Brief Definitive Report

CD81 Expressed on Human Thymocytes Mediates Integrin Activation and Interleukin 2–dependent Proliferation

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Summary

Lymphocyte recognition of antigen by the antigen-specific T cell receptor (TCR) and coreceptor complexes rapidly alters the cell's adhesive properties facilitating high avidity cell–ligand interactions necessary for lymphocyte development and function. Here, we report the expression of CD81 (target of antiproliferative antigen [TAPA]-1) on human thymocytes and the physical association of CD81 with CD4 and CD8 T cell coreceptors. Antibody ligation of CD81 on thymocytes promotes the rapid induction of integrin-mediated cell–cell adhesion via lymphocyte function-associated molecule–1 (LFA-1). Cross-linking CD81 is also shown to be costimulatory with signaling through the TCR/CD3 complex inducing interleukin 2–dependent thymocyte proliferation. These data suggest that a CD81-mediated pathway in thymocytes is involved in the regulation of both cell adhesion and activation.

Thymic T cell selection, as well as the activation of mature T cells, is mediated by engagement of MHC-peptide antigen by the TCR and its coreceptors CD4 or CD8 (1–3). Signals resulting from TCR and CD4/CD8 engagement can also influence the activation state of integrins and integrin–ligand interactions promote cell–cell or cell–extracellular matrix adhesion, cell migration, activation, and effector functions (4, 5). Integrin-mediated adhesion to thymic epithelial and dendritic cells, and migration of these developing progenitors to cortex, medulla, and peripheral lymphoid compartments are critical features of normal T cell development (6). However, little is known about the molecular mechanisms that connect antigen receptor complex–generated signals to the functional activation of integrins expressed by thymocytes.

CD81 is a member of the recently described tetraspan family of proteins which play an important role in regulating cell adhesion, migration, and cell proliferation mediated by integrins (7–10). For example, CD9 associates with very late antigen (VLA)4 and VLA5 on B cell progenitors but not mature B cells, and it mediates heterotypic cell adhesion to bone marrow stromal fibroblasts (11). Another recently described tetraspan protein, CD63, has been shown to associate with VLA3 and VLA6 integrins (12). CD81 was originally named target of antiproliferative antigen (TAPA-1) because anti- CD81 antibodies inhibit the proliferation of many lymphoid cell lines. CD81 is expressed on B cells as part of the CD21/CD19/Leu13 complex which is the coreceptor for the B cell antigen receptor (BCR) and facilitates B cell activation at low doses of antigen-C3d (complement fragment) complexes (9, 13, 14). Ligation of CD81 on B cells activates VLA4-mediated binding to fibronectin on tonsilar sections (15). CD81 has been also reported to associate with CD4 and CD8 (coreceptors for the TCR) on T cell lines (16, 17). Although CD81 expression has been demonstrated on a variety of lymphoid cell lines, its expression and function on human thymocytes is unknown. The present report demonstrates the expression of CD81 on thymocytes and its influence on thymocyte adhesion and activation.

Materials and Methods

Thymocyte Preparation and Western Blotting. Thymocytes were prepared by mincing through steel mesh in cold HBSS (GIBCO BRL, Gaithersburg, MD), purified over Ficoll gradient, and washed two times in HBSS. Cells were lysed at 10^8 cells/ml in 1% NP-40, 20 mM Tris, 150 mM NaCl, 4 mM EDTA, 5 μM pepstatin, 1 mM PMSF, 10 μg/ml leupeptin for 1 h on ice, centrifuged at 14,000 *g* for 30 min, and soluble material diluted 1:1 with 2× Laemmli's without 2-ME. For Western blotting, the cell lysates were boiled for 3 min and run on 9% (or 4–10% gradient) polyacrylamide gel, transferred to polyvinylidine difluoride (PVDF) membranes (Gelman Sciences Inc., Ann Arbor, MI), blocked in 5% BSA/1% ovalbumin and blotted with 1 μg/ml mAb 5A6 (anti-CD81). Proteins were visualized by enhanced chemiluminescence (ECL; Pierce Chemical Co., Rockford, IL).

