A role for MALT1 activity in Kaposi’s sarcoma-associated herpes virus latency and growth of primary effusion lymphoma

L Bonsignore1, K Passelli1, C Pelzer1, M Perroud1, A Konrad2, M Thurau2, M Stürzl2, L Dai3,4, J Trillo-Tinoco5, L Del Valle5, Z Qin3,4 and M Thome1

INTRODUCTION

Primary effusion lymphoma (PEL) is an incurable malignancy that originates from B cells latently infected with Kaposi’s sarcoma-associated herpes virus (KSHV). Malignant growth of KSHV-infected B cells requires the activity of the transcription factor nuclear factor (NF)-κB, which controls maintenance of viral latency and suppression of the viral lytic program. Here we show that the KSHV proteins K13 and K15 promote NF-κB activation via the protease mucosa-associated lymphoid tissue lymphoma translocation protein-1 (MALT1), a key driver of NF-κB activation in lymphocytes. Inhibition of the MALT1 protease activity induced a switch from the latent to the lytic stage of viral infection, and led to reduced growth and survival of PEL cell lines in vitro and in a xenograft model. These results demonstrate a key role for the proteolytic activity of MALT1 in PEL, and provide a rationale for the pharmacological targeting of MALT1 in PEL therapy.

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Primary effusion lymphoma (PEL) is an incurable malignancy that develops in immunodeficient patients as a consequence of latent infection of B-cells with Kaposi’s sarcoma-associated herpes virus (KSHV). Malignant growth of KSHV-infected B cells requires the activity of the transcription factor nuclear factor (NF)-κB, which controls maintenance of viral latency and suppression of the viral lytic program. Here we show that the KSHV proteins K13 and K15 promote NF-κB activation via the protease mucosa-associated lymphoid tissue lymphoma translocation protein-1 (MALT1), a key driver of NF-κB activation in lymphocytes. Inhibition of the MALT1 protease activity induced a switch from the latent to the lytic stage of viral infection, and led to reduced growth and survival of PEL cell lines in vitro and in a xenograft model. These results demonstrate a key role for the proteolytic activity of MALT1 in PEL, and provide a rationale for the pharmacological targeting of MALT1 in PEL therapy.

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protease that controls antigen receptor-induced NF-κB activation in lymphocytes via both, its scaffold and protease activities. As a scaffold, MALT1 promotes the activation of the IKK complex, most likely via recruitment of the ubiquitin ligase TRAF6\(^\text{18,20}\) and the linear ubiquitination chain assembly complex.\(^\text{21,22}\) The protease activity of MALT1 massively enhances the NF-κB response by the cleavage of A20 and RelB, which negatively regulate the canonical NF-κB pathway in lymphocytes.\(^\text{23,24}\)

MALT1 was originally identified as a proto-oncogene in B-cell lymphomas of the mucosa-associated lymphoid tissue, in which MALT1 is constitutively activated as a result of chromosomal translocations that lead to MALT1 overexpression or formation of an oncogenic apoptosis inhibitor protein-2-MALT1 fusion protein.\(^\text{25}\) Naturally, MALT1 is activated upon antigen triggering of the B-cell receptor (BCR)/CD79 complex. This initiates a signaling cascade that activates Bruton’s tyrosine kinase (BTK) and induces the formation of the CBM signaling complex formed by CARMA1 (also known as CARD11), BCL10 and MALT1, which is required for MALT1 activation.\(^\text{26}\) Constitutive MALT1 activity is observed in diffuse large B-cell lymphomas (DLBCL) of the activated B-cell (ABC) subtype,\(^\text{27,28}\) but whether MALT1 inhibition may also be effective against virally induced activated B-cell (ABC) subtype,\(^\text{27,28}\) which are characterized by observations in diffuse large B-cell lymphomas (DLBCL) of the B-cell receptor (BCR)/CD79 complex. This initiates a signaling cascade that activates Bruton’s tyrosine kinase (BTK) and induces the formation of the CBM signaling complex formed by CARMA1 (also known as CARD11), BCL10 and MALT1, which is required for MALT1 activation.\(^\text{26}\) Constitutive MALT1 activity is observed in diffuse large B-cell lymphomas (DLBCL) of the activated B-cell (ABC) subtype,\(^\text{27,28}\) but whether MALT1 inhibition may also be effective against virally induced tumors with constitutive NF-κB activation has not been explored.

