GRAIL Is Up-regulated in CD4+ CD25+ T Regulatory Cells and Is Sufficient for Conversion of T Cells to a Regulatory Phenotype*

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GRAIL (gene related to anergy in lymphocytes) is an ubiquitin-protein isopeptide ligase (E3) ubiquitin ligase necessary for the induction of CD4+ T cell anergy in vivo. We have extended our previous studies to characterize the expression pattern of GRAIL in other murine CD4+ T cell types with a described anergic phenotype. These studies revealed that GRAIL expression is increased in naturally occurring (thymically derived) CD4+ CD25+ T regulatory cells (mRNA levels 10-fold higher than naive CD25− T cells). Further investigation demonstrated that CD25+ Foxp3+ antigen-specific T cells were induced after a “tolerizing-administration” of antigen and that GRAIL expression correlated with the CD25+ Foxp3+ antigen-specific subset. Lastly, using retroviral transduction, we demonstrated that forced expression of GRAIL in a T cell line was sufficient for conversion of these cells to a regulatory phenotype in the absence of detectable Foxp3. These data demonstrate that GRAIL is differentially expressed in naturally occurring and peripherally induced CD25+ T regulatory cells and that the expression of GRAIL is linked to their functional regulatory activity.

CD4+ CD25+ T cells, which comprise from 5 to 10% of peripheral blood CD4+ T cells in normal adult mice and humans, are a subset of regulatory CD4+ T cells (CD25+ Tregs) with an “anergic” phenotype in vitro. CD25+ Tregs have been shown to play a critical role in the prevention of organ-specific autoimmune disease (1). Mutations in the coding region of the transcription factor, Foxp3, result in aberrant development and function of CD25+ Treg cells in mice and humans, implicating Foxp3 as the master genetic regulator of CD25+ Treg cells (2–4). The absence of Foxp3 protein is associated with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome in the majority of, but not all patients, suggesting that other genes may be important for CD25+ Treg function (5). Furthermore, there is increasing evidence that antigen-specific CD25+ Tregs can be induced by antigen exposure in a non-inflammatory context and play an essential role in modulating adaptive immune responses, and clearly defining the similarities and differences between naturally occurring and induced CD25+ Treg cells is a high priority for making inroads into potential therapeutics for multiple disease states (6–8).

Characterization of cells with intrinsic anergic properties (termed recessive tolerance) was formally undertaken in the late 1980s after the Schwartz laboratory described a model system in vitro for induction of anergic T cell clones that, following engagement of their T cell receptor, required new protein and mRNA synthesis (reviewed in Ref. 9). This model system has led to numerous studies to identify “anergy factors.” Our published work using this in vitro T cell anergy paradigm demonstrated the expression of a novel E3 ubiquitin ligase GRAIL, in T cell clones under anergy-inducing conditions (10). The development of immune unresponsiveness in vivo to neoantigens predates the development of the in vitro T cell clone model by over 20 years (11). An intriguing observation in the early 1970s suggested that there is development de novo of a suppressor population when neoantigen is presented in a non-inflammatory context. Our recent published work demonstrated a correlation between CD4+ T cell anergy in vitro and in vivo and GRAIL expression (12) that is complemented by studies from Heissmeyer et al. (13). These findings, coupled with several recent reports demonstrating an induction of CD25+ Treg cells in vivo after antigen presentation to naive CD4+ T cells in a tolerizing fashion, led us to pursue the role of GRAIL- and antigen-induced tolerance in vivo (6–8).

In the current study, we investigated the mRNA and protein levels of GRAIL in naturally occurring and induced CD25+ Treg cells. We demonstrated that both naturally occurring CD25+ Treg cells and antigen-specific CD25+ Foxp3+ CD4+ T cells induced in vivo after receiving antigen in a tolerizing fashion express GRAIL as a bulk population. Furthermore, retroviral-mediated forced expression of GRAIL in an antigen-specific CD4+ T cell line was sufficient for conversion of the transduced cells to a suppressor cell phenotype. These data suggest that...
GRAIL, along with and separable from Foxp3, might be involved in differentiation and biological function of CD4+ T cells into a regulatory phenotype.

