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Claudia Volpi, Pere Santamaria, Maria C. Fioretti and Paolo  
Puccetti

*J Immunol* 2006; 176:6752-6761; ;  
doi: 10.4049/jimmunol.176.11.6752  
<http://www.jimmunol.org/content/176/11/6752>

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# The Combined Effects of Tryptophan Starvation and Tryptophan Catabolites Down-Regulate T Cell Receptor $\zeta$ -Chain and Induce a Regulatory Phenotype in Naive T Cells<sup>1</sup>

Francesca Fallarino,\* Ursula Grohmann,\* Sylvaine You,<sup>†</sup> Barbara C. McGrath,<sup>‡</sup> Douglas R. Cavener,<sup>‡</sup> Carmine Vacca,\* Ciriana Orabona,\* Roberta Bianchi,\* Maria L. Belladonna,\* Claudia Volpi,\* Pere Santamaria,<sup>§</sup> Maria C. Fioretti,\* and Paolo Puccetti<sup>2\*</sup>

Tryptophan catabolism is a tolerogenic effector system in regulatory T cell function, yet the general mechanisms whereby tryptophan catabolism affects T cell responses remain unclear. We provide evidence that the short-term, combined effects of tryptophan deprivation and tryptophan catabolites result in GCN2 kinase-dependent down-regulation of the TCR  $\zeta$ -chain in murine CD8<sup>+</sup> T cells. TCR  $\zeta$  down-regulation can be demonstrated in vivo and is associated with an impaired cytotoxic effector function in vitro. The longer-term effects of tryptophan catabolism include the emergence of a regulatory phenotype in naive CD4<sup>+</sup>CD25<sup>-</sup> T cells via TGF- $\beta$  induction of the forkhead transcription factor Foxp3. Such converted cells appear to be CD25<sup>+</sup>, CD69<sup>-</sup>, CD45RB<sup>low</sup>, CD62L<sup>+</sup>, CTLA-4<sup>+</sup>, BTLA<sup>low</sup> and GITR<sup>+</sup>, and are capable of effective control of diabetogenic T cells when transferred in vivo. Thus, both tryptophan starvation and tryptophan catabolites contribute to establishing a regulatory environment affecting CD8<sup>+</sup> as well as CD4<sup>+</sup> T cell function, and not only is tryptophan catabolism an effector mechanism of tolerance, but it also results in GCN2-dependent generation of autoimmune-preventive regulatory T cells. *The Journal of Immunology*, 2006, 176: 6752–6761.

The immunoregulatory enzyme IDO mediates conversion of the essential amino acid tryptophan to metabolic by-products collectively known as kynurenines (1, 2). IDO contributes to maternal tolerance in pregnancy (3), control of allograft rejection (4), and protection against autoimmunity (5), inflammatory pathology (6, 7), and allergy (8). In both humans (9) and mice (10), IDO-expressing tolerogenic dendritic cells (DCs)<sup>3</sup> are found in tumor-draining lymph nodes, possibly resulting in Ag-specific anergy. IDO expression by certain tumors could contribute to immune unresponsiveness by the host (11, 12). In peripheral tolerance, modulation of tryptophan catabolism is thought to function as a general mechanism of action of regulatory T cells (Tregs) that express CTLA-4 (13, 14). Several cell types in addition to DCs (4) respond to CTLA-4 engagement of B7 receptor molecules

with the release of IFN- $\gamma$  and activation of IDO, including CD4<sup>+</sup> T cells (15) and polymorphonuclear leukocytes (16).

The wide spectrum of physiopathologic conditions in which IDO appears to be at work suggests that multiple mechanisms are used by this effector system to down-regulate T cell and inflammatory responses. IDO can deplete tryptophan in local tissue microenvironments, and thus prevent T cell proliferation (2), but can also generate immunotoxic kynurenines resulting in T cell apoptosis (1). Neither mechanism alone can explain the diversity (3, 5, 6, 8), durability (4), and Ag specificity (10) of the IDO-mediated suppressive effect. Naive CD4<sup>+</sup> T cells and Th2 cells are resistant to apoptosis induced by kynurenines (17), and CD8<sup>+</sup> T cells are resistant to activation blockade by tryptophan depletion when exposed to strong antigenic stimuli (15).

Reversible impairment of T cell function through down-regulation of the TCR  $\zeta$ -chain could be a physiological means of down-regulating exacerbated immune responses (18). This phenomenon has also been observed in several pathologic settings, including chronic infection, neoplasia, and autoimmune disease (19). Tregs are crucial in regulating self-reactive T cells in the periphery and ameliorating established inflammation in experimental models (20–22). They are usually described as CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>-</sup> cells that inhibit the activation and proliferation of CD4<sup>+</sup>CD25<sup>-</sup> lymphocytes via mechanisms ranging from cell-cell contact to the production of the inhibitory cytokines IL-10 and TGF- $\beta$  (23). In vitro-expanded Ag-specific Tregs can suppress autoimmune diabetes (24).

In a microbial infection model in which exacerbation of the immune response is avoided through the action of Tregs, blockade of IDO results in an impaired generation of Tregs and in overwhelming immunopathology (16). Successful treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite associated with the development of Treg activity has recently been reported (25). Thus, it is possible that besides generalized cell

\*Department of Experimental Medicine, University of Perugia, Perugia, Italy; <sup>†</sup>Institut National de la Santé et de la Recherche Médicale Unité 580-Institut Fédératif de Recherche Necker Enfants-Malades, Hôpital Necker, Paris, France; <sup>‡</sup>Department of Biology, The Pennsylvania State University, University Park, PA 16802; and <sup>§</sup>Julia McFarlane Diabetes Research Centre, University of Calgary, Calgary, Alberta, Canada

Received for publication January 26, 2006. Accepted for publication March 16, 2006.

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<sup>1</sup> This work was supported by the Italian Association for Cancer Research. P.S. is supported by grants from the Canadian Institutes of Health Research and is a Scientist of the Alberta Heritage Foundation for Medical Research.

<sup>2</sup> Address correspondence and reprint requests to Dr. Paolo Puccetti, Department of Experimental Medicine, Section of Pharmacology, University of Perugia, Via del Giochetto, Perugia 06126, Italy. E-mail address: plopec@tin.it

<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; BTLA, B and T cell lymphocyte attenuator; GITR, glucocorticoid-inducible TNFR; 3-HAA, 3-hydroxyanthranilic acid; LT, low tryptophan; LT-K, LT with kynurenine; 1-MT, 1-methyl-DL-tryptophan; Treg, regulatory T cell.

cycle arrest due to sustained tryptophan deprivation, IDO contributes to fine-tuning immune responsiveness by multiple mechanisms that are coordinate but differentiated for specific T cell subsets. We report that early down-regulation of the TCR  $\zeta$ -chain in CD8<sup>+</sup> cells and longer-term induction of a tolerogenic phenotype in naive CD4<sup>+</sup> T cells result from the combined effects of tryptophan starvation and tryptophan metabolites. Both of these effects require the general control non-derepressing 2 (GCN2) protein kinase, which is responsive to starvation for amino acids. The CD8<sup>+</sup> T cells display impaired function *ex vivo*, whereas the CD4<sup>+</sup> Tregs are shown to prevent disease in an experimental setting of diabetes transfer.

## Materials and Methods

### Mice

Eight- to 10-wk-old DBA/2 (H-2<sup>d</sup>) and 4-wk-old NOD (K<sup>d</sup>, I-A<sup>g7</sup>, D<sup>b</sup>) mice were purchased from Charles River Breeding Laboratories. *Gcn2*<sup>-/-</sup> mice, homozygous for the GCN2 targeted mutation, and wild-type *Gcn2*<sup>+/+</sup> controls were as described (26). NOD-SCID mice were bred in our animal facility under specific pathogen-free conditions to be used at ages 8–10 wk. Transgenic 8.3-NOD mice (27) and BDC2.5-NOD mice (28) were as described. Colorimetric strips were used to monitor glycosuria and hyperglycemia (>300 mg/dl) in NOD-SCID mice (4). Only female mice were used in this study, and all *in vivo* experiments were done in compliance with National and Perugia University Animal Care and Use Committee guidelines.

