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RETRACTED: Pro-inflammatory Effect of Downregulated CD73 Expression in EAE Astrocytes

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CD73, an ectonucleotidase, participates in the regulation of immune responses by controlling the conversion of extracellular AMP to adenosine. In this study, we investigated whether any type of brain cells, especially neuroglia cells, exhibit altered CD73 expression, localization or activity upon experimental autoimmune uveitis (EAU) induction and whether altered CD73 manipulates the activation of effector T cells that interact with such cell types. First, the amount of cell membrane-exposed CD73 was detected by flow cytometry in various types of brain cells collected from either naïve or EAE mice. Compared to that in astrocytes from naïve control mice, the amount of membrane-bound CD73 was significantly decreased in astrocytes from EAE mice, while no significant differences were detected in other cell types. Thereafter, wild-type and $CD73^{-/-}$ astrocytes were used to study whether CD73 influences the function of inflammatory astrocytes, such as the production of cytokines/chemokines and the activation of effector T cells that interact with astrocytes. The results indicated that the addition of exogenous AMP significantly inhibited cytokine/chemokine production by wild type astrocytes but had no effect on $CD73^{-/-}$ astrocytes and that the effect of AMP was almost completely blocked by the addition of either a CD73 inhibitor (APCP) or an adenosine receptor A1 subtype (ARA1) antagonist (DPCPX). Although the addition of AMP did not affect $CD73^{-/-}$ astrocytes, the addition of adenosine successfully inhibited their cytokine/chemokine production. The antigen-specific interaction of astrocytes with invading CD4 cells caused CD73 downregulation in astrocytes from mice that underwent EAE induction. Collectively, our findings support the conclusion that, upon EAE induction, likely due to an interaction with invading $CD4^{+}$ cells, astrocytes lose most of their membrane-localized CD73; this inhibits the generation of adenosine in the local microenvironment. As adenosine has anti-inflammatory effects on astrocytes and CNS-infiltrating effector T cells in EAE, the downregulation of CD73 in astrocytes may be considered a pro-inflammatory process for facilitating the pathogenesis of EAE.

Keywords: astrocytes, CD73, EAE, downregulation, adenosine

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Abbreviations: ADA, adenosine deaminase; ARA1, Adenosine receptors A1 subtype; CFA, Complete Freund's adjuvant; CNS, Central nervous system; EAE, Experimental autoimmune encephalomyelitis; LPS, Lipopolysaccharide; WT, Wild type.

INTRODUCTION

Adenosine triphosphate (ATP) and its metabolites have been shown to have a great impact on the regulation of the immune response. Under pathological conditions, such as hypoxia and inflammation, ATP is mainly released from cells and acts on P2X or P2Y receptors to mediate pro-inflammatory effects (Matute et al., 2007; Cicko et al., 2017; Le Duc et al., 2017). Two membrane-bound enzymes, CD39 and CD73, are capable of quickly converting ATP to adenosine. CD39 converts ATP to AMP via ADP, and CD73 converts AMP to adenosine (Eltzschig et al., 2006; Antonioli et al., 2013). Unlike ATP, adenosine is generally considered an anti-inflammatory stimulus (Liu et al., 2015; Konrad et al., 2017; Bekisz et al., 2018). Four types of adenosine receptor (AR) subtypes, named ARA1, ARA2A, ARA2B, and ARA3, have been identified. These AR subtypes are differentially distributed across the body and mediate different biological effects (Palmer and Stiles, 1997; Klinger et al., 2002; Sawynok, 2007; Ramakers et al., 2011).

In the CNS, ARA2A are the most commonly reported AR impacting on the regulation of neuroinflammation. The inhibition of ARA2A affords neuroprotection through the control of microglial reactivity (Rebola et al., 2011; Santiago et al., 2014; Madeira et al., 2016), and the non-selective AR antagonist, caffeine, has shown an identical protective effect against neuroinflammation through an antagonism of ARA2A (Brothers et al., 2010; Boia et al., 2017). In addition to ARA2A, other ARs have also been reported in the CNS (Gessi et al., 2013; Janes et al., 2014; Cervetto et al., 2017; Kashfi et al., 2017; Merighi et al., 2017). ARA1 is the second most abundant G-protein-coupled receptor in the brain and has the ability to control excitability and synaptic transmission in brain circuits as well as to regulate neuroinflammation (Fredholm et al., 2005; Liu et al., 2018).

It is interesting that although there is solid evidence supporting the pro-inflammatory effect of ARA2A in controlling neuroinflammation, the systemic administration of an ARA2A antagonist produces paradoxical outcomes in experimental autoimmune encephalomyelitis (EAE), a type of autoimmune neuroinflammation; it either exacerbates or alleviates EAE (Dai and Zhou, 2011; Ingwersen et al., 2016; Rajasundaram, 2018). This is caused by the opposing functions of A2AR signaling in brain cells and invading T cells. CNS-infiltrating T cells in EAE, mainly Treg, Th1, and Th17 cells, have high-affinity ARA2A on their surfaces, and the activation of this AR mediates robust anti-inflammatory effects (Liu et al., 2016; Montes et al., 2016; Ravani et al., 2017). This means that the utilization of adenosine by different cell types may produce opposing outcomes in a single disease and that as an autocrine or paracrine stimulus (Blackburn et al., 2009; Zhou et al., 2009), the microenvironment in which adenosine is generated has a critical role in deciding which cell type has priority regarding the use of adenosine. For this reason, cells, including B cells (Kim et al., 2017), retinal pigment epithelium (RPE) (Chen et al., 2014), and breast cancer cells (Jadidi-Niaragh et al., 2016), that control extracellular adenosine generation through membrane-bound CD73 have been widely studied for their immune regulatory effects, especially when they interact with T cells. Here, we wanted to determine if neural cells, particularly neuroglial cells, which are the first to encounter and have intensive interactions with invading T cells, can regulate neuroinflammation through surface-expressed CD73 upon the induction of EAE.

