Functional Association of CD9 with the Fcγ Receptors in Macrophages

Keisuke Kaji, Sunao Takeshita, Kensuke Miyake, Toshiyuki Takai and Akira Kudo

*J Immunol* 2001; 166:3256-3265; 
doi: 10.4049/jimmunol.166.5.3256
http://www.jimmunol.org/content/166/5/3256

This information is current as of September 15, 2017.

References
This article cites 40 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/166/5/3256.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Functional Association of CD9 with the Fcγ Receptors in Macrophages

Keisuke Kaji,* Sunao Takeshita,* Kensuke Miyake,† Toshiyuki Takai,‡ and Akira Kudo2*  

CD9, a member of the tetraspan family of proteins, is highly expressed on macrophages. Although a clear function for the molecule has yet to be described, we have found that the anti-CD9 mAb activates mouse macrophages. The rat anti-CD9 mAb, KMC8.8, but not the F(ab′)2, induced tyrosine phosphorylation of proteins including syk and cbl and induced cell aggregation in the mouse macrophage cell line, J774, suggesting that co-cross-linking of CD9 and FcγR was required for the signal. Co-cross-linking of CD9-FcγR with KMC8.8 on macrophages from three different FcR-deficient mice, FcR γ-chain −/−, FcγRIIB−/−, and FcγRIIa−/−, revealed that FcγRIIa is specific and crucial for syk phosphorylation. Although both KMC8.8 and the anti-FcγRIIB/III mAb, 2.4G2, evoked similar phosphorylation patterns, only KMC8.8 induced cell aggregation. Additionally, KMC8.8 treatment led to reduce levels of TNF-α production and p42/44 extracellular signal-related kinase phosphorylation relative to 2.4G2 stimulation. Immunofluorescence staining showed that co-cross-linking of CD9-FcγR with KMC8.8 induced filopodium extension before cell aggregation, which was followed by simultaneous colocalization of CD9, FcγRIIB/III, Mac-1, ICAM-1, and F-actin at the cell-cell adhesion site. Moreover, KMC8.8 treatment of FcγR-deficient macrophages revealed that the colocalization of CD9, FcγRIIa, Mac-1, and F-actin requires co-cross-linking of CD9-FcγRIII, whereas co-cross-linking of CD9-FcγRIIB induced the colocalization of only CD9 and FcγRIIB. Our results demonstrate that co-cross-linking of CD9 and FcγRs activates macrophages; therefore, CD9 may collaborate with FcRs functioning in infection and inflammation on macrophages. The Journal of Immunology, 2001, 166: 3256–3265.

The most important accessory cells in immune responses are phagocytes of the monocytic and myelocytic lineage, represented by the macrophage. In phagocytosis by macrophages, FcγRs (Fc receptor for IgG) are one of the most important receptor families. FcγRs recognize IgGs, which are produced in response to pathogen invasions, and mediate phagocytosis of the IgG-opsonized pathogens. During this process, cross-linking of FcγRs by the immune complex induces a wide variety of immune responses: Ab-dependent cellular cytotoxicity, release of numerous inflammatory mediators, and expression changes of cell surface proteins involved in cell-cell adhesion and Ag presentation (1–3).

Three classes of FcγR (FcγRI, FcγIIb, and FcγIII) are expressed on mouse macrophages and share a highly homologous extracellular portion for the IgG binding domain (1, 2). However, there are structural and functional differences in the various receptor family members. FcγRI and FcγRII exist as oligomeric complexes in which the α-chain, bearing the IgG binding domain, associates with γ-chain dimers that bear an immunoreceptor tyrosine-based activation motif (ITAM); these receptors do not contain intrinsic tyrosine kinase activity (2, 4). On cross-linking of FcγRI and FcγRIII receptors, nonreceptor tyrosine kinases including members of the Src and Syk/ZAP70 families are activated, resulting in the phosphorylation of the γ-chain ITAM. This is followed by tyrosine phosphorylation of downstream effectors, such as phospholipase Cγ, phosphatidylinositol 3-kinase, mitogen-activated protein kinase (MAPK), and cbl (2, 5, 6). Although both FcγRI and FcγRIII function as phagocytic receptors and transduce similar signals, the binding activity to IgGs is different; FcγRI specifically binds to IgG2a with a high affinity, whereas FcγRIII binds to IgG1, IgG2a, and IgG2b with a low affinity (2, 3). In contrast, FcγRIIB is a monomeric receptor containing the immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits the phosphatases Src homology 2 protein-1 and Src homology 2 domain-containing inositol phosphatase, and it is unknown how FcγRIIB contributes to phagocytosis in macrophages (2, 7).

