precursor whose mRNA is expressed in the brain. The new peptide, whose sequence appears to be strictly conserved across mammalian species (ox (not shown), mouse, rat and man) (see legend to figure 4), must be of widespread importance. Because its first demonstrated physiological action appears to be increased reactivity to pain in intact animals, the new peptide might be named nociceptin. □

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Negative regulation of T-cell adhesion and activation by CD43

Manjunath, N., Mariangela Correa, Margarett Ardman & Blair Ardman*

Department of Medicine, Division of Haematology-Oncology, New England Medical Center Hospitals, 750 Washington Street, Boston, Massachusetts 02111, USA

CD43 is a cell-surface sialoglycoprotein expressed by a variety of haematopoietically derived cells, including T lymphocytes[1–9]. Earlier observations of defective CD43 expression by T lymphocytes from boys with the X-chromosome-linked Wiskott–Aldrich syndrome suggested the importance of CD43 in lymphocyte function[10,11]. Subsequent studies have suggested that CD43 facilitates leukocyte adhesion[12–14] and has a co-stimulatory role during T-cell activation[15]. To define the physiologically relevant function(s) of CD43, we have generated CD43-knockout mice. We report here that CD43-deficient T cells from such mice show a marked increase in their *in vitro* proliferative response to concanavalin A, anti-CD3, the superantigen SEB and allostimulation. Additionally, CD43-deficient T cells show a substantial enhancement in homotypic adhesion and in their ability to bind different ligands, including fibronectin and the intercellular adhesion molecule ICAM-1. Vaccinia-virus-infected CD43-knockout mice mounted an augmented anti-vaccinia cytotoxic T-cell response compared with their wild-type littermates, yet developed an increased virus load. We conclude that CD43 negatively regulates T-cell activation and adhesion and is important for viral clearance.

CD43-knockout mice were generated by homologous recombination via embryonic stem-cell chimaeras (Fig. 1). Although CD43 is expressed from a very early stage in haematopoiesis[8,9] and the pattern of expression varies in a lineage-specific manner[16–19], no developmental defect was observed in the mutant mice. The mice develop and breed normally. Haematocrit values were normal, as were blood leukocyte and platelet counts. Histology of bone marrow, thymus, spleen and lymph nodes from CD43-knockout mice resembled that of wild-type mice. Flow-cytometric analysis of thymocytes and splenocytes revealed no gross immunophenotypic abnormalities other than the loss of CD43 expression. T-lymphocyte numbers, CD4 and CD8 subset proportions, levels of CD3, CD4 and CD8 expression, and B-cell ($B220^+$, IgM^+) numbers in spleens from CD43-knockout mice were comparable to those of their wild-type littermates. T-lymphocyte expression of activation markers, including interleukin-2α chain, Ia antigen and lymphocyte function-associated antigen-1 (LFA-1), were also comparable. Surface-antigen expression on marrow cells from the knockout mice was similar to that of wild-type mice, with the exception that most $B220^+$ cells in knockout marrow were $Mac\text{-}1^+$, a finding of uncertain functional relevance. We conclude that CD43 is not essential for haematopoiesis.

Previous studies have shown that monoclonal antibodies to human CD43 can be co-stimulatory for T cells[12] or activate them directly[20]. More recently, expression of human CD43 by a murine T-cell hybridoma was observed to enhance its response to antigen[15]. We therefore tested T cells from the CD43-knockout mice and their wild-type littermates for their ability to proliferate in response to T-cell activators. Splenocytes were stimulated with the T-cell mitogen concanavalin A (conA), the anti-CD3 monoclonal antibody 145-2C11, or the superantigen staphylococcal enterotoxin B (SEB) at various concentrations. Thymocytes were stimulated with anti-CD3 and phorbol-12-myristyl-13-acetate (PMA). In each case, T cells from knockout mice showed substantially enhanced proliferation compared with those from their wild-type littermates (Fig. 2*a*). Moreover, enhanced proliferation was evident at all concentrations of stimulants tested, although maximal enhancement was seen with suboptimal concentrations of stimulants (not shown). To assess the dynamics of proliferation of $CD43^+$ and $CD43^-$ T cells, different cell numbers were tested for proliferation at different time points using various concentrations of conA. At 48 h, the enhancement in proliferation of the CD43-deficient T cells was most striking when either conA concentration or cell number was limiting (Fig. 2*b*). By contrast, under optimal stimulatory conditions (2×10^5 cells, 2 μg ml^{-1} conA), only a modest increase (50%) in ^3H-thymidine incorporation by the CD43-deficient cells was observed. However, at 24 h, duplicate cultures under identical stimulatory conditions showed a 300% increase in ^3H-thymidine incorporation by the CD43-deficient cells (not shown). These results suggest an accelerated kinetics of T-cell activation in the absence of CD43.

