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Contact-dependent interference with invariant NKT cell activation by HSV-infected cells

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Abstract

Invariant CD1d-restricted natural killer T (iNKT) cells play important roles in generating protective immune responses against infections. Here, we have investigated the role of human iNKT cells in HSV-1 infection and their interaction with epidermal keratinocytes. These cells express CD1d and are the primary target of the virus. Keratinocytes loaded with α -GalCer could stimulate IFN- γ production and CD25 upregulation by iNKT cells. However, both α -GalCer- and cytokine-dependent activation of iNKT cells was impaired after coculture with HSV-1-infected cells. Interestingly, CD1d downregulation was not observed on infected keratinocytes, which were also found to inhibit TCR-independent iNKT cell activation. Further examination of the cytokine profile of iNKT/keratinocyte cocultures showed inhibition of IFN- γ , IL-5, IL-10, IL-13 and IL-17 secretion, but upregulation of IL-4 and TNF- α following the infection. Moreover, cell-to-cell contact between infected keratinocytes and iNKT cells was required for the inhibition of activation, as the cell-free supernatants containing virus did not affect activation. Productive infection of iNKT cells was however not required for the inhibitory effect. Following coculture with infected cells, iNKT cells were no longer responsive to further stimulation with α -GalCer-loaded CD1d. We found that exposure to HSV-1-infected cells resulted in impaired TCR signalling downstream of ZAP70. Additionally, infected cells upregulated the expression of the negative T cell regulator, galectin-9; however, blocking experiments indicated that the impairment of iNKT cell responses was independent of galectin-9. Thus, interference with activation of human iNKT cells by HSV-1 may represent a novel immunoevasive strategy utilized by the virus to avoid immune clearance.

Introduction

HSV causes life-long infections that are characterised by the alternating periods of latency in neuronal ganglia and reactivation in peripheral tissues such as skin and mucosa. HSV infections, of which the commonest are cold sores and genital herpes, can vary in frequency and severity from asymptomatic virus shedding to severe ulcerations. The exact host or viral factors that contribute to these differences are poorly defined. However, levels of local cellular immunity in the host appear to be important for the resolution of HSV infections in the skin and mucosa (1, 2). Therefore, timely activation and recruitment of virus-specific CD4⁺ and CD8⁺ T cell effectors by innate immune components that respond rapidly at the onset of infection is required.

Natural killer T (NKT) cells, a unique subset of lymphocytes with innate-like functions, have been suggested to provide the necessary activation signals leading to improved cellular antiviral responses (3, 4). NKT cells, which co-express various T and NK cell markers, recognise self and non-self glycolipid antigens that are presented on the non-polymorphic MHC class I-like molecule, CD1d (5). The majority of human NKT cells express an invariant TCR, which consists of V α 24 chain preferentially paired with V β 11 chain. As the identity of the endogenous glycolipid ligand that is responsible for iNKT cell selection and development in thymus remains elusive, characterisation of several exogenous glycolipids that iNKT cells can respond to, such as α -galactosyl ceramide (α -GalCer) and its derivatives, has greatly facilitated the research into the role and function of iNKT cells in health and disease (6, 7). Although iNKT cells can display cytotoxic activity much like the NK cells (8-10), their major function is thought to be rapid release of cytokines leading to stimulation or suppression of immune reactions (11). Within hours of activation, iNKT cells secrete copious amounts of immunopolarising cytokines, such as IFN- γ , IL-4 and TNF- α , which in turn can recruit and activate other immune cell types such as neutrophils, dendritic cells, conventional T cells, B cells and NK cells (11-16). This ability to respond rapidly at the onset of the immune response underscores the iNKT cells' role in immune response regulation. Consequently, iNKT cells have been demonstrated to play a critical role in several immune processes, from prevention of autoimmunity to protection against various pathogens, including bacteria and viruses (17). iNKT cells can directly respond to CD1d-bound glycolipids of certain bacteria, such as *Borrelia burgdorferi* or some members of *Sphingomonas* species (18-21). However, as viruses do not encode for unique glycolipids, indirect modes of iNKT cell activation have been proposed (22). In such setting, iNKT cells can respond to self-glycolipids in the presence of innate cytokines, such as IL-12 and IL-18, secreted by virus infected antigen-presenting cells (22-26).

