

CD152 (CTLA-4) Determines the Unequal Resistance of Th1 and Th2 Cells against Activation-induced Cell Death by a Mechanism Requiring PI3 Kinase Function

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Abstract

Survival of antigen-experienced T cells is essential for the generation of adaptive immune responses. Here, we show that the genetic and antibody-mediated inactivation of CD152 (cytotoxic T lymphocyte antigen 4) in T helper (Th) effector cells reduced the frequency of nonapoptotic cells in a completely Fas/Fas ligand (FasL)-dependent manner. CD152 cross-linking together with stimulation of CD3 and CD28 on activated Th2 cells prevented activation-induced cell death (AICD) as a result of reduced Fas and FasL expression. Apoptosis protection conferred by CD152 correlated with the up-regulation of Bcl-2 and was mediated by phosphatidylinositol 3 kinase, which prevented FasL expression through the inhibitory phosphorylation of Forkhead transcription factor FKHRL1. We show that signals induced by CD152 act directly on activated T lymphocytes and, due to its differential surface expression on activated Th1 and Th2 cells, induce resistance to AICD mainly in Th2 cells.

Key words: costimulation • apoptosis • survival • signal transduction • FasL

Introduction

The decision between survival and apoptosis of T cells is of particular importance for adaptive immune responses to ensure that a defined number of specialized T cells remain in the organism, thus maintaining homeostasis and memory. The primary form of apoptosis of clonally expanded T cells is activation-induced cell death (AICD), mainly controlled by the Fas (CD95) system (1, 2). Fas-mediated AICD is considered to be the primary mechanism in the deletion of mature CD4 T cells in the periphery.

In the absence of appropriate costimulation, TCR signaling induces Fas and Fas ligand (FasL) expression (3). Ligation of Fas initiates the recruitment of Fas-associated death domain and caspase-8 that triggers the proteolytic caspase cascade, resulting in the cleavage of various proteins and apoptosis (4). Several mechanisms exist to stop death processes either at the receptor, mitochondrial, or caspase level. Survival mechanisms are also mediated by phosphatidylinositol 3 kinase (PI3 K), which activates protein kinase B/Akt, an antiapoptotic kinase that inactivates proapoptotic molecules such as Bad and Forkhead transcription factor FKHRL1 (5, 6).

CD28 costimulatory signaling and cytokines have been suggested to up-regulate the antiapoptotic molecules Bcl-x_L and c-FLIP during an immune response (7–9). Importantly, some effector T cells survive and contribute to long-term memory (10).

Cytotoxic T lymphocyte antigen 4 (CTLA-4, CD152) is a major down-regulator of immune responses (11–13). In most circumstances, the two homologues CD28 and CD152, which share common ligands, fulfill opposing functions during T cell activation. Triggering of CD28 strongly up-regulates IL-2 production and T cell proliferation, which is counterregulated by CD152-mediated inhibition of IL-2 transcription and cell cycle progression (14). CD28 also leads to the stabilization of IL-2 mRNA and up-regulation of Bcl-x_L, events that are not counterregulated by CD152 (15). The underlying mechanisms responsible for these effects are unclear. CD28 has been reported to bind to PI3 K, the adapters Grb-2/GADS, and the phosphatase PP2A, whereas CD152 binds to PI3 K and the phos-

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Abbreviations used in this paper: AICD, activation-induced cell death; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; dB-cAMP, dibutyryl cAMP; FasL, Fas ligand; tg, transgenic; PI, propidium iodide; PI3 K, phosphatidylinositol 3 kinase; TUNEL, TdT-mediated dUTP nick-end labeling.

phatases PP2A and SHP-2 (16–19). The coupling of CD28 and CD152 to different signaling mediators might indicate that CD152 does not simply counteract CD28-mediated costimulation.

During suboptimal activation of the T cell, CD152 engagement leads to impaired T cell activation and to G1 cell cycle arrest by inhibition of cyclin D3, CDK4, and CDK6 production (11–14, 20). Although CD28 and CD152 bind to the same ligands, B7.2 and B7.1, on APCs, CD152 binds with a 20–50-fold higher affinity. CD28 is constitutively expressed on CD4⁺ T cells, whereas CD152 is inducible reaching maximal surface levels 48 h after T cell stimulation (21). CD152 is transported from intracellular vesicles to the cell membrane, where it is expressed at the immunological synapse (22). This restricted regulation of CD152 trafficking and localization represents a major control point for its inhibitory function. It has been proposed that CD152 prevents some T cell clones with a high-affinity TCR by decreasing their competitive advantage over clones with a low-affinity TCR (22). Suggestive evidence and correlations have also shown that CD152 ligation in previously activated ConA blasts or α CD3-stimulated T cells might induce apoptosis to terminate the T cell response (23, 24).

After initial activation, naive CD4⁺ cells differentiate into Th1 and Th2 cells that secrete different cytokines. Interestingly, Th2 cells are substantially more resistant to AICD than Th1 cells (25, 26). Whether the expression of Fas or other apoptotic regulators is involved in this differential sensitivity after antigenic restimulation is controversial (27). Although Fas-mediated apoptosis is functional in CD152^{-/-} T cells in vivo (28), the role of CD152 in AICD of activated primary T cells that are prone to AICD remains unknown. Suggestive stainings for surface CD152, which were, however, only performed in long-term T cell clones, indicate that surface expression of CD152 might be present in Th2 rather than in Th1 cell clones (29). CD152 has also been proposed as an inhibitor of Th2 cell differentiation (30, 31). Owing to its low surface expression, detection of surface CD152 on primary T cells by conventional techniques has been difficult. Therefore, the role of CD152 in the response of differentiated primary Th1 and Th2 cells is almost entirely unknown.

Here, we investigated the functional role of CD152 on individual primary Th1 and Th2 cells using a novel sensitive immunofluorescent labeling technique. We show for the first time that, after antigenic restimulation, CD152 surface expression is induced at high frequencies in Th2 cells compared with Th1 cells. Interestingly, CD152 rendered activated T cells resistant to AICD, whereas inhibition of CD152 sensitized cells for apoptosis. We identified that apoptosis protection by CD152 was dependent on the suppression of the Fas system and mediated by PI3 K, which induced the up-regulation of Bcl-2 and the inactivation of FKHRL1, a transcription factor regulating FasL expression. Thus, CD152 regulates AICD by targeting PI3 K and, due to higher frequencies of CD152 surface expres-

sion in Th2 than in Th1 populations, induces resistance to AICD mainly in Th2 cells. This novel activity of CD152 could explain the resistance of Th2 cells to apoptosis and might be important for T cell homeostasis.

Materials and Methods

Mice. Mice transgenic (tg) for the DO11.10 α/β -TCR (OVA-specific TCR^{tg/tg}) on a BALB/c background (a gift from D.Y. Loh, Washington University School of Medicine, St. Louis, MO), CD152 knockout mice on a C57/B6 background (a gift from J.P. Allison, University of California at Berkeley, Berkeley, CA), and C57/B6 and BALB/c mice were bred under pathogen-free conditions in the animal facility of the Bundesgesundheitsamt in Berlin and used at the age of 5–7 wk. Offspring of CD152^{-/-} mice were used at the age of 3 wk and genotyped by PCR using the following oligonucleotide primers: CD152, 5'-TGGAGTCCTTCATAGTTAGG-3' and 5'-GCAAGATGGTGAGTGTGATGTT-3'; and Neo, 5'-CATAGTTAGTCTGTGATGC-3' and 5'-CGTCAAGAAGGCGATAGAAGGCG-3'.

Antibodies, Cytokines, and Reagents. The following antibodies against murine antigens were used: α CD152 (UC10-4F10-11; Becton Dickinson), α CD25 (BD Biosciences), α TCR^{tg} (KJ1-26.1), α FasL (MFL3; BD Biosciences), and α Fas (Jo2; BD Biosciences) in their respective forms of FITC, PE, and Cy5 conjugates. α CD28 (37.51), α CD3 (145-2C11), α CD4 (GK-1.5/4), α CD8 (196), α CD62L (MEL-14), α CD152 (UC10-4F10-11), control antibodies (560-31.1B9; provided by J.P. Allison), α IL-12 (C17-8.6), and α IFN- γ (AN18.17.24) were purified from hybridoma supernatants with protein G and controlled by HPLC and FACS[®] analysis. α CD152 Fab fragments were prepared with the Immunopure Fab preparation kit (Pierce Chemical Co.). The fragments were controlled by HPLC and by Ag-specific T cell stimulations. The α FasL Ab (3C82; BioCheck) was used at a concentration of 20 μ g/ml in neutralization experiments. Control antibodies such as IgG1 (R3-34), FITC-conjugated ham IgG2 (α KLH, HA4/8), PE-conjugated ham IgG, and annexin-V-PE conjugates as well as α IL-2 (JES-61A12) and α IL2R α (3C7) that were used at 20 μ g/ml were obtained from BD Biosciences. Recombinant IL-12 was purchased from R&D Systems and used at 10 ng/ml. IL-4 (supernatant of myeloid cell line P3X63 Ag.8.653 transfected with murine IL-4 cDNA) was added at an equivalent of 30 ng/ml to the cultures. IL-2 was purified from supernatants of X63.IL-2.6 cells. IL-2 and IFN- γ were detected in culture supernatants by ELISAs (R&D Systems) with a detection limit of 2 pg/ml. Dibutyryl cAMP (dB-cAMP), forskolin, wortmannin, and LY294002 were obtained from Sigma-Aldrich and used at 0.5 mM and 60, 1, and 30 μ M, respectively. L-mimosine (ICN Biomedicals) was used at 60 μ g/ml. Magnetic microbeads α CD4, α CD62L, α CD90, and α FITC multisort microbeads were purchased from Miltenyi Biotech. Sulfate polystyrene latex microspheres of 5 μ m \pm 0.1 μ m diameter were obtained from Interfacial Dynamics.

