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Article

The E3 Ubiquitin Ligase GRAIL Regulates T Cell Tolerance and Regulatory T Cell Function by Mediating T Cell Receptor-CD3 Degradation

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SUMMARY

T cell activation is tightly regulated to avoid autoimmunity. Gene related to anergy in lymphocytes (GRAIL, encoded by *Rnf128*) is an E3 ubiquitin ligase associated with T cell tolerance. Here, we generated and analyzed GRAIL-deficient mice and found they were resistant to immune tolerance induction and exhibited greater susceptibility to autoimmune diseases than wild-type mice. GRAIL-deficient naive T cells, after activation, exhibited increased proliferation and cytokine expression than controls and did not depend on costimulation for effector generation. Moreover, GRAIL-deficient regulatory T (Treg) cells displayed reduced suppressive function, associated with increased Th17 cell-related gene expression. GRAIL-deficient naive and Treg cells were less efficient in downregulating T cell receptor (TCR)-CD3 expression after activation and exhibited increased NFATc1 transcription factor expression; GRAIL expression promoted CD3 ubiquitinylation. Our results indicate that GRAIL, by mediating TCR-CD3 degradation, regulates naive T cell tolerance induction and Treg cell function.

INTRODUCTION

T cell activation is tightly regulated to ensure effective elimination of invading pathogens as well as maintaining tolerance against self-tissues. T cells are regulated by extracellular signals, especially the positive and negative costimulatory molecules on antigen-presenting cells (APCs), and also by delicate intracellular signal transducers and regulators. E3 ubiquitin ligases, including cbl-b and Itch, have been shown to play important roles in regulation of T cell tolerance (Heissmeyer and Rao, 2004; Liu et al., 2005). GRAIL is a type I transmembrane protein localized to endosomal compartment with homology to RING finger proteins whose expression was previously associated with T cell anergy induction (Anandasabapathy et al., 2003; Heissmeyer et al., 2004; Seroogy et al., 2004). The GRAIL mRNA was initially determined to be induced in anergic T helper 1 (Th1) cells (Anandasabapathy et al., 2003). Previously, we reported that T cells activated in the absence of both CD28 and ICOS costimulation developed into tolerant T cells, associated with markedly upregulated GRAIL expression (Nurieva et al., 2006). Consistent with the notion that GRAIL regulates T cell anergy, overexpression of GRAIL in T cell hybridomas or in primary cells reduced T cell cytokine expression (Anandasabapathy et al., 2003). Moreover, expression of an enzymatic inactive form of GRAIL in primary T cells prevented T cell anergy (Seroogy et al., 2004). In addition to anergic $CD4^+$ T cells, enhanced amount of GRAIL was detected in regulatory T (Treg) cells, and overexpression of GRAIL in Ova-specific $CD4^+$ T cell line was reported to convert these cells to a regulatory phenotype in the absence of detectable Foxp3 expression (MacKenzie et al., 2007). Despite the above interesting preliminary data on GRAIL expression and function, the physiological function of GRAIL in immune regulation is not well understood, in part because of lack of genetic studies.

In the current study, we generated and analyzed mice deficient in *Rnf128* (encoding GRAIL). GRAIL-deficient mice exhibited impairments in peripheral tolerance induction and greater susceptibility to autoimmune diseases. Naive T cells lacking GRAIL showed greatly enhanced proliferation and cytokine production after T cell receptor (TCR) activation and did not depend on CD28 and ICOS for their effector cytokine expression. We also found that lack of GRAIL abrogated suppressive function of Treg cells in an interleukin-21 (IL-21)-dependent manner. Both naive and Treg cells from GRAIL-deficient mice were less efficient in downregulation of their TCR-CD3 expression and exhibited increased NFATc1 transcription factor expression after TCR activation. Moreover, GRAIL promotes CD3 ubiquitinylation. Our results thus indicate GRAIL as an essential regulator of T cell tolerance by regulating naive T cell tolerance and Treg cell function.

