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Enhanced CD4+ T Cell Proliferation and Th2 Cytokine Production in *DR6*-Deficient Mice

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Summary

We have found that DR6, a member of the TNF receptor family, is highly expressed in resting T cells and downregulated in activated T cells. *DR6*-targeted mutant mice were generated and showed normal development. However, *DR6*$^{-/-}$ CD4$^+$ T cells hyperproliferated in response to TCR-mediated stimulation and protein antigen challenge. Activated *DR6*$^{-/-}$ CD4$^+$ T cells exhibited upregulated CD25 expression and enhanced proliferation in response to exogenous IL-2 stimulation. In addition, increased CD28 and reduced CTLA-4 expression were observed in these cells. Enhanced Th2 cytokine production by activated *DR6*$^{-/-}$ CD4$^+$ T cells was associated with the increased transcription factor NF-ATc in nuclei. DR6, therefore, functions as a regulatory receptor for mediating CD4$^+$ T cell activation and maintaining proper immune responses.

Introduction

Tumor necrosis factor (TNF) is a key mediator involved in immune regulation and inflammation. TNF family members mediate their biological functions through structurally related but functionally distinct receptors that belong to the TNF receptor (TNFR) family. The interactions between TNF family ligands and their receptors are involved in modulating a number of signaling pathways in the immune system, such as cell proliferation, differentiation, apoptosis, and survival (Wallach et al., 1999). While much attention has been focused on identifying the factors that are involved in regulating T cell activation and differentiation, the signaling molecules in this process have yet to be completely determined. Interaction between the T cell receptor (TCR) and specific MHC-peptide complex on antigen-presenting cells (APC) is required for T cell activation. Additionally, a costimulatory signal is provided by ligation of coreceptors on T cells with accessory molecules on APC (Lenschow et al., 1996b; Fallarino et al., 1998). The combination of these two signals induces optimal T cell activation.

Among these costimulatory molecules, ligation between CD28 on T cells and B7-1 (CD80) and/or B7-2 (CD86) on APC has been well characterized as a critical costimulatory interaction, resulting in increased IL-2 production and T cell activation. In vivo studies have also shown that CD28/CD86-mediated costimulation may be important for Th2 cell differentiation (Lenschow et al., 1996a; Khattri et al., 1999). However, recent data have suggested that other molecules can act either in synergy with CD28 or in a CD28-independent manner for T cell activation and differentiation (Gravestein and Borst, 1998; Gramaglia et al., 1998; Saoulli et al., 1998; Bachmann et al., 1999; Watts and DeBenedette, 1999). In vitro studies have suggested that an efficient T cell response requires the combined use of several ligand-receptor pairs, including several TNF/TNFR family members, such as CD40, CD27, CD30, 4-1BB, and OX-40. These molecules have been shown to deliver a costimulatory signal for T cell proliferation, cytokine production, and Th1/Th2 differentiation when engaged by their corresponding ligands or specific agonist antibodies (Gravestein and Borst, 1998; Grewal and Flavell, 1998; Chen et al., 1999; Flavell, 1999; Watts and DeBenedette, 1999). Observations from these studies suggest that TNF/TNFR family members can act as important accessory molecules for promoting an optimal T cell immune response when combined with TCR signals.

While many molecules including TNF/TNFR family members may be important for positive regulation of T cell immune responses, some negative regulatory molecules are also required to balance these responses. For instance, CTLA-4, which is expressed on activated T cells and also binds to CD80 and CD86, is believed to deliver a negative signal to the T cells. *CTLA-4*-deficient mice develop a lymphoproliferative syndrome and autoimmune disease (Khattri et al., 1999; Fraser et al., 1999; Nakaseko et al., 1999). These negative signals are believed to be essential for regulating homeostasis of immune responses.

Several transcription factors are required to promote T cells to be fully activated to proliferate and differentiate into effector cells. One of the most critical transcription factors is nuclear factor of activated T cells (NF-ATc). NF-ATc translocates to the nucleus in response to intracellular calcium signaling following T cell activation and thereby plays a key role to enhance TCR-mediated T cell growth and differentiation (Kuo and Leiden, 1999; Kiani et al., 2000). *NF-ATc*-deficient T cells exhibited a defect in peripheral T cell proliferation and Th2 differentiation (Ranger et al., 1998a; Yoshida et al., 1998). Therefore, regulating the transcriptional activity of NF-AT family members is a key step in controlling T cell functions, especially effector functions.

DR6 was identified as a death domain-containing receptor within the TNFR superfamily (Pan et al., 1998). The extracellular cysteine-rich motifs of DR6 have about 40% homology with osteoprotegerin (OPG) and TNFR2. The expression of human DR6 was observed in a number of tissues, including lymphoid tissues (Pan et al., 1998). However, the physiological functions of DR6 remain unknown. In the present study, we have generated *DR6*-deficient mice by gene targeting. Our findings demon-

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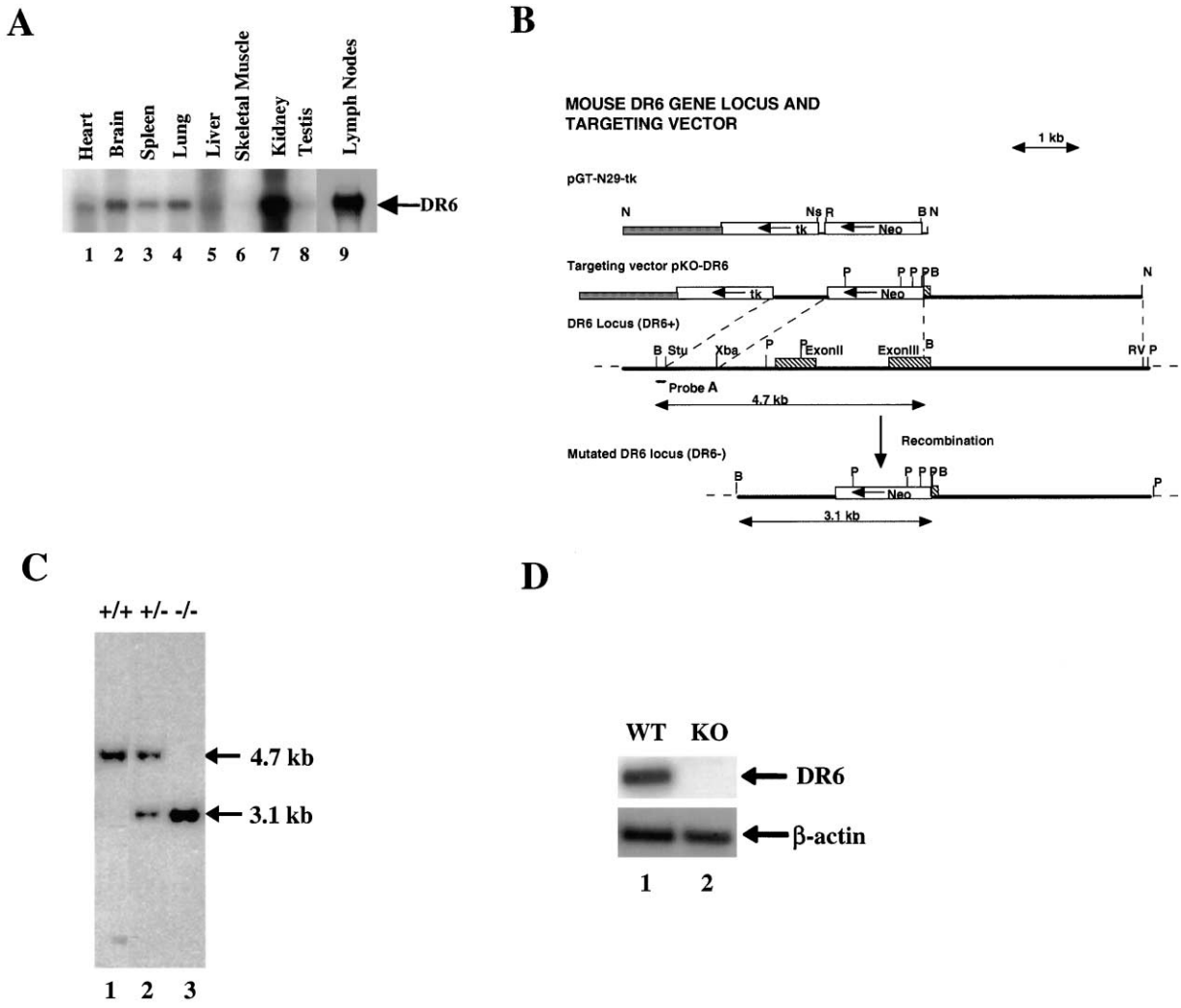

Figure 1. Generation of DR6-Deficient Mice by Gene Targeting

(A) Northern blot analysis of *DR6* transcripts in adult mouse tissues. The blot was also probed with a labeled β-actin gene to ensure that each lane has the same amount of RNA and that the RNA was intact (data not shown).

