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***APOE* genotype-dependent modulation of astrocyte chemokine CCL3 production**

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Abstract

Apolipoprotein E (apoE) is well known as a regulator of cholesterol homeostasis, and is increasingly recognized to play a prominent role in the modulation of innate immune response, including cell-to-cell communication and migration. Three common alleles Alzheimer's disease (AD) is a slowly progressive neurodegenerative disorder characterized by neuroinflammation that appears to be an important component of the pathophysiology of the disease. Astrocytes are the majority cell type in brain, exerting significant influence over a range of central nervous system activities, including microglial-mediated neuroinflammatory responses. As the resident innate immune effector cells of the brain, microglia respond to soluble chemical signals released from tissue during injury and disease by mobilizing to lesion sites, clearing toxic molecules, and releasing chemical signals of their own. While microglial-mediated neuroinflammation in the AD brain remains an area of intense investigation, the mechanisms underlying reinforcement and regulation of these aberrant microglial responses by astrocytes are largely unstudied. Moreover, although inheritance of *APOE* $\epsilon 4$ represents the greatest genetic risk factor for sporadic AD, the mechanism by which apoE isoforms differentially influence AD pathophysiology is unknown. Here we show that *APOE* $\epsilon 4$ genotype specifically modulates astrocyte secretion of potent microglial chemotactic agents, including CCL3, thus providing evidence that *APOE* modulation of central nervous system (CNS) innate immune response is mediated through astrocytes.

Keywords

astrocytes; *APOE*; Alzheimer's Disease; chemokine; neuroinflammation

Introduction

Alzheimer's disease (AD) is characterized by extracellular deposition of amyloid (A) β peptides in the form of plaques, which often are a focal point for microglia accumulation and activation (Prokop et al., 2013). Microglia represent the principle cellular effector of innate immunity in brain, and exacerbated or otherwise dysfunctional microglial response to insult contributes to the extent of pathology in many central nervous system (CNS) disorders, including amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), spinal cord injury, stroke, Parkinson's disease (PD), and AD (Festoff et al., 2006; Howell et al.,

2010; Malm et al., 2008; Neumann et al., 2006; Seabrook et al., 2006; Van Den Bosch et al., 2002; Wu et al., 2002; Yrjanheikki et al., 1998). In order to mount an effective response to spatially diverse tissue insults or damage, microglia must be able to bind soluble chemical signals, migrate to lesion sites, and actively neutralize and clear neurotoxic molecules and cellular debris, all the while synthesizing and secreting their own cytokine signals for purposes of synergistic immune support. While innate immune activation is not intrinsically deleterious, a delicate balance of pro- and anti-inflammatory activities must be maintained within temporal and spatial constraints so that injury to healthy tissue is averted. Although the precise mechanisms of AD pathogenesis are not known, aberrant microglial innate immune response in AD mediates neurotoxicity (Cimino et al., 2009; Maezawa et al., 2006b; Maezawa et al., 2011). This observation is supported through experimental extrinsic modulation of microglial function, which results in altered A β clearance and reduced neurotoxicity in transgenic models of AD (Cameron et al., 2012; Cramer et al., 2012; Griciuc et al., 2013; Xue et al., 2012; Yamanaka et al., 2012). While *in situ* regulation of microglia-mediated neuroinflammation in AD remains incompletely understood, astrocytes are critical modulators of microglial innate immune response.

Astrocytes far outnumber microglia in the CNS and subserve diverse functions, including maintenance of normal brain physiology through regulation of cerebral blood flow (Mulligan and MacVicar, 2004; Takano et al., 2006; Zonta et al., 2003) and local fluid and electrolyte balance (Benfenati et al., 2011; Leiserson et al., 2011), neurotransmitter clearance and recycling (Seifert et al., 2006), formation of glycogen-derived metabolites for neuron utilization (Suh et al., 2007), direct modulation of neuronal signaling (Halassa and Haydon, 2010; Perea and Araque, 2010), and maintenance of neuronal health via neurotrophic factor synthesis and secretion (Bsibsi et al., 2006). Accumulating evidence also identifies astrocytes as significant contributors to the CNS inflammatory milieu (Medeiros and LaFerla, 2013), responding to a variety of sterile insults, including aggregated A β , the pathologic hallmark of AD. Such activation results in robust elaboration of cytokines (Carrero et al., 2012; Garwood et al., 2011), which are diffusible cell-to-cell signaling molecules of the broader immune system. Chemokines, a chemotaxis-inducing cytokine subset, are diffusible regulators of both local and regional innate immune cell trafficking that directly influence microglial movement, cytokine release, and phagocytosis, and contribute to the pathophysiology of neurodegenerative diseases (Conductier et al., 2010). In experimental AD, A β clearance is modulated by manipulation of chemokine signaling (El Khoury et al., 2007; Kiyota et al., 2013), indicating that chemokines represent potentially important mechanistic and therapeutic targets in AD.

In brain, apolipoprotein (apo) E, a molecule critically important for cholesterol homeostasis, is primarily secreted by astrocytes and is known to influence innate immune activity both inside and outside the CNS (Cudaback et al., 2011; Vitek et al., 2009; Wang et al., 2009; Zhu et al., 2012). In humans, *APOE* is a polymorphic locus with the three common alleles (*APOE* ϵ 2, ϵ 3, and ϵ 4) encoding three unique protein isoforms (apoE2, apoE3, and apoE4, respectively), each differing at only two amino acid positions (112 and 158), with cumulative Cys-to-Arg changes imparting unique biochemical and cellular signaling properties to each (Zhong and Weisgraber, 2009). *APOE* ϵ 4 genotype is the greatest genetic

risk factor for sporadic AD (Harold et al., 2009). Neuroinflammation is a key component in most, if not all, progressive neurodegenerative disorders, including AD, PD, traumatic brain injury, MS, and ALS (Das et al., 2012; Evans et al., 2012; Keene et al., 2011; Nolan et al., 2013; Tourdias and Dousset, 2012), and inflammatory response in brain is known to be *APOE* genotype dependent. Maeda and colleagues (Sullivan et al., 1997; Xu et al., 1996) previously generated transgenic “humanized” mice homozygous for *APOE* ϵ 2, ϵ 3, or ϵ 4, providing a useful animal model for investigation of *APOE* influence of inflammatory response. Using these mice, we have previously reported *APOE* genotype-dependent modulation of microglial migration (Cudaback et al., 2011), as well as paradoxical rank order differences in cytokine secretion between microglia (Maezawa et al., 2006b) and astrocytes (Maezawa et al., 2006a), although the precise regulatory mechanism of these differential responses has not been identified. Astrocytes can broadly or selectively influence microglial activity through cytokine and chemokine secretion. As a first step toward understanding mechanisms of *APOE*-dependent modulation of microglia-mediated innate immune response, we hypothesized that chemokine expression in astrocytes is differentially *APOE* genotype-dependent. We tested this hypothesis in primary astrocyte cultures derived from wild type (wt), *APOE* deficient, and humanized *APOE* mice, and in human brain tissue.

