MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NF-κB dependent proliferative advantage and resistance against FAS-induced cell death in B cells

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Abstract

The most frequent recurring translocations in MALT B-cell Non-Hodgkin’s lymphoma, t(11;18)(q21;q21) and t(14;18)(q32;q21), lead to formation of an *API2-MALT1* fusion or *IgH*-mediated *MALT1* over-expression. Various approaches have implicated these proteins in NF-κB signaling, but this has not been shown experimentally in human B cells. Immunohistochemistry showed that MALT1 is predominantly expressed in normal and malignant germinal center B-cells, corresponding to the differentiation stage of MALT lymphoma. We expressed MALT1 and API2-MALT1 in human B-cell lymphoma BJAB cells, and found both transgenes in membrane lipid rafts along with endogenous MALT1 and two binding partners involved in NF-κB signaling, BCL10 and CARMA1. API2-MALT1 and exogenous MALT1 increased constitutive NF-κB activity and enhanced IKK activation induced by CD40 stimulation. Both transgenes protected BJAB cells from FAS-induced death, consistent with increases in NF-κB cytoprotective target gene expression, and increased their proliferation rate. Expression of a dominant-negative IκBα mutant showed that these survival and proliferative advantages are dependent on elevated constitutive NF-κB activity. Our findings support a model in which NF-κB signaling, once activated in a CD40-dependent immune response, is maintained and enhanced through deregulation of MALT1 or formation of an API2-MALT1 fusion.
Introduction

Four recurrent chromosomal translocations have been described in non-Hodgkin’s B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT) type. Two of them, t(14;18)(q32;q21) and t(11;18)(q21;q21), are found in 30-50% of extranodal MALT lymphomas. The former juxtaposes the \textit{MALT1 (MLT1 or paracaspase 1)} gene to the IgH promoter region, resulting in the deregulation of MALT1 expression, while the latter creates a fusion between MALT1 and the inhibitor of apoptosis gene \textit{API2 (cIAP2/HIAP1)}.\textsuperscript{1-6} Unlike the low grade indolent MALT lymphomas which are dependent on the continuous presence of \textit{Helicobacter pylori}, MALT lymphomas with translocations targeting the \textit{MALT1} gene can be found in cases without \textit{H. pylori} and are refractory to treatment against \textit{H.pylori}.\textsuperscript{7-8} The other two much rarer translocations, t(1;14)(p22;q32) and t(1;2)(p22;p12), target the \textit{BCL10} gene on the short arm of chromosome 1.\textsuperscript{9,10}

MALT1 was independently identified as a member of the human paracaspase family and an interacting partner of BCL10.\textsuperscript{11} \textit{In vitro}, MALT1 synergizes with BCL10 to enhance NF-\kappaB activation\textsuperscript{12}, and the association of the two proteins has been shown to mediate IKK activation by facilitating the ubiquitylation of the NEMO/IKK\gamma sub-unit by the ubiquitin-conjugating enzyme UBC13.\textsuperscript{13} BCL10 also interacts with a group of proteins that contain an N-terminal CARD domain and show overall structural homology to MAGUK (membrane associated guanylate kinase) proteins.\textsuperscript{14-17} These proteins were subsequently termed CARMA (CARD containing MAGUK) proteins, of which CARMA1 (CARD11) expression is predominantly lymphoid-specific. It has been shown recently that MALT1 not only interacts with BCL10 directly, but also associates with CARMA1, suggesting that a complex containing MALT1, CARMA1 and BCL10 plays an important role in lymphoid cell signaling.\textsuperscript{18} The critical function of MALT1 in lymphocyte signaling
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has been revealed by the analyses of MALT1-deficient mice\textsuperscript{19,20}, whose T cells fail to proliferate in response to CD3/CD28 co-stimulation or to PMA and ionomycin. Similarly, B cells of \textit{MALT1}\textsuperscript{-}\textsuperscript{-} mice fail to proliferate in response to IgM, CD40 or LPS stimulation. The lymphoid compartments of these mice showed severe reduction of marginal zone B cells and a lack of germinal center formation in spleens, as well as deregulated maturation of T cell subsets in the thymus. The phenotype of \textit{MALT1}\textsuperscript{-}\textsuperscript{-} mice closely resembles that of mice lacking \textit{Bcl10}, in particular with respect to the lack of marginal zone B cells, and in the lack of response to T and B cell stimuli leading to NF-κB activation.\textsuperscript{21,22} Consistently, phenotypes of mice lacking \textit{CARMA1} resemble those of \textit{MALT1}\textsuperscript{-}\textsuperscript{-} and \textit{Bcl10}\textsuperscript{-}\textsuperscript{-} deficient mice.\textsuperscript{23-26} These observations confirm the \textit{in vitro} data that MALT1 and CARMA1 both interact with BCL10\textsuperscript{12,13,27} and show that this ternary complex is critical in B- and T-cell stimulation leading to NF-κB activation and downstream immunological responses.\textsuperscript{28}

The above evidence strongly suggests that translocations involving BCL10 and MALT1 lead to elevated NF-κB activity in MALT B-cell lymphoma. In addition, it has been proposed that chronic antigenic stimulation caused by \textit{H. pylori} infection triggers T cell-mediated B cell expansion early in the disease process, \textit{via} activation of the CD40 and NF-κB pathway.\textsuperscript{29,30} We therefore investigated the involvement of MALT1 and its fusion gene product, API2-MALT1, in NF-κB signaling in the presence and absence of CD40 stimulation. Genetic analyses of immunoglobulin gene hypermutation of MALT lymphoma cells suggest that the tumor has a germinal center/post germinal center origin\textsuperscript{31}, and the development of MALT lymphoma may be caused by inhibition of germinal center B cell apoptosis.\textsuperscript{32} Using a germinal center/post germinal center-derived B lymphoma cell line, we examined the effect of stably expressed MALT1 or API2-MALT1
on the NF-κB pathway and on apoptosis induced by FAS (CD95/APO1), which is a mediator of cell death in the germinal center counterbalanced by CD40 signaling.\textsuperscript{32,33} This study is the first report addressing the functional consequence of MALT1 and API2-MALT1 over-expression in human B cell lymphoma cells. Our results support a model of MALT lymphomagenesis in which cytoprotection mediated by NF-κB, once activated in a CD40-dependent immune response (e.g. against \textit{H. pylori}), is maintained and enhanced through deregulation of MALT1 or formation of a API2-MALT1 fusion.