(B) Strategy for the targeted disruption of the *DR6* gene. The pGT-N29-tk vector, the targeting vector pKO-DR6, the genomic region at the *DR6* locus, and the predicted structure of the mutated *DR6* gene are illustrated. The restriction fragments used to construct the targeting vector are indicated (1.2 kb StuI-XbaI fragment and 3.5 kb BamHI-EcoRV fragment). The coding regions of the exons are presented as striped. Restriction enzyme sites (B, BamHI; N, NotI; Ns, NsiI; P, PstI; R, EcoRI; RV, EcoRV; Stu, StuI; Xba, XbaI) and the probe A for Southern blot analysis are indicated.

(C) Germline transmission of the *DR6* disruption is indicated by Southern analysis of mouse genomic DNA as the presence of all three expected genotypes (+/+, +/−, and −/−). The upper band (4.7 kb) corresponds to the wild-type allele, and the lower band (3.1 kb) corresponds to the mutant allele.

(D) Absence of the *DR6* transcript in the *DR6*-deficient mice. Poly(A)$^+$ RNA (0.5 μg per lane) from kidneys of 5-week-old mice was analyzed for *DR6* transcripts with a 524 bp DR6 cDNA probe derived from exon 4 of full-length *DR6* cDNA.

strate that DR6 is an important regulator for both T cell activation and Th cell differentiation.

Results

Generation of *DR6*-Deficient Mice

Like human DR6, murine DR6 is ubiquitously expressed in mouse tissues, and the highest expression was observed in kidney and lymph node (Figure 1A). To determine the physiological functions of DR6 in vivo, we generated *DR6*-deficient mice using homologous recombination in embryonic stem (ES) cells. We designed a targeting vector to replace exons II and III of the *DR6* gene, including the translation initiation site, with a neomycin resistance (pGK-*neo*') cassette (Figure 1B). Two targeted mutant ES clones identified by Southern Blot were injected into C57BL/6 blastocysts and resulted in chimeric mice that transmitted the disrupted *DR6* allele through the germline. Heterozygotes were intercrossed to generate homozygous mutant mice that were identified by Southern blot analyses of genomic DNA. BamHI bands, 3.1 and 4.7 kb, were detected as mutated and wild-type alleles, respectively (Figure 1C). The null mutation of *DR6* was demonstrated by the absence of *DR6*

Table 1. Immunofluorescent Profiles of T Cell Subpopulations

	Thymus		Spleen		Lymph Node	
T Cell Subpopulation	WT (1.6×10^8)[a]	*DR6* KO (1.4×10^8)	WT (1.2×10^8)	*DR6* KO (1.3×10^8)	WT (5.9×10^7)	*DR6* KO (6×10^7)
$CD4^+$ (%)	9.4 ± 0.3[b]	16.0 ± 0.2	20 ± 0.3	22.2 ± 0.4	47.5 ± 0.4	48.8 ± 0.2
$CD8^+$ (%)	2.5 ± 0.3	1.9 ± 0.1	13.0 ± 1	16.0 ± 2	15.5 ± 3	16.0 ± 2
$CD4^+$ $CD8^+$ (%)	83.7 ± 4	77.3 ± 5	0.62 ± 0.09	0.52 ± 0.07	4.9 ± 0.6	5.5 ± 0.4

Data represent the mean ± SEM of quadruplicate samples. There were four mice in each group.
[a] Total cell numbers.
[b] Percentage of individual cell population.

expression, as determined by Northern analysis of mRNA isolated from kidneys of wild-type and homozygous mutant mice (Figure 1D). Homozygous *DR6*-deficient mice were born at the expected Mendelian ratio and were fertile. Histological analysis revealed no significant changes in major organs, including lymphoid tissues up to 6 months of age. The cellularity in spleen and lymph nodes was comparable between wild-type and *DR6*-deficient mice (Table 1). These results indicate that DR6 is not essential for normal mouse development and lymphoid organogenesis.

We further examined whether lymphoid cell lineage development was affected in the absence of DR6. T cells, B cells, monocytes, neutrophils, and natural killer cells from thymi, lymph nodes, and spleens were analyzed by flow cytometry analysis (Table 1 and data not shown). No significant differences in cell numbers of different subpopulations in immune system were observed. The ratio between T and B cell populations, the percentages of $CD4^+$ and $CD8^+$ populations, as well as the activation stage of B and T cell populations also appeared to be normal (data not shown), suggesting that the absence of DR6 had no effect on cell lineage development in the immune system.

$CD4^+$ T Cell Proliferation Is Enhanced in *DR6*-Deficient Mice upon TCR Stimulation In Vitro

Since several TNF/TNFR family members were demonstrated to deliver a costimulatory signal for T cell immune response, we decided to examine if disruption of *DR6* affected T cell functions in vitro. Purified T cells from spleens were cultured in microtiter plates coated with different suboptimal doses of anti-CD3 and anti-CD28 antibodies, and T cell proliferation was determined by the [^{3}H]thymidine incorporation after a 48 hr incubation. Interestingly, the proliferation of T cells from *DR6*-deficient mice was enhanced 2- to 4-fold in response to anti-TCR/anti-CD28 compared with wild-type T cells (Figure 2A). The level of IL-2 secreted in these primary cultures also was examined after 24 hr incubation and showed no significant differences (Figure 2B). These results suggest that DR6 might negatively regulate T cell receptor-mediated cell growth, and the enhanced *DR6*-deficient T cell proliferation was not due to the higher IL-2 production.

To determine if enhanced T cell growth is CD28 dependent, we further analyzed the proliferative response of *DR6*-deficient T cells in the absence of CD28 signal. Purified T cells isolated from spleens of *DR6*-deficient mice and wild-type littermates were stimulated only in the presence of different concentrations of anti-CD3. Again, T cells from *DR6*-deficient mice showed about a 4-fold enhanced proliferation compared with control T cells (Figure 2C).

To further examine if this enhanced T cell growth applies to the subpopulations of *DR6*-deficient T cells, $CD4^+$ and $CD8^+$ T cell populations were purified and cultured in 96-well plates coated with anti-CD3 and anti-CD28. As shown in Figure 3A, the absence of DR6 had a profound hyperproliferative effect on the $CD4^+$ T cells but not the $CD8^+$ subset. To determine if DR6 is directly involved in TCR-mediated T cell growth, soluble DR6 was added to the wild-type T cell proliferation assay. Hyperproliferation of wild-type T cells as well as $CD4^+$ T cells was observed in a soluble DR6 concentration-dependent manner compared with the control IgG1-treated group (Figures 3B and 3C). These data suggest that DR6 is an important factor in regulating TCR-mediated T cell proliferation. To test if DR6 expression is also affected through a TCR-mediated pathway, we analyzed DR6 expression in resting versus activated $CD4^+$ T cells. Cells were harvested at 0, 24, and 48 hr after incubation with anti-CD3 and anti-CD28, and the expression of DR6 was determined by real-time PCR. Resting $CD4^+$ T cells expressed the highest level of DR6. However, after 48 hr activation, expression of DR6 was significantly reduced in $CD4^+$ T cells (Figure 3D). These data are consistent with the effects of *DR6* deficiency observed in vitro.

Increased T cell proliferation after stimulation could be due to either increased cell growth or reduction of cell death after disruption of *DR6*. Therefore, we next performed an activation-induced cell death (AICD) assay. No difference was observed between wild-type and *DR6*-deficient T cells in this assay (Figure 3E). These data suggested that the increased growth of *DR6*-deficient $CD4^+$ T cells was due to enhancement of T cell proliferative response after in vitro stimulation, which was supported by markedly increased BrdU labeling in *DR6*-deficient T cells in vitro (data not shown). These results were consistent with our observation that the number of T cells remained at similar level in naive wild-type and *DR6*-deficient mice and support the hypothesis that DR6 serves as a negative regulator for $CD4^+$ T cell growth after TCR stimulation.

