IFN-γ Production by Th1 Cells Generated from Naive CD4⁺ T Cells Exposed to Norepinephrine

Michelle A. Swanson, William T. Lee and Virginia M. Sanders

*J Immunol* 2001; 166:232-240; doi: 10.4049/jimmunol.166.1.232

http://www.jimmunol.org/content/166/1/232

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**  This article cites 40 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/166/1/232.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
IFN-\(\gamma\) Production by Th1 Cells Generated from Naive CD4\(^+\) T Cells Exposed to Norepinephrine\(^1\)

Michelle A. Swanson,* William T. Lee,‡ and Virginia M. Sanders\(^2*\)

During activation in vivo, naive CD4\(^+\) T cells are exposed to various endogenous ligands, such as cytokines and the neurotransmitter norepinephrine (NE). To determine whether NE affects naïve T cell differentiation, we used naive CD4\(^+\) T cells sort-purified from either BALB/c or DO11.10 TCR-transgenic mouse spleens and activated these cells with either anti-CD3/anti-CD28 mAbs or APC and OVA\(_{23-32}\) peptide, respectively, under Th1-promoting conditions. RT-PCR and functional assays using selective adrenergic receptor (AR) subtype antagonists showed that naïve CD4\(^+\) T cells expressed only the β\(_2\) AR subtype to bind NE and that stimulation of this receptor generated Th1 cells that produced 2- to 4-fold more IFN-\(\gamma\). This increase was due to more IFN-\(\gamma\)-produced per cell upon restimulation instead of more IFN-\(\gamma\)-secreting cells, as determined by IFN-\(\gamma\)-specific immunofluorescence and enzyme-linked immunospot. In contrast, Th1 cell differentiation was unaffected when naive T cells were exposed to NE and activated either in the presence of a neutralizing anti-IL-12 mAb or by APC from IL-12-deficient mice. Moreover, the addition of IL-12 to the IL-12-deficient APC cultures restored the ability of NE to increase Th1 differentiation. Taken together, these results indicate that a possible link may exist between the signaling pathways used by NE and IL-12 to increase naive CD4\(^+\) T cell differentiation to a Th1 cell. The Journal of Immunology, 2001, 166: 232–240.

C

D4\(^+\) Th cells play an important role in directing many aspects of the adaptive immune response. Th1 cells direct cellular immune responses through the production of high amounts of IL-2 to promote proliferation and expansion of CD4\(^+\) T cells (1), and IFN-\(\gamma\) to promote expression of MHC class I and II by APCs (2), activation of macrophages (reviewed in Ref. 3), and production of IgG2a by B cells (4). The production of these cytokines by Th1 cells is critical for providing cellular protection from intracellular pathogens (5) and for promoting delayed-type hypersensitivity and contact hypersensitivity responses (6). In addition to providing protection, Th1 cells also promote the development and/or progression of certain autoimmune diseases, such as insulin-dependent diabetes (7, 8) and experimental autoimmune encephalomyelitis (EAE) (9). Due to the ability of Th1 cells to be either protective or detrimental to the organism, it is critical to understand all of the physiological mechanisms that regulate the number of Th1 cells generated, as well as the level of cytokines that they produce.

Cells of both the Th1 and Th2 phenotype are derived from naive CD4\(^+\) T cells that are capable of initially producing only IL-2 (10) and that do not yet serve as T helper cells for effector responses (11). Many groups have shown that cytokines produced within the microenvironment of naive CD4\(^+\) T cells influence their differentiation into Th1 or Th2 cells. For example, naive CD4\(^+\) T cells activated in the presence of either the cytokine IL-12 or IFN-\(\gamma\) differentiate into Th1 effector cells (12, 13). In contrast, naive CD4\(^+\) T cells activated in the presence of IL-4 differentiate into Th2 cells (14). Therefore, the cytokine environment in which naive CD4\(^+\) T cells are activated profoundly influences the effector pathway into which they differentiate.

Within secondary lymphoid organs, cytokines represent only one type of factor found in the immediate microenvironment of naïve T cells. Another endogenous factor found within this microenvironment is norepinephrine (NE),\(^3\) a neurotransmitter that is released from sympathetic nerve terminals that end adjacent to CD4\(^+\) T cells (15) within the first 24 h after Ag exposure (16, 17). Thus, NE is released and available within the immediate microenvironment of naive CD4\(^+\) T cells during the critical time period of T cell activation and differentiation (18). Over the past two decades, the findings from many studies suggest that NE regulates CD4\(^+\) T cell function (reviewed in Ref. 19). Initial studies indicated that heterogeneous populations of CD4\(^+\) T cells express the β\(_2\) AR, which binds NE to generate a rise in the intracellular concentration of cAMP, activation of protein kinase A, and an inhibition of IL-2 production and proliferation (reviewed in Ref. 19). Findings from our laboratory indicated that resting (Ref. 20; A. Kohn, M. A. Swanson, and V. M. Sanders, manuscript in preparation) and activated (Ref. 21; A. Kohn, M. A. Swanson, V. M. Sanders, manuscript in preparation) clones of Th1 cells express the β\(_2\) AR, whereas clones of Th2 cells do not. Furthermore, stimulation of the β\(_2\) AR on clones of Th1 cells before or after cell activation decreases the level of both IL-2 and IFN-\(\gamma\) (20) or IL-2 alone (21), respectively, whereas the level of cytokine produced by newly-generated Th1 cells was not influenced by either a β\(_2\) AR-specific agonist or a cAMP analog exposure until the third round of restimulation (Ref. 22; Kohn et al., manuscript in preparation). Collectively, these data showed that effector CD4\(^+\) T cells differentially express the β\(_2\) AR and that stimulation of this receptor affects the level of cytokine produced by Th1 cells. The above data also showed that the β\(_2\) AR-induced change in cytokine

