Polarization of Tissue-Resident T<sub>FH</sub>-Like Cells in Human Hepatoma Bridges Innate Monocyte Inflammation and M2b Macrophage Polarization

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The existence, regulation, and functions of IL21<sup>+</sup> immune cells are poorly defined in human cancers. Here, we identified a subset of protumorigenic IL21<sup>+</sup> T<sub>FH</sub>-like cells in human hepatocellular carcinoma. These cells were the major source of IL21 in tumors and represented about 10% of the CD4<sup>+</sup> T-cell population at levels comparable with the T<sub>FH</sub> cells present in lymph nodes. However, these T<sub>FH</sub>-like cells displayed a unique CXCR5−PD-1<sup>−/−</sup>BTLA−CD69<sup>hi</sup> tissue-resident phenotype with substantial IFN<sub>γ</sub> production, which differed from the phenotype of T<sub>FH</sub> cells. Toll-like receptor 4 (TLR4)–elicited innate monocyte inflammation was important for IL21<sup>+</sup> T<sub>FH</sub>-like cell induction in tumors, and activation of STAT1 and STAT3 was critical for T<sub>FH</sub>-like cell polarization in this process. Importantly, the T<sub>FH</sub>-like cells operated in IL21−IFN<sub>γ</sub>-dependent pathways to induce plasma cell differentiation and thereby create conditions for protumorigenic M2b macrophage polarization and cancer progression. Thus, induction of T<sub>FH</sub>-like cells links innate inflammation to immune privilege in tumors.

SIGNIFICANCE: We identified a novel protumorigenic IL21<sup>+</sup> T<sub>FH</sub>-like cell subset with a CXCR5−PD-1−BTLA−CD69<sup>hi</sup> tissue-resident phenotype in hepatoma. TLR4-mediated monocyte inflammation and subsequent T-cell STAT1 and STAT3 activation are critical for T<sub>FH</sub>-like cell induction. T<sub>FH</sub>-like cells operate via IL21−IFN<sub>γ</sub> pathways to induce plasma cells and create conditions for M2b macrophage polarization.
neutrophils through epithelial cell–derived CXC chemokines, and the neutrophils subsequently stimulate the metastatic and proangiogenic activity of cancer cells at the edge of an invading tumor (9–11). These observations reveal a fine-tuned collaborative action between cancer cells and different types of immune cells in the designated areas of tumors, which reroutes the inflammatory response in a cancer-promoting direction. Thus, to understand the roles and potential regulating mechanisms of inflammation in tumor immunopathogenesis, it is essential to evaluate the interaction networks of inflammatory molecules/cells within a tumor.

IL21 belongs to the IL2 family of cytokines, and it plays a critical role in both autoimmune and inflammatory diseases (12). Blockade of IL21 signals significantly attenuates the progression of a range of inflammatory diseases in mice (13, 14). Despite its actions in other systems, IL21 has been identified as being secreted by T follicular helper (TFH) cells and functioning as one of the most important stimulators of B-cell proliferation, isotype switching, and differentiation (15, 16). TFH cells mainly exhibit a CXCR5⁺PD-1⁺ICOS⁺BTLA⁺ phenotype and depend on expression of the master regulator transcription factor BCL6 (17, 18). Nevertheless, IL21 is also produced by neutrophils in human lymph nodes (19). To date, the existence, regulation, and functions of IL21⁺ immune cells are poorly defined in human cancers. Furthermore, although not directly related to IL21, recent studies in mice have demonstrated that accumulation of mature B cell–derived autoantibodies in premalignant tissues can regulate recruitment and polarization of myeloid cells by activating Fcγ receptors, which in turn promotes neoplastic progression and cancer metastasis (20, 21). These findings suggest that the factors that participate in B-cell maturation may not represent the host defense against the malignancy, but instead reflect effects that are rerouted to disease progression.

The precise mechanisms of IL21⁺ T helper (T_H) cell polarization in local tissues are not yet clear. A related issue that should also be addressed concerns the nature of the antigen-presenting cells (APC) that can induce IL21⁺ T_H cells. It is known that dendritic cells (DC) in germinal centers can support T_FH cell differentiation via an IL12/STAT4-dependent pathway (22). However, differentiation and maturation of DCs are defective in most solid tumors (23, 24). Inasmuch as macrophages (Mϕ) constitute an abundant population of APCs in solid tumors (25, 26), it is vital to determine whether Mϕ are the APCs primarily responsible for mediating the responses displayed by IL21⁺ T_H cells in human cancers.

In this study, we identified a novel subset of protumorigenic IL21⁺ TFH-like cells with B-cell helper function in human HCC. We found that these cells are the major source of IL21 in tumors and represent about 10% of the entire CD4⁺ T-cell population, and they exhibit a unique CXCR5⁻PD-1⁻BTLA⁻CD69⁺ IFNγ-producing tissue-resident phenotype that differs from the phenotype of conventional TFH cells. Also, Toll-like receptor 4 (TLR4)–mediated innate monocyte/Mϕ activation is important for IL21⁺ TFH-like cell induction in tumors, and blockade of STAT1 and STAT3 activation markedly inhibits generation of TFH-like cells. Moreover, TFH-like cell–mediated B-cell maturation contributes to protumorigenic M2b monocyte/Mϕ polarization in the tumor.

