

# Both plasmacytoid dendritic cells and monocytes stimulate natural killer cells early during human herpes simplex virus type 1 infections

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## Introduction

Herpes simplex virus type 1 (HSV-1) is a member of the  $\alpha$ -herpes virus subfamily with a seroprevalence of 70–80%.<sup>1</sup> It is characterized by a short replication cycle, high cytopathogenicity and distinct neurotropism.<sup>2</sup> Primary

## Summary

Herpes simplex virus type 1 (HSV-1), a member of the herpes virus family, is characterized by a short replication cycle, high cytopathogenicity and distinct neurotropism. Primary infection and reactivation may cause severe diseases in immunocompetent and immunosuppressed individuals. This study investigated the role of human plasmacytoid dendritic cells (pDC) in the activation of natural killer (NK) cells for the control of herpetic infections. Within peripheral blood mononuclear cells, UV-inactivated HSV-1 and CpG-A induced CD69 up-regulation on NK cells, whereas infectious HSV-1 was particularly active in inducing NK cell effector functions interferon- $\gamma$  (IFN- $\gamma$ ) secretion and degranulation. The pDC-derived IFN- $\alpha$  significantly contributed to NK cell activation, as evident from neutralization and cell depletion experiments. In addition, monocyte-derived tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) induced after exposure to infectious HSV-1 was found to stimulate IFN- $\gamma$  secretion. A minority of monocytes was shown to be non-productively infected in experiments using fluorescently labelled viruses and quantitative PCR analyses. HSV-1-exposed monocytes up-regulated classical HLA-ABC and non-classical HLA-E molecules at the cell surface in an IFN- $\alpha$ -dependent manner, whereas stress molecules MICA/B were not induced. Notably, depletion of monocytes reduced NK cell effector functions induced by infectious HSV-1 ( $P < 0.05$ ). Altogether, our data suggest a model in which HSV-1-stimulated pDC and monocytes activate NK cells via secretion of IFN- $\alpha$  and TNF- $\alpha$ . In addition, infection of monocytes induces NK cell effector functions via TNF- $\alpha$ -dependent and TNF- $\alpha$ -independent mechanisms. Hence, pDC and monocytes, which are among the first cells infiltrating herpetic lesions, appear to have important bystander functions for NK cells to control these viral infections.

**Keywords:** cytokines; dendritic cells; human; natural killer cells; viral.

infections cause lytic lesions at oral or genital mucocutaneous sites, followed by transport of the virus to trigeminal or dorsal root ganglia, where lifelong latency is established. Reactivations occur frequently, usually causing self-limiting oral or genital lesions. Under conditions of immunosuppression, but also in immunocompetent

Abbreviations: FCS, fetal calf serum; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; INF, infectious; NK, natural killer; ODN, oligodeoxynucleotide; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cells; TNF, tumour necrosis factor; UV, UV-inactivated

individuals, primary infection and reactivation may cause severe sequelae such as encephalitis, acute retinal necrosis or systemic disease.

Studies in children suffering from severe herpes simplex infections revealed the central role of type I interferon (IFN) production and signalling for the innate immune control of these viruses.<sup>3</sup> Early studies by Fitzgerald-Bocarsly identified the 'interferon-producing cells' as an important accessory cell population for the cytolytic killing of HSV-infected fibroblasts.<sup>4</sup> These cells were subsequently characterized as plasmacytoid dendritic cells (pDC), the major producers of type I interferons in the blood.<sup>5,6</sup> The role of pDC in the immune control of HSV infections was confirmed in murine models. Local footpad and corneal HSV-1 infections in MyD88<sup>-/-</sup> and Toll-like receptor 9<sup>-/-</sup> mice resulted in decreased IFN- $\alpha$  production, but mice were still able to control the infection.<sup>7</sup> Increased pathogenesis in genital HSV-2 infections was observed after antibody-dependent pDC depletion<sup>8</sup> and in IFN- $\alpha$  receptor knockout mice.<sup>9</sup> Recently, specific depletion of pDC in CLEC4C-DTR transgenic mice corroborated the crucial role of these cells in IFN- $\alpha$  production, secretion of pro-inflammatory cytokines, and survival in systemic, but not local, HSV infections.<sup>10</sup>

Along with pDC, natural killer (NK) cells are important in inducing innate anti-HSV responses.<sup>11,12</sup> NK cells were first identified as killing tumour cells without previous activation.<sup>13</sup> In follow-up studies, it became clear that tumour cells, virus-infected and allogeneic cells induced NK cell effector functions via the 'missing-self' HLA repertoire at the cell surface.<sup>14</sup> NK cells comprise a CD16<sup>+</sup> CD56<sup>dim</sup> subset, which accounts for the majority of blood NK cells, migrates to the site of infection and is mostly cytolytic. The minor CD16<sup>(+)</sup> CD56<sup>bright</sup> subset migrates to lymphatic tissue and mostly secretes cytokines, in particular IFN- $\gamma$ .<sup>15</sup> A murine model of ocular HSV-1 infection showed anterior-to-posterior spread of HSV-1 after NK cell depletion.<sup>16</sup> Interleukin-15 deficient (IL-15<sup>-/-</sup>) mice lacking NK cells were found to be 100-fold more susceptible to genital HSV-2 infection, while mice lacking IFN- $\gamma$  were only 10-fold more susceptible than control mice.<sup>17</sup> NK cell depletion resulted in increased HSV-1 titres in the lung after intranasal inoculation of mice.<sup>18</sup>

Follow-up studies addressed the interplay of pDC and NK cells, with continuing discussions about the role of soluble and cell-associated factors. Human NK cell activation and cytolytic functions were reported to be induced by pDC-derived type I IFN upon stimulation with influenza virus, CpG and poly (I:C).<sup>19</sup> Other studies described how pDC-derived IFN- $\alpha$  and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) were responsible for CpG-induced NK cell activation and IFN- $\gamma$  secretion,<sup>20</sup> whereas NK cell degranulation and cytotoxicity required direct contact with pDC.<sup>21</sup> In recurrent human HSV-2 lesions, infiltrating pDC were

detected in close proximity to activated T lymphocytes and NK cells.<sup>22</sup> Murine models confirmed that NK cell activation required type I IFN signalling as IFN- $\alpha$  receptor knockout mice lacked IFN- $\gamma$  production in vaginal HSV-2 infections.<sup>9</sup> In systemic HSV infections of CLEC4C-DTR mice, pDC were shown to be important for NK cell activation, IFN- $\gamma$  production, and degranulation.<sup>10</sup>

Studies addressing the cross-talk between NK cells and pDC in human HSV infections are still limited. Therefore, we used sucrose gradient-purified HSV-1 to analyse in detail the induction of NK cell activation by ultraviolet-inactivated (HSV<sub>UV</sub>) and infectious (HSV<sub>INF</sub>) virus within the peripheral blood mononuclear cell (PBMC) context. Plasmacytoid DC and monocytes were involved in HSV-1-induced NK cell activation, but infection of monocytes additionally induced NK cell effector functions. In these processes, IFN- $\alpha$  and TNF- $\alpha$  were determined as crucial cytokines. Our findings appear to be important for the control of herpes virus infections as monocytes, NK cells and pDC are among the first cells infiltrating herpetic lesions.<sup>22</sup>

## Material and methods

### Isolation and cultivation of cells

The PBMC were isolated from EDTA-anticoagulated blood of healthy volunteers using standard Biocoll density gradient centrifugation (Biochrom AG, Berlin, Germany). A total of 21 different donors (13 female, eight male) were included in the study (age range 25–55 years). Plasmacytoid DC were purified or depleted from PBMC using the CD304 MicroBead Kit with MS/LS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany), as previously described.<sup>23,24</sup> Monocytes were purified or depleted from PBMC by positive selection using CD14 MicroBeads, and NK cells by negative selection using the NK Cell Isolation Kit (both Miltenyi Biotec) according to the manufacturer's recommendations. The purity of isolated NK cells was regularly above 93%. Cell viability was analysed by trypan blue staining. Cells were cultivated in RPMI-1640 (Invitrogen, Darmstadt, Germany), supplemented with 10% heat-inactivated (56°, 60 min) fetal calf serum (FCS; Sigma-Aldrich, Munich, Germany), 0.3 mg/ml glutamine, 200 U/ml penicillin and 90 U/ml streptomycin. The study was approved by the Ethical Committee of the Medical Faculty, Friedrich-Alexander-Universität Erlangen-Nürnberg (No. 3299).

