Blocking CC Chemokine Receptor (CCR) 1 and CCR5 During Herpes Simplex Virus Type 2 Infection *In Vivo* Impairs Host Defence and Perturbs the Cytokine Response

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Abstract

Elimination of viral infections is dependent on rapid recruitment of leucocytes to infected areas. Chemokines constitute a class of cytokines that regulate migration of leucocytes to sites of infection. In this work, the expression and function of CC chemokine receptor (CCR)1 and CCR5 and their ligands during a generalized herpes simplex virus type 2 (HSV-2) infection in mice were studied. Many CCR1 and CCR5 ligands were expressed in infected organs after intraperitoneal infection. In particular, CC chemokine expression in the liver preceded the expression of CCR1 and CCR5, suggesting recruitment of cells bearing these receptors, which correlated with a decrease in viral titres. Administration of Met-RANTES, a CCR1 and CCR5 antagonist, led to impaired antiviral response with significantly higher viral titre in the liver on days 1 and 6 after infection. This observation was accompanied by a decreased and shortened recruitment of natural killer cells to the peritoneum of infected mice treated with the antagonist. Despite this reduced recruitment of antiviral leucocytes in mice receiving Met-RANTES, peritoneal cells from these mice produced markedly enhanced levels of pro-inflammatory cytokines. Altogether, the results suggest that CCR1 and/or CCR5 are important for both viral clearance and eventual control of the immune response.

Introduction

Establishment of an efficient immune response to infections is highly dependent on rapid recruitment of cells of the immune system to infected areas. In this process, chemokines play a pivotal role [1, 2]. Chemokines constitute a group of small, secreted peptides with chemotactic and pro-inflammatory activities. They are classified into CC, CXC, C and CX_3C chemokines, based on the numbers and mutual placement of cysteines in the N-terminal region of proteins [3]. Chemokine receptors are seven-transmembrane spanning, G-protein-coupled receptors that are expressed primarily on leucocytes [1, 4]. The pattern of chemokine receptor expression is highly cell type dependent and is also affected by the state of differentiation and activation of the cells [5, 6]. Many chemokine receptors can bind several different chemokines, and likewise, most chemokines can work as ligand for more

than one receptor. For instance, CC chemokine receptor (CCR) 1 is a receptor for macrophage inflammatory protein (MIP)-1α/CCL3 and MIP-1γ/CCL9, regulated upon activation, normally T-cell expressed and secreted (RANTES)/CCL5 and monocyte chemoattractant protein (MCP)-3/CCL7, whereas CCR5 serves as a receptor for CCL3, MIP-1β/CCL4, CCL5 and MCP-2/CCL8 [3, 7]. Although chemokines were first recognized as chemotactic molecules for leucocytes [8], more recently, a number of other functions have been elucidated. These include leucocyte activation, induction of cytokine and chemokine expression, leucocyte degranulation and also developmental roles [3, 9–13].

Chemokines have been demonstrated to play beneficial as well as deleterious roles in many kinds of diseases and pathologic conditions, including infections, autoimmune diseases and transplant rejections [2, 3, 14, 15]. Concerning

the role of chemokines in host defence against viral infections, most attention has been drawn to CCR1 and CCR5 and their ligands, because these receptors seem to be associated with a T helper 1 (Th1)-type of immune response, which is generally considered to be protective against viruses [16–18]. Using mice deficient in specific genes of the chemokine system, it has been shown that CCL3 and CCR1 are important for resistance against certain viral infections [19, 20]. Also, CCR5 deficiency in humans increases the susceptibility to hepatitis C virus infections [21], and mice lacking this receptor display increased mortality rate after influenza virus infection [22]. However, lack of CCR5 can also be associated with beneficial modulation of the immune response, as evidenced by other studies in CCR5-deficient (CCR5$^{-/-}$) mice. Infection with a neurotropic coronavirus resulted in reduced demyelination in the central nervous system (CNS), which correlated with reduced recruitment of macrophages into the brain of CCR5$^{-/-}$ when compared with wild-type mice [23]. These results indicate that although chemokines in many instances are necessary for mounting an efficient immune response, in other cases they may contribute to an overwhelming immune response, eventually causing tissue damage.

Herpes simplex virus (HSV) is an alpha-herpesvirus, able to infect most cell types and establish latent infections in neurones. Infection by HSV-1 may give rise to cold sore and, in rare cases, encephalitis, whereas HSV-2 is mainly responsible for genital and neonatal herpes. We have previously shown that HSV infection *in vitro* leads to expression of many inflammatory mediators [24–26], whereas only a small subset of chemokines are produced [27]. In this study, we have evaluated the role of CCR1, CCR5 and their ligands during a generalized HSV-2 infection *in vivo* in mice. The results presented here show that CCR1 and/ or CCR5 are essential for establishment of an efficient antiviral response during a systemic HSV-2 infection. Moreover, our data point towards an immunoregulatory role of these two chemokine receptors at later stages when the virus has been cleared.

Materials and methods

Reagents. The growth media used were Eagle's minimal essential medium (MEM) and RPMI-1640 (both Bio-Whittaker, Verviers, Belgium) supplemented with antibiotics (penicillin 200 IU/ml, streptomycin 200 µg/ml, nystatin 25 IU/ml and Garamycin 40 µg/ml) and indicated concentrations of lipopolysaccharide-free fetal calf serum (FCS) (Bio-Whittaker). RPMI-1640 was also supplemented with 10 mM glutamine and 10 mM HEPES (Bio-Whittaker). Heparin was obtained from Leo Pharma (Copenhagen, Denmark), and Trizol was from Invitrogen (Carlsbad, CA, USA). Oligo-(dT), deoxynucleotide triphosphates, Expand Reverse Transcriptase and dithiothreitol were from Roche (Basel, Switzerland). DNA oligonucleo-

tides were provided by DNA Technology, and Taq 2000 DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). Recombinant cytokines and capture and detection antibodies used in enzyme-linked immunoadsorbent assays (ELISAs) were from BD Pharmingen, San Diego, CA, USA [interleukin-4 (IL-4) and IL-12] or R&D Systems, Minneapolis, MN, USA [CCL3, CCL5, IL-10 and interferon-γ (IFN-γ)], and Streptavidin–horseradish peroxidase (HRP) and the TMB substrate system were provided by R&D Systems. Recombinant tumour necrosis factor-α (TNF-α) and IFN-α/β were obtained from PBL Biomedical Laboratories (Piscataway, NJ, USA). Met-RANTES was from R&D Systems, and fluorescein isothiocyanate (FITC)-conjugated antibodies used for flow cytometry were purchased from BD Pharmingen. Collagenase IV and metrizamide were from Sigma-Aldrich (St Lewis, MS, USA), and DNAse I was from Roche.