MATERIALS AND METHODS

Animals and Reagents

Female C57BL/6 (B6) and CD73$^{-/-}$ mice were purchased from Nanjing University Animal Institute (China) and housed and maintained in the animal facilities of Tianjin Medical University. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed. The mice used in the experiments were all 12- to 14-week-old females. The ARA1 antagonist DPCPX, the ARA2A antagonist SCH58261, the ARA2B antagonist MRS1754, the ARA3 antagonist MRS1220, and 5′-AMP were purchased from Tocris (R&D, United States). Primary antibodies against mouse CD73 and Na^+/K^+-ATPase were purchased from Santa Cruz (United States). PE-, FITC- or APC-labeled antibodies against mouse GFAP, CD73, CD45, CD24, and GLAST and the isotope control of the anti-CD73 antibody were purchased from eBioscience (United States).

The Induction of EAE and Histological Staining of Spinal Cords

Experimental autoimmune encephalomyelitis was induced in female C57BL/6 mice (12 weeks old) by active induction (Miller et al., 2010). Briefly, mice were injected (i.p.) with 150 ng pertussis toxin. Two hours before they were immunized, the mice were given subcutaneous injections of 200 μl of 150 μg myelin oligodendrocyte glycoprotein (MOG35-55) emulsified in complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO, United States) in six locations on the flank and one location at the tail base. The mice were monitored every other day for the development of clinical symptoms. When typical EAE symptoms appeared, usually 12–14 days after immunization, the mice were euthanized together with naïve mice, which served as controls. Spinal sections were used to investigate CD73 levels and astrocytes by histology. Following an initial perfusion with PBS, the mice were perfused transcardially with 4% paraformaldehyde, and the spinal cords were removed. The tissues were processed and embedded in paraffin wax. The sections were immunostained to visualize astrocytes and CD73. Briefly, after the sections were deparaffinized and dried overnight at 37°C, they were rehydrated in Tris-buffered saline (TBS = 0.05 M Tris–HCl at pH 7.6 and 0.15 M NaCl). Following antigen-retrieving and endogenous peroxidase-block, 3-μm thick sections were stained with FITC- or PE-conjugated monoclonal antibodies against mouse GFAP (Novus Biologicals, United States) and mouse CD73 (Biolegend, United States). Green and red fluorescence were visualized under a fluorescence microscope and photographed.

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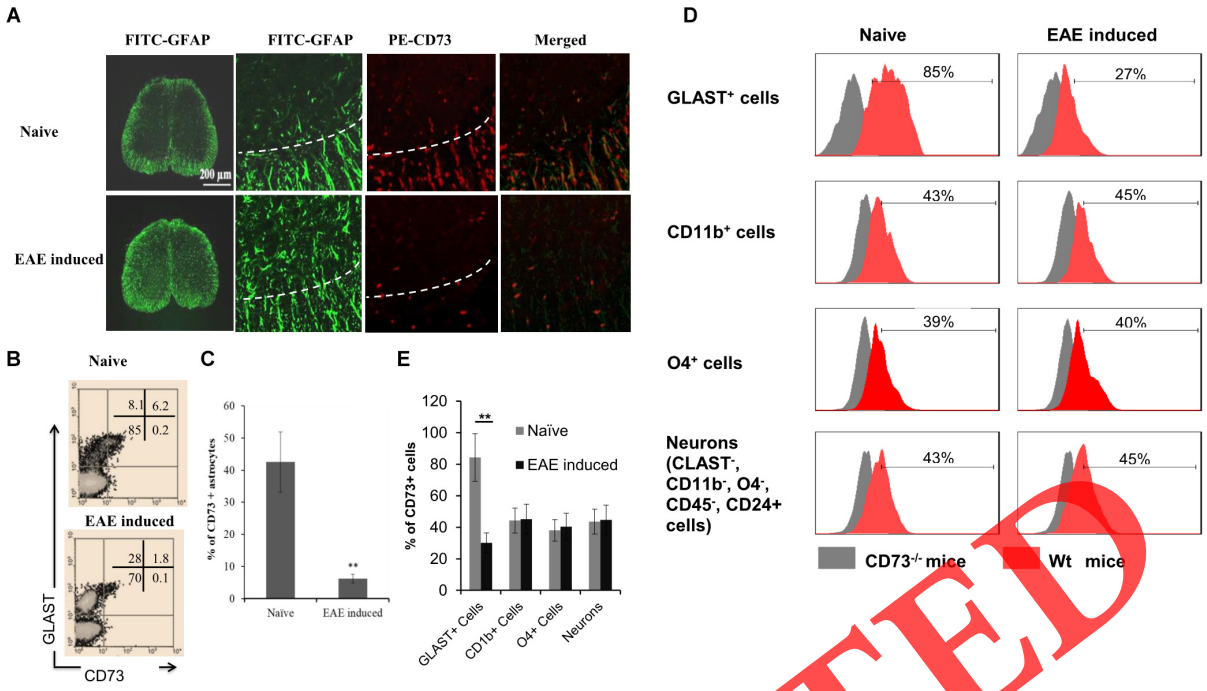

FIGURE 1 | Downregulated CD73 expression in EAE-astrocytes. Spinal cords were isolated from naïve and EAE-induced B6 mice, and the samples were fixed, embedded, and sectioned. Sections were fluorescence stained with GFAP (green fluorescence) and CD73 (red fluorescence). A single cell suspension was prepared from the brains of naïve and EAE-induced B6 mice. The percentage of $CD73^+$ cells in total $GLAST^+$ cells was analyzed by flow cytometry. Thereafter, astrocytes, microglial cells and oligodendritic cells were individually labeled with anti-mouse GLAST, O4, and CD11b antibodies, respectively, while neurons were defined as the $GLAST^-CD11b^-O4^-CD45^-CD24^+$ population. The fluorescence density of CD73 in these four types of cells was evaluated, with $CD73^{-/-}$ mice as a control. **Indicates statistically significant differences ($p < 0.01$). **(A)** Immunohistological staining of spinal cord sections from naïve and EAE mice. **(B)** Flow cytometry analysis of $CD73^+$ and $GLAST^+$ cells in a single cell suspension prepared from brains. **(C)** The percentage of $CD73^+$ cells in total $GLAST^+$ cells ($n = 6$). **(D)** Fluorescence density of an anti-CD73 PE-conjugated antibody in different types of brain cells from naïve and EAE-induced mice. **(E)** The percentage of $CD73^+$ cells in different types of neural cells from naïve and EAE-induced mice ($n = 6$).

