

Endothelial expression of fractalkine (CX3CL1) is induced by Toll-like receptor 3 signaling in cultured human glomerular endothelial cells

(培養ヒト腎糸球体内皮細胞におけるフラクタルカイン(CX3CL1)の発現はトル様受容体 3 経路により誘導される。)

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## Abstract

**Background:** Endothelial expression of membrane-bound fractalkine/CX3CL1 (Fkn) reportedly acts as a strong mediator of inflammation. Toll-like receptor 3 (TLR3) axes are thought to play some roles in the development of chronic glomerulonephritis (CGN) including lupus nephritis (LN). However, detailed mechanism of TLR3-mediated Fkn expression in glomerular endothelial cells (GECs) remains to be elucidated.

**Methods:** We examined the effect of polyinosinic-polycytidylic acid (poly IC) on Fkn expression in cultured human GECs. Fkn mRNA and protein levels were quantified by real-time PCR and enzyme-linked immunosorbent assay, respectively. To further elucidate the effects of poly IC on this signaling pathway, we used small interfering RNA (siRNA) to knockdown expression of TLR3, nuclear factor (NF)- $\kappa$ B p65, interferon (IFN)- $\beta$ , and IFN regulatory factor 3 (IRF3). We then analyzed whether pretreatment of chloroquine or dexamethasone inhibits poly IC-induced Fkn expression.

**Results:** We found that poly IC-induced Fkn expression in GECs, and that this involved NF- $\kappa$ B, IFN- $\beta$ , and IRF3. Pretreating cells with chloroquine, but not dexamethasone attenuated poly IC-induced Fkn expression in GECs.

**Conclusion:** Since the activation of TLR3/NF- $\kappa$ B/IFN- $\beta$ /Fkn and TLR3/IRF3/Fkn axes is involved in inflammatory reactions in GECs, intervention of glomerular TLR3 signaling may be

a suitable therapeutic strategy for treating CGN especially LN.

## Introduction

Fractalkine/CX3CL1 (Fkn) is a chemokine that induces chemotaxis and activation of cells expressing its receptor, CX3CR1 [1]. Fkn exists in either a membrane-bound or soluble form and the former reportedly acts as a strong adhesion molecule to circulating CX3CR1-positive leukocytes, such as activated monocytes and platelets on inflammatory endothelium [2]. Recently, it has been reported that use of anti-Fkn antibody clearly ameliorates arthritis and joint destruction in a mouse model of rheumatoid arthritis, suggesting a pivotal role for Fkn in the inflammatory process in autoimmune diseases [3]. Glomerular Fkn expression is reportedly regulated by proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [4], suggesting a role in glomerular inflammation. Indeed, mesangial Fkn expression has been reported to correlate significantly with histopathological disease activity in a rat model of prolonged proliferative glomerulonephritis (GN) [5] and also in a mouse model of lupus and in patients with lupus nephritis (LN) [6-8]. Thus, regional expression of membrane-bound form of Fkn is thought to play a pivotal role in the initiation and development of glomerular inflammation in GN, including LN [6-8].

It has been reported that antiviral host defenses may trigger development of inflammatory renal disease or lead to worsening of pre-existing renal disease [9]. This suggests that an inflammatory response involving Toll-like receptor (TLR) 3 activation and associated signaling pathways in

residual glomerular cells is involved in the pathogenesis of some forms of GN [10, 11]. Actually, some previous studies have revealed the expressions of TLR3 in resident renal cells [10, 12]. Accordingly, the TLR3-mediated intracellular signaling cascade activates transcription factors, such as interferon regulatory factor (IRF) 3 and nuclear factor kappa B (NF- $\kappa$ B) and the subsequent release of adhesion molecules, cytokines, and chemokines [13, 14], which play a pivotal role in innate and adaptive immune responses in residual glomerular cells [10, 11, 15].

Glomerular endothelial cells (GECs) are reported to express various adhesion molecules in response to proinflammatory cytokines and thereby play a pathophysiological role in hemodynamic disturbances and their interaction with intrinsic mesangial cells (MCs) [16]. Furthermore, GECs are directly exposed to circulating viral particles in the glomerulus [17].

Endothelial TLR3 is thought to play a role in the development of GN; however, little is known of the specific molecular mechanisms underlying the initiation of glomerular inflammation via activation of endothelial TLR3 signaling [17-19]. We previously reported that expression of the membrane-bound form of Fkn is induced via TLR3/IRF3 signaling in MCs [20]. Although glomerular Fkn expression contributes to the development of some forms of GN, including LN [5-8], the details regarding the glomerular endothelial signaling pathways involved in Fkn expression remain to be elucidated. In this study, we therefore examined endothelial Fkn expression in relation to TLR3 signaling in GECs. Further, since the implication of glomerular

Fkn expression has primarily been studied mainly in the context of LN [6-8], we also examined whether pretreatment with drugs typically used in the treatment of LN, such as the anti-malarial agent, chloroquine [21, 22] or the corticosteroid, dexamethasone (DEX) could restore TLR3-mediated Fkn expression in GECs.

## **Materials and Methods**

### **Reagents**

Polyinosinic-polycytidylic acid (Poly IC) as a TLR3 agonist and lipopolysaccharide (LPS) of *Escherichia coli* as a TLR4 agonist were obtained from Sigma (St. Louis, MO, USA). R848 as a TLR7 agonist and CpG as a TLR9 agonist were obtained from InvivoGen (San Diego, CA, USA) and Novus Biologicals (Centennial, CO, USA), respectively. Chloroquine diphosphate and DEX were purchased from Wako Pure Chemical (Osaka, Japan). Small-interfering RNAs (siRNAs) against TLR3, IFN- $\beta$ , and NF- $\kappa$ B p65 dNTP mix Moloney murine leukemia virus (MMLV) reverse transcriptase, and Lipofectamine RNAi MAX were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Non-silencing negative control siRNA and siRNA against IRF3 were purchased from Qiagen (Hilden, Germany). The illustra RNAspin Kit was purchased from GE Healthcare (Buckinghamshire, UK). SsoAdvanced Universal SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). Oligo (dT)<sub>18</sub> Primer and additional oligonucleotide

primers for PCR were custom synthesized by Greiner (Atsugi, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for CX3CL1 and IFN- $\beta$  were purchased from R&D systems (Minneapolis, MN, USA) and PBL Assay Science (Piscataway, NJ, USA), respectively. Anti-NF- $\kappa$ B p65 antibody and anti-IRF3 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA) and Immuno-Biological Laboratories (Gunma, Japan), respectively.

## **Cells**

GECs were purchased from ScienCell (Carlsbad, CA, USA) and were cultured using Endothelial Growth Medium-2 (EGM-2; Lonza, Walkersville, MD, USA). Poly IC was dissolved in phosphate-buffered saline (PBS), pH 7.4, and the cells were treated with 0.5-50  $\mu$ g/mL poly IC for up to 24 h. The cells were also treated with 1  $\mu$ g/mL LPS, 5  $\mu$ g/mL R848, or 100  $\mu$ g/mL CpG for up to 24 h. In RNA interference experiments, cells were transfected in the six-well plates with 30 pmole of siRNA (against TLR3, IFN- $\beta$ , p65, or IRF3 or non-silencing negative control siRNA) per well, using the Lipofectamine RNAi MAX reagent according to the supplier's protocol. The cells were 30-50% confluent at the time of transfection. After 48 h incubation, the cells were treated with poly IC as indicated. Effective knockdown of these molecules was confirmed previously [17, 18].

Chloroquine and DEX (dissolved in PBS and in ethanol, respectively) were added to cells at 10

µg/mL and 10 µM, respectively; following incubation for 1 h, cells were stimulated with 30 µg/mL poly IC [21, 22].

### **Quantitative real-time polymerase chain reaction (RT-PCR) analyses**

Total RNA was extracted from the cells and purified using an illustra RNAspin kit according to the manufactures' instruction. Single-stranded complementary DNA (cDNA) was synthesized from 1 µg of total RNA using Oligo(dT)<sub>18</sub> Primer and MMLV reverse transcriptase. Fkn (CX3CL1), IFN-β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified using SsoAdvanced Universal SYBR Green Supermix. GAPDH was used as an internal control. PCR was performed using the following primers:

CX3CL1-forward: 5'-GACCCCTAAGGCTGAGGAAC-3',

CX3CL1-reverse: 5'-CTCTCCTGCCATCTTTCGAG-3',

IFN-β-forward: 5'-CCTGTGGCAATTGAATGGGAGGC-3',

IFN-β-reverse: 5'-CCAGGCACAGTGACTGTACTCCTT-3',

TLR3-forward: 5'-CTCAGAAGATTACCAGCCGCC-3',

TLR3-reverse: 5'-CCATTATGAGACAGATCTAATG-3',

GAPDH-forward: 5'-GCACCGTCAAGGCTGAGAAC-3'



GAPDH-reverse: 5'-ATGGTGGTGAAGACGCCAGT-3'.