---

\(^1\) This work was supported by research funds from the National Institutes of Health Grant AI37326 (to V.M.S.) and AI35583 (to W.T.L.). M.A.S. is a recipient of the Arthur J. Schmitt Dissertation Fellowship.

\(^2\) Address correspondence and reprint requests to Dr. Virginia M. Sanders, Department of Cell Biology, Neurobiology, and Anatomy, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153. E-mail address: vsander@lumc.edu

\(^3\) Abbreviations used in this paper: NE, norepinephrine; AR, adrenergic receptor; cRPMI, complete RPMI.
production was modest, a finding that is not surprising because the role of the sympathetic nervous system and NE is to make minor adjustments in cellular activity to maintain overall homeostasis. However, to date only one study exists to suggest the presence of a functional β2AR on naive CD4+ T cells (22), and none exist to indicate whether stimulation of this receptor would affect naive T cell differentiation into Th1 cells.

To address this deficiency in the literature, naive CD4+ T cells were sort-purified from either BALB/c or DO11.10 TCR-transgenic mice on the BALB/c genetic background (23) and IL-12-deficient mice, the data indicate that the NE-induced effect is dependent on the presence of IL-12. These results may have important implications for understanding endogenous mechanisms that promote the development of optimal Th1-directed responses in vivo.

Materials and Methods

Animals

Female pathogen-free BALB/c mice obtained from Taconic Farms (Germantown, NY) were used between 7 and 12 wk of age. Upon arrival, mice were housed in the American Association of Laboratory Animal Care (AALAC)-accredited Animal Research Laboratory at Loyola University Chicago Medical Center (Maywood, IL). All mice were housed under a 12-h light-dark cycle. Mouse microisolator cages contained within a laminar flow system to maintain a pathogen-free environment. All mice were provided with autoclaved food and deionized water ad libitum. Female and male DO11.10 TCR-β-γ transgenic mice on the BALB/c genetic background (23) and IL-12-deficient mice (p35−/−) obtained from The Jackson Laboratory (Bar Harbor, ME) were housed under similar conditions at the Wadsworth Center (Albany, NY).

Reagents and Abs

Chicken OVA323–339 peptide was synthesized by the Loyola University Chicago Molecular Analysis Facility, Terbutaline, naldole, phenol, formaldehyde, NE, and 8-bromo-cAMP were purchased from Sigma (St. Louis, MO) and dissolved and diluted in culture medium immediately before addition to cultures. Hamster anti-mouse CD3 mAb was affinity purified in our laboratory from hybridoma (clone 145-2C11) supernatant. Hamster anti-mouse CD28 mAb (clone 37.51) was affinity purified from hybridoma supernatant (clone 37.51, provided by Dr. James Allison, University of California, San Francisco, CA). The following Abs were used as capture Abs in the cytokine ELISA and were either purchased from PharMingen (San Diego, CA) as affinity-purified Abs or affinity purified from supernatants of hybridomas grown in our laboratory: biotin-labeled rat anti-mouse L-selectin (clone MEL-14), biotin-labeled rat IgG2a (clone R35-95), FITC-labeled rat anti-mouse CD4 (clone GK1.5), FITC-labeled RlgG2b, and PE-labeled streptavidin. Sheep anti-rat IgG-coated magnetic beads were purchased from Dynal (Lake Success, NY). Neutralizing rat anti-mouse IFN-γ (clone XMG1.2) was purchased from PharMingen. PBS and HBSS were purchased from Life Technologies (Grand Island, NY).

Isolation of naive CD4+ T cells

Naive CD4+ T cells were isolated from the spleens of either BALB/c or DO11.10 mice. Spleen cells were first treated with 0.8% NH4Cl, washed in HBSS/5% FCS, and added to 100 mm2 polystyrene tissue culture plates (Corning Glass, Corning, NY) coated with goat anti-mouse Ig Ab. After a 30-min incubation at 4°C, nonadherent spleen cells were washed and enriched for CD4+ T cells by staining with mouse anti-mouse 5E6 mAb, rat anti-mouse α light chain mAb, rat anti-mouse Mac-3 mAb, rat anti-mouse B220 mAb, and rat anti-mouse CD8b mAb in HBSS/1% FCS for 30 min at 4°C. Cells were washed twice in HBSS/1% FCS and incubated with sheep anti-rat IgG bound to magnetic beads in HBSS/1% FCS for 30 min at 4°C. CD4+ T cells were negatively selected and stored at 4°C overnight.

