Constitutive activation of the MAL T1 paracaspase in conventional T cells of Malt1\(^{TBM/TBM}\) (TRAF6 Binding Mutant = TBM) mice causes fatal infection and autoimmunity, but the involved targets and underlying molecular mechanisms are unknown. We genetically rendered a single MAL T1 substrate, the RNA-binding protein (RBP) Roquin-1, insensitive to MAL T1 cleavage. These Roquin-1 insensitive to MAL T1 mice showed normal immune homeostasis. Combining Roquin-1 insensitive to MAL T1 alleles with those encoding for constitutively active MAL T1 (TBM) prevented spontaneous T cell activation and restored viability of Malt1\(^{TBM/TBM}\) mice. Mechanistically, we show how antigen/MHC recognition is translated by MAL T1 into Roquin cleavage and derepression of Roquin targets. Increasing T cell receptor (TCR) signals inactivated Roquin more effectively, and only high TCR strength enabled derepression of high-affinity targets to promote Th17 differentiation. Induction of experimental autoimmune encephalomyelitis (EAE) revealed increased cleavage of Roquin-1 in disease-associated Th17 compared to Th1 cells in the CNS. T cells from Roquin-1 insensitive to MAL T1 mice did not efficiently induce the high-affinity Roquin-1 target IkB\(\alpha\) in response to TCR stimulation, showed reduced Th17 differentiation, and Roquin-1 insensitive to MAL T1 mice were protected from EAE. These data demonstrate how TCR signaling and MAL T1 activation utilize graded cleavage of Roquin to differentially regulate target mRNAs that control T cell activation and differentiation as well as the development of autoimmunity.

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Antigen recognition by T cells activates MALT1, and the scaffold function of MALT1 induces JNK/AP-1 and IKK/NF-kB signal transduction (1). Simultaneously, MALT1 paracaspase function is activated to cleave specific target proteins (2). Up to now, 30 protein substrates have been predicted to be cleaved by MALT1, and cleavage of almost 20 proteins has been confirmed in cleavage assays (3). Substrates include regulators of NF-kB signal transduction like RELB, CYLD, HOIL, BCL10, TNFAIP3, and MALTI itself (1). Another set of targets are RNA-binding proteins (RBPs) like Roquin-1, Roquin-2, Regnase-1, Regnase-2, Regnase-4, and N4BP1 (3–6). Recently, additional proteins including TAB3, CASP10, CILK1, ILDR2, and TANK were predicted to harbor candidate cleavage sites and were tested for being potential targets (3). Interestingly, genetic manipulation in mice causing either loss-of-function or constitutive activation of the MALT1 paracaspase induced inflammatory or autoimmune disease (7–10). At this point, it is unclear whether deregulation of NF-kB or the posttranscriptional regulators drive the observed phenotypes. Furthermore, these phenotypes could relate to unknown targets and involve deregulation of single or multiple targets.

Posttranscriptional gene regulation exerts essential control over immune responses. In fact, human patients as well as mouse models with hypomorphic mutations in the Roquin-1 gene develop severe autoimmune or inflammatory diseases (11, 12). The Roquin-1 encoded RNA-binding protein Roquin-1 and its paralog Roquin-2 work redundantly in T cells (13) and decrease mRNA stability or translation efficiency of their targets including Icos, Trf4, Tnfafs4 (Ox40L), Tnf, and Nfkb1d (IkB\(\alpha\)) (13–16). These targets are recognized through the amino-terminal ROQ domain, which binds with high affinity to a well-defined constitutive decay element (CDE), originally identified in the Tnf\(3'-UTR\) (15, 17–21). The ROQ domain also interacts with CDE-like elements of a relaxed consensus, with U-rich stem-loop structures and alternative decay elements (ADE), as well as with the low-affinity linear binding elements (LBE) (14, 22, 23). While the amino-terminus of Roquin interacts with RNA, the carboxy-terminus recruits the CCR4–NOT complex to induce target mRNA

Significance

We show that autoimmunity, caused by conventional T cells expressing constitutively active MAL T1 paracaspase, is triggered by cleavage of a single substrate, the RNA-binding protein Roquin-1. MAL T1 translates graded TCR signal strength into binary cell fate decisions, as decreasing Roquin activities are associated with differential derepression of low- and high-affinity targets. As an example of this regulation, we find that Th17 differentiation requires high TCR strength, strongly reduced Roquin activity and the concomitant induction of the modulator of transcription, IkB\(\alpha\), a high-affinity Roquin target. Together, we show that interfering with a distinct TCR strength integrating signaling pathway selectively mitigates T cell–driven immunopathology.
deadenylation and degradation (11, 15, 24, 25). Complete loss of Roquin-1 expression causes perinatal lethality, while combined conditional ablation of Roquin-1 and Roquin-2 encoding alleles in T cells leads to aberrant T cell activation and differentiation. In these mice, excessive T follicular helper (Tfh) and Th17 differentiation were observed (4, 13, 26). In T cells, the TCR-activated paracaspase MALT1 cleaves Roquin-1 and Roquin-2 and separates RNA-binding and CCR4-NOT recruitment functions (4). The MALT1 paracaspase is critical for Th17 differentiation, and Malt1+/−/− (C472A paracaspase mutant) mice are less susceptible to experimentally induced autoimmune encephalomyelitis (EAE) (8, 10). Surprisingly, Malt1+/−/− mice suffer from spontaneous IFNγ-mediated autoinflammation as well as an IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked)-like imbalance of increased T effector (Teff) to Treg cell ratios (8-10, 27, 28). On the other hand, mutations of the TRAF6 binding sites in MALT1 activity in conventional T cells was found responsible (7). T o determine tissues and inflammation, for which the MALT1 activity in B activation. At the same time, these mutations rendered Continuous Roquin gene expression (33, 36), and the molecules and mechanisms that can translate graded TCR signal strengths into binary cell fate decisions are mostly unknown.

