CCR6 activation links innate immune responses to mucosa-associated lymphoid tissue lymphoma development

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Abstract

The genesis of extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT) is driven by oncogenic co-operation among immunological stimulations and acquired genetic changes. We previously identified recurrent *CCR6* mutations in MALT lymphoma, with majority predicted to result in truncated proteins lacking the phosphorylation motif important for receptor desensitization. Functional consequences of these mutational changes, the molecular mechanisms of CCR6 activation and how this receptor signaling contributes to MALT lymphoma development remain to be investigated. In the present study, we demonstrated that these mutations impaired CCR6 receptor internalization and were activating changes, being more potent in apoptosis resistance, malignant transformation, migration and intracellular signaling, particularly in the presence of the ligands CCL20, HBD2 (human β defensin 2) and HD5 (human α defensin 5). CCR6 was highly expressed in malignant B cells irrespective of the lymphoma sites. HBD2 and CCL20 were constitutively expressed by the duct epithelial cells of salivary glands, and also those involved in lymphoepithelial lesions (LEL) in salivary gland MALT lymphoma. While in the gastric setting, HBD2, and HD5, to a less extent CCL20, were highly expressed in epithelial cells of pyloric and intestinal metaplasia respectively including those involved in LEL, which are adaptive responses to chronic *Helicobacter pylori* infection. These findings suggest that CCR6 signaling is most likely active in MALT lymphoma, independent of its mutation status. The observations explain why the emergence of malignant B cells and their clonal expansion in MALT lymphoma are typically around LEL, linking the innate immune responses to lymphoma genesis.

Introduction

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is a broad group of low-grade B-cell lymphomas from various mucosal sites. Despite sharing similar histological and biological characteristics, the lymphoma at various sites shows divergence in its etiology and mutation profile.[1] As the lymphoma derives from a background of chronic inflammatory disorder, the emergence and expansion of the neoplastic B-cell clone are most likely the result of oncogenic cooperation between the persistent antigenic stimulations and acquired somatic genetic changes. There is increasing evidence showing that the acquired genetic changes affect the signaling pathways critical for the function of marginal zone B cells, and corroborate with the antigenic drive or stimuli generated by the local microenvironment in lymphoma development.[1]

A classic example is that overexpression of MALT lymphoma associated oncogenes such as *BCL10*, *MALT1* or *BIRC3-MALT1* alone is insufficient for lymphoma development, but capable of inducing lymphoma-like lesions in the presence of immunogenic stimulations in various animal models.[2,3] Among immunological stimulations, chronic B-cell receptor (BCR) activation, and T-cell help, particularly CD40/CD40L co-stimulation, are the signaling pathways known to be critically involved in the development of gastric MALT lymphoma.[4-8] This notion has now been extended to MALT lymphoma of other sites, with more receptor signaling identified to be involved in

lymphomagenesis. For example, in thyroid MALT lymphoma that invariably arises in a background of Hashimoto's thyroiditis, *CD274* (PD-L1) and *TNFRSF14* are commonly inactivated by mutation and/or deletion, and their inactivation eliminates their inhibitory regulation of T-helper cells, thus may enable T cells to provide more help to malignant B cells.[9] Similarly, in salivary gland MALT lymphoma that is closely associated with lymphoepithelial sialadenitis (myoepithelial sialadenitis), *GPR34* is activated by somatic mutations or t(X;14)(p11;q32), and also a paracrine stimulation via ligands generated by the lymphoepithelial lesion.[10-12]

Apart from GPR34, CCR6 is another G protein-coupled receptor (GPCR) which is recurrently mutated in MALT lymphoma.[12] But unlike *GPR34*, *CCR6* mutation is not restricted to MALT lymphoma of the salivary glands, but also seen in those of the stomach and thyroid.[12] The majority of *CCR6* mutations are nonsense changes or frameshift indels that are clustered in the C-terminal region, resulting in truncated products lacking the C-terminal phosphorylation motif (Figure 1A),[12] which is responsible for binding to β-arrestin and receptor desensitization. The remaining mutations are missense changes including R159S and Y352C affecting the second intracellular loop and a putative C-terminal phosphorylation site respectively.[12] It is unclear how these mutations affect CCR6 function and whether CCR6 signaling is also maintained by microenvironmental stimuli in MALT lymphoma, independent of its genetic changes.

CCR6 is a member of class-A GPCR superfamily, which transduces ligand stimulation to intracellular signaling through G proteins. CCR6 is expressed in a range of leukocytes including mature B cells and their derived lymphomas, with the protein expression seen in 84-100% of MALT lymphoma.[13-16] The ligands for CCR6 include CCL20 and β defensins.[13] CCL20, also known as macrophage inflammatory protein 3α (MIP-3α), is produced by a range of cell types including macrophages and endothelial cells, and its expression is typically upregulated by inflammatory cytokines.[13] β defensins are small, cationic, antimicrobial peptides, and produced by epithelial cells and leukocytes, particularly the former.[17,18] Among human β defensins (HBD), HBD1-3 have been shown to act as a ligand for CCR6, and these defensins, like CCL20, are highly upregulated during inflammatory responses, to recruit and activate leukocytes.[17,18] The CCR6/CCL20 axis is critical for humoral immune responses, particularly at mucosal sites, while the role of CCR6 and HBD interaction in B-cells and their derived lymphomas remain to be investigated.[17,18]

In order to understand the oncogenic action of CCR6 and its mutants, we investigated their receptor signaling and transformation potential *in vitro*, and their responses to stimulation by CCL20, HBD2 and human α defensin 5 (HD5). We also investigated the expression of CCR6, CCL20, HBD2 and HD5 in primary MALT lymphoma tissue specimens, and identified evidence supporting paracrine stimulation of lymphoma B cells via CCR6 by CCL20/HBD2/HD5 released by the inflamed epithelial cells.

Methods

CCR6 expression constructs

The *CCR6* wild-type and mutants (R159S and W335X) were cloned separately into pcDNA5/FRT (*Online Supplementary Figure S1*) and pIRESpuro vectors. All the above constructs contained a HA-tag at the N-terminus of CCR6, and a separate set of these constructs with an additional C-terminal GFP-tag were also generated.

Generation of a single copy *CCR6* stable expression isogenic cell lines

Flp-InTRex293 host cells (genetically engineered from HEK293 to enable targeted integration of a single copy expression vector) were used to generate isogenic cell lines that stably express a single copy of the wild-type or mutant *CCR6* expression construct, thus permitting direct comparison in their expression levels and response to ligand stimulations. Expression of recombinant CCR6 was confirmed (*Online Supplementary Figure S2*).

Generation of CCR6 enriched expression DG75 line

DG75 cells overexpressing the wild-type or mutant *CCR6* were generated and recombinant protein expression was confirmed by flow cytometry (*Online Supplementary Figure S3*).

Effect of CCR6 expression on cytotoxic challenge

The isogenic Flp-InTRex293 cells and the DG75 B cells that express recombinant *CCR6*, were subjected to staurosporine treatment in the presence of CCL20 or vehicle for 16 hours. Level of cell death was determined by flow cytometry analysis of annexin V.

Soft agar colony formation assay, wound scratch healing assay and transwell migration assays

These experiments were performed using the Flp-InTRex293 cells that carried a single copy of the wild-type or mutant *CCR6* expression construct, together with parental cells (please refer to the *Online Supplementary Appendix* for details)

Analysis of CCR6 internalization

For time-lapse microscopy, the Flp-InTRex293 cells that carried a single copy of wild-type or various mutant *CCR6*

with C-terminal GFP-tag were treated with CCL20 (50 nM) in FluoroBrite medium (Gibco) while recording by time-lapse microscopy for 30 minutes (*Online Supplementary Figure S4*). The CCR6 expression was quantified using Image J (https://theolb.readthedocs.io/en/latest/imaging-/measuring-cell-fluorescence-using-imagej.html).

