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Short Communication

Adoptive Transfer of Experimental Allergic Encephalomyelitis after *in Vitro* Treatment with Recombinant Murine Interleukin-12

Preferential Expansion of Interferon-γ-Producing Cells and Increased Expression of Macrophage-Associated Inducible Nitric Oxide Synthase as Immunomodulatory Mechanisms

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In an adoptive transfer model of experimental allergic encephalomyelitis, stimulation of lymph node cells with proteolipid protein and recombinant murine interleukin (rmIL)-12 before cell transfer accelerated the onset and exacerbates clinical disease.* In vitro *stimulation with proteolipid protein in the presence of rmIL-12 was associated with an increase in interferon-γ-producing cells and a decrease in IL-4-producing cells, indicating a preferential expansion of Th1 effector cells. This was supported by the finding that severe disease with rapid onset could be transferred with as few as 10 × 10^6 rmIL-12-stimulated lymph node cells. Immunohistochemical analysis confirmed that the accelerated onset of disease after* in vitro *stimulation with rmIL-12 coincided with an acute inflammatory response in the central nervous system. At peak disease, both control and rmIL-12 treatment groups exhibited extensive cellular infiltration with characteristic perivascular cuffing. No notable differences in either the cellular composition or cytokine expression within the lesions were seen between groups. However, the frequency of macrophages that stained positively for inducible nitric oxide synthase was increased in animals challenged with rmIL-12-treated lymph node cells. The results suggest that, in addition to promoting the preferential expansion of interferon-γ-producing cells by rmIL-12* in vitro, *secondary* in vivo *effects leading to macrophage activation and inducible nitric oxide synthase expression may contribute to the severe and protracted course of central nervous system inflammation in this model. (Am J Pathol 1996, 148:375–382)

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS) that is widely used for studying immunoregulatory mechanisms involved in the pathogenesis of multiple sclerosis.[1] In susceptible strains of mice, disease can be induced by injection of the encephalitogenic proteins myelin basic protein or proteolipid protein (PLP) in complete Freund's adjuvant.[2,3] Alternatively, EAE can be passively trans-

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ferred to naive animals using T lymphocytes, T cell lines, or T cell clones sensitized to myelin basic protein or PLP by stimulation *in vitro* with antigen before cell transfer.[4,5] The clinical course of disease is characterized by weight loss and a progressive paralysis, which commonly leads to complete hind limb paralysis. This paralytic episode coincides with an acute perivascular inflammatory response in the CNS that is composed predominantly of infiltrating macrophages and T cells.[6] In most species, disease remits spontaneously with the sequence of recovery being the reverse of that of onset.

Adoptive transfer experiments using T cell clones have shown the effector cells in EAE to be $CD4^+$ T cells that secrete interleukin (IL)-2 and interferon (IFN)-γ typical of a Th1 phenotype.[7] This is consistent with the cytokine profiles observed in the inflammatory lesions during early and peak disease during which high levels of T-cell-derived IL-2 and IFN-γ mRNA are observed.[8] In addition to the requirements for a Th1 cytokine profile for successful transfer of disease, VLA-4 expression also appears to be a prerequisite, as autoreactive Th1 T cell clones that lack this adhesion molecule were found to be nonencephalitogenic.[9] This is consistent with data indicating a pivotal role for cellular adhesion molecules in facilitating the passage of autoreactive cells across the blood brain barrier. In this respect, administration of neutralizing antibodies against either VLA-4 or VCAM-1 have been shown to block the transfer of EAE.[7,10] Although it is clear that $CD4^+$ T cells are required for disease induction, there is increasing evidence to suggest that macrophages play a central role in promoting late stage tissue damage. This is supported by the findings that *in vivo* depletion of macrophages before the onset of clinical symptoms, or inhibition of macrophage-derived inflammatory mediators such as tumor necrosis factor (TNF)-α, IL-1, or nitric oxide (NO) all prevent the development of disease.[11–14]

