# T cell–derived inducible nitric oxide synthase switches off TH17 cell differentiation

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T cell–derived inducible nitric oxide synthase switches off $T_{H17}$ cell differentiation

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ROTYT 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 ROYt, leading to the suppression of ROYt–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.

Abbreviations used: eNOS, endothelial NOS; 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-$\beta$ 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). ROYt, 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 RORa, 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...
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.

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 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 ± 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 ± 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 ± 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.
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 TH17 differentiation and highlight the importance of T cell–derived iNOS in switching off TH17-dependent immune responses.

**RESULTS**

**iNOS deficiency enhances TH17 cell differentiation**

To investigate the function of NO in TH17 cell differentiation, we first assessed the characteristics of CD4+ T cells from WT or iNOS−/− mice. Naive CD4+ T cells from WT or iNOS−/− mice were primed in vitro for 3 d under neutral (TH0) or TH17 (IL-6 plus TGFB) 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 CD4+ T cells from iNOS−/− T cell cultures was significantly greater than cells from WT cultures (Fig. 1 A). These observations correlated with enhanced...
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 Th17 cytokines, IL-17 and IL-21, were significantly enhanced in iNOS−/− Th17 cells (Fig. 1 C). To rule out the possibility that the enhanced Th17 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 Th17 cell differentiation, Th1 and Th2 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 Th17 conditions was comparable (Fig. 2 B). Collectively, these results indicate that Th17 cell differentiation is enhanced in CD4+ T cells deficient in iNOS, suggesting that NO plays a negative role in Th17 cell differentiation.

To investigate whether the enhancement of Th17 cell differentiation was the result of Treg 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 Th10 or Treg (TGF-β) polarizing conditions. There were no significant differences between the FOXP3+ CD4+ T cell populations of WT and iNOS−/− mice under Treg inducing conditions (Fig. 3 A). In addition, production of IL-10, another inhibitory cytokine, was comparable in WT and iNOS−/− Th17 cells (Fig. 3 B). Thus, the enhanced Th17 cell differentiation in iNOS−/− mice was not a result of the alterations of either TGF-β-derived Treg or IL-10 production.

### iNOS is induced in activated CD4+ T cells

The enhanced Th17 cell differentiation of iNOS−/− T cells cultured under Th17 polarizing conditions prompted us to think that iNOS expression in T cells could be responsible for reduced Th17 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 Th10 or Th17 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 B). 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 µg/ml LPS for 24 and 48 h and Western blotting was performed. The results
were primed in vitro for 3 d under T<sub>H</sub>0 or T<sub>H</sub>17 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<sup>+</sup>T cells from eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup> CD4<sup>+</sup>T cell cultures were comparable to cells from WT cell cultures (unpublished data). CD4<sup>+</sup>T cells developed normally in eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup> mice (unpublished data). These results suggest that iNOS is expressed in activated T cells and may play a role in T<sub>H</sub>17 cell differentiation.

Figure 4. iNOS is expressed in activated CD4<sup>+</sup>T cells. (A) Naive CD4<sup>+</sup>T cells from WT and iNOS<sup>−/−</sup> 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<sup>+</sup>T cells were then purified and iNOS expression was determined by Western blotting. (B) Naive CD4<sup>+</sup>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<sup>+</sup>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<sup>+</sup>T cells from WT and iNOS<sup>−/−</sup> 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<sup>+</sup>T cells did not express iNOS protein at all (unpublished data). These results suggest that NO derived from iNOS expressed by activated CD4<sup>+</sup>T cells plays a negative role in T<sub>H</sub>17 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<sub>H</sub>17 cell differentiation. Naive CD4<sup>+</sup>T cells from eNOS<sup>−/−</sup>, nNOS<sup>−/−</sup>, and WT mice were primed in vitro for 3 d under T<sub>H</sub>0 or T<sub>H</sub>17 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<sup>+</sup>T cells from eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup> CD4<sup>+</sup>T cell cultures were comparable to cells from WT cell cultures (unpublished data). CD4<sup>+</sup>T cells developed normally in eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup> mice (unpublished data). These results suggest that iNOS is expressed in activated T cells and may play a role in T<sub>H</sub>17 cell differentiation.
Figure 5. NO suppresses Th17 cell differentiation. (A) Naive CD4+ T cells from B6 mice were differentiated under Th17 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 ± SD from three independent experiments. *, P < 0.05 versus cells added with L-NIL. (B) The cells prepared in A were differentiated under Th17 polarizing conditions in the presence of SNAP (10, 100, or 200 µM) 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 ± SD from three independent experiments. *, P < 0.05 versus Th17 cells only. The results are representative of three independent experiments. (D) Naive CD4+ T cells from iNos−/− mice were differentiated under Th17 polarizing conditions in the presence of 0.5 mM L-NIL or 100 µM 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<sub>17</sub> cell differentiation

