Published in final edited form as:
J Immunol. 2013 July 1; 191(1): 164–170. doi:10.4049/jimmunol.1202580.

Nitric Oxide-induced regulatory T cells inhibit Th17 but not Th1 cell differentiation and function

Wanda Niedbala[*], **Anne-Gaelle Besnard**[*], **Hui R Jiang**[*,§], **Jose C Alves-Filho**[*,+], **Sandra Y Fukada**[*,+], **Daniela Nascimento**[*,+], **Akio Mitani**[*,¶], **Peter Pushparaj**[*,ξ], **Mohammed H Alqahtani**[ξ], and **Foo Y Liew**[*]

[*]Centre of Immunology, Institute of Infection, Immunity and Inflammation, University of Glasgow, 120 University place, Glasgow G12 8TA

[§]Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde, Glasgow, UK

[+]Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, 14049-900, Ribeirão Preto, Brazil

[¶]Department of Periodontology, School of Dentistry, Aichi Gakuin University, Nagoya 464-8651, Japan.

[ξ]CEGMR, King Abdulaziz University, Jeddah, Saudi Arabia.

Abstract

Nitric oxide (NO) is a free radical with pleiotropic functions. We have shown earlier that NO induces a population of $CD4^+CD25^+Foxp3^-$ regulatory T cells (NO-Tregs) which suppress the functions of $CD4^+CD25^-$ effector T cells in vitro and in vivo. We report here an unexpected finding that NO-Tregs suppressed Th17 but not Th1 cell differentiation and function. In contrast, natural Tregs (nTregs), which suppressed Th1 cells, failed to suppress Th17 cells. Consistent with this observation, NO-Tregs inhibited the expression of RORγt but not T-bet, whereas nTregs suppressed T-bet, but not RORγt expression. The NO-Tregs-mediated suppression of Th17 was partially cell-contact-dependent and was associated with IL-10. In vivo, adoptively transferred NO-Tregs potently attenuated experimental autoimmune encephalomyelitis (EAE). The disease suppression was accompanied by a reduction of Th17, but not Th1 cells in the draining lymph nodes, and decrease in the production of IL-17, but increase in IL-10 synthesis. Our results therefore demonstrate the differential suppressive function between NO-Tregs and nTregs and indicate specialization of the regulatory mechanism of the immune system.

Introduction

Nitric oxide (NO) mediates a variety of biological functions, including vascular relaxation, platelet aggregation, neurotransmission, microbicidal, tumoricidal and immune regulation (1-5). As such NO is associated with some of the most important immunopathologies such as rheumatoid arthritis (RA), diabetes, systemic lupus erythematosus (SLE), multiple sclerosis (MS) and septic shock (6-10). NO is derived from the guanidino nitrogen atom(s) and molecular oxygen in a reaction catalyzed by the enzyme nitric oxide synthase (NOS).

Address correspondence to: Foo Y Liew or Wanda Niedbala, Centre of Immunology, Institute of Infection, Immunity and Inflammation, University of Glasgow, 120 University Place, Glasgow G12 8TA, UK, foo.liew@glasgow.ac.uk. wanda.niedbala@glasgow.ac.uk. Tel: +44 131 330 8411, Fax: +44 141 330 4297..

The authors declare no conflict of financial interest

Europe PMC Funders Author Manuscripts

There are three forms of NOS: the neuronal form (nNOS or NOS1), the inducible form (iNOS or NOS2) and the endothelial derived form (eNOS or NOS3). NOS1 and NOS3 produce amounts of NO required for physiological functions. NOS2, normally exists at a low level, is activated by a number of immunological stimuli such as IFNγ, TNF-α and LPS, and catalyzes the high output of NO, which can be cytotoxic and kill intracellular pathogens.

We have previously shown that low dose of NO selectively enhances Th1 cell differentiation and expansion under Th1 polarizing conditions. This was mediated by enhancing the expression of IL-12 receptor (IL-12R) β2 through a cGMP-dependent pathway (11). We have also reported that NO can induce a subset of $CD4^+CD25^+Foxp3^-$ regulatory T cells (NO-Tregs) from $CD4^+CD25^-$ T cells via p53, IL-2 and OX40 in a cGMP-independent manner (12). NO-Tregs suppress the proliferation and expansion of $CD4^+CD25^-$ effector cells in vitro and markedly attenuate the effector cell-mediated colitis and collagen-induced arthritis in the mouse in an IL-10-dependent manner. More recently, we reported that NO can also directly suppress the differentiation and function of polarized human and mouse Th17 cells via the down-regulation of the aryl hydrocarbon receptor leading to attenuation of experimental autoimmune encephalomyelitis (EAE) (13). These results therefore indicate that NO is a key player in the modulation of inflammatory disease. To further explore the role of NO in immune regulation, we investigated the potential role of NO-Tregs in affecting the differentiation and function of different subsets of T-helper (Th) cells, particularly Th17 and Th1 cells.

Th17 cells are now defined as a distinct subset of Th cells that produce IL-17 with a signature transcription factor RORγt (retinoid-related orphan receptor γt). Th17 cells are associated with pathogenesis of human autoimmune diseases including MS, RA, psoriasis and inflammatory bowel disease (14-17). Thus there likely exist rigorous endogenous control mechanisms to limit Th17 proliferation and function.

We report here that NO-Tregs effectively suppress the differentiation and expansion of Th17 but not Th1 cells. Unexpectedly, we found that natural Tregs (nTregs) while effective in suppressing Th1 are relatively ineffective in suppression of Th17. NO-Tregs suppress Th17 via a cell contact-dependent mechanism and also partially via IL-10. In vivo, NO-Tregs markedly attenuate EAE in association with the reduction of Th17 but not Th1. Together these results support a key role of NO in the regulation of immune system, and further indicate compartmentalization of the regulatory mechanism in inflammatory disease.

Materials and Methods

Mice and reagents

Wild-type C57BL/6 and BALB/c mice were purchased from Harlan Olac. *Il10*$^{-/-}$ mice of the C57BL/6 background were obtained from the National Institute of Medical Research, Mill Hill, London, UK. Males and females mice were used at the age of 6-10 weeks. All animal experiments were performed according to the University of Glasgow guidelines and the UK Home Office regulations. NOC-18 {(Z)-1-[2-(2-Aminoethyl)-N-(2-ammononioethyl) amino]diazen-1-ium-1,2-diolate}, also known as DETA-NONOate, was purchased from Enzo Life Sciences, UK. Anti-CTLA4 was purchased from eBioscience.

