

Histamine- or VEGF-induced tissue factor expression and gap formation between vascular endothelial cells are synergistically enhanced by LPS, TNF- α , IL-33 or IL-1 β

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Short running title:

Expression of TF by molecules

Abbreviations: CSU; chronic spontaneous urticaria, TF; tissue factor, HUVEC; human umbilical vein endothelial cells, LPS; Lipopolysaccharides, TLR; Toll-like receptor.

Authors' contributions

AK, YY, ST, RS, AT, KO and MH designed the study and wrote the manuscript. AK, YY, TK, KI and KU contributed to data collection. All authors read and approved the final manuscript.

Abstract

The pathogenesis of chronic spontaneous urticaria (CSU), also called chronic idiopathic urticaria (CIU), has been considered to be associated with the activation of the extrinsic blood coagulation cascade. However, the trigger for the extrinsic coagulation cascade in patients with CSU remains unclear. We previously reported that histamine and lipopolysaccharide (LPS) synergistically induced the expression of tissue factor (TF), a trigger for the extrinsic coagulation cascade, in human umbilical vein endothelial cells (HUVECs). Since the elevation of TNF- α , IL-33, IL-1 β and VEGF in serum has also been observed in patients with CSU, we examined the effects of LPS, TNF- α , IL-33, IL-1 β , VEGF and histamine on TF expression in HUVECs by RT-PCR and flow cytometry, and its activity to trigger the extrinsic coagulation cascade and induce inter-cellular gap formation of HUVECs in the presence of plasma by Actochrome[®] TF activity assay and impedance sensor, respectively. The expression of TF mRNA and surface protein of TF on HUVECs in response to histamine or VEGF were synergistically enhanced by the treatment with LPS, TNF- α , IL-33 or IL-1 β . Moreover, the activation of the extrinsic coagulation pathway and inter-cellular gap formation of HUVECs in response to histamine or VEGF were also synergistically increased in the presence of TNF- α and LPS. Thus, TF expression on vascular endothelial cells was strongly enhanced by co-stimulation with CSU-related molecules in blood. Blocking a common pathway of LPS, TNF- α , IL-33 and IL-1 β , and/or that of VEGF and histamine may be an effective therapeutic measure for patients with severe and refractory CSU.

Key words: chronic spontaneous urticaria (CSU), tissue factor (TF), endothelial cells, vascular hyperpermeability, extrinsic coagulation cascade

Introduction

Chronic spontaneous urticaria (CSU), also called chronic idiopathic urticaria (CIU), is a common skin disorder characterized by daily or almost daily recurring wheal and flare with itch in association with histamine and other chemical mediators, such as leukotriene, released from mast cells and/or basophils^{1,2}. The increase of plasma histamine concentration and the effectiveness of H1 anti-histamines suggest a critical role of histamine released from skin mast cells and/or basophils and its receptor, histamine H1 receptor, in the pathogenesis of CSU³. The presence of autoantibodies against IgE or the high affinity receptor (FcεRI) on mast cells and basophils may be detected in around 30% of patients with CSU^{4,5}. However, the results might be streakly dependent on the method and donors of the cells used in the test⁶. IgE antibodies against self-molecules, such as dsDNA, thyroid peroxidase (TPO) and IL-24 have also been detected in patients with CSU⁷⁻⁹. The rapid effect of omalizumab, an anti-human IgE monoclonal antibody (IgG) suggests the involvement of autoantigens¹⁰. However, the continuous presence of autoantibodies and/or autoantigens cannot explain diurnal and local occurrence of wheals observed in CSU. Recently, the blood coagulation cascade¹¹ and infections^{12,13} have been suggested to be involved in CSU. Several reports suggest that heparin, an anticoagulant which inhibits activities of coagulation factors, may be effective in the treatment of CSU¹⁴⁻¹⁶. Moreover, oral anticoagulant drugs, such as warfarin which blocks the activity of coagulation factors, including VII, X and II (prothrombin), may improve clinical symptoms of patients with CSU unresponsive to antihistamines¹⁷. Asero and our group showed that plasma levels of prothrombin fragment 1+2 (PF1+2) and D-dimer in patients with CSU are higher than those in normal controls and correlate with disease severities^{18,19}. Moreover, Takeda et al., reported the elevation of extrinsic coagulation potential in the patients with CSU compared to healthy donors²⁰. However, the molecular mechanism that initiates the extrinsic coagulation cascade and

the relationship of infections to wheal formation induced by histamine released from mast cells and/or basophils have remained unclear. We previously reported that histamine and lipopolysaccharide (LPS), an endotoxin derived from the outer membrane of Gram-negative bacteria such as *H. pylori*, synergistically induced the expression of tissue factor (TF) in human umbilical vein endothelial cells (HUVECs) *in vitro*²¹. Moreover, we clarified that a large amount of TF expressed on HUVECs activates the extrinsic coagulation pathway and produces activated coagulation factors, such as Xa and IIa (thrombin), followed by inter-cellular gap formation of HUVECs via protease activated receptor (PAR)-1²¹. Furthermore, eosinophils have been identified as a source of TF in the lesion of CSU^{22,23}. To date, several blood molecules have been suggested as potential biomarkers to reflect the severity and activity of CSU. These include VEGF, histamine, D-dimer, FDP, (PF1+2), C-reactive protein (CRP), substance P (SP), and proinflammatory cytokines including TNF- α , IL-1 β , IL-6, IL-17, IL-31 and IL-33 in plasma in patients with CSU²⁴⁻²⁹. In this study, we examined the effects of LPS, TNF- α , IL-33, IL-1 β , VEGF and histamine on the expression of TF in HUVECs and its activity to trigger the extrinsic coagulation cascade and induce inter-cellular gap formation of HUVECs in the presence of plasma.

Methods

Reagents

LPS, ECGS and BSA were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Anti-TF-FITC and Actochrome[®] TF activity assay kit from SEKISUI DIAGNOSTICS (Lexington, MA). HUVECs from ATCC. Factor VIII deficient human plasma from COSMO BIO Co., Ltd. (Tokyo, Japan). Histamine was from Wako Pure Chemical Industries, Ltd. (OSAKA, Japan). Recombinant human TNF- α , IL-33, IL-1 β and VEGF were purchased from R & D systems (Minneapolis, MN).

Cells

HUVECs were cultured in DMEM/F12 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 40 μ g/ml endothelial cell growth supplement (ECGS) and heparin. The day before the experiments, HUVECs were harvested using trypsin and cultured in 96-wells plates for TF activity assay, 48-well plates for mRNA isolation, glass bottom dish for actin cytoskeleton staining, RepCell[®] (CellSeed Inc. Tokyo, Japan) for flow cytometry and E-plate for impedance assay.

Real-time PCR

Total RNA was extracted from HUVECs using the RNeasy Mini kit (Qiagen, Venlo, Netherlands), and cDNA was generated using the QuantiTect Reverse transcription kit (Qiagen). Levels of mRNA expression of TF and GAPDH were evaluated using ABI 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems). The specific primer pairs were as follows, forward primer for TF, 5'-GGAACCCAAACCCGTCAATC-3'; reverse primer for TF, 5'-

GTCCGAGGTTTGTCTCCAGGTA-3', and forward primer for GAPDH,
5'-GAAGGTGAAGGTCGGAGTCA-3'; reverse primer for GAPDH,
5'-GAAGATGGTGATGGGATTTC-3'. Expression of GAPDH was measured as an internal
control to calibrate gene expression.

