

GCN2 Kinase in T Cells Mediates Proliferative Arrest and Anergy Induction in Response to Indoleamine 2,3-Dioxygenase

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Summary

Indoleamine 2,3 dioxygenase (IDO) catabolizes the amino acid tryptophan. IDO-expressing immunoregulatory dendritic cells (DCs) have been implicated in settings including tumors, autoimmunity, and transplant tolerance. However, the downstream molecular mechanisms by which IDO functions to regulate T cell responses remain unknown. We now show that IDO-expressing plasmacytoid DCs activate the GCN2 kinase pathway in responding T cells. GCN2 is a stress-response kinase that is activated by elevations in uncharged tRNA. T cells with a targeted disruption of GCN2 were not susceptible to IDO-mediated suppression of proliferation *in vitro*. *In vivo*, proliferation of GCN2-knockout T cells was not inhibited by IDO-expressing DCs from tumor-draining lymph nodes. IDO induced profound anergy in responding wild-type T cells, but GCN2-knockout cells were refractory to IDO-induced anergy. We hypothesize that GCN2 acts as a molecular sensor in T cells, allowing them to detect and respond to conditions created by IDO.

Introduction

Indoleamine 2,3-dioxygenase (IDO) is a potent immunoregulatory enzyme. As recently reviewed (Grohmann et al., 2003; Mellor and Munn, 2004), expression of IDO allows certain macrophages and dendritic cells (DCs) to inhibit T cell proliferation *in vitro* and *in vivo*. Transfection with IDO confers suppressor activity on tumors and tumor cell lines, and delays the rejection of grafted cells and tissues. Endogenous IDO contributes to maternal tolerance toward the allogeneic fetus, self-tolerance in NOD mice, and control of autoimmune encephalitis, experimental asthma, and inflammatory bowel disease. IDO may also serve as a downstream suppressor mechanisms used by certain Tregs, and by the immunosuppressive agent CTLA4-Ig. In tumor-bearing hosts, IDO-expressing DCs are found in tumor-draining lymph nodes (TDLNs) of both humans and mice (Munn et al., 2004a; Munn et al., 2002). The IDO⁺ plasmacytoid

DCs (PDCs) from murine TDLNs actively suppress T cell proliferation *in vitro*, and create profound antigen-specific anergy *in vivo* (Munn et al., 2004a).

Despite growing recognition of its biologic importance, the molecular mechanism(s) by which IDO functions to regulate T cell responses remain unclear. Two hypotheses have been advanced: either that IDO generates immunosuppressive metabolites of tryptophan (Grohmann et al., 2003) or that IDO locally depletes tryptophan to the point that T cells are unable to proliferate (Mellor and Munn, 2004). These are not mutually exclusive possibilities, and each may play a role in the biology of IDO. However, it is impossible to directly measure tryptophan or its metabolites in living cells, so these hypotheses have remained largely speculative. To circumvent this impasse, we focused instead on identifying specific molecular pathways in T cells that responded to the conditions created by IDO and that therefore might mediate its downstream effects.

IDO degrades the essential amino acid tryptophan, so we hypothesized that IDO might affect pathways known to respond to amino acid metabolism. One possibility was the nutrient-sensitive mTOR kinase pathway (Fingar and Blenis, 2004). However, we, like others (Fox et al., 2005), found that that inhibitors of mTOR such as rapamycin did not recapitulate the profound proliferative arrest seen with IDO-mediated suppression. In yeast, there exists a second amino acid-sensitive pathway which is mediated by the kinase GCN2 (Hinnebusch, 1994). GCN2 contains a regulatory domain that binds the uncharged form of transfer RNA (tRNA). Amino acid insufficiency causes a rise in uncharged tRNA, which activates the GCN2 kinase domain and initiates downstream signaling (Dong et al., 2000). Recently, the mammalian homolog of GCN2 has been identified and shown to have similar signaling properties (Harding et al., 2000a; Sood et al., 2000).

Activation of GCN2 initiates a characteristic stress-response program conserved from yeast to mammals (Harding et al., 2003; Natarajan et al., 2001). This pathway, which has been termed the integrated stress response (ISR), can trigger cell-cycle arrest, differentiation, compensatory adaptation, or apoptosis, depending on the cell type and the initiating stress (Crosby et al., 2000; Harding et al., 2000b; Niwa and Walter, 2000; Rao et al., 2004). In the current study, we test the hypothesis that expression of IDO by APCs activates the GCN2 kinase pathway in responding T cells, generating an intracellular signal that mediates key biologic effects of IDO.

Results

Tryptophan Supplementation Reverses IDO-Mediated Suppression

We first asked whether tryptophan supplementation could prevent IDO-mediated suppression during mixed leukocyte reactions (MLRs). IDO⁺ PDCs (B220⁺CD11c⁺) were isolated from murine TDLNs (Munn et al., 2004a).

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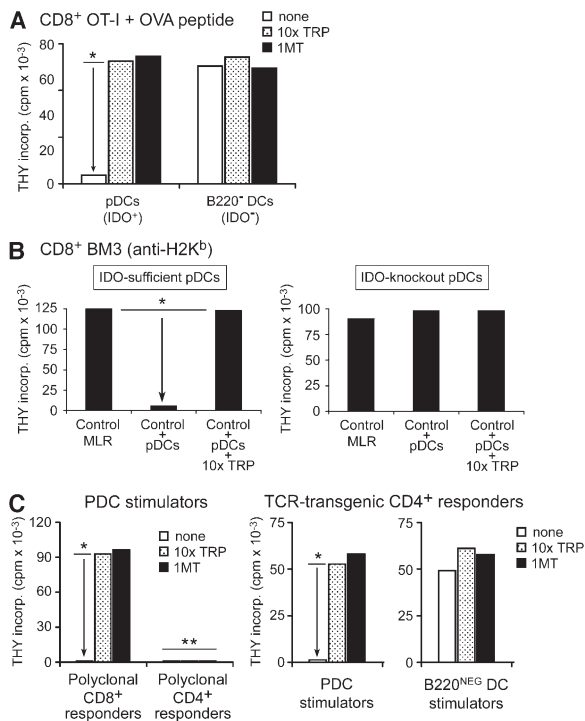


Figure 1. Exogenous Tryptophan Prevents IDO-Mediated Suppression

(A) IDO⁺ PDCs (CD11c⁺B220⁺) or nonsuppressive conventional DCs (CD11c⁺B220^{NEG}) were isolated from TDLNs, and used to present cognate SIINFEKL peptide to OT-I T cells in MLRs. Cultures received 250 μ M tryptophan (10x TRP), 200 μ M 1MT, or no additive. The arrow indicates the component of IDO-mediated suppression (* p < .01 by ANOVA, compared to all other groups).

