JCB Article

# Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes

**Yoshito Takeda,[1] Isao Tachibana,[1] Kenji Miyado,[2] Masatoshi Kobayashi,[2] Toru Miyazaki,[3] Toshiki Funakoshi,[1] Hiromi Kimura,[1] Hiroyuki Yamane,[1] Yoshiyuki Saito,[1] Hiroyuki Goto,[1] Tsutomu Yoneda,[1] Mitsuhiro Yoshida,[1] Toru Kumagai,[1] Tadashi Osaki,[1] Seiji Hayashi,[1] Ichiro Kawase,[1] and Eisuke Mekada[2]**

[1]Department of Molecular Medicine, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan
[2]Research Institute for Microbial Disease, Osaka University, Osaka 565-0871, Japan
[3]Center for Immunology, University of Texas Southwestern Medical Center, Dallas, TX 75390

Tetraspanins CD9 and CD81 facilitate the fusion between gametes, myoblasts, or virus-infected cells. Here, we investigated the role of these tetraspanins in the fusion of mononuclear phagocytes. Expression of CD9 and CD81 and their complex formation with integrins were up-regulated when blood monocytes were cultured under normal conditions. Under fusogenic conditions in the presence of Con A, CD9 and CD81 up-regulation was inhibited, and their complex formation with integrins was down-regulated. Anti-CD9 and -CD81 antibodies, which were previously shown to inhibit the fusion of gametes, myoblasts, and virus-infected cells, unexpectedly promoted the fusion of monocytes and alveolar macrophages. However, these effects were not due to altered cell adhesion, aggregation, or cytokine production. When stimulated in vitro or in vivo, alveolar macrophages and bone marrow cells of CD9- and CD81-null mice formed larger numbers of multinucleated cells than those of wild-type mice. Finally, CD9/CD81 double-null mice spontaneously developed multinucleated giant cells in the lung and showed enhanced osteoclastogenesis in the bone. These results suggest that CD9 and CD81 coordinately prevent the fusion of mononuclear phagocytes.

## Introduction

The tetraspanin proteins comprise at least 28 distinct members of transmembrane proteins that include CD9, CD37, CD53, CD63, CD81, CD82, and CD151. All these proteins share a characteristic structure that spans the membrane four times and thereby forms two extracellular loops. Tetraspanins complex with transmembrane proteins such as CD4, CD8, CD19, CD21, CD46, major histocompatibility complex class I and II proteins, and integrins. They also bind to intracellular signaling molecules including phosphatidylinositol 4-kinase, phosphatases, and small GTP-binding proteins. It is thought that, by facilitating the formation of these multimolecular complexes, tetraspanins play roles in cell activation, proliferation, differentiation, motility, fusion, and apoptosis (Boucheix and Rubinstein, 2001).

Although their multifunctional characteristics and multipartnerships with other proteins have been increasingly reported, the definitive biological functions of tetraspanins still remain elusive. In this respect, studies of tetraspanin knockout mice have revealed the cellular functions for which a given tetraspanin is essential. One of these is a role of CD9 in gamete membrane fusion; CD9 knockout mice were infertile because CD9-null eggs were incapable of fusing with sperm (Miyado et al., 2000). Tetraspanins may play a more general role in cell–cell fusion because additional reports have implicated tetraspanins in other fusion events. Antibodies against CD9 and CD81 inhibit the fusion of myoblasts, and CD9 transfection into myoblast-derived sarcoma cells enhances syncytium formation (Tachibana and Hemler, 1999). Anti-CD81 and -CD82 mAbs perturb the fusion of cells infected with human T cell leukemia virus type 1 (Fukudome et al., 1992). CD9 overexpression renders cells more susceptible to feline immunodeficiency virus and canine distemper virus, leading to elevated syncytium formation (Löffler et al., 1997; Willett et al., 1997). These results suggest that tetraspanins facilitate the fusion between gametes, myoblasts, and virus-infected cells. However, it is still unknown

Address correspondence to Isao Tachibana, Dept. of Molecular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-3833. Fax: 81-6-6879-3839. E-mail:itachi02@imed3.med.osaka-u.ac.jp

Key words: integrins; osteoclasts; cell fusion; macrophages; multinucleated giant cells

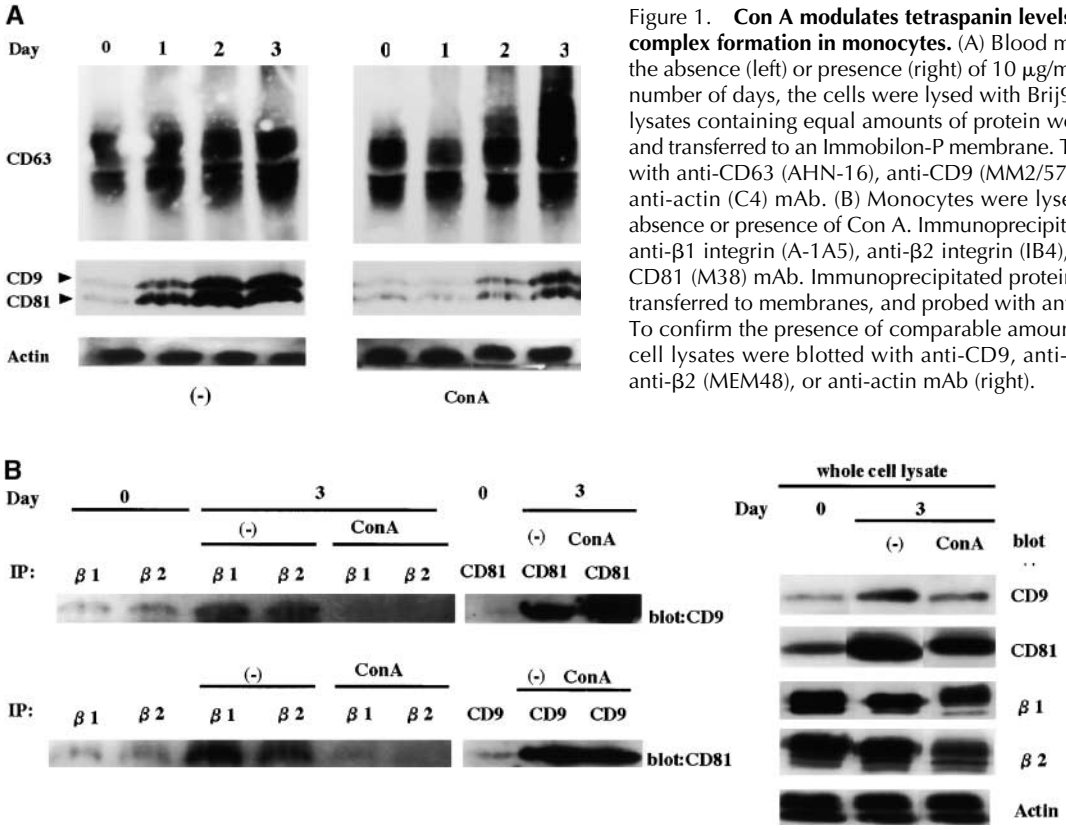


Figure 1. **Con A modulates tetraspanin levels and integrin–tetraspanin complex formation in monocytes.** (A) Blood monocytes were cultured in the absence (left) or presence (right) of 10 μg/ml Con A. After the indicated number of days, the cells were lysed with Brij99 lysis buffer. Whole-cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to an Immobilon-P membrane. The membranes were blotted with anti-CD63 (AHN-16), anti-CD9 (MM2/57) plus anti-CD81 (M38), or anti-actin (C4) mAb. (B) Monocytes were lysed at d 0 or at d 3 in the absence or presence of Con A. Immunoprecipitations were performed with anti-β1 integrin (A-1A5), anti-β2 integrin (IB4), anti-CD9 (BU16), or anti-CD81 (M38) mAb. Immunoprecipitated proteins were electrophoresed, transferred to membranes, and probed with anti-CD9 or -CD81 mAb (left). To confirm the presence of comparable amounts of each protein, whole-cell lysates were blotted with anti-CD9, anti-CD81, anti-β1 (A-1A5), anti-β2 (MEM48), or anti-actin mAb (right).

whether they play a similar role in cell fusion into other multinucleated cells, such as multinucleated giant cells (MGCs)* or osteoclasts.

Syncytia formed after the fusion of mononuclear phagocytes are called MGCs or osteoclasts. Multinucleation via cell fusion appears to endow monocytes/macrophages with the capacity to digest and resorb extracellular infectious agents, foreign materials, and other components that are too large to be internalized (Vignery, 2000). The presence of MGCs is a hallmark of granulomas, which are formed in inflammatory sites of tuberculosis, fungal infection, HIV infection, sarcoidosis, Crohn's disease, and tumors (Anderson, 2000; James, 2000). The physiological meanings of MGCs still remain unknown, but possible roles in the host defense against bacterial infection have been suggested; MGCs may limit the cell-to-cell spread of *Mycobacterium tuberculosis* (Byrd, 1998) and may have stronger candidacidal activity than macrophages (Enelow et al., 1992). Osteoclasts are formed by the fusion of mononuclear progenitors of the monocyte/macrophage lineage. These polykaryons are characterized by the presence of tartrate-resistant acid phosphatase (TRAP) activity and have a crucial role not only in physiological bone remodeling, but also in local bone disorders such as osteoporosis and bone tumors. However, the actual cut-off line that discriminates between osteoclasts and MGCs remains controversial (Vignery, 2000).

*Abbreviations used in this paper: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; BAL, bronchoalveolar lavage; MGC, multinucleated giant cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; sRANKL, soluble RANKL; TRAP, tartrate-resistant acid phosphatase.

