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Absence of Functional Inducible NO Synthase Enhances the Efficacy of Tolerance Induced by High Dose Antigen Feeding

Daniel A. Kahn,*† D. Clay Archer,‡§ and Carolyn J. Kelly2*§§

Recent studies have suggested that IL-12 and IFN-γ may impair the ability of fed Ag to induce systemic tolerance. Because both of these cytokines can function to directly or indirectly induce inducible NO synthase (iNOS) expression, we have investigated whether the functional expression of iNOS regulates oral tolerance. C57BL/6J wild-type or C57BL/6J NOS2−/− mice were gavaged with a single dose of 20 mg of keyhole limpet hemocyanin (KLH), followed by s.c. immunization with KLH/CFA. In the absence of feeding Ag, several parameters of the immune response were more robust in C57BL/6J NOS2−/− mice following KLH/CFA immunization, including the magnitude of the delayed-type hypersensitivity response, the proliferative response, and the production of IFN-γ and IL-2 by Ag-activated draining lymph node cells. These heightened responses in the C57BL/6J NOS2−/− mice are still effectively inhibited by feeding KLH. Feeding KLH to the C57BL/6J NOS2−/− mice elicited heightened TGF-β1 production by Ag-activated lymphocytes, as well as augmented total IgG, IgG1, and IgG2a responses to KLH/CFA compared with those seen in Ag-fed wild-type mice. Feeding Ag to the NOS2−/− mice suppressed proliferative responses and IFN-γ production, while increasing IL-4 production and the IgG1/IgG2a ratio even following a booster immunization of KLH/CFA. Administering L-Nω-(1-iminoethyl)-lysine · 2HCl to wild-type mice during the period of Ag feeding reproduced the high TGF-β1 production seen in Ag-activated lymphocytes from Ag-fed NOS2−/− mice. Feeding KLH is followed by transient up-regulation of NOS2 mRNA expression in the Peyer’s patches of wild-type mice. Selective inhibition of NOS2 may be a simple way to augment tolerogenic mucosal immune responses. The Journal of Immunology, 2000, 165: 6116–6122.

Initial exposure to Ags at mucosal surfaces can lead to systemic tolerance. Mucosal exposure to certain doses of Ag characteristically elicits T cell expression of cytokines such as IL-4, IL-10, and TGF-β1 (extensively reviewed in Ref. 1). These cytokines can be “suppressive” to cellular immune reactions and antagonize the expression of Th1-like cytokines (2, 3). At other doses of fed Ag, systemic tolerance is the result of clonal deletion and/or anergy (4, 5). The gut immune response to ingested Ag is centered in the Peyer’s patch, where activated T cells produce predominately IL-4, IL-10, and TGF-β1 (6). Following Ag feeding, such T cells migrate from the Peyer’s patch to mesenteric lymph nodes to peripheral sites (1). Because feeding Ag can modulate autoimmune disease processes (7–10), regulatory mechanisms that influence immune responses in the Peyer’s patch may determine the success of oral tolerance treatment strategies.

The role played by IFN-γ in the development of oral tolerance is controversial. Peyer’s patches from wild-type mice fed a large dose (25–250 mg) of OVA produce large amounts of IFN-γ (11, 12). Mice lacking IFN-γ fail to be systemically tolerized to OVA following feeding. These studies suggest that IFN-γ plays an important role in the regulation of the gut immune reaction responsible for the development of systemic tolerance. Other studies support the opposite conclusion. Targeted mutant mice lacking the IFN-γ receptor can be orally tolerized (13). Treatment of mice with IFN-γ i.p. before the feeding of Ag blocks the induction of systemic tolerance (14). Treatment of mice fed large doses of OVA with anti-IL-12 blocks the production of IFN-γ in the Peyer’s patch and augments induction of systemic tolerance as evidenced by increased production of TGF-β1 and IL-10 in the periphery (12). In the aggregate, these studies suggest a negative regulatory role for IFN-γ on the immune reactions in the gut that lead to systemic tolerance.