RESULTS
Identification of IL21⁺ Tissue-Resident TFH-Like Cells in Human HCC

Inflammatory signature genes are upregulated in human HCC (5). Analyzing the subset compositions of HCC-infiltrating inflammatory T_H cells revealed that IL21⁺ T_H cells represented a prominent component in CD4⁺ T cells at levels even higher than those of regulatory T cells (Supplementary Fig. S1A and S1B). We subsequently found that IL21⁺ cells accumulated in the peritumoral stroma of HCC and occurred at levels that were positively correlated with both the patients'
tumor-node-metastasis (TNM) stages (Fig. 1A) and the CD138+ plasma cell density in the same areas (Fig. 1B and C). To further investigate the source of IL21, we prepared single-cell suspensions from 12 normal blood samples, four normal liver samples (tissue distal to a liver hemangioma), and 30 HCC specimens paired with blood samples (Supplementary Tables S1 and S2; Supplementary Fig. S1A). Approximately 85% of IL21-secreting cells in the tumor tissue were CD3+ T+ cells, not CD14+ monocytes/Mφ, CD15+ neutrophils, CD19+ B cells, or CD56+ natural killer (NK) or NKT cells (Fig. 1D;
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Supplementary Fig. S1C and S1D; the percentage and absolute number of IL21+ T helper (Th) cells were significantly increased in tumor tissues compared with levels found in normal and HCC blood and normal liver tissues (Fig. 1E). Analogous changes in proportions of IL21+ Th cells were observed in samples from patients with HCC who were or were not infected with hepatitis B virus (HBV) or hepatitis C virus (HCV; Fig. 1F). Furthermore, the IL21+ Th cells expressed substantial amounts of trans-specific transcription factor BCL6 (Fig. 1G). These findings prompted us to further investigate and compare the phenotypic and functional features of HCC-infiltrating IL21+ Th cells and lymph node Th cells.

In general, the proportion of IL21+ Th cells was even larger in HCC tissues than in non–tumor-draining lymph nodes from patients with gastric cancer (N = 16; Fig. 1E and F; Supplementary Table S3). Interestingly, the tumor IL21+ Th cells exhibited a unique CXCR5+ PD-1+BTLA− CD69hi tissue-resident activated phenotype, whereas Th cells from lymph nodes appeared as CXCR5+ PD-1+BTLA− (Fig. 1H and I). Indeed, the proportion of CXCR5+ cells was basically reduced in tumor CD4+ T cells compared with peripheral Th cells (Supplementary Fig. S1E), which is consistent with a recent observation in liver cancer (27). Most strikingly, over 75% of the IL21+ Th cells isolated from HCC tissues were positive for IFNγ, although most IL21+ Th cells from blood and lymph nodes did not produce IFNγ; IL4, or IL17 (Fig. 1J and K). Notably, the IFNγ+IL21+ Th cells from tumor tissues simultaneously expressed BCL6 and T-bet and mainly displayed a CXCR5+PD-1+BTLA+ phenotype (Supplementary Fig. S1F–S1H). We further investigated whether IFNγ+IL21+ Th cells had B-cell helper function. Conditioned medium (CM) from tumor CD4+ T cells (approximately 20% of which were IL21+), but not CM from paired blood CD4+ T cells, effectively promoted the expansion of CD138+CD38+plasma cells (Fig. 1L; Supplementary Fig. S1I). ELISA also demonstrated marked production of IgM, IgA, and IgG by circulating B cells exposed to CM from tumor CD4+ T cells (Fig. 1M). In support of our hypothesis, antibody (Ab) blockade of IL21 in CM from tumor CD4+ T cells effectively inhibited the generation of CD138+CD38+CD27+ plasma cells and production of immunoglobulins (Fig. 1L and M; Supplementary Fig. S1I). Unexpectedly, such CM-mediated plasma cell differentiation was also abolished by an anti-IFNγ Ab (Fig. 1L and M; Supplementary Fig. S1I), revealing a previously unrecognized function of Th1 cytokine. Therefore, we examined the role of recombinant IL21 and IFNγ in plasma cell differentiation. IL21 promoted plasma cell differentiation in a dose-dependent manner (Fig. 1N). IFNγ alone, at a concentration up to 25 ng/mL, had only a marginal effect, although it did synergistically increase and accelerate the IL21-mediated plasma cell differentiation (Fig. 1N). Together, these data show that IFNγ+IL21+ Th1 cells represent a novel and functional tissue-resident Th1-like subset in human tumors.

Monocytes/Mφ Polarize IL21+ Th1-Like Cells in Cancer Microenvironments

Inasmuch as the IL21+ Th cells in tumor tissues exhibited characteristics distinct from such cells in peripheral blood and lymph nodes, we next investigated the effects of HCC environments on Th1-like cell generation. FACS analyses revealed that over 50% of IL21+ Th1 cells isolated from HCC tissue, but none of those obtained from normal blood or liver, showed substantial expression of KIT6, indicating that they were proliferating (Fig. 2A; Supplementary Fig. S2A). In HCC peritumoral stroma, the main site of IL21+ Th1 cells (Fig. 1A), there was pronounced accumulation of monocytes/Mφ (Fig. 2B), and the density of these cells was positively correlated with the density of IL21+ cells (R = 0.695, P < 0.01; Fig. 2C).

