Modulation of Hepatic Granulomatous Responses by Transgene Expression of DAP12 or TREM-1-Ig Molecules

Hitoshi Nochi,*[†] Naoko Aoki,* Kensuke Oikawa,* Mitsuru Yanai,* Yumi Takiyama,* Yoshiaki Atsuta,* Hiroya Kobayashi,* Keisuke Sato,* Masatoshi Tateno,* Takeo Matsuno,[†] Makoto Katagiri,* Zhou Xing,[‡] and Shoji Kimura[§]

From the Departments of Pathology* and Orthopaedic Surgery[†] and the School of Nursing,[§] Asahikawa Medical College, Asahikawa, Japan; and the Department of Pathology and Molecular Medicine,[‡] McMaster University, Hamilton, Ontario, Canada

DAP12 (also known as KARAP) is a novel ITAM-bearing transmembrane adapter molecule that is expressed on the cell surface of natural killer cells, monocytes, dendritic cells, and macrophages. Several myeloid cell-specific DAP12-associating receptors, such as TREM receptor family, SIRP- β 1, and MDL-1 have been identified. The *in vivo* function of DAP12 and its associating molecules in inflammation has remained primarily unknown. To investigate DAP12 signaling during chronic inflammation, we constructed two adenoviral gene transfer vectors to express FLAG/DAP12 (Ad-FDAP12) and the extracellular domain of mouse TREM-1 and the Fc portion of human IgG1 (Ad-TREM-1 Ig), respectively, and observed their modulatory activities in a mouse model of hepatic granulomatous inflammation elicited by zymosan A. Mice were injected with zymosan A intravenously and 24 hours after zymosan A, they were injected with Ad-FDAP12 or Ad-TREM-1 Ig. Zymosan A-induced hepatic granuloma formation peaked at day 7 and markedly declined by day 10. Although adenoviral-mediated DAP12 gene transfer did not enhance granuloma formation by day 7, it sustained and enhanced granuloma formation beyond day 7. However, an anti-FLAG monoclonal antibody used to potentiate the signaling of adenoviral-derived DAP12, enhanced granuloma formation at day 7. In sharp contrast to the effect by Ad-FDAP12, transgene expression in the liver of soluble form of extracellular domain of TREM-1 as an antagonist of DAP12 signaling, remarkably inhibited zymosan A-induced granuloma formation at all time points examined. Our findings thus suggest that both DAP12 and TREM-1 are involved in the development of granulomatous responses in the liver. (*Am J Pathol* 2003, 162:1191–1201)

DAP12 (KARAP) is a novel immunoreceptor tyrosine-based activation motif (ITAM)-bearing transmembrane adapter molecule.^{1,2} It is expressed on the cell surface of natural killer cells and associated noncovalently with the activating types of killer immunoglobulin-like cell receptors (KARs).^{2–5} Although the expression of KARs is restricted to natural killer and T-cell subsets,⁶ DAP12 is expressed in a wide variety of cell types, including peripheral blood granulocytes, monocytes, macrophages, and dendritic cells.^{2,4,7} Several myeloid cell-specific DAP12-associating receptors have been identified.^{8–10} These receptors include the C-type lectin superfamily and Ig superfamily; the former corresponds to the myeloid DAP12-associating lectin 1 (MDL-1)⁸ and the latter includes triggering receptor expressed on myeloid cells (TREM)-1, TREM-2, and TREM-3^{9,11,12} and signal regulatory protein β 1 (SIRP- β 1)¹⁰ (Figure 1A). The role of DAP12 and its associating receptors in inflammatory and immune responses still remains to be understood.¹³ More recently, Bouchon and colleagues¹⁴ demonstrated that blockade of TREM-1 protects mice against lipopolysaccharide (LPS)-induced shock and suggested a critical function of TREM-1 in acute inflammatory responses to bacteria. Furthermore, Sjolín and colleagues¹⁵ observed that functional DAP12-deficient mice suffered weakened host defense against murine CMV infection. These observations suggest that DAP12 signaling may play a critical regulatory role in immune responses during infection and inflammation.

Previously, we reported that signaling through the DAP12 ITAM motif was very important for terminal differentiation of the murine M1 leukemia cell line.^{16,17} We observed the vigorous morphological change of M1 cells to macrophages including giant cell formation after stimulation through DAP12. However, the role of DAP12 in the macrophage differentiation and activation during inflammation *in vivo* has not yet been established.

