**ORIGINAL RESEARCH**

**CD39/CD73 upregulation on myeloid-derived suppressor cells via TGF-β-mTOR-HIF-1 signaling in patients with non-small cell lung cancer**

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**ABSTRACT**

CD39/CD73-adenosine pathway has been recently defined as an important tumor-induced immunosuppressive mechanism. We here documented a fraction of CD11b^+CD33^+ myeloid-derived suppressor cells (MDSCs) in peripheral blood and tumor tissues from non-small cell lung cancer (NSCLC) patients expressed surface ectonucleotidases CD39 and CD73. Tumor TGF-β stimulated CD39 and CD73 expression, thereby inhibited T cell and NK cell activity, and protected tumor cells from the cytotoxic effect of chemotherapy through ectonucleotidase activity. Mechanistically, TGF-β triggered phosphorylation of mammalian target of rapamycin, and subsequently activated hypoxia-inducible factor-1α (HIF-1α) that induced CD39/CD73 expression on MDSCs. CD39 and CD73 on MDSCs, therefore, linked their immunosuppressive and chemo-protective effects to NSCLC progression, providing novel targets for chemo-immunotherapeutic intervention.

**Abbreviations:** CFSE, carboxyfluorescein succinimidyl ester; CR, complete response; HIF-1, hypoxia-inducible factor-1; MDSC, myeloid-derived suppressor cells; mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; SD, stable disease; TCM, tumor-conditioned medium

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**ARTICLE HISTORY**

Received 1 February 2017 Revised 9 April 2017 Accepted 11 April 2017

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such as CD4+Foxp3+ regulatory T cells (Tregs). Little information is known about the expression, regulation, and function of CD39/CD73 on myeloid cells particularly in the context of human immunity.

TGF-β is a pleiotropic cytokine that are present at elevated levels in many types of tumors, and plays a pivotal role in the control of tumor progression. TGF-β signals via type I (TβRI) and type II (TβRII) receptors, a transmembrane serine/threonine kinase complex that ultimately phosphorylates the receptor-activated Smads (R-Smads), which include Smad2 and Smad3. Studies from our laboratory and by others showed that multiple additional signaling pathways are modulated by TGF-β and participate in tumor growth. Given the multifaceted effects on varied immune cells, TGF-β sustains a delicate balance between immunosuppression and immunoa ctivation. In myeloid cells from mouse tumor models, TGF-β has been reported to promote recruitment of MDSCs into tumors. Further, TGF-β signaling can directly induce the generation of CD39⁺/CD73⁺ myeloid cells in tumors, thereby exerting tumor-promoting roles of this pleiotropic player. However, the major downstream signaling pathways how TGF-β induces CD73/CD39 expression on these myeloid cells especially in human tumor microenvironment remains elusive.

The present study using human NSCLC as a model system, evaluated the phenotypic and functional characteristics of CD39⁺/CD73⁺ MDSCs in human peripheral blood and tumor in situ. We demonstrate that CD39⁺/CD73⁺ MDSCs induced by tumor microenvironmental TGF-β are a distinct inflammatory subpopulation with enriched suppressive molecular signatures, and accumulation of this novel population is closely correlated with disease progression and chemotherapeutic response in the NSCLC patients. Furthermore, the molecular mechanisms of CD39 and CD73 promoted by TGF-β on MDSCs were explored.

