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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

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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.

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).

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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

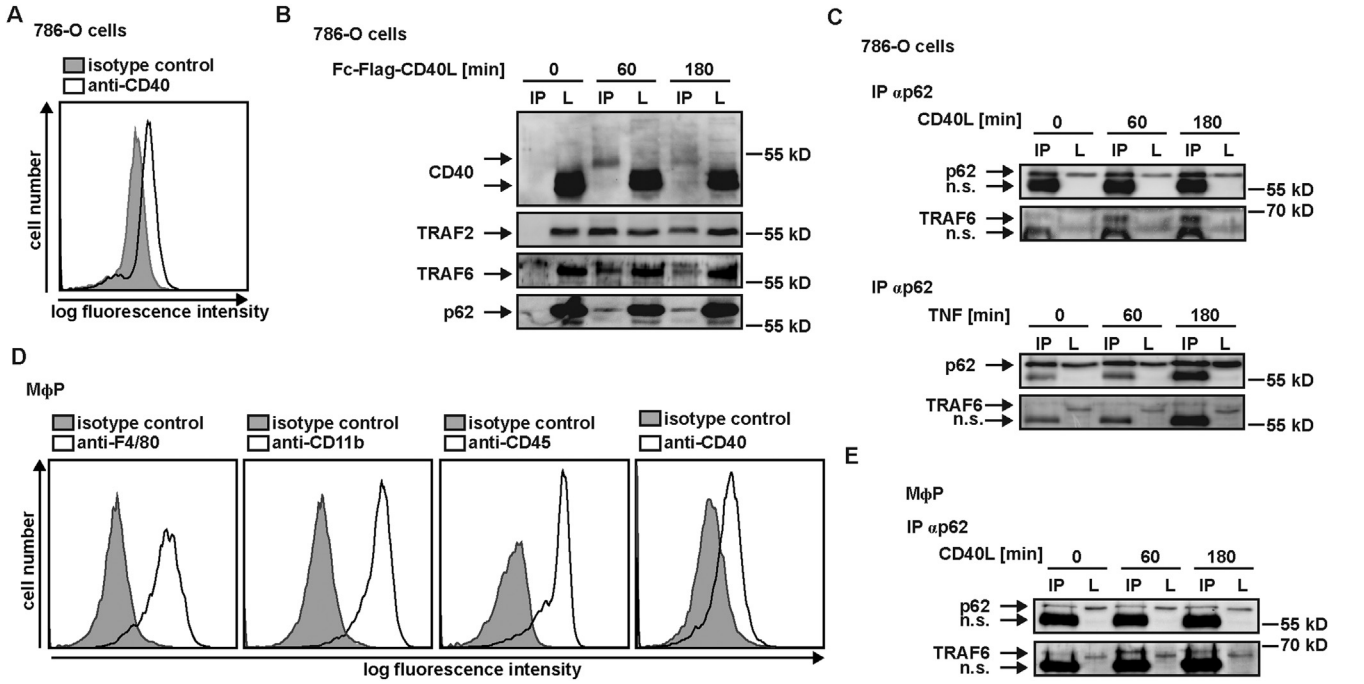

Fig. 1. CD40 activation induces formation of a TRAF6-p62 complex. (A) Cell surface expression of CD40 on 786-O cells was measured using flow cytometry. (B) CD40 signaling complexes in 786-O cells were induced by stimulation with 500 ng/mL Fc-Flag-CD40L for 1 and 3 h. Proteins associated with Fc-Flag-CD40L were immunoprecipitated using protein G sepharose ("IP") and analyzed together with the corresponding lysates ("L") by western blotting for the presence of the indicated proteins using specific antibodies. (C) 786-O cells were challenged with 100 ng/mL CD40L (upper panel) or 500 ng/mL TNF (lower panel) for 1 and 3 h. Following cell lysis, p62 was precipitated using a p62-specific antibody and protein A agarose beads. (D) Expression of characteristic macrophage surface antigens was measured using flow cytometry 5 days after initiating the differentiation of HoxB8-immortalized macrophage progenitors. CD40 expression was measured on IFNγ-activated macrophages ("MΦP"). (E) After differentiation of HoxB8-immortalized progenitors into macrophages ("MΦP"), cells were challenged with 100 ng/mL murine CD40L for 1 and 3 h. Following cell lysis, p62 was precipitated using a p62-specific antibody and protein A agarose beads.

consequences on CD40-mediated activation of the classical NFκB pathway, p38 MAPK and Akt (Fig. 2A). Treatment with p62-specific siRNA significantly reduced p62 levels. Of note, this had no negative effect on CD40L-induced activation of the classical NFκB pathway, as seen by intact phosphorylation of IκBα and unaltered expression of the NFκB target genes interleukin-8 (IL8) and IL6 (Fig. 2B). Additionally, CD40L-induced phosphorylation of p38 and Akt, indicative for activation of the respective pathways, was equally effective in 786-O cells treated with p62-specific or control siRNA.