Cell culture

$CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were purified from the pool of spleens and lymph nodes of naive mice by negative selection (routine purity >98%) using an AutoMacs (Miltenyi Bioscience). Culture medium was RPMI-1640 supplemented with 10% (vol/vol) FCS (LONZA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 M 2-mercaptoethanol. To obtain NO-Tregs, $CD4^+CD25^-$ T cells were cultured in medium at

2.5×10^4 cell/200 μl in 96-well round-bottom plate (Nunc) with soluble anti-CD3 (1 μg/ml, R&D System) and equal numbers of APC (antigen-presenting cells, mitomycin-C-treated spleen cells) for 5-6 days in the presence of 200 μM of NOC-18 added at the beginning of culture. The cells were then harvested, washed and added at graded numbers to other cell cultures as indicated. For Th17 cell differentiation, $CD4^+$ T cells (5×10^5 cells/ml) were cultured in round-bottom 96-well plates with equal numbers of APC and soluble anti-CD3 (1 μg/ml), TGF-β (1 ng/ml), IL-6 (10 ng/ml), IL-1β (10 ng/ml), anti-IFNγ (10 μg/ml) and anti IL-4 (10 μg/ml) for up to 5 days. For Th1 cell differentiation, $CD4^+$ T cells were cultured in round bottom 96-well plates with equal numbers of APC in the presence of soluble anti-CD3 (1 μg/ml), IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) for 4 days. For Th2 cell differentiation, $CD4^+$ T cells (5×10^5 cells/ml) were cultured in 96-well round-bottom plates with equal number of APC, anti-CD3 (1 μg/ml), IL-4 (10 ng/ml) and anti-IFNγ (10 μg/ml) for 4 days. For Th9 cell differentiation, $CD4^+$ T cells (5×10^5 cells/ml) were cultured for up to 6 days in flat-bottom 96-well plates with plate-bound anti-CD3 (3 μg/ml) and soluble anti-CD28 (1.5 μg/ml), TGF-β (5 ng/ml) and IL-4 (10 ng/ml). For suppression assay, NO-Tregs or nTregs were added at the beginning of the polarization culture or established T cells at various Tregs to effector cell ratios. The mixed cell populations were cultured for up to 6 days in 96-well round bottom plates. In some experiments, NO-Tregs cells were treated with mitomycin-C before being added to the suppression culture. In other experiments, NO-Tregs were placed in the upper well and the effector cells in the bottom well of a transwell (Corning) culture. All cytokines and antibodies were obtained from R&D Systems. Supernatants were collected and cytokine concentrations were determined by ELISA, and the cells were harvested for FACS analysis. Cell division was determined by labeling $CD4^+$ T cells with CFSE (Molecular Probes, Inc.), and fluorescence density was determined by FACS.

EAE induction and clinical evaluation

C57BL/6 mice were immunized subcutaneously on the back with 100 μg of MOG_{35-55} peptide (Sigma Genosys) in 50 μl of PBS emulsified with an equal volume of CFA (total 125 μg of *Mycobacterium tuberculosis*, strain H37RA, Difco, Detroit MI). Each mouse also received intraperitoneally 100 ng/200 μl of Pertussis toxin (PTX, Sigma, UK) in PBS on days 0 and 2 post immunization. EAE was scored according to a 0 - 5 scale as follows: 0, no clinical sign; 1, complete loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb involvement; 5, moribund.

Histological and cellular evaluation of EAE

Mice were euthanized in CO_2 chamber and their spinal cords flushed out with PBS by hydrostatic pressure using a syringe attached to a G18 size needle; the brains were also harvested. For histology analysis, the tissues were then placed in 10% buffered formalin fixative overnight before transferred to 75% ethanol. Tissues were then embedded in paraffin wax. Spinal cord and brain sections were stained with standard haematoxylin and eosin (H&E). For FACS analysis, brains and spinal cords were cut into small pieces and digested with collagenase A (2 mg/mL, Roche Diagnostics) and DNase (1 mg/mL, Sigma-Aldrich) at 37°C for 40 min. Mononuclear cells were isolated by passing the tissue through a cell strainer (100 mm), followed by a Percoll gradient (70/30%) centrifugation. Mononuclear cells were removed from the interphase, washed, and resuspended in culture medium for further analysis. Draining lymph nodes (DLN) were also harvested, passed through a cell strainer and resuspended in culture medium for cellular analysis ex vivo by FACS.

FACS analysis

For intracellular cytokine staining, the cells were re-stimulated for 4 h with 50 ng/ml phorbol-12-myristate-13-acetate and 500 ng/ml ionomycin (both from Sigma) in the presence of GolgiStop (BD Bioscience). The cells were first stained for CD4, then permeabilized with Perm/Fix solution (eBioscience), and finally stained with anti-IL-17A, anti-IFNγ, anti-IL-4, anti-IL-9, anti-RORγt, anti-T-bet, or anti-GATA3 (all from eBioscience). Data were acquired using a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). Isotype-matched antibodies (directly conjugated) were used as controls.

ELISA

ELISAs were carried out with paired antibodies for murine IL-9, IL-10 and IL-17A and IFNγ according to the manufacturer's instructions (all from BD Bioscience). Sensitivity of the assays was <20 pg/ml.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0. Comparisons between 2 groups were performed using a 2-tailed unpaired Student's t test. Multiple groups were compared using a 2-way ANOVA followed by a Bonferroni's post-test. Values for all measurements are expressed as mean ± SEM. $p<0.05$ was considered statistically significant. All experiments were performed at least two times.

Results

NO-Tregs suppress the induction of Th17 cells

We first investigated the possibility that NO-Tregs may inhibit the induction of Th17 cells. $CD4^+CD25^-$ T cells were purified from the lymph node and spleen of BALB/c mice. The cells were polarized to NO-Tregs with soluble anti-CD3 antibody and mitomycin-C-treated spleen cells (as Antigen presenting cells, APC) in the presence of 200 μM of NOC-18 (a stable NO donor, 100 μM of NOC-18 constantly release 200 nM of NO with half life of 20 h) (18) as described previously (12). The cells were harvested on day 5 or 6 and checked for the NO-Tregs phenotype, which were $CD4^+CD25^+Foxp3^-$, producing IL-10 but not IFNγ, IL-4, IL-17 or IL-2 (ref. 12, and data not shown). NO-Tregs were washed and added at the beginning of the culture of polarization to Th17 cells. Naïve $CD4^+$ T cells were labeled with CFSE and cultured with APC, anti-CD3/CD28 plus TGF-β, IL-6, IL-1β, IL-23, anti-IFNγ and anti-IL-4 with graded $CD4^+$: NO-Tregs ratios. The replication of Th17 cells and the production of IL-17 were determined on day 4 by FACS and ELISA respectively. NO-Tregs inhibited the polarization of Th17 cells in a cell ratio-dependent manner (Fig. 1A, B). The inhibition of Th17 polarization was confirmed by the reduction in the concentration of IL-17A in the culture supernatant (Fig. 1C). Furthermore, NO-Tregs also inhibited the expression of RORγt, the signature transcription factor of Th17 (ref. 19) (Fig. 1D). These results indicate that NO-Treg may be potent regulatory T cells for Th17 development.