In subsequent experiments, we purified CD14+ monocytes/Mφ from tumor tissues and paired peripheral blood samples (Supplementary Fig. S2B), and then cultured those cells with autologous peripheral T cells ex vivo. Although monocytes/Mφ from both sources markedly expanded Th1 cells, only those obtained from HCC tissues elicited robust proliferation of IL21+ Th1 cells (Fig. 2D; Supplementary Fig. S2C) and production of IL21 (Fig. 2E). Approximately 60% of the expanded IL21+ Th1 cells were positive for IFNγ (Fig. 2D), which concurred with the cytokine profiles of the Th1-like cells from tumor tissue (Fig. 1J and K). In support of these observations, using a pSIF-H1-CopGFP-shBCL6 lentiviral vector to silence the Th1-specific transcription factor BCL6 (Supplementary Fig. S2D), and then cultured those cells with peripheral T cells (Fig. 2H), although only a small fraction of these cells can express IFNγ (Fig. 2I), suggesting that data acquired in mouse cancer models only partially reflect the findings in human cancers. As expected, depletion of monocytes/Mφ significantly reduced the percentage and absolute number of IL21+ Th1 cells in tumor tissues, whereas the effects of control Ab were negligible (Fig. 2J and K; Supplementary Fig. S2G). Correspondingly, the proportion of plasma cells declined sharply with decreasing infiltration of the IL21+ Th1 cells in tumors (Fig. 2K; Supplementary Fig. S2G). Depletion of monocytes/Mφ also partly attenuated tumor growth in liver (Fig. 2L; Supplementary Fig. S2H).

Innate Monocyte Activation Is Essential for IL21+ Th1-Like Cell Differentiation

The above-mentioned results suggested that monocytes/Mφ educated by cancer environments acquired the ability to expand IL21+ Th1-like cells. Consistent with this, we found that healthy blood monocytes exposed to culture supernatant from primary HCC cells (HCC-SN) rapidly induced Th1-like cells, producing significant amounts of IL21 and...
IFNγ (Fig. 3A and B). Similar results were obtained using monocytes treated with culture supernatants from established hepatoma cell lines, including QGY-7703 and HepG2 (Fig. 3C). In contrast, the expansion was only marginally affected by monocytes cultured in medium alone or in supernatant from normal liver cells (LO2; Fig. 3C). Moreover, an activated status was exhibited by monocytes that were derived from HCC tissues or exposed to either culture supernatants from primary HCC or established hepatoma cells, as shown by determining the upregulation of HLA-DR, CD80, and CD86 (Supplementary Fig. S3A–S3C).

We previously showed that environmental hyaluronan (HA) fragments from hepatoma cells induced protumorigenic myeloid cells by interacting with TLR4 or CD44 (11, 28), which


Figure 3. Innate monocyte activation is required for IL21+ Tfh-like cell differentiation. A–D, purified monocytes (MO) were left untreated, stimulated with 20% culture supernatants from primary HCC cells, hepatoma QGY-7703 or HepG2 cells, or normal liver L02 cells, or with 20 ng/mL LPS or 25 μg/mL intermediate HA fragments for 1 hour, and were washed and cultured with autologous T cells (1:5) for 9 days (A, C, and D) or indicated times (B) as described in Methods. Cytokines in TH cells were detected by FACS. Results represent mean ± SEM of four independent experiments (N = 4). E, exposing healthy blood monocytes to 20 ng/mL LPS, 25 μg/mL intermediate HA fragments, or 20% culture supernatants from primary HCC cells or hepatoma QGY-7703 or HepG2 cells for 30 minutes induced activation of AKT, MAPKs, and NF-κB. Results represent three separate experiments (N = 3). F and G, blocking TLR4 or CD44 by neutralizing Ab or blocking HA by peptide-1 (PEP1) in the exposure to HCC-SN attenuated the ability of monocytes to induce IL21+ Tfh-like cells. Data represent mean ± SEM of four independent experiments (N = 5). H, knockout of TLR4 impaired hepatoma-mediated IL21+ Tfh cell generation in Hepa1-6 tumors (n = 8 each group) as determined by FACS. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Role of Inflammatory Cytokines and STAT Signals in IL21+ Tfh-Like Cell Differentiation