The sizes of the expected PCR products for CX3CL1, IFN- $\beta$ , TLR3 and GAPDH were 205, 370, 287 and 142 bp, respectively.

### **Enzyme-linked immunosorbent assay (ELISA)**

The concentrations of CX3CL1 and IFN- $\beta$  protein in the cell-conditioned medium were measured using commercially available ELISA kits according to the suppliers' recommended protocols.

### **Western blotting**

After the incubation, the cells were lysed with Laemmli's buffer. The lysates were subjected to polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane, and incubated with primary antibodies: anti-NF- $\kappa$ B p65 (1:1500), anti-IRF3 (1:300) or anti-actin (1:3000) antibody. After incubation with a secondary antibody labeled with horseradish peroxidase, the protein bands were detected using a chemiluminescence substrate.

### **Statistical analysis**

All experiments were performed at least 3 times. Values of real-time PCR and ELISA are reported

as mean  $\pm$  SD. Statistical significance was evaluated using unpaired *t*-tests. All analyses were performed using GraphPad Prism Software Version 7 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### *Poly IC also LPS and CpG induce the expression of Fkn in cultured human GECs*

In unstimulated GECs, Fkn mRNA expression level was less than the detection limit. When the cells were treated with various concentration of poly IC, the expression of Fkn mRNA (Fig. 1A) and protein (Fig. 1B) were induced in a concentration-dependent manner. Poly IC also induced the expression of Fkn mRNA and protein in GECs in a time-dependent manner as follows: Fkn mRNA expression gradually increased after poly IC stimulation, peaked at 16 h, and decreased at 24 h (Fig. 2A); and Fkn protein accumulated in the conditioned medium for up to 24 h in a time-dependent manner (Fig. 2B). When the cells were treated with LPS, or CpG, the expression of Fkn mRNA was also induced in a time-dependent manner, whereas treatment with R848 had no effect in this cell type (Fig. 2C).

### *TLR3, IFN- $\beta$ , NF- $\kappa$ B and IRF3 are involved in the poly IC-induced expression of Fkn in GECs*

To examine the role of TLR3, IFN- $\beta$ , NF- $\kappa$ B and IRF3 in poly IC-induced Fkn expression, we next performed the RNA interference experiments to knockdown expression of these molecules. Knockdown of TLR3, IFN- $\beta$ , p65, or IRF3 resulted in the significant inhibition of poly IC-induced Fkn mRNA (Fig.3A and 4A) and protein (Fig. 3B and 4B) expression.

***Poly IC-induced Fkn expression was attenuated by chloroquine, but not by DEX***

We examined the effect of chloroquine and DEX on the poly IC-induced Fkn expression. Pretreatment of cells with 10  $\mu$ g/mL chloroquine significantly suppressed the expression levels of Fkn mRNA (Fig. 5A) and protein (Fig. 5B) induced by poly IC. Chloroquine pretreatment also resulted in significantly lower levels of IFN- $\beta$  mRNA (Fig. 5C) and protein (Fig. 5D) in poly IC-exposed cells. Conversely, pretreatment of cells with DEX did not affect the poly IC-induced expression of Fkn mRNA (Fig. 6A) or protein (Fig. 6B) despite of a slight inhibition of IFN- $\beta$  mRNA expression (Fig. 6C). Chloroquine pretreatment also reduced poly IC induced-TLR3 mRNA expression (Fig. 5E) whereas it was not affected by DEX (Fig. 6D). In this experimental setting, neither chloroquine nor DEX affected the expression of NF- $\kappa$ B p65 and IRF3 protein (Figs. 5F and 6E). To further address this issue, we tried to examine immunofluorescence staining of p65 using chloroquine and DEX-treated GECs as we examined in previous report using MCs [21]. However, it was impossible due to unexpected cell fragility. Thus, this issue remains to be studied in the future.

## Discussion

It has been reported that glomerular Fkn expression and subsequent infiltration of CX3CR1-positive leukocytes, such as activated monocytes may play an important role in the development of prolonged glomerular inflammation in animal models and human GN, including LN [5-8]. Accordingly, glomerular Fkn expression and subsequent recruitment of circulating inflammatory cells expressing the Fkn receptor are believed to contribute to the initiation and progression of GN. Given the implication of TLR3 signaling in residual glomerular cells [11, 15], we previously examined the TLR3 signaling cascades in cultured human MCs and GECs following induction of a “pseudoviral” infection status by poly IC [18-25]. GECs are thought to mediate recruitment of circulating neutrophils into the glomeruli firstly [26, 27], and also interact directly with circulating viral particles in the glomerulus [17]; therefore, detailed mechanisms of these phenomena induced by the proinflammatory chemokine via TLR3 signaling are still to be examined [17-19]. The TLR3 signaling cascade begins with activation of the TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF)-dependent pathways via NF- $\kappa$ B and IRF3 (TLR3/TRIF/NF- $\kappa$ B and TLR3/TRIF/IRF3, respectively) [13]. TLR3-mediated induction of these phenomena is an area of particular interest. We previously found that the TLR3/IRF3 axis, but not the TLR3/NF- $\kappa$ B axis was involved Fkn expression in cultured human MCs [20]; however, to our knowledge, a detailed examination of TLR3-mediated Fkn expression in cultured human GECs has not yet been

performed.

In the present study, we found that poly IC treatment induced the expression of Fkn in GECs in almost along with a time- and dose-dependent manner. We previously showed that poly IC induced TLR3 signaling in MCs and GECs, and that TLR3 signaling resulted in the induction of IFN- $\beta$ , but not of IFN- $\alpha$  at least in our experimental settings [18-25]. Thus, in the present study, we examined the role of IFN- $\beta$  in GECs and confirmed that siRNA-mediated knockdown of IFN- $\beta$  clearly suppressed the poly IC-induced expression of Fkn. Interestingly, we found that knockdown of either NF- $\kappa$ B p65 or IRF3 almost equally inhibited the poly IC-induced expression of Fkn in GECs. These results suggest that TLR3, NF- $\kappa$ B, IRF3, and IFN- $\beta$  are, at least equally, involved in the poly IC-induced expression and regulation of Fkn in GECs. Notably, these results differ from our previous findings in MCs, as described above [20], suggesting that there may be different TLR3-mediated signaling cascades associated with Fkn expression in GECs and MCs. The discrepancy between GECs and MCs may partly be due to different cell type, although this will require further examination.

To date, the implication of TLR3 signaling in residual glomerular cells has been studied in some forms of GN, including LN [11, 15, 21-23, 28], we next examined the effect of pretreatment with the antimalarial agent, chloroquine on TLR3-mediated Fkn expression in GECs. It has been reported that membrane-bound Fkn acts as a strong adhesion molecule to macrophages in

activated endothelial cells [2]. Recently, anti-Fkn antibody has been considered an attractive candidate for future treatment of autoimmune diseases [3]. Thus, we consider that intervening in the endothelial TLR3/Fkn signaling cascades is a possible therapeutic strategy that could be beneficial in the treatment of glomerular inflammation as postulated in our recent paper describing that chloroquine effectively inhibited TLR3-mediated plasminogen activator inhibitor-1 in GECs [22]. Interestingly, chloroquine pretreatment clearly decreased the poly IC-induced expression of IFN- $\beta$  mRNA and protein in GECs whereas DEX treatment resulted in only a slight decrease in the IFN- $\beta$  mRNA expression which did not effect on the Fkn expression. However, in this study, we did not confirm the beneficial effect of chloroquine on the poly IC-induced nuclear translocation of NF- $\kappa$ B p65 and IRF3, leading to a decrease in Fkn expression in GECs. Since it has previously been reported that Shiga toxin-mediated Fkn expression in GECs depends on the activations of NF- $\kappa$ B and p38 MAPK [29], this issue should be determined in the future. Despite limitations, our results suggest that chloroquine inhibits endothelial TLR3 signaling during the early phase of IFN- $\beta$  production, as also postulated in the context of MCs and GECs [21, 29]. On the other hand, pretreatment with DEX did not show such effects, indicating a unique mode of action for chloroquine in TLR3 signaling in GECs. However, we did not determine detailed in-depth molecular mechanisms by which chloroquine inhibits TLR3 signaling in GECs or how these may differ from those in MCs, partly because of the fragility of cultured GECs we used that

was a potential barrier to our further experiment. Thus, this issue may require further in-depth investigation including the phosphorylation status of NF- $\kappa$ B and IRF3 inhibitors using such as a human GECs cell line.