Mice and virus. The mice used in this study were 3- to 4-week-old female BALB/c and C57BL/6 mice from Taconic M&B (Ry, Denmark). The virus used was the MS strain of HSV-2. Mice were infected intraperitoneally with 1×10^6 plaque-forming units (PFU) of HSV-2 suspended in 100 µl of phosphate-buffered saline (PBS) on day 0. Mice receiving Met-RANTES were injected intraperitoneally on days 0, 2 and 4 with 8.3 µg of Met-RANTES in 100 µl of PBS per mouse. Control animals were injected with PBS on the days indicated above. Mice were killed on days 1, 3 and 6, and peritoneal cells (PCs) were harvested by lavage of the peritoneal cavity with cold PBS (pH 7.4) supplemented with 2% FCS and heparin 20 IU/ml. Samples of liver, spleen and brain were frozen at the time of harvest, except for livers used for flow cytometry, which were kept in 10 ml of cold RPMI supplemented with 5% FCS, until further treatment.

Natural killer cell cytotoxicity assay. YAC-1 cells were loaded with BATDA [bis-(acetoxymethyl)2,2′:6′,2″-terpyridine-6,6″-dicarboxylate] by incubating the cells for 20 min at 37 °C in RPMI supplemented with 10 µM BATDA. The cells were washed four times and seeded in 96-well plates with 5×10^3 cells per well.

PCs were added to the target cells in the indicated ratios, to reach a final volume of 200 µl per well, and effector and target cells were brought into contact by centrifugation for 5 min at $50 \times g$. The cells were incubated for 2 h at 37 °C, after which the cells were resuspended and centrifuged to allow even distribution of the released BATDA. From each well, 25 µl was mixed with 200 µl of a 50 µM Eu^{3+} solution. Fluorescence was measured, and per cent cell lysis was calculated as:

$$100\% \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}.$$

Plaque assay. Samples of liver, spleen and brain were weighed, thawed and homogenized three times for 5 s in

MEM media supplemented with 2% FCS just before use in the plaque assay. After homogenization, the organ suspensions were pelleted by centrifugation at $1600 \times g$ for 30 min and the supernatants were used for plaque assay as well as reverse transcriptase polymerase chain reaction (RT-PCR). Plaque assay was carried out on Vero cells that were seeded in MEM supplemented with 5% FCS at a density of 1×10^6 cells in 5-cm diameter plates and left to settle overnight. The cells were infected by incubation for 1 h at 37 °C with 100 µl of serial dilutions of the organ suspensions and 400 µl of medium, during which the tissue-culture plates were rocked every 15 min to ensure even distribution of virus. The organ suspensions were subsequently removed, 8 ml of medium with 0.2% human immunoglobulin (Ig) was added to each plate and the cells were incubated for 2 days and stained with 0.03% methylene blue to allow quantification of plaques.

RT-PCR. Total RNA was extracted from PCs and organ homogenates with Trizol according to the recommendations of the manufacturer. Briefly, cells were lysed in Trizol and chloroform was added, followed by phase separation by centrifugation. RNA was precipitated with isopropanol and pelleted by centrifugation. Pellets were washed with 70% ethanol and redissolved in RNAse-free water. Two to four micrograms of RNA was subjected to reverse transcriptase using oligo(dT) as primer and Expand Reverse Transcriptase. The cDNA was amplified by PCR using the following primers: CCR1, 5′-CCA TCC ACA GGA GAA CAT GA-3′ (sense), 5′-GCC CCA TTT TGG TTT ATT CA-3′ (antisense); CCR5, 5′-TGG CTT CTC CAC ACA AAC AA-3′ (sense), 5′-CGG AGC TTG AGA AAA ACC AG-3′ (antisense); CCL3, 5′-GAA GAG TCC CTC GAT GTG GCT A-3′ (sense), 5′-CCC TTT TCT GTT CTG CTG ACA AG-3′ (antisense); CCL4, 5′-TTC TCT CTC CTC CTG CTT GTG G-3′ (sense), 5′-CAC ATA CTC ATT GAC CCA GGG C-3′ (antisense); CCL5, 5′-ATA TGG CTC GGA CAC CAC TC-3′ (sense), 5′-GAT GCC GAT TTT CCC AGG AC-3′ (antisense); CCL7, 5′-GTG TCC CTG GGA AGC TGT TA-3′ (sense), 5′-AGA AAG AAC AGC GGT GAG GA-3′ (antisense); CCL8, 5′-GGG TGC TGA AAA GCT ACG AG-3′ (sense), 5′-TCC AGC TTT GGC TGT CTC TT-3′ (antisense); CCL9, 5′-TTC CCC AGT GAC CAC CTA AG-3′ (sense), 5′-GGT GCT CTT AAC CAC CAA GC-3′ (antisense); β-actin, 5′-CCC ACT CCT AAG AGG AGG ATG-3′ (sense), 5′-AGG GAG ACC AAA GCC TTC AT-3′ (antisense). The products spanned 250 bp (CCR1), 203 bp (CCR5), 561 bp (CCL3), 237 bp (CCL4), 330 bp (CCL5), 194 bp (CCL7), 201 bp (CCL8), 251 bp (CCL9) and 215 bp (β-actin).

ELISA. Murine CCL3, CCL5, IL-4, IL-10, IL-12 and IFN-γ were detected by ELISA. Maxisorp plates were coated overnight at 4 °C (CCL3, CCL5, IL-4 and IL-12) or at room temperature (IL-10 and IFN-γ), with 100 µl of anti-murine capture antibody (IL-4: 1 µg/ml; CCL5: 2 µg/ml; CCL3, IL-10 and IFN-γ: 4 µg/ml; IL-12: 6 µg/ml) in PBS (IL-10 and IFN-γ) or coating buffer [$NaHCO_3$ 35 mM, Na_2CO_3 15 mM, sodiumazid 0.02%, pH 9.6 (CCL3, CCL5, IL-4 and IL-12)]. After blocking with 300 µl of 1% bovine serum albumin (BSA) in PBS (IL-4 and IL-12) or blocking buffer [PBS with 5% sucrose and 0.05% sodiumazid, pH 7.4 (CCL3, CCL5, IL-10 and IFN-γ)], successive twofold dilutions of recombinant murine cytokine or culture supernatants were added to the wells in duplicates, 100 µl each, and incubated overnight at 4 °C (IL-4) or 3 h at room temperature (CCL3, CCL5, IL-10, IL-12 and IFN-γ). Subsequently, wells were incubated for 2 h at 20 °C with 100 µl of biotinylated, anti-murine detection antibody (IL-4 and IL-12: 1 µg/ml; IL-10 and IFN-γ: 0.4 µg/ml; CCL5: 0.1 µg/ml; CCL3: 50 ng/ml) in a 0.1% suspension of BSA in TBS [Trizma 20 mM, NaCl 150 mM, Tween-20 0.05%, pH 7.3 (CCL3, CCL5, IL-4, IL-10 and IFN-γ)] or blocking buffer (IL-12). Streptavidin–HRP diluted 1 : 200 in TBS with 0.1% BSA (CCL3, CCL5, IL-4, IL-10 and IFN-γ) or PBS with 1% BSA (IL-12) was added and incubated at 20 °C for 20 min. As substrate was added, 100 µl/well of the TMB system and plates were incubated in the dark. After 20 min, the colour reaction was stopped with 50 µl of 2 M H_2SO_4 and absorbance was measured at 450 nm. Between each step, the plates were washed three times with PBS 0.05% Tween-20. The detection limit of the ELISA assays was: 7.8 pg/ml (CCL3, CCL5 and IL-4) and 15.6 pg/ml (IL-10, IL-12 and IFN-γ).

