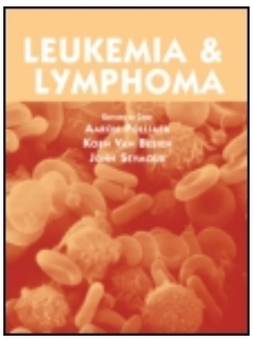

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The potential evasion of immune surveillance in mucosa associated lymphoid tissue lymphoma by DcR2-mediated up-regulation of nuclear factor-κB

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Abstract

This study investigated expression profiles of tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) pathway components and mechanisms underlying TRAIL-induced apoptosis in mucosa associated lymphoid tissue (MALT) lymphoma. Genetic aberrations including translocations and trisomies were assessed by reverse transcription polymerase chain reaction and fluorescence *in situ* hybridization. Expression of TRAIL, death receptors 4 and 5, decoy receptors 1 and 2, and FADD-like interleukin-1β-converting enzyme (FLICE) inhibitory protein was analyzed by immunohistochemistry. All 32 patients under study showed some alterations in TRAIL pathway mainly involving loss of death receptors (37.5%), gain of decoy receptors (3.1%) or both (59.4%). Decoy receptor 2 (DcR2) was highly expressed in patients with normal cytogenetic status as compared to those with cytogenetic aberrations ($p = 0.005$). Moreover, DcR2 expression correlated significantly with nuclear factor-κB (NF-κB) expression ($R = 0.372$, $p = 0.047$). High expression of DcR2 in patients with normal cytogenetic status and its significant correlation with NF-κB expression provides a potential clue to evasion of immune surveillance in cytogenetically normal MALT lymphomas.

Keywords: MALT lymphoma, TRAIL, NF-κB, DcR2

Introduction

Extranodal marginal zone B-cell lymphoma of the mucosa associated lymphoid tissue (MALT lymphoma) is a distinct lymphoma entity and accounts for roughly 7% of all newly diagnosed lymphomas. In terms of pathogenesis, MALT lymphoma predominantly arises in mucosal sites triggered by chronic antigenic stimulation, as exemplified by *Helicobacter pylori* (HP) infection in gastric MALT lymphoma [1] or autoimmune conditions including Sjögren syndrome or chronic autoimmune thyroiditis in both gastric as well as extragastric MALT lymphoma [1–3]. The cell of origin is thought to be the marginal zone B-cell, which is a relatively mature step and is thought to precede the plasma cell in the course of B-cell development. The marginal zone is fairly heterogeneous regarding the B-cell population, with a predominance of monoclonal plasma cells, a feature commonly termed plasmacytic differentiation or PCD [1]. In fact, up to 30% of MALT lymphomas show monoclonal immunoglobulin production, and this feature of PCD is also repeatedly found in (mostly extragastric) MALT lymphomas, suggesting a relationship with multiple myeloma [1–3].

The understanding of the biology of MALT lymphoma has significantly improved, in part through the application of standard cytogenetic analyses. The most common translocations identified in MALT lymphomas include t(11;18)/*BIRC3–MALT1*, t(1;14)/*IGH–BCL10* and t(14;18)/*IGH–MALT1*. Molecular investigations have suggested that these three disparate translocations seem to affect a common pathway, resulting in the constitutive activation of nuclear factor-κB (NF-κB) [4]. Variable frequencies of MALT lymphoma-associated genetic aberrations are seen in MALT lymphomas of different sites [5]. However, the vast majority of MALT lymphomas are negative for any of the above-mentioned translocations and the underlying pathogenesis remains unclear [5].

Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is known to be a stimulator of apoptosis and thus a potential therapeutic drug. Its clinical significance is based on the ability to trigger cell death in tumor cells while simultaneously having no effect on normal cells [6]. To

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date five TRAIL receptors have been identified in humans, including functional death receptors (DR4 and DR5), decoy receptors (DcR1 and DcR2) and osteoprotegerin (OPG). An intracellular adaptor protein (FADD) further relays the pro-apoptotic signal to caspase-8, leading to formation of the death inducing signaling complex (DISC), activation of effector caspases and consequently to apoptotic cell death [7–10]. FADD-like interleukin-1β-converting enzyme (FLICE) inhibitory protein (FLIP) inhibits the activation of caspase-8, and thus might be involved in a resistance mechanism to TRAIL induced apoptosis [11,12]. Additionally, due to its expression on the surface of cytotoxic T cells and natural killer cells, TRAIL is also being extensively studied in cancer immune surveillance [13,14].

Tumor cells have an ability to escape host innate and adaptive immune responses [15], which, in terms of TRAIL resistance, include mutation or down-regulation of death receptors, methylation of caspase-8 encoding genes and overexpression of $FLIP_L$ [16–18]. Previously, we could demonstrate functional silencing of DR4, as well as an association of TRAIL expression in the tumor microenvironment with overall survival in ovarian cancer [19,20]. Recently, we found a loss of death receptors and/or increase in $FLIP_L$ expression in prostate cancer, with a direct association of TRAIL expression in the tumor microenvironment with recurrence-free survival rate [21].

Extensive data have been generated in multiple myeloma, which arises from malignant plasma cells, on the role of TRAIL and the respective receptors. In an Affymetrix microarray-based study, DR4 and DR5 were found to be expressed in 71% and 98% of myeloma cell samples, respectively. TRAIL was expressed in the majority of primary multiple myeloma cells and FLIP expression was observed in all samples under study [22].

It has already been shown that TRAIL sensitivity of myeloma cells does not correlate with the expression of decoy receptors [23]. Both DR4 and DR5 may contribute to TRAIL-induced apoptosis in myeloma cells. The TRAIL-sensitive multiple myeloma cells have the highest levels of DR4 and DR5, while partially sensitive and TRAIL-resistant cell lines show reduced levels of DR5 but normal expression of DR4 [24]. TRAIL induces apoptosis in human multiple myeloma cell lines as well as in freshly obtained samples from patients with multiple myeloma [25]. In addition, TRAIL also exhibits antimyeloma activity in nude mice xenografted with human plasmacytomas [26]. Moreover, inhibition of the inhibitory protein FLIP sensitizes TRAIL-resistant multiple myeloma cells to TRAIL induced apoptosis [27].

