Roquin-dependent gene regulation in immune-mediated diseases and future therapies

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Abstract

The RNA-binding proteins Roquin-1/2 and Regnase-1 exert essential regulation by controlling proinflammatory mRNA expression to prevent autoimmune disease. More recently, inhibition of this post-transcriptional gene regulatory program has been demonstrated to enable enhanced anti-tumor responses by tumor antigen-specific CD8$^+$ T cells. In this review we describe the functions of these RNA-binding proteins and the phenotypes that arise in association with genetic inhibition or inactivation. We discuss how inducible inactivation of the system reprograms CD4$^+$ and CD8$^+$ T cell fates by changing cell metabolism, activation, differentiation or effector/memory decisions. We furthermore outline what we need to know to precisely modulate this system in order to dampen autoimmune reactions or boost the efficacy of adoptively transferred T cells or chimeric antigen receptor (CAR) T cells in cancer immunotherapies.

Keywords: autoimmunity, autoinflammation, cancer therapy, Regnase-1, post-transcriptional gene regulation

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Introduction

The post-transcriptional mechanisms that control mRNA half-life provide crucial gene regulation in the immune system. This level contributes especially to the rapid changes of gene expression during immune cell activation and differentiation, which are essential for immune responses to invading pathogens (1,2). Dysregulation of these pathways is often associated with systemic autoimmunity or autoinflammation and the development of immune-related diseases (reviewed in (3,4)). Post-transcriptional regulation of mRNAs involves binding of *trans*-acting factors to *cis*-elements, which are typically linear sequence motifs, modified nucleotides or secondary structures within the untranslated regions (UTRs). These interactions and additional binding to effector molecules can stabilize or destabilize the target mRNAs or stimulate or inhibit their translational output (1,5-7). Well-known examples for such *trans*-acting factors are the RNA-binding protein (RBP) families of Roquin and Regnase, which have been shown to regulate an overlapping target set and to prevent overactivation of immune cells (8-12).

The Roquin family is made up of the two paralogues, Roquin-1, encoded by *Rc3h1*, and Roquin-2, encoded by *Rc3h2* (10,13), which are localized in the cytoplasm and are enriched within P-bodies, but re-localize to stress granules upon cell stress. These proteins serve critical functions during homeostasis, activation and differentiation of adaptive immune cells, and, at least in T cells, Roquin-1 and Roquin-2 are functionally redundant (9,10,14-17). Biochemical approaches to determine RNA recognition by Roquin RBPs have shown that these proteins contain a novel RNA-binding domain (RBD) (Fig. 1A). This ROQ domain recognizes prototypic hairpins with a characteristic pyrimidine-purine-pyrimidine (Y-R-Y) tri-loop structure called CDE (constitutive decay element) and can also interact with lower affinity with Y-R-Y sequences in a U-rich linear context.

The ROQ domain also binds to hexaloop structures with GUUYUA sequences called ADE (alternative decay elements) or to hairpins with U-rich loops of variable length (6,18-25). In addition, Roquin proteins contain split $HEPN_N$ (higher eukaryotes and prokaryotes nucleotide-binding N-terminal) and $HEPN_C$ (higher eukaryotes and prokaryotes nucleotide-binding C-terminal) domains that encompass the ROQ domain as well as a CCCH-type zinc finger (Znf) (Fig. 1A). The HEPN and Znf domains are thought to stabilize interactions with double-stranded RNA (dsRNA) (16,26) or direct the RBPs to U-rich sequences (24), respectively, but their physiologic contribution to motif recognition in general has not been determined. Interestingly, the binding of Roquin proteins to stem–loops can be stabilized by the co-factor Nufip2 (nuclear FMR1 interacting protein 2) (27). Roquin-mediated mRNA repression is achieved through the recruitment of the Ccr4-NOT deadenylase complex and its

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interaction with the activators of decapping Edc4 (enhancer of mRNA decapping 4) and Rck/DDX6 (DEAD-box protein 6) (15,23,28).

Although the target set of Roquin overlaps with that of Regnase-1, it was shown that Regnase-1 regulates mRNA targets through a distinct pathway. Regnase-1 recognizes similar stem–loops, which then need to be dissolved by the helicase Upf1 (UP-frameshift 1; previously called regulator of nonsense transcripts 1), before Regnase-1 can endonucleolytically cleave the mRNA, further involving factors of nonsense-mediated decay (11,29).

Roquin-1/2 and Regnase-1 play a key role in T cells, since conditional T cell-specific inactivation of Roquin-1 and Roquin-2 or inactivation of Regnase-1 recapitulated in large parts the phenotypes of mouse lines with systemic expression of a hypomorphic Roquin-1 mutant or systemic Regnase-1 loss-of-function (LOF), respectively (9,10,12,30). As some of their best-described targets have strong immune-modulatory functions, it is not surprising that Roquin and Regnase-1 abundance and activity are tightly regulated. In T cells, this regulation is achieved through proteolytic cleavage by the paracaspase MALT1 (mucosa-associated lymphoid tissue lymphoma translocation 1 gene), which is activated following T cell receptor (TCR) and co-stimulatory signalling. MALT1 cleaves Roquin-1/2 and Regnase-1 proteins, leading to derepression of their target mRNAs (Fig. 1B) (8,12,31,32). Additionally, both Roquin-1/2 as well as Regnase-1 proteins can regulate the Regnase-1-encoding mRNA (*Zc3h12a*; zinc finger CCCH-type containing 12A), thus creating an important negative feedback loop (8,33-35).

Roquin proteins play important roles in cell-fate decisions of several distinct $CD4^+$ T cell subsets, including T helper 1 (T_H1), T_H17 and T follicular helper (T_{FH}) as well as T follicular regulatory (T_{FR}) cells (Fig. 2A), thus shaping the immune response against specific classes of pathogens (8,10,36). Partial inhibition or complete LOF of Roquin proteins leads to accumulation of the aforementioned T cell subsets associated with inflammatory disease in mice and humans (8-10,37).