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H. N. and N. A. contributed equally to this work.

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Address reprint requests to Naoko Aoki, Room 4H13-HSC, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main St. West, Hamilton ON, L8N 3Z5, Canada. E-mail address: aok@asahikawa-med.ac.jp.

Figure 1. A: Illustration of the mode by which Ad-FDAP12 and Ad-TREM-1 Ig vectors modulate the DAP12-mediated signaling pathway in myeloid lineage cells or monocytes/neutrophils. The membrane-anchored protein FDAP12 derived from Ad-FDAP12 is flagged in the diagram with circular shapes that distinguish from the endogenous DAP12 molecule. The signal through DAP12-associating molecules (TREM family, SIRP β 1, MDL-1) is transmitted to both FDAP12 and endogenous DAP12. TREM-1 Ig, the adenoviral-derived extracellular domain of TREM-1 that was linked to the human Ig Fc portion (dotted in the diagram), serves as an inhibitor of DAP12 signaling pathway by competing with an as yet unidentified ligand for binding to the TREM-1 molecule. **B:** Construction of adenovirus vectors. The elements inserted into the adenoviral genome are illustrated in two bars (top) for Ad-FDAP12 and Ad-TREM-1 Ig vectors, respectively. The pAxCawt cosmid vector containing the above insert was co-transfected into 293 cells with restriction enzyme-digested DNA-TPC (Ad genome tagged with 55-kd terminal protein) to generate recombinant adenoviruses. EC, Extracellular domain; CAG promoter, cytomegalovirus enhancer and chicken β -actin promoter; G poly A, rabbit β -globin poly A signal; FLAG, 24 nucleotides coding for eight defined amino acids (DYKDDDDK) serving as a tag; ApR, ampicillin-resistance gene; cos, cos site of λ phage; ori, replication origin.

To study the role of DAP12/TREM-1 signaling during chronic inflammation, we constructed two adenoviral gene vectors (Figure 1B): Ad-FDAP12 (allowing increased expression of FLAG-DAP12) and Ad-TREM-1 Ig (allowing expression of an antagonist of the DAP12-signaling pathway-soluble form of extracellular domain of TREM-1), and investigated their respective modulatory effect in a mouse model of zymosan A-induced hepatic granuloma.¹⁸ Zymosan A (zymosan, zymocel, β -glucans), which is composed of β -1,3 polyglucose, causes very strong stimulation of macrophages, neutrophils, and natural killer cells.¹⁹ We hypothesized that DAP12 signaling could enhance granulomatous responses of monocytes/macrophages whereas TREM-1 Ig will suppress inflammatory response via its antagonistic effect on DAP12 signaling *in vivo* (Figure 1A). In this study, we

demonstrated that zymosan A-induced granuloma formation was sustained and enhanced at later times by adenoviral-mediated DAP12 gene transfer. In contrast, gene transfer of a DAP12-signaling inhibitor, extracellular domain of TREM-1 markedly inhibited granuloma formation. Our results suggest that the DAP12-signaling pathway plays an important role in chronic inflammation and granuloma formation.

Materials and Methods

Mice, Cells, and Culture Conditions

C57BL/6NCrj female mice were purchased from Sankyo Labo Service Corp. (Sapporo, Japan). Experimental mice were used at 7 to 8 weeks of age. The murine myeloblastic leukemic cell line, M1, was obtained from Riken Gene Bank (Wakou, Japan). Cells were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% fetal calf serum and 5×10^{-5} mol/L 2-mercaptoethanol.

Antibodies

Rabbit anti-mouse DAP12 polyclonal antibody was generated by immunizing a rabbit (Japanese White) with GST-mouse DAP12 cytoplasmic domain fusion protein as described previously.¹⁶ Rat anti-murine F4/80 monoclonal antibody (mAb) (Cl:A3-1) was purchased from BMA Biomedicals (August, Switzerland). Mouse anti-FLAG mAb (M2) was purchased from Sigma (St. Louis, MO). Control mouse IgG1 was purchased from Chemicon International Inc. (Temecula, CA).