### Reagents and treatments

The IDO inhibitor 1-methyl-DL-tryptophan (1-MT) was purchased from Sigma-Aldrich to be used *in vitro* at the concentration of 20  $\mu$ M. To inhibit IDO activity *in vivo*, 1-MT or placebo pellets (150 mg/pellet, 7-day release at 0.9 mg/h; Innovative Research of America) were implanted as described (4). NRP-A7 (KYNKANAF), a synthetic peptide mimotope recognized in the context of the MHC class I H-2K<sup>d</sup> molecule by 8.3-CD8<sup>+</sup> T cells (29), was as described (13). FITC-conjugated NRP-A7 pentamer and negative control peptide pentamer were prepared by ProImmune and validated by staining 8.3-NOD spleen cells according to manufacturer's instructions. Pentamer-positive 8.3-CD8<sup>+</sup> T cells were recovered by magnetic-activated sorting using anti-FITC MicroBeads (Miltenyi Biotec). Ab reagents for ELISAs of IL-2, IL-4, IL-10, and IFN- $\gamma$  have been described (14). A specific TGF- $\beta$  ELISA kit was purchased from Promega. Blockade *in vitro* of IL-10 and CTLA-4 was achieved by the respective use of SXC-1 (rat IgM) and 4F10 (hamster IgG) monoclonals (13). Purified anti-TGF- $\beta$  2G7 (mouse IgG2b) was a gift from M. Kopf (Swiss Federal Institute of Technology, Zurich, Switzerland). Neutralizing Abs were added at 10 (anti-IL-10) or 50  $\mu$ g/ml (anti-CTLA-4, anti-TGF- $\beta$ ) and isotype controls were included in all assays. Standard IMDM (Invitrogen Life Technologies), containing 50  $\mu$ M tryptophan, and low tryptophan (LT, 5  $\mu$ M) medium (30), both with and without kynurenes (17), were used as culture media. Tryptophan and kynurenine concentrations were measured by HPLC (13).

### Cell sorting and suppression assay

CD11c<sup>+</sup>CD8<sup>+</sup> splenic DCs were purified by the sequential use of CD11c and CD8 $\alpha$  MicroBeads (Miltenyi Biotec), as described (13), and were referred to as IDO<sup>-</sup> DCs to distinguish them from IDO<sup>+</sup> DCs, i.e., CD11c<sup>+</sup>CD8<sup>+</sup> cells treated overnight with 200 U/ml IFN- $\gamma$  so as to induce expression of IDO transcript, protein, and function (4). CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from lymph nodes by magnetic-activated cell sorting as described (13, 14). The purity of either T cell fraction was >95%. For assay of regulatory activity, CD4<sup>+</sup>CD25<sup>-</sup> cells were cocultured with irradiated T cell-depleted splenocytes and CD4<sup>+</sup>CD25<sup>+</sup> cells for 3 days in the presence of soluble anti-CD3 (1  $\mu$ g/ml) (14). Proliferation was measured by incorporation of [<sup>3</sup>H]thymidine according to standard procedures.

### Flow cytometry, pentamer staining, and immunoblot analysis

In all FACS analyses, cells were treated with rat anti-CD16/32 (2.4G2) for 30 min at 4°C for blockade of Fc receptors before assaying on an EPICS flow cytometer using EXPO 32 ADC software (Beckman Coulter). The following FITC-labeled Abs were used in cell surface staining (BD Pharmingen): anti-CD4 (GK1.5), anti-CD69 (H1.2F3), and anti-CD45RB (16A). FITC-labeled anti-Foxp3 (FJK-16s) was from eBioscience. PE-labeled anti-CD62L (MEL-14; BD Pharmingen), anti-glucocorticoid-inducible TNFR

(GITR, 108619; R&D Systems), and anti-CD4 (GK1.5; BD Pharmingen) were also used. Detection of the surface molecule B and T cell lymphocyte attenuator (BTLA) was accomplished by the use of biotinylated anti-BTLA (a gift from K. Murphy, Washington University, St. Louis, MO) and Streptavidin-FITC. Total CTLA-4 expression was analyzed as described (13). For pentamer staining, spleen and lymph node cells were incubated for 30 min at room temperature with FITC-conjugated H-2K<sup>d</sup> pentamers before FACS analysis. For intracellular  $\zeta$ -chain staining, cells were washed and fixed for 5 min with 2% paraformaldehyde at room temperature, permeabilized and incubated for 30 min with PE-labeled anti-CD3 (6B10.2; Santa Cruz Biotechnology). All FACS analyses included the use of isotype control Abs. For CFSE or CellTrace Far Red DDAO-SE (Molecular Probes) labeling, the fluorescent dyes in the form of 5 mM stock solutions were added to 2  $\times$  10<sup>7</sup> CD4<sup>+</sup> T cells/ml to a final concentration of 2 (CFSE) or 1  $\mu$ M (DDAO-SE). The cells were incubated at room temperature for 10 min and washed twice with the same volume of FCS and then with culture medium. Immunoblot of TCR  $\zeta$ -chain and  $\epsilon$ -chain involved total T cells or CD4<sup>+</sup> and CD8<sup>+</sup> fractions purified by magnetic-activated sorting (Miltenyi Biotec), which were analyzed as described (18), using monoclonal anti-CD3 $\zeta$  (6B10.2) and polyclonal anti-CD3 $\epsilon$  (M20; both from Santa Cruz Biotechnology) in combination with the appropriate Abs conjugated to HRP, followed by ECL.

### Analysis of lymphocyte function

For mitogen-induced cell proliferation and cytokine induction, CD4<sup>+</sup> or CD8<sup>+</sup> T cells (1  $\times$  10<sup>6</sup>/ml) were cultured in the presence of 1  $\mu$ g/ml plate-bound anti-CD3 $\epsilon$  (145-2C11), 2.5  $\mu$ g/ml Con A (Sigma-Aldrich), or 2 ng/ml PMA (Sigma-Aldrich) combined with 0.2  $\mu$ M calcium ionophore (Sigma-Aldrich). In T cell-DC cocultures, T cells (1  $\times$  10<sup>6</sup>) were stimulated with DCs (at a DC to T cell ratio of 1:5 and in the presence of 1  $\mu$ g/ml soluble anti-CD3), and cultures were assayed for T cell proliferation by incorporation of [<sup>3</sup>H]thymidine or cell-free supernatants were analyzed for kynurenine or cytokine contents. The lytic activity of CD8<sup>+</sup> T cells against <sup>51</sup>Cr-labeled, NRP-A7-pulsed RMA-S/K<sup>d</sup> target cells was measured as described (29).

### Foxp3 expression

Real-time PCR were run on a Chromo4 Four-Color Real-Time Detector (Bio-Rad) using *Foxp3*-specific primers (5'-CCCAGGAAAGACAGCAACCTT-3'; 5'-TTCTCACAACCAGGCCACTTG-3'). *Gapdh* (5'-GCTTCCGTGTTCCCTACCC-3'; 5'-CAGTGGGCCCCTCAGATGC-3') was the normalizer gene. Conventional RT-PCR was performed using the following primers: *Foxp3*, 5'-CAGCTGCTACAGTGGCCCCTAG-3' and 5'-CATTTGCCAGCAGTGGGTAG-3'; *Gapdh*, 5'-AGCACAGTCCATGCATCAC-3' and 5'-TCCACCACCTGTTGCTGTA-3'. Specific T cell populations were permeabilized and stained with FITC-labeled anti-Foxp3 according to the manufacturer's instructions.

