

T cell–derived inducible nitric oxide synthase switches off T_H17 cell differentiation

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ROR γ t is necessary for the generation of T_H17 cells but the molecular mechanisms for the regulation of T_H17 cells are still not fully understood. We show that activation of CD4⁺ T cells results in the expression of inducible nitric oxide synthase (iNOS). iNOS-deficient mice displayed enhanced T_H17 cell differentiation but without major effects on either T_H1 or T_H2 cell lineages, whereas endothelial NOS (eNOS) or neuronal NOS (nNOS) mutant mice showed comparable T_H17 cell differentiation compared with wild-type control mice. The addition of N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL), the iNOS inhibitor, significantly enhanced T_H17 cell differentiation, and S-nitroso-N-acetylpenicillamine (SNAP), the NO donor, dose-dependently reduced the percentage of IL-17-producing CD4⁺ T cells. NO mediates nitration of tyrosine residues in ROR γ t, leading to the suppression of ROR γ t-induced IL-17 promoter activation, indicating that NO regulates IL-17 expression at the transcriptional level. Finally, studies of an experimental model of colitis showed that iNOS deficiency results in more severe inflammation with an enhanced T_H17 phenotype. These results suggest that NO derived from iNOS in activated T cells plays a negative role in the regulation of T_H17 cell differentiation and highlight the importance of intrinsic programs for the control of T_H17 immune responses.

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Abbreviations used: eNOS, endothelial NOS; IBD, inflammatory bowel disease; iNOS, inducible NOS; L-NIL, N6-(1-iminoethyl)-L-lysine dihydrochloride; MS, multiple sclerosis; NOS, nitric oxide synthase; nNOS, neuronal NOS; RA, rheumatoid arthritis.

IL-17-producing T helper cells (T_H17) are a recently identified T helper cell subset, which is clearly distinct from T_H1 and T_H2 cells. T_H17 cells mediate proinflammatory and autoimmune responses through the production of T_H17 signature cytokines including IL-17A, IL-17F, and IL-22 (Liang et al., 2006; Weaver et al., 2006; Bettelli et al., 2007; Zheng et al., 2007). Synergy between the cytokines TGF- β and IL-6 induces in vitro development of T_H17 cells (Veldhoen et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007), whereas IL-23 promotes the survival and expansion of T_H17 cell

populations (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Weaver et al., 2006). ROR γ t, a member of the orphan nuclear receptor family, has been identified as the master transcription factor for T_H17 cell development (Ivanov et al., 2006). Other transcription factors, including ROR α , STAT3, IRF4, and IRF8, are also involved in the control of T_H17 cell differentiation (Ouyang et al., 2011). In addition, the differentiation of T_H17 cells is also regulated

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J. Yang and R. Zhang contributed equally to this paper.

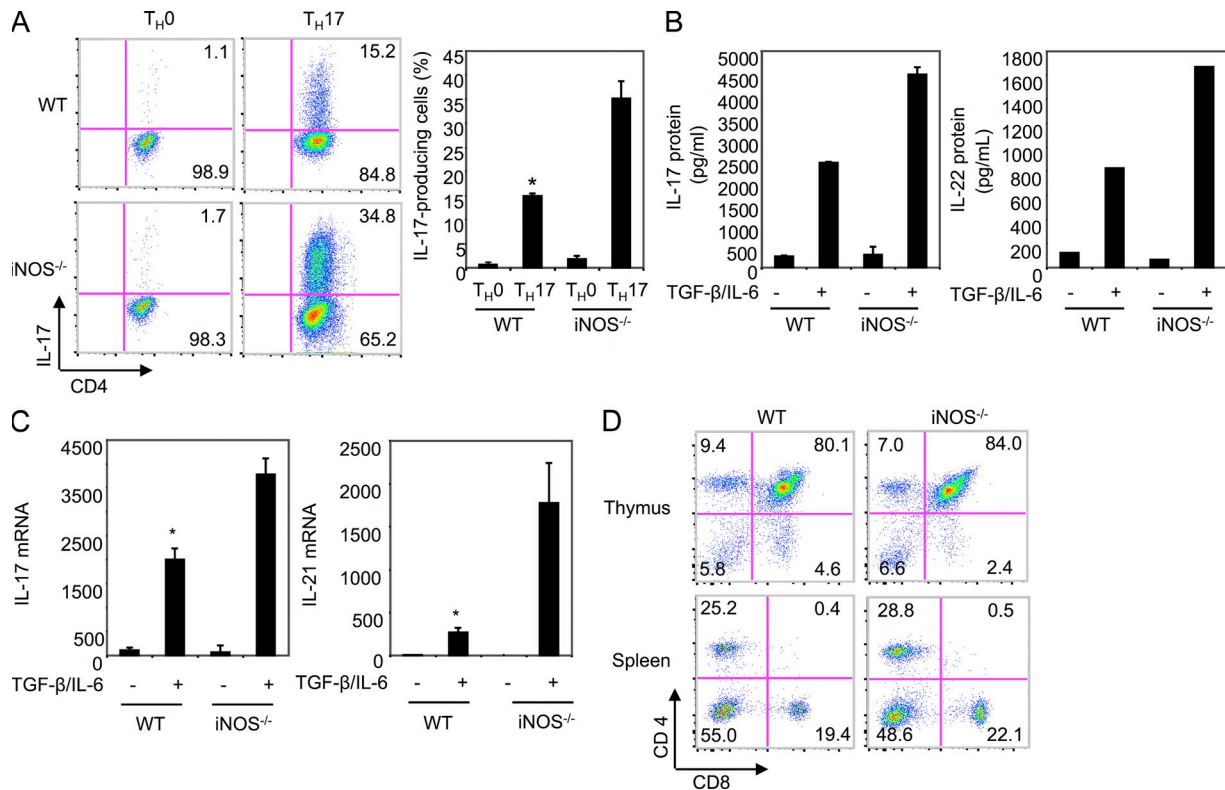


Figure 1. Enhanced T_H17 cell differentiation in iNOS-deficient mice. (A) Naive CD4⁺ T cells from WT or iNOS^{-/-} mice were differentiated under T_H0 and T_H17 polarizing conditions for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular IL-17 and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ T cells and the percentages of IL-17-producing CD4⁺ T cells are shown. Each bar represents mean $\pm$ SD from three independent experiments. *, $P < 0.05$ versus iNOS^{-/-} cells. (B) The cells prepared in A were restimulated with PMA/ionomycin for 12 h and the supernatants were analyzed for IL-17 and IL-22 by ELISA. Each bar represents mean $\pm$ SD of at least three independent measurements. (C) The cells prepared in A were restimulated with PMA/ionomycin for 5 h and mRNA expression of indicated genes was determined by qPCR. Data present mean $\pm$ SD of measurements from two independent experiments, performed in triplicate. The data shown were normalized to levels of ubiquitin expression as analyzed by qPCR. *, $P < 0.05$ versus iNOS^{-/-} cells. (D) Thymus and spleen cells from iNOS^{-/-} and WT controls were prepared and the cells were stained for surface CD4 and CD8 and analyzed by flow cytometry. The data shown were normalized to levels of ubiquitin expression as analyzed by qPCR. *, $P < 0.05$ versus iNOS^{-/-} cells. The results are representative of three independent experiments.

by several positive- and negative-feedback loops involving IL-21, IL-23R, IL-10, and IL-27 (Harrington et al., 2005; Stumhofer et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Gu et al., 2008; Yang et al., 2008; McGeachy et al., 2009), indicating that intrinsic genetic programs may contribute to the regulation of T_H17 lineage commitment.

There is increasing evidence that T_H17 cells are involved in the pathogenesis of various autoimmune/inflammatory diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel diseases (IBDs), and asthma (Korn et al., 2009). Thus, a more complete understanding of the molecular mechanisms involved in the regulation of T_H17 immune responses should provide insights into the pathogenesis and treatment of these and possibly other inflammatory diseases. Although the activation program for T_H17 cell differentiation has been well established, the intrinsic down-regulation for T_H17 cell differentiation has not been fully understood.

Nitric oxide (NO) is one of the smallest known bioactive products of mammalian cells and it can be produced by many mammalian cell types (Moncada et al., 1991). Three distinct

isoforms of NO synthase (NOS) have been identified: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS; Griffith and Stuehr., 1995). It has been demonstrated that NO plays many important roles in neurotransmission, vascular functions, host defense, and immune regulation (Bogdan, 2001; Calabrese et al., 2007). These enzymes are products of different genes, with different regulation, localizations, and catalytic properties. nNOS and eNOS are primarily expressed in neurons and endothelial cells, and they are calcium dependent. iNOS can be induced by cytokines and other stimuli in many cell types and it is calcium independent. It is clear that NO is an important proinflammatory cytotoxic mediator that defends the host against various pathogens by inactivating and destroying infectious agents (Bogdan et al., 2000). Interestingly, NO also plays critical roles in immune suppression (Xiong et al., 1996; Niedbala et al., 2006). Previously, we and other groups reported that NO suppresses IL-12 production from dendritic cells and macrophages (Xiong et al., 2004), suggesting that NO may control the generation of T_H1 immune responses by regulating IL-12 expression.

