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Chemokine receptors in gastric MALT lymphoma: loss of CXCR4 and upregulation of CXCR7 is associated with progression to diffuse large B-cell lymphoma

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Chemokine receptors have a crucial role in the development and progression of lymphoid neoplasms. To determine the chemokine receptor expression profile in gastric mucosa-associated lymphoid tissue (MALT) lymphoma, we performed an expression analysis of 19 chemokine receptors at mRNA levels by using real-time RT-PCR, as well as of five chemokine receptors—*CCR8*, *CCR9*, *CXCR4*, *CXCR6* and *CXCR7*—by immunohistochemistry on human tissue samples of *Helicobacter pylori*-associated gastritis, gastric MALT lymphoma and gastric extranodal diffuse large B-cell lymphoma originating from MALT lymphoma (transformed MALT lymphoma). Following malignant transformation from *H. pylori*-associated gastritis to MALT lymphoma, an upregulation of *CCR7, CXCR3* and *CXCR7*, and a loss of CXCR4 were detected. The transformation of gastric MALT lymphomas to gastric extranodal diffuse large B-cell lymphoma was accompanied by upregulation of *CCR1*, *CCR5*, *CCR7*, *CCR8*, *CCR9*, *CXCR3*, *CXCR6*, *CXCR7* and *XCR1*. Remarkably, *CXCR4* expression was exclusively found in nodal marginal B-cell lymphomas and nodal diffuse large B-cell lymphomas but not at extranodal manifestation sites, ie, in gastric MALT lymphomas or gastric extranodal diffuse large B-cell lymphomas. Furthermore, the incidence of bone marrow infiltration (16/51 with bone marrow involvement *vs* 35/51 with bone marrow involvement; Spearman $\rho = 0467$ $P < 0.001$) positively correlated with *CXCR4* expression. *CXCL12*, the ligand of *CXCR4* and *CXCR7*, was expressed by epithelial, endothelial and inflammatory cells, MALT lymphoma cells and was most strongly expressed by extranodal diffuse large B-cell lymphoma cells, suggesting at least in part an autocrine signaling pathway. Our data indicate that *CXCR4* expression is associated with nodal manifestation and a more advanced stage of lymphomas and hence, might serve as useful clinical prognostic marker.
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The marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) shows histological features more closely resembling the mucosa-associated tissue than those of peripheral lymph nodes. MALT lymphoma arises with the highest frequency in the MALT of the gastrointestinal tract. For the development of MALT lymphomas, a long-lasting chronic inflammation induced by *Helicobacter pylori* gastritis provides the pathogenetic background at these sites.[1]

Chemokines, also known as proinflammatory, chemotactic cytokines, represent a large superfamily of peptides with diverse biological functions involved in organogenesis, hematopoesis and inflammatory processes. Chemokines interact with a target cell by binding to G-protein-coupled chemokine receptors.[2–4] The homeostatic transport of precursor

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B cells to secondary lymphoid tissue is essential for B-cell development. *CCR6, CCR7, CXCR3, CXCR4* and *CXCR5* have a crucial role in this homing process, and therefore, the group of these five chemokine receptors is called B-cell homeostatic chemokine receptors.[5–7] The activation-dependent chemokine receptors, which are expressed on effector leukocytes (including activated effector/memory T cells) in response to inflammatory cytokines, exert an essential role in inflammation and are responsible for migration toward a chemokine gradient produced by inflamed cells.[2] Further, different patterns of chemokine receptor expression identified in different malignant B-cell subsets suggest that these chemokine receptors are functional and have a crucial role in malignant B-cell circulation leading to the emergence of monoclonal B cells and, eventually to transformation.[8–11] The development of chemokine antagonists and their use in clinical trials for a variety of diseases shows the potential of chemokine receptors for molecular-targeted therapy.[12]

The aim of the present study was to identify expression patterns of 19 chemokine receptors of non-neoplastic stomach specimens, *H. pylori*-associated gastritis, gastric MALT lymphomas and of extranodal diffuse large B-cell lymphoma arising from MALT lymphoma (transformed MALT lymphoma) in the stomach. Here, we show that the chemokine receptor expression profiles of gastric MALT lymphomas differ substantially to those of extranodal diffuse large B-cell lymphoma. Our data provide evidence that the stepwise development of gastric MALT lymphoma from a non-neoplastic event to *H. pylori*-associated gastritis to MALT lymphoma and finally to overt extranodal diffuse large B-cell lymphoma is accompanied by deregulation of activation-dependent and B-cell homeostatic chemokine receptors. Most importantly, *CXCR4* expression correlates with bone marrow infiltration and lymhomagenesis at nodal sites, whereas loss of *CXCR4* and gain of *CXCR7* is associated with the high-grade progression to the aggressive extranodal diffuse large B-cell lymphoma. Hence, *CXCR4* expression correlates with a more advanced stage and could therefore serve as useful clinical prognostic marker.

Materials and methods

Materials

Lymphoma entities were classified according to the WHO classification of lymphoid neoplasms.[13] Methylene-stained sections of formalin-fixed, paraffin-embedded lymphoma tissue containing >80% lymphoma cells were manually macrodissected by an experienced pathologist using a disposable, sterile, 30-gauge needle under direct light microscopic visualization and were further processed for RNA isolation. Attention was paid that cases of extranodal diffuse large B-cell lymphoma comprised at least—established by morphology and immunohistochemistry—one focus of a low-grade lymphoma component considering these lymphomas as 'transformed MALT lymphomas'.[14]

The determination of the chemokine receptors mRNA expression profiles was performed on 10 gastric MALT lymphomas, 10 extranodal diffuse large B-cell lymphoma of the stomach, 6 *H. pylori*-associated gastritis, 6 non-neoplastic stomachs, as well as on 16 nodal diffuse large B-cell lymphoma, 6 nodal marginal B-cell lymphomas and on 4 samples of peripheral blood B cells.

For immunohistochemical analysis of *CCR8*, *CCR9*, *CXCR4*, *CXCR6*, *CXCR7* and *CXCL12* (*SDF-1α*), 21 gastric MALT lymphomas (4 with bone marrow involvement and 17 without), 6 extranodal diffuse large B-cell lymphomas and 4 *H. pylori*-associated gastritis, and additionally for CXCR4 and CXCR7, 10 nodal diffuse large B-cell lymphomas and 6 nodal marginal zone B-cell lymphomas were used. To obtain knowledge about the expression profile of these four chemokine receptors on B cells of the marginal zone, four spleen samples from healthy donors were immunohistochemical analyzed.

The study was in agreement with the guidelines for the use of human material in research, issued by the local ethics committee.