Flow Cytometry. Thymocytes were incubated on ice for 30 min in HBSS with 1 μg/ml 5A6 (CD81) or mouse IgG control, washed, and labeled 30 min on ice with goat anti–mouse FITC conjugate (Fig. 1), or clone CH/4 (CD69) tricolor (Caltag Laboratories, San Francisco, CA), anti-CD25 biotin (Olympus Corp.,

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Lake Success, NY), streptavidin PE (Sigma Chemical Co., St. Louis, MO) or with appropriate directly conjugated isotype controls (Fig. 4 B), for assessment on a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data analysis was performed with Winmdi software, available as freeware for Windows (http://facs.scripps.edu/software.html).

Thymocyte Biotinylation. 2×10^8 thymocytes/ml in PBS were incubated 30 min at room temperature with 2 mg/ml sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) (Pierce Chemical Co.), washed three times in PBS, and used for lysis.

Immunoprecipitation. 150 μl of thymocyte lysate at 2×10^8 cell equivalents/ml was incubated with 2 μg of the indicated mAb for 2 h at 4°C and then for 2 additional h with rabbit anti–mouse adsorbed protein A–Sepharose. Antibodies used were: UPC-10 (IgG control), OKT11 (CD2), OKT3 (CD3), OKT4 (CD4), OKT8 (CD8) (American Type Culture Collection [ATCC], Rockville, MD), 5A6 (CD81; gift of Shoshana Levy, Stanford University, Stanford, CA). Immunoprecipitates were centrifuged at 14,000 *g* for 10 s, washed three times in lysis buffer containing 0.1% detergent, resuspended in Laemmli's reagent without 2-ME, run on PAGE, and Western blotted as described above. Streptavidin blotting was performed using 1:10,000 dilution of horseradish peroxidase–conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL).

Aggregation Assay. Thymocytes were cultured at 2×10^5 cells/well in 96-well culture plates. Inhibition studies were done by preincubations with the indicated blocking antibodies at 10 μg/ml at 4°C for 30 min. The precoated cells were then warmed to 37°C in a humidified incubator, and 200 ng/ml of anti-CD81 antibody were added. After 1.5 h of incubation the cells were photographed. Blocking antibodies were anti-VLA-4 (clone HP2/1; gift of Dr. Sanchez-Madrid, The Princess Hospital, Madrid, Spain), anti-LFA-1, and anti-intercellular adhesion molecule (ICAM)-1 (clones 25.3 and 84H10; Coulter Corp., Hialeah, FL). All cultures were done in AIM V serum-free media (GIBCO). As control, a mouse anti-rat IgG was used (clone 18.5; ATCC). All cultures were done in AIM V serum-free media.

Costimulation Assay. Culture wells containing immobilized antibody were precoated overnight with 1 μg/ml OKT3 in PBS at 4°C, rinsed, and coated with the indicated concentrations of anti-CD81 for 4 h at 37°C. Soluble anti-CD81 antibody was added at the indicated concentrations in media simultaneously with the addition of cells. Anti-CD25 (anti-Tac mAb; gift of Dr. T. Waldman and Caroline Goldman, National Institutes of Health) was also added simultaneously with cells at the indicated concentrations. Cultures were performed in AIM V serum-free media. All coated wells were blocked with heat-aggregated BSA (60°C for 5 min) to cover any remaining binding sites on the plastic before addition of any soluble antibodies to the cultures.

Results

CD81 is expressed at high levels on human thymocytes as determined by Western blotting (Fig. 1 *A*) and flow cytometric analysis (Fig. 1 *B*). Western blotting of a thymocyte lysate with anti-CD81 results in a single band at the expected size of 26 kD under nonreducing conditions. Flow cytometric analysis demonstrates that CD81 is expressed on >99% of all thymocytes, indicating that it is expressed at all stages of thymocyte development.

