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Monkeypox virus viral chemokine inhibitor (MPV vCCI), a potent inhibitor of rhesus macrophage inflammatory protein-1

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ABSTRACT

Monkeypox virus (MPV) is an orthopoxvirus with considerable homology to variola major, the etiologic agent of smallpox. Although smallpox was eradicated in 1976, the outbreak of MPV in the U.S. highlights the health hazards associated with zoonotic infections. Like other orthopoxviruses, MPV encodes a secreted chemokine binding protein, vCCI that is abundantly expressed and secreted from MPV infected cells. EMSA data shows vCCI efficiently binds rhesus MIP-1 α (rhMIP-1 α) at near one to one stoichiometry. In vitro chemotaxis experiments demonstrate that vCCI completely inhibits rhMIP-1 α mediated chemotaxis, while in vivo recruitment assays in rhesus macaques using chemokine-saturated implants show a decrease in the number of CD14⁺ cells responding to rhMIP-1 α when vCCI is present, suggesting vCCI is effectively inhibiting chemokine function both in vitro and in vivo. More importantly, we demonstrate that vCCI can diminish the severity of the acute phase and completely inhibit the relapsing phase of experimental allergic encephalomyelitis (EAE) disease. These data represent the first in vitro and in vivo characterization of vCCI emphasizing its function as a potent inhibitor of rhMIP-1 α . Furthermore, the ability of vCCI to inhibit relapsing EAE disease represents a novel therapeutic approach for treating chemokine-mediated diseases.

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1. Introduction

Monkeypox virus (MPV) is a member of the genus orthopoxvirus, which includes variola major, the etiologic agent of smallpox [1–3]. Monkeypox virus and variola major share considerable homology, approximately 85% at the genomic level, and cause similar disease manifestations in infected humans. Although variola major is no longer a worldwide threat, MPV is as the virus naturally infects rodents and primates in sub-Saharan Africa, and since its discovery, thousands of cases of human MPV infection have been reported. The disease is primarily transmitted from animals to humans, either through animal bites or through direct contact with animal body fluids. Person-to-person transmission is rare (less than 1/3 of reported cases), and is acquired through close contact and exposure to aerosol droplets or contaminated body fluids [1,4]. More importantly, MPV infection of humans is clinically indistinguishable from small pox, sharing similar pathology and disease progression, and without proper medical attention, a 1–10% mortality rate [5]. Further complicating diagnosis, the early

stages of human MPV infection are often misdiagnosed as chicken pox, caused by varicella-zoster virus. Although smallpox was officially eradicated in 1976 by world-wide vaccination, recent cases of MPV in the United States indicates that MPV should be considered as an re-emerging zoonotic infection that poses a threat to the millions of non-vaccinated individuals.

The *poxviridae* family is characterized as large, DNA viruses that are highly species specific and cause disease in a wide variety of organisms. Many poxviruses encode proteins that inhibit normal chemokine function, collectively, these proteins are referred to as viral chemokine binding proteins (vCBPs) [6–9]. Members of the orthopoxvirus and leporipoxvirus genera express a secreted, 35 kDa protein, commonly referred to as vCCI, vCBP-I, or 35 kDa, that binds to human and rodent CC and CXC chemokines with high affinity, competitively inhibiting their normal interaction with cellular chemokine receptors [10]. Members of the myxomavirus genus also encode a secreted CC chemokine inhibitor (referred to as T7 or vCBP-II), additionally, these proteins have also been shown to effectively scavenge γ -IFN [11]. As a result of their inhibitory nature, all of these secreted proteins function as anti-inflammatory proteins during viral infection. All vCBPs represent a structurally unique family that does not share homology to any known cellular chemokine receptors, or any other mammalian or eukaryotic proteins [12–15]. To date, two animal models have been used to

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investigate the effect vCCI has on poxvirus pathogenesis. Expression of vCCI during experimental vaccinia infection in mice has shown to greatly reduce the number of infiltrating cells in the lungs of vaccinia infected mice [16]. Additionally, skin lesions from rabbits infected with rabbitpox show reduced infiltrates, compared to a vCCI knockout virus [14].

Chemokines belong to a superfamily of small (8–14 kDa) proteins that possess similar structural and functional properties [17]. The chemokine family is further divided into the following subtypes: C, CC, CXC, and CX₃C, based on the position of conserved cysteines located in the N-terminus of the protein. Most of the known chemokines (~94%) belong to the CXC or CC subtypes. Chemokines impose function by binding to seven transmembrane G-protein-coupled receptors (GPCRs) and glycosaminoglycans (GAGs), initiating downstream signaling events leading to adhesion, contraction, and actin polymerization [17,18]. Although primarily known for their ability to mediate recruitment of effector leukocytes and lymphocytes during injury or pathogenic insult, chemokines are also critically involved in a variety of cellular processes, such as the development of secondary lymphoid tissue, organogenesis, angiogenesis, and hematopoiesis [17,19]. As a fundamental component of both the innate and adaptive immune responses, chemokines have been targeted by many viruses who have obtained the ability to modulate and mimic chemokine function.