**Material and Methods**

**Immunohistochemical staining**

Formalin-fixed, paraffin-embedded tissue blocks from 65 non-Hodgkin’s B-cell lymphomas and 12 normal lymphoid tissues were retrieved from the archives of the Department of Pathology at Geneva University Hospital. Lymphoid tumors were classified according to the Revised European-American classification of lymphoid neoplasms. After deparaffinization, sections were incubated for 10 minutes in methanol plus peroxide at room temperature, followed by washing with phosphate-buffered saline solution. Slides were heated in citrate buffer using a pressure cooker for 3 minutes. Incubation with the primary antibody, monoclonal αMALT1 antibody directed to the C terminal part of MALT1, was carried out for 1 hour at room temperature.\textsuperscript{5} Immunoreactivity detection was done by using the Envision system and visualized by the avidin-biotin-peroxidase complex (DAKO).

**Cell lines and retroviral constructs**
EBV-negative BJAB human B cell lymphoma cell lines were grown in RPMI-1640 with 10% fetal bovine serum. The human high-grade B-cell lymphoma cell lines, SU-DHL-6, OCI-Ly3, and OCI-Ly7 were maintained as described previously. Full length MALT1 or API2-MALT1 joining nucleotides 1-2048 of cIAP2 to nucleotides 814-2475 of MALT1 with c-terminal myc followed by 6 X His epitope tags in pCDNA3.1 vector (Invitrogen) were subcloned into pMSCV-IRES-EGFP retroviral vector. Following viral infection, EGFP-positive cells were selected by FACS-assisted cell sorting. Selected single cell clones were re-infected with empty retroviral vector vIRES-puromycin or vector carrying S32G/S36A mutant of IkBα with N-terminal FLAG epitope tag. A similar retroviral vector (vIRES-hygromycin) carrying MALT1 or API2-MALT1 cDNA and hygromycin resistance was also generated. Infected cells were selected with 3µg/ml puromycin (Sigma) or 500µg/mL hygromycin (Invitrogen). The IkBα-luciferase reporter cDNA was assembled in pBluescript, excising from pIkB-EGFP (Clontech) its Kozak and IkBα sequence (which lacks a stop codon and is followed by a BamHI site) and inserting in frame the luciferase coding region from pGL3 (Promega), and transferred to vIRES-puromycin. The study was performed in accordance with ethical standards of the Helsinki Declaration of the World Medical Association and conformed with Geneva University Hospital ethical committee guidelines at the time of commencing the study.

**Sucrose gradient raft fractionation**

An established protocol for raft isolation was used. Briefly, 50 x 10^6 cells were washed once in cold TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2, and 1 mM EGTA), then lysed in 680µl TKM buffer supplemented with 1% Triton TX-100 and protease inhibitors. This lysate was incubated on ice for 1 hour, before mixing with 820µl
TKM buffer containing 73% (wt/vol) sucrose to give a final volume of 1.5ml in 40% sucrose. This is transferred to a SW41 centrifugation tube, and overlaid with 6 ml TKM containing 35% sucrose and then 3.5 ml TKM containing 5% sucrose. The gradient is centrifuged at 250,000g for 16 hour at 4°C. Following centrifugation, 11 fractions, of 1 ml each, were collected from the top of the centrifugation tube. All fractions were immunoblotted for LYN (Santa Cruz) to confirm the presence of lipid rafts in fractions 3 to 5. The pooled fractions were diluted in TKM and further centrifuged at 250,000g for 2 hours. The pellet containing the Triton-resistant membrane proteins was boiled in denaturing sample buffer, and subjected to SDS-PAGE analysis.

**SDS PAGE and immunoblotting**

30-50µg of protein from cell lysates were analysed by SDS PAGE and immunoblotted with the appropriate antibodies: rabbit polyclonal anti-CARMA1 antibody against the N-terminus of CARMA1 (AL-220, Alexis Biochemicals) and monoclonal anti-BCL10 antibody (A-6, Alexis Biochemicals); rabbit anti human BclXL antibody (A-3535, DAKO); polyclonal antibodies against API2 and actin (H-85, Santa Cruz).

**IKK activity and nuclear NF-κB measurements**

BJAB cells stably infected with vIRES-puromycin vector containing the IκB-luciferase reporter alone or re-infected with vIRES-hygromycin vector carrying either MALT1 or API2-MALT1 cDNA were analyzed. Briefly, triplicates of 150,000 cells per well were either incubated in medium only (controls) or in the presence of 10ng/ml TNFα (R&D Systems), or 40ng/ml PMA with 2µM ionomycin (Sigma), or 0.1µg/ml CD40L (Alexis) cross-linked with 2.5µg/ml M2 anti-FLAG antibody (Sigma) for 4h before analysis. Collected cells were lysed with 50µl of Glo Lysis buffer (Promega) and mixed with 50µl of Bright Glo stable substrate (Promega) and emission was measured in a luminometer.
The values obtained for untreated controls were taken as 100% and the stimulated samples were expressed as percentage of the corresponding controls.

