ORIGINAL RESEARCH

CD13\(^{hi}\) Neutrophil-like myeloid-derived suppressor cells exert immune suppression through Arginase 1 expression in pancreatic ductal adenocarcinoma

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ABSTRACT

Perineural invasion and immunosuppressive tumor microenvironment are the distinct features of pancreatic ductal adenocarcinoma (PDAC). Heterogeneous myeloid-derived suppressor cells (MDSCs) are potent suppressors of antitumor immunity, posing obstacles for cancer immunotherapy. Increasing evidences have demonstrated the accumulation of MDSCs in PDAC patients. However, the role of MDSCs in perineural invasion of PDAC and the existence of novel MDSC subsets during PDAC remain unclear. This study found that lymphocytic perineural cuffs were frequently present in chronic pancreatitis (CP) tissues and adjacent non-neoplastic pancreatic tissues (ANPTs), but not in PDAC with perineural invasion. Meanwhile, we found that neutrophil-like MDSCs (nMDSCs), but not monocyte-like MDSCs (mMDSCs), were significantly increased in PBMCs and tumor tissues of PDAC patients. Further observation identified two distinct subsets of nMDSCs, CD13\(^{hi}\) and CD13\(^{low}\) nMDSCs in PDAC patients, which have not been reported previously. Despite a similar morphology, CD13\(^{hi}\) nMDSCs expressed higher levels of CD11b, CD33, CD16 and arginase 1 but lower levels of CD66b than CD13\(^{low}\) nMDSCs. Importantly, CD13\(^{hi}\) MDSCs, compared with CD13\(^{low}\) nMDSCs, more effectively suppressed alloreactive T cell responses via an arginase-1-related mechanism. After tumor resection, the circulating CD13\(^{hi}\) nMDSCs were decreased markedly. PDAC patients with more CD13\(^{hi}\) nMDSCs had a shorter overall survival than those with less CD13\(^{hi}\) nMDSCs. To conclude, we identified two novel MDSC subsets with different characteristics and functions in PDAC, demonstrated the association of the two MDSC subsets with cancer progression, and explored their roles in perineural invasion and immune escape of PDAC.

Abbreviations: ANPTs, adjacent non-neoplastic pancreatic tissues; CP, chronic pancreatitis; MDSCs, myeloid-derived suppressor cells; mMDSCs, monocyte-like MDSCs; nMDSCs, neutrophil-like MDSCs; PDAC, pancreatic ductal adenocarcinoma; PNI, perineural invasion; ROS, reactive oxygen species; TAM, tumor-associated macrophages; Treg, regulatory T cells; WBC, white blood cells

Introduction

Myeloid-derived suppressor cells (MDSCs) are defined as a heterogeneous population of activated immature myeloid cells, which are significantly expanded in tumor-bearing mice and cancer patients. MDSCs inhibit adaptive T cell response and innate immunity against tumor and impair the effectiveness of anticancer immunotherapeutic approaches. In tumor-bearing mice, MDSCs are identified as cells that co-express Gr-1 and CD11b and two main MDSC subsets have been characterized: monocytic MDSCs (also known as monocyte-like MDSCs) and granulocytic MDSCs (also known as neutrophil-like MDSCs). In cancer patients, MDSCs are highly heterogeneous and different phenotypes of MDSCs are increased in tumors of different origins. Similar to mouse MDSCs, human MDSCs include monocytic MDSCs and granulocytic MDSCs. Due to the lack of a specific marker, human MDSC phenotypes are described by the combination of several myeloid markers. Therefore, it is still a research focus to identify novel human MDSC subsets in cancer based on their phenotypes and immunosuppressive function or other new function, and such study will contribute to the standardization of MDSCs immunophenotyping and the understanding of MDSCs.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common, most rapidly progressive and deadly malignancies worldwide. Due to late diagnosis, 94% of patients die within 5 y of diagnosis, and currently there has been no effective treatment for this disease. Perineural invasion (PNI) is a prominent feature of pancreatic cancer, which contributes to metastatic spread, pain generation and poor prognosis of patients. Based on our own experience and previous studies, ...
PDAC has the highest incidence of PNI (80~100%) among cancers. The mechanisms of PNI in PDAC have been studied extensively, yet without convincing conclusions. Peripheral nerve, cancer cells, tumor microenvironment and related factors or signaling are thought to be involved in PNI in PDAC. Recent studies indicated that GDNF from nerve or CD68⁺ endoneurial macrophage induced PNI in PDAC through GDNF-GFRα1-RET signaling. And CD68⁺ or CD163⁺ tumor-associated macrophages (TAM) density was correlated with PNI in PDAC, and high-level TAM infiltration further worsened the prognosis, suggesting that macrophages in tumor microenvironment of PDAC might be involved in PNI development. However, whether other immune cells such as MDSCs in tumor microenvironment are involved in this process is still unknown.

PDAC is known for its marked immunosuppression, which may promote tumor progression and invasion and may be a reason for poor clinical response to immunotherapies. Immune suppression in tumor microenvironment of PDAC is mainly mediated by immune suppressor cells, which include TAM, regulatory T cells (Treg) and MDSCs. The role of MDSCs in PDAC has been investigated during the past few years. A large number Gr-1⁺ CD11b⁺ MDSCs were found in the spleen, tumor and bone marrow in mouse models of PDAC. In a genetically engineered mouse model of PDAC, tumor-derived GM-CSF was necessary and sufficient to drive the development of Gr-1⁺ CD11b⁺ cells that suppressed antigen-specific T cells. In patients with pancreatic adenocarcinoma, the frequency of CD11b⁺ CD33⁺ CD15⁺ MDSCs in peripheral blood, bone marrow and tumor were increased. A recent study demonstrated a significant increases of circulating and tumor-infiltrating granulocytic (Lin⁻ HLA-DR⁻ CD33⁻ CD11b⁺ CD15⁻) MDSCs in patients with pancreatic cancer. However, whether there are novel MDSC subsets in PDAC needs to be further investigated.

