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Malt1-Induced Cleavage of Regnase-1 in CD4$^+$ Helper T Cells Regulates Immune Activation

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SUMMARY

Regnase-1 (also known as Zc3h12a and MCPIP1) is an RNase that destabilizes a set of mRNAs, including *Il6* and *Il12b*, through cleavage of their 3′ UTRs. Although Regnase-1 inactivation leads to development of an autoimmune disease characterized by T cell activation and hyperimmunoglobulinemia in mice, the mechanism of Regnase-1-mediated immune regulation has remained unclear. We show that Regnase-1 is essential for preventing aberrant effector CD4$^+$ T cell generation cell autonomously. Moreover, in T cells, Regnase-1 regulates the mRNAs of a set of genes, including *c-Rel*, *Ox40*, and *Il2*, through cleavage of their 3′ UTRs. Interestingly, T cell receptor (TCR) stimulation leads to cleavage of Regnase-1 at R111 by Malt1/paracaspase, freeing T cells from Regnase-1-mediated suppression. Furthermore, Malt1 protease activity is critical for controlling the mRNA stability of T cell effector genes. Collectively, these results indicate that dynamic control of Regnase-1 expression in T cells is critical for controlling T cell activation.

INTRODUCTION

Immune cell activation is accompanied by dynamic changes in expression of genes related to inflammation (Beutler, 2009; Iwasaki and Medzhitov, 2010; Takeuchi and Akira, 2010). Concomitantly, immune reactions are tightly controlled to prevent harmful pathologies, such as septic shock or autoimmune disease, due to sustained inflammation. Gene expression is controlled at multiple checkpoints, including signal transduction, transcription, and messenger RNA (mRNA) stability. Among these, transcriptional regulation has been studied extensively in the context of immune responses (Vallabhapurapu and Karin, 2009). Transcription factors, such as NF-κB and AP-1, are sequestered in the cytoplasm in resting immune cells and translocate to the nucleus in response to various stimuli, such as Toll-like receptor (TLR) ligands, Tumor necrosis factor (TNF), and Interleukin-1β (IL-1β) in innate immune cells, and antigen receptor stimulation in acquired immune cells (Hayden and Ghosh, 2008). Nuclear NF-κB and AP-1 are, in turn, responsible for transcriptional activation of a set of genes controlling inflammation. Thus, signal-induced transcriptional activation is a common feature of both innate and acquired immunity.

On the other hand, recent studies have revealed that control of gene expression at the mRNA level is as important as transcriptional control (Anderson, 2008; Hao and Baltimore, 2009). It is widely accepted that microRNA is critical for fine-tuning immune reactions, both in innate and adaptive immune cells (O'Connell et al., 2010; O'Neill et al., 2011). Furthermore, a set of RNA binding proteins has been shown to be important for controlling the stability of mRNAs related to immune activation, such as cytokines. For instance, tristetraprolin, a protein with tandem CCCH-type zinc-finger motifs, destabilizes TNF mRNA by binding with AU-rich elements present in its 3′ UTR (Carballo et al., 1998). Furthermore, another CCCH-type zinc-finger molecule,

Roquin, is critical for suppressing expression of mRNA encoding Inducible T cell coactivator (*Icos*) by binding to its 3′ UTR (Glasmacher et al., 2010; Vinuesa et al., 2005) or through a microRNA (Yu et al., 2007). A point mutation in Roquin resulted in augmented expression of ICOS on follicular helper T cells, leading to an autoimmune disease by facilitating the production of autoantibody-producing B cells (Linterman et al., 2009). Thus, regulation of mRNA decay is critical for suppressing aberrant activation of not only innate immune cells but also acquired immune cells, including T cells.

We have previously shown that a set of mRNAs, including *Il6* and *Il12b*, is destabilized by a novel ribonuclease (RNase) named Regnase-1 (also known as Zc3h12a and MCPIP1) in response to TLR ligands in macrophages (Matsushita et al., 2009). Furthermore, we found that Regnase-1 is phosphorylated by IκB kinases (IKKs) in response to TLR ligands or IL-1β stimulation via the MyD88-dependent signaling pathway, leading to rapid degradation of Regnase-1 in an ubiquitin/proteasome-dependent fashion (Iwasaki et al., 2011). The degradation of Regnase-1 releases the "brake" on *Il6* mRNA expression and enables rapid and robust induction of *Il6* mRNA when needed. Interestingly, Regnase-1-deficient mice showed severe systemic inflammation characterized by T cell and B cell activation. These mice also showed hyperimmunoglobulinemia and produced autoantibodies. However, the induction mechanism of these inflammatory diseases is not well understood. Moreover, in addition to macrophages, Regnase-1 is expressed in various immune cells, including T and B cells.

In this study, we investigated the role of T cells in the induction of autoimmune disease in Regnase-1-deficient mice by generating a conditional *Regnase-1* allele. T-cell-specific deletion of Regnase-1 induces T and B cell activation as well as the development of autoimmune disease. Regnase-1 was degraded in response to T cell receptor (TCR) stimulation in a manner different from that in response to TLR/IL-18R stimulation. Malt1 cleaves Regnase-1 at R111 in response to TCR stimulation, and the protease activity of Malt1 is necessary for prolonging the half-life of Regnase-1 target mRNAs. Thus, Regnase-1 is critical for preventing autoimmunity in a resting T-cell-intrinsic manner and is dynamically regulated in the course of T cell activation.

RESULTS

Lack of Regnase-1 in T Cells Recapitulates Autoimmune Disease in *Regnase-1*$^{-/-}$ Mice

To investigate the role of IL-6 in the development of autoimmune inflammatory disease in the absence of Regnase-1, we generated mice lacking both Regnase-1 and IL-6. The massive increase in splenic plasma cells and immunoglobulin M/immunoglobulin D (IgM/IgD) double-negative B cells observed in *Regnase-1*$^{-/-}$ mice was reduced in mice lacking both Regnase-1 and IL-6 (Figures S1A and S1B available online). In contrast, generation of effector/memory T cells was unchanged, even in the absence of IL-6 (Figure S1C). Furthermore, deficiency in the IL-12p40 subunit did not change the accumulation of plasma cells or the activation of T cells (Figures S1D and S1E), though Th1 cell development and production of IFN-γ required IL-12 (Figures S1F and S1G). These results demonstrate that activation of T cells in *Regnase-1*$^{-/-}$ mice cannot be explained simply by the overproduction of a set of cytokines. Because we have previously shown that Regnase-1 is expressed in various hematopoietic cells, such as T cells and B cells (Iwasaki et al., 2011), we hypothesized that Regnase-1 acts cell autonomously in T cells. To examine the role of Regnase-1 in lymphocytes, we established floxed Regnase-1 (*Regnase-1*$^{fl/fl}$) mice (Figure S2A). The mice were crossed with mice expressing Cre under the control of a CD4 promoter (CD4-Cre). Recombination of the *Regnase-1* locus in CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ T cells was confirmed by Southern blot analysis (Figures S2B and S2C). An immunoblot analysis showed abrogation of Regnase-1 expression in T cells, but not in B cells (Figure S2D).

Interestingly, CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ mice started to die 8 weeks after birth, and most of them died within 17 weeks (Figure 1A). T-cell-specific deletion of Regnase-1 led to the development of severe splenomegaly (Figure 1B). The total numbers of splenic T cells were elevated, and most splenic T cells from CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ mice were CD62L^{-}CD44^{hi} effector/memory T cells (Figure 1C). Splenic T cells stimulated with anti-CD3ε and/or anti-CD28 antibodies, and phorbol myristate acetate (PMA) plus ionomycin had elevated production of IFN-γ, IL-17, and IL-4 (Figures 1D and 1E), suggesting that T cells were polarized to various helper T cell subsets. *Regnase-1*$^{-/-}$ CD4^{+} and CD8^{+} T cells showed elevated expression of Ki67, indicating that Regnase-1-ablated T cells exhibit accelerated cell-cycle progression (Figure 1F). Consistent with T cell activation, CD19^{dull}CD138^{hi} plasma cells accumulated more in CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ mice than in control mice (Figure 1G). Furthermore, most of CD19^{+} B cells did not express IgM or IgD (Figure 1H). All subtypes of immunoglobulins were highly elevated in the sera of CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ mice (Figure 1I), and anti-nuclear antibodies were also detected (Figure 1J). To further confirm that Regnase-1 in T cells are responsible for the pathology, we crossed floxed *Regnase-1* mice with Lck-Cre mice, which express Cre in T cells from early in the thymic T cell development (Figure S2E). Lck-Cre^{+}*Regnase-1*$^{fl/fl}$ mice also showed a massive increase in effector CD4^{+} T cells (Figure S1H), and elevation of plasma cells as well as class switched B cells (Figure S1I). These data indicate that deletion of Regnase-1 in T cells led to the development of a severe autoimmune inflammatory disease.