Cell Isolation. Isolation of naive CD62L^{high}CD4⁺ T cells from OVA-specific TCR^{tg/tg} mice was performed using two-parameter high-gradient magnetic cell separation (MACS) according to the manufacturer's instructions. CD4⁺ cells were isolated by positive selection on an AUTOMACS system to a purity of >99.3%, and CD62L^{high} CD4⁺ cells were positively selected on AUTOMACS to a purity of >99% as determined by flow cytometry. Isolated cells were stimulated with 1 μ g/ml OVA₃₂₃₋₃₃₉ peptide (Neosystem) and APCs (2 \times 10⁶ cells/ml) in RPMI 1640 medium con-

taining 10% fetal calf serum, 0.3 mg/ml glutamine, and 10 μ M 2-mercaptoethanol. CD90⁺ cell-depleted splenocytes from syngeneic BALB/c mice were used as APCs (1.5 \times 10⁶ cells/ml).

Differentiation of Primary Th1 and Th2 Cells. Naive (CD4⁺CD62L⁺) CD4 cells were differentiated in a primary stimulation into Th1 or Th2 cells in the presence of OVA₃₂₃₋₃₃₉ and T cell-depleted splenocytes as APCs. For Th1 polarization, IL-12 and α IL-4 were added to the cultures and for Th2 polarization, IL-4, α IL-12, and α IFN- γ were used during a primary stimulation period of 6 d. Cultures were split on day 3 after the onset of the primary stimulation. Dead cells were removed by Ficoll gradient centrifugation on day 6. Restimulation (secondary stimulation) was performed with OVA₃₂₃₋₃₃₉ and APCs. Polarities of Th1 and Th2 cells were routinely checked 3 d after the secondary Ag stimulation by mitogen stimulation with PMA/ionomycin for 6 h and incubation with brefeldin A for the last 2 h. To this end, intracellular IFN- γ and IL-4 were stained in fixed (2% formaldehyde) and permeabilized (0.5% saponin) cells and analyzed by FACS[®].

Cell Stimulation. CD4 T cells (10⁶ cells/ml) were stimulated at a ratio of 1:1 with Ab-coupled microspheres. Cross-linking of CD152 on CD4 cells was performed using latex microspheres coated with antibodies as described previously (14). In brief, 10⁷ microspheres/ml were suspended in PBS with 0.1 μ g/ml α CD3, 2 μ g/ml α CD28, α CD152, or a hamster control Ab (NF18, 2.9 μ g/ml) and incubated for 1.5 h at 37°C, followed by washing with PBS and blocking with 10% fetal calf serum. In some experiments, 10⁷ microspheres/ml were suspended in PBS with 0.5 μ g/ml α CD3, 1 μ g/ml α CD28, α CD152, or a hamster control Ab (NF18, 4.5 μ g/ml). In case of CD152 cross-linking in the presence of inhibitors, secondary Th2 cells restimulated with Ag and APCs for 48 h were used. After removal of dead cells, Th2 cells were sorted for CD4 expression and replated with Ab-coated microspheres with or without CD152 cross-linking in the presence of 50 U/ml IL-2 and the cell cycle inhibitors L-mimosine or dB-cAMP. IL-2 was added during replating to avoid apoptosis caused by growth factor deprivation or the addition of L-mimosine alone. For inhibition of PI3 K, Th2 cells were restimulated with plate-bound antibodies (2 μ g/ml of each α CD3 and α CD28) for 48 h. Cells were pretreated with wortmannin, LY294002, or vehicle controls for 30 min before they were replated with microspheres coated with 0.5 μ g/ml α CD3, 1 μ g/ml α CD28, 4.5 μ g/ml α CD152, or control antibodies in the presence of IL-2. For analyses of cells from CD152^{-/-} mice, splenocytes were stimulated with 1.5 μ g/ml Con A. After removal of dead cells, CD152^{-/-} cells were restimulated with 2 μ g/ml α CD3 and T cell-depleted APCs from syngeneic CD152^{+/+} mice.

Measurement of Cell Cycling and Apoptosis. Cell cycle progression was measured by labeling 10⁷ cells/ml T cells with 10 μ M 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) in PBS/0.1% BSA for 6 min at room temperature. The reaction was stopped by resuspending the cells in RPMI 1640 medium. To measure cell death, cells were stained with annexin-V-PE and propidium iodide (PI) according to the manufacturer's instructions to exclude early apoptotic and late apoptotic/dead cells, respectively. The frequency of nonapoptotic cells was taken into consideration for all experiments. Routinely, staining was controlled by calcium chelation with 2 mM EGTA. Apoptotic DNA fragmentation was measured by TdT-mediated dUTP nick-end labeling (TUNEL) staining using the FLOWTACS kit (R&D Systems). For measurement of caspase activation, cells were incubated for 20 min with the permeable substrate FITC-VAD-fmk (CaspACE; Promega) at a final concentration of 5 μ M, followed by subsequent flow cytometric analysis.

Four-color Cytometric Analysis of Bcl-2 and CD152 Surface Expression. Surface expression of CD152 was detected using magnetofluorescent liposomes (32). T cells were incubated with unconjugated hamster α CD152 Ab at 1 μ g/ml for 15 min at 4°C. Cells were washed twice, incubated with Cy5 dye-filled liposomes, and conjugated to α ham Fab fragments for 30 min at 4°C. Unbound liposomes were removed by washing the cells twice with PBS-BSA. The specificity of CD152 staining was controlled by isotype control Ab conjugated with Cy5-filled liposomes as well as by incubation of cells with Cy5-filled liposomes only. The expression of Bcl-2 was detected using a Cytofix/Cytoperm staining kit obtained from BD Biosciences (3F11-PE). Cytometric analyses were performed using a FACS-calibur[™] (Becton Dickinson) and CellQuest[™] software. Dead cells were excluded by forward and side scatter gating and PI staining in surface staining analyses.

Immunoblotting. Phosphorylation-induced inactivation of Forkhead transcription factor FKHRL1 was detected by immunoblot analysis as described previously (33). In brief, Th2 cells were cultured under the indicated conditions in the presence and absence of CD152 ligation and lysed after 24 h in sample buffer. Proteins (25 μ g/lane) were separated on a SDS-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane. Membranes were blocked for 1 h with 5% milk powder in TBS containing 50 mM NaF and immunoblotted for 1 h with a phosphorylation-specific polyclonal Ab recognizing phosphorylated Thr-32 of FKHRL1 (Santa Cruz Biotechnology, Inc.). Specificity of the Ab was controlled by incubation of the Ab with a FKHRL1 peptide before immunoblotting. Membranes were washed four times with TBS/0.02% Triton X-100 and incubated with a peroxidase-conjugated goat anti-rabbit Ab for 1 h. After extensive washing, the reaction was developed by enhanced chemiluminescent staining.

Results

CD152 Is Predominantly Expressed on the Surface of Th2 Cells. CD152 is not expressed on the surface of resting T cells and difficult to detect even after stimulation of T cells (22, 24, 34, 35). To analyze CD152 expression in different Th populations, we stimulated naive (CD4⁺CD62L⁺) OVA-specific CD4⁺ T cells in a primary stimulation with the peptide OVA₃₂₃₋₃₃₉ and congenic APCs using Th1 and Th2 culture conditions, respectively. On day 6, cells were restimulated with congenic APCs and Ag. Polarization of primary Th1 and Th2 cells resulted in a purity of >99.5% in both effector populations, as assessed by determining IFN- γ producers in the Th2 and IL-4 producers in the Th1 population after stimulation with PMA and ionomycin on day 3 of a secondary Ag-specific stimulation (Fig. 1 A). To unambiguously identify T cells expressing surface CD152, we used a staining technique based on CD152-specific immunofluorescent liposomes, which increased the detection sensitivity at least 1,000-fold (32). Expression of surface CD152 was evaluated at various time points after primary activation of naive T cells in Th1 or Th2 conditions and a subsequent secondary Ag stimulation (Fig. 1, B and C). Specific surface expression of CD152 was detected with similar frequencies in primary Th1 and Th2 cultures after primary stimulation and peaked after 48 h. Thereafter,

