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Stimulation of Fas (CD95) induces production of pro-inflammatory mediators through ERK/JNK-dependent activation of NF-κB in THP-1 cells

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ABSTRACT

Although Fas is known to be an apoptosis triggering molecule, accumulating studies indicate that Fas has non-apoptotic functions in certain cases. In an effort to identify the role of Fas in macrophage function, the human macrophage-like cell line THP-1 was analyzed after treatment with agonistic anti-Fas monoclonal antibody or co-incubation with FasL-expressing cells. Stimulation of Fas induced the expression of pro-inflammatory mediators such as matrix metalloproteinase (MMP)-9 and IL-8. The specificity of the reaction was confirmed by the transfection of Fas-specific siRNAs which resulted in a suppression of Fas expression as well as the responsiveness to the agonistic antibody. Utilization of various signaling inhibitors and ELISA-based NF-κB DNA binding assay demonstrated that the signaling initiated from Fas is mediated by mitogen activated protein kinases (MAPKs) including extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) which induce subsequent activation of NF-κB. Furthermore, mixed cell culture experiment demonstrated that Fas can be activated through interaction with membrane-bound form of FasL during cell-to-cell interaction. These data indicate that Fas plays a role as an activation inducing molecule through interaction with its counterpart and Fas-mediate events are mediated by ERK/JNK MAPKs which subsequently activate NF-κB for the transcriptional activation of pro-inflammatory mediators.

1. Introduction

Fas (CD95), as a member of TNF receptor superfamily, is a type I transmembrane protein [1] that works as a receptor for FasL (CD95L/CD178), a type II homotrimeric transmembrane protein that belongs to the TNF superfamily [1,2]. Fas, upon engagement with FasL, induces caspase-dependent apoptotic cell death [3–6]. In contrast to FasL, whose expression is restricted to several cell types including $CD4^+$ T helper 1 cells, activated $CD8^+$ cells, natural killer cells and monocytes, constitutive expression of Fas has been detected in many cell types (reviewed in [7]). Although Fas-mediated apoptosis has been extensively studied during tissue injury and organ dysfunction [8–10], accumulating evidence indicates that Fas-mediated signaling can enhance pro-inflammatory responses [11,12] though activation of myeloid differentiation factor 88 (MyD88)-dependent, caspase-independent signaling pathway [13–15]. In addition, Fas-mediated signaling pathway exhibited cross-talk with IL-1R1 and/or TLR4 pathway through MyD88 [16]. In addition to these pro-inflammatory effects, stimulation of Fas enhanced proliferation of fibroblasts [17,18] and T cells [19] in certain conditions. Interestingly, Fas-mediated enhancement of T-cell proliferation appears to involve caspase activation without subsequent apoptosis induction [20–23]. These observations demonstrate the existence of complex regulatory mechanism for Fas-mediated signaling as well as the differential effect of Fas-mediated signaling in various cell types.

In order to test the influence of Fas-mediated signaling in macrophage activities, human macrophage-like cell line THP-1 (which expresses both Fas and FasL) was stimulated through treatment with agonistic anti-Fas monoclonal antibody (mAb) or incubation with FasL expressing cells. Experimental data demonstrated the signaling, initiated from Fas, was mediated by mitogen activated protein kinase (MAPK) including ERK and JNK and NF-κB. We recently reported the existence of FasL-mediated cellular activation in THP-1 cells [24]. This raises an intriguing question about how the cells cope with constant activation signals that are evident through cell-to-cell interaction during cell culture. These questions were pursued and the possible implications of the resulting findings to the Fas/FasL-mediated regulation of normal and pathologic immune reactions are discussed.

2. Materials and methods

2.1. Monoclonal antibodies and reagents

Anti-Fas (clone DX-2) and anti-FasL (clone NOK-1) mAbs were purchased from Biolegend (San Diego, CA); Polyclonal antibodies

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against ERK1/2 (p42/44 MAPK), phospho-ERK1/2, JNK, p38 and phospho-p38 and mAb to phospho-JNK (G9) were purchased from Cell Signaling (Danvers, MA); mAb against NF-κB p65 subunit (clone F-6) came from Santa Cruz (Santa Cruz, CA); mouse IgG_1 was from BD Biosciences (HI111) (San Jose, CA); SB203580, LY294002, JNK inhibitor I (JNK-I1, a cell-permeable fusion protein containing 20 AA of the JNK-binding domain of islet-brain and HIV-TAT_{48-57}[25]) and its negative control containing only HIV-TAT were obtained from Calbiochem International Inc. (La Jolla, CA). Lipopolysaccharide (LPS), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), ethyl pyruvate and sulfasalazine were purchased from Sigma (St. Luis, MO); U0126 was purchased from Cell Signaling. THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD).

