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Requirement for CD28 May Not Be Absolute for Collagen-Induced Arthritis: Study with HLA-DQ8 Transgenic Mice

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CD28 is required to achieve optimal T cell activation to an Ag. To determine the role CD28 costimulation plays in collagen-induced arthritis, we have generated DQ8 transgenic, CD28-deficient mice. DQ8 mice deficient for CD28 had comparable numbers of CD4 and CD8 T cells as DQ8.CD28+/+ mice. DQ8.CD28−/− mice develop collagen-induced arthritis with delayed onset and less severity than DQ8.CD28+/+ mice. T cells from DQ8.CD28−/− mice did not respond to type II collagen efficiently in vitro, although the response to DQ8-restricted peptides was similar to that in the parent mice. There was no functional defect in T cells as observed by proliferation with Con A. Cytokine analysis from in vitro study showed the production of high levels of the inflammatory cytokine, IFN-γ, in response to type II collagen. We observed an increase in CD4+CD28−NK2G2+ cells after immunization, suggesting an important role for cells bearing this receptor in the disease process. CD28−/− mice also have an increased number of DX5+ cells compared with CD28+/+ mice, which can lead to the production of high levels of IFN-γ. DQ8.CD28−/− mice had an increased number of cells bearing other costimulatory markers. Cells from DQ8.CD28−/− mice exhibited a lower proliferation rate and were resistant to activation-induced cell death compared with DQ8.CD28+/+ mice. This study supports the idea that CD28 plays a crucial role in the regulation of arthritis. However, in the absence of CD28 signaling, other costimulatory molecules can lead to the development of disease, thus indicating that the requirement for CD28 may not be absolute in the development of arthritis.


In autoimmune diseases such as rheumatoid arthritis (RA), CD4 T cells have been suggested to initiate the disease after activation by an Ag (autoantigen). Optimal T cell activation and proliferation require presentation of an Ag by APCs in the context of MHC molecules and a costimulatory signal. CD28, a T cell surface receptor, has been shown to be one of the key costimulatory molecules for T cell activation (1, 2). CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xL (3) and may contribute to the persistence of cells at the site of inflammation. In RA, CD4 T cells lacking CD28 have been shown to expand clonally with prolonged survival (4). The longevity of CD4+CD28− cells is due to defects in the apoptotic pathway (5). These T cells can proliferate upon stimulation with autologous mononuclear cells and produce large amounts of IFN-γ (4, 6, 7). There is evidence that CD4+CD28− T cells are autoreactive in other autoimmune diseases (7, 8). These studies suggest an important role for CD28 in disease regulation and pathogenesis,

Although evidence implicating CD4+CD28− T cells directly in autoimmunity in RA is insufficient. Recent studies in human patients have described a large proportion of CD4+CD28− cells expressing NKG2D, a C-type lectin (9). The cells expressing NK markers can stimulate autoreactivity. The question that remains unresolved is whether the lack of CD28 expression on CD4 cells and the expression of NK markers is due to the disease process, or CD4+CD28− cells are involved in the initiation of the disease process in patients.

Animal models provide a way to study the roles of various molecules in vivo by knocking out the molecule in question or by blocking it and studying its effect on the disease process. Blockade of the B7/CD28 pathway has been shown to induce anergy and inhibit autoimmune diseases such as diabetes and experimental autoimmune encephalomyelitis (10–12). Additionally, blocking CD28 signaling with CD28 F(ab) ameliorates MBP-induced experimental autoimmune encephalomyelitis (13). However, in the absence of CD28 signaling, other costimulatory molecules can become dominant (14). T cells from CD28−/− mice have greatly reduced in vitro responses, but can be primed to Ag and mount primary and secondary responses. This is supported by the observation that CD28-deficient mice develop autoimmune diseases (15–18), which is attributed to costimulation through other costimulatory pathways (18). Thus, even in the absence of CD28, pathogenic T cells can result in disease initiation.

Collagen-induced arthritis (CIA) is an experimental model that shares histologic similarities with RA. Similar to RA, both T and B cells are involved in the pathogenesis of CIA. Type II collagen (CII)-specific CD4+ T cells have been reported to be fundamental in the initiation and perpetuation of the disease in murine CIA (19, 20). CD28-deficient DBA/1 mice do not develop CIA, suggesting an absolute requirement for costimulation provided by CD28 for
autoantigen-specific CD4+ T cell activation and development of disease in this model (21). The prevention and amelioration of collagen-induced arthritis by blockade of the CD28 costimulatory pathway have been observed (22), but not confirmed by others (23).

