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ORIGINAL ARTICLE

Differential expression of NF-κB target genes in MALT lymphoma with and without chromosome translocation: insights into molecular mechanism

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Mucosa-associated lymphoid tissue (MALT) lymphoma is characterized by t(11;18)(q21;q21)/API2-MALT1, t(1;14)(p22;q32)/BCL10-IGH and t(14;18)(q32;q21)/IGH-MALT1, which commonly activate the nuclear factor (NF)-κB pathway. Gastric MALT lymphomas harboring such translocations usually do not respond to *Helicobacter pylori* eradication, while most of those without translocation can be cured by antibiotics. To understand the molecular mechanism of these different MALT lymphoma subgroups, we performed gene expression profiling analysis of 21 MALT lymphomas (13 translocation-positive, 8 translocation-negative). Gene set enrichment analysis (GSEA) of the NF-κB target genes and 4394 additional gene sets covering various cellular pathways, biological processes and molecular functions have shown that translocation-positive MALT lymphomas are characterized by an enhanced expression of NF-κB target genes, particularly toll like receptor (TLR)6, chemokine, CC motif, receptor (CCR)2, cluster of differentiation (CD)69 and B-cell CLL/lymphoma (BCL)2, while translocation-negative cases were featured by active inflammatory and immune responses, such as interleukin-8, CD86, CD28 and inducible T-cell costimulator (ICOS). Separate analyses of the genes differentially expressed between translocation-positive and -negative cases and measurement of gene ontology term in these differentially expressed genes by hypergeometric test reinforced the above findings by GSEA. Finally, expression of TLR6, in the presence of TLR2, enhanced both API2-MALT1 and BCL10-mediated NF-κB activation *in vitro*. Our findings provide novel insights into the molecular mechanism of MALT lymphomas with and without translocation, potentially explaining their different clinical behaviors.

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Introduction

Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) originates from the MALT acquired as a result of chronic inflammatory or autoimmune disorders.[1] The etiological factors underlying these chronic inflammatory disorders have a pivotal role in MALT lymphomagenesis. This is best exemplified by the causative role of *Helicobacter pylori* infection in gastric MALT lymphoma as shown by the compelling evidence from the epidemiological, laboratory and particularly clinical studies, which show long-term complete remission of the lymphoma following *H. pylori* eradication in ~70% of cases.[1] In spite of this, the molecular mechanisms underlying the lymphoma development are not fully understood. Stimulations of antigen receptor by autoantigen, and co-stimulatory molecule CD40 by *H. pylori*-specific T cells are believed to have an important role. Recent studies on MALT lymphoma-associated chromosome translocations provide further insights into its molecular pathogenesis.

t(11;18)(q21;q21)/*API2-MALT1*, t(1;14)(p22;q32)/*BCL10-IGH* and t(14;18)(q32;q21)/*IGH-MALT1* are specifically associated with MALT lymphoma albeit occurring at considerately variable frequencies in different anatomic sites.[2–5] Although these translocations involve different oncogenes, molecularly their encoded products commonly activate the canonical nuclear factor (NF)-κB pathway,[6–8] accounting for their critical role in lymphomagenesis. Nonetheless, overexpression of these oncogenes alone is insufficient for malignant transformation as both *Eμ-API2-MALT1* and *Eμ-BCL10* transgenic mice developed splenic marginal zone hyperplasia, but not lymphoma.[9,10] Thus, other molecular events are required to cooperate with these chromosome translocations in MALT lymphoma development.

The above chromosomal translocations are always mutually exclusive and t(11;18), the most frequent translocation in MALT lymphoma, occurs often as the sole cytogenetic abnormality.[11] Several studies suggest that there is a potential cooperation between MALT lymphoma-associated oncogenic products and immunological stimuli in lymphomagenesis. In the *Eμ-API2-MALT1* transgenic mice, immunization with the Freund's complete adjuvant led to development of a splenic marginal zone lymphoma-like hyperplasia.[12] *In vitro* assay showed that CD40 stimulation enhanced both API2-MALT1 and MALT1-induced NF-κB activation.[13] However, the extent of potential cooperation between MALT lymphoma-associated oncogenic products and immune surface receptor signaling is unknown.

In spite of the presence of a potential overlap in the molecular mechanism of MALT lymphoma with and without translocation as discussed above, there are important differences in the clinical and histological presentations between these different subgroups. Clinically, gastric MALT lymphomas with t(11;18) or t(1;14) are significantly associated with advanced stages and resistance to *H. pylori* eradication.[14,15] Histologically, t(11;18)-positive MALT lymphomas seem to be more monotonous, lacking apparent transformed blasts.[16] These distinct

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clinico-pathological characteristics may indicate important differences in molecular mechanisms between MALT lymphomas with and without translocation. To analyze this and understand further the molecular mechanism of MALT lymphoma, we studied the transcriptional profiles of a well-characterized series of cases with different translocation status and further validated the genes identified and the hypothesis generated.

Materials and methods

Patient materials

Fresh frozen tissues from 24 well-characterized MALT lymphomas (Supplementary Table S1), 7 follicular lymphoma (FL) and 7 mantle cell lymphoma (MCL) were used for gene expression microarray analysis. The MALT lymphomas were nine positive for t(11;18)/*API2-MALT1* (eight gastric and one pulmonary), four positive for t(1;14)/*BCL10-IGH* or t(1;2)/*BCL10-IGκ* (three gastric and one pulmonary), two positive for t(14;18)/*IGH-MALT1* (one hepatic and one ocular adnexal) and nine gastric cases negative for all known MALT lymphoma-associated translocations. The percentage of tumor cells was estimated on hematoxylin and eosin stained slides and crude microdissection was performed to ensure that at least 70% tumor cells was used for expression microarray analysis.

In addition, 73 cases of MALT lymphoma, including 18 positive for t(11;18), 8 positive for t(1;14), 9 positive for t(14;18) and 38 negative for these translocations, were used for validation of the expression microarray findings. The use of archival tissues for research was approved by the local research ethics committees of the authors' institutions.

Gene expression microarray

RNA extraction, synthesis of labeled complementary RNA by *in vitro* transcription and hybridization to Affymetrix (Affymetrix UK Ltd., High Wycombe, UK) GeneChip HG-U133A (MALT lymphoma) or Affymetrix H133 plus 2 (FL and MCL), quality control analysis, microarray data normalization and nonspecific filtering are detailed in Supplementary Methods. All microarray data have been deposited with Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, GSE18736).

Unsupervised clustering

This was performed using Pearson's correlation coefficient and average linkage as the similarity measure and clustering algorithm respectively within Genespring GX 7.3.1. Separate clustering was performed among all MALT lymphoma, FL and MCL cases and also within the MALT lymphoma group.