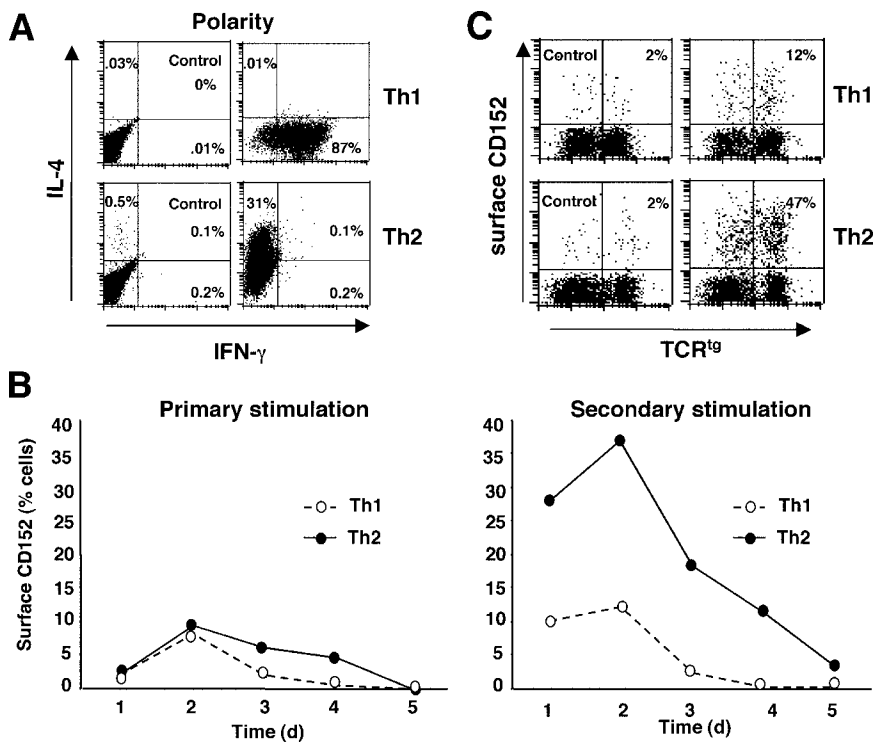


Figure 1. Detection of CD152 surface expression on Th1 and Th2 cells by enhanced fluorescence liposome staining. (A) Polarization of Th1 and Th2 cells. CD4⁺CD62L⁺ OVA-specific TCR^{lg} T cells were stimulated with 1 μ g/ml OVA₃₂₃₋₃₃₉ and T cell-depleted splenocytes. Th cell polarization was routinely determined by intracellular FACS[®] staining for IFN- γ and IL-4 on day 3 after the onset of an Ag-specific restimulation using PMA/ionomycin stimulation for 6 h (see Materials and Methods). Stainings with isotype-matched control antibodies are shown (left). (B) CD152 surface expression in differentiating and established Th1 and Th2 cells. The percentage of surface CD152-expressing CD4⁺ cells was detected by staining with an α CD152 Ab and α hamIgG-specific liposomes. Cell analysis was performed at different time points after a primary Ag-specific stimulation of naive T cells in their Th1 or Th2 milieu as indicated, followed by a secondary Ag stimulation of the established Th1 and Th2 cells. The specificity of the stainings was controlled with isotype-matched control antibodies. Indicated frequencies are values subtracted from background staining ranging from 0.5 to 2%. Surface CD152⁺ TCR^{neg} cells are CD19⁺ B cells (analysis not depicted). (C) Representative data of CD152 staining with the enhanced liposome FACS[®] technique showing surface CD152⁺ cells 48 h

after secondary stimulation. The indicated values show the frequencies of surface CD152⁺-expressing cells among all TCR^{lg} CD4⁺ cells. Stainings with isotype-matched control antibodies are shown (left). One out of five similar experiments is shown.

the frequency of surface CD152-expressing cells declined in Th2 cultures until day 5, whereas surface CD152-expressing Th1 cells were hardly detectable (1%) 3 d after stimulation. After secondary stimulation, >40% of the Th2 cells expressed CD152 and only ~10% of the Th1 cells displayed CD152 on their surface 48 h after restimulation (Fig. 1, B and C). Thus, both primary Th1 and Th2 cells are able to express surface CD152, but CD152 is expressed at a four- to fivefold higher frequency in Th2 cells, indicating that CD152 might play a more prominent role in Th2 than in Th1 cells.

Inactivation of CD152 Induces Susceptibility to AICD Mainly in Th2 Cells. To examine the role of CD152 during AICD, serological inactivation of CD152 was performed by adding neutralizing α CD152 Fab fragments to Th1 and Th2 cells during primary and secondary stimulations. Non-apoptotic cells were evaluated after different times after restimulation using double staining with PI and annexin-V. PI was used to identify dead and late apoptotic cells. Annexin-V stains phosphatidylserine in a Ca²⁺-dependent way, which is exposed at early stages of apoptosis. The specificity of the staining was controlled by chelating Ca²⁺ with EGTA demonstrating no unspecific annexin-V staining (unpublished data). After restimulation of Ag-experienced cells for 6 d, only 36% of the Th1 cells remained viable, whereas 57% of the Th2 cells survived after the onset of secondary Ag stimulation (Fig. 2 A). Serological blockade of CD152 by α CD152 Fab fragments led to reduced frequencies of surviving cells and, interestingly, only low

numbers of Th1 (22%) and Th2 cells (29%) remained viable (Fig. 2 A). The small difference in cell survival of 5–10% between activated, CD152-blocked Th1 and Th2 cells was highly reproducible. In principle, similar effects of CD152 in Th1 and Th2 cells were found after 3 d of restimulation, although cell death was clearly less pronounced at this earlier time point (Fig. 2 A). A survival-promoting effect of CD152 was confirmed when cell death was analyzed by measurement of DNA fragmentation using TUNEL staining or by the flow cytometric detection of caspase activation (Fig. 2, B and C). Thus, CD152 blockade enhanced the susceptibility to apoptosis in differentiated Th cells, which was more pronounced in Th2 cells compared with Th1 cells.

We further used cells from mice harboring a genetic inactivation of CD152. After polyclonal stimulation of splenocytes with ConA, substantially more CD152^{-/-} splenocytes were killed on day 5 compared with wild-type cells (Fig. 3 A). Excluding early apoptotic (annexin-V⁺, PI⁻) and dead (PI⁺) T cells, ~30% more of the wild-type cells than CD152^{-/-} cells remained viable. A comparable difference between CD152^{-/-} and CD152-expressing cells was found, when dead cells were removed and CD4⁺ T cells were restimulated with α CD3 for a further 48 h (Fig. 3 B). Enhanced apoptosis of CD152^{-/-} cells was also confirmed by TUNEL staining (Fig. 3 C). Thus, although it must be considered that CD152^{-/-} cells differ even after activation from wild-type cells, in the presence of functional CD152, less apoptosis occurred after primary and secondary cell

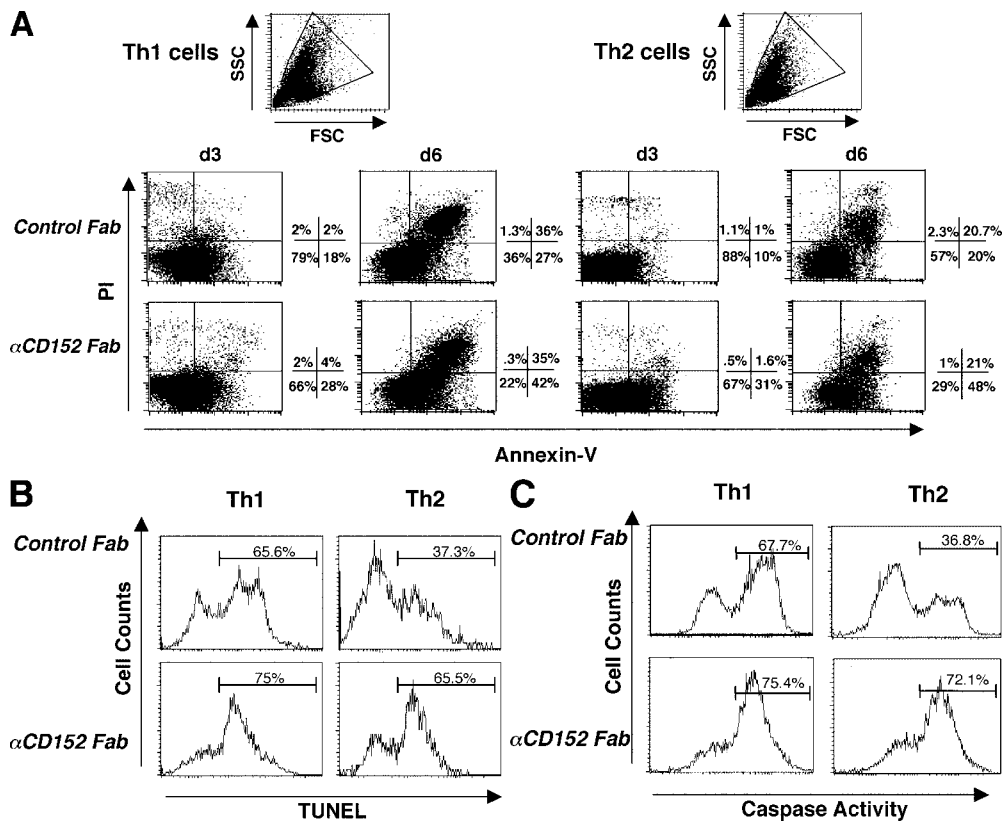


Figure 2. Th1 cells are more sensitive to AICD than Th2 cells, and genetic or serological blockade of CD152 further enhances apoptosis sensitivity. (A) AICD in Th1 and Th2 cells and the effect of CD152 inhibition. CD4⁺CD62L⁺TCR^{tg/tg} splenocytes were stimulated with OVA₃₂₃₋₃₃₉ and T cell-depleted APCs for 1 wk under their respective Th1 or Th2 culture conditions. Polarized Th1 and Th2 cells were restimulated in the presence of 200 μ g/ml of neutralizing α CD152 Fab or hamster control Fab fragments. 3 and 6 d later, cultures were stained with annexin-V and PI to detect early apoptotic cells and late apoptotic/dead cells, respectively. The data shown represent one out of three similar experiments. Stainings were also performed on day 4 or 5 after restimulation with similar results (not depicted). Gatings in forward scatter versus side scatter dot plots are depicted (top). (B and C) Effect of CD152 blockade on apoptotic DNA fragmentation and caspase activation. Th1 and Th2 cells were stimulated and restimulated in the presence of

