Interleukin-1α Promotes Matrix Metalloproteinase-9 Expression, Cellular Motility, and Local Invasiveness of Ameloblastoma Cells

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Conflict of interest

There is no conflict of interest about this study.

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Ethics approval statement

Not applicable.

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Not applicable.

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Not applicable.

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Abstract

Aim: Although ameloblastoma is a benign tumor, its local invasiveness and recurrence rate are both high. Thus, regulation the invasiveness of ameloblastoma cells into surrounded tissue is required for understanding of its pathogenesis. Ameloblastoma cells also secrete several MMPs; however, the factors inducing their secretion remain unclear. We previously suggested that IL-1α derived from ameloblastoma cells triggers the production of inflammatory cytokines by stromal fibroblasts. In this study, we estimated whether IL-1α affect the behavior of ameloblastoma cells.

Methods: The gene expression of MMP-9 was assessed by Real-Time RT-PCR. The secretion of MMP-9 was assessed by ELISA. The motility of AM-3 ameloblastoma cells and Raw264.7 macrophage derived cells, and invasiveness of AM-3 cells were calculated by using Boyden chamber. Invasiveness of AM-3 cells toward HFF-2 fibroblasts were assessed using modified Double Layered Collagen Gel Hemisphere (DL-CGH).

Results: The mRNA expression and secretion of MMP-9 by AM-3 ameloblastoma cells were significantly increased by a stimulation with IL-1α. The motilities of AM-3 and RAW264.7

macrophage derived cells and the invasiveness of AM-3 cells were significantly enhanced by IL-1α and suppressed by an IL-1 receptor antagonist (IL-1Ra). The invasiveness of AM-3 cells towards HFF-2 fibroblasts in a Double-layered Collagen Gel Hemisphere model was suppressed by a treatment with IL-1Ra or an anti-IL-1α neutralizing antibody.

Conclusion: IL-1α itself or the IL-1α-dependent production of unidentified chemo attractants by stromal cells may be important for the local invasiveness of ameloblastoma cells and IL-1α might be a therapeutic target of the ameloblastoma.

Keywords

Ameloblastoma, AM-3, Invasion, Tumor microenvironment, Macrophage, Fibroblast

1. Introduction

Ameloblastoma is a common odontogenic tumor that mainly occurs in patients in their 30s (1,2). It shows aggressive invasion, and jaw resection is often selected as the first-line treatment. Since ameloblastoma has a high recurrence rate, an adequate safety margin needs to be established, which results in facial deformity, tooth loss, nerve damage, and, ultimately, a reduced quality of life (3,4). Thus, it is required to regulate the invasiveness of tumor cells. In spite of the trials of additional therapy such as radiation and molecularly targeted drugs (5-7) , it is still difficult to control local invasiveness of the ameloblastoma.

Ameloblastoma has been reported to secrete a number of substances, such as matrix metalloproteinase (MMP)-9, interleukin (IL)-1α, IL-1β, IL-6, and IL-8 (8-10). Among these factors, the MMP-9 has been examined as the causative proteins for invasion by destroying surrounding bone. IL-1α produced by cancer cells promotes its growth rate, metastasis, and various phenomena in cancer microenvironment (11). IL-1 affected cell proliferation, invasiveness, and the secretion of various factors by fibroblasts in systemic sclerosis (12), and affected cell proliferation and invasiveness of fibroblast-like synoviocytes in rheumatoid arthritis(13). Considering these findings, IL-1α is related to various pathogenesis of diseases. We recently reported that IL-1α was highly expressed in the contents of the cyst cavity of ameloblastoma and the supernatant of an ameloblastoma-derived cell line (8). Furthermore,

ameloblastoma altered the release of various inflammatory factors by osteoblasts via IL-1α and the osteogenic potential of osteoblasts (14,15).

Ameloblastoma consists of a tumor parenchyma and abundant stroma, which includes various cells, such as fibroblasts and macrophages. We previously reported the invasiveness of AM-3 cells was induced in a 3D coculture with fibroblasts; however, the factors contributing to this invasiveness remain unclear. In this study, we examined whether IL-1α derived from ameloblastoma cells affect the behaviors of stroma cells and ameloblastoma cells to regulate MMP-9 production, cellular motility, and local invasiveness of this tumor.

2. Materials & Methods

2.1. Reagents

Defined keratinocyte serum-free medium (D-KSFM) was obtained from Thermo Fisher Scientific (Waltham, MA, USA), F-12 medium from Nissui Corp. (Tokyo, Japan), and Y-27632 (ROCK inhibitor) from Ado Q Bioscience (Irvine, CA, USA). Hydrocortisone and insulin were purchased from Wako Pure Chemical (Osaka, Japan), recombinant human epidermal growth factor (EGF) from Invitrogen Corp. (Carlsbad, CA, USA), and IL-1α and an IL-1Ra (receptor antagonist) from PEPRO TECH. (Cranbury, NJ, USA). An IL-1α neutralizing antibody was supplied by R&D systems (Minneapolis, MN, USA). Acid-soluble collagen type I collagen was purchased from Nitta Gelatin Inc. (Osaka, Japan).

2.2. Cell culture

AM-3 ameloblastoma cells were established from human follicular ameloblastoma (16). Green fluorescent protein (GFP)-labeled AM-3 cells were maintained with D-KSFM or F medium (Dulbecco's Modified Eagle medium (DMEM)/Ham's F-12 = 1 : 3) supplemented with 5% fetal bovine serum (FBS), insulin (10 μg/mL), Y-27632 (5 μM), recombinant human EGF (0.2 μg/mL), adenine-HCl (0.3 mg/mL), and hydrocortisone (2 μg/mL) (17). DsRed-labeled HFF-2 fibroblasts were maintained with DMEM supplemented with 10% FBS. RAW264.7 cells were purchased from the American Type Culture Collection and cultured with DMEM containing 10% FCS.

2.3. Preparation of AM-3-derived conditioned medium (CM)

A total of 3×10^5 AM-3 cells was cultured in a 60-mm dish with 3 mL of D-KSFM which contained no ROCK inhibitor. After 24 h, CM was collected and stored at -80℃ for migration assay experiments.

2.4. Real-Time RT-PCR

Total RNA was obtained from cells using NucleoSpin RNA (Takara Bio Inc., Shiga, Japan) and reverse transcribed using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio Inc.,

Shiga, Japan). cDNA was used for each real-time RT-PCR with Powertrack® SYBR® Green Master Mix and the StepOne Real-Time RT PCR system (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions with the following primers: β-actin, 5'-TGGCACCCAGCACAATGAA-3' (forward) and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (reverse); MMP-9, 5'-ACGCACGACGTCTTCCAGTA-3' (forward) and 5'-CCACCTGGTTCAACTCACTCC-3' (reverse). The expression of each mRNA was normalized using β-actin as a loading control.

2.5. ELISA

The expression of MMP-9 was assessed using the human (total) MMP-9 Quantikine ELISA kit® (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

2.6. Migration assay (for single cell)

A chemotaxis assay was performed using a Boyden chamber containing a membrane with 8-μm pores (Chemotaxicell®, KURABO, Osaka, Japan). The lower surface of the filters was coated with 10 μg/mL fibronectin for 2 h. Cells were resuspended in D-KSFM or DMEM and applied to the upper chamber. The same medium containing some chemoattractant reagents was applied to the lower chamber. After 2 h, cells on the upper surface were wiped with a swab and those that

migrated to the lower side were fixed with 4% paraformaldehyde in PBS, stained with propidium iodide, and counted under microscopic observation.

