

Letter to the Editor

Matrix metalloproteinase 12 is produced by M2 macrophages and plays important roles in the development of contact hypersensitivity

To the Editor:

Recent studies have suggested that M2 macrophages play key roles in the suppression of T_H1 responses and the orchestration of tissue repair^{1,2}; however, the roles of M2 macrophages in contact hypersensitivity (CHS) remain unknown. To address the roles of M2 macrophages in CHS, we first examined the phenotype of mannose receptor (MR)-positive (MR⁺) F4/80⁺ cells in the skin lesion of 2,4-dinitrofluorobenzene (DNFB)-induced CHS. We found that MR⁺ F4/80⁺ cells as well as MR⁻ F4/80⁺ cells were increased in the sites of CHS and were positive for both CCR2 and MerTK (see Fig E1 in this article's Online Repository at www.jacionline.org), suggesting that MR⁺ F4/80⁺ cells were monocyte-derived macrophages.³ We also found that MR⁺ F4/80⁺ cells expressed other M2 macrophage markers such as dectin-1, SR-A, arginase 1, and Fizz1 (see Fig E2 in this article's Online Repository at www.jacionline.org), whereas MR⁻ F4/80⁺ cells expressed M1 macrophage markers such as TNF- α and iNOS (Fig E2). These results suggest that MR⁺ F4/80⁺ cells in the sites of CHS represent monocyte-derived M2 macrophages.

To examine the roles of M2 and M1 macrophages in CHS, we next performed cell transfer experiments of bone marrow (BM)-derived M2 and M1 macrophages. The purity of M2 and M1 macrophages was routinely more than 90% as evaluated by fluorescence-activated cell sorting analysis (see Fig E3 in this article's Online Repository at www.jacionline.org), and the expression of M2 markers or M1 markers in these cells was confirmed by using quantitative PCR analysis (see Fig E4 in this article's Online Repository at www.jacionline.org). Importantly, the intradermal injection of M2 macrophages but not of M1 macrophages significantly exacerbated ear thickness in DNFB-induced CHS (Fig 1, A) and significantly increased the numbers of various inflammatory cells (Fig 1, B; see Fig E5 in this article's Online Repository at www.jacionline.org) in the ear. Moreover, even if transferred to nonsensitized mice, M2 macrophages modestly increased ear thickness and the numbers of Ly-6G⁺ cells in the skin (see Fig E6 in this article's Online Repository at www.jacionline.org). Taken together, these results suggest that M2 macrophages could induce the accumulation of inflammatory cells into the skin in CHS.

To address the mechanisms underlying M2 macrophage-mediated exacerbation of CHS, we analyzed the transcriptome of MR⁺ F4/80⁺ cells and MR⁻ F4/80⁺ cells that were isolated from the skin in DNFB-induced CHS. RNA-sequencing analysis identified 189 genes that are more than 4-fold as high in MR⁺ F4/80⁺ cells than in MR⁻ F4/80⁺ cells (see Table E1 in this article's Online Repository at www.jacionline.org). Among these 189 genes, genes expressed at high levels (FPKM > 300) in MR⁺ F4/80⁺ cells are shown in Fig 1, C. When these 189 genes were classified according to the categories of Gene Ontology (GO) (see Fig E7 in this article's Online Repository at www.jacionline.org), 11 genes met the following 2 conditions: "extracellular region" of GO cellular component and "cytokine/

chemokine" or "proteolytic enzyme" of GO molecular function. Heat map analysis of these 11 genes is shown in Fig 1, D. Because of the expression profiles available, we focused on matrix metalloproteinase 12 (MMP12) among the 11 genes as a candidate gene that may be involved in M2 macrophage-mediated exacerbation of CHS. We confirmed that MMP12 mRNA was highly expressed in MR⁺ F4/80⁺ cells than in MR⁻ F4/80⁺ cells by using quantitative PCR analysis (Fig 1, E) and was highly expressed in BM-derived M2 macrophages than in BM-derived M1 macrophages (see Fig E8, A, in this article's Online Repository at www.jacionline.org). Furthermore, IL-4 induced MMP12 expression in STAT6-dependent mechanisms (Fig E8, B) and STAT6 directly bound to the promoter of MMP12⁴ (Fig E8, C). In contrast, IFN- γ did not induce the expression of MMP12 (Fig E8, D). Taken together, these results indicate that MMP12 is highly and preferentially expressed in M2 macrophages in the skin lesion of DNFB-induced CHS in an IL-4-Stat6-dependent manner.

To determine the roles of MMP12 in CHS, we next examined DNFB-induced CHS in MMP12-deficient (MMP12^{-/-}) mice. Importantly, MMP12^{-/-} mice showed reduced ear thickness (Fig 2, A), mild inflammatory change (Fig 2, B), and decreased numbers of various inflammatory cells in the skin (Fig 2, C; see Fig E9 in this article's Online Repository at www.jacionline.org) as compared with littermate wild-type (WT) mice, indicating that MMP12 plays crucial roles in the development of DNFB-induced CHS. Moreover, cell transfer experiments of BM-derived M2 macrophages of WT mice or MMP12^{-/-} mice into DNFB-challenged MMP12^{-/-} mice revealed that the intradermal injection of M2 macrophages of WT mice but not of MMP12^{-/-} mice reconstituted ear thickness (Fig 2, D) and the numbers of Ly-6G⁺ cells in the sites of CHS (Fig 2, E) in MMP12^{-/-} mice to the levels of DNFB-challenged WT mice. The intradermal injection of recombinant MMP12 also reconstituted ear thickness (see Fig E10, A, in this article's Online Repository at www.jacionline.org) and the numbers of Ly-6G⁺ cells in the sites of CHS (Fig E10, B) in MMP12^{-/-} mice. In contrast, the intradermal injection of M1 macrophages of WT mice and MMP12^{-/-} mice only modestly increased ear thickness (Fig 2, D) and the numbers of Ly-6G⁺ cells (Fig 2, E) in the sites of CHS in MMP12^{-/-} mice. Taken together, these results suggest that M2 macrophage-derived MMP12 exacerbates CHS.

To assess the mechanism of MMP12-mediated exacerbation of CHS, we next examined the effect of intradermal injection of recombinant MMP12 into nonsensitized WT mice and found that the injection of MMP12 but not of heat-inactivated MMP12 increased ear thickness (see Fig E11, A, in this article's Online Repository at www.jacionline.org), caused skin inflammation (Fig E11, B), and induced the expression of chemokines capable of inducing the recruitment of lymphocytes, neutrophils, and macrophages (Fig E11, C). These results suggest that the enzymatic activity of MMP12 is required for the induction of skin inflammation and that MMP12 induces skin inflammation presumably through the induction of chemokine expression.

Finally, we examined the effect of a selective MMP12 inhibitor MMP408⁵ on DNFB-induced CHS and found that

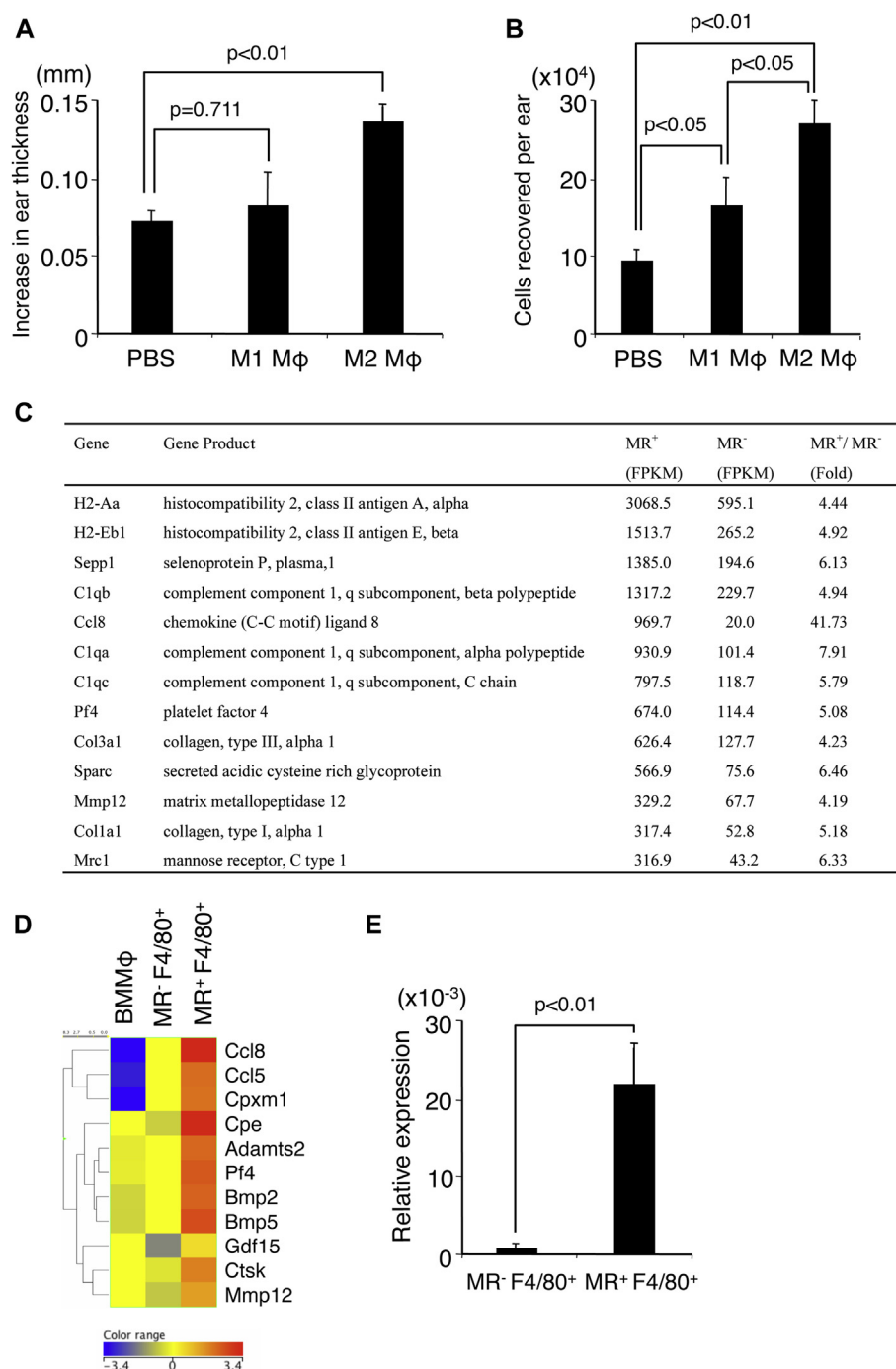


FIG 1. M2 macrophages express MMP12 and exacerbate DNFB-induced CHS. **A** and **B**, The effect of transplantation of BM-derived M1 and M2 macrophages on CHS was assessed by the increase in ear thickness (Fig 1, **A**) and the number of Ly-6G⁺ cells in the ear (Fig 1, **B**) as described in the [Methods](http://www.jacionline.org) section in this article's Online Repository at www.jacionline.org (n = 5). Shown are representative data of 3 independent experiments. **C-E**, Total RNA of MR⁻ F4/80⁺ cells and MR⁺ F4/80⁺ cells in the ear were isolated and subjected to RNA-sequencing analysis and quantitative PCR analysis. **C**, The list of top-ranked genes highly expressed in MR⁺ F4/80⁺ cells. **D**, The heat map analysis of 11 highly expressed genes that met the following 2 conditions, "extracellular region" and either "cytokine/chemokine" or "proteolytic enzyme," in MR⁺ F4/80⁺ cells. **E**, Relative expression of MMP12 by quantitative PCR (n = 4).

