Cellular Immune Suppressor Mechanisms in Patients with Hepatocellular Carcinoma

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Abstract
Hepatocellular carcinoma (HCC) has been shown to induce several immune suppressor mechanisms in patients. Our laboratory has been investigating different cellular mechanisms of immune suppression in patients with HCC. These suppressor mechanisms range from CD4+ regulatory T cells, functionally impaired dendritic cells, neutrophils, and monocytes to myeloid-derived suppressor cells. In vitro as well as in vivo studies have demonstrated that abrogation of the suppressor cells enhances or unmasks tumor-specific anti-tumor immune responses. We performed a literature search for immune suppressor cells in HCC, and here we provide a comprehensive summary of the latest studies in this field.

With the recent approval of anti-CTLA-4 immunoglobulin as an anti-cancer drug, immunotherapy has gained a lot of interest as a new treatment option for patients with cancer, including hepatocellular carcinoma (HCC). In fact, HCC represents an ideal candidate for potential immunotherapeutic approaches for a number of different reasons:
(i) HBV vaccination has been shown to protect against the development of HCC.
(ii) Type-I interferons have shown promising results for the treatment of HCC.
(iii) A number of correlative studies indicate a relation between spontaneous immune responses and patient outcomes.

We and others have previously demonstrated that spontaneous tumor-specific immune responses occur frequently in HCC patients. Both humoral and cellular tumor-specific immune responses to HCC can be detected [1–5]. Surprisingly, our data indicated that spontaneous immune responses in HCC patients occur at frequencies similar to those of immune responses to other tumors which are more immunogenic, such as melanoma. Simultaneous NY-ESO mRNA and antibody analysis for expression of NY-ESO in tumors and anti-NY-ESO antibody analysis in serum from patients with HCC revealed that at least 50% of HCC patients developed NY-ESO-specific antibody responses even in the absence of specific interventions aiming to prime immune responses (fig. 1). Therefore, if tumor-specific immune responses can already be detected without spe-
Specific vaccination approaches, this clearly questions the rationale for a vaccine approach aiming to increase tumor-specific immune responses [1, 5–8]. This data also suggests that tumor-specific cellular immune responses are potentially overshadowed by different suppressor mechanisms disabling effective anti-tumor immunity. In this article, we review and summarize the current knowledge on cellular suppressor mechanisms in patients with HCC (fig. 2).

**CD4⁺CD25⁺ Regulatory T Cells**

CD4⁺CD25⁺ regulatory T cells (Tregs) are a minor but functionally unique population of T cells which maintain immune homeostasis in immune tolerance and the control of autoimmunity. Absence of CD4⁺CD25⁺ Tregs is associated with severe signs of autoimmunity, which can be found both in mice and in humans [9, 10]. While in vitro Tregs can inhibit immune responses mediated by both CD4⁺ and CD8⁺ effector T cells by a contact-dependent and cytokine-independent mechanism [11–13], the mechanism of immune suppression is much more complex in vivo [14, 15]. Forkhead or winged helix family of transcription factor P3 (FOXP3) is not only essential for Treg development but also remains the best marker to identify these cells. However, a number of studies have shown that activation of human non-Tregs can also lead to expression of Foxp3 in vitro, so this marker needs to be used with caution [16]. Alternatively, it has been suggested that analysis of Foxp3 methylation status can be used to determine the presence of Tregs in humans [17].

Multiple investigations have demonstrated the pivotal role of Tregs in tumor immunology and their ability to suppress anti-tumor immune responses. Accordingly, targeting Tregs has been shown to boost anti-tumor immunity. Involvement of CD4⁺CD25⁺ Tregs in human cancer has been observed in peripheral blood and tumor tissues from patients with several types of cancer [18–20]. We and others have been able to demonstrate that Tregs are increased in peripheral blood and tumor-infiltrating lymphocytes of patients with HCC [21–23].

While initial investigations only demonstrated an increase in Treg frequencies in patients with HCC, follow-up studies have explored a potential correlation with disease progression and patients’ outcomes [24, 25]. One study demonstrated that an upregulation of Tregs was associated with a significantly reduced CD8⁺ T cell infiltration of tumors [26]. A correlation between poor survival and an increase in Tregs was also shown in the same report and supported by others [27, 28]. Finally, patients with advanced HCC had a higher percentage of intra-hepatic CD8⁺Foxp3 Tregs than did patients with early disease, suggesting that CD8⁺Foxp3⁺ Tregs also represent another immune escape mechanism [29].
Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of cells that consists of myeloid progenitor cells and immature myeloid cells [15, 16, 31, 32]. Natural suppressor cells (the initial name of MDSCs) were already described more than 25 years ago in patients with cancer [17, 33]. Murine MDSCs are characterized by coexpression of Gr-1 and CD11b. CD11b+Gr-1+ MDSCs represent approximately 2–4% of all nucleated splenocytes but can increase to up to 50% in tumor-bearing mice [18–20, 34, 35]. These cells are a mixture of immature myeloid cells, immature granulocytes, monocytes, macrophages, dendritic cells, and myeloid progenitor cells. MDSCs can be further subdivided into two major groups, granulocytic MDSCs (CD11b+Gr-1high) and monocytic MDSCs (CD11b+Gr-1low). Since MDSCs are such a heterogeneous population, efforts are ongoing to identify more markers to distinguish them better. Our laboratory has identified another marker, CD49d, to further characterize murine MDSCs. In the same report we showed that monocytic CD11b+CD49d+ MDSCs are more potent suppressors of antigen-specific T cells in vitro than CD11b+CD49d– granulocytic MDSCs and they suppress T cell responses through an NO-mediated mechanism [36].

