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CD43 cross-linking increases the Fas-induced apoptosis through induction of Fas aggregation in Jurkat T-cells

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Abbreviation: TcR, T-cell receptor

Abstract

CD43 (sialophorin, leukosialin) is a heavily sialylated surface protein expressed on most leukocytes and platelets including T cells. Although CD43 antigen is known to have multiple and complex structure, exact function of CD43 in each cell type is not completely understood. Here we evaluated the role of CD43 in Fas (CD95)-induced cell death in human T lymphoblastoid cell line, Jurkat. Crosslinking CD43 antigen by K06 mAb increased the Fas-mediated Jurkat cell apoptosis and the augmentation was inhibited by treatment with caspase inhibitors. Further, CD43 signaling of Jurkat cells induced Fas oligomerization on the cell surfaces implying that CD43 ligation have effects on early stage of Fas-induced T cell death. These also suggest that CD43 might play an important role in contraction of the immune response by promotion of Fas-induced apoptosis in human T cells.

Keywords: apoptosis; antigens, CD43; antigens, CD95; caspase; Jurkat cells; T-lymphocytes

Introduction

Lymphocyte homeostasis is a balance between lymphocyte proliferation and death. Tight control of apoptosis is essential for immune functions, because its altered regulation can result in cancers and autoimmunity. There is an active process leading to death caused by the stimulation of death-inducing molecules including Fas and TNF-receptor (TNFR)1, members of the expanding TNFR superfamily (Lenardo *et al.* 1999). Cross-linking of surface Fas molecules (CD95, Apo-1) by the Fas ligand (FasL) or agonistic anti-Fas Abs activates apoptotic death programs (Nagata and Goldstein, 1995; Wallach *et al.,* 1999; Kim *et al.,* 2000) via homo-oligomerization of these death receptors and results in the cleavage of several intracellular substrates (Medema *et al.,* 1997; Thornberry and Lazebnik, 1998; Muzio *et al.,* 1998).

There are several cell surface antigens and factors that might regulate the sensitivity of T cells to FasL and TNF. One of the most immunologically important means of facilitating T cell death by FasL or TNF is through TCR occupancy (Wong *et al.,* 1997). In addition, interleukin (IL)-2 also enhances Fas-mediated apoptosis in human T cells (Fournel *et al.,* 1996) and other antigens including CD45 and CD99 have been implicated in augmentation of Fas-mediated cell death (Klaus *et al.,* 1996; Jung *et al.,* 2003).

CD43 (sialophorin, leukosialin, or gp115) is a large sialoglycoprotein that is abundantly expressed by cells of hematopoietic origin, including both CD4+ and CD8+ T cells (Axelsson *et al.,* 1985; Borche *et al.,* 1987; Remold-O'Donnell *et al.,* 1987). This abundant surface protein CD43 is a transmembrane protein consisting of a highly O-glycosylated extracellular domain of 235 amino acids, a transmembrane domain of 23 amino acids, and an intracytoplasmic domain of 123 amino acids (Pedraza-Alva *et al.,* 1998). Posttranslational modifications result in two glycoforms of CD43. Resting T lymphocytes express a CD43 isoform of 113-122 kDa that contains O-linked tetrasaccharides attached to serine and threonine residues. Upon activation, T lymphocytes express a 125-135kDa form of CD43 carrying mainly O-linked hexasaccharides (Piller *et al.,* 1988; Piller *et al.,* 1991).

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 in each cell type remains unclear due to conflicting results. Cross-linking of CD43 may enhance T cell proliferation (Park *et al.,* 1991), or can act as a costimulatory molecule independent of

CD28 (Sperling *et al.,* 1995). On the contrary, T cells from CD43-deficient mice are hyper-responsive following both *in vivo* and *in vitro* activation, indicating a negative regulatory role of CD43 in down modulation of effector T cell response (Manjunath *et al.,* 1995; Thurman *et al.,* 1998). In addition, anti-apoptotic and proapoptotic functions of CD43 have been reported. Certain studies have shown that engagement of CD43 induces apoptosis of T cells and hematopoietic progenitor cells (Bazil *et al.,* 1995; Brown *et al.* 1996; Cermak *et al.,* 2002; Park *et al*., 2004). However, He and Bevan have reported that high level expression of CD43 inhibits T cell receptor (TCR)/CD3 mediated apoptosis (1999).