In macrophages, pro-inflammatory CD40 signaling plays a crucial role in the host response to pathogens [17]. We therefore also addressed the role of the TRAF6-p62 complex in macrophage CD40 signaling. Transient knockdown of gene expression using siRNA oligonucleotides is notoriously difficult in macrophages [18] and genetic deletion of the entire p62 gene is likely to have significant off-target effects as this multifunctional protein is involved in various cellular pathways (reviewed in Ref. [19]). We circumvented these issues by opting for macrophages derived from a gene-targeted mouse model expressing a truncated form of p62 (p62ΔST) [14]. Deletion of aa 69-251 removes the TRAF6 binding site [20], thereby abolishing TRAF6-p62 complex formation. Other domains of p62 with functions in autophagy and ubiquitin

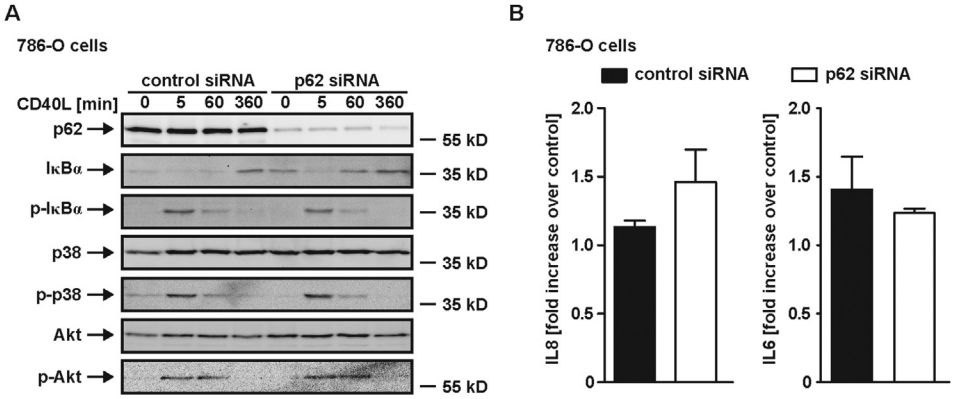

Fig. 2. p62 knockdown in 786-O cells does not negatively affect CD40-mediated NFκB activation. (A) 786-O cells were transfected with the indicated siRNA oligonucleotides. After 48 h, cells were challenged with CD40L (100 ng/mL) for the indicated periods of time. Activation of the classical NFκB-, p38-and Akt-signaling pathways was assessed by western blotting using specific antibodies. (B) 786-O cells were transfected with p62-specific or control siRNA and after 48 h stimulated with CD40L (100 ng/mL). Supernatants were collected after 6 h and levels of IL8 (left panel) and IL6 (right panel) were measured using ELISA. Results are depicted as fold increase over control.

recognition remain intact. In macrophages generated from HoxB8-immortalized progenitors of p62ΔST mice and corresponding littermate controls, p62 was detectable at the protein level in cell lysates (Fig. 3A). As expected, the migration pattern in SDS-PAGE of the truncated p62 variant indicated a lower molecular weight of approximately 43 kD.

We next investigated the impact of defective TRAF6-p62 interaction on CD40-mediated activation of NFκB and p38 signaling in macrophages. In wild-type macrophages, activation of the classical NFκB pathway, indicated by the occurrence of phosphorylated IκBα, was detectable as early as 5 min following CD40L stimulation and lasted for at least 60 min (Fig. 3B). In macrophages expressing p62ΔST, CD40L also triggered early IκBα phosphorylation, but phospho-IκBα levels vanished rapidly and, compared to wild-type macrophages, were significantly reduced 60 min post stimulation. Notably, CD40-mediated activation of p38 MAPK was comparably effective in p62ΔST and wild-type macrophages (Fig. 3B).

This pointed to a reduced capacity of p62ΔST macrophages to activate the classical NFκB pathway via CD40. In line with this, p62ΔST macrophages exhibited reduced ratios of phospho-IκBα/IκBα 60 min and 360 min after challenge with CD40L compared to their wild-type counterparts (Fig. 3C). Additionally, CD40L-induced secretion of IL6, a *bona fide* NFκB target gene [21], was impaired in p62ΔST macrophages (Fig. 3D). To test whether the observed alteration in NFκB signaling was a general phenomenon in p62ΔST macrophages or specifically related to disturbed TRAF6-p62 interaction, we also assessed activation of the classical NFκB pathway via TNFR1 in wild-type and p62ΔST macrophages (Fig. 3E). TNF-triggered phosphorylation of IκBα as a surrogate parameter for ongoing NFκB activation was detectable to a similar extent and with similar kinetics in wild-type and p62ΔST macrophages, which is in line with the TRAF6-independent route of TNFR1-mediated NFκB activation.

