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Disregulation of leukosialin (CD43, Ly48, sialophorin) expression in the B-cell lineage of transgenic mice increases splenic B-cell number and survival

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ABSTRACT **Leukosialin (also known as Ly48, CD43, and sialophorin) is a major cell surface sialoglycoprotein found on a variety of hematopoietically derived cells. The precise function of this molecule is poorly understood but it has been implicated in cell proliferation and intercellular adhesion. We developed a transgenic mouse model to assess leukosialin's function *in vivo*. Our approach was to alter mouse CD43 (mCD43) expression in the B-cell lineage where it is tightly regulated, by expressing it in peripheral B cells where it is normally absent. To drive expression of leukosialin in mature B cells, the immunoglobulin heavy chain enhancer was fused to the mCD43 gene. mCD43–immunoglobulin heavy chain enhancer transgenic mice display splenomegaly due to increased numbers of B cells. Transgenic B cells show a striking increase in their ability to survive *in vitro* compared to B cells from nontransgenic control mice. This prolonged survival is reflected in a decreased susceptibility to apoptosis. These observations suggest that mCD43 plays an important role in the regulation of B-cell survival. The alteration of the temporal expression, or "disregulation," of a gene in transgenic mice provides a general strategy for elucidating the *in vivo* role of other molecules involved in cell signaling and adhesion.**

Leukosialin [also designated as CD43, sialophorin and large sialoglycoprotein in humans, Ly48 or mouse CD43 (mCD43) in mice, and W3/13 in rats] is a major cell surface sialoglycoprotein found on T cells, granulocytes, macrophages, and both erythroid and B cells at specific stages of development (1). Altered expression of this molecule has been associated with the X chromosome-linked immunodeficiency disease Wiskott–Aldrich syndrome (2–4). Further interest in CD43 has been generated by the finding that anti-CD43 antibodies are present in AIDS patients, leading to speculation that these antibodies might contribute to human immunodeficiency virus-mediated immunodeficiency (5).

The reported association of CD43 with specific immunodeficiency diseases and the unusual structural features of this molecule have fueled speculations regarding its function. CD43 has an extracellular domain that is highly O-glycosylated, with carbohydrates making up >50% of its mass, and a relatively long intracellular domain (6, 7). DNA sequence comparison of rat, human, and mouse CD43 genes reveals that the transmembrane and intracellular domains are highly conserved compared to the extracellular portion, indicating differential selective pressures on either end of this protein (6–12). The evolutionarily more conserved intracellular/transmembrane domains may participate in critical intracellular events, such as signal transduction or interactions with conserved cytoskeletal structures (13–16). In contrast, the tightly regulated glycosylation of CD43 has led to the theory that the carbohydrate structures on the extracellular domain are the functional moieties, rather than the primary amino acid sequence itself, and that these carbohydrates interact with lectin-like receptors on cells. Although CD43 bears no sequence homology to any other known protein (GenBank release 85.0; EMBL release 40.0), many of the basic structural features described above are remarkably similar to those of the hematopoietic stem cell marker, CD34 (17).

The precise function of CD43 is unclear, although it appears to be involved in cell proliferation and cell adhesion. Monoclonal antibodies to human CD43 have been reported to stimulate T-cell proliferation (18, 19). Human CD43 has been reported to act as a cell adhesion molecule on T cells via binding to intercellular cell adhesion molecule 1 (20). Paradoxically, an antiadhesion role for this molecule has been suggested on theoretical grounds as well as from transfection and ablation studies in cell lines (21–23).

Considering the apparently complex function of CD43 and the potential intricacy of cell adhesion molecule function in general, an understanding of the true physiologic role of this protein may require study in the context of the whole animal. Consequently, we sought to develop an *in vivo* system in which the function of mCD43 could be examined. B cells demonstrate a unique developmental program for mCD43 expression that makes them particularly amenable for the *in vivo* manipulation of mCD43 within a physiologically relevant context. CD43 is present on the surface of early B-cell progenitors and on terminally differentiated plasma cells but is absent on mature B cells (24–30). We took advantage of this "ON–OFF–ON" expression pattern of mCD43 in the B-cell lineage and altered its temporal expression in transgenic (TG) mice by using the immunoglobulin heavy chain enhancer to drive mCD43 expression in mature B cells (31). We report here on the analysis of mCD43 expression in these TG mice and our investigation of the biological consequences of mCD43 "disregulation" *in vivo*.

