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Ammonia Drives Dendritic Cells into Dysfunction

Can Luo,*1 Guobo Shen,*1 Ning Liu,*1 Fengming Gong,* Xiawei Wei,* Shaohua Yao,* Dan Liu,† Xiu Teng,* Ning Ye,* Nan Zhang,* Xikun Zhou,* Jiong Li,* Li Yang,‡ Xia Zhao,‡ Li Yang,* Rong Xiang,* and Yu-quan Wei*

Ammonia levels are often elevated in patients with cirrhosis or tumors. Patients with these diseases are immunocompromised. In this study, we investigated the effects of ammonia on a member of the immune cell family, the dendritic cells (DCs). Our results demonstrated that ammonia diminished cell count, phagocytosis, and lymphocyte stimulation of DCs. Ammonia also induced DC swelling, excessive reactive oxygen species production, and mitochondrial damage, which may constitute the underlying mechanism of ammonia-induced DC dysfunction. In ammonium chloride (NH4Cl)–loaded mice, DCs exhibited lowered phagocytosis and a weakened immune response to the chicken OVA vaccine. DCs from patients with cirrhosis or ammonia-treated healthy human blood both exhibited diminished phagocytosis. Moreover, tumor cell conditioned medium drove DCs into dysfunction, which could be reversed by ammonia elimination. In a murine colon carcinoma model, we found that ammonia could regulate tumor growth involving DCs and their related immune response. These findings reveal that ammonia could drive DCs into dysfunction, which contributes to the immunocompromised state of patients with cirrhosis or tumors. The Journal of Immunology, 2014, 193: 000–000.

Ammonia concentration is often elevated in the peripheral blood of patients with cirrhosis (1). In healthy human blood, ammonia concentration ranges from 21 to 50 μM, whereas in patients with cirrhosis, it is always higher and variable, usually in the range of 60–200 μM. Patients with cirrhosis are always accompanied by increased susceptibility to infection, which accounts for an ~30% mortality (2). This is likely a result of their immunocompromised state (3). Such an immune dysfunction is associated with one of the abnormalities of cirrhosis, hyperammonemia. It has been shown that ammonia can impair neutrophil phagocytic function in liver disease (4, 5). However, other damaging effects of ammonia on the immune system are yet to be determined.

In comparison with normal cells, tumor cells rely on different energy metabolism pathways, such as glycolysis and glutaminolysis (6). Tumor cells exhibit elevated glutamine transportation and metabolism (7), because of which ammonia accumulates in the tumor microenvironment. Relatively few studies on ammonia in the tumor microenvironment are available. However, Christina et al. (8) demonstrated that ammonia levels in tumor cell cultures and human cancer cell line xenografts are ~2–5 mM. In addition, we tested ammonia levels in normal tissues and colon carcinoma tissues derived from humans. Our results showed that tumor ammonia levels were higher than those in normal tissues. Previous studies have shown that the production of lactic acid from glycolysis may facilitate the escape of tumor cells from immune surveillance (9, 10). However, few studies have looked at the relationship between ammonia and immunity.

Dendritic cells (DCs) are able to form a bridge between the innate and adaptive immune systems to fight against bacteria (11, 12). For immune disorders in patients with cirrhosis, the dysfunction of immune cells, especially DCs, has been described in previous studies (13, 14). However, the underlying mechanism is yet to be elucidated. Immature DCs can capture and process tumor Ags. Upon maturation, DCs move into secondary lymphoid organs, stimulate naïve T cells, and prime tumor Ag–specific T cells. This whole process is closely associated with antitumor activity (15, 16). However, the deficiency of DCs in tumors has been previously reported in human and mouse systems (17, 18). In this study, we investigated the influence of ammonia on DCs in cirrhosis and tumors. Our data revealed that ammonia could affect DC count, phagocytosis, and lymphocyte stimulation ability, as well as regulate immune response in cirrhosis and tumors, through a reactive oxygen species (ROS)–related pathway.

Materials and Methods

Ethics statement

All mouse experiments were carried out in strict accordance with the regulation of the Animal Care and Use Committee of Sichuan University. Experiments involving human subjects were undertaken with written informed consent from each patient and full approval of the Ethics Committee of Sichuan University.

