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ANATOMICAL PATHOLOGY

Peritumoural CCL1 and CCL22 expressing cells in hepatocellular carcinomas shape the tumour immune infiltrate

Gabriela M. Wiedemann[1,2], Natascha Röhrle[1], Marie-Christine Makeschin[4], Julia Fesseler[1], Stefan Endres[1], Doris Mayr[4], David Anz[1,3]

[1]*Center of Integrated Protein Science Munich (CIPS-M), Division of Clinical Pharmacology, Klinikum der Universität München, Munich, Germany;* [2]*Department of Medicine II, Klinikum rechts der Isar, Technische Universität München, Munich, Germany;* [3]*Department of Medicine II, University Hospital, Ludwig-Maximilians-Universität München, Munich, Germany;* [4]*Pathologisches Institut, Medizinische Fakultät der Ludwig-Maximilians Universität München, Munich, Germany*

Summary

Development, course of disease and prognosis of hepatocellular carcinomas (HCC) are strongly influenced by the immune system. Immunosuppressive regulatory T cells (Treg) have been shown to negatively impact disease progression and survival. To further understand the mechanisms of Treg attraction to HCC lesions, this study provides an analysis of Treg attracting chemokines in human HCC tissues. We analysed the expression of the Treg attracting chemokines CCL1 and CCL22 as well as the infiltration of FoxP3+ Treg and CD8+ T cells in paraffin-embedded tissue sections of 62 HCC patients. Expression of both chemokines was detected in 47 of 62 tissue slides. Chemokine expression was generally higher in tumour stroma and peritumoural liver tissue than in the tumour tissue itself. CD8+ T cells and FoxP3+ Treg were found at high levels in many tumour tissues. Intratumoural infiltration of Treg positively correlated with CCL22 levels in peritumoural liver tissue. In contrast, no correlation of Treg numbers and expression of CCL1 was detected. In summary, we describe here that the chemokines CCL1 and CCL22 are expressed in HCC tissues and, to a higher extent, in the stroma and peritumoural liver tissue. CCL22 may contribute to Treg recruitment and immunosuppression, whereas the role of CCL1 remains to be defined. It will be interesting to investigate the potential of these chemokines as drug targets for cancer therapy.

Key words: HCC; regulatory T cell; chemokines; CCL22; CCL1.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide.[1] Pathogenesis, disease progression and prognosis are strongly related to the immune system,[2] as HCC carcinogenesis is often associated with chronic viral hepatitis and inflammatory disorders of the liver. Immune cell infiltration in HCC tissues plays a crucial role for patient survival.[3] Treatment options in advanced disease remain limited: complete surgical removal cannot be performed at higher tumour stages and local ablative procedures such as trans-arterial chemoembolisation or percutaneous radiofrequency ablation often fail to prevent recurrence.[4] In the past years, progress in cancer immunological research, especially the development of checkpoint blockade antibodies, has provided new striking treatment strategies for several cancer types.[5] In the latest clinical studies, therapeutic efficacy of PD-1 blocking antibodies has been shown in a subgroup of HCC patients.[6] These studies suggest that immunotherapeutic strategies can be successful in HCC. However, a more detailed understanding of the interactions between HCC and the immune system is required in order to further advance immunotherapy in HCC.

Regulatory T cells (Treg) are a subpopulation of CD4+ T helper cells capable of suppressing cytotoxic T cell responses.[7] In many malignant diseases, immunosuppression by Treg is known to play a central role in tumour immune escape and high Treg to CD8 T cell ratios are often predictive of poor prognosis.[8,9] In HCC, elevated numbers of Treg in tumour, ascites and blood are associated with poor prognosis and higher disease stages.[3,10,11] Several mechanisms underlying intratumoural Treg accumulation have been described so far, one of them being chemokine-mediated Treg attraction. The chemokine which is to date best known for its potential of attracting Treg to tumour sites is CCL22.[12,13] We have previously shown that CCL22-expressing cells can be found inside HCC tissues.[14] Another chemokine which has more recently been found to play a role in intratumoural accumulation of Treg is CCL1: it drives conversion of Treg and enhances their suppressive function, inducing expression of FoxP3, IL-10 and the CCL1 receptor CCR8.[15,16] CCL1 has been found to be upregulated in breast cancer, and a high ratio of its receptor CCR8 to FoxP3+ cells is related to a poor prognosis.[17] So far, to our knowledge, the role of CCL1 on Treg in HCC has not been examined.

