C-Reactive Protein Is an Indicator of the Immunosuppressive Microenvironment Fostered by Myeloid Cells in Hepatocellular Carcinoma

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Background: C-reactive protein (CRP) is a widely used marker of systemic inflammation and predicts poor clinical outcomes in patients with hepatocellular carcinoma (HCC); however, its significance in the local immune response at the tumor site is not clear.

Methods: Serum CRP levels of 329 HCC patients were detected before resection. Paired paraffin-embedded tumor samples were used to quantify immune cell populations, such as CD11b+ myeloid cells, CD68+ macrophages (Mφs), CD15+ neutrophils, CD8+ T cells, and CD206+, CD204+, CD163+ and CD169+ Mφs, by immunohistochemistry. Enrichment scores for 34 types of immune cells based on transcriptome data from 24 HCC samples were calculated by xCell. Overall survival of patients was analyzed using the Kaplan-Meier method.

Results: Serum CRP levels were correlated with liver functions and tumor stages in patients with HCC. The densities of CD68+ tumor-associated macrophages (TAMs) and CD15+ tumor-associated neutrophils (TANs) were significantly higher in patients with elevated serum CRP levels than in those with low CRP levels (both \( p < 0.0001 \)). Further analysis of TAM subtypes revealed that serum CRP levels were associated with CD204+ and CD163+ Mφ densities (\( p < 0.0001 \) and \( p = 0.0003 \), respectively). Moreover, transcriptome data showed that CRP expression was associated with the expression of myeloid cell infiltration-related genes in HCC tumors. The combination of serum CRP with TAMs or TANs in both the nontumor and intratumor regions could represent a powerful criterion for predicting patient prognoses.

Conclusion: Serum CRP could serve as an indicator of an immunosuppressive TME in HCC, which could be of potential clinical application for treatment strategies targeting the TME.

Keywords: CRP, HCC, immune microenvironment, tumor associated macrophage, tumor associated neutrophil
INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms worldwide and is associated with an increased incidence and extremely poor survival (1). HCC is usually found in inflamed fibrotic and/or cirrhotic livers with extensive immune infiltration due to chronic viral infection. The immune status at a tumor site can largely influence the biologic behavior of tumors. However, there is still a lack of easy-to-use clinical markers to reflect the local immune response of HCC.

C-reactive protein (CRP) is a highly sensitive marker of systemic inflammation that is mainly released by hepatocytes in response to tissue injury, infection or inflammation. The circulating concentration of CRP is typically < 2 mg/L in healthy individuals and often increases to a high level in the presence of inflammation (2). Elevated levels of serum CRP have been associated with an increased risk of several chronic conditions, such as cardiovascular disease, atherosclerosis, and cancer (3). In patients with HCC, increased levels of CRP are correlated with reduced survival (4, 5). While the association between CRP and systemic inflammation in cancer patients is generally accepted (3–7), little is known about the significance of serum CRP levels and the local immune status of the tumor.

Leukocyte infiltration constitutes a major component of the tumor stroma, forming a highly complex, dynamic, and interactive immune contexture, which contributes to tumor development and progression (8, 9). Many of these infiltrating cells, including T cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMS) and tumor-associated neutrophils (TANS), are educated by tumor environmental signals and often acquire special phenotypic characteristics during tumor progression (8–10). Our previous studies showed that myeloid cells secrete various cytokines, such as IL-1β and TNF-α, to promote tumor cell autophagy and angiogenesis and correlate with poor clinical outcome in HCC patients (11–14). It has been reported that tumor and stromal cells, including TAMS and TANS, secreting inflammatory IL-1 and IL-6 could be responsible for the elevated synthesis and secretion of CRP by hepatocytes (4, 5). This evidence prompted us to hypothesize that the level of serum CRP might correlate with the immune context at the tumor site of HCC patients.

To explore the relationship between CRP and the local immune response of HCC, we examined the association between serum CRP and various immune cell infiltrates in the nontumor and intratumor regions of HCC tissues. Our results showed that patients with elevated serum CRP levels tended to have more immunosuppressive TAM and TAN infiltrates, which are important contributors to the tumor-promoting milieu and facilitate tumor progression. These results suggested that the serum CRP level might be a potential indicator of an immunosuppressive microenvironment in HCC tumors.