It can be expected that Roquin will control T cell-driven immune responses in many different types of infection. However, T cells lacking Roquin expression are strongly activated even in the absence of antigen and are prone to die, if additionally confronted with cognate antigen in experimental models of infection (V.H. and T.R., unpublished observations). In the following, we will therefore focus on the currently known disease phenotypes that spontaneously develop in association with Roquin LOF in human patients or mice and

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discuss how aberrant gene expression in T cells in the absence of Roquin proteins can be harnessed to improve anti-cancer immunotherapies.

Involvement of Roquin in human disease

Several reports have linked Regnase-1 function to a number of human inflammatory diseases and inflammation-driven cancers (35,38-44). For instance, reduced Regnase-1 levels in PBMCs have been associated with an increased disease severity in patients suffering from pulmonary hypertension (44). These expression changes were associated with an expansion of type 2 innate lymphoid cells in bronchoalveolar lavage of patients suffering from idiopathic pulmonary fibrosis and a concomitant increase in pro-fibrotic gene expression (43). Conversely, increased Regnase-1 levels have been reported in skin lesion biopsies of psoriasis patients (45,46), human ischemic heart disease samples (40) and in PBMCs of patients suffering from active myasthenia gravis (47). Whole exome sequencing of epithelial cells taken from patients with ulcerative colitis, identified *NFKBIZ* and *ZC3H12A* as two of the most frequently mutated genes in these patients (38,48). Notably, mutations in Regnase-1 were highly enriched in the DSGxxS motif as well as the S438 residue, phosphorylation of which is necessary for ubiquitination – mediated degradation of Regnase-1 (34). These mutations resulted in Regnase-1 gain-of-function. Organoids with these mutations showed a reduced susceptibility to IL-17A-induced epithelial cell death, imparting a selective advantage within the inflammatory milieu (38,48). Mechanisms of Regnase-1-mediated post-transcriptional gene regulation as well as the role of Regnase-1 in health and disease have been reviewed recently (49-51).

In contrast, very little is known about the link between Roquin malfunction and inflammation-associated diseases and neoplasia. The first indication for a potential role of Roquin-1 malfunction in human disease came from Ellyard and colleagues who observed that mice with the heterozygous *sanroque* mutation, a methionine to arginine substitution at position 199 (M199R) in Roquin-1 (*Rc3h1*$^{san/+}$) developed pathology reminiscent of human angioimmunoblastic T-cell lymphoma (AITL) (52). However, closer examination of AITL patients did not confirm associations of alterations in the sequence or expression level of Roquin; nor did it confirm changes in the Roquin target *ICOS* (inducible T cell co-stimulator) and therefore did not support an involvement of the human *RC3H1* gene in recurrent abnormalities associated with AITL (53).

To date only two deletion mutations have been reported for the human *RC3H1* gene. A large (111kb) hemizygous deletion on chromosome 1 in a Japanese patient resulted in severe hyperinflammation and clotting disorder. Cells of this patient lacked sequences encoding two thirds of the amino-

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terminal portion of *RC3H1* as well as the entire *SERPINC1* (serpin family C member 1) and *ZBTB37* (zinc finger and BTB domain containing 37)-encoding sequences, providing first indications that loss of Roquin function could affect the human immune response. It is, however, not clear how the loss of the other two genes contributed to the phenotype (54).

Reporting another mutation in *RC3H1,* Tavernier and colleagues (37) extensively characterized the hematopoietic system of a patient with a severe hyperinflammatory syndrome resembling hemophagocytic lymphohistiocytosis (HLH). HLH is characterized by prolonged fever, splenomegaly, hepatitis and neurological disorders and is thought to be driven by an uncontrolled expansion of $CD8^+$ T cells, macrophage activation and a resulting cytokine storm, with particularly high levels of interferon γ (IFNγ) and tumor necrosis factor α (TNFα) (55,56). Patients with familial HLH often lack perforin expression (57) and their cytotoxic T cells are subject to continuous activation by viral infections without being able to clear the pathogen. As a consequence, these cells produce large amounts of IFNγ and drive disease development and progression (58-60).

A homozygous nonsense mutation (R688*) in the *RC3H1* gene was identified in this patient, which resulted in a truncated Roquin-1 protein. Recruitment to stress granules upon arsenite treatment was not impaired, consistent with an intact N-terminus of Roquin-1 (14,37). The consanguineous parents were heterozygous carriers of this mutations in the *RC3H1* gene, and the mother exhibited a systemic lupus erythematosus (SLE) phenotype, whereas the father was affected by arthritis. These phenotypes differed and were much less severe as compared with the homozygous son (37). In a systematic comparison of the human R688* mutation with known mouse models (8-10), the authors furthermore identified differences, the most notable being the absence of T_{FH} cell accumulation or autoimmunity in this patient (37). Furthermore, the authors demonstrated that not all known Roquin targets were equally responsive to the R688* mutation, providing evidence for the ability of Roquin to regulate mRNA stability by different mechanisms and in redundant manners (6).

Interestingly, the patient carrying the R688* mutation did not show any signs of defective cytolytic activity, but systemic IFNγ levels were strongly elevated (37). One can speculate that Roquin LOF together with deregulation of IFNγ may cause other immune cells to produce proinflammatory cytokines and, thus, leads to a similar feed-forward amplification as has been described for familial HLH (58,60).

