CD73 Plays a Protective Role in Collagen-Induced Arthritis

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Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease affecting 0.5–1% of the world’s population. It is characterized by synovial infiltration of inflammatory cells secreting proinflammatory cytokines. Th1 cytokines play a predominant role in RA, in addition to IL-1, IL-6, and IL-17A. RA leads to progressive joint and bone destruction as well as synovial hyperplasia and chronic synovitis. Collagen-induced arthritis (CIA) is a commonly used mouse model of RA (1).

Extracellular adenosine is a potent immunosuppressor, and activation of adenosine receptors has been shown to protect against arthritic inflammation (2). Extracellular adenosine comes from two sources: transport of intracellular adenosine and hydrolysis of extracellular adenine nucleotides, including from the enzymatic activity of cytosolic 5'-nucleotidases, and from the hydrolysis of extracellular adenine nucleotides by ectonucleotidases including CD73. CD73 (ecto-5'-nucleotidase) is a GPI-anchored enzyme that dephosphorylates extracellular AMP to adenosine. CD73 is part of a complex system of enzymes, receptors, and transporters involved in the metabolism and function of purine nucleotides. Adenosine, by signaling through four distinct adenosine receptors (i.e., A1, A2A, A2B, and A3), exerts potent immune modulatory and tissue remodeling effects (3). Adenosine thus regulates many biological and pathological processes, including inflammation and cancer (3).

The role of CD73 in autoimmunity remains unclear. For instance, although CD73-deficient (CD73 knockout [KO]) mice spontaneously develop autoimmune nephritis and glomerulonephritis as they age (4), CD73 KO mice are also more resistant to experimental autoimmune encephalomyelitis (5). The precise role of CD73 in RA and its role on distinct CD73 expressing cell populations are not completely clear. A previous study reported that CD73 activity is increased in synovial fluids and positively correlated with inflammation in RA patients (6). Consistent with this, a recent study in juvenile RA patients reported a correlation between low CD73 expression and disease severity (7). In contrast, CD73 levels on joint-infiltrating immune cells have been shown to be decreased in some patients with juvenile RA (8).

In this study, we assessed the susceptibility of CD73 KO mice to CIA. We demonstrated that CD73 deficiency resulted in greater disease incidence, greater joint damage, upregulation of proinflammatory cytokines in the joints, and increased IFN-γ-producing T cells in lymph nodes. Using bone marrow chimera mice, our data also demonstrated that CD73 expression on nonhematopoietic cells plays a protective role in RA and suggests that strategies able to enhance CD73 activity or expression levels may be a valid therapeutic option. The Journal of Immunology, 2015, 194: 2487–2492.
Induction of CIA

Immunization-grade chicken type II collagen and CFA were purchased from Chondrex (Redmond, WA). Emulsion (final concentration of 2 mg/ml collagen) was prepared using a tissue homogenizer. Ten- to 12-wk-old male mice were injected intradermally into the tail (1.5 cm from the base) with a single injection of 50 μl emulsion. Following immunization, mice were monitored three times per week for development of joint swelling and/or redness. Disease was quantitated using a clinical score on a scale from 0 to 16 computed from combined assessment of all four paws (0, normal; 0.5, edema and erythema of one digit; and 1, edema and erythema of two or more digits, metatarsus, tarsus, or ankle).

Radiologic Evaluation of Arthritic Joints

Eight weeks after immunization, metatarsal, tarsal, and calcaneal imaging was performed using X-rays and reconstructed micro computerized tomography (CT) scans (Skyscan 1076; Skyscan, Antwerp, Belgium). Evaluation was performed blindly according to previously established criteria and was quantitated using the Larsen score (9).

Real-time PCR

Eight weeks after challenge, front paws (each paw was treated as a separate sample) were placed in Allprotect Tissue Reagent (Qiagen) and were stored at −80°C. RNA was isolated using RNeasy lipid tissue Mini- kit (Qiagen). cDNA synthesis was done using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Real-time PCR was performed using TaqMan Fast Advanced Master Mix and TaqMan probes for target genes using StepOne Plus Real-Time PCR System (Life Technologies, Burlington, ON, Canada). Probe for Gadd45 was used as endogenous control. Relative quantitation was calculated using the StepOne Plus Real-Time PCR software analysis relative quantitation function, using a randomly selected triplicate from the WT biologic group as a reference.

