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RAPID COMMUNICATION

De novo lipogenesis prolongs the lifespan and supports the immunosuppressive phenotype of neutrophils in HCC metastasis

Metastasis is the primary cause of HCC-related death. Neutrophils can activate the antitumor immune response and promote tumor cell clearance. However, most studies show that neutrophils support tumor progression and metastasis. The accumulation of neutrophils contributes to HCC progression and is associated with poor prognosis.[1] Metabolic reprogramming is one of the significant hallmarks of cancers, and immune cells in the tumor microenvironment (TME) undergo complex metabolic alterations.[2] Accumulating evidence indicates that high lipid content and increased fatty acid oxidation (FAO) support neutrophils' immunosuppressive functions of neutrophils.[2] However, the role of neutrophils lipid metabolism in HCC metastasis remains largely unclear. In the present study, we sought to evaluate lipid metabolism in metastasis-associated neutrophils (MANs) and found that *de novo* lipogenesis (DNL) endows neutrophils with a prolonged lifetime and immunosuppressive phenotype that contribute to HCC metastasis.

Increased neutrophil levels or an elevated peripheral neutrophil-to-lymphocyte ratio indicate poor clinical outcomes in HCC patients.[1] Consistent with previous studies, we found more intratumor CD66-positive cells in samples from metastatic HCC patients than those from non-metastatic HCC patients (Fig. 1A). Furthermore, the survival time of HCC patients was closely negatively associated with the number of intratumoral neutrophils (Fig. S1A,B).

Circulating neutrophils have a relatively short lifespan, but their longevity increases to more than a few days under specific conditions.[3] We treated peripheral neutrophils with different culture medium (CM) preparations at different time points. The data showed no apparent apoptosis in neutrophils at 6 h, but treatment with CM from Huh7 HCC cells with low metastatic potential and control media led to significant apoptosis in neutrophils 24 h later (Fig. S2A,B). Surprisingly, stimulation with CM from highly metastatic hepatoma cells, including HCCLM3 cells, prevented neutrophils from apoptosis to a large extent (Fig. 1B). Increased neutrophils in HCC tumors may be primarily due to direct recruitment from circulation, and our data suggested that the prolonged lifespan of neutrophils also comes into play in this course.

Although most studies have suggested a protumor role for neutrophils, few studies support the involvement of neutrophils in the early antitumor response.[4] Incubating Huh7 or HCCLM3 cells with neutrophils at a 1:20 ratio for 24 h, we found that neutrophils suppressed the viability of both HCC cell lines, but the inhibitory effect on Huh7 cells was more significant than that on HCCLM3 cells (Fig. 1C). Moreover, we found that more PKH26-labeled neutrophils adhered to Huh7 cells than HCCLM3 cells (Fig. S3A). Our data revealed that more neutrophils translocated into the basal chambers with HCCLM3-CM treatment than other CM treatments (Fig. S3B). Mechanistically, the cytokine profiles revealed increased chemokines such as IL-8, C-X-C motif chemokine ligand 1 (CXCL1) in Huh7-CM compared with that in HCCLM3-CM (Fig. S3C).

The short lifespan limits many functional experiments of neutrophils. HL60 cells can be differentiated into granulocyte-like cells by various agents and are commonly used to study neutrophil functions. We cocultured dHL60 cells with different HCC cells at a 20:1 ratio and found that dHL60 cells improved the colony formation ability of HCCLM3 cells but dramatically inhibited that of Huh7 cells (Fig. 1D). Upon stimulation, neutrophils secrete diverse mediators that are crucial for regulating the immune response to cancer cells. As shown in Figure 1E and S4A, HCCLM3-CM treatment notably upregulated the mRNA levels of *Il10*, *Pge2*, and *Arg1*, while there was no difference in the *Inos*, *Tnf*α, *and Tgf*β gene levels in dHL60 cells.

