Alternative splicing of MALT1 controls signalling and activation of CD4⁺ T cells

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MALT1 channels proximal T-cell receptor (TCR) signalling to downstream signalling pathways. With MALT1A and MALT1B two conserved splice variants exist and we demonstrate here that MALT1 alternative splicing supports optimal T-cell activation. Inclusion of exon7 in MALT1A facilitates the recruitment of TRAF6, which augments MALT1 scaffolding function, but not protease activity. Naive CD4⁺ T cells express almost exclusively MALT1B and MALT1A expression is induced by TCR stimulation. We identify hnRNP U as a suppressor of exon7 inclusion. Whereas selective depletion of MALT1A impairs T-cell signalling and activation, downregulation of hnRNP U enhances MALT1A expression and T-cell activation. Thus, TCR-induced alternative splicing augments MALT1 scaffolding to enhance downstream signalling and to promote optimal T-cell activation.
Antigenic stimulation of the T-cell receptor (TCR) together with a CD28 co-stimulatory receptor induces productive activation of naïve CD4⁺ T cells. MALT1 (mucosa-associated lymphoid tissue protein 1) bridges TCR/CD28 co-engagement to cellular downstream signalling pathways to promote T-cell activation and effector functions. As part of the CARMA1–BCL10–MALT1 (CBM) signalling complex, MALT1 channels upstream TCR signalling to the canonical IkB kinase (IKK)/nuclear factor-κB (NF-κB) signalling pathway. Three TRAF6-binding sites have been mapped on MALT1 (refs 3,4). MALT1 recruits TRAF6 to the CBM complex to promote MALT1 ubiquitination and to facilitate activation of the IKK complex. Besides its scaffolding function, MALT1 contains a paracaspase domain, and MALT1 proteolytic activity is induced on antigen stimulation in T cells. MALT1 proteolytic activity is not directly involved in controlling canonical NF-κB signalling. However, MALT1 cleavage of the deubiquitinases A20 and CYLD, the E3 ligase HOIL, the non-canonical NF-κB family member RelB or the RNA regulators Regnase-1 and Roquin has been associated with various functions for T-cell biology.

Alternative splicing is a crucial and ubiquitous mechanism that controls gene expression at the co- and post-transcriptional level. In mammals, most pre-mRNAs are prone to alternative splicing, which results in the generation of multiple transcripts and proteins with diverse functions. Extensive changes in splicing patterns have been shown to occur in the immune response and especially in antigen-dependent T-cell activation. Alternative splicing can act on multiple layers ranging from cell surface receptors, cytokines, signalling proteins to transcription factors, and thereby constitutes an essential regulatory mechanism for T-cell function. A well-studied example is the TCR-induced exon exclusion of the transmembrane phosphatase CD45, which creates a negative-feedback regulation that counteracts T-cell activation. However, in T cells, little is known how alternative splicing modulates expression and activity of intracellular signalling mediators and how this can influence T-cell signalling and activation.

Two conserved alternative splice isoforms of MALT1 have been assigned that differ only by inclusion (MALT1A) or exclusion (MALT1B) of exon7 that codes for 11 amino acids (aa 309–319 of human MALT1). However, neither expression nor functions of the two MALT1 alternative splice variants have been investigated. Here we identify heterogeneous nuclear ribonucleoprotein U (hnRNP U; SAF-A/SP120) as a factor that controls gene expression at the co- and post-transcriptional level. We wanted to validate that the putative binding motif in MALT1 exon7 is crucial for signalling in Jurkat T cells. For this, we reconstituted MALT1-deficient Jurkat T cells with MALT1A or MALT1B constructs mutated in one or more TRAF6-binding sites (Fig. 1b; Supplementary Fig. 1d). Whereas mutation of the TRAF6-binding motif 2 (T6BM2) next to the Ig3 in MALT1B does not affect signalling, activation of NF-κB, JNK and p38 completely relies on the presence of the most C-terminal T6BM3 (Supplementary Fig. 1e,f). In contrast, only the combined mutation of T6BM1 in exon7 and the C-terminal T6BM3 (T6BM1/3 EA) in MALT1A abrogates the activation of downstream signalling pathways (Fig. 1e,f). To prove that the mutations in individual or multiple T6BMs affect TRAF6 recruitment, we performed StrepTag-II purification of transduced Jurkat T cells (Fig. 1g). Constitutive BCL10 association and inducible CARMA1 recruitment were not affected by MALT1 exon7 inclusion or mutations of TRAF6 sites, but TRAF6 recruitment was diminished in MALT1B compared with MALT1A. In line with the effects on signalling, mutation of the binding site T6BM3 in MALT1B alone and the combined mutation of T6BM1/3 in

**Figure 1** | Conserved MALT1 exon7 enhances TRAF6 recruitment and NF-κB activation but not MALT1 activity. (a) Domain structure of MALT1 isoforms with different TRAF6-binding motifs (T6BMs) highlighted in orange and blue. Sequence conservation of T6BM1 in exon7 in different species is shown below. Protein domains are denoted by black boxes. DD, death domain, Ig, Immunoglobulin-like domain. (b) Schematics of the T6BMs in MALT1A and MALT1B. Different TRAF6-binding mutants were generated by glutamate (E) to alanine point mutations (A) as indicated. (c-h) MALT1-deficient Jurkat T-cell clone was reconstituted with StreptTag-II (mock) or MALT1-StreptTagII variants. (c) MALT1 expression was checked by western blot (WB). (d) Reconstituted cells were stimulated with P/I for the indicated time points. NF-κB signalling was analysed by electrophoretic mobility shift assay (EMSA) and WB. (e-f) Cells transduced with MALT1A wild-type or MALT1A mutants were stimulated with P/I for the indicated time points. NF-κB and MAPK signalling were analysed by WB and EMSA. (g) CBM complex formation as well as TRAF6 recruitment were investigated by Strept-T-PD after 30 min P/I stimulation. Binding of MALT1 to NEMO was monitored after NEMO IP. Modified MALT1 indicative of ubiquitination is marked by asterisk (*). (h) Proteins were precipitated by Strept-T-PD after 20 min P/I stimulation and active MALT1 was detected using fluorescent MALT1-ABP probe. Data are representative of at least three independent experiments.
**a** MALT1

**b** ex7

**c** MALT1

**d** MALT1

**e** MALT1

**f** MALT1

**g** MALT1

**h** MALT1
MALT1A abolished TRAF6 recruitment. Also, MALT1 ubiquitin modification was severely reduced only in the absence of any functional TRAF6-binding site, which prevented recruitment of ubiquitinated MALT1 to the IKK regulatory subunit NEMO (Fig. 1g). Thus, the mutagenesis of individual or multiple TRAF6-binding sites confirms that exon7 is significantly contributing to the activation of downstream signalling pathways.

