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9-*Cis*-retinoic acid suppresses inflammatory responses of microglia and astrocytes

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Abstract

Retinoic acid (RA) regulates a wide range of biologic process, including inflammation. Previously, RA was shown to inhibit the clinical signs of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). The current study investigated the effects of 9-*cis*-RA on primary mouse microglia and astrocytes, two cell types implicated in the pathology of MS and EAE. The studies demonstrated that 9-*cis*-RA inhibited the production of nitric oxide (NO) as well as the pro-inflammatory cytokines TNF- α , IL-1 β and IL-12 p40 by LPS-stimulated microglia. However, this retinoid had no effect on IL-6 secretion and increased MCP-1 production by LPS-stimulated microglia. In LPS-stimulated astrocytes, 9-*cis*-RA inhibited NO and TNF- α production but had not effect on IL-1 β , IL-6 and MCP-1 secretion. These results suggest that RA modulates EAE, at least in part, by suppressing the production of NO and specific inflammatory cytokines from activated glia and suggests that RA might be effective in the treatment of MS.

Keywords

Retinoic acid; Glia; NO; Cytokine; Chemokine

1. Introduction

Retinoids are vitamin A derivatives which play important roles in embryogenesis and, thereafter, for maintenance of functions such as vision, growth and reproduction (Zetterstrom et al., 1999). Retinoic acid (RA) is an active metabolite of retinoids and regulates a wide range of biologic processes, including cell proliferation, differentiation, and morphogenesis (De Luca, 1991). It is well known that RA modulates target cell activity by binding to specific nuclear receptors: retinoic acid receptors (RAR α , β , γ) and retinoid X receptors (RXR α , β , γ). All-*trans*-retinoic acid (tRA) and 9-*cis*-retinoic acid (9-*cis*-RA) are natural derivatives of vitamin A. Although RARs bind and are activated by both the 9-*cis*- and the all-*trans*-isomers of retinoic acid, RXRs are exclusively activated by the 9-*cis*-isomer (Allenby et al., 1993; Heyman et al., 1992; Levin et al., 1992).

RA has been shown to exert immunomodulatory and anti-inflammatory function in various cell types (Datta et al., 2001; Kim et al., 2004a,b; Mehta et al., 1994). Recent studies suggest that inhibition of NF- κ B translocation to the nucleus and suppression of JAK/STAT pathways may contribute to the anti-inflammatory mechanisms of RA (Choi et al., 2005; Dheen et al., 2005). Since brain inflammation is a risk factor of neurodegenerative disease, the anti-

inflammatory effect of RA may provide a novel therapeutic option for the treatment of neurodegenerative diseases, such as multiple sclerosis, Alzheimer's disease, Parkinson's disease and ischemic injury.

Several lines of evidence suggest that RA exerts beneficial effects on experimental autoimmune encephalomyelitis (EAE), which is an autoimmune disease characterized by central nervous system inflammation and demyelination, and represents an animal model of multiple sclerosis (MS). For example, oral administration of RA in the Lewis rats model of EAE prevented the subsequent development of clinic signs of disease (Massacesi et al., 1987, 1991). More recently, oral administration of retinoids were demonstrated to be effective in the treatment of a murine model of EAE (Racke et al., 1995). In vitro studies showed that RA was capable of influencing the differentiation of multipotential T cells to a Th2-like phenotype (Lovett-Racke and Racke, 2002), which provide a potential mechanism for RA's beneficial effects in EAE.

Microglia reside in the CNS, where they respond to pathogens and other foreign materials. In response to these agents, microglia becomes activated resulting in increased proliferation, altered morphology, increased phagocytosis, and increased production of a variety of cytokines including TNF- α and IL-1 β , as well as increased synthesis of NO (Benveniste, 1997). Although molecules including NO, TNF- α , and IL-1 β are toxic to pathogens, these agents can also be toxic to CNS cells including myelin-producing oligodendrocytes and neurons, which are compromised in the course of multiple sclerosis (Raine, 1997; Trapp et al., 1998). In addition to MS, chronically activated microglia are believed to contribute to the development of other neuroimmunological and neurodegenerative diseases including Alzheimer's disease (McGeer and McGeer, 1999; Sriram and Rodriguez, 1997). Chronically activated astrocytes are also believed to contribute to the pathology of neuroimmunological diseases, including MS (Holley et al., 2003; Zeinstra et al., 2003). Activated astrocytes produce a variety of pro-inflammatory cytokines, chemokines, and NO (De Keyser et al., 2003; Dong and Benveniste, 2001) and contribute to glial scarring that occurs at sites of MS plaques (Fawcett and Asher, 1999). Therefore, agents that block the activation of microglia and astrocytes may protect against the development of MS.

The aim of the present study was to investigate the anti-inflammatory effects of the natural agonist of RXR and RAR: 9-*cis*-RA on activated microglia and astrocytes, two cell types which upon activation may contribute to the pathology of MS and EAE.

2. Materials and methods

2.1. Reagents

The RXR agonist 9-*cis*-retinoic acid and lipopolysaccharide were obtained from Sigma (St. Louis MO). The cytokines IFN- γ or TNF- α were obtained from R&D Systems (Minneapolis, MN). DMEM media, glutamine, trypsin, and antibiotics used for tissue culture were obtained from BioWhittaker (Walkersville, MD). OPI medium supplement was obtained from Sigma (St Louis, MO). GM-CSF was obtained from BD Pharmingen (San Diego, CA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). C57BL/6 mice were obtained from Harlan (Indianapolis, IN), and bred in-house.