To determine whether the proliferation of CD43-deficient T cells was also enhanced in response to a natural antigen, alloreactivity of T cells was tested. Again, proliferation of T cells from the CD43-knockout mice was more than double that of cells from their wild-type counterparts ($23.1 \pm 1.8 \times 10^3$ c.p.m. compared with $9.4 \pm 0.7 \times 10^3$ c.p.m., respectively). A similar increase in CD43-deficient T-cell proliferation was also seen after secondary and tertiary stimulation (not shown), indicating an increase in the proliferative ability of the CD43-deficient T cells rather than in the number of lymphocytes in the allospecific precursor pool.

To investigate possible *in vivo* consequences of CD43-deficiency, cytotoxic T-cell response to vaccinia virus infection in the knockout mice was compared with that in their wild-type littermates. Seven days after infection with a high virus dose (10^6 $TCID_{50}$ (half-maximal tissue culture infective dose)), comparable levels of anti-vaccinia cytotoxic T-lymphocyte (CTL) activity were found in both mouse groups (not shown). By contrast, when limiting amounts of virus (2×10^3 $TCID_{50}$) were used, a significantly greater (fourfold) CTL response was consistently observed in the CD43-deficient mice (Fig. 3*a*). These

results are consistent with a decreased threshold for T-cell activation during an immune response. To determine the effect of the enhanced CTL activity in viral clearance, lungs and gonads from the mice were analysed for viral content. Surprisingly, infectious virus was recovered from all four CD43-knockout mice but from none of their wild-type littermates (Fig. 3*b*). These results suggest that defects involving tissue localization of effector cells or post-lysis viral clearance by phagocytes might occur in the absence of CD43.

The intercellular adhesion molecule ICAM-1 is reported to be a ligand for CD43 (ref. 14), suggesting that CD43 may mediate intercellular adhesion. In other studies, however, leukocytes from CD18-deficient (LAD) individuals showed no binding to ICAM-1, despite surface CD43 expression[21], and the anti-CD43 monoclonal antibody previously observed to block CD43 binding to ICAM-1 did not block binding of $CD43^-$ lymphoid cells to ICAM-1 or ICAM-3[22]. We have observed that CD43 expression impedes homotypic and heterotypic adhesion of cells, and diminishes cell binding to purified ligands[23,24]. As interaction of T lymphocytes with other cells (for example, antigen-presenting cells) is required for optimal activation[25,26], the enhanced sensitivity of the CD43-deficient T lymphocytes to activation might reflect increased adhesive properties. Thus, cells from the CD43-knockout mice and wild-type littermates were compared in homotypic adhesion assays and for their ability to bind purified ligands, including murine fibrinogen and soluble, recombinant ICAM-1[27]. PMA-treated thymocytes from knockout mice showed substantially increased homotypic adhesion compared with thymocytes from wild-type littermates (Fig. 4*a*). Furthermore, binding of CD43-deficient splenic T cells was nearly 100% greater than binding of $CD43^+$ cells to fibronectin (Fig. 4*b*), and 50% greater to ICAM-1 (Fig. 4*c*). The binding of $CD43^+$ and $CD43^-$ cells to ICAM-1 was mediated through LFA-1 because in both cases it was completely blocked by the anti-LFA-1 antibody M17/4. Taken together, these data indicate that loss of CD43 expression by T lymphocytes results in hyperadhesive properties and suggest that the increased sensitivity of CD43-deficient T cells to activation is related to their enhanced adhesiveness.