The innate immunity-related pathogenesis of LN is complex with involvements of TLR3, TLR4, TLR7 and TLR9 [10, 30]. Indeed, it has been reported that overexpressions of glomerular TLR3, TLR7, and TLR9 were seen in biopsy specimens of patients with LN and these phenomena correlated to clinicopathological indices [12]. With respect to TLRs-mediated proinflammatory chemokines, Fkn is already known to be related with TLR4 signaling [31]. Therefore, we examined whether a TLR4 agonist LPS, a TLR7 agonist R848, and a TLR9 agonist CpG effect on the Fkn expression in GECs. We observed that Fkn mRNA expression was induced by LPS and CpG, but not by R848. This suggests that expression of Fkn in GECs can be regulated by signaling via multiple TLRs [31]. With respect to TLR3 in intrinsic glomerular cells, it has been reported that activation of TLR3 and downstream immune responses can be induced by both infectious pathogens and endogenous ligands [10, 11, 13, 30], and possibly leading to development of “pseudo” antiviral immunity in at least in part of the pathogenesis of LN [11, 30], although this theory remains speculative. Thus, we believe that identifying the specific mechanisms of endothelial TLR3 signaling in GECs will aid the development of possible therapeutic strategies for LN in the future [28]. We found that TLR3 signaling contributes to



expression of Fkn in GECs. However, further studies, including *in vivo* murine models, will be needed to confirm and expand on our preliminary findings.

### **Acknowledgement**

This study was supported by Grants-in-Aid of the Japan Society for Promotion of Science (JSPS KAKENHI Grant Number: 16K10055 to H. T.).

### **Declaration of Conflicting Interests**

The authors declare that there is no conflict of interest.

### **Statement of Ethics**

This study was not involved human and animal subjects which required ethical approval.

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### Figure legends

**Fig.1.** Polyinosinic-polycytidylic acid (poly IC) induces the expression of fractalkine (CX3CL1) mRNA and protein in cultured human glomerular endothelial cells (GECs) in a concentration-dependent manner.

Human glomerular endothelial cells (GECs) were cultured, and were treated with 0.5–50 µg/mL of poly IC. Data is shown as means±SD (n=3).

(A) After 16 h incubation, RNA was extracted from the cells. The RNA was reverse-transcribed, and the cDNA was subjected to quantitative real-time PCR analysis for fractalkine and GAPDH. In untreated cells, fractalkine mRNA was not detected, and the data is therefore shown as an arbitrary unit.

(B) The conditioned medium was collected after 24 h incubation and the concentration of fractalkine protein in the medium was determined using an ELISA.

**Fig.2.** Poly IC induces the expression of fractalkine mRNA and protein in GECs in cultured human GECs in a time-dependent manner.

GECs were stimulated with 30 µg/mL poly IC for up to 24 h. The culture medium was collected and RNA was extracted. Data is shown as means±SD (n=3).

Expression of fractalkine mRNA (A) and protein (B) was examined using RT-PCR and ELISA,

respectively. GECs were treated with 1 µg/mL LPS (C, upper panel), 5 µg /mL R848 (C, middle panel) or 100 µg/mL CpG (C, lower panel) for up to 24 h, and expression of fractalkine mRNA was analyzed by RT-PCR.

**Fig.3.** Knockdown of TLR3 or IFN-β decreases the poly IC-induced expression of fractalkine in cultured human GECs.

The cells were transfected with siRNA against TLR3 or IFN-β or a non-silencing negative control siRNA and were incubated for 48 h. Then the cells were stimulated with 30 µg/mL poly IC. Data is shown as means±SD (n=3, \**p* < 0.01 by *t*-test).

(A) RNA was extracted from the cells after 16 h incubation, and real-time RT-PCR was performed.

(B) The medium was collected after 24 h incubation, and fractalkine ELSIA was performed.

**Fig.4.** Knockdown of NF-κB p65 or IRF3 decreases the poly IC-induced expression of fractalkine in cultured human GECs.

The cells were transfected with siRNA against p65, IRF3 or a non-silencing negative control siRNA. After 48 h incubation, the cells were stimulated with 30 µg/mL poly IC. Data is shown as means±SD (n=3, \**p* < 0.01 by *t*-test). RNA and the medium were collected from the cells, and real-time RT-PCR (A) and ELISA (B) were performed as in Fig. 3.



**Fig.5.** Pretreatment of cells with chloroquine suppresses the expression of fractalkine, IFN- $\beta$  and TLR3 in cultured human GECs treated with poly IC.

After pretreatment with 10  $\mu$ g/mL chloroquine for 1 h, the cells were treated with 30  $\mu$ g/mL poly IC. Data is shown as means $\pm$ SD (n=3, \* $p$  < 0.01 by  $t$ -test).

(A) After incubating for 16 h, RNA was extracted, and real-time RT-PCR was performed for fractalkine.

(B) After incubating for 24 h, culture medium was collected and fractalkine ELISA was performed.

(C) After incubation for 2 h, the cells were subjected to real-time RT-PCR for IFN- $\beta$ . Because IFN- $\beta$  mRNA was not detected in untreated cells, therefore the data is shown as arbitrary unit.

(D) The medium was collected after 24 h incubation, IFN- $\beta$  ELISA was performed.

(E) After incubating for 16 h, the cells were subjected to real-time RT-PCR for TLR3.

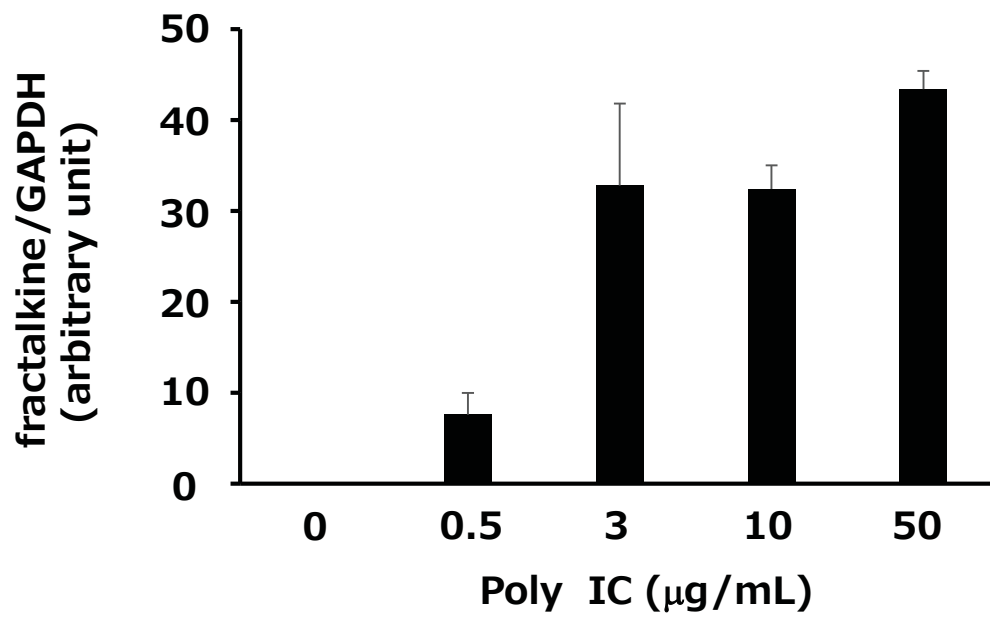
(F) The cells were lysed after 24 h incubation, and the lysates were subjected to western blotting for NF- $\kappa$ B p65, IRF3 and actin.

**Fig. 6.** Pretreatment with dexamethasone does not inhibit poly IC-induced fractalkine expression in cultured human GECs. Data is shown as means $\pm$ SD (n=3, \* $p$  < 0.01 by  $t$ -test).

Cells were pretreated with 10  $\mu$ M dexamethasone (DEX) for 1 h. Then the cells were stimulated with 30  $\mu$ g/mL poly IC for 2 h (C), 16 h (A, D) or 24 h (B, E) 16 h (A) or 24 h (B). Real-time RT-

PCR (A, C and D), ELISA (B) and western blotting (E) were performed as above.