In the present study, we found that lymphocytic perineural cuffs were frequently found in CP tissues and ANPTs, but not in PDAC with PNI, which suggests that MDSCs might be involved in PNI of PDAC. We also identified two novel MDSC subsets in PDAC, CD13⁺ and CD13⁻ CD11b⁺ CD33⁻ CD14⁻ CD15⁺ neutrophil-like MDSCs (nMDSCs). CD13⁺ nMDSCs suppressed alloreactive T cell responses more

Figure 1. Pathological changes in chronic pancreatitis, ANPT and PDAC tissues. Pathological changes in chronic pancreatitis (CP), ANPT and PDAC tissues were analyzed by H&E staining. Perineural cuffs containing inflammatory immune cells (indicated by star) were prominent in CP (A) and ANPT tissues (B). Such perineural cuffs were absent in PDAC tissues with perineural invasion (C). The triangle indicates the nerve branch and the square in C indicates the invading cancer cells. Scale bar = 100 μm.
efficiently than CD13\textsuperscript{low} nMDSCs, likely due to a higher expression of Arg 1. After tumor resection, the number of CD13\textsuperscript{hi} nMDSCs decreased significantly but CD13\textsuperscript{low} nMDSCs increased, with the overall number of non-specific nMDSCs unchanged. PDAC patients with more CD13\textsuperscript{hi} nMDSCs had shorter overall survival than those with less CD13\textsuperscript{hi} nMDSCs. These results indicate a close relationship of the quantity and quality of MDSC subsets with cancer progression, suggesting that MDSC subsets may play roles in PNI and immune escape of PDAC.

Results

Absence of perineural cuffs containing inflammatory lymphocytes in PDAC tissues

Inflammatory cells are frequently found to aggregate around peripheral nerves in chronic pancreatitis (CP) tissues and in adjacent non-neoplastic pancreatic tissues (ANPTs) of PDAC. In order to study the mechanisms that mediated PNI of PDAC, we assessed pathological features and tumor microenvironment of PDAC with PNI. We found that perineural cuffs containing inflammatory immune cells were prominent in 46 of 61 (75.4%) CP (Fig. 1A) and 41 of 62 (66.1%) ANPT tissues (Fig. 1B), but absent in intratumoral nerves invaded by PDAC (0/62, Fig. 1C, Table S2). Analysis of immune cell infiltrating perineural cuffs in CP tissues and ANPT tissues by immunohistochemistry showed that they are predominantly CD3\textsuperscript{T} and CD20\textsuperscript{B} lymphocytes (Fig. S1). Therefore, PNI of PDAC tissues coincides with the absence of inflammatory lymphocytes infiltration.

Infiltration of CD11\textsuperscript{b+} CD33\textsuperscript{+} MDSCs in PDAC tissues

To explore the mechanisms of inflammatory lymphocytes absence in PDAC tissues with PNI, we hypothesized that immune-suppressive cells in PDAC tissues such as MDSCs mediated the effects. So, we first detected the presence of MDSCs in PDAC tissues by immunofluorescence and found that CD11\textsuperscript{b+} CD33\textsuperscript{+} MDSCs were present in PDAC tissues (Fig. 2A), CP tissues and ANPTs of PDAC tissues surrounding the nerve (Fig. S2) but absent in normal pancreatic tissues far from PDAC tissues in PDAC (data not shown). The numbers of CD11\textsuperscript{b+} CD33\textsuperscript{+} MDSCs in PDAC tissues were more than those in CP or ANPTs (Fig. 2B). These results indicate that MDSCs in PDAC might be involved in PNI of PDAC by suppressing the infiltration of inflammatory lymphocytes.