Treatment with CGS 21680

The A2A adenosine receptor agonist CGS 21680 was obtained from Tocris Bioscience. Solution for injection was prepared in DPBS 10% DMSO and 0.1 μg/g mouse weight was administered daily i.p. in 200 μl, starting on day 23 following injection of chicken collagen type II. Treatment was performed until the end of the experiment. Vehicle solution alone was administered as control.

Anticollagen II Ab ELISA

Maxisorp plates (Thermo Scientific) were coated overnight with collagen II (2 mg/ml in PBS) at 4°C. Blocking was done with 3% BSA in PBS-Tween (0.05%) for 1 h at room temperature. Plates were washed, and serum samples at different dilutions (prepared in 0.1% BSA PBS-Tween were plated overnight). Plates were washed, HRP-conjugated anti-mouse Ig Abs (1:5000 dilution; Southern Biotechnology Associates, Birmingham, AL) were added, and plates were incubated for 2 h at room temperature. After three washes, tetramethylbenzidine substrate (50 μl/well) was added. Reaction was stopped with 2 N hydrochloric acid (50 μl/well). Absorbance was read at 450 and 540 nm on Versamax microplate reader.

Intracellular staining

Inguinal lymph nodes (LNs) were harvested, single-cell suspension was prepared, and cells were restimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) and monensin in complete RPMI 1640 medium for 6 h. Following culture, cells were stained with anti-CD4 mAb and cell fixable viability dye EFluor506 and were fixed and permeabilized using BD Cytofix/ Cytoperm plus kit. Rat anti-mouse CD16/CD32 mAb (2.4G2), anti-mouse CD4 FITC (RM4-4), anti-mouse IFN-γ PE (XMG1.2), anti-mouse IL-17A PE (TC11-18H10), and isotype control rat IgG1PE mAbs were obtained from BD Biosciences. Acquisition was done using the LSR Fortessa II, and analysis was done using FlowJo software.

Bone marrow transplantation chimeras

Eight- to 10-wk-old CD73 KO (CD45.2+) or WT B6 (CD45.2+) or B6.CD45.1 (CD45.1+) hosts were lethally irradiated (1100 rad whole body, first day 2 × 350 rad in 4-h interval, next day 400 rad). Hosts were reconstituted within 24 h of last irradiation by i.v. injection of 10⁶ bone marrow donor cells coming from femurs and tibias of either CD73 KO (CD45.2+) or B6.CD45.1 (CD45.1+) mice. Donor and host cells expressed different allelic forms of CD45. Donor cell engraftment was confirmed by flow cytometry 7 wk after transplantation and was followed by collagen type II intradermal injection, as described previously. Clinical monitoring for the development of arthritis was performed until day 50.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software, using Student t test, χ², ANOVA, or Mann–Whitney U test, as indicated.

![FIGURE 1](http://www.jimmunol.org/)

CD73-deficient mice are more susceptible to CIA. (A) CD73 KO and control WT mice were immunized intradermally with chicken type II collagen and followed for signs of arthritis. Incidence of arthritis in one of four independent experiments is shown (p = 0.012 by log-rank test). (C) Cumulative incidence of arthritis on day 48 of four independent experiments (p = 0.015 by χ² test). (B) Larson score quantification of radiological signs of arthritis using X-ray imaging of hind legs (n = 20–22/group) 8 wk postimmunization (symbols represent individual paws; p = 0.017 by Student t test). (D) Representative reconstructed CT images of CIA-affected joints and reconstructed three-dimensional images of CIA-affected joints showing irregular joint spaces and cortical bone defects (thick arrows) or bone apposition (thin arrows).
Results

CD73-deficient mice are more susceptible to CIA

CIA is a widely used mouse model of RA that involves both T cell– and B cell–specific responses. To assess whether CD73 plays a protective role in RA, we evaluated CIA progression in CD73-deficient (CD73 KO) and WT C57BL/6 mice. As shown in Fig. 1A and 1C, we observed a statistically significant increase of cumulative incidence of arthritis in CD73 KO mice. We next investigated joint damage by X-ray imaging of hind legs of WT and CD73 KO mice immunized with chicken collagen. As shown in Fig. 1B and 1D, CD73 KO mice had significantly more severe joint damage compared with WT mice 8 wk post immunization. Representative reconstructed CT and 3D images showing irregular joint spaces, defects of cortical bones, and bone apposition are shown in Fig. 1D. Our data thus demonstrated that CD73 expression plays a protective role in the incidence and severity of CIA in mice.