**EXPERIMENTAL PROCEDURES**

**Animals and Adoptive Transfer**—BALB/c mice were obtained from Harlan-Sprague (Madison, WI). DO11.10 and DO11.10 Rag2−/− mice (14) were bred under specific pathogen-free conditions at the University of Wisconsin animal facility (Madison, WI). Foxp3-IRE5-GFP knock-in mice were a generous gift from Dr. Talal Chatila (UCLA, Los Angeles, CA). The Animal Care Committee at the University of Wisconsin approved all experimental protocols involving the use of mice. The adoptive transfer of DO11.10 (DO11) T cells into BALB/c mice and subsequent immunization followed a previously described protocol (12).

**Flow Cytometry**—Single cell suspensions were stained using the following antibodies according to the manufacturer’s protocol: KJ.126 PE (Caltag, Burlingame, CA); CD4 Cy7 APC (RM-5), Foxp3 PE (FIK-165) (both from eBioscience, San Diego, CA); CD25 APC (PC61), CTLA4 PE (UC10-4B9), CD103 PE (M290) (all from Pharmingen); GITR PE (FAB5241P, R + D, Minneapolis, MN). Acquisition was done on a LSR II (BD Biosciences), and sorting was done on a Vantage SE equipped with a FACS-Diva digital upgrade. Analysis was done using FlowJo software (TreeStar, San Carlos, CA).

**Cells**—An OVA323–339-specific T cell line was derived from a DO11 T cell receptor transgenic mouse by serially stimulating the cells with 0.4 μg/ml OVA323–339 peptide and irradiated APCs from a BALB/c mouse every 8–14 days in supplemented RPMI 1640 as described previously (12).

**Retrovirus Production and T cell Transduction**—Ectropic retrovirus was produced using Phoenix-E cells and a modified CaPO4 precipitation transfection protocol as described previously (15). The DO11.10 T cell line was transduced 24 h after stimulation by spin-fecction with virus supernatant containing 5 μg/ml Polybrene and 10 units/ml murine IL-2 as described previously (16).

**Suppressor Assays**—DO11 Rag2−/− donors were used for isolation of OVA323–339-specific naive CD4 T cells by flow cytometry. For the bead suppressor assay, 5 μm latex beads (Interfacial Dynamics) were coated with 2.5 μg/ml anti-CD3 (145–2C11, Pharmingen) and 1.25 μg/ml or 3.75 μg/ml anti-CD28 (37.51, Pharmingen) as described previously (17). Cells and beads were co-cultured at a ratio of 1:1 in U-bottom 96-well microtiter plates. Cells were pulsed at 72 h for 12–16 h with 1 μCi of [3H]thymidine. For the peptide activation suppressor assays, cells were cultured in a 96-well flat bottom microtiter plate with 0.4 μg/ml OVA323–339 and irradiated BALB/c splenocytes at a concentration of 3 × 105/well. Cells were pulsed at 72 h for 12–16 h with 1 μCi of [3H]thymidine. The percentage of suppression versus DO11 parent cell line was calculated by the following equation: 100 − (mean cpm GRAIL-expressing DO11 T cells co-cultured with naive CD25− DO11 T cells/cpm DO11 parent T cells co-cultured with naive CD25− DO11 T cells × 100). For the transwell assay, 50,000 naive DO11 Rag2−/− cells were co-cultured with an equal number of DO11 T cells or GRAIL-expressing DO11 T cells either together or apart in a transwell plate (Corning 3413) and stimulated with 0.4 μg/ml OVA323–339 and 5 × 104 irradiated BALB/c splenocytes. After 72 h, triplicate aliquots were removed and pulsed with [3H]thymidine for an additional 12–16 h.

**Enzyme-linked Immunosorbent Assay**—Cell culture supernatants from transduced or control DO11 T cells were collected after activation with anti-CD3/anti-CD28-coated beads and analyzed for the presence of TGF-β using an enzyme-linked immunosorbent assay kit obtained from Promega (Madison, WI). To measure total TGF-β in the sample, 200-μl aliquots of sample were acid-treated with 2 μl of 1 N HCl for 15 min and then neutralized with an equal volume of 1 N NaOH. IFN-γ and IL-10 were assayed as reported previously (10).

