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The Gene Related to Anergy in Lymphocytes, an E3 Ubiquitin Ligase, Is Necessary for Anergy Induction in CD4 T Cells[1]

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Acquisition of the anergy phenotype in T cells is blocked by inhibitors of protein synthesis and calcineurin activity, suggesting that anergic T cells may have a unique genetic program. Retroviral transduction of hemopoietic stem cells from TCR transgenic mice and subsequent reconstitution of syngeneic mice to express the E3 ubiquitin ligase, gene related to anergy in lymphocytes (*GRAIL*), or an enzymatically inactive form, H2N2 *GRAIL*, allowed analysis of the role of *GRAIL* in T cell anergy in vivo. Constitutive expression of *GRAIL* was sufficient to render naive CD4 T cells anergic, however, when the enzymatically inactive form H2N2 *GRAIL* was expressed, it functioned as a dominant negative of endogenous *GRAIL* and blocked the development of anergy. These data provide direct evidence that a biochemical pathway composed of *GRAIL* and/or *GRAIL*-interacting proteins is important in the development of the CD4 T cell anergic phenotype in vivo. ***The Journal of Immunology*****, 2004, 173: 79–85.**

Peripheral CD4 T cell tolerance in vivo was described over 40 years ago (1). It was not until systems were devised that rendered T cells unresponsive to rechallenge with specific Ag in a conventional fashion in vitro that a mechanistic understanding of this form of tolerance began to unfold. The working paradigm put forth was that CD4 T cells require two signals to become fully activated. If a CD4 T cell received a signal through the TCR (signal one) in the absence of a costimulatory signal (signal two), the cell was rendered unresponsive to Ag rechallenge, a phenomenon called anergy. By definition, this meant that the cell no longer responded to its cognate Ag (either by proliferation or IL-2 production) when presented in a conventional fashion (signal one and signal two) in Ag recall challenge. Further studies using in vitro anergy systems demonstrated that acquisition of the anergy phenotype was an active process, because blocking protein synthesis and disrupting calcineurin activity inhibited development of the anergy phenotype (2). Based on these observations, it has been hypothesized that there might be a unique genetic program that defined the anergy phenotype at the molecular level. To date, several genes have been described as putative "anergy factors", e.g., *tob1* (3), *lymphotactin* (4), *cbl-b* (5), and $p27^{kip1}$ (6, 7). At present, a role for these anergy-associated genes is only indirectly implicated in the development of anergy.

Animal models of anergized CD4 T cells in vivo have been developed that use paradigms similar to those described by systems studied in vitro. CD4 T cells have been rendered unresponsive in vivo following i.v. administration of soluble peptide or superantigen (presumed to mimic signal one without signal two) (8, 9). It is not clear whether any of these animal model systems lead to a phenotype and genotype of anergy that mimics the systems described in vitro. Previous work from our laboratory demonstrated that the E3 ubiquitin ligase, gene related to anergy in lymphocytes (*GRAIL*),[5] is up-regulated in T cell clones rendered anergic in vitro (10). Additionally, we demonstrated that *GRAIL*, but not the E3 ubiquitin ligase inactive form, H2N2 *GRAIL*, where the two histidines in the ring finger motif are replaced by asparagine, inhibited IL-2 transcription in T cell hybridomas (10). In this current study, we expand on that observation by retrovirally transducing hemopoietic stem cells (HSC) to study the role of *GRAIL* and H2N2 *GRAIL* (which acts as a dominant negative) in naive T cells of lethally irradiated and transgenic bone-marrow-reconstituted mice. These studies provide the first direct evidence of the requirement for a specific anergy-related protein in the development of the anergy phenotype in CD4 T cells.

Materials and Methods

Mice

BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, ME). TCR transgenic DO11.10 mice were provided by Dr. D. Lewis (Stanford, CA; Ref. 11). All mice were kept under specific pathogen-free conditions in the Department of Comparative Medicine at Stanford University School of Medicine (Stanford, CA).

Injections of mice

For staphylococcal enterotoxin B (SEB) experiments, BALB/c mice received i.v. injections of 50 μg of purified SEB (Toxin Technology, Sarasota, FL) or endotoxin-free buffered saline (PBS). For OVA soluble peptide tolerizing experiments, gender- and age-matched BALB/c mice were injected i.v. with 1.5–5 $\times$ 10^6 DO11.10 $CD4^+$ cells pooled from lymph node and spleen from reconstituted bone marrow chimeric mice (see below) or unmanipulated DO11.10 mice. The day following cell transfer, mice were

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[5] Abbreviations used in this paper: *GRAIL*, gene related to anergy in lymphocytes; HSC, hemopoietic stem cells; IRES, internal ribosomal entry site; PVDF, polyvinylidene difluoride; QPCR, quantitative PCR; SEB, staphylococcal enterotoxin B; pep, peptide only.

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immunized. Tolerizing immunization consisted of an i.v. injection of 300 μg of $OVA_{323–339}$ peptide (Anaspec, San Jose, CA) in endotoxin-free buffered saline. Priming immunization consisted of an i.v. injection of 300 μg of OVA containing 25 μg of LPS (*Escherichia coli* 026:B6; Sigma-Aldrich, St. Louis, MO).

