Exposure to a Dysfunctional Glucocorticoid Receptor from Early Embryonic Life Programs the Resistance to Experimental Autoimmune Encephalomyelitis Via Nitric Oxide-Induced Immunosuppression

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*J Immunol* 2002; 168:5848-5859; doi: 10.4049/jimmunol.168.11.5848
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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Glucocorticoid (GC) hormones play a central role in the bidirectional communication between the neuroendocrine and the immune systems and exert, via GC receptors (GR), potent immunosuppressive and anti-inflammatory effects. In this study, we report that GR deficiency of transgenic mice expressing GR antisense RNA from early embryonic life has a dramatic impact in programming the susceptibility to experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. GR deficiency renders mice resistant to myelin oligodendrocyte glycoprotein-induced EAE, and such mice do not develop clinical or histological signs of disease compared with EAE-susceptible wild-type mice. Resistance to EAE in GR-deficient mice is associated not with endogenous GC levels, but with a significant reduction in spleen and lymph node cell proliferation. The use of NO inhibitors in vitro indicates that NO is the candidate immunosuppressor molecule. GR-deficient mice develop 3- to 6-fold higher nitrite levels in the periphery and are resistant to NO inhibition by GCs. Specific inhibition of NO production in vivo by treatment with the inducible NO synthase inhibitor, 1-NNm-(1-iminoethyl)-lysine, suppressed circulating nitrites, increased myelin oligodendrocyte glycoprotein-specific cell proliferation, and rendered GR-deficient mice susceptible to EAE. Thus, life-long GR deficiency triggers inducible NO synthase induction and NO generation with consequent down-regulation of effector cell proliferation. These findings identify a novel link among GR, NO, and EAE susceptibility and highlight NO as critical signaling molecule in bidirectional communication between the hypothalamic-pituitary-adrenocortical axis and the immune system. The Journal of Immunology, 2002, 168: 5848–5859.

By binding to cognate receptors, glucocorticoids (GCs)6–8 exert a negative feedback action at the pituitary, hypothalamic, and extrahypothalamic levels and are key regulators of the bidirectional communication between the neuroendocrine and immune systems (1–4). As a consequence of this interaction, GC secretion from the adrenal glands is increased during an immune response, and this mechanism limits the magnitude of the inflammatory reaction to an immunogenic stimulus. Malfunction of this interaction has been suggested to contribute to the pathogenesis of a number of autoimmune and inflammatory experimental diseases, such as thyroiditis, arthritis, and experimental autoimmune encephalomyelitis (EAE) (3, 5–8). In its chronic form, EAE is a well-accepted experimental model for the human disease multiple sclerosis (MS), an immune-mediated disorder of the CNS leading to a progressive decline of motor and sensory functions and neurological disability (9).

Endogenous GCs are vital in determining EAE severity. Activation of the hypothalamic-pituitary-adrenocortical (HPA) axis during clinical disease is crucial for the recovery from EAE, since suppression of this response results in lethal EAE and renders resistant strains of animals susceptible to disease induction, while, conversely, treatment with exogenous GCs can block disease (6, 10, 11). Of major concern is that early life events, including neonatal exposure to GCs or pathogens, alter the developmental programming of the HPA axis and stress response as well as to modulate the susceptibility to inflammatory and autoimmune diseases (12–17).

GC receptors (GRs; type II), which are primarily involved in feedback regulation of the HPA axis (1, 2), mediate the immune regulatory effects of GCs at multiple levels (2). After ligation, translocation of a GR complex to the nucleus and binding to GC-responsive elements occurs, resulting in inhibition of the transcription of a plethora of inflammatory mediators, such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-12, TNF-α, and GM-CSF (18). Among
the many proteins regulated by GC is the inducible NO synthase (iNOS or NOS2) gene. Suppression of this protein decreases the production of NO, a key molecule in the inflammatory response (19–21). An important molecular mechanism underlying most GC anti-inflammatory and immunosuppressive activity is inhibition of AP-1 (Jun-Fos heterodimers) and NF-kB (p65-p50 heterodimers) families of transcription factors (22, 23). The cytokine-dependent stimulation of iNOS responsible for NO production is mediated by NF-kB activation and is suppressed by GCs (24, 25).

Administration of synthetic GCs is used to control the symptoms of MS, although long-term therapy has failed to show a beneficial effect on either the progression of disability or the rate of relapse (26). The exact mechanisms of GC action in MS or EAE are unknown, although reduction of blood-brain barrier changes, decrease in inflammatory cells, suppression of cytokine production, altered adhesion molecule expression, and reduction of edema in MS lesions have been reported (26, 27).

Impaired GR function has been considered an important factor in the development and/or maintenance of various pathological conditions in humans, including (familial) GC resistance (28) and major depression (29). Long-lasting dysfunction/dysregulation of central GR expression are also known to accompany early neonatal experiences (12, 13) with potential consequences with respect to immune regulation (14–17). A transgenic (Tg) mouse expressing antisense RNA directed against GR was created to serve as an animal model for the study of neuroendocrine changes occurring in stress-related disorders (30). These mice show reduced GR mRNA in the brain, pituitary, thymus, and spleen; reduced brain, thymic, and splenic GR binding; and reduced HPA axis sensitivity to GCs (30–33). As a consequence of the impaired GR function, regulation of the HPA axis in these animals is disturbed (31–35). At the immunological level, GR-deficient mice show sex-dependent alterations in both trafficking and responsiveness of T lymphocytes during postnatal development of thymus and spleen, characterized by increased proliferative capacity in response to T- and B-dependent mitogens (31, 32, 36). In addition, a partial blockade of T cell differentiation and profound alterations of the thymic microenvironment, which persisted in adulthood, were observed, thus suggesting potential consequences for the programming of neuroendocrine-immune reactions in later life (36, 37).

