NKT Cells Inhibit Antigen-Specific Effector CD8 T Cell Induction to Skin Viral Proteins

Stephen R. Mattarollo, Michelle Yong, Christina Gosmann, Allison Choyce, Dora Chan, Graham R. Leggatt and Ian H. Frazer

*J Immunol* 2011; 187:1601-1608; Prepublished online 8 July 2011;
doi: 10.4049/jimmunol.1100756
http://www.jimmunol.org/content/187/4/1601

Supplementary Material  http://www.jimmunol.org/content/suppl/2011/07/08/jimmunol.1100756.DC1

References  This article cites 24 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/187/4/1601.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
NKT Cells Inhibit Antigen-Specific Effector CD8 T Cell Induction to Skin Viral Proteins

Stephen R. Mattarollo, Michelle Yong, Christina Gosmann, Allison Choyce, Dora Chan, Graham R. Leggett, and Ian H. Frazer

We recently demonstrated that CD1d-restricted NKT cells resident in skin can inhibit CD8 T cell-mediated graft rejection of human papillomavirus E7-expressing skin through an IFN-γ–dependent mechanism. In this study, we examined the role of systemically derived NKT cells in regulating the rejection of skin grafts expressing viral proteins. In lymph nodes draining transplanted skin, Ag-specific CD8 T cell proliferation, cytolytic activity, and cytokotoxicity were impaired by NKT cells. NKT cell suppression was mediated via CD11c+ dendritic cells. Inhibition of CD8 T cell function did not require Foxp3+ regulatory T cells or systemically derived NKT cells in regulating the rejection of skin grafts expressing viral proteins. In lymph nodes draining transplanted skin, NKT cells reduce the capacity of draining lymph node-resident APCs to cross-present Ag to CD8 T cell precursors, as evidenced by impaired expansion and differentiation to Ag-specific CD8 T effector cells. Therefore, in the context of viral Ag challenge in the skin, systemic NKT cells limit the capacity for effective priming of adaptive immunity.

Specialized immune regulatory cells are found in chronic skin infections and epithelial tumors and contribute to local mechanisms inhibiting generation and function of immune effector cells targeted at viral- or tumor-specific Ags (1, 2). Recently, using a murine model in which skin expressing human papillomavirus (HPV) 16 E7 oncoprotein as a transgene in basal keratinocytes is grafted into an immune-competent host, we showed that NKT cells present in skin locally suppress the function of systemically generated E7-specific effector CD8 T cells. This functional inhibition of CD8 T cells prevented rejection of E7-expressing skin (3). Local NKT cell-mediated regulation required production of IFN-γ by the NKT cells, and this was induced following CD1d-dependent NKT cell activation by a population of myeloid cells recruited to the skin graft.

In addition to regulation of local immune effector functions, NKT cells possess the capacity to promote or inhibit priming of the adaptive-immune response via the release of cytokines that can license APCs toward immunogenic or tolerogenic activity (4, 5). We recently showed that systemically derived NKT cells promote the generation of OVA-specific CD8 T cells in skin-draining lymph nodes (DLNs) following grafting of skin expressing OVA as a transgene in keratinocytes. NKT cell assistance in priming of CD8 T cells resulted in more effective CD8 T cell-mediated rejection of grafted K5mOVA-transgenic skin (6). Because NKT cells locally inhibit skin graft rejection when the grafted skin expresses E7 as a transgene, we wished to investigate the effects of systemically derived NKT cells on initiation of an adaptive-immune response to E7 cross-presented from skin. We demonstrate in this article that, in contrast to the effects of systemically derived NKT cells on priming of OVA-specific CD8 T cells following grafting of OVA-transgenic skin, NKT cells stimulated by grafting with skin expressing E7 or expressing another viral Ag, the herpes simplex gpβ, inhibit CD8 T cell-mediated rejection of skin grafts. For E7-transgenic skin, we further show that systemically derived NKT cells achieve suppression of graft rejection by inhibiting the ability of APCs in the skin-DLNs to promote proliferation and acquisition of effector functions by Ag-specific CD8 T cells. Unlike the local inhibitory effects of skin-resident NKT cells on CD8 T cell effector function, the regulatory function of systemically derived NKT cells is independent of IFN-γ.