Here, we show that MALT1-insensitive Roquin-1 alleles rescued fatal autoimmunity in mice with constitutively active MALT1. Strong TCR signals were needed to sufficiently decrease the activity of Roquin and enable expression of its high-affinity target Nfkbid/iκBNS, which is required for Th17 differentiation. Indeed, Roquin-1 cleavage was critical for Th17 cell–driven immunopathology since mice expressing MALT1-insensitive Roquin-1 were protected from EAE.

Results

Continuous Roquin-1 Cleavage Triggers Autoimmunity. Recently, mutations in MALT1 that block the TRAF6 binding motifs (T6BM) were shown to inactive antigen-receptor-induced NF-κB activation. At the same time, these mutations rendered the MALT1 protease constitutively active. These mice developed autoimmunity, and showed leukocyte infiltration into multiple tissues and inflammation, for which the MALT1 activity in conventional T cells was found responsible (7). To determine the importance of signal-induced cleavage of Roquin-1, we created a MALT1-insensitive allele (Re3h1p/Mins) by CRISPR/Cas9 genome editing in the mouse germline. We introduced two point mutations in exons 10 and 11 through homology-directed repair exchanging arginine 510 and arginine 579 to alanine (SI Appendix, Fig. S1A). The mutations blocked the main as well as alternative MALT1 cleavage sites of Roquin-1 (4), as confirmed in immunoblots of Th1 cells from Re3h1p/Mins mice stimulated with PMA and ionomycin (P/I) but did not affect cleavage of Regnase-1 (SI Appendix, Fig. S1B). The founder and all backcrossed Re3h1p/Mins or Re3h1p/Mins mice appeared normal and showed normal thymocyte development (SI Appendix, Fig. S1C). There was a small reduction in thymic Treg cell (CD4−SP; Foxp3+) frequencies, which was not significant in absolute numbers as there were slightly more thymocytes in Re3h1p/Mins compared to wild-type mice (SI Appendix, Fig. S1D). Peripheral Treg cells of Re3h1p/Mins mice were not reduced (SI Appendix, Fig. S1E), and Re3h1p/Mins mice did not show activation of peripheral CD4+ or CD8 T cells (SI Appendix, Fig. S1F).

To detect Roquin-1 cleavage on the single-cell level, we generated a monoclonal antibody that does not recognize full-length Roquin-1 but specifically binds the free carboxy-terminus of truncated Roquin-1 (aa 1-510) or Roquin-2 (aa 1-509), which arise due to MALT1-dependent cleavage (SI Appendix, Fig. S2 A–C). Importantly, the 20G6 hybridoma supernatant was able to detect the P/I-induced cleavage of endogenous Roquin protein in immunoblots of extracts from stimulated wild-type but not Re3h1p/Mins thymocytes (SI Appendix, Fig. S2D).

We validated this antibody further by intracellular staining and flow cytometry showing increased cleavage of Roquin after P/I stimulation of WT thymocytes as compared to unstimulated controls (SI Appendix, Fig. S2E) or stimulated thymocytes from Re3h1p/Mins (SI Appendix, Fig. S2F) or Malt1−/− genotypes (SI Appendix, Fig. S2G). Using recombinant mouse iKBNS protein, we also generated a monoclonal antibody against this target of Roquin. The 4C1 hybridoma supernatant worked in immunoblots and flow cytometry and detected transfected mouse iKBNS (SI Appendix, Fig. S2H) or PMA-induced endogenous iKBNS protein in lymphocytes from wild-type but not Nfkbid−/− mice (SI Appendix, Fig. S2I) and in P/I-stimulated A20 B cells (SI Appendix, Fig. S2J) or OVA323-330-stimulated OTII T cells (SI Appendix, Fig. S2K).

To determine whether phenotypes arising due to constitutive MALT1 protease activity depended on the cleavage of Roquin-1, we combined Malt1+/−/− with Re3h1p/Mins alleles. We confirmed constitutive proteolytic activity of MALT1 by detecting the aa1-510 cleavage product of Roquin in immunoblots of extracts from unstimulated lymph node cells of Malt1+/−/− mice and showed that this specific signal was undetectable in controls from WT, Re3h1p/Mins or Malt1+/−/−; Re3h1p/Mins mice (Fig. 1A). The Roquin target iKBNS was strongly induced in cells from Malt1+/−/−; Re3h1p/Mins mice, which was abolished by combined Re3h1p/Mins alleles (Fig. 1A). Single-cell analysis showed that Roquin, also in CD4+ T cells from Malt1+/−/−; Mins/Mins mice, exhibited constitutive cleavage (Fig. 1B).

The combination with homozygous alleles encoding for MALT1-insensitive Roquin-1 fully rescued mortality in all Malt1+/−/−; Re3h1p/Mins mice. In contrast, Malt1+/−/− or Malt1+/−/−; Re3h1−/− mice had to be killed between day 17 and day 20 of life because of their severe burden, and only one Malt1+/−/−; Re3h1p/Mins mouse survived (Fig. 1C). Consistent with restored viability in Malt1+/−/−; Re3h1p/Mins mice we found slightly reduced spleen sizes, no rescue of splenocyte numbers (Fig. 1D) but significantly reduced frequencies and numbers of activated T cells as determined by CD3+CD69+ measurements (Fig. 1E), as well as a partial rescue in frequencies of effector memory T cells as compared to Malt1+/−/− mice (Fig. 1F).

The upregulation of Roquin targets in T cells from Malt1+/−/−; Re3h1p/Mins was prevented in T cells from Malt1+/−/−; Re3h1p/Mins mice as shown for iKBNS (Fig. 1G) and Osx40, IRF4, and ICOS (Fig. 1H and SI Appendix, Fig. S1G).

Together, these data show that cleavage of Roquin-1 and Roquin-2 and derepression of Roquin targets can be detected on the single-cell level. Inactivating Roquin-1 cleavage does not
have an obvious impact in the absence of challenge. However, constitutive Roquin-1 cleavage leads to spontaneous activation of T cells, T cell effector function, and the development of autoimmunity.