IFN-α/β bioassay. IFN-α/β bioactivity was measured by an L929-cell-based bioassay. L929 cells (2×10^4 cells/well in 100 µl) in MEM with 5% FCS were incubated overnight at 37 °C in successive twofold dilutions of samples or murine IFN-α/β as standard. Subsequently, vesicular stomatitis virus (VSV/V10) was added to the wells and the cells were incubated for 2–3 days. The dilution mediating 50% protection was defined as 1 U/ml of IFN-α/β.

TNF-α bioassay. TNF-α bioactivity was measured by a bioassay. L929 cells were seeded in 96-well tissue-culture plates at a concentration of 2×10^4 cells/well in 100 µl of MEM with 10% FCS and allowed to settle overnight at 37 °C. Supernatants were removed, and serial twofold dilutions of the samples or recombinant TNF-α as standard were added and incubated at 38.5 °C with actinomycin D (1 µg/ml) for 18 h. Cells were fixed in 10% formaldehyde and stained with crystal violet, and TNF titres were determined from the dilution causing 50% cytotoxicity. The bioassay was specific for TNF-α because the activity was neutralized with a neutralizing antibody against TNF-α.

Nitrite assay. Nitric oxide (NO) was measured by detection of nitrite, a stable product of NO oxidation. Culture supernatant or sodium nitrite as standard was mixed (100 µl of each) with Griess reagent [equal volumes of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% p-aminobenzene sulfanilamide diluted in 2.5% phosphoric acid]. The mixture was incubated for 10 min

at room temperature, to allow the colour to develop, and absorbance was measured at 540 nm in a microplate reader.

Measurements of ex vivo production of cytokines and NO. To assay the *ex vivo* production of cytokines and NO, PCs were harvested as described. After washing, the cells were counted and seeded in 96-well tissue-culture plates at a concentration of 1.75×10^6 cells/ml in 200-µl RPMI/ well and left for 24 or 48 h at 37 °C. Supernatants were harvested and analysed for the presence of cytokines and NO.

Measurement of cytokines in organ homogenates. Organs were harvested in RPMI-1640 medium supplemented with 10% FCS and homogenized. The suspensions were centrifuged at $5000 \times g$ for 30 min, and the supernatants were harvested for measurement of cytokines.

Isolation of intrahepatic leucocytes. Intrahepatic leucocytes (IHLs) were isolated essentially as described by Kakimi et al. [28]. Livers were homogenized manually with a glass homogenizer in RPMI with 5% FCS and incubated for 40 min at 37 °C with 0.02% (w/v) collagenase IV and 0.002% (w/v) DNAse I. Cells and tissue were precipitated by centrifugation at $720 \times g$ for 10 min, and the digestion procedure was repeated on the pellet. Cells were resuspended in RPMI and underlaid with 24% (w/v) metrizamide in PBS and centrifuged for 20 min at $1500 \times g$. IHLs were isolated at the interface, and erythrocytes were lysed in NaCl 0.2% for 30 s, followed by addition of an equal volume of NaCl 1.6%. The leucocytes were pelleted by centrifugation for 10 min at $180 \times g$ and resuspended in RPMI.

Flow cytometry. PCs were counted and resuspended in RPMI supplemented with 5% FCS to a concentration of 10×10^6 cells/ml. PCs were incubated on ice, in the dark for 40 min with FITC-conjugated anti-mouse monoclonal CD3ε (155-2611), NK-1.1 (PK136) or Ly-6G (RB6-865) antibodies at a concentration of 16 µg/ml. For isotype standard FITC-conjugated monoclonal mouse IgG$_1$, κ was used in the same concentration. Samples were washed twice in RPMI with 5% FCS and fixed in PBS with 1% paraformaldehyde. IHLs were resuspended to a concentration of 2×10^6 cells/ml and treated as PCs, using an antibody concentration of 10 µg/ml. Data were acquired by flow cytometer with an excitation emission of 530 nm, and analysed using CELLQuest^{TM} software.

Statistical analysis. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was estimated with Student's t-test for unpaired observations. P-Values of less than 0.05 were considered significant.

Results

Expression of CCR1, CCR5 and their ligands during HSV–2 infection in BALB/c and C57BL/6 mice

This laboratory has previously shown that HSV-2 infection of murine PCs *in vitro* results in induction of CCL5

expression [27]. To investigate the chemokine expression patterns after an *in vivo* infection and the association with viral burden, age- and sex-matched BALB/c and C57BL/6 mice were infected intraperitoneally with 1×10^6 PFU of HSV-2. In both mouse strains, a generalized infection developed (Fig. 1A), with peak viral titres in the spleen and liver on days 1 and 3. The infection led to a focal, necrotizing hepatitis with large macroscopic liver lesions clearly visible on day 6 after infection (data not shown). In the brain, virus was detectable from day 6, and BALB/c mice showed lower viral load than C57BL/6 mice.

When examining the expression of the CC chemokines and their receptors (Fig. 1B,C), we observed a large degree of overlap between the two mouse strains. In the liver, CCL9 was expressed constitutively, whereas CCL5 and CCL8 were induced at late time points during infection. Importantly, induction of CCL5 expression preceded the observed decrease in viral titres in the liver on day 6, which correlated with upregulation of CCR1 and CCR5, indicating an accumulation in the liver of immune cells bearing these receptors.

The chemokine expression pattern in the spleen revealed a difference between the two mouse strains, even though viral titres were very similar. Peak viral titres were seen day 1 after infection, followed by a gradual decrease over the 6 days of infection. CCL9 expression was induced only in C57BL/6 mice late during infection, in contrast to a weak constitutive expression in BALB/c mice. CCL5 was expressed constitutively in the spleen of both strains. Of the receptors, only CCR1 was expressed in the spleen, with constitutive expression in BALB/c and inducible expression in C57BL/6 mice. In the brain, CCL3 was found to be expressed constitutively in small amounts in BALB/c mice and, together with RANTES, to be weakly induced on day 6 of the infection. No chemokines were detected in brains of C57BL/6 mice. Equivalently, neither of the receptors was upregulated, but CCR5 was expressed constitutively in both strains.

Altogether, these results show that a generalized HSV-2 infection develops similarly in 3- to 4-week-old BALB/c and C57BL/6 mice and that these two mouse strains also mount chemokine responses to the infection with a large degree of overlap, although differences are apparent. In the remaining part of this study, we have concentrated on BALB/c mice.