**Results**

**CD39⁺CD73⁺ MDSCs are highly enriched in the tumor and peritumoral stroma**

Previous studies reported the accumulation of CD11b⁺CD14⁻ granulocytic (G-MDSC) and CD11b⁺CD14⁺ monocytic MDSC (M-MDSC) in NSCLC patients. To analysis of the clinical data, we found that lin⁻CD14⁻CD11b⁺ cells but not lin⁻CD14⁺CD11b⁻ were significantly increased in peripheral blood of NSCLC patients (Fig. S1). Moreover, the frequency of lin⁻CD14⁻CD11b⁻ but not lin⁻CD14⁺CD11b⁺ cells was associated with tumor size (Table S1). Less is known about the influence of MDSCs on chemotherapeutic response. We found that the percentage of lin⁻CD14⁻CD11b⁺ G-MDSC and lin⁻CD14⁺CD11b⁻ M-MDSC significantly decreased with chemotherapy cycles in stable disease (SD) and partial response (PR) groups, whereas progressive disease (PD) group showed no significant change (Fig. S2). To evaluate the potential role of CD39/CD73 in tumor immunopathology, we examined their surface expression on circulating MDSCs from NSCLC patients and healthy donors, and on infiltrating MDSCs freshly isolated from tumor and adjacent nontumor lung tissues of patients with various performance status (n = 35 in each group). CD39/CD73 was detected on MDSCs from NSCLC patients by flow cytometry (Fig. 1A). The frequencies of CD39⁺ (Fig. 1B and C) and CD73⁺ MDSCs (Fig. 1D and E) in peripheral blood from NSCLC patients were not significantly higher than those from healthy donors. However, there were more CD73⁺ MDSCs in tumor tissues than those in blood from patients or healthy donors (Fig. 1D and E). Interestingly, tumor tissues and adjacent nontumor tissues comprised a greater proportion of CD39⁺CD73⁺ MDSCs than blood from NSCLC patients (Fig. 1F and G). As shown in Fig. S3, the mean fluorescence intensity (MFI) of CD39⁺ or CD73⁺ MDSCs in peripheral blood from NSCLC patients was significantly higher than that from healthy donors. We also found the markedly elevated levels of CD39 expression on both MDSC subsets in tumor tissues compared with the surrounding non-cancerous tissues (Fig. S3A and B). In addition, expression of CD39/CD73 on both MDSC subsets was positively correlated with the levels of CD33 and CD124 (IL-4R) that was common phenotypic markers of MDSCs (Fig. S4). Given the tumor-promoting roles of CD39/CD73 and MDSCs, we subsequently examined the relationship between CD39⁺/CD73⁺ MDSCs and clinicopathological parameters (tumor size, node involvement, metastasis, and tumor stage). Notably, the prevalence of CD39⁺CD73⁺ M-MDSC was specifically correlated with the node involvement, metastasis, and tumor stage except for tumor size (Table 1). Collectively, these data suggest that CD39/CD73 expression identifies a novel MDSCs subpopulation that increases with progression of NSCLC, and might thus serve as an independent predictor of poor prognosis.

**CD39 and CD73 expression identifies a distinct inflammatory subpopulation in activated MDSCs with enriched suppressive molecular signatures**

To study the nature of MDSCs from NSCLC patients, we sorted the two subsets of MDSCs (Fig. 2A) and measured the level of inhibitory molecules by qRT-PCR (Fig. 2B). We found that MDSCs from NSCLC patients expressed higher level of inhibitory molecules, such as arginase 1 (ARG1), HIF-1α, COX-2, and IL-10 (Fig. 2C) than those from healthy donors. We then compared the phenotypes of CD39⁺CD73⁺ MDSCs with other corresponding subpopulations. Notably, CD39⁺CD73⁺ MDSCs from NSCLC patients produced greater amount of inflammatory cytokines IL-10 and TNF-α compared with its counterparts (CD39⁻CD73⁻ or CD39⁻CD73⁺ MDSCs (Fig. 2D). Similarly, there were significantly elevated levels of HIF-1α, COX-2, IL-10, and TGF-β on CD39⁺CD73⁺ MDSCs, compared with the CD39⁻CD73⁻, CD39⁺CD73− or CD39⁻CD73− MDSCs (Fig. 2E). These data suggest that CD39⁺CD73⁺ MDSCs from NSCLC patients may represent a novel cell subpopulation with enriched suppressive molecular signatures.

**MDSCs suppress both T cell and NK immunity and protect tumor cells against drug-induced apoptosis via ectonucleotidase activity**