2.2. Cell culture

Primary mouse microglia cultures were obtained through a modification of the McCarthy and de Vellis protocol (McCarthy and de Vellis, 1980). Briefly, cerebral cortices from 1- to 3-day-old C57BL/6 mice were excised, meninges removed, and cortices minced into small pieces. Cells were separated by trypsinization followed by trituration of cortical tissue. The cell suspension was filtered through a 70- μ m cell strainer to remove debris. Cells were centrifuged

at $153 \times g$ for 5 min at 4 °C, resuspended in DMEM medium containing 10% FBS, 1.4 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, OPI medium supplement, and 0.5 ng/mL recombinant mouse GM-CSF, and plated into tissue culture flasks. Cells were allowed to grow to confluence (7–10 days) at 37 °C/5% CO₂. Flasks were then shaken overnight (200 rpm at 37 °C) in a temperature-controlled shaker to loosen microglia and oligodendrocytes from the more adherent astrocytes. These less adherent cells were plated for 2–3 h and then lightly shaken to separate oligodendrocytes from the more adherent microglia. Microglia were seeded in 96-well plates (4×10^4 cells/well) and incubated overnight at 37 °C/5% CO₂. Astrocyte medium contained all the substances described in the defined medium above except GM-CSF. After shaking to remove microglia and oligodendrocytes, astrocytes were recovered by trypsinization, seeded in 96-well plates (5×10^4 cells/well) and incubated overnight at 37 °C/5% CO₂. These panning procedures were repeated to obtain primary microglia and astrocytes of greater than 95% purity as determined by immunohistochemistry. The following day, microglia and astrocytes were treated with 9-*cis*-retinoic acid for 1 h and then stimulated with LPS or IFN- γ plus TNF- α for 24 h. Finally, tissue culture supernatants were collected for nitrite or enzyme-linked immunosorbent assay (ELISA) assays.

2.3. Nitric oxide production

Levels of the NO derivative nitrite were determined in the culture medium by Griess reaction as described previously (Drew and Chavis, 2001). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 550 nm. A standard curve using NaNO₂ was generated for each experiment for quantitation.

2.4. Cell viability assays

Cell viability was determined by MTT reduction assay as described previously (Drew and Chavis, 2001). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 570 nm. Results were reported as percent viability relative to untreated cultures.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cytokine (TNF- α , IL-1 β , IL-6 and IL-12 p40) and chemokine (MCP-1) levels in tissue culture media were determined by ELISA as described by the manufacturer (OptEIA Sets, Pharmingen, San Diego, CA). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Cytokine and chemokine concentrations in media were determined from standards containing known concentrations of the proteins.

2.6. Statistics

Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test to determine the significance of difference.

3. Results

3.1. Effects of 9-*cis*-retinoic acid on NO production by cytokine-stimulated microglia

Activated microglia produce NO that may be toxic to oligodendrocytes, cells which are compromised in MS. The cytokines IFN- γ and TNF- α are believed to be important modulators of disease activity in MS patients. Here, we showed that IFN- γ plus TNF- α increased nitrite production by primary mouse microglia. The RXR agonist 9-*cis*-RA inhibited cytokine-induced nitrite production in a dose dependent manner. This retinoid is a potent inhibitor of NO production by microglia, significantly inhibiting NO production at concentrations as low as 5 nM (Fig. 1A). 9-*cis*-RA was not toxic to the cells at the concentrations examined as

demonstrated by MTT reduction assays (Fig. 1B). Thus, the inhibition of 9-*cis*-RA on nitrite production was not due to effects on cell viability.

3.2. Effects of 9-*cis*-retinoic acid on NO production by LPS-stimulated microglia

LPS is a potent activator of microglia. The present studies demonstrate that 9-*cis*-RA significantly inhibited LPS-induction of NO production by primary microglia. This inhibition occurred over a wide range of RA concentrations (from 5 nM to 5 μ M) and occurred in a dose-dependent manner from 5 nM to 1 μ M (Fig. 2A). Interestingly, 9-*cis*-RA did not significantly inhibit LPS-induction of NO production by microglia at the highest dose examined (10 μ M) and only weakly at 5 μ M. This retinoid did not affect cell viability in these studies (Fig. 2B).

3.3. Effects of 9-*cis*-retinoic acid on cytokine and chemokine production by microglia

Activated microglia are known to secrete a variety of proinflammatory cytokines and chemokines. These molecules may contribute to the pathogenesis of neuroinflammatory disorders including MS. We demonstrate that upon LPS-stimulation, activated microglia produce proinflammatory cytokines including TNF- α , IL-1 β , IL-6, and IL-12 p40. 9-*cis*-RA inhibited LPS-induction of TNF- α , IL-1 β , and IL-12 p40 in a dose-dependent manner (Fig. 3). Also, 9-*cis*-RA had no effect on IL-6 production (Fig. 3). Interestingly, 9-*cis*-RA alone significantly increased production of the chemokine MCP-1 by microglia. In addition, 9-*cis*-RA significantly increased LPS-stimulated production of this chemokine (Fig. 4).