Materials and Methods

Reagents and materials

Recombinant mouse CCL3 was purchased from R & D Systems (Minneapolis, MN, USA). Double-stranded polyinosinic-polycytidylic acid (PIC) and 9-cis-retinoic acid were purchased from Sigma (St Louis, MO, USA); lipopolysaccharide (LPS) was purchased from Calbiochem (La Jolla, CA, USA); Pam3CSK4 (Pam3) and CpG oligonucleotides were purchased from Invivogen (San Diego, CA, USA); low endotoxin recombinant human receptor associated protein (RAP) was purchased from Innovative Research (Novi, MI, USA); and T0901317 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Animals

Wild type) C57BL/6 and *apoe* $-/-$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Humanized *APOE* mice were a generous gift from Dr. Maeda (Sullivan et al., 1997; Xu et al., 1996). In brief, these transgenic mice had homozygous targeted replacement (TR) of the mouse apolipoprotein E gene with human *APOE* ϵ 2, *APOE* ϵ 3, or *APOE* ϵ 4. All mice were housed in a temperature-controlled specific pathogen-free facility with a strict 12-h light/dark cycle and with free access to food and water, and used with approval of the University of Washington Animal Care and Use Committee.

Primary cell culture

Primary murine mixed glial cultures were generated from 0- to 3-day old pups as previously described (Cudaback et al., 2012). Microglia were isolated from mixed glial cultures by gentle agitation, yielding primary microglial cultures of >99% purity as determined by Iba-1 immunostaining, while astrocyte cultures were generated via enzymatic detachment with

trypsin (> 95% pure as identified by immunostaining for glial fibrillary acidic protein). Primary astrocytes were allowed to reach confluence (3 d) prior to treatment. Optimal agonist concentrations and treatment times were previously determined using identical primary culture methodologies (Cudaback et al., 2012). Serum-free culture media was used for all cell treatments.

ELISA and Luminex

Murine chemokine (C-C motif) ligand (CCL) 3 in medium from treated astrocytes was quantified by ELISA (R & D Systems). Levels of 32 mouse cytokines/ chemokines in medium were determined using a custom mouse MILLIPLEX MAP kit that measures CCL2, CCL3, CCL4, CCL5, chemokine (X-C motif) ligand (CXCL) 1, CXCL2, CXCL9, CXCL10, eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon (IFN) γ , Interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), macrophage-colony stimulating factor (M-CSF), tumor necrosis factor (TNF)- α , and vascular endothelial growth factor (VEGF) (Millipore, Billerica, MA, USA).

Human brain tissue

Post-mortem parietal lobe was obtained from the Neuropathology Core of the Alzheimer's Disease Research Center (ADRC) at the University of Washington (UW). Tissue was dissected from human brain at autopsy, flash frozen using liquid nitrogen, and stored at -80°C prior to assay. Sample inclusion criteria were restricted as follows: 1) probable sporadic AD diagnosis during life, 2) confirmed post-mortem neuropathologic diagnosis of AD (Braak stage of V or VI), 4) post-mortem interval of less than or equal to 10 hours to limit mRNA degradation of brain samples, and 5) *APOE* genotype of $\epsilon 3/\epsilon 3$ or $\epsilon 4/\epsilon 4$. All brain samples were de-identified and coded so that all analyses were performed blinded to personal identifiers and *APOE* genotype. Written informed consent was obtained from all participants and the use of human tissue was approved by the UW Institutional Review Board.

qPCR

Total RNA was isolated from cultured primary astrocytes, TR *APOE3* and TR *APOE4* mouse cerebra, and post-mortem human parietal cortex using an RNeasy extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Age-matched (12 week old) LPS-injected TR *APOE3* and TR *APOE4* animals were anesthetized and transcardially perfused with ice-cold PBS. Brains were harvested and cerebra rapidly dissected, flash frozen in liquid nitrogen, and stored at -80°C until assayed. RNA (1 μg) was reversed-transcribed using an Advantage RT-for-PCR kit (Clontech, Mountain View, CA, USA), and quantitative expression of mouse or human apoE and CCL3 mRNAs, and mouse TLR2, TLR3, TLR4, TLR9, ABCA1, ABCG1, CCL3 mRNAs were determined by qPCR using gene-specific TaqMan Gene Expression Assays run on an ABI 7900HT (Applied Biosystems, Carlsbad, CA). Quantification of gene expression was calculated using the

delta-delta cycle threshold ($\Delta\Delta$ ct) method with normalization to 18 S rRNA for murine samples and GAPDH for human brain tissue.

Immunofluorescence

Primary microglia from wt mice were prepared for immunofluorescence staining as described previously (Maezawa et al., 2006a). Primary antibodies were to Iba-1 (Wako, Richmond, VA, USA) (1:1000); chemokine (CC motif) receptor (CCR) 1 (LifeSpan Biosciences, Seattle, WA) (1:500) and CCR5 (R & D Systems) (1:500). For secondary antibodies, we used cy3-conjugated donkey antibodies to rabbit IgG (1:400) and cy2-conjugated donkey antibodies to rat IgG (1:400) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Images from primary microglial cells were examined using a Nikon fluorescence microscope and images captured using a Nikon Super Coolsan 4000 ED (Nikon, Melville, NY).

