ANTXR-1 and -2 independent modulation of a cytotoxicity mediated by anthrax toxin in human cells

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ABSTRACT. Several animal models have shown that anthrax toxin (ATX) elicits a cytotoxic effect on host cells through anthrax toxin receptor (ANTXR) function. In this study, compared with mouse cells, cells obtained from humans exhibited low sensitivity to ATX-mediated cytotoxicity, and the sensitivity was not correlated with expression levels of ANTXRs. ATX treatment also induced a cytotoxic effect in other cultured human cells, human embryonic kidney (HEK) 293 cells, that express ANTXRs at undetectable levels. Furthermore, ectopic expression of ANTXRs in HEK293 cells did not affect the sensitivity to ATX treatment. These findings suggest that there is an ANTXR-independent cytotoxic mechanism in human cells.
KEY WORDS: anthrax, ANTXRs, cell death, macrophage, toxin

'Anthrax' is a systemic hemorrhagic syndrome in several animals including domestic and wild-life animals and in humans [1, 21]. Because of its high mortality rate, especially in humans, the disease is viewed as an ecologic and economic problem world wide and particularly in developing countries [4, 9, 16, 21]. Anthrax disease is caused by infection of *Bacillus anthracis*, which is a gram-positive rod-like-shaped bacterium. The bacterium is introduced into the host body through a cut on the epithelial barrier of skin, intestine or respiratory tract. In the infected host body, the bacterium reaches the red pulp of the spleen engorged with blood and highly proliferates, causing bacteremia [20].

B. anthracis has two virulent factors encoded on two plasmids, pXO1 and pXO2, and the presence of these two plasmids is essential for virulence of the bacterium, since it is known that the bacterium missing one of these two plasmids can not exhibit full virulence in the host body. pXO1 encodes toxic proteins, namely anthrax toxin (ATX), and pXO2 encodes proteins for forming a poly-D-glutamic acid capsule outside the cell wall to escape from host immune reactions, such as phagocytosis by macrophages. ATX encoded by pXO1 is highly secreted by *B. anthracis* at its exponential growth stage, and the production of ATX causes dysfunction of several host homeostatic systems, including the innate immune system and cardiovascular system, and liver edema by cytotoxic effects on several cell types [13,

19]. It is well known that macrophages are highly susceptible to the cytotoxic effect of ATX. This toxic effect on macrophages causes enhancement of proliferation of the bacterium in the host body, because macrophages play an essential role in host immune defense against bacterial infection by their phagocytotic activity, antigen presentation and release of inflammatory cytokines for activating other immunological cells [5, 19].

ATX consists of three components; protective antigen (PA), lethal factor (LF) and edema factor (EF). PA secreted by the bacterium immediately binds to its specific receptors expressed on the host cell surface and forms a pore-shaped homo-oligomer to introduce LF and EF into the host cell cytosol [22]. Since LF has a direct protease activity against host intracellular molecules for cell survival, such as mitogen-activated kinase kinases (MKKs), and since EF is known as an adenylate cyclase that catalyzes the formation of intracellular cAMP, it is thought that the sensitivity of ATX-mediated cytotoxicity is dependent on PA binding of the cells.

Previously, two genes, encoding type-I transmembrane proteins, Tem-8/ANTXR-1 and CMG-2/ANTXR-2, have been isolated as gene responsibile for ATX-mediated cytotoxicity in Chinese hamster ovary (CHO) cells [3, 18]. While their cytoplasmic tails should not be sufficient for their role in the cytotoxic function of the PA-oligomer [12], these two proteins conserve a von Willebrand factor (vWF)-like structure in their extracellular region for their association with PA [17, 18]. Therefore, these two receptors should play a role as scaffolding proteins for the formation of a toxin channel consisting of PA homo-oligomers on the host cell membrane. In fact, it has been demonstrated that deficiency of the ANTXR-2 gene, but not that of the ANTXR-1 gene, protects mice against ATX challenge [14]. More recently, although both ANTXR-1 and -2 were shown to be ubiqui-