Bone marrow chimeras

Retroviral transduction of bone marrow cells was achieved as previously described (12). One day after transduction, cells were sorted for GFP expression and i.v. injected into BALB/c mice after receiving 800 rad of whole-body irradiation. Each animal received 3×10^5 sorted cells i.v.

FACS analysis and sorting

The following Abs were used for FACS analysis: Fc block (2.4G2; BD Pharmingen, San Jose, CA), CD4 (RM4-5; BD Pharmingen, and eBioscience, San Diego, CA), CD8 (53-6.7; eBioscience), anti-clonotypic Ab for DO11.10 (KJ1.26; Caltag Laboratories, Burlingame, CA), Sca-1 (D7; eBioscience), c-kit (2B8; eBioscience), and biotinylated lineage mixture (clones 145-2C11, RA3-6B2, RB6-8C5, 53-6.7, GK1.5, M1/70; all eBioscience). For analysis, LSR (BD Pharmingen) or Moflops (DakoCytomation, Fort Collins, CO) was used for flow cytometry. For sorting, cells were sorted using a modified FACStar flow cytometer (BD Pharmingen; modified at Stanford FACS Facility, Stanford, CA). For SEB experiments, CD4$^+$Vβ8$^+$ cells were purified using magnetic microbeads (Miltenyi Biotec, Auburn, CA). CD8 cells were initially eliminated using anti-CD8 microbeads; the negative population was stained with PE-conjugated anti-Vβ8 (MR5-2; BD Pharmingen). The stained cells were washed and incubated with anti-PE microbeads according to manufacturer's recommendations (Miltenyi Biotec). Alternatively, CD4$^+$ cells were positively selected with magnetic microbeads, then stained with anti-Vβ8 for flow cytometry sorting. Purity was checked by flow cytometry and was routinely >95%. All FACS data analysis was done using FlowJo software (Tree Star, San Carlos, CA).

In vitro proliferation assay

Single-cell suspensions were made from lymph node and spleen, and cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin plus 100 μg/ml streptomycin, 2 mM L-glutamine (all Life Technologies, Gaithersburg, MD), and 50 μM 2-ME (Sigma-Aldrich). Cells were cultured in 96-well flat-bottom plates with sorted CD4$^+$ T cells or pooled lymph node and spleen cell from recipients with irradiated BALB/c splenocytes at a concentration of 5×10^5/well. The number of KJ1.26$^+$ T cells for each group of recipients was determined by FACS analysis and proliferation was normalized for KJ1.26 input as previously described (6). Purified OVA or SEB was added at the stated concentrations. Cells were pulsed at 72 h for 12–16 h with 1 μCi of [^{3}H]thymidine. For assay of IL-2 production, supernatant was removed from cultures at 48 h and assayed immediately or stored at −20°C before assay.

IL-2 ELISA

Assay for IL-2 protein was performed as previously described (13). The lower limit of detection for the assay was 20 pg/ml.

Real-time quantitative PCR (QPCR)

Total RNA was extracted from cell pellets using Qiagen RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed using random hexamer primers and TaqMan reverse transcription reagent kit following the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Real-time QPCR for *GRAIL* and *rRNA* as the normalizing gene was performed as previously described using the ABI Prism 5700 Sequence Detection System (Applied Biosystems) (10). Probes and primer sets specific for *rRNA* and target mRNAs were purchased from Applied Biosystems. *GRAIL* primers were designed to span intron-exon regions with a 76-bp amplicon size. The following primers were used: *GRAIL* forward primer, 5′-AAAGCAGGAAGCAG AGGCAG; *GRAIL* reverse primer, 5′-TGTTTCAAGGTGCGCAGCT; and TaqMan hybridization probe, FAM-5′-AAAGGCAGAT GCTAAAAAAGC TATTGGAAAGCTT–TAMRA. Commercially available *rRNA* primer and probe was used (Applied Biosystems). Murine liver cDNA was used to generate the standard curve (murine *GRAIL* is highly expressed in liver tissue). All samples were analyzed in triplicate. *GRAIL* arbitrary units are expressed as the mean of triplicate normalized values ± SD as previously described (13).

Immunoblot analysis

Cells were washed with cold PBS and lysed in buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, and a protease inhibitor mixture (Sigma-Aldrich). Lysates were centrifuged at 12,000 rpm for 20 min and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Cleared lysates were resolved by SDS-PAGE and adsorbed to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Proteins were detected with a *GRAIL*-specific rabbit polyclonal Ab (12) or mouse monoclonal actin-specific Ab (Sigma-Aldrich; clone AC-15). Blots were developed with HRP-conjugated secondary Abs (Zymed Laboratories, San Francisco, CA) and ECL (Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation assays

293 cells were grown in DMEM with 10% FCS + 100 U/ml penicillin + 100 μg/ml streptomycin, 2 mM L-glutamine (all Life Technologies). P3 constructs were used for retroviral production as previously described (10). 293 cells were infected with P3-wild-type *GRAIL*-internal ribosomal entry site (IRES)-GFP virus and sorted by FACS for GFP expression. GFP-positive cells were then cotransfected with a HA-tagged USP8, a FLAG-tagged ubiquitin, or a *GRAIL*-H2N2-V5 construct as indicated (12). Forty-eight hours after transfection, cells lysates were prepared and immunoprecipitated with HA-agarose beads. The immunoprecipitates were washed several times, resolved by SDS-PAGE, transferred to PVDF, and probed with anti-FLAG-HRP Ab (Sigma-Aldrich).