Peripheral CD4^{+} T Cell Activation Is Sufficient for the Pathology in CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ Mice

We next investigated thymic T cell development in CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ mice. The subsets of thymic T cells as well as splenic CD4^{+} and CD8^{+} subsets were indistinguishable between CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ and control mice (Figures S3A and S3B). Furthermore, the TCRβ repertoire of peripheral CD4^{+} T cells was comparable between CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ and control mice (Figure S3C). These results suggest that thymic T cell development is not affected by deletion of Regnase-1 in T cells. To further investigate whether peripheral CD4^{+} T cells are pathogenic in CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ mice, we transferred splenic CD4^{+} T cells from CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ or control mice into congenic CD45.1^{+} mice. Mice that received *Regnase-1*-deficient CD4^{+} T cells showed severe splenomegaly (Figure 2A). CD4^{+} T cells from CD4-Cre^{+}*Regnase-1*$^{fl/fl}$ mice survived

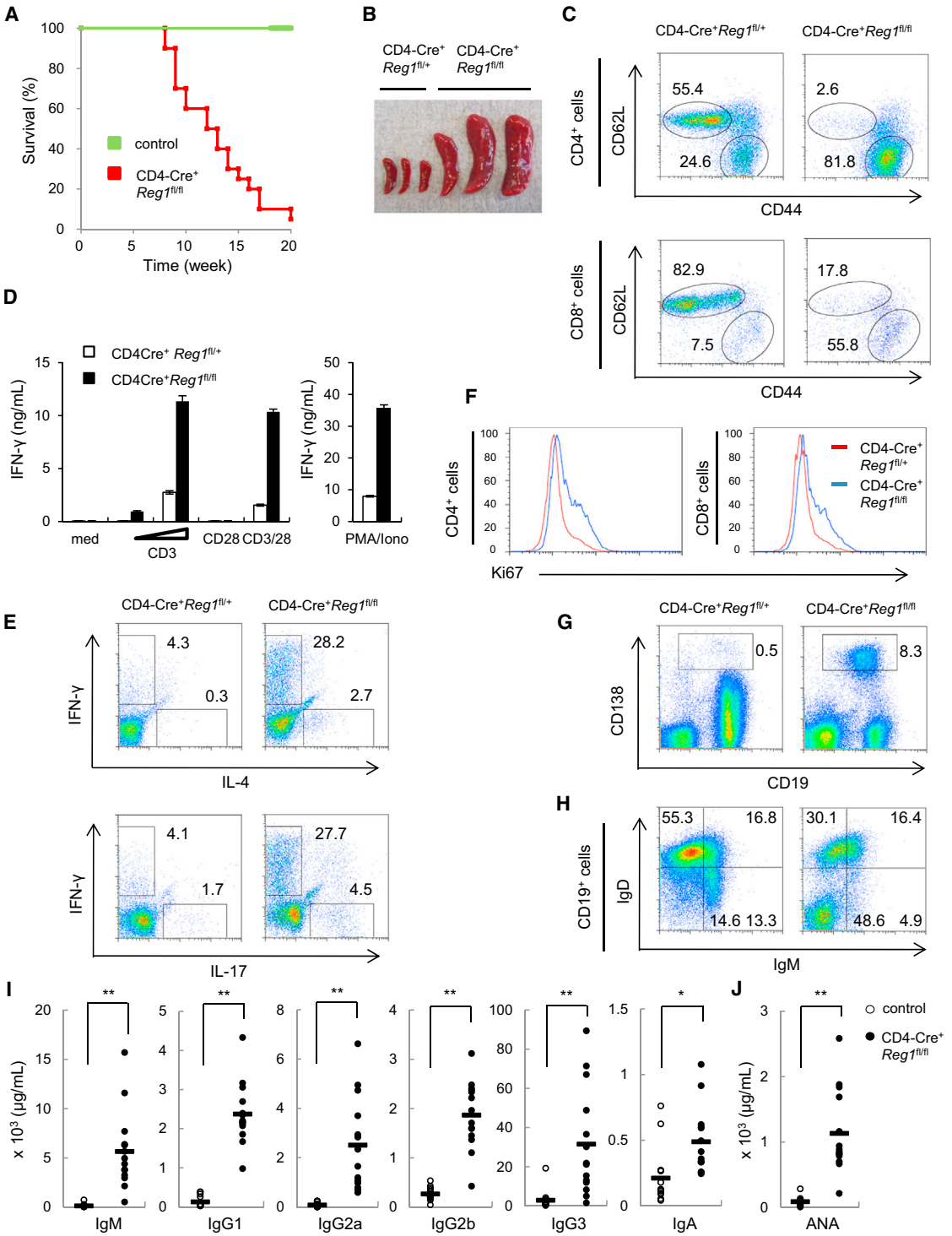

Figure 1. T-Cell-Specific Deletion of Regnase-1 Causes Autoimmune Inflammatory Disease in Mice

(A) Survival rates of control (CD4-Cre$^+$ *Regnase-1*$^{+/+ \text{ or } fl/+}$) and CD4-Cre$^+$ *Regnase-1*$^{fl/fl}$ mice at the indicated periods (n = 20).

(B) Spleens from three representative mice with the indicated genotype.

(C) Flow cytometric analysis of expression of CD62L and CD44 in splenic CD4$^+$ and CD8$^+$ T cells.

(D) Production of IFN-γ in response to CD3ε and CD28, and PMA plus ionomycin in splenic T cells. Error bars indicate SD of triplicates.

(E and F) Intracellular staining of IFN-γ, IL-4, and IL-17 in splenic CD4$^+$ T cells after stimulation with PMA and ionomycin (E), and expression of Ki67 in splenic CD4$^+$ and CD8$^+$ unstimulated T cells (F).

(G and H) Flow cytometric analysis of proportion of plasma cells in the spleen (G) and expression of IgM and IgD in splenic CD19$^+$ B cells (H).

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for 3 months in the recipient mice, although $CD4^+$ T cells from control mice were not detected (Figure 2B). Surprisingly, transfer of splenic $CD4^+$ T cells from CD4-Cre^+*Regnase-1*$^{fl/fl}$ mice alone led to the increase of recipient $CD45.1^+CD62L^{lo}CD44^{hi}$ effector/memory T cells as well as $CD19^{dull}CD138^{hi}$ plasma cells (Figures 2C and 2D). These results indicate that *Regnase-1*$^{-/-}$ peripheral $CD4^+$ T cells have the ability to activate immune cells. Next, we utilized the IFN-inducible Cre-expressing system (Mx-Cre). Treatment of Mx-Cre^+*Regnase-1*$^{fl/fl}$ mice with polyinosinic:polycytidylic acid (poly I:C) rapidly reduced Regnase-1 expression (Figure S2F), followed by an accumulation of $CD44^{hi}$ $CD4^+$ T cells in the blood (Figure 2E). In contrast, poly I:C did not change $CD44^{hi}$ T cells in control mice. Then $CD4^+$ T cells isolated from untreated Mx-Cre^+*Regnase-1*$^{fl/fl}$ or control spleen were transferred into $CD45.1^+$ mice, and recipient mice were immediately treated with poly I:C (Figure 2F). $CD4^+$ T cells that lost Regnase-1 expression peripherally survived for 3 months in the spleen (Figure 2G). Furthermore, the mice showed increased levels of $CD45.1^+CD62L^{lo}CD44^{hi}$ effector/memory T cells and an accumulation of $CD19^{dull}CD138^{hi}$ plasma cells (Figures 2H and 2I). The proportion of $FoxP3^+CD4^+$ regulatory T (Treg) cells was comparable between control and CD4-Cre^+*Regnase-1*$^{fl/fl}$ splenocytes (Figure S3D). In addition, Treg cells purified from Mx-Cre^+*Regnase-1*$^{fl/fl}$ splenocytes 10 days after poly I:C treatment were competent in suppressing effector T cells, suggesting that Treg cells are not functionally defective in the absence of Regnase-1 (Figure S3E). These results demonstrate that Regnase-1 plays a pivotal role in preventing aberrant generation of effector $CD4^+$ T cells peripherally in a cell-autonomous fashion.

Recognition of Antigens by TCR Is Required for Pathology under Regnase-1-Deficient Conditions

To examine the requirement of TCR-mediated antigen recognition in T cell activation under Regnase-1-deficient conditions, we generated Regnase-1-deficient mice harboring a TCR specific to ovalbumin (OVA) in $CD4^+$ T cells (OT-II^+*Regnase-1*$^{-/-}$). Surprisingly, Regnase-1-deficient T cells stained with an I-Ab $OVA_{323-339}$ tetramer showed a $CD62L^{hi}CD44^{lo}$-naive phenotype (Figure 2J), indicating that aberrant activation of T cells under Regnase-1-deficient conditions requires TCR signaling. Furthermore, immunization of Regnase-1-deficient mice harboring OVA-specific $CD4^+$ T cells resulted in a highly enhanced transition of naive to effector/memory $CD4^+$ T cells (Figure 2K). To further investigate whether the aberrant activation is T cell intrinsic, we adoptively transferred OT-II^+*Regnase-1*$^{-/-}$-naive $CD4^+$ T cells into $CD45.1^+$ congenic mice. Five days after immunization with OVA with aluminum adjuvant (Alum), the number of $CD45.2^+$ OT-II^+ cells lacking Regnase-1 was about 3- to 6-fold higher than that of OT-II^+ control cells (Figure 2L). Then we examined the effector responses of OT-II^+ T cells by recall assay in vitro after stimulation with $OVA_{323-339}$ peptide. Splenocytes from mice receiving OT-II^+ T cells lacking Regnase-1 showed highly enhanced proliferative responses (Figure 2M) and production of IFN-γ (Figure 2N) than mice receiving control cells. These results demonstrate that Regnase-1 is critical for suppressing excess immune responses in T cells downstream of the TCR.

