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Expression of genes for cytokines and cytokine-related functions in leukocytes infected with Herpes simplex virus: comparison between resistant and susceptible mouse strains



Abstract

Cytokines and chemokines play an important role in the first line of defence against viral infections. Moreover, these groups of proteins also contribute

significantly to regulation of the acquired immune response. Therefore, knowledge of the expression of cytokines, chemokines and factors involved in their action may provide information about the immune reaction responsible for elimination of viral infections and for immune-mediated pathology. Using cDNA arrays, we have evaluated the expression of cytokines and genes related to cytokine function in resting murine peritoneal cells and in inflammatory macrophages infected with Herpes simplex virus (HSV)-1 and -2. To allow comparison, the experiments were performed using both the resistant mouse strain C57BL/6 and the susceptible strain BALB/c. The work identified a group of genes that is differentially expressed during HSV infection of cells from the two strains. Another group of genes was affected by HSV-1 but not HSV-2 infection and vice versa. Further analysis of these genes may provide new information about host defense against viral infections and could also lead to identification of the molecular basis for the pathological differences between infections with HSV-1 and -2.

Full text

Herpes simplex virus (HSV) is an enveloped DNA virus of the *Herpes viridae* family [1]. Two types of HSV exist, HSV-1 and HSV-2, which exhibit 83% homology in the protein-coding regions of the DNA [2], which share many biological functions [1]. The primary target cells are epithelial cells of mucocutaneous membranes. In these cells, the virus replicates efficiently and causes cell lysis. Following the initial replication at the site of entry, the virus gains access to the sensory neurons where it resides in a non-replicating state known as latency, from which the virus can be reactivated [3]. Although HSV-1 and HSV-2 can give rise to a highly overlapping spectrum of clinical symptoms, some differences are noticeable. For instance, genital HSV-1 infections are clinically less severe and reactivate with lower frequency than similar HSV-2 infections [4, 5]. Moreover, HSV-2, but not HSV-1, gives rise to a pronounced focal necrotizing hepatitis after intraperitoneal infection of mice [6].

The ability of the organism to mount an efficient immune response against HSV infections is dependent on both the innate and adaptive immune responses [7, 8]. The innate immune response to HSV infections serves a dual role. Firstly, it serves the purpose of controlling virus replication until the adaptive response develops. Secondly, it also plays a pivotal role in the process of T cell differentiation and activation. As for the adaptive immune response, it is now

well established that CD4⁺ Th1 cells are of particular importance in the elimination of HSV infections [8].

Our understanding of the functions of different leukocyte subtypes in the innate immune response to viral infections has grown considerably in recent years. Firstly, macrophages contribute to the early non-specific response, mainly through production of free radicals and cytokines [7, 9-11]. *In vivo* macrophage-depletion studies have shown that this cell type is also crucial for bridging between the innate and the adaptive immune system [12]. Secondly, natural killer cells contribute to the first line of defense against viral infections [13]. This function seems to be mediated largely through two activities, namely production of interferon (IFN)- α and cytotoxic activity against virus-infected cells [13]. Thirdly, the natural, IFN- α / β -producing cells, which appear to be responsible for a large pool of the early IFN- α / β , have now been identified as plasmacytoid immature dendritic cells (DC), which, following infection, differentiate into mature DCs [14]. Finally, $\gamma\delta$ T cells have been suggested to play an important role in the early host defense against many viruses, including HSV-1 [7, 15, 16]. This is mainly due to the ability of $\gamma\delta$ T cells to produce IFN- α at early stages of the infection.

The optimal antiviral function of the above-described innate leukocytes relies heavily on cytokine production and action, working in an auto- and paracrine manner [13, 16-19]. Cytokines and cytokine-induced genes are important for the ability of the organism to raise an antiviral response. Mice deficient in IFN- α or interleukin (IL)-6 are unable to control HSV-1 and HSV-2 infections and succumb at normally sub-lethal virus doses [20, 21]. Cytokines work through multiple mechanisms of action such as (i) direct antiviral activity through induction of various enzymes, (ii) activation and recruitment of other cells of the innate immune system, and (iii) stimulation of T cell differentiation and activation.

The invention of cDNA arrays has allowed investigators to examine expression of a very large number of genes at the same time. By this means Huang *et al.* compared gene expression in DCs after exposure to bacteria, fungi, and viruses and found that a set of core genes was expressed in response to all pathogens, while a subset of genes was regulated in a more pathogen-specific manner [22]. In this study, we have taken the cDNA array approach and analyzed the expression pattern of genes encoding cytokines and proteins related to cytokine action during the early stages of HSV-1 and HSV-2 infection in resting

murine PCs and inflammatory PCs, which are highly macrophage-enriched. This cell type has previously been shown to play an important role in resistance against HSV infections [7, 12], and knowledge of gene expression in macrophages during HSV infection is therefore of interest. Also, by comparing the relatively resistant mouse strain C57BL/6 with the relatively susceptible BALB/c strain, the results of the present work have identified proteins that potentially play important roles in the host response against HSV infections.

MATERIALS AND METHODS

Mice and cell cultures

The mice used in our experiments were specific pathogen-free, 7 to 8-week-old female C57BL/6 and BALB/c mice. The mice were from M&B and were kept in our animal facilities between delivery (at 4-6 weeks of age) and the time of the experiments. Resting peritoneal cells (PC) were harvested by lavage of the peritoneal cavity with cold PBS supplemented with 2% fetal calf serum (FCS, Hyclone) and 20 IU/ml of heparin (Leo Pharmaceuticals). The cells were washed once in RPMI 1640 medium with 5% FCS and antibiotics and seeded at a density of 7×10^6 cells per 10 ml in the same medium. The cells were left overnight before infection. Thioglycollate (TG)-elicited macrophages were induced by injection of 2 ml of 10% TG into the peritoneal cavity. Five days later PCs were harvested and seeded as described above.

Virus and infections

The viruses used in our experiments were the 17+ strain of HSV-1 and the MS strain of HSV-2. The virus stocks used were produced by infecting Vero cells with 0.01 p.f.u. per cell (or leaving the cells uninfected for generation of mock preparations), and incubation at 37° C until a complete cytopathic effect was observed. After three rounds of freezing (- 70° C) and thawing (room temperature), virus was separated from cell debris by centrifugation for 30 min at 4,500 x g (at 4° C). The virus was pelleted by ultracentrifugation at 4,500 x g for 1 hour. Re-suspension was done in PBS, supplemented with 0.1% BSA and sonication for 30 sec, three times at 40 W. The virus was quantified by plaque titration in Vero cells and stored at - 70° C. The murine PCs were infected at a multiplicity of infection (m.o.i.) of 1.

