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Canonical Type I IFN Signaling in Simian Immunodeficiency Virus-Infected Macrophages Is Disrupted by Astrocyte-Secreted CCL2

Luna Alammar Zaritsky,* Lucio Gama,* and Janice E. Clements*†

HIV-associated neurologic disorders are a mounting problem despite the advent of highly active antiretroviral therapy. To address mechanisms of HIV-associated neurologic disorders, we used an SIV pigtailed macaque model to study innate immune responses in brain that suppress viral replication during acute infection. We previously reported that during acute infection in brain, noncanonical type I IFN signaling occurs, where IFN-β mRNA is induced while IFN-α is simultaneously suppressed. Two downstream IFN-stimulated genes, MxA and TRAIL, also show differential expression patterns. In this study, we show that differential signaling is due to interactions between macrophages and astrocytes. Astrocytes produce high levels of CCL2 upon SIV infection, which binds to CCR2 receptors on macrophages, leading to a selective suppression of IFN-α and the IFN-stimulated gene TRAIL while simultaneously inducing IFN-β and MxA. The interactions between chemokine and cytokine pathways are a novel finding that may specifically occur in the CNS. The Journal of Immunology, 2012, 188: 000–000.

Type I IFNs are the first line of defense against viral infections, including HIV and SIV. Canonical type I IFN signaling begins with the production of IFN-β upon stimulation of pattern recognition receptors (PRRs). IFN-β is then secreted and binds the IFN receptor in an autocrine and paracrine manner where it activates the JAK/STAT pathway, leading to the expression of IFN-α and a plethora of antiviral IFN-stimulated genes (ISGs). IFN-α is then secreted and binds to the IFN receptor where it perpetuates the IFN signaling cascade in a positive feedback loop (1).

Our group has made the novel discovery that type I IFN signaling in the CNS in response to SIV infection occurs in a more complex, noncanonical manner (2). Using an accelerated, consistent pigtailed macaque SIV model of HIV-associated neurologic disease, we have shown that whereas IFN-β is induced during acute SIV infection in brain, IFN-α is simultaneously downregulated and associated with a suppression of signaling modulators downstream of the IFN receptor, including Tyk2, STAT1, and IRF7. This results in differential regulation of ISGs as well, with MxA being induced along with IFN-β, whereas TRAIL is suppressed along with IFN-α. These results indicate that suppression of JAK/STAT signaling leading to IFN-α and TRAIL does not result in complete shutdown of antiviral signaling, as IFN-β and MxA are still induced. Rather, accessory signaling pathways downstream of the IFN receptor exist, which enables IFN-β to upregulate a subset of antiviral genes, such as MxA. Other groups have also found that despite the suppression of the JAK/STAT pathway, IFN-β still upregulates a subset of antiviral ISGs through alternative pathways, such as MAPK and PI3K (3). These results reveal that highly regulated mechanisms controlling IFN signaling occur during acute SIV infection in the brain, where the JAK/STAT pathway leading to IFN-α and TRAIL is suppressed, whereas other IFN-β–stimulated pathways downstream of the IFN receptor are induced.

Why this differential regulation occurs in the CNS and not in peripheral tissues, such as the lung, is most likely a consequence of the effects IFNs and their ISGs have in the brain. Whereas IFN-β has been associated with neuroprotection and the stimulation of anti-inflammatory mediators, IFN-α and Tyk2 have been associated with neurotoxicity, inflammation, cell death, and neuroinflammatory diseases such as HIV dementia and multiple sclerosis (4–10). TRAIL is a gene associated with cell death as well. Therefore, this differential pattern of IFN signaling in brain may be an evolutionary mechanism to induce an antiviral response mediated by IFN-β without inducing the inflammatory and destructive effects mediated by IFN-α, TRAIL, and the JAK/STAT pathway.