IL-12 is novel cytokine that has been shown to be a potent stimulator of IFN-γ production from natural killer and T cells.[15] In addition, results from a variety of *in vitro* assays as well as *in vivo* animal models have demonstrated preferential development of Th1-specific immune responses after IL-12 treatment.[16–18] In the context of EAE, we have shown that, in an adoptive transfer model of disease, the addition of recombinant murine (rm)IL-12 during *in vitro* stimulation of lymph node cells (LNCs) with PLP exacerbates clinical signs and symptoms after cell transfer.[19] To determine whether the addition of rmIL-12 during *in vitro* stimulation with PLP alters the balance of Th1 and Th2 cells, the frequency of IFN-γ- and IL-4-producing cells was determined before cell transfer. We have also carried out an extensive immunohistochemical analysis to determine whether rmIL-12 treatment alters the nature of the inflammatory response in the CNS. The results indicate that, in addition to promoting the preferential expansion of Th1 effector cells, treatment with rmIL-12 before cell transfer increases the production of macrophage-derived inducible nitric oxide synthase (iNOS) in the CNS throughout the course of disease. These findings are consistent with the proposed Th1 effector mechanism in EAE and support a central role for macrophages in mediating target tissue damage in this model.

Materials and Methods

Adoptive Transfer of PLP-Sensitized LNCs

EAE was adoptively transferred using LNCs from SJL/J mice (Jackson Laboratory, Bar Harbor, ME) immunized with PLP (residues 139 to 151) in complete Freund's adjuvant. The protocol for adoptive transfer has been described in detail previously.[19] LNCs were stimulated *in vitro* with either PLP alone (2 μg/ml) or PLP and rmIL-12 (20ng/ml). After incubation for 96 hours, cells were washed twice, and either 10 or 30 $\times$ 10^6 cells were injected intraperitoneally into naive mice.

Determination of IFN-γ- and IL-4-Producing Cells

The frequency of IFN-γ- and IL-4-producing cells after *in vitro* stimulation of LNCs with PLP and IL-12 was determined by ELISPOT assay. The capture and detection antibodies used were clones BVD4–1D11 and BVD6–24G2, respectively, for IL-4 (Pharmingen, San Diego, CA) and R46A2 and XMG1.2 for IFN-γ. The conditions of the assay have been described in detail elsewhere.[20]

Clinical Evaluation of Disease

After the transfer of cultured LNCs, recipient mice were monitored for clinical signs of EAE and scored on the following scale: 0.5, distal limp tail; 1.0, complete limp tail; 1.5, limp tail and hind limb weakness (unsteady gait); 2.0, partial hind limb paralysis; 3.0, complete bilateral hind limb paralysis.

Immunohistochemistry

Mice were sacrificed either 6 days after cell transfer or after they had developed complete hind limb paralysis. Brains were cross-sectioned at the third ventricle, cryopreserved in OCT (Tissue Tek, Miles Laboratories, Elkhart, IN), and stored at −70°C. The 5 to 7 μm thick cryosections of brain tissue were cut and immediately fixed in ice-cold acetone, air dried, and stored at −20°C. Methods for immunoperoxidase staining using streptavidin/horseradish peroxidase/aminoethyl carbazole have been described.[6]

