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NKT Cells Determine Titer and Subtype Profile of Virus-Specific IgG Antibodies during Herpes Simplex Virus Infection

Martin J. Raftery,1*1,1 Eike Wolter,1*1,2 Simon Fillatreau, † Helga Meisel, *, Stefan H. E. Kaufmann, ‡ and Günther Schönrich*

Innate NKT cells (iNKT cells) are innate lymphocytes that recognize lipid-derived Ags presented by the MHC class I–related protein CD1d. In this study, we analyzed the role of iNKT cells in the generation of Abs against HSV type 1 (HSV-1). In sera from healthy humans, we found a correlation between HSV-1–specific IgG titers and proportions of CD4+ iNKT cells. In HSV-1–infected iNKT cell–deficient mice, the amount of specific IgM and IgG Abs were significantly reduced compared with wild-type mice. Moreover, iNKT cell–deficient mice were unable to upregulate CD1d on B cells and failed to establish an IFN-γ–driven subtype profile of HSV-1–specific IgG Abs. In spleens of HSV-1–infected wild-type mice, the percentage of iNKT cells expressing CCR6, a marker for inflammatory iNKT cells secreting IFN-γ, was significantly decreased at 6 mo postinfection, suggesting that these cells were released from the spleen to other tissues. Finally, in vitro experiments showed that in the absence of CD1d–restricted cells, HSV-1 induced markedly lower IFN-γ production in splenocytes from naïve mice. Taken together, our results indicate that iNKT cells shape the Ab response to HSV-1 infection and provide a basis for rational development of antiviral vaccines. The Journal of Immunology, 2014, 192: 4294–4302.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are enveloped dsDNA viruses that belong to the family of herpesviruses (1). HSV-1 and HSV-2 are ubiquitous and cause painful recurrent mucocutaneous infections affecting more than a billion people in all parts of the world (2). Neonates and immunosuppressed hosts develop serious symptoms in response to these common pathogens (3, 4). After primary infection of epithelial tissue, HSV-1 and HSV-2 persist by establishing latency in neuronal ganglia. From there, they can reactivate to migrate down the axon infecting skin and mucosa which is served by the affected neurons. HSV-1– and HSV-2–associated diseases represent a heavy medical burden that could be relieved by an efficient prophylactic strategy. Accordingly, considerable effort has been put into the development of HSV-1 and HSV-2 vaccines (5). To achieve this goal, it is of importance to understand how the B cell response against these viruses is regulated.

Innate NKT cells (iNKT cells) show phenotypic and functional features of both NK cells and T cells. They represent innate lymphocytes that express a semi-invariant TCR consisting of invariant TCR-α-chain, Vα14 in mice and Vα24 in humans. In essence, these iNKT TCRs function as pattern recognition receptors and can detect a limited number of different endogenous and pathogen-derived lipid Ags bound to a MHC class I–like molecule, called CD1d (6). Exogenously added α-galactosylceramide (αGalCer), a synthetic glycolipid originally derived from a marine sponge, binds to CD1d and acts as a potent stimulator of iNKT cells resulting in rapid release of huge amounts of cytokines such as IFN-γ or IL-4 (7).

Accumulating evidence suggest that iNKT cells participate in defense against viral pathogens, including human herpesviruses (8). Experiments with human cells have shown that HSV-1 interferes with CD1d Ag presentation and NKT cell activation (9–11). Finally, there is direct evidence from murine HSV-1 and HSV-2 infection models that iNKT cells contribute to virus control (12, 13). How iNKT cells affect antiviral responses remains unclear.

Recent work has demonstrated that αGalCer-stimulated iNKT cells can be used as vaccine adjuvants (14). Whether iNKT cells also contribute to virus-specific Ab production under physiological conditions in the absence of αGalCer is an important issue that has not been addressed. In this study, we investigated the role of iNKT cells in HSV-1–induced B cell responses in vivo.