Here, we propose another proapoptotic function of CD43 through the mechanism that modulates Fas induced cell death in human T lymphoblastoid cell line. CD43 cross-linking increases the Fas-induced apoptosis through the induction of Fas aggregation in Jurkat cells. Therefore, we come to a conclusion that CD43 is related with the Fas-induced apoptosis in Jurkat cell, and this might have some implications in activation induced cell death (AICD) of human T cells.

Materials and Methods

Cells and reagents

Jurkat cells were maintained in RPMI-1640 containing 10% fetal calf serum (FCS, Hyclone, Logan, UT), 2 mM L-glutamine, 50 μM β-mercaptoethanol (ME), 10 mM HEPES and 100 U/ml each of penicillin and streptomycin. Antibodies against Fas protein were purchased from Medical & Biological Laboratory (clone CH11; Nagoya, Japan) and Santa Cruz Biotechnology (clone C-20; Santa Cruz, CA). Anti-mouse Ig antisera coupled to horseradish peroxidase (HRP) were also from Santa Cruz Biotechnology. The caspase inhibitors, z-VAD-fmk, z-DEVD-CHO, and z-IETD-fmk were from Calbiochem (La Jolla, CA). Anti-human CD43 mAb, K06, was produced and purified as previously described (Park *et al*., 2004).

Flow cytometric analysis

Jurkat cells were plated in 24-well plate (Falcon, Becton-Dickinson, Franklin Lakes, NJ) at 2×10^5 cells/well in a final volume of 1ml of RPMI medium. These cells were treated by anti-Fas mAb, CH11, with or without anti-CD43 mAb, K06. At various time points, cells were harvested and used for apoptosis or western blot analyses. To evaluate the rate of apoptosis, cells were incubated with FITC-conjugated Annexin V. For the detection of early and late apoptosis by flow cytometry, double staining of Anneixn V and the vital dye 7-AAD was performed. The cells were washed three times with Annexin V-binding buffer (0.1 M HEPES/NaOH [pH7.4], 1.4 M NaCl, 25 mM $CaCl_2$) and were incubated for 30 min at room temperature in the dark in Annexin V-binding buffer containing FITC-conjugated annexin V and 20 μg/ml 7-AAD. This double-staining shows both early (Annexin V^+/7-AAD^-) and late apoptotic cells (Annexin V^+/7-AAD^+). Stained cells were analyzed using a FACScalibur (Becton-Dickinson, Mountain View, CA) and data were analyzed using Cellquest software.

Treatment of caspase inhibitors

Jurkat cells were treated with caspase inhibitors, 20 μM caspase inhibitor I (z-VAD-fmk), 20 μM caspase-3 inhibitor (z-DEVD-CHO), or 50 μM caspasse-8 inhibitor (z-ITED-fmk), for 2 h prior to the addition of CH11 and/or K06 mAbs. After 5 h incubation, cells were harvested and analyzed to evaluate the effect of caspase inhibitors on Jurkat cell death.

Detection of Fas aggregation

Analysis of SDS-stable Fas microaggregates was performed as described previously (Algeciras-Schimnich *et al*., 2002). In brief, 3×10^6 cells were stimulated with 5 μg/ml K06 for 2 h at 37°C and the cells were washed with PBS and lysed in lysis buffer (50 mM HEPES [pH7.4], 150 mM NaCl, 10 mM NaF, 10 mM iodoacetamine, 200 μM Na_3VO_4, 1% NP-40). Cell extracts were separated by SDS-PAGE, transferred electrophoretically onto Immobilon P (Millipore, Billerica, MA) and probed with C-20 Ab against Fas. The specific bands were visualized using ECL system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Confocal analysis

Jurkat cells were pretreated with K06 mAb and rabbit anti-mouse polyclonal antibody, and then adhered to the poly-L-lysine coated slide. As a negative control, isotype matched irrelevant mAb was treated. The cells were fixed with paraformaldehyde and were incubated with biotinylated anti-Fas mAb (Pharmingen, Becton-Dickinson) and streptavidin-alexa (Molecular Probes, OR). The stained preperations were analyzed on a confocal microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, CA).

Results

Engagement of anti-CD43 mAb enhances Fas-mediated cell death in Jurkat cells

In order to evaluate the role of CD43 in Fas-mediated apoptosis of T cells, Jurkat cells were treated with anti-CD43 mAb, K06, along with an agonistic anti-Fas mAb, CH11, and phosphatidylserine exposure was analyzed by Annexin-V staining (Figure 1). When the cells were treated with K06 mAb alone, apoptosis of Jurkat cells was not observed as previously reported (Park *et al.*, 2004). In the cells treated with anti-Fas mAb CH11, Fas-induced cell death was observed in about 20% of the cells as previously described (Wallach *et al.,* 1999). When the cells were co-ligated with CH11 and K06, the percentage of apoptotic cells was higher than that of the cells treated with CH11 alone (Figure 1). These data showed that signal through CD43 alone did not induce the cell death in Jurkat cells, but engagement of CD43 antigen increased the Fas-mediated apoptosis of Jurkat cells.