To investigate the mechanism behind this differential IFN signaling, we examined the two cell types involved in HIV and SIV infection in brain: macrophages and astrocytes. HIV and SIV infection of the CNS occurs during early stages of disease, as indicated by production of viral mRNA and protein both in brain tissue and cerebrospinal fluid (11–13). This occurs via a “Trojan horse” mechanism, where infection of certain subpopulations of peripheral monocytes results in immune activation and upregulation of adhesion molecules such as VCAM-1, facilitating transmigration across the blood–brain barrier (14–16). These monocytes accumulate in the perivascular regions and undergo differentiation into macrophages, which is where early HIV and SIV replication takes place (17–20). Although induction of cytokines such as IFN-β in macrophages has been shown to be protective against SIV infection in the brain (21, 22), uncontrolled viral replication can lead to the production of proinflammatory...
cytokines, excitatory amino acids, and molecules mediating oxidative stress, all of which ultimately lead to neuronal death (23, 24). Astrocytes are the most numerous cells in the brain and have been reported to express relatively high levels of TLR3 and the downstream signaling adapter molecules, indicating that astrocytes, as well as macrophages, play a major role in the immunological response to infection (25–27). HIV/SIV infection in astrocytes has been reported in brain tissue of humans and macaques, as well as in vitro (13, 28). Upon infection, astrocytes are the major producers of MCP-1, or CCL2, which is a chemokine that recruits monocytes expressing CCR2 into the CNS (14). Certain populations of macrophages/microglia in brain have been shown to express CCR2 during SIV/HIV infection (29). Importantly, CCL2 has also been reported to protect neurons from apoptosis as well as inhibit glutamate production in response to HIV infection, making it an important neuroinflammatory modulator (30).

Perhaps the most significant immunological role astrocytes play in the CNS is their immunomodulatory effects on macrophages and microglia. Microglia, the resident macrophages of the brain, have been shown to exhibit increased inflammatory activity in vitro than that observed in pathologic conditions in the brain (31, 32), largely because microglia are usually cultured alone, which does not accurately portray the CNS microenvironment. However, by coculturing astrocytes with macrophages or by exchanging supernatants, it was found that astrocytes release soluble factors that attenuate the inflammatory response in macrophages. These effects include reducing the expression of inducible NO synthase and IL-12 (33, 34), increasing expression of antioxidant enzymes (35), and inhibiting IFN-γ–induced microglial activation (36). Also, HIV-infected monocytes that interact with astrocytes have been reported to secrete decreased levels of TNF-α and eicosanoids upon LPS stimulation (37).

Because astrocytes have been shown to attenuate the inflammatory response in macrophages, we examined if these cells could selectively down modulate IFN-α, as this cytokine is a potent neuroinflammatory mediator. We found that the differential IFN response observed in vivo is attributed to astrocytic production of CCL2. When SIV-infected macrophages are exposed to CCL2, there is specific suppression of IFN-α and TRAIL, while upregulation of IFN-β and MxA is maintained. This not only illustrates the novel role CCL2 plays in selectively suppressing IFN-α expression in SIV-infected macrophages but also suggests that the immunomodulatory effects that astrocytes exert on macrophages are a key factor in maintaining an anti-inflammatory environment during acute viral infection in the brain.

Materials and Methods

Cell culture and infections

Macrophages. Primary rhesus macaque astrocytes (Cambrex, Walkersville, Md.) were cultured as previously described (39). Cells were infected with SIV/17E-Fr at an MOI of 0.05 for 6 h and then washed three times with sterile PBS. Cells were then cultured in DMEM, 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 0.5 mg/ml gentamicin, and 2 mM Na pyruvate. Half of the media was replaced with fresh D10 every 5 d for 30 d. Infected and uninfected cells were harvested on days 5–7, 10, 14, 21, and 25. RNA was isolated using the RNeasy kit and treated with DNase as described earlier. For polynosinic:polycytidylic acid (poly(IC))-treated and IFN-β–treated astrocytes, cells were treated with either 10 μg/ml polyIC or 100 U/ml IFN-β. Cells were harvested at 24, 48, and 72 h posttreatment, and RNA was isolated as described earlier.

Supernatant exchange reactions. Primary MDMs were infected as indicated earlier. After washing off virus, cells were cultured with either normal macrophage media, supernatants from uninfected astrocytes, or supernatants from infected astrocytes. Astrocytes supernatants including time-point–specific uninfected controls, were collected at 14–21 d.p. when cells were harvested. Ultracentrifugation was done at 40,000 rpm for 2 h at 4°C on both sets of supernatants using the Sorvall Discovery 100SE (rotor TH641) (Thermo-Fischer Scientific) to pellet virus and cellular debris. Supernatants were then passed through an 0.22-μm filter unit, and a p27 ELISA (Zephyros) was done to verify no infectious virus was present. Infected and uninfected cells were harvested 48 h.p.i., and RNA was isolated as described earlier.