One possible mechanism by which IFN-γ may act as a negative regulator is through the induction of inducible NO synthase (iNOS). IFN-γ, among other proinflammatory cytokines, is a potent positive regulator of iNOS gene transcription (15). NO, in turn, can play an important role in the regulation of immune responses (16–19). In these studies, we have investigated whether functional expression of iNOS modulates oral tolerance. Our results show that mice genetically lacking iNOS or mice treated with a selective iNOS inhibitor have augmented induction of systemic tolerance to fed Ags, reminiscent of the effects of anti-IL-12 treatment. Furthermore, mice fed a single large dose of Ag have significantly increased levels of iNOS mRNA expression in the Peyer’s patch. The ability to augment tolerance by inhibiting expression of iNOS may be beneficial to mucosal tolerance strategies for the treatment of autoimmune diseases.
Materials and Methods

Mice

Male C57BL/6 and C57BL/6NOS2−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were used between 4 and 6 wk of age. Mice were housed and handled in accordance with Department of Veterans Affairs and National Institutes of Health guidelines under Institutional Animal Care and Use Committee approved protocols.

Reagents

Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (San Diego, CA). Freund’s adjuvant and Mycobacterium tuberculosis were obtained from Difco (Detroit, MI). CFA was prepared as 4 mg/ml M. tuberculosis in a 1:1 (v/v) emulsion of Freund’s adjuvant and PBS. l-Nil (L-iminoethyl)-lysine - 2HCl (l-NIL) was obtained from Alexis Biochemicals (San Diego, CA). Mice treated with l-NIL were given 50 μg/ml in the drinking water for a total of 6 days or −167 μg/day/mouse.

Feeding, immunization, and assessment of delayed-type hypersensitivity (DTH)

For feeding and immunization, mice were anesthetized with methoxyflurane obtained from Mallinckrodt (Mundelein, IL). The mice were allowed to recover on room air. Mice were gavaged with either 20 mg KLH in 250 μl PBS or 250 μl PBS with a 20-gauge feeding needle. In some experiments, mice were given 50 μg/ml l-NIL in the drinking water, starting the day before gavage and continuing until day 5 after gavage. Mice were immunized s.c. at the base of the tail with 100 μg KLH in 200 μl CFA. Some mice were given a second s.c. flank injection of 100 μg KLH in 200 μl CFA in a site distinct from the initial injection. To assess DTH, all mice involved in the studies were given 50 μg KLH in 50 μl PBS intradermally in the left foot pad and 50 μl PBS in the right foot pad 5 days after the last immunization. Foot pad swelling was measured with a micrometer (Mitsutoyo, Japan) by an observer blinded to the experimental design and was recorded as the difference between the left and right foot pad.

Proliferation assays

Draining flank lymph nodes were harvested and prepared into a single cell suspension with a metal screen. Cells were plated in 96-well tissue culture plates (Falcon/Becton Dickinson, Franklin Lakes, NJ) at 1 × 105 cells/ml in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FCS (Gemini Bio-Products, Calabasas, CA), 1.5 × 10−5 M 2-ME (Life Technologies) 130 U/ml penicillin, 130 μg/ml streptomycin, and 2.5 mM l-glutamine (Omega Scientific, Tarzana, CA). Cells were cultured at 37°C with 5% CO2 in a humidified incubator. Cells were cultured at 37°C with 5% CO2 in a humidified incubator. Cells from the spleen or mesenteric lymph nodes were cultured in RPMI 1640. Lymph node cells were cultured on plastic Petri dishes to assess spontaneous proliferation.

Cytokine ELISAs

Culture supernatant concentrations of IFN-γ were determined by sandwich ELISA with Abs purchased from PharMingen (San Diego, CA). Levels of IL-2, IL-4, and IFN-γ concentrations were determined from culture supernatants on day 5. Ab dilutions, which maximized signal to noise, were determined for each Ab pair. Briefly, 96-well Maxisorp microtiter plates (Nunc-Nalgene, Naperville, IL) were coated with the appropriate capture Ab (0.1 M carbonate buffer, pH 9.5) overnight at 4°C. Plates were blocked for 1 h at room temperature (RT) with PBS containing 10% FCS and 0.05% Tween 20 (Sigma, St. Louis, MO). Plates were incubated for 1 h at RT with PBS containing 4% BSA (Sigma) and 0.05% Tween 20. Plates were incubated with serum samples for 2.5 h at 37°C. Plates were developed with anti-IgG (Calbiochem, San Diego, CA), anti-IgG1 (Callab, Burlingame, CA), or anti-IgG2a (PharMingen) alkaline phosphatase conjugates at 37°C for 2.5 h. Color was developed by incubating plates with p-nitrophenylphosphate disodium purchased from Sigma (1 mg/ml in 1 M carbonate buffer, pH 9.6) at RT for equal amounts of time. Color development was evaluated in a microplate reader at 405 nm.