To determine whether CD81 is associated with CD4, CD8, or other surface receptors, thymocytes were surface

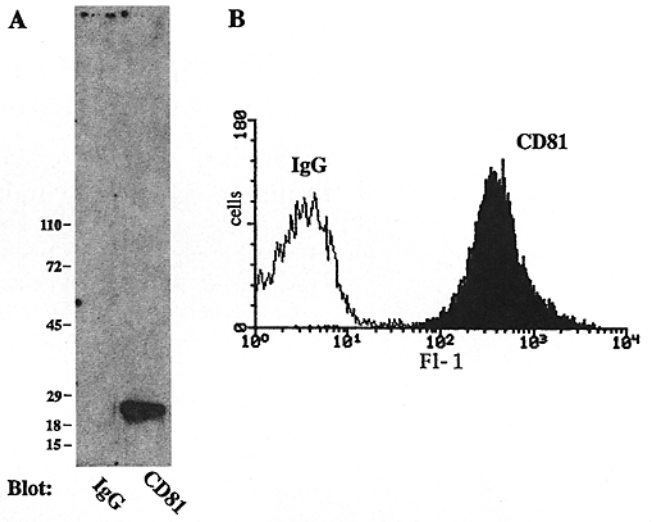

Figure 1. Demonstration of CD81 expression on human thymocytes. Thymocyte lysate was resolved on 4–10% gradient PAGE under nonreducing conditions, transferred to a PVDF membrane, and blotted with control IgG or with anti-CD81 (mAb 5A6) as indicated (*A*). Total thymocytes were stained with anti-CD81 or IgG control and goat anti–mouse FITC and assessed by flow cytometry (*B*). Data represent experiments from two thymic donors

biotinylated, lysed in (3-[(3-cholamidopropyl) dimethyl/ammonio]-1-propane-sulfonate) (CHAPS) detergent, immunoprecipitated with antibodies against CD2, CD3, CD4, CD8, CD81, or Ig control, and then analyzed by Western blotting with anti-CD81 (Fig. 2 *B*). These samples were also blotted with streptavidin to visualize the biotinylated target antigen of each immunoprecipitation, as well as coprecipitating cell surface molecules (Fig. 2 *A*). The results indicate that CD81 is prominently associated with CD4 and CD8, weakly associated with CD3, and not detectable in association with CD2. The immunoprecipitation of CD81 and visualization of associated surface proteins reveals that CD81 may be also associated with a number of other cell surface proteins. None of these associations were seen in NP-40 lysates (Todd, S.C., S.G. Lipps, L. Crisa, D.R. Salomon, and C.D. Tsoukas, unpublished observations). Note that CD81 on thymocytes is not biotinylated under these conditions, and thus is not visualized in the immunoprecipitated lysates.

To assess whether CD81 is capable of influencing the adhesive properties of thymocytes, these cells were incubated with anti-CD81 Ab. Ligation of thymocyte CD81 with as little as 250 ng/ml Ab, without secondary Ab cross-linking, rapidly induces (within 1 h of treatment) cell–cell adhesion resulting in cell aggregation (Fig. 3, row *A*). The rapid aggregation kinetics suggest that adhesion is mediated by the activation of preexisting receptors rather than the result of new protein synthesis. Culture of thymocytes with up to 1,000 ng/ml anti-CD2, -CD3, -CD4, or -CD8, has little effect (Fig. 3, row *B*). This result indicates that aggregation is not simply a consequence of cross-linking highly expressed surface proteins thereby creating a bridge between adjacent cells.