### Statistical analysis

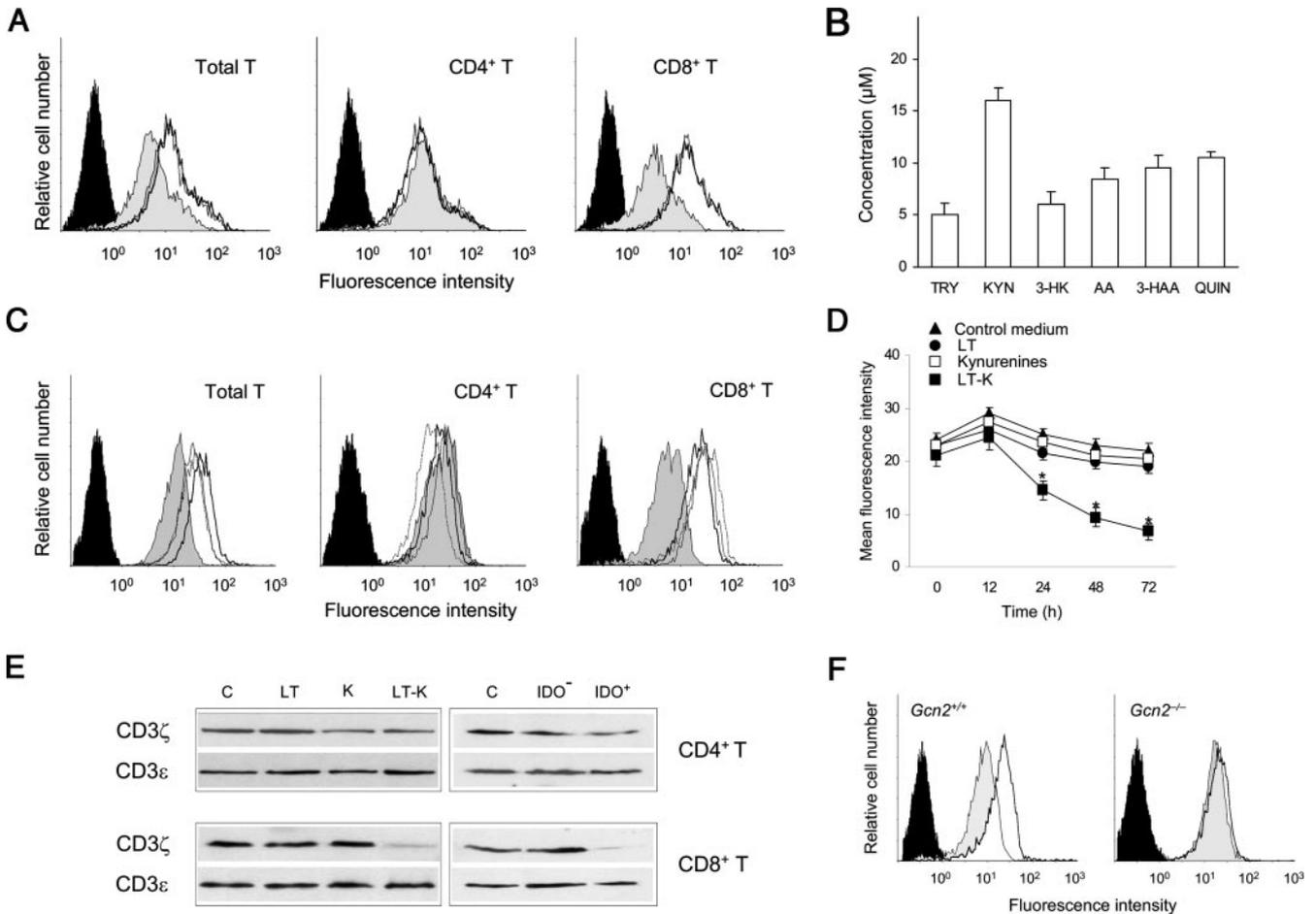
In all assays, the Student's *t* test was used for analysis of *in vitro* and *in vivo* data.

## Results

### Tryptophan catabolism down-regulates TCR $\zeta$ -chain in CD8<sup>+</sup> T cells

Arginine consumption by macrophages has been reported to modulate the expression of TCR  $\zeta$ -chain (CD3 $\zeta$ ) in T lymphocytes in the absence of apoptosis (31). We have previously shown that, contrary to Th1 cells, naive T cells do not undergo apoptosis when cultured with IDO<sup>+</sup> DCs for 48 h (17). We assessed CD3 $\zeta$  expression in CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes from naive mice after culturing these cells with IDO<sup>+</sup> DCs in the presence or absence of 1-MT, a specific enzyme inhibitor (Fig. 1A). FACS analysis revealed a noticeable decrease of CD3 $\zeta$  in CD8<sup>+</sup> T cells cultured with DCs in the absence of 1-MT. Under these conditions, the initial 50  $\mu$ M tryptophan concentration dropped to  $\sim$ 5  $\mu$ M by 24 h, a time when the concentrations of the major tryptophan catabolites L-kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid (3-HAA), and quinolinic acid were in the low micromolar range (Fig. 1B).

To investigate whether  $\zeta$ -chain down-regulation occurred by the direct action of tryptophan catabolism and to identify the possible



**FIGURE 1.** Culture of splenic T cells or purified CD8<sup>+</sup> T cells with IDO<sup>+</sup> DCs, or with LT and kynurenines, results in selective CD3ζ down-regulation that is concomitant with tryptophan consumption and kynurenine production and is mediated by GCN2. *A*, Total T cells and purified CD4<sup>+</sup> or CD8<sup>+</sup> fractions from DBA/2 mice were cultured for 48 h with soluble anti-CD3 (1 μg/ml) and IDO<sup>-</sup> DCs (thick line histogram) or IDO<sup>+</sup> DCs with (thin line histogram) or without (gray-filled histogram) 1-MT. Cells were labeled for surface CD3, CD4, or CD8 with the respective FITC-labeled reagents, and were then fixed, permeabilized and stained for total ζ-chain expression by means of PE-labeled anti-mouse CD3ζ. Filled histogram indicates isotype controls. Data are from one experiment representative of four. *B*, Concentrations of tryptophan (TRY) and different kynurenines (mean ± SD of three independent experiments) were measured in 24-h supernatants of total T cells and IDO<sup>+</sup> DCs. The initial tryptophan concentration was 50 μM. In parallel cocultures established in the presence of 1-MT, the final concentration of tryptophan was ~30 μM, whereas kynurenines were undetectable. *C*, Total T cells or purified CD4<sup>+</sup> and CD8<sup>+</sup> fractions were activated by plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (0.5 μg/ml) in control medium (thick line histogram) or medium containing LT for 48 h, in the absence (thin histogram) or presence (gray-filled histogram) of kynurenines. The effect of addition of kynurenines to control medium was also investigated (dotted line histogram). Cells were fixed, permeabilized and stained for total ζ-chain by means of PE-labeled anti-CD3ζ. Filled histogram indicates isotype controls. *D*, CD8<sup>+</sup> cells cultured as in *C* were assayed for ζ expression at 12, 24, 48, and 72 h. Results were expressed as fluorescence intensity values (mean ± SD) of three independent experiments. \*, *p* < 0.01, LT-K vs control medium. *E*, CD4<sup>+</sup> and CD8<sup>+</sup> T cells cultured as in *C* in control medium (C) or in LT, kynurenines (K), or both (LT-K) were lysed and analyzed by immunoblot with anti-ζ or anti-ε. The effect of T cell coculture with IDO<sup>+</sup> or IDO<sup>-</sup> DCs was also investigated. Selected densitometry data (CD3ζ to CDε ratios) were as follows: CD4<sup>+</sup> T cell in control medium, 1.1; CD4<sup>+</sup> T cell in LT-K, 0.9; CD4<sup>+</sup> T cell with IDO<sup>+</sup>, 1.0; CD8<sup>+</sup> T cell in control medium, 1.0; CD8<sup>+</sup> T cell in LT-K, 0.3; and CD8<sup>+</sup> T cell with IDO<sup>+</sup>, 0.2. *F*, FACS analysis of CD3ζ expression in CD8<sup>+</sup> cells cultured as in *C* with LT-K (gray-filled histogram) or control media using wild-type or *Gcn2*<sup>-/-</sup> mice as donors. Filled histograms indicate isotype controls. All experiments were performed at least three times.

milieu responsible for this effect, we cultured CD4<sup>+</sup> and CD8<sup>+</sup> T cells in medium containing a LT (5 μM) concentration, a mixture of 3-hydroxykynurenine, 3-HAA, and quinolinic acid, each at 10 μM, or a combination of LT with kynurenines (LT-K). Plate-bound anti-CD3 and anti-CD28 were present in the cultures. FACS analysis revealed that the LT-K culture conditions, but not LT or kynurenines alone, decreased CD3ζ, and did so mostly in CD8<sup>+</sup> T cells (Fig. 1C). The LT-K conditions had no effect on the expression of other TCR components, including TCRαβ and TCRε, and did not affect costimulatory molecules, including CD28 (data not shown). The effect of LT-K was time-dependent (Fig. 1D) and slowly reversible, as it required reculturing the cells for at least 72 h in control medium for 90% recovery of the original ζ expres-

sion (data not shown). The effect was also demonstrable by immunoblot analysis with anti-ζ but not anti-ε Abs, and was likewise observable using IDO<sup>+</sup> DCs in place of LT-K (Fig. 1E).