ELISA-based measurement of nuclear NF-κB sub-units, was performed according to the manufacturer's protocol (Active Motif, Rixensart, Belgium). Briefly, 3µg of BJAB nuclear extract was used per sample point in triplicates in the 96-well plate pre-coated with consensus oligonucleotides for all NF-κB sub-units (p50, p65/RelA, RelB, p52 and c-Rel). After washing to remove non-specific binding, specific antibodies to NF-κB sub-units were added. After antibody binding, the plate was washed again before adding a horseradish peroxidase conjugated secondary antibody. The peroxidase substrate was added and colorimetric change was measured at OD450.

**FACS analyses for FAS and CD83 expression**

For FAS or CD83 expression, 5-10 x 10^5 BJAB cells were incubated in 100µl FACS buffer (PBS/1%BSA/0.05% sodium azide) with 1µg/ml 2R2 anti-FAS antibody, (Alexis), the recommended concentration of anti-CD83 antibody (Pharmingen), or the corresponding isotype control IgG (Jackson ImmunoResearch). The cells were washed twice and incubated in 1:700 dilution of PE- or FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch) for 30min. The cells were washed again and re-suspended in 300µl of the same buffer for FACS analyses for the presence of FAS or CD83 using FacSCAN (Becton Dickinson).

**Cytotoxic assays**

The cytotoxic assays were performed using the tetrazolium compound [3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], (MTS) (Promega) and phenazine methosulfate (PMS) (Sigma-Aldrich), as described
previously. Briefly, 50,000 cells in a volume of 100 µl were subjected to serial dilutions of soluble FAS-L (gift of Pascal Schneider, Lausanne, Switzerland). Where stated, CD40L-FLAG (Alexis) cross-linked with anti-FLAG antibody (Sigma) or anti-CD154 monoclonal antibody (Pharmingen) was added to the medium with various concentrations of FAS-L. After overnight incubation, cell viability was determined by incubation with MTS/PMS reagent for 2 h, followed by measuring the absorbance at 490 nm. In experiments with BJAB cells expressing the IκBα super repressor, FAS-L at indicated concentrations was used in triplicate wells under the same experimental conditions.

\( ^{3}\text{H}\)-thymidine incorporation

Exactly 5000 cells per well were plated in triplicates in 96-well culture plate. The cells were incubated for 24 h before pulsing with 0.5 µCi/well \( ^{3}\text{H}\)-thymidine (Amersham) for another 12 h. Cells were harvested with a cell harvester (Inotech), and the cpm counted with a Wallac Trilux scintillation counter (Perkin Elmer).

Quantitative PCR and RNA preparation

Total RNA was isolated from cells with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. To eliminate DNA contamination, the final preparations were treated with RNAsase-free DNAsase (Roche). Reverse transcription of 0.5-1 µg of total RNA was carried out using the cDNA first strand synthesis kit (Roche), and 1 µl of a 100 fold dilution of this reaction was used to proceed real time PCR in iQ™ SYBR Green Supermix (Biorad). mRNA expression was evaluated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis using an iCycler (Biorad). For each target, PCR reactions were performed in triplicate in two independent experiments. The primers used are listed in Table 1. Selected primers for API2 detect
only endogenous *API2* but not the *API-MALT1* fusion, while *MALT1* primers detect all species (endogenous, over-expressed *MALT1* and the *API2-MALT1* fusion). Expression levels of target mRNAs were normalized to the endogenous mRNA levels of *GAPDH* gene (ΔCT method). The thermal cycling conditions for all reactions were 95°C for 10 min followed by 40 cycles of 95°C, 15 sec and 60°C 1 min.

**Results**

**Expression of MALT1 in normal and malignant lymphoid tissues**

To investigate the localization of MALT1 in human normal and neoplastic lymphoid tissues, we performed immunohistochemical studies using a monoclonal antibody against the C terminal part of MALT1. MALT1 is expressed predominantly in B-cells in the germinal center (white pulp) and marginal zone of the spleen, the germinal centre of tonsils, and the medulla of the thymus. In contrast, mantle zone B cells and interfollicular T cells of tonsils express relatively low amounts of MALT1 ([Fig. 1, A-C](#)). We also stained 6 cases of MALT lymphoma, which showed strong staining in infiltrating neoplastic lymphocytes in the gastric mucosa ([Fig. 1D](#)). MALT1 was strongly expressed in large and small follicular cells in all 20 cases of follicular lymphomas examined ([Fig. 1E](#)), and in large B cell blasts in all 20 cases of DLBCL ([Fig. 1F](#)). Overall, these findings are consistent with a germinal center/marginal zone origin for MALT lymphoma, and with deficiencies in marginal zone B cells and germinal centers in MALT1-deficient mice.