2.2. Gelatin zymogram, ELISA and Western blot analysis

THP-1 cells (1×10^6/ml) were activated by adding antibodies to the reaction medium (RPMI1640 supplemented with 0.1% FBS). The culture supernatants were collected 24 h after activation for the analysis of MMP-9 and MMP-2 activities using gelatin zymogram and the measurement of IL-8 concentrations using double sandwich ELISA as described previously [26,27]. Western blot analysis was also performed as described previously [28,29].

2.3. SiRNA transfection

Fas-specific siRNAs (a pool of three target-specific 20–25 nt siRNAs) and control siRNA (a random base sequence with no known specificity to human genes) were purchased from Santa Cruz (Santa Cruz, CA). SiRNAs were transfected into the THP-1 cells using DharmaFECT (Dharmacon Inc.) as previously described [29]. Down-regulation of cell surface Fas was measured at 3, 5 and 10 days after transfection.

2.4. Flow cytometry

Flow cytometry was performed on the FACS-calibur system (Becton–Dickinson, Mountain View, CA). Cells (5×10^5) were pelleted and incubated with 0.3 μg of antibodies in 30 μl of FACS solution (a PBS containing 0.5% BSA and 0.1% Sodium Azide) for 20 min on ice. Cells were washed twice and incubated with 0.3 μg of FITC-labeled goat anti-mouse IgG in a 30 μl FACS solution. For background fluorescence, the cells were stained with an isotype-matching control antibody. The fluorescence profiles of 1×10^4 cells were collected and analyzed.

2.5. ELISA-based measurement of NF-κB DNA binding activity

NF-κB binding activity was measured following the previously described method [24]. Briefly, streptavidin-coated 96-well culture plates were used to immobilize biotin-labeled double-stranded oligonucleotides which contain the consensus NF-κB binding site (0.02 nm/well). Whole cell lysates or nuclear lysates were then added into the NF-κB oligo plates for 1 h incubation. The plates were then sequentially incubated with a mAb specific to NF-κB p65 subunit, HPR-labeled secondary antibody and tetramethylbenzidine (chromogen). Absorbance (450–540 nm) was then measured and the values were normalized by subtracting the background values. Utilization of either whole cell lysate or nuclear lysate resulted in the same pattern. For blocking, cell lysates were pre-incubated with 1.0 nm/sample of double-stranded oligonucleotides containing wild type or mutant NF-κB binding sequences.

2.6. Statistical analysis

All data are presented as mean values ± SEM, with the number of independent experiments indicated in the figure legends. All analyses were performed using SPSS software with one-way ANOVA or the paired or unpaired Student's *t*-test, as appropriate. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. THP-1 cells express Fas and triggering of it induces the expression of pro-inflammatory mediators

Macrophages are known to express both Fas and FasL [7]). When THP-1 cells were tested for the expression of each of them using flow cytometry, their expression on the cell surface was detected (Fig. 1). Stimulation of FasL on the surface of THP-1 cells resulted in an inflammatory activation of the cells [24]. In order to find out whether stimulation of Fas also leads to the inflammatory activation of the cells, THP-1 cells were treated with agonistic anti-Fas mAb. As a positive control, bacterial lipopolysaccharide (LPS) and anti-FasL were used to stimulate the cells. As shown in Fig. 2, anti-Fas mAb treatment resulted in the induction of MMP-9 and IL-8 expression in a dose-dependent manner. The expression levels of MMP-2 were also slightly induced but the extent of induction was far less than that of MMP-9. As expected, LPS and anti-FasL treatment induced similar effect while isotype-matching mouse antibody did not (Fig. 2). This indicates that the pro-inflammatory responses are induced by specific interaction between Fas and anti-Fas mAb. Fab fragments of anti-Fas or anti-FasL mAb, which have only one binding site for the antigen, failed to stimulate the cell (data not shown), indicating that the stimulation requires antibody-mediated cross-linkage of Fas or FasL.