Previous studies from our laboratory have shown that Aβo.DQ8 mice elicit a vigorous CD4+-mediated, DQ8-restricted cellular response after immunization with CII that progresses to a severe form of CIA (24, 25). In this study we investigated the role of CD28 in the initiation of CIA in transgenic mice expressing an RA-associated HLA class II molecule, DQ8 (DQA1*0301, DQB1*0302). We observed a delayed onset of CIA in DQ8.CD28+/− mice, suggesting that CD28 is not an absolute requirement for pathogenic response by T cells. Even though cells from CD28-deficient mice proliferated at a slower rate than those from DQ8.CD28+/+ mice, a defect in activation-induced cell death (AICD) led to survival and accumulation of activated cells in CD28-deficient mice. We have attempted to elucidate the mechanisms involved in the initiation of arthritis in CD28-deficient mice. Our data suggest that the expression of NK markers on CD4+CD28− cells occurs during the effector phase after immunization.

Materials and Methods

Mice

Aβo.DQ8.CD28+/−, Aβo.DQ8.CD28−/−, and Aβo.DQ8.CD28+/+ mice were generated by mating Aβo.DQ8 mice (26) with Aβo.mCD28−/− deficient mice (provided by Dr. T. W. Mak, Ontario Cancer Institute and University of Toronto). All mice were typed by PCR and flow cytometry for phenotypic expression of DQ8 and DQ8 molecules. For all experimental groups, parental mice and negative littermates were included as controls. Mice of both sexes (8–12 wk of age) used in this study were bred and maintained in the pathogen-free Immunogenetics Mouse Colony, Mayo Clinic, in accordance with the animal use and care committee. All mice used in this study lacked endogenous class II molecules (Aβo).

Flow cytometry

Expression of HLA-DQ, H2A, CD28, CD3, CD4, CD8, B220, and TCR Vβ-chain molecules on PBLs was analyzed by flow cytometry using FACS IV (BD Biosciences) as described previously (27). Abs used for staining were IVD12 (anti-DQB1), HB163 (anti-Aβ), KT4-10 (anti-CD4), MR9-4 (anti-Vβ5.1), MR9-8 (anti-Vβ5.1.2), 44-22-1 (anti-Vβ6), F23.1 (anti-Vβ8.2.3), KJ-16 (anti-Vβ8.1), F23.2 (anti-Vβ8.1), KT11 (anti-Vβ11), and KJ23 (anti-Vβ17). For staining CD3, CD4, CD8, CD28, and B220 cell surface markers, conjugated Abs were used (BD Pharmingen). Conjugated Abs for CD5, CD40, OX-40, CD81, CD137, CD152, DX5, NK1.1, NKG2A, LY49A, and LY49D (BD Pharmingen) and NKG2D (eBiosciences) were also used. All cell surface markers were performed with cells pooled from two mice per strain, and experiments were repeated two or three times.

Induction and evaluation of CIA

Pure native chie CII was obtained by multiple-step purification as described previously (27). To induce CIA, 8- to 12-wk-old transgenic mice and negative littermates were immunized with 100 μg of CII emulsified 1/1 with CFA H37 Ra (Difco) intradermally at the base of the tail. Animals received a booster of 100 μg of CII emulsified in IFA 28 days after initial immunization. Mice were monitored for the onset and progression of CIA from 3–14 wk postimmunization. The arthritis severity of mice was evaluated as described previously with a grading system for each paw from 0–3 (28). The mean arthritic score was determined using arthritic animals only.

Histopathology

Mice were killed after 10 wk of immunization, and paws were decalcified and fixed. Sections were stained with H&E and examined histologically for mononuclear infiltration and bone erosion.

Anti-collagen Abs

Mice were bled on day 35 postimmunization, and the level of anti-mouse CII and anti-chick CII Abs in sera were determined using standard ELISA technique as described previously (29). Briefly, microtiter plates were coated overnight with chick or mouse CII (2 μg/well in KPO4, pH 7.6) at 4°C, washed, and blocked with 1% BSA in PBS/0.05% Tween 20. Sera were added in 4-fold dilutions (1/100 to 1/6000) and incubated overnight at 4°C. The plates were washed, and peroxidase-conjugated goat anti-mouse IgG (Organon Teknika) was added for another overnight incubation at 4°C. After washing, O-phenylenediamine was added, and the colorimetric change was measured at 410 nm. All assays were performed in duplicate and were quantitated against a standard curve obtained with a known positive serum, arbitrarily determined to equal 100 Ab units (units per milliliter).