**MALT1 and API2-MALT1 are components of lipid rafts of malignant B-cells.**

Detergent-resistant lipid rich microdomains, or rafts, on the plasma membrane are believed to be signaling platforms harboring molecular complexes that are essential
for signal transduction. The in vitro binding partner of MALT1, BCL10, is recruited to rafts by CARMA1 upon T cell receptor stimulation. We therefore examined several B-cell lymphoma cell lines established from DLBCL patients for MALT1 localization. We found that a pool of MALT1 is constitutively present in rafts, together with CARMA1 and BCL10 (Fig. 2A). To study the biological consequence of MALT1 and cIAP-MALT1 expression in B cells, we generated BJAB cells stably expressing MALT1 or API2-MALT1 using retroviral vectors (Fig. 2B). Expression of the transgenes was confirmed in the transfected cell populations and compared with that of the endogenous levels (Fig. 2C). Similar expression levels of exogenous and endogenous MALT1 was observed (Fig. 2C, second panel). The presence of its binding partner, BCL10, was detected in similar levels in transfected and parental BJAB cells (Fig. 2C, lower panel). Individual cell clones were selected expressing either MALT1 or API2-MALT1 and used in some of the following experiments. Like endogenous MALT1 in DLBCL cells, exogenously expressed MALT1 and API2-MALT1 in BJAB cells were also found to be constitutively present in lipid rafts (Fig. 2D).

Effects of exogenous MALT1 and API2-MALT1 on constitutive and CD40-mediated NF-κB activity.

We examined the effect on NF-κB activity of API2-MALT1 or MALT1 overexpression in BJAB cells, using an ELISA-based assay to quantify NF-κB sub-units (p50, p65/RelA, RelB, p52 and c-Rel) in nuclear extracts. In unmodified BJAB cells, there were relatively high amounts of p50 and c-Rel compared to p65 (Fig. 3) and other sub-units (data not shown). After stimulation of CD40, which is known to activate the IKK complex, nuclear c-Rel was relatively unchanged but there were increases in p50 and p65/RelA (Fig. 3A-C). Analysis of unstimulated cells showed that basal NF-κB
activity was increased by API2-MALT1 (p50 and p65/RelA) or exogenous MALT1 (p50 only). The response to CD40 stimulation was also increased by API2-MALT1 (p50, c-Rel, and p65/RelA), and MALT1 produced a modest but significant rise in nuclear c-Rel after CD40 stimulation.

**MALT1 and API2-MALT1 act in the CD40 inducing NF-κB pathway upstream of IKK complex activation.**

To address at which point the transgenes affect the CD40 pathway to NF-κB activation, we created a cell-based assay for IKK activity in BJAB cells by stably expressing an IκBα-luciferase fusion reporter gene. The assay derives from observations that the exogenous IκBα-reporter protein (like endogenous IκB) is degraded as a result of IKK activity, but its transcription (unlike that of endogenous IκB) is relatively independent of NF-κB activity, i.e., its production can be regarded as constitutive. Therefore, the level of the reporter protein (easily measured as luciferase activity) can serve as an indicator of IKK activity, falling as IKK activity increases and vice versa. As expected, BJAB cells stably expressing the reporter alone showed a substantial decline in the reporter level after stimulation by PMA and ionomycin, indicating an acute increase in IKK activity, with less of a response to TNFα (Fig. 3D). BJAB cells also expressing MALT1 or API2-MALT1 showed a similar pattern and degree of response to these agents, indicating that these transgenes had no effect on the respective pathways by which these agents increase IKK activity. In contrast, it was clear that these transgenes further augmented the increase in IKK activity caused by CD40 signaling in BJAB cells. The fact that API2-MALT1 was the more effective transgene, despite similar levels of expression shown by Western blots, is in agreement with NF-κB activation abilities observed in 293T cells (data not shown).
API2-MALT1 and MALT1 expression protect BJAB cells from FAS-mediated apoptosis

The above indicates that MALT and API2-MALT1 can function in the CD40 signaling pathway, which blocks apoptosis induced by FAS (APO1) in B-cell lines and primary cells from the germinal center stage. We found that BJAB cells expressing MALT1 or API2-MALT1 were more resistant to FAS-induced apoptosis than were parental mock-infected cells (Fig. 4A). This difference in FAS sensitivity was not due to differences in expression of FAS, which was essentially the same between the three cell lines (Fig. 4D). WT cells were more resistant to FAS in the presence of CD40 ligand (CD154) (Fig. 4B), approaching the resistance displayed by unstimulated cells expressing either transgene. The specificity of protection caused by CD40L (CD154) on WT cells was further confirmed when the sensitivity to FAS-L shifted back to that of untreated cells in the presence of an anti-CD154 antibody (Fig. 4C).

Constitutive NF-κB activation is critical for MALT1 and API2-MALT1 mediated FAS resistance and proliferation.

It is perhaps surprising that CD154 did not further increase the FAS resistance of cells expressing MALT1 or API2-MALT1 (Fig. 4B), since these cells respond to CD40 stimulation in several other measures, e.g. in the ELISA assay for nuclear sub-units, and are in fact even more responsive than WT cells. However, this finding suggests that FAS resistance imparted by the transgenes is due to their impact on constitutive NF-κB activity, an effect shown by the ELISA assay in the absence of stimulation. To examine this, BJAB cells expressing no transgene, MALT1, or API2-MALT1 were secondarily infected with a "super-repressor" (SR) form of IκBα, bearing mutations in the two serine
phosphorylation sites (S32G, S36A). This mutant cannot be phosphorylated by IKK and subsequently degraded, and acts thus as a dominant negative in the NF-κB pathway.\textsuperscript{36} MALT1- and API2-MALT1-expressing empty vector cells were resistant to FAS-mediated apoptosis even at 2µg/ml FAS-L. In contrast, the corresponding lines co-expressing SR were significantly less resistant, similar to their parental cells (Fig. 5A). In the parental cells, the super-repressor had no effect on sensitivity to FAS-L. Given that all the SR+ lines express similar levels of the super-repressor (Fig. 5D), these results indicate that MALT1- or API2-MALT1- mediated resistance to FAS-induced apoptosis is dependent on their induction of constitutive NF-κB activity.