To further enrich the CD4+ T cell population for naive CD4+ T cells, cells were stained with biotin-labeled rat anti-mouse L-selectin mAb or isotype control biotin-labeled rat IgG2a in HBSS/1% FCS for 30 min at 4°C. Cells were washed and incubated with PE-labeled streptavidin and either FITC-labeled rat anti-mouse CD4 mAb or the isotype control FITC-labeled rat IgG2b in HBSS/1% FCS for 30 min at 4°C. Cells were washed three times with PBS and resuspended in PBS. Naive CD4+ T cells (CD4+ and L-selectinhigh) were sorted on a Becton Dickinson FACStar plus (San Jose, CA). Purity of sorted populations was >99%, as determined by postsort analysis. After sorting, cells were either immediately used for RNA isolation or placed into culture for the generation of primary Th1 cells.

Culture conditions for activation-induced IL-2 production

Naive CD4+ T cells were activated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb. Ninety-six-well, flat-bottom microtiter plates (Costar 3596; Cambridge, MA) were coated overnight at 4°C with anti-CD3 mAb (10 μg/ml) in PBS in a final volume of 50 μl. Cells in complete RPMI (cRPMI) were plated at 1 × 106 cells/ml in a final volume of 0.2 ml to which soluble anti-CD28 mAb (5 μg/ml) was added per well and incubated in an atmosphere of 5% CO2 at 37°C in a humidified incubator. Pharmaco logical agents were added at the time of cell activation. Supernatants were collected from culture wells at 24 h and immediately frozen at −80°C until analyzed for cytokine levels.

RT-PCR analysis of naive CD4+T cells

RNA was isolated by Trizol (1 ml/1 × 105 cells; Life Technologies) treatment and phenol/chloroform extraction from either 1 × 105 cells from the whole brain or sort-purified naive CD4+ T cells that were isolated from BALB/c mice. RNA samples were then treated with DNase 1 Amplification Grade (Life Technologies) at a concentration of 1 U/1 μg RNA for 10 min at room temperature. cDNA synthesis was performed with 50 μl of murine leukemia virus in a reaction buffer containing 25 mM MgCl2, 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 10 μM each dNTP, 60 μM random hexamers, and 20 U Rnase Inhibitor (Perkin-Elmer, Norwalk, CT). A reverse transcription reaction was performed for 1 cycle (42°C 15 min, 95°C 5 min). PCR of cDNA was performed with 5 U AmpliTaq DNA polymerase (Perkin-Elmer), 25 mM MgCl2, 10× PCR buffer, 10 μM primer, and double distilled H2O. cDNA was denatured for 2 min at 95°C and then amplified for 35 or 25 cycles (95°C 45 s, 58°C 45 s, 72°C 2 min) for the β2AR and β-actin, respectively. Sequences of the sense and antisense oligonucleotides were as follows: 5′-AAACTCTGGTAGCAGTGGTAATGTTC-3′ and 5′-ATGAGGTAGTCTGTCAGGT-3′ for the β2AR, 5′-AGCGGTTCCATAGATGTCGC-3′ and 5′-CCTGCTCTTATGTTGTCTTTTACG3′ for the β3AR, 5′-CGAAGACAGGCAAGAAGGGGAC-3′ and 5′-TCTGCTCATCGTGGTTGGGTAAAC-3′ for the β1AR, 5′-AACTCTGCTGAGAAGGGGAC-3′ and 5′-TCTGCTCATCGTGGTTGGGTAAAC-3′ for the β1AR, 5′-AGCGGTTCCATAGATGTCGC-3′ and 5′-CCTGCTCTTATGTTGTCTTTTACG3′ for the β3AR, 5′-CAAGAAGATCATAAGAAGGGGAC-3′ and 5′-CCTGCTCTTATGTTGTCTTTTACG3′ for the β3AR. Amplification products were run on a 99% agarose gel and stained with 5 μg/ml ethidium bromide for 5 min.

The β1-actin primer was served as an internal control to ensure the efficiency of both the reverse transcription reaction and the amount of RNA used.
Culture conditions for generation of primary Th1 cells

Naive CD4+ T cells from DO11.10 transgenic mice were incubated at 2.5 × 10^6 cells/ml in the presence of OVA_{323-339} peptide at a final concentration of 0.6 μM and irradiated (3300 rad) BALB/c or IL-12-deficient spleen cells at 2.5 × 10^6 cells/ml. Naive CD4+ T cells from BALB/c mice were incubated at 3–5 × 10^6 cells/ml in the presence of 5 μg/ml immobilized hamster anti-mouse CD3 mAb (clone U4-4D5) and 5 μg/ml soluble hamster anti-mouse CD28 mAb (clone 37.51). Both culture conditions were in a final volume of 1 ml cRPMI in a flat-bottom tissue culture-treated polystyrene 24-well plate (Corning Glass) for 5 days in the absence or presence of pharmacologic agents. In indicated experiments, Th1 cells were generated by activating naive T cells in the presence of 1.066 ng/ml recombinant mouse IL-2 and varying concentrations of recombinant mouse IL-12 (0.5–4 ng/ml). Cells were placed into an atmosphere of 5% CO₂ at 37°C in a humidified incubator. On day 3 of primary culture, 1 ml cRPMI was added to all cultures and on day 5, the resulting Th1 cells were collected, and equivalent numbers of Th1 cells were restimulated to assess IFN-γ production.