Here, we have identified a key role for MALT1 downstream of the viral NF-κB inducers K13 and K15 of KSHV, and demonstrated that MALT1 activity is critical for viral latency and cellular growth of PEL cells in vitro and in a xenograft model. These findings suggest that therapeutic inhibition of MALT1 may be used as a rational strategy to treat PEL by purging the reservoir of latently infected cells driving this malignancy.

Figure 1. MALT1 activity is required for survival of PEL cell lines. (a) Cell viability analysis of BJAB (GCB DLBCL), HBL-1 (ABC DLBCL), BC-3 and BCBL-1 (PEL) cells treated with 3.125, 6.25, 12.5, 25 or 50 nM of the BTK inhibitor ibritinib for 72 h, with the indicated concentrations of the MALT1 inhibitor thioridazine for 48 h, or with staurosporine (STS) for 3 h. (b) and (c) Western blot analysis of the indicated cell lines to assess expression of BTK (b), or MALT1, BCL10, CARMA1 and cleavage of BCL10 (c). Tubulin blot serves as a loading control. Data are means ± s.d. of duplicate (ibritinib) or triplicate (all others) measurements (a) and representative of two independent experiments (a–c).
KSHV reactivation assays
HEK293T cells (3 × 10^5 cells/well) were seeded in a six-well plate, and infected 24 h later with rKSHV219 which expresses green or red fluorescent protein dependent on the latent or lytic viral state, respectively, in the presence of 8 μg/ml polybrene (S2667, Sigma-Aldrich). On day 2, cells were washed and on day 4, supernatant was harvested and used to infect wild-type HEK293T cells, previously seeded at 1 × 10^5 cells/well in a 12-well plate. On day 7, HEK293T cells were harvested and analyzed by flow cytometry for green fluorescent protein expression.

Cell viability assay
Cells (2.5 × 10^5/ml) were treated with indicated concentrations of thioridazine, staurosporin, ibrutinib or vehicle alone (phosphate-buffered saline or dimethyl sulfoxide) for 48, 3 and 72 h, respectively, and cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Dübendorf, Switzerland) and phenazine methosulfate (PMS, Sigma-Aldrich, at 9 μg/ml), according to the manufacturer’s instructions. Reduction of MTS to formazan was measured at 492 nm with Capture 96 Software (Basel, Switzerland) on a LEDETECT 96 microplate spectrophotometer (Dynamica Scientific, Zürich, Switzerland). Cell viability of ibrutinib-treated cells was assessed using WST-1 assay (Roche Diagnostics) following the manufacturer’s instructions.

Antibodies
Antibodies used for western blotting include anti-BTK (D3H5, Cell Signaling Technology/Bioconcept, Allschwil, Switzerland), anti-BCL10 (H197, Santa Cruz Biotechnology/Labforce AG, Muttenz, Switzerland), anti-CARMA1 (1D12, Cell Signaling Technology/Bioconcept), anti-Tubulin (B-5-1-2, Sigma-Aldrich), anti-Strep-HRP (IBA BioTAGnology/Lucerna-Chem AG, Luzern, Switzerland). Affinity-purified antibodies specific for MALT1 and cleaved BCL10 have been previously described. A monoclonal antibody directed against the Myc epitope (clone 9E10) was kindly provided by R Iggo (Bordeaux, France).

Protein expression vectors
Expression vectors for the 86 myc-tagged ORFs of KSHV in a pcDNA4 background have been previously described. Point mutants of K13 or K15 expression constructs were generated by quick-change PCR using PfuUltra high-fidelity DNA polymerase AD (Agilent Technologies, Morges, Switzerland) and verified by sequencing. Tagged MALT1 and BCL10 expression constructs and the eYFP–Leu-Val-Ser-Arg–eCFP construct have been previously described.