We have used these Tg mice to verify the impact of a dysfunctional GR from early embryonic life on the susceptibility to EAE. Counter to the expected predictions, here we report that the long-lasting GR deficiency of Tg mice programs the resistance to EAE induction and that iNOS/NO has a chief role in this protection through NO-induced immunosuppression.

Materials and Methods

Mice

Female B6C3F1 (C57B female × C3H male) (H-2b) wild-type (Wt) and Tg (line 1.3) mice, in which a transgene, driven by a neurofilament promoter, was inserted in the genome constitutively expressing antisense RNA against the GR (30), were bred at the OAS Institute (Troina, Italy). The GR mRNA levels and GR binding capacity in the brain, pituitary, and immune organs of these Tg mice are decreased by 40–50% compared with those in Wt mice (30, 32).

Mice were housed five per cage in a temperature- (21–23°C), humidity- (60%), and light- (12-h light, 12-h dark cycle, lights on at 0600 h) controlled room. Food and tap water were available ad libitum. Studies were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health) and were approved by the review boards of the OAS Institute (Troina, Italy).

Induction of EAE

EAE Wt and GR-deficient mice were immunized on days 0 and 7 s.c. in the flank with 200 µl emulsion containing 300 µg myelin oligodendrocyte glycoprotein (MOG) peptide aa 36–50 (EYGVYRSPEVRVYHL) (38, 39) added to PBS and emulsified in CFA supplemented with 500 µg heat-inactivated Mycobacterium tuberculosis (Difco, Detroit, MI) as reported previously (40). Wt and GR-deficient mice injected with vehicle (PBS) or CFA (Sigma-Aldrich, St. Louis, MO) alone served as sham-immunized and control immunized mice, respectively. Mice were also injected i.v. with 500 ng pertussis toxin derived from Bordetella pertussis (Sigma-Aldrich, Poole, U.K.) in 500 µl PBS in the tail vein immediately after and 48 h after Ag immunization. Mice were weighed before immunization and from day 8 were weighed and examined for neurological signs of EAE as follows: 0, no signs; 0.5, paresis (tail weakness) or partial paralysis; 1, complete tail paralysis; 2, paraparesis, hind limb weakness; 2.5, partial hind limb paralysis; 3, complete hind limb paralysis; 3.5, paraplegia; 4, quadriplegia; and 5, death.

Pathological examination

Five-micrometer wax sections of the CNS were stained with either H&E or Lulu Fast Blue/Cresyl Fast Violet for myelin evaluation (41).

Corticosterone (cort) measurement

Groups of 8–10 Wt and GR-deficient mice injected with PBS, CFA, or MOG36–50 in CFA were killed between 0800 and 1200 h at times after immunization. Great care was taken to keep the mice undisturbed before the experiment. Trunk blood was collected after decapitation, and plasma was stored at −80°C for hormones and nitrite assays. Cort was assayed using a specific RIA (ICN Biomedical, Costa Mesa, CA) (32). Results are represented as the mean ± SEM. No significant differences were observed between the control groups sacrificed at different time points after PBS injection, and thus results were pooled and expressed as preimmunization levels.

T cell proliferation assays

Splenocytes and lymph node cells (LNC) were isolated and processed as previously described (32, 42). Briefly, single-cell suspensions of splenocytes and LNC were collected from at least four individual mice per group, and 0.2 ml containing 2 × 106 cells/ml was cultured in 96-well microtiter plates (Costar, Cambridge, MA) in nutrient medium: RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 1-glutamine, 2-ME, penicillin G (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated FCS. The cultures were incubated at 37°C in humidified 5% CO2/95% O2 with Con A (0.5–40 µg/ml), MOG36–50 (0.5–40 µg/l), or LPS (0.5–500 ng/ml). After 72 h, 1[H]thymidine (25 Ci/mmol; 1 µCi/well; New England Nuclear, Boston, MA) was added, and the cultures were incubated for an additional 6 h. The cells were harvested, and the proliferation index was calculated as the mean counts per minute of triplicate wells containing Con A or MOG36–50 divided by the mean counts per minute of triplicate wells of cells only. For the time-course study, groups of four Wt and GR-deficient mice, immunized with MOG36–50 in CFA or CFA only, were sacrificed at 3, 7, 10, and 20 days, and spleen and LN cells were cultured in the presence or the absence of Con A or MOG36–50 (2.5 µg/ml). Wt and GR-deficient mice injected with PBS only were sacrificed at the same time points and used as controls.

Isolation and culture of peritoneal macrophages

On day 10 after immunization with MOG36–50, peritoneal macrophages were isolated from Wt and GR-deficient mice following i.p. injection of 10 ml RPMI 1640 medium, which was collected after gentle massage of the peritoneum for 5 min (43, 44). The cell suspension was adjusted to 1 × 106 cells/well, and the adherent macrophages were cultured in RPMI 1640 supplemented with 1 mM l-glutamine, 0.5% HEPES, penicillin G (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated FCS in 24-well plates at 37°C. The macrophages were cultured for 24 h either alone (basal level) or in the presence of 100 ng/ml LPS. The effect of LPS activation was studied in the absence or the presence of the NO synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) (10−4 M), the inactive enantiomer Nω-nitro-arginine methyl ester (L-NMMA), the inactive enantiomer Nω-nitro-L-arginine, and the specific inhibitor of iNOS, L-NAME (100 µg/ml). These studies were also conducted in the presence or the absence of the GR antagonist mifepristone, RU-486 (17β-hydroxy-11β(34)-dimethylamino-phenol)-17α(propynyl)-extra-9-dien-3-one), at a concentration of 10−6 M (provided by Roussel-UCLAF, Hoechst, Romainville, France). After 24 h the supernatants were collected for nitrite analysis. The number of macrophages was determined after harvesting by trypsinization.
in Ca2- and Mg-free PBS and washing in RPMI medium. Peritoneal macrophages from sham-injected Wt and GR-deficient mice were used as controls.