### Generation of viral stocks

Vero cells deficient for IFN- $\alpha$  and IFN- $\beta$  genes<sup>25</sup> were infected with a clinical HSV-1 isolate<sup>26</sup> for 2 hr, washed and incubated in Dulbecco's modified Eagle's medium

(Invitrogen) with 10% FCS and supplements as described above. After 3–4 days of infection, the cell culture was subjected to two freeze–thaw cycles. Lysates were cleared by centrifugation at 440 g for 5 min, and the resulting supernatants were centrifuged at 50 000 g at 4° for 90 min. Viral pellets were incubated in the residual liquid overnight at 4°, resuspended, dounced 20 times and then loaded onto a continuous gradient (30% to 15% sucrose in virus standard buffer, 0.05 M Tris–HCl, 0.012 M KCl, 0.005 M EDTA, 0.1% BSA). After centrifugation at 50 000 g for 30 min, the visible viral layer was harvested and centrifuged at 78 000 g for 90 min. Virus pellets were filtered through 0.22- $\mu$ m pores and stored at –80° (HSV<sub>INF</sub>). Aliquots of the viral stocks were completely inactivated (HSV<sub>UV</sub>) by application of 1 Joule/cm<sup>2</sup> using the Bio-Link 254 UV cross-linker (Vilber Lourmat, Eberhardzell, Germany). The autofluorescing HSV-1 166v isolate, which expresses a green fluorescent protein (GFP)-fused VP22 protein,<sup>27</sup> was propagated on Vero cells, and the HSV-1<sub>d106S</sub> isolate,<sup>28</sup> which expresses GFP under the control of the CMV promoter, was propagated on complementing E11 cells. Stocks of 166v were only filtered through 0.22- $\mu$ m pores, whereas supernatants containing HSV-1<sub>d106S</sub> were sucrose-purified as described above. The 50% tissue culture infective dose was determined for all viral stocks.

#### Generation of pDC supernatants

A total of  $5 \times 10^5$  pDC were exposed to HSV-1<sub>INF</sub> (multiplicity of infection 1) at 37° for 3 hr, washed with Dulbecco's PBS and incubated with trypsin EDTA at 37° for 15 min. Cells were washed and cultivated in RPMI-1640 media with supplements including 20 ng/ml IL-3 (R&D Systems, Wiesbaden-Nordenstadt, Germany) for 18 hr before removal of supernatants.

#### Stimulation of cells

Either PBMC or respectively depleted cells were plated at a density of  $1 \times 10^6$  cells/500  $\mu$ l in 24-well flat-bottom plates (Greiner, Frickenhausen, Germany). Cells were stimulated using the endotoxin-free CpG-A oligodeoxynucleotide (ODN) 6016 (5'-T\*C-G-A-C-G-T-C-G-T-G-G\*G\*G\*G-3', where \* represents phosphorothioate and - represents a phosphodiester bond) at 0.75  $\mu$ M, provided by Coley Pharmaceutical GmbH – A Pfizer Company (Düsseldorf, Germany); HSV-1<sub>INF</sub> at a multiplicity of infection of 0.5 or a corresponding volume of HSV<sub>UV</sub>. Recombinant human IL-2 (Roche-Pharma, Grenzach-Wyhlen, Germany) was used as a positive control stimulus at a concentration of 100 U/ml in a separate culture condition. Supernatants were harvested at the indicated time periods after stimulation and stored at –20°, whereas cells were immediately processed for FACS

analysis. NK cells were plated at a density of  $2.5 \times 10^5$  cells/500  $\mu$ l and stimulated with increasing concentrations of recombinant human IFN- $\alpha$ 2b (hrIFN- $\alpha$ ) (Miltenyi Biotec) or pDC supernatants containing corresponding levels of IFN- $\alpha$ 2a/2b for 3–18 hr.

#### Neutralization experiments

Neutralization experiments were performed using murine IgG1 antibodies to IL-1 $\beta$  (clone 8516) and TNF- $\alpha$  (clone 28 401) together with the mouse IgG1 isotype control (clone 11 711) (all R&D Systems); and the murine IgG2a antibody to the human IFN- $\alpha$  receptor (clone MMHAR-2) together with the mouse IgG2a isotype control (clone PPV-04) (both Acris, Herford, Germany). The concentration of antibodies used for neutralization was 15  $\mu$ g/ml throughout the study.

#### Infection experiments

A total of  $5 \times 10^5$  monocytes were exposed to HSV-1<sub>INF</sub>, HSV-1<sub>UV</sub> and HSV-1 166v (multiplicity of infection 1) for 24 and 48 hr, and subsequently analysed for the expression of GFP, CD14 (clone 61D3; AbD Serotec, Düsseldorf, Germany), CD33 (clone WM53), CD64 (clone 10-1), HLA-ABC (clone W6/32), HLA-E (clone 3D12), and stress-induced molecules MICA/MICB (clone 6D4; all Biolegend, London, UK). Cellular contaminations within the monocyte preparation were identified using CD1c (clone L161; Biolegend), CD3 (clone UCHT; Biolegend or AbD Serotec, Düsseldorf, Germany), CD19 (clone HIB19; Biolegend), CD56 (clone HCD56; Biolegend), and CD123 (clone AC145; Miltenyi Biotec).

#### FACS analysis

Cells were incubated on ice for 10 min and harvested by thorough pipetting. They were washed once with FACS buffer (Dulbecco's PBS plus 1% FCS and 1 mM EDTA) and incubated with FcR blocking reagent (Miltenyi Biotec) at 4° for 10 min. Then, cells were stained with antibodies against specific cell surface markers at 4° for 20 min, washed, and fixed using a 4% paraformaldehyde solution. In all experiments, NK cells were identified as positive for CD56 and negative for CD3 and CD14. Within PBMC, pDC were identified as positive for CD303 (Miltenyi Biotec) and negative for CD3 and CD14; monocytes as positive for CD14; T cells as positive for CD3, negative for CD14, and positive for CD4 (clone RPA-T4; Biolegend), CD8 (clone RPA-T8; Biolegend, or clone MEM-31; Immunotools, Friesoythe, Germany), or TCR- $\gamma/\delta$  (clone 11F2; Miltenyi Biotec); B cells as positive for CD19 and negative for CD3 and CD14. Respective isotype antibodies were used as controls. Activation of cells was evaluated via FITC- or Alexa Fluor 700-labelling of

CD69 (clone FN50 obtained from Miltenyi Biotec or Biolegend, respectively). Cellular degranulation was investigated by adding 5  $\mu$ l of the Alexa Fluor 488-labelled CD107a antibody (clone eBioH4A3; eBioscience, Frankfurt, Germany) to the cell culture 1.5 hr before cell harvest. Live and dead cell staining was performed using a Fixable Violet Dead Cell Stain Kit (Invitrogen). Cells were collected using the multiparameter LSR-II flow cytometer together with the FACSDIVA software for automatic compensation and measurement of samples (BD Biosciences, Heidelberg, Germany). Data were analysed using the FCS EXPRESS 3 Software (De Novo Software, Los Angeles, CA).

**Quantification of cytokines**

IFN- $\alpha$ 2a/2b levels in the cell culture supernatants were measured using an ELISA module set (eBioscience); samples with values above the linear range were diluted as appropriate. Other cytokines were quantified using the Th1/Th2 11 plex RTU FlowCytomix Multiplex assay and FLOWCYTOMIXPRO software according to the manufacturer’s recommendations (eBioscience). IFN- $\gamma$  and TNF- $\alpha$  production by different cells was determined using the respective Secretion Assay Detection Kits (Miltenyi Biotec). In brief, harvested cells were washed with cold cytokine buffer, resuspended in cold media with supplements, and incubated on ice for 5 min after addition of the

cytokine catch reagent antibody. Thereafter, cells were incubated in warm media with supplements in a micro-tube shaker for 45 min. The reaction was stopped by adding cold cytokine buffer, followed by FACS labelling.