Adenovirus Vectors and Zymosan A-Induced Hepatic Granuloma Formation

A fusion protein consisting of the extracellular domain of mouse TREM-1 and the Fc portion of human IgG1 was referred to as TREM-1 Ig (the human Ig Fc molecule was used both to prolong the half-life of soluble TREM-1 molecule and to serve as a tag). Recombinant adenovirus containing LacZ (Ad-LacZ), FLAG-DAP12 (Ad-FDAP12), or TREM-1 Ig (Ad-TREM-1 Ig) was generated by the COS-TPC method²⁰ using the Adenovirus Expression Vector kit (Takara, Shiga, Japan) (see Figure 1B for details). These viral vectors were deleted of E1A and E1B, as well as the E3 region. Encoded cDNA was expressed under the control of the CAG promoter.²¹ Mice were injected with 350 μ l of phosphate-buffered saline (PBS) containing 350 μ g of zymosan A from *Saccharomyces cerevisiae* (Nacaratessque, Inc., Kyoto Japan) into right retro-orbital plexus. Twenty-four hours after zymosan A injection, mice were injected with 1×10^9 plaque-forming units of viral vector in 100 μ l of PBS into the left retro-orbital plexus. Mice were killed by cervical dislocation under diethyl ether anesthesia at days 3, 5, 7, and 10 after zymosan A injection.

Localization of Transgene Expression in the Liver by LacZ Histochemical Staining

For X-gal staining, mouse liver was washed with 20 ml of PBS and fixed with 20 ml of fixation reagent (1% formalin, 0.2% glutaraldehyde, 0.002% Nonidet P-40 in PBS) using liver perfusion. Then, the liver was removed and soaked in fixation reagent for 30 minutes with gentle shaking, followed by three washes: 1) PBS for 10 minutes, 2) 1% Triton X in PBS for 10 minutes, and 3) PBS for 10 minutes. X-gal staining was performed with staining reagent (0.5 mmol/L $MgCl_2$, 5 mmol/L $K_4[Fe(CN)_6]$, 5 mmol/L $K_3[Fe(CN)_6]$, 0.05% X-gal in PBS) at 37°C for 2 days. Stained liver was observed with a stereoscope.

Immunoprecipitation, Electrophoresis, and Blotting

Cells were lysed in lysis buffer (0.5% Triton X-100, 50 mmol/L Tris, pH 8.0, 140 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid) containing the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). Lysates were clarified by centrifugation and immunoprecipitated with anti-DAP12 Ab bound to rProtein A Sepharose Fast Flow (Amersham Pharmacia Biotech AB, Uppsala Sweden) for 1 to 2 hours at 4°C. The resulting immunocomplexes were washed and run on 4 to 12% NuPage bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Novex, San Diego, CA) under reducing conditions. Proteins were then blotted onto Immobilon-P (Millipore, Bedford, MA), blocked in 5% skim milk, and probed with rabbit anti-DAP12 Ab or anti-FLAG mAb (M2) (Sigma) followed by donkey anti-rabbit IgG-horseradish peroxidase (Amersham Pharmacia Biotech AB) or sheep anti-mouse IgG-horseradish peroxidase (Amersham Pharmacia Biotech AB). The ECL system (Amersham Pharmacia Biotech AB) was used for detection.

Light Microscopy and Immunohistochemistry

Liver tissues fixed in 10% formaldehyde were embedded in paraffin. Paraffin sections were cut at 3- μ m thick and slides were stained with hematoxylin and eosin (H&E) for light microscopy. Hepatic granulomas were defined as being composed of more than 10 cells according to a previous report.¹⁸ In an immunohistochemical study, after deparaffinization and inhibition of the endogenous peroxidase activity, the sections were stained by Histofine SAB-PO Kit (Nichirei, Tokyo, Japan) using the antibodies mentioned above. Hematoxylin was used for nuclear staining.

Assays for Differentiation by DAP12 Signaling

To immobilize antibodies, the SonicSeal slide wells (Nalge Nunc International Corp., Naperville, IL) were incubated with anti-FLAG mAb (M2) (20 μ g/ml in PBS) overnight at 4°C and washed with culture medium twice. M1 cells were incubated with LPS (10 μ g/ml) from *Esch-*

erichia coli serotype 0111:B4 (Sigma) overnight (15 to 17 hours) and washed with culture medium. Then, the cells were infected with a multiplicity of infection of 25 of Ad-LacZ and Ad-FDAP12 for 1 hour, and were transferred to anti-FLAG mAb (M2)-coated SonicSeal slide wells. The cells were cultured for 3 days and were stained with H&E.