Several lines of evidence point to the importance of iNKT cells in HSV infection. iNKT cells are an early source of IFN- γ in herpes infection (27), which is important for the T cell-mediated suppression of HSV (28-30). Increased disease severity and impaired clearance of HSV has been observed in iNKT cell deficient mice (31, 32), although the contribution of iNKT cells to disease resolution may depend on the virus strain as shown by Cornish et al. (33). More recently, however, two human *in vitro* studies have provided indirect evidence for the role of iNKT cells in anti-HSV immunity. HSV-1 was shown to downregulate CD1d expression on the surface of infected dendritic cells and monocytes (34, 35). This is, at least in part, due to two viral proteins, Us3 and gB, which act to inhibit CD1d recycling (36). Indeed, other viral pathogens including HIV-1, vaccinia virus and Kaposi's sarcoma-associated herpesvirus also have the capacity to interfere with CD1d antigen presentation (37-40). However, whether HSV-1 can also inhibit iNKT cell activation directly has not been addressed to date. As HSV infection is primarily restricted to the epidermal layers, we were interested in examining the effects of HSV on skin-resident CD1d-expressing keratinocytes and how this infection might modulate the ability of iNKT cells to generate an appropriate antiviral immune response.

Materials and methods

Culture of iNKT cells

PBMCs of healthy donors were cultured in RPMI1640 (Invitrogen, Paisley, UK) supplemented with 10% FCS (Invitrogen), 2.05 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES (Hyclone, Logan, UT), and 100 ng/ml of α -GalCer (Enzo Life Sciences, Plymouth Meeting, PA) in order to stimulate proliferation of donor iNKT cells. After initial 24 h of culture, the medium was supplemented with 10 ng/ml of recombinant human IL-2 (Peprotech, London, UK). Ten to 14 days later, iNKT cells were purified by immunomagnetic cell sorting. Briefly, cells were incubated with a biotinylated

anti-TCR V α 24 mAb (Beckman Coulter, Marseille, France) for 30 min at 4°C, washed twice, then incubated with streptavidin-conjugated MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for further 15 min at 4°C, washed once then purified using a MACS LS column (Miltenyi Biotech). The purity of isolated iNKT cells was assessed by flow cytometry and routinely exceeded 95%. Purified cells were restimulated with gamma-irradiated (40 Gy) allogeneic monocytes loaded with α -GalCer and maintained in culture medium supplemented with IL-2 (8).

Primary human keratinocyte culture

Human skin was obtained after consent from patients undergoing breast reduction surgery. Keratinocytes were isolated as previously described with minor modifications (41). Briefly, adipose and breast tissue were removed from skin and sheets passed through a skin graft mesher (Zimmer, Swindon, UK). Skin was then incubated epidermis-side down for 90 min at 37°C in 1.2 WU/ml of dispase (Roche Diagnostics, Indianapolis, IN). The epidermis was separated from the dermis, washed and incubated for 30 min at 37°C in 0.25% trypsin versene solution (Invitrogen). Single cell suspensions obtained after the trypsin digest were resuspended in EpiLife medium to which human keratinocyte growth factor supplement (Invitrogen) and 50 μ g/ml of gentamycin (Sigma) were added. A total of 5×10^5 cells were seeded in a 75-cm² flask and maintained in EpiLife medium. The cultures were split into fresh flasks when keratinocytes reached 70-80% confluency.

Culture of CD1d expressing cell lines

To obtain stable CD1d transfectants, the CD1d gene was cloned into the pcDNA3.1/Zeo(1) expression vector (Invitrogen) and transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Stable 293T cells expressing CD1d were selected using 50 μ g/ml zeocin (Invitrogen) and CD1d expression was confirmed by flow cytometry.

Virus

HSV-1 (strain F) stocks were prepared by infecting 80–90% confluent monolayers of African green monkey kidney (VERO) cells. Infected cells were incubated in DMEM (Invitrogen) supplemented with 5% FCS and were harvested after 2 days and subjected to two freeze-thaw cycles to lyse cells. After centrifugation at $20,000 \times g$ for 10 min to pellet cell debris, clarified supernatant was aliquoted and stored at -80°C until use. Virus titers were determined by plaque assay on VERO cell monolayers. Briefly, 10-fold dilutions of virus were adsorbed onto VERO cells for 1 h at 37°C. The inoculum was then removed and fresh medium containing 0.5% carboxymethyl cellulose (Sigma) was added. Two days later, cells were fixed and stained with a 0.1% crystal violet solution to determine the number of plaques.

