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Short communication

Reoxygenation of hypoxia-differentiated dentritic cells induces Th1 and Th17 cell differentiation

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ABSTRACT

Dendritic cells (DCs) are often exposed to various oxygen tensions under physiological and pathological conditions. However, the effects of various oxygen tensions on DC functions remain unclear. In this study, we showed that hypoxia-differentiated DCs expressed lower levels of MHC-II molecule, co-stimulatory molecules (CD80, CD86) and proinflammatory cytokines (IL-1β, IL-6, and TNF-α), but higher levels of immunoregulatory cytokine transforming growth factor-beta (TGF-β) than normoxia-differentiated DCs. Unexpectedly, re-exposure of hypoxia-differentiated DCs to saturated oxygen (reoxygenation) completely restored their mature phenotype and function. Specifically, the reoxygenated DCs induced naïve CD4$^+$ T cells to differentiate into Th1 and Th17 effector cells, but deceased the generation of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs). The data indicate that hypoxic microenvironment suppresses the maturation and function of murine DCs. Reoxygenation of hypoxia-differentiated DCs however results in complete recovery of their mature phenotype and function, and has strong ability to drive immune response toward a proinflammatory direction, suggesting reoxygenated DCs may contribute to inflammation of ischemia-reperfusion injury.

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1. Introduction

Hypoxia exists in many ischemia conditions such as organ transplantation, trauma, hypovolemic shock, liver surgery and cardiovascular diseases, the following return of blood flow and oxygen delivery in the hypoxic tissues lead to rapid change of oxygen tension, which often exacerbates the damage of hypoxic organ and induces ischemia-reperfusion injury (IRI) (Kutala et al., 2007; Li and Jackson, 2002; Wang et al., 2002; Yamauchi and Kimura, 2008). There are considerable data suggesting an important role of innate and adaptive immune response in IRI (Boros and Bromberg, 2006). Dendritic cells (DCs), the professional antigen presenting cells (APCs), are the initiator to drive adaptive immune responses. Recent studies have provided novel insights into the potential role of DCs in mediating immune responses in IRI. Accumulation of DCs was observed in liver and kidney within 1 h and peaked at 24 h after IRI (Zhou et al., 2005). Increased number of DCs expressing more mature phenotype was identified in ischemic kidney in the early phase (within 24 h) after IRI, while these activated DCs with a strong capacity of inducing T cell proliferation migrated from the kidney into the renal lymph node 1 day after IRI (Dong et al., 2005). DCs isolated from liver after IRI also exhibited a mature phenotype (Loi et al., 2004). These data suggest that activated DCs are involved in IRI. However, the mechanism of DC activation during IRI is largely unknown.

It has been suggested that reoxygenation (re-exposure to saturated oxygen) after hypoxia is a crucial step mediating organ injury in IRI, where oxygen availability leads to a dramatic shift of oxygen tension (Cao et al., 2006; Leonard et al., 2006; Li and Jackson, 2002; Saikumar et al., 1998; Wang et al., 2002). Previous studies have demonstrated that hypoxia inhibits the activation and cytokine secretion of CD4$^+$ T cells (Caldwell et al., 2001), but increases the phagocytosis of macrophages (Anand et al., 2007). Several studies have indicated that hypoxia affects differentiation, maturation

Abbreviations: DC, dendritic cell; Tregs, regulatory T cells; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; IFN, interferon; LPS, lipopolysaccharides; HIF-1α, hypoxia induced factor-1 alpha; MLR, mixed lymphocyte reaction; IRI, ischemia-reperfusion injury.

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(Jantsch et al., 2008; Mancino et al., 2008), migration (Zhao et al., 2005; Qu and Yang, 2005; Yang et al., 2006; Zhao et al., 2008), antigen uptake of DCs (Elia et al., 2008), and their capability to promote adaptive immunity (Jantsch et al., 2008; Mancino et al., 2008). In addition, hypoxia significantly alters the expression profile of cytokines, chemokines and chemokine receptors (Bosco et al., 2008; Elia et al., 2008; Mancino et al., 2008; Ricciardi et al., 2008). However, the conclusions from different reports are controversial. Furthermore, previous researches have failed to consider the impact of reoxygenation on hypoxia-differentiated DCs.

Although the direct or indirect role of $CD4^+$ effector T cells in IRI has been investigated (Huang et al., 2007; Marques et al., 2006), but which subsets of $CD4^+$ T cells are involved in the pathogenic mechanism of IRI is not fully understood. Th17 cells and regulatory T cells (Tregs) have been described as two new subsets of $CD4^+$ T cells in addition to Th1 and Th2 types (Bettelli et al., 2006; Harrington et al., 2006; Mosmann et al., 1986; Sakaguchi, 2004; Sakaguchi et al., 1995). Th17 cells producing IL-17 mediate host defensive mechanisms to extracellular bacteria infections, and are involved in the pathogenesis of several organ specific autoimmune diseases which have historically been associated with Th1 responses (Afzali et al., 2007; Korn et al., 2007). Tregs expressing CD25 and Foxp3 have an anti-inflammatory role and maintain tolerance to self-components by contact-dependent suppression or releasing anti-inflammatory cytokines such as IL-10 and TGF-β (Chen et al., 2003; Rudensky and Campbell, 2006; Sakaguchi, 2003, 2004; Sakaguchi et al., 1995; von Boehmer, 2005; Xiao et al., 2008). DCs may drive either Th effector cell differentiation or induce Treg generation upon the different microenvironment and cytokine milieu (Belkaid and Oldenhove, 2008; Luo et al., 2007; Tang et al., 2006). It remains largely unknown however whether diverse oxygen tension influences the ability of DCs in T cell differentiation.