Migration assay

Microglial migration was assayed using a previously described modified Boyden chamber technique (Cudaback et al., 2011). Microglia were recovered in serum-free DME/F-12 media, stained with a near-infrared nuclear dye DRAQ5 (Axxora, San Diego, CA, USA), and then loaded into the upper wells of a 96-well chemotaxis chamber. Recombinant CCL3 (R&D Systems) was serially diluted in serum-free media and loaded into lower wells in triplicate. Basal migration was assessed by quantification of cells migrating toward CCL3 vehicle only (phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA)). Migration was assessed over 3 h at 37°C and 5% CO₂. Filters were processed and migrating cells quantified.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

APOE genotype differentially modulates PIC-induced chemokine secretion from astrocytes

Microglia respond to sterile insult in brain by migrating to lesion sites and secreting both pro- and anti-inflammatory cytokines (Aguzzi et al., 2013). Human apoE isoform and/or *APOE* genotype are known to modulate microglial function, including migration and cytokine release (Cudaback et al., 2011; Maezawa et al., 2006b; Vitek et al., 2009; Yang et al., 2013; Zhu et al., 2012). Because astrocyte activation results in elaboration of chemokines that influence microglial activation, we hypothesized that this process might be *APOE* genotype-dependent. Selective activation of the toll-like receptor (TLR) class of cell-surface receptors is commonly used to mimic the innate immune activation profile associated with a variety of neurodegenerative diseases, including AD (Maezawa et al., 2006b). We treated cultured primary astrocytes derived from humanized *APOE* mice with the TLR3-specific agonist PIC, and assayed the culture supernatants for various chemokines. This initial multiplex screen revealed *APOE* genotype-dependent differential production of several disease-relevant chemokines (Table 1). We have previously shown that astrocytes

derived from humanized *APOE* mice display an *APOE* genotype-dependent rank order difference of $\epsilon_2 > \epsilon_3 > \epsilon_4$ for pro-inflammatory cytokine secretion in response to LPS (Maezawa et al., 2006a). Interestingly, fold inductions over baseline for many of the chemokines assayed in this initial screen displayed a U-shaped profile with ϵ_3 less than both ϵ_2 and ϵ_4 , including CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CXCL2 (MIP-2 α). This observation is reminiscent of previous work from our lab that demonstrated *APOE* genotype-dependent modulation of microglial migration to various chemotactic agents (Cudaback et al., 2011), specifically identifying an inverted U-shape in this microglial response, such that ATP- and C5a-induced migration of primary microglia derived from TR *APOE2* and TR *APOE4* mice was significantly reduced compared to TR *APOE3* cells. This preliminary screen suggests that *APOE* genotype regulates the chemokine environment of brain through differential modulation of astrocyte-derived chemokine secretion. We chose to focus subsequent experiments on astrocyte secretion of CCL3 because it exhibited pronounced *APOE*-dependent modulation (Table 1) and has been repeatedly implicated in a variety of neurodegenerative disorders (Israelsson et al., 2008; Kalkonde et al., 2007; Levine et al., 2009; Mines et al., 2007; Takami et al., 1997), especially AD (Azizi et al., 2014; Flex et al., 2014; Geppert et al., 2010; Li et al., 2008; Man et al., 2007).

Microglia express receptors for CCL3 and dose-dependently migrate to CCL3

We next assessed the functional relevance of CCL3 in the context of microglial response. CCL3 synthesis in brain regulates transit of peripheral immune cells across the blood brain-barrier (Babcock et al., 2003; Chui and Dorovini-Zis, 2010), especially in multiple sclerosis (Balashov et al., 1999; Simpson et al., 1998). Because ligation of the cell-surface chemokine receptors CCR1 and CCR5 mediates chemotaxis along peripheral CCL3 concentration gradients, we first determined whether microglia express these same receptors. Immunofluorescence staining of primary microglial cultures revealed robust expression of both CCR1 and CCR5 (Figure 1A) on cells positive for Iba-1, which comprised almost all of the cells in the assay thus confirming the purity of the cultures.

Although little is known about the significance of CCL3 expression in the CNS, others have shown that primary cultures of human and rat microglia migrate in response to beta-chemokines, including CCL3 (Cross and Woodroffe, 1999; Peterson et al., 1997). We found that recombinant CCL3 dose-dependently stimulated migration of mouse primary wild-type microglia in a modified Boyden chamber assembly (Figure 1B), eliciting a maximal chemotactic response of greater than 400% over basal migration (Figure 1C). These data confirm previous reports in other mammalian cell culture systems and implicate CCL3 as a potential modulator of resident microglia recruitment in the central nervous system.

APOE genotype differentially modulates CCL3 secretion by astrocytes in response to various TLR agonists

We hypothesized that *APOE* genotype-dependent differences in astrocyte chemokine production might influence microglia innate immune function, so we evaluated *APOE* genotype-dependent production of CCL3 by astrocytes. Because it is possible that the differential secretion of CCL3 by humanized astrocytes outlined in Table 1 is specific to PIC or TLR3 agonists, we used ELISA to validate our initial multiplex findings and expanded

the stimulants to include agonists for TLR2 (Pam3), TLR4 (LPS), and TLR9 (CpG). Figure 2 confirms the U-shaped observation initially made in PIC-treated humanized astrocytes, and broadens the finding to include similar responses from both LPS- and CpG-stimulated cells. PIC- and LPS-stimulated CCL3 secretion by astrocytes was robust in comparison to other agonists (Figure 2), and so subsequent experiments focused on one or both of these two agonists. CCL3 was below the level of assay detection in supernatant from control (vehicle-treated) cultures, so induced chemokine values are presented as the amount of CCL3 (pg) per amount of total cellular protein (μ g). Interestingly, treatment of primary astrocytes with the TLR2 agonist, Pam3, resulted in significantly reduced CCL3 overall compared to other activators, and failed to reproduce the U-shaped chemokine response (Figure 2). These data suggest a previously unreported *APOE* genotype-dependent differential pattern of astrocyte CCL3 secretion specific to TLR3 and TLR4 activation.