CD9, which is highly expressed in macrophages, is a cell surface glycoprotein belonging to the transmembrane 4 superfamily (TM4SF). The TM4SF is a group of cell surface proteins, including at least 16 members such as CD37, CD53, CD63, CD81, and CD82. The structure of these proteins is typified by four hydrophobic domains spanning the cell membrane and short N- and C-terminal cytoplasmic domains (around 5–14 aa in length) (8, 9). CD9 was reported to be associated with various integrin family molecules, CD5, CD19, and other TM4SF proteins on the cell surface and has been postulated to participate in the regulation of cell growth, motility, and signaling (8–16). Besides monocytes/macrophages, CD9 is expressed on certain hematopoietic lineage cells such as platelets, subpopulations of lymphocytes, eosinophils, and basophils and on some other cell lineages such as endothelial cells, myoblasts, and neuroblasts (8, 15, 16). Additionally, CD9 is expressed in oocytes, and CD9-deficient female mice showed serious sterility caused by a defect in the gamete fusion

*Department of Life Science, Tokyo Institute of Technology, Yokohama, Japan; †Department of Immunology, Saga Medical School, Saga, Japan; and ‡ Department of Experimental Immunology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan

Received for publication July 18, 2000. Accepted for publication December 19, 2000. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by a grant-in-aid for scientific research from the Japan Ministry of Education, Science, Sports, and Culture.

Address correspondence and reprint requests to Dr. Akira Kudo, Department of Life Science, Tokyo Institute of Technology, 2-12-1, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan.

Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; TM4SF, transmembrane 4 superfamily; DIDS, detergent-insoluble glycolipid membranes; Ctxs, cholera toxin; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; BSS, buffer saline solution.

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00
process (17–20). However, no abnormalities were detected in any other tissues in CD9-deficient mice (18–20), suggesting that the function(s) of CD9 may be compensated by the presence of other TM4SF proteins.

In human platelets, CD9 is thought to functionally associate with FcγRIIA, an isoform of FcγRII bearing the ITAM in the cytoplasmic tail, which is not found in mice, and co-cross-linking of CD9 and FcγRIIA induces cell aggregation and activation. Moreover, in various types of cells, CD9 is proposed to be involved in signal transduction coupled with cell activation, proliferation, and adhesion, as mAbs against CD9 can induce the activation of T cells, the homotypic aggregation of pre-B cells, the adhesion and proliferation of Schwann cells, and can inhibit the migration of leukemia cells (13, 21–23). However, in mouse macrophages, the function of CD9 has not yet been investigated, and the association of molecules with CD9 on macrophages are not known, despite their high level of CD9 expression.

In the present study, we found that an anti-CD9 mAb, KMC8.8, induced much less TNF-α production and p42/44 MAPK phosphorylation than FcγRIIB/III cross-linking. Finally, our results suggest that CD9 on mouse macrophages functionally associates with FcγRs and may modify signals for phagocytosis and inflammatory responses.

Materials and Methods

Reagents

The monoclonal rat anti-mouse CD9 Ab, KMC8.8, was reported previously (24), the isotype of which is IgG2aκ. KMC8.8 and anti-IgM mAb (MA6), as an isotype (IgG2aκ)-matched control mAb, were purified from ascitic fluids as described (24), and KMC8.8 was biotinylated by the manufacturer’s instruction (Pierce, Rockford, IL). F(ab’2), of KMC8.8 were generated by Takara Shuzo (Tokyo, Japan). Anti-FcγRIIB/III mAb (2.4G2), anti-β1 integrin mAb, biotinylated anti-ICAM-1 mAb, and FITC-conjugated anti-Mac-1 mAb were obtained from BD PharMingen (San Diego, CA). Anti-syk mAb, anti-cbl-b mAb, anti-phosphorylated p42/44 MAPK mAb, anti-p42 MAPK mAb, and HRP-conjugated anti-phosphotyrosine mAb (PY99) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-rat Fab polyclonal Abs, HRP-conjugated anti-rabbit IgG, anti-mouse IgG Ab, and FITC-conjugated anti-rat IgG Ab were purchased from...
Detection of protein tyrosine phosphorylation by Western blotting