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Roquin-mediated gene regulation in autoimmunity

The original identification of the murine Roquin-1 protein already revealed its involvement in disease, when Chris Goodnow, Carola Vinuesa and colleagues (10) uncovered the Roquin-1-encoding gene in an N-ethyl-N-nitrosourea (ENU) screen. In this random mutagenesis they looked for single base-pair mutations that caused humoral autoimmunity detectable by standard clinical tests for antinuclear antibodies (ANAs). Mice carrying the homozygous M199R (*sanroque)* mutation in the *Rc3h1* gene developed a severe T_{FH}-driven SLE-like disease and aged *sanroque* mice exhibited signs of comorbid Sjögren's syndrome (SS) (Fig. 2A) (10,61,62). In addition to ANAs, the autoimmune phenotype was further characterized by splenomegaly, lymphadenopathy, hypergammaglobulinemia, strong expansion of activated $CD44^{hi}CD4^{+}$ and $CD8^{+}$ T cells, accumulation of T_{FH} and germinal center (GC) B cells as well as spontaneous GC formation. On the macroscopic level these mice developed glomerulonephritis accompanied by immune complex deposition, necrotizing hepatitis and autoimmune thrombocytopenia (10). Interestingly, these changes occurred despite normal thymic T cell development and an expanded T regulatory (Treg) cell compartment and could be attributed to predominantly T cell-intrinsic functions of Roquin-1 (10,14,61,63).

ICOS was identified as the first directly regulated target of Roquin (10,14,15,64). In *sanroque* mice, ICOS expression strongly increased on activated and naive $CD4^{+}$ T cells and was found to promote aberrant T_{FH} accumulation and GC formation by substituting for CD28 signalling during T cell priming (63,64). Interestingly, heterozygous ICOS deletion in *sanroque* mice partially ameliorated autoimmunity (64), whereas complete ICOS ablation seemed to exacerbate splenomegaly, augment ANA production and lead to a decrease in Treg cells (65). This suggested that additional mechanisms are involved in the development of autoimmune manifestations in the *sanroque* mouse.

Further direct Roquin targets important for T_{FH} differentiation were described later on, including Interferon regulatory factor 4 (*Irf4*) (8) and Ox40 (TNF receptor superfamily, member 4; encoded by *Tnfrsf4*) (9). Interfering with T_{FH} formation by deletion of SLAM-associated protein (Sap; encoded by *Sh2d1a)* significantly attenuated lupus-like disease, and adoptive transfer of T_{FH} cells harbouring the *sanroque* mutation induced GC formation in recipient mice, further corroborating the driving role of pathogenic T_{FH} cells in the *sanroque* (*Rc3h1*$^{san/san}$) mouse strain (61).

Rc3h1$^{san/san}$ $CD4^{+}$ T cells produced high levels of IFNγ, which was later proposed to be a Roquin target (10,65). Since abrogation of *Ifng* signalling in the *sanroque* mouse significantly improved disease phenotypes, it was suggested that excess IFNγ was able to induce $CD4^{+}$ T cell proliferation and T_{FH} differentiation via induction of the master transcription factor Bcl6 (B-cell lymphoma 6) (65).

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However, conclusive evidence for direct Roquin-mediated regulation of *Ifng* mRNA is lacking to date. The absence of interleukin-27 (IL-27) signalling in *sanroque* mice led to significantly reduced renal inflammation, a reduction in IFNγ-producing T cells and lower frequencies of T_{FH} and GC B cells, suggesting IL-27 as another driver of the *sanroque* phenotype (66).

In addition to T cells, the *sanroque* mutation affects other cell types, especially B cells (33,67). For instance, the *sanroque* mutation and additional deletion of the transcriptional co-activator Obf1, a factor required for GC formation and efficient affinity maturation (68,69), led to an increase in ANAs and in immunoglobulin M (IgM) anti-dsDNA antibodies compared with Obf1-sufficient *sanroque* counterparts. Autoantibodies were presumably produced by pre-existing autoreactive B cells and extrafollicular plasma cells, which receive help from the overactive T cell compartment, thus accelerating autoimmune disease in these mice (70).

Combination of the *sanroque* mutation with the TCR^+ hen egg lysozyme $(HEL)^+$ double-transgenic mouse, a model for type I diabetes, significantly accelerated the onset of diabetes. In this model, high frequencies of islet-reactive $CD4^+$ T cells are generated, which recognize the HEL neoantigen expressed by β cells of the pancreas. Pathology in this system was driven by an expansion of islet-reactive $Rc3h1^{san/san}$ TCR^+ HEL^+ T_H1 cells, increased GC formation and production of anti-HEL immunoglobulin G2a (IgG2a) autoantibodies (71).

Roquin-mediated gene regulation in inflammation

In another mouse line, i.e. $Rc3h1^{gt/gt}$ mice, a random insertion of a gene-trap vector between exons 1 and 2 of the *Rc3h1* gene showed lack of Roquin-1 detection that was associated with hepatitis and chronic inflammation along the small intestine and a strong dysregulation of chemokines that may contribute to the observed inflammation (72). Complete systemic deletion of Roquin-1, either by gene-trap targeting or by deletion of the floxed exons 4–6 using germline cre-deleter mice ($Rc3h1^{-/-}$), led to severe developmental defects, including impaired caudal spine closure and perinatal death associated with lung pathology (67,72). Interestingly, a small number of $Rc3h1^{gt/gt}$ mice that survived until adulthood developed an autoinflammatory phenotype, which was not observed in $Rc3h1^{-/-}$ mice.