Along with their role in mediating inflammation due to injury or pathogen, some chemokines can play key roles in the progression of many auto-immune and neurodegenerative diseases, such as rheumatoid arthritis, Grave's disease, multiple sclerosis, Alzheimer's disease, human immunodeficiency virus-associated dementia, Type 1 diabetes, and Parkinson's disease [20]. Most auto-immune diseases involve autoreactive lymphocytes that can express chemokines, such as IL-8, MCP-1, MIP-1 α , MIP-1 β , and RANTES, which promote the recruitment of inflammatory cells. It is this influx of inflammatory cells and their secreted products which mediate the auto-immune destruction of host cells and tissue, thus promoting disease. Current therapies for treating chemokine-mediated diseases generally involve suppression of the host immune system, but as with any immunosuppressive regime, there is substantial risk for secondary infection. Initially developed as possible blocking agents for HIV infection, small molecule antagonists for chemokine receptors are currently being evaluated in both animal models and clinical trials for effectiveness in treating chemokine-mediated diseases, but to date, no therapies exist that specifically target the chemokine protein itself [21].

Infection of rhesus macaques with MPV represents an excellent non-human primate model for variola and the goal of this study was to biologically characterize MPV encoded vCCI, a protein found to be present in the MPV virion by proteomic analysis [22], and how it may contribute to MPV pathogenesis. As such, these data represent a foundation for further studies into the biological significance of MPV vCCI in a non-human primate model. Here we provide the first evidence that MPV vCCI is expressed and secreted during MPV infection and that MPV vCCI interacts with rhesus MIP-1 α (rhMIP-1 α) in vitro and in vivo inhibiting normal chemokine function. Additionally, we tested the utility of MPV vCCI in treating chemokine-mediated disease and show that MPV vCCI can inhibit relapsing EAE in mice, which represents a novel therapeutic approach for treating disease mediated by chemokine function.

2. Materials and methods

2.1. Protein alignments

Protein alignments were preformed using ClustalW from MacVector version 9.0 software (Accelrys, Inc., Madison, WI). A Blosom

scoring matrix was used in pairwise alignment of each sequence, with a gap introduction penalty of 10 and a gap extension of 0.1.

2.2. Virus, cell culture, and MPV vCCI specific antibodies

Human monkeypox virus (MPX V79-I-005, herein referred to MPV) was provided by Dr. Inger Damon (Center for Disease Control and Prevention, Atlanta, GA) and propagated in BSC₄₀ cells (African green monkey kidney cells—American Type Culture Collection (ATCC), Manassas, VA) cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 1% penicillin, streptomycin, and L-glutamine (Invitrogen, Carlsbad, CA). Viral titers were determined by plaque assay. HeLa cells and primary rhesus fibroblasts were maintained in DMEM and human THP-1 cells were maintained in RPMI 1640 (Mediatech, Herndon, VA), both supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, streptomycin, L-glutamine. RPMI 1640 was further supplemented with HEPES, and sodium pyruvate, 2% sodium bicarbonate (Invitrogen, Carlsbad, CA). MPV vCCI specific monoclonal antibodies were made on-site in the monoclonal antibody core at the Vaccine and Gene Therapy Institute (Beaverton, OR) using purified recombinant MPV vCCI (see below) as antigen.

2.3. Immunofluorescence analysis

Approximately 0.8×10^5 BSC₄₀ cells were seeded onto 12 mm glass cover slips (Fisher Scientific, Pittsburg, PA). The following day, cells were either infected with MPV at a multiplicity of infection (MOI) = 1 or mock and at 24 h post-infection, cells were fixed with 4% paraformaldehyde in PBS at 25 °C for 20 min. Fixed cells were then permeabilized with 0.2% Triton-X 100 in PBS. Staining for MPV vCCI was preformed using mouse monoclonal antibodies (Clone #11A3.4.2), followed by a biotinylated horse anti-mouse secondary antibody (Dako, Cuppertino, CA). The 11A3.4.2 clone was used specifically for immunofluorescence because of its low background in this application. Visualization was performed using streptavidin conjugated to Alexa-488 (Invitrogen, Carlsbad, CA) followed by a nuclear counterstain with a Hoechst dye (Sigma, St. Louis, MO).

2.4. Immunoprecipitation and Western blot analysis

BSC₄₀ cells (2.5×10^6) were infected with MPV at MOI = 10. Following 24 h of incubation, supernatants were clarified and concentrated 10-fold via 5000 MWCO Amicon® Ultra centrifugal filtration device (Millipore, Bedford, MA), while infected cells were washed with PBS and lysed in ice cold RIPA buffer (PBS, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). An MPV vCCI-specific mouse monoclonal antibody (clone #3D1) was added to the concentrated supernatants at 12.5 μ g/ml and incubated for 1 h at 4 °C with agitation. One hundred milliliter of Protein A/G-plus agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the mixture and allowed to incubate for 1 h at 4 °C with agitation. Protein bound agarose was pelleted and washed twice with cold PBS. Bound proteins were denatured by adding 2 $\times$ NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA) and heating to 70 °C for 10 min. Proteins (15 μ L load) were resolved on 4–12% NuPAGE® Bis-Tris polyacrylamide gels and wet transferred to PVDF membranes at 30 V for 1 h. Protein blots were probed using our anti-MPV vCCI mouse monoclonal antibody (clone #3D1) followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Bands were visualized using chemiluminescence. For the co-immunoprecipitation of rhMIP-1 α with MPV vCCI, 6 μ g of recombinant MPV vCCI was mixed with increasing