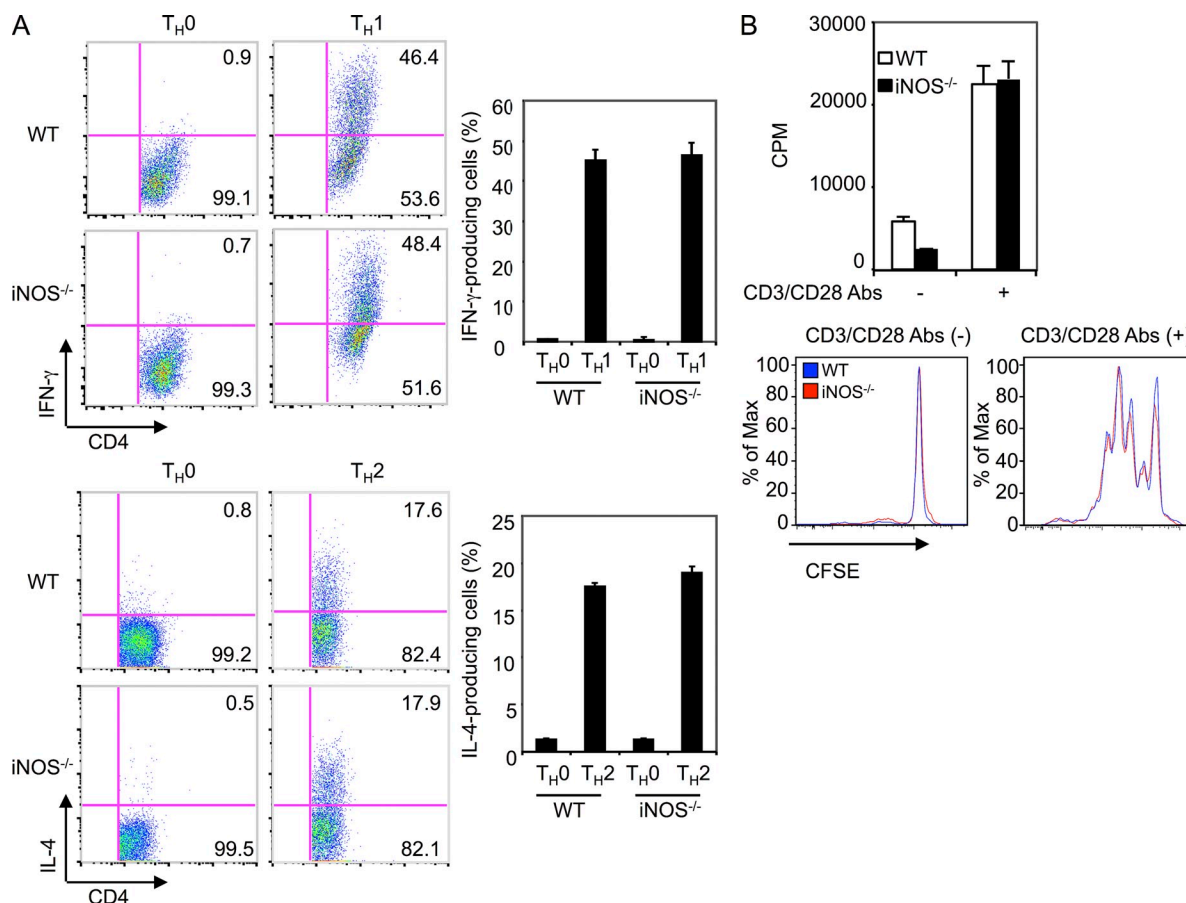


Figure 2. T_H1 and T_H2 differentiation in *iNOS*^{-/-} CD4⁺ T cells. (A) Naive CD4⁺ T cells from WT or *iNOS*^{-/-} mice were differentiated under T_H1 or T_H2 conditions for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h and stained for intracellular IFN-γ or IL-4 by flow cytometry. Each bar represents mean ± SD from three independent experiments. (B) Naive CD4⁺ T cells from spleens and lymph nodes of WT and *iNOS*^{-/-} mice were prepared and the cells were activated with anti-CD3 and anti-CD28 antibodies for 3 d. [³H]-Thymidine was added during the last 8 h of culture. Then the cells were collected and were counted with a β-counter. Alternatively, naive CD4⁺ T cells were labeled with CFSE and the cells stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 3 d. T cell proliferation was analyzed by flow cytometry. Data represent mean ± SD from two independent experiments, performed in triplicate.

In addition, *iNOS*-deficient mice are more susceptible than WT mice to the development of inflammatory diseases such as EAE (Bogdan., 1998; Niedbala et al., 2011). More and more evidence indicates that NO affects T helper cell differentiation (Nath et al., 2010; Lee et al., 2011; Niedbala et al., 2011), suggesting that NO may dictate T cell immune responses. However, it is still not clear whether T cells express NOS, and if so, what functions T cell-derived NO might serve.

In the present study, we show that mice deficient in *iNOS* exhibited enhanced T_H17 cell differentiation while exhibiting no significant effects on T_H1 or T_H2 cells. We demonstrated that *iNOS* protein was induced in activated CD4⁺ T cells and that use of an *iNOS*-selective inhibitor, N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL), significantly increased the percentage of IL-17-producing CD4⁺ T cells in cell cultures from WT mice, whereas an NO donor, S-nitroso-N-acetylpenicillamine (SNAP), dose-dependently suppressed IL-17 production in WT and *iNOS*^{-/-} T cell cultures. In addition, the tyrosine residues of RORγt were nitrated resulting in the inhibition of RORγt-mediated IL-17 promoter activation.

Transfer of naive T cells from *iNOS*-deficient mice induced more severe colitis in *Rag1*^{-/-} mice than T cells from normal controls. These findings suggest that NO plays a critical suppressive role in the control of T_H17 differentiation and highlight the importance of T cell-derived *iNOS* in switching off T_H17-dependent immune responses.

RESULTS

iNOS deficiency enhances T_H17 cell differentiation

To investigate the function of NO in T_H17 cell differentiation, we first assessed the characteristics of CD4⁺ T cells from *iNOS*-deficient mice. Naive CD4⁺ T cells from *iNOS*^{-/-} or WT control mice were primed in vitro for 3 d under neutral (T_H0) or T_H17 (IL-6 plus TGF-β) polarizing conditions. The cells were then restimulated with PMA/ionomycin and examined for the percentages of IL-17-producing cells by intracellular staining using flow cytometry. Notably, the frequency of IL-17-producing cells generated from *iNOS*^{-/-} T cell cultures was significantly greater than cells from WT cultures (Fig. 1 A). These observations correlated with enhanced

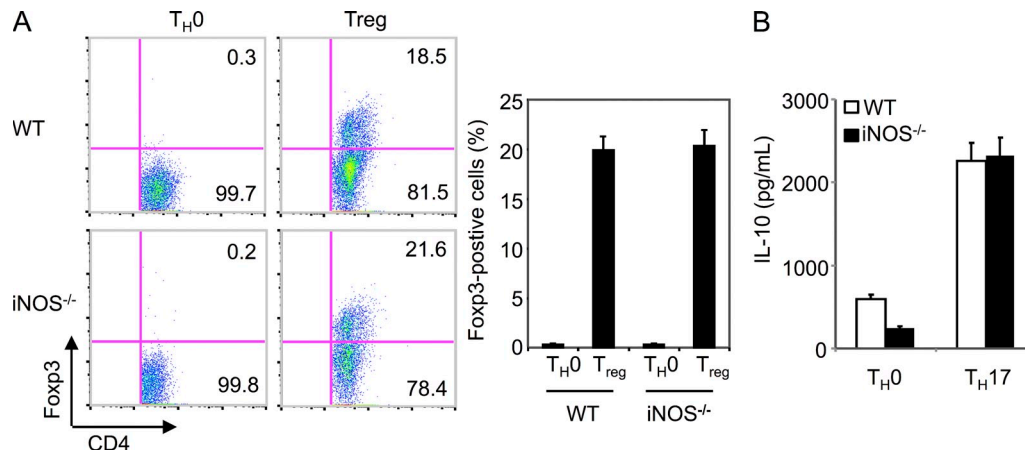


Figure 3. There are no changes of T_{reg} differentiation and IL-10 production in *iNOS*^{-/-} mice. (A) Naive CD4⁺ T cells from WT or *iNOS*^{-/-} mice were differentiated under T_H0 and T_{reg} polarizing conditions for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular FOXP3, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ cells and the percentages of FOXP3-positive CD4⁺ cells are shown. Each represents mean $\pm$ SD from three independent experiments. (B) Naive CD4⁺ T cells from WT or *iNOS*^{-/-} mice were differentiated under T_H0 and T_H17 polarizing conditions for 3 d. Cells were then restimulated with PMA/ionomycin for 24 h and the supernatants were analyzed for IL-10 by ELISA. Data represent mean $\pm$ SD from three independent experiments.

IL-17, IL-22, and IL-9 secretion by *iNOS*^{-/-} T_H17 cells as determined by ELISA (Fig. 1 B). In addition, transcript levels of the signature T_H17 cytokines, IL-17 and IL-21, were significantly enhanced in *iNOS*^{-/-} T_H17 cells (Fig. 1 C). To rule out the possibility that the enhanced T_H17 cell differentiation was a result of abnormal T cell development, we analyzed CD4⁺ T cells from spleens and lymph nodes of WT and *iNOS*^{-/-} mice (Fig. 1 D). In contrast to the dramatic effect of iNOS deficiency on T_H17 cell differentiation, T_H1 and T_H2 differentiation were not noticeably affected in *iNOS*^{-/-} T cell cultures (Fig. 2 A). Furthermore, when we polarized naive CD4⁺ T cells under conditions with TGF- β /IL-6 plus IL-23, we found that IL-17 single-positive cells were significantly increased in *iNOS*^{-/-} T cell cultures, but there was no clear difference in the number of IFN- γ single-positive cells between WT and *iNOS*^{-/-} T cell cultures, whereas IL-17/IFN- γ double-positive cells were just minimally increased (unpublished data). *iNOS*^{-/-} mice had normal numbers of CD4⁺ T cells (unpublished data) and exhibited comparable expression of T cell activation markers CD62L, CD44, CD25, and CD69 to relative cells from WT mice (unpublished data). In addition, [³H]-thymidine incorporation assays and CFSE dilution showed that the proliferation of CD4⁺ T cells from *iNOS*^{-/-} or WT control mice cultured under T_H17 conditions was comparable (Fig. 2 B). Collectively, these results indicate that T_H17 cell differentiation is enhanced in CD4⁺ T cells deficient in iNOS, suggesting that NO plays a negative role in T_H17 cell differentiation.

To investigate whether the enhancement of T_H17 cell differentiation was the result of T_{reg} dysfunction in *iNOS*-deficient mice, we examined FOXP3⁺CD4⁺ T cells in these mice. Naive CD4⁺ T cells from the spleens and lymph nodes of WT and *iNOS*^{-/-} mice were activated in vitro for 3 d under neutral T_H0 or T_{reg} (TGF- β) polarizing conditions.