Isolation of total RNA

After the appropriate period of infection, cells were lysed in Trizol (Life Technologies) and total RNA was isolated following the recommendations of the manufacturer. To eliminate possible trace amounts of genomic DNA, the RNA was treated with DNase I (Stratagene) for 90 min at 25° C. The amount of DNase used was 1 unit per 1 µg RNA. Subsequently, the RNA was isolated by two rounds of extraction with acidic phenol, one extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and two extractions with chloroform:isoamyl alcohol (24:1). To precipitate the RNA, 0.1 volume of 3 µM sodium acetate, pH 5.2 and 3 volumes of 96% ethanol was added, mixed and left overnight at - 2° C. The tubes were centrifuged at 15,000 x g for 30 min (at 4° C). The pellet was washed in 80% ethanol, dried, and redissolved in water containing 0.1% DEPC. The RNA was quantified by OD measurement at 260 nm.

Radioactive labelling of cytokine-related RNAs

Total RNA was labelled using the cDNA Labeling and Hybridisation Kit from R&D Systems and [³²P]-dATP (Hartmann Analytics), following the instruction provided with the kit. Briefly, 1-2 µg of total RNA was mixed with 4 µl of murine cytokine-specific primers (R&D Systems) and heated to 90° C for 2 min, followed by slow cooling to 42° C. The remaining components were added, mixed, and incubated for 2.5 hours at 42° C. The labelled cDNA was separated from unincorporated radioactivity by centrifugation through Sephadex G-25 spin-columns (Roche).

cDNA array analysis

The cDNA arrays (Mouse Cytokine Expression Array, R&D Systems) were rinsed in 0.2 x SSPE and prehybridized for 2 hours at 65° C in hybridisation buffer (5 x SSPE, 2% SDS, 5 x Denhardt's reagent, 100 µg/ml Salmon testes DNA). The hybridisation solution was made by adding the labeled cDNA to 5 ml hybridisation buffer which was heated for 10 min at 90° C. The pre-hybridisation solution was discarded and exchanged with the hybridisation solution. Hybridisation was allowed to occur for 16-20 hours at 65° C. Unhybridized cDNA was washed off the array by 2 x 20 min washes with 0.5 x SSPE, 2% SDS and 2 x 20 min washes with 0.1 x SSPE, 2% SDS. All washing steps were carried out at 65°

C with 150 ml washing buffer used per wash. The arrays were sealed in plastic bags and subjected to autoradiography.

Analysis of results

Hybridisation intensities were determined by densitometric measurement of the X-ray films after the array was aligned to the grid reference template to determine the target localization for each of the duplicate 514 murine cDNAs, 8 housekeeping genes, and 6 negative controls. The background was determined individually for each gene and subtracted from the duplicate spots to yield the average hybridisation intensity. Data were discarded when significant deviations between duplicate experiments were found, or when the signal intensity was not above the background level. For comparison between arrays, hybridization intensities were normalized to the housekeeping genes prior to comparison between samples from HSV and mock-treated cells. Only those genes which reproducibly showed at least threefold upregulation or downregulation were considered differentially expressed.

RT-PCR

Two µg of RNA were subjected to reverse transcription using oligo(dT)₁₅ and Expand Reverse Transcriptase (both from Roche). The cDNA was amplified by 30 cycles of PCR with the following primers: MCP-1: 5'-ACA AGA GGA TCA CCA GCA GCA G-3' (sense), 5'-AAG GCA TCA CAG TCC GAG TCA C-3' (antisense); β-actin: 5'-CCA ACC GTG AAA AGA TGA CC-3' (sense), 5'-GCA GTA ATC TCC TTC TGC ATC C-3' (anti-sense). The products spanned 319 bp (MCP-1) and 616 bp (β-actin), respectively.

ELISA

Murine IL-12 p40 and RANTES were detected by ELISA. Maxisorp plates were coated overnight at 4° C with primary antibody (6 µg/ml anti-IL-12 p40 (BD Pharmingen) and 0.5 µg/ml anti-RANTES (Peprotech)) in coating buffer (15 mM Na₂CO₃; 35 mM NaHCO₃; 0.2% sodium azide, pH 9.6). After blocking for 2 hours at 37° C with PBS pH 7.4 containing 1% w/v BSA, samples and standard dilutions of cytokines (concentration range: 3.9-2,000 pg/ml for IL-12 p40 and 100-6,000 pg/ml for RANTES) were added to the wells and the plates were incubated at 37° C for 1 hour or overnight at 4° C. Subsequently, the wells were incubated for 1

hour at 37° C with a biotin-labelled detection-antibody (2 µg/ml anti-IL-12 p40 (BD Pharmingen) and 0.25 µg/ml anti-RANTES (Peprotech)) in blocking buffer. Finally, HRP-conjugated streptavidin, diluted in blocking buffer, was added and incubated for 20 min at room temperature and the result was visualized by the TMB system (R&D Systems). After 10 min, the colour reaction was stopped with 5% H₂SO₄. Between each step the plates were washed 3 times with PBS containing 0.05% v/v Tween 20. The results were quantified by reading at 450 nm.

Griess assay

PCs were infected with HSV-1 or -2 and treated with IFN-* (10 IU/ml) (BD Pharmingen). Supernatants were harvested 48 hours after infection/stimulation and assayed for NOS activity by the Griess assay. Briefly, 100 µl culture supernatant was mixed with 100 µl Griess reagent (equal volumes of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% p-aminobenzene sulfanilamide (both from Sigma)), in a 96-well microtiter plate. After 10 min incubation at room temperature, the absorbance was measured at 540 nm. Two fold dilutions of sodium nitrite in the same medium as the experimental samples were used to generate a standard curve.

Reproducibility of data

Experiments were performed twice with a high degree of reproducibility. Occasional unreproducible results (*i.e.* genes induced in one experiment and repressed in the second and *vice versa*) are not presented.

Statistical analysis

Statistical significance was estimated with Student's t-test for unpaired observations. Results from repeated experiments were pooled and used for the calculations in order to obtain the largest number of observations available for evaluation of statistical significance. A p-value of < 0.05 was considered significant.