Gene set enrichment analysis (GSEA)

GSEA was used to identify gene sets differentially regulated between MALT lymphoma with (13 cases) and without (8 cases) chromosome translocation.[17] As the original GSEA only identifies the gene set showing either uniformly up- or down-regulation, for the gene sets showing both up- and down-regulated genes, absolute GSEA was additionally performed.[18] A total of 4395 gene sets were analyzed (Supplementary Table S2) and they included (1) NF-κB target genes, collated from online database, published works and careful bioinformatic search (Supplementary Table S3); (2) biological pathways involved in inflammatory and immune responses from human immunome database,[19] gene ontology (GO) and ingenuity and (3) gene sets from Molecular Signature database. The GSEA results were ranked according to the nominal *P*-value (<0.05) and false discovery rate ($\leqslant 0.25$) as described previously.[17]

For the gene sets differentially regulated between MALT lymphoma with and without translocation, leading edge analysis was performed to identify the biologically important gene subset.[17] When generating gene sets, for each sample, only the maximum expression value of the multi-probes for a given gene was used for GSEA as described previously.[17]

Analysis of differential gene expression in MALT lymphomas with and without translocation

For identification of differentially expressed genes between MATL lymphoma with and without translocation, the MAS5 normalized and filtered data set was used as suggested previously.[20] The genes differentially expressed (one-way analysis of variance test, $P<0.05$) between translocation-positive and -negative MALT lymphomas were identified, and those showing >2.5-fold differences were selected for functional annotation using GO.

Functional annotation using GO

To assess the biological implication of differential gene expression in MALT lymphomas with and without translocation, we measured the representation of GO terms (association of gene products with their related biological processes and molecular functions) in the above differentially expressed genes using Genespring and hypergeometric tests provided in the R package (GOstats, version 2.8.0, http://www.bioconductor.org). This allowed us to examine whether any GO term was over- or under-represented as compared with what can occur by chance. Independent analyses of GO categories were performed for overexpressed genes in both translocation-positive and -negative MALT lymphoma.

Quantitative reverse-transcription PCR (qRT-PCR), immunohistochemistry and western blot analysis

Please see description in the Supplementary Methods (Supplementary Table S4–S5).

NF-κB reporter assay

The potential cooperation between BCL10 (or API2-MALT1) and TLR6 expression in NF-κB activation was analyzed *in vitro* using a Luciferase Reporter Assay and the experimental details are given in the Supplementary Methods.

Results

Transcriptional profiling defines MALT lymphoma as a distinct entity

The microarray data from 21 MALT lymphomas (13 translocation-positive and 8 translocation-negative), 5 FL and 7 MCL passed the microarray hybridization quality control and were further analyzed and presented below (Supplementary Table S6). The standard normalization and filtering across all these cases yielded a set of 2629 probes. As expected, *CD10* and *BCL6* were found most highly expressed in FL, *CCND1* most highly expressed in MCL, and *MALT1* most highly expressed in MALT lymphoma with t(14;18) or t(11;18) (Supplementary Figure S1). Unsupervised hierarchical clustering showed that MALT lymphomas were clustered as a single branch, irrespective of their origin from different anatomic sites. Within the MALT

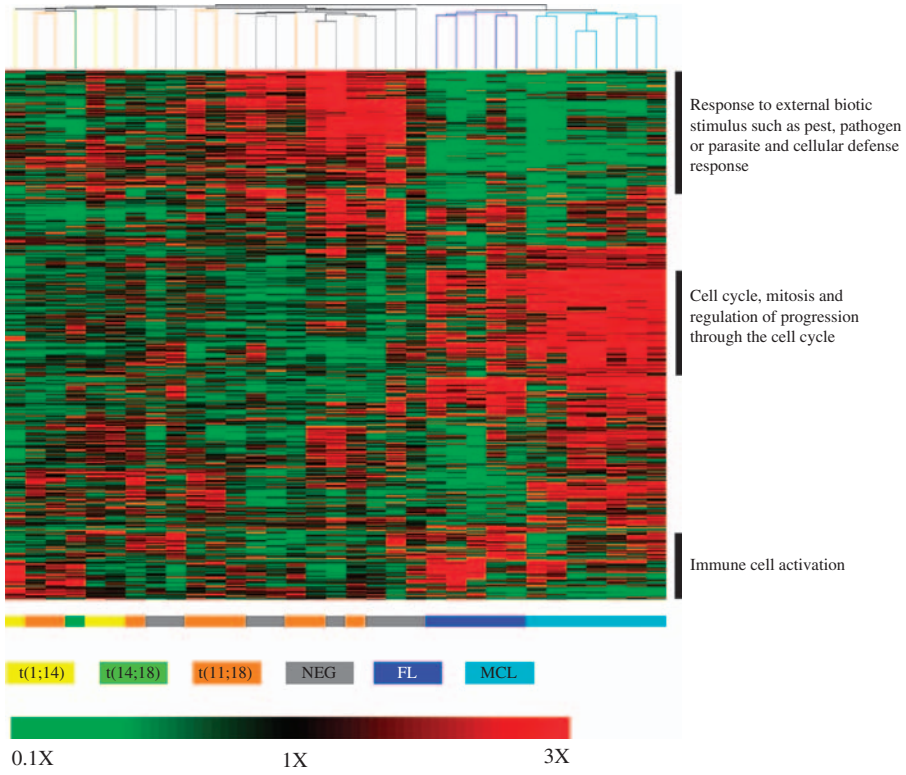

Figure 1 MALT lymphoma shows distinct gene expression profiles from FL and MCL. After standard normalization and filtering, a set of 2629 probes were obtained and used for unsupervised hierarchical clustering analysis. All MALT lymphomas are clustered as single branch, clearly separated from both FL and MCL. Nonetheless, within MALT lymphoma group, cases with and without chromosome translocation are intermingled together. The gene tree was ordered according to biological processes defined by GO, and the gene clusters enriched for a particular biological process in a lymphoma subtype as shown by hypergeometric testing are indicated. The gene sets for cell cycle (GO:7049) and regulation of progression through cell cycle (GO:74) are highly enriched in MCL, whereas those for immune cell activation (GO:45321, GO:46649) are enriched in FL. The gene sets for immune response to biotic stimulus (GO:9607), external biotic stimulus such as pest, pathogen or parasite (GO:9613) and cellular defense response (GO:6952) are highly enriched in MALT lymphoma. The chromosome translocation status of MALT lymphoma, FL and MCL are indicated by different color scheme. On the heatmap, red represents upregulated genes and green downregulated genes, with the scale showed at the bottom of the figure. FL, follicular lymphoma; MCL, mantle cell lymphoma; NEG, translocation-negative MALT lymphoma.

lymphoma group, translocation-positive cases were intermingled with translocation-negative cases (Figure 1), indicating that the translocation status did not have major effect on the hierarchical clustering. We also repeated the unsupervised hierarchical clustering analysis exclusively on MALT lymphoma cases using a set of 6893 variant probes derived from U133A and very similar results were found (Supplementary Figure S2).