Antibodies Tested

Antibodies to cell surface antigens tested included CD4 (RM4–5), CD8 (53.7), B220 (RA3–6B2), and NK1.1 (PK136) (PharMingen, San Diego, CA) and Mac-1 (M1–70) (Boehringer Mannheim, Indianapolis, IN). Cytokine antibodies tested were anti-IFN-γ (XMG1.2), TNF-α (MP6-XT3), IL-2 (S4B6), IL-10 (XSC-1) (PharMingen), and IL-1β (rabbit polyclonal) (Endogen, Boston, MA). Other antibodies included VCAM-1 (MVCAM.A.429), ICAM-1 (CD54) (3E2) (PharMingen), and iNOS (rabbit polyclonal) (Upstate Biotechnology Inc., Lake Placid, NY). All antibodies are rat anti-murine monoclonals unless otherwise specified. Rat anti-mouse IgG1, IgG2a, and IgG2b (PharMingen) were tested along with the rat monoclonal primary antibodies to test for nonspecific isotype binding, and normal rabbit serum at a dilution of 1:1000 was tested along with the rabbit primary antibodies. No primary reagent controls were also done with each staining run. Biotinylated secondary antibodies included rabbit anti-rat IgG (mouse serum adsorbed) at 1:50, goat anti-rabbit at 1:150 (Vector Laboratories, Burlingame, CA), and anti-hamster IgG (PharMingen, clone G94–56) at 1:50.

Results

LNCs from PLP-immunized mice were stimulated *in vitro* with either PLP alone or PLP and rmIL-12 (20ng/ml). After 96 hours in culture, either 30 or 10 × 10^6 cells were transferred to naive SJL/J mice, which were monitored for clinical signs of EAE. As reported previously, the addition of rmIL-12 during *in vitro* antigen stimulation enhances the encephalitogenicity of the LNCs, with the mice that received 30 × 10^6 LNCs all progressing to complete hind limb paralysis by day 9 (Figure 1a). As the majority of mice fail to recover from disease after this regimen,[19] mice with complete hind limb paralysis that lasted for greater than 3 days were sacrificed for immunohistochemical analysis. To determine whether the addition of rmIL-12 during *in vitro* antigen stimulation would allow disease transfer with fewer cells, mice were injected with 10 × 10^6 PLP-stimulated LNCs, and subsequent disease progression was determined. In control animals, this suboptimal number of cells resulted in mild disease with only two of five mice reaching a score of 2 (partial hind limb paralysis) and with the remaining mice developing only mild disease. In contrast, mice injected with 10 × 10^6 LNCs stimulated *in vitro* with PLP and rmIL-12 developed severe disease with all mice (five of five) progressing to complete hind limb paralysis (Figure 1a).

As the effector cell responsible for initiating disease transfer has been shown to possess a Th1-type cytokine profile, we analyzed the frequency of IFN-γ- as well as IL-4-producing cells after *in vitro* stimulation with rmIL-12. Using an ELISPOT assay, we observed a twofold increase in IFN-γ-producing cells with a concomitant threefold reduction in IL-4-producing cells after stimulation with PLP and rmIL-12