The mechanisms of the fusion of mononuclear phagocytes are not well understood, but previous papers have shown that several membrane proteins, such as CD44, CD47, CD98, macrophage fusion receptor, P2X$_7$ receptor, ADAMs, and integrins, are involved (Vignery, 2000; Namba et al., 2001). In the present paper, we show that tetraspanins CD9 and CD81 play a preventive role in the fusion of mononuclear phagocytes.

## Results

### Con A modulates tetraspanin levels and integrin–tetraspanin complex formation in monocytes

MGCs can be generated in vitro in different ways by stimulating human blood monocytes or alveolar macrophages with cytokines (Fais et al., 1994), phorbol myristate acetate (Hassan et al., 1989), lectins (Chambers, 1977), conditioned media (Abe et al., 1991), or mAbs (Tabata et al., 1994). We isolated monocytes from human peripheral blood and allowed them to attach to culture plate surfaces in the presence of serum for 3 d, but the monocytes were not able to fuse into MGCs. However, on stimulation with Con A, cell–cell fusion occurred and many syncytia were formed within 3 d of incubation (see following paragraph). We examined the expression of six tetraspanin proteins (CD9, CD63, CD81, CD82, CD151, and NAG-2) by flow cytometry, and confirmed that all of these tetraspanins except NAG-2 were present on blood monocytes (unpublished data). To analyze the expression in detail, the time courses of CD9, CD63, and CD81 expression were examined by immunoblotting (Fig. 1 A). When blood monocytes were cul-

tured under normal conditions, levels of CD9 and CD81 were up-regulated, reached a peak at ~2 d, and were sustained until 3 d after incubation. CD63 also appeared to be gradually up-regulated (Fig. 1 A, left). Notably, when monocytes were cultured in the presence of Con A, the up-regulation of CD9 and CD81 was inhibited compared with that under normal conditions. In contrast, the up-regulation of CD63 was enhanced in the presence of Con A (Fig. 1 A, right). Control anti-actin blots showed that comparable amounts of protein were loaded in each lane.

The up-regulation of tetraspanin–integrin complex formation during myoblast fusion has been reported (Tachibana and Hemler, 1999). Among integrins, the β1 subfamily most commonly associates with tetraspanins, but a β2 integrin, αLβ2, also complexes with tetraspanins in hematopoietic cells. Tetraspanins also form complexes with other tetraspanins (Boucheix and Rubinstein, 2001). In freshly isolated blood monocytes, CD9 and CD81 associated with β1 and β2 integrins and with each other as shown in coimmunoprecipitation experiments (Fig. 1 B, left). During the culture under normal conditions, the formation of tetraspanin–integrin and CD9–CD81 complexes was up-regulated (compare d 3 with d 0, "–"). Notably, during multinucleation under fusogenic conditions containing Con A, the formation of tetraspanin–integrin complexes was instead down-regulated. On the other hand, the up-regulation of the CD9–CD81 complex formation was not affected by the presence of Con A (compare d 3 with d 0, "Con A"). In control immunoblot-

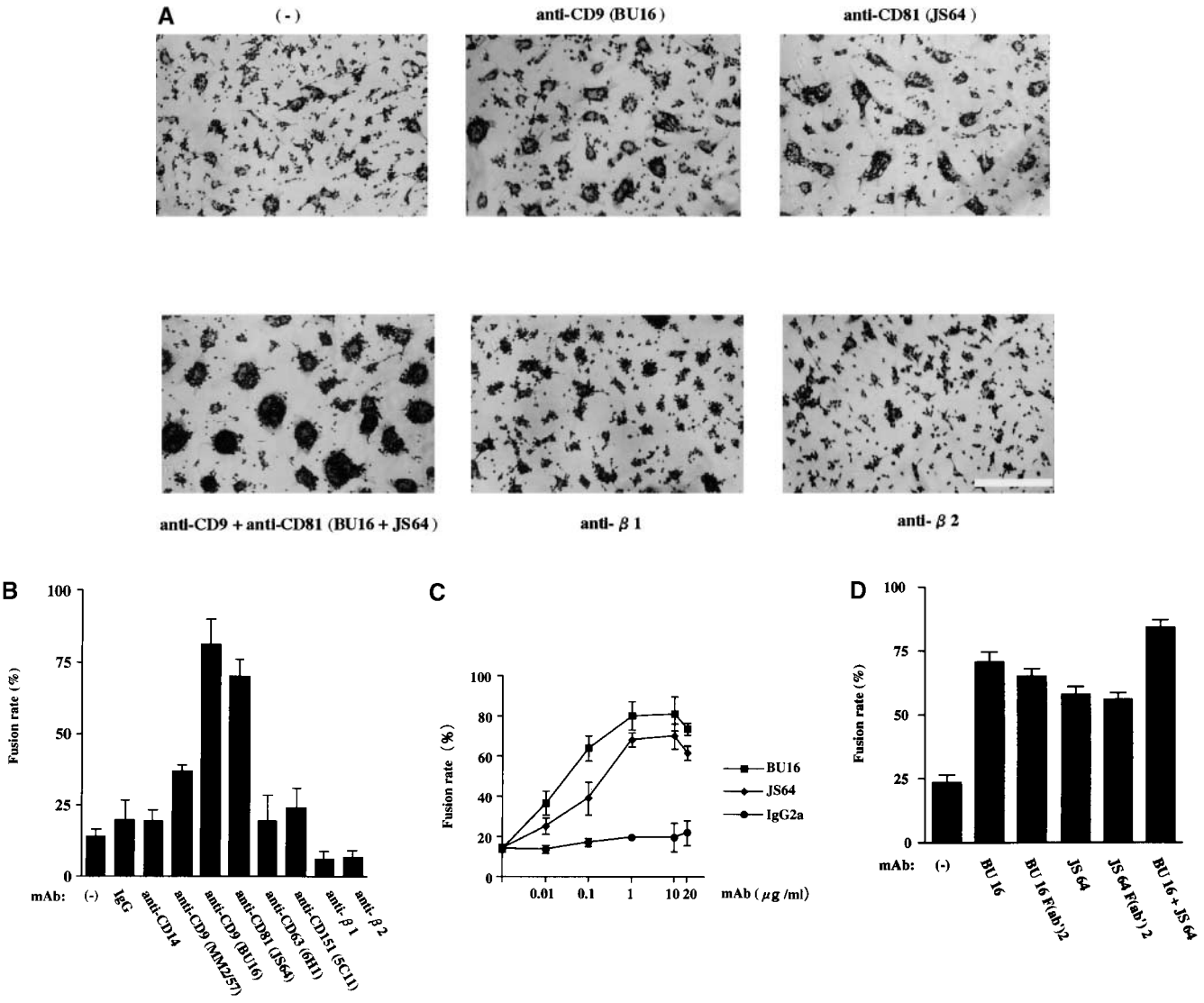


Figure 2. **Anti-CD9 and -CD81 mAbs promote the fusion of blood monocytes.** (A) Blood monocytes were induced to fuse into MGCs in culture medium containing 10 μg/ml Con A for 3 d in the absence or presence of 10 μg/ml of the indicated mAbs. Nuclei were then visualized using Wright stain. Bar, 250 μm. (B) Monocytes ($2 \times 10^5$) were plated into the wells of a 96-well tissue culture plate and induced to fuse in the absence or presence of 10 μg/ml of the indicated mAbs. Fusion rates were determined by calculating the percentages of the number of nuclei within MGCs (three or more nuclei per cell) per total number of nuclei. (C) Monocytes were induced to fuse in the absence or presence of increasing concentrations of anti-CD9 (BU16), anti-CD81 (JS64) mAbs, or isotype-matched IgG, and then fusion rates were determined. (D) Monocytes were induced to fuse in the absence or presence of 10 μg/ml of the indicated mAbs (in the case of BU16 + JS64, 10 μg/ml of each was used). Fusion rates were then determined. Each bar and data point represent the mean ± SD.

ting using whole-cell lysates (Fig. 1 B, right), the up-regulation of CD9 and CD81 under normal conditions was confirmed as already shown in Fig. 1 A. The presence of Con A inhibited this up-regulation, but even under these conditions, higher levels of CD9 and CD81 appeared at d 3 compared with the level at d 0. The expression of β1 and β2 integrins was not much affected by these culture conditions. Thus, the down-regulation of CD9 and CD81 in β1 or β2 immunoprecipitates under fusogenic conditions was not due to the reduction of total CD9, CD81, β1, or β2 proteins.

### Anti-CD9 and -CD81 mAbs promote the fusion of blood monocytes/alveolar macrophages

Anti-CD9 and -CD81 antibodies were previously shown to inhibit the fusion of sperm and egg (Takahashi et al., 2001), myoblasts (Tachibana and Hemler, 1999), or virus-infected cells (Fukudome et al., 1992; de Parseval et al., 1997). To examine the role of tetraspanins in monocyte fusion, we added anti-tetraspanin mAbs to the monocyte culture under fusogenic conditions containing Con A. Unexpectedly, anti-CD9 (BU16) and anti-CD81 (JS64) mAbs dramatically promoted monocyte fusion (Fig. 2). The fusion rates in the presence of BU16 and JS64 were elevated 3.5-fold and fourfold relative to those in control IgG cultures, respectively. Moreover, MGCs formed in the presence of these mAbs were larger in size than the control MGCs (Fig. 2 A). Another anti-CD9 mAb, MM2/57, also promoted the fusion, although its effect was weaker than those of BU16 and JS64. On the other hand, anti-CD14, -CD63, and -CD151 mAbs had little, if any, fusion-promoting effects. Anti-integrin β1 and β2 mAbs significantly inhibited the fusion (Fig. 2, A and B), consistent with previous reports (Most et al., 1990; Tabata et al., 1994). To examine the dose dependency, fusion rates in the presence of various concentrations of BU16, JS64, and isotype-matched IgG were determined (Fig. 2 C). The fusion-promoting effects of BU16 and JS64 were dose dependent, reached a plateau at 1–10 μg/ml, and decreased slightly at 20 μg/ml. Meanwhile, the isotype-matched control IgG had little effect on monocyte fusion even at 20 μg/ml. To exclude the possibility that the effects of BU16 and JS64 were mediated by Fc receptors, we generated divalent $F(ab')_2$ fragments and examined their effects on monocyte fusion. As shown in Fig. 2 D, these $F(ab')_2$ fragments exerted similar degrees of fusion-promoting effects as untreated mAbs. Finally, the coaddition of anti-CD9 and -CD81 mAbs appeared to have additive effects on the monocyte fusion (Fig. 2, A and D).