3.4. Effects of 9-*cis*-retinoic acid on NO, cytokine and chemokine production by astrocytes

Activated astrocytes, like activated microglia, have also been implicated in the course of MS as they are able to produce NO and many proinflammatory cytokines and chemokines that may contribute to disease progression. Here, we examined the ability of 9-*cis*-RA to modulate the inflammatory activity of activated primary mouse astrocytes. These cells produced NO after stimulation by LPS (Fig. 5). Treatment with relatively low concentrations of 9-*cis*-RA significantly suppressed NO (Fig. 5A). Higher concentrations of 9-*cis*-RA demonstrated a clear dose-dependent suppression of NO production by primary astrocytes (Fig. 5C). 9-*cis*-RA alone or in combination with LPS was not toxic to the astrocytes at all concentrations examined, indicating that the NO suppression observed was not due to effects on cell viability (Fig. 5B and D). LPS also stimulated the production of TNF- α , IL-1 β , IL-6 and MCP-1 by primary astrocytes (Fig. 6A–D). 9-*cis*-RA inhibited TNF- α production by astrocytes in a dose-dependent manner (Fig. 6A). However, 9-*cis*-RA did not suppress IL-1 β , IL-6 and MCP-1 secretion by activated astrocytes (Fig. 6B–D). In fact, 9-*cis*-RA did not inhibit production of these molecules at concentrations as high as 50 μ M (data not shown). Interestingly, in contrast to that observed with microglia, 9-*cis*-RA alone did not stimulate MCP-1 expression in astrocytes (data not shown).

4. Discussion

Multiple sclerosis (MS) is an organ-specific autoimmune disease, characterized pathologically by cell-mediated inflammation, demyelination and variable degrees of axonal loss (Lim and Giovannoni, 2005). It is generally believed that T lymphocytes react against myelin components, leading to damaged myelin sheaths with impaired nerve conduction (Hohlfeld and Wekerle, 2001; Steinman et al., 2002). However, pathological features of MS have also been attributed to chronically activated glia present in the CNS parenchyma (Benveniste, 1997; De Keyser et al., 2003; Smith, 2001; Sriram and Rodriguez, 1997).

Microglia activation in MS and EAE is thought to contribute directly to CNS damage through several mechanisms. Microglia can be activated to express a wide range of cytokines and chemokines, such as IL-1, IL-6, TNF- α , LT, macrophage inflammatory protein (MIP)-1 α , and

MCP-1 (Lee et al., 1993; Walker et al., 1995). Activated microglia are also able to secrete serine proteinases, matrix metalloproteinases (MMP) 2 and 9, and free radicals (Colton and Gilbert, 1993; Colton et al., 1993). In addition, activated microglia serve as the major antigen-presenting cell (APC) in the CNS and are capable of expressing class II major histocompatibility complex (MHC) antigens and co-stimulatory molecules that are critical for antigen presentation and T-cell activation (Benveniste, 1997).

Numerous studies have confirmed the role of microglia as important APCs within the CNS, while the ability of astrocytes to serve as APCs remains controversial. However, the ability of astrocytes to produce a wide variety of cytokines and chemokines is not in question. Both in vitro and in vivo studies have documented the ability of astrocytes to produce cytokines including IL-1, IL-6, IL-10, IFN- α , IFN- β ; TNF- α ; TGF- β , and chemokines including RANTES, IL-8, and MCP-1 (Dong and Benveniste, 2001). As a potent source of immunologically relevant cytokines and chemokines, astrocytes play a pivotal role in CNS diseases including Alzheimer's disease, MS, Parkinson's disease and brain injury/trauma. Therefore, treatments that suppress the activation of microglia and astrocytes may alleviate inflammation in the CNS and provide potential avenues for the treatment of these diseases.

It has been shown that the administration of retinoids in a murine model of EAE resulted in an improved clinical course, even when given after disease onset (Racke et al., 1995). The same group further demonstrated that RA was capable of promoting the differentiation of human T cells toward a Th2 phenotype which is believed to protect against development of MS (Lovett-Racke and Racke, 2002). Here, we wished to determine if RA alters the function of activated microglia and astrocytes and to explore an additional potential mechanism by which RA may block development of EAE or MS.

Our current studies demonstrated that 9-*cis*-RA inhibited NO production by microglia stimulated with either cytokines (combination of IFN- γ and TNF- α) or with LPS and also suppressed NO production by astrocytes stimulated with LPS. The free radical NO is a major mediator in autoimmune diseases and is involved in the killing of oligodendrocytes (Steinman et al., 2002). Since high levels of NO are believed to contribute to the pathology associated with MS and EAE, our results suggest that 9-*cis*-RA could ameliorate the disease. However, it should be noted that the role of NO in EAE and MS is controversial. For example, mice in which the inducible nitric oxide synthase gene is inactivated are more susceptible to EAE (Fenyk-Melody et al., 1998; Kahl et al., 2004; Sahrbacher et al., 1998; van der Veen et al., 2003). Thus, NO may be protective or alternatively destructive in these diseases, which may depend upon the concentration of NO as well as the time at which the molecule is produced in these multiphasic diseases.

MS lesions are associated with increased levels of cytokines, including TNF- α and IL-1 β (Cannella and Raine, 1995; Samoilova et al., 1998). TNF- α has been implicated in the disease process of MS due to its ability to mediate oligodendrocyte damage, leading to demyelination observed in MS (Selmaj et al., 1991; Selmaj and Raine, 1988). In addition, TNF- α and IL-1 β induce astrocyte proliferation (Giulian and Lachman, 1985; Selmaj et al., 1990), which could elicit the astrogliosis associated with MS and contribute to impairment of the blood-brain barrier. Here, our studies showed that 9-*cis*-RA potently inhibited the production of TNF- α by either microglia or astrocytes, and IL-1 β by microglia, which suggest that an additional mechanism by which 9-*cis*-RA may have beneficial effects on EAE or MS. However, like with NO, these same cytokines may also protect against disease. For example, in some studies, TNF- α knockout mice were more susceptible to EAE (Kassiotis et al., 1999; Sean Riminton et al., 1998). In addition, IL-1 β and TNF- α contribute to remyelination (Arnett et al., 2001; Mason et al., 2001).