Cell migration and chemokine expression in the peritoneum during a HSV–2 infection

To investigate the association between chemokine expression and migration of cells during HSV-2 infection *in vivo*, PCs were harvested, counted and examined for the expression of CCR1, CCR5 and their ligands at different time points after infection. The HSV-2 infection resulted in a rapid and significant accumulation of cells in the peritoneal cavity (Fig. 2A), with peak cell numbers seen
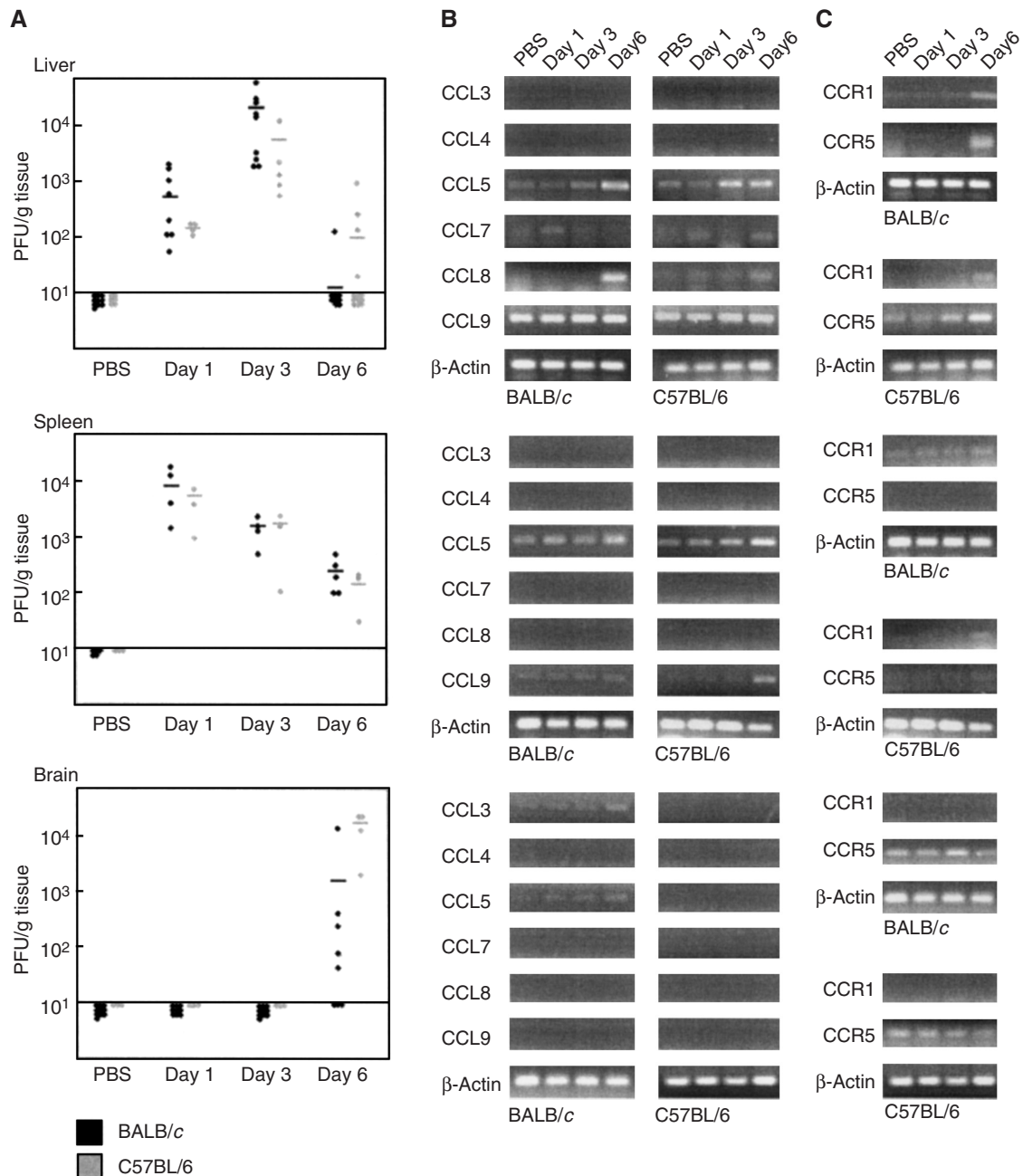
Figure 1 Viral titres and expression of CCR1, CCR5 and their ligands during herpes simplex virus type 2 (HSV-2) infection. BALB/c and C57BL/6 mice were infected intraperitoneally with 1×10^6 plaque-forming units (PFU) of HSV-2, and liver, spleen and brain were harvested at the indicated time points after infection. (A) HSV-2 titres were determined in liver, spleen and brain using plaque assay. Mean values are shown together with values of the individual measurements. In this assay, the limit of detection was 10^1 PFU/g tissue (marked with a thin line). (B and C) Total RNA was extracted from organ homogenates. Expression of chemokines (B), as well CCR1 and CCR5 (C), was detected by reverse transcriptase polymerase chain reaction. PBS, phosphate-buffered saline.

on day 3, reaching in average 11.7×10^6 ($\pm 2.2 \times 10^6$) PCs/mouse compared to 2.6×10^6 ($\pm 3.7 \times 10^5$) in control mice. In a different set of experiments, IHLs were harvested from mice 6 days after infection. While 5.0×10^5 ($\pm 0.95 \times 10^5$) leucocytes were recovered from the livers of non-infected mice, this number was increased

to 9.3×10^5 ($\pm 3.1 \times 10^5$) cells after infection (data not shown).

Several of the CC chemokines were expressed by PCs during the HSV-2 infection (Fig. 2B). CCL5 and CCL9 were expressed constitutively in low amounts. CCL5 expression was strongly induced on day 1, and the level
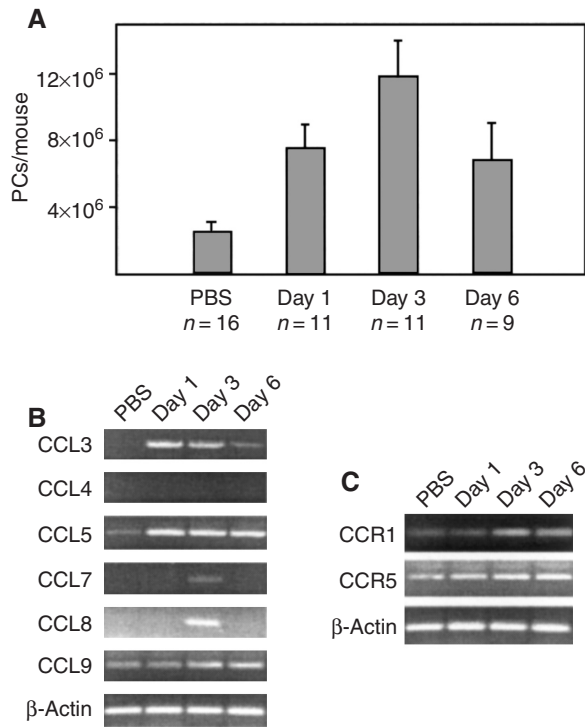
Figure 2 Accumulation of leucocytes in the peritoneal cavity and expression of CCR1, CCR5 and their ligands in peritoneal cells (PCs) during herpes simplex virus type 2 (HSV-2) infection. (A) PCs were harvested as described and counted. Values are mean ± SEM. (B and C) Total RNA was extracted from PCs, and the expression of CCR1, CCR5 and chemokines was detected by reverse transcriptase polymerase chain reaction.

remained elevated through the 6 days of the experiment. CCL3 was induced on day 1, after which the expression gradually decreased, while the expression of CCL7 and CCL8 was observed on day 3 only. Importantly, among the chemokines expressed, CCL3 and CCL5 were strongly

induced prior to the peak in accumulation of leucocytes in the peritoneal cavity. Also correlating with the PC migration was the upregulation of CCR1 on day 3, whereas CCR5 was expressed constitutively throughout the infection (Fig. 2C).

