Peli1 negatively regulates T-cell activation and prevents autoimmunity

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Abstract

T-cell activation is subject to tight regulation to avoid inappropriate responses against self-antigens. Here we show that genetic deficiency in an ubiquitin ligase, Peli1, causes hyper activation of T cells and renders T cells refractory to suppression by T regulatory cells and transforming growth factor (TGF)-β. As a result, *Peli1* knockout mice spontaneously develop autoimmunity, characterized by multiorgan inflammation and autoantibody production. Peli1 deficiency results in accumulation of nuclear c-Rel, a member of the NF-κB family of transcription factors with pivotal roles in T-cell activation. Peli1 negatively regulates c-Rel by mediating its K48 ubiquitination. These results identify Peli1 as a critical factor in the maintenance of peripheral T-cell tolerance and reveal a novel mechanism of c-Rel regulation.

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INTRODUCTION

T cells are primary players in adaptive immune responses against invading pathogens. Upon antigen engagement, the T cell receptor (TCR) signals T cells activation in cooperation with signals delivered by costimulatory molecules, most importantly CD28 [1]. Activated CD4+ and CD8+ T cells differentiate into various effector T cells that participate in pathogen clearance either directly or indirectly, by providing help for activation of other immune cells. In contrast to their efficient response to foreign antigens, T cells are normally tolerant to antigens of self-tissues, thus preventing the development of autoimmunity [2]. T cell tolerance to self-antigens is controlled by different mechanisms. In addition to the developmental deletion or inactivation of autoreactive T cells by mechanisms of central tolerance [2], peripheral T cells are tightly controlled by both extrinsic and intrinsic factors [3]. Among the extrinsic factors, T regulatory (Treg) cells suppress naïve T-cell activation through both physical interaction and secretion of immunosuppressive cytokines, such as TGFβ and IL-10 [4–7]. Intrinsic factors include various molecules that negatively regulate the TCR and CD28 signals [8]. Thus, autoimmunity may occur due to either Treg defects or impaired negative regulation of TCR-CD28 signaling.

Several negative regulators of the TCR-proximal signaling have been described [9], although relatively less is known about the regulation of downstream signaling events. A critical downstream signaling event triggered by the TCR-CD28 signals is activation of the NF-κB pathway, a family of transcription factors required for T cell activation and differentiation [10, 11]. Mammalian NF-κB is composed of five members, RelA, RelB, c-Rel, p50, and p52, which form various dimeric complexes and transactivate target genes via binding to an enhancer element, κB. In resting T cells, NF-κB proteins are sequestered in the cytoplasm by inhibitory proteins, termed IκBs. Canonical pathway of NF-κB activation involves phoshorylation of IκBα by the IκB kinase (IKK) and subsequent IκBα degradation, which triggers the nuclear translocation of NF-κB dimers. An important NF-κB family member involved in T cell activation is c-Rel, which mediates cytokine production, proliferation, and differentiation of T cells [12–17]. Deficiency in c-Rel renders T cells more susceptible to tolerance induction [18]. In contrast to the rapid and transient nature of RelA activation, the induction of c-Rel nuclear translocation is delayed and more persistent and critically dependent on CD28 costimulation [19–21]. Although RelA is subject to tight control by IκBα-mediated feedback regulation, the negative regulation of c-Rel activation remains unclear.

Ubiquitination has emerged as a critical mechanism that regulates T-cell activation [9, 22]. The Cbl family of ubiquitin ligases mediates lysine (K) 48 ubiquitination and degradation of TCR-proximal signaling factors, thereby negatively regulating T-cell activation [23, 24]. On the other hand, ubiquitin ligases that catalyze K63-linked ubiquitin chains mediate IKK activation and positively regulate NF-κB signaling [22]. More recently, a new family of E3 ligases, termed Peli (or Pellino), has been shown to catalyze formation of both K63 and K48 ubiquitin chains [25–27]. Mammalian Peli family is composed of three members, Peli1, Peli2, and Peli3, which share strong sequence homology and structural domains [28, 29]. The E3 ubiquitin ligase function of Peli proteins is dependent on their C-terminal RING domain [25–27]. *In vitro* studies suggest that Peli proteins interact with IRAK1 and mediate

activation of NF-κB and MAP kinases by Toll-like receptors (TLRs) and interleukin 1 receptor (IL-1R) [28, 29]. In addition, Peli1 has an essential role in mediating NF-κB activation by TRIF-dependent TLRs, such as TLR3 and TLR4, although Peli1 is dispensable for NF-κB activation by the MyD88-dependent TLRs and IL-1R [30]. Since Peli1 possesses both K63 and K48 E3 ligase activities, it remains an intriguing question whether it mediates distinct biological functions.

In the present study, we describe a new function of Peli1 in the regulation of T-cell activation and homeostasis. We found that Peli1 serves as a critical negative regulator of T cell activation and prevents the development of autoimmunity. This function of Peli1 is mediated through targeting c-Rel for K48 ubiquitination and degradation. *Peli1* deficiency does not affect the activation of IKK but causes accumulation of nuclear c-Rel in the activated T cells. Consequently, the Peli1-deficient T cells are hyperresponsive to TCR-CD28 stimulation, and the *Peli1* knockout (KO) mice develop autoimmunity. These data establish Peli1 as a critical factor that controls T cell homeostasis and peripheral tolerance and highlight a novel mechanism of c-Rel regulation. Since Peli1 does not effect upstream TCR signaling, it may be exploited as a therapeutic target for manipulating T cell responses.

RESULTS

Peli1 is a negative regulator of T cell activation

As an approach to assess the physiological functions of Peli1, we searched for its expression profile in the BioGps database (http://biogps.gnf.org). *Peli1* showed abundant expression in lymphocytes, and neither *Peli2* nor *Peli3* displayed such an expression pattern. Real-time RT-PCR confirmed that *Peli1* was abundantly expressed in lymphocytes, particularly T cells (Supplementary Fig. 1a). Furthermore, the level of Peli1 was induced along with activation of T cells by agonistic anti-CD3 and anti-CD28 antibodies (Supplementary Fig. 1b, c).

To assess the function of Peli1 in T cell activation, we stimulated the wild-type (*Peli1*$^{+/+}$) and *Peli1* knockout (*Peli1*$^{-/-}$) T cells *in vitro* using anti-CD3 and anti-CD28. In contrast to its positive role in TRIF-dependent TLR signaling [30], Peli1 displayed a potent negative function in T cell activation. The *Peli1*$^{-/-}$ CD4$^+$ T cells were hyperresponsive to CD3-CD28 stimulation, as revealed by their heightened production of the major T cell cytokines IL-2 and interferon-γ (IFN-γ) (Fig. 1a). This phenotype was even more striking in *Peli1*$^{-/-}$ CD8$^+$ T cells (Fig. 1b). In response to both anti-CD3 and anti-CD28 and T cell mitogens (PMA plus ionomycin) activation, the *Peli1*$^{-/-}$ CD8$^+$ T cells produced higher amounts of IL-2 and IFN-γ than the wild-type CD8$^+$ T cells (Fig. 1b, c). Moreover, the hyperresponsive phenotype was also readily detected with purified naïve *Peli1*$^{-/-}$ T cells (Fig. 1d and data not shown).

