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A potent endocytosis inhibitor Ikarugamycin up-regulates TNF production

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ABSTRACT

Ikarugamycin (IK) is an antibiotic which has been reported to have a variety of functions, such as inhibition of clathrin-mediated endocytosis (CME), anti-tumor effects and regulation of the immune system. Whether IK influences cytokine production is poorly understood. We have investigated the relationship between IK and production of tumor necrosis factor-α (TNF). TNF plays a pivotal role in pathogenesis of many diseases. Although the dynamics of soluble TNF (sTNF) has been widely explored so far, the functions of the membrane form of TNF (mTNF) have not been fully elucidated. We demonstrated that IK increases the amount of mTNF and prolongs the duration of TNF expression. This effect is unrelated to the shedding activity of disintegrin and metalloproteinase domain-containing protein 17 (ADAM 17). Our results revealed that there is a mechanism to terminate inflammation at the cellular level which IK dysregulates. Furthermore, IK can be a tool to study TNF signaling due to its effect of increasing mTNF expression.

1. Introduction

Ikarugamycin (IK) is an antibiotic with antiprotozoal activity, which belongs to the polycyclic tetramate macrolactams. It was first isolated from Streptomyces sp. By Jomon et al., in 1972 [1]. However, IK was not commonly used as an antibiotic because it exhibited strong cytotoxicity and caused hemorrhage [1]. IK also has various functions besides antibiotic activities.

IK prevents clathrin-mediated endocytosis (CME) [2,3], a major pathway for cells to internalize surface molecules. CME is important for regulating cellular signaling and homeostasis. For example, IK prevented the internalization of the low-density lipoprotein (LDL) [4] and removal of CD4 from the cell surface by HIV Nef [5]. IK caused redistribution of the clathrin heavy chain and the adaptor protein 2 [6]. However, its precise mechanism of action remains unclear.

Additionally, like other polycyclic tetramate macrolactams, IK exhibits anti-tumor activities on several cancer cell lines through various pathways [7–9]. Dhaneesha et al. reported that IK caused DNA damage through binding to the minor groove of DNA, cell arrest and ultimately apoptosis [8]. Jiang et al. demonstrated that IK showed anti-tumor effects on pancreatic cancer cell lines by disrupting glucose metabolism [9]. Finally, IK showed anti-inflammatory functions through induction of A20 [10]. As mentioned above, the influence of IK ranges broadly from DNA to protein level, and therefore it has an enormous unknown potential to impact biological activities.

Since there are few reports about the effect of IK on the immune

Abbreviations: IK, ikarugamycin; CME, clathrin-mediated endocytosis; TNF, tumor necrosis factor-α; sTNF, soluble TNF; mTNF, membrane form of TNF; TNFR, TNF receptor; ADAM 17, disintegrin and metalloproteinase domain-containing protein 17; LPS, lipopolysaccharide; TAPI-1, TNF alpha processing inhibitor-1.

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system, we decided to explore the influence of IK on production of cytokines, particularly tumor necrosis factor-α (TNF). TNF was first identified as a substance which caused necrosis of tumor cells [11]. It is now recognized as a major pro-inflammatory cytokine. Although TNF is necessary for a defense against infection [12], its excessive production leads to chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases. There have been many biological agents to target against TNF for the treatment of these diseases.

Bacterial infection or stimulation with lipopolysaccharide (LPS) increase the production of TNF in monocytes and macrophages. TNF is first synthesized as a 26 kDa type II transmembrane precursor [13]. Translation of TNF mRNA is carried out in the endoplasmic reticulum. Post-transcriptional regulation of TNF occurs through the TRIF/p38 MAPK/MK2/3 pathway [14–17]. Newly synthesized TNF is transported to Golgi, and the membrane form of TNF (mTNF) exits from the trans-Golgi network to recycling endosomes and, is carried to the cell surface [18]. mTNF receives post-translational modifications, such as palmitoylation and phosphorylation [19,20]. Murine TNF has a potential *N*-linked glycosylation site [21]. mTNF is cleaved into 17 kDa soluble mature form by a sheddase named disintegrin and metalloproteinase domain-containing protein (ADAM) 17 that is also called TNF converting enzyme (TACE) [22–24].