### A. RT-PCR



### B. ELISA

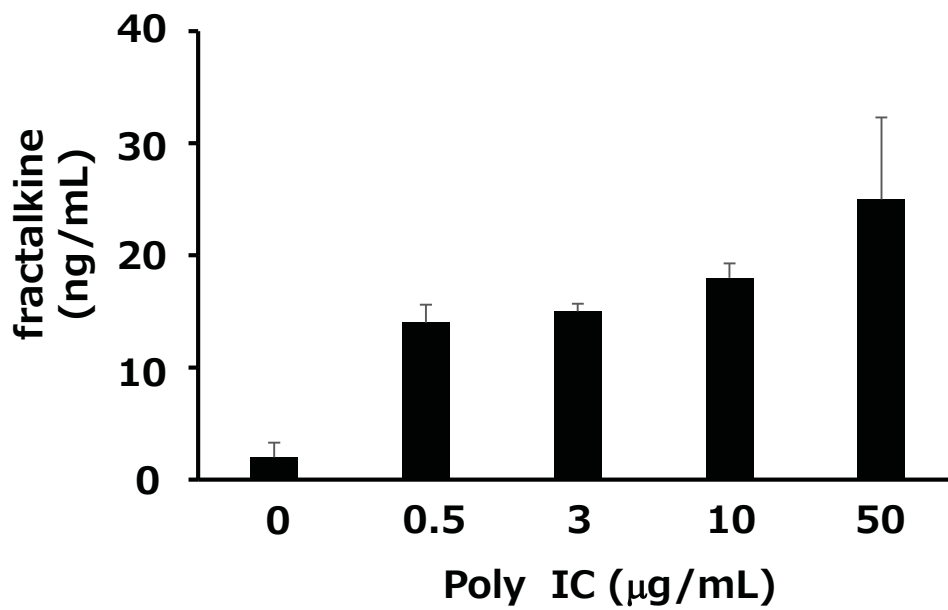
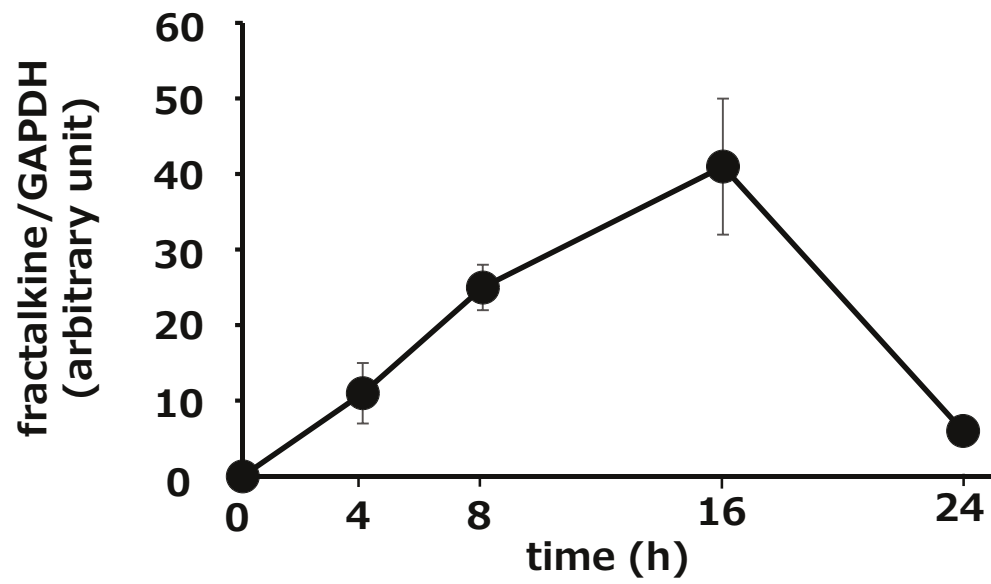