2.7. Invasion assay (for single cell)

Cellular invasiveness was examined using a collagen gel assay according to the manufacturer's protocol (Cat no. CBA-100; Cell Biolabs Inc., San Diego, CA, USA). Briefly, AM-3 cells (1.0×10^6 cells/ml in F medium without serum) were applied to the upper chamber and F medium with 10% FBS to the lower chamber. Cells were then incubated at 37°C. After 24 h, cells on the upper surface were wiped with a swab and those on the lower surface were fixed with 4% paraformaldehyde in PBS, stained with propidium iodide, and counted under microscopic observation.

2.8. Collective invasion model of ameloblastoma cells and fibroblasts using a double-layered collagen gel hemisphere (DL-CGH)

We assessed collective cell invasion by AM-3 cells towards HFF-2 cells using a DL-CGH model according to a previous study with minor modifications (17,18). Briefly, GFP-labeled AM-3 cells resuspended in F-12 medium containing 10% FCS or DsRed- labeled HFF-2 cells resuspended in DMEM containing 10% FCS were mixed with diluted type-I collagen solution to prepare a "50%

cell collagen mixture" (final cell density of 3.0×10^6 cells/mL each). Five microliters each of the "50% cell collagen mixture" of AM-3 cells and HFF-2 cells were plated separately, but adjacently on the same well (6-well tissue culture test plates, Orange Scientific Cat # 5530500). The outer layers of these "50% cell collagen mixtures" were coated with 70 µl of collagen solution, as described in Figure 5Aa and b. Cells embedded in collagen gels were maintained in F-12 medium containing 10% FCS and observed under a microscope. We defined the axis for observations by connecting the centers of AM-3 cells and HFF-2 cells on day 0. The protrusion of AM-3 cells along the axis was estimated on day 5. Optical sectioning images of DL-CGH were obtained by Apotome 2 (Carl-Zeiss, Jena, Germany).

2.9. Proliferation assay

AM-3 ameloblastoma cells (4×10^4 cells) were seeded in a 60mm-diameter dish with F medium containing 10% FCS in the presence or absence of IL-1Ra (100 ng/mL). Cells were counted at days 1, 3, and 5. HFF-2 cells (4×10^4 cells) were seeded in a 60mm-diameter dish with DMEM containing 10% FCS in the presence or absence of IL-1α (100 pg/mL). Cells were counted at days 1, 3, and 5.

2.10. Other assays

Protein concentrations were determined using a BCA protein assay (Thermo Fisher Scientific) with BSA as a standard.

2.11. Statistical analysis

The Kruskal-Wallis test was used for comparisons among the five groups. Statistical comparisons between two groups were performed using the Mann-Whitney-U test and false discovery ratios were calculated by the Benjamini-Hochberg method.

3. Results

3.1. IL-1α up-regulated MMP-9 expression in AM-3 cells

We previously reported that the cystic fluid of human ameloblastoma cases contained inflammatory cytokines, such as IL-1α(8). Furthermore, AM-3, an ameloblastoma cell line, is known to produce inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF) (8). The gene expression of MMP-9 in AM-3 cells was significantly up-regulated by the treatment with IL-1α and this response was suppressed by IL-1Ra (receptor antagonist) (Figure 1A). IL-1Ra also suppressed the endogenous expression of MMP-9. The secretion of the MMP-9 protein was up-regulated by the treatment with IL-1α, and this response was suppressed by IL-1Ra. Furthermore, IL-1Ra down-regulated the endogenous secretion of

MMP-9 protein (Figure 1B and Supplemental Table 1). These results suggest that IL-1α contributed to the high expression of MMP-9 in AM-3 cells.

3.2. IL-1α promoted the migration of AM-3 and RAW264.7 cells

Previous studies reported that AM-3 cells secreted IL-1α, which accumulated in ameloblastoma cystic fluid (8). Therefore, the present study investigated the mechanisms by which this cytokine affects the behavior of ameloblastoma cells. The motility of ameloblastoma cells was evaluated using the Boyden Chamber technique. IL-1α significantly stimulated the motility of AM-3 cells, while IL-1Ra antagonized this effect (Figure 2). Furthermore, the treatment with IL-1α or AM-3-derived CM enhanced the motility of RAW264.7 cells, while that with IL-1Ra suppressed this response by AM-3 CM (Figure 3). These results indicate that IL-1α might promote motility of AM-3 and RAW264.7 cells. Endogenous secretion of IL-1α by AM-3 cells was sufficient for the migration of RAW264.7 cells in the tumor microenvironment.

3.3. IL-1α promoted the invasiveness of AM-3 cells

Pathologically, the destruction of the extracellular matrix (ECM) is required for the invasion of ameloblastoma cells. Since IL-1α enhanced the migration of AM-3 cells, we investigated their invasive ability using invasion assays in which the membrane was coated with type-IV collagen.

The invasiveness of AM-3 cells was significantly enhanced by IL-1α, and this response was suppressed by additional treatment of IL-1Ra (Figure 4). Invasiveness was also evaluated using a modified DL-CGH model (Figure 5A). In a co-culture of AM-3 cells and HFF-2 cells, AM-3 cells were significantly more invasive towards HFF-2 cells than a monoculture of AM-3 cells on day 5 (Figure 5B a, b). Furthermore, the collective cell invasion can be observed in DL-CGH (Supplemental Figure 1). The treatment of the co-culture with IL-1Ra or anti-IL-1α suppressed the collective invasiveness of AM-3 cells significantly more than the control group (Figure 5B b-e), whereas IL-1α and anti-IL-1α didn't significantly affect the proliferation of HFF-2 and AM-3 cells, respectively (Supplemental Figure 2).

4. Discussion

Ameloblastoma is a benign odontogenic tumor that develops in the jawbone and is characterized by active invasion with the destruction of surrounding bone. Regardless of its aggressive invasiveness and recurrence rate, the mechanisms underlying its invasion remain unclear. Jaw resection has a low recurrence rate, but impairs the quality of life (19). Although limited information is currently available on the application of chemotherapy and radiation therapy to the treatment of ameloblastoma, surgical resection is still the first-line treatment for this tumor (5-7).

The MMP family are proteases, and MMP-9 is expressed in a number of malignant tumors

(20-22). Since MMP-9 is involved in tumor cell invasion, it has potential as a biomarker for the malignancy of various tumors (23). MMP-9 was shown to be secreted by ameloblastoma cells (9). Although we previously demonstrated that Wnt3a up-regulated MMP-9 secretion in AM-3 cells, we were unable to rule out whether other factors triggered the secretion of MMP-9. Kubota et al. showed that IL-1α was expressed in odontogenic keratocysts and that cultured cells of this disease showed the expression of MMP-9 when treated with IL-1α (24). IL-1α, along with IL-1β, IL-6, and TNF, is a soluble factor involved in bone destruction in rheumatoid arthritis and contributed to the induction of osteoclasts and bone destruction (25-28). We reported that the cystic fluid of ameloblastoma and the CM of ameloblastoma cells included IL-1α (8).

In the present study, the gene expression and protein production of MMP-9 in AM-3 cells was increased by the treatment with IL-1α and decreased by that with IL-1Ra. Furthermore, IL-1Ra reduced the endogenous gene expression and protein production of MMP-9. These results indicate that IL-1α secreted by AM-3 cells acts autocrinally to promote the expression of MMP-9.