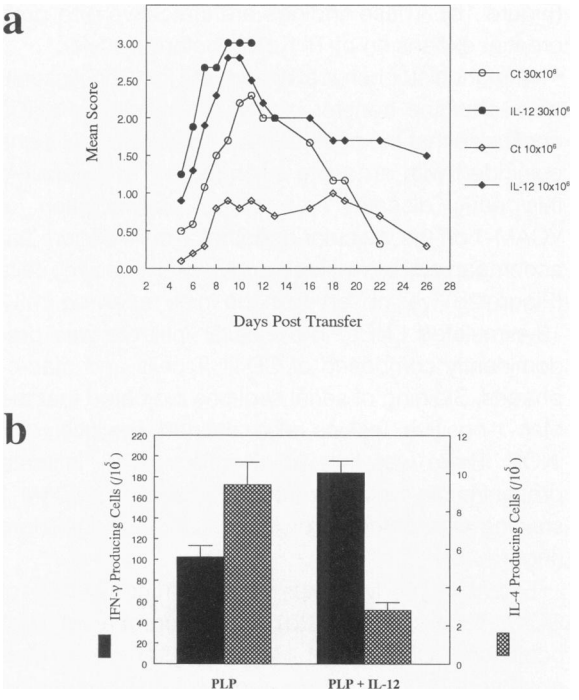

Figure 1. *Adoptive transfer of EAE after* in vitro *stimulation with PLP and rmIL-12* **a:** *LNCs from PLP-immunized SJLJ mice were stimulated* in vitro *with either PLP alone (open symbols) or PLP and 20 ng/ml rmIL-12 (closed symbols) as described in Materials and Methods. After* in vitro *stimulation, either 10 × 10^6 cells (◇, ◆; n = 5) or 30 × 10^6 cells (○, ●; n = 8) were injected intraperitoneally into naive mice. Mice receiving 30 × 10^6 cells were sacrificed on day 6 or after developing complete hind limb paralysis for immunohistochemistry.* **b:** *Enumeration of IFN-γ- and IL-4-producing cells after* in vitro *stimulation with PLP and IL-12. The results are expressed as mean ± SD calculated from the limiting dilution assay. The results are representative of two independent experiments.*

(Figure 1b). These findings are indicative of a preferential expansion of Th1 cells before transfer.

Immunohistochemical analysis of the CNS tissue 6 days after the transfer of rmIL-12-stimulated LNCs confirmed that the accelerated onset of clinical signs coincided with an acute inflammatory response. At this early disease time point, up-regulation of VCAM-1 on the vascular endothelial cells (Figure 2a) accompanied by infiltrating ICAM-1-positive cells (Figure 2b) was observed in the mice receiving rmIL-12-stimulated LNCs. The cellular infiltrate was predominantly composed of $CD4^+$ T cells and macrophages. Staining of serial sections indicated that the Mac-1-positive lesions also stained positively for iNOS. There was little or no inflammatory infiltrate present in the control group, although some VCAM-1 staining was detected on the vascular endothelium (Figure 2c).

Extensive perivascular infiltration composed of $CD4^+$ T cells (Figure 2d) and macrophages, with occasional $CD8^+$, $NK1.1^+$, and $B220^+$ cells, was observed in both groups of mice sacrificed during the period of complete hind limb paralysis (days 9 to 12). Using a panel of antibodies against T-cell- and macrophage-derived cytokines we could detect no apparent differences in the cytokine profiles expressed within the lesions with high amounts of IFN-γ (Figure 2e), TNF-α, IL-1β, and IL-2 (not shown) observed. Enumeration of the inflammatory foci in mice receiving rmIL-12-stimulated LNCs revealed a trend toward greater numbers of lesions compared with controls (Table 1) despite the fact that the cellular composition and cytokine expression within the lesions were similar.

In contrast to the intralesional cytokine profiles that were comparable between groups, immunohistochemical staining revealed a twofold increase in the percentage of iNOS-positive foci in the IL-12 treatment group (65.9% *versus* 30.5%; Table 1 and Figure 2f). Staining of serial sections demonstrated iNOS protein localized to regions of macrophage infiltration (Figure 2g). In the control mice, although there were a similar number of Mac-1-positive lesions (51.2% in control *versus* 60.2% in the IL-12 group), the frequency of macrophage containing foci that stained positively for iNOS was reduced (Figure 2, h and i).

Discussion

IL-12 is a novel heterodimeric cytokine that enhances the production of IFN-γ from natural killer cells and T lymphocytes and promotes $CD4^+$ Th1-specific immune responses both *in vitro* and *in vivo*. We have previously shown in an adoptive transfer model of EAE that the addition of rmIL-12 during *in vitro* stimulation of LNCs with PLP exacerbates disease after subsequent cell transfer.[19] To determine whether rmIL-12 increases the frequency of Th1 cells after stimulation with antigen, ELISPOT assays for IFN-γ and IL-4 were performed before cell transfer. The results demonstrated a twofold increase in IFN-γ-producing cells and a concomitant threefold reduction in IL-4-producing cells after rmIL-12 stimulation *in vitro*. This observation, coupled with the finding that rmIL-12 treatment allows the transfer of severe disease with fewer cells, suggests an increase in the frequency of autoreactive Th1 cells. Although the frequency of IL-4-producing cells in control cultures was low compared with IFN-γ-producing cells, given the ability of exogenous IL-4 treatment as well as IL-4-producing T cell clones to inhibit disease transfer,[21,22] it is likely that the additional inhibition of this suppressive cytokine by rmIL-12 treatment *in vitro* may contribute to the increased severity of disease.