Antibodies and fluorescent reagents

Expression of surface and intracellular markers on iNKT cells and keratinocytes was assessed with multicolour flow cytometry using following mAbs: CD3 (IgG1, clone UCHT1, Pacific Blue, FITC and APC conjugate) from Dako (Glostrup, Denmark), V α 24 (IgG1, clone C15, FITC, PE and APC conjugate) and TCR V β 11 (IgG2a, clone C21, FITC and PE conjugate) from Beckman Coulter, IFN- γ (IgG2b, clone 25723.11, PE and APC conjugate), CD1d (IgG1, clone CD1d42, PE conjugate), CD25 (IgG1, clone 2A3, PE conjugate), galectin-1 (IgG1, clone 6C8.4-1) from Invitrogen. Rabbit polyclonal galectin-9 antibody was from Abcam (Cambridge, UK), and the rat anti- T cell immunoglobulin mucin 3 (TIM-3) mAb was from R&D Systems (IgG2a, clone 344823, PE conjugate, Minneapolis, MN). Virus infection was assessed using a viral glycoprotein specific mAb (IgG2a, Virostat,

FITC conjugate, Portland, ME). Cell viability was assessed using the near-IR Live/Dead stain and annexin V-Pacific Blue (both from Invitrogen).

Coculture experiments

Keratinocytes were infected at multiplicity of infection (MOI) of 5 for 24 h before being cocultured with iNKT cells for further 6 to 18 h at 37°C. In some experiments, keratinocytes and iNKT cells were separated by a 3- μ m pore membrane (Costar). For α -GalCer stimulation experiments, keratinocytes were incubated with 100 ng/ml α -GalCer one hour prior to HSV-1 infection. For cytokine-mediated stimulation of iNKT cells exogenous IL-12 (100 ng/ml, PeproTech) and IL-18 (200 ng/ml, R&D Systems) were added for the duration of the coculture. In blocking experiments, iNKT cells or keratinocytes were incubated with 10 μ g/ml of TIM-3 antibody 2E2 (R&D Systems) 1 h prior to coculture with infected cells.

Cytokine analysis from cell culture supernatants

NKT cells were cultured alone or in contact with uninfected or HSV-1-infected keratinocytes with or without α -GalCer as detailed above. Cell-free supernatants were collected in duplicate at 24 h and 48 h after coculture and used in a multiplex cytokine assay (Invitrogen). Supernatants of iNKT cells stimulated with immobilised CD3 mAb and soluble CD28 were used as a positive control. Supernatants from cultures containing keratinocytes alone were also included in the assay.

Phospho-specific flow cytometry

TCR-induced signalling events were examined by flow cytometry according to the manufacturer's protocol (BD Phosflow). Briefly, iNKT cell activation was triggered by TCR cross-linking with a purified mouse anti-human CD3 mAb (UCHT1, 10 μ g/ml, BD) and goat-anti mouse Ig (5 μ g/ml, BD). The activation was terminated by fixation in BD Cytotfix solution for 15 min. Cells were then washed, permeabilised with BD Phosflow Permeabilisation Buffer III and labelled with phospho-specific antibodies for pZAP70/Syk (Tyr319, PE conjugate, BD Phosflow), pSLP-76 (Tyr128, Alexa Fluor 488 conjugate, BD Phosflow) and pERK1/2 (Thr202/Tyr204, Alexa Fluor 647 conjugate, Cell Signalling Technologies). Cells were acquired on BD LSR Fortessa flow cytometer (BD Immunocytometry Systems) followed by analysis using FlowJo software (Tree Star, Ashland, OR).