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Flow Cytometry Analysis of CD73 in Mouse Brain Cells

After mouse dissections, brain structures were removed, the meninges were carefully stripped off and a single cell suspension was prepared (Pösel et al., 2016). The tissues were washed in phosphate buffered saline (PBS) containing 0.6% glucose, and the dissected tissue was minced and sieved through a 100-μm cell strainer. The strained cells were centrifuged at 286 × *g* for 5 min at 4°C, and the supernatant was carefully discarded. The pellet was resuspended in 1 ml of digestion buffer (Liberase with a low thermolysin concentration (up to 2 U/ml) in Hank's balanced salt solution), and the suspension was incubated under slow continuous rotation at 37°C for 1 h. The cell suspension was sieved through a 70-μm cell strainer and rinsed thoroughly with 3 ml of HBSS containing DNase. The cell suspension was centrifuged at 286 × *g* for 5 min, and the supernatant was discarded. The cell pellet was resuspended in 5 ml of 25% density gradient medium of iodixanol solution (OptiPrep, Sigma-Aldrich, United States) and centrifuged at 521 × *g* for 20 min at 18°C. The myelin layer and the supernatant were aspirated, and the pellet was washed with 10 ml of HBSS. Cells were resuspended in flow cytometry staining buffer, incubated with anti-mouse CD16/CD32 antibodies to block FcR, and then subjected to staining with different combinations of cell surface markers and CD73 and analyzed by flow cytometry. Anti-mouse GLAST, CD11b, and O4 antibodies were used to label astrocytes, oligodendrocytes and microglial cells, respectively, and neurons were screened out as the $GLAST^-CD11b^-O4^-CD45^-CD24^+$ population.

Astrocyte Purification and CD73 Activity Assay

Single-cell suspensions of mouse brains collected from naïve and EAE-mice were prepared, and astrocytes were isolated by autoMACS (Miltenyi Biotec, Germany), which separated Na $CD73^{hi}$ astrocytes from EAE $CD73^{low}$ astrocytes. Briefly, cells were first incubated with a biotin-conjugated anti-mouse GLAST (ACSA-1) antibody and were then incubated with magnetic microbeads conjugated to an anti-biotin antibody. $GLAST^+$ cells were sorted by positive selection on an autoMACS separator column to obtain naïve $CD73^{hi}$ astrocytes (Na $CD73^{hi}$ Astro.) and EAE-induced $CD73^{low}$ astrocytes (EAE $CD73^{low}$ Astro.). The purity of the sorted astrocytes was verified by staining with a PE-conjugated anti-mouse GFAP antibody and analysis by flow cytometry. Freshly isolated astrocytes were thoroughly washed and suspended in HBSS at a density of 5×10^5 cells/ml.