Quantitative RT-PCR

All primer and probe sequences as well as quantitative RT-PCR (qRT-PCR) protocols are described elsewhere (2). All genes were normalized to 18S rRNA. SIV-infected samples were expressed as a fold induction over each sample’s respective uninfected control. In the case where uninfected samples were given a treatment, all treated samples were expressed as a fold induction over untreated controls.

Western blots and Abs

All Western blot protocols and Abs are described elsewhere (2). Quantitations were performed using ImageQuant TL 7.0 software on scanned images.

CCL2 ELISA

CCL2 was measured in the supernatants of SIV-infected and UI macrophages (48 h.p.i.), and of SIV-infected and UI astrocytes (14 d.p.i.) using the Quantikine Human CCL2 ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol.

CCL2 neutralization

Infected astrocyte supernatants were incubated with 50 μg/ml of anti-human CCL2 neutralizing Ab (R&D Systems) or nonspecific goat IgG for 1 h at room temperature. The supernatants were then added to uninfected and SIV-infected MDMs after virus had been washed off. Cells were harvested 48 h.p.i., and RNA was isolated using the RNeasy kit (Qiagen) as described earlier.

Chemokine and growth factor treatment

Uninfected and SIV-infected MDMs were untreated or treated with either 100 ng/ml recombinant human CCL2 (ProSpec, East Brunswick, NJ), 0.2 ng/ml recombinant human TGF-β (R&D Systems), or 10 ng/ml recombinant human CCL3 (ProSpec). Cells were harvested 48 h.p.i., and RNA was isolated using the RNeasy kit (Qiagen) as described earlier.

CCL2 inhibitor: propagermanium

Bis[2-carboxyethylgermanium(IV) sesquioxide] was purchased from Sigma Aldrich (St. Louis, MO) and made into a 10 mg/ml stock solution in water. (Other commonly used names: propagermanium and 2-carboxyethylgermaneousquioxide.) A final concentration of 100 μM was used to treat infected and uninfected MDMs. For samples that were treated with both propagermanium and CCL2, cells were incubated with propagermanium 45 min before adding CCL2.

Statistical analysis

For mRNA comparisons, either one-way ANOVA with Bonferroni posttest for multiple comparisons or two-way ANOVA with Bonferroni posttest for multiple comparisons was performed depending on the experimental conditions. For protein comparisons that only consisted of two groups...
Results

Type I IFN signaling is upregulated in SIV-infected MDMs

Because macrophages are the major target cells responsible for SIV replication in brain, we examined SIV infection of pigtailed macaque macrophages in vitro. Macaque MDMs were infected with SIV (MOI 0.05) and harvested at 24, 48, and 72 h p.i. Levels of Gag p27 were measured in cell supernatants to quantify viral production (Fig. 1A), and qRT-PCR was performed for IFN-α and IFN-β. We found that at 48 h p.i., both IFN-β and IFN-α mRNA are significantly upregulated 5-fold ($p < 0.01$) and 2-fold ($p < 0.01$), respectively (Fig. 1B, 1C), coinciding with the start of viral detection in the supernatant. mRNA levels of two ISGs, TRAIL and MxA, were also quantitated. We show that at 48 h p.i., both mRNA are signif-

icantly upregulated (2) (Figs. 1D, 1E), contrary to our in vivo observations where MxA mRNA is upregulated in the same pattern as IFNB, whereas TRAIL, like IFN-α, is not induced (2). Because the 48-h time point exhibited the least amount of variability in gene expression between replicates, this time point was chosen for subsequent experiments. By 72 h p.i., cell death started occurring in infected cells, which may have accounted for the high degree of variability. At 48 h p.i., the transcription factor STAT1, which is phosphorylated upon IFN receptor activation, is not only translationally upregulated ($p = 0.0111$) but also has an increase in tyrosine phosphorylation (Y701) ($p = 0.0271$) (Fig. 1F). Both p-STAT1 and STAT1 are induced by ~75% upon SIV infection, indicating that there is a translational and posttranslational upregulation of IFN signaling upon SIV infection. These results indicate that both IFN-α and IFN-β are upregulated in SIV-infected MDMs in vitro, and downstream IFN signaling is activated.