***APOE* genotype differentially modulates CCL3 gene expression in astrocytes**

We considered whether *APOE* genotype-dependent differences in CCL3 levels were regulated at the synthetic or secretory level, so we next evaluated the time dependence of PIC-stimulated chemokine secretion from humanized astrocytes. The U-shaped 18-hour differences in CCL3 concentration from astrocyte-conditioned media originally observed were relatively conserved over the time course (Figure 3A), but quantitative PCR (qPCR) analysis of a similar time course for PIC-induced CCL3 mRNA nearly mirrored the time-dependent trends seen in chemokine secretion (Figure 3A and B). As expected, the time-dependence for mRNA induction preceded that seen with chemokine secretion. Although these findings do not absolutely rule out *APOE* genotype-dependent differential modulation of chemokine secretion, mRNA data indicates that *APOE* genotype modulation of CCL3 in astrocytes is mediated at the level of gene expression.

***APOE* genotype differentially modulates apoE isoform production by astrocytes**

We next considered the possible mechanism by which *APOE* genotype may be modulating chemokine production by astrocytes. Because it is possible that *APOE* genotype-dependent variations in TLR expression underlie the observed differences in humanized astrocyte responses to immune activation, we first measured the basal expression of TLR2, TLR3, TLR4, and TLR9 mRNAs in these cultured cells. Specifically, we found that astrocyte expression of TLR2, TLR3, and TLR4 did not vary with *APOE* genotype (Supplemental Figure 1A, B, and C). Interestingly, while TLR9 expression differed significantly between all astrocyte genotypes (Supplemental Figure 1D), the expression pattern did not mirror the CpG-stimulated CCL3 secretion profile of astrocytes, suggesting an alternate mechanism for their observed *APOE* genotype-dependent differential chemokine secretion. Indeed, previous work has shown that apoE suppresses inflammatory responses in a variety of cells types (Kelly et al., 1994; Laskowitz et al., 1997; Zhu et al., 2010), including TLR-induced cytokine secretion from astrocytes (Lynch et al., 2001). Furthermore, multiple laboratories have identified rank order differences of $\epsilon 2 > \epsilon 3 > \epsilon 4$ in brain apoE expression from humanized mice that parallel the genetic risk for AD in human populations (Riddell et al., 2008; Ulrich et al., 2013). These observations led us to hypothesize that reduced apoE4 protein levels, rather than specific functional differences between apoE isoforms, are responsible for *APOE* genotype-dependent differences in CCL3 expression. Indeed,

therapies that increase apoE result in increased A β clearance and result in recovery of behavioral deficits in experimental animal models of AD (Cramer et al., 2012; Mandrekar-Colucci et al., 2012; Terwel et al., 2011).

We therefore investigated whether the astrocyte-specific patterns of *APOE* genotype-dependent differences in CCL3 elaboration correlate with levels of apoE synthesis by astrocytes. Conditioned media from untreated primary cultures of humanized *APOE* ϵ 2, ϵ 3, and ϵ 4 astrocytes was assayed for apoE isoform concentrations. Not surprisingly, the *APOE* genotype rank order of apoE secretion by astrocytes over the entire time course was apoE2>apoE3>apoE4 (Figure 4A), corroborating previously published *in vivo* data (Riddell et al., 2008; Ulrich et al., 2013). In addition, apoE secretion from all three genotypes was similarly time-dependent, with the rate of apolipoprotein accumulation not differing significantly between genotypes (Figure 4A). To confirm that this pattern of differential apoE isoform secretion from astrocytes did not result from *APOE* genotype-dependent differences in ubiquitous cell secretory machinery, we also measured total cell-associated apoE. The amounts of each apoE isoform from corresponding humanized astrocyte cell lysates were found to follow the same rank order pattern, with *APOE* ϵ 4 astrocytes synthesizing the least apoE after 18 hours (Figure 4B). In addition, the amounts of cell-associated apoE derived from the three astrocyte genotypes did not change significantly over the same 24-hour period used for analysis of secreted apoE isoforms (Supplemental Figure S2). Importantly, treatment of astrocytes with either LPS or PIC did not affect secreted or cell-associated apoE levels (data not shown). Also, while *APOE* genotype-dependent immunomodulatory differences between ϵ 3 and ϵ 4 astrocytes inversely correlated with the levels of corresponding apoE isoform expression, this relationship did not hold with ϵ 2 astrocytes, since comparably elevated apoE2 levels failed to similarly temper TLR-induced CCL3 secretion compared to ϵ 4 astrocytes. These data suggest that while apoE levels may account for some of the difference in TLR-dependent CCL3 synthesis observed between ϵ 3 and ϵ 4 astrocytes, additional unidentified mechanisms of immunomodulation are likely at play, especially with regard to ϵ 2-specific astrocyte responses.

TLR-induced CCL3 secretion from humanized astrocytes is RAP-independent

Secreted apoE is known to bind cell-surface receptors (Holtzman et al., 2012), thereby initiating signal transduction cascades that subsequently alter a variety of cellular processes, including cell motility (Cudaback et al., 2011) and chemical secretion (Keene et al., 2011; LaDu et al., 2001). To investigate whether the differential CCL3 secretion observed between humanized astrocytes resulted from differential interaction of the apoE isoforms and cognate cell-surface receptors, we evaluated the effects of the apoE receptor antagonist, receptor associated protein (RAP), on apoE-dependent modulation of astrocyte CCL3. Primary cultures of ϵ 2, ϵ 3, or ϵ 4 astrocytes were stimulated with either PIC or LPS for 18 hours in the presence or absence of RAP, and conditioned media was assayed for CCL3 concentration using ELISA. RAP failed to suppress *APOE* genotype-dependent differences in CCL3 secretion from primary ϵ 2, ϵ 3, and ϵ 4 astrocytes (Figure 5). In addition, administration of RAP alone did not stimulate the secretion of CCL3 from humanized astrocytes (data not shown). These data suggest that while TLR-induced CCL3 secretion

from humanized astrocytes is differentially modulated by apoE isoforms, the precise mechanism does not appear to depend upon RAP-sensitive apoE receptors.