For flow cytometry analysis, the Flp-InTRex293 cells with a single copy of the wild-type or mutant *CCR6* expression construct were treated with CCL20 (50 nM), and an aliquot (100 μL) was taken out at the indicated times for measuring surface CCR6.[10,19]

Dual luciferase reporter assay

The firefly reporter plasmids for *CRE* (cAMP/PKA), *SRF-RE* (RhoA), *SRE* (MAPK/ERK), *NFAT-RE* (Calcium/Calcineurin), TCF/LEF-RE (Wnt), *ISRE* (JAK/STAT1/2), *AP1* (MAPK/JNK), *NFκB, CSL* (NOTCH) and the Renilla pRL-TK control vector were from our previous studies.[10,20,21] Reporter assays were optimized before data collection (*Online Supplementary Figure S5*). For detailed methodology, please see the *Online Supplementary Appendix*.

Prediction of coupling probabilities of CCR6

Coupling probability of G protein α to CCR6 wild-type and its various mutants was estimated by using an online machine-learning program PRECOG (predicting coupling probabilities of G-protein coupled receptors) (http://precog.russelllab.org) (*Online Supplementary Figure S6*).[22]

Immunohistochemistry

Local ethical guidelines were followed for the use of archival tissues for research with ethical approval (05-Q1604-10).

The expression of CCR6, CCL20, HBD2, TFF2 (trefoil factor 2) and HD5 in MALT lymphoma, lymphoepithelial sialadenitis and normal salivary glands, was investigated by immunohistochemistry on formalin-fixed paraffin-embedded tissue sections (*Online Supplementary Table S1*). Please refer to the *Online Supplementary Appendix* for details.

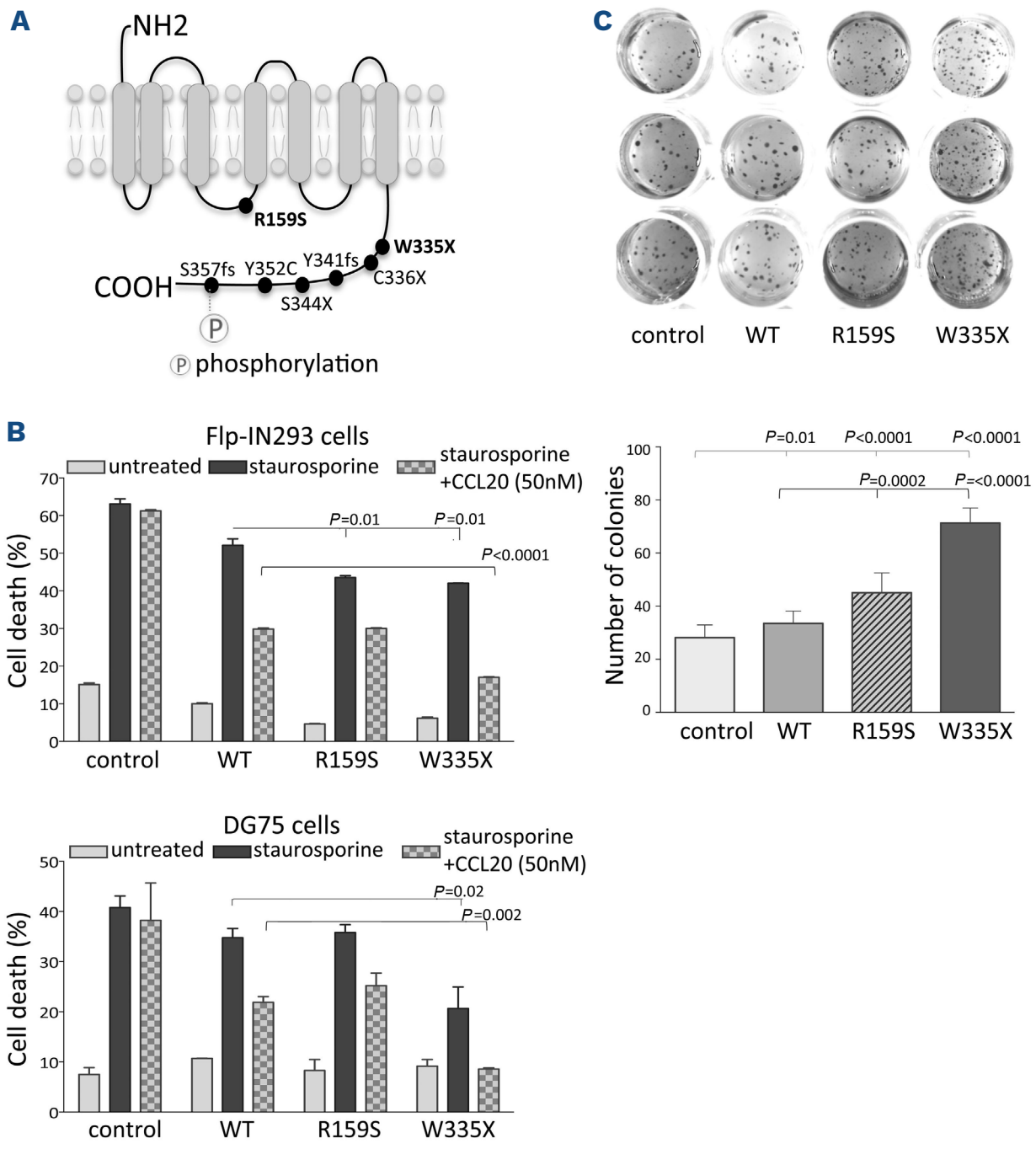


Figure 1. CCR6 mutants confer increased resistance to apoptosis and greater transforming capacity. Schematic presentation of CCR6 mutations seen in MALT lymphoma. (A) Effect of CCR6 mutation on cell survival induced by staurosporine. Isogenic Flp-IN293 cells (top panel) that stably expressed a single copy of CCR6 or its mutants, and DG75 cells (below) that express CCR6 or its mutants were treated with 1.5 nM staurosporine in the presence or absence of CCL20 stimulation for 16 hours, cell death was then measured by flow cytometry analysis of annexin V binding. (B) Transforming potential of CCR6 and its mutants determined by soft agar colony formation assay. Isogenic Flp-INTRex293 cells that stably expressed a single copy of CCR6 or its mutants were grown on soft agar for 3 weeks and colonies were stained with a crystal violet (top) and quantified (below).

The data (mean + standard deviation) presented in (B and C) are from at least 3 independent experiments. Statistical significance is analyzed by a two-tailed unpaired *t*-test, with significance indicated. WT: wild-type.

Results

CCR6 mutants confer resistance to cell death

In order to investigate whether *CCR6* mutations conferred more resistance to cytotoxic damages, we investigated their effect on staurosporine induced cell death. In the absence of any ligand stimulation, both isogenic Flp-InTRex293 and DG75 cells expressing the CCR6 truncation, and the Flp-InTRex293 cells expressing the R159S mutant showed a significantly reduced cell death than those expressing the wild-type respectively (Figure 1B). In the presence of CCL20 ligand stimulation, all cell lines overexpressing CCR6 showed a significant reduction of cell death, and the Flp-InTRex293 and DG75 cells expressing the CCR6 truncation mutant displayed a significantly reduced cell death than those expressing the wild-type.

CCR6 mutants show enhanced transforming potential

In order to test whether mutation potentiated CCR6 transforming ability, we performed soft agar colony formation assay using the isogenic Flp-InTRex293 cells expressing the wild-type or mutant CCR6. The cells expressing the CCR6 W335X truncation, to a lesser extent the R159S mutant, showed a significantly higher number of colonies than those expressing the wild-type (Figure 1C).