Immunohistochemistry, macrophages are enriched in the tumor microenvironment of ameloblastoma (29). In this study, IL-1α secreted by AM-3 cells promoted the migration of RAW264.7 macrophage-derived cells. We previously reported that AM-3 cells promoted

osteoclast differentiation by RAW264.7 cells (16). These findings indicate that IL-1α produced by ameloblastoma promotes the accumulation of macrophage-like cells in the microenvironment and the formation of osteoclasts from macrophages.

IL-1α is known as one of the factors that promote the migration and invasion of cancer cells (30,31). Cervical cancer cases with the positive expression of IL-1α were more likely to have short survival times and a high rate of metastasis, and IL-1α was identified as a prognostic factor (32). The expression of IL-1 or IL-6 was previously shown to correlate with an aggressive phenotype (10). Therefore, IL-1α may promote the migration and invasion of ameloblastoma cells. Ameloblastoma is surrounded by stromal cells. We showed that the migration of AM-3 cells was promoted by the treatments with HFF-2 and MC3T3 CM (8,14). The present results suggest that IL-1α promoted the motility and invasiveness of ameloblastoma cells, similar to malignant tumors. In a co-culture of AM-3 and HFF-2 cells, AM-3 cells invaded HFF-2 cells and this behavior was significantly antagonized by IL-1Ra or anti-IL-1α. We previously showed that IL-1α induced fibroblasts to secrete IL-6 and IL-8, and these cytokines promoted the migration of AM-3 cells (8). We also demonstrated that some factors secreted by osteoblasts promoted the migration of AM-3 cells (14). In this study we have shown that IL-1α secreted by AM-3 cells acts autocrinally to produce MMP-9 and that IL-1-induced cytokines from surrounding cells in the tumor microenvironment may

promote the invasion of ameloblastoma cells. These phenomena are consistent with the pathological finding of ameloblastoma cells invading the stroma and bone, similar to tuft formation. Recently, BRAF and Wnt signals were reported to contribute to the cell proliferation and migration of ameloblastoma cells, respectively (33-35). Our findings suggested that IL-1α might be the novel therapeutic target of ameloblastoma in addition to these molecules.

Malignant tumors metastasize using epithelial-mesenchymal transition and disordered growth, whereas ameloblastoma does not (17). On the other hand, aggressive invasion to the surrounding tissue is a common characteristic of ameloblastoma and malignant tumors. In conclusion, the present study revealed a role for IL-1α in the invasiveness and the induction of MMP-9 expression in ameloblastoma cells. IL-1α secreted from ameloblastoma promotes the expression of MMP-9, which destroys the ECM. Furthermore, IL-1α may intervene between tumors and the surrounding tissue to promote invasion by tumor cells into the ECM. Although the factors that induce local invasion by ameloblastoma cells remain unclear, IL-1α may be the candidate and a novel therapeutic target to control local invasion of ameloblastoma.

This study indicated that IL-1α triggered MMP-9 secretion by ameloblastoma in addition to Wnt pathways (16), and the effect of IL-1 receptor antagonist to suppress invasion potential of

ameloblastoma. Therefore, further animal and clinical studies are needed to establish the practical usability of IL-1α antagonizing reagents for the treatment of ameloblastoma.

5. Conclusion

The present results suggests that IL-1α promote secretion of MMP-9 from AM-3 ameloblastoma cells, cellular motility and invasiveness of ameloblastoma cells.

Author contributions

YO, TF, and SK contributed to the concept underlying the study and designed the study. YO and MK acquired data. YO, HK, and YN contributed to the analysis and interpretation of data. YO drafted the manuscript. YO, TF, MK, HK, MI, KO,TK, KI, YN, TK, NN, and SK revised the content of the manuscript. All authors read and approved the final manuscript.

Abbreviations

IL Interleukin

IL-1 Ra IL-1 receptor antagonist

MMP Matrix metalloproteinase

GFP Green fluorescent protein

D-KSFM Defined keratinocyte serum-free medium

DMEM Dulbecco's Modified Eagle medium

ECM Extracellular matrix

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Figure Legends

Figure 1A

IL-1α stimulated the gene expression of MMP-9 in AM-3 cells

AM-3 cells were maintained D-KSFM including no Y-27632.

Semiconfluent AM-3 cells were treated with/without IL-1α (5 ng/ml) in the presence or absence of IL-1Ra (100 ng/ml). After 24 h, the mRNA levels of MMP-9 were analyzed. Results are the means ± SE of at least six independent experiments. $*p < 0.05$.

Figure 1B

IL-1α stimulated the production of the MMP-9 protein by AM-3 cells

AM-3 cells were maintained D-KSFM including no Y-27632.

Semiconfluent AM-3 cells were treated with/without IL-1α (5 ng/ml) in the presence or absence of IL-1Ra (100 ng/ml). After 5 days, CM was collected, and the concentration of MMP-9 was quantified by ELISA. Results are the means ± SE of at least four independent experiments. $* p < 0.05$.

Figure 2

The motility of AM-3 cells was stimulated by IL-1α.

In brief, F medium containing Y-27632 (5 μM) were applied in the upper chamber, and serum-free F medium containing Y-27632 (5 μM) were applied in lower chamber.

In addition, AM-3 cells were treated with IL-1α (5 ng/ml) (b, c) or without (a, d) in the presence (c, d) absence (a, b) of IL-1Ra (100 ng/ml). Reagents were added to media at the beginning of the migration assay. After 2 h of migration, the number of migrated AM-3 cells was counted in three arbitrary views. Representative microscopic images are presented. Bars, 100 μm. (e) Relative migration activities of AM-3 cells. Results are the means ± SE of at least six independent experiments. * $p < 0.05$.

Figure 3

The motility of RAW264.7 cells was stimulated by AM-3-derived IL-1α.

Mixture of DMEM containing 5% FCS with pure D-KSFM (50:50, v/v) were applied in the upper chamber. Mixture of DMEM with pure D-KSFM or AM-3CM (50:50, v/v) were applied in the lower chamber. These reagents include no ROCK inhibitor.

RAW264.7 cells migrated for 6h in the presence of CM derived from AM-3 cells treated with D-KSFM (a, b) or AM-3-derived CM (c, d). IL-1α (5 ng/ml) (b), IL-1Ra (100 ng/ml) (d) were added to media at the beginning of the migration assay. Representative microscopic images are presented. Bars, 100 μm. (e) Relative migration activities of RAW264.7 cells. Results are the means ± SE of

at least five independent experiments. * $p < 0.05$.

Figure 4

IL-1α promoted the invasiveness of AM-3 cells.

F medium containing Y-27632 (5 μM) were applied in the upper chamber, and serum-free F medium containing Y-27632 (5 μM) were applied in lower chamber.

AM-3 cells invaded for 24 h. IL-1α (5 ng/ml) (b, c) and IL-1Ra (100 ng/ml) (c, d) were added to the upper chamber at the beginning of the invasion assay. Representative microscopic images are presented. Bars, 100 μm. (e) Relative invasion activities of AM-3 cells. Results are the means ± SE of at least six independent experiments. * $p < 0.05$.

Figure 5A

A schematic of the monoculture of AM-3 cells and modified DL-CGH model containing AM-3 and HFF-2 cells. (a) A mono-culture of AM-3 cells. (b) A modified DL-CGH model of the co-culture.

The internal layer contains collagen with AM-3 or HFF-2 cells, whereas the outer layer contains collagen only. This collagen hemisphere was maintained by F medium.

Microscopic images of co-cultured AM-3 cells and HFF-2 cells on day 5 (c, d, f), showing (c)

GFP-AM-3 cells (d) and DsRed-HFF-2 cells. (e), A double-stained image of AM-3 cells and HFF-2 cells on day 0. (f), A double-stained image of AM-3 cells and HFF-2 cells on day 5. Both cell types on day 0 were superimposed on microscopic images on day 5.

