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# An association between RBMX, a heterogeneous nuclear ribonucleoprotein, and ARTS-1 regulates extracellular TNFR1 release

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ABSTRACT

The type I, 55-kDa tumor necrosis factor receptor (TNFR1) is released to the extracellular space by two mechanisms, the constitutive release of TNFR1 exosome-like vesicles and the inducible proteolytic cleavage of TNFR1 ectodomains. Both pathways appear to be regulated by an interaction between TNFR1 and ARTS-1 (aminopeptidase regulator of TNFR1 shedding). Here, we sought to identify ARTS-1-interacting proteins that modulate TNFR1 release. Co-immunoprecipitation identified an association between ARTS-1 and RBMX (RNA-binding motif gene, X chromosome), a 43-kDa heterogeneous nuclear ribonucleoprotein. RNA interference attenuated RBMX expression, which reduced both the constitutive release of TNFR1 exosome-like vesicles and the IL-1β-mediated inducible proteolytic cleavage of soluble TNFR1 ectodomains. Reciprocally, over-expression of RBMX increased TNFR1 exosome-like vesicle release and the IL-1β-mediated inducible shedding of TNFR1 ectodomains. This identifies RBMX as an ARTS-1-associated protein that regulates both the constitutive release of TNFR1 exosome-like vesicles and the inducible proteolytic cleavage of TNFR1 ectodomains.

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Tumor necrosis factor (TNF, TNFSF2) activity can be regulated by extracellular TNF receptors that function as TNF-binding proteins. The 55-kDa, type I TNF receptor (TNFR1) can be released from cells by two pathways. The first involves proteolytic cleavage of TNFR1 ectodomains to generate 27–34 kDa soluble TNFR1 (sTNFR1) [1]. The second involves the constitutive release of full-length TNFR1 within the membranes of 20–50 nm exosome-like vesicles that are capable of binding TNF [2]. The translocation of intracytoplasmic TNFR1 vesicles appears to play an important role in both pathways. We previously identified ARTS-1 (Aminopeptidase Regulator of TNFR1 Shedding), also known as endoplasmic reticulum-associated aminopeptidase 1, as a type II integral membrane protein that binds full-length TNFR1 and regulates both the constitutive release of TNFR1 exosome-like vesicles and the IL-1β-mediated inducible proteolytic cleavage of soluble TNFR1 ectodomains [3,4]. Nucleobindin 2 (NUCB2), a putative DNA- and calcium-binding protein, was subsequently identified as a calcium-dependent ARTS-1 binding partner that associates with cytoplasmic TNFR1 prior to its commitment to either release pathway [3]. Here, we sought to identify additional ARTS-1-associated proteins that regulate TNFR1 release. Using a mass spectroscopy approach, we show that RBMX (RNA-binding motif protein, X-linked), a 43-kDa RNA-binding motif protein (heterogeneous nuclear ribonucleoprotein (hnRNP) G), associates with ARTS-1 and regulates both the constitutive release of TNFR1 exosome-like vesicles and the IL-1β-mediated inducible proteolytic cleavage of TNFR1 ectodomains. This identifies an unexpected role for a hnRNP in the regulation of TNFR1 release pathways.

## Methods

*Cells and reagents*. NCI-H292 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA). HUVEC were grown in EGM-2 medium (Cambrex, East Rutherford, NJ). Recombinant human IL-1β was from R&D Systems (Minneapolis, MN). Goat polyclonal anti-RBMX, murine monoclonal anti-TNFR1 (H5), and murine monoclonal anti- β-tubulin (D10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

*Immunoblotting*. Immunoblotting was performed as previously described [3,4]. For immunoblots of HUVEC-conditioned medium, cells were grown in exosome-depleted medium. For immunoblots of HUVEC-conditioned medium following IL-1β stimulation, cells were incubated in 1 ml of EGM-2 medium without fetal bovine serum or supplements for 2 h. Proteins present in 0.5 ml of conditioned

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medium were precipitated with 10% trichloroacetic acid. Densitometry was performed using NIH Image Software (version 1.63).

*Immunoprecipitations*. Immunoprecipitations of NCI-H292 cellular proteins were performed as previously described [4]. Samples of membrane proteins (1 mg) were incubated overnight at 4 °C with 1 μl of rabbit anti-ARTS-1 antibody or non-immune serum, followed by addition of 200 μl of immobilized protein A/G beads (Pierce, Rockford, IL) for 2 h. After washing six times in lysis buffer, bound proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue R-250. Bands were excised and proteins were identified by MALDI-MS peptide mass mapping.

HUVEC were lysed in buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, and 0.1% Triton X-100, with aprotinin (2 μg/ml) and AEBSF (1 mg/ml). Protein A/G beads were blocked with 1% ovalbumin and incubated with 1 μl of rabbit anti-ARTS-1 antibody or pre-immune serum. Samples (300 μg) of HUVEC lysates were treated with or without SUPERase-In RNase inhibitor (1 U/μl) or RNase cocktail (0.4 U/μl), which contains RNases A and T1 (Ambion, Austin, TX). Lysates were incubated with antibody-coated protein A/G beads, washed with ice-cold PBS, followed by Western blotting. Proteins remaining in supernatants were precipitated with 10% trichloroacetic acid prior to Western blotting.