RESULTS

To examine the expression of the genes on the cDNA arrays, resting and TG-elicited mouse PCs were left overnight to settle and were then infected with HSV-1 or HSV-2 at an m.o.i. of 1. After 24 hours of infection, total RNA was harvested and analysed on the arrays. The data are presented as fold induction/repression relative to mock infection. We used a 3 fold induction/repression as the cut-off limit. Only gene expression levels reproducibly found to be changed more than 3 fold by HSV infection are presented. The lowest degree of induction/repression found in two independent experiments is shown. The results therefore represent a conservative estimate of how HSV-1 and -2 infection affects expression of cytokine genes and genes related to cytokine function in resting and inflammatory leukocytes.

The cDNA array used in this study was the commercially available Mouse Cytokine Expression Array. This array allows detection of 514 genes encoding cytokines, chemokines, and proteins related to their functions, including receptors, adhesion molecules, integrins, tumour necrosis factor (TNF) superfamily members, signal transduction proteins, and proteases. For a full list of all the cDNA species on the array, visit the website <http://www.rndsystems.com>.

Expression of cytokine genes and genes related to cytokine function in resting PCs from C57BL/6 and BALB/c mice after HSV infection

The C57BL/6 mouse strain is relatively resistant to HSV infection, whereas the BALB/c strain is susceptible [23]. In order to identify host factors that potentially contribute to this phenomenon, we compared the expression of cytokines and genes related to cytokine function in resting PCs from these two mouse strains after HSV infection. Moreover, with the aim of characterizing the small, yet clinically significant, differences between HSV-1 and HSV-2 infections we also compared the host response to the two HSV types.

Prior to the cDNA array analysis, the composition of the PC population was characterized by Giemsa staining. We found that macrophages constituted 76% of the cell population, while granulocytes and lymphocytes contributed with 4 and 20%, respectively. Moreover, the cells were indeed infected after exposure to HSV as demonstrated by detection of immediate-early viral gene transcription by RT-PCR (data not shown). However, the virus replicated poorly in the resting

PCs, since only 1.1×10^3 p.f.u of virus were recovered from the cell culture supernatants 24 h after infection with 1×10^6 p.f.u.

Figure 1 shows the effect of HSV-1 and -2 infection on gene expression in resting PCs from C57BL/6 mice. Among the 514 genes analysed, only 14 were altered more than 3-fold by HSV-1 infection. By contrast, HSV-2 infection of resting PCs from C57BL/6 mice altered expression of 36 genes by more than 3-fold. Among the genes induced/repressed by both HSV-1 and -2 (see *Table 1*) were the CC chemokine RANTES/CCL5 and the TNF receptor II (TNFRSF 1B). The induction of RANTES expression was confirmed by ELISA (Figure 2a). A large group of genes was induced or repressed more than 3-fold by HSV-2 infection but not by HSV-1 infection. However, HSV-1 did, in most cases, affect expression levels of these genes, but not beyond the 3-fold threshold. This emphasises the higher stimulatory potency of HSV-2 than HSV-1. A few genes induced or repressed by HSV-2 infection were not affected at all by HSV-1 and *vice versa*. For instance, nitric oxide synthase type 2 (NOS²/iNOS) was expressed at high levels in HSV-2-infected but not HSV-1-infected PCs. This was confirmed by a NOS activity assay (Figure 2b), which also illustrates that, in the presence of IFN-*, both viruses stimulated NOS activity to the same extent.

We next examined how HSV-1 and -2 infection of PCs from BALB/c mice affected the expression of genes on the arrays. In parallel with what was found with PCs from C57BL/6, HSV-2 was a stronger stimulator of gene induction/repression than HSV-1 in cells from BALB/c mice (Figure 3). However, the difference between the two HSV types was not as large as that which was seen in C57BL/6 mice. While HSV-1 infection led to 3-fold induction/repression of 18 genes, HSV-2 infection affected the expression of 22 genes. Many of the genes that were induced/repressed in HSV-infected cells from C57BL/6 mice were affected in the same manner after HSV infection in resting PCs from BALB/c (*Table 1*). A noticeably large portion of the TNF superfamily genes was similarly regulated in HSV-infected PCs from the two mouse strains. However, a few genes were differentially regulated between C57BL/6- and BALB/c-derived PCs. As an example hereof, PCs from C57BL/6 mice up-regulated expression of RANTES/CCL5, whereas PCs from BALB/c mice did not (Figure 2a).

Kinetics of gene expression in HSV-infected, resting PCs from BALB/c mice

The results shown above in *Table 1* were obtained for RNA from cells infected for 24 hours. Since induction/repression of some genes may have peaked at earlier time points, we decided to do a time-course experiment to follow how expression of the genes on the cDNA array developed during the first 24 hours after HSV-2 infection. We chose to do the experiment with resting PCs from BALB/c mice. The results are presented in Table 2. With very few exceptions, the genes affected after 24 hours of HSV-2 infection are identical to those presented in *Table 1*. Most mRNA species found to be affected after 24 hours of infection, started to accumulate/decline after 8 hours. For all of these genes, the maximal effect was observed after 16 or 24 hours of infection. Two notable exceptions are apparent, namely TNF- α and IFN- β . They were induced within the first 3 hours of infection, peaked soon after, and were down-modulated to basal levels after 24 hours of infection. These results therefore show that assessment of gene expression 24 hours post-infection allows detection of most HSV-regulated genes in resting PCs. However, the induction, expression, and down-modulation of a small subset of genes, is already complete within 24 hours.

Expression of genes for cytokines and cytokine-related functions in HSV-infected inflammatory leukocytes from C57BL/6 and BALB/c mice

The experiments presented above were performed on populations of resting PCs. For comparison, we also examined how HSV-1 and -2 infection affected gene expression in PCs elicited by TG (TG-PCs). TG injection into the peritoneum elicits an inflammatory response characterized by an initial influx of neutrophils followed by recruitment of macrophages, which phagocytose the apoptotic neutrophils [24]. When harvested 5 days after TG injection, the PC population consisted of 89% macrophages, 6% granulocytes, and 5% lymphocytes.