To examine whether the elevated mRNA expression of CCR1 and CCR5 ligands (Figs 1 and 2) was also apparent at the protein level, we harvested PCs and organs for measurement of CCL3 and CCL5 by ELISA. As seen in Fig. 3A,E, both chemokines were expressed in a virus-inducible manner by PCs, with CCL5 expression displaying a more sustained kinetics. In the organs, only CCL5 expression in the liver at day 6 after infection was significantly elevated (Fig. 3F).

Thus, expression of CCL5 in the liver and CCL3 and CCL5 by PCs after intraperitoneal viral infection correlates with clearance of virus from the liver and migration of cells to the peritoneal cavity, respectively.

Effect of Met–RANTES on *in vivo HSV-2 infection*

The experiments shown above revealed that accumulation of CCR1- and CCR5-expressing cells in the liver correlates with decreases in viral titres (Fig. 1) and that the expression of chemokines preceded recruitment of leucocytes (Figs 2 and 3). Therefore, we wanted to investigate the effect of Met-RANTES on the HSV-2 infection. This chemokine derivative works as an antagonist for CCR1 and CCR5 [29] and also to a lesser extent for CCR3 [30].

In the spleen, no effect of Met-RANTES on viral titres was detected on day 1 after infection (Table 1), whereas on days 3 and 6, a modest, but not a significant, increase in viral titres was seen in mice treated with HSV-2 + Met-RANTES versus HSV-2 alone. The infection of the liver was highly affected by administration of Met-RANTES. This was seen both early and late during the infection. On
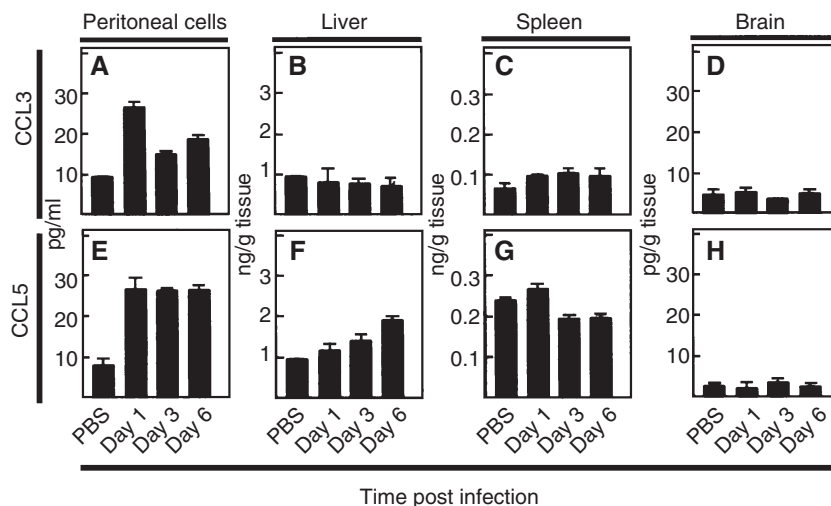

Figure 3 Chemokine protein production during infection *in vivo*. Mice were infected intraperitoneally, and peritoneal cells (PCs) and organs were harvested at the indicated time points after infection. Supernatants were harvested from PCs cultured 24 h *ex vivo* and from centrifuged organ homogenates for measurement of CCL3 (A–D) and CCL5 (E–H) by enzyme-linked immunoadsorbent assays. Results are shown as mean ± SEM. $n = 3$–6.

Table 1 Effect of Met-RANTES on viral titres in herpes simplex virus type 2 (HSV-2)-infected BALB/c mice

Time of infection	Spleen*		Liver		Brain	
	HSV-2	HSV-2 + Met-RANTES	HSV-2	HSV-2 + Met-RANTES	HSV-2	HSV-2 + Met-RANTES
Day 1†	$16,000 \pm 7200$	$13,000 \pm 1700$	600 ± 210	3100 ± 1300	BD‡	BD
Day 3	3200 ± 910	8400 ± 3500	$27,000 \pm 13,000$	$44,000 \pm 17,000$	BD	BD
Day 6	410 ± 140	1700 ± 1100	17 ± 17	1500 ± 560	140 ± 79	760 ± 340

Mice were infected intraperitoneally with 1.0×10^6 plaque-forming units (PFU) of HSV-2 and received either Met-RANTES (8.3 µg per injection) or phosphate-buffered saline on days 0, 2 and 4. On successive days, mice were killed and spleen, liver and brain were harvested. Viral titres in the organs were determined on Vero cells infected with serial dilutions of homogenates of the organs.
*The results are presented as PFU per gram tissue ($\pm$SEM).
†Numbers of mice per group: 3–9.
‡BD, below the limit of detection.

day 1, viral titres were significantly increased in mice treated with HSV-2 + Met-RANTES as compared with mice receiving HSV-2 alone. On day 3, no significant difference in viral titres in the liver was observed between the two groups. On day 6, mice receiving Met-RANTES again had a significantly higher viral load as compared with the group of mice receiving HSV-2 only. Moreover, the livers from mice receiving Met-RANTES and infected for 6 days were highly fibrotic and displayed more and larger necrotic plaques, as compared with livers from mice given HSV-2 alone (data not shown). In the brain, virus was not detected in either group of mice on days 1 and 3 after infection. On day 6, however, we found higher viral titres in the Met-RANTES group. This difference, however, did not reach statistical significance ($P = 0.07$). Altogether, these results show that blocking of CCR1 and CCR5 affects the antiviral response to HSV-2 infection, reducing the ability of the host to clear the infection.

Effect of Met–RANTES on leucocyte migration during HSV-2 infection

Given the finding above that Met-RANTES affected the clearance of HSV-2, we next wanted to investigate whether this impaired antiviral response correlated with a changed pattern of leucocyte migration to sites of infection. We therefore examined the total number of leucocytes, as well as selected leucocyte subsets, in the peritoneal cavity at the indicated time points after infection. This was done by flow cytometry using FITC-labelled monoclonal antibodies against surface antigens on natural killer (NK) cells, granulocytes (neutrophils) and T lymphocytes.