RESULTS

Correlation of Serum CRP Level and Patient Characteristics

The patients’ clinical characteristics were summarized in Table 1. Considering that serum CRP is mainly synthesized by hepatocytes,
TABLE 1 | Characteristics of patients.

| Characteristics                  | No. (%) |
|----------------------------------|---------|
| Age, yr                          | 50      |
| range                            | 22-78   |
| Gender                           |         |
| male                             | 288 (87.5) |
| female                           | 41 (12.5)  |
| HBV infection                    |         |
| no                               | 46 (14.0)  |
| yes                              | 239 (72.6)* |
| HCV infection                    |         |
| no                               | 312 (96.0) |
| yes                              | 3 (0.9)*   |
| Alpha-fetoprotein                |         |
| ≤25 ng/ml                        | 122 (37.1) |
| >25 ng/ml                        | 196 (59.6)* |
| Child-Pugh score                 |         |
| A                                | 305 (92.7) |
| B                                | 12 (3.6)  |
| C                                | 1 (0.3)*   |
| Tumor number                     |         |
| single                           | 243 (75.0) |
| multiple                         | 79 (24.4)* |
| Tumor size                       |         |
| ≤5 cm                            | 93 (28.3)  |
| >5 cm                            | 231 (70.2)* |
| TNM stage                        |         |
| I–II                             | 213 (64.7) |
| III–IV                           | 105 (31.9)* |

*Information of the indicated characteristics is not available for some patients.

we analyzed the correlation between serum CRP levels and liver function. The patients were divided into two groups according to the median CRP level (Figure 1A) and the results showed that CRP level significantly correlated with the circulating level of glutamic-oxaloacetic transaminase (AST, p = 0.005; Figure 1B and Supplementary Table 1). However, no significant difference was found between CRP levels and Child-Pugh score (Figure 1C), which might be due to the limited cases of Child-Pugh B (12/329) and C (1/329) in the study cohort. Moreover, the serum level of CRP was positively correlated with TNM stage (p < 0.001; Figure 1D), suggesting that an elevated level of serum CRP was associated with disease progression.

Circulating CRP Level and Mφ Subtypes in HCC

Mφs are versatile plastic cells that have multiple functions and phenotypes in response to environmental signals (15–17). To assess different subtypes of Mφ and their correlations with serum CRP level, we examined 4 common markers that are mainly expressed on Mφs (Figure 3A). Of these, CD206⁺, CD204⁺ and CD163⁺ Mφs are suggested to represent immunosuppressive Mφs that promote tumor progression, whereas CD169⁺ Mφs display antitumor properties in HCC (18–20). The results showed that the densities of CD204⁺ and CD163⁺ Mφs in the NT region of HCC tissues were significantly higher in patients with an elevated serum CRP level than in those with a lower level (p < 0.0001 and p = 0.0003 for CD204⁺ and CD163⁺ Mφs, respectively; Figure 3B). No significant correlation was found between CD206⁺ or CD169⁺ Mφ densities and serum CRP levels in either NT or IT regions. Collectively, these results suggest that serum CRP levels might be an indicator of an increased density of immunosuppressive Mφs in HCC tissues.

CRP Expression and Immune Status in HCC

To further characterize the association of serum CRP level and the immune status of the tumor microenvironment (TME) in HCC tissues, we analyzed gene expression microarray data of 24 HCC samples from our previous study (21). Serum CRP data were available for 17 samples, and the samples were divided into two groups with the same cutoff value (Figure 1A, red dots). The overall type-specific enrichment scores for 34 types of immune cells based on transcriptome data were calculated by xCell, a gene signature-based method. Statistics showed that tumor tissues from patients with higher levels of serum CRP had higher infiltration of myeloid cells, such as alternatively activated Mφs (M2, p = 0.04; Figure 4A) and basophils (p = 0.02; Figure 4B), while no significant difference in the enrichment score of other major immune cell types was found (Figures 4C–I and Supplementary Table 2).