Differential expression of NF-κB target genes in MALT lymphoma with and without chromosome translocation

As expected, the absolute GSEA revealed that a subset of the NF-κB target genes was over-represented in the translocation-positive MALT lymphomas, whereas another subset was enriched in translocation-negative cases ($P=0.011$, false discovery rate $=0.005$, Figure 2a, Supplementary Table S7). Leading edge analysis showed that 19 core genes accounted for the significant enrichment in translocation-positive cases and the top 10 genes included *CCR2A*, *BCL2*, *CD69*, *TLR6*, *TFEC*, *IRF4*, *PRDM2*, *REL*, *CCR7* and *CCR5*. Whereas, 34 core genes underscored the significant enrichment in translocation-negative cases and the top 10 genes were *PTGS2*, *PLAU*, *NR4A3*, *PTGIS*, *IL8*, *CD86*, *CCL2*, *CCL11*, *CXCL5* and *CXCL1*.

NF-κB target genes potentially underpins the differential representation of significant gene sets between MALT lymphomas with and without chromosome translocation

To gain further insights into the potential difference in molecular mechanisms between translocation-positive and -negative MALT lymphomas, we performed GSEA, in which indicated absolute GSEA, on 4394 gene sets covering various cellular pathways, biological processes and molecular functions. A total of 33 gene sets (not including those with very general term or those containing <20 genes) were differentially over-represented between MALT lymphomas with and without translocation ($P<0.05$, false discovery rate <0.20, Table 1).[17] As there was a considerable overlap among the gene sets associated with the related cellular pathways or biological processes, they were grouped according to their involvement in the NF-κB activation pathway, inflammation/immune responses, chemokine and cell migration, G protein-coupled receptor (GPR) signaling and cell proliferation/apoptosis (Table 1). Leading edge analysis was performed to identify the core subset genes that underscored the significant enrichment and were thus most likely biologically important. Interestingly, the NF-κB target genes were frequently presented in each of these core subset genes, often on top of the list (Supplementary

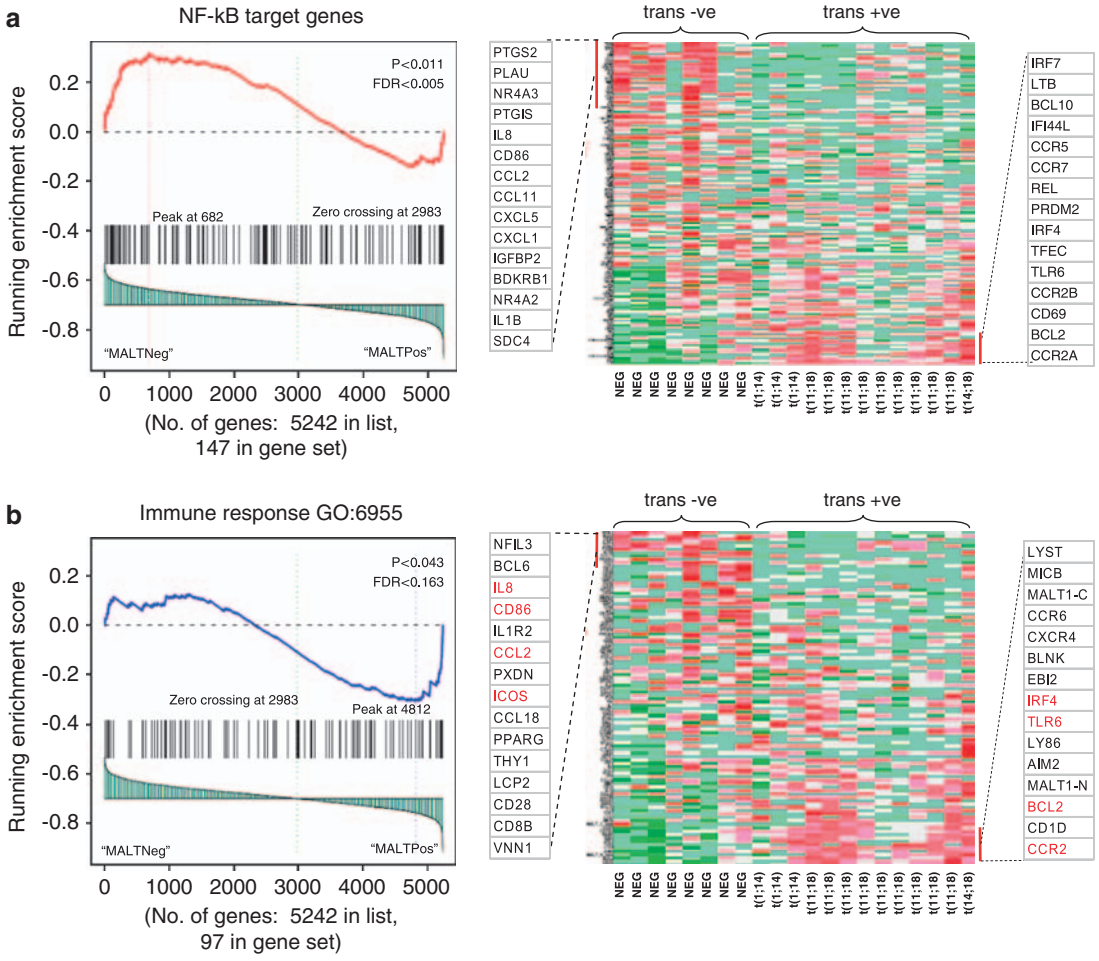

Figure 2 GSEA of NF-κB target genes (**a**) and immune response genes (GO:6955) (**b**) in MALT lymphomas with and without chromosome translocation. Left panel shows the distribution of NF-κB target genes or immune response genes according to their rank position. Right panel shows heatmap illustration of their expression between MALT lymphoma with and without chromosome translocation. The top 15 leading edge core genes are shown. Trans –ve: translocation-negative MALT lymphoma; trans +ve: translocation-positive MALT lymphoma.

