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_J Immunol_ 2012; 188:4158-4170; Prepublished online 30 March 2012; doi: 10.4049/jimmunol.1103450

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_The Journal of Immunology_ is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Herpes Simplex Virus Antigens Directly Activate NK Cells via TLR2, Thus Facilitating Their Presentation to CD4 T Lymphocytes

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NK cells infiltrate human herpetic lesions, but their role has been underexplored. HSV can stimulate innate immune responses via surface TLR2, which is expressed on monocyte-derived dendritic cells (DCs) and NK cells. In this study, UV-inactivated HSV1/2 and immunodominant HSV2 glycoprotein D peptides conjugated to the TLR2 agonist dipalmitoyl-S-glyceryl cysteine stimulated CD4 T lymphocyte IFN-γ responses within PBMCs or in coculture with monocyte-derived DCs. NK cells contributed markedly to the PBMC responses. Furthermore, NK cells alone were activated directly by both Ags, also upregulating HLA-DR and HLA-DQ and then they activated autologous CD4 T lymphocytes. Using Transwells, Ag-stimulated NK cells and CD4 T lymphocytes were shown to interact through both cell-to-cell contact and cytokines, differing in relative importance in different donors. A distinct immunological synapse between Ag-stimulated NK cells and CD4 T lymphocytes was observed, indicating the significance of their cell-to-cell contact. A large proportion (57%) of NK cells was also in contact with CD4 T lymphocytes in the dermal infiltrate of human recurrent herpetic lesions. Thus, NK cells stimulated by TLR2-activating HSV Ags can present Ag alone or augment the role of DCs in vitro and perhaps in herpetic lesions or draining lymph nodes. In addition to DCs, NK cells should be considered as targets for adjuvants during HSV vaccine development. The Journal of Immunology, 2012, 188: 4158–4170.

In cutaneous herpes simplex lesions of humans and in murine models, keratinocytes, dendritic cells (DCs), and infiltrating lymphocytes, especially HSV-specific CD4 and CD8 T lymphocytes, were shown to play a central role in controlling primary and recurrent HSV infections (1). In human recurrent lesions, monocytes and CD4 T lymphocytes infiltrate first, followed by CD8 T lymphocytes, which appear to clear HSV infection (1–3). HSV infection of keratinocytes in vitro and in vivo induces the secretion of a sequence of chemokines and cytokines (first IFN-β and β chemokines and then IL-12, followed by IL-1β and IL-6) (4). The β chemokines probably attract monocytes and CD4 and CD8 T lymphocytes into lesions. IFN-α/β and IL-12 may entrain Th1 patterns of cytokine response from HSV Ag-stimulated CD4 (and CD8) T lymphocytes, especially IFN-γ. IFN-α and IFN-γ synergize to inhibit infection of keratinocytes after transmission from axon termini (5). HSV1/2 downregulate MHC class I expression by keratinocytes. This is reversed by IFN-γ, mainly from early infiltrating CD4 T lymphocytes, thus allowing CD8 T lymphocytes to recognize infected keratinocytes (1, 4). IFN-γ also stimulates MHC class II (MHC II) expression on keratinocytes, allowing recognition by CD4 T lymphocytes. Thus, Th1, rather than Th2, patterns of response are important for immune and vaccine control of HSV (6). Furthermore, resident memory CD8 T lymphocytes can be induced to proliferate directly in (murine) skin without the requirement to migrate to the lymph nodes. This requires DC interactions with CD4 and CD8 T lymphocytes (7). Infrequent “sentinel” CD4 and CD8 T lymphocytes are present in the dermis between lesions. Residual dermal DCs, plasmacytoid DCs (pDCs), and CD4 and CD8 T lymphocytes persist in the dermis between lesions. CD8 T lymphocytes are most superficial, with some present at the dermo–epidermal junction (8).

Mucocutaneous HSV infection is usually confined to the epidermal layer. Therefore, Langerhans cells (LCs) are likely to be involved in initial HSV Ag uptake (9), as shown by older murine studies (10). However, recent studies in mice demonstrated that migrating dermal CD103+, langerin-expressing DCs are the major transporters of HSV Ag out of skin and, together with resident CD8+ DCs, are the major presenters of HSV Ag to CD8 T lymphocytes in lymph nodes (11, 12), probably after Ag transfer from LCs (13). No human equivalent of the CD103+ dermal DC subset has been described, nor has a similar sequence of events, although it was shown that HSV-infected monocyte-derived DCs (MDDCs) undergo apoptosis and can be taken up by bystander uninfected MDDCs, which can then present HSV Ag to CD8 T lymphocytes (14).

More recently, the importance of other innate immune effectors, both humoral and cellular, such as macrophages, pDCs, NK cells, and γδ T lymphocytes has been re-emphasized, either in direct immune control or via modulation of adaptive immune responses (15, 16). Recently, pDCs were demonstrated to sequester the upper dermis of recurrent herpes lesions where they show

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Received for publication December 9, 2011. Accepted for publication February 16, 2012.

This work was supported by the National Health and Medical Research Council of Australia (Program Grant 358399 and Project Grant 632638).

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Abbreviations used in this article: DC, dendritic cell; gD, HSV glycoprotein D; gD2, HSV2 glycoprotein D; gD2-24-4, HSV2 glycoprotein D peptide 24-4; gD2-30-5, HSV2 glycoprotein D peptide 30-5; TLR2, human TLR2; IS, immunological synapse; LC, Langerhans cell; MDDC, monocyte-derived dendritic cell; MHC II, MHC class II; MOI, multiplicity of infection; Pam2Cys, dipalmitoyl-S-glyceryl cysteine; pDC, plasmacytoid dendritic cell; RH10, RPMI 1640 supplemented with 10% human AB serum; RT, room temperature; UV-HSV, UV-inactivated HSV.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1103450
evidence of IFN-\(\alpha\) production and are in contact with activated NK cells and lymphocytes (17). They produce IFN-\(\alpha\) in response to stimulation with HSV, to limit viral spread (18–21), and can present Ag to naive effector and regulatory lymphocytes (21–24).