To test whether splicing affects MALT1 proteolytic activity in response to T-cell stimulation, we measured MALT1 protease activation in reconstituted Jurkat T cells using fluorescently labelled MALT1 activity-based probe (MALT1-APB; Fig. 1h). As expected, MALT1 activation was detected on P/I stimulation and active MALT1 was heavily modified, which most likely corresponds to previously described active MALT1 ubiquitin conjugates. However, MALT1A and MALT1B were activated to a similar extent and also cleavage of the MALT1 substrate CYLD was not altered in MALT1A- or MALT1B-expressing cells (Supplementary Fig. 1g), revealing that MALT1 protease activation is not affected by inclusion or exclusion of exon7.

To test whether the MALT1 splice variants exert differential effects on CBM downstream signalling in primary T cells, we purified CD4+ T cells from Malt1–/– mice and reconstituted the cells by retroviral transduction with either MALT1A or MALT1B. Reconstituted T cells were expanded and transduced with either MALT1A or MALT1B. Reconstituted T cells were expanded and transduced cells were purified by the co-expressed surface marker Thy1.1 yielding 80–90% Thy1.1-positive T cells (Supplementary Fig. 2a). As expected, the absence of MALT1 completely abolished NF-κB and JNK signalling on T-cell stimulation (Supplementary Fig. 2b). Expression of MALT1A and MALT1B was comparable to endogenous MALT1 levels in T cells from wt mice (Fig. 2a). To determine induction of NF-κB and MAPK signalling, reconstituted T cells were restimulated with anti-CD3/CD28 or P/I (Fig. 2b,c; Supplementary Fig. 2c,d). Expression of both the MALT1 isoforms rescued NF-κB signalling on anti-CD3/CD28 or P/I stimulation. Congruent with our observations in MALT1-deficient Jurkat T cells, IκBx degradation was more rapid and NF-κB activation was stronger in cells expressing MALT1A when compared with MALT1B. Also, JNK phosphorylation was rescued on MALT1 expression and MALT1A was more potent in mediating JNK activation than MALT1B after anti-CD3/CD28 or P/I stimulation. MALT1 was also able to rescue the partial defect in p38 activation that has been observed earlier in MALT1-deficient T cells, but p38 activation did not rely on the MALT1 isoforms. Moreover, despite a reduction in ERK amounts in MALT1A- or MALT1B-expressing cells, no differences in the extent of inducible ERK phosphorylation were detected using the two splice variants.

To investigate whether the TRAF6-binding motifs in the C terminus and exon7 contribute to differential effects on MALT1 downstream signalling in primary T cells, we performed reconstitutions using a series of MALT1 point mutations to destroy T6BMs individually or in combination (Fig. 1b) and determined the effects on NF-κB signalling in primary T cells. Mutation of the C-terminal TRAF6 motifs (T6BM2/3 EA) in

Figure 2 | MALT1A promotes stronger NF-κB and JNK activation on T-cell stimulation. (a–c) CD4+ T cells from MALT1-deficient mice (C57BL/6 j) were reconstituted with mock, MALT1A or MALT1B. (a) MALT1 expression levels of T cells from wt mice or reconstituted Malt1−/− mice were determined by western blot (WB). NF-κB (b) and MAPK signalling (c) were analysed by WB after stimulation with anti-CD3/CD28 as indicated. NF-κB DNA binding was determined by electrophoretic mobility shift assay (EMSA). NF-κB signal was quantified relative to OCT1 control. (d,e) MALT1-deficient mice (C57BL/6 j) were reconstituted with MALT1A, MALT1B or different TRAF6-binding mutants as indicated (Fig. 1b). IκBx phosphorylation and degradation were analysed by WB after P/I stimulation and DNA binding of NF-κB was monitored by EMSA. NF-κB signal was quantified relative to OCT1 control. Data are representative of at least three independent experiments.
MALTA1 led to a similar reduction in P/I-triggered NF-κB signalling activation as exclusion of exon7 in MALT1B (Fig. 2d). Moreover, NF-κB signalling was decreased on destruction of the T6BM1 in exon7 MALT1A (T6BM1 EA; Fig. 2e). Only the combined destruction of T6BM1s in either MALT1A (T6BM1/2/3 EA) or MALT1B (T6BM2/3 EA) completely abolished the NF-κB response (Fig. 2d,e). Thus, in primary T cells, intact TRAF6-binding sites in the C terminus of MALT1B are sufficient to induce moderate downstream signalling, but cooperation between C-terminal and exon7-encoded TRAF6-binding motifs promote optimal NF-κB signalling.