It is interesting that THP-1 cells are expressing both Fas and its ligand (FasL) at the same time. Although it is believed that the stimulation of the cells with anti-Fas mAb induced cellular changes through direct interaction with Fas, expression of both the interacting counterparts simultaneously may complicate the situation. For example, it is possible that the mAb induced cellular activation through blocking normal interaction between ligands and receptors present on the cells. In order to make sure that the anti-Fas-mediated cellular responses are truly mediated by Fas, the expression levels of Fas was suppressed by transfection with siRNA targeting Fas (siFas). As shown in Fig. 3A, the expression of Fas was not detected in siFas transfectants while the expression of FasL was detected at normal levels. This demonstrates the specificity of siRNA-mediated suppression of Fas expression. Transfection of control siRNA did not affect the expression levels of both Fas and FasL. When these cells were stimulated with anti-Fas mAb, siFas transfectants failed to respond to the treatment while control siRNA-transfected cells responded at a normal extant. The responsiveness toward LPS and anti-FasL was not affected in

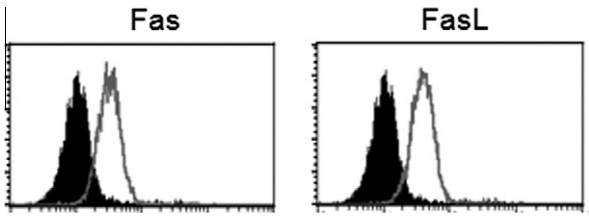

Fig. 1. THP-1 cells express both Fas and FasL on the cell membrane. Cells were stained with mAbs specific for Fas or FasL for flow cytometry analysis. Histograms from specific staining (open area) and background staining (filled area, stained with mouse IgG) are shown.

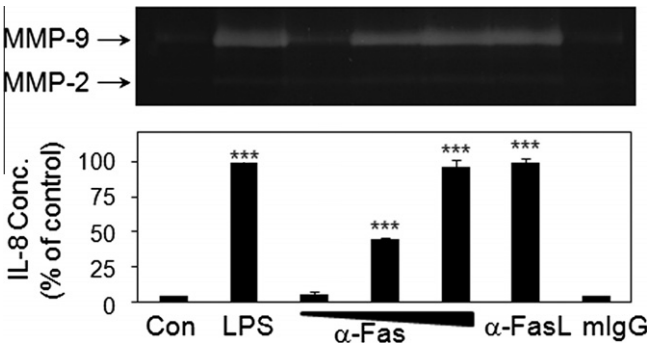

Fig. 2. Stimulation of either Fas or FasL using agonistic mAb induces the expression of pro-inflammatory mediators in THP-1 cells. Cells were treated with 0.1, 0.3 or 1 μg/ml of anti-Fas mAb or 1 μg/ml of LPS, anti-FasL mAbs or mouse IgG for 24 h. Culture supernatants were then collected and MMP-9/MMP-2 activities were analyzed using gelatin zymogram and IL-8 concentrations were measured using double sandwich ELISA. Data points are represented as percentage of LPS-treated positive control ($n = 3$, $^{***}p < 0.001$ when compared with negative control).

both control siRNA and siFas transfectants, indicating that the cellular responsiveness to agents that ligate cell surface molecules other than Fas was not affected by the siFas transfection. These data confirm that the triggering of Fas can lead to the inflammatory activation of THP-1 cells. Apoptotic cell death (such as DNA fragmentation or nuclear breakdown) was not observed in THP-1 cells that were treated with anti-Fas mAb (data not shown).

3.2. Stimulation of Fas induces production of IL-8 through activation of MAPK, PI3K and NF-κB

The signaling adapters that are involved in Fas-mediated cellular signaling were then analyzed. When specific inhibitors of MAPK (U0126 for ERK1/2 [30,31], SB203580 for p38 [32,33] and JNK-I1 for JNK [25]) were used for the pretreatment of the cells before stimulation with anti-Fas mAb, the expression of IL-8 was inhibited (Fig. 4). In contrast, negative control of JNK inhibitor did not exhibit any inhibitory effect, demonstrating the specificity of the inhibition. Fas-mediated induction of IL-8 expression was also inhibited by LY294002, the well-known PI3K-specific inhibitor [34,35]. All of the three NF-κB inhibitors (sulfasalazine, ethyl pyruvate and TPCK) exhibited similar inhibitory effect on IL-8 expression.