Cytokine ELISAs

Cytokine ELISAs were performed as described in detail elsewhere (21). Briefly, 96-well U-bottom flexible microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with rat anti-mouse cytokine capture Ab before adding cytokine-containing supernatant or recombinant standard to each well and incubating plates for 1–2 h at 37°C in a humidified atmosphere. Plates were washed, and a biotinylated rat anti-mouse cytokine-detecting Ab was added to each well. Plates were incubated for 45 min at 37°C in a humidified atmosphere and washed before addition of alkaline phosphatase-conjugated streptavidin (Southern Biotechnology Associates). After incubation at 37°C for 30 min in a humidified atmosphere, plates were washed and developed with p-nitrophenyl phosphate (Sigma) in diethanolamine. OD was determined on a UVmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm. The background from wells containing only cRPMI was subtracted from all groups. The data were analyzed using Softmax version 2.3 software (Molecular Devices).

Intracellular staining for IFN-γ

Naive T cells were activated under Th1-promoting conditions in the absence or presence of NE, and after 5 days the number of viable cells was counted using trypan blue dye exclusion. The cells were washed in cRPMI, and equivalent numbers of Th1 cells that had been generated in the absence or presence of NE were restimulated with plate-immobilized hamster anti-mouse CD3 mAb (clone U4-4D5) in the presence of PBS/1% BSA/0.5% saponin at 37°C in a humidified incubator. After incubation at 37°C for 30 min in a humidified atmosphere, plates were washed and developed with p-nitrophenyl phosphate (Sigma) in diethanolamine. OD was determined on a UVmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm. The background from wells containing only cRPMI was subtracted from all groups. The data were analyzed using Softmax version 2.3 software (Molecular Devices).

IFN-γ enzyme-linked immunospot (ELISPOT) assay

Naive T cells were activated under Th1-promoting conditions in the absence or presence of NE, and after 5 days the number of viable cells was counted using trypan blue dye exclusion. The cells were washed in cRPMI, and equivalent numbers of Th1 cells that had been generated in the absence or presence of NE were restimulated by irradiated APCs (3300 rad) pulsed with 0.6 μM OVA_{323-329} peptide in a sterile 96-well Millipore Multiscreen-HA (Bedford, MA) coated with purified rat anti-mouse IFN-γ mAb (2 μg/ml). NE was not added to restimulated cells. Brefeldin A (Sigma) was added at a final concentration of 2.5 μg/ml for the last 2 h of culture. Cells were washed extensively with PBS/0.05% azide, fixed in 1% fresh paraformaldehyde, and subsequently incubated with biotin-conjugated rat anti-mouse IFN-γ mAb, followed by streptavidin–alkaline phosphatase (Sigma) in diethanolamine. OD was determined on a UVmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm. The background from wells containing only cRPMI was subtracted from all groups. The data were analyzed using Softmax version 2.3 software (Molecular Devices).

Results

Analysis of βAR expression by naive CD4+ T cells

A recent study from our laboratory suggested that NE decreased the level of IL-2 produced by naive CD4+ T cells by stimulating the βAR, but not the αAR (22). Therefore, to determine the βAR subtype that was expressed on naive T cells, we isolated RNA from sort-purified naive CD4+ T cells and performed RT-PCR analysis using primers specific for either the β₁, β₂, or β₃ AR. The RT-PCR showed that a β₂ AR-specific band was amplified from the RNA of naive CD4+ T cells. cDNA from whole brain lysates, which are known to express all βAR receptors, was used as a positive control for the PCR (Fig. 1). To determine whether a functional binding site on the naive T cell surface was expressed, we assessed the ability of specific antagonists to block the NE-induced decrease in the level of IL-2 produced by these cells. As shown in Fig. 2, naive T cells activated in the presence of CD8+ cells. Small, high-density resting B cells were enriched using Percoll gradient centrifugation and collected at the 66–70% interface. In a total volume of 0.2 ml, 1 × 10⁷ B cells were exposed to LPS (50 μg/ml; Sigma) and dilutions of supernatants from either Th1 cells generated in the absence or presence of NE and in the absence or presence of 10 μg/ml anti-IFN-γ (clone XMG1.2). Ig-containing supernatants were collected after 8 days, and IgG2a levels were analyzed by ELISA.