Figure 2. MALT1 inhibition promotes KSHV lytic reactivation. (a and b) RT-PCR analysis of the expression of indicated viral lytic genes in BCBL-1 and BC-3 cells treated with vehicle or 5 or 10 μM thioridazine (a), or transduced with MALT1 silencing or control vectors (b). MALT1 protein expression was assessed by western blot. Tubulin blot serves as a loading control. (c) Assessment of production of infectious virus particles by 293 T cells stably transduced with a silencing vector for MALT1 or a control vector, and infected with rKSHV219. MALT1 protein expression in the initial 293 T cell population was monitored by western blot. Tubulin blot serves as a loading control. Data in (a) and (c) represent means ± s.d. of two (a) or three (c) independent experiments. Data in (b) represent means ± s.d. of triplicate measurements, representative of two independent experiments. ns: not significant; *P < 0.05; **P < 0.01 (unpaired t-test).
Transfection and transduction of cells, gene silencing

Transient transfection of HEK293T cells and lentiviral transduction of HEK293T, BCBL-1 and BC-3 cells were performed using previously described methods.\textsuperscript{34,38} For silencing of MALT1 in HEK293T, BCBL-1 and BC-3 cells, cells were transduced with a lentiviral vector (pAB286.1, a kind gift of R Iggo, Bordeaux, France) containing short hairpin RNA sequences specific for BCL10 (5′-GTAGAGAAAGACCTGAAGA-3′)\textsuperscript{38} or MALT1 (5′-GTCACAAGATTGAGTTC-3′).\textsuperscript{38}

Cell lysis, immunoprecipitation and immunoblot analysis

Cells were lysed in lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors (Complete; Roche Diagnostics) and phosphatase inhibitors (NaF, Na4P2O7 and Na3VO4). For immunoprecipitation, cleared lysates, followed by incubation for 2 h at 4 °C. Beads were washed (Sigma-Aldrich). StrepTactin Sepharose beads (IBA) were then added to lysates were precleared for 60 min with Sepharose 6B beads (Sigma-Aldrich) for 60 min with Sepharose 6B beads (Sigma-Aldrich). StrepTactin Sepharose beads (IBA) were then added to cleared lysates, followed by incubation for 2 h at 4 °C. Beads were washed five times with Triton 1% lysis buffer. Samples were boiled in reducing sodium dodecyl sulfate sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblot as described.\textsuperscript{34}

NF-\textit{kB} luciferase reporter assay

Activation of gene transcription was assessed by transient transfection of HEK293T cells with a NF-\textit{kB} firefly luciferase reporter construct together with a Renilla luciferase vector (pRL-TK). Cells were lysed in passive lysis buffer (Promega) and luciferase activity assessed using dual luciferase assay (Promega) on a TD-20/20 luminometer (Turner Design, Fisher Scientific, Reinach, Switzerland).

Analysis of MALT1 protease activity by flow cytometry

Forster resonance energy transfer-based determination of MALT1 protease activity in HEK293T cells was essentially done as described.\textsuperscript{39} In brief, 293 T cells were transfected with the indicated combinations of expression constructs for MALT1, BCL10, and K13, K15 or the K15 Y481F mutant, and the proportion of cells with MALT1-dependent reporter cleavage was assessed by flow cytometry. Expression of tagged proteins and of MALT1 autophosphorylation was assessed by western blot. Tubulin blot serves as a loading control. Three isoforms are detected for Myc-tagged K13, as previously described.\textsuperscript{16} Asterisk indicates a non-specific band detected by anti-Myc, FL, full-length. (b) 293 T cells were transfected with the indicated combinations of expression vectors for BCL10 and catalytically inactive MALT1 (C464A), together with K13, K15 or the K15 mutant Y481F, and protein expression and monoubiquitination of MALT1 was monitored by western blot (MALT1-Ub, open arrowhead). The relative increase in MALT1 monoubiquitination by the co-transfected K13 or K15 constructs was quantified by ImageJ. Migration of endogenous (endog., gray arrowhead) and overexpressed MALT1 (MALT1, black arrowhead) isoforms are indicated. Tubulin blot serves as a loading control. Data in a represent means ± s.d. of triplicate measurements, and are representative of two independent experiments. Data in b are representative of two independent experiments.