The diagram shows Western blot analysis and flow cytometry analysis of Roquin cleavage in WT, Mins/Mins, TBM/TBM, and combined Malt1-TBM/TBM; Rc3h1-Mins/Mins (TBM/TBM; Mins/Mins) mice. Spontaneous Roquin cleavage and upregulation of IκBα is prevented in TBM/TBM; Mins/Mins mice. The Kaplan-Meier plot shows survival of WT, TBM/TBM, and TBM/TBM; Mins/Mins mice (n = 5–51). Combining Malt1-insensitive Roquin-1 rescues survival of mice with constitutive MALT1 activity. The representative spleen sizes and quantification of spleen weights and numbers of splenocytes show significant differences among the groups. The quantification of frequencies and numbers of CD69+CD3+ cells in spleens demonstrates a significant increase in TBM/TBM and TBM/TBM; Mins/Mins mice. The representative plots and quantifications of peripheral CD4+ T cell activation from spleens of WT, Mins/Mins, TBM/TBM and TBM/TBM; Mins/Mins mice showing naive (CD62Lhi CD44−), central memory (CD62Lhi CD44+), and effector memory (CD62Llo CD44+) T cells (n = 6–10). The representative plots of the direct Roquin target IκBα expression in splenic CD4+ T cells shows a significant increase in TBM/TBM and TBM/TBM; Mins/Mins mice. The quantification of the direct Roquin target expression OX40, IRF4, or ICOS expression in splenic CD4+ T cells (n = 6–10). Error bars represent mean ± SEM. CM: central memory, EM: effector memory, MFI: median fluorescence intensity. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Cleavage of Roquin by MALT1 Is Correlated with TCR Signal Strength. We aimed to correlate Roquin cleavage to T cell responses by analyzing cleavage of Roquin in ex vivo stimulated CD4+ T cells. We found no Roquin cleavage in cells with...
gMFI values (per target) were normalized to maximum levels observed in iDKO CD4αNS (4C1) expression for OTII loaded bone marrow derived dendritic cells (BMDCs) for 18 h followed by flow cytometry to detect OX40 and IκB expression. To determine Roquin cleavage in T cells recognizing antigen, we tested OT-II T cells stimulated with cognate OVA123–339 peptide loaded splenocytes. Cleavage of Roquin and upregulation of IκBNS occurred in a concentration-dependent manner and correspondingly increased in the range of 0.1–10.0 µg/mL OVA antigen (SI Appendix, Fig. S3 B and C), while CD69 upregulation showed stronger increases already at lower concentrations (SI Appendix, Fig. S3 D). We also tested a mutated OVA123–339 peptide replacing histidine 331 with arginine (OVA R9), which is known to have a lower functional avidity for activating OT-II T cells without retroviral transduction. Data are presented as mean ± SD. Statistical analysis was performed using an unpaired Student’s t test, two-tailed. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. (C) In vitro coculture of naive WT OT-II CD4+ T cells with OVA123–339 peptide-loaded bone marrow derived dendritic cells (BMDCs) for 18 h followed by flow cytometry to detect OX40 and IκBNS (4C1) expression for OT-II T cells (pregated on CD4+CD25+). OX40, IκBNS (4C1), and surface CD69 expression were analyzed in stimulations with increasing concentrations of WT OVA123–339. CD69 expression (Fig. 2 A). (D) Representative plots of and (E) quantification of Roquin cleavage (20G6) and CD69 expression in T cells stimulated with the indicated anti-CD3 and fixed anti-CD28 (10 µg/mL) concentrations for 18 h. (F) Quantification of Roquin target expression in OT-II T cells from cocultures shown in (E). Data are presented as mean ± SEM (n = 3–6). (F) Representative plots of OX40 and IκBNS (4C1) expression in IDKO CD4+ T cells retrovirally reconstituted with WT or mutant GFP-Roquin-1 constructs. (G) Relative quantification of target expression as in (F) in GFP+ cells and within 7 equal-sized gates corresponding to increasing GFP-Roquin-1 expression. WT and mutant GFP-Roquin-1 constructs are color-coded, and individual gMFI values (per target) were normalized to maximum levels observed in iDKO CD4+ T cells without retroviral transduction. Data are presented as mean ± SEM (n = 3–4). Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

**Roquin Targets Exhibit Different Functional Avidities.** We then asked whether increasing doses of antigen and concomitantly decreased Roquin activity differentially affected Roquin targets. We focused on IκBNS (Nfkbid) and OX40 (Tnfrsf4) in OT-II T cells stimulated with antigen doses ranging from 0.01 to 10 µg/mL OVA123–339 (Fig. 2 C). We found OX40 expression fully induced at 0.1 µg/mL OVA antigen (Fig. 2 C and D), while maximum responses of IκBNS required at least 10-fold higher concentrations, similar to CD69 (Fig. 2 C and D). The 3′UTR of Nfkbid harbors 6 stem-loop structures and two of these motifs are prototypic