The reasons for these differences are unclear and may relate to the mouse background, different microbiota in the housing facilities or even to the differential targeting of Roquin-1 itself, which may cause expression of a truncated Roquin-1 protein. In fact, conditional targeting of exon 2 in the

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genes encoding either Roquin-1 or Roquin-2 (*Rc3h1*$^{rin/rin}$ and *Rc3h2*$^{rin/rin}$ mice, respectively) produced truncated proteins lacking the N-terminal RING (really interesting new gene) finger, since an alternative translation initiation (Met133 in the Roquin-1^{Ringless} protein) occurred. Although the importance of the E3 ligase for Roquin protein function is still unclear, truncations that deleted the RING finger impaired the localization of Roquin proteins to stress granules in T cells (17,73). Homozygous germline deletion of the RING domain recapitulated perinatal lethality, lung pathology and neural-tube defects observed in *Rc3h1*$^{-/-}$ and *Rc3h1*$^{gt/gt}$ mice (17,67,72). The cell types and deregulated genes that cause these phenotypes upon Roquin inactivation or RING finger deletion are currently unknown.

Conditional deletion of Roquin-1-encoding alleles in T cells or in the entire hematopoietic system had only mild effects and did not cause the break of self-tolerance observed in *sanroque* mice (67). This phenotypic disparity can be explained by Roquin-2 redundantly regulating target mRNAs in the absence of Roquin-1 (9,17). In fact, the *sanroque* phenotype may only reveal the effect of this mutation because the mutated Roquin-1 protein is expressed at levels several times higher than Roquin-2 and outcompetes any compensation by the Roquin-2 protein (9). Indeed, combined ablation of Roquin-1 and Roquin-2 in T cells resulted in many phenotypes similar to the ones observed in the *Rc3h1*$^{san/san}$ mice, including lymphadenopathy, splenomegaly, activation of $CD4^+$ and $CD8^+$ T cells and an increase in T_{FH} cells as well as strong upregulation of *Icos* and *Ox40*. In contrast to the *sanroque* mouse, Roquin-1/2 deficiency did not induce ANAs, anti-dsDNA antibodies or rheumatoid factors (8) presumably because of a strongly perturbed splenic microarchitecture that may prevent sufficient B cell help (9).

Despite the lack of apparent autoimmunity, mice with Roquin-1/2 deficiency in T cells developed pathologies in multiple organs, including thickening of arterial walls with collagen deposition and severe inflammation in the lung (8), underlining the importance of adequate Roquin function in T cells for the prevention of spontaneous lung inflammation. In contrast to *sanroque* mice, these mice exhibited an accumulation of T_H17 cells in the lung, revealing a hitherto unrecognized role of Roquin proteins in the control of T_H17 differentiation (8). Roquin-deficient Treg cells acquired a T_{FR} phenotype and lost their suppressive capacity in a T cell transfer-induced colitis model (36), indicating that insufficient Treg cell function may further contribute to the strong inflammatory phenotype of mice that lack Roquin-1/2 function in all T cells.

Although a direct link between Roquin LOF and neoplasia has not yet been described, the absence or dysfunction of Roquin proteins results in severe autoimmunity and chronic inflammation in both mice and humans. Given that chronic inflammation, which can induce genetic instabilities and

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alterations rendering tissues more susceptible to neoplastic transformations (74), is one of the well-established hallmarks for cancer development, it would be of great interest to investigate the link between Roquin-mediated inflammation and neoplasia in the future.

Roquin-1 and Regnase-1 cooperation prevents autoimmunity

Only recently, it was demonstrated that autoimmunity in the *sanroque* mouse is explained by impaired interaction of Roquin-1 with Regnase-1 (33). Conditional ablation of Regnase-1 alone recapitulates the autoimmune phenotype of the *sanroque* mouse, including accumulation of autoantibodies (12). Similarly, introducing individual point mutations into Roquin-1 that had been predicted and validated to be crucial for Roquin/Regnase-1 interaction, completely reproduced the *sanroque* phenotype *in vivo* (33). These data suggested that the interaction between Roquin and Regnase proteins is necessary to maintain immune homeostasis and prevent autoimmunity in mice.

Interestingly, it is not only impaired Roquin function that has detrimental effects on the immune system. Mice with enforced overexpression of Roquin-1 in T cells developed stronger collagen-induced arthritis with higher systemic levels of proinflammatory cytokines (75) and were more susceptible to the development of T cell-mediated hepatitis (76). These findings are surprising in light of other published studies suggesting that Roquin LOF and the subsequent deregulation of Roquin targets promote autoimmunity and inflammation.

Since the *Zc3h12a* mRNA is repressed by Roquin (8,33) and Regnase-1 expression itself is required for the repression of a number of proinflammatory molecules and prevention of tissue inflammation (12,30,35), we speculate that the autoimmune and autoinflammatory phenotypes described above could potentially be explained by reduced Regnase-1 levels caused by Roquin-1 overexpression. In line with this notion, therapeutically increased Regnase-1 levels may represent a promising strategy to ameliorate autoimmune disease in the future. Recently, it was demonstrated by Osamu Takeuchi, Ka Man Tse and colleagues that combined "morpholino" oligonucleotides can unfold two Roquin/Regnase-recognized mRNA hairpins in the 3′-UTR of *Zc3h12a*. This treatment reduced negative feedback regulation, elevated Regnase-1 levels and, in a preclinical model, intracranial application of these oligonucleotides attenuated the development of experimental autoimmune encephalomyelitis (EAE) (35).

Another possible way to therapeutically enhance Roquin and Regnase function is the inhibition of the MALT1 paracaspase. Such a strategy with small-molecule inhibitors for the MALT1 paracaspase was originally devised to exploit the observed dependency of diffuse large B-cell lymphoma (DLBCL)

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cancers on MALT1 proteolytic activity for tumor growth (77,78). However, one caveat of targeting the MALT1 paracaspase is that mice with genetic inactivation of the paracaspase develop severe autoimmunity due to decreased Treg cell development and function (31,79,80).

Conversely, it was recently described that Treg-specific inhibition of Carma1 (CARD-containing MAGUK protein 1), Bcl10 (B-cell lymphoma/leukemia 10) or of the paracaspase function of MALT1 affects the differentiation of effector Treg cells and, within tumors, reprograms these cells to produce IFNγ, which was shown in preclinical cancer models to protect from tumor growth in synergy with checkpoint blockade (81,82).