βAR ELISA

Cytokine ELISAs were performed as described in detail elsewhere (21). Briefly, 96-well U-bottom flexible microtiter plates (Dynatech Laboratories) were coated with goat anti-mouse Ig capture Ab before adding Ab-containing supernatant or known quantities of mouse IgG2a (myeloma UPC-10; Sigma) to each well and incubating plates for 1–2 h at 37°C in a humidified atmosphere. Plates were washed and an alkaline phosphatase-conjugated goat anti-mouse IgG2a-detecting Ab was added to each well. Plates were incubated for 45 min at 37°C in a humidified atmosphere. Plates were washed and developed with p-nitrophenyl phosphate (Sigma) in diethanolamine. OD was determined on a UVmax kinetic microplate reader (Molecular Devices) at a wavelength of 405 nm. The background from wells containing only cRPMI was subtracted from all groups. The data were analyzed using Softmax version 2.3 software (Molecular Devices).

Statistics

Data were first analyzed by a one-way ANOVA to determine whether an overall statistically significant change existed before using the two-tailed, unpaired t test. The p values were adjusted for multiple comparisons, and an asterisk indicates significant differences from control when the p value was <0.05. Statistics were performed using StatView (Abacus Concepts, Berkeley, CA).
To address this possibility, sort-purified naive CD4 \(^+\) T cells (16), may also influence naive T cell differentiation. It was possible that NE, which is released into the spleen after Ag administration, may also influence naive T cell differentiation, naive T cells express the \(\beta_2\)-AR protein, which is functional on the surface of naive CD4 \(^+\) T cells.

\(\beta_2\)-AR antagonist metoprolol produced a decreased amount of IL-2, an effect similar to that of cells exposed to NE alone. In contrast, naive CD4 \(^+\) T cells exposed to NE in the presence of the \(\beta_2\)-AR-specific agonist ICI 118,551 produced a level of IL-2 that was equivalent to that produced by control cells exposed to medium alone (Fig. 2). Also, the addition of either antagonist alone did not significantly decrease the amount of IL-2 produced by naive T cells. Thus, these data suggest that only the \(\beta_2\)-AR protein appears to be expressed and functional on the surface of naive CD4 \(^+\) T cells.

IFN-\(\gamma\) production by Th1 cells generated from naive T cells activated in the presence of NE and Th1-promising conditions

Naive T cell differentiation is influenced by many of the factors found within its microenvironment, e.g., cytokines and prostaglandins (24, 25). Therefore, because naive T cells express the \(\beta_2\)-AR, it was possible that NE, which is released into the spleen after Ag administration (16), may also influence naive T cell differentiation. To address this possibility, sort-purified naive CD4 \(^+\) T cells were activated under Th1-promoting conditions, which included the addition of IL-12 in the absence or presence of NE. Also, because \(\beta_2\)-AR stimulation decreased the level of IL-2 produced by naive T cells (Fig. 2), exogenous IL-2 was added during Th1 differentiation to enhance overall cell yield. After 5 days, the number of cells in all cultures was equivalent (data not shown) and, when the resultant Th1 cells derived from naive cells activated in the presence of 10 \(^{-7}\) M or 10 \(^{-6}\) M NE were restimulated, the level of IFN-\(\gamma\) produced was increased in comparison to Th1 cells derived from unexposed naive cells (Fig. 3B). In contrast, it appears that NE exposure of naive T cells, while having an immediate effect on IL-2 production (Fig. 2), does not affect the level of IL-2 produced when the resulting effector cells are restimulated (data not shown). In addition, the \(\beta_2\)-AR antagonist ICI 118,551, prevented the NE-induced increase in IFN-\(\gamma\) (Fig. 3A).

To confirm that stimulation of the \(\beta_2\)-AR influences Th1 differentiation, naive T cells were exposed to a range of concentrations of the \(\beta_2\)-AR-selective agonist, terbutaline, during Th1 differentiation. In a manner similar to NE, the resultant Th1 cells generated in the presence of 10 \(^{-7}\) M terbutaline produced more IFN-\(\gamma\) after restimulation in comparison to unexposed controls (Fig. 3D), an effect that was blocked by the concomitant addition of nadolol (Fig. 3C). Also, the addition of either antagonist alone did not significantly increase the amount of IFN-\(\gamma\) produced by the resulting Th1 cells (Fig. 3, A and C). These results further confirm that the \(\beta_2\)-AR expressed on naive CD4 \(^+\) T cells binds NE to increase Th1 differentiation.

**FIGURE 2.** IL-2 production by naive CD4 \(^+\) T cells activated in the presence of NE and \(\beta_2\)-AR-selective agonist, terbutaline, during Th1 differentiation. In a manner similar to NE, the resultant Th1 cells generated in the presence of 10 \(^{-7}\) to 10 \(^{-6}\) M terbutaline produced more IFN-\(\gamma\) after restimulation in comparison to unexposed controls (Fig. 3D), an effect that was blocked by the concomitant addition of nadolol (Fig. 3C). Also, the addition of either antagonist alone did not significantly increase the amount of IFN-\(\gamma\) produced by the resulting Th1 cells (Fig. 3, A and C). These results further confirm that the \(\beta_2\)-AR expressed on naive CD4 \(^+\) T cells binds NE to increase Th1 differentiation.