In brief, the expression profiles of TRAIL components and the mechanisms underlying TRAIL-induced apoptosis have been vastly explored in multiple myeloma, but no investigations have been performed so far in MALT lymphoma. In view of this, we initiated a pilot study to assess the potential role of the TRAIL pathway in MALT lymphomas with and without cytogenetic abnormalities.

Materials and methods

Patient cohort

Paraffin embedded specimens from 32 patients diagnosed and treated at the Medical University of Vienna were assessed. Written and oral informed consent was obtained from all patients involved in the study, and approval was acquired from the Ethics Board of the Medical University of Vienna for further use and processing of the tumor specimens and clinical data. In all patients, a diagnosis of MALT lymphoma had been established by a reference hematopathologist at our institution (L.M.). Fifteen patients were especially selected due to the presence of plasmacytic differentiation of the MALT lymphoma, characterized by a profound presence of monoclonal plasma cells.

In addition, assessment of MALT lymphoma-associated genetic aberrations including t(11;18)(q21;q21), t(14;18)(q32;q21) involving *IGH* and *MALT1*, t(1;14)(p22;q32), t(3;14)(q14;q32) involving *FOXP1* and *IGH*, and trisomies 3 and 18 was performed in these patients. t(11;18)(q21;q21), involving *BIRC3* and *MALT1*, was assessed by reverse transcriptase polymerase chain reaction (RT-PCR), and t(14;18)(q32;q21) involving *IGH* and *MALT1*, t(1;14)(p22;q32) involving *BCL10* and *IGH*, t(3;14)(q14;q32) involving *FOXP1* and *IGH*, and trisomies 3 and 18 were investigated by fluorescence *in situ* hybridization (FISH).

Reverse transcriptase PCR

RNA was isolated from archival formalin-fixed, paraffin-embedded lymphoma tissues. Total RNA was extracted from 10 μm sections with a high pure RNA paraffin kit (Roche Diagnostics, Mannheim, Germany). First-strand cDNA was synthesized from 1 μg total RNA with a superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) using random hexamers as primers. RT-PCR for detection of the BIRC3–MALT1 fusion transcript was performed according to Inagaki *et al.* [28], with one modification: first round RT-PCR products were amplified in a second round separately and not as multiplex nested PCRs in order to discriminate the various fusion signals. Where indicated, PCR products were sequenced using dRhodamine dye terminators on an ABI Prism 310 (PE Applied Biosystems, Foster City, CA) [5].

Fluorescence *in situ* hybridization

Formalin-fixed, paraffin-embedded tissues were used for FISH. It was performed on interphases with the following probe sets: for detection of the t(14;18)(q32;q21) involving *IGH* and *MALT1*, P1 artificial chromosome (PAC) 152M5 (SpectrumOrange labeled) spanning the *MALT1* gene and flanking regions and bacterial artificial chromosome (BAC) 158A2 were used; for rearrangements of *BCL10*, BACs RP11-1077C10 and RP11-36L4 (SpectrumGreen) centromeric to *BCL10* and RP11-1080I1 and RP11-40K4 (SpectrumOrange) telomeric to *BCL10* were used [29]. In cases with rearrangement of *BCL10*, FISH with a dual-color, break apart rearrangement probe for *IGH* (Vysis, Downer's Grove, IL) was performed; FISH with BAC 158A2 for *IGH* and BACs RP11-1080I1 and RP11-40K4 telomeric to *BCL10* were used

to confirm the t(1;14)(p22;q32). For detection of trisomies 3 and 18, we applied centromere-specific probes for chromosomes 3 (SpectrumOrange) and 18 (SpectrumGreen) (Vysis). The cut-off value for the diagnosis of each probe set was the mean percentage of cells with a false-positive signal constellation plus three standard deviations, as assessed on tissue from 20 reactive lymph nodes [5].

Immunohistochemistry

MALT lymphoma biopsies from 32 patients were processed for immunohistochemistry. Standard tissue fixation and paraffinization procedures were followed and all the tissues were processed simultaneously. The immunohistochemistry protocol was standardized before applying on samples under study. All the tissues were processed synchronously under the same conditions of time, temperature, pH, etc. and were concurrently incubated to avoid any external/conditional influence. The paraffinized tissues were cut into 3–4 μm thick slices and were deparaffinized by heating at 60°C and subsequent rehydration in xylene and graded alcohols. Antigen retrieval was performed with DEPP-9 epitope retrieval solution (EB-depp9-250; Eubio, Vienna, Austria) followed by treatment with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. After blocking with 10% secondary antibody host serum for 30 min, the sections were incubated in primary antibodies (rabbit polyclonal DR4 [H-130] sc-7863 [1:300 dilution]; goat polyclonal DR5 [C-20] sc-7191 [1:300 dilution]; goat polyclonal $FLIP_L$ [C-19] sc-7108 [1:300 dilution]; goat polyclonal TRAIL [K-18] sc-6079 [1:300 dilution]; NF-κB [p65] sc-109 [1:300 dilution], Santa Cruz Biotechnology, Inc.; goat polyclonal DcR1 ALX-210-744 [1:1000 dilution], Alexis; rabbit polyclonal DcR2 ab2019 [1:100 dilution], Abcam) for 1 h at room temperature. Primary antibody dilutions were made in 10% secondary antibody host serum. The sections, after two phosphate buffered saline (PBS) washes, were incubated in respective biotinylated secondary antibodies (biotinylated anti-rabbit immunoglobulin G [IgG] [BA-1000]; biotinylated anti-goat IgG [BA-5000]; Vector Laboratories, Inc., Burlingame, CA) diluted 1:200 in 10% serum for 30 min at room temperature followed by 45 min incubation in StreptABComplex/HRP (K0377 Dako, Denmark). The sections were again washed twice with PBS and incubated in Dako Liquid DAB+ Substrate Chromogen System (K3468; Dako, Carpinteria, CA) until the development of a brown color. This was followed by counterstaining with Meyer's hematoxylin, dehydration and mounting using Eukitt mounting medium (O. Kindler GmbH, Freiburg, Germany). Staining analysis was performed on an Olympus BX50F microscope (Olympus Optical Co. Ltd., Tokyo, Japan) using Cell Imaging Software for Life Science Microscopy (Soft Imaging Solutions GmbH, Münster, Germany).