We also analyzed gene expression differences between groups divided by the CRP expression level in 24 HCC tumor tissues (Figure 5A). Overall, the CRP high-expressing group had 967 upregulated and 779 downregulated genes compared to the CRP low-expression group (Figure 5B). Gene Ontology (GO) analysis of the differentially expressed genes showed that these genes were enriched in immune regulation processes, such as T cell signaling, leukocyte aggregation and Mφ chemotaxis (Figure 5C). Furthermore, analysis of 366 HCC tumor samples from TCGA demonstrated positive associations of tissue CRP...
level and genes related to myeloid cells, including CD68 and CD14, as well as chemokines and receptors for the chemotaxis of monocytes or neutrophils, such as CCL2, CCR2, and CXCL1 (Figure 5D). Collectively, these results suggest that CRP level positively correlates with myeloid cell infiltration in HCC tissues.

Combined Prognostic Power of CRP Levels and TAM/TAN Density

The above results suggest a relationship between serum CRP level and myeloid cell infiltration in HCC tissues. Next, we analyzed their clinical value in predicting patient prognoses in the cohort of 329 HCC patients. Consistent with previous reports from other groups, serum CRP levels alone were correlated with poor OS in HCC patients (Figure 6A). A fitting model was presented with a nomogram to predict the prognosis of HCC patients using serum CRP level, tumor-infiltrating TAMs and TANs (Figure 6B). The total points of nomogram for each patient were calculated, and patients were divided into two groups according to the median value of total points. The Kaplan-Meier curves showed that the total points of nomogram could notably predict OS of HCC patients (Figure 6C). Multivariate analysis revealed that the total points of nomogram was an independent prognostic factor for OS (Table 3). In addition, the associations between the total points of nomogram and clinicopathological variables were analyzed, and the results showed that the total points of nomogram was significantly correlated with AST level, tumor number, tumor size and TNM stage (Supplementary Tables 3). Taken together, these results indicate that the combination of serum CRP levels with myeloid infiltrations is closely associated with disease progression and could represent a powerful criterion for predicting prognoses in HCC patients.

DISCUSSION

Serum CRP has been widely used as a highly sensitive biomarker of systemic inflammation in clinical settings. In the present study, we applied in situ immunostaining and gene expression analysis and found that patients with elevated serum CRP levels often have high densities of CD68⁺ TAMs and CD15⁺ TANs in HCC tissues. These myeloid cells displayed immunosuppressive properties, and the combination of serum CRP with TAMs or TANs could represent a powerful criterion for predicting prognoses in HCC patients. These findings reveal a novel biological value of serum CRP and suggest that the evaluated level of serum CRP might serve as a potential indicator of immunosuppressive myeloid cell infiltration in HCC tissue.
CRP is an acute phase inflammatory marker of systemic inflammation that is mainly produced by hepatocytes in response to infection, liver injury or inflammatory cytokine release. Over the past few decades, emerging evidence has shown that serum CRP is associated with the risk of several chronic conditions, such as cardiovascular disease, atherosclerosis and cancer (3, 22, 23). The clinical value of serum CRP has also been explored in tumor settings, such as ovarian cancer, breast cancer, colorectal cancer, lung cancer, prostate cancer, renal cell carcinoma, and pancreatic cancer (24–31). In patients with HCC, several studies have reported that high serum CRP levels predict poor clinical outcomes in the case of both resectable and unresectable tumors (4, 5, 32, 33). Although the predictive power of CRP has been intensely investigated, its biological significance and underlying mechanisms remain largely unknown.
mechanisms are not fully understood. In the present study, we confirmed the negative prognostic role of CRP in HCC. Furthermore, we found that serum CRP was closely associated with the immune status of the TME. Specifically, patients with higher serum CRP levels often had more TAMs and TANs infiltrating the TME. Due to the tumor-promoting roles of TAMs and TANs, these results might partially explain why serum CRP is an indicator of a progressive tumor.