Competitive RT-PCR

Total RNA was prepared from Peyer’s patches and non-Peyer’s Patch from the RNeasy Mini kit (Qiagen, Valencia, CA) and stored at −70°C with 40 U RNase-OUT inhibitor purchased from Life Technologies. cDNA was prepared from 2 μg of each sample using a kit from Life Technologies (Superscript II preamp) according to the manufacturer’s instructions. The cDNA was used in a PCR with serial dilutions of a known molar amount of a competitive template for iNOS (Clontech, Palo Alto, CA) and β-actin. The resulting levels of iNOS were normalized to the levels of β-actin. The primers for the murine iNOS were 5′-CCCTTCCAGGTTTCTGCGAC CAGC-3′ and 3′-GOTTCCGTGCAGACTCGTG-5′. The primers for the murine β-actin were 5′-AATTGTCCTGCAAGGACTTGTG-3′ and 3′-AAGCCTGCTAAGGAGACACA-5′. The competitive template for β-actin was a generous gift from Daniel P. Gold (Sidney Kimmel Cancer Institute, San Diego, CA). The PCR buffer for both the iNOS and β-actin amplifications contained a final concentration of 2 mM MgCl2. The PCR conditions used for the iNOS and β-actin amplification were 38 cycles of 45 s at 94°C, 45 s at 55°C, and 2 min at 72°C followed by a 7-min 72°C step. PCR products were resolved on a 1.4% agarose Tris-boric acid-EDTA gel. Band intensity was evaluated on a digital imaging system (IL-1000 v2.02; Alpha Innotech, San Leandro, CA).

Statistics

 Differences were statistically analyzed using a one-way ANOVA with a Bonferroni/Dunn post-test. Analysis was accomplished with Statview v4.5 (Abacus Concepts, Berkeley, CA).

Results

Oral tolerance is preserved in the absence of iNOS

Because the role of NO in mucosal tolerance had not been examined, we studied the effect of NO generated through iNOS on high dose (20 mg of KLH) oral tolerance. As shown in Fig. 1A, this protocol of feeding KLH had no effect on the magnitude of the Ag-specific DTH in wild-type C57BL/6J mice. The iNOS−/− mice had a more robust DTH to KLH than wild-type mice, consistent with previous observations regarding their heightened T cell responses both in vivo and in vitro (3, 18, 19). Despite the magnitude of the DTH response in the iNOS−/− mice, feeding KLH to these mice reduced the DTH response by almost 50%.

Feeding KLH resulted in suppression of the proliferative response to KLH in draining lymph node cells from both the wild-type and iNOS−/− mice (Fig. 1B). The in vitro proliferative response following immunization with KLH/CFA in the iNOS−/− mice, feeding KLH to these mice reduced the proliferative response to near baseline values. Because KLH can be mitogenic to spleens from the same animals in these experimental groups. The proliferative response to Ag was markedly higher in spleens from the iNOS−/− mice, compared with those from wild-type mice. However, in the spleen, we did not observe any feeding-dependent alterations in the proliferative response to KLH (data not shown).

