Agonist antibody activates death receptor 6 downstream signaling involving TRADD recruitment

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ABSTRACT

Death receptor 6 (DR6) is a member of the death domain-containing receptors that belong to the TNFR superfamily. To date, the ligand for DR6 is still not clearly defined. Here, we developed a functional agonist monoclonal antibody (DQM3) against DR6, which bound to the first cysteine-rich domain. Importantly, DR6 signaling could be clearly activated by DQM3, which was dependent on its intracellular death domain. In addition, we demonstrated that the association between DR6 and TRADD was enhanced upon DQM3 stimulation and TRADD was involved in DR6-induced signaling activation. Taken together, our findings provide new insight into a novel mechanism by which DR6 induces downstream signaling in response to an agonist antibody.

1. Introduction

Death receptors are a subset of the tumor necrosis factor (TNF) receptor superfamily characterized by the presence of a conserved death domain (DD) in their cytoplasmic regions [1,2]. There are eight death receptors which have been identified in humans, including TNFR1, Fas (DR2, CD95 or APO-1), DR3, TRAIL-R1 (DR4), TRAIL-R2 (DR5), DR6, p75NTR (NGFR) and EDAR. In addition to primarily inducing apoptosis, engagement of death receptor under certain circumstances may initiate multiple non-apoptotic signaling pathways, including activation of nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAPK) cascades, which promote the regulation of cell differentiation, proliferation, cytokine and chemokine production, and the modulation of immune responses [3,4].

Abbreviations: CRD, cysteine-rich domain; DD, death domain; DR, death receptor; FADD, Fas-associated death domain; JNK, c-Jun N-terminal kinase; N-APP, N-terminal amyloid precursor protein; siRNA, small interfering RNA; TNFR, tumor necrosis factor receptor; TRADD, TNFR-associated death domain protein; TRAF, tumor necrosis factor receptor-associated factor

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2. Materials and methods

2.1. Reagents and plasmid construction

The full-length DR6 (DR6-FL) and DR6 mutants including extracellular DR6 (DR6-EX), DR6 (1-414), DR6 (1-498), ATRAF (Δ371-414) and ΔDD (ΔA15-498) were cloned into mammalian expression vector. DR6 mutants DR6ΔCRD1-4, DR6ΔCRD2-1, DR6ΔCRD2, DR6ΔCRD1, DR6ΔA77-88 and DR6ΔA50-66 were generated from pIRE2-EGFP-DR6-FL. A 5’ kbp fragment of the human IL-6 gene promoter was amplified from human genomic DNA and ligated into the vector pGL4.20 (Promega). pNF-κB-Luc was purchased from Clontech. The full-length TRADD and dominant negative form of TRADD (TRADD-DN) lacking the death domain were epitope tagged with an N-terminal Flag tag and cloned into the vector pT73.

Mouse monoclonal antibodies against human DR6 were generated using the standard techniques from splenocytes of mice immunized with the protein DR6-EX fused with human IgG1-Fc expressed in HEK293T cells. The rabbit polyclonal anti-TRADD antibody was purchased from Abcam. The mouse monoclonal anti-Flag M2 antibody was from Sigma. Antibodies against phospho-SAPK/JNK (Thr183/Tyr185) and SAPK/JNK were purchased from Cell Signaling Technology. Mouse monoclonal antibodies against human DR6 were generated using the standard techniques from splenocytes of mice immunized with the protein DR6-EX fused with human IgG1-Fc expressed in HEK293T cells. The rabbit polyclonal anti-TRADD antibody was purchased from Abcam. The mouse monoclonal anti-Flag M2 antibody was from Sigma. Antibodies against phospho-SAPK/JNK (Thr183/Tyr185) and SAPK/JNK were purchased from Cell Signaling Technology. The mouse monoclonal anti-Flag M2 antibody was from Sigma. Antibodies against phospho-SAPK/JNK (Thr183/Tyr185) and SAPK/JNK were purchased from Cell Signaling Technology.

2.2. Cell culture and transfection

The human embryonic kidney cell line HEK293T was cultured in a 5% CO2 incubator at 37 °C with complete DMEM (Gibco) medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine and 100 U/ml penicillin/streptomycin. For transfection, HEK293T cells were transfected with indicated plasmids using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Empty vector was used to equalize the total amount of DNA in all transfections. Subsequently, reporter gene assays, flow cytometry analysis and co-IP experiments were preformed.