Materials and Methods

Buffy coats

Buffy coats from healthy human donors latently infected with different herpesviruses were obtained from the Deutsches Rotes Kreuz (German Red Cross, Dresden, Germany).
**ELISAs**

The following ELISAs were used: EBV-specific human IgG, varicella zoster virus (VZV)–specific human IgG and HSV-1–specific human IgG (all from Dade Behring); CMV–specific human IgG and human IgM (all from Medac); human IgG specific for EBV nuclear Ag (DiaSorin); and human herpes virus 6 (HHV-6)–specific human IgG (PanBio). Indeterminate results (OD 0.10–0.20) were regarded as negative. Statistical significance was calculated by Pears or the Mann–Whitney test.

For detection of HSV–specific Abs in murine or human sera, Nunc Maxisorp 96–well plates were coated overnight at 4°C with lysates from HSV-1–infected Vero cells, diluted 1:200 in coating buffer (ph 9.4). Lysates were made by applying three cycles of freeze–thaw using dry ice and a water bath heated at 37°C. Lysed cells were centrifuged and supernatants harvested. Sonification for 15 min at 37°C served to separate viral particles from cellular debris and ultracentrifugation (two times for 2 h, 30,000 rpm) in a SW 3 Ti Rotor (Beckman Coulter) were used to enrich for viral proteins. Plates were blocked with 20% FCS in PBS overnight at 4°C. Sera from C57BL/6 (wild-type [WT]) and C57BL/6 Jα18 knockout (KO) mice, either infected (HSV-1 F strain) or uninfected or uninfected were collected at different time points and stored at −20°C until used. Sera were diluted 1:200 in PBS, and plates were incubated in duplicates overnight at 4°C. Subsequently, HRP–conjugated goat anti–mouse Abs detecting IgM, IgG1, IgG2a, IgG2b, IgG2c, or IgG3 (all from Southern Biotechnology Associates) were applied. For detection of HSV–1 specific–human IgG HRP–conjugated mouse anti–human IgG Abs (Southern Biotechnology Associates) were used as secondary reagent. Dilutions were 1:2000, except for IgG2c (1:1000); incubation was done for 2 h on a shaker at room temperature. Prior to analysis, 3′,3′,5′,5′–tetramethylbenzidine was added to each well, serving as a substrate for HRP. OD values were measured on a plate reader (Tecan Sunrise), wavelength 450 nm, using Magellan software. Mean values from duplicates were obtained and results from uninfected mice were subtracted from those of infected mice to exclude background. Statistical significance was calculated using Prism software (Mann–Whitney test).

**IFN-γ stimulation assay**

Spleen cells (2 × 10^6 ml/l from C5BL/6 WT and C57BL/6 CD1d KO mice were incubated in RPMI 1640 complete medium with 10% FCS in 96–well plates and blocked with assay diluent for 2 h at room temperature. Prior to analysis, 3′,3′,5′,5′–tetramethylbenzidine was added to each well, serving as a substrate for HRP. OD values were measured on a plate reader (Tecan Sunrise), wavelength 450 nm, using Magellan software.

**Abs and tetratramers**

For phenotypic analyses of cell markers by flow cytometry, the following reagents were used: anti–human CD4 mAb clone MEM241 (ImmuNoTools); anti–human CD8 mAb clone 3B5 (ImmuNoTools); anti–human CD3 mAb clone UCHT1 (BD Biosciences); mAb (clone 6B11) specific for the invariant V-3′,5′,5′–tetramethylbenzidine substrate solution. OD values were measured on a plate reader (Tecan Sunrise), wavelength 450 nm, using Magellan software.

**Flow cytometry**

Cells in suspension were washed once with ice–cold FACS wash solution (PBS with 1% heat–inactivated FCS and 0.02% sodium azide) before being resuspended with the first Ab in ice–cold blocking solution (PBS with 10% heat–inactivated FCS and 0.2% sodium azide) for 1 h. Cells were then washed in ice–cold FACS wash solution and in experiments with directly labeled Abs were washed twice. Subsequently, the staining was completed with fluorophore–coupled secondary Ab in ice–cold blocking solution. Subsequently, cells were again washed in ice–cold FACS wash and then resuspended in 100 μl PBS with 0.37% formaldehyde. Flow cytometry was performed on a FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software. For determination of human iNKT cell subsets, at least 1 × 10^6 cells were acquired.