After finding that activated monocytes/Mφ promoted expansion of IL21+ Tfh cells in tumors, we evaluated the effect of tumor-elicited proinflammatory responses of monocytes/Mφ on development of IL21+ Tfh-like cells. Exposure of monocytes to culture supernatants from primary HCC and established hepatoma cells led to substantial production of IL1β, IL6, IL23, TNFα, and TGFβ, but not IL12p70 (Fig. 4A). Real-time PCR showed that CD14+ cells from HCC tumors displayed marked upregulation of IL1β, IL6, IL23, TNFα, and TGFβ, but not IL12p70 (Fig. 4B). Therefore, we used neutralizing Abs that effectively abolished the individual roles of above-indicated cytokines in our monocyte-expanded IL21+ Tfh cell system. Expression of IL21 in CD4+ T cells was inhibited 40% to 70% by blocking IL1β and IL23, but was not reduced by blocking IL6, TNFα, TGFβ, or IL12p70 (Fig. 4C). Interestingly, blocking IL1β primarily decreased the proportion of IFNγ-expressing IL21+ Tfh cells, whereas an anti-IL23 Ab partially reduced proportions of total IL21+ Tfh cells (Fig. 4D). Comparably, recombinant IL1β effectively increased the prevalence of IFNγ+ IL21-expressing Tfh cells, whereas IL23 promoted expansion of both IFNγ+ and IFNγ− IL21-producing Tfh cells (Fig. 4E).
STAT proteins play crucial roles in cytokine-specifying TH cell differentiation, and IL12p70-mediated STAT4 activation is essential for TH1 cell differentiation in lymph nodes (22, 29). However, exposing T cells to CM from monocytes preincubated with HCC-SN did not elicit activation of STAT4 but instead triggered pronounced phosphorylation of STAT1 and STAT3 (Fig. 4F), thus revealing additional STAT signals in IL21+ TH cells. Correspondingly, recombinant IL1β effectively promoted STAT1 activation in T cells, whereas recombinant IL23 was more potent in triggering STAT3 activation in those cells (Supplementary Fig. S4). Analogously, abolishing STAT1 activation by treating with fludarabine or using a pSIF-H1-CopGFP-shSTAT1 lentiviral vector effectively suppressed the expansion of IFNγ+ IL21-producing TH cells mediated by HCC-SN–treated monocytes; in parallel, suppressing STAT3 signals by the specific inhibitor AG490 or by the pSIF-H1-CopGFP-shSTAT3 lentiviral vector potently reduced proportions of total IL21+ TH cells (Fig. 4G and H). Notably, silencing STAT3 also effectively suppressed the expression of transcription factor BCL6 in TH cells, whereas inhibition of STAT1 marginally affected BCL6 expression (Fig. 4I). Together, these observations suggest that cytokine production profiles of activated monocytes determine the “content” of STAT signals and subsequent BCL6-mediated IL21+ TH cell differentiation in tumors.
High Infiltration of IL21+ TFH-Like Cells Predicts Poor Patient Survival and Induces Protumorigenic M2b Mφ Polarization and Hepatoma Growth

To investigate the impact of IL21+ TFH-like cells on clinical features of human HCC, we collected and analyzed clinical data on 185 patients with HCC (Supplementary Table S2) and divided these subjects into two groups according to the median value of IL21+ cell density. We found a striking inverse association between IL21+ cell density in the peritumoral stroma and both overall and disease-free survival (OS and DFS; both \( P < 0.001 \); Fig. 5A). By contrast, IL21+ cells in the intratumoral area were not related to either OS or DFS (Fig. 5A). Using the same cutoff point, similar results were obtained in patients with early stages of HCC (\( N = 116 \)) and in patients from a validation cohort of HCC (\( N = 78 \); Supplementary Table S2; Supplementary Fig. S5A and S5B). The density of peritumoral stromal IL21+ cells was also associated with tumor size \( (P = 0.001) \), vascular invasion \( (P = 0.036) \), and \( \alpha \)-fetoprotein level \( (P = 0.018) \); Supplementary Table S4). In multivariate analysis, the number of IL21+ cells in the peritumoral stroma was an independent prognostic factor for both OS and DFS (Supplementary Table S5). Considering the colocalization of CD68+ cells and IL21+ cells in the HCC peritumoral stroma (Fig. 2B) and the protumorigenic role of monocytes/Mφ in liver (Fig. 2K), we investigated whether IL21+ cells affect the polarization of protumorigenic monocytes/Mφ in tumors. Figure 5B and C show that IL21+ cell density was associated with an M2b protumorigenic phenotype of monocytes/Mφ in HCC tissue. More precisely, substantial infiltration of IL21+ cells was positively correlated with higher expression of the M2b marker CCL1 and the M2 marker IL10 in CD14+ cells derived from invading HCC tissue. We subsequently injected anti-IL21 neutralizing Ab or recombinant IL21 into the peritoneum of mice bearing Hepa1-6 hepatoma or H22-associated ascitic hepatoma. Primarily, manipulation of IL21 signals significantly affected the plasma cell differentiation in tumors in both models (Fig. 5D). In support, injection of anti-IL21 neutralizing Ab did effectively reduce the number of peritoneal IL21+ Mφ in vivo, and the addition of recombinant IL21 significantly enhanced both of those processes (Fig. 5D–F).

Inasmuch as the level of CD138+ plasma cells was positively associated with the patients’ TNM stage (Supplementary Fig. S5C), we afterward eliminated the plasma cell differentiation in tumors by applying an anti-CD20 Ab to deplete B cells (Supplementary Fig. S5D). Application of anti-CD20 Ab did attenuate the M2b Mφ polarization and hepatoma growth (Fig. 5G and H; Supplementary Fig. S5E and S5F). More importantly, such treatment completely abolished the effects of anti-IL21 neutralizing Ab or recombinant IL21 on M2b Mφ polarization and hepatoma progression (Fig. 5G and H; Supplementary Fig. S5E and S5F). Supporting our hypothesis that IL21-mediated protumorigenic monocyte/Mφ polarization and plasma cell maturation are interrelated in tumors, adoptive transfer of tumor plasma cells significantly reinstated the Mφ polarization and aggressive hepatoma growth at levels comparable to those exhibited by hepatoma-bearing mice without treatment of anti-CD20 Ab (Fig. 5G and H; Supplementary Fig. S5E and S5F).