Tables S8–S16, Supplementary Figure S3). Figure 2b shows the results of GSEA of immune response genes (GO:6955) with the top 15 leading edge core genes indicated in the heatmap illustration. Several NF-κB targets such as *CCR2*, *BCL2*, *TLR6* and *IRF4* were enriched in translocation-positive MALT lymphoma, whereas *IL8, CD86 CCL2* and *ICOS* were over-represented in translocation-negative cases (Supplementary Table S12).

Differential gene expression between MALT lymphomas with and without chromosome translocation

Using one-way analysis of variance test ($P<0.05$) and a 2.5-folds change as the threshold, we identified 26 and 62 genes significantly overexpressed in translocation-positive and translocation-negative MALT lymphoma respectively (Supplementary Table S17). To assess the biological implication of this differential gene expression in MALT lymphoma with and without chromosome translocation, we measured the representation of GO terms in the above gene sets using hypergeometric tests. Among the genes overexpressed in translocation-positive MALT lymphoma, the GO terms assocaited with NF-κB pathway activation, chemokine/GPR signaling, and antigen presentation were significantly over-represented (Supplementary Table S18). Although among the genes overexpressed in translocation-negative MALT lymphoma, the GO terms related to immune/defense response were significantly over-represented (Supplementary Table S18). These findings from analysis of differentially expressed genes between MALT lymphomas with and without translocation reinforce the above observations by GSEA.

Validation of gene expression by qRT-PCR and immunohistochemistry

To confirm the differential expression of the key candidate genes between MALT lymphoma with and without translocation identified by the above microaray analyses, we analyzed the expression of *MALT1, BCL10, TLR6, CD69, CCR2A, CCR5, CD86* and *NR4A3* by qRT-PCR of the microdissected tumor cells from formalin-fixed paraffin-embedded tissue and wherein possible by immunohistochemistry in a total of 58 cases including 16 used in gene expression profiling. As expected, *MALT1* was most highly expressed in cases with t(14;18)/*IGH-MALT1* and *BCL10* was highest expressed in those with t(1;14) (Supplementary Figure S4). In keeping with the expression microarray data, *TLR6, CD69* and *CCR2A* were highly expressed in t(1;14) or t(11;18)-positive MALT lymphomas and

Table 1 Gene sets differentially over-represented between MALT lymphoma with and without chromosome translocation

Gene sets	*Source*	*SIZE*	*ES*	*NES*	*NOM P-val*	*FDR q-val*	*Tag %*	*Gene %*	*Leading edge core set genes*
NF-κB related									
NF-κB target genes	Supplementary Table S3for details	147	0.44	1.64	0.0109	0.0050	0.361	0.250	Supplementary Table S7
Positive regulation of IKK NF-κB cascade	GO:43123	43	0.49	1.80	0.0041	0.0754	0.395	0.209	Supplementary Table S8
Regulation of IKK NF-κB cascade	GO:43122	40	0.50	1.68	0.0148	0.1235	0.400	0.206	
Inflamation and immune response									
Response to chemical stimulus	GO:42221	118	0.42	1.64	0.0000	0.1388	0.475	0.356	Supplementary Table S9
Defense response	GO:6952	110	0.43	1.62	0.0079	0.1449	0.382	0.223	Supplementary Table S10
B-cell activation	GO:42113	21	0.58	1.57	0.0183	0.1661	0.381	0.080	
Innate immune response	GO:45087	42	0.45	1.55	0.0371	0.1503	0.548	0.359	Supplementary Table S11
Immune response	GO:6955	97	0.44	1.50	0.0431	0.1633	0.433	0.299	Supplementary Table S12
Chemokine and cell migration									
Cation homeostasis	GO:55080	47	0.47	1.66	0.0022	0.1280	0.362	0.193	Supplementary Table S13
Cellular cation homeostasis	GO:30003	46	0.46	1.63	0.0044	0.1376	0.348	0.193	
Locomotory behavior	GO:7626	46	0.52	1.64	0.0166	0.1432	0.370	0.125	Supplementary Table S14
GPR signaling and MAPK pathway									
Peptide GPCRs	MSD-C2 (Supplementary Table S2)	20	0.71	2.07	0.0000	0.0096	0.550	0.153	Supplementary Table S15
GPCRDdb class A rhodopsin like	MSD-C2 (Supplementary Table S2)	38	0.57	2.08	0.0000	0.0100	0.421	0.163	
Cyclic nucleotide mediated signaling	GO:19935	26	0.55	1.83	0.0000	0.0379	0.308	0.134	
G protein signaling coupled to cyclic nucleotide second messenger	GO:7187	26	0.55	1.83	0.0000	0.0379	0.308	0.134	
Regulation of kinase activity	GO:43549	72	0.43	1.85	0.0000	0.0744	0.333	0.186	
Regulation of protein kinase activity	GO:45859	71	0.43	1.86	0.0000	0.0838	0.338	0.186	
Negative regulation of catalytic activity	GO:43086	29	0.49	1.86	0.0000	0.1257	0.483	0.275	
Positive regulation of catalytic activity	GO:43085	66	0.40	1.74	0.0021	0.0876	0.273	0.178	
Regulation of MAPK activity	GO:43405	34	0.48	1.84	0.0024	0.0623	0.500	0.303	Supplementary Table S16
G protein coupled receptor protein signaling pathway	GO:7186	96	0.42	1.63	0.0041	0.1337	0.302	0.185	
G protein signaling coupled to camp nucleotide second messenger	GO:7188	19	0.55	1.70	0.0043	0.1108	0.316	0.134	
Positive regulation of signal transduction	GO:9967	51	0.43	1.63	0.0143	0.1399	0.471	0.348	
Proliferation and apoptosis									
Growth	GO:40007	24	0.52	1.74	0.0022	0.0930	0.458	0.255	
Regulation of growth	GO:40008	22	0.51	1.64	0.0044	0.1409	0.455	0.255	
Regulation of cell growth	GO:0001558	21	0.50	1.62	0.0065	0.1415	0.429	0.255	
Anti-apoptosis	GO:6916	70	0.38	1.50	0.0397	0.1514	0.300	0.218	
Others									
B-cell lymphoma	GeneGo	71	0.43	1.53	0.0489	0.1516	0.507	0.340	
Regulation of transferase activity	GO:51338	74	0.42	1.84	0.0000	0.0519	0.432	0.303	
Regulation of translation	GO:6417	26	0.52	1.77	0.0000	0.0699	0.615	0.364	
Lian myeloid diff receptors	MSD-C2 (Supplementary Table S2)	17	0.71	1.87	0.0000	0.1504	0.647	0.156	
Zhan MM CD138 CD2 BS rest	MSD-C2 (Supplementary Table S2)	24	0.57	1.83	0.0000	0.1947	0.625	0.267	
Behavior	GO:7610	57	0.51	1.69	0.0043	0.1138	0.333	0.125	
Ion homeostasis	GO:50801	50	0.45	1.59	0.0174	0.1728	0.340	0.193	

Abbreviations: ES, enrichment score; FDR, false dicovery rate; Gene %, the percentage of genes in the gene list before (for positive ES) of after (for negative ES) the peak in the running enrichment score; GO, gene ontology; GPCRs, G protein-coupled receptors; GPR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; MSD, molecular signature database (http://www.broadinstitute.org/gsea/msigdb/index.jsp); NES, normalised ES; NF, nuclear factor; NOM, nominal; Tag %, the percentage of gene tags before (for positive ES) of after (for negative ES) the peak in the running enrichment score.