Increased Th2 Cytokine Production in *DR6*-Deficient Mice

When activated by APCs, naive $CD4^+$ T cells undergo clonal proliferation and produce IL-2. The activated $CD4^+$ T cells may then become Th1 or Th2 effector cells,

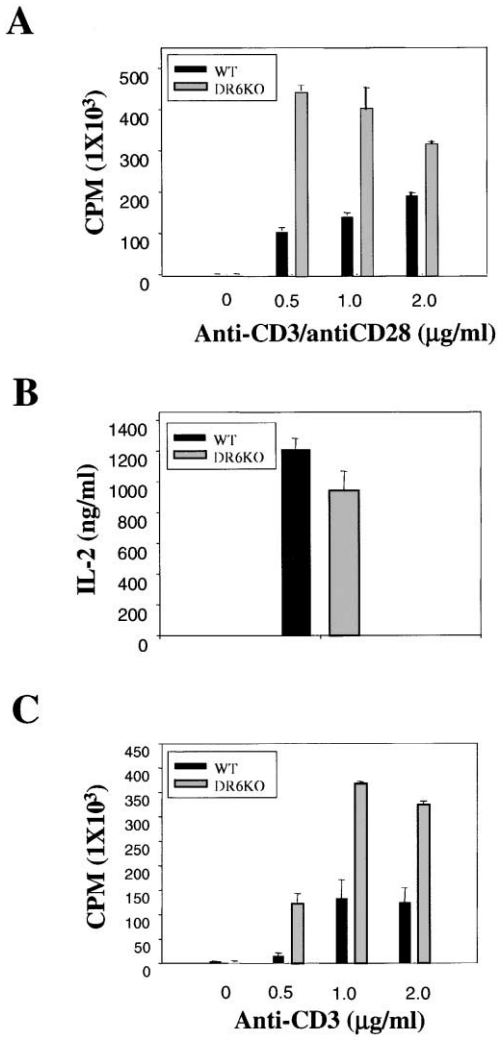

Figure 2. Enhanced Proliferation of Peripheral *DR6*-Deficient T Cells in Response to Anti-TCR/Anti-CD28 or Anti-TCR Stimulation

(A) Purified T cells from spleen were cultured for 48 hr in a 96-well plate coated with different concentrations of anti-CD3 and anti-CD28. [^{3}H]thymidine was added to the culture for the last 12 hr of incubation. The amount of [^{3}H]thymidine incorporation was examined and used to determine the proliferation of peripheral T cells in response to cross-linking TCR and CD28. (B) IL-2 secretion from the purified T cells was examined by ELISA after 24 hr stimulation with anti-CD3/anti-CD28. (C) Purified T cells from spleen were stimulated with anti-CD3 for 48 hr. [^{3}H]thymidine incorporation was used to estimate T cell responses after stimulation. The above data represent five independent experiments. *DR6*-deficient T cells were labeled as *DR6* KO for this figure and the rest of figures in this paper.

which mediate inflammatory or humoral responses, respectively. Although the polarization of Th cell differentiation is, at least in part, determined by the cytokine environment, signals from the TCR-CD3 complex and from the costimulatory factor CD28 may also affect cytokine production by mechanisms not yet understood (Constant and Bottomly, 1997). To investigate if DR6 has effects on Th cell differentiation, we measured cytokine production by purified CD4$^+$ T cells after stimulation with anti-CD3 and anti-CD28. Compared with wild-type T cells, *DR6*-deficient CD4$^+$ T cells secreted about 5-fold higher levels of Th2 cytokines, including IL4, IL-5, IL-10, and IL-13, while the secretion of Th1 cytokines, such as IL-2 and IFN-γ, remained comparable to the control CD4$^+$ T cells (Figure 4A). These data demonstrated that disruption of *DR6* resulted in a cytokine production profile characteristic of Th2 cells.

To exclude the possibility that the increased secretion of Th2 cytokines was due to the enhanced T cell proliferation, the cells were stimulated with anti-CD3/anti-CD28 for 48 hr, washed, counted, and then seeded at the same number for another 12 hr stimulation. Under these conditions, significantly higher Th2 cytokine secretion was observed in *DR6*-deficient T cells, whereas Th1 cytokine secretion remained at a similar level (Figure 4B). This result indicates that enhanced secretion of Th2 cytokines from *DR6*-deficient T cells was not due to expansion of the cells. To confirm this, we examined the Th differentiation of wild-type and *DR6*-deficient CD4$^+$ T cells in vitro. Purified CD4$^+$ T cells from *DR6*-deficient mice stimulated under Th2 condition showed dramatically increased IL-4 production compared to wild-type CD4$^+$ T cells (Figure 4C). In contrast, under Th1 differentiation condition, there were no obvious differences in the production of the Th1 cytokine IFN-γ between wild-type and *DR6*-deficient mice. Strikingly, *DR6*-deficient CD4$^+$ T cells produced 300-fold higher IL-4 compared with wild-type T cells, even under Th1 condition (Figure 4C). These data demonstrate that absence of DR6 during an ongoing immune response stimulates the establishment of a Th2 response.

T Cell Priming and Cytokine Production Are Enhanced in *DR6*-Deficient Mice

To define the in vivo effects of *DR6* deficiency on antigen-specific T cell activation and proliferation, *DR6*-deficient mice and their wild-type littermates were immunized with the protein antigen keyhole limpet hemocyanin (KLH). CD4$^+$ T cells were then purified from draining lymph nodes, and in vitro T cell proliferative recall responses were examined in the presence of APCs and KLH. As shown in Figure 5A, significantly enhanced T cell proliferative response was observed with *DR6*-deficient CD4$^+$ T cells. At a concentration of 100 μg/ml of KLH, the T cell proliferative response was 35-fold higher than the control T cells (Figure 5A). Similarly enhanced responses were observed with another protein antigen, hen egg lysozyme (HEL) (Figure 5B). However, it was not clear from these data whether the enhanced proliferative responses in *DR6*-deficient mice occurred at the level of the APC, the CD4$^+$ T cell, or both. To address this issue, KLH-primed CD4$^+$ T cells purified from wild-type and *DR6*-deficient mice were examined for their recall response in the presence of wild-type or *DR6*-deficient APC. The T cell recall response for *DR6*-deficient CD4$^+$ T cells remained at a similar level in the presence of wild-type APC (data not shown), indicating that DR6 is important to balance antigen-specific T cell responses during both the priming and effector phases of T cell activation. In addition, we also measured cytokine production from the primed T cell culture. The production levels of the Th1 cytokine (IFN-γ) and Th2 cytokines (IL-4, IL-5, IL-10) were significantly higher in *DR6*-deficient CD4$^+$ T cells compared to those of wild-type CD4$^+$ T