Generation of DCs

BALB/c mouse bone marrow–derived DCs were generated as previously described. Briefly, bone marrow cells were harvested from the femur and tibia of 8- to 12-wk-old mice. Cells were cultured in RPMI 1640
(Life Technologies) with recombinant murine GM-CSF [Sigma-Aldrich (Shanghai) Trading Company, Shanghai, China] at the concentration of 20 ng/ml. The medium was refreshed every 2–3 d. After 7–10 d of culture, nonadherent and loosely adherent cells were harvested and stained with anti-CD11c for purity verification. The purity was >90%, and the cells were ready to use as DCs. In this study, we used ammonium chloride [NH₄Cl, Sigma-Aldrich (Shanghai) Trading Company, Shanghai, China] for ammonia studies, as used by previous researchers (4, 8, 19).

We used 35 μM, 75 μM, 150 μM, 2.5 mM, or 5 mM NH₄Cl from day 0 or day 7. DCs were simulated by 1 mM incubated in the presence of 35 μM, 75 μM, 150 μM, 2.5 mM, or 5 mM NH₄Cl to induce maturation. Then the cells were washed, washed with PBS, and stained with anti-CD11c–PE, anti-MHC-II–FITC, anti-CD80–FITC, and anti-CD86–FITC (BD Biosciences) for phenotype analysis. In addition, we sorted three populations of DCs, including terminally differentiated mature DCs (CD11c+CD86+), less differentiated immature DCs (CD11c+CD86−), and DC precursors (CD11c−CD86−) (20). Sorted DCs were cultured with or without NH₄Cl for 48 h and stained with Annexin V/PI (KeyGEN BioTECH, Nanjing, China) for DC viability analysis. The Annexin V–positive cells were counted as apoptotic DCs. Sorted DCs were also labeled with 2 μM CFSE [Sigma-Aldrich (Shanghai) Trading Company] in PBS.0.1% BSA for 10 min at 37˚C and washed twice with complete medium. Cells were then seeded in 24-well plates (Costar) at 5 × 10⁶/ml and cultured with or without NH₄Cl. Proliferation was analyzed by flow cytometry after 48 h.

**FITC–dextran uptake by DCs**

To test DC Ag uptake ability, DCs were harvested at day 7 and cultured in the presence of 10⁶ cells per ml5, 150 μM, 2.5 mM, or 5 mM NH₄Cl for 48 h. Then DCs were seeded in 24-well plates at a density of 10⁶ cells per milliliter, and cultured with 1 mg/ml FITC–dextran [Sigma-Aldrich (Shanghai) Trading Company] at 37˚C for 1 h, washed with PBS, and stained with anti-CD11c. Spontaneous FITC–dextran incorporation was assessed by incubating DCs with FITC–dextran at 4˚C. DC Ag uptake ability was determined by FITC mean fluorescence intensity (MFI) of CD11c-positive cells.

**MLR**

After culturing DCs with 35 μM, 75 μM, 150 μM, 2.5 mM or 5 mM NH₄Cl for 48 h, DCs were irradiated at 50 Gy and plated in 96-well plates at a density of 2 × 10⁴ cells per well. Allogeneic lymphocytes suspensions were prepared from C57BL/6 spleens, using an EZ-Sep Mouse Lymphocyte Separation Kit (Dakewe Biotech Company, Shenzhen, China) according to the manufacturer’s instructions. Briefly, spleens were homogenized through a cell strainer into density gradient separation medium. Then the suspended cells were centrifuged at 800 × g for 30 min at room temperature. The mononuclear cell–enriched fraction was collected, and after erythrocyte lysis, the cells were washed and added to the culture at the density of 2 × 10⁷. After 5 d of culture, the cells were pulsed for 16 additional hours with [3H]thymidine at 1 μCi; thereafter, cells were collected onto filter paper using a cell harvester. The dried filters were counted directly in a β counter. The data were expressed as mean ± SD of five replicates.

**Transmission electron microscopy analysis**

DCs were pretreated with 75 μM and 2.5 mM NH₄Cl for 48 h, fixed with 3% glutaraldehyde for ~4 h. The cells were washed twice with PBS, post fixed with 1% OsO₄ for 1 h, dehydrated in ascending grades of ethanol (70%, 90%, 100%), embedded in Epon 812 and polymerized at 60˚C for 24 h. Ultrathin sections were cut (50–70 nm) and stained with uranyl acetate followed by lead citrate. Then the samples were analyzed by transmission electron microscope (Hitachi, Tokyo, Japan).

**Detection of ROS**

To measure the generation of ROS, DCs pretreated with or without the Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (25 μM, 15 min) [Sigma-Aldrich (Shanghai) Trading Company] were cultured with NH₄Cl for 4 h; then 10 μM dichlorodihydrofluorescein diacetate [Sigma-Aldrich (Shanghai) Trading Company] was added. In the cell plasma, dichlorodihydrofluorescein diacetate is converted to dichlorodihydrofluorescein in the presence of ROS.DCF is oxidized to the fluorescent DCF. After incubation at 37˚C for 30 min, the cells were harvested, washed twice with PBS, and analyzed by flow cytometry (BD) for DCF fluorescence.