When comparing the response of TG-PCs from C57BL/6 and BALB/c mice to HSV infection, we observed that the difference between the two mouse strains is much less apparent than after HSV infection of resting PCs (compare *Table 3* with *Table 1*). Many of the genes displaying differential expression in HSV- versus mock-treated resting PCs behaved in the same way in TG-elicited PCs. However, another set of genes, which was not affected by HSV infection in resting PCs, was induced/suppressed in TG-elicited PCs. This group contains IL-11, IL-12 p40, monocyte chemoattractant protein-1 (MCP-1), and caspase-1, which cleaves pro-IL-1 β and pro-IL-18 to generate bioactive cytokines [25]. The induction of

expression of IL-12 p40 and MCP-1 after HSV infection of TG-PCs, but not resting PCs, was confirmed by ELISA and RT-PCR, respectively (Figure 2c and d).

Finally we found that viral infection of TG-elicited PCs did not alter the expression of a subset of genes that was affected by HSV infection of resting PCs. For instance, expression of the TNF superfamily members THANK and OX40 was not changed after HSV-2 infection in TG-PCs, which is in contrast to that which is observed after HSV-2 infection of resting PCs.

DISCUSSION

In the present study, we have examined the expression of genes encoding cytokines and proteins related to cytokine function during HSV-1 and -2 infection of resting leukocytes and TG-elicited macrophages from mice. We examined cell populations from both the relatively resistant C57BL/6 mouse strain and the relatively susceptible strain BALB/c. The key findings of this study are discussed below.

First, a number of genes are induced in both resting and TG-elicited PCs from both mouse strains. These include proteins previously reported to support antiviral defense, such as IL-15, IL-18, and STAT1 [21, 26, 27] as well as proteins yet to be studied in the context of viral infections (*e.g.* integrin $\beta 5$). It is interesting to note that HSV-infected leukocytes upregulate the expression of STAT1, thus enhancing the responsiveness to IFN-*, which starts accumulating 2 days after infection [28].

An important conclusion from this work is that resting PCs from C57BL/6 mice react with a more vigorous proinflammatory response to HSV infections than do resting PCs from BALB/c mice. We have identified specific genes that are induced/repressed differentially in the two mouse strains after HSV infection. Some of these may be important for the establishment of an efficient immune response. For instance, the CC chemokine RANTES/CCL5 was induced in PCs from C57BL/6 mice but not from BALB/c mice. This may imply a role for RANTES in the host response against HSV infections. At least two roles for RANTES are possible. First, the chemokine could stimulate the immune response by attracting leukocytes to the infected area. Second, RANTES has been reported to directly promote Th1 differentiation, and through this route support the antiviral defense [29]. As to the cellular source of RANTES, results from this

laboratory have shown that macrophages produce large amounts of this chemokine after HSV infection [30].

A third finding of this study is that HSV-2 infection generally affected expression of cytokines and cytokine-related genes more than HSV-1 infection (see Table 4 for comparison of gene expression in cells infected with HSV-1 and HSV-2). This phenomenon was not restricted to the two virus strains used in the array experiments since four HSV-1 strains (17+, KOS, F, HFEM) all stimulated expression of RANTES, IL-12p40 and NOS² less potently than the two HSV-2 strains MS and 333 (data not shown). The subset of genes differentially regulated by HSV-1 and -2 infections may provide important information about the subtle differences between the pathogenesis of some HSV-1 and -2 infections [4-6]. The observation that HSV-2, but not HSV-1, induced NOS₂ expression in resting PCs from C57BL/6 mice is interesting. In the presence of large amounts of IFN-*, however, this and previous studies have shown that both types of HSV promote NOS2 activity [31]. Together with reports that NOS2 plays a role in neurological damage [32] and that the neurological sequelae are much more severe after neonatal HSV-2 than HSV-1 infection [33], our finding may suggest a role for NO in this observed difference in pathogenesis.

A fourth group of genes was found not to be affected by HSV infection in resting PCs, but to be induced or repressed in TG-elicited PCs. This latter cell population is highly enriched in inflammatory macrophages [24]. IL-12 p40 represents one such gene. Macrophages have previously been shown to produce large amounts of this subunit of IL-12 and IL-23 after HSV-2 infection [34], and there is ample evidence supporting a beneficial role of IL-12 p40 in clearance of HSV-1 and -2 infections [21, 35, 36].

When examining the kinetics of expression of the genes represented on the cDNA array, we found that they fall into two classes. The first class of genes, consisting only of TNF-* and IFN-β, was induced within 3 hours of infection, peaked early, and returned to basal levels of expression after 24 hours. Genes belonging to this group are induced by mechanisms involving viral glycoproteins and virus-induced events occurring immediately after viral entry into the cell [11]. For the second class of genes, expression was affected by HSV after 8 hours of infection and throughout the 24 hours of the experiment. This group included all the remaining HSV-regulated genes on the array. There are at least two possible reasons why expression of these genes is affected with a delayed

kinetics relative to the first class. Firstly, it is possible that the viral replication cycle has to reach a certain stage before induction/repression is initiated, as previously shown for induction of RANTES expression [30]. Secondly, expression of some of the genes in this group is regulated by genes from the first group of HSV-regulated genes. For instance, HSV-2-induced expression of NOS²/iNOS in macrophages is dependent on autocrine expression of TNF-* [37].

In this work the peritoneal cell populations were not sub-fractionated and we therefore do not know which cell types are responsible for expression of the mRNA species found to be differentially expressed after HSV infection. Moreover, with the virus concentration used in this study, some cells remain uninfected. Therefore, we still have to learn how this cell population contributes to expression of proinflammatory genes after HSV infection.

CONCLUSION

In conclusion, this work has identified a number of genes encoding cytokines and proteins related to cytokine function that are induced or repressed after HSV infection of leukocytes. Further studies are required to identify their roles in clearance of HSV infections and in viral pathogenesis and pathology.