Experiments with iNOS<sup>−/−</sup> mice indicated that NO may be involved in the negative regulation of T<sub>17</sub> 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<sup>+</sup> T cells cultured under T<sub>17</sub> conditions. Naive CD4<sup>+</sup> T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under T<sub>17</sub> 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<sup>+</sup> T cell cultures (Fig. 5 A), which mimicked the iNOS<sup>−/−</sup> CD4<sup>+</sup> 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<sup>+</sup> 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<sub>17</sub> cell differentiation in both WT and iNOS<sup>−/−</sup> cell cultures, whereas L-NIL had no significant effect on the percentage of IL-17–producing cells and IL-17 protein release in iNOS<sup>−/−</sup> CD4<sup>+</sup> 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<sup>+</sup> T cells in WT T cell cultures but not in iNOS<sup>−/−</sup> T cell cultures (Fig. 5 E). To test whether L-NIL and SNAP affects T<sub>17</sub> or T<sub>12</sub> cell differentiation, naive CD4<sup>+</sup> T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under T<sub>17</sub> or T<sub>12</sub> conditions in the presence of 0.5 mM L-NIL or 50 µM SNAP. We did not find a significant effect of L-NIL or SNAP on T<sub>17</sub> or T<sub>12</sub> 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<sub>17</sub> cell differentiation.

NO suppresses RORγt-mediated IL-17 transcription

The above findings prompted us to probe the molecular basis for NO control of T<sub>17</sub> cell differentiation. Because many studies have demonstrated a critical role for RORγt in T<sub>17</sub> cell differentiation both in vitro and in vivo (Ivanov et al., 2006), we asked if NO might affect RORγt expression, resulting in the control of T<sub>17</sub> cell differentiation. First we examined RORγt expression in iNOS-deficient CD4<sup>+</sup> T cells. Naive CD4<sup>+</sup> T cells from iNOS<sup>−/−</sup> or WT littermate mice were primed in vitro for 3 d under T<sub>17</sub> or T<sub>12</sub> polarizing conditions. The cells were then restimulated with PMA/ionomycin and examined for the percentages of RORγt-positive cells by intracellular staining using flow cytometry. Interestingly, the percentage of RORγt-positive cells in iNOS<sup>−/−</sup> CD4<sup>+</sup>

Figure 6. NO suppresses T<sub>17</sub> cell differentiation. Naive CD4<sup>+</sup> T cells from WT or iNOS<sup>−/−</sup> mice were differentiated under T<sub>17</sub> polarizing conditions in the presence of 200 µM 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<sup>+</sup> cells and the percentages of IL-17–producing CD4<sup>+</sup> 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 RORγt, STAT3, IRF4, and AHR protein were similar in iNOS<sup>−/−</sup> and WT mice (Fig. 7 B). These results suggest that enhanced T<sub>17</sub> cell differentiation in iNOS<sup>−/−</sup> mice is not the result of a change in RORγt expression at the protein level. To further assess whether NO might directly affect RORγt protein expression, we transfected RORγt into 293T cells treated with different concentrations of SNAP for 40 h and then examined RORγt expression by Western blotting. The results showed that the treatment with SNAP did not affect the levels of RORγt expression (Fig. 7 C) or nuclear translocation (Fig. 7 C).

We then proceeded to analyze whether NO modulates posttranslational modification of RORγt protein. First, we considered if NO affects RORγt ubiquitination. To test this, we cotransfected T7-RORγt 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-RORγt and HA-ubiquitin. SNAP treatment had no significant effect on
the pattern of ubiquitination (unpublished data), suggesting that RORγt ubiquitation 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 T17-polarizing 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−/− cells (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 T17-polarizing conditions. We found that anti-nitrotyrosine antibody coimmunoprecipitated RORγt from lysates of T17-cells (Fig. 8 A), suggesting that tyrosine residues of RORγt were nitrated under T17 conditions. To confirm these results, 293T cells were transfected with T7-RORγt plasmid for 40 h. Cell lysates were immunoprecipitated with anti-nitrotyrosine antibody and analyzed by Western blotting. Representative FACS dot plots are gated on CD4+ cells. (E) Naive CD4+ T cells from WT mice were cultured under T17-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 ± SD from three independent experiments.
Thus, tyrosine residues in the region from residue 169 to 491 of RORγt 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 NAPDH p47-phox (unpublished data), as reported previously (Jackson et al., 2004). To examine the effect of NO on RORγt at the functional level, we cotransfected an IL-17 promoter reporter and RORγt expression plasmids into 293T cells in the presence of various doses of SNAP for 40 h, suggesting that NO-induced alterations of tyrosine residues may affect RORγt activation. To analyze which part of RORγt is sensitive to NO, we generated three T7-tagged RORγt truncation mutants (Δ5–166, Δ169–491, and Δ342–491). 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 RORγt mutant Δ5–166 (Fig. 8 C) but clearly increased nitrotyrosine levels of RORγt truncation mutants Δ169–491 and Δ342–491 (Fig. 8 C). Thus, tyrosine residues in the region from residue 169 to 491 of RORγt 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 NAPDH p47-phox (unpublished data), as reported previously (Jackson et al., 2004). To examine the effect of NO on RORγt at the functional level, we cotransfected an IL-17 promoter reporter and RORγt expression plasmids into 293T cells in the presence of various doses of SNAP for.