Because NF-κB activation in malignant lymphomas has been shown to increase proliferation as well as survival,\textsuperscript{41} we examined the effect of MALT1 and API2-MALT1 on proliferation as measured by incorporation of $^3$[H] thymidine into DNA. Both transgenes conferred a modest but significant growth advantage to pools and single clones expressing the transgenes (Fig. 5B). Because this advantage was largely eliminated by the super-repressor IκBα (Fig. 5C), it can be attributed to constitutive NF-κB activation. In contrast, expression of the super-repressor did not affect proliferation of the parental BJAB cells.

**MALT1 and API2-MALT1 specifically enhance CD40 stimulated NF-κB activation**

The effect of the super-repressor (SR) as an effective inhibitor of NF-κB activation in these lines was further demonstrated by measuring surface expression of CD83, a known direct target of NF-κB.\textsuperscript{42,43} Among cell lines infected in parallel with the empty second vector, and in keeping with other results described above, the CD83 increase after 4h of CD40 stimulation was augmented by the transgenes (Fig. 6A), although they did not affect the response to PMA/Ionomycin (Fig. 6B). A similar pattern
of enhancement by transgenes was seen over a spectrum of lower doses and shorter duration of CD40 stimulation, and in experiments using transient transfection of an NF-κB-driven luciferase reporter plasmid (data not shown). In sharp contrast, all SR lines were completely unresponsive to CD40 stimulation (Fig. 6A) i.e. CD83 remained virtually undetectable and lower than in the non-SR lines without stimulation (Fig. 6C). The effect of the transgenes on CD40 signaling was not explained by baseline CD83 levels or levels of surface CD40 itself, which were little affected by the transgenes and only modestly reduced by the super-repressor (Fig. 6D). Furthermore, reduced CD40 was unlikely to be the only effect of the super-repressor on NF-κB activation by the CD40 pathway, since the CD83 response to PMA/ionomycin stimulation was also markedly reduced in SR lines.

**MALT1 and API2-MALT1 enhance expression of distinct cytoprotective NF-κB target genes induced by CD40 signaling**

Many NF-κB target genes protect against apoptotic stimuli in B cells and other cell types; in particular, CD40 signaling increases B-cell survival through NF-κB mediated up-regulation of BclX<sub>L</sub> and BFL1/A1. We therefore compared expression of a series of known anti-apoptotic NF-κB target genes (API1, API2, BclX<sub>L</sub>, hA20, Bfl1/A1, and Bcl2) in BJAB cells expressing MALT1, API2-MALT1 or vector only (Fig. 7A). While resting mRNA levels of API2 and hA20 were significantly higher in MALT1-expressing cells than in others, that of Bcl2 was significantly higher in API2-MALT1-expressing cells, suggesting that there may be differences in how MALT1 and API2-MALT1 produce constitutive NF-κB activation. CD40 stimulation of these lines produced significant elevations in mRNA levels of most targets (Fig. 7B), but BclX<sub>L</sub> rose significantly only in the presence of API2-MALT1 or over-expressed MALT1, confirming the previous
suggestion that both transgenes are involved in CD40 mediated NF-κB activation. The protein levels of BclXL and API2 rose significantly following 24h of CD40 stimulation, consistent with the rise in their mRNA levels (Fig. 7C). In the same experiment, no change due to transgenes and/or CD40 stimulation was found in the mRNA levels of several other known cytoprotective NF-κB target genes (cFLIP, hXIAP, SURVIVIN and Gadd45β, data not shown), showing that these do not mediate the observed changes in FAS resistance in BJAB cells, and that target gene expression is influenced by cell type.

Discussion

It has been known for some time that a small set of recurring chromosomal translocations is associated with MALT B-cell lymphomas, but the functional consequences of these genetic alterations have only recently come to light. Chromosomal translocations t(11;18)(q21;q21) and t(14;18)(q32;q21), found in up to 50% of MALT lymphomas, lead to over-expression of MALT1 or formation of an API2-MALT1 fusion protein.7,8 Although there was reason to believe that these would lead to NF-κB activation in MALT B-cell lymphoma, and immunostains of primary MALT lymphomas have shown evidence of NF-κB activation,45 this has not been shown experimentally in human B cells. Here we demonstrate that both MALT1 and API2-MALT1 confer constitutive NF-κB activity in a human B cell line, resulting in increased proliferation and resistance to FAS-induced apoptosis. Furthermore, both transgenes act upstream of the IKK complex to enhance NF-κB activation induced by CD40. Many lymphoid malignancies have been associated with constitutive NF-κB activation and poor prognosis, and elevated NF-κB activity in MALT B-cell lymphoma would favor malignant B-cell proliferation and survival.41,46-48 Our results are consistent with recent
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findings that NF-κB activation by API2-MALT transforms NIH 3T3 cells and inhibits p53-induced cell death.49

Based on previous studies and our results presented here, we propose the following model for the role of MALT1 and API2-MALT1 in MALT lymphomagenesis (Fig. 8). In early stages, the immune response triggered by *H. pylori* infection induces NF-κB activation in B cells via CD40/CD40L interaction with T cells, increasing the expression of specific cytoprotective genes. The acquisition of recurring chromosomal translocations results in the expression of API2-MALT1 or over-expression of MALT1, producing constitutive NF-κB activation and enhancing the response to CD40 stimulation. Both of these are potentially advantageous to lymphoma cells, but constitutive NF-κB activity would confer independence from the immune response to *H. pylori*, and we have shown that it alone may be sufficient for enhanced proliferation and resistance to FAS-induced apoptosis. Finally, because the API2-MALT1 fusion is itself regulated by NF-κB, a positive feedback loop may be maintained that further supports bacterium- and CD40-independent NF-κB activation, leading to progression of MALT lymphoma.7,8 It should be noted that at least parts of this model may not be restricted to gastric MALT lymphoma or *H. pylori* infection, although those have generated much of the research implicating CD40 and antigen drive in MALT lymphoma. MALT1 translocations are found in tumors from other sites, and recently similar causal associations have been found between *Chlamydia psittaci* and MALT lymphoma of the orbit and between *Campylobacter jejuni* and a MALT lymphoma-like immunoproliferative lesion of the small intestine.50,51