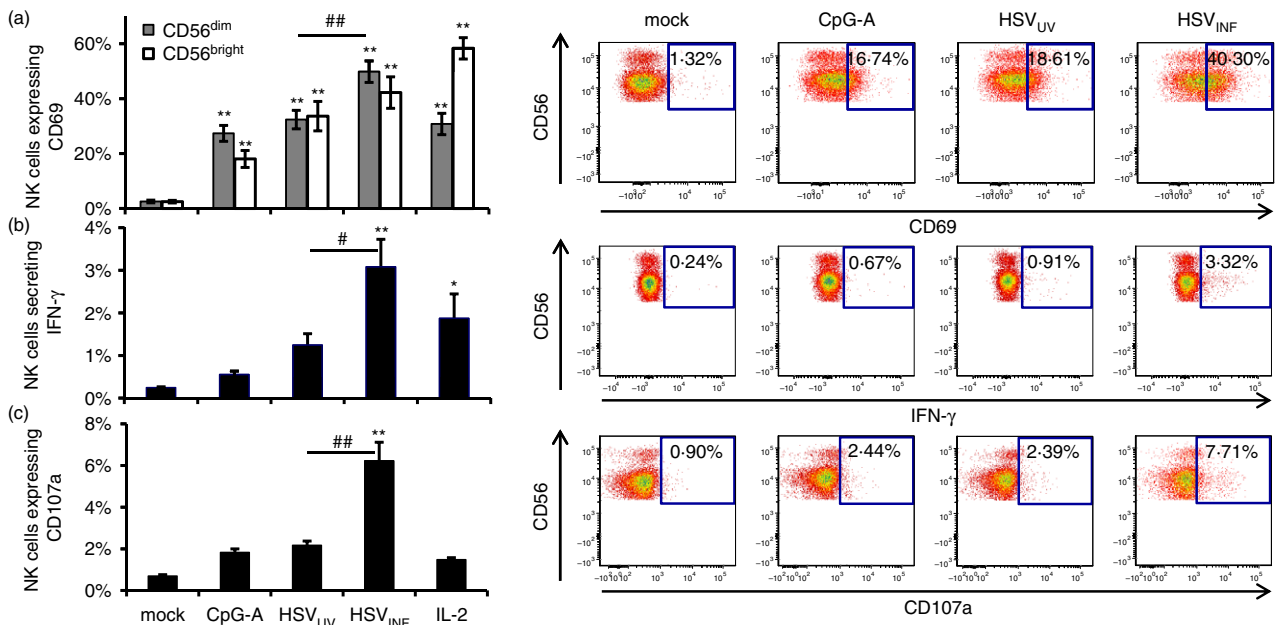
**Statistics**

All experiments were performed using cells of different donors. The Student’s *t*-test was used for comparisons of two datasets, the Tukey HSD test for three or more datasets to account for multiple comparisons. Two-sided *P*-values  $\leq 0.05$  were considered significant.

**Results**

**Stimulation of PBMC with HSV-1 leads to NK cell activation**

To analyse the potential of HSV-1 to induce NK cell activation and effector functions within the PBMC context, PBMC were exposed to HSV<sub>UV</sub> and HSV<sub>INF</sub>. IL-2 and CpG-A served as control stimuli, representing direct<sup>29</sup> and pDC-dependent<sup>30</sup> NK cell activation, respectively. As evident from flow cytometry analyses, all stimuli significantly up-regulated CD69 expression on NK cells compared with mock at 12 hr post stimulation (*P* < 0.01) (Fig. 1a). In these experiments, HSV<sub>INF</sub> was significantly



**Figure 1.** Stimulation of peripheral blood mononuclear cells (PBMC) with herpes simplex virus type 1 (HSV-1) induces natural killer (NK) cell activation. A total of  $1 \times 10^6$  PBMC were exposed to CpG-A (ODN 6016), UV-inactivated and infectious HSV-1 (HSV<sub>UV</sub> and HSV<sub>INF</sub>), and interleukin-2 (IL-2). At 12 hr post stimulation, NK cell activation, cytokine secretion and degranulation were determined via CD69 expression, interferon- $\gamma$  (IFN- $\gamma$ ) secretion, and CD107a surface expression, respectively. The percentages of NK cells expressing these markers are given as mean and standard error of 15 independent experiments. \**P* < 0.05, \*\**P* < 0.01 versus mock; #*P* < 0.05, ##*P* < 0.01 HSV<sub>UV</sub> versus HSV<sub>INF</sub> (Tukey HSD). A representative FACS plot is shown on the right.

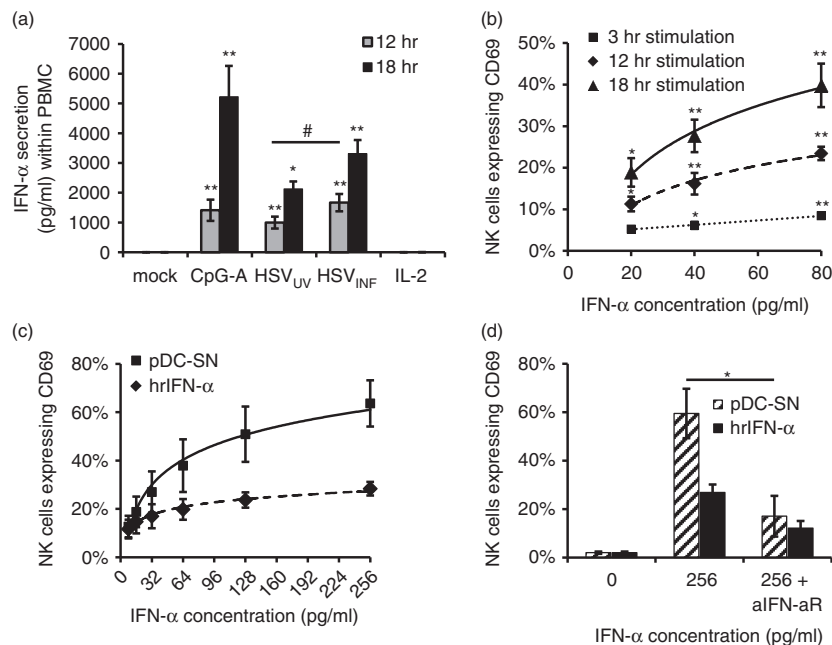
more active than HSV<sub>UV</sub> on CD56<sup>dim</sup> NK cells ( $P < 0.01$ ). At 18 hr post stimulation, all stimuli were similarly active on CD56<sup>dim</sup> NK cells, whereas CD56<sup>bright</sup> NK cells were more pronouncedly activated by HSV<sub>INF</sub> compared with HSV<sub>UV</sub> ( $P < 0.05$ ) (data not shown). This indicates faster and more potent NK cell activation by HSV<sub>INF</sub> versus HSV<sub>UV</sub>. In contrast, the ratio of CD56<sup>bright</sup> to CD56<sup>dim</sup> NK cells and hence NK cell differentiation was not affected by any of the stimuli used (data not shown).

To find out whether CD69 up-regulation reflected induction of NK cell effector functions, we simultaneously evaluated IFN- $\gamma$  secretion and surface expression of CD107a. This lysosomal-associated membrane protein 1 is only expressed at the cell surface after degranulation. The correlation between CD107a surface expression and cytokine secretion as well as cytotoxicity was demonstrated for NK cells.<sup>31</sup> At 12 hr post stimulation, CpG-A and HSV<sub>UV</sub> were not significantly different from the mock control in inducing IFN- $\gamma$  secretion or degranulation (Fig. 1b,c). Interleukin-2 induced IFN- $\gamma$  secretion ( $P < 0.05$ ) but no degranulation, while HSV<sub>INF</sub> induced significant IFN- $\gamma$  secretion and degranulation compared with mock and HSV<sub>UV</sub> ( $P < 0.05$ ) (Fig. 1b,c). These data

underline the importance of viral infectivity for the induction of NK cell effector functions.