**Mice**

C57BL/6 WT mice were purchased from Charles River Laboratories. C57BL/6 Jα18 KO mice (15) were bred at the Robert Koch Institute (Berlin, Germany) under specific pathogen–free conditions. Mice were all female and 10–20 wk old at the time of i.p. infection with a sub lethal dose of HSV-1 F strain (1 × 10^6 PFU). For in vitro experiments, cells were prepared from C57BL/6 CD1d KO mice (16) that had been bred at the Deutsche Rheumafor schungszentrum (Berlin, Germany) under specific pathogen–free conditions. Mice were females and 6–12 wk old at the time of killing. All experiments were performed after approval by the local animal ethics commission.

**Western blot**

Lysates from HSV–1–infected Vero cells were made by applying three cycles of freeze–thaw procedure using dry ice and a water bath heated at 37°C. Lysed cells were centrifuged, and supernatants were harvested. At this point, the uninfected control lysates were frozen and stored until use.

Lysates from the HSV–1–infected cells were solubilized for 15 min to separate viral particles from cellular debris and ultracentrifugation (two times for 2 h, 30,000 rpm) was used to enrich for viral proteins. Denaturation of the sample for 10 min at 95°C was performed prior to 1:1 dilution with loading buffer. Proteins (1 μg/μl) were then separated using a 12% SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The gel was loaded from left to right such that one lane with uninfected lysate was running next to one lane with a HSV–1–infected lane. PVDF membrane was blocked with 3% milk powder in TBS for 1 h. The membrane was cut into strips, according to the loading pattern, that is, each strip consisted of two lanes, one run with infected lysate and the other run with uninfected lysate as a negative control.

Each strip was incubated at 4°C overnight separately with serum from one individual mouse (diluted 1:2000). Subsequently, strips were washed five times with TBS containing 0.02% Tween (TBST) and incubated with HRP–coupled secondary Ab for 1 h. Secondary Abs (Southern Biotechnology Associates) were either goat anti–mouse–specific at a dilution of 1:2000 for 1 h. Before detection with a charge–coupled device camera (Pegcam), the strips were washed five times with TBST buffer and visualized using Super Signal West Dura Extended Duration Substrate kit (Pierce), according to manufacturer’s protocol.

**Cells and virus**

Spleen and liver cells from C57BL/6 WT and C57BL/6 Jα18 KO mice were isolated using cell strainers and several washing steps and in addition by density gradient centrifugation (Optiprep) for liver cells. The iNKT cell hybridoma DN3A4-12 has been described in detail previously (17). HSV–1 F strain was propagated on VeroE6 cells as described elsewhere (18). MCMV (Smith strain) was obtained from U. Koszinowski (Max von Pettenkofer–Institut, Ludwig–Maximilians–Universität, Munich, Germany).

**Results**

HSV–1–specific Ab titers correlate with the proportion of human CD4^+ iNKT cells in human peripheral blood

First, we analyzed whether a correlation exists between iNKT cells and virus–specific IgG titers. For this purpose, peripheral blood samples from 21 normal healthy human donors were grouped according to their seropositivity for HSV–1 (19 of 21), VZV (21 of 21), EBV (21 of 21), human CMV (11 of 21), or HHV–6 (12 of 21). In these groups, the percentages of total iNKT cells in CD3^+ PBMCs were similar (Fig. 1B, upper graphs). Human iNKT cells can be divided into a CD4^+ subset and CD4^- subsets (19, 20). Intriguingly, within the group of HSV–1–seropositive donors, a significant correlation between the HSV–1–specific IgG titers and the proportion of CD4^+ iNKT cells was observed (Fig. 1B, lower graphs). Representative plots and the gating strategy is shown in Fig. 1A. Taken together, these data suggest that iNKT cells play a role in the humoral immune response against HSV–1.

