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Grail controls Th2 cell development by targeting STAT6 for degradation

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Abstract

T helper (Th)-2 cells are the major players in allergic asthma; however, the mechanisms that control Th2-mediated inflammation are poorly understood. Here we find that enhanced expression of Grail, an E3 ubiquitin ligase, in Th2 cells depends on IL-4-signaling components, Stat6 and Gata3 that bind to and transactivate the *Grail* promoter. Grail-deficiency in T cells leads to increased expression of Th2 effector cytokines *in vitro* and *in vivo* and Grail deficient mice are more susceptible to allergic asthma. Mechanistically, the enhanced effector function of Grail-deficient Th2 cells is mediated by increased expression of Stat6 and IL-4 receptor α -chain. Grail interacts with Stat6 and targets it for ubiquitination and degradation. Thus, our results indicate that Grail plays a critical role in controlling Th2 development through a negative feedback loop.

CD4⁺ T helper (Th) cells are critical components in adaptive immunity. After activation, CD4⁺ T helper cells differentiate into various effector subsets characterized by expression of lineage-specific transcriptional factors and cytokines that determine their effector functions¹. Th2 cells produce interleukin (IL)-4, IL-5 and IL-13, which are important for immunity against extracellular parasites and provide help to B cells for antibody production¹⁻². However, abnormal Th2 activation is responsible for allergic inflammatory diseases such as asthma³.

Work in the past decade has revealed complex regulation of the Th2 cell differentiation program⁴⁻⁶. The cytokine IL-4 is the determining factor for Th2 differentiation^{4,6-7}. IL-4 binding to the IL-4 receptor (IL-4R) results in Signal transducer and activator of transcription 6 (Stat6) recruitment, phosphorylation, dimerization and translocation to the nucleus, where Stat6 activates the transcription factor Gata3, which is considered a Th2 master regulator⁸⁻¹⁰. T cell receptor (TCR) and costimulatory receptor signals are important for early IL-4 production during Th2 differentiation by regulating expression of the nuclear factor of activated T cells (NFAT), activating protein (AP)-1 protein JunB, and Interferon

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AUTHOR CONTRIBUTIONS

A.S. and R.N. formulated the hypothesis, designed all experiments and wrote the manuscript. A. S. performed most of the experiments. R.N. performed the overexpression and co-immunoprecipitation experiments and helped in analysis of flow cytometry results. A.A and L.O. assisted with the cloning and i.v injections for adoptive transfer respectively.

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regulatory factor 4 (IRF4)^{11–16}. In addition to IL-4, IL-2 and IL-21 cytokines are also involved in initiation of Th2 differentiation^{17–18}. However, the factors and mechanisms that control abnormal Th2 development and protect from Th2-mediated autoimmunity are poorly understood.

Recent evidence has suggested that E3 ubiquitin ligases including Cbl-b, Itch and Grail are crucial regulators of T cell activation¹⁹. Grail is a type I transmembrane protein localized to the endosomal compartment whose expression is associated with T cell anergy induction²⁰. Recently we reported that *Grail* KO mice were resistant to immune tolerance induction *in vitro* and *in vivo*²¹. In addition to tolerant T cells, *Grail* mRNA is upregulated during normal T cell activation, suggesting that Grail function might not be restricted to T cell anergy^{21–22}. *Grail* expression has also been linked to hyporesponsive Th2 cells in a model of chronic murine schistosomiasis²³. In fact, overexpression of Grail in T cell hybridomas dramatically limits production of Th2 essential cytokines such as IL-2 and IL-4²⁰, suggesting the role of Grail in controlling Th2 programming.

In the current study, high expression of *Grail* in *in vitro* differentiated Th2 cells compared to other T helper lineages is selectively induced upon IL-4 stimulation in a time dependent manner and depends on Th2-specific factors Stat6 and Gata3 that bind to and transactivate the Grail promoter. Grail deficiency in T cells leads to enhanced Th2 development *in vitro* and *in vivo*; *Grail* KO mice are more susceptible to allergic asthma. Both naïve T cells and Th2-polarized cells from *Grail* KO mice exhibit increased Stat6 transcription factor expression. Moreover, Grail interacts with Stat6 and promotes Stat6 ubiquitination and degradation. Our results suggest an important link between the Th2 specific expression of Grail and its role in control of Th2 development and Th2-mediated pathogenesis and immunity.

RESULTS

Regulation of *Grail* expression in T helper 2 cells

Although it is known that Grail is expressed in anergic cells generated both *in vivo* and *in vitro*, its expression is also upregulated during T cell activation^{21–22}. To further dissect the role of Grail in T helper cell activation and differentiation, FACS-sorted naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells were differentiated *in vitro* under Th0, Th1, Th2, and Th17 polarizing conditions and analyzed for *Grail* mRNA expression. Consistent with a previous study which showed upregulation of *Grail* in Th2 cells during chronic schistosomiasis²³, *Grail* mRNA level was significantly increased in Th2 cells compared to other T helper subsets (Fig. 1a), suggesting a possible role of Grail in Th2 cell development.

We next checked whether this increase is mediated by active transcriptional machinery in the Grail promoter region. Acetyl histone H3 lysine9/14 (AcH3) and histone H3 lysine 4 methylation (H3k4) are typically associated with active transcription while H3 lysine 27 methylation (H3k27) is mainly associated with gene silencing^{24–25}. Naïve CD4⁺ T cells differentiated under Th0 and Th2 conditions *in vitro* were assessed for abundance of AcH3, H3k4 and H3k27 in Grail promoter by chromatin immunoprecipitation (ChIP) assay. The *Grail* promoter region was significantly enriched with active histone modifications (AcH3

and H3k4) in the Th2 cells compared to Th0 cells (Fig. 1b), suggesting a role for IL-4 signaling in regulation of chromatin modification in the *Grail* locus. The IL-4 gene promoter region was analyzed as positive control (Supplementary Fig. 1). To further dissect the role of IL-4 in *Grail* regulation, we analyzed *Grail* expression in naïve CD4⁺ T cells activated with anti-CD3/28 and treated with IL-4 or anti-IL-4 for 24, 48 or 72 hrs. This analysis showed that *Grail* was expressed as early as 24 hours after T cell activation and IL-4 was essential for this induction, because blockade of IL-4 in Th0 condition decreased *Grail* expression (Fig. 1c). *Grail* expression was further increased in a time dependent manner and correlated with the level of *il4* expression. The above results suggest that *Grail* is highly expressed in Th2 cells in an IL-4 dependent manner.

As *Grail* expression is significantly increased upon IL-4 treatment, we assessed whether its expression is regulated by Stat6, a transcription factor which promotes Th2 differentiation in an IL-4-dependent manner²⁶. Naïve CD4⁺ T cells from WT and *Stat6* KO mice were cultured under Th2 conditions for four days and then assessed for *Grail* mRNA expression (Fig. 2a). Absence of Stat6 led to a significantly decreased expression of *Grail* in Th2 cells. Similarly, we found significantly low mRNA level of *Grail* in Stat6 deficient memory CD4⁺ T cells compared to WT cells (Supplementary Fig. 2a). To determine the exact role of Stat6 in regulating *Grail* expression, we assessed the status of histone modifications at the *Grail* promoter locus in WT and Stat6 deficient Th2 cells by ChIP analysis. As expected Stat6 deficient Th2 cells showed decreased active histone modifications (AcH3 and H3k4) and increased abundance of inactive H3k27 modifications in the *Grail* promoter locus (Fig. 2b). To check if this effect is mediated by direct binding of Stat6 to the *Grail* promoter locus, we performed ChIP assay with Stat6 and control antibodies (Fig. 2c). Stat6 binding was enriched in the Th2 cells compared to Th0 cells in the *Grail* promoter. Apart from Stat6, we also checked binding of Gata3, a transcription factor downstream of Stat6 and also IRF4 and Jun B transcription factors which are known to play important functions in Th2 cell differentiation^{14,16} (Fig. 2c). Similar to Stat6, Gata3 showed increased binding to the *Grail* promoter in Th2 cells compared to Th0 cells, whereas we observed moderate binding of IRF4 and JunB to *Grail* promoter locus which was not Th2 cell specific (Fig. 2c). Binding of the above factors was also analyzed at various control loci (Supplementary Fig.2b). The above factors were then over-expressed by retroviral transduction into naïve CD4⁺ T lymphocytes. Upon over-expression, both Stat6 and Gata3 facilitated a significant increase in *Grail* expression whereas IRF4 and JunB did not (Fig. 2d, upper panel). Expression of *il4* was also checked to validate our overexpression system (Fig. 2d, lower panel).