In this study, we investigated the expression of the Treg attracting chemokines CCL1 and CCL22 in HCC tissues. We

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analysed paraffin-embedded tissue sections of 62 HCC patients who underwent surgical tumour removal for chemokine expression and CD8 and Treg cell infiltration.

METHODS

Patients

Paraffin-embedded HCC tissues of 62 patients were analysed by immunohistochemistry. One healthy control liver was analysed as reference. All cases were retrieved from the archives of the Institute of Pathology of the Ludwig-Maximilians-Universität (Munich, Germany). Patients' ages ranged from 20 to 98 years, and 85.7% of patients were male (Table 1). All patients were treated surgically at the same institution (Klinikum der Universität München, Munich, Germany) between 2008 and 2013. For patients' characteristics see Table 1.

Immunohistology

All paraffin-embedded specimens were cut at 2–3 µm and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Germany). After deparaffinisiation and rehydration, immunohistochemical assays were performed with standard methods. The following antibodies were used: rabbit polyclonal anti-CCL1 (Atlas Antibodies, Sweden), rabbit polyclonal anti-CCL22 (Peprotech, USA), monoclonal mouse anti-FoxP3, clone 236A/E7 (Abcam, USA), monoclonal mouse anti-CD8a, clone C8/144B (Cell Marque, USA). Appropriate horseradish peroxidase-conjugated and alkaline phosphatase-conjugated secondary antibodies were used for detection. The amounts of tumour-infiltrating CCL1+, CCL22+, FoxP3+ and CD8+ cells were evaluated quantitatively. The whole surface of the tissue sections was analysed and tissues were divided in tumour epithelium, peritumoural stroma and tumour-free surrounding liver tissue. Tissues were scanned for areas with high immune cell infiltration and 10 high power fields (HPF, 40× magnification) per slide were analysed for absolute infiltrating cell numbers. Digital images were obtained at 40× magnification on a Zeiss microscope (Zeiss, Germany) and processed with Adobe Photoshop (Adobe, USA) for adjustment of size and contrast. No colour adjustments were made.

Histological subtype was defined according to standard pathology criteria. Grades of differentiation G1 (highly differentiated) to G3 (poorly differentiated) were defined microscopically by experienced pathologists.

Table 1 Patients' characteristics

Characteristics	*n*/Total	%
Age (years)		
≤50	10/63	15.9
>50	48/63	76.2
NA	5/63	7.9
Sex		
Male	54/63	85.7
Female	9/63	14.3
Sample origin		
Primary tumour	57/63	90.5
Recurrent tumour	4/63	6.3
Metastasis	2/63	3.2
Histological subtype		
HCC	56/63	88.9
Fibrolamellar HCC	3/63	4.8
HCC + CCC	2/63	3.2
HCC or HCC + CCC	1/63	1.6
Clear cell	1/63	1.6
Tumour status		
pT1	27/63	42.9
pT2	21/63	33.3
pT3	8/63	12.7
pT4	1/63	1.6
NA	6/63	9.5
Nodal status		
pN0	17/63	27.0
pN1	2/63	3.2
NA	44/63	69.8
Grade		
G1	11/63	17.4
G2	28/63	44.4
G3	17/63	27.0
NA	7/63	11.1
Resection margin		
R0	54/63	85.7
R1	5/63	7.9
NA	4/63	6.3

CCC, cholangiocellular carcinoma; HCC, hepatocellular carcinoma; NA, not applicable.