We examined T cell proliferation using dilution of the CFSE dye. Both total and naïve *Peli1*$^{-/-}$ CD8$^+$ T cells displayed markedly enhanced proliferation ability (Supplementary Fig. 2a, b). Notably, the loss of Peli1 rendered T cells capable of responding to TCR stimulation in the absence of CD28 ligation. Whereas wild-type T cells were only weakly stimulated at low doses of anti-CD3 antibody, the *Peli1*$^{-/-}$ T cells displayed strong proliferation ability when stimulated with even low doses of CD3 antibody. The effect of

Peli1 deficiency on CD4$^+$ T cell proliferation was less prominent (data not shown). Taken together, these results demonstrate a critical role for Peli1 in negatively regulating T cell activation.

Peli1 deficiency causes spontaneous activation of T cells *in vivo*

To assess the role for Peli1 in regulating T cell homeostasis *in vivo*, we analyzed the activation status of CD4$^+$ and CD8$^+$ T cells based on their surface expression of activation markers, CD44 and CD62L. In wild-type mice, the majority of T cells isolated from the spleen were in a naïve state, characterized by a CD44^{lo}CD62L^{hi} phenotype (Fig. 2a)., *Peli1$^{-/-}$* mice did not show obvious abnormality in the frequency of naïve and memory T cells at an young age (5–8 weeks; data not shown) and only displayed a moderate increase in memory CD44^{hi}CD62L^{lo} T cells at an older age (10 weeks;Fig. 2a). However, at 6 months of age or older, *Peli1$^{-/-}$* mice had a higher frequency of memory T cells and a concomitant decrease in naïve T cells in the spleen (Fig. 2a, b). A similar phenotype was detected in peripheral lymph nodes (Supplementary Fig. 3), although it was moderate in the mesentery lymph nodes (Supplementary Fig. 3). The absolute number of memory T cells was also higher in the spleen and peripheral lymph nodes of the *Peli1$^{-/-}$* mice (Supplementary Fig. 4). These results suggest that Peli1 plays a critical role in maintaining the homeostasis of T cells *in vivo*, in line with the *in vitro* hyperresponsiveness of the Peli1-deficient T cells.

Peli1 is a T cell intrinsic regulator of activation

To examine whether the role of Peli1 in T cell regulation is cell intrinsic, we performed mixed bone marrow adoptive transfer experiments. Under such experimental conditions, wild-type and *Peli1$^{-/-}$* T cells develop and are homeostatically maintained in the same *in vivo* environment. Ten weeks following mixed bone marrow adoptive transfer, we analyzed the frequency of memory and naïve T cells derived from the *Wild-type* and *Peli1$^{-/-}$* bone marrow. Compared to wild-type T cells, *Peli1$^{-/-}$* T cells had a marked increase in memory T cells (CD44^{hi}CD62L^{lo}) and concomitant decrease in naïve T cells (CD44^{lo}CD62L^{hi}) (Fig. 2c). This phenotype was detected in different peripheral lymphoid organs, including peripheral lymph nodes (Fig. 2c), spleen (Supplementary Fig. 5a), and mesentery lymph nodes (Supplementary Fig. 5b). Therefore, Peli1 has a T cell intrinsic role in the negative regulation of T cell activation.

Peli1$^{-/-}$ T cells are refractory to suppression by Tregs and TGF-β

Since T$_{reg}$ cells play a critical role in maintaining peripheral T cell tolerance, we asked whether Peli1 influences T$_{reg}$ development. We analyzed the frequency of T$_{regs}$ in the thymus and the peripheral lymphoid organs (spleen and lymph nodes) based on their expression of the transcription factor Foxp3 and the surface expression of CD4$^+$ and CD25. *Peli1$^{-/-}$* mice did not show a reduction in the frequency of T$_{reg}$ cells (Fig. 3a). We detected a slightly higher frequency of T$_{reg}$ cells in both the thymus and the peripheral lymphoid organs of young *Peli1$^{-/-}$* mice (Fig. 3a, b). This phenotype became more prominent in older *Peli1$^{-/-}$* mice (6 months; Fig. 3b), probably due to the higher percentage of memory T cells in the mutant mice. Notwithstanding, these results suggest that *Peli1* deficiency does not cause a defect in T$_{reg}$ development.

The strength of TCR-CD28 signaling impacts target T cell sensitivity to T_{reg} suppression [5]. Because $Peli1^{-/-}$ T cells are hyperresponsive to TCR-CD28 stimulation (Fig. 1), we examined whether the mutant T cells are more resistant to T_{reg}-mediated suppression. Wild-type or $Peli1^{-/-}$ naïve CD4$^+$ T cells were activated in the presence of increasing ratios of T_{reg} cells derived from wild-type mice, and the proliferation of the target T cells was measured based on CFSE dilutions. As expected, the proliferation of wild-type CD4$^+$ T cells was suppressed by the T_{reg} cells in a dose dependent manner (Fig. 3c), while $Peli1^{-/-}$ T cells were less sensitive to T_{reg}-mediated suppression (Fig. 3C). On the other hand, loss of Peli1 did not affect the effector function of T_{reg} cells (Supplementary Fig. 6).

Because T_{reg} cells are known to inhibit target cell activation by secreting TGF-β [31, 32], we examined the effect of TGF-β on CD3 and CD28-mediated T cell activation. TGF-β effectively suppressed the activation of wild-type T cells (Fig. 3d), while under the same conditions $Peli1^{-/-}$ T cells were markedly less susceptible to TGF-β-mediated suppression (Fig. 3d). This phenotype was particularly prominent for CD8$^+$ T cells, although it was also detected for CD4$^+$ T cells (2.8 fold inhibition of $Peli1^{-/-}$ as compared to 7.5 fold inhibition of wild-type CD4$^+$ T cells; Fig. 3d).

Peripheral T-cell tolerance also involves the induction of anergy, as a result of TCR stimulation in the absence of costimulation [41]. We examined the role of Peli1 in regulating T cell anergy using a well-characterized *in vivo* oral tolerance model. We crossed wild-type and $Peli1^{-/-}$ mice to transgenic mice expressing the ovalbumin-specific TCR (OT-II) and induced T cell tolerance by feeding the mice with OVA peptide. As expected, OVA peptide induced T cell anergy in *Wild-type* OT-II mice, as indicated by a 37.1% inhibition of antigen-stimulated T cell proliferation (Fig. 3e). Peli1-deficiency reduced, although did not completely block (14.4% inhibition), the induction of T cell anergy (Fig. 3e). Collectively, these data suggest that *Peli1* deficiency renders CD4$^+$ and CD8$^+$ T cells less susceptible to suppression by T_{reg} cells and TGF-β as well as to anergy induction, a phenotype that has also been observed with T cells lacking some other negative regulators of T cell activation[5, 33, 34].

$Peli1^{-/-}$ mice spontaneously develop autoimmunity

Since T cell abnormal activation is often associated with autoimmunity, we examined potential autoimmune phenotypes of the $Peli1^{-/-}$ mice. The peripheral lymph nodes of the mutant mice were enlarged as early as 10 weeks of age (Fig. 4a). At the age of 6 months, mutant mice also displayed moderate splenomegaly (Supplementary Fig. 7). Moreover, old $Peli1^{-/-}$ mice has increased cellularity in the spleen and peripheral lymph nodes, although this phenotype was moderate in mesentery lymph nodes (Fig. 4b). Histology analyses detected severe immune cell infiltration in multiple organs, including kidney, liver, and lung (Fig. 4c). Both CD4$^+$ and CD8$^+$ T cells were readily detected by immunohistochemistry assays in the inflammatory lesions of the $Peli1^{-/-}$ mice (Fig. 4d), and the lesions also contained B220$^+$ B cells (Supplementary Fig. 8). These results are typical of autoimmune diseases associated with aberrant T cell responses. To further examine the autoimmune status of the $Peli1^{-/-}$ mice, we investigated the presence of serum autoantibodies and immune complex deposition in kidney glomeruli. $Peli1^{-/-}$ mice contained significantly

higher amounts of antinuclear antibodies (ANA) in the serum (Fig. 4E) and also displayed severe immune complex deposition in the kidney glomeruli (Fig. 4F).