topical application of MMP408 to the ear significantly suppressed ear thickness (see Fig E12, **A**, in this article's Online Repository at www.jacionline.org), inflammatory responses (Fig E12, **B**), and the recruitment of inflammatory cells in the

ear (Fig E12, **C**). In contrast, WAY170523, an inhibitor for MMP13, did not suppress DNFB-induced CHS (see Fig E13 in this article's Online Repository at www.jacionline.org). These results indicate that the inhibition of MMP12 activity at the

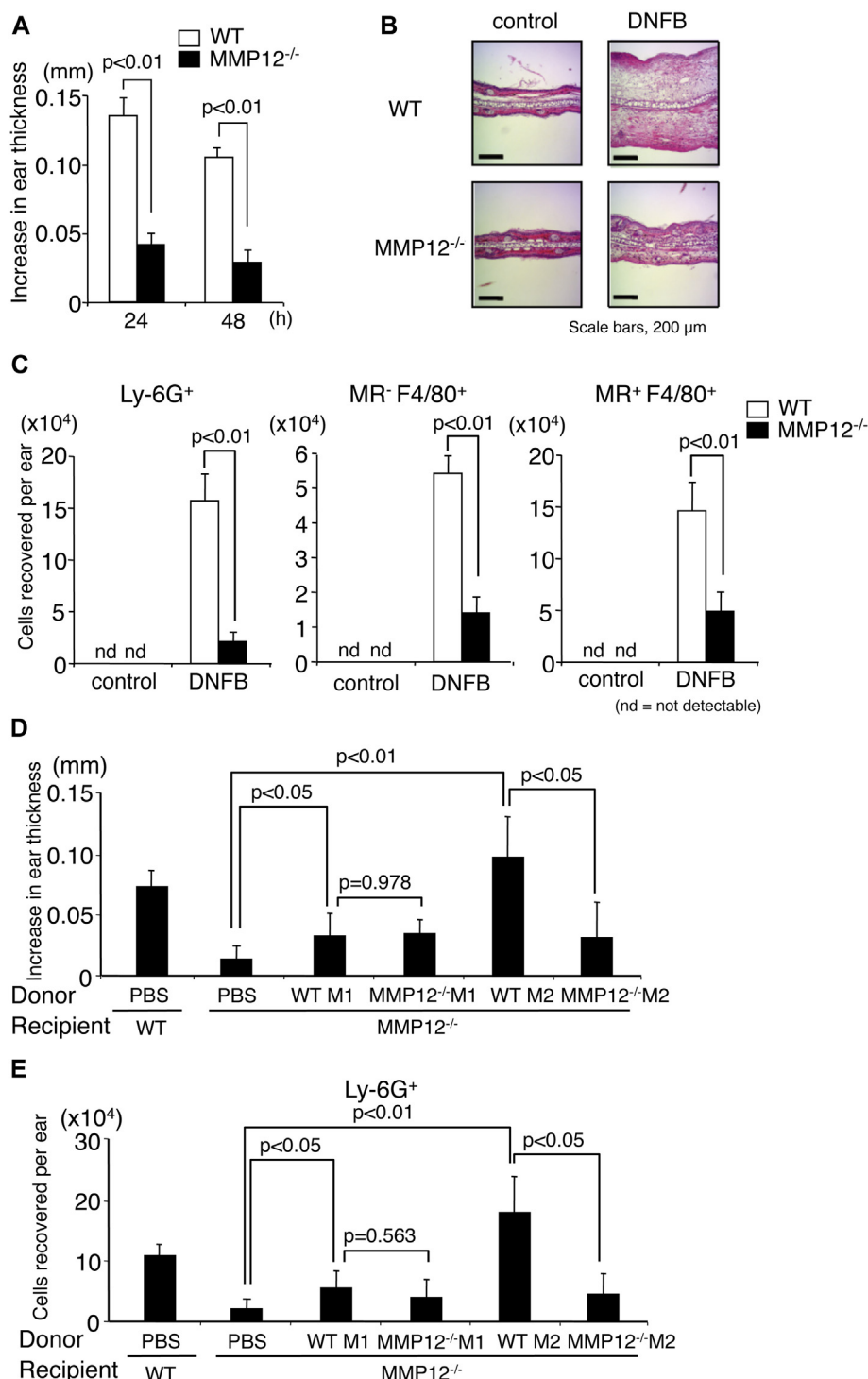


FIG 2. M2 macrophage-derived MMP12 exacerbates CHS. **A-C**, CHS in MMP12^{-/-} mice was assessed by the increase in ear thickness (Fig 2, A), histological analysis (Fig 2, B), and the numbers of inflammatory cells in the ear (Fig 2, C) as described in the [Methods](#) section in this article's Online Repository at www.jacionline.org (n = 5). **D** and **E**, BM-derived M1 or M2 macrophages of MMP12^{-/-} mice or WT mice were injected into the ear at 4 hours after the challenge. Ear swelling (Fig 2, D) and the number of Ly-6G⁺ cells (Fig 2, E) were examined at 48 hours after the challenge. Shown are representative data of 3 independent experiments.

site of CHS could be applicable for the treatment of contact dermatitis.

In conclusion, we show here that MR⁺ M2 macrophages play important roles in the development of CHS by producing

MMP12. Although further studies are needed, our data provide new insights into the pathogenesis of contact dermatitis and raise the possibility that MMP12 inhibitors have therapeutic potential against contact dermatitis.

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Online Repository

MMP-12 is produced by M2 macrophages and plays important roles in the development of contact hypersensitivity

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Online Repository Methods

Mice

STAT6^{-/-} mice¹ were backcrossed onto C57BL/6 mice (Japan SLC Inc., Shizuoka, Japan) over ten generations. MMP12^{-/-} mice² on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed in microisolator cages under specific pathogen-free conditions and all experiments were performed according to the guidelines of Chiba University.

DNFB-induced CHS

Mice were sensitized on the shaved abdomen with 30 µl of 0.5% 2, 4-dinitrofluorobenzene (DNFB, Sigma-Aldrich, St Louis, MO) in acetone. Five days later, mice were painted with 0.2% DNFB in acetone to the right ear (10 µl to each side) and acetone alone (as a negative control) to the left ear. Ear swelling was quantified by three consecutive measurements of ear thickness using calipers at 24 h and 48 h after the challenge.

Isolation of leukocytes from ear skin

Ear skin was cut into small pieces and incubated in RPMI 1640 medium containing Liberase TL (0.5 mg/ml, Roche, Basel, Switzerland) for 1 h at 37°C. Single cell suspensions were separated from undigested tissue by passing through a 40 µm nylon cell strainer.³

FACS analysis

Cells were stained and analyzed on the FACSCalibur (Becton Dickinson, San Jose, CA) using CELLQuest software. The following antibodies were used: anti-F4/80 (BM8, BioLegend, San Diego, CA), anti-MR (MR5D3, BioLegend), anti-MerTK (R&D Systems, Minneapolis, MN), anti-CCR2 (475301, R&D Systems), anti-CD11b (M1/70, eBioscience, San Diego, CA), anti-FcγRI (CD64) (X54-5/7.1, BioLegend), anti-dectin-1 (RH1, BioLegend),

anti-scavenger receptor class A (SR-A) (Lifespan Biosciences, Seattle, WA), anti-CD4 (RM4-5, BioLegend), anti-CD8 α (53-6-7, BD Biosciences, San Jose, CA), **anti-CD11c (N418, eBioscience)**, **anti-Siglec-F (E50-2440, BD Biosciences)**, and anti-Ly-6G (1A8, BioLegend). Before staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2, BioLegend). Negative controls consisted of isotype-matched directly conjugated, nonspecific antibodies (BD Biosciences).

FACS sorting

MR⁻ F4/80⁺ cells and MR⁺ F4/80⁺ cells were isolated by FACS Aria cell sorter (Becton Dickinson). The resultant cells were at least >90% pure for each cell population by FACS analysis.

Bone marrow (BM)-derived macrophages

To prepare bone marrow (BM)-derived macrophages, BM cells were cultured in DMEM supplemented with 10% FBS in the presence of M-CSF (10 ng/ml, R&D systems) for 5 days.⁴ Cells were then cultured for 2 days with IFN- γ (10 ng/ml, R&D systems) for M1 induction or with IL-4 (10 ng/ml, R&D systems) for M2 induction.

RNA-sequencing analysis

Total RNAs were prepared from MR⁻ F4/80⁺ cells (**1 x 10⁶ cells**) and MR⁺ F4/80⁺ cells (**1 x 10⁶ cells**) isolated from DNFB-exposed skin and BM-derived macrophages using a PureLink RNA mini kit (Invitrogen, Carlsbad, CA). RNA-sequencing libraries were prepared using a SureSelect Strand Specific RNA Library Preparation kit (Agilent technologies, Santa Clara, CA). Sequencing was performed on an Illumina HiSeq 1500 using a TruSeq Rapid SBS kit (Illumina, San Diego, CA) in a 50-base single-end mode. mRNA profiles of respective samples were calculated from approximately 3.5 x 10⁷ reads successfully mapped on the mouse genome (**GRCm38/mm10, using TopHot v1.3.2;**

<http://ccb.jhu.edu/software/tophat/index.shtml>) with a Cufflinks software (v2.0.2, <http://cufflinks.cbc.umd.edu/>). mRNA levels were expressed as FPKM (fragments per kilobase of exon model per million mapped fragments).

Data integration of RNA-sequencing analysis

Log-transformed FPKM values were normalized and used for clustering analysis with GeneSpring software (ver. 12.6) (Agilent technologies).

Selection of highly and preferentially expressed genes in MR⁺ F4/80⁺ cells in CHS

Genes more than 4-fold as high in MR⁺ F4/80⁺ cells as those in MR⁻ F4/80⁺ cells (189 genes) were classified according to categories of Gene ontology (GO) cellular component. Among 189 genes, 11 genes that meet following two conditions, “extracellular region” on GO cellular component and “cytokine/chemokine” or “proteolytic enzyme” on GO molecular function, were subjected to heat map analysis. Genes mainly expressed in erythrocytes were excluded.