CCR5 was highly expressed in t(1;14)-positive cases in comparison with translocation-negative cases (Supplementary Figure S4). Conversely, *CD86* and *NR4A3* were significantly highly expressed in translocation-negative MALT lymphomas (Supplementary Figure S4).

In keeping with the above qRT-PCR data, immunohistochemistry showed that most translocation-positive MALT lymphomas showed strong to moderate homogeneous BCL2 and CD69 staining in >70% tumor cells, often in most tumor cells, whereas majority of translocation-negative cases showed heterogeneous staining in 30–70% cells or a negative result (Figure 3). Similarly, western blot analyses showed that TLR6 was highly expressed in translocation-positive MALT lymphoma in comparison with the translocation-negative

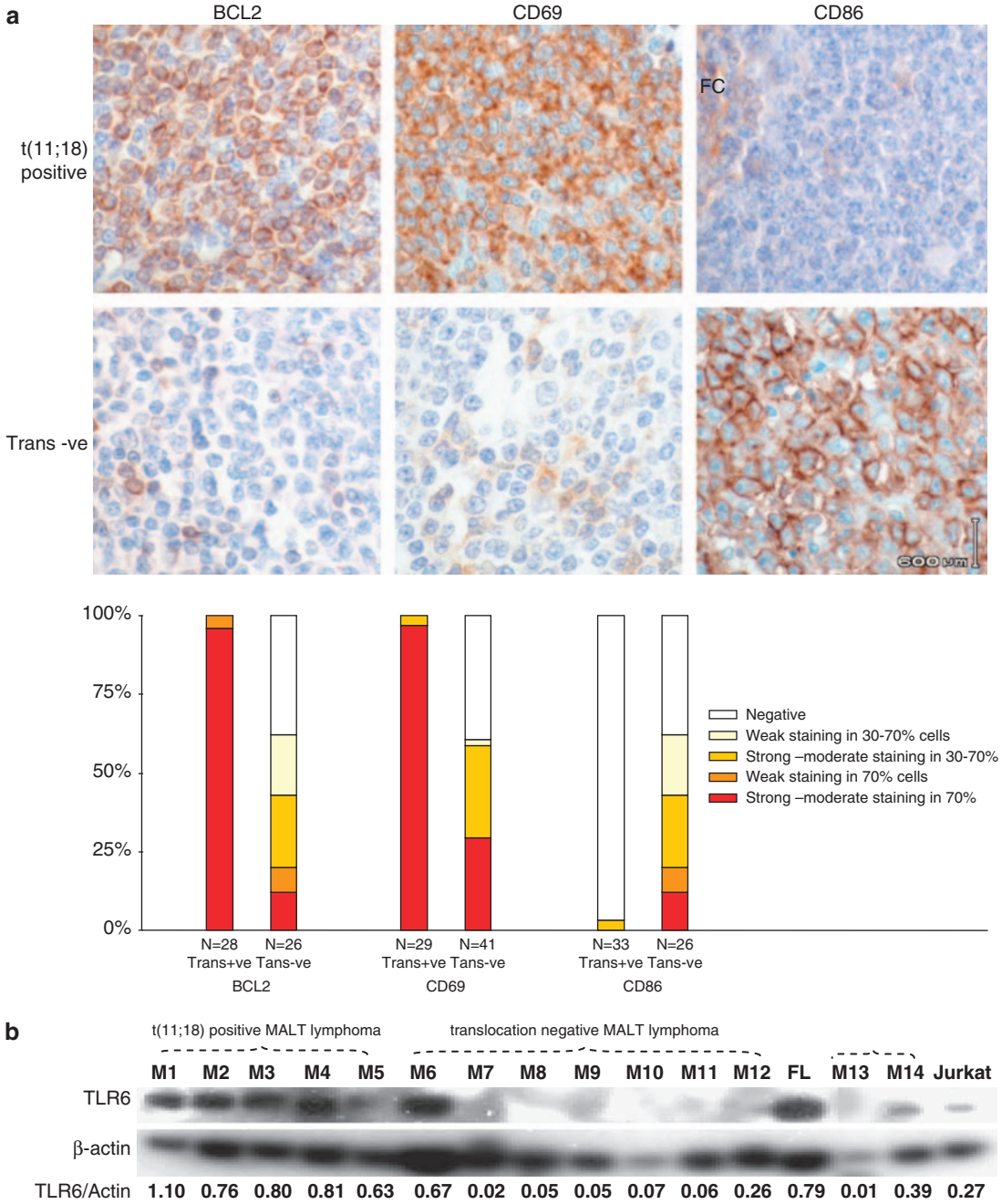

Figure 3 BCL2, CD69, CD86 and TLR6 protein expression in MALT lymphomas with and without chromosomal translocation. (**a**) Top panel: examples of BCL2, CD69 and CD86 immunohistochemistry in MALT lymphomas with and without chromosome translocation. Original magnification ×400 for all panels. Lower panel summaries BCL2, CD69 and CD86 immunohistochemical results in MALT lymphoma with and without chromosome translocation. BCL2 and CD69 are more strongly and homogeneously expressed in translocation-positive than translocation-negative MALT lymphoma ($P=6.9\times10^{-5}$, $P=2.2\times10^{-4}$ respectively by Fisher's exact test), whereas CD86 is more strongly expressed in translocation-negative than translocation-positive MALT lymphoma ($P=6.4\times10^{-7}$ by Fisher's exact test). FC, follicle center; Trans+ve, translocation-positive; Trans −ve, translocation-negative. (**b**) Western blot analysis shows that TLR6 is highly expressed in translocation-positive MALT lymphoma, but at low levels in translocation-negative cases. M, MALT lymphoma; FL, follicular lymphoma.