α CD152 Fab or hamster control Fab fragments as described in A. On day 5, DNA fragmentation (B) was measured by TUNEL staining and caspase activation (C) by labeling of cells with the fluorogenic caspase substrate FITC-VAD-fmk, followed by flow cytometric analysis. The data represent the results from one out of two similar experiments. Similar results were obtained from stainings on day 6 after restimulation (not depicted).

stimulation, which supports the idea that CD152 could be involved in apoptosis resistance.

CD152-induced Apoptosis Resistance in Preactivated T Cells Is Independent of IFN- γ and IL-2. Because CD152 blockade may lead to enhanced IFN- γ production (35), which has been implicated in AICD (36), IFN- γ levels were analyzed in the cultures using a sensitive ELISA. After serological inactivation of CD152, enhanced IFN- γ production was detectable in supernatants of Th1 but not Th2 popula-

tions (Fig. 4 A). Thus, at least in Th2 cells, enhanced IFN- γ production was unlikely to be responsible for the reduced survival in the absence of CD152 engagement.

Blockade of CD152 during antigenic stimulation of primary T cells also enhances IL-2 synthesis, which could affect apoptosis induction (26, 37, 38). After neutralization of CD152 with Fab fragments, we found enhanced IL-2 levels in supernatants of Th1 cells, but also Th2 cells, increasing from 0.01 to 2 ng/ml (unpublished data). The en-

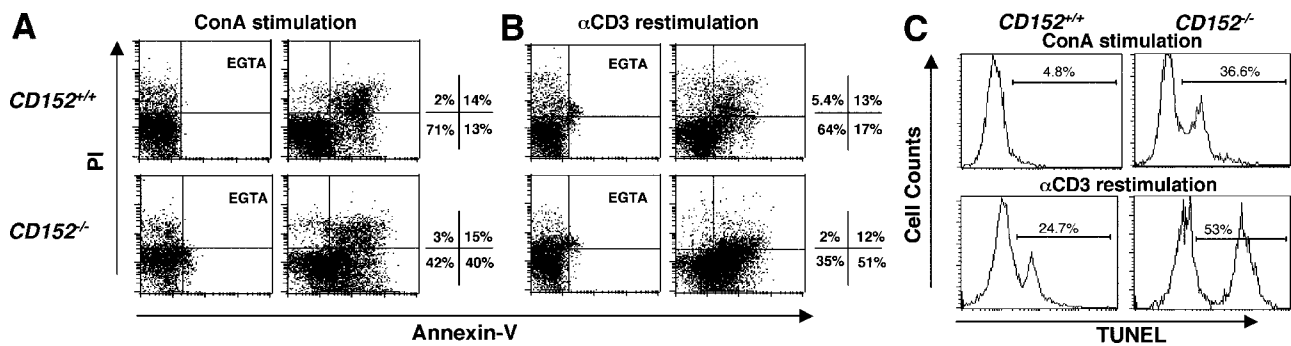


Figure 3. CD152^{-/-} T cells are more susceptible to AICD. Splenocytes from CD152^{-/-} and CD152^{+/+} mice were stimulated with 1.5 μ g/ml ConA. Annexin-V/PI stainings were performed 96 h after the onset of a primary stimulation with 2 μ g/ml α CD3, which was performed after removal of dead cells for a further 48 h (B). The frequency of viable cells is indicated together with an annexin-V staining control performed in the presence of EGTA (left). (C) Increased AICD in CD152^{-/-} T cells compared with WT cells was also observed when apoptosis was measured by TUNEL staining after primary and secondary stimulation with ConA and α CD3, respectively.

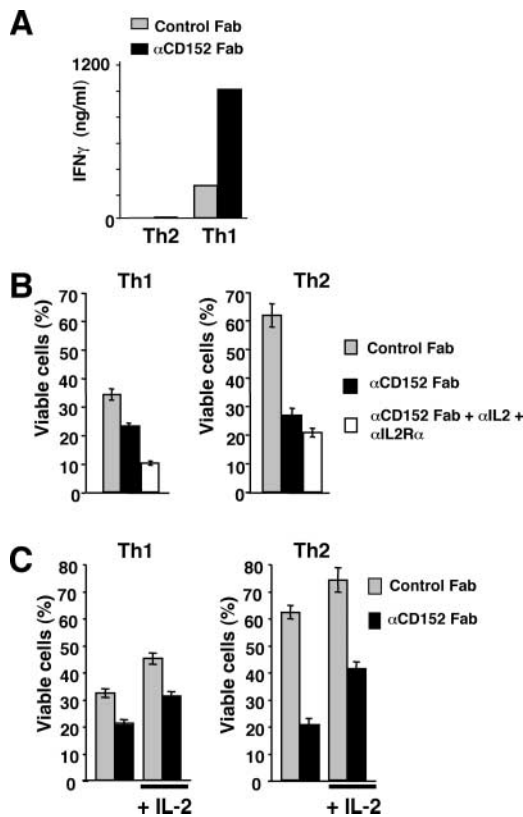


Figure 4. CD152 blockade enhances AICD in both T cell subsets independently of IFN- γ and IL-2 induction. (A) Effect of CD152 blockade on IFN- γ production in Th1 and Th2 cells. CD4⁺CD62L⁺ TCR^{tg/tg} splenocytes were stimulated in the presence and absence of 200 μ g/ml of α CD152 or hamster control Fab fragments. Supernatants were collected on day 6 after stimulation, and the amount of IFN- γ was detected using a sensitive ELISA with a detection limit of <2 pg/ml. (B) The effect of neutralizing α IL-2 and α IL-2R α antibodies. Polarized Th1 and Th2 cells were restimulated as described in Fig. 2 A in the presence of 200 μ g/ml of neutralizing α CD152 Fab (black bars) or hamster control Fab fragments (gray bars). Some cultures (white bars) additionally received 20 μ g/ml of each α IL-2 and α IL-2R α to inhibit IL-2 signaling. 6 d after secondary stimulation, cells were stained with annexin-V and PI to determine the number of viable cells. The data represent one out of three similar experiments. Stainings were also done on days 4 and 5 with similar results (not depicted). (C) Effect of exogenous IL-2. Th1 and Th2 cells, generated and restimulated as described in Fig. 2 A, were treated with 50 U/ml of exogenous IL-2. Black and gray bars show cells incubated with solely α CD152 Fabs or ham IgG Fabs (200 μ g/ml), respectively. The data represent the results from one out of two similar experiments.

hanced apoptosis in CD152-blocked Th2 cells was not reversed by inhibition of IL-2 signaling (Fig. 4 B). Furthermore, addition of exogenous IL-2 even slightly enhanced survival of Th1 and Th2 cells (Fig. 4 C), but the CD152 effect was still clearly detectable, which indicated that the susceptibility to apoptosis conferred by CD152 blockade was not due to effects of IL-2.

CD152 Induces Resistance in Activated Th2 Cells Independently of Cell Cycle Arrest. In the next experiments, we asked whether CD152-mediated protection against AICD was a consequence of altered cell cycle progression or a direct effect of CD152. Because CD152 exerts its main function at its peak expression, we monitored its effect on an

equally activated CD4 T cell population 48 h after the onset of cell stimulation. To avoid potential effects of IFN- γ on AICD (36), we focused on Th2 cells and induced AICD in the presence of IL-2 as described previously (8). Th2 cells were restimulated for 48 h with congenic APCs and OVA, and Ab-coupled microspheres were used to cross-link CD152 in combination with CD3 and CD28 coligation. Individual nonapoptotic cells were determined 3 d (Fig. 5, top) and 4 d later (not depicted) with similar results. 21% more Th2 cells remained resistant to AICD after stimulation with α CD3, α CD28, and α CD152 compared with CD3/CD28-stimulated T cells. Thus, CD152 engagement of Th2 cells triggered enhanced resistance to AICD, which was even observed in the presence of α CD28.