It has been suggested that structural features of CD43 are consistent with an anti-adhesive function[3,28]. The mucin-like extracellular CD43 domain probably exists as an unfolded structure[3], and has been measured to extend 45 nm in length[29], a distance greater than spanned by most cell-surface adhesion

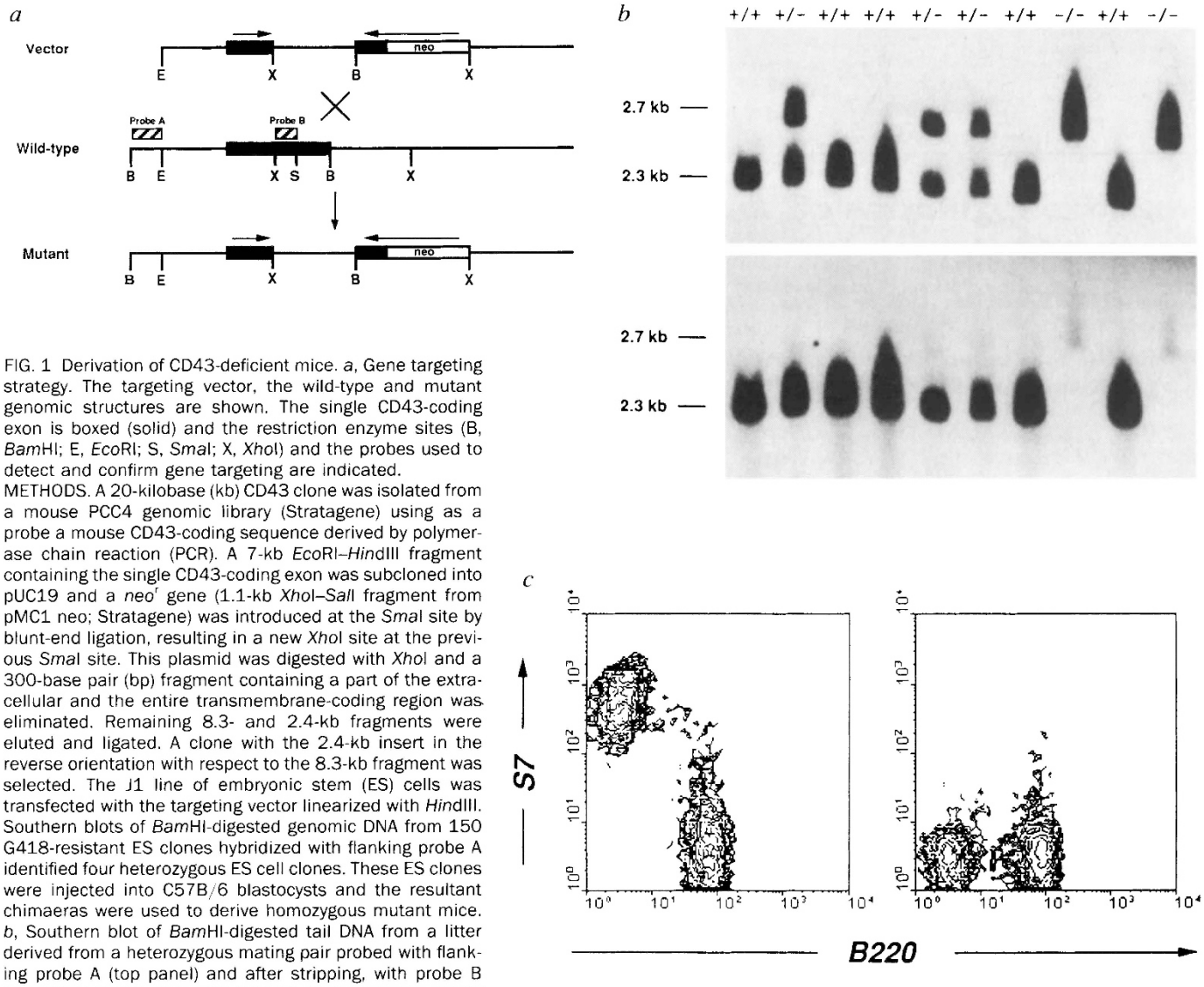

FIG. 1 Derivation of CD43-deficient mice. *a*, Gene targeting strategy. The targeting vector, the wild-type and mutant genomic structures are shown. The single CD43-coding exon is boxed (solid) and the restriction enzyme sites (B, *Bam*HI; E, *Eco*RI; S, *Sma*I; X, *Xho*I) and the probes used to detect and confirm gene targeting are indicated.
METHODS. A 20-kilobase (kb) CD43 clone was isolated from a mouse PCC4 genomic library (Stratagene) using as a probe a mouse CD43-coding sequence derived by polymerase chain reaction (PCR). A 7-kb *Eco*RI–*Hin*dIII fragment containing the single CD43-coding exon was subcloned into pUC19 and a neo^r gene (1.1-kb *Xho*I–*Sal*I fragment from pMC1 neo; Stratagene) was introduced at the *Sma*I site by blunt-end ligation, resulting in a new *Xho*I site at the previous *Sma*I site. This plasmid was digested with *Xho*I and a 300-base pair (bp) fragment containing a part of the extracellular and the entire transmembrane-coding region was eliminated. Remaining 8.3- and 2.4-kb fragments were eluted and ligated. A clone with the 2.4-kb insert in the reverse orientation with respect to the 8.3-kb fragment was selected. The J1 line of embryonic stem (ES) cells was transfected with the targeting vector linearized with *Hin*dIII. Southern blots of *Bam*HI-digested genomic DNA from 150 G418-resistant ES clones hybridized with flanking probe A identified four heterozygous ES cell clones. These ES clones were injected into C57B/6 blastocysts and the resultant chimaeras were used to derive homozygous mutant mice. *b*, Southern blot of *Bam*HI-digested tail DNA from a litter derived from a heterozygous mating pair probed with flanking probe A (top panel) and after stripping, with probe B containing CD43 sequence deleted from targeting vector (bottom panel). The positions of the wild-type (2.3 kb) and mutant (2.7 kb) alleles are indicated. *c*, Flow-cytometric analysis of splenocytes for CD43 expression. Cells were stained with anti-B220 (B-cell-specific) and S7 (CD43-specific) monoclonal antibodies and analysed on a FACSCAN (Becton-Dickinson) flow cytometer. Left, +/+ splenocytes; right −/− splenocytes.