J774 cells and bone marrow macrophages were washed and resuspended at 1 x 10^7 cells/ml in DMEM or αMEM containing 10 μg/ml indicated mAb. In some experiments, J774 cells were pretreated with 10 μg/ml anti-FcγRIIB/III mAb, 2.4G2 on ice for 20 min before stimulation with anti-rat IgG Ab or anti-CD9 mAb, KMC8.8. After 1–20 min incubation at 37°C, cells were washed with cold PBS and lysed with TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM NaVO₃, 2 mM PMSF, 1 μg/ml aprotinin, 100 μg/ml leupeptin) on ice for 30 min. The cell lysate (30 μg/lane) was separated by 7.5% or 10% SDS-PAGE in reducing conditions, electrophoretically transferred to a nitrocellulose membrane, and then reacted with Abs. Bound Abs were visualized by chemiluminescence by using an ECL immunoblotting kit (Amersham, Buckinghamshire, U.K.).

Immunoprecipitation

J774 cells were lysed with TNE buffer before or after stimulation with KMC8.8 for 5 min at 37°C. Four micrograms of anti-syk or cbl-b mAb plus protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) were added to lysates, and they were incubated for 2 h at 4°C under constant agitation. After washing beads with TNE buffer, the immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting.

Aggregation assay

J774 cells were incubated with Abs at 37°C for the indicated time under constant agitation in 1 mM CaCl₂/HCMF (10 mM HEPES-buffered Ca²⁺, Mg²⁺-free Hank’s solution) containing 1% BSA. After incubation for t minutes, the total number of aggregated particles and cells in the cell suspension (Nₜ) was counted with a counter chamber. The ratio of aggregation was represented by the index Nₜ/N₀, where N₀ is the initial cell number before aggregation (27).

TNF-α measurement

J774 cells (5 x 10⁶) were cultured with 10 μg/ml of KMC8.8 or 2.4G2 plus anti-rat IgG Ab in 100 μl DMEM containing 10% FBS for various times (0–24 h) at 37°C. The amount of TNF-α in the collected supernatant was measured with an ELISA kit (Amersham) according to the manufacturer’s instructions.

Cholesterol extraction

To remove cholesterol, suspended J774 cells (5 x 10⁶ cells/ml) were incubated for 15 or 30 min at 37°C in the presence of 20 mM MβCD in BSA-containing buffer saline solution (BSA/BSS: 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 1 mg/ml BSA; Ref. 28) and then washed with BSA/BSS before stimulation or analysis by flow cytometry. The cells stained with anti-CD9 mAb or anti-FcγRIIB/III mAb followed by FITC-conjugated anti-rat IgG were analyzed by using FACS Calibur (Becton Dickinson, San Jose, CA).

Immunohistochemistry and laser-scanning confocal microscopy

J774 cells or bone marrow macrophages cultured on a coverslip were stimulated with KMC8.8 at 37°C for 0, 5 and 60 min. After washing with PBS, cells were fixed in 4% paraformaldehyde/PBS for 30 min at 25°C and then permeabilized and blocked with 0.1% Triton X-100 and 1% skim-milk in PBS. Immunofluorescence staining was conducted by using Abs to CD9, α-integrin, β₁ integrin, F-actin, and biotinylated anti-Mac-1 mAb, respectively. Microscopy was performed with a laser-scanning confocal microscope (CSU10; Yokogawa Electric, Tokyo, Japan), and captured images were processed and superimposed by using the IPLab software package (Scanalytics, Billerica, MA).

Cells

The mouse macrophage cell line J774 was cultured in DMEM with 10% FBS. M-CSF-dependent bone marrow macrophages were prepared from wild-type mice and mice deficient in the FcRγ chain, FcγRIIB, and FcγRIII as described previously (25, 26). In brief, the bone marrow cells from femurs and tibias of adult mice were cultured in M-CSF-producing cell line CMG14-12 (final ~35,000 U/ml of M-CSF) for 3 days. After removing cells in suspension, adherent M-CSF-dependent bone marrow macrophages were harvested with 0.02% EDTA/PBS treatment and refed or used for experiments.