Reprogramming of T cells by Roquin or Regnase-1 deficiency

Adoptive T cell therapies have brought new hope in fighting cancers that are typically resistant to conventional therapy (83). Although these immunotherapies have proven successful for the treatment of some malignancies, such as CD19-expressing acute lymphoblastic leukemia, treatment of solid tumors still poses significant challenges (reviewed in (84-86)): cancer cells in solid tumors are less accessible, exhibit great heterogeneity and can lack cancer-specific antigens (reviewed in (85-87)). Moreover, they create an immunosuppressive and hypoxic microenvironment with low levels of nutrients and promote T cell exhaustion (reviewed in (86,88)). The exhausted state is characterized by a loss of cytotoxicity, an inability to produce effector cytokines and an elevated expression of a variety of inhibitory receptors and involves a distinct transcriptional program and epigenetic landscape (89,90). Consequently, adoptively transferred tumor-specific T cells show poor trafficking to the tumor site and often lack effective activation and proliferation (reviewed in (84,91)). Overcoming exhaustion and increasing T cell activation and persistence are therefore key to successful treatment of solid tumors (reviewed in (88,91)).

Whereas inactivation of Roquin and Regnase-1 leads to autoimmunity (10,12,30) loss of either has been shown to promote tumor antigen-specific T cell responses, suggesting that these RBPs as well as their regulated mRNAs are attractive targets for adoptive cell therapies (ACT).

Adoptive transfer of $CD4^+$ or $CD3^+$ T cells to characterize the behavior of naive polyclonal T cells after inducible knockout of Roquin-1/2 or Regnase-1 showed that a large proportion of these T cells became spontaneously activated and proliferated inside wild-type hosts, likely without having seen antigen (Fig. 3). For Regnase-1 deficient $CD4^+$ T cells a very long persistence has been observed, whereas Roquin-1/2 deficient T cells disappeared faster than Regnase-1 deficient cells did (12,33). A

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striking feature of Roquin-1/2 loss was the acquisition of an effector memory (EM) phenotype ($CD44^+$, $CD62L^-$) for $CD4^+$ and $CD8^+$ T cells, whereas knockout of Regnase-1 led to the differentiation into EM but also into central memory (CM) T cells ($CD44^+$, $CD62L^+$) (Fig. 3). In general, deletion of Roquin-1/2 or Regnase-1 in T cells was associated with an increased production of effector molecules such as granzyme B, TNFα and INFγ. Additionally, Regnase-1 deficiency was associated with a significant increase in IL-2^+ cells. Roquin-1/2 deficient $CD8^+$ T cells performed better in *in vitro* killing assays (33).

A key player in metabolic reprogramming is the PI3K–mTOR (phosphatidylinositol 3-kinase – mechanistic target of rapamycin) and Akt (AKT serine/threonine kinase) pathway, a coordinator of glycolysis, lipid-synthesis and oxidative phosphorylation. mTORC1 activity affects T cell fates (92) and the early phase of activation, as well as the late phase of T cell exhaustion (93). Both Roquin and Regnase-1 have been shown to suppress mTOR activity in conventional T cells and Treg cells and in intestinal epithelial cells, respectively (36,94). Elevated mTORC1 activity in $CD8^+$ T cells inactivates FOXO1 (forkhead box protein O1), which leads to elevated T-bet (T-box transcription factor TBX21) and reduced Eomes (eomesodermin) expression, favoring effector rather than memory differentiation (95,96). Moreover, upon loss of Roquin-1/2, Itch (itchy E3 ubiquitin protein ligase), an E3 ubiquitin ligase, is upregulated, which is known to induce ubiquitination and degradation of FOXO1 (36). In-depth investigations of how Roquin and Regnase-1 control metabolism as well as a comprehensive identification of targets that drive metabolic reprogramming will be required to exploit this regulatory program for the improvement of ACT.

Roquin and Regnase-1 functions in tumor-specific $CD8^+$ T cells

Adoptive T cell transfer of tumor antigen-specific T cells into mice with subcutaneously injected tumor cells revealed that $CD8^+$ T cells elicited improved tumor control when they harbored deletion of Roquin-1 and Roquin-2 encoding alleles or CRISPR/Cas9 gene editing that inactivated Roquin-1. In the presence of tumor antigen, the T cells persisted and limited the tumor growth, presumably owing to their strong proliferation, enhanced cytokine production and high cytotoxicity (Fig. 2B) (33,97). The improved persistence and superior tumor killing was attributed to increased *Irf4* expression in the absence of Roquin proteins, since combined deletion of *Irf4* and Roquin-1 attenuated the positive effects observed in $CD8^+$ T cells (97).

Similar to inactivation of Roquin in tumor-specific cytotoxic T cells, Regnase-1 deficient $CD8^+$ T cells showed superior inhibition of tumor growth (33,97-100). This ability was related to the increased

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expression of the basic leucine zipper ATF-like transcription factor (BATF), which is upregulated in Roquin-1/2 or Regnase-1 knockouts (33,100). BATF is important for maintaining mitochondrial fitness in $CD8^+$ cells (100), regulating metabolic reprogramming, differentiation of effector $CD8^+$ T cells and survival (101), as well as promoting effector functions in the tumor microenvironment (102).

Overexpression of *BATF* has been implicated in improved tumor control not only in conventional T cells (100), but also in chimeric antigen receptor (CAR) T cells by promoting their survival and expansion (103). Additionally, an increase in the transcription factor BATF3 in $CD8^+$ T cells resulted in metabolic changes and in increased numbers of tumor-infiltrating $CD8^+$ T cells. However, in contrast to BATF, BATF3 did not affect cytokine production. Instead it promoted memory formation and counteracted apoptosis by suppressing the proapoptotic factor BIM (Bcl-2-like 11; encoded by *Bcl2l11*) (104).

