Brief Definitive Report

High Level Expression of CD43 Inhibits T Cell Receptor/CD3-mediated Apoptosis

By You-Wen He and Michael J. Bevan

From the Department of Immunology and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195

Summary

In a screen designed to identify genes that regulate T cell receptor (TCR)/CD3-mediated apoptosis, we found that high level expression of CD43 protected T cell hybridomas from activation-induced cell death. The protection appears to result from its capacity to block Fas-mediated death signals rather than from inhibition of the upregulation of Fas and/or Fas ligand after T cell stimulation. We found that peripheral CD4+ T cells can be divided into two subsets based on the level of CD43 surface expression. The CD4+CD43low subset exhibits a naive T cell phenotype, being CD62LhighCD45R BhighCD44low, whereas CD4+CD43high cells exhibit a memory phenotype, being CD62LlowCD45R BlowCD44high. Recent studies have demonstrated that engagement of TCR and Fas induces naive CD4+ T cells to undergo apoptosis, and the same treatment enhances the proliferation of memory CD4+ T cells. We confirm here that peripheral CD4+CD43high T cells are resistant to TCR/CD3-mediated cell death. These results suggest that the expression levels of CD43 on naive and memory CD4+ T cells determine their susceptibility to Fas-dependent cell death and that high level expression of CD43 may be used as a marker to define CD4+ memory T cells. Expression of CD43 provides a novel mechanism by which tumor cells expressing abnormally high levels of CD43 may escape Fas-mediated killing.

Key words: CD43 • activation-induced apoptosis • memory CD4+ T cells • Fas

After generation of a successful T cell response, a majority of the activated T cells die, and a minority survive to become resting memory cells (1). Memory T cells differ from naive T cells not only in the expression of surface markers but also in their functionality. Compared with naive T cells, memory T cells express lower levels of CD62L and CD45R B and higher levels of CD44. Memory T cells also respond faster, have a lower activation threshold, and secrete a wider range of cytokines than naive T cells. In addition, recent studies have demonstrated that, compared with naive CD4+ T cells, memory CD4+ T cells are resistant to TCR-mediated, Fas-dependent apoptosis (2–4). Engagement of TCR or TCR plus Fas on naive CD4+ T cells induces these cells to undergo apoptosis whereas Fas engagement may enhance the proliferation of memory CD4+ T cells (2–4). Studies on activation-induced cell death (AICD) of T cell hybridomas have been fruitful for understanding the molecular mechanisms of T cell apoptosis. After TCR stimulation, T cell hybridomas upregulate Fas and FasL (ligand). Autocrine stimulation of Fas by FasL triggers the death pathway via the recruitment of adaptors to the cytoplasmic tail of Fas (5). CD43 (leukosialin, sialophorin) is a highly glycosylated transmembrane protein expressed on the surfaces of all hematopoietic cells except mature B cells and erythrocytes (6). Previous studies suggest that CD43 may regulate multiple cellular functions, such as cell adhesion, activation, and proliferation as well as cell survival and apoptosis. However, the precise function of CD43 remains unknown due to conflicting results. Cross-linking of CD43 may enhance T cell proliferation (7), and it can act as a co-stimulatory molecule independent of CD28 (8). However, T cells from CD43-deficient mice are hyperresponsive after both in vivo and in vitro activation, indicating a negative regulatory role for CD43 in T cell activation (9). Although an anti-CD43 mAb has been shown to inhibit T cell binding to lymph node and Peyer’s patch high endothelial venule and homing from the blood into second lymphoid tissues (10), T cells from CD43-deficient mice homed significantly more frequently to secondary lymphoid organs compared with wild-type T cells (11). Certain studies have shown that engagement of CD43 induces apoptosis of T cells and hematopoietic progenitor cells (12, 13); CD43 was also shown to promote B cell survival when ectopically expressed (14).