Another hallmark of CD152 signaling is the induction of G1 cell cycle arrest in lymphocytes (14). Because cell cycle arrest might induce resistance to apoptosis (39), we performed similar experiments in the presence of L-mimosine or dB-cAMP, two drugs inducing G1 arrest (Fig. 5). As reported previously (40), measurement of CFSE staining revealed that both drugs arrested cell cycle progression (unpublished data), and further analysis was only performed in nonproliferating cells. In the presence of IL-2, L-mimosine and dB-cAMP caused a moderate increase in the viability of cells that had been restimulated solely with α CD3 and α CD28. Nevertheless, CD152 ligation induced a comparable increase in resistance against AICD (~20%), regardless of whether mimosine or dB-cAMP was used. Replating of the L-mimosine-treated cells without IL-2 caused dramatic cell death that could not be analyzed. CD152 ligation of dB-cAMP-treated cells was protective also without IL-2, although to a lesser extent than in the presence of IL-2 (unpublished data). Thus, CD152-induced resistance of Th2 cells against apoptosis was not caused indirectly by reduced IL-2 production or G1 arrest, but rather by a direct CD152 effect.

CD152 Regulates Apoptosis in a Fas/FasL-dependent Manner. Because AICD is mainly mediated by the Fas system, we investigated whether CD152 interfered with Fas/FasL interaction. Th1 and Th2 effectors were generated under our standard conditions by stimulation of naive T cells with Ag plus APCs in the presence or absence of neutralizing α CD152 Fab fragments. Upon Ag-specific restimulation, Fas/FasL interaction was blocked with the FasL-specific Ab 3C82. Ag-stimulated Th1 cells showed only a 14% increase in the number of cells undergoing AICD after CD152 inhibition, whereas the blockage of CD152 in Th2 cells resulted in ~30% more cell death 6 d after restimulation (Fig. 6 A). Analysis on day 5 gave similar results (unpublished data). Prevention of Fas signaling by α FasL reversed the sensitizing effect induced by CD152 blockade completely, and also substantially inhibited AICD. Despite different frequencies of Th1 and Th2 cells undergoing AICD, in both populations the CD152-mediated effect was dependent on the Fas/FasL pathway.

Inactivation of CD152 during Ag Stimulation of Th2 Cells Up-regulates FasL. Previous data on the expression of Fas and FasL in Th1 and Th2 cells are controversial, due to the

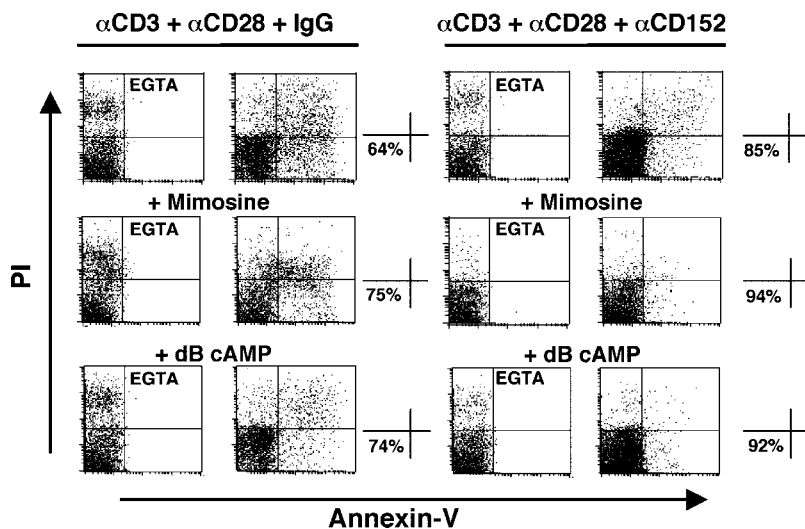


Figure 5. Cross-linking of CD152 on Th2 cells inhibits AICD independently of cell cycle arrest. CD4⁺CD62L⁺ TCR^{tg/tg} T cells were stimulated with OVA₃₂₃₋₃₃₉ and T cell-depleted splenocytes under Th2 conditions. On day 2 after Ag-specific restimulation, CFSE-labeled CD4 cells were replated either with microspheres coated with αCD3, αCD28, and hamIgG (left) or with microspheres coated with αCD3 and αCD28 plus αCD152 (right). Cells were cultured in the presence or absence of either dB-cAMP or L-mimosine, which induced a G1 arrest. On day 3 after restimulation with the respective antibodies coupled to microspheres, annexin-V/PI stainings were performed, and the number of nonapoptotic cells was determined. An annexin-V staining control performed in the presence of EGTA is shown (left). The data represent results from two experiments with similar results.

use of unspecific antibodies, insufficient T cell polarization, or the use of different Th effector cell clones and cell lines. Therefore, we analyzed Fas and FasL expression using polarized, pure Th1 and Th2 cells upon restimulation with APCs and Ag, which allowed physiological TCR signaling in the presence of dynamic B7 expression. In unstimulated cells, FasL was not detectable and also expression of Fas was low. Both Th cell populations showed increased surface expression of Fas and FasL after the onset of secondary stimulation, but at different densities (Fig. 6 B). Fas was strongly expressed 8 h after restimulation and steadily declined thereafter. In general, Th1 cells showed a higher density of Fas molecules per cell compared with Th2 cells over the entire time course. Serological inactivation of CD152 enhanced the expression of Fas in both populations (Fig. 6 B). Also FasL was expressed at lower levels in Th2 cells. Blockade of CD152 during stimulation substantially increased FasL expression in Th2 cells, whereas it had no enhancing effect in Th1 cells. Thus, CD152 modulated expression of Fas in Th1 and Th2 cells, whereas FasL levels were regulated by CD152 mainly in Th2 cells. Consistently, FasL was also expressed to substantially higher levels in CD152^{-/-} T cells compared with wild-type cells after restimulation with APCs and αCD3 (Fig. 6 C). Therefore, these results suggest that down-regulation of FasL expression in Th2 cells might contribute to CD152-mediated resistance against AICD.

CD152-induced Resistance to Apoptosis Is Dependent on PI3 Kinase. CD152 binds to and activates PI3 K and, therefore, we examined whether PI3 K was necessary for apoptosis resistance induced by CD152. Th2 cells were stimulated for 48 h with plate-bound αCD3 and αCD28, before CD152 was cross-linked together with αCD3 and αCD28 coupled to microspheres. Because inhibition of PI3 K might prevent IL-2 production (41) and replating could cause growth factor deprivation leading to cell death, cells were supplied with 50 U/ml of exogenous IL-2. When cells were treated with the PI3 K inhibitors LY294002 or wortmannin, inhibition of PI3 K activity completely pre-

vented the survival effect of CD152 ligation. The survival effect of CD152 ligation was attenuated by LY294002 also in the absence of IL-2 addition (unpublished data). Thus, T cells restimulated with αCD3/αCD28 plus αCD152 in the presence of nonapoptotic cells as T cells stimulated with only αCD3/αCD28 (Fig. 7 A). The inhibitors themselves were not cytotoxic as determined by incubation of nonstimulated T cells for 2 d (Fig. 7 A, inset). Also, LY294002 did not affect the increased cell death induced by neutralizing αCD152, but only reduced the number of viable Th2 cells in control cultures, where CD152 signaling was still functional (Fig. 7 B).

PI3 K-mediated generation of the second messenger PIP₃ leads to activation of the survival kinase Akt (42). Akt phosphorylates and thereby inhibits several proapoptotic factors, including the Forkhead transcription factor FKHRL1, which is involved in transactivation of the FasL promoter (43) and, therefore, might also contribute to CD152-mediated down-regulation of FasL. Therefore, phosphorylated and inactivated FKHRL1 was analyzed in Ag-primed Th2 cells using a phosphorylation-specific Ab. Unstimulated cells revealed only low amounts of phosphorylated FKHRL1, whereas restimulation of Th2 cells resulted in a strong increase of FKHRL1 phosphorylation (Fig. 7 C, left). Most importantly, when cells were restimulated and CD152 was blocked by αCD152 Fab, a significant decrease of FKHRL1 phosphorylation was observed. The specificity of the analysis was verified by competition with FKHRL1 peptide as well as by experiments in the presence of LY294002, both of which resulted in disappearance of the phosphospecific protein band (Fig. 7 C, right).

Another consequence of PI3 K-triggered Akt activation is induction of Bcl-2 expression, which was recently observed in αCD3-stimulated T cells (44). Inhibition of CD152 strongly reduced the amounts of Bcl-2 in activated Th2 cells upon restimulation with Ag and APCs, which was evident in particular on day 1 after restimulation (Fig. 8 A). In addition, experiments with CD152 cross-linking