RNA Isolation, cDNA Synthesis and Real-Time PCR

Total RNA isolation and cDNA synthesis of formalin-fixed, paraffin-embedded tissue samples of peripheral B isolated from blood of four healthy donors was performed as previously described.[15,16]

The real-time PCR and calculation of the expression levels were performed according to a protocol previously reported by Seidl *et al*.[17] The nucleotide acid sequences for the primers and probes used for the determination of the expression levels of 19 chemokine receptors are shown in the Supplementary Table S1.

Immunohistochemical Analyses for CCR1, CCR5, CXCR6 and XCR1

Formalin-fixed, paraffin-embedded tissue was stained after pretreatment with Target Retrieval Solution (Dako, Glostrup, Denmark) using the DakoCytomation automated immuno-stainer and iView detection system (Ventana Medical System, Tucson, AZ, USA). Primary antibodies to *CCR8* (1:400), *CCR9* (1:250) and *CXCR4* (1:200) were purchased from Abcam (Cambridge, Great Britain); *CXCR6* (1:100) from MBL (Woburn, MA, USA); *CXCR7* (1:100) from Acris (Herford, Germany); *CD3* (1:50), *CD20* (1:200), *CD68* (1:200), *MIB-1* (1:200) from Dako; *CXCL12* (1:50) from R&D (Minneapolis, MN, USA); *Vs38C* (1:100) from Dako; and *XCR1* from Acris (1:1000; Hiddenhausen, Germany). For control purposes, tissues known to contain the

respective antigens were included (positive controls). Replacement of the primary antibody by normal serum always revealed negative results (negative controls). Scoring of tissue slides and determination of the immunoreactive score (IRS) was performed as previously described by our group.[16]

Statistical Analysis

Statistical analysis was performed by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) as previously described by our group.[16]

Results

The Chemokine Receptor Expression Pattern is not Influenced by Intratumoral Inflammatory Cells

The occurrence of mRNA transcripts for *CCR1, CCR5, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6* and *CXCR7* in pheripheral B cells was consistent with previously published data,[18,19] demonstrating the suitability of the real-time PCR assays.

Analyses of the intratumoral T cells (*CD3+*), macrophages (*CD68+*) and plasma cells (*Vs38C+*) are included in Table 1 to further document the morphological variation of the two lymphoma categories. No correlation was observed between the percentage of *CD3+*, *CD68+* and *Vs38C+* cells and the chemokine receptor expression profile, suggesting that the chemokine receptor expression pattern is determined by the lymphoma cells and not by intratumoral inflammatory cells.

The Proliferation Rate of MALT Lymphomas and Extranodal diffuse large B-Cell Lymphomas Correlates with CCR9 Expression

To study whether the chemokine receptor expression profile has any impact on the proliferation rate of the gastric MALT lymphomas and extranodal diffuse large B-cell lymphomas, *Ki67* staining was carried out on 10 gastric MALT lymphomas and on 10 extranodal diffuse large B-cell lymphomas of the stomach and correlated to the chemokine receptor expression profile. The proliferation rate of the gastric MALT lymphomas was on average 5.7% (range: 3–7%) and 67% for the extranodal diffuse large B-cell lymphomas (range: 30–90%; Table 1). On comparing the proliferation rate of both lymphoma entities to the chemokine receptor mRNA expression profile, a significant positive correlation (Spearman ρ 0.893 and $P<0.001$) for *CCR9* and a tendency of a positive correlation (Spearman ρ 0.651 and $P=0.081$) for *CXCR7* was observed.

Differential Expression of CC Chemokine Receptor Levels During MALT Lymphomagenesis

CCR7—as member of the B-cell homeostatic chemokine receptors—[5–7]was most highly expressed in all

Table 1 Percentage of positive cells of *CD3, CD20, CD68, Vs38c, CCR8, CCR9, CXCR4, CXCR6, CXCR7* and *Ki67* in *H. pylori*-associated gastritis, gastric MALT lymphoma, gastric extranodal diffuse large B-cell lymphoma and splenic marginal B cells derived from healthy donors by IHC

	CD3 %	*CD20* %	*CD68* %	*Vs38C* %	*CCR8*		*CCR9*		*CXCR4*		*CXCR6*		*CXCR7*		*Ki67* %
					%	*React.*	%	*React.*	%	*React.*	%	*React.*	%	*React.*	
HP	47.5 (10–80)	5.5 (1–10)	1 (—)	15.25 (1–40)	5.75 (1–20)	+	42.5 (20–60)	++	40 (30–50)	+	5.5 (1–10)	+	19 (1–40)	++	NA
MALT	6.2 (<5–10)	85 (80–97)	7.2 (<5–20)	5.5 (<5–20)	39 (5–80)	+	10[a] (0–20)	+	2.4 + (<1–5)	+	20.4 (0–80)	+	62 (10–90)	++	5.7 (3–7)
eDLBCL	5 (<5–10)	90 (85–99)	8.5 (<5–20)	2.6 (<5–5)	53.66 (2–80)	+	58.33[b] (30–80)	+	12.5 (0–50)	+	23.33 (0–70)	+	83.3 (70–90)	++	67 (30–90)
Marginal B cells	NA	NA	NA	NA	50	++	10	+	100	++	20	++	100	++	NA

Abbreviations: eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; IHC, immunohistochemistry; MALT, gastric MALT lymphoma; NA, denotes not applicable; <5, only single cells were positive; +; denotes weak positive staining; ++, denotes moderate to strong positive staining; (), defines range.
[a]$P<0.05$ *vs H. pylori*-associated gastritis.
[b]$P<0.05$ *vs* MALT lymphoma.

extranodal diffuse large B-cell lymphoma samples (Table 2 and Figure 1). At lower levels, *CCR7* was also detectable in 7 of the 10 MALT lymphoma samples, in all *H. pylori*-associated gastritis samples and in all peripheral B cells, but was entirely lacking in the non-neoplastic stomach control samples.

CCR1, CCR5, CCR8 and *CCR9*—as members of the activation-dependent chemokine receptors—[2] showed a similar expression profile in the lymphoma specimens (Table 2 and Figure 1). All four receptors were most highly expressed in the majority of extranodal diffuse large B-cell lymphoma samples. *CCR1* and *CCR5* were additionally expressed in 8 of the 10 gastric MALT lymphoma samples in all *H. pylori*-associated gastritis and in the majority of non-neoplastic stomach samples, as well as in peripheral B cells. *CCR8* and *CCR9* were expressed in 4 of the 10 and in 2 of the 10 MALT lymphoma samples. *CCR8* expression was only found in half of *H. pylori*-associated gastritis (three out of six) and in a minority of non-neoplastic stomach samples (one out of six), and its expression was entirely lacking in peripheral B cells. *CCR9* expression was undetectable in *H. pylori*-associated gastritis and non-neoplastic stomach specimens, whereas it was expressed in all peripheral B cells.

mRNA expression of *CCR2, CCR3, CCR4, CCR6* and *CCR10* expression was either heterogeneous or missing and thus no significant differences within the study groups were observed.