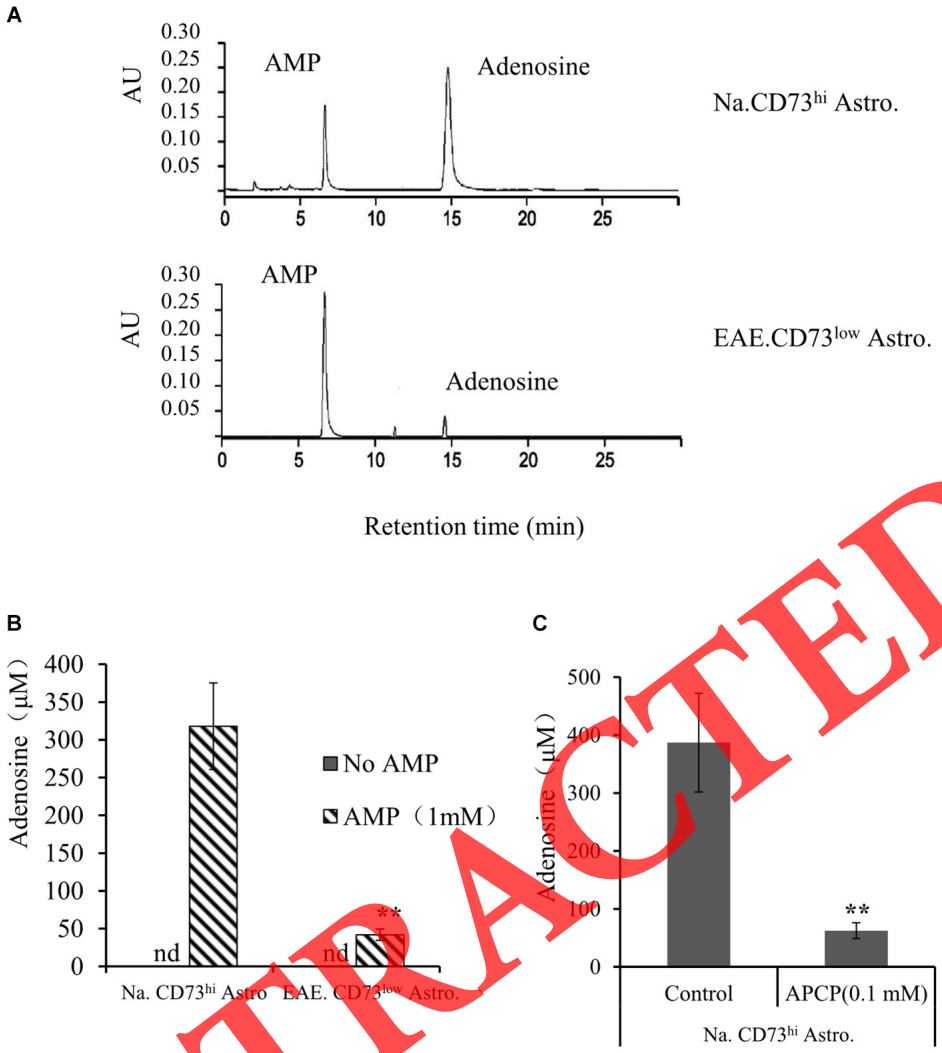

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FIGURE 2 | Decreased ability of EAE astrocytes to convert 5′-AMP to adenosine. Brain astrocytes were isolated from naïve and EAE-induced B6 mice by autoMACS. The enzymatic activity of CD73 was represented as the ability to convert 5′-AMP (1 mM) to adenosine in 1 h. Adenosine levels in the reactions were measured by an HPLC-based method. **Indicates statistically significant differences ($p < 0.01$). **(A)** HPLC detection of adenosine levels in reactions with Na.CD73^{hi} – and EAE.CD73^{low} astrocytes. **(B)** Detected adenosine levels in reactions containing naïve and EAE astrocytes, with or without the addition of 5′-AMP ($n = 6$). **(C)** Detected adenosine levels in reactions containing naïve CD73hi astrocytes, with or without the addition of a CD73 inhibitor, APCP (0.3 mM) ($n = 6$).

A 100-μl aliquot of the suspension was incubated with or without 1 mM 5′-AMP (a substrate of CD73) at 37°C for 1 h in the presence of pentostatin (2.5 μM), an adenosine deaminase (ADA) inhibitor. The reaction was terminated upon the addition of 5 μl of HCl (12 N). The concentration of adenosine in the reaction was measured by an HPLC-based assay, and the enzymatic activity of CD73 in the purified astrocytes was represented as the conversion of AMP to adenosine in 1 h. For the HPLC analysis of adenosine, a reverse-phase HPLC column (Agilent Technologies, C18, 5 μm particle size, 250 × 4.6 mm column dimensions) was used, and adenosine was eluted by a 0–50% methanol/H2O gradient mobile phase (1 ml/min). The UV absorption (wavelength of 254 nm) was recorded to obtain the HPLC peaks, which allowed identifying different purines by their different retention time during the elution. The concentration of adenosine in the samples was calculated by referring their corresponding peak areas to the peak areas of adenosine standards. To ensure that the detected adenosine was predominantly, if not entirely, the result of the catalysis of 5′-AMP and not the result of spontaneous release from dead cells, the viability of the purified astrocytes before and after the reaction was tested by trypan blue staining. Control experiments, to which 5′-AMP substrate was not added, were carried out. CD73 activity in Na.CD73^{hi} astrocytes was also evaluated with APCP, a CD73 inhibitor. Na.CD73^{hi} astrocytes were pretreated with APCP of different concentration (0, 0.02, 0.05, 0.1, 0.3 0.5, and 1.0 mM) for 30 min and then subjected to the assay.

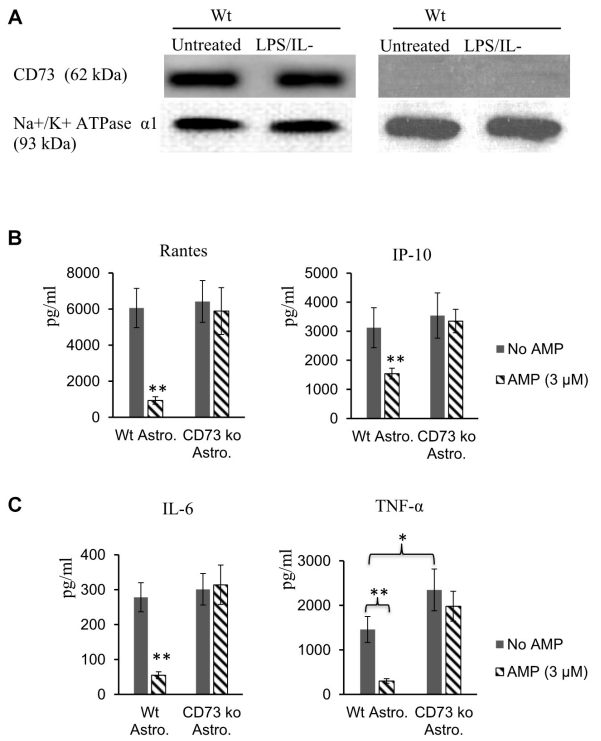

FIGURE 3 | AMP decreases cytokine/chemokine production by wt astrocytes. Astrocytes were purified from the brains of wt and CD73$^{-/-}$ mice, cultured *in vitro* and treated with LPS (100 ng/ml) plus IL-1β (10 ng/ml), with or without the addition of 5′-AMP (3 μM). Cytokine and chemokine levels in the supernatant were detected by ELISA 2 days later. CD73 levels in the cells before and after the treatment were detected by Western blotting. **Indicates statistically significant differences ($p < 0.01$). **(A)** CD73 levels in wt and CD73$^{-/-}$ astrocytes were detected by Western blotting with Na$^+$/K$^+$-ATPase as the reference. **(B)** RANTES and IP-10 levels in the medium were determined by ELISA ($n = 6$). **(C)** IL-6 and TNF-α levels in the medium were determined by ELISA ($n = 6$).

Cytokine/Chemokine Production From wt and CD73$^{-/-}$ Astrocytes

Medium was collected from cultured Wt and CD73$^{-/-}$ astrocytes after they were exposed to a combination of TNF-α (10 ng/ml) and LPS (10 ng/ml) for 48 h, with or without the addition of 5′-AMP (3 μM). The concentrations of CCL2, CXCL8, and IP-10 in the medium were detected by ELISA (kits from R&D Systems, United States).

Interaction of Cultured Astrocytes With CD4$^+$ Cells

Isolated naïve astrocytes were cultured in DMEM/F12 medium supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. When the cells reached 80% confluency, usually after 12–14 days in culture, the astrocytes were pretreated with LPS (50 ng/ml) plus IFN-γ (10 ng/ml) for 1 day and then added with effector T cells isolated from EAE mice. Two-step autoMACS isolation was applied to purify CD4$^+$CD25$^-$ effector T cells from the spleens of EAE mice. A negative selection kit for CD4 cells (Miltenyi Biotec, Germany) was used firstly, followed by a CD25$^+$ cell depletion procedure. Briefly, splenocytes were incubated for 20 min at 4°C in a cocktail of biotin-conjugated antibodies to label all non-CD4 cells and were then incubated with streptavidin-conjugated microbeads (Miltenyi Biotec, Germany) for 15 min at 4°C. The bound and unbound cells were separated using an autoMACS separator column, and the unbound cells were considered CD4$^+$ cells. The CD4$^+$ cells were further incubated with a FITC-conjugated anti-mouse CD25 antibody and anti-FITC antibody-conjugated microbeads, and then CD25$^+$ cells were removed by running a depletion protocol on an autoMACS separator.