Staining analysis and statistics

The staining analysis was performed by three independent blinded investigators, one a trained pathologist. The staining intensity was determined on the basis of strength of the 3,3′-diaminobenzidine (DAB) signal. Dark brown intense staining was taken as strong (scale 3), medium brown was considered moderate (scale 2), light brown was taken as weak (scale 1) and an absence of signal was considered negative (scale 0). All these signals were confirmed by the pathologist. The cells were counted and grouped according to the percentage of positive cells as $<10\%$, 10–30% and $>30\%$ positively stained cells. For TRAIL and $FLIP_L$, staining was considered positive if more than 10% of the cells showed moderate or strong staining; for TRAIL receptors and NF-κB, moderate or strong staining in more than 30% cells was considered positive, as described previously [20,30]. Pair-wise correlations of all TRAIL proteins and their association with patients' age were calculated using Spearman correlation coefficients denoted by *R*. Mann–Whitney *U*-test was used to assess differences in TRAIL protein expression between genders and status. Kruskal–Wallis test was used for comparing TRAIL expression levels in patient groups having normal cytogenetic status, translocations and trisomies. Patient survival was compared between groups with high and low DR5 and NF-κB expression, as well as PCD status using Kaplan–Meier curves and log-rank test statistics.

Results

Patient description

Out of the 32 patients assessed, 13 had MALT lymphoma of the gastrointestinal tract (11 gastric and two small-bowel lymphoma) and nine suffered from lymphoma of the salivary glands (eight of the parotid and one of the submandibular gland). In the remaining patients, the lymphoma was located in the ocular adnexa in three cases, in the liver in two cases, in the dermis in two cases and in the palate, thyroid and subglottis in one patient each. Twenty-one patients were female, and 11 were male, with age ranging from 31 to 77 years (median 63.5 years). Fifteen patients had MALT lymphoma with PCD while 17 had no PCD. Seventeen of the patients (53%) had a normal cytogenetic status, seven patients (22%) had translocation t(11;18), seven patients (22%) had trisomy 3, 8 and/or 18 and one patient had trisomy 3 along with t(14;18) (Figure 1, Table I). Eleven patients had a history of HP infection, whereas no evidence of hepatitis B, C or human immunodeficiency virus (HIV) infections was found (Table I). One patient had previously undergone surgery, two patients had had radiotherapy while two patients had had chemotherapy. Three patients had undergone HP-eradication while five had chemotherapy along with HP-eradication therapy. One patient died because of myelodysplastic syndrome (MDS), one due to septicemia, and two died as a result of lymphoma progression. For the remaining patients, the cause of death was not clear.

Loss of death receptors in MALT lymphoma

We stained the MALT lymphoma tissues with TRAIL pathway proteins including DR4, DR5, DcR1, DcR2, $FLIP_L$ and TRAIL. Reactive plasma cells were taken as positive controls while the same tissues without primary antibody served as negative controls (Figure 2).

Interestingly, we saw a loss of DR4 and DR5 expression in 18/30 (60%) and 29/32 (91%) patients, respectively. DR4 was moderately expressed in seven (23%) and strongly expressed

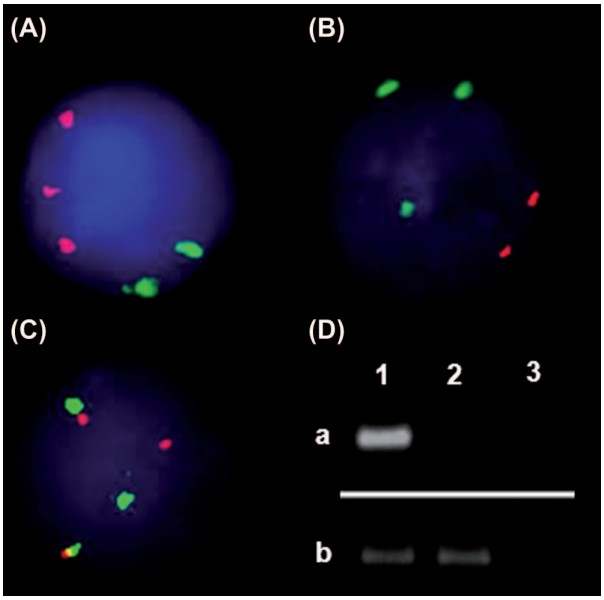

Figure 1. Determination of cytogenetic status of patients with MALT lymphoma. (A, B) FISH with centromeric probes for chromosomes 3 (red) and 18 (green) revealing trisomy 3 (A) and trisomy 18 (B). (C) FISH with probes spanning IGH (green) and MALT1 (red) show two fusions of a case with t(14;18)/*IGH–MALT1*. (D) RT-PCR demonstrates t(11;18)/*BIRC3–MALT1* in a case of MALT lymphoma (row 1). Row 2 is a negative control, row 3 water; line (a) delineates RT-PCR for t(11;18), line (b) positive control. FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcriptase polymerase chain reaction.

Table I. Sex, age, infected organ, cytogenetics, plasmacytic differentiation and *H. pylori* infection status of MALT lymphomas.