Like DCs, NK cells can bridge the innate and adaptive immune responses. In humans, there are two main NK subsets: the first and well-characterized CD56dimCD16+ NKs make up \(>95\%\) of those in blood and readily lyse malignant or virally infected cells but are low cytokine secretors. In contrast, the more recently described CD56brightCD16- NK cells produce large amounts of cytokines, such as TNF-\(\alpha\) and IFN-\(\gamma\), upon stimulation, but they only acquire cytokotaxicity after prolonged activation. DCs and NK cells often colocalize and are able to interact both at sites of inflammation and in lymph nodes (25, 26). In DC–NK cell interactions, cytokines, such as IL-12, IFN-\(\alpha\), and IL-15, are important, but direct cell-to-cell contacts also seem to be essential in promoting full NK activation by DCs (27). The CD56brightCD16- subset can also act as accessory APCs via upregulated HLA-DR and secretion of IFN-\(\gamma\), thus enhancing CD4 T lymphocyte responses (28, 29). Interactions among DCs, NK cells, and CD4 T lymphocytes can enhance such responses (22, 30). IFN-\(\gamma\) production by NK cells can be induced by IL-12 secreted by TLR-stimulated DCs (31). Cross-talk between DCs and NK cells in viral infection was reported (32). However, it has not been fully explored how the trio of NK cells, DCs, and CD4 T lymphocytes interact with each other, especially physically.

TLRs on the surface and in endosomes of dendritic, NK, and other innate immune cells are key sensors of viral pathogens leading to activation of the innate and adaptive immune responses. Human LCs express TLRs 1, 2, 3, 5, 6, and 10 (33, 34); dermal DCs and MDDCs express TLRs 1, 2, 3, 4, 6, 8, and 10 (23); and NK cells express TLRs 1, 2, 3, 5, 6, 7, 8, and 9 (31, 35). Some strains of HSV1 and HSV2, as well as human CMV, interact with TLR2 at the surface of many cell types via an unknown mechanism, as well as with TLR9 in the endosomes (particularly of pDCs) via unmethylated CpGs in viral DNA. UV-inactivated HSV2 requires initial interaction with TLR2 prior to full activation via TLR9, whereas infectious HSV2 can interact directly with TLR9 (18). IFN-\(\alpha\) production by pDCs via TLR9 (18–20) also activates NK cells, which, in turn, contribute to the Th1 adaptive immune response (36). TLRs 2 and 9 also synergize in control of HSV in the mouse brain (37).

HSV glycoprotein D (gD) is a highly immunogenic molecule containing both B and T cell epitopes capable of inducing neutralizing Abs and CD4 T lymphocyte Th1 responses in the majority of the human outbred population. Therefore, it has been used as an immunogen in human vaccine trials. However, gD alone does not appear to interact with surface TLRs on innate immune cells (38). In consort studies of couples discordant for genital herpes, a vaccine consisting of HSV2 gD (gD2) and the TLR4 agonist d-monophosphoryl lipid A showed 73–74% efficacy in preventing HSV-infected individuals, providing an explanation for the broad T cell recognition in humans. In general, short peptides are weak immunogens, so they need to be administered with adjuvants. To enhance the immunogenicity of our gD2 peptides, they were conjugated to dipalmitoyl-S-glycerol cysteine (Pam2Cys) (42), which binds specifically to and stimulates via TLR2 and its heterodimeric partner TLR6 and induces maturation in DCs (43–46) (some HSV strains also target TLR2 naturally).

In preliminary experiments, we determined that these lipopeptides markedly enhanced HSV-specific CD4 T lymphocyte IFN-\(\gamma\) responses. Therefore, we examined the mechanism of these responses and then compared them with those of endogenous TLR2 stimulation by whole HSV as UV-inactivated Ag. Initially, the HSV2 186 strain, previously shown to stimulate sequentially via TLR2 and 9, was used, followed by an HSV1 strain also shown to stimulate via TLR2. Both types of Ags, UV-inactivated HSV (UV-HSV) and Pam2Cys–gD2 peptides, are also known to stimulate maturation of human MDDCs (47, 48). Because of the prominent infiltration of NK cells into herpetic lesions, we also examined the role of NK cells in modulating the immune response of CD4 T lymphocytes to these Ags. We found that NK cells contributed significantly to the unseparated PBMC IFN-\(\gamma\) responses to HSV Ag and enhanced MDDC–CD4 T lymphocyte responses to UV-HSV and Pam2Cys-conjugated HSV peptides. Furthermore, NK cells alone were capable of direct presentation of these HSV Ags to CD4 T lymphocytes in the absence of DCs. The mechanism was determined to be through both direct NK cell–CD4 T lymphocyte physical interaction and cytokines. These studies are relevant to such DC–NK cell–CD4 T lymphocyte interactions in pairs and triplets in herpes lesions, as well as to adjuvant choice and their mechanisms of action in HSV vaccine development.

**Materials and Methods**

**Synthesis of gD2 peptides**

gD2-24-4 and -30-5 were synthesized using Fmoc chemistry, as previously described (42). The amino acid sequences of the immunodominant CD4 T lymphocyte epitopes in gD2 were QQGVTVDSIGML and PEDPED-. gD2-24-4 and -30-5 were synthesized using Fmoc chemistry, as previously described. The reasons for these differences are unclear and indicate the need for increased understanding of the human immune response and control of HSV infection, including innate immunity and its key role in directing the adaptive immune response.