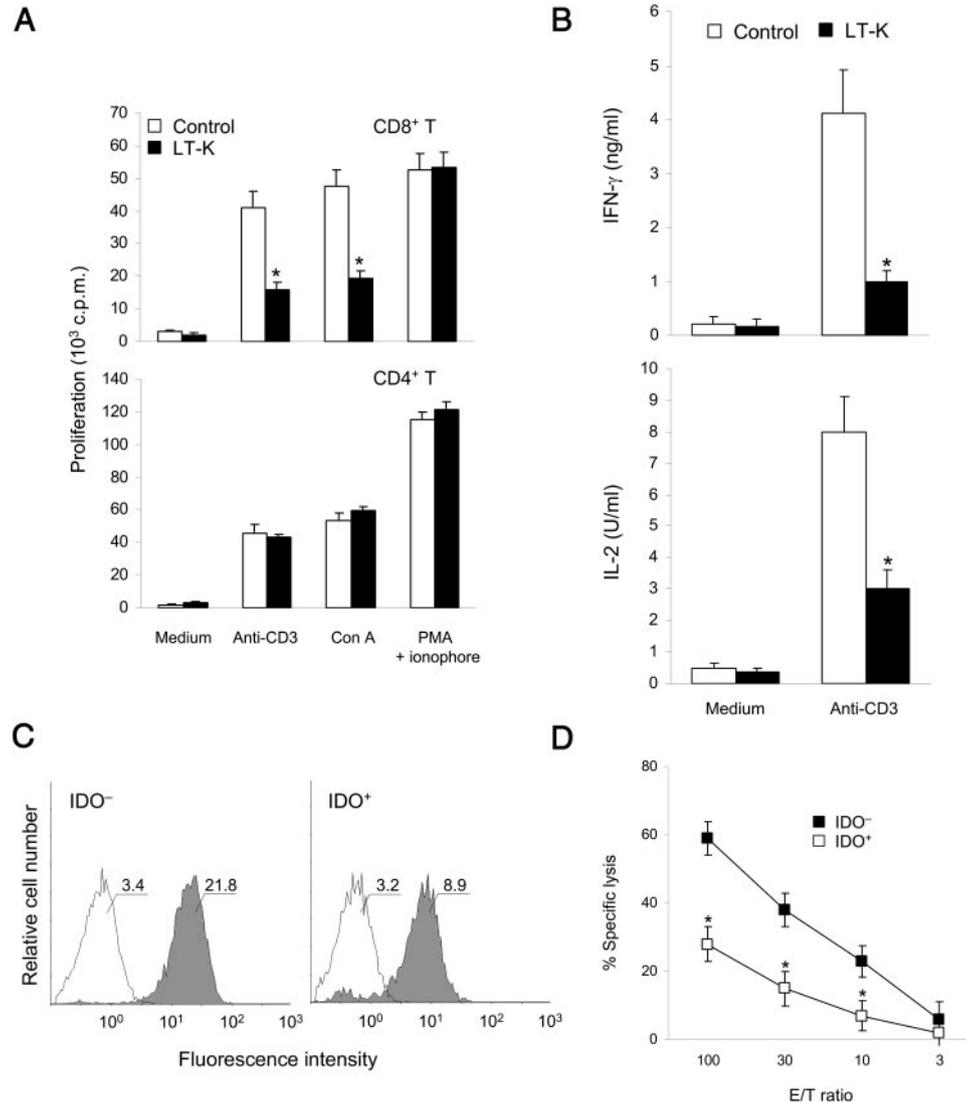
The GCN2 stress-response kinase is activated by starvation for amino acids and functions to control transcriptional and translational programs that couple cell growth to amino acid availability (26). Recent evidence indicates that GCN2 activation could be an upstream mechanism in the immunoregulatory effects of IDO (32). To ascertain whether down-regulation of CD3ζ by tryptophan catabolism requires GCN2, we used *Gcn2*<sup>-/-</sup> mice as a source of CD8<sup>+</sup> T cells that were exposed to LT-K medium (Fig. 1F). In contrast to the *Gcn2*<sup>+/+</sup> mice, we observed no changes in CD3ζ expression in the T cells lacking GCN2.

*TCR  $\zeta$ -chain down-regulation is associated with impaired function*

We investigated whether the diminished expression of CD3 $\zeta$  induced by LT and kynurenines in CD8<sup>+</sup> T cells correlates with an impaired immune function in vitro. Purified CD8<sup>+</sup> and CD4<sup>+</sup> T cells were exposed to LT-K for 72 h, washed and recultured for 48 h in the presence of anti-CD3, Con A, or a combination of PMA and calcium ionophore (Fig. 2A). Proliferation was significantly reduced by LT-K conditioning upon activation of CD8<sup>+</sup> cells, but not CD4<sup>+</sup> cells, with anti-CD3 or Con A. No effect was found on activation with PMA and calcium ionophore in either cell type, demonstrating a selective impairment of TCR-mediated signaling

events in the CD8<sup>+</sup> cells conditioned by LT-K. Under the same conditions, the production of IFN- $\gamma$  and IL-2 by CD8<sup>+</sup> T cells in response to anti-CD3 was severely impaired (Fig. 2B). The cytokine response of CD4<sup>+</sup> T cells was apparently unaffected (data not shown).

NOD mice expressing the 8.3-TCR $\alpha\beta$  transgene (8.3-NOD) (27) possess a prevalent population of autoreactive CD8<sup>+</sup> T cells that recognize a  $\beta$  cell autoantigen and are reactive to the islet-related NRP-A7 peptide (29, 33, 34). To ascertain whether  $\zeta$ -chain down-regulation occurs in vivo, we used pentamer technology to transfer NOD-SCID mice with a combination of NRP-A7-pulsed, IDO<sup>+</sup> DCs and NRP-A7 pentamer-positive 8.3-CD8<sup>+</sup> T cells. The



**FIGURE 2.** TCR  $\zeta$ -chain down-regulation by tryptophan catabolism correlates with impaired T cell function in vitro and occurs in vivo. *A*, Purified CD8<sup>+</sup> or CD4<sup>+</sup> T cells were precultured for 72 h in control or LT-K medium in the presence of plate-bound anti-CD3 and anti-CD28, and were then exposed for 48 h to plate-bound anti-CD3, Con A, or PMA and calcium ionophore, to be assayed for cell proliferation as measured by [<sup>3</sup>H]thymidine incorporation (mean  $\pm$  SD of triplicate samples). \*,  $p < 0.001$ , LT-K vs control in one experiment representative of three. *B*, Cultures of CD8<sup>+</sup> T cells established as in *A* and recultured with anti-CD3 were assayed for IFN- $\gamma$  and IL-2 contents in 48-h supernatants (mean  $\pm$  SD of triplicate samples). \*,  $p < 0.005$ , LT-K vs control in one experiment representative of three. *C*, IDO<sup>-</sup> and IDO<sup>+</sup> DCs pulsed with NRP-A7 were injected s.c. into NOD-SCID mice ( $1 \times 10^6$ ), which also received NRP-A7 pentamer-positive 8.3-CD8<sup>+</sup> T cells ( $5 \times 10^6$ ) i.v. The injection of IDO<sup>-</sup> or IDO<sup>+</sup> NRP-A7-pulsed DCs was repeated after 1 wk and, after 48 h, CD8<sup>+</sup> cells were recovered from the spleen and lymph nodes; of these cells, 95.4 and 97.3% were pentamer-positive cells in the spleen and lymph nodes, respectively. The CD8<sup>+</sup> T cells were pooled and assayed for CD3 $\zeta$  expression by FACS analysis as in Fig. 1C. Isotype control treatment (open histogram) is indicated. The mean channel fluorescence intensity is indicated for one experiment representative of three. The mean fluorescence value  $\pm$  SD for the three experiments were  $22.3 \pm 4.1$  and  $9.5 \pm 2.0$  for IDO<sup>-</sup> and IDO<sup>+</sup> DC treatments, respectively ( $p < 0.01$ ). *D*, Portions of the CD8<sup>+</sup> cells recovered as in *C* were assayed for lytic activity to NRP-A7-pulsed RMA-S/K<sup>d</sup> target cells at different E:T ratios (mean  $\pm$  SD of replicate samples). \*,  $p < 0.01$ , IDO<sup>-</sup> vs IDO<sup>+</sup> DC treatment of the prospective donors of CD8<sup>+</sup> cytotoxic effectors. One experiment representative of three is shown.

CD8<sup>+</sup> cells were harvested from the spleen and lymph nodes, and were analyzed for CD3 $\zeta$  expression (Fig. 2C) and for ability to lyse peptide-pulsed target cells (Fig. 2D). Noticeable down-regulation of the  $\zeta$ -chain and impaired lytic activity were observed in the CD8<sup>+</sup> T cells recovered from NOD-SCID mice immunized with IDO<sup>+</sup> DCs at the time of T cell transfer.

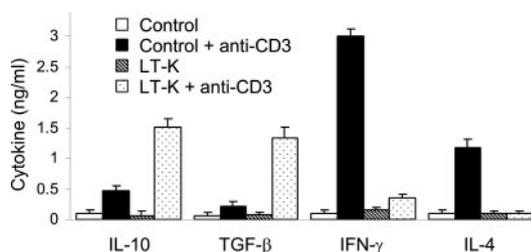
#### *Tryptophan catabolism converts CD25<sup>-</sup> Foxp3<sup>-</sup> into CD25<sup>+</sup> Foxp3<sup>+</sup> cells*

Despite the lack of LT-K effects on proliferation and cytokine production by CD4<sup>+</sup> T cells following a 48-h exposure, we asked whether a prolonged exposure to LT and kynurenines might act on naive CD4<sup>+</sup> T cells to change their functional properties. When CD4<sup>+</sup> T cells exposed to LT-K for 7 d were recultured for 24 h with anti-CD3 in standard medium, they produced little or no IFN- $\gamma$  and IL-4; yet, they produced noticeable amounts of IL-10 and TGF- $\beta$  (Fig. 3). A similar pattern of cytokine production was observed on assaying CD4<sup>+</sup> T cells exposed to IDO<sup>+</sup> DCs in place of LT-K (data not shown).