(B) Dominant suppression is blocked by 10x tryptophan. TDLN PDCs derived from IDO-sufficient hosts (left panel) mediated dominant suppression when added to MLRs stimulated by nonsuppressive APCs from the same LN ("Control MLR", BM3 responders). In contrast, TDLN PDCs from IDO-knockout hosts (right panel) showed no suppression (* p < .01 by ANOVA).

(C) Polyclonal CD4⁺ T cells contain a nonspecific suppressor activity, unrelated to IDO. (Left panel) Comparison of sorted CD8⁺ versus CD4⁺ polyclonal T cell responders, using TDLN PDCs plus anti-CD3 mitogen as stimulators (* p < .01 by ANOVA versus the two other CD8 groups; ** p < .01 versus the CD8 groups). (Right panel) Naive TCR-transgenic CD4⁺ T cell responders from female A1 mice, with cognate HY peptide presented by either IDO-expressing B220⁺ PDCs versus nonsuppressive B220^{NEG} conventional DCs, both from TDLNs (* p < .01 by ANOVA versus the two other groups).

Responder cells in MLRs were purified CD8⁺ TCR-transgenic T cells: either OT-I (recognizing an ovalbumin peptide) or BM3 (recognizing H2K^b as an alloantigen). **Figure 1A** shows that TDLN PDCs effectively suppressed proliferation of OT-I cells, and this was abrogated by supplementation excess L-tryptophan (10x the normal concentration in RPMI medium) or by blocking IDO activity with the pharmacologic inhibitor 1-methyl-D-tryptophan (1MT). In contrast, MLRs stimulated by conventional DCs (CD11c⁺B220^{NEG}) from the same LNs showed no suppression, and neither 1MT nor 10x tryptophan had any effect on proliferation.

The absence of T cell proliferation was not due to a failure of the PDCs to present antigen, since (as shown

above) the PDCs were excellent APCs as long as IDO was blocked. Rather, the IDO⁺ PDCs appeared to be actively and dominantly suppressive, as shown in **Figure 1B**. IDO⁺ PDCs were mixed with an excess of non-suppressive (CD11c⁺B220^{NEG}) DCs, and the mixed population used as stimulators for BM3 T cells. The target H2K^b alloantigen was constitutively expressed at high levels on both populations of DCs, so effective antigen presentation was assured. Despite this, the IDO⁺ PDCs dominantly suppressed all T cell proliferation in the mixed cultures. This occurred in an IDO-dependent fashion, since suppression was reversed by 10x tryptophan and 1MT, and TDLN PDCs derived from mice with a targeted disruption of the IDO gene (IDO-KO mice, right panel) showed no suppression.

The preceding studies focused on TCR-transgenic CD8⁺ responder cells. Nontransgenic (polyclonal) CD8⁺ T cells showed a similar pattern IDO-mediated suppression (**Figure 1C**, left panel). However, as shown in the same panel, polyclonal CD4⁺ T cells displayed a high level of nonspecific regulatory/suppressor activity, which inhibited essentially all proliferative responses. This was not IDO-mediated (since it was not reversed by 1MT or 10x tryptophan), and it was observed only when PDCs from TDLNs were used as APCs for polyclonal CD4⁺ T cells. (In other experiments, data not shown, conventional B220^{NEG} DCs stimulated normal proliferation of CD4⁺ cells.) Thus, the nonspecific suppression appeared to represent a Treg-like activity, similar to the potent CD4⁺ Treg activity that we have previously described in TDLNs (Munn et al., 2004a). This suppressor activity was present in the polyclonal CD4⁺ repertoire, but it was not an intrinsic feature of all CD4⁺ T cells, because naive TCR-transgenic CD4⁺ T cells (recognizing a male HY peptide, and taken from female mice so as to be obligately naive) displayed no such suppressor activity (**Figure 1C**, right panel). These naive CD4⁺ T cells responded to IDO-expressing PDCs identically to CD8⁺ T cells. However, because the presence of nonspecific suppressor activity potentially complicated the interpretation of results using CD4⁺ cells, in subsequent experiments we used purified CD8⁺ responder cells. This allowed us to unambiguously assess the specific effects of IDO.

CHOP Is a Marker for GCN2 Activation in T Cells

There is no technique to directly measure the kinase activity of GCN2 in intact cells. Therefore, in order to ask whether IDO activated GCN2 in T cells, we wished to identify a downstream marker whose expression was dependent on GCN2 and that could serve as a "reporter" for intracellular GCN2 activation. The CHOP gene (also known as gadd153) is a well-accepted marker for GCN2 activation, and CHOP protein can be readily detected by specific antisera (Harding et al., 2000a). While CHOP can be induced by other stresses as well, its induction by amino acid deprivation is known to be specifically mediated by GCN2 (Harding et al., 2000a). To define the mechanistic role of GCN2 in T cells, we used mice homozygous for a targeted deletion in the GCN2 locus (GCN2-KO), as described in Experimental Procedures.

Spleen cells were isolated from GCN2-KO mice or

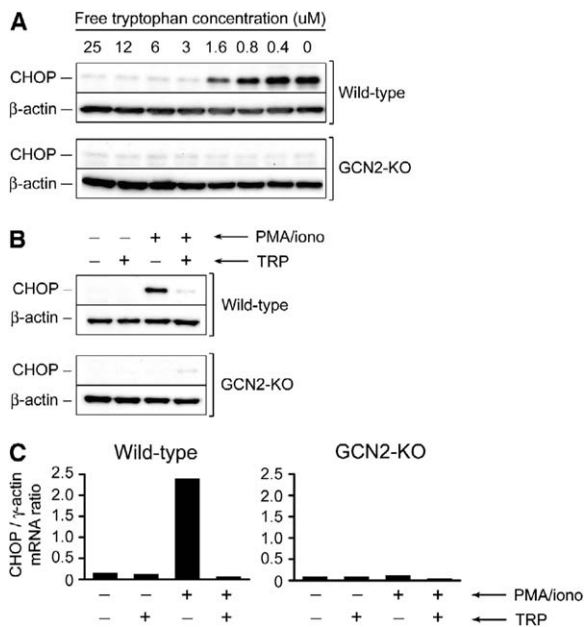


Figure 2. GCN2-Mediated Upregulation of CHOP in T Cells

(A) GCN2-dependent upregulation of CHOP in tryptophan-deficient medium. Splenocytes cultured in chemically defined medium with graded amounts of tryptophan. Cells were activated for 24 hr using either PMA + ionomycin (shown) or immobilized anti-CD3 + anti-CD28 antibodies (which gave similar results). CHOP expression was analyzed by western blot. Experiments were performed on wild-type cells (upper blot) or GCN2-KO cells (lower blot). Membranes were stripped and reblotted for β-actin as a loading control. One of two identical experiments.

(B) CHOP upregulation requires simultaneous T cell activation and tryptophan withdrawal. Splenic T cells from wild-type or GCN2-KO mice were cultured with or without activation (PMA+ionomycin) in the presence or absence of tryptophan (25 μM). CHOP was measured by western blot after 24 hr with β-actin as a loading control. One of three experiments.