Quantitative real-time PCR analysis

Total RNA was prepared, and reverse transcription was performed as described previously.⁵ The expression levels of MMP12, MR, CXCL1, CXCL2, CCL2, CCL3, Arg1, Fizz1, CD36, TNF- α , and iNOS were determined by quantitative real-time PCR (qPCR) with a standard protocol on an ABI PRISM7300 instrument (Applied Biosystems, Foster City, CA) using a SYBR green reagent (Power SYBR Green PCR Master Mix, Applied Bioscience). The levels of each expression were normalized to the levels of β -actin.

Sequences of PCR primers:

MMP-12:

forward primer: GAG TCC AGC CAC CAA CAT TAC,

reverse primer: GCG AAG TGG GTC AAA GAC AG.

CXCL1:

forward primer: CTG GGA TTC ACC TCA AGA ACA TC,

reverse primer: CAG GGT CAA GGC AAG CCT C.

CXCL2:

forward primer: CCA ACC ACC AGG CTA CAG G,

reverse primer: GCG TCA CAC TCA AGC TCT G.

CCL2:

forward primer: TTA AAA ACC TGG ATC GGA ACC AA,

reverse primer: GCA TTA GCT TCA GAT TTA CGG GT.

CCL3:

forward primer: TTC TCT GTA CCA TGA CAC TCT GC,

reverse primer: CGT GGA ATC TTC CGG CTG TAG.

Arg1:

forward primer: CTC CAA GCC AAA GTC CTT AGA G,

reverse primer: AGG AGC TGT CAT TAG GGA CAT C.

Fizz1:

forward primer CCA ATC CAG CTA ACT ATC CCT CC,

reverse primer ACC CAG TAG CAG TCA TCC CA.

CD36:

forward primer : AGA TGA CGT GGC AAA GAA CAG,

reverse primer: CCT TGG CTA GAT AAC GAA CTC TG.

TNF- α :

forward primer: CCC TCA CAC TCA GAT CAT CTT CT,

reverse primer: GCT ACG ACG TGG GCT ACA G.

iNOS:

forward primer: GTT CTC AGC CCA ACA ATA CAA GA,

reverse primer: GTG GAC GGG TCG ATG TCA C.

β -actin:

forward primer: GCT CTG GCT CCT AGC ACC AT,

reverse primer: GCC ACC GAT CCA CAC AGA GT.

Chromatin immunoprecipitation (ChIP)-qPCR assays

ChIP-qPCR assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). In brief, after cross-linking with 1% formaldehyde for 10 min at 37°C, cells were lysed in SDS buffer (50 mM Tris-Cl, PH 8.1, 10 mM EDTA, 1 % SDS, and protease inhibitors). Lysates were sonicated using Bioruptor (Cosmo Bio Co., Tokyo, Japan) and diluted with ChIP dilution buffer (20 mM Tris-Cl, PH 8.1, 1 mM EDTA, 150 mM NaCl, and 0.3 % Triton X-100). Lysates were incubated with anti-STAT6 antibody (D3H4, Cell Signaling Technology, Denver, MA) or control rabbit IgG at 4°C overnight and then added with protein A/G agarose for 2 h at 4°C. After reverse formaldehyde cross-linking, DNA was recovered and DNA fragments were amplified by PCR with specific primers (mouse MMP12 promoter: forward, 5'- TGTAGAAACCAGATGACAACTTCCA -3', reverse, 5'- GCTGACGAGCTGAGTTTGATCTC-3') with a SYBR Green reagent. Data were expressed as percent input for each ChIP fraction.

The effect of intradermal injection of M1 and M2 macrophages on CHS

BM-derived M1 or M2 macrophages (5×10^5 cells in 20 μ l of PBS) from MMP12^{-/-} mice or littermate wild type (WT) mice were injected intradermally into the ear of DNFB-sensitized MMP12^{-/-} mice or WT mice at 4 h after the challenge with DNFB. As a control, PBS was injected to the ear of DNFB-sensitized MMP12^{-/-} mice. The degree of CHS was estimated by quantification of increase in the ear thickness and of recruitment of inflammatory cells into the ear at 48 h after the challenge with DNFB.

The effects of MMP inhibitors on CHS

A MMP12 inhibitor, MMP408 (Merck KGaA, Darmstadt, Germany) and a MMP13 inhibitor, WAY170523 (R&D systems), were dissolved in white petrolatum (Maruishi Pharmaceutical Co., Osaka, Japan). Mice were sensitized with DNFB, and five days later, mice were challenged with DNFB on the ear. MMP408 (0.5 %), WAY170523 (0.5 %), or vehicle was painted on the ear at 10 min, 24 h, and 48 h after the challenge.

The effects of intradermal injection of recombinant murine MMP12

Recombinant murine MMP12 (R&D systems) was activated by *p*-aminophenylmercuric acetate (Sigma-Aldrich) as described elsewhere.⁶ The enzymatic activity of activated MMP12 was evaluated by using the fluorogenic peptide substrate Mca-KPLGL-Dpa-AR-NH₂ (R&D systems) according to the manufacturer's instructions. The enzymatic activity of activated MMP12 was >100 pmol/min/μg, whereas that of heat-inactivated MMP12 was < 5 pmol/min/μg. Endotoxin levels of the solution were < 0.1 pg/mL (<0.001 EU/mL) by Limulus test.⁷ Activated MMP12 (2 μg/ml [20 μl]), heat-inactivated MMP12 (2 μg/ml [20 μl]), or vehicle was injected intradermally into the ear of non-sensitized C57BL/6 mice.

Data Analysis

Data are summarized as means ± SD. The statistical analysis of the results was performed by unpaired t-test. P values <0.05 were considered significant.

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Online Repository Figure Legends

Figure E1. M2 macrophages are increased in the inflammatory sites of DNFB-induced CHS.

(A-C) C57BL/6 mice were sensitized with DNFB and 5 days later, mice were challenged with DNFB on the ear. (A) The numbers of MR⁻ F4/80⁺ cells (left panel) and MR⁺ F4/80⁺ cells (right panel) in the ear were examined at the indicated time points after DNFB challenge. Shown are means $\pm$ SD, n = 6 mice in each group. **p<0.01. (B) Two days after the challenge, leukocytes were isolated from the ear and the expression levels of MerTK and CCR2 on MR⁻ F4/80⁺ cells and MR⁺ F4/80⁺ cells were analyzed by FACS. As controls, the expression levels of MerTK and CCR2 on skin resident macrophages (CD11b⁺ CD64^{high} cells) and CD11b⁺ dendritic cells (CD11b⁺ CD64⁻ cells) which were isolated from normal skin were analyzed. Isotype-matched control staining for CCR2 (rat IgG2b) and MerTK (goat IgG) were also shown (lower panels). Shown are representative data of three independent experiments.

Figure E2. M2 macrophage-related molecules are preferentially expressed in MR⁺ F4/80⁺ cells in the inflammatory sites of DNFB-induced CHS.

C57BL/6 mice were sensitized and challenged with DNFB as described in Fig E1. (A) Two days after the challenge, the expression levels of dectin-1 and SR-A on MR⁻ F4/80⁺ cells and MR⁺ F4/80⁺ cells were analyzed by FACS. (B) Two days after the challenge, MR⁻ F4/80⁺ cells and MR⁺ F4/80⁺ cells were isolated from the ear by FACS sorting. Total RNAs of the cells were prepared and subjected to qPCR analysis for Arg1, Fizz1, CD36, TNF- α , and iNOS. Data are means $\pm$ SD of relative expression. n = 4. *p<0.05, **p<0.01.

Figure E3. The purity of BM-derived M1 and M2 macrophages.

BM-derived macrophages were prepared from BM cells of C57BL/6 mice. Cells were then cultured for 2 days under M1- or M2-skewing conditions as described in the Online Repository Methods. The purity of M1 macrophages (MR⁻ F4/80⁺ cells) and M2 macrophages (MR⁺ F4/80⁺ cells) was analyzed by flow cytometry (upper panels). The expression levels of MerTK and CCR2 on M1 and M2 macrophages were analyzed by flow cytometry (middle panels). Lower panels show isotype-matched control staining for CCR2 and MerTK. Shown are representative data of three independent experiments.

Figure E4. Gene expression profiles of BM-derived M1 and M2 macrophages.

BM-derived macrophages were cultured for 2 days under M1- or M2-skewing conditions as described in the Online Repository Methods. Total RNAs were prepared and subjected to qPCR analysis for Arg1, Fizz1, CD36, TNF- α , and iNOS. Data are means $\pm$ SD of relative expression. n = 4. *p<0.05, **p<0.01. nd = not detectable.

Figure E5. M2 macrophages exacerbate DNFB-induced CHS.

C57BL/6 mice were sensitized and challenged with DNFB as described in Fig E1. **BM-derived M1 and M2** macrophages were prepared as described in the **Online Repository Methods**. After washing, **M1 macrophages (5x10⁵ cells in 20 μ l of PBS)**, **M2 macrophages (5x10⁵ cells in 20 μ l of PBS)**, or PBS alone (as a control) were injected intradermally into the ear at 4 h after the challenge with DNFB. Forty-eight hours after the challenge with DNFB, the numbers of **CD4⁺ cells**, **CD8⁺ cells**, **Siglec-F⁺ CD11c⁻ cells (eosinophils)**, **MR⁻ F4/80⁺ cells**, and **MR⁺ F4/80⁺ cells** in the ear were examined. Shown are means $\pm$ SD, n = 5 mice in each group. *p<0.05, **p<0.01. **Shown are representative data of three independent experiments.**

Figure E6. M2 macrophages modestly induce skin inflammation even in non-sensitized mice.

(A-B) BM-derived M1 and M2 macrophages were prepared as described in the Online Repository Methods. After washing, M1 macrophages (5×10^5 cells in 20 μ l of PBS), M2 macrophages (5×10^5 cells in 20 μ l of PBS), or PBS alone (as a control) were injected intradermally into the ear of non-sensitized C57BL/6 mice. **(A)** Ear swelling was quantified at 48 h after the injection. Shown are means $\pm$ SD of the increase in the ear thickness from the baseline. $n = 5$ mice in each group. $**p < 0.01$. nd = not detectable. **(B)** Forty-eight hours after the injection, the numbers of Ly-6G⁺ cells, MR⁻ F4/80⁺ cells, and MR⁺ F4/80⁺ cells in the ear were examined. Shown are means $\pm$ SD. $n = 5$ mice in each group. $*p < 0.05$, $**p < 0.01$. nd = not detectable. Shown are representative data of two independent experiments.

Figure E7. RNA-sequencing analysis for M2 macrophages isolated from the inflammatory sites of DNFB-induced CHS.

C57BL/6 mice were sensitized and challenged with DNFB as described in Fig E1. Two days after the challenge, MR⁻ F4/80⁺ cells and MR⁺ F4/80⁺ cells were isolated from the ear by FACS sorting. Total RNAs of these cells and bone marrow-derived macrophages (as a control) were prepared and subjected to RNA-sequencing analysis and to qPCR analysis. Genes more than 4-fold as high in MR⁺ F4/80⁺ cells as those in MR⁻ F4/80⁺ cells (189 genes) were classified according to categories of Gene ontology (GO) cellular component.