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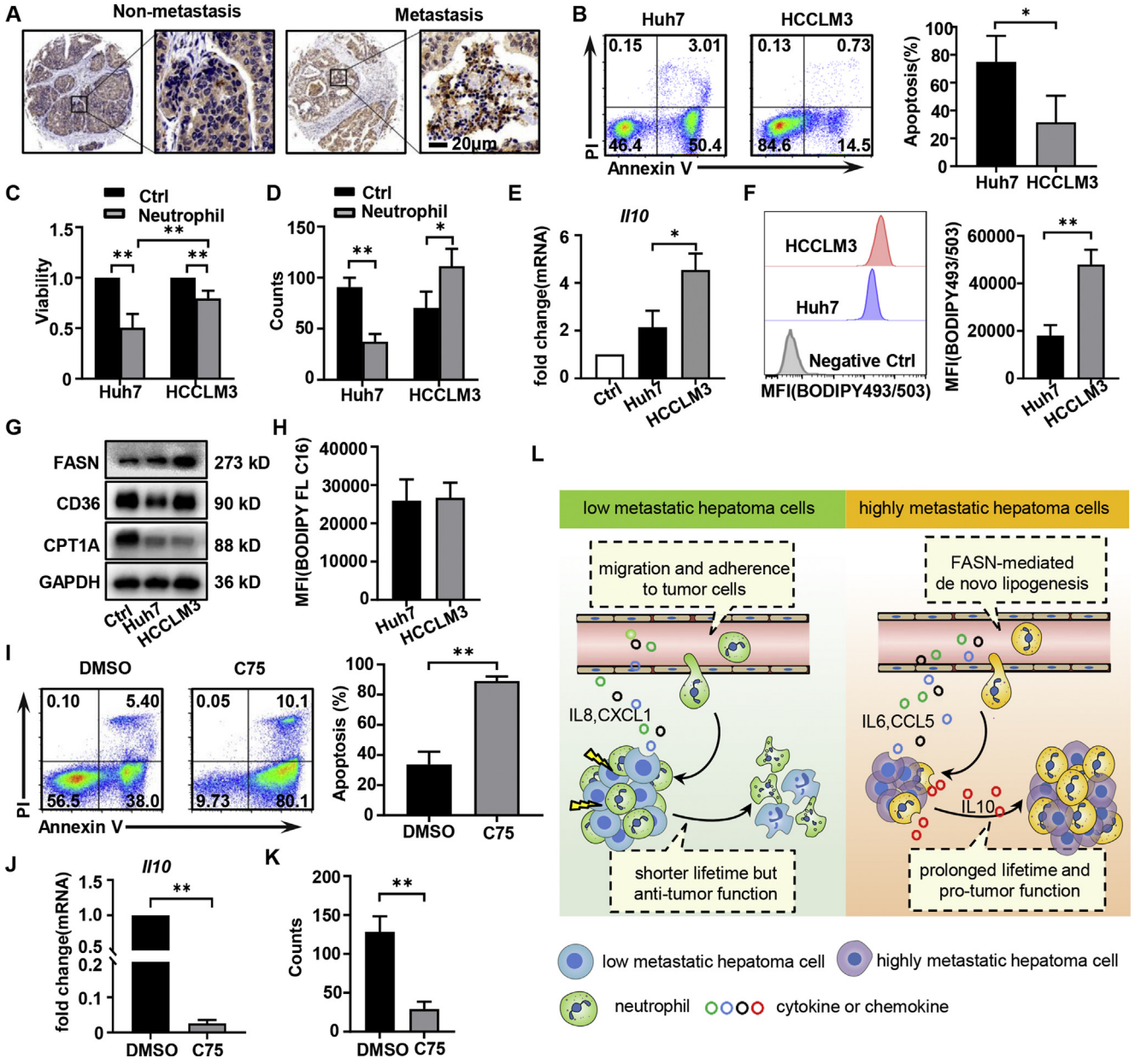