Figure 3. K13 and K15 induce MALT1 protease activity. (a) 293 T cells were transfected with a eYFP-LVSR-eCFP reporter plasmid and the indicated combinations of expression constructs for MALT1, BCL10, and K13, K15 or the K15 Y481F mutant, and the proportion of cells with MALT1-dependent reporter cleavage was assessed by flow cytometry. Expression of tagged proteins and of MALT1 autophosphorylation was assessed by western blot. Tubulin blot serves as a loading control. Three isoforms are detected for Myc-tagged K13, as previously described.\textsuperscript{16} Asterisk indicates a non-specific band detected by anti-Myc, FL, full-length. (b) 293 T cells were transfected with the indicated combinations of expression vectors for BCL10 and catalytically inactive MALT1 (C464A), together with K13, K15 or the K15 mutant Y481F, and protein expression and monoubiquitination of MALT1 was monitored by western blot (MALT1-Ub, open arrowhead). The relative increase in MALT1 monoubiquitination by the co-transfected K13 or K15 constructs was quantified by ImageJ. Migration of endogenous (endog., gray arrowhead) and overexpressed MALT1 (MALT1, black arrowhead) isoforms are indicated. Tubulin blot serves as a loading control. Data in a represent means ± s.d. of triplicate measurements, and are representative of two independent experiments. Data in b are representative of two independent experiments.
HEK293T cells were transfected with the eYFP-Leu-Val-Ser-Arg–eCFP reporter construct, together with indicated combinations of KSHV ORFs, MALT1 and BCL10 expression constructs. After 6 h of transfection, cells were washed and 24 h later resuspended in flow cytometry buffer (2% fetal bovine serum in phosphate-buffered saline) and analyzed with an LSR II flow cytometer (BD Biosciences, Allschwil, Switzerland) containing 405-, 488-, 561- and 640-nm lasers. To measure the eCFP and Forster resonance energy transfer signal, the transfected cells were excited with a standard 450/50 filter for collection of the eCFP fluorescence and a 585/42 filter for Forster resonance energy transfer fluorescence, respectively, and for each sample at least 10,000 highly eYFP+ cells were counted.

PEL xenograft model
Aliquots of 10^7 BCBL-1 cells were diluted in 200 μL sterile phosphate-buffered saline, and 6–8 week-old male non-obese diabetic/severe-combined immunodeficiency mice (Jackson Laboratory, Bar Harbor, ME, USA) received intraperitoneal injections with a single-cell aliquot. The MALT1 inhibitors mepazine or thioridazine (10 mg/kg body weight, respectively), or vehicle alone, was administered using an insulin syringe for intraperitoneal injection. Drug was administered either 24 h or 28 days (allowed to establish tumor expansion) after BCBL-1 injection, 3 times/week. Two experiments, with six mice per group for each experiment, were performed. The PEL expansion in vivo was confirmed by testing the expression of cell-surface markers including CD45, CD138, EMA and intranuclear expression of the viral protein LANA within ascites tumor cells, using IFA and flow cytometry as described previously.41 Weights were recorded weekly as a surrogate measure of tumor progression, and ascites fluid volumes were tabulated for individual mice at the completion of each experiment. Spleen sections were stained by hematoxylin and eosin for identification of tumors infiltrating along vascular channels. Images were collected at ×400 magnification using a Olympus BX61 microscope equipped with a high resolution DP72 camera and CellSense image capture software. All protocols were approved by the Louisiana State University Health Science Center Animal Care and Use Committee in accordance with national guidelines (IACUC, No. 3237).