The inhibition of IL-8 expression by inhibitors of MAPK and PI3K indicates that Fas-mediated signaling events are mediated by these signaling adapters. In order to confirm their involvement, THP-1 cells were treated with anti-Fas mAb and the activation MAPKs was tested by Western blot analysis using mAbs specific for the phosphorylated form of ERK1/2, p38 and JNK. The phosphorylation levels of AKT, the main substrate of PI3K, were also tested. As shown in Fig. 5, triggering of Fas with specific mAb resulted in the phosphorylation of these signaling adapters within 3 min and continued for up to 60 min. Phosphorylation of AKT, ERK, JNK or P38 was detected without anti-Fas treatment over the time-course of the assay in THP-1 cells (data not shown).

Although stimulation of Fas or FasL appears to have same outcome (induction of MMP-9 and IL-8), the signaling pathways induced by each of them appear to be different. In case of FasL, the signaling was mediated by all three of the MAPKs but not by PI3K [24]. In contrast, Fas-mediated signaling appears to be mediated by both MAPKs and PI3K. The time points of MAPK activation also differ slightly. In case of FasL, phosphorylation of ERK and p38 was detected within 3 min while phosphorylation of JNK started 20 min after activation. In contrast, Fas-mediated signaling activated all three MAPKs within 3 min after activation. Although

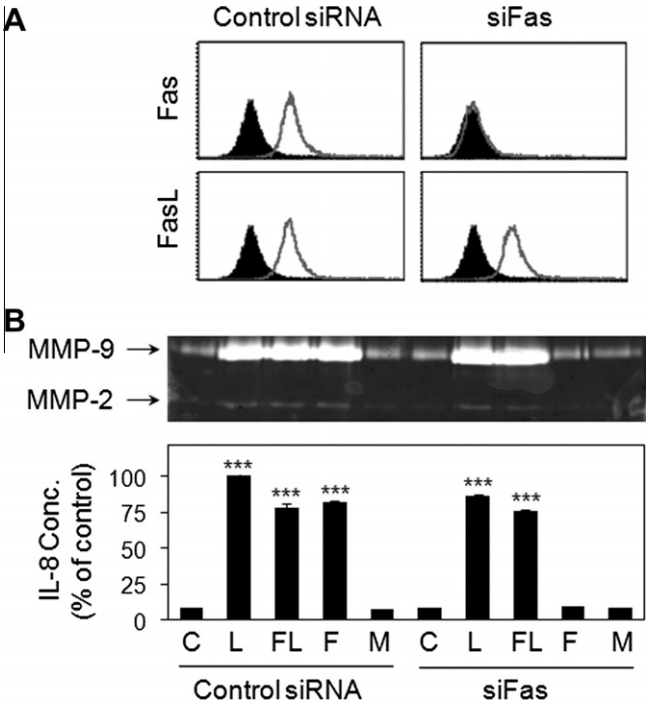

Fig. 3. Transfection of siFas resulted in the abolishment of Fas expression as well as the responsiveness toward anti-Fas mAb. (**A**) Control siRNA and siFas transfectants were stained with Fas or FasL specific mAbs for flow cytometry analysis. Histograms from specific staining (open area) and background staining (filled area, stained with mouse IgG) are shown. (**B**) Control siRNA and siFas transfectants were treated with 1 μg/ml of LPS (L), anti-FasL mAb (FL), anti-Fas mAb (F), or mouse IgG (M) for 24 h. Culture supernatants were then collected for the measurement of MMP activities and IL-8 concentrations. (**C**) No treatment control. Data points are represented as percentage of positive control ($n = 3$, $^{***}p < 0.001$ when compared with negative control).

MMP-9 and IL-8 were commonly activated by ligation of either Fas or FasL, Fas may induce other yet-unidentified cellular responses that are different from those induced by FasL stimulation and vice versa.

3.3. Fas-mediated activation of NF-κB requires the involvement of ERK1/2 and JNK

Since inhibitor assay in Fig. 4 indicated that NF-κB is involved in the Fas-mediated cellular activation, its functional activity was then tested using ELISA-based NF-κB DNA binding assay. For that, oligonucleotides containing the consensus NF-κB binding sequence were immobilized and incubated with cellular extracts that contain NF-κB. Binding of NF-κB was detected using a mAb specific for the p65 subunit of NF-κB. DNA binding activity of NF-κB was transiently upregulated 10 min after stimulation with anti-Fas mAb. The specificity of the assay was demonstrated by successful suppression of the binding by excess amount of free oligonucleotides containing the wild type NF-κB binding site but not by mutant oligonucleotides (Fig. 6A).