FIGURE 2. No effects of F(ab’)₂ of KMC8.8 in tyrosine phosphorylation and cell aggregation. A, J774 cells were incubated with 10 μg/ml of F(ab’)₂ or the whole of anti-CD9 mAb KMC8.8 for 1 or 5 min at 37°C. After lysis of cells, tyrosine-phosphorylated proteins were detected by anti-phosphotyrosine mAb PY99 in Western blotting. To reveal equal loading, bands were stripped and reprobed with anti-syk Ab. Data are representative of three similar experiments. B, J774 cells were incubated with a control mAb, F(ab’)₂ of KMC8.8 and KMC8.8, respectively, for 60 min in 1 mM CaCl₂/HCMF at 37°C under constant agitation. The ratio of aggregation, Nₜ/N₀, was calculated. Results are presented as mean ± SD of three experiments.

Jackson ImmunoResearch (West Grove, PA), Southern Biotechnology Associates (Birmingham, AL), Bio-Rad (Hercules, CA), and ICN Pharmaceuticals-Cappel Products (Costa Mesa, CA), respectively. FITC-conjugated cholera toxin (CTx) and methyl-β-cyclodextrin (MβCD) were obtained from Sigma (St. Louis, MO). Rhodamine-conjugated phalloidin and FITC-conjugated streptavidin were purchased from Molecular Probes (Eugene, OR).
Results

Protein tyrosine phosphorylation and cell aggregation induced by anti-CD9 mAb in the mouse macrophage cell line J774

In human platelets, anti-CD9 mAbs induce protein tyrosine phosphorylation and cell aggregation (10–12). To investigate the function of CD9 on macrophages, the effect of an anti-CD9 mAb, KMC8.8, on the mouse macrophage cell line, J774 was examined. After incubation with the mAb for 5 min at 37°C, J774 cells were lysed, and tyrosine-phosphorylated proteins were detected by an anti-phosphotyrosine mAb, PY99, in Western blot analysis. As shown in Fig. 1A, KMC8.8 induced the tyrosine phosphorylation of 55-, 60-, 72-, and 120-kDa proteins, and the same signals were obtained by cross-linking with an anti-FcγRIIB/III mAb, 2.4G2. Neither a control mAb nor a mAb against β1 integrin, which associates with CD9 in B cells and endothelial cells (9), induced protein tyrosine phosphorylation, despite being the same isotype as KMC8.8. CD43 and CD44 are expressed on J774 cells at a similar level to CD9, and mAbs against both surface molecules did not induce tyrosine phosphorylation. Furthermore, incubation with KMC8.8 did not induce tyrosine phosphorylation in macrophages derived from CD9-deficient mice (data not shown). Immunoprecipitation followed by Western blot analysis confirmed that the phosphorylated proteins of 72 and 120 kDa were a nonreceptor tyrosine kinase, syk, and an adapter protein, cbl, respectively (Fig. 1B). Moreover, KMC8.8 induced time-dependent cell aggregation in the presence of 1 mM CaCl2 (Fig. 1, C and D, right), and the same result was obtained in the absence of CaCl2 (data not shown).

In the previous report, the activation of human platelets induced by anti-CD9 mAbs required Fcγ receptor expression (10–12). Therefore, to avoid FcγR engagement in signal transduction, cross-linking by F(ab′)2 of KMC8.8 on J774 cells was examined. The incubation with F(ab′)2 of KMC8.8 for 5 min did not induce protein tyrosine phosphorylation (Fig. 2A) and did not cause cell aggregation after 60 min (Fig. 2B). The results suggest that the protein tyrosine phosphorylation and cell aggregation induced by KMC8.8 require co-cross-linking of CD9 and FcγRI with a whole molecule of the anti-CD9 Ab on J774 cells.

Specific induction of protein tyrosine phosphorylation by co-cross-linking of FcγRIII and CD9 with KMC8.8

On mouse macrophages, three types of FcγR, FcγRI, FcγRIIIB, and FcγRIII, are expressed. FcγRI and FcγRIII, but not FcγRIIIB,
transduce tyrosine phosphorylation signals through the ITAM of the γ subunit (2), whereas FcyRIIB transduces dephosphorylation signals through the ITIM. We tried to identify which FcγR.