MATERIALS AND METHODS

Antibodies. Anti-mCD43 (S11, S7, S15, and 3E8) and anti-Thy-1 (T40/70.4) monoclonal antibodies were obtained as described (32–34). Anti-CD45 R B220 (14.8), anti-major histocompatibility complex class II (M5-114.15.2), anti-CD4 (GK1.5), and anti-CD8 (53-6.72) were obtained from the American Type Culture Collection. R-phycoerythrin (R-PE)-labeled anti-B220 (RA3-6B2) was purchased from Pharmingen. Goat anti-rat immunoglobulin conjugated with fluorescein isothiocyanate (FITC) was purchased from Southern

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Abbreviations: TG, transgenic; NTG, nontransgenic; FITC, fluorescein isothiocyanate; R-PE, R-phycoerythrin; PI, propidium iodide; mCD43, mouse CD43; mCD43–Ig heavy chain enhancer, mCD43–immunoglobulin heavy chain enhancer.

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Biotechnology Associates. Normal rat and goat antisera were purchased from Jackson ImmunoResearch.

Generation of DNA Construct and TG Mice. A 2.3-kb *Bam*HI DNA fragment containing the full coding sequence of mCD43 and 1 kb of 5′ noncoding sequence was isolated from a mCD43 genomic clone. This fragment was ligated into pBluescript KS+/− (Stratagene) with a 0.8-kb *Bam*HI–*Bgl* II fragment from pKV461 (35) containing polyadenylylation/splice signals, and a 1-kb *Xba* I fragment, containing the immunoglobulin heavy chain enhancer (31). The transgene construct was excised and isolated on a continuous 10–40% (wt/vol) sucrose gradient. Transgene fractions were pooled, dialyzed against 5 mM Tris·HCl/0.15 mM EDTA (pH 7.4), before use. TG mice were generated by microinjection as described (36).

Flow Cytometry. Single-color cell surface flow cytometry was performed as described (37). Two-color cell surface flow cytometry was performed in two steps using first a rat primary antibody followed by FITC-conjugated goat anti-rat IgG. A directly conjugated rat anti-mCD45R-R-PE monoclonal antibody was added in the presence of 5% (vol/vol) rat serum. Cells were then analyzed using an Epics Profile flow cytometer (Coulter).

Cellular Studies. Spleens were removed, weighed, dissociated into single-cell suspensions, and treated with hypotonic ammonium chloride to lyse red blood cells. Nucleated viable cells were counted using trypan blue (Sigma). Purified B cells were isolated by depleting spleen cells of macrophages by adherence. Subsequently, T lymphocytes were depleted using anti-T-cell antibodies and complement as described (38). The resulting cells were >95% $B220^+$ and <5% Thy-1^+ by flow cytometry. B cells were plated at 1×10^6 cells per ml per well and cultured in RPMI 1640 medium with 5% (vol/vol) newborn calf serum. Wells were harvested on day 2, 6, 8, 10, 12, or 14 and viable cells were counted using trypan blue; or wells were harvested at 24, 48, and 72 hr, fixed in 70% ethanol, and stained for DNA content with propidium iodide (PI; Sigma), as described (39, 40). After PI staining, the cells were analyzed on an Epics Profile flow cytometer.

RESULTS

Disregulation of mCD43 Within the B-Cell Lineage. To better understand the role mCD43 plays *in vivo*, we decided to alter the temporal expression of the gene in a cell lineage where it is physiologically relevant. In the B-cell lineage, mCD43 has a well-regulated pattern of expression. The mCD43 gene is expressed early in development, shut off as the B cells mature, and reexpressed as B cells differentiate into plasma cells (24–30). Due to the tight regulation of mCD43 in the B-cell lineage, we hypothesize that it plays an important role in B-cell development and function. Therefore, by altering or "disregulating" mCD43's temporal expression, we can better understand its function in the B-cell compartment by defining the loss or gain of function that results. The fusion of the well-characterized immunoglobulin heavy chain enhancer to the mCD43 gene (mCD43–Ig heavy chain enhancer gene) is expected to drive its expression in the mature B-cell compartment (Fig. 1). The hybrid construct was microinjected into fertilized mouse eggs, implanted into pseudopregnant females, and allowed to develop to term (36). Of the 57 progeny screened, 6 animals were TG. Three founders, designated 7, 53, and 82, were expanded into lines and examined for effects of mCD43 transgene expression on B cells.