Tissue sections and immunohistochemistry

Spleens were removed and fixed in buffer formalin followed by paraffin embedding. Tissue sections were stained with H&E. For immunohistochemistry, sections were incubated with *GRAIL*-specific rabbit polyclonal Ab, followed by biotinylated anti-rabbit IgG (Zymed Laboratories), and HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA), and developed with DAB substrate (Vector Laboratories). Imaging was done using an inverted Nikon scope equipped with Spot camera and software (Nikon, Melville, NY).

Results

GRAIL *expression is up-regulated in T cells during anergy induction in vivo*

Recently, our laboratory demonstrated that *GRAIL*, a gene encoding an E3 ubiquitin ligase, is up-regulated in T cell anergy induction in vitro following signaling through the TCR alone or with ionomycin (10). To ascertain whether there was an in vivo correlate to our findings with *GRAIL* in models of T cell anergy in vitro, we investigated the expression levels of *GRAIL* in CD4 T cells rendered anergic in vivo. The adoptive transfer of a small number of CD4$^+$ TCR transgenic T cells into immunocompetent, nontransgenic, syngeneic mice, followed by a tolerizing immunization (soluble peptide delivered i.v.) has been a model widely used to study anergy in vivo (8)(Fig. 1*A*). Using this model, we looked at the mRNA expression levels of *GRAIL* in the adoptively transferred CD4$^+$ TCR transgene-positive (KJ1.26$^+$) cells and studied their functional phenotype. *GRAIL* mRNA levels were consistently elevated in the sorted CD4$^+$KJ1.26$^+$ cells following peptide tolerizing immunization (Tolerized) when compared with untreated (Naive) or peptide plus LPS-immunized (Primed) mice at various time points after immunization (Fig. 1*B*). The SEB in vivo anergy model was used to study the mRNA and protein levels of *GRAIL* (9). SEB administration results in the initial expansion of reactive CD4$^+$Vβ8$^+$ T cells, followed by a contraction in cell numbers (Ref. 9, and data not shown). The remaining CD4$^+$Vβ8$^+$ T cells are anergic as demonstrated by their inability to respond to SEB restimulation in vitro (Fig. 1*C*). Similar to our results in the DO11.10 in vivo anergy model, *GRAIL* mRNA was up-regulated in CD4$^+$Vβ8$^+$ T cells anergized by SEB administration in vivo (data not shown). Additionally, CD4$^+$Vβ8$^+$ cells from SEB- or PBS-treated animals were sorted and assayed for *GRAIL* protein expression using a *GRAIL*-specific polyclonal Ab. This analysis