A Set of Effector Genes Is Highly Expressed in $CD4^+$ T Cells Lacking Regnase-1

To understand the molecular basis for pathogenicity of $CD4^+$ T cells under Regnase-1 deficiency, we examined gene expression profiles in $CD4^+$ T cells from $CD4Cre^+$-*Regnase-1*$^{fl/fl}$ and control mice. We found that Regnase-1 deficiency in $CD4^+$ T cells upregulated the expression of many genes (Figure 3A; Table S1) (424 genes were upregulated more than 10-fold under Regnase-1 deficiency, in contrast to 157 genes downregulated in the cells). Among the genes involved in T cell activation, Regnase-1 deficiency increased the expression of surface molecules, including *Icos*, *Tnfr2*, *Ox40*, and *Il2rb1*, and cytokines, such as *Il6* and *Il2* (Table S1). The transcriptome data were confirmed by quantitative PCR analysis (Figure 3B), and surface expression of T cell activation markers, including ICOS, CD40L, TNFRII, OX40, IL-2Rα, CD69, and CTLA4, but not CD28, were highly upregulated in $CD4^+$ T cells from CD4-Cre^+*Regnase-1*$^{fl/fl}$ mice compared with wild-type (Figure 3C). T cell activation is known to be regulated by a set of transcription factors belonging to the NF-κB and NF-AT families (Rao et al., 1997; Sun et al., 2000). When we checked the expression of genes in these transcription factor families, the expression of *c-Rel* mRNA was higher in T cells lacking Regnase-1 compared with control cells. On the other hand, the expression of other NF-κB members and NF-ATc1 were not altered between control and *Regnase-1*$^{-/-}$ T cells, based on the transcriptome analysis. Furthermore, c-Rel, but not RelA, was more abundantly expressed in the absence of Regnase-1 even at the protein level in whole-cell lysates and in nuclear proteins (Figure 3D). These results indicate that c-Rel is negatively regulated by Regnase-1.

To exclude the possibility that the difference in the gene expression is simply due to the difference in the proportion of naive and effector $CD4^+$ T cells in the absence of Regnase-1, we sorted $CD62L^{hi}CD44^{lo}$ and $CD62L^{lo}CD44^{hi}$ $CD4^+$ T cells from Mx-Cre^+*Regnase-1*$^{+/+}$ and Mx-Cre^+*Regnase-1*$^{fl/fl}$ mice 10 days after poly I:C treatment. At this period, more numbers of $CD62L^{hi}CD44^{lo}$-naive $CD4^+$ T cells remained in Mx-Cre^+ *Regnase-1*$^{fl/fl}$ mouse splenocytes (Figure S4A). We examined the gene expression in the sorted cells by a microarray analysis. The expression of c-Rel and a set of costimulatory molecules were elevated both in naive and effector $CD4^+$ T cells from Mx-Cre^+*Regnase-1*$^{fl/fl}$ mice (Figure S4B; Table S2).

Then we examined the role of Regnase-1 in the TCR signaling pathways. First, the levels of TCRβ expression on the cell surface were not altered even in the absence of Regnase-1 (Figure S4C). TCR-induced tyrosine phosphorylation was not enhanced even in the absence of Regnase-1 in OT-II^+CD4^+ T cells (Figure S4D). Furthermore, MAP kinase activation or the NF-ATc1-DNA binding activity to TCR stimulation was not increased in the absence

(I) Hypergammaglobulinemia in CD4-Cre^+ *Regnase-1*$^{fl/fl}$ mice. Serum immunoglobulin levels were measured.

(J) Production in antinuclear antibodies (ANA) in CD4-Cre^+ *Regnase-1*$^{fl/fl}$ mice.

Bars indicate the mean, and statistical significance was determined using the Student's t test in (I) and (J). *$p < 0.01$, **$p < 0.001$. Data are representative of two (F), three (D and E), or five (C, G, and H) independent experiments. See also Figures S1 and S2.

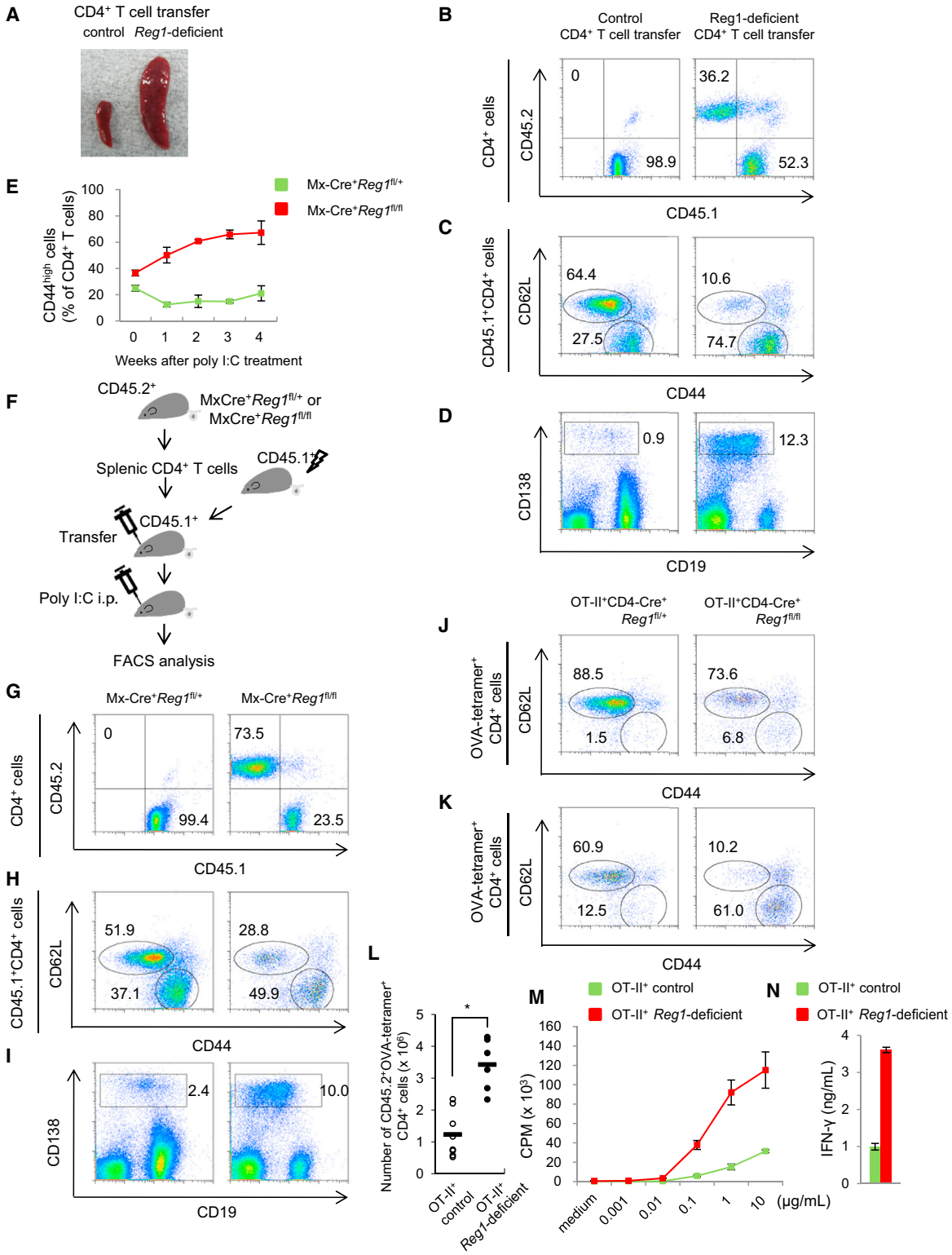

Figure 2. Mature CD4⁺ T Cells Are Responsible for the Cause of the Disease

(A) Spleens from mice that received CD4⁺ T cells from control or CD4-Cre⁺ *Regnase-1*$^{fl/fl}$ mice.

(B–D) Representative flow cytometric analysis of splenocytes. Splenic CD4⁺ T cells from CD4-Cre⁺ *Regnase-1*$^{fl/+}$ or CD4-Cre⁺ *Regnase-1*$^{fl/fl}$ mice were transferred into CD45.1⁺ congenic mice. Three months later, the population of CD45.1 and CD45.2 in CD4⁺ T cells (B), expression of CD62L and CD44 in CD45.1⁺CD4⁺ T cells (C), and proportion of plasma cells (D) in the spleen were analyzed. Representative data of two independent experiments.

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of Regnase-1 (Figures S4E and S4F). Whereas many genes upregulated in T cells lacking Regnase-1 are known to be regulated by NF-κB, activation of the NF-κBp65 subunit in response to stimulation with anti-CD3ε Ab and PMA plus ionomycin was not enhanced, but impaired, in the absence of Regnase-1 in $CD4^+$ T cells (Figure S4G). In contrast, OT-II^+ splenic T cells lacking Regnase-1 showed enhanced proliferation compared with wild-type OT-II^+ T cells in response to anti-CD3ε Ab stimulation (Figure S4H). Furthermore, TCR-induced production of effector cytokines, such as IFN-γ, IL-17, and IL-4, was also elevated in the absence of Regnase-1 (Figure S4I). Collectively, Regnase-1 suppresses T cell effector functions downstream of the TCR signaling pathways.

Furthermore, we knocked down Regnase-1 expression in Jurkat cells (Figure 4A) and examined changes in mRNA expression in response to PMA and ionomycin stimulation. Knockdown of Regnase-1 increased expression of *c-Rel*, *Ox40*, and *Il2* mRNA after stimulation with PMA plus ionomycin (Figure 4B). In contrast, *IκBα* mRNA levels were comparably induced between parent and Regnase-1 knockdown Jurkat cells. Furthermore, the half-lives of *c-Rel*, *Ox40*, and *Il2* mRNA, but not *IκBα* mRNA, were extended in Regnase-1 knockdown cells compared to cells treated with control small interfering RNA (siRNA) (Figure 4C). Reciprocally, overexpression of Regnase-1 in Jurkat cells resulted in the inhibition of the expression of *c-Rel*, *Ox40*, and *Il2* mRNA (Figure 4D). In contrast, the expression of Regnase-1 RNase-inactive form (D141N) failed to suppress these mRNAs, indicating that the RNase activity of Regnase-1 is essential for suppressing a set of mRNAs.