There are two types of TNF receptors (TNFR), TNFR1 and TNFR2 [25,26]. TNFR1 is stimulated by both mTNF and soluble TNF (sTNF), whereas TNFR2 is mostly activated by mTNF [27,28]. While TNFR1 mediates inflammatory response [29,30], the function of TNFR2 has not been completely understood since the mechanism of mTNF regulation remains largely unclear. Hence a precise knowledge of mTNF functions will contribute to understand the role TNFR2.

The mechanism of production and regulation of sTNF has been vigorously explored compared to those of mTNF. However, there are a few reports about the fate of the mTNF after surface expression. It was demonstrated that mTNF is endocytosed under the treatment of the metalloprotease inhibitor BB-3103 [31]. In addition, the complex of mTNF and anti-TNF agents is endocytosed via clathrin-mediated pathway [32].

In this study, we examined the effect of IK on the TNF production, which indicates that there is a post-transcriptional mechanism terminating TNF production to suppress excessive inflammation at the cellular level.

2. Materials and methods

2.1. Cell culture and reagents

RAW264 murine macrophages cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, USA) with 10% fetal bovine serum, penicillin-streptomycin and L-glutamine at 37 °C. Cells were stimulated with 100 ng/ml of LPS (14,945–81, Invivo Gen, San Diego, USA), 5 μg/ml of Brefeldin A (Bio Legend, San Diego, USA), 10 μM of TAPI-1 (PEPTIDE INSTITUTE, Japan) and 5 μM of ikarugamycin from two commercial sources (Cayman Chemical, Ann Arbor, USA; Abcam, Cambridge,UK) were used.

2.2. CRISPR/Cas9 system

The target array of exon 1 of ADAM17 was determined using the CRISPRdirect system. crRNA and tracrRNA were fused into sgRNA, which was then combined with Cas9 (Takara Bio, Kusatsu, Japan). Cas9-sgRNA RNPs were electroporated into RAW264 cells, using 4D-Nucleofector™ system (LONZA, Basel, Switzerland). The sequences of crRNA and tracrRNA (purchased from FASMAC, Atsugi, Japan) are as follows: crRNA, 5′-AGCAGCACUCCAUAAGGAAA-3'; tracrRNA, 5′-AAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU3'.

2.3. Western blot

Cells were resuspended in lysis buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40 supplemented with Halt Protease Inhibitor [Thermo Fisher Scientific, Waltham, USA], 10 μM MG-132 and 10 mM *N*-ethylmaleimide). PNGase F (New England Biolabs, Ipswich, USA) were used for de-glycosylation experiments. Samples were loaded into 4–12% Bis-Tris precast gels (Thermo Fisher Scientific). Anti-TNF antibody (#11948, Cell Signaling Technology, Danvers, USA) and Anti-ADAM17 antibody (ab75609, abcam) were diluted with Western BLoT Immuno Booster (Takara) as primary antibodies. Relative protein levels were quantified using ImageJ (National Institutes of Health, Bethesda, USA) and normalized to β-actin levels.

2.4. ELISAs

TNF concentrations in the culture medium were measured by an ELISA kit (430901, Bio Legend) according to the manufacturer's instruction.

2.5. Real time quantitative PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN). qPCR was performed with the QuantiTect SYBR Green Master Mix (QIAGEN) and the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Gene expression was normalized with the levels of GAPDH. Sequences of the primers to measure mRNAs are as follows: TNFα, 5′-GACCCTCACACTCAGATCATCTTC-3′ and 5′-CGCTGGCTCAGCCACTCC-3'; GAPDH, 5′-GCCATGAGGTCCACCACCCTG-3′ and 5′-CTACTGGCGCTGCCAAGGCTGT-3'.

2.6. Flow cytometry

Surface expressions of TNF on RAW264 cells were analyzed by flow cytometry using APC anti-mouse TNF (506308, Bio Legend). Data were acquired using the FACS CantoII flow cytometer (BD Biosciences, Franklin Lakes, USA) and analyzed using FlowJo software (BD Biosciences).

2.7. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, permeabilized with digitonin and blocked with Blocking One (Nacalai Tesque, Kyoto, Japan). Cells are incubated with anti-TNF antibody (#11948, Cell Signaling Technology. Images were acquired with a Fluorescence Microscope (KEYENCE, Osaka, Japan).