amounts of recombinant rhMIP-1 α (from 0.1 μ g to 2 μ g). Following 10 min room temperature incubation, 10 μ g of our anti-MPV vCCI mouse monoclonal antibody (clone #3D1) was added to the reaction and immunoprecipitation was carried out as described above. Western blot analysis for rhMIP-1 α was conducted in a similar fashion as described for MPV vCCI using a cross-reactive human MIP-1 α polyclonal antibody (#BAF270—R&D Systems, Minneapolis, MN).

2.5. Cloning and expression of recombinant MPV vCCI

The coding sequence for MPV-J1L was isolated from MPV genomic DNA via PCR using primers specific for MPV-J1L which also contained a 6 $\times$ -histidine tag (underlined region) and restriction sites for NdeI (5'-CATATGATCCTACCACTCTTCAGCA-3') and XhoI (5'-CTCGAGTCATCAGTGGTGGTGGTGGTGACACATGCTTTGAGTTTGT-3'). A non-sense mutation (in quotation marks) was introduced into an internal NdeI site via site directed mutagenesis using the following primers: 5'-AACAAACATCA"CATGGGAATCG-3' and 5'-CGATTCCTAT"GTGATGTTTGT-3'. A 6 $\times$ -histidine tagged rhMIP-1 α was isolated in a similar manner from another expression plasmid (graciously provided by Dr. Todd Reinhart, University of Pittsburgh) also using NdeI (5'-CATATGGCTGACACCCGACCTC-3') and XhoI (5'-CTCGAGTCATCAGTGGTGGTGGTGGTGACAGGCACTCAGCTCTAGGTC-3'). The resulting products were cloned into pRSETb (Invitrogen, Carlsbad, CA) for expression. Rosetta 2[®] DE3 cells (Novagen, Madison, WI) were transformed with the pRSETb expression plasmids. Expression cultures were set up by diluting overnight cultures 1:20 into 1 L of LB media without antibiotic and incubated for 3 h at 37 °C with agitation. At 3 h, the temperature of the cultures was reduced to 25 °C and protein expression was induced with 0.5 μ M isopropyl-D-thiogalactoside (IPTG—Fisher, Fair Lawn, NJ) with continued agitation for 6 h. Cells pellets were harvested by centrifugation (5000 $\times$ g for 12 min) and stored at -80 °C until use.

2.6. Purification of recombinant MPV vCCI

Induced cell pellets were resuspended in lysis buffer (300 mM NaCl, 50 mM NaPO₄, 20 mM Tris-HCl, 0.1 mM PMSF, 3 mM ME, pH8.0) and lysed by two freeze/thaw cycles, incubation with 1 mg/ml lysozyme, 5 μ g/ml DNAase, and 5 μ g/ml RNAase for 30 min on ice, and then sonicated 30 s (3 $\times$). Lysates were separated into soluble and insoluble fractions by centrifugation at 20 000g for 60 min at 4 °C. Proteins were purified via immobilized metal affinity chromatography (IMAC) by applying the soluble fraction to pre-equilibrated BD Talon[®] metal affinity resin (Clontech Laboratories Inc., Mountain View, CA) (1 ml resin per 2 L culture), where it was incubated on a rotator at RT for 1 h. Protein-bound resin was pelleted and washed (2 $\times$) with 20 ml wash buffer (300 mM NaCl, 50 mM NaPO₄, 20 mM Tris-HCl, 10% glycerol, 3 mM ME, pH 7.5). Protein was eluted from the resin by adding 3 ml elution buffer (300 mM NaCl, 50 mM NaPO₄, 20 mM Tris-HCl, 250 mM imidazole, 3 mM ME, pH 7.0) and incubated on a rotator at room temperature for 5 min (3 $\times$). Eluted protein was 0.22 μ m filtered and run over a HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare, Piscataway, NJ) pre-equilibrated in running buffer (20 mM NaPO₄, 150 mM NaCl, 3 mM ME, pH7.0). Pooled fractions were further purified and concentrated by binding to a HiTrap Q FF column and eluted with a 0–1 M NaCl gradient over 20 ml. Protein purity and size were determined on 4–12% Bis-Tris NuPAGE[®] gels and the purest fractions were pooled together. Endotoxin levels were assessed using a limulus amoebocyte lysate (LAL) assay (Cambrex, Walkersville, MD), followed by endotoxin removal using AffinityPak[™] Detoxi-Gel[™] endotoxin removal gel (Pierce, Rockford, IL). Protein concentration was determined by absorbance spectroscopy.

Purified proteins were lyophilized and stored at -80 °C, while reconstituted protein was kept at -20 °C.

2.7. Electrophoretic mobility shift assays

Purified recombinant rhMIP-1 α and MPV vCCI were incubated together at room temperature for 10 min. Samples were resolved on a non-denaturing, non-reducing 12% polyacrylamide gel at 30 mA. Bands were visualized using SimplyBlue[®] SafeStain (Invitrogen, Carlsbad, CA).