Increased numbers of neutrophil-like MDSCs in patients with PDAC

MDSCs are one of several important immuno-suppressive cells during cancer development, yet the details of MDSC subsets are unknown. Human MDSCs are heterogeneous in phenotype and usually include monocyte-like MDSCs (mMDSCs) and nMDSCs.\textsuperscript{1,21} We investigated the numbers of different MDSC subsets in PBMCs from patients with PDAC by multi-parameter flow cytometry analysis and compared it to healthy donors. Representative flow cytometry dot plots demonstrated the presence of CD11\textsuperscript{b+} CD14\textsuperscript{−} HLA-DR\textsuperscript{−} nMDSCs in PBMCs of both patients with PDAC and healthy donors (Fig. 2C). Collective data confirmed that the numbers of nMDSCs in PBMCs of patients with PDAC (n = 36, mean = 6.10 × 10\textsuperscript{6}/L, SEM = 0.51 × 10\textsuperscript{6}/L, p = 0.0844) was comparable to that in PBMCs of healthy donors (n = 13, mean = 7.25 × 10\textsuperscript{6}/L, SEM = 0.46 × 10\textsuperscript{6}/L) (Fig. 2C). Furthermore, representative flow cytometry dot plots showed the existence of a multitude of CD11\textsuperscript{b+} CD33\textsuperscript{+} CD14\textsuperscript{−} CD15\textsuperscript{−} nMDSCs in PBMCs of the patient with PDAC (Fig. 2D). The numbers of nMDSCs in PBMCs of patients with PDAC (n = 36, mean = 61.29 × 10\textsuperscript{6}/L, SEM = 11.37 × 10\textsuperscript{6}/L, p = 0.0068) were increased significantly compared with those in healthy donors (n = 13, mean = 12.20 × 10\textsuperscript{6}/L, SEM = 3.00 × 10\textsuperscript{6}/L) (Fig. 2D). More importantly, we found the existence of CD45\textsuperscript{+} HLA-DR\textsuperscript{−} CD11\textsuperscript{b+} CD33\textsuperscript{+} CD14\textsuperscript{−} CD15\textsuperscript{−} nMDSCs by multi-parameter flow cytometry analysis in infiltrating immune cells of PDAC tissues (Fig. 2E) and there were no infiltrating immune cells found in normal pancreatic tissues far from PDAC tissues (data not shown). Collective data indicated that their numbers in PDAC tissues (n = 10, mean = 18.62 × 10\textsuperscript{6}/g, SEM = 3.27 × 10\textsuperscript{6}/g, p = 0.0131) were higher than that in CP tissues (n = 8, mean = 9.41 × 10\textsuperscript{6}/g, SEM = 0.87 × 10\textsuperscript{6}/g) (Fig. 2F). These data collectively demonstrate that the numbers of nMDSCs but not mMDSCs are significantly higher in patients with PDAC, suggesting a distinct profile of MDSC subsets during PDAC development.

Phenotype and identification of CD13\textsuperscript{hi} or CD13\textsuperscript{low} neutrophil-like MDSC subsets

Considering the existence of abundant nMDSCs in PBMCs and tumor tissues of patients with PDAC, we further analyzed the phenotype of these MDSCs by multi-parameter flow cytometry and first focused on the expression of CD13, a neutrophil-related marker. Representative flow cytometry dot plots clearly demonstrated two distinct subpopulations of CD11\textsuperscript{b+} CD33\textsuperscript{+} CD14\textsuperscript{−} CD15\textsuperscript{−} nMDSCs in patients with PDAC, CD13\textsuperscript{hi} and CD13\textsuperscript{low} cells (Fig. 3A), which have not been reported before. We proposed that CD13\textsuperscript{hi} and CD13\textsuperscript{low} nMDSCs in PBMCs of PDAC patients compared with healthy donors (PDAC: n = 36, mean = 2.79 × 10\textsuperscript{6}/L, SEM = 0.46 × 10\textsuperscript{6}/L, p = 0.0079) (Fig. 3B). By flow cytometry analysis, both CD13\textsuperscript{hi} nMDSCs and CD13\textsuperscript{low} nMDSCs exhibited neutrophil-like characteristics in FSC/SSC, different from CD11\textsuperscript{b+} CD14\textsuperscript{hi} CD15\textsuperscript{−} monocytes (Fig. 3C). CD13\textsuperscript{hi} nMDSCs expressed higher levels of CD11b, CD33 and CD16 and lower levels of CD66b than CD13\textsuperscript{low} nMDSCs, although they both expressed low levels of HLA-DR, CD14 and CD124 (Fig. 3C). Collective data demonstrated that the numbers of CD13\textsuperscript{hi} nMDSCs in PBMCs of patients with PDAC were significantly more than those in healthy donors (PDAC: n = 36, mean = 53.42 × 10\textsuperscript{6}/L, SEM = 10.29 × 10\textsuperscript{6}/L; Healthy donors: n = 13, mean = 10.02 × 10\textsuperscript{6}/L, SEM = 3.05 × 10\textsuperscript{6}/L, p = 0.0079) (Fig. 3D) and there were the increasing trend numbers of CD13\textsuperscript{low} nMDSCs in PBMCs of PDAC patients compared with healthy donor (PDAC: n = 36, mean = 13.28 × 10\textsuperscript{6}/L, SEM = 2.94 × 10\textsuperscript{6}/L; Healthy donors: n = 13, mean = 6.19 × 10\textsuperscript{6}/L, SEM = 1.92 × 10\textsuperscript{6}/L, p = 0.0834) (Fig. 3E). Overall the
numbers of CD13\textsuperscript{hi} nMDSCs in PBMCs of patients with PDAC were higher than those of CD13\textsuperscript{low} nMDSCs (Figs. 3D and E). Consistently, both CD45\textsuperscript{+} HLA-DR\textsuperscript{−} CD11b\textsuperscript{+} CD33\textsuperscript{+} CD14\textsuperscript{−} CD15\textsuperscript{+} CD13\textsuperscript{hi} and CD13\textsuperscript{low} nMDSC subsets were detected in PDAC tumor tissues (Fig. 4A). The numbers of CD13\textsuperscript{hi} nMDSC subsets in PDAC tumor tissues (n = 10, mean = 16.77 \times 10^3/g, SEM = 3.08 \times 10^3/g, p = 0.037) were more than those in CP tissues (n = 8, mean = 8.08 \times 10^3/g, SEM = 0.92 \times 10^3/g) (Fig. 4B). Thus, we identified two different nMDSC subsets according to the expression levels of CD13 in PDAC. To demonstrate whether these two nMDSC subsets can differentiate toward the other, isolated PBMC cells from PDAC patients were stimulated with inflammatory cytokines or not and flow cytometry analysis demonstrated that the percentages of these two populations were similar irrespective of whether TNF-\alpha or IL-1\alpha was used (Fig. 3), indicating that CD13\textsuperscript{hi} nMDSC subsets did not lose CD13 or CD13\textsuperscript{low} nMDSC subsets did not acquire CD13 even in response to inflammatory stimuli such as TNF-\alpha or IL-1\alpha.

