Overexpression of CD82 on human T cells enhances LFA-1/ICAM-1-mediated cell-cell adhesion: functional association between CD82 and LFA-1 in T cell activation

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We previously demonstrated that CD82, expressed on both T cells and antigen-presenting cells (APC), plays an important role as a co-stimulatory molecule especially in the early phase of T cell activation. We also showed that the CD82 expression level is up-regulated on activated T cells and memory T cells. This up-regulation enhances both T cell-T cell and T cell-APC interactions. In this study, we further investigated the mechanism of CD82-mediated cell-cell adhesion. The enhanced adhesion between CD82-overexpressing Jurkat cells was completely blocked by anti-ICAM-1/LFA-1 monoclonal antibodies. Increased interaction of LFA-1 with ICAM-1 was further confirmed by enhanced adhesion of CD82-overexpressing Jurkat cells to immobilized ICAM-1-Ig. CD82 co-immunoprecipitated with LFA-1 from Jurkat cells and CD82 and LFA-1 colocalized at an adhesion foci. These results suggest that the T cell stimulation via anti-CD3 cross-linking or phorbol myristate acetate treatment up-regulates CD82 expression, leading to the colocalization of CD82 and LFA-1, and results in enhanced interaction between LFA-1 and ICAM-1. In addition, a blocking experiment using monoclonal antibodies suggested that CD82 and LFA-1 molecules on APC are also important for the optimal activation of T cells. This is the first report that describes the enhancement of cell-cell interaction through LFA-1 and ICAM-1 by the over-expression of another cell surface molecule, CD82.

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1 Introduction

T cell activation through antigen (Ag)-presenting cells (APC) is critical for an Ag-specific immune response. It is well established that T cell receptor (TCR) occupancy by an Ag/MHC complex (signal 1) and a co-stimulation signal from accessory molecules (signal 2) are essential requirements for inducing optimal T cell activation [1]. Although the most prominent co-stimulatory molecule is CD28 [2–5], a number of other molecules have been suggested to activate T cells independently of the CD28/B7 pathway [6–17], indicating the existence of multiple pathways of T cell co-stimulation. Therefore, it is very important to understand how signal 2 from these accessory molecules interacts after the input of signal 1. However, it is not clear how redundant the different molecules are in exhibiting overlapping functions, and exactly how they co-stimulate T cell activation.

Among the accessory molecules, LFA-1 (CD11a/CD18) is known to function as a cell adhesion molecule as well as a co-stimulatory molecule [18–20]. It is expressed on T and B cells, granulocytes, macrophages, and dendritic cells. The natural ligands for LFA-1 are ICAM-1, 2, and 3 (CD54, 102 and 50), which also are expressed on most hematopoietic cells [21]. LFA-1 and ICAM-1 have been shown to play an important role in enhancing cell-cell contact. Interestingly, stimulation of T cells by anti-CD3 mAb cross-linking or PMA treatment increases the activity of LFA-1 for ICAM-1 immediately without changing

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Abbreviations: PBT: Peripheral blood T cells **TM4SF:** Transmembrane 4 superfamily **CsA:** Cyclosporin A

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their expression levels [22]. Therefore, it is likely that LFA-1 is critical for the establishment of the initial T cell-APC interaction and, after the activation step by signal 1, to strengthen cell-cell contact.

Recently, we demonstrated that CD82, which is expressed on both T cells and APC, has a functional role in the early phase of T cell activation by U937 cells [23]. We also demonstrated that CD82 expressing was up-regulated by anti-CD3 cross-linking stimulation or PMA treatment, and that CD82-overexpressing T cells enhance not only homotypic but also heterotypic cell-cell adhesion. In addition, we and other investigators showed that the cross-linking stimulation signal by immobilized anti-CD82 mAb co-stimulated T cells [23–25] and that the signal acts synergistically with the CD28-mediated T cell co-stimulation signal [23].

On the other hand, transmembrane 4 superfamily (TM4SF) proteins [26], including CD82, were reported to associate with some integrins, such as VLA-3 ($\alpha3\beta1$), VLA-4 ($\alpha4\beta1$) and VLA-6($\alpha6\beta1$) [27, 28]. Therefore, it is deduced that CD82 may associate with other integrins. Integrins are composed of $\alpha\beta$ heterodimers, and integrin-mediated cell adhesion is activated by inside-out signaling with modulation of the conformation of these heterodimers [22]. However, it is still unknown how the activity of these heterodimers is modulated.

In this report, we demonstrate that up-regulation of CD82 on Jurkat T cells can enhance ICAM-1/LFA-1-mediated cell adhesion. These results may account for the functional roles of CD82 in the early phase of T cell activation through APC.

2 Results

2.1 CD82-overexpression-induced homotypic cell aggregation in Jurkat cells is completely blocked by anti-ICAM-1/anti-LFA-1 mAb

Our previous analyses revealed that the expression level of CD82 was up-regulated on activated/memory T cells or by TCR signal/PMA treatment [23]. Then we established CD82-overexpressing T cell clones by transducing CD82-cDNA stably into Jurkat cell lines to analyze the functional roles of CD82. All four CD82-overexpressing clones revealed enhancement of homotypic cell aggregation compared with parental cells (Fig. 1 a, b). We next analyzed which molecules were involved in CD82-mediated cell adhesion using mAb reactive with cell adhesion molecules. As shown in Fig. 1, this homotypic cell aggregation was inhibited by anti-CD54, anti-CD11a, and CD18 mAb, respectively (Fig. 1 e–h). Anti-