For direct interactions, LPS/IFN-γ-pretreated astrocytes were thoroughly washed with DMEM/F12, incubated with 10 μg/ml MOG_{35-55} for 30 min, and washed again. Approximately 5×10^5 CD4$^+$ cells were added to each well of astrocytes in a 24-well cell culture plate. Two days later, cells attached to the bottom of the plate were harvested, and the amount of membrane-bound CD73 in GLAST$^+$ cells was analyzed by flow cytometry. For separated coculture, astrocytes were seeded in a cell culture insert, cultured in 24-well cell culture plates and treated with LPS/IFN-γ for 1 day. Isolated CD4$^+$CD25$^-$ cells were preactivated with a plate-bound anti-CD3 antibody (1 μg/well) and a free anti-CD28 antibody (1 μg/well) for 1 day. Astrocyte-seeded inserts were then transferred to the wells with preactivated CD4$^+$CD25$^-$ cells. Cells in the inserts were harvested 2 days later, and CD73 levels in GLAST$^+$ cells were analyzed by flow cytometry.

Statistical Analysis

A two-tailed *t*-test was used to statistically analyze the differences between two groups. One-way ANOVA and Tukey's *post hoc* analysis were used for multiple groups. A *P*-value < 0.05 was considered significant.

RESULTS

Downregulated CD73 Expression in Astrocytes From EAE-Induced Mice

Immunofluorescence staining of the spinal cord sections in **Figure 1A** indicate that, compared with those collected from naïve controls, the spinal cords collected from EAE-induced mice exhibited increased staining of GFAP (green fluorescence) but a decreased amount of CD73 (red fluorescence). Meanwhile, most of the red fluorescence was colocalized with green fluorescence when merged. The flow cytometry results in **Figures 1B,C** show that the amount of CD73 in astrocytes was significantly decreased after EAE induction. With CD73$^{-/-}$ cells as a control, the amount of CD73 in different types of neural cells was evaluated before and after EAE induction. As shown in **Figures 1D,E**, the relative amount of membrane CD73 was detected in astrocytes, oligodendrocytes, microglial cells and neurons by flow cytometry. Among these cell cultures, astrocytes had the highest CD73 intensity and were the only cell type that showed significant CD73 downregulation after EAU induction compared with astrocytes from corresponding naïve controls.

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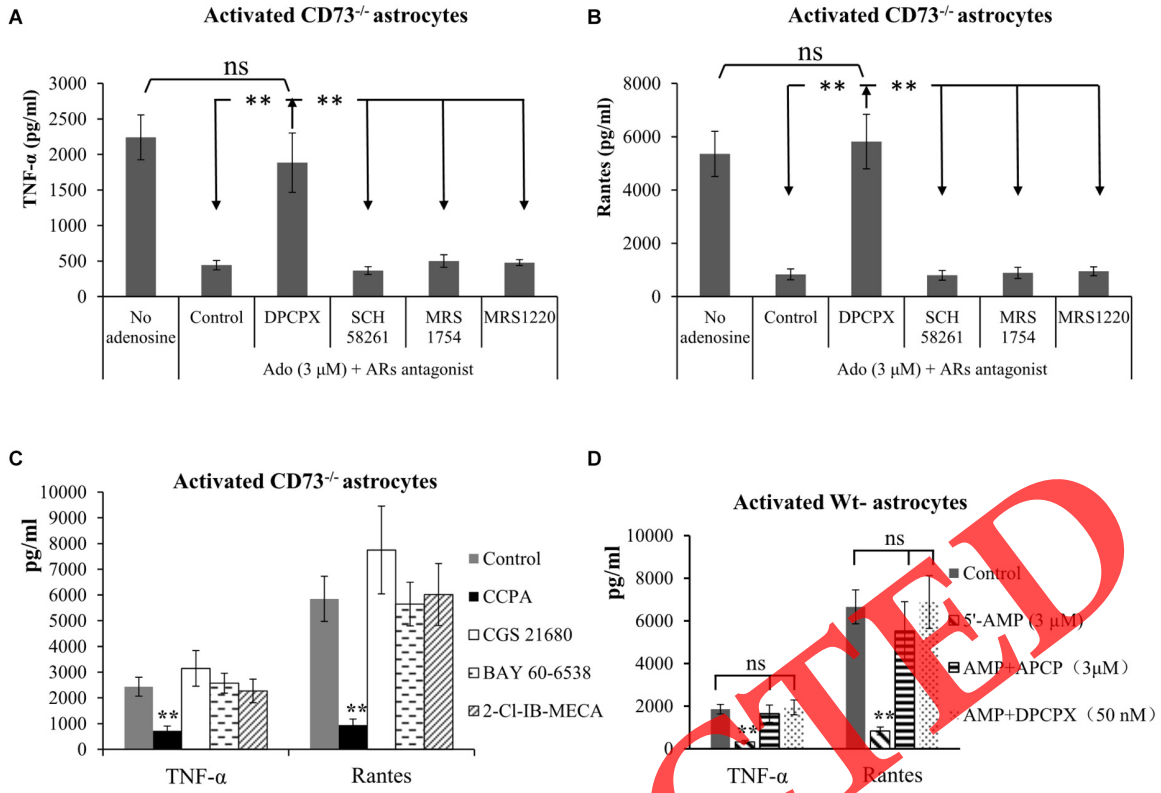