To further determine whether the binding of Stat6 and Gata3 to the *Grail* promoter resulted in its transactivation, we performed dual luciferase reporter assay by overexpression of the above factors along with the *Grail* promoter construct in EL-4 cells. As expected both Stat6 and Gata3 transactivated the *Grail* promoter (Fig. 2e). To further assess whether Gata3 is required for Stat6 activity, we used *Grail* promoter with a mutated Gata3 binding site (*GrailP-mu*) (Fig. 2e). We found that endogenous activity of *Grail* promoter was significantly lower when Gata3 site was mutated, suggesting that Gata3 is required for *Grail* expression. However Stat6 is still able to transactivate the *GrailP-mu*. Overall, these data suggest that Stat6 and Gata3 could independently regulate *Grail* expression. To further confirm these observations, we overexpressed Stat6 and Gata3 in both WT and Stat6

deficient cells. We found that over-expression of either of these two factors can rescue *Grail* expression in Stat6 deficient cells (Fig. 2f). We also checked the expression of *Il4*, *Stat6* and *Gata3* to validate our overexpression system (Supplementary Fig. 2c). Together these findings indicate that *Grail* expression is induced by IL-4 and both Stat6 and Gata3 are sufficient to induce *Grail*.

Enhanced Th2 response by Grail-deficient T cells *in vitro*

The enhanced *Grail* expression in Th2 cells led us to further check the role of Grail in Th2 cell development. Naive CD4⁺ T cells from WT and *Grail* KO mice were activated with anti-CD3 and anti-CD28 in the presence or absence of IL-4 (Fig. 3a). Enhanced expression level of the Th2-specific factors was detected in both IL-4-treated and untreated Grail-deficient cells compared to WT cells. Similarly, *in vitro* differentiated Grail deficient Th0 and Th2 cells showed increased expression of Th2 specific factors (IL-4, IL-5, IL-13 and *Gata3*) compared to WT cells (Fig. 3b and c). As expected Grail-deficient Th2 cells showed increased active histone modifications (AcH3 and H3k4) in the IL-4 and *Gata3* promoter locus and decreased abundance of inactive H3k27 modifications (Supplementary Fig. 3, right panel), suggesting that Grail deficient cells more profoundly acquire a Th2 phenotype.

Furthermore, elevated expression of Th2 cytokines by Grail deficient Th0 and Th2 cells functionally associated with enhanced IL-4R expression (Fig. 3d–e and Supplementary Fig. 4a), because IL-4 blockade under Th0 condition diminished difference in *Il4r* expression between WT and Grail deficient cells. Interestingly, Grail deficiency in naïve T cells already led to a slight enhancement in IL-4R expression, which was significantly enhanced 24 hours after activation and peaked at 48 hours (Fig. 3d–e). In addition, analysis of IL-4R/*Il4R* promoter locus in naïve Grail deficient CD4⁺ T cells showed both active and inactive histone modifications (Supplementary Fig. 3), suggesting that in the absence of Grail, IL-4R is poised for rapid transcription. Upon Th2 differentiation *Il4R* promoter locus still maintained a more open chromatin configuration in Grail-deficient cells compared to WT (Supplementary Fig.3).

IL-2 and IL-21 dependent Th2 responses in Grail KO T cells

The above results indicate the role of Grail in regulation of IL-4R expression at early stage of Th2 cell programming. In addition, we found enhanced expression of *Nfatc1* in Grail deficient T cells in an IL-4-dependent manner (Supplementary Fig. 4b). Role of NFATc1 is well known in regulation of the cytokines IL-2 and IL-21, which play a role in early Th2 development^{17–18}. As expected, mRNA expression of both *Il2* and *Il21* was increased in Grail deficient CD4⁺ T cells as early as 24 hours of activation with anti-CD3/28 in an IL-4-dependent manner, because IL-4 blockade diminished this difference (Fig. 4a and Supplementary Fig. 4b). To check whether the increased IL-2 and IL-21 levels aid in the increased Th2 responses seen in *Grail* KO Th2 cells, we blocked both IL-2 and IL-21 expression in WT and *Grail* KO CD4⁺ T cells by respective antibodies for 48 hrs. We found that blockade of either IL-2 or IL-21 led to decreased expression of Th2 cytokines IL-4, IL-5 and IL-13 and the *Il4ra* more prominently in Grail KO cells compared to WT cells (Fig. 4b). Since Grail resides in the endosome, we next treated the WT or Grail deficient T cells activated in the presence or absence of IL-4 with an endosome inhibitor Latrunculin B.

mRNA analysis 48hrs post treatment showed that expression of *Il4r* and most Th2 specific genes were upregulated upon Latrunculin B treatment (Fig. 4c–d), supporting the critical role of Grail in controlling Th2 development.

Grail promotes Th2 responses *in vivo*

To analyze the function of Grail *in vivo*, FACS-sorted memory CD4⁺CD44⁺ T cells from WT and *Grail* KO mice were analyzed. As measured by quantitative RT-PCR (qRT-PCR), Grail deficient CD4⁺ CD44⁺ effector memory cells had significantly higher expression of major Th2 specific genes like *Il4* and its receptor (Supplementary Fig. 5a) compared to WT cells. Higher IL-4, IL-5 and IL-13 protein levels were also detected by ELISA in the Grail deficient memory cells compared to WT cells (Supplementary Fig. 5b), supporting Grail as an essential factor in Th2 differentiation *in vivo*.

To further determine the role of Grail in Th2 responses *in vivo*, WT and *Grail* KO mice were immunized intraperitoneally with Ova and the adjuvant aluminum hydroxide (alum). After restimulation with Ova, T cells from *Grail* KO mice had significantly higher expression of IL-2, IL-4, IL-5 and IL-13 (Fig. 5a). Similarly, intracellular staining revealed a dramatic increase in CD4⁺IL-4⁺ cells in both the spleen and lung lymph nodes of *Grail* KO mice compared to wild-type mice (Fig. 5b). In addition, analysis of sorted CD4⁺CD44^{hi} cells from immunized mice showed that Grail deficiency led to enhanced expression of Th2 specific factors (IL-4, IL-5, IL-13, and Gata3) both at mRNA and protein levels (Fig. 5c).

To check whether the increased Th2 responses in *Grail* KO mice are T cell intrinsic, we transferred naïve WT and Grail KO CD4⁺ cells into *TCR-β* KO mice followed by Ova in alum immunization. Consistent with above data, mice reconstituted with *Grail* KO CD4⁺ T cells showed increased expression of Th2 cytokines both at mRNA and protein levels compared to mice that received WT CD4⁺ T cells (Fig. 5d–e). To further demonstrate the role of Grail in T cells, we generated mixed bone marrow chimeras by transferring a mixture of congenic CD45.1⁺ WT and CD45.2⁺ Grail-deficient bone marrow cells into sub-lethally irradiated *Rag1* KO mice. Eight weeks after reconstitution, we immunized mice with Ova protein in alum. CD4⁺CD44^{hi} Grail deficient cells exhibited enhanced Th2-specific cytokine expression compared to WT cells (Fig. 5f). The above results confirm that Grail expression in T cells is required to control Th2 programming *in vivo*.

As Th2 cells are pathogenic, we next subjected WT and *Grail* KO mice to allergic asthma using a standard protocol²¹. Histology analysis revealed that Grail deficiency resulted in significantly more inflammatory cell infiltration into the lung tissue (Fig. 6a). Consistent with histological analysis, the numbers of total cells, eosinophils, neutrophils and lymphocytes in bronchoalveolar fluid (BALF) were significantly increased in *Grail* KO mice compared to WT mice (Fig. 6b). In addition, we also observed increased levels of Th2 cytokines in BALF and lungs of *Grail* KO mice compared to their WT counterparts (Fig. 6c–d). Moreover, after restimulation *ex vivo* with Ova protein, spleen and lung-associated lymph node cells from *Grail*-KO mice produced more Th2 cytokines than did cells from WT mice (Fig. 6e–g and Supplementary Fig. 5c), suggesting that Grail is required to control the generation of proallergic helper T cells.