Statistics

Statistical analysis was performed with GraphPad Prism 5.0 software. Infiltration rates were analysed with two-tailed student's *t* test, grading was analysed by Mann–Whitney non-parametric test. Correlation between infiltration rates was calculated with Pearson's product-moment correlation coefficient.

Ethics

The retrospectively registered study was approved by the ethics committee of the Ludwig-Maximilians-Universität München.

RESULTS

CCL1 and CCL22 expressing cells and are present at high numbers in HCC and surrounding tissues

It has been shown that infiltration of CD8+ cytotoxic T cells and immunosuppressive Treg correlates with survival and prognosis in HCC patients.[3] To dissect the mechanisms of immune cell attraction, we analysed paraffin-embedded tissue sections of 62 HCC patients for expression of the Treg attracting chemokines CCL1 and CCL22 as well as for infiltration of FoxP3+ and CD8+ cells. Cells expressing CCL1 and CCL22 were present in many of the tissue sections and showed a typical cytoplasmic staining (Fig. 1A). Moreover, CD8+ T cells and FoxP3+ Treg were identified in nearly all tissue sections analysed at different numbers (Fig. 1B). Morphologically, chemokine expressing cells were not tumour cells but rather resembled tumour infiltrating immune cells (Fig. 1C). To determine the localisation of infiltrating immune cells we analysed tumour epithelium, tumour stroma (including peritumoural and intratumoural stroma) and peritumoural liver tissue separately. The areas were defined by typical histomorphological criteria. Interestingly, chemokine expressing cells as well as infiltrating T cells could be found at highest numbers in peritumoural and intratumoural stromal areas and not inside the tumour epithelium itself. Cell counts of each cell type analysed within one patient and area are displayed in heatmaps (Fig. 1D). For all cell types, patients with high intratumoural infiltration mostly showed high numbers of the same cell type in peritumoural stroma and healthy liver as well (Fig. 1D). Mean cell numbers (chemokine expressing cells detected in 10 HPF) of CCL1-expressing cells were 11 in tumour, 43 in stroma, and 24 in tumour-free surrounding liver. For CCL22, means were 2 (tumour), 44 (stroma) and 23 (liver) (Fig. 1D). A similar pattern was found for T cell infiltration with 167 CD8+ cells in tumour, 538 in the stroma and 423 in the surrounding liver. For FoxP3+ cells numbers were 42 (tumour), 121 (stroma) and 22 (liver) (Fig. 1D). In the healthy control liver cell infiltration was generally lower (cells/10 HPF were 29 for CCL1, 1 for CCL22, 25 for CD8 and 5 for FoxP3). Immune cells in peritumoural liver were most abundantly found in portal areas. Hence, immune cell infiltrates in HCC were mostly localised within tumour-associated stromal areas and not inside the tumour epithelium.