2.8. In vitro chemotaxis inhibition assay

Inhibition of THP-1 cell migration was carried out using Transwell[®] plates (6.5 mm $\times$ 3.0 μ m pore, Corning, New York, NY) equilibrated in assay media (RPMI 1640 supplemented with 0.5% heat-inactivated fetal bovine serum) for 1 h prior to assay. Ten minutes prior to beginning the assay, 10⁻⁹ M rhMIP-1 α was mixed with increasing amounts of MPV vCCI and incubated at 25 °C. The protein mixture was then added to 600 μ L of assay media in the lower chamber. THP-1 cells (5 $\times$ 10⁵) suspended in 100 μ L were added to the upper chamber of the transwell and incubated for 4 h at 37 °C with 5% CO₂. Migrated cells were counted using CyQuant cell proliferation assay kit (Invitrogen, Carlsbad, CA).

2.9. In vivo chemotaxis assay

In vivo recruitment assay was adapted from a previously published angiogenesis assay [23]. Gelfoam[®] plugs (Pharmacia & Upjohn Company, Kalamazoo, MI) were cut 5 mm³ and rehydrated overnight in PBS at 4 °C. On the day of implantation, plugs were briefly dried between two pieces of filter paper and soaked with (a) 500 ng of rhMIP-1 α , (b) 500 ng of rhMIP-1 α plus 1.5 μ g MPV vCCI (1:1 molar ratio), or (c) PBS mixed with 0.4% agarose warmed to 42 °C. The soaked implants were stored at 4 °C until implantation. For implantation, rhesus macaques are anesthetized with ketamine (15–20 mg/kg i.m.), placed in oblique ventral recumbency, and the hair clipped from the mid-scapular region to the shoulder. Skin prep was performed in routine fashion with betadine scrub and solution, followed by placement of a medium fenestrated drape. A 5–10 mm skin incision was made in the lateral mid-scapular region, the skin is undermined with a Kelly forceps for a distance of approximately 2–3 cm from the incision, and the Gelfoam[®] implants were inserted in the undermined space. The skin was then closed with several simple interrupted sutures. Spacing between implants was maximized to avoid potential functional overlap. The implants remained in the animal for 7 days, at which time, the Gelfoam[®] plugs and surrounding tissue were excised and cryopreserved in tissue freezing media (Triangle Biomedical Sciences, Durham, NC) and stored at -80 °C for later sectioning. All aspects of the experimental implantation studies were performed according to institutional guidelines for animal care and use at the OHSU, West Campus.

2.10. Immunohistochemistry

Ten micrometer sections of the cryopreserved samples were cut and mounted onto Superfrost[®]/Plus slides (Fisher Scientific, Pittsburgh, PA) at RT overnight. Slides were fixed with ice-cold acetone for 10 min and then washed three times with tris-buffered saline (pH 7.4) + 0.1% Tween-20 (TBST) to remove freezing media. Slides were blocked with PBS + 1% BSA and 10% donkey serum at RT for 1 h, followed by PBS + 0.3% H₂O₂. A CD14-specific mouse monoclonal primary antibody (Clone# M5E2—BD Pharmingen, San Diego, CA) diluted in PBS + 1% BSA was incubated on the

sections overnight at RT. Following TBST washes, sections were incubated with horse anti-mouse secondary antibody conjugated to horse radish peroxidase for 1 h at RT. CD14 specific staining was visualized using a DAB substrate kit (Dako, Cuppertino, CA) and counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA).

2.11. Experimental allergic encephalomyelitis (EAE) model

Our EAE model strictly follows the published protocol of Stromnes and Goverman [24] and was performed according to institutional guidelines for animal care and use at the OHSU, West Campus. Briefly, on day zero, 8-week-old, female SJL/J mice (Jackson Labs, Bar Harbor, MA) were injected subcutaneously (s.c.) with 200 µg of myelin proteolipid peptide residues 139–151 (PLP_{139–151}); Peptides Intl., Louisville, KY) emulsified in complete Freud's adjuvant (Sigma, St. Louis, MO), and 100 ng of pertussis toxin (List Biological Laboratories, Inc., Campbell, CA) was given intraperitoneally (i.p.), these mice serve as positive controls. Each mouse in the experimental group received an additional 25 µg of MPV vCCI i.p.. Mice receiving 25 µg MPV vCCI alone or buffer alone serve as negative controls. On day 3, an additional boost of 100 ng of pertussis and 25 µg of MPV vCCI were given to the appropriate groups. Mice were monitored daily and disease was scored using the following scale: 0—Normal, 0.5—Partially limp tail, 1.0—Paralyzed tail, 2.0—Hind limb paresis, 2.5—One hind limb paralyzed, 3.0—both hind limbs paralyzed, 3.5—Hind limbs paralyzed; fore limbs weak, 4.0—Fore limbs paralyzed, 5.0—Moribund. Additional care was given to mice exhibiting disease, such as, soaked chow and the administration of s.c. fluids to mice exhibiting a 25% reduction in weight.