IFN-\(\gamma\) production by Th1 cells generated from naive T cells activated by APC and peptide in the presence of NE

A previous study showed that \(\beta_2\)-AR stimulation of PBMC inhibited the level of IL-2 produced unless the cells were activated in the presence of autologous accessory cells (26). This finding suggested that any \(\beta_2\)-AR-induced effect on the T cell may be masked by costimulatory signals provided by the APC. Thus, to test the possibility that NE would not influence Th1 differentiation when an APC was used to activate naive T cells, sort-purified naive CD4 \(^+\) T cells were obtained from DO11.10-transgenic mice activated by irradiated APC and peptide under Th1-promoting conditions plus IL-2 in the absence or presence of NE. After 5 days, the number of cells in culture was equivalent (data not shown) and, in a manner equivalent to polyclonally activated naive T cells, the level of Th1 differentiation in the presence of 10 \(^{-7}\) or 10 \(^{-6}\) M NE concentrations was increased in comparison to unexposed controls (Fig. 4).

To confirm the ELISA results that the supernatants from restimulated Th1 cells contained more IFN-\(\gamma\), we measured the ability of these supernatants to increase the amount of IgG2a Ab produced by LPS-activated B cells (27). LPS-activated B cells produced significantly more IgG2a when exposed to supernatants from restimulated Th1 cells derived in the presence of NE when compared with B cells exposed to control supernatants (Fig. 5), and this increase in IgG2a production was abrogated by the addition of a neutralizing anti-IFN-\(\gamma\) mAb (clone XMG1.2). These data suggest that IgG2a production in this system is enhanced by the IFN-\(\gamma\) from the supernatants of the Th1 cells and confirms that Th1 cells generated in the presence of NE produce more biologically active IFN-\(\gamma\) after restimulation in comparison to Th1 cells generated in the absence of NE. It is important to note that the increase in the amount of IgG2a was not due to any residual NE in the Th1 supernatants because NE was added only during the Th1-promoting conditions and has a half-life of only 6–7 h in culture (Bruce Fuchs, unpublished observation) and also because the Th1 cells were washed extensively before restimulation. Therefore, these results suggest that NE added to naive T cells activated either Ag-specifically or polyclonally under Th1-promoting conditions in the presence of NE increases Th1 differentiation.

The cellular process for the NE-dependent increase in IFN-\(\gamma\) production

Because NE increased Th1 differentiation, it was possible that the process by which this occurred was due to either an increased number of resultent Th1 cells producing IFN-\(\gamma\) after restimulation, an increased amount of IFN-\(\gamma\) being produced per Th1 cell after restimulation, or a combination of both. To test these possibilities, sort-purified naive CD4 \(^+\) T cells were activated with APC and peptide under Th1-promoting conditions in the absence or presence of NE. The number of Th1 cells that produced IFN-\(\gamma\) after restimulation was analyzed by intracellular immunofluorescence staining for IFN-\(\gamma\), as well as by the IFN-\(\gamma\) ELISPOT technique. A slight, but consistent increase in the mean fluorescence intensity (2474 (Th1/medium) vs 2741 (Th1/NE) was measured in Th1 cells.

**Activation Conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Anti-CD3/CD28</th>
<th>NE (10^4 M)</th>
<th>Metoprolol (10^4 M)</th>
<th>ICI118,551 (10^4 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Downloaded from http://www.jimmunol.org/ by guest on November 17, 2017
generated in the presence of NE, with no difference in the percentage of IFN-γ-producing cells; 75 vs 76%) (Fig. 6A). In addition, even though the Th1 cells generated in the presence of NE secreted more IFN-γ after restimulation, the IFN-γ ELISPOT confirmed that both groups produced equivalent numbers of IFN-γ-secreting cells (Fig. 6B). Therefore, these data suggest that the process by which NE increases Th1 differentiation is due to increasing the amount of IFN-γ produced per Th1 cell.

**NE requires the presence of IL-12 to increase Th1 differentiation**

Unlike most exogenous factors that influence the level of Th1 differentiation by increasing the number of cytokine-secreting cells produced (28), NE appears to use a unique process that involves more IFN-γ produced per cell. Many different receptor-induced signals have been shown to positively regulate Th1 differentiation, e.g., stimulation of the TCR, IFN-γR, and the IL-12R (12, 13, 29). Because high levels of TCR stimulation have been shown to positively regulate TCR-induced signal transduction, such as Lyn, Syk, or Zap-70. Furthermore, preliminary data suggest that stimulation of the β2AR on naive T cells does not influence CD3e expression or TCR-induced proliferation (data not shown). Therefore, we hypothesized that NE was influencing the cytokine signals responsible for directing Th1 differentiation. Initial studies suggested that IFN-γ-directed Th1 differentiation was not modulated by NE because the addition of a neutralizing anti-IFN-γ mAb to naive T cells during differentiation did not abrogate the ability of NE to positively influence Th1 differentiation (data not shown). This left the possibility that NE was positively regulating IL-12-directed Th1 differentiation. The data show that only Th1 cells generated in the presence of NE and IL-12 produced significantly more IFN-γ after restimulation and that this effect occurred in an IL-12 concentation-dependent manner (Fig. 7A). This result suggests not only that NE requires IL-12 to positively regulate Th1 differentiation, but also that the level of IL-12 influences the degree to which NE influences naive CD4+ T cell differentiation to generate Th1 cells that produce increased levels of IFN-γ after restimulation.