Accumulating evidence suggests that Tregs, including those characterized by IL-10 and TGF- $\beta$  secretion, represent a dedicated T cell lineage, and that the forkhead family transcription factor Foxp3 functions as the Treg cell lineage specification factor (35). We used real-time PCR analysis for measuring Foxp3 transcript expression in unfractionated CD4<sup>+</sup> T cells exposed to IDO<sup>-</sup> or IDO<sup>+</sup> DCs or to LT or LT-K for 1, 3, or 6 days (Fig. 4A). The results demonstrated an 8- to 40-fold increase in Foxp3 transcription at 3–6 days of cell culture with IDO<sup>+</sup> DCs or in the LT-K medium. The effect of culturing CD4<sup>+</sup> T cells with IDO<sup>+</sup> DCs on Foxp3 transcript expression was negated by the presence of 1-MT.

The CD25 molecule, which is expressed by 5–10% of CD4<sup>+</sup> T cells in naive mice, is able to operationally if not specifically differentiate naturally present autoimmune-preventive Tregs from other T cells (22). Using RT-PCR, we examined Foxp3 transcripts in CD25<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> fractions after cell culture in LT-K for 1, 3, or 6 days (Fig. 4B). We obtained evidence for the appearance of Foxp3 transcripts in the CD25<sup>-</sup> fraction at 3–6 days of culture. Foxp3 expression in the CD25<sup>+</sup> cells remained unchanged. The presence of Foxp3 protein in the CD25<sup>-</sup> fraction after LT-K exposure was confirmed by flow cytometry (Fig. 4C).

We used FACS analysis to investigate the possible conversion of CD25<sup>-</sup> cells into CD25<sup>+</sup> cells in concomitance with Foxp3 appearance, and to evaluate the expression of a series of phenotypic and functional markers including CD69, CD45RB, CD62L, CTLA-4, BTLA, and GITR. At 1 wk of LT-K exposure, >90% of the original CD25<sup>-</sup> cells appeared to express the CD25 molecule. The converted cells were CD69<sup>-</sup>, CD45RB<sup>low</sup>, CD62L<sup>+</sup>, CTLA-



**FIGURE 3.** Tryptophan catabolism and tryptophan catabolites favor the emergence of cells producing IL-10 and TGF- $\beta$ . CD4<sup>+</sup> T cells were cultured for 7 days in control or LT-K medium in the presence of plate-bound anti-CD3 and anti-CD28 and were then activated for 24 h with anti-CD3 to be assayed for the production of IL-10, TGF- $\beta$ , IFN- $\gamma$ , and IL-4. Results are means  $\pm$  SD of replicate samples. One experiment representative of three is shown.

4<sup>+</sup>, BTLA<sup>low</sup>, and GITR<sup>+</sup>, thus expressing markers associated with the function of CD4<sup>+</sup>CD25<sup>+</sup> Tregs and subsets thereof in different experimental settings (28, 36–39) (Fig. 4D).

Next we investigated whether the conversion of CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> Tregs by LT-K could be the result of expansion of residual CD4<sup>+</sup>CD25<sup>+</sup> cells or selective CD4<sup>+</sup>CD25<sup>+</sup> cell survival. Using individual populations or combinations of CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells we examined proliferation, cell division, and viable cell recovery data at different times of LT-K exposure. Purified CD4<sup>+</sup>CD25<sup>-</sup> T cells but not CD4<sup>+</sup>CD25<sup>+</sup> proliferated in LT-K medium, by both [<sup>3</sup>H]thymidine uptake and trypan blue dye exclusion analysis (Fig. 5A). Coculturing CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> cells with 10% DDAO-SE-labeled CD4<sup>+</sup>CD25<sup>+</sup> cells in LT-K revealed selective proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells (Fig. 5B). Thus, neither expansion of residual CD4<sup>+</sup>CD25<sup>+</sup> cells nor selective CD4<sup>+</sup>CD25<sup>+</sup> cell survival contributed significantly to the conversion of CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> Tregs by LT-K.

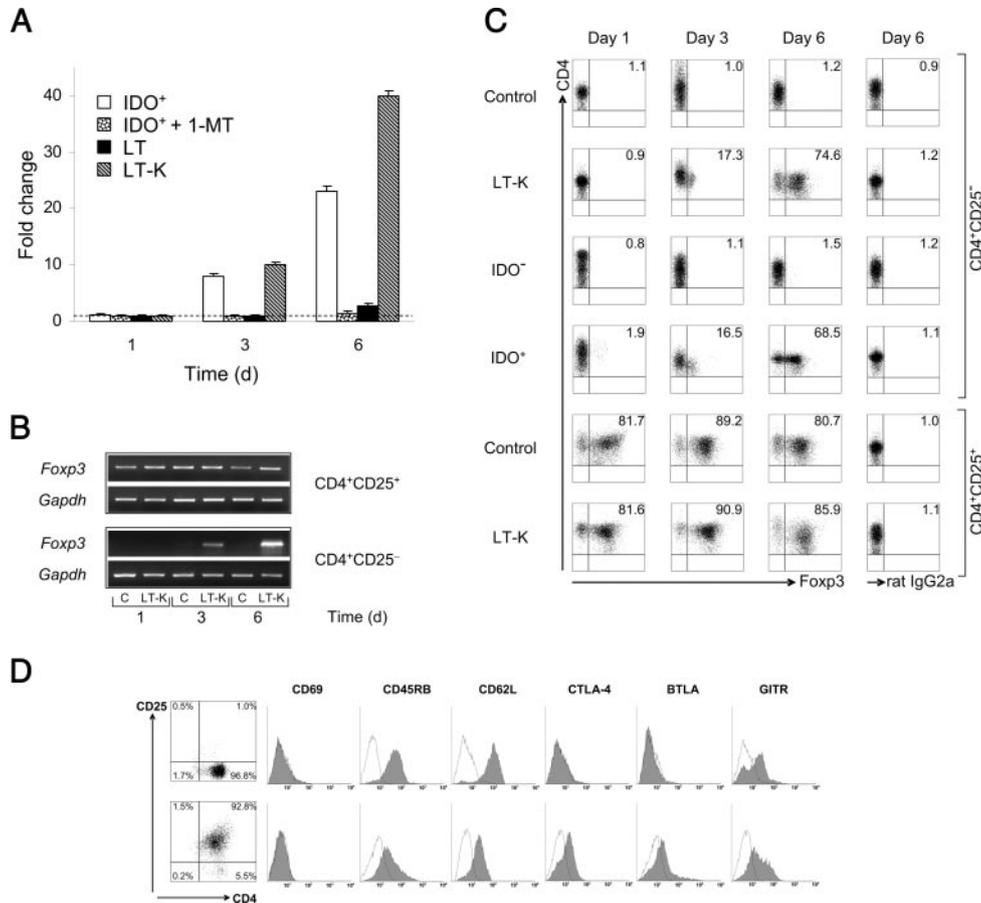
#### *Converted CD4<sup>+</sup>CD25<sup>+</sup> cells mediate CTLA-4 and IL-10-dependent suppression*

To investigate whether the CD4<sup>+</sup>CD25<sup>+</sup> Foxp3-expressing cells induced by LT-K display regulatory function in vitro, we cocultured various numbers of those cells with responder CD4<sup>+</sup>CD25<sup>-</sup> T lymphocytes from naive mice in the presence of anti-CD3 and T cell-depleted splenocyte samples as APCs (Fig. 6A). CD4<sup>+</sup>CD25<sup>-</sup> cells were exposed to LT-K or cultured with IDO<sup>+</sup> DCs for 7 days (a time when no viable CD8<sup>+</sup> T cells would be recoverable from parallel cultures; Fig. 5A), and cells were then assayed for suppressive activity after separation into CD25<sup>+</sup> and CD25<sup>-</sup> fractions. The CD4<sup>+</sup>CD25<sup>+</sup> T cells cultured in LT-K or with IDO<sup>+</sup> DCs for 7 days greatly inhibited the proliferation of responder T cells in contrast to the minority fraction that had remained CD25<sup>-</sup> after LT-K or IDO<sup>+</sup> DC treatment. (These cells did not express Foxp3 transcripts, data not shown.) Suppression was comparable to that of natural Tregs, i.e., CD4<sup>+</sup>CD25<sup>+</sup> Foxp3-expressing T cells isolated from lymph nodes of naive mice.

