement, thus preventing Akt phosphorylation, mTOR is inactive, and Otub1 protein is absent, leading to the maintenance of GRAIL, inhibiting proliferation. Rapamycin blocks the activity of mTOR, prevents protein expression of Otub1, leading to the maintenance of GRAIL, inhibiting proliferation.

Table 1

GRAIL-interacting proteins

GRAIL Substrate	Reference
Otubain-1	Soares *et al. Nature Immunol (2004) 1:45-54*
Otub-1 ARF	Soares *et al. Nature Immunol (2004) 1:45-54*
USP8	Soares *et al. Nature Immunol (2004) 1:45-54*
RhoGDI	Su *et al J. Immunol 2006; 177:7559-7566*
CD83	Su *et al. J Immunol. 2009 183 (1): 438-44*
CD81	Lineberry *et al. JBC (2008) vol 283: 28497-28505*
CD151	Lineberry *et al. JBC (2008) vol 283: 28497-28505*
CD40L	Lineberry *et al. J Immunol 2008, 181:1622-1626*