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Extracellular adenosine produced by ecto-5′-nucleotidase (CD73) regulates macrophage pro-inflammatory responses, nitric oxide production, and favors *Salmonella* persistence

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ABSTRACT

Surface enzymes CD39 (nucleoside triphosphate dephosphorylase) and CD73 (ecto-5′-nucleotidase) mediate the synthesis of extracellular adenosine that can regulate immune responses. Adenosine produced by CD39/CD73 acts via adenosine receptors (ARs). CD73 is expressed by a variety of cell types and mediates anti-inflammatory responses. Because efficient innate immune responses are required for clearance of *Salmonella* infection, we investigated the role of CD73 in macrophage function, including phagocytosis, intracellular killing of *Salmonella*, and anti-bacterial pro-inflammatory responses to *Salmonella*-whole cell lysate (ST-WCL) or *Salmonella* infection. Additionally, RAW 264.7 macrophage mRNA expression of CD39, CD73, and all ARs were measured by qPCR after ST-WCL treatment. Pro-inflammatory cytokine mRNA and nitric oxide (NO) production were quantitated in the ST-WCL treated macrophage with and without CD73-inhibitor (APCP) treatment. Phagocytosis and intracellular killing by peritoneal macrophages from CD73-deficent mice were also evaluated using *E. coli* BioParticles® and GFP-*Salmonella* infection, respectively. CD73, CD39, and A_{2B}AR mRNA were predominantly expressed in RAW cells. ST-WCL treatment significantly reduced CD73 expression, suggesting endogenous down-regulation of CD73, and an enhanced pro-inflammatory response. ST-WCL treated and CD73-inhibited macrophages produced more NO and a higher level of pro-inflammatory cytokines than CD73-competent macrophages (e.g. IL-1β, TNF-α). Phagocytosis of *E. coli* BioParticles® was significantly higher in the macrophages treated with APCP and in the peritoneal macrophages from CD73-deficent mice as compared to APCP-untreated, and CD73-competent macrophages. Internalized bacteria were more efficiently cleared from macrophages in the absence of CD73, as observed by fluorescence-microscopy and *Salmonella*-DNA measurement by qPCR from the infected cells. CD73 down-regulation or CD73-inhibition of macrophages during *Salmonella* infection can enhance the production of pro-inflammatory cytokines and NO production, improving intracellular killing and host survivability. Extracellular adenosine synthesized by CD73 suppresses antibacterial responses of macrophages, which may weaken macrophage function and impair innate immune responses to *Salmonella* infection.

1. Introduction

Non-typhoidal *Salmonella* spp. is a major cause of hospitalization and death, accounting for an estimated one million foodborne illnesses annually [1]. In immunosuppressed individuals, these infections can lead to bacteremia and even death. Numerous host immune components are involved in the fight against infection. *Salmonella* infection can cause a blunted early immune response that plausibly facilitates long-term pathogen survival [2]. The dampened inflammatory response to systemic *Salmonella* infection also reduces immune-mediated damage to host tissues, which may outweigh the immediate risk posed by the pathogen itself [3]. During salmonellosis, clearance of bacteria requires the production of pro-inflammatory cytokines by T helper (T_h) cells and nitric oxide (NO) production by macrophages. Phagocytic cells, including macrophages, are a critical line of defense against infection. The ability of pathogens to survive and even replicate within

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phagocytic cells is an effective method of evading the defense mechanism of the host. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium* [4]. Macrophages function in the innate host defense against salmonellosis by executing efficient phagocytosis of pathogens and production of pro-inflammatory cytokines (e.g. IL-1β, TNFα). The clearance of *Salmonella* also requires the production of IFN-γ by CD4 T cells [5], which play an important role in amplifying inflammatory responses induced in the gut [6].

Adenosine (Ado) is a purine nucleoside implicated in the control of the complex and multifaceted host responses to infection [7–10]. Adenosine accumulates in inflamed or hypoxic tissues largely due to the action of CD39 (nucleoside triphosphate dephosphorylase) mediating the dephosphorylation of ATP to ADP and then to 5′-AMP, the substrate for CD73 (ecto-5′-nucleotidase). CD73 then catalyzes the terminal reaction to convert 5′-AMP to adenosine [11]. CD73 is a ubiquitously expressed protein in immune cells and tissues. The numerous responses controlled by adenosine are mediated by four G-protein–coupled receptors (A_1, A_{2A}, A_{2B}, and A_3). In this respect, the effect of adenosine on mononuclear phagocytes appears to be a central event. Adenosine A_1, adenosine A_2, and adenosine A_3 receptors are present on cultured monocyte/macrophage cells [12,13]. As a result of interaction between adenosine and adenosine receptors it is likely that adenosine produced through the expression of CD39 and CD73 in macrophages can also regulate innate responses. Previous reports suggest that various adenosine agonists may be able to modulate monocyte/macrophage functions, such as phagocytosis [14], NO production [15] and chemotaxis [16].

The primary aim of this study is to determine if CD73/Ado regulates macrophage function in host defense during *Salmonella* infection. Recently we reported that during murine salmonellosis, a lack of CD73 expression results in a greater inflammatory response and improved bacterial clearance [17]. Little is known, however, about the role of CD73 in controlling the innate host response to infection with *Salmonella* species. In the present study, we examined the expression CD73 in macrophages after *Salmonella* infection and evaluated the role of CD73 in regulating inflammation and bacterial persistence.