tously expressed in several tissues, including the thymus, stomach, skeletal muscles, heart, kidney, lung, liver, brain and uterus, it was demonstrated that smooth muscle cell-specific deletion of the ANTXR-2 gene protected mice against ATX challenge [13]. In addition, it was reported that there was a difference in resistance to ATX-mediated cytotoxicity between mouse strains, such as A/J versus C3H [6, 15]. These findings suggested that ATX-mediated cytotoxicity is dependent on the expression of ANTXRs and that there are cell type-dependent and/or genetic background-dependent mechanisms regulating sensitivity to ATX of host cells. As mentioned above, elucidation of a mechanism regulating the sensitivity to ATX would be important for understanding the pathogenicity in animals as well as humans. However, the details are not clear. In this study, we demonstrated that human monocyte-like cells exhibited greater resistance to ATX-mediated cytotoxicity than did cells obtained from mice. Neither the expression of ANTXR-1 nor that of ANTXR-2 was correlated with sensitivity to ATX-mediated cytotoxicity of human monocyte-like cells. It was also demonstrated that HEK293 cells, which expressed both ANTXR-1 and -2 at undetectable levels, exhibited sensitivity to ATX-mediated cytotoxicity. While the human homolog of ANTXRs was functional for introduction of ATX into cells, ectopic expression of these receptors did not affect the cytotoxicity of ATX in HEK293 cells. These findings indicated that there is a possibility for the presence of an ANTXR-independent cytotoxic mechanism in human cells.

MATERIALS AND METHODS

Cell culture: Cultured cells derived from human monocytes (K562, THP1 and U937 cells) and murine monocyte-derived Raw264.7 and J774.1 cells were maintained in RPMI1640 medium (Wako Jyunyaku Kogyo, Sapporo, Japan) with 10% fetal calf serum (FCS). Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (Wako Jyunyaku Kogyo) supplemented with 10% FCS at 37°C in 5% CO_2.

Reagents: Recombinant full-length protective antigen (PA), lethal factor (LF), edema factor (EF) and fluorescein-conjugated PA were purchased from List Biological Laboratories (Campbell, CA, U.S.A.). Anti-TEM8/ANTXR-1 and anti-CMG2/ANTXR-2-specific polyclonal antibodies were from R&D Systems (Minneapolis, MN, U.S.A.).

Cell viability assay: Cells seeded in a 96-well culture plate were treated with the indicated amount of PA in the presence of 300 *ng*/m*l* LF. After the indicated periods of incubation, viability of the cells was examined by a WST assay using Cell counting kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Means obtained from samples were depicted as a percentage of that from samples treated with the medium alone at the indicated time point.

Western blotting: Western blotting was done as previously described [7]. Briefly, cells were lysed with RIPA buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate and 5 mM EDTA). After centrifugation at 16,000 × g for 1 hr at 4°C, the supernatants of

samples were collected and boiled in SDS-sample buffer and fractionated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bradford, MA, U.S.A.). After blocking by PBS containing 0.1% Tween20 and 5% skim milk, the membranes were incubated with the indicated Abs. Visualization of immunoblots was performed by enhanced chemiluminescence (GE Healthcare Biosciences, Piscataway, NJ, U.S.A.).

Real time polymerase chain reaction (RT-PCR): Total RNA of cells was isolated with Trizol reagent (Life Technologies, Carlsbad, CA, U.S.A.), according to manufacturer's instruction. 0.5 μg of the total RNA was reverse-transcribed using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) with random primer and oligo-dT primer. The resulted cDNA was subjected to RT-PCR with CFX96 (Bio-Rad, Hercules, CA, U.S.A.) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara bio, Otsu, Japan). Copy number of the indicated mRNA was calculated using standard curve obtained from plasmid that contains the targeted sequence. Sequence of primer sets for detecting mouse GAPDH mRNA was described previously [8]. Sequences of the other primer sets were listed below.

