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Translational Regulation of Autoimmune Inflammation and Lymphoma Genesis by Programmed Cell Death 4

Anja Hilliard,* Brendan Hilliard,* Shi-Jun Zheng,2* Honghong Sun,* Takashi Miwa,† Wenchao Song,‡ Rüdiger Göke,‡ and Youhai H. Chen3*

Both inflammatory diseases and cancer are associated with heightened protein translation. However, the mechanisms of translational regulation and the roles of translation factors in these diseases are not clear. Programmed cell death 4 (PDCD4) is a newly described inhibitor of protein translation. To determine the roles of PDCD4 in vivo, we generated PDCD4-deficient mice by gene targeting. We report here that mice deficient in PDCD4 develop spontaneous lymphomas and have a significantly reduced life span. Most tumors are of the B lymphoid origin with frequent metastasis to liver and kidney. However, PDCD4-deficient mice are resistant to inflammatory diseases such as autoimmune encephalomyelitis and diabetes. Mechanistic studies reveal that upon activation, PDCD4-deficient lymphocytes preferentially produce cytokines that promote oncogenesis but inhibit inflammation. These results establish that PDCD4 controls lymphoma genesis and autoimmune inflammation by selectively inhibiting protein translation in the immune system. The Journal of Immunology, 2006, 177: 8095–8102.

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4 Abbreviations used in this paper: 4E-BPs, 4E-binding proteins; eIF, eukaryotic initiation factors; 5′ UTR, 5′ untranslated regions; PDCD4, programmed cell death 4; ES cells, embryonic stem cells; STZ, streptozotocin; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein.

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least five generations to a C57BL/6 background. PDCD4<sup>+/−</sup> littermates were used as controls in all experiments.

**Reagents and ELISA**

Streptozotocin (STZ) was purchased from Sigma-Aldrich. The following reagents were purchased from BD Pharmingen: purified rat anti-mouse IL-4, IL-10, and IFN-γ mAbs and recombinant mouse IL-4, IL-10, and IFN-γ. Quantitative ELISA was performed using paired mAbs specific for corresponding cytokines per the manufacturer’s recommendations.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections (5-μm thick) were treated with sodium citrate (pH 6.0) at 90–95°C for 20 min to retrieve antigenic epitopes. Sections were blocked for endogenous peroxidase with 3% H<sub>2</sub>O<sub>2</sub>, and endogenous avidin and biotin with an avidin-biotin blocking kit (Vector Laboratories). They were then incubated overnight at 4°C with rabbit anti-c-Myc (Santa Cruz Biotechnology), anti-Bcl-2 (eBioscience), normal rabbit Ig. After washing, the sections were further incubated overnight at 4°C with biotinylated anti-rabbit Ig (Southern Biotechnology Associates). After treatment with the biotin-avidin peroxidase complexes (ABC standard kit, Vector Laboratories), color development was conducted with the nickel-diaminobenzidine reagent (Pierce). Sections were blocked for endogenous peroxidase with H<sub>2</sub>O<sub>2</sub>. After treatment with the biotin-avidin peroxidase complex, color development was conducted with the nickel-diaminobenzidine reagent (Pierce). Sections were blocked for endogenous peroxidase with H<sub>2</sub>O<sub>2</sub>. After treatment with the biotin-avidin peroxidase complex, color development was conducted with the nickel-diaminobenzidine reagent (Pierce).

**Somatic hypermutation analysis**

DNA was isolated from formalin-fixed tissues using the genomic DNA isolation kit (Wizard). To determine the clonality of the B cell population, Ig gene hypermutation analysis was performed as previously described (19, 20). Briefly, a primer derived from the most commonly used variable H chain gene family, JH558, and a reverse primer in the JH3 intron were used to amplify Ig gene sequences from tumors and normal lymphoid tissues. Three major products for the three JH genes amplified with the JH3 primer are expected from polyclonal normal spleen and lymph node tissues. One major amplified product is indicative of a clonal B cell population (19, 20).