**Immunoblot**—CD4+ CD25+ T cells were selected using magnetic MicroBeads according to the manufacturer’s instructions (Miltenyi). Cells were stained with CD25-PE in a saturating concentration followed by several washes and then incubated with anti-PE magnetic MicroBeads. CD25-bearing cells were isolated using a Quadramacs, and purity was confirmed by flow cytometry (>92% CD4+ CD25+). Protein was extracted, resolved on SDS-PAGE, and transferred to polyvinylidene difluoride as described previously (18). The membrane was probed with a rabbit anti-GRAIL-specific polyclonal antibody raised against the N-terminal portion of murine GRAIL (18). The membrane was stripped and reprobed with anti-Foxp3 (eBioscience; FJK-16s) and anti-glyceraldehyde-3-phosphate dehydrogenase (Abcam; polyclonal) as a loading control. Antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and the ECL Western blot detection system (Amersham Biosciences). Bands were detected on Kodak BioMax Light film (GE Healthcare). Jurkat cells transduced with murine GRAIL were used as a positive control.

**Real-time Quantitative PCR (QPCR)**—RNA from sorted cell populations was isolated using TRIZol reagent according to the manufacturer’s instructions (Invitrogen) and treated with RNase-free DNase I (Ambion). cDNA was made using an iScript kit according to the manufacturer’s instructions (Bio-Rad). Real-time QPCR triplicate samples were run with SYBR green on an iCycler (Bio-Rad) using the following primer pairs: murine GRAIL primers, forward, 5′-GGCGCATGCAGCAAAT-GAA-3′, and reverse, 5′-GTGCAACATGGGGAAACAAA-3′; and murine Foxp3 primers, forward, 5′-CACCCTATGCCAC-CCTTATCC-3′, and reverse, 5′-CGGAACATGCCAGTAAAC-CAAA-3′. For normalization, the cyclophilin gene was used. Cyclophilin primers are as follows: forward, 5′-GGCTTCTCTCTGCAGACTGT-3′, and reverse, 5′-GGACCCCTTAGC-CATAATCC-3′. Liver cDNA (GRAIL and cyclophilin) and T cells transduced with murine Foxp3 cDNA were used to generate standard curves for quantitation of transcript and gauge of reaction efficiency.

**Confocal Microscopy**—Lymph nodes from Foxp3 GFP knock-in mice were isolated, and single cell suspensions were prepared. CD25+ cells were bead-purified using biotinylated anti-CD25 (PC61) and streptavidin MicroBeads (Miltenyi Biotech). Following isolation, cells were allowed to adhere to poly-L-lysine-coated coverslips for 10 min at 37°C. Cells were the
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fixed in 3% formalin, quenched with 0.15 M glycine, permeabilized with 0.3% Triton-X, and washed with phosphate-buffered saline. Cells were incubated in blocking buffer (5% goat serum with 0.1% saponin in phosphate-buffered saline) for 30 min at room temperature followed by rabbit anti-GRAIL primary antibody for 2 h at room temperature and then washed extensively. Finally, cells were incubated with Alexa Fluor 594-conjugated anti-rabbit secondary antibody (Invitrogen) for 45 min at room temperature and washed extensively, nuclei were stained with 4',6-diamidino-2-phenylindole (Invitrogen), and coverslips were mounted with Prolong Gold Antifade reagent (Invitrogen) on glass slides and imaged with a Bio-Rad MRC-1024 confocal microscope and LaserSharp 5.2 software (W.M. Keck Laboratory for Biological Imaging, University of Wisconsin).