Image quantification and statistical analysis
Quantification of the relative proportion of monoubiquinated MALT1 in different samples was performed using ImageJ, and signal intensity of monoubiquitinated MALT1 was normalized to the band corresponding to unmodified MALT1. Two-tailed Student’s t-test was used for statistical analysis.

RESULTS
MALT1 activity is required for survival of PEL cell lines
An attractive target for the treatment of ABC DLBCL with chronic BCR signaling is BTK, a kinase that acts downstream of the BCR and upstream of the CBM complex.23 To assess if the survival of PEL cells depends on BTK activity, we treated PEL cell lines BC-3 and BCBL-1 with the BTK inhibitor ibrutinib. As controls, we used HBL-1 cells, which are derived from DLBCL of the ABC type and characterized by constitutive BCR signaling, and BJAB cells, which are derived from

Figure 4. K13 and K15 promote NF-κB activation via MALT1. (a and b) 293 T cells stably transduced with silencing constructs for MALT1 (a), BCL10 (b) or a control vector were transfected with increasing concentrations of K13 or K15 and an NF-κB reporter construct, and NF-κB activation was monitored by luciferase assay. Expression of transfected constructs and silencing efficiency was monitored by western blot. Tubulin expression serves as a loading control. Three isoforms are detected for Myc-tagged K15, as previously described.16 Asterisk indicates a non-specific band detected by anti-Myc. Mock controls for K15 belong to the same experiment in (a) and (b). Data represent means ± s.d. of triplicate measurements, and are representative of two independent experiments.
DLBCL of the GCB subtype in which BCR signaling is not activated. As expected, ibrutinib induced cytotoxicity in HBL-1 cells, but not in BJAB cells. Interestingly, both PEL cell lines were insensitive to BTK inhibition (Figure 1a), and did not express detectable levels of BTK (Figure 1b), whereas BTK expression was easily detectable in the cell lines derived from ABC and GCB DLBCL. Similar results were obtained with two additional PEL cell lines (BC-1 and BCP-1) (Supplementary Figure 1).

Next, we tested whether the CBM complex, and in particular MALT1 activity, might be relevant for PEL cell survival, by treating PEL cell lines with the MALT1 protease inhibitor thioridazine. MALT1 activity, which can be monitored by the cleavage of BCL10 and by MALT1 autoprocessing, was clearly detectable in HBL-1 cells but also, to a lower degree, in the PEL cell lines BC-3 and BCBL-1 (Figure 1c). Treatment with the MALT1 inhibitor thioridazine impaired both BCL10 cleavage and autoprocessing in BCBL-1 cells (Supplementary Figure 2B). Treatment with the MALT1 inhibitor thioridazine impaired both BCL10 cleavage and autoprocessing in BCBL-1 cells (Supplementary Figure 2C). We also found evidence for a weak

**Figure 5.** K13 but not K15 physically interacts with MALT1. (a) 293 T cells were co-transfected with the indicated combinations of tagged expression constructs for MALT1 and BCL10, K13 or K15, and protein expression and binding of K13 or K15 to precipitated MALT1 or BCL10 was monitored by western blot. (b) Schematic representation of different MALT1 constructs used to map the MALT1-K13 interaction (ΔDD-Ig1, deleted of the DD and first Ig; ΔDD-Ig1/Ig2, deleted of the DD and first two Igs; PD, protease domain alone). (c) 293 T cells were co-transfected with the indicated combinations of tagged expression constructs for K13 and MALT1 (ΔDD-Ig1/Ig2; ΔDD-Ig1/Ig2; PD), and protein expression and binding of K13 to precipitated MALT1 constructs was monitored by western blot. Three isoforms are detected for Myc-tagged K15, as previously described. Asterisk indicates a non-specific band detected by anti-Myc. Data in all figure panels are representative of two independent experiments.