CCR6 mutants show enhanced migration ability

In the absence of ligand stimulation, the isogenic Flp-InTRex293 cells expressing the CCR6 W335X truncation mutant showed an enhanced migration capacity than those expressing the wild-type as shown by the wound healing assay (Figure 2A). In the presence of CCL20 stimulation, both CCR6 W335X and R159S mutants displayed a significantly greater response to wound healing than the wild-type.

Similar results were also seen from the transwell assays. Under the CCL20 stimulation, both CCR6 mutants displayed a significantly greater migration capacity than the wild-type (Figure 2B). While under the HBD2 stimulation, both mutants showed a similar trend, but only the truncation mutant reached a significant difference in comparison with the wild-type.

CCR6 mutants show delayed internalization

Receptor internalization following ligand stimulation downregulates GPCR signaling. In order to investigate whether mutation affected CCR6 internalization, we monitored the protein membrane expression in isogenic Flp-InTRex293 cell lines that expressed a single copy of wild-type or mutant CCR6 following CCL20 stimulation. In comparison with the wild-type CCR6, the W335X truncation mutant showed a significant higher level of membrane retention by time lapse microscopy (Figure 3A; *Online Supplementary Figure S4*). Similarly, flow cytometry analysis of these isogenic cell lines also demonstrated a significantly higher level of surface retention of the truncation mutant than the wild-type following CCL20 stimulation (Figure 3B). In both assays, there was no significant difference in surface CCR6 retention between the R159S missense mutant and the wild-type. In line with this, the microscopic appearance also showed a higher level of CCR6 W335X expression than both CCR6 R159S and CCR6 wild-type (Figure 3A).

CCR6 mutants show enhanced signaling capacity

Following ligand stimulation, GPCR may activate multiple cellular signaling through interaction with G-proteins. In order to investigate the signaling pathways activated by CCR6 and whether this was potentiated by mutation, we examined the activities of nine signaling pathways in DG75 cells using dual luciferase report assays (Figure 4; *Online Supplementary Figure S7*). In the absence of ligand stimulation, both the wild-type and mutant CCR6, particularly the truncation mutant showed a low level of enhanced NFAT RE (calcium/calcineurin) and TCF-LEF (Wnt/β-catenin) luciferase reporter activities in comparison with the control (Figure 4). In the presence of CCL20 stimulation, both CCR6 wild-type and mutant displayed a significantly increased SRE (MAPK/ERK) than their respective controls. Additionally, the two mutants, particularly the W335X truncation change, exhibited significantly greater SRF-RE (RhoA) reporter activities than the wild-type CCR6. The CCR6 W335X mutant also showed small, but significantly enhanced CSL (Notch), NFκB and CRE (cAMP/PKA) reporter activities than the wild-type (*Online Supplementary Figure S7*).

We also performed SRE (MAPK/ERK) and SRF-RE (RhoA) reporter assays using the isogenic Flp-InTRex293 cells that expressed a single copy of wild-type or mutant CCR6 (Figure 5A). In the presence of CCL20 or HBD2 stimulation, both CCR6 wild-type and mutants showed significantly enhanced SRE (MAPK/ERK) and SRF-RE (RhoA) reporter activities than their respective controls. Furthermore, the CCR6 W335X truncation, to a lesser extent the R159S mutant where indicated, displayed significantly greater reporter activities than the wild-type. As expected, the SRF-RE (RhoA) reporter activities could be eliminated in the presence of the Rho-associated protein kinase ROCK inhibitor Y27632 (Figure 5A).[23]

In order to explore whether other defensin family members may also serve as ligand for CCR6,[18] we modeled CCR6 and ligand binding using molecular docking software (https://bioinfo3d.cs.tau.ac.il/PatchDock/). This revealed that HD5 had a predicted binding affinity to CCR6, similar to CCL20, and subsequent reporter assays confirmed that HD5 is capable of stimulating CCR6 and its mutants (Figure 5B).

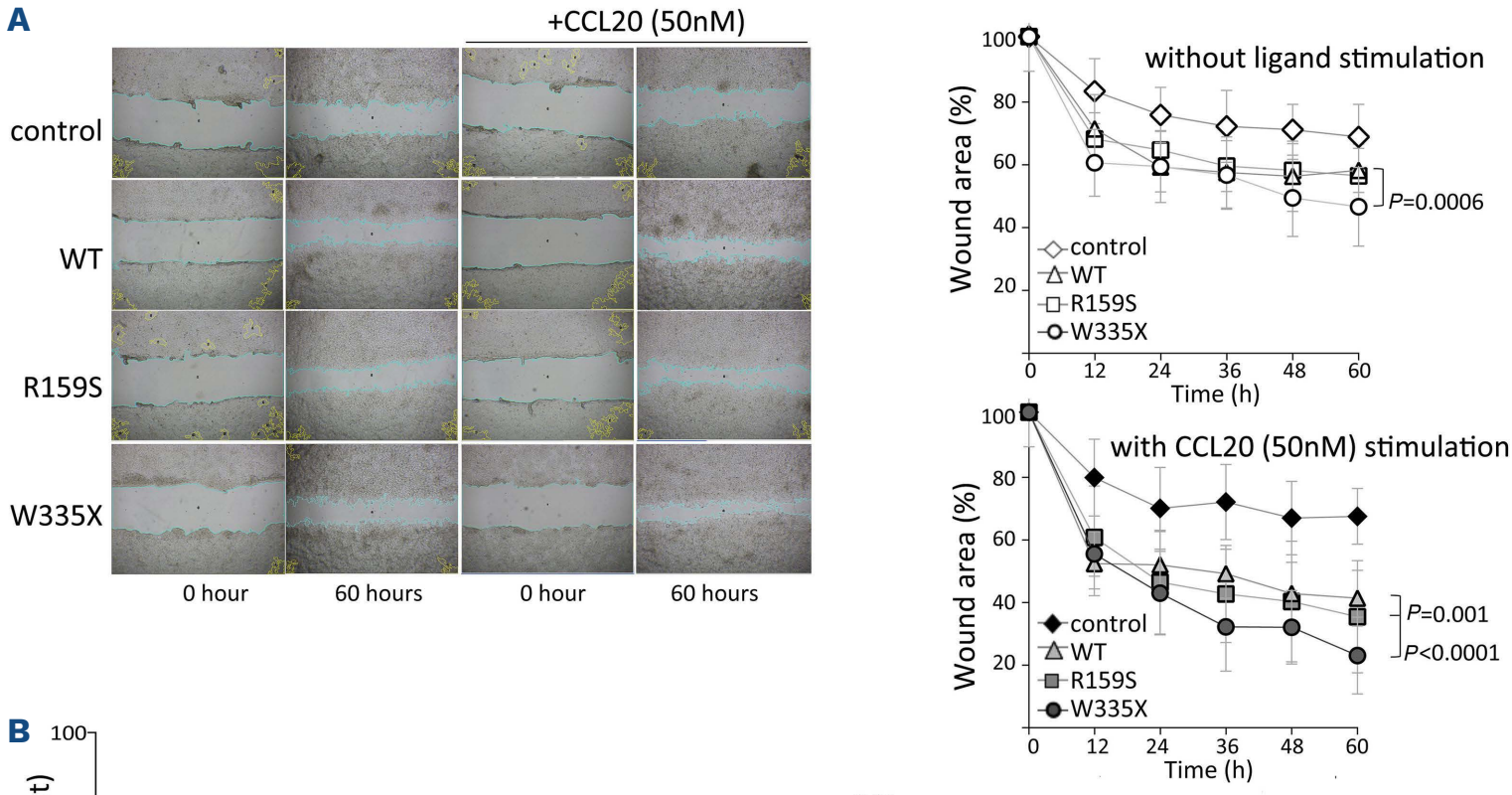


Figure 2. Effect of CCR6 mutation on cell migration. (A) Isogenic Flp-IN293 cells (left) that stably expressed a single copy of CCR6 or its mutants, together with their parental cell line, were measured for their migration capacity using wound closure assay (left panel). For each cell line, at least 19 images of the wound are taken at the time points indicated (right panel), and wound closure is presented as percentage of the initial scratched area. The data presented (mean ± standard deviation) are from 3 independent experiments. (B) Similarly, the isogenic Flp-IN293 cells that stably expressed a single copy of CCR6 or its mutants, together with their parental cell line, were assessed for their migrating capacity using transwell assays. The data presented (mean + standard deviation) are from 3 independent experiments. Statistical significance is analyzed by one-way ANOVA with significant differences indicated. WT: wild-type.