Having established the important roles of IL21+ T cells in inducing protumorigenic Mφ and hepatoma growth, we also investigated the effects of these cells on liver tumorigenesis using a diethylnitrosamine (DEN)-induced autonomous mouse hepatoma model. Consistent with the findings acquired in Hepa1-6 and H22 hepatoma models, the proportions of IL21+ Tc1 cells and plasma cells in DEN-induced hepatoma were significantly increased compared with those in normal liver (Supplementary Fig. S5G). Accordingly, the expression of M2 markers CCL1 and IL10 was markedly upregulated in monocytes/macrophages derived from this hepatoma model (Supplementary Fig. S5G). More strikingly, in such a model, injecting anti-IL21 neutralizing Ab for the final 2 months almost completely abolished the liver tumorigenesis (data not shown), which further revealed the importance of IL21+ T cells in liver cancer.

Fcγ Receptor–TLR Cross-talk Is Required for M2b Mφ Polarization

We finally determined whether and how TFH-like cell-induced plasma cell differentiation contributed to M2b Mφ polarization. CM from tumor TFH-like cell-induced plasma cells (PC-CM) did not influence the activation route of normal blood monocytes/Mφ, but it evidently triggered the expression of M2 markers, and even further enhanced the expression of IL1β, IL6, and IL23 in HCC-SN–treated monocytes (Fig. 6A), which indicates an M2b phenotype consistent with the monocyte phenotype in tumors in situ (Figs. 4B and 5C). This is further supported by the finding in monocytes that PC-CM–augmented HCC-SN–elicited activation of IκBα, JNK, and p38 (Figs. 3E and 6B). It is noteworthy that Ab-mediated engagement of Fcγ receptor can regulate spleen tyrosine kinase (SYK) activation (30). Consistently, adding an SYK inhibitor, R406, selectively suppressed PC-CM–mediated activation of IκBα, JNK, and p38 (Fig. 6C). These data suggest that HCC-SN–associated TLR4 signal cooperates with plasma cell–elicited SYK activation to induce M2b Mφ. Indeed, knockout of TLR4 in mice bearing Hepa1-6 hepatoma or H22-associated ascitic hepatoma substantially decreased the expression of M2 markers in monocytes from tumor tissues (Fig. 6D); peritoneal injection of SYK inhibitor R406 for the final 2 days could suppress the IL21-mediated M2 marker upregulation in tumor monocytes (Fig. 6E).

We further probed the mechanisms involved in the induction of M2b Mφ polarization by using protein G to specifically deplete IgG complexes in PC-CM (Supplementary Fig. S6), which successfully inhibited the M2b marker upregulation (Fig. 6F). More interestingly, we found that the interactions between IgG complex and FcγIla receptor were correlated with the additional increase in inflammatory cytokines, whereas the cross-talk between IgG complex and FcγIIb receptor mainly upregulated the M2 markers IL10 and CCL1 (Fig. 6G). Neither FcγIIa receptor nor FcγIII receptor participated in M2b Mφ polarization (Fig. 6G). Together, these findings suggest that IL21+ TFH-like cell-mediated plasma cell differentiation collaborates with HCC environments to induce protumorigenic M2b Mφ polarization.

DISCUSSION

Here, we identified a subset of novel protumorigenic IL21+ TFH-like cells and applied multiple complementary strategies...
Figure 5. High density of IL21+ T<sub>FH</sub>-like cells predicts poor survival of patients with HCC and induces M2b M<sub>ϕ</sub>polarization. **A**, patients were divided into two groups according to the median IL21+ lymphocyte density in nontumoral liver, peritumoral stroma, and intratumoral tissues: low (N = 93, black) and high (N = 92, red) density. Cumulative OS and DFS time were calculated using the Kaplan–Meier method and analyzed by the log-rank test. **B**, representative distribution of IL21+ (green) and CCL1+ (red) cells in HCC samples (N = 8). Scale bar, 50 μm. **C**, real-time PCR–determined relative fold changes of CCL1 and IL10 in monocytes isolated from tumor tissue from 18 patients with HCC. Patients were divided into two groups according to the median proportion of IL21+ TH cells. **D–F**, effect of anti-mouse IL21 Ab or recombinant IL21 on plasma cell and M2 M<sub>ϕ</sub>differentiation (**D**), monocyte/M<sub>ϕ</sub>cytokine production (**E**), and tumor growth (**F**) in liver bearing Hepa1-6 or H22 hepatoma (N = 5 each group). Scale bar, 100 μm. **G and H**, depletion of B cells by anti-CD20 Ab suppressed tumor growth (**G**) and M2b M<sub>ϕ</sub>polarization (**H**) in liver bearing hepatoma Hepa1-6, an effect that was abrogated by adoptive transfer of tumor plasma cells (N = 5). Relative fold changes in the indicated markers in monocytes were determined by real-time PCR. Data represent mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with PBS.