When comparing the two groups of mice, there was no statistically significant difference in total numbers of PCs on any of the days examined between the HSV-2-infected mice and mice receiving HSV-2 and Met-RANTES (Fig. 4M), although, in the latter group, a tendency towards decreased leucocyte migration to the peritoneal cavity at days 3 and 6 was observed. The number of

NK-1.1-positive cells, however, was significantly decreased on day 1 in mice given HSV-2 + Met-RANTES ($0.83 \times 10^6 \pm 0.15 \times 10^6$ cells/mouse) as compared with the group of mice given HSV-2 alone ($3.8 \times 10^6 \pm 0.19 \times 10^6$ cells/mouse) (Fig. 4N,G–I for a representative example for day 1 after infection). On day 3, the two groups displayed similar numbers of NK cells, but again on day 6, a tendency towards less NK cells in mice given HSV-2 + Met-RANTES was seen. Significantly less Ly-6G-positive granulocytes were also seen on day 1 in the group receiving both HSV-2 and Met-RANTES (Fig. 4J–L,O, for a representative example). There was no significant difference between the two groups with respect to CD3$^+$ T lymphocytes in the peritoneal cavity (Fig. 4D–F,P).

These results show that uncoupling of the CCR1 and CCR5 receptor systems profoundly affects the leucocyte migration patterns to infected areas during a generalized HSV-2 infection. This could contribute to the observed impaired ability of Met-RANTES-treated mice to clear the infection.

NK cell activity in HSV–infected mice

In the light of the above finding, we wanted to examine whether NK cell activity at the site of infection was also affected by Met-RANTES treatment. We, therefore, harvested PCs on day 1 from mice infected with HSV-2 in the presence or absence of co-treatment with Met-RANTES and assayed for NK cell cytotoxic activity towards YAC-1 cells. As shown in Fig. 5, HSV-2 infection did lead to elevated NK cell activity, thus in line with the above-observed recruitment of NK cells. Interestingly, NK cell activity of PCs from mice infected with HSV-2 and co-treated with Met-RANTES was strongly inhibited as compared with infected mice not receiving Met-RANTES. These results thus demonstrate that Met-RANTES inhibits recruitment and, hence, cytotoxic activity of NK cells at the site of infection.
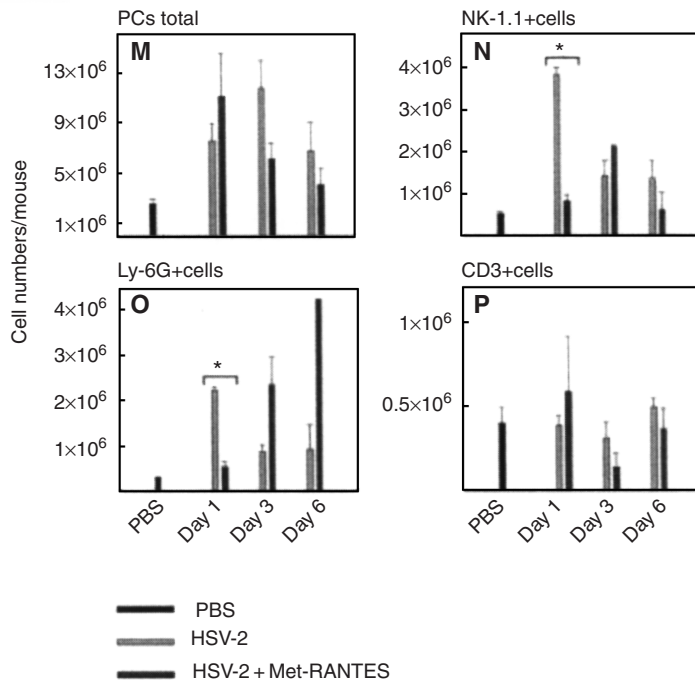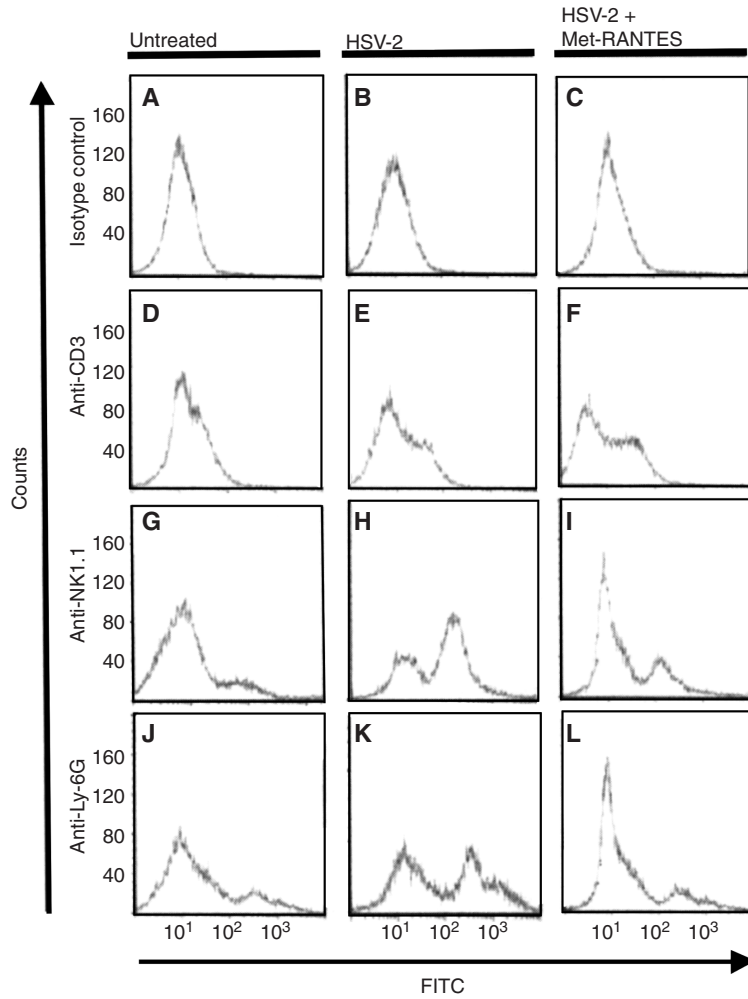
Figure 4 Effect of Met-RANTES on total leucocyte numbers and accumulation of selected leucocyte subsets in the peritoneal cavity during herpes simplex virus type 2 (HSV-2) infection. (A–L) Representative example of flow cytometric data on peritoneal cells (PCs) harvested day 1 after infection and stained with isotype control (A–C), anti-CD3 (D–F), anti-NK-1.1 (G–I) or anti-Ly-6G (J–L). (M) Total number of PCs after intraperitoneal HSV-2 infection. (N–P) Accumulative flow cytometric data on PCs harvested at the indicated time points after infection. (N) Anti-NK-1.1. (O) Anti-Ly-6G. (P) Anti-CD3. Results are shown as mean ± SEM. $^*P < 0.05$.
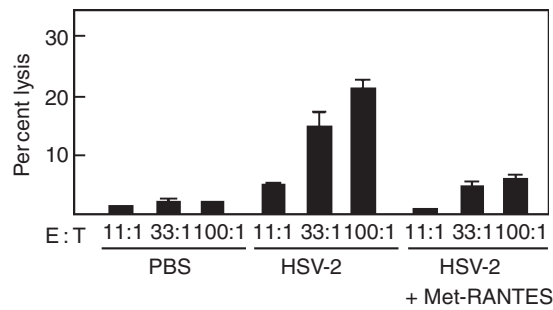
Figure 5 Natural killer (NK) cell activity in herpes simplex virus-infected mice. Three mice per group were infected intraperitoneally in the presence or absence of Met-RANTES or were treated with phosphate-buffered saline. Peritoneal cells (PCs) were harvested 24 h after infection and analysed for NK cell activity on YAC-1 cells in the indicated ratios of effector cells (E) and target cells (T). Results are shown as mean ± SEM.