Results

HSV-1 inhibits activation of iNKT cells by epidermal keratinocytes

Keratinocytes are highly susceptible to HSV-1 infection and are the major virus target during primary and recurrent herpes infection. Since keratinocytes express CD1d on their surface (42), we set out to determine the effects of HSV-1 infection on CD1d expression on human epidermal keratinocytes and their ability to activate iNKT cells. As expected, keratinocytes induced iNKT cell activation as measured by IFN- γ production. iNKT cell activation by keratinocytes was dependent on the presence of α -GalCer, as iNKT cells exposed to unpulsed keratinocytes did not produce any IFN- γ (Fig. 1A). In contrast, α -GalCer-dependent production of IFN- γ and activation, as measured by CD25 upregulation, was inhibited in cocultures with HSV-1-infected keratinocytes (Fig. 1A and 1B). Several studies have demonstrated that the requirement for exogenous glycolipid antigens such as α -GalCer for the activation of iNKT cells can be overcome in the presence of pro-inflammatory cytokines such as IL-12 and/or IL-18 (22-26). In this scenario, iNKT cells recognise endogenous glycolipids bound to CD1d. Thus, we examined if HSV-1 infection also affected cytokine-driven iNKT cell activation. Indeed, whilst the cytokines IL-12 and

IL-18 induced iNKT cell activation even in the absence of α -GalCer, iNKT cells cocultured with HSV-1-infected keratinocytes failed to upregulate their CD25 expression (Fig. 1B, middle panel). Moreover, CD1d-independent iNKT cell activation by PMA/ionomycin was also inhibited in the presence of HSV-1-infected keratinocytes (Fig. 1B, lower panel). Interestingly, CD25 upregulation was absent from iNKT cells which were pre-activated with immobilized CD3 mAb for 4 and 6 h prior to coculture with infected keratinocytes (Fig. 1C). This indicated that HSV-1 can also interfere with prior TCR-mediated activation. As HSV-1 infection can lead to apoptosis of conventional T lymphocytes (43, 44), we considered this as a possible explanation for the inability of iNKT cells to respond following α -GalCer stimulation. However, no induction of apoptosis, measured by annexin V expression, was observed after the exposure of iNKT cells to either cell-free HSV-1 (MOI 10) or HSV-1-infected keratinocytes (Fig. 1D).

HSV-1 has previously been shown to interfere with CD1d recycling and thereby its expression on the surface of antigen-presenting cells (34, 36). Thus, it was possible that the observed impairment of iNKT cell activation was due to the same mechanism. Indeed, we were able to confirm the previous findings on HSV-1-infected dendritic cells (Fig. 1E). In contrast, HSV-1 infection of keratinocytes did not result in the inhibition of CD1d expression (Fig. 1E), suggesting that this effect may be cell type dependent (Fig. 1E). Collectively, these data suggest a novel strategy utilised by HSV-1 to interfere with iNKT cell activation.

HSV-1 alters the cytokine pattern of iNKT cells following stimulation with α -GalCer

The above results demonstrate that IFN- γ production was inhibited by HSV-1. Thus, it was of interest to determine if the virus caused a general suppression of cytokine secretion or a change in the pattern of cytokine release. To this end we tested cell-free supernatants from iNKT cell/keratinocyte cocultures in the presence or absence of α -GalCer as well as supernatants from iNKT cells stimulated with CD3 antibody using the Luminex technology. The multiplex cytokine assay confirmed the initial data that IFN- γ production was suppressed by HSV-1 (Fig. 2). In contrast, production of IL-4 and TNF-alpha was increased in HSV-1-infected cocultures, which suggested a reversal of the pattern of cytokine release (Fig. 2). Interestingly, IL-10 production was also inhibited by the virus as was the release of several other Th1- and Th2-type cytokines including IL-5, IL-13 and IL-17 (Fig. 2). These results show that HSV-1 alters the cytokine pattern of iNKT cells and inhibits the secretion of soluble factors that are critical for the adequate protection against herpes infection.

NKT cells are resistant to HSV-1 infection

Since HSV-1 infected keratinocytes did not display reduced levels of CD1d on their surface, a possible explanation for the failure of iNKT cells to respond to α -GalCer stimulation could be their direct infection by HSV-1. Activated T lymphocytes have previously been shown to be susceptible to HSV-1 infection (45). Therefore, we examined if human iNKT cells could support HSV-1 infection. Infection was detected using a mAb against one of the structural proteins of HSV-1, which is typically expressed on the surface of infected cells following viral DNA replication. No expression of viral antigens was observed by flow cytometry when iNKT cells were infected with cell-free HSV-1 (Fig. 3A). The resistance of iNKT cells to HSV infection was further demonstrated by the lack of viral protein expression by iNKT cells even in the presence of high titres of cell-free virus, compared to keratinocytes, which were highly susceptible (Fig. 3A).

