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Adenosine A_{2A} receptor, a potential valuable target for controlling reoxygenated DCs-triggered inflammation

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ABSTRACT

Dendritic cells (DCs) exposed to various oxygen tensions under physiopathological conditions are the critical immune cells linking innate and adaptive immunity. We have previously demonstrated that reoxygenation of hypoxia-differentiated DCs triggers complete DCs activation and inflammatory responses, so restraining the activation of reoxygenated DCs is important to suppress inflammatory responses in diseases caused by oxygen redelivery such as ischemia-reperfusion injury. In the current study, we showed that reoxygenation of hypoxia-differentiated DCs led to predominant expression of high levels of adenosine receptor $A_{2A}R$ on reoxygenated DCs as compared to those on hypoxic or normoxic DCs. Agonist CGS21680 targeting $A_{2A}R$ could effectively inhibit the maturation and activation of reoxygenated DCs through downregulating the expression of MHC class II molecules and CD86. In response to CGS21680 treatment, reoxygenated DCs exhibited a decrease in proinflammatory cytokines IL-1β, IL-6 and TNF-α, and an increase in immune-regulatory cytokine TGF-β. These data suggest the critical role of $A_{2A}R$ signaling pathway in inhibiting the maturation and proinflammatory function of reoxygenated DCs, thereby proposing $A_{2A}R$ as a potential valuable target for controlling reoxygenated DCs-triggered inflammation.

1. Introduction

Hypoxia resides in ischemic conditions including organ transplantation, trauma, hypovolemic shock, liver surgery and cardiovascular diseases, while the return of blood flow to the ischemic tissues, which is accompanied by the redelivery of oxygen (also known as reoxygenation), absolutely accelerates the damage of hypoxic organ through inducing ischemia reperfusion injury (IRI) (Kutala et al., 2007; Li and Jackson, 2002; Wang et al., 2002; Yamauchi and Kimura, 2008). Considerable data have demonstrated the important roles of innate and adaptive immune responses in IRI (Boros and Bromberg, 2006). As the most potent antigen presenting cells (APCs) linking innate immunity with adaptive immunity, dendritic cells (DCs) play the curial roles in IRI. It has been reported that activated DCs are involved in the pathologic process of some organs' IRI such as kidney and liver (Castellaneta et al., 2014; Dong et al., 2007; Jang et al., 2009; Zhai et al., 2011). However, there are few data elucidating how DCs are activated in the organs of IRI. We have reported in previous study that the change of oxygen tension maybe an important causal factor triggering the tolerance or activation of immune responses through regulating the maturation of DCs. Hypoxic microenvironment drives immune tolerance via suppressing the maturation of DCs; while reoxygenation of hypoxia-differentiated DCs results in complete recovery of their mature phenotype and function, and then drives immune response toward a proinflammatory direction, thus suggesting reoxygenation contributes to DCs activation and subsequent inflammation in IRI (Wang et al., 2010). So it is important to make clear and restrict the activation of reoxygenated DCs with regard to the control of IRI.

Previous studies have indicated the accumulation of extracellular adenosine can trigger and translate hypoxia-related signal into immune cells through the adenosine receptors (ARs), which are definitely involved in the regulation of DCs function (Sitkovsky and Lukashev, 2005; Yang et al., 2010). To date, four ARs (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) have been identified on most immune cells including DCs (Sitkovsky and Lukashev, 2005; Sitkovsky et al., 2004). Regarding the immunoregulatory function of ARs on DCs, studies from different models draw differential conclusions; and also different ARs pathways regulate DCs function in different ways. In normoxic conditions, adenosine upregulates the

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expression of CD80, CD86 and HLA-DR on DCs, but inhibits the release of TNF-α, IL-12, as well as the capacity to induce Th1 polarization (Panther et al., 2003). While Sergey et al. show that adenosine is an important factor affecting differentiation of myeloid DCs, signaling through $A_{2B}R$ skews DCs differentiation toward a distinct DCs population that produces high levels of angiogenic factors and Th2-type cytokines, phenotype associated with promotion of angiogenesis, tumor growth, immune suppression, and tolerance (Novitskiy et al., 2008). Some in vivo animal models points out the anti-inflammatory effects of ARs signaling in IRI, which provide potential therapeutic targets for the control of IRI. Injection of $A_{2A}R$ agonist ATL146e reduced the infarct size in B6 mice after 24 h of reperfusion, but had no effect in $A_{2A}R$ knock-out mice (Yang et al., 2005). More interestingly, mice with $A_{2A}R$-deficient DCs were more susceptible to kidney IRI and were not protected from injury by $A_{2A}R$ agonists (Li et al., 2012). Furthermore, Thiel found that the possibility of iatrogenic exacerbation of acute lung injury upon oxygen administration due to the oxygenation-associated elimination of $A_{2A}R$-mediated lung tissue-protecting pathway (Thiel et al., 2005). All these suggested the important roles of ARs pathways in DCs function or in IRI. However, it remains largely unknown about the function of ARs pathway in the metergasis of reoxygenated DCs, which is important for exploring the effective strategy to regulate the immune inflammation mediated by reoxygenated DCs.

