IL-17–producing T helper cells (TH17) are a recently identified T helper cell subset, which is clearly distinct from TH1 and TH2 cells. TH17 cells mediate proinflammatory and autoimmune responses through the production of TH17 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 TH17 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 TH17 cell populations (Bettelli et al., 2006; Mangan et al., 2006; Weaver et al., 2006). RORγt, a member of the orphan nuclear receptor family, has been identified as the master transcription factor for TH17 cell development (Ivanov et al., 2006). Other transcription factors, including RORα, STAT3, IRF4, and IRF8, are also involved in the control of TH17 cell differentiation (Ouyang et al., 2011). In addition, the differentiation of TH17 cells is also regulated by intrinsic programs for the control of TH17 immune responses.

RORγt is necessary for the generation of TH17 cells but the molecular mechanisms for the regulation of TH17 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 TH17 cell differentiation but without major effects on either TH1 or TH2 cell lineages, whereas endothelial NOS (eNOS) or neuronal NOS (nNOS) mutant mice showed comparable TH17 cell differentiation compared with wild-type control mice. The addition of N6-(1-iminoethyl)-l-lysine dihydrochloride (L-NIL), the iNOS inhibitor, significantly enhanced TH17 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 RORγt, leading to the suppression of RORγt–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 TH17 phenotype. These results suggest that NO derived from iNOS in activated T cells plays a negative role in the regulation of TH17 cell differentiation and highlight the importance of intrinsic programs for the control of TH17 immune responses.
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 Th17 lineage commitment.

There is increasing evidence that Th17 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 Th17 immune responses should provide insights into the pathogenesis and treatment of these and possibly other inflammatory diseases. Although the activation program for Th17 cell differentiation has been well established, the intrinsic down-regulation for Th17 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 Th17 immune responses by regulating IL-12 expression.

Figure 1. Enhanced Th17 cell differentiation in iNOS-deficient mice. (A) Naive CD4+ T cells from WT or iNOS−/− mice were differentiated under Th0 and Th17 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 \( \text{Rag1}^{-/-} \) 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

\( \text{iNOS}^{-/-} \) 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 \( \text{Th}_{17} \) cell differentiation while exhibiting no significant effects on \( \text{Th}_{1} \) or \( \text{Th}_{2} \) 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 \( \text{iNOS}^{-/-} \) T cell cultures. In addition, the tyrosine residues of ROR\( \gamma \)-t were nitrated resulting in the inhibition of ROR\( \gamma \)-t-mediated IL-17 promoter activation.

Figure 2. \( \text{Th}_{1} \) and \( \text{Th}_{2} \) differentiation in \( \text{iNOS}^{-/-} \) CD4+ T cells. (A) Naive CD4+ T cells from WT or \( \text{iNOS}^{-/-} \) mice were differentiated under \( \text{Th}_{1} \) or \( \text{Th}_{2} \) conditions for 3 d. Cells were then restimulated with PMA/ionomycin for 5 h and stained for intracellular IFN-\( \gamma \) or IL-4 by flow cytometry. Each bar represents mean \( \pm \) SD from three independent experiments. (B) Naive CD4+ T cells from spleens and lymph nodes of WT and \( \text{iNOS}^{-/-} \) mice were prepared and the cells were activated with anti-CD3 and anti-CD28 antibodies for 3 d. \( [\text{H}] \)-Thymidine was added during the last 8 h of culture. Then the cells were collected and counted with a \( \beta \)-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 \( \pm \) SD from two independent experiments, performed in triplicate.

In addition, \( \text{iNOS}^{-/-} \) 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 \( \text{Th}_{17} \) cell differentiation while exhibiting no significant effects on \( \text{Th}_{1} \) or \( \text{Th}_{2} \) 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 \( \text{iNOS}^{-/-} \) T cell cultures. In addition, the tyrosine residues of ROR\( \gamma \)-t were nitrated resulting in the inhibition of ROR\( \gamma \)-t-mediated IL-17 promoter activation. Transfer of naive T cells from iNOS-deficient mice induced more severe colitis in \( \text{Rag1}^{-/-} \) mice than T cells from normal controls. These findings suggest that NO plays a critical suppressive role in the control of \( \text{Th}_{17} \) differentiation and highlight the importance of T cell–derived iNOS in switching off \( \text{Th}_{17} \)-dependent immune responses.