Fig. 1C depicts the effect of Ag feeding on the Ab response to KLH in the wild-type and knockout mice. Feeding KLH augmented the total IgG, IgG1, and IgG2a responses in both wild-type and iNOS−/− mice. However, in the spleen, we did not observe any feeding-dependent alterations in the proliferative response to KLH (data not shown).
On day 12, mice were challenged with 50 mg KLH. Mice were immunized s.c. in the base of the tail with 100 μl PBS in the right foot pad. On day 14, foot pad swelling was assessed by an observer blinded to the experimental design. Draining lymph node cells from boosted iNOS−/− mice were fed 20 mg KLH in 50 μl PBS in the left foot pad and with 50 μl PBS in the right foot pad. On day 14, foot pad swelling was assessed by an observer blinded to the experimental design. ANOVA with Bonferroni/Dunn post-test; *, p < 0.0005. B. The effect of Ag feeding on draining lymph node proliferative response to Ag. Draining lymph nodes were taken from the mice described in A on day 14. Cellular proliferation assay was conducted at 1 × 10⁶ cells/ml with the indicated concentrations of KLH. Cultures were pulsed with 1 μCi/well [3H]tdR on day 4 and incorporation assayed on day 5. ANOVA with Bonferroni/Dunn post-test; *, p = 0.0013; **, p = 0.0919. C. Feeding induced alterations of the humoral immune response. Sera from the mice described in A were collected on day 14. KLH-specific Ig response was determined by ELISA. The OD values shown here are from a serum dilution determined to be on the linear part of the OD vs dilution curve. The same dilution was used for all samples. ANOVA with Bonferroni/Dunn post-test; *, p = 0.0147; **, p = 0.0004; ***, p = 0.0003; †, p = 0.0048.

FIGURE 1. A. The effect of oral tolerance on DTH. C57BL/6J NOS2+/+ (n = 5) and C57BL/6J NOS2−/− (n = 5) mice were fed 20 mg KLH (filled columns) or PBS vehicle control (open columns). On day 7, mice were immunized s.c. in the tail with 100 μg KLH in CFA. On day 12, mice were challenged with 50 μg KLH in 50 μl PBS in the left foot pad and with 50 μl PBS in the right foot pad. On day 14, foot pad swelling was assessed by an observer blinded to the experimental design. ANOVA with Bonferroni/Dunn post-test; *, p < 0.0005. B. IL-4 (Fig. 2B), IFN-γ (Fig. 2C), and TGF-β1 (Fig. 2D). The supernatants were not acid activated for the TGF-β1 ELISA. Thus, the observed concentrations reflect the levels of bioactive TGF-β1 produced in the cultures. Lymphocytes from immunized iNOS−/− mice produced far more IL-2 and IFN-γ, and less TGF-β1 in response to Ag than did wild-type mice. Again, despite the augmented T(H1)-like response in iNOS−/− mice, feeding KLH suppresses the production of IL-2 and IFN-γ to below the limits of detection. The role of iNOS or fed Ag on the production of IL-4 is less clear because none of the groups tested reached statistical significance (Fig. 2B). Feeding KLH to iNOS−/− (but not wild-type) mice augments the production of TGF-β1 (Fig. 2D) in the draining lymph nodes, consistent with the paradigm that oral exposure to Ag induces TGF-β1-secreting T cells (T(H1)) (20, 21).

FIGURE 1. A. The effect of oral tolerance on DTH. C57BL/6J NOS2+/+ (n = 5) and C57BL/6J NOS2−/− (n = 5) mice were fed 20 mg KLH (filled columns) or PBS vehicle control (open columns). On day 7, mice were immunized s.c. in the tail with 100 μg KLH in CFA. On day 12, mice were challenged with 50 μg KLH in 50 μl PBS in the left foot pad and with 50 μl PBS in the right foot pad. On day 14, foot pad swelling was assessed by an observer blinded to the experimental design. ANOVA with Bonferroni/Dunn post-test; *, p < 0.0005. B. The effect of Ag feeding on draining lymph node proliferative response to Ag. Draining lymph nodes were taken from the mice described in A on day 14. Cellular proliferation assay was conducted at 1 × 10⁶ cells/ml with the indicated concentrations of KLH. Cultures were pulsed with 1 μCi/well [3H]TdR on day 4 and incorporation assayed on day 5. ANOVA with Bonferroni/Dunn post-test; *, p = 0.0013; **, p = 0.0919. C. Feeding induced alterations of the humoral immune response. Sera from the mice described in A were collected on day 14. KLH-specific Ig response was determined by ELISA. The OD values shown here are from a serum dilution determined to be on the linear part of the OD vs dilution curve. The same dilution was used for all samples. ANOVA with Bonferroni/Dunn post-test; *, p = 0.0147; **, p = 0.0004; ***, p = 0.0003; †, p = 0.0048.

and iNOS−/− mice, but the effect was more marked for each determination in the iNOS−/− mice. Only in the absence of iNOS did the augmentation of the Ab response reach statistical significance in all three groups. In particular, the increase in IgG1 was greatest in iNOS−/− mice fed KLH.