Recently, a high density of promiscuous immunodominant peptide epitopes in gD2 for CD4 T lymphocytes was identified in our laboratory (41). The identified immunodominant gD2 peptide 24-4 (gD2-24-4) and gD2 peptide 30-5 (gD2-30-5) promiscuously bound to a broad range of HLA-DR molecules and were recognized by both HSV1- and HSV2-infected individuals, providing an explanation for the broad T cell recognition in humans. In

**PBMC preparation**

Blood was collected from HSV1- and/or HSV2-seropositive healthy donors and donor serotype-matched HSV1 or HSV2 Ags in individual experiments. Informed consent was obtained from all of the blood donors, and the study was approved by the Ethics Committee for Sydney Western Area Health Service Research. PBMCs were prepared by Ficoll-Hypaque (GE Healthcare Biosciences) density gradient.

**Depletion of CD8+ and CD56+ lymphocytes from PBMCs**

To examine the immunogenicity of Pam2Cys–gD2 peptides and whether Pam2Cys–gD2 peptides target only CD4 T lymphocytes as intended, CD8 T lymphocytes and CD56- NK cells were positively depleted using CD8 and CD56 MicroBeads (Miltenyi Biotec), respectively. The same starting number of PBMCs was used as undepleted PBMC control for CD8 depletition as well as for CD56 depletition. After cell depletion, the resulting

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cells for each depletion were resuspended in an equal volume of RPMI 1640 (Invitrogen) supplemented with 10% human AB serum (RH10; Sigma-Aldrich) to a final concentration of 1 × 10^6 cells/ml based on the number of undepleted PBMCs. Cells were cultured for 40 h in a 96-well ELISPOT plate with 5 μg/ml PHA, UV-HSV1 (strain F) or UV-HSV2 (strain 186) at multiplicity of infection (MOI) 0.5, 10 μM peptide, or DMSO (Sigma-Aldrich) as negative control. UV-HSV was prepared as previously described (14). Viral infectivity was not observed after UV inactivation by plaque assay. To measure the immune response after the incubation, IFN-γ ELISPOT was performed, as previously described (41).

TLR2-binding assay using HEK-Blue TLR2 cells

To investigate the mechanism of UV-HSV binding to NK cells, TLR2 was tested as one of the receptor candidates for UV-HSV. HEK-Blue human TLR2 (hTLR2) cells and HEK-Blue Null cells (parental cell line of HEK-Blue hTLR2 cells) were purchased from InvivoGen. They were cultured and used for assays, according to the manufacturer’s instructions. Before the assay, TLR2 expression on both cells was examined by flow cytometry using FITC-conjugated hTLR2 mAb. Pam2CSK4 (10 μM; InvivoGen), Pam2Cys–gD2-30-5 (10 μM), UV-HSV1 (MOI 0.5), UV-HSV2 (MOI 0.5), and water and TLR7/8 agonist Resiquimod (5 μM; InvivoGen) as negative control were added to the cells and cultured for 20 h at 37˚C, 5% CO₂. Supernatants were collected for measuring NF-negative control were added to the cells and cultured for 20 h at 37˚C, 5% CO₂. Supernatants were collected for measuring NF

Preparation of peptide-specific CD4 T cell line

To increase the frequency of immunological synapse (IS) formation between Pam2Cys–gD2-30-5–stimulated NK cells and CD4 T lymphocytes, a gD2-30-5–specific CD4 T cell line was generated. PBMCs of HSV1–infected individuals were pulsed with 10 μM gD2-30-5 in 100 μl serum-free RPMI 1640 for 1 h at 37˚C, 5% CO₂ and cocultured with autologous PBMCs at a 1:2 ratio at a cell concentration of 1 × 10^6 cells in RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin (50 μU/ml and 50 μg/ml, respectively; Gibco-BRL). After 1 wk, cells were restimulated with irradiated (3000 rad) peptide-sensitized autologous PBMCs, as above. Restimulated cells were cultured in RPMI 1640 supplemented with 10% FBS, IL-2 (10 U/ml; Roche), and penicillin/streptomycin at 1 × 10^6 cells/ml. Restimulation was repeated once a week until enough cells were obtained. Medium was replenished twice a week.

Detection and quantification of NK cell–CD4 T lymphocyte conjugates

To confirm that NK cells can directly contact CD4 T lymphocytes, purified and stimulated NK cells were cocultured with purified CD4 T lymphocytes, or gD2-30-5–specific CD4 T cell line as follows. Purified NK cells were pulsed with 10 μM Pam2Cys–gD2-30-5 or UV-HSV1 at MOI 0.5, or without Ag as negative control, in 100 μl serum-free RPMI 1640 for 1 h at 37˚C, 5% CO₂. After the incubation, cells received a 10× volume of serum-free RPMI 1640 and were centrifuged for 5 min at 300 × g. After decanting the supernatant, NK cell pellet was resuspended and added to CD4 T lymphocytes at a 2:1 ratio. The combined NK cells and CD4 T lymphocytes were briefly centrifuged at 10,000 × g and incubated for 5 min at 37˚C. After the conjugation, cells were gently resuspended; serum-free RPMI 1640 was added to the cells, and they were loaded onto the prepared Cell Tak (BD)–coated cover slips through “gummy squares” (eight-well flexiPERM removable and reusable TC chambers; ISCO BioExpress). Cells were centrifuged for 3 min at 300 × g. The gummy square was removed before cells were fixed and labeled.