We also examined the role of Peli1 in inducible autoimmunity and we used EAE (experimental autoimmune encephalomyelitis) as an experimental model. To avoid a contribution from innate immune cells, which also express Peli1, EAE was induced using a T cell transfer model, in which wild-type and $Peli1^{-/-}$ T cells were transferred into $Rag1^{-/-}$ mice. Consistent with their hyperresponsive phenotype, $Peli1^{-/-}$ T cells induced EAE with much higher disease scores than wild-type T cells (Supplementary Fig. 9a). Consistently, $Peli1^{-/-}$ T cells produced significantly higher frequency of inflammatory T_H17 and T_H1 cells (Supplementary Fig. 9b). These findings suggest that Peli1 has an important role in preventing T cell mediated autoimmunity.

Peli1 deficiency causes hyper-activation of late-phase NF-κB

To obtain molecular insights into Peli1 function, we analyzed the effects *Peli1* deficiency on TCR and CD28-stimulated signaling events. We purified T cells from young (5–8 weeks) wild-type and $Peli1^{-/-}$ mice, since at such young ages they did not show differences in the frequency of memory and naïve T cells (data not shown). The activation of NF-κB by anti-CD3 or anti-CD3 plus anti-CD28 was strongly enhanced in $Peli1^{-/-}$ T cells (Fig. 5a). We noticed that the hyper-activation of NF-κB in $Peli1^{-/-}$ T cells was particularly evident for the late time point of cell stimulation, while during the early phase loss of Peli1 had no obvious effect on the activation of NF-κB (Fig. 5b–d). Furthermore, in contrast to the hyper-activation of NF-κB, the activation of another T-cell transcription factor, AP-1, was largely normal (Fig. 5b), thus suggesting a specific role for Peli1 in the control of the NF-κB pathway. Similar results were obtained using purified CD4$^+$ and CD8$^+$ T cells (Fig. 5c and data not shown). Of note, the hyper-activation of NF-κB was also seen in $Peli1^{-/-}$ T cells stimulated with the T cell mitogens PMA and ionomycin (Fig. 5c), which are known to activate the PKC and calcium pathways independently of the TCR-proximal signaling steps, suggesting that Peli1 might not affect TCR proximal signaling. To exclude that the enhanced NF-κB activation in $Peli1^{-/-}$ T cells was not due to the presence of more memory T cells, we repeated the experiment using sorted naïve T cells (Fig. 5d). As in the case of total T cells, *Peli1* deficiency promoted the delayed activation of NF-κB without appreciably altering the early phase NF-κB activation.

To understand how Peli1 negatively regulates NF-κB activation, we examined the effect of *Peli1* deficiency on the activation of IKK, the protein kinase mediating activation of NF-κB. $Peli1^{-/-}$ T cells did not display elevated IKK activation in response to T cell mitogens (Fig. 5e). and even more, we detected a moderately reduced level of IKK activation in the $Peli1^{-/-}$ T cells (Fig. 5e). *Peli1* deficiency did not promote mitogen-stimulated activation of the MAPK ERK (Fig. 5e). In addition, following TCR and CD28 cross-linking, we did not detect appreciable differences in the activation of Zap-70 or ERK (Supplementary Fig. 10). Taken together with the predominant effect of *Peli1* deficiency on late-phase NF-κB activation, these findings suggest that Peli1 may specifically regulate a downstream step in NF-κB activation.

Lost of Peli1 selectively promotes activation of c-Rel

c-Rel is a late-phase NF-κB family protein activated in T cells[19] and is crucial for the induction of genes involved in the activation and differentiation of T cells as well as in the prevention of T cell anergy [12, 14, 16, 35]. An antibody supershift assay revealed abundant c-Rel in the late-phase NF-κB complex (Fig. 6a). We thus examined the effect of *Peli1* deficiency on TCR plus CD28-stimulated nuclear expression of c-Rel and other NF-κB members. *Peli1*^{−/−} T cells had markedly more nuclear c-Rel than the control wild-type T cells (Fig. 6b). In contrast to c-Rel, the nuclear level of RelA and RelB were comparable between the wild-type and *Peli1*^{−/−} T cells (Fig. 6c). Hyper-activation of c-Rel was also seen in *Peli1*^{−/−} T cells stimulated with PMA and ionomycin (Fig. 6c). Moreover, this phenotype was also detected in purified CD4^{+} and CD8^{+} T cells (Fig. 6c and data not shown) as well as in naïve T cells (Fig. 6d).

To address whether the detected phenotype was directly caused by the loss of Peli1, we knocked down Peli1 in the EL4 T cell line by shRNA. As seen in primary T cells (Supplementary Fig. 1c), the level of Peli1 was low in untreated EL4 cells but was greatly induced in response to mitogen stimulation (Fig. 6e). Moreover, Peli1 knockdown led to a marked enhancement in c-Rel activation (Fig. 6f), while overexpression of Peli1 in EL4 cells inhibited the activation of c-Rel (Fig. 6g). Of note, modulation of c-Rel activation required the C-terminal RING domain of Peli1, since a mutant lacking this region (Peli1 C) did not inhibit c-Rel (Fig. 6g). Collectively, these results demonstrate a role for Peli1 in regulating c-Rel activation by TCR and CD28 signals.

Peli1 mediates K48 ubiquitination of c-Rel

A previous study reported that c-Rel undergoes ubiquitination and degradation in leukemia T cell lines with constitutive NF-κB signaling [36]. To examine whether TCR plus CD28 signals induce the ubiquitination of c-Rel and whether this event is regulated by Peli1, we stimulated T cells with anti-CD3 and anti-CD28 antibodies and then incubated them with the proteasome inhibitor MG132 to block degradation of ubiquitinated c-Rel. In wild-type T cells, c-Rel was ubiquitinated in response to TCR plus CD28 stimulation (Fig. 7a). Because the accumulation of ubiquitinated c-Rel was detected only in the presence of the proteasome inhibitor MG132, it is likely that c-Rel undergoes ubiquitin-dependent degradation in activated T cells. However, the induction of c-Rel ubiquitination was largely blocked in the *Peli1*^{−/−} T cells (Fig. 7a). These results suggest the involvement of ubiquitination in the regulation of c-Rel during normal T-cell activation and establish Peli1 as an essential E3 that mediates c-Rel regulation.

We further examined if a physical interaction occurred between Peli1 and c-Rel. Endogenous Peli1 and c-Rel were co-precipitated by an anti-Peli1 antibody but not by a pre-immune serum (Fig. 7b), suggesting the two proteins interact in T cells. A stronger binding of c-Rel to Peli1 was detected in CD3 plus CD28-stimulated T cells, probably due to the increase in c-Rel and Peli1 protein levels. In HEK293 cells transfected with the two proteins, Peli1 bound to c-Rel and the C-terminal RING domain of Peli1 was dispensable for this association (Fig. 7c and Supplementary Fig. 11). Moreover, Peli1 expression in HEK293 cells led to potent induction of c-Rel ubiquitination (Fig. 7d). Since the ubiquitin

ligase function of Peli1 requires its C-terminal RING domain, a Peli1 mutant lacking its C-terminal region (Peli1 C) largely lost its ability to induce c-Rel ubiquitination (Fig. 7d), despite its ability to bind c-Rel (Fig. 7c). Similar results were obtained with a T cell line, EL4 (Supplementary Fig. 12). The defective c-Rel ubiquitination by Peli1 C was consistent with the inability of Peli1 C to inhibit c-Rel activation (Fig. 6g).