Flow Cytometry for Quantitation of Macrophage Differentiation

Cultured M1 cells (1×10^6) were blocked with 50% goat serum for 1 hour at 4°C and then incubated with saturating amounts of fluorescein isothiocyanate-conjugated rat anti-mouse Mac-1 (M1/70) (PharMingen, San Diego, CA) and phycoerythrin-conjugated anti-mouse MHC Class II (M5/114.15.2) (PharMingen) for 30 minutes in staining buffer (PBS, 1% fetal calf serum, 0.1% sodium azide) at 4°C. Dead cells were gated out using 2 μ g/ml of propidium iodide at the last step of staining. The fluorescence intensity was analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was prepared using Sepasol-RNA I (Nacalai-tesque, Inc.) according to the manufacturer's protocol. cDNA was prepared from 1 μ g of total RNA by using the first-strand cDNA synthesis kit for RT-PCR (AMV) (Roche). Primers used were as follows: FLAG-DAP12 forward (5'-GCG AAT TCC GCG TCA TGG CCT TAC CAG TGA-3'), FLAG-DAP12 reverse (5'-ACC CTG TGG ATC TGT ATT-3'), TREM-1 forward (5'-CGG AAT TCG AGC TTG AAG GAT GAG GAA GGC-3'), TREM-1 reverse (5'-AAT CCA GAG TCT GTC ACT TGA AGG TCA GTC-3'), β -actin forward (5'-ACC CAC ACT GTG CCC ATG TA-3'), β -actin reverse (5'-CGG AAC CGC TCA TTG CC-3'). PCR was performed under the conditions of 1 minute at 94°C, 30 cycles (5 seconds at 94°C, 30 seconds at 60°C, 90 seconds at 72°C), 7 minutes at 72°C.

Isolation of F4/80-Positive Cells

We performed collagenase perfusion using a buffer (140 mmol/L NaCl, 10 mmol/L HEPES, 5 mmol/L $CaCl_2$, 2H₂O) including 400 U/ml of collagenase type 4 (Sigma). Hepatocytes were removed by centrifugation at $50 \times g$.²² Then cells containing Kupffer cells and monocytes were collected and labeled with F4/80 followed by goat anti-rat IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). We purified F4/80-positive cells using the MACS system (Miltenyi Biotec).

Statistical Analysis

The results were analyzed using one-way analysis of variance, post hoc test, and the Mann-Whitney *U*-test. All data in this study are expressed as the mean $\pm$ SD and *P* < 0.05 is considered significant.

Figure 2. Morphological change and cell-surface phenotypic analysis of Ad-FDAP12-infected M1 cells stimulated with immobilized anti-FLAG mAb. **A:** Ad-LacZ-infected cells were used as a control. After pretreatment with LPS (10 μ g/ml) for 15 to 17 hours, M1 cells were infected by adenoviral vector. Cells were cultured on the sonic seal slide wells coated with anti-FLAG mAb for 3 days without LPS. Cells were stained with H&E and observed under the microscope. **B:** After pretreatment with LPS (10 μ g/ml) for 15 to 17 hours, M1 cells were infected by adenoviral vectors and cultured on the dish coated with anti-FLAG mAb for 3 days without LPS. Cells were collected and stained with fluorescein isothiocyanate-conjugated anti-Mac-1 and phycoerythrin-conjugated anti-MHC class II monoclonal antibodies. Samples were analyzed by flow cytometry. Original magnification, $\times 200$ (**A**).

Results

Stimulation of DAP12 Facilitated Macrophage Differentiation in Ad-FDAP12-Infected M1 Cells

Mouse leukemic M1 cells infected with Ad-FDAP12 could transport FLAG-DAP12 to the cell surface during LPS stimulation because of the concomitant expression of associate molecule of DAP12 (data not shown). Without the associated molecule, the surface expression of DAP12 was not detectable in our system using flow cytometry analysis (data not shown). To investigate the effect of Ad-FDAP12 gene transfer on macrophage differentiation, we pretreated M1 cells with LPS to induce DAP12-associating molecules on the cell surface overnight before infection with Ad-FDAP12 and stimulation by immobilized anti-FLAG mAb. In contrast to Ad-LacZ, Ad-FDAP12-infected M1 cells showed morphological changes suggestive of macrophage differentiation on stimulation via DAP12 (Figure 2A). To verify macrophage differentiation, we also performed fluorescence-activated cell sorting analysis by using mAbs against murine MHC class II and macrophage surface molecule Mac-1. Indeed, Ad-FDAP12-infected cell population contained at least 100% more macrophages that expressed bright Mac-1 or both Mac-1 and MHC class II (28%) whereas the control cell population contained $\sim 14\%$ of macrophages (Figure 2B).