Other studies in humans have identified T<sub>FH</sub> cells that exhibit a CXCR5<sup>+</sup>PD-1<sup>+</sup>BTLA<sup>+</sup> phenotype (15, 31), and it has been suggested that such cells play a crucial role in aiding B cells by releasing IL21 (16). Furthermore, several recent investigations have demonstrated that IL21 can directly promote the generation and proliferation of human antigen-specific cytotoxic T-cell responses in vitro or ex vivo (32, 33), which implies
TFH-like cells synergistically increased IL21-mediated plasma cell differentiation and immunoglobulin production, which represents a previously unrecognized effect of T<sub>H1</sub> cytokine type that differed from the phenotype of conventional T<sub>FH</sub> cells. Interestingly, we found that the IFN<sub>y</sub> released by these T<sub>H1</sub>-like cells synergistically increased IL21-mediated plasma cell differentiation and immunoglobulin production, which represents a previously unrecognized effect of T<sub>H1</sub> cytokine...
IFNγ. Indeed, Perez-Shibayama and colleagues (34) recently observed that IFNγ was required for optimal germinal center reactions and generation of IgM-producing B cells, and, more importantly, such T~FH~like cell-elicited generation of immunoglobulin-secreting plasma cells also led to M2b polarization in tumors. Consistent with this, the density of IL21’ TH cells in peritumoral stroma was advanced with correlated disease stages and poor survival in patients with HCC. These findings together imply that it is not IL21 per se, but rather the immune network of IL21 that determines the ability of IL21 to facilitate or prevent tumor growth. In other words, a better understanding of the immune network of IL21 in human tumor environments would be helpful for developing rational design of novel immune-based anticancer therapies that can restore the antitumorigenic function of IL21.

Despite recent advances in understanding the differentiation of T~FH~ cells in immune organs (35–37), little is known about the mechanisms underlying regulation of IL21’ TH cells in local tissues. Our results obtained in four sets of experiments provide evidence that TLR4-mediated innate activation of monocytes/Mφ plays a dominant role in the development of IL21’ TH like cells in 10 times higher in peritumoral stroma than in cancer nests, and there were significant correlations between the densities of IL21’ TH cells and oncocytes/Mφ in peritumoral stroma, where most of the oncocytes/Mφ were activated. Second, compared with CD14’ monocytes/Mφ isolated from blood, such cells derived from tumor tissues induced significantly greater expansion of IL21’ TH cells exhibiting phenotypic features more similar to the phenotype of tumor-infiltrating T~FH~like cells (i.e., a remarkable proportion of IL21’IFNγ’ T~FH~ cells). Third, healthy blood monocytes exposed to HCC-SN produced significant amounts of pro-inflammatory IL1β, IL6, and IL23 and subsequently markedly induced IL21’ TH like cells, and such processes were clearly reversed by blocking TLR4, but not by blocking TLR2. Fourth, knockout of TLR4 in C57BL/10 mice bearing Hepa1-6 hepatoma largely diminished the levels of IL21’ TH cells in liver in vivo. Therefore, TLR4-mediated activation of monocytes/Mφ in tumors may serve as a novel route to promoting IL21’ TH like cell expansion in human cancer. This hypothesis is compatible with previous studies showing that activated DCs are involved in differentiation and expansion of T~FH~ cells (22, 38).

IL12-triggered STAT4 phosphorylation is vital for DC-mediated T~FH~ cell differentiation in the germinal center (22, 37). However, in our study, such a pathway was not active in T cells cultured with tumor-derived monocytes/Mφ, and inhibiting STAT1 and STAT3 activation effectively attenuated the induction of T~FH~ like cells. More precisely, blocking STAT3 activation selectively impeded IL21 expression in T cells conditioned by tumor monocytes/Mφ, whereas the STAT1 inhibitor was more efficient in reducing the generation of IFNγ’ T~FH~ like cells, which supports the general view that STAT1 participates in regulating T~FH~ differentiation (39). Involvement of STAT3 in T~FH~ like cell polarization is supported by an investigation showing that STAT3 played a critical role in inducing BCL6 (40), an essential protein in T~FH~ like cell polarization in our HCC system (Fig. 2F). We also identified the key cytokines secreted by tumor monocytes that were responsible for T~FH~ like cell induction: IL1β played a more dominant role in the induction of IFNγ’IL21’ T~FH~ cells, and IL23 stimulated virtually all IL21’ T~FH~ cells. These results reveal that an inflammatory cytokine milieu facilitates development of T~FH~ like cells and hence might contribute to B-cell-mediated cancer immunoediting. This concurs with data reported by Schmitt and colleagues (37) showing that an inflammatory cytokine milieu further augments TGFβ-mediated initiation of T~FH~ differentiation in human tonsils. M2 Mφ can be further divided into subsets M2a, M2b, and M2c based on gene expression profiles (41). M2b Mφ are rather unique in that they produce high levels of anti-inflammatory IL10 and CCL1 chemokine, but they also express significant amounts of TNFα, IL1β, and IL6, which indicates a more complex role in the inflammatory response than has previously been recognized. Binding of Fcγ receptors by IgG complexes leads to M2b polarization during tumor progression (20). The presence of IgG-producing B cells and TLR4-mediated monocyte activation in tumors increases the likelihood of engagement of this pathway, that is, the probability that T~FH~like cell-conditioned B cells will encounter tumor-activated monocytes in the peritumoral stroma. Perhaps the M2b Mφ-derived IL10 serves to control T-cell activation and helps avoid hyper-immune activation (41). More importantly, enhanced release of the inflammatory cytokines TNFα, IL1β, IL6, and IL23 by M2b Mφ might result in Th17-mediated inflammation (8) that in turn stimulates the metastatic and proangiogenic activity of cancer cells by recruiting neutrophils (9–11). These activated monocytes would thereby repurpose the inflammatory response away from antitumor immunity and toward tissue remodeling and proangiogenic pathways.

