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Gene therapy with CCL2 (MCP-1) mutant protects CVB3-induced myocarditis by compromising Th1 polarization

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ABSTRACT

Viral myocarditis, which is most prevalently caused by Coxsackievirus B3 (CVB3) infection, affects about 5–20% of the world population and lacks efficient treatments. We previously reported that monocyte chemotactic protein-1 (MCP-1, CCL2) was significantly induced during CVB3 infection and greatly contributed to the myocardic inflammation and injury. Herein a CCL2 mutant with removed chemotactic activity was administrated and its therapeutic effect on CVB3-induced myocarditis was explored. A dominant negative CCL2 mutant, lacking the N-terminal amino acids 2–8 ($CCL2^{\Delta 2-8}$), was genetically constructed and intramuscularly injected into BALB/c mice after CVB3 infection, severity of myocarditis was evaluated by weight loss, survival rate, serological indices and pathological observation. Systemic and local Th1/Th2 cytokine profiles were also assessed. Mice receiving $pCCL2^{\Delta 2-8}$ exhibited a profound attenuation of myocarditis compared to pcDNA3.1 or non-treated mice, as evidenced by invariant body weight, decreased serum CK-MB level, reduced myocardial inflammatory infiltration and increased survival. This effect was not attributable to the efficient viral clearance, but associated with weakened Th1 immune responses, as evidenced by significantly reduced $CD4^+IFN\text{-}\gamma^+$ T cell frequency and Th1 cytokine level systemically and locally. Strategy of blocking *in vivo* CCL2 activity could effectively alleviate the severity of CVB3-induced myocarditis and may present an alternative therapeutic approach against viral myocarditis.

1. Introduction

Coxsackievirus B3 (CVB3), a small RNA virus belonging to *Enterovirus* genus of Picornaviridae, is believed to be a principle etiologic agent causing acute and fulminant myocarditis in human (Henke et al., 2008; Tam, 2006). Although the pathogenesis of CVB3 has been extensively studied in the murine model, the clear mechanisms underlying the interaction of CVB3 and host need to be further elucidated.

Viral myocarditis is characterized by robust leukocytes infiltration in myocardium leading to tissue injury. Leukocytes migration from blood to the heart tissue is a critical reflection of host defense in response to viral infection (Yuan et al., 2009) and always occurs in a stepwise fashion. Myocardial inflammation usually appears noticeable at day 4 and day 5 post CVB3 infection and reaches the maximum from day 7 to 14 (Fairweather and Rose, 2007). The main inflammatory cell subsets comprise macrophages and T lymphocytes, which are responsible for both virus clearance and subsequent tissue injury (Huber, 2005; Li et al., 2009). Chemokines, such as RANTES (Poffenberger et al., 2009), MIP-1α (Cook et al., 1995), MIP-2 (Kishimoto et al., 2001), have been shown to participate in the above cell migration processes, thus play important roles in the pathophysiology process of CVB3-induced myocarditis.

Monocyte chemoattractant protein-1 (MCP-1, or CCL2), a member of CC chemokines, plays a major role in the migration of monocytes, memory T cells and activated natural killer cells (Rollins, 1996). Interaction between CCL2 and its receptor CCR2 has been reported extensively involved in many inflammatory or infectious heart diseases. Göser et al. (2005) reported that CCL2 was critical to the induction of myosin-induced experimental autoimmune myocarditis in BALB/c mice. Marino et al. (2005) also found that CCL2 was closely correlated with the severity of Trypanosoma cruzi-elicited myocarditis in rats. Our previous study demonstrated that CCL2 was the most abundantly upregulated chemokine mediating the migration and infiltration of mononuclear cells into heart tissue during murine CVB3-induced myocarditis (Shen et al., 2004). All these research works strongly suggest a central role of the CCL2/CCR2 pathway in the pathophysiology process of myocarditis. Blockade of CCL2/CCR2 signaling thus

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appears to be a potential tool to prevent and treat CVB3-induced myocarditis.

However, CCL2 is also considered as a critical active factor promoting virus clearance (Dessing et al., 2007; Hokeness et al., 2005), it remains unclear whether blockade of CCL2/CCR2 pathway would accelerate CVB3 replication and aggravate viral myocarditis. Additionally, there are already some hints that CCL2 is involved in the development of Th2 inflammation (Ip et al., 2006; Luther and Cyster, 2001; Nakajima et al., 2001), so blocking CCL2/CCR signaling pathway may bias the host immune response to Th1 profile, which was considered detrimental to CVB3 myocarditis (Huber and Pfaeffle, 1994; Jiang et al., 2008). Therefore, it is necessary to explore whether blockade of the CCL2/CCR2 pathway is a useful gene therapy against CVB3-induced myocarditis.

There are several approaches to block CCL2/CCR2 signaling pathway, including blocking CCL2 or its receptor CCR2 by neutralizing antibody respectively. Recently, new molecular inhibitor of RANTES based on the mutation of its functional domain has exhibited potent ability to block the native RANTES signaling pathway and subsequent cell migration (Cai et al., 2004), and has shed light on the molecular design of new CCL2 antagonist. The amino-terminus is essential for CCL2-mediated cell migration, so we designed several plasmids encoding different deletion mutation in this domain and found that the one lacking the first amino acids 2–8 ($CCL2^{\Delta 2-8}$) processed the most potent CCL2 antagonism activity (data not shown). In the present study, 100 μg of $CCL2^{\Delta 2-8}$ plasmid was intramuscularly injected into BALB/c mice at day 0 and day 3 after CVB3 infection, and the effectiveness of this strategy in exempting mice from CVB3-induced myocarditis was observed.