RESULTS

GRAIL mRNA Is Increased in Naturally Occurring CD25+ T Regulatory Cells—We have previously reported that GRAIL is expressed in anergic CD4+ T cells using in vitro and in vivo model systems (10, 12). Furthermore, GRAIL function was found to be a necessary requirement for the induction of an anergic phenotype in CD4+ T cells in vivo (12). To expand on these observations, we began to study the expression profile of GRAIL in other anergic CD4+ T cells. During this survey, we found that both GRAIL mRNA and protein are expressed in naturally occurring CD25+ Tregs. At the RNA level, GRAIL expression was 10-fold higher in the CD25+ population than the CD25- naive CD4+ T cells (Fig. 1A). Using a GRAIL-specific polyclonal antibody, we were able to demonstrate at the protein level that GRAIL was preferentially expressed in the CD25+ Treg subset of lymphocytes, thus corroborating the mRNA data (Fig. 1B). To further solidify that GRAIL and Foxp3 are expressed in the same cell, confocal microscopy was utilized. We took advantage of transgenic mice that had GFP knocked-in to the 3′-untranslated region of the Foxp3 locus that allows GFP as a surrogate marker of Foxp3 protein expression. Staining of the CD25+ T cells from these mice with the GRAIL polyclonal antibody revealed dual expression of Foxp3 and GRAIL within the same cell (Fig. 1C). The cellular localization of GRAIL is as previously demonstrated or predicted; GRAIL, which is resident in endosomes, is present in the cytoplasm in a vesicular pattern. These data suggested that GRAIL, in addition to Foxp3, is differentially expressed in CD25+ Treg cells and may be involved in CD25+ Treg function or differentiation.

GRAIL Expression Segregates with the CD25+, Foxp3-expressing Antigen-specific Tolerized Cells in Vivo—We have previously shown that GRAIL is expressed in antigen-specific anergic T cells induced following antigen administration in a tolerizing fashion in vivo (12), and in this study, we have demonstrated that GRAIL is expressed in naturally occurring CD25+ Treg cells (Fig. 1). There is accumulating evidence demonstrating that CD25+ antigen-specific Treg cells can be induced from naive CD4+ CD25- T cells following oral or intravenous administration of antigen under non-inflammatory conditions similar to the methodology used in the adoptive transfer tolerance model of our previous studies that demonstrated that GRAIL was necessary for the development of the CD4+ T cell anergic phenotype in vivo (6). To determine whether GRAIL (and Foxp3) expression in antigen-specific anergic T cells segregated with CD25 expression, we used the in vitro adoptive transfer model described previously to explore CD25 and GRAIL expression (12). In our experiments, CD25+ antigen-specific CD4+ T cells were induced and detected by flow cytometry on day 12 after administering antigen in a tolerizing fashion (Fig. 2A). Transfer of DO11 naive T cells from either a Rag2−/− or a non-Rag background and antigen challenge demonstrated comparable up-regulation of CD25 expression (Fig. 2A). Since T cell receptor transgenic T cells from mice on a Rag2−/− background lack naturally occurring CD25+ Treg cells, we concluded that this population of CD4+ CD25+ T cells was induced by antigen under non-inflammatory conditions. These data are consistent with the published work by Thorstenson et al. (6), with CD25+ antigen-specific cells appearing between days 3 and 8 after administering antigen in a tolerizing immunization. We carried our studies out longer and found that the CD25+ antigen-specific CD4+ T cells persisted at least 70 days after administration of antigen in a tolerizing fashion (data not shown), which is consistent with data suggesting that CD25+ Treg cells do not require persistence of antigen for survival (8).
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**Figure 2.** GRAIL expression segregates with the CD25 expressing, Foxp3⁺, antigen-specific tolerated cells. 5 x 10⁶ CD4⁺ KJ1.26⁺ from DO11.10 or DO11.10 Rag2⁻/⁻ mice were transferred intravenously to age- and gender-matched BALB/c mice. The following day, recipients were immunized in a tolerizing fashion with soluble peptide (300 μg) intravenously (Peptide) or immunized in a priming fashion with soluble peptide (300 μg) and lipopolysaccharide (25 μg) intravenously (Peptide/LPS). 12 days after immunization, mice were sacrificed, lymph node and spleen tissue was removed, and single cell suspensions were made for sorting and analysis as described under “Experimental Procedures.” A, CD25 and KJ1.26 expression within CD4 gated cells. The percentage in the upper left quadrant represents the percentage of CD25⁺ CD4⁺ KJ1.26⁺ cells/total KJ1.26⁺ cells. B, cells were sorted for CD4 and KJ1.26 expression (± CD25 expression). RNA was prepared, and real-time QPCR for GRAIL mRNA was done as described under “Experimental Procedures.” C, real-time QPCR for Foxp3. cDNA from sorted cells in panel B was used for Foxp3 mRNA quantitation as described under “Experimental Procedures.” D, Foxp3 protein analysis on tolerized DO11.10 CD4⁺ cells by flow cytometry. 12 days after receiving a tolerizing immunization (as described above), pooled lymph node and spleen cells were stained for Foxp3 protein as described under “Experimental Procedures.” Dashed histogram, CD4⁺ KJ1.26⁺ CD25⁺ Foxp3⁺ histogram; solid histogram, CD4⁺ KJ1.26⁺ CD25⁺ Foxp3⁺ histogram. The data are representative of 2–4 independent experiments.