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constitutive cleavage of A20, corresponding to previously described bands of ~ 65, 55 and 43 kD that are detectable with various intensities in the PEL cell lines (Supplementary Figure 2D). Surprisingly, the PEL cell lines did not express CARMA1, a scaffold protein that is essential for antigen receptor-induced MALT1 activation (Figure 1c and Supplementary Figure 2A). We also did not detect expression of the CARMA1 homologs CARMA3 (CARD10) and CARD9 (data not shown). Collectively, this suggests that MALT1 activity is essential for the survival of PEL cells, and that MALT1 activation in PEL cells must occur independently of BCR-BTK-CARMA1 signaling.

Inhibition of MALT1 activity promotes KSHV reactivation in PEL cell lines

To assess the mechanism underlying PEL cell death upon MALT1 inhibition with thioridazine (Figure 1a), we next monitored the expression of viral lytic genes in the two PEL cell lines BCBL-1 and BC-3. Upon inhibition of MALT1, we observed an increase in the mRNA expression of viral lytic genes such as ORF57 (MTA), gB and K8.1 in two PEL cell lines (Figure 2a). An even stronger effect was obtained upon MALT1 silencing (Figure 2b), which affects both the scaffold and the catalytic function of MALT1. MALT1 silencing also favored the upregulation of the lytic KSHV program and release of infectious virus particles from 293 T cells infected with a previously described recombinant KSHV (Figure 2c). Thus, MALT1 is constitutively active in PEL cell lines, and inhibition of MALT1 activity compromises viral latency and leads to lytic reactivation of KSHV, which specifically drives PEL cell death.

The latency-associated viral genes K13 and K15 promote MALT1 protease activity.

To identify the mechanisms by which KSHV activates MALT1 in latently infected PEL cells, we screened a validated expression library of 86 ORFs encoded by KSHV for their capacity to induce MALT1 protease activity upon co-expression in 293 T cells. For this screen, we made use of a previously established, Forster resonance energy transfer-based reporter assay for MALT1 activity, which is based on the MALT1-dependent cleavage of an eYFP-LVSR-eCFP reporter construct that can be monitored by flow cytometry (Supplementary Figure 3). Among the most potent KSHV genes that increased MALT1 activity were the KSHV-specific genes K13 and K15 (Supplementary Figure 3), which are known as NF-κB-inducing proteins expressed during viral latency (Supplementary Figure 4). When tested individually, both K13 and K15 promoted MALT1 activation in a dose-dependent manner, as evident from reporter cleavage and induction of MALT1 autoprocessing, which is a consequence of MALT1 activation (Figure 3a). K15-induced MALT1 activation was abolished by mutation of Y481 (Figure 3a), a tyrosine residue that is part of an SH2-binding motif required for K15-mediated NF-κB activation (Supplementary Figure 5A). In contrast, mutation of a previously reported TRAF2/3-binding site in K13, which is also

Figure 6. MALT1 inhibition prevents growth of PEL in a xenograft model. (a) Overview of the time course of the experiment, indicating time points of injection of PEL cells, treatment and schedule of monitoring. (b) Analysis of the overall size of PEL-injected animals and respective spleens under conditions of treatment with vehicle and either the MALT1 inhibitor mepazine (upper panel) or thioridazine (lower panel). (c) Analysis of the tumor-related gain in body weight (g) or ascites volume (ml) of PEL-injected animals under conditions of treatment with vehicle or the MALT1 inhibitors mepazine or thioridazine. Data shown in (b) represent individual animals (n = 5 per group) from one out of two independent experiments. Data in (c) represent means ± s.d. *P < 0.01; **P < 0.001 (unpaired t-test).
K13 and K15-dependent NF-κB activation is impaired upon MALT1 silencing