**Fig. 2.** Correlating Roquin cleavage and target derepression with TCR strength. (A) Representative plots and (B) quantification of Roquin cleavage (20G6) and CD69 expression in T cells stimulated with the indicated anti-CD3 and fixed anti-CD28 (10 µg/mL) concentrations for 18 h. (B) Quantifications display MFI (median fluorescence intensity) of CD69, cleaved Roquin (20G6), and IκBNS (4C1) (n = 5). Data presented as mean ± SD. Statistical analysis was performed using an unpaired Student’s t test, two-tailed. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. (C) In vitro coculture of naive WT OT-II CD4+ T cells with OVA123–339 peptide-loaded bone marrow derived dendritic cells (BMDCs) for 18 h followed by flow cytometry to detect OX40 and IκBNS (4C1) expression for OT-II T cells (pregated on CD4+CD25+) (TCR). OX40, IκBNS (4C1), and surface CD69 expression were analyzed in stimulations with increasing concentrations of WT OVA123–339. (D) Quantification of Roquin target expression in OT-II T cells from cocultures shown in (C), depicting the fold change (fc) in gMFI values of OX40 or IκBNS (4C1) after stimulation in comparison to unstimulated cells. Data are presented as mean ± SEM (n = 3–6). (E) Representative plots of OX40 and IκBNS (4C1) expression in IDKO CD4+ T cells retrovirally reconstituted with WT or mutant GFP-Roquin-1 constructs. (F) Relative quantification of target expression as in (E) in GFP+ cells and within 7 equal-sized gates corresponding to increasing GFP-Roquin-1 expression. WT and mutant GFP-Roquin-1 constructs are color-coded, and individual gMFI values (per target) were normalized to maximum levels observed in iDKO CD4+ T cells without retroviral transduction. Data are presented as mean ± SEM (n = 3–4). Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
high-affinity CDEs (14, 15, 18), while the 3′-UTR of Tnfrsf4 has one ADE and one CDE-like stem-loop only, and both of them have lower affinity as compared to a CDE (13, 22, 39). We hypothesized that the molecular basis for the differential derepression lies in the encoded binding site affinities of target mRNAs for the RNA-binding domain of Roquin. To test this, we mutated residues in the ROQ domain of Roquin-1 (K220A, K239A, Y250A and R260A), which are known to contribute to the binding to CDE-, ADE-, and LBE-type motifs (14, 18, 22). We used tamoxifen-inducible double knockout (iDKO) of Roquin-1 and Roquin-2 encoding alleles in Cd4+ T cells (22, 40, 41) (SI Appendix, Fig. S4A) and transduced these T cells with doxycycline-inducible retroviral expression vectors to analyze reconstitution with wild-type or mutant Roquin-1 proteins fused to GFP (11, 22) (SI Appendix, Fig. S4B). The mutant proteins were expressed equally well compared to wild-type GFP-Roquin-1, and they fully or partially retained the ability to repress all or some target mRNAs (Fig. 2E and F and SI Appendix, Fig. S4C). We analyzed target expression by flow cytometry in intervals of increasing GFP expression, normalized to target levels in GFP-negative iDKO cells (SI Appendix, Fig. S4B). As expected, reconstitution with wild-type Roquin-1 was most effective to repress targets and the triple alanine mutant (K220A; K239A; R260A) was least effective (Fig. 2E and F), since targets like Ox40 and IkBNS did not respond to the GFP-marked expression of the triple alanine mutant. Consistent with the Y250A mutation being selectively involved in the interaction with ADE-type stem-loops as found in the Tnfrsf4 (Ox40) 3′-UTR (22), this mutant was almost inactive to repress Ox40 but fully active to repress IkBNS (Fig. 2E and F). The double-mutant K220; R260A important for ADE- and CDE-type stem-loops (18, 22) discriminated between different targets as it was almost inactive to repress Ox40, while fully active to repress IkBNS (Fig. 2E and F). Other targets of Roquin-1, including ICOS and Regnase-1, exhibited an intermediate responsiveness as compared to the differential regulation of Ox40 or IkBNS (Fig. 2E and F and SI Appendix, Fig. S4C). Together, these data demonstrate that the extent of MALT1-induced Roquin cleavage correlates with TCR signal strength and target derepression. Targets with low avidity binding sites like Ox40 are derepressed at weaker and those with high functional avidity like IkBNS at higher TCR signal strength.

Roquin Loss of Function and High TCR Signal Strength Promote Th17 Fate Decisions. Since the high-affinity Roquin-1 target Nkfkdil/IkBNS plays a prominent role in Th17 differentiation (42), we hypothesized that increasing inactivation of the Roquin system by conditional gene targeting could differentially affect T helper cell fate decisions. To test this, we combined the Cd4-Cre transgene with different combinations of floxed and wild-type alleles of Rch3h₁ and Rch3h₂ to gradually decrease Roquin expression as confirmed in immunoblots (Fig. 3A), using a monoclonal antibody (3F12) that detects Roquin-1 and Roquin-2 equally well (SI Appendix, Fig. S5). Since both paralogs act redundantly in T cells (13), this genetic titration allowed us to test the effect of decreasing Roquin activity on target derepression and T cell differentiation. Consistent with Tnfrsf4 (Ox40) being a lower affinity target than Nkfkdil mRNA (encoding for IkBNS), we found full derepression of Ox40 levels already upon genetic inactivation of Roquin-1 encoding alleles, while IkBNS became increasingly derepressed (Fig. 3B and C). In fact, derepression of IkBNS was gradual and only fully observed upon combined ablation of Roquin-1 and Roquin-2 encoding alleles (Fig. 3A and B). Targets like ICOS, IRF4, and Regnase-1, which also promote Th17 differentiation, were derepressed in a manner more similar to Nkfkdil/IkBNS than Tnfrsf4/Ox40 (SI Appendix, Fig. S6A). Testing Th1 vs. Th17 cell differentiation, we observed that partial or complete inactivation of the system did not significantly affect the frequencies of IFNγ-producing cells under Th1 culture conditions (SI Appendix, Fig. S6B). Also, heterozygosity of floxed alleles for both paralogs did not enhance Th17 cell differentiation (Fig. 3D and E). However, complete inactivation of the system strongly facilitated the development of IL-17A-producing T cells, and the expression of only one Rch3h2 wild-type allele encoding for Roquin-2, which was determined to be lower expressed in Cd4+ T cells than Roquin-1 (13) (Fig. 3A), already greatly diminished this effect (Fig. 3D and E). We then analyzed whether Th1 and Th17 commitment also reacted differentially to antigen availability. Indeed, when cocultured with OVA23-33γ-loaded BMDCs, full differentiation of naive OT-II T cells into Th1 cells was achieved already at low (0.1 µg/mL) peptide loading concentrations (SI Appendix, Fig. S6C), while Th17 differentiation occurred only at much higher antigen concentrations (1–10 µg/mL) (Fig. 3F and G). This pattern of differentiation correlated with the expression of the Th1-defining transcription factor T-bet at lower and the Th17 subset-defining transcription factor RORγt at higher antigen concentrations (SI Appendix, Fig. S6D). These data show how decreased Roquin activity facilitates Th17 differentiation but does not increase Th1 differentiation under Th1 polarizing conditions.