Immunohistochemical analysis confirmed that the accelerated onset of clinical signs in mice receiving rmIL-12-stimulated LNCs was due to early infiltration of cells into the CNS, which was apparent 6 days after transfer when control mice showed minimal signs of disease. Perivascular cuffing of infiltrating cells stained positively for ICAM-1 and were associated with VCAM-1-positive endothelial cells. At this early time there was minimal if any evidence of cellular infiltration in the control mice, although VCAM-1 staining was detected on the vascular endothelium.

Despite the accelerated onset and increased severity of disease observed in mice receiving rmIL-12-stimulated LNCs, immunohistochemical examination of the CNS at peak disease (ie, complete hind limb paralysis) revealed no gross differences in either the cellular composition or cytokine profiles within the lesions between the two groups. In both cases there was extensive perivascular infiltration

Figure 2. *Immunohistochemical characterization of the inflammatory lesions after the adoptive transfer of PLP-stimulated LNCs with or without rmIL-12. Tissue from mice with EAE was harvested and sectioned as described in Materials and Methods. Immunoperoxidase staining from representative sections is shown.* **A**: *VCAM-1.* **B**: *ICAM-1 staining 6 days after the transfer of rmIL-12-stimulated LNCs.* **C**: *VCAM-1 staining from a control animal on day 6.* **D**: *CD4.* **E**: *IFN-γ.* **F** *and* **G**: *iNOS and Mac-1 staining, respectively, at peak disease from a mouse injected with rmIL-12-stimulated LNCs.* **H** *and* **I**: *iNOS and Mac-1 staining, respectively, at peak disease in control mice. iNOS and Mac-1 staining were performed on serial sections. Original magnification, ×400 for all sections except* **f** *and* **g**, *which was ×200.*