In Jurkat T cells, we observed the effects of exon7 on downstream signalling, but not on paracaspase activity, suggesting that inclusion of exon7 is primarily modulating MALT1 scaffolding function. Interleukin (IL)-2 expression critically depends on MALT1 scaffolding and partially on MALT1 cleavage activity5,21–23. Indeed, IL-2 production after anti-CD3/CD28 stimulation was rescued in T cells retrovirally reconstituted with either mock, MALT1A or MALT1B, but the number of IL-2-producing cells was increased in MALT1A transduced cells (Fig. 3a,b). Therefore, the stronger potency of MALT1A to promote downstream signalling correlates with a more robust IL-2 production. TTH17 differentiation was completely abolished in MALT1 protease defective mice and is thus apparently independent of MALT1 scaffolding function22,24. To investigate whether inclusion of exon7 influences TTH17 differentiation, we adenovirally transduced naive CD4+ T cells expressing a signalling-deficient coxsackie adenovirus receptor (CARA1) from MALT1/−−/−R26/CAG-CARAs1stop−8Cd4-Cre mice12,25 using green fluorescent protein (GFP) as infection marker (Supplementary Fig. 3a). We optimized ex vivo differentiation under Th1- or Th17-inducing conditions and found that in MALT1−/− T cells in the presence of anti-CD3 and irradiated antigen-presenting cells (APCs; Supplementary Fig. 3b). Interestingly, also TTH1 differentiation was diminished in the absence of MALT1, indicating that depending on the conditions MALT1 does not only control TTH17 differentiation24. Using these conditions, we monitored the number of IFNγ-producing TTh1 cells and IL-17A-expressing TTh17 cells in MALT1-deficient cells after viral rescue (Fig. 3c,d). Both the MALT1 splice variants induced Th17 and Th1 differentiation to a similar extend. In contrast, the catalytically inactive MALT1A C464A mutant was unable to promote Th1 and Th17 differentiation. Thus, the biochemical and functional data provide evidence that alternative MALT1 splicing modulates MALT1 scaffolding and downstream signalling, but does not affect MALT1 protease activity and cell fate decisions.

TCR signalling upregulates MALT1A in CD4+ T cells. To elucidate whether the differences in MALT1 signalling strength observed on inclusion or skipping of exon7 are relevant for activation of CD4+ T cells, we next investigated the expression of MALT1 splice variants on RNA level. We designed primer pairs that either flank exon7 (ex6–ex9/10) to amplify MALT1A/B simultaneously or exon spanning primers that selectively amplify either MALT1A (ex5–ex7/8) or MALT1B (ex5–ex6/8; Supplementary Fig. 4a). Semi-quantitative PCR (qPCR) demonstrated that MALT1A and MALT1B are expressed at equivalent levels in Jurkat T cells, but differentially expressed in murine tissues (Fig. 4b). Whereas MALT1A and MALT1B were present...
in murine brain and liver, the lymphoid organs spleen and thymus contained almost exclusively the shorter MALT1B variant, indicating that MALT1 is prone to alternative splicing in different cells and tissues. To better compare MALT1 isoform expression, we amplified either MALT1A or MALT1B by RT-PCR. In murine CD4\(^+\) T cells, MALT1B transcripts were more abundant and expressed ~100-fold more than MALT1A mRNA, which was hardly detectable (Fig. 4c). Next, we analysed the changes in mRNA expression of the two MALT1 isoforms after TCR/CD28 stimulation by semi-qPCR or qPCR (Fig. 4d–f). Again, MALT1A transcripts in CD4\(^+\) T cells purified from spleen or thymus were almost undetectable, but MALT1B was induced by a factor of 10–12 on anti-CD3/CD28 or anti-CD3 stimulation. P/I stimulation was not sufficient to induce MALT1A, suggesting that proximal TCR signalling events are critical. MALT1B levels were only moderately induced (2–4-fold) on anti-CD3 or anti-CD3/CD28 stimulation by semi-qPCR or qPCR (Fig. 4d–f).
ligation, which was also seen for overall MALT1 expression (Supplementary Fig. 4b). Induction of MALT1A, but not MALT1B expression, was also observed on stimulation of primary OT-II T cells with the ovalbumin (OVA) peptide-loaded APCs (Fig. 4g). Thus, TCR ligation induces alternative MALT1 splicing and inclusion of exon7. Despite the higher expression of MALT1B, TCR signalling triggers a relative increase of MALT1A transcript levels in CD4+ T cells.

To confirm that the induction of MALT1A was also seen in vivo, BALB/c mice were immunized by intravenous injection of sheep erythrocytes. After 7 days, naive T cells (CD62L+CD44hi), activated effector memory T cells (CD62L−CD44hi), and T-follicular helper cells (TFH CXCR5+PD1−) were purified. Whereas MALT1B transcripts were not changed in the three T-cell subsets, the MALT1A splice variant was highly expressed in activated T cells and TFH cells, but low in naive T cells (Fig. 4h). Thus, alternative MALT1A splicing is also induced in vivo, leading to a MALT1A upregulation in activated CD4+ T-cell subsets.

To address whether TCR-induced alternative splicing and exon7 inclusion also enhanced MALT1A protein amounts, we generated a rabbit polyclonal antibody against exon7 (aa 309–316), which is identical in human and murine MALT1A. On overexpression of MALT1, the antibody recognized exclusively Flag-MALT1A in western blots or after immunoprecipitation (IP) and did not cross-react with Flag-MALT1B (Supplementary Fig. 4c). To detect endogenous MALT1A, we had to enrich MALT1A by IP and used the higher affinity pan-MALT1 antibody for immunoblot detection (Fig. 4i). MALT1A protein was hardly detectable in CD4+ T cells, but anti-CD3/CD28 stimulation induced MALT1A expression in T cells from different mouse strains. Again, anti-CD3 stimulation alone was able to induce MALT1A expression. Owing to the high MALT1 expression levels, total MALT1 protein expression was largely unchanged after stimulation and we only observed a modest upregulation in C57BL/6 mice. Taken together, the results demonstrate that TCR ligation leads to an alteration in the MALT1 isoform ratio and an increase in the relative amounts of MALT1A in activated T cells.