Immunofluorescence microscopy

Cells loaded on cover slips were fixed with 3% paraformaldehyde (ProSciTech) for 20 min at room temperature (RT) and then neutralized with 50 mM NH₄Cl three times for 3 min each. Cells were permeabilized with 0.05% Triton X-100 (Sigma-Aldrich) for 1 min at RT and washed once with PBS. After permeabilization, cells were left for 15 min in PBS supplemented with 1% fish skin gelatin (Sigma-Aldrich) and 0.02% saponin (Sigma-Aldrich) and then for 30 min in Image-IT FX signal enhancer (Invitrogen). For visualizing ISs, the actin-binding molecule talin was labeled with mouse anti-human talin mAb (Abcam) as primary Ab for 30 min at RT and then with Alexa Fluor 633-conjugated goat anti-mouse IgG (Molecular Probes) for 30 min as secondary Ab. For the secondary staining to differentiate NK cells and CD4 T lymphocytes, mouse anti-human CD4 Ab (BD Pharmingen) was used to label CD4 T lymphocytes for 30 min, followed by Alexa Fluor 633-conjugated goat anti-mouse IgG (Molecular Probes) for 30 min. Mouse anti-human IgG (Sigma-Aldrich) was used as primary Ab for negative control staining for primary and secondary staining. All washes between steps were carried out with 10% goat serum (Sigma-Aldrich) in PBS. After staining, ProLong Gold Antifade reagent (Invitrogen) was added to the stained cells, and glass slides were mounted onto cell-loaded coverslips. Slides were visualized through an ×100 1.35 NA oil–immersion lens with an inverted Olympus IX-70 microscope (DeltaVision Image Restoration Microscope; Applied Precision/Olympus) and a Photometrics CoolSnap QE camera.

Immunostaining of histologic sections of herpetic lesion biopsies

Punch biopsies were taken from recurrent genital herpes lesions ≤4 d after onset. Samples were obtained under a protocol approved by the ethics committee for Sydney West Area Health Service. Written informed-
sent was obtained prior to sample donation. The biopsies were snap-frozen in OCT, cut into 5-μm sections, and placed on slides. Slides were kept at −80°C until required for immunohistochemical staining. Slides were fixed with ice-cold methanol/acetone (1:1) prior to staining. Abs used for microscopy were mouse mAb for CD56 (1:200; Invitrogen) and goat polyclonal Ab for CD4 (1:100; R&D Systems). Slides were incubated at 37°C with primary Abs for 45 min. Following washing, the secondary Ab Alexa Fluor 546 donkey anti-mouse (1:400; Molecular Probes) and Alexa Fluor 488 donkey anti-goat (1:400; Molecular Probes) were added for 30 min at 37°C. Excess secondary Ab was washed away, cells were mounted using ProLong Gold Antifade supplemented with DAPI (Invitrogen), coverslips were applied, and slides were refrigerated until imaged with an Olympus IX-70 microscope.

Statistical analysis
Outcome variables were log transformed to stabilize the variance prior to analysis. Repeated measures ANOVA with multiple pairwise comparisons was used to assess the effects of treatment within a group. SPSS version 20 was used to analyze data. A 5% significance level with two-sided tests was used throughout.

Results

Conjugation of immunodominant gD2 peptides to the TLR2 agonist Pam2Cys enhanced IFN-γ responses by CD4 T lymphocytes in MDDC–CD4 T lymphocyte cocultures and contributed to the better effect in PBMCs and NK cells

Two 12 mer analogs from gD2-24-4 and -30-5, previously defined as MHC II restricted, immunodominant and broadly recognized by CD4 T lymphocytes of HSV1- and HSV2-seropositive subjects across multiple HLA-DR types, were selected for conjugation to Pam2Cys, a TLR2 agonist. The effects of lipidated and nonlipidated peptides on PBMC and CD4 T lymphocyte IFN-γ responses were examined and compared. After conjugation to Pam2Cys, the immunogenicity of gD2-24-4 and -30-5 peptides for CD4 T lymphocytes in MDDC–CD4 T lymphocyte culture was significantly enhanced, as measured by IFN-γ ELISPOT (Fig. 1A). As expected, this enhancement of PBMC IFN-γ responses was not generally diminished by CD8 T lymphocyte depletion alone (n = 3, p = 0.256; ANOVA, followed by multiple pairwise comparisons); however, unexpectedly, this immune response was significantly decreased by depletion of CD56 lymphocytes whether they were stimulated with Pam2Cys–gD2-24-4 (p = 0.005, ANOVA) or UV-HSV1 or UV-HSV2 Ag (p = 0.050 and 0.030, respectively) (Fig. 1B). This suggests that NK cells contribute directly or indirectly to PBMC IFN-γ production.

UV-HSV1 and UV-HSV2 strains bound to TLR2

UV-HSV Ag was used because certain clinical strains of HSV2 (including HSV2 strain 186) were also shown to stimulate cells via TLR2 (18). Therefore, we first determined the TLR2-stimulating activity of UV-HSV1 strain F and HSV2 strain 186 with a TLR2-transfected HEK293 responder cell line in comparative studies with Pam2Cys–gD2 peptides. To examine the physical binding of UV-HSV to NK cells and subsequent stimulation, HEK-Blue hTLR2 cells were used with HEK-Blue Null cells as negative control. Surface TLR2 expression was detected on HEK-Blue hTLR2 cells but not on HEK-Blue Null cells (Fig. 2A). When a TLR2 agonist binds to TLR2 on HEK-Blue hTLR2 cells, NF-κB–induced embryonic alkaline phosphatase is secreted into the supernatant, which is detected by spectrophotometry. UV-HSV1 and UV-HSV2 bound to TLR2 expressed on HEK-Blue hTLR2 cells as did Pam2Cys–gD2-30-5 and Pam2CSK4 as positive control, but not on HEK-Blue Null cells. Negative controls included the TLR7/8 agonist Resiquimod (Fig. 2B).