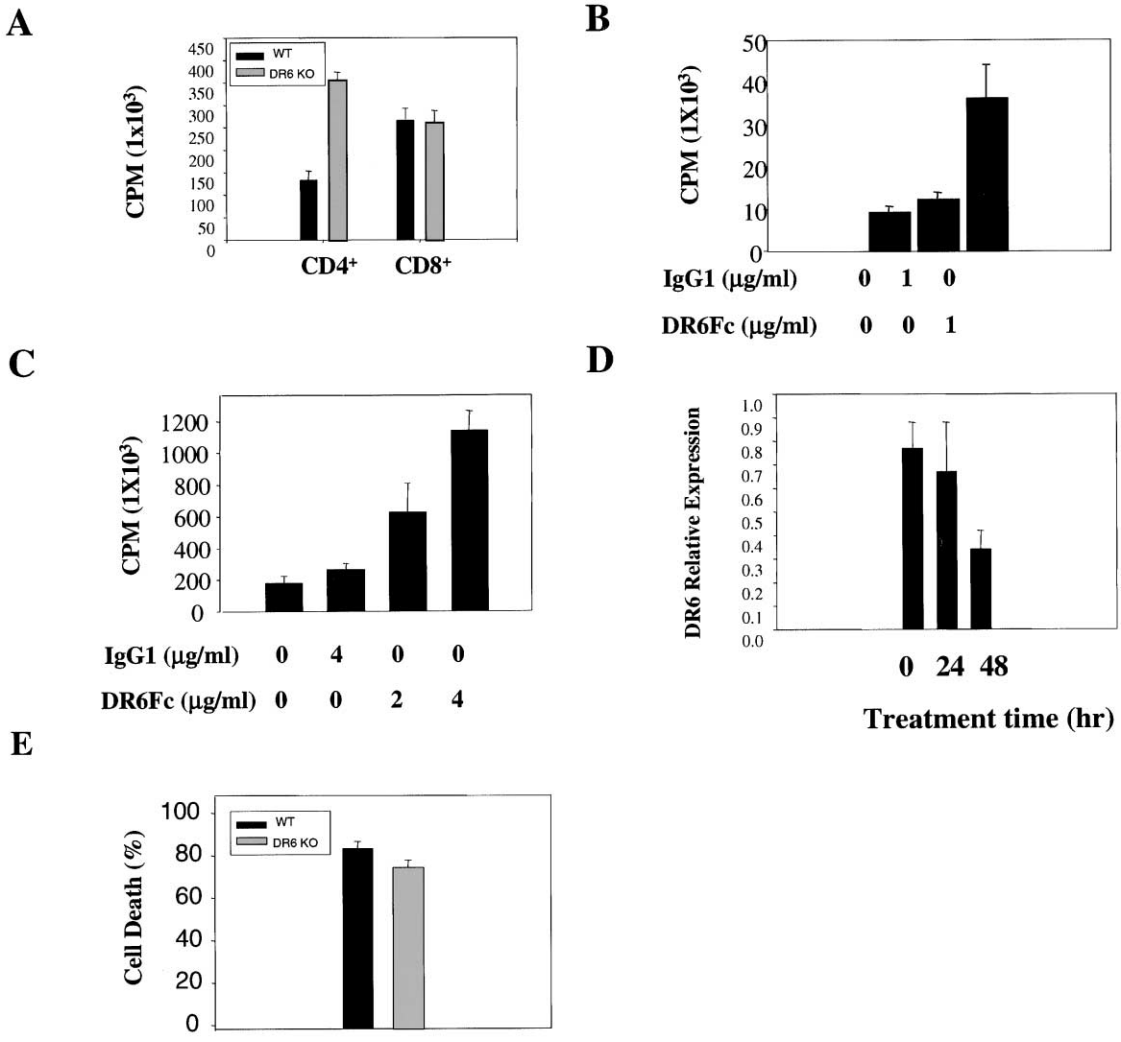

Figure 3. Enhanced Proliferation of *DR6*-Deficient CD4$^+$ T Cells

(A) CD4$^+$ and CD8$^+$ T cells were purified as described in the Experimental Procedures. The purified subpopulations of T cells were cultured for 48 hr with plate-bound anti-CD3 and anti-CD28. Proliferation was determined after 48 hr by [^{3}H]thymidine incorporation. (B) Soluble DR6 and IgG1 were used to treat wild-type T cells to block the interaction between the putative DR6 ligand and DR6 in the presence of anti-CD3. The cells were treated for 48 hr and subsequently pulsed with [^{3}H]thymidine. (C) Soluble DR6 and IgG1 were used to treat wild-type CD4$^+$ T cells to block the interaction between the putative DR6 ligand and DR6 in the presence of anti-CD3. The above data represents five independent experiments. (D) Real-time PCR to determine the expression level of DR6 in CD4$^+$ T cells before and after stimulation with 1 μg/ml anti-CD3/anti-CD28 at different time points. (E) The T cells from wild-type and *DR6*-deficient mice were treated as described, and the percentage of cells undergoing apoptosis was determined by staining with Annexin V and measurement by flow cytometry. Three other independent experiments showed similar results in this study.

cells (Figure 5C). The adjuvant we used in this study can strongly promote Th1 response, which may explain the higher Th1 response of CD4$^+$ T cells from *DR6*-deficient mice. Together, these results suggest that DR6 is critically involved in regulating Th cell immune response in vivo.

CD25 and CD28 Expression Is Upregulated and CTLA-4 Is Downregulated in Activated *DR6*-Deficient T Cells

Signaling through the IL-2 receptor plays a pivotal role for T cell growth. As shown in Figure 2B, secretion of IL-2 was not increased in the absence of DR6. To determine if IL-2 receptor (CD25) expression was altered in *DR6*-deficient T cells, we examined the expression level of CD25 on activated CD4$^+$ T cells. T cells from wild-type and *DR6*-deficient mice were activated with anti-CD3 and anti-CD28 for 24 hr, and cell surface expression of CD25 was measured by flow cytometry analysis. Strikingly, CD25 level was significantly higher in *DR6*-deficient T cells (Figure 6A), suggesting that enhanced IL-2 signaling might account for the hyperproliferation observed in *DR6*-deficient T cells. To confirm this hypothesis, T cells were activated for 12 hr with anti-CD3 and anti-CD28, washed, and stimulated with IL-2 for 48 hr. T cell proliferation was measured by [^{3}H]thymidine incorporation. As shown in Figure 6B, IL-2 stimulated a significantly higher proliferative response in *DR6*-deficient T