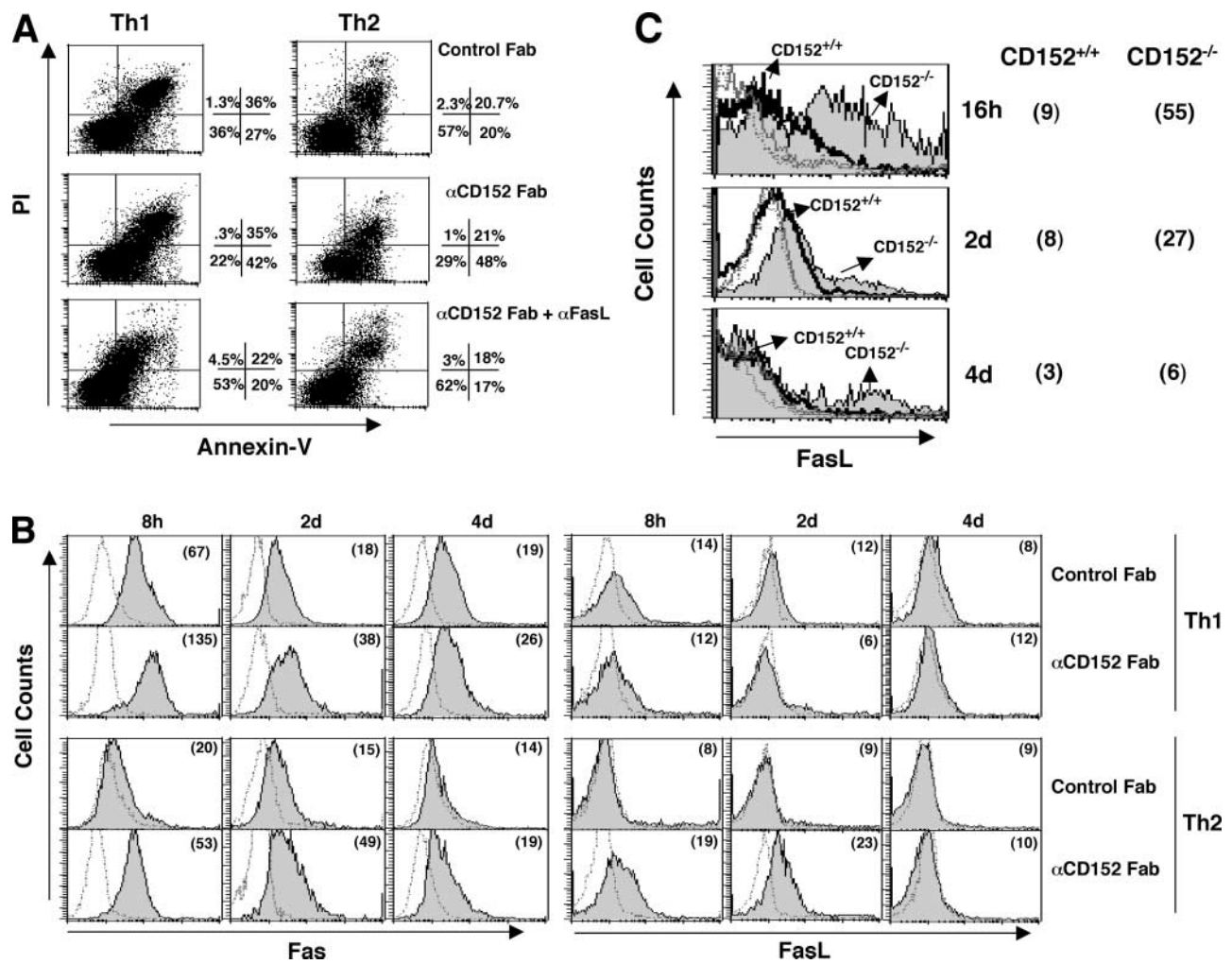


Figure 6. Fas/FasL interaction is involved in the apoptosis-sensitizing effect of CD152 inhibition. (A) FasL-neutralizing antibodies protect against cell death induced by α CD152 Fab fragments. Polarized Th1 and Th2 cells were restimulated as described in Fig. 2 A with or without blocking of CD152. Control ham IgG Fabs and α CD152 Fabs were used at 200 μ g/ml in primary and secondary stimulations. FasL signaling was blocked during secondary Ag-specific stimulation by using the neutralizing α murine FasL moAb 3C82 (20 μ g/ml). After 6 d of secondary stimulation, cell viability was assessed by annexin-V and PI staining. Data from one out of three similar experiments are shown. Measurements were also performed on day 5 with similar results (not depicted). (B) Blockade of CD152 up-regulates Fas and FasL expression. Fas and FasL molecules were stained on the surface of Th1 and Th2 cells at different time points after secondary stimulation with or without blocking of CD152. All stainings were controlled using isotype-matched control antibodies (dotted lines). The histograms from one out of three similar experiments are shown. Geometric means of fluorescence intensity are indicated in parentheses. (C) FasL is up-regulated in activated CD152^{-/-} T cells. Stainings were performed on CD4⁺ cells from CD152^{-/-} or CD152^{+/+} mice at the indicated time points after the onset of the secondary stimulation. Dotted gray lines show stainings with isotype-matched control antibodies. Geometric means of FasL expression in CD152^{-/-} and WT T cells are indicated in parentheses.

were performed. In primary Th2 cells as well as in Th2 cells preactivated for 48 h and restimulated with α CD3/ α CD28-coupled microspheres, CD152 activation resulted in increased Bcl-2 expression (Fig. 8 B). The induction of Bcl-2 expression by CD152 was completely reversed by LY294002. Thus, Bcl-2 expression was regulated by CD152-mediated PI3 K activity during CD3/CD28 stimulation on primary activated Th2 cells. Altogether, our data show that CD152 is predominantly expressed on activated Th2 cells and contributes to enhanced resistance of activated Th2 cells against AICD by down-regulating Fas/FasL expression, an event closely associated with CD152/PI3 K-mediated up-regulation of Bcl-2.

Discussion

Our results identify enhanced CD152 surface expression and signaling in Th2 cells as an important functional difference between Th1 and Th2 cells. CD152 cross-linking in the Th2 population revealed that a fraction of cells was rescued from apoptosis corresponding to the number of Th2 cells expressing surface CD152. The heterogeneity of CD152 expression in individual primary T cells has not been analyzed thus far. Our paper shows clearly that Th1 and Th2 cells differ in the frequency of CD152 expression and, therefore, are remarkably different in their susceptibility to AICD. Although more Th1 effectors than Th2 cells underwent Fas/FasL-mediated AICD, mainly Th2 effectors

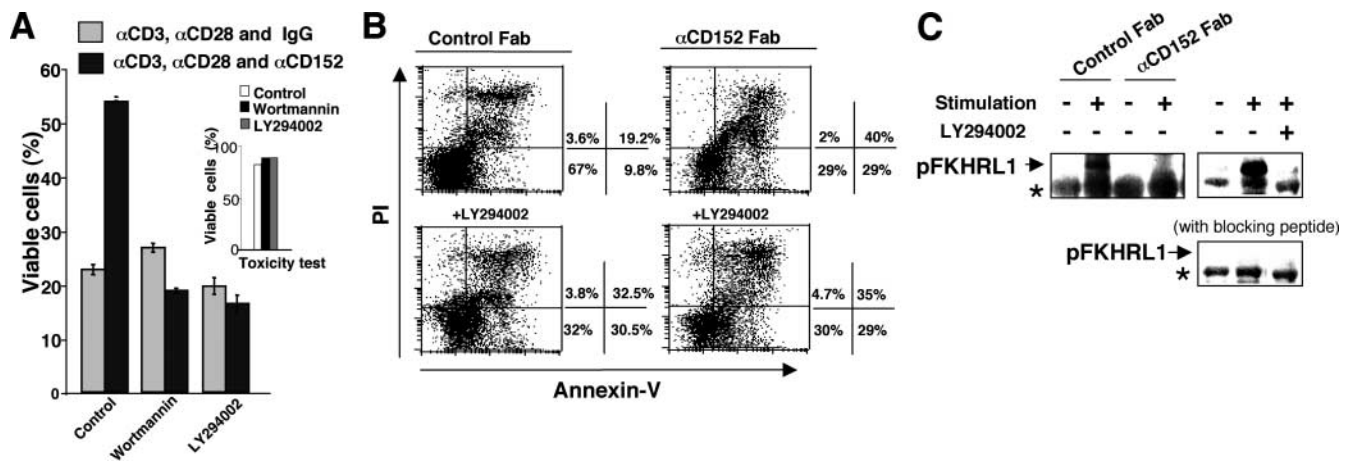


Figure 7. CD152-mediated inhibition of apoptosis requires a functional PI3 K pathway. (A) Inhibition of PI3 K prevents the protecting effect triggered by CD152. Th2 cells were generated as described in Fig. 2 A, and on day 2 after secondary stimulation, they were replated with αCD3/αCD28/IgG- or αCD3/αCD28/αCD152-coated microspheres in the presence or absence of the PI3 K inhibitors wortmannin or LY294002. 4 d after replating cell viability was determined by annexin-V/PI staining. The inset in A shows a toxicity control in which unstimulated cells were treated with the PI3 K inhibitors for 2 d. (B) Polarized Th2 cells were restimulated as described in Fig. 2 A with or without blocking of CD152. To inhibit PI3 K, cultures were incubated with LY294002 during secondary stimulation. After 6 d of secondary stimulation, cell viability was assessed by annexin-V/PI staining. (C) Inhibition of CD152 signaling impairs Ag/APC-induced phosphorylation of Forkhead transcription factor FKHRL1 (left). Th2 cells were generated and restimulated as aforementioned. After 24 h, FKHRL1 phosphorylation was determined in whole cell lysates by immunoblotting using a phosphorylation-specific FKHRL1 Ab. The FKHRL1-specific protein band is indicated by an arrow, and the asterisks denote a nonspecific protein band that served as a control for equal protein loading. Additional controls, including incubation of cells in the presence of LY294002 or competition of the Ab with the immunizing FKHRL1 peptide confirmed the specificity of the immunoblot analysis (right). The data shown represent one out of two to four experiments.