The presence of NK cells enhanced DC–CD4 T lymphocyte responses to Pam2Cys-conjugated gD2 peptides

To directly examine whether NK cells contribute to CD4 T lymphocyte IFN-γ responses to gD2-24-4 Ag presentation by DCs, MDDCs were cultured with autologous NK cells and CD4 T lymphocytes with lipidated or nonlipidated gD2-24-4. The lipopeptide-enhanced responses by all three paired combinations of NK cells, MDDCs, and CD4 T lymphocytes, as well as by NK cells alone but not by CD4 T lymphocytes alone. The immune response was the greatest when all three cell types were cultured together (Fig. 3A, 3B). To further investigate the specific role of NK cells in the NK cell–CD4 T lymphocyte IFN-γ response to Pam2Cys–gD2-24-4 and UV-HSV2 strain 186, both subsets were purified from PBMCs of HSV1/2-seropositive subjects and cultured alone or together in the presence of gD2-24-4, Pam2Cys–

![Figure 1](http://example.com/figure1.png)

**Figure 1.** HSV Ags stimulate CD4 T lymphocyte (IFN-γ) responses through DCs and NK cells. (A) Conjugation of two immunodominant HSV gD2 peptides (gD2-24-4, left panel, gD2-30-5, right panel) to Pam2Cys enhanced CD4 T lymphocyte IFN-γ responses when cocultured with MDDCs. Mean ± SD of triplicates. Representative of three different donors. (B) Immune response of HSV-seropositive PBMCs, CD8 lymphocyte-depleted PBMCs, or CD56 lymphocyte-depleted PBMCs to HSV Ags. Stimulation of CD4 T lymphocyte responses with HSV Ags requires NK cell participation. The immune responses were measured by IFN-γ ELISPOT. Mean ± SD of triplicates. Representative experiments from three donors. Negative control, no Ag; Pam24-4, Pam2Cys–gD2-24-4; Pam 30-5, Pam2Cys–gD2-30-5; positive control, PHA-stimulated PBMCs; UV-1, UV-HSV1; UV-2, UV-HSV2; 24-4, gD2-24-4; 30-5, gD2-30-5.

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gD2-24-4, or UV-HSV2. However, the NK cell–CD4 T lymphocyte combination again produced more IFN-γ than NK cells alone and in the absence of other APCs (Fig. 3C). Thus, NK cells produce IFN-γ alone and probably stimulate CD4 T lymphocytes to enhance their production with both UV-HSV2 and Pam2Cys–gD2-24-4.

**Both Pam2Cys–gD2 peptide and UV-HSV directly activated NK cells**

Whether NK cells are activated by Pam2Cys–gD2 peptide or UV-HSV Ag was determined by measuring expression of the early activation marker CD69 on the surface of NK cells, after stimulation by these HSV Ags. Both types of Ags directly and specifically activated NK cells, similar to the IFN-γ responses (by ELISPOT) (Fig. 4 compared with Fig. 3). Both CD56brightCD16– and CD56dimCD16+ NK cell subsets were activated, but activation was greater in the former (data not shown).

**Pam2Cys–gD2 peptide and UV-HSV Ag-pulsed NK cells activated CD4 T lymphocytes**

To confirm the activation of CD4 T lymphocytes by NK cells pulsed with Pam2Cys–gD2 peptide and UV-HSV, the expression of CD69 on CD4 T lymphocytes was examined by flow cytometry after coculturing with Ag-stimulated NK cells. The activation of CD4 T lymphocytes was significantly enhanced by Pam2Cys conjugation of the gD2 peptides (Fig. 5A) and by UV-HSV1 stimulation (Fig. 5C), although there was considerable individual variation.
CD4 T lymphocytes were not directly activated by the Ag (Fig. 5B), as also shown in Fig. 3B.

Expression of HLA-DR, HLA-DQ, and TLR2 on blood NK cells and their upregulation by HSV Ags

To examine the mechanism of direct stimulation of CD4 T lymphocytes by NK cells stimulated with Pam2Cys–gD2 peptide and UV-HSV (Fig. 5), the expression of TLR2, HLA-DR, and HLA-DQ on resting blood NK cells in PBMCs was examined by flow cytometry (Fig. 6A). All three molecules were expressed at low and variable levels (1–8%) on resting NK cells and on both the CD56bright and CD56dim NK subsets (Fig. 6B). A variable proportion (up to 4%) of these cells coexpressed TLR2, HLA-DR, or HLA-DQ. However, after NK cell isolation and culture, HLA-

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** NK cells alone can be activated with HSV Ags. (A and C) NK cells were pulsed or not with UV-HSV1 and incubated for 2 d at 37°C, 5% CO₂. NK cell activation was measured as surface CD69 expression by flow cytometry (A; shaded curve: isotype control; dashed line: no Ag; solid line: UV-HSV1). (B) NK cells were also cultured overnight in the presence of gD2-30-5, Pam2Cys–gD2-30-5, or DMSO as negative control. (B and C) Left panels. Percentage of CD69-expressing NK cells. Right panels. Mean fluorescence intensity (MFI; geometric means) of CD69 expressed on NK cells. Box-and-whisker plots show median and 25th and 75th percentiles of proportions of CD69⁺ NK cells. Pam30-5, Pam2Cys–gD2-30-5; 30-5, gD2-30-5.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** CD4 T lymphocyte activation by HSV Ags presented by NK cells. (A) NK cells were cultured overnight with CD4 T lymphocytes in the presence of gD2-30-5, Pam2Cys–gD2-30-5, or DMSO as negative control. (B) Effects of naked peptide and lipopeptide on CD4 T lymphocytes alone. As a control, CD4 T lymphocytes were also incubated with the above Ags overnight. (C) NK cells were pulsed with UV-HSV1 for 1 h and washed before NK and CD4 T lymphocytes were cocultured for 2 d at 37°C, 5% CO₂. Activation of CD4 T lymphocytes was measured as CD69 expression by flow cytometry. Box-and-whisker plots show median and 25th and 75th percentiles of proportions of CD69⁺ CD4 T lymphocytes. Pam30-5, Pam2Cys–gD2-30-5; 30-5, gD2-30-5.
DR and HLA-DQ expression decreased, reducing the proportion of TLR2+MHC II+ NK cells (M. Kim and N. Osborne, unpublished observations).