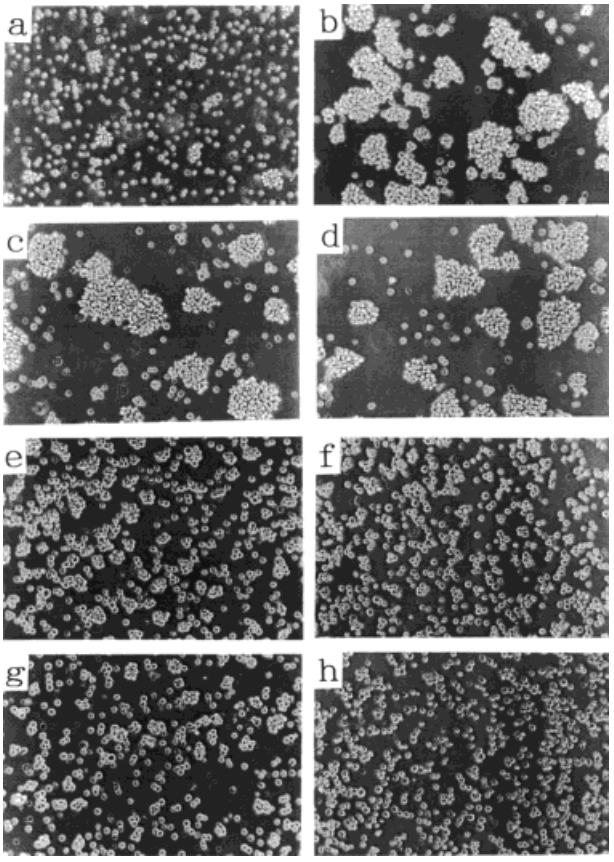

Figure 1. Morphological appearance of Jurkat cells after a 4-h incubation. (a) Parental Jurkat cells, (b) CD82-overexpressing Jurkat cells (c–h) CD82-overexpressing Jurkat cell pretreated with: (c) 53H5 (anti-CD82 mAb), (d) Lia 1/2 (anti-CD29 mAb), (e) 17H5 (anti-CD54 mAb), (f) TS1/22 (anti-CD11a mAb) (g) TS1/18 (anti-CD18 mAb), or (h) 17H5-TS1/22. Cell aggregates were dissociated by pipetting at the start of culture and observed after 4 h of incubation. (a)–(h) × 100.

CD82 or anti-CD29 ($\beta1$-integrin) mAb (Lia 1/2) showed no inhibitory effect (Fig. 1 c, d) and that $\beta1$-integrin did not participate in this aggregate formation was further supported by the lack of an inhibitory effect of another anti-CD29 mAb (P4C10) or RGD peptide (data not shown).

We next compared the expression levels of these adhesion molecules between parental and CD82-overexpressing Jurkat cells. As shown in Fig. 2, parental Jurkat cells expressed ICAM-1, 2, 3 (CD54, 102, 50), LFA-1(CD11a/CD18), Mac-1(CD11b/CD18), and VLA-1, 2, 3, 4, 5, 6 (CD49a, b, c, d, e, f/CD29), but not VCAM-1(CD106). CD82-overexpressing Jurkat cells also expressed these molecules, and we could not find any differences in these expression levels between parental

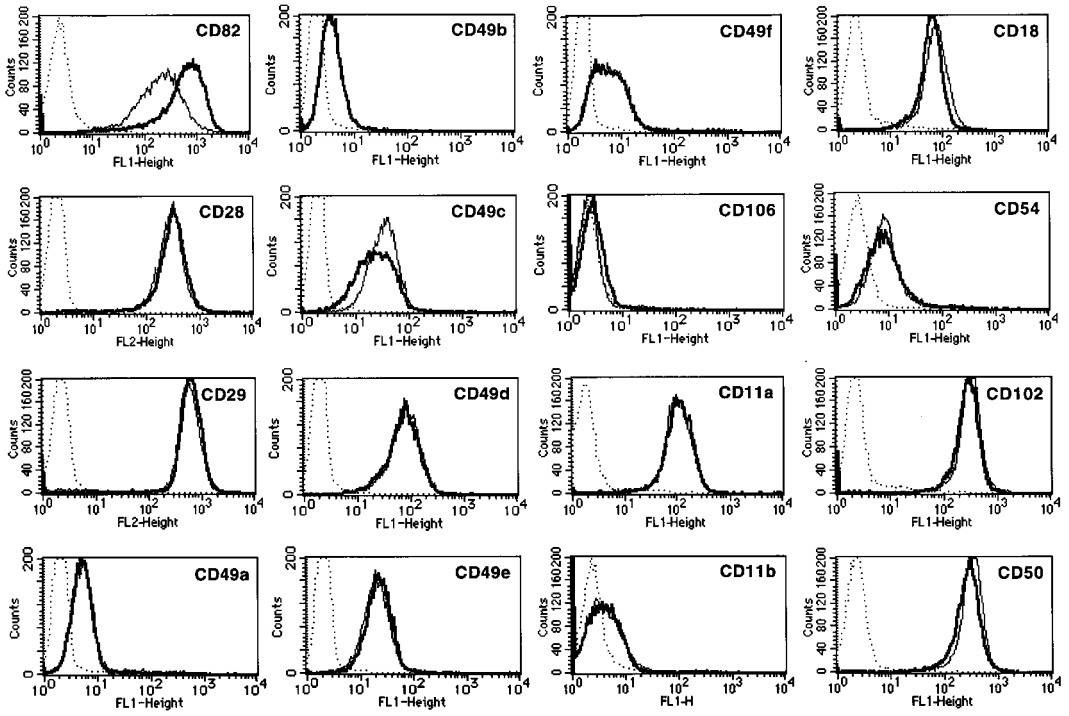

Figure 2. Flow cytometric analysis of expression of CD82 and other major molecules on parental and CD82-overexpressing Jurkat cells. Expression of CD82, CD28, CD29, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD106, CD11a, CD11b, CD18, CD54, CD102, and CD50 on parental (——) or CD82 cDNA-transfected (╍) Jurkat cells are shown. Log fluorescence intensity is represented on the horizontal axis, and cell number, on the vertical axis.

and CD82-overexpressing cells except for CD82. We also compared the expression levels of other TM4SF proteins including CD9, CD37, CD53, CD63 and CD81, and there were no differences in the expression of these molecules between parental and CD82-overexpressing cells (data not shown).