However, when iNKT cells were cocultured in direct contact with HSV-1-infected keratinocytes, greater than 50% of iNKT cells were viral antigen positive as detected by flow cytometry (Fig. 3B). In contrast, there was little or no expression of the viral antigen by

iNKT cells if they were separated from keratinocytes by a Transwell membrane (Fig. 3B). These differences could be explained by the ability of HSV-1 to infect more efficiently via cell-to-cell contact rather than as cell-free virions. However, there was still a possibility that the viral antigen detected on the surface of iNKT cells was not due to *de novo* production in iNKT cells but rather due to transfer of viral proteins from the membrane of keratinocytes. Expression of the majority of viral glycoproteins and other structural proteins is dependent on viral DNA replication. Viral DNA polymerase inhibitors can effectively block this step in virus replication. Therefore, to examine if the expression of viral glycoprotein on the surface of iNKT cells was due to direct infection, iNKT cells were incubated with infected keratinocytes in the presence of a viral DNA polymerase inhibitor, phosphonoacetic acid (PAA). Viral antigen expression on iNKT cells was unaltered by the presence of PAA, indicating that the expression was not due to productive infection (Fig. 3B). Together, the above results show the resistance of human iNKT cells to HSV-1 infection, and suggest that the inhibition of iNKT cell activation is a distinct mechanism mediated by permissive cells such as keratinocytes.

HSV-1-infected keratinocytes induce a sustained inhibition of iNKT cell activation

Next, we were interested if iNKT cell inactivation was sustained beyond contact with HSV-1-infected cells. Thus, we transferred iNKT cells after 24-h incubation with uninfected or infected keratinocytes to cultures containing α -GalCer-pulsed CD1d-expressing 293T cells. iNKT cell activation was inhibited only in cocultures where direct contact between the infected keratinocytes and iNKT cells was allowed (Fig. 3A). In contrast, when iNKT cells were separated from the keratinocytes by a membrane, only allowing contact with the cell-conditioned medium and cell-free virus, no inhibition of IFN- γ production was seen (Fig. 4B). These results collectively indicate that inhibition of iNKT cell activation was a specific event dependent on close cell-to-cell contact and that this inhibition was sustained following the removal of HSV-1-infected cells.

HSV-1 infection of keratinocytes induces galectin-9 expression

Since inhibition of iNKT cell activation was mediated via close contact with HSV-1-infected cells in the absence of direct virus infection of iNKT cells (Fig. 4), we were interested in possible factors expressed by infected keratinocytes that could affect iNKT cell activation. A number of recent studies have revealed several members of galectin family as important regulators of cellular immunity (46-48). Two of the members, namely galectin-1 and galectin-9, have been shown to suppress Th1 responses through inhibition of IFN- γ secretion in activated Th1-type T lymphocytes (46-48). Thus, we examined the expression patterns of the two galectins by keratinocytes. We found that while there was no significant expression of either galectin on the surface of uninfected keratinocytes, there was a significant upregulation of galectin-9 but not galectin-1 following HSV-1 infection (Fig. 5A). We confirmed the flow cytometry findings with fluorescence confocal microscopy, which revealed that galectin-9 was specifically expressed by HSV-1-infected keratinocytes with perinuclear, cytosolic and membrane staining (Fig. 5B). Inhibition of IFN- γ secretion by galectin-9 is believed to be mediated through its binding to TIM-3, which is expressed on activated but not naïve T lymphocytes (46, 47). Therefore, we examined the relative expression of TIM-3 on purified iNKT cells. Resting iNKT cells already had a detectable expression of TIM-3 on the surface, consistent with their constitutive “memory” phenotype. As has been shown previously for conventional T cells (46), iNKT cell activation lead to upregulation of TIM-3 expression (Fig. 5C). However, TIM-3 blockade using the mAb 2E2 (49, 50) and galectin-9 blockade using the mAb 9MI-3 (50), failed to rescue the production of IFN- γ (Fig. 5 and Supplementary Fig. 1), suggesting that other inhibitory signals were responsible for the inhibition of iNKT cell activation.