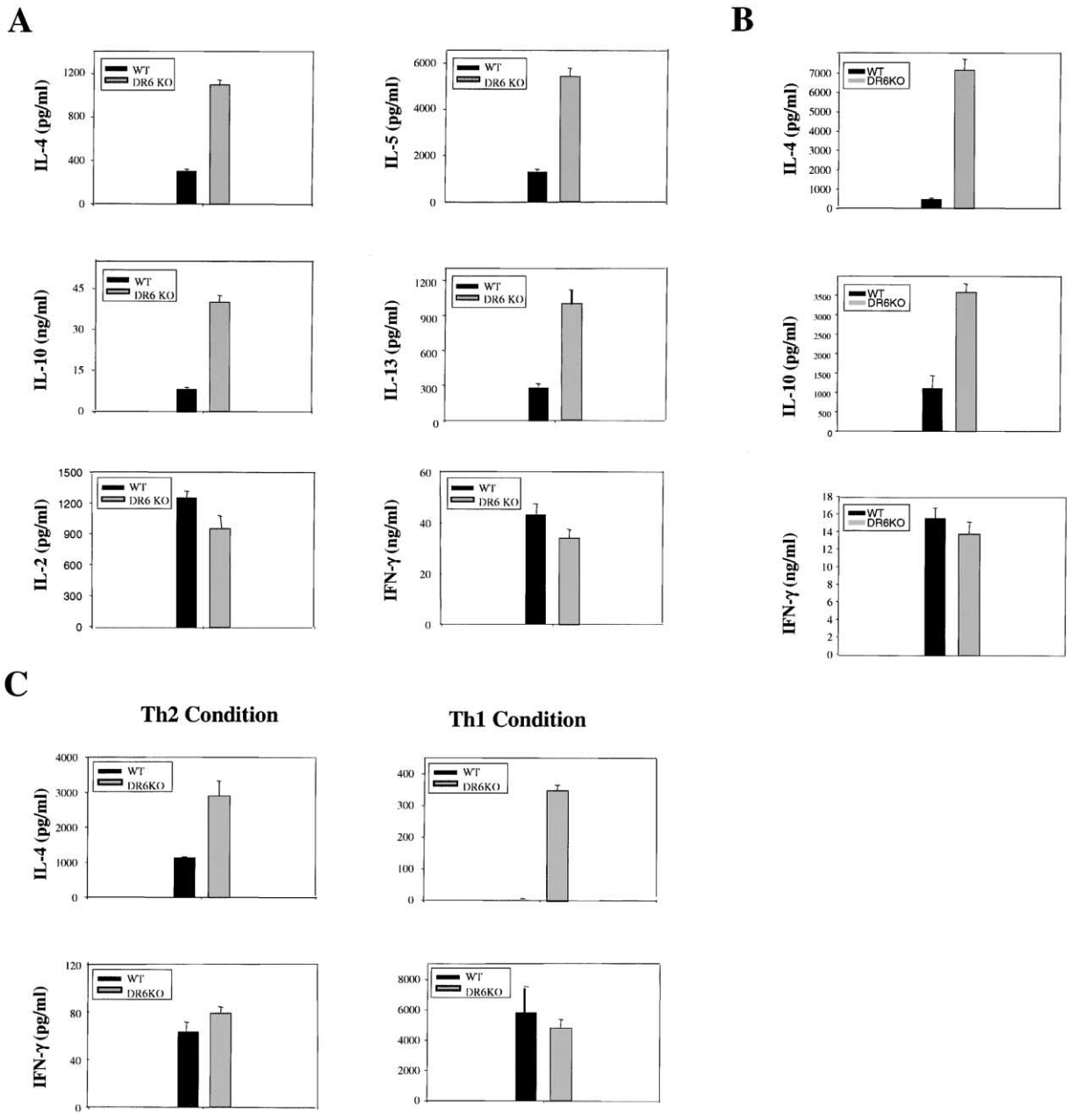

Figure 4. Increased Th2 Cytokine Production in *DR6*-Deficient Mice

(A) Purified $CD4^+$ T cells from WT and *DR6*-deficient mice were stimulated with plate-bound anti-CD3 plus anti-CD28 for 48 hr. The supernatant was harvested to determine cytokine levels by ELISA. (B) Th1 and Th2 cytokine production from wild-type and *DR6*-deficient T cells after normalization with cell numbers. (C) Purified $CD4^+$ T cells were differentiated under Th1 conditions and Th2 conditions as described, and the cytokine secretions after differentiation were determined by ELISA. The data represent the results from three independent experiments.

cells compared with wild-type T cells, indicating that enhanced proliferation observed in activated *DR6*-deficient T cells could be due to the higher expression level of CD25.

In addition to CD25, which is required for IL-2-mediated T cell growth, CD28 and CTLA-4 are also involved in regulating T cell activation, proliferation, and TCR-mediated immune responses (Fallarino et al., 1998; Fraser et al., 1999). To determine if the absence of DR6 affects cell surface expression of these two important receptors after stimulation, the expression of CTLA-4 and CD28 were examined before and after TCR stimulation. No difference was observed before stimulation (data not shown). Upon stimulation, the cell surface level of CTLA-4 was clearly downregulated in *DR6*-deficient T cells (Figure 6C). In contrast, CD28 expression was upregulated and exhibited two times higher than wild-type T cells in activated *DR6*-deficient T cells (Figure 6D). Furthermore, the effect of *DR6* deficiency on the expression of these key molecules on activated T cells

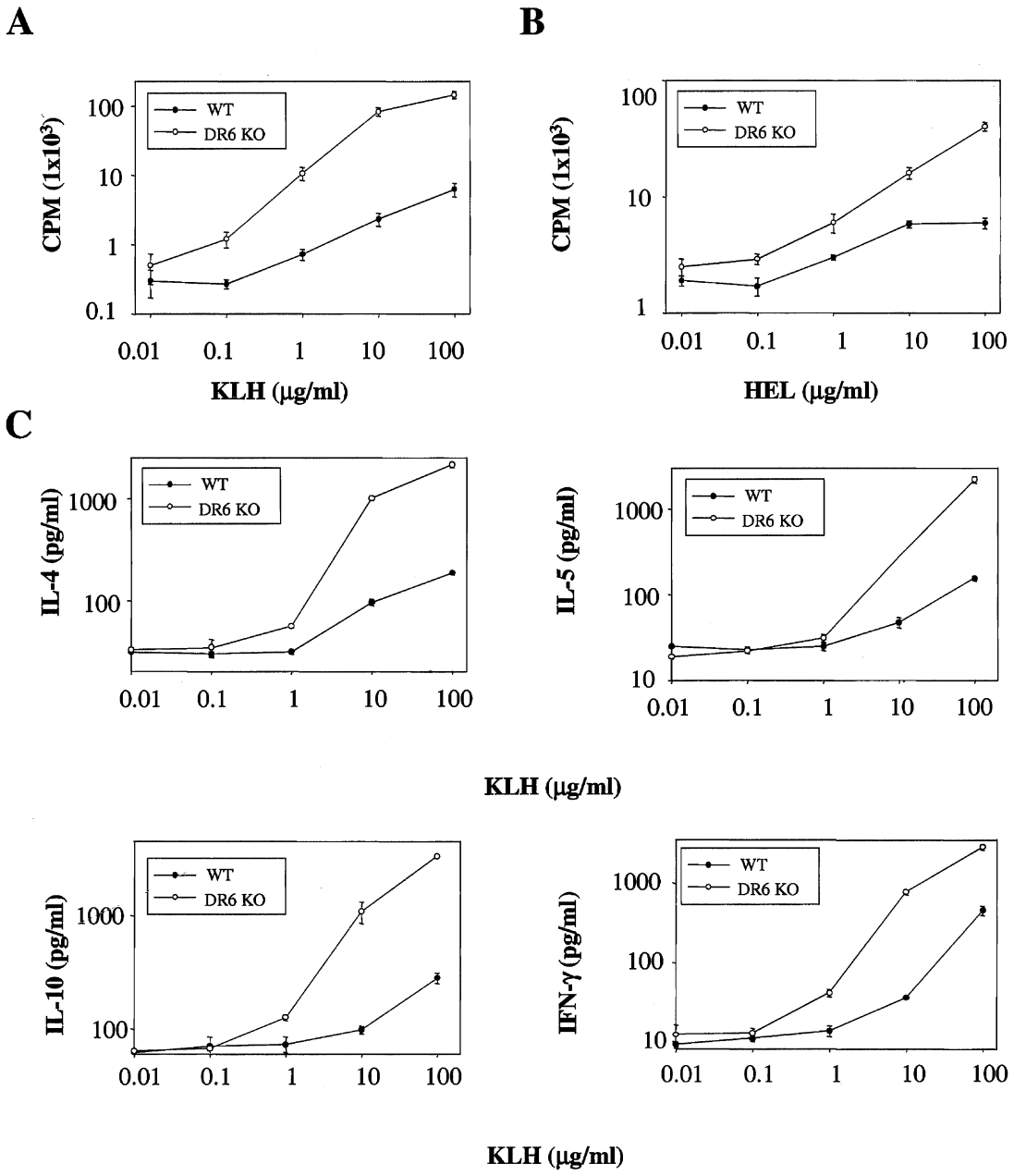

Figure 5. Enhanced In Vivo CD4$^+$ T Cell Priming, In Vitro T Cell Recall Responses, and Cytokine Production in the Absence of DR6

(A) CD4$^+$ T cells from *DR6*-deficient mice have enhanced recall proliferation in responses to protein antigens. Wild-type mice and *DR6*-deficient mice were immunized with KLH in the hindfootpads. At 9 days after immunization, draining lymph nodes were extracted and subjected to an in vitro challenge with various concentrations of KLH in the presence of antigen-presenting cells. After culturing for 4 days, [^{3}H]thymidine uptake was measured. (B) Wild-type and *DR6*-deficient mice were immunized with another protein antigen, HEL, and the CD4$^+$ T cell recall responses were measured as above. (C) The supernatants from in vitro T cell recall responses of wild-type and *DR6*-deficient mice were collected, and IL-4, IL-5, IL-10, and IFN-γ levels were measured by ELISA. The data represent the results from four mice in each group.

appears to be specific, since other activation markers, such as CD69, did not show significant differences between wild-type and *DR6*-deficient T cells (data not shown). Together, the higher cell surface expression of CD25, combined with the distinct surface expression level of CTLA-4 and CD28, may contribute to the observed increase in CD4$^+$ T cell proliferation and Th2 cytokine production in activated *DR6*-deficient T cells both in vivo and in vitro.