Flow cytometry

TF expression on the surface of HUVECs was measured in living cells by flow cytometric
analysis. HUVECs cultured in RepCel[®] were treated in the presence or absence of LPS, TNF- α ,
VEGF and/or histamine for 6 hours, and then harvested by temperature reduction at 20 °C for
30 min. Detached cells were stained with anti-TF-FITC (American Diagnostica, Stamford, CT)
or FITC-conjugated mouse IgG1 (Myltenyi Biotec), an isotype control. All tubes were
incubated for 10 min at room temperature and then 30 min on ice in the dark. The sample in
each tube was suspended with PBS and then added with Sytox (Life technologies, Carlsbad,
CA) to reveal and eliminate dead cells. The fluorescence intensity of individual cells was
analyzed using an Attune acoustic focusing cytometer (Life technologies).

TF activity assay

The biological activity of TF on cells was measured according to the instructions of an
Actochrome[®] TF activity assay kit, as described in our previous work¹⁷. Briefly, HUVECs were
seeded in 96-well plate, stimulated with LPS, TNF- α , VEGF and/or histamine for 6 hr. The cells
were washed with PBS twice and exposed to 75 μ l of assay buffer, 25 μ l of factor VIIa and 25 μ l
of factor FX for 15 min at 37 °C. Then 25 μ l of Spectrozyme factor Xa substrate was added and
incubated at 37 °C for 30 min, and absorbance at 405 nm of the wells were measured using a
microplate reader.

Impedance-based analysis of vascular permeability

Impedance analysis was performed as described in our previous manuscript¹⁷. Briefly, HUVECs were seeded on E-Plates (Rhoche Applied Science, Upper Bavaria, Germany) at a density of 50,000 cells/well. On Day 2, cells were treated in the presence or absence of LPS, TNF- α , VEGF and/or histamine. On Day 3, cells were washed and treated with DMEM/F12 with 2% BSA without any supplements and FCS. The E-plate was then set onto iCelligence (Roche Applied Science) and impedance termed “cell index” (CI) was measured every 10 seconds for indicated time in the presence or absence of Factor VIII deficient plasma.

Statistical analysis

Difference among each group was tested using a one-way ANOVA followed by Tukey’s test using GraphPad PRISM ver.6 (GraphPad Software, San Diego, CA).

Results

Effects of LPS, TNF- α , IL-33, IL-1 β , histamine and VEGF on TF expression in HUVECs

We first investigated the effect of CSU-associated molecules, LPS, TNF- α , IL-33, IL-1 β , histamine and VEGF, on the TF mRNA expression in HUVECs. For convenience, we termed these molecules as TF-inducers. As shown in Fig.1, the expression of TF mRNA in HUVECs in response to histamine or VEGF were synergistically enhanced by the treatment with LPS, TNF- α , IL-33, or IL-1 β (Fig.1 A-H, Supplementary Fig.1A). Moreover, histamine-induced TF-mRNA expression enhanced by the treatment with LPS, TNF- α , IL-33, or IL-1 β were clearly blocked by the treatment with H1 antihistamine, desloratadine to the levels of TF expression induced by the enhancers (supplementary Fig. 2). However, only additive enhancement of TF mRNA expression was observed in HUVECs by the co-stimulation with histamine and VEGF, LPS and TNF- α , IL-33 and LPS, LPS and IL-1 β , IL-33 and TNF- α , IL-33 and IL-1 β , or IL-1 β and TNF- α (Fig.1 I-O, Supplementary Fig.1B). These results suggest that LPS, TNF- α , IL-33 and IL-1 β can significantly enhance VEGF- or histamine-induced TF mRNA expression of vascular endothelial cells. Since the synergistic enhancement of histamine- or VEGF-induced TF mRNA expression HUVECs by IL-1 β or IL-33 was somewhat weaker than that by TNF- α or LPS, we focused on the effect of LPS, TNF- α , VEGF and histamine for the subsequent experiments. We then studied the expression of TF proteins on the surface of HUVECs by means of flow cytometry. As shown in Fig.2, histamine- or VEGF-induced TF expression were synergistically enhanced by treatment with LPS or TNF- α . However, only an additive enhancement of TF protein expression on the surface of HUVECs was observed by co-stimulation with histamine and VEGF, or LPS and TNF- α (Fig.2). These results suggest that TNF- α and LPS can also significantly enhance VEGF- or histamine-induced surface TF expression on vascular endothelial cells

Definition of S/A ratio as an indicator of synergistic or additive effect of co-stimulation with TF-inducers on the TF expression in HUVECs

We then defined an indicator, S/A ratio, which shows the synergistic or additive effect of the combinations of TF inducers on TF expression of HUVECs. The S/A ratio was calculated according to the following mathematical formula.

$$\text{S/A ratio} = \frac{[\text{TF mRNA expression by A+B}]}{[\text{TF mRNA expression by A}] + [\text{TF mRNA expression by B}]}$$

When the combination of TF-inducers shows only additive effect for TF expression, the S/A ratio should be around 1. On the other hand, when co-stimulation with TF-inducers shows a synergistic effect for TF expression, the S/A ratio should be substantially higher than 1. We tentatively defined synergistic effect as 2 or more of S/A ratio. When HUVECs were treated with VEGF (10 ng/ml) and LPS (100 ng/ml), the S/A ratio was 7.40 which is remarkably higher than 1 (table1, Supplementary Fig.1A). Therefore, co-stimulation with VEGF and LPS induces a synergistic effect for TF mRNA expression. When HUVECs were treated with TNF- α (10 ng/ml) and LPS (100 ng/ml), the S/A ratio was 1.06 (around 1), suggesting the effect was additive (table1, Supplementary Fig.1B). The S/A ratios of all combinations of the TF-inducers are summarized in table 1. Based on the results of Table 1, we divided TF-inducers into two groups. Group 1 contains LPS, TNF- α , IL-33 and IL-1 β . Group 2 contains histamine and VEGF. Thus, the synergic expression of TF in HUVECs was induced by co-stimulation of two types of molecules, one in Group 1 and another in Group 2 TF-inducers (Supplementary Fig.3).

Synergistically expressed TF on HUVECs activates extrinsic coagulation pathway

The potential of synergistically expressed TF on HUVECs for the activation of the extrinsic coagulation pathway was evaluated by Spectrozyme[®] FXa. As shown in Fig.3, the activity of TF expressed on HUVECs was increased by co-stimulation with LPS and histamine (A), LPS+VEGF (A), TNF- α and histamine (B), and TNF- α and VEGF (B), respectively. These results suggest that synergistically-expressed TF on the surface of HUVECs by co-stimulation with Group 1 and Group 2 TF-inducers effectively activates the extrinsic coagulation pathway, resulting in the activation of coagulation factors, such as VIIa, Xa and possibly thrombin (IIa).