### HSV-1 activates NK cells in part via IFN- $\alpha$ induction

IFN- $\alpha$  was reported as a major stimulus for NK cell activation.<sup>4</sup> Therefore, we investigated whether HSV-1 activates NK cells within PBMC via induction of IFN- $\alpha$  production. CpG-A, HSV<sub>UV</sub> and HSV<sub>INF</sub> induced significant IFN- $\alpha$  production at 12 and 18 hr post stimulation ( $P < 0.05$ ) (Fig. 2a). HSV<sub>INF</sub> was more active than HSV<sub>UV</sub> at 12 hr post stimulation ( $P < 0.05$ ), which was no longer significant at 18 hr post stimulation. The effect of IFN- $\alpha$  on purified NK cells was evaluated in more detail performing time-course experiments. Supernatants of HSV<sub>INF</sub>-stimulated pDC containing IFN- $\alpha$ 2a/2b concentrations as low as 20 and 40 pg/ml significantly up-regulated CD69 compared with mock at 12 and 3 hr post stimulation, respectively ( $P < 0.05$ ) (Fig. 2b). To compare the effect of pDC supernatants to human recombinant IFN- $\alpha$ 2b (hrIFN- $\alpha$ ), both solutions were adjusted to similar concentrations of IFN- $\alpha$ 2a/2b. Serial dilutions confirmed IFN- $\alpha$  as a potent stimulus for NK cell activation and revealed a more pronounced activity of pDC supernatants (Fig. 2c). This



**Figure 2.** Herpes simplex virus type 1 (HSV-1) activates natural killer (NK) cells in part via interferon- $\alpha$  (IFN- $\alpha$ ). (a) IFN- $\alpha$  production after stimulation of peripheral blood mononuclear cells with CpG-A, UV-inactivated and infectious HSV-1 (HSV<sub>UV</sub> and HSV<sub>INF</sub>), and interleukin-2 (IL-2) for 12 and 18 hr. Results obtained by ELISA represent mean and standard error of 15 and 7 independent experiments, respectively. \* $P < 0.05$ , \*\* $P < 0.01$  versus mock; # $P < 0.05$  HSV<sub>UV</sub> versus HSV<sub>INF</sub> (Tukey HSD). (b) Time- and concentration-dependent up-regulation of CD69 on purified NK cells after exposure to supernatants obtained from HSV<sub>INF</sub>-stimulated plasmacytoid dendritic cells (pDC-SN). Data obtained by flow cytometry represent mean and standard error of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  versus mock (Tukey HSD). (c) Comparison of CD69 up-regulation on purified NK cells after exposure to pDC-SN and human recombinant interferon- $\alpha$ 2b (hrIFN- $\alpha$ ) for 18 hr. (d) Effect of a neutralizing antibody to the IFN- $\alpha$  receptor (aIFN-aR) on activation of purified NK cells. Data represent mean and standard error of four independent experiments. \* $P < 0.05$ , mock versus aIFN-aR (Student's *t*-test).

activity was significantly reduced by neutralizing the IFN- $\alpha$  receptor ( $P < 0.05$ ) (Fig. 2d), indicating that IFN- $\alpha$  and other type I IFNs are the main soluble factors in pDC-induced NK cell activation.

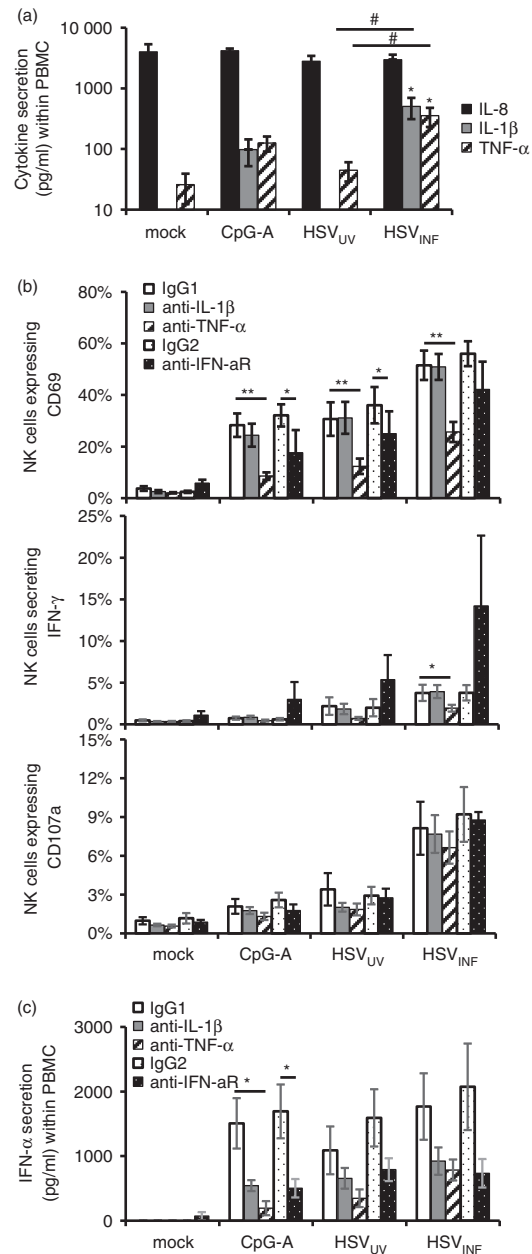
### TNF- $\alpha$ plays a major role in HSV-1-induced NK cell activation

To find out whether other cytokines besides type I IFNs are involved in NK cell activation, we performed a Th1/Th2 multiplex cytokine bead array on supernatants of PBMC stimulated with CpG-A, HSV<sub>UV</sub> and HSV<sub>INF</sub>. Neither stimulus induced significant secretion of IL-2, IL-4, IL-5, IL-10, IL-12p70, IFN- $\gamma$  or TNF- $\beta$ , whereas CpG-A up-regulated IL-6 ( $P < 0.05$ ) (data not shown). Interleukin-8 was secreted in all samples including the mock control, and two cytokines, namely IL-1 $\beta$  and TNF- $\alpha$ , were significantly increased in HSV<sub>INF</sub>-stimulated PBMC compared with mock and HSV<sub>UV</sub> ( $P < 0.05$ ) (Fig. 3a). To investigate the role of these cytokines in HSV<sub>INF</sub>-induced NK cell activation, PBMC were stimulated in the presence of neutralizing antibodies against IL-1 $\beta$ , TNF- $\alpha$  or the isotype control. Neutralization of TNF- $\alpha$  significantly reduced CD69 up-regulation on NK cells induced by CpG-A and HSV-1 at 12 hr post stimulation ( $P < 0.01$ ) (Fig. 3b). TNF- $\alpha$  was also significantly involved in HSV<sub>INF</sub>-induced IFN- $\gamma$  secretion by NK cells ( $P < 0.05$ ), whereas it had no effect on NK cell degranulation (Fig. 3b). These findings evidence a crucial role for TNF- $\alpha$  in HSV<sub>INF</sub>-induced NK cell activation and IFN- $\gamma$  secretion, while neutralization of IL-1 $\beta$  had no effect.

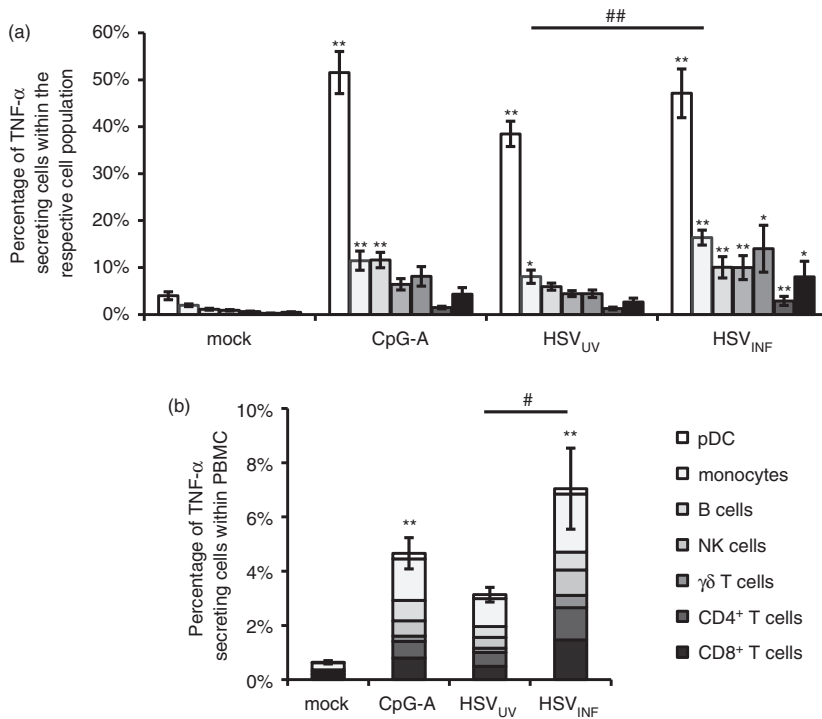
To study the contribution of pDC-derived IFN- $\alpha$  in HSV<sub>INF</sub>-induced NK cell activation, PBMC were stimulated in the presence of a neutralizing antibody against the IFN- $\alpha$  receptor. This blocking diminished CpG-A-induced and HSV<sub>UV</sub>-induced CD69 up-regulation ( $P = 0.05$  and  $P < 0.01$ , respectively), but had no effect on NK cell effector functions (Fig. 3b). Neutralization of the IFN- $\alpha$  receptor decreased IFN- $\alpha$  secretion within PBMC (Fig. 3c), which was consistent with the known autocrine loop.<sup>32</sup> A similar effect was observed with neutralization of IL-1 $\beta$  and TNF- $\alpha$ , suggesting that all three cytokines are involved in the secretion of large amounts of IFN- $\alpha$  upon stimulation with CpG-A and HSV-1.