In vitro T cell proliferation

Mice were immunized with 200 μg of CII emulsified 1/1 in CFA (Difco) intradermally at the base of the tail and one hind footpad. Ten days postimmunization, draining popliteal, caudal, and lumbar lymph nodes were removed and prepared for in vitro culture. Lymph node cells (LNCs; 1 × 106) were challenged by adding 100 μl of medium (negative control), Con A (20 μg/ml; positive control; final concentration, 10 μg/ml; Sigma-Aldrich), and native collagen (50 μg/ml). For inhibition experiments, culture supernatant containing mAbs (25 μg/ml Ab) GKL1.5 (anti-CD4), IVD12 (anti-HLA-DQ), or Ly2 (anti-CD8) was added to the cells challenged in vitro with CII at 50 μg/ml. The cells were incubated for 48 h at 37°C. During the last 18 h, the cells were pulsed with [3H]thymidine, and tritium incorporation was determined by liquid scintillation counting. Results are calculated as Δ cpm (mean cpm of triplicate cultures containing Ag− mean cpm of medium).

Delayed-type hypersensitivity response

Mice (four or five mice in each group) were primed with 100 μg of CII as performed for in vivo induction of CIA. Five days later, 5 or 50 μg of CII emulsified in IFA was injected in the left footpad after measuring thickness using a caliper with 0.05-mm increments (Schnettlaster; Dyer). The right footpad was injected with PBS in IFA. Swelling/thickness were measured after 24 h and 48 h using the caliper.

Measurement of cytokines

Capture ELISA was performed for measuring cytokines IFN-γ, TNF-α, TGF-β, IL-10, and IL-4 in culture supernatants using kits according to the manufacturer’s instructions (BD Pharmingen). Cells isolated from two or three primed mice were cultured in vitro as described above, and supernatants were pooled. The experiment was repeated twice.

Immunoblotting

Spleen cells from two mice per strain were collected by centrifugation at 200 × g for 5 min and washed with cold PBS. Also, 1 × 106 splenic cells were prepared for in vitro culture. Whole-cell lysates were prepared using cold lysis buffer (50 mM Tris [pH 7.2], 100 mM NaCl, 1 mM DTT, 1% Nonidet P-40, and protease inhibitor mixture). Lysates were centrifuged at 10,000 × g, and the supernatants were analyzed for the presence of Bcl-2, Bcl-xL, BAX, caspase-3, FLICE inhibitory protein, and poly(ADP-ribose) polymerase (PARP) by immunoblotting using Abs against Bcl-2, Bcl-xL, BAX, FLIP (G-11; Santa Cruz Biotechnology), caspase-3, and PARP (New England Biolabs).

Cell division and cell cycle

Mice were killed on day 7 after immunization with CII. For every experiment, pooled LNCs from two or three mice were collected and stained with conjugated CD3-FITC mAb (BD Pharmingen) according to the manufacturer’s instructions. For cell division, equal numbers of CD3+ cells were stained with cell division tracking dye, CFSE, and cultured in vitro for 48–72 h with or without CII. Cells were stained with propidium iodide and CD3 Abs after in vitro culture for 24 h and were analyzed by FACS for cell cycle of CD3+ cells.

AICD

To study the sensitivity of the proliferating cells to CII-induced AICD, cells pooled from two or three mice per strain were stained with CD3-PE and annexin V conjugated with FITC (BD Pharmingen) after in vitro stimulation for 24 and 96 h in the presence of CII and were analyzed by FACS.

Statistical analysis

Differences in the incidence of arthritis between groups were analyzed using χ2 test with Yates’ correction. Ab levels and mean scores for arthritic mice were compared using Student’s t test. Differences in cytokines and apoptotic cells were determined by Student’s t test.
CD28 is not an absolute requirement for the development of CIA

CD28 transgenic mice deficient in CD28 (CD28−/−), heterozygous for CD28 (CD28+/−), and homozygous for CD28 (CD28+/+) were immunized with chick CII and monitored for the onset and progression of arthritis. CD28−/− and CD28+/− mice developed CIA with similar incidence and severity (Fig. 1). However, CD28−/− mice had a lower incidence of arthritis compared with CD28+/− mice (11 of 25 (44%) and 11 of 15 (73%), respectively), although not statistically significant (p = 0.069). CIA onset was delayed in CD28−/− mice with milder arthritis compared with CD28+/− mice (p < 0.05). However, histopathology correlated with the clinical score of individual paws and was similar in both mice. CD28-deficient mice produced similar amounts of anti-CII Abs as CD28+ mice (17.4 ± 3.1 and 18.9 ± 5.9, respectively; Fig. 1C). The level of Abs to mouse CII was lower in CD28−/− compared with CD28+/− (10.3 ± 3.6 and 12.7 ± 4.6, respectively; not significant).

