Biochemical and Biophysical Research Communications 464 (2015) 330–335

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

p62 regulates CD40-mediated NFκB activation in macrophages through interaction with TRAF6

Kristina Seibold, Martin Ehrenschwender*

Institute of Clinical Microbiology and Hygiene, University Hospital of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

ARTICLE INFO

Article history:
Received 18 June 2015
Accepted 24 June 2015
Available online 29 June 2015

Keywords:
p62
CD40
CD40L
TRAF6

ABSTRACT

CD40 is a member of the tumor necrosis factor (TNF) receptor family. Activation-induced recruitment of adapter proteins, so-called TNF-receptor-associated factors (TRAFs) to the cytoplasmic tail of CD40 triggers signaling cascades important in the immune system, but has also been associated with excessive inflammation in diseases such as atherosclerosis and rheumatoid arthritis. Especially, pro-inflammatory nuclear factor κB (NFκB) signaling emanating from CD40-associated TRAF6 appears to be a key pathogenic driving force. Consequently, targeting the CD40-TRAF6 interaction is emerging as a promising therapeutic strategy, but the underlying molecular machinery of this signaling axis is to date poorly understood.

Here, we identified the multifunctional adaptor protein p62 as a critical regulator in CD40-mediated NFκB signaling via TRAF6. CD40 activation triggered formation of a TRAF6-p62 complex. Disturbing this interaction tremendously reduced CD40-mediated NFκB signaling in macrophages, while TRAF6-independent signaling pathways remained unaffected. This highlights p62 as a potential target in hyper-inflammatory, CD40-associated pathologies.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily. Together with its ligand CD40L (also known as CD154), this receptor–ligand pair represents an important pathway in the immune system and promotes, for example, isotype switching of immunoglobulins and T cell activation. CD40 is primarily expressed on antigen presenting cells, but can also be found on non-immune cells and various cancer cells. Pathophysiologically, CD40 has been implicated in the pathogenesis of (chronic) inflammatory diseases such as inflammatory bowel disease [1], psoriasis [2] or rheumatoid arthritis [3]. In context of cancer, CD40 signaling seems to be a double-edged sword. Activation of CD40 is on the one hand capable to trigger apoptotic cell death in transformed cells [4]. On the other hand, interaction of CD40-expressing tumor cells with CD40L-expressing T-cells can promote tumor growth by increasing TGFβ production and Th17 differentiation [5]. Understandably, the emerging role of CD40 in disease sparked interest in therapeutic exploitation.

Binding of CD40L induces CD40 homo-trimerization with concomitant recruitment of intracellular adapter proteins called TNF-receptor associated factors (TRAFs) that elicit signaling. CD40 can interact with TRAF2, TRAF3, TRAF5 and TRAF6. Depending on which TRAFs are recruited, CD40 is capable to activate signaling pathways such as nuclear factor κB (NFκB), c-Jun N-terminal kinase (JNK) or the p38 mitogen-activated protein (MAP) kinase (MAPK) pathway [6]. The cytoplasmic tail of CD40 harbors two independent TRAF binding sites [6]. TRAF2, TRAF3 and TRAF5 bind to a membrane distal region, whereas TRAF6 binding occurs at a distinct membrane proximal domain. In macrophages, TRAF6 is an essential mediator of CD40-activated pro-inflammatory pathways such as NFκB [7]. Recent studies reported that genetic ablation of CD40-TRAF6 interaction or small-molecule-mediated inhibition reduced atherosclerotic lesions [8] and obesity-associated insulin resistance in mice [9,10]. Targeting TRAF6-associated NFκB activation is therefore emerging as a potential therapeutic approach. Notably, TRAF6 is also involved in NFκB activation emanating from interleukin-1 receptor (IL1R) [11] and receptor activator of NFκB (RANK) [12], another member of the TNF-receptor family. In these signaling pathways, however, efficient NFκB activation critically depends on complex formation of TRAF6 with the multifunctional protein p62 (also known as sequestosome-1). Mechanistically, p62

* Corresponding author.
E-mail address: martin.ehrenschwender@ukr.de (M. Ehrenschwender).

http://dx.doi.org/10.1016/j.bbrc.2015.06.153
0006-291X/© 2015 Elsevier Inc. All rights reserved.

links TRAF6 to the inhibitor of nuclear factor κB kinase (IKK) complex [11], thereby essentially releasing NFκB from the inhibitory molecule inhibitor of nuclear factor κB α (IκBα). In pro-inflammatory CD40 signaling via the TRAF6 axis, the role of p62 has to date not been addressed. In this study, we showed that ligand-induced activation of CD40 triggered recruitment of p62 and TRAF6 to the cytoplasmic tail of the receptor. Disruption of the TRAF6-p62 complex impaired the CD40-mediated activation of the classical NFκB signaling pathway in murine macrophages, thereby pointing to a (patho-) physiologically important role of p62 in pro-inflammatory CD40 signaling.