### Monocytes contribute to HSV-1-induced TNF- $\alpha$ production

Since TNF- $\alpha$  appeared to be a key effector molecule in the HSV<sub>INF</sub>-induced NK cell activation, we analysed the secretion of this respective cytokine upon stimulation of PBMC using flow cytometry analyses. Seven individual cell populations, namely pDC, monocytes, B cells, NK cells,  $\gamma\delta$  T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, responded to HSV<sub>INF</sub> exposure with significant TNF- $\alpha$  secretion compared with mock



**Figure 3.** Herpes simplex virus type 1 (HSV-1) activates natural killer (NK) cells in part via tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). (a) Secretion of interleukin-8 (IL-8), IL-1 $\beta$  and TNF- $\alpha$  in peripheral blood mononuclear cells (PBMC) stimulated with CpG-A, UV-inactivated and infectious HSV-1 (HSV<sub>UV</sub> and HSV<sub>INF</sub>) for 18 hr. Data were obtained using a T helper type 1 (Th1)/Th2 11 multiplex cytokine bead array. Only data with significant differences between HSV<sub>INF</sub> and HSV<sub>UV</sub> are shown. Results represent mean and standard error of seven independent experiments. \* $P < 0.05$  versus mock; # $P < 0.05$  HSV<sub>UV</sub> versus HSV<sub>INF</sub> (Tukey HSD). (b) Percentage of NK cells expressing CD69, interferon- $\gamma$  (IFN- $\gamma$ ), and CD107a, and (c) IFN- $\alpha$  production after stimulation of PBMC in the presence of neutralizing antibodies to IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\alpha$  receptor (anti-IFN- $\alpha$ R). Results represent mean and standard error of five independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  neutralizing antibody versus respective isotype control (Student's *t*-test).



**Figure 4.** Plasmacytoid dendritic cells (pDC) and monocytes are major sources of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) upon exposure to herpes simplex virus type 1 (HSV-1). (a) Percentages of pDC, monocytes, B cells, natural killer (NK) cells,  $\gamma\delta$  T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells secreting TNF- $\alpha$  at 18 hr post stimulation, analysed by flow cytometry for the individual cell populations. (b) Overall TNF- $\alpha$  secretion within peripheral blood mononuclear cells (PBMC), considering the frequency of each cell type within the PBMC population. Data represent mean and standard error of five independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  versus mock; # $P < 0.05$ , ## $P < 0.01$  HSV<sub>UV</sub> versus HSV<sub>INF</sub> (Tukey HSD). The figure legend applies to both figures.

( $P < 0.05$ ) (Fig. 4a). Plasmacytoid DC and monocytes also responded to stimulation with CpG-A and HSV<sub>UV</sub> ( $P < 0.05$ ), and B cells to CpG-A ( $P < 0.01$ ). A significant difference between HSV<sub>UV</sub> and HSV<sub>INF</sub> stimulation was only observed for monocytes ( $P < 0.01$ ).

The percentage of cells secreting TNF- $\alpha$  was highest among pDC. However, they are a rare cell population within PBMC. To adjust for the different frequencies of individual cell populations within PBMC, we multiplied the percentage of TNF- $\alpha$ -secreting cells within an individual cell population by the frequency of this cell population within PBMC (Fig. 4b). When the percentages of all TNF- $\alpha$  secreting cell populations were assembled, significant TNF- $\alpha$  secretion was only observed after stimulation with CpG-A and HSV<sub>INF</sub> ( $P < 0.01$ ). HSV<sub>INF</sub> induced significantly more TNF- $\alpha$  than HSV<sub>UV</sub> ( $P < 0.05$ ), which confirmed the data obtained in the bead array. In this analysis, monocytes were identified as most numerous TNF- $\alpha$  producing cell population upon stimulation with HSV<sub>INF</sub>.

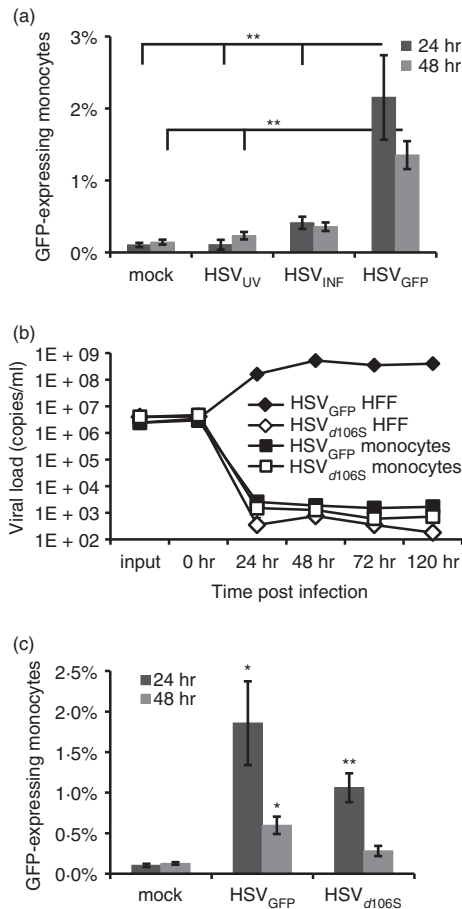
### Monocytes can be infected by HSV-1

As TNF- $\alpha$  secretion by monocytes was significantly affected by HSV-1 infectivity (Fig. 4a), and HSV-1 may infect monocytes as reported in early studies,<sup>33</sup> we decided to investigate monocytes as potential target cells for HSV-1 infection. Monocytes were isolated from PBMC and exposed to HSV<sub>UV</sub>, HSV<sub>INF</sub> and HSV<sub>GFP</sub> expressing a GFP-VP22 fusion protein.<sup>27</sup> The percentage of monocytes expressing GFP in flow cytometry analyses was significantly higher after exposure to HSV<sub>GFP</sub> than to

mock, HSV<sub>UV</sub> and HSV<sub>INF</sub> at 24 hr ( $P < 0.01$ ) and 48 hr ( $P < 0.01$  for mock and HSV<sub>UV</sub>, n.s. for HSV<sub>INF</sub>) (Fig. 5a). GFP expression was already observed at 12 hr post infection (data not shown). The percentage of infected monocytes declined from 24 to 48 hr, indicating abortive rather than productive infection, in concordance with early observations by other groups.<sup>33,34</sup> To investigate whether productive infection occurred, supernatants of HSV-1-infected monocytes were analysed for HSV-1 DNA. Quantitative PCR analyses showed replication of HSV<sub>GFP</sub> in control fibroblasts, while HSV-1 DNA dropped sharply in supernatants of these cells after exposure to infectious, but non-replicative HSV<sub>d106S</sub>.<sup>28</sup> In monocytes, replication kinetics of HSV<sub>GFP</sub> and HSV<sub>d106S</sub> were similar, confirming non-productive infection in these cells (Fig. 5b). Since VP22 is a tegument protein present within viral particles, the observed fluorescence might have resulted from viruses sticking to the outside of exposed monocytes. Therefore, experiments were repeated using HSV<sub>d106S</sub>, which expresses GFP under the control of the cytomegalovirus promoter. Cells exposed to this virus only fluoresce if they have been infected and express GFP. Infection rates with HSV<sub>d106S</sub> were similar to HSV<sub>GFP</sub> (Fig. 5c), confirming actual infection of a minority of monocytes by HSV-1.