**Mitochondrial permeability transition**

Calcine acetoxyethyl (calcine/AM) goes through the cell membrane freely; after de-esterification, it becomes fluorescent. Treating cells with cobalt chloride could quench the fluorescence in the cell except in mitochondria. With induction of mitochondria permeability transition (MPT), cobalt can enter the mitochondria and extinguish the fluorescence. The cells were pretreated in the presence or absence of BAPTA (25 μM, 15 min) or ROS scavenger superoxide dismutase (SOD) (25 μM, 30 min) [Sigma-Aldrich (Shanghai) Trading Company]. After 48 h exposure of NH₄Cl, cells were washed with HBSS and were cultured in HBSS medium containing calcine/AM (1 mM) [Sigma-Aldrich (Shanghai) Trading Company] and cobalt chloride (1 mM) [Sigma-Aldrich (Shanghai) Trading Company] at 37˚C for 20 min. Then, the cells were washed and suspended at the same density for each group. Each group had the same number of cells plated in 24-well plates and then centrifuged at 2000 rpm for 3 min, and images were captured by the fluorescence microscope.

**Measurement of mitochondrial potential**

Cells were treated with or without NH₄Cl, BAPTA (25 μM, pretreated for 15 min), and SOD (25 μM, pretreated for 30 min). At 48 h later, cells were harvested and incubated at a density of 1 × 10⁷/ml in PBS with 1 μM rhodamine-123 (Rh123) [Sigma-Aldrich (Shanghai) Trading Company]. Rh123 is a cationic lipophilic green fluorescent. Disruption of mitochondrial membrane potential is associated with a lack of Rh123 retention and a decrease in fluorescence. Following 30-min incubation at 37˚C, samples were washed and analyzed by FACS. Each group had the same number of cells tested.

**Chronic ammonia-loading experiments**

BALB/c mice were served with 0.28 M NH₄Cl solution instead of water. At 1 wk later, mice were sacrificed and the plasma was collected. Plasma ammonia was measured by the Ammonia Assay Kit [Sigma-Aldrich (Shanghai) Trading Company]. A total of 100 μl 5 mg/ml FITC–dextran was injected i.v. into control and ammonia-loaded mice. At 1 h later, spleen tissues were miniced, single-cell suspension was gained by stillstanding. After erythrocyte lysis, the cells were washed with PBS and stained with anti-CD11c and anti-MHCII for the gating of spleen DCs. The phagocytosis ability of DCs was measured by the percentage of FITC-positive cells. Mice were immunized with model Ag chicken OVA [Sigma-Aldrich (Shanghai) Trading Company] emulsified in CFA [Sigma-Aldrich (Shanghai) Trading Company] at 100 μg per animal, and boosted two times, 14 and 21 d later. Serum was taken at day 13 and day 28, the OVA-specific total IgG, IgG1, IgG2a, IgG2b, and IgG3 were determined through ELISA.

**Measurement of human DC FITC–dextran uptake**

Venous blood was collected aseptically from patients with cirrhosis and healthy volunteers (n = 8 for both groups), and the phagocytic function of DCs was analyzed. For the study of ammonia’s influence on normal DCs, healthy human whole blood was cultured with 35 μM, 75 μM, 150 μM, 2.5 mM, and 5 mM NH₄Cl at 37˚C for 90 min before phagocytosis. A total of 3 ml heparinized whole blood was cultured with FITC–dextran at 1 mg/ml at 37˚C for 1 h. Spontaneous FITC–dextran incorporation was assessed by incubation at 4˚C. PBMCs were collected through density gradient centrifugation. The cells were washed with PBS, then stained with anti-HLA-DR (BioLegend) and anti-human lineage Ab mixture-1 (CD3, CD14, CD16, CD19, CD20, CD56) (BioLegend). Then the cells were washed and analyzed by FACS. DCs were identified as HLA-DR+Lin-; dextran uptake was determined by the FITC MFI value of DCs.

