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Differential expression of CD43 isoforms on murine T cells and their relationship to acute intestinal graft versus host disease: studies using enhanced-green fluorescent protein transgenic mice

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Abstract

Three mAb (R2/60, S7 and 1B11) were used to study the expression of murine CD43 on peripheral T cells and intestinal intraepithelial lymphocytes (IEL) from normal mice, and from mice during acute graft versus host disease (GVHD). In the spleen, essentially all T cells expressed the R2/60 and S7 antigens, whereas the 1B11 antigen was expressed on about half of the $CD8^+$ cells and ~15% of $CD4^+$ T cells. Interestingly, a significant proportion of resting splenic B cells expressed the 1B11 and R2/60 antigens, but not the S7 antigen. The majority of IEL expressed R2/60 antigen; however, the S7 and 1B11 markers were differentially expressed on CD8α, CD8β, TCRαβ and TCRγδ cells. Immunoprecipitation and Western blotting analyses identified characteristic 115 and 130 kDa reactive components from IEL lysates with mAb S7 and 1B11 respectively, and reactivity to both molecular entities by mAb R2/60. During acute intestinal GVHD induced by injecting $CB6F_1$ athymic nude mice with spleen cells from C57BL/6 enhanced-green fluorescent protein transgenic mice, 80–90% of donor T cells in the intestine epithelium expressed all CD43 isoforms; however, the level of expression of the 130 kDa CD43 antigen increased significantly and the level of the 115 kDa antigen decreased on GVHD donor T cells compared to cells at the time of transfer. Using EL4 cells, a similar shift in the expression of CD43 isoforms occurred experimentally following treatment with neuraminidase, suggesting that the type of CD43 isoform expressed on T cells is strongly influenced by conditions which affect membrane charge. The significance of these findings for intestinal immunopathology is discussed.

Introduction

The small intestine epithelium in rodents and humans contains a diverse population of T cells collectively referred to as the intraepithelial lymphocytes (IEL). In mice these cells are primarily $CD8^+$ cells which include both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ subsets that express either a CD8αα homodimer or a CD8αβ heterodimer molecular complex (1). While the exact function of the IEL remains unclear, their strategic location at the surface of the intestinal lumen suggests a protective role in the defense against the entry and dissemination of harmful foreign antigens and infectious agents. That possibility is further underscored by the fact that a significant proportion of murine (2,3) and human (4) IEL are activated cytotoxic T cells. Perhaps related to that also, it is noteworthy that the intestine is a site particularly susceptible to inflammatory enteropathies (5) and to tissue damage resulting from graft versus host disease (GVHD) (6).

CD43 is a marker expressed on peripheral T cells (7,8), on thymocytes (9), and on early myeloid and lymphoid hematopoietic precursors within the bone marrow (10). Because of its potential for extensive *O*-linked glycosylation

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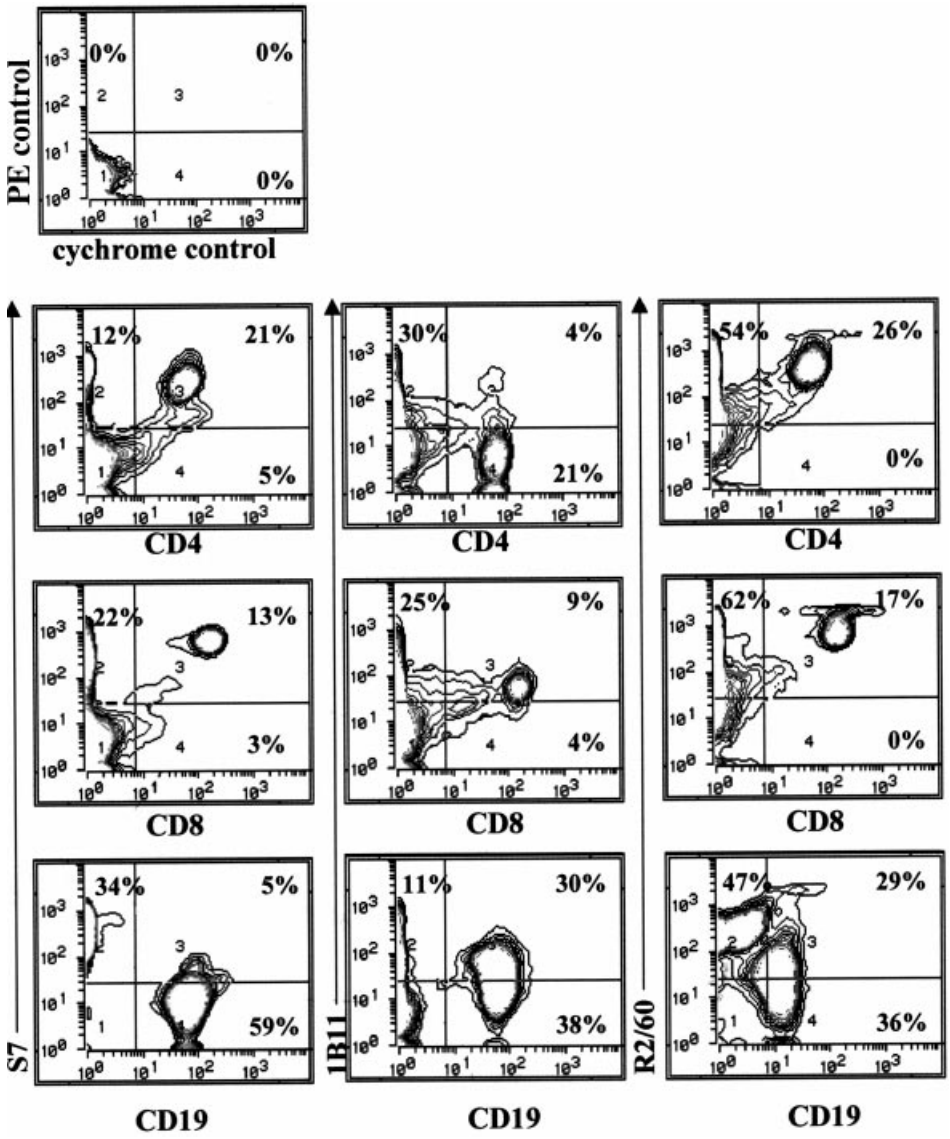


Fig. 1. Expression of CD43 isoforms on murine spleen cells. mAb R2/60 and S7 are reactive with all Thy-1$^+$ lymph node T cells from normal mice, whereas mAb 1B11 is principally reactive with CD8$^+$ but not CD4$^+$ peripheral T cells.

(11), CD43 can exist in a variety of molecular isoforms generated through post-translational modifications of the 35–45 kDa core protein. In mice these variations have been identified by mAb which reveal two principal membrane-bound CD43 molecules on T cells: one consisting of the widely distributed 115 kDa molecular component recognized by mAb S7 (8) and the other consisting of a 130 kDa component recognized by mAb 1B11 that is expressed on developing thymocytes and on resting CD8$^+$ but not CD4$^+$ resting T cells, and may be associated with T cell activation in mice (9). Functionally, CD43 has been shown to be involved in a number of T cell-mediated responses, including co-stimulation for proliferation (13–15), enhanced cytotoxicity by CD8$^+$ T cells (16) and leukocyte adhesion (17,18). However, T cell proliferative responses are augmented rather than depressed in CD43 knockout mice (11), further suggesting that the role of CD43 in the regulation of T cell function is complex and not fully understood.

Despite the diverse functional properties of CD43 on murine T cells described above, few studies have been done which have explored the role of CD43 in GVHD. In one study (19) the 130 kDa 1B11-reactive isoform was up-regulated and the 115 kDa S7-reactive isoform was unchanged or slightly reduced on CD8$^+$ T cells in the spleen during an acute GVHD response, although the expression of CD43$^+$ T cells in the intestine was not explored. In the present study we have examined the expression of CD43 isoforms on intestinal IEL and splenic lymphocytes from normal mice using mAb S7 and 1B11, and a recently characterized anti-CD43 mAb (R2/60) generated in our laboratory (14–16). Additionally, we have examined the expression of those markers on donor T cells during an acute GVHD in a semi-allogeneic experi-