In sum, our data indicate a differential role for p62 in CD40-mediated NFκB signaling. CD40L-induced NFκB activation was unaffected in p62-depleted 786-O cells, but disruption of the TRAF6-p62 interaction in macrophages resulted in impaired NFκB activation.

4. Discussion

CD40 signaling has been implicated in the development and progression of inflammatory and autoimmune diseases (reviewed in Ref. [17]). In macrophages, CD40L-induced NFκB signaling and production of IL6 critically depends on interaction of CD40 with TRAF6, as CD40 constructs lacking the TRAF6 binding domain exhibited a dramatically reduced pro-inflammatory capacity [7]. Recent studies further substantiated these findings *in vivo* by demonstrating that inhibition of CD40-TRAF6 signaling significantly ameliorated the course of inflammation-driven diseases such as atherosclerosis and obesity-induced insulin-resistance. Notably, interfering with CD40-TRAF2/-TRAF3/-TRAF5 interaction had no beneficial effect [8,9]. Targeting the CD40-TRAF6 interaction (e.g. with small molecules [10]) or inhibiting TRAF6 function could therefore represent a promising therapeutic approach. Interestingly, TRAF6 critically depends on interaction with p62 for efficient induction of pro-inflammatory IL1R- and RANK-signaling, as p62 links TRAF6 to the NFκB-activating IKK complex [11,12]. We therefore hypothesized that p62 might also be involved in TRAF6-dependent CD40 signaling.

The observed CD40L-induced TRAF6-p62 interaction in the renal adenocarcinoma cell line 786-O (Fig. 1C, upper panel) and in macrophages (Fig. 1E) corroborated our hypothesis and was reminiscent of an earlier study, reporting RANK-ligand-induced RANK-p62 interaction [12]. Surprisingly, siRNA-mediated downregulation of p62 in 786-O cells did not negatively affect CD40L-induced activation of NFκB, p38 and Akt (Fig. 2). This could potentially reflect an incomplete reduction of p62 to levels that still allow TRAF6 function, despite the western blot analysis suggested high knock-down efficacy (Fig. 2A). Additionally, depending on the cell