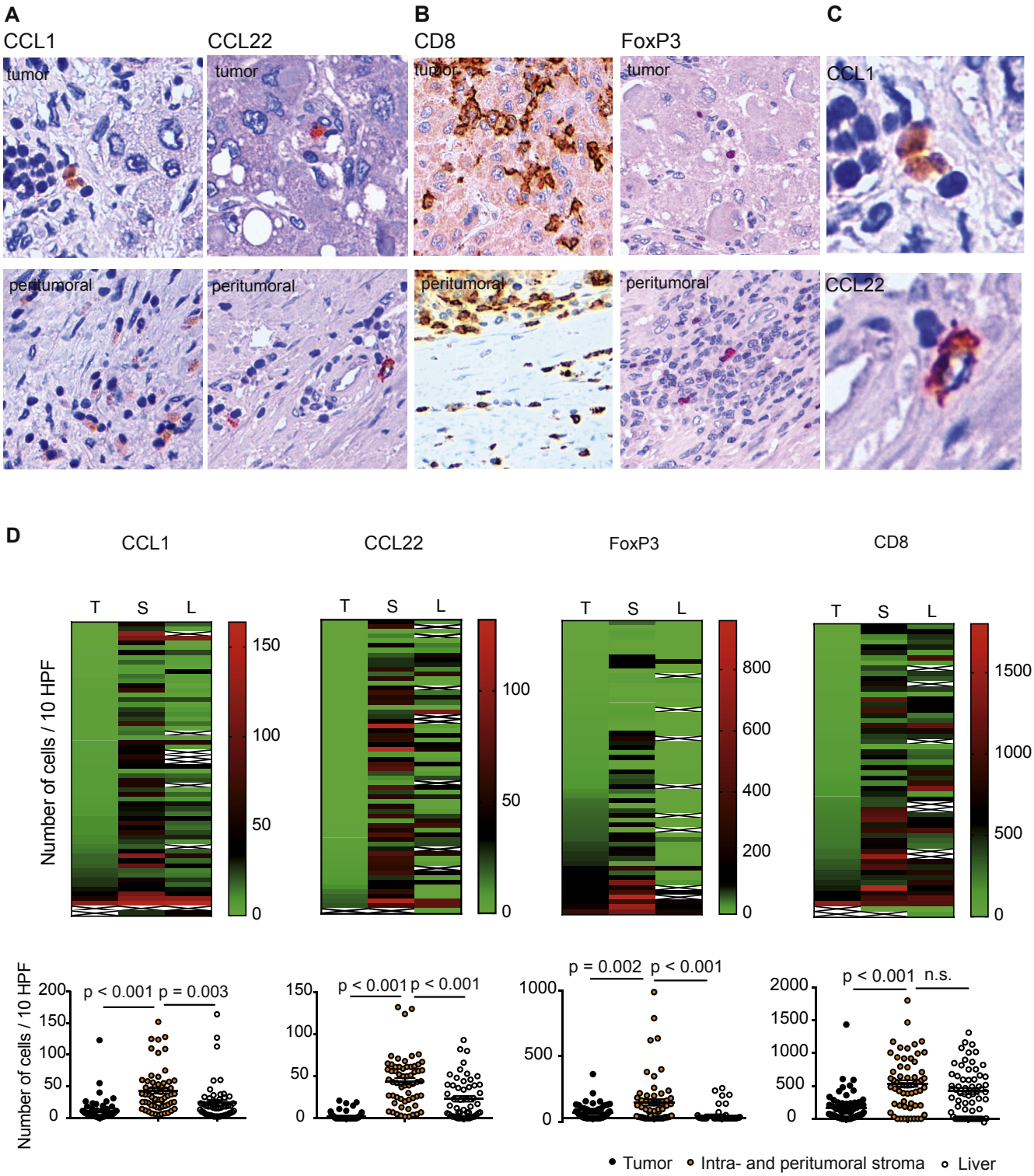

Fig. 1 Presence of chemokine expressing cells and CD8+ and FoxP3+ T cells in HCC. (A–C) Whole tissue sections were screened for CCL1 or CCL22 expressing cells (A) or CD8+ T cells or FoxP3+ Treg (B), respectively. Pictures were taken at 40× magnification on a Zeiss microscope and adjusted in size and contrast with Adobe Photoshop. (C) Zoom on chemokine expressing cells. (D) Distribution of chemokine expressing cells and T cells on tissue samples is displayed in heatmaps and dot plots. Whole tissue sections were screened for areas with positively stained cells. 10 areas with high cell infiltrates (high power fields, HPF) were counted for positive cells at 40× magnification. Y-axes shows number of positive cells per 10 HPF. T, tumour; S, peri- and intratumoural stroma; L, liver. Each line in the heatmap represents one patient. Crossed-out fields: data were not available on slide.