The relative rate of invasion was expressed as the ratio of the radius of AM-3 cells on day 0 (X) and the length of the protrusion of AM-3 cells on day 5 (Y) along the axis (e, f).

Figure 5B

The invasiveness of AM-3 cells was regulated by IL-1α in the co-culture with HFF-2 cells.

Y-27632 (5μM) was included in the collagen layers and medium.

The DL-CGH method was used in this assay. AM-3 cells were monocultured (a) or co-cultured with HFF-2 fibroblasts (b), co-cultured with the treatment of IL-1Ra (100 ng/ml) (c) or the anti-IL-1α antibody (1 μg/ml) (d). Representative microscopic images are presented. (e) Relative rates of invasion were estimated as described in Figure 5A. Results are the means ± SE of at least six independent experiments. * $p < 0.05$.

Supplemental Figure 1

Microscopic images of 3D collective invasion (DL-CGH) model. AM-3 GFP and HFF-2 DsRed cells were seeded as described in Figure 5 Legend, and the images were captured at DAY5.

(a) Fluorescent image of AM-3 GFP cells using an GFP filter (ex: 470/40nm, em: 525/50nm, dichroic: 495nm)

(b) Fluorescent image of HFF-2 DsRed cells using an TRITC filter (ex: 545/45nm, em: 605/70nm, dichroic: 565nm)

(c) Merged fluorescent image of AM-3 GFP and HFF-2 DsRed cells using an GFP filter (ex: 470/40nm, em: 525/50nm, dichroic: 495nm) and TRITC filter (ex: 545/45nm, em: 605/70nm, dichroic: 565nm)

(d) The magnified Z-stack image of yellow-boxed area in (A) was obtained by using an all-in-one fluorescence microscope (BZ-X700, KEYENCE, Osaka, Japan) according to the user manual.

Supplemental Figure 2

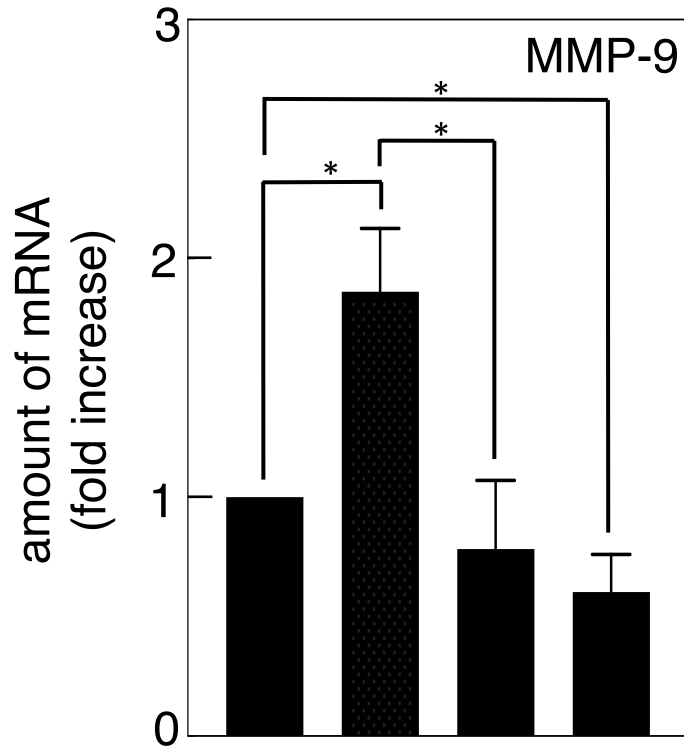
The effect of IL-1a to the proliferation of AM-3 cells and HFF-2 cells.

(A) AM-3 cells were seeded and cultured in the F medium with 5% FCS as a control (●) or in the presence of anti-IL-1a (100ng/ml) (○). Cells were counted at DAY 1,3, and 5. Statistical analyses were carried out using the parametric independent t-test. Asterisks (*) indicate statistically not significant results. Results are the means ±SE of at least three independent experiments. ($p < 0.05$)

(B) HFF-2 cells were seeded and cultured in the DMEM with 5% FCS as a control (●) or in the presence of IL-1a (100pg/ml) (○). Cells were counted at DAY 1,3, and 5. Statistical analyses were

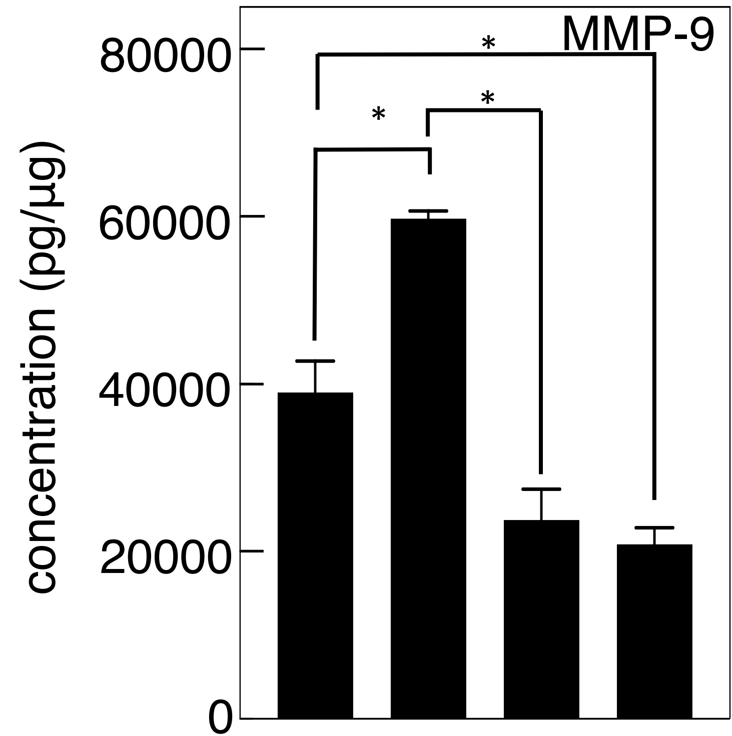
carried out using the parametric independent t-test. Asterisks (*) indicate statistically not significant results. Results are the means ±SE of at least three independent experiments. ($p < 0.05$)