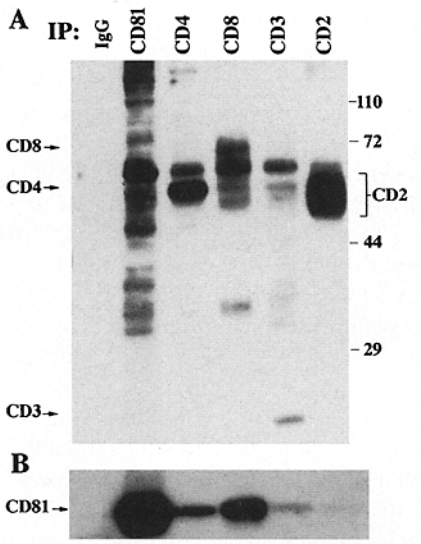

Figure 2. CD81 is physically associated with CD4 and CD8 on thymocytes. Thymocytes were surface biotinylated, lysed in 1% CHAPS, soluble material was subjected to immunoprecipitation as indicated, resolved on 9% PAGE under nonreducing conditions, transferred, and blotted with streptavidin (*A*) or with anti-CD81 (*B*). Arrows or brackets indicate expected molecular weights for indicated antigens under nonreducing conditions. Results represent experiments from three thymic donors.

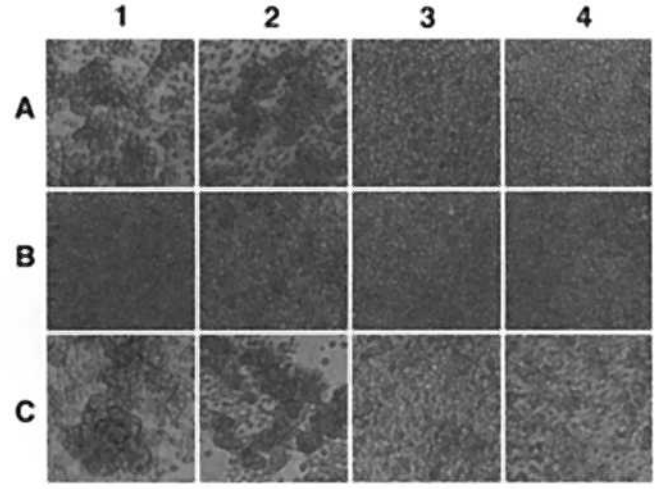

Figure 3. CD81 ligation by antibody induces LFA-1–mediated cell–cell adhesion. Thymocytes were cultured with a titration of anti-CD81 antibody, shown in row *A*, at 500 ng/ml (A1), 250 ng/ml (A2), 50 ng/ml (A3), or 10 ng/ml (A4). Row *B* shows thymocytes cultured with Abs to other highly expressed cell surface proteins at 1,000 ng/ml of anti-CD2 (B1), anti-CD3 (B2), anti-CD4 (B3), or anti-CD8 (B4). Row *C* shows thymocytes treated with 200 ng/ml anti-CD81 after prior incubation with 10 μg/ml rabbit anti–mouse control (C1), anti-VLA-4 (C2), anti–LFA-1 and anti–ICAM-1 (C3), or anti–VLA-4, anti–LFA-1 and anti-ICAM-1 (C4). In all cases, thymocytes were cultured for 1.5 h and photographed. The data represent experiments from three or more thymic donors.

CD81-induced aggregation does not occur in the absence of either Ca^{2+} or Mg^{2+} or when cells were maintained at 4°C (Todd et al., unpublished observations). Such divalent cation and temperature dependence is characteristic of integrin-mediated adhesion. Integrins expressed on thymocytes include the beta-2 family member lymphocyte function-associated molecule-1 (LFA)-1, CD11a/CD18) which interacts with the ICAMs of the Ig supergene family, and the beta-1 family member VLA-4 (CD49d/CD29) which serves as a receptor for fibronectin and vascular cell adhesion molecule- (VCAM)-1 (18). To assess whether these integrins are mediating the CD81-induced adhesion, thymocytes were pretreated at 4°C with blocking concentrations of specific integrin Abs and then stimulated with anti-CD81 as described above. The results indicate that Ab blocking of LFA-1 and ICAM-1 prevents aggregation whereas anti-VLA-4 does not (Fig. 3, row *C*). Treatment with anti–LFA-1 alone strongly inhibits aggregation while anti–ICAM-1 alone has little effect (Todd, S.C. et al., unpublished observations). This may indicate that LFA-1 is interacting with ICAM-2 or ICAM-3, as well as with ICAM-1.