We have previously shown that Regnase-1 controls the expression of mRNAs via their 3′ UTR. To examine whether mRNAs encoding proteins controlling T cell activation are regulated by Regnase-1, we generated a set of luciferase reporter constructs with 3′ UTRs from genes encoding cytokines, cell-surface molecules, and transcription factors. By coexpression of these reporters and Regnase-1 or its nuclease-inactive form in HEK293 cells, we could identify genes whose 3′ UTRs are regulated by Regnase-1 nuclease activity. In addition to *Il6* and *Il12b*, *Il1b* and *Il2* are destabilized by Regnase-1 overexpression (Figure S5A). When we examined cell-surface molecules involved in T cell activation, *Icos*, *Cd44*, *Ox40*, and *Tnfr2* mRNAs were downregulated by Regnase-1 expression (Figure S5B), consistent with upregulation of these genes in T cells lacking Regnase-1. Among transcription factors involved in T cell activation, *c-Rel* mRNA 3′ UTR was profoundly inhibited by wild-type, but not D141N, Regnase-1 expression (Figure S5C). In contrast, the 3′ UTR of NF-κB family members other than *c-Rel* were unaffected by Regnase-1 overexpression (Figure S5C), indicating that c-Rel is the sole Regnase-1 target among NF-κB family members. Although NFATc1 mRNA 3′ UTR was inhibited by Regnase-1 overexpression, NFATc1 mRNA expression was increased only modestly in the absence of Regnase-1 in T cells (Figure S5D; Table S1). Collectively, Regnase-1 appears to control multiple mRNAs for suppressing T cell effector function.

To examine whether mRNAs differentially expressed in Regnase-1-deficient T cells are directly regulated by Regnase-1, we examined mRNAs encoding *Il2*, *Ox40*, and *c-Rel*. The Tet-off assay revealed that overexpression of wild-type, but not D141N mutant, Regnase-1 accelerated the degradation of *Il2*, *Ox40*, and *c-Rel* mRNAs (Figure 4E), which indicates that Regnase-1 directly destabilizes *Il2*, *Ox40*, and *c-Rel* mRNAs in a manner dependent on their RNase activity. Then we examined sequences responsible for Regnase-1-mediated suppression in *c-Rel* 3′ UTR. We transfected HEK293 cells with luciferase reporter constructs with the 3′ UTR of *c-Rel*. In addition to the entire 3′ UTR of *c-Rel* mRNA, the first half of the 3′ UTR recapitulates Regnase-1-mediated suppression (Figure 4F). We found putative stem-loop structures in the sequences of the first half of *c-Rel* 3′ UTR (Figure 4G). Addition of these sequences from the *c-Rel* 3′ UTR to β-globin 3′ UTR resulted in reduction of the luciferase activity in response to Regnase-1 overexpression (Figure 4F), suggesting that this stem-loop structure is responsible for Regnase-1-mediated inhibition of c-Rel expression.

Deletion of c-Rel Partially Rescued T Cell Effector Function in *Regnase-1*$^{-/-}$ Mice

Given that some of the genes upregulated in Regnase-1-deficient T cells are not direct Regnase-1 targets, we hypothesized that transcription factor(s) regulated by Regnase-1 further control T cell activation. c-Rel has been shown to be a key factor in T cell activation associated with autoimmune disease (Hilliard et al., 2002). Therefore, we generated mice lacking both c-Rel and Regnase-1. The differentiation of $CD4^+$ T cells from naive to effector/memory cells as well as surface CD69 expression observed in *Regnase-1*$^{-/-}$ mice was partially inhibited by the

(E) Percentage of $CD44^{hi}$ among $CD4^+$ T cells in the blood from Mx-Cre^+ *Regnase-1*$^{fl/+}$ or Mx-Cre^+ *Regnase-1*$^{fl/fl}$ after treatment with poly I:C (n = 3). Error bars indicate SD.

(F) Schematic diagram of generation of mice transferred with $CD4^+$ T cells from Mx-Cre^+*Regnase-1*$^{fl/+}$ or Mx-Cre^+*Regnase-1*$^{fl/fl}$.

(G–I) Flow cytometric analysis of splenocytes of the mice transferred as in (F). Population of CD45.1 and CD45.2 in $CD4^+$ T cells (G), expression of CD62L and CD44 (H) in $CD45.1^+CD4^+$ T cells, and proportion of plasma cells (I).

(J and K) Flow cytometric analysis of splenocytes of the mice with the indicated genotype. Expression of CD62L and CD44 without treatment (J) and 1 week after immunization (K).

(L–N) OT-II^+ control or *Regnase-1*-deficient naive $CD4^+$ T cells were adoptively transferred to $CD45.1^+$ wild-type mice and immunized with OVA in Alum. The number of OVA-induced donor $CD4^+$ T cells was examined, and FACS analysis was performed using pooled populations of splenocytes and LN cells at day 5 after immunization (L). Bars indicate the means and statistical significance was determined using the Student's t test in (L). Splenocytes were restimulated with various doses of OVA peptide, and recall proliferation was assessed by thymidine incorporation at 72 hr (M). IFN-γ production was measured in culture supernatant at 48 hr (N). Error bars indicate SD.

Open and closed circles represents OT-II^+ control (n = 8) and *Regnase-1*-deficient (n = 6) $CD4^+$ T cells, respectively. *$p < 0.001$. Similar results were obtained in two independent experiments. See also Figure S3.

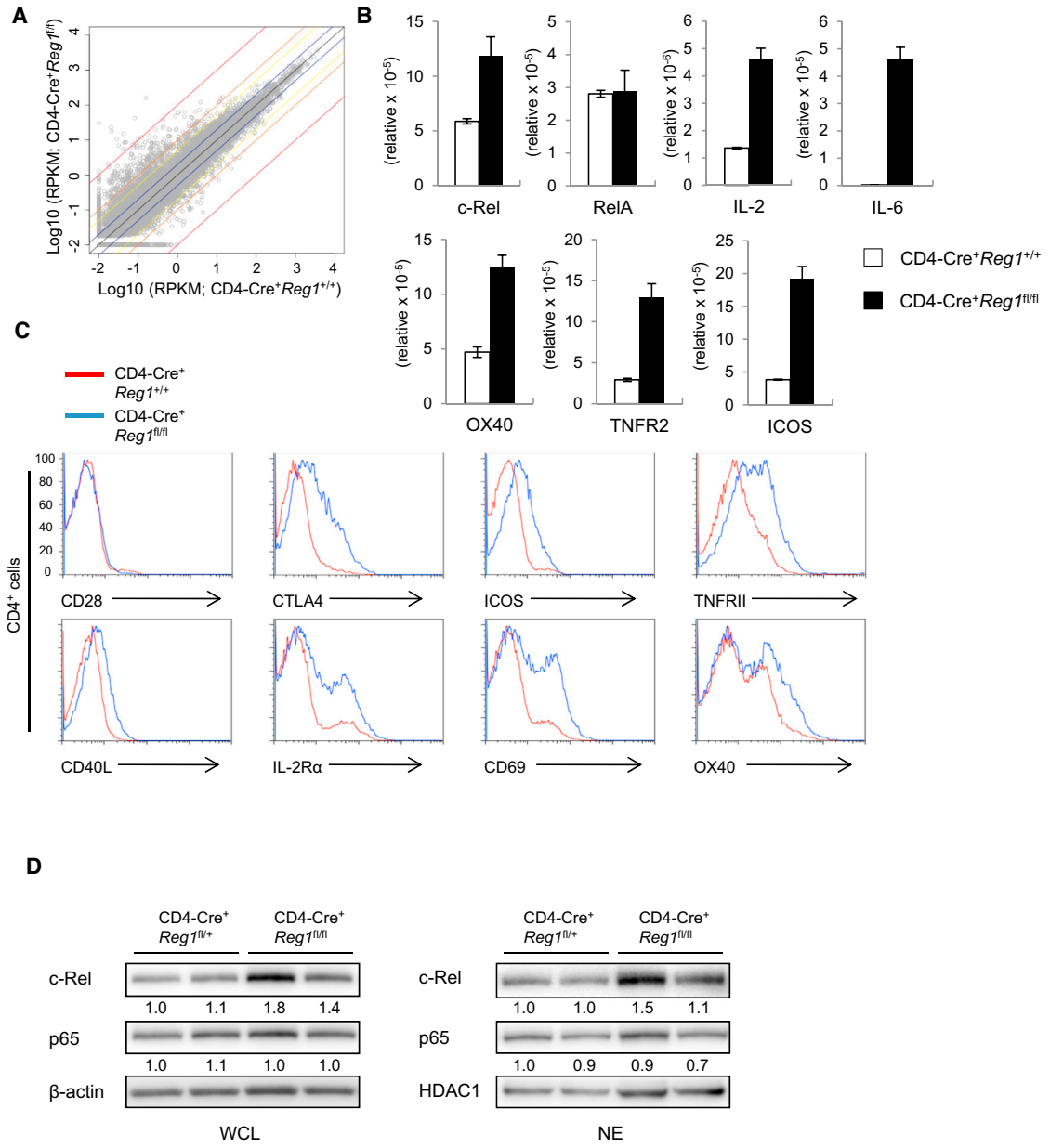

Figure 3. Altered Gene Expressions of Cytokines, Surface Costimulatory Molecules, and Transcription Factors in the Absence of Regnase-1

(A) Scatterplot of gene expression profile in CD4$^+$ T cells from wild-type and CD4-Cre$^+$*Regnase-1*$^{fl/fl}$ mice. The red, orange, yellow, and blue lines represent 100-, 10-, 5-, and 2-fold differences, respectively.