**FIGURE 4.** Different effect of anti-CD9 mAb from that of anti-FcγRIIB/III mAb in cell aggregation. A, J774 cells were incubated with or without 10 μg/ml anti-FcγRIIB/III mAb 2.4G2 for 20 min at 4°C. After washing, either none or 10 μg/ml anti-rat IgG Ab or anti-CD9 mAb KMC8.8 were reacted to cells for 1–20 min at 37°C. Phosphotyrosine was detected by PY99 in Western blotting. To reveal equal loading, blots were stripped and reprobed with anti-syk Ab. Data are representative of three similar experiments. B, J774 cells were incubated with a control mAb, anti-FcγRIIB/III 2.4G2, plus anti-rat IgG Ab, anti-CD9 mAb KMC8.8, for 60 min in 1 mM CaCl2/HCMF at 37°C under constant agitation. The ratio of aggregation, NAg/Nno, was calculated. Results are presented as mean ± SD of three experiments.

**FIGURE 5.** TNF-α production or p42/44 MAPK phosphorylation by cross-linking by anti-CD9 mAb or anti-FcγRIIB/III mAb. A, J774 cells (5 × 10⁶) were cultured with 10 μg/ml of anti-FcγRIIB/III mAb 2.4G2 plus anti-rat IgG Ab (solid line) or anti-CD9 mAb KMC8.8 (dotted line) for 0, 12, 18, and 24 h (37°C, 5% CO2). The amount of TNF-α in the collected supernatant was measured with an ELISA kit. Results are presented as mean ± SD of two experiments. When error bars were not seen, their sizes were smaller than the symbols. B, J774 cells were incubated with 10 μg/ml of a control mAb, 2.4G2, plus anti-rat IgG Ab or KMC8.8 for 5 min at 37°C. Phosphotyrosine was detected by PY99 in Western blotting. Phosphorylated p42/44 MAPK and p42 MAPK were detected with the corresponding Ab on the same membrane after stripping. Data are representative of three similar experiments. C, After western blotting described above, levels of phosphorylated p42 MAPK and p42 MAPK were quantified by densitometric analysis, and the specific phosphorylation was calculated by dividing the densitometric values for phosphorylated p42 MAPK by those for p42 MAPK. Data are the mean (±SD) of values relative to the specific phosphorylation in cells treated with control mAb from three independent experiments.

FcγR γ-chain−/−, FcγRIIB−/−, and FcγRIII−/− mice. These macrophages express functional FcγRI, FcγRIIB, and FcγRIII (wild-type), FcγRIIB (FcγR γ-chain−/−), FcγRI and FcγRIII (FcγRIIB−/−), or FcγRI and FcγRIIB (FcγRIII−/−), as the FcγR γ-chain is critical in facilitating either surface expression or ligand binding of the FcγRI and FcγRIII (30). Stimulation with KMC8.8 for 5 min at 37°C induced tyrosine phosphorylation of proteins, including syk, in bone marrow macrophages from wild-type and FcγRIIB−/− mice (Fig. 3B, lanes 2 and 6) but not from FcγR γ-chain−/− and FcγRIII−/− mice (Fig. 3B, lanes 4 and 8). These results demonstrate that co-cross-linking of FcγRIII and CD9 by
KMC8.8 induces tyrosine phosphorylation signals. Mouse FcγRI shows 10-fold higher binding affinity to Fc region of mouse IgG2α than FcγRIIB and FcγRIII (2). The isotype of rat anti-CD9 mAb, KMC8.8, is IgG2α; however, the binding affinity of mouse FcγRs is to the Fc region of rat IgG2α is not well known. Thus, we examined whether co-cross-linking of FcγRs and CD9 with KMC8.8-F(ab')2 followed by mouse anti-rat Fab polyclonal Abs induces tyrosine phosphorylation signals. Treatment of macrophages from wild-type, FcR γ-chain−/−, FcγRIIB−/−, and FcγRIII−/− mice with KMC8.8-F(ab')2 followed by the addition of mouse anti-rat Fab Abs induced tyrosine phosphorylation except for macrophages from FcR γ chain−/− (Fig. 3C). Moreover, in macrophages lacking FcγRIIB containing the ITIM, the phosphorylation level of syk was stronger than that in wild-type macrophages (Fig. 3, B and C). These results may suggest that co-cross-linking of any combination of FcγR and CD9 transduces some signals or enhances FcR signals.

Because no specific Ab to each FcγR is available, it is difficult to compare the effects of co-cross-linking between CD9 and each FcγR. Therefore, we compared the effects of FcγR-CD9 cross-linking by KMC8.8 with that of FcγRIIB/III cross-linking by the anti-FcγRIIB/III Ab, 2.4G2.