It is well documented that the conversion of LFA-1 from the low to high affinity state results from cell activation and is associated with protein kinase C (PKC)-mediated phosphorylation of the cytoplasmic domains of LFA-1 and adjacent cytoskeletal elements (19–21). Therefore, we performed a series of experiments to determine if PKC activation also mediates CD81- induced thymocyte aggregation. First, the addition of phorbol ester, PMA (10 ng/ml) induces thymocyte aggregation within 1 h which is specifically blocked by anti–LFA-1 but not anti–VLA-4 antibodies (Todd, S.C. et al., unpublished observations). This result is consistent with a previous report indicating that LFA-1–mediated adhesion to thymic epithelial cell lines was induced by PMA (22). Both anti-CD81 and PMA-induced aggregation are inhibited by the PKC inhibitors calphostin C and H7 at approximate EC_{50}s of 250 nM and 20 μM, respectively. In contrast, aggregation is not inhibited by the tyrosine kinase inhibitor tyrphostin. These findings implicate PKC as a mediator of the CD81 signals that lead to LFA-1 activation.

The functionality of CD81 on thymocytes was further investigated by assessing its capacity to influence cell proliferation. In view of the antiproliferative effects of anti-CD81 on lymphoid cell lines, we assessed its effect on spontaneously proliferating thymocytes. Freshly isolated thymocytes contain a population of precursor ($CD3^{lo}$) cells that are undergoing spontaneous cell division (23). The proliferation of these precursors in vivo generates a large pool of $CD4^+8^+$ cells which are the subjects of thymic selection. This proliferation can be measured when cells are plated at 5×10^5/well and pulsed overnight with [^{3}H]thymidine. In a representative experiment, control IgG treatment yielded 30,813 ± 7,757 cpm (mean ± SD of quadruplicates) while cultures treated with anti-CD81 yielded 33,130 ± 8385 cpm (mean ± SD of quadruplicates) after 24 h in culture. Similarly, anti-CD81 did not effect naturally occurring proliferation when measured at 48 h. Thus, proliferation of the naturally cycling population of thymocyte precursors does not appear to be inhibited by CD81 binding.