Recently IL-6 has been shown to play an important role in EAE. IL-6 deficient mice are completely resistant to EAE, while the introduction of IL-6 in these mice renders them susceptible to EAE induction (Eugster et al., 1998). However, in our current studies, 9-*cis*-RA failed to show any suppression on IL-6 production by either microglia or astrocyte.

CD4⁺ Th1 cells are believed to contribute to the development of MS and EAE. IL-12 and IL-23 stimulate the differentiation of T cells toward a Th1 phenotype. The current studies demonstrate that 9-*cis*-RA inhibits IL-12 p40 production by microglia. Thus, 9-*cis*-RA may drive T cell differentiation toward a Th2 phenotype which protects against disease by indirect effects on microglia. Future studies will determine the effects of RA on expression of the IL-12 holoprotein, which consists of a heterodimer of IL-12 p40 and p35 subunits, and IL-23 holoprotein, which consists of a heterodimer of p40 and p19 subunits. Finally, the function of IL-12 p40 remains controversial. This molecule has alternatively been suggested to be anti-inflammatory or pro-inflammatory (Gran et al., 2004).

MCP-1 is a member of the CC chemokine family and is a potent monocyte chemoattractant secreted by a variety of cell types in response to proinflammatory stimuli. There is an apparent correlation between the expression of MCP-1 in the CNS and the degree of CNS inflammation and onset and severity of EAE (Hulkower et al., 1993; Kennedy et al., 1998). Astrocytes appear to be the major producers of MCP-1 within the injured/diseased CNS (Dorf et al., 2000; Huang et al., 2000). In the present study, 9-*cis*-RA did not alter MCP-1 production by astrocytes, but significantly increased MCP-1 secretion by microglia. It has been reported that 9-*cis*-RA induced MCP-1 secretion in human monocytic THP-1 cells, but inhibited THP-1 cell migration (Zhu et al., 1999). Additional studies demonstrated that RXR ligands inhibited MCP-1-directed migration of monocytes (Kintscher et al., 2000). In contrast, one recent study showed that 9-*cis*-RA increased the expression of both CCR1 and CCR2 mRNA and protein, and the migration of THP-1 cells and peripheral blood monocytes (Kim et al., 2004a,b). These conflicting results cannot be presently explained. However, the different cell culture and assay systems used in the studies may be responsible for the disparate results. To our best knowledge, the current studies are the first report regarding the effects of 9-*cis*-RA on MCP-1 production by microglia. The mechanisms regulating MCP-1 expression in glia, and the relevance to MS, need to be further explored.

9-*cis*-RA is the natural ligand for retinoid X receptors (RXRs). RXRs are known to regulate the expression of a variety of genes following formation of heterodimers with other nuclear receptors including peroxisome proliferators-activated receptors (PPARs) and liver-X-receptors (LXRs). Interestingly, PPAR agonists have been demonstrated to block the development of EAE (Diab et al., 2002; Feinstein et al., 2002; Lovett-Racke et al., 2004; Natarajan and Bright, 2002; Niino et al., 2001). Furthermore, PPAR and RXR agonists exert additive anti-inflammatory effects in suppressing EAE (Diab et al., 2004). This suggests the intriguing possibility that a combination of these agonists may be effective in the treatment of MS. It should be noted that the anti-inflammatory effects of 9-*cis*-RA in the current studies could result from receptor-dependent or receptor-independent mechanisms. For example, 9-*cis*-RA, in combination with PPAR agonists, could act in a receptor-dependent manner by stimulating the formation of RXR/PPAR heterodimers which could bind peroxisome proliferators response elements (PPREs) present in the promoter elements of PPAR-responsive genes. In addition, since RXRs are known to form heterodimers with both PPARs and LXRs, the relative abundance of the ligands for these receptors could determine the exact heterodimers that form and thus differentially alter gene expression. PPAR/RXR and LXR/RXR heterodimers are known to regulate gene expression through interaction with a complex of proteins including co-activator and co-repressor molecules. Some of these co-activators and co-repressors play an important role in regulating both PPAR and LXR responsive genes, and these molecules are in limited supply. Therefore, the RXR agonist 9-*cis*-RA may differentially

regulate the expression of PPAR and LXR responsive genes through competition for a limited supply of common co-activators and co-repressors (reviewed in Li and Palinski, 2006). In addition, PPARs are known to regulate gene expression through a receptor-dependent mechanism which does not involve binding to PPREs. This is termed receptor-dependent transrepression and is believed to involve the interaction of PPARs with other transcription factors. PPAR- γ agonists are believed to suppress many immune responses through this mechanism. For example, these ligands inhibit transcription factors including NF- κ B, AP-1, and STAT-1 from activating gene expression by receptor-dependent trans-repression (Kielian and Drew, 2003). Finally, 9-*cis*-RA may regulate the immune response through receptor-independent mechanisms. The PPAR- γ agonist 15d-PGJ₂ has been demonstrated to regulate gene expression through receptor-independent mechanisms. For example, this prostaglandin inhibits NF- κ B activity by suppressing IKK activation in response to inflammatory stimuli. This inhibits the degradation of I- κ B and, thus, prevents the translocation of NF- κ B to the nucleus (Castrillo et al., 2000; Rossi et al., 2000). Furthermore, this prostaglandin also directly inhibits NF- κ B binding to NF- κ B DNA-response elements independent of PPARs (Straus et al., 2000). Future studies will investigate the molecular mechanisms by which 9-*cis*-RA regulates the expression of pro-inflammatory molecules.