Next, we isolated alveolar macrophages from human lungs and examined the effects of these mAbs on the fusion of these cells. Like blood monocytes, alveolar macrophages were capable of fusing into MGCs in response to stimulation by Con A. Anti-CD9 and -CD81 mAbs enhanced the fusion of alveolar macrophages as well as that of blood monocytes. In contrast, anti-integrin β1 and β2 mAbs inhibited their fusion (unpublished data).

### Anti-CD9 and -CD81 mAbs do not affect cell adhesion, aggregation, proliferation, or cytokine production

The effects of anti-CD9 and -CD81 mAbs may have been due to altered cell adhesion or aggregation, both of which are prerequisite for cell–cell fusion. To examine these possibilities, we performed adhesion and aggregation assays. As shown in Fig. 3 A, anti-β1 and -β2 mAbs significantly in-

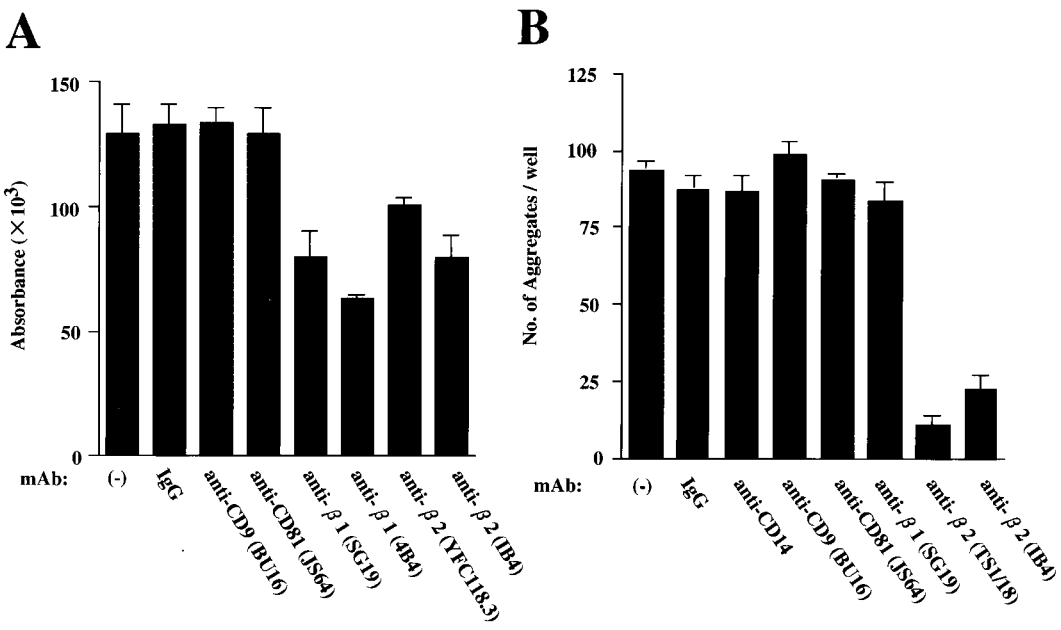


Figure 3. **Anti-CD9 and -CD81 mAbs do not affect monocyte adhesion or aggregation.** (A) Blood monocytes ($2 \times 10^5$) were suspended in RPMI 1640 containing 10 μg/ml Con A, and were allowed to adhere to the wells of a 96-well culture plate for 12 h in the absence or presence of 10 μg/ml of the indicated mAbs. Nonadherent cells were removed, and the remaining adherent cells were evaluated using an MTT assay. (B) Monocytes ($2 \times 10^5$) were cultured in RPMI 1640 containing 5 μg/ml Con A in the absence or presence of 10 μg/ml mAbs for 12 h on the wells of a 96-well nontissue culture-treated plate. The numbers of cell aggregates (>4 cells/aggregate) were determined under a light microscope. Each bar represents the mean ± SD.

hibited monocyte adherence to the tissue culture–treated surfaces. This is consistent with a previous report showing that β1 and β2 integrins mediate adhesion to culture surfaces during macrophage fusion (McNally and Anderson, 2002). Meanwhile, anti-CD9 and -CD81 mAbs, and control IgG had no effect on the monocyte adherence. In aggregation assays, no mAb had a significant effect on the monocyte aggregation that was induced by 10 μg/ml Con A (unpublished data). However, when a lower concentration of Con A (5 μg/ml) was used, anti-β2 integrin mAbs markedly inhibited the monocyte aggregation (Fig. 3 B). On the other hand, neither anti-β1 integrin mAb, anti-CD9 and -CD81 mAbs, anti-CD14 mAb, nor control IgG affected the monocyte aggregation. We also examined whether anti-CD9 and -CD81 mAbs alter the proliferation of monocytes using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and observed that these mAbs had no effect on monocyte proliferation (unpublished data).

Previous reports suggested that activated macrophages secrete cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, and that these cytokines play an important role in MGC formation (Anderson, 2000). Accordingly, we investigated whether anti-CD9 and -CD81 mAbs affect the production of these cytokines by Con A–stimulated monocytes. Blood monocytes were cultured for 3 d with Con A in the absence or presence of mAbs, and then the concentrations of TNF-α, IL-1β, and IL-6 in culture supernatants were determined using enzyme-linked immunosorbent assays. The supernatants of monocytes cultured without Con A (normal conditions) were also examined in parallel. As compared with normal conditions, Con A remarkably enhanced the production of these cytokines by monocytes. The up-regulation of TNF-α, IL-1β, and IL-6, was 23-, 59-, and 48-fold, respectively (380 to 8730 pg/ml for TNF-α, 50 pg/ml to 2970 pg/ml for IL-1β, and 230 pg/ml to 10980 pg/ml for IL-6). The presence of anti-tetraspanin, integrin, or control mAbs had no significant effect on this up-regulation (unpublished data).

## Soluble GST–CD9 large extracellular loop protein inhibits monocyte fusion

There was a possibility that the results of the antibody-blocking experiments were caused by steric hindrance to molecules adjacent to the tetraspanins. Thus, to extend the observations made in the antibody experiments, we studied effects of a recombinant GST fusion protein that contained the large extracellular loop of human CD9 (GST–CD9; Shimizu et al., 2002). Various concentrations of GST–CD9 were added to the monocyte culture under fusogenic conditions containing Con A. As shown in Fig. 4, GST–CD9 inhibited monocyte fusion, and this inhibitory effect was dose-dependent in the range of 0.2–20 μg/ml. On the other hand, neither GST alone nor GST–murine CD9 fusion protein (GST–mCD9) had significant fusion-inhibitory effect even at 20 μg/ml.

## Anti-CD9 and -CD81 mAbs promote the fusion of murine alveolar macrophages and bone marrow cells

To further extend our observations on cell fusion of the monocyte/macrophage lineage, murine alveolar macrophages

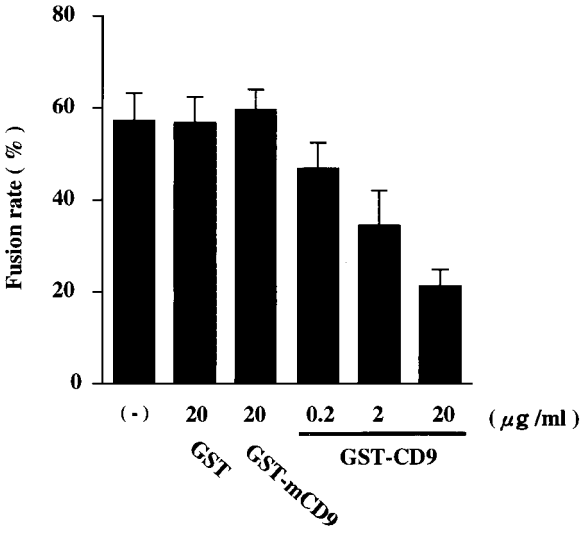


Figure 4. **Soluble GST–CD9 large extracellular loop protein inhibits monocyte fusion.** Blood monocytes were induced to fuse into MGCs in culture medium containing Con A for 3 d in the absence or presence of the indicated concentrations of GST, GST–mCD9, or GST–CD9. Fusion rates were then determined. A higher concentration (15 μg/ml) of Con A was used in order to show the dose-dependent, fusion-inhibitory effect of GST–CD9. Each bar represents the mean ± SD.

were isolated by bronchoalveolar lavage (BAL) and induced to fuse into MGCs by the addition of 1α,25-dihydroxyvitamin $D_3$ ($1\alpha,25(OH)_2D_3$) and culture supernatants of Con A–stimulated spleen cells as described previously (Abe et al., 1983). We performed an immunofluorescence study because CD9 protein on murine alveolar macrophages was resistant to fixation and permeabilization procedures. As compared with control IgG (Fig. 5 A), staining of CD9 was positive in freshly isolated murine alveolar macrophages (Fig. 5 B), and it was up-regulated after 3 d of incubation under normal culture conditions, especially at the cell periphery and cell–cell contacts (Fig. 5 C). Conversely, fusogenic culture conditions appeared to inhibit this CD9 up-regulation (Fig. 5 D). CD9 expression was relatively weak in unfused macrophages and MGCs, and its distribution was more obvious at the perinuclear region than at the cell periphery in MGCs (Fig. 5 D, inset). The findings of immunoblotting were consistent with those of the immunofluorescence. Up-regulation of CD9 was observed under the normal culture conditions, whereas the fusogenic conditions suppressed this up-regulation (Fig. 5 E).