Consistent with previous results, we found that both G-MDSC and M-MDSC subsets from NSCLC patients displayed a greater capacity to inhibit autologous CD8⁺ T cells
proliferation in a dose-dependent manner (Fig. 3A). We and others have reported that tumor cells and Treg cells suppress T cell immunity through the enzymatic activity of CD39/CD73,29,30,43 implying a similar mechanism in MDSCs. To test this possibility, we purified both MDSCs subsets from NSCLC patients and cocultured with autologous CD8\(^+\) T cells in the presence or absence of CD39/CD73 enzymatic inhibitors (ARL67156 and/or APCP). As expected, blockade of CD39 and CD73 activity significantly attenuated such T cell suppression mediated by both MDSCs subsets (Fig. 3B). Similar results were observed in NK cells. As shown in Fig. 3C, both MDSCs subsets induced the apoptosis of NK cells, and this effect was alleviated by blockade of CD39 and CD73 activity. Moreover, IFN-\(\gamma\) production from NK cells was inhibited by MDSCs. However, addition of CD39 and CD73 inhibitors restored the ability of NK cells to produce IFN-\(\gamma\). These findings indicate that CD39 and CD73 ectonucleotidase activity contributes to MDSC-mediated immune suppression in vitro. To test the effect of CD39/CD73\(^+\) MDSCs on NK cells function in vivo, we treated CD39/CD73\(^+\) MDSCs with CD39/CD73 inhibitors or DMSO and then injected them together with NK cells into NOD/SCID mice bearing established A549 tumors. As shown in Fig. 3D, NK therapy alone inhibited tumor growth. NK cell-mediated antitumor effect was abrogated by addition of CD39/CD73\(^+\) MDSCs, but could be restored by treating these MDSC with CD39/CD73 inhibitors, suggesting a role of CD39/CD73 on MDSC in suppressing in vivo antitumor activity of NK cells.

As MDSCs may affect drug-induced tumor cell apoptosis, we examined the role of CD39/CD73 in this specific process.
A549 tumor cells pretreated by docetaxel, were cocultured with or without both MDSCs subsets pretreated with or without CD39/CD73 inhibitors. As expected, either MDSCs subset weakened the drug-induced tumor cell apoptosis (Fig. 3E). Interestingly, blockade of either CD39 or CD73 activity, abrogated the inhibitory effect mediated by both MDSCs subsets. Moreover, an additive effect was observed when both MDSCs subsets were treated with both CD39 and CD73 inhibitors (Fig. S5A). These data suggest that MDSCs may promote the chemoresistance of tumor cells via CD39/CD73 activity.

### TGF-β induces CD39/CD73 expression on MDSCs via activation of mTOR-HIF-1α pathway

Because tumor MDSCs expressed higher levels of CD39/CD73, we hypothesized that tumor-derived factors may stimulate CD39/CD73 expression on MDSCs. To test this, we incubated normal blood MDSCs with control medium or tumor-conditioned medium (TCM). TCM induced significant CD39/CD73 expression on both MDSCs subsets (Fig. S5A). As exogenous TGF-β has been shown to stimulate CD39/CD73 expression on T cells and dendritic cells, we determined whether TGF-β from tumor cells contributed to the induction of CD39/CD73 expression on MDSCs. As expected, addition of a neutralizing monoclonal antibody against TGF-β significantly blocked TCM-mediated CD39/CD73 upregulation (Fig. S5A). The expression of CD39 and CD73 was upregulated within 48 h and reduced subsequently after exposure to rhTGF-β (Fig. S5B). In support further, rhTGF-β induced significant expression of CD39 and CD73 on both MDSCs subsets in a dose-dependent manner, though the effect is more pronounced on CD73 than CD39 (Fig. S5C–H). Moreover, tumor cells secreted a great amount of TGF-β (Fig. S5I). Thus, these data suggest an important role of TGF-β from tumor cells in inducing CD39/CD73 expression on MDSCs.

We next investigated the mechanism by which TGF-β promoted CD73/CD39 expression on MDSCs. Several lines of evidence indicate that TGF-β can stimulate mammalian target of rapamycin (mTOR) activation in cell type-dependent manner. Indeed, we observed rapid phosphorylation of downstream mTOR effectors 4E-BP1 and S6K (Fig. 4A) together with Smad2 and Smad3 (Fig. 4B) in both MDSC subsets after TGF-β treatment, and these effects could be diminished by addition of Smad2/3 inhibitors, suggesting a link between mTOR and TGF-β-Smad2/3 signaling. Importantly, repressing the mTOR pathway by rapamycin treatment abrogated the TGF-β-mediated induction of CD39/CD73 expression on both MDSC subsets (Fig. 4C). We further analyzed activation of mTOR and HIF-1α, both CD39 and CD73 are direct target genes of HIF-1α.52 As shown in Fig. 4D and E, TGF-β enhanced HIF-1α expression on MDSCs in hypoxia (1.5% O2–5% CO2) (not shown) or in the presence of the hypoxia mimetic CoCl2. However, this TGF-β-induced effect could be impaired by addition of rapamycin. As anticipated, CoCl2 treatment promoted CD39/CD73 expression, whereas disruption of HIF-1α by inhibitors (MeoE2) reduced CD39/CD73 expression on MDSCs (Fig. 4F). Thus, our data suggest TGF-β may induce CD39/CD73 expression on MDSCs via activation of mTOR-HIF-1α pathway.