The expression of HLA-DR and HLA-DQ molecules on the surface of cultured NK cells was then examined, after stimulation with both types of HSV Ags, and shown to be significantly increased (Fig. 7). When NK cell subsets were examined, the levels of HLA-DR and HLA-DQ and the proportion of MHC II-expressing NK cells were significantly increased and to a greater degree by lipopeptide on the CD56bright CD162 NK subset compared with the CD56dim CD16+ NK subset (Fig. 7A).

Pam2Cys–gD2 peptide and UV-HSV Ag-stimulated NK cells activated CD4 T lymphocyte responses through cell-to-cell contact and cytokine effects

To determine whether CD4 T lymphocytes needed to directly contact NK cells to be activated in an Ag-dependent manner, they were cultured separately or together in the same compartment in Transwells. Because NK cells were postulated to be APCs, the peptide was always added to the chamber containing NK cells. After overnight culture, cells were harvested and labeled for flow cytometry. In all five donors, CD69 expression was enhanced to a greater degree on CD4 T lymphocytes when they were cocultured with NK cells in the same chamber (Fig. 8B); there was an overall significant decrease in expression when the two cell types were separated, although this showed marked interdonor variation in magnitude (1.69-fold decrease; 95% confidence interval, 1.20–2.37; p = 0.013, paired t test) (Fig. 8A). This indicates that there was little feedback effect of activated CD4 T lymphocytes on activation of NK cells.

Thus, cell-to-cell contact between NK and CD4 T lymphocytes varied between donors with regard to the magnitude of its importance for CD4 T lymphocyte activation, being complemented by the humoral (or cytokine) effects (similar results were observed with UV-HSV Ag stimulation; data not shown).

**NK cells formed ISs with CD4 T lymphocytes after stimulation with Pam2Cys–gD2 peptide and UV-HSV1**

To examine the physical contact between NK cells and CD4 T lymphocytes, highly purified NK cells were pulsed with Pam2Cys–gD2-30-5, UV-HSV1 Ag, or controls for 1 h and then washed prior to examining for conjugate formation with CD4 T lymphocytes. After 5 min of coculture, NK cells and CD4 T lymphocytes were fixed/permeabilized and labeled for immunofluorescence microscopy. A very low background level of homotypic and heterotypic cell-conjugate formation was observed between NK cells and CD4 T lymphocytes in control cultures (i.e., CD4–CD4, NK–NK, and NK–CD4 T lymphocyte conjugates formed) (Fig. 9A). However, the proportion of NK cell–CD4 T lymphocyte conjugates in the Pam2Cys–gD2-30-5–stimulated cocultures was significantly greater than in controls. As expected, these proportions were very low in the lipopeptide-stimulated NK cells. Therefore, to increase the frequency of IS formation between NK cells and CD4 T lymphocytes, gD2-30-5–stimulated CD4 T cell lines were generated. As a result, ISs were more frequent and recognized by polarization of the actin-binding molecule talin and also the CD4 molecule to the CD4 T lymphocyte–NK cell contact region with the Pam2Cys–gD2-30-5–stimulated CD4 T cell line (1 IS/104 cell pair for gD2-specific peptide). Similar ISs were observed in UV-HSV1–pulsed NK
cells with CD4 T lymphocytes (1 IS/3 \times 10^5 cell pair) (Fig. 9B, 9C, respectively). Although the cells partnering CD4 T lymphocytes were of similar size and CD4^+ and, therefore, very unlikely to be contaminating DCs, this possibility was excluded by their quantification in the purified NK population. Contaminating CD11c^+ myeloid DCs or BDCA2^+ pDCs were undetectable in the 2% of non-CD56^+CD3^+ NK cells (as were CD19^+ B lymphocytes; i.e., a maximum concentration \( \leq 10^{-6} \)). If the maximum concentrations of Ag-specific CD4 T lymphocytes were \( \leq 5 \times 10^5 \) in the cell lines and \( \leq 2 \times 10^{-3} \) in UV-HSV Ag-stimulated CD4 T lymphocytes, then the frequency of any IS formed by potential contaminating cells with CD4 T lymphocytes was calculated to be >3 logs lower than that actually observed.

NK cells and CD4 T lymphocytes were in contact in the mononuclear dermal infiltrates in recurrent herpetic skin lesions

To determine whether these direct NK cell–CD4 T lymphocyte interactions may occur in vivo, biopsies from three recurrent herpetic lesions at \( \leq 4 \) d after appearance were stained for the two cell types. CD4 T lymphocytes were highly abundant in the infiltrate. CD56^+ NK cells were also detected, with up to 27 NK cells/field of view (×40 magnification). For each biopsy, the number of CD56^+ NK cells was counted in six to eight fields of view. An average of 57.5% of the CD56^+ NK cells was found to be contacting a CD4 T lymphocyte in the lesions (Fig. 10). In normal skin control biopsies, no infiltrating CD4 T lymphocytes or CD56^+ NK cells were observed in the dermis.
cytotoxic. In this study, we determined that NK cells may play a pivotal role in interacting with CD4 T lymphocytes, as well as DCs, to augment their response to HSV Ag and, specifically, to gD2 peptides, especially after TLR2 stimulation on both DCs and NK cells.

In general, short peptides, such as the immunodominant gD2 12–24 and gD2-24-4 used in this study, are poor immunogens and need to be administered with adjuvants to maximize their immunogenicity. Such adjuvants are often TLR agonists, administered concurrently with Ag, which act on DCs and macrophages via TLRs (e.g., d-monomophosphoryl lipid A and CpG oligonucleotides) (52). However, the TLR2 agonist Pam2Cys acts as a self-adjuvant when directly conjugated to peptides. It then binds specifically to TLR2 on APCs and directs peptide uptake by DCs, to augment their response to HSV Ag and, specifically, to gD2 peptides, marked by TLR2 stimulation on both DCs and NK cells.