**Tumor cell conditioned medium and tumor tissue coculture**

Supernatant was collected from 72-h culture of CT-26 tumor cells as tumor cell conditioned medium (CM). After rotary evaporation and reconstitution with an equal volume of water, we have ammonia-eliminated evaporated CM (eCM) with less ammonia than CM. To demonstrate the effect of NH₄Cl, we measured ammonia levels in CM and eCM, and added NH₄Cl into eCM to restore its ammonia level. We took s.c. CT-26 tumor tissue from mice, minced it into ~1-mm³ cubes, washed it with HBSS, and cultured with DCs. Ammonia was generated during the conversion of glutamine to α-ketoglutarate (α-KG) in mitochondria. Glutaminolysis can be circumvented by direct addition of α-KG (as a cell-permeable di-methyl ester) to the culture medium. We added 5 mM α-KG [Sigma-Aldrich (Shanghai) Trading Company] to the tumor tissue culture medium to eliminate ammonia production. After incubation in different conditions as above, DC count, allogenic stimulation, and ROS production were ana-
lyzed, as mentioned before. In addition, CT-26 cells were irradiated at 50 Gy to induce apoptosis and were labeled with PKH67 [Sigma-Aldrich (Shanghai) Trading Company]. After culturing these tumor cells and DCs at a ratio of 1:1 at 37°C overnight, the cells were washed with PBS and stained with anti-CD11c. Spontaneous uptake control was performed by culturing tumor cells and DCs at 4°C. Apoptotic tumor cell uptake was evaluated; the double positive fraction represented DCs that phagocytosed the tumor cells.

**In vivo tumor studies**

To evaluate the effect of ammonia on tumor in vivo, $1 \times 10^6$ CT-26 cells were injected s.c. (100 μl) into the flank region of BALB/c mice. Animals were examined daily until the tumor became palpable, after which they were intratumor (i.t.) injected with 100 μl PBS, 20 mM NH$_4$Cl, or 750 mg/kg α-KG daily. The diameter, in two dimensions, was measured every 2 d using calipers. After 2 wk of treatment, DCs and T cells were analyzed by FACS. For tumor-infiltrating DC phagocytosis analysis, FITC–dextran (5 mg/ml) was injected directly i.t. (50 μl volume) into CT-26-bearing animals. DCs were gated as CD11c$^+$ MHC-II$^+$ cells among the CD45$^+$ population; FITC fluorescent percentages of DCs were determined 3 h, 6 h, 12 h, and 24 h after FITC–dextran injection. For detecting DCs and T cells in vivo, tumor tissues were minced and digested with 1 mg/ml collagenase I (Life Technologies) at 37°C for 2 h. Tumor-draining lymph nodes were collected and made into single-cell suspensions. After erythrocyte lysis, the cells were washed with PBS and stained with anti-CD45, anti-MHCII, and anti-CD11c; anti-CD4, anti-CD8, and anti-IFN-γ. All Abs were from BD Biosciences (BD).

**Statistical analysis**

Descriptive and analytical statistics were performed using the SPSS (version 16) software package. Descriptive data were presented as mean ± SD. Comparison between different conditions was performed using the one-way ANOVA and Student t test analysis. A $p$ value $< 0.05$ was considered statistically significant.

**FIGURE 1.** The effect of ammonia on DC count, phagocytosis, and allogenic lymphocyte stimulation. DCs derived from BALB/c bone marrow cells were cultured with or without 5 mM NH$_4$Cl. (A) CD11c, MHCII, CD80, and CD86 expression was analyzed for differentiation determination; (B) after stimulation with LPS 1 μg/ml, MHCII, CD80, and CD86 expression of CD11c-positive cells was analyzed. (C) Cell count of DCs after 48-h culture of NH$_4$Cl. The sorted three groups, including terminally differentiated mature DCs (CD11c$^+$ CD86$^+$), less differentiated immature DCs (CD11c$^+$ CD86$^-$), and DC precursors (CD11c$^-$ CD86$^-$), were cultured with NH$_4$Cl for additional 48 h. (D) Cells were stained with Annexin V/PI for viability analysis, and the Annexin V positive fraction was measured; (E) CFSE-labeled DCs were analyzed for proliferation analysis. (F) Following 1-h culture with FITC–dextran 1 mg/ml at 37°C, the FITC MFI of CD11c$^+$ positive cells was analyzed; (G) spleen lymphocytes from C57BL mice were cultured with irradiated DCs for 5 d, cells were then harvested, and $^3$H-thymidine uptake was measured. Bars represent mean ± SD from three independent experiments.*$p < 0.05$ (one-way ANOVA).
Results

Ammonia diminishes DC count, phagocytosis, and allogenic stimulatory activity

To assess the influence of ammonia on DC differentiation, NH₄Cl was added on culture day 0. After 7 d of culture, the expression of CD11c, and the costimulatory molecules CD80, CD86, and MHCI, was detected. There was no significant difference in expression between the control and NH₄Cl groups (Fig. 1A). To assess the effect of ammonia on DC maturation, LPS was added and the cells were analyzed for CD80, CD86, and MHCI expression. Still, no significant difference between each group was found (Fig. 1B). These results confirmed that ammonia had no effect on DC differentiation and maturation phenotype.