FIGURE 4 | The effects of AMP, adenosine, selective ARs agonists or antagonists on wt- and CD73$^{-/-}$ astrocytes. Wt- and CD73$^{-/-}$ astrocytes were activated with LPS (100 ng/ml) plus IL-1β (10 ng/ml), with or without the addition of 5′-AMP (3 μM), adenosine (300 nM), selective ARA1 agonist/antagonist: CCPA (50 nM)/DPCPX (50 nM), selective ARA2A agonist/antagonist: CGS 21680 (259 nM)/SCH 58261 (100 nM), selective ARA2B agonist/antagonist: BAY 60-6538 (100 nM)/MRS 1754 (100 nM) and selective ARA3 agonist/antagonist: 2-Cl-IB-MECA (100 nM)/MRS1220 (5 μM). Culture medium was collected 2 days later, and RANTES and TNF-α levels in the supernatant were detected by ELISA. TNF-α **(A)** and Rantes **(B)** in the medium of activated CD73$^{-/-}$ astrocytes treated with adenosine(Ado) and ARs antagonists or ARs agonists **(C)**. **(D)** TNF-α and Rantes in the medium of activated Wt-astrocytes treated with AMP, CD73 inhibitor and ARA1 antagonist. **Indicates statistically significant differences ($p < 0.01$). ns: no significance.

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EAE-CD73^{low} Astrocytes Have a Decreased Ability to Convert AMP to Adenosine

AutoMACs-isolated astrocytes were verified to have a purity of 92.4–93.1%, as determined by fluorescence-labeled anti-GFAP antibody staining and flow cytometry analysis. The enzymatic activity of CD73 in these purified astrocytes was represented by the ability of the astrocytes to convert 5′-AMP to adenosine, and adenosine levels were detected by HPLC-based analysis. The results shown in **Figure 2A** indicate that EAE-CD73^{low} astrocytes had a significantly decreased ability to generate adenosine from the supplied 5′-AMP. **Figures 2B,C** confirm that the detected adenosine almost completely resulted from the conversion of 5′-AMP and not from spontaneous release by astrocytes. When 5′-AMP was not added, no adenosine was detected in the samples, and Na-CD73^{hi} astrocyte-induced adenosine production was significantly inhibited by the addition of APCP. APCP at 0.1 mM inhibited by nearly 50% CD73 activity, assessed as adenosine production from 1 mM 5′-AMP substrate in 1 h; and CD73 activity in Na-CD73hi astrocytes was almost completely inhibited when APCP was used at concentration of 0.3 mM or higher. Meanwhile, there was no significant difference in the cell viability of Na-CD73^{hi} astrocytes before and after the assay (85.5 ± 8.1% vs. 82.7 ± 10.4%) was detected.

CD73 Regulates Cytokine/Chemokine Production From Astrocytes by Converting AMP to Adenosine Acting on ARA1

To detect whether downregulated CD73 produces any changes in astrocytes in EAE, we compared the cytokine and chemokine production from Wt and CD73$^{-/-}$ astrocytes, with or without the addition of AMP (3 μM). LPS/IL-1β treatment did not affect the amount of CD73 in wt astrocytes, which is confirmed in **Figure 3A**. The addition of AMP significantly inhibited RANTES, IP-10, IL-6, and TNF-α production from Wt astrocytes but had no effect on CD73$^{-/-}$ astrocytes (shown in **Figures 3B,C**). The inhibitory effect of the addition of 5′-AMP to Wt astrocytes was nearly completely blocked by both APCP (3 μM), a CD73 inhibitor and DPCPX (50 nM), a selective ARA1 antagonist (**Figure 4D**). If the effect of 5′AMP on inhibiting cytokines/chemokine production from wt-astrocytes