**Inhibition of iNOS**

To evaluate the effects of macrophages on splenocyte or LNC proliferation, peritoneal macrophages from Wt and GR-deficient mice were isolated on day 10 following immunization with MOG36-50, and the cell suspension was added to lymphoid cell preparations according to the method described by Willenborg et al. (46). Briefly, cells were washed three times and added (1–4 × 10^5 cells in 100 μl) to LNC or spleen cell cultures (2 × 10^5 cells in 100 μl) collected on day 10 of immunization. The cultures were incubated with 5 μg/ml CM and cell proliferation was assessed as described above. The effect of antagonism of iNOS on the mixed cultures was conducted using t-NNAME, t-NMMA, or t-NIL (2 μg to 2 mg/ml) in the presence of MOG36-50 (5 μg/ml), and after 3 days in culture proliferation was assessed. Supernatants were harvested and stored at −70°C until assayed.

**Nitrite/nitrate assay**

The production of NO, as measured by the formation of the stable decomposition product nitrite, was determined in cell-free supernatant and plasma (43, 44). Briefly 100 μl supernatant was mixed with 100 μl Griess reagent (1% sulfanilamide plus 0.1% N-1-naphthylethenediamine in 2.5% H3PO4). For nitrite and nitrate studies, plasma aliquots were diluted 1/5 with phosphate buffer, and 0.6 ml was incubated at room temperature for 2 h with 0.05 ml nitrate reductase (1 U/ml; Sigma) and 0.05 ml NADPH (1 mM). Excess NADPH was oxidized by addition of 0.05 ml phenazine methosulfate (80 mM). The samples were deproteinized using 0.1 ml zinc acetate (0.5 M) and 0.1 ml NaOH (0.5 M), followed by centrifugation (1900 × g for 10 min). Nitrite levels were determined in the supernatant using Griess reagents. After 10 min at room temperature, the OD at 540 nm was measured. In parallel, a sodium nitrite/nitrate standard curve (1–200 μM) was generated. Samples were tested in triplicate, and results represent the mean ± SE of at least four to six mice per group.

**Effect of macrophage-derived factors on lymphoid cell proliferation**

Conditioned medium (CM) from encephalitogenic myelin basic protein-sensitized LNC cultures induces the expression of iNOS/NO in vitro (47). To examine whether macrophage-derived factors can influence lymphoid cell proliferation and to study the involvement of iNOS/NO, peritoneal macrophages from Wt and GR-deficient mice sensitized with MOG36-50 (day 10) were cultured for 24 h. The cells were pelleted, and the supernatants (referred to as CM) were either used directly or snap-frozen at −80°C (47). Supernatants from macrophage cultures established from healthy Wt and GR-deficient mice were used as controls.

The effects of CM on the proliferative capacity of Wt and GR-deficient mouse LNC or splenocyte cultures (taken on day 10) were determined by adding CM (diluted 50 or LPS, and the proliferative response was measured. In preliminary dose-response studies, 75%–100% CM gave maximal effects, and this concentration was selected for all experiments.

**Endogenous GC levels are not associated with resistance to active EAE**

GR-deficient mice exhibit an aberrant HPA axis response to stress, demonstrated both in vivo and in vitro (31–35), and thus one possible factor contributing to the EAE resistance could be the endogenous levels of plasma cort. However, similar levels of cort were present in the sham (PBS)-injected (i.e., preimmunization levels; Fig. 3A) Wt and GR-deficient mice. Following immunization with CFA alone (data not shown) or MOG36-50 in CFA (Fig. 3A) Wt mice exhibited a robust plasma cort response that was sustained during the course of EAE (p < 0.01 vs preimmunization levels). Likewise, plasma cort levels in GR-deficient mice following immunization with CFA alone (data not shown) or MOG36-50 in CFA were significantly (p < 0.01) increased above levels in sham-immunized animals (Fig. 3A); however, these were significantly lower than those in Wt mice during days 1–12 following MOG immunization (p < 0.05). After this time the levels in Tg mice increased to within the range observed in Wt mice.

The marked increase in plasma cort of Wt mice was reflected by a profound thymic atrophy (days 12–40), whereas in GR-deficient mice, thymus weight was reduced to a lesser extent; it was significantly (p < 0.01) higher compared with that in Wt mice (days (100 μg/ml) (19, 48) 0–1 days after immunization for 8 consecutive days. Solutions were prepared daily, fluid consumption in both t-NIL-treated and untreated mice was monitored due to changes in water consumption during the development of disease symptoms, and doses of t-NIL were adjusted accordingly (48). Alternatively, t-NIL (3 mg/kg) was administered i.p. daily (48). Comparison between oral and i.p. delivery of inhibitors yielded similar levels of inhibition of NO production. Mice were weighed and examined twice a day for signs of EAE, and blood from the tail vein was sampled for determination of plasma nitrite/nitrate levels. On day 10 after MOG sensitization, groups of saline- and t-NIL-injected mice (four mice per group) were sacrificed, and the proliferation of spleen and draining lymph node cells was measured.

**Results**

Expression of GR antisense RNA confers resistance to active EAE

In contrast to female Wt B6C3F1 (C57B × C3H) mice, which developed relapsing remitting EAE (35 of 38), only one of 35 GR-deficient mice developed clinical or histological signs of disease (Fig. 1A). Wt mice exhibited typical signs of EAE, with an onset of 13.4 ± 1.7 days and a mean maximal score of 3.2 ± 0.3 at 16.0 ± 0.8 days. Following a period of remission, 18 of 35 mice exhibited relapses of neurological deficits (mean day of onset, 33.3 ± 4.5; Fig. 1A). The GR-deficient mouse that exhibited neurological signs showed tail weakness after 12 days (maximal score, 0.5) for 2 days and did not show signs of relapse until it was sacrificed on day 30 (Fig. 1A). From 12 days (12.2 ± 0.9 days) after immunization, Wt, but not Tg, mice showed a progressive loss of body weight. At the end of the experimental protocol the mean weight of Wt mice was significantly reduced (20.5 ± 0.4 g; p < 0.01) compared with that of controls (CFA and PBS-treated; 32.5 ± 1.2 g) or Tg mice (34.5 ± 2.9 g; Fig. 1B). Histological examination of the CNS of Wt mice at the peak of neurological disease (days 16–20) showed the presence of typical signs of EAE, i.e., subpial inflammation in the cord with marked lesions of demyelination (Fig. 2, A and B). In contrast, in GR-deficient mice, neither inflammation nor demyelination was present at this time (Fig. 2, C and D).