Following another 48-h culture with NH₄Cl from day 7, we examined DC count, phagocytosis, and allogenic stimulatory activity. We found that NH₄Cl diminished the cell count of DCs at the concentration of 2.5 mM and 5 mM (Fig. 1C). To further clarify this effect, we tested viability and proliferation of the sorted three groups of DCs, including terminally differentiated mature DCs (CD11c⁺CD86⁺), less differentiated immature DCs (CD11c⁺CD86⁻), and DC precursors (CD11c⁻CD86⁻). NH₄Cl didn’t induce apoptosis of DCs in any groups but inhibited the proliferation of immature DCs and DC precursors (Fig. 1D, 1E).

In terms of DC phagocytosis of FITC–dextran, the 75 μM, 150 μM, 2.5 mM, and 5 mM NH₄Cl groups showed reduced phagocytic ability, whereas the 35-μM group was comparable to control (Fig. 1F). Inhibition of allogenic stimulatory activity was significant only in the 2.5 mM and 5 mM NH₄Cl groups, whereas the other concentrations tested showed no influence (Fig. 1G). These data suggest that ammonia could inhibit phagocytosis of DCs at 75 μM, 150 μM, 2.5 mM, and 5 mM; ammonia could also diminish the cell count and allogenic stimulation of DCs at a concentration of 2.5 mM and 5 mM.

Ammonia induces DC swelling, associated with ROS overproduction and related mitochondria dysfunction

After revealing the effects of ammonia on DC function, we investigated the underlying mechanism. DCs treated with 75 μM and 2.5 mM NH₄Cl for 24 h were harvested for FACS analysis. The results showed that 75 μM and 2.5 mM NH₄Cl both raised the mean forward scatter (FSC) value of the DCs (Fig. 2A). This finding suggests that the volume of DCs increased after ammonia treatment. From transmission electron microscopy analysis, we observed that mitochondria in the 75 μM NH₄Cl–treated DCs remained similar to the control but swelled in the 2.5 mM NH₄Cl–treated DCs (Fig. 2B). The swelling phenomenon of ammonia-treated DCs was in accordance with ammonia’s effect on astrocytes in hepatic encephalopathy (21). It has been previously shown that a close relationship exists between cell swelling and...
oxidative stress (22), and that ROS could induce MPT and even mitochondria membrane potential loss (19). Therefore, we investigated whether ammonia could induce ROS production and related mitochondria membrane permeability transition and mitochondria membrane potential loss in DCs. To investigate ROS production, DCF fluorescence of DCs induced by ROS was determined after incubation with NH₄Cl. The mean green fluorescence intensity value of the NH₄Cl-treated DCs was higher than that of the control DCs. In addition, the 2.5-mM group showed higher ROS production than the 75-mM group. In astrocytes, an ammonia-induced increase in intracellular Ca²⁺ activates free radical–producing enzymes that ultimately contribute to the mechanism of astrocyte swelling (23). Therefore, we also investigated whether Ca²⁺ played a role in ammonia-induced ROS overproduction in DCs. We found that, by adding BAPTA, a calcium chelator, NH₄Cl failed to induce ROS overproduction (Fig. 2C). Our results showed that although 75 µM NH₄Cl had no effect, 2.5 mM NH₄Cl induced DC MPT and mitochondria membrane potential loss after 48 h of treatment. This could be reversed by the addition of calcium chelator BAPTA or ROS scavenger SOD (Fig. 2D, 2E). These results indicate that 75 µM NH₄Cl induced a small increase of ROS but was not high enough to change mitochondria, which remained intact. The higher concentration of 2.5 mM NH₄Cl, however, could induce a larger increase of ROS, MPT, and mitochondrial membrane potential loss. The addition of a calcium chelator or ROS scavenger resulted in the protection of DCs from this fate.

**Chronic ammonia loading diminished DC phagocytosis and Ab response**

Because ammonia could inhibit DC Ag uptake in vitro, we created a hyperammonemia mouse model to study whether ammonia had the same effect in vivo. After a 1-wk treatment, the plasma ammonia level of ammonia-loaded mice was 202.3 ± 14.0 µM, and the control was 38.8 ± 8.5 µM (Fig. 3A). To determine DC phagocytosis in vivo, we injected FITC–dextran i.v. and analyzed spleen DCs for FITC fluorescence. Spleen DCs were gated by MHCII and CD11c (Fig. 3B), and their FITC fluorescent percentages were calculated (Fig. 3C). The spleen DCs from ammonia-loaded mice had significantly lower FITC positive percentage than did control (Fig. 3D). This finding suggests that ammonia reduced DC phagocytosis in vivo. In addition, we tested the expression of costimulatory molecules CD80 and CD86 on spleen DCs; however, no significant difference was observed (data not shown).