High stromal infiltration of Treg is correlated with high grade tumours

Histological grading of HCC is of significance for patients' prognosis and therapy.[18] We next analysed if infiltration by T cells and Treg attracting chemokine expression diverged between tumours of different differentiation grades or different size. We found no differences in chemokine expression or T cell infiltration from T1 to T4 stages (data not shown). Regarding the grading, in our patient group 11 tumours were classified G1, 28 were G2 and 17 were G3. FoxP3+ Treg were more abundant in higher grade tumours, whereas CD8 T cell infiltration did not significantly change in G1–G3 tumours (Fig. 2). By contrast, CCL1 expression in tumour and stroma as well as CCL22 expression in stroma seemed to decrease with higher grading; however, changes in chemokine-expressing cells did not reach statistical significance (Fig. 2). In summary, we found that high grade tumours showed more pronounced infiltration with immunosuppressive FoxP3+ Treg, whereas chemokine expressing cells tended to decrease in high grade tumours.

Peritumoural but not intratumoural chemokine expression correlates with immune cell infiltration

We next wanted to find out whether numbers of CCL1 or CCL22 expressing cells correlated with intratumoural infiltration of FoxP3+ cells. We found no correlation between intratumoural expression of CCL1 or CCL22 and infiltration with Treg or CD8 T cells (Table 2). However, CCL22 expression in the peritumoural liver significantly correlated with Treg numbers in tumour epithelium, tumour stroma and peritumoural liver (Fig. 3A). Moreover, we found chemokine as well as CD8 T cell and Treg numbers in the tumour and peri- and intratumoural stroma mostly positively correlated with the respective numbers in the liver (Table 2). In the HCC tissues examined, we found no correlation between CCL1 expression and Treg infiltration, but numbers of CCL1 expressing cells in the peritumoural liver positively correlated with intratumoural CD8+ T cells (Fig. 3B). Thus, although both chemokines are expressed in HCC, CCL22 rather than CCL1 may promote HCC Treg infiltration.

We next analysed ratios of FoxP3/CD8 T cells and CCL22+/CCL1+ cells in our tissue sections. We found the

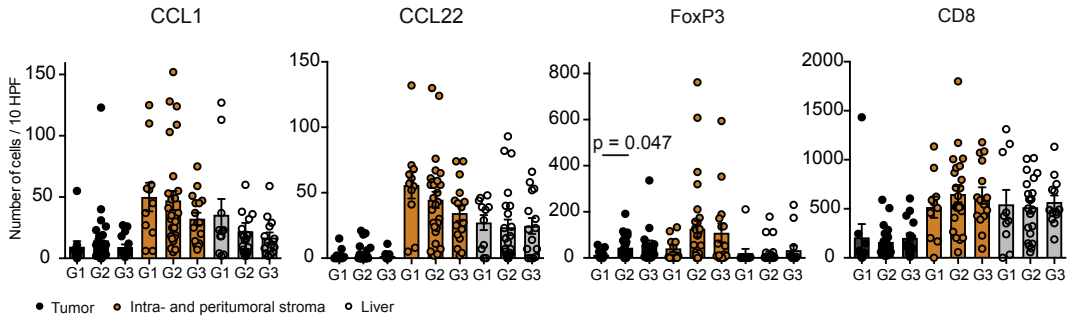

Fig. 2 Immune cell infiltration according to tumour grading. Differentiation grade of tumours was defined microscopically and number of infiltrating cells per 10 HPF is depicted according to tumour grade and localisation.