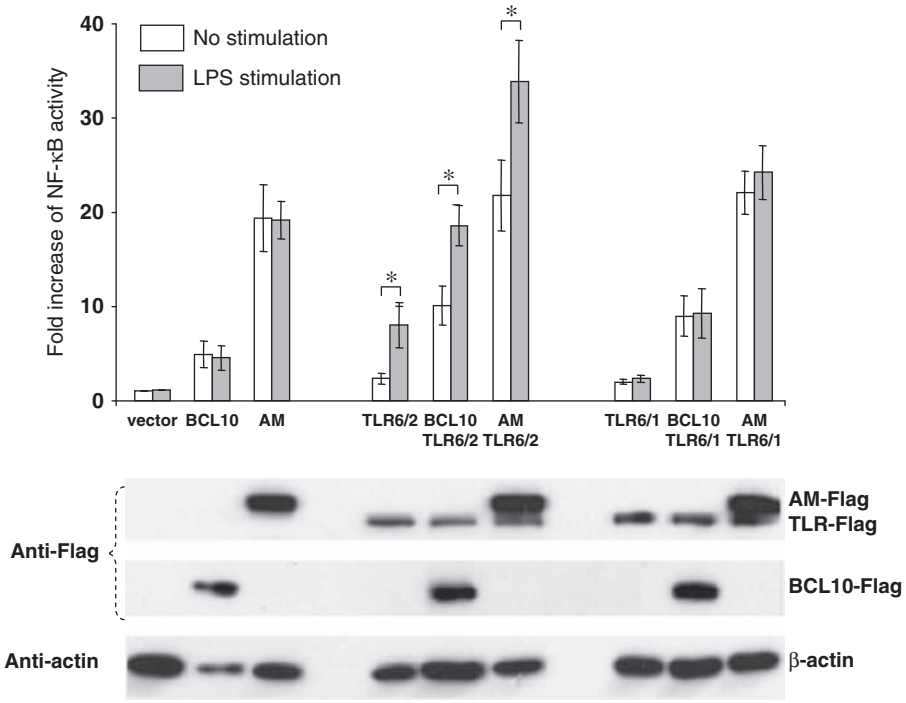

Figure 4 TLR6 enhances BCL10 and API2-MALT1-mediated NF-κB activation, in presence of TLR2 but not TLR1, in Jurkat T cells. Jurkat T cells were co-transfected with vector (pIRESpuro2) or plasmids containing *BCL10, API2-MALT1* (AM), *TLR6, TLR2* and *TLR1* as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multiple cell plates, cultured for 20 h and then treated with LPS or vehicle alone for 6 h. NF-κB activities were measured in quadruplet experiments and recorded as fold increase in the vector control. Western blot in the lower panel shows appropriate expression of various expression constructs. $*P<0.01$ by Student's *t*-test.

cases (Figure 3). In contrast, most translocation-positive MALT lymphomas showed no CD86 staining, whereas the majority of translocation-negative cases showed heterogeneous CD86 staining albeit variable in both positivity and intensity (Figure 3).

TLR6 expression enhances NF-κB activation by BCL10 and API2-MALT1 in vitro

Among the genes highly expressed in translocation-positive MALT lympoma, *TLR6*, *CCR2A*, *CD69* and *BCL2* were particularly interesting and we selected *TLR6* for further functional investigation because overexpression of this pattern recognition receptor may sensitize the response of tumor cells, particularly those with translocation, to stimulation by microbial antigens. To attest this, we performed a series of NF-κB reporter assays in Jurkat T cells, which are known not responding to lipopolysaccharides (LPS) stimulation, thus ideal for analyzing TLR signaling. Expression of TLR6 alone did not enhance BCL10 or API2-MALT1-induced NF-κB activation in Jurkat cells even in the presence of LPS stimulation (Supplementary Figure S5). TLR6 functions through the formation of heterodimer with its family member and typically forms heterodimer with TLR2 in responding to stimulation by bacterial antigen.[21] We next analyzed whether co-expression of TLR6 and TLR2 could enhance BCL10 or API2-MALT1-mediated NF-κB activation in the presence of LPS stimulation. As expected, TLR6/2 co-expression, in the presence of LPS stimulation, were synergistic with BCL10 and API2-MALT1 in activating the NF-κB pathway (Figure 4). In contrast, there was no cooperation between co-expression of TLR6/1 and BCL10 or API2-MALT1 in NF-κB activation (Figure 4).

Discussion

This study showed that MALT lymphoma was characterized by distinct expression profile in comparison with FL and MCL, in line with the recent finding by Chng *et al*.[22] Although unsupervised clustering analyses showed considerable overlap in the gene expression profiles between MALT lymphomas with and without chromosome translocation, there was important difference in the expression of NF-κB target genes between the two subgroups. By exhaustive GSEA of various molecular pathways and biological processes, we also showed that the gene sets related to inflammation, immune responses, chemokine and GPR signaling are differentially over-represented between these different subgroups. Importantly, several of these molecular pathways or biological processes also lead to NF-κB activation. These findings were reinforced by independent analyses of differentially expressed genes between MALT lymphomas with and without translocation using hypergeometric tests. Our observations provide several novel insights into the molecular mechanisms of both translocation-positive and -negative MALT lymphomas and potentially explain their different clinical and histological presentations.

Molecular mechanism of translocation-positive MALT lymphoma

In comparison with translocation-negative MALT lymphoma, GSEA and leading edge analyses revealed a common core subset of genes that were overexpressed in translocation-positive cases and a high proportion of them are NF-κB target genes involving multiple related biological processes or molecular pathways. The top examples included immune

receptors such as TLR6, TLR7, CD69 and CD1D, and chemokine receptor such as CCR2, CXCR4, CCR6 and CCR7, the apoptosis inhibitor BCL2, and positive regulators of the NF-κB pathway such as REL and molecules involved in GPR signaling (Figure 2, Supplementary Tables S7–S16). The overexpression of CCR2, BCL2, CD69 and TLR6 in translocation-positive cases was further confirmed in a large cohort of MALT lymphomas by qRT-PCR and/or immunohistochemistry/western blot analysis. All these molecules are expected to promote tumor cell survival and proliferation either directly or indirectly. Among these, the overexpression of the above immune surface receptors and chemokine receptors is particularly interesting.