(B) Quantitative PCR analysis of the expressions of a set of genes in splenic CD4$^+$ T cells from mice with the indicated genotype. Error bars indicate SD.

(C) Expression of surface markers in CD4$^+$ T cells from mice with the indicated genotype.

(D) Immunoblot analysis of whole-cell lysate and nuclear extract of control or *Regnase-1*$^{-/-}$ T cells. The protein levels of c-Rel and p65 were quantified as a ratio to a loading control by densitometry.

See also Figure S4 and Tables S1 and S2.

lack of c-Rel (Figures S6A and S6B). Furthermore, generation of CD138$^+$ plasma cells depends on the presence of c-Rel (Figure S6C). Because the levels of c-Rel expression was partially reduced in *Rel*$^{+/-}$ T cells compared with wild-type (Figure S6D), we examined the effect of the decrease of c-Rel expression in Regnase-1 deficiency by generating *Regnase-1*$^{-/-}$*Rel*$^{+/-}$ mice. Indeed, decrease, but not abrogation, of c-Rel also partially rescued the increase of effector T cells and plasma cells and surface expression of CD69 induced by Regnase-1 deficiency (Figure S6E). These results indicate that increased c-Rel expression contributes to the activation of T cells and subsequent B cell activation under Regnase-1 deficiency.

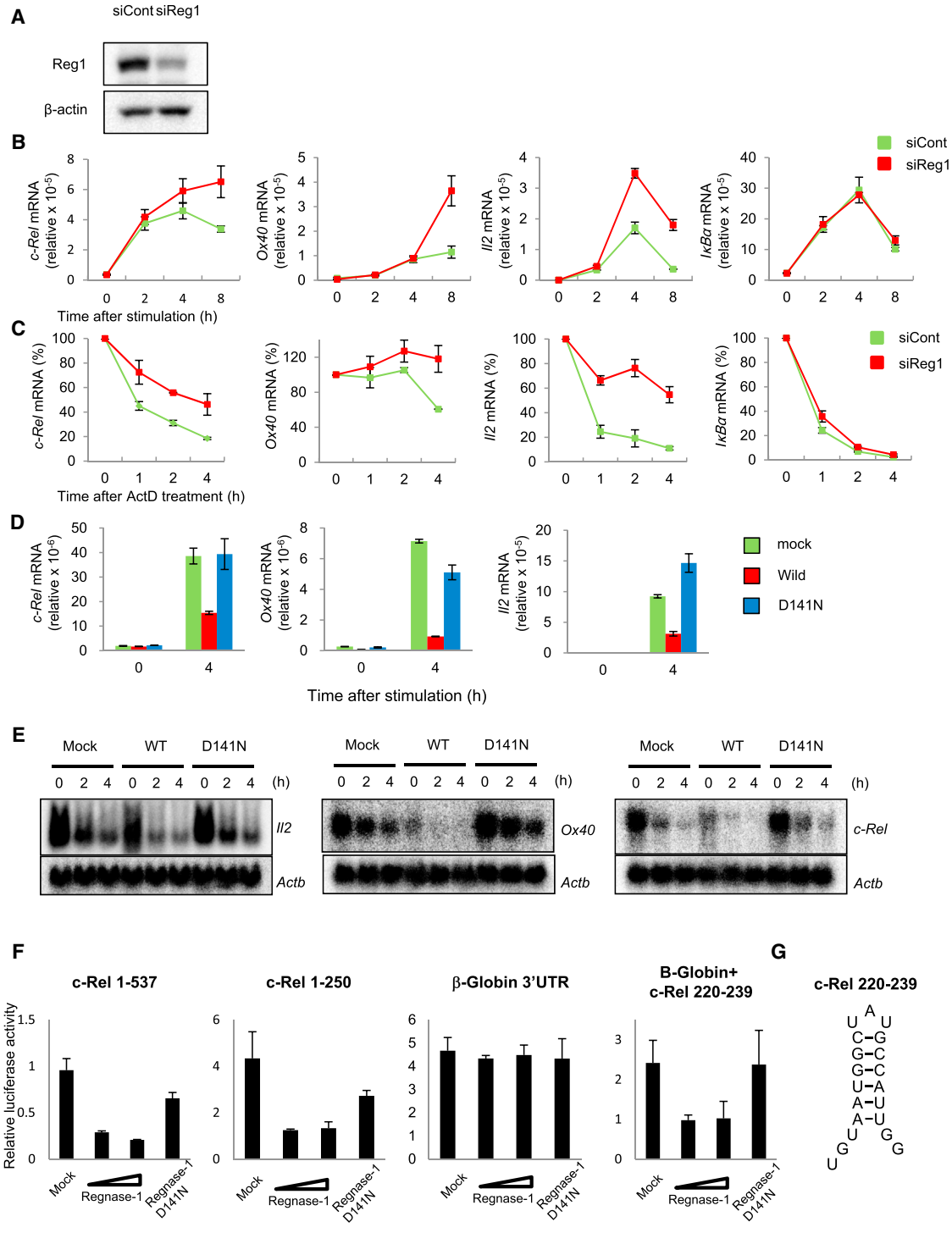

Figure 4. Identification of Regnase-1 Target mRNAs in T Cells

(A) Immunoblot analysis of Regnase-1 in Jurkat cells transfected by electroporation with Regnase-1-specific siRNA (si-Reg1) and control siRNA (si-Cont).

(B) Quantitative PCR analysis of mRNAs encoding *c-Rel*, *Ox40*, *Il2* and *IκBα* in Jurkat cells electroporated with si-Reg1 or si-Cont, then, 24 hr later, stimulated with PMA plus ionomycin for 0-8 hr. Error bars indicate SD. Data are representative of three independent experiments.

(C) Quantitative PCR analysis of mRNAs encoding *c-Rel*, *Ox40*, *Il2*, and *IκBα* in Jurkat cells electroporated as in (E), stimulated for 4 hr with PMA plus ionomycin, and then treated for 0–4 hr with actinomycin D (ActD). Error bars indicate SD. Data are representative of three independent experiments.

(D) Jurkat cells were electroporated with wild-type or D141N Regnase-1 or mock and then stimulated with PMA plus ionomycin for 4 hr. Gene expression was measured by quantitative PCR. Error bars indicate SD. Data are representative of two independent experiments.

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Regnase-1 Is Cleaved and Degraded in a Malt1-Dependent Manner

Then we investigated the change in Regnase-1 expression in T cells. Stimulation of Th1 cells with IL-18, which activates signaling pathways overlapping with those of TLR/IL-1R, induced the disappearance of Regnase-1 expression (Figure 5A). Treatment with a proteasome inhibitor, MG132, resulted in the suppression of Regnase-1 degradation, suggesting that the MyD88-dependent signaling induces degradation of Regnase-1 in T cells in a manner similar to that in macrophages. In addition, stimulation of naive $CD4^+$ T cells with anti-CD3ε plus anti-CD28 antibodies or PMA plus ionomycin led to the reduction in Regnase-1 (Figures 5B and 5C), although the decrease of Regnase-1 was not inhibited by MG132 treatment (Figure 5D). Furthermore, coculture of OT-II^+ T cells with OVA-pulsed DCs resulted in the decrease in Regnase-1 expression (Figure 5E). These results indicate that the TCR signaling decreases Regnase-1 expression independent of the IKK-dependent mechanisms or the proteasome system. In contrast, TCR stimulation led to the appearance of a protein smaller than full-length Regnase-1 that was detected by anti-Regnase-1 antibody 15 min after PMA and ionomycin stimulation (Figure 5F). Stimulation of *Regnase-1*$^{-/-}$ $CD4^+$ T cells with PMA and ionomycin did not produce the small protein (Figure 5F), suggesting that TCR signaling leads to the cleavage of Regnase-1. The change in Regnase-1 expression was induced by PMA, but not ionomycin alone (Figure 5G). Given the fact that PMA activates the complex of Carma1, Bcl10, and Malt1, also known as paracaspase, downstream of PKCθ for activating NF-κB (Thome, 2008), we examined the involvement of this complex in the decrease of Regnase-1. Interestingly, *Bcl10*$^{-/-}$ or *Malt1*$^{-/-}$ $CD4^+$ T cells stimulated with PMA and ionomycin did not decrease Regnase-1 or generate cleaved protein (Figures 5H and 5I). In addition, treatment of Jurkat cells with zVRPR-fmk, a compound inhibiting the protease activity of Malt1, prevented the degradation of Regnase-1 (Figure 5J). Furthermore, degradation of Regnase-1 induced by anti-CD3ε plus anti-CD28 antibodies in $CD4^+$ T cells was also suppressed by zVRPR-fmk treatment (Figure 5K). These results indicate that Regnase-1 is degraded in a manner dependent on the protease activity of Malt1. Of note, the Malt1 inhibitor failed to suppress IL-18-mediated degradation of Regnase-1 in $CD4^+$ T cells (Figure 5A), indicating that the mechanism of Regnase-1 degradation in T cells is different depending on the stimuli.