Th17 Differentiation Requires Roquin-1 Cleavage and IkBNS Derepression. We then aimed to find out whether Th17 differentiation required the TCR-induced cleavage of Roquin-1 by MALT1. To answer this question, we cultured naïve Cd4+ T cells from homozygous Rch3h₁[Min/Min] as well as heterozygous and WT genotypes under Th1 or Th17 skewing conditions and analyzed IL-17A and IFN-γ production after P/I restimulation. MALT1-insensitive Roquin-1 had no effect on the frequency of IFNγ-producing T cells cultured under Th1 conditions (SI Appendix, Fig. S7A). However, the frequency of IL-17A-producing cells gradually decreased in heterozygous and homozygous Rch3h₁[Min/Min] as compared to wild-type mice (Fig. 3H and I). Analyzing Th1 vs. Th17 differentiation of Cd4+ T cells from Rch3h₁[Min/Min] in more detail, we found a trend toward less proliferation under Th17 and significantly less proliferation under Th1 differentiation conditions (SI Appendix, Fig. S7B). There was no difference in cell death (SI Appendix, Fig. S7C) and no difference of T-bet under Th1 but strongly reduced RORγt expression under Th17 differentiation conditions in Cd4+ T cells from Rch3h₁[Min/Min] as compared to control cells (SI Appendix, Fig. S7D). Importantly, the decreased differentiation into Th17 cells did not promote the reciprocal Treg cell differentiation program as tested under iTreg conditions (SI Appendix, Fig. S8A). We also investigated the importance of Roquin-1 cleavage for Th1 vs. Th1 fate decisions. However, differentiation into the Th17 cell fate appeared more sensitive to Roquin cleavage, since adoptive transfer of SMARTA TCR transgenic Cd4+ T cells from WT or Rch3h₁[Min/Min] followed by subsequent LCMV Armstrong infection did not alter Th1 vs. Th17 differentiation choices significantly. Eight days post infection, we found only a tendency of slightly more Th1 (CXC5R5; PSGL1⁺) and less Th1 (CXC5R5; PSGL1⁺) differentiation in Rch3h₁[Min/Min] compared to wild-type SM(t)g T cells (SI Appendix, Fig. S8B).

We then asked how inactivation of the MALT1 paracaspase affected Th1/Th17 differentiation by analyzing naïve Cd4+ T differentiation from WT, Malt1−/− and Malt1Pmns−/− mice. Similar to naïve T cells expressing cleavage-resistant Roquin-1, T cells with inactive MALT1 paracaspase showed very similar Th1 but strongly reduced Th17 differentiation (SI Appendix, Fig. S8C and Fig. 3J and K). Finally, we determined the contribution of
Fig. 3. Decreasing Roquin activity and increasing TCR signal strength promote Th17 fate decisions. (A) Western blot analysis of Roquin-1 and Roquin-2 and IκBNS in extracts from Th1-polarized CD4+ T cells cultured for 6 d. Depicted is a representative of three experiments. Quantification of (B) IκBNS and (C) Ox40 expression determined in flow cytometry analyses of Th1 cells after 6d of in vitro culture. Data are presented as fold change of geometric mean fluorescence intensity (gMFIs) (n= 5–7). (D) Naive CD4+ T cells from WT, heterozygous Rc3h1fl/+;Rc3h2fl/+;Cd4-Cre, Rc3h1fl/fl;Rc3h2fl/+;Cd4-Cre or Rc3h1fl/fl;Rc3h2fl/fl;Cd4-Cre (i.e., double knockout; DKO) mice were activated with anti-CD3 and anti-CD28 antibodies and cultured for 3.5 d in vitro under Th17 polarizing conditions. T helper cell differentiation was assessed by i.c. cytokine staining of IL-17A production after P/I stimulation (n= 4) (D and E). (F) Naive WT OT-II transgenic T cells were cocultured with OVA323–339 peptide-loaded BMDCs for 3.5 d in vitro under Th17 polarizing conditions. OT-II T cell differentiation (pregated on TCR Vα2+Vβ5+) in response to increasing concentrations of peptides is shown in a representative contour plot (F) or quantification (G) of IL-17A producing OT-II T after restimulation (n= 3–7). Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001. (H and I) Naive CD4+ T cells from WT, Malt1+/−, and Malt1PM/− mice were activated with anti-CD3 and anti-CD28 antibodies and cultured for 3.5 d in vitro under Th17 polarizing conditions. T helper cell differentiation was assessed by i.c. cytokine staining of IL-17A production after P/I stimulation. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001. (J and K) In vitro Th17 differentiation of WT, Nfkbidfl/+;Cd4-Cre, and Nfkbidfl/fl;Cd4-Cre T cells as described in (H and I) (n= 2–3). Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. Data are presented as mean ± SEM. i.c.: intracellular, P/I: PMA/Ionomycin. Statistical analysis was performed using an unpaired Student’s t test, one-tailed. *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001.
heterozygous or homozygous deletion of IκBNS encoding Nfkbid alleles with Cdh4-Cre. Complete inactivation of IκBNS strongly decreased the potential of naive CD4+ T cells to commit to Th17 differentiation and heterozygosity already caused a significant reduction (Fig. 3 L and M). Consistent with the greater importance for Th17 differentiation, we detected stronger cleavage of Roquin and IκBNS expression under Th17 as compared to Th1 differentiation conditions (SI Appendix, Fig. S8D).

Together, these data show that specifically Th17 differentiation depends on MALT1-mediated cleavage of Roquin-1, and this dependency can be explained by the corresponding upregulation and increased activity of the Roquin-1 target IκBNS.

MALT1-insensitive Roquin-1 Protects from Experimental Autoimmune Encephalomyelitis. We then analyzed how induction of experimental autoimmune encephalomyelitis (EAE) that relies
on Th17 responses in the central nervous system (CNS) of mice involves cleavage of Roquin. At the peak of MOG-induced EAE, CD4+ CD4+ T cells were detected in the CNS (Fig. 4A). Consistent with the accumulation of antigen-specific T cells in the CNS, we detected stronger cleavage of Roquin as well as enhanced expression of hNGFR in the CNS compared to the spleen (Fig. 4B). We discriminated Th1 and Th17 cells by staining for CXCR3 and CCR6 chemokine receptors, respectively (43) (Fig. 4C). Indeed, comparing both subsets of CNS localized effector cells, we found increased Roquin cleavage in CCR6+ Th17 cells (Fig. 4D). Furthermore, we addressed this finding using an IL-17A reporter mouse, which expresses the hNGFR under the control of the cis-regulatory regions of the IL-17A locus. Again, the results confirmed higher expression in CNS-localized IL-17A-producing hNGFR+ compared to hNGFR− CD4+ T cells at the peak of EAE (Fig. 4E).