NO-Tregs suppress Th17 but not Th1 cells differentiation, whereas nTregs do the opposite

We next compared the suppressive function of NO-Tregs with nTregs. NO-Tregs were prepared as above and nTregs ($CD4^+CD25^+$) were purified from the spleen and LN of naïve BALB/c mice. $CD4^+$ T cells were labeled with CFSE and polarized to Th17 as above in the presence of NO-Tregs or nTregs for 4 days. While NO-Treg strongly suppressed the differentiation of Th17 cells, nTregs had only a modest effect (Fig. 2A). Furthermore, while NO-Tregs markedly inhibited the number of $IL\text{-}17^+ROR\gamma t^+$ T cells, nTregs did not (Fig. 2B). We then compared the suppressive effect of NO-Tregs and nTregs on Th1 cell

differentiation. Naïve $CD4^+$ T cells were polarized to Th1 cells in cultures containing anti-CD3/CD28 plus IL-12 and anti-IL-4 in the presence of NO-Tregs or nTregs. As expected nTregs potently suppressed the differentiation of $IFN\gamma^+$ T cells (Fig. 2C) and the expression of T-bet on these cells, the signature transcription factor of Th1. In contrast, NO-Tregs failed to affect the differentiation of Th1 cells and T-bet expression (Fig. 2D). These results therefore demonstrate a differential effect of the suppressive functions of NO-Tregs and nTregs.

NO-Tregs suppress established Th17 but not Th1 cells

We next investigated the relative suppressive effect of NO-Tregs and nTregs on established Th17, Th1 cells. Th17 and Th1 cells were cultured with 2 rounds of polarization as above, harvested on day 4, washed and re-cultured with NO-Tregs or nTregs at various Tregs : Th17/Th1 ratios as described in *Materials and Methods*. Cells were harvested on day 4 and were then analyzed by FACS, cell proliferation (^{3}H-thymidine uptake) and by ELISA (for cytokines in the culture supernatants). NO-Tregs potently decreased the percent of $IL\text{-}17^+ROR\gamma t^+$ cells, whereas nTregs registered only a modest and not significant inhibition of Th17 cells (Fig. 3A). This differential suppressive effect was evident across a range of Tregs : Th17 ratio (Fig. 3B) and was supported by the results of the relative concentrations of IL-17A in the culture supernatants (Fig. 3C) and cellular proliferation (Fig. 3D). Under the present culture condition, NO-Tregs are about 16× more effective (on cell for cell basis) than nTregs in suppressing Th17 cells. In contrast, while nTregs potently decreased the percent of established Th1 ($IFN\gamma^+$/T-bet^+) cells, NO-Tregs did not (Fig. 3E). Again, this differential suppressive effects of NO-Tregs and nTregs were observed over a range of Tregs : Th1 ratios (Fig. 3F) and confirmed by the concentrations of IFNγ in the culture supernatants (Fig. 3G) and cellular proliferation (Fig. 3H). Under this culture condition, nTregs are about 8× more effective than NO-Tregs in suppressing Th1. We also examined the relative suppressive effects of NO-Tregs and nTregs on established Th2 cells and Th9 cells. NO-Tregs and nTregs were equally suppressive against Th2 (Supplementary Fig. 1) and Th9 cells (Supplementary Fig. 2). Together, these results corroborate the finding that NO-Tregs and nTregs have differential suppressive effects on the proinflammatory Th17 and Th1 cells.

Th17 suppression by NO-Tregs partially depends on cell contact and IL-10

We next investigated the mechanism by which NO-Tregs inhibit the differentiation of Th17 cells. We first tested the potential role of CTLA4, which has been reported to be associated with the suppressive activity of nTregs (20). NO-Tregs were added to the polarization culture of Th17 cells at 1:1 ratio in the presence of a neutralizing anti-CTLA4 antibody. The suppressive effect of NO-Tregs was not affected by anti-CTLA4 (Fig. 4A). We also tested the requirement of metabolically active NO-Tregs in the suppressive function. NO-Tregs were pre-treated with mitomycin C, washed and then added to the polarizing culture of Th17 cells. The mitomycin-C treatment did not affect the suppressive activity of NO-Tregs (Fig. 4A). These results were confirmed by the relative concentrations of IL-17A in the culture supernatants (Fig. 4B). Since mitomycin-C did not affect the function of NO-Tregs, we tested the possibility that NO-Tregs assert their suppressive activity via a cell contact-dependent mechanism. Th17 cells and NO-Tregs were cultured together or separated by a semi-permeable membrane in a transwell culture under Th17 polarizing condition. The suppressive effect of NO-Tregs was partially reversed when cultured in a transwell system (Fig. 4C). This result was also evident in the level of IL-17A in the culture supernatant (Fig. 4D). IL-10 has been shown to be important in nTregs-mediated suppression of effector cells (21). We therefore ascertained the potential role of IL-10 in NO-Tregs-mediated suppression of Th17 cells. NO-Tregs were derived from WT or $IL\text{-}10^{-/-}$ mice of the BALB/c background and cultured with $CD4^+$ T cells under Th17 polarizing condition. NO-Tregs

from IL-10$^{-/-}$ mice were significantly less suppressive than those from the WT mice (Fig. 4E, F). These results therefore indicate that the suppression of Th17 cells by NO-Tregs requires both cell-contact and the presence of IL-10.