The in vivo delayed-type immune response was tested by immunizing primed mice with 5 or 50 μg of CII. A similar response was observed after 24 and 48 h in both strains when mice were immunized with 50 μg of CII. However, with the lower dose of Ag, the response was milder and delayed at both time points in CD28−/− mice compared with CD28+/− mice (24 h, 5.7 ± 0.3 and 1.75 ± 0.2; 48 h, 6.2 ± 0.3 and 2.25 ± 0.3, respectively; p < 0.05).

CD28-deficient T cells can respond in vitro and produce high amounts of IFN-γ

Cells isolated from CD28-deficient mice presented CII in a dose-dependent manner in vitro, although the T cell response was much lower than that in CD28+/+ and CD28+/− mice (Fig. 2A). In vitro T cell proliferation in response to known DQ8-restricted peptides (30) was also lower in CD28-deficient than CD28+/+ mice (Fig. 2B).

Various cytokines, IFN-γ, TNF-α, IL-10, IL-4, and TGF-β, were measured in supernatants of splenic cells cultured in vitro for 24 and 96 h in the presence of CII (Fig. 3). Interestingly, CD28−/− mice produced lower amounts of inflammatory cytokines than CD28+/− mice after 24 h in vitro culture in response to CII (p < 0.02). However, incubation for a longer period resulted in significantly higher amounts of IFN-γ and TNF-α in CD28−/− mice compared with CD28+/− mice (p < 0.04). Similarly, Th2 cytokines, IL-4 and TGF-β, and the regulatory cytokine, IL-10, were produced in higher amounts in CD28−/− mice than in CD28+/+ mice after longer incubation with CII in vitro; only the difference in IL-4 levels was statistically significant (p < 0.05).

Table II. Vβ T cell repertoire in DQ8 transgenic CD28+/+ and CD28−/− mice

<table>
<thead>
<tr>
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<th>DQ8.CD28+/+</th>
<th>DQ8.CD28−/−</th>
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<tr>
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a. Pooled spleen cells were isolated from three to four naive mice and analyzed by FACS for expression of HLA-DQ8, CD4, and CD8 after conjugated Ab-specific staining. Numbers indicate the percentage of cells positive for the marker.
of CII for 24 and 96 h, then stained with annexin V. Cultured cells were stained with CD3-PE and analyzed by FACS for percent cell death in CD3⁺ cells (Fig. 7). Lower numbers of apoptotic CD3⁺ cells were observed in CD28⁻/⁻ compared with CD28⁺/+ mice, although the difference was significant only after 96-h in vitro culture \((p < 0.05)\). Decreased apoptosis indicated a defect in AICD in DQ8.CD28⁻/⁻ mice.

Because CD28⁻/⁻ mice showed decreased Ag-specific proliferation and AICD, we analyzed the presence of proteins that regulate apoptosis. Mice primed with CII were killed after 2–3 wk of immunization, and spleen cells were collected. Whole-cell extracts were prepared and analyzed for the expression of the Bcl-2 family of proteins. CD28⁻/⁻ mice had higher levels of antiapoptotic proteins (Bcl-2 and Bcl-xL); however, the level of the proapoptotic protein, BAX, was similar to that in CD28⁺/+ mice (Fig. 8). To further define the role of apoptosis in disease development and its relation to accumulation of cells, we studied apoptosis markers, caspase-3 and PARP. There was an increased activation of caspase-3, as apparent by enhanced cleavage of the protein, in CD28⁺/+ compared with CD28⁻/⁻ mice. However, cleavage of PARP was not significantly different between the two strains. Activation of cells on CII-precoated plates led to high levels of FLIP expression in primed CD28⁻/⁻ cells (Fig. 8), whereas isotype control cultures had low levels of FLIP (data not shown).

