Molecular Immunology 51 (2012) 255–262

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

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

Involvement of Notch signaling pathway in regulating IL-12 expression via c-Rel in activated macrophages

Natt Boonyatecha[a,1], Naunpun Sangphech[a,1], Wipawee Wongchana[a], Pathipark Kueanjinda[c], Tanapat Palaga[a,b,c,*]

[a] Graduate Program in Biotechnology, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand
[b] Department of Microbiology, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand
[c] Medical Microbiology Interdisciplinary Program, Graduate School, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand

ARTICLE INFO

Article history:
Received 10 November 2011
Received in revised form 23 February 2012
Accepted 3 March 2012
Available online 29 March 2012

Keywords:
Notch signaling
Macrophages
IL-12p40
c-Rel

ABSTRACT

Macrophages play an important role both in innate and adaptive immune responses. Treatment with interferon (IFN) γ together with lipopolysaccharide (LPS) activates pro-inflammatory macrophages which secrete various pro-inflammatory cytokines including IL-12. IL-12 promotes a Th1 type immune response by directly controlling the differentiation of $CD4^+$ T helper 1 cells. Activation of Notch signaling pathway was reported in activated macrophages but the involvement of this signaling pathway in IL-12 expression has not been documented. In this study, we investigated the role of Notch signaling in regulating expression of the IL-12/IL-23 subunit, IL-12p40. Using a gamma-secretase inhibitor (GSI) to inhibit Notch signaling, we observed a profound decrease in *il12p40* mRNA levels and IL-12p70 secretion upon IFNγ/LPS stimulation. On the other hand, overexpression of activated form of Notch1 in activated RAW264.7 macrophage-like cell lines significantly increased the level of *il12p40* mRNA. GSI treatment did not affect the expression of *irf5*, a master regulator of *il12p40* transcription in macrophages. Detailed analysis of the signaling cascades that were affected by this inhibition showed that c-Rel nuclear translocation was inhibited and Erk1/2 activation was compromised by GSI treatment. Addition of exogenous tumor necrosis factor (TNF) α only partially rescued the expression of *il12p40* in the presence of GSI. Unexpectedly, inhibition of Notch signaling using a dominant negative (DN) Mastermind-like (MAML) transcription co-activator, did not affect c-Rel nuclear localization upon activation or *il12p40* mRNA levels, suggesting that the transcriptional activity of Notch signaling is dispensable for the activation of c-Rel. These results strongly suggest that Notch signaling in activated macrophages is involved in regulating the expression of *il12p40* directly via c-Rel and indirectly via TNFα production.

1. Introduction

Macrophages play important roles in host defense by contributing to both innate and adaptive immune responses. Their pro-inflammatory roles are well recognized; they can produce pro-inflammatory cytokines and mediators upon encountering danger signals, such as pathogen associated molecular patterns (PAMPs) (Zhang and Mosser, 2008). Macrophages also function as antigen presenting cells (APCs) by presenting peptide antigens to $CD4^+$ T helper cells. Through their critical role as APCs, macrophages create a microenvironment and express cell surface molecules that instruct $CD4^+$ T helper cells to differentiate into appropriate helper T cell lineages. In addition to their pro-inflammatory role, recent evidence suggests that macrophages also have a contrasting role in resolving inflammation during wound healing and tissue repair (Martinez et al., 2009; Mosser and Edwards, 2008).

The immune system must have the flexibility to mount the most effective and efficient responses to diverse invading pathogens. Therefore, most immune cells, including macrophages, are highly plastic as they can readily change phenotypes in response to changing environmental signals (Biswas and Mantovani, 2010; Stout and Suttles, 2004). Previous studies have identified two distinct subsets of macrophages based on their cytokine production and biological functions (Mosser, 2003). Macrophages stimulated with IFNγ are highly inflammatory and produce chemical mediators, including

Abbreviations: APC, antigen presenting cell; BMM, bone marrow derived macrophages; DMSO, dimethyl sulfoxide; DN, dominant negative; GSI, gamma secretase inhibitor; IFN, interferon; LPS, lipopolysaccharide; MAML, Mastermind-like; N^{IC}, intracellular Notch1; PAMP, pathogen associated molecular pattern; qPCR, quantitative real-time RT-PCR; TNF, tumor necrosis factor.

* Corresponding author at: Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand. Tel.: +66 2 2185070; fax: +66 22527576.
E-mail address: tanapat.p@chula.ac.th (T. Palaga).

[1] Equal contribution.

0161-5890/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2012.03.017

nitric oxide through the expression of inducible nitric oxide synthetase (iNOS). In contrast, TGFβ-treated macrophages stimulate the expression of arginase1 instead of iNOS, which generates ornithine and urea from arginine (Mills et al., 2000). Because these two macrophage subsets influence T helper cells in opposing ways, the former was originally termed "M1" and the latter was termed "M2", which is in parallel with the Th1/Th2 paradigm (Mills et al., 2000). It is currently believed that macrophage activation results in a spectrum of phenotypes, ranging from a pro-inflammatory phenotype at one end to an anti-inflammatory phenotype at the other (Mantovani et al., 2004; Mosser and Edwards, 2008). IFNγ-primed macrophages treated with LPS are equivalent to M1 macrophages which express iNOS and produce IL-12. This type of activated macrophages promotes a Th1 type immune response.

IL-12 is a heterodimer cytokine of IL-12p40 and p35 subunit. IL-12p40 expression in macrophages is under the regulation of various signaling pathways, including NF-κB, PU.1 and c-Rel (Grazia Cappiello et al., 2001; Laderach et al., 2003; Sanjabi et al., 2000). Recently, IRF5 was shown to be a master regulator of M1-specific genes, such as *il12p40*, *il12p35* and *il23p19*, while it represses the expression of *il10*, an M2-specific marker gene (Krausgruber et al., 2011).