The NF-κB DNA binding assay was then performed in the presence of MAPK or PI3K inhibitors in order to identify the upstream signaling adapters that are responsible for the Fas-mediated activation of NF-κB. Interestingly, Fas-mediated activation of NF-κB DNA binding was inhibited by U0126 and JNK-I1 while SB203580 and LY294002 were not inhibitory (Fig. 6B). These data indicate that ERK1/2 and JNK are involved in the Fas-mediated activation of NF-κB, while PI3K and p38 are involved in Fas-mediated induction of IL-8 expression through pathway(s) that does not involve NF-κB.

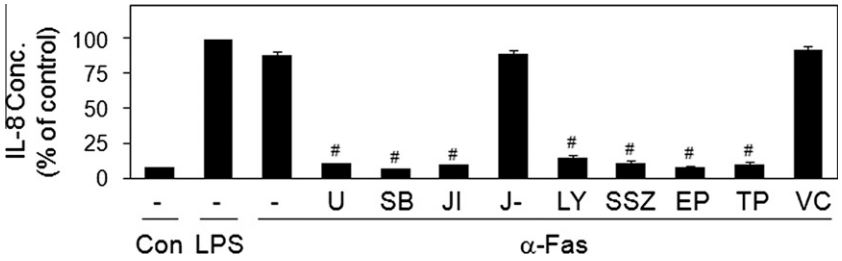

Fig. 4. Fas-mediated expression of IL-8 requires the involvement of MAPK, PI3K, and NF-κB. THP-1 cells were pretreated with 5 μM of SB203580 (SB), 10 μM of U0126 (U), JNK-I1 (JI), negative control of JNK-I1 (J-), 5 mM of ethyl pyruvate (EP), 1 mM of sulfasalazine (SZ), 1 μM TPCK (TP), 20 μM of LY294002 (LY), or 0.1% DMSO (vehicle control, VC) for 30 min. Cells were then treated with 1 μg/ml of anti-Fas mAb for 24 h. Culture supernatants were then collected for the measurement of IL-8 concentration using ELISA. Data points are represented as percentage of LPS-treated samples ($n = 3$, $^{\#}p < 0.001$ when compared with samples treated with anti-Fas mAb only).

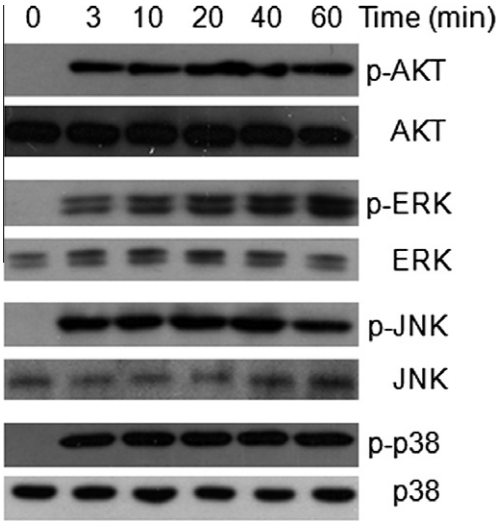

Fig. 5. Stimulation of Fas results in the phosphorylation of AKT, ERK, JNK and p38. THP-1 cells were stained with anti-Fas mAb for indicated times. Cell lysates were then obtained for Western blot analysis using antibodies specific for AKT, ERK1/2, JNK and p38 and their phosphorylated forms.

It is interesting that the NF-κB activation peaks at 10 min after activation while ERK and JNK activation lasts longer than that. It is well known that activation/nuclear localization of NF-κB does not last long due to multiple regulatory mechanisms such as restoration of IκB levels by new synthesis and modification (acetylation and phosphorylation) of NF-κB which regulate its nuclear localization and/or its transcription activation activity [36–38]. Due to these regulatory mechanisms, activation status of NF-κB does not last long even with the continued activation of ERK and JNK MAPK. Continued activation of ERK and JNK MAPKs may induce yet-unidentified Fas-mediated cellular activation in addition to NF-κB activation.