RESULTS

\( \text{iNOS} \) deficiency enhances \( \text{Th}_{17} \) cell differentiation

To investigate the function of NO in \( \text{Th}_{17} \) cell differentiation, we first assessed the characteristics of CD4+ T cells from \( \text{iNOS}^{-/-} \) or WT control mice. Naive CD4+ T cells from \( \text{iNOS}^{-/-} \) T cell cultures were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 3 d. T cell proliferation was analyzed by flow cytometry. Data represent mean \( \pm \) SD from two independent experiments, performed in triplicate.

In the present study, we show that mice deficient in iNOS exhibited enhanced \( \text{Th}_{17} \) cell differentiation while exhibiting no significant effects on \( \text{Th}_{1} \) or \( \text{Th}_{2} \) 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 \( \text{iNOS}^{-/-} \) T cell cultures. In addition, the tyrosine residues of ROR\( \gamma \)-t were nitrated resulting in the inhibition of ROR\( \gamma \)-t-mediated IL-17 promoter activation. Transfer of naive T cells from iNOS-deficient mice induced more severe colitis in \( \text{Rag1}^{-/-} \) mice than T cells from normal controls. These findings suggest that NO plays a critical suppressive role in the control of \( \text{Th}_{17} \) differentiation and highlight the importance of T cell–derived iNOS in switching off \( \text{Th}_{17} \)-dependent immune responses.
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 (unpublished data). To rule out the possibility that the enhanced T\(_{H17}\) cell differentiation in iNOS\(^{-/-}\) mice was not a result of the alterations of either TGF-\(\beta\)-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\(_{H17}\) 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-iNOS 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 protein was induced in 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 \(\mu\)g/ml LPS for 24 and 48 h and Western blotting was performed. The results

![Figure 3.](image)

There were 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 ± 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 ± SD from three independent experiments.
were primed in vitro for 3 d under T\textsubscript{H0} or T\textsubscript{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\textsuperscript{+} T cells from eNOS\textsuperscript{−/−} and nNOS\textsuperscript{−/−} CD4\textsuperscript{+} T cell cultures were comparable to cells from WT cell cultures (unpublished data). These results suggest that iNOS is expressed in activated T cells and may play a role in T\textsubscript{H17} cell differentiation.

demonstrated that macrophage cell line 264.7 cells expressed high level of iNOS protein after LPS stimulation, whereas LPS-stimulated CD4\textsuperscript{+} T cells did not express iNOS protein at all (unpublished data). These results suggest that NO derived from iNOS expressed by activated CD4\textsuperscript{+} T cells plays a negative role in T\textsubscript{H17} cell differentiation.

Because T\textsubscript{H17} 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\textsubscript{H17} cell differentiation. Naive CD4\textsuperscript{+} T cells from eNOS\textsuperscript{−/−}, nNOS\textsuperscript{−/−}, and WT mice were primed in vitro for 3 d under T\textsubscript{H0} or T\textsubscript{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\textsuperscript{+} T cells from eNOS\textsuperscript{−/−} and nNOS\textsuperscript{−/−} CD4\textsuperscript{+} T cell cultures were comparable to cells from WT cell cultures (unpublished data). CD4\textsuperscript{+} T cells developed normally in eNOS\textsuperscript{−/−} and nNOS\textsuperscript{−/−} mice (unpublished data). These results suggest that iNOS is expressed in activated T cells and may play a role in T\textsubscript{H17} cell differentiation.