**Statistical analysis**

Data were analyzed by two-way ANOVA, with group and time as independent variables, and by Student’s t test. Comparisons a posteriori between different experiments were made using the Newman-Keuls test (49).
T cell proliferation is down-regulated in GR-deficient mice

To determine whether EAE resistance was related to modulation of the immune response, the T cell proliferative responses to Con A and MOG36-50 in the spleen and LN were examined (Fig. 4, A and B). Although the proliferative responses following PBS injection of GR-deficient mice were higher \((p < 0.05)\) than those of Wt mice (Fig. 4, A and B, preimmunization) following MOG36-50 immunization, the responses to both Con A and MOG were dramatically reduced in GR-deficient mice compared with Wt mice \((p < 0.01)\) especially on days 7–20 postimmunization, when the proliferation index of both splenocytes and LNC was ~3- to 7-fold lower in GR-deficient compared with Wt mice (Fig. 4, A and B). These results indicate a dramatic down-regulation of cell proliferative capacity. Furthermore Wt mice responded to Con A and MOG36-50 in a dose-dependent manner (Fig. 4C), particularly between 2.5 and 10.0 \(\mu\)g/ml, while GR-deficient mice responded to the lower concentrations of 0.5 and 1.2 \(\mu\)g/ml Con A, and higher concentrations resulted in a sharp reduction of proliferative capacity (Fig. 4C). This shift in the proliferative dose-response curve of GR-deficient mice LNC suggests the presence of powerful counterinhibitory mechanisms down-regulating T cell responses in Tg mice early during EAE induction.

**GR-deficient mouse macrophages inhibit T cell proliferation**

Macrophages of EAE-resistant animal strains can down-regulate Ag-driven T cell proliferation (46, 50). To examine whether the EAE resistance of GR-deficient Tg mice involves the macrophage compartment, peritoneal macrophages or CM from macrophage cultures were incubated with splenocytes or LNC in proliferation assays. Addition of peritoneal macrophages or CM prepared from control Wt or Tg mouse macrophage cultures did not influence the proliferative effect of LNC or spleen cell cultures from Wt and GR-deficient mice (data not shown). Similarly, macrophages or their conditioned media from MOG-immunized Wt mice failed to modify the proliferative response of MOG36-50-immunized mice (Fig. 5, A and B). In direct contrast, addition of equal numbers of peritoneal macrophages from GR-deficient mice to Wt LNC cultures inhibited the MOG-driven cell proliferation in a dose-dependent manner (Fig. 5A). Similarly, CM prepared from these Tg macrophage cultures established on day 10 after MOG36-50 immunization (Tg-CM) significantly inhibited \((p < 0.01)\) the proliferation of LNC from Wt mice in response to increasing concentrations of MOG36-50 (Fig. 5B), whereas Wt-CM was unable to modify the MOG proliferative response of Wt LNC preparations (Fig. 5B). These results indicated that factors produced by macrophages of GR-deficient Tg mice, but not Wt mice, powerfully counter-regulate LNC proliferation.

**Inhibition of iNOS inhibits the suppression of T cell proliferation by GR-deficient macrophages**

Among the many inflammatory mediators inhibited by GCs, is macrophage-derived NO (24, 25), a free radical synthesized in a reaction catalyzed by iNOS. Since iNOS/NO is known to inhibit proliferation of different cell types (20, 21, 46, 50–52), we investigated whether the GR deficiency of Tg mice influences endogenous NO, by application of L-NAME and L-NMMA or the specific iNOS inhibitor, L-NIL (45, 48). It was observed that both Tg-CM (Fig. 5B) and Tg macrophages (Fig. 5C) lost the ability to inhibit Ag-driven LNC proliferation when NOS inhibitors (L-NAME, L-NMMA, L-NIL), but not inactive enantiomers \((N^\bullet\text{monomethyl}-d-arginine, \text{not shown})\) were applied with the macrophage cultures. Thus, in the mixed culture condition, NOS inhibition efficiently counteracted the Tg macrophage-induced decrease in the Wt LNC proliferative response to MOG36-50 (Fig. 5C). Both L-NAME and L-NMMA dose-dependently reversed the reduction of the Wt LNC proliferative response to MOG resulting from addition of Tg macrophages, but L-NIL was ~10- to 50-fold more potent (Fig. 5C). Likewise, L-NIL \((20 \mu\)M) sharply counteracted the Tg-CM-induced inhibition of the Wt LNC proliferative response to increasing concentrations of MOG36-50 (Fig. 5B). In contrast, addition of NOS inhibitors to Wt macrophages failed to modify the proliferative response of Wt cells in both CM (Fig. 5B) and mixed culture conditions (Fig. 5C).
To further investigate this phenomenon, the ability of NOS inhibitors, NO scavengers, or NO donors to directly affect MOG- or LPS-driven LNC or spleen cell proliferation was studied. It was observed that the low proliferation of mitogen-stimulated LNC from GR-deficient mice was significantly increased by direct application of L-NIL (20 μM) to levels comparable to those measured in Wt mice, whereas the iNOS inhibitor did not affect LNC proliferation of Wt-sensitized mice cultures (Fig. 5D). Further evidence for the contribution of NO in T cell down-regulation of GR-deficient mice was revealed when the NO scavenger ferrous hemoglobin (10^{-6} M) was added to Tg LNC or spleen cultures stimulated by LPS (50 ng/ml; 6500 ± 1050 cpm in the presence compared with 1600 ± 630 cpm in the absence of NO scavenger). In contrast, a sharp inhibition of the Wt spleen cell proliferative response to LPS (50 ng/ml) was measured in the presence of the NO donor spermine NONOate (spermine NO complex), in which a complete suppression was observed at 1 mM (750 ± 95 cpm in the presence compared with 7500 ± 1200 cpm in the absence of the NO donor). Together, these findings indicate that the GR deficiency of Tg mice up-regulates iNOS/NO generation after MOG36-50 sensitization, pointing to NO as the candidate immunosuppressor molecule.