Since MALT1 exon7 is conserved in mammals, we asked whether differences in MALT1A and MALT1B levels are also seen in human T cells purified from peripheral blood. Human CD4+ T cells expressed more MALT1B mRNA compared with MALT1A (Fig. 4j). Again, anti-CD3/CD28 stimulation induced a relative increase in MALT1A expression. Even though the effects were not as pronounced as in splenic murine CD4+ T cells, the data reveal that MALT1 is prone to alternative splicing in human T cells.

hnRNPs negatively regulate inclusion of MALT1 exon7. To corroborate our findings that MALT1 is prone to alternative splicing, we searched for RNA-binding proteins that influence the relative expression of MALT1A and MALT1B. Since both the MALT1 isoforms are expressed constitutively in Jurkat T cells (Fig. 4a), we initiated two independent small interfering RNA (siRNA) screens after transfection of smart pool siRNA against hunRNP U, affecting transcript levels on TCR stimulation in primary T cells, we used adenoviral small hairpin RNA (shRNA) knockdown in murine embryonic fibroblasts (MEFs; Supplementary Fig. 6a,b). Also, in murine T cells, AMO treatment abolished anti-CD3- or anti-CD4+ T cells. Infected CD4+ T cells were sorted based on co-expression of GFP and an efficient downregulation of hnRNPU was obtained with two independent shRNAs (Fig. 5f; Supplementary Fig. 5e). Indeed, increased MALT1A expression after anti-CD3 stimulation was detected after downregulation of hnRNPU (Fig. 5g). In contrast, MALT1B expression was not altered. We asked whether enhanced MALT1A expression might be correlated with a decrease in hnRNPU after T-cell activation. However, on RNA level, the MALT1A suppressor hnRNPU was induced, but at the same time other splicing factors that enhanced MALT1A in the screens (for example, hnRNPR, SRFS3 and SRFS9) were increased (Supplementary Fig. 5f). Thus, TCR-induced alternative splicing of exon7 and the relative expression of MALT1A/B transcripts in CD4+ T cells is regulated by hnRNPU, but alternative MALT1 splicing seems to be regulated by a complex network of negatively and positively acting splicing factors.

MALT1 supports hnRNPs and hnRNPs counteract T-cell activation. To test a potential influence of alternative MALT1 splicing on cellular signalling in T cells, we designed a morpholino oligomer (MO) targeting the exon7-intron7 boundary of the MALT1 pre-mRNA to selectively prevent alternative inclusion of exon7 and thus MALT1A expression (Fig. 6a). MALT1A MO (AMO) suppressed MALT1A mRNA and protein expression in mouse embryonic fibroblasts (MEFs; Supplementary Fig. 6a,b). Also, in murine T cells, AMO treatment abolished anti-CD3- or anti-CD4+/CD28-stimulated induction of MALT1A transcripts without affecting transcript levels of MALT1B (Fig. 6b,c). Supplementary Fig. 6c).

Since freshly isolated CD4+ T cells lack considerable MALT1A expression, no changes in NF-κB signalling and JNK activation were observed in AMO-treated cells stimulated with
anti-CD3/CD28 or P/I (Supplementary Fig. 6d,e). To test whether induction of MALT1A on TCR ligation can augment T-cell signalling, we pretreated CD4+ T cells with anti-CD3 to induce MALT1A expression and restimulated the cells with P/I, taking advantage of the fact that P/I bypasses upstream TCR stimulation that may be altered due to the prestimulation. NF-kB was enhanced after 4 h TCR prestimulation, and the activation of NF-κB and JNK was augmented on secondary P/I restimulation, while the activities of ERK and p38 were unaffected in these experimental conditions (Fig. 6d,e). To test whether MALT1A induction is required to augment NF-κB and JNK signalling on restimulation, we cultured the purified CD4+ T cells in the presence of AMO during anti-CD3 pretreatment to prevent MALT1A induction (Fig. 6b). Again, TCR stimulation augmented
NF-κB activation and slightly enhanced JNK phosphorylation on secondary P/I stimulation in untreated or in cMO-treated T cells (Fig. 6f,g). In contrast, inhibition of MALT1A by AMO severely reduced NF-κB activation and mildly impaired JNK signalling. Both the pathways are activated after AMO treatment to a similar degree as in CD4⁺ T cells without prestimulation. Again, ERK and p38 activation were not affected, revealing that MALT1A induction is selectively affecting NF-κB and JNK signalling. Since JNK activation in Jurkat T cells has been linked to CYLD cleavage by MALT1 paracaspase10, we checked for changes in MALT1 paracaspase activity and CYLD cleavage in primary T cells depending on the induction of MALT1A (Fig. 6h; Supplementary

**Figure 6 | Induction of MALT1A augments T-cell signalling.** (a) Scheme for vivo morpholino (MO)-induced disruption of MALT1A expression. MO is designed to bind the 3'-splice site of exon7/intron7 in MALT1 pre-mRNA to prevent splicosomal recognition. (b,c) CD4⁺ T cells from BALB/c mice were treated with morpholino against MALT1A (AMO), control MO (cMO) or kept untreated for 18 h before anti-CD3 or anti-CD3/CD28 stimulation for 6 h. MALT1 mRNA levels were analysed by qPCR using isoform-specific primers. HMBS served as internal control and relative induction was determined by comparing stimulated to unstimulated cells. (d,e) CD4⁺ T cells from BALB/c mice were pretreated with anti-CD3 for 4 h and afterwards stimulated with P/I for the indicated times. Phosphorylation and degradation of IκBα (d) as well as MAPK activation (e) were analysed by WB. Electrophoretic mobility shift assay (EMSA) was used to detect DNA binding of NF-κB (d). (f-h) Untreated, AMO- or cMO-treated CD4⁺ T cells were pretreated with anti-CD3 (4 h) before stimulation with P/I. (f,g) NF-κB and MAPK signalling were analysed by EMSA and WB. NF-κB signal was quantified relative to OCT1 control. (h) CYLD cleavage was monitored by WB. Data are representative of two (h) or three independent experiments (b–g). In b and c, the mean ± s.d. (n = 3) is depicted.
Inhibition of MALT1A induction did not alter MALT1 protease activation. CYLD cleavage was reduced after anti-CD3 pretreatment, but morpholino treatment did not alter CYLD cleavage after P/I restimulation, revealing that augmented JNK signalling is not caused by enhanced MALT1 activity.