2.8. Statistical analysis

The results are presented as means ± SEM from three to seven independent experiments (Indicated as N in the figure legend of each result) performed with single well per sample. Each sample was analyzed in triplicate. Statistical analysis was performed using Prism 9.0 (GraphPad, San Diego, USA). Data are considered significant when p values are less than 0.05. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Results

3.1. Evidence for existence of an ADAM17-independent regulatory mechanism

Wild type (WT) RAW264 cells were stimulated with LPS in the presence or absence of Brefeldin A for 8 h. Brefeldin A inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus [33]. mTNF was detected as two bands by western blotting (Fig. 1A). Upper

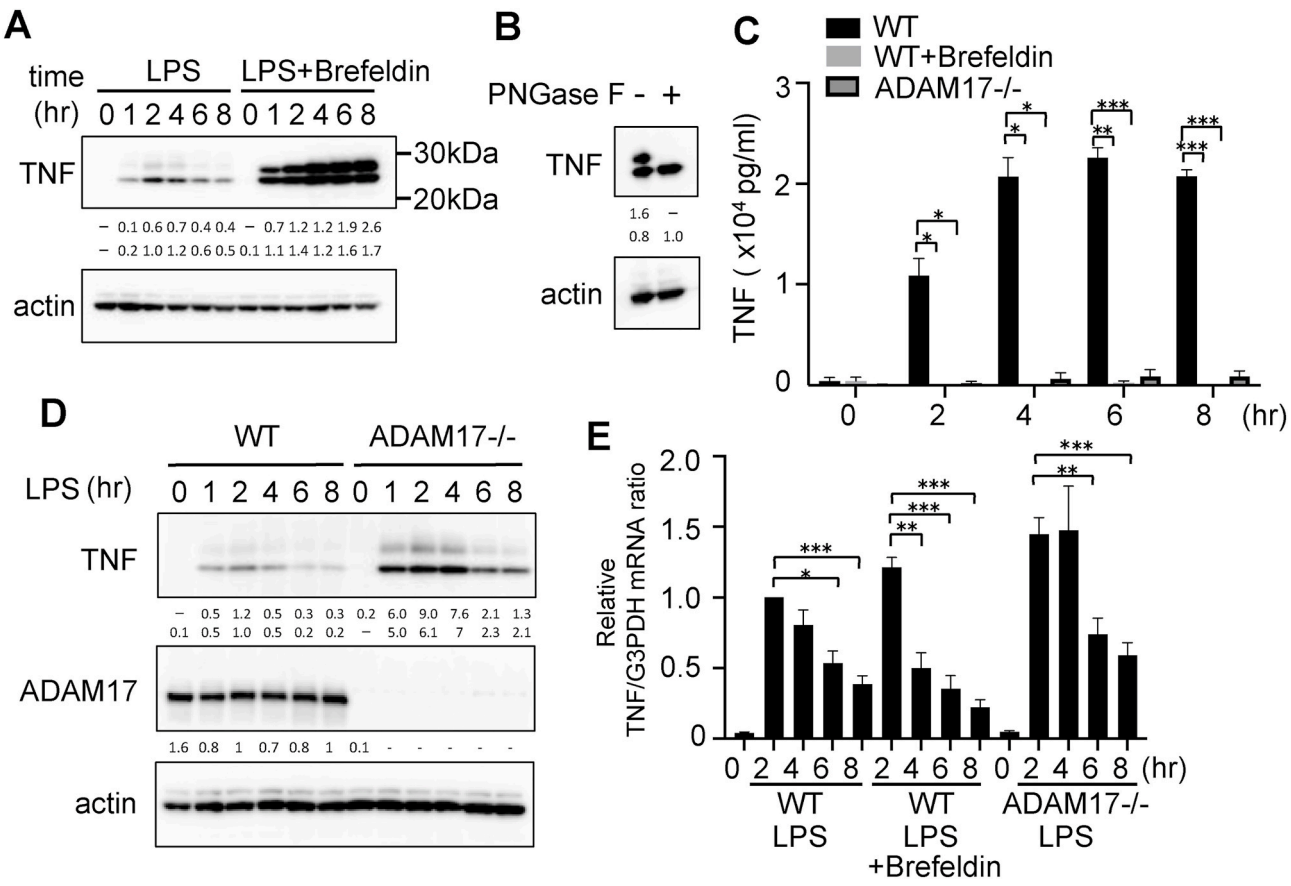

Fig. 1. Kinetics of TNF. (A–E)100 ng/ml of LPS and 5 μg/ml of Brefeldin A were used. (A) WT RAW264 cells were incubated with LPS or with LPS and Brefeldin A. Whole cell lysates were analyzed for the level of mTNF by Western blotting. (B)WT RAW264 cells were incubated with LPS and Brefeldin A for 4 h. Whole cell lysates were processed with PNGase F and analyzed by Western blotting. (C) WT RAW264 cells were incubated with LPS or LPS plus Brefeldin A. ADAM-17 KO RAW264 cells were stimulated with LPS. The amount of the sTNF into the supernatant was measured by ELISA assay. (D) Comparison of expression of TNF between WT and ADAM-17 KO RAW264 cells stimulated with LPS. Whole cell lysates were analyzed by Western blotting. (E) The relative TNF/GAPDH ratios were measured by qPCR. A, B and D: Data are representative from three independent experiments. (C and E) Two way ANOVA followed by Tukey's multiple comparisons test, SEM from four (C) and six (E) independent experiments.

band disappeared after processing with PNGase (personal communication with Cell Signaling Technology Inc.) (Fig. 1B), indicating that upper band is a glycosylated form of mTNF. mTNF in cells treated with only LPS alone reached at the maximum level at 2 h after stimulation then began to decline, while that in cells treated with LPS and Brefeldin A continued to increase (Fig. 1A). On the other hand, release of soluble TNF (sTNF) from cells into culture supernatant was suppressed under the treatment with Brefeldin A (Fig. 1C).