Effect of Met–RANTES on cytokine expression by PCs during HSV–2 infection

As shown above, treatment with Met-RANTES strongly affects recruitment of different leucocyte subsets to the peritoneal cavity during HSV-2 infection. Moreover, because chemokines are also known to have a role in the activation of leucocytes [10, 11], we wanted to examine the effect of Met-RANTES on cytokine levels during the *in vivo* HSV-2 infection. Mice were infected intraperitoneally, and PCs were harvested on day 1, 3 or 6 after infection and cultured for 1 or 2 days before harvest of supernatants for measurement of cytokines and NO.

As seen in Fig. 6A, levels of IFN-α/β peaked at day 1 and there was no significant difference detected during the infection between mice receiving HSV-2 alone and mice given HSV-2 + Met-RANTES. However, the levels of four potent pro-inflammatory mediators, the cytokines IFN-γ, IL-12 and TNF-α and the radical NO, were strongly affected by co-treatment with Met-RANTES (Fig. 6B–E). IFN-γ levels were statistically indistinguishable on days 1 and 3 between the two groups of mice, but on day 6, the levels were significantly higher in the group treated with HSV-2 + Met-RANTES when compared with mice given HSV-2 alone. For TNF-α, no upregulation was seen on days 1 and 3 in either group, nor on day 6 in the HSV-2-infected group. In the group receiving HSV-2 + Met-RANTES, however, the levels of TNF-α were significantly elevated on day 6. IL-12 was also detected at higher concentrations on day 6 in the group of mice given HSV-2 + Met-RANTES than in the group given HSV-2 alone, but this difference was not statistically significant ($P = 0.07$). Finally, the levels of NO, an inflammatory mediator which is part of the innate immune response to many viruses [31, 32], were significantly higher on day 6 in the HSV-2 + Met-RANTES group.

Next, we wanted to investigate whether these increased concentrations of Th1-associated cytokines and inflammatory
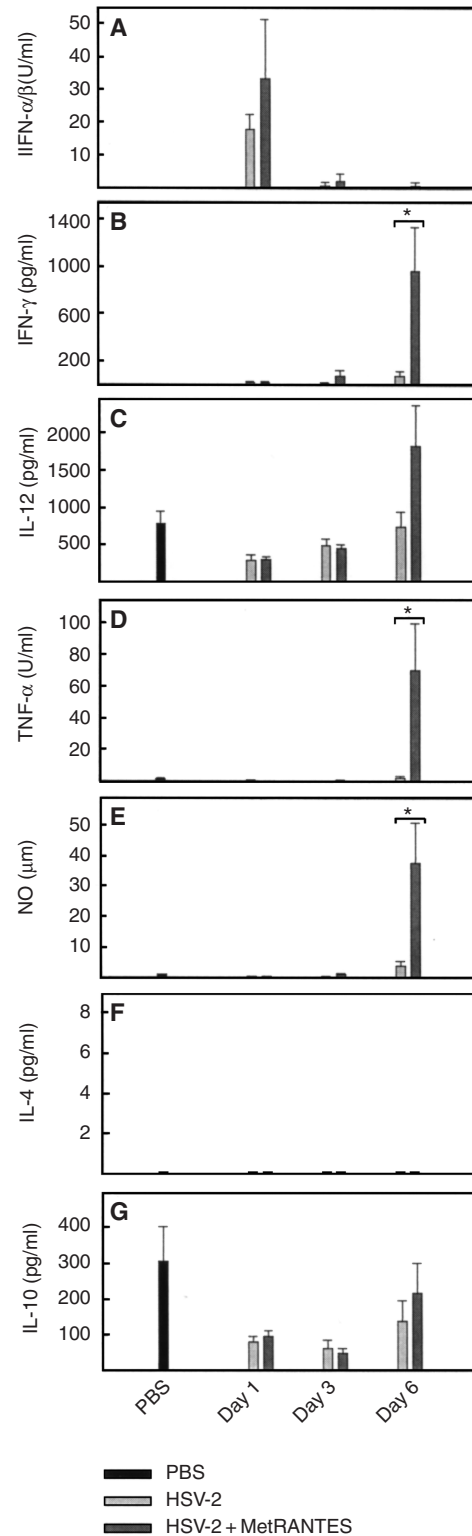

Figure 6 Effect of Met-RANTES on cytokine expression in peritoneal cells (PCs) during herpes simplex virus type 2 (HSV-2) infection. PCs were harvested and cultured as described. Supernatants were harvested after 24 h for the measurement of (A) interferon-α (IFN-α)/β, (B) IFN-γ, (C) IL-12, (D) tumour necrosis factor-α (TNF-α), (F) IL-4 and (G) IL-10 or after 48 h for measurement of (E) NO. Results are shown as mean ± SEM; $n = 3$–6; *$P < 0.05$.

mediators in virus-infected mice treated with Met-RANTES were associated with changes in the levels of Th2-associated cytokines or cytokines with an anti-inflammatory effect. We therefore examined the supernatants for the content of IL-4 and IL-10 but found no significant differences between the two groups in the concentration of either cytokine during the infection (Fig. 6F,G). Met-RANTES alone did not affect expression of any of the cytokines examined (data not shown).

This set of experiments therefore shows that blocking CCR1 and CCR5 leads to a strong upregulation of Th1-associated cytokines and inflammatory mediators after 6 days of infection and that this upregulation appears not to be mediated by a parallel down-modulation of Th2 cytokines.

Discussion

Following viral infections, the organism responds by mounting a pro-inflammatory host response aiming at eliminating the virus. Central for this response is recruitment of leucocytes to sites of infection, a process where chemokines play pivotal roles.

Several studies have investigated the role of chemokines and their receptors in virus infections. The results have shown that during some viral infections, a specific chemokine or chemokine receptor is necessary for generation of an efficient antiviral response, whereas for other viral infections, the chemokine system contributes to an excessive immune response, causing tissue damage [22, 33–35]. In this study, we show that inflammatory CC chemokines are important for viral clearance during a generalized HSV-2 infection in mice and that this group of chemokines also plays immunoregulatory roles in the host response to HSV-2 infection.