**Induction and evaluation of type 1 diabetes**

To induce diabetes, mice were injected i.p. for five consecutive days with 40 mg/kg STZ. Mice were tested in a blinded manner every other day for urinary glucose levels using the Keto-Diastix kit (Bayer). They were considered diabetic if the urinary glucose levels equaled or exceeded 500 mg/dl on at least two consecutive tests. To obtain histological profiles of the pancreas, mice were sacrificed 30 days after the first STZ injection. Pancreata were collected, fixed in 10% formalin, and embedded in paraffin. Five micrometer-thick sections were stained with hematoxylin/eosin and examined by microscopy. Pancreatic inflammation was graded as follows (21): 0, no inflammation; 1, peri-insulitis with mononuclear cell infiltration affecting ≤25% of the circumference; 2, peri-insulitis with mononuclear cell infiltration affecting >25% of the circumference; 3, mild to moderate insulitis with intraislet mononuclear cell filtration but good preservation of islet architecture; 4, severe insulitis with numerous intraislet inflammatory cells and loss of normal islet architecture.

**Induction and evaluation of autoimmune encephalomyelitis**

Mice were immunized with myelin oligodendrocyte glycoprotein (MOG) 38–50 peptide to induce experimental autoimmune encephalomyelitis (EAE) as described (22), and scored as follows: 0, no disease; 1, limp tail; 2, weak hind limbs; 3, paralyzed hind limbs; 4, paralyzed hind limbs and weak forelimbs; and 5, moribund or dead. To determine the degree of CNS inflammation, mice were sacrificed 36 days after the immunization. Formalin-fixed, paraffin-embedded spinal cord sections were stained with Luxol fast blue and cresyl violet. The degree of inflammation was scored by a blinded observer as follows: 0, no inflammation or demyelination; 1, mild infiltration; 2, moderate infiltration; and 3, extensive infiltration with demyelination throughout the white matter.

**Cell culture**

For cytokine assays, splenocytes were cultured at 1.5 × 10<sup>6</sup> cells/well in 0.2 ml of DMEM with 10% FBS in the presence or absence of MOG38–50 peptide, anti-CD3 mAb, and anti-CD28 mAb. Culture supernatants were collected 40 h later, and cytokine concentrations were determined by ELISA.

**Western blot**

Thymus or isolated splenocytes were homogenized in PBS supplemented with protease inhibitors. The homogenate was centrifuged at 2300 × g for 10 min at 4°C. The supernatant was then fractionated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk and 0.1% skim milk, the membrane was incubated with rabbit anti-PDCD4 Ab (specific for a peptide consisting of aa 1–20 of the PDCD4 protein), anti-c-Myc mAb, anti-Bcl-2 mAb, anti-β-actin mAb, and HRP-labeled secondary Abs (Amersham Biosciences). Color was developed using ECL Western blotting detection reagents (Amersham Biosciences).

**Protein microarray studies**

Splenocytes isolated from 6–7-wk-old male PDCD4<sup>+/−</sup> (<i>n</i> = 4) and PDCD4<sup>−/−</sup> (<i>n</i> = 4) mice were cultured on a 24-well plate coated with anti-mouse CD3 mAb (2 μg/ml) in complete DMEM containing 1 μg/ml LPS. Forty-eight hours later, supernatants were collected and tested for cytokines using the RayBiotech cytokine Ab microarray. The strengths of the signals were determined by densitometry.
Statistical analysis
The significance of the differences in disease severity and immune parameters was determined by Mann-Whitney U test and ANOVA, respectively.

Results
Generation of PDCD4-deficient mice
PDCD4-deficient mice were generated by deleting both the third and the fourth exons of the PDCD4 gene through homologous recombination (Fig. 1). The 2.2-kb PDCD4 mRNA and the 60-kDa PDCD4 protein were completely absent in lymphoid organs of mice homozygous for the gene mutation while organs heterozygous for the gene mutation expressed roughly half of the wild-type levels (Fig. 1c and d). Mice carrying the PDCD4 gene mutation developed normally and exhibited no abnormalities with regard to body weight, body temperature, $O_2$ consumption, and physical activities during the first few months of their lives. No spontaneous infectious diseases were detected in these mice or their littermate controls throughout this study.