We investigated whether the expression of CTLA-4, TGF- $\beta$ , or IL-10 has any role in mediating the regulatory function of the CD25<sup>+</sup> cells induced by LT-K. Abs to CTLA-4, TGF- $\beta$ , or IL-10 were added, either singly or in combination, to the cocultures of Treg and responder CD25<sup>-</sup> T cells in the proliferation assay (Fig. 6B). The Abs had limited (anti-IL-10, anti-CTLA-4) or no (anti-TGF- $\beta$ ) effect when used singly, but the combination of anti-IL-10 and anti-CTLA-4 resulted in ablation of the regulatory properties of the Tregs generated by LT-K exposure.

#### *GCN2 and TGF- $\beta$ are required for generation of Foxp3<sup>+</sup> Tregs by LT-K*

Naive CD4<sup>+</sup>CD25<sup>-</sup> T cells can be converted to a CD25<sup>+</sup> Treg phenotype by TGF- $\beta$ , which is thought to act through the induction of Foxp3 (37). Although the production of this cytokine seemed to have little role in the in vitro downstream effects of the T cells converted by LT-K, we asked whether autocrine TGF- $\beta$  is involved in Foxp3 induction. We also examined whether activation of the GCN2 kinase is a prerequisite for the induction of Foxp3 and Treg function by LT-K. Gcn2<sup>-/-</sup> and wild-type mice served as a source of CD4<sup>+</sup>CD25<sup>-</sup> cells that were used for generating Tregs by 7-day exposure to LT-K in the presence of anti-CD3 and anti-CD28, with or without anti-TGF- $\beta$ . Foxp3 transcript expression (Fig. 6C) and ability to inhibit the proliferation of responder T cells (Fig. 6D) were examined in the recovered cells according to the conditions described above. The GCN2 kinase and TGF- $\beta$  appeared to be required for the expression of Foxp3 and regulatory



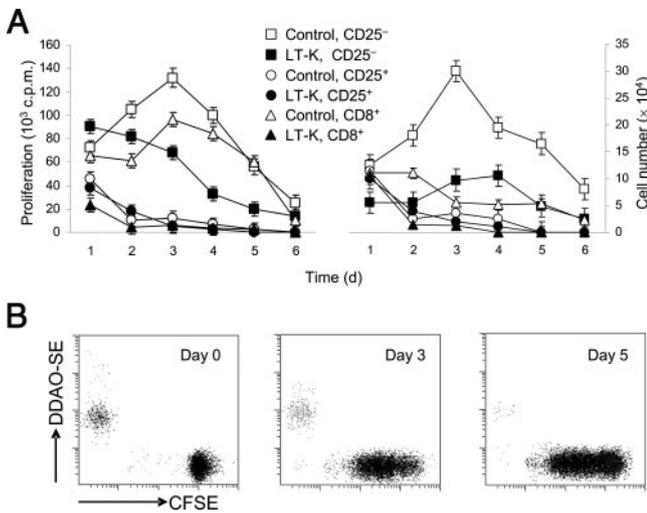
**FIGURE 4.** Induction of *Foxp3* transcripts in CD4<sup>+</sup> T cells cultured with IDO<sup>+</sup> DCs or in LT-K medium for different times. **A**, Sorted CD4<sup>+</sup> lymphocytes ( $3 \times 10^5$ ) were exposed to IDO<sup>-</sup> or IDO<sup>+</sup> DCs ( $1 \times 10^5$ ) and soluble anti-CD3 and anti-CD28 in LT or LT-K media for 1, 3, or 6 days. A group of CD4<sup>+</sup> T cells was cultured with IDO<sup>+</sup> DCs in the presence of 1-MT. *Foxp3* mRNA levels were quantified by real-time PCR using *Gapdh* normalization. Data (mean  $\pm$  SD of triplicate samples in one of three experiments) are presented as normalized *Foxp3* transcript expression in the samples relative to normalized *Foxp3* transcript expression in the respective control cultures, i.e., cells exposed to IDO<sup>-</sup> DCs or maintained in standard medium (fold change = 1; dashed line). **B**, CD4<sup>+</sup> T cells were fractionated and CD25<sup>+</sup> and CD25<sup>-</sup> fractions were cultured as in **A** in control (C) or LT-K medium for 1, 3, or 6 days. *Foxp3* message expression was analyzed by RT-PCR. On examining data as normalized *Foxp3* transcript expression in CD4<sup>+</sup>CD25<sup>-</sup> cell samples relative to normalized *Foxp3* transcript expression in control cells (i.e., maintained in standard medium; fold change = 1), the 6-day fold change was 8.3. One experiment representative of three is shown. **C**, FACS analysis of *Foxp3* expression in permeabilized CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> cells cultured in control medium or exposed to LT-K or IDO<sup>+</sup> DCs for 1, 3, or 6 days as in **A**. Isotype controls were included in all assays and are shown for day 6. The dot plots were gated on CD4<sup>+</sup> cells. Number (upper right quadrant) indicates percentage of double-positive cells. Data are representative of three experiments. **D**, Cytofluorometric analysis of naive CD4<sup>+</sup> T cells cultured in LT and kynurenines. Sorted CD4<sup>+</sup>CD25<sup>-</sup> cells were activated by plate-bound anti-CD3 and anti-CD28 in LT-K medium for 7 days (bottom), to be stained with Abs to CD25 (dot plot) or CD69, CD45RB, CD62L, CTLA-4, BTLA, and GITR (histograms). Also depicted is the profile of the original CD4<sup>+</sup>CD25<sup>-</sup> population (top). Number indicated is percentage of cells in each quadrant. Control cultures were stained with isotype-matched Ab (open histogram).

properties in CD4<sup>+</sup> T cells exposed to LT-K. Although it is possible that the anti-TGF- $\beta$  would also neutralize any TGF- $\beta$  present in the bovine sera added to the media, it should be noticed that the DCs did not produce significant amounts of TGF- $\beta$  in vitro in response to LT-K (data not shown).

#### Tregs induced by LT-K protect hosts from diabetes transfer

Clonotypic T cell NOD mice expressing the BDC2.5-TCR $\alpha\beta$  transgene (BDC2.5/NOD) are characterized by a prevalent population of CD4<sup>+</sup> T cells that recognize a mimotope homologous to a peptide of glutamic acid decarboxylase 65 (40–42). Most BDC2.5/NOD mice do not develop spontaneous diabetes, but do develop insulinitis accompanied by the appearance of activated islet-specific T cells. Evidence suggests that these cells are actively prevented from causing disease by a CTLA-4-dependent immunoregulatory mechanism and by the action of CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>-</sup> Tregs (43). At variance with BDC2.5/NOD mice, BDC2.5 TCR

transgenic mice backcrossed into the NOD-SCID background show fulminant diabetes, strongly arguing for the regulatory role of endogenously rearranged T cells lacking the transgenic TCR (44). Rapid diabetes also develops in NOD-SCID mice adoptively transferred with spleen cells or purified CD4<sup>+</sup> T cells from BDC2.5/NOD donors (45). In this setting, however, onset of disease is prevented by the cotransfer of CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> cells from prediabetic NOD mice (45). Using the cotransfer model, we investigated whether the Tregs generated by LT-K in vitro are capable of protecting NOD-SCID mice from the induction of diabetes by BDC2.5 transgenic T cells. We tested CD4<sup>+</sup>CD25<sup>-</sup> cells cultured for 7 d in LT-K medium in comparison with purified CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> cells from NOD mice (Fig. 7A). The results showed that the Tregs generated by LT-K prevented transfer of diabetes in a durable and dose-dependent fashion and to an extent comparable to the control Treg population. However, when the recipients of the cotransfer received 1-MT pellets at the time of



**FIGURE 5.** Proliferation and viable cell recovery of T cell subsets cultured in LT and kynurenines. *A*, Purified CD4<sup>+</sup>CD25<sup>-</sup> T cells ( $5 \times 10^4$ ), but neither CD4<sup>+</sup>CD25<sup>+</sup> nor CD8<sup>+</sup> T cells, proliferate in LT-K medium, by both [<sup>3</sup>H]thymidine uptake and trypan blue dye exclusion analysis. *B*, Coculturing CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> cells ( $6 \times 10^5$ ) with 10% DDAO-SE-labeled CD4<sup>+</sup>CD25<sup>+</sup> cells for different times reveals selective proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells, as determined by flow cytometry. All culture conditions included the use of plate-bound anti-CD3 and anti-CD28.

cell transfer, neither the CD4<sup>+</sup>CD25<sup>+</sup> Treg population generated by LT-K nor the control Treg population was capable of protecting the hosts from diabetes transfer, and the proportions of disease-free animals at 30 days were 0% and 25% for the two treatments, respectively (Fig. 7*B*). This result demonstrates that the Tregs induced by LT-K protect NOD-SCID mice from diabetes transfer via IDO-dependent mechanisms, and so does the CD25<sup>+</sup>CD62L<sup>+</sup> subset of Tregs from early prediabetic NOD mice.