Modulation of cytokine expression by Ag feeding is exaggerated in iNOS−/− mice

Cytokine determinations were performed on supernatants derived from draining lymph node cultures set up in parallel with the above-described proliferation assays (Fig. 2). We assayed the supernatants for IL-2 (Fig. 2A), IL-4 (Fig. 2B), IFN-γ (Fig. 2C), and TGF-β1 (Fig. 2D). The supernatants were not acid activated for the TGF-β1 ELISA. Thus, the observed concentrations reflect the levels of bioactive TGF-β1 produced in the cultures. Lymphocytes from immunized iNOS−/− mice produced far more IL-2 and IFN-γ, and less TGF-β1 in response to Ag than did wild-type mice. Again, despite the augmented T(H1)-like response in iNOS−/− mice, feeding KLH suppresses the production of IL-2 and IFN-γ to below the limits of detection. The role of iNOS or fed Ag on the production of IL-4 is less clear because none of the groups tested reached statistical significance (Fig. 2B). Feeding KLH to iNOS−/− (but not wild-type) mice augments the production of TGF-β1 (Fig. 2D) in the draining lymph nodes, consistent with the paradigm that oral exposure to Ag induces TGF-β1-secreting T cells (T(H1)) (20, 21).

We additionally analyzed cytokine expression in supernatants derived from activated splenocytes set up in parallel with the proliferation assays. The levels of IL-2 produced by activated splenocytes from the iNOS−/− are significantly higher than those of the wild type. This is consistent with the higher proliferative responses in these spleens. However, we did not detect any significant effects of feeding on the cytokine levels produced by Ag-activated splenocytes (data not shown).

Tolerance from single high dose Ag feeding persists in iNOS−/− mice despite a boosting immunization

Previously used protocols for assessing the role of IFN-γ in oral tolerance have included a single high dose feeding followed by two s.c. immunizations (11). Because such boosted immune responses may be fundamentally different from nonboosted responses, we treated wild-type and iNOS−/− mice as before (Fig. 1) but then boosted them 2 wk following the initial injection of KLH/CFA immunization is a potent stimulus of iNOS expression in the wild type, the effect of feeding is unclear due to the lack of proliferative response to immunization alone. The failure in wild-type mice to make a proliferative immune response with boosting may reflect potent NO dependent negative regulation, because we have found that CFA immunization is a potent stimulus of iNOS expression in the lymph node (D.K. and C.K., unpublished observations).

In both wild-type and iNOS−/− mice, the effect of feeding on the pattern of cytokines produced is consistent with deviation from a T(H1)- to a T(H2)-like profile (Fig. 3, B and C). The amount of IL-4 produced in the PBS-gavaged mice is similar between the two strains (Fig. 3B). Draining lymph nodes from wild-type mice fed KLH make 3- to 4-fold higher levels of IL-4 compared with unfed wild-type mice. In the absence of iNOS, the feeding-induced augmentation of IL-4 is exaggerated. Feeding results in a 7- to 10-fold increase in the amount of IL-4 produced in draining lymph nodes. iNOS−/− mice produce ~40 fold more IFN-γ than wild-type mice.
Despite the overwhelmingly Th1-like response in iNOS−/− mice, KLH feeding significantly reduces the amount of IFN-γ 4- to 5-fold.

Feeding-induced immune deviation in iNOS−/− mice is also evident in the composition of the Ag (KLH)-specific Ab response (Fig. 3D). Both wild-type and iNOS−/− mice produced an Ab response of similar magnitude in terms of total IgG. In iNOS−/− mice fed KLH, the ratio of IgG1/IgG2a changed from 3.89 in unfed mice to 7.29 in KLH-fed mice. The shift in the ratio was due to statistically significant increases in IgG1 and decreases in IgG2a with KLH feeding. This trend was not observed in wild-type mice.