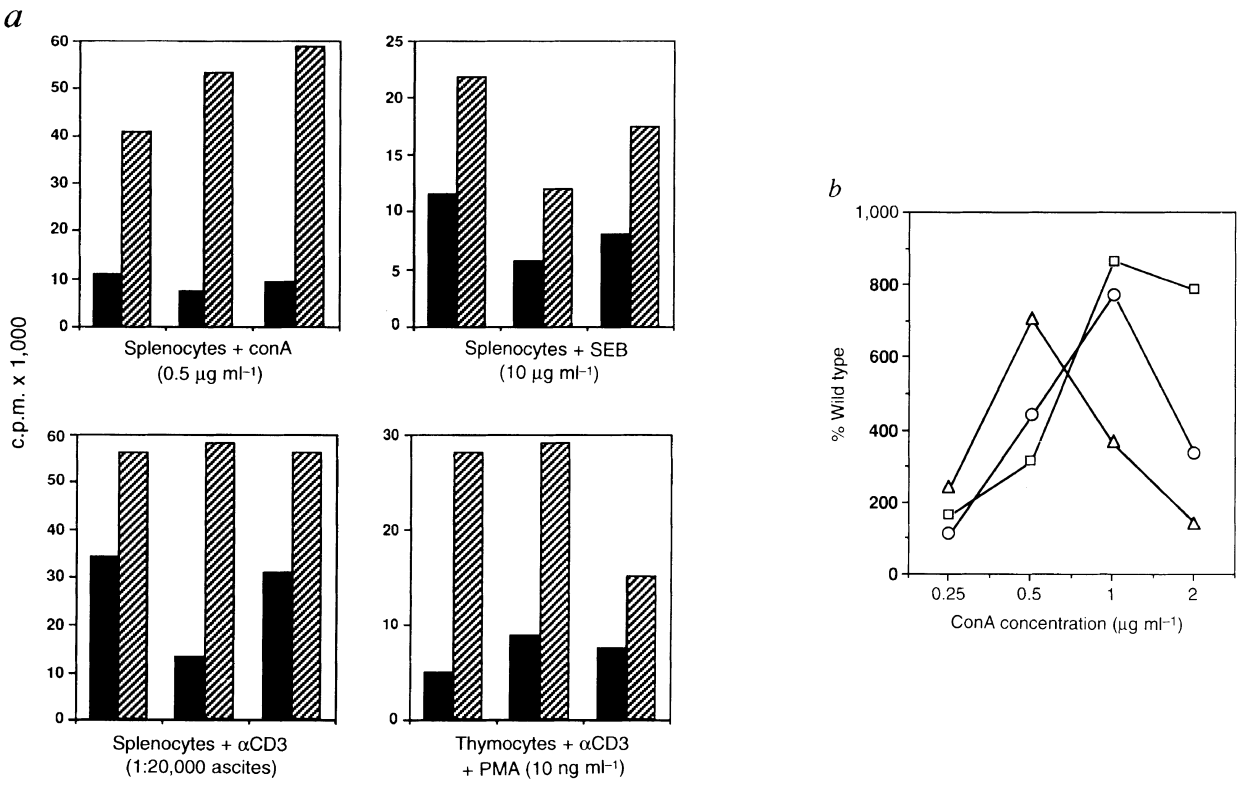

FIG. 2 Proliferative response of T lymphocytes from CD43-knockout mice. *a*, Data from three pairs (representative of six pairs tested) of wild-type (+/+; ■) littermates and CD43-knockout (−/−; ▨) mice are shown. 2×10^5 splenocytes per well were stimulated in 96-well culture plates with the indicated concentrations of conA, SEB or the anti-CD3 ascites 145-2C11. Thymocytes (2×10^5 per well) were stimulated with 145-2C11 and PMA. The cultures were pulsed with ^{3}H-thymidine for 42–48 h (0.5 μCi per well) and cells were collected onto 96-well GF/C plates and counted in a Packard microplate scintillation counter. Results shown are after subtraction of ^{3}H-thymidine incorporation in nonstimulated cultures. All assays were done in triplicate. *b*, Dynamics of proliferative response. Varying numbers of splenocytes from +/+ and −/− mice were stimulated with different concentrations of conA as indicated and proliferation was measured as described above. Data are shown as per cent counts obtained with +/+ splenocytes. △, 2.0×10^5 splenocytes; ○, 1.0×10^5; □, 0.5×10^5.