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Figures

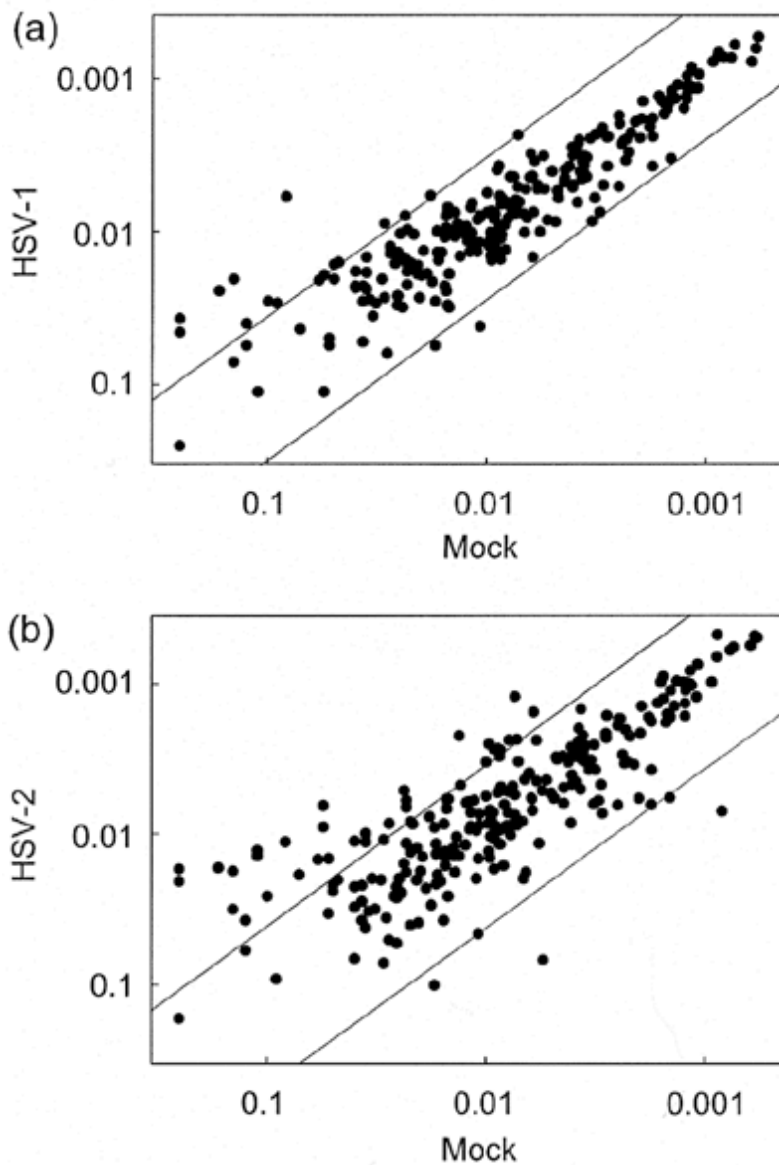


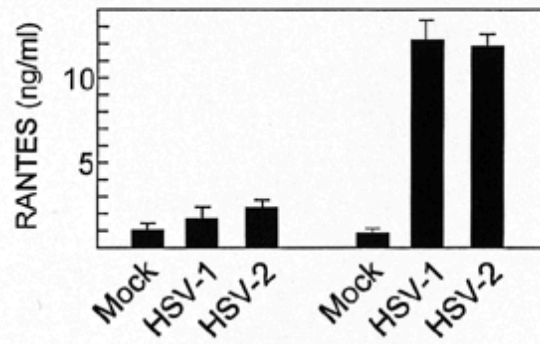
Figure 1

Effect of infection with HSV-1 and -2 on the expression of genes encoding cytokines and proteins related to cytokine function in resting PCs from C57BL/6 mice.

The relative levels of expression of the genes represented on the cDNA array were quantified and plotted as shown. The diagonal lines in the graphs represent 3-fold changes in expression levels relative to mock infection. **a:** HSV-1 infection *versus* mock infection. **b:** HSV-2 infection *versus* mock infection.

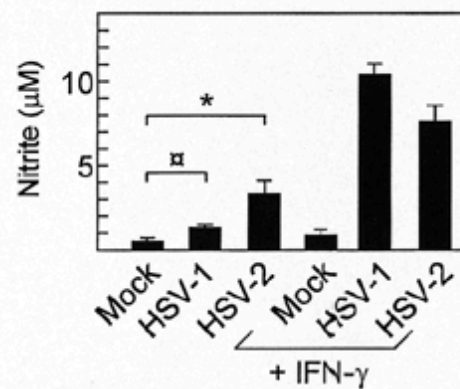
Mouse strain (rPCs): BALB/c C57BL/6

(a)



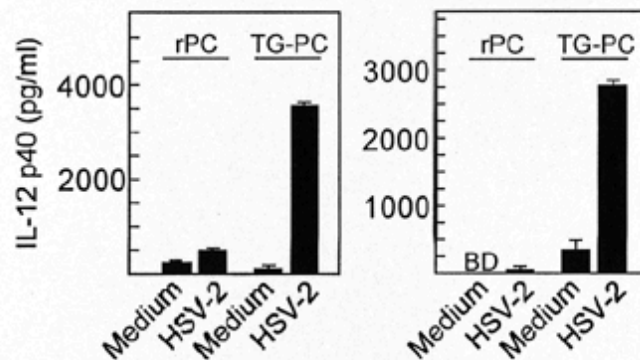
Mouse strain (rPCs): C57BL/6

(b)



Mouse strain: BALB/c C57BL/6

(c)



(d)

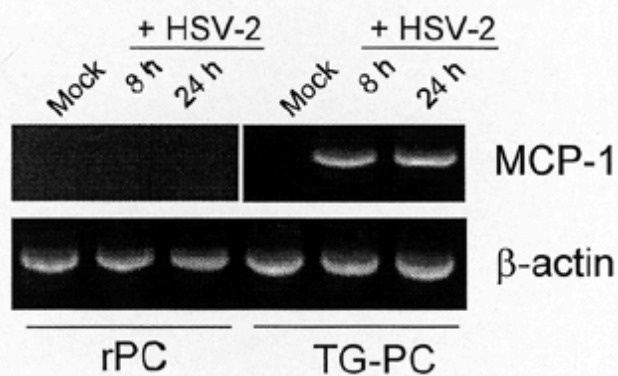


Figure 2

Production of RANTES, nitric oxide, IL-12p40, and MCP-1 during HSV infection of peritoneal cells.

a: Resting PCs from BALB/c and C57BL/6 mice were infected with HSV-1 or -2 (m.o.i. 1) or treated with mock preparation. Twenty-four hours later, supernatants were harvested and analysed for the presence of RANTES protein by ELISA. Results are shown as means of triplet cultures $\pm$ SEM. **b:** Resting PCs from C57BL/6 mice were stimulated with IFN-* (10 IU/ml) and infected with HSV-1 or -2 (m.o.i. 1) or treated with mock preparation as indicated. Forty-eight hours later, supernatants were harvested and analysed for NOS activity by the Griess assay. Results are shown as means of triplet cultures ($\pm$ SEM. * $p < 0.05$; o, $p > 0.05$. **c:** PCs from untreated or TG-treated BALB/c and C57BL/6 mice were infected with HSV-2 (m.o.i. 1) and incubated for 24 hours. Supernatants were harvested and IL-12 p40 content was quantified by ELISA. Results are shown as means of four cultures $\pm$ SEM. BD, below detection limit. **d:** PCs from untreated or TG-treated C57BL/6 mice were mock-treated or infected with HSV-2 (m.o.i. 1). After the indicated times of infection, total RNA was harvested and MCP-1 and β -actin was measured by RT-PCR.