Nitrite/nitrate levels are elevated in GR-deficient mice

As an indirect measure of NO formation, endogenous plasma nitrite/nitrate production in vivo during EAE and by macrophages and splenocytes in vitro was examined. In GR-deficient mice, circulating nitrite and nitrate levels were elevated 3–20 days after immunization (Fig. 6A), whereas in Wt mice, levels were 3- to 4-fold lower before and during the acute disease compared with days 30 and 40. Similarly, macrophage and splenocyte cultures of GR-deficient mice established 3, 7, 10, and 20 days following MOG36-50 immunization exhibited 3- to 5-fold higher LPS-induced NO production (Fig. 6B). In peritoneal macrophage cultures, NO was maximally increased from day 7, whereas in splenocytes, LPS-stimulated NO reached a maximal concentration on days 10–20. In contrast, in Wt macrophage and splenocyte cultures, nitrite levels were significantly lower, although a progressive increase was observed (Fig. 6B).

In summary, during the critical phase of EAE induction, endogenous NO levels were significantly increased in GR-deficient mice compared with their Wt susceptible counterparts in both in basal and activated conditions.

Regulation of macrophage NO production by GC is reduced in GR-deficient mice

Next, we investigated the ability of GCs to regulate NO production in macrophage cultures from mice 10 days after MOG immunization compared with direct inhibition of iNOS by L-NIL.

Basal nitrite production from peritoneal macrophages of GR-deficient mice was significantly higher than that of Wt mice (p < 0.01; Fig. 7C). Both basal and LPS-induced production of nitrites were inhibited by L-NAME, L-NMMA (data not shown), and L-NIL (Fig. 6C) in a dose-dependent manner. However, L-NIL was ~10- to 50-fold more potent, with an IC50 of 8 μM, compared with L-NMMA (90 μM) or L-NAME (700 μM), whereas the inactive enantiomer of L-NAME, N⁴-monomethyl-L-arginine was ineffective. These findings clearly supported the hypothesis that iNOS activation was responsible for higher nitrite formation of Tg macrophage cultures. As a comparison administration of cort to LPS-stimulated macrophages induced a dose-dependent inhibition of NO formation in macrophage cultures from Wt mice, with an IC50 of 4.7 × 10^{-9} M and complete prevention of NO formation at 10^{-7} M. This effect was mediated by GRs, since pretreatment of

FIGURE 2. Histopathology of Wt (A and B) and GR-deficient (C and D) mice following MOG36–50 immunization. Inflammation and perivascular cuffs (PVC) of inflammatory cells were found in Wt (A), but not Tg (C), mice. Demyelination (arrow) in the subpial regions of the spinal cord was observed in Wt (B, arrow), but not Tg (D), mice. NB demyelination was limited to the CNS and was not observed in the peripheral nerves (B, PN white arrow).
macrophage cultures with the GR antagonist, RU486 (10⁻⁶ M), completely counteracted cort-induced inhibition of nitrite production (28.8 ± 3.5 μM after RU486 compared with 6.9 ± 0.7 μM in the absence of RU486; p < 0.01). In macrophage cultures from GR-deficient mice, cort-induced inhibition of NO formation was reduced compared with that of Wt cultures (IC₅₀ = 6.78 × 10⁻⁶ M). Moreover, cort was unable to totally suppress NO formation even under the highest concentrations (10⁻⁶ – 10⁻⁷ M), whereas in the presence of l-NIL, NO levels were comparable to those in Wt cultures, again supporting iNOS activation in macrophages cultures of GR-deficient mice. Thus, the high basal production of nitrite levels in Tg macrophages was not suppressed by exogenous application of cort, and macrophage sensitivity to the cort inhibitory effect was reduced.

In addition, supernatants from mixed cultures of Wt LNC and Tg macrophages assayed for nitrite generation demonstrated a sharp increase in NO concentration (from a basal level of 25 ± 6 to 134 ± 14 μM) 36 h after addition Tg macrophages. In contrast, mixed cultures of Wt LNC with Wt macrophages resulted in a marginal increase in nitrite production (36 ± 8 μM).
Specific iNOS inhibition reverts the resistance of GR-deficient mice into susceptibility