For mouse ANTXR-1
Forward; 5′-CTCGATGCCTTGTGGGTTCT-3′
Reverse; 5′-AGTTGATACAGCGTCCCGTG-3′
For mouse ANTXR-2
Forward; 5′-GTCCGCTGGGGAGATAAAGG-3′
Reverse; 5′-CAAACGACCCTTGATTGGCG-3′
For human ANTXR-1
Forward; 5′-CAATCAAGGGAAAACTCGATGCC-3′
Reverse; 5′-TACTTGGCTGGCTGGTTGTT-3′
For human ANTXR-2
Forward; 5′-AGCGGGAGGGAGTCTCAG-3′
Reverse; 5′-CCACACTCCCAGACTTGTCC-3′
For human GAPDH
Forward; 5′-AAGGTGAAGGTCGGAGTCAACG-3′
Reverse; 5′-GCCATGGGTGGAATCATATTGG-3′

Flowcytometry: Flowcytometric assay to examine the expression of indicated proteins on the cell surface was done as described previously [8]. Briefly, cells were incubated in ice-cold PBS containing 10% FCS and the indicated antibody. After 1 hr of incubation, the cells were washed twice with ice-cold PBS containing 10% FCS. After washing, specific binding of the indicated antibody was detected by Alexa Fluor 647-conjugated affinitypure F (ab') 2 fragment rabbit anti-goat IgG (Jackson Immunoresearch Laboratories, Westgrove, PA, U.S.A.). Non-specific goat IgG was used for a control. Fluorescent signals of the cells were analyzed by a flow cytometer (FACS Canto, BD Biosciences, San Jose, CA, U.S.A.).

PA-binding assay: To detect PA-binding ability of the cells, cells were washed twice with ice-cold PBS, scraped and resuspended in ice-cold PBS (4×10^6 cells/m*l*). Cell suspensions (each 25 μl) were reacted with 1 μg/m*l* of FITC-PA in 1.5 m*l* tubes and incubated at 4°C for 60 min in the dark. The cells were then washed twice with 2 m*l* ice-cold PBS and re-suspended in 0.5 m*l* ice-cold PBS for flowcytometric analysis as described above.

Construction of plasmids: cDNA encoding human AN-TXR-1 transcript variant 1 or human ANTXR-2 transcript variant 1 was isolated from cDNA library obtained from human uterus (Clontech, Palo Alto, CA, U.S.A.) by PCR. The cDNA was inserted into the mammalian expression plasmid (pcDNA3.1 myc/his ver.B, Life Technologies) to configure a $3'$ in frame fusion to myc/his tag of the plasmid.

Generation of HEK293 cells stably expressing ANTXR-1 or -2: HEK293 cells (1×10^6 cells in a 10 cm tissue culture dish) were transfected with an empty or expression plasmid for human ANTXR-1 or -2 by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. At 48 hr after transfection, the cells were selected in the presence of 500 μg/ml G418. Two weeks after the selection, expression level of the protein in cells was analyzed by Western blotting as described above.

siRNA treatment: Small interfering RNA (siRNA) pools used in this study were siGENOME SMARTpool for human ANTXR-1, human ANTXR-2 and non-targeting #1 control (Dharmacon, Lafayette, CO, U.S.A.). Cells (5×10^6 cells) were suspended in 0.5 ml of pre-warmed Opti-MEM-I (Life Technologies) containing 120 pmoles of the indicated siRNA. The cells were electroporated in 4 mm cuvettes using a Gene Pulser XCell (Bio-rad). Pulse conditions were square-wave pulse, 300 V, 10 ms. Electroporation efficiency was validated as above 80% with flow cytometry using 20 μg of plasmid for expressing enhanced green fluorescent protein (pEGFP-N1, Clontech). 48 hr after the electroporation, the cells were subjected to the indicated assay.

Statistics: Statistical comparisons were performed by student's *t*-test. *P*-value <0.05 was considered as significant. Error bar means standard deviations in each graph. Asterisk or NS means "Significant" or "NOT significant", respectively.

RESULTS

Sensitivity to ATX-mediated cytotoxicity has been confirmed in several cell types derived from mice and humans. Murine (Raw264.7 or J774.1) or human (THP1, U937 or K562) cells were treated with 300 *n*g/ml of LF and with the indicated concentration of PA, and viabilities of the cells were examined 24, 48 or 72 hr after the treatment (Fig. 1A). In the case of murine cells, ATX treatment showed a cytotoxic effect in a PA dose-dependent manner at 24 hr after treatment. Comparing with murine cells, human U937 and K562 cells had exhibited resistance to ATX-mediated cytotoxicity in the presence of a high concentration (500 *n*g/ml) of PA. Human THP1 cells also had low sensitivity compared with the sensitivity of murine Raw264.7 and J774.1 cells. In the further assay assessing cell count of U937 and K562 cells (Fig.1B), the presence of ATX was not capable to affect growth of these cell lines that were tested. These findings suggest that compared with murine cell lines, human monocyte-like cells are resistant to cytotoxicity mediated by ATX.