Figure E8. IL-4 induces MMP12 expression in M2 macrophages in a STAT6-dependent manner.

(A) M1 and M2 macrophages were prepared from BM cells of C57BL/6 mice and the expression levels of MMP12 were determined by qPCR analysis. Data are means $\pm$ SD of relative expression. $n = 4$. $**p < 0.01$. **(B, C)** BM-derived macrophages were prepared from STAT6^{-/-} mice and WT mice and cultured in the presence or absence of IL-4 for 24 h. **(B)** The expression levels of MMP12 were evaluated by qPCR analysis. Data are means $\pm$ SD of relative expression. $n = 4$. $**p < 0.01$. **(C)** After cells were re-stimulated with or without IL-4

for 2 h, ChIP-qPCR assay for MMP12 promoter was performed with anti-STAT6 antibody or control rabbit IgG. Data are means $\pm$ SD of % input for each ChIP fraction. $n = 4$. $*p < 0.05$.

(D) BM-derived macrophages were prepared from C57BL/6 mice and cultured in the presence or absence of IFN- γ for 24 h. The expression levels of MMP12 were evaluated by qPCR analysis. Data are means $\pm$ SD of relative expression. $n = 4$.

Figure E9. The recruitment of T cells is attenuated in CHS in MMP12-deficient mice.

MMP12-deficient (MMP12^{-/-}) mice and littermate WT mice were sensitized and challenged with DNFB as described in Fig E1. Forty-eight hours after the challenge, the numbers of CD4⁺ cells and CD8⁺ cells in the ear were examined. Shown are means $\pm$ SD. $n = 5$.

$**p < 0.01$. nd = not detectable. Shown are representative data of three independent experiments.

Figure E10. Intradermal injection of MMP12 reconstitutes the reduced DNFB-induced CHS in MMP12-deficient mice.

(A, B) MMP12^{-/-} mice and littermate WT mice were sensitized and challenged with DNFB as described in Fig E1. Recombinant MMP12 or PBS (as negative control) was injected intradermally into the ear of MMP12^{-/-} mice at 4 h after the challenge with DNFB. Ear swelling **(A)** and the numbers of Ly-6G⁺ cells, MR⁻ F4/80⁺ cells, and MR⁺ F4/80⁺ cells in the ear **(B)** were examined at 48 h after the challenge with DNFB. Shown are means $\pm$ SD. $n = 5$ mice in each group. $**p < 0.01$. Shown are representative data of three independent experiments.

Figure E11. Intradermal injection of recombinant MMP12 causes skin inflammation.

(A-C) Recombinant MMP12, heat-inactivated MMP12, or vehicle was injected intradermally into the ear of non-sensitized C57BL/6 mice. **(A)** Ear swelling was quantified at 6 and 24 h after the injection. Shown are means $\pm$ SD of the increase in the ear thickness from the

baseline. $n = 5$ mice in each group. $*p < 0.05$, $**p < 0.01$. (B) Before and 6 and 24 h after the injection, histological analyses of the ear were performed. Representative photomicrographs with HE staining are shown. Scale bars, 200 μm . $n = 5$ mice in each group. (C) Before and 6 and 24 h after the injection, mRNA levels of CXCL1, CXCL2, CCL2, and CCL3 in the ear were determined by qPCR analysis. Data are means $\pm$ SD of relative expression. $n = 4$, each. * $**$ significantly different from the mean value at 0 h, $*p < 0.05$, $**p < 0.01$. nd = not detectable. Shown are representative data of three independent experiments.

Figure E12. Topical application of a selective MMP12 inhibitor suppresses CHS.

(A-C) C57BL/6 mice were sensitized and challenged with DNFB as described in Fig E1. Where indicated, MMP408, a selective MMP12 inhibitor, or vehicle was painted on the ear at 10 min, 24 h, and 48 h after the challenge with DNFB. (A) Ear swelling was quantified at 48 h and 72 h after the challenge. Shown are means $\pm$ SD of the increase in the ear thickness from the baseline. $n = 5$ mice in each group. $**p < 0.01$. (B) Seventy-two hours after the challenge, histological analyses of the ear were performed. Representative photomicrographs with HE staining are shown. Scale bars, 200 μm . $n = 5$. (C) Seventy-two hours after the challenge, the numbers of CD4⁺ cells, CD8⁺ cells, Ly-6G⁺ cells, MR⁻ F4/80⁺ cells, and MR⁺ F4/80⁺ cells in the ear were examined. Shown are means $\pm$ SD. $n = 5$. $**p < 0.01$. nd = not detectable. Shown are representative data of three independent experiments.

Figure E13. Effect of topical application of a selective MMP13 inhibitor on CHS.

(A-B) C57BL/6 mice were sensitized and challenged with DNFB as described in Fig E1. Where indicated, WAY170523, a selective MMP13 inhibitor, or vehicle was painted on the ear at 10 min, 24 h, and 48 h after the challenge with DNFB. (A) Ear swelling was quantified at 48 h and 72 h after the challenge. Shown are means $\pm$ SD of the increase in the ear thickness from the baseline. $n = 5$ mice in each group. ns = not significant. (B) Seventy-two hours after the challenge, the numbers of CD4⁺ cells, CD8⁺ cells, Ly-6G⁺ cells, MR⁻ F4/80⁺

cells, and MR⁺ F4/80⁺ cells in the ear were examined. Shown are means $\pm$ SD. n = 5. nd = not detectable. Shown are representative data of two independent experiments.

Online Repository Table E1

The list of genes that were more than 4-fold as high in MR⁺ F4/80⁺ cells as those in MR⁻

F4/80⁺ cells in DNFB-induced CHS

	MR ⁺ F4/80 ⁺ (FPKM)	MR ⁻ F4/80 ⁺ (FPKM)	BMDM (FPKM)	MR ⁺ F4/80 ⁺ / MR ⁻ F4/80 ⁺ (fold)
H2-Aa	3068.51	595.15	86.96	4.44
H2-Eb1	1513.73	265.22	42.88	4.92
Sepp1	1385.04	194.64	170.31	6.13
C1qb	1317.22	229.70	472.28	4.94
Ccl8	969.67	20.02	0.16	41.73
C1qa	930.89	101.38	137.23	7.91
C1qc	797.49	118.70	310.02	5.79
Pf4	673.96	114.35	144.43	5.08
Col3a1	626.35	127.65	11.00	4.23
Sparc	566.87	75.62	53.93	6.46
Hba-a1	564.30	24.31	0.21	20.01
Beta-s	559.78	18.93	0.09	25.48
Bgn	340.40	57.87	62.49	5.07
Hbb-b1	335.26	12.32	0.00	23.45
Mmp12	329.21	67.71	171.49	4.19
Col1a1	317.43	52.84	34.25	5.18
Mrc1	316.91	43.20	53.88	6.33
Cd300lh	245.27	13.10	53.99	16.14
Col1a2	239.98	40.79	29.70	5.07
Ms4a7	228.43	22.95	40.52	8.58
Pltp	215.14	15.18	25.26	12.21
Ccl5	156.52	32.68	6.19	4.13
Eln	149.65	4.549	0.04	28.35
Postn	99.66	8.532	14.74	10.07
Col8a1	95.76	4.63	4.01	17.82
Col6a2	93.27	13.77	10.78	5.84
Timp2	93.09	19.75	52.38	4.06
Ctgf	86.24	13.65	43.31	5.44
Col6a1	80.78	12.49	12.08	5.58
Cyt11	80.69	1.92	0.00	36.21
Gas6	80.13	10.80	77.05	6.39
Clu	78.16	6.21	3.62	10.85
Lum	76.87	14.40	0.07	4.60

Krt14	64.82	10.23	0.00	5.46
Clec10a	60.49	8.27	3.84	6.30
Cbr2	59.86	1.68	6.28	30.69
Myoc	55.82	6.17	0.01	7.80
Serpina3n	51.19	10.43	0.03	4.23
Scd1	50.80	2.10	12.96	20.85
Ctsk	50.63	10.13	19.43	4.31
Krt5	47.53	6.37	0.00	6.43
Folr2	45.62	2.32	134.45	16.96
Fcgrt	45.59	5.92	6.15	6.63
Col2a1	45.09	1.27	0.74	30.60
Mgl2	43.83	1.83	1.14	20.59
Chad	43.29	2.31	0.07	16.12
Smoc2	42.84	2.10	1.92	17.58
Fmod	42.47	4.04	1.17	9.05
Dpt	40.64	7.64	0.02	4.59
Fxyd3	39.51	1.29	0.00	26.41
Timp3	37.48	3.22	11.54	10.04
Lpl	34.78	5.37	1289.59	5.58
Cd163	34.36	1.24	2.40	23.79
Rcn3	33.71	7.06	8.48	4.11
Abi3bp	33.21	6.13	0.03	4.67
Col6a3	31.75	6.72	4.83	4.07
Cd72	31.65	3.95	175.77	6.90
Col5a1	31.20	6.10	4.65	4.41
Krt10	30.22	0.99	0.48	26.04
Penk	28.38	5.73	1.26	4.27
Comp	27.17	2.16	0.14	10.82
Lgals7	27.13	3.36	0.00	6.96
Lect1	27.09	0.62	0.00	23.34
Cpe	26.43	1.55	3.64	14.68
Enpp2	25.01	1.23	1.04	17.48
Hspb1	24.66	3.13	2.79	6.78
Ppic	24.57	4.95	9.23	4.27
Acan	23.46	0.82	0.00	20.22
Clec3b	23.39	2.45	0.10	8.24
Aspn	22.01	2.93	0.22	6.48
Prep	21.74	4.05	0.32	4.63
Hebp1	21.42	2.83	14.98	6.51
Npl	21.30	1.43	6.05	12.82
Scara3	21.25	3.01	0.78	6.08

Cbr1	21.11	4.40	25.39	4.14
Tmem37	20.78	3.31	7.24	5.40
Fads1	20.55	4.32	44.90	4.10
Fxyd2	19.71	1.78	4.44	9.52
Krt1	19.68	1.22	0.00	13.85
Sfrp2	19.12	3.42	2.08	4.81
Frmd4b	18.96	4.02	21.23	4.06
Mfap4	18.16	1.92	0.00	8.16
Fkbp9	18.13	3.62	6.76	4.31
Anxa8	17.85	3.67	0.02	4.19
Col12a1	17.84	3.52	4.66	4.37
Vcam1	17.83	3.17	5.73	4.85
Cilp	17.02	1.87	0.00	7.83
Col9a3	16.83	0.48	0.02	14.50
Gdf15	16.39	3.36	15.38	4.20
Gpr34	15.72	1.48	0.31	9.15
Cd4	15.63	0.32	1.26	13.47
Loxl4	15.51	0.65	0.45	13.36
Igf1	15.00	1.49	91.42	8.68
Adamts2	14.49	2.87	3.52	4.35
Ogn	14.35	2.74	0.93	4.52
Col16a1	14.34	1.90	1.19	6.50
Col11a2	14.00	0.78	0.22	12.06
Krt16	13.69	2.01	0.00	5.87
Nid2	13.61	2.90	3.71	4.05
Clec3a	13.56	0.17	0.00	11.69
Pcolce2	12.7	1.65	1.01	6.64
Nr1h3	12.65	2.58	6.78	4.22
Fxyd1	12.56	1.56	0.21	6.96
Col9a1	12.19	0.14	0.00	10.50
Maged2	11.94	2.55	10.04	4.03
Sox9	11.94	1.01	0.20	10.24
Dkk3	11.65	0.85	2.38	10.04
Scamp5	11.63	0.63	12.03	10.02
Dpep1	11.50	1.23	1.30	8.06
Serpine2	11.40	2.41	10.49	4.07
Tns1	11.38	1.62	4.90	6.04
Krtdap	11.36	1.67	0.00	5.85
Nfatc2	11.33	2.32	11.97	4.22
Gbgt1	11.31	1.84	0.75	5.28
Fcrls	11.28	0.35	19.16	9.72