3.4. Interaction with FasL-expressing cells can induce Fas-mediated cellular activation

Stimulation of Fas with antibody is not a natural way of activation. Cell surface receptors can be activated by interaction with soluble ligands or membrane-bound ligands during cell-to-cell communication. In order to mimic this natural way of activation, siFasL transfectants (which express Fas but not FasL [24]) were mixed with siFas transfectants (which express FasL but not Fas). When these two cell populations were mixed, IL-8 expression was induced. Since both Fas and FasL are capable of inducing cellular activation, it was not clear which cell population responded (Fig. 7). For that, one of the two cell populations were fixed with 4% paraformaldehyde and mixed. When siFas transfectants were fixed, siFasL transfectants (which express Fas) responded and vice versa. These data demonstrate that both the receptor (Fas) and the membrane-bound form of ligand (FasL) are capable of inducing cellular activation through interaction with their natural counterparts during cell-to-cell interaction.

3.5. Incubation of THP-1 cells with equal amounts of anti-Fas and anti-FasL mAb resulted in a significant reduction of IL-8 production in comparison to anti-Fas or anti-FasL mAb alone

Co-expression of ligands and their cognate receptors on the surface of macrophage has been detected (both *in vivo* and *in vitro*) for most members of the TNF superfamily. These include FasL/Fas, GITRL/GITR, LIGHT/TR2, BAFF/BAFF-R, TL1A/DR3 and CD40L/CD40 [29,39–41]. Interaction between these ligands and cognate

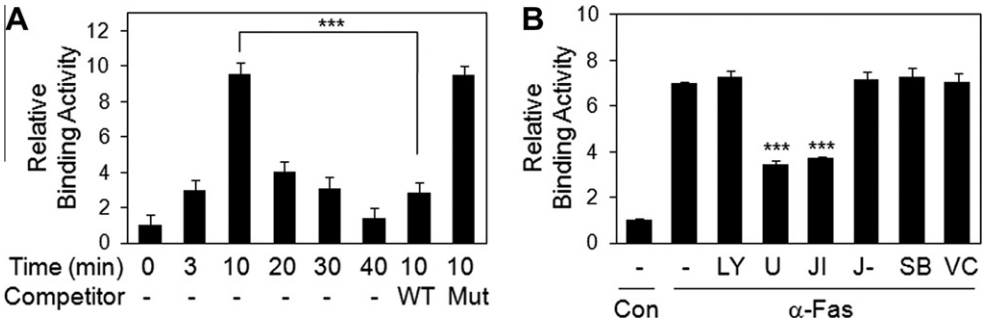

Fig. 6. ERK and JNK activities are involved in Fas-mediated activation of NF-κB in THP-1 cells. (**A**) cells were stimulated with anti-Fas mAb for indicated times. Cell lysate were obtained and subjected to ELISA-based NF-κB DNA binding assay using mAb against p65. For blocking experiment, cell lysates were pre-incubated with 50-fold excess of wild type (WT) or mutant (Mut) form of consensus NF-κB binding sequence ($n = 3$, $^{***}p < 0.001$). (**B**) THP-1 cells were pre-treated with 20 μM of LY294002 (LY), 5 μM of SB203580 (SB) and 10 μM of U0126 (U), JNK-I1 (JI), or negative control of JNK-I1 (J-) for 30 min. DMSO (0.1%) was used as a vehicle control (VC). Cells were then treated with 1 μg/ml of anti-Fas mAb for additional 10 min. Cell lysates were then collected and used for the ELISA-based NF-κB binding assay using mAb against p65 subunit ($n = 3$, $^{***}p < 0.001$ when compared samples treated with only anti-Fas mAb).

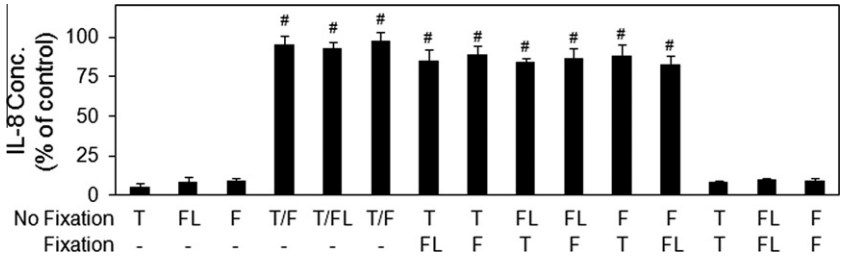

Fig. 7. Interaction between membrane-bound form of Fas and FasL results in a reciprocal activation of each other. Two of the three cell types [THP-1 cells (T), siFas transfectants (F) and siFasL transfectants (FL)] were co-incubated at a combinatorial fashion with or without fixation. Fixation was done with 4% paraformaldehyde for 5 min on ice after which the cells were washed twice with PBS. Culture supernatants were collected 24 h after co-incubation for the measurement of IL-8 concentrations. Data points are represented as percentage of LPS-treated samples which was set at 100% (not shown in the figure) ($n = 3$, $^{\#}p < 0.001$ when compared with samples from THP-1 cells alone).