We examined the expressions of ANTXR-1 and -2 in ATX-sensitive murine Raw264.7 cells and insensitive human THP1, U937 and K562 cells by Western blotting using anti-ANTXR-1 (Fig.2A, left panel) or -2 (Fig.2A, right panel)

specific polyclonal antibodies. Specificities of the polyclonal antibodies were confirmed by their specific detection for human ANTXR-1 or -2 ectopically expressed in HEK293 cells (represented in right side of each panel). An ANTXR-1 Ab specific band was observed in Raw264.7 cells, but not in human cells, except for HEK293 cells transduced with plasmid encoding human ANTXR-1 cDNA (Fig.2A, left panel). In a further assay using an anti-ANTXR-2-specific polyclonal antibody, diverse ANTXR-2 expression levels were observed in tested human cells (Fig. 2A, right panel). Interestingly, the expression level of ANTXR-2 in ATX-insensitive K562 cells was high compared with the levels in the other cells. The same results were obtained from RT-PCR assay detecting mRNA of the indicated genes (Fig. 2B). As mentioned above, ANTXRs scaffold PA on the cell surface. Therefore, we examined the functions of ANTXRs, such as expression on the cell surface and binding activity for PA in human cells. In flowcytometrical assay using allophycocyanin (APC)-conjugated anti-ANTXR-1 (upper panels in Fig. 2C) or -2 (lower panels, in Fig. 2C) antibodies, surface expression of ANTXR-1 was undetectable in all tested human cells (Fig. 2C, upper panels). A high expression level of ANTXR-2 on the surface of ATX-insensitive K562 cells was detected, suggesting that surface expression level of ANTXRs does not correlate with their sensitivity for ATX-mediated cytotoxicity (Fig. 2C, lower panels). To examine PA-binding capacity of ANTXR-2 expressed on human cells, the indicated human cells were incubated with fluorescein-conjugated recombinant PA (FITC-PA) at 4°C for preventing non-specific endocytosis of the cells. At 30 min after incubation, the cells were washed with ice-cold PBS. Fluorescence signals of FITC-PA on the cells were detected by flowcytometry. As shown in Fig. 3D, FITC-PA specific fluorescent signal was detected as comparative level of ANTXR-2 expression (Fig. 2C, lower panels) in all tested human cells. These findings indicated that expressions of ANTXRs should correlate with PA-binding, but not ATX-mediated cytotoxic effect in human monocyte-like cells.