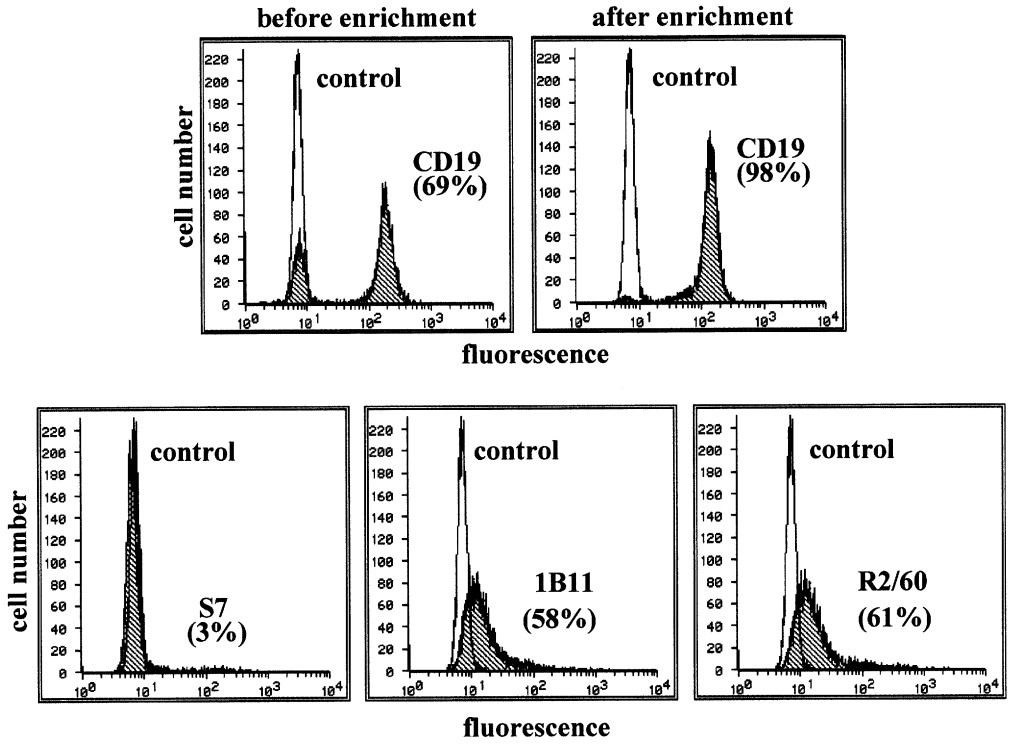


Fig. 2. Reactivities of mAb S7, 1B11 and R2/60 on purified splenic B cells. Note the nearly identical staining pattern for mAb 1B11 and R2/60, and the lack of expression of the S7-reactive antigen.

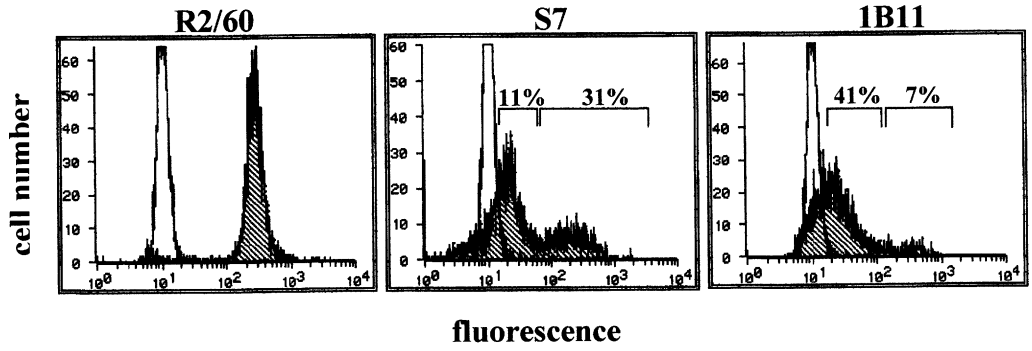


Fig. 3. Reactivities of mAb S7, 1B11 and R2/60 on freshly-isolated murine intestinal IEL. Open histograms indicate position of control staining.

mental system using H-2^b spleen cells from transgenic mice expressing a green fluorescent protein [enhanced-green fluorescent protein (EGFP)] adoptively transferred into non-transgenic congenitally athymic H-$2^{b/d}$ $CB6F_1$ nude mice. These studies indicate that, although most IEL in normal mice express CD43 as determined by reactivity with mAb R2/60, the S7 and 1B11 isoforms are differentially expressed on intestinal IEL. During GVHD, however, essentially all donor T cells which migrate to the intestine express both CD43 isoforms. The significance of these findings for understanding the involvement of CD43 on intestinal IEL under normal and disease states is discussed.

Methods

Mice and cell isolations

Congenitally athymic $CB6F_1$ nude mice were purchased from the Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice and EGFP-Tg C57BL/6 mice (20) were raised at the University of Tulsa; animals were housed under conventional conditions and used at 8–10 weeks of age.

Spleen cells and lymphoid cells from inguinal, popliteal and mesenteric lymph nodes were isolated by pressing tissues through a 60 mesh stainless steel screen. Small intestine IEL were isolated according to previously reported procedures

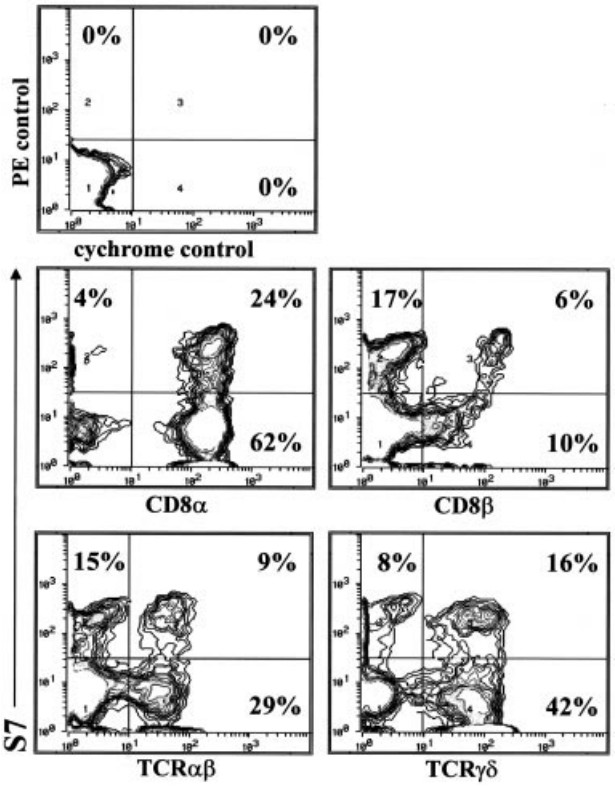


Fig. 4. Two-color flow cytometric analyses of S7 expression on intestinal CD8α, CD8β, TCRαβ and TCRγδ IEL subsets indicating that a significant proportion of $CD8^+$ IEL of either TCRαβ or TCRγδ type fail to express the S7 marker.

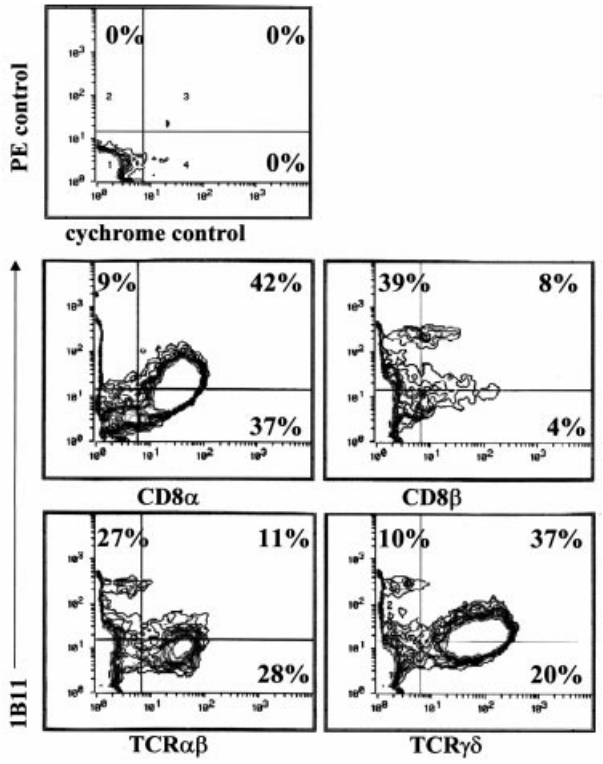


Fig. 5. Two-color flow cytometric analyses of 1B11 expression on intestinal CD8α, CD8β, TCRαβ and TCRγδ IEL subsets indicating that the 1B11 marker, which is expressed on most $CD8^+$ peripheral T cells, is expressed on only about half of the total IEL.

(21). Purification of splenic B cells was done using published protocols (22). Briefly, spleen cells were collected and incubated at 1×10^8 cells/10 ml in 100 mm tissue culture plates (Fisher Scientific, Dallas, TX) at 37°C in 5% CO_2 for 1 h to remove adherent cells. Non-adherent cells were recovered and erythrocytes were lysed with 0.83% ammonium chloride. Cells were washed, layered onto a discontinuous gradient consisting of 50, 60, 70 and 80% Percoll (Pharmacia, Uppsala, Sweden), and centrifuged for 20 min at 600 *g*. B cells were collected from the 60–70% Percoll interface, and residual T cells were removed by treatment with anti-Thy-1.2 (J1j.10), anti-CD4 (GK1.5), anti-CD8 (3.155) and anti-Mac1α (M1/70.15.11.5.HL) mAb (ATCC, Rockville, MD; all reagents) plus guinea pig complement (Accurate Chemicals, Westbury, NY).

Data displays are representative of multiple experiments consisting of a total of 21 mice studied individually for phenotypic analyses of spleen cells and IEL, 13 mice used for GVHD analyses and eight mice for immunoprecipitation/Western blotting experiments.