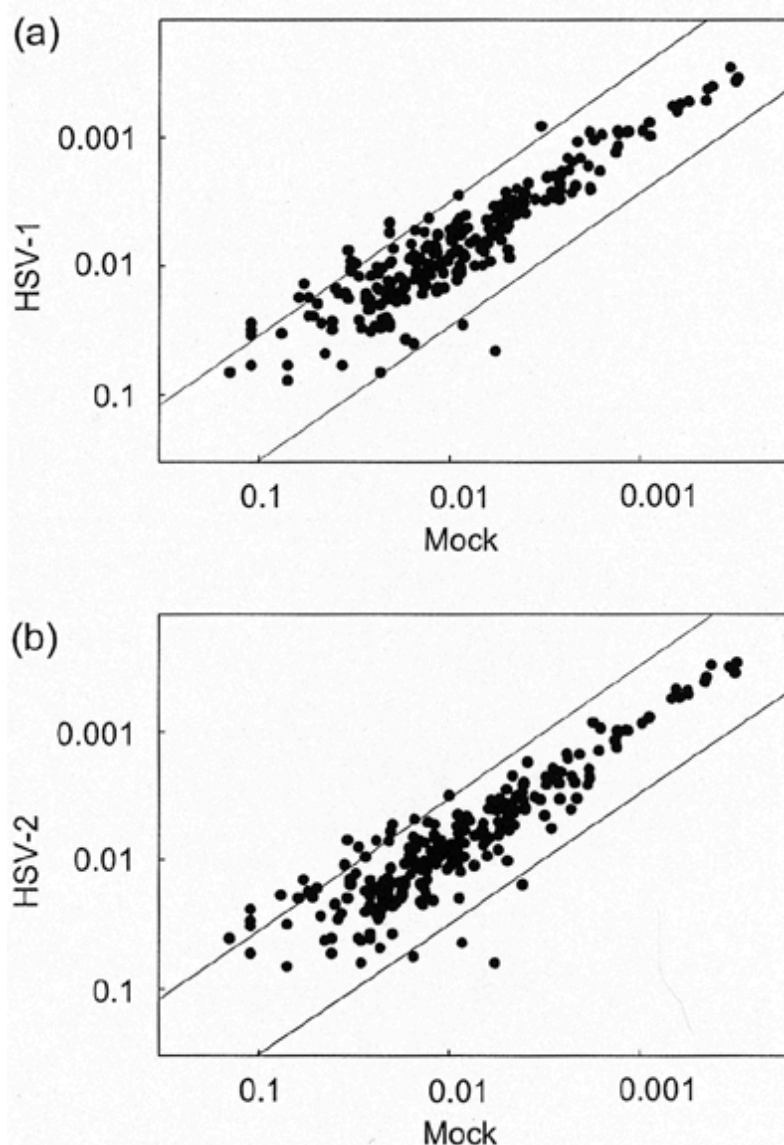


Figure 3

Effect of infection with HSV-1 and -2 on the expression of genes encoding cytokines and proteins related to cytokine function in resting PCs from BALB/c mice.

The relative levels of expression of the genes represented on the cDNA array were quantified and plotted as shown. The diagonal lines in the graphs represent 3-fold changes in expression levels relative to mock infection. **a:** HSV-1 infection *versus* mock infection. **b:** HSV-2 infection *versus* mock infection.

Table 1
Effect of HSV-1 and -2 infection on gene expression in resting peritoneal cells from C57BL/6 and BALB/c mice^a

Gene group	Gene name	Induction ratio (HSV/mock) ^b C57BL/6		Induction ratio (HSV/mock) BALB/c	
		HSV-1	HSV-2	HSV-1	HSV-2
Cytokines	Oncostatin M	6.75	1.80	2.72	1.73
	PDCF- β	1.00	8.02	1.31	1.67
	PDCF- α	0.99	1.03	4.56	1.08
	Osteopontin	0.56	0.73	0.13	0.09
	IL-15	1.19	3.38	1.03	1.26
	IL-16	1.85	3.88	2.41	3.46
Cytokine receptors	IL-18	1.79	3.03	2.55	3.05
	IL-1 receptor 1	1.75	3.95	0.92	1.03
	IL-1 receptor antagonist	2.37	3.28	1.00	1.34
	IL-2R γ	1.29	3.02	1.12	1.23
	IL-6R α	2.00	4.57	2.34	1.80
	IL-18R	2.42	3.60	1.67	2.89
Chemokines	RANTES/CCL5	7.85	9.53	1.39	1.42
	BCA-1/CXCL13	7.04	8.08	0.99	1.23
	Eotaxin/CCL11	BD ^c	BD	0.39	0.28
	MCP-1/CCL2	BD	BD	0.92	0.27
Chemokine receptors	D6	0.47	0.28	0.75	0.89
	CCR8	0.26	1.24	1.34	1.17
TNF superfamily	TRAIL/TNFRSF10	2.50	4.09	2.14	3.57
	THANK/TNFRSF13B	0.92	0.23	4.00	4.66
	TNFR1/TNFRSF1A	1.67	2.15	3.67	2.17
	TNFR2/TNFRSF1B	5.50	12.25	2.31	4.15
	LTR β /TNFRSF3	2.88	6.22	2.21	3.29
	OX40/TNFRSF4	1.52	6.04	1.95	3.22
	Fas/TNFRSF6	1.05	4.30	2.11	3.77
	RANK/TNFRSF6	2.12	1.45	3.07	2.23
TGF- β superfamily	BMP-3	1.15	3.33	3.81	2.23
	BMP-4	0.50	8.61	1.02	2.67
	ALK-1	1.67	1.82	3.23	2.40
	Inhibin A	1.00	8.66	0.67	1.21
Adhesion proteins	VCAM-1	3.07	2.38	2.67	2.21
	VAP-1	3.05	2.30	0.89	1.05
Binding proteins	IGF binding protein 2	0.31	0.17	0.78	1.02
	Hip	2.89	1.68	3.56	3.18
Cell surface proteins	WIF-1	1.42	3.91	2.76	3.80
	Toll-like receptor 2	2.57	3.59	1.48	2.84
	Calectin-9	1.38	0.95	4.29	4.18
Signal transduction	Jak2	3.43	4.85	2.15	2.79
	SMAD3	1.42	3.09	2.51	4.18
	SMAD4	2.13	1.59	2.22	3.81
	STAT1	3.50	3.90	3.22	3.44
Developmental factors	SLIT-1	3.10	0.78	4.05	0.99
EGF family	ErbB2	3.13	2.38	3.25	2.05
Eph family	Ephrin-B2	2.50	3.38	2.28	3.22
FGF family	FGF receptor 1	1.64	3.58	1.58	2.41
	FGF-1	1.14	1.04	3.28	2.47
Integrins	Integrin- α 5	4.07	18.07	3.17	3.11
	Integrin- β 2	1.49	4.64	0.30	0.20
	Integrin- β 4	0.97	5.54	1.16	1.23
Neurotrophic group	CNTF	3.32	2.71	3.32	2.71
	Neurturin	2.51	1.83	2.93	3.70
NO metabolism	NOS γ /iNOS	0.98	6.11	1.00	2.01
Proteases and related factors	BMP-1	2.40	3.51	4.59	4.93
Weight regulation	Leptin receptor	1.30	3.14	1.19	2.15
NPY Y5R		1.92	3.18	3.56	2.11