Itm2a	10.99	1.52	0.89	6.25
Ecm2	10.11	0.48	0.04	8.71
Susd5	10.06	0.43	0.02	8.67
Sbsn	9.96	1.73	1.11	4.95
Col11a1	9.78	0.50	0.12	8.43
Crispld1	9.50	0.61	0.01	8.18
Leprel2	9.44	1.95	2.11	4.18
Arhgap19	9.35	0.96	14.22	8.06
Itga9	9.26	0.86	0.48	7.98
Fzd9	9.23	0.30	0.01	7.95
Col9a2	9.04	0.27	0.00	7.79
Ppp1r1b	8.99	0.33	0.00	7.75
Boc	8.94	1.31	0.23	5.86
Mfi2	8.91	0.32	0.00	7.68
Hpgds	8.79	1.19	71.51	6.39
Enpp1	8.70	1.87	6.57	4.01
Mfap2	8.42	0.57	0.26	7.25
Col27a1	8.37	0.60	0.23	7.21
Rpl26	8.26	1.48	15.72	4.79
Alas2	8.24	0.43	0.10	7.10
Hapln1	7.88	0.43	0.00	6.79
Trpv4	7.84	1.27	0.88	5.33
Col14a1	7.82	1.56	0.07	4.32
C1qtnf1	7.75	1.67	0.15	4.01
Sorbs3	7.73	0.96	1.29	6.66
Podn	7.66	1.45	0.05	4.54
Fgfr3	7.62	0.26	0.13	6.57
Cd109	7.59	1.57	7.10	4.16
Fbln1	7.51	1.16	0.47	5.60
Frzb	7.43	0.23	0.02	6.40
Synpo	7.37	1.58	0.70	4.01
Ephx1	7.33	1.46	9.92	4.32
Dnm1	7.25	0.89	0.23	6.25
Srpx2	7.18	1.48	3.94	4.19
Gprc5c	7.01	0.64	0.29	6.04
Spic	7.00	1.17	0.52	5.15
Slc25a10	6.91	1.18	14.19	5.03
Lhpp	6.82	1.20	11.42	4.88
Bmp5	6.79	1.01	0.47	5.78
Hba-a2	6.65	0.00	0.00	5.73
Slc13a3	6.59	1.17	1.10	4.85

Gdpd1	6.56	0.53	5.16	5.65
Aldh7a1	6.44	1.06	5.80	5.22
Phyhd1	6.31	0.54	2.28	5.44
Fgfr1	6.31	1.18	1.17	4.63
Has2	6.29	1.00	0.58	5.41
Cd209d	6.26	0.04	0.00	5.41
Gpc3	6.25	1.20	0.05	4.48
Spr2a2	6.15	0.82	0.00	5.31
Lrrc17	6.08	1.00	0.23	5.24
Ucma	6.04	0.78	0.00	5.21
Gpr162	5.99	0.18	0.54	5.16
S100b	5.95	0.13	0.05	5.13
Dbp	5.64	0.86	1.43	4.86
Omd	5.59	0.19	0.03	4.82
Srpx	5.59	1.16	0.10	4.15
Cd209a	5.55	0.25	0.92	4.78
Smoc1	5.54	0.51	1.46	4.77
Cspg4	5.52	0.67	3.68	4.76
Kank1	5.52	0.38	0.11	4.76
Meg3	5.44	1.11	0.03	4.24
Slc46a3	5.43	1.08	4.75	4.31
Cdon	5.41	0.99	0.44	4.66
Muc1	5.39	0.21	0.00	4.64
Sult1a1	5.34	0.39	0.32	4.60
Cthrc1	5.32	0.83	0.96	4.58
Bmp2	5.28	0.86	0.32	4.55
Npr2	5.25	0.73	0.41	4.52
Fap	5.08	1.00	0.61	4.37
Krt2	5.06	0.08	0.00	4.36
Itgb1	4.83	0.60	0.00	4.16
Dnase1l3	4.72	0.93	0.03	4.07
Ghr	4.71	0.70	1.63	4.06

Fig.E1

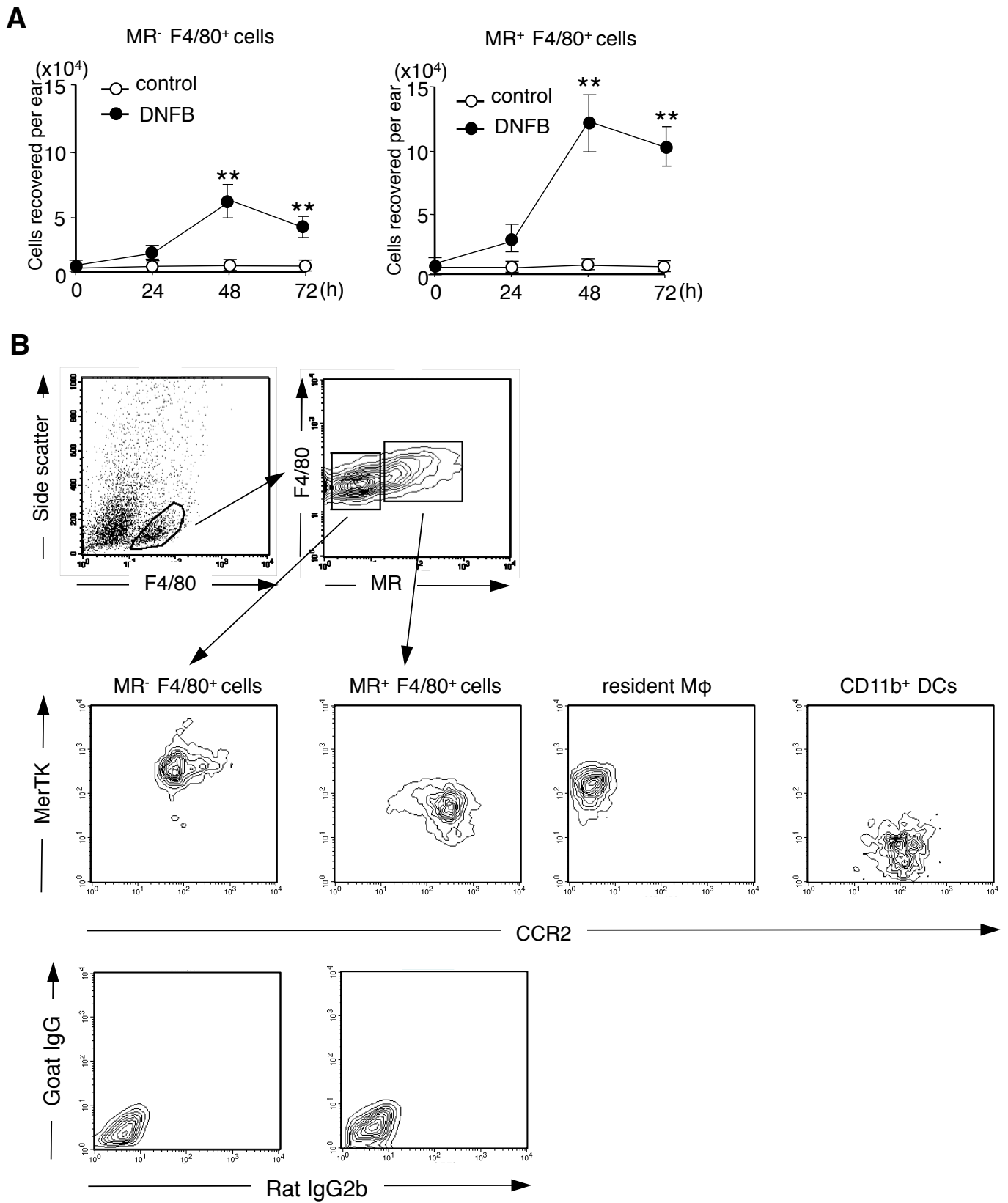
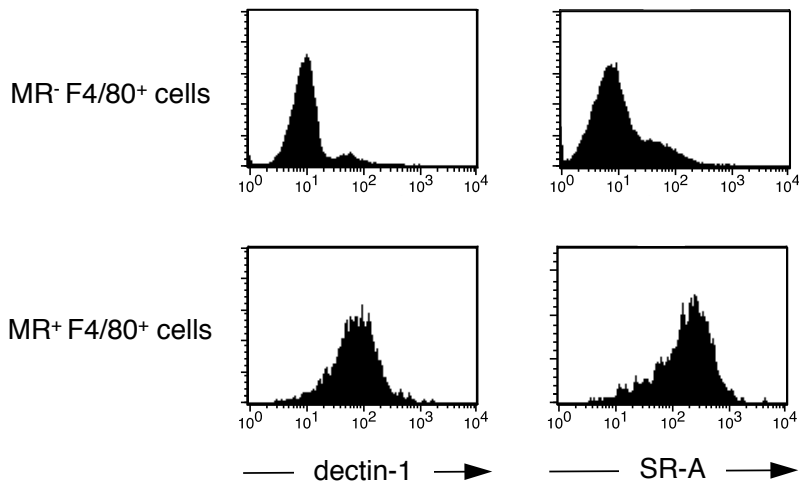