Fig.1. Hirono et al

**A. RT-PCR**



**B. ELISA**

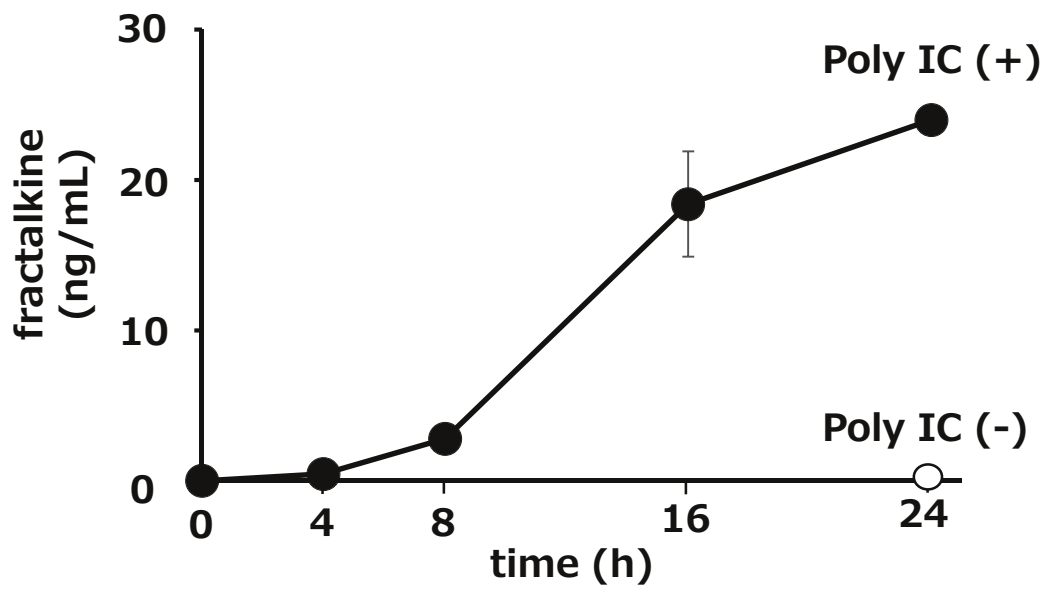


Fig 2AB. Hirono et al

C.RT-PCR

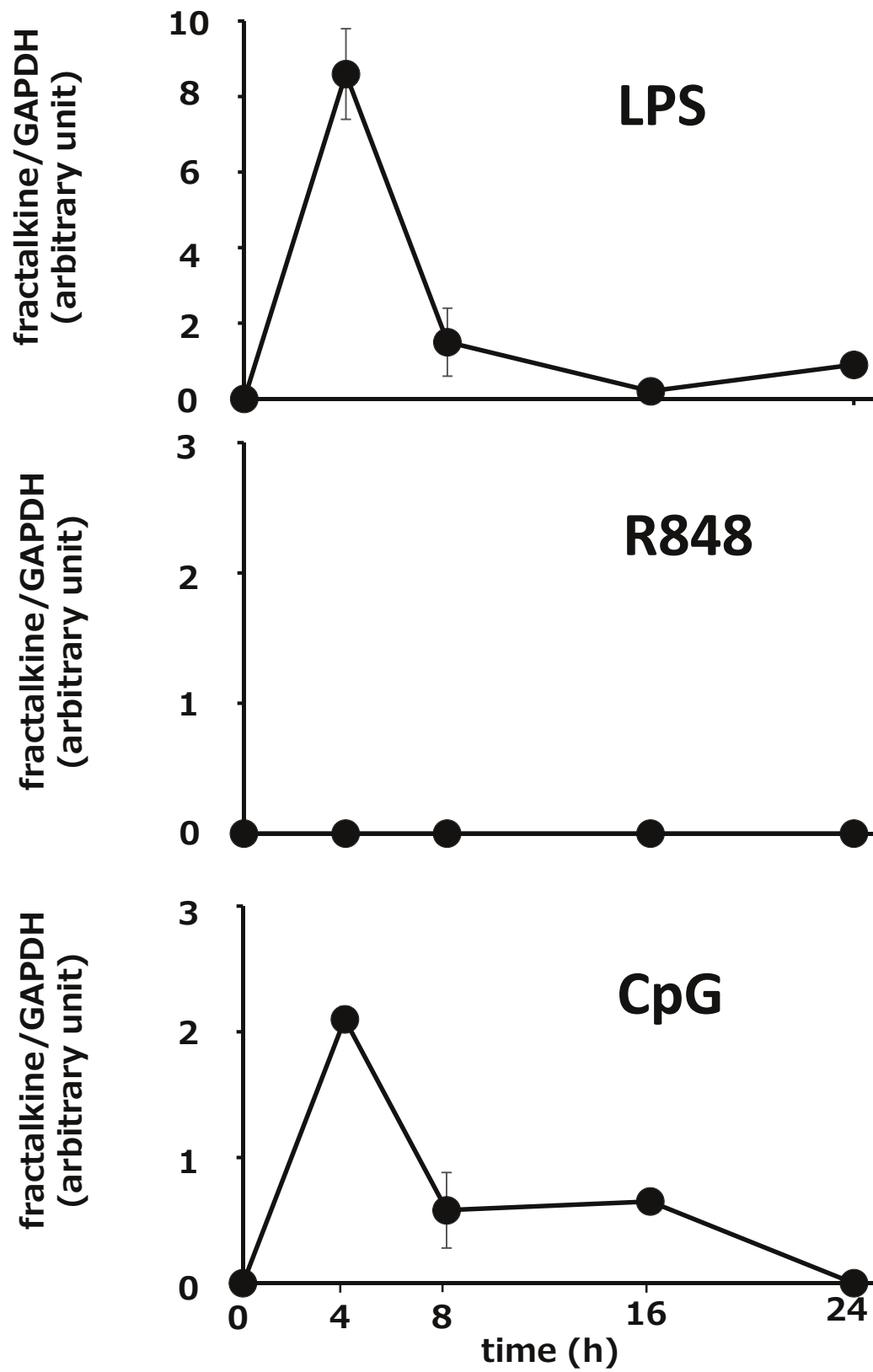
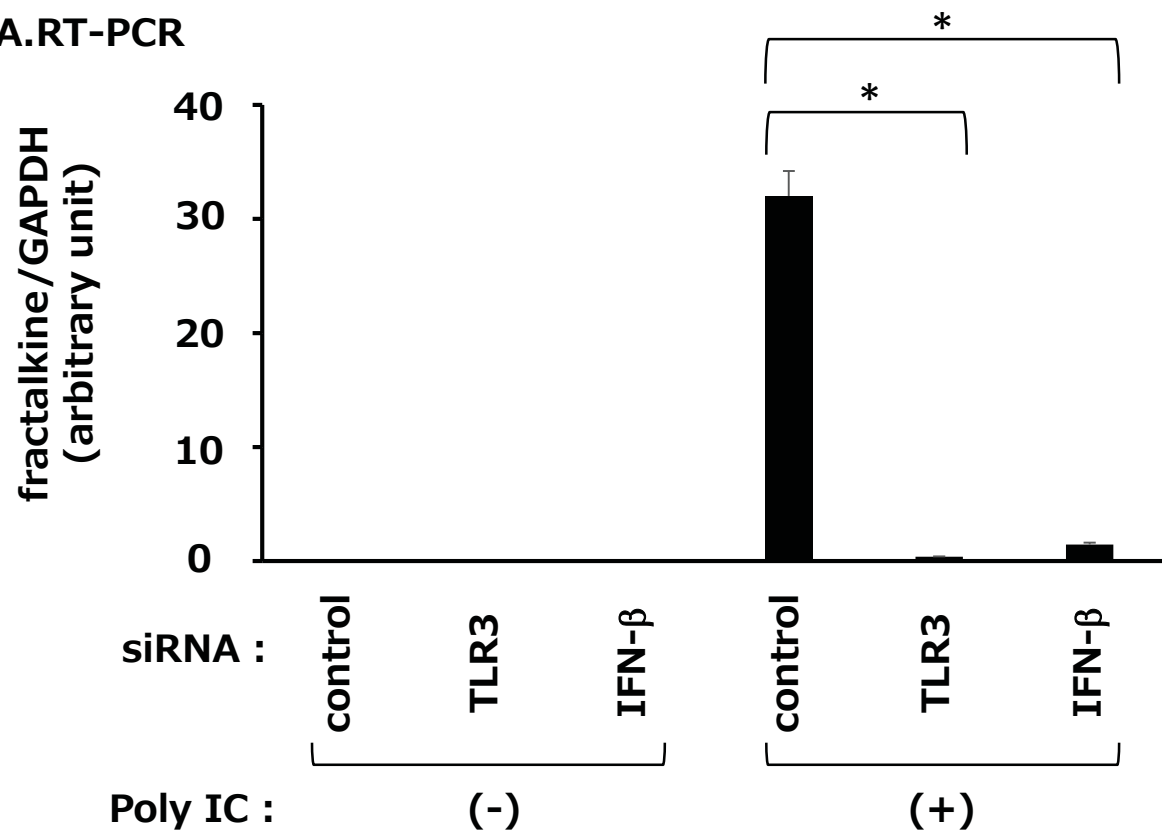


Fig. 2C. Hirono et al.

### A. RT-PCR



### B. ELISA

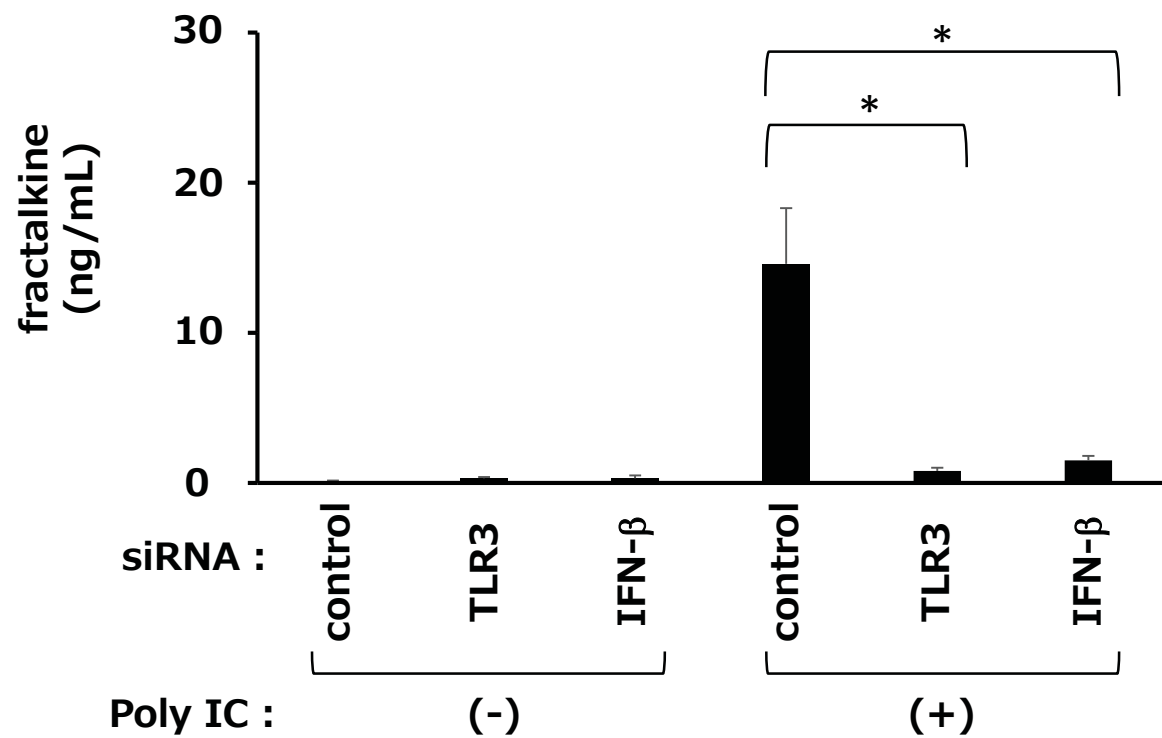
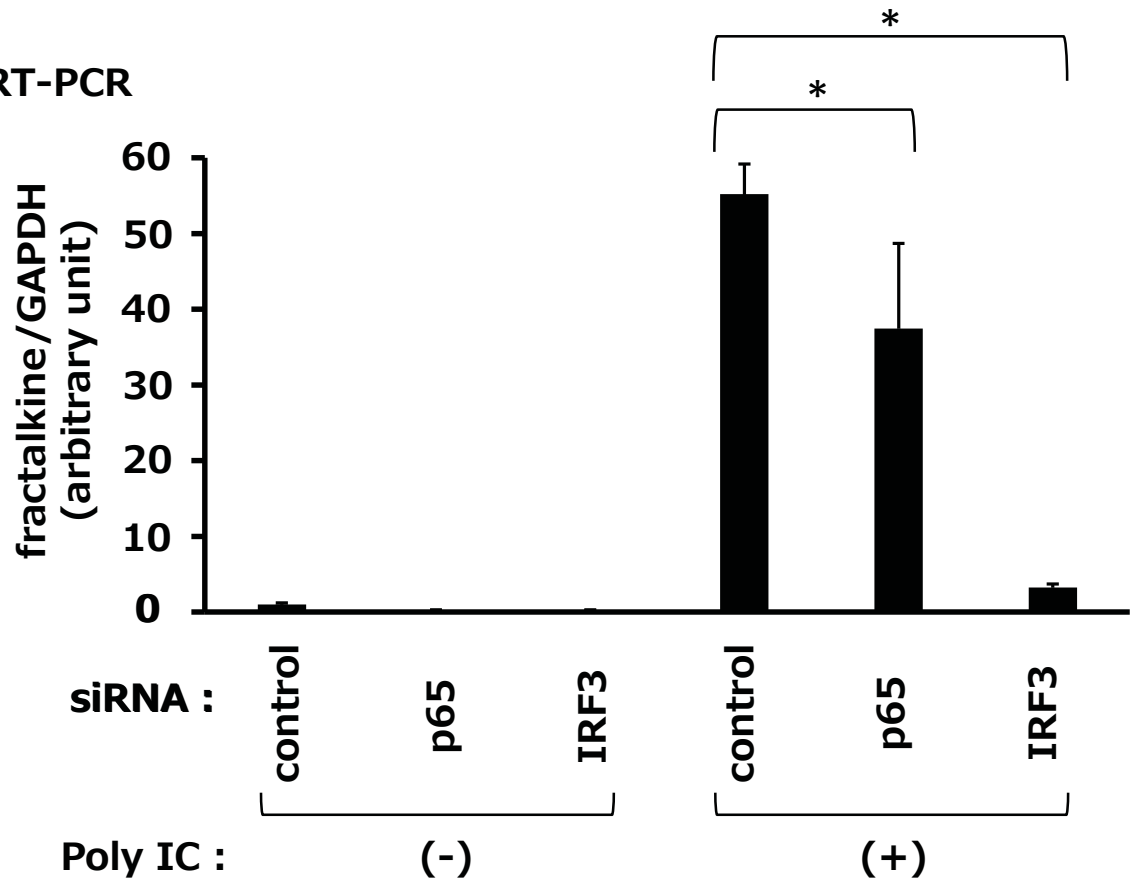