In summary, the development of gastric MALT lymphoma is associated with increased *CCR7* expression, whereas during high-grade transformation upregulation of *CCR1, CCR5, CCR8* and *CCR9* occurs.

Differential Expression of CXC, CX3C and XC Chemokine Receptors during MALT Lymphomagenesis

In the group of the B-cell homeostatic chemokine receptors—*CXCR3*, *CXCR4* and *CXCR5*—[5–7] *CXCR4* expression was differentially regulated when compared with CXCR3 and CXCR5 (Table 2 and Figure 2). *CXCR3* and *CXCR5* were most highly expressed in all samples of the extranodal diffuse large B-cell lymphoma and in the peripheral B cells, but were undetectable in stomach control samples. *CXCR3* expression was found in some (2 of 6) *H. pylori*-associated gastritis—and in MALT lymphoma samples (5 of 10)—whereas CXCR5 was expressed in all *H. pylori*-associated gastritis and in 8 of 10 MALT lymphoma samples. The expression profiles of *CXCR3* and *CXCR5* were consistent with previously reported results in non-Hodgkin's lymphoma.[8,9] *CXCR4* expression was detectable in the majority of peripheral B cells (three of four) and *H. pylori*-associated gastritis (five of six) samples, in three of 10 extranodal diffuse large B-cell lymphoma samples, whereas its expression was lacking in all non-neoplastic stomach samples and MALT lymphoma samples.

The activation-dependent chemokine receptors were also predominantly expressed in extranodal diffuse large B-cell lymphoma samples, *CXCR6* in all specimens, CXCR7 in 9 of the 10 extranodal diffuse large B-cell lymphoma samples and XCR1 in 8 of the 10 extranodal diffuse large B-cell lymphoma samples (Table 2 and Figure 2). *CXCR6* and *CXCR7* expression was also found in all pCD19+ samples, but was virtually absent in control samples, and was moderately expressed in *H. pylori*-associated gastritis samples and MALT lymphoma samples. On the contrary, *XCR1* expression was detected in all non-neoplastic stomach samples, in 3 of 6 *H. pylori* samples and in 2 of 10 MALT lymphoma samples.

CXCR1, CXCR2 and *CX3CR1* were heterogeneously expressed and no significant differences within the analyzed groups were observed.

In summary, the development of gastric MALT lymphoma is associated with increased *CXCR3* and *CXCR7* expression, and loss of *CXCR4*. During high-grade transformation upregulation of *CXCR6, CXCR7* and *XCR1* is observed.

Immunohistochemical Analyses Confirms Loss of CXCR4 and Upregulation of CXCR7 in the Development of Gastric MALT Lymphoma

Owing to their consistently high mRNA expression level in all MALT- and extranodal diffuse large B-cell lymphoma samples compared with their pCD19+ counterpart cells, immunohistochemical analyses of five chemokine receptors (*CCR8*, *CCR9*,

Table 2 mRNA expression of CC and CXC receptors in peripheral CD19+ B cells, non-neoplastic stomach, *H. pylori*-associated gastritis, gastric MALT lymphoma and extranodal diffuse large B-cell lymphoma

	CCR1	*CCR5*	*CCR7*	*CCR8*	*CCR9*	*CXCR3*	*CXCR4*	*CXCR5*	*CXCR6*	*CXCR7*	*XCR1*
B cell	4/4	4/4	4/4	0/4	4/4	4/4	3/4	3/4	4/4	4/4	0/4
Stomach	4/6	6/6	0/6	1/6	0/6	0/6	0/6	0/6	1/6	0/6	6/6
HP	6/6	6/6	6/6	3/6	0/6	2/6	5/6	6/6	5/6	4/6	3/6
MALT	8/10	8/10	7/10	4/10	2/10	5/10	0/10	8/10	5/10	7/10	2/10
eDLBCL	10/10	10/10	10/10	10/10	9/10	9/10	3/10	10/10	10/10	9/10	8/10

Abbreviations: B cell, peripheral B cells; eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; MALT, gastric MALT lymphoma; Stomach, non-neoplastic gastric tissue.

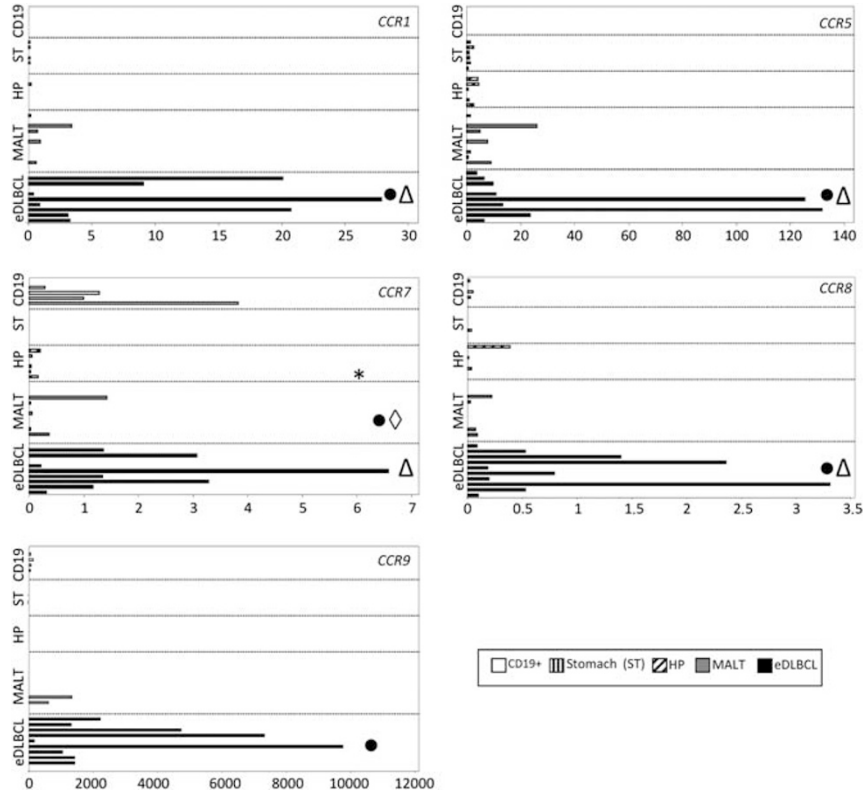

Figure 1 CC chemokine receptor mRNA profile of CD19+ cells, non-neoplastic stomach, *H. pylori*-associated gastritis, gastric MALT lymphoma and extranodal diffuse large B-cell lymphoma. Each bar represents a specimen. Values of gene expression are calculated as relative expression. Dots, significantly deregulated chemokine receptors compared with CD19+ cells; asteriks, significantly deregulated chemokine receptors of *H. pylori*-associated gastritis compared with stomach; rhombuses, significantly deregulated chemokine receptors of MALT lymphoma compared with *H. pylori*-associated gastritis; triangles, significantly deregulated chemokine receptors of *H. pylori* extranodal diffuse large B-cell lymphoma compared with MALT lymphoma. CD19 denotes peripheral B cells; eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; MALT, gastric MALT lymphoma; ST, non-neoplastic stomach.