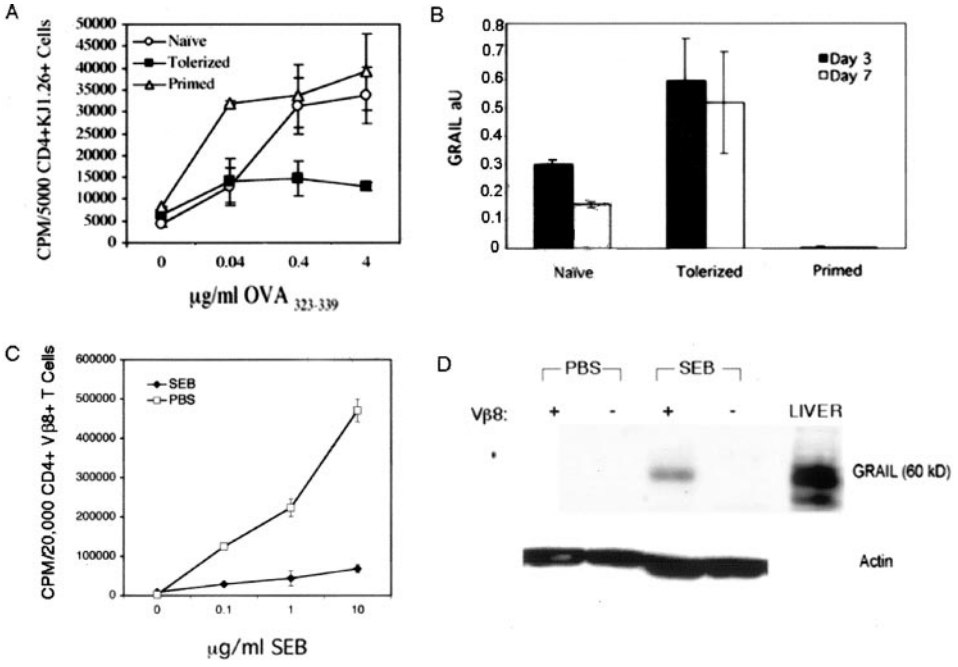

FIGURE 1. *GRAIL* is up-regulated in tolerized CD4 T cells. *A*, A total of 5×10^6 DO11.10 CD4 T cells were transferred i.v. to gender and age-matched BALB/c recipients. The following day mice were either not immunized (○, Naive), or immunized with 300 μg $OVA_{323–339}$ peptide i.v. (■, Tolerized), or immunized with 300 μg $OVA_{323–339}$ plus 25 μg LPS i.v. (△, Primed). Lymph nodes (cervical, axillary, inguinal) and spleen were removed 12 days after immunization and restimulated in vitro with irradiated BALB/c splenocytes and $OVA_{323–339}$ in a dose-response fashion. Each Ag concentration reflects the mean of triplicate samples, and error bars represent SD. One representative experiment of six is shown. *B*, Transferred DO11.10 CD4 T cells were isolated from recipient BALB/c mice at the indicated days after immunization and sorted using the KJ1.26 Ab. Real-time QPCR data are presented as the normalized arbitrary unit (aU) of *GRAIL*, (see *Materials and Methods*), and represent the mean of triplicate samples ± SD. Data are one representative experiment of four independent experiments. *C*, Eight days after infusion of PBS (□) or SEB (♦) to BALB/c mice, lymph nodes and spleen were removed for proliferation assays in vitro. $CD4^+V\beta8^+$ cells were added to each well with 5×10^5 irradiated BALB/c splenocytes and SEB in a dose-response fashion. Each SEB concentration reflects the mean cpm of triplicate samples ± SD. Data are from one representative experiment of four independent experiments. *D*, *GRAIL* immunoblot analysis of $CD4^+V\beta8^+$-selected cells (+) 24 h after treatment of BALB/c mice with PBS or SEB. Lanes labeled "−" represent $CD8^-$ and $CD4^+V\beta8^-$ cells. Endogenous *GRAIL* migrates at 60 kDa as denoted by arrow (positive control is liver extract). Data are one representative of two experiments performed.

demonstrated a direct correlation between *GRAIL* mRNA and protein levels, as *GRAIL* was found to be elevated in the $CD4^+V\beta8^+$ T cells from SEB-treated mice and not in PBS-treated naive $CD4^+V\beta8^+$ T cells (Fig. 1*D*). We conclude from these data that *GRAIL* mRNA and protein expression was up-regulated during the induction of anergy in CD4 T cells in vivo, thus providing a genetic commonality between anergy induction in vitro and in vivo and implicating *GRAIL* as an anergy factor.

Forced expression of GRAIL *in bone marrow results in normal thymic development with diminished peripheral lymphoid tissue reconstitution*

GRAIL is expressed at the protein and mRNA level in multiple tissues (10). However, Northern blot analysis of hemopoietic tissues demonstrated low to no detectable *GRAIL* mRNA in peripheral lymphoid tissue and low levels of mRNA detected in the thymus and bone marrow (C.H., unpublished observations). To further study the role of *GRAIL* in T cell anergy, we examined the effects of constitutive *GRAIL* expression in primary CD4 T cells by transducing bone marrow cells from DO11.10 TCR transgenic mice with a retroviral vector containing wild-type *GRAIL*, or the E3 ubiquitin ligase-inactive form of *GRAIL* (H2N2 *GRAIL*), or the retroviral vector-expressing GFP only, and analyzed cells following bone marrow reconstitution in lethally irradiated BALB/c mice. The retroviral constructs contained GFP downstream of an IRES sequence and the gene of interest; thus expression of the transgene could be followed by GFP expression (14). FACS analysis of the transduced cells before transfer into lethally irradiated donors demonstrated transduction of HSC ($Sca\text{-}1^+$, $c\text{-}kit^+$, biotinylated $lineage^-$) in all groups. GFP^+ short-lived cells (i.e., neutrophils) were found as far out as 150 days after transplant, demonstrating that long-term HSC were transduced (data not shown). No abnormalities were noted in thymic development using DO11.10 bone marrow for reconstitution in any of the experimental groups (Fig. 2, *A–C*). However, the wild-type *GRAIL*-reconstituted mice were found to be lymphopenic compared with H2N2-*GRAIL*- and control-reconstituted mice (mean absolute lymphocyte count in peripheral blood measured serially after engraftment 753 for wild-type *GRAIL* vs 2543 for H2N2 *GRAIL* and 3876 for GFP only; $p < 0.05$, each group $n = 6$). Staining of splenic tissue from reconstituted mice demonstrated poorly developed white pulp areas in wild-type *GRAIL* mice compared with H2N2 *GRAIL* and control mice, particularly in the periarteriolar lymphatic T cell sheath region or T cell area (Fig. 2, *D–I*). Immunohistochemistry on splenic tissue from reconstituted mice confirmed the expression of *GRAIL* protein in the wild-type and H2N2 *GRAIL* mice with no detectable endogenous *GRAIL* in the GFP only control mice (Fig. 2, *J–L*). Because *GRAIL* is a gene involved in conveying an anergic phenotype in CD4 T cells it may not be surprising that peripheral lymphocyte numbers would be diminished. This would suggest that the *GRAIL* substrate important in the anergic phenotype pathway is present in naive T cells or that *GRAIL* may play a separate and distinct role in normal homeostatic proliferation in addition to its role in anergy.