Fig.3. Hirono et al

### A. RT-PCR



### B. ELISA

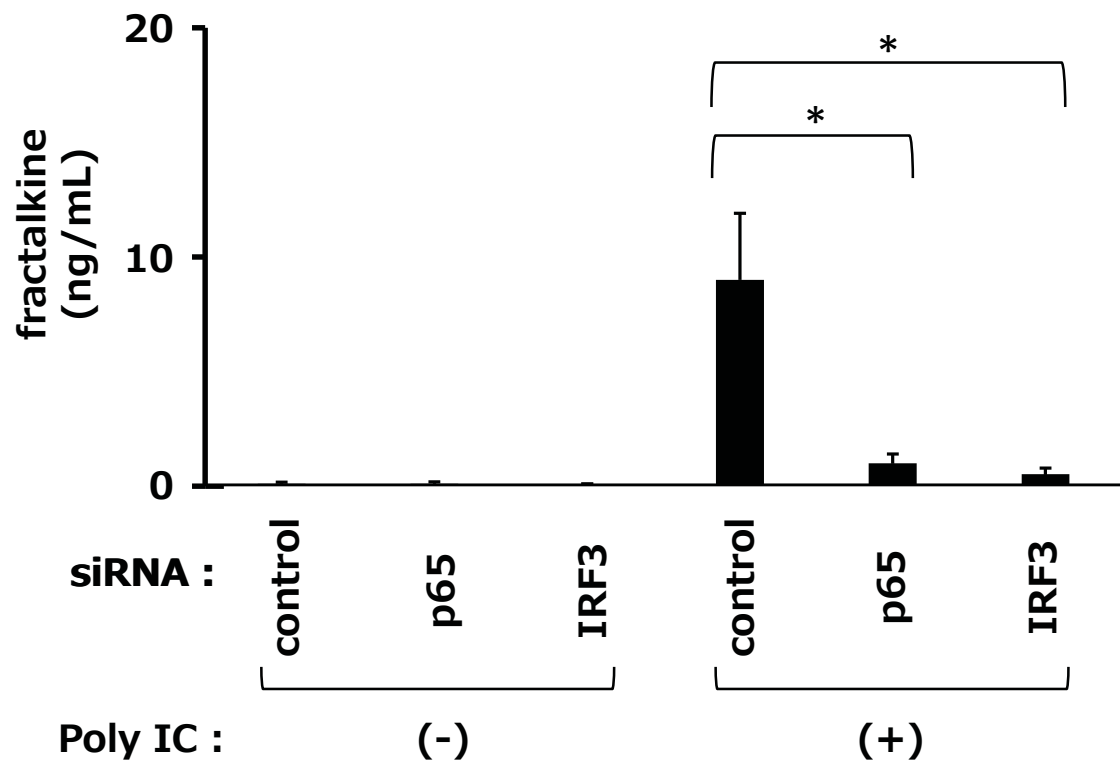
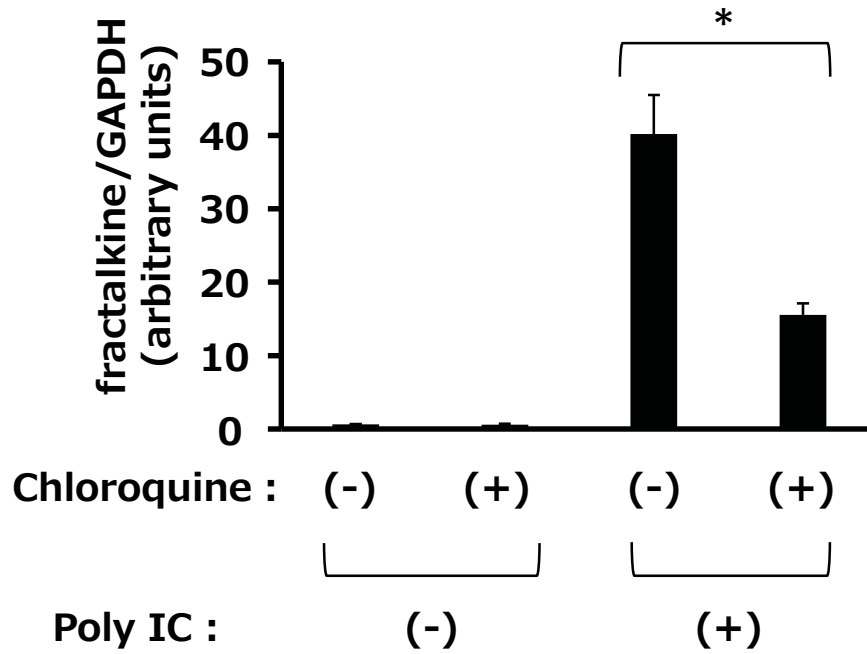