NO-Tregs attenuate EAE

Finally, we investigated the role of NO-Tregs in vivo, using the EAE model, a disease in mice closely associated with Th17 (refs. 15, 22-24). EAE was induced in C57BL/6 mice by priming with MOG_{35-55} peptide. The EAE mice were injected intraperitoneally with 1×10^6 NO-Tregs on day 10 after immunization, when the disease became apparent. Mice given NO-Tregs developed significantly less severe EAE compared to the control mice injected with PBS alone (Fig. 5A). The disease attenuation by NO-Tregs was confirmed by histological examination, which showed a marked reduction of leukocyte infiltration in the sub-meningeal area of the CNS of the mice receiving NO-Tregs compared to the control mice (Fig. 5B). The draining lymph node (DLN) cells were harvested on day 20 and analyzed by FACS ex vivo. The LN of the NO-Tregs recipients showed a marked reduction in the frequency of IL-17$^+$ T cells compared to the PBS control mice. There was however no significant difference in the frequency of IFNγ$^+$ or IL-17$^+$/IFNγ$^+$ T cells between the two groups of mice (Fig. 5C-E). We also analyzed the CNS cells of these mice by FACS. There was a significant reduction of the frequency of CD4$^+$IL-17$^+$ (Th17) cells in the NO-Tregs recipients compared to controls (Fig. 5F-H). There was however, no different in the frequency of CD4$^+$IFNγ$^+$ (Th1) and IL-17+IFNγ+ double positive cells in the CNS of the two groups (data not shown). Serum from NO-Tregs recipients also produced less IL-17A but more IL-10 compared to the cells from the control mice (Fig. 5I). These results therefore show that NO-Tregs are able to attenuate EAE and that this effect is associated with the suppression of Th17 cells but not Th1 cells.

Discussion

The existence and function of regulatory T cells are arguably one of the most satisfying explanations of the maintenance of homeostasis of the immune system. Their key roles in immune regulation demand the possibility that Tregs exist in different forms to meet the challenges of distinct subsets of effector cells, mediating over exuberance of immune stimulations. Thus in addition to nTregs, several other forms of Tregs have been described. Tregs are now known to be induced from CD4$^+$CD25$^-$ T cells by specific antigens in human and mice (25-27). Tregs with a Th1-like phenotype have also been reported (28), whereas Tregs induced by IL-10 (Tr1) mediated their suppressive function in the absence of Foxp3 (ref. 29). A subset of TGF-β-induced Tregs (Th3) has also been described (30). NO-Tregs are distinct from these subsets of Tregs in several important aspects. NO-Tregs are CD4$^+$CD25$^+$, Foxp3$^-$, GITR$^+$ and CD27$^+$, with a Th2-like phenotype and ameliorate inflammatory diseases in an IL-10-dependent manner (12). NO-Tregs differ from nTregs in that NO-Tregs do not express Foxp3. NO-Tregs are also different from Th3 in that NO-Tregs do not produce TGF-β. NO-Tregs are distinct from Th2 because Th2 are not suppressive against CD4$^+$CD25$^-$ effector cells. NO-Tregs are also different from Tr1 in that the induction of NO-Tregs is IL-10-independent. It is important to note that IL-10 is the only detectable cytokine produced by NO-Tregs (12). This finding excludes the possibility that NO-Tregs may suppress Th17 via the production of IL-2, which has been shown to be a key inhibitor of Th17 differentiation (31).

Natural Tregs have been reported to suppress Th17 (ref. 32). However, this suppression required a relatively high nTreg : Th17 ratio. In our hands, on a cell for cell basis, NO-Tregs are at least 16× more powerful than nTregs in suppressing Th17. In sharp contrast, nTregs are approximately 8× more effective than NO-Tregs in suppressing Th1. The exact molecular mechanism for this reciprocal opposite effect is not clear, but the suppressions

parallel the effect on the respective signature transcription factors of Th17 (RORγt) and Th1 (T-bet). It should also be noted that despite intensive research over a decade, the molecular mechanism for nTregs-mediated suppression of effector cells remains elusive and controversial. NO-Tregs suppress Th17 in a cell contact-dependent manner and is partially dependent on IL-10. We have explored other possibilities by blocking the usual suspects (ICAM-1, LFA-1, OX40) with negative results (data not shown). It may be that IL-10 needs to act in a short range, effective only when NO-Tregs and Th17 are in close contact.

The 200-400 nM dose of NO used in our study is likely to occur in vivo in sites of acute infection and inflammation (18) and has been used routinely in experiments in vitro (4, 13). Although human macrophages produced only a low level of NO, humans do produce substantial amounts of NO comparable to that of rodents in vivo, and the role of NO and iNOS in humans is not in dispute. It has been recently reported that NO is produced in large amounts in the mouse by non-hematopoietic stroma cells such as fibroblastic reticular cells and lymphatic endothelial cells (33). The production of NO by these cell types in humans remains to be explored. Thus it is likely that the findings reported here are equally applicable to humans.

We have demonstrated that NO-Tregs are effective in attenuating ongoing EAE. We have elected to test NO-Tregs in EAE because both Th17 and Th1 have been implicated in this disease model (22-24, 34). NO-Tregs-mediated EAE attenuation is accompanied by a marked reduction in the frequency of Th17 cells in the DLN. In contrast, the frequency of Th1 cells remains unchanged. These results are consistent with the notion that NO-Tregs selectively suppress Th17 but not Th1 in vivo. It has been well documented that nTregs can suppress EAE probably through the suppression of Th1 cells (reviewed in 23, 24). We have demonstrated earlier that Th17 and Th1 cells infiltration in the draining LN and spleen reflect the infiltration of these cells in the CNS (35). It should be note that the disease attenuation by NO-Tregs was incomplete, compatible with a role for other effector cells such as Th1 in EAE. For this matter, we did not test the effect of NO-Tregs from IL-$10^{-/-}$ mice, as the EAE model would not be sensitive enough to measure this partial effect.

Data reported here provide important functional characterization of NO-Tregs, which selectively suppress Th17 over Th1 cells. Additionally, we demonstrate the contrasting function of nTregs, which selectively suppress Th1 over Th17. The molecular mechanism involved is likely the selective inhibition of RORγt by NO-Tregs, and the reduction of T-bet expression by nTregs. Natural Tregs exist under physiological condition for the maintenance of immune balance. High dose of NO is induced during infections, which also activates Th17 cells that can lead to a range of inflammatory autoimmune disorders. The selective suppression of Th17 by NO-Tregs would ensure the rapid and economic local suppression of excessive Th17 to avoid collateral damage, while leaving the general surveillance nature of Th1 intact. Our finding here of the compartmentalization of Treg activities demonstrates an important biological function which may be of relevance to the evolution of the immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Wellcome Trust, the Medical Research Council, UK; the European Union and the HiCi grant of the King Abdulaziz University, Saudi Arabia.