^a Resting peritoneal cells were treated with mock preparation or infected with HSV-1 or 2 at a m.o.i. of 1. Twenty-four hours later, total RNA was isolated, cleared for potential DNA contamination and analysed for the presence of specific mRNAs using cDNA arrays.

^b Normalized mRNA levels from infected cells were compared against the levels in mock-treated cells and -fold induction was calculated. The figures are shown as mRNA levels in infected cells relative to mock-infected.

^c BD, below detection limit after 30 days of exposure.

Table 2
Kinetics of expression of genes encoding cytokines and proteins related to cytokine function after HSV-2 infection in resting peritoneal cells from BALB/c mice^a

Induction ratio (HSV-2/mock) ^b					
Gene group	Gene name	3 h	8 h	16 h	24 h
Cytokines	Osteopontin	1.17	0.89	0.20	0.15
	IFN- β	2.40	3.21	2.03	1.42
	IL-6	1.07	1.57	3.01	2.75
	IL-16	0.74	2.03	2.71	3.41
	IL-18	1.42	1.73	3.79	2.83
Cytokine receptors	IL-6R α	0.99	1.31	3.44	2.41
Chemokines	Eotaxin/CCL11	1.21	0.59	0.44	0.29
	MCP-1/CCL2	1.05	0.70	0.73	0.33
TNF superfamily	TNF- α /TNFSF2	4.03	2.41	1.07	0.70
	TRAIL/TNFSF10	0.89	1.07	3.50	4.03
	THANK/TNFSF13B	0.89	4.21	4.89	4.56
	TNFR2/TNFRSF1B	1.21	2.97	3.21	4.06
	LTR β /TNFRSF3	1.01	2.19	3.42	3.31
	OX40/TNFRSF4	0.79	2.93	4.01	4.40
	Fas/TNFRSF6	1.49	2.90	3.30	3.04
	RANK/TNFRSF11A	0.63	2.44	3.04	2.70
TGF- β superfamily	BMP-3	1.22	2.60	3.11	3.02
Binding proteins	WIF-1	1.07	2.70	3.56	3.65
Cell surface proteins	Callectin-9	1.90	2.79	4.00	4.73
Signal transduction	SMAD3	0.99	3.91	3.79	4.22
	SMAD4	2.07	3.46	3.73	3.69
	STAT1	1.31	1.75	3.51	3.47
Eph family	Ephrin-B2	1.13	2.91	3.71	3.97
Integrins	Integrin- α 5	0.99	2.03	4.07	4.19
	Integrin- β 2	1.23	0.73	0.19	0.24
Neurotrophic group	CNTF	0.91	1.99	3.14	2.79
	Neurturin	1.33	2.79	3.11	4.03
Proteases and related factors	BMP-1	1.14	3.33	5.01	4.83

^a Resting peritoneal cells were treated with mock preparation or infected with HSV-2 at a m.o.i. of 1. After the indicated times of infection, total RNA was isolated, cleared for potential DNA contamination and analysed for the presence of specific mRNAs using cDNA arrays.

^b Normalized mRNA levels from infected cells were compared against the levels in mock-treated cells and -fold induction was calculated. The figures are shown as mRNA levels in infected cells relative to mock-infected.

Table 3
Effect of HSV-1 and -2 infection on gene expression in TG-elicited peritoneal cells from BALB/c and C57BL/6 mice^a

Gene group	Gene name	Induction ratio (HSV /mock) ^b		Induction ratio (HSV /mock)	
		C57BL/6		BALB/c	
		HSV-1	HSV-2	HSV-1	HSV-2
Cytokines	PDCF- α	3.07	1.53	4.17	2.41
	IL-1 α	3.50	3.75	3.91	5.34
	IL-11	3.13	4.20	5.91	6.06
	IL-12 p40	3.40	4.20	6.53	7.02
	IL-15	1.25	5.25	0.79	1.16
	IL-16	1.33	3.78	1.93	4.72
	IL-18	1.20	3.60	1.43	4.29
Cytokine receptors	MCSF R	3.14	1.66	3.92	2.43
	IL-1 receptor antagonist	3.22	7.00	4.01	3.37
	IL-2R γ	5.20	3.20	2.03	3.37
Chemokines	RANTES/CCL5	4.20	7.60	1.87	1.91
	MCP-1/CCL2	9.50	12.00	3.20	3.71
Chemokine receptors	CCR8	0.90	3.10	1.03	1.38
TNF superfamily	TRAIL/TNFRSF10	3.04	4.18	4.20	4.16
	TNFR1/TNFRSF1B	4.30	4.13	2.89	3.19
	LTRb/TNFRSF3	3.25	5.15	2.93	4.01
	Fas/TNFRSF6	2.61	4.03	2.04	3.29
Apoptosis-related proteins	Caspase-1	4.00	5.50	4.31	4.40
Cell surface proteins	Callectin-9	4.57	4.42	3.05	3.73
Signal transduction	STAT1	3.17	3.50	4.23	3.91
Developmental factors	SLIT-1	9.57	1.71	5.03	1.01
	WNT-5a	3.00	3.88	2.71	1.30
FGF family	FGF-1	1.78	3.00	1.01	1.05
Integrins	Integrin- α 5	3.60	4.20	4.49	5.71
	Integrin- β 2	0.32	0.32	0.21	0.17
NO metabolism	NOS ³ /iNOS	0.89	3.83	0.99	3.11
	PIN	3.40	3.80	1.30	2.71
Proteases and related factors	MMP-12	0.17	0.15	0.31	0.22

^a Resting peritoneal cells were treated with mock preparation or infected with HSV-1 or 2 at a m.o.i. of 1. Twenty-four hours later, total RNA was isolated, cleared for potential DNA contamination and analysed for the presence of specific mRNAs using cDNA arrays.