Malt1-Mediated Regnase-1 Cleavage Increases Stability of *c-Rel*, *Ox40*, *and Il2* mRNAs in T Cells

Then we transfected HEK293 cells with an N-terminal Flag-tagged and a C-terminal HA-tagged Regnase-1-expressing plasmid, together with Malt1 and Bcl10. Cotransfection of Regnase-1 with Bcl10 and Malt1 in HEK293 cells led to the cleavage of Regnase-1, producing an ~15 kDa N-terminal and 55 kDa C-terminal fragments (Figure 6A). A fusion protein comprised of the N terminus of inhibitor of apoptosis 2 (API2) and the C terminus of Malt1 (API2-Malt1) generated in MALT lymphoma by a translocation is proteolytically active to cleave NF-κB-inducing kinase (NIK) (Rosebeck et al., 2011). Coexpression of Regnase-1 and API2-Malt1 resulted in the generation of similar fragments (Figure 6B). These findings suggest that Malt1 cleaves Regnase-1 at a single site. Because Malt1 is an arginine-specific protease, we expressed mutant Regnase-1 constructs whose arginine residues present in the N terminus (R111, R130, R136, R158, and R214) (Figure 6C) were substituted with alanine together with Flag-tagged API2-Malt1 in HEK293 cells. We found that Regnase-1 with substitution of alanine for arginine at position 111 (R111A) was resistant to API2-Malt1-mediated cleavage, although other mutant Regnase-1 proteins were cleaved by API2-Malt1 (Figure 6B). These results indicated that Regnase-1 is cleaved at the site of R111 by Malt1. Consistently, wild-type, but not R111A, Regnase-1 was degraded 30 min after PMA/ionomycin stimulation in Jurkat cells (Figure 6D), indicating that cleavage of Regnase-1 by Malt1 at R111 is critical for inducing Regnase-1 degradation in response to TCR stimulation. To further investigate the role of Malt1 protease activity in controlling mRNA stability, primary $CD4^+$ T cells pretreated with zVRPR-fmk were stimulated with PMA/ionomycin, and the kinetics of mRNA degradation was examined after stopping new transcription. As shown in Figure 6E, mRNAs for *c-Rel*, *Ox40*, and *Il2*, but not mRNA for *IκBα*, were destabilized in response to treatment with the Malt1 inhibitor, indicating that the Malt1 protease activity is critical for increasing the stability of a set of mRNAs. Finally, we examined whether cleavage of Regnase-1 in T cells is induced in vivo in response to antigen stimulation. When OT-II mice harboring TCR specific to OVA were immunized with OVA together with alum adjuvant, Regnase-1 levels decreased and cleaved products increased in $CD4^+$ T cells 12 hr after immunization (Figure 6F). The immunization resulted in no cleavage of Regnase-1 in $CD4^+$ T cells from nontransgenic animals, indicating that TCR signaling is required for the cleavage of Regnase-1.

DISCUSSION

We have previously shown that Regnase-1 controls *Il6* and *Il12b* mRNA via their 3′ UTR (Matsushita et al., 2009). However, lack of IL-6 and IL-12p40 only modestly rescued aberrant plasma cell development and Th1 induction, respectively. In contrast, T cells lacking Regnase-1 were spontaneously activated and showed effector/memory phenotype. It is noteworthy that mice lacking Regnase-1 in macrophages and neutrophils also developed a fatal inflammatory disease (data not shown), indicating that Regnase-1 is essential for suppressing aberrant activation

(E) HEK293 tet-off cells were cotransfected with pTREtight-Il2, pTREtight-Ox40, or pTREtight-c-Rel containing CDS plus 3′ UTR together with wild-type or D141N Regnase-1 expression plasmid or control (empty) plasmid. Total RNA was prepared after dox treatment, and *Il2*, *Ox40*, *c-Rel*, and *Actb* levels were determined by northern blot analysis. Representative data of two independent experiments are shown.

(F) Luciferase activity of HEK293 cells transfected for 48 hr with luciferase reporter plasmids, together with control plasmid (Mock) or expression plasmid for wild-type or D141N Regnase-1. Error bars indicate SD. Data are representative of two independent experiments.

(G) Predicted stem-loop structure of the Regnase-1-responsive element in the 3′ UTR of *c-Rel* mRNA. See also Figure S5.

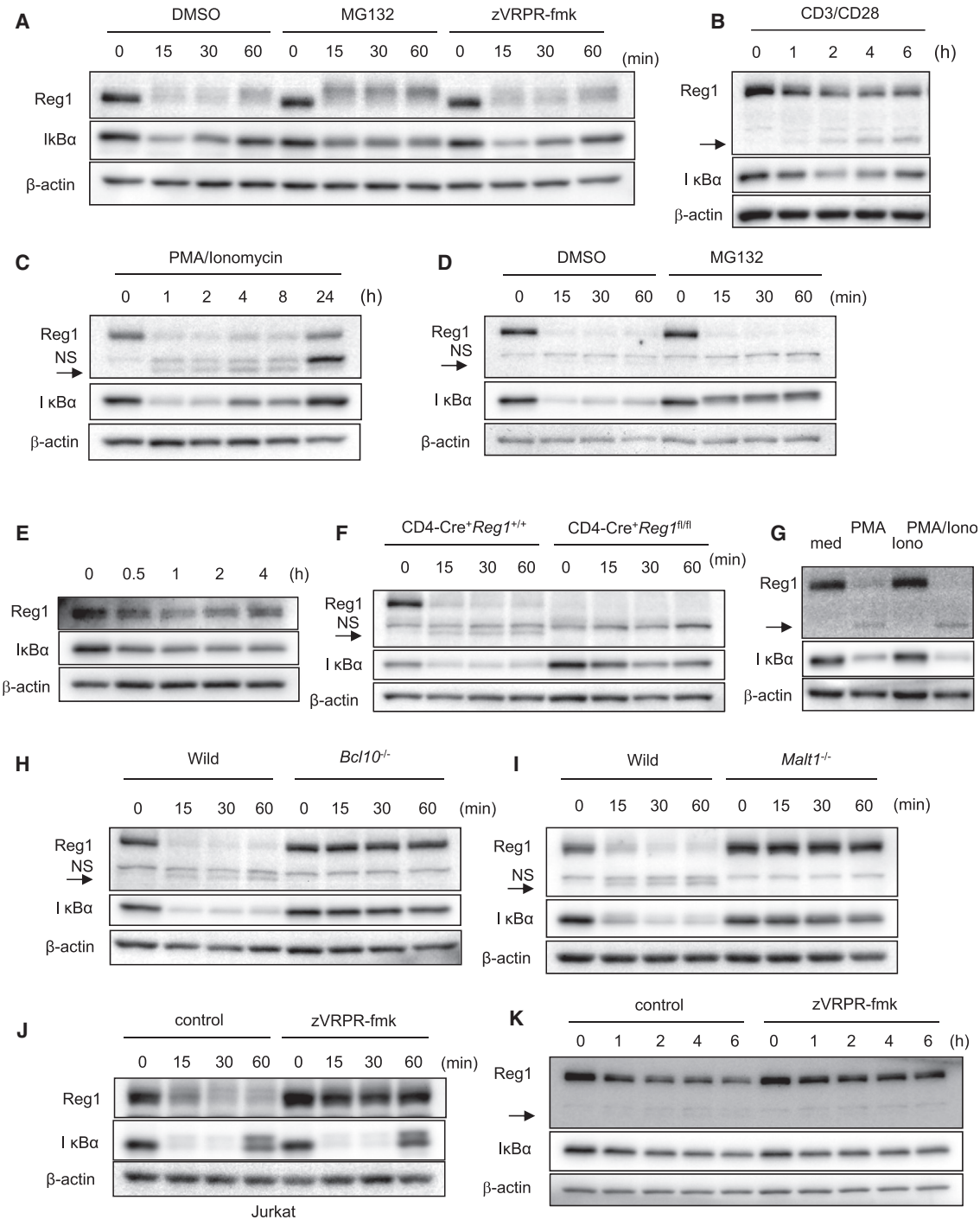

Figure 5. Regnase-1 Is Cleaved and Degraded in a Malt1-Dependent Manner in T Cells

(A–K) Immunoblot analysis of Regnase-1, IκBα, and β-actin in whole-cell lysates from indicated cell types after treatment with indicated stimuli.

(A) Th1 cells were pretreated with 0.1% dimethyl sulfoxide (DMSO), the proteasome inhibitor MG-132 (10 μM), or the Malt1-specific inhibitor zVRPR-fmk (100 μM) and then stimulated with IL-18 for 0–60 min.

(B and C) Wild-type $CD4^+$ T cells were stimulated with anti-CD3ε plus anti-CD28 (B), or with PMA plus ionomycin (C) for the indicated time.

(D) Wild-type $CD4^+$ T cells were pretreated with 0.1% DMSO or MG132 (10 μM) and then stimulated as in (C) for 0–60 min.

(E) $CD4^+$ T cells were isolated from OT-II^+ mice and cocultured with OVA-pulsed DC for indicated time.

(F) $CD4^+$ T cells from CD4-Cre^+ *Regnase-1*$^{+/+}$ or CD4-Cre^+ *Regnase-1*$^{fl/fl}$ mice were stimulated as in (C) for 0–60 min.

(G) Wild-type $CD4^+$ T cells were stimulated with PMA, ionomycin, or both for 30 min.

(H and I) $CD4^+$ T cells were prepared from wild-type and *Bcl10*$^{-/-}$ (H), or *Malt1*$^{-/-}$ mice (I) and then stimulated as in (C) for 0–60 min.