In Malt1−/− T cells, expression of MALT1A promoted a more robust T-cell signalling and IL-2 production when compared with MALT1B (Figs 2 and 3). To test whether MALT1A induction is also required for robust T-cell activation, AMO- and cMO-treated CD4⁺ T cells were stimulated with anti-

Fig. 6f).
CD3/CD28 for 6 h and surface expression of activation markers CD25 and CD69 as well as induction of IL-2 were examined. Indeed, prevention of MALT1A induction led to reduced surface expression of CD25 and CD69 (Fig. 7a,b). Further, induction of IL-2 mRNA as well as the number of IL-2-expressing cells were decreased in AMO-treated CD4⁺ T cells (Fig. 7c-e), reflecting that TCR-triggered induction of MALT1A is necessary to promote optimal T-cell activation.

To provide evidence that alternative splicing and MALT1A induction is involved in augmented T-cell activation, we determined the effects of knocking down the splicing factor hnRNP U that counteracts inducible MALT1A production in CD4⁺ T cells (Fig. 5g). Indeed, CD25 surface expression was significantly enhanced in GFP⁺ cells containing the adenosival hnRNP U shRNA, but not in the GFP⁻ cells (Fig. 7f-h). The weaker differences in CD69 upregulation after hnRNP U knockdown is most likely due to the rapid localization of intracellular CD69 at the cell surface on T-cell activation 26. We sorted GFP expressing CD4⁺ T cells to determine IL-2 expression on mRNA and protein level. Again, sh-hnRNP U augmented IL-2 induction and enhanced the number of IL-2-expressing cells (Fig. 7i,j). Taken together, prevention or enhancement of MALT1A induction by morpholinos or sh-hnRNP U, respectively, exerted opposing effects on T cell activation, suggesting that MALT1 alternative splicing controls relative MALT1A and MALT1B levels and thus functions as a molecular switch to tune T-cell activation.

### Discussion

MALT1 paracaspase controls adaptive and innate immune signalling pathways and is a key regulator of T-cell activation 27. Despite the fact that two conserved MALT1 splice variants exist in mammals neither expression nor the functional relevance of these MALT1 isoforms has been explored. Here we show that MALT1 is prone to alternative splicing. While murine CD4⁺ T cells express almost exclusively the shorter isoform MALT1B, the longer exon7 containing variant MALT1A is induced on TCR stimulation. Lower MALT1A expression and induction of MALT1A after T-cell stimulation was also observed in human CD4⁺ T cells from peripheral blood. We find differences in the levels of MALT1A/B in human and murine cells, but the different sources, purification protocols and stimulatory conditions preclude a quantitative comparison. We identified the RNA-binding protein hnRNP U as a negative regulator of exon7 inclusion in human Jurkat and murine primary T cells and we show that MALT1A upregulation enhances T-cell signalling and activation. Despite the induction of MALT1A, MALT1B remained the more abundant isoform, suggesting that not the absolute amounts, but the shifted balance in MALT1A versus MALT1B expression is defining downstream effects. The relevance of MALT1 alternative splicing is also underscored by the observation that MALT1A transcripts are elevated in vivo in T cells with a more activated phenotype such as effector memory T cells and T follicular cells.

Alternative splicing is an important mechanism regulating cell function during the immune response 28. Global analysis of splicing events using exon array demonstrated extensive alternative exon usage in many immune regulators during T-cell activation 29. Alternative exon7 usage of MALT1 in T cells was never reported using exon arrays, most likely because the short exon7 cannot be detected in global analyses. TCR-induced inclusion of exon7 generates the hyperactive MALT1A isoform that provides an additional and functional TRAF6-binding motif that boosts NF-κB activation and slightly enhances JNK signalling in primary T cells. Also, in Jurkat T cells MALT1A was slightly more effective in triggering downstream signalling, but interestingly deletion of exon7 in MALT1B exerted a more severe effect than point mutation of T6BM1, suggesting that the role of exon7 may not be restricted to TRAF6 recruitment. Further, whereas in Jurkat T cells one of two T6BM1s was largely sufficient to trigger full-NF-κB signalling, decreased signalling was seen after mutation of individual sites in CD4⁺ T cells. Thus, modulation of signalling strength by alternative MALT1 splicing seems to be more important in primary T cells, presumably to enhance weaker TCR signals. Constitutively expressed MALT1A seems to function as a molecular rheostat that limits the signalling strength in naive CD4⁺ T cells and MALT1A induction in activated T cells is required to overcome a threshold and to achieve a full response. Thus, we propose that TCR-induced MALT1 splicing is part of a positive feed-forward mechanism that is tuning T-cell signalling to allow productive T-cell activation.