CD25⁺ subset, strongly suggesting that this population represents induced CD25⁺ Treg cells.

**Forced Expression of GRAIL in a T Cell Line Is Sufficient for Conversion to a Suppressor Phenotype in a Contact-dependent Manner—**As a first step to studying the role of GRAIL in the suppressor cell phenotype, we enforced GRAIL overexpression in an OVA-specific CD4⁺ T cell line by retroviral transduction. Maintenance of this cell line in vitro required frequent supplementation of the culture media with IL-2 in addition to serial stimulation with antigen + APC (OVA₃₂₃₋₃₃₉ peptide), similar to the requirements necessary for the maintenance of Foxp3-transduced T cells (20).

Not surprisingly, the GRAIL-expressing DO11 T cell line (DO11 GRAIL) demonstrated diminished proliferative capacity in response to antigen + APC when compared with the retrovirally transduced parent cell line expressing the marker protein alone, GFP (DO11 GFP) or non-transduced DO11 T cells (Fig. 3A: cells alone; the non-transduced parent cell line was phenotypically comparable with DO11 GFP; data not shown). Co-cultivation of the GRAIL-expressing DO11 T cell line and naive CD25⁺ CD4⁺ T cells from a DO11.10 Rag2⁻/⁻ mice with the APC + OVA₃₂₃₋₃₃₉ peptide demonstrated suppression of the naive responder cells (~50% response when compared with responder naive cells alone and 90% reduction when compared with co-cultivation with DO11 GFP cells, Fig. 3A). The suppressor capacity of the GRAIL-expressing T cell line was compared with that of naturally occurring CD25⁺ Treg cells from DO11 mice using a previously described anti-CD3/anti-CD28-conjugated bead suppression system (17). The GRAIL-expressing T cell line suppressed naïve CD25⁻ CD4⁺ T cell proliferation as well or better than the naturally occurring CD25⁺ Treg cells when co-cultured at a 1:1 ratio in this system (Fig. 3B). Consistent with previous data, stronger co-stimulation (the addition of IL-2 or higher CD28 density on the beads) partially restored the proliferative response of naïve CD4⁺ CD25⁻ T cells (Fig. 3B, last two experimental groups). The addition of IL-2 resulted in a 19-fold increase in the proliferative response.
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A DO11.10 T cell line was transduced with ecotropic retrovirus constitutively expressing wild-type GRAIL upstream of an IRES sequence and GFP as described under "Experimental Procedures." Transductants were sorted based on GFP expression and maintained in culture with IL-2 (50 units/ml) every third day and restimulated with irradiated BALB/c splenocytes and OVA323–339 peptide every 8–14 days. A DO11.10 T cell line was transduced with ecotropic retrovirus constitutively expressing wild-type GRAIL upstream of an IRES sequence and GFP as described under "Experimental Procedures." Transductants were sorted based on GFP expression and maintained in culture with IL-2 (50 units/ml) every third day and restimulated with irradiated BALB/c splenocytes and OVA323–339 peptide every 8–14 days.

The proliferative response of naive CD4+ cells from DO11.10 Rag2−/− mouse, DO11.10 T cell line expressing GFP only (DO11 GFP), and GRAIL-expressing DO11.10 T cell line (DO11 GRAIL). All cells went through the flow cytometer, and DO11.10 T cell lines were sorted for GFP. 10,000 sorted cells were cultured in a 96-well flat bottom tissue culture plate with 3 × 105 irradiated BALB/c splenocytes with (0.4 μg/ml) or without (0) OVA323–339 peptide either alone or at a ratio of 1:1 with naive DO11.10 Rag2−/− T cells as noted. After 72 h of culture, wells were pulsed with 1 μCi of [3H]thymidine and harvested 12 h later. Mean CPM ± S.D. of triplicate wells are shown. 