Prediction of Gα coupling probabilities of CCR6

In general, there was a similar profile of Gα coupling probabilities among the wild-type CCR6 and its mutants as shown by the machine-learning program PRECOG although a higher binding probability for $G\alpha_{i3}$ and $G\alpha_{15}$ was found for the truncation mutant (*Online Supplementary Figure S6*).[22,24] The predicted Gα coupling profile was in line with the observed signaling activities measured by reporter assays (Figure 4).[24-27]

CCR6 and its ligand expression in mucosa-associated lymphoid tissue lymphoma

In order to investigate whether CCR6 signaling was operational in MALT lymphoma, we investigated CCR6 and its ligands (CCL20, HBD2, HD5) expression in primary lymphoma tissue specimens by immunohistochemistry.

Among the 29 cases of MALT lymphoma examined, strong CCR6 expression in malignant B cells was seen 26 cases, independent of the *CCR6* mutation status and lymphoma sites (Figures 6 and 7), although all the mutants cases (n=3, p.C336X, p.S344X, p.Y352C) examined showed strong, more uniform staining (*Online Supplementary Figure S8*). Interestingly, CCR6 expression was high in the malignant B cells involving in lymphoepithelial lesions, but down-regulated in those undergoing plasmacytic differentiation (Figure 6).

The expression of CCR6 ligands HBD2 and CCL20 was investigated in MALT lymphoma (salivary gland: n=14; stomach: [n=29 for HBD2; n=11 for CCL20]; thyroid: n=79), lymphoepithelial sialadenitis (n=5), chronic gastritis (n=3) and normal salivary gland tissues (n=2), and HD5 only in gastrointestinal tissues in light its known expression in Paneth cells.[28] These ligands were not expressed in malignant B cells, but found in epithelial cells with their ex-

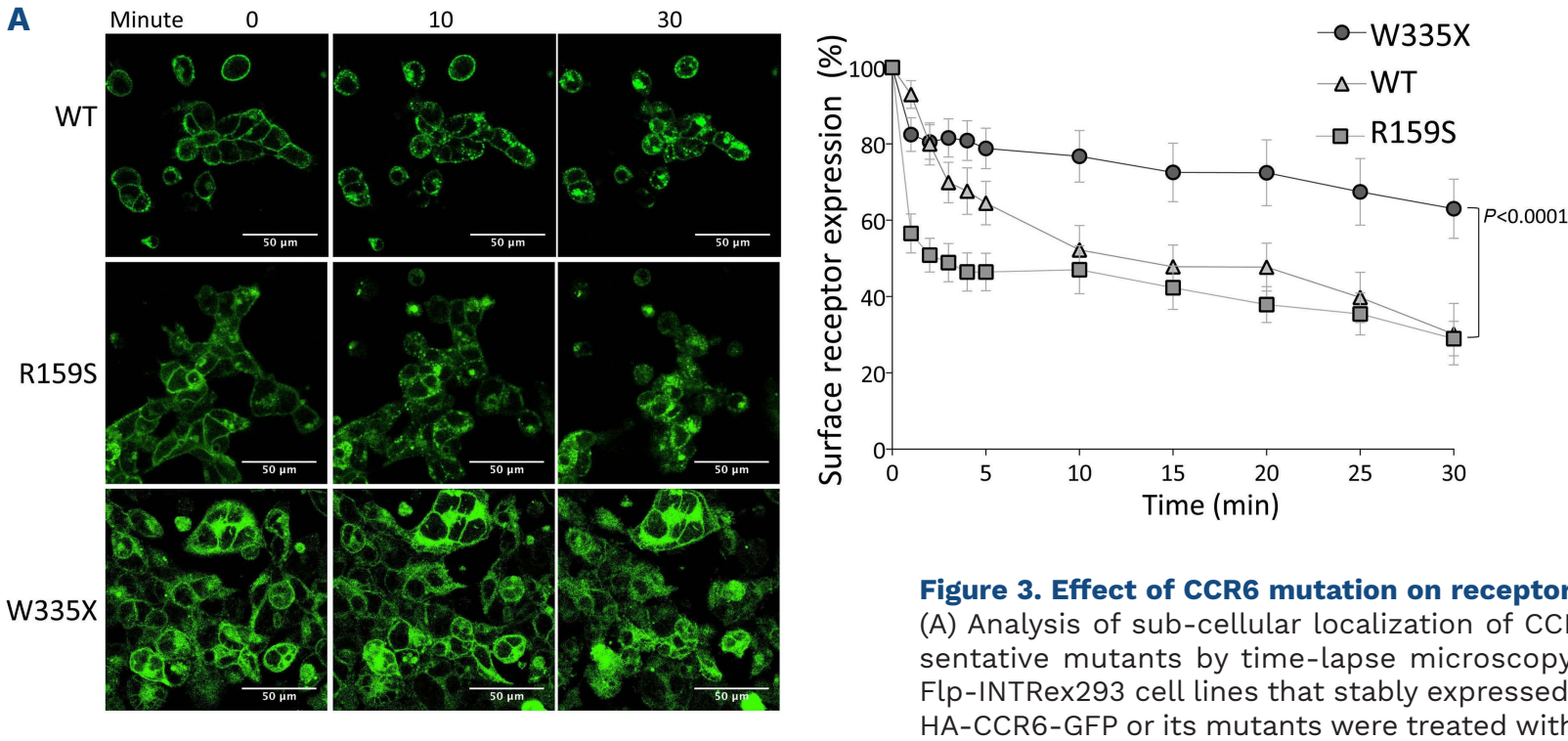


Figure 3. Effect of CCR6 mutation on receptor internalization. (A) Analysis of sub-cellular localization of CCR6 and it representative mutants by time-lapse microscopy (n=3). Isogenic Flp-INTRex293 cell lines that stably expressed a single copy of HA-CCR6-GFP or its mutants were treated with CCL20 (50 nM), and CCR6 subcellular localization was monitored for 30 minutes. Shown are images at the indicated times following ligand stimulation (left). Please also refer to the *Online Supplementary Figure S4* for the video record. The CCR6 membrane expression in individual cells was qualified at the indicated times using Image J and normalized to the 0 time point (right). Comparison between wild-type (WT) CCR6 and its various mutants was performed with Prism6 non-linear regression analyses, with significantly difference indicated. (B) The experiment is similarly carried out as above but with CCR6 surface expression analyzed by flow cytometry using an anti-HA antibody. The CCR6 surface expression is normalized to the time 0 point in each cell line. The data (mean ± standard deviation) presented are from 3 independent experiments. Statistical comparison between wild-type CCR6 and its mutants was carried out using Prism6 non-linear regression analyses (sigmoidal model fitting), with significant differences indicated.