Different effects of anti-CD9 and anti-FcγRIIB/III mAbs on macrophage cell aggregation and TNF-α production

J774 cells were stimulated with KMC8.8, 2.4G2 alone, or 2.4G2 plus anti-rat IgG Ab, and then tyrosine-phosphorylated proteins were detected by Western blot analysis. An anti-FcγRIIB/III mAb, 2.4G2, induced time-dependent protein tyrosine phosphorylation, and stronger phosphorylation was induced by super cross-linking of 2.4G2 with a secondary Ab or with KMC8.8 alone (Fig. 4A). Moreover, in an aggregation assay, unlike incubation with KMC8.8, super cross-linking of 2.4G2 with anti-rat IgG Ab did not induce cell aggregation after 60 min (Fig. 4B), despite the similar high level of tyrosine phosphorylation (Fig. 4A). The cross-linking of FcγRs by IgG immune complexes triggers a wide variety of effector functions including phagocytosis, Ab-dependent cellular cytotoxicity, and release of inflammatory mediators like IL-1, IL-10, and TNF-α (6, 31, 32). We stimulated J774 cells with KMC8.8 or 2.4G2 plus anti-rat IgG Ab for 0, 12, 18, and 24 h and measured the TNF-α production in the supernatant with an ELISA kit. FcγRIIB/III cross-linking by 2.4G2 plus anti-rat IgG Ab induced the production of TNF-α from J774 cells rapidly, whereas FcγR-CD9 co-cross-linking by KMC8.8 induced less TNF-α (Fig. 5A). An increase in TNF-α mRNA expression was observed in J774 cells cultured with 2.4G2 plus anti-rat IgG Ab for 12 h but not with KMC8.8 (data not shown). It has been reported that the activation of p42 MAPK is necessary for TNF-α synthesis induced by FcγR cross-linking (6); therefore, the phosphorylation of p42/44 MAPK was examined. As shown in Fig. 5B, 2.4G2 plus anti-rat IgG Ab induced the phosphorylation of p42/44 MAPK after stimulation for 5 min. However, KMC8.8 did not induce but rather reduced it, although other major tyrosine-phosphorylated proteins were much the same in both treatments (Fig. 5, B and C). These results suggest that cross-linking of FcγR and CD9 with KMC8.8 induces different signals from that of FcγRIIB/III with 2.4G2.

Induction of filopodium extension and colocalization of CD9, FcγR, Mac-1, ICAM-1, and F-actin after CD9 cross-linking

KMC8.8 induced aggregation of J774 cells in a suspension culture. To observe the process of cell aggregation, J774 cells adhered on a coverslip were stimulated with KMC8.8. After fixing the cells, localization of CD9 or F-actin was examined by staining with KMC8.8, followed by FITC-conjugated anti-rat IgG Ab or rhodamine-conjugated phalloidin. Time-dependent filopodium extension was observed by staining of F-actin in J774 cells (Fig. 6). Additionally, localization of CD9 at filopodia was also observed (Fig. 6). Moreover, after Ab treatment, many cells adhered to neighboring cells as if cells had moved toward the cells attached on the extended filopodia, and CD9 and F-actin were densely colocalized at the cell-cell adhesion sites (Fig. 6, arrow). CD9 has not been reported to be associated with actin filaments or to function in cell-cell or cell-matrix adhesion directly, but CD9 can make complexes with CD5, CD19, heparin-binding-epidermal growth factor, or β1 integrin on the plasma membrane (8, 9, 13–16, 33). Therefore, we examined whether other molecules are colocalized at the cell-cell adhesion site after stimulation with KMC8.8. As shown in Fig. 7, the localization of FcγRIIB/III, Mac-1 (α5β1 integrin), and ICAM-1 at the cell-cell adhesion site was observed after stimulation, and these molecules were colocalized with CD9 or F-actin at this site as detected by double staining (Fig. 7A, arrow). In contrast, β1 integrin did not change localization after stimulation, while F-actin did concentrate at the cell-cell adhesion site (Fig. 7B, arrow).

In addition, localization of the ganglioside GM1, used as a marker of DIGs, was also observed at the cell-cell adhesion site with FITC-conjugated CTx β subunit, suggesting that CD9 existed at DIGs (Fig. 7A).