Generation of GVHD

Whole EGFP spleen cells (50×10^6) injected i.p. or whole lymph node cells (10×10^6) injected i.v. were transferred into non-transgenic $CB6F_1$ nude mice. Between 9 and 21 days later mice were sacrificed and lymphoid tissues were recovered for flow cytometric analyses and histopathology.

Immunoprecipitation and Western blot analyses

For immunoprecipitation experiments, 10×10^6 IEL were lysed at 4°C in 1 ml of detergent buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 μg/ml of aprotinin, leupeptin and pepstatin, 1% NP-40, and 0.25% deoxycholate (Sigma, St Louis, MO; all reagents). Clarified lysates were mixed overnight at 4°C with 8 μg/ml of S7 or 1B11 mAb and immune complexes were collected with agarose-conjugated anti-rat IgG (Sigma). Precipitated materials were boiled in 2×Laemmli's sample buffer (BioRad, Hercules, CA), electrophoresed through 7.5% SDS–polyacrylamide gel (BioRad) and subjected to Western blotting with mAb R2/60 as described below.

For direct R2/60 Western blotting, 20×10^6 IEL were lysed in 200 μl lysis buffer and 25 μl of clarified lysates were mixed with 25 μl of 2×sample buffer and electrophoresed through 7.5% acrylamide gel. Electrophoresed materials from these samples, and those described above from the S7 and 1B11 immunoprecipitation experiments, were transferred electrophoretically to PVDF membranes, blocked with 5% non-fat dry milk in PBS and reacted overnight at 4°C with 1:75 dilution of R2/60 culture supernatant or with J1j.10 mAb as a control reagent. Membranes were washed with PBS and reacted for 2 h at room temperature with 1:5000 dilution of horseradish peroxidase-conjugated rabbit anti-rat IgM antibody (Zymed, South San Francisco, CA).

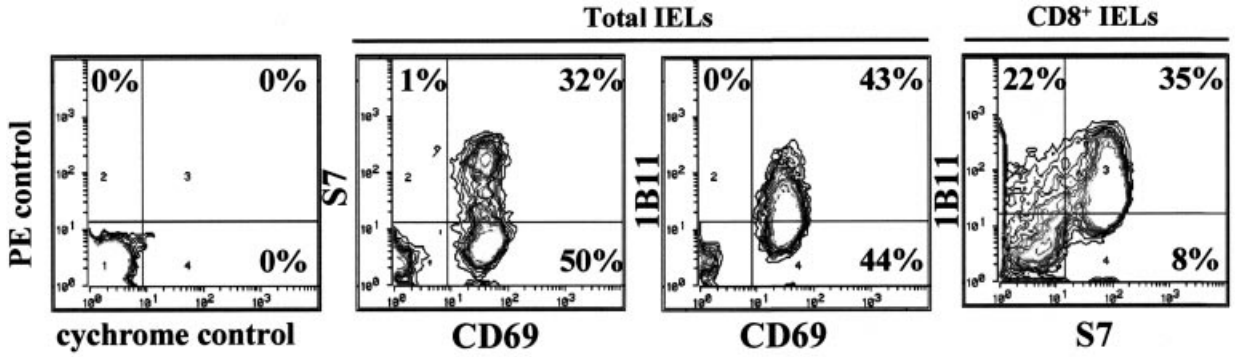


Fig. 6. Multicolor staining for CD69 expression on $S7^+$ and $1B11^+$ IEL, and S7 and 1B11 expression on $CD8^+$ IEL. CD69 and S7 or 1B11 expression on total IEL. Three-color staining after gating onto $CD8^+$ IEL (not shown) and analyzed for expression of S7 and 1B11 isoforms.

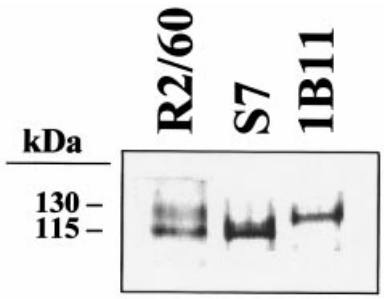


Fig. 7. Immunoprecipitation and/or Western blot analyses of IEL cell lysates with mAb S7, 1B11 and R2/60. Direct Western blot analyses of IEL cell lysates using mAb R2/60 identified both the 115 and 130 kDa components (left lane). S7 (middle lane) or 1B11 (right lane) precipitated products were electrophoresed, transferred to immunoblotting membranes and reacted with mAb R2/60; note the presence of the characteristic 115 and 130 kDa products for S7 and 1B11 respectively.

Enzymatic treatment of cells

EL4 cells (5×10^6) were washed once in HBSS, pelleted by centrifugation and resuspended in 470 μl of HBSS. Then 30 μl *Clostridium perfringens* neuraminidase (Sigma; cat. no. N2133) at 10 U/ml was added for a final concentration of 0.6 U/ml. Control cells received HBSS. Cells were incubated at 37°C for 30 min, washed twice with serum-supplemented media, and stained with mAb S7, 1B11 and R2/60 for flow cytometric analyses.

Histopathology

Mice were sacrificed, and intestinal tissues were removed and fixed in Bouin's solution. After dehydration in ethanol followed by xylene washing, tissues were embedded in paraffin, cut into 5 μm sections, stained with hematoxylin and eosin and mounted for microscopic analyses.

Antibodies and flow cytometry

Antibodies and reagents used in this study were: FITC-labeled anti-CD4, anti-CD8α, anti-CD8β, anti-CD43 (S7), anti-CD69, anti-$\alpha_E\beta_7$ and anti-Thy-1; phycoerythrin-(PE)-labeled anti-CD43 (S7 and 1B11); biotin-labeled anti-TCRαβ and anti-TCRγδ; anti-CD16 and anti-CD32 mAb were used for Fc receptor blocking; species-specific control antibodies; streptavidin-labeled Cy-Chrome Red-670 (PharMingen, San Diego, CA; all reagents). For staining with mAb R2/60, affinity-purified antibody from tissue culture supernatants was biotinylated using published protocols (23). Cells (1×10^6) were cultured for 30 min at 4°C with an appropriate fluorochrome- or biotin-labeled mAb in 100 μl HBSS containing 0.01% NaN_3, washed and fixed in 2% paraformaldehyde (Sigma) or washed and reacted with streptavidin–cychrome Red-670. Expression of cell surface markers was analyzed using an Epics 751 flow cytometer (Coulter Electronics, Hialeah, FL) interfaced to a CICERO data acquisition and analyses system (Cytomation, Fort Collins, CO) using 5000 events for single-color analyses and 50,000 events for multi-color analyses. Immuno-Check Alignment Fluorospheres (Epics Division, Coulter) were used for daily optical alignment. Standardization for compensation was done using a flow cytometry compensation kit (Sigma Immunochemicals).

Results

On peripheral T cells, anti-CD43 mAb reveal diverse patterns of CD43 expression

Freshly isolated spleen cells were assayed by flow cytometric analyses for reactivity with mAb R2/60, S7 and 1B11. As shown in Fig. 1, nearly all $CD4^+$ splenic T cells were reactive with mAb S7 and R2/60; in contrast, most $CD4^+$ cells were non-reactive with mAb 1B11. That pattern was generally similar for $CD8^+$ splenic T cells, although slightly more of those cells were weakly reactive with mAb 1B11. Interestingly, a significant population of splenic B cells also expressed the 1B11 and R2/60 antigens, whereas <10% of those cells expressed the S7 antigen (Fig. 1). Because expression of the 1B11 and R2/60 antigens has not been previously described for B cells, experiments were done to more thoroughly explore this using highly enriched splenic B cell preparations stained for single-color analyses with the three anti-CD43 mAb. As seen in Fig. 2, few B cells expressed the S7 antigen although slightly more than half of the total B cells were $1B11^+$ and $R2/60^+$. In these experiments, the possibility that reactivity of 1B11 and R2/60 mAb was due to binding by Fc receptor expressed on B cells was effectively ruled out by pretreatment of cells with Fc receptor blocking