Synergistic expression of TF on HUVECs increases intra-cell gap formation of cells in the presence of plasma

In order to evaluate the effect of TF-expressed on HUVECs on gap formation of HUVECs in the presence of plasma, we employed impedance analysis. Impedance sensors detect sensor surface impedance, which reflects the area of adhesion and morphology of the cells on the electrodes as Cell Index (CI)¹⁷. This technique can sensitively monitor the changes, such as disruption of tight and adherent junctions without any labeling, in full agreement with a permeability assay using a Boyden chamber¹⁷. When gap formation of HUVECs is induced, CI decreases¹⁷. The impedance assay revealed that the treatment of HUVEC with LPS and VEGF, VEGF and LPS synergistically induced cell contractions in the presence of factor VIII deficient-plasma (Fig.4). These results suggest that co-stimulation with Group 1 and Group 2 TF-inducers synergistically activates coagulation factors, and then induce gap formation of vascular endothelial cells.

Effect of NF- κ B inhibitor and PKC activator on the TF inducer-expressed TF in HUVECs

The mechanism of synergistic expression of TF on HUVECs by co-stimulation with Group 1 and Group 2 TF inducers was further investigated with respect to intracellular signal transductions. LPS, TNF, IL-1 β and IL-33 are known to activate the intracellular MyD88-, NF- κ B-, and MAP kinase-dependent pathways³⁰, whereas histamine and VEGF mainly activate PLC, PKC, MAP kinase and increase the concentration of calcium in cells³¹. Treatment with Bay-11-7082, an NF- κ B inhibitor, clearly inhibited the expression of TF in HUVECs in response to LPS, IL-33, IL-1 β and TNF- α (Group 1 TF-inducers), but not that induced by histamine or VEGF (Group 2 TF-inducers) (Fig.5A). On the other hand, phorbol 12-myristate 13-acetate (PMA), PKC activator, synergistically activated LPS, IL-1 β , IL-33 or TNF- α (Group 1 TF-inducers) induced TF expression, but not histamine or VEGF induced TF expression (Fig.5B). These results revealed that signal transductions by histamine and VEGF are related to PMA-induced signal transduction. Moreover, calcineurin inhibitors, such cyclosporine and FK506, did not block histamine- and VEGF-induced TF expression, suggesting that histamine and VEGF induced TF expression may be through a calcineurin-NFAT independent pathway (data not shown). Thus, the activation of different signal pathways such as group 1 and group 2 TF-inducers, are required for the synergistic TF expression on HUVECs.

Discussion

In this study, we demonstrated that the expression of TF mRNA and surface protein of TF on HUVECs in response to histamine or VEGF (Group 2 TF-inducer) were synergistically enhanced by treatment with TNF- α , LPS, IL-33 or IL-1 β (Group 1 TF-inducer). Moreover, TF activity and inter-cellular gap formation of HUVECs in response to histamine or VEGF were also synergistically increased in the presence of TNF- α or LPS.

Several studies have already suggested that histamine, TNF- α , VEGF, IL-1 β , IL-33 and TLR ligands, such as LPS, induce the expression of TF on the surface of vascular endothelial cells³²⁻³⁶. However, the physiological concentrations of these molecules are insufficient by themselves to induce TF expression for the activation of the extrinsic coagulation cascade in CSU patients. We here successfully divided TF-inducers into 2 groups. Group 1 TF-inducer, LPS, TNF, IL-1 β and IL-33, seems to be proinflammatory molecules. On the other hand, Group 2 TF-inducers, histamine and VEGF, increase vascular permeability by themselves. While intracellular signal transductions of all Group 1 TF-inducers are NF- κ b dependent pathway, that of Group 2 TF-inducers are NF- κ b independent. Therefore, co-stimulation with different group TF-inducers might be crucial for a sufficient amount expression of TF to activate the extrinsic coagulation cascade followed by gap formation of vascular endothelial cells.

Increase of TF expression and activation of blood coagulation system are also observed in other diseases, such as sepsis. The reason for no clinically apparent clots formation in CSU may be due to an only local TF expression by vascular endothelial cells and consequent vascular permeability in CSU. However, several kinds of blood cells, such as eosinophils and monocytes, also express TF on their surface. Moosbauer et al showed the increase of TF on the surface of eosinophils in response to several mediators and/or cytokines, such as platelet activating factor (PAF) and IL-5³⁷. Cugno et al showed the enhancement of TF expression on eosinophils in the

lesion of CSU³⁸. Furthermore, autoantibodies against the low-affinity IgE receptor (FcεRII: CD23), which can activate eosinophils, are detected in around 70% of patients with CSU³⁹. We recently reported that TF expression levels on monocyte of patients with CSU are significantly enhanced compared with that of healthy donors⁴⁰. Thus, TF expressed on eosinophils and monocytes may also be involvement of the pathogenesis of CSU together with TF-expressing endothelial cells. Basophils do not express TF on the surface of plasma membrane, but may contribute to the expression of TF on endothelial cells by releasing histamine. Autoantibodies (IgGs) against IgE antibody or the high affinity IgE receptor (FcεRI), and IgE against self-molecules, such as dsDNA and IL-24 have been detected in patients with CSU. Moreover, we recently reported that the concentrations of IgE antibodies in sera of patients with CSU are significantly higher than that in sera of healthy donors⁴¹. All these autoantibodies, autoreactive IgE and high concentrations of IgE antibodies may activate human peripheral basophils and induce histamine release, cellular polarization, and upregulation of surface antigens (CD203c) in the absence of exogenous antigens (allergens)⁴¹. In addition, human basophils also express phosphatidylserine (PS), known as an activator of the extrinsic coagulation cascade, on their surface during degranulation (Yanase, et al., unpublished observation), suggesting that basophils themselves may contribute to the activation of the extrinsic cascade together with VIIa/VII, TF and Ca²⁺ in the blood.

Although an approximately ten-fold higher amount of histamine is required for edema formation recognized as wheal as compared with that for flare⁴², the detailed underlying mechanism of mast cells and/or basophils activation in dermis has been unclear. As we have described above, a substantial population of patients have IgG autoantibodies against FcεRI or IgE which can activate mast cells⁴. Alternatively, several types of IgE that bind to autoantigens, such as dsDNA, TPO and IL-24 have been found in patients with CSU⁷⁻⁹. Moreover, the

337 presence of IgE against staphylococcal enterotoxins has been confirmed in the sera of patients
338 with CSU⁴³. Other histamine releasing factors (HRF) in plasma may also contribute to
339 activation of skin mast cells⁴⁴. Bossi et al showed that plasma from patients with CSU causes
340 the release of histamine from human mast cell lines, LAD2 and HMC-1, by an
341 IgE-FcεRI-independent mechanism⁴⁵. Cugno, et al reported the presence of a molecule that is
342 smaller than 30 kDa, in the plasma of patients with CSU, that induced histamine release from
343 mast cells⁴⁶. Several reports demonstrated that PAR-2 agonists, such as trypsin, activate human
344 mast cells^{47,48}. Moreover, the expression of PAR-2 on human mast cells is up-regulated in
345 urticarial lesion compared with non-urticarial lesions⁴⁹. Furthermore, Vliagoftis reported the
346 expression of PAR-1 on bone marrow-derived mast cells (BMMCs)⁵⁰. Therefore, the production
347 of activated coagulation factors, such as Xa and IIa known as PAR agonists, by various
348 combinations of TF inducers may induce not only intercellular gap formation, but also activate
349 skin mast cells via a leakage of plasma with various mast cell activators in patients with CSU,
350 resulting in a long and robust edema formation, recognized as wheals.