A previous study suggested that stimulation of the β2AR on LPS-activated macrophages decreases IL-12 production and subsequently decreases naive T cell differentiation into Th1 cells (30). Thus, to determine whether endogenous levels of IL-12 produced by APC were sufficient for NE to increase Th1 differentiation, sort-purified naive T cells from DO11.10-transgenic mice were activated by APC and peptide in the absence of IL-12 (28). NE was added to the cultures to promote Th1 differentiation before the addition of exogenously added IL-12 but in either the absence or presence of NE for 5 days. A neutralizing rat anti-mouse IL-4 mAb was also added to the cultures to promote Th1 differentiation because the genetic background of the DO11.10 mouse (BALB/c) defaults to a Th2 pathway in the absence of Th1-promoting
cytokines (31). Under these conditions, Th1 cells generated in the presence of NE produced more IFN-γ after restimulation (Fig. 7B). This effect was dependent on the endogenous IL-12 that was produced by the APC, because the addition of a neutralizing anti-IL-12 mAb, as opposed to a species- and isotype-matched control Ab (data not shown), abrogated the NE-induced increase (Fig. 7B). The possibility existed that the addition of NE to the APC was increasing IL-12 production to subsequently enhance Th1 differentiation. To test this possibility, sort-purified naïve T cells from DO11.10-transgenic mice were activated by APC and peptide in either the absence or presence of NE, and supernatants from these cultures were harvested every day for 5 days. The level of IL-12 produced between the two groups was equivalent throughout the culture period (Fig. 7C). Therefore, the increase in Th1 differentiation in naïve T cells activated by APC in the presence of NE is not due to an increase in the amount of IL-12 produced by the APC.

To further confirm that NE required IL-12 to enhance Th1 differentiation, sort-purified naïve T cells from DO11.10-transgenic mice were activated by IL-12-deficient APC and peptide in the absence or presence of NE for 5 days. The Th1 cells generated in this IL-12-deficient system and in the presence of NE produced equivalent amounts of IFN-γ after restimulation in comparison to controls (Fig. 7D). However, if recombinant IL-12 was added to parallel cultures, the Th1 cells generated in the presence of NE and recombinant IL-12 produced significantly more IFN-γ after restimulation in comparison to Th1 cells generated in the presence of IL-12 alone (Fig. 7D). In combination, these results suggest that NE positively regulates IL-12-directed naïve CD4+ T cell differentiation into Th1 cells.

**Discussion**

Over the past 10 years, great strides have been made in understanding the immune factors that drive naïve CD4+ T cells to differentiate into Th1 cells and that influence the level of IFN-γ.
produced by this effector subset (reviewed in Ref. 24). In vivo, naive CD4\(^+\) T cells are exposed to endogenous ligands that are not part of the immune system itself, but nevertheless, are found within the microenvironment in which these cells reside, e.g., the sympathetic neurotransmitter NE. Factors such as NE within the naive T cell microenvironment may provide a mechanism by which the sympathetic nervous system is able to fine-tune the magnitude and/or duration of an immune response to maintain homeostasis within the organism. Previous studies from our laboratory have addressed how NE affects the level of IFN-\(\gamma\) produced from naive CD4\(^+\) T cells stimulated through the \(\alpha_2\)AR during the first 48 h of T cell activation, a time during which a number of cellular and molecular events occur to allow naive CD4\(^+\) T cells to become competent to produce IFN-\(\gamma\) (18, 32–34). Therefore, \(\beta_2\)AR stimulation of naive T cells, as compared with fully differentiated Th1 cells, may influence the level of IFN-\(\gamma\) produced by using different mechanisms, e.g., acting in coordination with different signaling pathways and/or transcription factors. Furthermore, \(\beta_2\)AR stimulation on naive CD4\(^+\) T cells may also influence the activity of molecular factors that allow for the induction of the IFN-\(\gamma\) promoter, factors that may not be active in fully differentiated Th1 cells.

Even though the concentrations of NE and terbutaline used in this study appear to be high at first glance, recent findings suggest that the concentrations used are not unrealistic in a physiological situation. CD4\(^+\) T cells have been shown to be adjacent to NE-containing nerve terminals found in the spleen (15). Recent studies from our laboratory by Kohm et al. (16) indicate that NE turnover increased in the spleen within 18 h after injection of the soluble protein Ag, trinitrophenyl-keyhole limpet hemocyanin, suggesting that NE was released in the spleen following the administration of Ag. Therefore, CD4\(^+\) T cells are localized to a microenvironment...
that may be exposed to very high concentrations of NE after injection of Ag in vivo. It has been estimated that the concentration of NE that is reached at such nerve terminal endings is between 0.3 and 3 mM (35). Therefore, the concentrations used in this study are well below the maximal concentration that a naive T cell may see in vivo. Because naive T cells may see a range of NE during their initial activation in vivo, we exposed naive T cells to a range of concentrations and found that naive T cells exposed to concentrations of NE at $10^{-7}$ and $10^{-6}$ M during Th1 differentiation generate Th1 cells that produce more IFN-γ after restimulation (see Fig. 3).