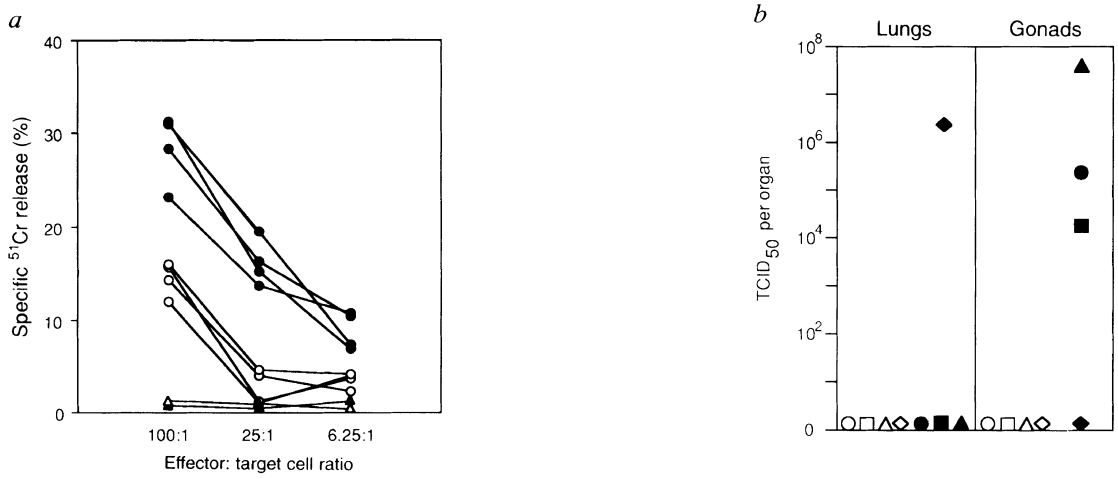

FIG. 3 CTL response to vaccinia virus infection. *a*, Mice were infected by intraperitoneal injection with 2×10^3 $TCID_{50}$ of vaccinia virus (WR strain). On day 7 after infection, splenocytes were depleted of B cells with B220-coated magnetic beads and assayed for specific lysis of MC57G target cells uninfected (triangles) or infected (circles) with vaccinia virus (10 $TCID_{50}$ per cell) for 3 h before labelling with ^{51}Cr. The percentage specific release of ^{51}Cr was calculated from the (experimental release − spontaneous release) × 100/total release − spontaneous release). *b*, Vaccinia virus titre. $TCID_{50}$ in lungs and gonads obtained from mice killed for CTL assay was measured on CV1 indicator cells. Different symbols represent individual mice (solid symbols indicate −/− mice, open symbols indicate +/+ mice).