mCD43–Ig Heavy Chain Enhancer TG Mice Express mCD43 in Mature B Cells. To determine whether the mCD43 transgene was expressed in peripheral B cells, spleen cells were isolated, stained with R-PE-labeled anti-B220 (RA3-6B2) and FITC-labeled anti-mCD43 (S11) monoclonal antibodies, and analyzed by two-color flow cytometry (Fig. 2). Nontransgenic

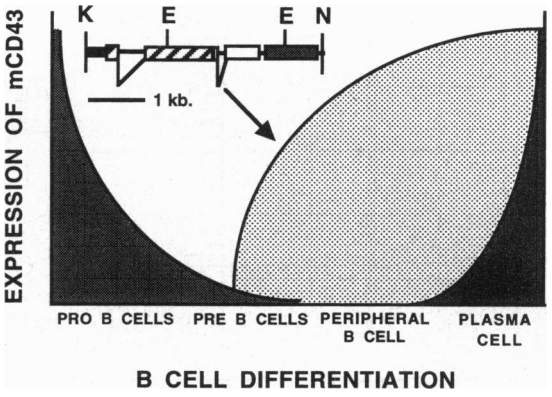

FIG. 1. mCD43–Ig heavy chain enhancer TG construct and model of disregulation of mCD43 in the B-cell compartment. The mCD43-injected construct is the mCD43 genomic clone (hatched bar) and its promoter. To the 3′ end of the mCD43 gene, the simian virus 40 (SV40) splice/polyadenylylation signal (open bar) and the immunoglobulin heavy chain enhancer (solid bar) were added to complete the construction. Endogenous mCD43 (solid area) and predicted transgene expression (shaded area) in the immunoglobulin-positive B-cell compartment are shown.

(NTG) splenic B cells ($B220^+$) are essentially negative for mCD43 expression (Fig. 2*A*) as reported (24, 26–30). In the three TG lines examined, however, >95% of $B220^+$ B cells expressed mCD43, as indicated by the large double-positive population (Fig. 2 *B–D*). Similar results were obtained with two other anti-mCD43 monoclonal antibodies (S7 and S15; data not shown). In every TG line, regardless of the level of mCD43 expression on B cells, essentially all $B220^+$ or immunoglobulin-positive cells were also mCD43$^+$.

Expression of mCD43 Results in Increased Spleen Weight and Increased Numbers of B Cells. TG mice revealed marked splenomegaly compared to NTG mice. Spleens from TG

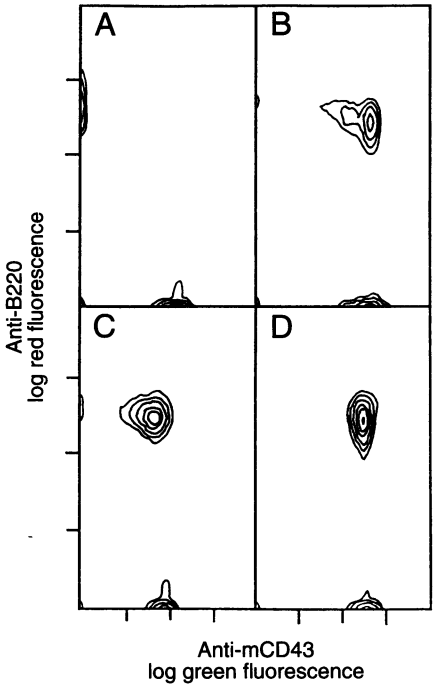

FIG. 2. Two-color flow cytometry of total splenocytes from mCD43–Ig heavy chain enhancer TG and NTG littermate mice. Anti-mCD43 (S11) was added followed by FITC-labeled goat anti-rat immunoglobulin. Cells were then labeled with R-PE-labeled anti-B220 (RA3-6B2) to define B cells in the splenocyte preparations. (*A*) NTG control. (*B*) Line 82. (*C*) Line 53. (*D*) Line 7.