We hypothesized that the decrease of mTNF is due to the sTNF release from cells. Next, we tested whether mTNF also remains increasing without ADAM17 which converts mTNF into sTNF, using ADAM 17 knock out (KO) RAW264 cells. A significantly lower amount of sTNF was produced from ADAM17 KO (Fig. 1C). However, mTNF of ADAM-17 KO cells began to decrease 4 h after LPS stimulation (Fig. 1D). The amount of TNF mRNA increased transiently after stimulation and started to decrease afterwards in the presence or absence of Brefeldin A (Fig. 1E). These results suggested that decrease of mTNF is mediated not only by the shedding activity of ADAM17, but also by another ADAM17-imdependent mechanism by which mTNF levels are decreased after exiting from the Golgi apparatus and before being released from the plasma membrane.

3.2. . *IK increases the expression of mTNF*

To investigate another regulatory mechanism of mTNF, we used ikarugamycin (IK) which is known as an inhibitor of clathrin-mediated endocytosis (CME). WT RAW264 cells were incubated with LPS in the presence or absence of IK. mTNF levels in cells stimulated with LPS alone began to decrease after 2 h, whereas those in cells stimulated with LPS and IK remained increasing even after 4 h (Fig. 2A). The difference in the amounts of sTNF between the two groups was greater at later time points (Fig. 2B). In contrast to the protein levels, the amounts of TNF mRNA increased transiently after the LPS stimulation in the presence or absence of IK, then began declining. Next, we investigated whether IK increases surface expression of mTNF by fluorescence-activated cell sorting (FACS). Surface expression of mTNF of cells was augmented by IK treatment (Fig. 2D). Moreover, duration of TNF expression was extended in cells treated with IK.

3.3. *IK increased the amount of mTNF and extended the duration of mTNF expression by a mechanism independent of ADAM17*

Next, we compared the effect of IK and TNF alpha processing inhibitor-1 (TAPI-1), which inhibits activities of ADAM17 and another metalloprotease [24]. WT RAW264 cells were divided into eight groups; untreated or treated with IK, TAPI-1 or IK and TAPI-1 with or without LPS. Both IK and TAPI caused retention of mTNF (Fig. 3A). The levels of sTNF are higher in cells treated with IK than that with LPS alone, whereas TAPI prevented the release of sTNF (Fig. 3B). IK treatment resulted in a slightly increased production of mRNA of TNF even without LPS (Fig. 3C). IK and TAPI increased the surface expression of mTNF synergistically (Fig. 3D). TAPI causes the increase of mTNF likely via