To elucidate whether Th17 responses were affected by Roquin cleavage in vivo, we challenged Rc3h1fl/fl;Mins/Mins mice and wild-type counterparts with MOG35−55 peptide in complete Freund’s adjuvant to induce active EAE. We observed a reduced average clinical score in Rc3h1fl/fl;Mins/Mins compared to wild-type mice when following both groups for 15 d after MOG immunization (Fig. 4F). Analyzing the CNS from selected mice of both groups that showed signs of disease (Fig. 4G and H), we found a variable amount of infiltrated CD4+ T cells (Fig. 4G), which, upon ex vivo stimulation with P/I, showed similar frequencies of IFNγ positive cells (Fig. 4H). However, in contrast to wild-type T cells, Rc3h1fl/fl;Mins/Mins CD4+ T cells from the CNS were not able to produce high amounts of IL-17 or IFNγ, and much fewer cells produced IL-17 (Fig. 4F). Further support for the inability of Rc3h1fl/fl;Mins/Mins CD4+ T cells to differentiate into Th17 cells was obtained by demonstrating a greatly attenuated RORγt expression in CD4+ T cells isolated from the inflamed CNS (SI Appendix, Fig. S8E). To analyze whether the protection of Rc3h1fl/fl;Mins/Mins from EAE is a T cell–intrinsic phenotype, we generated Rc3h1h−/−;Mins/Mins; 2D2 transgenic mice, transfected 2D2 T cells of either genotype into Rag1−/− mice, and immunized the host mice with MOG peptide. Here, Rc3h1h−/−;Mins/Mins 2D2 T cells were significantly less efficient in inducing EAE than their 2D2 wild-type counterparts, proving that cleavage-resistant Roquin-1 in T cells is responsible for protection from EAE in Rc3h1h−/−;Mins/Mins mice (Fig. 4I).

These findings reveal the physiologic importance of TCR-induced and MAL11-executed cleavage of Roquin for the development of autoimmune disease. In summary, our data promote a concept in which the TCR signal strength is correlated with the degree of Roquin inactivation. Exceeding a specific threshold enables sufficient induction of hNGFR expression as a prerequisite for Th17 cell differentiation in vitro and in vivo.

Discussion

There is growing therapeutic interest in manipulating MAL11, and inhibitors of the MAL11 paracaspase are currently investigated in clinical trials. The therapeutic aims are to deprive hematologic tumor cells from an essential growth signal or to modulate the balance of Treg and conventional T cells in antitumor immunity (44). Preclinical studies have also studied inactivation of the MAL11 targets Roquin-1/2 or Regnase-1 or inhibition of Roquin-1/Regnase-1 cooperation in tumor-specific T cells or CAR T cells (40, 45–48). For the success and safety of these approaches, we now need a comprehensive understanding of the function and regulation of these factors in the different cell types.

In this study, we define the molecular and cellular consequences of selectively inhibiting Roquin-1 cleavage by MAL11. We identify a major role for Roquin-1 cleavage in the phenotype of mice expressing a constitutively active MAL11 paracaspase (7). We propose that the autoimmune/autoinflammatory phenotype of these mice results from constitutive cleavage and thereby loss-of-function of Roquin-1, which triggers activation of naïve T cells as well as their differentiation into effector cells. Indeed, acute loss-of-function of Roquin-1 and Roquin-2 proteins is associated with spontaneous CD4+ and CD8+ T cell activation, as well as preferential Th1 and Th17 differentiation (40). From this work, it becomes clear that T cell quiescence is not primarily defined by the absence of foreign antigen recognition, but rather requires the TRAF6/MAL11 interaction and high Roquin activity to maintain naïve T cells constantly exposed to tonic TCR signals, in a quiescent state. The Roquin-1-regulated targets that control T cell quiescence are currently unknown, and it will be interesting to find out whether they include poised mRNAs, which are kept untranslated until the naïve T cell recognizes antigen (49, 50). Unfortunately, the mortality of MAL11−/−/B6m−/− mice during adolescence precludes a detailed analysis of T helper cell differentiation. Nevertheless, Th1 and Th17 responses likely contribute to the observed phenotype since elevated autoantibodies and T helper cell cytokines are present in the sera of these mice (7).