2.3. Flow cytometry analysis

The cells for flow cytometry analysis are harvested and incubated on ice with blocking buffer for 20 min, and then incubate the cells with anti-DR6 mAbs (5 μg/ml) or isotype control antibodies (BioLegend) on ice for 30 min. Add the PE-conjugated goat anti-mouse IgG (BioLegend) to the cells and incubate in the dark on ice for 30 min. Add the viability dye 7-AAD (Invitrogen) to each sample to exclude dead cells from analysis. Wash the stained cells once and resuspend in staining buffer prior to flow cytometry analysis on FACS Calibur (BD Biosciences). Data were analyzed using Summit software.

2.4. Luciferase assay

HEK293T cells in 24-well plates were transiently transfected with reporter plasmid encoding the firefly luciferase gene under control of the NF-κB or IL-6 promoter together with various expression plasmids for DR6 or DR6 mutants. The pRL-TK encoding Renilla luciferase served as an internal transfection control. Anti-DR6 mAb was added 24 h after transfection, and isotype control antibody also served as a negative control. 16 h later, luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

2.5. RNA interference

Double strand siRNA oligonucleotides targeting human TRADD were purchased from GeneChem (Shanghai, China), and the sequences are: sense, 5’-GGAGGAUGCGCUGCGAAAUUU-3’; antisense, 5’-AAUUUCGCCACGCAUUCCUC-3’ [15]. The siRNA with a scrambled sequence was used as a negative control. HEK293T cells were transfected with siRNA using Lipofectamine 2000 according to the manufacturer’s instructions.

2.6. Co-immunoprecipitation and Western blot analysis

For co-immunoprecipitation, 2 x 10^6 of HEK293T cells were transfected with the indicated expression plasmids for DR6-FL or DR6-EX and flag-tagged TRADD. 24 h later, anti-DR6 mAb was added for stimulation for the indicated times. After treatment, cells were washed twice with cold PBS and then lysed for 30 min on ice in 500 μl of lysis buffer. The lysates were precipitated with anti-DR6 mAb overnight at 4 °C. Then, Protein G beads (GE Health) were added and incubated for 4 h at 4 °C under gentle rotation. The beads were washed five times with lysis buffer and eluted by boiling the beads for 5 min in 2 x SDS loading buffer. The eluted proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes followed by immunoblot analysis with the indicated antibodies.

2.7. Statistical analysis

GraphPad Prism software was used for statistical analysis. All samples were performed in at least duplicate, with each experiment repeated at least three times. Data are shown as mean ± s standard errors of mean (S.E.M.) of three independent experiments. Differences between groups were determined using an unpaired, two-tailed, Student’s t test. P-values < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)) were considered statistically significant.

3. Results

3.1. DR6 induces the activation of NF-κB

In addition to inducing apoptosis in some cell lines, DR6 was also reported to induce NF-κB activation [5]. Overexpression of death receptors can mimic ligand activation and trigger receptor activation and downstream signaling events. Consistent with previous observation, we found that in transfection studies, DR6-FL induced NF-κB activation in a dose dependent manner, but DR6-EX lacking the intracellular domain did not (Fig. 1A). IL-6 represents a pleiotropic inflammatory cytokine, which is predominantly regulated by NF-κB. We wondered whether DR6 could induce the activation of target gene regulated by NF-κB. As shown in Fig. 1B, overexpression of DR6-FL resulted in significantly upregulation of IL-6 luciferase activity (up to 6-fold) in a dose dependent manner. However, in contrast to DR6-FL, DR6-EX had no obvious effect on IL-6 activation, indicating that DR6-induced downstream signaling activation is dependent on cytoplasmic domain.