351 In conclusion, we here demonstrated that the combinations of different TF-inducers
352 synergistically express TF on vascular endothelial cells, result in the activation of the extrinsic
353 blood coagulation cascade, producing activated coagulation factors, and finally induced gap
354 formation between endothelial cells (plasma leakage). Further studies on the mechanism of
355 synergistic TF expression may allow us to clarify crucial points in the pathogenicity of CSU and
356 find new therapeutic targets for this disease.

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Conflict of Interest (COI): The authors declare no conflict of interest.

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Figure legends

Fig.1 Effects of histamine, VEGF, LPS, TNF- α , IL-33 and IL-1 β on TF expression of HUVECs

Relative expression levels of TF mRNA in response to TF-inducers, LPS+histamine (A), LPS+VEGF (B), TNF- α +histamine (C), TNF- α +VEGF (D), IL-33+histamine (E), IL-33+VEGF (F), IL-1 β +histamine (G), IL-1 β +VEGF (H), VEGF+histamine (I), LPS+TNF- α (J), IL-33+LPS (K), LPS+IL-1 β (L), IL-33+TNF- α (M), IL-1 β and IL-33 (N) and IL-1 β +TNF- α (O). All figures are representative results of three independent experiments. Data represent mean \pm SEM.

Fig.2 Surface expression levels of TF on HUVECs in response to TF inducers.

TF expression on the surface of HUVECs in response to TF-inducers, VEGF (10 ng/ml), TNF α (10 ng/ml), LPS (100 ng/ml) and or histamine (His) (10 μ M). All figures are representative results of three independent experiments.

Fig.3 Activation of the extrinsic coagulation pathway by TF expressed on the surface of HUVECs

The biological activity of TF expressed on HUVECs in the presence or absence of TF inducers were measured by the production of Spectrozyme factor Xa substrate. VEGF (10 ng/ml), TNF- α (10 ng/ml), LPS (100 ng/ml) and histamine (10 μ M). The figure are representative results of three independent experiments. Data represent mean \pm SEM.

Fig.4 Intercellular gap formation between cells by TF expressed HUVECs

CI changes of HUVECs pre-treated with or without histamine (10 μ M) and/or LPS (1 ng/ml) and/or TNF- α (1 ng/ml) and/or VEGF (10 ng/ml) for 18 hr, and then washed and exposed to VIII-deficient human plasma. All figures are representative results of three independent

experiments.

Fig.5 Effects of NF- κ B inhibitor or PKC activator on TF expression of HUVECs

Effects of indicated concentrations of NF- κ B (A) or PMA (B) on the expression of TF mRNA in HUVECs in response to TF-inducers; LPS (100 ng/ml), histamine (10 μ M), TNF- α (10 ng/ml), IL-1 β or histamine (10 μ M). Data represent mean \pm SEM. Difference between two groups was considered significant with $P < 0.001$ (***).

Table 1. Summary of S/N ratio of TF expression of HUVECs in response to co-stimulation with TF-inducers

When the combination of TF-inducers shows only additive effect for TF expression, the S/A ratio is around 1. On the other hand, when co-stimulation with TF-inducers shows synergistic effect for TF expression, the S/A ratio is much higher than 1 (light gray highlighted area).