Increased expression of proinflammatory cytokines in joints of CD73-deficient mice

We next assessed the levels of proinflammatory cytokines in the joints of CIA-challenged mice. Quantitative PCR analysis of front paws 8 wk after challenge revealed that CD73 deficiency was associated with a significant increase in IL-1β, IFN-γ, TNF-α, and IL-6 levels in the joints (Fig. 2A). In contrast, no differences were observed in TGF-β, IL-17A, and IL-10 levels. These results suggested increased Th1 response in CD73 KO mice. To further assess whether CD73 deficiency increased Th1 response, draining LN cells were isolated 8 wk after collagen immunization, activated with PMA and ionomycin, and analyzed by flow cytometry for intracellular IFN-γ expression. As shown in Fig. 2B, IFN-γ–producing CD4+ T cells were significantly increased in draining LN of CD73 KO mice.

CD73 on nonhematopoietic cells plays an important role in the control of CIA

CD73 is expressed on both hematopoietic and nonhematopoietic cells (10). Notably, CD73 is expressed at high levels on human joint synovial cells (Supplemental Fig. 1). We thus investigated whether the protective effect of CD73 in the mouse model of CIA was dependent on CD73 expression on hematopoietic or nonhematopoietic cells. For this purpose, we performed bone marrow transplantation between CD73 KO and WT mice. Following hematopoietic reconstitution (Fig. 3A), mice were immunized with chicken type II collagen and followed for clinical signs of arthritis. As shown in Fig. 3B–D, CD73 deficiency in nonhematopoietic cells was associated with increased susceptibility to CIA.

FIGURE 2. Increased expression of proinflammatory cytokines and Th1 responses in CD73-deficient arthritic mice. (A) CD73 KO and control WT mice were immunized intradermally with chicken type II collagen, and 8 wk later, RNA was isolated from joints of front paws and expression levels of inflammatory cytokines assessed by real-time PCR (means ± SEs are shown. *p < 0.05, ***p < 0.001 by Student t test). (B) Draining lymph node LN cells were isolated 8 wk after immunization, activated for 5 h in vitro with PMA and ionomycin, stained for CD4 and intracellular IFN-γ, and analyzed by flow cytometry, gating on all viable cells. Absolute numbers of IFN-γ–producing CD4+ T cells were statistically higher in CD73 KO mice compared with WT mice (means ± SEs are shown. *p < 0.05 by Student t test). Analysis was done on samples from experiment shown in Fig. 1A.

FIGURE 3. CD73 deficiency in nonhematopoietic cells is associated with increased susceptibility to CIA. (A) Bone marrow chimera mice were generated by i.v. injection of 10⁶ bone marrow cells into lethally irradiated hosts as described in Materials and Methods. Seven weeks after transplantation, peripheral blood cells from all chimeric mice [shown in (C)] were analyzed for CD45.1 and CD45.2 expression, and the percentage of donor cells was determined (means ± SEs are shown). Mice were then immunized intradermally with chicken type II collagen and followed for clinical signs of arthritis. (B) Cumulative incidence of CIA observed on day 45 in two independent experiments [shown in (C) and (D); statistical analysis was done using the χ² test]. (C and D) Incidence of arthritis in the two independent bone marrow chimera experiments.
hematopoietic cells was not associated with increased susceptibility to CIA. Fig. 3B shows cumulative incidence of two independent experiments shown ($p = 0.024$ by $\chi^2$ between WT to WT and WT to KO). Our bone marrow chimera studies thus demonstrated that CD73 expression by nonhematopoietic cells is required for protection against CIA in mice.

**Discussion**

RA is a chronic autoimmune disease with significant morbidity and mortality, despite treatment. In this study, we demonstrated that CD73, which participates in the formation of extracellular adenosine, plays a protective role in the development of CIA, a commonly used mouse model of RA. CD73 KO mice developed greater...
incidence of arthritis, greater disease severity and mounted an enhanced Th1 immune response associated with an increased production of IL-1β, IFN-γ, TNF-α, and IL-6 levels in the joints.