No.	Sex	Age	Organ	Cytogenetic status	PCD	*H. pylori*
1	F	43	Thyroid	Normal	Yes	NA
2	F	47	Parotid	Normal	No	Yes
3	F	64	Dermis	Normal	Yes	NA
4	M	39	Submandibularis	Normal	Yes	Yes
5	M	69	Gastric	Normal	Yes	Yes
6	F	68	Parotid	Normal	No	Yes
7	M	55	Gastric	Normal	Yes	No
8	F	64	Gastric	Normal	Yes	NA
9	M	60	Parotid	Normal	No	No
10	F	55	Gastric	Normal	No	NA
11	M	77	Gastric	Normal	No	Yes
12	F	64	Gastric	Normal	No	NA
13	M	67	Eye, base of skull	Normal	Yes	NA
14	F	64	Gastric	Normal	Yes	Yes
15	M	63	Dermis	Normal	Yes	Yes
16	F	42	Parotid	Normal	Yes	NA
17	M	60	Small intestine	Normal	Yes	NA
18	F	59	Liver	Trisomy 3	No	No
19	F	36	Parotid	Trisomy 3	No	Yes
20	F	62	Liver	Trisomy 3, t(14;18)	No	Yes
21	F	46	Parotid	Trisomy 3	No	NA
22	F	66	Palatal arch, right	Trisomy 3, 18	Yes	Yes
23	F	65	Parotid, orbit	Trisomy 3, 18	No	No
24	F	67	Eye	47,XX,+8	Yes	No
25	F	65	Gastric	t(11;18)	No	No
26	M	31	Parotid	t(11;18)	No	No
27	F	69	Subglottic	t(11;18)	No	Yes
28	F	74	Small intestine	t(11;18)	Yes	NA
29	F	48	Gastric	t(11;18)	No	No
30	F	70	Gastric	t(11;18)	No	No
31	M	36	Gastric	t(11;18)	No	NA
32	M	74	Conjunctiva (eye)	Trisomy 18	Yes	NA

MALT, mucosa associated lymphoid tissue; PCD, plasmacytic differentiation; NA, no information available.

in five (17%) cases, while DR5 was moderately expressed in only three (9%) of the patients [Figure 3(A), Table II].

Differential expression of decoy receptors

A considerable percentage of MALT lymphoma tissues (16/30) were positive for DcR1 expression, with eight (27%) having moderate and eight (27%) having strong expression. On the other hand, 12 (41%) of the tissues were positive for DcR2 expression. Interestingly, the vast majority (10/12, 83%) of this population were strongly positive for DcR2 expression [Figure 3(A), Table II].

Diminished $FLIP_L$ and TRAIL expression

In the present study, normal reactive plasma cells showed a significant expression of $FLIP_L$; however, we found a loss of this protein in MALT lymphomas. Only 4/31 (13%) showed positive $FLIP_L$ expression, while the remaining 27 (87%) had weak or no expression at all. At the same time, we also observed a loss of TRAIL expression in patients with MALT lymphoma, as compared to the normal reactive plasma cells, with 26/31 (84%) having negative and only five (16%) with positive expression [Figure 3(A), Table II). Moreover, the loss of TRAIL expression significantly correlated with the presence of HP infection ($R = -0.52$, $p = 0.042$).

In general, all 32 patients investigated had some alteration in the TRAIL pathway, mainly involving loss of death receptors or high decoy receptor presence [Figure 3(B)]. Further division of the patient cohort into groups with and without cytogenetic aberrations disclosed an increased decoy receptor expression in the group with normal cytogenetic status (12/17, 70%) with or without loss of death receptors, as compared to the cytogenetic aberrations group [6/15, 40%; Figure 3(B)].

Additionally, we also determined the correlation of TRAIL components' expression with survival for patients with MALT lymphoma using Kaplan–Meier curves, and observed a trend toward worse survival outcome for the patients with loss of DR5 expression. However, this correlation did not reach statistical significance [$p = 0.484$; median follow-up time 76 (DR5 negative) and 64 months (DR5 positive); Supplementary Figure 1(A) to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.953149].

DR4 is coexpressed with the decoy receptors

Since the expression of DR4 in MALT lymphoma was higher (12/30, 40%) as compared to DR5 (3/32, 9%), we decided to correlate this expression with the inhibitory proteins DcR1, DcR2 and $FLIP_L$ to understand the escape of these lymphomas from TRAIL mediated immune surveillance. Interestingly, DR4 was found to be coexpressed with either DcR1 (3/12, 25%), DcR2 (2/12, 17%) or both of the decoy receptors (7/12, 58%) (Figure 4). The Spearman correlation coefficient for DR4 coexpression with DcR1 and DcR2 was $R = 0.376$ ($p = 0.040$) and $R = 0.713$ ($p < 0.001$), respectively. This coexpression might cause inhibition of DR4 signaling through decoy receptor overexpression.