3.2. DR6 mAbs bind to different antigenic epitopes in CRD domain

Although the N-APP has been reported to function as a DR6 ligand, triggering axon pruning, neuron death and axonal degeneration, results from two other groups indicate that the N-APP may not activate DR6 in other cell types[13,14]. Accordingly, we also demonstrated that N-APP had no effect on DR6-induced NF-κB and IL-6 activation in HEK293T cells (data not shown). Therefore, we generated three monoclonal antibodies (DQM2, DQM3 and DQM5) against DR6, which could bind HEK293T cells transfected with DR6-FL (Fig. 2A).
DR6, a type I transmembrane protein, has four highly conserved cysteine-rich domains (CRDs) and a stalk region in the extracellular portion [16]. To investigate the binding sites of anti-DR6 mAbs, we constructed a set of DR6 mutants with deletion of certain extracellular domains (Fig. 2B). As shown in Fig. 2C, in flow cytometry analysis with HEK293T cells expressing DR6 mutants, all three mAbs could still bind ΔCRD3-4, but not ΔCRD1-2 (top two panels). Additionally, we found these mAbs could bind ΔCRD2, but not ΔCRD1 (third and fourth panels), suggesting that the binding sites were located on CDR1. To narrow down the binding sites of these mAbs, further studies showed that DQM3 and DQM5 could still bind DR6Δ67-88, while DQM2 could not (fifth panel). It was worth to point out that the mutant DR6Δ67-88 severely decreased the cell surface localization of DR6 and led to its intracellular retention (data not shown). Moreover, all three anti-DR6 mAbs could not bind DR6Δ50-66 (bottom panel). Additionally, the deletion of stalk region between CRDs and the transmembrane domain did not affect their binding (data not shown). Taken together, these results
demonstrate that all three antibodies bind to the CRD1 of DR6, and the binding sites of DQM3 and DQM5 are located on amino acids 50-66 of CRD1.

3.3. DQM3, but not DQM2 and DQM5, activates DR6 downstream signaling

We next asked whether DR6 could induce increased activation of downstream signaling upon anti-DR6 mAbs stimulation, and we sought to compare the effects of three anti-DR6 mAbs on DR6-induced signaling activation by reporter gene assays. As shown in Fig. 3A, only DQM3 could upregulate IL-6 promoter-driven luciferase activity, while DQM2 and DQM5 could not. In addition, DR6-induced IL-6 promoter activation was shown in a dose-dependent manner in response to DQM3 stimulation (Fig. 3B). These results indicate that the DQM3 may serve as an agonist antibody specific to DR6, which will be used in further dissecting the downstream signaling of DR6.

To further understand the potential mechanism through which DR6 mediates downstream signaling, we next examined the impact of DR6 on NF-κB and JNK activation. DR6-FL induced activation of both JNK and NF-κB, as indicated by the phosphorylation of JNK and NF-κB inhibitor IκBα (Fig. 3C). However, DR6-EX showed considerably impaired induction of NF-κB, but not JNK activation (Fig. 3C and Supplementary Fig. 1A), suggesting that DR6-induced NF-κB activation is intracellular domain dependent, whereas JNK activation is not. Furthermore, DQM3 stimulation could enhance DR6-induced NF-κB activation (Fig. 3D, third panel). In contrast, the JNK pathway was not affected upon ligation of DQM3, as demonstrated by the similar level of phosphorylated JNK (Fig. 3D and Supplementary Fig. 1B).

3.4. Death domain is essential for DR6-induced signaling upon the agonist antibody stimulation

The death domain is an essential protein-binding domain that accounts for the ability of TNFR1 to induce the activation of the NF-κB pathway and activation of the apoptotic caspase cascade [17]. In order to investigate the mechanism by which DR6 transmits signals upon the agonist antibody stimulation, a panel of DR6 mutants were constructed (Fig. 4A and Supplementary Fig. 2). They were designed to delete large portions of DR6 intracellular domains responsible for cytosolic signal propagation. Signal activation induced by DR6 truncations was assessed by luciferase reporter assay. In response to DQM3 stimulation, DR6-FL resulted in a 3.6-fold increase of IL-6 promoter activity over removal of cytoplasmic domain (DR6-EX) (Fig. 4B). In contrast to DR6-EX, DR6 (1-414) had no effect on induction of IL-6 activity in cells treated with DQM3. However, removal of the N-terminal fragment following DD termed DR6 (1-498), and deletion of the TNFR-associated factor (TRAF) binding motif (ΔTRAF) had almost the same ability as DR6-FL to induce IL-6 promoter activation upon DQM3 stimulation (Fig. 4B). Interestingly, deletion of the DD (ΔDD) showed considerably impaired induction of IL-6 promoter activity comparing to DR6-FL. These results suggest that death domain is essential for DR6-induced downstream signaling upon the agonist antibody stimulation.

3.5. TRADD is involved in DR6-induced signaling activation

Ligation of TNFRI by TNF recruits the TNFR associated death domain (TRADD), which serves as the adaptor protein [17]. Pan et al. have demonstrated that DR6 doesn't interact with the adaptor

![Fig. 3](image-url)
protein FADD, but has a weak interaction with TRADD [5]. However, whether TRADD is involved in DR6 signaling is still unknown. We showed that transfection of TRADD alone resulted in induction of IL-6 promoter activity, but TRADD-DN did not (Fig. 5A). Furthermore, in cells with co-transfection of DR6 and TRADD, the IL-6 promoter activity increased significantly up to 6-fold over transfection of DR6 alone, but the cells co-transfected with DR6 and TRADD-DN did not have clear effect (Fig. 5B). We found that DR6-induced IL-6 activity could not be further upregulated upon DQM3 stimulation, suggesting TRADD was sufficient for the signaling pathway induced by DR6 (Fig. 5C).