There were no significant differences between the FOXP3⁺ CD4⁺ T cell populations of WT and *iNOS*^{-/-} mice under T_{reg}-inducing conditions (Fig. 3 A). In addition, production of IL-10, another inhibitory cytokine, was comparable in WT and *iNOS*^{-/-} T_H17 cells (Fig. 3 B). Thus, the enhanced T_H17 cell differentiation in *iNOS*^{-/-} mice was not a result of the alterations of either TGF- β -derived T_{reg} or IL-10 production.

iNOS is induced in activated CD4⁺ T cells

The enhanced T_H17 cell differentiation of *iNOS*^{-/-} T cells cultured under T_H17 polarizing conditions prompted us to think that iNOS expression in T cells could be responsible for reduced T_H17 cell differentiation in WT mice. To address this question, naive CD4⁺ T cells from the spleens and lymph nodes of WT and *iNOS*^{-/-} mice were activated in vitro for 3 d under T_H0 or T_H17 polarizing conditions. Western blotting showed that iNOS protein was indeed induced in WT CD4⁺ T cells but not in *iNOS*^{-/-} T cells (Fig. 4 A). In addition, stimulation of OT-II cells with OVA peptide (323–339) resulted in significant induction of iNOS protein as determined by Western blotting (Fig. 4 A). Staining cultured CD4⁺ T cells with anti-CD4 and anti-iNOS antibodies demonstrated that CD4⁺ T cells expressed iNOS after stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 4 B). Furthermore, real time RT-PCR experiments revealed the expression of iNOS mRNA at 6 and 12 h after TCR engagement (Fig. 4 C). In addition, single cell FACS analysis showed that iNOS was expressed in activated WT CD4⁺ T cells but not in *iNOS*^{-/-} CD4⁺ T cells (Fig. 4 D), and iNOS/IL-17 double-positive cells were present in WT T cells but not in *iNOS*^{-/-} T cells (unpublished data). To rule out the possibility that myeloid cells may have been contaminated in the CD4⁺ T cell cultures, we stimulated CD4⁺ T cells with 1 μ g/ml LPS for 24 and 48 h and Western blotting was performed. The results

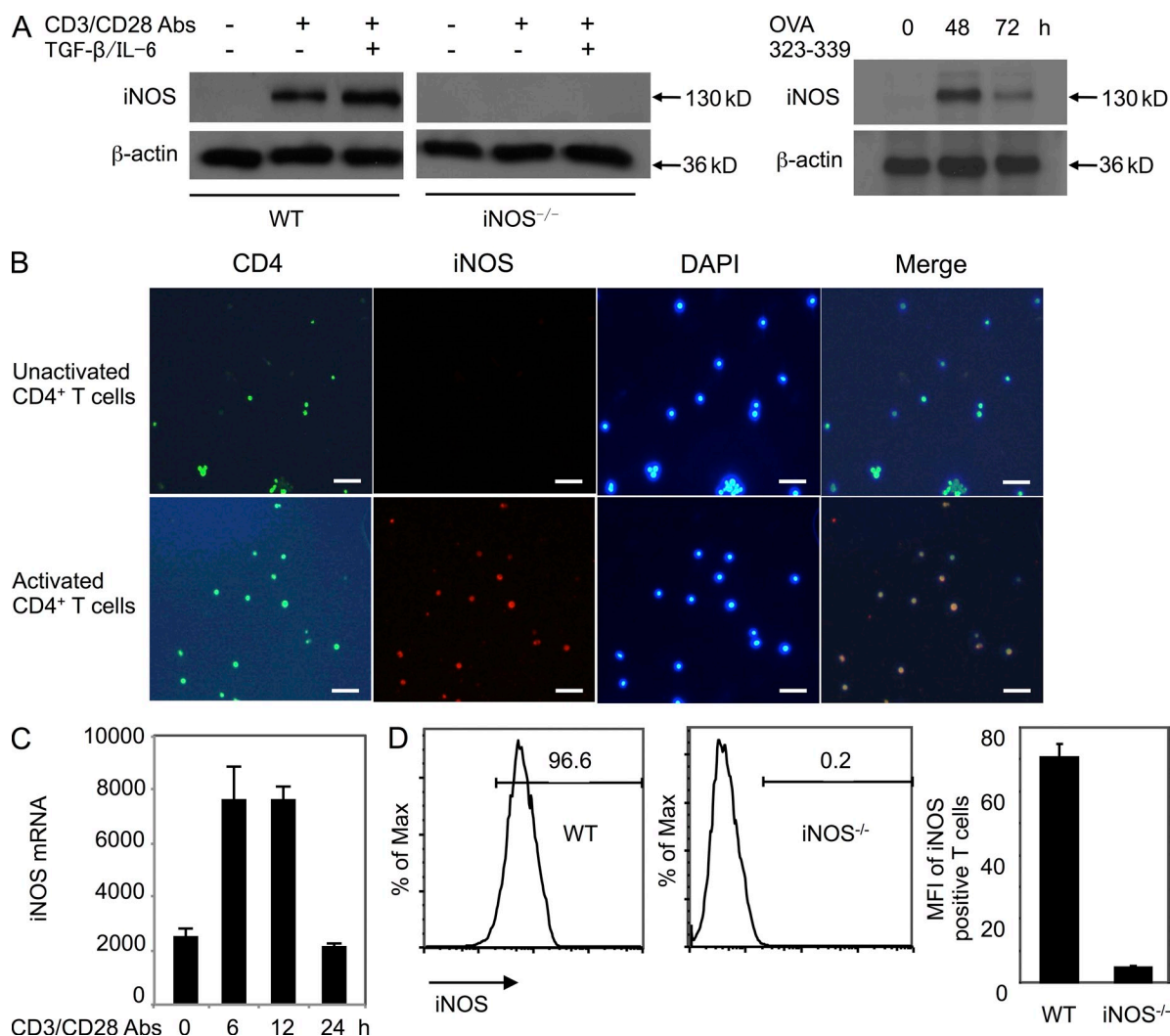


Figure 4. iNOS is expressed in activated CD4⁺ T cells. (A) Naive CD4⁺ T cells from WT and *iNOS*^{-/-} mice were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of 10 ng/ml IL-6 plus 5 ng/ml TGF-β. 3 d after stimulation, the cell lysates were collected and iNOS protein expression was analyzed by Western blotting. Alternatively, spleen cells were prepared from OT-II mice and the cells were activated with OVA peptide at 1.0 μg/ml for different period of time. CD4⁺ T cells were then purified and iNOS expression was determined by Western blotting. (B) Naive CD4⁺ T cells from B6 mice were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 72 h, and cells were fixed and stained for iNOS, CD4, and DAPI followed by microscopic analysis. Bars, 100 μm. (C) Naive CD4⁺ T cells from WT mice were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for various time intervals (6, 12, and 24 h). Total cellular RNA was extracted and iNOS mRNA expression was analyzed by qPCR. Each bar represents mean ± SD from three independent experiments. (D) Naive CD4⁺ T cells from WT and *iNOS*^{-/-} mice were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 72 h, and iNOS expression was analyzed by a single cell flow cytometry. Each bar represents mean ± SD from three independent experiments.

showed that macrophage cell line 264.7 cells expressed high level of iNOS protein after LPS stimulation, whereas LPS-stimulated CD4⁺ T cells did not express iNOS protein at all (unpublished data). These results suggest that NO derived from iNOS expressed by activated CD4⁺ T cells plays a negative role in T_H17 cell differentiation.

Because T cells have also been reported to express eNOS and nNOS (Williams et al., 1998; Ibiza et al., 2006), we further wanted to explore whether eNOS or nNOS is involved in the regulation of T_H17 cell differentiation. Naive CD4⁺ T cells from *eNOS*^{-/-}, *nNOS*^{-/-}, and WT mice

were primed in vitro for 3 d under T_H0 or T_H17 polarizing conditions. The cells were then restimulated with PMA/ionomycin and examined for the percentages of IL-17-producing cells by intracellular staining using flow cytometry. The percentages of IL-17-producing CD4⁺ T cells from *eNOS*^{-/-} and *nNOS*^{-/-} CD4⁺ T cell cultures were comparable to cells from WT cell cultures (unpublished data). CD4⁺ T cells developed normally in *eNOS*^{-/-} and *nNOS*^{-/-} mice (unpublished data). These results suggest that iNOS is expressed in activated T cells and may play a role in T_H17 cell differentiation.