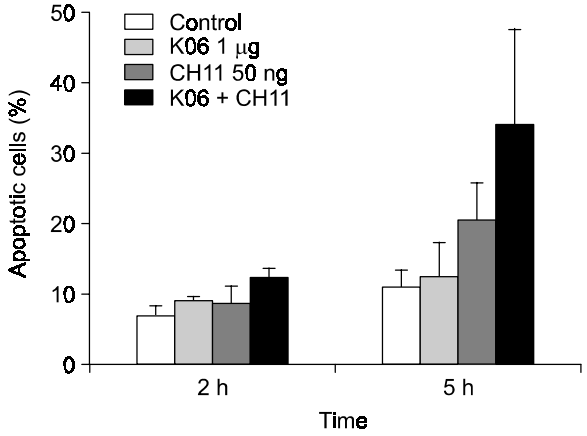

Figure 1. Augmentation of Fas-mediated apoptosis in Jurkat cells by anti-CD43 mAb, K06. Jurkat cells were incubated with 1 μg/ml K06 in the presence, or absence of 50 ng/ml anti-Fas mAb (CH11). At the time point indicated, cells were harvested and analyzed apoptosis using Annexin-V staining and flow cytometry. When the cells were treated with K06 and CH11, the degree of apoptotic cells was higher than CH11 alone. The values were average and standard deviation of five independent experiments.

To evaluate whether the augmentation of Fas-mediated cell death by the CD43 ligation is dependent on the amounts of CH11 mAb, we added various concentrations of CH11 mAb to the Jurkat cells. When the cells were treated with 50ng/ml anti-Fas mAb CH11 alone, 16% of cells appeared to be in early apoptosis (Annexin V^+/7-AAD^-, Figure 2). The percentage of early apoptotic cells was increased to 38% when the cells were co-ligated with CH11 and K06 mAbs (Figure 2). However, the percentage of late apoptosis (Annexin V^+/7AAD^+) was not significantly changed upon K06 engagement (1.7% vs. 3.4%). This implies that K06 ligation signals the initial step of apoptosis in Fas mediated T cell death. Maximum effect of CD43 mediated augmentation of Fas induced cell death was observed when 50 ng/ml of CH11 mAb was added to the Jurkat cells. In addition, enhanced cell death was not proportional to the CH11 mAb added (Figure 2).

The augmentation of Fas-mediated apoptosis via anti-CD43 mAb (K06) is related to early events of caspase activation