To bolster the above findings, we examined whether TRADD deficiency had effects on DR6-induced signaling activation. We used siRNA targeting TRADD to suppress its expression in HEK293T cells (Fig. 5D) and then examined the ability of DR6 to activate IL-6 luciferase reporter in response to DQM3 stimulation. As shown in

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**Fig. 4.** Death domain is essential for DR6-induced signaling upon DQM3 stimulation. (A) Schematic representation of DR6 mutants used in this study. The deletion mutants targeting intracellular domain include DR6-EX (1-370), DR6 (1-414), DR6 (1-498), DR6-ΔTRAF (Δ371-414) and DR6-ΔDD (Δ415-498). (B) HEK293T cells were transfected with IL-6 reporter plasmid alone or together with a series of expression vectors encoding DR6 or its mutants as indicated above. 24 h later, cells were stimulated with DQM3 or isotype control antibody. Cell lysates were prepared 16 h after antibody incubation and relative luciferase activity of stimulus versus control was calculated as the mean ± S.E.M. Similar results were obtained in three independent experiments. **P < 0.001; *P < 0.01; N.S., not significant.

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**Fig. 5.** TRADD is involved in DR6-induced IL-6 activation. (A and B) HEK293T cells were transiently transfected with IL-6 reporter plasmid, Renilla, and increasing amounts of expression vectors for TRADD or TRADD-DN (A) or together with DR6-FL (B). 24 h later, promoter activity was measured by luciferase assay. (C) HEK293T cells were co-transfected with IL-6-Luc, Renilla, DR6-FL and TRADD as described in (B), and then stimulated with DQM3. 16 h later, luciferase activity was measured. (D) HEK293T cells were co-transfected with TRADD siRNA (si-TRADD) or negative control siRNA (si-NC) and expression plasmid for TRADD. Western blot analysis was performed to verify the efficiency of siRNA. (E) TRADD siRNA-treated cells were transfected with IL-6 reporter plasmid and DR6-FL. 24 h after transfection, cells were subjected to DQM3 stimulation for 16 h, and then luciferase activity was monitored as described above. Results are shown as the mean ± S.E.M. **P < 0.05; ***P < 0.01.
Fig. 5E, silencing the expression of TRADD could significantly inhibit the DR6-induced IL-6 promoter activity in cells treated with control antibody or DQM3. The effect of DQM3 ligation on siRNA-control group (1.6-fold) was comparable to that of siRNA-TRADD group (1.4-fold). These data further support our conclusion that TRADD is critically involved in DR6 signaling both in the presence and absence of DQM3.

3.6. TRADD is a putative adaptor molecule in DR6 downstream signaling

Previous studies demonstrate that TRADD is a crucial signal adapter molecule that mediates intracellular responses from TNFR1 [18,19]. To further investigate a potential role for TRADD in DR6 signaling, we sought to confirm the possible interaction of DR6 with TRADD by co-immunoprecipitation. As shown in Fig. 6A, TRADD was only co-immunoprecipitated with DR6 in cells induced for DR6-FL expression (lane 3, second panel), but not in cells with induction of DR6-EX expression (lane 4). These data suggest that DR6 interacts with TRADD in HEK293T cells.

It has been shown that the death domain-containing C-terminus of TRADD (195–312) was essential and sufficient for interaction with the DD of TNFR1 and induction of apoptosis and NF-κB activation [17]. To further validate the interaction of TRADD with DR6, HEK293T cells were transfected with DR6 and TRADD or TRADD-DN, and the association was determined as described above. As shown in Fig. 6B, the results demonstrated that TRADD, but not TRADD-DN co-immunoprecipitated with DR6 (compare lane 1 with lane 2, second panel), indicating that the DD of TRADD was necessary for the interaction between DR6 and TRADD.

Next, we asked whether DQM3 could enhance the association between DR6 and TRADD. As shown in Fig. 6C, the time course study indicated that DR6–TRADD interaction could be enhanced as soon as 15 min of DQM3 stimulation (second panel). Taken together, these findings demonstrate that there is a possibility that TRADD serves as an adaptor molecule that mediates DR6 downstream signaling.