The percentages of HIF-1α+ G-MDSCs and HIF-1α+ M-MDSCs were markedly elevated in lung tumor tissues compared with match adjacent non-tumor tissues and peripheral blood (Fig. S6A). However, the prevalence of HIF-1α+ M-MDSC was specifically correlated with clinicopathological parameters including the tumor size, node involvement; metastasis and tumor stage (Table S2). We subsequently examined the relationship between HIF-1α expression and CD39/CD73 expression in MDSCs. Notably, the percentage of HIF-1α+ cells was positively correlated with that of CD73+ or CD39+ cells in both MDSC subsets (Fig. S6B).

### Predictive value of CD39+/CD73+ MDSCs for chemotherapeutic response

Given the importance of CD39+/CD73+ MDSCs in the tumor chemoresistance described above, we examined whether assessment of CD39+/CD73+ MDSCs in NSCLC patients may be valuable in predicting response to neoadjuvant chemotherapy. We compared the change of CD39+/CD73+ MDSCs frequency

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Table 1. Correlations between CD39 and CD73 expression and clinicopathological parameters (mean ± sem). Note: The “boldface values” means it has statistical significance.

| Characteristics | CD11b+CD14-MDSC | CD11b+CD14-MDSC |
|-----------------|-----------------|-----------------|
|                  | CD39+ | CD73+ | CD39+CD73+ | CD39+ | CD73+ | CD39+CD73+ |
| Tumor size (T)   |       |       |           |       |       |           |
| T1               | 27.6  | 2.9   | 11        | 2.6   | 1.3   |           |
| T2               | 29.3  | 5.2   | 1.3       | 2.3   | 0.5   |           |
| T3               | 28.3  | 5.2   | 1.2       | 1.5   | 0.7   |           |
| T4               | 29.0  | 2.0   | 0.6       | 1.9   | 0.5   |           |
| P                | 0.99  | 0.18  | 0.77      |       |       |           |
| Node (N) involvement |      |       |           |       |       |           |
| N0               | 24.5  | 5.2   | 1.3       | 2.2   | 0.6   |           |
| N1–N3            | 31.8  | 2.5   | 3.2       | 2.3   | 0.4   |           |
| P                | 0.27  | 0.08  | 0.86      |       |       |           |
| Metastasis       |       |       |           |       |       |           |
| M0               | 20.0  | 7.8   | 2.0       | 2.6   | 0.5   |           |
| M1               | 34.9  | 3.7   | 1.7       | 2.2   | 0.4   |           |
| P                | 0.02  | 0.33  | 0.97      |       |       |           |
| Stage            |       |       |           |       |       |           |
| I–II             | 18.0  | 9.6   | 2.6       | 2.4   | 0.6   |           |
| III–IV           | 33.2  | 3.6   | 1.4       | 2.1   | 0.4   |           |
| P                | 0.01  | 0.18  | 0.56      |       |       |           |
| Note: The “boldface values” means it has statistical significance.
between that from before chemotherapy and that after chemotherapy, among groups in response to chemotherapy. As shown in Fig. 5A–F, the percentage of CD39⁺CD73⁺ MDSCs was decreased with chemotherapy cycles in SD and PR groups, whereas there was a trend toward the increase in the percentage of CD39⁺CD73⁺ MDSCs in PD group. More importantly, the increased percentages of CD39⁺CD73⁺ MDSCs in both subsets were significantly correlated with response to chemotherapy in NSCLC patients following 4 cycles of chemotherapy (Fig. 5G and H), suggesting that the changes of CD39⁺CD73⁺ MDSCs frequency in NSCLC patients may be sufficient to predict the chemotherapeutic response.

**Discussion**

MDSCs accumulate in many cancer patients and suppress anti-tumor immunity, thereby limiting the efficacy of cancer immunotherapy. However, human MDSCs comprise a diverse and heterogeneous population of myeloid cells that have been poorly characterized to date.⁶⁻⁷⁻⁹⁻¹⁰ The present study demonstrates that a distinct fraction of activated MDSCs expresses CD39 and CD73 to inhibit NK and T cell immunity, which is linked with the disease progression and chemoresistance in patients with NSCLC.