Materials and Methods

Mice

C57BL/6 mice and HPV16-E7–transgenic C57BL/6 mice (designated K14E7) (3) were obtained from the Animal Resources Centre (Perth, Australia). K14gB-transgenic mice, in which HSV gpB is driven off the K14 promoter, were kindly provided by F. Carbone (University of Melbourne, Melbourne, Australia). IFN-γ knockout (KO) mice were purchased from The Jackson Laboratories (Bar Harbor, ME), IL-10−/− mice were sourced from the Australian National University (Canberra, Australia), and IL-17−/− mice were provided by Y. Iwakura (University of Tokyo, Tokyo, Japan). NKT cell-deficient CD1d−/− and Ju18−/− mice were obtained from M. Smyth (Peter MacCallum Cancer Centre, Melbourne, Australia) and maintained locally at the Princess Alexandra Hospital Biological Research Facility. HPV16 E7–specific TCR β-chain transgenic C57BL/6 mice (E7TCRβ) were generated in our laboratory by G. Leggatt (7) and crossed with CD45.1 congenic C57BL/6.SJL-Ppopc mouse (Animal Resources Centre) to generate mice bearing CD45.1+E7 TCR cells. DEREG mice were provided by T. Sparwasser (TWINCORE, Hannover, Germany) (8).

To generate K14E7–transgenic crosses with DEREG mice (K14E7 × DEREG), heterozygous K14E7 mice were crossed with DEREG mice to an F1 generation. All mice were housed under specific pathogen-free conditions at the Princess Alexandra Hospital Biological Research Facility, were sex-matched for all experiments, and were used at 6–10 wk of age.
age. All animal procedures were approved by the University of Queensland Animal Ethics Committee.

Reagents and flow cytometry

The HPV16-E7 peptide containing the H-2Dd-restricted CTL epitope, with the amino acid sequence RAHYNIVTF (GF001), was purchased from Auspep (Melbourne, Australia) with >80% purity, dissolved in 100% DMSO, and stored at −20°C. Anti-mouse mAbs to CD3 (145-2C11), CD4 (RM4-4), CD8 (53-67), CD25 (PC61), CD69 (H1.2F3), CD11c (HL3), CD45.1 (A20), CD45.2 (104), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL1), TCRβ (H57-597), MHC class II (M5/1415.2), IFN-γ (XM1.2), and associated isotopic control IgGs were purchased from BD Biosciences (San Diego, CA), eBioscience (San Diego, CA), and BioLegend (San Diego, CA). Preparation of α-galactosylceramide (αGalCer)-loaded CD1d tetramer is described elsewhere (9) and was kindly provided by D. Godfrey (University of Melbourne). Cells were stained at predetermined optimal concentrations of Ab for 30 min at 4°C. For intracellular staining of cytokines, permeabilization and fixation of cells were conducted using the BD Cytofix/Cytoperm kit, according to the manufacturer’s instructions (BD Biosciences). Monensin (BioLegend) was added to the cells to inhibit cytokine release from the Golgi/endoplasmic reticulum complex. Stained cells were acquired on a FACSCalibur or FACSCanto flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Skin grafting and in vivo cell depletions

The process of grafting donor ear skin onto the flanks of recipient mice, as well as the assessment of graft acceptance versus rejection, is described in detail elsewhere (10, 11). Anti-CD4 (GK1.5), anti-CD8 (53-5.8), and anti-CD25 (PC61) mAbs were purified in-house, from supernatant taken from hybridoma cell line culture, by protein elution using G-protein columns (Thermo Fisher Scientific, Rockford, IL). CD4+, CD8+, or CD25+ cells were depleted from donor and/or host mice, as indicated, prior to skin grafting by i.p. injection of 500 μg GK1.5 (×1), 100 μg 53-5.8 (×3), or 500 μg PC61 (×2) Abs, respectively. An equal volume of isotype-matched rat IgG Ab was used for control treatments. Maintenance treatments were given weekly to recipient mice to continue cell depletion for the duration of the experiment. Foxp3+ cells were depleted from DEREG and K14E7 × DEREG mice by three administrations of 1 μg diphtheria toxin (DT) in 1 week, prior to grafting.

Adoptive transfers of NKT cell populations

For bulk reconstitution experiments (providing a source of NKT cells), 5 × 10^7 splenocytes isolated from C57BL/6 mice were injected i.v. into Jα18Ko recipients 3 d prior to grafting with K14E7 skin. For pure NKT cell transfers, mononuclear cells pooled from liver, thymus, and inguinal lymph nodes of wild-type (WT), IL-10−/−, IFN-γ−/−, or IL-17−/− C57BL/6 mice were sorted by flow cytometry (MoFlo, BD Biosciences) based on dual CD3+ and CD1d-tetramer+ staining. CD3+CD1d-tetramer+ T cells were purified using G-protein microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in vitro for 5 d with 0.01 μM GF001 peptide and 2 ng/ml IL-2 to restimulate CD8 T cells prior to purification. Isolated CD8 T cells were then cocultured for 24 h with GF001-pulsed EL4 cells, used as targets in a standard chromium-release assay, as previously described (13).