Fig.1

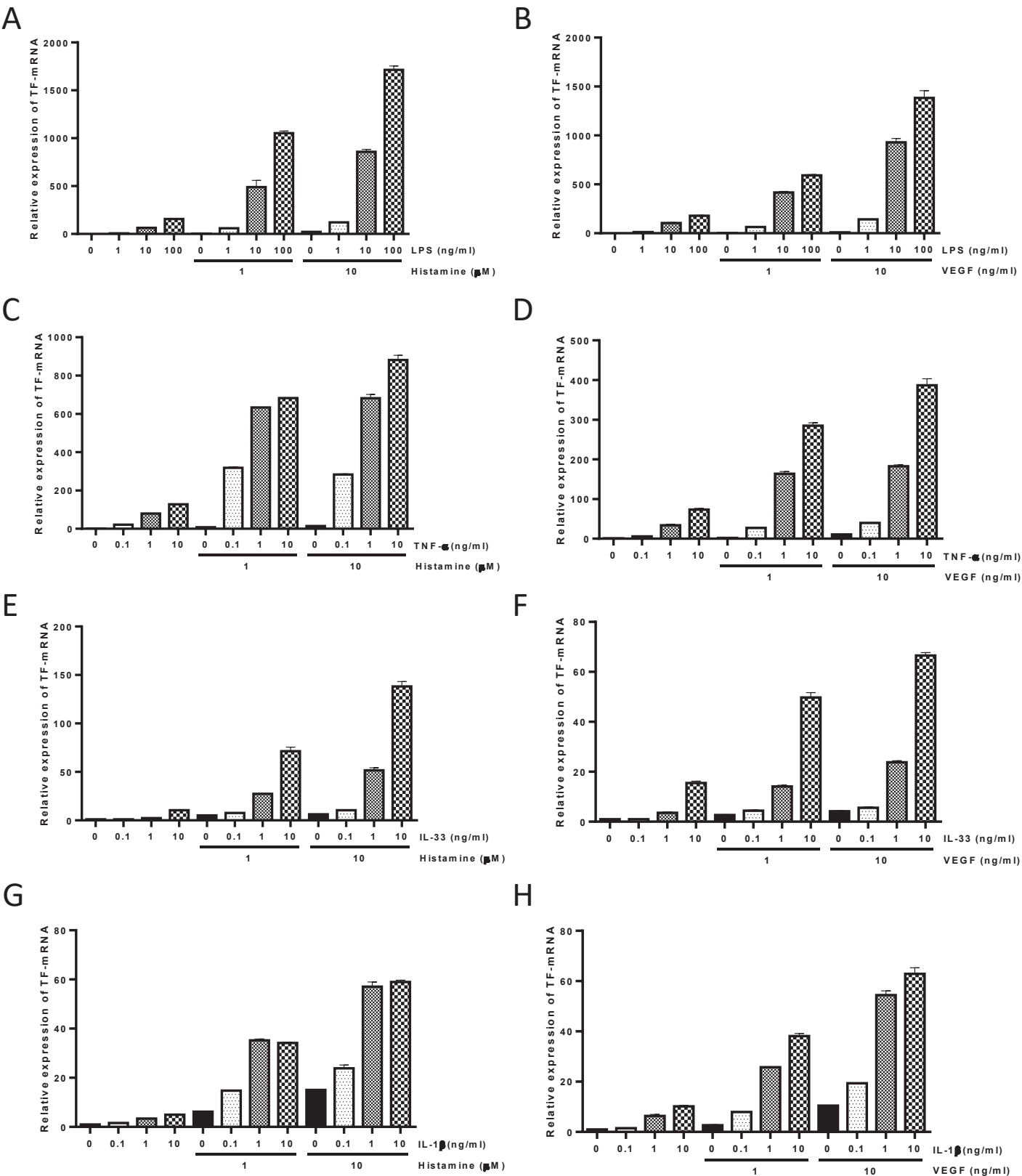


Fig.1

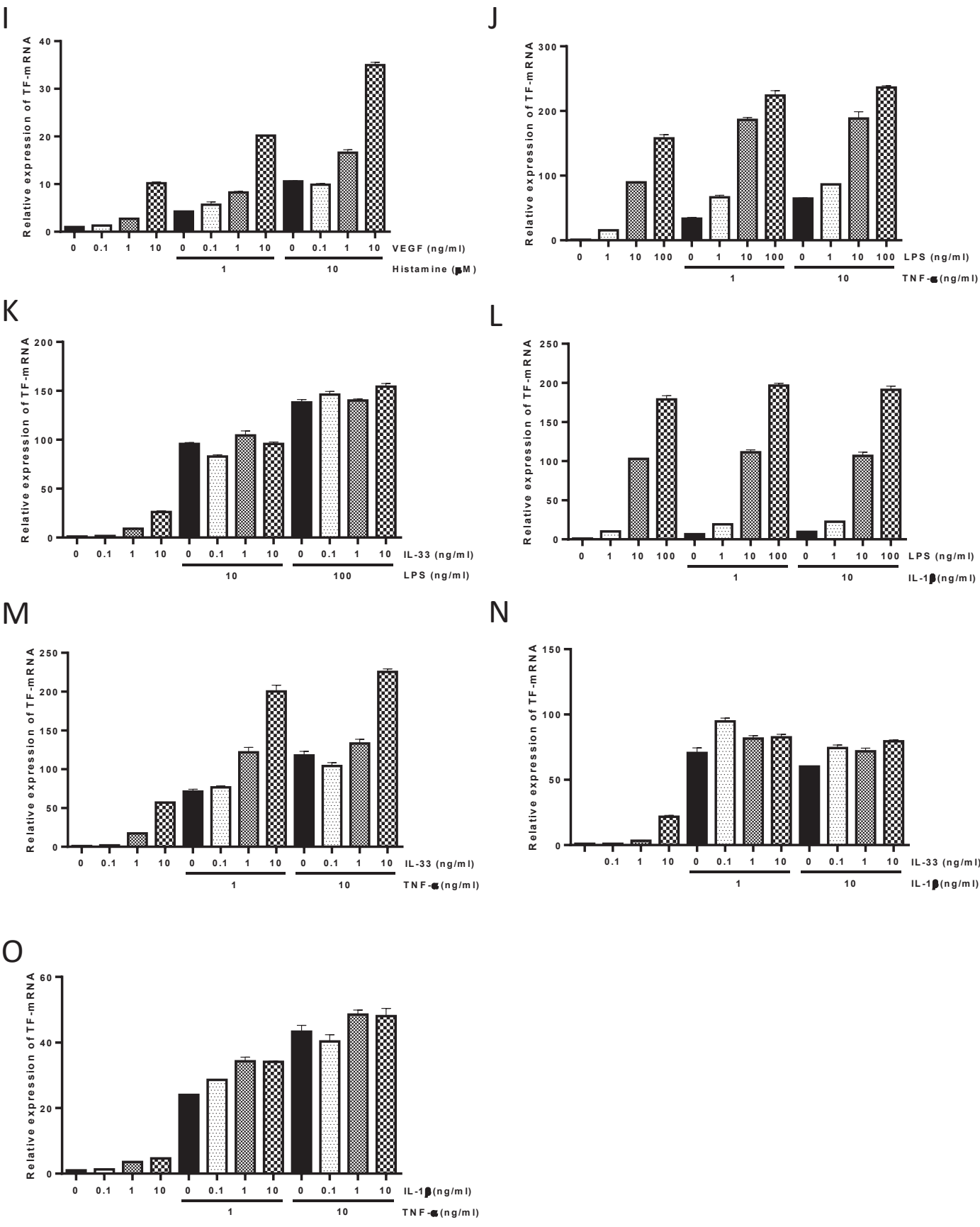
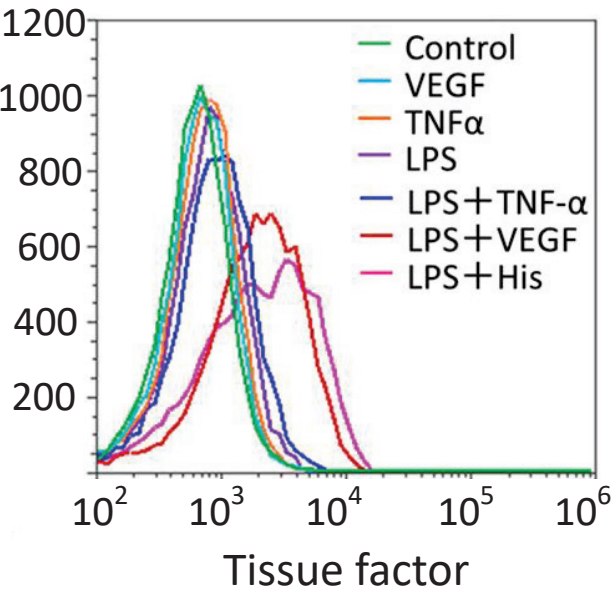