Absence of DR6 Results in Increased NF-ATc Nuclear Translocation and DNA Binding Activity

To further understand the potential mechanism through which DR6 regulates T cell immune response, we next examined the impact of *DR6* deficiency on NF-ATc and NF-κB, which are key transcription factors involved in TCR-mediated signaling. Upon T cell activation, NF-ATc is dephosphorylated and translocated into nucleus to activate gene transcription, whereas NF-κB is translo-

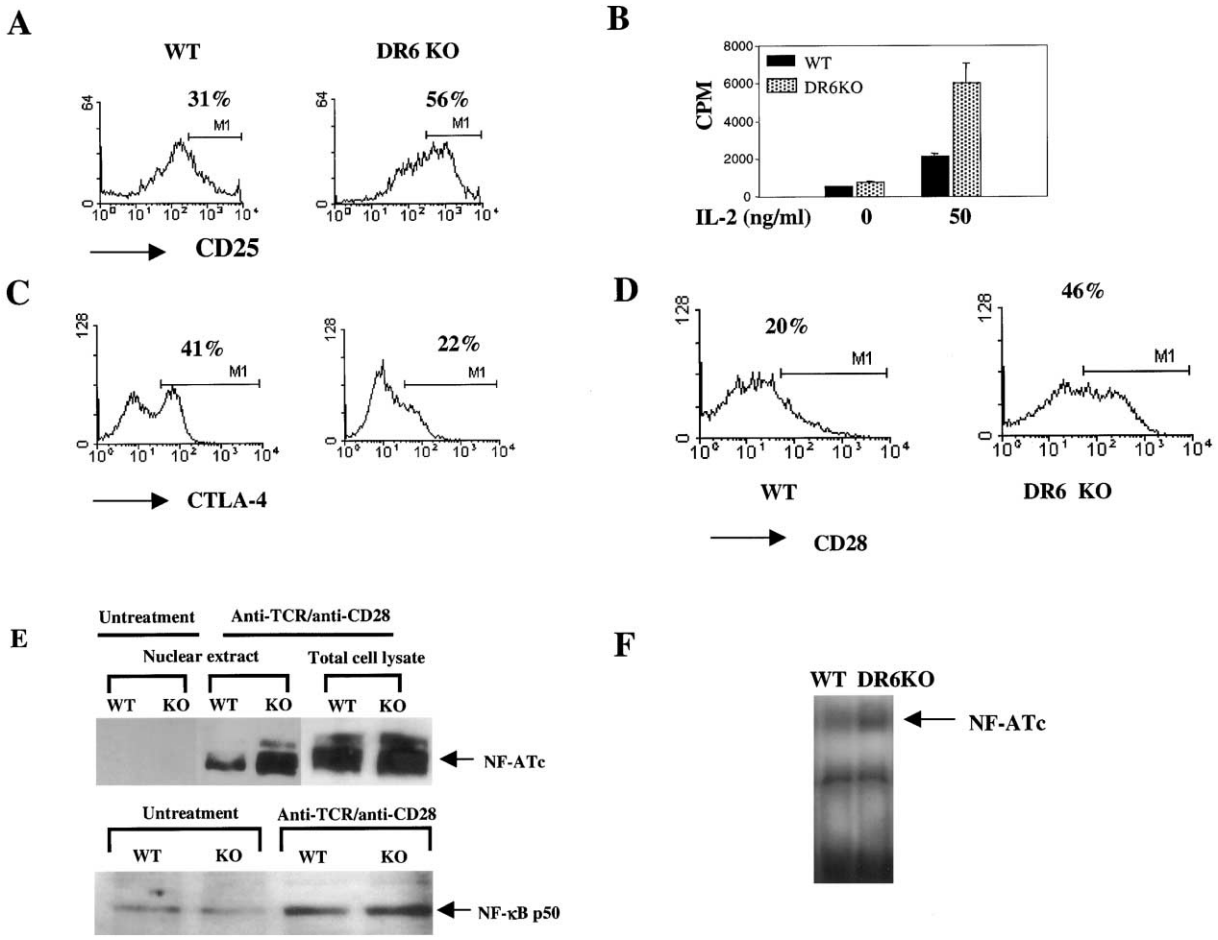

Figure 6. Potential Mechanisms for Enhanced T Cell Growth and Upregulating Th2 Cytokine Production in the Absence of DR6

(A) Cell surface expression of CD25 from wild-type (left panel) and *DR6*-deficient $CD4^+$ T cells (right panel) after 24 hr stimulation with 0.5 μg/ml anti-CD3 and anti-CD28.

(B) Enhanced T cell proliferation in response to exogenous IL-2. $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 (0.5 μg/ml) for 12 hr, and cells were washed and incubated with 50 ng/ml IL-2 for another 48 hr.

(C) The surface expression of CTLA-4 on wild-type (left panel) and *DR6*-deficient $CD4^+$ T cells (right panel) after 72 hr stimulation with anti-CD3 (1 μg/ml) was measured by flow cytometry.

(D) The surface expression of CD28 on wild-type (left panel) and *DR6*-deficient $CD4^+$ T cells (right panel) after 72 hr stimulation with anti-CD3 (1 μg/ml) was measured by flow cytometry.

(E) The protein levels of NF-ATc and NF-kB p50 in the nuclear extracts were measured by immunoblot analysis. Nuclear extracts were made from $CD4^+$ T cells before and after stimulation with anti-TCR/anti-CD28 (1 μg/ml) for 72 hr. Nuclear extracts were prepared as described in Experimental Procedures, and immunoblot analyses were performed with specific antibodies.

(F) Nuclear NF-ATc from activated wild-type and *DR6*-deficient $CD4^+$ T cells was functional as confirmed by gel mobility shift assay. The middle bands were nonspecific binding, since they were not competed by cold homologous oligonucleotide.

cated into nucleus after disassociating from phosphorylated IκB (Olsson et al., 1999). Purified $CD4^+$ T cells from wild-type and *DR6*-deficient lymph nodes were stimulated with anti-TCR, and nuclear extracts were subsequently prepared and subjected to Western blot analyses. These studies showed that the level of NF-ATc in the nucleus was dramatically increased in *DR6*-deficient T cells. The increased NF-ATc in the nucleus of *DR6*-deficient T cells is not due to the enhanced NF-ATc in these cells, since no significant difference was observed for total and cytoplasmic NF-ATc levels upon stimulation (Figure 6E and data not shown). Consequently, higher DNA binding activity of NF-ATc from *DR6*-deficient T cells was observed in the gel mobility shift assay (Figure 6F), and the specificity of DNA binding was confirmed by homologous oligonucleotide competition and anti-NF-ATc supershift assays (data not shown). In contrast, the NF-κB pathway was not affected, as demonstrated by the similar nuclear level of p50 Rel A protein after stimulation in both wild-type and *DR6*-deficient $CD4^+$ T cells (Figure 6E). Nuclear level of another member of the NFAT family, NF-ATp, was also tested in our study, and no significant difference was observed (data not shown). These data provided convincing evidence that DR6 can specifically mediate NF-ATc activation and its DNA binding activity in nucleus upon TCR stimulation.

Discussion

DR6 is a member of the TNF receptor family. A recent study has suggested that it may be involved in mediating

apoptosis and activation of NF-kB and JNK signaling in vitro (Pan et al., 1998). However, the physiological functions of DR6 still remain unknown. To determine the in vivo function of DR6, we generated *DR6*-deficient mice by gene targeting and extensively investigated the impact of *DR6* deficiency on modulating T cell immune responses. Our results show that DR6 is not required for normal development. However, deficiency of *DR6* led to hyperproliferation of $CD4^+$ T cells in response to TCR stimulation and protein antigen challenges. Enhanced Th2 cytokine production was also observed in *DR6*-deficient mice. DR6 appears to mediate these effects by regulating key molecules involved in T cell activation and differentiation upon stimulation of T cells. Thus, DR6 functions as a critical molecule in regulating the TCR signaling pathway and plays an important role in mediating T cell functions.

Recently, interactions between TNF and TNF receptor family members, including CD40/CD40L, CD30/CD30L, CD27/CD27L, 4-1BB/4-1BBL, RANK/RANKL (OPGL), HVEM/LIGHT, and OX40/OX40L, have also been demonstrated in mediating T cell costimulation and/or antigen-presenting cell activation (Reviewed by Watts and DeBenedette, 1999; Tamada et al., 2000). In this study, we demonstrated that the absence of DR6 resulted in enhanced $CD4^+$ T cell growth in a CD28-independent manner in vitro. Under in vitro conditions investigated, mature peripheral T cells from *DR6*-deficient mice displayed a significantly stronger $CD4^+$ T cell proliferative response than cells from wild-type littermates upon activation with suboptimal concentrations of anti-TCR or anti-TCR combined with anti-CD28 costimulation, whereas $CD8^+$ T cell growth was unaffected. Interestingly, DR6 is expressed in both $CD4^+$ and $CD8^+$ T cells. The highest expression of DR6 was observed in the resting $CD4^+$ and $CD8^+$ T cells. DR6 expression level was dramatically reduced after 48 hr stimulation (Figure 3D and data not shown). Our in vitro study only showed profound effect of *DR6* deficiency on $CD4^+$ T cells. The explanation for this observation is not immediately apparent and could be due to difference in TCR signaling mechanism or difference of effector molecules coupled to DR6 between these two subsets of T cells. For example, the expression level of DR6 ligand, yet to be identified, on $CD8^+$ T cells in our in vitro study may be below the threshold that is required for delivering the inhibitory effect of DR6 on T cell growth.