*RNA interference and quantitative real-time RT-PCR*. The RBMX siRNA duplex (GUGGAAGUCGAGACAGUUAUU and UAACUGUC UCGACUUCCACUU), was purchased from Dharmacon (Lafayette, CO). The control siRNA duplex that targets green fluorescent protein (GFP) was purchased from Qiagen-Xeragon (Germantown, MD). HUVEC were transfected with 25 nM siRNA using DharmaFECT #1 (Dharmacon, Chicago, IL) and assayed 2 days post-transfection.

*RBMX expression plasmid*. The full-length coding sequence of RBMX was generated by RT-PCR of HUVEC mRNA, cloned into pcDNA3.1/V5-His TOPO TA (Invitrogen), and sequence verified. HUVEC, grown in 6-well plates, were transfected with plasmids using FuGENE 6, (Roche, Indianapolis, IN), as previously described [5].

*Quantification of extracellular TNFR1 by ELISA*. To assess constitutive TNFR1 release, HUVEC were transfected with siRNA targeting RBMX or GFP for 1 day and fresh, exosome-depleted medium was added for an additional 24 h. To assess IL-1β-induced TNFR1 release, HUVEC were transfected with siRNA targeting RBMX or GFP for 2 days and medium with IL-1β, but without FBS or supplements, was added for 2 h. Conditioned medium was collected and cleared of cells and debris prior to TNFR1 quantification using a Quantikine sandwich ELISA kit (R&D Systems).

*Statistical analysis*. Data were analyzed by a paired Student's *t* test. A $P$ value $\leqslant 0.05$ was considered significant.

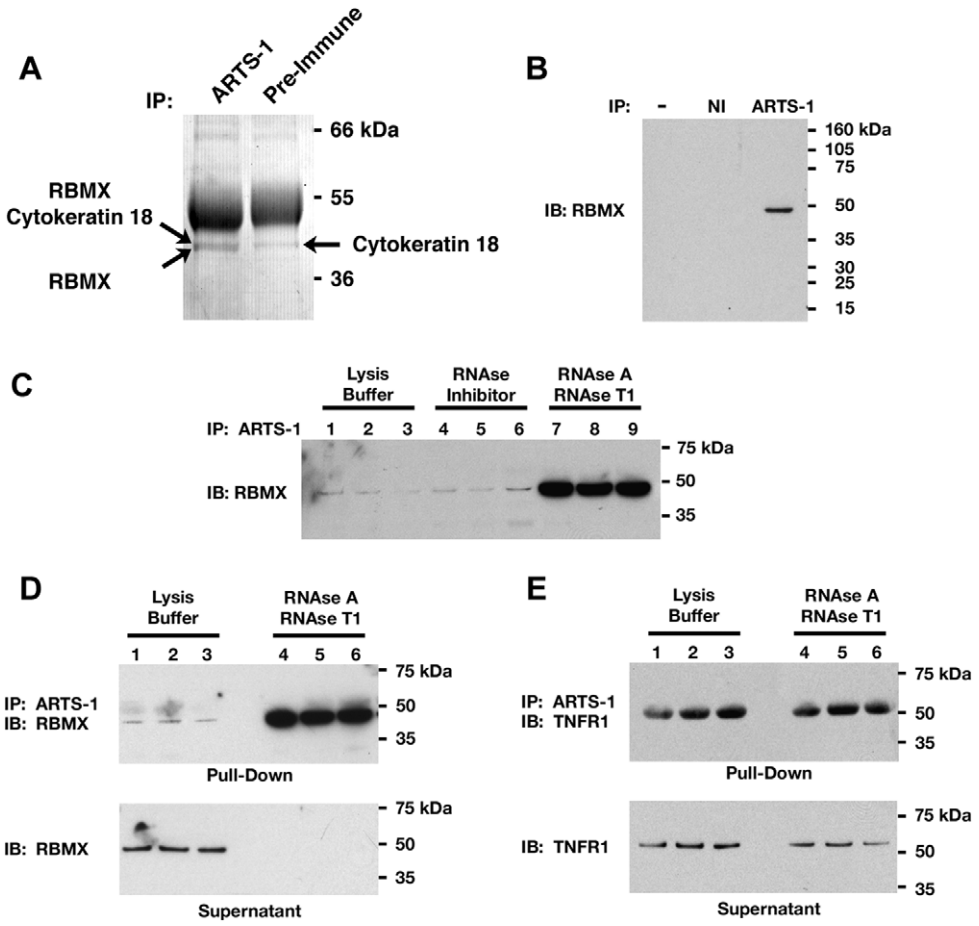


**Fig. 1.** Co-immunoprecipitation of endogenous RBMX and ARTS-1. (A) NCI-H292 cell membrane proteins were immunoprecipitated (IP) with anti-ARTS-1 antibodies or pre-immune serum and stained with Coomassie blue. This gel is representative of two experiments. (B) HUVEC lysates were immunoprecipitated with the anti-ARTS-1 antibody or non-immune serum (NI) and RBMX was detected by Western blotting. (C) HUVEC lysates, in triplicate, were incubated with RNase inhibitor or a mixture of RNases A and T1 for 1 h prior to immunoprecipitation with the anti-ARTS-1 antibody. RBMX was detected by Western blotting. (D) Immunoprecipitation of proteins in HUVEC lysates were performed, in triplicate, as in (C). Proteins pulled-down by the ARTS-1 immunoprecipitation are shown in the top panel, while proteins remaining in lysates are shown in the bottom panel labeled supernatant. (E) Immunoprecipitation of proteins in HUVEC lysates, performed in triplicate, as described in (C) and (D). TNFR1 was detected by immunoblotting.

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