Table 2 Pearson's correlation coefficients and *p* values

	CCL1 liver	CCL1 stroma	CCL1 tumour	CCL22 liver	CCL22 stroma	CCL22 tumour	FoxP3 liver	FoxP3 stroma	FoxP3 tumour	CD8 liver	CD8 stroma	CD8 tumour
CCL1 liver												
CCL1 stroma	**<0.001** *0.781*											
CCL1 tumour	**<0.001** *0.693*	**<0.001** *0.555*										
CCL22 liver	0.9112 *0.016*	0.466 *0.101*	0.105 *0.226*									
CCL22 stroma	.3390 *0.131*	0.072 *0.230*	0.159 *0.183*	0.052 *0.266*								
CCL22 tumour	0.750 *0.0440*	0.861 *−0.023*	0.109 *0.207*	0.419 *0.112*	**0.024** *0.286*							
FoxP3 liver	0.583 *−0.077*	0.782 *−0.039*	0.832 *0.030*	**0.005** *0.385*	0.787 *−0.038*	0.880 *0.021*						
FoxP3 stroma	0.919 *−0.014*	0.620 *0.065*	0.525 *0.083*	**0.014** *0.337*	0.377 *0.114*	0.608 *0.066*	**<0.001** 0.644					
FoxP3 tumour	0.363 *−0.126*	0.524 *−0.082*	0.971 *0.005*	**0.025** *0.309*	0.728 −0.045	0.316 *0.129*	**<0.001** *0.663*	**<0.001** *0.737*				
CD8 liver	0.143 *0.215*	0.073 *0.2556*	0.483 *−0.102*	0.076 *0.261*	0.873 *0.023*	0.139 *−0.212*	**0.004** *0.407*	**0.028** *0.312*	**0.056** *0.273*			
CD8 stroma	0.899 *0.019*	0.380 *0.121*	0.490 *−0.094*	0.059 *0.275*	0.893 *−0.018*	0.202 *−0.173*	**0.008** *0.373*	**<0.001** *0.535*	**0.002** *0.398*	**0.004** *0.401*		
CD8 tumour	**0.035** *0.305*	**0.018** *0.321*	0.120 *0.214*	0.455 *0.110*	0.410 *0.1133*	0.991 *0.002*	0.199 *0.187*	**0.020** *0.314*	**0.005** *0.377*	**0.003** *0.423*	**<0.001** *0.463*	

p values regular, significant values bold; Pearson's r italics.