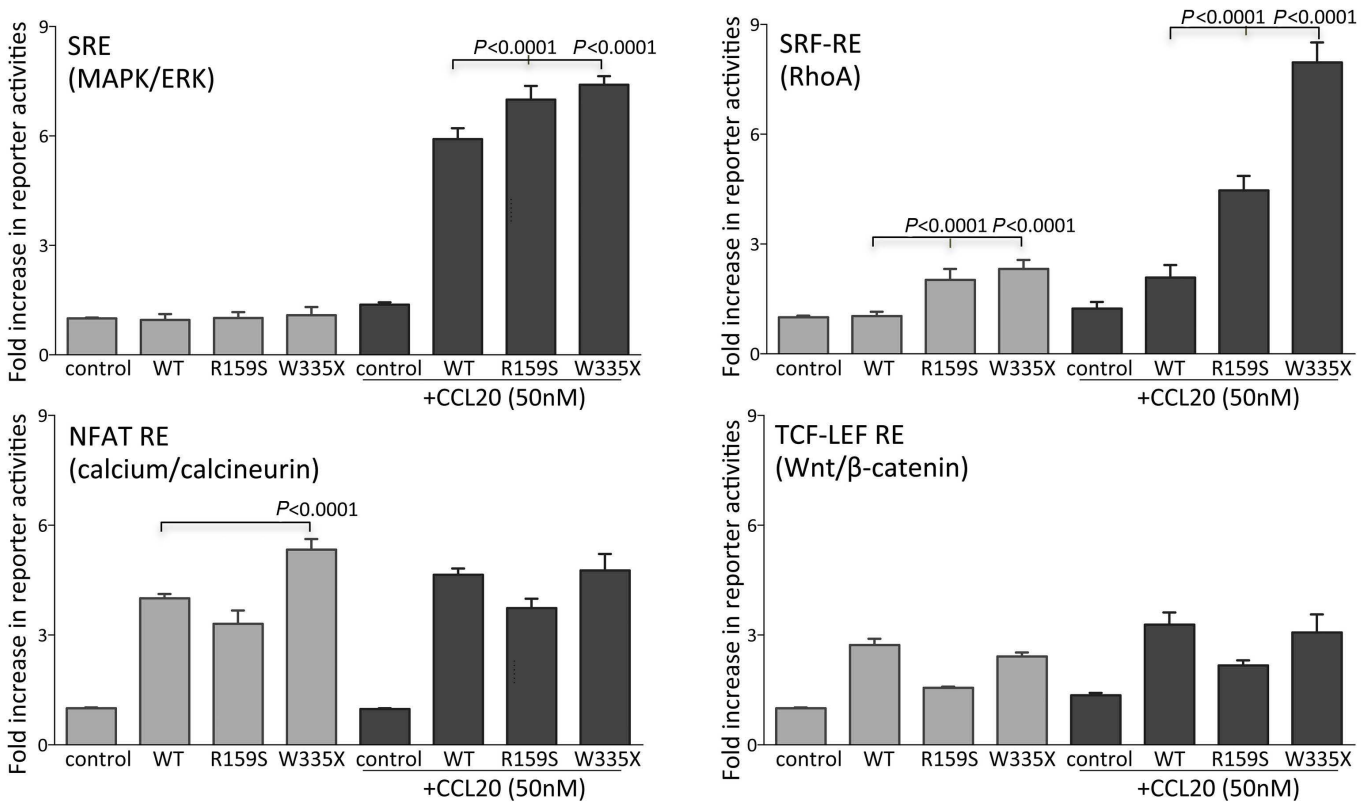
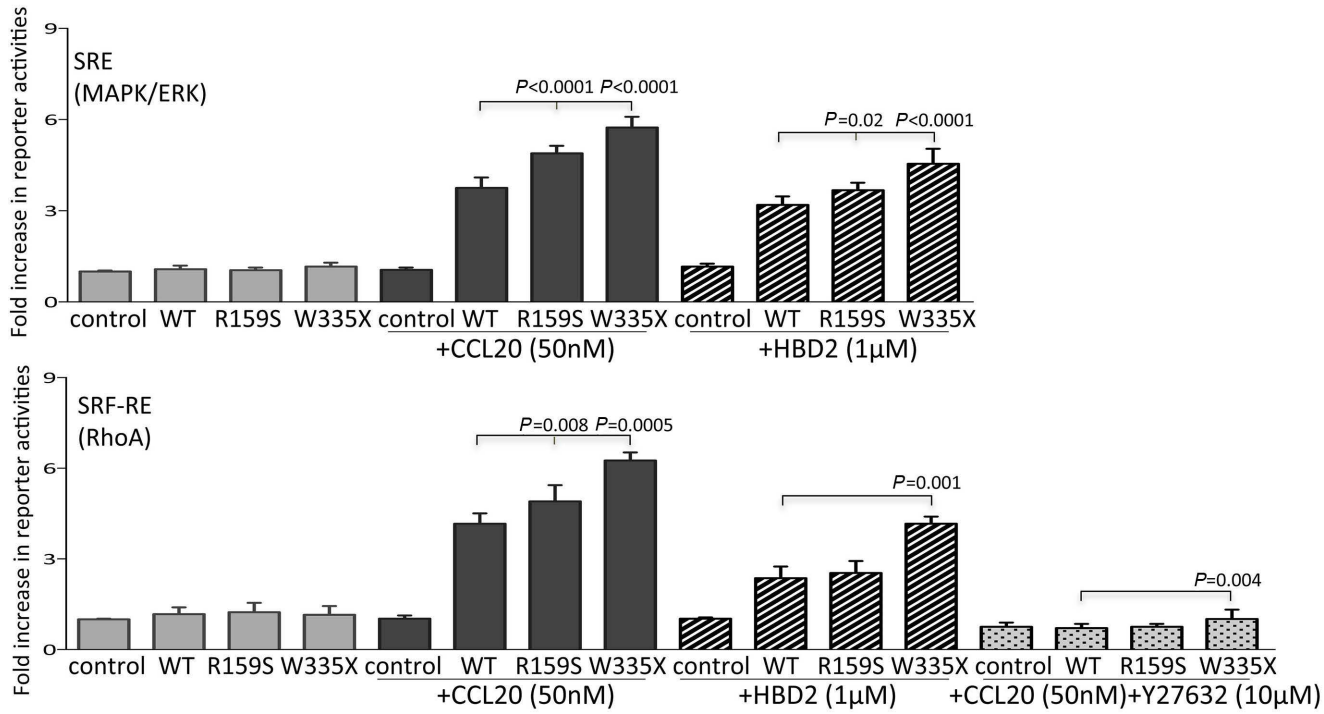


Figure 4. Comparison of CCR6 and its representative mutants in activation of various cellular signaling using reporter assays. This was carried out in DG75 B-cell lymphoma cell line transiently co-transfected with CCR6 expression constructs and reporter plasmids. Data (mean + standard deviation) are from at least 2 independent experiments performed in triplicate. Comparisons between two groups were assessed using one-way ANOVA with significant differences indicated. WT: wild-type.

pression patterns varying among different mucosal sites where MALT lymphoma occurred (Figures 6 and 7; *Online Supplementary Figures S9* and *S10*). In normal salivary glands including those adjacent to MALT lymphoma lesion, HBD2 was expressed moderately in the ductal, but not in acinar epithelial cells, while CCL20 was expressed moderately in the ductal, and strongly in acinar epithelial cells (*Online Supplementary Figure S9*). In salivary gland MALT lymphoma where acini were obliterated, and ducts involved by LEL, weak HBD2 and CCL20 expression were seen in the ductal epithelial cells of LEL (Figure 6A).