Dendritic cell functional assay

Dendritic cells (DCs) were isolated from skin-DLNs of grafted or nongrafted mice by FACS, based on dual CD11c+/CD1d+ staining. CD11c+CD1d+tetramer+ cells were depleted (Fig. 1 a) and associated isotype control IgGs were purchased from BD Biosciences. Maintenance treatments were given weekly to recipient mice to continue cell depletion for the duration of the experiment. Foxp3+ cells were depleted from DEREG and K14E7 × DEREG mice by three administrations of 1 μg diphtheria toxin (DT) in 1 week, prior to grafting.

In vivo proliferation assay and immunizations

To assess HPV16 E7-specific CD8+ T cell proliferation in vivo, CD45.1+ E7TCRB+ splenocytes were labeled with 2.5 μM CFSE and injected i.v. (1 × 10^7) into the tail vein of WT, CD1d−/−, or Jα18Ko mice. Seven days later, recipient spleens were harvested and CFSE dilution in CD45.1+CD8+ cells was assessed by flow cytometry. Percentages of proliferation were generated using ModFit LT software (Verity Software House, Topsham, ME). To determine Ag-specific CD8 T cell responsiveness to soluble Ag challenge, mice were immunized s.c. at the tail base with 50 μg GF001 peptide, 20 μg keyhole limpet hemocyanin (Sigma, Castle Hill, NSW, Australia), and 20 μg QuilA adjuvant (Soperfos Biotech, Vedbaek, Denmark).

In vitro assays of dendritic and CD8 T cell function

CD8+ T cell cytokine production and cytokotoxicity, CD8 T cells were isolated from skin-DLNs by MACS separation using CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). For detection of cytokine secretion, CD8 T cells were restimulated in vitro for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin prior to collecting culture supernatant. Secreted levels of IFN-γ, TNF-α, and IL-2 were detected by Th1/Th2 cytometric bead array, according to the manufacturer’s protocol (BD Biosciences). Samples were analyzed on a FACSArray (BD Biosciences). For IFN-γ ELISPOT, cell suspensions isolated from skin-DLNs of immunized or grafted recipients were cultured overnight in complete RPMI 1640 medium in the presence of 5 ng/ml recombinant mouse IL-2 (BD Biosciences), with or without 0.01 μM GF001 peptide. The IFN-γ ELISPOT procedure was described previously (12). For assessment of Ag-specific cytotoxicity, cell suspensions isolated from spleens and skin-DLNs of immunized mice were cultured in vitro for 5 d with 0.01 μM GF001 peptide and 2 ng/ml IL-2 to restimulate CD8 T cells prior to purification. Isolated CD8 T cells were then cocultured for 24 h with GF001-pulsed EL4 cells, used as targets in a standard chromium-release assay, as previously described (13).

Statistics

Kaplan–Meier plots were used to analyze skin graft survival, and a log-rank test was performed to assess the statistical significance of differences between survival curves. For all other data for which statistics were performed, a two-tailed t test or nonparametric Mann–Whitney U test, as indicated, was used for assessment of differences between groups; p values < 0.05 were considered significant. Prism (GraphPad Software, La Jolla, CA) software was used to prepare graphs and for statistical analysis.