Fig.2

A



B

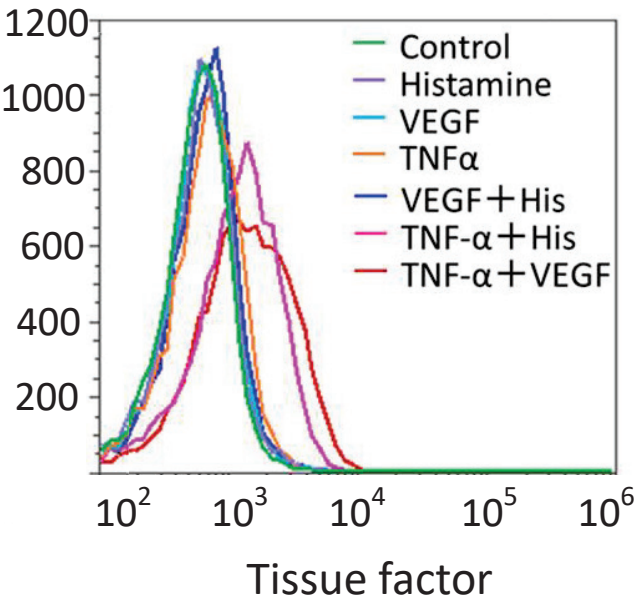


Fig.3

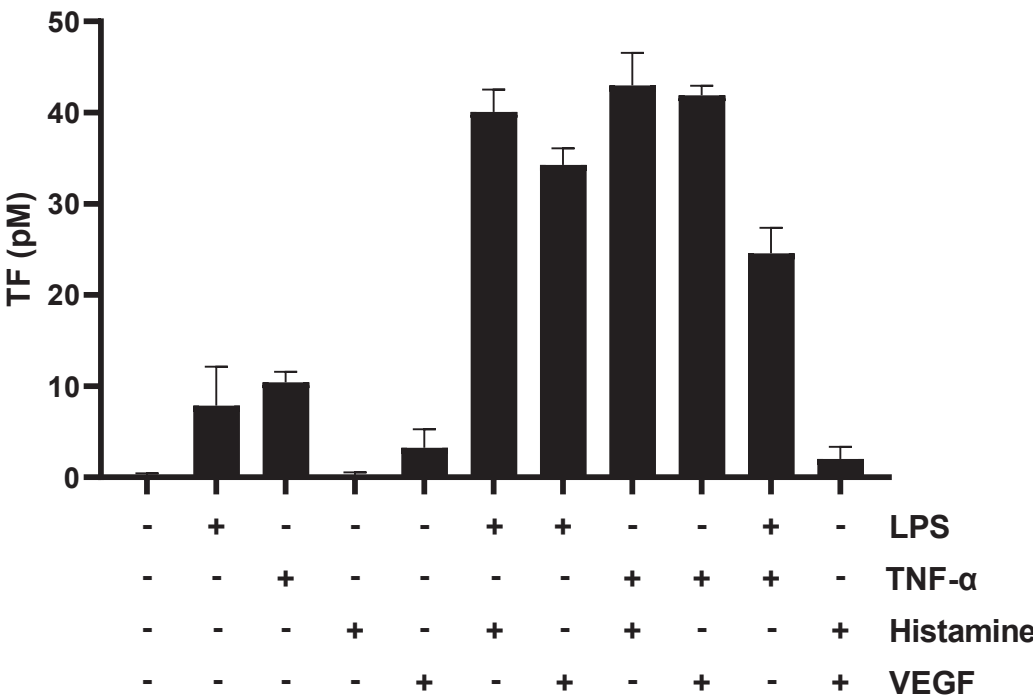


Fig.4

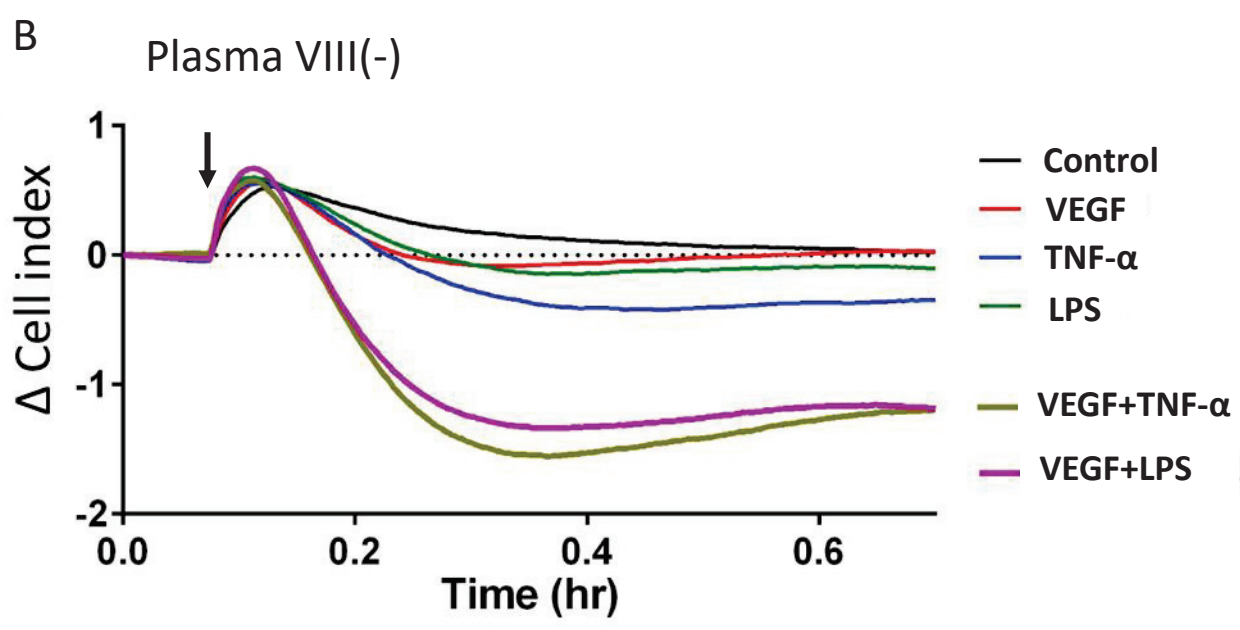
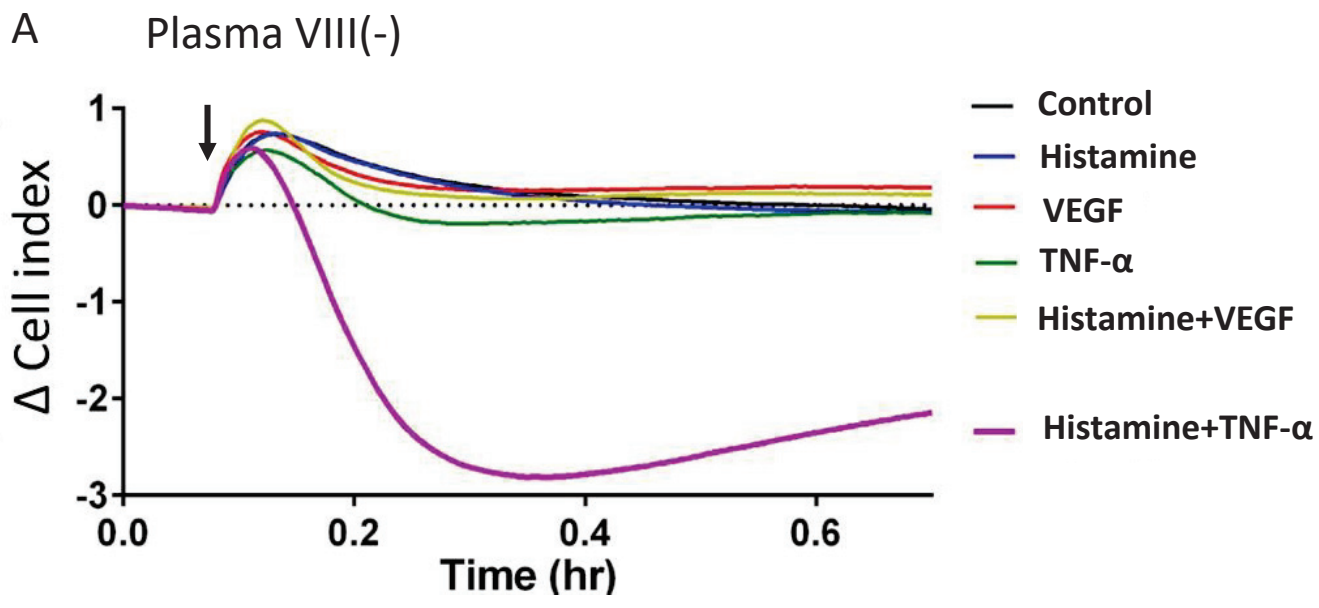
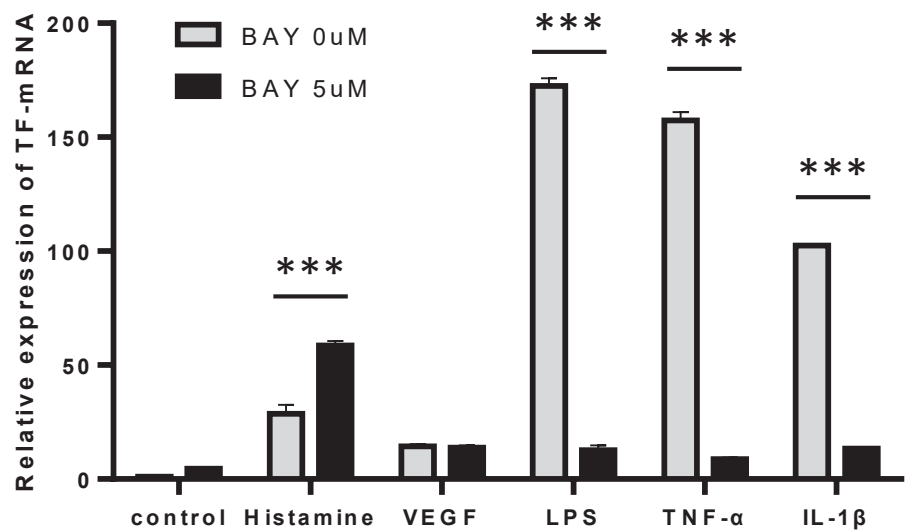