Constitutive NFκB activation by API2-MALT1 or over-expressed MALT1 is a primary feature of the proposed model. Normal mature B cells require some degree of constitutive NF-κB activity for survival, as shown by their disappearance from mice after conditional deletion of NEMO or IKK-beta. 52 Deficiencies in germinal centers and
splenic marginal zone B cells are features of mice with germline inactivation of members of the NF-κB family, as they are of mice with germline inactivation of MALT1, BCL10, or CARMA. Since there is *in vitro* evidence that these proteins can produce IKK activation, it is plausible that they normally function in a common pathway contributing to constitutive NF-κB activity in B cells. Using a human B cell line corresponding to the germinal center stage of B cell differentiation, we have shown that API2-MALT1 or over-expressed MALT1 produces increased constitutive NF-κB activity, resulting in increased proliferation and resistance to FAS-induced apoptosis. These benefits are plausible given that many NF-κB target genes have anti-apoptotic functions, and some (e.g. *cyclin D2*) are associated with cell-cycle progression. However, this may not fully model the consequences of increased constitutive NF-κB activity in MALT lymphoma or other types of lymphoma in which it is found. In particular, while the IκBα super-repressor (IκBα-SR) merely abrogates the effects attributable to API2-MALT or over-expressed MALT1 in BJAB cells, returning them to a state comparable to that of wild-type cells, it is lethal to cell lines from lymphoma types characterized by increased constitutive NF-κB activity. In other words, increased constitutive NF-κB activity is not merely advantageous but an essential feature of the biology of these types of lymphoma; whether this is the case for MALT lymphoma remains unknown.

The mechanisms of constitutive IKK activation by API2-MALT and over-expressed MALT1 may be multifaceted and somewhat different, related to our findings that the two transgenes had both similar and different effects by several measures. *In vitro* studies showed that IKK activation by MALT1 or BCL10 requires both proteins, and this was supported by studies using embryonic fibroblasts from MALT1 knockout mice. Using purified proteins, Sun *et al.* successfully reconstituted the pathway from BCL10 to IKK activation, in which oligomerization of MALT1 by a BCL10/CARMA1/MALT1
complex leads to the recruitment of a ubiquitin ligase (TRAF6), followed by activation of a kinase (TAK1) which activates the IKK complex and NF-κB. In contrast, BCL10 is not required for IKK activation by API2-MALT, and BCL10 was not among several proteins found to bind to API2-MALT in one study. API2-MALT is more stable than MALT1 or API2, because it lacks domains of those proteins which promote proteasomal degradation. Subcellular localization is also likely to be a major factor affecting the function of API2-MALT and MALT1. We found that endogenous MALT1 in several DLBCL cell lines, and exogenously expressed MALT1 and API2-MALT1 in BJAB cells, are constitutively present in lipid raft fractions, together with BCL10 and CARMA1. This is contrary to previous studies using primary T-cells which showed that BCL10 is only recruited into rafts upon TCR stimulation. Studies using cells from DLBCL patients have shown that pre-assembled signaling complexes in rafts, named signalosomes, contain molecules of the CD40 signaling pathway and may be responsible for elevated constitutive NF-κB activity. These observations suggest that MALT1 and API2-MALT1 may be pre-assembled into so-called signalosomes, contributing to their ability to activate IKK constitutively and/or enhance CD40 signaling. However, it is likely that there are additional regulating factors since DLBCL cell lines with similar findings on lipid raft analysis for MALT1, CARMA, and Bcl10 (Fig. 2A) have substantially different levels of constitutive NF-κB activity and a starkly different response to IκBα-SR expression.

A second key feature of our proposed model is that both API2-MALT and over-expressed MALT1 enhance the effect of CD40 stimulation on IKK activation. This is consistent with one study of germline inactivation of MALT1, which found that MALT1 was required for the proliferative response of mouse B cells to CD40 stimulation. CD40 signaling has previously been implicated to play an essential role in MALT lymphomagenesis, and is more significant given our finding that API2-MALT or
MALT1 over-expression can potentiate CD40 signaling to NF-κB. CD40 signaling affects multiple points in the normal B cell response to antigen, including B-T cell interaction and germinal center formation, entry, and clonal selection. \(^{33,58}\) Immunoglobulin gene hypermutation indicates that cells of MALT lymphoma traverse this same process, at least initially, so there are many opportunities for enhanced CD40 signaling to promote survival, although questions remain about B-cell physiology. For example, CD40 signaling can block spontaneous \textit{in vitro} apoptosis of normal human germinal center B cells, which originates from complexes centered around FAS (highly expressed at this differentiation stage). \(^{59}\) However, \textit{lpr} mice with inactivating mutations in FAS do not show a simple reduction in apoptosis of germinal center B cells, but rather more subtle changes in number and autoreactivity of memory cells, implying a more modest role for FAS in germinal center physiology. \(^{60}\) Nonetheless, resistance to FAS-induced apoptosis has been implicated as a factor in MALT lymphomagenesis, in that FAS mutations have been found in some primary tumors, \(^{61}\) and we have shown that FAS killing of BJAB cells is opposed by the constitutive effects of API2-MALT or MALT1 over-expression.