The hypomorphic sanroque allele of Rc3h1 (encoding for Roquin-1sm) exhibits an accumulation of Th1 and Th1 effector cells (11, 12, 51). In contrast, combined deletion of Roquin-1 and Roquin-2 encoding alleles in peripheral T cells promotes Th1 and Th17 differentiation (4). Interestingly, we did not detect significant differences in Th1 vs. Th1 cell fate decisions in LCMV Armstrong infections in T cells from mice expressing MAL11-insensitive Roquin-1. These findings could suggest that Th1 and Th1 fate decisions may not be different in their dependence on the expression of high-affinity targets of Roquin-1. Instead, we show that Th17 cell fate decisions depend on the cleavage of Roquin-1 by MAL11. Our mouse model of MAL11-insensitive Roquin-1 (Rc3h1h−/−;Mins/Mins) suggests that repression of IkBα/Nfkbid by Roquin-1 is a major mechanism for integrating TCR signaling into Th17 differentiation. This conclusion is further supported by the graded conditional inactivation of the Roquin-1 and Roquin-2 encoding alleles in T cells. Only their complete inactivation led to full derepression of IkBα and full Th17 differentiation. Moreover, Rc3h1h−/−;Mins/Mins and Rc3h1h−/−;Mins/Mins genotypes limited IkBα upregulation during activation of CD4+ T cells, and Rc3h1h−/−;Mins/Mins mice showed impaired Th17 differentiation in vitro as well as in vivo during EAE. Low levels of IkBα in Rc3h1h−/−;Mins/Mins mice can explain the reduced Th17 differentiation as we also found a comparable impairment in the heterozygous Nfkbid−/−;Cd4−Cre as well as an even more pronounced reduction of Th17 differentiation in the Nfkbid−/−;Cd4−Cre genotype. Only antigen signals of high strength induced the extensive cleavage of Roquin that was required for full upregulation of IkBα, which did not affect Th1 but was necessary for Th17 differentiation and experimental induction of Th17-driven autoimmunity. The functional avidity of the Nfkbid mRNA for Roquin binding involves on the one hand the high affinity of individual binding sites of the CDE and ADE type and on the other hand the high number of at least seven Roquin-recognized stem-loops and LBEs in its 3′UTR (14, 15). These binding sites have been proposed to serve accessory, redundant, or cooperative functions and confer induced Nfkbid mRNA decay and also translational inhibition of IkBα (14). Translational inhibition affects only a subset of Roquin targets, which are enriched in four or more Roquin binding sites (14). Of course, also other high or intermediate affinity targets like
Nfkbia, Ifnγ, and Icos may, following their derepression at intermediate or high TCR strength, additionally promote Th17 commitment (42, 52–55). Conversely, also the low avidity target Ovx40 can shape this program further since Ovx40 was demonstrated to inhibit Th17 differentiation via activation of noncanonical NF-kB signaling and RelB-mediated recruitment of epigenetic repressors to the Il17 locus (56). In our experiments Ovx40 was already fully induced at low TCR signal strength or intermediate levels of Roquin inactivation and could inhibit Th17 commitment in response to T cell stimulations that induce suboptimal IkBNS levels. Together, we propose that the MALT1/Roquin/IkBNS axis establishes a unique posttranscriptional threshold of TCR strength in Th17 differentiation since blocking Roquin-1 cleavage by MALT1 did not shift the cells toward Tfh differentiation as it was previously observed for TCR strength- and Ick-dependent control of Th17 differentiation (31, 57).

Here, we describe a mouse model that genetically renders one substrate of MALT1 insensitive to proteolytic cleavage. These Rch3h1/Mins/Mins mice shared some phenotypes with Malt1-KO/PM mice including a milder reduction in thymic Treg frequencies (8–10) and to a similar extent an impairment of Th17 differentiation (8). At present, it is possible that the system is even more strongly involved than evident from Rch3h1/Mins/Mins mice, since the lack of Roquin-1 cleavage may also be compensated by cleavage of the functionally redundant Roquin-2 or potentially through cleavage of the functionally cooperating Regnase-1 protein. Future work will answer these questions by generating MALT1-insensitive Roquin-2 (Rch3h2) or Regnase-1 alleles and by combining them with Rch3h1/Mins/Mins alleles. Of note, targets of MALT1 in the NFκB or other pathways could also be crucial for the phenotypes of Malt1-KO/PM mice that are not shared in Rch3h1/Mins/Mins mice (58, 59).

Importantly, our mouse model reveals the impact of the MALT1 protease activity on Th17 differentiation and Th17-driven autoimmunity segregates with cleavage of the Roquin-1 substrate. In active EAE, we detect stronger cleavage of Roquin in CNS-localized CD4+ T cells that produce IL-17A compared with CNS-localized CD4+ cells that are negative for IL-17A expression. This finding prompts the question of whether chronically activated pathogenic CD4+ T cells in Th17-promoting diseases show accumulations of MALT1 paracaspase activity in Th17 cells. To verify this (56), we tested whether chronic activation or Regnase-1 deletion could also be crucial for the phenotypes of MALT1 alleles and by combining them with MALT1-KO/PM mice. Of note, targets of MALT1 in the NFκB or other pathways could also be crucial for the phenotypes of MALT1-KO/PM mice that are not shared in Rch3h1/Mins/Mins mice (58, 59).

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Materials and Methods

**Animals.** All experiments involving mice and rats were performed in accordance with the regulations and with approval by the local government (Regierung von Oberbayern, ROB). Mice and rats were housed in specific pathogen-free barrier facilities in standard cages on a 12-h light/dark cycle with ad libitum access to food and water in accordance with institutional, state, and federal guidelines of the Helmholtz Zentrum München and the Ludwig-Maximilians-Universität München.

**T Cell Cultures.** In vitro cultivation of primary murine CD4+ T cells, single-cell suspensions in T cell isolation buffer (PBS supplemented with 2% FCS and 1 mM EDTA) were generated from spleens and peripheral lymph nodes. Ethnicytes were lysed using a TAC-lysis buffer (13 mM Tris and 140 mM NaCl, pH 7.2). CD4+ T cells were isolated using the negative selection EasySep™ Mouse CD4+ T cell isolation Kit (StemCell). A total of 4 × 10^5 to 10^6 cells were activated in vitro in 6-well plates by plate-bound and goat anti-hamster (IgG-cross-linked) (MP Biomedicals, #69894) anti-CD3 (0.25 µg/mL; clone 2C11, in-house) and anti-CD28 antibodies (2.5 µg/mL; clone 37.5 N, in-house). Cells were cultured for 6 d in T cell medium (DMEM medium (Gibco) supplemented with 10% FCS, 10 units/mL penicillin, 10 µg/mL streptomycin (both Thermofisher), 10 mM HEPES (Gibco), 50 µM β-mercaptoethanol (β-ME; Invitrogen), 1x nonessential amino acids (NEAAs; Gibco), and 200 units/mL hIL-2 (Novartis)) under Th1-polarizing conditions (10 µM anti-IL-4 (clone 11B11, in-house) and 10 ng/mL hIL-12 (BD Pharmingen)). Naive CD4+ T cells were isolated using the EasySep™ Mouse Naive CD4+ T cell isolation Kit (StemCell). For anti-CD3 titrations with constant anti-CD28, total CD3+ T cells were isolated and cultured on 96-well U-bottom plates with plate-bound anti-CD3 and anti-CD28 concentrations for 18 h. T cells were maintained in T cell medium (RPMI 1640 + Glutamax (Gibco) supplemented with 10% FCS, 10 units/mL penicillin (Thermofisher), 10 µg/mL streptomycin (Thermofisher), 5 µM β-mercaptoethanol (β-ME; Invitrogen) and 5ng/mL hIL-7 (ImmunoTools) (Fig. 2A, SI Appendix, Fig. S3).