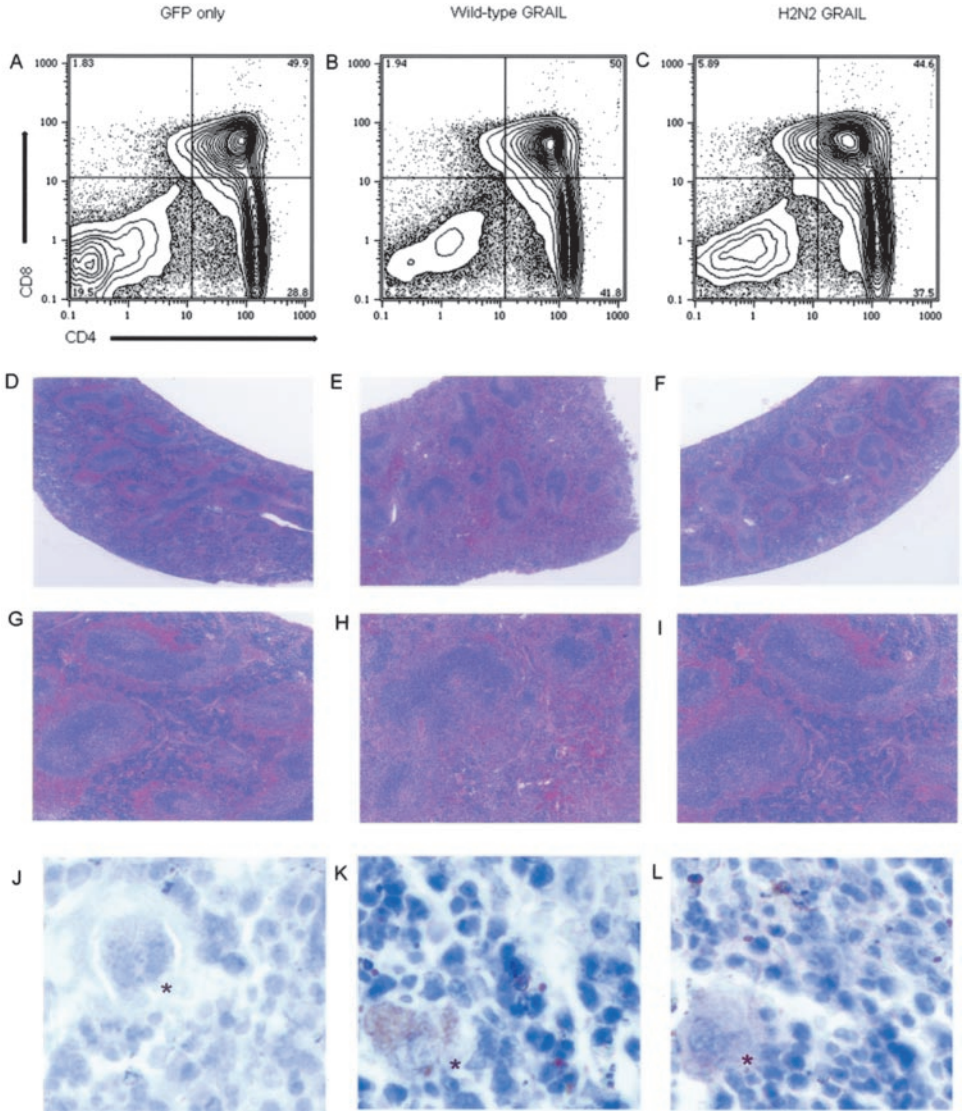

FIGURE 2. Constitutive expression of *GRAIL* during lymphopoiesis results in diminished peripheral lymphoid tissue development with no impact on thymopoiesis. Thymic and peripheral lymphoid tissue was analyzed in reconstituted lethally irradiated BALB/c mice 4–8 wk after transfer of transduced bone marrow cells: GFP only (*A*, *D*, *G*, *J*), wild-type *GRAIL* (*B*, *E*, *H*, *K*), and H2N2 *GRAIL* (*C*, *F*, *I*, *L*). *A–C*, Flow cytometry from thymus, CD4, and CD8 expression on GFP$^+$ gated cells. *D–I*, H&E staining from spleen of reconstituted mice (original magnification, *D–F*, ×10; *G–I*, ×40). *J–L*, Immunohistochemistry in splenic tissue using *GRAIL* polyclonal Ab in reconstituted mice (original magnification, ×60). *GRAIL*-expressing cells are stained brown, with highest level of expression detected in megakaryocytes (*).

Constitutive expression of GRAIL *in naive CD4 T cells is sufficient to convey an anergic phenotype*

Lymph node and spleen DO11.10 CD4 cells from wild-type *GRAIL*-, H2N2 *GRAIL* (H2N2)-, and control (DO11)-reconstituted mice were sorted based on GFP expression and stimulated in vitro with OVA peptide and irradiated splenocytes from BALB/c mice as APCs. Constitutive expression of wild-type *GRAIL* in naive T cells resulted in diminished IL-2 production consistent with our previous work (10). This effect was dependent on intact *GRAIL* E3 ubiquitin ligase activity, because IL-2 production was unaffected in H2N2 *GRAIL* transduced T cells. Additionally, proliferation was diminished in wild-type *GRAIL* transduced T cells, but not in H2N2 *GRAIL* or GFP-expressing T cells (an anergy parameter that could not previously be assayed in the transduced hybridomas) (Fig. 3, *A* and *B*). The addition of exogenous IL-2 or stimulation with PMA/ionomycin did not overcome the block in proliferation in the *GRAIL*-expressing T cells (data not shown). The levels of *GRAIL* mRNA in sorted KJ1.26$^+$CD4$^+$GFP$^+$ cells from reconstituted mice were found to be ~10-fold higher than levels detected in CD4 T cells rendered anergic in vivo (Fig. 3*C*). Importantly, in these experiments, surface TCR expression levels as measured by flow cytometry were found to be comparable between the samples (Fig. 3*C*). These data indicated that constitutive expression of the wild-type *GRAIL* in naive CD4 T cells was sufficient to convey the anergy phenotype.