In this study, we investigated the expression and function of four ARs on the reoxygenated DCs. The results have demonstrated that reoxygenated DCs express high levels of $A_{2A}R$ compared to normoxic or hypoxic DCs; of note, $A_{2A}R$ is the most predominant receptor on reoxygenated DCs as compared to other ARs. $A_{2A}R$ agonist CGS21680 significantly inhibits the upregulation of costimultory molecules CD86 and MHC class II molecules (MHC-II) in reoxygenated DCs. More importantly, in response to CGS21680 treatment, reoxygenated DCs exhibited marked decrease in proinflammatory cytokines IL-1β, IL-6 and TNF-α, but increase in immunoregulatory cytokine TGF-β, suggesting that $A_{2A}R$ pathway maybe a potential target to control the inflammation mediated by reoxygenated DCs.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice (6–8 weeks) used in the experiments were purchased from Shanghai SLAC Laboratory Animal co. Ltd (Shanghai, China) and maintained in the specific pathogen-free animal facility at Shandong University (Jinan, China). All animal studies were approved by the Animal Care and Utilization Committee of the Shandong University.

2.2. Reagents and antibodies

Recombinant murine GM-CSF and IL-4 were obtained from PeproTech Inc. (New Jersey, USA), 7-AAD viability staining solution, FITC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse MHC-II, CD80, CD86 and anti-mouse CD16/CD32 purified mAb were purchased from eBioscience (San Diego, CA, USA). Lipopolysaccharides (LPS), adenosine, and different ARs agonists including N^6-cyclopentyladenosine (CPA, A_1R agonist), 2-*p*-(2-Carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS21680, $A_{2A}R$ agonist), *N*-ethylcarboxamidoadenosine, (NECA, $A_{2B}R$ agonist), N^6-(3-Iodobenzyl)-9-[5-(methylcarbamoyl)-β-D-ribofuranosyl] adenine, (IB-MECA, A_3R agonist) were purchased from Sigma-Aldrich (Saint Louis, USA). All the ARs agonists were dissolved with DMSO (Solarbio, Shanghai, China) follow the instructions, using DMSO with the same dilution as vehicle control in the experiments. SYBR Premix Ex Taq™ Real time PCR test kit was purchased from TaKaRa Biotechnology CO.LTD (Dalian, China).

2.3. Generation and treatment of bone marrow-derived DCs

DCs were generated from the bone marrow (BM) precursors of C57BL/6 mice as described in references (Inaba et al., 1992; Lutz et al., 1999) under normoxic, hypoxic or reoxygenated conditions, respectively. In brief, BM cells were flushed from femurs and tibias with RPMI 1640 medium and treated with RBC lysis buffer for 5 min, washed twice with phosphate buffered saline (PBS), then cultured in complete RPMI 1640 medium supplemented with 50 ng/ml GMCSF and 20 ng/ml IL-4. Half of the medium was replaced every two days. At day 7, LPS (1 μg/ml) was administrated to induce maturation for 24 h. DCs morphology and phenotypes (CD11c) were determined by light microscopy and flow cytometry (FCM). For different culture conditions, the cells were maintained in a humidified incubator (HERAcell, Germany) at 37 °C under normoxic (21% O_2, 5% CO_2, 74% N_2) or hypoxic (1% O_2, 5%CO_2, 94% N_2) conditions, respectively. Hypoxia/reoxygenation was performed by transferring hypoxia-differentiated DCs to normoxic condition during the last 24 h of culture in the absence or presence of different ARs agonists. In some experiments, normoxic DCs were treated with different ARs agonists for 24 h. To clarify the toxic effects of ARs agonists on DCs, cell viability were tested by 7-AAD staining, which showed more than 90% viability of DCs.

2.4. Real time PCR

Total cellular RNA was extracted from cells using a modified Trizol one-step extraction method, and reverse-transcribed into cDNA using Reverse Transcription System (Promega, USA) according to the manufacturer's protocol. The real time PCR was performed to determine the relative mRNA levels of interested genes. Primers used in the experiments includes: A_1R, sense: 5′-CAT TGG GCC ACA GAC CTA CT-3′, antisense: 5′-ACC GGA GAG GGA TCT TGA CT-3′; $A_{2A}R$, sense: 5′-AAC CTG CAG AAC GTC AC-3′, antisense: 5′-GTC ACC AAG CCA TTG TAC CG-3′; $A_{2B}R$, sense: 5′-CAT TAC AGA CCC CCA CCA AC-3′, antisense: 5′-AGG ACC CAG AGG ACA GCA AT-3′; A_3R, sense: 5'-ATA CCA GAT GTC GCA ATG TGC-3', antisense: 5'-GCA GGC GTA GAC AAT AGG GTT-3, β-actin, sense: 5′-TGC GTG ACA TCA AAG AGA AG-3′, antisense: 5′-TCC ATA CCC AAG AAG GAA GG-3′.

2.5. Flow cytometry

For the staining of surface molecules, DCs were blocked with anti-CD16/CD32 mAb and stained with FITC-conjugated anti-CD11c, PE-conjugated anti-MHC class II, CD80, and CD86 mAbs. Flow cytometry (FCM) data acquisition and analysis were performed on a Cytomics FC500 Flow Cytometer (Beckman Coulter, CA, USA).

2.6. RT-PCR

Total cellular RNA was extracted from cells using a modified Trizol one-step extraction method, and reverse-transcribed into cDNA using Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's protocol. PCR was performed using specific primers. Primers for amplification of each gene are as follows: IL-1β, sense: 5′-GCA ACT GTT CCT GAA CTC A-3′, antisense: 5′-CTC GGA GCC TGT AGT GCA G-3′; IL-6, sense: 5′-TTC TTG GGA CTG ATG CTG-3′, antisense: 5′-CTG GCT TTG TCT TTC TTG TT-3′; TNF-α, sense: 5′-ATG AGC ACA GAA AGC ATG ATC-3′, antisense: 5′-TAC AGG CTT GTC ACT CGA ATT-3′; TGF-β, sense: 5′-GGC GGT GCT CGC TTT GTA-3′, antisense: 5′-CGT GGA GTT TGT TAT CTT TGC T-3′;