## Discussion

The immunosuppressive pathway of tryptophan catabolism has been implicated, with either protective or disease-promoting roles, in a variety of physiopathologic conditions, ranging from pregnancy (3) to transplantation (4), from autoimmunity (5) and inflammation (6, 7, 46) to neoplasia (11, 12). Two major theories have been proposed to explain how tryptophan catabolism creates tolerance. One theory posits that tryptophan breakdown suppresses T cell proliferation by dramatically reducing the supply of this indispensable amino acid in local tissue microenvironments (2). The other theory postulates that the downstream metabolites of tryptophan catabolism act to suppress immune cells, probably by proapoptotic mechanisms (1). In addition, it has been suggested that quinolinate, a tryptophan catabolite, functions to replenish nicotinamide adenine dinucleotide in leukocytes, which is depleted by oxidative stress during an immune response (47). Recently, it has been proposed that the Treg phenotype could result, in part, from a biochemical reaction involving the modulatory activity of the tryptophan catabolite 3-HAA (48). These are not necessarily excluding possibilities and each might have a role in the biology of IDO (32). Reconciling, however, these different possibilities could be crucial to an integral role of tryptophan catabolism in mediating aspects of immune regulation that are both differentiated and interdependent (49).

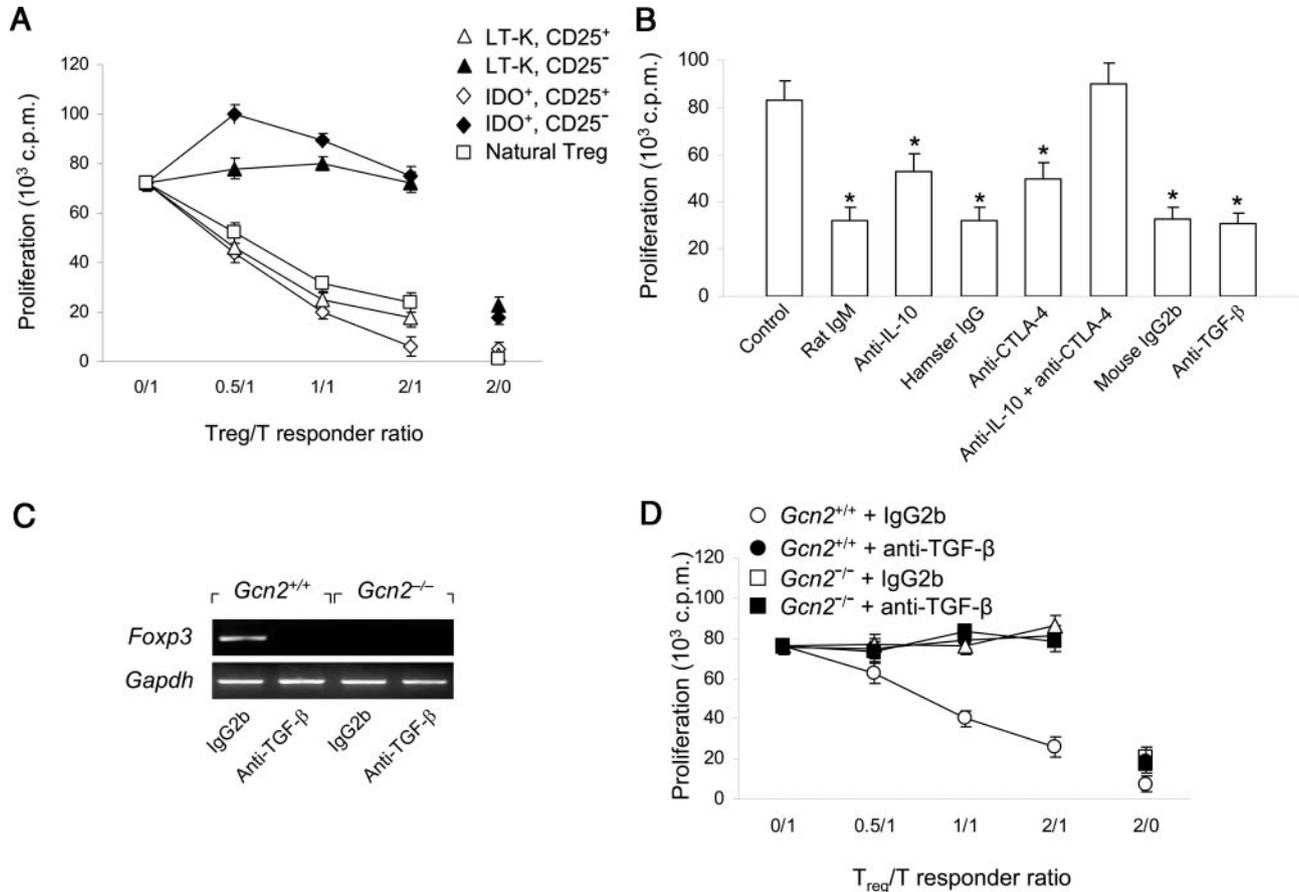
Presumably due to their expression of tryptophanyl-tRNA-synthetase, which counteracts IDO-induced tryptophan depletion, naive CD8<sup>+</sup> T cells are resistant to cell cycle arrest by tryptophan catabolism when exposed to strong antigenic stimuli (15), and are

likewise resistant to kynurenine-induced apoptosis (17). Yet, Ag-specific class I-restricted responses are severely and reversibly impaired under conditions of IDO activation in vivo (10, 11). TCR  $\zeta$ -chain down-regulation and impaired in vitro T cell function have been described in cancer and autoimmune and infectious diseases (19). Although down-regulation of  $\zeta$ -chain could be a normal mechanism for controlling an excessive and potentially hazardous immune response, the continuous presence of pathogen or tumor Ags and chronic inflammation most likely exploits this mechanism to impair immune responses to chronic diseases (18), many of which are characterized by sustained IDO expression (1, 2). We found that a LT concentration, incapable of impairing T cell reactivity per se (30), synergized with physiologic concentrations of kynurenines (17) in down-regulating T cell CD3 $\zeta$  expression in a specific and reversible fashion. The effect was demonstrable in vivo and required the activity of the GCN2 kinase. Although a causal link was not established between down-regulation of CD3 $\zeta$  expression and impaired function of CD8<sup>+</sup> T cells, the data suggested an association of the former effect with a reduced cytotoxic activity in vitro.

In eukaryotes, the mechanism for recognizing indispensable amino acid deficiency follows the conserved general control system, wherein uncharged transfer RNA induces phosphorylation of eukaryotic initiation factor 2 via the GCN2 kinase. This basic mechanism of nutritional stress management functions primarily to couple cell growth to amino acid availability (26). It has been proposed that GCN2 acts as a molecular sensor in T cells, allowing them to detect and respond to conditions created by IDO (32). Although amino acid deficiency alone is necessary and sufficient to activate a GCN2 response, our current data demonstrate that CD3 $\zeta$  down-regulation requires the combined effects of tryptophan depletion and tryptophan catabolites. This clarifies the qualitative nature of the stress conditions created by IDO in vivo and, at the same time, explains the specificity of the T cell response to this particular form of 'nutritional' stress. In the case of conditions generated by tumoral IDO (11), the exquisite sensitivity of TCR-expressing T cells to LT-K effects could explain why tryptophan catabolism likely represents an efficient mechanism used by tumors for immune evasion in the absence of any gross effect of nutrient depletion, which would be detrimental to the tumor itself.

CD4<sup>+</sup>CD25<sup>+</sup> Tregs are crucial in controlling autoimmune and inflammatory pathology, but little is known about their development and maintenance (20–23). Although it appears very likely that most CD4<sup>+</sup>CD25<sup>+</sup> Treg cell development occurs in the thymus, accumulating evidence suggests that these cells can also develop in the periphery (50, 51), and that they do so under natural conditions, i.e., with the expression of a natural TCR repertoire and exposure to the natural endogenous Ag load (38). We found that mature peripheral CD4<sup>+</sup>CD25<sup>-</sup> T cells, that are relatively resistant to CD3 $\zeta$  down-regulation by LT-K, can indeed convert to a CD4<sup>+</sup>CD25<sup>+</sup> Treg phenotype through a process requiring GCN2 and leading to a gradual decrease in IL-2 production and up-regulation of IL-10 and TGF- $\beta$ . Although the converted CD4<sup>+</sup>CD25<sup>+</sup> cells themselves fail to proliferate after stimulation, they can suppress proliferation of responder cells in vitro. The converted cells express high amounts of *Foxp3* transcript and Foxp3 protein. Autocrine TGF- $\beta$  appears to be a prerequisite for the induction of Foxp3 and Treg function.