Figure 1A, Ono *et al.*



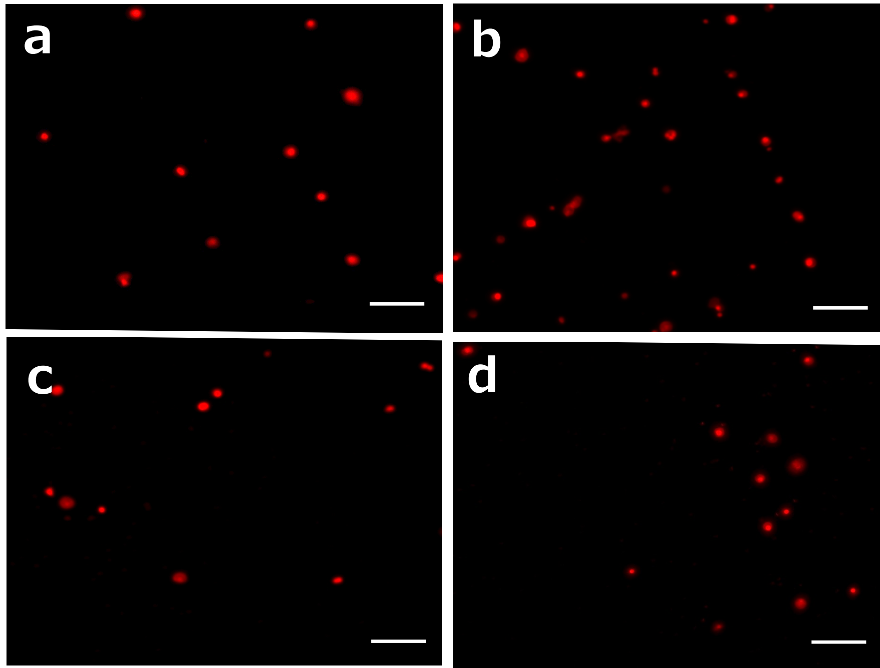
IL-1α	-	+	+	-
IL-1 Ra	-	-	+	+

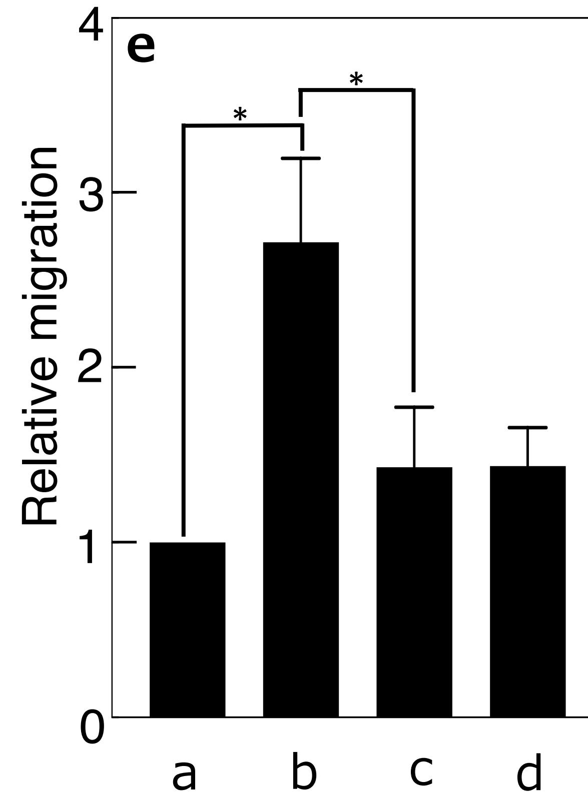
Figure 1B, Ono *et al.*



IL-1α	-	+	+	-
IL-1 Ra	-	-	+	+

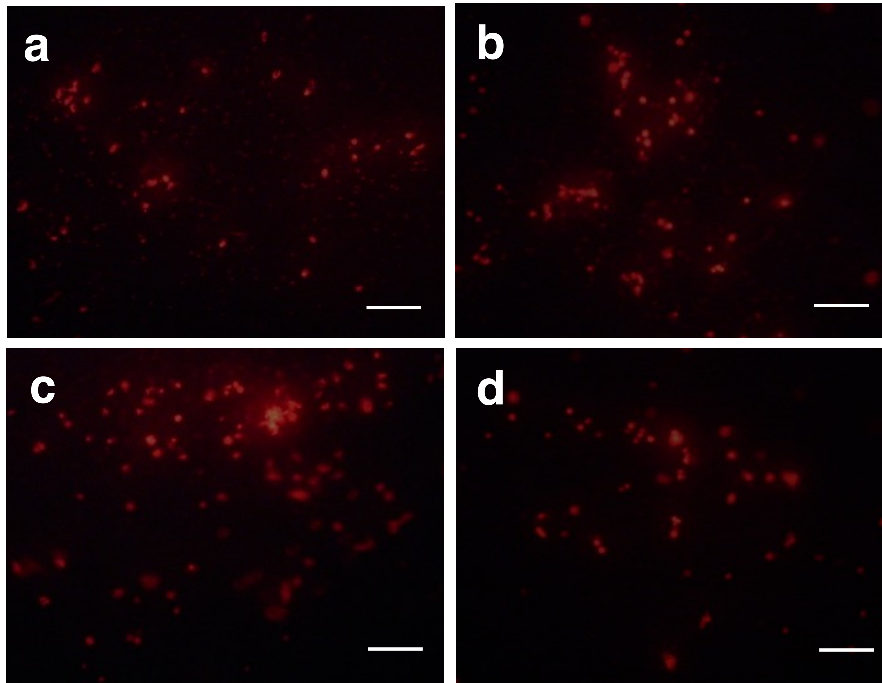
Figure 2,Ono *et al.*

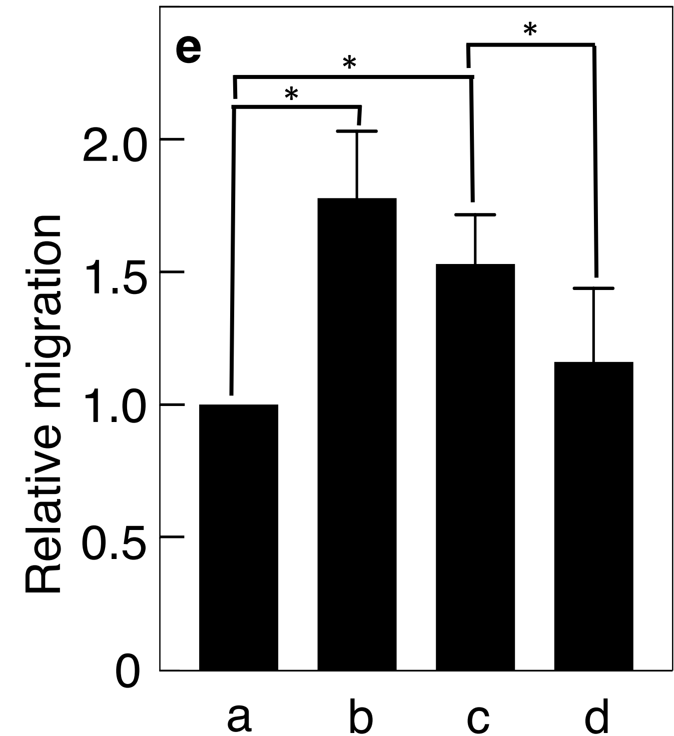