**Discussion**

We report in this study that the costimulation by CD28 is not an absolute requirement for the initiation of CIA in DQ8 transgenic mice. However, CD28-deficient mice developed a milder disease, with delayed onset and lower incidence, than CD28⁺/+ mice, confirming that CD28 costimulation is the major pathway for optimal activation for cellular and humoral immune responses. Lack of costimulation by CD28 is compensated by the other costimulatory molecules, as evidenced by an increased number of CD40-, CD81-, CD137-, and OX-40-positive cells in CD28-deficient mice in the present data. This is in confirmation of previous studies demonstrating that CD28 is not required for all T cell responses in vivo (31, 32) and with other autoimmune models, such as experimental autoimmune encephalomyelitis and proteoglycan-induced...
arthritis, which can also be induced in the absence of CD28 (15, 17, 18, 33). However, it is not in confirmation with a previous study showing that the DBA/1 mice deficient in CD28 are resistant to CIA (21). One of the reasons DBA/1.CD28-deficient mice did not develop arthritis could be that they did not produce Th1 cytokines when challenged with CII. Moreover, CIA in DBA/1 mice is not CD4 dependent, unlike that in DQ8 mice (25). Our observations with DQ8 transgenic mice deficient in CD28 showed low levels of cytokine production when cultured for a short term; however, an extended period of in vitro culture led to the production of high amounts of both Th1 and Th2 cytokines. In human patients with RA, CD4+ CD28− cells have been shown to produce high amounts of IFN-γ (4, 5). Clonal expansion of CD4+ CD28− cells in RA patients led to the thought that these cells might be important in pathology. However, the Ag specificities of those populations have not been identified, and autoreactivity is only presumed due to the autologous MLR proliferation. Even though there is no direct evidence implicating these cells in RA, recent studies suggesting the expression of NK markers on CD4+ CD28− cells in patients with arthritis lend support to an important role of these cells in pathogenesis (9, 34). If these cells were required for autoreactivity leading to the development of arthritis, we would have observed a much more severe disease in CD28-deficient animals. Because this was not the case, our data suggest and lend support to the findings in humans that the presence of clonally expanded CD4+ CD28− cells in RA might be the result of rapid division due to the disease. The requirements for CD28 costimulation for CD4 and CD8 cells are markedly different. Although CD4 cells require costimulation for activation, CD8 T cells require CD28 costimulation for the induction of peripheral tolerance (35). To understand the costimulatory requirements for CD28-deficient mice, we investigated alternative costimulatory molecules that could have been used for the activation of T cells. Immunization with CII led to the activation of T cells in CD28−/− as well as CD28+/+ mice, which was indicated by high production of IFN-γ. However, delayed-type hypersensitivity was defective in CD28−/− mice at lower doses of Ag, although higher doses could reverse the defect, probably by recruiting enough CD4+ CII-reactive cells. These data suggest a role for CD28 in in vivo trafficking, as shown in a previous study (12). We observed high amounts of IFN-γ after long term cultures in vitro, suggesting that IFN-γ production in vivo by Ag-specific cells is the result of other costimulatory pathways. Because naive CD4+ CD28− cells do not produce IFN-γ, this suggests that activation by Ag-specific exposure is required. After immunization, other costimulatory molecules can lead to activation of T cells. However, the effector immune response takes longer to reach a threshold for the development of disease, which might explain the delayed onset of arthritis. Costimulation of T cells from CD28−/− mice have increased numbers of cells expressing other costimulatory markers compared with CD28+/+ mice. The expression of costimulatory markers was analyzed by FACS after staining with specific Abs. The data are the mean ± SE of two experiments performed with pooled supernatants from two or three mice each. Comparison between CD28+/+ and CD28−/− for cytokine levels: *, p < 0.05; **, p < 0.01.
mice can occur via 4-1BB/4-1BBL interaction and can sustain T cell effector function and production of cytokines (36). Our data confirm recent findings demonstrating that involvement of the CD40-CD40L and OX40-OX40L costimulatory pathways in the absence of CD28 signaling can lead to a delayed response and development of autoimmune disease (18).