In the series of the assay regarding function of ANTXRs, the other human cell line, HEK293 cells in which expression levels of ANTXR-1 and -2 were undetectable level (Fig. 2A and 2B), exhibited high sensitivity against ATX challenge (Fig. 3A). In addition, a cytotoxic effect was not observed in the absence of PA, suggesting that the detected cytotoxic effect was dependent on PA. To clarify the functional association of ANTXRs with the cytotoxic effect mediated by ATX, HEK293 cells ectopically expressing ANTXR-1 or -2 were established, and the sensitivity of these cells was compared with that of HEK293 cells transduced with an empty vector as control cells. The results presented in Fig. 3B and 3C clearly demonstrated that neither the ectopic expression of ANTXR-1 nor that of ANTXR-2 significantly affected the sensitivity to cytotoxicity mediated by ATX consisting of PA with LF (Fig. 3B) or EF (Fig. 3C). In addition, neither knockdown treatments on ANTXR-1 nor ANTXR-2 could not significantly affect sensitivity to ATX-mediated cytotoxicity of THP1 cells (Fig. 3D). These findings indicated that expression of ANTXR-1 and -2 should not have a significant
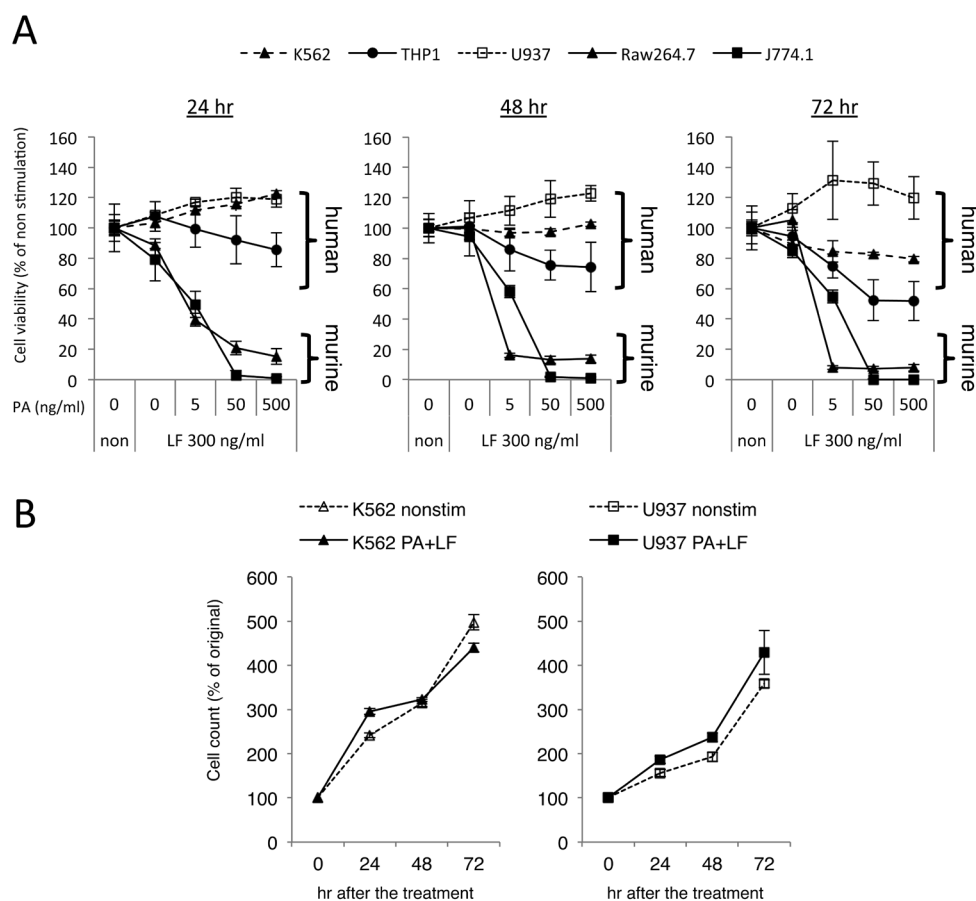
A

B

Fig. 1. Monocyte like-human cells have lower sensitivity to Anthrax toxin-mediated toxicity than do murine cells. (A) The indicated cells were treated with the combination of PA at the indicated concentrations and 300 *n*g/m*l* of LF. After the indicated periods of incubation, viabilities of the cells were examined as described in *Materials and Methods*. Error bar means ± standard deviation (SD). N=3. (B) K562 or U937 cells were cultured in the presence or absence of PA (500 *n*g/m*l*) and LF (300 *n*g/m*l*). After the indicated periods of the incubation, number of the cells was assessed with cell counting kit-8. The value represented a percentage ratio against mean of original. Error bar means ± SD. N=3.

effect on the sensitivity to a cytotoxic effect mediated by ATX in human cell lines, such as HEK293 and THP1 cells.

DISCUSSION

In the present study, we demonstrated that human monocyte-like cells were more resistant to ATX-mediated cytotoxicity than that obtained from mice (Fig. 1) and that this resistance observed in human cells should not correlate with the expression level and PA-binding of ATX-specific receptors including ANTXR-1 and -2 (Fig. 2). The results regarding receptor expression independent cellular sensitivity against ATX were consistent with the observation in human lung cells [11]. It was previously reported that macrophages isolated from a C3H mouse had high sensitivity to ATX-mediated cytotoxicity but that those from an A/J mouse were insensitive [6]. Differences in the sensitivity to ATX of macrophages isolated from several mouse strains were also