The suppressor assay was set up using latex beads coated with anti-CD3/anti-CD28 as described under "Experimental Procedures." The proliferative response of the cells alone cultured with an equal number of conjugated latex beads (1:1) with IL-2 (10,000 naive T cells were co-cultured with an equal number of conjugated latex beads (coated with 2.5 μg/ml anti-CD3 and 3.75 μg/ml anti-CD28) and CD25+, DO11, or DO11 GRAIL cells as denoted. (1:1) IL2 is the same with the addition of 50 units/ml IL-2; (1:1) 3 × anti-CD28; 10,000 naive T cells were co-cultured with an equal number of conjugated latex beads (coated with 2.5 μg/ml anti-CD3 and 3.75 μg/ml anti-CD28) and CD25+, DO11, or DO11 GRAIL cells as denoted. Mean CPM ± S.D. of triplicate wells are shown. 

in cells co-cultured with naturally occurring CD25+ Treg cells and GRAIL-expressing DO11 T cell line to superphysiologic expression of GRAIL based on real-time QPCR data (data not shown). The suppressive effect mediated by GRAIL-expressing DO11 T cells was titratable demonstrated by decreased suppression of naive CD25− CD4+ T cells at lower suppressor naïve cell ratios (Fig. 3C). We assessed the role of cell contact for suppression in the GRAIL-expressing DO11 T cells using a transwell system. This demonstrated that suppression was 4-fold greater if the cells were in contact when compared with separation by a permeable membrane (Fig. 3D).

GRAIL-expressing DO11 T Cells Express Increased Levels of TGF-β, Surface CD25, GITR, and Total CTLA-4, and Biological Suppressor Function Is Separate from Foxp3 Expression In Vitro—To explore the potential mechanism for suppressor function of the GRAIL-expressing DO11 T cells, surface receptor expression and cytokine production was analyzed for various proteins that have been suggested to be important for suppressor function of naturally occurring CD25+ Treg cells (21, 22). Analysis of IL-10 production demonstrated detectable levels produced by the parent cell line, and interestingly, undetectable levels in the supernatant from activated GRAIL-expressing DO11 T cells (Fig. 4A). Published analysis of naturally occurring CD25+ Treg cells and T cells ectopically expressing Foxp3 have increased levels of IL-10 (20); however, our data suggest that the suppressive mechanism in the GRAIL-expressing DO11 T cells did not involve IL-10.

Although the parent T cell line produces considerable amounts of IFN-γ, we found that the levels of IFN-γ in the culture supernatant were consistently higher in the GRAIL-expressing DO11 T cell line (Fig. 4A). A recent report by Stock et al. (23) has described the development of a suppressor T cell that expresses Foxp3 and IFN-γ, providing evidence that IFN-γ does not interfere with suppressor function. The cytokine that has most consistently been associated with suppressor T cells is TGF-β. Enzyme-linked immunosorbent assay analysis of the supernatant from GRAIL-expressing
DO11 T cells demonstrated significantly increased levels of TGF-β when compared with the parent DO11 T cell line (Fig. 4A). Flow cytometric analysis of known markers of CD25 Treg cells revealed an expression profile similar to naturally occurring CD25 Treg cells and Foxp3-expressing T cells, with the exception of CD103 (23). The GRAIL-expressing T cells had elevated levels of surface CD25, GITR, and total CTLA-4 (Fig. 4B). We assayed our GRAIL-expressing T cell line for Foxp3 protein by flow cytometry and found no detectable Foxp3 protein (Fig. 4C). Likewise, we assayed a Foxp3-expressing DO11 T cell line (retroviral expression vector kindly provided by Dr. A. Rudensky) for GRAIL mRNA by real-time QPCR and found no up-regulation of GRAIL mRNA in these cells despite demonstrable suppressor activity as previously published (data not shown). These data suggest that either GRAIL or Foxp3 expression by itself is sufficient but not necessary for regulatory T cell function in vitro.