FIGURE 1. Type I IFN signaling is upregulated in SIV-infected MDMs. Primary rhesus astrocytes were infected with SIV (MOI 0.05) and harvested at 2, 7, 10, 14, and 21 d p.i. for mRNA analysis. As we and other groups have shown (39, 40), whereas virus infection in macrophages takes place during the course of days, infection in astrocytes takes longer because restrictive factors specific to this cell type severely slows down viral replication in vitro (Figs. 1A, 2A). Unlike macrophages, which displayed an activated IFN response, no significant induction of IFN-α, IFN-β, or MxA mRNA was observed for any of the time points (Fig. 2A–D), nor was there an increase in STAT1 protein or phosphorylation status in infected cells (Fig. 2E). TRAIL mRNA was undetectable. To determine why this inhibition of IFN was occurring, we measured IRF7 mRNA and protein, as this is an important transcription factor in IFN-α and IFN-β production that is both transcriptionally and translationally induced upon viral infection (41). IRF7 mRNA was undetectable in infected and uninfected cells, and there was no detectable change in protein at 14 d p.i. (Fig. 2H). To ensure that the astrocytes were immunologically functional, uninfected cells were treated with polyI:C to test pathogen sensing activity and with IFN-β to test stimulation through the IFN receptor. Both treatments significantly upregulated all gene expression by 24 h posttreatment (Fig. 2F, 2G), indicating that the astrocytes were able to induce a full innate immune response. These results indicate that the IFN response is specifically downregulated in SIV-infected astrocytes.

Astrocytes release soluble factors that specifically suppress IFN-α induction in SIV-infected macrophages

We found that type I IFN signaling is upregulated in SIV-infected macrophages in vitro, whereas SIV-infected astrocytes exhibit the
opposite effect. In brain tissue, however, there is an intermediate response. It has been reported that proteins released by astrocytes have anti-inflammatory effects on stimulated macrophages. Because IFN-α is a more potent inflammatory cytokine in the CNS than IFN-β, we determined if soluble factors released by astrocytes could specifically downregulate IFN-α in SIV-infected macrophages while maintaining the IFN-β response. We opted to perform a series of supernatant exchange experiments instead of coculturing the cells for two reasons: The two primary cell types were from different donor macaques, and obtaining mixed primary glial cultures directly from adult primate brain is extremely costly and complicated. Therefore, we studied the effects of astrocytes on macrophages by doing a series of astrocyte supernatant exchange experiments. Supernatants were collected from either uninfected or SIV-infected astrocytes and cleared of virus by ultracentrifugation. Gag p27 ELISAs were done to ensure no in-

FIGURE 2. Type I IFN signaling is downregulated in SIV-infected astrocytes. Primary rhesus astrocytes were infected with SIV. RNA was isolated at 2, 7, 10, 14, and 21 d p.i. (A) p27 levels were measured in the supernatants by ELISA. (B-D) qRT-PCR analysis was done for (B) IFN-α, (C) IFN-β, and (D) MxA. TRAIL mRNA was undetectable. All qRT-PCR data are expressed as a fold induction over uninfected controls. All genes are normalized to 18S rRNA. Data are expressed as the mean of three independent experiments (each done with duplicate technical replicates) with 95% confidence interval. (E) Protein lysates were made at 5, 10, 14, 21, and 25 d.p.i. from uninfected controls (C) and SIV-infected (I) primary rhesus astrocytes and probed for GAPDH, STAT1, and p-STAT1 (Y701) via Western blot. Quantitations were performed on four replicate Western blots at 14 d.p.i. normalized to GAPDH. Blot shown is representative of all experiments. (F) Astrocytes were stimulated with polyI:C (10 μg/ml), and RNA was isolated at 24, 28, and 72 h p.i. qRT-PCR was done on IFN-α, IFN-β, and MxA. Values are normalized to 18S rRNA and are expressed as a fold induction over untreated controls. (G) Primary rhesus astrocytes were treated with IFN-β (100 U/ml), and RNA was isolated 24, 48, and 72 h.p.i. qRT-PCR was done for IFN-α and MxA. Values are normalized to 18S rRNA and are expressed as a fold induction over untreated controls. Data for astrocyte treatments are expressed as the means of duplicate independent experiments (each done with three technical replicates) with 95% confidence interval. (H) Western blot for IRF7 and GAPDH was performed on control (C) and infected (I) astrocytes 14 d.p.i. Blot is representative of three replicates. For all mRNA comparisons, one-way ANOVA with Bonferroni posttest for multiple comparisons was performed. Significant (p < 0.05) pairwise comparisons are shown.
fectious virus was present (data not shown). All macrophages were infected with SIV in normal macrophage media and then cultured with one of three different media conditions: normal macrophage media, uninfected astrocyte supernatant, or SIV-infected astrocyte supernatant. Cells were then harvested at 48 h p.i., and mRNA expression of IFN-α, IFN-β, MxA, and TRAIL was measured.