The beneficial effects of BATF overexpression in CAR T cells were dependent on its interaction with *IRF4*, since a mutation that inhibited the BATF/Irf4 interaction was unable to promote antitumor immunity, whereas co-expression of BATF and Irf4 in T cells significantly attenuated exhaustion (103). In order to bind DNA and regulate gene expression, BATF needs to heterodimerize with a Jun family member (105). Interestingly, the c-Jun transcription factor has also been identified as a potential therapeutic target that could counteract exhaustion by driving expansion, enhancing functions and suppressing terminal differentiation, thereby supporting anti-tumor responses of CAR T cells (106). BATF, together with IRF4 and Jun, mediates the expression of T-bet and Blimp-1 (B-lymphocyte-induced maturation protein 1) (107) and promotes the acquisition of cytotoxic functions (108).

One concern in CAR T cell therapy is that the chimeric receptor exerts tonic signaling and such antigen-independent clustering of CAR single-chain variable fragments leads to exhaustion (109). Exhausted T (Tex) cells result from chronic antigen encounter and are also the progeny of precursor exhausted T (Tpex) cells which, unlike Tex, are still capable of self-renewal (93,110). This property depends on the expression of the T cell factor 1 (TCF1) transcription factor in T cells (110). TCF1 also plays a crucial role in CM formation (111) and is elevated in naive and memory $CD8^+$ T cells (112,113). Recently, TCF1 was identified as a novel target of Regnase-1, and upregulation of TCF1 in the absence of Regnase-1 was implicated in the formation of Tpex, supporting the persistence and longevity of CAR T cells (98). However, during activation of T cells, an early loss of TCF1 expression is crucial for appropriate effector T cell differentiation (113).

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Roquin-deficient CD8$^+$ T cells, on the other hand, exhibit low TCF1 expression but strong effector functions and high KLRG1 (killer cell lectin like receptor G1) expression (33), implying that they are prone to terminal exhaustion, which, however, was not observed in tumor-infiltrating Roquin-deficient T cells. Together these aspects suggest that even though Regnase-1-deficient T cells may persist better in the tumor microenvironment, Roquin-deficient T cells may unfold a greater cytotoxicity potential. Detailed characterizations of Roquin and Regnase-1 deficient CD8$^+$ T cells exposed to persistent antigen are required to elucidate Roquin and Regnase-1 function in the prevention of exhaustion.

One criterion for targets that control antitumor responses should be that their mRNAs are bound by Roquin-1/2 and Regnase-1 proteins and exhibit cooperative regulation by both factors (33). An in-depth analysis of the post-transcriptional mechanisms will therefore identify critical nodes of gene regulation that allow selective modulation to improve adoptive T cell or CAR T cell therapy to fight cancer.

Concluding remarks

As outlined in this review, Roquin and Regnase proteins not only play crucial roles in the prevention of autoimmunity and inflammation, but their inhibition can also have significant contributions to anti-tumor responses. Therapeutically induced Roquin or Regnase-1 LOF in CD8$^+$ T cells can, on the one hand, be beneficial in combating cancers and improve T cell/CAR T cell therapies. On the other hand, continuous inactivation of the system results in severe autoimmunity or inflammation in both mice and humans. In this respect treatment of autoimmune disease may profit from strategies that actually strengthen the system.

Roquin and Regnase-1 proteins act in concert, regulating shared and exclusive target sets, and control their own expression levels via negative autoregulation. Therapeutic strategies that modulate the availability of both components or disrupt Roquin-1/Regnase-1 interaction itself will be of particular interest for future therapeutic interventions. Possible therapeutic approaches may entail the inhibition of the protease function of MALT1, the abrogation of negative regulation by small oligonucleotides that unfold specific hairpin structures in their own mRNAs or target mRNAs, the targeting of the RBPs themselves by gene editing or gene silencing approaches, and the targeting of either the Roquin/Regnase-1 interaction or the Regnase-1 catalytic activity by small molecules.

Since the discovery of Roquin and Regnase-1 functions in the immune system (10,30) more than a decade ago, we have learned a great deal about their importance, function and regulation. However,

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to be able to exploit this system in personalized therapies, we need to gain an in-depth understanding of Roquin and Regnase-1 mediated gene regulation. Moreover, we need to comprehend how targeting the system upstream, at the level of the RBPs or even very downstream at the level of direct and indirect Roquin and Regnase-1 targets, will affect cellular functions and whether durable or transient manipulations are required and tolerated.

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Abbreviations

Abbreviation	Definition
ACT	adoptive cell therapies
ADE	alternative decay elements
AITL	angioimmunoblastic T-cell lymphoma
Akt	AKT serine/threonine kinase
ANAs	antinuclear antibodies
BATF	basic leucine zipper ATF-like transcription factor
Bcl10	B-cell lymphoma/leukemia 10
Bcl6	B-cell lymphoma 6
BIM	Bcl-2-like 11
Blimp-1	B-lymphocyte-induced maturation protein 1
CAR	Chimeric antigen receptor
Carma1	CARD-containing MAGUK protein 1
CDE	constitutive decay element
CM	central memory
DLBCL	diffuse large B-cell lymphoma
dsDNA	double-stranded deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
Edc4	enhancer of MRNA decapping 4
EM	effector memory
ENU	N-ethyl-N-nitrosourea
EOMES	eomesodermin
FOXO1	forkhead box protein O1
GC	germinal center
HLH	hemophagocytic lymphohistiocytosis
ICOS	inducible T cell co-stimulator