iNKT cells regulate the quantity of HSV–1–specific Abs in mice

To analyze a possible causal relationship between iNKT cells and virus–specific Ab titers, we switched to a mouse model of HSV–1 infection.
Whereas CD1d-expressing human cells lost their iNKT cell-stimulatory capacity already at a low infectious dose, CD1d-expressing murine cells appeared to be rather resistant to HSV-1-associated evasion mechanisms that target iNKT cells (Supplemental Fig. 1). This made the mouse model especially valuable for detecting differences between iNKT cell-deficient (Ja18 KO) and WT mice. Postinfection (p.i.) with a sublethal dose of HSV-1 a kinetic analysis of HSV-1-specific Ab production in Ja18 KO and WT mice was performed. HSV-1-specific IgM serum titers were enhanced in WT mice as compared with Ja18 KO mice until day 35 when they returned to baseline levels in both types of mice (Fig. 2A). Moreover, the increase in HSV-1-specific IgG serum titers in WT mice was significantly stronger and remained at higher levels through 11 wk p.i. as compared with Ja18 KO mice (Fig. 2B). Finally, the presence of HSV-1-specific Abs in sera from mice at day 35 p.i. was visualized by Western blot analysis (Fig. 2C). At day 21 p.i. in all four sera from HSV-1-infected WT mice, strong reactivity against HSV-1 proteins of ~120, 80, and 50 kDa was clearly visible. In contrast, only one of four sera from HSV-1-infected Ja18 KO mice showed reactivity against these viral proteins. Taken together, our results indicate that in the absence of iNKT cells, the abundance of specific IgM and IgG Abs generated in response to HSV-1 is markedly decreased.
Hematopoietic cells including B cells express CD1d molecules (21). CD1d expression on B cells has been described as essential for stimulation of Ab production by iNKT cells (22). Thus, we determined whether HSV-1, which establishes persistent infections, modulates expression of CD1d molecules on B cells. For this purpose, HSV-1–infected and uninfected mice were sacrificed, and B cells were isolated from liver and spleen. B lymphocytes, which express CD19, can be further divided into a subset constitutively expressing CD5+ and a CD5− subset (23). CD5+ B cells, also referred to as B-1a cells, appear to be mainly T cell independent and produce IgM and low-affinity polyreactive IgG. In contrast, CD5− B cells comprise mostly conventional B cells. Expression of CD1d was significantly upregulated on both B cell subsets from liver of HSV-1–infected WT mice (Fig. 3A). In addition, CD5+CD19− lymphocytes (non-B lymphocytes) in the spleen from HSV-1–infected WT mice also significantly increased CD1d expression (data not shown). In contrast, HSV-1 infection of Ja18 KO mice did not result in CD1d upregulation on any of these cell types (Fig. 3B). In both Ja18 KO and WT mice peripheral B cells showed a tendency for increased CD80 expression 2 and 7 d p.i. (Supplemental Fig. 2). At 21 d p.i., these changes returned to normal levels in both types of mice. This finding underlines the specificity of the observed differences in CD1d expression of B cells from Ja18 KO and WT mice. Collectively, these results suggest that B cells and other lymphocytes increase CD1d lipid Ag presentation in response to HSV-1 infection only if iNKT cells are present.