	a	b	c	d
IL-1α	**-**	**+**	**+**	**-**
IL-1 Ra	**-**	**-**	**+**	**+**

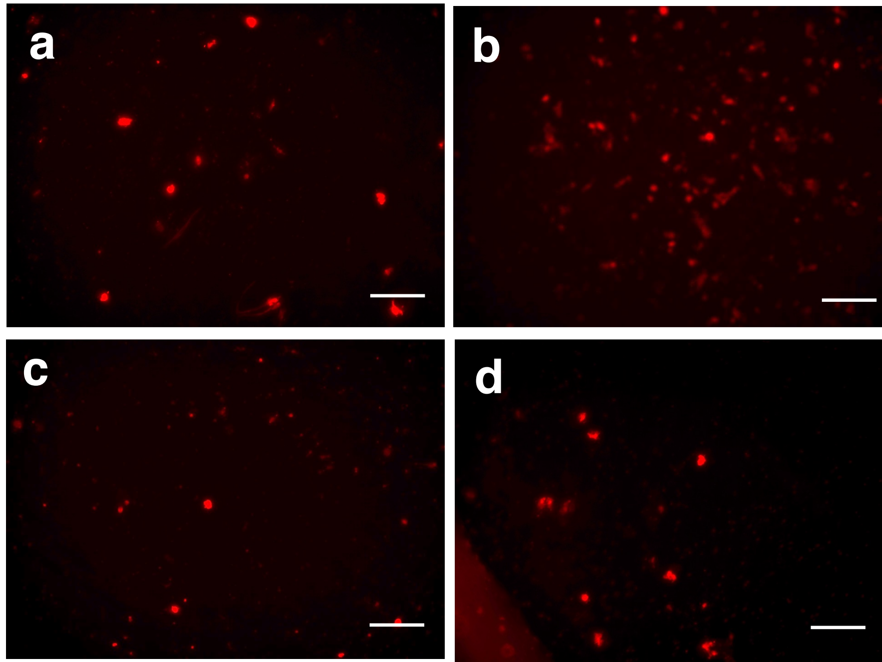
Figure 3,Ono *et al.*

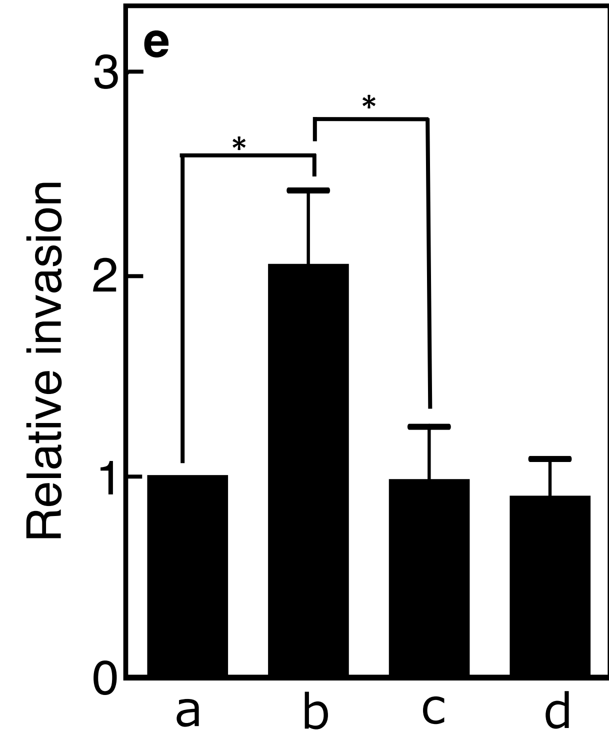




D-KSFM	**+**	**+**	**-**	**-**
IL-1α	**-**	**+**	**-**	**-**
AM-3CM	**-**	**-**	**+**	**+**
IL-1 Ra	**-**	**-**	**-**	**+**