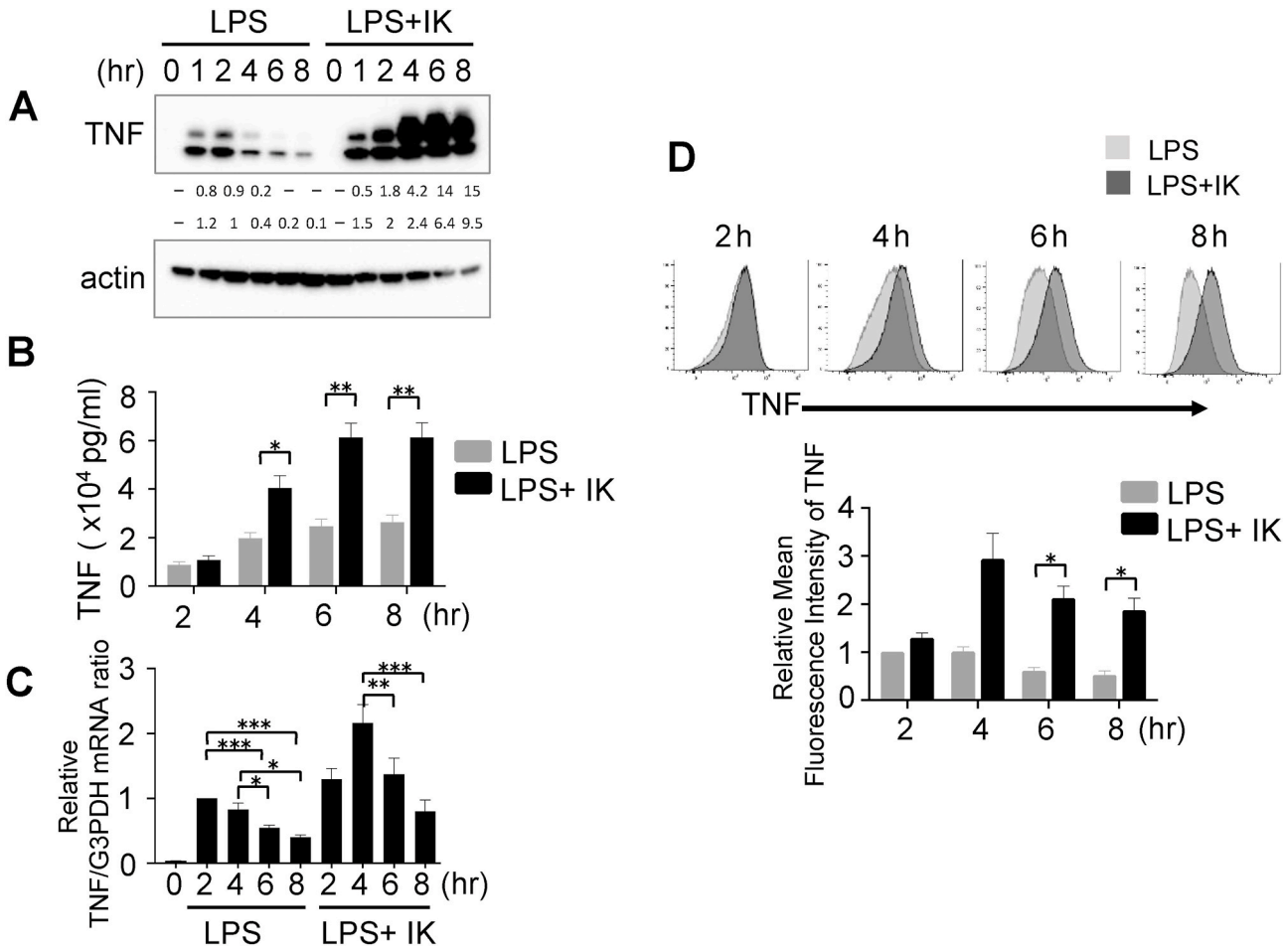

Fig. 2. WT RAW264 cells were stimulated with either 100 ng/ml of LPS or 100 ng/ml of LPS plus 5 μM of IK. (A) The whole cell lysates were immunoblotted for mTNF. (B) The amount of the sTNF into the supernatant was measured by ELISA assay. (C) The relative TNF/GAPDH ratios were measured by qPCR. (D) Surface expression of TNF was analyzed by FACS. A: Data are representative from three independent experiments. B: two way ANOVA followed by Bonferroni's multiple comparisons test, SEM, from seven independent experiments. C: two way ANOVA followed by Tukey's multiple comparisons test, SEM, from seven independent experiments. D: two way ANOVA followed by Šídák's multiple comparisons test, SEM, from four independent experiments.

inhibition of the shedding activity, while IK induces mTNF via a different mechanism.