In cells cultured in normal media and infected astrocyte supernatants, IFN-β mRNA is significantly upregulated upon SIV infection compared with that of uninfected controls (p < 0.05 for both conditions) (Fig. 3B). Normal macrophage media, as well as SIV-infected astrocyte supernatant, yielded a 2.5-fold induction of IFN-β mRNA, whereas culturing with uninfected astrocyte supernatants yielded a 1.5-fold induction, although not significantly (p > 0.05). IFN-α mRNA expression was significantly upregulated (p < 0.001) in SIV-infected macrophages cultured with normal macrophage media (Fig. 3C). IFN-α expression in macrophages cultured with uninfected astrocyte supernatants also was increased above uninfected although not significantly (p > 0.05). In contrast, SIV-infected macrophages cultured with infected astrocyte supernatant show no induction of IFN-α mRNA compared with uninfected controls (p > 0.05) (Fig. 3C).

**FIGURE 3.** Astrocytes release soluble factors that specifically suppress IFN-α induction in SIV-infected macrophages. Primary pigtailed MDMs were infected with SIV and then cultured with either normal macrophage media (normal), uninfected astrocyte supernatants [astro (ui)], or infected astrocyte supernatants [astro (inf)]. Cells were harvested at 48 h p.i. (A) Levels of p27 were measured by ELISA in the supernatants. (B–E) RNA was isolated from cells, and qRT-PCR analysis was done for (B) IFN-β, (C) IFN-α, (D) MxA, and (E) TRAIL. Values are expressed as a fold induction over the respective uninfected controls. All values are normalized to 18S rRNA. Graphs represent means of three independent experiments (each done with duplicate technical replicates) with 95% confidence interval. One-way ANOVA with Bonferroni posttest for multiple comparisons was performed for mRNA comparisons. Significance (p < 0.05) or lack thereof is shown for pairwise comparisons discussed in the text.
thermore, the induction pattern of downstream ISGs showed that MxA RNA was significantly upregulated in all media conditions ($p < 0.05$ for normal media, $p < 0.001$ for both astrocyte conditions), with the highest fold inductions (>50-fold) in macrophages cultured with either type of astrocyte supernatant (Fig. 3D). TRAIL mRNA levels were significantly upregulated almost 800-fold in cells cultured in normal macrophage media ($p < 0.001$), but SIV-infected macrophages cultured with infected or uninfected astrocyte supernatant showed no significant upregulation ($p > 0.05$ for both) (Fig. 3E). Taken together, SIV-infected macrophages cultured with infected astrocyte supernatants most closely resembles the expression pattern in brain, where IFN-α and TRAIL are suppressed and IFN-β and MxA are significantly induced. These results indicate that SIV-infected astrocytes secrete soluble factor(s) that, when cultured with SIV-infected macrophages, lead to a strong suppression of IFN-α and TRAIL mRNA while not suppressing IFN-β and MxA.