It is well known that caspases play a pivotal role in

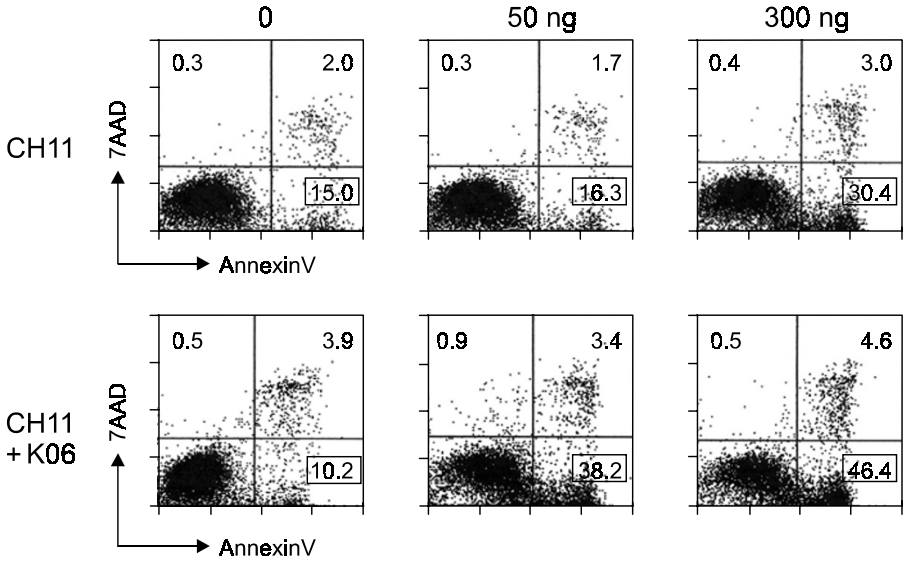

Figure 2. The effect of CH11 concentration on the K06-amplified Fas-induced apoptosis. K06-amplified Fas-induced apoptosis was not increased by the increment of CH11 concentration (50 or 300 ng/ml). Jurkat cells (2×10^5/ml) were incubated with indicated amounts of CH11 with or without anti-CD43 mAb (K06). The K06 mAb was used as 5 μg/ml. After 5 h incubation, cell death was assayed by Annexin-V and 7AAD staining. This result was representative of five independent experiments.

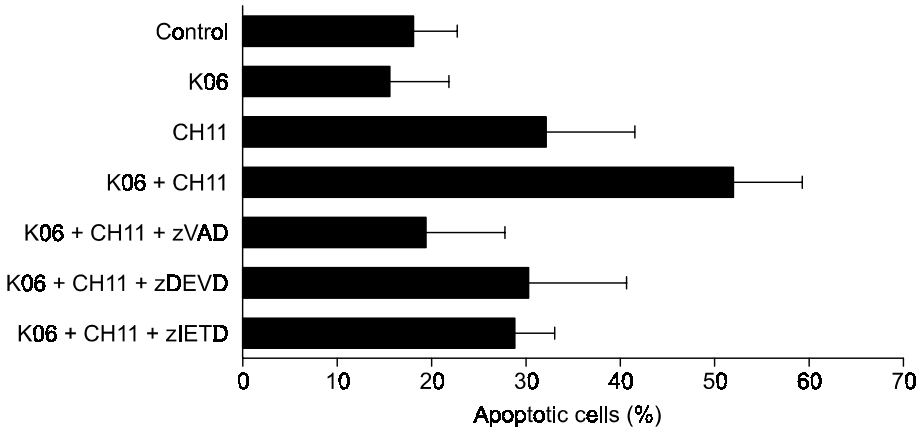

Figure 3. Effects of caspase inhibitors on the K06-amplified apoptosis. Cells (2×10^5/ml) were incubated with K06 (5 μg/ml) and CH11 (100 ng/ml) for 5 h. 20 μM of zVAD-fmk, 20 μM of zDEVD-fmk, or 50 μM of zIETD-fmk was added to block the caspase activation. After 5 h incubation, the degree of apoptosis was determined by using Annexin-V staining and analyzed by flow cytometry. The values in this figure were average and standard deviation of three independent experiments.

Fas-mediated apoptosis. On binding to cognate ligands or agonistic antibodies, Fas aggregates initiate the activation of caspase-8, which is followed by a cascade of caspase activation. To clarify which step in pathway of Fas-mediated apoptosis is involved in the CD43-amplified Fas-induced cell death, we added the caspase inhibitors, z-VAD, z-DEVD and z-IETD. Treatment with pan caspase inhibitor, z-VAD-fmk, blocked completely the K06-mediated augmentation of Fas-mediated cell death (Figure 3). Next, a caspase-8 inhibitor, z-IETD-fmk, and a caspase-3 inhibitor, z-DEVD-fmk, were used to block specific events in the Fas pathway. As shown in Figure 3, both caspase-3 and caspase-8 inhibitors also diminished the K06-mediated augmentation of Fas-induced apoptosis. These findings imply that K06-mediated apoptosis of Jurkat cells is dependent on Fas signal and the K06 signal affects early events of Fas-mediated apoptosis.

Ligation of anti-CD43 mAb (K06) induces aggregation of Fas molecules on the cell surface

When Fas interacts with FasL or agonistic anti-Fas Abs, Fas molecules are trimerized and caspase-8 is subsequently activated (Chinnaiyan *et al.,* 1995). When Jurkat cells were treated with K06 mAb, no increment of Fas expression on the cell surface was observed by flow cytometric analysis (data not shown). Furthermore, as shown in Figure 3, CD43 ligation seemed to have an effect on the early stage of Fas-induced apoptosis pathway, possibly upstream of caspase 8 activation.