4. Discussion

Death receptor 6, characterized by the intracellular death domain, belongs to the TNF receptor superfamily. DR6 has been shown to play an apparent role in regulating transcription factor activation, apoptosis, maturation and immune responses. Upon ligand binding and activation, members of the death receptor family trigger different signaling pathways. Previous studies demonstrate that DR6 is highly expressed in human brain and it has been shown to induce axon pruning, neuron death and axonal degeneration through binding to its recently described ligand N-APP [12]. However, Klíma and colleagues showed that N-APP could not bind to DR6 and mediate the activation of NF-κB and JNK [13]. In particular, a recently published report proposed a potential role of DR6 in the regulation of oligodendrocyte survival, maturation and myelination through a mechanism independent of N-APP [14]. We also found that N-APP had no effect on DR6-induced NF-κB and IL-6 promoter activation, and it was difficult to definitively address the molecular signaling mechanisms of this receptor. Thus, we generated a functional agonist antibody against DR6, which could serve as a useful tool in the studies on DR6-induced signaling pathways. Importantly, we showed that DR6 signaling could be clearly activated by this agonist antibody, which was dependent on the death domain.

Upon binding of their specific ligands, DD-containing adaptor proteins are recruited by death receptors leading to the assembly

![Figure 6](https://example.com/fig6.png)

**Fig. 6.** DR6 interacts with TRADD in co-immunoprecipitation assays. (A and B) HEK293T cells in 60-mm dishes co-transfected with DR6-FL/EX and Flag-tagged TRADD or TRADD-DN were harvested 48 h after transfection. Total cell lysates were immunoprecipitated with anti-DR6 (DQM3) and immunoblotted with anti-TRADD (A) or anti-Flag (B). Western blot analysis of DR6, TRADD and Flag was performed on whole cell lysates as a control (Input). (C) HEK293T cells were co-transfected with expression vectors for DR6-FL and Flag-tagged TRADD, and then stimulated or not with DQM3 for the indicated times. The interaction of TRADD with DR6 was assessed after DQM3 stimulation by co-immunoprecipitation. The immunoprecipitates and cell extracts were analyzed by immunoblot. Results representative of at least three independent experiments are shown. β-Actin was used as loading control for each fraction. *, Heavy chain (DQM3); IP, immunoprecipitates; IB, immunoblot.
of protein complex that serves as a binding platform for signal-transducing proteins. It has been demonstrated that TRADD, the first protein recruited to TNFR1, serves as a central adaptor to recruit additional mediators such as the serine/threonine kinase RIP and TRAF2, activating NF-κB and JNK survival pathways [17,20,21]. We hypothesized it was likely that DR6 mediated downstream signaling using a similar mechanism as TNFR1. In this study, we showed that TRADD was also involved in DR6-induced signaling, which was significantly inhibited when the expression of TRADD was silenced (Fig. 5E). To further dissect the role of TRADD in DR6-induced signaling activation, we confirmed the physical interaction of DR6 with TRADD. Nevertheless, further studies are still needed to discover other unknown mediators involved in DR6 signaling.

Mimicking ligand induced receptor activation by overexpressing DR6 in some cell lines such as HeLa and HEK293 cells demonstrated that DR6 could induce apoptosis. Moreover, DR6-induced apoptosis was independent of FADD and could be inhibited by the caspase inhibitor Z-VAD [22]. Recently, it has been shown that DR6 induces apoptosis not through type I or type II pathways of Fas-mediated apoptosis, but via a unique pathway that exclusively depends on the mitochondrial pathway and probably through interacting with Bax [23]. In our study, we found that DR6 induced more cell death if co-transfected with TRADD or stimulated with the agonist antibody in HEK293T cells (data not shown).

Various types of cancer cells express death receptors and activation of them on tumor cells by agonists can exert antitumor activity [24]. Wilson et al. detected DR5 expression in tumor endothelial cells, and treatment of tumor-bearing mice with DR5 ligand-induced apoptosis, which disrupts the integrity of tumor blood vessels and decreases tumor growth [25]. DR6 is highly expressed on many tumor cell lines and clinical tumor samples [5,22,26]. Therefore, DR6 signaling may play a crucial role in tumorigenesis by affecting the generation of anti-tumor activity. Understanding the mechanism of DR6-induced signaling pathway makes DR6 to be a potential therapeutic target for treating inflammatory and autoimmune disease or cancer [27,28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.12.010.

References