Much evidence is now available for the diversity of CD4<sup>+</sup> Tregs that control distinct pathologies (52). Treg subsets defined by the CD25, CD62L, and CD45RB markers overlap only partially, suggesting that these subsets are functionally distinct or that a relevant Treg subset represents only a minority of the cells composing the three populations defined by these markers. As a result, the Treg



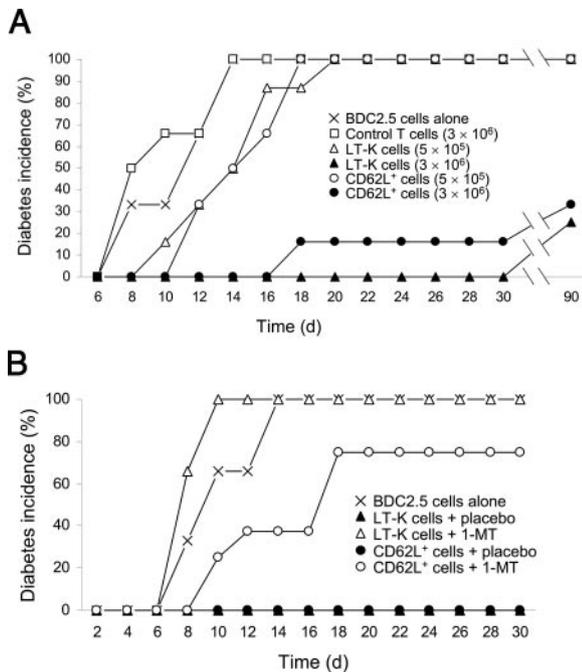
**FIGURE 6.** The CD4<sup>+</sup>CD25<sup>+</sup> cells induced by LT-K have regulatory function in vitro that is dependent on CTLA-4 expression and IL-10 production, and require GCN2 and autocrine TGF-β for the induction of Foxp3. *A*, Sorted CD4<sup>+</sup>CD25<sup>-</sup> cells were activated by plate-bound anti-CD3 and anti-CD28 in LT-K media or cocultured with IDO<sup>+</sup> DCs in the presence of soluble anti-CD3 for 7 days. Cells were then recovered and fractionated, and various numbers of CD25<sup>+</sup> or CD25<sup>-</sup> cells were cocultured with 1 × 10<sup>5</sup> naive CD4<sup>+</sup>CD25<sup>-</sup> cells and anti-CD3 and accessory cells at different Treg to responder cell ratios. Natural Tregs were assayed for comparison. Proliferation was measured as [<sup>3</sup>H]thymidine incorporation (mean ± SD) at 48 h. *B*, CD4<sup>+</sup>CD25<sup>-</sup> cells were exposed to LT-K to generate a CD25<sup>+</sup> fraction that was purified and assayed for regulatory activity as described in *A*, in the presence of Abs to IL-10, CTLA-4, or TGF-β or of isotype controls. Control, CD4<sup>+</sup>CD25<sup>-</sup> cells cultured in the absence of a CD25<sup>+</sup> fraction. Significantly different (\*, *p* < 0.01) from control. In both *A* and *B*, one experiment is reported of three performed with similar results. *C*, CD4<sup>+</sup>CD25<sup>-</sup> cells were obtained from *Gcn2*<sup>-/-</sup> or wild-type mice to be activated with anti-CD3 and anti-CD28 in control or LT-K media, with or without anti-TGF-β. At 7 days, cells were recovered and *Foxp3* mRNA levels were quantified by RT-PCR using *Gapdh* normalization. *D*, Portions of the cells cultured in LT-K as described in *C* were admixed with CD4<sup>+</sup>CD25<sup>-</sup> cells (1 × 10<sup>5</sup>) and accessory cells in the presence of anti-CD3 at different Treg to responder cell ratios. After 48 h, proliferation was assessed by [<sup>3</sup>H]thymidine incorporation (mean ± SD). One experiment representative of three.

population that protects NOD mice from fulminant diabetes appears to be CD25<sup>+</sup>CD62L<sup>+</sup>, shows a variable expression of CD45RB, and is distinct from the Treg subset protecting from colitis or gastritis (28). Conversion of peripheral CD4<sup>+</sup>CD25<sup>-</sup> naive T cells to CD4<sup>+</sup>CD25<sup>+</sup> Tregs by TGF-β induction of Foxp3 results in the appearance of a CD25<sup>+</sup>CD45RB<sup>low</sup>CTLA-4<sup>+</sup> subset that is unresponsive to TCR stimulation, produces neither Th1 nor Th2 cytokines, expresses TGF-β, and inhibits normal T cell proliferation in vitro (37). Similar to this Treg population, we found that cell conversion in our model system with LT-K was further characterized by the presence of CD62L, GITR and secreted IL-10, with little or no expression of CD69 or BTLA.

Among Foxp3-expressing Treg populations, CD4<sup>+</sup>CD25<sup>+</sup> T cells are known to function through different mechanisms, including cell-cell contact, membrane or soluble TGF-β, and secreted IL-10 (21, 23, 52). We found that the Tregs generated by LT and kynurenines would suppress naive T cell proliferation in vitro by a combination of CTLA-4-dependent and IL-10-dependent effects, as demonstrated by the blocking effect of neutralizing Abs. In vivo, we found that the CD4<sup>+</sup>CD25<sup>+</sup> Tregs induced by LT-K prevented

transfer of diabetes to an extent comparable to the control Treg population from prediabetic NOD mice. The protection transferred by both populations required tryptophan catabolism in the host. This indicates that modulation of tryptophan catabolism could represent an important mechanism of action not only for developmentally controlled 'professional' Tregs (52), as suggested by previous data (13), but also for T cells that are induced in the periphery to express a regulatory phenotype.

In conclusion, our data demonstrate that, before T cell cycle arrest due to a critical shortage in tryptophan supply, the early control of an immune response by IDO involves adjustable and versatile effects, including CD3ζ down-regulation and Treg generation, operated by two distinct signals, i.e., tryptophan deficiency and kynurenine production. Although the mechanisms of kynurenine action are largely unknown (17, 25, 53, 54), this observation clearly distinguishes the activity of IDO as a truly immunoregulatory enzyme from the effects on T cells of a mere starvation for amino acids, and paves the way to the possible use of synthetic kynurenines as therapeutic agents. Defining one possible means by which Tregs are physiologically generated and maintained in the periphery could provide



**FIGURE 7.** Tregs generated in vitro by LT and kynurenines protect NOD-SCID mice from developing fulminant diabetes. **A**, Eight- to 12-wk-old NOD/BDC2.5 splenocytes ( $1 \times 10^6$ ) were injected i.v. either alone or in combination with two different doses ( $5 \times 10^5$  or  $3 \times 10^6$ ) of unfractionated cells derived from an originally  $CD4^+CD25^-$  population exposed to LT-K for 7 days in the presence of anti-CD3 and anti-CD28.  $CD4^+CD25^-$  cells cultured in standard medium (control T cells) and  $CD62L^+$  T cells isolated from 4-wk-old prediabetic NOD mice were also used for comparison. The incidence of diabetes is indicated over time for the different groups ( $n \geq 6$ ), as revealed by glycosuria and hyperglycemia. One representative experiment of three conducted is shown. Breaks in lines and axis indicate scale interruption. **B**, Naive  $CD4^+CD25^-$  cells were cultured with LT-K for 7 days as indicated in **A**. Sorted  $CD25^+$  cells or  $CD62L^+$  T cells from prediabetic NOD mice ( $2 \times 10^6$ ) were cotransferred with diabetogenic NOD/BDC2.5 splenocytes ( $1 \times 10^6$ ) into NOD-SCID mice that had been implanted with placebo or 1-MT pellets. The animals were then monitored for diabetes incidence. One of three experiments with similar results.

new opportunities for designing ways to induce or abrogate tolerance in physiopathologic conditions.

## Acknowledgments

We thank L. Chatenoud (Institut National de la Santé et de la Recherche Médicale Unité 580, Paris, France) for the gift of transgenic BDC2.5/NOD mice, T. Fortunati for performing surgical procedures, and G. Andrielli for technical assistance.

## Disclosures

The authors have no financial conflict of interest.

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