2. Materials and methods

2.1. Reagents

Adenosine 5'-(α,β-methylene) diphosphate (APCP) and lipopolysaccharides (LPS) (from *Salmonella enterica* serotype Enteritidis) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

2.2. Salmonella growth conditions, Salmonella whole cell lysate preparation

Salmonella enterica serotype Typhimurium (LT-2 strain) or Green Fluorescent Protein (GFP)-labeled *Salmonella enterica* serotype Enteritidis (SE338 strain) were cultured on LB agar plates (BBL, Becton and Dickinson, MD) at 37 °C for 18 h. The number of inoculated bacteria was estimated spectrophotometrically. *Salmonella* LT-2 whole cell lysate (WCL) was prepared as an antigen by sonication of *Salmonella* LT-2 strain and collection of the resulting supernatant.

2.3. Cell culture

The murine macrophage-like cell line RAW 264.7 (ATCC TIB-71) was obtained from American Type Culture Association (ATCC), (Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, Gaithersburg, MD) containing 10% heat-inactivated defined-Fetal Bovine Serum (FBS), (HyClone™, Thermo Scientific, Logan, Utah) at 37 °C under 5% CO_2/95% air. Cells were washed gently with PBS (pH 7.4) and were removed from the flask by scraping or trypsinization. The cells were then plated and cultured in 24-well plates with DMEM at a density of 10^5 cells per well for antimicrobial analysis as described below.

2.4. Mouse peritoneal macrophages

Wild-type, C57BL/6 ($CD73^{+/+}$) black mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CD73-deficient (CD73-KO or $CD73^{-/-}$) mice inbred onto the C57BL/6 background were kindly provided by Dr. Linda Thompson. The animal portion of the study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The protocol was approved through the Food and Drug Administration, Center for Food Safety and Applied Nutrition, Institutional Animal Care and Use Committee (FDA-CFSAN-IACUC). All mice were maintained in FDA Module-1 animal facility. Mice were used to generate thioglycollate-elicited peritoneal macrophages. Wildtype and CD73-deficient mice were injected intraperitoneally with 1 mL 3% thioglycollate broth (Rewel, San Diego, CA). Three days later, mouse peritoneal lavage was collected in 10 mL ice-cold PBS. The lavage was centrifuged at 170 × g for 10 min at 4 °C and suspended in DMEM containing 10% FBS and 10 μg/mL gentamicin (Gibco, Gaithersburg, MD). For bactericidal assay, 5×10^5 cells were seeded in 24-well plates or four-well chamber slides, respectively. After 4 h incubation at 37 °C under 5% CO_2, non-adherent cells were removed by washing with PBS (pH 7.4). Adherent cells were incubated at 37 °C under 5% CO_2 overnight in DMEM containing 10% FBS and used for subsequent assays.

2.5. Macrophage stimulation assay

RAW 264.7 cells (5×10^5 cell/well) were cultured in 24-well flat-bottom tissue culture plates (Costar, Corning, NY) in DMEM supplemented with 10% heat-inactivated FBS, at 37 °C under 5% CO_2. Cells were stimulated with *Salmonella*-WCL (7 μg/mL) in the presence or absence of APCP (100 μM), a CD73 enzyme-inhibitor. Supernatant and/or trypsinized cells were collected at time intervals up to 24 h of incubation and used in subsequent assays.

2.6. Infection of cultured macrophages

Salmonella LT-2 strain or GFP-*Salmonella* were grown overnight at 37 °C in BHI broth; the culture was centrifuged and the pellet washed with PBS (pH 7.4) two times. The optical density was measured at 600 nm in order to estimate bacterial cell density. Based on this reading, the bacterial suspension was diluted to 10^7 CFU/mL in DMEM. The procedure for macrophage infection was followed as described previously [18]. Briefly, the bacterial suspension was added to wells at an MOI of 2:1 for RAW 264 cells or peritoneal macrophages. Plates were centrifuged at 130 × *g* for 5 min to initiate infection and incubated at 37 °C under 5% CO_2 for 1 h. Subsequently, plates were washed twice with pre-warmed PBS (pH 7.4) and incubated in media containing 100 μg/mL gentamicin for1 h. For RAW cells, APCP (100 μM) was added as required and plates were cultured in DMEM containing 50 μg/mL gentamicin for the remainder of the experiment. For infection of peritoneal macrophages, bacteria were opsonized with 10% normal mouse serum at 37 °C for 30 min before infection. After macrophage infection supernatant was collected and/or cells trypsinized and collected at different time intervals up to 24 h of incubation and used in subsequent assays.

2.7. Phagocytosis assay

A pH-sensitive, heat-killed fluorescein-conjugated pHrodo® *Escherichia coli* (BioParticles®) was used according to the manufacturer's protocol (Invitrogen, Molecular Probes, Carlsbad, CA) to analyze levels of phagocytosis by macrophages *in vitro*. Approximately 10^5 macrophages were pretreated with 100 μM APCP for 30 min. The media was removed and replaced with the BioParticle® suspension and allowed to