**Properties of CD13\textsuperscript{hi} and CD13\textsuperscript{low} nMDSCs**

To investigate the properties and functions of the two novel subsets of nMDSCs, we sorted CD13\textsuperscript{hi} and CD13\textsuperscript{low} nMDSCs for further analysis, with CD11b\textsuperscript{+} CD14\textsuperscript{hi} CD15\textsuperscript{−} monocytes served as control cells or antigen-presenting cells. By morphology, both CD13\textsuperscript{hi} nMDSCs and CD13\textsuperscript{low} nMDSCs showed the characteristics of neutrophils and were polymorphonuclear (Fig. 5A). It is well known that MDSCs usually express high levels of Arg 1, iNOS or ROS. Western blot analysis showed that both subsets of nMDSCs in patients with PDAC expressed Arg 1 but not iNOS, and expression of Arg 1 in CD13\textsuperscript{hi} nMDSCs was higher than CD13\textsuperscript{low} nMDSCs, while monocytes did not express Arg 1 or iNOS (Fig. 5B, Fig. 5C and data not shown).
Figure 3. Phenotype and identification of neutrophil-like MDSC subsets in PBMC of patients with PDAC. (A) Phenotype of neutrophil-like MDSCs (nMDSCs) in PBMC of patients with PDAC was analyzed by multi-parameter flow cytometry. PBMCs were obtained from patients with PDAC, and then stained using fluorochrome-labeled antibodies against CD11b, CD14, HLA-DR, CD33, CD15, CD13 (one of neutrophil markers) and CD16. Representative multi-parameter dot plots of CD13 expression of nMDSCs from patients with PDAC are shown. nMDSCs can be divided into two subsets including CD13hi and CD13low nMDSCs according to CD13 expression. Numbers in plots indicate the percentages of gated populations. (B) Monocytes in PBMC of patients with PDAC were defined as CD14hi CD11bhi CD15− population. Representative dot plots of monocytes in PBMC of patients with PDAC are shown. Numbers in plots indicate the percentages of gated populations. (C) Three populations including monocytes, CD13hi nMDSCs and CD13low nMDSCs in PBMC of patients with PDAC are compared in phenotype. Representative histograms of several markers expression including FSC/SSC, CD11b, CD33, CD16, HLA-DR, CD13, CD66b, CD124, ROS and CD14 are shown. Numbers in the right of histograms indicate gMFI of several markers expression. (D and E) The numbers of nMDSC subsets in PBMCs were calculated as “the frequency of cells in PBMCs × (the numbers of WBC cells in blood — the numbers of granulocytes in blood).” The numbers of CD13hi nMDSCs (D) or CD13low nMDSCs (E) in PBMCs from healthy donors (n = 13) were compared with those from patients with PDAC (n = 36) and the data was statistically analyzed by unpaired t test.
Flow cytometry analysis showed that both subsets of MDSCs in patients with PDAC expressed similar levels of ROS (Fig. 3C). In terms of cytokines secretion, CD13hi nMDSCs produced more IL-6 and IL-1β than CD13low nMDSCs after LPS stimulation and both of them produced very low levels of TNF-α, IL-10, IL-12, IL-4 and IFNγ irrespective of LPS stimulation or not (Fig. 5D and data not shown). As control cells, monocytes produced high levels of TNF-α, IL-6, IL-10 and IL-1β after LPS stimulation (Fig. 5D). Overall the major difference between CD13hi nMDSCs and CD13low nMDSCs was the level of Arg 1 expression.

**Immuno-suppressive activity of CD13hi and CD13low nMDSCs**

The most important function of MDSCs is to suppress antigen-presenting cells induced T cell responses. We established monocytes-initiated alloreactive T cell proliferation by assaying the dilution of CFSE expression in CD4+ or CD8+ T cells and investigated the effects of CD13hi and CD13low nMDSCs on alloreactive CD4+ or CD8+ T cell proliferation. We found that both CD13hi nMDSCs and CD13low nMDSCs had a very weak ability to initiate alloreactive T cell proliferation, but could suppress monocytes-initiated alloreactive CD4+ or CD8+ T cell proliferation (Figs. 6A–C). The inhibition of monocytes-initiated alloreactive T cell proliferation by CD13hi nMDSCs was much stronger than that by non-specific nMDSCs or CD13low nMDSCs (Figs. 6A–C). Arg 1 specific inhibitor was able to abolish the suppression of monocytes-initiated alloreactive T cell proliferation by CD13hi nMDSCs (Figs. 6A–C), suggesting the strong immuno-suppressive functions is mediated by Arg-1-related mechanism.

**The relationship between CD13hi or CD13low nMDSCs and tumor development and progression**

Furthermore, we also investigated the relationship between the quantity of these two kinds of nMDSCs and clinical characteristics of patients with PDAC, including age, gender, tumor stage, location of tumor and jaundice, and we found that there were no significant relationships between age, gender or tumor stage and the frequencies or numbers of non-specific nMDSCs, CD13hi
nMDSCs or CD13\textsuperscript{low} nMDSCs in patients with PDAC (Tables S3–5). However, the association between tumor location or jaundice and the frequencies or numbers of non-specific nMDSCs, CD13\textsuperscript{hi} nMDSCs or CD13\textsuperscript{low} nMDSCs was significant in patients with PDAC (Fig. S4 and Tables S3–5). These results indicated that location of PDAC tumor in head of pancreas and related jaundice might contribute to increased numbers of CD13\textsuperscript{hi} nMDSCs or CD13\textsuperscript{low} nMDSCs in patients with PDAC.