3. Results

3.1. Protein alignment of various poxvirus vCCI sequences

The predicted product of the MPV ORF-J1L is a 27.6 kDa protein, MPV vCCI. The amino acid sequence of MPV vCCI was aligned with other vCCI sequences encoded by variola virus (VARV), cowpox virus (CPV), rabbitpox virus (RPV), and vaccinia strain Copenhagen (VV COP) to determine the level of amino acid sequence homology. Fig. 1 shows the protein alignments for all five proteins and confirms conserved homology between them. On average, MPV vCCI shares approximately 85.8% similarity and 82.5% identity with the other chemokine inhibitors (Table 1). Although highly homologous, there is one area of divergence from amino acid 72–94, where the vCCIs of MPV and CPV differ from the other viral vCCIs. Based on previously published studies, these similarities suggest that MPV vCCI likely functions in a similar manner as the vCCIs of CPV and VV, and will inhibit CC chemokine function in an infected host.

3.2. Expression and purification of recombinant MPV vCCI

The DNA sequence encoding MPV vCCI was amplified by PCR and a 6×-histidine tag was placed in frame at the C-terminus for purification purposes. After a multi-step purification protocol, SDS-PAGE on fractions from anion exchange chromatography shows purified recombinant MPV vCCI. Despite having a predicted molecular weight of 27.6 kDa, MPV vCCI migrates roughly 5–6 kDa higher on SDS-PAGE, which is consistent with other vCCI species, like VARV, CPV, and VV COP, and is more than likely the result of charged residues in the primary sequence (data not shown).

Fig. 1. Amino acid comparison of MPV vCCI to vCCIs encoded by variola virus (VARV), cowpox virus (CPV), rabbitpox virus (RPV), and vaccinia Copenhagen strain (VV COP). Alignments were performed with ClustalW using Blosom scoring matrix. Dark shaded boxes indicate either: (1) identical residues, or (2) unique residues to MPV vCCI. Lightly shaded boxes represent similar residues to MPV vCCI.

Table 1
Homology of MPV vCCI to CPV vCCI, RPV vCCI, VARV vCCI, and VV COP vCCI

vCCI	% Identical to MPV vCCI	% Similar to MPV vCCI
CPV	79	84
RPV	85	89
VARV	83	87
VV COP	83	83

3.3. MPV vCCI is expressed and secreted during MPV infection

To determine if MPV vCCI protein is expressed during MPV infection, an immunofluorescence assay was preformed on MPV infected BSC₄₀ cells using an MPV vCCI specific mouse monoclonal antibody (11A3.4.2). As shown in Fig. 2A, MPV infected cells begin to stain positive for MPV vCCI, as early as 24 h post infection. Positive cells show an intense cytoplasmic staining as compared to mock infected cells.

Next, to determine if MPV vCCI is secreted from MPV infected cells, Western blot analysis was preformed on clarified/concentrated supernatants and cellular lysates from BSC₄₀ cells infected with MPV for 24 h. Western blot analysis shows the presence of a MPV vCCI specific band at, or near the apparent molecular weight of ~35 kDa in supernatant from infected samples, but not in supernatants from mock samples (Fig. 2B). Recombinant MPV vCCI was loaded as a positive control. Taken together, these data clearly demonstrate MPV vCCI is expressed and secreted during MPV infection, either via active transport or during cell lysis.

3.4. MPV vCCI interacts with rhesus MIP-1 α

To assess the ability of MPV vCCI to bind rhMIP-1 α , we utilized a modified electrophoretic mobility shift assays (EMSA) to visualize differences in MPV vCCI mobility with and without

rhMIP-1 α present (Fig. 3A). Because of its small size and amino acid content, rhMIP-1 α does not stain at the concentrations used (lanes 2 and 5). Therefore, if MPV vCCI is forming a complex with rhMIP-1 α , we should see an increase in the apparent molecular weight (MW_{app}) of MPV vCCI. Compared to free MPV vCCI (lane 4), MPV vCCI runs at a higher MW_{app} when incubated with rhMIP-1 α (lane 3). Moreover, to address MPV vCCI aggregation as a possible explanation for the shift in molecular weight, twice the amount MPV vCCI was loaded (lane 1), and although some “smearing” is observed, the higher molecular weight band is not observed. To confirm the presence of both MPV vCCI and rhMIP-1 α , the shifted band (lane 3) was excised and in-gel trypsin digest was performed, followed by mass spectrophotometry. Following analysis of unique peptide hits, the presence of two species, MPV vCCI and rhMIP-1 α , was confirmed (data not shown).

To further demonstrate the formation of the MPV vCCI: rhMIP-1 α complex, we set up a titration assay where increasing amounts of MPV vCCI were incubated against a fixed amount of rhMIP-1 α . Fig. 3B shows that with limiting amounts of MPV vCCI, the only species present is the higher MW_{app} species (lanes 1 and 2). As MPV vCCI begins to be in excess, the presence of the free MPV vCCI begins to be seen (lanes 4 and 5). As seen in Fig. 3A, 2 $\times$ MPV vCCI was loaded to verify that aggregation was not the reason for the shifted band (lane 8).