MALT1 acts as a molecular scaffold protein to recruit the IKK complex to the CBM complex 30, but it is also a protease which is activated on T-cell stimulation 31,32. Since exon7 contains a functional TRAF6-binding site and TRAF6-dependent MALT1 ubiquitination drives NF-κB signalling, the data suggest that exon7 inclusion augments MALT1 scaffolding. The E3 ligase TRAF6 can catalyse MALT1 poly-ubiquitination and trigger IKK activation 33,34, but its role in T-cell activation has been questioned as TRAF6 ablation does not significantly affect TCR signalling to NF-κB and MAPK 35,36. Nevertheless, our mutagenesis underscores an essential requirement of the TRAF6-binding motifs for MALT1 scaffolding function, suggesting that another E3 ligase may compensate for the loss of TRAF6. Clearly, inclusion of exon7 in MALT1A does not enhance protease function, which is in line with the results obtained by comparing Malt1⁺/− and MALT1 paracaspase mutant (Malt1PM) mice 21-23,30. CD4⁺ T cells from both Malt1⁻/− and Malt1PM mice are unable to differentiate into Tfh cells 32,34. Congruently, we show in reconstitution experiments in vitro that catalytically inactive
MALT1 does not promote Th17 development, but both MALT1 splice variants support Th17 differentiation. Further, our data indicate that even though Th11 differentiation is not completely relying on MALT1, the catalytic activity augments Th11 development. Thus, differentiation processes are apparently primarily driven by MALT1 protease function, which is required to remove the critical post-transcriptional regulators Roquin and Regnase-1 that counteract upregulation of Th11 differentiation factors such as Ifi6 or Icn5. In contrast, proteolytic activity of MALT1 is largely dispensable for direct downstream signalling to NF-κB and JNK2,21,22,30, providing further evidence that the differential effects of MALT1 splice variants are caused by the scaffolding function. JNK activation has been connected to MALT1-catalysed CYLD cleavage. However, MALT1 activity and CYLD cleavage were not affected by blocking MALT1 induction and JNK activation was also not impaired in T cells from Malt1/mice, revealing that JNK signalling in naive CD4⁺ T cells is not relying on MALT1 protease activity. Thus, biochemical and functional evidence demonstrates that alternative splicing modulates MALT1 scaffolding function, but not protease activity.

We identified several RNA-binding factors that influence splicing of MALT1 by either promoting inclusion (for example, hnRNP R, hnRNP LL and SRSF3) or skipping (for example, hnRNP H1, hnRNP A1, U2AF1 and hnRNP U) of exon7. Together with a minigene approach, the results indicate that MALT1 exon7 splicing is controlled by a complex machinery of positive and negative trans-acting factors as well as cis-acting RNA elements. In the screen, we identified hnRNP U as the most potent negative regulator of exon7 inclusion in Jurkat T cells. hnRNP U knockdown enhanced TCR-induced upregulation of MALT1A and T-cell activation, supporting the conclusion that alternative splicing of MALT1 modulates T-cell responses. hnRNP U is able to bind DNA and RNA, and it has been implicated in a variety of biological processes. Recent genome-wide studies revealed that hnRNP U is an important regulator of alternative splicing34,35. hnRNP U can promote exon skipping or inclusion in a highly context-dependent manner and it can act in conjunction with other splicing regulators. Since the initial search already indicated that multiple RNA-binding proteins impact on alternative MALT1 splicing, it seems reasonable to assume that MALT1A and MALT1B levels will be regulated by a network of splicing factors. TCR stimulation induces the expression of hnRNP U, but at the same time putative MALT1A promoting splice factors such as hnRNP R, SRSF3 and SRSF9 are increased, suggesting that expression and competition of a complex network of positive and negative splice factors may control MALT1 splicing. Clearly, a more detailed understanding about these factors and their cis-acting elements is required to elucidate the mechanism how TCR signalling impacts on MALT1 splicing. hnRNP U was suggested to regulate splice site selection either by modulating the core splicing machinery or by directly targeting the pre-mRNA. The processes are not mutually exclusive and future studies must determine the exact mechanism how hnRNP U regulates exon7 splicing of MALT1. Little is known how cellular signalling pathways influence splicing. AKT signalling was shown to control hnRNP U- and hnRNP L-dependent Caspase-9 splicing. Recently, JNK signalling was shown to induce alternative splicing of multiple targets including the MAP kinase kinase 7 (MKK7) upon T-cell activation. It will be interesting to unravel if similar molecular mechanisms link TCR stimulation to MALT1 splicing.

We show that alternative MALT1 splicing impacts on signalling strength and induction of the hyperactive splice variant MALT1A pushes more robust T-cell activation. Such positive feed-forward regulation may facilitate to surpass thresholds for cellular decisions and they even can place on the single-cell level. Thus, the relative MALT1A and MALT1B expression may trigger different programs in individual cells. Interestingly, intrinsic characteristics of T-cell signalling determine the fate of naive CD4⁺ T cells. Strong TCR signalling favours Th1 over Th11 development, but the underlying molecular mechanisms that translates the magnitude of TCR signalling into a response remains elusive. We find that induced MALT1 splicing augments TCR signalling to NF-κB and JNK, and that MALT1 expression in vivo is indeed high in Th1 cells. We propose that MALT1 alternative splicing constitutes an early bifurcation point to translate TCR signalling into different T-cell activation states that may favour distinct fate decisions and effector functions in an immune response.

Methods
Murine Malt1PM6 mice were from Jackson laboratory and BALB/c as well as OT-II (C57BL/6Tg(TcrαTcrβ)1222Cbn/Crl) mice were from Charles River. Malt1−/− and Malt1−/− R26:CAG-CARΔ1top-ΔCd4-Cre mice (C57BL/6 genetic background) were described. All experiments were performed using 8–16-week-old (C57BL/6, BALB/c, OT-II) or 10–30-week-old (Malt1−/− and Malt1−/− R26:CAG-CARΔ1top-ΔCd4-Cre) male or female mice. All mice were housed in accordance with established guidelines of the Regional Ethics Committee of Bavaria and animal protocols were approved by local authorities.