CXCR6 and *CXCR7*, and of *CXCR4*)—because of the complete lack in gastric MALT lymphomas and in the majority of extranodal diffuse large B-cell lymphomas—were performed. As controls, marginal zone B cells of four spleens derived from healthy donors were included. When comparing protein levels with mRNA expression, a significant positive correlation for *CCR8* (Spearman $\rho = 0.732$, $P < 0.01$), for *CXCR4* (Spearman $\rho = 0.573$, $P = 0.03$), for *CXCR6* (Spearman $\rho = 0.780$, $P < 0.01$) and for *CXCR7* (Spearman $\rho = 0.720$, $P < 0.01$) was found, whereas for *CCR9*, a clear positive trend (Spearman $\rho = 0.486$, $P = 0.066$) was observed, thereby validating our RT-PCR results.

Expression of all five receptors was found on marginal zone B cells with different percentages: *CCR8* and CXCR6 were strongly expressed on 50 and 20% of marginal zone B cells, CCR9 moderately on 10% of marginal zone B cells, and CXCR4 and CXCR7 strongly on all marginal zone B cells (Table 1).

In *H. pylori*-associated gastritis, inflammatory cells exhibited a moderate expression of *CCR8* (5.75%), *CXCR6* (5.5%) and *CXCR7* (19%), whereas more cells stained positive for *CXCR4* (40%) and *CCR9* (42.5%; Tables 1 and 3 and Figure 3).

In gastric MALT lymphoma specimens, 39% of malignant cells showed moderate *CCR8* expression; however, considerable expression was also evident on centroblasts in reactive lymphatic follicles (IRS: 1.64; Tables 1 and 3 and Figure 3). *CCR9* imunohistochemical reactivity was moderately apparent in only 10% of malignant B cells in the majority of MALT lymphomas (IRS: 0.93; Tables 1 and 3 and Figure 3). Like *CCR8, CCR9* reactivity was also found on centroblasts in reactive lymph follicles. A total of 20.4% of MALT lymphoma cells, centroblasts of reactive lymphatic follicles and endothelial cells of the surrounding tissue moderately expressed *CXCR6* (IRS: 1.02; Tables 1 and 3 and Figure 3). It was also obvious that blasts in the lymphomas showed a stronger *CCR8, CCR9* and *CXCR6* expression than the infiltrating small neoplastic lymphocytes. In all gastric MALT lymphomas, on average 2.4% of malignant B cells showed a moderate *CXCR4* expression (IRS: 0.14), whereas CXCR7 was strongly expressed on 62% of malignant B cells (IRS on average 11; Tables 1 and 3 and

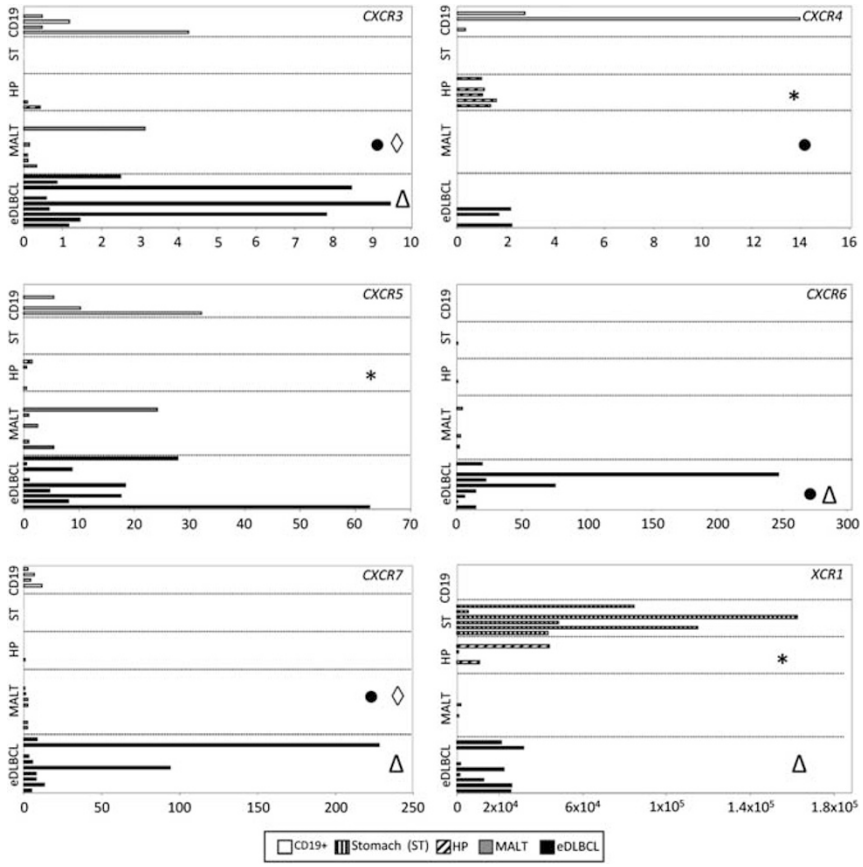

Figure 2 CXC chemokine receptor and XCR1 mRNA profile of pCD19+ cells, non-neoplastic stomach, *H. pylori*-associated gastritis, gastric MALT lymphoma and extranodal diffuse large B-cell lymphoma. Each bar represents a specimen. Values of gene expression are calculated as relative expression. Dots, significantly deregulated chemokine receptors compared with CD19+ cells; asteriks, significantly deregulated chemokine receptors of *H. pylori*-associated gastritis compared with stomach; rhombuses, significantly deregulated chemokine receptors of MALT lymphoma compared with *H. pylori*-associated gastritis; triangles, significantly deregulated chemokine receptors of *H. pylori* eDLBCL compared with MALT lymphoma. CD19 denotes peripheral B cells; eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; MALT, gastric MALT lymphoma; ST, non-neoplastic stomach.

Figure 3). Considerable expression of *CXCR4* and *CXCR7* was also evident on endothelial cells and on centroblasts in reactive lymphatic follicles.

The majority of malignant B cells in extranodal diffuse large B-cell lymphoma moderately expressed *CCR8* (53.7%, IRS on average 5.01; Tables 1 and 3 and Figure 3) and *CCR9* (58.3%, IRS on average 1.275; Tables 1 and 3 and Figure 3), whereas moderate *CXCR6* (23.3%, IRS 2.35; Tables 1 and 3 and Figure 3) and *CXCR4* (12.5%, IRS on average 0.39; Tables 1 and 3 and Figure 3) expression was present on a minority of extranodal diffuse large B-cell lymphoma cells. The only chemokine receptor that was strongly expressed on 83.3% of extranodal diffuse large B-cell lymphoma cells was *CXCR7* (IRS: 14.8; Tables 1 and 3 and Figure 3).