Initially, we compared the two mouse strains BALB/c and C57BL/6 and found that the infection developed similarly in 3- to 4-week-old mice of the two strains. With regard to expression of chemokines, the two strains also showed similar patterns. Previous studies have shown that the C57BL/6 mouse strain is more resistant to HSV infection than BALB/c mice, but because this resistance does not develop until 5–6 week of age, our results do not contradict these earlier findings [36–38].

Studies by others have shown that, in particular, CCL3 and CCL5 are induced during viral infections [33, 39–42]. Moreover, CCL3 seems to be essential for defence against several virus infections, including influenza virus, pneumonia virus of mice (PVM) and murine cytomegalovirus (MCMV) [19, 20, 34]. Salazar-Mather et al. [43] demonstrated that IFN-α/β-induced accumulation of NK cells in the liver during MCMV infection is dependent on CCL3. NK cells have long been recognized to represent a major mediator of early defence against many viruses, including herpesviruses [44]. In our studies, we found that expression of CCL5 was induced in the liver and peritoneal cavity (seen at the levels of both mRNA and protein) after HSV-2 infection and, weakly, in brain and spleen after HSV-2 infection (found at the level of mRNA but not protein). CCL3 expression was induced in the peritoneal cavity (mRNA and protein) but only weakly in the brain (mRNA not protein) and not in detectable amounts in the liver or spleen. The induction of, notably, CCL5, but also some of the other chemokines examined, was detected immediately prior to, or coincident with, peak leucocyte numbers in the peritoneum as well as upregulation of CCR1 and CCR5 in the liver and spleen, an indication of accumulation of immune cells bearing these receptors. Also, in the liver, the elevation in cells expressing CCR1 and CCR5 correlated with a decrease in viral titres. Our findings therefore suggest that the pattern of chemokine expression evoked by virus infections differs between viruses and may suggest that other CCR1 and CCR5 ligands than CCL3 could be important for antiviral defence.

We also examined the effect of the CCR1 and CCR5 antagonist Met-RANTES on HSV-2 infection. In vitro, Met-RANTES has been shown to antagonize the chemotactic effect of CCL3 and CCL5 on T cells, monocytes and eosinophils [29, 45, 46]. In vivo, murine studies have focused on the effect of Met-RANTES on inflammatory diseases, e.g. experimental autoimmune encephalomyelitis, rheumatoid arthritis and colitis, and results have shown some effect on clinical and inflammatory parameters [47–49]. As to infections, Stumbles and colleagues [50] found that treatment with Met-RANTES in rats inhibited dendritic cell (DC) influx to tracheal epithelium in response to bacterial challenge but had no effect on the DC response to Sendai virus infection. We found that co-treatment with Met-RANTES led to significantly higher HSV-2 titres in liver on day 1 and day 6 after infection. In particular, the marked reduction in viral titres in the liver between day 3 and day 6 in HSV-2-infected BALB/c mice, which correlates with our finding that CCR1 and CCR5 were upregulated between days 3 and 6, was less pronounced in infected mice co-treated with Met-RANTES. This could indicate that recruitment of a CCR1$^+$/CCR5$^+$ cell population is important for mounting an efficient antiviral response in the liver. However, T-cell migration to the liver was not affected, and accumulation of NK cells was increased in Met-RANTES-treated mice 6 days after infection (Sørensen and Paludan, unpublished data). Another possible explanation for the increased viral titres in the liver could be a higher viral load delivered to the liver, owing to impaired initial antiviral response in the peritoneal cavity, where accumulation of NK cells and granulocytes was significantly decreased on day 1. Both these cell types have been documented to possess antiviral activity towards HSV [44, 51–53]. Third, direct chemokine-mediated activation of antiviral mechanisms in the liver, e.g. activation of Kupffer's cells, may also have been blocked by Met-RANTES. Higher viral titres were also detected in the brain (day 6) and spleen (days 3 and 6),

although these differences did not reach statistical significance. Again, the reason could be increased viral load delivered to organs, owing to impaired initial response in the peritoneal cavity. However, the blocked CCR5 activity in the brain could also be important, as CCR5 has been shown to have a role in the antiviral response and virus-mediated pathology in CNS as well as immunity to non-viral pathogens in CNS [23, 54]. Altogether, our results show that Met-RANTES, which blocks CCR1 and CCR5, impairs the antiviral response to a generalized HSV-2 infection. CCR1 and CCR5 have been previously found to contribute to antiviral defence in respiratory infection with PVM [20]. In other viral infections, however, CCR1 and CCR5 have not been found to play important roles in antiviral response. For instance, CCR1 deficiency had no effect on titres or leucocyte migration in respiratory syncytial virus infection [55], and viral titres were also unaffected by CCR5 deficiency in lymphocytic choriomeningitis virus infection in mice [56].

Co-treatment with HSV-2 + Met-RANTES also resulted in significantly elevated expression of inflammatory mediators in PCs as compared with PCs from mice given HSV-2 alone. This observation could be explained by alternations in important regulatory mechanisms following treatment with Met-RANTES, hence suggesting immunoregulatory roles for CCR1 and CCR5. Alternatively, it could simply be a cellular response to ongoing viral replication and increased antigen load in mice co-treated with Met-RANTES. However, we did not detect virus in the peritoneal cavity at day 6 after infection of either mouse infected with HSV-2 alone or after co-treatment with Met-RANTES (data not shown). Moreover, the literature and our results argue against prolonged presence of virus as responsible for the elevated production of pro-inflammatory mediators. First, IFN-α/β production was upregulated only on day 1 in mice given HSV-2 + Met-RANTES, contrary to a more sustained expression expected if the infection persisted in the peritoneum. Second, studies from this laboratory have shown that the capacity of HSV-2 to induce the production of IFN-α/β in PCs is independent of viral replication [57], thus arguing that not even viral antigen is present in the peritoneal cavity day 6 after infection. Finally, studies by other groups have indicated important immunoregulatory roles for CCR1 and CCR5 in infection with viral and nonviral pathogens [12, 56, 58, 59]. For instance, it was demonstrated that CCR5-deficient mice display enhanced macrophage infiltration, tissue damage and mortality after influenza virus infection, despite unchanged viral titres, hence strongly indicating a possible regulatory role for CCR5 [22]. Also, regulatory T cells express CCR5 and are recruited to inflamed areas by the ligands of this receptor to participate in the regulation of the immune response [60]. Therefore, we believe that blocking of CCR1 and CCR5, in addition to impeding central antiviral activities, uncouples important immunoregulatory processes and hence leads to impairment of some of the postinfectious control mechanisms.

In summary, we have shown that inflammatory CC chemokines are expressed during a generalized HSV-2 infection and contribute to antiviral defence. At the same time, expression of these chemokines may also play important parts in the eventual control of the pro-inflammatory response and hence ensure that such a response with a potent intrinsic capacity to cause tissue damage is down-modulated when the infection has been cleared.

Acknowledgments

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