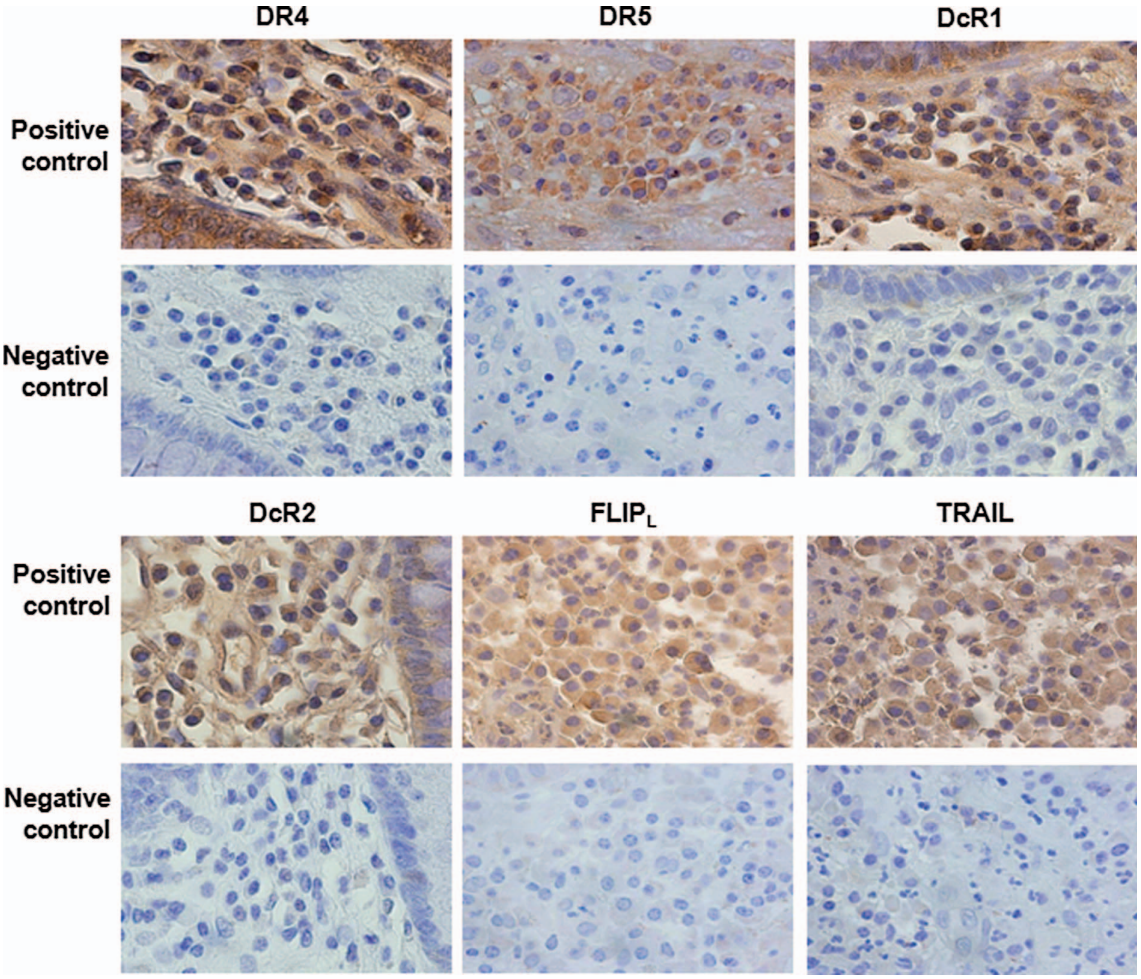

Figure 2. Negative and positive controls for immunohistochemistry. Reactive plasma cells were taken as positive controls while negative controls were the same tissues stained without primary antibodies. Magnification, × 60. DR4, death receptor 4; DR5, death receptor 5; DcR1, decoy receptor 1; DcR2, decoy receptor 2; FLIP$_L$, long form of FLICE inhibitory protein; TRAIL, TNF related apoptosis inducing ligand.

Plasmacytic differentiation and TRAIL components' expression

PCD is a phenomenon characterized by the abundant presence of monoclonal plasma cells in MALT lymphoma. We determined the expression of TRAIL proteins in the groups with (15/32, 47%) and without PCD (17/32, 53%). Higher DcR2 expression was found in the group with PCD as compared to the group with no PCD (p = 0.046 [*t*-test]; $p = 0.069$ [Mann–Whitney *U*-test]). Additionally the expression of FLIP$_L$ was lower in the PCD group as compared to the non-PCD group ($p = 0.039$ [*t*-test]; $p = 0.059$ [Mann–Whitney *U*-test]) [Figures 5(A) and 5(B)]. We also performed a survival analysis for patients with and without PCD and found a trend toward favorable survival for patients with PCD; however, the analysis did not reach statistical significance [$p = 0.278$; median follow-up time 50 (with PCD) and 78.5 months (without PCD); Supplementary Figure 1(B) to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.953149].

DcR2 inversely correlates with cytogenetic abnormalities in MALT lymphoma

Since the pathogenetic mechanisms of MALT lymphoma in patients without genetic anomalies remain somewhat elusive, we divided our patient population into groups with either normal cytogenetic status or with translocations and/or trisomies. Upon applying the Kruskal–Wallis test, we observed low DcR2 expression in patients with cytogenetic abnormalities and significantly elevated DcR2 expression in the tumors without any cytogenetic anomaly ($p = 0.005$) [Figure 5(C)].

This result might indicate a potential role of DcR2 in the pathogenesis of MALT lymphomas without known genetic abnormalities. Since DcR2 is known to cause evasion from TRAIL mediated immune surveillance through NF-κB induction [31], we decided to investigate whether DcR2 might have a similar role of NF-κB regulation in the lymphomas without aberrations.

DcR2 expression significantly correlates with NF-κB expression

We stained the tissue specimens with an NF-κB antibody and observed high NF-κB expression in 24 out of 30 evaluable tissues (80%). Among these 24, 15 (62.5%) had strong and nine (37.5%) had moderate NF-κB expression [Figure 6(A)]. We further correlated NF-κB expression with TRAIL components using Spearman correlation and found a significant positive correlation of DcR2 expression with NF-κB ($R = 0.372$, $p = 0.047$). Moreover, we found a trend toward worse survival outcome for patients with high NF-κB expression; however,