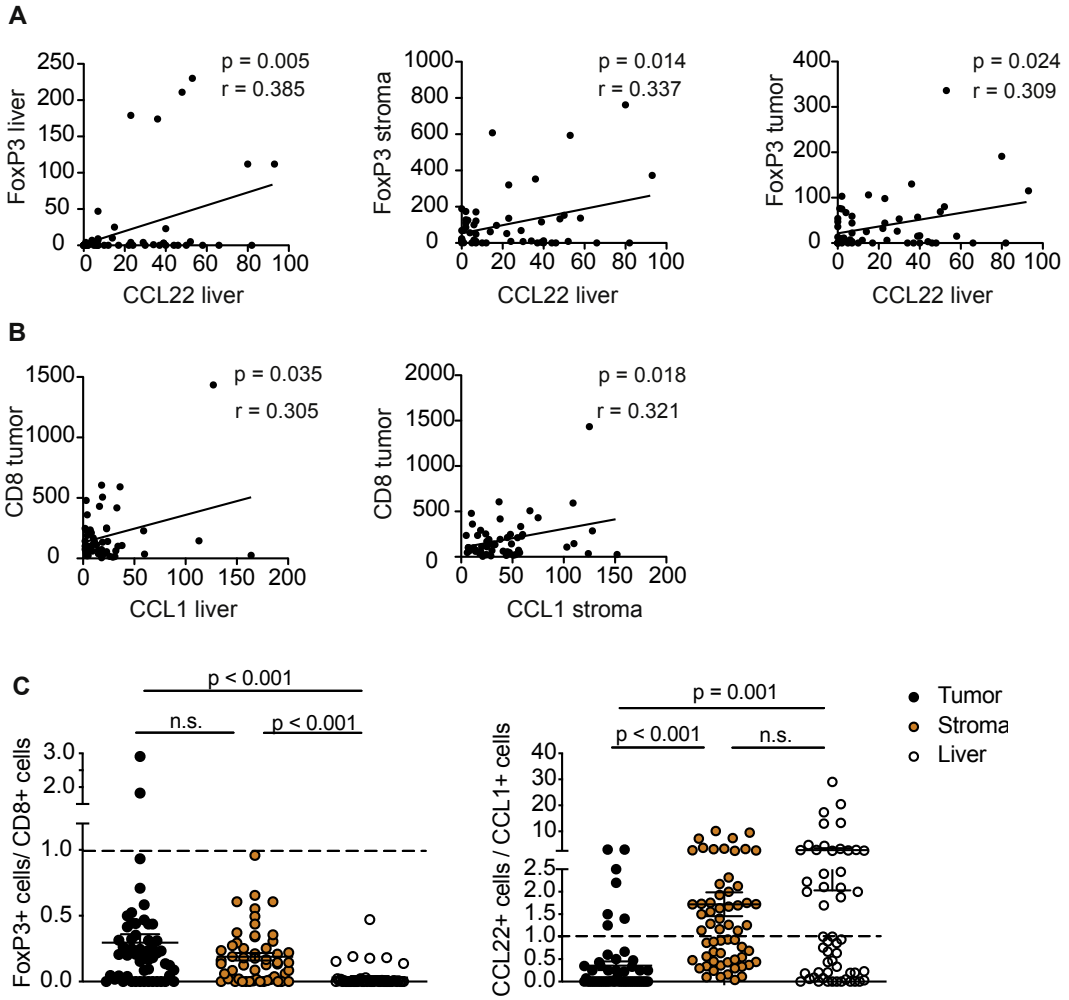

Fig. 3 (A, B) Correlation of chemokine expression and CD8 T cell and Treg infiltration. Numbers of infiltrating cells per 10 HPF are depicted on x and y axes. Correlation was calculated with Pearson's product-moment correlation coefficient. p, *p* value; r, correlation coefficient. (C) Ratios of FoxP3/CD8 numbers and CCL22/CCL1+ cells numbers within each patient and tissue area.

highest FoxP3/CD8 ratios in tumours and tumour-surrounding tissues, whereas the CCL22/CCL1 ratios were lowest inside the tumour (Fig. 3C).

DISCUSSION

Inflammation is a hallmark of HCC and the composition and dimension of immune cell infiltrates are critical for HCC development and progression.[2,19] The presence of FoxP3+ Treg cells, as well as a high ratio of Treg to CD8+ cytotoxic T cells, is a predictor of poor prognosis in HCC patients.[3] One important mechanism by which immune cells are attracted to the tumour site is the establishment of chemokine gradients. Despite the undeniable impact of Treg infiltration on HCC prognosis, only little is known so far on how they are attracted to the tumours: one study shows a role for the CCL20-CCR6 axis,[10] whereas CCL22 has been described to be expressed in hepatitis B and C associated cancers.[11,20] Moreover, we have recently depicted the presence of CCL22 expressing immune cells in HCC tumours and tumour stroma.[14] CCL22 was discovered to play a role in Treg attraction to ovarian cancers more than a decade ago and since then its role has been confirmed in many other cancers, including gastrointestinal cancers.[12,21,22] In many of these cancer entities, high levels of CCL22 are related to high infiltration with Treg and poor prognosis.[12,21,22] More recently, the chemokine CCL1 was found to promote Treg attraction and *de novo* conversion and its presence in murine breast cancers was associated with an accumulation of CCR8+ Treg.[16,23] In this model, blockade of CCL1 improved immunotherapy by reducing intratumoural Treg numbers.[16] Yet, to our knowledge, expression of CCL1 in HCC tissues has not been analysed previously.

In our patient cohort, both CCL1 and CCL22 were expressed in HCC tissue sections. A role for CCL22 in hepatitis B virus (HBV) induced HCC has been described before in one study.[11] However, presence, morphology and localisation of CCL22 expressing cells have not been studied in HCC so far. We show here that CCL22+ cells are generally more abundant in tumour-surrounding stromal areas than within the tumour epithelium and that their peritumoural presence is positively correlated with intratumoural FoxP3 numbers. Also, in accordance with the literature, CCL22 expressing cells are morphologically of myeloid origin.[9,17] As has been described for many tumour entities, we believe that myeloid cell-derived CCL22 may attract immunosuppressive Treg to HCC tissues and thereby foster tumour immune escape.