RESULTS

Generation of GRAIL-Deficient Mice

In order to understand the physiological function of GRAIL, we generated mice deficient in *Rnf128* by replacing part of exon 4

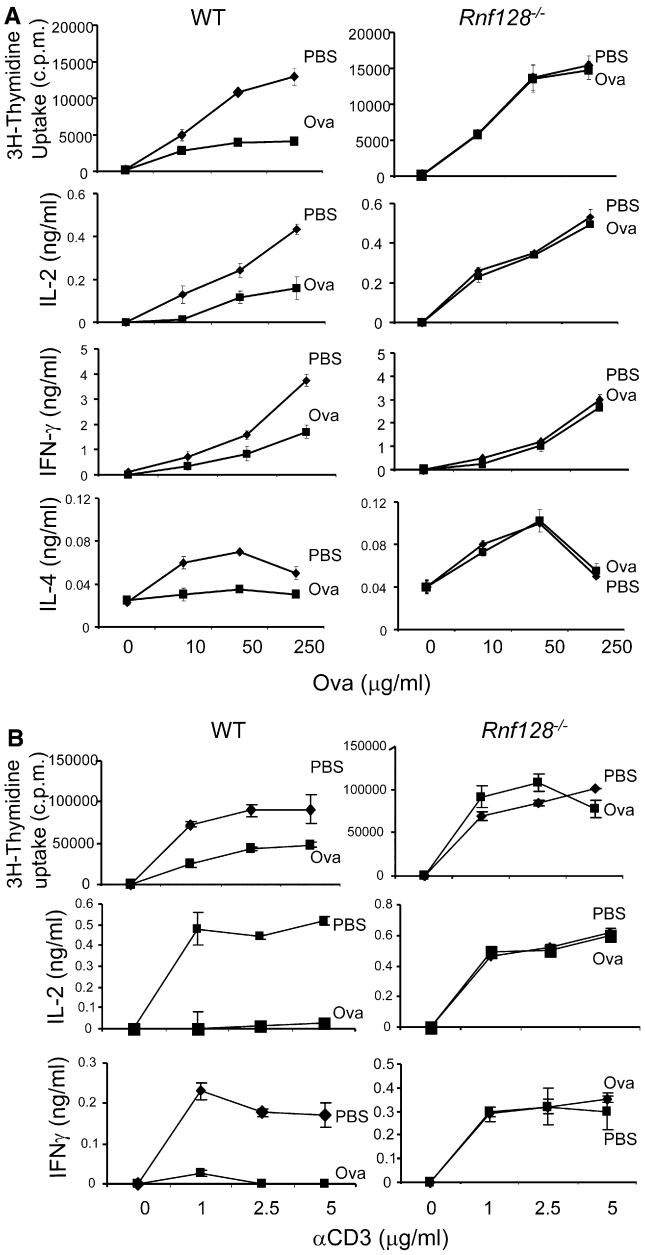

Figure 1. GRAIL Is Required for T Cell Tolerance Induction In Vivo

(A) WT and *Rnf128*$^{-/-}$ mice were fed five times with OVA or PBS. Seven days after the last feeding, all mice were immunized with OVA in CFA. Seven days later, mice were sacrificed and analyzed. Spleen cells from these mice were stimulated with the indicated concentration of Ova. Proliferation was assayed after 3 days of treatment by adding [^{3}H]-thymidine to the culture for the last 8 hr. IL-2 was measured 1 day later, and effector cytokines (IFN-γ and IL-4) were measured after 4 days of treatment. Each experimental group consisted of three mice. The graph shows means ± standard deviation (SD). Data are a representative of two individual experiments.