**Figure 4. iNOS is expressed in activated CD4\textsuperscript{+} T cells.** (A) Naive CD4\textsuperscript{+} T cells from WT and iNOS\textsuperscript{−/−} 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\textsuperscript{+} T cells were then purified and iNOS expression was determined by Western blotting. (B) Naive CD4\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} T cells from WT and iNOS\textsuperscript{−/−} 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.
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 Th17 cell differentiation

Experiments with iNOS−/− mice indicated that NO may be involved in the negative regulation of Th17 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 Th17 conditions. Naive CD4+ T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under Th17 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 Th17 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 affect Th1 or Th2 cell differentiation, naive CD4+ T cells from spleens and lymph nodes of WT mice were activated in vitro for 3 d under Th1 or Th2 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 Th1 or Th2 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 Th17 cell differentiation.

NO suppresses RORγt-mediated IL-17 transcription

The above findings prompted us to probe the molecular basis for NO control of Th17 cell differentiation. Because many studies have demonstrated a critical role for RORγt in Th17 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 Th17 cell differentiation. First we examined RORγt 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 Th0 or Th17 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−/− CD4+ 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−/− and WT mice (Fig. 7 B). These results suggest that enhanced Th17 cell differentiation in iNOS−/− 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 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 Th17-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−/− 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 coinmunoprecipitation experiments using cell lysates from primary CD4+ T cells cultured under Th17-polarizing conditions. We found that anti-nitrotyrosine antibody coinmunoprecipitated RORγt from lysates of Th17 cells (Fig. 8 A), suggesting that tyrosine residues of RORγt were nitrated under Th17 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...
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 9. iNOS deficiency promotes the Th17 immune responses in experimental colitis. CD4+CD45RBhi T cells were purified from spleens and lymph nodes of WT or iNOS−/− mice and 5 × 10⁵ 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+CD45RBhi 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−/− cells), and sections of colons with colitis (D) from Rag1−/− mice (n = 5–6 mice in each group) on day 35 after naive 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.
Published June 24, 2013

examined the expression of TH17 and TH1 cytokines in EAE (MOG35-55) peptide in complete Freund’s adjuvant.

...IFN-γ cells than control mice (Fig. 8 E), whereas the percentage of sections from mice reconstituted with T cells from WT mice significantly higher pathological scores than those observed in sections from mice reconstituted 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 T cells 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 T17 cell differentiation in vivo.

**DISCUSSION**

T17 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 T17 cells will help to dissect mechanisms for the control of T17 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 T17 condition led to more efficient T17 cell differentiation without major effects on either the T17 or T12 cell lineages. In vivo, transfer of CD4+ CD45RBhi 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 T17 immune responses (Xiong et al., 2004). Huang et al. (1998) suggested that the enhanced T17 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-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

iNOS regulates T17 cell differentiation in vivo

Accumulating evidence indicates that T17 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 T17 cell development in vivo, we performed adoptive transfer experiments using CD4+CD45Rbhi 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 T17 immune response.

To further understand the role of iNOS in T17 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 naive T cells polarized under TH17 condition led to more efficient T17 cell differentiation without major effects on either the T17 or T12 cell lineages. In vivo, transfer of CD4+ CD45RBhi 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.

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that iNOS expressed by activated CD4+ T cells negatively regulates T_{H}17 cell differentiation. The results are consistent with recent studies showing that the NO donors NOC-18 and S-nitrosoglutathione suppressed T_{H}17 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_{H}17 cell development.