FIGURE 6. Filopodia extension and colocalization of CD9 and F-actin at the cell-cell adhesion site induced by anti-CD9 mAb. J774 cells were stimulated with anti-CD9 mAb KMC8.8 at 37°C for 0, 5, and 60 min. After fixation, permeabilization, and blocking, CD9 and F-actin were stained with FITC-conjugated anti-rat IgG Ab or rhodamine-conjugated phalloidin. Right panels represent superimposed images. Upper three panels indicate the adhesive sections to a coverslip observed by using a confocal microscopy. The lowest panels show images of the middle section of the cells. After stimulation with the Ab, CD9 and F-actin were colocalized at the cell-cell adhesion site (arrows). Data are representative of four similar experiments.
Diminution of the signal from FcγRIIB/III or FcγR-CD9 cross-linking after extraction of cholesterol from DIGs

DIGs are postulated to represent plasma membrane domains that may function as centers for signal transduction and membrane trafficking (28, 29). However, FcγR has not been reported to be involved in DIGs, whereas Src family kinases, which have been implicated in the FcγR signals, exist in DIGs (28, 29). Therefore, we tested the effects of disrupting DIGs on FcγR-CD9 cross-linking by extracting cholesterol with MjβCD (28). The MjβCD treatment (20 mM for 30 min at 37°C) did not change the amount of FcγRIIB/III and CD9 on the cell surface (Fig. 8A). However, tyrosine phosphorylation signals induced by both 2.4G2 plus anti-rat IgG or KMC8.8 were diminished in a time-dependent manner (Fig. 8B). This result suggests that DIGs may function in FcγR signaling in J774 cells. The result that KMC8.8 induces strong signals of tyrosine phosphorylation, filopodium extension and cell aggregation without super cross-linking by a second Ab may be explained by localization of CD9 to a specific membrane structure like DIGs as reported in T cell activation induced by anti-CD9 mAb (13).

The localization of Mac-1 and F-actin at the cell-cell adhesion site induced by co-cross-linking of FcγRII and CD9

To investigate the contribution of FcγR to the colocalization of CD9, F-actin, FcγRIIB/III, and Mac-1 at the cell-cell adhesion site (Figs. 6 and 7), we examined the localization of these proteins by immunostaining before or after stimulation with KMC8.8. In both wild-type and FcγRIIB-/- bone marrow macrophages, CD9, F-actin, FcγRIIB/III (in wild type), or FcγRII (in FcγRIIB-/-) and Mac-1 were colocalized at the cell-cell adhesion site after stimulation (Fig. 9A and C, arrow). However, in FeR γ-chain-/- and FcγRIII-/- bone marrow macrophages, CD9 and FcγRIIB were colocalized at the cell-cell adhesion site, but F-actin and Mac-1 were not (Fig. 9, B and D, arrow). These results suggest that FcγRIII is crucial for the localization of F-actin and Mac-1 at the cell-cell adhesion site after stimulation with KMC8.8. In addition, it is possible that FcγRIIB can transduce some signal by cross-linking with CD9, because FcγRIIB and CD9 are colocalized at the cell-cell adhesion site in FcγRIII-deficient bone marrow macrophages.

Discussion

The interaction of FeR with immune complexes at the cell surface induces a wide variety of immune responses. Our studies demonstrate that CD9, a member of the tetraspan family, is functionally correlated with FcγRs on the macrophage. In a previous report, an Ab against another tetraspan molecule, CD82, activated the human monocyte cell line U937 and induced an increase in intracellular calcium after FcR-CD82 co-cross-linking (34). In addition, an interaction between FeR and CD9 has been reported on human platelets; a monoclonal anti-CD9 Ab treatment resulted in phosphorylation of syk, and an intact anti-CD9 Ab, but not F(ab’)2, induced rapid platelet aggregation (35). Moreover, recombinant FcγRIIa inhibited Fe-dependent anti-CD9-induced platelet aggregation in a dose-dependent manner (10). These results have not been observed in mouse cells, and it may result from the fact that human platelets express ITAM-containing FcγRIIA but mouse platelets do not. We demonstrated that treatment of an anti-CD9 mAb, KMC8.8, but
cross-linking with an anti-CD9 Fab(ab\textsuperscript{'})\textsubscript{2} followed by mouse anti-rat Fab Abs induced phosphorylation of syk in macrophages from Fc\textgamma {RIII}\textsuperscript{−/−} but not from FcR \( \gamma \)-chain\textsuperscript{−/−} mice. These results suggest that CD9 functionally associates with all Fc\textgamma {Rs} and modifies the signals.