The ability of CD81 to provide costimulation in concert

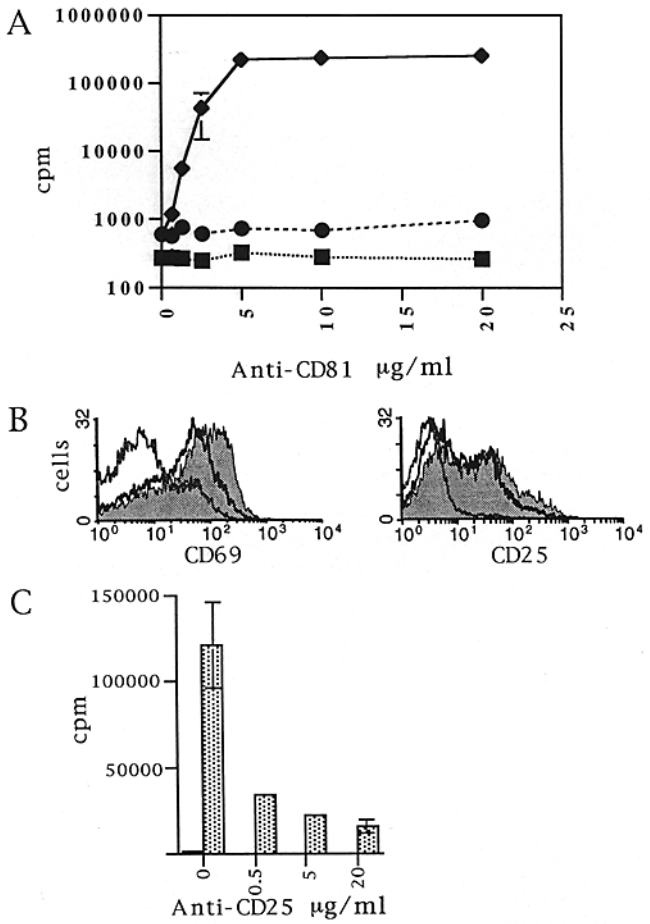

Figure 4. Co-cross-linking of CD3 with CD81 stimulates IL-2–mediated thymocyte proliferation. (*A*) Thymocytes were cultured with the indicated concentrations of immobilized anti-CD81 alone (*squares*), or with 1 μg/ml immobilized anti-CD3 and indicated concentrations of soluble (not prebound to culture well) anti-CD81 (*circles*), or with immobilized anti-CD3 and indicated amounts of immobilized anti-CD81 (*diamonds*). Cultures, performed in replicates of four, were pulsed with [^{3}H]thymidine (1 μCi/well) and harvested on day 4 of culture. These results represent experiments from two thymic donors. (*B*) Thymocytes cultured overnight (17 h) with media (*gray line*), anti-CD3 alone (*dark line*), or anti-CD3 with anti-CD81 (*shaded histogram*) were analyzed for CD69 and CD25 expression as indicated. (*C*) Thymocytes were stimulated with anti-CD3 and anti-CD81 in the presence of indicated concentrations of anti-CD25 (Tac) Ab known to block the binding of IL-2 to its receptor. Anti-CD3 alone yielded 1,024 cpm in the experiment shown.

with CD3 cross-linking was measured as [^{3}H]thymidine incorporation after 4 d in culture. Microtiter plate wells were coated with anti-CD3 (1 μg/ml) and increasing concentrations of anti-CD81 (Fig. 4 *A*). Alternatively, cells were cultured with immobilized anti-CD3 and soluble anti-CD81 (free Ab added to cultures after blocking the plastic surfaces to prevent fixation). The results illustrate that cross-linking of CD3 and CD81 (both Abs fixed to the culture well) promotes a powerful proliferative response in thymocytes. In contrast, immobilized anti-CD3 and soluble anti-CD81, immobilized anti-CD3 alone, or immobilized anti-CD81 alone, have little effect. Moreover, the physical proximity of CD81 and CD3 at the same cell surface is required for stimulation. This was demonstrated by the lack of proliferation when anti-CD81–coated thymocytes were plated onto immobilized anti-CD3 and subsequently the CD81 was cross-linked by addition of rabbit anti–mouse Ab (Todd, S.C. et al., unpublished observations).

Flow cytometric analysis of anti-CD3/anti-CD81–stimulated thymocytes on day 3 of culture demonstrated that 76% are $CD8^+CD4^-$. That proliferation is attributed to mature thymocytes was expected, since these cells bear a functionally competent TCR/CD3 complex. We have demonstrated that this is the thymocyte population proliferating to the combination of CD3 cross-linking and fibronectin or laminin costimulation (24, 25).

Thymocytes cultured under the same conditions as those in Fig. 4 *A* (media, anti-CD3 alone, or anti-CD3 and anti-CD81), were phenotypically characterized for the expression of the activation markers CD69 and CD25 (IL-2R). Anti-CD3 alone induces an increased expression of both these activation markers as early as 17 h of culture and expression of CD69 is further enhanced by coculture with anti-CD81 (Fig. 4 *B*). By day 3, the expression of both CD25 and CD69 is substantially higher on the CD81 costimulated thymocytes which is consistent with the observed proliferation in these cultures (not shown). Three-color flow cytometric analysis after 17 h of culture demonstrated that in both anti-CD3 alone and anti-CD3/anti-CD81–treated cultures the activated, $CD69^+$ cells are predominantly $CD4^{-/lo}CD8^+$. The specific activation of cells in transition to the mature $CD8^+CD4^-$ stage is consistent with the expansion of the $CD4^-CD8^+$ population observed on day 3 in the presence of CD81 cross-linking (not shown).