Increasing evidences have demonstrated that MDSCs interact closely with the tumor. For example, the mobilization or generation of MDSCs can be promoted by tumor and the growth of tumor induced by MDSCs.\textsuperscript{21,22} So, we wondered whether there is a relationship between these two kinds of nMDSCs and tumor progression in patients with PDAC. We assessed the changes of the numbers of CD13\textsuperscript{hi} nMDSCs and CD13\textsuperscript{low} nMDSCs after surgical excision of tumor in 13 patients with PDAC. As shown in Fig. 7A, in two representative patients with PDAC, the numbers of CD13\textsuperscript{hi} nMDSCs in PBMCs decreased significantly and the numbers of CD13\textsuperscript{low} nMDSCs in PBMCs increased 7 d post-operation. Collective data demonstrated that the numbers of CD13\textsuperscript{hi} nMDSCs in PBMCs decreased notably from (83.17 ± 19.27) × 10\textsuperscript{6}/L to (18.62 ± 6.09) × 10\textsuperscript{6}/L (n = 13, p = 0.0052), the numbers of CD13\textsuperscript{low} nMDSCs in PBMCs increased from (14.59 ± 4.76) × 10\textsuperscript{6}/L to (131.30 ± 54.03) × 10\textsuperscript{6}/L (n = 13, p = 0.0209) 7 d after operation and the numbers of non-specific nMDSCs remained unchanged (p = 0.1879) (Figs. 7B and C). These data indicate that the phenotype of circulating nMDSC subsets shifted toward CD13\textsuperscript{low} nMDSCs which had lower immunosuppressive activity after the tumor was removed, maybe being beneficial to PDAC patients. Correspondingly, PDAC patients with more CD13\textsuperscript{hi} nMDSCs (the frequency of CD13\textsuperscript{hi} nMDSCs >1%) had shorter overall survival than those with less CD13\textsuperscript{hi} nMDSCs (the frequency of CD13\textsuperscript{hi} nMDSCs <1%) (Fig. 7D), suggesting that the numbers of CD13\textsuperscript{hi} nMDSCs may serve as an independent factor to predict worse prognosis in PDAC.
**Discussion**

This study demonstrated that MDSCs might be involved in PNI of PDAC and identified two novel MDSC subsets in PDAC, CD13$^{hi}$ nMDSCs and CD13$^{lo}$ nMDSCs, which were different in phenotypes and immunosuppressive function despite of their similarity in morphology. Importantly, CD13$^{hi}$ nMDSCs expressed higher levels of Arg 1 than CD13$^{lo}$ nMDSCs, which endowed CD13$^{hi}$ nMDSCs with stronger immunosuppressive function.

**Figure 6.** Immunosuppressive function of neutrophil-like MDSC subsets in PBMC of patients with PDAC. Monocytes, non-specific nMDSCs (MDSCs), CD13$^{hi}$ nMDSCs and CD13$^{lo}$ nMDSCs in PBMCs from patients with PDAC are sorted. CD3$^+$ T cells in PBMCs from healthy donor are purified and stained with CFSE. Then, CD3$^+$ T cells are co-cultured with monocytes and/or nMDSCs at the ratio of 10:1 (T cells: monocytes or nMDSCs). In some groups, Arg-1-specific inhibitor nor-NOHA is used. After 7 d, cells are collected and stained with fluorochrome-labeled antibodies against CD4$^+$ and CD8$^+$ for flow cytometry. Representative histograms of CFSE dilution in CD4$^+$ T cells (A) or CD8$^+$ T cells (B) in various groups are shown. Numbers in histograms indicate the percentages of CFSE$^{lo}$ cells in CD4$^+$ or CD8$^+$ T cells. (C) The results in triplicate cultures in each group were expressed as proliferating CFSE$^{lo}$ T cells (% Mono/T group) and the value in Mono/T group was set as 100%. The data are shown as mean ± SD in one representative experiment and analyzed by ANOVA. Similar results are obtained in three independent experiments. **p < 0.01.
Figure 7. Changes of nMDSC subsets in PBMC of PDAC patients after operation and the association between CD13<sup>hi</sup> nMDSC and OS. The frequencies or numbers of CD13<sup>hi</sup> and CD13<sup>low</sup> nMDSCs were quantitated in the PB of patients with PDAC before operation and 7 d after operation by multi-parameter flow cytometry. PBMCs were obtained from patients with PDAC (n = 13), and then stained for MDSCs using fluorochrome-labeled antibodies against CD11b, CD14, CD33, CD15 and CD13. (A) Representative dot plots of CD13<sup>hi</sup> and CD13<sup>low</sup> nMDSCs in PBMCs from two patients with PDAC are shown. Numbers in plots indicate the percentages of gated populations and the percentages in bracket indicate the frequency of CD13<sup>hi</sup> and CD13<sup>low</sup> nMDSCs in PBMCs. (B and C) The numbers of CD13<sup>hi</sup> and CD13<sup>low</sup> nMDSCs in PBMCs from patients with PDAC before operation were compared with that 7 d after operation and the data was statistically analyzed by paired Student’s t test. The “before-after” graph and “scatter plot” graph of the data are shown in B and C, respectively. (D) Kaplan–Meier curves for 36 PDAC cases including 15 cases (CD13<sup>hi</sup> nMDSCs <1%) and 21 cases (CD13<sup>hi</sup> nMDSCs >1%) were shown. The p value was determined using the log-rank test.
activity. Furthermore, surgical treatment of PDAC patients altered the quality of non-specific nMDSCs—CD13$^{hi}$ nMDSCs were decreased and CD13$^{low}$ nMDSCs with lower immunosuppressive activity were increased, highlighting the relationship between the quantity and quality of MDSC subsets and PDAC progression.