In order to confer specificity, a co-immunoprecipitation assay was preformed on a mixture MPV vCCI and rhMIP-1 α using an anti-MPV vCCI monoclonal. As shown in Fig. 3C, as increasing amounts of rhMIP-1 α were added to the incubation mixture, more rhMIP-1 α is co-immunoprecipitated with MPV vCCI (lanes 4 through 8). This effect is dependent on MPV vCCI, as rhMIP-1 α alone does not immunoprecipitated with the MPV vCCI antibody (lane 3). Taken together, these data show that MPV vCCI binds and forms a complex with rhMIP-1 α .

Fig. 2. Expression of MPV vCCI during MPV infection. (A) Immunofluorescence analysis on MPV infected (panel A) or mock infected (panel B) BSC₄₀ cells fixed at 24 h.p.i. Cells were stained with a mouse anti-vCCI monoclonal antibody, followed by a biotinylated horse anti-mouse secondary antibody, and visualized using an alexa-488 conjugated to streptavidin. Nuclear staining was preformed using Hoescht stain. All images were taken with 20 $\times$ objective. (B) Secretion of MPV vCCI during MPV infection. Samples of supernatants and lysates from MPV (lanes 1 and 3) and Mock (lanes 2 and 4) infected BSC₄₀ cells were resolved on 4–12% Bis–Tris NuPAGE® gels and transferred to PVDF. Western blot analysis was preformed using a mouse anti-vCCI monoclonal antibody (3D1) and an HRP-conjugated goat anti-mouse secondary antibody. Purified MPV vCCI was used as a positive control (lane 5).

Fig. 3. MPV vCCI binds rhesus MIP-1 α . (A) Purified MPV vCCI and rhMIP-1 α were mixed together at a 1:1 molar ratio and incubated for 10 min at room temperature. Purified MPV vCCI alone and rhMIP-1 α alone were used as controls. Reactions were resolved on a 12% native PAGE gel and stained with SimplyBlue™ Safe Stain. (B) MPV vCCI was titrated from limiting to excess, into a reaction mixture with a fixed amount of rhMIP-1 α . MPV vCCI alone and rhMIP-1 α alone were used as controls. (C) Co-immunoprecipitation of rhMIP-1 α with MPV vCCI. Increasing amounts of rhMIP-1 α (0.1 μ g–2.0 μ g—lanes 4–8) were incubated with a fixed amount of MPV vCCI (6 μ g), as a result more rhMIP-1 α co-elutes with immunoprecipitated MPV vCCI. 3 μ g of rhMIP-1 α (lane 2) and 6 μ g of MPV vCCI (lane 1) were used as positive controls. As a negative control, MPV vCCI immunoprecipitation was preformed on 3 μ g of rhMIP-1 α alone (lane 3). Proteins were resolved on 4–12% Bis–Tris NuPAGE® gels.

3.5. MPV vCCI inhibits rhesus MIP-1 α mediated chemotaxis of THP-1 cells

In order to assess the inhibitory properties of MPV vCCI, we first utilized an in vitro transwell assay using human THP-1 cells, a pre-monocytic cell line. We used THP-1 cells for their consistency, as opposed to isolating cells from different rhesus macaques and dealing with animal to animal variability. Furthermore, we have previously determined that THP-1 cells are fully responsive to rhMIP-1 α with maximum chemotaxis occurring at 10^{-9} M (data not shown). Fig. 4 shows that with increasing concentrations of MPV vCCI, rhMIP-1 α mediated chemotaxis is reduced to levels similar to PBS controls. The use of heat inactivated MPV vCCI restores rhMIP-1 α mediated migration confirming that the observed effect is mediated by MPV vCCI. These findings clearly show that MPV vCCI is binding to rhMIP-1 α and effectively inhibiting chemotaxis.

3.6. MPV vCCI inhibits rhMIP-1 α mediated chemotaxis of CD14 $^{+}$ cells during in vivo recruitment assays

To better understand the in vivo function of MPV vCCI, we designed an in vivo assay to observe whether or not MPV vCCI could effectively inhibit rhMIP-1 α mediated recruitment. To introduce our samples into a macaque in a controlled setting, we modified a previously published angiogenesis protocol by Fan et al. [23]. Gelfoam is an inert, sponge-like material used as a hemostatic material during surgery. When a soluble agent, such as a chemokine, is absorbed into Gelfoam in the presence of 0.4% agarose, it can be handled as a solid and once implanted is slowly released into the external environment over time. Based on previous work in our laboratory that showed rhMIP-1 α mediates recruitment of CD14 $^{+}$ cells during in vivo recruitment assays, rhMIP-1 α was incubated with MPV vCCI at a 1:1 molar ratio prior to absorption into Gelfoam® plugs. Following surgical implantation, incubation, and

Fig. 4. Inhibition of rhesus MIP-1 α mediated migration of Human THP-1 cells. 5×10^5 THP-1 cells suspended in 100 μ L of assay media (RPMI 1640 + 0.5% fetal bovine serum) were placed in 3 μ m pore size transwell inserts and placed in 24-well culture plates containing 600 μ L assay media with 10^{-9} M rhMIP-1 α plus increasing concentrations of MPV vCCI or 10^{-7} M heat inactivated MPV vCCI (Δ MPV vCCI). PBS was used as a negative control. Following a 4 h incubation at 37 °C (5% CO₂), THP-1 cells migrating through the transwell were counted using a CyQuant[®] cell proliferation assay kit (Molecular Probes, Eugene, OR). Represented data are the average number of migrated cells of 3 wells (2500 $\times$) $\pm$ SEM.