Our results provide important insights into loop manipulation of inflammatory monocyte-mediated immuno-suppression in human tumors (Supplementary Fig. S7). Soluble factors derived from cancer cells promote innate activation of newly recruited monocytes by triggering TLR4. After interacting directly with these activated monocytes, the T~FH~ cells acquire a tissue-resident T~FH~ like phenotype and operate via IL21–IFNγ-dependent pathways to induce plasma cell differentiation and thereby create conditions for M2b Mφ polarization. Thus, induction of T~FH~ like cells links innate inflammation to immune privilege in tumors. Accordingly, studying the mechanisms that can selectively modulate functional activities of inflammatory stromal cells may lead to a novel strategy for anticancer therapy (26, 42–44).

METHODS

Patients and Specimens

Lever and HCC samples and samples of non–tumor-draining lymph nodes from individuals with gastric cancer were obtained from patients undergoing curative resection at the Cancer Center of Sun Yat-sen University (Supplementary Tables S1–S3). None of the patients had received antitumor therapy before sampling, and those with concurrent autoimmune disease, HIV, or syphilis were excluded. Paired fresh blood samples taken on day of surgery and tumor tissue from 30 patients with HCC who underwent surgical resections between December 2011 and June 2014 were used to isolate peripheral and tissue-infiltrating leukocytes (Cohort 1; Supplementary Tables S1 and S2). One hundred eighty-five patients with HCC (Cohort 2, Supplementary Table S2) and 78 patients with HCC...
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The counting of CD138+ cells was performed by two independent observers who were blinded to the clinical outcome. For immunofluorescence analysis, frozen sections were stained with mouse anti-human CD4 (1:100; Neomarker) plus rabbit anti-human CCL1 (5 μg/mL; Novus), or goat anti-human IL21, or goat anti-human CCL1 (5 μg/mL; R&D Systems) plus rabbit anti-mouse CCL1 (2 μg/mL; R&D Systems), followed by Alexa Fluor 488–conjugated anti-rabbit IgG (Molecular Probes) plus Alexa Fluor 555–conjugated anti-goat IgG (Molecular Probes). Positive cells were quantified using ImagePro Plus software or detected by confocal microscopy.

**Evaluation of Immunohistochemical Variables**

The procedure for evaluation of immunohistochemical variables was established in several of our previous studies (45, 46). In this study, we used the same staining procedure of IHC for each marker in paraffin-embedded and formalin-fixed HCC samples. Analysis was performed by two independent observers who were blinded to the clinical outcome. At low-power field (×100), the tissue sections were screened, and the 5 most representative fields were selected using a Leica DM IRB inverted research microscope (Leica Microsystems). For evaluating the density of tissue-infiltrating IL21+ cells, the respective areas of non-tumoral liver, peritumoral stroma, and intratumoral region were then scanned at ×400 magnification (0.146 mm² per field). The number of nucleated IL21+ cells in each area was then counted manually and expressed as cells per field. Positively stained cells that are smaller than the size of circulating T cells (10 μm) were excluded from counting. There was a significant linear correlation between the counting data of two independent observers (P = 1.99 × 10−38, R = 0.878; P = 3.12 × 10−39, R = 0.911; and P = 2.33 × 10−40, R = 0.893, respectively), and the average counting by two investigators was applied in the following analysis to minimize interobserver variability. The same standard was applied to the counting of CD138+ cells and CD68+ cells.

**Isolation of Mononuclear Cells from Peripheral Blood and Tissues**

Details are provided in Supplementary Methods.

**Immunoblotting**

Proteins were extracted from cells as previously described (47). The Abs used are listed in Supplementary Table S6.

**FACS**

Details are provided in Supplementary Methods. The fluorochrome-conjugated Abs used are listed in Supplementary Table S7.

**ELISA**

Details are provided in Supplementary Methods.

**Real-Time PCR**

Details are provided in Supplementary Methods. The specific primers used to amplify the genes are listed in Supplementary Table S8.

**Preparation of Culture Supernatant from Primary HCC Cells and Established Hepatoma Cell Line Cells**

Culture supernatants were acquired by culture of completely digested HCC tumor or hemangioma liver biopsy specimens. All specimens were from individuals without concurrent autoimmune disease, HBV, HCV, HIY, or syphilis. The digested tumor or liver cells were washed in medium containing polymyxin B (20 μg/mL; Sigma-Aldrich) to exclude endotoxin contamination. Thereafter, 107 digested cells were resuspended in 10 mL of complete medium and cultured in 100-mm dishes. After 2 days, the supernatants were harvested, centrifuged, and stored at −80°C.

The human hepatoma cell lines HepG2 and QGY-7703 and normal liver cell line L02 were obtained in January 2013 from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) within 6 months of the experiments being carried out. The cells were authenticated by short tandem repeat profiling and were confirmed to be Mycoplasma-negative before use, and they were maintained in DMEM supplemented with 10% FCS. Culture supernatant was prepared as previously described (28).

**Construction of Viral Vectors**

Details are provided in Supplementary Methods.

**Ex Vivo Plasma Cell Induction**

Details are provided in Supplementary Methods.