Tumor-associated myeloid cells, including TAMs and TANs, are major immunosuppressive components of the TME and contribute to tumor progression by promoting genetic instability, nurturing cancer stem cells, supporting metastasis,
and suppressing protective adaptive immunity (34). We and other groups have demonstrated that the number of CD68+ TAMs in HCC is negatively correlated with patient prognosis (11, 35), and TAN density is also associated with poor prognosis (36–38). The present study revealed the tight association of serum CRP with TAMs and TANs infiltrating the tissues, especially TAMs expressing CD204 and CD163 with immunosuppressive properties. This conclusion is supported by the following observations: 1) patients with elevated serum CRP levels were likely to have more CD204+ and CD163+ tissue-infiltrating Mψs; 2) serum CRP selectively correlated with enrichment scores of M2 Mψs and basophils from the gene expression microarray; and 3) CRP expression also positively correlated with chemokines and chemokine receptors associated with myeloid cell mobilization. It has been reported that CRP was mainly expressed by hepatocytes in response to inflammatory factors, and could promote the formation of immunosuppressive immune cells (6, 39, 40). Together with our findings, it is possible that the secreted inflammatory cytokines in HCC tissues could upregulate the synthesis and secretion of CRP by hepatocytes, which further promote the accumulation of myeloid cells. The regulating mechanisms of CRP secretion and myeloid cell infiltration deserve further investigations.

We noted that serum CRP correlated with TAMs and TANs in the NT but not the IT regions of HCC tissues, suggesting that the tumor phenotype is not only dictated by genetic and epigenetic alterations in the tumor cells per se but is also influenced by molecular crosstalk between tumor cells and the surrounding microenvironment, especially the infiltrating immune cells. This is particularly relevant for liver cancer, where the majority of HCCs (up to 90%) develop in the background of chronic liver inflammation caused by hepatitis B or C virus, alcoholic steatohepatitis, or nonalcoholic steatohepatitis (41). Accordingly, it has been reported that a predominant Th2-like cytokine profile occurs in the liver milieu and that a shift toward anti-inflammatory/immunosuppressive responses plays a vital role in promoting tumor progression and venous metastases of HCC (42). Our previous studies have also revealed that monocytes/Mψs in the peritumoral region of HCC could effectively suppress tumor-specific T cell immunity via PD-L1-PD1 and induce cancer cell autophagy in the invading edge region by secreting TNF-α and IL-1β, which resulted in epithelial-mesenchymal transition of cancer cells and tumor metastasis (11, 13). These results suggest the importance of the liver microenvironment in the aggressive malignancy of HCC. However, the context of immune reactions in the TME is tissue

**FIGURE 4** | Enrichment score of 34 immune cell subtypes calculated by xCell. (A–I) Immune cell enrichment score of M2 Mψs (A), basophils (B), Mψs (C), monocytes (Mo) (D), NK cells (E), DCs (F), CD4+ T cells (G), CD8+ central memory T cells (Tcm) (H), Tregs (I), and between low and high serum CRP level groups. Student’s t tests were applied, and *P < 0.05. ns, not significant.
FIGURE 5 | CRP expression in situ and immune status of HCC patients. (A) Twenty-four patients were divided into two groups based on in situ CRP expression, with 10 high-expressing and 14 low-expressing patients. (B) Volcano plot of gene expression. Upregulated genes (fold change > 2, \( P < 0.01 \), highlighted in red) and downregulated genes (fold change < 0.5, \( P < 0.01 \), highlighted in blue) were identified in the high CRP group compared to the low CRP group. (C) Top twenty GO terms from the GO enrichment analysis using the differentially expressed genes between the low CRP versus high CRP groups. (D) TCGA data showing the associations between the expression levels of CRP and myeloid-related genes in HCC tissues. Correlations were determined by Pearson correlation and linear regression analysis.
specific and depends on multiple factors. How to manipulate local inflammation, thus lead to the reactivation of immune status in HCC tissue deserves further study.

In conclusion, our results provide insights into the association between serum CRP and myeloid cell infiltration in HCC. Given the increasing appreciation of the TME as a critical regulator of tumor immunity and immunotherapy (43, 44), the serum CRP level might serve as a simple indicator of the immune status in the tumor site and thus has a potential application for treatment strategies targeting the immunosuppressive TME for patients with HCC.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Institutional Review Board of Sun Yat-sen University Cancer Center. The patients/participants provided
their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS
YW designed research. YW, ZL, and ZH performed research. YW analyzed data. XY and ZH collected human samples. YW, LZ, and JX wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.774823/full#supplementary-material

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