Antibodies and reagents. The following antibodies were used for stimulation, fluorescence-activated cell sorting (FACS) staining and western blot and: anti-CD3 (145-2C11), anti-CD28 (37.51), Streptavidin-PE, anti-CXCR5 (RF8822, unconjugated) and anti-NEMO (C036) (both from BD Biosciences); anti-Thy1.1-APC (HS51), anti-IL-2-ITC or anti-IL-2-APC (JES6-5H4), anti-CD25-PE (PC6.5), anti-CD69-APC (HL.2F3), anti-CD2-APC (RPA-2.10), anti-TCR-β-Biotin (H57-597), anti-CD4-PerCP (RM4-5) and anti-CD1-ITC (J43; all from ebioscience); anti-BCL10 (EP6801), biotinylated goat anti-rat IgG, anti-hnRNP U (306) and anti-Tra6 (EP91Y; all from Abcam); anti-CYLD AlexaFluor 647 (MEL-14; from Biolegend); anti-CYLD-AlexaFluor 488 (IM7; Caltag); anti-MALT1 (B12), anti-MALT1 (H00), anti-β-Actin (I-19), anti-p38 (C-20), anti-CYLD (E10) and anti-NEMO (FL-419; IL); anti-CD4 (4G7; all from Santa Cruz Biotechnology); anti-CARM1 (1D2), anti-MALT1 (2494), anti-p-ERK (9101), anti-hnRNP U (9211), anti-TCR-(9255), anti-TCR-(9355), anti-TCR-(9352) (all from Cell-signalling); anti-ERK (422704; Calbiochem); rabbit anti-syrian hamster IgG (dianova) and anti-Flag-M2 (from Sigma-Aldrich); horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch); and rabbit polyclonal antibody was raised against peptide CDJELVMRFVNCLC from MALT1A. For differentiation experiments, the following antibodies, reagents and cytokines were used: anti-CD3 (145-2C11), anti-CD28 (37N), anti-IL-12 (1C7.8), anti-IL-4 (B11B1), anti-IFN-γ (Xmg1.2) and anti-CD63/2 (24G2; produced by V.H. and E. Kremmer); anti-IL-2 (JES6-5H4; Milenyl); anti-CD45.2-APC-Cy7 (30-F11), anti-CD4-PE-Cy7 (GK1.5), anti-IFN-γ-APC (XM12), anti-IL-17A-PE (EbioB7F; all from ebioscience); indol-1 for live/dead-cell viability assays (Life Technologies); recombinant mouse IL-12 (BD Biosciences) and recombinant mouse IL-6 and TGF-β (R&D Systems). The following reagents were used: recombinant human TNF-α (Biomol); Phorbol 12-myristate 13-aceta (PMA/P) and ionomycin (Iono/I; both from Calbiochem); mouse anti-CD3/CD28 coated beads (anti-CD3: 13.2; anti-CD28: 2.4D); anti-p38 (C-20), anti-CYLD (E10) and anti-NEMO (FL-419; IL); anti-CD4 (4G7; all from BD Biosciences); anti-CD8 (53-67.25; anti-CD28: 37.51); anti-TCR-(9255), anti-p-ERK (9101), anti-hnRNP U (9211), anti-TCR-(9255), anti-TCR-(9255) and 26.6 μg ml⁻¹; Life Technologies); human T-Activator CD3/CD28 Dynabeads (Life Technologies); Protein G Sepharose (GE Healthcare); Strep-Tactin Sepharose (IBA); brefeldin-A (Sigma); OVA peptide (aa 323–339; IQAVHAAHAEINGR; Biotrend), LumiGlo reagent (Cell Signalling), vivom-Mo: AMO, GAACCAAGAGGCTTTGCCTTCACTTICA and startCodon (cma), and GACCAAGGAGGCTTTGCCTTCACTTICA ATTATA (both from GeneTools). For siRNA knockdown experiments, the following siRNAs and shRNAs were used: ON-TARGETplus SMARTpool siRNA library (qPCR) and siGENOME SMARTpool siRNA library (radioactive PCR; all from Dharmacon). ON-TARGETplus Non-targeting pool (si-control) and ON-TARGETplus SMARTpool si-hnRNP U were used for further knockdown experiments. For verification of the SMARTpool experiments, the following individual siRNAs were used: siRNA negative control (si-control), si-hnRNP U #1: 5’-ACA GAAAGGGAGGAAGAUAU-3’, si-hnRNP U #2: 5’-GAAGAAGAUGUGAGAUU-5’, si-hnRNP U #3: GAUAUGACUGAUAGAUAAGAUU (all Eurogentec). For adenosinergic knockdown, experiments, shRNA against hnRNP U (sh-hnRNP U #1: 5’-CCATAACTGTGCAGTTGAATT-3’, sh-hnRNP U #2: 5’-CCCTGTTGCTACTACAAGATG-3’, sh-hnRNP U #2: 5’-GGCTGTTGCTACTACAAGATG-3’, sh-hnRNP U #25: 5’-GATCTTCTATGATTGATCCAGCA-3’).
Cell culture and stimulation. Jurkat T cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS and 100 μU/ml penicillin/streptomycin (P/S, Life Technologies). Stimulation of Jurkat T cells was initiated by the addition of Phorbol 12-myristate 13-acetate (PMA) (200 ng/ml) and ionomycin (300 ng/ml) and TNP-α (10 ng/ml). DMEM with 10% FCS, and MEM with 10% FCS were supplemented with 30 μM β-mercaptoethanol (Life Technologies) was used for culture of Phoenix packaging cells. Human embryonic kidney (HEK) 293 cells and MEFs were cultured in MEM with 10% FCS, 100 μU/ml P/S, 1% FBS. For serological reconstitution experiments, CD4+ T cells from spleen and lymph nodes were isolated using mouse CD4+ T-cell-specific Dynabeads (Life Technologies). Differentiation experiments were performed using the naive CD4+ T-cell isolation kit (Miltenyi). For other experiments, isolation of murine CD4+ T cells was performed by negative magnetic-activated cell sorting (MACS) selection using the CD4+ T-cell isolation kit II (Miltenyi). Primary T cells were cultured in RPMI medium supplemented with 10% heat-inactivated FCS, 1% P/S, 1% NEAA (Life Technologies), 1% HEPES, 1% L-glutamine, 1% sodium pyruvate (Life Technologies), 1% P/S, 1% NEAA (Life Technologies), 1% L-glutamine, and 1% sodium pyruvate (Life Technologies). MEFs were cultured in DMEM with 10% FCS and 100 U/ml penicillin/streptomycin. Written consent and approval by the ethics board was achieved by serial dilutions and was followed by an appropriate expansion medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Life Technologies). To analyse differences in surface marker expression after adenosiviral knockdown, cells (0.1-0.5 × 10^6) were stimulated using anti-CD3 precoated 96-well plates and anti-CD28 (1 μg/ml) and 0.1 μg/ml and 0.1 μg/ml of CD4+ T cells from spleen and lymph nodes were detached and primary virus was collected by three freeze–thaw cycles. Primary virus was amplified in HEK293A cells using an multiplicity of infection (MOI) of 50. CD4+ T cells from Balb/c mice were transduced with GFP control, MALT1A, MALT1B or MALT1A C464A virus using an MOI of 50. CD4+ T cells from R26/CAG-CARA1top-Bsd-cre mice were infected with control- or sh-hnrnp U #1 and #2 (identified above) were purchased from Sirion Biotech and amplified in HEK293A cells (200 μl). Viruses were harvested and titered using a plaque assay. For differentiation experiments and reconstitution, CD4+ T cells from Balb/c mice were transduced with GFP control, MALT1A, MALT1B or MALT1A C464A virus using an MOI of 50. CD4+ T cells from R26/CAG-CARA1top-Bsd-cre mice were infected with control- or sh-hnrnp U virus (MOI of 50). After 5 h of infection, cells were washed twice with PBS, resuspended in primary T-cell medium and rested overnight before staining with GFP. CD4+ T cells were sorted by FACS (BD) and stimulation. Transduction efficiency was determined by FACS. T-cell differentiation experiments were conducted as indicated above.