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IFNγ	interferon γ
IgG2a	immunglobulin G 2a
IgM	immunglobulin M
IL-2	interleukin-2
IL-27	interleukin-27
Irf4	interferon regulatory factor 4
Itch	itchy E3 ubiquitin protein ligase
KLRG1	killer cell lectin like receptor G1
LOF	loss-of-function
MALT1	mucosa-associated lymphoid tissue lymphoma translocation 1 gene
mTOR	mechanistic target of rapamycin
Nufip2	nuclear FMR1 Interacting Protein 2
Ox40	tumor necrosis factor receptor superfamily, member 4
PI3K	phosphatidylinositol 3-kinase
RBD	RNA-binding domain
RBP	RNA-binding protein
RING	really interesting new gene
Sap	SLAM-associated protein
SERPINC1	serpin family C member 1
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
T-bet	T-box transcription factor TBX21
TCF1	T cell factor 1
TCR	T cell receptor
Tex	Exhausted T cells
T_{FH}	T follicular helper
T_{FR}	T follicular regulatory

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T$_H$1	T helper 1
T$_H$17	T helper 17
TNFα	tumor necrosis factor α
Tpex	precursor exhausted T cells
Treg	T regulatory
Upf1	regulator of nonsense transcripts 1
UTR	untranslated region
ZBTB37	Zinc finger and BTB domain containing 37

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Figure legends

Fig. 1. Regulation of Roquin proteins in T cells. **(A)** Domain organization of Roquin-1 and its paralogue Roquin-2. Sequence similarity and MALT1 cleavage sites are indicated. **(B)** Mechanism of Roquin- and Regnase-1-mediated gene repression in T cells. In naive T cells, Roquin-1/2 and Regnase-1 repress their target mRNAs by binding to specific stem–loop structures in their 3'UTRs. Upon TCR activation and co-stimulation via CD28, Roquin-1/2 and Regnase-1 are cleaved by the MALT1 paracaspase and their target mRNAs are released. Both RBPs regulate transcripts of important immune modulators which comprise various transcription factors, cytokines and co-stimulatory molecules. PRR: Proline rich region, RING: really interesting new gene, HEPN: higher eukaryotes and prokaryotes nucleotide-binding, ZnF: CCCH-type zinc finger, UTR: untranslated region, CDS: coding sequence, TCR: T cell receptor.

Fig. 2. Roquin proteins control cell fate decisions in T cells. **(A)** Consequences of Roquin dysfunction in T cells (upper panel) and their exploitation for cancer therapy (lower panel). Disruption of the interactions between Roquin-1/2 and Regnase-1 by the *sanroque* mutation leads to the development of autoimmune disease, characterized by excess T_{FH} differentiation, increased ICOS and increased IFNγ production. In contrast, Roquin loss-of-function in T cells causes aberrant T cell activation and spontaneous accumulation of T_{FH} and T_H17 cells, overexpression of ICOS and Ox40 and systemic inflammation in mice. **(B)** In $CD8^+$ T cells, interfering with Roquin and Regnase-1 function and the associated changes in metabolic programs have high potential for the improvement of adoptive T cell therapies, because of an improved killing capacity and reduced exhaustion of these cells. RBP: RNA-binding protein, T_{FH}: T follicular helper cells, ICOS: inducible T cell costimulatory, ANA: anti-nuclear antibodies, SLE: systemic lupus erythematosus, SS: Sjörgren's syndrome.

Fig. 3. Reprogramming of T cells by Roquin or Regnase-1 deficiency. Experimental setup used to study the behavior of naive polyclonal T cells after inducible deletion of Roquin-1/2 or Regnase-1. Naive $CD4^+$ or $CD3^+$ T cells were adoptively transferred into congenicaly marked wild-type recipients and deletion was induced in these cells by Tamoxifen treatment of the recipient mice. Deletion of Roquin-1/2 or Regnase-1 leads to metabolic reprogramming and a spontaneous activation and proliferation of $CD4^+$ and $CD8^+$ T cells, presumably in an antigen independent manner. Roquin-1/2- and Regnase-1-deficient T cells acquire an EM phenotype, whereas Regnase-1-deficient T cells in

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addition exhibit a CM phenotype. Loss-of-function of either protein leads to spontaneous T_{FH} differentiation in lymphoreplete hosts and, in the case of Regnase-1 deficiency, leads to transfer of autoimmunity, as assessed by increased production of anti-nuclear antibodies. Cells deficient in either Roquin-1/2 or Regnase-1 undergo proliferation after adoptive transfer. Only the absence of Regnase-1 can support long-term persistence of the transferred cells in the lymphoreplete host, whereas Roquin-1/2-deficient T cells disappear, potentially due to activation-induced cell death. EM: effector memory, CM: central memory.

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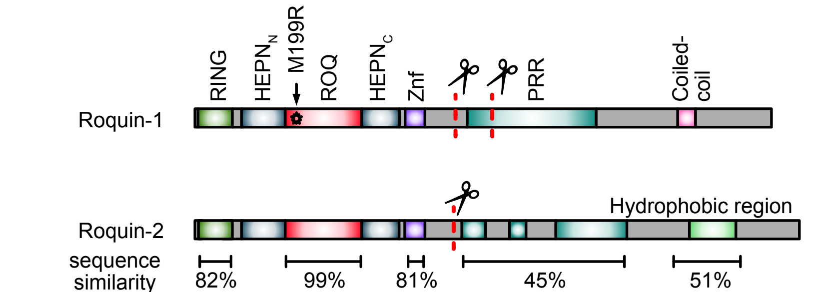

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(A) Roquin dysfunction and disease phenotypes

Roquin$^{san/san}$:

T_{FH}

$CD8^{+}$

↑ T cell activation

↑ IFNγ production

↑ T_{FH} differentiation

↑ ICOS expression

✗ Impaired interaction with Regnase-1

↑ Autoimmunity (SLE, SS)