9-*cis*-RA appears to differentially regulate the production of pro-inflammatory molecules by microglia relative to astrocytes. In general, this molecule more potently suppresses the production of pro-inflammatory molecules in microglia than astrocytes. For example, 9-*cis*-RA potently inhibited the production of NO, TNF- α , IL-1 β , and IL-12 p40 in response to LPS, but only inhibited NO and TNF- α production by astrocytes. Previously, we demonstrated that 9-*cis*-RA in combination with PPAR- α (Xu et al., 2005) or PPAR- γ agonists (Diab et al., 2004) suppressed NO production by microglia more than either agonist alone. This might suggest that 9-*cis*-RA suppresses NO production in a receptor-dependent manner in microglia. However, additional studies are required to determine if 9-*cis*-RA regulates the expression of pro-inflammatory molecules by receptor-dependent and/or receptor-independent mechanisms in glia. Differential expression of pro-inflammatory molecules by glia may also result from differential expression of critical signaling molecules, transcription factors, co-activator, or co-repressor proteins by these cells. However, the mechanisms resulting in differential expression of these molecules is currently unknown and is currently under investigation. Interestingly, 10 μ M 9-*cis*-RA potently inhibited LPS-induction of TNF- α , IL-1 β and IL-12 p40 by microglia, without affecting NO production. The mechanisms by which high-dose 9-*cis*-RA differentially regulates the production of NO relative to the pro-inflammatory cytokines examined has not been elucidated. It is possible that the 9-*cis*-RA ligand concentration may alter interactions with other nuclear receptors or co-activator/co-repressor molecules which may result in differential expression of the genes that encode these pro-inflammatory molecules. However, this potential mechanism of action of 9-*cis*-RA must be examined empirically. The current studies demonstrate that 9-*cis*-RA also differentially regulates the expression of MCP-1 in microglia relative to astrocytes. Interestingly, this agent alone stimulates the expression of MCP-1 in primary microglia. In addition, 9-*cis*-RA and LPS appear to induce the expression of this chemokine in an additive manner in these cells. However, 9-*cis*-RA alone or in combination with LPS appears to have no effect on MCP-1 expression by astrocytes. Future studies will determine the molecular mechanisms by which 9-*cis*-RA differentially regulates the expression of MCP-1 in glia.

In summary, we have demonstrated that 9-*cis*-RA blocked the production of NO by both primary mouse microglia and astrocytes. This retinoid also inhibited microglial production of the pro-inflammatory cytokines TNF- α and IL-1 β . In addition, 9-*cis*-RA inhibited IL-12 p40 production by microglia which could influence T cell phenotype. The agent also inhibited production of NO and TNF- α by astrocytes. Since activated microglia and astrocytes both

contribute to the pathology of MS and EAE, the present study suggests that 9-*cis*-RA may be effective in the treatment of MS.

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References

- Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc. Natl. Acad. Sci. U. S. A* 1993;90:30–34. [PubMed: 8380496]
- Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat. Neurosci* 2001;4:1116–1122. [PubMed: 11600888]
- Benveniste EN. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J. Mol. Med* 1997;75:165–173. [PubMed: 9106073]
- Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann. Neurol* 1995;37:424–435. [PubMed: 7536402]
- Castrillo A, Diaz-Guerra MJ, Hortelano S, Martin-Sanz P, Bosca L. Inhibition of IkappaB kinase and IkappaB phosphorylation by 15-deoxy-Delta(12,14)-prostaglandin J(2) in activated murine macrophages. *Mol. Cell. Biol* 2000;20:1692–1698. [PubMed: 10669746]
- Choi WH, Ji KA, Jeon SB, Yang MS, Kim H, Min KJ, Shong M, Jou I, Joe EH. Anti-inflammatory roles of retinoic acid in rat brain astrocytes: suppression of interferon-gamma-induced JAK/STAT phosphorylation. *Biochem. Biophys. Res. Commun* 2005;329:125–131. [PubMed: 15721283]
- Colton CA, Gilbert DL. Microglia, an in vivo source of reactive oxygen species in the brain. *Adv. Neurol* 1993;59:321–326. [PubMed: 8380516]
- Colton CA, Keri JE, Chen WT, Monsky WL. Protease production by cultured microglia: substrate gel analysis and immobilized matrix degradation. *J. Neurosci. Res* 1993;35:297–304. [PubMed: 8350390]
- Datta PK, Reddy RS, Lianos EA. Effects of all-*trans*-retinoic acid (atRA) on inducible nitric oxide synthase (iNOS) activity and transforming growth factor beta-1 production in experimental anti-GBM antibody-mediated glomerulonephritis. *Inflammation* 2001;25:351–359. [PubMed: 11831438]
- De Keyser J, Zeinstra E, Frohman E. Are astrocytes central players in the pathophysiology of multiple sclerosis? *Arch. Neurol* 2003;60:132–136. [PubMed: 12533101]
- De Luca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 1991;5:2924–2933. [PubMed: 1661245]
- Dheen ST, Jun Y, Yan Z, Tay SS, Ling EA. Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia. *GLIA* 2005;50:21–31. [PubMed: 15602748]
- Diab A, Deng C, Smith JD, Hussain RZ, Phanavanh B, Lovett-Racke AE, Drew PD, Racke MK. Peroxisome proliferator-activated receptor-gamma agonist 15-deoxy-Delta(12,14)-prostaglandin J (2) ameliorates experimental autoimmune encephalomyelitis. *J. Immunol* 2002;168:2508–2515. [PubMed: 11859145]
- Diab A, Hussain RZ, Lovett-Racke AE, Chavis JA, Drew PD, Racke MK. Ligands for the peroxisome proliferator-activated receptor-gamma and the retinoid X receptor exert additive anti-inflammatory effects on experimental autoimmune encephalomyelitis. *J. Neuroimmunol* 2004;148:116–126. [PubMed: 14975592]
- Dong Y, Benveniste EN. Immune function of astrocytes. *GLIA* 2001;36:180–190. [PubMed: 11596126]
- Dorf ME, Berman MA, Tanabe S, Heesen M, Luo Y. Astrocytes express functional chemokine receptors. *J. Neuroimmunol* 2000;111:109–121. [PubMed: 11063828]
- Drew PD, Chavis JA. The cyclopentone prostaglandin 15-deoxy-Delta(12,14) prostaglandin J2 represses nitric oxide, TNF-alpha, and IL-12 production by microglial cells. *J. Neuroimmunol* 2001;115:28–35. [PubMed: 11282151]