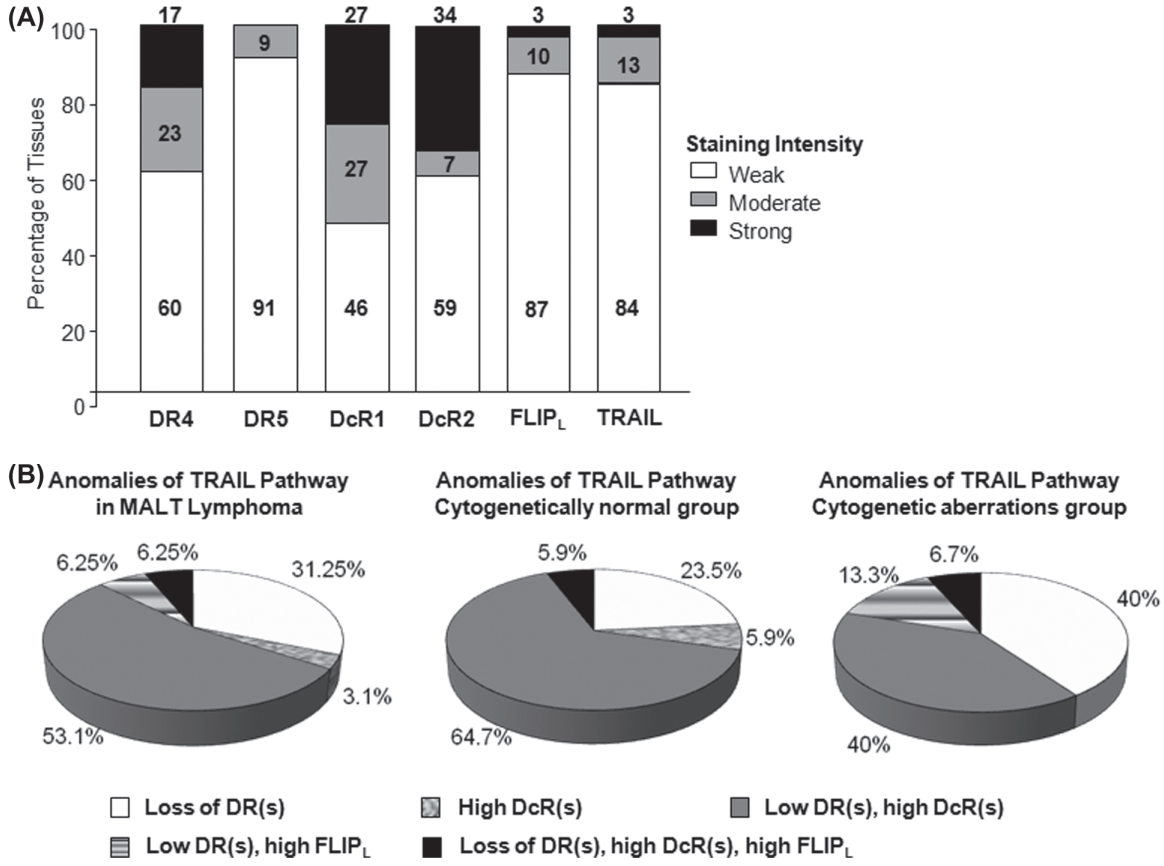

Figure 3. Expression and distribution of TRAIL components in MALT lymphoma. (A) Stained tissues were graded on the basis of staining intensity as weak, moderate and strong. (B) Patient samples were distributed into groups of specific TRAIL pathway anomalies and all of them showed some alterations in TRAIL pathway including loss of death receptors (DRs), high DcRs and/or high FLIP$_L$. Most of the tissues showed high DcR1/2 expression along with the loss of DRs. Further subdivision of patients with MALT lymphoma according to cytogenetic aberrations depicted higher expression of DcRs in the group with no known cytogenetic aberrations. DR4, death receptor 4; DR5, death receptor 5; DcR1, decoy receptor 1; DcR2, decoy receptor 2; FLIP$_L$, long form of FLICE inhibitory protein; TRAIL, TNF related apoptosis inducing ligand.

the analysis did not reach statistical significance [$p = 0.291$; median follow-up time 66 (low NF-κB) and 78.5 months (high NF-κB); Supplementary Figure 1(C) to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.953149].

On the basis of these observations we propose a model suggesting the possible mechanism underlying MALT lymphoma pathogenesis with respect to the TRAIL pathway in the patient group having no known cytogenetic anomalies [Figure 6(B)].

Discussion

MALT lymphoma accounts for about 7% of all newly diagnosed lymphomas, but the pathogenetic events responsible for this disease remain unknown in a significant percentage of patients. Chronic antigenic stimulation with subsequent NF-κB activation seems to be at least in part responsible for MALT lymphoma formation. The cytogenetic aberrations in MALT lymphoma include translocations t(11;18)/*BIRC3–MALT1*, t(1;14)/*IGH–BCL10* and t(14;18)/*IGH–MALT1*, which may be responsible for NF-κB activation [4]. In addition, trisomies 3, 8 and 18 have also been reported in a number of MALT lymphomas. A majority of MALT lymphomas, however, are still negative for any of the described anomalies. To further elucidate a potential underlying pathogenetic mechanism, we have determined the expression of TRAIL components for their potential role in MALT lymphoma pathogenesis.

TRAIL is known for its property of selective apoptosis. This selectivity is attained at the initial stage by the interplay of death and decoy receptors on the surface of cells.

Table II. Expression of TRAIL components in MALT lymphoma.

Marker	DR4	DR5	DcR1	DcR2	FLIP$_L$	TRAIL	NF-κB
Weak	18 (60%)†	29 (91%)	14 (46%)	17 (59%)	27 (87%)	26 (84%)	6 (20%)
Moderate	7 (23%)	3 (9%)	8 (27%)	2 (7%)	3 (10%)	4 (13%)	9 (30%)
Strong	5 (17%)	0 (0%)	8 (27%)	10 (34%)	1 (3%)	1 (3%)	15 (50%)
n*	30	32	30	29	31	31	30

TRAIL, TNF related apoptosis inducing ligand; DR4, death receptor 4; DR5, death receptor 5; DcR1, decoy receptor 1; DcR2, decoy receptor 2, FLIP$_L$, long form of FLICE inhibitory protein; MALT, mucosa associated lymphoid tissue; NF-κB, nuclear factor κB.
*n = total number of patients.
†No. of patients (percentage).

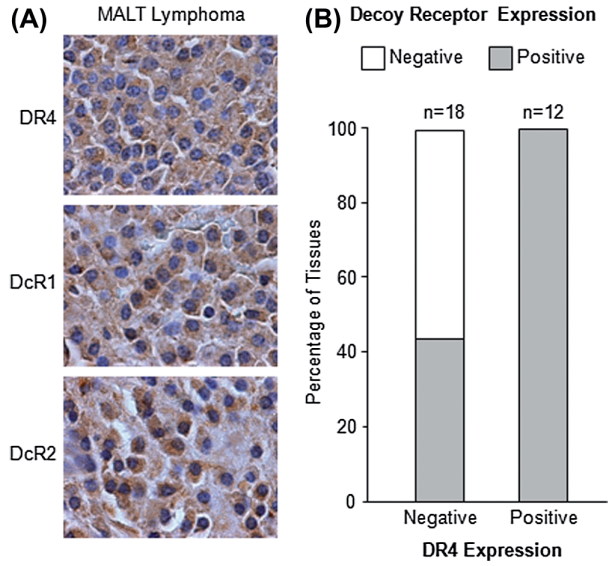

Figure 4. Coexpression of DR4 with decoy receptors. (A) Representative stainings of DR4, DcR1 and DcR2 in MALT lymphoma. Magnification, ×100. (B) All of the tissues having positive DR4 also showed an increase in any one or both of the decoy receptors. DR4, death receptor 4; DcR1, decoy receptor 1; DcR2, decoy receptor 2.