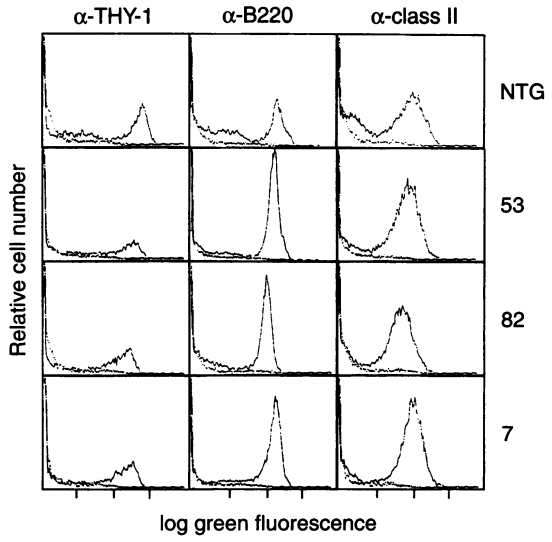

FIG. 3. Flow cytometry analysis of TG and NTG littermate mice. Splenocytes were stained for the expression of the indicated panel of markers. For each panel, the total number of cells recovered, percent Thy-1$^+$, percent B220$^+$, and percent class II$^+$ are as follows: NTG, 1.4 $\times$ 10^8 cells, 34%, 52%, and 59%; line 53, 3.7 $\times$ 10^8 cells, 18%, 72%, and 74%; line 82, 1.0 $\times$ 10^8 cells, 22%, 62%, and 63%; line 7, 2.3 $\times$ 10^8 cells, 23%, 71%, and 72%. In all histograms, 10,000 events were analyzed and compared against samples stained only with the second antibody (dotted line). α-, Anti-.

animals weighed significantly more than spleens from their age-matched NTG littermates (Table 1). For example, line 7 spleens weighed 1.6 times as much as NTG spleens and this increase is significant ($P \leq 0.0001$). This increase in mass is also reflected in a significant ($P < 0.0277$) 1.9-fold increase in cell number (Table 1). We performed flow cytometry to determine which cells accounted for this increase in cell number. Cells were stained with anti-Thy-1 (T24/40.7), as a T-cell marker, or separately with anti-B220 (14.8) or anti-major histocompatibility complex class II (M5114), as a B-cell

Table 1. Increased spleen weight and cell number of mCD43–Ig heavy chain enhancer transgenic mice compared to nontransgenic controls

	NTG	TG line 82	TG line 53	TG line 7
Spleen weight, mg	90 ± 2.9 (n = 33)	105 ± 3.4 (n = 24)	130 ± 7.5 (n = 10)	140 ± 9.4 (n = 27)
No. of cells	9.2 ± 1.0 (n = 13)	14 ± 1.1 (n = 8)	17 ± 1.7 (n = 8)	18 ± 2.6 (n = 10)

Data for spleen weight are the mean ± SEM; n represents the number of animals per group. Data for cell number are for mononuclear cells and are expressed as number $\times$ 10^{-7} (mean ± SEM). Significant difference was determined by using an independent unpaired Student's t test with the Bonferroni adjustment. P values for spleen weights are as follows: NTG vs. TG 82, $P \leq 0.0014$; NTG vs. TG 53, $P \leq 0.0001$; NTG vs. TG 7, $P \leq 0.0001$, when comparing the NTG to TG lines. P values for spleen cell numbers are as follows: NTG vs. TG 82, $P \leq 0.0277$; NTG vs. TG 53, $P \leq 0.0009$; NTG vs. TG 7, $P \leq 0.0035$, when comparing the NTG to TG lines.

marker. The calculated percent of cells positive for each marker is shown in Fig. 3. These results demonstrate there is an increased percentage of B cells ranging from 62 to 72% in the TG spleens compared to 52% in the NTG tissue. Similar results were obtained in at least four additional experiments per line. These results suggest that most, if not all, of the increase in cells is due to an expansion of B cells, which is due to the expression of the mCD43 transgene.

The Increased Number of B Cells in the TG Animals Appears Due to Their Increased Longevity. The increased number of cells in the TG spleen could be due to increased proliferation or to decreased death. We have examined purified B cells by [^{3}H]thymidine incorporation assays and found no evidence of increased proliferation in the TG lines compared to control mice (data not shown). In contrast, cultured TG B cells exhibited a dramatic difference in their ability to survive (Fig. 4*A*). NTG B cells die rapidly under standard culture conditions, as reported (41), whereas a significantly larger fraction of TG B cells survive. Approximately 10% of the TG B cells are alive at day 10 vs. <1% of NTG B cells. Flow cytometric analyses of the B cells surviving from the TG cultures showed that >90% were B220$^+$ (data not shown). In