A third key aspect of our model is that since API2 is regulated by NF-κB, \(^{62}\) API2-MALT is still subject to regulation by NF-κB at the API2 locus and has the potential to establish a positive feedback loop, as has previously been speculated. \(^{12}\) This could be critical in enabling gastric MALT lymphoma with t(11;18)(q21;q21) not to require ongoing stimulation by \textit{H. pylori} infection, and would be consistent with the observation that cases with this translocation do not regress with eradication of infection. In contrast, endogenous \textit{MALT1} mRNA levels in BJAB cells were not changed by CD40 stimulation or \(\text{iκBα-SR}\) expression (data not shown), confirming that endogenous MALT1 is not an NF-κB target gene in BJAB cells and cannot be part of a positive feedback loop.
In summary, we have used a B cell line model to provide evidence that MALT1 and its fusion protein API2-MALT1 contribute to MALT lymphomagenesis through constitutive and CD40-stimulated increases in NF-κB activity. To study the roles of MALT1 and its fusion protein API2-MALT1 in lymphomagenesis in vivo, transgenic mouse models over-expressing these proteins would be informative. Due to the role of NF-κB as a common downstream mediator of a large number of signal transduction pathways, its targeted therapeutic inhibition may be difficult to achieve without untoward side effects. Identification and functional characterization of the upstream activators of NF-κB in particular types of lymphoma could provide more specific therapeutic targets. Since germline inactivation of the MALT1 gene in mice seems almost exclusively to affect the lymphoid system, blocking MALT1 function may be associated with controllable side effects.

ACKNOWLEDGEMENTS

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**Fig. 1** Expression of MALT1 in normal human lymphoid tissues.

Sections of normal reactive tonsil (A), spleen (B) and thymus (C) were stained with antibodies against MALT1. The germinal center (GC) of the tonsil, the white pulp (WP) and red pulp (RP) of the spleen, and the medulla (M) and cortex (C) of the thymus are indicated. The same staining was performed with sections of gastric MALT lymphoma (D), follicular lymphoma (E), and diffuse large B cell lymphoma (DLBCL) (F).

**Fig. 2** Constitutive expression of MALT1 in lipid rafts of human B cell lymphoma cells.

(A) Western blotting shows MALT1, as well as CARMA1 and BCL10, to be located in both lipid raft (R) and soluble fractions (S) in DLBCL cell lines separated by sucrose gradient centrifugation. Arrowheads mark the band of the expected full-length size for each protein. The origin of the smaller band on the MALT1 blots, not observed in blots from BJAB cells, is uncertain. The multiple bands of BCL10 represent phosphorylation isoforms as they disappear after phosphatase treatment (data not shown). The partitioning of LYN, a B-cell-specific protein of the Src protein kinase family known to be constitutively present in rafts, confirms effective separation of raft and non-raft protein fractions. (B) Structure of C-terminal myc-tagged MALT1, and API2-MALT1 transgenes and pMSCV retroviral vectors used in the study. Solid arrows show positions of the most common breakpoints found in t(11;18) that gives rise to the API2-MALT1 fusion gene, which is used for all of the following experiments. (C) Lysates from
equivalent cell numbers of pools of BJAB cells transfected with API2-MALT1 or MALT1 were immunoblotted for myc, MALT1, and BCL10. The exogenous API2-MALT1 and MALT1 are detected by anti-myc and with the MALT1 antibody (marked by *). (D) Lipid raft and soluble fractions of lysates from pools of MALT1- and API2-MALT1 expressing BJAB cells were immunoblotted for myc and LYN.

**Fig. 3** Constitutive and CD40-stimulated NF-κB activation in BJAB cells expressing MALT1 or API2-MALT1.

BJAB cells transfected with pMSCV-IRES-EGFP vector alone, or single clones transfected with vector carrying MALT1 or API2-MALT1 were untreated (open bars) or treated with 0.1µg/ml cross-linked CD40L for 4h (filled bars). 2µg of nuclear protein extracts from each cell line was used in an ELISA assay measuring (A) p50, (B) c-Rel and (C) p65/RelA subunits of NF-κB. The results represent triplicates of one of three representative experiments.

(D) Enhanced NF-κB activation by API2-MALT1 and MALT1 upon CD40L but not PMA/ionomycin or TNFα stimulation. BJAB cells expressing IκBα reporter only (WT), or reporter with MALT1 or API2-MALT1, were untreated or treated for 4h with TNFα, PMA/ionomycin, or cross-linked CD40L. Lysates were used in a luciferase assay to measure the amount of exogenous IκBα-luciferase, inversely related to the activity of IKK. Each bar represents the reporter level as a percentage normalized to the corresponding untreated cells. Values are obtained from triplicates of one of several representative experiments.

**Fig. 4** Protection from FAS-induced apoptosis by MALT1 and API2-MALT1.
(A) Parental BJAB cells with \( vIRES-puromycin \) vector alone (WT), or single clones of cells expressing MALT1 or API2-MALT1, were challenged for 16h with serial dilutions of soluble FAS-L, alone or also with (B) 0.1\( \mu \)g/ml cross-linked CD40L or (C) a CD40L neutralizing antibody (anti-CD154). Results shown are representative of 4 different experiments. (D) FACS analyses of FAS expression in the three cell lines.

**Fig. 5** NF-\( \kappa \)B activation by MALT1 and API2-MALT1 is necessary for resistance to FAS-mediated apoptosis and proliferation.