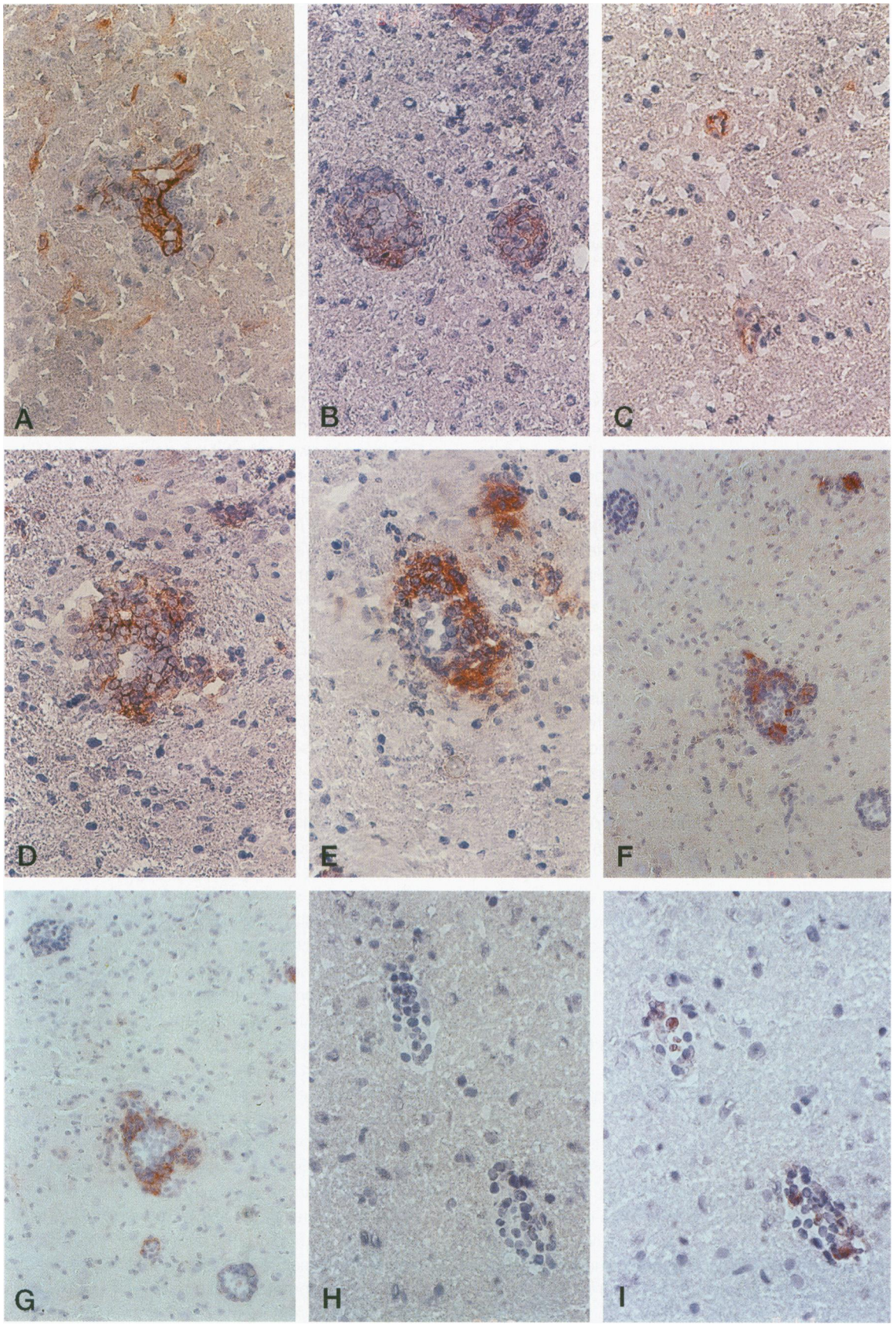
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Table 1. *Immunohistochemical Analysis of EAE Brains at Early and Peak Disease*

Tissue	n	CD4	CD8	Mac-1	NK1.1	B220	ICAM-1	VCAM-1	iNOS	Total foci	% Mac-1 + foci	% iNOS + foci
CT, day 6	4	–	ND	–	ND	ND	–	+*	–	0	0	0
CT, peak	8	+++	+/–	+++	+/–	+/–	+++	+++*	++	22 (2–109)	51 (0–88)	31 (0–49)
IL-12 day 6	4	+++	ND	ND	+++	+++*	+++			67 (15–121)	84 (78–90)	77 (67–89)
IL-12, peak	10	+++	+/–	+++	+/–	+/–	+++	+++*	+++	35 (11–76)	60 (18–77)	66 (42–81)

Mice injected with PLP-stimulated LNCs (with or without rmIL-12) were sacrificed on day 6 (before clinical disease in controls) or days 10 to 13 (at the time of peak disease), and tissue was harvested for immunohistochemical analysis. Cross sections of the brain were evaluated for the relative amount and intensity of staining (± moderate to +++ extensive) with the indicated antibodies. The total number of foci per cross section as well as the percentage of iNOS and Mac-1-positive lesions were evaluated by counting a minimum of four cross sections per animal. Mean number as well as the range are presented. ND, not determined.

* Staining localized to vascular endothelium.