Figure 8. NO suppresses RORγt-mediated IL-17 transcription. (A) Naive CD4+ T cells from B6 mice were cultured under Th0 or Th17-polarizing conditions for 60 h and the cell lysates were then immunoprecipitated with an anti-nitrotyrosine antibody and blotted with anti-RORγt. Data are representative of three independent experiments. (B) 293T cells were transfected with T7-tagged RORγt plasmid for 40 h in the presence of 100 µM SNAP. Cell lysates were immunoprecipitated with anti-nitrotyrosine antibody and immunoblotted with anti-T7 antibody. (C) 293T cells were transfected with T7-tagged RORγt truncation mutant plasmids (Δ5–166, Δ169–491, and Δ342–491) for 40 h in the presence of 100 µM 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 RORγt plasmid in the presence of SNAP (50, 100, or 200 µM) or 293T cells were transfected with an IL-17A promoter reporter construct and WT RORγt or different RORγt mutant plasmids for 30 h. Luciferase assays were performed and luciferase activities were normalized to β-galactosidase activity. Data indicate mean ± SD of triplicate cultures and are representative of three independent experiments.
Figure 9. iNOS deficiency promotes the Th17 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 ± 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 ± 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 µm. (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 ± 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 RORγt-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 RORγt, corresponding to Tyr369 and Tyr382 of human RORγt, that are important for ligand binding (Fig. S1, A and B). We found that mutating these two tyrosine residues significantly impaired RORγt-mediated IL-17 promoter activation, suggesting that Tyr346 and Tyr359 are critical for RORγt 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 RORγt.

iNOS regulates T\textsubscript{h}17 cell differentiation in vivo

Accumulating evidence indicates that T\textsubscript{h}17 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\textsubscript{h}17 cell development in vivo, we performed adoptive transfer experiments using CD4\textsuperscript{+}CD45R\textsuperscript{b} cells from WT and iNOS\textsuperscript{−/−} mice to induce colitis in Rag\textsuperscript{1}/− mice. Rag\textsuperscript{1}/− mice reconstituted with iNOS\textsuperscript{−/−} 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 Rag\textsuperscript{1}/− mice reconstituted with iNOS\textsuperscript{−/−} 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\textsuperscript{−/−} 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\textsubscript{h}17 immune response.

To further understand the role of iNOS in T\textsubscript{h}17 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\textsuperscript{−/−} mice with the myelin oligodendrocyte glycoprotein (MOG\textsubscript{35-55}) peptide in complete Freund’s adjuvant. iNOS\textsuperscript{−/−} 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\textsuperscript{+} T cells from WT but not from iNOS\textsuperscript{−/−} mice in these experiments (Fig. 10 B). We then examined the expression of T\textsubscript{h}17 and T\textsubscript{h}1 cytokines in EAE mice. Infiltrating CD4\textsuperscript{+} 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\textsuperscript{+} 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\textsubscript{h}17 cell differentiation in vivo.