Figure 3. Ad-LacZ transgene expression in the liver after intravenous gene transfer by retro-orbital plexus injection. Mice were injected with or without 1×10^9 plaque-forming units of Ad-LacZ into left retro-orbital plexus. Two days after injection, the mice were killed and the livers were analyzed by X-gal staining. Stained liver was observed with a stereoscope.

Hepatic Adenoviral-Mediated Gene Transfer

Because signaling through DAP12 may affect monocyte differentiation and activation (Figure 2),^{16,17,23,24} we set out to investigate the role of DAP12 during inflammation *in vivo* using adenoviral vectors and zymosan A-induced hepatic granuloma formation system. Additionally, we also examined the effect of Ad-TREM-1 Ig that would block the signal through TREM-1/DAP12 in monocytes and neutrophils (Figure 1A).¹⁴ We first investigated transgene expression in the liver after the delivery of an adenoviral vector expressing a marker gene coding for LacZ via the retro-orbital plexus route. This route of gene transfer was previously shown to lead to transgene expression predominantly in the liver.²⁵ Two days after intravenous gene transfer, the mouse liver injected with Ad-LacZ showed abundant LacZ staining (Figure 3). In contrast, the control mouse had little LacZ staining. LacZ expression in the liver lasted for at least 10 days after gene transfer (data not shown). These results suggest that transgene can be significantly expressed in the liver after intravenous adenoviral gene transfer.

Prolongation of Zymosan A-Induced Hepatic Granuloma Formation by Ad-FDAP12 and Inhibition by Ad-TREM-1 Ig

Because application of high-titer adenoviral vectors in mice may result in severe liver injury^{26–28} and thus confound granuloma formation by zymosan, we determined appropriate doses of zymosan A and adenoviral vectors in trial experiments. The peak of zymosan A-induced hepatic granuloma formation was observed at 7 days after a single injection with 350 μ g of zymosan A and it vanished by 11 days after the administration (data not shown). Thus, this dose of zymosan was chosen for our following experiments (in contrast to larger doses often used in other studies¹⁸) to keep a relatively short course of granulomatous responses that would allow the observation of the effect of adenoviral-mediated transgene expression after a single delivery of viral vector (a dose of 1×10^9 plaque-forming units of adenoviral vector was chosen).

To investigate the role of DAP12 and TREM-1 in zymosan A-induced granuloma formation, we administered the control vector (Ad-LacZ), Ad-FDAP12, or Ad-TREM-1 Ig

Figure 4. Zymosan A-induced hepatic granuloma formation in Ad-LacZ (**A, D**), Ad-FDAP12 (**B, E**), and Ad-TREM-1 Ig (**C, F**)-infected mice at day 3. Liver sections were subjected to H&E staining (**A–C**) and immunostaining for F4/80 (**D–F**). Original magnifications, $\times 100$.

to groups of mice at day 1 after zymosan A injection. Under lower magnifications of light microscopy, at days 3 and 5, the number of granulomas was similar between control and Ad-FDAP12 groups whereas the number of granulomas was slightly less in Ad-TREM-1 Ig-treated mice (Figures 4 and 5). By day 7, although the number of granulomas in the control and Ad-FDAP12 groups markedly increased and remained similar, the number of granulomas in Ad-TREM-1 Ig-treated mice was minimal (Figure 6) (the size of granuloma in Ad-TREM-1 Ig group also

tended to be smaller; see Figure 9 for more details). By day 10, granulomas in the control group primarily disappeared, close to the constant low level of granuloma formation in Ad-TREM-1 Ig-treated mice (Figure 7). In sharp contrast, the number of granulomas in Ad-FDAP12-treated mice continued to increase (Figure 7). The number of granulomas was also enumerated at four different time points in three groups of mice (Figure 8) and the results were in line with morphological observations. Under higher magnification of light microscopy, the discrete

Figure 5. Zymosan A-induced hepatic granuloma formation in Ad-LacZ (**A, D**), Ad-FDAP12 (**B, E**), and Ad-TREM-1 Ig (**C, F**)-infected mice at day 5. Liver sections were subjected to H&E staining (**A–C**) and immunostaining for F4/80 (**D–F**). Original magnifications, $\times 100$.