Because ammonia could suppress DC Ag uptake, the crucial step initiating adoptive immunity, it is possible that ammonia could affect the systemic immune response to Ags. Thus, we tested Ab production in response to chicken OVA vaccine. OVA-specific total IgG, IgG1, and IgG2b titers were reduced with ammonia loading after prime and second immunizations, although this was not statistically significant. However, OVA-specific IgG2a and IgG3 titers were significantly decreased after prime and second immunizations in ammonia-loaded mice (Fig. 3D). These results suggest that ammonia may restrain Ab production in response to OVA vaccine, especially Th1-associated Abs.

**Reduced phagocytosis ability of DCs exposed to ammonia and from patients with cirrhosis**

Because hyperammonemia is a feature of cirrhosis, we analyzed whether DCs from patients with cirrhosis had impaired Ag uptake ability, we collected i.v. blood from patients with cirrhosis with high levels of ammonia (Table I) and healthy human volunteers (n = 8 for both groups). After treatment of 3 ml of whole blood...
with FITC–dextran, PBMCs were analyzed by FACS. Human DCs were gated by Lineage1 and HLA-DR (Fig. 4A), and their FITC fluorescence was tested (Fig. 4B). In contrast to healthy human DCs, those from patients with cirrhosis showed a remarkably weakened dextran uptake (Fig. 4C). After treatment with 75 μM, 150 μM, 2.5 mM, or 5 mM NH₄Cl, healthy human DCs also exhibited a decreased dextran phagocytosis compared with control (Fig. 4D). This finding suggests that in cirrhosis, human DCs are limited in their phagocytosis capability, as well as with ammonia treatment alone.

**Inhibited DC function in cultures with CM and tumor tissue**

In the current study, we have demonstrated that DC count and immune function were inhibited with the level of ammonia associated with tumors. Therefore, we studied whether ammonia had the same effect in CM and tumor tissue coculture. The results indicated that CM and tumor tissue coculture diminished DC count and allogenic lymphocyte stimulation, and that evaporation and α-KG supplementation could reverse this effect (Fig. 5A, 5C). To better study phagocytosis of tumor Ags, we prepared apoptotic CT-26 cells to mimic tumor Ags for DC uptake. After CM and tumor tissue coculture, the percentage of tumor Ag phagocytosed by DCs was reduced by approximately half compared with control, which could be partially rescued by ammonia elimination (Fig. 5B). Transmission electron microscopy analysis revealed that DC mitochondria swelled when cultured with tumor cell CM (Fig. 5D). In addition, CM and tumor tissue could provoke ROS overproduction in DCs, which could also be ameliorated by ammonia elimination (Fig. 5E). Ammonia concentrations in CM and eCM were 3.12 ± 0.23 and 0.19 ± 0.06 mM, respectively. To demonstrate the effect of ammonia, we reconstituted eCM to reach the amount of ammonia in CM. The reconstituted eCM showed an inhibition effect similar to that of CM (Fig. 5A–C, 5E). These data suggest that CM or tumor tissue could provoke ROS overproduction, induce mitochondrial swelling, and suppress DC survival and function, whereas ammonia elimination could effectively reduce these effects.

**Ammonia-affected tumor growth is associated with immune response**

In the current study, we have demonstrated that ammonia could induce DCs into an immune-disabled status using tumor-associated levels in vitro. This observation motivated us to study whether ammonia could affect tumors in vivo. The results demonstrated that tumor growth was accelerated after ammonia injection and was decreased by suppressing ammonia production with α-KG (Fig. 6A). We also assessed the immune cell changes in tumors and tumor-draining lymph nodes by FACS. This assessment demonstrated that tumor-infiltrating and tumor-draining lymph node DCs were decreased in the NH₄Cl-treated group and increased in the α-KG–treated group (Fig. 6B). The phagocytosis ability of DCs was weakened in the NH₄Cl-treated group and

**Table I. Patient characteristics**

<table>
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<tr>
<th>Characteristics</th>
<th>Amount</th>
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<td>Age (years)</td>
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<td>Venous Ammonia (μM)</td>
<td>91.8 ± 26.3</td>
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</tbody>
</table>