CD73-mediated adenosinergic effects can now be recognized as among the important immunosuppressive regulatory pathways in the tumor microenvironment.⁴⁸ We and others have reported that tumor cells and Tregs coexpress CD73 and CD39, and produce extracellular adenosine in the tumor-bearing mice and cancer patients.²⁷⁻²⁹⁻³¹⁻⁴⁹⁻⁵¹ Although MDSCs in the mouse system were found to express CD39/CD73,⁴¹⁻⁵² the role of CD39 and CD73 in human MDSCs in tumor immunity and clinical relevance remains elusive. There are two major populations of
MDSCs currently defined in lung cancer patients, namely, granulocytic CD33+CD11b+CD14–/CD15+ cells and monocytic CD33+CD11b+CD14+CD15– cells. In line with the previous studies, we found that MDSCs increased in patients with NSCLC, and they were indeed immune suppressive. Of note, we observed that majority of CD11b–CD14+ cells express CD39 in the peripheral blood and tumor tissues. In contrast, CD73 expression displayed considerable heterogeneity. Although only a minor proportion of either G-MDSC or M-MDSC expressed CD73, the frequency of CD73-positive cells was elevated in lung lesions. Moreover, both MDSCs subsets infiltrating lesions of NSCLC patients were distinctly enriched in CD39+CD73+ subpopulation.

Studies in mouse models have revealed that expression of CD73/CD39 promotes tumor cells to evade cytotoxic T cell responses. However, the regulatory mechanisms and the function of CD73/CD39 remain to be defined in human
immunology. We found that recombinant and tumor environmental TGF-β stimulate CD39/CD73 expression on MDSCs. Moreover, in vitro TGF-β blockade study demonstrated that TGF-β produced from tumor cells is essential for CD39/CD73 induction. Other cells such as tumor-associated macrophages and Treg cells may be the additional source for TGF-β. It is also possible that autocrine TGF-β released from MDSCs may stimulate CD39/CD73 expression. These data provide novel mechanisms for how tumor environmental TGF-β induce MDSC-mediated immune dysfunction via CD39/CD73. This notion is compatible with previous murine studies showing a TGF-β-dependent upregulation of CD39/CD73 expression on activated T cells, and myeloid cells.

Our results revealed CD39/CD73 as one of the nonredundant suppressive mechanisms used in MDSC-mediated suppression of NK and T cell activities. It is important to explore whether A2AR and/or A2BR pathway is involved in CD39/CD73-mediated MDSC activities. Nevertheless, several suppressive mechanisms seem to be involved because blockade of CD39 and CD73 did not completely reverse immunosuppression. Indeed, it has been reported that MDSCs from NSCLC patients suppressed T cells via arginase, ROS, and the IL-13/IL-4R axis, and myeloid cells.

Interestingly, we also showed that CD39 and CD73 expression was correlated with CD33 and IL-4R expression on MDSCs. More strikingly, CD39+/CD73+ MDSCs expressed higher levels of typical MDSC-associated suppressive factors including HIF-1α, COX-2, IL-10, TNF-α, and TGF-β, compared with its counterparts, indicating a possibility of MDSC and CD73 as prospective functional markers defining MDSCs. Thus, CD39 and CD73 may distinguish a novel cell subpopulation with enriched suppressive molecular signatures from MDSCs in NSCLC patients. As a recent study reported that circulating MDSCs in colorectal cancer patients expressed CD39 but lack CD73, CD39/CD73 expression among MDSCs populations in cancer patients is likely diverse and can differ in phenotype and location, probably depending on factors secreted by the tumor.

The frequency and function of MDSCs could be modulated by extracellular adenosine. Intriguingly, we showed that the MDSCs immunosuppressive activity was at least partially dependent on CD39/CD73-mediated adenosinergic effect. This suggests that adenosine generated by CD39 and CD73 expressed on MDSCs can inhibit antitumor activities of effector cells such as NK cells and effector T cells via paracrine signaling. On the other hand, extracellular adenosine may promote the numbers and sustain immunosuppressive potential of...
MDSCs in an autocrine manner. Thus, our observations on the upregulation of ectonucleotidase expression on MDSCs upon tumor TGF-β induction demonstrate a previously unappreciated role of tumor-infiltrating CD39⁺CD73⁺ MDSCs in limiting antitumor immunity through both autocrine and paracrine adenosine signaling mechanisms.