To examine the effects of anti–mouse tetraspanin mAbs, anti–mouse CD9 and CD81 mAbs were added to murine alveolar macrophages under fusogenic conditions. Because murine alveolar macrophages formed smaller numbers of MGCs than human monocytes/macrophages, we evaluated their fusion by determining the numbers of MGCs instead of fusion rates. As shown in Fig. 6, anti–mouse CD9 and CD81 mAbs promoted the fusion of murine macrophages. Moreover, the combination of anti-CD9 and -CD81 mAbs exerted an additive effect. The effect of anti-CD9 mAb, KMC8, was not mediated by Fc portion–Fc receptor interaction, because the $F(ab')_2$ fragments exerted a quite similar effect (Fig. 6 B).

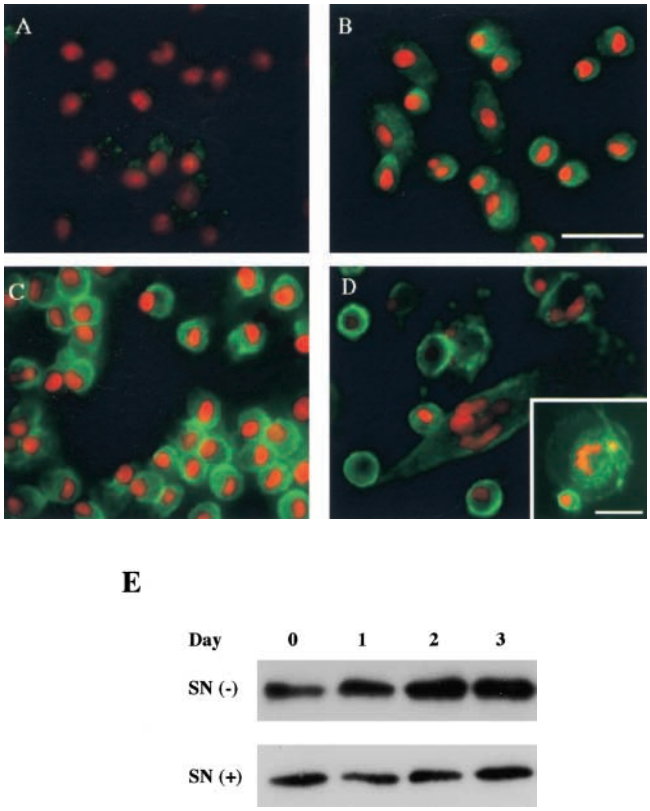


Figure 5. **Modulation of the expression and distribution of CD9 in murine alveolar macrophages under fusogenic conditions.** Murine alveolar macrophages were cultured in DME containing 5% human serum for 3 d under normal conditions (C) or fusogenic conditions containing $1\alpha,25(OH)_2D_3$ and culture supernatant from Con A–stimulated splenocytes (D). Macrophages were immunostained at d 0 (A and B) and at d 3 (C and D) with control mAb (A) or anti-CD9 mAb (B–D). Cells and nuclei were visualized with FITC-conjugated anti–rat immunoglobulin and propidium iodide. Typical CD9 distribution in an MGC is shown in the inset. Bar, 250 μm (100 μm for the inset). (E) Murine alveolar macrophages were cultured in the absence or presence of $1\alpha,25(OH)_2D_3$ and supernatant (SN) from Con A–stimulated splenocytes. After the indicated number of days, cells were lysed, and whole-cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred to a membrane, and probed with anti-CD9 mAb (KMC8).

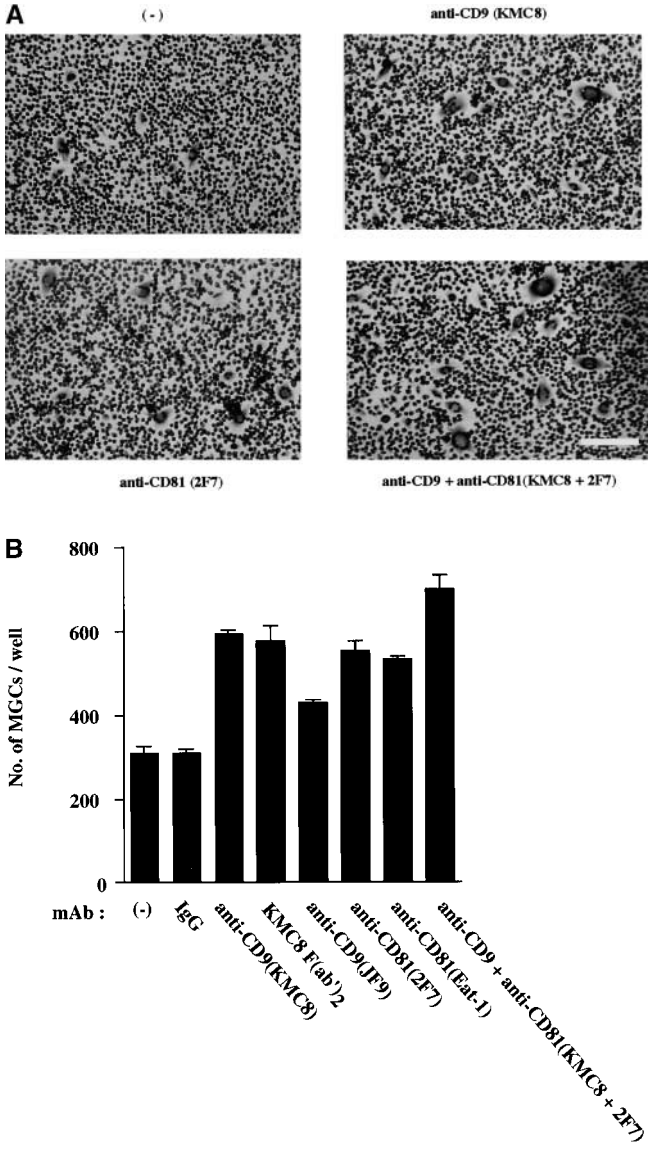


Figure 6. **Anti-CD9 and -CD81 mAbs promote the fusion of murine alveolar macrophages.** (A) Murine alveolar macrophages were induced to fuse into MGCs under fusogenic conditions containing $1\alpha,25(OH)_2D_3$ and culture supernatant from Con A–stimulated splenocytes for 3 d in the absence or presence of 10 μg/ml of the indicated mAbs. Nuclei were then visualized using Wright stain. Bar, 250 μm. (B) Murine alveolar macrophages ($3 \times 10^5$) were plated into the wells of a 96-well culture plate and induced to fuse in the absence or presence of 10 μg/ml control IgG or various anti-CD9 and -CD81 mAbs (in the case of KMC8 + 2F7, 10 μg/ml of each was used). The numbers of MGCs per well were determined. Each bar represents the mean ± SD.

Next, we examined the effects of anti–mouse CD9 and CD81 mAbs in the fusion of osteoclast progenitor cells. Murine bone marrow cells were isolated from tibiae and induced to fuse by the addition of soluble RANKL (sRANKL) and M-CSF (Li et al., 2002). Like alveolar macrophages, the addition of these mAbs promoted the fusion of bone marrow cells. Relative to control IgG, numbers of TRAP-positive multinucleated cells in the presence of anti-CD9 mAb (KMC8) and anti-CD81 mAb (2F7) increased fourfold and twofold, respectively (unpublished data).

## Enhanced cell fusion by CD9- and CD81-null alveolar macrophages and bone marrow cells after in vitro or in vivo stimulation

To determine more definitively the roles of CD9 and CD81 in monocyte/macrophage fusion, we investigated MGC formation of CD9- and CD81-deficient murine alveolar macrophages. Alveolar macrophages were isolated by BAL from the lungs of wild-type, CD9-null, and CD81-null mice, but no MGCs were present in these mice (unpublished data). Equal numbers of collected macrophages were then induced to fuse into MGCs in vitro. Notably, after the stimulation, CD9 (−/−) and CD81 (−/−) alveolar macrophages formed threefold and fourfold more MGCs than wild-type macrophages, respectively (Fig. 7 A). To further examine if the deficiency of CD9 or CD81 alters MGC formation in vivo, we used a *Propionibacterium acnes*–induced lung inflammation