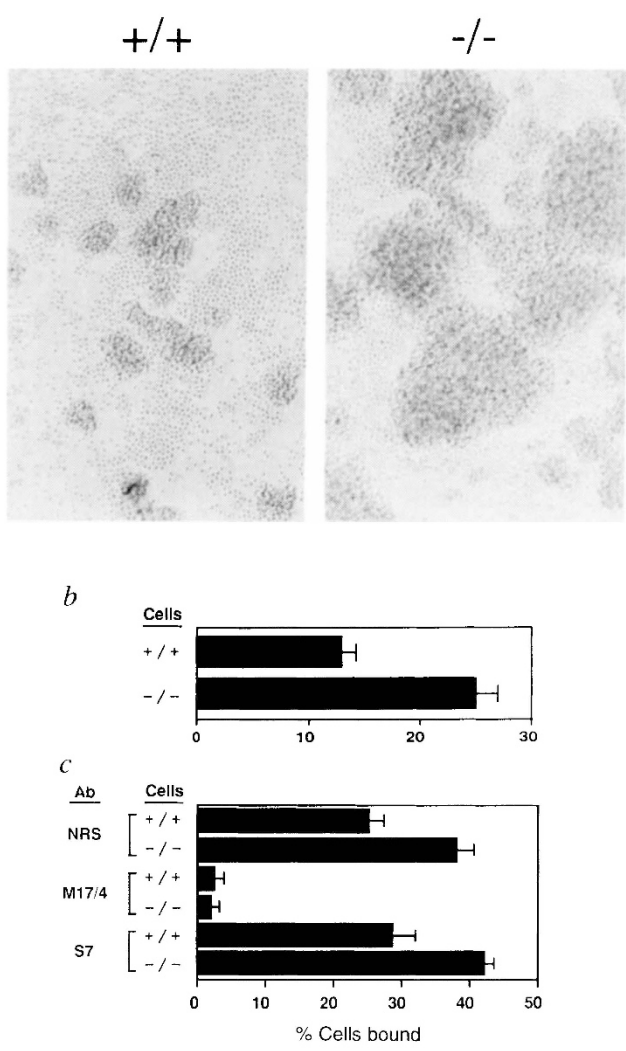

FIG. 4 *a*, PMA-induced homotypic adhesion of thymocytes; *b*, Binding of splenic T cells to fibronectin, and *c*, to ICAM-1.
METHODS. For homotypic adhesion assays, 2×10^6 thymocytes from $+/+$ and $-/-$ mice in 1 ml of medium (RPMI 1640 with 10% FBS) were added to individual wells in a 24-well plate. PMA (40 ng ml^{-1} final concentration) was added to each well and cells were photographed (200× magnification) after 1 h incubation in a humidified incubator at 37 °C, 5% CO_2. For adhesion to purified ligands, splenocytes from $+/+$ and $-/-$ mice were depleted of B cells and macrophages using anti-B220 monoclonal antibody (clone RAB-632; Pharmingen) coupled to immunomagnetic beads followed by plastic adherence for 2 h at 37 °C. Non-bead-bound, non-adherent cells were then treated with anti-CD3 ascites (mAb 145-2C11 at 1:1,000 dilution) for 30 min at 4 °C. Cells were labelled with carboxyfluorescein dye (BCECF; Molecular Probes), washed in adhesion buffer (RPMI, 5% FBS) and resuspended at 10^6 per ml in adhesion buffer containing PMA at 40 ng ml^{-1}. Polystyrene plates (96-well flat-bottom; Linbro) were prepared by coating overnight at 4 °C with murine fibronectin or sICAM-1 (ref. 25), both at 4 μg ml^{-1} in 0.02 M Tris-buffered saline (TBS), pH 7.2, or with buffer alone. The binding assay was carried out as described[24]. For antibody inhibition studies, cells were incubated with a rat anti-mouse LFA-1 (clone M17/4; Pharmingen), rat anti-mouse CD43 (clone S7) at 20 μg ml^{-1}, or normal rat serum (1:500) at room temperature for 10 min before adding to ligand-coated wells. The percentage of bound cells was calculated from (fluorescence of cells in ligand-coated wells − fluorescence of cells in buffer-coated wells) × 100/(fluorescence of cells added to the wells). All tests were performed in triplicate. Data shown are from one experiment and are representative of three separate assays.

molecules and their respective counter-receptors[28]. Moreover, the substantial number of sialic acid residues in the CD43 extracellular domain[3,5,30] probably gives the molecule a gross negative charge. These features might explain the ability of CD43 to retard T-lymphocyte interactions, which in turn might impede cell activation. It is also possible, however, that CD43 acts more directly to suppress T-cell activation. Structure function analysis using cytoplasmic domain truncations should elucidate whether the anti-adhesive and -proliferative phenotypes are related and clarify the mechanism of CD43-regulated T-cell activation. □

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