ANA Production

Glomerulonephritis and kidney damage

exacerbated disease in type I diabetes models

Roquin-1/2 deficiency:

$CD8^{+}$

$T_{H}17$

T_{FH}

↑ T cell activation

↑ IL-17A and IFNγ production

↑ T_{FH} and $T_{H}17$ differentiation

↑ ICOS, Ox40 expression

↑ mTOR signaling

→ metabolic reprogramming

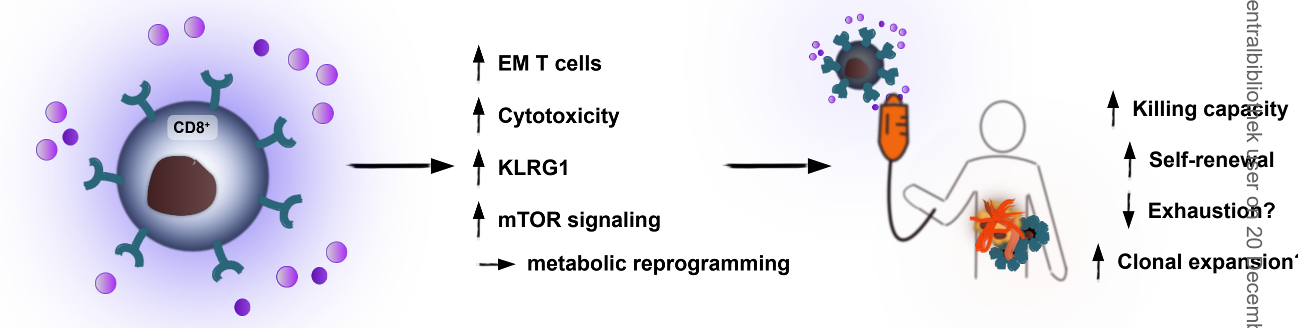
↑ Systemic inflammation

Lung damage

arterial wall thickening

gastritis

(B) Roquin and Regnase-1 manipulation for cancer therapy



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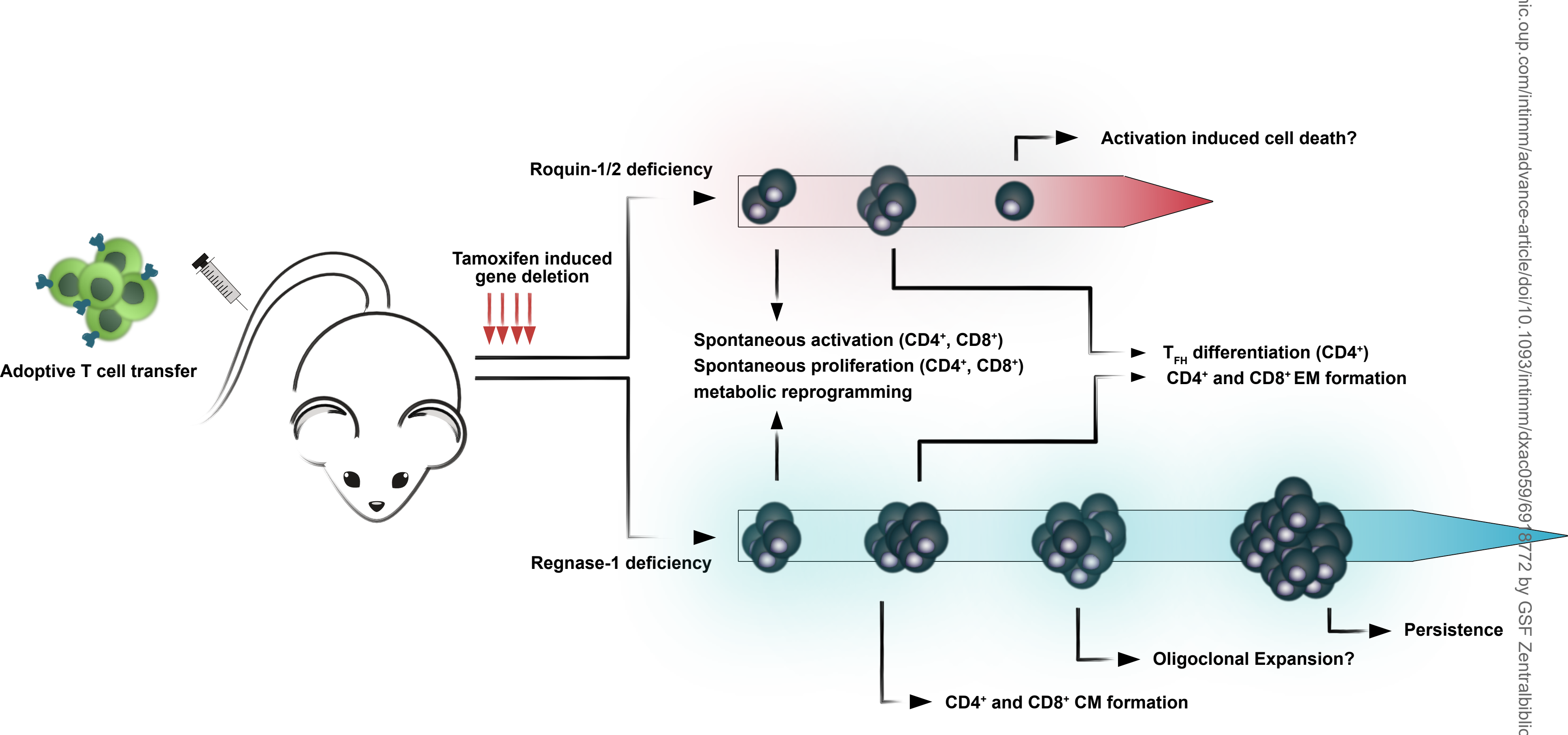

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