Grail controls Stat6 expression in Th2 cells

The above results suggest that Grail deficiency leads to Th2-hyperresponsiveness and Th2-mediated inflammation. Recently, we reported that Grail regulates CD4⁺ T cell activation by targeting TCR-CD3 complex via proteasome-mediated degradation²¹. Owing to its high expression in Th2 cells, first we assessed whether Grail controls Th2 cells by targeting CD3 ζ . However, analysis of TCR β down-regulation in WT and *Grail* KO Th2 cells upon anti-CD3 stimulation did not show difference in TCR β levels between WT and *Grail* KO cells (Supplementary Fig. 6). Next, we analyzed the protein level of Th2 transcriptional factors such as Stat6 and Gata3 in naïve WT and *Grail* KO CD4⁺ T cells either left unstimulated or stimulated with anti-CD3/28 in the presence or absence of IL-4 (Fig. 7a and Supplementary Fig. 10a). Stat6 protein levels were increased in naïve CD4⁺ T cells in *Grail* KO mice compared to WT mice. This increase was maintained after 24hrs of anti-CD3/28 stimulation either in the presence or absence of IL-4 (Fig. 7a and Supplementary Fig. 10a). Moreover, fully committed Th2 cells from *Grail* KO mice showed a more dramatic increase in Stat6 protein expression (Fig. 7b and Supplementary Fig. 10b). In addition to Stat6, Gata3 protein expression was also significantly enhanced in Grail-deficient Th2 cells (Fig. 7b and Supplementary Fig. 10b), whereas we did not detect enhanced Gata3 protein level at early steps of Th2 development (Supplementary Fig. 9 and 10g), suggesting that Grail potentially controls Gata3 expression through Stat6. To further dissect the expression and activation kinetics of Stat6 in IL-4-primed cells, we isolated naïve CD4⁺ T cells from WT and *Grail* KO mice and activated them with anti-CD3/28 and IL-4 for different time points. Interestingly, analysis of phospho-Stat6 level revealed significant activation of Stat6 in Grail deficient T cells activated in the presence of only anti-CD3/28 compared to WT cells (Fig. 7c and Supplementary Fig. 10c). This increase was further enhanced in the presence of IL-4. The total Stat6 levels were also increased in Grail-deficient T cells (Fig. 7c and Supplementary Fig. 10c), suggesting that Grail may regulate Th2 programming by targeting Stat6.

To check whether Grail directly targets Stat6, we overexpressed Stat6 together with WT or Grail mutant which lacks amino acids encompassing the RING domain (amino acids 277–317) and hence the E3 ubiquitin ligase activity in HEK293T cells along with HA-tagged ubiquitin. After immunoprecipitation of Stat6 with Stat6 antibody and reprobing with HA-HRP antibodies, we observed the formation of higher molecular weight ubiquitin conjugates in the presence of WT Grail, indicating polyubiquitin chain formation (Fig. 7d and Supplementary Fig. 10d). This ubiquitination was decreased upon lowering the concentration of WT Grail and was not present upon overexpression of the Grail mutant (Fig. 7d and Supplementary Fig. 10d). Thus, Stat6 is a potential substrate for Grail mediated ubiquitination in Th2 cells. In further investigation, we checked the endogenous ubiquitination of Stat6 in WT and Grail deficient CD4⁺ T cells activated with anti-CD3/28 and IL-4 in the presence of MG132 to block any proteasome degradation. T cell lysates were then immunoprecipitated with Stat6 antibodies and immunoblotted with ubiquitin antibodies. Compared to WT cells, ubiquitination of Stat6 in Grail deficient cells was substantially reduced (Fig. 7e and Supplementary Fig. 10e), suggesting that Grail regulates Stat6 expression and function through ubiquitin-dependent proteasome degradation pathway. Moreover, immunoprecipitation experiments performed in HEK293T cells transfected with

expression vectors for Stat6 and Grail-GFP revealed an interaction between Grail and Stat6 (Fig. 7f and Supplementary Fig. 10f), thus suggesting that ubiquitination of Stat6 by Grail is mediated by their physical interaction. However we were unable to detect interaction between endogenous Grail and Stat6 proteins due to unavailability of efficient Grail antibodies. Further experiments with the endogenous Grail would be required to establish the physical interaction between Grail and Stat6 in a physiological setting.

DISCUSSION

The molecular mechanisms leading to generation of pro-allergic Th2 cells are gradually becoming clearer. While most of the previous studies have focused on active regulators of the Th2 differentiation programme, including transcription factors like Stat6, Gata3, JunB, IRF4, c-Maf, and NFAT⁷, the mechanisms that keep Th2 cell generation and function under control remain poorly understood. In the current study, we have shown the essential role of Grail in regulating Th2 cell development and Th2-mediated pathogenic responses. *Grail* is preferentially expressed in Th2 cells, targets Stat6 for ubiquitination and restricts expression of Th2 cytokines. As a result, Grail deficient mice are more susceptible to Th2 dependent allergic responses like asthma.

It has been reported that *Grail* mRNA is upregulated during normal T cell activation even under non tolerant conditions^{21–22}. As reported previously²³, our analysis of *Grail* expression in *in vitro* differentiated different T helper subsets showed that *Grail* is highly expressed in Th2 cells, suggesting the potential role of Th2 cytokines in regulation of *Grail* expression (Fig. 1a). We tested a panel of cytokines that are known to promote Th2 development including IL-2, IL-4 and IL-21^{1,17–18,26} and found that *Grail* expression selectively depends on the presence of IL-4 (Supplementary Fig.8a). Consistent with that, upon IL-4 blockade, T cells stimulated with ant-CD3 and CD28 failed to upregulate *Grail* expression (Fig. 1c). Moreover, we observed correlation between *Grail* and *Il4* expression (Fig. 1c and Supplementary Fig. 7) which suggests that Grail could serve as a marker of pro-allergic cells where IL-4 signaling determines Grail expression. In fact, Stat6 and Gata3, major Th2-specific transcription factors⁷, bind to and transactivate the Grail promoter (Fig. 2e) and are necessary and sufficient to induce *Grail* as evident from overexpression experiments (Fig. 2d). Higher *Grail* expression was also detected in Th9 cells (Supplementary Fig.8b). This could be mediated by the IL-4-IL-4R-Stat6 signaling pathway which is important for the development of Th9 cells as well^{27–28}.

In addition to Stat6 and Gata3, transcription factors like JunB and IRF4 are also known to be involved in the Th2 developmental process; however they did not contribute to Th2-specific *Grail* expression (Fig. 2 c–d). Previously, it has been proposed that NFATc1 has the potential to induce Grail in anergic T cells²⁹. However, blockade of NFATc1 activity by CsA during Th2 development did not affect *Grail* expression and we did not detect transactivation of Grail promoter activity by overexpression of NFATc1 (Supplementary Fig. 2d–e). It is also possible that NFATc1 could cooperate with Stat6 and Gata3 to enhance and stabilize Grail expression in Th2 cells.

Since *Grail* is induced in Th2 cells by IL-4 signaling, *Grail* may be involved in differentiation of Th2 cells. Indeed, *Grail*-deficiency in CD4⁺ T cells enhanced Th2-biased differentiation *in vitro* and *in vivo* (Fig. 3, 5–6). Interestingly *Il4r* expression was also enhanced in the *Grail* deficient T cells 24 hours after IL-4 treatment and peaked at 48 hours, suggesting the role of *Grail* in early stages of Th2 cell programming by regulating *Il4r* expression (Fig. 3d and e and Supplementary Fig. 4a). Moreover, analysis of naïve CD4⁺ T cells from *Grail* deficient mice revealed that the IL-4R promoter region is enriched in both permissive H3K4me3 and the restrictive H3K27me3 modifications (Supplementary Fig. 3). This bivalent chromatin could allow minimal IL-4R expression in naïve cells but nonetheless make it poised for rapid transcriptional activation in response to subsequent IL-4 signaling favoring Th2 differentiation. IL-2 and IL-21 are known to aid in early events of Th2 cell differentiation¹⁷. We found that *Grail* deficient CD4⁺ T cells expressed substantially high amounts of IL-2 and IL-21 compared to WT cells 24 hours after IL-4 treatment (Fig.4a). In addition, these cytokines facilitate the rapid expression of *Il4r* and Th2 cytokines in *Grail* deficient CD4⁺ T cells. Our studies for the first time indicate a critical role of *Grail* in controlling of *Il4r* expression and Th2 programming.