HSV-1 inhibits ERK activation in iNKT cells

Considering that inhibition of IFN- γ response occurred after contact with infected cells, we wanted to examine if there was interference with the TCR signal transduction in iNKT cells. Using phospho-specific flow cytometry, we examined activation of TCR proximal factor ZAP70 and its substrate SLP76 following the engagement of TCR receptor and subsequent ERK phosphorylation, which was shown to be required for TCR-mediated IFN- γ secretion (51-53). Our results indicate that the levels of ZAP70 and SLP76 activation were unchanged by the virus, whereas ERK1/2 phosphorylation was reduced (Fig. 6). This was also reflected in the reduced CD25 upregulation 24 h later (data not shown). These results indicate that the contact with HSV-1-infected cells directly suppresses iNKT cell activation via the TCR downstream of ZAP70.

Discussion

HSV has developed multiple strategies to avoid immune recognition, from inhibition of MHC class I and II presentation to interference with dendritic cell maturation (54). Here, we show yet another mechanism of immune evasion that directly targets iNKT cells. Coculture with HSV-infected keratinocytes rapidly led to inhibition of iNKT cell activation and IFN- γ secretion. Considering the importance of IFN- γ in the control of HSV infection, interference with its production, especially in the early stages of the response, represents a powerful immunoevasive strategy. Inhibition of IFN- γ secretion by HSV-1 in conventional T lymphocytes has been attributed to selective production of IL-10 (55). However, in our experiments IL-10 production, which was induced in response to α -GalCer, was inhibited by the virus suggesting a distinct interference mechanism. Indeed, production of other cytokines was inhibited in HSV-1-infected cocultures including IL-5, IL-13 and IL-17. In contrast, the levels of TNF- α and IL-4 were raised in HSV-infected cocultures. This increase in the two cytokines could be speculated to be of some benefit to the host at the site of herpetic lesion by acting on infiltrating macrophages and/or resident dendritic cells and promoting their activation (56). These antigen-presenting cells may subsequently enhance activation of lymphocytes, including iNKT cells, through secretion of pro-inflammatory cytokines such as IL-12 and IL-18 (57-59). However, our data demonstrated that HSV-1 is also able to prevent this cytokine-driven iNKT cell activation. Although HSV can inhibit CD1d expression on antigen-presenting cells (34, 35), we found that inhibition of iNKT cell activation was a distinct phenomenon, since iNKT cells exposed to HSV-infected cells did not respond to further stimulation. Interestingly, this occurred in the absence of productive infection as we found that iNKT cells did not support virus replication even at high MOI. The fact that HSV-1-mediated inhibition of iNKT cell activation is independent of CD1d downregulation was further supported by the observation that PMA/ionomycin-induced activation was inhibited by HSV-1 and that the activation of ZAP70 and SLP76, which are proximal to the TCR, was unaffected by the virus. In contrast, ERK1/2 activation was inhibited following co-incubation with the infected cells. This is in agreement with the observed reduction in IFN- γ production by iNKT cells, as its synthesis in response to TCR engagement has been shown to be dependent on signal transduction through MEK/ERK pathway (51-53). In conventional T lymphocytes, HSV-1 was shown to inhibit LAT phosphorylation which also leads to inhibition of signalling through ERK (60), however, we have not been able to obtain reliable detection of LAT phosphorylation to confirm that the same is true for iNKT cells. TCR activation can lead to LAT-independent ERK activation (61, 62), suggesting that HSV-1 may target other parts of the TCR signalling cascade in order to inhibit iNKT cell activation. Our results also indicate that the virus is able to override prior TCR-mediated activation of iNKT cells which may have implications for the possible anti-herpes therapies utilizing glycolipids.