TLR are critical in surveillance of microbial infection by recognizing pathogen-associated molecular patterns such as LPS and bacterial lipopeptides. In mouse model, it has been shown that TLR signaling promotes marginal zone B-cell activation and migration.[23] TLR6 typically forms heterodimers with TLR2 on the cell surface to recognize bacterial antigens.[21] TLR2/TLR6 signaling activates not only IkappaB kinase (IKK) complex that leads to activation of the NF-κB transcriptional factor, but also the mitogen-activated protein kinase (MAPK) p38 and Jun amino-terminal kinase that lead to activation of the activator protein 1 (AP-1) transcriptional factor.[24] Hence, overexpression of TLR6 in translocation-positive MALT lymphoma could potentially augment the NF-κB activity mediated by MALT lymphoma-associated oncogenic products and also activate the MAPK pathways. In this study, we tested the former hypothesis and showed indeed that expression of TLR6, in presence of TLR2, could enhance both BCL10 and API2-MALT1-mediated NF-κB activation *in vitro* and this effect was particularly significant on LPS stimulation. A role of TLR signaling in the pathogenesis of translocation-positive MALT lymphoma is also suggested by the followings: (1) *H. pylori* infection is invariably associated with translocation-positive gastric MALT lymphoma; (2) *H. pylori* activates NF-κB through both the classical and alternative pathway in B lymphocytes and this effect is dependent on LPS but not cag pathogenecity island;[25] (3) *H. pylori*-associated LPS-induced NF-κB activation requires TLR2/TLR6 or TLR2/TLR1 complex.[26] Taken together, these findings suggest that there is a potential biological cooperation between MALT lymphoma translocation and TLR signaling in the lymphomagenesis.

CD69, a type II transmembrane glycoprotein, is a potential co-stimulatory receptor and may also have an immunoregulatory role.[27] Although the precise function of CD69 in B cells is largely unknown, it is a well-described activation marker in several cell types, and its expression is upregulated in marginal zone B cells on TLR stimulation.[23] CD69 is frequently expressed in low-grade B-cell lymphomas, and in FL, its expression is associated with poor treatment outcome.[28,29] Our finding of enriched expression of CD69 in translocation-positive MALT lymphoma further implicates its role in lymphoma pathogenesis.

CCR are GPRs and mediate immune cells migration and their retention in the inflammatory site. As B-cell homeostatic chemokine receptor, CCR7, CCR6 and CXCR4 are crucial for this homing process. For example, CCR7 has a central role in the regulation of normal mucosal lymphocyte re-circulation and homeostasis, particularly in the stomach,[30] and CXCR4 is critical for B-cell homing to the Peyer's patches and splenic marginal zone.[31] Although the specific role of CCR2 in B-cell trafficking and homing is unclear, it forms heterodimer with CXCR4,[32] thus potentially having a role in mature B-cell homing process. In both low-grade B-cell lymphomas and classic Hodgkin lymphomas, CCR7 and CXCR4 overexpression were associated with a wide lymph node spread, supporting their role in lymphoma pathogenesis.[33–35] In addition to homing process, CCR signaling may also promote cell survival and proliferation through its activation of MAPK pathways. In this context, it is noteworthy that GPR is also targeted by chromosomal translocation in MALT lymphoma. A recent study reported deregulation of GPR34 expression by t(X;14)(p11;q32) in a salivary gland MALT lymphoma.[36] Importantly, expression of GPR34-induced activation of both the NF-κB and MAPK pathways *in vitro*.[36] In keeping with these findings, our GSEA also showed that several gene sets related to GPR signaling and MAPK pathways were enriched in translocation-positive MALT lymphoma.

As discussed above, several molecular pathways including signaling through TLR, and chemokine receptor may be operational in translocation-positive MALT lymphomas and contribute to the activation of the NF-κB pathway (Figure 5). Together with MALT lymphoma-associated oncogenic products, they cause relentless NF-κB activation, leading to the prolonged survival of tumor cells even in the case of obliteration of microbe-mediated immune responses, such as *H. pylori* eradication in gastric MALT lymphoma. In this regard, it is to be noted that the apoptosis inhibitor BCL2 was remarkably uniformly overexpressed virtually in all tumor cells in nearly all translocation-positive cases. In contrast, the protein was heterogeneously expressed, at a much lower level, in tumor cells of translocation-negative cases.

In spite of the above overwhelming evidence of NF-κB activation in translocation-positive MALT lymphoma, there was considerable heterogeneity in the expression of NF-κB target genes among these lymphomas. Not all translocation-positive MALT lymphomas showed uniform overexpression of the leading edge core set of the NF-κB target genes described above, nor each of the translocation-negative cases showed a complete lack of expression of these NF-κB target genes (Figure 2, Supplementary Figure S4). This is also consistent with the clinical response of gastric MALT lymphoma to *H. pylori* eradication therapy. Although most of t(11;18)-positive gastric MALT lymphomas do not respond to *H. pylori* eradication, there are occasional cases responsive to the antibiotic treatment,[37] suggesting that not all translocations have the same biological effect. Equally, the majority of translocation-negative gastric MALT lymphomas can be cured by *H. pylori* eradication, but there are 10–20% cases that are negative for *MALT1*, *BCL10* and *FOXP1* involved translocations, and do not respond to *H. pylori* eradication,[37] suggesting presence of other unknown genetic abnormalities that may also target the NF-κB pathway.

Molecular mechanism of translocation-negative MALT lymphoma

In contrast to translocation-positive MALT lymphoma, translocation-negative cases were characterized by expression of a strong inflammatory gene signature. GSEA and leading edge analysis also revealed common core subset genes involving several related biological processes or molecular pathways, which were enriched in translocation-negative MALT lymphoma. The top examples included proinflammatory cytokines IL8 and IL1β, molecules involved in B- and T-cell interaction such as CD86, CD28 and ICOS, several chemokine and chemokine receptors and NR4A3 (also known as MINOR) (Figure 2, Supplementary Tables S7, S9, S10, S12, S14).

IL8 and IL1β are the hallmark of proinflammatory cytokine profile in response to *H. pylori* infection. IL8 is critical for neutrophil infiltration and activation, whereas IL1β induces gastrin release, inhibits acid secretion and promote apoptosis of epithelial cells.[38] The finding of overexpression of these