**DISCUSSION**

The functional outcome of naive CD4 T cells following engagement with antigen in a non-inflammatory environment in vivo has been thought to follow one of several pathways in peripheral lymphoid tissues. In general, this engagement was thought to result in clonal ignorance, apoptosis, or anergy. The anergic phenotype has in general referred to a form of tolerance indicating a cell-intrinsic defect in lack of responsiveness, particularly for neoantigens. Accumulating evidence has suggested that these cells require persistence of antigen to maintain this anergic phenotype has in general referred to a form of tolerance thought to result in clonal ignorance, apoptosis, or anergy. The peripheral lymphoid tissues. In general, this engagement was thought to follow one of several pathways in vivo can lead to the induction of CD25 Treg cells extrathyrmically from naive, conventional CD4 T cells (6-8). The study from Thorstenson et al. (6) reported the induction of CD4 CD25 antigen-specific T cells when low dose antigen was delivered in a non-inflammatory context. The functional characteristics of these cells in vitro implicated suppressor capabilities (6).

Our findings support the hypothesis that the anergic phenotype seen in CD4 T cells in vivo in response to a neoantigen challenge under non-inflammatory conditions is due to the acquisition of a suppressor phenotype by the antigen-activated anergic CD4 T cells. This is based upon the induction of Foxp3 expression in the antigen-reactive T cells as well as their ability to be used in functional suppressor assays as regulatory T cells. Interestingly, the anergy-related E3 ubiquitin ligase, GRAIL, also segregates with these CD25 antigen-specific regulatory T cells. Thus, in addition to Foxp3, GRAIL may be another gene product involved in CD25 Treg cell function, and by itself (and separable from Foxp3), it seems to be sufficient to convey regulatory function to T cells that over express GRAIL.

Our study complements and expands upon the observation by Thorstenson et al. (6) by demonstrating that these induced CD25 Treg cells are long-lived and express genotypic markers of naturally occurring CD25 Treg cells, namely Foxp3 and GRAIL. In our experiments, low dose (5 μg/animal) or high dose (300 μg/animal) antigen did not result in a large disparity in numbers of CD4 CD25 antigen-specific T cells between the groups. We cannot reconcile these data with the antigen dose-response Treg cell induction published by Thorstenson et al. (6) with low dose favoring induction of CD4 CD25 antigen-specific cells, but among potential explanations could be that they might have been using a peptide that had some lipopolysaccharide contamination or perhaps was more aggregated, thus making it more immunogenic. Nonetheless, the observation that non-inflammatory exposure to antigen can result in long-lived suppressor T cells may have tremendous therapeutic potential. A better understanding of the conditions that favor this outcome could be exploited for treatment of multiple dis...

3 C. Seroogy, unpublished data.
ease states that result from inappropriate and unregulated adaptive immune responses.

Interestingly, we have consistently observed that the GRAIL mRNA levels were 100-fold higher in the induced CD25+ expressing population when compared with the naturally occurring CD25+ Treg cell population (Fig. 1A versus Fig. 2B). This suggests that GRAIL may be up-regulated in activated CD25+ Treg cells, or alternatively, that GRAIL is differentially expressed between naturally occurring and induced CD25+ Treg cells. This observation is in contrast to that of Foxp3 expression that does not appear to be differentially regulated with the activation status of CD25+ Treg cells (26). It is also possible that GRAIL is differentially regulated between naturally occurring CD25+ Treg cells and induced CD25+ Treg cells, an intriguing possibility since very little is known about the genotypic and functional differences between these important T cell subsets. Studies are ongoing in our laboratory to further define the expression kinetics of GRAIL after CD25+ Treg cell activation and more formal characterization of the differences and similarities between naturally occurring and induced CD25+ Treg cells.

The suppressor characteristics of GRAIL-expressing DO11 T cells share similarities with previously described features of naturally occurring CD25+ Treg cells and T cells with enforced expression of Foxp3 in that the effect is titratable and suppression is maximal when cell contact is maintained. Moreover, cytokine and surface receptor expression appear to overlap, with all of these cells sharing the expression of CTLA-4, CD25, and GITR as well as increased production of TGF-β, with all of these cells sharing the expression of CTLA-4, CD25, cytokine and surface receptor expression appear to overlap, naive CD25+ the suppressor function of CD25+ T cells share similarities with previously described features of T Regulatory Cells

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