References

1. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993; 329:2002–2012. [PubMed: 7504210]
2. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell. 1994; 78:915–918. [PubMed: 7522969]
3. Liew FY. Regulation of lymphocyte functions by nitric oxide. Curr Opin Immunol. 1995; 7:396–399. [PubMed: 7546406]
4. Macphail SE, Gibney CA, Brooks BM, Booth CG, Kim BF, M. Y, Chung HT, Simmons RL, Billiar TR. Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. J Biol Chem. 2000; 275:10954–10961. [PubMed: 10753895]
5. Flanagan, Coleman JW. Nitric oxide regulation of human peripheral blood mononuclear cells: critical time dependence and selectivity for cytokine versus chemokine expression. J Immunol. 2003; 171:4809–4815. [PubMed: 14568959]
6. Lukic ML, Stosic-Grujicic S, Ostojic N, Chan WL, Liew FY. Inhibition of nitric oxide generation affects the induction of diabetes by streptozocin in mice. Biochem Biophys Res Commun. 1991; 178:913–920. [PubMed: 1831356]
7. McInnes IB, Leung BP, Field M, Wei XQ, Huang FP, Sturrock RD, Kinninmonth A, Weidner J, Mumford R, Liew FY. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. J Exp Med. 1996; 184:1519–1524. [PubMed: 8879223]
8. Smith KJ, Lassmann H. The role of nitric oxide in multiple sclerosis. Lancet Neurol. 2002; 1:232–241. [PubMed: 12849456]
9. Rios-Santos F, Alves-Filho JC, Souto FO, Spiller F, Freitas A, Lotufo CM, Soares MB, Dos Santos RR, Teixeira MM, Cunha FQ. Down-regulation of CXCR2 on neutrophils in severe sepsis is mediated by inducible nitric oxide synthase-derived nitric oxide. Am J Respir Crit Care Med. 2007; 175:490–497. [PubMed: 17138957]
10. Nagy G, Clark JM, Buzas EI, Gorman CL, Cope AP. Nitric oxide, chronic inflammation and autoimmunity. Immunol Lett. 2007; 111:1–5. [PubMed: 17568690]
11. Niedbala W, Wei XQ, Campbell C, Thomson D, Komai-Koma M, Liew FY. Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. Proc Natl Acad Sci U S A. 2002; 99:16186–16191. [PubMed: 12451176]
12. Niedbala W, Cai B, Liu H, Pitman N, Chang L, Liew FY. Nitric oxide induces CD4+CD25+ Foxp3 regulatory T cells from CD4+CD25 T cells via p53, IL-2, and OX40. Proc Natl Acad Sci U S A. 2007; 104:15478–15483. [PubMed: 17875988]
13. Niedbala W, Alves-Filho JC, Fukada SY, Vieira SM, Mitani A, Sonego F, Mirchandani A, Nascimento DC, Cunha FQ, Liew FY. Regulation of type 17 helper T-cell function by nitric oxide during inflammation. Proc Natl Acad Sci U S A. 2011; 108:9220–9225. [PubMed: 21576463]
14. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med. 2003; 198:1951–1957. [PubMed: 14662908]
15. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005; 201:233–240. [PubMed: 15657292]
16. Ahern PP, Izcue A, Maloy KJ, Powrie F. The interleukin-23 axis in intestinal inflammation. Immunol Rev. 2008; 226:147–159. [PubMed: 19161422]
17. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med. 2009; 361:888–898. [PubMed: 19710487]
18. Keefer LK, Nims RW, Davies KM, Wink DA. “NONOates” (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. Methods Enzymol. 1996; 268:281–293. [PubMed: 8782594]
19. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006; 126:1121–1133. [PubMed: 16990136]

20. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med. 2000; 192:303–310. [PubMed: 10899917]
21. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med. 1999; 190:995–1004. [PubMed: 10510089]
22. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005; 6:1133–1141. [PubMed: 16200068]
23. Steinman L. A rush to judgment on Th17. J Exp Med. 2008; 205:1517–1522. [PubMed: 18591407]
24. Weiner HL. A shift from adaptive to innate immunity: a potential mechanism of disease progression in multiple sclerosis. J Neurol. 2008; 255(Suppl 1):3–11. [PubMed: 18317671]
25. Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S, Horwitz DA. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25-precursors. J Immunol. 2002; 169:4183–4189. [PubMed: 12370347]
26. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25-naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. 2003; 198:1875–1886. [PubMed: 14676299]
27. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25-T cells. J Clin Invest. 2003; 112:1437–1443. [PubMed: 14597769]
28. Stock P, Akbari O, Berry G, Freeman GJ, Dekruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. Nat Immunol. 2004; 5:1149–1156. [PubMed: 15448689]
29. Vieira PL, Christensen JR, Minaee S, O'Neill EJ, Barrat FJ, Boonstra A, Barthlott T, Stockinger B, Wraith DC, O'Garra A. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol. 2004; 172:5986–5993. [PubMed: 15128781]
30. Fukaura H, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Hafler DA. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. J Clin Invest. 1996; 98:70–77. [PubMed: 8690806]
31. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'Shea J. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007; 26:371–381. J. [PubMed: 17363300]
32. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. Cell. 2010; 140:845–858. [PubMed: 20303875]
33. Lukacs-Kornek V, Malhotra D, Fletcher AL, Acton SE, Elpek KG, Tayalia P, Collier AR, Turley SJ. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. Nat Immunol. 2011; 12:1096–1104. [PubMed: 21926986]
34. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, Anderton SM. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. J. Immunol. 2008; 181:3750–3754. [PubMed: 18768826]
35. Jiang HR, Milovanović M, Allan D, Niedbala W, Besnard AG, Fukada SY, Alves-Filho JC, Togbe D, Goodyear CS, Linington C, Xu D, Lukic ML, Liew FY. IL-33 attenuates EAE by suppressing IL-17 and IFN-γ production and inducing alternatively activated macrophages. Eur J Immunol. 2012; 42:1804–1814. [PubMed: 22585447]