Since Peli1 is able to catalyze both K48-and K63-linked ubiquitin chains [26], we examined whether Peli1-induced c-Rel ubiquitination involved K63-or K48-linked ubiquitination chains by employing ubiquitin mutants harboring K to R mutations at K63 (K63R) and K48 (K48R). Whereas the K63R ubiquitin mutant was competent in mediating Peli1-stimulated c-Rel ubiquitination, the K48R ubiquitin mutant was defective in this modification (Fig. 7e). This result was not due to the differential expression of these ubiquitin mutants (Fig. 7f). Thus, Peli1 induces the K48-ubiquitination and degradation of c-Rel in activated T cells.

DISCUSSION

Peli family of proteins are regulators of IL-1R and TLR signaling [28, 29]. Peli1 has a critical role in regulating IKK activation by TRIF-dependent TLR signaling, while being largely dispensable for IKK activation by IL-1R and the MyD88-dependent TLRs [30]. In the present study, we show that Peli1 is unique in its high levels of expression in lymphocytes and that it plays an important role in the negative regulation of T-cell activation and maintenance of peripheral immune tolerance. In contrast to its role in TLR signaling, Peli1 is largely dispensable for activation of IKK by TCR signals, but mediates the ubiquitination and degradation of c-Rel. *Peli1*-deficient T cells are hyper-responsive to TCR and CD28 stimulation *in vitro* and display an activated phenotype *in vivo*, and *Peli1*$^{-/-}$ mice develop severe autoimmune symptoms. Although a previous work suggests c-Rel constitutive ubiquitination in transformed T-cell lines [36], whether this event occurs inducibly during normal T-cell activation has remained unknown. Our work not only identified Peli1 as a c-Rel ubiquitin ligase but also demonstrated ubiquitin-dependent degradation of c-Rel as a mechanism to negatively regulate T-cell activation.

The opposing functions of Peli1 in TLR and TCR signaling pathways are intriguing. However, similar functions have been reported for another E3 ubiquitin ligase, TRAF6. Like Peli1, TRAF6 functions as a negative regulator in T cells, but has a positive role in regulating TLR signaling [34]. Precisely how TRAF6 negatively regulates T cell activation is unclear, but TRAF6-deficient T cells have higher Akt activation [34]. Our current study revealed that the negative role of Peli1 in T cell activation is not due to IKK hyper-activation but rather involves accumulation of a specific NF-κB protein, c-Rel. Our data suggested that Peli1 mediates K48 ubiquitination of Peli1, in agreement with previous studies showing that Peli1 can catalyze both K48 and K63-linked ubiquitin chains [26]. Dual specificity in ubiquitin chain conjugation has been reported for several other E3 ubiquitin ligases, such as the SCF$^{\beta TrCP}$, c-IAP1 and c-IAP2, and Parkin [37–40]. It remains to be investigated whether Peli1 mediates K48 ubiquitination of additional targets.

T cell activation requires both TCR and CD28 co-stimulatory signals, and stimulation of naïve T cells in the absence of the CD28 signal leads to T cell anergy [41]. Accumulating

evidence suggests that c-Rel is a critical factor that integrates the TCR and CD28 signals and mediates T cell activation. c-Rel activation is strictly dependent on the CD28 signal [19, 20], and c-Rel is a major transcription factor that binds to the CD28-responsive element in the promoter region of IL-2 and other T-cell cytokine genes [19, 42, 43]. Indeed, c-Rel is required for both the initial activation and subsequent differentiation of T cells [12–14, 17, 35]. Moreover, c-Rel deficiency renders T cells sensitive to tolerance induction and prevents the development of autoimmune inflammation [14, 18, 35]. Consistent with these previous studies, we found that the hyper-activation of c-Rel in $Peli1^{-/-}$ T cells is associated with aberrant T cell activation. In contrast to the requirement for TCR and CD28 co-stimulation for normal T cell activation, $Peli1^{-/-}$ T cells could be activated by the TCR signal in the absence of CD28 co-stimulation. Thus, Peli1, likely through regulating the magnitude of c-Rel activation, controls the threshold of T cell response and maintains the requirement of CD28 co-stimulation, a mechanism that may contribute to prevention of autoimmunity.

We found that $Peli1$-deficient naïve T cells were less susceptible than control T cells to Treg-mediated suppression. These mutant T cells also displayed a high level of resistance to TGFβ-mediated suppression. This functional phenotype may be due to the hyper-activation of $Peli1$-deficient T cells, because similar phenotypes have been seen with T cells lacking other negative regulators [33, 34]. T cells with hyper-active NF-κB, due to the lack of the IκB-like molecule p105, are also resistant to Treg-mediated suppression [44]. It is thus likely that the hyper-activation of c-Rel contributes to the resistance of $Peli1^{-/-}$ T cells to Treg- and TGFβ-mediated suppression. Of course, our data do not exclude the possible involvement of Peli1 in an additional mechanism of T cell regulation.

In summary, we show that the E3 ubiquitin ligase Peli1 is a novel negative regulator of T cell activation, which contributes to the maintenance of peripheral T cell tolerance. Our data suggest that Peli1 mediates K48 ubiquitination of c-Rel, which appears to prevent aberrant accumulation of this key NF-κB factor during T-cell activation. This is in line with its abundant expression in T cells. Thus, despite their structural homology, the Peli family members possess non-redundant functions, probably due to their differential profile of expression. Our current findings further emphasize the importance of E3 ubiquitin ligases in the regulation of immune tolerance and establish Peli1 as a potential therapeutic target in the treatment of immunological disorders.

METHODS

Mice

$Peli1$ KO mice (in C57BL/6x129/sv genetic background) were described [30]. Where indicated, the mice were backcrossed to C57BL/6 background for 6 generations. For producing experimental animals, $Peli1$ heterozygous ($Peli1^{+/-}$) mice were bred to generate age-matched $Peli1$ homozygous knockout ($Peli1^{-/-}$) and wild-type mice. B6.SJL, $Rag1^{-/-}$, Foxp3-GFP, and OTII TCR transgenic mice (in C57BL/6 background) were purchased from The Jackson Laboratory. Mice were maintained in specific pathogen-free facility, and all animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Plasmids, antibodies and reagents

HA-Peli1 and HA-Peli1 C expression vectors (pSG5-KHA2M1 based) were obtained from Dr. Seok Hee Park [45]. Peli1 C (called Pellino-1 NM in the original paper [45]) encodes the N-terminal 280 amino acids and lacks the C-terminal RING domain. Peli1 and Peli1 C were subcloned into a modified pRV-GFP retroviral vector (pRV100G). Etag-Peli1 was provided by Dr. Rudi Beyaert, and pcDNA-HA-ubiquitin and GST-IκBα(1–54) were described [30, 46]. HA-tagged ubiquitin mutants harboring lysine-to-arginine point mutations (K48R and K63R) were provided by Dr. Zhijian Chen. Lentiviral vector pLKO.1 and pLKO.1-*Peli1* shRNA were from Sigma.

Antibodies for p50 (D17), c-Rel (sc-70), c-Rel (sc-71, used for supershift), RelB (N-17), IKKα/β (H-470), IKKγ (FL419), LaminB (C20), HSP60 (H1), ubiquitin (P4D1), Peli1/2 (F-7), Tubulin (TU-02), Zap70 (1E7.2), HDAC1, (ERK (K-23), and phopho-ERK (P-ERK, E-4) were from Santa Cruz. The anti-HA-HRP (3F10) was from Roche, and the anti-p65 (E495) and anti-phospho-Zap70 (Tyr319) were from Cell Signaling. The polyclonal Peli1 antibody used in IP was generated at Cocalico Biologicals (Reamstown, PA) by immunizing rabbits with a recombinant Peli1 protein. Other antibodies were as described [30, 44, 47]. The recombinant human TGFβ was purchased from PeproTech. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma.