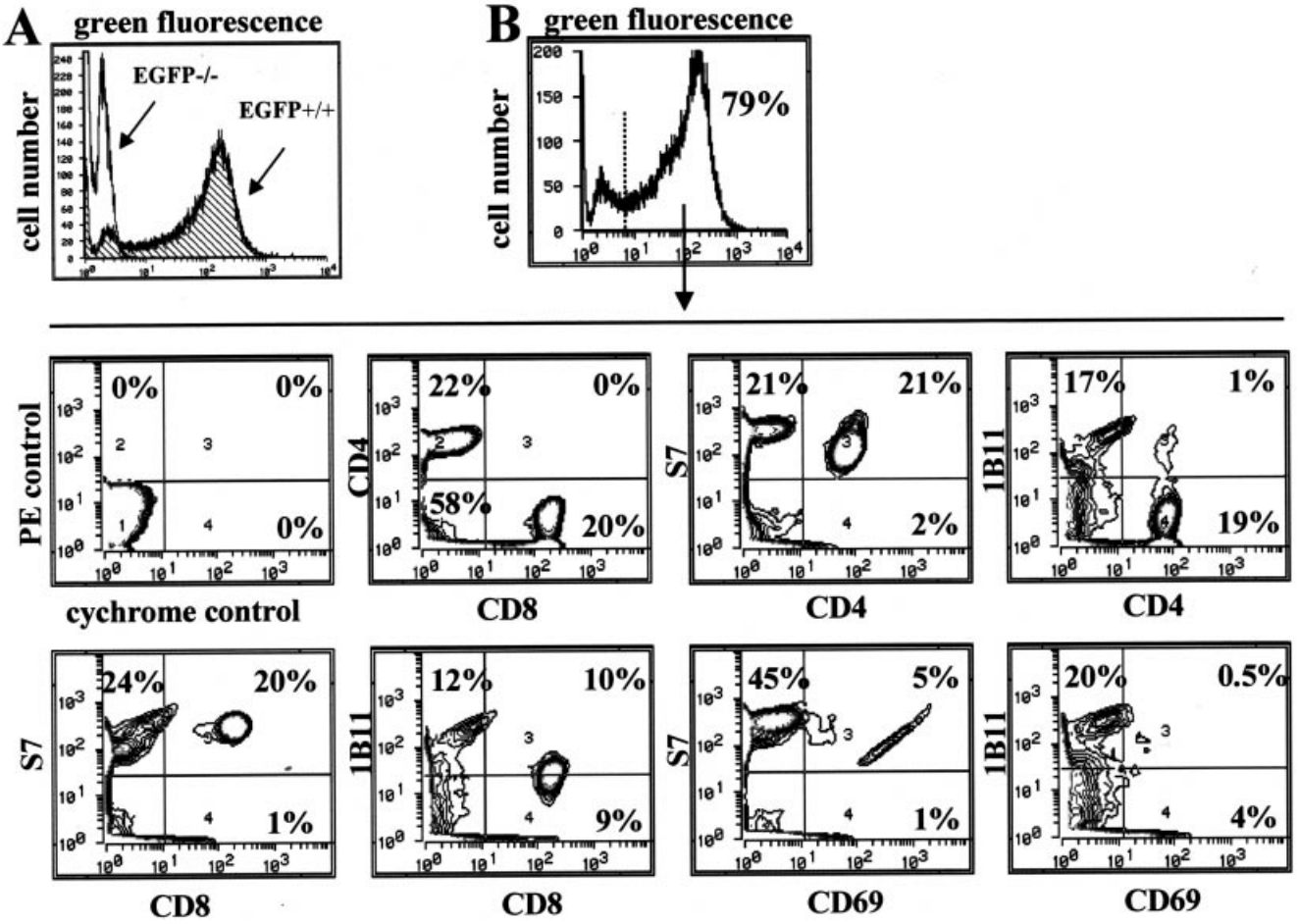


Fig. 8. (A) Comparison of endogenous green fluorescence on spleen cells from EGFP-Tg mice and non-transgenic C57BL/6 mice indicating high levels of green fluorescence in transgenic mice. (B) Phenotypic analyses of EGFP-Tg donor spleen cells at the time of cell transfer into $CB6F_1$ nude mice. Note the low levels of 1B11 antigen expression on $CD8^+$ T cells, the expression of the S7 antigen on both $CD4^+$ and $CD8^+$ T cells, and the non-activated phenotype of those cells based on CD69 expression.

antibodies prior to staining, because S7 and 1B11 are both rat IgG2a/κ antibodies despite the differences in reactivities of those reagents, and because the control antibody used for these analyses also was a PE-labeled rat IgG2a/κ antibody. Collectively, these findings indicate that the CD43 isoforms recognized by mAb S7 and 1B11 are differentially expressed on resting T cells and B cells, and that mAb R2/60 appears to recognize an antigen associated with either isoform.

Intestinal T cells display complex patterns of CD43 expression distributed across CD8α, CD8β, TCRαβ, and TCRγδ subsets

Intestinal IEL consist of $CD8^+$ T cells that include populations with properties typical of T cells found in other peripheral immune compartments, as well as subsets with features that are unique to the intestine (1). Thus, we were interested in analyzing the distribution of CD43 on murine IEL. As shown in Fig. 3, mAb R2/60 reacted with >90% of murine IEL, indicating that as with splenic T cells, most IEL also express CD43. However, unlike the expression pattern for R2/60, about half of the IEL were reactive with mAb S7 and 1B11 (Fig. 3).

To gain additional information about the reactivity patterns of S7 and 1B11 among IEL, two-color staining was done in conjunction with CD8α, CD8β, TCRαβ and TCRγδ since those markers define the primary IEL subsets in mice. $CD4^+$ IEL generally comprise only ~5–15% of the IEL; B cells are normally not present in the gut epithelium (1). As seen in Figs 4 and 5, both S7 and 1B11 isoforms were expressed on all four subsets, although in each case this consisted of $CD43^+$ and $CD43^-$ populations, indicating that expression of those antigens on IEL is not exclusively linked to a particular subset. Analyses of IEL using three-color staining of $CD8^+$ cells in conjunction with mAb S7 and 1B11 revealed that a high proportion of $CD8^+$ IEL that expressed CD43 co-expressed both isoforms, although $S7^+$ $1B11^-$ and $S7^-$ $1B11^+$ cells also were present (Fig. 6). Because murine IEL are known to have a high proportion of activated T cells (1), we explored the extent to which the S7 and 1B11 isoforms were associated with activated IEL based on expression of CD69, an early activation marker of T cells. As shown in Fig. 6, CD69 was expressed on all $S7^+$ and $1B11^+$ IEL, indicating that those IEL were recently activated T cells.

Finally, to characterize the molecular entities recognized by the three anti-CD43 mAb used here, immunoprecipitation and

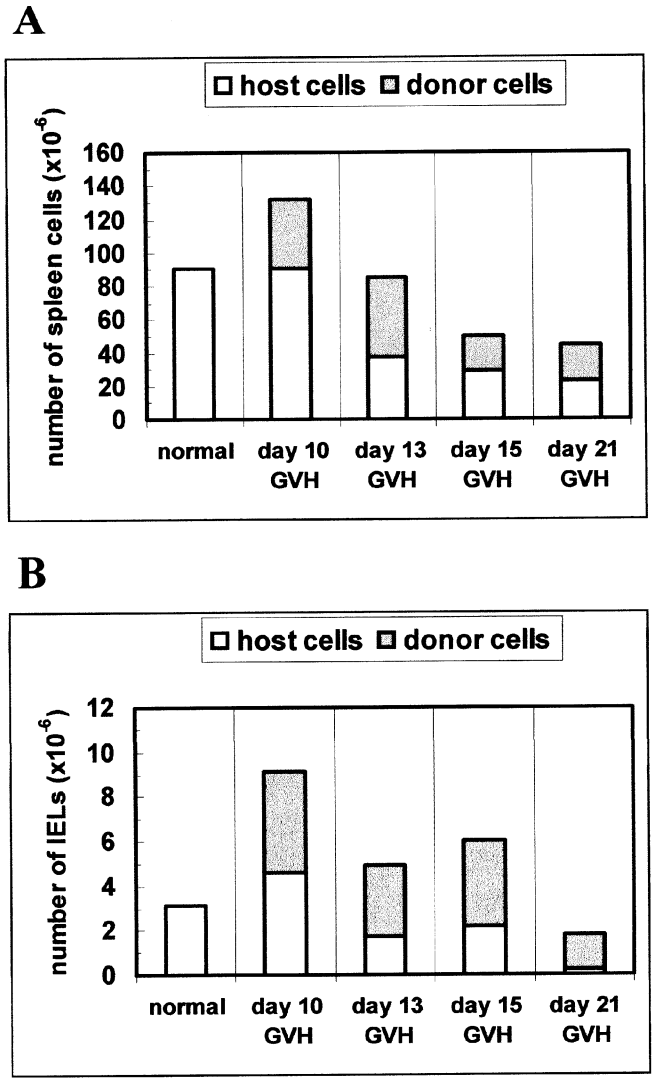


Fig. 9. Numbers of cells and relative proportion of EGFP$^+$ (donor cells) and EGFP$^-$ (host cell) in the spleen (A) and the intestine (B) of $CB6F_1$ nude mice between days 10 and 21 post-cell transfer.

Western blotting experiments were done with freshly isolated IEL cell lysates. S7 and 1B11 precipitated products were electrophoresed on polyacrylamide gels, transferred to immunoblotting membranes and reacted with mAb R2/60. As shown in Fig. 7, mAb S7 and 1B11 precipitated 115 and 130 kDa components respectively, further confirming that both CD43 molecular isoforms are present among lymphoid cells of the gut epithelium. Moreover, Western blotting of IEL lysates by mAb R2/60 identified both the 115 and 130 kDa components in IEL lysates (Fig. 7). Taken together, these findings strongly suggest that mAb R2/60 is reactive with an epitope that is shared by both the 115 and 130 kDa CD43 isoforms.