Fig.4. Hirono et al

### A. RT-PCR



### B. ELISA

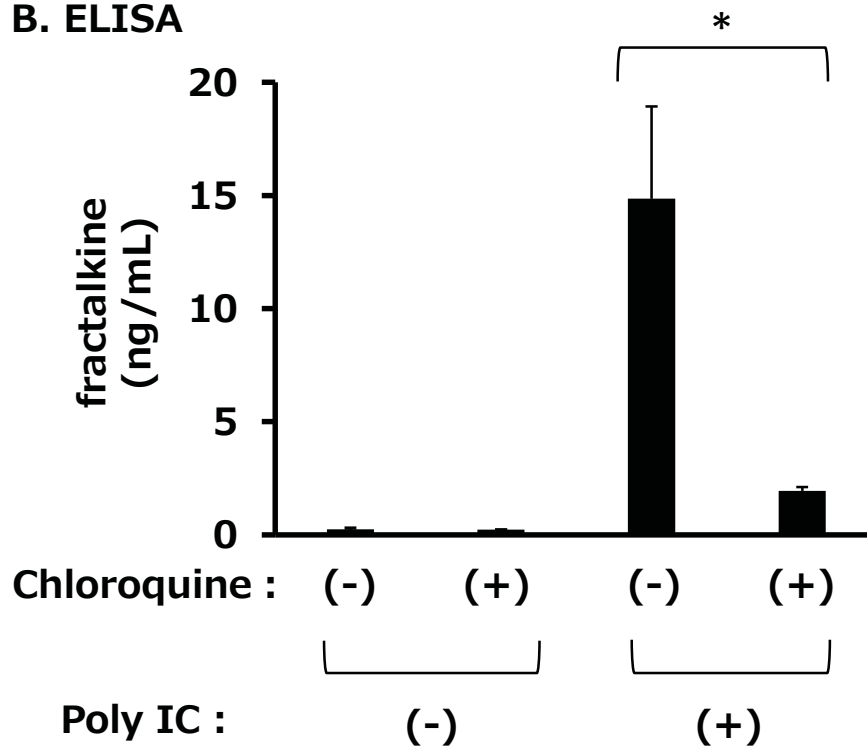
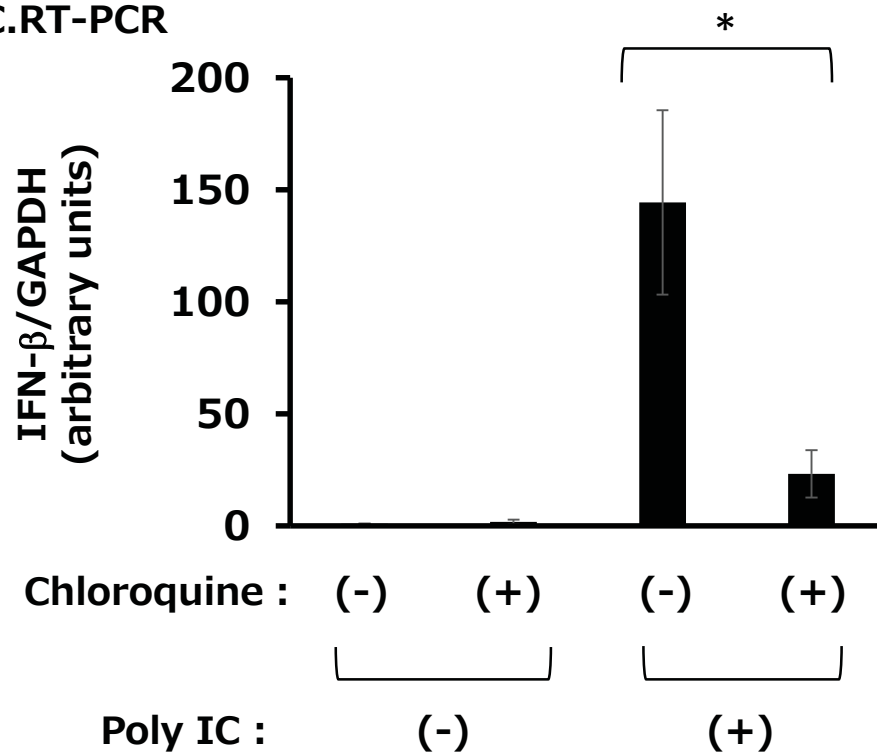


Fig.5AB. Hirono et al



### C. RT-PCR



### D. ELISA

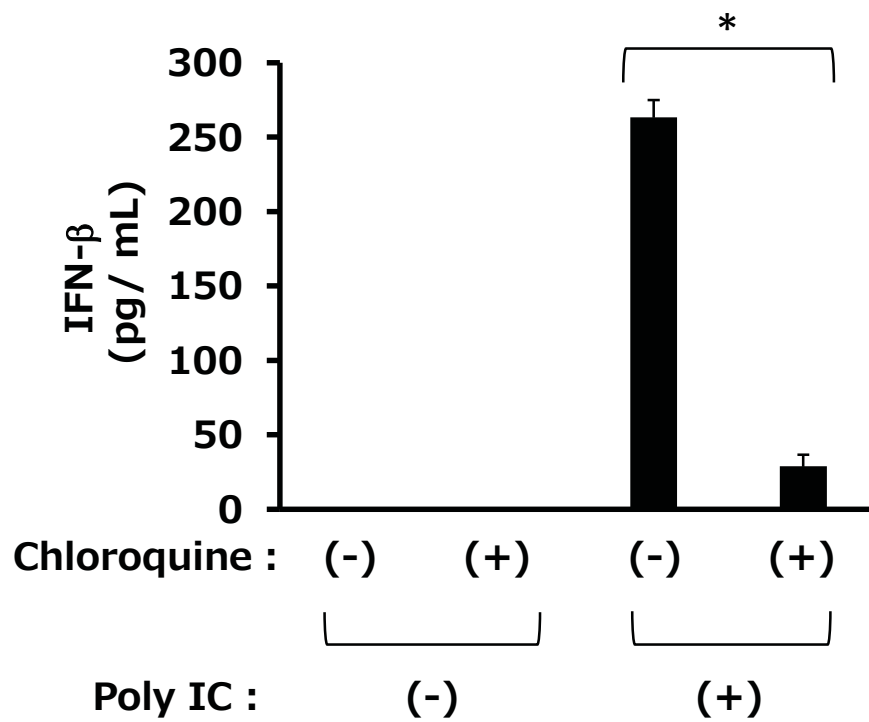
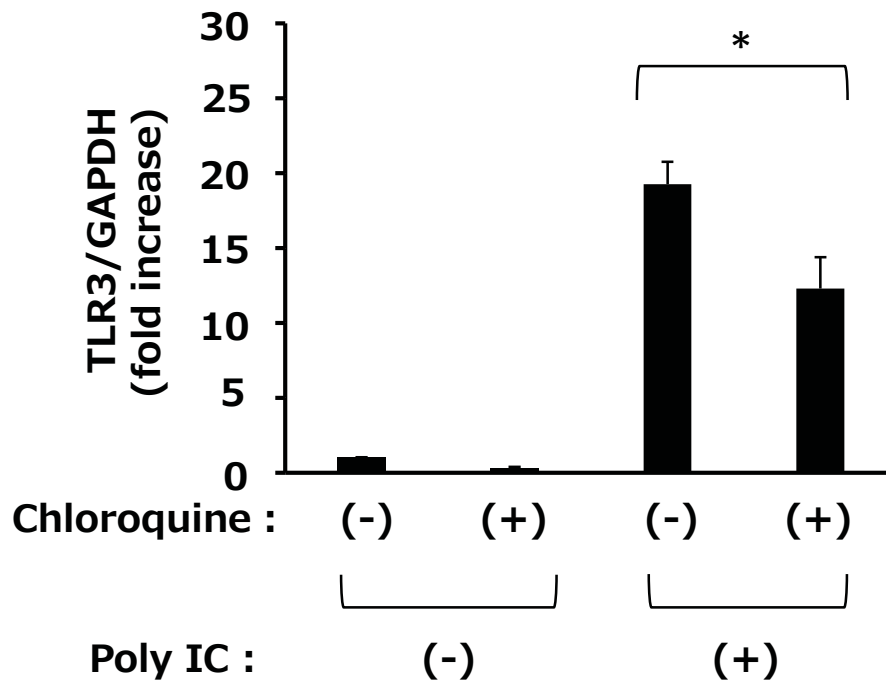


Fig.5CD. Hirono et al

### E. RT-PCR



### F. western blotting

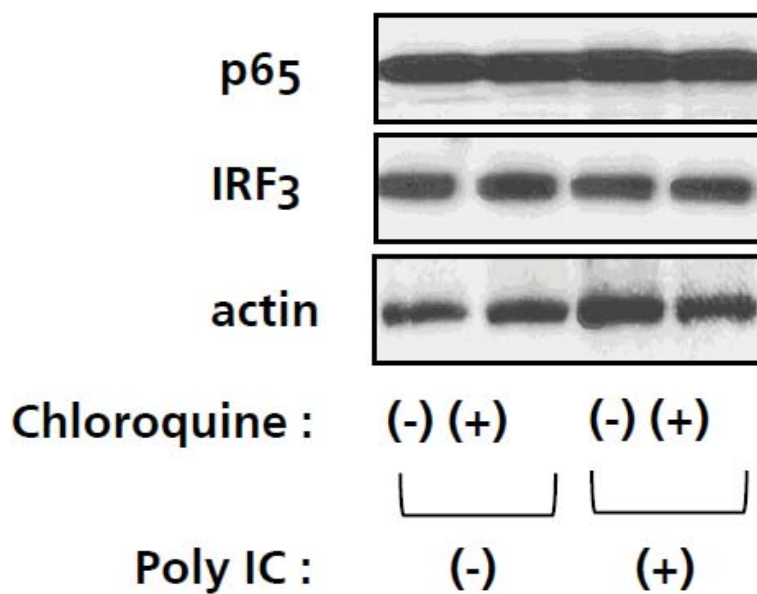
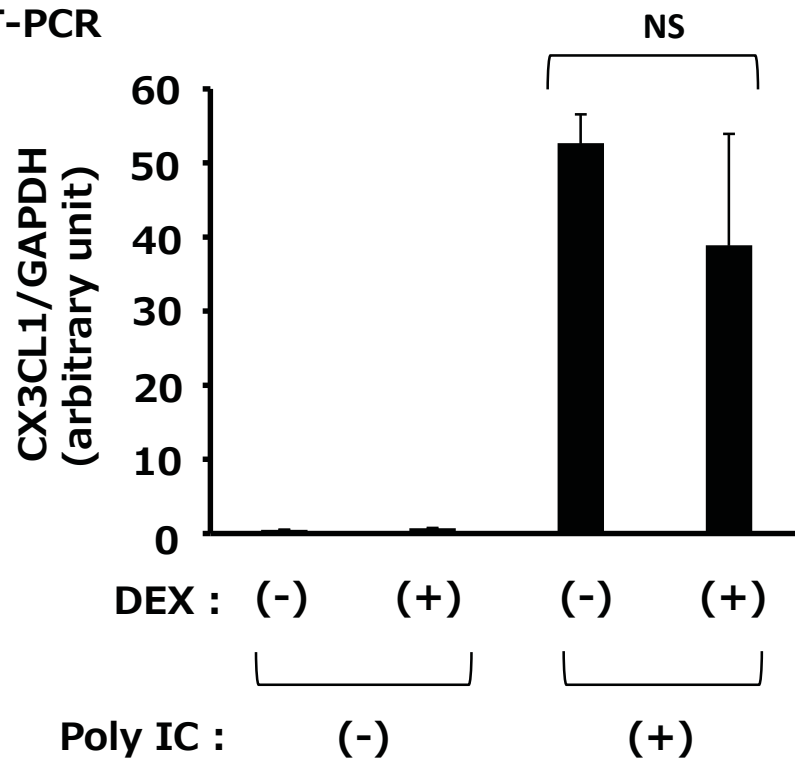