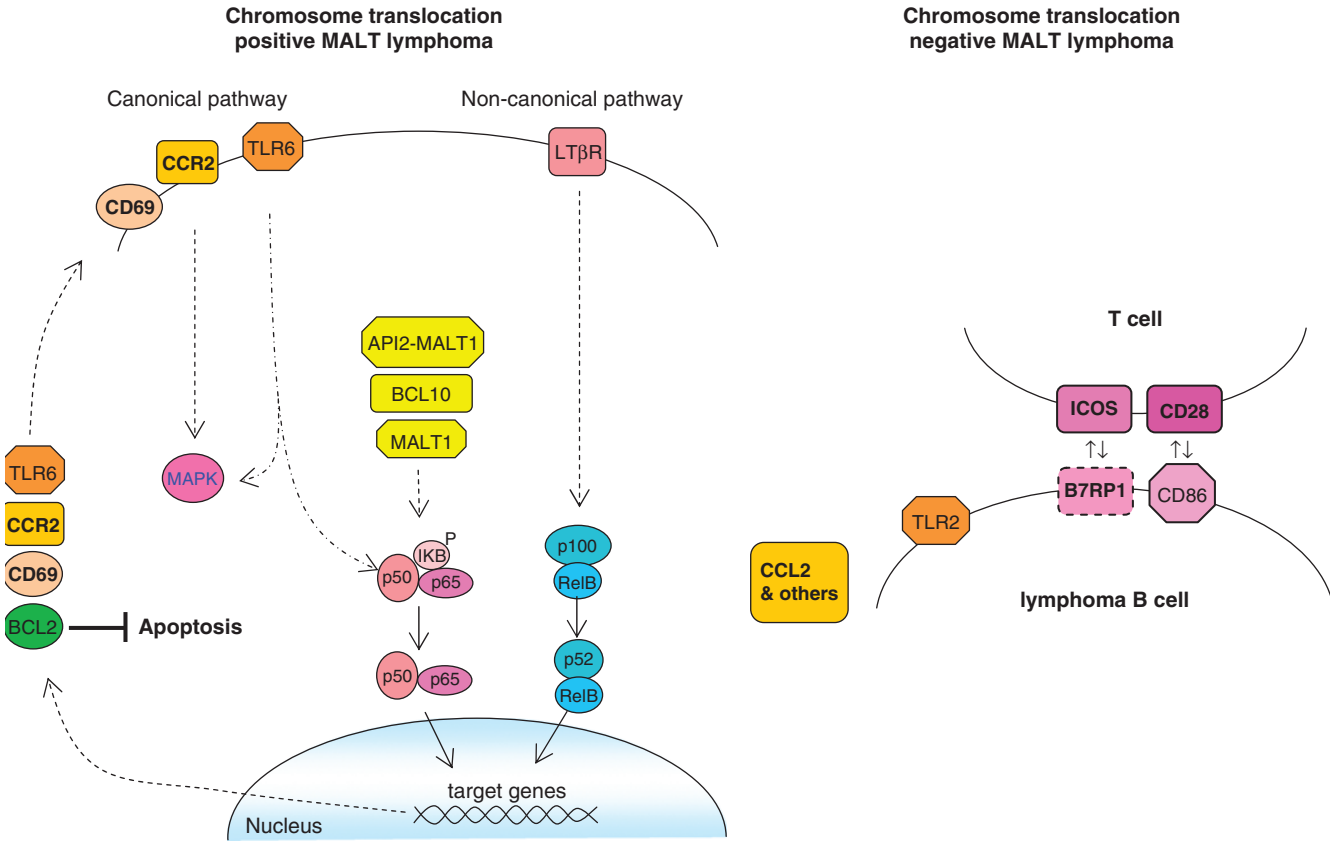

Figure 5 Summary and hypothesis on molecular mechanism of MALT lymphoma with and without chromosomal translocation. In translocation-positive MALT lymphoma, overexpression of API2-MALT1, BCL10 and MALT1 activates the canonical NF-κB pathway, leading to enhanced expression of the NF-κB target genes, particularly *TLR6*, *CCR2*, *CD69* and *BCL2*. Overexpression of TLR6 may provide a further positive feedback to the activation of the NF-κB pathway. Similar positive feedback may also be expected from the CCR2 signaling, and in addition both TLR6 and CCR2 may trigger activation of the MAPK pathway. The pathogenic implication of enhanced CD69 expression is currently unknown. Overexpression of BCL2 is expected to promote the tumor cell survival. In essence, the above chromosome translocations cause constitutive NF-κB activation with expression of its target genes forming a potential positive feedback loop, and the relentless NF-κB activation, in the case of gastric MALT lymphoma, confers its resistance to *H. pylori* eradication. In translocation-negative MALT lymphoma, the ongoing inflammatory and immune responses maintain active cognate B and T cell interaction through co-stimulating molecules CD86/CD28, B7RP1/ICOS, which are the major determinants of tumor cell survival and thus explain, in the cases of gastric MALT lymphoma, their responses to *H. pylori* eradication.

proinflammatory cytokines in translocation-negative gastric MALT lymphomas, indicates the presence of active *H. pylori* infection. In keeping with this, translocation-negative gastric MALT lymphomas show a higher number of blast cells than translocation-positive cases.[16] In addition, a number of chemokines and chemokine receptors were highly expressed in the translocation-negative cases. This may reflect the trafficking and retention of various immune cells in response to an active *H. pylori* infection.

Most importantly, GSEA showed enriched expression of the surface molecules involved in B- and T-cell interaction namely CD86, CD28 and ICOS in translocation-negative gastric MALT lymphoma. Although residual reactive follicles may be present and contribute to the high CD86, CD28 and ICOS expression in translocation-negative cases, the germinal center markers CD10 and BCL6 were expressed in much low levels in MALT lymphoma (Supplementary Figure S1), and more importantly overexpression of CD86 in tumor cells was clearly shown by qRT-PCR and immunohistochemistry. In line with our finding, a previous study showed significantly higher CD86 expression in gastric MALT lymphomas that responded to *H. pylori* eradication than those resisted to the therapy (66% vs 10%).[39] Although the chromosome translocation status in these cases is not available, it is most likely that the cases responded to *H. pylori* were translocation-negative.[14] Taken together, these findings suggest that there is an active immune response to *H. pylori* infection in translocation-negative gastric MALT lymphoma, and this most likely underscores the tumor cell survival and expansion, and thus determines their response to *H. pylori* eradication (Figure 5).

In summary, this study shows that (1) translocation-positive MALT lymphoma is in general characterized by an enhanced expression of NF-κB target genes, particularly *CCR2*, *TLR6* and *BCL2*; (2) the oncogenic products of MALT lymphoma-associated translocation may cooperate with signaling from several surface receptors including TLR6 and chemokine receptors in activation of the NF-κB pathways; and (3) translocation-negative MALT lymphoma is featured by active inflammatory and immune responses to *H. pylori* infection, and tumor cell interaction with infiltrating T cells through co-stimulating molecules (especially CD86/CD28) may have an important role in their survival and clonal expansion.

Conflict of interest

The authors declare no conflict of interest.

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Authors contribution

RAH designed the experiment, collected and analyzed the data. AA, HY and LG contributed to the design and experimental data collection and analysis; ARF, BS, AC, MR, IW, CDWP, KAM, LdL and PGI provided lymphoma cases; MQD designed, analyzed the data and wrote the paper.

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