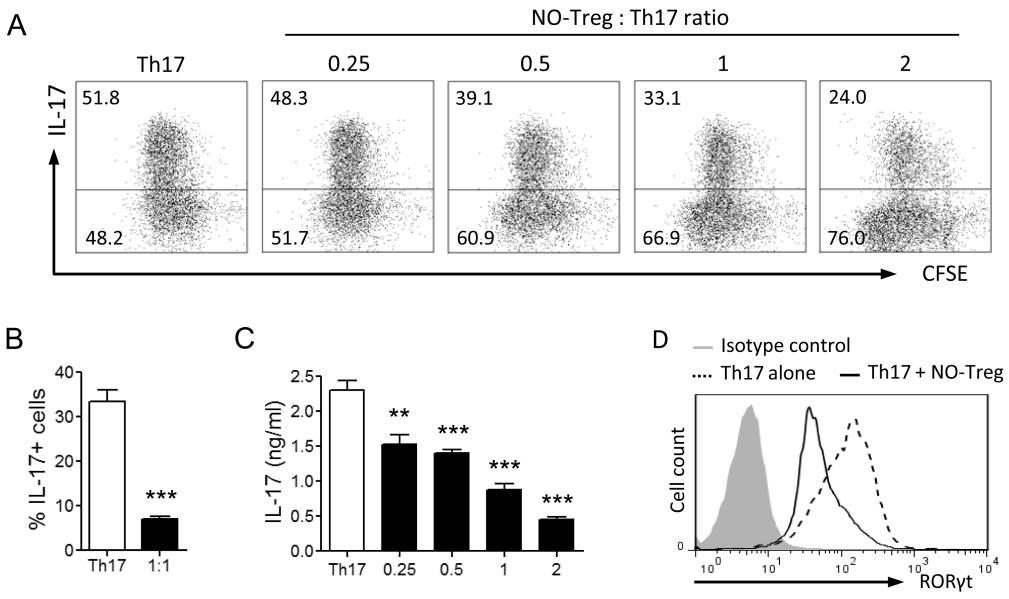

FIGURE 1.

NO-Tregs suppress the polarization of Th17. $CD4^+$ T cells from BALB/c mice were labeled with CFSE and cultured under Th17 polarizing condition in the presence of graded ratio of NO-Tregs. (**A**) The cells were cultured with added anti-IL-2 and harvested on day 4 of culture and analyzed for IL-17 and CFSE by FACS. The effector cells were gated for $CFSE^+$ cells only. Results are representative of 3 experiments. (**B**) The percent of $IL\text{-}17^+$ cells cultured in the absence of anti-IL2 in the presence or absence of NO-Tregs at 1:1 ratio were pooled from 6 experiments (data are mean ± SEM). (**C**) Concentrations of IL-17A in the culture supernatant were determined by ELISA. Supernatants were collected on day 4 of culture set up as in (A). Data are mean ± SEM, pooled from 3 experiments. (**D**) FACS analysis of RORγt expression in $CD4^+$ cells following 4 days of culture under Th17 condition with or without NO-Tregs at 1:1 ratio. **p<0.01, ***p<0.001.

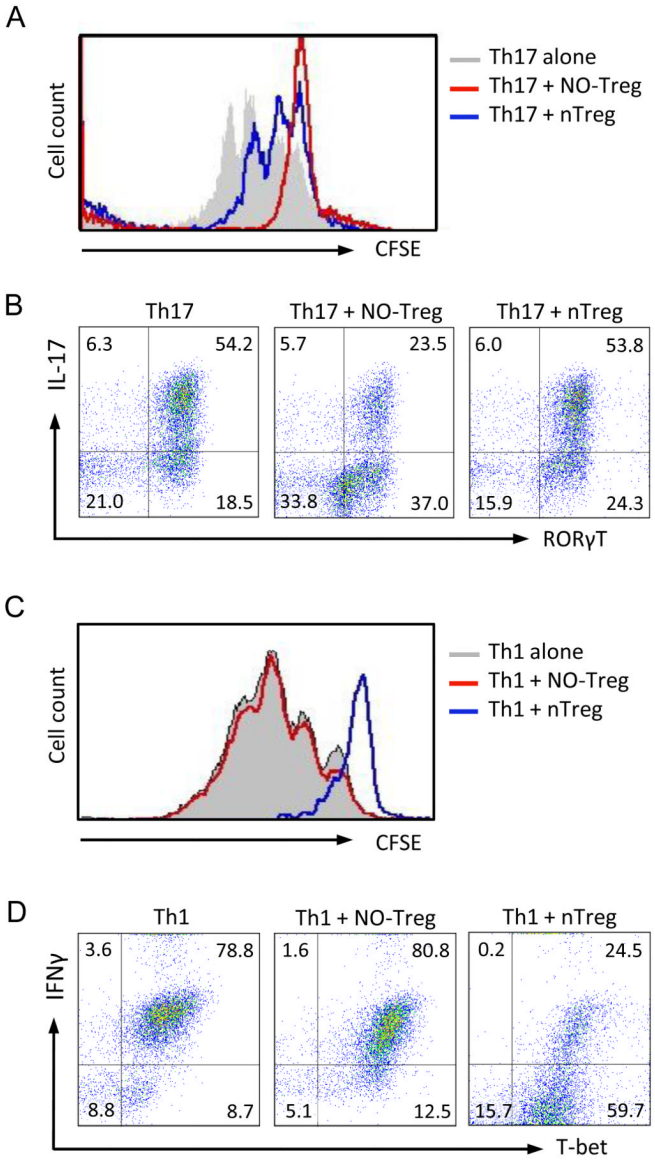

FIGURE 2.

NO-Tregs suppress Th17 but not Th1 whereas nTregs inhibit Th1 but not Th17 differentiation. (**A**) $CD4^+$ T cells were labeled with CFSE and polarized to Th17 in the presence of NO-Tregs or nTregs at 1:1 ratio. Cells were harvested on day 4 of culture and analyzed for CFSE dilution by FACS. Data are representative of 3 experiments. (**B**) $CD4^+$ T cells were polarized under Th17 condition in the presence of NO-Tregs or nTregs as above and stained for IL-17 and RORγt. Data are representative of 3 experiments. Similar results were obtained on days 3 and 5. (**C**) $CD4^+$ T cells were labeled with CFSE and polarized to Th1 in the presence of NO-Tregs or nTregs at 1:1 ratio. Cells were harvested on day 4 of culture and analyzed for CFSE dilution by FACS. Data are representative of 3 experiments. (**D**) $CD4^+$ T cells were polarized under Th1 conditions in the presence NO-Tregs or nTregs. The cells were harvested on day 4 and stained for IFNγ and T-bet. Data are representative of 3 experiments. Similar results were obtained on days 3 and 5.