The importance of RORγt in T_{H}17 cell development has been well studied in mice. RORγt^{−/−} mice fail to develop lymph nodes or Peyer’s patches and T_{H}17 cell development is severely impaired, indicating that RORγt is a master transcription factor in T_{H}17 cell differentiation (Ivanov et al., 2006). Interestingly, RORγt expression in iNOS^{−/−} CD4+ T cells cultured under T_{H}17 conditions was comparable to WT CD4+ T cell cultures, implying that the enhanced T_{H}17 cell differentiation observed is not the result of increased RORγt protein levels. Instead, we found that the NO donor SNAP suppressed RORγt-mediated IL-17 promoter activation in a dose-dependent manner, suggesting that NO...
may control RORγt activity during IL-17 gene transcription. How might NO control RORγt activity? NO has been shown to directly affect the activity of many proteins via tyrosine nitration. We have clearly shown that tyrosine residues in RORγt are nitrated and that such nitration of RORγt significantly impaired the binding of RORγt to the promoter region of the IL-17 gene resulting in the inhibition of IL-17 transcription. Our RORγt 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 RORγt 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 RORγt ligand binding activity and formation. Our further mutation experiments demonstrated that Tyr346 and Tyr359 of mouse RORγt, corresponding to Tyr369 and Tyr382 residues of human RORγt, are critical for RORγt transcriptional activation. Thus, the results suggest that Tyr346 and Tyr359 could be the target for NO on RORγt transcriptional function. Recently, Niedbala et al. (2011) reported that NO donor S-nitrosylation of p65, which limits its binding activity. Our further mutation experiments demonstrated that Tyr346 and Tyr359 of mouse RORγt, corresponding to Tyr369 and Tyr382 residues of human RORγt, are critical for RORγt transcriptional activation. Thus, the results suggest that Tyr346 and Tyr359 could be the target for NO on RORγt transcriptional function. Recently, Niedbala et al. (2011) reported that NO donor S-nitrosylation of p65, which limits its binding activity. Our further mutation experiments demonstrated that Tyr346 and Tyr359 of mouse RORγt, corresponding to Tyr369 and Tyr382 residues of human RORγt, are critical for RORγt transcriptional activation. Thus, the results suggest that Tyr346 and Tyr359 could be the target for NO on RORγt transcriptional function. Recently, Niedbala et al. (2011) reported that NO donor

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, microglias, 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/6 J (B6, stock #000664) and iNOS-deficient mice (B6.129P2-Nos1tm2pir/J, stock #002684) and iNOS−/− mice (B6.129S4-Nos1tm1Wj, 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 (L243), CD8 (53–6.7), CD3e (145-2C11), CD25 (PC61.3), CD44 (IM7), CD62L (MECA-367), CD45RB (30H11), 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 (JE6-6A12), RORγt (B2D), IL-4 (11B11), IL-10 (JES5-16E3), and Foxp3 (FJK-16S) were purchased from eBioscience. Antibodies for AHR (BML-SA210) was purchased from Enzo Life Sciences.

CD4+ T cell preparation and differentiation in vitro. Naive CD4+ T cells (CD62L+CD44hi) 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 restimulated for 48 h and were then restimulated for 5 h with PMA plus ionomycin in the presence of brefeldin A before intracellular staining. Cells were fixed from Santa Cruz Biotechnology, Inc.

Intracellular staining and flow cytometry. Cells were primed 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

Published June 24, 2013
RNA isolation and quantitative real-time RT–PCR (qPCR). Total RNA was extracted using an RNaseasy 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’-CTCCAGAACGGGCTCTCAGACTAC-3’; IL-17a antisense, 5’-GGTCTAAAGGCTCCGGGCT-3’; RORγt sense, 5’-CGGCCTTAGACCACTTGGTGGA-3’; RORγt antisense, 5’-GGCTTGAAGCGGTCCTAC-3’; RORγt sense, 5’-TGCGAGTGGGCCACATTACA-3’; iNos sense, 5’-CCGAACGCAAACACTCACATTACA-3’; iNos antisense, 5’-GCTTTAAACGCCTGGGCT-3’; ubiquitin sense, 5’-TGCTTATTATTTCGCTGCA-3’; and ubiquitin antisense, 5’-GCAAAGTGCTGAGCTGAGAT-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 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-actin (Sigma-Aldrich) antibodies were used according to the manufacturers’ instructions. Secondary antibodies were from Santa Cruz Biotechnology, Inc.

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.

We express our thanks to Dr. Feng Hong for technical support and Mr. Zihan Zheng for reading of our manuscript.

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