Astrocytes release a higher concentration of CCL2 than macrophages, which can selectively suppress IFN-α in response to SIV infection

To identify the soluble factors in the infected astrocyte supernatants that lead to differential type I IFN expression in SIV-infected macrophages, we examined levels of CCL2, which is one of the major chemokines released by activated astrocytes in brain in response to HIV infection (42). We have previously shown in our macaque model that activated astrocytes in brain are associated with an upregulation of CCL2 protein (43). Although we have shown this in vivo, we wanted to confirm it in our astrocytes in vitro. CCL2 protein was measured by ELISA in the supernatants of uninfected and SIV-infected astrocytes between days 15 and 20 p.i. We found that CCL2 was upregulated by 55% upon SIV infection with a trend toward significance ($p = 0.0678$) (Fig. 4A). CCL2 was also measured in uninfected and infected macrophage supernatants, and, although macrophages did upregulate CCL2 upon SIV infection ($p = 0.0026$), both infected and uninfected macrophages produced at least 10 times less CCL2 than that of astrocytes (Fig. 4A), suggesting that culturing macrophages with astrocyte supernatants creates an entirely different immunological environment than what macrophages are exposed to when cultured alone.

To determine if CCL2 was responsible for differential IFN regulation, supernatants from SIV-infected astrocytes were incubated in the presence or absence of a neutralizing Ab to CCL2. Primary SIV-infected MDMs were then cultured with either the CCL2 neutralized or non-neutralized astrocyte supernatant. In macrophages cultured with infected supernatants without the CCL2 neutralizing Ab, there was no significant induction of IFN-α mRNA upon SIV infection ($p > 0.05$), whereas when cultured with supernatants containing the neutralizing Ab to CCL2, IFN-α mRNA was significantly upregulated 7-fold ($p < 0.001$) upon SIV infection (Fig. 4B). This was not seen in the presence of a nonspecific IgG. IFN-β mRNA was up-

**FIGURE 4.** Astrocytes release a higher concentration of CCL2 than macrophages, which can selectively suppress IFN-α in response to SIV infection. (A) Levels of CCL2 were measured in the supernatants of uninfected (UI) and SIV-infected (SIV) primary astrocytes and primary MDMs. Graphs represent mean levels in supernatants from at least three different experiments with SE. An unpaired $t$ test was performed to compare levels of CCL2 in uninfected and SIV-infected macrophages for both astrocytes and macrophages. (B and C) SIV-infected astrocyte supernatants were incubated with either a neutralizing Ab to CCL2 (NA) or a control goat IgG. SIV-infected MDMs were then cultured with either infected astrocyte supernatants (SIV), infected astrocyte supernatants with CCL2 neutralizing Ab (SIV + NA), or infected astrocyte supernatants with control IgG (SIV + IgG). Cells were harvested 48 h p.i., and RNA was isolated. qRT-PCR analysis was done for (B) IFN-α and (C) IFN-β. Graphs represent means of two independent experiments (each done with duplicate technical replicates) with 95% confidence interval. One-way ANOVA with Bonferroni posttest was performed. Significance ($p < 0.05$) or lack thereof is shown for comparisons discussed in the text.
regulated in both media conditions, but only significantly in media without neutralizing Ab (Fig. 4C). These data suggest that CCL2 production by astrocytes is responsible for the selective suppression of IFN-α mRNA in SIV-infected macrophages.

**Recombinant CCL2 differentially regulates the IFN response**

We have shown that CCL2 is highly secreted by astrocytes upon SIV infection and that this chemokine selectively down-modulates IFN-α mRNA, as its neutralization restores IFN-α expression upon SIV infection. To examine whether CCL2 alone would reproduce this effect, recombinant CCL2 was added to macrophages in culture to mimic the astrocyte supernatant exchange experiments. Primary macaque MDMs were infected with SIV for 6 h and then incubated in the presence or absence of recombinant CCL2 (100 ng/ml) in normal macrophage media. Treating SIV-infected macrophages with CCL2 significantly downregulated both IFN-α and TRAIL mRNA expression (p < 0.001 for both genes) (Fig. 5A, 5D). However, neither IFN-β nor MxA mRNA induction in SIV-infected macrophages was affected by treatment with CCL2 (p > 0.05 for both genes) (Fig. 5B, 5C).

To test the specificity of the CCL2 effect, primary macrophages were also treated with CCL3, which is another chemokine, and TGF-β (10 ng/ml and 0.2 ng/ml, respectively), which is a cytokine that astrocytes are known to produce (37). Infection of cells treated with either of these proteins resulted in significant upregulation of IFN-α (p < 0.01 for TGF-β, p < 0.05 for CCL3) (Fig. 5E). These data indicate that CCL2 is able to specifically downmodulate mRNA expression of IFN-α and TRAIL in SIV-infected macrophages while having no effect on the mRNA expression of IFN-β and Mx. Therefore, CCL2 causes a noncanonical IFN signaling cascade in SIV-infected macrophages, where IFN-α and certain ISGs (i.e., TRAIL) are selectively suppressed transcriptionally.