2. Materials and methods

2.1. Mice and virus

Male inbred BALB/c (H-2^d) mice 6–8 weeks of age were obtained from the experimental animal centre of Chinese Academy of Science (Shanghai, China), and housed in the pathogen-free mouse colonies. All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals of the Laboratory Animal Ethical Commission of Soochow University. CVB3 (Nancy strain) was a gift from Professor Yingzhen Yang (Key Laboratory of Viral Heart Diseases, Zhongshan Hospital, Shanghai Medical college of Fudan University) and was maintained by passage through Hela cells (ATCC number: CCL-2). Viral titer was routinely determined prior to infection by a 50% tissue culture infectious dose ($TCID_{50}$) assay on Hela cell monolayers according to previously published procedures (Henke et al., 1995).

2.2. Construction of $CCL2^{\Delta 2-8}$ plasmid

The human CCL2 gene was amplified from cDNA of PMA-activated THP1 by reverse transcriptase–polymerase chain reaction (RT-PCR) according to the published sequence (GenBank ID: NM_002982) using a forward primer (5′-TCGGATCCGCCACCATGAAAGTCTCTGCCGCCCTTCTG-3′) engineered with a restrict enzyme site BamHI and a reverse primer (5′-GACTCGAGTCAAGTCTTCGGAGTTTGGGTTTGCTT-3′) engineered with an XhoI site, and then subcloned into eukaryotic expression plasmid pcDNA3.1 (Invitrogen). $CCL2^{\Delta 2-8}$ cDNA with a FLAG tag in the carboxyl-terminal was constructed by recombinant PCR using full-length CCL2 cDNA as template, and then cloned into pcDNA3.1 plasmid. 24 nucleotides encoding FLAG epitope (DYKDDDDK) were added directly at the C-terminus of $CCL2^{\Delta 2-8}$ gene sequence by primer design. All sequences were confirmed by double-stranded DNA sequencing.

2.3. Expression and antagonizing effect of $pCCL2^{\Delta 2-8}$ on CCL2-mediated chemotaxis in vivo and in vitro

The chemotaxis assay *in vitro* was conducted using a modified 48-well Boyden chamber migration assay. WEHI-274.1 (murine monocytic cell line) cells were transferred to upper chambers (1×10^5 cells/50 μl). The supernatant from pCCL2 or $pCCL2^{\Delta 2-8}$ transfected 293T cells was pretreated with or without anti-CCL2 (10 μg/ml) antibody before adding to the lower chamber. After 3 h incubation, migration was expressed as cell number per five high-power fields in the polycarbonate chemotaxis membrane, with duplicate wells being counted for each of three experiments. The chemotaxis ability of CCL2 or $CCL2^{\Delta 2-8}$ was evaluated by the chemotactic index [(number of cells migrating in experimental well)/(number of cells migrating in medium well)].

The chemotaxis assay *in vivo* was conducted as follows: mice were injected with 100 μg $pCCL2^{\Delta 2-8}$, 100 μg pCCL2, 100 μg $pCCL2^{\Delta 2-8}$ plus 100 μg pCCL2 or 100 μg pcDNA3.1 at the site of femoral muscle. 3 days later, the muscle tissues were removed and fixed in 10% buffered formalin, sectioned and stained with hematoxylin and eosin (HE). Sections were observed using a Nikon Eclipse TE2000-S microscope (Nikon) with magnification ×200, and evaluated for the antagonizing effect of $CCL2^{\Delta 2-8}$ on CCL2-mediated chemotaxis. At the same time, mouse serum was collected, electrophoresed on 12% SDS-PAGE gels and then transferred to the PVDF membrane. The membrane was probed with goat anti-human CCL2 (Santa Cruz) or mouse anti-FLAG antibody (Santa Cruz), followed by horseradish peroxidase (HRP) conjugated rabbit anti-goat (Dako) or goat anti-mouse antibody (SouthernBiotech). The signals were developed by chemiluminescence (Pierce).

2.4. CVB3 infection and $pCCL2^{\Delta 2-8}$ gene therapy

8 mice in each group were infected by an intraperitoneal injection with 10^3 $TCID_{50}$ CVB3 at day 0, and then immediately treated with intramuscular injection of 100 μg $pCCL2^{\Delta 2-8}$ or pcDNA3.1 at day 0 and day 3. Infected mice without treatment were used as control.

2.5. Tissue histopathology and myocarditis grading

8 days following CVB3-infection, the heart tissues were collected, sectioned and stained with HE. Sections were examined by two independent investigators in a blinded manner, and the severity of myocarditis was assessed by a previously described 0–4 scale (Grabie et al., 2003), in which 0 = no inflammation; 1 = one to five distinct mononuclear inflammatory foci with involvement of 5% or less of the cross-sectional area; 2 = more than five distinct mononuclear inflammatory foci, or involvement of over 5% but not over 20% of the cross-sectional area; 3 = diffuse mononuclear inflammation involving over 20% of the area, without necrosis; and 4 = diffuse inflammation with necrosis.

2.6. Intracellular staining

8 days following CVB3-infection, splenocytes were isolated and reactivated *in vitro* with 10 μg/ml of plate-bound anti-CD3 (eBioscience) antibody and 2 μg/ml soluble anti-CD28 antibody (eBioscience) for 8 h. For the final 4 h, 1 μg/ml of GolgiStop (BD Pharmingen) was added. Cells were harvested and stained with FITC-conjugated anti-mouse CD4 (eBioscience). After washing, cells were fixed with fixation buffer (eBioscience) for 20 min at 4 °C and permeabilized with permeabilization solution (eBioscience) for 20 min at room temperature, and then stained with PE-conjugated anti-mouse IFN-γ or anti-mouse IL-4 (eBioscience). The percentage