By contrast, although highly abundant in HCC surrounding stroma and liver, CCL1 expression did not correlate with

Treg infiltration. Conversely, CCL1 expression is related to CD8 T cell accumulation in our patient cohort. CCL1 has been described to attract and activate Th2 cells,[24] but it has also been shown that activated CD8+ T cells express the cognate receptor CCR8 and migrate towards CCL1 chemokine gradients in the skin.[25] Moreover, CCL1 has been reported to play a role in the expansion of immunosuppressive myeloid cell compartments in human renal and urothelial cancer.[26] Thus, we can only suggest a role for CCL1 in HCC immunoediting, and the exact effects will have to be further elucidated.

We also analysed the effects of chemokine expression and immune cell infiltration on patient disease-free survival (DFS) and overall survival (OS). We could see no significant effects on patient survival of any of the chemokines or immune cells analysed. It has been shown before that high numbers of Treg are associated with poor survival in HCC.[3] The fact that we did not see any correlation might be due to a patient cohort that is too small. Moreover, the causes leading to HCC development were not the subject of this study. In order to investigate the effect of chemokines and Treg in HCC more closely, a larger patient cohort should be examined and patients' characteristics such as the grade of liver cirrhosis and its cause (chronic hepatitis, chronic alcohol abuse, inherited liver diseases) should be evaluated for their effects on intratumoural chemokine expression and immune cell infiltration.

In our patient cohort, chemokine expressing cells as well as Treg and CD8+ T cells were preferentially located in the intra- and peritumoural stroma and not inside the tumour tissue itself. FoxP3/CD8 ratios were significantly higher in tumour and peritumoural stroma than in the surrounding liver, whereas the opposite was true for CCL22/CCL1 ratios. This again highlights the differential properties of tumours and their surrounding tissues and suggests differing preferential locations of tumour-associated immune cells. CCL1+, CCL22+, and CD8+ cells were found at higher numbers in non-tumourous tumour-surrounding liver than inside the tumour epithelium. Several studies have described the accumulation of immune cells at tumour borders and in the surroundings of tumours rather than inside the tumour.[27,28] Bell *et al.* suggested, that important immune crosstalk such as priming of T cells by mature dendritic cells takes place in the peritumoural stromal areas of breast tumours.[27] We observed significant correlations between numbers of CCL22 expressing cells in peritumoural liver and stroma and hepatic infiltration with FoxP3+ Treg cells. We have recently described tumour-derived soluble Il-1alpha[14] as a cause of systemic CCL22 induction in tumour-bearing mice. A similar paracrine mechanism might also account for increased CCL22 expression in tissues close to the tumour. The positive correlation between high infiltration of CD8 T cells and Treg in the liver with their respective presence in the tumour and peritumoural stroma also suggests that the tumour immune environment extends across the borders of the tumour. It would be intriguing to see if this is caused by underlying inflammation and disease of the liver or by the tumour itself. Further studies including a higher number of patients and detailed information on the history of liver disease will help to answer this question.

It is well known that chemokines play a versatile role in HCC related inflammatory infiltrates and also for prediction of patient survival.[29] Chemokines predominantly expressed in HCC tissues comprise CCL2, CCL5, CXCL9, CXCL10, CXCL16, and they are expressed in HCC cells themselves, endothelial cells or tumour-associated immune cells.[30–33] Their pleiotropic functions range from increase of certain cytokines to the inhibition of angiogenesis as well as chemotaxis of several immune cell subpopulations.[31,34,35] Our data expand the already complex knowledge on HCC-related chemokine expression to two new candidates, CCL1 and CCL22. We have shown their abundant expression in HCC and HCC surrounding stroma and liver. While the exact function of CCL1 in HCC remains to be elucidated, our data indicate that CCL22 expression in the tumour surrounding liver might be an important parameter for the intratumoural accumulation of immunosuppressive Treg. Taken together, both chemokines are interesting new targets for immunotherapy and require further investigation.

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Address for correspondence: David Anz, MD, Department of Medicine II, Klinikum der Universität München, Ziemssenstraße 1, 80336 Munich, Germany. E-mail: d.anz@lmu.de

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