Histology and immunohistochemistry

Organs were processed for hematoxylin-eosin staining and immunohistochemistry as described [44,50]. Cryostat sections (6 um) of kidnews were fixed in aceton for 10 min and then stained with Alexa Fluor 594-conjugated goat anti-mouse IgG (invitrogen). The glomeruli were visualized by DAPI counterstaining.

Flow cytometry analyses and cell sorting

Splenic and lymph node cell suspensions were subjected to flow cytometry analyses and cell sorting as previously described [48] using a LSRII flow (BD Bioscience) and FACSAria (BD Bioscience), respectively.

Cell culture, ELISA, and proliferation assays

293T cell transfection and primary T cell isolation from immune organs were as described [30, 44]. T cells were stimulated in replicate wells in 96-well plates (1×10^5 cells/well). Cell-culture supernatants were subjected to ELISA (eBioScience), and the cells were labeled for 6 h with ^{3}H-thymidine for proliferation assays. ELISA of serum ANA was performed using a commercial kit (Alpha Diagnostic International). T-cell proliferation assays based on CFSE (carboxyl fluorescent succinimidyl ester) dilution were as described [47].

In vitro Treg assays

Treg cells were isolated from spleen of Foxp3-GFP mice by flow cytometric cell sorting based on cell surface markers (CD4$^+$CD8$^-$CD25$^+$GFP$^+$). The purity is about 97%. For isolating APC, splenocytes of wild-type mice were depleted of CD4$^+$ and CD8$^+$ T cells by

MACS sorting. Naïve CD4$^+$ T cells were isolated from spleen of wild-type and *Peli1*$^{-/-}$ mice by first depleting CD25$^+$ cells and then selecting CD4$^+$ T cells by MACS sorting. The purified naive CD4$^+$ T cells (called Teffector cells) were labeled with 10μM CFSE for 15 min at 37°C, and the CFSE-labeled T cells (5×10^4) were co-cultured with Treg cells (at different Treg-to-Teffector ratios) in the presence of anti-CD3 (1 μg/ml) plus γ-irradiated APCs (5×10^4) in 96-well plates for 4 days. The proliferation of the Teffector cells was analyzed based on CFSE dilution.

Mixed bone marrow transfer

Rag1$^{-/-}$ mice were exposed to 900 cGy total body irradiation and, 6 h later, were intravenously transferred with 2×10^6 bone marrow cells mixed from *Peli1*$^{-/-}$ (CD45.1$^-$; backcrossed to B6 background for 6 generations) and *Wild-type* B6.SJL (CD45.1$^+$) mice in 1:1 ratio. After 10 weeks, recipient mice were sacrificed for flow cytometry analysis of *Peli*1$^{-/-}$ and *Wild-type* T cells by gating on CD45.1$^-$ and CD45.1$^+$ cells.

Oral tolerance induction

Wild-type and *Peli1*$^{-/-}$ mice were crossed with OTII TCR transgenic mice and intragastrically injected with 20 mg of ovalbumin (Grade V; Sigma, St. Louis, MO) or PBS daily for 4 days. 6 days after the last injection, spleen CD4$^+$ T cells were stimulated *in vitro* in the presence of irradiated APCs pulsed with OVA$_{323-339}$ peptide. After 72 h, cell proliferation was measured by flow cytometry based on CFSE dilution.

IB, *in vitro* kinase assay, and ubiquitination assay

IB and kinase assays were as described [49]. For ubiquitination assays, T cells or transfected HEK293 cells were lysed in RIPA buffer supplemented with 4 mM N-ethylmaleimide (NEM). The cell lysates were immediately boiled for 5 min in the presence of 1% SDS and then diluted 10 times with RIPA buffer. Ubiquitinated c-Rel was isolated by IP and detected by IB.

Analysis of TCR-proximal signaling

Spleen T cells were resuspended in RPMI media supplemented with 10% FCS and incubated on ice for 15 min with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml). The cells were washed once with 500 μl of cold RIPA media and stimulated by crosslinking at 37°C with goat anti-hamster Ig (45 μg/ml) [48].

Statistical analysis

Two-tailed unpaired t test statistical analysis was performed using the Prism software. P value <0.05 and <0.01 means significance and very significance, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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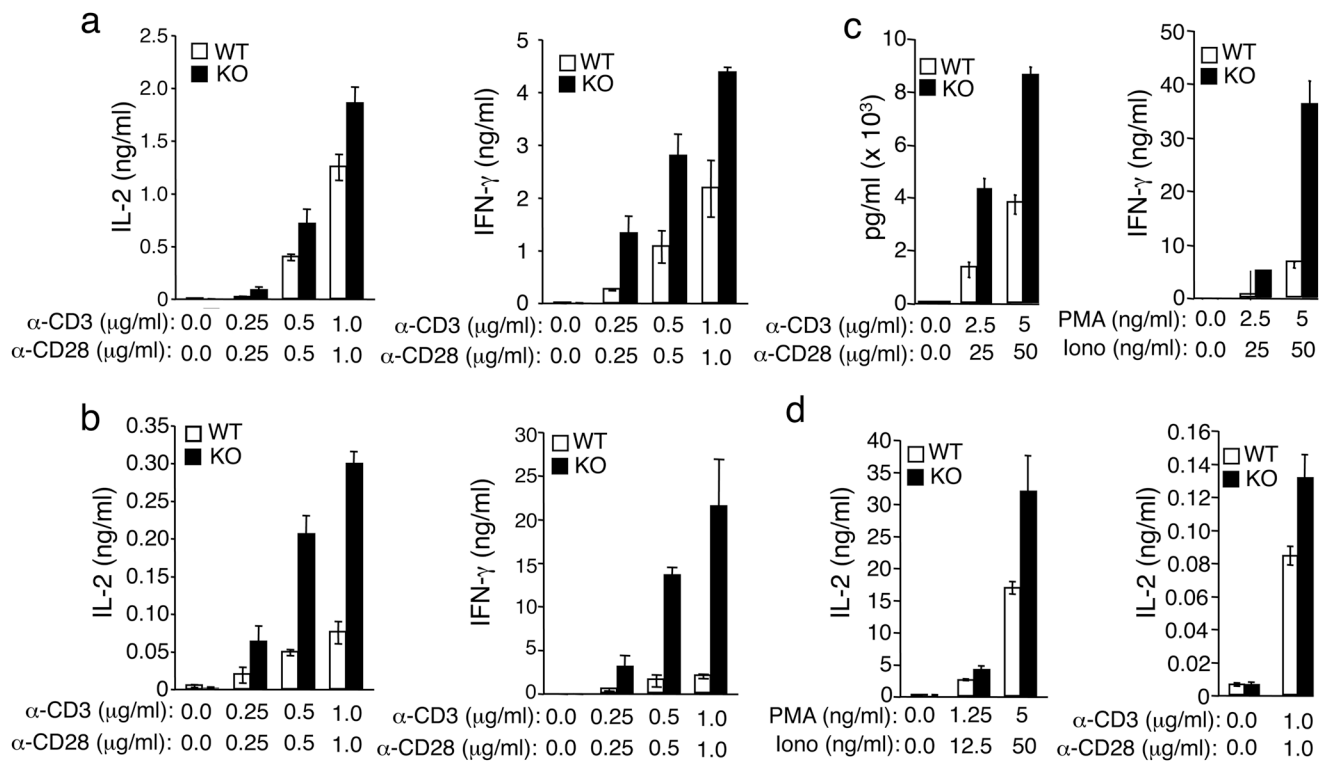**Figure 1. *Peli1*-deficient T cells are hyper-responsive to TCR and CD28 signals**
(**a– d**) Enzyme-linked immunosorbent assay (ELISA) of IL-2 and IFN-γsecretion by CD4^{+}
T cells (**a**), CD8^{+} T cells (**b**, **c**), or naïve CD4^{+} T cells (**d**) purified from splenocytes of age-
and sex-matched wild-type (WT) and *Peli1*^{−/−} (KO) 7 week old mice and stimulated for 48
h with the indicated doses of plate-bound anti-CD3, anti-CD3 plus anti-CD28, or PMA plus
ionomycin. Data are representative of 4 independent experiments performed using multiple
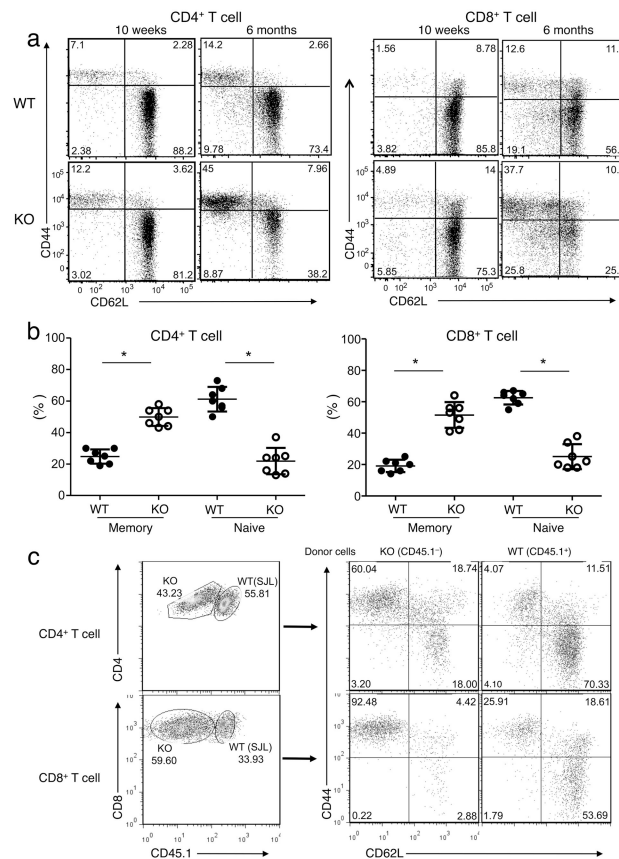mice.