- Eugster HP, Frei K, Kopf M, Lassmann H, Fontana A. IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *Eur. J. Immunol* 1998;28:2178–2187. [PubMed: 9692887]
- Fawcett JW, Asher RA. The glial scar and central nervous system repair. *Brain Res. Bull* 1999;49:377–391. [PubMed: 10483914]
- Feinstein DL, Galea E, Gavrilyuk V, Brosnan CF, Whitacre CC, Dumitrescu-Ozimek L, Landreth GE, Pershadsingh HA, Weinberg G, Heneka MT. Peroxisome proliferator-activated receptor-gamma agonists prevent experimental autoimmune encephalomyelitis. *Ann. Neurol* 2002;51:694–702. [PubMed: 12112074]
- Fenyk-Melody JE, Garrison AE, Brunnert SR, Weidner JR, Shen F, Shelton BA, Mudgett JS. Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J. Immunol* 1998;160:2940–2946. [PubMed: 9510198]
- Giulian D, Lachman LB. Interleukin-1 stimulation of astroglial proliferation after brain injury. *Science* 1985;228:497–499. [PubMed: 3872478]
- Gran B, Zhang GX, Rostami A. Role of the IL-12/IL-23 system in the regulation of T-cell responses in central nervous system inflammatory demyelination. *Crit. Rev. Immunol* 2004;24:111–128. [PubMed: 15581393]
- Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 9-*cis*-retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 1992;68:397–406. [PubMed: 1310260]
- Hohlfeld R, Wekerle H. Immunological update on multiple sclerosis. *Curr. Opin. Neurol* 2001;14:299–304. [PubMed: 11371751]
- Holley JE, Gveric D, Newcombe J, Cuzner ML, Gutowski NJ. Astrocyte characterization in the multiple sclerosis glial scar. *Neuropathol. Appl. Neurobiol* 2003;29:434–444. [PubMed: 14507335]
- Huang D, Han Y, Rani MR, Glabinski A, Trebst C, Sorensen T, Tani M, Wang J, Chien P, O'Bryan S, Bielecki B, Zhou ZL, Majumder S, Ransohoff RM. Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. *Immunol. Rev* 2000;177:52–67. [PubMed: 11138785]
- Hulkower K, Brosnan CF, Aquino DA, Cammer W, Kulshrestha S, Guida MP, Rapoport DA, Berman JW. Expression of CSF-1, c-fms, and MCP-1 in the central nervous system of rats with experimental allergic encephalomyelitis. *J. Immunol* 1993;150:2525–2533. [PubMed: 8450228]
- Kahl KG, Schmidt HH, Jung S, Sherman P, Toyka KV, Zielasek J. Experimental autoimmune encephalomyelitis in mice with a targeted deletion of the inducible nitric oxide synthase gene: increased T-helper 1 response. *Neurosci. Lett* 2004;358:58–62. [PubMed: 15016434]
- Kassiotis G, Pasparakis M, Kollias G, Probert L. TNF accelerates the onset but does not alter the incidence and severity of myelin basic protein-induced experimental autoimmune encephalomyelitis. *Eur. J. Immunol* 1999;29:774–780. [PubMed: 10092079]
- Kennedy KJ, Strieter RM, Kunkel SL, Lukacs NW, Karpus WJ. Acute and relapsing experimental autoimmune encephalomyelitis are regulated by differential expression of the CC chemokines macrophage inflammatory protein-1alpha and monocyte chemoattractant protein-1. *J. Neuroimmunol* 1998;92:98–108. [PubMed: 9916884]
- Kielian T, Drew PD. Effects of peroxisome proliferator-activated receptor-gamma agonists on central nervous system inflammation. *J. Neurosci. Res* 2003;71:315–325. [PubMed: 12526021]
- Kim BH, Kang KS, Lee YS. Effect of retinoids on LPS-induced COX-2 expression and COX-2 associated PGE(2) release from mouse peritoneal macrophages and TNF-alpha release from rat peripheral blood mononuclear cells. *Toxicol. Lett* 2004a;150:191–201. [PubMed: 15093674]
- Kim IS, Kim YS, Jang SW, Sung HJ, Han KH, Na DS, Ko J. Differential effects of 9-*cis*-retinoic acid on expression of CC chemokine receptors in human monocytes. *Biochem. Pharmacol* 2004b;68:611–620. [PubMed: 15276068]
- Kintscher U, Goetze S, Wakino S, Kim S, Nagpal S, Chandraratna RA, Graf K, Fleck E, Hsueh WA, Law RE. Peroxisome proliferator-activated receptor and retinoid X receptor ligands inhibit monocyte chemotactic protein-1-directed migration of monocytes. *Eur. J. Pharmacol* 2000;401:259–270. [PubMed: 10936484]