It has been reported that Smad3/mTORC1 interaction to promote HIF-1 expression was a key step in normoxic kidney fibrogenesis. Our work showed that TGF-β stimulation can cause normoxic expression of HIF-1α, and this process could be important for CD39/CD73 expression on MDSCs from NSCLC patients. In support, HIF-1α expression elevated in
MDSCs was positively in correlation with CD39/CD73 expression. As no change in HIF stability or mRNA expression in MDSCs was found after exposure of TGF-β (not shown), we hypothesized that mTOR, a known activator of HIF translation, might play a major role in TGF-β-induced normoxic HIF-1α expression and, consequently, CD39/CD73 induction. Indeed, we found that Smad2, Smad3, and mTOR signaling were simultaneously activated in MDSCs after exogenous TGF-β. This observation led us to identify possible cross-talk between Smad signaling and mTOR activation in MDSCs. Upon binding of TGF-β to TGF-βRII on extracellular surface, it triggers phosphorylation of TGF-βRI that activates either Smad-dependent or -independent pathway. In our result, TGF-β-induced phosphorylation of mTOR was inhibited by SB253344 implying that this induction is dependent on Smad2/3. Further, we demonstrate that exogenous TGF-β-induced HIF-1α activation is mTOR dependent and is crucial for CD39/CD73 induction under normoxic conditions. In contrast, hypoxia-induced HIF-1α activation does not require mTOR activity. These results suggest the importance of a TGF-β-mTOR-HIF-1 pathway in at least one aspect of MDSCs suppression via CD39/CD73 activity. In addition, there is an important consideration for the activation of autophagy, one of the main consequences of the mTOR inhibition by rapamycin. This pathway contributes to antitumor immunity due to its active role in ATP secretion from dying cells and to manifold activities in immune cells. As recently shown, administration of autophagy inducers increases ATP secretion and slows down tumor growth in an ATP (and thus CD39 and CD73)-dependent manner. Thus, whether rapamycin effect on CD39 and CD73 expression depends on autophagy warrants further investigation.

Our study also demonstrated that NSCLC CD11b+ MDSCs protected tumor cells from the cytotoxic effect of docetaxel, consistent with the previous report. Blocking the enzymatic activity of both CD39 and CD73 appears to completely reverse the protective effect of NSCLC MDSCs, suggesting an essential role of CD39 and CD73 in MDSC-mediated chemo-protective effect. In agreement, patients with higher levels of CD39+CD73+ MDSCs showed the poor response to chemotherapy. Furthermore, having established CD39/CD73 as an indicator of in situ activity of MDSCs, we used this marker for an assessment of their impact on tumor progression or chemo-therapeutic response in NSCLC patients. Notably, the prevalence of CD39+CD73+ MDSCs was correlated with the node involvement, metastasis, tumor stage, and chemotherapeutic response.

Taken together, we document for the first time that a fraction of peripheral blood and tumor-infiltrating MDSCs expresses CD39 and CD73 to inhibit T cell and NK cell activities, and protect tumor cell from the cytotoxic effect of chemotherapy. The ectoenzymatic activity of CD39 and CD73 is required for MDSC-mediated suppressive and chemo-protective effects. TGF-β induced the expression of CD39/CD73 via TGF-β-mTOR-HIF-1 pathway on MDSCs. Our study provides the strong evidence showing CD39 and CD73 as prospective functional markers defining MDSCs. Pharmacological targeting CD39/CD73 or TGF-β-mTOR-HIF-1 pathway using small molecule inhibitors and monoclonal antibodies are currently under clinical development. Our study thus contributes new insights into the potential for CD39/CD73 blockade as MDSC-targeting strategies to promote the conventional chemoinmunotherapy of NSCLC.

**Methods**

**Donor recruitment and blood sample preparation**

From November 2012 to November 2013, 72 patients with NSCLC from the First Affiliated Hospital of Zhengzhou University were enrolled. The patients have not been treated with anticancer drugs, radiotherapy, or surgery in the beginning of the study and have no other systemic diseases. Clinical stages were classified according to the International Union against Cancer. Peripheral blood was collected from 72 patients with NSCLC and 45 healthy donors with similar gender and age distribution, respectively. Tumor tissue and adjacent non-tumor tissue (at least 3 cm away from the tumor site) were obtained from 41 patients with NSCLC for the isolation of tumor- and nontumor-infiltrating leukocytes. All patients gave written informed consent. The whole consent procedure was in accordance with the standard defined by Institutional Review Boards of the First Affiliated Hospital of Zhengzhou University. Of these 72 patients, they were to received cisplatin (75 mg/m²) plus docetaxel (30 mg/m² on day 1 and day 8) every 3 weeks for 4 cycles. Tumor response was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria and classified as complete response (CR), PR, SD, or PD. Clinicopathologic information, including patient characteristics, tumor size, clinical staging, node involvement, and metastasis status was summarized in Table S1.