Donor T cells that migrate to the intestinal epithelium during acute GVHD express all CD43 isoforms with high levels of CD43 1B11 expression

To assess the extent to which CD43 isoforms may be involved in intestinal inflammation, GVHD was induced by adoptive transfer of spleen cells or lymph node cells from EGFP-Tg (H-2^b) mice into non-transgenic $CB6F_1$ (H-$2^{b/d}$) nude mice. Figure 8(A) indicates the levels of endogenous green fluorescence on lymph node cells from donor EGFP$^+$ mice compared to cells from non-transgenic $CB6F_1$ host mice, thus demonstrating the feasibility of using this system to discriminate donor versus host cells in recipient animals. As seen in Fig. 8(B), donor EGFP spleen cells prior to injection into $CB6F_1$ animals displayed a normal cellular profile of S7$^+$ and 1B11$^+$ cells in a non-activated state based on anti-CD69 staining.

Spleen cells and IEL from recipient $CB6F_1$ mice were studied between days 10–21 post-cell transfer—a time of donor cell infiltration into the intestine (24) and slightly after the time of peak 130 kDa CD43 expression in the spleen during acute GVHD (19). This is seen in Fig. 9(A), which indicates the presence of donor EGFP$^+$ cells in the spleen of recipient mice during the period of study. Phenotypic analyses of spleen cells 10 days post-cell transfer revealed a notable increase in the level of CD43 1B11 expression on both CD4$^+$ and CD8$^+$ donor cells (Fig. 10) compared to donor cells at the time of cell transfer (Fig. 8). Note, also, that by day 10, >90% of the EGFP$^+$ cells in the spleen were T cells (Fig. 10), indicating that few donor B cells were retained as long-lived cells following adoptive transfer.

In the intestine, donor cells were detected throughout days 10–21 post-cell transfer (Fig. 9B), and the intestinal epithelium displayed histopathological features typical of GVHD as seen by an erosion of the epithelial cell layer and pockets of mononuclear inflammatory cells (Fig. 11). Phenotypic analyses of isolated small intestine IEL indicated that, unlike IEL in normal mice, 80–90% of donor EGFP$^+$ cells expressed the antigens recognized by R2/60, S7 and 1B11 mAb (Fig. 12). Because approximately two-thirds of the S7$^+$ and 1B11$^+$ cells co-expressed the $\alpha_E\beta_7$ integrin, a molecular complex that is involved in cell adhesion between IEL and epithelial cells (25,26), and within the intestinal mucosa is expressed predominantly on IEL (25,27,28), and because <5% of IEL isolated throughout the period of GVHD were B cells (data not shown), it is unlikely that donor EGFP$^+$ cells were contaminants from the lamina propria as a consequence of pathology.

About half of the S7$^+$ cells and two-thirds of the 1B11$^+$ donor cells in the intestine expressed the CD69 activation marker (Fig. 12). Most interesting, however, was the finding that the 1B11 antigen was expressed at high levels on donor CD4$^+$ and CD8$\alpha\beta^+$ T cells that had migrated to the intestine, and that there was a decrease in the level of expression of the CD43 S7 isoform (Fig. 12) compared to IEL from mice without GVHD (Figs 4 and 5). These differences are readily evident in single-color staining profiles for S7 and 1B11 expression on EGFP$^+$ intestinal T cells during GVHD (Fig. 13).

Previous *in vitro* studies suggest that expression of the 130 kDa CD43 isoform is normally masked by sialic acid residues and that desialylation leads to increased expression of the 1B11 antigen (9,29). Thus, in an effort to understand the basis for the observed changes in the expression of the S7 and 1B11 antigens during GVHD, EL4 cells, which are known to be S7$^+$ and 1B11$^-$ (9), were treated with neuraminidase; enzyme-treated and untreated cells were stained for reactivity with mAb S7, 1B11 and R2/60. As shown in Fig. 14,

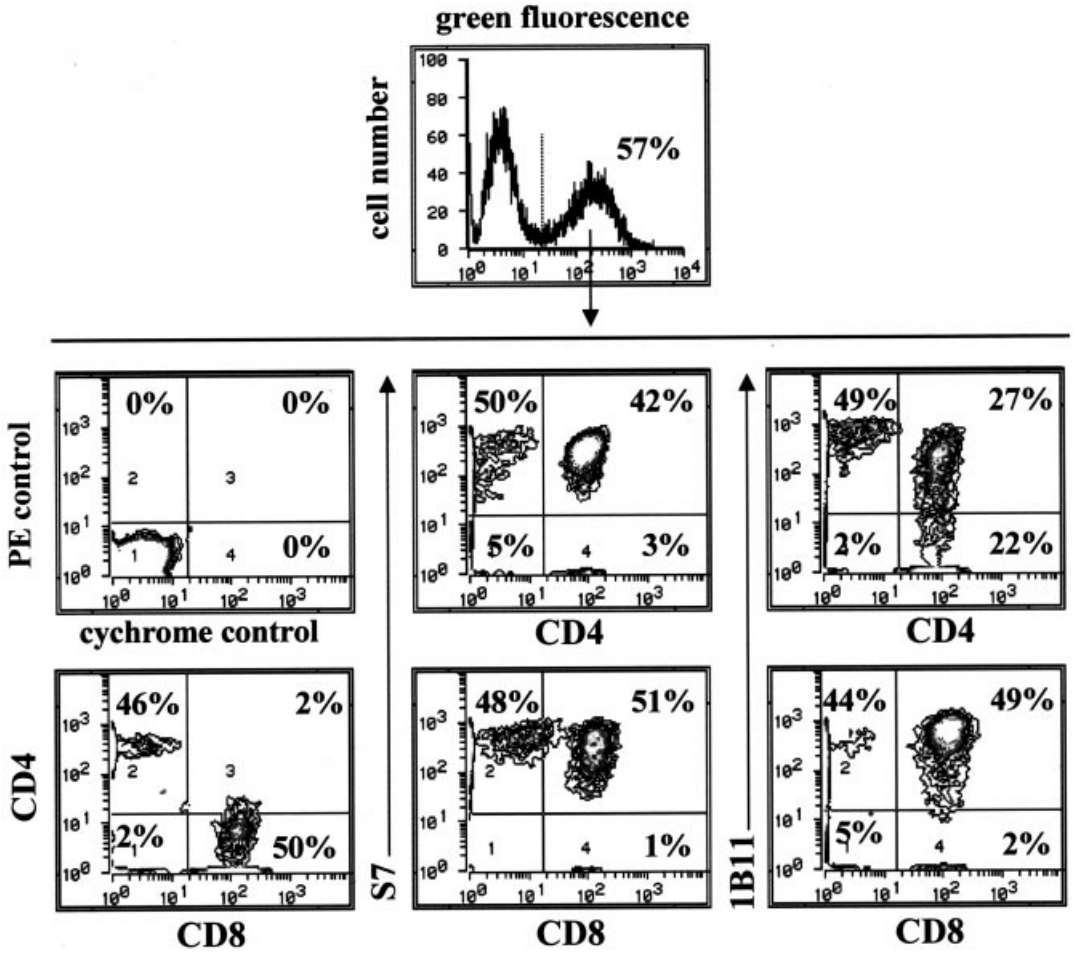


Fig. 10. Phenotypic analyses of EGFP$^+$ cells from the spleen of CB6F$_1$ nude mice 10 days after cell transfer. Note the increase in the 1B11 antigen expression on CD4$^+$ and CD8$^+$ EGFP$^+$ cells compared to donor cells at the time of transfer (Fig. 8B).

untreated EL4 cells were highly reactive with mAb S7 and R2/60, but had low levels of reactivity with mAb 1B11. In contrast, after neuraminidase treatment, reactivity of mAb S7 was significantly reduced, whereas reactivity with mAb 1B11 was markedly increased, although there was no change in reactivity with mAb R2/60. A similar pattern to this also was observed using freshly isolated IEL; however, neuraminidase treatment was less efficient, probably due to the neutralization of enzymatic activity by proteases released from intestinal cells. Regardless, these findings imply that the increase in expression of the 1B11 antigen on donor T cells in the intestine during GVHD may involve changes in surface membrane sialylation resulting in alterations in the expression of CD43 isoforms.