Additionally, immunohistochemical analyses for *CXCR4* and *CXCR7* of Peyer's patches in non-neoplastic small intestine of four donors was performed. Around 80 (range 70–90%) and 100% of lymphocyte in the Peyer's patches express *CXCR4* and *CXCR7* (data not shown), indicating that the loss of *CXCR4* is a specific phenomenon for gastric MALT lymphomas and extranodal diffuse large B-cell lymphomas of the stomach.

CXCL12 is Expressed in *H. pylori*-Associated Gastritis, Gastric MALT Lymphomas and Extranodal Diffuse Large B-cell Lymphoma of the Stomach

To further investigate a potential autocrine stimulation of *CXCR4* and *CXCR7*, the expression level of their ligand *CXCL12*[20–22] was analyzed by immunohistochemistry.

In *H. pylori*-associated gastritis, strong *CXCL12* expression was found in epithelial cells and in inflammatory cells (macrophages, dendritic cells and reactive blasts; Figure 4a). Additionally, single lymphoma cells of gastric MALT lymphoma and a significantly higher amount of around 18% (single cells up to 30%) lymphoma cells of extranodal diffuse large B-cell lymphoma of the stomach strongly express *CXCL12* ($P<0.01$; Figure 4a).

Table 3 Immunhistochemical analyses of *CCR8, CCR9, CXCR4, CXCR6* and *CXCR7* in *H. pylori*-associated gastritis, gastric MALT lymphomas and extranodal diffuse large B-cell lymphoma of the stomach

	CCR8				*CCR9*				*CXCR4*				*CXCR6*				*CXCR7*			
	0	+	++	*IRS*	*0*	+	++	*IRS*	*0*	+	++	*IRS*	*0*	+	++	*IRS*	*0*	+	++	*IRS*
HP	4/4 (100%)			0.3		1/4 (25%)	3/4 (75%)	1.275		4/4 (100%)		0.6		4/4 (100%)		0.075			4/4 (100%)	0.3
MALT	4/5 (80%)	1/5 (20%)		1.64	1/5 (20%)	1/5 (20%)	3/5 (60%)	0.93	3/5 (60%)	1/5 (20%)	1/5 (20%)	0.14[a]	1/5 (20%)	4/5 (80%)		1.02		2/5 (40%)	3/5 (60%)	11[a]
eDLBCL	6/6 (100%)			5.01		5/6 (83%)	1/6 (17%)	1.275[b]	1/6 (17%)	4/6 (66%)	1/6 (17%)	0.39	1/6 (17%)	5/6 (83%)		2.35		1/6 (17%)	5/6 (83%)	14.83

Abbreviations: eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; IRS, immunoreactive score; MALT, gastric MALT lymphoma.
[a]$P<0.05$ *vs H. pylori*-associated gastritis.
[b]$P<0.05$ *vs* MALT lymphoma.

Manifestation Site of Nodal and Extranodal Lymphomas is Guided by Differential CXCR4 and CXCR7 Expression

The RNA expression profiles of *CXCR4* and *CXCR7* in extranodal and nodal lymphomas were determined: 10 gastric MALT lymphomas and 10 extranodal diffuse large B-cell lymphoma were compared with 6 nodal marginal zone B-cell lymphomas and 16 nodal diffuse large B-cell lymphomas. Transcripts of *CXCR4* were almost exclusively found in all nodal marginal zone B-cell lymphomas and nodal diffuse large B-cell lymphoma but only in three extranodal diffuse large B-cell lymphomas and in none of the MALT lymphomas (Figure 4b). Immunohistochemical analyses of *CXCR4* and *CXCR7* in extranodal and nodal NHLs confirmed the significantly lower *CXCR4* expression in extranodal *vs* nodal lymphomas (45-fold reduction in extranodal diffuse large B-cell lymphoma compared with nodal diffuse large B-cell lymphoma, $P=0.01$; 66-fold reduction in gastric MALT compared with nodal marginal zone B-cell lymphomas, $P=0.016$; Figure 4c).

CXCR4 is Associated With Bone Marrow Infiltration

As *CXCR4* has a role in cell homing of malignant hematopoiesis, hematopoetic stem cells, as well as cancer cells of solid tumors to the bone marrow,[23–28] *CXCR4* expression and bone marrow infiltration were determined. A significant correlation of *CXCR4* expression and bone marrow involvement comprising all lymphoma entities (bone marrow involvement ($n=16/51$) *vs* ($n=35/51$ w/o bone marrow involvement; Spearman $\rho=0.467$ $P<0.001$, Table 4) was found. However, probably due to small sample size, no significant difference in *CXCR4* expression levels between gastric MALT lymphomas with bone marrow involvement ($n=4$) and those without bone marrow involvement ($n=21$) was observed ($P=0.835$). In contrast, in nodal lymphomas (nodal diffuse large B-cell lymphoma and nodal marginal zone B-cell lymphomas), *CXCR4* expression was significantly ($8\times$) higher expressed in the group of lymphomas with bone marrow infiltration ($n=12$) compared with those without bone marrow infiltration ($n=22$; $P=0.025$), indicating the potential role of *CXCR4* in the dissemination properties of nodal lymphomas.

Chemokine Receptor Patterns in the Model of Gastric MALT Lymphomagenesis

In a model of gastric MALT lymphomagenesis from a benign lesion to overt malignancy, four of five B-cell homeostatic chemokine receptors[5–7] (*CCR7, CXCR3, CXCR4* and *CXCR5*) and seven activation-dependent chemokine receptors (*CCR1, CCR5, CCR8, CCR9, CXCR6, CXCR7* and *XCR1*) were differentially expressed ($P<0.001$; Figures 5a and b).

