Molecular Immunology 48 (2011) 720–727

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Short communication

IL-10 inhibits the starvation induced autophagy in macrophages via class I phosphatidylinositol 3-kinase (PI3K) pathway

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ARTICLE INFO

Article history:
Received 7 August 2010
Received in revised form 25 October 2010
Accepted 25 October 2010
Available online 20 November 2010

Keywords:
Akt
Autophagy
IL-10
Mammalian target of rapamycin
p70S6K
Phosphatidylinositol 3-kinase

ABSTRACT

Autophagy is an important process which maintains cellular homeostasis under stressful conditions such as starvation and pathogenic invasion. Previous observations have indicated that several cytokines are important regulators of the autophagic process. Among the various cytokines, IL-10 has a unique property which functions to suppress overall immunity. However, the functional role of IL-10 during the autophagic process has not been studied. In this study, we examined the effect of IL-10 during starvation induced autophagy of murine macrophages (J774). The results clearly indicated that IL-10 and IL-10 receptor signaling inhibits autophagy induction of murine macrophage. Further experiments revealed that IL-10 activates the class I phosphatidylinositol 3-kinase (PI3K) pathway, which results in the phosphorylation of p70S6K through the activation of Akt and a mammalian target of the rapamycin complex 1 (mTORC 1). These results will advance our understanding of the physiological function of IL-10 during the autophagic process of macrophage.

1. Introduction

Autophagy is an active degradative process to remove or turnover bulk cytoplasmic constituents through the endosomal and lysosomal fusion system resulting in the formation of autophogosomes in eukaryotic cells (Baehrecke, 2005; Yang and Klionsky, 2009). Particular stressful conditions such as starvation and pathogenic invasion induce autophagy to remove long lived proteins and dysfunctional organelles or for the degradation of constituents (Yang and Klionsky, 2009). In addition, autophagy is used to present specific exogenous antigens through the MHC class II to stimulate adaptive immunity (Deretic, 2006). Thus, autophagy plays an important role during the early stage of infection with specific pathogens such as *Mycobacterium tuberculosis* (Gutierrez et al., 2004) and virus (Tallóczy et al., 2002).

Previous observations have suggested that cytokines are important regulators of the autophagic process. For example, IFN-γ induces or promotes autophagy in immune cells (macrophages) as well as non-immune cells (Inbal et al., 2002; Gutierrez et al., 2004; Pyo et al., 2005). In contrast to IFN-γ, IL-13 strongly inhibits the induction of autophagy (Petiot et al., 2000; Arico et al., 2001). Thus, T helper type 1 (Th1) and Th2 cytokines seem to be antagonistics of autophagy induction. However, the function of IL-4, typical the Th2 cytokine, on autophagy induction is controversial because IL-4 enhances autophagy in non-immune cells despite the fact that IL-4 signaling is very similar to IL-13 signaling (Wright et al., 1997; Yamamoto et al., 2006).

IL-10 was first described as a cytokine synthesis inhibitory factor that has an anti-inflammatory function (Mosmann et al., 1986). IL-10 is expressed by various cell types including B cells, Th2 cells, mast cells, monocytes, macrophage, dendritic cells and regulatory T cells (O'Garra et al., 2008). Although IL-10 is classified as a Th2 type cytokine, IL-10 has been shown to suppress a broad range of inflammatory responses and is known to be an important factor in maintaining homeostasis of overall immune responses (O'Garra and Murphy, 2009). Thus, novel therapies using IL-10 have been developed for several human diseases such as allergic responses, autoimmune diseases and graft rejection (O'Garra et al., 2008; Akdis and Akdis, 2009; O'Connell et al., 2010). However, the functional role of IL-10 during the autophagic process has not been studied.

Abbreviations: EBSS, Earle's balanced salts solution; EGFP.LC3, EGFP fusion LC3B protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIV-1, human immunodeficiency virus-1; mTORC, mammalian target of rapamycin complex; PI3K, phosphatidylinositol 3-kinase; Th, T helper type.

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doi:10.1016/j.molimm.2010.10.020

In this study, we examined the effect of IL-10 during starvation induced autophagy of murine macrophage (J774). The results clearly indicated that IL-10 inhibits the autophagy induction of murine macrophage through the class I phosphatidylinositol 3-kinase (PI3K) pathway.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

Mouse macrophage cell lines, P338D1, RAW264.7 and J774, mouse B cell line, A20, mouse mast cell line, P815, mouse fibroblast cell line, L929, mouse myoblast cell line, C2C12, and mouse Leydig cell line, MLTC were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37 °C and 5% CO_2 in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin (Invitrogen). The recombinant mouse IL-10, PE-conjugated anti-IL-10 receptor and PE-conjugated isotype control (ratIgG1κ) were purchased from BD Biosciences (San Jose, CA, USA). FITC-conjugated anti-$CD11^b$ and FITC-conjugated isotype control (ratIgG2bκ) were purchased from eBioscience, Inc. (San Diego, CA, USA). Rapamycin (99% purity by HPLC) was purchased from LC Laboratories (Woburn, MA, USA). LY294002, purified anti-Akt (cat. # 9272), purified anti-phosphorylated Akt (cat. # 4058S), purified anti-LC3B (cat. # 2775S), purified anti-p70S6K (cat. # 9202) and purified anti-phosphorylated p70S6K (cat. # 9234S) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Purified anti-β-actin (cat. # A5441) was purchased from Sigma (St. Louis, MO, USA).