Discussion

Due to its multiplicity of functions, many with seemingly contradictory properties, the CD43 molecule represents one of the most complex and arcane lymphocyte accessory molecules used by the immune system. The negatively charged mucin-like nature of CD43 imparts many anti-adhesive characteristics upon CD43$^+$ cells, thus making it difficult to envision how CD43 might participate in intercellular interactions between lymphocytes, particularly on activated cells where such interactions would be expected to be of paramount importance. A possible mechanism for this, as recently proposed (30), is that CD43 interactions are regulated to a large degree by specific ligands expressed on other hematopoietic cells or on tissue cells. According to this model, CD43$^+$ cells exist simultaneously in two functional states: one which exerts repulsive forces between the CD43$^+$ cell and ligand-negative cells, the other which promotes and facilitates intercellular interactions due to direct engagement of CD43 with ligand-positive cells (30). Although at present there is little direct evidence to support a mechanistic model such as this, and the complete nature of the CD43 ligand(s) remains to be defined, this scenario nonetheless provides a feasible explanation as to how cells bearing highly sialylated residues can operate in a manner conducive to intercellular commun-

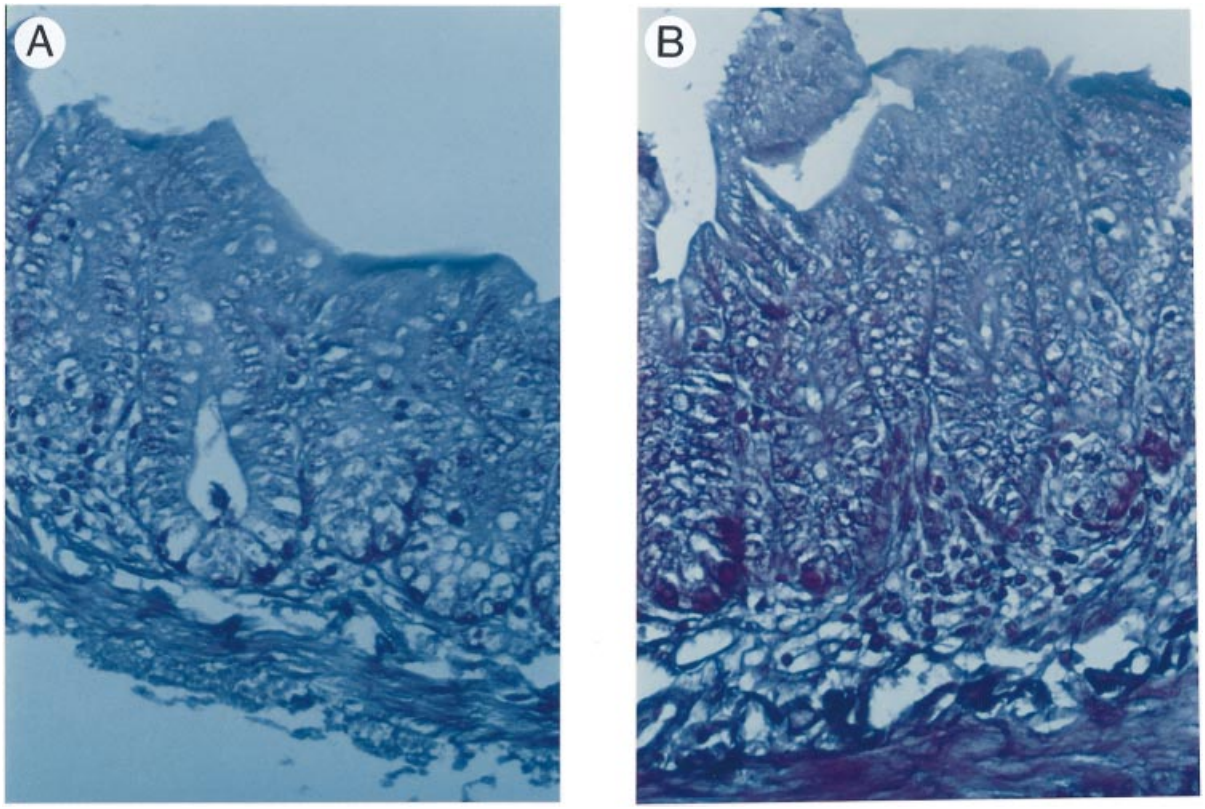


Fig. 11. Histopathological analyses of small intestine tissues from C57BL/6 mice injected with EGFP lymph node cells 15 days previously. Note the villus disorganization in the apical regions and the foci of mononuclear cells below villus crypts. ×200.

ication. From a functional point of view, CD43 on T cells has been shown to be involved in cell signaling leading to T cell activation. Moreover, this occurs in a CD28-independent manner—a point which may have considerable significance for intestinal IEL as discussed later.

The findings reported here identify several previously unknown properties of CD43. Using three mAb to CD43, we demonstrate that although essentially all murine T cells from normal mice express CD43 as shown by reactivity with mAb R2/60, expression of the 115 kDa isoform recognized by mAb S7 and the 130 kDa isoform recognized by mAb 1B11 can vary significantly between and within immunological compartments, most notably on lymphoid cells of the small intestine where the S7 and 1B11 antigens were found to be differentially expressed across several phenotypic subsets. In fact, murine IEL are an extremely complex cell population comprised of both thymus-derived and extrathymic $CD8^+$ T cells, the latter most likely developing from within specialized compartments of the intestinal mucosa (31). Those differences in developmental lineages are reflected, at least in part, by the expression of CD8αα or CD8αβ in conjunction with TCR usage. Thus, CD8αα IEL expressing either TCRαβ or TCRγδ are probably extrathymic T cells (32), whereas in normal mice some TCRαβ, CD8αβ IEL undoubtedly enter the intestinal mucosa from the peripheral T cell pool in a manner similar to that demonstrated here. However, the empirical observations described in the present study indicate that CD43 expression does not correlate with developmental lineage since all of the major IEL subsets consisted of both isoform-positive and isoform-negative cells for either isoform. This further reinforces the notion that CD43 on IEL is probably linked to effector-related activities as discussed below.

Our findings, based on staining of IEL, indicate that mAb S7 does not recognize a pan-CD43 antigen, as previously believed. By comparison, the likelihood that mAb R2/60 is specific for all CD43 isoforms in mice is empirically evident in several ways. First, both the S7 and the 1B11 immunoprecipitated products obtained from IEL lysates were reactive with mAb R2/60 by Western blotting, and in each case this resulted in a characteristic 115 kDa band from S7 precipitates and a 130 kDa band from 1B11 precipitates. This indicates that mAb R2/60 is reactive with both molecular species of CD43 and suggests that it is probably specific for a shared CD43 determinant. Secondly, direct immunoblotting of IEL lysates using mAb R2/60 identified both the 115 and the 130 kDa CD43 components. Thirdly, flow cytometric analyses of CD43 expression by mAb R2/60 within peripheral immunological compartments generally correlated with the total reactivities of mAb S7 and 1B11. Fourthly, neuraminidase treatment of EL4 cells caused a reciprocal shift in S7 and 1B11 expression, but did not alter the overall reactivity of mAb R2/60.

Within the intestine epithelium of normal mice, CD43 (S7 and 1B11) was almost exclusively expressed on $CD69^+$ cells, indicating a strong correlation between CD43 expression and gut T cell activation. Moreover, the majority of,