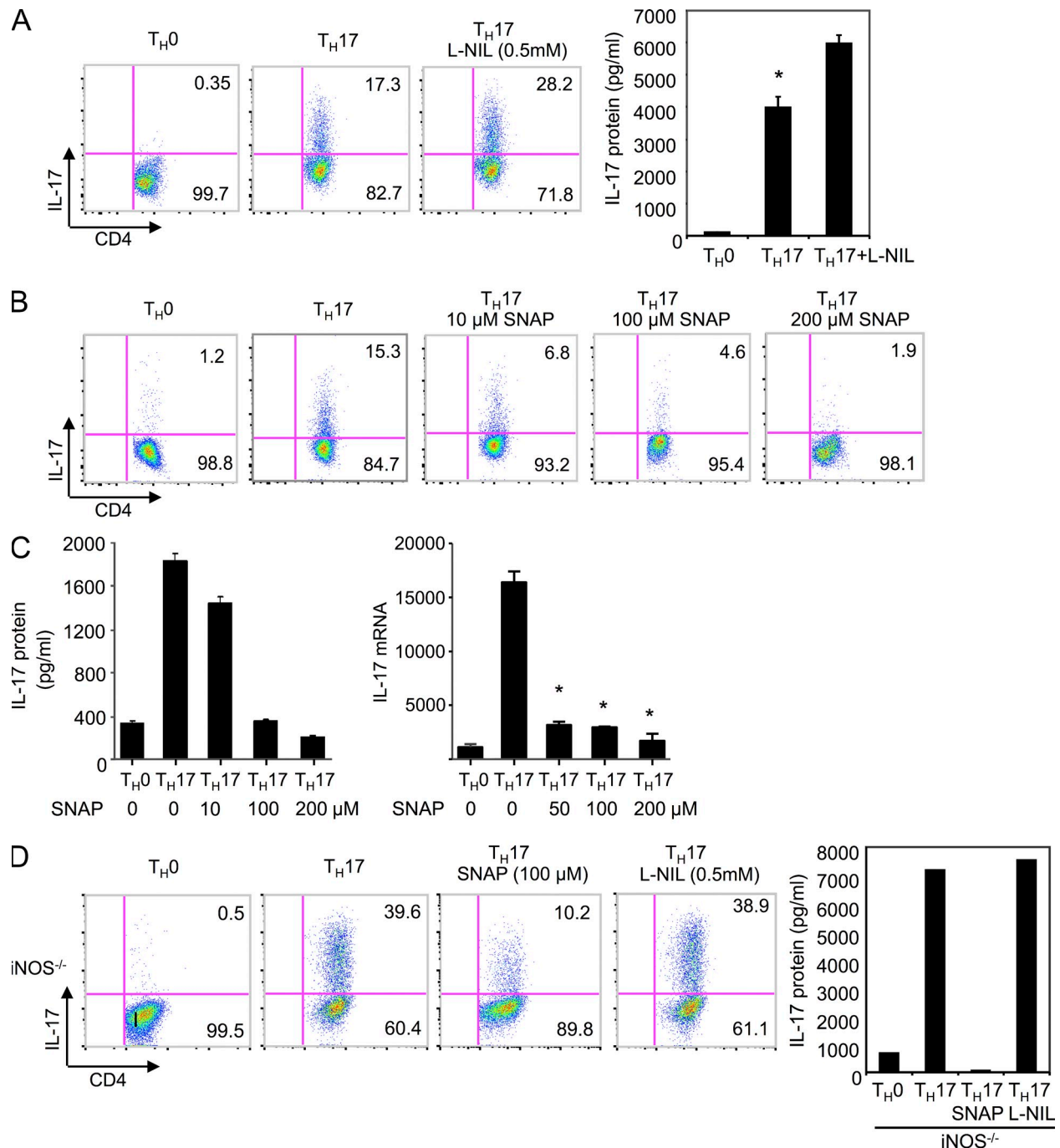


Figure 5. NO suppresses T_H17 cell differentiation. (A) Naive CD4⁺ T cells from B6 mice were differentiated under T_H17 polarizing conditions in the presence of 0.5 mM L-NIL for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular IL-17, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ cells and the percentages of IL-17-producing CD4⁺ cells are shown. Each bar represents mean $\pm$ SD from three independent experiments. *, $P < 0.05$ versus cells added with L-NIL. (B) The cells prepared in A were differentiated under T_H17 polarizing conditions in the presence of SNAP (10, 100, or 200 μ M) for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular IL-17, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ cells and the percentages of IL-17-producing CD4⁺ cells are shown. (C) The cells prepared in A were restimulated with PMA/ionomycin for 12 h and the supernatants were analyzed for IL-17 by ELISA or the cells prepared in A were restimulated with PMA/ionomycin for 5 h and mRNA expression of indicated genes was determined by qPCR. The data shown were normalized to levels of ubiquitin expression as analyzed by qPCR. Each bar presents mean $\pm$ SD from three independent experiments. *, $P < 0.05$ versus T_H17 cells only. The results are representative of three independent experiments. (D) Naive CD4⁺ T cells from *iNOS*^{-/-} mice were differentiated under T_H17 polarizing conditions in the presence of 0.5 mM L-NIL or 100 μ M SNAP for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular IL-17, and analyzed by flow cytometry. Representative FACS

The effect of L-NIL, a pharmacologic iNOS inhibitor, on T_H17 cell differentiation

Experiments with *iNOS*^{-/-} mice indicated that NO may be involved in the negative regulation of T_H17 cell differentiation. We next tested this by adding an iNOS inhibitor to WT T cell cultures. We used L-NIL, a selective iNOS inhibitor, to treat CD4⁺ T cells cultured under T_H17 conditions. Naive CD4⁺ T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under T_H17 polarizing conditions in the presence of 0.5 mM L-NIL. Addition of L-NIL significantly enhanced the percentage of IL-17-producing cells and IL-17 protein release in WT CD4⁺ T cell cultures (Fig. 5 A), which mimicked the *iNOS*^{-/-} CD4⁺ T cell cultures. We next used SNAP, which releases NO spontaneously and is widely used as an NO donor. SNAP dose-dependently reduced the percentage of IL-17-producing CD4⁺ T cells (Fig. 5 B). Similarly, IL-17 protein release and IL-17 mRNA expression were inhibited by SNAP in a dose-dependent manner (Fig. 5 C). In addition, SNAP suppressed T_H17 cell differentiation in both WT and *iNOS*^{-/-} cell cultures, whereas L-NIL had no significant effect on the percentage of IL-17-producing cells and IL-17 protein release in *iNOS*^{-/-} CD4⁺ T cell culture (Fig. 5 D). Furthermore, we also used 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), an NO scavenger. We demonstrated that PTIO treatment significantly increased the percentage of IL-17-producing CD4⁺ T cells in WT T cell cultures but not in *iNOS*^{-/-} T cell cultures (Fig. 6). To test whether L-NIL and SNAP affects T_H1 or T_H2 cell differentiation, naive CD4⁺ T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under T_H1 or T_H2 conditions in the presence of 0.5 mM L-NIL or 50 μ M SNAP. We did not find a significant effect of L-NIL or SNAP on T_H1 or T_H2 cell differentiation (unpublished data). In addition, the compound SNAP or L-NIL had no significant effect on cell viability and proliferation (unpublished data). Thus, these results suggest that NO derived from iNOS expressed in T cells selectively inhibits T_H17 cell differentiation.

NO suppresses ROR γ t-mediated IL-17 transcription

The above findings prompted us to probe the molecular basis for NO control of T_H17 cell differentiation. Because many studies have demonstrated a critical role for ROR γ t in T_H17 cell differentiation both in vitro and in vivo (Ivanov et al., 2006), we asked if NO might affect ROR γ t expression, resulting in the control of T_H17 cell differentiation. First we examined ROR γ t expression in *iNOS*-deficient CD4⁺ T cells. Naive CD4⁺ T cells from *iNOS*^{-/-} or WT littermate mice were primed in vitro for 3 d under T_H0 or T_H17 polarizing conditions. The cells were then restimulated with PMA/ionomycin and examined for the percentages of ROR γ t-positive cells by intracellular staining using flow cytometry. Interestingly, the percentage of ROR γ t-positive cells in *iNOS*^{-/-} CD4⁺

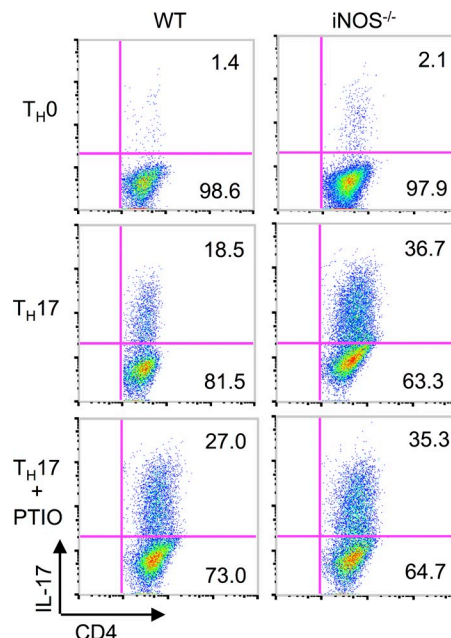


Figure 6. NO suppresses T_H17 cell differentiation. Naive CD4⁺ T cells from WT or *iNOS*^{-/-} mice were differentiated under T_H17 polarizing conditions in the presence of 200 μ M PTIO for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular IL-17, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ cells and the percentages of IL-17-producing CD4⁺ cells are shown. The data are representative of three similar experiments.

T cell cultures was comparable to that in WT cell cultures (Fig. 7 A). This was confirmed by Western blotting experiments showing that the levels of ROR γ t, STAT3, IRF4, and AHR protein were similar in *iNOS*^{-/-} and WT mice (Fig. 7 B). These results suggest that enhanced T_H17 cell differentiation in *iNOS*^{-/-} mice is not the result of a change in ROR γ t expression at the protein level. To further assess whether NO might directly affect ROR γ t protein expression, we transfected ROR γ t into 293T cells treated with different concentrations of SNAP for 40 h and then examined ROR γ t expression by Western blotting. The results showed that the treatment with SNAP did not affect the levels of ROR γ t expression (Fig. 7 C) or nuclear translocation (Fig. 7 C).

We then proceeded to analyze whether NO modulates posttranslational modification of ROR γ t protein. First, we considered if NO affects ROR γ t ubiquitination. To test this, we cotransfected T7-ROR γ t and HA-ubiquitin overexpression plasmids into 293T cells in the presence of the NO donor SNAP at different concentrations for 40 h. Cell lysates were analyzed by immunoprecipitation with anti-T7 antibody and immunoblotted with anti-HA mAb. A high molecular mass smear typical of ubiquitinated protein was detected only in samples cotransfected with T7-ROR γ t and HA-ubiquitin. SNAP treatment had no significant effect on

dot plots gated on CD4⁺ cells and the percentages of IL-17-producing CD4⁺ cells are shown. Or the cells were restimulated with PMA/ionomycin for 12 h and the supernatants were analyzed for IL-17 by ELISA. The results are representative of three independent experiments.