### Monocytes up-regulate MHC-I, not MICA/B, upon exposure to infectious HSV-1

Down-regulation of MHC-I molecules by HSV-1 might provide an explanation for the activation of NK cells by



**Figure 5.** Herpes simplex virus type 1 (HSV-1) infects a minority of monocytes non-productively. (a) Percentages of purified monocytes expressing green fluorescent protein (GFP) as analysed by flow cytometry at 24 and 48 hr post infection with HSV<sub>GFP</sub>, which codes for a GFP-fused VP22 protein. UV-inactivated and infectious HSV-1 (HSV<sub>UV</sub> and HSV<sub>INF</sub>) were included as controls. Data represent mean and standard error of 11 independent experiments. \*\* $P < 0.01$  as indicated (Tukey HSD). (b) Quantitative PCR analyses of HSV-1 DNA in supernatants of HSV<sub>GFP</sub>- and HSV<sub>d106S</sub>-infected human foreskin fibroblasts (HFF) and monocytes at indicated time-points post infection. Results were reproduced with monocytes of three donors. (c) Purified monocytes were infected in parallel with HSV<sub>GFP</sub> and HSV<sub>d106S</sub>, which expresses GFP under the control of the cytomegalovirus promoter. Data represent mean and standard error of six independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  versus mock (Student's *t*-test).

HSV-1-infected monocytes. Therefore, we investigated the expression of classical HLA-ABC and non-classical HLA-E molecules on monocytes. HLA-ABC molecules were up-regulated on monocytes upon stimulation with HSV<sub>INF</sub> and HSV<sub>GFP</sub> compared with HSV<sub>UV</sub> at 24 and 48 hr post stimulation ( $P < 0.01$ ), with rising kinetics from 24 to 48 hr (Fig. 6a). Similar data were obtained using HSV<sub>d106S</sub> (data not shown) and investigating HLA-E up-regulation (Fig. 6b). These findings indicate that MHC-I

molecules are up-regulated and not down-regulated on monocytes upon exposure to infectious HSV-1. Since IFN- $\alpha$  was reported to induce up-regulation of MHC-I molecules on cells,<sup>35</sup> monocyte supernatants were analysed for IFN- $\alpha$  production. Reproducible secretion was observed only after stimulation with HSV<sub>INF</sub> and HSV<sub>GFP</sub>, but not HSV<sub>UV</sub> (Fig. 6c). To test the hypothesis that IFN- $\alpha$  was responsible for HLA-ABC and HLA-E up-regulation, we conducted neutralization experiments infecting monocytes with HSV<sub>GFP</sub> in the presence of the IFN- $\alpha$  receptor antibody or the respective isotype. Neutralization of the IFN- $\alpha$  receptor significantly prevented up-regulation of HLA-ABC (Fig. 6d) and HLA-E (data not shown) (all  $P < 0.05$ ) and increased monocyte infection at 48 hr post infection (Fig. 6e). Consequently, IFN- $\alpha$  restricts HSV-1 infection in exposed monocytes and up-regulates MHC-I molecules on the cell surface. We also analysed expression of stress molecules MICA/MICB, because they are known to trigger NK cell activation.<sup>14</sup> Monocytes did not express MICA/MICB upon exposure to HSV-1 (Fig. 6f). Hence, HSV-stimulated monocytes do not contribute to NK cell activation via MHC-I down-regulation or expression of MICA/MICB.

### Monocytes mediate NK cell effector functions upon HSV-1 infection

To quantify the actual contribution of monocytes in comparison to pDC to HSV<sub>INF</sub>-induced NK cell activation, we conducted cell depletion experiments. Depletion of monocytes and pDC significantly reduced CD69 up-regulation induced by HSV<sub>UV</sub> and HSV<sub>INF</sub> ( $P < 0.05$ ) (Fig. 7a). For HSV<sub>INF</sub> (not HSV<sub>UV</sub>), depletion of monocytes had a much stronger effect than depletion of pDC ( $P < 0.01$ ), confirming the important role of pDC and particularly monocytes in HSV<sub>INF</sub>-induced NK cell activation. Further, monocytes (not pDC) were responsible for inducing IFN- $\gamma$  secretion ( $P < 0.05$ ) (Fig. 7b) and degranulation ( $P < 0.01$ ) (Fig. 7c) after exposure of PBMC to HSV<sub>INF</sub>, indicating that monocytes are indispensable for the induction of NK cell effector functions by HSV<sub>INF</sub>. Depletion of pDC and monocytes significantly reduced IFN- $\alpha$  production ( $P < 0.05$ ) (Fig. 7d), pinpointing both cell populations as major contributors to IFN- $\alpha$  secretion. Altogether, our data reveal that pDC-triggered NK cell activation by HSV<sub>INF</sub> depends on the infection of monocytes, which eventually induces NK cell effector functions via TNF- $\alpha$ -dependent and TNF- $\alpha$ -independent mechanisms.

### Discussion

Our systematic analysis of cytokine induction and NK cell activation by different stimuli identified HSV-1 as a potent and fast inducer of NK cell activation in the



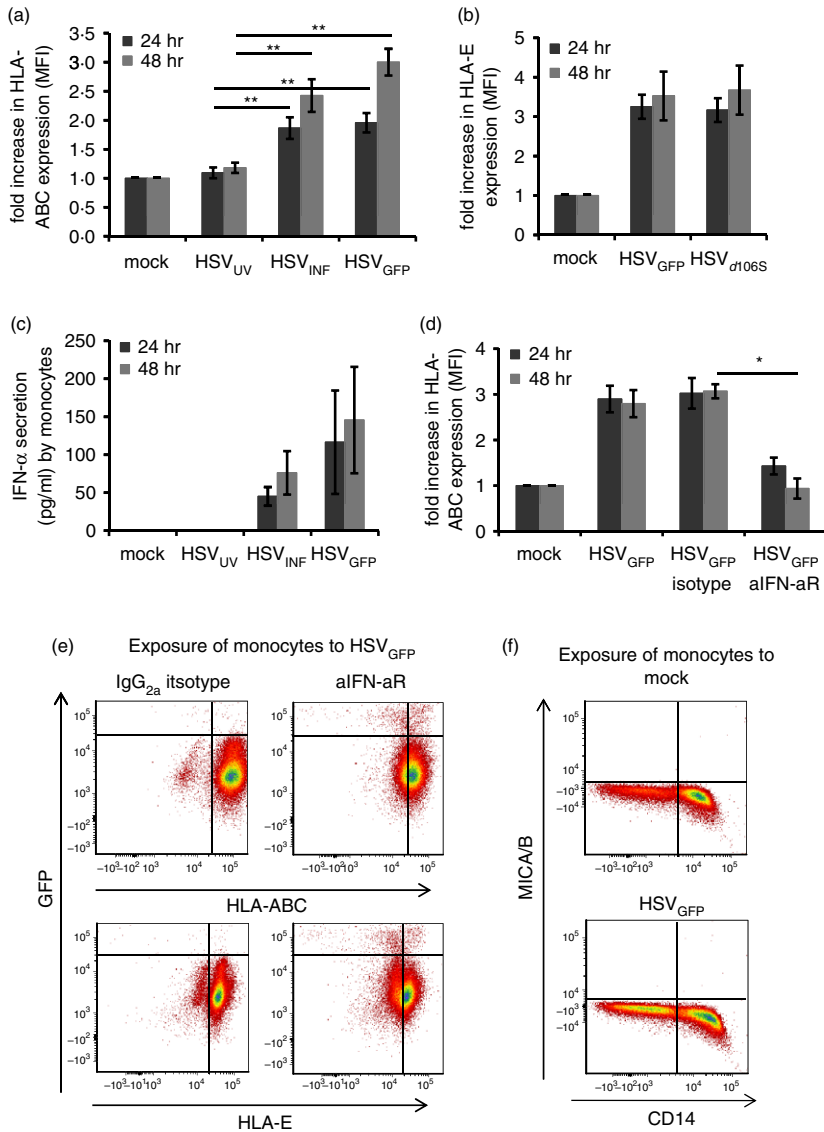
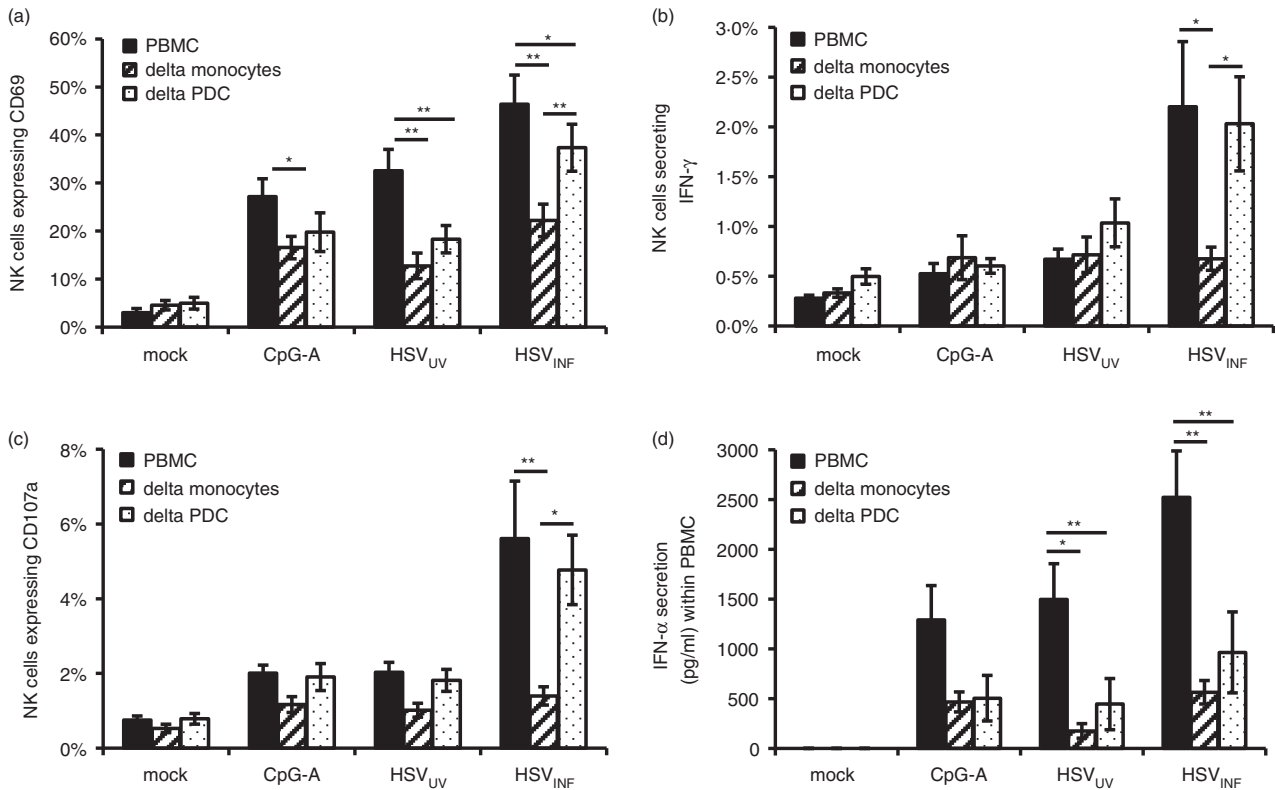