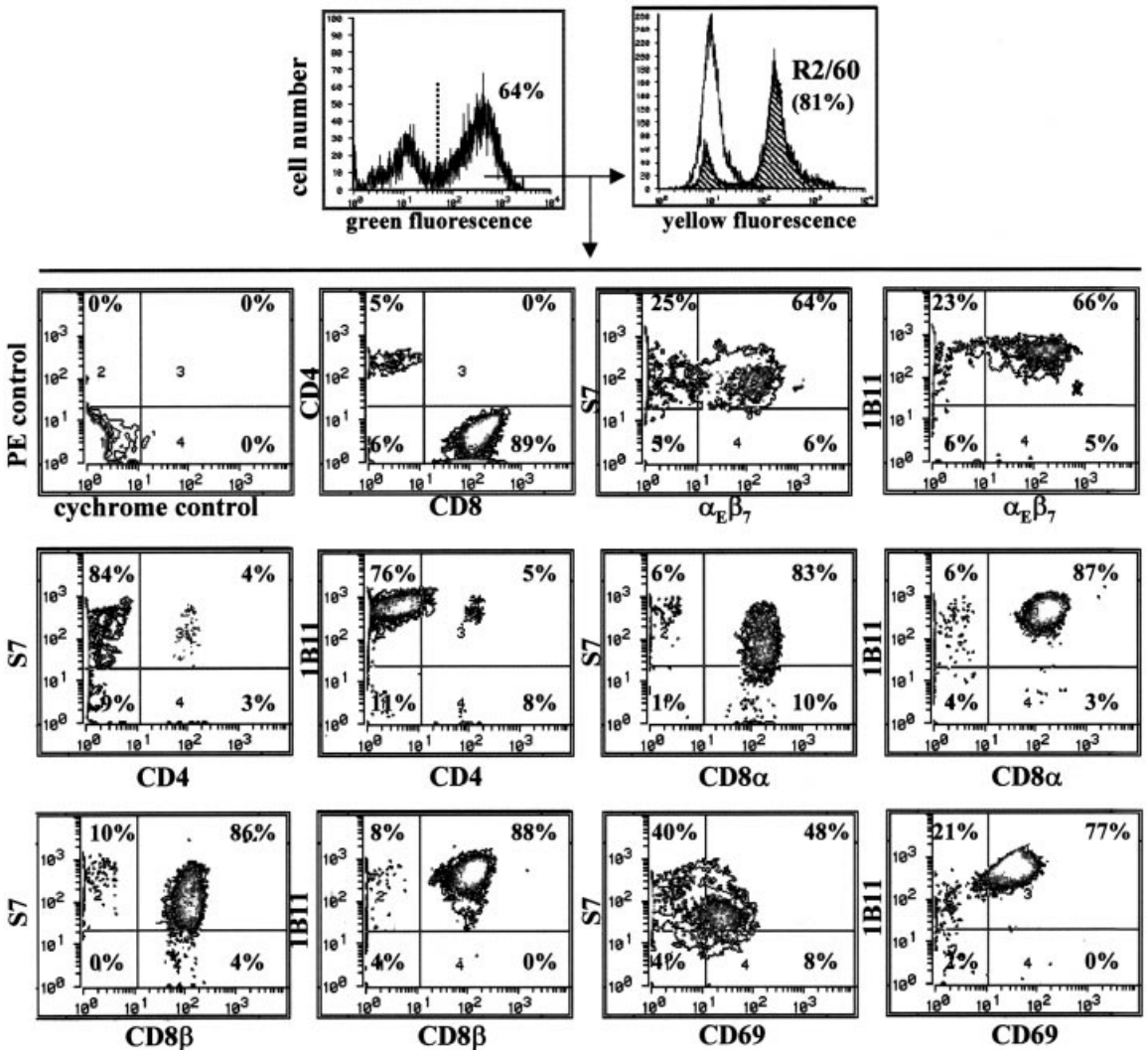


Fig. 12. R2/60, S7 and 1B11 expression on EGFP$^+$ cells isolated from the small intestine of C57BL/6 host mice injected 13 days previously with EGFP lymph node cells. Note the increase in CD43 1B11 antigen expression on CD8$^+$ donor cells and the presence of the CD69 activation marker on 1B11$^+$ cells compared to donor cells at the time of transfer (Fig. 8B).

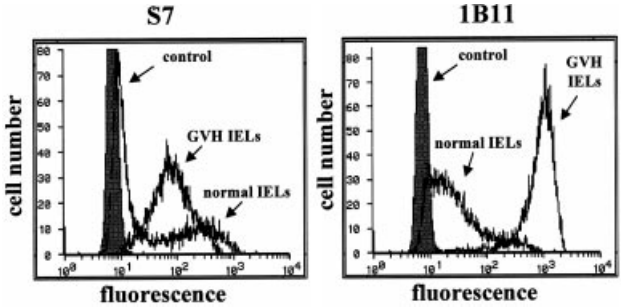


Fig. 13. Comparison of S7 and 1B11 fluorescence intensity on IEL from normal non-diseased mice and donor EGFP$^+$ IEL during acute GVHD. Note the reduction in the level of S7 antigen expression and the marked increase in level of the 1B11 antigen expression on donor cells during acute GVHD.

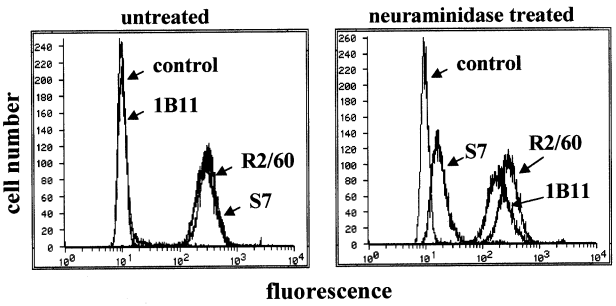


Fig. 14. Reactivity of mAb R2/60, S7 and 1B11 on untreated and neuraminidase-treated EL4 cells. Note the reversal of reactivities of S7 and 1B11 mAb following enzyme treatment, suggesting that under normal conditions the 1B11 epitope is masked by surface sialic acid residues.

though not all, IEL co-expressed both the S7 and 1B11 isoforms, thus demonstrating simultaneous expression of those CD43 isoforms on the same IEL. Although the meaning of the differential expression of S7 and 1B11 antigens on intestinal IEL remains unclear and will require additional work to elucidate, both S7 and R2/60 mAb have been shown to augment the proliferation of T cells from other tissues (13–15). Additionally, because murine IEL lack CD28 expression (33), an important co-stimulatory molecule of murine T cells, signals for optimal cell proliferation by IEL would be largely restricted to subsets expressing the 115 kDa CD43 component, thus implying that there may be significant qualitative differences in the capacity of IEL to undergo cellular expansion following TCR triggering. We are currently addressing this through functional studies of activation/tolerance using IEL subsets based on the expression of CD43 isoforms.

The findings regarding CD43 expression on T cells during acute GVHD were of interest for several reasons. The expression of CD69 on $CD43^+$ donor T cells present in the intestine during acute GVHD indicates that those cells were recently activated T cells. Moreover, >99% of donor T cells in the intestine expressed Thy-1 and were $CD45RB^{hi}$ (data not shown). High levels of CD45RB expression are indicative of a non-memory cell population and on IEL Thy-1 expression has been linked to activated T cells (34,35), thus further suggesting that donor cells present in the intestine at the time of GVHD are derived from naive cells present in the donor cell inoculum. Consistent with that, it has been reported that IEL expressing the CD44 memory phenotype, although present in the intestine of normal mice and in mice during acute GVHD (36; M. D. Armstrong and J. R. Klein, unpublished), do not appear to be involved in GVHD as determined from cell depletion experiments (36).

Perhaps the most interesting finding observed during GVHD was the shift in CD43 1B11 expression from low to high density and the reduction in S7 antigen expression, following *in vivo* activation of donor effector cells; this was evident for both $CD4^-CD8^+$ and $CD4^+CD8^-$ T cells, both within the spleen and the intestine of recipient mice 10–15 days post-cell transfer. Based on the neuraminidase experiments using EL4 cells, the shift in S7 and 1B11 expression appears to reflect alterations in surface sialic acid residues resulting in an unmasking of the 1B11 epitope. Moreover, those findings are consistent with prior studies demonstrating that the S7 reagent reacts with an *N*-acetyl neuraminic acid-dependent epitope, whereas the 1B11-reactive antigen may not be dependent upon neuraminic acid (29,37). This raises the possible scenario that activation of T cells under normal conditions results in two events: the first being the removal of S7 determinants which then expose 1B11 antigens and the second being a concomitant overall increase in surface glycosylation. It is also known, however, that expression of the 1B11 epitope correlates with β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) activity, a key enzyme that gives rise to the formation of 'core 2 branches' of *O*-linked carbohydrates (9,12,29,37). Interestingly, C2GnT activity is elevated in activated T cells during GVHD (9), and transgenic mice which overexpress C2GnT have impaired adhesion of T cells to ICAM-1 and fibronectin (37). Based on that it has been proposed that under normal conditions expression of core 2 *O*-glycans attached to mouse CD43 might provide a mechanism that helps to modulate excessive T cell activation and curtail constitutive T cell proliferation (37). The possibility must be considered, therefore, that the increased levels of 1B11-reactive glycoform on IEL during GVHD have beneficial effects for the host during disease by negatively regulating T cell responses to antigens in a manner analogous to process by which CTLA-4 expression on activated peripheral T cells competes with CD28 for binding to B7 ligands, ultimately leading to T cell inactivation (38,39).

Two additional points emerge from this study. The first pertains to the finding of 1B11 antigen expression on resting B cells, which to our knowledge is the first report of this kind. Although the functional significance of this remains to be determined, it is possible that the expression of the highly glycosylated 1B11 moiety on B cells is involved in maintaining a state of non-responsiveness between resting T cells and resting B cells. In fact, empirical evidence for this exists from studies which demonstrate poor T cell:B cell interactions when B cells express heavily sialylated glycocalyx (22,40). An interesting feature of the 1B11 B cell staining pattern, however, was the fact that a minor population of the total B cells did not express the 1B11 antigen. Given that the purification technique used for enrichment of B cells selects mostly, although not necessarily exclusively, for resting B cells (40–42), the 1B11 marker may prove useful for further defining B cell subsets in mice in ways not appreciated here-to-fore.

Finally, this study is the first to demonstrate the efficacy of using transgenic EGFP mice for analyses of cell trafficking by accurately discriminating donor versus host cells in adoptive transfer experiments. Clearly, this system has direct application for *in vivo* studies of GVHD, and will likely have heuristic value for other immunological experimental systems involving lymphocyte trafficking, homing and cell migration.

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Abbreviations

C2GnT	β-1,6-*N*-acetylglucosaminyltransferase
EGFP	enhanced-green fluorescent protein
GVHD	graft versus host disease
IEL	intraepithelial lymphocytes
PE	phycoerythrin

References

1 Klein, J. R. and Mosley, R. L. 1994. Phenotypic and cytotoxic characteristics of intraepithelial lymphocytes. In Kiyono, H. and McGhee, J. R., eds, *Mucosal Immunology: Intraepithelial Lymphocytes*, p. 33. Raven Press, New York.

2 Kawamoto Y., Sasaki, K., Kato, Y., Kojima, K., Tsuji, T. and Miyama, A. 1996. Rapid killing of murine lymph node T cell blasts by intestinal intraepithelial lymphocytes *in vitro*. *Eur. J. Immunol.* 26:653.