Data are presented as mean ± standard error. M, male; PSC, primary sclerosing cholangitis.
enhanced in the α-KG–treated group (Fig. 6C). Because ammonia could significantly restrain the Th1-associated immune response according to our results above, and Th1 immune activity plays as a crucial role in the antitumor immune response, we analyzed IFN-γ production of lymphocytes in tumor-draining lymph nodes. Compared with controls, in the IFN-γ–positive lymphocyte pool, CD4+ and CD8+ lymphocyte numbers were reduced in the NH4Cl group and augmented in the α-KG group (Fig. 6D). These results further confirmed that ammonia could promote tumor growth and inhibit Th1-associated immune response. However, decreasing ammonia could decelerate tumor growth and boost Th1-related immune response.

Discussion
In cirrhosis, ammonia level is often elevated owing to liver dysfunction. In cancer, ammonia level is elevated because of glutamine addiction (1, 8). Both of these diseases are accompanied by an immunocompromised state. It is conceivable that ammonia may participate in the inhibition of immune response in patients with cirrhosis and tumors. Several observations have been made in this study concerning the effects of ammonia on DCs in cirrhosis and tumors. We found that ammonia could diminish DC count, Ag uptake, and allogenic lymphocyte stimulation. We also found the possible underlying mechanism was that ammonia causes DC swelling and mitochondrial dysfunction because of ROS overproduction. In vivo, ammonia was shown to regulate DC phagocytosis and Ab production in response to OVA vaccine. DCs from patients with cirrhosis showed a reduced phagocytosis, as did ammonia-cultured DCs. In addition, CM and tumor tissue culture experiments showed that ammonia in the tumor environment could drive DCs into dysfunction. Still, ammonia could regulate DCs, DC-associated immune response, and tumor growth. Thus, this study indicates that ammonia can negatively affect DCs and induce them to change into a disabled state, which may be an additive explanation of the immunocompromised state in cirrhosis and tumors.

In this study, both the levels of ammonia associated with cirrhosis and tumors caused DC swelling and inhibited DC phagocytosis. Several studies have clarified the mechanisms involved in phagocytosis and show that swelling could reduce phagocytic function. The phagocytic activity of human monocytes exposed to 20% v/v Oxypherol decreased with induction of cell swelling (24). Channel blocker–treated microglia (monocyte-derived cells from the brain) exhibited decreased phagocytosis with increased cell volume (25). In addition, hyponatremia and hyperammonemia have been shown to cause neutrophil cell swelling–related phagocytic dysfunction (4). Our observation was consistent with these findings, suggesting that ammonia caused the inhibition of phagocytosis by DCs, which likely resulted from ammonia-induced DC swelling. Even so, whether ammonia-caused DC
phagocytic suppression could be rescued by DC volume normalization needs further investigation.

In addition, we demonstrated DC mitochondrial damage induced by tumor-associated levels of ammonia, along with DC count and allogenic lymphocyte stimulation suppression. Several studies have reported the central role of mitochondria in DCs. As differentiation occurs, DCs consistently exhibit a much larger number of mitochondria consistently (26). Rotenone has been shown to prevent the increase of mitochondrial number and inhibit DC differentiation (27). Furthermore, mitochondrial membrane potential–disrupted DCs were unable to activate allogenic T cells (28). These findings clarified the importance of mitochondria in DCs. Because ammonia could induce mitochondrial damage, it is quite probable that the ammonia induced DC count and allogenic lymphocyte stimulation suppression was a result of this. However, further elucidation is necessary to determine whether DC function can be regained by recovering or preventing the mitochondrial damage.

To identify the molecular mechanism of ammonia-induced DC dysfunction, we analyzed ROS level in DCs. It has been shown previously that ammonia can induce oxidative stress, cell swelling, and mitochondrial damage in astrocytes and epithelial cells (21, 22, 29). In the current study, we found that ammonia could induce oxidative stress and mitochondrial damage in DCs, and that this mitochondrial damage could be ameliorated by ROS control. These data suggest that ROS are the key factors in ammonia-induced mitochondrial damage in DCs. For cell swelling caused by ammonia, ROS are thought to be involved (30, 31). Astrocyte swelling involves the generation of ROS, which promotes further astrocyte swelling, resulting in the generation of an autoamplificatory-signaling loop (22). For DCs, we also observed the overproduction of ROS and cell swelling. Because Ca2+ is a known inducer of ROS (23), we investigated whether it may be responsible for the production of ROS in DCs. Our data confirmed the importance of Ca2+ in ammonia-induced ROS overproduction. Although we found that ROS participated in ammonia-induced DC dysfunction, ROS are also known to participate in important processes in DCs, such as maturation, Ag presentation, cytokine production and secretion (32–34). Because ROS plays double sides, restoring DC function by ROS inhibition is impractical. A better understanding of ROS regulation and the participation of other molecules is needed.