As a consequence, we next assessed whether the aggregation of Fas molecules on the cell surface could be induced by K06 mAb treatments. Jurkat cells were treated with 2 or 10 μg of K06 mAb for 30 min or 2 h, and western blot analysis was performed to visualize Fas-aggregates. As shown in Figure 4A, formation of SDS- and 2-ME-stable, high M_r aggre-

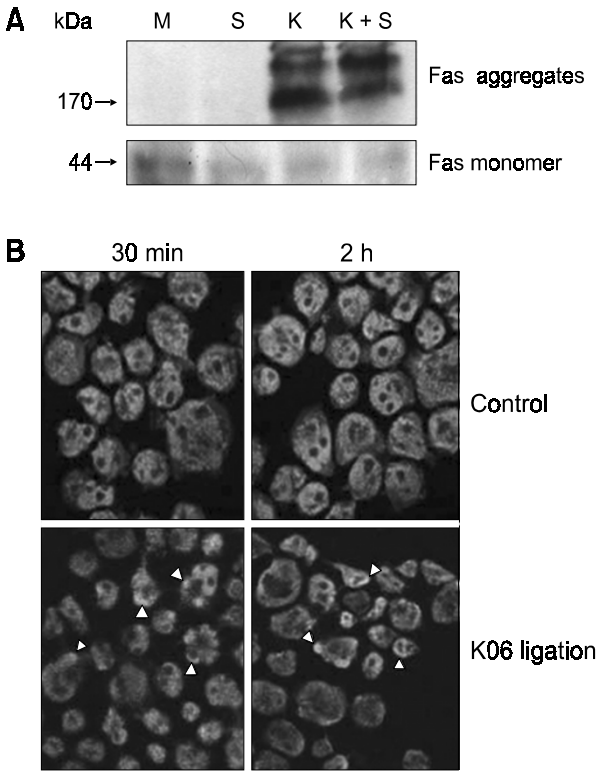

Figure 4. K06 induced aggregation of Fas molecules. (A) Jurkat cells (5×10^6/ml) were treated as indicated condition for 5 h (M: media only, S: secondary Ab only (anti-mouse Ig 20 μg/ml), K: K06 (10 μg/ml), K + S: (K06 and secondary Ab)). Total cell lysates were prepared and western blot analysis was performed. (B) Confocal analysis of immunostained Jurkat cells using biotinylated-mAb against Fas. The cells were pretreated with K06 10 μg/ml and secondary antibody (K06 ligation) or the secondary Ab (anti-mouse Ig) 20 μg/ml only (control) for indicated time. At each time point, cells were harvested and staining was applied using anti-Fas Ab. Arrow heads indicate patch-shaped aggregation of the Fas molecules on the cell surface.

gates (> 200 kDa) of Fas antigens were detected in lysates of K06 only or K06 and the secondary Ab treated cells. The formation of the high M_r aggregates increased with time and the amount of mAb added (data not shown), while aggregation of Fas was not observed in the control cells treated with irrelevant mAb. These results indicate that CD43 ligation via the K06 epitope induced Fas aggregation in the absence of Fas-engagement. Confocal analysis also revealed the Fas aggregation induced by K06 mAb treatment. After Jurkat cells were treated with anti-CD43 mAb, K06, for indicated time points, Fas molecules were visualized and analyzed by confocal microscopy. The aggregated Fas molecules were located on the cell membrane as patch-shape as compared with those in the negative control (Figure 4B). Therefore, the CD43 signal via the K06 mAb induced aggregation of Fas molecules in Jurkat T cells without FasL or agonistic anti-Fas mAb treatment.

Discussion

Receptor mediated apoptotic signaling in hematopoietic cells is carried out primarily by the death receptors of the tumor necrosis factor family. However, the cross-linking of other cell surface receptors such as major histocompatibility complex class I, CD45, CD47, and CD99 could also induce apoptosis (Klaus *et al.,* 1996; Skov *et al.,* 1997; Pettersen *et al.,* 1999; Pettersen *et al.,* 2001). It has been controversial whether ligation of CD43 with mAb induces apoptosis of hematopoietic cells, especially in T cells (Bazil *et al.,* 1995; Brown *et al.,* 1996; He and Bevan, 1999; Cermak *et al.,* 2002). Treatment of Jurkat cells with anti-CD43 mAb, J393, alone killed 25-50% of the cell population, while concomitant engagement of the CD3-TcR complex significantly potentiated this effect and a blocking antibody to CD95/Fas receptor could not prevent J393/CD43-induced apoptosis (Brown *et al.,* 1996). Moreover, ligation with anti-CD43 mAb induced tyrosine phosphorylation and inhibited the DNA binding activities of the transcription factors, such as AP-1 and NF-κB (Brown *et al.,* 1996; Cermak *et al.,* 2002). However, opposite results have been reported that high level expression of CD43 inhibits T cell receptor (TCR)/CD3 mediated apoptosis (He and Bevan, 1999).