FIGURE 8. Pam2Cys–gD2 peptide-stimulated NK cells activated CD4 T lymphocytes through cell-to-cell contact and cytokine effects. CD4 T lymphocytes from five donors were cocultured with autologous NK cells in the lower compartment of Transwells, or NK cells were cultured separately from CD4 T lymphocytes in the upper chamber. Pam2Cys–gD2-24-4 or the control gD2-24-4 peptides were always added to the chamber containing NK cells. The pore size of the Transwell membrane was 0.4 μM to inhibit cell migration between the upper and lower chambers. After overnight culture, cells were harvested and labeled. Activation of both NK cells (A) and CD4 T lymphocytes (B) was measured by CD69 expression. Pam24-4, Pam2Cys–gD2-24-4; 24-4, gD2-24-4.

Discussion

Both CD4 and CD8 T lymphocytes are key effectors in human cutaneous herpetic lesions and persist as sentinels between lesions: CD8 T lymphocytes at the derмо-epidermal junction and CD4 T lymphocytes deeper in the dermis (3, 49). They show different kinetics of recruitment into clinical recurrent lesions and apparently different, but overlapping, functions. Early in inflammation, CD4 T lymphocytes are the major IFN-γ-producing cells, although they are capable of cytolytic function against infected keratinocytes in vitro (1). IFN-γ plays an important role in HSV-infected sites by restoring MHC class I downregulation of HSV-infected cells and upregulating MHC II molecules, allowing the later-infiltrating CD8 T lymphocytes to recognize and infect cells. However, they also can secrete IFN-γ. Recently, the load of HSV2 shed in vaginal secretions was shown to be inversely proportional to the density of lesional CD8 T lymphocytes, although this could just represent the density of all key immune effectors (3).

NK cells are also present in the dermis of cutaneous herpetic lesions, in contact with other infiltrating immune cells, especially pDCs (17), as well as in cells expanded from genital sites of HSV2 infection ex vivo (2). An important role for NK cells in HSV infection was suggested previously: Dalloul et al. (50) observed severe HSV2 infection in patients with myelodysplasia and undetectable NK cells and pDCs in the blood. Pandakumar et al. (51) demonstrated that mice lacking or depleted of NK cells were susceptible to HSV-induced lesions and that NK cell supplementation enhanced the function of anti-HSV CD8 T lymphocytes. Nevertheless, the role of NK cells in human HSV immunology has been underinvestigated, despite the likely contribution of both NK cell subsets to control of HSV. The minor CD56dimCD16+ subset produces IFN-γ, and the major CD56brightCD16+ subset is cytotoxic. In this study, we determined that NK cells may play a pivotal role in interacting with CD4 T lymphocytes, as well as DCs, to augment their response to HSV Ag and, specifically, to gD2 peptides, especially after TLR2 stimulation on both DCs and NK cells.

In general, short peptides, such as the immunodominant gD2 12–24mers, gD2-24-4 and gD2-30-5 used in this study, are poor immunogens and need to be administered with adjuvants to maximize their immunogenicity. Such adjuvants are often TLR agonists, administered concurrently with Ag, which act on DCs and macrophages via TLRs (e.g., d-monomophosphoryl lipid A and CpG oligonucleotides) (52). However, the TLR2 agonist Pam2Cys acts as a self-adjuvant when directly conjugated to peptides. It then binds specifically to TLR2 on APCs and directs peptide uptake by the same cells (53–55). After uptake of these in PBMCs, pre-
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also coexpressed on a small proportion of unstimulated NK cells, as suggesting that both cell types were sources for IFN-α production and IFN-γ expression, strongly reflecting expression of TLR2 on NK cells, and HLA-DR expression was upregulated to the greatest extent on the CD56brightCD16− NK subset.

To determine whether direct contact between NK cell and CD4 T lymphocytes was required for CD4 T lymphocyte activation, a Transwell system was used. In all five donors, cell-to-cell contact enhanced CD4 T lymphocyte activation; although there was a considerable variation in the magnitude of this effect between donors, overall it was significant. Where the effect of contact was minor, activation of CD4 T lymphocytes probably occurred through NK cytokine production, in particular IFN-γ, as previously described (64). Cytokines, such as IL-12 and IL-15 from DCs, IFN-γ from NK cells, and IL-2 from CD4 T lymphocytes, may all contribute to these interactions. There may be several reasons for these differences in the relative importance of contact and cytokines between individual donors. These may include individual variations in cytokine production, TLR2 expression on NK cells, or expression of cell-adhesion molecules between NK cells and CD4 T lymphocytes. Separation of NK cells and CD4 T lymphocytes had little effect on HSV Ag/TLR2-induced NK activation, arguing against a significant role for activated CD4 T lymphocyte feedback on NK activation.

To define the mechanism of Ag presentation to CD4 T lymphocytes by contacting NK cells pulsed with lipopeptide or whole UV-HSV Ag, their HLA-DR and HLA-DQ expression was examined. Both MHC II molecules were significantly upregulated on NK cells, and HLA-DR expression was upregulated to the greatest extent on the CD56brightCD16− NK cell subset (data not shown). Expression of MHC II molecules on human NK cells as mRNA and protein was reported (67, 68) but not previously shown to be upregulated by lipopeptide or a viral pathogen, although similar lipopeptides induce maturation and upregulate MHC II on DCs (71).