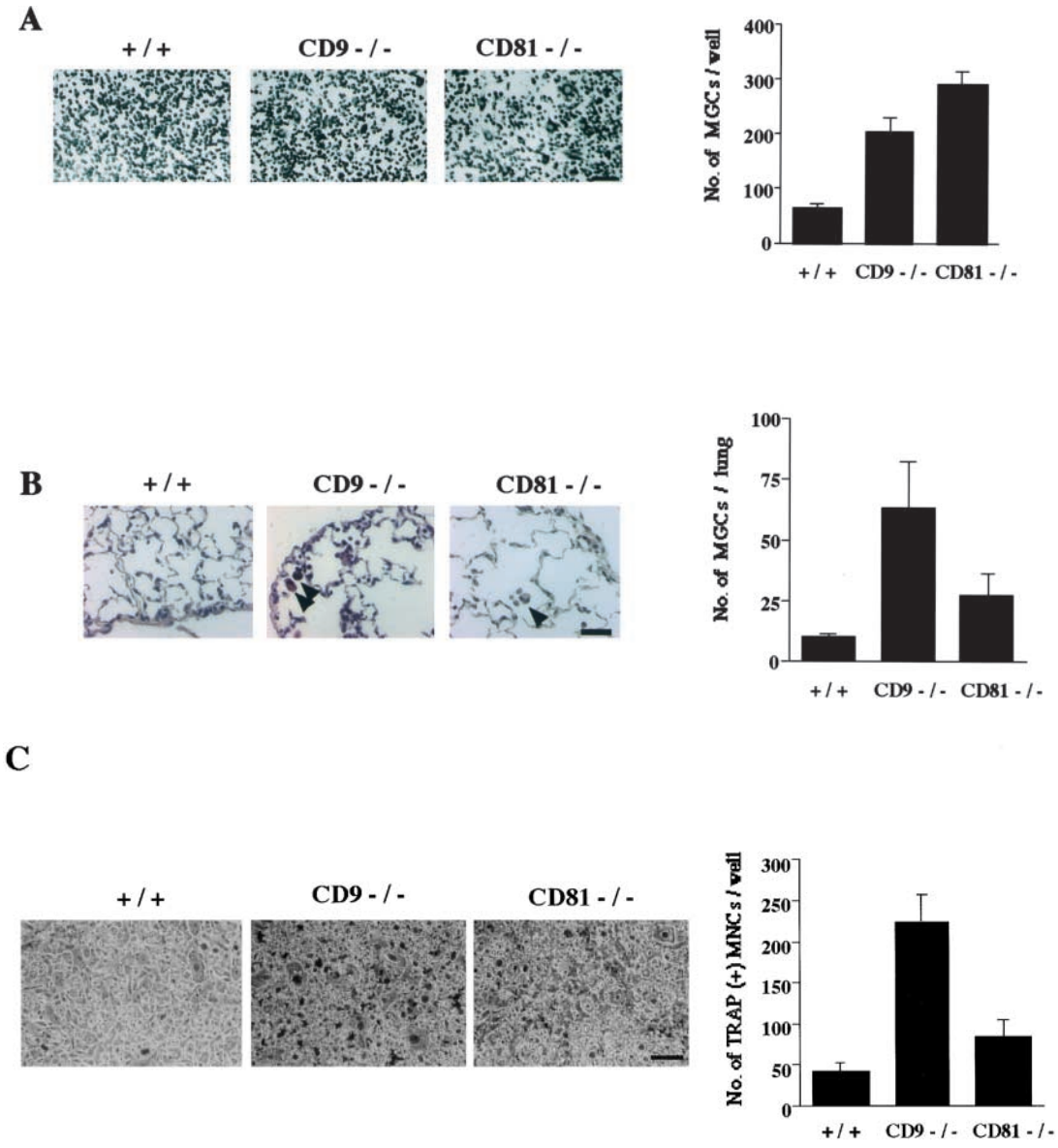


Figure 7. **Enhanced cell fusion by CD9- and CD81-null murine alveolar macrophages and bone marrow cells after in vitro or in vivo stimulation.** (A) Alveolar macrophages ($3 \times 10^5$) from wild-type, CD9 (−/−), and CD81 (−/−) mice were plated into the wells of a 96-well culture plate and induced to fuse into MGCs by a 3-d incubation with $1\alpha,25(OH)_2D_3$ and splenocyte-conditioned medium. Nuclei were then visualized using Wright stain (left). The numbers of MGCs per well were determined (right). (B) 300 μg heat-killed *P. acnes* was administered intratracheally to wild-type, CD9 (−/−), and CD81 (−/−) mice. After 7 d, lung paraffin sections were stained with hematoxylin and eosin (left). Arrowheads indicate MGCs. In a separate experiment, alveolar macrophages were isolated by BAL from the lung, and the numbers of MGCs per lung were determined (right). Assays were done in triplicate for each animal tested. Two additional experiments gave similar results. (C) Bone marrow cells ($2 \times 10^6$) from wild-type, CD9 (−/−), and CD81 (−/−) mice were plated into the wells of a 24-well culture plate and induced to fuse by a 7-d incubation with sRANKL and M-CSF. Cells were then stained for TRAP (left). The numbers of TRAP-positive multinucleated cells (MNCs) per well were determined (right). Bar, 100 μm (A–C). Each bar represents the mean ± SD.

model (Itakura et al., 2001). Wild-type and mutant mice were challenged by intratracheal injection of heat-killed *P. acnes*. After 7 d, lung paraffin sections were prepared and analyzed to detect MGCs. Although infiltration of inflammatory cells consisting of neutrophils, macrophages, and lymphocytes into the alveolar space was reported ~3 d after *P. acnes* injection (Itakura et al., 2001), most of cells remaining at d 7 were macrophages. Remarkably, although few MGCs were present in wild-type mice, substantial numbers of MGCs were formed in the airspace of CD9- and CD81-null mice (Fig. 7 B, left). In a separate experiment to quantify the MGC formation, alveolar macrophages were separated by BAL after *P. acnes* injection, and the number of MGCs was determined (Fig. 7 B, right). The total numbers of cells isolated from wild-type and mutant mice were not significantly different (unpublished data), but nonetheless, sixfold and threefold more MGCs were detected in CD9- and CD81-null mice compared with wild-type mice, respectively.

We also isolated bone marrow cells from wild-type, CD9-null, and CD81-null mice and induced these cells to fuse in

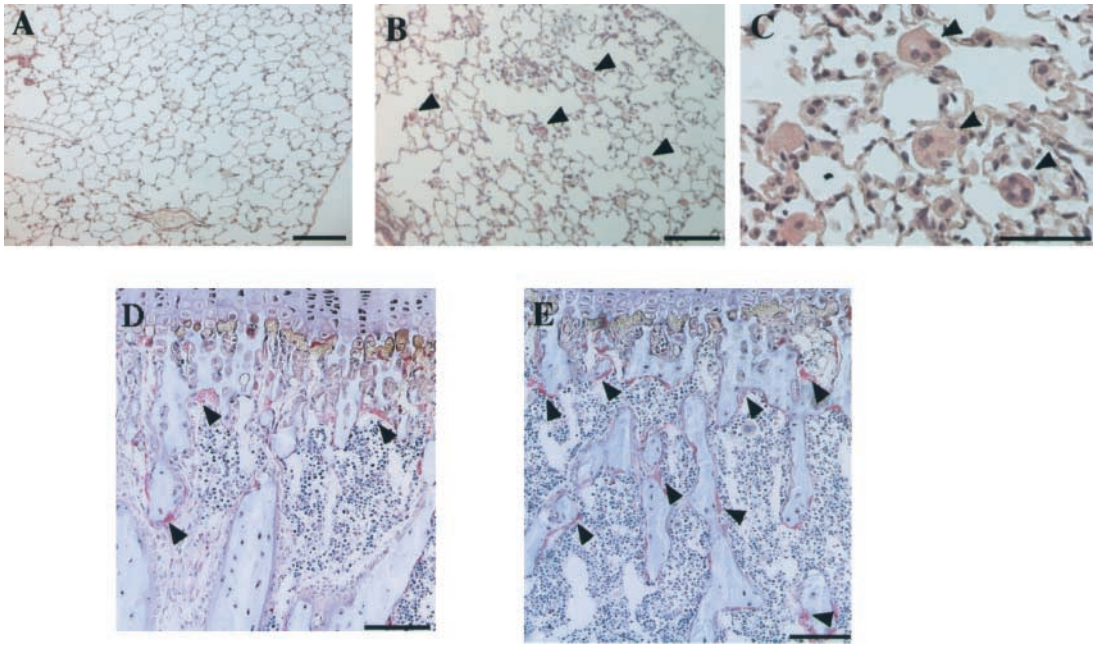


Figure 8. **Histological sections of the lung and bone from wild-type and CD9/CD81 double-null mice.** Lung sections from wild-type (A) and CD9 (−/−)/CD81 (−/−) mice (B and C) aged 8 wk were stained with hematoxylin and eosin. Bone sections of the proximal tibia from wild-type (D) and CD9 (−/−)/CD81 (−/−) mice (E) were stained for TRAP. Arrowheads indicate typical MGCs (B and C) and osteoclasts (D and E). Bars: 250 μm for A and B, 100 μm for C–E.

vitro by the addition of sRANKL and M-CSF. TRAP-positive multinucleated cells generated from CD9- and CD81-null marrow cells were larger in size and increased sixfold and twofold in number, respectively, compared with those from wild-type marrow cells (Fig. 7 C).

### CD9/CD81 double-null mice spontaneously develop MGCs in the lung and showed enhanced osteoclastogenesis in the bone

Because CD9 and CD81 appear closely related in view of amino acid sequence (the identity in the aligned human sequences is ∼31%; Boucheix and Rubinstein, 2001), one may compensate for the function of the other in CD9 or CD81 single-null mice. Therefore, we generated CD9/CD81 double-null mice by intercrossing CD9 (+/−)/CD81 (+/−) mice. CD9/CD81 double-null mice were viable, but smaller in size than wild-type mice and displayed osteopenic phenotype in association with angular kyphotic deformity of the spine (unpublished data). Fig. 8 shows hematoxylin- and eosin-stained lung sections and TRAP-stained bone sections from wild-type and double mutant mice. Notably, alveolar airspace enlargement with the infiltration of macrophages and a smaller number of lymphocytes into the space and septa was observed in CD9/CD81 double-null mice (Fig. 8, B and C). More importantly, although no MGCs were detected in wild-type (Fig. 8 A) or CD9 (+/−)/CD81 (+/−) mice (unpublished data), eosin-stained MGCs were spontaneously formed in the alveolar space of double mutant mice (Fig. 8, B and C). Most of these MGCs were large, round, eosin-stained cells containing 3 to ∼10 nuclei, and resembled those detected in CD9 or CD81 single-null mice that had been stimulated by intratracheal injection of *P. acnes* (Fig. 7 B). Few neutrophils or eosinophils were present, thus ruling out the occurrence of bacterial or parasitic infection. Findings from bones of CD9/CD81 double-null mice were also remarkable (Fig. 8 E). When compared with the wild-type (Fig. 8 D) and single-null littermates, TRAP-positive osteoclasts in tibiae increased 1.5-fold in number per millimeter bone surface (wild type, 1.78 ± 0.19/mm; CD9 (−/−) mice, 1.79 ± 0.70/mm; CD81 (−/−) mice, 1.67 ± 0.22/mm; CD9 (−/−)/CD81 (−/−) mice, 2.61 ± 0.29/mm; $P < 0.005$ versus wild type in the *t* test, $n = 4$). Dual-energy X-ray absorptiometry on femurs showed that bone-mineral density of double mutant mice were significantly reduced (wild-type, 25.1 ± 1.8 mg/cm$^2$; CD9 (−/−) mice, 26.1 ± 0.4 mg/cm$^2$; CD81 (−/−) mice, 25.5 ± 1.7 mg/cm$^2$; CD9 (−/−)/CD81 (−/−) mice, 22.0 ± 1.5 mg/cm$^2$; $P < 0.05$ versus the wild type).