- Lee SC, Liu W, Dickson DW, Brosnan CF, Berman JW. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. *J. Immunol* 1993;150:2659–2667. [PubMed: 8454848]
- Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzisen C, Rosenberger M, Lovey A. 9-*cis*-retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature* 1992;355:359–361. [PubMed: 1309942]
- Li AC, Palinski W. Peroxisome proliferator-activated receptors: how their effects on macrophages can lead to the development of a new drug therapy against atherosclerosis. *Annu. Rev. Pharmacol. Toxicol* 2006;46:1–39. [PubMed: 16402897]
- Lim ET, Giovannoni G. Immunopathogenesis and immunotherapeutic approaches in multiple sclerosis. *Expert Rev. Neurotherapeutics* 2005;5:379–390.
- Lovett-Racke AE, Racke MK. Retinoic acid promotes the development of Th2-like human myelin basic protein-reactive T cells. *Cell. Immunol* 2002;215:54–60. [PubMed: 12142036]
- Lovett-Racke AE, Hussain RZ, Northrop S, Choy J, Rocchini A, Matthes L, Chavis JA, Diab A, Drew PD, Racke MK. Peroxisome proliferator-activated receptor alpha agonists as therapy for autoimmune disease. *J. Immunol* 2004;172:5790–5798. [PubMed: 15100326]
- Mason JL, Suzuki K, Chaplin DD, Matsushima GK. Interleukin-1beta promotes repair of the CNS. *J. Neurosci* 2001;21:7046–7052. [PubMed: 11549714]
- Massacesi L, Abbamondi AL, Sarlo F, Amaducci L. The control of experimental allergic encephalomyelitis with retinoic acid. Further studies. *Riv. Neurol* 1987;57:166–169.
- Massacesi L, Castigli E, Vergelli M, Olivetto J, Abbamondi AL, Sarlo F, Amaducci L. Immunosuppressive activity of 13-*cis*-retinoic acid and prevention of experimental autoimmune encephalomyelitis in rats. *J. Clin. Investig* 1991;88:1331–1337. [PubMed: 1918383]
- McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol* 1980;85:890–902. [PubMed: 6248568]
- McGeer EG, McGeer PL. Brain inflammation in Alzheimer disease and the therapeutic implications. *Curr. Pharm. Des* 1999;5:821–836. [PubMed: 10526088]
- Mehta K, McQueen T, Tucker S, Pandita R, Aggarwal BB. Inhibition by all-*trans*-retinoic acid of tumor necrosis factor and nitric oxide production by peritoneal macrophages. *J. Leukoc. Biol* 1994;55:336–342. [PubMed: 8120450]
- Natarajan C, Bright JJ. Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. *Genes Immun* 2002;3:59–70. [PubMed: 11960303]
- Niino M, Iwabuchi K, Kikuchi S, Ato M, Morohashi T, Ogata A, Tashiro K, Onoe K. Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by an agonist of peroxisome proliferator-activated receptor-gamma. *J. Neuroimmunol* 2001;116:40–48. [PubMed: 11311328]
- Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, Raine CS, McFarlin DE, Scott DE. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. *J. Immunol* 1995;154:450–458. [PubMed: 7527821]
- Raine CS. The Norton Lecture: a review of the oligodendrocyte in the multiple sclerosis lesion. *J. Neuroimmunol* 1997;77:135–152. [PubMed: 9258244]
- Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 2000;403:103–108. [PubMed: 10638762]
- Sahrbacher UC, Lechner F, Eugster HP, Frei K, Lassmann H, Fontana A. Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur. J. Immunol* 1998;28:1332–1338. [PubMed: 9565373]
- Samoilova EB, Horton JL, Hilliard B, Liu TS, Chen Y. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. *J. Immunol* 1998;161:6480–6486. [PubMed: 9862671]
- Sean Riminton D, Korner H, Strickland DH, Lemckert FA, Pollard JD, Sedgwick JD. Challenging cytokine redundancy: inflammatory cell movement and clinical course of experimental autoimmune encephalomyelitis are normal in lymphotoxin-deficient, but not tumor necrosis factor-deficient, mice. *J. Exp. Med* 1998;187:1517–1528. [PubMed: 9565643]