Cell lysis and IP. For analysis of expression levels or activation of signalling pathways, cells (1×10^5) were lysed in high-salt buffer (20 mM HEPES (pH 7.9), 350 mM NaCl, 20% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM dithiothreitol (DTT), 10 mM sodium fluoride, 8 mM β-glycerophosphate, 300 μM sodium vanadate and protease inhibitor cocktail). For binding studies, cells (1×10^5) were lysed in-co-IP buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, 10 mM sodium fluoride, 8 mM β-glycerophosphate, 300 μM sodium vanadate and protease inhibitor cocktail). Lysate controls were mixed with 500 μl of S80-loading dye and boiled. IP was carried out using Protein G agarose (1 μg/ml) for 2 h at 4°C. After antibody incubation, Protein G Sepharose (20 μl 1:1 suspension) was added and incubated for 1–2 h at 4°C. For Strep-tagged proteins, precipitation was performed using Strep-Tactin Sepharose (30 μl 1:1 suspension) at 4°C. Overnight. Bound complexes were washed with ice-cooled wash buffer (2×10 μl 2× S80-loading dye. Lysates and IPs were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and analysed by western blot.

Western blot. Proteins were transferred onto polyvinylidene difluoride membranes for immunodetection using electrophoretic semi-dry transfer system. After transfer, membranes were blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature and incubated with specific primary antibody (indicated above, dilution 1:1,000 in 1.5% BSA/PBS-T) overnight at 4°C. Membranes were washed in PBS-T before addition of HRP-coupled secondary antibodies (indicated above, 1:7,000 in 0.75% BSA in PBS-T; 1 h, room temperature). HRP was detected by enhanced chemiluminescence using the LumiGlo reagent (Cell Signaling) according to the manufacturer’s instructions. Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 7.

Electrophoretic mobility shift assay. For electrophoretic mobility shift assays, double-stranded NF-κB or OCT1 binding sequences (HEK or OCT1 see reagents) were labelled with [α-32P] dATP using Klenow Fragment (NEB). To monitor DNA binding, whole-cell lysates (3–6 μg) were incubated for 30 min at room temperature with shift buffer (HEPES (pH 7.9; 20 mM), KCl (120 mM), Ficoll (4%), DTT (5 mM), BSA (10 μg) and poly-dI-dC (2 μg), Roche) and radioactive probe (2×10^6 c.p.m.) were separated on a 5% polyacrylamide gel in TBE buffer, vacuum-dried and exposed to autoradiography. To detect NF-κB fold induction, intensities of NF-κB bands were quantified relative to OCT1 signal using ImageJ software. Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 7.

Flow cytometry. For surface protein staining, unspecific binding was blocked with anti-CD16/32 (dilution 1:50) in FACS buffer (PBS with 2% FCS and 0.01% sodium azide) and cells were stained with monoconal antibodies (dilution 1:100). For intracellular IL-2 staining, cells were detached and primary virus was collected by three freeze–thaw cycles. Primary virus was amplified in HEK293A cells using an multiplicity of infection (MOI) of 50 and virus lysate was collected by freeze–thaw cycles after cytophatic effect. For adenosiviral knockdown, adenosiviruses encoding control or sh-hnrnp U #1 and #2 (identified above) were purchased from Sirion Biotech and amplified in HEK293A cells (200 μl). Viruses were harvested and titered using a plaque assay. For differentiation experiments, CD4+ T cells from Balb/c mice were transduced with GFP control, MALT1A, MALT1B or MALT1A C464A virus using an MOI of 50. CD4+ T cells from R26/CAG-CARA1top-Bsd-cre mice were infected with control- or sh-hnrnp U virus (MOI of 50). After 5 h of infection, cells were washed twice with PBS, resuspended in primary T-cell medium and rested overnight before staining with GFP. CD4+ T cells were sorted by FACS (BD) and stimulation. Transduction efficiency was determined by FACS. T-cell differentiation experiments were conducted as indicated above.

Immunization and isolation of different T-cell subsets. For isolation of different T-cell subsets, BALB/c mice were immunized intravenously with ~2×10^6 sheep erythrocytes (Aciia AG, Mörfelden, Germany). After 1 week, the spleens were
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Author contributions

I.M. and R.A.G. did most of the experiments. D.H., T.G., T.S., A.B., J.K., A.O., A.C.E., U.G. and A.G. performed or contributed to specific experiments. M.S.-S., J.R., T.B., V.H. and F.H. provided the reagents, advice, and design and supervision of the experiments. D.K. conceived the project and designed the experiments. I.M., R.A.G. and D.K. wrote the paper. All authors have been reading and approving the manuscript.

Additional information

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