(A) BJAB cells expressing no primary transgene (WT), or expressing MALT1 or API2-MALT1, were secondarily infected with empty vector or I\( \kappa \)B\( \alpha \) super-repressor (SR) and incubated in triplicates with soluble FAS-L. Cell viability was assayed after 16h. The results are representative of 2 independent experiments. (B) Cell proliferation was measured by incorporation of 3[H]-thymidine measured in single clones or pools of BJAB cells expressing no transgene (WT), MALT1 or API2-MALT1. Results were obtained from triplicate wells and are shown as cpm. (C) The same BJAB cells as in panel A with empty second vector (open bars) or I\( \kappa \)B\( \alpha \) super-repressor (solid bars) were assayed for 3[H]-thymidine incorporation as above. The data shown are 1 of 3 independent experiments. (D) Lysates of equal numbers of the above BJAB cells were immunoblotted against the N-terminal FLAG epitope tag of SR-I\( \kappa \)B\( \alpha \) to show specific and equal expression.

**Fig. 6** Effect of MALT1 and API2-MALT1 on NF-\( \kappa \)B activation as measured by CD83 expression.

FACS measurement of surface markers in BJAB lines with no transgene (dotted line), MALT1 (thin line), or API2-MALT1 (bold line), secondarily infected with empty
vector (upper panels) or IκBα super-repressor (lower panels). Histograms show CD83 expression after 4h incubation with crosslinked CD40L (A), PMA+ionomycin (B), or medium alone (C). Histogram (D) shows resting CD40 expression in the 6 lines.

**Fig. 7** Constitutive and CD40-enhanced transcription of cytoprotective genes.

(A) Quantitative PCR of cDNAs from unstimulated BJAB cells with no transgene (closed bars), with API2-MALT1 (open bars) or MALT1 (cross-hatched bars). Relative expression levels were normalized to GAPDH mRNA expression. Standard deviation bars were obtained from 2 independent experiments, each performed in triplicates. (B) In parallel, the same cells above were stimulated with cross-linked CD40L for 4h and total RNA was extracted for quantitative RT-PCR analyses as described. (C) BJAB cells with and without the transgenes were stimulated with CD40 ligand for 0, 4h or 24h, and 30µg of lysate was charged per lane and immunoblotted for BclXₐ and API2.

**Fig. 8** Model of the role of MALT1 and API2-MALT1 in the NF-κB activation pathway leading to MALT lymphoma.

*H. pylori* infection induces a chronic T-cell dependent immune reaction leading to CD40/CD40L interaction. Stimulation of B cells through CD40 activates IKK and NF-κB via a complex containing MALT1, BCL10, and CARMA. Over-expression of MALT1 or expression of API2-MALT1 as a consequence of chromosomal translocations [t(11;18)(q21;q21) or t(14;18)(q32;q21)] results in elevated constitutive and CD40-stimulated IKK activity, increasing transcription of NF-κB target genes and favoring survival and proliferation. In cases with t(11;18)(q21;q21), API2-MALT feeds back
positively to sustain high constitutive NF-κB activity and reduce dependence on *H. pylori* infection.

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Fig. 1

A, B, C, D, E, F
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Fig. 3

A  
\( \text{p50} \)

\[ \begin{array}{cccc}
\text{OD 450} & - & + & - & + \\
\text{WT (EGFP)} & 0.5 & 1.5 & 0.5 & 2.5 \\
\text{MALT1} & 1.0 & 2.0 & 1.0 & 3.0 \\
\text{API2-MALT1} & 2.5 & 3.5 & 2.5 & 4.0 \\
\end{array} \]

B  
\( \text{c-Rel} \)

\[ \begin{array}{cccc}
\text{OD 450} & - & + & - & + \\
\text{WT (EGFP)} & 0.5 & 1.5 & 0.5 & 2.5 \\
\text{MALT1} & 1.0 & 2.0 & 1.0 & 3.0 \\
\text{API2-MALT1} & 2.5 & 3.0 & 2.5 & 4.0 \\
\end{array} \]

C  
\( \text{p65/RelA} \)

\[ \begin{array}{cccc}
\text{OD 450} & - & + & - & + \\
\text{WT (EGFP)} & 0.2 & 0.4 & 0.2 & 0.6 \\
\text{MALT1} & 0.4 & 0.8 & 0.4 & 0.6 \\
\text{API2-MALT1} & 0.6 & 0.8 & 0.6 & 1.0 \\
\end{array} \]
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Fig. 3D
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Fig. 4

A: Cell viability [OD₄₅₀] vs. ng/ml Fas-L for constitutive.

B: Cell viability [OD₄₅₀] vs. ng/ml Fas-L for + CD40L.

C: Cell viability [OD₄₅₀] vs. ng/ml Fas-L for + anti-CD154.

D: Flow cytometry histograms for IgG, WT, MALT1, API2-MALT1.
Fig. 5

A

B

C

D

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Fig. 6

A. CD40 stimulation

B. PMA+ionomycin

C. Control

D. Surface CD40

+ SR

+ SR
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Fig. 7

A  constitutive

B  + CD40L (4h)

C

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Table 1. RT-PCR primers used for quantification of putative cytoprotective NF-κB target genes.

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<td>5'-aat tgc cgt cac cgt tct gc t-3'</td>
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<td>hXAIP1</td>
<td>5'-ggt gtt ttc tca gta gtt ctt acc aga ca-3'</td>
<td>5'-atg cta aat ggt atc cag ggt gc-3'</td>
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MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NF-κB dependent proliferative advantage and resistance against FAS-induced cell death in B cells

Liza Ho, R E Davis, Beatrice Conne, Richard Chappuis, Margaret Berczy, Paulette Mhawech, Louis M Staudt and Juerg Schwaller