In this study, we evaluated the functional role of CD43 in T cell death using anti-CD43 mAb, K06, we produced (Park *et al*., 2004) and found another mechanism for CD43 to enhance apoptosis in Jurkat cells. Although CD43 ligation through the K06 epitope failed to induce apoptosis in Jurkat cells (Park *et al.,* 2004), K06 signal enhanced Fas-mediated cell death (Figure 1). Apparently, this implies that the cell-specific environment, the nature of the CD43 epitope involved, or cell type-dependent changes in the glycosylation of CD43 could affect the final outcome of CD43-mediated signaling.

Next, we dissected the mechanisms of enhancement of Fas-mediated apoptosis by K06 signal in Jurkat cells. First, we considered the possibility that the expression of Fas molecules on the cell surface is enhanced by the K06 treatment. However, K06 engaged Jurkat cells showed no increment of Fas expression on the cell surface as analyzed by flow cytometric analysis (data not shown). These indicate that increased apoptosis of Jurkat cells by CD43 cross-linking is not resulted from up-regulation of Fas molecules on the cell surfaces.

Fas ligation via the FasL or an agonistic anti-Fas Ab induces Fas oligomerization, recruits Fas-associated adaptor and effector proteins leading to formation of large Fas surface clusters, and results in caspase activation (Wallach *et al.,* 1999; Algeciras-Schimnich *et al.,* 2002). To understand if the caspases are involved in K06-amplified Fas-induced apoptosis, caspase inhibitors are added prior to the addition of mAbs. Caspase-8 inhibitor as well as caspase-3 inhibitor was able to inhibit the augmentation of Fas-induced cell death (Figure 3). Therefore, the signaling pathway of K06 induced augmentation in Fas-mediated apoposis might be involved in the initial signaling event, possibly caspase-8 activation or the earlier events. To confirm whether CD43 signal could induce Fas oligomerization before CH11 ligation, we performed western blot analysis and confocal microscopic examination. As shown in Figure 4A and B, oligomerized Fas molecules were readily observed on cross-linking of anti-CD43 mAb, K06, alone. There have been some conflicts whether Fas aggregation correlates with Fas-mediated apoptosis. However, not only the Fas oligomerization by Fas ligation but preassociated receptor complex of Fas have been revealed necessary for Fas signaling and dominant interference in human disease. In addition, Siegel *et al*. also demonstrated that Fas complexes from surface-cross linked cells showed only partial recruitment of FADD and no recruitment of caspase-8, with no cleavage of the downstream caspase substrate poly (ADP-ribose) polymerase (2000). This implies that Fas signal through the Fas molecule is requisite to complete apoptosis and therefore pre-associated receptor complex of Fas by CD43 cross-linking itself was not able to induce apoptosis in our system. Further, the formation of Fas aggregates by cross-linking of CD43 might increase the CH11-mediated apoptosis of Jurkat

cells without the change of Fas expression level on the cell surface (Figure 4 and data not shown).

In addition, the Fas cell membrane polarization in long term activated T lymphocytes, through an ezrin-mediated association with the actin cytoskelecton, is a key intracellular mechanism in rendering human T lymphocytes susceptible to the Fas-mediated apoptosis (Parlato *et al.,* 2000). Further, the cytoplasmic tail of CD43 has been shown to interact with members of the ezrin-radixin-moesin (ERM) family of cytoskelectal adaptor molecules (Yonemura *et al.,* 1998). Most recently, it has been shown that CD43 is excluded from the immunological synapse during T cell activation, and this exclusion appears to be mediated by an ezrin-radixin-moesin-dependent mechanism(s) (Sperling *et al.,* 1998; Allenspach *et al.,* 2001; Delon *et al.,* 2001). Based on these findings and our present data, we propose an alternate mechanism; CD43 molecule might regulate the activation induced cell death in human T cells through ezrin and Fas molecules. Full T cell activation involves a complete membrane reorganization event. In parallel with the formation of the immunological synapse at the contact sites between the T cell and the APC, a second “anti-synapse” forms at the distal pole in which CD43 anchored with ezrin is located. Another signal through CD43 might promote association of Fas and cytoskeleton molecules, ezrin, and results in aggregation of Fas molecules on the cell surface. These would render activated human T lymphocytes susceptible to Fas-mediated apoptosis and play an important role in maintaining T cell homeostasis.

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