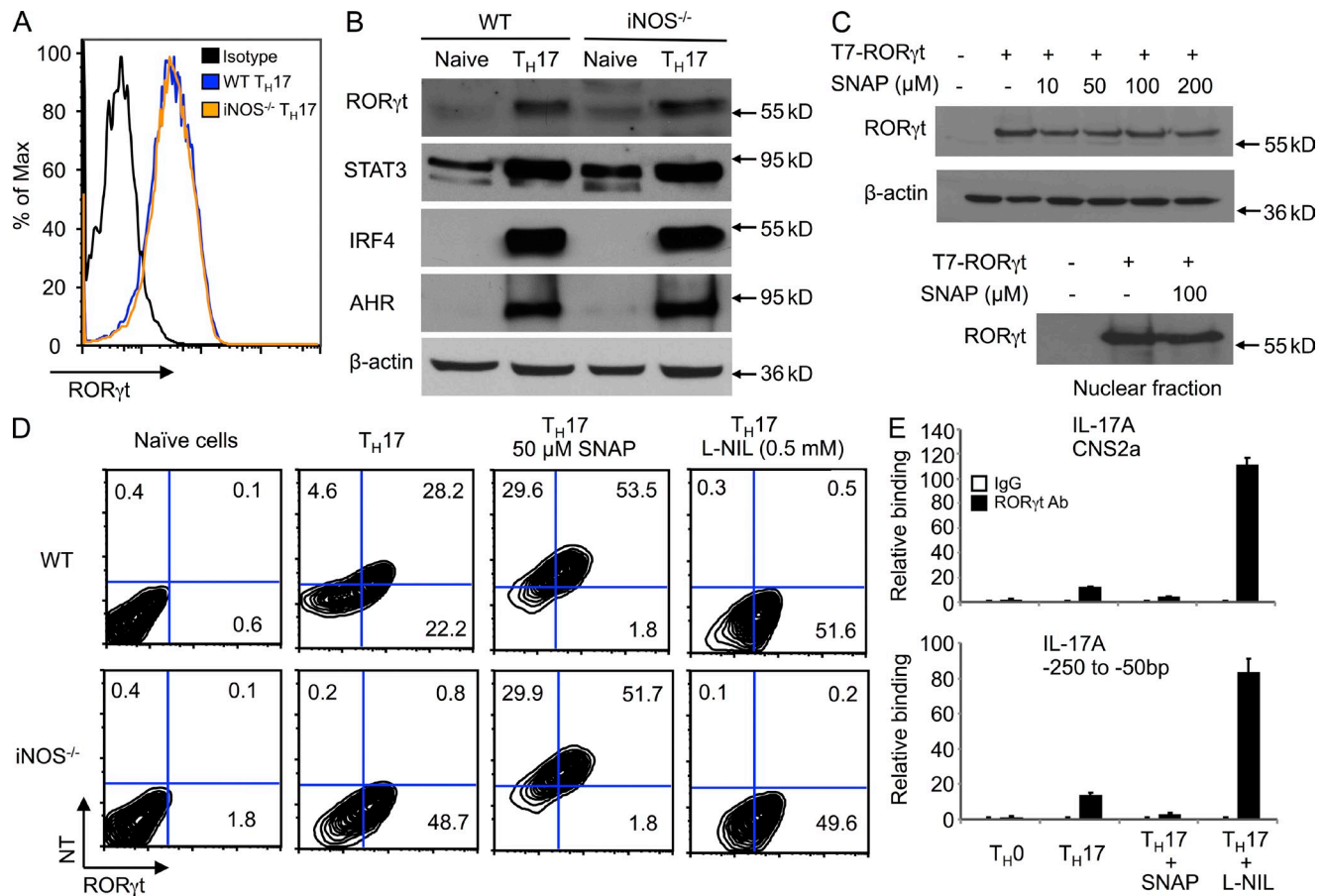


Figure 7. NO suppresses RORγt-mediated IL-17 transcription. (A) Naive CD4⁺ T cells from WT or $iNOS^{-/-}$ mice were differentiated under T_H17 polarizing conditions for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular RORγt, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ cells and the percentages of RORγt-positive CD4⁺ cells are shown. (B) The cells prepared in A were lysed and cell lysates were prepared. The expression of RORγt and other indicated proteins was analyzed by Western blotting. (C) 293T cells were transfected with T7-tagged RORγt plasmid for 40 h in the presence of SNAP (10, 50, 100, or 200 μM). Cell lysates were collected and RORγt protein expression was analyzed by Western blotting. Alternatively, 293T cells were transfected with T7-tagged RORγt plasmid for 40 h in the presence of 100 μM SNAP. Nuclear protein was extracted and RORγt protein expression was analyzed by Western blotting. (D) Naive CD4⁺ T cells from WT or $iNOS^{-/-}$ mice were differentiated under T_H17 polarizing conditions in the presence of 50 μM SNAP or 0.5 mM L-NIL for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular RORγt and nitrotyrosine, and analyzed by flow cytometry. Representative FACS dot plots are gated on CD4⁺ cells. (E) Naive CD4⁺ T cells from WT mice were cultured under T_H17 -polarizing conditions in the presence of 50 μM SNAP or 0.5 mM L-NIL for 60 h, followed by ChIP assay. 3 μg anti-RORγt antibody or isotype-matched IgG as control antibody were used in the immunoprecipitation step. PCR was used to quantify the amount of precipitated DNA with primers flanking the CNS2 and -250 to -50 regions of the IL-17 promoter. Each bar represents mean $\pm$ SD from three independent experiments.

the pattern of ubiquitination (unpublished data), suggesting that RORγt ubiquitination is not the target for the effect of NO on RORγt.

The mouse amino acid sequence of RORγt has 15 tyrosine residues (unpublished data), which may be subject to nitration induced by NO. To investigate this possibility, naive CD4⁺ T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under T_H17 conditions in the presence of L-NIL or SNAP. Flow cytometric analyses clearly revealed a population of RORγt⁺Nitrotyrosine⁺ cells in WT but not in $iNOS^{-/-}$ cell cultures (Fig. 7 D). Treatment with SNAP resulted in an expansion of this cell population (Fig. 7 D), which was reversed in the presence of L-NIL (Fig. 7 D). ChIP analysis indicated that SNAP treatment suppressed RORγt

binding to the promoter region of IL-17 gene (Fig. 7 E), whereas L-NIL treatment significantly enhanced the binding of RORγt there (Fig. 7 E). To further analyze the effect of NO on nitration of tyrosine residues in RORγt, we performed coimmunoprecipitation experiments using cell lysates from primary CD4⁺ T cells cultured under T_H0 or T_H17 conditions. We found that anti-nitrotyrosine antibody coimmunoprecipitated RORγt from lysates of T_H17 cells (Fig. 8 A), suggesting that tyrosine residues of RORγt were nitrated under T_H17 conditions. To confirm these results, 293T cells were transfected with T7-RORγt in the presence of SNAP for 40 h. Cell lysates were immunoprecipitated with anti-nitrotyrosine antibody and immunoblotted with anti-T7 antibody. SNAP treatment clearly induced tyrosine nitrosylation

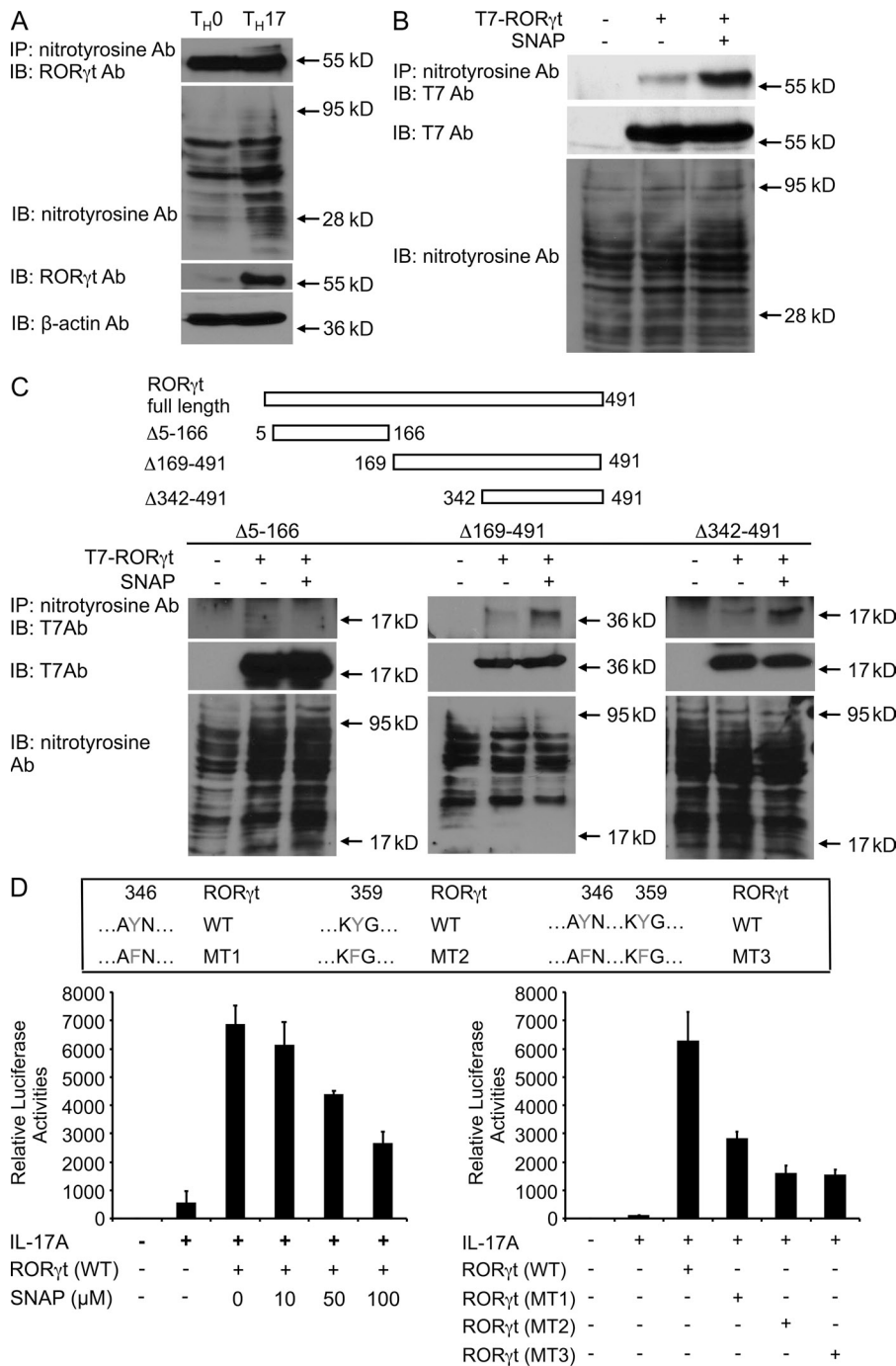


Figure 8. NO suppresses ROR γ t-mediated IL-17 transcription. (A) Naive CD4⁺ T cells from B6 mice were cultured under T_H0 or T_H17-polarizing conditions for 60 h and the cell lysates were then immunoprecipitated with an anti-nitrotyrosine antibody and blotted with anti-ROR γ t. Data are representative of three independent experiments. (B) 293T cells were transfected with T7-tagged ROR γ t plasmid for 40 h in the presence of 100 μ M SNAP. Cell lysates were immunoprecipitated with anti-nitrotyrosine antibody and immunoblotted with anti-T7 antibody. (C) 293T cells were transfected with T7-tagged ROR γ t truncation mutant plasmids (Δ 5-166, Δ 169-491, and Δ 342-491) for 40 h in the presence of 100 μ M SNAP. Cell lysates were immunoprecipitated with anti-nitrotyrosine antibody and immunoblotted with anti-T7 antibody. (D) 293T cells were cotransfected with an IL-17A promoter reporter construct containing the 6-kb promoter and a ROR γ t plasmid in the presence of SNAP (50, 100, or 200 μ M) or 293T cells were transfected with an IL-17A promoter reporter construct and WT ROR γ t or different ROR γ t mutant plasmids for 30 h. Luciferase assays were performed and luciferase activities were normalized to β -galactosidase activity. Data indicate mean $\pm$ SD of triplicate cultures and are representative of three independent experiments.

of ROR γ t (Fig. 8 B), suggesting that NO-induced alterations of tyrosine residues may affect ROR γ t activation. To analyze which part of ROR γ t is sensitive to NO, we generated three T7-tagged ROR γ t truncation mutants (Δ 5-166, Δ 169-491, and Δ 342-491). The mutants were transfected into 293T cells in the presence of SNAP for 40 h and cell lysates were immunoprecipitated with anti-nitrotyrosine antibody and immunoblotted with anti-T7 antibody. SNAP treatment had no significant effect on ROR γ t mutant Δ 5-166 (Fig. 8 C) but clearly increased nitrotyrosine levels of