Our previous studies as well as others indicate that *Grail* negatively regulates T cell responses by targeting components of TCR signaling pathway for degradation^{21–22}. However, analysis of TCR β down-regulation in WT and *Grail* deficient Th2 cells upon anti-CD3 stimulation did not show difference in TCR β levels between WT and *Grail* KO cells (Supplementary Fig. 6), indicating that TCR signaling is not responsible for the profound Th2 phenotype in *Grail*-deficient cells. Next, we analyzed the protein level of Th2 transcriptional factors such as Stat6 and Gata3 in naïve and IL-4 treated WT and *Grail* KO CD4⁺ T cells. We found increased Stat6 protein level in naïve *Grail* KO CD4⁺ T cells compared to WT cells and this increase was maintained in *Grail* KO cells upon IL-4 stimulation (Fig.7a–b and Supplementary Fig. 10a–b). We further determined that *Grail* interacts with Stat6 (Fig. 7f and Supplementary Fig. 10f) and the down-regulation of elevated Stat6 levels by *Grail* is dependent on E3 ligase activity and mediated by proteasome-mediated degradation (Fig. 7d–e and Supplementary Fig. 10d–e). This result suggests that *Grail*, located in endosomal compartment, may target Stat6 via ubiquitination and proteasome-mediated degradation. Increased level of Stat6 could explain why *Grail* deficient cells can spontaneously acquire a Th2 phenotype. In fact, we saw significantly enhanced Gata3 expression in fully polarized *Grail* deficient Th2 cells compared to WT cells (Fig. 7b and Supplementary Fig. 10b). The augmented total Stat6, phospho-Stat6 and Gata3 levels in the absence of *Grail* could further lead to increase in the transcription of Th2 signature cytokines by direct binding to their promoters or by regulating the chromatin accessibility to other trans-activating factors. We also found increased abundance of active histone markers (AcH3 and H3k4) and decreased levels of suppressive markers (H3k27) in the *il4*, *il4R* and *Gata3* promoter locus in *Grail* KO Th2 cells (Supplementary Fig. 3). Stat6 is known to influence the expression of an array of genes by regulating their chromatin structure³⁰. Further investigation is needed to check whether *Grail* directly targets and influences the expression and functional status of chromatin modifiers involved in Th2 gene expression.

In summary, our studies for the first time indicate the Th2 specific expression of Grail and its critical role in controlling Th2 responses. *Grail* expression is mainly mediated by IL-4 and IL-4 signaling components. It controls Th2 responses by targeting Stat6 transcription factor for degradation in activated Th2 cells. Thus, the results suggest Grail as a potential marker of allergic inflammation and provide a molecular mechanism explaining the generation of peripheral Th2 tolerance, which has implications in the prevention and treatment of allergic diseases. In addition to Grail, other E3 ubiquitin ligases such as Itch has been implicated in regulation of Th2 development by targeting JunB³¹. These E3 ligases could potentially cooperate with each other to play a synergistic role in promoting Th2 cell tolerance. Future studies in mice deficient in both Grail and Itch will potentially have therapeutic implications for allergic diseases.

METHODS

Mice and cell lines

Grail KO mice were generated as described before²¹. OT II TCR transgenic, TCR- β KO, Rag1 KO, Stat6 KO and C57BL/6J mice were obtained from Jackson laboratory. All mice were housed in the SPF animal facility at M.D. Anderson Cancer Center and the animal experiments were performed using protocols approved by Institutional Animal Care and Use Committee. The EL4 (murine lymphoma) and HEK293T (human fibroblast) cell lines were purchased from ATCC and maintained in DMEM medium supplemented with 10% fetal bovine serum and antibiotics.

T cell differentiation

T cell differentiation was performed as described previously.^{21,32} In brief, naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells were flow cytometry-sorted from lymph nodes and spleens of various mice and activated with anti-CD3 and anti-CD28 (BD Pharmingen, 2 μ g/ml) under Th0 (hIL-2 - Peprotech, 50U/ml), Th1 (anti-IL-4 - Bioexcel, 10 μ g/ml, IL-12 - Peprotech, 10ng/ml, hIL-2-50U/ml), Th2 (anti-IFN- γ -Bioexcel, 10 μ g/ml, IL-4 - Peprotech, 10ng/ml, hIL-2-50U/ml) and Th17 (anti-IFN- γ -10 μ g/ml, anti-IL-4-10 μ g/ml, IL-6 - Peprotech, 20ng/ml, TGF- β -Peprotech, 2ng/ml) polarizing conditions for 4 days. Cells were restimulated with anti-CD3 for 4 hrs before mRNA analysis

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was prepared from naïve or differentiated T cells using TriZol reagent (Invitrogen). cDNA was synthesized using Superscript reverse transcriptase and oligo(dT) primers (Invitrogen), and gene expression of beta-Actin (*Actb*), *Grail*, *Il21*, *Il4*, *Gata3*,^{21,33-34}, *Il2* (F-GCGCACCCACTTCAAGCTCCA; R-CTGTGGCCTGCTTGGGCAAGT), *Il5* (F-CGCTCACCGAGCTCTGTTG; R-CCAATGCATAGCTGGTGATTTTT), *Il13* (F-GCTTATTGAGGAGCTGAGCAACA; R-GGCCAGGTCCACACTCCATA) and *Stat6* (F-AGTCACTATAAGCCCGAACAG; R-GCCATTCCAAGATCATAAGGT) was examined with a Bio-Rad iCycler Optical System using iQ SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to Beta-Actin gene.

Retroviral transduction

Retroviral transduction was performed as described previously²¹. Briefly, naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from OT-II mice were sorted by flow cytometry and activated with Ova peptide and irradiated WT splenic antigen-presenting cells (APCs). Twenty-four hours after activation, cells were infected by retroviruses expressing Stat6, Gata3, IRF4 or JunB or control empty vector (containing only IRES-GFP). Four days after infection, GFP⁺ cells were flow cytometry-sorted and restimulated with anti-CD3 for 4 hrs, and mRNA expression were analyzed by qRT-PCR analysis. For over expression experiments in Fig. 2f and Supplementary Fig. 2b naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells were sorted by flow cytometry from C57BL/6J (WT) and *Stat6* KO mice by flow cytometry and activated with anti-CD3 and anti-CD28 for 24hrs prior to infection.

Chromatin Immunoprecipitation (ChIP) assay

Naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells were isolated and activated under Th0 or Th2 differentiation conditions. Four days later cells were restimulated with anti-CD3 (2µg/ml) for 4 hours and fixed with 1% formaldehyde for 10 minutes at 37°C. Chromatin immunoprecipitation was performed using the Millipore ChIP kit. Briefly, chromatin was sheared (500bp-100kb) and immunoprecipitated using the Abs to the following: Ach3 (06-599), H3k4me3 (07-473), H3k27me3 (074-449), Rabbit IgG (12-370) (Millipore, 2.5µg/sample), Stat6 (sc-981), IRF4 (sc-6059), JunB (sc-73) and Gata3 (sc-22206) (Santa Cruz Biotechnology, 5µg/sample). Following reversal of cross-links, the presence of selected DNA sequences was assessed by qRT-PCR using the following primer sequences: *Grail* promoter (primer 1 - F-TTTTAAGAGGCGGATCCCCGA; R-GCTGGGACTAGGTGCGAACCAG); primer 2 - F-AAATCGAGGCGCAGAGAAGTAA; R-CTAAAATGAGGCCAGCCACCT), IL-4 promoter (Histone and Stat6 ChIP respectively)³⁵⁻³⁶, HS2 locus³⁷, CNS-3 (IL-10 locus)³⁸, CNS+6.45(IL-10 locus)³⁹.

Immunoblot (IB) analysis and ubiquitination assay

Western blot analysis and ubiquitination assay was performed as described previously²¹. Briefly, whole T cell lysates were prepared by lysing cells in triton lysis buffer. The amounts of protein were determined by Bio-Rad protein assay to ensure equal protein loading for immunoblot analysis with antibodies to Stat6, phospho-Stat6 and Gata3 (Santa Cruz Biotechnology, 1:1000 dilution) and beta-Actin (Pierce, 1:1000 dilution).

For ubiquitination assays, HEK293T cells were transfected in 6-well plates with retroviral expression vector containing Stat6 (kindly provided by Mark Boothby, Vanderbilt University); bicistronic retroviral vector pGFP-RV containing either Grail or Grail mutant (Grail-M), and pcDNA-HA-ubiquitin. Stat6 protein in the cell lysates was immunoprecipitated using anti-Stat6 antibody (5µg/sample), followed by detection of ubiquitinated Stat6 by IB using anti-HA-HRP (Santa Cruz Biotechnology, 1:1000 dilution). For detecting endogenous ubiquitination of Stat6, cells were lysed in kinase lysis buffer supplemented with 1 mM N-ethylmaleimide (NEM). Stat6 protein was immunoprecipitated using Stat6 specific antibody, and the ubiquitin-conjugated Stat6 was detected by IB using anti ubiquitin (Santa Cruz Biotechnology, 1:500 dilution).

For coimmunoprecipitation experiments, HEK293T cells were transfected in 6-well plates with retroviral expression vectors containing Stat6 and Grail-GFP. Stat6 protein in the cell lysates was immunoprecipitated using anti-Stat6 antibody (5µg/sample), followed by detection of Grail by IB using anti-GFP antibody (Santa Cruz Biotechnology, 1:500 dilution).

Ovalbumin (Ova) Immunization

Male WT (C57BL/6J) and *Grail* KO mice (6–8 weeks old, n=5per group) were immunized with Ova protein (1.0 mg/ml) emulsified in aluminum hydroxide (Alum) intraperitoneally. Seven days after immunization, mice were sacrificed and analyzed individually. Spleen cells from Ova-immunized mice were stimulated in 96-well plates as triplicates with or without different concentrations of Ova protein. IL-2 production was determined 24 hours after T cell activation by ELISA. Effector cytokines were analyzed 3 days later by ELISA (PharMingen).