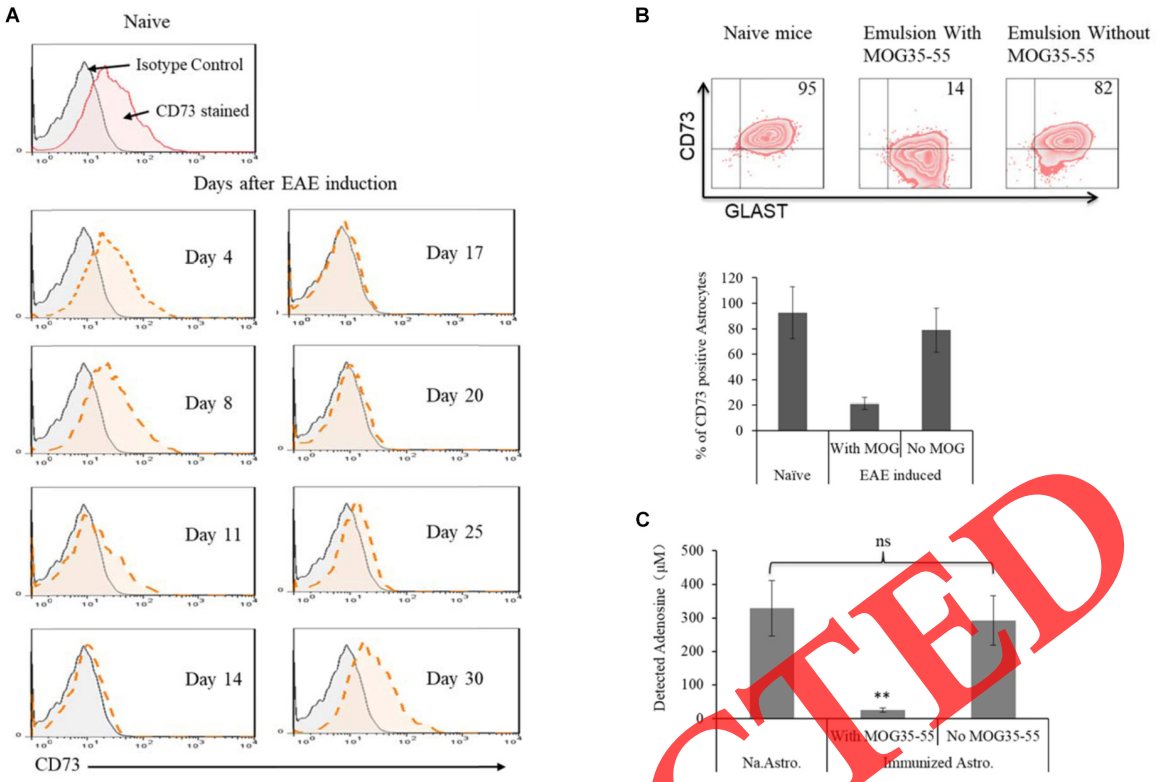

FIGURE 5 | Gradual downregulation of CD73 in astrocytes following EAE induction. EAE-induced B6 mice were euthanized on different days after immunization. A single cell suspension of brain tissues was prepared, and CD73 levels in GLAST-positive cells were evaluated by flow cytometry. B6 mice were immunized with or without MOG_{35-55} in the emulsion, and CD73 levels in astrocytes were evaluated by flow cytometry. **(A)** CD73 density in $GLAST^+$ cells. **(B)** A representative FACS result (upper panel) and the mean percentage of CD73-positive cells (lower panel, $n = 6$). **(C)** CD73 enzymatic activity, as represented by adenosine generation ($n = 6$). **Statistically significant difference ($p < 0.01$). ns: no significance.

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was mediated by CD73 generated adenosine, adenosine or some selective ARs agonist/antagonist should be effective in $CD73^{-/-}$ astrocytes. As expected, in $CD73^{-/-}$ astrocytes the addition of adenosine (300 nM) effectively mimicked the inhibitory effect of AMP observed in wt-astrocytes, which could also be blocked by DPCPX but not by selective ARs antagonists for ARA2A, ARA2B, and ARA3 (**Figures 4A,B**). Meanwhile, selective ARA1 agonist, CCPA (50 nM), showed the same effect as adenosine in $CD73^{-/-}$ astrocytes; and other selective ARs agonists did not show significant effect (**Figure 4C**).

MOG_{35-55} Is Necessary for EAE-Induced CD73 Downregulation in Astrocytes

To study the mechanism of EAE-induced CD73 downregulation, we investigated the relative density of CD73 in astrocytes on multiple days after EAE induction. **Figure 5A** shows that a decreased CD73 density in astrocytes was first observed approximately 11 days after EAE induction, and the most significant CD73 loss occurred 14 to 17 days after EAE induction. CD73 levels began to be restored on the 20th day and increased to almost the same level as that in naïve astrocytes by around 30 days after EAE induction. **Figure 5B** reveals that, when EAE was induced virtually in the absence of MOG_{35-55}, the downregulation of CD73 in astrocytes was completely blocked, while the astrocytes isolated from these sham mice exhibited the same level of CD73 activity as naïve astrocytes (**Figure 5C**).

CD73 Downregulation *in vitro* Is Induced by Interactions With $CD4^+$ Cells

To test whether CD73 downregulation in astrocytes is induced by their interactions with $CD4^+$ cells *in vitro*, coculture experiments were performed. **Figures 6A,B** indicate that the antigen-specific direct interaction between preactivated astrocytes and EAE-primed $CD4^+$ cells is capable of inducing CD73 downregulation in astrocytes. When these cells were cocultured in a two-chamber system, no CD73 downregulation was induced in astrocytes. However, the separately cultured CD4+ cells were more activated than the astrocytes that interacted with $CD4^+$ cells and produced a larger amount of inflammatory cytokines (**Figures 6C,D**).

DISCUSSION

CD73 is widely expressed in the CNS and performs different functions. As early as the 1990s, Schoen et al. reported that CD73, as a synaptic enzyme, attenuates the glutamatergic