(C) CHOP is induced at the mRNA level by tryptophan withdrawal. Experiments were performed as in the previous panel, and CHOP-specific mRNA measured by quantitative real-time RT-PCR. Data are normalized to γ-actin expression in each sample. One of five experiments.

wild-type controls, then cultured in tryptophan-free medium for 24 hr and tested for CHOP induction. Controls received graded amounts of added tryptophan. All groups were activated either with phorbol myristate acetate (PMA) and ionomycin, or with TCR cross linking (anti-CD3 antibody), both of which gave identical results. Figure 2A shows that activated wild-type T cells upregulated CHOP when the concentration of tryptophan fell below ~3 μM, whereas GCN2-deficient T cells showed no expression of CHOP. CHOP induction occurred only if the T cells were activated, either by anti-CD3/CD28 crosslinking, or by PMA+ionomycin (Figure 2B). This requirement for both T cell activation and tryptophan withdrawal was also seen at the level of CHOP mRNA (Figure 2C). In all cases, CHOP induction was strictly dependent on an intact GCN2 pathway. Thus, we concluded that CHOP expression served as an informative downstream marker of GCN2 activity in activated T cells.

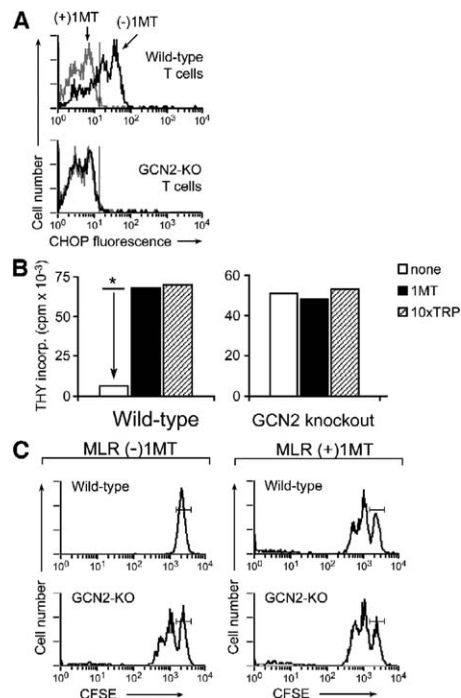


Figure 3. GCN2 Is Required for Suppression of T Cells by IDO-Expressing PDCs

(A) Upregulation of CHOP by IDO during MLRs. Sorted polyclonal CD8⁺ T cells from wild-type mice (upper panel) or GCN2-KO mice (lower panel) were activated in MLRs with IDO⁺ TDLN PDCs, using anti-CD3 mitogen. MLRs were performed without 1MT (black line) or with 1MT (grey line). After 24 hr, intracellular CHOP expression was measured by FACS. Histograms show CHOP staining in the gated CD8⁺ T cell population in each MLR. Vertical bars show the 2 SD cutoff for each isotype-matched negative control. (The negative control histograms are omitted for clarity, but were identical to the "(+)1MT" histograms). One of three experiments.

(B) GCN2-KO T cells are resistant to suppression by IDO. Sorted CD8⁺ T cells were isolated from either wild-type or GCN2-KO spleens as shown. TDLN PDCs (CD11c⁺B220⁺) were added and MLRs stimulated by anti-CD3 mitogen, with or without 1MT or 10x tryptophan. (*p < .01 by ANOVA versus the other wild-type groups.)

(C) Sorted CD8⁺ T cells were labeled with CFSE, used as responders in anti-CD3 mitogen assays with TDLN PDCs. Wild-type or GCN2-KO cells were tested with or without 1MT, as indicated. After 48 hr, cells were analyzed for cell division by CFSE dye dilution. The marker identifies the nondividing peak in each histogram. One of three experiments.

GCN2 Is Activated by IDO

To ask whether GCN2 was activated when T cells were exposed to IDO, we examined intracellular CHOP induction in T cells during MLRs. Polyclonal CD8⁺ T cells were isolated from wild-type or GCN2-KO mice, mixed with IDO⁺ PDCs from TDLNs, and activated with anti-CD3 mitogen. CHOP was assessed by intracellular immunofluorescent staining and flow cytometry. Experiments were performed with and without 1MT to block IDO activity. Figure 3A shows that the majority of wild-type T cells had upregulated CHOP after 24 hr in MLRs. CHOP induction was IDO dependent, since T cells showed no CHOP expression when IDO activity was inhibited by 1MT (identical results were obtained using PDCs from IDO-knockout mice, data not shown). CHOP

induction was also strictly GCN2 dependent, since GCN2-KO T cells showed no CHOP expression under any conditions (Figure 3A, lower panel).

GCN2-KO T Cells Are Not Suppressed by IDO⁺ PDCs

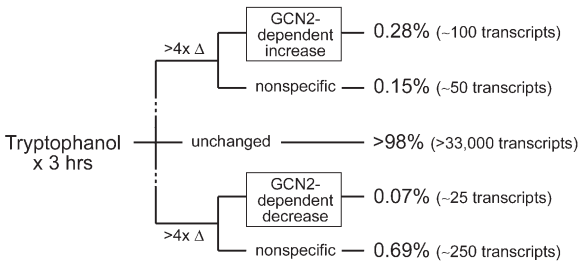
We next asked whether GCN2 was required in order for T cell proliferation to be suppressed by IDO. Sorted CD8⁺ T cells from wild-type or GCN2-KO mice were used as responders in MLRs. Figure 3B shows that TDLN PDCs potently suppressed proliferation of wild-type T cells, whereas GCN2-KO T cells proliferated normally in the presence of the same PDCs, and showed no effect of 10× tryptophan or 1MT. Identical results were also seen in an allogeneic-MLR model (data not shown) using PDCs from F1(CBA×B6) tumor-bearing mice and allogeneic T cells from 129 background responders (wild-type and GCN2-KO). Figure 3C shows CFSE dye-dilution studies, confirming that GCN2-KO T cells proliferated normally in the presence of IDO⁺ PDCs. In all of the preceding experiments, the IDO⁺ PDCs were derived from GCN2-sufficient (wild-type) mice, so the role for GCN2 was played out specifically in the responding T cells.

GCN2 Activates a Selective and Specific Regulatory Pathway

Like other eIF2α kinases, GCN2 activates the ISR pathway. In principle, this pathway is able to selectively modulate the expression of specific target genes (Harding et al., 2003). However, no such studies had been performed in T cells. We used microarrays to analyze expression of >34,000 full-length mouse genes before and after activation of GCN2. T cells were stimulated for 40 hr with PMA+ionomycin, then treated for 3 hr with 0.25 mM tryptophanol. Tryptophanol is a competitive inhibitor of the tryptophanyl-tRNA synthetase enzyme; by raising the pool of uncharged tRNA^{TRP} it acts as a pharmacologic activator of GCN2 (Jiang et al., 2003).