To examine the in vivo relevance of increased NO production in GR-deficient mice, the NOS inhibitors, L-NAME and L-NIL, were administered during the induction phase of EAE. Wt and GR-deficient mice were injected i.p. with L-NAME for 8 consecutive days, whereas L-NIL was administered by the oral route. L-NAME treatment alone of nonimmunized Wt and GR-deficient mice failed to induce neuropathological effects (data not shown). In Wt mice immunized with MOG, L-NAME treatment resulted in a significant exacerbation of the clinical signs (mean EAE score, 3.55 ± 0.29) compared with saline-treated Wt mice (2.35 ± 0.35; p < 0.01; Table I). Body weight was also significantly decreased in L-NAME-treated mice (17.05 ± 1.5 g on days 35–40) compared with untreated MOG-immunized Wt mice (22.70 ± 1.20 g; p < 0.05; Table I). In addition, the onset of EAE in L-NAME-treated Wt mice was significantly earlier (7.4 ± 0.9 days) compared with that (12.8 ± 1.5 days) in saline-treated Wt mice (p < 0.01; Table I) and whereas 6 of 8 Wt saline-injected mice exhibited clinical relapse, 8 of 10 L-NAME-treated mice exhibited relapses. In GR-deficient mice, L-NAME treatment significantly increased the number of mice with EAE (10 of 10 mice) compared with untreated GR-deficient (1 of 10; Table I; p < 0.01) and the clinical mean EAE score (3.5 ± 0.4) compared with saline-treated Tg mice (1 of 10; EAE score, 1.5; p < 0.01). In addition, L-NAME treatment significantly decreased body weight in MOG-immunized Tg mice.
mean body weight, 18.00 ± 2.0 g) compared with untreated Tg mice (30.5 ± 2.50 g; p < 0.01; Table I). The onset of disease in GR-deficient mice given L-NAME was 9.5 ± 1.2 days, which was significantly earlier than that in untreated mice (18.0 days). Although the severity of disease declined (mean score, 1.5 ± 0.5), the GR-deficient mice did not fully recover for the entire observation period (35–40 days).

Having established that L-NAME precipitated EAE in GR-deficient mice, we evaluated whether the specific iNOS inhibitor, L-NIL, had comparable effects. The appearance of clinical signs in both Wt and Tg mice after L-NIL administration was accelerated compared with untreated Wt MOG-sensitized mice (Table I; mean EAE score for L-NIL-treated WT mice, 3.45 ± 0.32 compared with 2.70 ± 0.24 in untreated mice; p < 0.01). The mean EAE score for L-NIL-treated Tg mice was 3.50 ± 0.30 compared with
that in untreated mice (in eight of eight a clinical score of 0.0; \( p < 0.01 \); Table I). The onsets of disease in Wt and GR-deficient mice given \( \alpha \)-NIL were 8.0 ± 0.5 and 8.7 ± 1.0 days, respectively, significantly earlier than in untreated mice (\( p < 0.01 \); Table I). Although Wt recovered from clinical disease, GR-deficient mice did not fully recover for the entire observation period (35–40 days). This effect was specific for Tg mice, since Wt mice treated with \( \alpha \)-NIL fully recovered, and subsequently five of eight mice exhibited clinical relapses. Finally, body weight was less severely affected by \( \alpha \)-NIL compared with \( \alpha \)-NAME in both Wt and GR-deficient mice (Table I).

No significant differences were observed when \( \alpha \)-NIL treatment was given i.p. compared with oral administration in either time of appearance or severity of disease.

\( \alpha \)-NIL treatment suppressed the up-regulated plasma nitrate and nitrite levels of MOG-sensitized GR-deficient mice and reduced the already low plasma nitrite concentrations of Wt MOG-immunized mice (Fig. 7A). A comparable inhibition of nitrite levels was observed after \( \alpha \)-NAME treatment (not shown). Furthermore, \( \alpha \)-NIL treatment significantly increased both basal and Ag-driven proliferation of splenocytes and LNC (Fig. 7B). Together these findings strongly support that the GR deficiency of Tg mice is responsible for iNOS-derived NO up-regulation and identifies GR-NO interaction as the key mechanism responsible for the EAE-resistant phenotype.

**Discussion**

The present study demonstrates that defective GR expression, binding capacity, and signaling in genetically modified mice are associated with a dramatic reduction in the susceptibility to EAE. Furthermore, in vitro studies revealed that the increased NO production by macrophages from GR-deficient mice during EAE was responsible for the decreased T lymphocyte proliferation. Moreover, in vivo treatment of GR-deficient mice with specific inhibitors or antagonists of NO during EAE induction resulted in susceptibility to EAE in Tg mice identical with that in Wt mice, thus supporting the indication that EAE resistance was linked to increased NO production as a result of GR deficiency. The dual effects of NO in EAE have been described previously, including its down-regulatory role (19–21, 44, 46, 50, 53–55). However a direct link among impaired GR, increased endogenous NO production, and EAE resistance has, to our knowledge, not been reported. In view of the critical importance of GRs during the development of thymus-dependent immune functions (32, 37, 56, 57), it is unknown whether early embryonic life exposure to a dysfunctional GR may directly and/or indirectly influence the programming of the mononuclear phagocyte response to inflammatory signals in adult life and/or interfere with endogenous molecules involved in or synergizing with NO production (20, 21).

The absence of a correlation between endogenous production of GCs and the EAE resistance of GR-deficient mice observed in the present study clearly indicates that in this strain and strain of mice, the plasma cort response following MOG\(_{36-55}\) immunization is not a predictor of disease susceptibility. The transgenic mouse used in the present work has been characterized as a valuable model to study the consequences of life-long central GR dysfunction for HPA axis regulation and other GC-controlled brain functions (30, 34, 35). In previous reports GR-deficient mice displayed a reduced GC negative feedback (33, 34), exaggerated adrenocorticotropic hormone (ACTH) response to stress, and exogenously administrated corticotropin-releasing hormone (35), but maintained normal early morning levels of both ACTH and cort, because of the hyposensitivity of the adrenal gland to ACTH (34). Hence, these Tg mice show reduced GR capacity, which is not compensated for by elevations in circulating GC levels (35). In addition, plasma cort concentrations increase to the same extent in Wt and GR-deficient mice after acute IL-1\( \beta \) challenge (58). In Wistar rats treated during neonatal life with dexamethasone, the susceptibility to EAE in adult life is increased in the face of a reduced cort response to endotoxin challenge, an impaired macrophage capability to respond to LPS, and an increased expression of proinflammatory cytokines in the spleen (15). Early life exposure to Gram-negative bacterial endotoxin results in a remarkable protection from adjuvant-induced arthritis, while no difference in the cort response to immune challenge is observed (16). In addition, splenocyte proliferative response was sharply reduced in these animals (16). It is interesting to note that animals exposed to Gram-negative bacterial endotoxin early in life display decreased GR binding in hypothalamus, frontal cortex, and hippocampus in adult life (12, 13), but whether a reduction/dysfunction of GRs is also present in immune tissues is unknown. Although a number of studies indicate that the predisposition and/or severity of inflammatory and autoimmune diseases is not always associated with HPA axis reactivity (16, 17, 59, 60), an altered dialogue between the HPA axis and the immune system during development may program long-term effects in the mechanisms regulating immune responsiveness to inflammation, thereby contributing to modulate the severity or predisposition to inflammatory disorders (15–17). The present work demonstrates that activation of macrophage iNOS/NO in a result of GR deficiency in Tg mice represents one crucial regulatory mechanism.