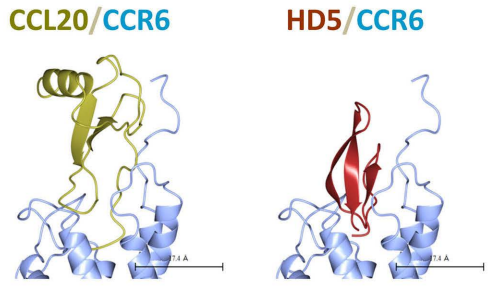
In chronic gastritis including those in gastric MALT lymphoma, HBD2 was expressed weakly in gastric epithelial cells, but strongly in a subset cell population of the inflamed epithelia, particularly in those of basal glands, which were also strong positive for TFF2, a marker of pyloric metaplasia [29] (Figure 7A; *Online Supplementary Figure S10*). HBD2 was at very low level or negative in the Paneth cells of intestinal metaplasia (*Online Supplementary Figure S10*). Similarly, CCL20 expression was weakly expressed in gastric epithelial cells, but moderately in a subset cell population of the inflamed epithelia (*Online Supplementary Figure S10*). In gastric MALT lymphoma, the HBD2 and CCL20 expression pattern in LEL was very simi-

A



B

Known or putative CCR6 ligands	Ligand prediction by molecular docking*	
	Binding affinity (ΔG, kcal mol-1)	Dissociation Constant (M)
CCL20	-9.6	1.8E-07
HBD2	-7.8	3.3E-06
HD5	-10.0	**8.7E-08**



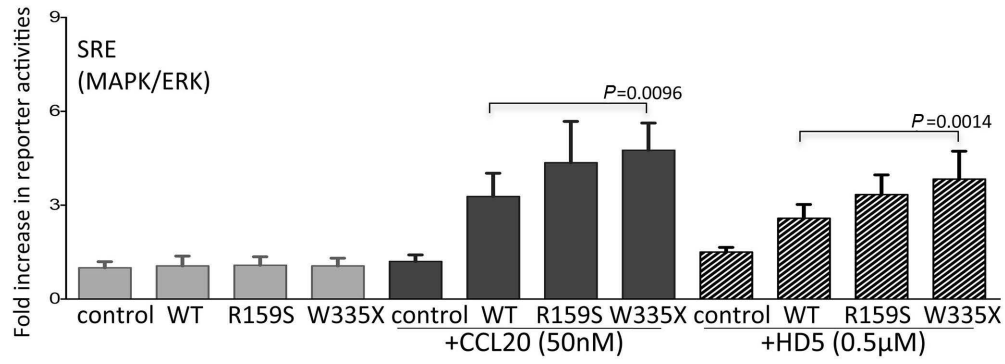


Figure 5. Response of CCR6 and its representative mutants to HBD2 and HD5 stimulation as measured by reporter assays using isogenic Flp-InTRex293 cell lines that stably express a single copy of CCR6 or its mutant. (A) CCR6 and its mutants show similar response profile to CCL20 and HBD2 stimulation as measured by SRF-RE reporter assay, and the receptor mediated RhoA activation can be effectively abrogated by the Rho-associated protein kinase ROCK inhibitor Y27632 (Miltenyi Biotec). (B) Structural modeling of GPCR and ligand binding. HD5 is predicted to have a binding affinity to CCR6, similar to CCL20 using molecular docking software (https://bioinfo3d.cs.tau.ac.il/PatchDock/), and is capable of stimulating CCR6 and its mutants as shown by reporter assay. Data (mean + standard deviation) presented are from at least 3 independent experiments. Comparisons between various groups are assessed using one-way ANOVA, with significant differences indicated. WT: wild-type.

lar to those of inflamed epithelia with HBD2 highly expressed in a subset of the epithelial cells in glands with high TFF2 expression, including those involved in LEL (Figure 7B).

Intestinal metaplasia is a frequent finding in gastric MALT lymphoma, and as expected, HD5 was strongly expressed in the Paneth cells of intestinal metaplasia, including those involved in LEL (Figure 7B). This was particularly prominent in small intestinal MALT lymphoma (*Online Supplementary Figure S11*).

There was no apparent expression of HBD2 and CCL20 in the normal thyroid epithelial cells. In thyroid MALT lymphoma, both protein expression were weak or negative in the epithelial cells of LEL.

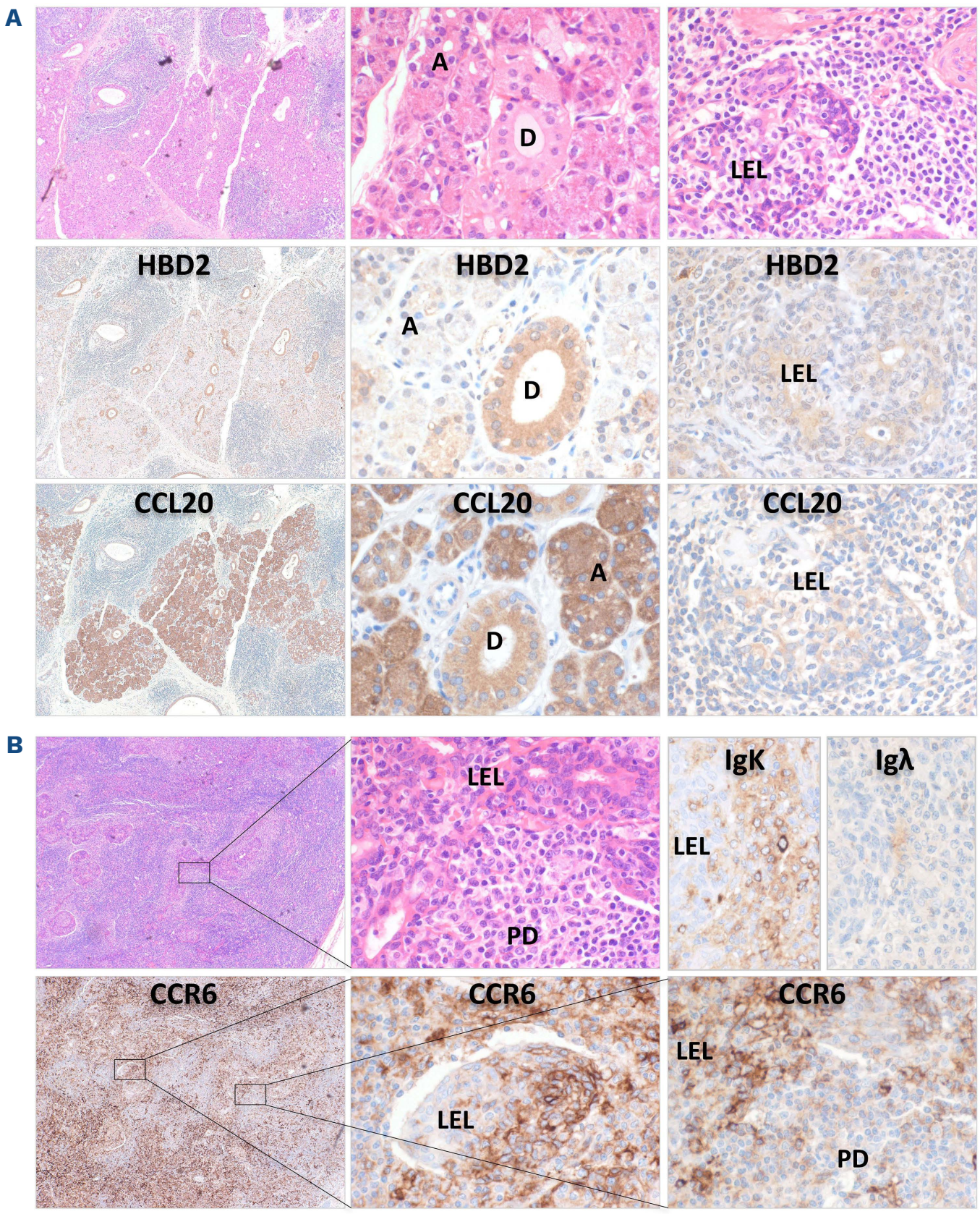


Figure 6. HBD2, CCL20 and CCR6 expression in a representative case of salivary gland mucosa-associated lymphoid tissue lymphoma. (A) Adjacent tissue shows lymphoepithelial sialadenitis and uninvolved salivary glands. HBD2 is expressed moderately in the ductal (D), but not in acinar (A) epithelial cells, and weakly in the epithelial cells involved in lymphoepithelial lesions (LEL). CCL20 is expressed moderately in the ductal, strongly in acinar epithelial cells, and weakly in those involved in LEL. (B) Area of mucosa-associated lymphoid tissue (MALT) lymphoma shows expansion of malignant B cells surrounding LEL, which frequently display plasmacytic differentiation (PD). The neoplastic nature of the B-cell is indicated by IgK light chain restriction. The malignant B cells, including those forming LEL, show strong CCR6 expression, but those undergoing plasmacytic differentiation display weak to negative staining.