In this study, we investigated the phenotype and function of murine BM-DCs generated under hypoxic or reoxygenated conditions. We demonstrated that hypoxic microenvironment suppressed the maturation and function of DCs. Reoxygenation of hypoxia-differentiated DCs resulted in complete recovery of their mature phenotype and function, and drove them to initiate Th1 and Th17 cell differentiation, suggesting that DCs play a critical role in the inflammation of IRI.

2. Materials and methods

2.1. Mice

Female BALB/$c^{H\text{-}2d}$ and C57BL/$6^{H\text{-}2b}$ 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 Shandong University, China.

2.2. Reagents and antibodies

Recombinant murine GM-CSF and IL-4 were obtained from PeproTech Inc. (NJ, USA). FITC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse MHC class II, CD80, CD86, Foxp3, IL-4, IL-17A mAbs, purified anti-mouse CD3 mAb, and Brefeldin A (BFA) were purchased from eBioscience (San Diego, CA, USA). PE-Cy5-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD25, IFN-(mAbs were from BD Biosciences (San Jose, CA, USA). Lipopolysaccharides (LPS), ionomycin and phorbol myristate acetate (PMA) were purchased from Sigma–Aldrich (Saint Louis, USA). Mouse CD4 MicroBeads were obtained from Miltenyi Biotec (Auburn, CA, USA).

2.3. Generation of bone marrow-derived DCs

DCs were generated from the bone marrow (BM) precursors of C57BL/6 mice as described by Inaba et al. (1992) and Lutz et al. (1999). 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 GM-CSF and 20 ng/ml IL-4. Half of the medium was replaced at days 3 and 5. At day 7, the suspended and loosely adherent cells were collected as immature DCs ($CD11c^+$ cells were about 80%). To induce maturation, the immature DCs were stimulated with LPS (1 μg/ml) for additional 24 h. Cells showed DC morphology and phenotype as determined by light microscopy and flow cytometry (FCM), respectively. For normoxic culture condition, cells were maintained in a humidified incubator (HERAcell, Germany) containing 21% O_2, 5% CO_2, 74% N_2 at 37 °C. For hypoxic culture condition, cells were incubated in a humidified hypoxic incubator (HERAcell, Germany) flushed with a gas mixture of 1% O_2, 5% CO_2, and 94% N_2 at 37 °C. To ensure that cells were exposed to hypoxic condition, the medium for hypoxic culture was balanced in hypoxic condition at least for 24 h and the time for replacing medium was kept as short as possible. In some experiments, hypoxia-differentiated DCs were transferred from hypoxia to normoxia for different periods of time to allow reoxygenation (Wang et al., 2002). To avoid the effect of various oxygen tensions on phenotype of DCs, cells were fixed before staining. In addition, to indicate the status of hypoxia or normoxia, the expression of hypoxia induced factor-1 alpha (HIF-1α), a major regulator for cell adaptation to hypoxia, was detected by Western blot.

2.4. 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′; MMP9, sense: 5′-ACC CTG TGT GTT CCC GTT-3′, antisense: 5′-CCG TCT ATG TCG TCT TTA TTC A-3′; TGF-β1, sense: 5′-GGC GGT GCT CGC TTT GTA-3′, antisense: 5′-CGT GGA GTT TGT TAT CTT TGC T-3′; β-actin, sense: 5′-TGC GTG ACA TCA AAG AGA AG-3′, antisense: 5′-TCC ATA CCC AAG AAG GAA GG-3′. RT-PCR was performed in at least three independent experiments, mRNA levels for different genes were normalized to the house keeping genes, β-actin using Quantity One® Version 4.3.1 (Bio-Rad, Hercules, CA, USA).

2.5. T cell proliferation assay

$CD4^+$ T cells from the spleen of C57BL/$6^{H\text{-}2b}$ or BALB/$c^{H\text{-}2d}$ mice were isolated via positive selection by Mouse CD4 MicroBeads as the manufacturer's protocols. The purity of $CD4^+$ T cells was >95%. To assay the proliferation of $CD4^+$ T cells, DCs were induced from precursors in BM of C57BL/$6^{H\text{-}2b}$ mice in the presence of IL-4 and GM-CSF for 5–7 days in hypoxia, stimulated by LPS for maturation for 18 h in hypoxia and then these dendritic cells were divided into two groups: one was transferred into normoxia for additional 6 h, describes as reoxygenated DCs (H/R), the other was still kept in hypoxia for additional 6 h, named as hypoxic DCs (H). Meanwhile, normoxia-differentiated DCs (N) were used as control. After treated with mitomycin C (Mancino