reported [15]. These findings and our results suggested that the sensitivity to ATX might be regulated by a mechanism dependent on genetic background. A mechanism regulating ATX-mediated cytotoxicity is divided by two phases consisting of a receptor dependent ATX-uptake and intracellular toxin reaction. Since some MKK-inhibitor insensitive human leukemic cell lines are resistant to ATX mediated cytotoxicity, it was suggested that a sensitivity to ATX-mediated cytotoxicity might associate with its survival dependency on ATX targets, such as MKK [10]. ATX also targets and activates the other host intracellular protein, Nlrp1b, which stimulates Caspase-1-dependent pyroptotic cell death in murine macrophages [2]. However, in our experimental condition, the ATX-treatment was not capable of activating Caspase-1 in Raw264.7 cells, which had exhibited their high sensitivity to ATX-mediated cytotoxicity (data not shown). In the study that reported the association of the pyroptotic mechanism with the cytotoxic effect of ATX-treatment, the
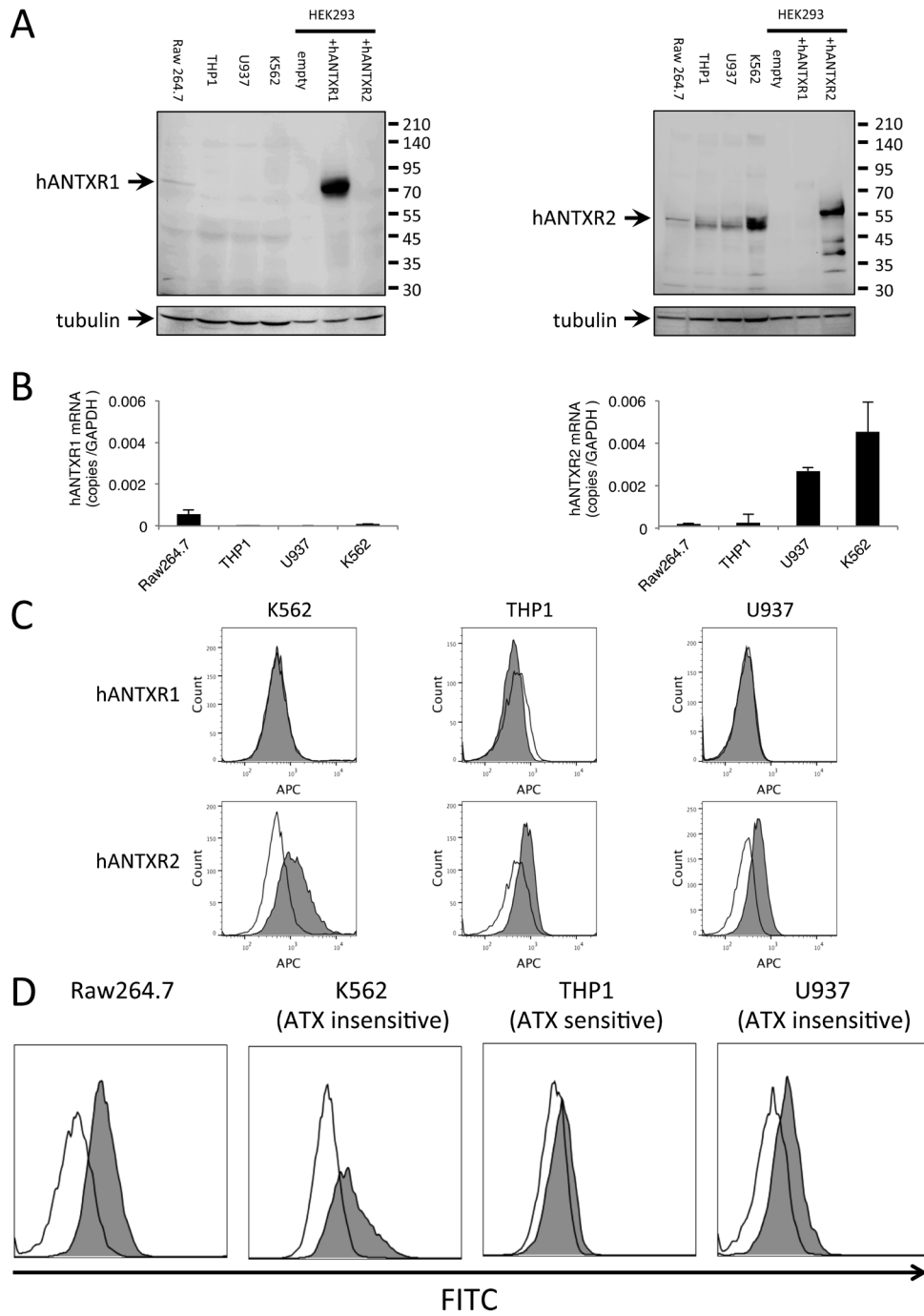
Fig. 2. Expression of ANTXR-1 and -2 and PA-binding in human monocyte-like cells. (A) Protein expression of both ANTXR-1 (left panel) and ANTXR-2 (right panel) in murine (Raw264.7) or human monocyte-like cells (THP1, U937 or K562) was examined by Western blotting. The expressions were also analyzed in human embryonic kidney (HEK) 293 cells transfected with empty or the plasmid for expressing hANTXR-1 or -2. Tubulin protein was used for a loading control. (B) ANTXR-1 or -2 mRNA production of the indicated cells was also analyzed with RT-PCR. Copy number of the indicated mRNA was shown after normalized by that of GAPDH mRNA in each cell line. Error bar means ± SD. N=3. (C) Surface