ROR γ t truncation mutants Δ 169-491 and Δ 342-491 (Fig. 8 C). Thus, tyrosine residues in the region from residue 169 to 491 of ROR γ t are sensitive to nitration by NO. Because NADPH oxidase is required for the formation of peroxynitrite, which is involved in the tyrosine nitration, we found that T cells expressed NADPH p47-phox (unpublished data), as reported previously (Jackson et al., 2004). To examine the effect of NO on ROR γ t at the functional level, we cotransfected an IL-17 promoter reporter and ROR γ t expression plasmids into 293T cells in the presence of various doses of SNAP for

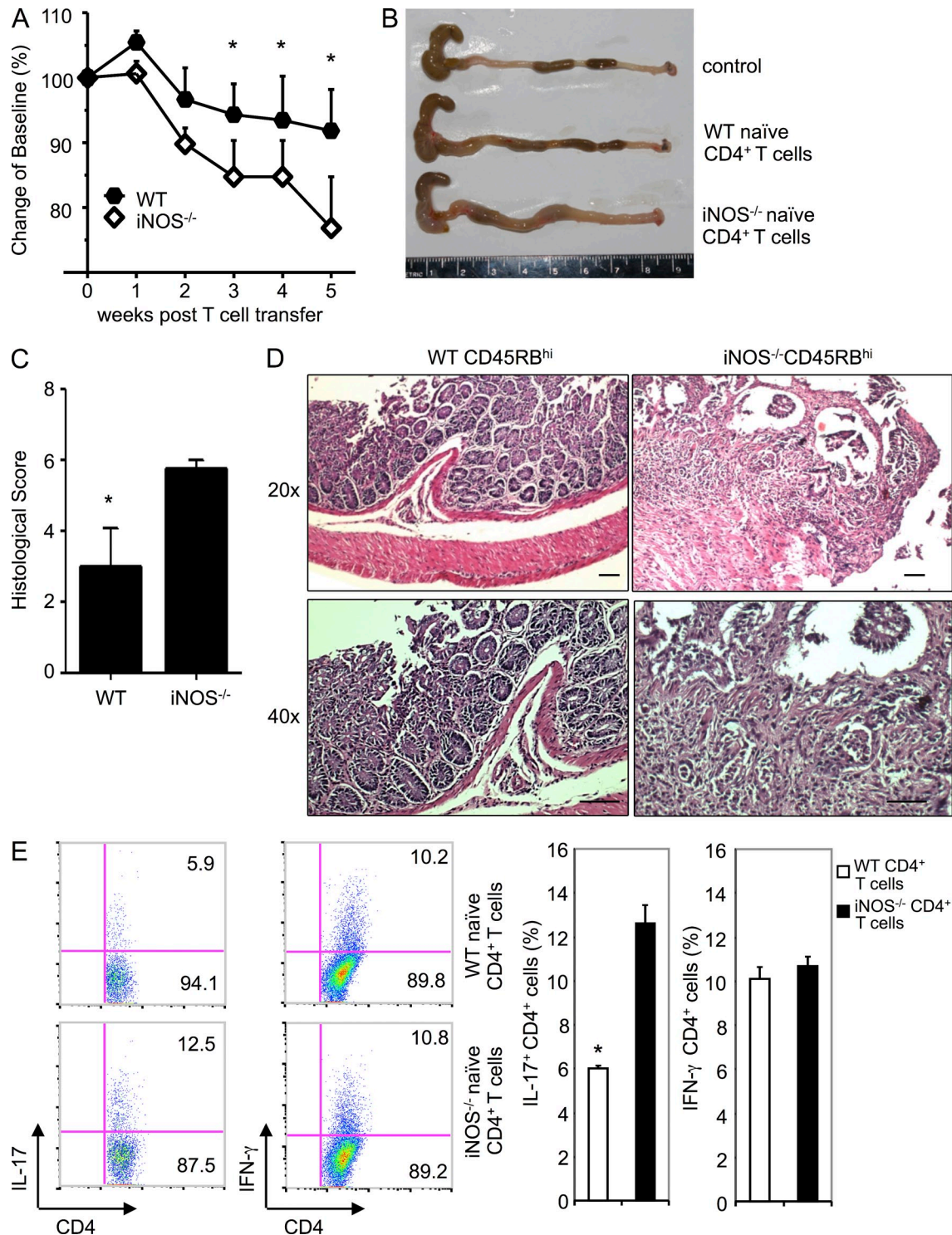


Figure 9. iNOS deficiency promotes the T_H17 immune responses in experimental colitis. CD4⁺CD45RB^{hi} T cells were purified from spleens and lymph nodes of WT or *iNOS*^{-/-} mice and 5×10^5 cells were injected (i.p.) into recipient *Rag1*^{-/-} mice. Body weight change was monitored every week and mice were sacrificed 5 wk later. (A) Changes in body weight of *Rag1*^{-/-} mice ($n = 5-6$ mice per group) after i.p. transfer of WT or *iNOS*^{-/-} CD4⁺CD45RB^{hi} T cells were recorded. Data are presented as the mean $\pm$ SD of the percentage of initial body weight and are representative of two similar experiments. *, $P < 0.05$ versus recipients of *iNOS*^{-/-} cells. Morphology of intestines (B), disease scores (C; each bar represents mean $\pm$ SD of measurements made from five mice; *, $P < 0.05$ versus recipients of *iNOS*^{-/-}), and sections of colons with colitis (D) from *Rag1*^{-/-} mice ($n = 5-6$ mice in each group) on day 35 after naïve T cell transfer was as described above. Bars, 100 μ m. (E) The percentage of IL-17-producing cells from mesenteric lymph nodes of *Rag1*^{-/-} mice in B, C, and D (white column, transfer with WT cells; black column, transfer with *iNOS*^{-/-} cells). Each bar represents mean $\pm$ SD of measurements made from five mice. *, $P < 0.05$ versus recipients of *iNOS*^{-/-} cells.

36 h and analyzed them for IL-17 promoter activation. The data showed that SNAP suppressed ROR γ t-mediated IL-17 promoter activation in a dose-dependent manner (Fig. 8 D). Furthermore, we have analyzed two tyrosine residues Tyr346 and Tyr359 of mouse ROR γ t, corresponding to Tyr369 and Tyr382 of human ROR γ t, that are important for ligand binding (Fig. S1, A and B). We found that mutating these two tyrosine residues significantly impaired ROR γ t-mediated IL-17 promoter activation, suggesting that Tyr346 and Tyr359 are critical for ROR γ t transcriptional function (Fig. 8 D). Thus, the results indicate that NO suppresses IL-17 expression at the transcriptional level by nitration of tyrosine residues in ROR γ t.

iNOS regulates T_H17 cell differentiation in vivo

Accumulating evidence indicates that T_H17 cells are involved in the pathogenesis of various autoimmune/inflammatory diseases, including MS, RA, IBD, and asthma (Weaver et al., 2006; Wilke et al., 2011). To further assess the effects of iNOS on T_H17 cell development in vivo, we performed adoptive transfer experiments using CD4⁺CD45Rb^{hi} cells from WT and *iNOS*^{-/-} mice to induce colitis in *Rag1*^{-/-} mice. *Rag1*^{-/-} mice reconstituted with *iNOS*^{-/-} T cells began losing weight earlier and lost significantly more weight than mice transferred with WT cells (Fig. 8 A). Parallel histological studies of colon sections from *Rag1*^{-/-} mice reconstituted with *iNOS*^{-/-} T cells revealed more severe inflammatory cell infiltrates and significantly higher pathological scores than those observed in sections from mice reconstituted with T cells from WT mice (Fig. 9, B–D). In addition, mice reconstituted with *iNOS*^{-/-} cells had significantly higher percentages of IL-17-producing cells than control mice (Fig. 8 E), whereas the percentage of IFN- γ -producing cells was comparable (Fig. 9 E). Thus, iNOS deficiency in T cells promotes intestinal inflammation in a T cell-mediated model of colitis, suggesting that iNOS expressed in T cells may play a negative role in the regulation of T_H17 immune response.

To further understand the role of iNOS in T_H17 cell function in vivo, we extended our studies to include experimental autoimmune encephalomyelitis (EAE), a mouse model of human MS. Previous studies showed that NO will suppress EAE (Zielasek et al., 1995; Okuda et al., 1997; Fenyk-Melody et al., 1998). To investigate the role of iNOS expressed by T cells on the development of EAE, we immunized WT and *iNOS*^{-/-} mice with the myelin oligodendrocyte glycoprotein (MOG_{35–55}) peptide in complete Freund's adjuvant. *iNOS*^{-/-} mice developed EAE with an accelerated time course and greater severity than WT mice as indicated by disease scores (Fig. 10 A). As expected, iNOS mRNA expression was significantly induced in CD4⁺ T cells from WT but not from *iNOS*^{-/-} mice in these experiments (Fig. 10 B). We then examined the expression of T_H17 and T_H1 cytokines in EAE mice. Infiltrating CD4⁺ T cells from the central nervous systems of *iNOS*-deficient mice with EAE expressed significantly higher percentage of IL-17-producing and IFN- γ -producing CD4⁺ T cells and expressed higher levels of transcripts for

IL-17 and IFN- γ compared with cells from WT mice (Fig. 10, C and D). Thus, iNOS deficiency promotes inflammation in central nervous system in EAE, further confirming that NO negatively regulates T_H17 cell differentiation in vivo.

DISCUSSION

T_H17 cells are a new member of the still-growing family of T helper cell subsets, which play critical roles in the pathogenesis of autoimmune and inflammatory diseases. Therefore, understanding the intrinsic suppressing programs for T_H17 cells will help to dissect mechanisms for the control of T_H17 immune responses and elucidate the mechanism involved in the development of human inflammatory diseases including IBD, MS, and RA. In the present study, we demonstrated that iNOS-deficient naive CD4⁺ T cells polarized under T_H17 condition led to more efficient T_H17 cell differentiation without major effects on either the T_H1 or T_H2 cell lineages. In vivo, transfer of CD4⁺CD45Rb^{hi} cells into *Rag1*^{-/-} mice induced more severe colitis than transfer of control cells. In addition, mice reconstituted with *iNOS*^{-/-} T cells had a significantly higher percentage of IL-17-producing CD4⁺ T cells than mice transferred with WT cells. These results suggest that iNOS derived from activated T cells selectively regulates T cell differentiation.