Loss of death receptors can lead to failure of apoptosis and consequent carcinogenesis, but at the same time, numerous cancers have shown up-regulated death receptors probably as a natural defense mechanism to counter transformation [21]. However, in such instances, up-regulated decoy receptors and/or $FLIP_L$ potentially nullify the apoptotic property of death receptors and thereby prevent apoptosis and favor survival.

We have reported a general loss of TRAIL components in MALT lymphoma with DR4 expression only in 12/30 (40%), DR5 in 3/32 (9%), inhibitory protein $FLIP_L$ in 4/31 (13%) and TRAIL in 5/31 (16%) of patients, which is different from our previous findings in prostate and ovarian cancers [20,21]. The reason for this difference might be related to the lymphoid tissue of origin or an altered tumor–microenvironment interaction in lymphoid malignancies in general.

$FLIP_L$ is a splice variant of c-FLIP and has a long catalytically inactive caspase-like protease domain, crucial for its ubiquitylation and degradation, in addition to the two death effector domains [32]. It is normally present in the cell cytoplasm. In the present study, we report a loss of $FLIP_L$ from the lymphoma cells. The absence of $FLIP_L$ is

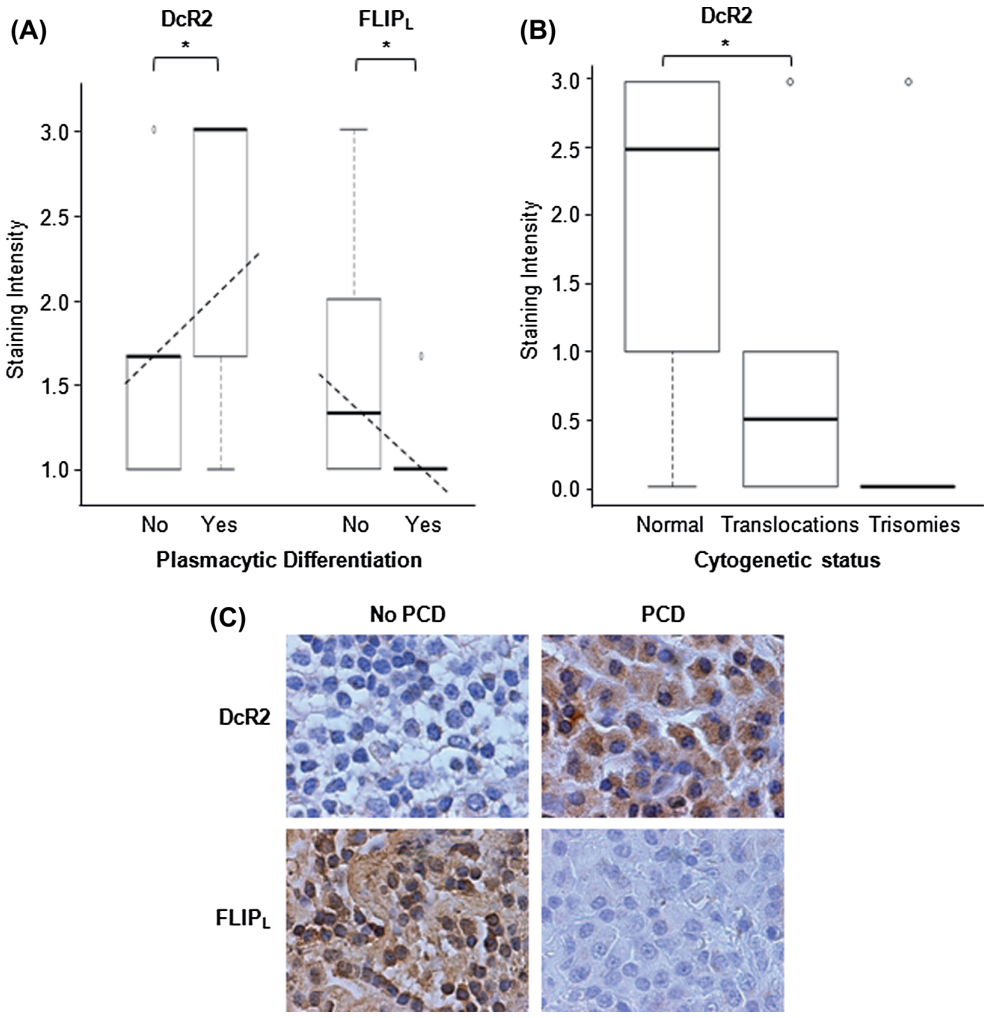

Figure 5. Effect of plasmacytic differentiation and cytogenetic status on TRAIL components' expression. (A) Expression of DcR2 and $FLIP_L$ in groups with and without plasmacytic differentiation was determined. (B) Representative stainings of DcR2 and $FLIP_L$ in MALT lymphomas with and without PCD. Magnification, ×100. (C) Expression levels of DcR2 in groups with and without cytogenetic abnormalities were compared using Kruskal–Wallis test. $^*p < 0.05$. DcR2, decoy receptor 2; $FLIP_L$, long form of FLICE inhibitory protein; PCD, plasmacytic differentiation.