Fig.5

A



B

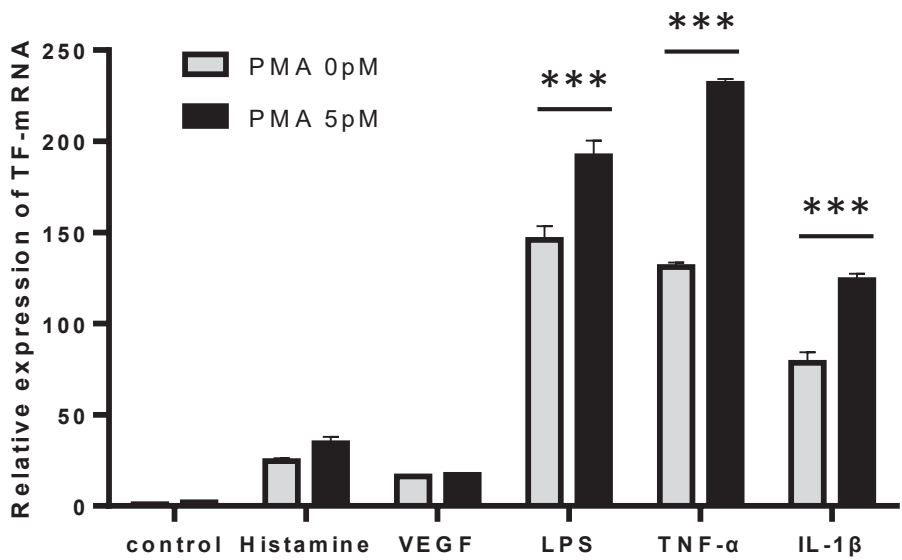
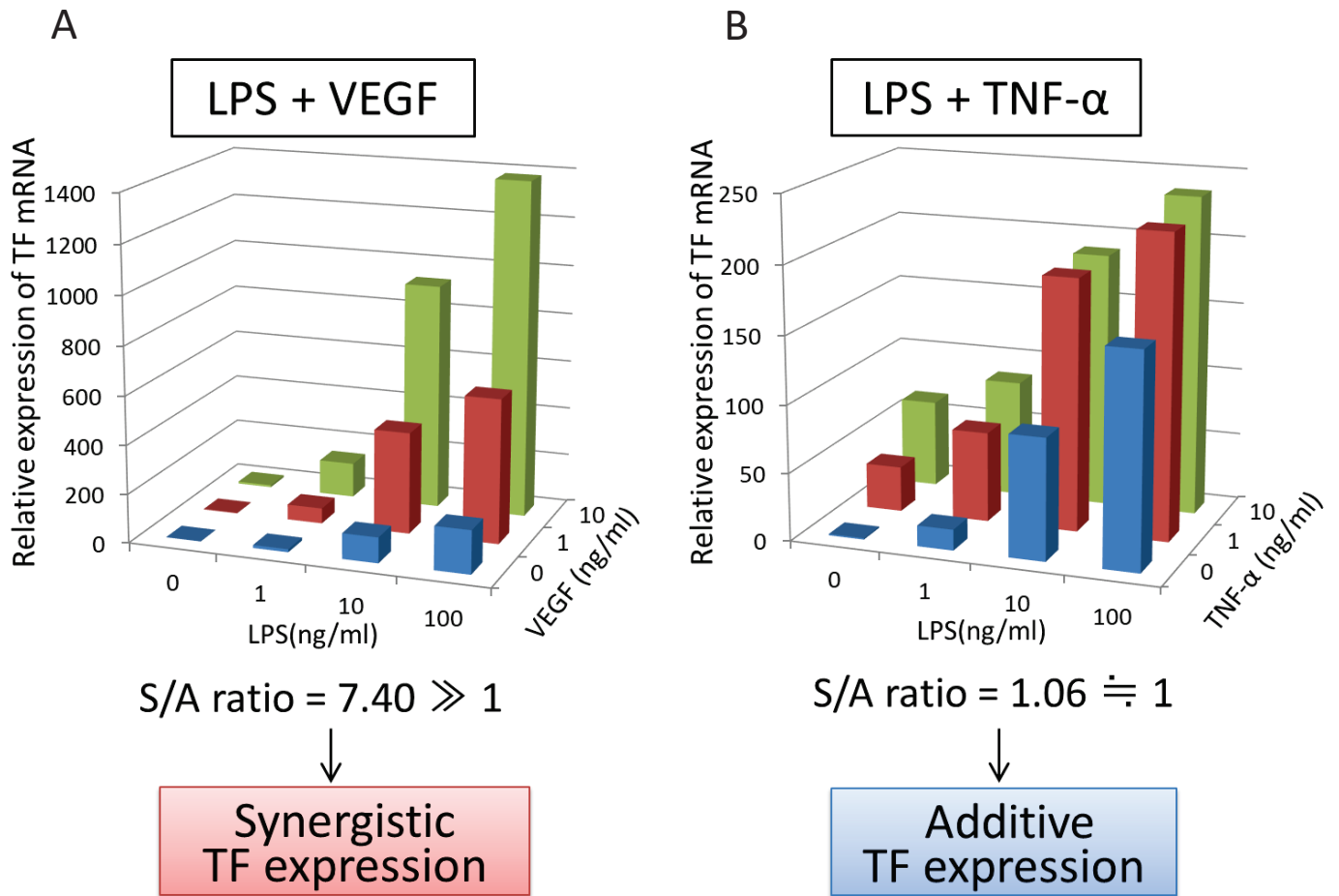


Table.1

	LPS	TNF- α	IL-1 β	IL-33	Histamine	VEGF
LPS		1.06	1.01	0.94	9.63	7.40
TNF- α			1.00	1.29	6.18	4.59
IL-1 β				0.97	2.94	3.04
IL-33					8.26	3.38
Histamine						1.68
VEGF						