Figure 2. Peli1 has a cell autologous role in regulating T cell homeostasis *in vivo*
(**a**) Frequency of naïve (CD44^{lo}CD62L^{hi}) and memory (CD44^{hi}CD62L^{lo}) CD4$^+$ and CD8$^+$ T cells in total splenocytes from age- and sex-matched Wild-type (WT) and Peli1$^{-/-}$ (KO) mice (10 week and 6 month old) gated on CD3$^+$CD25 cells. Data are representative of 5 independent experiments with multiple mice. (**b**) Spleen CD4$^+$ and CD8$^+$ memory and naïve T cells were measured as in **a** using age-matched wild-type and Peli1$^{-/-}$ mice (6–8 month old). Data are presented as mean value of multiple mice (each circle represents an individual mouse). *P<0.001 (two-tailed unpaired t test). (**c**) Bone marrow cells (2×10^6) from Peli1$^{-/-}$ (CD45.1$^-$; backcrossed to B6 background) and wildtype B6.SJL (CD45.1$^+$) mice were mixed in 1:1 ratio and adoptively transferred into γ-irradiated *Rag1*$^{-/-}$ mice. After 10 weeks, recipient mice were sacrificed for flow cytometry analyses using peripheral lymph node cells. The frequency of *Peli1*$^{-/-}$ and wild-type (SJL) CD4$^+$ and CD8$^+$ T cells were determined based on expression of CD45.1 (left panels). The frequency of memory and naïve T cells within the *Peli1*$^{-/-}$ and wild-type (SJL) CD4$^+$ and CD8$^+$ T-cell populations were determined based on CD44 and CD62L markers (naïve: CD44^{lo}CD62L^{hi}; memory: CD44^{hi}CD62L^{lo}). Data are representative of four recipients of each group.
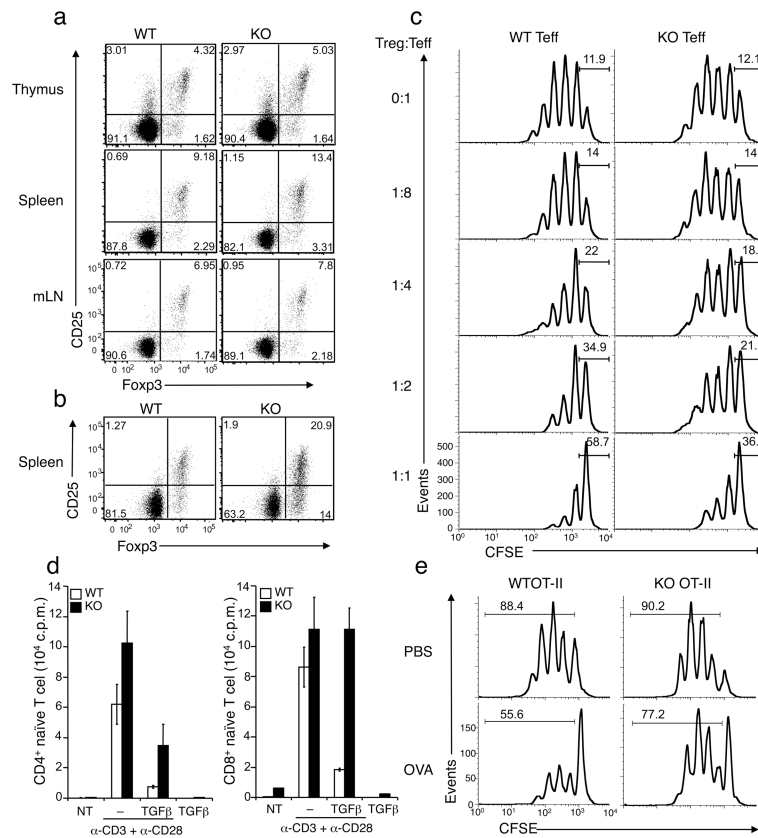
Figure 3. *Peli1* deficiency renders naïve T cells refractory to suppression by Tregs and TGFβ