Mobilization of Th1-type iNKT cells in HSV-1–infected mice

Next, we determined whether HSV-1 has impacts on the distribution of iNKT cells. For this purpose, HSV-1–infected WT mice were sacrificed at 7 d to 6 mo p.i., and percentages of iNKT cells within the T cell population of blood, spleen, and liver were determined (Fig. 4A). The proportion of iNKT cells in the peripheral blood of HSV-1–infected WT mice was significantly increased compared with uninfected WT mice that served as control. A slight increase also was observed in the liver of HSV-1–infected WT mice. In contrast, the percentage of iNKT cells in the spleen was significantly diminished after HSV-1 infection. Nearly all iNKT cells in the blood of both HSV-1–uninfected and –infected WT mice expressed CCR6, which is characteristic for Th1-type inflammatory iNKT cells secreting IFN-γ (24). However, the percentage of CCR6+ iNKT cells in the spleen of HSV-1–infected WT animals was significantly decreased compared with uninfected control WT mice 6 mo p.i. (Fig. 4B). In contrast, the percentage of CCR5+ iNKT cells in blood, spleen, and liver did not change after HSV-1 infection (Fig. 4C) demonstrating the specificity of the observed change in percentage of CCR6+ iNKT cells. In sum, these observations indicate that Th1-type iNKT cells from the spleen are mobilized and may home to other tissue sites during HSV-1 infection.

Virus-induced IFN-γ production in naive mice is CD1d dependent

We then examined whether CD1d-restricted NKT cells are required for HSV-1–induced IFN-γ production. For this purpose, splenocytes from naive WT and CD1d KO mice were infected in vitro with live or UV-inactivated HSV-1; uninfected splenocytes served as negative control. For purposes of comparison, splenocytes infected with MCMV were included. To achieve maximum release of IFN-γ, splenocytes were treated with αGalCer or LPS. After 48 h, the concentration of IFN-γ in the supernatant was measured (Fig. 5). WT cells stimulated with αGalCer released considerable amounts IFN-γ (mean: 507 pg/ml), whereas this cytokine was undetectable in the supernatant of αGalCer-treated CD1d KO splenocytes. Treatment with LPS also resulted in profound IFN-γ release (mean: 309 pg/ml), which was markedly diminished in the

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**FIGURE 2.** Requirement of iNKT cells for efficient HSV-1–specific IgM and IgG responses in mice. C57BL/6 WT and C57BL/6 Ja18 KO mice were infected with HSV-1 (F strain, 1 × 10⁶ PFU i.p.). Blood was drawn from the tail vein at the indicated timepoints after HSV-1 infection and levels of HSV-1-specific IgM (A) and IgG (B) Abs were determined by ELISA as described in Materials and Methods. ELISA measurements were done in duplicates. In parallel, sera from uninfected mice were analyzed in the same way. Mean OD values from uninfected mice were regarded as background and subtracted from those of HSV-1–infected mice. Each experimental group of mice consisted of four to six animals, and values shown correspond to mean ± SEM. Statistical analyses were done applying Prism software (mean ± SEM; **p < 0.005 unpaired Student t test). (C) Western blot analysis of HSV-1–specific IgG responses. Lysates from HSV-1–infected (“+”) and –uninfected (“−”) Vero cells were separated by SDS-PAGE and transferred onto a PVDF membrane as described in Materials and Methods. The membrane was cut into strips with each strip consisting of one “+” and one “−” lane as a negative control. Each strip was incubated separately with serum from one individual WT mouse (strip 1–4) and Ja18 KO mouse (strip 5–8), respectively, collected 21 d p.i. HSV-1–specific Abs were visualized with a HRP-coupled goat-anti-mouse IgG secondary Ab. Four WT and four Ja18 KO mice were used in each group.

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B cells from iNKT cell–deficient mice fail to upregulate CD1d in response to HSV-1

Hematopoietic cells including B cells express CD1d molecules (21). CD1d expression on B cells has been described as essential...
absence of CD1d-restricted immune cells (mean: 39 pg/ml). No IFN-γ was detected in uninfected and untreated splenocytes. Post-infection of WT mice with MCMV or HSV-1, production of IFN-γ was approximately an order of magnitude lower than after αGalCer stimulation. Importantly, in response to live HSV-1 splenocytes devoid of CD1d-restricted immune cells produced far less IFN-γ compared with WT cells (mean: 8 versus 60 pg/ml). UV-inactivated HSV-1 induced only marginal IFN-γ secretion in splenocytes compared with live HSV-1 (Fig. 5). These data demonstrate that HSV-1–induced early IFN-γ production in naive mice requires viral replication and potentiation by iNKT cells.