Adoptive transfer and mixed bone marrow chimera studies

Adoptive transfer and bone marrow chimera experiments were done as described previously⁴⁰. Briefly, FACS sorted naïve CD4⁺ T cells from 8 week old male WT or *Grail* KO mice were intravenously transferred into 6–8 week old male *TCR-β* KO mice (10 × 10⁶ cells/mouse, n=4 per group). The recipient mice were immunized i.p. with Ova in alum 24 hrs post transfer. Seven days after immunization, spleen cells from the recipient mice were isolated and CD4⁺CD44^{hi} effector memory cells were sorted for analysis.

To generate mixed bone marrow chimera, T cell-depleted bone marrow cells were obtained from 6–8 weeks old male B6.SJL (CD45.1) or *Grail* KO mice and mixed 1:1 ratio before being transferred into irradiated male (6–8 weeks old, n=10) *Rag1* KO mice (10 × 10⁶ cells/mouse, 750 rad). Eight weeks later, the recipient mice were immunized with Ova in alum and analyzed as above.

Asthma induction

Asthma induction was done as described previously^{21,41}. Briefly, male WT (C57BL/6J) and *Grail* KO mice (6–8 weeks old, n=5per group) were immunized twice intraperitoneally at 2 weeks interval with 0.2 ml saline containing 100 µg Ova in Alum. On day 14 mice were intranasally challenged with Ova followed by three more challenges at day 26, 27 and 28. 24 hours post the last challenge mice were sacrificed and the BALF, lungs, spleens and the lung lymph nodes were harvested for further analysis.

Statistical analysis

The results shown are mean ± SEM. Student's t-test was performed to detect between-group differences. A P value of <0.05 was rendered statistically significant. P values: *<0.05, **<0.01, ***<0.001 and ****<0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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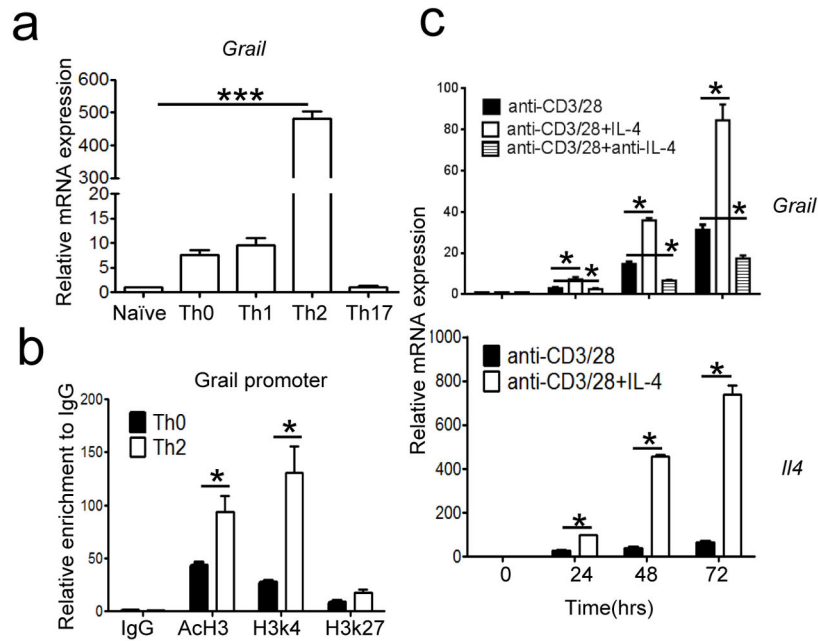


Figure 1. Selective expression of *Grail* in T helper 2 cells

(a) Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells from C57BL/6 mice were activated with anti-CD3 and anti-CD28 and cultured for 4 days under Th0, Th1, Th2 and Th17 polarizing cell conditions. *Grail* mRNA expression was analyzed by qRT-PCR analysis. (b) Chromatin immunoprecipitation (ChIP) analysis of histone H3 acetylation (AcH3), trimethyl histone H3 lysine 4 (H3k4) and trimethyl histone H3 lysine 27 (H3K27) methylation at *Grail* promoter locus in $CD4^+$ T cells polarized under Th0 and Th2 conditions. The data from each replicate were normalized to the input control and the graphs represent fold enrichment of the indicated proteins to control antibody (rabbit IgG) at the designated locus. (c) Naïve $CD4^+$ T cells were cultured under indicated conditions and *Grail* and *Il4* mRNA expression was analyzed in different time points by qRT-PCR. The data shown in A and C were normalized by the expression of a reference gene *Actb*. The results shown are mean \pm SEM. P values: * <0.05 , *** <0.001 , Student's t-test was performed to detect between-group differences. t test. The data are representative of at least three independent experiments with consistent results.

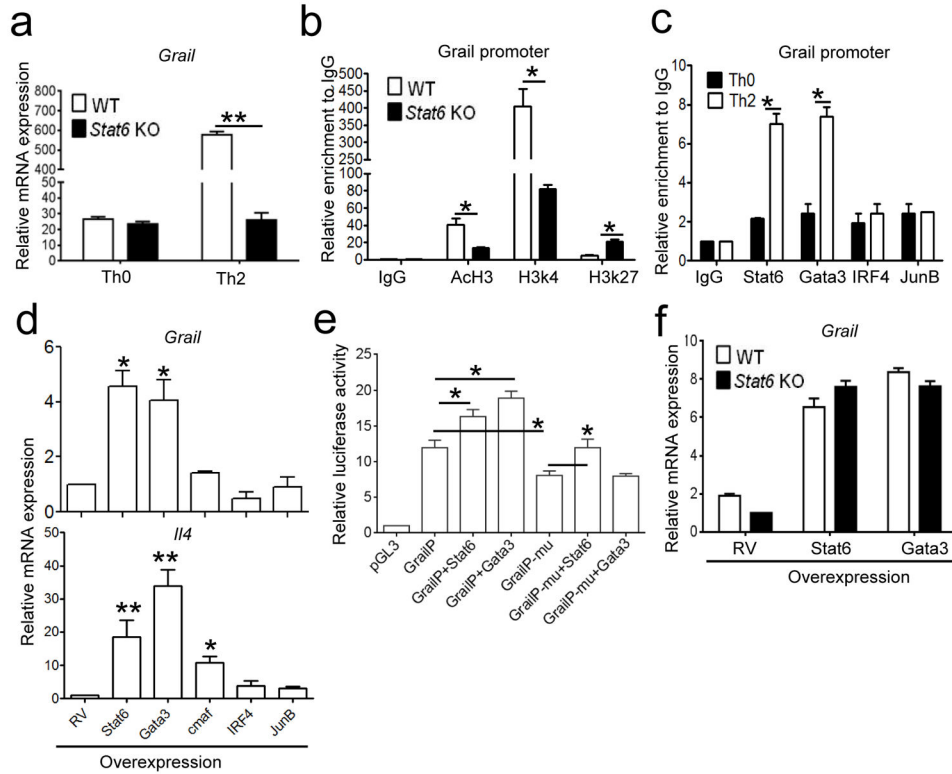


Figure 2. Regulation of *Grail* expression in Th2 cells

(a) Naïve CD4⁺ T cells from WT and *Stat6* KO mice were cultured for 4 days under Th0 and Th2 conditions and analyzed for *Grail* mRNA expression by qRT-PCR. (b) ChIP analysis of histone H3 acetylation (AcH3), trimethyl histone H3 lysine 4 (H3k4) and trimethyl histone H3 lysine 27 (H3k27) at *Grail* promoter locus in CD4⁺ T cells from WT and *Stat6* KO mice polarized under Th2 condition. The data from each replicate were normalized to the input control and the graphs represent fold enrichment of the indicated proteins to control antibody (rabbit IgG) at the designated locus. (c) ChIP assay to check binding of indicated transcription factors to the *Grail* promoter locus in naïve CD4⁺ T cells polarized under Th0 and Th2 conditions. (d) *Grail* and *Il4* mRNA expression in naïve CD4⁺ T lymphocytes infected with bicistronic retroviruses expressing the indicated factors. (e) Luciferase assay in EL-4 cells transfected with *Grail* promoter (*Grail*-P) containing luciferase vector or with a mutated *Gata3* site (*Grail*P-mu) promoter along with vectors containing the indicated factors. Luciferase activity is expressed relative to the expression of cotransfected renilla luciferase plasmid as a control for transfection efficiency. Relative luciferase units are expressed as a fold difference to the control (vector only, pGL3) value. (f) *Grail* mRNA expression in naïve CD4⁺ T lymphocytes from WT and *Stat6* KO mice infected with bicistronic retroviruses expressing the indicated factors. The data shown in a, d and f were normalized by the expression of a reference gene *Actb*. The results shown are mean ± SEM. P values: * < 0.05, ** < 0.01, Student's t-test was performed to detect between-group differences. The data are representative of least three independent experiments with consistent results.