To confirm this hypothesis, ADAM-17 KO RAW264 cells were stimulated with LPS in the presence or absence of IK (Fig. 4A). ADAM-17 KO cells treated with IK resulted in a substantial increase of mTNF in a time-dependent manner compared to cells without IK. Immunocytochemistry also confirmed that IK increases the expression of TNF (Fig. 4B). The production of sTNF in ADAM-17 KO cells increased at 2 h after IK stimulation (Fig. 4C). This indicates that there is additional enzyme to cleave mTNF other than ADAM17. The amounts of TNF mRNA were transiently increased after the LPS stimulation with or without IK (Fig. 4D). We also analyzed the expression of TNF on the surface of ADAM-17 KO RAW cells by FACS (Fig. 4E). While surface expression of mTNF on cells stimulated with LPS alone continued to decline after 4 h post-stimulation, that on cells stimulated with LPS and IK remained at high levels (Fig. 4D). From these results, we conclude that IK has a function to increase mTNF and to extend the expression of TNF, which is independent of the ADAM17 activity.

4. Discussion

In this study, we demonstrated that LPS-dependent mTNF expression begins decreasing after reaching the maximum level at 2 h s, while Brefeldin A prevents the decline of mTNF (Fig. 1A). Although the release of sTNF was impaired in ADAM-17 KO cells, mTNF levels in these cells began decreasing 4 h after stimulation (Fig. 1C and D). From these results, we conclude that there is an ADAM17-independent regulatory mechanism of TNF expression, which presumably occurs between the Golgi apparatus and the plasma membrane.

We then discovered that IK increases the retention of mTNF. Since IK does not prevent stimulated cells from releasing sTNF, this function of IK is caused through a different pathway other than inhibition of ADAM-17. It is known that IK prevents CME [2,3]. There is a report that the complex of mTNF and anti-TNF agents is endocytosed through a clathrin-dependent pathway [32]. Therefore, we hypothesized that TNF protein is endocytosed through clathrin-dependent pathway in the steady state and IK inhibits CME resulting in the accumulation of TNF on the cell surface. However, further studies are necessary to prove this. Also this report has the limitation of using only a chemical method and immortalized cell lines, further studies will be required by employing genetic engineering methods such as confirming with primary macrophages from transgenic or knockout mice.

There are several other potential mechanisms through which IK increases the amount of TNF. CME of mTNF may trigger a negative feedback loop that modulates TNF expression since this is reversed by IK. Alternatively, mRNA of TNF substantially increased 4 h after stimulation with LPS plus IK may indicate a self-feedback system where newly synthesized TNF stimulates further production of mRNA of TNF (Figs. 2C and 4C). Rossol et al. reported that reverse signaling through TNFR1/2 b y mTNF leads to activation of monocytes [34]. Furthermore,