Spontaneous lymphoma development in PDCD4-deficient mice
Starting from ~80 wk of age, many PDCD4$^{-/-}$ mice developed abdominal masses, which eventually led to their demises (Fig. 2a).
Upon autopsy, severe disseminated lymphoma was detected in the lymph nodes and spleen of these mice, with frequent metastasis to the liver and kidney. Combined histochemical and flow cytometric analyses of affected organs revealed that the lymphoma was primarily of B cell lineage with diffuse, but not follicular, patterns (Figs. 2 and 3). Destruction of normal lymph node and spleen architecture and invasion of the capsule and adjacent fat were common. The abnormal cells in the lymph node, spleen, and extranodal sites were small in size, and expressed high levels of B220, surface IgM, IgD, Bcl-2, Bcl-6, and c-Myc, but not the early B cell marker CD43; nor did they express any T cell markers such as CD4, CD8, or CD3, or myeloid markers such as CD11b, CD11c, or F4/80 (Figs. 2 b–f, 3, 4a). Although normal B cells also express Bcl-2, Bcl-6, and c-Myc, lymphoma cells expressed increased levels of these proteins (Fig. 3a). Ig variable gene mutation analysis revealed that lymphoma cells but not normal lymph node cells harbored the same gene modifications, indicating that the lymphoma cells were clonally derived (Fig. 4b).

By 106 wk of age, the vast majority of PDCD4<sup>−/−</sup> mice (86%) had succumbed to lymphoma. At this point, all mice were sacrificed and full autopsy was performed. Lymphoma was detected in ~14% of PDCD4<sup>−/−</sup> littermate controls as well (Fig. 2a), consistent with the previous observation that C57BL/6 mice were resistant to spontaneous tumors. In addition to lymphoma, multiorgan cysts that involved kidney, ovary, or prostate were also detected in ~50% of PDCD4<sup>−/−</sup> mice, as compared with 14% in the PDCD4<sup>+/+</sup> littermates (p < 0.01). However, no other types of tumors were detected in these mice throughout this study. The vast majority of PDCD4<sup>−/−</sup> mice were healthy at 106 wk of age. By contrast, the median life span of PDCD4<sup>−/−</sup> mice was reduced to 101 wk. The expected life span of control C57BL/6 mice is 115–150 wk (23).

Reduced autoimmune diseases in PDCD4-deficient mice

Although it is known that PDCD4 is expressed at high levels in lymphoid tissues (9), the preferential development of lymphoma in PDCD4<sup>−/−</sup> mice was unexpected and prompted us to further evaluate the lymphoid compartment of 6–12-wk-old mice that had not developed tumors. We found no significant differences between PDCD4<sup>−/−</sup> and PDCD4<sup>+/+</sup> mice in the weight and microstructure of lymphoid organs. By flow cytometry, we found that the percentages of cells expressing CD4, CD8, B220, CD11b, and CD11c in the spleen, thymus, blood, and mesenteric lymph node of PDCD4<sup>−/−</sup>, PDCD4<sup>+/+</sup>, and PDCD4<sup>−/−</sup> mice were similar (Fig. 5a and A. Hilliard and Y. H. Chen, unpublished observations). One of the functions of the lymphoid cells is to initiate and sustain inflammation, a basic pathological process associated with increased protein translation. To determine whether PDCD4 deficiency affects the ability of lymphoid cells to initiate and sustain inflammation, we studied two models of inflammatory diseases caused by self-reactive lymphocytes: type I diabetes induced by toxin STZ and EAE induced by MOG. Unexpectedly, we found that PDCD4<sup>−/−</sup> mice were significantly resistant to the development of both diseases (Fig. 6). Specifically, the incidence of diabetes in PDCD4<sup>−/−</sup> mice was 5% as compared with 59% in PDCD4<sup>+/+</sup> mice 4 wk after the STZ injection. For EAE, the incidence was 100% for both groups, but the disease severity as measured by clinical score was significantly reduced in PDCD4<sup>−/−</sup> mice. The mortality was reduced from 24% (n = 19) in the PDCD4<sup>−/−</sup> group to 14% (n = 16) in the PDCD4<sup>+/+</sup> group. Consistent with these clinical findings, histological examination of the pancreas and spinal cord revealed dramatic differences between the two groups. In PDCD4<sup>+/+</sup> mice treated with STZ, severe intra-isletic and peri-insulitis, which is characterized by edema, monocyte infiltration, and tissue injury, was noted in most islets. This was significantly reduced or completely absent in PDCD4<sup>−/−</sup> mice (Fig. 6, c and d). Similarly, PDCD4<sup>−/−</sup> mice developed a much-reduced degree of spinal cord inflammation as compared with PDCD4<sup>+/+</sup> mice (Fig. 6e). Macroscopic and microscopic examination of the spleens and mesenteric lymph nodes of the PDCD4<sup>−/−</sup> mice revealed no signs of lymphoproliferation or tumor. The total number of cells recovered per spleen was not significantly different between PDCD4<sup>−/−</sup> and PDCD4<sup>+/+</sup> mice.