2. Materials and methods

2.1. Western blots

Following cell lysis, proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes and incubated with primary antibodies of the specificity of interest. Antigen-antibody complexes were visualized using secondary horseradish peroxidase-conjugated antibodies. Antibodies specific for IκBα, phospho-IκBα, Akt, phospho-Akt, p38 and phospho-p38 were obtained from Cell Signaling (Beverly, MA, USA), anti-TRAF6 and anti-CD40 from Santa Cruz (Santa Cruz, CA, USA), anti-tubulin from Dunn Labortechnik (Asbach, Germany) and anti-p62 from Sigma (Steinheim, Germany).

2.2. Cell culture and generation of murine macrophages

786-O cells were a gift from Harald Wajant (University Hospital of Wuerzburg, Germany) and cultivated in RPMI 1640 medium containing 10% (v/v) fetal calf serum (FCS). Hoxb8-immortalized macrophage progenitors were generated as described previously [13], a plasmid for estrogen-regulated HoxB8 expression (3HA-ERHBH-HoxB8) was provided by Hans Haecker (St. Jude Children's Research Hospital, Memphis, USA). Macrophage progenitors were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, 1 μM β-estradiol, 1% (v/v) L-glutamine, 30 μM 2-mercaptoethanol and 10 ng/mL stem cell factor. Differentiation into macrophages was initiated by estradiol withdrawal and addition of 10% (v/v) M-CSF containing L929-conditioned media for five days. Macrophages were activated by addition of 200 U/mL IFNγ overnight. C57BL/6 mice expressing a truncated p62 variant (deletion of aa 69-251) [14] and corresponding littermate controls were a kind gift of André Gessner (University Hospital of Regensburg, Germany).

2.3. Flow cytometry

Cell surface expression of CD45, CD11b, F4/80 and CD40 was assessed by flow cytometry following standard procedures and using specific antibodies from BD Bioscience (Heidelberg, Germany).

2.4. Recombinant proteins

Human Fc-Flag-CD40L was provided by Harald Wajant (University Hospital of Wuerzburg, Germany) and was produced as described previously [15]. Murine and human CD40L were purchased from ImmunoTools (Friesoythe, Germany). Human TNF was a kind gift from Daniela Maennel (University Hospital of Regensburg, Germany).

2.5. siRNA-transfection

Knockdown of p62 was performed using specific siRNA oligonucleotides (Cell Signaling, Beverly, MA, USA) and Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

2.6. Immunoprecipitation

Immunoprecipitation of Fc-Flag-CD40L was performed as described previously [16]. For immunoprecipitation of p62, cell lysates were cleared by centrifugation, incubated with 5 μg anti-p62 antibody (Sigma, Steinheim, Germany) overnight at 4 °C and precipitated using Protein A agarose beads (Roche, Mannheim, Germany). Beads were washed five times and bound proteins were released in Laemmli sample buffer by heating samples at 96 °C for 5 min.

2.7. Determination of cytokine production

Interleukin-8 (IL8) and IL6 were quantified in collected supernatants using ELISA (BD Bioscience, Heidelberg, Germany) according to manufacturer's instructions.

2.8. Statistics

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Prism, San Diego, CA, USA). All results are depicted as mean ± standard error of the mean (SEM) from at least 3 independent experiments. Differences were considered statistically significant when $p \leq 0.05$ using two-way ANOVA.

3. Results

3.1. CD40 activation induces formation of a TRAF6-p62 complex

In endogenously CD40-expressing 786-O cells (Fig. 1A), immunoprecipitation of Fc-tagged CD40L demonstrated a ligand-induced association with CD40, TRAF2, TRAF6 and p62 (Fig. 1B). The apparently higher molecular weight of CD40 in the immunoprecipitates has been reported previously and most likely reflects mono-ubiquitinylation [16]. Additionally, immunoprecipitation of p62 revealed CD40L-induced recruitment of TRAF6 (Fig. 1C, upper panel). Expectedly, immunoprecipitation of p62 in TNF-treated 786-O cells exhibited no TRAF6 association, as TNF-receptor 1 (TNFR1) does not interact with TRAF6.

CD40 signaling has an important role in macrophages [17] and we therefore also analyzed CD40L-induced TRAF6-p62 interaction in murine macrophages derived from HoxB8-immortalized macrophage progenitors [13]. Typical murine macrophage surface markers such as F4/80, CD11c and CD45 were detectable 5 days after differentiation into macrophages was initiated (Fig. 1D). CD40 expression was also verified by flow cytometry (Fig. 1D). Immunoprecipitation of p62 in CD40L-treated macrophages demonstrated a ligand-induced association with TRAF6 (Fig. 1E). This was in line with our previous results and collectively these data pointed to a CD40L-induced recruitment of p62 to the TRAF6-containing CD40-signaling complex.

3.2. Disruption of the TRAF6-p62 complex differentially affects CD40-mediated NFκB activation in cancer cells and macrophages

To assess the functional role of the TRAF6-p62 complex in pro-inflammatory CD40 signaling, we diminished p62 expression in 786-O cells using siRNA oligonucleotides and examined the

Download English Version:

https://daneshyari.com/en/article/10751860

Download Persian Version:

https://daneshyari.com/article/10751860

Daneshyari.com