Considering that close cellular contact was required for the inhibition of IFN- γ secretion, we focused our attention on putative inhibitory receptors that could be expressed on infected cells and suppress iNKT cell activation. We discovered that HSV-1-infected cells upregulate expression of galectin-9, a β -galactosidase-binding protein, which negatively regulates cellular immunity. Galectin-9 binding to its receptor TIM-3 on the surface of activated conventional T lymphocytes leads to multiple inhibitory effects including suppression IFN- γ secretion and apoptosis (48). In addition, in chronic HIV-1 and HCV infections, exhausted CD8⁺ T cells express elevated levels of TIM-3 (49, 63). More recently, induction of galectin-9 was also seen in HSV infection in mice, and animals deficient in galectin-9 had improved antiviral CD8 T cell responses (64). Although we found that human iNKT cells expressed TIM-3, our blocking experiments failed to prove that this axis was responsible for the inhibition of iNKT cell responses. While the reasons for this are unclear at present, it is possible that the signalling events that lead to suppression of activation of iNKT cells vary from those of conventional T lymphocytes. Support for this type of scenario comes from studies in chronic HIV-1 infection in which elevated expression of inhibitory receptor PD-1 is observed in functionally impaired CD8 T lymphocytes as well as iNKT cells (65-67). In these studies, blockade of PD-1 and its ligands, PD-L1 and PD-L2, led to improved CD8 T cell responses but failed to restore iNKT cell responses. Interestingly, we have also examined the contribution of the PD-1 in the suppression of iNKT cell activation by HSV-infected cells, but did not see any effect of receptor blockade (alone or in combination with TIM-3 blocking) on restoration of IFN- γ (Supplementary Fig. 1). However, another explanation for the inability of TIM-3 and/or PD-1 blocking to rescue the IFN- γ production by iNKT cells may be that inhibition mediated by HSV may be due to another more dominant inhibitory pathway. Optimal activation of Th1 responses by iNKT cells appears to be dependent on the localisation of CD1d/glycolipids complexes to lipid rafts (68). Therefore, it can be speculated that HSV-1 infection of keratinocytes may lead to redistribution of CD1d away from such structures and thus compromise the adequate iNKT activation. While iNKT cells were resistant to productive infection by cell-free HSV-1, we cannot exclude the possibility that the virus entered the cells. This is a likely scenario, as we observed transfer of viral glycoproteins from infected cells to iNKT cells even in the presence of a viral DNA replication inhibitor. It is thus possible that the viral structural proteins may be involved in the inhibition of iNKT cell activation. This has been shown for conventional T lymphocytes where transfer of virus from infected fibroblasts lead to inhibition of T cell activation (60).

The presence of multiple pathways of iNKT cell interference such as inhibition of activation and CD1d downregulation suggests an important role for these cells in the defence against HSV infection. As evidence for the role of iNKT cells in human HSV infection is lacking to date, data from murine models are our only reference. In this system, contribution of iNKT cells in the control of HSV is still open to debate, as conflicting data exist (31-33). There is no information available on the effects of HSV infection on CD1d expression in the mouse or the effects of such infection on murine iNKT cells. However, assuming such immunoevasive mechanism exist in the murine system, the general contribution of iNKT cells to the resolution of herpes infection may be underrated. A good evidence for the possible adjuvant role of iNKT cells in HSV infection comes from a recent study which showed that α -GalCer, when used as adjuvant, induces highly protective immune responses to genital HSV challenge (69). Together, these findings underscore the importance of iNKT cells in the generation of effective anti-HSV immune responses and further highlight the complex interactions between a highly host-adapted pathogen, such as HSV-1, and the immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

α-GalCer	α-galactosyl ceramide
MOI	multiplicity of infection
NKT cells	natural killer T cells
PAA	phosphonoacetic acid
TIM-3	T cell immunoglobulin mucin-3

FIGURE 1.