Figure 6. Zymosan A-induced hepatic granuloma formation in Ad-LacZ (**A, D**), Ad-FDAP12 (**B, E**), and Ad-TREM-1 Ig (**C, F**)-infected mice at day 7. Liver sections were subjected to H&E staining (**A–C**) and immunostaining for F4/80 (**D–F**). Original magnifications, $\times 100$.

granuloma structure and kinetic influx of inflammatory cells were revealed (Figure 9). At day 3, the major cell types were polymorphonuclear leukocytes and monocytes/macrophages, regardless of treatment given (Figure 9; A, E, I). By day 5, the main cell type within the granuloma was mononuclear cells including macrophages and lymphocytes (Figure 9; B, F, J). At day 7, many macrophage-derived epithelioid cells were seen, suggestive of mature granuloma formation (Figure 9; C, G, K). By day 10, many cells in the control group under-

went apoptosis (Figure 9D), which was associated with diminishing granuloma whereas many epithelioid cells were still seen in the granuloma of Ad-FDAP12-treated mice (Figure 9H).

To specifically examine the kinetic changes in the accumulation of Kupffer cells and macrophages in the liver and granuloma, we performed immunohistochemical staining using the F4/80 mAb that recognizes monocytes/macrophages. At day 3, immunostaining revealed only clusters of Kupffer cells in the sinusoids of liver in both

Figure 7. Zymosan A-induced hepatic granuloma formation in Ad-LacZ (**A, D**), Ad-FDAP12 (**B, E**), and Ad-TREM-1 Ig (**C, F**)-infected mice at day 10. Liver sections were subjected to H&E staining (**A–C**) and immunostaining for F4/80 (**D–F**). Original magnifications, $\times 100$.

Figure 8. Enumeration of granulomas in the liver throughout time in Ad-LacZ-, Ad-FDAP12-, and Ad-TREM-1 Ig-infected mice ($n = 4$). The results were analyzed using one-way analysis of variance and post hoc test. Granulomas were calculated in randomly selected 10 fields at an original magnification of $\times 200$.

control and Ad-FDAP12-treated mice (Figure 4, D and E). At days 5 and 7, although there was increasing accumulation of F4/80-positive cells within the granuloma, there was a decreased number of Kupffer cells in these two groups (Figures 5 and 6, D and E). By day 10, the F4/80 positivity markedly decreased in the control whereas macrophages within the granuloma of Ad-FDAP12 group demonstrated strongest staining for F4/80 (Figure 7, D and E). In comparison, Ad-TREM-1 Ig treatment led to decreased F4/80-positive cells starting from day 3 through day 7 (Figures 4F, 5F, and 6F). However, by day 10, although there was a lack of granuloma formation in the liver of these mice, there was an increased number of F4/80-positive Kupffer cells (Figure 7F).

Figure 9. Higher magnification of zymosan A-induced hepatic granuloma. **A–D:** Ad-LacZ-treated mice; **E–H:** Ad-FDAP12-treated mice; and **I–L:** Ad-TREM-1 Ig-treated mice. PMN, Polymorphonuclear leukocyte; Mφ, macrophage; LC, lymphocyte; Epi, epithelioid cell. Liver sections were subjected to H&E staining and observed at a high magnification of $\times 400$.

Figure 10. A: Gene expression of TREM-1 and FLAG-DAP12 in Ad-LacZ-, Ad-FDAP12-, and Ad-TREM-1 Ig-infected and zymosan A-injected mice. RT-PCR analysis was performed on total RNA extracted from mouse liver. Every liver of four mice in each group represents the same results. **B:** FLAG-DAP12 and endogenous DAP12 protein expression in F4/80-positive cells from Ad-FDAP12-infected mouse liver 3 days after zymosan A injection. F4/80-positive cells from four Ad-FDAP12-infected mice were collected. Lysates prepared from 1×10^6 cells were immunoprecipitated with anti-DAP12 polyclonal antibodies and analyzed by Western blotting using anti-DAP12 polyclonal antibodies or anti-FLAG mAb.