Figure 4 summarizes the results of a representative microarray experiment comparing T cells from wild-type versus GCN2-KO mice, with and without tryptophanol. The analysis focuses on transcripts that changed ≥4-fold (up or down) in the wild-type cells following treatment with tryptophanol (versus control cells receiving buffer only). Of the transcripts showing a change in the wild-type cells, the GCN2-dependent subset of these was defined as those which failed to change in GCN2-KO cells (treated identically with tryptophanol). Overall, more than 98% of transcripts remained unchanged (<2-fold difference) across all treatment groups. Only 0.28% of transcripts in this experiment showed a GCN2-dependent increase at the ≥4-fold level, and only 0.07% showed a GCN2-dependent decrease. (Changes falling between 2- and 4-fold were considered ambiguous, but represented <1% of total transcripts.)

Several examples of genes showing GCN2-dependent upregulation are listed in Figure 4. (The few transcripts showing GCN2-dependent downregulation were almost all unknown sequences, and are not listed.) As expected, CHOP was one of the genes upregulated by



Examples of genes showing selective GCN2-dependent upregulation

Gene	Symbol	Fold increase*	Description
CHOP	Ddit3	7x	ISR-responsive gene
Gadd45α	Gadd45a	8x	ISR-responsive gene
Herp	Herpud1	5x	ISR-responsive gene
4E-BP1	Eif4ebp1	4x	translational control
eIF4G	Eif4g1	6x	translational control
JAK1	Jak1	6x	signal transduction
NFκB-2	Nfkb2	7x	signal transduction
FK506-BP8	Fkbp8	23x	anti-apoptosis
IFNγ-R2	Ifngr2	17x	IFN-gamma receptor

* Fold increase in wild-type cells after treatment with tryptophanol, compared to wild-type cells without tryptophanol.

Figure 4. Selective Regulation of Gene Expression by GCN2

Nylon wool-enriched T cells (from wild-type or GCN2-KO) mice were activated for 40 hr with PMA + ionomycin, then treated for 3 hr with 0.25 mM tryptophanol to activate GCN2. (The concentration of tryptophanol was chosen as the dose that reduced the rate of proliferation by 50%.) Control cells received buffer alone without tryptophanol. Microarray hybridization was performed for each sample, as described in the text. A second, identical experiment produced similar results. The examples in the table showed the same pattern of changes in both experiments.

tryptophanol, and its induction as measured by microarray corresponded closely to the value obtained by quantitative RT-PCR on the same samples (9-fold for the experiment shown).

Activation of the GCN2 Pathway In Vivo

We next asked whether IDO activated the GCN2 pathway in vivo. For this we employed two complementary models: one in which IDO expression was genetically defined in the APCs, and a second in which GCN2 expression was genetically defined in the T cells. The first model was a graft-versus-host reaction mediated by BM3 T cells, in which host IDO is known to be strongly induced (Munn et al., 1998). Figures 5A–5D show that CHOP was upregulated in a population of cells in spleen in this model, and that CHOP induction was dependent on host IDO (since no cells upregulated CHOP in IDO-KO hosts).

To ask whether CHOP induction was mediated specifically via GCN2 in responding T cells, we used a model of genetically defined T cells responding to antigen presented by adoptively transferred TDLN DCs in vivo (Munn et al., 2004a). Responder T cells were taken from OT-I mice bred onto either the GCN2-KO background (OT-I^{GCN2-KO}) or wild-type background (OT-I^{WT}). Figure

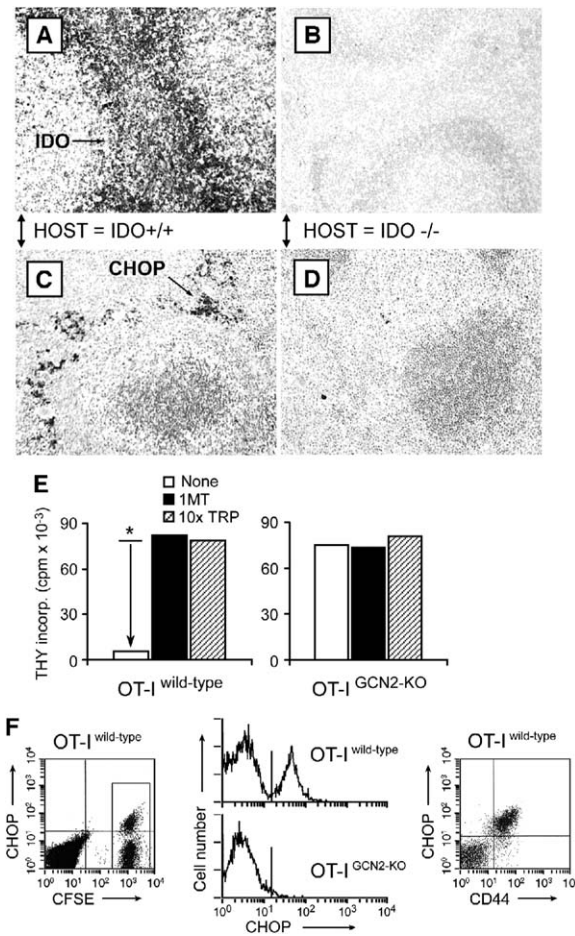


Figure 5. IDO-Induced GCN2-Dependent Upregulation of CHOP In Vivo

(A–D) IDO-dependent induction of CHOP in vivo. BM3 T cells were injected into allogeneic F1(CBA×B6) hosts expressing the H2K^b target antigen. Hosts were either wild-type for IDO, or homozygous IDO knockout (IDO^{-/-}). After 4 days, spleens were stained for IDO expression (dark chromogen, panels [A] and [B]) or for CHOP expression (dark chromogen, panels [C] and [D]). Negative controls (each primary antibody neutralized with the immunizing peptide) were comparable to the IDO-KO hosts, and are not shown. (×200). (E) CD8⁺ T cells were sorted from OT-I mice bred onto the GCN2-KO background (OT-I^{GCN2-KO}) or wild-type (OT-I^{WT}). MLRs were stimulated with IDO⁺ TDLN PDCs plus SIINFEKL peptide. (*p < .01 by ANOVA versus all other groups.)

(F) Upregulation of CHOP in vivo. CFSE-labeled OT-I^{WT} or OT-I^{GCN2-KO} cells were transferred into GCN2-KO recipients. DCs were isolated from TDLNs (sorted CD11c⁺ cells, as described in Experimental Procedures) and injected subcutaneously into each recipient animal. Three days later the draining inguinal LNs were harvested and stained for intracellular CHOP expression versus CD8 or CD44. The left-hand dot plot shows expression of CHOP versus CFSE in the OT-I^{WT} cells. The middle histograms compare CHOP expression (gated on CFSE⁺ CD8⁺ cells) in OT-I^{WT} versus OT-I^{GCN2-KO} T cells. The right-hand dot plot shows expression of CHOP versus CD44 in OT-I^{WT} cells (gated on CFSE⁺). One of three experiments.