**In Vitro Culture System for Monocytes/Mϕ**

Purified CD14+ healthy blood monocytes were left untreated or were stimulated with 20 ng/mL LPS, 25 μg/mL intermediate HA fragments (Sigma), or 20% culture supernatants from primary HCC cells or established hepatoma cells in the presence or absence of 20 μg/mL anti-CD44 (LabVision), anti-TLR2 (eBioscience), or anti-TLR4 Ab (eBioscience), or anti-TLR4 Ab (eBioscience), or 100 or 200 μg/mL HA-specific blocking peptide (PEP1, GAHWQFQALTAVNQ) or a control peptide (CPEP, WRHGFAALTAVNQ) for indicated time. Expression of inflammatory cytokines and surface markers was determined by ELISA, FACS, or real-time PCR, and activation of the PI3K/AKT, MAPK, and NF-kB pathways was analyzed by immunoblotting. In some experiments, before exposure to HCC-SN, the monocytes were left untreated or were preincubated with a blocking Ab (10 μg/mL; eBioscience or BD) against CD4, CD8, CD3, or 20 μg/mL anti-TLR4 Ab, or 1 μmol/L SYK inhibitor R406 (Selleck), and then stimulated with CM from tumor Tαi cell-induced plasma cells, or with CM depleted of IgG by Dynabeads protein G (Life Technologies).

**In Vitro T-cell Culture System**

In 2-day incubations, purified autologous T cells, naïve T cells, memory T cells (Miltenyi Biotec), or T cells infected with viral particles containing a pSIF-H1-CopGFP-shBCL6, pSIF-H1-CopGFP-shSTAT1, or pSIF-H1-CopGFP-shSTAT3 lentiviral vector were treated in different ways: left untreated; cultured with CD14+ cells (5:1) derived from HCC blood or tumor or with blood monocytes that had been exposed for 5 hours to various stimulants; exposed to medium supplemented with recombinant IL1β (2 ng/mL), IL6 (10 ng/mL), IL23 (1 ng/mL), TNFα (5 ng/mL), or TGFβ (2 ng/mL; R&D Systems) in the presence of 2 μg/mL anti-CD3 and 2 μg/mL anti-CD28 (eBioscience). Thereafter,
In Vivo Regulation of Tfh-Like Cells and Plasma Cell Differentiation

Murine Hepa1-6 or H22 hepatoma cells (10^6) in 25 μL Matrigel (Corning) were injected under the hepatic capsule of 5- to 7-week-old female C57BL/6 mice for 2 days. Thereafter, the mice were injected with Ab against CSF1R (10 mg/kg; Bio X Cell) every 3 days. In parallel, control animals were injected with isotype Ab. After 21 days, the peripheral and tumor-infiltrating leukocytes were isolated and stimulated at 37°C for 5 hours with Leukocyte Activation Cocktail, then stained with a fluorochrome-conjugated conjugated Ab for mouse CD4, CD3, and IL21 and analyzed by FACS. Also, some of the peripheral and tumor-infiltrating leukocytes were analyzed immediately after isolation to determine the proportion of CD138^+ plasma cells.

In the second set of in vivo experiments, 5- to 7-week-old female C57BL/6 mice were preinjected with anti-mouse CD20 Ab (10 mg/kg; eBioscience) or buffer alone for 3 days. Thereafter, Hepa1-6 or H22 hepatoma cells (10^6) in 25 μL Matrigel were injected under the hepatic capsule. The mice were subsequently injected every 3 days with Abs against mouse CD20 in the presence or absence of anti-mouse Ab IL21 or recombinant IL21 (both from eBioscience; 10 mg/kg) or 10^5 FACS-sorted tumor CD138^+ plasma cells harvested from mice bearing the same hepatoma. In some cases, R406 (1 mg/kg) was injected for the final 2 days of the 21-day tumor bearing. Afterward, part of the tumor tissues were prepared for frozen sections and subjected to immunofluorescent staining and confocal microscopy, the tumor-infiltrating monocytes were isolated (Miltenyi Biotec) and the expression of CCL1 and IL10 in these cells was analyzed by ELISA or real-time PCR.

The authors thank Ms. Patricia Ödman for linguistic revision of the manuscript.

Acknowledgments

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by project grants from the National Natural Science Foundation of China (81422036 and 31470855), the National Key Research and Development Plan of China (2016YFA0502600), the Guangdong Natural Science Funds for Distinguished Young Scholar (2013050014639), the Foundation for the Author of National Excellent Doctoral Dissertation of PR China (201230), the Project Supported by Guangdong Province Higher Vocational Colleges & Schools Pearl River Scholar Funded Scheme (2016), and the Fundamental Research Funds for the Central Universities (15lgj09).

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Received March 17, 2016; revised August 9, 2016; accepted August 9, 2016; published OnlineFirst August 16, 2016.

REFERENCES


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No potential conflicts of interest were disclosed.

Grant Support

This work was supported by project grants from the National Natural Science Foundation of China (81422036 and 31470855), the National Key Research and Development Plan of China (2016YFA0502600), the Guangdong Natural Science Funds for Distinguished Young Scholar (2013050014639), the Foundation for the Author of National Excellent Doctoral Dissertation of PR China (201230), the Project Supported by Guangdong Province Higher Vocational Colleges & Schools Pearl River Scholar Funded Scheme (2016), and the Fundamental Research Funds for the Central Universities (15lgj09).

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Tissue-Resident TFH-Like Cells in Human HCC


Polarization of Tissue-Resident T\(_{FH}\)-Like Cells in Human Hepatoma Bridges Innate Monocyte Inflammation and M2b Macrophage Polarization

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Cancer Discov  Published OnlineFirst August 16, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-16-0329

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