Increasing evidences demonstrate that the close interaction between tumor microenvironment and cancer plays vital roles in cancer development and progression. PDAC is one of the most stroma-rich cancers and stromal components in PDAC outnumber cancer cells. PDAC stroma comprises cellular and acellular components, including pancreatic stellate cells, fibroblasts, immune cells, peritumoral nerves, blood vessels, extracellular matrix and soluble proteins such as cytokines and growth factors. Our present study found that in ANPTs of PDAC tissues, abundant inflammatory lymphocytes aggregated around peripheral nerves, similar to pathological changes in CP tissues. Interestingly, such perineural cuffs containing inflammatory lymphocytes were absent in PDAC tissues with PNI. This indicates that tumor microenvironment may contribute to PNI of PDAC tissues and further immune escape by suppressing the infiltration of inflammatory lymphocytes.

PNI is a distinct route of tumor metastasis, defined as the presence of tumor cells in the perineurium space of local peripheral nerves. PNI is frequently found in many human cancers, especially in pancreas and prostate cancer, associated with tumor recurrence and pain in advanced cancer patients. It is known that growth factors, cytokines and chemokines from peripheral nerves, malignant cells and local tumor microenvironment play roles in the development of PNI in pancreatic cancer. Molecules mediating PNI include TGF-$\alpha$, NGF family, GDNF family, CX3CL1 chemokine, MMPs, CD74 and other cell-surface molecules. Cells involved in PNI are mainly perineural nerves and PDAC cells although two studies indicated that macrophages such as TAM and endoneurial macrophage were also involved in PNI. To our knowledge, this study is the first of this kind to explore the potential roles of MDSCs in PNI. To further elucidate the association between MDSCs and PNI in PDAC, we need to analyze the difference of MDSC numbers in PNI and non-PNI tissues in PDAC, so as to demonstrate the exact roles of MDSCs and the underlying mechanisms in PNI of PDAC in vitro and in vivo.

In previous studies, human MDSC subsets in various cancers including PDAC were divided into monocyte-like MDSC subsets characterized by the expression of CD14 and neutrophil-like MDSC subsets characterized by CD15. However, detailed phenotype analysis was not performed. Knowing that CD15$^{+}$ neutrophil-like MDSC subsets are abundant in PDAC, we focused on neutrophil-related cell surface molecules and found two distinct nMDSC subsets expressing different levels of CD13. CD13, also known as aminopeptidase N, is expressed on cell surface of human neutrophils. CD13 can regulate TNF-$\alpha$-induced apoptosis via inhibiting TNFRI shedding, degrade interleukin-8 (IL-8) and inactivate its chemotactic activity. Although it is unknown whether CD13 directly mediates the functions of nMDSCs, nMDSCs with different levels of CD13 expressed different levels of CD16, CD11b, CD33, CD66b and Arg 1. High expression of Arg 1 in CD13 high subsets likely accounts for a better immunosuppressive function. Notably, CD13$^{hi}$ nMDSC subsets also consistently expressed very high levels of CD16 (Fc RIII) of phagocytes. So, CD16 can also be used to discriminate nMDSC subsets, similar to CD13.

The mechanisms of MDSCs-induced suppression of T cell responses include: (1) the expression of arginase 1, inducible NOS or reactive oxygen species (ROS); (2) activation and expansion of Treg by MDSCs; (3) deprivation of nutrients such as L-cysteine required for T cell activation; and others. We found that both CD13$^{hi}$ nMDSCs and CD13$^{low}$ nMDSCs expressed Arginase 1 and ROS, but not inducible NOS, and CD13$^{hi}$ nMDSCs expressed higher levels of Arginase 1 than CD13$^{low}$ nMDSCs, which correlates well with stronger immunosuppressive activity by CD13$^{hi}$ nMDSCs. The two kinds of nMDSCs expressed similar levels of ROS but did not express inducible NOS, a marker of nMDSC subsets.

Based on the fact that the numbers of CD13$^{hi}$ nMDSCs was reduced after tumor resection, we hypothesized that CD13$^{hi}$ nMDSCs could be used as a prognostic factor for PDAC. Indeed, PDAC patients with more CD13$^{hi}$ nMDSCs had shorter overall survival than those with less CD13$^{hi}$ nMDSCs. This indicates that CD13$^{hi}$ nMDSCs might be an independent indicator for prognosis in PDAC, which will be helpful to provide potential basis for PDAC treatment.