The induction of oral tolerance in wild-type mice is altered by in vivo treatment with an iNOS-specific inhibitor

We next examined whether the effects of Ag feeding seen in iNOS−/− mice could be reproduced in animals made transiently iNOS deficient by in vivo administration of a selective iNOS inhibitor, L-NIL (22) (Fig. 4). In contrast to iNOS−/− mice, feeding KLH to mice treated with L-NIL did not significantly suppress Ag-specific proliferative responses in draining lymph node cells. However, consistent with our findings in iNOS−/− mice, feeding Ag to the L-NIL-treated mice substantially decreased the amount of IFN-γ produced by Ag-reactive cells (Fig. 5C). Lymphocytes from KLH-fed mice treated with L-NIL additionally secreted three times more bioactive TGF-β1 as did unfed L-NIL-treated mice (Fig. 5D).

Feeding significantly altered the IgG subtype composition. The ratio of IgG1/IgG2a was reduced nearly 3-fold by feeding. The observed reduction was primarily due to a feeding induced increase in IgG2a. The levels of IgG1 were unaffected by feeding (Fig. 5A).

Ingested Ag transiently induces the expression of iNOS selectively within the Peyer's patch

When wild-type mice that were deprived of food for 18 h were gavaged with 20 mg KLH, iNOS mRNA was up-regulated by 6 h.
in the Peyer's patch (Fig. 6). However, by 12 h postgavage, iNOS mRNA had subsided to baseline levels. Single cell suspensions prepared from the Peyer's patches 6 h after feeding were cultured for an additional 24–96 h, and the supernatants were analyzed for nitrite and nitrate by Greiss reaction. We could not detect any elevation in NO endproducts in the Peyer's patches derived from fed animals (data not shown). This likely reflects the relative insensitivity of the assay to lower concentrations of NO endproducts. Because intestinal epithelia can produce iNOS in response to a variety of signals (23, 24), surrounding non-Peyer's patch tissue was evaluated for iNOS expression to control for possible contamination of the sample. iNOS mRNA was below the limits of detection in non-Peyer's patch intestine in all experimental groups (data not shown).

Discussion

Previous studies have supported the notion that NO can be an important immunoregulator. However, such studies have largely supported the paradigm that, in the absence of functional iNOS, Th1-like responses are accentuated. In these experiments, we have found that functional iNOS is additionally an important modulator of a regulatory response, i.e., the induction of tolerance by oral Ag administration. iNOS−/− mice demonstrated augmented immune responses to KLH/CFA, as demonstrated by the enhanced DTH responses, enhanced Ag-specific proliferative responses, and enhanced production of IFN-γ and IL-2. However, the iNOS−/− mice similarly demonstrate augmented regulatory responses following Ag feeding. This is demonstrated by the partial inhibition of DTH in the iNOS−/− by Ag feeding, and the inhibition of proliferative responses and Th1 cytokine production, and augmented TGF-β production. Inhibition of Th1 cytokine production, and augmented TGF-β, is also seen when the animals are fed Ag while transiently treated with a selective iNOS inhibitor. Finally, we have shown that iNOS mRNA is transiently up-regulated within the Peyer's patches following high dose Ag feeding. In addition, significant alterations in Ig titers and composition with feeding were observed under conditions (genetic or pharmacological) of iNOS deficiency. Feeding increases the serum titers of KLH-specific Igs in both wild-type mice and iNOS knockout mice. Similar to the alternations in cytokine production, the increase in Ab concentrations is exaggerated in the knockout compared with the wild type. In the single feed and immunization protocol, the trend in both strains is for the Ab concentrations to increase. However, only in the knockout does the increase reach statistical significance. One plausible interpretation is that these changes reflect systemic immune deviation as a consequence of feeding. The more substantial increase in KLH-specific IgG1 concentrations observed in the knockout compared with the wild type is perhaps a reflection of the dramatic suppressive effect that feeding has on the production of IFN-γ in the knockout. In contrast, the
to heightened T H 1-like responses, and that NO selectively inhibits proliferation (independent of feeding Ag) is the draining lymph node hypothesized.