3 Mosley, R. L., Wang, J., Hamad, M. and Klein, J. R. 1994. Functional heterogeneity of murine intestinal intraepithelial lymphocytes: studies using TCR-$\alpha\beta^+$ IEL lines and fresh IEL

isolates reveal multiple cytotoxic subsets differentiated by CD5, CD8α/α, and CD8α/β expression. *Dev. Comp. Immunol.* 18:155.
4 Sarnacki, S., Jarry, B. B. and Cerf-Bensussan, N. 1991. Human intestinal intraepithelial lymphocytes, a distinct population of activated T cells. *Immunol. Res.* 10:302.
5 Simpson, S. J., Hollander, G. A., Mizoguchi, E., Allen, D., Bhan, A. K., Wang, B. and Terhorst, C. 1997. Expression of pro-inflammatory cytokines by TCRαβ$^+$ and TCRγδ$^+$ T cells in an experimental model of colitis. *Eur. J. Immunol.* 27:17.
6 Felstein, F. M. and Mowat, A. M. 1988. Experimental studies of immunologically mediated enteropathy: IV. Correlation between immune effector mechanisms and type of enteropathy during GvHR in neonatal mice of different ages. *Clin. Exp. Immunol.* 72: 108.
7 Baecher, C. M., Infante, A. J., Semcheski, K. L. and Frelinger, J. G. 1988. Identification and characterization of a mouse cell surface antigen with alternative molecular forms. *Immunogenetics* 28:295
8 Gulley, M. L., Ogata, L. C, Thorson, J. A., Dailey, M. O. and Kemp, J. D. 1988. Identification of a murine pan-T cell antigen which is also expressed during terminal phases of B-cell differentiation. *J. Immunol.* 140:3751.
9 Jones, T. A., Federsppiel, B., Ellies, L. G., Williams, M. J., Burgener, R., Duronio, V., Smith, C. A., Takei, F. and Ziltener, H. J. 1994. Characterization of the activation-associated isoform of CD43 on murine T lymphocytes. *J. Immunol.* 153:3426.
10 Moore, T., Huang, S., Terstappen, L. W., Bennett, M. and Kumar, V. 1994. Expression of CD43 on murine and human pluripotent hematopoietic stem cells. *J. Immunol.* 153:4978.
11 Manjunth, N., Correa, M., Ardman, M. and Ardman, B. 1995. Negative regulation of T-cell adhesion and activation by CD43. *Nature* 377:535.
12 Barran, P., Fellinger, W., Warren, C. E., Dennis, J. W. and Ziltener, H. J. 1997. Modification of CD43 and other lymphoycte *O*-glycoproteins by core 2 *N*-acetylglucaosaminyltransferase. *Glycobiology* 7:129.
13 Rosenstein, Y., Park, J. K., Hahn, W. C., Rosen, F. S., Bierer, B. E. and Burakoff, S. J. 1991. CD43, a molecule defective in Wiskott–Aldrich syndrome, binds ICAM-1. *Nature* 354:233.
14 Sperling, A. I., Green, J. M., Mosley, R. L., Smith, P., Klein, J. R., Bluestone, J. A. and Thompson, C. B. 1995. CD43 is a murine T cell costimulatory receptor that functions independently of CD28. *J. Exp. Med.* 182:139.
15 Hamad, M., Mosley, R. L., Wang, J. and Klein, J. R. 1996. Stimulation via the CD43 coreceptor augments T cell proliferation during the early phase of antigen-induced activation. *Dev. Comp. Immunol.* 20:77.
16 Mosley, R. L., Hamad, M., Whetsell, M. and Klein, J. R. 1994. A novel marker of murine bone marrow hematopoietic stem cells that is expressed on peripheral T cells and is associated with a functionally important molecule on activated cytotoxic T lymphocytes. *Hybridoma* 13:353.
17 Axelsson, B., Youseffi-Etemad, R., Hammerstrom, S. and Perlmann, P. 1988. Induction of aggregation and enhancement of proliferation and IL-2 secretion in human T cells by antibodies to CD43. *J. Immunol.* 141:2912.
18 Nong, Y. H., Remold-O'Donnell, E., LeBien, T. W. and Remold, H. G. 1989. A monoclonal antibody to sialophorin (CD43) induces homotypic adhesion and activation of human monocytes. *J. Exp. Med.* 170:259.
19 Ellies, L. G., Tomlinson Jones, A., Williams, M. J. and Ziltener, H. J. 1994. Differential regulation of CD43 glycoforms on CD4$^+$ and CD8$^+$ T lymphocytes in graft-versus-host disease. *Glycobiology* 4:885.
20 Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. 1997. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* 407:313.
21 Mosley, R. L. and Klein, R. L. 1992. A rapid method for isolating murine intestine intraepithelial lymphocytes with high yield and purity. *J. Immunol. Methods* 156:19.
22 Croft, M., Joseph, S. D. and Miner, K. T., 1997. Partial activation of naïve CD4 T cells and tolerance induction in response to peptide presented by resting B cells. *J. Immunol.* 159, 3257.
23 Harlow, E. and Lane, D. 1988. *Antibodies, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24 Tsuzuki, T., Yoshikai, Y., Ito, M., Mori, N., Ohbayashi, M. and Asai, J. 1994. Kinetics of intraepithelial lymphocytes during acute graft-versus-host disease in mice. *Eur. J. Immunol.* 24:709.
25 Cepek, K. L., Parker, C. M., Madara, J. L. and Brenner, M. B. 1993. Integrin $\alpha_E\beta_7$ mediates adhesion of T lymphocytes to epithelial cells. *J. Immunol.* 150:3459.
26 Roberts, K. and Kilshaw, P. J. 1993. The mucosal T cell integrin $\alpha_{M290}\beta_7$ recognizes a ligand on mucosal epithelial cell lines. *Eur. J. Immunol.* 23:1630.
27 Lefrancois, L., Barrett, T. A., Havran, W. L. and Puddington, L. 1994. Developmental expression of the $\alpha_{IEL}\beta_7$ integrin on T cell receptor γδ and T cell receptor αβ cells. *Eur. J. Immunol.* 24:635.
28 Ni, J. and Hollander, D. 1995. Expression of β7 integrins and other cell adhesion molecules on mouse lymphocytes and their modulation by a new cytokine, IL-2 receptor-inducing factor. *Cell. Immunol.* 164:150.
29 Fukuda, M. 1992. Cell surface carbohydrates in hematopoietic cell differentiation and malignancy. In Fukuda, M., ed., *Cell Surface Carbohydrates and Cell Development*, p. 127. CRC Press, Boca Raton, FL.
30 Ostberg, J. R., Barth, R. K. and Frelinger, J. G. 1998. The Roman god Janus: a paradigm for the function of CD43. *Immunol. Today* 19:546.
31 Saito, H., Kanamori, Y., Takemore, T., Nariuchi, H., Kubota, E., Takahashi-Iwanaga, H., Iwanaga, T. and Ishikawa, H. 1998. Generation of intestinal T cells from progenitors residing in gut cryptopatches. *Science* 280:275.
32 Guy-grand, D., Vanden Broecke, C., Briottet, C., Malassis-Seris, M., Selz, F. and Vassalli, P. 1992. Different expression of the recombination activity gene RAG-1 in various populations of thymocytes, peripheral T cells and gut-thymus-independent intraepithelial lymphocytes suggests two pathways of T cell receptor rearrangement. *Eur. J. Immunol.* 22:505.
33 Boll, G., Rudolphi, A., Spiess, S. and Reimann, J. 1995. Regional specialization of intraepithelial T cells in the murine small and large intestine. *Scand. J. Immunol.* 41:103.
34 Barrett, T. A., Tatsumi, Y. and Bluestone, J. A. 1993. Tolerance of T cell receptor γ/δ cells in the intestine. *J. Exp. Med.* 177:1755.
35 Gramzinski, R. A., Adams, E., Gross, J. A., Goodman, T. G., Allison, J. P. and Lefrancois, L. 1993. T cell receptor-triggered activation of intraepithelial lymphocytes *in vitro*. *Int. Immunol.* 5:145.
36 Tanaka, T., Ohtsuka, Y., Yagita, H., Shiratori, Y., Omata, M. and Okumura, K. 1996. Involvement of α1 and α4 integrins in gut mucosal injury of graft-versus-host disease. *Int. Immunol.* 8:1183.
37 Tsuboi, S. and Fukuda, M. 1997. Branched O-linked oligosaccharides ectopically expressed in transgenic mice reduce primary T-cell immune responses. *EMBO J.* 16:6364.
38 Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A. and Sharpe, A. H. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541.
39 Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., Thompson, C. B., Griesser, H. and Mak, T. W. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science* 270:985.
40 Bagriacik, E. and Miller, K. S. 1999. Cell surface sialic acid and the regulation of immune cell interactions: the neuraminidase effect reconsidered. *Glycobiology* 9:267.
41 Frohman, M. and Cowing, C. 1985. Presentation of antigens by B cells: functional dependence on radiation dose, interleukins, cellular activation, and differential glycosylation. *J. Immunol.* 134:269.
42 Croft, M. and Swain, S. L. 1995. Recently activated naïve CD4 T cells can help resting B cells and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. *J. Immunol.* 154:4269.