In humans, MDSCs are also poorly characterized and are also divided into monocyctic and granulocytic populations. We have identified human monocytic CD14+HLA-DRlow/neg MDSCs in patients with HCC [37] and described an increase in the frequency of CD14+HLA-DRlow/neg MDSCs in peripheral blood and ascites in these patients. CD14+HLA-DRlow/neg MDSCs failed to induce proliferative T cell responses and did not mature into dendritic cells in vitro. Apart from their ability to suppress nonspecific T cell responses, MDSCs also masked AFP-specific T cell responses [37].

In order to better understand the biology and the clinical relevance of human MDSCs, we examined the interaction of MDSCs with other immune cells in more detail. Natural killer (NK) cells represent an important cell type in the context of HCC. NK cells are impaired in function in HCC patients [38]. We have demonstrated that MDSCs are potent suppressors of NK cells in patients with HCC [39]. In addition, we showed that human MDSCs induced a T regulatory phenotype when cocultured with CD4+ T cells [37]. Interestingly, while MDSCs induce FoxP3+ Tregs, CD14+HLA-DR+ cells induced a different T helper subtype, i.e. Th17 cells [40].

We have been able to identify S100A9 as one potential marker which can be used to distinguish MDSCs from monocytes [41]. Unfortunately, S100A9 is expressed intracellularly and cannot be used to isolate cells for functional studies. Genetic comparison of CD14+HLA-DRneg/low MDSCs and CD14+HLA-DR+ monocytes revealed a number of differences including higher expression of ATRA-related genes in MDSCs. ATRA is an important factor for the generation of Tregs [42], which correlates with the induction of the T regulatory phenotype by MDSCs.

Th17 Cells

It has been shown that Th17 cells are associated with poor outcomes in HCC [43]. In other tumors, conflicting reports have been published on the role of Th17 cells in tumor immunity [43, 44]. We have shown an increase in the frequency of Th17 cells in peripheral blood and Th17-related cytokines (IL-17, IL-23) in tumor supernatants from HCC patients. This observation prompted us to investigate whether Th17 cells have an effect on CD8+ T cell function. In vitro studies demonstrated that Th17 cells inhibit IFN-γ production and proliferation by CD8+ T cells. Further analysis revealed that only the CCR4+CCR6+ subpopulation of Th17 cells was responsible for this effect, which prompted us to examine CCR4+CCR6+ Th17 cell populations in HCC patients in more detail. Interestingly, we found only an increase in CCR4+CCR6+ Th17 cells in peripheral blood from patients with HCC but not in CCR4neg/CCR6+ Th17 cells [45]. Due to the low frequency of CCR4+CCR6+ Th17 cells in peripheral blood,
it was not possible to isolate enough cells for functional studies in vitro. However, we were able to examine CCR4+CCR6+ CD4+ T cells from peripheral blood of HCC patients and compare their function with CCR4neg CCR6+ CD4+ T cells. In order to eliminate any potential effect of Tregs, CCR4+CCR6+ or CCR4neg CCR6+ CD4+ T cells were isolated from peripheral blood of patients with HCC and g-irradiated. Irradiated cells were cultured with autologous CD3+CD8+ T cells at different ratios. CD8+ T cell proliferation was analyzed. The data shown are the cumulative results of 7 independent experiments. The white bars represent the proliferation of CD3+CD8+ T cells alone. * p < 0.05.

Fig. 3. CCR4+CCR6+ CD4+ T cells from patients with HCC suppress proliferation of CD8+ T cells. CCR4+CCR6+ or CCR4neg CCR6+ CD4+ T cells were isolated from peripheral blood of patients with HCC and g-irradiated. Irradiated cells were cultured with autologous CD3+CD8+ T cells at different ratios. CD8+ T cell proliferation was analyzed. The data shown are the cumulative results of 7 independent experiments. The white bars represent the proliferation of CD3+CD8+ T cells alone. * p < 0.05.

Monocytes

The role of monocytes in the tumor microenvironment of HCC has been thoroughly studied by Zheng’s groups. It has been shown that expression of PD-L1 on the surface of monocytes and macrophages in the peritumoral stroma suppresses T cell responses [47]. In further studies, they also demonstrated that monocytes not only suppressed T cell function directly but also induced IL-17-secreting effector CD8+ T cells (Tc17 cells) [48] as well as Th17 cells [49].

In summary, HCC has developed multiple cellular and molecular pathways to evade potent anti-tumor immune responses. In this review, we have summarized the cellular immune escape mechanisms in HCC. We believe that it will be important in the future to test treatment modalities which will target this cell population, especially in trials which aim to enhance anti-tumor immune responses. Initial studies targeting Tregs using low-dose cyclophosphamide and a recently published study evaluating the effect of anti-CTLA4 treatment in HCC [50] not only provide preliminary evidence that it is possible to augment anti-tumor immunity but also demonstrate that this type of approach is safe and needs further development and evaluation in the near future.

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