(legend continued on next page)

of multiple immune cells in a cell-autonomous fashion. Treg cell populations and T cell suppression activity were not impaired in Regnase-1-deficient T cells, suggesting that Treg cells are not the cause of the pathology in the absence of Regnase-1. Thymic CD4 and CD8 single positive T cell populations and TCRβ repertoires were similar in wild-type mice and mice with Regnase-1-deficient T cells, suggesting that Regnase-1 is dispensable for thymic T cell development. Therefore, it is likely that Regnase-1 controls the activation of naive to effector T cells peripherally in a cell-autonomous manner. However, further studies are required for assessing the role of Regnase-1 in thymic T cell negative/positive selection.

The Regnase-1 nuclease domain has been reported to act as a deubiquitinase for suppressing NF-κB activation in addition to its nuclease activity (Liang et al., 2010). In contrast, we have previously shown that NF-κB activation in response to TLR4 stimulation as well as IL-1R stimulation was comparable between wild-type and Regnase-1-deficient macrophages (Matsushita et al., 2009). Similarly, we did not observe an enhancement in NF-κB-DNA binding activity in Regnase-1-deficient T cells compared with wild-type cells (Figure S4G). These results indicate that Regnase-1 is not critical for suppressing NF-κB, either in macrophages or T cells. Thus, we believe that Regnase-1 functions as an RNase, degrading mRNAs downstream of the TLR or TCR signaling pathways inducing activation of transcription factors.

We identified a set of mRNAs upregulated in Regnase-1-deficient T cells with transcriptome analysis. Because many transcription factors and surface cytokine/chemokine receptors are highly upregulated, both in naive and effector Regnase-1-deficient T cells, it is assumed that there are many genes upregulated secondarily, in addition to primarily regulated genes. The complexity makes it difficult to clarify Regnase-1 target mRNAs responsible for T cell activation. We found that *c-Rel* mRNA was a unique target of Regnase-1 among NF-κB family members. c-Rel has been shown to be involved in autoimmune diseases (Campbell et al., 2000) and also contributes to differentiation into Th1 cells (Hilliard et al., 2002). However, deletion of c-Rel failed to completely block the T cell activation observed in *Regnase-1*$^{-/-}$ mice. These results indicate that enhanced expression of c-Rel alone cannot fully explain the phenotype of *Regnase-1*$^{-/-}$ mice. Regnase-1 is likely to control the expression of multiple genes responsible for the development of autoimmune diseases.

T cells lacking Regnase-1 highly express *Icos* mRNA and protein on the cell surface. Indeed, ICOS seems to be one promising Regnase-1 target for suppression (Figure S5B). This observation is reminiscent of San/San mice, which harbor a point mutation in Roquin gene (Yu et al., 2007). The mutant mice show follicular T cells were expanded because of excess expression of ICOS. However, the lack of ICOS under Regnase-1 deficiency failed to rescue spontaneous activation of T cells and pathology (data not shown), suggesting that Roquin and Regnase-1 control T cell activation by different mechanisms.

We have previously reported that TLR and IL-1R signaling induces IKK-mediated phosphorylation of Regnase-1, triggering its degradation via the ubiquitin-proteasome system in macrophages and HeLa cells (Iwasaki et al., 2011). IL-18-induced degradation of Regnase-1 was inhibited by a proteasome inhibitor, suggesting that MyD88-dependent signaling induces Regnase-1 degradation in T cells via IKK activation. In contrast, the change in Regnase-1 expression in response to the TCR signaling was totally independent of the proteasome system. We have previously shown that IKK activation by TNF stimulation is not sufficient to induce degradation of Regnase-1 (Iwasaki et al., 2011). Phosphorylation of Regnase-1 by IRAK1 downstream of MyD88 appears to be required for the phosphorylation of Regnase-1 by IKKs. Thus, TCR-mediated activation of IKKs might be insufficient to induce degradation of Regnase-1 because MyD88 and IRAK1 are dispensable for the TCR signaling pathway.

We identified Malt1 as a protease responsible for Regnase-1 cleavage in response to TCR stimulation. Malt1 forms a complex with Bcl10 and CARMA1 and plays an important role in activation of NF-κB in T cells in response to TCR stimulation (Thome, 2008). Several reports have identified Malt1 cleavage target proteins that are involved in T cell activation. These include A20, Bcl10, CYLD, and NIK (Coornaert et al., 2008; Rebeaud et al., 2008; Rosebeck et al., 2011; Staal et al., 2011). Because A20 and CYLD are deubiquitinases targeting K63-type polyubiquitin chains and are responsible for suppressing NF-κB activation, inactivation of A20 and CYLD by Malt1 is expected to potentiate NF-κB activation. On the other hand, cleaved Bcl10 is highly potent to signal. Nevertheless, treatment with Malt1 protease inhibitor did not affect IκBα degradation in response to TCR stimulation; although, the presence of Malt1 is required for IκBα degradation (Figures 5H and 5I). In this regard, the protease activity of Malt1 seems to play a supportive, but not essential, role in NF-κB signaling. On the other hand, Malt1 protease activity is essential for TCR-mediated Reganse-1 degradation.

Treatment with the Malt1 protease inhibitor results in destabilization of a set of mRNAs. These data indicate that Malt1 is critical not only for activating NF-κB-mediated transcriptional control but also for prolonging mRNA half-lives by cleaving Regnase-1. Given that the Malt1 protease activity is critical for controlling the stability of a set of TCR-inducible mRNAs, it is tempting to speculate that the major role of Malt1 protease activity is to regulate mRNA stability by cleaving Regnase-1.

We showed that Regnase-1 was cleaved by Malt1 at the position R111. Because the position R111 is N-terminal to the RNase domain, the C-terminal product of cleaved Regnase-1 is expected to be enzymatically active. Indeed, a luciferase reporter assay revealed that a Regnase-1 construct corresponding to the C-terminal part of cleaved protein is still potent to suppress gene expression via *Il6* and *c-Rel* 3′ UTRs, although the deleted protein was less active than wild-type Regnase-1 protein (data not shown). However, the expression levels of cleaved

(J) Jurkat cells were stimulated with PMA plus ionomycin for 0–60 min in the absence or presence of zVRPR-fmk.
(K) CD4$^+$ T cells were pretreated with Z-VRPR-fmk and then stimulated as in (B) for the indicated time.
Arrows indicate a faster migrating form of Regnase-1. NS, nonspecific band. Data are representative of two (E–K) or three (A–D) independent experiments. See also Figure S6.

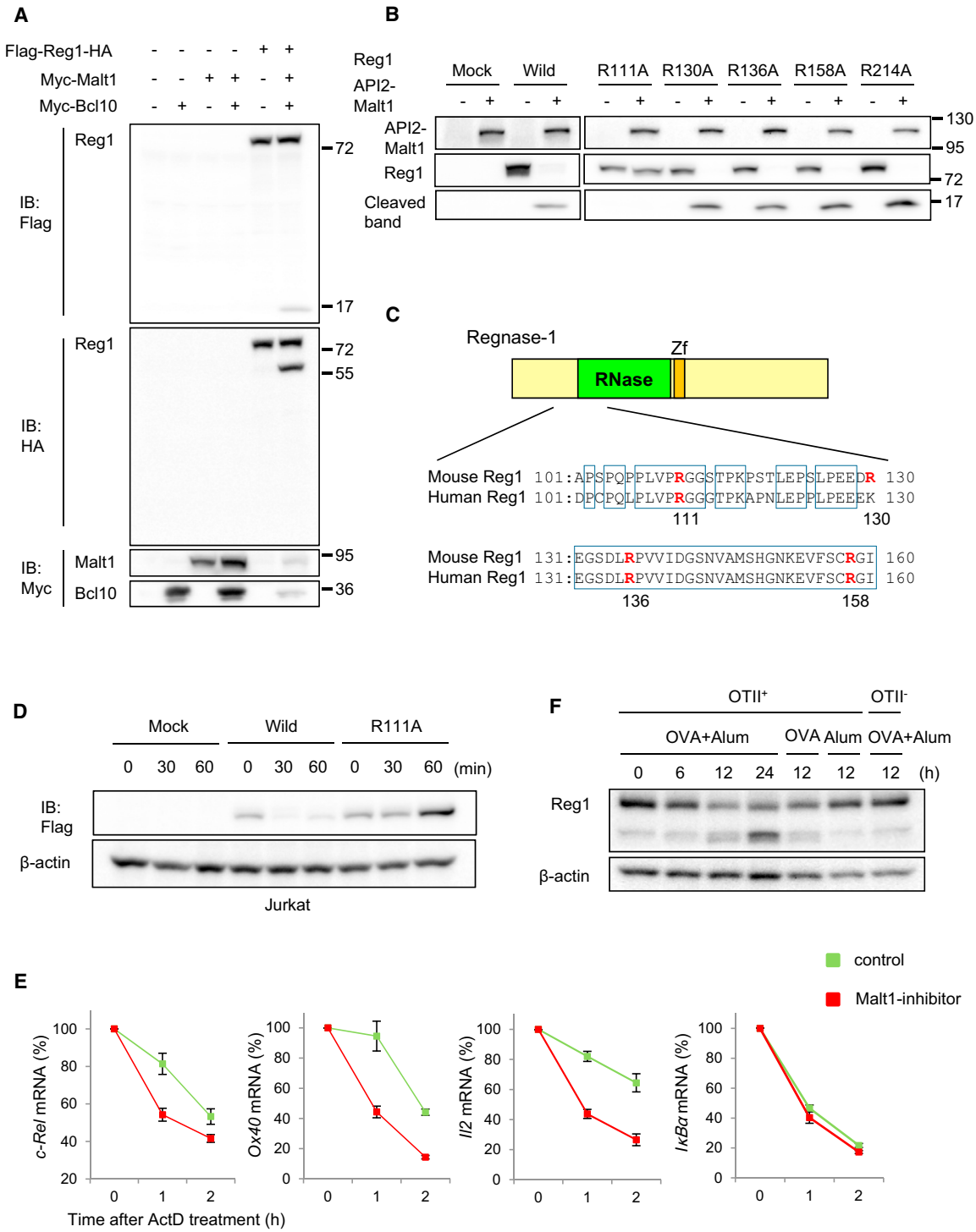

Figure 6. Protease Activity of Malt1 Is Critical for the Degradation of Regnase-1

(A) Immunoblot analysis of lysates of HEK293 cells transfected to express Flag- and HA-Regnase-1 and/or Myc-Malt1 or Myc-Bcl10 (above lanes), probed with anti-Flag, anti-HA, or anti-Myc.