**iNKT cells are required for generating a Th1-like subtype profile of HSV-1–specific IgG Abs**

We determined the HSV-1–specific IgG subtype profile because it can give clues regarding the cytokine driving IgG subtype selection (25). For example, IFN-γ stimulates the synthesis of IgG2a/c and IgG3 subtypes. Thus, the time kinetics of HSV-1–specific IgG subtype appearance in serum from WT- and iNKT-deficient mice were assessed. No significant difference was detected in HSV-1–specific IgG1 responses between Jα18 KO and WT mice (Fig. 6A). The HSV-1–specific IgG2b response reached the plateau phase earlier and remained higher throughout the observation period of 11 wk in WT mice as compared with Jα18 KO mice (Fig. 6B). Strikingly, the HSV-1–specific IgG2c response in WT mice increased over 35 d and remained elevated, whereas this IgG subtype was virtually undetectable in iNKT cell–deficient mice (Fig. 6C). Finally, the HSV-1–specific IgG3 response in WT mice reached its maximum at day 21 p.i. and subsequently declined to a basal level at 7 wk p.i., whereas in iNKT cell–deficient mice, the HSV-1–specific IgG3 response was hardly detectable throughout the observation period (Fig. 6D). In addition, we analyzed the relationship between HSV-1–specific human IgG2 levels and CD4+ iNKT cells in peripheral blood from normal seropositive donors (Fig. 6E, 6F).
A positive correlation could be identified between the percentage CD4\(^+\) NKT cells within the T cell population and the antiviral IgG2 responses (Fig. 6F). Altogether, these results show that iNKT cells are required to generate an IFN-\(\gamma\)-driven IgG subtype profile during HSV-1 infection.

**Discussion**

We observed that in peripheral blood of healthy human donors the serum titer of HSV-1–specific IgG Abs correlate with the proportion of CD4\(^+\) iNKT cells within the iNKT cell population. This iNKT cell subset potently produces both Th1 and Th2 cytokines upon stimulation (19, 20) and is able to provide direct help for B cell proliferation and Ab production in a CD1d-restricted fashion (26). A recent report demonstrated that most iNKT cells that enhance follicular Ab responses surface express the CD4 molecule (27). HSV-1 is known to interfere with CD1d Ag presentation (10, 11, 28) and to impair TCR signaling in iNKT cells (9). In contrast, mice are more resistant to HSV-associated immune evasion mechanisms. This implies that the comparative analysis of iNKT cell–dependent B cell responses against HSV-1 in WT and iNKT cell–deficient mice is not obscured by viral countermeasures. Using CD1d- or iNKT cell–deficient mice, others have shown involvement of iNKT cells in the production of Abs against bacteria and parasites (29–32). In contrast to these pathogens, viruses are not considered to harbor CD1d ligands. Nevertheless, we found that the virus-specific IgM response was significantly reduced in \(J_{a}18\) KO mice as compared with WT mice after HSV-1 infection. Furthermore, there were significantly less HSV-1–specific IgG Abs generated in iNKT cell–deficient mice. Thus, iNKT cells contribute to virus-specific B cell responses in absence of exogenous CD1d ligands, such as \(\alpha\)GalCer.