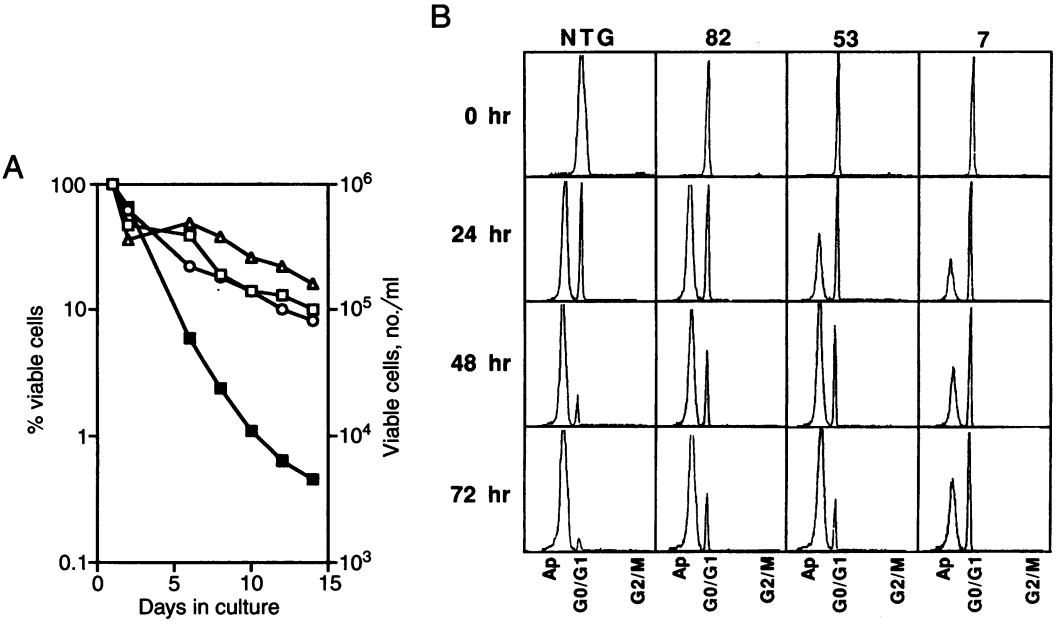

FIG. 4. Prolonged survival and cell cycle analysis of purified B cells from mCD43–Ig heavy chain enhancer TG and NTG littermate mice. (*A*) Purified splenic B cells were assayed for viability. NTG control (■), line 82 (□), line 53 (○), and line 7 (△). (*B*) DNA content was determined by PI staining and flow cytometry. Ap, apoptotic cells; G_0/G_1, resting lymphocytes; G_2/M, dividing cells. For each panel, the highest peak was normalized to full scale.

six separate experiments, line 7, the highest-expressing TG line, displayed this increased survival phenotype whereas the NTG B cells always died rapidly. Line 82 was tested four times and always displayed the prolonged survival phenotype. Interestingly, line 53, which expresses the transgene at the lowest level, survived longer three out of five times.

B Cells from TG Mice Show Decreased Apoptosis *in Vitro*. The enhanced survival of TG B cells may be due to their resistance to apoptosis since normal nonactivated B cells in culture undergo apoptosis. An assay utilizing PI staining to measure apoptotic DNA fragmentation was used to address directly whether the TG B cells were resistant to apoptosis (39, 40). B cells were cultured *in vitro* for 24, 48, or 72 hr and then were stained with PI and analyzed by flow cytometry (Fig. 4*B*). The results demonstrate that, although nearly all NTG B cells rapidly undergo apoptosis, a significant fraction of TG B cells remains in the G_0/G_1 stage. This is consistent with the cell survival results described above and indicates that the expression of the transgene *in vivo* results in the development of B cells that are more resistant to apoptosis.

DISCUSSION

Our experimental approach relied on the rationale that the immunoglobulin heavy chain enhancer would drive expression from the heterologous mCD43 promoter. This strategy was based on the fact that the mCD43 promoter is already active in parts of the B-cell lineage (27–30) and that the immunoglobulin heavy chain enhancer can support augmented expression even from heterologous promoters (42). Our finding that the mCD43–Ig heavy chain enhancer fusion gene is expressed at high levels in mature peripheral B cells indicates that the immunoglobulin enhancer sequence exerts a dominant influence on mCD43 transgene expression, with a developmental program consistent with that of the immunoglobulin heavy chain itself (31). Preliminary studies on bone marrow cells are consistent with expression of the mCD43 transgene in pre-B cells in addition to the peripheral B cells. Further, flow cytometry analyses also indicate that mCD43 transgene expression extends to nearly all $CD5^+$ B cells in the peritoneum (data not shown), whereas endogenous CD43 expression is seen on only a subset of $CD5^+$ peritoneal B cells (43, 44).