DISCUSSION

T\textsubscript{h}17 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\textsubscript{h}17 cells will help to dissect mechanisms for the control of T\textsubscript{h}17 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 naïve CD4\textsuperscript{+} T cells polarized under T\textsubscript{h}17 condition led to more efficient T\textsubscript{h}17 cell differentiation without major effects on either the T\textsubscript{h}1 or T\textsubscript{h}2 cell lineages. In vivo, transfer of CD4\textsuperscript{+} CD45RB\textsuperscript{hi} cells into Rag\textsuperscript{1}−/− mice induced more severe colitis than transfer of control cells. In addition, mice reconstituted with iNOS\textsuperscript{−/−} T cells had a significantly higher percentage of IL-17–producing CD4\textsuperscript{+} 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\textsubscript{h}1 immune responses (Xiong et al., 2004). Huang et al. (1998) suggested that the enhanced T\textsubscript{h}1 immune responses in iNOS KO mice were a result of increased production of IL-12 by iNOS\textsuperscript{−/−} 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\textsuperscript{−/−} 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-κB 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 Thüring et al. (1995) did not find iNOS expression in T cells clones or T cells from naive and L. major–infected mice. In the present study, we clearly demonstrated
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In lymph nodes or Peyer’s patches and Th17 cell development is severely impaired, indicating that RORγt is a master transcription factor in Th17 cell differentiation (Ivanov et al., 2006). Interestingly, RORγt expression in iNOS−/− CD4+ T cells cultured under Th17 conditions was comparable to WT CD4+ T cell cultures, implying that the enhanced Th17 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 derived from iNOS in activated T cells controls T cell differentiation by selectively suppressing Th17 cell development. The importance of RORγt in Th17 cell development has been well studied in mice. RORγt−/− mice fail to develop lymph nodes or Peyer’s patches and Th17 cell development.

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.
Cells were stimulated with IFN-γ and IL-17 expression was significantly higher in 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 H17 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 RORγt 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-Nos2tm1Unc/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-Nos3tm1Lun/J, stock#002684) and iNOS−/− mice (B6.129S4-Nos1tm1H2/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), NO2 (6/iNOS/NO type II), 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), RORγ (B2D), IL-4 (11B11), IL-10 (JES-5D1), and Foxp3 (FJK-16S) were purchased from eBioscience. Antibody for AHR (BML-SA230) 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−) 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 μg/ml anti-CD3 (145-2C11; BD) and 2 μg/ml of soluble anti-CD28 (37.51; BD). The cells were rested for 48 h and 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 0 cells. Cells were stimulated to differentiate into T H17 cells by supplementing with IL-12 plus anti–IL-4 or into T H17 cells by supplementation with IL-4 and anti–IFN-γ. For T H17 cell differentiation, cells were stimulated with 5 ng/ml TGF-β1, 20 ng/ml IL-6, 10 ng/ml and IL-23 (all from R&D Systems) in the presence of 10 μg/ml anti–IL-4 antibody (11B11; BD) and 10 μg/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′-CCTCAGAGGCGCCTCAGACTAC-3′; IL-17a antisense, 5′-AGGTTCTTTCTCCGCCATTGACACAG-3′; IL-21 sense, 5′-CGCCTCTTGATTAGATCGTCG-3′; IL-21 antisense, 5′-GCCCCCTTCATCTTGGTGA-3′; RORγt sense, 5′-CCGCTGAGAGGCTTCTCAC-3′; RORγt antisense, 5′-TGGCAGAGTTGCCACATTACA-3′; iNos sense, 5′-CCGAAGCAACACATCAGATTCA-3′; iNos antisense, 5′-GGCTTAAAGGCCTGGCGGCT-3′; ubiquitin sense, 5′-TGCTTATTAGATTCCGTCGCA-3′; and ubiquitin antisense, 5′-GCAGTGCTACGTAAGGCGGCTAAA-3′.

Transfection and luciferase reporter assay. 293T cells were transiently transfected with an IL-17 promoter luciferase reporter plasmid together with RORγt in the presence of SNAP at different concentrations. For each transfection, 2.0 µg of plasmid was mixed with 100 µl DMEM (without serum and antibiotics) and 4.0 µl 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 µg/ml anti-CD3 and 2 µg/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 µl 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 supernatants were electrophoresed on Criterion 4%–12% SDS-PAGE gels. Proteins were transferred to a nitrocellulose membrane and were blocked with 5% BSA in TBS containing 0.1% Tween-20 at 4°C overnight. Membranes were incubated with primary antibodies (anti-iNOS, anti-T7, and anti-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 (Poirier et al., 1993; Totuka et al., 2007). In brief, purified CD4+CD45RB+ T cells from WT and iNos−/− mice were injected intraperitoneally into Rag1−/− recipients (5 × 106 cells per mouse in 200 µl sterile PBS per injection). Mice were weighed every week throughout the course of experiments. After 5 wk, mice were sacrificed and colon tissues were excised. Tissues were fixed in 10% buffered formalin and paraffin embedded. The sections (5 µm) 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 submucosalis propria was scored separately as described previously (Totuka 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, Tgfβ17 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 µg of anti-RORγt (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 2000× 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 µl emulsion containing 100 µg myelin oligodendrocyte glycoprotein (MOG)35-55 peptide (MEGWSYRSPFSRVRHVLYRNGK), 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 ~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 RORγt. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122494/DC1.

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