Selective disruption of cytokine production in PDCD4-deficient mice

Results described above indicate that PDCD4 is involved in regulating both oncogenesis and inflammation. To explore the mechanisms whereby PDCD4 mediates this effect, we examined protein synthesis in PDCD4<sup>−/−</sup> and PDCD4<sup>+/+</sup> cells using several techniques. First, we cultured naïve splenocytes and thymocytes with [35S]methionine, and measured the amounts of radioactive amino
acid incorporated into proteins to gauge the rate of protein synthesis. We found that the rate of protein synthesis in PDCD4−/− splenocytes was increased 2-fold as compared with wild-type cells under this condition (Fig. 7, a and b). Second, we used protein microarray, Western blot, and ELISA that collectively detected ~50 proteins to identify molecules that might be affected by the PDCD4 gene mutation. We found that the expression of the vast majority of these proteins was not significantly different between PDCD4+/+ and PDCD4−/− splenocytes (Table I and Fig. 8). However, three growth factors/cytokines, i.e., IL-4, IL-10, and IFN-γ, were significantly increased in PDCD4−/− cells (Fig. 7, c–e). The increase was due to the posttranscriptional regulation because the mRNA levels of these genes were not significantly affected by the PDCD4 deficiency (Fig. 9). Spontaneous and anti-CD3- or LPS-induced apoptosis of splenocytes and thymocytes was not significantly affected by PDCD4 gene mutation when tested using 6–12-wk-old mice (Fig. 5b and our unpublished study).

To further test the theory that PDCD4 regulates the expression of growth factors/cytokines in lymphocytes, we also determined the levels of cytokine expression by MOG-specific T lymphocytes isolated from mice that were immunized for EAE. Thus, splenocytes were collected from both control and PDCD4−/− mice 35 days after immunization with MOG peptide and tested in vitro for their cytokine production in response to the peptide. As shown in Fig. 10, splenocytes of both groups produced IL-4 and IFN-γ, but the levels of these cytokines were significantly increased in PDCD4−/− groups in response to MOG peptide. No significant differences were observed for T cell proliferation and IL-2 production under these conditions (data not shown). These results indicate that PDCD4 deficiency affected T cell activation and cytokine production in response to MOG peptide. The MOG peptide used in this study, MOG38–50, contains only a T cell epitope. By ELISA, we were not able to detect any anti-MOG38–50 Abs in the blood 4 wk after immunization with the MOG peptide (our unpublished data).

Discussion

Results reported here indicate that PDCD4 serves as an endogenous protein translation inhibitor and helps promote inflammation but suppress tumor genesis in vivo. The preferential development of lymphoma in PDCD4−/− mice is surprising, given the ubiquitous nature of the PDCD4 gene expression. However, preferential lymphoma development has also been reported for mice that are deficient in other tumor suppressor genes such as p53. Mice homozygous for the p53 gene mutation develop lymphomas around 12 wk of age (24, 25), whereas our PDCD4−/− mice develop the disease around 85 wk. This difference in latency may indicate that PDCD4 is a much weaker tumor-suppressor gene than p53. The PDCD4 effect on inflammation is also unexpected because translation-inhibiting drugs such as rapamycin can inhibit inflammatory responses associated with graft rejection, type 1 diabetes (2), and EAE (3). However, the mode of action of rapamycin may differ significantly from that of PDCD4. Rapamycin binds to mTOR and inhibits translation through at least two mechanisms (26, 27): 1) by inhibiting ribosomal protein p70 S6 kinase (p70S6k); and 2)
by activating eIF 4E-BPs. Although the latter effect also reduces the functions of the initiating complex, the spectrum of rapamycin effect on protein translation is likely different from that of PDCD4. It has been reported that, when over-expressed, PDCD4 regulates cap-dependent translation through interacting with eIF4A4 and eIF4G (14). Data reported here establish that endogenous PDCD4 mediates highly selective inhibition of a group of proteins that are important for inhibiting inflammation and promoting oncogenesis. Whether tumor-suppressor gene mutations affect the development of inflammatory diseases in humans needs now to be investigated.