To obtain primary mouse macrophages, peritoneal macrophages from female C57BL/6 mice (8 weeks old) were isolated as previously described (Hoover and Nacy, 1984). Briefly, mice were intraperitoneally injected with 1 ml of 3% Brewer thioglycollate (BD Biosciences). Three days after injection, cells from peritoneal cavity were isolated by flushing out the peritoneal cavity using 10 ml of PBS without calcium and magnesium. Cells were then plated on the culture dish and incubated at 37 °C and 5% CO_2 in DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin for 4 h. Subsequently, nonadherent cells were removed by gently washing three times with warm PBS. Adherent cells were counted, stained with FITC-conjugated anti-$CD11^b$ antibody and analyzed using flow cytometry. More than 90% were $CD11^b$ positive cells.

2.2. Expression of EGFP fusion LC3B protein (EGFP.LC3)

A cDNA segment corresponding to the rat *Lc3b* was purchased from Thermo Scientific Open Biosystems (Huntsville, AL, USA) and was then subcloned through *Bgl*II and *Eco*RI restriction enzyme sites into pEGFPC1 vector (Clontech Laboratories, Inc., Mountain View, CA, USA) to make cDNA encoding a EGFP fusion LC3B protein (EGFP.LC3). To express EGFP.LC3 using a retroviral system, cDNA encoding EGFP.LC3 was further subcloned through a *Not*I restriction enzyme site into a pLNCX2 retroviral vector (Clontech). The EGFP.LC3 cDNA was sequenced by the dideoxy-mediated chain termination method using a 373 automatic sequencer (PerkinElmer, Wellesley, MA, USA). The full-length cDNA encoding EGFP.LC3 consisted of a 1110 bp of open reading frame and was found to be identical to the open reading frame of *egfp* cDNA (GenBank access NM_U55763.1) and rat *Lc3b* cDNA (GenBank access NM_BC083556). The cDNA encoding EGFP.LC3 in the pLNCX2 was introduced into the 293GPG retrovirus packaging cell line via transient transfection with Lipofectamine (Invitrogen). After three days, the supernatants were harvested and infected with J774 cells or C2C12 cells using polybrene (1 μg/ml).

2.3. Autophagy induction and reagent treatment

Autophagy was induced by amino acid and serum starvation. For starvation, cells were washed three times with PBS and incubated in Earle's balanced salts solution (EBSS; Invitrogen) at 37 °C for 2 h (Gutierrez et al., 2004). For IL-10 and/or rapamycin treatment, cells were incubated with IL-10 (100 ng/ml) and/or rapamycin (100 ng/ml) for 10 h before starvation. The cells were then washed three times with PBS and incubated in EBSS with IL-10 (100 ng/ml) and/or rapamycin (100 ng/ml). For LY294002 treatment, cells were incubated with LY294002 (50 μM) for 1 h during a 2-h starvation period.

2.4. RT-PCR analyses

For the RT-PCR analyses, total RNAs from various cells were isolated using a Trizol reagent (invitrogen) according to the manufacture's protocol and reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit with oligo-dT primers and Super-Script RT (Invitrogen). After cDNA synthesis, PCR was conducted using a PTC-100 Thermal Cycler (MJ Research) for 30 cycles of 1 min at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, followed by a 10 min final extension step at 72 °C. Primers for mouse *Il10r* were designed based on the cDNA sequence of mouse *Il10r* (GenBank access NM_008348). The forward primer used was 5′-CCTGGATCTGTATCACCGAAGC-3′, and the reverse primer used was 5′-CTCCGACCACTCTGCCTTGTTA-3′. The resulting RT-PCR product was 400 bp in length. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) primers were used as an internal control in equal amounts to that of the mRNA used. Primers for *Gapdh* were designed based on the cDNA sequence of mouse *Gapdh* (GenBank access NM_008084). The primer sequences for mouse *Gapdh* were as follows: 5′-ATGACCACAGTCCATGCCATC-3′ (sense), 5′-CCTGCTTCACCACCTTCTTG -3′ (anti-sense), resulting in a 271 bp RT-PCR product. The resulting PCR products were loaded onto a 1.5% agarose gel containing ethidium bromide, and were visualized using ultraviolet light.

2.5. Western bolt analyses

Cells were harvested, washed in PBS, centrifuged, and resuspended in a cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100) supplemented with 200 μg/ml phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (EMD Chemicals, Inc., Gibbstown, NJ, USA). The cell lysates were subsequently resolved by 15% SDS polyacrylamide gel electrophoresis, transferred onto Immobilon P membranes (Millipore Corporation, Billerica, MA, USA), and immunoblotted with the appropriate antibody. Immunoreactive bands were visualized using an ECL system (GE Healthcare, Buckinghamshire, UK). The autoradiograph was imaged on i-MAX™ image analysis system (i-MAX-D500, CoreBio system, South Korea). To determine the LC3B II/LC3B I ratio, appropriate bands appeared on immunoblot were quantified using ImageJ software version 1.43u (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin.

2.6. Flow cytometry analyses

Flow cytometry analyses were performed to determine the cell surface expression of the IL-10 receptor on various mouse cell lines and $CD11^b$ on peritoneal macrophages. Briefly, cells were harvested and washed three times with PBS and incubated with each relevant antibody on ice in Hank's balanced salt solution (Invitrogen) containing 2% FBS and 0.1% sodium azide (Sigma). After washing in the same buffer, all stained cells were analyzed via flow cytome-