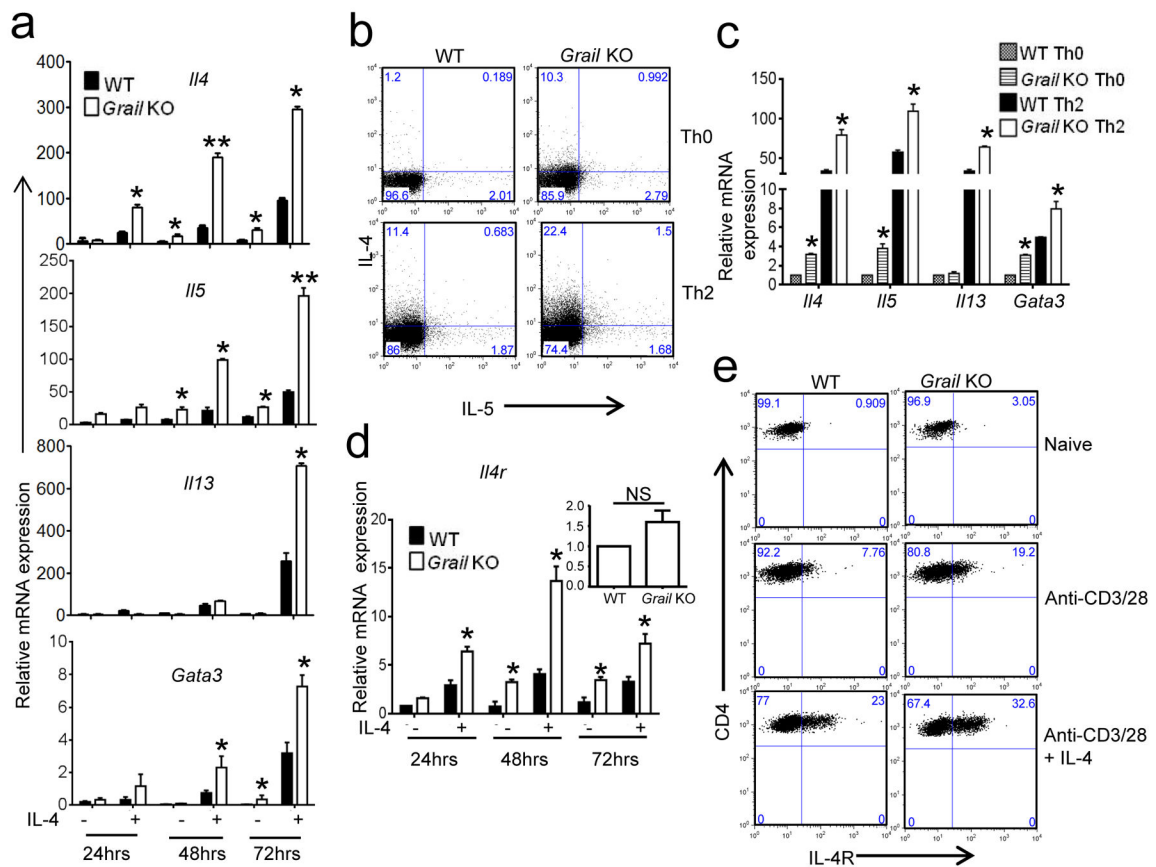


Figure 3. Grail deficiency upregulates Th2 cytokines and IL-4 receptor *in vitro*

(a) Naïve CD4⁺ T cells from WT and *Grail* KO mice were activated with anti-CD3 and anti-CD28 in the presence or absence of IL-4 for 24, 48 and 72 hours and analyzed for expression of Th2 specific genes by qRT-PCR. (b–c) Naïve CD4⁺ T cells from WT and *Grail* KO mice were cultured under Th2 conditions for 4 days and analyzed for IL-4 and IL-5 expression by intracellular cytokine staining (b). Numbers in dot plot quadrants represent the percentages. mRNA levels of the indicated genes was analyzed (c). (d) Naïve CD4⁺ T cells from WT and *Grail* KO mice were activated as in (a) and analyzed for *Il4r* expression by qRT-PCR. (e) Naïve CD4⁺ T cells from WT and *Grail* KO mice were activated for 48 hours as indicated and analyzed for IL-4R expression by FACS. Numbers in dot plot quadrants represent the percentages. The data shown in a, c and d were normalized by the expression of a reference gene *Actb*. The results shown are mean ± SEM. P values: *<0.05, **<0.01, Student's t-test was performed to detect between-group differences. The data are representative of at least three independent experiments with consistent results.

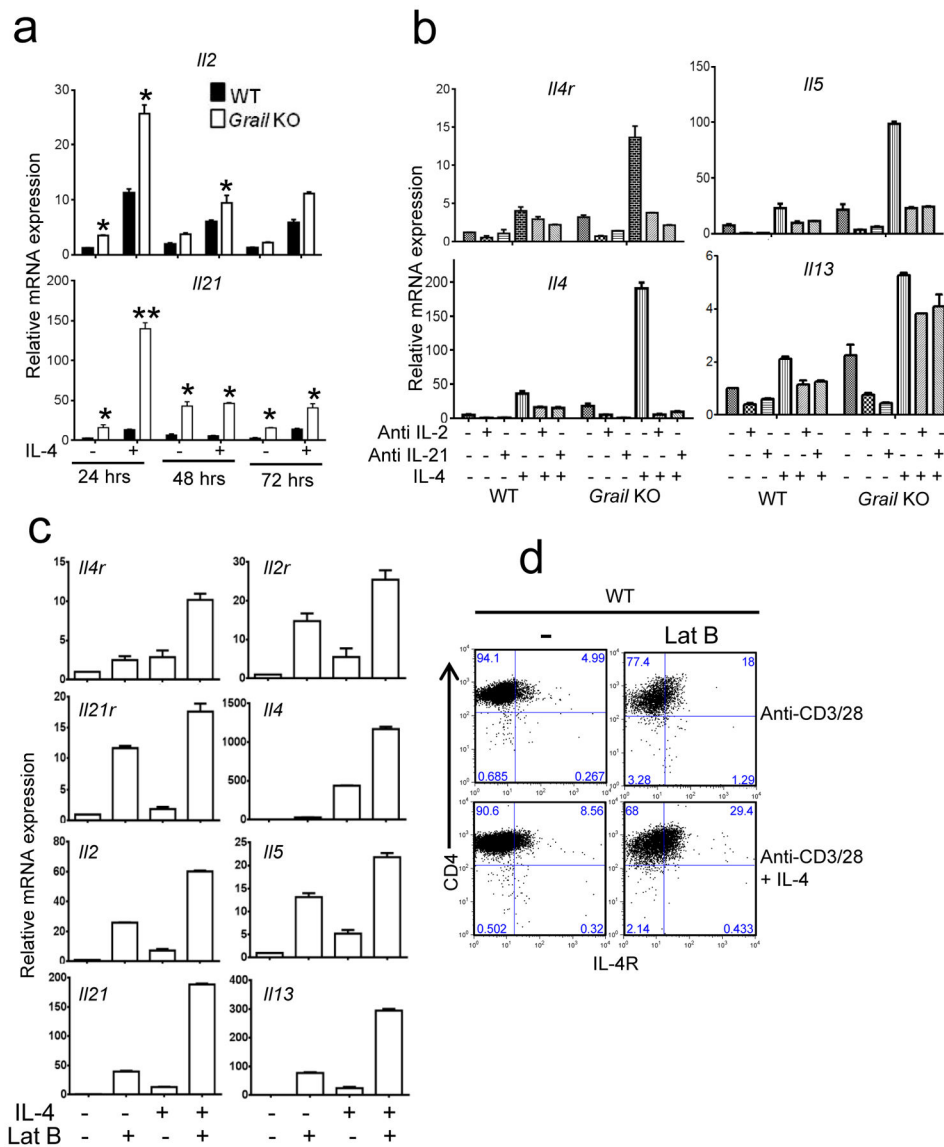


Figure 4. Grail controls IL-4 receptor expression in Th2 cells

(a) Naïve CD4⁺ T cells from WT and *Grail* KO mice were activated with anti-CD3 and anti-CD28 in the presence or absence of IL-4 for 24, 48 and 72 hours and analyzed for expression of *I12* and *I121* by qRT-PCR. (b) Naïve CD4⁺ T cells from WT and *Grail* KO mice were activated with anti-CD3 and anti-CD28 along with the indicated conditions for 48 hrs and the mRNA expression of the indicated genes was analyzed. (c–d) Naïve CD4⁺ T cells from WT mice were activated with anti-CD3 and anti-CD28 under indicated conditions and treated with an endosome inhibitor, Latrunculin B for 48 hours. (c) mRNA expression of Th2-specific genes was analyzed by qRT-PCR. (d) IL-4R protein expression was analyzed by FACS. Numbers in dot plot quadrants represent the percentages. The data shown in a, b and c were normalized by the expression of a reference gene *Actb*. The results shown are mean ± SEM. P values: * <0.05 , ** <0.01 , Student's t-test was performed to detect between-