Fig.5 EF. Hirono et al

### A. RT-PCR



### B. ELISA

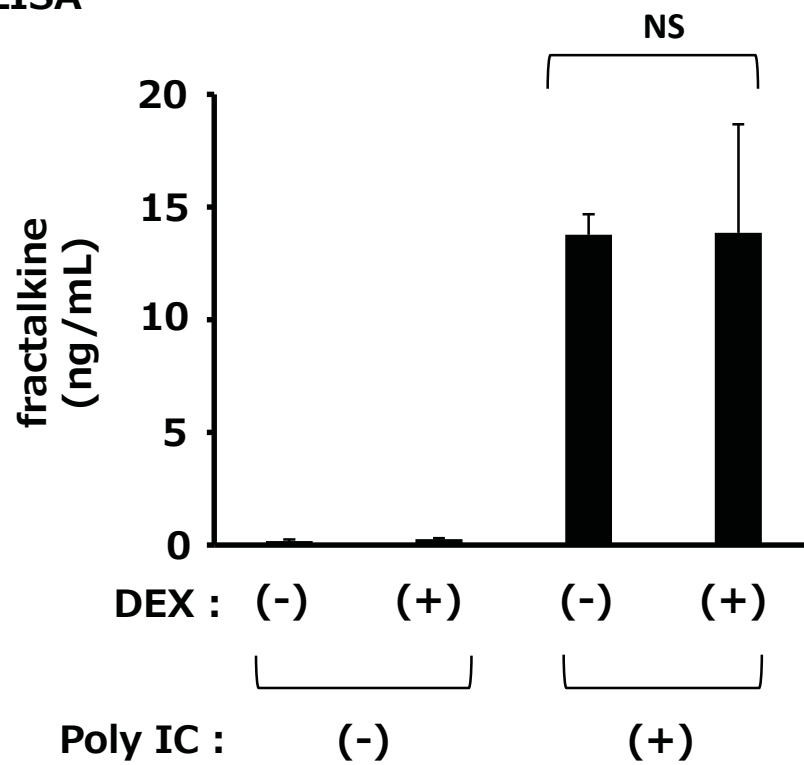
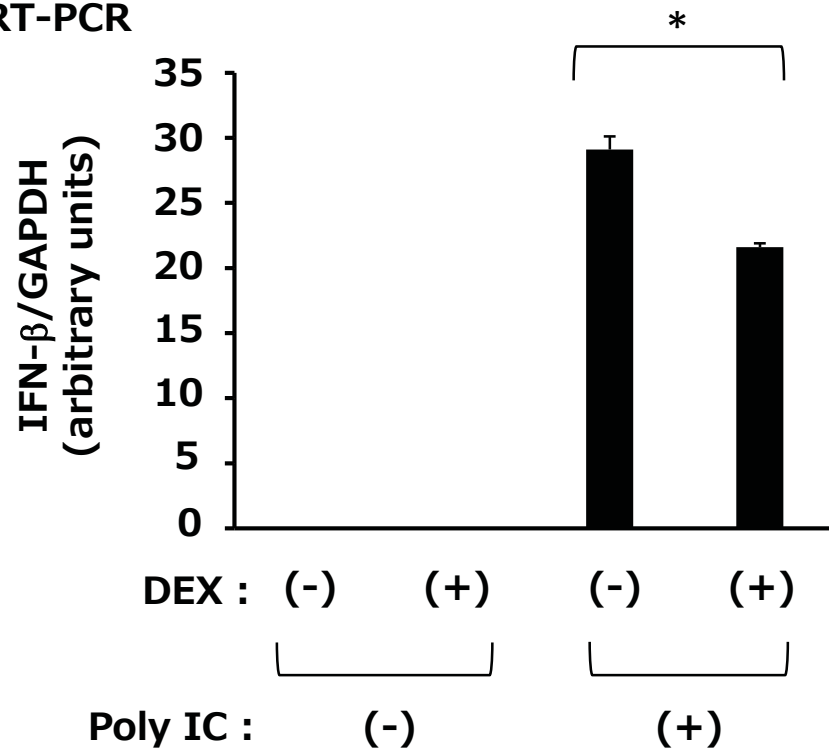


Fig.6AB. Hirono et al

C. RT-PCR



D. RT-PCR

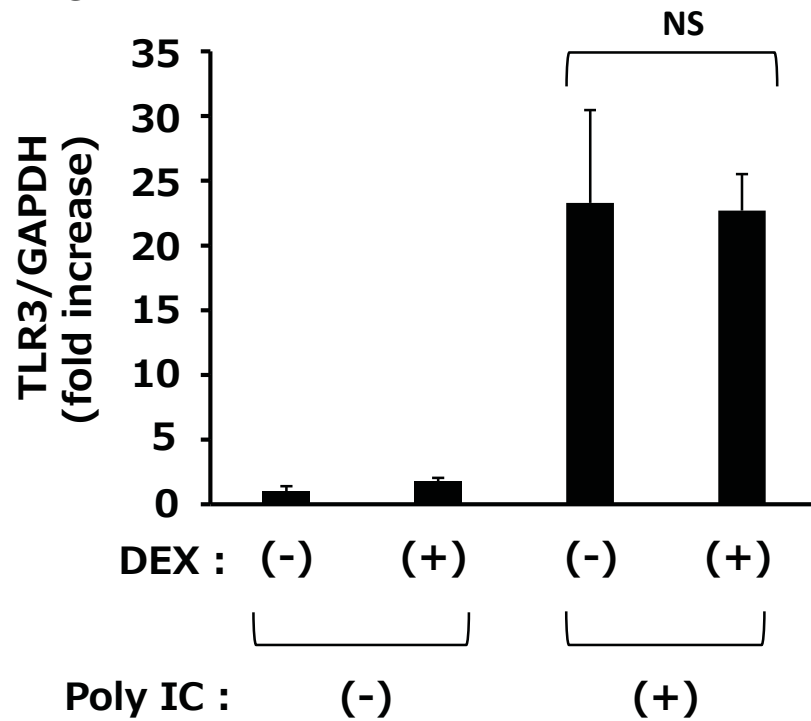


Fig.6. CD Hirono, et al.

**E. western blotting**

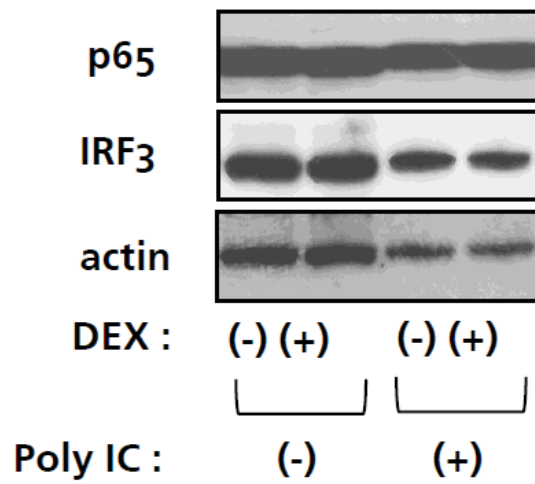


Fig.6. E Hirono, et al.