subsequent removal of the protein-saturated implants, frozen sections of the Gelfoam[®] implants and surrounding tissue were analyzed by immunohistochemistry using a CD14 specific antibody. In Fig. 5A–D, the Gelfoam[®] implants can be differentiated from surrounding tissue by its intense H and E (dark blue/purple) staining pattern. As compared to rhMIP-1 α alone, our data suggests that MPV vCCI inhibits rhMIP-1 α mediated recruitment of CD14⁺ cells, as indicated by a decrease in DAB positive (dark grey/brown) staining in and around the Gelfoam implant (Fig. 5B). In an effort to quantify the levels of inhibition, the number of DAB positive pixels for each image was normalized to the PBS control and graphed as a migration index. Fig. 5E shows significant inhibition of rhMIP-1 α mediated recruitment. These findings are consistent with our in vitro data and clearly indicated the MPV vCCI is a potent inhibitor of rhMIP-1 α , both in vitro and in vivo.

3.7. MPV vCCI inhibits relapsing experimental allergic encephalomyelitis (EAE)

In order to assess the ability of MPV vCCI to treat a chemokine-mediated disease, we utilized the well described EAE mouse model. Four groups of mice were used for our experiment: Group (1) Positive controls—mice that received PLP_{139–151} only; Group (2) Experimental group—mice that received recombinant MPV vCCI and PLP_{139–151}; Group (3) MPV vCCI alone—mice receive MPV vCCI alone; and Group (4) Buffer alone—mice receive buffer alone. Groups 3 and 4 serve as negative controls. Fig. 6 shows that on day 12, both group 1 and 2 began to exhibit early signs of acute EAE and by day 16 the disease had peaked and both groups began to resolve the disease with complete recovery occurring by day 20. Interestingly, although administration of MPV vCCI did not stop or delay the onset of EAE, animals that received MPV vCCI showed a slight reduction in severity during the acute phase of disease. On day 24, animals of group 1 began to show signs of EAE relapse, lasting approximately 6 days. While the majority of animals fully recovered from EAE relapse, one animal developed chronic EAE, thus the consistent score from day 30 on. More importantly, none of the animals that received MPV vCCI showed any signs of relapse, which was confirmed in a second cohort of animals. Animals receiving MPV vCCI alone or buffer alone, showed no clinical signs of EAE or other pathologies. These data suggest that administration

Fig. 5. In vivo inhibition of rhesus MIP-1 α mediated chemotaxis. Gelfoam[®] sponges containing agarose-embedded (A) rhMIP1 α or (B) rhMIP1 α + MPV vCCI or (C) PBS were implanted s.c. in the back of a rhesus macaque (≥ 8 cm apart), where they remained for 7 days before being harvested, sectioned, and stained. CD14 staining shows a clear reduction in CD14⁺ infiltrates (lack of dark grey/brown coloration) in the Gelfoam[®] sponges containing rhMIP1 α + MPV vCCI, as compared to rhMIP1 α . An isotopically matched primary antibody was used on a section of rhMIP1 α -containing Gelfoam[®] as an antibody control (D). Quantification of CD14⁺ infiltrates (E) was performed by comparing the number of DAB⁺ pixels in each image and normalizing to PBS and represented as a migration index $\pm$ SEM. All images (A–D) were taken using a 20 $\times$ objective and are the same size, 345,000 pixels.

of recombinant MPV vCCI is capable of reducing, and possibly inhibiting, chemokine-mediated disease.

4. Discussion

Chemokines play an important role in mediating the recruitment of leukocytes to sites of infection, and ultimately establishing effective innate and adaptive immune responses. As a result, many viruses encode proteins which subvert normal chemokine function. In this report, we sought to biologically characterize MPV vCCI both in vitro and in vivo. We show that MPV vCCI is expressed and secreted during MPV infection and that MPV vCCI efficiently inhibits rhMIP-1 α mediated chemotaxis in both in vitro and in vivo assays. Furthermore, we have shown that MPV vCCI has the ability to halt relapsing EAE in mice, suggesting that MPV vCCI might represent a novel therapeutic for the treatment of chemokine-mediated disease.

The MPV vCCI staining pattern seen during MPV infection is consistent with the cytoplasmic replication of poxviruses and

Fig. 6. MPV vCCI inhibits relapsing EAE. Following induction of EAE by administration of PLP_{139–151} peptide $\pm$ MPV vCCI, mice ($n = 4$) were observed on a daily basis and scored for disease using the following scale: 0—normal, 0.5—partially limp tail, 1.0—paralyzed tail, 2.0—hind limb paresis, 2.5—one hind limb paralyzed, 3.0—both hind limbs paralyzed, 3.5—hind limbs paralyzed; fore limbs weak, 4.0—fore limbs paralyzed, 5.0—moribund. Represented values are the average scores for all mice within each group. Symbols represent the four groups: $\blacklozenge$ —PLP_{139–151} peptide; $\blacksquare$ —PLP_{139–151} peptide + MPV vCCI; $\blacktriangle$ —MPV vCCI alone control; $\bullet$ —buffer alone control.

represents the first time that any vCCI has been visualized using immunofluorescence. Furthermore, immunoprecipitation from infected supernatants and cell lysates clearly shows the presence of MPV vCCI. Interestingly, the presence of a single band in the lysate and a broadened band in the supernatant suggests that the secreted form of MPV vCCI may undergo some post-translational modification.