$CD4^+$ T cells from *DR6*-deficient mice showed enhanced proliferation in response to protein antigens and TCR stimulation. The higher proliferation could be due to increased TCR signaling through increased T cell receptor expression. However, no difference of cell surface expression of TCR/CD3 complex was observed between wild-type and *DR6*-deficient T cells before and after stimulation (data not shown). Furthermore, combination of ionomycin and PMA also enhanced *DR6*-deficient $CD4^+$ T cell proliferation (data not shown). This suggests that the phenotypes observed in *DR6*-deficient T cells might be due to its effect on either downstream TCR signaling or through regulating other key molecules on T cells involved in controlling T cell growth. In this study, we showed that both IL-2 receptor (CD25) and costimulatory receptor (CD28) are more dramatically upregulated and resulted in at least a 2-fold increase, whereas inhibitory receptor (CTLA-4) is significantly downregulated in activated *DR6*-deficient T cells compared with wild-type T cells. Furthermore, *DR6*-deficient T cells showed augmented cell proliferation and cell cycle progression in response to exogenous IL-2 (Figure 6B and data not shown). These results suggest that enhanced T cell proliferation in response to plate-bound antibodies is at least partially due to the increased expression of IL-2 receptor, whereas the increased CD28 and reduced CTLA-4 expression could play an important role when APCs deliver the T cell activating signals (e.g., in vivo foreign antigen challenge). Another possibility is that $CD4^+$ T cell hyperproliferation could be the result of reduction of activation-induced cell death, since DR6 is a death domain-containing receptor, and it was shown to be able to induce apoptosis in some cell types from in vitro study (Pan et al., 1998). However, no difference in activation-induced cell death was observed between wild-type and *DR6*-deficient T cells (Figure 3E). These data indicate that DR6 may function as a regulatory receptor rather than as an apoptosis modulator for TCR-mediated signaling.

CD28 is one of the most critical costimulatory molecules and plays an important role in stimulating T cell proliferation and Th2 cell differentiation (Lenschow et al., 1996a, 1996b; Khattri et al., 1999). In contrast, CTLA-4, which is homologous to CD28, binds to the same ligand and delivers a negative signal to T cells that leads to the inhibition of T cell proliferation (Fallarino et al., 1998; Oosterwegel et al., 1999). Furthermore, the $CD4^+$ T cell subset present in *CTLA-4*-deficient mice showed predominantly Th2 responses and secreted high levels of IL-4 and IL-5 upon TCR stimulation. In contrast, CTLA-4 costimulation resulted in differentiation of naive $CD4^+$ T cells toward Th1 and inhibits Th2 subset differentiation by affecting IL-4 production (Oosterwegel et al., 1999; Nariuchi and Kato, 2000). Therefore, reduction of CTLA-4 expression or increased CD28 expression should result in increases in both T cell proliferation and Th2 differentiation, which was demonstrated in our *DR6*-deficient mice. Previous studies have demonstrated that *CTLA-4*-deficient mice have a dramatic accumulation of T cells in lymph nodes, and these T cells exhibited an activated phenotype (Madelbrot et al., 1999). However, no significant difference was observed in the numbers of thymus and peripheral T cells in naive *DR6*-deficient mice. In contrast, T cells from draining lymph nodes of *DR6*-deficient mice were about three times more abundant than those of their wild-type littermates after KLH challenge (data not shown), which is consistent with the observation that CTLA-4 is downregulated only after T cells are activated (see Figure 6C). These data strengthen the evidence that DR6 may modulate signals generated through the TCR/CD28/CTLA-4 pathway.

Development and responses of immune system are well coordinated by an interacting network of transcription factors. One of the most important transcription factors, NF-ATc, has been demonstrated to play a critical role in promoting peripheral T lymphocyte proliferation and Th2 cell differentiation. NF-ATc is also necessary for optimal IL-4 gene expression, which is essential for Th2 cell polarization (Yoshida et al., 1998; Ranger et al., 1998a; Kuo and Leiden, 1999). We showed here that the nuclear level of NF-ATc was significantly increased in activated *DR6*-deficient $CD4^+$ T cells, and the accumulated NF-ATc in *DR6*-deficient T cells was functional

as shown by the enhanced DNA binding activity (Figure 6F). Interestingly, NF-ATc is also involved in upregulating CD25 expression in activated T cells (Schuh et al., 1998), which is consistent with our observation. Furthermore, nuclear level of NF-ATp, which is another member of NFAT family to serve as a negative regulator for T cell proliferation and Th2 cell differentiation (Ranger et al., 1998b), exhibited no significant difference in our study.

NF-ATc and *NF-ATp* double-knockout T cells have been shown to be deficient in global cytokine production and T cell effector functions (Peng et al., 2001), further supporting that NF-ATc and NF-ATp are essential for the completion of T cell differentiation. However, the functions of NF-ATc and NF-ATp in regulating the production of effector cytokines may be largely redundant, as shown by the previous work from NFAT single-mutant T cells (Hodge et al., 1996; Ranger et al., 1998a). Thus, the increased nuclear level of NF-ATc in the activated *DR6*-deficient T cells makes a major contribution to the enhanced Th2 cytokine production, implicating that DR6 plays a predominant role in Th cell differentiation. Our data demonstrate that DR6 is an important regulatory factor involved in controlling TCR-mediated T cell differentiation through the NF-ATc pathway. NF-ATc has been shown to be dephosphorylated by calcineurin in cytosol and translocated into the nucleus upon TCR activation. The translocated NF-ATc can then be phosphorylated and exported out of the nucleus by GSK3 (Beals et al., 1997). NF-ATc is believed to be involved in JNK signaling, whereas *JNK1*/*JNK2*-deficient T cells showed higher nuclear levels of NF-ATc and produce increased Th2 cytokines (Dong et al., 1998, 2000; Chow et al., 2000). Interestingly, overexpression of DR6 was shown to activate JNK in vitro (Pan et al., 1998), and JNK was demonstrated to inhibit targeting of the protein phosphatase calcineurin to NF-ATc (Chow et al., 2000). Therefore, absence of DR6 may, to some extent, reverse the inhibitory effect of JNK on calcineurin to NF-ATc and promote nuclear translocation of NF-ATc.

Together, we provided convincing evidence that DR6 functions as a regulatory molecule in $CD4^+$ T cells for TCR-mediated cell proliferation and differentiation, by mediating nuclear levels of an important transcription factor, NF-ATc, and several critical molecules, such as CD25, CD28, and CTLA-4, on activated T cells. Modulation of DR6/DR6 ligand interaction might be a potential strategy for the treatment of Th2-associated diseases, such as atopy and asthma. Thus, understanding the DR6/DR6L signaling pathway may yield insights into the control of diverse immune responses, including rheumatoid arthritis, graft-versus-host disease, and various other autoimmune disorders.

Experimental Procedures

Generation of *DR6*-Deficient Mice

The sequence of the murine *DR6* cDNA was deposited in GenBank, with an accession number AF322069. Using the sequence of *DR6* cDNA as a probe, we isolated mouse genomic DNA clones corresponding to the *DR6* locus from mouse strain 129/Sv (FixII phage library, Stratagene Inc., La Jolla, CA). An 8.1 kb Stu-EcoRV genomic DNA fragment corresponding to *DR6* was identified. A targeting vector (pKO-DR6) was constructed in the vector pGT-N29 (New England BioLabs, Beverly, MA) by replacing an internal 3.4 kb XbaI-BamHI fragment of *DR6* (encompassing the translation initiation site) with a neomycin resistance cassette (pGK-*neo*). The neomycin resistance cassette was placed in the antisense orientation to *DR6* transcription. A 2.0 kb MCl-tk cassette was added to the 5′ end of the Stu-EcoRV *DR6* genomic fragment.