(B) Cleavage of mouse Regnase-1 in HEK293 cells transfected with Flag-tagged wild-type or a series of mutant Regnase-1 with substitution of alanine for arginine, and/or Flag-API2-Malt1. N-terminal fragment of Regnase-1 was analyzed by immunoblot with anti-Flag antibody.

(C) Domain structure of mouse Regnase-1 and alignment of N-terminal RNase domain in mouse and human Regnase-1.

(D) Immunoblot analysis of Regnase-1 and β-actin in Jurkat cells transfected with the plasmids expressing wild or R111A Regnase-1, or control plasmid (Mock) and then stimulated with PMA plus ionomycin.

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Regnase-1 protein were much less than those of the full-length protein expressed in unstimulated cells, suggesting that the cleaved protein is unstable and further degraded by a cellular protease.

In summary, we have demonstrated that Regnase-1 is essential for suppressing an unwanted T-cell-mediated immune reaction by targeting multiple mRNAs encoding transcription factors, surface molecules, and cytokines. Dynamic regulation of Regnase-1 by TCR signaling contributes to robust T cell activation. Manipulation of Malt1-mediated Regnase-1 expression appears to be an important control point for T-cell-mediated immune responses in vivo.

EXPERIMENTAL PROCEDURES

Generation of Regnase-1-Floxed Mice

The targeting vector was constructed by inserting a 3.2 kb fragment containing exons 4 to 6 of the *Regnase-1* (*Zc3h12a*) gene flanked by loxP sites, 1.0 kb of a 3′ sequence, 5.7 kb of a 5′ sequence, and a neomycin (neo)-resistant gene flanked by loxP sites into a pBluescript vector. The targeting vector was linearized and transfected into GSI-I embryonic stem (ES) cells by electroporation, and G418-resistant clones were screened for homologous recombination by PCR and Southern blot analysis. The ES cells were transiently transfected with CMV-Cre to eliminate the neo gene. The successfully recombinated clones were microinjected into blastocytes derived from C57BL/6 mice and transferred to pseudopregnant females. Mating of chimeric male mice to C57BL/6 female mice resulted in the transmission of the floxed allele to the germline. The *Regnase-1*$^{fl/+}$ mice were crossed with the indicated Cre-expressing mice. See also Figure S2.

Mice

CD4-Cre, Mx-Cre, or OT-II TCR transgenic mice have been described previously (Barnden et al., 1998; Kühn et al., 1995; Lee et al., 2001). *c-Rel*$^{-/-}$, *Bcl10*$^{-/-}$, and *Malt1*$^{-/-}$ mice were provided by S. Gerondakis, S. Morris, and V.M. Dixit, respectively (Köntgen et al., 1995; Ruefli-Brasse et al., 2003; Xue et al., 2003). All mice were housed under specific pathogen-free conditions. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases, Osaka University.

Plasmids, Antibodies, and Reagents

The plasmid expressing Regnase-1 has been described. Point mutations of the gene encoding Regnase-1 (R111A, R130A, R136A, R158A, and R214A) were made with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The complementary DNAs (cDNAs) encoding Bcl10 and Malt1 were ligated to the vector pcDNA3.1(+)-Myc for expression. Full-length 3′ UTR of *c-Rel* (1–536) and 3′ UTR of β-globin (1–130), with or without a *c-Rel* 3′ UTR (220–239) sequence, were inserted in the pGL3 vector. *c-Rel*, *Ox40*, or *Il2* CDS+3′ UTR was inserted in pTREtight vector (Clontech, Mountain View, CA, USA). Expression plasmid for Flag-tagged API2-Malt1 was provided by L.M. McAllister-Lucas. Antibodies for immunoblot analysis were as follows: anti-Flag (M2 [A8592]; Sigma-Aldrich, St. Louis), anti-hemagglutinin (3724; Cell Signaling Technology, Danvers, MA, USA), anti-c-Myc (M4439; Sigma-Aldrich), IκBα (C-21; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (C-11; Santa Cruz Biotechnology). Polyclonal rabbit antibody to Regnase-1 has been described previously. Anti-mouse CD3ε and CD28 were from BD Pharmingen (San Diego). Actinomycin D, PMA, ionomycin, and OVA (A5503) were from Sigma-Aldrich. Imject Alum was from Pierce (Rockford, IL, USA). I-A^{d} OVA helper peptide was from MBL. MG132 was from Calbiochem (Darmstadt, Germany). Z-VRPR-fmk was from Alexis (Farmingdale, NY, USA). Poly I:C has been described. The mouse IFN-γ and ANA antibody ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) and Alpha Diagnostic (Owings Mills, MD, USA), respectively.

Transfer of CD4^{+} T Cells

CD4^{+} T cells were isolated from CD4-Cre^{+} *Regnase-1*$^{fl/+}$ and CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ splenocytes using the autoMACS system. The cells were intravenously transfused into partially irradiated CD45.1^{+} mice. Three months after the transfer, the mice were sacrificed for experiments. For inducible deletion of Regnase-1, 8- to 12-week-old MxCre^{+}*Regnase-1*$^{fl/fl}$ mice were intraperitoneally (i.p.) injected with 50 μg of poly I:C. We used Mx-Cre^{+} *Regnase-1*$^{fl/fl}$ mice when we transferred unrecombinased CD4^{+} T cells into CD45.1^{+} mice followed by injection with 50 μg of poly I:C per head.

Immunization and Recall Stimulation

CD62L^{hi}CD44^{lo} CD4^{+} T cells (1×10^{6}) from OT-II^{+} control and CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ mice were adoptively transferred into CD45.1^{+} C57BL/6 mice. The mice were immunized i.p. with 100 μg of OVA protein complexed to Imject Alum (Thermo Scientific, Rockford, IL, USA) at the day of transfer. At day 5 after immunization, the mice were sacrificed. For in vitro recall responses, 3×10^{5} splenocytes were stimulated with various doses of $OVA_{323-339}$ peptide.

RNA Sequencing

Total RNA was prepared from fluorescence-activated cell sorting (FACS)-sorted CD4^{+} T cells from CD4-Cre^{+} *Regnase-1*$^{+/+}$ or CD4-Cre^{+} *Regnase-1*$^{fl/fl}$ mice. For RNA sequencing, sample preparation was performed using the TruSeq RNA Kit (Illumina, San Diego). cDNA cluster generation and sequencing were carried out by cBot and HiSeq2000, respectively (Illumina). For processing of RNA sequencing (RNA-seq) data, quality of RNA-seq tags was checked using FASTQC. The 3′ ends of tags were trimmed, and the 30 most 5′ bases were mapped to the mouse genome (mm9). Mapping was done using TopHat (v. 1.4.0) (Trapnell et al., 2009) and Bowtie (v. 0.12.7) (Langmead et al., 2009). University of California Santa Cruz (UCSC) annotations of transcripts were used for the initial mapping of tags to the transcriptome. Tags that could be uniquely mapped with at most two mismatches were converted to counts of tags per base in the genome, which were in turn used to calculate reads per kilobase per million (RPKM) values for each transcript (Mortazavi et al., 2008). In order to facilitate comparison of gene expression levels between samples, RPKM values were normalized using quantile normalization.

Statistical Analysis

All data are presented as mean and SD. Statistical analysis was performed with the Student's t test. Statistical significance was defined as a p value of <0.05.

For further additional details about the experimental procedures, please refer to the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.04.034.

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(E) Quantitative PCR analysis of mRNAs encoding *c-Rel*, *Ox40*, *Il2*, and *IκBα* among total RNA from primary CD4^{+} T cells stimulated with PMA plus ionomycin, followed by treatment for 0–4 hr with ActD. Error bars indicate SD.

(F) Immunoblot analysis of Regnase-1 and β-actin in CD4^{+} T cells from OT-II^{-} or OT-II^{+} mice immunized with OVA and/or Alum for the indicated period.

Data are representative of two (D and F), three (A and E), or five (B) independent experiments.

S.W. Morris and S. Yamasaki for *Bcl10*$^{-/-}$ mice, V.M. Dixit and N. Kayagaki for *Malt1*$^{-/-}$ mice, S. Gerondakis and T. Kurosaki for *c-Rel*$^{-/-}$ mice, and L.M. McAllister-Lucas for the Flag-API2-Malt1 plasmids. This work was supported by the Cabinet Office, Government of Japan and the Japan Society for the Promotion of Science (JSPS) through the funding program for World-Leading Innovative R&D on Science and Technology (FIRST Program) and the Grant-in-Aid for Specially Promoted Research. This work was also supported by the JSPS Grant-in-Aid for Young Scientists (A) (OT) and a grant-in-aid for Scientific Research on Innovative Areas "Genome Science" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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