**Cocultures.** Bone marrow (BM) cells from the tibia and femur of wild-type mice were differentiated to mature BM-derived dendritic cells (BMDCs) for 6 d in T cell medium supplemented with 20 ng/mL GM-CSF (Cell Signaling Technologies) and kept at 37 °C 5% CO2. On day 6 of differentiation, lipopolysaccharide (LPS, 100 ng/mL, Sigma) was added for a 24 h to induce maturation of BMDCs, and full maturation was confirmed by surface CD1c and MHCI expression. Mature BMDCs were loaded with different concentrations of Ovalbumin peptides, OVA257-264 (wild type or R9 mutant; BIO TREND), for 2–3 h at 37 °C 5% CO2, and cocultured with naïve CD4+ OTII-T cells at a 1:1 ratio.

For mixed lymphocyte stimulations, 50,000 naïve CD4+ OTII-TCR-transgenic T cells were cocultured with 50,000 congenically marked splenocytes loaded with different OVA peptides. Cells were supplemented with 5 ng/mL murine hIL-7 (ImmunoTools) and kept at 37 °C for 18 h (SI Appendix, Fig. S3E).

**In Vitro Differentiation of Naive CD4+ T Cells.** A total of 200,000 naïve CD4+ T cells were cultured in 96-well flat bottom plates for 3.5 d under TGF-β (0 ng/mL) or TGF-β and IL-1α (10 ng/mL) conditions. Differentiated cells were washed 1× by adding T cell medium without additional cytokines. Differentiated cells were washed with cold PBS and subsequently stimulated for 5 h with 20 nM PMA and 1 µM ionomycin. After 2.5 h, 10 µg/mL brefeldin A (Brefa) was added.

**Immunophenotyping.** Single-cell suspensions of isolated lymphocytes were stained for viability (Fixable Viability dye (eF780)) and LIVE/DEAD Fixable Blue (Invitrogen), followed by surface staining for 20 min protected from light at 4 °C. Cells were fixed in 2% formaldehyde (FA) for cytokine staining or 4%PFA for 20G6 staining for 15 min at RT. For transcription factor staining, cells were fixed in 4% PFA for 1 h and washed with FACS buffer. Flow cytometry data were acquired with BD LSRFortessa (5-laser), the BD FACSCanto (3-laser), or the Cytoflex S (4-laser, Beckman Coulter) flow cytometers.

**LCMV Infection of Mice.** LCMV Armstrong virus was propagated in Baby hamster kidney–21 cells (BHK–21) virus titers were determined by plaque assays on Vero cells as described previously (60). To assess T cell–intrinsic Th vs. Th1 differentiation, × 10^5 SMARTA TCR-tg (Smtg), Rch3h7 × 10^5 or Smtg (Rch3h7/Mins/Mins) CD4+ T cells were adoptively transferred into congenically marked C57BL/6 WT recipients. One day after transfer, recipient mice were infected with 2×10^6 pfu LCMV Armstrong intraperitoneally (i.p.) and killed 8 d postinfection (dpi).

**Experimental Autoimmune Encephalomyelitis (EAE).** The active EAE disease model was induced on day 0 by subcutaneous (s.c.) injection of 200 µg MOP peptide (Auspep Pty Ltd.) emulsified in CFA (BD Difco™ Adjuvants) at the tail base of age-matched mice. 100 ng of pertussis toxin (PTX, in PBS; Sigma) was intravenously (i.v.) injected on day 0 and on day 2 (200 ng total dose). Mice were checked on a daily basis and scored starting at day 9 after MOG/CFA and PIX injections. Clinical scores were documented up until day 15 after induction resembling the peak of disease scores in wild-type counterparts. To induce EAE in a T cell conditional Mins/Mins setting, 1,500 CD4+ CD25+ T cells from 2D2–TCR transgenic Rch3h7/Mins/Mins mice were mixed into Rag1−/− recipients. EAE was induced as described above.

On day 15, mice were killed by isoflurane inhalation, perfused with PBS and CNS samples from spinal cords and brains were isolated for ex vivo analysis of
T cells. Briefly, CNS tissue was cut into small pieces and digested by incubation with 1 mg/mL DNase I (Sigma) and 2.5 mg/mL collagenase D (Sigma) in DMEM medium supplemented with 10% FCS, 10 units/ml penicillin, 10 μg/ml streptomycin, 10 mM HEPES, 50 mM β-ME, and 1x NEAs for 90 min at 37°C. CNS-infiltrating mononuclear cells were isolated by Percoll gradient centrifugation (37%, 70% Percoll in PBS buffer; GE Healthcare).

Western Blotting. T cells were washed in ice-cold PBS were subsequently lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% (v/v) NP-40, 1.5 mM MgCl₂, 1 mM DTT supplemented with 1x Complete, EDTA-free Protease Inhibitor Cocktail (Roche) for 15 min on ice (4°C). Lysates were clarified for 15 min at 10,000 x g, and protein concentration was determined by Bradford assay (Bio-Rad). Equal amounts of protein lysates (30-50 μg) were denatured at 95°C for 5 min in 4 × Laemmli buffer (314 mM Tris, 50% glycerol, 5% SDS, 5% mercaptoethanol, and 0.1% bromophenol blue, pH 6.8), size-separated by SDS-PAGE, transferred to a MeOH-activated PVDF membrane, and incubated with respective primary and HRP-conjugated secondary antibodies (CST). Signal detection was performed with ECL western blotting reagents (Novex Invitrogen, Amersham GE or in-house produced ECL) and DB-8 medical X-ray films. Detailed information on methods and results is also available at ref. 61 and https://edoc.ub.uni-muenchen.de/29277/7/Schmidt_Henrik.pdf.

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix.

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