## Discussion

To induce fusion between monocytes in vitro, we used cultures containing Con A, because Con A readily induces monocyte fusion in a dose- and time-dependent manner (Takashima et al., 1993). A time-course analysis based on immunoblotting revealed that the up-regulation of the CD63 level was moderate under normal culture conditions, whereas stronger elevation of CD63 was observed under fusogenic conditions containing Con A. On the other hand, although CD9 and CD81 were up-regulated under normal conditions, their up-regulation was rather inhibited under fusogenic conditions. The findings of immunofluorescence and immunoblotting analysis of murine alveolar macrophages were in line with this. CD9 distribution into the cell periphery and cell–cell contacts was less marked under fuso-

genic conditions. Moreover, CD9 staining was more obvious at the perinuclear area than at the cell periphery in MGCs. Consistent with these findings, a recent paper reported CD9 down-regulation in murine peritoneal macrophages stimulated with IFN-γ, which is a cytokine capable of promoting fusion of macrophages (Wang et al., 2002). These results suggest that the functions of CD9 and CD81 may be suppressed under fusogenic conditions, and that the functional contribution of these tetraspanins to monocyte/macrophage fusion might be different from that of CD63.

All of anti-tetraspanin mAbs used in fusion assays of the present work were previously shown to inhibit cellular functions in vitro. In particular, the anti–human CD9 mAbs MM2/57 and BU16 were reported to inhibit virus-induced fusion (Löffler et al., 1997). The anti–mouse CD9 mAbs KMC8 and JF9 were reported to prevent sperm–egg fusion (Miyado et al., 2000; Takahashi et al., 2001). KMC8 and anti–mouse CD81 mAb 2F7 inhibited/delayed the fusion of the myoblast cell line C2C12 (Tachibana and Hemler, 1999). These anti-CD9 and -CD81 mAbs unexpectedly promoted the fusion of mononuclear phagocytes. It is unlikely that the effects were mediated by Fc portion–Fc receptor interactions, because isotype-matched control IgG had no effect on the fusion, and $F(ab')_2$ fragments had similar effects to untreated mAbs. These results raise the possibility that CD9 and CD81 facilitate the fusion of virus-infected cells, sperm–egg, and myoblasts, while preventing the fusion of monocytes/macrophages via the same epitopes. A previous work used a soluble GST–CD81 extracellular loop fusion protein to demonstrate that this protein bound to neurons but not to astrocytes, and that it blocked neuron-induced astrocyte proliferative arrest, thus suggesting the presence of a ligand for CD81 on neurons (Kelic et al., 2001). In the present work, the GST–CD9 extracellular loop fusion protein inhibited monocyte fusion in a dose-dependent manner. This result further supports the involvement of CD9 protein in monocyte fusion, and may reflect a functional interaction between the CD9 extracellular loop and its putative ligand on an apposed cell surface.

With regard to genuine tetraspanins (Boucheix and Rubinstein, 2001), mice lacking CD9 (Miyado et al., 2000), CD37 (Knobeloch et al., 2000), CD81 (Maecker and Levy, 1997; Miyazaki et al., 1997), and Tssc6 (Tarrant et al., 2002) have been produced by gene targeting. CD9-null mice are infertile due to defective fusion capacity of their eggs. CD81-null mice are deficient in T cell–dependent IgG production and in Th2 cytokine secretion (Maecker et al., 1997). Recently, CD81-null mice were also reported to have reduced reproductive capacity after repeated backcrosses (Deng et al., 2000). Moreover, exogenously overexpressed CD81 rescued the fusibility of CD9-null eggs (Kaji et al., 2002). Thus, CD9 and CD81 may share an essential role in gamete fusion processes. Meanwhile, no abnormal findings in other cell fusion events and no morphological abnormalities have been reported in tetraspanin knockout mice. However, given that several different tetraspanins form complexes with each other, the loss of a particular tetraspanin may be compensated by other tetraspanins. We presumed that single tetraspanin knockout mice under particular conditions or multiple tetraspanin knockout mice may reveal novel tetraspanin functions. In fact, in the present work, in response to in vitro and in vivo stimulation, CD9- and CD81-null macrophages and bone marrow cells displayed enhanced fusion capacity. Furthermore, CD9/CD81 double-null mice spontaneously developed MGCs in the lung and showed increased osteoclastogenesis in the bone. It is tempting to presume that osteopenic phenotype of CD9/CD81 double-null mice is due to enhanced osteoclastogenesis. However, bone mass is maintained under a balance between bone resorption and formation. In fact, CD9 is present on osteoblast progenitors, and this CD9 molecule is also likely to be involved (Hayashi et al., 2000). Thus, detailed morphometric analysis and studies to evaluate the activities of osteoclasts and osteoblasts are obviously required to elucidate mechanisms in the osteopenic phenotype; such studies are currently in progress. Collectively, our data obtained using mAb and knockout mouse experiments suggest that CD9 and CD81 function to inhibit the fusion of mononuclear phagocytes, and that these tetraspanins may be able to compensate for each other.

Because the roles of CD9 and CD81 in monocyte/macrophage fusion proposed based on the present paper are contradictory to the previous hypothesis that these tetraspanins facilitate cell–cell fusion, the mechanisms of the tetraspanin contribution to cell–cell fusion now appear to be complex. One possible reason for this may be that the functions of tetraspanins are dependent on the cell lineage. Notably, although lectins such as Con A and phytohemagglutinin induce the fusion of macrophages, these lectins paradoxically inhibit the fusion of virus-infected cells, myoblasts, and gametes (Chambers, 1977). This evidence may indicate a fundamental distinction between macrophage and nonmacrophage cell fusion.

Integrins are well known to form complexes with tetraspanins, and tetraspanins may modulate the adhesive functions of integrins during cell–cell fusion (Boucheix and Rubinstein, 2001). In fact, it was shown in previous papers that the fusion of blood monocytes involves β1 and β2 integrins (Most et al., 1990; Tabata et al., 1994). In the present work, both anti-β1 and -β2 integrin mAbs inhibited the fusion of monocytes, probably due to blocking of monocyte adherence and aggregation, respectively. However, these adhesive and aggregative functions are not likely to be modified by tetraspanins because neither anti-CD9 nor anti-CD81 mAb altered monocyte adhesion or aggregation. We also observed that β1 and β2 integrins were complexed with CD9 and CD81 in freshly isolated blood monocytes, and this complex formation was up-regulated under normal culture conditions. Complexes between CD9 and CD81 were also increased; these up-regulations may at least partly reflect the elevated levels of total CD9 and CD81 proteins, but are consistent with the previous report that CD9 and β1 integrins assemble into a molecular complex during maturation of monocytes (Kurita-Taniguchi et al., 2001). However, under fusogenic conditions, the complex formation of tetraspanin (CD9 or CD81)–integrin (β1 or β2) was down-regulated, whereas tetraspanin–tetraspanin (CD9–CD81) complex formation occurred normally. A mild detergent (Brij99) was used in coimmunoprecipitation experiments, indicating that the molecular complexes in cell lysates could

be part of raft-like membrane microdomains (Claas et al., 2001). Possibly, the different regulation of tetraspanin–integrin and tetraspanin–tetraspanin complexes might reflect differential localization of these complexes into distinct microdomains. Further studies will be needed to clarify the contribution of tetraspanin–integrin complexes to monocyte/macrophage fusion.

Other proteins involved in the fusion of mononuclear phagocytes, such as ADAMs (Namba et al., 2001), CD44, CD47 (Vignery, 2000), and CD98 (Tabata et al., 1994), may be affected by CD9 and CD81. An interaction between CD9 and ADAMs was suggested in the fusion between sperm and eggs (Zhu and Evans, 2002). Both CD44 and CD47 were reported to associate with CD9 (Jones et al., 1996; Longhurst et al., 1999). CD98 associates with β1 integrins and is important in integrin activation (Fenczik et al., 2001). Tetraspanins may indirectly influence CD98 function through their participation in integrin complexes. Roles of these proteins in tetraspanin-regulated monocyte/macrophage fusion remains unknown, but the fact that CD9 associates with multiple monocyte/macrophage fusion-related proteins further supports the involvement of tetraspanins in this particular type of fusion.

In conclusion, the present paper has demonstrated a novel fusion-regulatory function of tetraspanins. While facilitating the fusion of gametes, myoblasts, and virus-infected cells, CD9 and CD81 are essential to prevent the fusion of mononuclear phagocytes. Given that multinucleation endows cells with more resorptive capacity for extracellular components such as bone or infectious agents (Vignery, 2000), functional alterations of tetraspanins may contribute to the progression of osteoporosis, infection, and granulomatous diseases.