Fig.E2

A



B

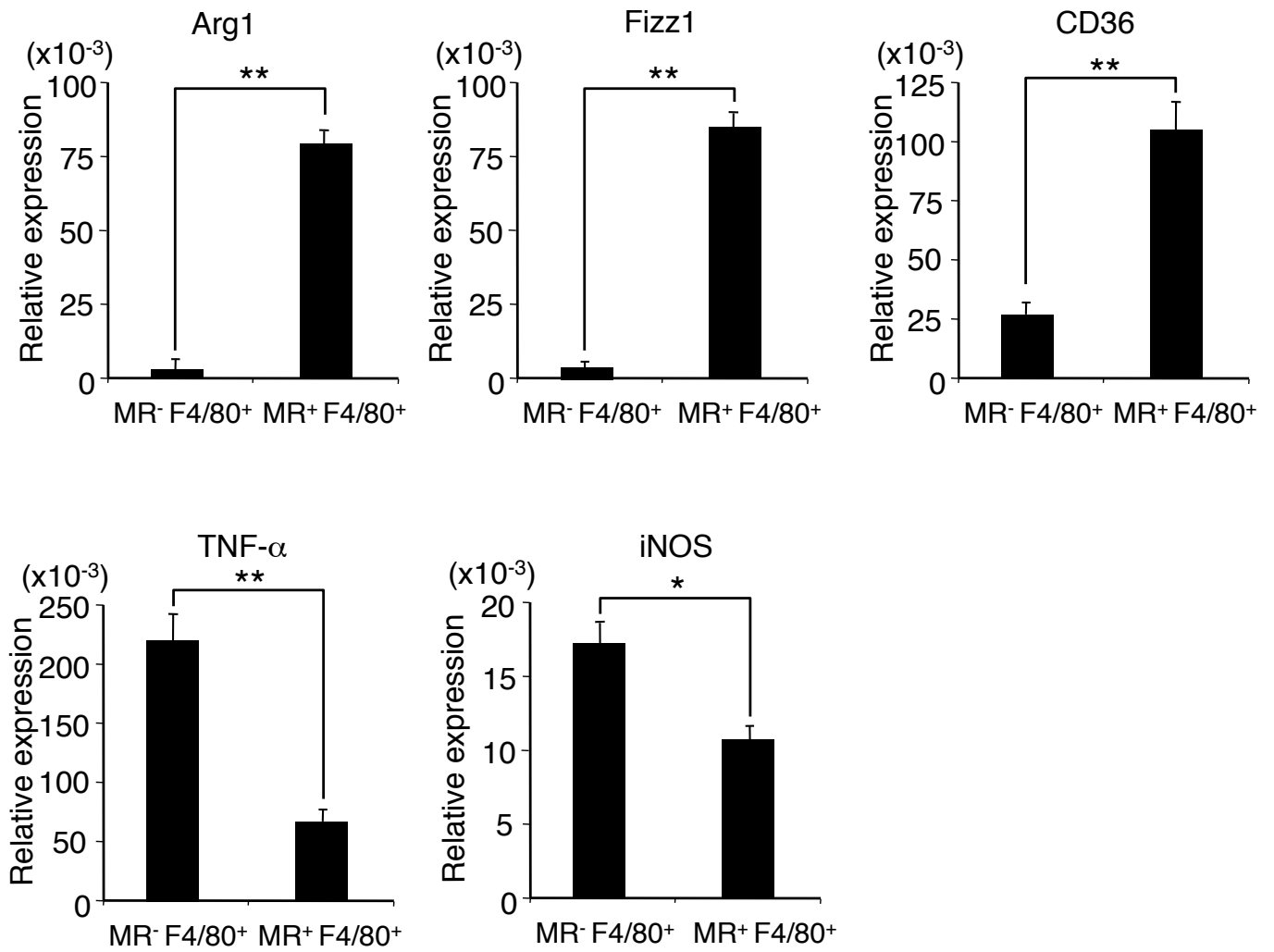


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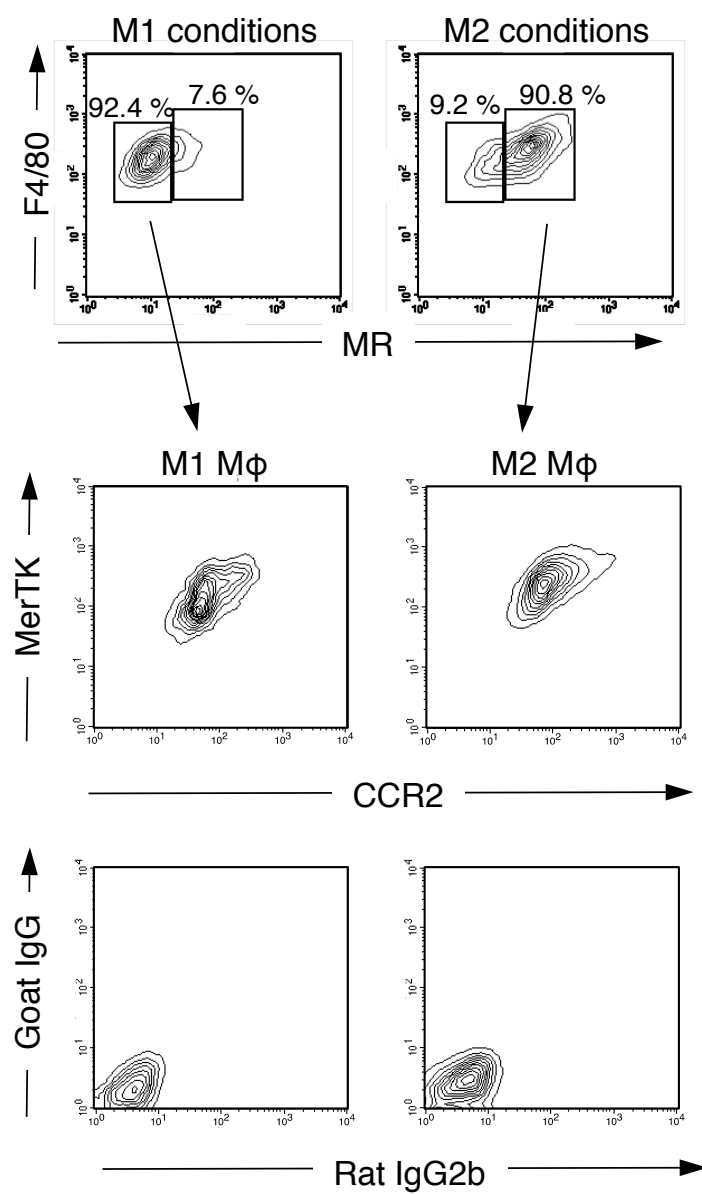