**Table I. \( \alpha \)-NAME and \( \alpha \)-NIL reverse the resistance of GR-deficient mice to EAE**

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Treatment</th>
<th>No. of EAE</th>
<th>Body Weight</th>
<th>Clinical Score</th>
<th>Day of Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>Untreated</td>
<td>8/8</td>
<td>22.70 ± 1.20</td>
<td>2.35 ± 0.33</td>
<td>12.80 ± 1.50</td>
</tr>
<tr>
<td>GR-deficient</td>
<td>Untreated</td>
<td>1/10</td>
<td>17.05 ± 1.50*</td>
<td>3.55 ± 0.20**</td>
<td>7.40 ± 0.90**</td>
</tr>
<tr>
<td></td>
<td>( \alpha )-NAME</td>
<td>10/10</td>
<td>30.5 ± 2.50</td>
<td>1.5 ± N/A</td>
<td>18.00 ± 2.50</td>
</tr>
<tr>
<td>Wt</td>
<td>Untreated</td>
<td>8/8</td>
<td>18.00 ± 2.00**</td>
<td>3.5 ± 0.40**</td>
<td>9.50 ± 1.20**</td>
</tr>
<tr>
<td>GR-deficient</td>
<td>Untreated</td>
<td>8/8</td>
<td>20.0 ± 1.0</td>
<td>2.7 ± 0.24</td>
<td>13.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>( \alpha )-NIL</td>
<td>8/8</td>
<td>21.0 ± 0.5</td>
<td>3.45 ± 0.32**</td>
<td>8.0 ± 1.0**</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>0/8</td>
<td>29.5 ± 1.50</td>
<td>0.0 ± 0.0</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>( \alpha )-NIL</td>
<td>0/8</td>
<td>23.0 ± 1.50**</td>
<td>3.50 ± 0.30**</td>
<td>8.7 ± 1.50**</td>
</tr>
</tbody>
</table>

\( * \ p < 0.05 \) and \( ** \ p < 0.01 \) compared with untreated within each treatment group by Newman-Keuls test (50).

5856 GLUCOCORTICOID RECEPTOR DEFICIENCY, NO, AND EAE

Wt and GR-deficient mice were treated or not with the NOS inhibitor \( \alpha \)-NAME (125 mg/kg, twice a day, i.p.), 24 h after the second injection of Ag, for 8 consecutive days or the specific iNOS inhibitor \( \alpha \)-NIL (100 \( \mu \)g/ml in the drinking water) for 8 consecutive days. Mice were examined twice a day for signs of EAE and their body weights were recorded daily until sacrifice (35–40 days). Results are the mean ± SEM. Mortality (score = 5) was observed within the first 3 wk only in \( \alpha \)-NAME-treated Wt (2 of 10) and GR-deficient (2 of 10) mice.
lower in Tg compared with EAE-susceptible Wt mice. Since NO is involved in the modulation of neuroendocrine functions (61), and during inflammation NO can restrain the HPA axis (4), the up-regulated nitrite levels of GR-deficient mice may at least in part contribute to reduce the cort concentration in response to MOG immunization. Interestingly, PVG rats, which have a very low susceptibility to myelin basic protein-induced EAE (50) develop higher serum levels of NO (50), and spleen cell cultures stimulated in vitro with IFN-γ produce significantly higher levels of NO than the EAE-susceptible Lewis rat (50). Consistent with this finding, NO has been shown to play a critical role in the recovery of Lewis rats from EAE and the maintenance of resistance to reinduction of disease (53). Moreover, rodents immunized with CFA are resistant to subsequent attempts to induce autoimmune diseases through mechanisms requiring a functional iNOS gene (54). Rapid induction of Th1-type cytokines and iNOS are also responsible for the protective effects of high dose Ag therapy in EAE (62). The results of the present study thus indicate that NO is involved in the control of susceptibility to EAE in this sex and mouse strain, and that such control is directly linked to GR.

GR-deficient mice have previously been shown to mount higher T cell responses to T-dependent mitogens during postnatal maturation of thymus and spleen (32). However, in this study MOG-immunized adult GR-deficient mice showed a marked reduction of T cell responses despite the higher basal proliferative capacity of GR-deficient lymphocytes. In sharp contrast with Wt mice responses, application of Con A or MOG in vitro induced a significant and long-lasting down-regulation of cell proliferation, especially after the second immunization challenge, suggesting that after a first priming signal such an inhibitory mechanism(s) was more actively stimulated in GR-deficient mice cultures. Although different events, including alteration of cytokine production, changes in T cell sensitivity to proapoptotic signals, and/or the production of suppressor molecules (20, 21), may participate in the immunosuppression of Tg mice, the final common pathway appears to be macrophage activation of iNOS/NO, as demonstrated by studies ex vivo and in vitro, using NO inhibitors, NO scavengers, or NO donors. In addition, the ability of L-NIL treatment in vivo to sharply increase MOG-reactive LNC and spleen cell proliferation and revert the EAE resistance of GR-deficient mice further supports the participation of iNOS/NO in counteracting MOG reactive cell expansion during the early induction phase of EAE (46, 55). Several endogenous and exogenous molecules can induce (LPS, IFN-γ, TNF-α, and IL-1β) or repress (TGF-α, IL-4, IL-8, IL-10, corticosteroids, and estrogens) iNOS activity. Thus, complex interplays between hormones and cytokines may interact in vivo to temporally and spatially induce differential expression of iNOS in organs and tissues of Tg mice, leading to inhibition of autoreactive cell proliferation. That NO is involved in T cell regulation is well documented, since both aggravation and suppression of EAE have been attributed to NO (19–21; see Ref. 55 for review). Mice lacking the ligand-binding chain of the IFN-γ receptor (IFN-γR−/−) develop severe and usually fatal EAE as opposed to their Wt IFN-γR−/+ counterparts, which are resistant to EAE (46). Willenburg et al. (46) showed that reduced NO production by peripheral macrophages correlated with increased autoreactive T cell proliferation of IFN-γR−/− mice. In contrast, peritoneal exudate from IFN-γR−/-/−-resistant mice produced high levels of NO and down-regulated Ag-driven cell proliferation of IFN-γR−/− mice (46).