Many studies have demonstrated that NO can play a dual role in the modulation of immune responses (Niedbala et al., 2006). NO derived from iNOS in macrophages and other innate immune cells is proinflammatory and an essential component of host defenses against various pathogens including bacteria, parasites, and viruses (Bogdan et al., 2000). However, mounting evidence indicates that NO can also contribute to immune suppression. We and other groups previously reported that IL-12 mRNA and protein expression were significantly increased in iNOS KO mice, suggesting that NO may suppress IL-12-mediated T_H1 immune responses (Xiong et al., 2004). Huang et al. (1998) suggested that the enhanced T_H1 immune responses in iNOS KO mice were a result of increased production of IL-12 by *iNOS*^{-/-} macrophages after infection with *Leishmania major*. In addition, Giordano et al. (2011) reported that expression of inflammatory cytokines, including TNF, IL-6, IL-12p70, and IL-23, was up-regulated in *iNOS*^{-/-} bone marrow-derived dendritic cells. Collectively, these results indicate that iNOS expressed in innate immune cells, including macrophages and dendritic cells, can modulate inflammatory cytokine production. Although the exact molecular mechanisms responsible for this regulation are not fully understood, NO-mediated control of NF- κ B activation may be involved (Xiong et al., 2004).

It is well established that iNOS is expressed in different cell types including macrophages, dendritic cells, NK cells, and by both primary tumor cells and tumor cell lines (Bogdan., 2001). In addition, there is a controversy for the expression of iNOS in T cells. Vig et al. (2004) reported that T cell blasts expressed iNOS, which plays an important role in immune memory, whereas Thüning et al. (1995) did not find iNOS expression in T cell clones or T cells from naive and *L. major*-infected mice. In the present study, we clearly demonstrated

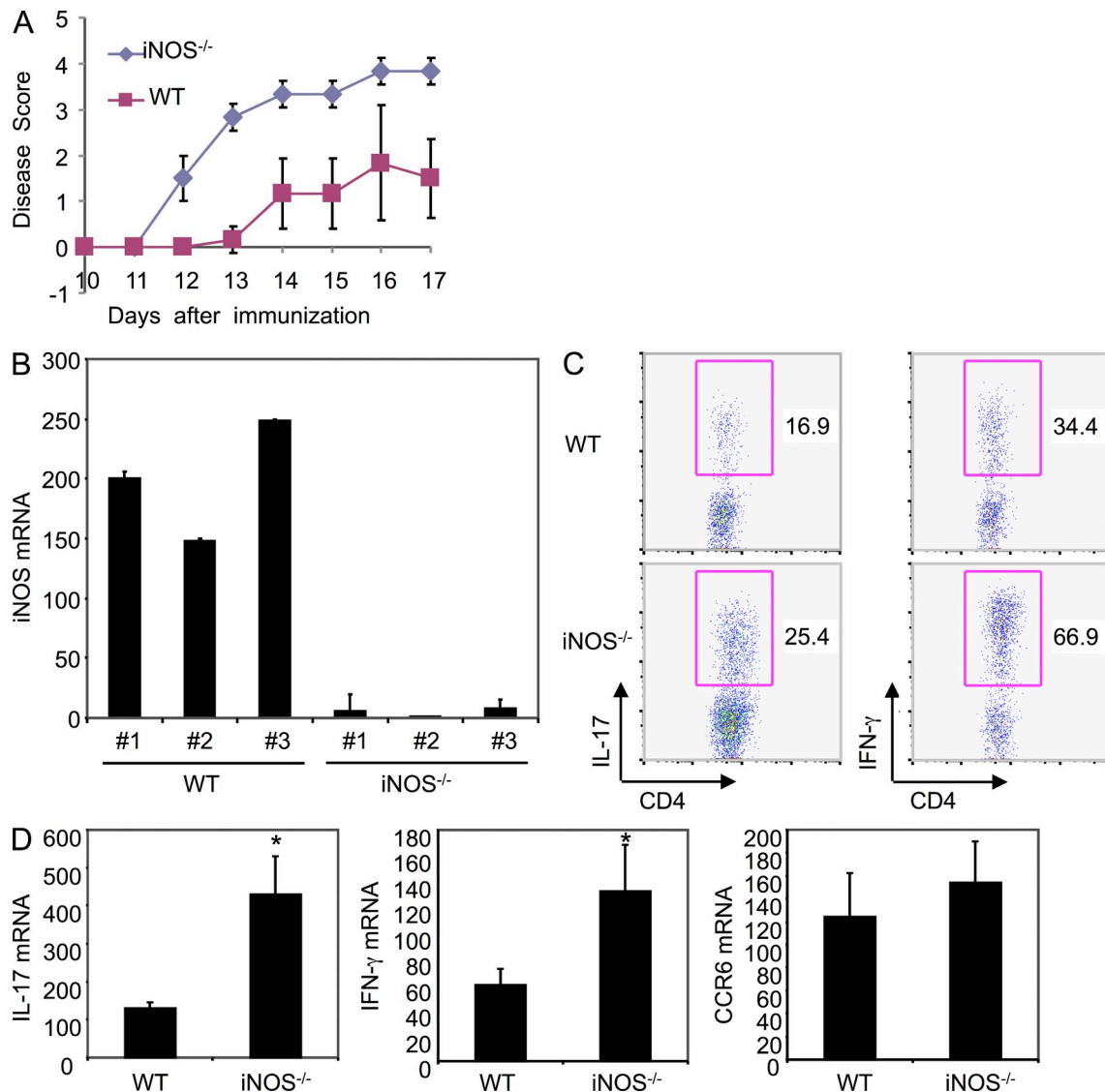


Figure 10. iNOS is expressed in CD4⁺ T cells infiltrated in CNS of WT mice with EAE and *iNOS*^{-/-} mice develop more severe EAE.

(A) *iNOS*^{-/-} or WT mice were induced for EAE, and disease development was scored. Data are mean ± SD ($n = 15$ mice per group) pooled from three experiments. The score was based on a five-point scale. (B) CD4⁺ T cells were purified from CNS of WT and *iNOS*^{-/-} mice with EAE. Total RNA was extracted and iNOS mRNA expression was determined by qPCR. Each bar represents mean ± SD from two independent experiments. (C) WT or *iNOS*^{-/-} mice were induced for EAE and CD4⁺ T cells were purified from CNS of *iNOS*^{-/-} or WT mice with EAE. Cells were then restimulated with PMA/ionomycin for 5 h, stained for intracellular IL-17 or IFN-γ, and analyzed by flow cytometry. Representative FACS dot plots gated on CD4⁺ cells and the percentages of IL-17-producing or IFN-γ-producing CD4⁺ cells are shown. (D) WT or *iNOS*^{-/-} mice were induced for EAE and CD4⁺ T cells were purified from CNS of *iNOS*^{-/-} or WT mice with EAE. Total RNA was extracted and qPCR was performed for the analysis of IL-17, IFN-γ, and CCR6 mRNA expression. Each bar represents mean ± SD from three independent experiments. *, $P < 0.05$ versus WT cells.

that iNOS expressed by activated CD4⁺ T cells negatively regulated T_H17 cell differentiation. The results are consistent with recent studies showing that the NO donors NOC-18 and S-nitrosoglutathione suppressed T_H17 cell differentiation (Nath et al., 2010; Niedbala et al., 2011). Thus, our observations support the notion that NO derived from iNOS in activated T cells controls T cell differentiation by selectively suppressing T_H17 cell development.

The importance of RORγt in T_H17 cell development has been well studied in mice. *RORγt*^{-/-} mice fail to develop

lymph nodes or Peyer's patches and T_H17 cell development is severely impaired, indicating that RORγt is a master transcription factor in T_H17 cell differentiation (Ivanov et al., 2006). Interestingly, RORγt expression in *iNOS*^{-/-} CD4⁺ T cells cultured under T_H17 conditions was comparable to WT CD4⁺ T cell cultures, implying that the enhanced T_H17 cell differentiation observed is not the result of increased RORγt protein levels. Instead, we found that the NOS donor SNAP suppressed RORγt-mediated IL-17 promoter activation in a dose-dependent manner, suggesting that NO

may control ROR γ t activity during IL-17 gene transcription. How might NO control ROR γ t activity? NO has been shown to directly affect the activity of many proteins via tyrosine nitration. We have clearly shown that tyrosine residues in ROR γ t are nitrated and that such nitration of ROR γ t significantly impaired the binding of ROR γ t to the promoter region of the IL-17 gene resulting in the inhibition of IL-17 transcription. Our ROR γ t mutant studies showed the tyrosine residues between amino acids 169 and 491 are possible targets for nitration by NO. Combined with our knowledge of the human ROR γ t crystal structure and its ligand binding domain with digoxin (Fig. S1), we found that several tyrosine residues are indeed in this region, with Tyrosine369 and Tyrosine382 being located near the ligand binding pocket. As such, the tyrosine nitration may well affect ROR γ t ligand binding activity and formation. Our further mutation experiments demonstrated that Tyr346 and Tyr359 of mouse ROR γ t, corresponding to Tyr369 and Tyr382 residues of human ROR γ t, are critical for ROR γ t transcriptional activation. Thus, the results suggest that Tyr346 and Tyr359 could be the target for NO on ROR γ t transcriptional function. Recently, Niedbala et al. (2011) reported that NO donor NOC-18 inhibits AHR protein expression in T_H17 cells, and they conclude that NO suppresses T_H17 cell development by inhibiting AHR protein expression. However, we did not find significant difference of AHR protein expression in WT and iNOS^{-/-} CD4⁺ T cells under T_H17 conditions, suggesting that AHR protein expression does not explain the effect of T cell-derived iNOS on T_H17 cell differentiation.

A previous study showed that tyrosines of I κ B α are nitrated as a consequence of NO synthase activation, leading to dissociation of I κ B α from NF- κ B (Yakovlev et al., 2007). Other studies have shown that nitration of a specific tyrosine in proteins can have structural and functional significance (Radi, 2004; Ischiropoulos and Gow., 2005; Ji et al., 2006). Thus, our study documents a novel mechanism for the regulation of T_H17 cell development by nitration of ROR γ t tyrosine residues. In addition, nitrosylation has also been reported to regulate the activation of transcription factors (Pannu and Singh., 2006; Prasad et al., 2007). Khan et al. (2005, 2006) reported that a physical NO carrier, GSNO, regulates NF- κ B activation via S-nitrosylation of p65, which limits its binding activity. Our future studies explore whether S-nitrosylation of ROR γ t is also involved in the regulation of IL-17 transcription.