Cancer cells and tumor-associated stromal cells release multiple tumor-derived soluble factors that contribute to the expansion and activation of MDSCs. These soluble factors include GMCSF, GCSF, MCSF, stem cell factor (SCF), VEGF, IL3, IL1$\beta$, IL6, S100A8, S100A9 and others. They can promote myelopoiesis or are pro-inflammatory and they can contribute to the expansion of MDSCs through a variety of transcription factors, with STAT3 playing a major role. One in-vitro study demonstrated that pancreatic cancer-associated stellate cells could produce MDSCs-promoting cytokines including IL-6, VEGF and M-CSF, promoting the differentiation of CD15$^{+}$ CD11b$^{+}$ CD33$^{+}$ MDSCs in a STAT3-dependent manner. Knowing that tumor-derived soluble factors play important roles in the expansion of MDSCs and CD13$^{hi}$ nMDSCs is decreased significantly after tumor resection, the mechanisms mediating the expansion of CD13$^{hi}$ nMDSCs will probably be attributed to tumor or tumor-related events. Interestingly, we found that patients with PDAC in the head of pancreas or PDAC patients with jaundice had more CD13$^{hi}$ nMDSCs and CD13$^{low}$ nMDSCs than patients with tumor in the body/tail of pancreas or without jaundice. However, jaundice often coincides with the tumor in the head of the pancreas pressing on the bile duct in PDAC, which is prone to confuse their roles in MDSC expansion in PDAC. Furthermore, due to relatively small sample size in this study, it requires more samples from PDAC patients to compare the numbers of MDSC subsets in PDAC in the head of pancreas with or without jaundice, confirming the exact roles of jaundice or tumor location in MDSC expansion in PDAC. Given that PDAC patients with jaundice almost always have cancer in the head of pancreas and only about half with cancer in the head of pancreas had jaundice, both tumor resection and alleviating obstructive jaundice might contribute to the reduction of CD13$^{hi}$ nMDSCs after surgery. Previous studies demonstrated that increased number and enhanced functions of peripheral neutrophils were found in
obstructive jaundice,32 and the present study is the first report showing that jaundice might be related to nMDSCs expansion in cancer patients.

To summarize, we identified two MDSC subsets based on their CD13 expression and explored the possible roles that MDSCs may play in PNI in PDAC and during PDAC development. Our study will further enhance our knowledge about the role of MDSCs in cancers and clarify the association between novel MDSC subsets and PDAC development and progression.

Materials and methods

Samples and patients

Heparinized venous blood samples were obtained from 36 patients with PDAC before surgery, 13 patients with PDAC 7 d post-surgery and 13 age-matched healthy donors. Tissue specimens of CP, ANPT and PDAC were collected from 61 patients with CP and 62 patients with PDAC receiving the surgery for histological assessment. All patients were diagnosed with PDAC for the first time in Changhai Hospital, Shanghai, China. The general information of the PDAC patients and healthy donors are summarized in Table S1. All samples were collected with the informed consent of the participants and all the experiments were approved by the Ethics Committee of Second Military Medical University. The 36 patients receiving MDSC analysis were followed up after surgical treatment for 24 mo.

Reagents

The antibodies for flow cytometry, including fluorochrome-labeled antibodies against human CD15 (HI98), CD16 (3G8), CD45 (H130), CD13 (WM15), CD33 (WM53), CD11b (ICRF44), CD14 (HCD14), HLA-DR (L243), CD66b (G10F5), CD124 (G077F6), CD4+ (OKT4) and CD8+ (HIT8a) were from Biologend (San Diego, CA). Antibodies against human Arginase 1 (sc-166920) and NOS 2 (sc-7271) were from Santa Cruz. CFSE were from Invitrogen (Eugene, OR). DCFDA cellular ROS detection assay kit was from Abcam (Cambridge, UK). CBA reagents of TNF-α, IL-6, IL-1β, IL-10, IL-12, IL-2, IL-4 and IFNγ were from BD Bioscience. Microbead-conjugated mAbs to human CD3 were from MiltenyiBiotec (Bergisch Gladbach, Germany). Arginase-1-specific inhibitor nor-NOHA was from EMD Millipore (Billerica, MA).

Immunohistochemical staining

Formaldehyde-fixed tissues were made into 4-μm sections and were mounted onto poly-L-lysine-coated slides. The slides were incubated with mouse anti-human CD3 antibody or anti-human CD20 antibody (1:30, DAKO, Glostrup, Denmark) in a humid incubator at 4°C overnight. The secondary antibody system (PV9000, Golden Bridge International, Beijing, China) was applied according to the manufacturer’s instructions. A positive staining result was recorded when the membrane and/or cytoplasm of cells was stained yellow or brown. Three high-power fields (HPF) per slide were randomly chosen to measure the proportion of immunopositive cells in perineural cuffs.

Immunofluorescence double staining

Paraffin-embedded PDAC tissues were made into 4-μm sections. After de-paraffin, sections were subjected to antigen retrieval for 20 min, and then blocked by goat serum for 20 min. For co-localization of CD11b/CD33, rabbit anti-human CD11b or mouse anti-human CD33 (1:50, Jackson, West Grove, PA, USA) was added and maintained at 4°C overnight. After washing with PBS, FITC or rhodamine-conjugated anti-rabbit or mouse secondary antibodies (1:50, Abcam, Hong Kong) was added and incubated at room temperature for 30 min. Fluorescence was observed after drying by a microscope (Leica DM 2000, Leica Microsystems, Germany) and the photographs were acquired by software LAS V3.7 (Leica Microsystems, Germany). Five random HPFs per slide were chosen for cell counting and the average of CD11b+ CD33+ cells per slide was calculated.

Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from the peripheral blood of patients with PDAC and healthy donors by density gradient centrifugation at 800 × g for 30 min using Ficoll-Paque Plus (17–1440–03, GE Healthcare, Piscataway, NJ, USA). The buoyant layer was recovered and washed in RPMI-1640 medium plus 10% fetal bovine serum and antibiotics.