Expression of the enzymatically inactive form of GRAIL*, H2N2* GRAIL*, blocks the induction of anergy in CD4 T cells in vivo*

We hypothesized that under anergizing conditions in vivo, when endogenous *GRAIL* was up-regulated, forced expression of H2N2 *GRAIL* in T cells might abrogate the development of an anergy phenotype if *GRAIL* was central to this process. In these studies, 1.5–2 × 10^6 CD4$^+$KJ1.26$^+$ transduced cells from reconstituted mice or unmanipulated DO11.10 mice were transferred to BALB/c mice. The following day, a subset of the mice was immunized. Eight days following immunization, lymph node and spleen cells

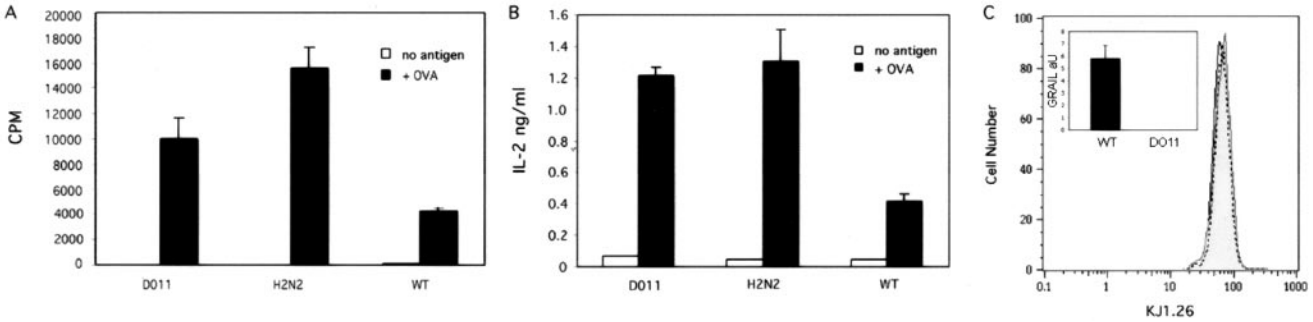

FIGURE 3. Constitutive expression of *GRAIL* renders CD4 T cells anergic. $CD4^+KJ1.26^+$ T cells were sorted from lymph node and spleens of lethally irradiated BALB/c mice reconstituted with DO11.10 bone marrow cells transduced to express wild-type (WT) *GRAIL* plus GFP, H2N2 *GRAIL* plus GFP (H2N2), or mock transduction (DO11). *A*, A total of 6000 sorted cells were cultured with 5×10^5 irradiated BALB/c splenocytes with 0.4 μg/ml $OVA_{323–339}$ peptide (+ OVA) or no Ag. The data are mean cpm of triplicate samples ± SD pulsed with 1 μCi of [^{3}H]thymidine at 72 h. *B*, Mean IL-2 (nanograms per milliliter of triplicate culture supernatant samples ± SD) harvested at 48 h. *C*, TCR levels on sorted cells. Shaded histogram, mock transduced; dashed histogram, H2N2 transduced; solid line histogram, wild-type transduced. *Inset*, *GRAIL* mRNA levels in wild-type-reconstituted mice and control DO11.10 mice CD4 T cells by real-time QPCR. Data are one representative of three independent experiments. aU, arbitrary unit (see *Materials and Methods*).

were removed for phenotypic analysis. Administration of i.v. soluble OVA (pep (peptide only), tolerizing immunization) to mice that had received unmanipulated DO11.10 T cells or GFP-only-expressing cells, resulted in the expected anergy phenotype (40% and 30% reduction in proliferation vs naive, respectively; Fig. 4*A*). Addition of LPS to the immunization regimen resulted in a productive immune response with a higher proliferative capacity compared with the response of naive DO11.10 T cells (Fig. 4*A*). There was almost no detectable IL-2 production in the DO11.10 control cells or GFP-only transduced DO11.10 T cells following a tolerizing immunization, consistent with an anergy phenotype (Fig. 4*B*). In marked contrast, delivery of a tolerizing immunization to the H2N2-*GRAIL*-expressing DO11.10 T cell recipients demonstrated a blockade in the ability of these cells to be anergized. The proliferative capacity and IL-2 production of these cells was comparable to the fully responsive OVA plus LPS-immunized DO11.10 recipients (Fig. 4*B*). Of note, the proliferative response and IL-2 production was equivalent in the unimmunized GFP-only or H2N2-*GRAIL*-expressing DO11.10 T cells, demonstrating that there was no increased background proliferative response or IL-2 production in the DO11.10 H2N2-*GRAIL*-expressing cells (data not shown). These data demonstrated that interfering with *GRAIL* function during the induction phase of a tolerizing response abrogated the development of the anergy phenotype in CD4 T cells, providing strong evidence that *GRAIL* is an essential anergy factor.