In a search for genes that regulate TCR/CD3-mediated AICD, we found that CD43 can protect T cells when expressed at high levels. This protection appears to be due to a blockade of Fas function by CD43. Our findings shed new light on the significance of CD43 expression in lymphoid and nonlymphoid tissues.
Materials and Methods

Cell Lines. A subclone of DO 11.10 T hybridoma cells with a low survival rate in PMA plus ionomycin was used for expression cloning. αN X-Ampo is a retrovirus packaging cell line (15). DO 11.10 cell line expressing a tailless version of human CD2 (DO 11.10HC-D2) as control was described previously (16). DO 11.10 cells expressing high levels of CD43 were generated by transduction of mouse CD43 cDNA using the pMX retrovirus vector, followed by FACS® sorting for CD43 expression. Cells were cultured in DMEM containing 10% FCS, 2 mM glutamine, 25 mM HEPES, 50 μM β-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Expression cloning. Expression cloning was performed as described (16). In brief, a thymocyte cDNA library in the retroviral vector pMX (17) was transfected into the DO 11.10 cell line using CaPO4 precipitation, and retrovirus-containing supernatant was harvested 2 d later. DO 11.10 cells were infected with the retrovirus supernatant. A total of 5 x 106 DO 11.10 cells were infected, as assessed using control pMX-hCD2 retrovirus infection. 2 d after the infection, DO 11.10 cells were plated into 96-well plates (5 x 104 cells/well) and selected for survival and growth in the presence of MA (10 ng/ml) plus ionomycin (0.2 μg/ml). Resistant clones were identified after 2–4 wk in culture. cDNA inserts were amplified by reverse transcriptase–PCR and sequenced.

Flow Cytometric Analyses. Before staining, cells were incubated with biotinylated mAbs, or PE–anti-CD3 (145-2C11), FITC– or PE–anti-CD4 (GK1.5), FITC–, PE–, or anti-CD45R B (16A), and biotin–anti-CD43. Cells were cultured in 100 U/ml human IL-2 for an additional 2 d. The cells were then harvested and plated on an anti-CD3–coated plate. 16–24 h later, cells were quantitated by trypan blue exclusion and FACS® analysis.

Results

Identification of CD43 as an Inhibitor of AICD. We employed an expression cloning strategy to identify genes that regulate AICD in a T cell hybridoma. We infected DO 11.10 cells with a thymocyte cDNA library packaged in a retrovirus and selected for cells that could grow in the presence of PMA plus ionomycin. cDNA inserts from resistant clones were sequenced, revealing that one of the inserts encoded a full length CD43. To confirm the antiapoptotic effect of CD43, we transduced DO 11.10 cells with either the full length CD43 or a tailless human CD2 as control. DO 11.10 cell lines expressing high levels of CD43 were established by fluorescence-activated cell sorting. As shown in Fig. 1 A, DO 11.10hCD2 control cells express a low level of endogenous CD43 on their surfaces. After transduction with pMX.CD43, we isolated DO 11.10 cell lines expressing ~6–20-fold higher surface levels of CD43 compared with control cells (Fig. 1 A). Interestingly, DO 11.10CD43high cells expressed two- to threefold lower levels of CD3 on their surfaces compared with DO 11.10hCD2 control cells (Fig. 1 A). The lower CD3 surface staining was not due to non-specific blockade of cell surface accessibility, as the expression levels of other surface molecules such as CD2 and CD5 were not changed (data not shown).

To examine the effect of high level expression of CD43 on activation-induced apoptosis, we measured [3H]thymidine uptake of DO 11.10hCD2 control and DO 11.10CD43high cells in the absence or presence of anti-CD3 mAb or PMA plus ionomycin. [3H]thymidine incorporation by these cells was highly correlated with their viability, measured by either propidium iodide uptake or trypan blue exclusion (16). As shown in Fig. 1 B, DO 11.10 cells expressing high levels of CD43 were protected from anti-CD3–induced death. In contrast, control cells were readily induced to undergo apoptosis. We further determined whether the antiapoptotic effect of DO 11.10CD43high cells could be due to a lowered TCR/CD3 surface expression by using PMA plus ionomycin as a stimulus for AICD, which bypasses the TCR. DO 11.10CD43high cells were also refractory to apoptosis induced by PMA plus ionomycin (Fig. 1 B). The protection from AICD by CD43 correlated with its expression level, and the dosing effect was more pronounced when these cells were stimulated with anti-CD3, possibly due to some steric hindrance of CD43 imposed on the interaction of TCR/CD3 with the plate-bound anti-CD3 mAb. Next, we tested the effect of high level expression of CD43 on apoptosis induced by other stimuli. DO 11.10 cells expressing high levels of CD43 were protected from apoptosis only in the presence of low amounts of staurosporine and were not protected from ceramide-induced killing (Fig. 1 B).