Direct cell-to-cell contact between NK cells and CD4 T lymphocytes, resulting in Ag presentation, is likely to occur via ISs. MHC–peptide complexes reach a peak ∼5 min after coculture and remain maximally concentrated for 10–20 min (72). Adhesion molecules in the clusters increase the avidity of the APC–T cell interaction, thus facilitating more efficient TCR engagement. They are polarized to the contact region between APCs and lymphocytes (73). The actin-binding molecule talin is one of the molecules that polarizes to ISs. In DC–NK cell ISs, F-action polymerization occurs within DCs, whereas in cytolytic NK cell–K562 conjugates, F-actin polymerization occurs in the NK cells (74). Talin polarization is present in ISs formed by noncytolytic and cytolytic NK cells but is transient in noncytolytic ISs (∼1 min) when interacting with target cells (75). If true for interactions with CD4 T lymphocytes, this could lead to underestimates of ISs in our system. CD4 molecules are also involved in IS formation within 5 min (76). In our study, ISs were observed as colocalization of CD4 and talin at the contact region with the expected kinetics, 5 min after coculture, of CD4 T lymphocytes and NK cells stimulated with Pam2Cys–gD2-30-5 or UV-HSV Ag. To our knowledge, this is the first report of physical contact between NK cells and CD4 T lymphocytes through ISs.

At all time points, significantly more NK–CD4 cell conjugates were observed with Pam2Cys–gD2 peptide or UV-HSV Ag stimulation than in control unstimulated cultures. Low proportions of NK–NK and CD4–CD4 cell conjugates were also found in Pam2Cys–gD2 peptide-stimulated samples and are probably nonspecific. As previously reported, unstimulated naïve lymphocytes...
and DCs can form conjugates in the absence of Ag, which may play a major role in the maintenance of naïve lymphocytes in vivo (77). Specific NK cell–CD4 T lymphocyte IS formation, shown by talin and CD4 polarization to the IS, was rare and reflects the low frequency of circulating memory CD4 T lymphocytes specific for single gd2 peptides. The mean frequency of whole-HSV Ag-specific memory CD4 T lymphocytes reported to be ~0.2% of total circulating CD4 T lymphocytes (78). Less than 5% of NK cells (probably mostly the CD56<sup>high</sup>CD16<sup>-</sup> subset) were involved in ISs, also correlating with the proportion of NK cells expressing HLA-DR (data not shown). Calculation of the approximated specificities of whole-HSV Ags for human T lymphocytes in seropositive patients suggests that 1 in 10,000 cells should form specific ISs, consistent with our experimental results. In an attempt to increase the frequency of IS formation, UV-HSV1 or a gd2-30-5–specific CD4 T cell line was used to study IS formation with NK cells. IS formation between UV-HSV1 or Pam2Cys–gd2 peptide-stimulated NK cells and CD4 T lymphocytes was observed in different HSV-seropositive donors. The mechanism of processing of particulate viral Ags, like HSV, by NK cells is unclear and is being investigated. Presumably, this involves endocytosis, cathepsin-mediated peptide cleavage, and combination with MHC II molecules in vesicular compartments, as with other APCs (79).

Both CD56<sup>high</sup>CD16<sup>-</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cell subsets are found in lung, liver, and skin and circulate through peripheral blood. However, the minor subset CD56<sup>high</sup>CD16<sup>-</sup> NK cells are more abundant in secondary lymphoid organs, accounting for up to 75% of NK cells in lymph nodes and 50% in the spleen (25). CD56<sup>dim</sup>CD16<sup>+</sup> NK cells are found in T cell-enriched areas in human lymph nodes and tonsils and, thus, can directly contact lymphocytes in their vicinity (29). Postinfection with Leishmania, major IFN-γ–secreting NK cells were found to contact the same DCs as Ag-specific CD4 T lymphocytes in the lymph node (80). However, a direct interaction between NK cells and CD4 T lymphocytes in response to pathogens has not been shown previously. In this study, we demonstrate a new role for NK cells as APCs in the context of HSV peptide conjugated to Pam2Cys and with UV-HSV1 or UV-HSV2, and also show IS formation between NK and CD4 T lymphocytes. However, the exact mechanism of HSV binding to TLR2 is unknown. With human CMV, it is via the major envelope glycoproteins, but that does not seem to be the case for HSV1 or HSV2, and it also appears to be strain dependent (18, 38).

In summary, NK cell depletion markedly decreased IFN-γ secretion of PBMCs in response to an immunogenic HSV2 peptide conjugated to the TLR2 agonist Pam2Cys, and the presence of NK cells enhanced the resulting immune response to HSV Ags. NK cells alone responded to whole-HSV–inactivated Ags and HSV lipopeptides through TLR2 molecules, then stimulated NK cells induced CD4 T cell activation though cytokine and cell-to-cell contact via MHC II molecules on NK cells. The significance of their physical contact was shown by the observation of IS formation between them in vitro and contact between them in recurrent herpetic lesions in vivo.

These findings indicate the importance of interactions between DCs, NK cells, and CD4 T lymphocytes in pairs and triplets in human viral immunology using HSV as an example, as well as the multiple roles of NK cells that extend beyond cytotoxicity and cytokine production to Ag presentation. Furthermore, there are practical consequences to developing vaccines for HSV. TLR2 (and other TLRs) on NK cells, as well as on DCs, must be considered a potential target for adjuvants to augment protective CD4, and perhaps CD8, T lymphocyte responses.

**Acknowledgments**

We thank Dr. Stuart G. Turville for training M.K. and N.R.O. for Delta-Vision Microscopy and Fabienne Brilot-Turville for providing the protocol for conjugation experiments. We also thank Dr. Karen Byth-Wilson for statistical analysis.

**Disclosures**

The authors have no financial conflicts of interest. A.L.C. has served as a consultant to GlaxoSmithKline on the development of vaccines for genital herpes, but no commercial funding was received for this project.

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