These data demonstrate that up-regulation of CD82 enhances LFA-1/ICAM-1-mediated cell adhesion without changing the expression level of LFA-1/ICAM-1 and other representative cell adhesion molecules.

2.2 CD82-overexpression enhances LFA-1/ICAM-1-mediated cell adhesion

Our results indicate that CD82 modulates the affinity or avidity of intercellular adhesion mediated by ICAM-1 and LFA-1. To investigate whether either ICAM-1 or LFA-1 molecules functionally associate with CD82 in Jurkat cells, we compared the binding activity of LFA-1 for immobilized ICAM-1 protein using parental and CD82-overexpressing Jurkat cells. For this purpose, we produced a fusion protein of the extracellular portion of human ICAM-1 and the Fc portion of human IgG1 (ICAM-1-Ig) as a substitute for the soluble ICAM-1, and immobilized ICAM-1-Ig on plates. As shown in Fig. 3 a, CD82-overexpressing Jurkat cells adhered to the ICAM-1-Ig coated plate more than parental cells. In addition, pretreatment of CD82-overexpressing Jurkat cells with anti-CD11a or anti-CD18 mAb, but not with anti-CD29 mAb or CD82 (data not shown), strongly inhibited the binding activity of LFA-1, demonstrating the involvement of LFA-1 in these kinetics. We also compared the binding activity against extracellular matrix (ECM) using each cell line. There were no differences in cell adhesion activity against fibronectin, vitronectin, or laminin between parental and CD82-overexpressing Jurkat cells (data not shown). In contrast, CD82-overexpressing Jurkat cells adhered to collagen more strongly than parental Jurkat cells (Fig. 3 b). These results indicate that expression of CD82 on Jurkat cells is functionally associated with the binding activity of VLA family molecules for collagen as well as LFA-1 for ICAM-1. These results also indicate that VLA family molecules do not participate in the CD82-mediated Jurkat homotypic cell aggregation (Fig. 1), since pretreatment of CD82-overexpressing Jurkat cells with anti-CD29 mAb or RGD peptide strongly inhibited their binding to collagen (Fig. 3 b) but did not inhibit the homotypic aggregation (Fig. 1 d).

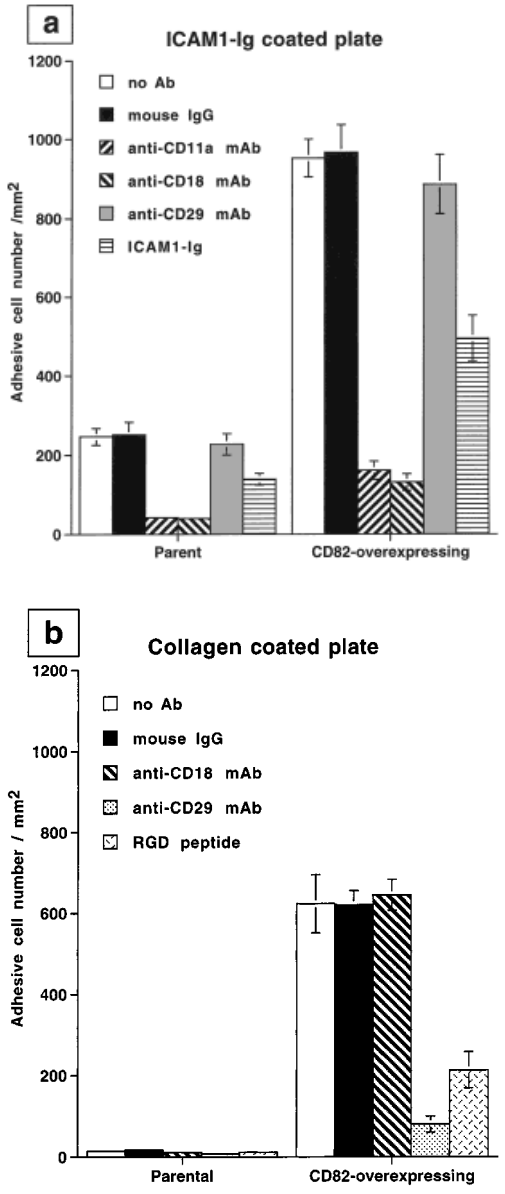

Figure 3. Comparison of cell-binding activity against immobilized ICAM-1-Ig fusion protein or ECM between parental and CD82-overexpressing Jurkat cells. Parental or CD82-overexpressing Jurkat cells (3×10^6/well) with or without pretreatment by blocking reagents were seeded and incubated in (a) a ICAM-1-Ig-coated, (b) collagen type1-coated 12-well plate for 1 h at 37 °C. After washing each well gently with PBS+ at 37 °C, the number of bound cells were counted using an objective micrometer.

2.3 CD82 is co-precipitated with LFA-1, but not with ICAM-1 on Jurkat cells

It has been reported that CD82 is associated with CD4 or the β1-integrin family, such as VLA-3, VLA-4, and VLA-6 [27, 29]. Thus far, the association of CD82 with LFA-1 has not been demonstrated. It has been reported that CD82 does not associate with LFA-1 in Raji cells [29]. To investigate whether CD82 associates with LFA-1 in Jurkat cells, we performed immunoprecipitation and blotting analyses. As shown in Fig. 4 a and b, CD82 was co-precipitated with CD11a and CD18 as well as CD4 and CD29 using both parental and CD82-overexpressing Jurkat cells. However, CD82 did not co-precipitate with either CD54 or CD28. To further confirm the association between CD82 and LFA-1, we performed a similar experiment using anti-CD18 mAb. As shown in Fig. 4 c, CD18 was co-precipitated with CD82. To make the case that the association between CD82 and LFA-1 is selective, we performed the co-precipitation analyses with other TM4SF proteins. As shown in Fig. 4 d, CD18 was not co-precipitated with CD9, CD37, CD53, CD63 or CD81 in CD82-overexpressing Jurkat cells. These results indicate that CD82 associates with CD11a/CD18 and makes a complex in Jurkat cells.