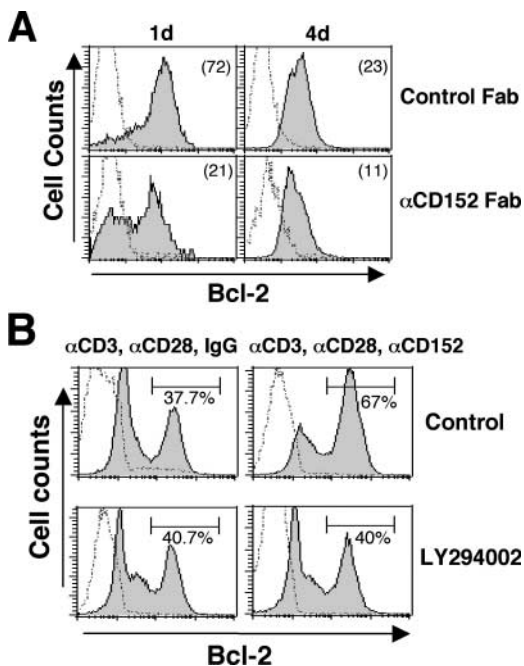


Figure 8. Inhibition of CD152 signaling in Th2 cells down-regulates Bcl-2 expression. (A) Th2 cells were generated and restimulated as described in Fig. 2 A. At the indicated days after the onset of Ag-specific secondary stimulation, Th2 cells were fixed and stained for Bcl-2 (solid lines). The geometric means of Bcl-2 expression are indicated in parentheses. The dotted lines show the staining obtained with an isotype-matched control Ab. (B) CD152 ligation up-regulates Bcl-2 expression by a PI3 K-dependent pathway. Th2 cells were restimulated and treated in the presence or absence of LY294002 as described in Fig. 7. On day 6 after replating, cells were fixed and stained with Bcl-2-specific (solid lines) or isotype-matched control antibodies (dotted lines). The percent-

age of cells with up-regulated Bcl-2 expression is indicated. The data shown represent one out of two to four experiments.

could escape this fate through CD152 engagement. We demonstrate that antiapoptotic consequences of CD152 ligation are associated with up-regulation of Bcl-2, phosphorylation of FKHRL1, and reduced Fas/FasL expression. Expression of CD152 on the surface of primary T cells is difficult to detect using conventional cytometric and microscopic techniques (14). Studies on CD152 expression have been generally performed intracellularly without discriminating CD152 surface expression, and mostly in Th1 and Th2 cell clones or cell lines. The sensitive liposome-based staining technique used in this work allowed the detection of as few as 100–200 surface molecules per cell. We show through CD152-specific liposome staining that only a fraction of primary cells, activated in an appropriate Th1 or Th2 milieu, express surface CD152. The induction of CD152 expression was rather similar during primary responses of naive T cells activated under Th1 or Th2 conditions. However, upon secondary stimulation a pronounced increase of surface CD152-expressing cells was consistently found in particular in the Th2 population. Hence, the differentiation history of an activated naive T cell apparently correlates with CD152 surface expression upon reencountering an Ag. The fact that activated Th1 and Th2 cells differentially express CD152 during an immune response was reflected by a functional analysis as follows. When CD152 was ligated at the time point of maximal expression after the onset of T cell activation, a substantial fraction of the Th2 cells remained viable.

age of cells with up-regulated Bcl-2 expression is indicated. The data shown represent one out of two to four experiments.

Our paper is the first demonstrating that CD152 ligation and genetic or serological ablation of CD152 signaling affects AICD in different primary T cell subsets. At first view, our data would concur with the observation that transfection of CD152 could inhibit apoptosis in a T cell hybridoma cell line (45). However, in this work, the hybridoma cells were not preactivated before CD152 ligation. As CD152 cross-linking at the beginning of stimulation leads to a general shutdown of T cell activation associated with impaired up-regulation of activation molecules, inducible FasL expression will be also abolished. Thus, it is very likely that in the reported experiments, T cell activation and not AICD was inhibited by CD152.

AICD of already activated T cells is mainly mediated by the expression of Fas/FasL. We provide several lines of evidence that CD152 affects AICD mainly by impinging on the Fas pathway. First, sensitization of activated T cells to AICD induced by blockade of CD152 was completely abolished by FasL antibodies. Second, CD152 ligation led to a strong inhibition of FasL in Th2 cells, whereas decreased Fas expression was seen in both effector cell populations. The inhibition of Fas/FasL expression and a consequently reduced AICD were observed at various time points after antigenic restimulation, suggesting that CD152 did not simply delay the induction of Fas and FasL expression. In accordance with this concept is our finding that FasL was also strongly up-regulated during the onset of activation in CD152^{-/-} T cells. It might be argued that up-regulation of Fas/FasL in CD152^{-/-} T cells is discrepant to the lymphoproliferative disease observed in CD152^{-/-} mice. However, CD152 deficiency also strongly affects cell cycle control and proliferative events that might overcome the up-regulation of proapoptotic mediators. Thus, experiments with CD152^{-/-} cells, which might be already in activated state *ex vivo*, must certainly be interpreted with caution. Data with Rag2^{-/-} CD152^{-/-} T cells suggested that CD152, even though being involved in tolerance induction, does not affect AICD (46). Whether these differences to our results are caused by differences in the activation history of cells, effects of CD152 on cell cycle progression or different experimental systems remains to be investigated. Nevertheless, and more in line with the present results, it has been shown that Rag2^{-/-} mice reconstituted with a mixture of CD152^{+/+} and CD152^{-/-} T cells do not show enhanced, but even reduced total numbers of lymphocytes after infection with LCMV and *Leishmania major* (47). This surprising observation does not only indicate that CD152 affects T cell survival in a nonautonomous fashion but eventually also via modulating the expression of a proapoptotic factor (47).

Our results identified PI3 K as the presumably crucial mediator of CD152-induced resistance against AICD. It has been demonstrated that Akt, a target of PI3 K, is an essential signaling intermediate in this antiapoptotic pathway. We show that cross-linking of CD152 during T cell activation induces Bcl-2, which might contribute to the early protection of T cells. PI3 K, either directly or through the

Akt pathway, is involved in the up-regulation of Bcl-2 after CD152 ligation. The PI3 K/Akt survival pathway has been recently reported to be required for the up-regulation of Bcl-2 by IL-2 (45). As CD152 inhibits IL-2 transcription during T cell activation, and Bcl-2 is up-regulated by IL-2, this effect is probably independent of IL-2 and directly due to CD152 signaling. PI3 K/Akt also inhibits the Forkhead factor FKHRL1 involved in FasL transcriptional regulation. Akt phosphorylates FKHRL1 leading to its retention in the cytoplasm (42). Our results demonstrate that antigenic restimulation of Th2 cells results in phosphorylation of FKHRL1, which was significantly impaired when CD152 signaling was blocked by α CD152 Fab. The dephosphorylation of FKHRL1 induced by CD152 inhibition should promote nuclear translocation and activation of target genes such as FasL. It is interesting to note that suppression of Akt has been recently found to induce FasL expression in smooth muscle cells (48).

In most analyses, only suppressive functions and down-regulation of gene expression have been described for CD152. Apart from TGF β , whose role in CD152 function is obscure, our data show for the first time that CD152 engagement is able to directly induce protein expression (e.g., of Bcl-2). The reason why most studies missed such an effect lies presumably in the fact that CD152 function has been generally examined after CD152 cross-linking at the onset of T cell responses, which will fully prevent T cell activation. Our results suggest that when CD152 is maximally expressed at the cell surface, CD152 might have more complex functions and actively determines a T effector cell's fate.

According to our data, T cells would be activated and primed to die unless they up-regulate surface CD152, which blocks AICD. As only a fraction of cells up-regulate surface CD152, and a similar fraction of cells responds to CD152 cross-linking, we assume that only those cells that express sufficient surface CD152 and receive appropriate additional signals will become resistant of apoptosis. That this restricted population being maintained after the T cell response fades away might be important to create the memory for the adaptive immune response. Although Bcl-2, a T cell memory marker (49, 50), is up-regulated by CD152 in a fraction of CD4 cells, future studies are needed to clarify the fate of them and their role in contribution to the memory cell compartment.