**CCL2-induced IFN-α suppression occurs through CCR2 signaling**

To examine whether CCL2 exerts its effect through its cognate receptor, CCR2, a small-molecule inhibitor of CCR2, propagermanium (100 μM), was used. Propagermanium is an organic small-molecule inhibitor that specifically blocks CCR2 without stimulating downstream signaling (44). CCL2-treated and untreated macrophages were infected in the presence or absence of inhibitor. Cells were harvested 48 h p.i., and qRT-PCR analysis was performed on RNA for IFN-α and IFN-β. Blocking the CCR2 receptor in the presence of CCL2 was able to restore IFN-α induction levels in SIV-infected macrophages (p < 0.01), whereas neither the presence of CCL2 nor the blocking of CCR2 affected mRNA levels of IFN-β (Fig. 6A, 6B). These data indicate that CCL2 mediates the selective suppression of IFN-α in SIV-infected macrophages via a CCR2-dependent mechanism.
The suppressive effects that high concentrations of CCL2 have on present in a particular microenvironment but also the concentration. signaling is affected by not only the type of soluble factors that are infection, the amount of CCL2 is 10 times less than the amount immediately recognized by PRRs.

The complete suppression of the type I IFN response in SIV-infected astrocytes is very striking. Because astrocytes are able to induce the IFN response in polyI:C-treated and IFN-β-treated cells, this suppression of signaling in astrocytes is specific to SIV infection. We report that there is a deficiency in IRF7 mRNA and protein induction upon SIV infection, which may be responsible for the inability to produce IFN-α and IFN-β. The exact intracellular sensor that recognizes HIV and SIV in astrocytes is currently unknown, although one group has suggested the mannose receptor as playing a role (45). Therefore, more work is needed to determine which upstream innate intracellular regulators are being affected during infection that leads to lack of IRF7 activation. It is already known that astrocytes contain restrictive factors that severely impair the kinetics of SIV and HIV replication (39, 40). It is therefore possible that these restrictive factors may somehow prevent activation of the innate IFN response by interfering with the production of viral intermediates that would otherwise be immediately recognized by PRRs.

Although macrophages do upregulate CCL2 protein upon SIV infection, the amount of CCL2 is 10 times less than the amount produced by infected astrocytes. This suggests that immunological signaling is affected by not only the type of soluble factors that are present in a particular microenvironment but also the concentration. The suppressive effects that high concentrations of CCL2 have on IFN-α mRNA that we see in vitro are consistent with what we see in vivo in our macaque model. During acute SIV infection, there is ∼10 times more CCL2 protein production in the cerebrospinal fluid than there is in the plasma (43). Furthermore, there is an almost 100-fold induction of CCL2 mRNA in brain (46), whereas there is no significant induction in peripheral tissues such as the lung (J. Clements, unpublished observations). We have previously found that suppression of IFN-α mRNA occurs in the brain during acute infection, but not in lung (2). Thus, the CNS, which contains a high concentration of CCL2, exhibits differential type I IFN regulation, whereas tissues in the periphery such as the lung, which are not exposed to as much CCL2, do not. These results provide valuable insight into how the compartmentalization of different tissues and cell types results in an entirely different immunological response.

Why this differential regulation of the IFN signaling pathway occurs is most likely due to type of tissue in which infection occurs. Astrocytes are cells that are exclusively found in the brain and are potent producers of CCL2 upon HIV infection (42, 47). Macrophages and microglia are strong producers of type I IFNs in response to viral infection in the CNS, but each IFN has very different effects. IFN-α has known neurotoxic and neuroinflammatory effects. Not only is it associated with HIV dementia and genetic diseases such as Aicardi-Goutières syndrome and Cree encephalitis, which are characterized by neurodegeneration and ongoing inflammatory processes, but also hepatitis C patients on IFN-α therapy have been known to suffer from depression, seizures, and EEG changes (4–6, 8, 10). IFN-β, however, has been associated with the secretion of anti-inflammatory cytokines and neurotropic growth factors (9, 48). Therefore, the high level of CCL2 production by astrocytes in the CNS may be an evolutionary mechanism that limits the production of proteins that lead to neuronal death, such as IFN-α and TRAIL, while allowing the induction of cytokines that can provide protection against pathogens without the destructive effects, such as IFN-β and MxA. This balance is extremely important in the CNS, as it is an organ composed of nonrenewable neurons. However, in the periphery, where cell turnover is particularly high and apoptosis can serve a protective purpose rather than a permanent loss of nonrenewable cells, this fine-tuned balance between inflammatory versus anti-inflammatory cytokine production is not as vital.