5E shows in vitro validation studies using these two cell types, verifying that OT-I^{WT} T cells were fully suppressed by IDO⁺ PDCs in MLRs (which was reversed

by 1MT and 10× tryptophan), while OT-I^{GCN2-KO} T cells showed no suppression.

Recipient mice were preloaded with a cohort of CFSE-labeled OT-I^{GCN2-KO} or OT-I^{WT} responder T cells. DCs were isolated from TDLNs as described in Experimental Procedures, pulsed with cognate SIINFEKL peptide, and injected subcutaneously into the preloaded recipients. All recipient mice in these experiments were homozygous GCN2-KO, so that GCN2 expression would be confined solely to the transferred T cells; this allowed us to ask if GCN2 expression in the T cells was both necessary and sufficient for IDO-mediated suppression to occur. Figure 5F shows that a significant fraction of the wild-type OT-I cells upregulated CHOP following exposure to TDLN DCs in vivo. In contrast, none of the GCN2-KO T cells expressed CHOP (middle histograms). Figure 5F further shows that the population of OT-I cells that became CHOP⁺ were specifically those that had encountered antigen (as indicated by upregulation of CD44, a marker for antigen encounter in OT-I cells [Kurts et al., 1996]). This latter finding was consistent with the data in Figure 2B, where we found that CHOP was expressed only when T cells simultaneously received both a GCN2-activating signal and a second signal simulating TCR engagement.

Functional Suppression of Wild-Type OT-I T Cells In Vivo

Figure 6 demonstrates that OT-I^{WT} are functionally suppressed by IDO⁺ TDLN DC in vivo. Control, nonsuppressive DCs (from lymph nodes of normal animals without tumors) produced a brisk proliferative response in CFSE-labeled OT-I^{WT} cells (Figure 6A, left panel), and the proliferating cells upregulated the 1B11 surface antigen (a differentiation marker expressed by activated/effector CD8⁺ T cells [Harrington et al., 2000]). In contrast, suppressive DCs from TDLNs elicited no proliferative response from OT-I^{WT} cells, and there was no upregulation of 1B11 (Figure 6A, right panel). The lack of response was not due to a failure of the TDLN DCs to present antigen, since mice receiving TDLN DCs showed a 6- to 8-fold selective accumulation of OT-I^{WT} T cells in the LNs draining the site of TDLN DC injection (Figure 6B, left panel), and the majority of recruited cells became antigen-experienced (CD44⁺, Figure 6B, right panel). Suppression of T cell proliferation was specifically mediated by IDO, as shown by the fact that administration of 1MT to the recipient mice abrogated the suppressive effect of TDLN DCs, and restored normal proliferation (Figure 6C).

GCN2 Is Required for Susceptibility to IDO-Mediated Suppression In Vivo

We next used this model to ask whether GCN2 was mechanistically required for IDO-mediated suppression in vivo. Host mice (GCN2-KO) were preloaded with either OT-I^{WT} or OT-I^{GCN2-KO} T cells, and all mice challenged with antigen-pulsed TDLN DCs. Figure 7A shows that both proliferation and upregulation of the 1B11 activation antigen were suppressed in OT-I^{WT} cells (just as in the preceding figure), whereas OT-I^{GCN2-KO} T cells proliferated normally, and uniformly upregulated 1B11 expression. Thus, GCN2-deficient T cells dis-

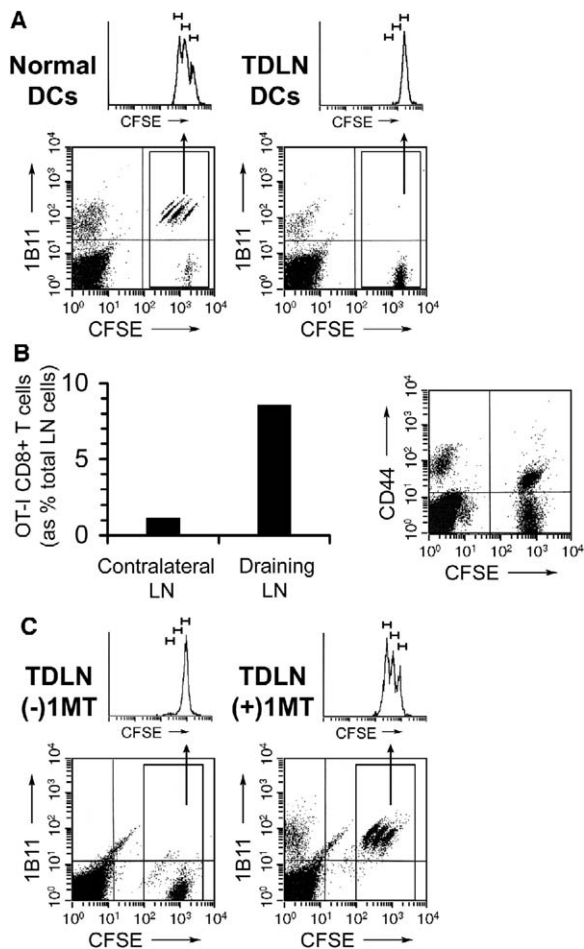


Figure 6. IDO-Mediated Suppression of Wild-Type OT-I In Vivo
(A) CFSE-labeled OT-I^{WT} T cells were adoptively transferred into wild-type hosts. Mice were injected with either CD11c⁺ TDLN DCs, or normal DCs (from inguinal LNs of animals without tumors). After 3 days, draining LNs were recovered and stained for 1B11 versus CFSE. The histograms show the fluorescence profiles of the CFSE-positive cells, with each 2-fold dilution indicated by a bar. One of three experiments.
(B) (Left) Preferential recruitment of OT-I^{WT} T cells into LNs draining the site of TDLN DC injection, compared to contralateral LNs from the same animals. (Right) Expression of CD44 antigen by the CFSE⁺ OT-I^{WT} cells in the draining LN. (OT-I cells in the contralateral LNs showed no CD44 expression, data not shown.) One of three experiments.
(C) Abrogation of IDO-mediated suppression by 1MT administration in vivo. CFSE-labeled OT-I^{WT} cells were adoptively transferred as in the previous panels, and all mice injected with TDLN DCs. One group was treated with 1MT by continuous timed-release pellets, beginning at the time of adoptive transfer. Control mice received pellets without 1MT. One of four experiments.

played the same indifference to IDO-mediated suppression in vivo that they had shown in vitro.