To test the specific association between CD82 and VLA family in CD82-overexpressing Jurkat cells, we also performed the co-precipitation analyses with CD49b, CD49c, CD49d, and CD49e. Although CD29 (β1-integrin) was co-precipitated with CD82, none of α-chain of VLA family co-precipitated with CD82 in CD82-overexpressing Jurkat cells (Fig. 4 e).

2.4 CD82 colocalizes with LFA-1 at the cell adhesion foci on CD82-overexpressing Jurkat cells

It is well known that LFA-1 localizes at the cell adhesion foci once cells are stimulated by anti-CD3 mAb cross-linking or PMA treatment [30]. Thus, we investigated the localization of CD82 and LFA-1 on parental and CD82-overexpressing Jurkat cells by immunofluorescence double-staining and confocal microscopy analysis. As shown in Fig. 5, CD11a on aggregating CD82-overexpressing cells was strongly stained at cell-cell contact sites (Fig. 5 c). However, CD11a on parental Jurkat cells was homogeneously stained at the cell surface (Fig. 5 a). A similar pattern for CD82 was observed for both parental and CD82-overexpressing Jurkat cells respectively (Fig. 5 b, d). Substitution of mouse IgG for the primary anti-adhesion molecule mAb was carried out as a control (Fig. 5 e, f). These data demonstrate that CD82 also localizes at the cell adhesion foci when cells showed ICAM-1/LFA-1-mediated cell adhesion by up-regulation of CD82. Therefore, CD82-overexpression affects the redistribution of LFA-1.

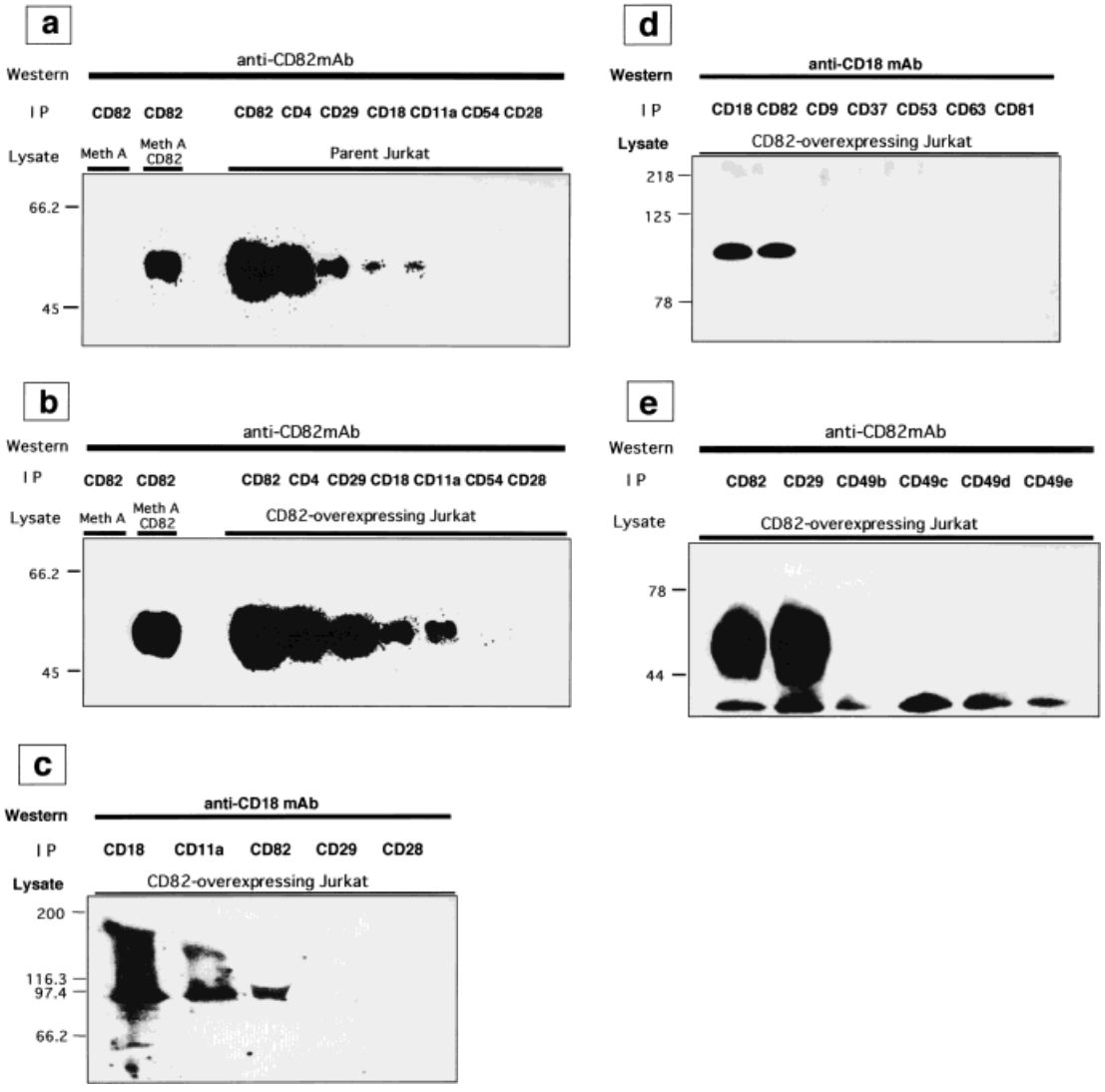

Figure 4. Co-precipitation between CD82 and LFA1 in Jurkat cells. Parental (a), CD82-overexpressing (b–e) Jurkat cells, and CD82 cDNA-transduced murine sarcoma cells (Meth A CD82) [left lanes of (a, b)] were lysed with 1 % Brij 96 lysis buffer. Aliquots of the lysates, each equivalent to 2×10^7 cells were immunoprecipitated (IP) with mAb as described in the figure and in Sect. 4.6. Co-precipitated proteins were subjected to SDS-PAGE under nonreducing conditions and analyzed by immunoblotting using biotin-labeled anti-CD82 mAb (a, b, e), or biotin-labeled anti-CD18 mAb (c, d). CD82 is observed as a 50–53-kDa band and CD18 is observed as a 95-kDa band in nonreducing SDS-PAGE.