High Level Expression of CD43 Does Not Inhibit T H ybridoma Activation and Upregulation of Fas and Fasl. Previous studies have demonstrated that CD43 acts as a negative regulator of T cell activation, presumably due to its highly charged nature and its large size (9). To examine the effect of high level expression of CD43 on T cell hybridoma activation, we stimulated control DO 11.10hCD2 and DO 11.10CD43high cells with either plate-bound anti-CD3 mAb or PMA plus ionomycin for 6 h and monitored the upregulation of Fas, Fasl, and CD69 by FACS® analysis.
sis. Before TCR stimulation, the expression of Fas on DO11.10CD4\(^{3}\)\(^{\text{high}}\) cells was lower compared with control cells (Fig. 2). However, both Fas and FasL were upregulated by anti-CD3 stimulation of DO11.10CD4\(^{3}\)\(^{\text{high}}\) cells (Fig. 2), even though these cells express lower levels of CD3 (Fig. 1 A). The cell activation marker CD69 was also upregulated to a similar level in both cell types (Fig. 2). Similarly, PMA plus ionomycin upregulated expression of these three markers in control and CD43\(^{\text{high}}\) cells (data not shown). These data indicate that high levels of CD43 expression did not obviously affect the activation of these T hybridoma cells and the upregulation of Fas and FasL and suggest that high level expression of CD43 may interfere with the death signal initiated through the Fas molecule.

**Figure 1.** High level expression of CD43 protects T hybridoma cells from AICD. (A) FACS\(^{\text{®}}\) analysis of surface expression of CD43 and CD3 on DO11.10CD2 control and DO11.10CD43\(^{\text{high}}\) cell lines. Shown are the histogram profiles of DO11.10CD2 control and two individual DO11.10CD43\(^{\text{high}}\) cell lines. Histograms on the left represent isotype-matched antibody staining for background control. (B) DO11.10CD2 control cells (○) and two DO11.10CD43\(^{\text{high}}\) (○, number 1; □, number 2) cell lines as shown in A were cultured in plates coated with 2C11, PMA (10 ng/ml) plus various amounts of ionomycin, or different concentrations of staurosporine and ceramide and assayed for their \(^{\text{[H]}}\)thymidine incorporation. Incorporation of individual cell lines cultured in medium alone is calculated as 100%.

**Figure 2.** Effect of high level expression of CD43 on the upregulation of Fas, FasL, and CD69. FACS\(^{\text{®}}\) analysis of cell surface expression of Fas, FasL, and CD69 in DO11.10CD2 control or DO11.10CD43\(^{\text{high}}\) cell line after TCR-mediated activation. Cells were activated on 2C11-coated plates for 5 h, and the cell surface phenotype was examined (filled curve, before activation; unfilled curve, after activation).
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CD41CD43low T cells express low levels of CD44 and high levels of CD62L and CD45RB. Peripheral CD41 and CD81 T cells from lymph nodes exhibited a similar phenotype to splenic T cells in the above analysis (data not shown). These results suggest that CD43 may be used as a marker to define memory CD41 T cells.

**CD41CD43high T Cells Are Resistant to TCR/CD3-mediated Cell Death.**

Recent studies have demonstrated that naive CD4+ T cells are more susceptible to TCR-mediated, Fas-dependent cell death compared with memory phenotype CD4+ T cells (2–4). To test whether the CD41CD43high primary cells are resistant to TCR/CD3-mediated cell death, we used an in vitro assay for AICD. Peripheral CD41CD43low T cells exhibit a naive phenotype, whereas CD41CD43high exhibit a memory phenotype, suggesting that the surface expression levels of CD43 on CD4+ naive and memory T cells determine their susceptibility to TCR-mediated, Fas-dependent cell death.

How is the inhibition of TCR/CD3-mediated cell death by high level expression of CD43 achieved? CD43 has been shown to have multiple, sometimes contradictory functions. This may be due to its structural features and the systems that have been used to study its function. CD43 is arguably the most abundant protein on the T cell surface (19). It extends 45 nm from the cell surface, at least six times the height of the TCR. It is also highly O-glycosylated and bears numerous sialic acid residues. The antiadhesive and antiproliferative effects mediated by CD43 are thought to result from a physical barrier formed by this highly negatively charged and rigid rod-like structure. However, this functional model of CD43 has been challenged by recent experiments showing that the cytoplasmic domain of CD43 plays an important role in its function (20, 21). Moreover, the extracellular portion of CD43 may also be highly dynamic and actively interact with other surface structures. In support of this notion, a recent study showed that CD43 moves away from the contact sites of T

**Discussion**

Several recent studies have demonstrated that CD4+ memory T cells are resistant to TCR-mediated, Fas-dependent cell death, whereas CD4+ naive T cells succumb (2–4). In this report, we show that high level expression of CD43 protects T cell hybridomas from AICD. Peripheral CD41CD43low T cells exhibit a naive phenotype, whereas CD41CD43high exhibit a memory phenotype, suggesting that the surface expression levels of CD43 on CD4+ naive and memory T cells determine their susceptibility to TCR-mediated, Fas-dependent cell death.