GCN2 Is Required for IDO-Mediated Anergy Induction

We have previously shown that IDO-expressing PDCs induce antigen-specific anergy in responding T cells in vivo (Munn et al., 2004a). Figure 7B shows an in vitro

assay demonstrating anergy induction in OT-I^{WT} cells by IDO. CD8⁺ T cells from OT-I^{WT} or OT-I^{GCN2-KO} mice were activated for 3 days using IDO⁺ TDLN PDCs. The suppressed cells (i.e., the OT-I^{WT} cells without 1MT) were then harvested, supplemented with fresh medium, and restimulated with irradiated B6 splenocytes plus SIINFEKL peptide. Figure 7B (right panel) shows that suppressed T cells had become completely refractory to restimulation. This was not due to carryover of the original IDO⁺ PDCs (most of which did not survive the initial MLR and replating step), as shown by the fact that 1MT had no ability to restore proliferation in these secondary MLRs. However, the anergic T cells were still fully viable, because brisk proliferation could be restored by the addition of exogenous IL-2 to the recall MLR (Figures 7B and 7C). Thus, IDO induced an unresponsive state consistent with classical anergy (Schwartz, 2003).

One drawback to this in vitro model was that it could not directly test whether the GCN2-KO T cells were resistant to anergy induction (because, in these experiments, groups that were actively proliferating at the end of the primary MLR had to be considered “nonanergic” by definition). We therefore addressed this question using the in vivo adoptive-transfer model. CFSE-labeled OT-I^{WT} or OT-I^{GCN2-KO} T cells were adoptively transferred into GCN2-KO hosts and all mice injected with peptide-loaded TDLN DCs. After 5 days, CFSE-labeled CD8⁺ T cells were recovered from the draining LNs by FACS sorting and restimulated with irradiated B6 splenocytes plus cognate antigen. Figure 7D shows that the OT-I^{WT} T cells had been rendered completely anergic to restimulation. (Although the OT-I^{WT} cells were anergic, they were still present and viable, as shown by their ability to be rescued by exogenous IL-2.) In contrast, OT-I^{GCN2-KO} T cells were unaffected by exposure to IDO-expressing DCs and remained fully responsive in the recall MLRs. Thus, GCN2 expression was required for IDO-mediated anergy induction in vivo.

Discussion

In the current study, we demonstrate that GCN2 is required for CD8⁺ T cells to sense and respond to conditions created by IDO. T cells lacking GCN2 proliferated normally in the presence of IDO⁺ PDCs both in vitro and in vivo and were not susceptible to IDO-induced anergy. These studies thus identify GCN2 as a downstream mediator for several key effects of IDO in our models. This constitutes the first elucidation of a specific molecular target for the immunoregulatory action of IDO in T cells.

GCN2 is one of a family of four related kinases (GCN2, PERK, HRI, and PKR), which share as their only known substrate the alpha subunit of translation initiation factor 2 (eIF2 α). Because each of the eIF2 α kinases are activated by different upstream stress signals, yet target a similar downstream pathway, this pathway has been referred to as the integrated stress response (ISR) (Dever, 2002; Harding et al., 2003; Rutkowski and Kaufman, 2004). The consequences to the cell of activating the ISR pathway depend on the nature of the stress, the specific initiating kinase, and the cell type involved.

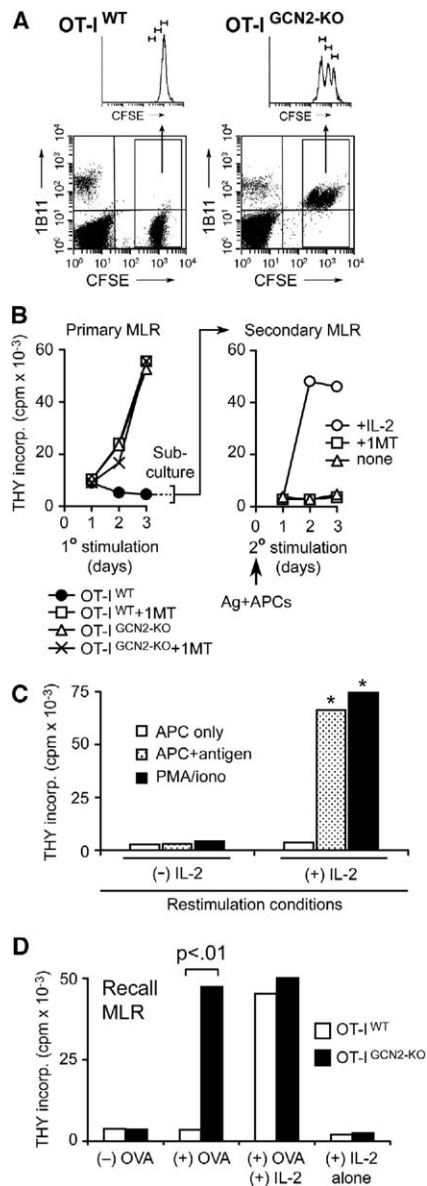


Figure 7. GCN2-KO T Cells Are Refractory to Suppression and Energy Induction In Vivo

(A) CFSE-labeled OT-I^{WT} or OT-I^{GCN2-KO} T cells were adoptively transferred into MHC-matched GCN2-KO hosts. All mice received TDLN DCs subcutaneously. After 3 days, draining LNs were harvested stained for 1B11 versus CFSE. One of four experiments.

(B) Sorted CD8⁺ T cells from OT-I^{WT} or OT-I^{GCN2-KO} mice were activated in vitro with TDLN PDCs plus SIINFEKL peptide, with or without 1MT. Proliferation was measured as thymidine incorporation on successive days during the primary MLR (left panel). On day 3, the OT-I^{WT} group without 1MT (the only nonproliferating group) was harvested and tested for energy in recall MLRs (restimulation with normal C57BL/6 splenocytes plus peptide antigen), as described in [Experimental Procedures](#). Recall MLRs were performed with or without 1MT or IL-2, as shown.

(C) Anergic T cells are rescued by exogenous IL-2, but not by PMA+ionomycin. OT-I^{WT} T cells were anergized for 3 days by PDCs + antigen as in the previous panel, then restimulated with either normal splenocytes + antigen, or with PMA+ionomycin, in the presence or absence of exogenous IL-2. (**p* < .05 by ANOVA versus all of the groups without IL-2.)

The result may be cell cycle arrest, lineage-specific differentiation, metabolic adaptation, or cell death (Niwa and Walter, 2000) (Anthony et al., 2004; Crosby et al., 2000; Harding et al., 2000a; Harding et al., 2000b; Harding et al., 2003; Rao et al., 2004; Zhang et al., 2002a; Zhang et al., 2002b). The biologic importance of this pathway is suggested by its hypothesized role in settings as diverse as neurodegenerative disorders, Type 2 diabetes, adaptation to oxidative stress, and response to viral infection (Forman et al., 2003; Harding et al., 2003; Ozcan et al., 2004).