Discussion

By using a range of *in vitro* functional assays, we have shown that the CCR6 C-terminal truncation and R159S mutations are activation changes, causing CCR6 constitutive activation and enhanced signaling upon ligand stimulation. We have also demonstrated the expression of CCR6 in malignant B cells and its ligands (HBD2, CCL20,

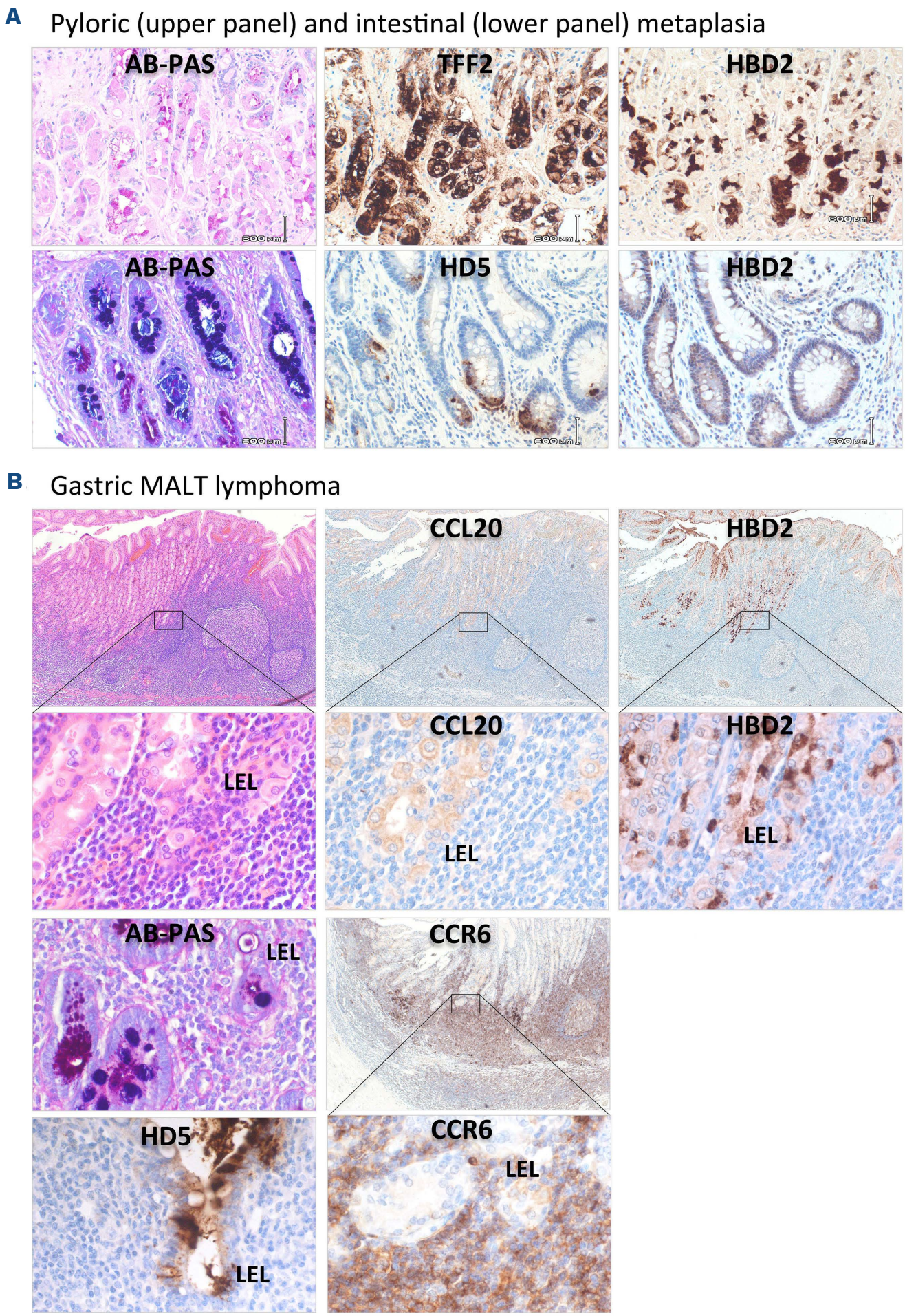


Figure 7. Expression of CCR6 ligands in pyloric and intestinal metaplasia, and gastric mucosa-associated lymphoid tissue lymphoma. (A) HBD2 is highly expressed in a subset of epithelial cells within glands which are consistent with pyloric metaplasia as identified by high TFF2 (trefoil factor 2) expression. While HD5, but not HBD2, is highly expressed in the Paneth cells of intestinal metaplasia identified by strong AB (Alcian blue) staining and goblet cells. (B) A representative case of gastric mucosa-associated lymphoid tissue (MALT) lymphoma showing CCL20, HBD2 and HD5 expression in the epithelial cells involved in lymphoepithelial lesion (LEL). CCR6 is highly expressed in malignant B-cells including those involved in LEL.

HD5) in the inflamed epithelial cells within the lymphoma microenvironment, suggesting that CCR6 signaling is operational in MALT lymphoma.

In vitro assays showed clear functional differences between the CCR6 W335X truncation and R159S mutants, with the former being more potent in apoptosis resistance, malignant transformation, migration and intracellular signaling, particularly in the presence of ligand stimulation. The CCR6 truncation mutant lacks the C-terminal phosphorylation motif,[12,19] which mediates binding to β-arrestin, triggering receptor desensitization and internalization.[30] As expected, the truncation mutant showed a higher level of membrane retention following ligand stimulation, and this most likely underpins its much potent oncogenic activities observed by *in vitro* assays.

It is unclear how the R159S mutation enhances CCR6 signaling. R159 is located in the second intracellular loop, and its change to serine may destabilize the receptor as suggested by an online protein stability prediction neural network (a predictive score of -1.153, much lesser than <0 required for a destabilizing change).[31] Destabilizing mutation can increase receptor flexibility and obviate the necessity for ligand binding to open G-protein binding pocket, and enhance basal signaling. [32] Interestingly, R159 is located in G-protein binding pocket based on the CCR6 structure in complex with a Go protein (*Online Supplementary Figure S12*).[33] Thus, R159 residue change may alter CCR6 structure and provide more favorable interacting pocket for G-protein activation.[32,34]

Among the nine intracellular signaling pathways examined, the two CCR6 mutants, and to a lesser extent the wild-type were highly sensitive to ligand stimulation in both SRE (MAPK/ERK) and SRF-RE (RhoA) reporter assays. SRE (MAPK/ERK) activation mainly drives cell proliferation, while SRF-RE (RhoA) signaling largely promotes cell migration.[35] Through activation of both cellular pathways, CCR6 signaling may help B cells to migrate to inflammatory sites, and maintain their activation, consequently contributing to malignant transformation and clonal expansion. In support of this, CCR6 is highly expressed in MALT lymphoma cells, independent of its mutation status. Moreover, both CCL20 and HBD2, the ligand of CCR6, are constitutively expressed in epithelial cells, including those involved in the LEL within MALT lymphoma. These findings suggest that CCR6 signaling is likely commonly involved in the pathogenesis of MALT lymphoma, and its activation may be maintained by persistent exposure to ligand stimulation, independent of *CCR6* mutation. Nonetheless, *CCR6* mutations can sensitize the receptor to ligand stimulation, thereby enhancing the receptor signaling and its pathogenic potential.