- Selmaj KW, Raine CS. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann. Neurol* 1988;23:339–346. [PubMed: 3132891]
- Selmaj KW, Farooq M, Norton WT, Raine CS, Brosnan CF. Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor. *J. Immunol* 1990;144:129–135. [PubMed: 2104886]
- Selmaj K, Raine CS, Cross AH. Anti-tumor necrosis factor therapy abrogates autoimmune demyelination. *Ann. Neurol* 1991;30:694–700. [PubMed: 1722388]
- Smith ME. Phagocytic properties of microglia in vitro: implications for a role in multiple sclerosis and EAE. *Microsc. Res. Tech* 2001;54:81–94. [PubMed: 11455615]
- Sriram S, Rodriguez M. Indictment of the microglia as the villain in multiple sclerosis. *Neurology* 1997;48:464–470. [PubMed: 9040740]
- Steinman L, Martin R, Bernard C, Conlon P, Oksenberg JR. Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu. Rev. Neurosci* 2002;25:491–505. [PubMed: 12052918]
- Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, Glass CK. 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proc. Natl. Acad. Sci. U. S. A* 2000;97:4844–4849. [PubMed: 10781090]
- Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med* 1998;338:278–285. [see comment]. [PubMed: 9445407]
- van der Veen RC, Dietlin TA, Hofman FM. Tissue expression of inducible nitric oxide synthase requires IFN-gamma production by infiltrating splenic T cells: more evidence for immunosuppression by nitric oxide. *J. Neuroimmunol* 2003;145:86–90. [PubMed: 14644034]
- Walker DG, Kim SU, McGeer PL. Complement and cytokine gene expression in cultured microglial derived from postmortem human brains. *J. Neurosci. Res* 1995;40:478–493. [PubMed: 7616608]
- Xu J, Storer PD, Chavis JA, Racke MK, Drew PD. Agonists for the peroxisome proliferator-activated receptor-alpha and the retinoid X receptor inhibit inflammatory responses of microglia. *J. Neurosci. Res* 2005;81:403–411. [PubMed: 15968640]
- Zeinstra E, Wilczak N, De Keyser J. Reactive astrocytes in chronic active lesions of multiple sclerosis express co-stimulatory molecules B7-1 and B7-2. *J. Neuroimmunol* 2003;135:166–171. [PubMed: 12576238]
- Zetterstrom RH, Lindqvist E, Mata de Urquiza A, Tomac A, Eriksson U, Perlmann T, Olson L. Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. *Eur. J. Neurosci* 1999;11:407–416. [PubMed: 10051741]
- Zhu L, Bisgaier CL, Aviram M, Newton RS. 9-*cis*-retinoic acid induces monocyte chemoattractant protein-1 secretion in human monocytic THP-1 cells. *Arterioscler. Thromb. Vasc. Biol* 1999;19:2105–2111. [PubMed: 10479651]

Fig. 1. 9-*cis*-RA inhibits IFN- γ plus TNF- α induction of NO in primary mouse microglia. Cells were pre-treated for 1 h with the indicated concentrations (μ M) of 9-*cis*-RA. IFN- γ (250 U/ml) and TNF- α (500 U/ml) were added as indicated, and 24 h later, the concentration of nitrite in the culture medium was determined (A). Cell viability was determined by MTT assay (B). Values represent the mean $\pm$ S.E.M. for a representative experiment run in triplicate. At least three independent experiments were conducted. *** $p < 0.001$ vs. IFN- γ - and TNF- α -treated cultures.

Fig. 2. 9-*cis*-RA inhibits LPS induction of NO in primary mouse microglia. Cells were pre-treated for 1 h with the indicated concentrations (μM) of 9-*cis*-RA. LPS (0.5 μg/ml) was added as indicated, and 24 h later, the concentration of nitrite in the culture medium was determined (A). Cell viability was determined by MTT assay (B). Values represent the mean ± S.E.M. for a representative experiment run in triplicate. At least three independent experiments were conducted. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. LPS-treated cultures.

Fig. 3. 9-*cis*-RA inhibits cytokine production by primary mouse microglia. Cells were pre-treated for 1 h with the indicated concentrations (μ M) of 9-*cis*-RA, then LPS (0.5 μ g/ml) was added as indicated, and 24 h later, TNF- α (A), IL-1 β (B), IL-6 (C) and IL-12p40 (D) levels were determined in culture medium by ELISA. Values represent the mean $\pm$ S.E.M. for a representative experiment run in triplicate. At least three independent experiments were conducted. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs. LPS-treated cultures.

Fig. 4.

9-*cis*-RA induces chemokine production by primary mouse microglia. Cells were pre-treated for 1 h with the indicated concentrations (μ M) of 9-*cis*-RA, then LPS (0.5 μ g/ml) was added as indicated, and 24 h later, MCP-1 levels were determined in culture medium by ELISA.

Values represent the mean $\pm$ S.E.M. for a representative experiment run in triplicate. At least three independent experiments were conducted. ## p < 0.01, ### p < 0.001 vs. control, *** p < 0.001 vs. LPS-treated cultures.

Fig. 5. 9-*cis*-RA inhibits LPS induction of NO in primary mouse astrocytes. Cells were pre-treated for 1 h with the indicated concentrations (μM) of 9-*cis*-RA. LPS (2.5 μg/ml) was added as indicated, and 24 h later, the concentration of nitrite in the culture medium was determined (A and C). Cell viability was determined by MTT assay (B and D). Values represent the mean ± S.E.M. for a representative experiment run in triplicate. At least three independent experiments were conducted. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. LPS-treated cultures.

Fig. 6. 9-*cis*-RA's effects on cytokine and chemokine production by primary mouse astrocyte. Cells were pre-treated for 1 h with the indicated concentrations (μ M) of 9-*cis*-RA, then LPS (2.5 μ g/ml) was added as indicated, and 24 h later, TNF- α (A), IL-1 β (B), IL-6 (C) and MCP-1 (D) levels were determined in culture medium by ELISA. Values represent the mean $\pm$ S.E.M. for a representative experiment run in triplicate. At least three independent experiments were conducted. *** $p < 0.001$ vs. LPS-treated cultures.