Figure 6. Herpes simplex virus type 1 (HSV-1) induces up-regulation of MHC-I, but not stress molecules MICA/B. (a) Fold increase in HLA-ABC or (b) HLA-E expression as analysed by flow cytometry after exposure of purified monocytes to HSV<sub>UV</sub>, HSV<sub>INF</sub>, HSV<sub>GFP</sub>, and HSV<sub>d106S</sub> for 24 and 48 hr. *\*\*P* < 0.01 compared with HSV<sub>UV</sub> (Tukey HSD). Results are representative of eleven (HLA-ABC) and three (HLA-E) independent experiments. (c) Interferon- $\alpha$  (IFN- $\alpha$ ) production after exposure of purified monocytes to HSV<sub>UV</sub>, HSV<sub>INF</sub>, and HSV<sub>GFP</sub> for 24 and 48 hr. Data represent eight independent experiments. (d) HLA-ABC expression on monocytes exposed to HSV<sub>GFP</sub> in the presence of a neutralizing IFN- $\alpha$  receptor antibody (aIFN-aR). Data represent three independent experiments. *\*P* < 0.05, *\*\*P* < 0.01 compared with the isotype control (Student's *t*-test). All merged data are presented as mean and standard error. (e) Representative example of GFP and HLA-ABC- and HLA-E expression in monocytes exposed to HSV<sub>GFP</sub> in the presence of aIFN-aR or IgG2a isotype. (f) Representative example for MICA/B expression on isolated monocytes 24 hr post stimulation.

context of human PBMC. Interestingly, we observed differences in the stimulating potential between HSV<sub>UV</sub> and HSV<sub>INF</sub>, concerning NK cell activation and cytokine secretion. HSV<sub>INF</sub> induced faster and stronger CD69 up-regulation than HSV<sub>UV</sub> and, in contrast to HSV<sub>UV</sub>, also caused IFN- $\gamma$  secretion and degranulation (Fig. 1). This stands in contrast to a study, where NK cell activity of HSV-1-stimulated PBMC was similar for infectious and UV-inactivated virus,<sup>36</sup> which may be explained by different methodical approaches: Ahmad *et al.* studied the increase of basic lytic activity against NK target cells in the absence of cytotoxicity-inducing target cells. In our experiments, only a minority of NK cells produced IFN- $\gamma$  and/or degranulated upon HSV<sub>INF</sub> stimulation. A similar low responsiveness of peripheral blood NK cells to classical NK cell stimulation was reported by others.<sup>14,37</sup> An explanation may be that we did not use stimulating

cytokines (e.g. IL-2) in addition to HSV<sub>INF</sub>. In conditions of inflammation, however, NK cell effector functions may be further augmented *in vivo*. Notably, we identified IFN- $\gamma$  production and degranulation in CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells. The CD56<sup>dim</sup> NK cell subset was originally identified to be mostly cytolytic.<sup>15</sup> Recent data, however, describe these cells as rapid producers of IFN- $\gamma$  upon antibody-mediated stimulation of natural killer receptors.<sup>38</sup> Our data show, for the first time, that CD56<sup>dim</sup> NK cells are also a source of IFN- $\gamma$  upon HSV<sub>INF</sub> stimulation.

Interferon- $\alpha$  was described by others as the main cytokine in the induction of NK cell activation after stimulation of human pDC with influenza virus, CpG and poly (I:C).<sup>19–21</sup> Our data identified HSV-1 as potent inducer of IFN- $\alpha$  production within human PBMC, with HSV<sub>INF</sub> being faster and more potent than HSV<sub>UV</sub> (Fig. 2a). Supernatants of HSV<sub>INF</sub>-stimulated pDC



**Figure 7.** Herpes simplex virus type 1 (HSV-1)-exposed monocytes induce natural killer (NK) cell effector functions. NK cell expression of (a) CD69, (b) interferon- $\gamma$  (IFN- $\gamma$ ), and (c) CD107a, as well as (d) IFN- $\alpha$  production after stimulation of peripheral blood mononuclear cells (PBMC) with CpG-A (ODN 6016), UV-inactivated and infectious HSV-1 (HSV<sub>UV</sub> and HSV<sub>INF</sub>). PBMC were either used as isolated or after depletion (delta) of monocytes or plasmacytoid dendritic cells (pDC). Data represent mean and standard error of eight independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  as indicated (Tukey HSD).

induced time- and dose-dependent NK cell activation, which was significantly reduced after neutralization of the IFN- $\alpha$  receptor (Fig. 2d). Sucrose-purified HSV-1 was not able to activate isolated NK cells (data not shown), which emphasizes the crucial role of pDC-derived IFN- $\alpha$  in the induction of NK cell activation by HSV-1.

The induction of IFN- $\alpha$  production was able to explain some but not all effects observed after stimulation of PBMC with HSV<sub>INF</sub>, in particular the induction of NK cell effector functions. Using a cytokine bead array, we identified TNF- $\alpha$  and IL-1 $\beta$  as further cytokines, which were significantly induced by HSV<sub>INF</sub> compared with HSV<sub>UV</sub> (Fig. 3a). Other groups have shown an important role of TNF- $\alpha$  in the NK cell activation induced by CpG-activated human pDC.<sup>19,20</sup> Studies with TNF- $\alpha$  knockout mice showed decreased survival rates in acute corneal HSV-1 infections and increased reactivation rates after UV light stimulation,<sup>39</sup> and lethal encephalitis after intranasal HSV-1 infection.<sup>40</sup> Our neutralization experiments support the role of this cytokine in the control of herpes viral infections, as TNF- $\alpha$  turned out to contribute significantly to HSV-1-induced NK cell CD69 up-regulation and IFN- $\gamma$  secretion (Fig. 3b).