We next investigated the expression of transferred gene at the liver in each group using RT-PCR (Figure 10A). The expression of Ad-FDAP12 and Ad-TREM-1 Ig was observed until day 7 and decreased at day 10. Taking one step further, we also examined whether Ad-FDAP12 gene transfer led to DAP12 transgene protein expression in Kupffer cells/macrophages. Little is known about whether adenovirus could effectively infect Kupffer cells.²⁹ We purified F4/80-positive cells from mouse liver in Ad-FDAP12-infected group. The cells taken from mouse liver showed 100% positivity for F4/80 by flow cytometry analysis (data not shown). Although the amount was less than that of endogenous DAP12, we were able to demonstrate the expression of FLAG-DAP12 protein in F4/80-positive cells (Figure 10B).

Potentiation of DAP12 Signaling Promoted Zymosan A-Induced Hepatic Granuloma Formation by Day 7

As shown in Figure 2, we demonstrated that DAP12 signaling using anti-FLAG mAb made M1 cells differentiated to macrophages vigorously. To induce potent signal via FLAG-DAP12 in Ad-FDAP12-infected monocytes and macrophages, we injected anti-FLAG mAb together with Ad-FDAP12. At day 7, a remarkable number of large and F4/80-positive mature granulomas were seen in anti-FLAG mAb-injected group (Figure 11, B and E). The number of granulomas in the anti-FLAG mAb-injected group was larger than that of isotype control Ig-injected group (Figure 12). Interestingly, the Ad-FDAP12, Ad-TREM-1 Ig, and anti-FLAG mAb-injected group revealed numerous inflammatory cells but the size of the granulo-

Figure 11. Zymosan A-induced hepatic granuloma formation in Ad-DAP12-infected mice was augmented by anti-FLAG mAb. Twenty-four hours after zymosan A injection, mice were infected with Ad-FDAP12 plus isotype control mouse IgG1 (**A, D**), Ad-FDAP12 plus anti-FLAG mAb (**B, E**), and Ad-FDAP12 plus Ad-TREM-1 Ig plus anti-FLAG mAb (**C, F**). At day 7, mice were killed and liver sections were subjected to H&E staining (**A–C**) and immunostaining for F4/80 (**D–F**). Original magnifications, $\times 100$.

Figure 12. Difference in number of zymosan A-induced granulomas between the anti-FLAG mAb-injected group and isotype control-injected group in Ad-FDAP12-infected mouse livers ($n = 4$). The results were analyzed using Mann-Whitney U -test. Granulomas were calculated in randomly selected 10 fields at an original magnification of $\times 200$.

mas remained very small (Figure 11, C and F). These granulomas looked different from the large mature granulomas observed in Ad-FDAP12 and the anti-FLAG mAb-injected group (Figure 11, B and E).

Discussion

DAP12 is known as a novel ITAM-bearing transmembrane adapter molecule and associated with several myeloid-specific associating molecules, such as the TREM family,⁹ SIRP- $\beta 1$,¹⁰ and MDL-1.⁸ Functional mutation in human DAP12 causes the Nasu-Hakola disease that is characterized by a combination of bone cysts and presenile dementia.³⁰ These results suggest that the DAP12 mutation may affect differentiation and activation of monocyte/macrophage lineage cells such as microglia in the brain and osteoclasts in the bone. Previously, we have demonstrated that DAP12 protein was detected in several organs and cell lines related to myeloid/macrophage lineage cells.¹⁶ Moreover, we investigated macrophage differentiation through the DAP12 ITAM motif using an *in vitro* model.¹⁷ In this study, we further extended our observations and demonstrated the role of DAP12 signaling *in vivo* during inflammation using a mouse model of zymosan A-induced hepatic granuloma formation.

It has been reported that Kupffer cells, together with monocytes, neutrophils, lymphocytes, and macrophages, have the critical role for mouse hepatic granuloma formation induced by zymosan.³¹ Zymosan A is composed of β -1,3 glucan and causes a very strong stimulation of macrophages, neutrophils, and natural killer cells through its receptor, mouse CR3 (CD11b/CD18, $\alpha M\beta 2$ integrin).¹⁹ Recently, it was reported that TLR2 and TLR6 were also involved in zymosan recognition by mouse macrophages.³² As shown in Figure 10B, we demonstrated that F4/80-positive cells that include Kupffer cells and monocytes expressed FLAG-DAP12. The signaling through DAP12 molecule on these F4/80-positive cells during early inflammatory phase may affect the magnitude and sustenance of granuloma formation. Such influence, as a result of increased DAP12 expression, on local microenvironment may act through at least