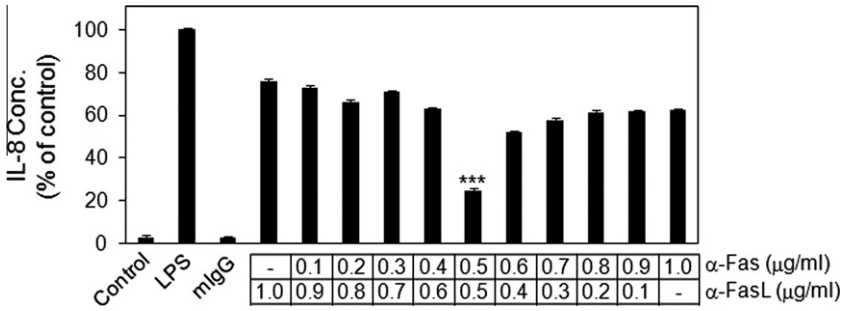

Fig. 8. Cellular response differs according to the ratio between anti-Fas and anti-FasL mAb. THP-1 cells were treated with mixture of anti-Fas and anti-FasL mAbs (total 1 μg/ml) as indicated for 24 h for the measurement of IL-8 in the culture supernatant. LPS or isotype matching mouse IgG were added at 1 μg/ml concentration as control ($n = 3$, $^{***}p < 0.001$ when compared with samples treated with 1 μg/ml of anti-Fas mAb).

receptors results in the cellular activation through a so called 'reverse signaling' that is initiated from the ligands [24,29,41–43] in addition to the 'forward signaling' that is initiated from the receptor [26,28,43–45]. Since both Fas and FasL are expressed on the surface of THP-1 cells, it can be expected that THP-1 cells are under constant stimulation. However, basal levels of IL-8 in THP-1 culture were not different with those of siFas transfectants or siFasL transfectants (Fig. 7). In addition co-incubation of THP-1 cells with fixed siFas or siFasL-transfected cells induced stimulation of the cells (Fig. 7). This indicates that the THP-1 cells, albeit retaining responsiveness toward stimulation of Fas and FasL, are not activated by cell-to-cell contact in homogeneous cell culture.

Base on the results it was hypothesized that Fas and FasL interact on the same cell, but remain inactive without crosslinking from an adjacent cell. Crosslinking may only occur when the Fas and FasL are no longer expressed in a 1:1 ratio, such as upon down-regulation by siRNA. In order to test the likelihood of the hypothesis, THP-1 cells were incubated with fixed total amount of mAb mixtures which contains anti-Fas and anti-FasL at various ratios. As shown in Fig. 8, cells incubated in a mAb mixture that contains equal amounts of anti-Fas and anti-FasL expressed significantly less amount of IL-8 when compared with cells treated with same total amounts of anti-Fas or anti-FasL mAb alone.

Same type of homogeneous cell population can be found in tissues undergoing acute or chronic inflammation where a large number of activated macrophages are in close contact with each other. The production of pro-inflammatory mediators from the initial contact could be beneficial for conduction of cellular functions by enhancing inflammatory processes and phagocytic removal of pathogens or antigenic particles. However, constant activation of the cells through cell-to-cell contact in the absence of pathogens and antigenic particles is expected to cause the exhaustion of cellular activity and, subsequently, the development of unresponsiveness toward a later encounter with real pathogens or antigenic particles. This indicates that the cells should develop some kind of mechanism that blocks the activation of the cell from routine interaction among cells within a homogeneous cell population. A situation similar to that of homogeneous culture of THP-1 cells may occur in these cells for the suppression of cellular response from routine cell-to-cell contact.

Current data provide the first experimental evidence showing that the Fas-mediated cellular activation can lead to the induction of IL-8 expression through a signaling pathway that depends on the activation of ERK/JNK MAPK and subsequently activation of NF-κB. PI3K and p38 MAPK also appear to be involved in the transduction of Fas-mediated cellular activation signal through non-NF-κB-dependent pathway(s). These activities of Fas, alone or in combination with various other mediators that are known to control inflammation, may regulate macrophage inflammatory responses during the development of normal immune reactions or chronic inflammatory diseases.

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