(**a**, **b**) Flow cytometry to determine the frequency of Tregs (percentage of $CD3^+CD4^+$ cells) in the indicated immune organs of 10 weeks (**a**) or 6 months (**b**) old mice. Data are representative of three independent experiments. (**c**) Naïve $CD4^+$ T cells (Teffector) prepared from *Wild-type (WT)* and *Peli1$^{-/-}$* (KO) mice (7 week) were activated *in vitro* in the presence of the indicated ratios (Treg-to-Teffector) of wild-type Treg cells. Teffector cell proliferation was analyzed based on CFSE dilution, showing the percentage of non-divided cells. Data are representative of two independent experiments. (**d**) Naïve $CD4^+$ and $CD8^+$ T cells were cultured for 48 h without (NT) or with the indicated inducers and subjected to proliferation assay based on ^{3}H-thymidine incorporation. Data are representative of three independent experiments. (**e**) Wild-type OT-II and *Peli1$^{-/-}$* OT-II mice were given 20 mg of oral OVA or PBS daily for 4 days. $CD4^+$ T cells from spleen were stimulated *in vitro* with OVA peptide-pulsed APCs and subjected to proliferation assay based on CFSE dilution.
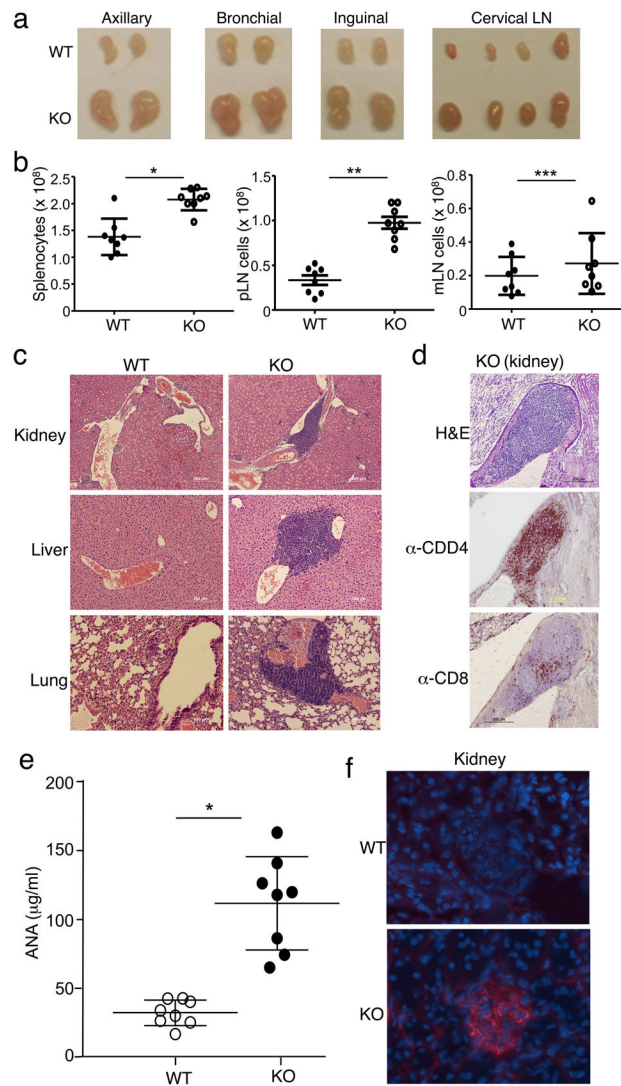
Figure 4. *Peli1⁻/⁻* mice spontaneously develop autoimmune symptoms

(**a**) Lymph nodes isolated from age- and sex-matched *Wild-type (WT)* and *Peli1⁻/⁻* (KO) mice (10 week old). (**b**) Total cell numbers were counted in the spleen, peripheral lymph nodes (pLN), and mesentery lymph nodes (mLN) of 6 month old mice. Data are presented as mean value of multiple mice (each circle represents one mouse). *P<0.0002, **P<0.0001, and ***p=0.3457 (two-tailed unpaired t test). (**c**) Hematoxylin-eosin staining of the indicated tissue sections from 6 month old WILD-TYPE and KO mice, showing immune cell infiltrations. Original magnification, 100x (kidney) and 200x (liver and lung). (**d**) Kidney tissue sections were subjected to either hematoxylin-eosin staining or immunohistochemistry using anti-CD4 or anti-CD8 antibodies. Original magnification, 20x. Data in **c** and **d** are representative of multiple mice. (**e**) ANA ELISA of sera collected from age-matched wild-type and *Peli1⁻/⁻* mice (8 each; 10 to 24 month old). *P<0.001 (two-tailed unpaired t test). (f) Immunofluorescence staining of kidney tissue sections of wild-type and *Peli1⁻/⁻* mice to detect immune-complex deposits (red). Glomeruli were visualized