Modulation of adenosine signaling is a promising new approach for treatment of RA. Small molecule agonists of adenosine receptors have already been shown to significantly suppress arthritic inflammation in preclinical and clinical studies (6). An alternative approach could be to develop small molecules able to enhance CD73 activity or expression. Another approach is to exploit the upregulated expression of CD73 in arthritic joints with a CD73-dependent prodrug, as was demonstrated recently (11). Modulators of CD73 activity, prodrugs that rely on CD73, or recombinant CD73 enzyme thus constitute potential alternatives to adenosine receptor agonists for treatment of RA. Of particular interest, previous work demonstrated a critical role for CD73 in the anti-inflammatory effects of MTX, one of the most commonly used antiarthritis drugs (12). Although this study was performed using a carrageenan air pouch model of inflammation, the antiarthritic effects of MTX might also depend on CD73 activity, although this has not been formally shown.

To our knowledge, our study provides the first evidence that CD73 expression is a homeostatic mechanism that downmodulates joint inflammation during CIA. Our observation that A2A adenosine receptor activation rescues the phenotype of CD73 KO mice suggests that enzymatic activity of CD73 plays a protective role against CIA. Our data thus support another independent study demonstrating a beneficial effect of CGS 21680 on CIA severity in WT mice (13). Our data also confirm the protective role of the A2A adenosine receptor in CIA in addition to the established protective role of the adenosine A3 receptor in this disease (14) and support the role of these receptors in rheumatoid arthritis (15). It would be most interesting to assess whether deregulated CD73 expression and/or CD73 gene polymorphisms are associated with increased susceptibility to RA. In juvenile RA, for instance, where MTX displays considerable heterogeneity response rates, reduction of CD73 activity on mononuclear cells has been reported (8). Although correlation between CD73 polymorphism and autoimmunity has not been reported, a recent study by S. Robson and colleagues (16) revealed that polymorphisms in CD39 (ectonucleoside triphosphate diphosphohydrolase 1), a cell membrane–expressed enzyme acting upstream of CD73 by converting ATP or ADP to AMP, is associated with increased susceptibility to inflammatory bowel disease in humans. Future work should investigate potential correlation between CD39/CD73 polymorphisms and susceptibility to RA.

The increased susceptibility of CD73 KO mice to CIA occurred despite a delayed anticollegen IgG response. Consistent with recent findings, our results suggest that CD73 KO mice exhibit impaired isotype switching (17). Notably, patients with common variable immunodeficiency with impaired class-switched Ab responses are selectively deficient in CD73 expression in B cells (17). The molecular basis of this defect has not been elucidated, but it may involve a decrease of activation induced deaminase activity from alterations of cytokine secretion known to regulate class-switch recombination (17). Our data thus demonstrated that the increased susceptibility of CD73 KO mice to CIA is not the consequence of an enhanced humoral response.

Instead, CD73 KO mice developed an enhanced Th1 immune response following collagen immunization, demonstrated by higher levels of IFN-γ-producing CD4+ T cells in draining LN and higher levels of proinflammatory cytokines in the joints. These observations are in line with what we have previously reported in tumor-bearing CD73-deficient mice (18). The CD73-adenosine axis thus appears to negatively regulate Th1 immune responses in general.

Although the altered B cell responses detected during the course of CIA in CD73 KO mice demonstrate altered function of immune cells involved in CIA pathogenesis, our bone marrow chimera experiments clearly demonstrated a protective role for CD73 expression on nonhematopoietic cells. Although we have not identified the identity of the cells responsible, two possibilities exist. First, CD73+ synovial cells in the joints could locally regulate arthritis. Accordingly, CD73 is highly expressed on synovial stromal cells (10). A second possibility is that CD73 expression on endothelial cells regulates joint inflammation. Joint inflammation could be enhanced as a consequence of increased trafficking of leukocytes across CD73-deficient endothelium, as was reported previously (19).

In conclusion, our study points toward an important role for CD73 in regulating RA and supports the rationale of enhancing CD73 expression or function for treatment of RA. This could include small molecule modulators of CD73 enzymatic function or therapeutic administration of recombinant CD73 enzyme. Our study also strengthens the view that the CD73-adenosine axis plays an important protective role in RA and could be a clinically relevant pathway to target therapeutically. In support of this, a recent meta-analysis found that elevated caffeine consumption (an A2A receptor antagonist) is associated with an increased risk of RA (20). Modulation of the CD73-adenosinergic pathway may thus constitute a promising approach for treatment of RA.

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Disclosures
J.S. is a paid consultant and own stocks for Surface Oncology, Inc.

References


Supplemental Figure 1. Human synovial tissue expresses CD73.
Data on CD73 mRNA expression were obtained from the Human body index transcriptional profiling study (GEO accession GSE7307). Statistical analysis was performed using the ANOVA test (*, p<0.05).