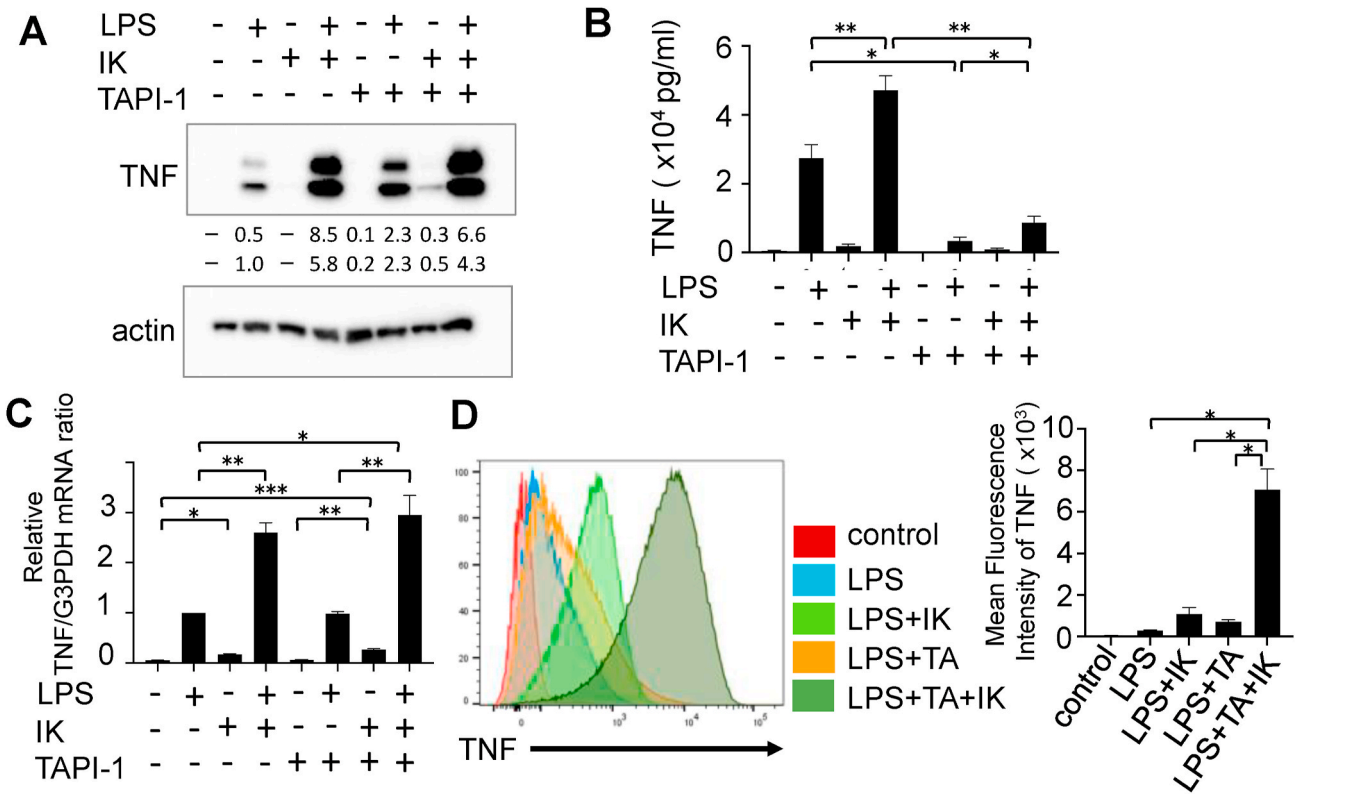

Fig. 3. An influence of IK and TAPI in RAW264 cells were analyzed. (A–D)100 ng/ml of LPS, 5 μM of IK and 10 μM of TAPI were used for 2 h s(A) or 4 h s (B-D). (A) Whole cell lysates were analyzed by Western blotting. (B) The amount of the sTNF into the supernatant was measured by ELISA assay. (C) The relative TNF/GAPDH ratios were measured by qPCR. (D) Surface expression of TNF was analyzed by FACS. A: Data are representative from three independent experiments. B, C and D: one way ANOVA followed by Tukey's multiple comparisons test, SEM, from four (B), five (C) and four (D) independent experiments.