A commonality among all vCCI research from both the leporipoxvirus and orthopoxvirus genera is their ability to bind α - and β -chemokines. Structural analysis has determined that binding and subsequent inhibition is much stronger with β -chemokines. In fact, vCCI binding to α -chemokines occurs with such a low affinity that many question whether it is physiologically relevant [10,12]. Our work on MPV vCCI is consistent with these previous results, in that MPV vCCI forms a complex with rhMIP-1 α resulting in a significant shift in MPV vCCI MW_{app}. Although there is some debate as to the exact stoichiometry, stoichiometric analysis suggests that vCCI binds MCP-1 at nearly 1:1 ratio [12,15]. Our data with rhMIP-1 α is supported by these findings, in that at a 1:1 ratio, all of the MPV vCCI is migrating at the higher MW_{app}, only when in excess does MPV vCCI migrate at the lower MW_{app}.

Structural analysis of rabbitpox virus vCCI (RPV vCCI) complexed with human MIP-1 β has provided significant insight as to a possible mechanism behind vCCI-mediated inhibition. Zhang et al. reported that RPV vCCI possess a number of important contacts with MIP-1 β . In particular, the highly conserved vCCI residues Ser-182 to Thr-187 make “extensive contacts” with the chemokine [25]. This region is critical for receptor binding, therefore high affinity association with vCCI appears to inhibit cc-chemokine receptor interaction. Along these lines, our in vitro inhibition assay clearly demonstrates the inhibitory power of MPV vCCI as it completely blocks rhMIP-1 α -mediated chemotaxis in a dose-dependent manner. This effect is dependent on MPV vCCI function, since heat inactivation of MPV vCCI restores rhMIP-1 α -mediated migration to near rhMIP-1 α alone levels. Although we have shown that MPV vCCI interacts with rhMIP-1 α at approximately 1:1 stoichiometry (Figs. 3B and 5), our in vitro inhibition assay requires 100-fold excess MPV vCCI for complete inhibition of rhMIP-1 α mediated migration (Fig. 4). More than likely, this discrepancy is the result of using the human THP cells in our in vitro inhibition assay. Subtle differences between human and rhesus GPCRs may explain the requirement for excess vCCI to be present in order to achieve complete inhibition in THP cells.

Our in vivo studies on vCCI further confirm that the inhibitory potential is not limited to the in vitro setting. Although several reports have studied VV vCCI in mice and guinea pigs showing that vCCI can inhibit leukocyte recruitment in these animals, these are not natural host for VV, therefore slight differences may exist in the natural host [10,12,16]. Graham et al. investigated the inhibitory potential of RPV vCCI in rabbits showing a marked increase in leukocyte infiltrates when rabbits were infected with a vCCI knockout virus [14]. For our in vivo studies, we performed two tests, both of which utilized purified recombinant MPV vCCI. The first involved an in vivo inhibition assay in rhesus macaques using protein saturated Gelfoam[®] plugs. Although rhMIP1- α alone induces significant recruitment of CD14⁺ cells, when complexed with MPV vCCI, CD14⁺ recruitment was drastically reduced. Secondly, we tested the ability of MPV vCCI to mitigate a chemokine-mediated disease. Experimental allergic encephalomyelitis (EAE) is an induced disease in mice that closely mimics multiple sclerosis in human. Previous work by Karpus et al. has shown that administration of neutralizing antibodies for MIP-1 α and MCP-1 causes a significant reduction in EAE disease, and therefore MIP-1 α and MCP-1 must play an integral part in the establishment and progression of EAE [26]. Prior to initiating the EAE study, we confirmed that MPV vCCI interacts with several mouse chemokines, namely MIP-1 α and MCP-1, via shift assay (data not shown). These results were consistent with work by Smith et al., who showed that CPV vCCI bound with high affinity to mouse MCP-1, MCP-5, MIP-1 α , MIP-1 β , C10, and Eotaxin [10]. As our EAE study progressed into the acute phase we observed that mice receiving MPV vCCI exhibited reduced severity of disease compared to mice that did not receive MPV vCCI. More importantly, as mice from our positive control group began to exhibit signs of relapsing-remitting EAE (around day 24); the mice that received MPV vCCI did not and remained free of relapse until the end of the study.

This represents the first time that any vCCI has been shown to inhibit or mitigate a chemokine-mediated disease. The implications of our in vitro and in vivo characterization are significant, laying the ground work for additional studies to investigate the effectiveness of MPV vCCI in treating chemokine-mediated disease in non-human primates. Furthermore, we feel as though MPV vCCI represents a legitimate therapeutic candidate for the treatment of chemokine-mediated disease.

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