**Antibodies and flow cytometry**

Fresh human peripheral blood mononuclear cells (PBMCs) and tumor- and nontumor-infiltrating leukocytes were stained with fluorescence-conjugated antibodies (CD3, 7AAD, CD14, CD11b, CD15, HLA-DR, CD39, CD73, IL-4R, IL-10, TNF-α, and HIF-1α BD Biosciences). PBMCs were stained according to the manufacturer’s instructions.

**Cell isolation and sorting**

Fresh tumor- and nontumor-infiltrating leukocytes were harvested as described previously. Lung biopsy specimens (n = 41) were cut into small pieces and digested in RPMI 1640 (Gibco, USA) supplemented with 0.25% trypsin (Gibco), 0.002% DNase I (Gibco), and 20% fetal bovine serum (FBS, Gibco) at 37°C for 20 min. Dissociated cells were filtered through a 100 μm mesh and isolated by Ficoll–Hypaque density gradient centrifugation (Beijing Chemical Reagent Company, China). The mononuclear cells were washed and resuspended in medium supplemented with 10% heat-inactivated FBS for FACS analysis. PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation, sequentially using the anti-CD14 and anti-CD11b MACS magnetic sorting system (Miltenyi Biotec, Germany) within 2 h of sample collection. CD14+CD11b+ and CD14−CD11b+ cells were enriched according to the
manufacturer’s instructions. The purity of the two subsets of cells was >95% and >88%, respectively, as confirmed by flow cytometry. CD8+ T cells were also enriched by MACS magnetic sorting system and the purity was >95%. CD39+CD73+ MDSCs and CD3+CD56+ NK cells were sorted using MoFlo-XDP (Beckman Coulter, USA). The purity of CD39+CD73+ MDSCs and NK cells was >95%, confirmed by flow cytometry (data not shown).

**Regulation of CD39 and CD73 expression**

Human lung adenocarcinoma A549 cell line was grown in RPMI 1640 with 10% FBS with 100 U/mL penicillin, 100 µg/mL streptomycin. TCM was prepared as described previously.\(^{33}\) Fresh blood MDSCs from healthy donors were cultured for the indicated times with TCM or different concentration (0.5, 1, and 2 ng/mL) of rhTGF-β (Sigma-Aldrich, USA).\(^{14}\) In some groups, before exposure to TCM, the cells were pretreated with neutralizing mAbs against TGF-β, at a concentration of 5 µM. In other groups, MDSCs were pretreated with rapamycin (10 nM) or DMSO (control solvent) for 30 min followed by TGF-β. After 24, 48, and 72 h, expression of CD39/CD73 on MDSCs was determined by flow cytometry. To determine the involvement of HIF-1 activity, MDSCs were cultured with or without 100 µM CoCl₂ or CoCl₂ plus 10 µM MeoE₂ for 48 h. The gene expression of CD39/CD73 in MDSCs was determined by qRT-PCR.

**Quantitative RT-PCR**

Total RNA from CD14+CD11b+ and CD14−CD11b+ cells was extracted using the TRIZOL Reagent (Invitrogen Life Technologies, CA, USA), and cDNA that was synthesized using the PrimeScript RT reagent kit with gDNA eraser (TaKaRa, Japan). Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (Takara) and assessed by Agilent Mx3005P. Relative gene expression was determined using SYBR Green I. Relative expression was calculated by the 2^{-ΔΔCt} method. Primer sequences for all gene-specific amplifications were shown in Table S3.