Fig.E4

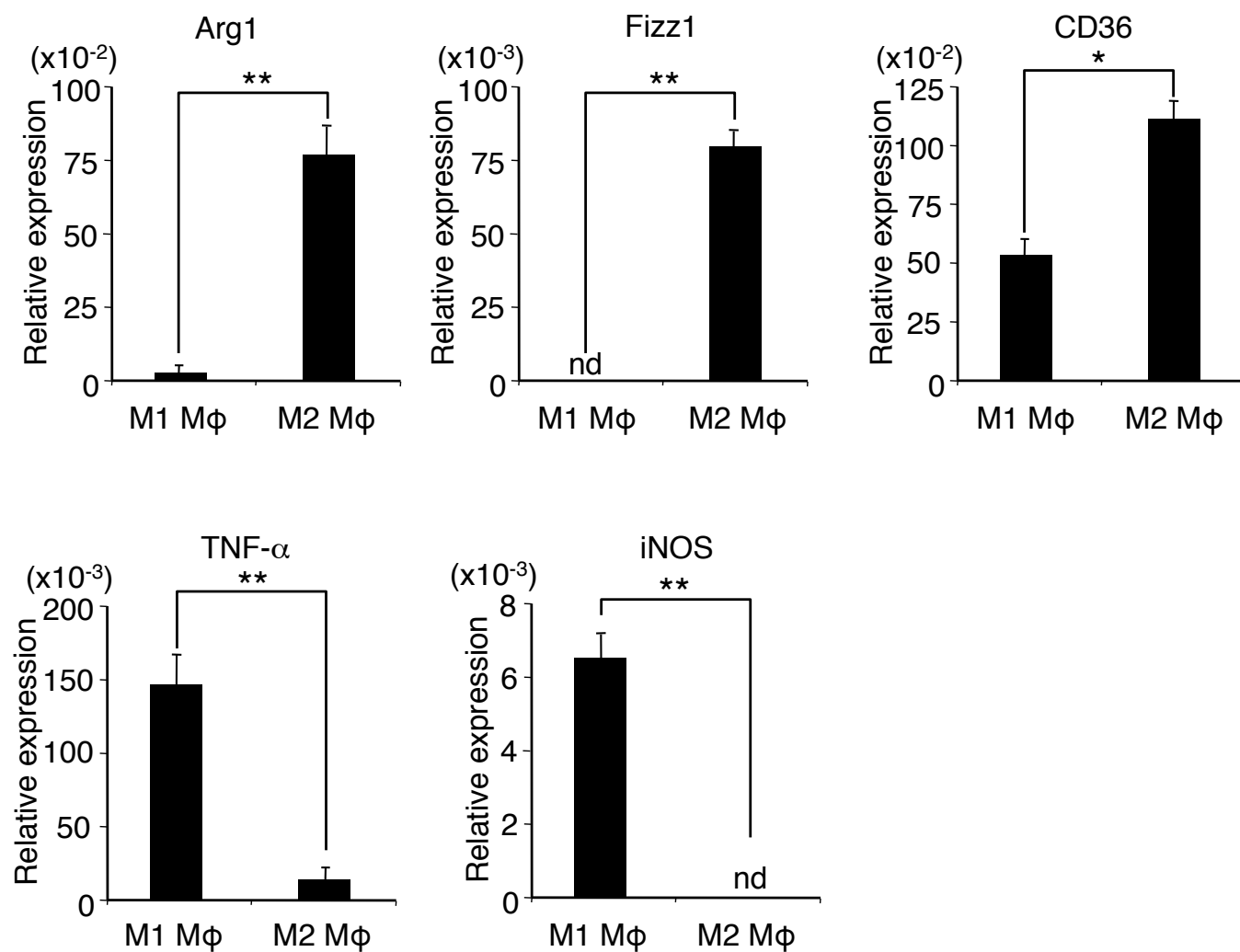
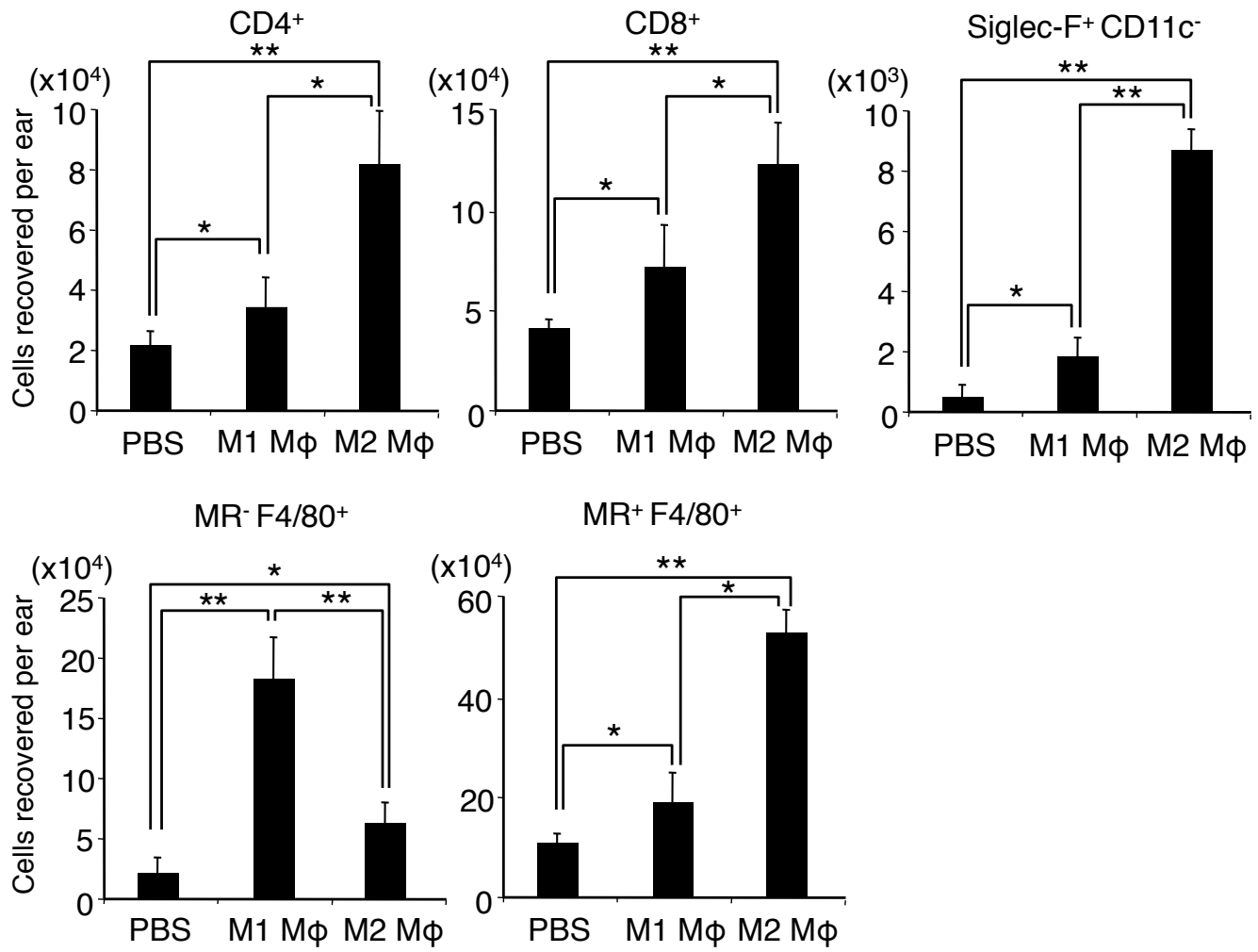
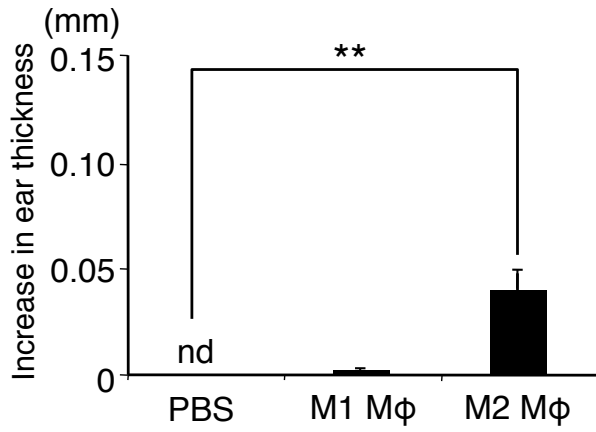
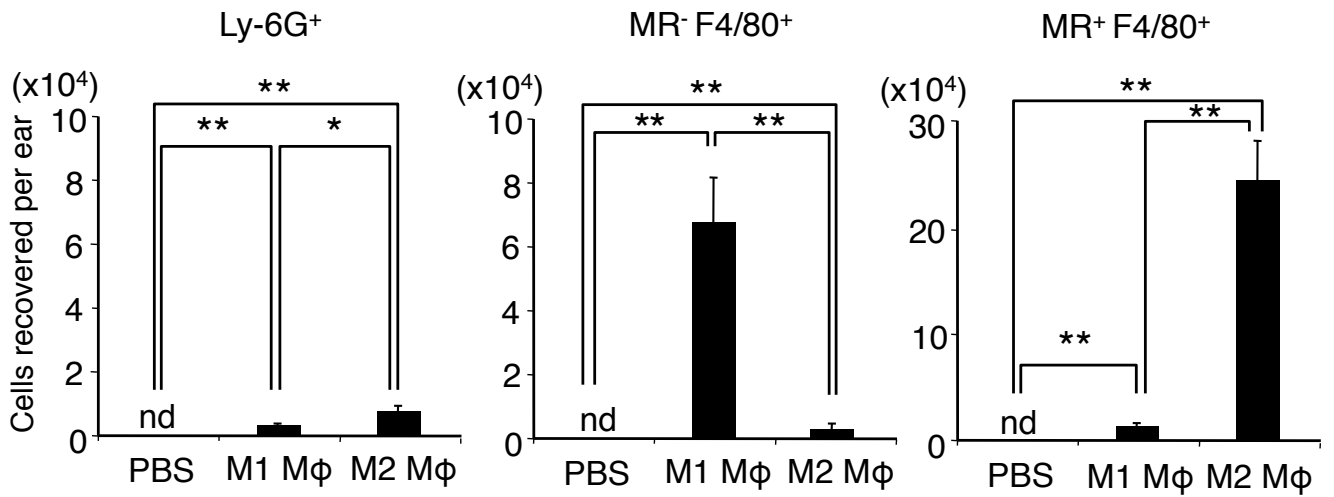
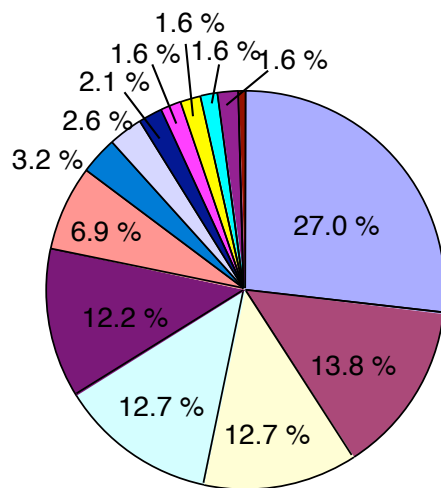


Fig.E5



A**B**

MR⁺ F4/80⁺ / MR⁻ F4/80⁺ > 4.00
189 genes



- extracellular region
- membrane
- extracellular matrix
- plasma membrane
- cytoplasm
- cellular component
- keratin filament
- nucleus
- cell
- mitochondrion
- intracellular
- extracellular space
- endoplasmic reticulum
- synapse