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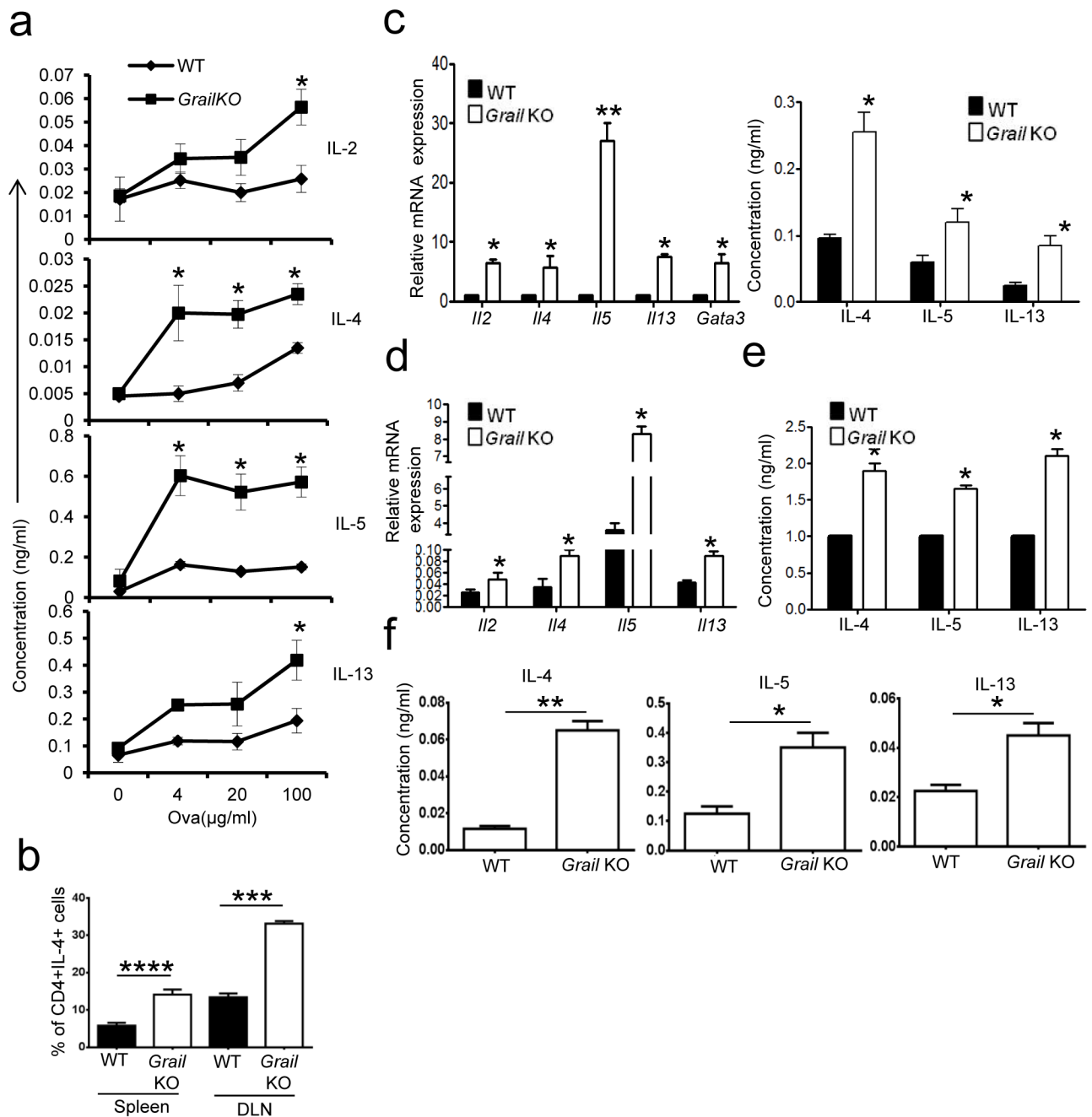


Figure 5. Grail deficiency leads to saturated Th2 responses *in vivo*

(a–c) Male WT and Grail KO mice (6–8 weeks, n=5 per group) were intraperitoneally injected with Ova in alum. (a) Seven days after immunization splenocytes were restimulated with different concentrations Ova and Th2 cytokines were measured by ELISA. (b) Percentage of Ova-specific CD4⁺IL-4⁺ cells in spleen and lung lymph nodes of WT and Grail KO mice. (c) CD4⁺CD44⁺ cells were sorted from the spleen and lung lymph nodes of immunized mice and expression of Th2 genes was analyzed by qRT-PCR and ELISA. The data shown were normalized by the expression of a reference gene *Actb*. (d–e) FACS sorted

naïve CD4⁺ T cells from WT and *Grail* KO mice were intravenously transferred into *TCR-β* KO mice (5 million cells per mouse, n=4 per group) followed by intraperitoneal injection with Ova in alum. Seven days later WT and *Grail* KO CD4⁺CD44⁺ cells were sorted and effector cytokine levels were measured by qRT-PCR analysis (d) and ELISA (e). (f) Mixed-bone marrow chimeric mice (n = 10) were generated by co-transfer of 10 million cells at a ratio of 1:1 WT (CD45.2) and *Grail* KO (CD45.1) bone marrow cells into *Rag1* KO mice. 8 weeks later, mice were immunized with Ova in alum. One week later and WT and *Grail* KO CD4⁺CD44⁺ cells were sorted and effector cytokine levels were measured by ELISA. The results shown are mean ± SEM. P values: *<0.05, **<0.01, ***<0.001, ****<0.0001, Student's t-test was performed to detect between-group differences.. The data are representative of at least three independent experiments with consistent results.