IL-4 is a well-known growth and differentiation factor for B lymphocytes whereas IL-10 is produced at high levels by and acts as an autocrine growth factor for Burkitt’s lymphoma cells (28). Mice deficient in IL-10 are resistant to the development of B cell lymphoma (29). Additionally, both IL-4 and IL-10 can effectively inhibit inflammatory diseases including type 1 diabetes and EAE (30–32). Therefore, it is likely that IL-4 and/or IL-10 produced by PDCD4−/− cells is responsible for the effects of PDCD4 deficiency on lymphoma genesis and inflammation. However, because PDCD4 likely regulates the expression of a much larger number of proteins, it is reasonable to assume that the roles of PDCD4 may not be mediated only by these proteins. Because it is well recognized that immune suppression is associated with increased incidence of tumors in humans, presumably due to reduced immune surveillance against tumor cells, it is also plausible that PDCD4 indirectly regulates lymphoma genesis by promoting cellular immunity. Further studies are needed to elucidate the precise mechanisms whereby PDCD4 suppresses tumor genesis.

Protein translation is a well-coordinated event regulated by a number of repressors and enhancers (4–7). PDCD4 shares no sequence homology with other translation repressors such as 4E-BPs and may represent a new class of endogenous protein translation repressors. Because inflammation and oncogenesis are both associated with enhanced protein translation, it is generally assumed that inhibiting translation may help to suppress both inflammatory diseases and cancer. Additionally, a tumor can develop following chronic inflammation, and certain inflammatory molecules such as NF-κB can also promote tumor development (33). However, our results indicate that promoting inflammation and suppressing tumor development can be mediated by the same molecule. This unexpected combination of the PDCD4 function may be explained by the unique group of molecules selectively targeted by PDCD4, i.e., cytokines that promote oncogenesis but inhibit inflammation. By contrast, NF-κB may target genes that promote both oncogenesis and inflammation. Thus, although inflammation may promote tumor development, the roles of tumor suppressors in these two

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<th>Method of Detection</th>
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<tr>
<td>Western blot</td>
<td>p27, cyclin D1, c-Myc, Bcl-2, Bcl-6, enolase, and β actin</td>
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<td>ELISA</td>
<td>IL-2, IL-5, IL-6, IL-12 p40, TNF-α</td>
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<td>6-Cysteine chemokine, cutaneous T-cell attracting chemokine, eotaxin, granulocyte CSF, granulocyte-macrophage CSF, IL-2, IL-3, IL-5, IL-6, IL-9, IL-12 p40, IL-12 p70, IL-13, IL-17, keratinocyte-derived chemokine (KC), leptin, MCP-1, MCP-5, MIP-1α, MIP-2, MIP-3β, RANTES, normal T cell expressed stem cell factor, thymus and activation-regulated chemokine, tissue inhibitor of metalloproteinases-1, TNF-α, thrombopoietin, and vascular endothelial growth factor</td>
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Table 1. Proteins whose expressions are not affected by PDCD4 gene mutation

FIGURE 7. Selective increase of protein synthesis in PDCD4−/− cells. a, Protein synthesis in splenocytes as measured by [35S]methionine incorporation. Splenocytes were isolated from PDCD4+/+ and PDCD4−/− mice (n = 5), and cultured for 48 h in DMEM containing 10% FCS. [35S methionine was then added to the culture to a final concentration of 10 μCi/ml. Protein synthesis was then determined by quantitative ELISA (21). The differences between PDCD4+/+ and PDCD4−/− culture supernatants were collected and cytokine concentrations were determined by quantitative ELISA (21). The differences between PDCD4+/+ and PDCD4−/− cultures are statistically significant for all the parameters in this figure except the control group in c–e (p < 0.01 as determined by ANOVA). Data are representative of three separate experiments.