**MDSC-mediated chemo-protective assay**

The assay was performed using A549 tumor cells treated or untreated with 11 nmol/L docetaxel as targets and MDSCs pretreated with ARL67165, a CD39 inhibitor (250 µmol/L, Sigma-Aldrich), and/or APCP, a CD73 inhibitor (100 µmol/L, Sigma-Aldrich), purified from peripheral blood from three patients with NSCLC, as effector cells. Briefly, target tumor cells were labeled with 5 mol/L carboxyfluorescein diacetate succinimydil ester (CFSE, Sigma-Aldrich) at 37°C in 5% CO₂ atmosphere for 15 min. The labeled tumor cells were washed three times, added to the effector cells in a 96-well U bottom plate at the 0:1, 1:1, and 4:1 E: T ratios, and incubated for 24 h. The cell apoptosis was determined by the Annexin V (BD Bioscience) and PI (Sigma-Aldrich) staining using flow cytometry as described previously.\(^{33}\)

**T cell suppression assay**

CD14+CD11b+ and CD14−CD11b+ cells purified from NSCLC patients were incubated at ratios of 1:1 and 1:2 with autologous CD8+ T cells stimulated by 30 ng/mL anti-CD3/ anti-CD28 (Miltenyi Biotech). To examine the importance of CD39/CD73 activity, MDSCs were pretreated with ARL67165 (250 µmol/L), and/or APCP (100 µmol/L) before the addition of T cells. After 72 h, T cell proliferation was measured by Ki-67 staining using flow cytometry (Biolgend, USA). Proliferation index was determined as follows: (% proliferated T cells in the presence of mAB — % proliferated T cells in the presence of control IgG)/(% proliferated T cells in T cells alone — % proliferated T cells in the presence of control IgG) × 100.

**NK cell suppression assay**

Sorted CD14+CD11b+ or CD14−CD11b+ MDSCs from peripheral blood of NSCLC patients were incubated with NK cells (10⁶ per well) at a ratio of 1:1. To determine the importance of CD39/CD73 activity, MDSCs were pretreated with ARL67165 (250 µmol/L), and/or APCP (100 µmol/L) for 30 min before adding the NK cells. To examine the apoptosis, cells were stimulated with 1000 IU/mL IL-2 for 3 d, and stained with Annexin V and PtdIns. To measure intracellular IFN-γ production, cells were stimulated with 1 µg/mL phorbol myristate acetate (PMA, Sigma-Aldrich), 50 ng/mL ionomycin (Sigma-Aldrich), and 5 µg/mL brefeldin A solution (BFA, Biolgend) for 4 h at 37°C before harvesting.

**Western blot analysis**

Cells were washed twice with ice-cold PBS and lysed on ice in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) containing serum protease and phosphatase inhibitors (Sigma). Cleared cell lysates were subjected to SDS-PAGE (8–10% polyacrylamide gels), transferred onto PVDF membranes (Millipore, Bedford, MA), and probed with antibodies as indicated: HIF1α, mTOR, CD39/CD73, Smad2/3 (Cell Signaling Technology). Densitometric analysis was performed using the NIH Image 1.61 program for Windows.

**Animal experiment**

Animal protocols were approved by the Review Board of the First Affiliated Hospital of Zhengzhou University. 1 × 10⁶ A549 cells in 100 µL of buffered saline were subcutaneously injected into the dorsal tissues of female NOD/SCID mice (4–6 week old, two tumors per mouse). 5 × 10⁶ NK cells were mixed with or without TCM- treated MDSCs in the presence or absence of CD39/CD73 inhibitors, as described in the previous section, and were subsequently injected into the peritoneum in 100 µL of buffered saline on day 7 after inoculation. Tumor size was measured twice weekly by two independent observers using calipers fitted with a vernier scale. Tumor volume was calculated based on three perpendicular measurements.
Statistics
Based on the distribution level, differences in mean ± SEM and correlation analysis were evaluated with parametric (Independentsample or paired t-test and Spearman’s test) or non-parametric (Wilcoxon and Spearman’s p test) tests. Due to the supportive and exploratory nature of these comparisons, no adjustments for multiplicity were performed for the multiple comparisons across end-points and sub-groups. The differences of clinicopathologic characteristics were compared by repeated-measures ANOVA. All analyses were performed at a significance level of 5% (p ≤ 0.05) using SPSS version 17.0 (SPSS, Inc.).

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Yanyan Sun and Xiangnan Li for assistance in sample collecting.

Funding
This study was supported by grants from the National Institutes of Health (CA149669 and CA208354), National Natural Science Foundation of China (Grant nos. 81171985 and 81717986), Research Grant from the Ministry of Public Health (No. 2011010001), the Basic and Advanced Technology Research Foundation from Science and Technology Department of Henan Province (Grant no.112300410153, Grant no.122300410155).

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