We have previously shown the regulatory function of CD8 cells in CIA (25). In an another autoimmune model, CD8+CD28+ T cells have been shown to regulate the inhibition of Th1 cell expansion (37). In this study we observed a decrease in CD8+CD28+ T cells bearing NKG2D, a C-type lectin-like activating factor, after immunization in CD28+/+ mice. However, a reverse trend was observed in CD28−/− mice. Ligand engagement of NKG2D activates NK cells and costimulates effector T cells (5, 38, 39), leading to a potent immune response and high amounts of IFN-γ production in CD28−/− mice. High amounts of IFN-γ are also produced by NK cells accumulated after immunization with CFA (40). CD28−/− mice have more DX5+ cells than CD28+/+ mice, which can become activated and produce high amounts of IFN-γ in CD28−/− mice. To explore the similarities with studies in human RA patients, we investigated the role of T cells bearing NK receptors in CIA. We observed an increase in the number of CD4+CD28−NKG2D cells after immunization. This increase can be explained by high production of TNF-α, as shown previously in human studies (9). In addition, there was a reduced frequency of T cells bearing LY49A (functional analog of human NK inhibitory receptor) after immunization. These data suggest that differential expression of NK markers occurs after initiation of the immune response following immunization. In humans, a similar situation can be envisaged where exposure to an Ag, viral or bacterial, can lead to activation of NKT cells or CD4 T cells, which then acquire some NK markers, whereas some other markers are lost. However, there are limitations in comparing the expression of NK receptors in humans and mice due to differential expression.

Both transgenic strains also produce the Th2 cytokines, IL-4 and TGF-β, and the regulatory cytokine, IL-10, in vitro when challenged with CIA. All Th2 cytokines have been associated with protection from arthritis in most of the experimental models studied (41, 42), although there is evidence that these cytokines can be involved in pathogenesis (43–45). IL-4 can be produced by CD4
Studies have shown that CD152 can induce resistance to AICD in Th2 cells (50). Recent studies have shown that CTLA-4 Ig therapy is efficacious in RA, suggesting that costimulation via CD152 might contribute to the pathogenic response (51). CTLA-4 has been shown to regulate cell cycle progression, and CD152 engagement during suboptimal activation of T cells can lead to G1 cell cycle arrest at an early phase (52, 53). There are data showing that Ag-induced cell death occurs from late G1 phase (54). This suggests that cells are arrested in early G1 phase in CD28−/− mice and may not be susceptible to apoptosis. Our data with annexin staining of CD3+ T cells confirm these data. Analyses of proteins involved in the regulation of apoptosis suggest increased amounts of antiapoptotic proteins, although there was no difference in the amounts of proapoptotic protein, BAX, between the two strains. Higher levels of FLIP after stimulation in vitro in CD28−/− mice could be related to protection from AICD, as suggested previously in RA patients (5, 55). This suggests that a balance between the various proteins and cytokines is needed to reach a threshold for disease to develop. Clonal expansion of CII-reactive cells with reduced apoptosis due to the imbalance of proteins involved in apoptosis and inflammatory cytokines might lead to organ-specific damage.

In conclusion, our data suggest 1) other costimulatory pathways can lead to the development of autoimmune arthritis in the absence of CD28 signaling; 2) CD4+CD28−/−NKG2D cells are activated after immunization with an autoantigen; 3) high IFN-γ and TNF-α production with increased survival and less AICD can lead to accumulation of CII-reactive T cells leading to pathology. Late production of both Th1 and Th2 cytokines explain the delayed onset with milder disease and lower disease incidence. The study demonstrates that CD28 is not an absolute requirement for the development of autoimmunity; however, an optimal response to an Ag preferably requires CD28.

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References


FIGURE 7. Less apoptosis of CD3+ cells was observed in CD28−/− mice compared with CD28+/+ mice. LNCs pooled from two or three mice per strain were obtained and cultured in vitro for 24 and 96 h in the presence of CII and stained with CD3 and annexin. Cell death was analyzed by FACS for annexin-positive cells. Data are plotted as the percent cell death per strain were obtained and cultured in vitro for 24 and 96 h in the presence of CII and stained with CD3 and annexin. Cell death was analyzed by FACS for annexin-positive cells. Data are plotted as the percent cell death of LNCs (A) and CD3+ cells (B) and are the mean ± SE of two experiments. *, p < 0.05.

FIGURE 8. Lanes 1 and 2, Proteins extracted from splenic cells of DQ8.CD28+/+ and DQ8.CD28−/− mice, respectively. Lysates were analyzed for the expression of various proteins involved in apoptosis (Bcl-2, Bcl-xL, BAX, poly(ADP-ribose) polymerase (PARP), caspase-3 (Cas-3), cleaved caspase-3, and cFLIP) using specific Abs.


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