We detected significantly increased CD1d surface levels on liver B cells after HSV-1 infection of WT mice. It has been shown that \(~50\%\) of the intrahepatic lymphocyte population in adult mice are conventional B cells (33). Moreover, in murine and to a lesser extent in human liver, iNKT cells account for as much as 20–35 and 10–15\%, respectively, of intrahepatic lymphocytes (34). It is conceivable that intrahepatic B cells and iNKT cells interact during HSV-1 infection. In accordance, HSV-1 after i.p. inoculation infects the liver starting at day 2 and may persist in non-neuronal tissue (35). Moreover, intrahepatic follicle-like structures have been observed and are functionally similar to those of lymph nodes with respect to B cell activation, expansion, and maturation (36). Thus, iNKT cell–mediated help for intrahepatic B cells

**FIGURE 4.** Decreased percentage of splenic CCR6\(^+\) iNKT cells in HSV-1–infected WT mice. Single-cell suspensions were made from spleen, liver, and blood of C57BL/6 WT mice at 7 d to 6 mo after HSV-1 infection. Cells were stained for CD3, iNKT TCR, CCR6, and CCR5 surface expression. For detecting iNKT TCR, CD1d-tetramers loaded with the \(\alpha\)GalCer equivalent PBS-57 were used. Percentages of iNKT cells within the CD3\(^+\) cell population are shown in (A). The percentages of CCR6\(^+\) and CCR5\(^+\) iNKT cells are shown in (B) and (C), respectively. Each group consisted of four to six mice, and values are shown correspond to mean ± SEM. Statistical analyses were done applying Prism software (*\(p\) < 0.05, **\(p\) < 0.005, ***\(p\) < 0.001; unpaired Student t test).
might contribute to the production of HSV-1–specific Abs. Conversely, iNKT cells may directly or indirectly upregulate CD1d on B cells. Supporting this view, we did not observe increased CD1d expression on intrahepatic B cells after HSV-1 infection of iNKT cell–deficient mice.

HSV-1 infection apparently leads to redistribution of iNKT cells. The percentage of iNKT cells within the CD3+ cell population 6 mo after HSV-1 infection was increased in the blood but reduced in the spleen. This suggests that HSV-1, which is detected in the spleen after i.p. injection (35), mobilizes iNKT cells from the spleen. In addition, the percentage of CCR6-expressing iNKT cells within the total iNKT cell population in the spleen decreased 6 mo p.i. CCR6 is required for trafficking to epithelial cells (37) and represents a Th1-associated chemokine receptor that mediates recruitment of inflammatory iNKT cells (24, 38). Besides iNKT cells, subsets of dendritic cells (DCs) and most B cells also express CCR6. Thus, CCL20, the single chemokine ligand for CCR6, could recruit these immune cells to epithelial or lymphoid tissue via peripheral blood where they interact.

Several mechanisms could be involved in activation of iNKT cells during HSV-1 infection. So far, a viral ligand for CD1d has not been identified but iNKT cell activation by an endogenously produced lipid Ag induced by danger signals has been demonstrated (39, 40). Moreover, viral danger signals promote synthesis of CD1d molecules in DCs resulting in a Th1-like iNKT cell response (41). In addition, B cells express CD1d (21) and could play a direct role in iNKT cell activation after HSV-1 infection. Finally, iNKT cells can be activated in a CD1d-independent manner by virus-induced IL-12 or type I IFN similar to NK cells (42).

We revealed a prominent role for iNKT cells in shaping the IgG subtype profile of HSV-1–specific Abs. In contrast to WT mice, hardly any HSV-1–specific IgG2c and IgG3 Abs were detectable in iNKT cell–deficient mutants. This is best explained by insufficient production of IFN-γ because this cytokine selectively facilitates the switch to IgG2a/c and IgG3, whereas IgG1 and IgG2b are promoted by IL-4 and TGF-β, respectively (25). In accordance, we observed that in the absence of CD1d-restricted cells naive lymphocytes produced far less IFN-γ in response to HSV-1. Thus, during persistent infection with HSV-1, CD1d-restricted T cells may serve as an important source for IFN-γ that has been shown to play a crucial role in the control of neurotropic viruses such as HSV-1 (43). iNKT cell–dependent production of IgG2a/c and IgG3 subtypes also was observed in a study analyzing the Ab response to a model haptenated lipid molecule (44). Activation of iNKT cells modulates function of Th2 cells in such a way that they produce more IFN-γ and less Th2 cytokines (45). This effect could also contribute to production of IgG2a/c and IgG3 during HSV-1 infection.