Finally, our data suggest that PDCD4 likely regulates the expression of a much larger number of proteins, it is reasonable to assume that the roles of PDCD4 may not be mediated only by these proteins. Because it is well recognized that immune suppression is associated with increased incidence of tumors in humans, presumably due to reduced immune surveillance against tumor cells, it is also plausible that PDCD4 indirectly regulates lymphoma genesis by promoting cellular immunity. Further studies are needed to elucidate the precise mechanisms whereby PDCD4 suppresses tumor genesis.

Protein translation is a well-coordinated event regulated by a number of repressors and enhancers (4–7). PDCD4 shares no sequence homology with other translation repressors such as 4E-BPs and may represent a new class of endogenous protein translation repressors. Because inflammation and oncogenesis are both associated with enhanced protein translation, it is generally assumed that inhibiting translation may help to suppress both inflammatory diseases and cancer. Additionally, a tumor can develop following chronic inflammation, and certain inflammatory molecules such as NF-κB can also promote tumor development (33). However, our results indicate that promoting inflammation and suppressing tumor development can be mediated by the same molecule. This unexpected combination of the PDCD4 function may be explained by the unique group of molecules selectively targeted by PDCD4, i.e., cytokines that promote oncogenesis but inhibit inflammation. By contrast, NF-κB may target genes that promote both oncogenesis and inflammation. Thus, although inflammation may promote tumor development, the roles of tumor suppressors in these two
types of diseases are likely dictated by the nature of their target molecules. The roles of other endogenous-translation repressors in cancer and inflammatory disease need now to be investigated. Recent genomic studies indicate that both inflammation and oncogenesis are associated with enhanced expression of myriad genes (34, 35). However, because mRNA levels do not always mirror those of proteins in the same cell, translational regulation plays an important role in determining the levels of proteins in the cell. Understanding the roles of translation regulators in protein synthesis during inflammation and oncogenesis is therefore crucial for elucidating the molecular mechanisms of the related diseases. Because different mRNAs have different 5'UTRs and are present at different levels in the cell, the effect of translation regulators is likely mRNA-specific. The gene knockout model reported here serves as a unique tool to address this issue. Using several complementary techniques, we found that PDCD4 deficiency preferentially affected a small number of proteins (Fig. 7). Using the Zuker mfold RNA structure program (36), we found that all these proteins (which include IFN-γ, IL-4, and IL-10) had structured GC-rich 5'UTRs. However, whether this is the only reason for the selectivity of the PDCD4 effect is not clear. More basic research is needed to unravel the mechanisms of the selectivity of translation factors. Because most endogenous translation repressors may regulate protein synthesis in a selective manner as described here, their roles in inflammation and cancer are likely dictated by the nature of the proteins they regulate. Thus, strategies targeting translation for the treatment of inflammatory disease and cancer would be most effective if they inhibit synthesis of only pathogenic proteins but not nonpathogenic ones.

**FIGURE 9.** Real-time PCR analysis of cytokine mRNA expression by splenocytes. Splenocytes were prepared and treated as in Fig. 7c. Total RNA was extracted, treated with DNase I, and purified with the RNeasy mini kit (Qiagen). The primers and probes for PCR were purchased from eBioscience. In the 20 μl PCR, 1 μl of cDNA and 1 μl of primers and probes for IL-4, IFN-γ, or internal control (18S RNA) were mixed with 10 μl of the TaqMan Fast Universal Master Mix (Applied Biosystems) and 8 μl of H2O. All PCR were performed on 7500 Fast System (Applied Biosystems). The relative gene expression levels were determined using 18S RNA as a control. Means and SDs of each group are shown.

**FIGURE 10.** Increased cytokine production by MOG-specific PDCD4-deficient T cells. Mice were treated as in Fig. 4b and sacrificed 35 d after the immunization. Splenocytes were cultured with or without 25 μg/ml MOG peptide and tested as described in Materials and Methods. Results are shown as means and SD of cytokine concentrations from a total of eight mice with four mice per group. The differences between the two groups are statistically significant for cultures with MOG peptide (p < 0.01). The experiments were repeated twice with similar results.
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Disclosures
The authors have no financial conflict of interest.

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