Next, we tested whether K13 and K15 promote NF-κB gene transcription via MALT1 and possibly via its binding partner BCL10 that is required for MALT1 activation in lymphocytes. Silencing of MALT1 (Figure 4a) or BCL10 (Figure 4b) partially affected both K13- and K15-induced NF-κB activation. Moreover, K13- or K15-induced NF-κB activation was strongly increased by co-expression of wild-type MALT1, but much less so by the catalytically inactive C464A mutant of MALT1 (Supplementary Figure 6). In addition, we have assessed the effect of the MALT1 inhibitor thioridazine on the expression of the NF-κB target genes cFLIP and c-IAP1, and found that MALT1 inhibition led to a reduced expression of these target genes and a correlating increase in caspase-3 activation (Supplementary Figure 6C). K13 most likely promoted NF-κB activation by direct or indirect physical recruitment of MALT1, as K13 interacted with MALT1 but not with BCL10 upon co-expression of the proteins in 293 T cells (Figure 5a). Under similar conditions, no physical interaction of K15 with MALT1 or BCL10 was detectable (Figure 5a). Using various MALT1 constructs deleted in individual domains (Figure 5b), we then mapped the region of MALT1 that interacted with K13. This revealed that the DD and the two N-terminal Ig domains of MALT1 were not necessary for the interaction, whereas the protease domain was both required and sufficient for the interaction with K13 (Figure 5c). Thus, K13 probably activates MALT1 and consequently NF-κB via binding to its protease domain, either directly or via an additional binding partner. K15 most likely activates MALT1 indirectly, via the Y481-dependent recruitment of additional signaling proteins.

MALT1 inhibitors prevent the growth of PEL in a xenograft model

To test whether MALT1 inhibition could be of interest for the treatment of PEL, we subsequently assessed the effect of two small molecule inhibitors of MALT1 on the growth of BCBL-1 cells in a xenograft model. BCBL-1 cells were injected...
intraperitoneally into non-obese diabetic/severe-combined immunodeficiency mice, and 24 h later, we started treatment of the mice with a 3 times per week intraperitoneal injection of the MALT1 inhibitors mepazine or thioridazine30 (both at 10 mg/kg) for 4 weeks. Establishment of PEL was monitored by measuring tumor-related gain in body weight, spleen size and ascites volume at the indicated time points (Figure 6a). Compared with mice treated with vehicle alone, mepazine- or thioridazine-treated mice showed an almost complete inhibition of tumor-related gain in body weight and spleen enlargement (Figures 6b and c), and of ascites formation (Figure 6c) measured after 4 weeks of treatment. Hematoxylin and eosin staining enabled us to observe a great amount of tumor infiltration into the spleen of vehicle-treated mice, whereas only small tumor nodules were dispersed in the spleen of mepazine- or thioridazine-treated mice (Supplementary Figure 7). Thus, MALT1 inhibition is highly efficient in preventing the development of xenografted PEL in vivo.

MALT1 inhibitors efficiently induce regression of established PEL tumors in vivo

Next, we assessed whether MALT1 inhibition was also efficient in mice with already established PEL. To test this, mice were inoculated with BCBL-1 cells at day 0, and treatment with vehicle, mepazine or thioridazine was initiated only after 28 days, when the mice had already well established PEL. Tumor-related gain in body weight, spleen size and ascites volume were monitored at the indicated time points (Figure 7a). Compared with mice treated with vehicle only, mepazine- or thioridazine-treated animals showed a significant reduction in tumor-related spleen enlargement and gain in body weight (Figures 7b and c), and a complete loss of measurable ascites volume (Figure 7c) upon 21 days of treatment. Therefore, we conclude that MALT1 inhibition by mepazine or thioridazine is highly efficient not only in preventing the establishment of PEL expansion, but also in promoting the regression of established PEL in this xenograft model.

DISCUSSION

In this study, we provide several lines of evidence for an essential role of the paracaspase MALT1 in the malignant growth of latently KSHV-infected PEL cells. First, we found that MALT1 was constitutively active in PEL cell lines in the absence of BCR/BTK/CARMA1 signaling. Second, MALT1 inhibition with a small molecule inhibitor induced the viral lytic program and impaired cell viability. Third, we showed that MALT1 expression and activity contributes to K13- and K15-driven NF-κB activation, which controls the growth and survival of latently KSHV-infected B cells and development of PEL. Finally, MALT1 inhibition prevented the development of xenografted PEL in vivo, and led to efficient regression of established PEL in this xenograft model. Collectively, these findings identify MALT1 as a key driver of KSHV latency, and as a potentially interesting target for the treatment of PEL (Figure 8).