^b Normalized mRNA levels from infected cells were compared against the levels in mock-treated cells and -fold induction was calculated. The figures are shown as mRNA levels in infected cells relative to mock-infected.

Table 4
Comparison of gene expression in cells infected with HSV-1 versus HSV-2

Classes of HSV-regulated genes	Mouse strain (Cell population)	
Genes induced by both HSV-1 and HSV-2		
Cytokines	IL-1 α	C* (TG-PC), B (TG-PC)
	IL-1 receptor antagonist	C (TG-PC), B (TG-PC)
	IL-11	C (TG-PC), B (TG-PC)
	IL-12p40	C (TG-PC), B (TG-PC)
	IL-2R γ	C (TG-PC)
Cytokine receptors	MCP-1/CCL2	C (TG-PC), B (TG-PC)
Chemokines	RANTES/CCL5	C (iPC, TG-PC)
	BCA-1/CXCL13	C (iPC)
TNF superfamily	TRAIL/TNFSF10	C (TG-PC), B (TG-PC)
	THANK/TNFSF13B	B (iPC)
	TNFR2/TNFRSF1B	C (iPC, TG-PC)
	LTR β /TNFRSF3	C (TG-PC)
Apoptosis-related	Caspase-1	C (TG-PC), B (TG-PC)
Signal transduction	Jak2	C (iPC)
	STAT1	C (iPC, TG-PC), B (iPC, TG-PC)
Integrins	Integrin- α 5	C (iPC), B (iPC, TG-PC)
Binding proteins	Hip	B (iPC)
	WNT-5a	C (TG-PC)
Cell surface proteins	Calectin-9	C (TG-PC), B (iPC, TG-PC)
NO metabolism	PIN	C (TG-PC)
Proteases	BMP-1	B (iPC)
Genes suppressed by both HSV-1 and HSV-2		
Cytokines	Osteopontin	B (iPC)
Integrins	Integrin- β 2	C (TG-PC), B (iPC, TG-PC)
Binding proteins	IGF binding protein-2	C (iPC)
Proteases	MMP-12	C (TG-PC), B (TG-PC)
Genes induced by HSV-1 but not HSV-2		
Cytokines	Oncostatin M	C (iPC)
	PDGF- α	C (TG-PC), B (iPC, TG-PC)
Cytokine receptors	MCSF R	C (TG-PC), B (TG-PC)
TNF superfamily	TNFR1/TNFRSF1A	B (iPC)
	RANK/TNFRSF1A	B (iPC)
TGF- β superfamily	BMP-3	B (iPC)
	ALK-1	B (iPC)
Adhesion proteins	VCAM-1	C (iPC)
	VAP-1	C (iPC)
Developmental factors	SLIT-1	C (iPC, TG-PC), B (iPC, TG-PC)
FGF family	FGF-1	B (iPC)
EGF family	ErbB2	C (iPC), B (iPC)
Neurotrophic group	CNTF	C (iPC), B (iPC)
Weight regulation	NPY Y5R	B (iPC)
Genes induced by HSV-2 but not HSV-1		
Cytokines	PDGF- α	C (iPC)
	IL-15	C (iPC, TG-PC)
	IL-16	C (iPC, TG-PC), B (iPC, TG-PC)
	IL-18	C (iPC, TG-PC), B (iPC, TG-PC)
	IL-1 receptor 1	C (iPC)
	IL-1 receptor antagonist	C (iPC)
Cytokine receptors	IL-2R γ	C (iPC), B (TG-PC)
	IL-6R α	C (iPC)
	IL-18R	C (iPC)
Chemokine receptors	CCR8	C (TG-PC)
TNF superfamily	TRAIL/TNFSF10	C (iPC), B (iPC)
	TNFR2/TNFRSF1B	B (iPC, TG-PC)
	LTR β /TNFRSF3	C (iPC), B (iPC, TG-PC)
	OX40/TNFRSF4	C (iPC), B (iPC)
	Fas/TNFRSF6	C (iPC, TG-PC), B (iPC, TG-PC)
TGF- β superfamily	BMP-3	C (iPC)
	BMP-4	C (iPC)
	Inhibin A	C (iPC)
Cell surface proteins	Toll-like receptor 2	C (iPC)
Binding proteins	WIF-1	C (iPC), B (iPC)
Signal transduction	SMAD3	C (iPC), B (iPC)
	SMAD4	B (iPC)
Eph family	Ephrin-B2	C (iPC), B (iPC)
FGF family	FGF receptor 1	C (iPC)
	FGF-1	C (TG-PC)
Integrins	Integrin- β 2	C (iPC)
	Integrin- β 4	C (iPC)
Neurotrophic group	Neurturin	B (iPC)
NO metabolism	NOS ^{2} /iNOS	C (iPC, TG-PC), B (TG-PC)
Proteases	BMP-1	C (iPC)
Weight regulation	Leptin receptor	C (iPC)
	NPY Y5R	C (iPC)
Genes suppressed by HSV-1 but not HSV-2		
Chemokine receptors	CCR8	C (iPC)
Genes suppressed by HSV-2 but not HSV-1		
Chemokines	Eotaxin/CCL11	B (iPC)
	MCP-1/CCL2	B (iPC)
Chemokine receptors	D6	C (iPC)
TNF superfamily	THANK/TNFSF13B	C (iPC)

* Abbreviations used in this column: C, C57BL/6; B, BALB/c; iPC, resting peritoneal cells; TG-PC, thioglycollate-elicited peritoneal cells.

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