HSV-1 inhibits CD1d-mediated iNKT cell activation by keratinocytes. Keratinocytes were either left uninfected or infected with HSV-1 at MOI of 1 in the presence or absence of α -GalCer. After 18 h, the cells were harvested, washed and resuspended in culture medium. (A) IFN- γ production by iNKT cells after coculture with keratinocytes. Uninfected or HSV-1-infected keratinocytes were cocultured for 6 h at 1:1 ratio with purified iNKT cells in the presence of brefeldin A. (B) Expression of activation marker CD25 on purified iNKT cells following 24-h culture with unpulsed or α -GalCer-pulsed keratinocytes by flow cytometry in the absence or presence of PMA/ionomycin or the combination of exogenous IL-12 and IL-18 (100 ng/ml and 200 ng/ml, respectively). (C) Expression of activation marker CD25 on purified iNKT cells which were activated via CD3/CD28 mAb stimulation for 4 h or 6 h prior to coculture with uninfected or HSV-1-infected keratinocytes for further 24 h. (D) iNKT cells' viability after 24 h incubation with 10 MOI of cell-free HSV-1 or HSV-1-infected keratinocytes. Viability was assessed by flow cytometry using staining with Pacific Blue-conjugated Annexin V and LIVE/DEAD near IR fixable stain. Events shown in the above plots were from the CD3⁺ gate. (E) Expression of CD1d by keratinocytes was analysed by flow cytometry using surface staining and compared to the expression found on uninfected and HSV-1-infected (5 PFU/cell) monocyte-derived dendritic cells. The results are representative of three independent experiments.

FIGURE 2. Cytokine profile of keratinocyte/NKT cell cocultures. Cell-free supernatants from iNKT cells activated with CD3/CD28 mAbs or iNKT cell/keratinocyte cocultures in the presence or absence of HSV-1 and/or α -GalCer were collected in duplicate at 24- and 48-h time points and various cytokine levels assessed in a multiplex cytokine assay. Representative results of one out of two donors are shown. Vertical bars represent standard deviation. White columns, 24-h time point. Black columns, 48-h time point.

FIGURE 3.

HSV-1 transfer from keratinocytes to iNKT cells occurs via cell-to-cell contact. (A) The proportion of HSV-1-infected iNKT cells and keratinocytes after 18 h infection with cell-free HSV-1. (B) Expression of viral antigens by iNKT cocultured with keratinocytes. Keratinocytes were either left uninfected or infected for 18 hours with HSV-1 at MOI of 1. The cells were then harvested, washed and cocultured for 24 h at 1:1 ratio with purified iNKT cells. Some keratinocytes were separated from iNKT cells by a 3.0- μ m Transwell membrane to prevent cell-to-cell contact or cultured in the presence of PAA to inhibit virus replication. Infection of iNKT cells was analysed by flow cytometry. Grey-tinted histograms, iNKT cells exposed to uninfected keratinocytes; clear histograms, iNKT cells exposed to HSV-1-infected keratinocytes. The results are representative of three independent experiments.

FIGURE 4.

Direct contact to HSV-1-infected keratinocytes renders human iNKT cells unresponsive. Activation of iNKT cells after co-culture with uninfected or HSV-1-infected keratinocytes for 24 h either in direct contact (A) or separated by a 3- μ m Transwell membrane (B). iNKT cells were then mixed with untreated or α -GalCer-pulsed CD1d-transfected 293T cells at 1:1 ratio in the presence of brefeldin A for 6 h. iNKT cell activation was assessed by flow cytometry. The results are representative of three independent experiments.

FIGURE 5.

HSV-1 infection induces galectin-9 expression by keratinocytes. Keratinocytes were either left uninfected or infected with HSV-1 at MOI of 1 for 18 hours. (A) Galectin-1 and -9 expression by uninfected and HSV-1-infected keratinocytes. (B) Cellular localisation of galectin-9 in HSV-1-infected keratinocytes. Harvested keratinocytes were cytospun onto glass slides, labelled with HSV-1 and galectin-9 antibodies and analysed by fluorescence confocal microscope (60x objective). (C) TIM-3 expression by iNKT cells before and after coculture with keratinocytes. (D) The effect of TIM-3 blocking on IFN- γ production by iNKT cells in coculture with keratinocytes. iNKT cells were pre-incubated with 10 μ g/ml of 2E2 mAb before coculture with keratinocytes. IFN- γ production was assessed 6 h later. The results are representative of three independent experiments.

FIGURE 6.

Contact with HSV-1-infected cells inhibits TCR signalling. Uninfected or HSV-1-infected cells were cocultured with iNKT cells for 2 h followed by TCR cross-linking with a UCHT-1 mAb. Activation of ZAP70, SLP76 and ERK1/2 were assessed using phospho-specific mAbs at 1 and 10 min after cross-linking, respectively. TCR cross-linking and PMA/ionomycin served as a positive control for ZAP70, SLP76 and ERK1/2, respectively. The results are representative of three independent experiments.