Supporting information

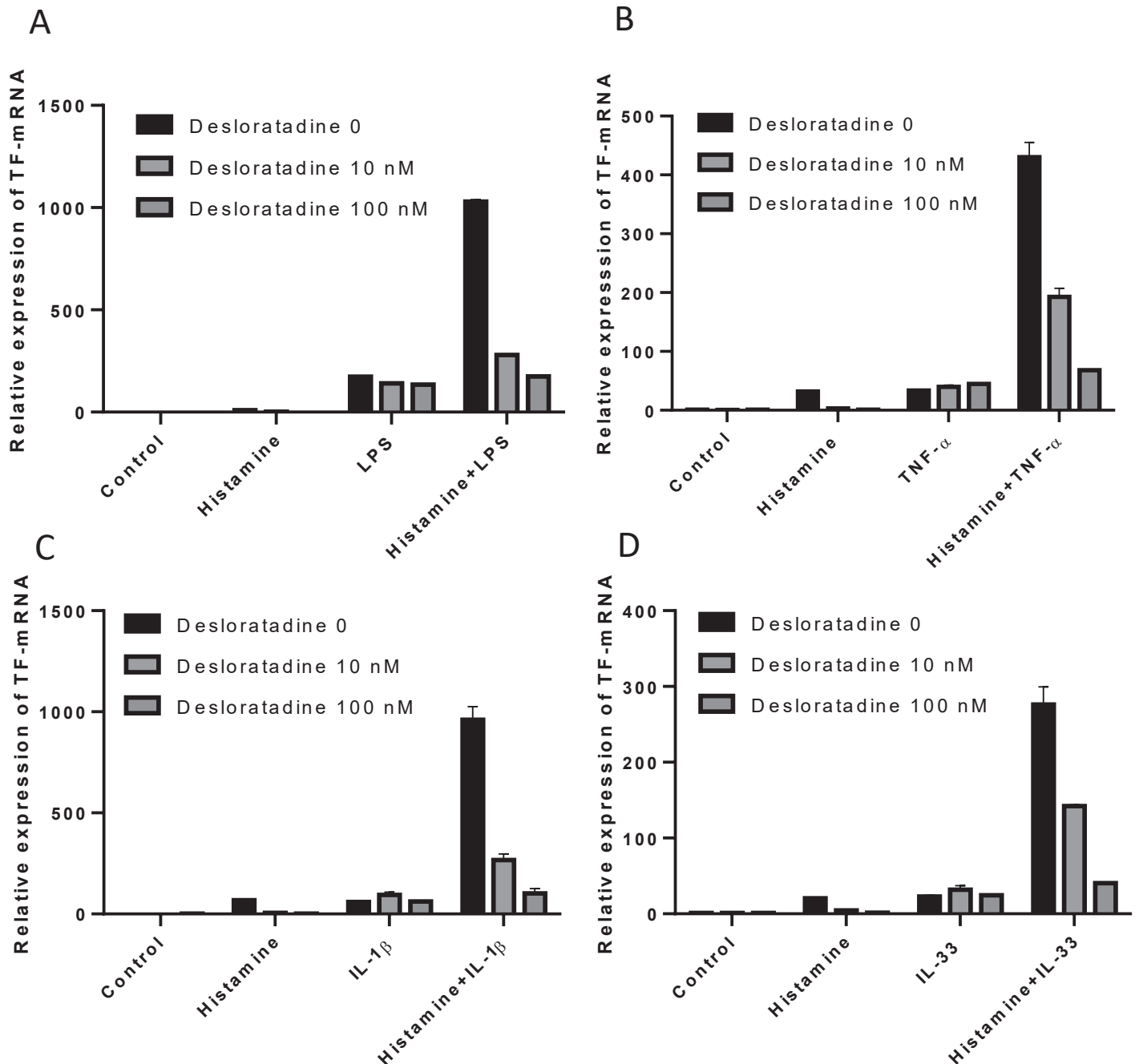
Figure. S2



Supplementary Fig.1. 3D graph of relative expression of TF of HUVECs in response to LPS+VEGF and LPS+TNF- α

Concentration-dependent synergistic expression of TF in HUVECs was observed in response to indicated concentrations of TF-inducers.

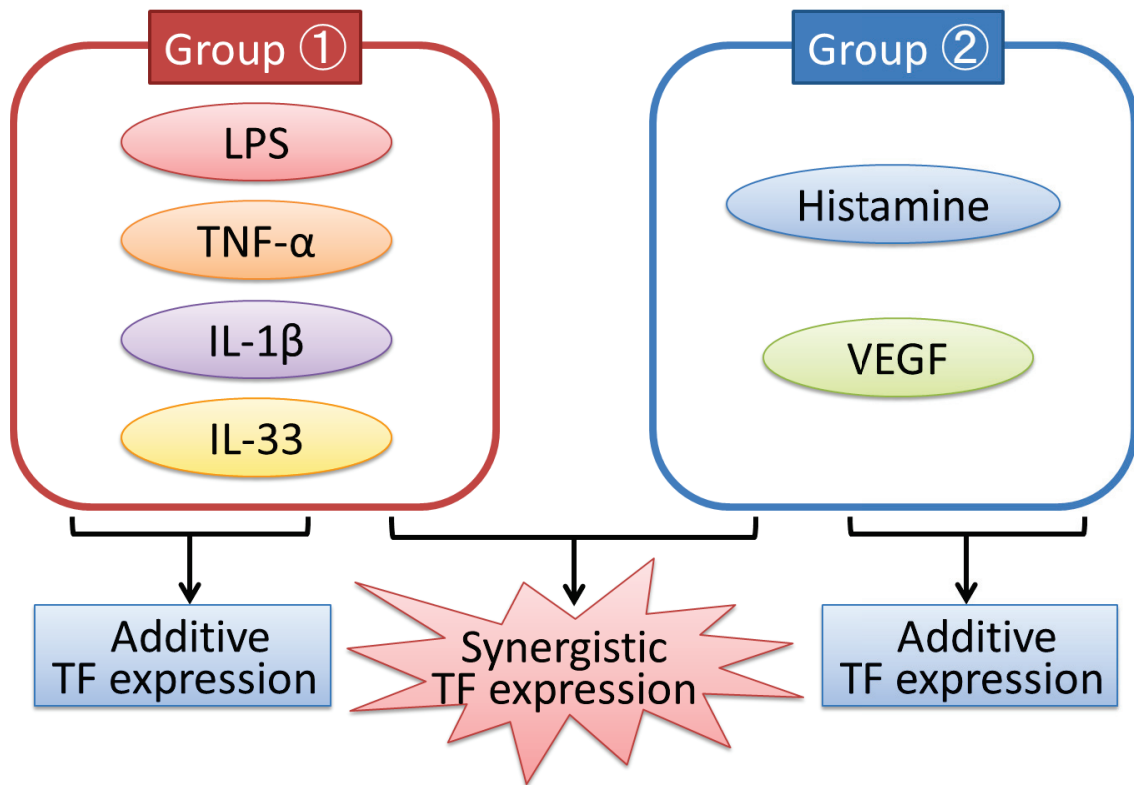
Figure. S2



Supplementary Fig.2. Effect of H1 antihistamine, desloratadine, on the TF mRNA expression in HUVECs.

(A) Effect of desloratadine on the TF mRNA expression stimulated with histamine (10 μ M) and/or LPS (100 ng/ml). (B) Effect of desloratadine on the TF mRNA expression stimulated with histamine (10 μ M) and/or TNF- α (100 ng/ml). (C) Effect of desloratadine on the TF mRNA expression stimulated with histamine (10 μ M) and/or IL-1 β (10 ng/ml). (D) Effect of desloratadine on the TF mRNA expression stimulated with histamine (10 μ M) and/or IL-33 (10 ng/ml).

Figure. S3



Supplementary Fig.3 Summary of additive or synergistic effect of TF inducers on the TF expression.

Group1 contains LPS, TNF- α , IL-1 β and IL-33. Group2 contains histamine and VEGF.