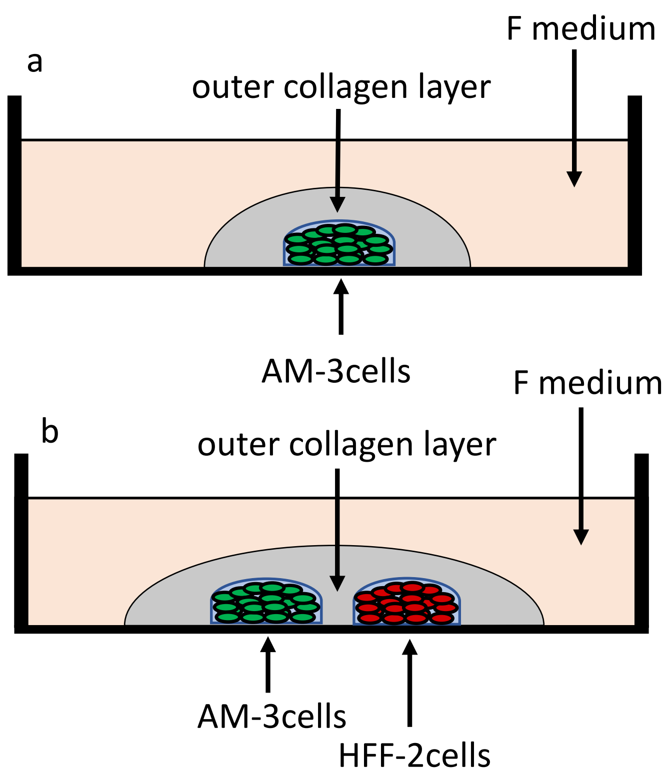
Figure 4,Ono *et al.*

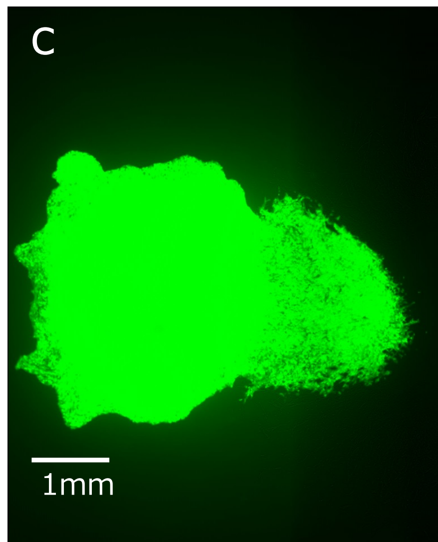


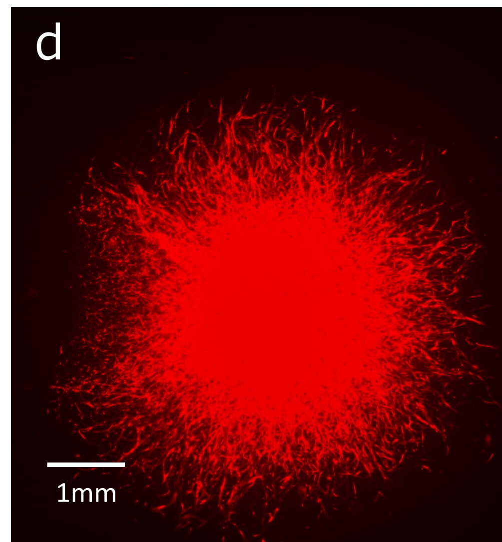


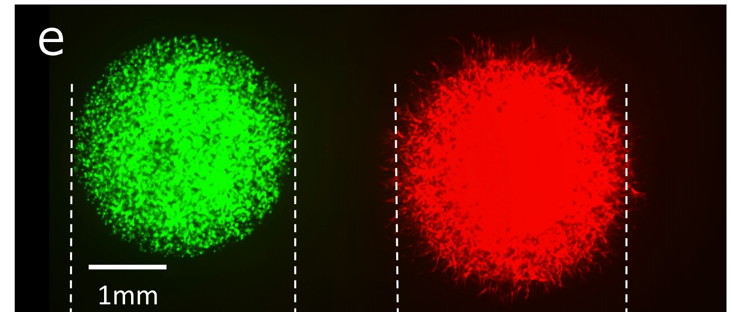
IL-1α	-	+	+	-
IL-1 Ra	-	-	+	+

Figure 5A,Ono *et al.*









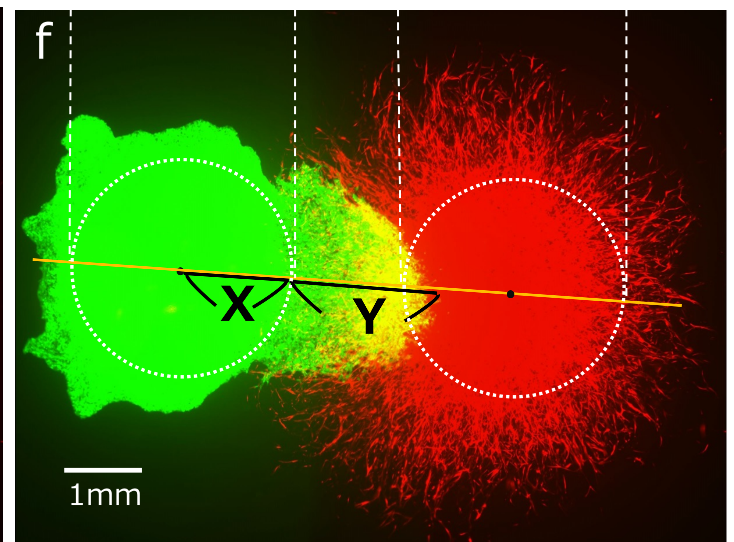
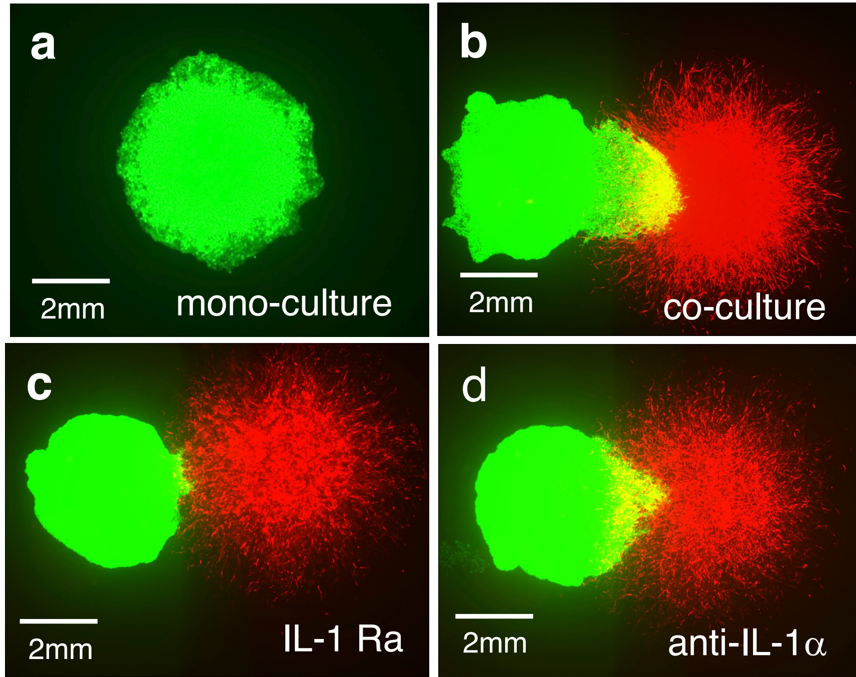
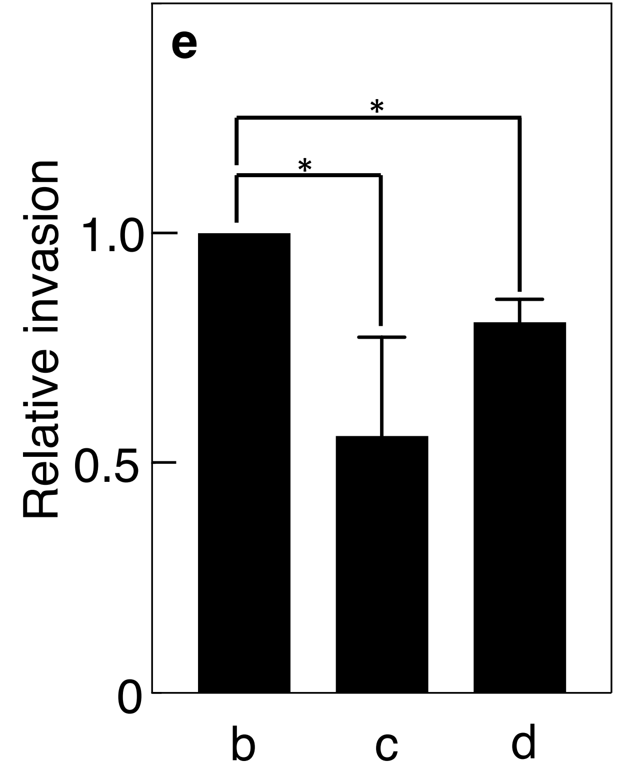