Activation of nuclear receptors in APOE ϵ 4 astrocytes simultaneously increases apoE production and reduces PIC-induced CCL3

Inheritance of *APOE* ϵ 4 significantly increases the risk for sporadic AD. Furthermore, while the mean allele frequency for *APOE* ϵ 4 is estimated at around 15% worldwide (Eisenberg et al., 2010), half of AD individuals carry at least one *APOE* ϵ 4 copy (West et al., 1994; Yu et al., 2007). While true that *APOE* ϵ 3 individuals also get AD, modulation of the *APOE* ϵ 4 immunophenotype to be more *APOE* ϵ 3-like represents a valuable approach with translational potential. Our data confirm decreased apoE production as well as increased synthesis and secretion of the pro-inflammatory cytokine CCL3 by *APOE* ϵ 4 compared to *APOE* ϵ 3 astrocytes, so we hypothesized that increasing apoE expression in *APOE* ϵ 4 astrocytes would suppress TLR-induced CCL3 secretion resulting in *APOE* ϵ 3-like astrocyte CCL3 expression. Previous reports show that activation of the nuclear receptor family members liver X receptor (LXR) and retinoid X receptor (RXR) leads to increased apoE levels *in vivo* (Terwel et al., 2011; Ulrich et al., 2013), presumably acting through astrocytes since they represent the principal apoE source in brain. LXR and RXR activation has also been shown to suppress neuroinflammation, increase A β clearance, and reverse behavioral deficits in mouse models of AD (Cramer et al., 2012; Cui et al., 2012; Mandrekar-Colucci et al., 2012). Therefore, we tested our hypothesis by treating TLR-stimulated *APOE* ϵ 4 astrocytes with TO901317, an LXR agonist, 9-*cis*-retinoic acid, an RXR agonist, or their combination, and then assayed cell lysates for apoE and conditioned media for apoE and CCL3. Although astrocyte-secreted apoE levels were unaffected by nuclear receptor agonist treatment (data not shown), co-administration of PIC or LPS with TO901317 resulted in increased cell-associated apoE4 and a concomitant suppression of CCL3 elaboration by *APOE* ϵ 4 astrocytes compared to corresponding stimulated controls (Figure 6A and B). Interestingly, while co-administration of PIC or LPS with 9-*cis*-retinoic acid alone had no effect on either apoE or CCL3 levels (data not shown), the combination of TO901317 and 9-*cis*-retinoic acid synergized to increase apoE levels above, and decrease CCL3 levels below, those concentrations resulting from TO901317 treatment alone (Figure 6A and B). This is especially surprising given a previous report showing equally robust suppression of chemokine secretion by either TO901317 or 9-*cis*-retinoic acid in LPS-stimulated primary murine astrocytes (Zhang-Gandhi and Drew, 2007). Importantly, nuclear receptor activation in *APOE* ϵ 4 astrocytes significantly increased the expression of expected target mRNAs, including the cholesterol transporters *ABCA1* and *ABCG1* (Supplemental Figure S3), confirming individual agonist efficacy in our system. Furthermore, these findings suggest that the inducible pool of apoE is appropriately lipidated, although it is noteworthy that neither TO901317 nor 9-*cis*-retinoic acid alone resulted in significant induction of *APOE* mRNA by these primary astrocytes (Supplemental Figures S3). Regardless, our data confirms the hypothesis that decreased apoE isoform expression by *APOE* ϵ 4 astrocytes contributes, in part, to the observed differential TLR-induced secretion of CCL3.

Peripheral inflammation differentially induces CCL3 expression in brain of APOE humanized mice

We next investigated whether immune challenge *in vivo* results in a similar pattern of APOE genotype-dependent difference in TLR-induced CCL3 secretion in brain. Twelve-week-old male APOE $\epsilon 3$ and $\epsilon 4$ mice were given intraperitoneal injections of LPS or saline, sacrificed after 4 hours, and their brains rapidly removed and processed for RNA isolation. As expected, qPCR analysis of total mRNA isolated from the brains of control (uninjected) mice revealed that CCL3 mRNA was below the level of assay detection in both APOE $\epsilon 3$ and $\epsilon 4$ animals, whereas peripheral LPS injection resulted in a rapid and robust induction of CCL3 message in brain (data not shown). LPS-induced CCL3 mRNA expression in APOE $\epsilon 4$ brain was significantly increased compared to that of APOE $\epsilon 3$ animals (Figure 7A), confirming our *in vitro* findings in primary astrocytes. While these data do not explicitly identify astrocytes as the lone cellular source of this disproportionate neuroinflammatory response (parenchymal microglia, LPS-activated endothelial cells at the blood-brain barrier), they certainly implicate astrocytes as important contributors.

Post-mortem AD brains show elevated CCL3 expression in APOE $\epsilon 4/4$ human subjects compared to $\epsilon 3/\epsilon 3$

Neuroinflammation represents one of the pathologic hallmarks of AD, and is characterized by microglial activation and astrogliosis (Klegeris et al., 2007). To investigate whether our current findings translated to human disease, we used qPCR analysis to compare the expression of CCL3 mRNA in post-mortem parietal lobe samples from human AD subjects. Specifically, we found CCL3 expression was increased nearly threefold in brains from homozygous APOE $\epsilon 4$ subjects (n=8) with a confirmed neuropathologic AD diagnosis compared to age-matched homozygous APOE $\epsilon 3$ AD individuals (n=8; Figure 7B). This observation expands our initial findings in humanized TR APOE mice and corresponding primary astrocyte cultures, suggesting relevance of astrocyte-derived CCL3 in human disease.

Discussion

We describe a novel, APOE-dependent pathway for modulation of CNS innate immune regulation that is dependent upon astrocyte production of a specific chemokine. We found that, under innate immune activating conditions, CCL3 production is reduced in APOE $\epsilon 3$ astrocytes in comparison with those from an APOE $\epsilon 2$ or APOE $\epsilon 4$ background. This effect is specific to TLR3 and TLR4 pathways, and is not dependent on direct activation of RAP-specific apoE receptors, although induced CCL3 levels are inversely proportional to the levels of apoE protein in culture. This effect is restricted to a subset of chemokines in astrocytes with the most robust APOE-dependent modulation seen for CCL3. In total, these data implicate a stimulus- and response-specific pathway for APOE-dependent astrocyte modulation of innate immune response in brain. We demonstrate modulation of mouse microglia migration by CCL3, confirming the potential significance of CCL3 signaling in brain reported by others (Okamura et al., 2012; Redell et al., 2012; Soares et al., 2013). Since there is chronic induction of innate immune response in AD, we predicted that AD

patients with *APOE* $\epsilon 4/\epsilon 4$ genotype would have chronically increased levels of CCL3, which we confirmed in cortical brain samples from *APOE* $\epsilon 4/\epsilon 4$ AD patients.