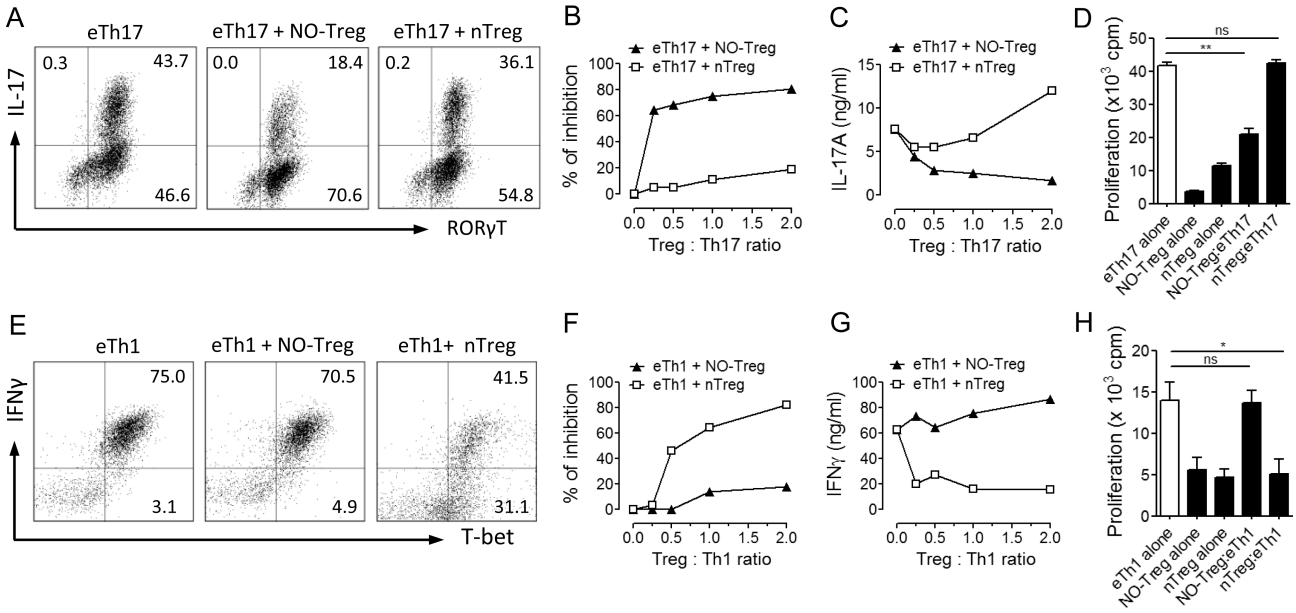

FIGURE 3.

NO-Tregs suppress established Th17 but not established Th1 whereas nTregs inhibit established Th1 but not established Th17. (**A-D**) $CD4^+$ T cells were polarized to Th17. After 2 rounds of culture, the cells were re-cultured for up to 6 days with anti-CD3 and IL-23 in the presence of graded ratio of NO-Tregs or nTregs. Cells were stained for IL-17 and RORγt on day 4 (**A, B**). The concentration of IL-17A in the culture supernatant was determined by ELISA (**C**). Cell proliferation of individual cell types or in a combination was determined by ^{3}H-thymidine uptake on day 3 (**D**). (**E-H**) $CD4^+$ T cells were polarized under Th1 condition. After 2 rounds of polarization, the cells were re-cultured with anti-CD3 in the presence of NO-Tregs or nTregs for up to 6 days. The cells were harvested on day 4 and stained for IFNγ and T-bet (**E, F**). IFNγ in the culture supernatant was determined by ELISA (**G**). Cell proliferation was determined by ^{3}H-Thymidine uptake (**H**). All data are representative of at least 3 experiments. Vertical bars are SEM, *p<0.05, **p<0.01, ns=not significant.

Europe PMC Funders Author Manuscripts

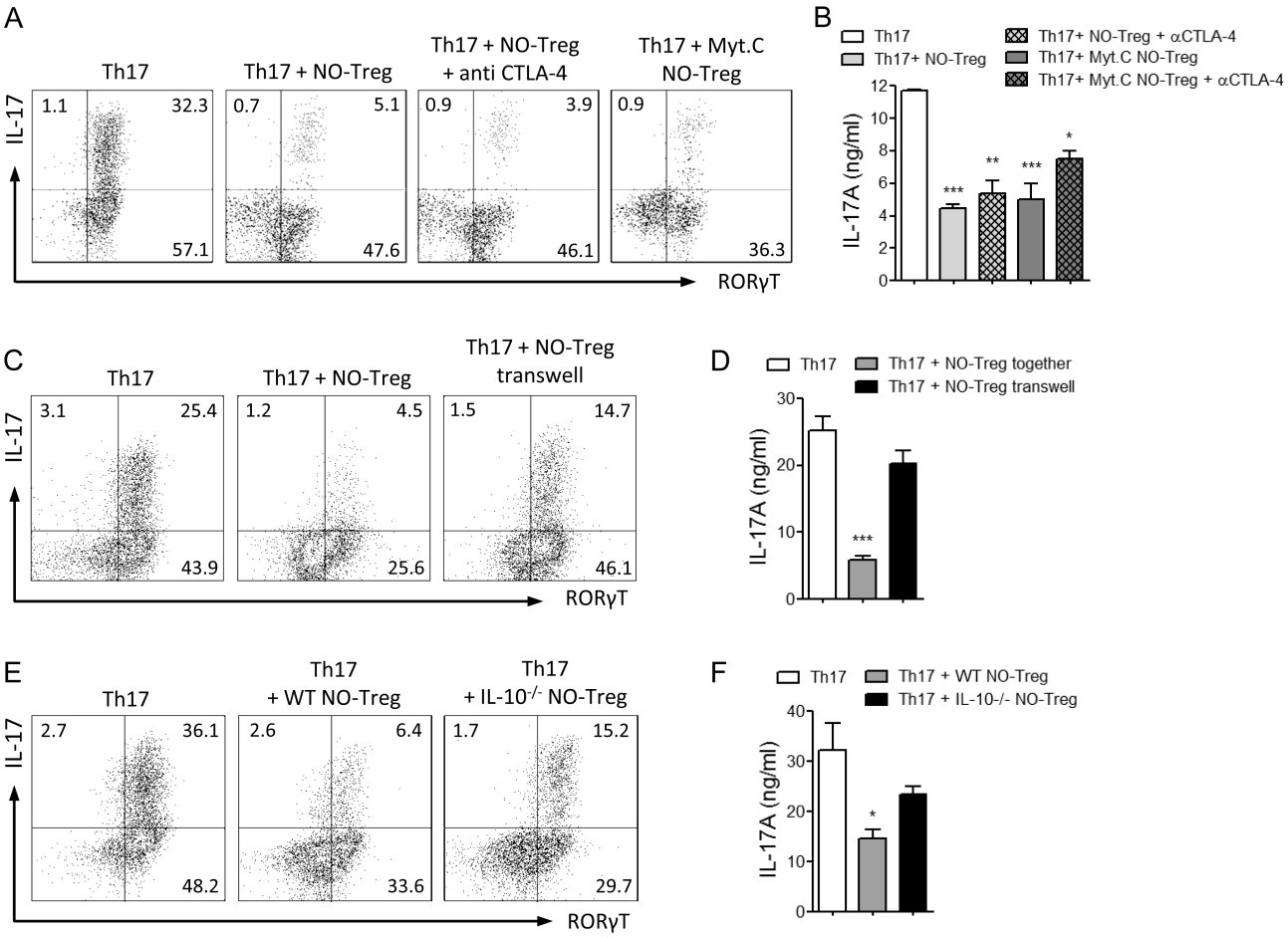

FIGURE 4.