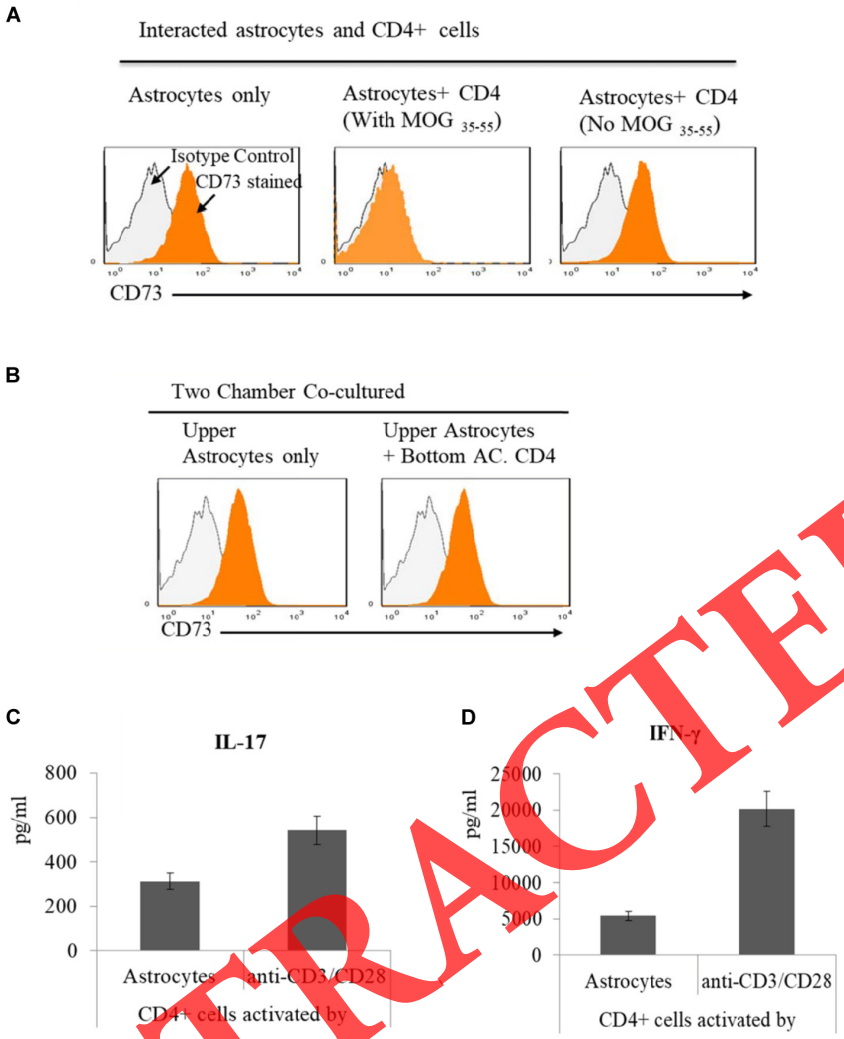

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FIGURE 6 | Antigen-mediated astrocyte-CD4 cell interactions account for the downregulation of CD73 in astrocytes. To study direct interactions, astrocytes were seeded in 24-well cell culture plates and pretreated with LPS (50 ng/ml) plus IFN-γ (10 ng/ml) for 1 day, pulsed with MOG_{35-55}, and added to *in vivo* primed $CD4^+$ cells. After 2 days, the CD73 density in astrocytes was analyzed by flow cytometry **(A)**. For separated cultures, astrocytes were seeded in Transwell inserts, with or without the seeding and activation of $CD4^+$ cells by anti-CD3 and anti-CD28 antibodies in the bottoms of the wells. CD73 expression in the astrocytes was analyzed by flow cytometry 2 days later **(B)**. IL-17 and IFN-γ levels in the medium of the activated $CD4^+$ cells were detected by ELISA (n = 4; **C,D**).

transmission of mossy fibers by producing adenosine (Schoen and Kreutzberg, 1994; Schoen et al., 1999). Thereafter, CD73 was reported to be located in the human hippocampus (Barros-Barbosa et al., 2016), microglial cells (Marina et al., 2017), and astrocytes (Chu et al., 2014) and to have different functions in different locations. We show in **Figure 1D** that CD73 can be widely detected in different brain cells. Astrocytes bear a greater CD73 density than other cells, and the amount of CD73 is significantly downregulated in astrocytes, but not in other neural cells, upon EAE induction. These results imply that the change in CD73 levels in astrocytes might be involved in the pathogenesis of EAE. **Figure 2** further demonstrates that downregulated CD73 expression in EAE astrocytes is associated with a decreased extracellular conversion of 5′-AMP to adenosine.

Subsequently, we aimed to determine whether CD73 downregulation results in any functional alterations in astrocytes regarding the regulation of EAE and especially the regulation of interactions with pathogenic effector T cells. It has been verified in many cases that CD73-bearing cells suppress T cell activation by controlling extracellular adenosine generation (Whiteside et al., 2011; Saze et al., 2013; Smyth et al., 2013). First, the production of pro-inflammatory cytokines and chemokines by Wt and $CD73^{-/-}$ astrocytes, in the presence or absence of 3 μM of 5′-AMP as a substrate of CD73, were compared. LPS and IL-1 were used to enhance cytokine and chemokine production from astrocytes (Tarassishin et al., 2014). The addition of 5′-AMP inhibited cytokine/chemokine production by Wt-astrocytes but had no effect on $CD73^{-/-}$ astrocytes. Additionally, we found

the following evidence: (i) the addition of a CD73 inhibitor or a selective ARA1 antagonist blocked the effect of 5′-AMP on wt-astrocytes; (ii) the addition of 5′-AMP did not show any effect on $CD73^{-/-}$ astrocytes; (iii) exogenous adenosine had an inhibitory effect on $CD73^{-/-}$ astrocytes, which could be blocked by the addition of ARA1 antagonist. Thus, we propose that CD73 has an anti-inflammatory effect on astrocytes by catalyzing 5′-AMP to adenosine and acting on ARA1.

For the mechanism study of EAE-induced CD73 downregulation, the outcomes of two different immunization strategies were compared. The purpose of these experiments was to determine whether the existence of antigen-specific T cells is critical for EAE-induced CD73 downregulation in astrocytes or whether CD73 downregulation can be simply evoked by pathogen-associated molecular patterns (PAMPs), such as TLR ligands or pertussis toxin in an emulsion. As expected, CD73 downregulation occurred only in MOG_{35-55}-treated emulsion-immunized mice. This implies that the interaction with invading T cells is necessary for EAE-induced CD73 downregulation in astrocytes; as astrocytes have been reported to be the local antigen-presenting cells (APCs) in the CNS, they present antigens to interact with invading T cells and mutually activate each other (Constantinescu et al., 2005; Chastain et al., 2011; Guerrero-García, 2017). To test this hypothesis, we evaluated the amount of CD73 in astrocytes on multiple days after EAE induction to determine whether CD73 downregulation occurs before or after T cell infiltration in the CNS. **Figure 5A** shows that CD73 downregulation in astrocytes initially occurs 11 days after EAE induction, when CD4 cells are already present in the CNS. CD4 cells can be found in the CNS as early as 8 days after EAE induction (Felix et al., 2004). The results in **Figure 6** further confirm that the antigen-specific interaction between astrocytes and $CD4^{+}$ cells is capable of inducing CD73 downregulation in cultured astrocytes.

In conclusion, this study indicates that the levels of CD73 are significantly decreased in astrocytes following EAE induction. Decreased CD73 levels hinder local extracellular adenosine generation from the conversion of 5′-AMP. Since adenosine has an anti-inflammatory effect on astrocytes by acting on their ARA1 adenosine receptor subtype, downregulated CD73 in astrocytes can be considered a mechanism that facilitates EAE. Further *in vivo* studies will be required to test the relative importance of this mechanism of astrocyte-mediated control of the reactivity of infiltrating T cells in comparison with the role of the enriched synaptic CD73 (Cunha et al., 2000) controlling the activity of glutamatergic synapses (Cunha et al., 1996; Rebola et al., 2008), that has also been proposed to contribute for MS (Peterson et al., 2001; Wegner et al., 2006; Dutta et al., 2011).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Animal research committee of Tianjin Medical University. Institutional approval of using mice for EAE experiments was obtained, and institutional guidelines regarding animal experimentation were followed.

AUTHOR CONTRIBUTIONS

SZ carried out or participated in most of the experiments. GL and JG carried out astrocytes isolation and culture. FK finished flow cytometry analysis and data analysis. SC organized figures and made draft. ZW designed the study and helped to draft the manuscript. FK conceived of the study, and participated in its design. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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