Neuroinflammation is a common component of AD neuropathology, and *APOE* represents the single greatest genetic risk factor for development of sporadic AD. These data implicate *APOE*-dependent alterations in astrocyte chemokine modulation of microglia innate immune response in the exacerbated or persistent brain inflammation characteristic of AD neuropathology. Epidemiologic evidence supports this notion (McGeer et al., 2006). Microglia are the principal effector cells of neuroinflammation in AD (Aguzzi et al., 2013), and considerable effort has been dedicated to understanding the relationship between *APOE* genotype and microglial contribution to AD pathophysiology (Cudaback et al., 2011; Jiang et al., 2008; Qin et al., 2006; Vitek et al., 2009), but the potential contribution of astrocytes in neuroinflammation has been largely overlooked. We demonstrate *APOE*-dependent modulation of astrocyte CCL3 secretion that potentially exerts indirect neurotoxicity through exacerbation of microglia innate immune response but may also exert direct neurotoxicity in AD through direct neurotoxicity of CCL3. Furthermore, while CNS expression of CCL3 receptors (CCR1 and CCR5) in humans has been reported (Mines et al., 2007), it remains unclear whether *in vivo* expression is restricted to microglia. Thus, CCL3 may represent a therapeutic target for diverse astrocyte-dependent neurotoxic pathways, but, in light of strong evidence that neuroinflammation-mediated neurotoxicity is mediated primarily through differential microglia innate immune response, we propose that *APOE* risk for development of AD is, at least in part, mediated through modulation of astrocyte-dependent orchestration of microglial recruitment and activation.

Microglia respond to CNS insults by migrating to lesion sites and initiating inflammatory activities that can be contextually beneficial or deleterious to the host. We previously reported apoE isoform-dependent microglial migration differences *in vitro* (Cudaback et al., 2011), work which we subsequently confirmed *in vivo* using bone marrow transplantation (Yang et al., 2013). Collectively, our findings suggest that *APOE* $\epsilon 4$ microglia are functionally less responsive to chemotactic stimuli than *APOE* $\epsilon 3$ cells. As the major cell type of the brain, astrocytes are poised to significantly contribute to the local inflammatory environment, specifically directing microglial responses through the secretion of chemokines, including CCL3 (Gonzalez-Perez et al., 2012). Here we show that CCL3 stimulates chemotaxis in primary microglia, and that astrocytes derived from TR *APOE* mice differentially secrete this potent chemokine in response to a variety of TLR activators. Of particular interest is the novel pattern (*APOE* $\epsilon 2$ and $\epsilon 4$ greater than $\epsilon 3$) of TLR-induced chemokine secretion by astrocytes observed in our initial screen. In particular, the marked U-shaped response to PIC activation for CCL3 secretion by astrocytes inversely mirrors our previous migration data for microglia, suggesting a possible association. For example, through some unknown developmental mechanisms, elevated CCL3 synthesis by *APOE* $\epsilon 2$ and $\epsilon 4$ astrocytes may have evolved to compensate for the corresponding reduction in migratory capacity displayed by the target microglia. We did not specifically investigate the *APOE* genotype-dependence of microglial chemotactic response to CCL3 as it was beyond the scope of our proposed study, but future studies are needed to address this possibility. In addition, since this U-shaped CCL3 expression pattern could not be generalized to all

chemokines screened, this interpretation is at least incomplete. Because previous studies focused on LPS innate immune activation, these findings may be less restricted to chemokine induction and more specific for PIC-mediated responses. This seems unlikely since LPS induced activation profiles in astrocytes are similar to those elicited by PIC stimulation. Furthermore, previous work from our laboratory identified *APOE* genotype-dependent differences in LPS-stimulated pro-inflammatory cytokine secretion from humanized astrocytes whose rank order of $\epsilon 2 > \epsilon 3 > \epsilon 4$ is the reverse of that observed in similarly treated microglia (Maezawa et al., 2006a). While understandable that cell-specific differences would likely exist across a diverse collection of similarly induced cytokines, it is somewhat surprising that, in the case of primary microglia, a multiplex screen identical to that described for astrocytes revealed a rank order secretory pattern for CCL3 similar to published results for pro-inflammatory cytokines, namely *APOE* $\epsilon 4 > \epsilon 3 > \epsilon 2$ (data not shown). These observations would suggest that astrocytes uniquely express CCL3 in this pattern and that the basis for this difference involves pathways that to date have not been associated with *APOE*.

It is frequently assumed that the *APOE* genotype-dependent differential modulation of various CNS functions observed result from the unique biochemical differences between apoE isoforms. Indeed, human apoE isoforms differentially engage cognate cell-surface receptors, as well as the pleiotropic neurotoxic peptide A β . However, our laboratory and others have identified a strong association between *APOE* genotype and the level of specific isoform expression. The current study reveals a novel mechanistic relationship between apoE concentration and TLR-induced CCL3 secretion by astrocytes. The level of apoE expression by *APOE* $\epsilon 3$ and $\epsilon 4$ astrocytes was found to inversely correlate with the extent of TLR-induced chemokine expression and secretion, a finding validated by increased CCL3 secretion from *apoe*^{-/-} astrocytes (data not shown). Furthermore, pharmacological modulation of apoE4 expression in *APOE* $\epsilon 4$ astrocytes inversely correlated with TLR-induced CCL3 secretion, such that increasing apoE4 expression significantly reduced CCL3 elaboration. Interestingly, while activation of LXR or the combination of LXR and RXR increased the amount of cell-associated apoE4 in primary astrocytes, secreted apoE was unchanged (data not shown). This suggests that in addition to modulating levels of apoE expression, possibly through ABCA1- and/or ABCG1-mediated lipidation, nuclear receptor agonists likely influence the subcellular distribution of apoE isoforms, potentially implicating a discrete pool of cellular apoE in the apoE concentration-dependent control of inflammatory responses by astrocytes. In addition, the fact that TLR-induced CCL3 secretion by astrocytes was RAP-independent supports the assertion that extracellular apoE concentrations have limited influence over innate inflammatory phenotypes. While this data strongly suggests that the particular isoform is less important than the absolute amount, the association did not hold for *APOE* $\epsilon 2$ astrocytes. These findings are again reminiscent of our earlier migration work in microglia, and we stress that the relevant epidemiologic correlate in AD is the comparison between *APOE* $\epsilon 3$ and $\epsilon 4$, and not *APOE* $\epsilon 2$. Also, when considering the evolutionary origin of *APOE* $\epsilon 2$, it is entirely possible that the mechanisms underlying *APOE* genotype-dependent immunomodulation diverged at the point in human history where the point mutation in *APOE* $\epsilon 3$ gave rise to *APOE* $\epsilon 2$, thus constituting a novel and as yet unidentified molecular mechanism. In addition to the primary astrocyte