Figure 1 The lifetime and phenotype of metastasis-associated neutrophils are highly dependent on lipogenesis. **(A)** HCC tissue array was subjected to CD66b immunostaining. **(B)** After treatment with hepatoma cell CM for 24 h, FCM measured apoptosis in peripheral neutrophils. **(C)** Cocultured with neutrophils at a 1:20 ratio, the proliferation of tumor cells was measured 48 h later. **(D)** Treatment with the indicated HCM for 24 h, RT-qPCR analyzed the levels of *Il10* mRNA in dHL60 cells. **(E)** Cocultured with dHL60 cells at a 1:20 ratio for 7—10 days, colonies of HCC cells were stained with 0.05% (v/v) crystal violet dye and analyzed by ImageJ software. **(F)** Treatment with the HCM for 24 h, levels of lipids in neutrophils were stained with BODIPY 493/503 and measured by FCM. **(G)** Expression of lipid metabolism proteins in dHL60 cells was detected with immunoblotting. **(H)** Ability of BODIPY FL C16 uptake by dHL60 cells. **(I)** Treated with HCCLM3-CM in combination with C75 (50 μM) for 24 h, and the apoptosis in neutrophils was measured. **(J)** Stimulated with HCCLM3-derived CM and C75 (50 μM) for 24 h, the mRNA levels of *Il10* in dHL60 cells were measured. **(K)** Coculturing HCCLM3 cells with dHL60 cells at a 1:20 ratio, colonies were stained with 0.05% (v/v) crystal violet dye 7—10 days later and analyzed by ImageJ software in the presence of C75 (50 μM). **(L)** A proposed model shows increased DNL prevents apoptosis and induces immunosuppressive function of neutrophils, which subsequently contributes to the colonization of metastatic HCC cells. *, $P < 0.05$; * *, $P < 0.001$. CM, culture medium; DNL, *de novo* lipogenesis; FCM, flow cytometry; MFI, mean fluorescence intensity.

High lipid content and increased FAO support the immunosuppressive functions of neutrophils.[5] We examined lipid levels in neutrophils after treatment with different CM preparations from hepatoma cells. As shown in Figure 1F and S5A, HCCLM3-CM stimulation accumulated neutral lipids (BODIPY 493/503) in neutrophils 24 h later. Mechanistically, HCCLM3-CM treatment upregulated FASN and CD36 expression 24 h later, whereas carnitine

palmitoyltransferase 1A (CPT1A) expression was significantly suppressed (Fig. 1G). Given that CD36 is responsible for exogenous lipid uptake, there was increased CD36 expression in dHL60 cells upon HCCLM3-CM stimulation. To differentiate intracellular lipid increments from an increase in lipids uptake or DNL, we measured the uptake of fluorescently labeled free fatty acids (BODIPY FL C16) by dHL60 cells treated with different HCM preparations. The data showed no difference in BODIPY FL C16 uptake between the two groups (Fig. 1H).

Given that FASN is responsible for increased lipids in MANs, we sought to evaluate the effect of inhibiting DNL on the apoptosis and immunosuppressive phenotype of MANs. As illustrated in Figure 1I, FASN inhibitor C75 treatment resulted in elevated apoptosis of neutrophils treated with HCCLM3-CM. However, synchronous palmitate supplementation did not alleviate the apoptosis of neutrophils treated with HCCLM3-CM in the presence of C75 (Fig. S6A). RT-qPCR analysis showed that C75 notably decreased *Il10* but not *Pge2* and *Arg1* mRNA in dHL60 cells treated with HCCLM3-CM (Fig. 1J, S6B). The growth of colonies represents the final phase of metastatic progression. As expected, C75 treatment could effectively suppress colony formation by HCCLM3 cells cocultured with dHL60 cells (Fig. 1K). Interestingly, although C75 could suppress the migration of both Huh7 and HCCLM3 cells, the proliferation inhibitory effect on Huh7 cells was more significant (Fig. S7A,B). The data indicated that fatty acid synthesis contributes less to the proliferation of HCCLM3 cells but displays a pivotal role in the protumor function of MANs.

Neutrophils make up a substantial proportion of the TME in multiple cancers, and many studies have proved that lipid metabolic rewiring contributes to the functional plasticity of tumor-associated neutrophils.[2] In the present research, we revealed that DNL does not prolong the lifespan of neutrophils but contributes to an immunosuppressive phenotype, which makes for colonization by highly metastatic HCC cells (Fig. 1L). In short, our findings suggest that targeting DNL in neutrophils may be a plausible therapeutic strategy for HCC metastasis.

Conflict of interests

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.12.008.

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