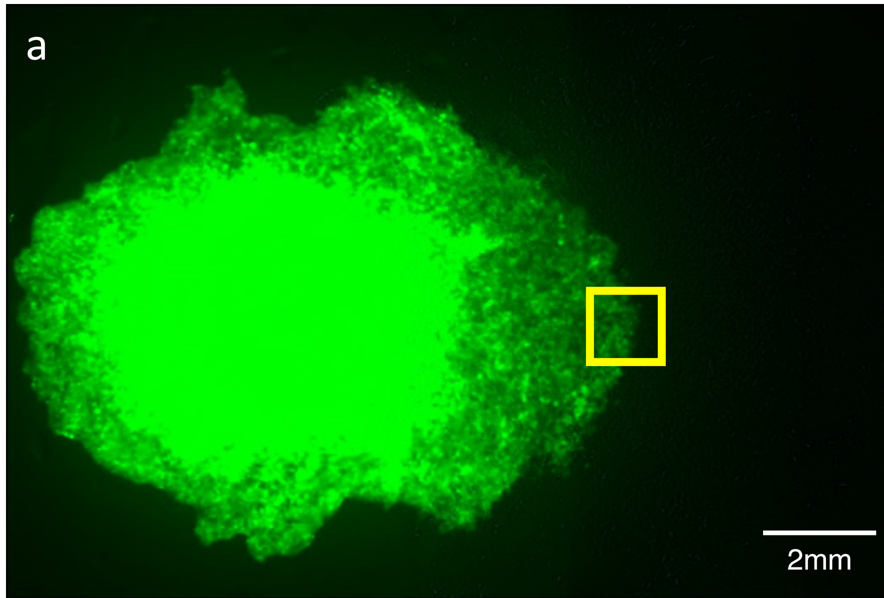
Figure 5B,Ono *et al.*

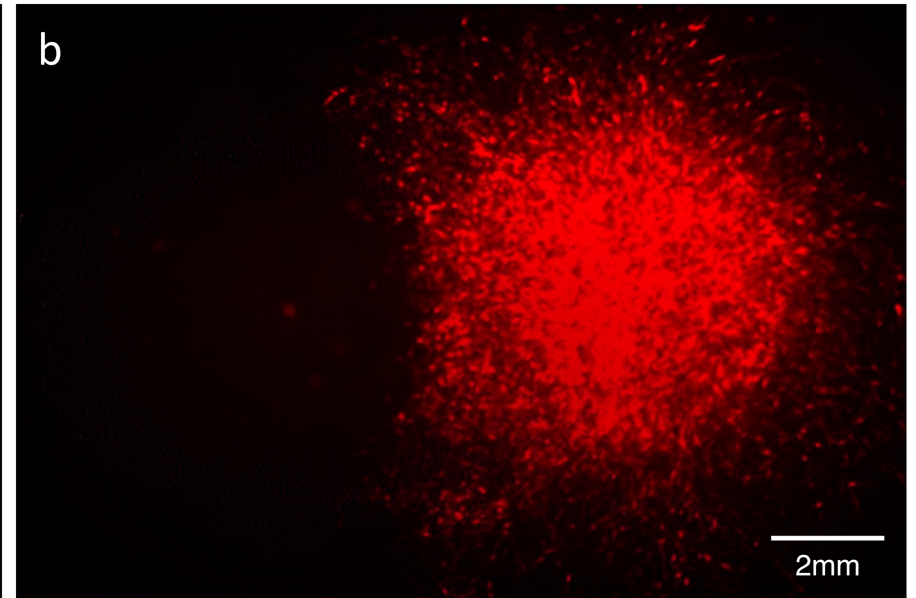


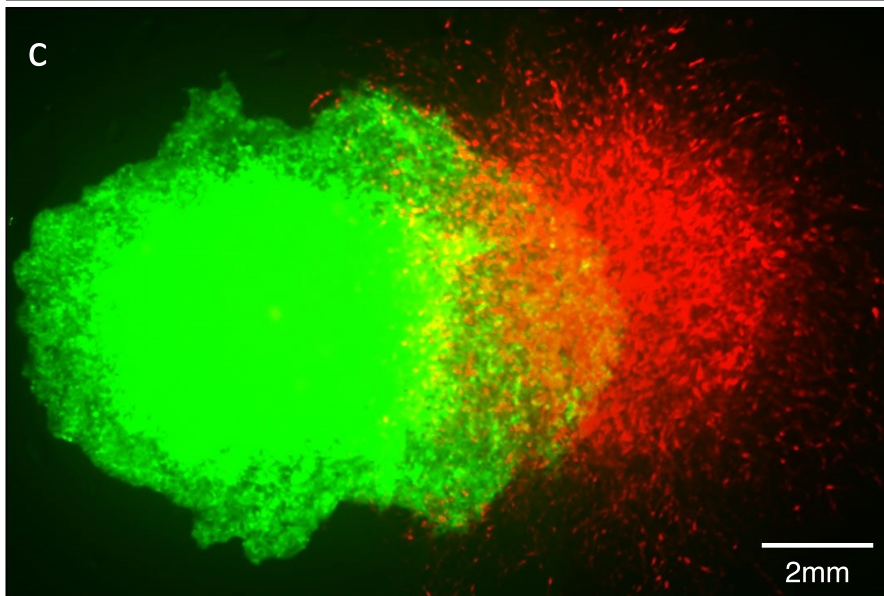


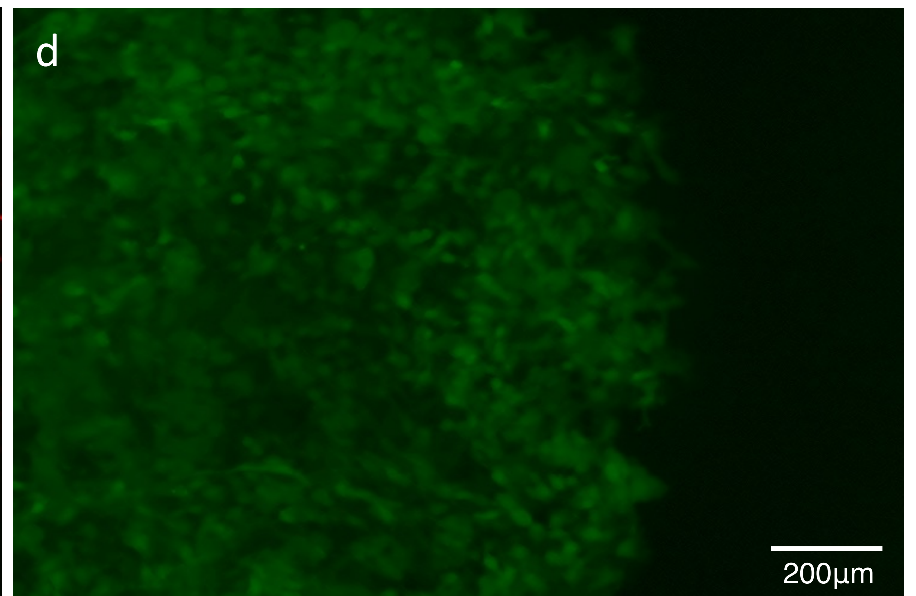
IL-1 Ra	**-**	**+**	**-**
anti-IL-1α	**-**	**-**	**+**

Supplemental Figure 1 Ono *et al.*

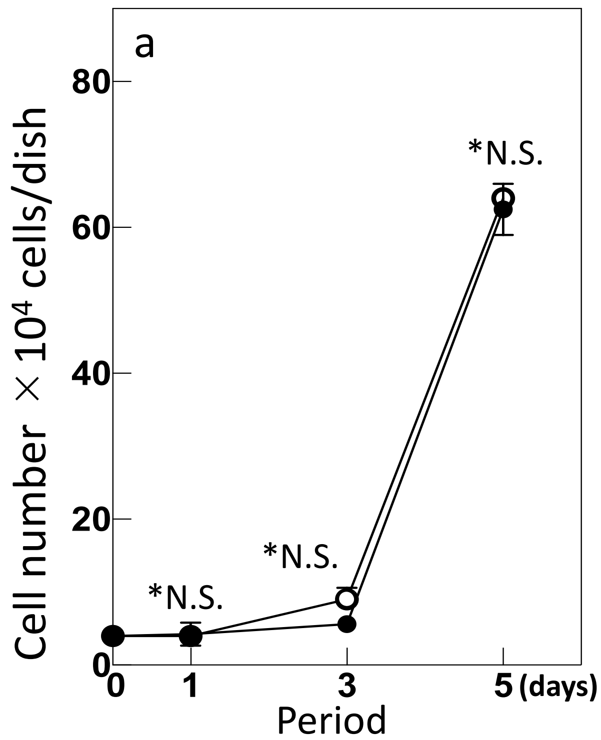


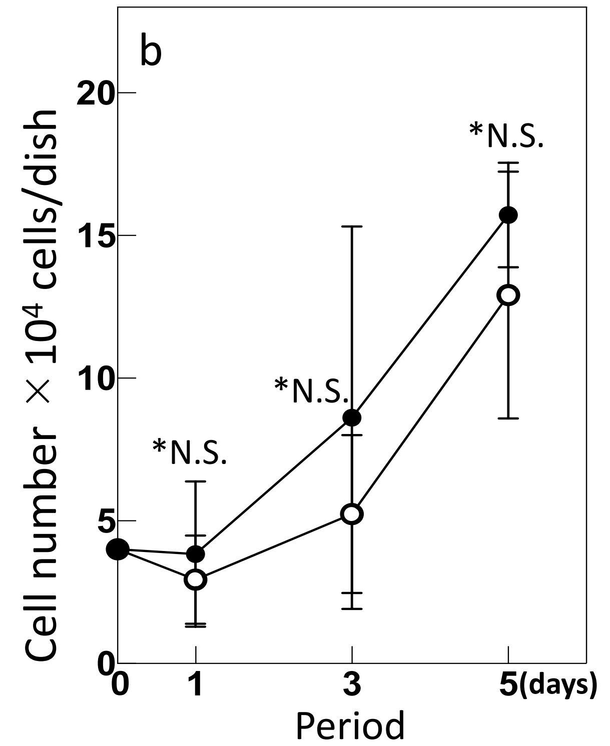






Supplemental
Figure 2 Ono *et al.*





Supplemental table 1

Secretion of MMP-9 protein in the culture medium

Treatment*	MMP-9 ELISA (pg/ml)	Total protein (μg/ml)	Standardized (pg/μg)#
Control	41618.2	0.956	38962.6
IL-1α	64558.3	0.967	59751.5
IL-1α＋Ra	23452.1	0.882	23797.8
Ra	21655.7	0.928	20885.7

* The condition of treatments was described in the legend part of Figure 1B.

MMP-9 secretion was standardized by the total protein concentration of the conditioned medium (CM)