2.5 Comparison of co-stimulatory activity between CD82 and LFA-1 on normal peripheral blood T cells

Our previous analyses demonstrated that cross-linking of CD82 by anti-CD82 mAb induces co-stimulatory activity in T cells [23]. However, in the presence of cyclosporin A (CsA), this CD82-mediated co-stimulation could not substitute for the CD82 signal. On the other hand, integrins, including LFA-1, have also been reported to have co-stimulatory activities [30, 31]. We, therefore, compared these co-stimulatory activities by performing a cross-linking stimulation assay using mAb against each molecule. For this purpose, we used normal human peripheral blood T cells (PBT) and not Jurkat cells, since the T cell activation pathway might be different between normal and leukemia cells. As shown in Fig. 6, cross-linking stimulation by anti-CD18 mAb as well as that by anti-CD82 mAb enhanced IL-2 production under conditions of suboptimal activation by PMA and calcium ionophore stimulation. However, these effects were suppressed by the addition of CsA. These data dem-

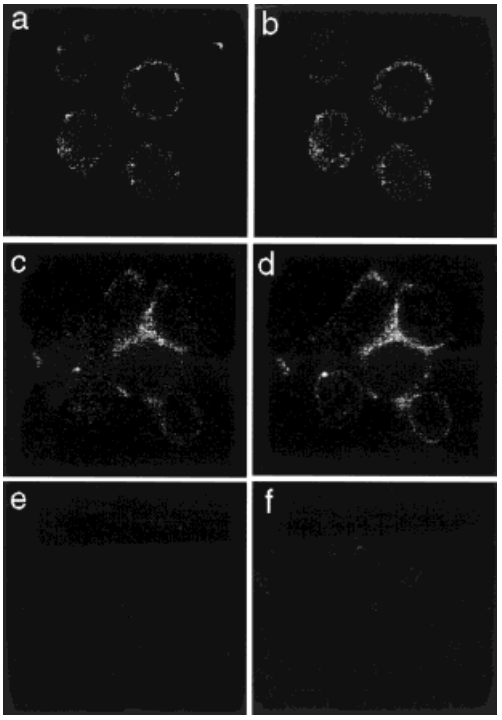

Figure 5. Colocalization of CD11a and CD82 analyzed by confocal microscopy. Parental (a, b) or CD82-over-expressing (c–f) Jurkat cells were fixed, double-stained with anti-CD11a (a, c), biotin-labeled anti-CD82 (b, d) mAb, or mouse IgG (e, f) followed by TRITC-conjugated goat anti-mouse IgG and FITC-conjugated streptavidin (a–d), or by TRITC- (e) and FITC-conjugated goat anti-mouse IgG (f). These were observed with a confocal laser microscope. The same field of cells was observed (600x).

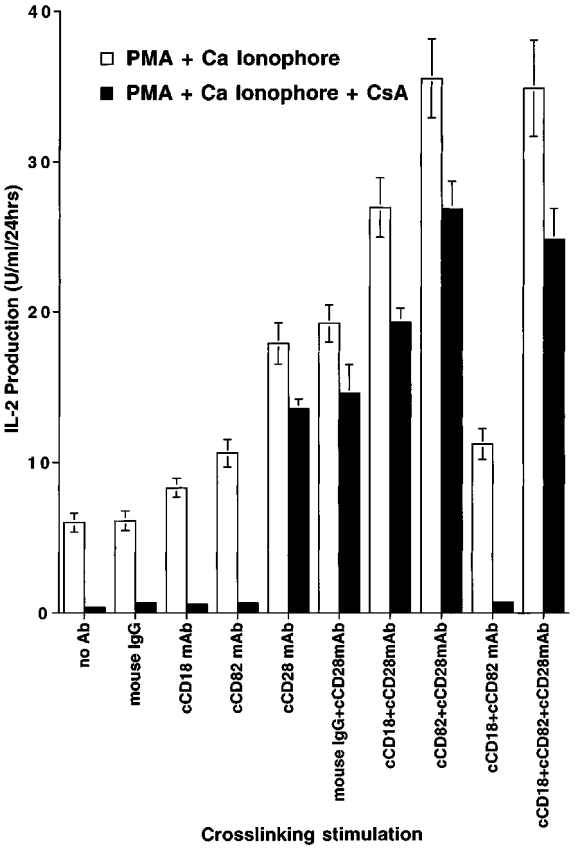

Figure 6. Comparison of CD18-, CD82-, and CD28-mediated T cell signal transduction. PBT (1×10^5/well) were stimulated with PMA (2 ng/ml) and/or calcium ionophore (250 ng/ml) in the presence or absence of cross-linking stimulation by mouse IgG, CD18, CD82 and/or CD28. CsA (100 ng/ml) was added at the start of the culture where indicated. IL-2 concentration in the supernatants was measured 24 h after the start of culture. Representative results of one of two experiments are shown.

onstrated that LFA-1-mediated co-stimulation also could not substitute completely for the CD28 signal. Furthermore, this CD18-mediated co-stimulation effect was not observed when PBT was stimulated simultaneously with anti-CD82 mAb. These results demonstrated that there is a similarity in the co-stimulatory activities of LFA-1 and CD82.