(B) WT and *Rnf128*$^{-/-}$ OT-II TcR transgenic mice (three mice per group) were injected twice with 500 μg of soluble Ova peptide to induce T cell tolerance or with PBS as control. Seven days after the second administration of the peptide, flow cytometry-sorted Vα2$^+$CD44$^+$CD4$^+$ T cells were isolated from spleen and restimulated with different concentrations of plate-bound anti-CD3. Proliferation, IL-2 production, and secretion of IFN-γ were assessed as in (A). The graph shows means ± standard deviation (SD). Data are a representative of two individual experiments with consistent results.

and all of exons 5 and 6 with the neomycin-resistant gene, which removes amino acids 283–385 encompassing most of the RING domain (amino acids 277–317) (Figure S1A). *Rnf128* gene targeting was confirmed by Southern blot and PCR analysis of genomic DNA from several embryonic stem cell clones (Figures S1B and S1C). The targeted ESCs were used to generate GRAIL-deficient mice. RT-PCR analysis of *Rnf128* expression in various tissues revealed that the appropriate regions of *Rnf128* were deleted (Figure S1D).

GRAIL-deficient male and female mice were viable, fertile, and grossly normal. Analysis of spleen and thymus of 6- to 8-week-old mice indicated a normal ratio of CD4$^+$ and CD8$^+$ T cells (Figure S1E). In addition, CD4$^+$ and CD8$^+$ T cells from spleen of GRAIL-deficient mice at this age did not show altered expression of activation markers CD69, CD44, and CD25 and naive T cells marker CD62L (Figure S1G). Furthermore, GRAIL-deficient mice displayed the same percentages of Treg cells in spleen and thymus as wild-type mice (Figure S1F). Thus, T cells in young GRAIL-deficient mice appear to develop normally.

GRAIL Is Required in Immune Tolerance Induction In Vivo

Next, we examined the role of GRAIL in oral tolerance, a form of peripheral tolerance. Wild-type (WT) and *Rnf128*$^{-/-}$ mice were given daily doses of 2 mg of ovalbulim (Ova) protein intragastrically for a total of five times, after subcutaneous immunization with Ova protein emulsified in complete Freud's adjuvant (CFA). Seven days after immunization, splenocytes were restimulated with different concentrations of Ova protein, and proliferation and cytokine production were examined. Whereas WT T cells from the Ova-fed group exhibited markedly reduced proliferation and IL-2, IFN-γ, and IL-4 production upon Ova protein restimulation, proliferation and cytokine production by GRAIL-deficient T cells from Ova- and PBS-fed mice were indistinguishable (Figure 1A).

To further ascertain the role of GRAIL in induction of CD4$^+$ T cell tolerance, *Rnf128*$^{-/-}$ mice were bred with OT-II TCR transgenic mice, and both WT and *Rnf128*$^{-/-}$ OT-II TCR transgenic mice were injected twice with a high dose of soluble Ova peptide to induce T cell tolerance or with PBS as control. Seven days after the second administration of the peptide, clonotypic CD4$^+$ T cells were isolated from spleen and restimulated with different concentrations of plate-bound anti-CD3. Although WT T cells from Ova-sensitized mice showed decreased proliferation, accompanied with markedly reduced IL-2 and IFN-γ production, *Rnf128*$^{-/-}$T cells from Ova- or PBS-treated mice were identical in their responses (Figure 1B). These data indicated that GRAIL controls the induction of antigen-specific CD4$^+$ T cell tolerance.

Rnf128$^{-/-}$ Mice Exhibit Increased Susceptibility to Autoimmune Diseases

Because GRAIL plays a critical role in regulating T cell tolerance in experimental models, we further investigated if deficiency of GRAIL leads to spontaneous autoimmunity in aged mice. *Rnf128*$^{-/-}$mice on C57BL/6 × 129 background at age of 2, 18–20, and 26–28 months, together with age- and sex-matched wild-type mice (Figures 2A and 2B) and *Rnf128*$^{-/-}$ mice on C57BL/6 background at age 18–20 months (Figures 2C and 2D),