Interleukin-1 $\beta$  costimulated IFN- $\gamma$  production of CD56<sup>bright</sup> NK cells together with IL-12 or, in particular, IL-15.<sup>41</sup> A mouse model of lethal encephalitis after intranasal HSV-1 infection confirmed its role *in vivo*.<sup>40</sup> Our neutralization studies did not reveal a direct impact of IL-1 $\beta$  on NK cell activation and effector functions, but rather an indirect effect via enhanced IFN- $\alpha$  production (Fig. 3c). Strikingly, all three cytokines affected IFN- $\alpha$  secretion, suggesting a positive feedback loop for IFN- $\alpha$  secretion, in which, besides IFN- $\alpha$  itself,<sup>32</sup> IL-1 $\beta$  and TNF- $\alpha$  are involved. Induction of high IFN- $\alpha$  levels by HSV-1 may imply a cross-talk between the involved cell populations through secretion of these cytokines. Other cytokines, which appear to contribute to NK cell activation, are IL-15 and IL-18, which were found to be of relevance in murine models of vaginal HSV-2 and intravenous HSV-1 infections.<sup>17,42</sup> Our experiments also showed that not all cytokines induced each other: neutralization of the IFN- $\alpha$  receptor increased the HSV<sub>INF</sub>-induced IFN- $\gamma$  production (Fig. 3b). These findings suggest that IFN- $\alpha$  inhibits production of IFN- $\gamma$ , which was similarly described in another study of murine cytomegalovirus-induced type I IFN production.<sup>43</sup> In this process,

signal transducer and activator of transcription 1 (STAT1) was described to be crucially involved.<sup>44</sup> In contrast, in lymphocytic choriomeningitis infection, type I IFNs activate STAT4 and thereby induce IFN- $\gamma$  production.<sup>45</sup> These findings suggest a plasticity of NK cell innate immune responses in different infection models.<sup>46</sup> To further investigate this process, we addressed the impact of combined blockade of TNF- $\alpha$  and IFN- $\alpha$  receptor on NK cell functions. Combination of blocking antibodies did not have additive effects on NK cell CD69 up-regulation compared with blockade with single antibodies, whereas IFN- $\gamma$  production after combined blockade was comparable to the isotype control (data not shown). These findings suggest opposing roles for TNF- $\alpha$  and IFN- $\alpha$  in HSV<sub>INF</sub>-induced IFN- $\gamma$  secretion.

Our study identified pDC and monocytes as potent TNF- $\alpha$  producers in response to HSV<sub>INF</sub> stimulation (Fig. 4). The significant difference in TNF- $\alpha$  secretion between HSV<sub>UV</sub>-exposed and HSV<sub>INF</sub>-exposed monocytes could in part explain the inability of HSV<sub>UV</sub> (in contrast to HSV<sub>INF</sub>) to induce significant IFN- $\gamma$  secretion, because neutralization of TNF- $\alpha$  significantly diminished HSV<sub>INF</sub>-induced IFN- $\gamma$  secretion. These observations suggest a crucial role for viral infectivity in the induction of pro-inflammatory cytokines and NK cell effector functions. Analyses of two different GFP-expressing HSV-1 isolates revealed infection of a minority of monocytes, yet without inducing a productive replication cycle (Fig. 5), in concordance with previous studies of monocyte infection by HSV-1.<sup>33,47</sup> The infected cells were indeed monocytes, as confirmed by staining of CD14 (data not shown). Low infection rates of monocytes were in part due to IFN- $\alpha$ , as blocking of the IFN- $\alpha$  receptor increased infection rates (Fig. 6e), which confirms previous studies reporting that type I interferons suppress HSV-1 replication *in vitro* and *in vivo*.<sup>48,49</sup> Another explanation for the low infection rates may be the lack of pre-stimulation, which has similarly been reported for T lymphocytes, whose infectability was enhanced after phytohaemagglutinin stimulation.<sup>50</sup> It was also recently reported that SAMHD1 restricts HSV-1 DNA replication in macrophages.<sup>51</sup> It should be noted, however, that a frequency of 1–2% infected monocytes in our experiments contrasted with roughly 20% of monocytes secreting TNF- $\alpha$  upon exposure to HSV<sub>INF</sub>. Hence, infection of monocytes appears to stimulate a number of uninfected bystander cells to secrete TNF- $\alpha$ .

Depletion experiments confirmed pDC as potent mediators of HSV-induced NK cell activation via IFN- $\alpha$  production (Fig. 7a,d). More importantly, these studies showed the relevance of monocytes in both NK cell activation and IFN- $\alpha$  secretion. Monocytes may contribute to high IFN- $\alpha$  levels via secretion of IFN- $\alpha$  itself, as observed for infected monocytes (Fig. 6c), and/or via secretion of IL-1 $\beta$  and TNF- $\alpha$ , thereby stimulating IFN- $\alpha$  secretion by pDC, as suggested by cytokine neutralization (Fig. 3c). A

new aspect in our study is that monocytes were identified as an indispensable cell population in the induction of NK cell effector functions by HSV<sub>INF</sub> within the PBMC context (Fig. 7b,c). We could furthermore determine TNF- $\alpha$  secretion as an important mechanism in the induction of IFN- $\gamma$ , but the exact process, in which degranulation was induced, remained elusive. Most likely, infected monocytes are directly recognized by NK cells as target cells and induce NK cell activation and effector functions through various possible mechanisms.

Induction of NK cell cytotoxicity via down-regulation of HLA molecules on infected cells was demonstrated for HSV-1 and HSV-2.<sup>52,53</sup> In our studies, we observed IFN- $\alpha$ -dependent overall up-regulation of HLA-ABC and HLA-E on monocytes. Unfortunately, we were not able to investigate HLA-A, HLA-B and HLA-C separately, because no specific antibodies are available. HLA up-regulation may protect monocytes from NK cell killing. In turn, monocytes may produce TNF- $\alpha$  to enhance NK cell killing of HSV-infected cells in inflamed tissue.<sup>54</sup> MICA, which was up-regulated on Toll-like receptor-stimulated monocytes,<sup>55</sup> was not induced by HSV-1, consistent with a study in which MICA in infected cells was down-regulated by late HSV-1 gene products.<sup>56</sup> Immediate early gene expression was found to be sufficient for NK cell-mediated lysis of HSV-infected fibroblasts.<sup>54</sup> Follow-up studies found that the HSV-1 immediate early protein ICP0 induced lysis of HSV-infected cells via the natural cytotoxicity receptors.<sup>57</sup> Yet, the induced molecules on target cells were not identified. Possible candidates may be CD244 (2B4, SLAMF4), which is up-regulated on NK cells and interacts with CD48 on macrophages;<sup>58</sup> the glucocorticoid-induced TNF receptor-ligand (GITRL), involved in the induction of NK cell cytotoxicity by CpG-stimulated pDC;<sup>59</sup> or B7-H6, a cell surface ligand for Nkp30, induced on pro-inflammatory monocytes upon stimulation with IL-1 $\beta$  and TNF- $\alpha$ .<sup>60</sup>

Altogether, our data propose a model in which the cross-talk between NK cells, pDC, and monocytes is mediated in part by IFN- $\alpha$  and TNF- $\alpha$ , eventually causing activation of effector NK cells secreting IFN- $\gamma$  and exhibiting degranulation. In this process, both HSV-1-stimulated pDC and monocytes initially activate NK cells, but only monocytes exposed to HSV<sub>INF</sub> induce effector functions via TNF- $\alpha$ -dependent and TNF- $\alpha$ -independent mechanisms. These data may stimulate further studies investigating the role of cell contact-dependent molecules in the induction of NK cell effector functions. Plasmacytoid DC accumulate in herpes virus-induced lesions in the skin<sup>61,62</sup> and mucosa,<sup>8</sup> as well as in draining lymph nodes,<sup>63</sup> where they encounter dendritic cells, NK and T cells.<sup>22,59,64</sup> Analysing the interactions between pDC, NK cells, and monocytes is important because these cells are among the first to infiltrate herpetic lesions,<sup>22</sup> and thereby may contribute to the efficient control of primary and recurrent HSV infections.

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**Disclosures**

The authors declare no financial or commercial conflict of interest.

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