2.6 CD82 and LFA-1 on APC are also important for optimal T cell activation

Previous studies demonstrated that IL-2 production was inhibited when either PBT or U937 cells were pretreated by anti-CD82 mAb (Fab) in studies of U937-dependent T cell activation [23]. These results demonstrate a requirement for expression of functional CD82 by both of these cells. LFA-1 is also expressed not only on T cells but also on APC, including U937 cells. Likewise, ICAM-1 is expressed on APC and activated/memory T cells. Therefore, we investigated the necessity of the expression of LFA-1 and ICAM-1 on T cells and APC in a U937-dependent T cell activation assay using various blocking mAb. As shown in Fig. 7, PBT were activated and produced IL-2 within 24 h when co-cultured with U937 cells (TCR signal was provided by immobilized OKT3), if either PBT or U937 cells were pretreated by control IgG or anti-CD29 mAb. In contrast, IL-2 production by PBT was strongly inhibited when either PBT or U937 cells were pretreated by anti-CD82 (Fab), anti-CD11a, or anti-CD54 mAb. These data demonstrate that expression of functional LFA-1 and CD82 on T cells and APC is important for optimal T cell activation.

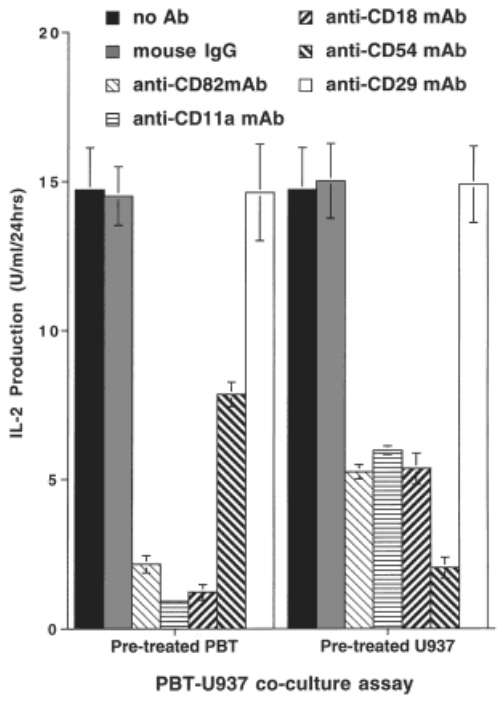

Figure 7. Effect of adhesion blocking reagents on PBT-U937 co-culture assay. PBT or U937 (1×10^5/well) were pre-treated with 10 μg/ml of adhesion blocking reagents (anti-CD82 Fab, anti-CD11a, anti-CD18, anti-CD54, anti-CD29, and control IgG) for 30 min at 37 °C and were co-cultured in the presence of immobilized OKT3. The concentration of IL-2 was measured after 24 h of co-culture. The data are expressed as the mean ± SD of triplicate samples. Representative data of two separate experiments are shown.

3 Discussion

In a previous report, we demonstrated that CD82 is up-regulated on T cells after activation by OKT3 or PMA treatment, and that the expression is higher on memory T cells compared with naive T cells [23]. CD82 up-regulation results in enhanced cell aggregate formation between T cells and APC as well as between T cells. We also determined that the formation of cell aggregates is enhanced solely by CD82 gene transduction, which validated that the expression level of CD82 is associated with the regulation of cell-cell interaction. In this report, we further analyzed the mechanism of CD82-induced cell-cell adhesion. The results of experiments using blocking mAb, immunoprecipitation, and confocal microscopy all suggested involvement of LFA-1-ICAM-1 interactions. LFA-1 belongs to the integrin superfamily and is composed ot CD11a/CD18 heterodimers. Its activity can be modulated by a structural modification of the complex without changes in its surface expression level [30]. The interaction between LFA-1 and ICAM-1 can be controlled in two ways, *i.e.*, through change of the affinity by Mg^{2+}-mediated conformational modification of the LFA-1 heterodimer or through alteration of the avidity by Ca^{2+}-mediated multimer formation of LFA-1 on the cell surface [22]. If we take into consideration that analysis by confocal microscopy revealed that CD82 and LFA-1 coexisted in adhesion foci and that CD82 was co-immunoprecipitated with LFA-1, it is tempting to speculate that CD82 expression regulates cell-cell contact by integrating LFA-1 into the focal adhesion complex and changing the avidity between LFA-1 and ICAM-1. However, it is also possible that an intracellular signal mediated by CD82 changes the conformation of LFA-1 and its affinity for ICAM-1 (a possibility that deserves further study).

Thus far, the roles of TM4SF proteins in cell adhesion have mainly been described in relation to their association with the β1-integrins. Recently, several investigators have reported that expression levels of TM4SF proteins are inversely correlated with the metastatic disposition of some human cancers [32–35]. As a possible explanation, Radford et al. [36] demonstrated that the expression level of the TM4SF protein CD63 is in good correlation with the affinity of β1-integrins for extracellular matrix. It is also reported that TM4SF proteins form a complex on the cell surface with integrins, especially the β1-integrin family, as revealed by immunoprecipitation analysis [27, 28, 36–41]. These observations are suggestive of a functional relationship between TM4SF and β1-integrins. Furthermore, it has been shown that cross-linking stimulation to TM4SF proteins such as CD53 and CD63 induces LFA-1/ICAM-1-mediated cell adhesion [42, 43]. Taken together with our observations, TM4SF may be a family of molecules that can regulate cell adhesion through β1- and/or β2-integrins. Physical and functional associations between TM4SF and integrins may be different in different cell types or conditions.

Analysis of T cell co-stimulatory activities of CD82 and LFA-1 using mAb revealed a similarity, in that they are CsA sensitive and synergistically amplified by anti-CD28 mAb. Results of immunoprecipitation indicated that CD82 and LFA-1 from a complex, and it is likely that cross-linking by anti-CD82 or anti-LFA-1 mAb results in the ligation of a similar complex of molecules and, hence, generation of similar co-stimulatory activities. So far, no enzyme activity nor associated protein has been identified in the intracellular portion of CD82, and it is possible that CD82 expressing may co-stimulate T cells through modifying the activity of LFA-1. If this is the case, it may be that CD82 can regulate both the intercellular and intracellular signals of LFA-1.