The down-regulatory effect of NO in primary EAE has been previously suggested to involve inhibition of T cell proliferation as well as inhibition of cell adhesion and migration (55). Such modulation may be dependent on the known functions of iNOS/NO, such as suppression of cytokine production, scavenging of superoxide, and/or apoptosis of macrophages or (encephalitogenic) T cells (20, 21). Inducible NOS-derived NO can modulate cytokine response of macrophages and T cells through activation/inactivation of ion channels, G proteins, protein tyrosine kinase, Janus kinases (Jak1, Jak2, Jak3, Tyk2), mitogen-activated protein kinases (i.e., extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, c-Jun NH2-terminal kinases), caspases (i.e., caspase 1, caspase 3), metalloproteases, and phosphoproteases (63). Thus, complex mechanisms may underlay the NO-mediated inhibition of MOG-reactive cell proliferation observed in the present study.

To establish a direct link between the GR deficiency and the increased NO production in GR-deficient mice, we evaluated the capability of exogenously applied GC to inhibit macrophage production of nitrite corresponding to the phase of maximal inhibition of autoreactive cell proliferation in Tg spleen and LNC cultures. Hence, in GR-deficient mice macrophage appeared resistant to GC inhibition, since cort was unable to suppress both basal and LPS-stimulated NO production, whereas in Wt mice a similar dose of cort significantly suppressed nitrite production. The inverse correlation between up-regulation of NO and decreased GR transcription and binding capacity in immune tissues (32) suggests that increased NO production after Ag priming may contribute to further lower GR expression. Our previous studies indicated the presence and uniqueness of the antisense mRNA transgene in different tissue (cortex, hypothalamus, pituitary, heart, liver, kidney, adrenal) extracts of Tg mice, thus suggesting a less stringent neuronal expression directed by the 2.4-kb human neurofilament i. gene promoter (30). Although not addressed in the present study, the decreased GR mRNA and binding capacity measured in thymus and spleen of Tg mice (31, 32) reflect transgene expression in immune tissues. The almost complete (70–90%) loss of GR mRNA transcription and binding capacity measured in lymphoid cells of GR-deficient compared with Wt mice (not shown) following Ag priming support this contention. Reaction of NO with sulfhydryl groups yields S-nitrosothiols (64), and nitrosylation of critical cysteine residues of the GR by NO has been demonstrated to cause a loss GR binding activity and GR signaling in L929 cells in vitro (64). Furthermore, incubation of GR with NO donors results in both time- and concentration-dependent decreases in ligand binding and GR signaling (64). It is therefore feasible that as well as the specific immunization paradigm (MOG and CFA) and the rodent strain, which are known to modulate NO production, GR down-regulation may also play a pivotal role in NO generation. Thus, the GR deficiency in the Tg mice may prime monocytes and macrophages to produce higher amounts of NO via iNOS induction directly or through increased production of Th1 cytokines (62). Our data argue that an excess production of NO can trigger a feedback loop involving loss of GR signaling, thus perpetrating or exacerbating NO output at a critical time during Ag priming, resulting in deletion and/or suppression of Ag-specific cells.

That iNOS is involved in EAE has been shown by Gold et al. (19) using the iNOS-specific inhibitor L-NIL, which induced a marked aggravation of neurological deficits in active myelin basic protein-induced EAE, whereas treatment of rats with adoptive EAE was protective. In another model of inflammation, i.e., streptococcal cell wall-induced arthritis in the rat, McCartney-Francis et al. (48) demonstrated that pharmacological inhibition of iNOS with L-NIL exacerbated streptococcal cell wall-induced arthritis, whereas the nonselective NO inhibitor, L-NMMA, was disease protective, suggesting different roles for constitutive and inducible NOS isoforms in the context of joint inflammation. In our study we demonstrate that L-NIL is more potent in inhibiting NO production
by LPS-stimulated peritoneal macrophages in vitro, but is also equally as effective as a nonselective inhibitor of NOS in suppressing circulating nitrates in vivo during EAE. Regardless of the route of administration and the dose regimen used, both l-NNAME and L-NIL treatment significantly accelerated the onset of and increased the severity of clinical EAE in GR-deficient and Wt mice. Interestingly, while Wt mice fully recovered from acute disease, the NOS inhibitors prevented full recovery in GR-deficient mice, suggesting that factors/mechanisms contributing to the recovery of Wt mice are not fully operative in mice with a dysfunctional GR.

In summary, this work identifies NO as a crucial mediator in EAE resistance of GR-deficient mice and highlights its prominent role as a key messenger of the HPA-immune axis. These results may explain the difference in the susceptibility to inflammatory diseases depending on prenatal experiences known to influence the expression and function of GRs and may thus have clinical implications for the development of autoimmune diseases in humans (65). Modulation of iNOS/NO by stress- or pharmacologically induced alterations at the GR level may also have potential therapeutic implications for such autoimmune diseases.

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