R1 ES cells were electroporated with Not-linearized pKO-DR6. Genomic DNA from transfectants resistant to G418 (300 μg/ml) (GIBCO-BRL, Rockville, MD) and gancyclovir (2 μM) were characterized by BamHI digestion and hybridization with probe A. This probe is a 200 bp BamHI-StuI fragment of the *DR6* locus. Chimeric mice were produced by microinjection of targeted mutant embryonic stem cells into 3.5-day-old C57BL/6 (B6) blastocysts. The contribution of embryonic stem cells to the germline of chimeric mice was assessed by breeding with B6 mice and screening for agouti offspring. Germline transmission of the *DR6* mutation to their F1 129/B6 offspring was confirmed by Southern analysis of tail DNA. F2 animals were maintained in sterilized microisolator cages and used for further analyses.

RNA Preparation and Northern Blot Analysis

Kidneys from wild-type and *DR6*-deficient mice were used for total RNA preparation with the TRIzol reagent protocol as recommended by the manufacturer (GIBCO-BRL). Northern blot analysis was performed with poly(A)$^+$ RNA from wild-type and *DR6*-deficient mouse kidneys. Poly(A)$^+$ RNA was prepared from the total RNA, using Oligotex (Qiagen, Santa Clarita, CA). The probe for the Northern blot was a PCR-amplified 524 bp fragment and corresponding to nucleotides 1340–1864 of the murine *DR6* cDNA. The forward primer and reverse primer were 5′-GGTATTGACATCTTGAAGCTTGTAGCA-3′ and 5′-GACCTGCCGCAACACTGTGTGTCC-3′, respectively. The probe was labeled with [^{32}P]d-CTP with Primer-it II labeling kit (Stratagene Inc.). Hybridization was performed according to the manufacturer (Clontech, Pola Alto, CA).

Real-Time Quantitative PCR

The expression pattern of *DR6* was determined by quantitative PCR analysis (Taqman®, Applied Biosystems, Foster City, CA) using gene-specific oligonucleotides for *DR6* (probe 5′-FAM-CCACGATG TCCCTTCCTCCACCTATG-TAMRA and primers 5′-TTCTCACCCT GAGCATATGGAA and 5′-TGTTGAGTTCATGCCTTGGG) or 18s rRNA (part 4310893E, Applied Biosystems). The PCR reactions included cDNA made from T cells stimulated at different time points and were performed in an ABI 7700 (Applied Biosystems) using standard conditions. The relative amount of either DR6 or 18s RNA in each sample was calculated from a standard curve made from a dilution series of one of the samples. The amount of DR6 RNA was then normalized to the amount of 18s rRNA.

Flow Cytometry Analysis

Characterization of lymphocyte populations in wild-type and *DR6*-deficient mice was determined by staining cells from bone marrow, thymus, lymph nodes, and spleen with fluorescent antibodies to specific cell surface markers. Markers including Ter119, CD41, CD220, CD45, and CD117 were used for bone marrow cell staining, while CD4, CD8, and CD3 were used as T cell markers for thymus cell staining. CD4, CD8, Thy1.2, TCR$\alpha\beta$, and TCR$\gamma\delta$ were used as T cell markers; CD220, CD19, and IgM were used as B cell markers; CD23 and IgD were used as B cell activation markers; NK1.1 was used as a nature killer cell marker; CD11c was used as a monocyte marker; Ly-6-G was used as a neutrophil marker; and CD69 and CD25 were used as T and B cell activation markers. Both FITC- and PE-conjugated anti-mouse antibodies were purchased from BD PharMingen (San Diego, CA).

Proliferation Assays

For T cell proliferation assays, purified T cells were stimulated for 48 hr or 72 hr and pulsed for the last 12 hr incubation with 1 μCi of [^{3}H]thymidine, and thymidine incorporation was quantitated using a scintillation counter. For IL-2 response assays, purified $CD4^+$ T cells were stimulated with suboptimal concentration of anti-CD3/anti-CD28 (0.5 μg/ml for both antibodies) for 12 hr. T cells were then washed and stimulated with 50 ng/ml IL-2 for 48 hr, and the

proliferation was measured by [^{3}H]thymidine incorporation. Soluble DR6 treatment was similar to the above procedure, except that only wild-type T cells or CD4$^+$ T cells and 0.2 μg/ml anti-CD3 were used. Soluble DR6 was obtained from R&D systems (Minneapolis, MN).

Activation-Induced Cell Death Assay

T cells were isolated and cultured with plate-bound anti-CD3 (10 μg/ml, BD PharMingen) for 24 hr, followed by culturing in media containing murine recombinant IL-2 (30 μg/ml; R&D systems) for 4 days. The activated lymphocytes were treated with plate-bound anti-CD3 (1 μg/ml; BD PharMingen) for 24 hr. Treated cells were then subject to apoptosis assay by Annexin V staining (BD PharMingen).

Cytokine ELISA

Quantitation of cytokines in cell culture supernatants after stimulation was performed by a sandwich ELISA as described (Hodge et al., 1996).

Preparation of Purified T Cells, CD4$^+$, and CD8$^+$ T Cells

Lymph node cells were purified through negative selection. In brief, cells were incubated with anti-CD8 or anti-CD4 and anti-I-A^b (BD PharMingen) for 30 min. The cell/antibody mixture was then incubated with goat anti-rat IgG (H&L)-labeled magnetic beads (PerSeptive Biosystems, Framingham, MA) for 20 min followed by magnetic field separation to remove MHC class II-, CD8-, or CD4-expressing cells. Cell purity was determined using FITC-labeled anti-CD4 or PE-labeled anti-CD8 followed by the flow cytometry analysis. This procedure generates 90%–96% CD4$^+$ or CD8$^+$ T cells. Alternatively, labeled CD4$^+$ and CD8$^+$ cells were sorted by a fluorescence cell sorter. Total T cells from spleen were purified using the same procedure, except that B220 and other MHC class II-expressing cells were removed in this process.

Th1 and Th2 Differentiation

Th1 and Th2 cell differentiation was performed according to the method of McKenzie et al. (1998). In brief, purified CD4$^+$ cells were cultured on anti-CD3- and anti-CD28-coated plates (1 μg/ml) in the presence of exogenous cytokines or anti-cytokine antibodies as indicated. IL-2 (10 ng/ml) was added to all cultures. For Th1 differentiation, IL-12 (5 ng/ml) and anti-IL-4 antibody (2 μg/ml; R&D Systems) were added to the culture. For Th2 differentiation, IL-4 (100 ng/ml) and anti-IFN-γ (2 μg/ml) were added to the culture.

In Vivo T Cell Priming and Recall Response

DR6-deficient female mice and wild-type F2 controls (6-week-old; four mice in each group) were immunized in the hindfootpads with 100 μg KLH or HEL in complete Freund's adjuvant (CFA). Draining lymph nodes were then isolated after 9 days. In vitro T cell recall proliferative responses were examined. CD4$^+$ T cells were purified as described earlier and cultured in the presence of APCs (irradiated splenocytes from wild-type and *DR6*-deficient mice with 3000 rads). Different concentrations of KLH or HEL were added for 4 days followed by [^{3}H]thymidine incorporation for 12 hr. Cytokine secretion by CD4$^+$ T cells from KLH-immunized mice was assayed by ELISA.

Western Blot Analysis

Nuclear extracts for Western blot analysis were prepared from wild-type and *DR6*-deficient CD4$^+$ T cells that had been stimulated with plate-bound anti-CD3/anti-CD28 (1 μg/ml) for 3 days. Nuclei were isolated as described (Liu et al., 1997). The blots were probed with antibodies specific to NF-ATc (BD PharMingen) and NF-κB p50 (Santa Cruz, CA), respectively. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies and developed by chemiluminescence.

Gel Mobility Shift Assay

GMSAs were performed as previously described (Liu et al., 1997), using 5 μg nuclear proteins and 0.5 μg poly [d (I-C)]. The following oligonucleotides were used as probes (Santa Cruz): first strand, 5′-gggCGCCCAAAGAGGAAAATTTGTTTCATA-3′; second strand, 5′-gggTATGAAACAAATTTTCCTCTTTGGGCG-3′. Small letters indicate linker nucleotides for labeling purpose. Cold homologous oligonucleotide was used for competition assay to test the specific binding, and anti-NF-ATc and anti-NF-ATp (BD PharMingen) were used for supershift assays.

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