Perhaps the most striking phenotypic change exhibited by mCD43–Ig heavy chain enhancer TG mice is the prolonged survival of their B cells in culture and the association of this effect with decreased apoptosis. This represents a distinct phenotype associated with mCD43 expression that could not have been easily identified from the transfection of established cell lines. In this regard, these TG animals are reminiscent of *Bcl-2* TG mice, which also demonstrate decreased apoptosis (45, 46). Whether mCD43 is normally involved in *Bcl-2*-mediated apoptotic processes is unknown; however, our preliminary experiments show no dramatic increase in either *Bcl-2* transcript or protein levels in B cells from the mCD43–Ig heavy chain enhancer TG mice (data not shown). Thus, the disregulation of mCD43 in this TG mouse system may point toward an alternative regulation of apoptosis either intersecting a common pathway downstream of Bcl-2 or, alternatively, a completely different pathway.

Our finding that the altered temporal expression of mCD43 in the B-cell lineage can affect programmed cell death is intriguing in light of the normal developmental expression pattern of mCD43. mCD43, as detected by S7, is turned off as pro-B cells develop into pre-B cells, which has led to the use of mCD43 as a marker for these developmental stages (30, 47–49). It has been estimated by others that >75% of developing B cells die at the pro-B to pre-B stage transition and during the pre-B stage (49, 50). One intriguing possibility, consistent with the increased number of B cells found in the mCD43–Ig heavy chain enhancer TG mice, is that mCD43 is delivering a signal that rescues B cells that would have normally undergone programmed cell death. mCD43 could play a role in inhibiting apoptosis by delivering a signal by itself or perhaps by acting as part of an adhesion cascade with other receptor ligands. Adhesion of early B-cell precursors to stromal elements of the bone marrow delivers signals important for their survival and maturation, and the subset of pre-B cells most easily cloned on stromal cell lines are $mCD43^+$ (51, 52). Thus it is possible that the carbohydrate on mCD43 interacts with a lectin-like receptor on stromal cells, analogous to the functional role proposed for CD34 (53, 54), and this interaction in the case of CD43 promotes B-cell expansion and development. Alternatively, mCD43 could act as an antiadhesion molecule due to its extensive glycosylation and sialic acid (22, 23). In this scenario, mCD43 could prevent programmed cell death of B cells by preventing the engagement of receptor–ligand pairs, such as the fas–fas ligand, that are involved in initiating apoptosis (55).

Our observation that B-cell expression of mCD43 in TG mice is associated with increased splenic B-cell number is another result that could not have been easily discernable from cell transfection studies. Despite this expansion, histology and immunocytochemistry of splenic sections revealed no gross alterations in the cytoarchitecture of TG spleens (data not shown), although the hematoxylin/eosin sections had fewer follicles and germinal centers. This may indicate that mCD43 TG mice are compromised in their ability to mount T-cell-dependent B-cell responses and memory responses. Preliminary studies indicate that these animals are less able to respond to keyhole limpet hemocyanin in both primary and secondary immunizations (unpublished data).

Many adhesion molecules are involved in signaling and these molecules do not act in isolation. There appears to be extensive "cross-talk" between cell surface receptors (56) whose expression must change during development. Therefore, *in vitro* studies of adhesion molecules may not simulate the dynamic *in vivo* microenvironment necessary to define their biological effects. The use of TG mice to study the disregulation of a putative adhesion/cell signaling molecule within a lineage where it normally functions provides a powerful *in vivo* approach that in turn may reveal functions not predicted by *in vitro* studies. Here we have shown that mCD43, previously used as a cell surface developmental marker (27–30, 47–49), is involved in programmed cell death in the B-cell lineage and results in increased B-cell numbers. Disregulation provides a complementary approach to gene "knockouts," achieved by homologous recombination, for understanding the function of developmentally regulated genes. The approach of using gene disregulation should also prove useful for dissecting the function of other adhesion molecules, particularly those with complex expression patterns, with aberrant expression during the pathogenesis of disease, or with highly regulated expression through ontogeny.

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