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References

1. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation dis-

- order in mice explained by defects in Fas antigen that mediates apoptosis. *Nature*. 356:314–317.
2. Lynch, D.H., M.L. Watson, M.R. Alderson, P.R. Baum, R.E. Miller, T. Tough, M. Gibson, T. Davissmith, C.A. Smith, K. Hunter, et al. 1994. The mouse Fas-ligand gene is mutated in *gld* mice and is part of a TNF family gene cluster. *Immunity*. 1:131–136.
 3. Lenardo, M., K.M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocyte apoptosis-immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* 17:221–253.
 4. Pinkoski, M.J., and D.R. Green. 2002. Lymphocyte apoptosis: refining the paths to perdition. *Curr. Opin. Hematol.* 9:43–49.
 5. Datta, S.R., A. Brunet, and M.E. Greenberg. 1999. Cellular survival: a play in three Acts. *Genes Dev.* 13:2905–2927.
 6. Vaux, D.L., and R.A. Flavell. 2000. Apoptosis genes and autoimmunity. *Curr. Opin. Immunol.* 12:719–724.
 7. Van Parijs, L., D.A. Peterson, and A.K. Abbas. 1998. The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. *Immunity*. 8:265–274.
 8. Van Parijs, L., A. Biuckians, and A.K. Abbas. 1998. Functional roles of Fas and Bcl-2-regulated apoptosis of T lymphocytes. *J. Immunol.* 160:2065–2071.
 9. Kirchhoff, S., W.W. Muller, M. Li-Weber, and P.H. Krammer. 2000. Up-regulation of c-FLIPshort and reduction of activation-induced cell death in CD28-costimulated human T cells. *Eur. J. Immunol.* 30:2765–2774.
 10. Kaech, S.M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell*. 111:837–851.
 11. Brunner, M.C. 1999. Costimulatory molecules and modulation. *The Immunologist*. 7:9–12.
 12. Chambers, C.A., M.F. Krummel, B. Boitel, A. Hurwitz, T.J. Sullivan, S. Fournier, D. Cassell, M. Brunner, and J.P. Allison. 1996. The role of CTLA-4 in the regulation and initiation of T-cell responses. *Immunol. Rev.* 153:27–46.
 13. Egen, J.G., M.S. Kuhns, and J.P. Allison. 2002. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat. Immunol.* 3:611–618.
 14. Brunner, M.C., C.A. Chambers, F.K. Chan, J. Hanke, A. Winoto, and J.P. Allison. 1999. CTLA-4-mediated inhibition of early events of T cell proliferation. *J. Immunol.* 162:5813–5820.
 15. Blair, P.J., J.L. Riley, B.L. Levine, K.P. Lee, N. Craighead, T. Francomano, S.J. Perfetto, G.S. Gray, B.M. Carreno, and C.H. June. 1998. CTLA-4 ligation delivers a unique signal to resting human CD4 T cells that inhibits interleukin-2 secretion but allows Bcl-X(L) induction. *J. Immunol.* 160:12–15.
 16. Prasad, K.V., Y.C. Cai, M. Raab, B. Duckworth, L. Cantley, S.E. Shoelson, and C.E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. USA*. 91:2834–2838.
 17. Chuang, E., K.M. Lee, M.D. Robbins, J.M. Duerr, M.L. Alegre, J.E. Hambor, M.J. Neveu, J.A. Bluestone, and C.B. Thompson. 1999. Regulation of cytotoxic T lymphocyte-associated molecule-4 by Src kinases. *J. Immunol.* 162:1270–1277.
 18. Schneider, H., K.V. Prasad, S.E. Shoelson, and C.E. Rudd. 1995. CTLA-4 binding to the lipid kinase phosphatidylinositol 3-kinase in T cells. *J. Exp. Med.* 181:351–355.
 19. Marengere, L.E., P. Waterhouse, G.S. Duncan, H.W. Mittrucker, G.S. Feng, and T.W. Mak. 1996. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science*. 272:1170–1173.
 20. Martin, M., H. Schneider, A. Azouz, and C.E. Rudd. 2001. Cytotoxic T lymphocyte antigen 4 and CD28 modulate cell surface raft expression in their regulation of T cell function. *J. Exp. Med.* 194:1675–1681.
 21. Linsley, P.S., J. Bradshaw, J. Greene, R. Peach, K.L. Bennett, and R.S. Mittler. 1996. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity*. 4:535–543.
 22. Egen, J.G., and J.P. Allison. 2002. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity*. 16:23–35.
 23. Scheipers, P., and H. Reiser. 1998. Fas-independent death of activated CD4(+) T lymphocytes induced by CTLA-4 crosslinking. *Proc. Natl. Acad. Sci. USA*. 95:10083–10088.
 24. Doyle, A.M., A.C. Mullen, A.V. Villarino, A.S. Hutchins, F.A. High, H.W. Lee, C.B. Thompson, and S.L. Reiner. 2001. Induction of cytotoxic T lymphocyte antigen 4 (CTLA-4) restricts clonal expansion of helper T cells. *J. Exp. Med.* 194:893–902.
 25. Zhang, X., T. Brunner, L. Carter, R.W. Dutton, P. Rogers, L. Bradley, T. Sato, J.C. Reed, D. Green, and S.L. Swain. 1997. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J. Exp. Med.* 185:1837–1849.
 26. Refaelli, Y., L. Van Parijs, C.A. London, J. Tschopp, and A.K. Abbas. 1998. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity*. 8:615–623.
 27. Watanabe, N., H. Arase, K. Kurasawa, I. Iwamoto, N. Kayagaki, H. Yagita, K. Okumura, S. Miyatake, and T. Saito. 1997. Th1 and Th2 subsets equally undergo Fas-dependent and -independent activation-induced cell death. *Eur. J. Immunol.* 27:1858–1864.
 28. Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K.P. Lee, C.B. Thompson, H. Griesser, and T.W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4*. *Science*. 270:985–988.
 29. Alegre, M.L., H. Shiels, C.B. Thompson, and T.F. Gajewski. 1998. Expression and function of CTLA-4 in Th1 and Th2 cells. *J. Immunol.* 161:3347–3356.
 30. Oosterwegel, M.A., D.A. Mandelbrot, S.D. Boyd, R.B. Lorschach, D.Y. Jarrett, A.K. Abbas, and A.H. Sharpe. 1999. The role of CTLA-4 in regulating Th2 differentiation. *J. Immunol.* 163:2634–2639.
 31. Kato, T., and H. Nariuchi. 2000. Polarization of naive CD4(+) T cells toward the Th1 subset by CTLA-4 costimulation. *J. Immunol.* 164:3554–3562.
 32. Maszyrna, F., H. Hoff, D. Kunkel, A. Radbruch, and M.C. Brunner-Weinzierl. 2003. Diversity of clonal T cell proliferation is mediated by differential expression of CD152 (CTLA-4) on the cell surface of activated individual T lymphocytes. *J. Immunol.* 171:3459–3466.
 33. Wesselborg, S., I.H. Engels, E. Rossmann, M. Los, and K. Schulze-Osthoff. 1999. Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. *Blood*. 93:3053–3063.
 34. Walunas, T.L., D.J. Lenschow, C.Y. Bakker, P.S. Linsley, G.J. Freeman, J.M. Green, C.B. Thompson, and J.A. Bluestone. 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. *J. Exp. Med.* 183:2541–2550.
 35. Krummel, M.F., and J.P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon ac-

- tivation of resting T cells. *J. Exp. Med.* 183:2533–2540.
36. Refaeli, Y., L. Van Parijs, S.I. Alexander, and A.K. Abbas. 2002. Interferon gamma is required for activation-induced death of T lymphocytes. *J. Exp. Med.* 196:999–1005.
 37. Van Parijs, L., Y. Refaeli, J.D. Lord, B.H. Nelson, A.K. Abbas, and D. Baltimore. 1999. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. *Immunity.* 11:281–288.
 38. Van Parijs, L., A. Ibraghimov, and A.K. Abbas. 1996. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity.* 4:321–328.
 39. Lissy, N.A., L.F. Van Dyk, M. Becker-Hapak, A. Vocero-Akbani, J.H. Mendler, and S.F. Dowdy. 1998. TCR antigen-induced cell death occurs from a late G1 phase cell cycle check point. *Immunity.* 8:57–65.
 40. Richter, A., M. Lohning, and A. Radbruch. 1999. Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J. Exp. Med.* 190:1439–1450.
 41. Eder, A.M., L. Dominguez, T.F. Franke, and J.D. Ashwell. 1998. Phosphoinositide 3-kinase regulation of T cell receptor-mediated interleukin-2 gene expression in normal T cells. *J. Biol. Chem.* 273:28025–28031.
 42. Scaffidi, C., S. Kirchhoff, P.H. Krammer, and M.E. Peter. 1999. Apoptosis signaling in lymphocytes. *Curr. Opin. Immunol.* 11:277–285.
 43. Brunet, A., A. Bonni, M.J. Zigmund, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell.* 96:857–868.
 44. Kelly, E., A. Won, Y. Refaeli, and L. Van Parijs. 2002. IL-2 and related cytokines can promote T cell survival by activating AKT. *J. Immunol.* 168:597–603.
 45. da Rocha, D.S., and C.E. Rudd. 2001. CTLA-4 blockade of antigen-induced cell death. *Blood.* 97:1134–1137.
 46. Greenwald, R.J., V.A. Boussiotis, R.B. Lorsch, A.K. Abbas, and A.H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. *Immunity.* 14:145–155.
 47. Bachmann, M.F., A. Gallimore, E. Jones, B. Ecabert, H. Acha-Orbea, and M. Kopf. 2001. Normal pathogen-specific immune responses mounted by CTLA-4-deficient T cells: a paradigm reconsidered. *Eur. J. Immunol.* 31:450–458.
 48. Sahara, T., H.S. Kim, L.A. Kirshenbaum, and K. Walsh. 2002. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. *Mol. Cell. Biol.* 22:680–691.
 49. Garcia, S., J. DiSanto, and B. Stockinger. 1999. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity.* 11:163–171.
 50. Grayson, J.M., A.J. Zajac, J.D. Altman, and R. Ahmed. 2000. Increased expression of Bcl-2 in antigen-specific memory CD8+ T cells. *J. Immunol.* 164:3950–3954.