Regulate a wider variety of immune responses than previously results suggest that NO generated through the iNOS isoform may mice significantly augment their IgG1 responses when fed Ag. Our immune response generated by feeding is more robust in the ab-

In this study, a powerful example of NO-mediated immune regulation (independent of feeding Ag) is the draining lymph node proliferative response seen in wild-type mice given a boosting immunization of KLH/CFA (Fig. 3). The lack of proliferation of the lymph node cells is clearly related to iNOS-derived NO because immune lymphocytes from lymph nodes of similarly boosted iNOS−/− mice proliferate briskly. Again, feeding KLH to these iNOS−/− mice leads to significant attenuation of the immune response. Because the observed suppression is 2 wk later than in the single immunization protocol and is following a boosting immunization, the effectiveness of fed KLH to regulate the immune response is remarkable for both the duration and degree of the tolerance. In the boosted mice, the mechanism of tolerance is consistent with immune deviation from T H 1 to T H 2 (Fig. 3) and previous studies supporting a requirement for IL-4 and IL-10 in oral tolerance (25). In contrast with the single immunization protocol, the levels of TGF-β1 were below the limits of detection suggesting that perhaps short term tolerance is due to induction of T H 3-like T cells, but that longer term tolerance is maintained by immune deviation.

Wild-type mice treated with the iNOS-specific inhibitor l-NIL only during the feeding period mount qualitatively similar responses to iNOS−/− mice. The failure of fed KLH to suppress the proliferative response of the draining lymph node cells to statistically significant levels may be due to incomplete iNOS suppression by l-NIL. Analogous to iNOS−/− mice, l-NIL-treated wild-type mice fed KLH suppress IFN-γ production, whereas augmenting TGF-β1 expression.

We were intrigued to see the transient nature of iNOS mRNA up-regulation following high dose Ag ingestion. Peyer’s patch expression of IFN-γ has been observed early (<6 h) in response to high and low doses of oral Ags (11, 12). Others have found no increase in IFN-γ expression in the Peyer’s patch after low doses of Ag (6). However, the consequences of IFN-γ treatment and IFN-γ receptor deficiency on oral tolerance induction are clear (13, 14). Furthermore, the ability to augment oral tolerance with anti-IL-12 treatment in vivo suggests a functional role for IFN-γ as a negative regulator. Although it is possible that expression of IFN-γ in the Peyer’s patch impairs oral tolerance induction solely by antagonizing the expression of IL-4, IL-10, and TGF-β1 (6, 11, 12), IFN-γ may additionally impair oral tolerance via iNOS-dependent mechanisms. Whether the transient induction of iNOS mRNA expression in the Peyer’s patch following Ag feeding is an “innate” response or one that requires Ag-specific T cells is not yet known. In our preliminary studies, where the animals were not fasted before Ag feeding, we observed high levels of iNOS mRNA in Peyer’s patches and gut epithelium in both Ag-fed and PBS-fed mice (D.A.K. and C.K., unpublished observations). The levels of iNOS mRNA in both tissues under nonfasting conditions were comparable to those seen in Fig. 6 with fed Ag at 6 h. These results, coupled to the kinetics depicted in Fig. 6, suggest to us that the up-regulation of iNOS is likely an innate response and one that occurs in response to multiple dietary Ags.

NO donors, such as nitroglycerin derivatives and nitroprusside, are already in clinical use for the treatment of angina and hypertension. Therefore, it is a valid question to ask whether the administration of NO donors to iNOS−/− mice might restore their responses to resemble wild-type mice. In our experience, it is difficult, with the currently available agents, to administer a large enough dose of a NO donor to a small rodent, systemically and chronically, to achieve the high local concentrations of NO present at sites of inflammation. Whether more sophisticated delivery systems that target an organ directly might accomplish that goal remains to be seen.

Although there have not been any direct studies addressing the physiology of iNOS expression in human Peyer’s patches, it is
clear that iNOS is expressed in human gut epithelium (24). Moreover, human Peyer’s patches express a pattern of cytokines similar to murine models including a preponderance of IFN-γ expression (26). The exaggerated nature of the tolerogenic immune response induced by oral Ag in the absence of iNOS suggests this could be useful as a model system for the dissection of the requirements and subtle characteristics of oral tolerance. Furthermore, the potential to augment tolerogenic immune responses to fed Ags by treatment with iNOS inhibitors may be important to hone oral tolerance treatment strategies of autoimmune disease.

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