In the case of T cells, little is known about the biologic role of the ISR (Beretta, 2004). One effect of ISR activation is a transient but generalized inhibition of mRNA translation (translational repression) (Hinnebusch, 2000). Thus, a key question in the current study was whether something as apparently “nonspecific” as translational repression could create antigen-specific regulation of T cell responses. However, the ISR is not simply a global repressor; it also actively upregulates a selective program of downstream response genes. The ISR pathway enhances (rather than represses) translation of certain selected genes, because phosphorylation of eIF2 α activates the translation of mRNAs containing a specific internal ribosomal entry site (Fernandez et al., 2002; Harding et al., 2000a). These GCN2-responsive mRNAs include potent transcriptional regulators such as GCN4p in yeast (Natarajan et al., 2001) and ATF4 in animal cells (Harding et al., 2000a; Lu et al., 2004; Vatter and Wek, 2004). This results in coordinated regulation of a conserved set of downstream stress-responsive genes (Harding et al., 2003).

We used microarray expression profiling to show that GCN2 affected a highly restricted set of transcripts in T cells, most of which were actively upregulated by the GCN2 pathway. Several of these genes (e.g., CHOP and Herp) are known to be induced by ISR activation in other cell types (Harding et al., 2003), while others have not been previously reported and may be specific to T cells. Further studies will be required to elucidate the specific downstream events by which GCN2 triggers cell-cycle arrest and energy induction. However, our microarray data clearly demonstrate that GCN2 can function as a selective and specific regulatory pathway in T cells.

Exactly how IDO creates the stress that activates GCN2 in T cells is not yet known. In the published literature, the only well-characterized and proven stimulus for GCN2 activation is a rise in uncharged tRNA, as would occur if IDO depleted the T cells of tryptophan. The fact that IDO-mediated suppression was reversed by 10 \times tryptophan might also be consistent with a

(D) Energy induction in vivo is GCN2 dependent. Labeled OT-I^{WT} and OT-I^{GCN2-KO} T cells were adoptively transferred into GCN2-KO hosts, and mice were injected with peptide-pulsed CD11c⁺ TDLN DCs. After 5 days, the labeled T cells (CFSE⁺ CD8⁺) were recovered from draining LNs by sorting, and used as responders in recall MLRs. Stimulator cells were irradiated C57BL/6 splenocytes. MLRs were performed with or without OVA peptide, and with or without exogenous IL-2, as shown. The bracket shows statistical comparison of OT-I^{WT} versus OT-I^{GCN2-KO} cells by ANOVA, showing that OT-I^{GCN2-KO} are not rendered anergic.

mechanism of tryptophan depletion. However, we cannot exclude the alternative possibility that a downstream metabolite produced by IDO might interfere with the acylation reaction by which tryptophan is ligated to its tRNA. Although speculative, such an inhibitory metabolite would also cause a rise in uncharged tRNA^{TRP} (by inhibiting the charging reaction), and its effect might be overcome by 10× tryptophan. With current technology, it is not possible to directly measure tryptophan or its metabolites in the local microenvironment of the T cell, so it is impossible to distinguish between these two speculative scenarios. For this reason, we chose to focus instead on the downstream response pathway in the T cell. This approach allowed us to demonstrate that – whether due to tryptophan depletion or inhibitory metabolites – a key biologic effect of IDO is to activate the GCN2 pathway. Thus, considered at the level of GCN2 activation, the T cell responds as if it were deprived of tryptophan, and this mediates biological responses in the T cell.

One important biological response mediated by GCN2 was the creation of anergy. Anergy is a critical outcome because it allows the immunoregulatory effects of IDO to be extended in time (i.e., beyond the initial physical encounter with the IDO-expressing APC). At the molecular level, we do not yet know how GCN2 participates in the induction of anergy, but a number of studies suggest that cell-cycle arrest during early T cell activation may causally contribute to anergy induction (Schwartz, 2003). One of the prominent effects of the ISR pathway is cell-cycle arrest (Niwa and Walter, 2000), and we speculate that this could be one way in which GCN2 contributes to anergy induction.

We have previously shown that the anergy created by IDO is antigen-specific (Munn et al., 2004a). This raises the question of how a “generalized” pathway such as GCN2 could result in antigen-specific regulation of T cells. No specificity would be conferred by tryptophan itself, because GCN2 would respond equally to any amino acid. However, in our system the particular amino acid that was actually affected by PDCs was tryptophan (as shown by the fact that adding tryptophan was sufficient to restore proliferation), and this occurred via IDO (as shown by the fact that suppression was lost with IDO-KO PDCs, and was blocked by 1MT). The expression of IDO itself is under tight biologic regulation, being expressed only by certain APCs, and induced in these APCs only by specific signals (Mellor and Munn, 2004). On the T cell side, we found that simply activating GCN2 (e.g., with tryptophan-deficient medium) was not sufficient to upregulate downstream response genes such as CHOP. Rather, the T cells also had to receive an additional activation signal via TCR crosslinking (or via PMA/ionomycin, which mimics the TCR signal). This suggests that the IDO/GCN2 pathway would affect only those T cells that simultaneously encountered their cognate antigen. Consistent with this hypothesis, upregulation of CHOP in vivo was found to be restricted to those responder cells that became antigen experienced (i.e., were CD44-positive). Thus, while the GCN2 pathway was not in itself specific, it was induced by IDO and acted in concert with TCR signaling, which together could con-

fer contextual and antigen specificity on the regulation of T cell responses.

Finally, our focus on the role of GCN2 in T cells does not mean that T cells are the only possible site of action for GCN2. GCN2 is widely expressed, and other cell types (including the APC itself) might respond to IDO via their own endogenous GCN2 pathway. We and others have shown that IDO can mediate potent cell-autonomous effects (i.e., effects which modify the biology of the IDO-expressing cell) (Li et al., 2004; Marshall et al., 2001; van Wissen et al., 2002). Thus, the IDO/GCN2 pathway might influence the biology of the APC itself, and perhaps even other bystander cells as well. The current study does not address these more speculative autocrine or paracrine effects, but they may prove important to certain potent but poorly understood phenomena such as IDO-mediated bystander suppression (Munn et al., 2004a) and IDO-mediated systemic immunosuppression in vivo (Mellor et al., 2003). In the current study, however, the key finding is the identification of GCN2 as a molecular sensor in T cells, which allows them to detect and respond to the immunoregulatory signal generated by IDO.

Experimental Procedures

Reagents

All reagents were from Sigma (St. Louis, MO) unless noted. 1-methyl-D-tryptophan (catalog number 45,248-3, Sigma) was prepared as a 20 mM stock solution in 0.1 N NaOH and adjusted to pH 7.4. L-tryptophan (T0254, Sigma) was prepared as a 25 mM stock. Both reagents were stored at 4°C and protected from light.