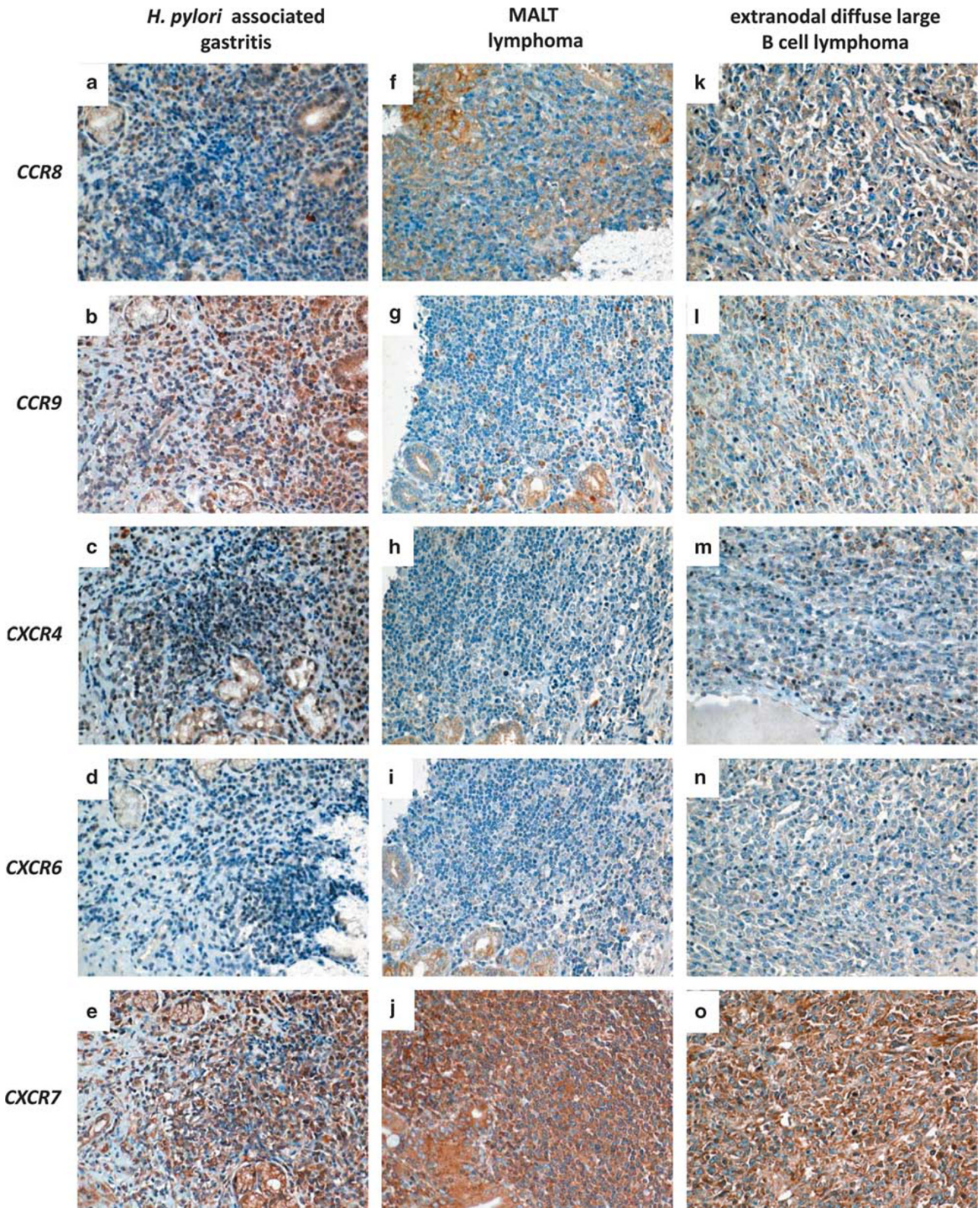

Figure 3 Immunohistochemistry of *CCR8, CCR9, CXCR4, CXCR6* and *CXCR7* in *H. pylori*-associated gastritis, gastric MALT lymphoma and extranodal diffuse large B-cell lymphoma. (**a–e**) Immunohistochemistry for CCR8, CCR9, CXCR4, CXCR6 and CXCR7 in *H. pylori*-associated gastritis demonstrating positivity at varying degree. (**f–j**) Expression levels for the above mentioned chemokine receptors on gastric MALT lymphoma cells; CXCR4 expression was missing on the majority of malignant B cells. (**k–o**) Strong expression of CCR8, CCR9 and CXCR7 was found on the majority of extranodal diffuse large B-cell lymphoma cells. CXCR6 was expressed only in a minority of extranodal diffuse large B-cell lymphomas, whereas CXCR4 expression was just detectable on a minority of extranodal diffuse large B-cell lymphoma cells.