It has been shown that iNKT cells can provide B cell help in a cognate fashion (27, 44, 46, 47). Accordingly, B cells may capture HSV-1 particles through their specific BCR. Subsequently, the same B cell may stimulate iNKT cells via CD1d molecules that present an endogenous or a still unknown virus-associated lipid Ag resulting in iNKT cell–mediated specific B cell help. In accordance, iNKT cell–dependent Ab responses have been demonstrated after internalization of lipid model Ags through the BCR (44, 46). The observation that coadministration of αGalCer can compensate for the absence of Th cells during vaccine-induced Ab responses (48) is compatible with a direct effect of iNKT cells on B cells.

Alternatively, iNKT cells may act indirectly through activation of bystander APCs, which subsequently activate Th cells (49). In line with this view, HSV-1 is a strong inducer of DC apoptosis (50, 51). The apoptotic debris has to be phagocytosed and presented by uninfected bystander DCs (52) to counteract this viral immune evasion mechanism (53). However, these cross-presenting DCs may require iNKT cells for full maturation and subsequent stimulation of HSV-1–specific CD4+ Th1 cells. In accordance, iNKT cells have been shown to enhance the T cell stimulatory capacity of DCs (54). Finally, iNKT cells may influence Ab production through controlling the maintenance of memory Th cells (45).

The iNKT-dependent polarization of the HSV-1–specific B cell response is of biological relevance because HSV-1–specific IgG2a/c Abs exert a more profound neutralizing capacity than HSV-1–specific IgG1 Abs (55). Thus, the lack of a Th1-polarized B cell response could explain the more prominent HSV-1–induced zosteriform lesions and impaired virus clearance that is observed in iNKT cell–deficient mice (13, 56). Supporting this view, neutralizing Abs can interfere with the development of HSV-1–induced zosteriform lesions (57). In addition, the HSV-1–specific IgG subtype profile in humans is Th1-polarized (58), which supports the involvement of IFN-γ in the observed iNKT driven isotype switching to IgG2. Thus, for the development of an efficient human vaccine, it will be mandatory to efficiently activate iNKT cells to produce sufficient IFN-γ such that it skews the IgG subtype profile in the right direction. In this regard, a more natural CD1d ligand than αGalCer could be of advantage as hyperstimulation of iNKT cells concomitantly results in secretion of large amounts of Th2-related cytokines, such as IL-4 and which could impair B cell memory (59).
Disclosures

The authors have no financial conflicts of interest.

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Supplementary figure 1. (A) B16 (murine melanoma) cells, CD1d-expressing B16 transfectants, and B cells isolated from spleens of C57BL/6 mice were infected with HSV-1 at different MOI, loaded with αGalCer (100 ng/ml), and co-cultured with iNKT hybridoma cells DN3A4-1.2 at a ratio of 1:5. After 24 h the concentration of murine IL-2 (mIL-2) in the supernatant was determined by ELISA. (B) In parallel CD1d-expressing human (HeLa) transfectants were treated exactly in the same way. The y-axis shows the amount of IL-2 released by 1.2 iNKT hybridoma cells whereas the x-axis gives the MOI used for infection with HSV-1. Experiments were done in triplicates and values shown correspond to mean ± SD. One experiment out of three is shown.
Supplementary figure 2. CD80 expression on peripheral B cells isolated from HSV-1 infected WT and Jα18 KO. At the time points indicated, blood was drawn from the tail vein of HSV-1-infected mice and uninfected control mice. Cells were stained with fluorochrome-conjugated antibodies to CD19 and CD80. Subsequently, the MFI of CD80 expression on CD19+ cells was measured by flow cytometry. The y-axis shows fold change of CD80 expression on B cells from HSV-1 infected animals as compared to B cells from uninfected control mice. Each experimental group of mice consisted of 5-6 animals. Statistical analyses were done applying Prism software.