The present study demonstrated that, for the optimal T cell activation by APC (U937), CD82, LFA-1, and ICAM-1 need to be functionally present on both cells (Fig. 7). In this study, IL-2 production was strongly inhibited when resting T cells were pretreated by anti-CD82 (Fab) mAb (Fig. 7). Our analysis also revealed that anti-CD82 (Fab) mAb did not block the cell aggregate formation between the cells that highly express CD82 (Fig. 1 c). In a preliminary study, we found that only pretreatment of T cells with anti-CD82 mAb, but not the simultaneous treatment of T cells, strongly blocked the U937-dependent T cell activation in PBT-U937 co-culture assay (unpublished data). Moreover, among the anti-TM4SF proteins (anti-CD9, CD37, CD53, CD63, CD81, and CD82) mAb, only anti-CD82 (Fab) mAb blocked allo-reactive T cell activation, and also the heterotypic cell aggregation in the MLR of PBT and U937 cells (unpublished data). These results demonstrated the very rapid kinetics of the CD82-mediated cell adhesion, indicating that anti-CD82 mAb blocks the CD82-mediated cell adhesion only when the structural modification of integrins is not yet induced. CD82 expression is up-regulated on U937 cells by an activation signal such as by LPS/IFN-γ treatment, and this up-regulation results in enhanced cell aggregate formation and higher allogeneicity of U937 cells in MLR [23]. This suggests that the CD82 expression level correlates with the functional activity of both T cells and APC. It is possible that the TM4SF protein expression level can regulate the activation of various cells through interacting with different integrins. Analysis of the interactions between integrins and TM4SF proteins on various cells will help us understand the roles of this family of proteins in the regulation of cell-cell interaction and intracellular signaling.

4 Materials and methods

4.1 Cells and cell culture

The human T cell lymphoma cell line, Jurkat cells (E6-1 clone), and CTLL-2 cells were purchased from American Type culture collection (Rockville, MD). U937 cells (humans histiocytic leukemia cell line) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Methylcholanthrene-induced murine sarcoma cell line (Meth A) was purchased from Riken Cell Bank (Tsukuba Science City, Japan). These cell lines were maintained in culture in RPMI 1640 supplemented with 2 mM L-glutamine, 10 % FBS, 50 mM 2-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin; 10 IU/ml human rIL-2 was added to maintain CTLL-2 cells in culture. Fresh normal human PBT were obtained from adult healthy donors, and $CD3^+$ T cells were purified using the Cellect Human T column (Biotex Laboratories, Alberta, Canada). The resulting population was consistently >98 % $CD3^+$ as checked by flow cytometry.

4.2 Monoclonal antibodies

Mouse anti-human CD82 mAb (IgG2a) was prepared from 53H5 hybridoma culture supernatant as described previously [23]. Mouse anti-human CD54 (ICAM-1) mAb (IgG1)-producing hybridoma (17H5) was also established in our laboratory with a similar procedure. Anti-CD3 mAb, anti-CD4 mAb, anti-CD11a mAb, and anti-CD18 mAb were prepared from OKT3, OKT4, TS1/22, and TS1/18 hybridoma culture supernatants (ATC), respectively. MEM48 (purified or biotin-anti CD18 mAb), Lia 1/2 (anti-CD29 mAb), B-T1 (anti-CD102), HP2/19 (anti-CD50), TS2/7 (anti-CD49a), AK7(anti-CD49b), 11G5(anti-CD49c), 44H6(antiCD49d), SAM-1(anti-CD49e), 4F10(ANTI-CD49F), mouse IgG Fab, and human IgG1 were purchased from Cosmo-bio Co. (Tokyo, Japan); TP 1/32 (anti-CD11a mAb) and DE9 (anti-CD29 mAb) were purchased from Upstate Biotechnology (Lake Placid, NY); 44 (anti-CD11b mAb), ML13 (anti-CD9mAb), MB371 (anti-CD37mAb), H129 (anti-CD53mAb), H5C6 (anti-CD63mAb), and JS81 (anti-CD81mAn) were purchased from PharMingen (San Diego, CA); P4C10 (anti-CD29 mAb) was purchased from Gibco-BRL (Rockville, MD). FITC-, PE-, and TRITC-conjugated goat anti-mouse IgG and mouse IgG were purchased from Cappel (Durham, NC); TN288 (anti-CD28 mAb) was a generous gift from Dr. Hideo Yagita (Juntendo University, Tokyo, Japan). The 53H5 Fab fragment was prepared by a Fab preparation kit (Pierce, Rockford, IL). Biotin-CD82mAb was prepared with the EZ-link NHS-LC-Biotinylation kit (Pierce). In summary, OKT3, 53H5, TN288, Lia 1/2, TS1/22, TS1/18, 44, 17H5, B-T1, and HP2/19 were used for immunofluorescence staining or adhesion blocking assays. TS1/18, 53H5, and TN288 were used for cross-linking stimulation assays. Biotin-53H5, biotin-MEM48, 53H5, OKT4, DE9, MEM48, TP1/32, 17H5, and TN288 were used for immunoprecipitation and immunoblotting analyses.

4.3 ICAM-1-Ig fusion proteins

We used ICAM-1-Ig fusion protein as a substitute for the soluble ICAM-1. The fusion protein of the extracellular portion of human ICAM-1 and the Fc portion of human IgG1 (ICAM-1-Ig) was produced according to the technique for CTLA4-Ig as described previously [23]. Briefly, cDNA of the extracellular domain of ICAM-1 (+1 to +1440) was synthesized by PCR using oligonucleotide AGTCAAGCTTCACCATGGCTCCCAGCAGCCC as the forward primer, AGTCGGATCCGGGCTCCTCATACCGGGGGGA as the reverse primer, and the ICAM-1 cDNA fragment, a generous gift from Dr. S. Wright Caughmann (Emory University, Atlanta, GA) [44], as the template. The HindIII/BamHI digest of the ICAM-1 fragment and the Bcl/XbaI-cleaved Fc fragment encoding the hinge, CH2, and CH3 were subcloned together into a HindIII/XbaI-cleaved expression vector, CDM8. The ICAM-1-Ig construct (pCDM8ICAM-1-Ig) and neomycin-resistant gene selection marker plasmid were co-transfected into P3U1 cells by electroporation. After selection by G418 (800 μg/ml) for a few weeks, ICAM-1-Ig secreting clones