Our data suggest that CD9 may be involved in Fc\textgamma {R}-mediated phagocytosis and enhance or modify it like Mac-1/CR-3, a major receptor in the phagocytosis of complement-opsonized targets (36, 37). Moreover, in our preliminary experiments, colocalization of FcR and CD9 was observed in the phagosome when IgG-opsonized particles were engulfed (data not shown). Recently, it has been demonstrated that the small G proteins Cdc42/Rac and Rho are necessary for Fc\textgamma {R}- and Mac-1-dependent phagocytosis, respectively (38), and the cross-talk of signal transduction among the small G proteins has been described (39). The excessive filopodium extension and concentration of F-actin at the cell-cell adhesion site induced by the CD9-Fc\textgamma {R} co-cross-linking are thought to be a result of the activation of the small G proteins, which regulates the actin cytoskeleton. In fact, CD9 has been reported to be associated with small G proteins (40). The CD9 and Fc\textgamma {R} co-cross-linking may specifically enhance the signals of small G proteins downstream of Fc\textgamma {R}, and the machinery could be involved in the Fc\textgamma {R}-Mac-1-dependent phagocytosis. We demonstrated the possibility that CD9 could work functionally in association with Fc\textgamma {R} in DIGs in J774 cells like CD9 in T cells behaving as a coreceptor of TCR in DIGs (13). In some cells, co-cross-linking between surface molecules is constitutively present in DIGs and ITAM-containing immunoreceptors, which induces recruitment to lipid raft and activation of the receptors. However, these phenomena may include some artifacts and do not reflect the biological function of these molecules. Concerning CD9 on bone marrow macrophages, M\textbeta {CD} does not cancel the activation with KMC8.8 out of accord with the experiment carried with J774 cells under the same condition (data not shown), so it is obscure whether DIGs contribute to the activation induced with KMC8.8. Therefore, further investigation is required.

We and others have shown that CD9-deficient mice exhibit defective cell fusion between sperm and eggs, but no significant immune system defects were observed (18–20). We revealed that the structure of microvilli on the egg plasma membrane seems to capture the sperm before fertilization evokes phagocytosis and that CD9 is localized at the sperm-egg adhesion site (20). Sperm-egg fusion must require tight adherence of the egg plasma membrane to the sperm, likely being mediated by the reorganization of the actin cytoskeleton. We suggest that CD9 functions in this process.

In vivo, the reorganization of the actin cytoskeleton after recognition of IgG immune complexes by Fc\textgamma {R} is extremely important, not only for internalization of the complex, but also for activation or stabilization of adhesion molecules, which lead to chemotaxis and invasion of the inflammatory site by monocytes/macrophages. CD9 has been reported to be involved in cell adhesion and motility in various types of cells, both of which require the reorganization of the cytoskeleton. Our findings demonstrate that co-cross-linking of CD9 and Fc\textgamma {R} activates macrophages; therefore, CD9 may play a role when Fc\textgamma {Rs} function in infection and inflammation on macrophages. The detailed function of CD9 on macrophages in vivo will be investigated in Ag-stimulated CD9-deficient mice.

Acknowledgments

We thank Drs. Christopher Paige and Heather Fleming for critical reading of the manuscript.
FIGURE 9. Localization of Mac-1 and F-actin at the cell-cell adhesion site induced by co-cross-linking of FcγRIII and CD9. A–D, Bone marrow macrophages from wild-type (A), FcR γ-chain−/− (B), FcγRIIB−/− (C), and FcγRIII−/− (D) mice were stimulated with anti-CD9 mAb KMC8.8 for 5 min at 37°C. Before or after stimulation with the Ab, CD9 and F-actin (A–D), FcγRIIB/III and F-actin (A), FcγRIIB and F-actin (B and D), FcγRIII and F-actin (C), Mac-1, and CD9 (A–D) were stained with Abs and reagents described as in Materials and Methods. Right panels present the superimposed images of the two left panels, respectively. Arrows indicate localization of each molecule detected with each Ab or the reagent at the cell-cell adhesion site. Data are representative of three similar experiments.