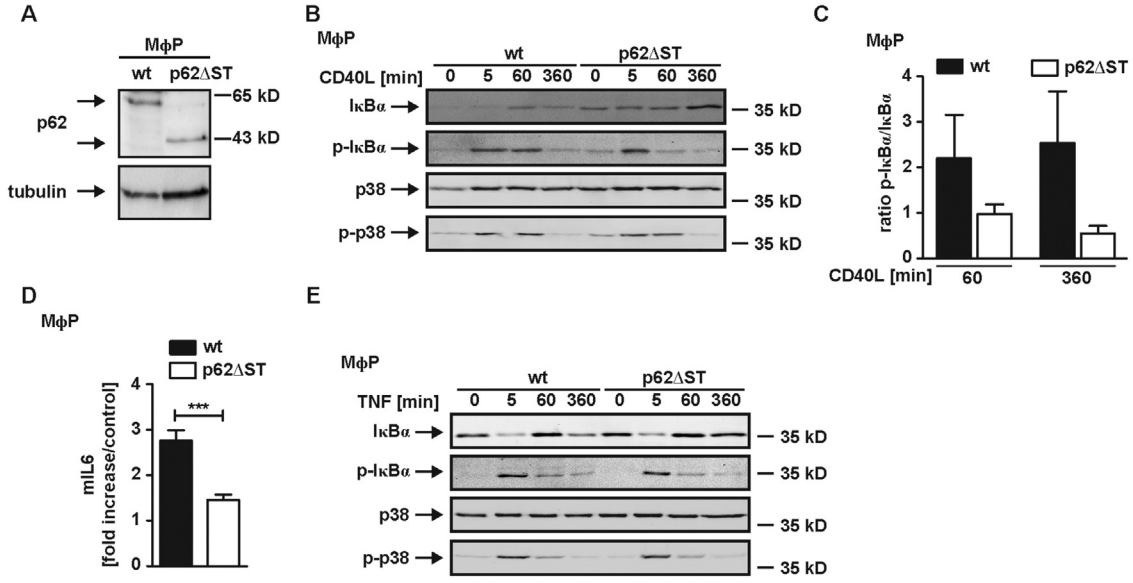

Fig. 3. Disruption of the TRAF6-p62 complex impairs classical NFκB signaling in macrophages. (A) p62 expression in macrophages ("MΦP") derived from HoxB8-immortalized progenitors of wild-type and p62ΔST mice was analyzed by western blot. The migration pattern of p62ΔST was in good agreement with the expected molecular weight. (B, E) Macrophages ("MΦP") derived from HoxB8-immortalized progenitors of wild-type and p62ΔST mice were activated with IFNγ and subsequently stimulated with (B) 100 ng/mL murine CD40L or (E) 100 ng/mL TNF for the indicated periods of time. Activation of the classical NFκB pathway and p38 signaling was analyzed by western blotting using specific antibodies for the indicated proteins. (C) Densitometric analysis of IκBα and phospho-IκBα levels from (B) and two additional independent experiments. (D) Wild-type and p62ΔST macrophages ("MΦP") were generated as previously, activated with IFNγ and subsequently stimulated with 100 ng/mL murine CD40L. Supernatants were collected after 6 h and levels of murine IL6 were measured using ELISA. Results are depicted as fold increase over control. *** indicates statistical significance with $p \leq 0.05$ using two-way ANOVA.

type, partly redundant functions of TRAF2 and TRAF6 in CD40 signaling have been reported. In non-hemopoietic cells, CD40-mediated NFκB and JNK activation depended on intact CD40-TRAF2 binding. Disruption of CD40-TRAF6 interaction reduced, but not abolished pro-inflammatory CD40 signaling [22]. In line with this, CD40-mediated NFκB activation was preserved in a murine B cell line lacking TRAF6, but defective when both TRAF2 and TRAF6 were absent [23]. Therefore, our observation that CD40L-induced NFκB signaling was intact in p62 depleted 786-O cells potentially points to an alternative, TRAF2-dependent mechanism of CD40-mediated NFκB activation in these cells. In agreement with our data, initiation of the NFκB pathway via TRAF2 remains functional even if the CD40-TRAF6 axis is compromised through the absence of p62.

However, a previous study elegantly demonstrated that macrophages do not exhibit a functional redundancy of TRAF proteins in the activation of pro-inflammatory pathways. Deletion of the TRAF2/TRAF3/TRAF5 binding site in the cytoplasmic tail of CD40 had no negative effect on CD40-mediated production of inflammatory cytokines [7], whereas NFκB activation was abrogated in macrophages expressing CD40 constructs defective in TRAF6 binding. Macrophages obviously fail to compensate a non-functional CD40-TRAF6 axis via TRAF2-mediated pro-inflammatory CD40 signaling, thereby providing an excellent model to address the functional role of p62 in the CD40-TRAF6 interaction. Notably, p62 is as a multifunctional adapter protein involved in a variety of pathways (e.g. autophagy, signal transduction cascades and tumorigenesis; reviewed in Ref. [19]) which renders a permanent full-knockout likely to result in off-target effects. To circumvent this potential limitation, we generated macrophages from a gene-targeted mouse expressing a truncated p62 variant (p62ΔST) [14]. Deletion of aa 69–251 removes the TRAF6 binding site [20], whereas the domains involved in ubiquitin recognition and autophagy remain functional.

In agreement with our hypothesis, the disturbed TRAF6-p62 interaction in p62ΔST-macrophages resulted in reduced CD40L-induced NFκB activation (Fig. 3B, C and D). Although early (5 min) CD40L-induced phosphorylation of IκBα was comparable in wild-type and p62ΔST macrophages, p62 deficiency caused impaired phospho-IκBα levels 60 min and 360 min following CD40L challenge concomitant with reduced production of the NFκB target gene IL6. It is tempting to speculate that the preserved rapid CD40L-induced IκBα phosphorylation in p62ΔST macrophages is attributable to a biphasic pro-inflammatory cytokine-induced NFκB activation with differential involvement of IKK complexes and other IκB molecules, as reported in previous studies [24,25].

Taken together, our data highlight p62 as a novel player in CD40L-induced, CD40-TRAF6-mediated NFκB activation in macrophages and enhance our understanding of pro-inflammatory CD40-signaling. Perspectively, targeting the CD40-TRAF6-p62 signaling axis could represent a promising therapeutic approach to attenuate pathologic inflammatory processes. A recent study using small molecules to interfere with CD40-TRAF6 interaction already revealed promising data in a mouse model of obesity-induced insulin resistance [10]. Perspectively, targeting TRAF6-p62 interaction could be equally fruitful.

Acknowledgments

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