Isolation of tissue-infiltrating immune cells in PDAC and CP

Tumor tissues and normal pancreatic tissues from PDAC patients and CP tissues were rinsed with PBS twice and then minced into 1–3 mm³ pieces. The minced tissues were re-suspended in enzymatic cocktail containing 1 mg/mL collagenase (Sigma-Aldrich), 0.1 mg/mL hyaluronidase (Sigma-Aldrich) and 0.1 mg/mL DNase (Sigma-Aldrich) and incubated in 37°C for 1.5 h. The cell suspension was passed through a Falcon 70 μm cell strainer (BD Biosciences) to remove any large aggregates and debris and was then subjected to centrifugation at 300 g for 5 min. The cell pellets were re-suspended in 36% Percoll solution, added onto 30% Percoll solution and centrifuged at 1,000 g for 15 min. The cells in the middle layer were collected and washed twice with PBS. The purified cells were tissue-infiltrating immune cells for cell counting or flow cytometry analysis.

Flow cytometry and cell sorting

The phenotypic characteristics of isolated PBMCs were examined by flow cytometry. PBMCs were pretreated with 50 μg/mL rat immunoglobulin G (IgG) whole molecules (Pierce, Rockford, IL, USA) and then incubated with the following antibodies in PBS, i.e., APC-conjugated anti-CD13, FITC-conjugated anti-CD15, PE-conjugated anti-CD16, PE-Cy7-conjugated anti-CD11b, PerCp-Cy5.5-conjugated anti-CD33, APC-Cy7-conjugated anti-CD14 and Pacific blue-conjugated anti-HLA-DR. After incubation at 4°C for 30 min, cells were washed once with PBS and cell phenotypes were analyzed by flow cytometry. Cytometric data were collected on an LSR II.
cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo Version 5.7.2 software (TreeStar).

To purify monocytes or nMDSC subsets from PBMCs of patients with PDAC, isolated PBMCs were costained with APC-conjugated anti-CD13, FITC-conjugated anti-CD15, PE-Cy7-conjugated anti-CD11b, PerCp-Cy5.5-conjugated anti-CD33 and PE-conjugated anti-CD14, and then CD11bhi or CD133lo MDSCs were co-cultured with sorted monocytes which were stained using anti-human CD3 magnetic microbead (Dako). The purity was confirmed by flow cytometry to be >97%.

Wright-Giemsa staining

Sorted cells were fixed in 2% formaldehyde for 10 min and then washed twice with PBS. Re-suspended cells were smeared on slides by cytopsin centrifugation. After being dried, the samples were stained with Wright-Giemsa dye solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for appropriate times to observe the cellular nuclear characteristics. The size and shape of cells were observed by a microscope (Leica DM 2000, Leica Microsystems, Germany) and the images were acquired by software LAS V3.7 (Leica Microsystems, Germany).

Western blotting

Cells were washed twice with cold PBS and lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail, and protein concentrations of the extracts were measured by BCA assay (Pierce). Proteins were separated on 10% SDS-PAGE gels, and transferred onto nitrocellulose membranes. The membranes were blocked in 5% non-fat milk and reacted with primary antibodies for Arginase 1 (sc-7271, Santa Cruz, CA, USA) and NOS 2 (sc-7271, Santa Cruz, CA, USA) and β-actin (sc-1616, Santa Cruz, CA, USA). Then, HRP-conjugated secondary antibodies were added to the membranes. Protein expressions were detected by enhanced chemiluminescence (ECL) Western blotting detection reagent (Pierce, Rockford, IL).

Cytokine assay

Sorted monocytes or nMDSCs (2 × 10^5 cells per mL) were cultured in RPMI-1640 medium with 10% (vol/vol) FCS in the presence or absence of 500 ng/mL LPS. After 24 h, the contents of TNF-α, IL-6, IL-1β, IL-10, IL-12, IL-2, IL-4 and IFNγ in cell supernatants were measured by cytometric bead array immunoassay (CBA; BD Biosciences) according to the manufacturer’s protocol.

Assay of alloreactive T cell response

To analyze the ability of nMDSC subsets to initiate alloreactive T cell responses or suppress monocytes-induced alloreactive T cell responses, CD3 T cells from healthy donor PBMCs were purified using anti-human CD3 magnetic microbead and labeled with CFSE (Invitrogen). Then, CD3 T cells (1 × 10^5/well) were co-cultured with sorted monocytes (1 × 10^5/well) and/or non-specific nMDSCs, CD13^hi or CD13^low nMDSCs (1 × 10^5/well) at the ratio of 10: 1 (T cells: monocytes or nMDSCs) in 96-well round-bottom plates in triplicate cells. In some groups, Arg-1-specific inhibitor nor-NOHA was used. After 7 d, cells were stained with anti-CD4-Percy-Cy5.5 and anti-CD8-PE and assayed by flow cytometry for CFSE dilution in CD4+ T cells and CD8+ T cells. The degree of T cell proliferation was determined by the percentages of CFSElow cells in gated CD4+ or CD8+ T cells. The results were expressed as proliferating CFSElow T cells (% Mono/T group) and the value in Mono/T group was set as 100%. In each experiment, blood sample from one PDAC patient was collected for purification of MDSC subsets or monocytes. And these experiments were repeated twice.

Statistical analysis

All the data were analyzed using GraphPad Prism version 5.01 statistic software (GraphPad Software, Inc., San Diego, CA, USA). Prior to the statistical analysis, it is always detected whether the assumptions for the selected method were verified. Statistical comparisons between two experimental groups were analyzed with Student’s t test and p < 0.05 was considered statistically significant. For statistical comparisons of more than two groups, ANOVA test followed by a post hoc analysis (Tukey’s multiple comparison test) was used and p < 0.05 was considered statistically significant.

Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

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