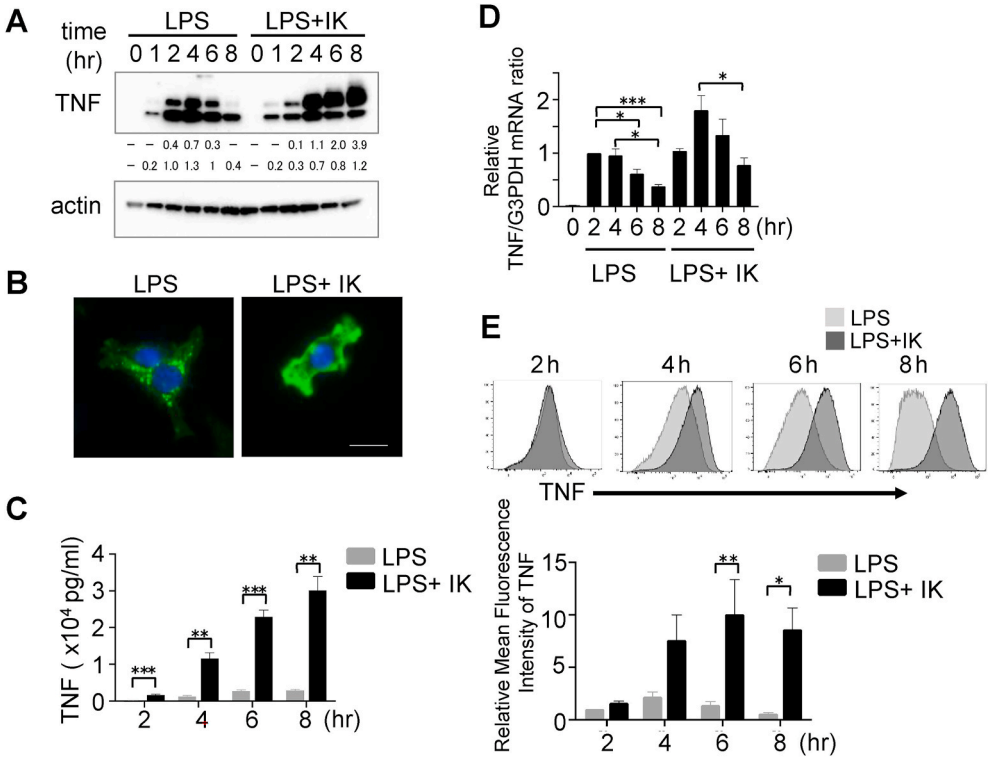

Fig. 4. ADAM-17 KO RAW264 cells were stimulated with either 100 ng/ml of LPS or 100 ng/ml of LPS plus 5 μM of IK. (A) Whole cell lysates were analyzed by Western blotting. (E) Representative immunocytochemistry staining of TNF (green) and DAPI (blue). KO RAW264 cells was stimulated for 4 h. (C) The amount of the sTNF into the supernatant was measured by ELISA assay. (D) The relative TNF/GAPDH ratios were measured by qPCR. (E) Surface expression of TNF was analyzed by FACS. Scale bars are 10 μm. A and B: Data are representative from three independent experiments. C: two way ANOVA followed by Bonferroni's multiple comparisons test, SEM, from six independent experiments. D: two way ANOVA followed by Tukey's multiple comparisons test, SEM, from seven independent experiments. E: two way ANOVA followed by Šídák's multiple comparisons test, SEM, from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IK itself might stimulate synthesis of TNF mRNA. Our data indicate that stimulation of cells with IK alone induces production of TNF mRNA (Fig. 3C). Since mTNF levels remained increasing even after mRNA began decreasing, there are additional mechanisms of maintaining the higher retention of mTNF other than inducing TNF mRNA levels. The influence of IK in each step of TNF production needs to be further investigated.

We also demonstrated that only a part of produced mTNF is converted into sTNF. The fate of uncleaved mTNF is currently unclear although endocytosis is one of the potential mechanism. This regulation of TNF may play an important role to control an excessive inflammation. In addition, we showed that even without the function of ADAM-17, sTNF is released sufficiently with stimulation of LPS and IK. Our results are consistent with a previous report with ADAM17-deficient fibroblasts [35], implying that this may be caused by the function of ADAM-10.

Finally, IK is useful not only for the understanding of TNF production but also a research tool. While Brefeldin A is useful for studying the intracellular cytokine staining, treatment with IK and TAPI renders surface TNF expression more feasibly detectable. Furthermore, the effective TNF induction by IK will contribute to the research of TNF signaling, especially TNFR2.

In conclusion, we report a novel function of IK, which is to increase not only sTNF but also the surface expression of mTNF, resulting in the persistence of inflammation for an extended period of time. Our data indicate that there is a regulatory mechanism which stops an excessive response to maintain homeostasis in the normal physiological state. Our results might ultimately lead to a discovery of effective medicines to treat inflammatory diseases. Furthermore, our findings indicate that IK can be a fundamental tool for future research of the TNF signaling.

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Declaration of competing interest

The authors have declared that no competing interests exist.

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