ANTXR-1 and -2 expression levels of the indicated cells were determined by flowcytometry using anti-ANTXR-1 and -2-specific antibodies. The open histogram is fluorescent activity of cells stained with non-specific goat antibody, and the closed histogram is fluorescent activity of cells stained with the indicated antibody. (D) The indicated cells were reacted with fluorescein (FITC)-conjugated recombinant PA (FITC-PA) or soy bean trypsin inhibitor at 4°C for preventing non-specific internalization. After washing twice with ice-cold PBS, fluorescent activity of the cells was examined by flowcytometry. The soybean trypsin inhibitor was used as a negative control. The Open histogram is fluorescent activity specific to the soybean trypsin inhibitor, and the closed histogram is fluorescent activity specific to FITC-PA.
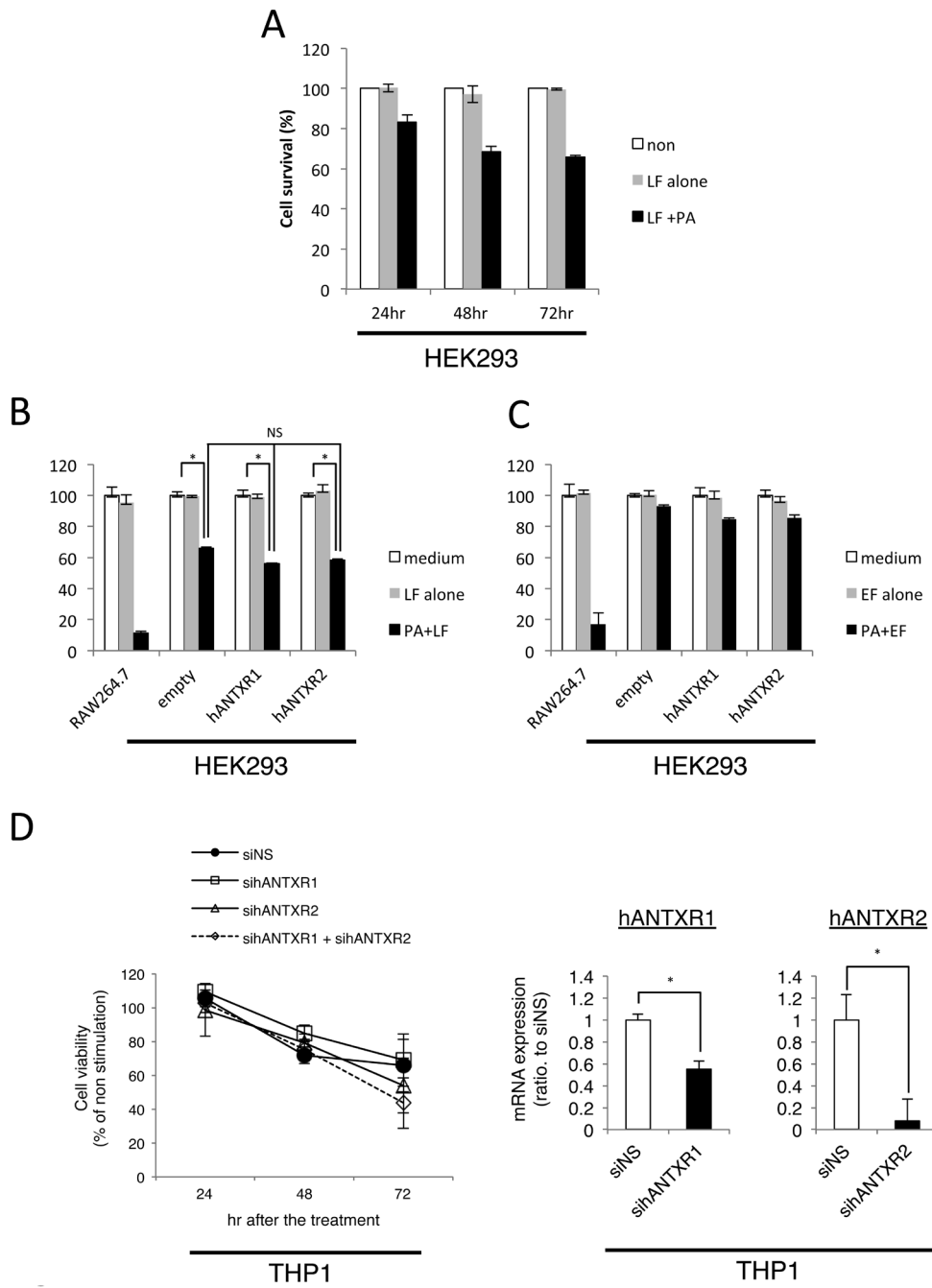
Fig. 3.   Expression of ANTXR-1 or -2 does not affect the sensitivity to anthrax toxin-mediated cytotoxicity in HEK293 cells.
(A) HEK293 cells were treated by the indicated combinations of 300 *n*g/m*l* LF and 500 *n*g/m*l* PA. At the indicated periods of treat-
ment, viability of the cells was examined as described in the legend of Fig. 1. HEK293 cells stably transduced with an empty control
or plasmid for expression of hANTXR-1 or -2 were incubated in a culture medium containing the indicated combinations of 500 *n*g/
m*l* LF (B) or 500 *n*g/m*l* EF (C) with 300 *n*g/m*l* PA. 48 hr after the incubation, viability of the cells was examined as described in the
legend of Fig. 1A. Murine Raw264.7 cells were used for a positive control. Error bar means ± SD. N=3. *(D, left p*anel) THP1 cells
were electroporated with the indicated combination of siRNA pools non-specific (siNS), specific for human ANTXR-1 (sihANTXR1)