We and others have reported that the well-conserved Notch signaling pathway plays a critical role during the macrophage response to danger signals via TLR signaling cascades (Fung et al., 2007; Hu et al., 2008; Monsalve et al., 2006; Palaga et al., 2008). These studies have elucidated that Notch signaling via its target gene *Hes*/*Hey* regulates the macrophage inflammatory response partly via the NF-κB and/or STAT pathways. Notch signaling is involved in cell fate determination and cellular differentiation in various cell types, such as neuronal cells, muscle cells, adipocytes and hematopoietic cells (Artavanis-Tsakonas et al., 1999). During helper T cell polarization, Notch signaling has been shown to regulate Th1/Th2 differentiation likely through direct regulation of the main lineage-specific transcription factors in T cells and selective expression of Notch ligands on APCs (Amsen et al., 2009; Osborne and Minter, 2007). Furthermore, Notch signaling directly regulates cytokine production such as IL-10 in T cells and IL-6 in macrophages (Rutz et al., 2008; Wongchana and Palaga, 2011). Because Notch signaling plays a role at critical steps of various effector cell functions and cytokine productions, we hypothesized that it might also be involved in the activation of macrophages. In this study, we show that the inhibition of Notch signaling affects the expression of *il12p40* mRNA. Furthermore, we provide evidence that Notch signaling regulates IL-12p40 expression directly via c-Rel and indirectly via TNFα production in activated macrophages.

2. Materials and methods

2.1. Animals and generation of bone marrow derived macrophages (BMM)

Female C57BL/6 (National Laboratory Animal Center, Mahidol University, Salaya, Thailand) were sacrificed, and bone marrow was obtained from their femurs. The cells flushed from femur cavities were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), 5% horse serum, HEPES with sodium pyruvate and 20% (v/v) L929-conditioned media for 9 days. Fresh medium was added to the culture at day 4. The cells were harvested at the end of the culture period using cold PBS and were subjected to cell surface staining with anti-F4/80 and CD11b antibodies (BioLegend, CA) to confirm the macrophage phenotype. All procedures involving laboratory animals were carried out according to the guidelines issued by Chulalongkorn University, and all animal protocols were reviewed by the IACUC (protocol review No. 0923013). The murine macrophage-like RAW 264.7 cell line (ATCC No. TIB-71) was used in this study. Cells were maintained in DMEM media (HyClone, UT, USA) supplemented with 10% (v/v) FBS (HyClone), 100 U/mL penicillin (General Drugs House Co. Ltd., Thailand), 0.4 mg/mL streptomycin (M&H Manufacturing Co. Ltd., Thailand), 1% (w/v) sodium pyruvate (HyClone) and 1% (w/v) HEPES (HyClone) at 37 °C and incubated in a humidified 5% (v/v) CO_2 incubator.

2.2. Activation of macrophages

BMMs or RAW264.7 cell line were activated by priming overnight with recombinant murine IFNγ (10 ng/mL) (R&D Systems, Minneapolis, MN, USA) and washed twice with cold PBS. Pre-warmed media and *Salmonella* LPS (100 ng/mL) (Sigma Aldrich, St. Louis, MO) were added to activate macrophages. In some experiments, recombinant murine TNFα (10 ng/mL) (BioLegend, San Diego, CA) were added to activated macrophages.

2.3. Gamma secretase inhibitor (GSI)

The GSIs, GSI (a kind gift from Dr. Todd Golde, University of Florida, FL, USA) or DAPT (Merck, NJ), have been used previously (Monsalve et al., 2009; Palaga et al., 2008). GSI was dissolved in DMSO to a final concentration of 50 mM and stored at −80 °C until use. For treatment of activated macrophages, cells were treated with GSI (25 μM) or vehicle control DMSO during the priming by IFNγ overnight and the stimulation with LPS.

2.4. Western blotting

Cells were treated as described, and cell lysates were harvested as described previously (Palaga et al., 2008). Upon separation via SDS-PAGE, Notch1 and cleaved Notch1 were detected using rabbit antibodies against Notch1 (C20) (Santa Cruz Biotech, Santa Cruz, CA, USA) and cleaved Notch1 (Val1744) (Cell Signaling Technology, Danvers, MA, USA). RIPA buffer with the addition of phosphatase inhibitor cocktail (Sigma Aldrich) was used to prepare cell lysates to detect phosphoproteins. Antibodies for detecting molecules in the MAPK pathways were from the MAPK and phospho-MAPK family antibody sampler kit (Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG were obtained from GE Healthcare (Buckinghamshire, UK). Signals were detected by chemiluminescence.

For separation of cytoplasmic and nuclear extracts, cells were treated as indicated and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL) were used to prepare the extracts according to the manufacturer's instruction. Western blot was carried out for detecting c-Rel as described above.

2.5. Quantitative real-time RT-PCR (qPCR)

Total RNA was isolated from cells treated as indicated using TRIzol reagent (Invitrogen, Paisley, UK). cDNA was prepared using reverse transcriptase (Fermentas, Glen Burnie, MD, USA) and random hexamers (Invitrogen). qPCR amplifications were performed with 1xMaxima^{TM} SYBR Green/ROX qPCR Master Mix (Fermentas) according to the manufacturer's protocol. Primers specific for *Hes1* have been described previously (Narayana and Balaji, 2008) and primers specific for *Hes5* were designed in this study. Primers used for the amplification of *il12p40*, *il23p19* and *irf5* have also been previously described (Edwards et al., 2006; Krausgruber et al., 2011; Palaga et al., 2008; Tada et al., 2000). β-Actin was used as a reference gene. qPCR was carried out using an MJ Mini personal Thermal cycler (BioRad, USA). The relative gene expression levels