by DAPI counterstaining (blue). Original magnification, 40x. Data are representative of 4 wild-type and 4 *Peli1*$^{-/-}$ mice.
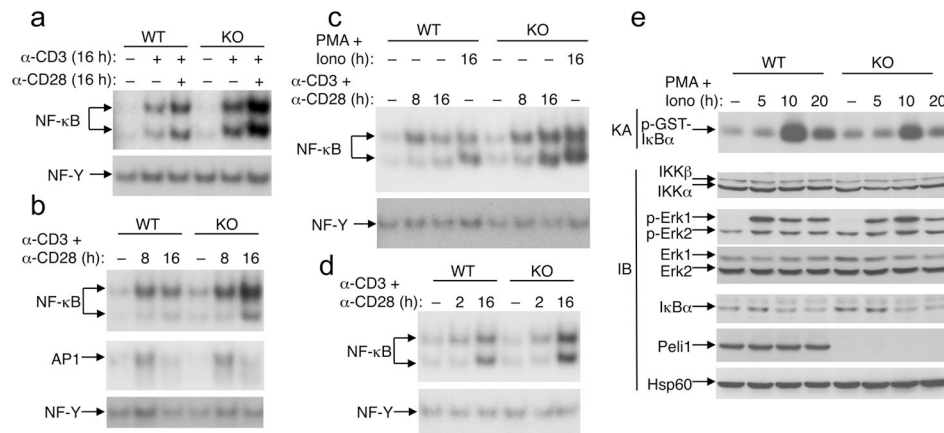
Figure 5. *Peli1* **deficiency causes hyper-activation of late-phase NF-κB**
(**a**) Total T cells isolated from *Wild-type (WT)* and *Peli1*^{−/−} mice (7 week old) were either not treated (−) or stimulated (+) with plate-bound anti-CD3 and anti-CD28 antibodies (1 μg/ml). Nuclear extracts were subjected to EMSA using ^{32}P-radiolabeled probes for NF-κB or NF-Y. (**b**, **c**) Total T cells (**b**) or CD8$^+$ T cells (**c**) were either not treated (NT) or stimulated for the indicated times with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) or PMA (5 ng/ml) plus ionomycin (100 ng/ml). Nuclear extracts were subjected to EMSA using probes for the indicated transcription factors. (**d**) Naïve T cells were stimulated as indicated and subjected to EMSA as in **a**. (**e**) Total T cells were stimulated with PMA plus ionomycin, and total cell lysates were subjected to protein kinase assay (KA) or IB to detect IKK activity (panel 1) and ERK phosphorylation (panel 3) or the expression level of the indicated proteins (other panels). Data are representative of 4 independent experiments performed using multiple mice.
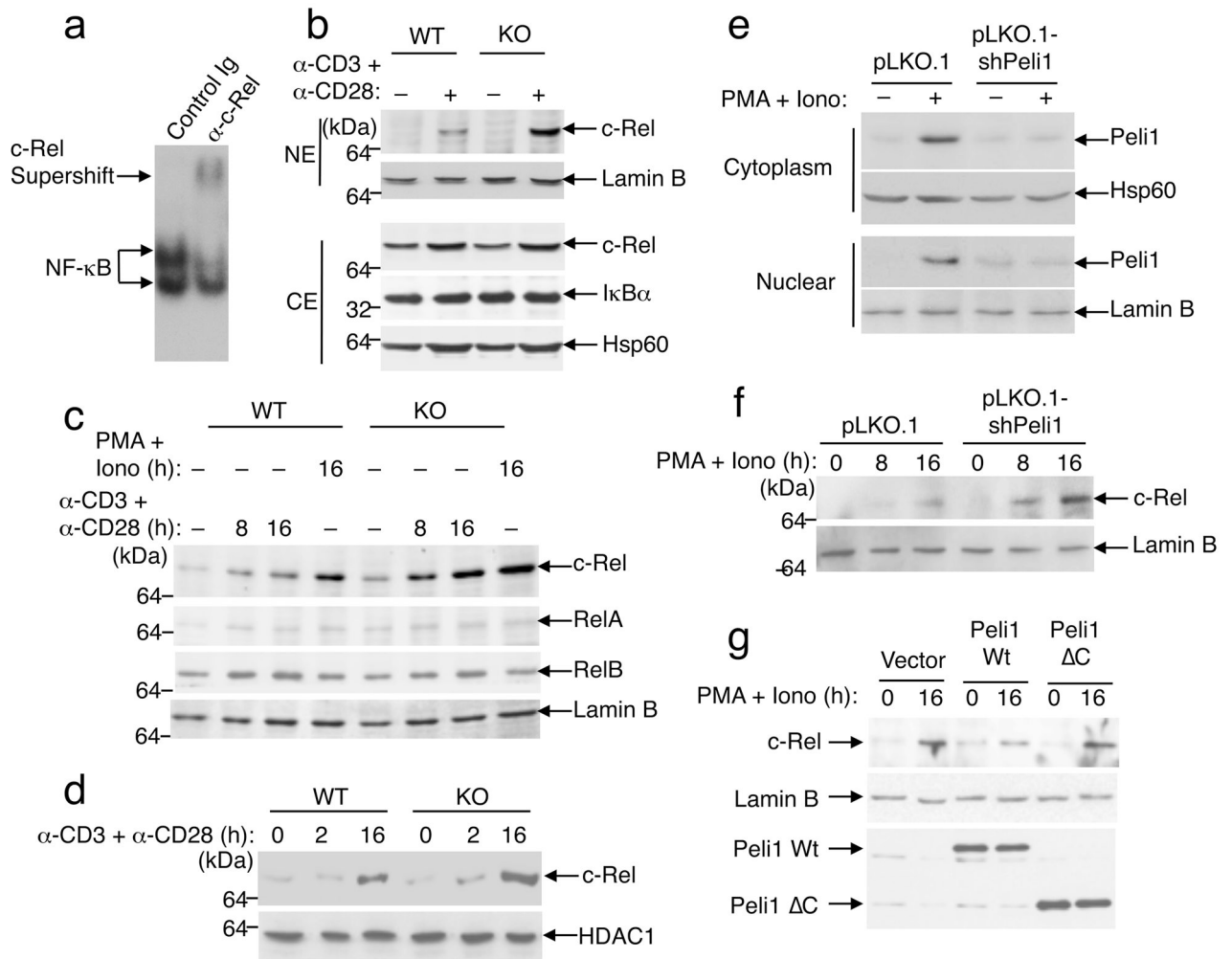
Figure 6. *Peli1* negatively regulates c-Rel
(**a**) EMSA was performed using nuclear extract from anti-CD3/CD28-stimulated (16 h)
Peli1$^{-/-}$ (KO) T cells, in the presence of control Ig or anti-c-Rel antibody. (**b**) Total T cells
from wild-type (WT) and *Peli1$^{-/-}$* mice (7 week old) were incubated for 16 h in the absence
(−) or presence (+) of plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml). Nuclear
(NE) and cytoplasmic (CE) extracts were subjected to IB to detect the indicated proteins. (**c**)
Purified CD8$^+$ T cells from wild-type and *Peli1$^{-/-}$* mice (7 week old) were either not treated
(−) or stimulated (+) with anti-CD3 plus anti-CD28 or PMA (5 ng/ml) plus ionomycin (100
ng/ml). Nuclear extracts were subjected to IB. (**d**) Naïve CD4$^+$ T cells were stimulated as
indicated. Nuclear extracts were subjected to IB to detect c-Rel and HDAC1. (**e**) EL4 T-cells
were infected with lentiviral vector pLKO.1 or pLKO.1 encoding Peli1 shRNA. The cells
were either not treated (−) or stimulated (+) with PMA (5 ng/ml) plus ionomycin (100
ng/ml) for 8 h, and cytoplasmic and nuclear extracts were subjected to IB. (**f**) The cells from
e were stimulated as indicated and subjected to IB. Data are representative of 4 independent
experiments performed using multiple mice. (**g**) EL4 cells were infected with pRV100G
vector or wild-type Peli1 or Peli1 C. Following stimulation, nuclear c-Rel and Lamin B and
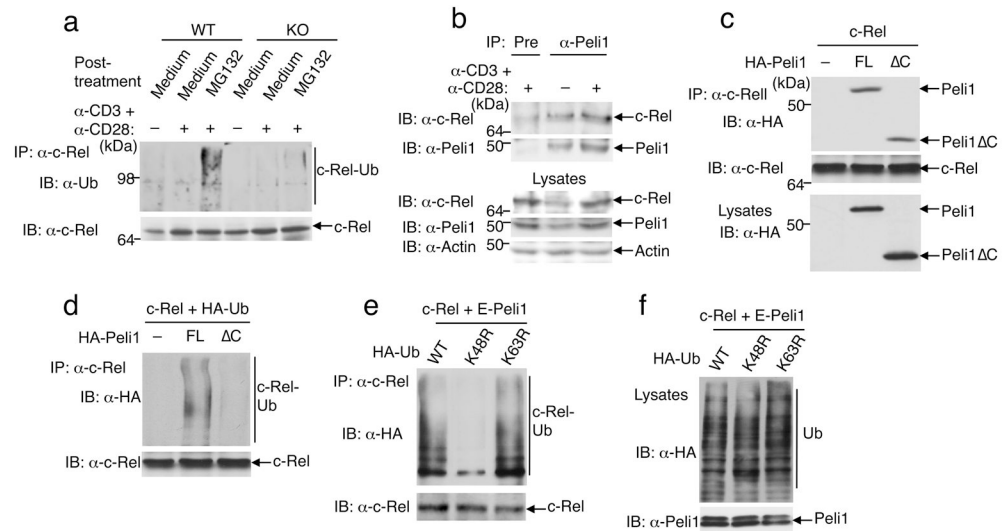whole-cell Peli1 proteins were detected by IB.

Figure 7. Peli1 induces c-Rel ubiquitination

(**a**) Wild-type (WT) and *Peli1*^{−/−} (KO) T cells were incubated for 16 h in the absence (−) or presence (+) of anti-CD3 plus anti-CD28 and then further incubated for 6 h with medium or MG132. Cell lysates were subjected to c-Rel IP, followed by IB. (**b**) Wildtype T cells were not treated (−) or stimulated (+) with anti-CD3 plus anti-CD28 for 16 h. Cell lysates were subjected to c-Rel-Peli1 coIP (top two panels) or direct IB (bottom three panels). Data in **a** and **b** are representative of 3 independent experiments performed using multiple mice. (**c**) HEK293 cells were transfected with c-Rel along with full-length (FL) Peli1 or Peli1 C. Total cell lysates were subjected to c-Rel-Peli1 coIP (top two panels) or Peli1 IB (bottom panel). (**d**) 293 cells were transfected as indicated. Immunoprecipitated c-Rel was subjected to IB to detect its conjugation with HA-ubiquitin and its expression level. (**e**, **f**) 293 cells were transfected with c-Rel and Etag-Peli1 along with HA-tagged wild-type (WT) or mutant forms of ubiquitin. Cell lysates were subjected to c-Rel ubiquitination (**e**) or direct IB (**f**) assays. Data in **c-f** are representative of 3 independent experiments.