NO-Tregs suppress Th17 in a cell contact-dependent manner. (**A**) $CD4^+$ T cells were polarized to Th17 in the presence of NO-Tregs, mitomycin-C-treated NO-Tregs or NO-Tregs + anti-CTLA4 antibody. The cells were harvested on day 4 and stained for IL-17 and RORγt. IL-17A concentration in the culture supernatants was determined by ELISA (**B**). (**C**) $CD4^+$ T cells were polarized to Th17 together with NO-Tregs or separated from NO-Tregs in transwells. The cells were harvested on day 4 and stained for IL-17 and RORγt. IL-17A concentration in culture supernatants was determined by ELISA (**D**). (**E**) $CD4^+$ T cells were polarized to Th17 in the presence of NO-Tregs derived from *Il10*$^{-/-}$ or wild-type (WT) mice. Cells were harvested on day 4 and stained for IL-17 and RORγt. IL-17A concentration in the culture supernatants was determined by ELISA (**F**). All experiments were repeated at least 3 times. Vertical bars are SEM, *p<0.05, **p<0.01, ***p<0.001.

Europe PMC Funders Author Manuscripts

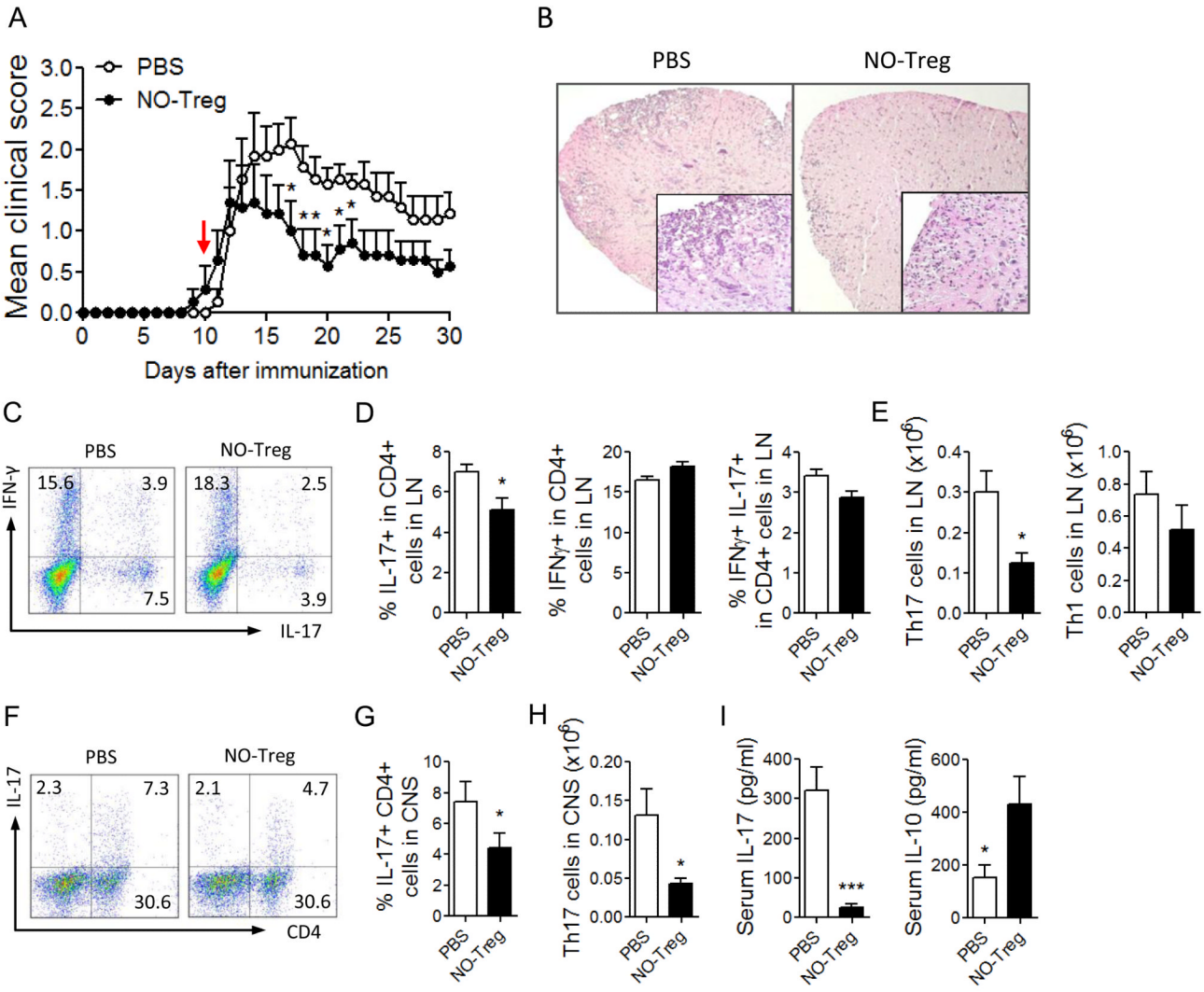

FIGURE 5.

NO-Tregs attenuate EAE and reduce the frequency of Th17 but not Th1 cells in vivo. C57BL/6 mice were immunized with MOG peptide to induce EAE. Mice were injected intravenously with NO-Tregs (1×10^6 cells) on day 10 after immunization (red arrow). Disease development was monitored daily and expressed as Mean clinical score (**A**). Mice were sacrificed on day 30 and the spinal cords were collected, fixed, and sections were stained with H&E (**B**). (**C-E**) DLN were collected 20 days after immunization and analyzed ex vivo by FACS. IL-17$^+$ and IFNγ$^+$ cells were gated for CD4$^+$ cell populations. Data are representative of 10 mice per group in 2 independent experiments. (**F-H**) In an additional experiment, CNS was collected on day 20 after immunization and analyzed as above. (**I**) Sera of mice collected 30 days after immunization were analyzed by ELISA. Vertical bars are SEM, n=5, *p<0.05, ***p<0.001.

Europe PMC Funders Author Manuscripts