Despite the fact that thymocytes activated with either anti- CD3 or anti-CD3/anti-CD81 express equal levels of IL-2R at 17 h, only those cultures costimulated with anti-CD81 demonstrate proliferation (Fig. 4 *A*). This suggested that CD81-mediated costimulation may be mediated by an IL-2–dependent pathway. IL-2–dependent proliferation would be consistent with the increased IL-2R expression observed in the anti-CD3/anti-CD81 activated cells by day 3. To test this hypothesis, cells were stimulated with anti-CD3 and anti-CD81 in the presence of increasing amounts of a blocking anti-IL-2R Ab. As shown in Fig. 4, anti-CD3/anti-CD81–stimulated proliferation is inhibited by 73% with 500 ng/ml of anti-IL-2R antibody. Thymocyte proliferation is unaffected by control Abs at concentrations up to 20 μg/ml.

Discussion

Lymphocyte recognition of antigen rapidly alters their adhesive state. Increased adhesion can support higher avidity interactions with APCs that may in turn facilitate the engagement and signaling by other counterreceptor pairs. Similarly, T cell development and selection requires TCR-CD4 or -CD8 recognition of MHC-peptide on thymic epithelial or dendritic cells, and possibly other molecules, including LFA-1. That integrin-mediated adhesion is im-

portant for thymocyte development is supported by evidence that Ab blockade of LFA-1/ICAM-1 interactions in mouse fetal thymic organ culture inhibits differentiation of $CD4^-CD8^-$ cells to $CD4^+CD8^+$ cells (26).

In the present investigation we demonstrate that a CD81-derived signal can regulate thymocyte cell–cell adhesiveness through activation of the integrin, LFA-1. In vivo, CD81 may be activated directly by an unknown ligand or indirectly as a result of associated receptor engagement. The latter model allows for the possibility that during antigen recognition CD4 or CD8 (or CD21 on B cells) are engaged causing activation of associated CD81 molecules which in turn regulate adhesion. The participation of CD81 in the antigen receptor complexes of both T and B lymphocytes suggests a common fundamental role for CD81 in lymphocyte activation.

A recent report has suggested that CD81 is expressed on murine thymic epithelium. In that model CD81 serves as a ligand for an unidentified receptor on early thymocytes which promotes the differentiation of $CD4^-CD8^-$ cells into $CD4^+CD8^+$ cells (27). The same report also states that fetal mouse thymocytes do not express CD81 (27). In contrast, we demonstrate that CD81 is highly expressed on human thymocytes at all stages of maturation (Fig. 1). Reconciliation of these data may involve ontogenic differences between fetal mouse and pediatric human thymus or appreciation of important molecular differences between the two species in CD81 structure or expression.

The present report is the first demonstration that CD81 is expressed on human thymocytes and that the colocalization and cross-linking of CD81 with CD3 on T cells promotes DNA synthesis. This event does not occur upon independent engagement of CD81 or CD3. That proliferation is IL-2 mediated suggests that CD81 can provide a costimulation similar to that described for CD28. The data presented here also demonstrate a novel functional relationship between a tetraspan family member, CD81, and the activation of the integrin LFA-1–mediating homotypic cell aggregation. In the context of thymocyte development, the regulation of adhesiveness will determine the location of a cell, the nature of interactions with neighboring thymocytes or stromal cells, and its migration within the cortex or medulla. It will also influence the avidity of the thymocyte for APCs involved in positive or negative selection. Together, these findings support a role for CD81 in the regulation of thymocyte adhesion and activation during T cell development.

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