## Materials and methods

### Antibodies and reagents

Mouse anti-CD9 mAbs (BU16 and MM2/57), anti-CD81 mAb (JS64), and anti-CD63 mAb (AHN-16) were purchased from Biodesign, Biosource International, Immunotech, and CHEMICON International, respectively. Mouse mAb against CD81 (M38) was a gift from Dr. O. Yoshie (Kinki University School of Medicine, Osaka, Japan). Mouse mAb against CD151 (5C11) and mouse anti-β1 integrin mAbs (A-1A5 and SG/19) were described previously (Berditchevski et al., 1995; Tachibana et al., 1996; Yoch et al., 1998). Another mouse anti-β1 integrin mAb (4B4) was obtained from Beckman Coulter. Mouse anti-β2 integrin mAbs (IB4, MEM48, YFC118.3, and TS1/18) were purchased from Ancell, Monosan, CHEMICON International, and Endogen, respectively. Rat anti–mouse CD9 mAb (KMC8) and anti-CD81 mAb (Eat1) were obtained from BD Biosciences. Hamster anti–mouse CD81 mAb (2F7) was purchased from Southern Biotechnology Associates, Inc. Rat anti–mouse CD9 mAb (JF9) was provided by Dr. P.W. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK). $F(ab')_2$ fragments of several mAbs were prepared using an ImmunoPure® $F(ab')_2$ preparation kit (Pierce Chemical Co.). Preparation of a recombinant GST fusion protein containing the large extracellular loop of CD9 was described previously (Shimizu et al., 2002). A GST fusion protein containing murine CD9 large extracellular loop residues 111–192 (GST–mCD9) was produced in the Mekada laboratory.

### Isolation of blood monocytes, alveolar macrophages, and bone marrow cells

Peripheral blood mononuclear cells were isolated from heparinized whole blood by Ficoll-Hypaque density gradient centrifugation. Mononuclear cells were collected and cultured on MSP plates (Japan Immunoresearch). The adherent monocytes were detached and suspended in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium). Human alveolar macrophages were collected by BAL from patients in Osaka University Hospital (Osaka, Japan) with informed consent. Murine alveolar macrophages were obtained by BAL from 6–8-wk-old ddY male mice. Lungs of mice were subjected to lavage six times with 1.0 ml saline. Collected cells consisting mostly of macrophages were suspended in DME containing 5% human serum. Murine bone marrow cells were obtained from tibiae of 8–12-wk-old C57BL/6 male mice and suspended in αMEM containing 10% FCS.

### Immunoblotting

Cells were lysed in lysis buffer containing 1% Brij99, 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Samples containing equal protein concentrations were electrophoresed on SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The membranes were probed with mAbs diluted at 1 μg/ml, followed by incubation with peroxidase-conjugated goat anti–mouse IgG (H+L; Bio-Rad Laboratories) diluted 1:2,000. Immunoreactive bands were visualized with Renaissance® Chemiluminescent Reagent (NEN Life Science Products).

### Immunoprecipitation

Lysates containing equal amounts of protein were incubated with anti-integrin and -tetraspanin mAbs. Immune complexes were collected with protein A-Sepharose (Sigma-Aldrich), separated by SDS-PAGE under non-reducing conditions, and transferred to an Immobilon-P membrane. Immunoblotting was performed with biotinylated mAbs followed by peroxidase-conjugated streptavidin (Zymed Laboratories).

### In vitro fusion assay of monocytes, macrophages, and bone marrow cells

For human blood monocyte/alveolar macrophage fusion experiments, $2 \times 10^5$ cells were suspended in 100 μl culture medium, plated in a 96-well tissue culture plate (Falcon), and induced to fuse into MGCs by the addition of 10 μg/ml Con A for 3 d. To estimate the degree of cell fusion in the absence or presence of 10 μg/ml mAb or recombinant GST–CD9 protein, nuclei were visualized using Wright stain. Fusion rates were determined by calculating the percentages of the number of nuclei within MGCs (three or more nuclei per cell) per total number of nuclei in six independent fields. Between 400 and 600 nuclei were counted in each field. For murine alveolar macrophage fusion, $3 \times 10^5$ macrophages were suspended in 50 μl DME containing 5% human serum, plated onto a 96-well plate, and then induced to fuse into MGCs by the addition for 3 d of 10 nM $1\alpha,25(OH)_2D_3$ and 50 μl culture supernatant that was obtained from cultures of Con A–stimulated murine spleen cells. The number of MGCs per well in triplicate cultures was determined. For murine bone marrow cells, $2 \times 10^6$ cells were suspended in 500 μl αMEM containing 10% FCS, plated onto a 24-well plate, and then induced to fuse by the addition for 7 d of 50 ng/ml sRANKL and 20 ng/ml M-CSF. Cells were then fixed and stained for TRAP (Li et al., 2002). The number of TRAP-positive multinucleated cells (three or more nuclei per cell) per well in triplicate cultures was determined.

### In vivo fusion assay of murine alveolar macrophages

300 μg heat-killed and sonicated *P. acnes* (ATCC 11828; American Type Culture Collection) was suspended in 100 μl PBS and administered intratracheally to CD9 (−/−), CD81 (−/−), and wild-type mice under anesthesia as described previously (Itakura et al., 2001). After 7 d, the mice were killed and lung paraffin sections were prepared to observe MGC formation in the lung. In separate experiments, alveolar macrophages were isolated by BAL as described above, and the number of MGCs per lung was determined.

### Cell adhesion assay

200,000 monocytes were suspended in 100 μl RPMI 1640 containing 10 μg/ml Con A and plated onto the wells of a 96-well tissue culture plate (Falcon). Cells were then allowed to adhere to the plate for 12 h in the absence or presence of 10 μg/ml mAbs. After nonadherent cells were removed by rinsing, the remaining adherent cells were evaluated in triplicate cultures using an MTT assay.

### Cell aggregation assay

200,000 monocytes were cultured in 100 μl RPMI 1640 with 5 μg/ml Con A in the absence or presence of 10 μg/ml mAbs for 12 h in wells of a 96-well nontissue culture-treated plate (Linbro). The number of cell aggregates (>4 cells/aggregate) was determined in six independent fields.

### Immunofluorescence

Murine alveolar macrophages were cultured in Lab-Tek® glass chamber slides (Nunc), fixed in 3% PFA, and then permeabilized with 0.5% Triton X-100. Nonspecific recognition of Fc receptors was blocked with 20% goat serum. The permeabilized cells were stained with 1 μg/ml control mAb (R35–95) or anti–mouse CD9 mAb (KMC8) and incubated with FITC-conjugated anti–rat immunoglobulin at a 1:50 dilution and 0.5 μg/ml propidium iodide (Sigma-Aldrich). Immunofluorescence images were obtained using an inverted immunofluorescence microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.).

### Mice

The generation of CD9 (−/−) mice and CD81 (−/−) mice was described previously (Miyazaki et al., 1997; Miyado et al., 2000). These mice were backcrossed more than five generations into the C57BL/6 background. CD9 (−/−)/CD81 (−/−) mice were produced by intercrossing CD9 (+/−)/CD81 (+/−) mice. The genotyping of all breeding pairs was confirmed by PCR analysis. The mice were maintained in a barrier facility, and all animal procedures were performed in accordance with the Osaka University (Osaka, Japan) guidelines on Animal Care. 7–11-wk-old mice matched for age and sex were used in all experiments.

### Histological analysis of lung and bone sections

1 ml 10% buffered formaldehyde was instilled into the lung of each mouse via an intratracheal cannula, and then the whole lung was excised and fixed in 10% buffered formaldehyde. The fixed lung was embedded in paraffin, sectioned sagittally, and stained with hematoxylin and eosin. Tibiae were fixed with ethanol, and the undecalcified bones were embedded in glycolmethacrylate. 3-μm longitudinal sections from the proximal parts were stained for TRAP. The number of TRAP-positive osteoclasts was determined as described previously (Liu et al., 2001).

The authors are grateful to Dr. O. Yoshie and Dr. P.W. Kincade for providing antibodies to tetraspanins. We also wish to thank Dr. S. Hirota for helpful comments on lung sections, Dr. T. Komori for helpful comments on bone sections, Dr. T. Iwaki for valuable suggestions on CD9/CD81 double knockout mice, and Y. Habe for secretarial assistance.

Submitted: 3 December 2002
Revised: 5 May 2003
Accepted: 5 May 2003

## References

Abe, E., C. Miyaura, H. Tanaka, Y. Shiina, T. Kuribayashi, S. Suda, Y. Nishii, H.F. DeLuca, and T. Suda. 1983. 1 alpha,25-dihydroxyvitamin D3 promotes fusion of mouse alveolar macrophages both by a direct mechanism and by a spleen cell-mediated indirect mechanism. *Proc. Natl. Acad. Sci. USA.* 80:5583–5587.

Abe, E., Y. Ishimi, C.H. Jin, M.H. Hong, T. Sato, and T. Suda. 1991. Granulocyte-macrophage colony-stimulating factor is a major macrophage fusion factor present in conditioned medium of concanavalin A-stimulated spleen cell cultures. *J. Immunol.* 147:1810–1815.

Anderson, J.M. 2000. Multinucleated giant cells. *Curr. Opin. Hematol.* 7:40–47.

Berditchevski, F., G. Bazzoni, and M.E. Hemler. 1995. Specific association of CD63 with the VLA-3 and VLA-6 integrins. *J. Biol. Chem.* 270:17784–17790.

Boucheix, C., and E. Rubinstein. 2001. Tetraspanins. *Cell. Mol. Life Sci.* 58:1189–1205.

Byrd, T.F. 1998. Multinucleated giant cell formation induced by IFN-gamma/IL-3 is associated with restriction of virulent *Mycobacterium tuberculosis* cell to cell invasion in human monocyte monolayers. *Cell. Immunol.* 188:89–96.

Chambers, T.J. 1977. Fusion of hamster macrophages induced by lectins. *J. Pathol.* 123:53–61.