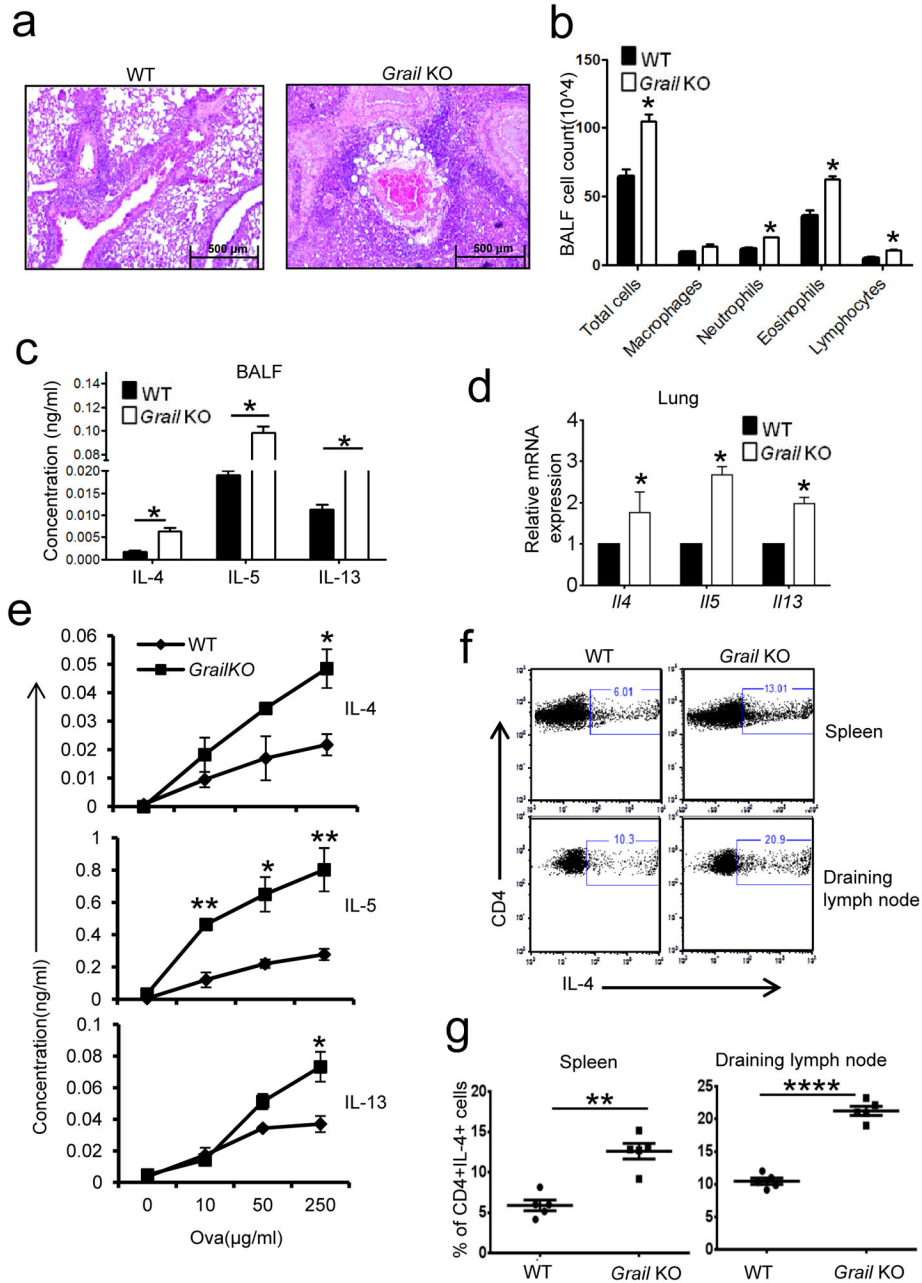


Figure 6. *Grail* deficient mice are more susceptible to allergic asthma

Groups of WT and *Grail* KO mice (6–8 weeks, n=5 per group) were intraperitoneally sensitized with Ova in alum with 2 weeks interval, followed by intranasal challenge with Ova. **(a)** Mononuclear cell infiltration in lung tissues from WT and *Grail* KO mice subjected to asthma was analyzed by H&E staining. Original magnification X20. Scale bar: 1cm=500 μ m. **(b)** Bronchoalveolar lavage fluids (BALF) were analyzed to measure airway infiltrating cells. **(c)** Protein levels of Th2 cytokines in BALF was analyzed by ELISA. **(d)** mRNA expression of effector cytokines in the lungs of asthmatic WT and *Grail* KO mice were analyzed by qRT-PCR. The data shown were normalized by the expression of a

reference gene *Actb*. (e) Lung lymph node cells from WT and *Grail* KO mice were restimulated with different concentrations Ova and Th2 cytokines were measured by ELISA. (f) IL-4 expression by CD4⁺ T cells from lung lymph nodes of WT and *Grail* KO mice following Ova restimulation was accessed by intracellular cytokine staining. Numbers in dot plot quadrants represent the percentages. (g) The percentage of CD4⁺IL-4⁺ cells in spleen and lung lymph nodes of asthmatic WT and *Grail* KO mice. The results shown in **b**, **c**, **d** and **e** are mean \pm SEM. P values: * <0.05 , ** <0.01 , Student's t-test was performed to detect between-group differences.. The data are representative of at least three independent experiments with consistent results.

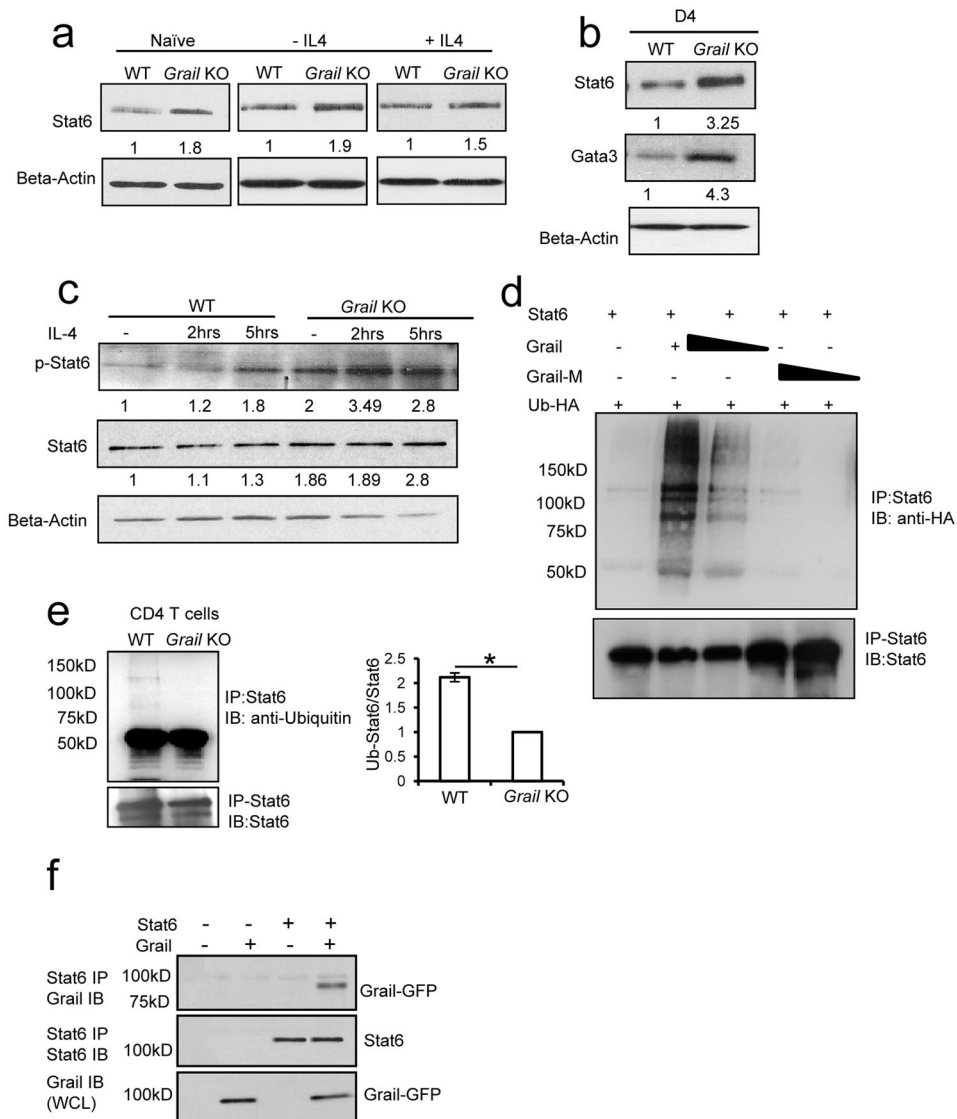


Figure 7. Grail negatively regulates Stat6 expression and activity

(a) Naïve CD4⁺ T cells from WT and *Grail* KO mice were activated with anti-CD3/anti-CD28 in the presence or absence of IL-4 for 24 hours, and Stat6 protein expression were detected by western blot analysis. (b) Naïve CD4⁺ T cells from WT and *Grail* KO mice were cultured for 4 days under Th2 differentiation conditions and analyzed for Stat6 and Gata3 expression by western blot analysis. The numbers in a and b depict relative immune blotting signals compared to WT after normalizing with Beta-actin levels. (c) Naïve CD4⁺ T cells from WT and *Grail* KO mice were isolated and serum starved for 12 hours followed by activation with anti-CD3/anti-CD28 in the presence or absence of IL-4 for the indicated time points and phospho-Stat6 and total Stat6 levels were determined by western blot analysis. Beta-actin levels were determined in the same samples as loading control. The numbers depict relative immune blotting signals compared to WT anti-CD3/CD28 only treated samples after normalizing with Beta-actin levels. (d) HEK293T cells were transfected with vectors encoding Stat6, HA-Ub and either Grail or a Grail mutant. The lysates were

subjected to immunoprecipitation (IP) using anti-Stat6 antibody. The blot was probed with anti-HA-HRP and re-probed for Stat6 expression. (e) CD4⁺ T cells from WT and *Grail* KO mice were activated with anti-CD3 and IL-4 along with MG132 (proteasome inhibitor) for 6 hrs. The cell lysates were then immunoprecipitated using anti-Stat6 antibody and the blot was probed with anti-ubiquitin or anti-Stat6 antibodies. Relative western blotting signals were calculated as a ratio of ubiquitinated and non-ubiquitinated Stat6. (f) HEK293T cells were either left untransfected or transfected with vectors encoding Stat6, Grail-GFP or both. The lysates were subjected to IP using anti-Stat6 antibody. The blot was probed with anti-GFP antibody (upper panel) and re-probed with anti-Stat6 antibody (middle panel) as a control. The WCL was immunoblotted with anti-GFP antibody (lower panel) for checking antibody efficacy. The results shown in the figures are representative blots of at least three independent experiments. P values: * <0.05 , Student's t-test was performed to detect between-group differences..