In studies that have assayed for the ability of endogenous factors to either positively or negatively influence Th1 differentiation, the increase in IFN-γ was due to an increase in the number of IFN-γ-producing cells (28, 36). We sought in this study to determine whether a similar mechanism was responsible for the NE-induced increase in IFN-γ after restimulation. The data suggest a unique model in which Th1 cells that are generated in the presence of NE produce more IFN-γ per cell. There is a paucity of data in the literature showing that treatment of a naive T cell can influence the amount of a cytokine produced per cell after restimulation. It is possible that NE positively regulates factors that influence the accessibility of the IFN-γ promoter in naive T cells, which is inherited in the subsequent Th1 effector cells. This possibility is supported by data showing that PGE$_2$ exposure of naive CD4$^+$ T cells during differentiation influences the methylation status of the IFN-γ promoter, as well as the level of IFN-γ produced after restimulation by the resultant Th1 cells (25).

Our experimental design allowed us to determine the effect of NE when the individual components required for Th1 differentiation were limiting. Our data indicate that NE does not independently promote Th1 differentiation, but rather influences IL-12-directed differentiation to generate Th1 cells that produce more IFN-γ after restimulation. Likewise, another factor, IFN-γ-inducing factor (IGIF) has been shown to augment IL-12-directed Th1 differentiation to produce Th1 cells that produce more IFN-γ (37). Although the modulation of IL-12-directed Th1 differentiation may be modest in both cases, increases in the amount of IFN-γ produced locally may have a profound effect in vivo.

The finding that NE requires IL-12 to modulate Th1 differentiation suggests that stimulation of the β$_2$AR on naive T cells may affect a component of the IL-12 pathway to result in an increased Th1 differentiation. These results are in contrast to the findings of previous studies showing that exposure to other cAMP-elevating ligands, such as PGE$_2$, during activation of human PBMCs by anti-CD3 mAb, decreases the expression of both chains of the IL-12 receptor on cell types other than naive CD4$^+$ T cells, e.g., memory and effector CD4$^+$ T cells, CD8$^+$ T cells, and NK cells (39). Also, in this published study, IL-12 was not added to cultures during the initial activation of the human PBMCs (38), a fact which may be important because a recent report suggests that the presence of IL-12 during CD4$^+$ T cell activation may influence the level of IL-12R expression (40). Therefore, differences in the ability of endogenous ligands to influence Th1 differentiation may be dependent on the cytokine milieu in which naive T cells reside during initial activation and differentiation.

In addition, our results suggest that the endogenous level of IL-12 produced by APC in vitro is necessary and sufficient for NE to influence Th1 differentiation in response to peptide Ag. Furthermore, exposure of naive T cells and APC to NE did not influence IL-12 production by APC when presenting peptide in the context of MHC II to the naive T cell (Fig. 7C). This is in contrast to a previous study that suggested that β$_2$AR stimulation of LPS-activated macrophages decreased both IL-12 production and subsequent Th1 differentiation (30). However, the ability of NE to influence macrophage function may be different when activation is induced by LPS vs MHC class II and Ag. In addition, the data with LPS-activated macrophages showed that a correlation existed between the addition of a β$_2$AR agonist and a decrease in IFN-γ-producing cells, but did not determine whether the addition of IL-12 would reverse the β$_2$AR-dependent decrease. Therefore, the data presented herein show that under physiological conditions in which naive T cells are activated with APC and peptide instead of mitogen, exposure of APC to NE does not influence the level of IL-12 produced, and this level is sufficient for NE to augment Th1 differentiation.

Because NE-containing sympathetic nerve terminals have been reported to end in the direct vicinity of T cells in secondary lymphoid tissue (15), it has been hypothesized that the nervous system influences T cell function. These data are the first to confirm that naive CD4$^+$ T cells express the β$_2$AR, a receptor for the neurotransmitter NE, and to suggest that a link may exist between the signaling pathways associated with a neurotransmitter receptor and the IL-12 receptor. Continuing studies will help us to better understand the role of the β$_2$AR on naive T cells in regulating the development of protective Th1-directed responses, as well as in exacerbating the intensity of Th1-mediated disease states.

**Acknowledgments**

We thank Patricia Simms for flow cytometric assistance, Adam Kohn for help with RT-PCR analysis, and Cris Kamperschroer and John Dye for their assistance with the IFN-γ ELISPOT. We also to thank Deborah J. Kasprowicz and Afshaneh Mozaffarian for helpful discussions.

**References**