work presented here, a previous study from our lab revealed similarly irreconcilable differences between apoE isoform-specific immunomodulatory differences in glia and the rank order genetic risk for AD of $\epsilon 2 > \epsilon 3 > \epsilon 4$, especially with regard to apoE2 (Cudaback et al., 2011). It is equally plausible that *APOE* genotype-dependent differences in TLR-stimulated signaling account for this unique pattern of chemokine secretion observed in primary astrocytes. However, TLR expression data from humanized astrocytes (Supplemental Figure S1), as well as the diverse pattern of chemokine secretion shown in Table 1, fails to support this hypothesis. Alternatively, differences in downstream signaling may underlie our observations. Indeed, we have previously reported that *APOE* genotype-dependent differences in pro-inflammatory cytokine secretion from astrocytes correlate with differences in nuclear factor-kappa B (NF- κ B) activity (Maezawa et al., 2006a). While such differences in NF- κ B activity do not clearly support the observed pattern of CCL3 secretion, it is possible that these reported signaling variations influence the induction of other chemokines reported here, especially CXCL1 and CXCL9. Moreover, it is interesting that CCL4 and CXCL2 display similarly U-shaped secretion profiles between *APOE* genotypes. But what, then, is the common thread linking TLR-mediated induction of these proinflammatory chemokines? Peripheral immunity studies have established significant functional overlap between CCL3, CCL4, and CXCL2, and while our observations may only be coincident with this fact, it is possible that common transcriptional regulatory pathways underlie this unique activation profile. Nuclear factor of activated T cells (NFAT) is a transcription factor known to regulate the induction of CCL3, CCL4, and CXCL2, including in primary astrocytes (Ahamed et al., 2004; Kim et al., 2011; Shiratori et al., 2010). Therefore we investigated the expression and nuclear translocation of known NFAT isoforms (NFATc1, NFATc2, NFATc3, and NFATc4), but found no significant differences between *APOE* genotypes of TLR-stimulated astrocytes (data not shown). Additionally, the induction of CCL3, CCL4, and CXCL2 expression in cellular effectors of peripheral innate immunity by various inflammatory agents has been shown to be under the control of CCAAT/enhancer-binding proteins (C/EBP) and cAMP response element binding protein (CREB) family members (Mayer et al., 2013; Yan et al., 2012a; Yan et al., 2012b), and therefore warrants future investigation in the context of our model system.

Disproportionate or persistent inflammatory responses in brain represent a significant contributor to the pathophysiology and disease progression of many neurodegenerative diseases. Astrocytes represent integral regulators of neuroinflammation, especially relating to the recruitment of microglia, and we have shown that *APOE* genotype exerts considerable influence over astrocyte coordination of these pathways. While our results focus on the potent chemokine CCL3, additional astrocyte-derived mediators of microglial recruitment and activation are likely to be differentially influenced by *APOE* genotype as well. The relevance of *APOE* genotype to AD risk is undeniable. While the absolute abrogation of inflammation is neither achievable nor advisable, a more targeted approach aimed at key inflammatory regulators may be the best therapeutic approach for efficacious modulation of neuroinflammation in AD. Astrocytes and the chemokines that they are induced to release represent just such a target. These data lend support to the notion that pharmacological modulation of inflammatory pathways in brain remains a viable valuable therapeutic target in neurodegenerative diseases such as AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Murine microglia express CCL3 receptors and display dose-dependent migration to CCL3. A) Cultured wild-type primary mouse microglia were plated onto glass chamber slides and stained with antisera directed against Iba-1, CCR1, and CCR5. Merged immunofluorescent images show microglial localization of both CCL3 receptors. B) Migration of wild-type microglia was measured using a modified Boyden chamber in the presence of increasing CCL. Data are expressed as mean $\pm$ SE percentage of the maximum wild-type response to CCL3; $n=9$ (i.e., 3 independent experiments were performed in triplicate). ANOVA for dose vs. time had $P<0.0001$. C) 100 μ M CCL3 stimulated wild-type microglial migration 400% compared to basal migration. Data are expressed as the mean $\pm$ SE percentage of wild-type basal migration; $n=9$ (i.e., 3 independent experiments performed in triplicate). *** $P<0.001$; student T test.

Figure 2.

Toll-like receptor (TLR)-mediated secretion of CCL3 by astrocytes displays *APOE* genotype-dependent differential induction. Murine astrocytes expressing apoE2, apoE3, or apoE4 were stimulated with TLR-selective agonists [1μg/ml Pam3 (TLR2), 20μg/ml PIC (TLR3), 100ng/ml LPS (TLR4), 1μM CpG (TLR9)] for 18h and supernatant amounts of CCL3 (pg/μg protein) quantified via ELISA. Unstimulated vehicle controls were below the level of detection. Data are expressed as average amount ± SE (n=3–5). Two-way ANOVA had $P < 0.0001$ for *APOE* genotype, TLR agonist, and interaction. Bonferroni-corrected paired comparisons between astrocyte *APOE* genotype were significant for PIC and LPS ($***P < 0.001$), and $P > 0.05$ for Pam3 and CpG.

Figure 3.

PIC-stimulated differential CCL3 secretion and mRNA expression by humanized astrocytes is time-dependent. A) Primary humanized astrocytes were stimulated with PIC (20μg/ml) for indicated times and supernatant amounts of CCL3 (pg/μg protein) quantified via ELISA. Data are expressed as average amount $\pm$ SE (n=3–6). Two-way ANOVA had $P < 0.0001$ for *APOE* genotype, time, and interaction. Bonferroni-corrected post tests had $P < 0.01$ for ε2 and ε4 greater than ε3 at 8, 12, and 18 hours. B) Total RNA was isolated from astrocytes treated for indicated times and CCL3 mRNA expression quantified using qPCR. Data are expressed as fold increases relative to unstimulated time-matched controls (n=4). Two-way ANOVA had $P < 0.0001$ for *APOE* genotype, time, and interaction. Bonferroni-corrected post tests had $P < 0.01$ for ε2 and ε4 greater than ε3 at 4, 6, and 8 hours. Note that in some cases error bars are smaller than symbols.