It has been demonstrated that T_H17 cells play critical roles in the pathogenesis of several inflammatory diseases, including IBD and MS (Weaver et al., 2006; Wilke et al., 2011). In the present study, we demonstrated that iNOS derived from T cells targets ROR γ t, resulting in impaired T_H17 cell development. We demonstrated that transfer of iNOS^{-/-} naive CD4⁺ T cells into *Rag1*^{-/-} mice induced more severe colitis with an enhanced T_H17 immune response. In addition, iNOS^{-/-} mice developed more severe EAE than WT mice, consistent with previous studies (Zielasek et al., 1995; Okuda et al., 1997; Fenyk-Melody et al., 1998; Niedbala et al., 2011), and IL-17 expression was significantly higher in iNOS^{-/-} mice.

Consistent with our in vitro results, iNOS transcripts were highly induced in T cells infiltrating the CNS of WT but not iNOS^{-/-} mice with EAE. Interestingly, we found that IFN- γ -producing CD4⁺ T cells were significantly increased in iNOS^{-/-} mice with EAE compared with WT mice with EAE, in a similar pattern to IL-17-producing CD4⁺ T cells. Because in this EAE model iNOS can be expressed by different cell types, including macrophages, dendritic cells, microglia, and T cells, it is therefore still not clear which iNOS-expressing cell contributes to the regulation of T_H1 cells. Collectively, iNOS expressed by activated T cells selectively regulates T_H17 cell development, resulting in the control of diseases development in colitis and EAE models.

Collectively, our studies clearly demonstrate that iNOS is expressed in activated CD4⁺ T cells, and NO derived from iNOS in activated CD4⁺ T cells suppresses T_H17 cell development. Based on these observations, we suggest a novel molecular mechanism for the inhibitory effects of NO on T_H17 differentiation that involves the suppression of ROR γ t activation. Our results support the concept that iNOS expressed by T cells may play an important role in the development of inflammatory diseases by controlling T_H17 immune responses.

MATERIALS AND METHODS

Mice. C57BL/6j (B6, stock#000664) and iNOS-deficient mice (B6.129P2-Nos2^{tm1Lau}/J, stock#002609) were obtained from The Jackson Laboratory and maintained in the barrier facility at the Mount Sinai School of Medicine. eNOS-deficient mice (B6.129P2-Nos3^{tm1unc}/J, stock#002684) and nNOS^{-/-} mice (B6.129S4-Nos1^{tm2ph}/J, stock#008519) were derived from The Jackson Laboratory. The animal study protocols were approved by the Institutional Animal Care and Use Committees of Mount Sinai School of Medicine.

Antibodies. The following antibodies were purchased from BD, as conjugated to FITC, PE, PE-Cy5, perCP-Cy5.5, or APC: CD4 (L3T4), NOS2 (6/iNOS/NOS typeII), CD8 (53-6.7), CD3e (145-2C11), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD45RB (C363-16A), IL-17 (TC11-18H10), IFN- γ (XMG1.2), and isotype controls. Antibodies for nitrotyrosine (1A6) and p47phox (mouse) were purchased from EMD Millipore. Antibodies for IL-2 (JES6-1A12), ROR γ (B2D), IL-4 (11B11), IL-10 (JES5-16E3), and Foxp3 (FJK-16S) were purchased from eBioscience. Antibody for AHR (BML-SA210) was purchased from Enzo Life Sciences. Antibodies for NOS1 (K-20), NOS2 (M-19), and NOS3 (C-20) were purchased from Santa Cruz Biotechnology, Inc.

CD4⁺ T cell preparation and differentiation in vitro. Naive CD4⁺ T cells (CD62L⁺CD44^{lo}) were prepared by fluorescence-activated cell sorting from spleens and lymph nodes of iNOS^{-/-} and WT littermates. The sorted cells were primed for 96 h with 1 μ g/ml anti-CD3 (145-2C11; BD) and 2 μ g/ml of soluble anti-CD28 (37.51; BD). The cells were rested for 48 h and were then restimulated for 5 h with PMA plus ionomycin in the presence of brefeldin A, and intracellular cytokines were measured by flow cytometry. Cells stimulated under neutral conditions were defined as T_H0 cells. Cells were stimulated to differentiate into T_H1 cells by supplementation with IL-12 plus anti-IL-4 or into T_H2 cells by supplementation with IL-4 and anti-IFN- γ . For T_H17 cell differentiation, cells were stimulated with 5 ng/ml TGF- β 1, 20 ng/ml IL-6, 10 ng/ml and IL-23 (all from R&D Systems) in the presence of 10 μ g/ml anti-IL-4 antibody (11B11; BD) and 10 μ g/ml anti-IFN- γ antibody (XMG1.2, BD).

Intracellular staining and flow cytometry. Cells were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin A before intracellular staining. Cells were fixed with IC Fixation Buffer (BD), incubated with

permeabilization buffer, and stained with PE-anti-mouse IL-17, APC-anti-IFN- γ , and PE-Cy 5.5 anti-mouse CD4 antibodies. Flow cytometry was performed on a FACSCalibur (BD).

RNA isolation and quantitative real-time RT-PCR (qPCR). Total RNA was extracted using an RNeasy plus kit (QIAGEN) and cDNA was generated with an oligo (dT) primer and the Superscript II system (Invitrogen), followed by analysis using iCycler PCR with SYBR Green PCR master Mix (Applied Biosystems). Results were normalized based on the expression of ubiquitin. The following primer sets were used: IL-17A sense, 5'-CTCCAGAAGGC-CCTCAGACTAC-3'; IL-17A antisense, 5'-AGCTTTCCTCCGC-ATTGACACAG-3'; IL-21 sense, 5'-CGCCTCCTGATTAGACTTCG-3'; IL-21 antisense, 5'-GCCCCCTTACATCTTGTGGA-3'; ROR γ t sense, 5'-CCGCTGAGAGGGCTTCAC-3'; ROR γ t antisense, 5'-TGCAG-GAGTAGGCCACATTACA-3'; iNOS sense, 5'-CCGAAGCAAACAT-CACATTCA-3'; iNOS antisense, 5'-GGTCTAAAGGCTCCGGGCT-3'; ubiquitin sense, 5'-TGGCTATTAATTATTCGGTCTGCA-3'; and ubiquitin antisense, 5'-GCAAGTGGCTAGAGTGCAGAGTAA-3'.

Transfection and luciferase reporter assay. 293T cells were transiently transfected with an IL-17 promoter luciferase reporter plasmid together with ROR γ t in the presence of SNAP at different concentrations. For each transfection, 2.0 μ g of plasmid was mixed with 100 μ l DMEM (without serum and antibiotics) and 4.0 μ l Lipofectamine 2000 reagent. The mixture was incubated at room temperature for 20 min and added to 12-well plates containing cells and complete medium. The cells were incubated for 30 h and harvested using reporter lysis buffer (Promega) for determination of luciferase activity. Cells were cotransfected with a β -galactosidase reporter plasmid to normalize experiments for transfection efficiency.

T cell proliferation assay. Naive CD4⁺ T cells were purified from spleens and lymph nodes of WT and iNOS^{-/-} mice. 10⁵ cells/well were cultured in the absence or presence of 1 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28 antibodies for 3 d in 96-well microplates. [³H]-Thymidine was added during the last 8 h of a 72-h culture. The cells were then harvested and counted with a β -counter.

Immunoblotting analysis. Cells were washed with cold phosphate-buffered saline and lysed for 15 min on ice in 0.5 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 280 mM NaCl, 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, and 1 mM dithiothreitol) containing protease inhibitors. Cell lysates were clarified by centrifugation (4°C, 15 min, 14,000 rpm), and protein was subjected to 10% SDS-PAGE and immunoblotting was performed. Anti-iNOS (Santa Cruz Biotechnology, Inc.), anti-T7 (MBL), and anti- β -actin (Sigma-Aldrich) antibodies were used according to the manufacturers' instructions. Secondary antibodies were from Santa Cruz Biotechnology, Inc.

T cell transfer colitis and histopathology. T cell transfer colitis was performed as previously described (Powrie et al., 1993; Totsuka et al., 2007). In brief, purified CD4⁺CD45RB^{hi} T cells from WT and iNOS^{-/-} mice were injected intraperitoneally into Rag1^{-/-} recipients (5 $\times$ 10⁵ cells per mouse in 200 μ l sterile PBS per injection). Mice were weighed every week throughout the course of experiments. After 5 wk, mice were sacrificed and colons tissues were excised. Tissues were fixed in 10% buffered formalin and paraffin embedded. The sections (5 μ m) of tissues samples stained with hematoxylin and eosin. All the slides were read and scored by an experienced pathologist without previous knowledge of the type of treatment. The degree of inflammation in the epithelium, submucosa, and submucularis propria was scored separately as described previously (Totsuka et al., 2007).

Chromatin immunoprecipitation (ChIP) assay. The ChIP procedure was performed using an assay kit according to the manufacturer's instructions (EMD Millipore). In brief, T_H17 cells were cross-linked by 1% formaldehyde for 10 min at 37°C. Nuclei were prepared and subjected to sonication to obtain DNA fragments. Chromatin fractions were precleared with protein A-agarose beads followed by immunoprecipitation overnight at 4°C with

3 μ g of anti-ROR γ (Santa Cruz Biotechnology, Inc.) or control antibody. Cross-linking was reversed at 65°C for 4 h, followed by proteinase K digestion. DNA was purified and subjected to qPCR. The input DNA was diluted 200 $\times$ before PCR amplification. The input and immunoprecipitated DNA were amplified by qPCR using primers encompassing the CNS2 and -50 to -250 regions of the mouse IL-17 promoter.

Induction of experimental autoimmune encephalomyelitis (EAE). iNOS^{-/-} and WT mice were immunized subcutaneously with 200 μ l emulsion containing 100 μ g myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK), complete Freund's adjuvant, and 0.4 mg Mycobacterium tuberculosis extract H37-Ra (Difco). Mice were given 100 ng pertussis toxin (list Biological Laboratories) intraperitoneally on days 0 and 2. Mice were sacrificed $\sim$ 3 wk later. EAE was scored as follows: 0, no diseases; 1, limp tail; 2, hind limb weakness; 3, partial paralysis and hind limb paralysis; 4, front and hind limb paralysis; 5, death.

Cytokine ELISA. Supernatants from cell cultures were collected after activation under various conditions and secreted cytokines in the supernatants were measured by ELISA kits with purified coating and biotinylated detection antibodies anti-IL-17 (R&D Systems) and anti-IL-10 (BD).

Statistical analysis. Statistical analysis was performed using Student's *t* test. *P*-values <0.05 were considered statistically significant.

Online supplemental material. Fig. S1 shows structural analysis of the ligand-binding domain of ROR γ t. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20122494/DC1>.

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The authors declare that they have no competing interests.

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