Mouse Models

All animal studies were approved by the institutional animal use committee of the Medical College of Georgia. GCN2-KO mice were prepared with a targeted deletion in the GCN2 locus, removing exon 12 of the murine *EIF2AK4* gene. This encodes for an essential region of the GCN2 kinase, abolishing protein phosphorylation by GCN2. The targeted mutation in the *EIF2AK4* locus deletes exon 12 and surrounding intronic sequences. It is identical to the previously described mutation (Harding et al., 2000a), except that the selection marker used in gene targeting of the W4 embryonic stem cells had been removed by Cre-mediated excision at flanking *loxP* sites. Mice with the targeted mutation were produced by blastocyst injection of the mutant ES cell clone and the mutation was maintained in the inbred 129svev (Taconic) strain background. The mutant allele produces little if any detectable mRNA (presumably due to frame-shifting by splicing of exons 11 to 13, data not shown) and is thus referred to here as GCN2-KO. Like previously described GCN2 “knockout” mice (Zhang et al., 2002b), mice homozygous for this mutant allele were viable, fertile, and appeared overtly indistinguishable from wild-type mice.

TCR-transgenic OT-I mice, recognizing the SIINFEKL peptide of ovalbumin presented by H2K^b (Hogquist et al., 1994), C57BL/6 background, were purchased from Jackson Laboratories (Bar Harbor, ME). The C57BL/6 and 129 backgrounds are MHC-matched (b-haplotype); there was no discernable effect of minor antigenic differences between the B6 and 129 backgrounds, and OT-I cells responded identically to APCs from B6 or 129 backgrounds. OT-I mice were backcrossed three generations with GCN2-KO mice and then self mated. BM3 mice (TCR-transgenic CD8⁺, CBA background) have been previously described (Tarazona et al., 1996). A1 mice (TCR-transgenic CD4⁺, CBA background, recognizing a male HY peptide on H2E^b) have been described (Zelenika et al., 1998).

Tumor Models

Tumors were initiated using 1 × 10⁶ B78H1-GM-CSF cells (an MHC-deficient subline of B16 melanoma transfected with GM-CSF

([Huang et al., 1994]; gift of S. Antonia, Moffitt Cancer Center, Tampa FL), implanted subcutaneously in the thigh as previously described (Munn et al., 2004a), using the following hosts: C57BL/6 mice; IDO-KO mice bred on the F1(B6xCBA) background (Mellor et al., 2003); or wild-type F1(CBAxB6) mice.

Flow Cytometry and Cell Sorting

TDLN PDCs were isolated as described (Munn et al., 2004a), using high-speed cell sorting for CD11c versus B220. Intracellular staining for CHOP was performed as described for intracellular IDO (Munn et al., 2004b), using monoclonal antibody sc-7351 (Santa Cruz Biotechnology, Santa Cruz, CA).

T Cell Proliferation

Responder CD8⁺ T cells were prepared from spleens by sorting for CD8 cells (clone 53.6.7) using a CD11c^{NEG}B220^{NEG} gate to exclude CD8α⁺ DCs. Sorted PDCs were mixed with 1 × 10⁵ responder T cells at a DC:T cell ratio of 1:40. Stimulator DCs were not irradiated. MLRs were performed as described (Munn et al., 2004a), using V-bottom culture wells to ensure cell-cell contact. Some experiments received 50 ng/ml PMA + 500 ng/ml ionomycin; or 1 μg/ml anti-CD3 antibody. OT-I cells received 100 nM SIINFEKL peptide in the MLRs. Where indicated, groups received 200 μM 1MT (D-isomer) or 250 μM L-tryptophan.

Gene Expression Microarrays

Splenic T cells (wild-type 129 or GCN2-KO) were nylon wool enriched and activated with PMA+ionomycin for 40 hr, then half of each group received tryptophanol (0.25 mM) or buffer control for an additional 3 hr. Total RNA was purified by RNeasy columns (Qiagen, Valencia, CA), labeled by the MCG Microarray Core Facility using standard Affymetrix protocols, and hybridized to mouse genome arrays (GeneChip 430A2.0, Affymetrix, Santa Clara, CA). The Affymetrix system used in these experiments generates a signal normalized to internal standards, which allows expression levels to be compared across chips. Two identical experiments were performed, for a total of eight array hybridizations.

Adoptive Transfer Studies

Recipient mice were prepared by intravenous injection of 5 × 10⁶ OT-I cells (either OT-I^{GCN2-KO} or OT-I^{WT}, nylon-wool enriched), labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) for 10 min at 37°C. Host mice were GCN2-KO littermates (on the same GCN2-KO B6x129 background as the OT-I^{GCN2-KO} mice but without the OT-I transgene), or wild-type C57BL/6. As in our previous publication (Munn et al., 2004a), the entire CD11c⁺ DC fraction (unsorted CD11c⁺B220⁺ cells) was used for injection because this gave better viability of the PDC population during peptide loading, washing, and injection. Since IDO-mediated suppression is dominant, the unfractionated CD11c⁺ preparation was still potentially suppressive. CD11c⁺ DCs were incubated with 100 nM SIINFEKL peptide for 30 min at 37°C, washed, and 5 × 10⁴ DCs injected subcutaneously into the anteromedial thigh of each recipient. Where indicated, mice received 1-methyl-D-tryptophan (NSC 721782, National Cancer Institute, Rockville, MD) via implantable copolymer pellets (Munn et al., 1998), or control pellets without 1MT. After 3 days, the inguinal LNs were harvested and analyzed by FACS for CFSE versus CD8 versus other markers (IB11, CD44, or intracellular CHOP). For in vivo anergy experiments, CFSE-labeled CD8⁺ T cells were sorted from the LNs on day 5, and used as responder cells in recall MLR assays (below).

Anergy Induction and Recall MLRs

For in vitro anergy induction, CD8⁺ OT-I^{WT} T cells were incubated for 72 hr with IDO⁺ TDLN PDCs plus SIINFEKL peptide (primary MLRs). Cells were harvested, washed, and resuspended at 2× the original seeding density, in 50% fresh medium plus 50% original conditioned medium. Recall MLRs were stimulated by C57BL/6 spleen cells plus SIINFEKL peptide, or by PMA + ionomycin, and replicate groups received recombinant mouse IL-2 as described (Munn et al., 2004a).

Quantitative Real-Time RT-PCR for CHOP

RNA analysis was performed on the LightCycler real-time quantitative PCR system (Roche Diagnostics, Indianapolis, IN), using SYBR Green RNA Amplification Kit (Roche), and quantitated against a standard curve prepared from RAW cells treated with thapsagargin (Harding et al., 2000a). Primers for mouse CHOP were 5'-TCC CTGCTTTTCACCTTG-3' (sense) and 5'-GCCCTGGCTCCTCTG TCA-3' (antisense). Primers for mouse γ-actin were 5'-GATGACG CAGATAATGTTT- (sense) and 5'-TCTCCTTTATGTCACGAAC-3' (antisense). Both primer pairs gave a single band, and the method was linear over a 3-log range.

Western Blots and Immunohistochemistry

Immunohistochemistry was performed as previously described (Munn et al., 2004a). Western blot for CHOP was performed using anti-gadd153/CHOP antibody (1:100 dilution) with PVDF membranes.

Statistical Analysis

Groups within a single experiment were compared by ANOVA. Experiments were repeated at least three times unless otherwise stated.

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