Claas, C., C.S. Stipp, and M.E. Hemler. 2001. Evaluation of prototype transmembrane 4 superfamily protein complexes and their relation to lipid rafts. *J. Biol. Chem.* 276:7974–7984.

de Parseval, A., D.L. Lerner, P. Borrow, B. Willett, and J.H. Elder. 1997. Blocking of feline immunodeficiency virus infection by a monoclonal antibody to CD9 is via inhibition of virus release rather than interference with receptor binding. *J. Virol.* 71:5742–5749.

Deng, J., V.P. Yeung, D. Tsitoura, R.H. DeKruyff, D.T. Umetsu, and S. Levy. 2000. Allergen-induced airway hyperreactivity is diminished in CD81-deficient mice. *J. Immunol.* 165:5054–5061.

Enelow, R.I., G.W. Sullivan, H.T. Carper, and G.L. Mandell. 1992. Cytokine-induced human multinucleated giant cells have enhanced candidacidal activity and oxidative capacity compared with macrophages. *J. Infect. Dis.* 166:664–668.

Fais, S., V.L. Burgio, M. Silvestri, M.R. Capobianchi, A. Pacchiarotti, and F. Pallone. 1994. Multinucleated giant cells generation induced by interferon-gamma. Changes in the expression and distribution of the intercellular adhesion molecule-1 during macrophages fusion and multinucleated giant cell formation. *Lab. Invest.* 71:737–744.

Fenczik, C.A., R. Zent, M. Dellos, D.A. Calderwood, J. Satriano, C. Kelly, and M.H. Ginsberg. 2001. Distinct domains of CD98hc regulate integrins and amino acid transport. *J. Biol. Chem.* 276:8746–8752.

Fukudome, K., M. Furuse, T. Imai, M. Nishimura, S. Takagi, Y. Hinuma, and O. Yoshie. 1992. Identification of membrane antigen C33 recognized by monoclonal antibodies inhibitory to human T-cell leukemia virus type 1 (HTLV-1)-induced syncytium formation: altered glycosylation of C33 antigen in HTLV-1-positive T cells. *J. Virol.* 66:1394–1401.

Hassan, N.F., N. Kamani, M.M. Meszaros, and S.D. Douglas. 1989. Induction of multinucleated giant cell formation from human blood-derived monocytes by phorbol myristate acetate in in vitro culture. *J. Immunol.* 143:2179–2184.

Hayashi, S., K. Miyake, and P.W. Kincade. 2000. The CD9 molecule on stromal cells. *Leuk. Lymphoma.* 38:265–270.

Itakura, M., A. Tokuda, H. Kimura, S. Nagai, H. Yoneyama, N. Onai, S. Ishikawa, T. Kuriyama, and K. Matsushima. 2001. Blockade of secondary lymphoid tissue chemokine exacerbates *Propionibacterium acnes*-induced acute lung inflammation. *J. Immunol.* 166:2071–2079.

James, D.G. 2000. A clinicopathological classification of granulomatous disorders. *Postgrad. Med. J.* 76:457–465.

Jones, P.H., L.A. Bishop, and F.M. Watt. 1996. Functional significance of CD9 association with beta 1 integrins in human epidermal keratinocytes. *Cell Adhes. Commun.* 4:297–305.

Kaji, K., S. Oda, S. Miyazaki, and A. Kudo. 2002. Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion. *Dev. Biol.* 247:327–334.

Kelic, S., S. Levy, C. Suarez, and D.E. Weinstein. 2001. CD81 regulates neuron-induced astrocyte cell-cycle exit. *Mol. Cell. Neurosci.* 17:551–560.

Knobeloch, K.P., M.D. Wright, A.F. Ochsenbein, O. Liesenfeld, J. Lohler, R.M. Zinkernagel, I. Horak, and Z. Orinska. 2000. Targeted inactivation of the tetraspanin CD37 impairs T-cell-dependent B-cell response under suboptimal costimulatory conditions. *Mol. Cell. Biol.* 20:5363–5369.

Kurita-Taniguchi, M., K. Hazeki, N. Murabayashi, A. Fukui, S. Tsuji, M. Matsumoto, K. Toyoshima, and T. Seya. 2001. Molecular assembly of CD46 with CD9, alpha3-beta1 integrin and protein tyrosine phosphatase SHP-1 in human macrophages through differentiation by GM-CSF. *Mol. Immunol.* 38:689–700.

Li, X., N. Udagawa, K. Itoh, K. Suda, Y. Murase, T. Nishihara, T. Suda, and N. Takahashi. 2002. p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function. *Endocrinology.* 143:3105–3113.

Liu, W., S. Toyosawa, T. Furuichi, N. Kanatani, C. Yoshida, Y. Liu, M. Himeno, S. Narai, A. Yamaguchi, and T. Komori. 2001. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J. Cell Biol.* 155:157–166.

Löffler, S., F. Lottspeich, F. Lanza, D.O. Azorsa, V. Meulen, and J. Schneider-Schaulies. 1997. CD9, a tetraspan transmembrane protein, renders cells susceptible to canine distemper virus. *J. Virol.* 71:42–49.

Longhurst, C.M., M.M. White, D.A. Wilkinson, and L.K. Jennings. 1999. A CD9, alphaIIbbeta3, integrin-associated protein, and GPIb/V/IX complex on the surface of human platelets is influenced by alphaIIbbeta3 conformational states. *Eur. J. Biochem.* 263:104–111.

Maecker, H.T., and S. Levy. 1997. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med.* 185:1505–1510.

Maecker, H.T., S.C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB J.* 11:428–442.

McNally, A.K., and J.M. Anderson. 2002. Beta1 and beta2 integrins mediate adhesion during macrophage fusion and multinucleated foreign body giant cell formation. *Am. J. Pathol.* 160:621–630.

Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, et al. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. *Science.* 287:321–324.

Miyazaki, T., U. Muller, and K.S. Campbell. 1997. Normal development but differentially altered proliferative responses of lymphocytes in mice lacking

CD81. *EMBO J.* 16:4217–4225.

Most, J., H.P. Neumayer, and M.P. Dierich. 1990. Cytokine-induced generation of multinucleated giant cells in vitro requires interferon-gamma and expression of LFA-1. *Eur. J. Immunol.* 20:1661–1667.

Namba, K., M. Nishio, K. Mori, N. Miyamoto, M. Tsurudome, M. Ito, M. Kawano, A. Uchida, and Y. Ito. 2001. Involvement of ADAM9 in multinucleated giant cell formation of blood monocytes. *Cell. Immunol.* 213:104–113.

Shimizu, T., T. Matsuishi, R. Iwamoto, K. Handa, H. Yoshioka, H. Kato, S. Ueda, H. Hara, T. Tabira, and E. Mekada. 2002. Elevated levels of anti-CD9 antibodies in the cerebrospinal fluid of patients with subacute sclerosing panencephalitis. *J. Infect. Dis.* 185:1346–1350.

Tabata, N., M. Ito, K. Shimokata, S. Suga, S. Ohgimoto, M. Tsurudome, M. Kawano, H. Matsumura, H. Komada, M. Nishio, et al. 1994. Expression of fusion regulatory proteins (FRPs) on human peripheral blood monocytes. Induction of homotypic cell aggregation and formation of multinucleated giant cells by anti-FRP-1 monoclonal antibodies. *J. Immunol.* 153:3256–3266.

Tachibana, I., and M.E. Hemler. 1999. Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance. *J. Cell Biol.* 146:893–904.

Tachibana, I., M. Mori, Y. Tanio, S. Hosoe, T. Sakuma, T. Osaki, K. Ueno, T. Kumagai, T. Kijima, and T. Kishimoto. 1996. A 100-kDa protein tyrosine phosphorylation is concurrent with beta 1 integrin-mediated morphological differentiation in neuroblastoma and small cell lung cancer cells. *Exp. Cell Res.* 227:230–239.

Takahashi, Y., D. Bigler, Y. Ito, and J.M. White. 2001. Sequence-specific interaction between the disintegrin domain of mouse ADAM 3 and murine eggs: role of beta1 integrin-associated proteins CD9, CD81, and CD98. *Mol. Biol. Cell.* 12:809–820.

Takashima, T., K. Ohnishi, I. Tsuyuguchi, and S. Kishimoto. 1993. Differential regulation of formation of multinucleated giant cells from concanavalin A-stimulated human blood monocytes by IFN-gamma and IL-4. *J. Immunol.* 150:3002–3010.

Tarrant, J.M., J. Groom, D. Metcalf, R. Li, B. Borobokas, M.D. Wright, D. Tarlinton, and L. Robb. 2002. The absence of Tssc6, a member of the tetraspanin superfamily, does not affect lymphoid development but enhances in vitro T-cell proliferative responses. *Mol. Cell. Biol.* 22:5006–5018.

Vignery, A. 2000. Osteoclasts and giant cells: macrophage-macrophage fusion mechanism. *Int. J. Exp. Pathol.* 81:291–304.

Wang, X.Q., G.F. Evans, M.L. Alfaro, and S.H. Zuckerman. 2002. Down-regulation of macrophage CD9 expression by interferon-gamma. *Biochem. Biophys. Res. Commun.* 290:891–897.

Willett, B., M. Hosie, A. Shaw, and J. Neil. 1997. Inhibition of feline immunodeficiency virus infection by CD9 antibody operates after virus entry and is independent of virus tropism. *J. Gen. Virol.* 78:611–618.

Yoch, R., F. Berditchevski, M.B. Harler, J. Reichner, and M.E. Hemler. 1998. Highly stoichiometric, stable, and specific association of integrin $\alpha 3\beta 1$ with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. *Mol. Biol. Cell.* 9:2751–2765.

Zhu, X., and J.P. Evans. 2002. Analysis of the roles of RGD-binding integrins, alpha(4)/alpha(9) integrins, alpha(6) integrins, and CD9 in the interaction of the fertilin beta (ADAM2) disintegrin domain with the mouse egg membrane. *Biol. Reprod.* 66:1193–1202.