were selected by ELISA assay using 17H5 and horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig (Fc) mAb. ICAM-1-Ig was purified by Hitrap protein A (Pharmacia Biotech, Uppsala, Sweden) from serum-free culture supernatants from the ICAM-1-Ig-secreting P3U1 cells. Purity was confirmed by SDS-PAGE and Coomassie Brilliant blue staining. In preliminary study, soluble ICAM-1-Ig fusion protein could not be shown to bind even to PMA (5 ng/ml)-stimulated Jurkat cells by immunofluoroscence staining analysis. In contrast, PMA-stimulated Jurkat cells adhered to immobilized ICAM-1-Ig (10 μg/ml) more than parental cells, and pretreatment of PMA-stimulated Jurkat cells with anti-CD11a or anti-CD18mAb strongly inhibited the binding activity against the immobilized ICAM-1-Ig fusion protein (data not shown).

4.4 Reagents

Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (A23187) were purchased from Sigma (St. Louis, MO). CsA was purchased from Sandoz (Tokyo, Japan). RGD peptide (Gly-Arg-Gly-Asp-Ser), human fibronectin, vitronectin, laminin, and collagen type 1 were purchased from Gibco-BRL (Gaithersburg, MD).

4.5 Stable transfection of CD82 cDNA

A human CD82 cDNA-inserted plasmid (pRxCD82) and neomycin-resistant gene-inserted plasmid (pGEMneo) were co-transfected into Jurkat cells by electroporation as described previously. We generated four independent CD82-overexpressing Jurkat clones, which showed almost identical levels in CD82 expression. Human CD82-expressing Meth A cells were generated by infecting RxCD82 with the BOSC23 retroviral transduction system [45]. At 48 h after infection, the CD82-expressing population was analyzed by flow cytometry (> 98 % $CD82^+$).

4.6 Immunoprecipitation and immunoblotting analyses

Sequential immunoprecipitation and immunoblotting analysis (immunoprecipitation-Western blotting) were performed in accordance with methods described previously [29] with minor modification. In brief, parental or CD82-overexpressing Jurkat cells were lysed on ice for 1 h with 1 % polyoxyethylene 10 oleyl ether (Brij 96) lysis buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM NaF). After centrifugation at 12 000 rpm for 15 min, supernatants were incubated with protein G-Sepharose for 6 h to remove nonspecific adsorption. Then lysates were incubated with protein G-Sepharose saturated with each mAb at 4 °C for 1 h. After washing extensively with lysis buffer, coprecipitated proteins were eluted using Laemmli sample buffer. Eluted proteins were separated by SDS-PAGE under nonreducing conditions and electrophoretically transferred to a nitrocellulose membrane. After treatment with blocking buffer (3 % skim milk in PBS), membranes were incubated with biotin-mAb, and then with Extravidin HRP (Sigma). For detection of the signals, the Supersignal CL-HRP substrate system from Pierce was used.

4.7 Immunofluorescence staining and microscopic analysis

Jurkat cells were fixed briefly with – 20 °C acetone, then blocked with blocking solution (PBS containing 2 % BSA) at room temperature for 30 min and incubated with anti-CD11a mAb (1 μg/ml) in blocking solution. After washing, cells were treated with TRITC-conjugated goat anti-mouse IgG. For double staining, biotin-53H5 (anti-CD82 mAb) and FITC-conjugated streptavidin were used for indirect staining of CD82. Cells were then prepared for deposition onto poly-L-lysine-coated glass slides by Cytospin 2 (Shandon Southern Products, GB). These samples were observed under a fluorescence microscope/laser scanning confocal microscope (Leitz DMRBF and Leica TCS4D; Leica, Germany). Optical sections of 512 × 512 pixels were digitally recorded. Images were printed with a Fujix Pictography 3000 printer (Fuji, Japan) using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

4.8 Immobilized ICAM-1-Ig or extracellular matrix binding assay/blocking assay

To analyze binding activity of Jurkat cells against ICAM-1 or ECM, we performed an immobilized ICAM-1-Ig or ECM binding assay. A 12-well flat-bottom microplate was coated with purified ICAM-1-Ig (10 μg/ml), fibronectin (5 μg/ml), vitronectin (5 μg/ml), laminin (10 μg/ml), or collagen type 1 (5 μg/ml) for 2 h at room temperature and washed three times with PBS. Parental or CD82-overexpressing Jurkat cell lines (1×10^6/well) were added and incubated for 1 h at 37 °C. After gently washing wells with 37 °C PBS with calcium and magnesium (PBS+), the number of bound cells was counted using an objective micrometer (Olympus, Tokyo, Japan). For blocking assay, soluble mAb (10 μg/ml) were pretreated with cells for 30 min at 37 °C before incubation for the binding assay.

4.9 Antibody cross-linking stimulation assay

A 96-well flat-bottom microplate was coated with 53H5, TS1/18, and/or TN288 for 2 h at room temperature and washed three times with PBS. PMA- (2 ng/ml) and calcium ionophore- (250 ng/ml) stimulated PBT (1×10^5/well) were cultured for 24 h in the presence or absence of CsA (100 ng/ml). Supernatants after 24 h stimulation were collected and used for quantitation of IL-2.

4.10 PBT-U937 co-culture assay/blocking assay

PBT (1×10^5/well) and U937 cells (1×10^5/well) were co-cultured for 24 h in an OKT3-coated 96-well flat-bottom plate. Supernatants after a 24-h stimulation were collected and used for quantitation of IL-2. For blocking assay, soluble mAb (10 μg/ml) were pretreated with cells for 30 min at 37 °C before co-culture assay.

4.11 Quantitation of IL-2

IL-2 assay was performed using the IL-2-dependent T cell line, CTLL-2. Recombinant human IL-2, which was a generous gift from Dr. Taichi Sekine (National Cancer Research Institute, Tokyo, Japan), was used as a standard.

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