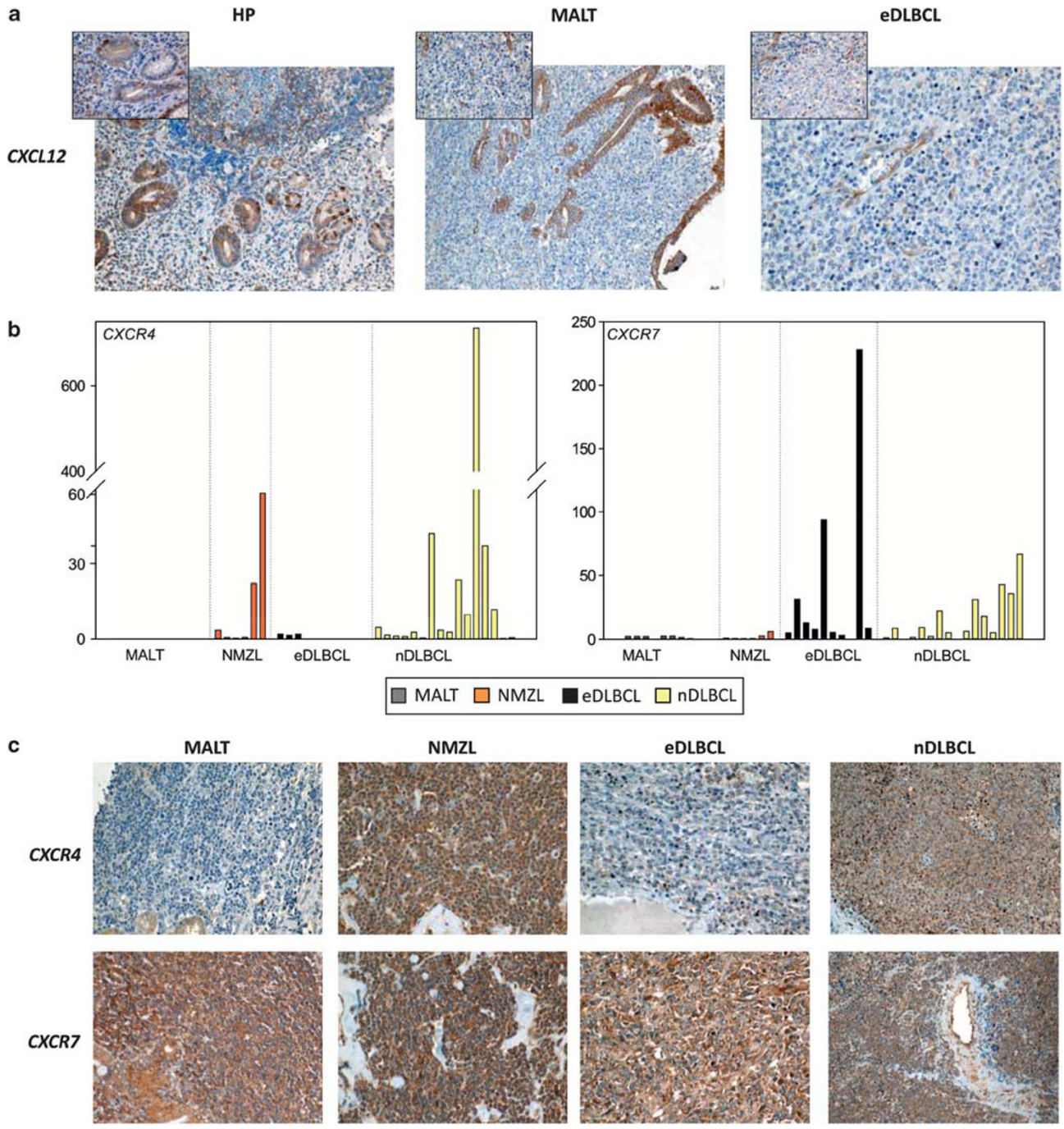

Figure 4 (**a**) CXCL12 expression in *H. pylori*-associated gastritis, gastric MALT lymphoma and gastric extranodal diffuse large B-cell lymphoma. (**b**) CXCR4 and CXCR7 expression in extranodal and nodal lymphomas on mRNA levels. Each bar represents a specimen. Values of gene expression are calculated as relative expression. (**c**) CXCR4 and CXCR7 immunohistochemical analyses in nodal and extranodal lymphomas. CXCR4 staining is restricted to nodal lymphomas, ie, diffuse large B-cell lymphoma and nodal marginal zone B-cell lymphoma; on the contrary CXCR4 is entirely lacking in lymphomas with extranodal manifestation, ie, MALT lymphoma and extranodal diffuse large B-cell lymphoma. eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; MALT, gastric MALT lymphoma; nDBLCL, nodal diffuse large B-cell lymphoma; NMZL, nodal marginal zone B-cell lymphoma.

During the development of *H. pylori*-associated gastritis, the B-cell homeostatic chemokine receptors *CCR7*, *CXCR4* and *CXCR5* were *de novo* expressed and the activation-dependent chemokine receptor XCR1 was downregulated ($P<0.001$). During the transformation to gastric MALT lymphoma from *H. pylori*-associated gastritis, *CCR7, CXCR3* and *CXCR7* were upregulated, whereas *CXCR4* lost its expression ($P<0.001$). Upregulation of *CXCR7* and absence of *CXCR4* in the majority of MALT lymphoma cases was confirmed by immunohistochemical analyses (Tables 1 and 3, Figure 3).

Table 4 *CXCR4* expression and bone marrow infiltration of gastric MALT lymphoma, nodal marginal zone B-cell lymphoma, gastric extranodal diffuse large B-cell lymphoma and nodal diffuse large B-cell lymphoma

	MALT		*NMZL*		*eDLBCL*		*nDLBCL*	
	%	*IRS*	*%*	*IRS*	*%*	*IRS*	*%*	*IRS*
CXCR4	2.8% (<1 – 5)	0.14	82.5% (50 – 90)	12.75	12.5% (0 – 50)	0.39	75% (50 – 90)	10.25
Bone marrow infiltration	19% (4/21)		67/% (4/6)[a]		0% (0/8)		50% (8/16)[b]	

Abbreviations: eDLBCL, extranodal diffuse large B-cell lymphoma; IRS, immunoreactive score; MALT, gastric MALT lymphoma; nDLBCL, nodal DLBCL; NMZL, nodal marginal zone B-cell lymphoma.
[a]$P<0.05$ compared with MALT lymphoma.
[b]$P<0.05$ compared with extranodal diffuse large B-cell lymphoma.

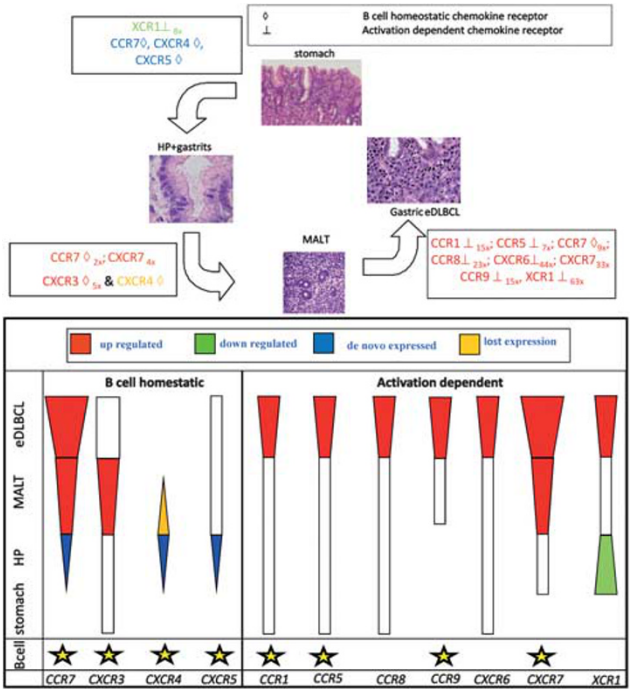

Figure 5 Overview of significant changes in the chemokine receptor expression profile during the gastric MALT lymphomagenesis for 19 chemokine receptors at mRNA levels. (**a**) In the development of *H. pylori*-associated gastritis, CCR7, CXCR4 and CXCR5 were *de novo* expressed and XCR1 was downregulated; progression to gastric MALT led to CCR7, CXCR3 and CXCR7 upregulation and loss of CXCR4; during the transformation of MALT to extranodal diffuse large B-cell lymphoma, CCR1, CCR5, CCR7, CCR8, CCR9, CXCR6, CXCR7 and XCR1 (numbers indicate magnitude of changes). (**b**) Graphical illustration of chemokine receptor expression divided into B-cell homeostatic, activation-dependent chemokine receptors. Yellow stars depict expression of chemokine receptors in pCD19+ cells. eDLBCL, gastric extranodal diffuse large B-cell lymphoma; HP, *H. pylori*-associated gastritis; MALT, gastric MALT lymphoma; Stomach, non-neoplastic stomach.

Transformation of gastric MALT lymphoma to extranodal diffuse large B-cell lymphoma was accompanied by significant deregulation of seven activation-dependent chemokine receptors: upregulation of chemokine receptors *CCR1*, *CCR5*, *CCR8*, *CCR9*, *CXCR6*, *CXCR7* and *XCR1* (Figures 1 and 2; Table 2); further, in the group of B-cell homeostatic chemokine receptors, *CCR7* and *CXCR3* were also significantly upregulated.