The immunomodulatory and protective effects of CCL2 on cells in the CNS have been well characterized. CCL2 has been reported to protect neurons and astrocytes from HIV-tat–induced and N-methyl-D-aspartate–induced apoptosis (30, 49). Furthermore, the treatment of microglia and neurons with CCL2 does not activate an inflammatory response, nor does it induce apoptosis, indicating that this chemokine does not inflict deleterious inflammatory effects (50). In addition, CCR2 deficiency in microglia has been

**FIGURE 6.** CCL2-induced IFN-α suppression occurs through CCR2 signaling. (A) and B) SIV-infected primary MDMs were treated with either propagermanium (prop), CCL2 (CCL2), CCL2 plus propagermanium (CCL2 + prop), or left untreated (UT). Cells were harvested 48 h p.i., and RNA was isolated. qRT-PCR analysis was done for (A) IFN-α or (B) IFN-β. Data represent means of three independent experiments (each done with two technical replicates) with 95% confidence interval. One-way ANOVA with Bonferroni posttest for multiple comparisons was performed on the SIV-infected group, and selected pairwise comparisons are shown. Results were considered significant with p < 0.05.
reported to accelerate disease progression in a mouse model of Alzheimer’s disease (51). Our findings that CCL2 suppresses signaling pathways leading to IFN-α, which is a known inflammatory modulator in the CNS, coincides with evidence in the literature of its neuroprotective role.

Although the finding that CCL2 plays a role in the differential regulation of type I IFNs is novel, cross-talk between IFN and CCL2 signaling pathways has been established. Mice lacking the IFN receptor have an impaired ability to produce CCL2 in the liver in response to CMV and to Listeria infection (52, 53), suggesting that IFN signaling is necessary for CCL2 expression. Conversely, in mice coinfected with influenza virus and pneumococcal pneumonia, increased type I IFN production leads to decreased CCL2 gene expression (54). This has also been supported by microarray analysis on mouse splenocytes showing that IFN-α treatment leads to decreased CCL2 gene expression (55). Furthermore, studies using the CCR2 inhibitor promegranate have shown that blocking this pathway in mice increases the production of type I IFNs in response to influenza infection (44, 56). Additionally, both NK cells and macrophages have increased IFN production after treatment with promegranate (57, 58). It is probable that the type of relationship between cytokines and chemokines largely depends on the cell type, pathogenic stimulus, and the presence of other cytokines and chemokines in that particular microenvironment. These studies, as well as ours, demonstrate that these signaling networks are not mutually exclusive and show constant interaction.

Examination of the CCR2 and IFN signaling pathways is required to obtain the mechanism behind their interactions. Binding of CCL2 to CCR2 leads to a conformational change in the receptor–ligand complex, which enables binding of heterotrimeric (αγ) G-proteins to certain receptor residues (59). This conformational change is also attributed to the activation of receptor-associated JAKs, resulting in receptor phosphorylation (60). Once bound, the α subunit disassociates from the complex, and different effector pathways, including calcium mobilization, cell migration, and increased cAMP levels, are activated. IFN signaling is initiated upon cellular pathogen sensing via PRRs and subsequent transcription of IFN-β, which is secreted and binds to the IFN receptor, leading to suppression of IFN-α and a subset of ISGs.

These results are no doubt of paramount importance to study of the neuroimmunological effects of viral infection, but they also provide valuable and novel information about basic immunological regulatory networks. Study of the interactions between cytokines and chemokines in different immunological environments provides valuable information about the pathogenesis of tissue-specific infections and will ultimately aid in the discovery of new treatments.

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