or ANTXR-2 (sihANTXR2). Fouty eight hr after the electroporation, the cells was incubated in the presence or absence of 500 *n*g/m*l*
LF and 300 *n*g/m*l* PA. After the indicated periods of the incubation, viability of the cells were assessed as described in Fig. 1A. *(D,
right* panel) Knockdown efficacies of the indicated siRNA treatment were assessed with RT-PCR as described in Fig. 2B. Error bar
means ± SD. N=3. Asterisk means significant (*P*-value<0.05).

authors used cells that have already been differentiated [2]. In the present study, we observed a significant cytotoxic induction mediated by ATX-treatment in undifferentiated condition (Fig.1A). Therefore, the association of the pyroptotic mechanism with cytotoxic induction during ATX-treatment might depend on a differentiation state of the cells that were tested. As the other possibility, it was demonstrated that HEK293 cells, expressing ANTXR-1 and -2 at undetectable levels, exhibited significant sensitivity to ATX-mediated cytotoxicity (Fig. 3A), and it was also demonstrated that high expression of human ANTXR-1 and -2 did not affect ATX-mediated cytotoxicity in HEK293 cells (Fig. 3B and 3C). These findings suggested that there should be an unknown mechanism (s) for ATX-mediated cytotoxicity, such as the function of a novel receptor for PA in HEK293 cells. Taken together, the findings suggested that sensitivity to ATX-mediated cytotoxicity in human cells is not modulated by surface expression and PA-scaffolding function of ANTXRs in human cells. Elucidation of the mechanism regulating the resistance to ATX in human cells would be beneficial for developing effective pharmacological agents for anthrax disease.

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