three potential mechanisms: first of all, the normal turnover of granuloma is perhaps affected by the rate of macrophage apoptosis and altered DAP12 expression may slow down granuloma turnover by decreasing macrophage apoptosis. Second, because it is believed that the maintenance of granuloma requires the continuous supply of newly arrived monocytes, increased DAP12 expression may enhance such supply from the peripheral blood and/or local Kupffer cell pool to sustain already formed granulomas. Third, Bouchon and colleagues²³ showed that the TREM-2/DAP12-mediated pathway regulates maturation of human dendritic cells. Therefore compulsory expression of DAP12 on clustering immature dendritic cells may cause their maturation and they may sustain microenvironments suitable for granuloma formation. More recently, KARAP/DAP12 transgenic mice were generated and they suffered massive inflammatory syndrome associated with neutrophilia and lung infiltration by multinucleated macrophages.³³ Hyperresponsiveness to experimental septic shock was also observed. These observations, together with our current findings, indicate that overexpression of DAP12 leads to myeloid cell activation and accumulation and may profoundly enhance an ongoing chronic tissue inflammatory response.

DAP12 alone is not sufficient for its expression and function at the cell surface.^{5,16} Thus, the combination of DAP12-associating molecule and DAP12 may account for transmitting a particular physiological signal via DAP12. It has been reported that the cellular distribution of TREM-1 and TREM-2 is limited to monocytes and granulocytes, and to macrophages and dendritic cells, respectively.⁹ It was also suggested that TREM-1 on monocytes and granulocytes plays a role in acute inflammation, contrasting the role of TREM-2 on macrophages and dendritic cells for chronic inflammation. Because, granulomas generated in early phase of inflammation after zymosan A injection consisted of monocytes and neutrophils (Figure 9), TREM-1 on these cells may have a critical role for initiation of granuloma formation through DAP12 signaling. In fact, granuloma formation was markedly inhibited throughout the entire course of observation (up to day 10) by TREM-1 Ig that blocked TREM-1/DAP12 signaling (Figure 1A). It was speculated that weakened TREM-1 Ig expression (Figure 10A) caused accumulation of Kupffer cells and inflammatory cell infiltration (Figure 7, C and F) at day 10. These results suggested that the signal through TREM-1/DAP12 on circulating monocytes and neutrophils was critical for Kupffer cell activation during granuloma formation.

Moreover, we demonstrated that the augmentation of DAP12 signaling using anti-FLAG mAb caused exaggeration of zymosan A-induced mouse hepatic granuloma formation. The result suggested that the direct signal from DAP12 activated Ad-FDAP12-infected cells, such as Kupffer cells and monocytes. In fact, when anti-FLAG mAb was not given as an agonist of DAP12-mediated signaling, we found no difference in the magnitude of granuloma formation between the control and Ad-FDAP12-treated groups up to day 7 (Figure 8), although there was enhanced earlier Kupffer cell accumulation

with Ad-FDAP12 treatment (Figure 4). These findings, in conjunction with the finding that treatment with DAP12-signaling inhibitor, Ad-TREM-1 Ig, markedly inhibited zymosan A-induced granuloma formation throughout the entire course of observation, strongly suggest that at the early phase of granuloma formation, endogenous DAP12 expressed by monocytes/macrophages may be sufficient to trigger signals via its partner-associating receptors, such as the TREM receptor family. On the other hand, the result observed in the group administered with Ad-FDAP12, Ad-TREM-1 Ig, and anti-FLAG mAb was noteworthy. Although we detected much inflammatory cell infiltration in this group, these inflammatory cells remained scattered and they were unable to be assembled to form granulomas. Direct DAP12 signal augmented inflammatory cell infiltration but TREM-1 Ig inhibited the granuloma formation. Thus inflammatory cell infiltration and granuloma formation were separate events, and considered to require different signals.

As mentioned above, it has been reported that the blockade of TREM-1/DAP12 signal by TREM-1 Ig protects mice against LPS-induced shock and microbial sepsis.¹⁴ Here we show that TREM-1 Ig is also able to inhibit a chronic inflammatory response such as zymosan A-induced granuloma formation, although it does not seem to suppress the infiltration of inflammatory cells caused by augmentation of DAP12 signaling (Figure 11). Although this molecule represents an attractive therapeutic agent used to dampen inflammatory diseases, the precise mechanism of the anti-inflammatory role by TREM-1 Ig remains to be fully understood.

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