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**Figure 3.** Phenotypic analysis of CD4+ T cells in the spleens of C57BL/6 mice. (A) Expression of CD43 on CD4 and CD8 T cells. Shown are the dot plot profiles of splenocytes double stained with anti-CD43 plus anti-CD4 or anti-CD8. Gated regions represent CD43low and CD43high cells. (B) Expression of CD62L, CD45RB, CD44, and CD43 on CD4+ T cells. Shown are histogram profiles of CD62L, CD45RB, and CD44 staining on CD4+CD43low and CD4+CD43high cells as gated in A.

**Figure 4.** CD4+CD43high T cells are resistant to TCR/CD3-mediated cell death. Splenocytes were activated with Con A, cultured in IL-2, and restimulated with plate-bound 2C11 for 18 h in the presence of IL-2. Live cells were electronically gated and analyzed for their expression of CD43 and CD4. Control cells were cultured in IL-2 alone.
cells and APCs, whereas CD45, similarly large in size and strongly negatively charged, does not (22). In addition, CD43 was reported to be physically associated with the TCR/CD3 complex (23). Several mechanisms can be envisioned for the anti-apoptotic effect caused by high level expression of CD43. First, highly expressed CD43 might block Fas signaling by physically preventing the interaction between FasL and Fas. Alternatively, it may prevent Fas from forming trimers. The fact that high level expression of CD43 on DO11.10 specifically decreases the expression of TCR/CD3 and Fas indicates that they may physically interact or form surface complexes with each other. Furthermore, high level expression of CD43 may interfere with the recruiting of death signaling molecule by Fas or inhibit the activation of caspases. Overexpression of Toso, a surface protein that contains an Ig domain, was shown to inhibit Fas signaling by upregulating the caspase-8 inhibitor cFLIP (cellular FLICE [FADD-like IL-1β-converting enzyme]-inhibitory protein; reference 15). We have not observed differences in the expression of cFLIP in DO11.10 control cells and CD43high-expressing cells (our unpublished observation). It is interesting to note that although CD43highCD4+ T cells from spleen and lymph nodes express six- to eightfold higher levels of CD43 on their surfaces than CD43lowCD4+ cells, these cells are much easier to activate by either anti-CD3 or Con A than CD43lowCD4+ cells (our unpublished data), in agreement with previous studies showing that memory T cells have a lower threshold for activation (1). These data further argue against the model that CD43 negatively regulates T cell activation by increasing the stimulation threshold or by physically hindering the interaction between T cells and APCs. Our attempt to investigate whether the cytoplasmic tail of CD43 is required for the inhibition of AICD was inconclusive. A tailless CD43 retaining two amino acids of the cytoplasmic tail can be expressed at only two- to threefold higher levels than the endogenous CD43 on DO11.10 cells, and this tailless version of CD43 had no obvious antiapoptotic effect (our unpublished data).

We propose that high level expression of CD43 be used as a surface marker to define CD43+ memory T cells in C57BL/6 mice. Our results further suggest that CD43 may protect activated cells from AICD. In addition, we note that, correlating with their resistance to Fas-mediated killing, CD43+ T cells uniformly express high levels of CD43.

The protection against Fas-mediated killing by high level expression of CD43 may provide a novel mechanism for its role in tumorigenesis. CD43 has been shown to be overexpressed in Friend erythroleukemia cells (24) and abnormally expressed in nonhematopoietic tumor lines such as colon carcinoma and adenoma cells (25-27). Surface expression of CD43 diminishes susceptibility of target cells to T cell-mediated cytolysis (28), and the obvious implication is that CD43 expression on tumor cells protects them against lymphoid effectors bearing FasL.

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Address correspondence to Michael J. Bevan, HHMI, Dept. of Immunology, University of Washington School of Medicine, Box 357370, Seattle, WA 98195. Phone: 206-685-3610; Fax: 206-685-3612; E-mail: mbevan@u.washington.edu

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