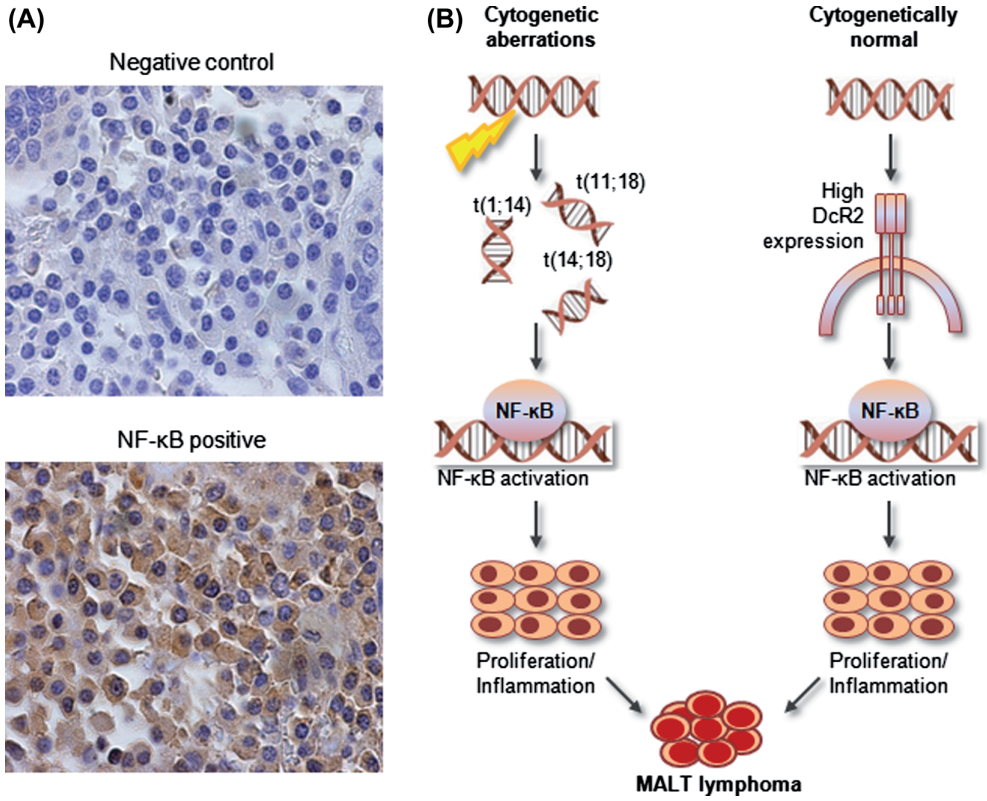

Figure 6. NF-κB expression and schematic representation of possible mechanism underlying evasion of immune surveillance in MALT lymphomas with normal cytogenetic status. (A) Expression of NF-κB in MALT lymphoma. Upper panel represents negative control and lower panel shows representative NF-κB staining in MALT lymphoma. Magnification, ×60. (B) Genetic aberrations such as t(11;18)/*BIRC3–MALT1* and t(14;18)/*IGH–MALT1* lead to NF-κB activation resulting in increased proliferation. A similar function of NF-κB activation is potentially being performed by DcR2 in the MALT lymphoma group with normal cytogenetic status. DcR2, decoy receptor 2; MALT, mucosa associated lymphoid tissue; NF-κB, nuclear factor-κB.

generally pro-apoptotic, since it allows caspase-8 activation and consequent apoptosis [33]; however, there was also a simultaneous loss of death receptors and TRAIL protein. Additionally, of the two death receptors, the presence of DR4 was dominant but had a significant coexpression with both the decoy receptors, which may nullify the apoptotic effect through simultaneous competition for TRAIL binding. Having enough evidence on the anti-apoptotic nature of FLIP [34], the role of FLIP as an inducer of apoptosis can also not be ignored [35]. Mice with T cell-specific deletion of c-FLIP have impaired development of mature T cells due to increased sensitivity of thymocytes to Fas-mediated apoptosis [36]. On the other hand, deletion of c-FLIP in B cells does not noticeably alter the maturation of peripheral B cell populations but leads to severe defects in *in vitro* responses to Toll-like receptor (TLR) 3 and 4 ligands, which are associated with perturbations in the NF-κB pathway [37]. According to a recent study in mice with B-cell specific deletion of c-FLIP, there was no effect on the primary development of mice; however, participation of c-FLIP-deficient B cells in the immune response was severely compromised, and the presence of c-FLIP was stated as a prerequisite for participation of B cells in the immune response [38]. Thus the absence of $FLIP_L$ might also be a potential cause of evasion of the immune surveillance mechanism in MALT lymphoma.

Additionally, we also determined the correlation of TRAIL component expression with survival for patients with MALT lymphoma using Kaplan–Meier curves and found two interesting correlations. First, we found a trend toward survival benefit for patients with high DR5 expression. Second, we observed a trend toward worse survival outcome for patients with high NF-κB expression (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.953149). However, both of these correlations did not reach statistical significance. In view of the limited number of patients included in the series and the generally excellent prognosis of MALT lymphoma, these correlations should be assessed in a larger cohort with a longer follow-up time period.

As little is known about pathogenetic mechanisms in MALT lymphomas without known cytogenetic abnormalities, we divided our patient cohort into two groups, one with and the other without any translocation/trisomy. We found a significantly higher expression of DcR2 in the group without genetic aberrations. This finding suggests a possible inhibition of TRAIL ligand-receptor binding by competing with death receptors and thus blocking TRAIL-mediated apoptosis in MALT lymphomas. DcR2 is a novel TRAIL receptor, which lacks a complete death domain needed for apoptotic signaling but has some of the cytoplasmic signaling region. Several members of the TNFR family contain cytoplasmic sequences associated with NF-κB activation. DcR2 also has an ability to directly induce NF-κB possibly through the residual cytoplasmic signaling region as determined in 293/

EBNA cells [31]. In view of this, we investigated the potential link between DcR2 expression and MALT lymphoma pathogenesis through NF-κB expression. Patients with MALT lymphoma with a normal genetic status showed a significantly higher expression of DcR2, which correlated significantly with NF-κB expression in our study. Thus apparently in cytogenetically normal patients, DcR2 has a potential role in up-regulating NF-κB, which could likely be involved in increased proliferation, survival and subsequent tumorigenesis. Since RelA (p65) is cytoplasmic and DcR2 is a cell surface receptor, hence targeting of DcR2 is virtually more feasible and could possibly facilitate the regulation of NF-κB at the cellular level.

In conclusion, our study is the first to explore the TRAIL pathway in MALT lymphoma. The results indicate that the TRAIL pathway is generally compromised in most of the MALT lymphoma samples evaluated. Moreover, DcR2 is highly up-regulated in cytogenetically normal patients, and this up-regulation is significantly correlated with higher NF-κB expression. Expression of individual elements of the TRAIL signaling pathway may harbor high prognostic significance. We propose that TRAIL-based therapies and specific targeting of individual TRAIL components could be successful in MALT lymphoma management, and advance our chance to obtain better insights into tumor immune surveillance in lymphoid malignancies.

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Supplementary material available online

Supplementary Figure showing further results.