H2N2 GRAIL *is a dominant-negative form of wild-type* GRAIL

Dominant-negative effects of RING motif mutants have been used in other model systems to study the role of E3 ubiquitin ligases in biological pathways (15). We reasoned that H2N2 *GRAIL* might act as a dominant-negative form of *GRAIL* in cells expressing wild-type *GRAIL*. To test this hypothesis, we used a system where *GRAIL* ubiquitinates an unknown substrate through its association with the deubiquitinating enzyme *USP8* (12). In this system, *GRAIL* mediates ubiquitination of a *USP8*-associated protein in a RING-dependent fashion (Fig. 5*A*). Transfection of H2N2 *GRAIL* into cells at increasing concentrations revealed a clear dominant interfering effect of H2N2 *GRAIL* over the wild-type *GRAIL*. This is demonstrated by a complete lack of ubiquitination of a *USP8*-associated protein at the highest H2N2 *GRAIL* concentration (Fig.

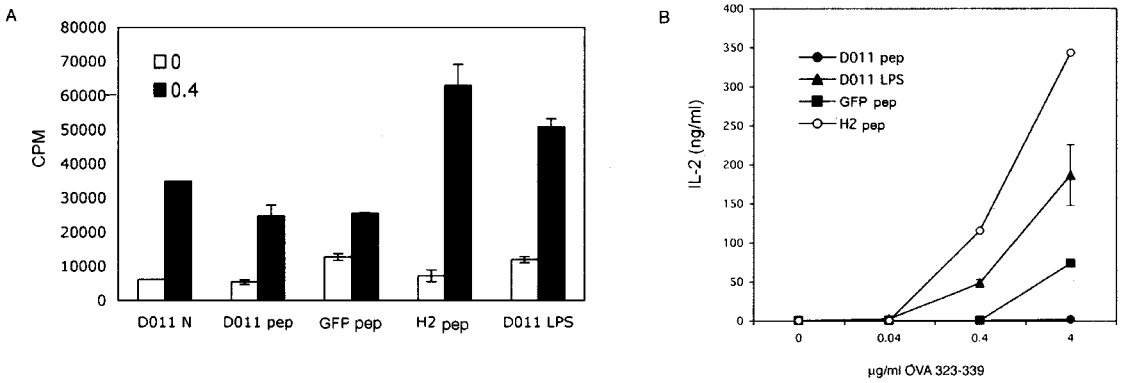

FIGURE 4. CD4 T cells expressing H2N2 *GRAIL* are resistant to the development of an anergy phenotype in vivo. A total of $1.5–2 \times 10^6$ $CD4^+KJ1.26^+$ cells was transferred to BALB/c mice i.v. from DO11.10 TCR transgenic mice (DO11), lethally irradiated BALB/c mice reconstituted with bone marrow cells transduced with H2N2 *GRAIL* plus GFP (H2 pep), or GFP only (GFP pep). *A*, Spleen and lymph node tissues were removed and pooled 8 days after immunization and restimulated with 5×10^5 irradiated BALB/c splenocytes per well and $OVA_{323–339}$ peptide in triplicate in a dose-response fashion. The no Ag (□, 0) and 0.4 μg/ml $OVA_{323–339}$ (■, 0.4) proliferative responses are shown. The data are mean cpm ± SD pulsed with 1 μCi of [^{3}H]thymidine at 72 h. *B*, Mean IL-2 (nanograms per milliliter of triplicate culture supernatant samples ± SD) harvested at 48 h. Data are representative of four independent experiments. ○, DO11 N (recipient of DO11.10 cells and no immunization); ●, DO11 pep (recipient of DO11.10 cells and peptide only (tolerizing) immunization); ▲, DO11 LPS (recipient of DO11.10 cells and immunization with peptide and LPS); ■, GFP pep (recipient of GFP-only-expressing cells and peptide only (tolerizing) immunization); ○, H2 pep (recipient of H2N2-*GRAIL*-expressing cells and peptide only (tolerizing) immunization).