The inflamed epithelial cells are likely the major source of HBD2 and CCL20 in MALT lymphoma, and there are important differences in their origin between salivary gland and gastric MALT lymphoma. In salivary gland MALT lymphoma, these ligands are constitutively expressed by ductal epithelial cells as shown in the present study and also supported by the literature,[36,37] and are likely more actively released when these epithelial cells undergo cellular stress or damage due to LEL. Importantly, it is these ductal glands that are preferentially invaded by B cells, forming LEL, and the LEL are a dynamic structure, with epithelial cells undergoing active regeneration. This provides a perpetual microenvironment for relentless production and release of HBD2 and CCL20, thus chronic stimulation of B cells via CCR6 (Figure 8). In line with these findings, the emergence of malignant B cells and their clonal expansion are always around the LEL, and this is known histologically as a halo appearance, an important feature for recognizing early lesion of salivary gland MALT lymphoma.[38,39]

In gastric MALT lymphoma, HBD2 and CCL20 are mainly expressed in the inflamed epithelial glands, particularly in a subset of epithelial cells within glands consistent with pyloric metaplasia, while HD5 is strongly expressed in the Paneth cells of intestinal metaplasia.[28] Defensins

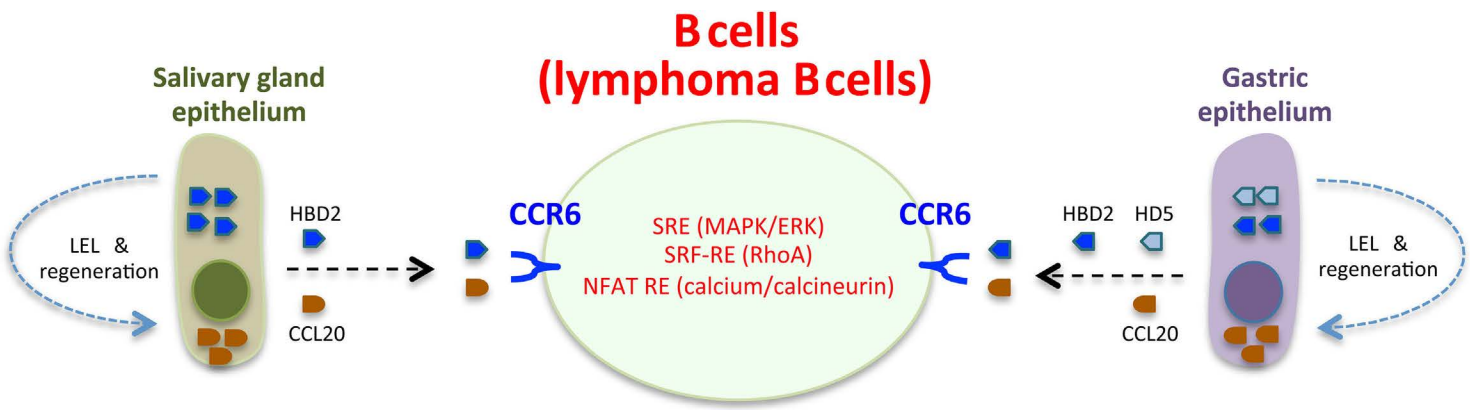


Figure 8. CCR6 activation links innate immune responses to mucosa-associated lymphoid tissue lymphoma development. HBD2 and CCL20 are constitutively expressed in salivary gland epithelial cells, and together with HD5 are induced to be expressed at a high level in the inflamed gastric epithelial cells, likely through the metaplastic process. These ligands are likely actively released when the epithelial cells are under stress or involved in lymphoepithelial lesions (LEL). LEL are a dynamic structure, undergoing active regeneration, thus providing an enduring source of ligands for CCR6 activation, and linking innate immune responses to mucosa-associated lymphoid tissue (MALT) lymphoma genesis.

are a group of antimicrobial peptides, better known in its high expression in the intestinal Paneth cells, and serve as innate responses to constrain bacterial colonization.[40] Most gastric MALT lymphomas arise in a background of chronic *H. pylori* gastritis, in which both pyloric and intestinal metaplasia are a common histological finding, often being multifocal.[29,41-43] These metaplasia are most likely an adaptive response to *H. pylori* infection, as the intestinal mucosa is evolutionarily developed to serve as a barrier to defend gut microbiota invasion. Apart from producing mucins and a high pH environment, intestinal metaplasia also harbors the Paneth cells that produce abundant antimicrobial peptides including defensins.[40] In support of this, the areas of gastric mucosa, which show intestinal metaplasia with defensins 5/6 expressing Paneth cells, have reduced *H. pylori* colonization, and these defensins also bear antibacterial activities *in vitro* at a low concentration.[28, 44] Similarly, HBD2, although not other family members, was induced and expressed at a high level in *H. pylori* associated gastritis, and HBD2 can inhibit *H pylori* growth *in vitro* in a dose-dependent manner.[44,45] Our findings of high HBD2 and CCL20 expression in inflamed gastric epithelia are in keeping with the above observations, and further bridge the innate immune responses to MALT lymphoma genesis (Figure 8).

Similar to salivary gland MALT lymphoma, the emergence of neoplastic B cells and their clonal expansion in the gastric form also start around the LEL, extending to the marginal zone of reactive B-cell follicles. The injured epithelial cells also undergo active regeneration. This together with broad mucosal involvement by the lymphoma cells may provide them enduring favorable environmental milieu. Apart from the survival advantage, the lymphoma cells close to LEL also frequently show histological features of plasmacytic differentiation and blast transformation, suggesting the presence of a plethora of immunological stimulations in this microenvironment. In this context, the CCR6 and CCL20/HBD2/HD5 interaction between the malignant B cells and inflamed epithelia identified in the present study may represent only one of the many crosstalks to be discovered between the two cell populations. Indeed, in salivary gland MALT lymphoma, we recently identified evidence supporting a paracrine stimulation of malignant B-cells via GPR34 by ligand generated by LEL.[10] In a proportion of cases, both CCR6 and GPR34 are potentially co-expressed in the same lymphoma cells (*Online Supplementary Figure S13*) and the two receptor signaling may cooperate in their oncogenic activities in the development of MALT lymphoma. In this context, it is worth noting that the two receptors activate different signaling, with CCR6 primarily SRE (MAPK/ERK) and SRF-RE (RhoA), while GPR34 predominantly NF-kB and AP1-RE (MAPK/JNK), hence potentially complementing in their biological activities.

In conclusion, our findings indicate that CCR6 mutations seen in MALT lymphoma are activation changes, and these mutants are highly sensitive to ligand stimulation, hence enhancing the receptor signaling. More importantly, CCR6 is commonly expressed in MALT lymphoma, independent of CCR6 mutation status, and its signaling is most likely operational in the lymphoma cells via relentless stimulation by HBD2 and CCL20 produced by inflamed epithelial cells, particularly those involved in LEL (Figure 8). In gastric MALT lymphoma, HBD2 and HD5 are mainly derived from pyloric and intestinal metaplasia, potentially linking innate immune responses to lymphoma genesis. These findings elegantly explain why the emergence of malignant B cells and their clonal expansion typically around the LEL at multiple mucosal sites.

Disclosures

No conflicts of interest to disclose.

Contributions

BK, DK, WZ and MQD set up the experimental design, collected and analyzed data; AW provided pathology input; BK prepared and wrote the manuscript; MQD provided research funding, set up the study design and coordination. All authors commented on the manuscript and approved its submission for publication.

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Data sharing statement

Not applicable as data generated in this study have been included in the published article files.

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