Figure 4.

Differential apoE expression by humanized astrocytes is *APOE* genotype-dependent. A) Primary humanized astrocytes were incubated in serum-free media for 0 – 24h as indicated and supernatant amounts of apoE (pg/μg protein) quantified via ELISA. Data are expressed as average amount $\pm$ SE (n=3–5). Two-way ANOVA had $P < 0.0001$ for genotype, time, and interaction. Note that in some cases error bars are smaller than symbols. B) Cell-associated apoE was determined similarly by analyzing whole-cell lysates from 18h astrocyte cultures. Data are expressed as average amount $\pm$ SE (n=6). ANOVA had $P < 0.0001$ and Bonferroni-corrected paired comparisons between astrocytes genotypes are noted above the columns (** $P < 0.01$, *** $P < 0.001$).

Figure 5.

Toll-like receptor (TLR)-stimulated CCL3 expression by humanized astrocytes is RAP-independent. RAP (1μM) or vehicle was co-administered to primary humanized astrocytes stimulated with either PIC (A) or LPS (B) for 18h. Assay of conditioned supernatants revealed that RAP failed to inhibit TLR-stimulated astrocyte secretion of CCL3. Unstimulated vehicle controls were below the level of detection, and treatment with RAP alone did not stimulate CCL3 release (data not shown). Data are expressed as mean $\pm$ SE percentage of maximum PIC or LPS stimulation (n=3). Two-way ANOVA for both PIC and LPS treatment had $P>0.05$ for genotype, treatment, and interaction.

Figure 6.

Nuclear receptor activation in TR *APOE4* astrocytes concomitantly increases cell-associated apoE and reduces CCL3 secretion. Primary humanized TR *APOE4* astrocytes were pretreated with 2 μ M RXR agonist 9-*cis*-retinoic acid, 20 μ M LXR agonist TO901317, or the combination of both. After 30 minutes cells were activated with either PIC or LPS and apoE (A,C) and CCL3 (B,D) were assayed at 18 hours using ELISA. Importantly, 9-*cis*-retinoic acid alone, as well as PIC and LPS alone had no effect on apoE levels (data not shown). In addition, CCL3 from unstimulated vehicle controls was below the level of detection, and LXR and RXR administration alone did not affect CCL3 secretion (data not shown). Data are expressed as mean $\pm$ SE percentage of maximum PIC or LPS stimulation (n=3). ANOVA had $P < 0.0001$ for all panels and Bonferroni-corrected paired comparisons between treatments are noted above the columns (ns=not significant, *** $P < 0.001$).

Figure 7.

Brain CCL3 shows *APOE* genotype-dependent differential expression *in vivo*. A) Adult TR *APOE3* and TR *APOE4* male mice (12 week old) received intraperitoneal injection of LPS (5mg/kg). Mice were sacrificed after 4 hours and total RNA isolated from cerebral cortex. qPCR analysis revealed greater CCL3 induction in the brains of LPS-challenged TR *APOE4* mice compared to similarly challenged TR *APOE3* animals. CCL3 mRNA expression was below the level of detection in brains from saline-treated controls for both genotypes (data not shown). Data are expressed as mean $\pm$ SE percentage of LPS-challenged TR *APOE3* animals (n=6–8). * $P < 0.05$; Student's t-test. B) Total RNA was isolated from parietal lobe of age-matched human subjects enrolled in the University of Washington Alzheimer's Disease Research Center brain donation program with previous clinical AD diagnosis and

homozygous for either *APOE* ϵ 3 or *APOE* ϵ 4. qPCR analysis revealed significantly increased CCL3 mRNA expression in the AD brains of *APOE* ϵ 4/ ϵ 4 individuals (n=7) compared to similarly diseased *APOE* ϵ 3/ ϵ 3 subjects (n=7). Data are expressed as mean $\pm$ SE percentage of CCL3 expression by *APOE* ϵ 3/ ϵ 3 individuals. * P <0.05; Student's t-test.

Table 1
Differential chemokine secretion by primary humanized astrocytes in response to 20 µg/ml PIC exposure

Primary astrocyte cultures prepared from neonatal TR APOE2, TR APOE3, and TR APOE4 mice were exposed to 20 µg/ml PIC or vehicle for 18 h and culture supernatants assayed with a 32-plex array for CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL9, CXCL10, eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), IFN γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), macrophage-colony stimulating factor (M-CSF), TNF- α , and vascular endothelial growth factor (VEGF). PIC exposure resulted in significant induction of the 10 chemokine analytes shown. Induction of CCL3 revealed a robust U-shaped profile such that chemokine induction TR APOE3 astrocytes was markedly reduced in comparison to astrocytes from either TR APOE2 or TR APOE4 mice.

	Concentration (pg/ml)					
	e2		e3		e4	
	vehicle	PIC	vehicle	PIC	vehicle	PIC
CCL2	90.4	21118.6	63.8	17141.2	399	22340.9
CCL3	62	2133	54.3	338.1	65.4	2182.1
CCL4	ND	5592.1	ND	711	ND	6063.1
CCL5	18	4040.7	ND	3515.5	161.6	3745.9
CXCL1	40.9	18759.2	59.7	21600.9	128.9	16913.9
CXCL2	71.3	3163.4	71.3	1270.4	69.7	4190.6
CXCL9	19.2	89.1	15.1	275.5	12.3	156.1
CXCL10	348.8	20729.1	199.7	17803.6	1717.4	19916.3
Eotaxin	12.7	42.5	12.4	77.6	14.3	78.3
LIF	ND	203.4	ND	75.8	ND	107.6

Data are expressed as chemokine amounts (pg/µg protein) from two independent experiments pooled from three replicate cultures and run in duplicate. ND: not detectable; PIC: polyinosinic-polycytidylic acid.