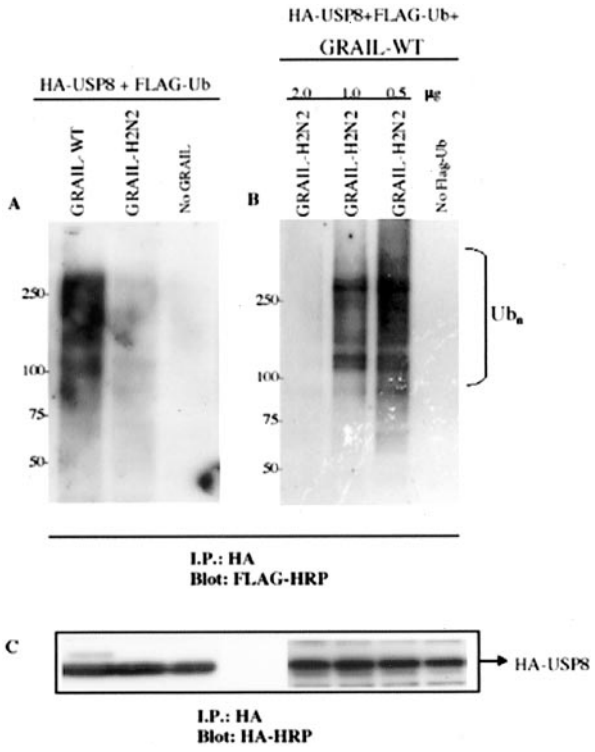

FIGURE 5. H2N2 *GRAIL* is a dominant-negative form of *GRAIL*. *A*, 293 cells were transduced with *GRAIL* wild-type (WT) or H2N2 *GRAIL*, and sorted for GFP expression. GFP-positive cells were then cotransfected with HA-tagged USP8 and a FLAG-tagged ubiquitin construct. Forty-eight hours after transfection, cells lysates were immunoprecipitated with HA-agarose beads. The immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF, and probed with anti-FLAG-HRP Ab to detect ubiquitinated targets. The *control lane* demonstrates the lack of endogenous ubiquitination in the absence of *GRAIL*. *B*, 293 cells were first infected with wild-type *GRAIL*-IRES-GFP retrovirus. Transduced cells were sorted for GFP expression and cotransfected with HA-tagged USP8, a FLAG-tagged ubiquitin construct, and the RING mutant H2N2 *GRAIL* (in a dose-dependent fashion). Forty-eight hours after transfection, cell lysates were prepared and immunoprecipitated with HA-agarose beads and processed as above. *C*, FLAG-ubiquitin blots were striped and reprobed with anti-HA Ab to demonstrate equivalent levels under all experimental conditions.

5*B*). Of note, in 293 cells there is no background ubiquitination of this unknown substrate.

Discussion

In this study, we have demonstrated that *GRAIL* expression is important for the development of an anergic phenotype in CD4 T cells in vivo. Constitutive expression of *GRAIL* in naive CD4 T cells was sufficient to render these cells anergic. The observation that constitutive expression of a dominant-negative form of *GRAIL*, H2N2 *GRAIL*, completely abrogated the development of the anergy phenotype in mice receiving a tolerizing regimen in vivo provides the strongest evidence for the essential role that *GRAIL* plays in the development of CD4 T cell anergy. Our current working hypothesis is that *GRAIL* expression is induced and stabilized when a T cell receives an "anergizing" signal and the expression of *GRAIL* results in ubiquitination of a substrate required for normal T cell activation. Although the substrate(s) for *GRAIL* is unknown at this time, one can speculate that the H2N2 mutant acts as a dominant negative by binding this substrate, in association with USP8 and blocking its ubiquitination, thus altering the biochemical pathway(s) that would have led to T cell unresponsiveness.

Ubiquitination of proteins has been an accepted method for targeting proteins for degradation for many years. Recently, this post-translational modification has gained a new level of appreciation in many areas of research based on observations that ubiquitination can exercise tight control by degradation-independent mechanisms, for example, recompartmentalization of cellular proteins (16) and modification of transcription factors resulting in altered gene expression (reviewed in Ref. 17). There is increasing evidence implicating the important role that E3 ubiquitin ligases play in T cell function. The identification and characterization of *GRAIL* as an E3 ubiquitin ligase that is necessary for the development of the anergy phenotype in T cells reiterates the important role of the ubiquitin pathway in controlling T cell functions, including T cell tolerance. Despite implications that other E3 ubiquitin ligases, including *cbl-b* and *itch*, may play a role in the development of anergy, with the exception of *GRAIL*, the relationship is indirect at this time (5, 18, 19). There are several aspects of *GRAIL* that distinguish it from the majority of E3 ubiquitin ligases, including *cbl-b* and *itch*. *GRAIL* is a transmembrane E3 ubiquitin ligase and shares this property with only three other structurally unrelated mammalian E3 ubiquitin ligases (20–22). Fluorescence microscopy studies have demonstrated that *GRAIL* localizes to recycling endosomes (10). We have previously reported that the overexpression of H2N2 *GRAIL* in 3T3 cells resulted in subtle phenotypic changes in the cellular morphology such as membrane ruffling and less punctate localization pattern suggesting two possibilities. Either expression of the dominant-negative form of *GRAIL*, H2N2 *GRAIL*, results in complete mislocalization of the protein, or its expression leads to changes in the endosomal structures as has been demonstrated with other dominant-negative proteins that localize to endosomes (23). We favor the latter because coexpression of wild-type *GRAIL* and H2N2 *GRAIL* affect the ubiquitination of its substrate. This observation, and the fact that *GRAIL* is membrane bound, may provide some clues to its function (e.g., it may serve to tightly control the fate of cell surface receptors). However, the effects of constitutive expression of *GRAIL* are demonstrable in T cells that express normal levels of TCR on their surface (Fig. 3), suggesting that the dominant effect of *GRAIL* is not related to alterations in constitutive TCR recycling. Mechanistic studies are ongoing to elucidate the biological function of *GRAIL*.

There is accumulating evidence that there are phenotypic differences between cells rendered anergic in vitro vs in vivo (24). Recent work has suggested a genetic commonality between these model systems (25). Our studies demonstrate that expression of *GRAIL* is an additional genetic commonality between in vitro anergy model systems and in vivo tolerance. Furthermore, *GRAIL* appears to fulfill the criteria for an anergy factor, and by using a dominant-negative form of *GRAIL*, we have been able to demonstrate a necessary requirement for *GRAIL* in anergy induction. Studies from our laboratory have begun to elucidate the complex regulation of cellular *GRAIL* protein levels, and further work in this area will lead to a more complete characterization of this important biochemical pathway in anergy development (12). By identifying a novel gene that is up-regulated in anergized T cells in vivo as well as in vitro, more systematic approaches can be taken to elucidate the conditions under which anergized T cells develop and persist. Understanding the molecular and biochemical basis of anergy has major implications for unraveling the pathogenesis of many human diseases and could potentially lead to new treatment regimens for such diverse disease states as autoimmunity, cancer, and allergy.

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