Patients with cirrhosis are in an immunosuppressive state and susceptible to infections. Approximately 30% of patients with cirrhosis are admitted to the hospital and 45% of those with gastrointestinal hemorrhage suffered from bacterial infections (35). DCs could present microbial Ags to T cells to activate an adaptive immune response, and the recently identified TNF/inducible NO synthase–producing DC subset could also mediate the innate immune defense against bacterial infection (36). The dysfunctional status of DCs in cirrhosis has been described as numerical abnormality, allostimulatory inability, and IL-12 low yield (37, 38). Our study demonstrated that ammonia could reduce DC phagocytosis and Ab response in mice, and that DCs from patients with cirrhosis showed reduced phagocytic ability, which was induced by ammonia. It has been reported that, in patients

**FIGURE 6.** Regulation of ammonia on tumor growth and immune response. CT-26 cells (1 × 10⁶) were injected into the flank region of BALB/c mice. When the tumor was palpable, PBS, 20 mM NH₄Cl, or 750 mg/kg α-KG was i.t. injected daily. (A) Tumor volume was measured every 3 d. (B) After 2 wk of treatment, mice were sacrificed and tumor-infiltrating DCs and tumor-draining lymph node (TDLN) DCs were analyzed. The panels were all gated on CD45⁺ cells. (C) To detect phagocytosis of tumor-infiltrating DCs, FITC–dextran was injected directly i.t. into CT-26–bearing animals. CD11c⁺MHCI⁺ tumor-infiltrating DCs were tested for FITC signal at 3 h, 6 h, 12 h, and 24 h after FITC–dextran injection. (D) To detect DC-associated antitumor T cell response, TDLN IFN-γ–secreting CD4⁺ cells (Th1 cells) and CD8⁺ T cells (CTLs) were detected. This experiment was repeated twice with five mice per group. For FACS analysis, dot plots from one representative experiment of three are shown. *p < 0.05 (one-way ANOVA).
with cirrhosis, immune response against influenza vaccination was inhibited, especially cell-mediated immunity (39), and the Ag-specific T cell response against bacterial Ags was also attenuated (40). Our study proposed ammonia as a possible cause, considering that increased ammonia levels in mice reduced the Th1 response to OVA vaccine. Thus, our data suggest that ammonia could participate in the formation of an immunocompromised state in cirrhosis. To control infection in patients with cirrhosis, ammonia could be a key factor that is worth further investigation.

It has been reported that tumor-derived factors such as vascular endothelial growth factor (VEGF), PGE2, and IL-10 inhibit DC differentiation and lymphocyte stimulation (41). In terms of the tumor-induced suppression of the endocytic activity of DCs, Cdc42, and Rac1 (Rho GTPase)–mediated pathway, which could regulate the cytoskeleton architecture, is thought to be involved (42). These data support our research, which provided ammonia as an inhibitor of DC phagocytosis, with cell volume regulating potential. The addiction of cancer cells to glutaminolysis is well known (43). Glutaminolysis provides tumor cells with glutamate for the TCA cycle, nitrogen for the biosynthesis, NADPH for fatty acid synthesis, and cellular redox balance (44, 45). Nevertheless, few studies have examined the metabolite of glutaminolysis, ammonia, in tumors. It has been reported that ammonia supports basal phagocytosis and protects tumor cells from TNF-α–induced cell death (8). In the current study, we found that ammonia could affect tumor growth, involving DC regulation, and that DC infiltration and phagocytosis were regulated by ammonia. Because DCs are the only APCs able to activate naive T cells (46, 47), we analyzed IFN-γ-secreting T cells in tumor lymph nodes. The results suggest that ammonia could also regulate these T cells. These data help to explain the tumor-regulating ability of ammonia. Our observations authenticate the immune regulatory potential of glutaminolysis, in addition to its known contributions to energy, nitrogen, and NADPH donation. Glutaminolysis has been an efficient tumor therapeutic target in previous studies for blocking tumor cell proliferation (43, 48). Our study suggests glutaminolysis as an efficient target because of its immune regulatory quality.

In conclusion, to the best of our knowledge, this is the first time that the relationship between ammonia and DCs in cirrhosis and tumors has been studied, and that ammonia has been shown to affect T cell proliferation (43, 48). Our study suggests glutaminolysis as an inhibitor of DC phagocytosis, with cell volume regulating potential.