Fig.E8

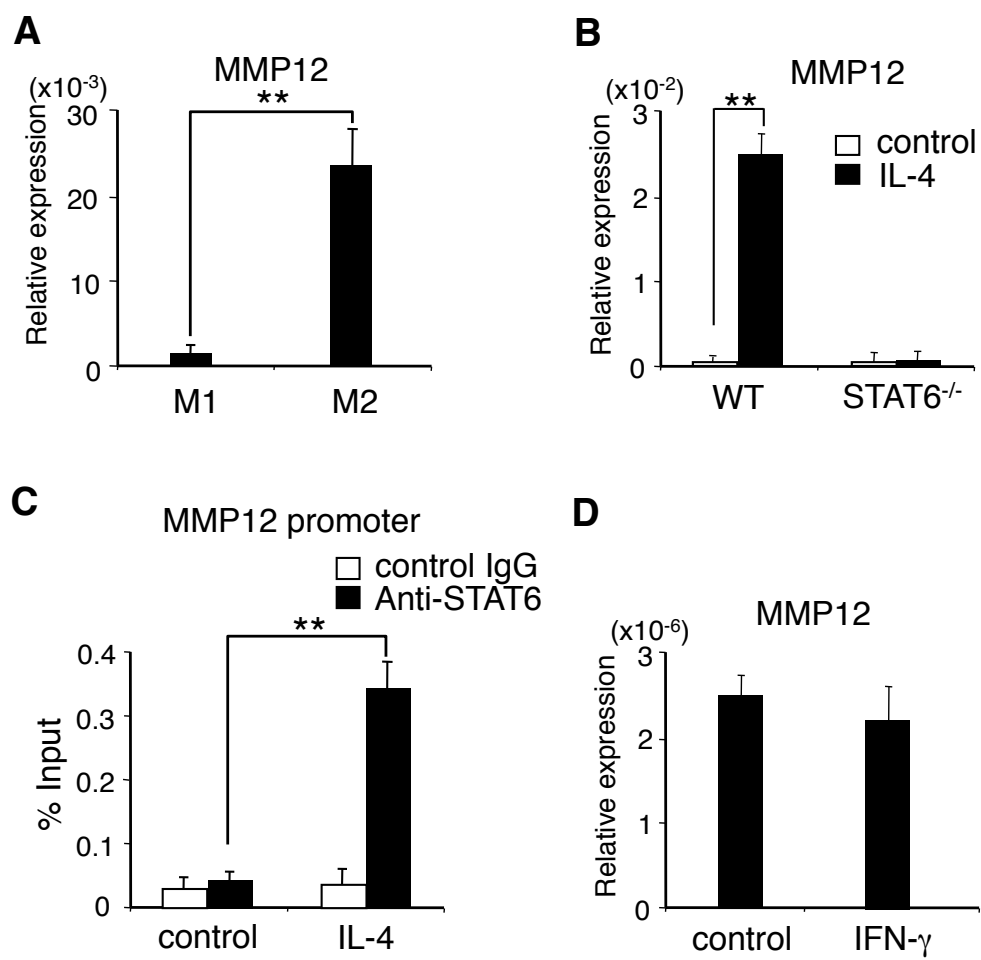


Fig.E9

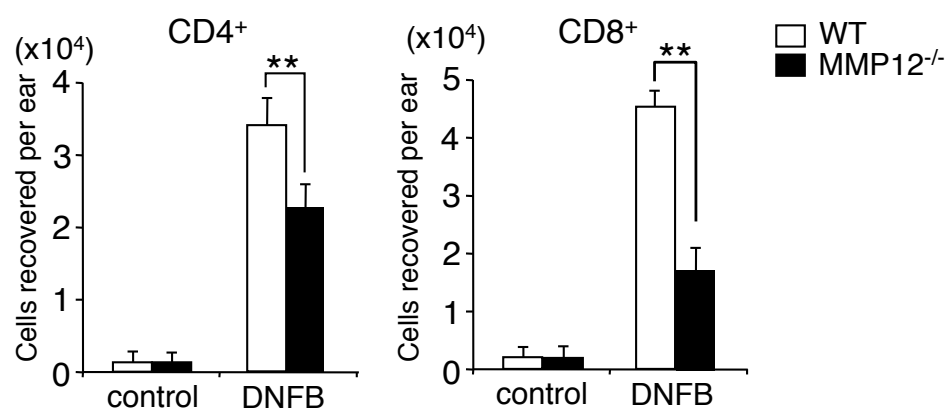
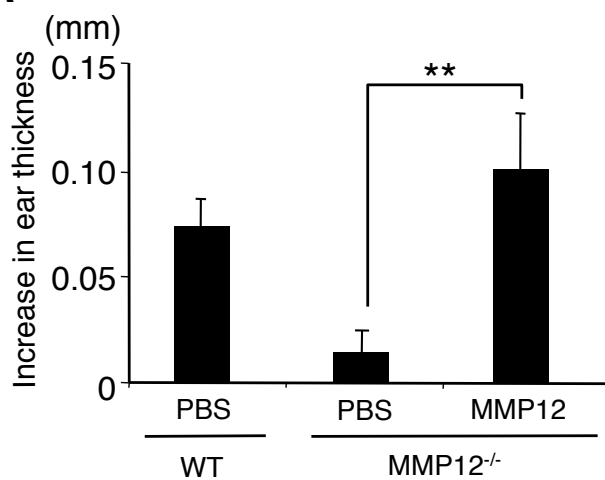
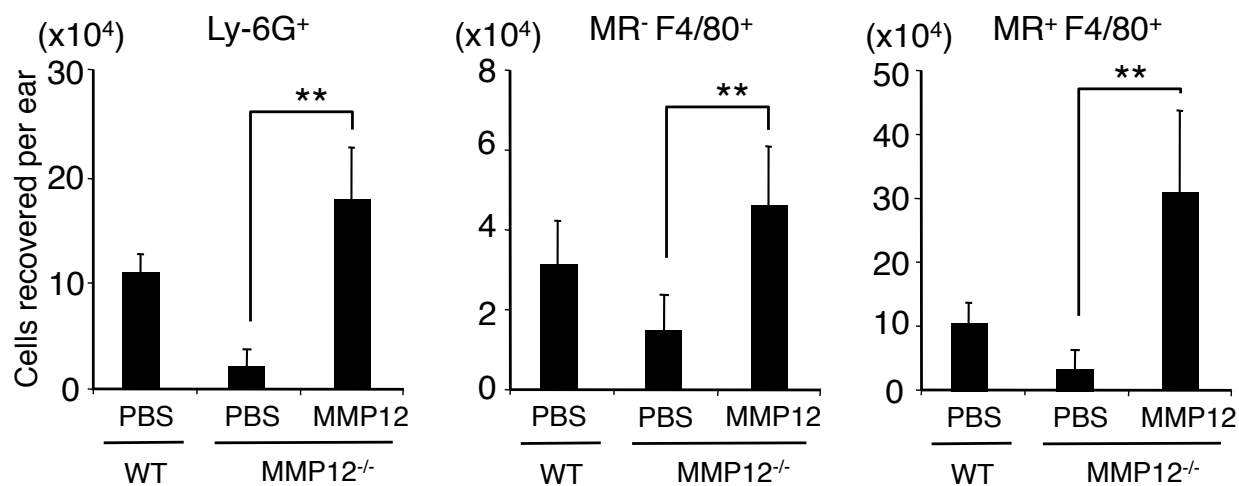


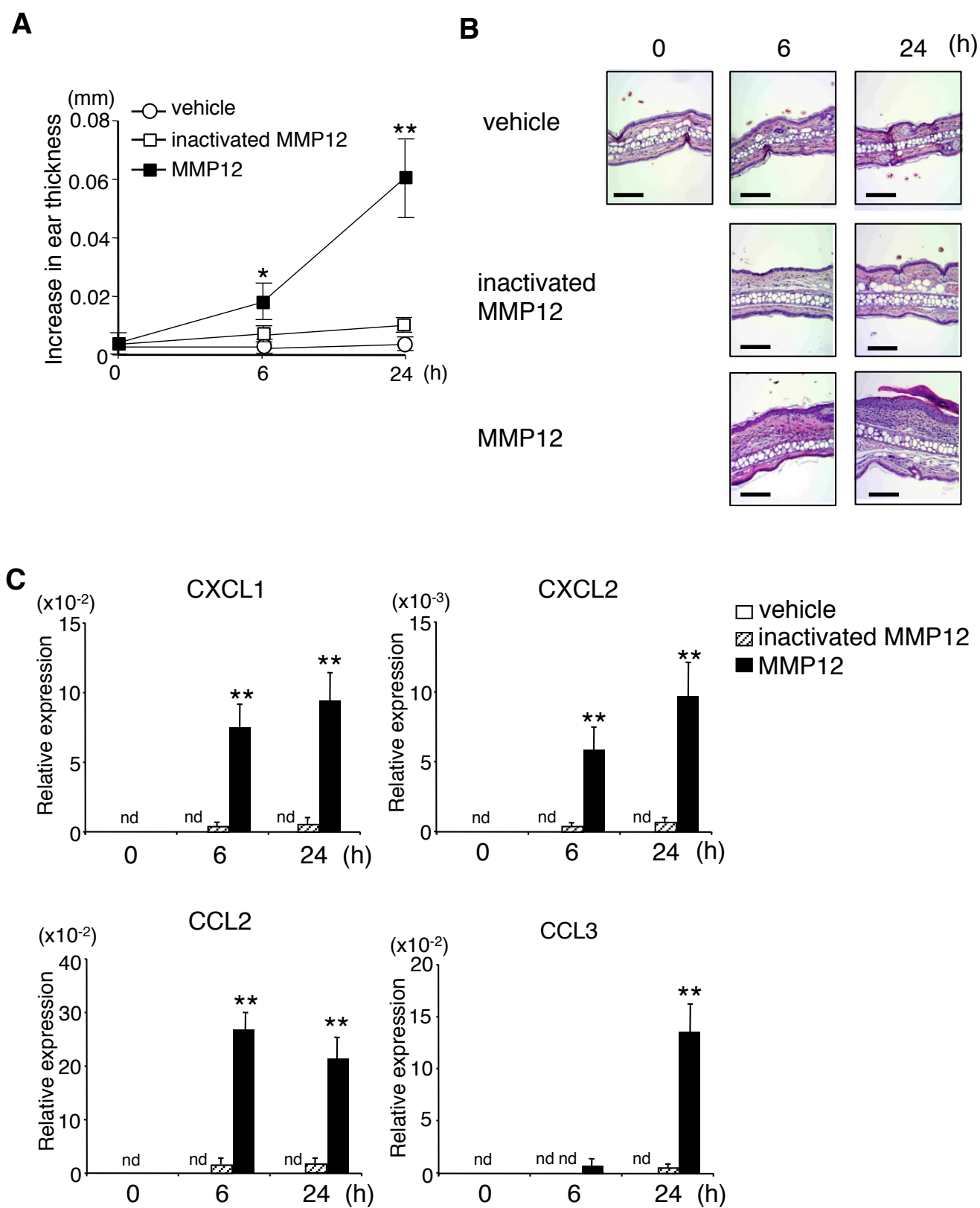
Fig.E10

A



B





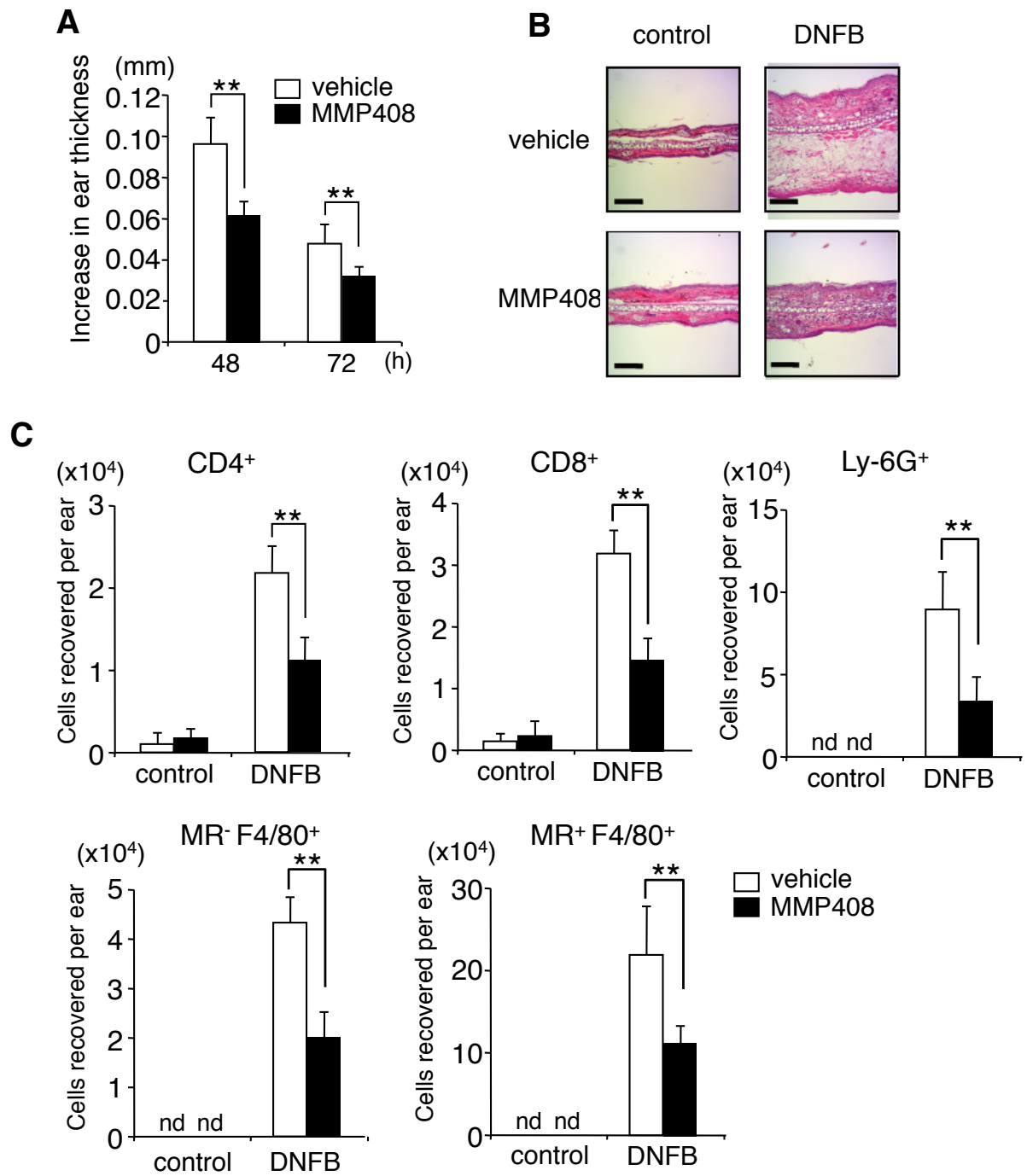
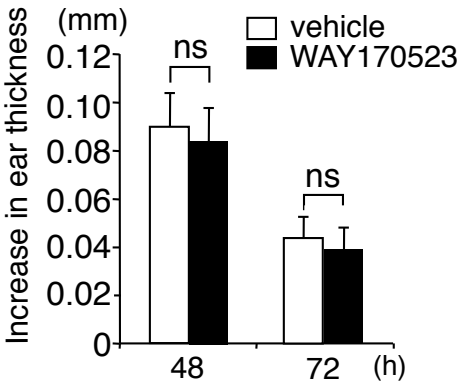


Fig.E13

A



B