composed predominantly of $CD4^+$ T cells and macrophages with $CD8^+$, $B220^+$, and $NK1.1^+$ cells also detected albeit to a lesser extent. In addition, immunohistochemical staining for the T-cell-derived cytokines IL-2 and IFN-γ as well as macrophage-derived IL-1β and TNF-α were also comparable between the two groups, suggesting that treatment with rmIL-12 did not alter the overall nature of the inflammatory response. Two observations were made, however, that are consistent with the severe and prolonged course of disease noted in mice receiving rmIL-12-stimulated LNCs. First, although there is a degree of experiment-to-experiment variability, enumeration of the inflammatory lesions at peak disease in mice receiving rmIL-12-stimulated LNCs revealed an increase in the number of foci relative to controls. This is consistent with the accelerated onset of disease observed in these mice and is most likely a direct consequence of *in vitro* activation/expansion of antigen-specific T cells by rmIL-12 treatment. It is noteworthy that this *in vitro* activation of autoreactive T cells by rmIL-12 is independent of the secondary production of either IFN-γ or TNF-α.[19] Second, a profound increase in the expression of iNOS in infiltrating macrophages was observed in the rmIL-12 group even though the frequency of macrophage-positive lesions was not increased. Marked iNOS expression was also apparent early after cell transfer (day 6) before the development of peak disease, indicating that macrophage activation occurs rapidly and thus may be a contributing factor throughout the course of EAE. In all instances, iNOS expression was restricted to infiltrating macrophages and was not detected on the vascular endothelial cells.

Cytokine-induced synthesis of NO from L-arginine via the iNOS pathway is known to have diverse biological consequences, including tissue damage during acute inflammation.[23] In the context of EAE, NO produced by activated microglia and astrocytes is directly cytotoxic to oligodendrocytes and is thought to contribute to myelin breakdown.[24] As *N*-nitro-L-arginine, an inhibitor of iNOS and NO production, has been shown to inhibit the development of EAE in mice,[14] exacerbation of disease as a consequence of increased NO production might well be expected.

Although IFN-γ is the principle mediator of macrophage-derived NO,[25] we could detect no difference in the immunohistochemical staining for this cytokine between treatment groups. However, a direct role for IFN-γ as a causative factor in the pathogenesis of EAE has yet to be established, as administration of neutralizing antibodies against IFN-γ potentiate disease progression[26] and fail to inhibit either the *in vitro*[19] or *in vivo* effects of IL-12 (J. Leonard, unpublished observation). In addition, administration of IL-4, which ameliorates clinical signs of EAE, does so in the absence of any effect on IFN-γ gene expression in the CNS.[21] As macrophage-derived NO production is regulated by a variety of cytokines that can interact synergistically, it is possible that the elevated expression of iNOS in mice receiving rmIL-12-stimulated LNCs reflects increased macrophage activation due to subtle changes in the local cytokine environment. Thus, although macrophages are not primary targets for IL-12 activity, the downstream synergistic effects of rmIL-12-stimulated cytokines such as IFN-γ and TNF-α may act to enhance macrophage function and thus affect the course of disease. In this respect, although it is clear from adoptive transfer experiments that $CD4^+$ T cells are required for the initiation of disease, there is a vast body of evidence that implicates macrophage/microglia cells as key components of late stage tissue damage. This is supported by the finding that depletion of macrophages before the onset of disease protects against subsequent development of clinical signs.[11] In addition, the anti-inflammatory effects of both IL-10 and IL-13

in the EAE model are thought to be due in part to inhibition of macrophage/microglial-derived inflammatory cytokines and NO production.[27,28]

Previous studies have demonstrated an increase in IL-10 mRNA late in the course of disease, which is thought to be involved in the resolution of the inflammatory response.[8] Using antibodies specific for murine IL-10, we were unable to detect any increase in the expression of this cytokine in control mice during the period of recovery. Due to the increased mortality rate in mice receiving rmIL-12-stimulated LNCs it was not possible to evaluate changes in this Th2-associated cytokine late in disease. However, as *in vitro* treatment with rmIL-12 resulted in a reduction in IL-4-producing cells *in vitro*, it is possible that similar inhibition of Th2-associated cytokines occurs *in vivo* and contributes to the increased expression of macrophage-associated iNOS and exacerbation of disease. Thus, *in vitro* expansion of autoreactive Th1 cells by rmIL-12 together with a reduction in IL-4-producing cells is likely to potentiate the early inflammatory response after cell transfer, with secondary *in vivo* effects leading to macrophage activation, NO production, and target tissue damage.

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