Among the latent KSHV genes previously demonstrated to have a capacity to activate NF-κB, K13 and K15 were able to potently promote MALT1 activation. K13 directly or indirectly interacted with MALT1 and in particular its protease domain, and may thus induce a conformational change in the protease domain that promotes MALT1 activation, for example, by rendering it accessible to monoubiquitination. The transmembrane protein K15 seemed to use a distinct mechanism that required Y481 in its cytoplasmic SH2-binding motif, and thus most likely mediates MALT1 activation by the recruitment of an SH2 domain-containing protein that remains to be identified (Figure 8). Both K13 and K15 have previously been described to activate NF-κB via IKK components,17,19 and MALT1 has been proposed to physically recruit and activate the IKK complex.20,47 Whether MALT1 and IKK

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Model summarizing the proposed role of MALT1 in K13- and K15-mediated NF-κB activation and maintenance of viral latency. K13 promotes MALT1 activation by a direct or indirect interaction with its protease domain, whereas K15-dependent MALT1 activation may depend on the recruitment of an unknown signaling protein through Y481 in its cytoplasmic domain. Whether MALT1 and IKK components are part of the same or distinct K13-/K15-induced signaling complexes, and whether IKK- and MALT1-dependent NF-κB activation by K13 and K15 happens in a simultaneous or sequential manner remains unclear. Treatment of PEL cell lines with MALT1 inhibitors induces a switch from the latent to the lytic state of viral infection by impairment of MALT1-dependent NF-κB activation, which promotes lytic cell death of primary effusion lymphoma cells.
components are part of the same or distinct K13–K15-induced signaling complexes, and whether IKK- and MALT1-dependent NF-kB activation by K13 and K15 happens in a simultaneous or sequential manner will have to be investigated in future studies.

Several different types of lymphomas are driven by oncogenic activation of the NF-kB pathway; signaling components driving NF-kB activation in tumor cells have thus gained attention as rational drug targets in lymphomas. A lymphoma type with constitutive MALT1-dependent NF-kB activation is the ABC DLBCL, in which MALT1 is activated by chronic BCR-BTK-CARMA1 signaling. The majority of ABC DLBCL cell lines therefore respond to inhibition of BCR signaling by the BTK inhibitor ibrutinib. In contrast, PEL cell lines lack significant surface BCR expression, and are resistant to treatment with Dasatinib, which inhibits upstream tyrosine kinase activity. This suggests that NF-kB activation in PEL does not rely on early BCR-signaling events, but rather on K13- and K15-mediated NF-kB activation that occurs in a BTK- and CARMA1-independent, but MALT1- and IKK-dependent manner. Inhibition of NF-kB activation by the IKK inhibitor Bay-11-7082 efficiently inhibits the development of xenografted PEL in vivo. Drugs targeting IKK-dependent NF-kB activation directly (such as IKK inhibitors) or indirectly (such as proteasome inhibitors) are under development or used in preclinical trials for PEL treatment, but adverse side-effects of these drugs due to systemic NF-kB inhibition in all cell types and/or NF-kB independent effects of proteasome inhibitors remain a concern. MALT1 activity, which appears to be important mainly for immune cells, might thus be a rational alternative target for the treatment of PEL. Possible adverse side-effects of long-term MALT1 inhibition include compromised regulatory T cells responses and autoimmunity as MALT1 is required during embryonic development for the generation of regulatory T cells. However, such side-effects have thus far not been reported during short-term treatments with MALT1 inhibitors. Our data using PEL cell lines and a xenograft model suggest that MALT1 inhibition may be efficient in purging the reservoir of latently infected PEL cells, promoting viral lytic replication. Therapeutic MALT1 inhibition should thus be combined with an anti-herpes virus drug that limits viral replication and spreading, such as the nucleoside analog ganciclovir.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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