Discussion

This study was designed to investigate the expression pattern of B-cell homeostatic and activation-dependent chemokine receptors in the development of gastric MALT lymphoma and extranodal diffuse large B-cell lymphoma of the stomach. We observed deregulated expression of 4 of 5 B-cell homeostatic B-cell receptors—*CCR7, CXCR3, CXCR4* and *CXCR5*—and of 7 of 13 activation-dependent chemokine receptors—*CCR1*, *CCR5*, *CCR8, CCR9, CXCR6, CXCR7* and *XCR1*—in both lymphoma entities. Some of these chemokine receptors have already been described in MALT lymphoma and in diffuse large B-cell lymphoma likewise by several authors.[9,11,16,29–31]

In our study, *CCR9* mRNA expression significantly correlated with the proliferation rate in both lymphoma entities and its protein expression was restricted to malignant B cells with a significantly higher occurrence on extranodal diffuse large B-cell lymphoma cells. Expression of *CCR9* was described to be localized on gut-homing B and T cells having a role in mucosal immune response.[32,33] *CCL25*—the ligand of *CCR9*[34]—was found to be induced by *H. pylori* infection leading to an accumulation of *CCR9*+ cells in the stomach in a mouse model of *H. pylori*-induced gastritis.[35] Immunohistochemical analyses of chemokine receptors showed aberrant expression of *CCR9* in mediastinal large B-cell lymphomas compared with diffuse large B-cell lymphomas, suggesting a role in the extranodal localization of lymphomas for this receptor.[32] Besides the homing properties, anti-apoptotic and proliferating effects have been described for *CCR9* in cancer cell lines.[33] As *CCR9* was expressed in MALT lymphoma and extranodal diffuse large B-cell lymphoma likewise, we hypothesize that *CCR9* might have a role in the extranodal lymphoma localization of the

stomach and that it may be involved in the transformation of the low proliferating MALT lymphoma to the high proliferating extranodal diffuse large B-cell lymphoma.

In the stepwise development from *H. pylori*-associated gastritis to gastric MALT lymphoma, three of five B-cell homeostatic chemokine receptors and two activation-dependent chemokine receptors were deregulated. In *H. pylori*-associated gastritis, recruitment of T- and B cells, dendritic cells and neutrophil granulocytes is mediated by a variety of chemokines interacting with their cognate receptors such as *CCR7, CCR5, CXCR1* and *CXCR4* expressed on infiltrating immunological cells.[30,36–41] Consistent with these findings, we also observed upregulated mRNA transcripts of *CCR8, CCR9, CXCR3* and *CXCR7*.

During the development of gastric MALT lymphoma from *H. pylori*-associated gastritis, *CXCR4* expression was lost and *CXCR7* was upregulated. Immunohistochemistry underscored the loss of *CXCR4* in MALT lymphoma cells. Additionally, *CXCR7* expression was associated with the proliferation rate of gastric MALT lymphomas and extranodal diffuse large B-cell lymphoma in our study. *CXCL12*—the ligand of *CXCR4* and *CXCR7*[20–22]—has been linked to multiple key processes in tumor cells, including proliferation, survival, migration, invasion and metastasis in more than 20 different types of cancer,[42–49] providing evidence for the importance of this chemokine signaling pathway in cancer. *CXCR7* expression was described in malignant cell types, in fetal liver cells and on tumor-associated blood vessels but not in normal vessels.[22,50] *CXCR7* transcripts were also detected in marginal zone B cells and their precursors but not in blood B cells.[51,52] Interaction of *CXCL12* with *CXCR7* promotes tumor growth, proliferation and pro-survival effects[18,50,53,54] in contrast to *CXCR4*, which mediates chemotaxis. [55] Our observation that *CXCR4* expression was lost during the transformation process of non-malignant marginal B cells to MALT lymphoma suggests that CXCL12 signals through binding to *CXCR7* in MALT lymphoma and extranodal diffuse large B-cell lymphoma cells instead of signaling through *CXCR4* and *CXCR7* in non-malignant marginal B cells. This may cause proliferative and pro-survival effects in the lymphoma cells. As pharmacological blockage of the interaction of CXCL12 with CXCR7 leads to retention of marginal zone B cells in the splenic marginal zone,[56] it might be speculated that *CXCR7–CXCL12* interaction is essential for the homing process of MALT lymphoma and extranodal diffuse large B-cell lymphoma cells to the gastric mucosa.

CXCR4 expression is associated with bone marrow infiltration in our cohort of nodal diffuse large B-cell lymhoma and nodal marginal zone B-cell lymphoma. *CXCR4* functions as a homing factor of malignant and normal hematopoietic stem cells of solid tumor cells to the bone marrow microenvironment, as well as of a Burkitt's lymphoma cell line subcutaneously implanted into NOD/SCID mouse.[23–28,57] In our cohort of extranodal and nodal lymphomas, we observed a lower frequency of bone marrow involvement in extranodal lymphomas as also described in other studies.[58,59] Our data indicate that the loss of *CXCR4* in extranodal lymphomas might explain the lower incidences of bone marrow involvement of gastric MALT lymphomas and extranodal diffuse large B-cell lymphomas compared with nodal marginal zone B-cell lymphomas and nodal diffuse large B-cell lymphomas. Bone marrow involvement is associated with a poor prognosis in nodal diffuse large B-cell lymphomas,[60] and therefore *CXCR4* might be a valuable prognostic marker for bone marrow infiltration of nodal diffuse large B-cell lymphomas and hence represents a potential new target for lymphoma therapies.

During the transformation from gastric MALT lymphoma to extranodal diffuse large B-cell lymphoma, upregulation of *CCR1, CCR5, CCR8* and *CXCR7*—representative for activation-dependent chemokine receptors—and of *CCR7*, a B-cell homeostatic chemokine receptor, was observed. Further, *CCR9* and *XCR1* were upregulated. A similar upregulation of *CCR1* and *CCR5* during the transformation process of extragastric MALT lymphoma was recently reported by us.[16] *CCR8* and XCR1 expression has not yet been described in gastric extranodal diffuse large B-cell lymphoma so far. As CCR8 mediates rescue from steroid-induced apoptosis via an *ERK*-dependent pathway, it is conceivable that anti-apoptotic effects may contribute to high-grade transformation.[58,59] Functional characterization of *XCR1* in oral squamous cell carcinoma cell lines show invasive and proliferative effects,[61] suggesting a crucial role of *XCR1* expression during transformation from MALT lymphoma to an aggressive lymphoma.

In summary, our data suggest that the development of gastric MALT lymphoma is associated with increased *CCR7*, *CXCR3* and *CXCR7* expression and a loss of CXCR4. The high-grade transformation is accompanied by upregulation of *CCR1*, *CCR5*, *CCR8*, *CCR9*, *CXCR7* and *XCR1*. In MALT lymphoma, the *CXCR7* pathway seems to be responsible for the homing process to the gastric mucosa, whereas homing to the bone marrow microenvironment in nodal diffuse large B-cell lymphoma, and marginal zone B-cell lymphoma seems to be guided by *CXCR4*. Also, for the site of lymphoma manifestation (nodal *vs* extranodal), a differential regulation of *CXCR4* and *CXCR7* seems to be key determinants. Exploring distinct pathways in gastric MALT lymphomagenesis is a crucial step for the future development of new targeted therapies.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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