Results

Host-type I NKT cells are critical in the inhibition of K14E7 graft rejection

We recently reported that a population of NKT cells resident in HPV16-E7–transgenic skin is capable of inhibiting K14E7 graft rejection (3). In addition, a previous report showed that systemically derived host NKT cells can regulate rejection of MHC-mismatched skin grafts (14). To address the role of host-derived NKT cells in the suppression of K14E7 skin graft rejection, we grafted native K14E7 skin onto NKT cell-deficient CD1d−/− recipients. We observed 100% rejection of K14E7 grafts on CD1d−/− recipients (Fig. 1 a), without rejection of WT C57BL/6 or littermate E7 CD1d−/− control grafts. K14E7 grafts were also rejected by type I NKT cell-deficient Jα18Ko recipients. These findings demonstrated a critical role for host-derived type I, invariant NKT cells in inhibiting HPV16-E7–specific effector T cell functions necessary and sufficient for skin graft rejection (11). To further confirm a role for systemically derived NKT cells in regulating effector functions necessary for graft rejection, we transferred 5 × 10^7 splenocytes from WT mice (equating to ~4 × 10^7 NKT cells) into Jα18Ko graft recipients; this was sufficient to prevent K14E7 graft rejection, as observed in control animals. Only one of eight (12.5%) grafts was rejected following NKT cell reconstitution (Fig. 1 b), supporting our observation that systemically derived type I NKT cells are, like locally resident NKT cells, fully competent to regulate the effector functions necessary for rejection of K14E7 skin grafts. For skin grafts expressing OVA or human growth hormone from a keratin promoter, which, unlike E7 grafts, are routinely rejected by immunocompetent recipients, graft rejection is mediated by CD8 effector T cells (6, 15). To establish whether CD8 T cells were critical for rejection of K14E7 grafts in NKT cell-deficient hosts, we depleted CD1d−/− mice of CD8 T cells using an anti-CD8 mAb prior to grafting with K14E7 skin. We observed a substantial reduction and delay in K14E7 graft rejection during the 6-wk period that the CD8 T cells were depleted (Fig. 1 c). To confirm that failure to reject K14E7 grafts was due to the absence of CD8 T cells, we permitted CD8 T cell recovery in NKT cell-deficient mice and then placed a second K14E7 graft on the contralateral flank of those that failed to reject the primary graft. With recovery of the CD8 T cell population, rejection of second grafts was complete and followed similar kinetics to primary K14E7 graft rejection on nondepleted...
CD1dKO recipients following systemic depletion of transferred from WT mice, as a source of NKT cells. Splenocytes transferred from Jo18KO mice were used as controls (n = 8 mice/group). C. K14E7 skin graft survival on CD1dKO recipients following systemic depletion of CD8 T cells, using anti-mouse CD8α (53-5.8) mAb or similar treatment with an isotype-matched rat IgG mAb. Anti-CD8α mAb-treated recipients that failed to reject the primary K14E7 graft received a second K14E7 graft on the contralateral flank on day 42 (n = 12 for rat IgG and anti-CD8α mAb-treated groups; n = 6 for second K14E7 graft group). *p < 0.0001, log-rank test.

Host NKT cell-mediated regulation of K14E7 skin graft rejection is independent of IFN-γ, IL-10, or IL-17 cytokine production and does not require Foxp3+ regulatory T cells

We previously observed that inhibition of K14E7 graft rejection by donor-derived skin-resident NKT cells requires IFN-γ (3). To determine whether immune suppression mediated by systemic host-derived NKT cells is dependent on IFN-γ production by NKT cells, we repopulated NKT cell-deficient Jo18KO mice with IFN-γ−/− or IFN-γ−/− NKT cells prior to grafting. NKT cell reconstitution led to complete inhibition of graft rejection in Jo18KO mice, regardless of their capacity for IFN-γ production (Fig. 3A). This observation was extended to other candidate cytokines, including IL-10 and IL-17, because transferred IL-10−/− and IL-17−/− NKT cells were equally capable of inhibiting graft rejection (Fig. 3A). Therefore, in contrast to suppression of CD8 T effector cell-mediated rejection of skin grafts by skin-resident NKT cells, which requires IFN-γ suppression of the generation of effector function for graft rejection mediated by host-resident NKT cells is independent of their ability to produce IFN-γ, IL-10, or IL-17.

Host NKT cells could be indirectly mediating suppression of HPV16-E7 graft rejection by CD8 T cells, by enhancing the function of other suppressive or regulatory cell populations in the skin. Therefore, we investigated the requirement for CD4+CD25+ Foxp3+ regulatory T cells (Tregs) in NKT cell-mediated regulation of skin graft rejection. Depletion of Tregs from donor K14E7 skin, WT recipients, or both, using an anti-CD25 mAb, did not enable rejection of K14E7 grafts (Fig. 3B). To confirm this finding, we grafted skin from DT-treated DEREG mice, allowing specific depletion of Foxp3+ Tregs without compromising effector cell function. Depletion of Foxp3+ cells from the graft donor or from both donor and host did not enable K14E7 graft rejection (Fig. 3C), confirming that interactions with traditional Foxp3+ Tregs are not critical for host NKT cell-mediated regulation of effector T cell functions necessary for graft rejection.

Host NKT cells suppress the generation of Ag-specific CD8 effector T cells

Rejection of K14E7 skin grafts in NKT cell-deficient recipients is mediated by effector CD8 T cells (Fig. 1). Therefore, we addressed whether NKT cells suppressed host immunity to HPV16-E7 by

FIGURE 1. Host type I NKT cells inhibit K14E7 graft rejection. A. Kaplan–Meier survival curves of HPV16 E7-expressing skin grafts (K14E7) transplanted onto naive WT C57BL/6 and NKT cell-deficient CD1dKO recipients (n = 10 mice/group). B. Survival of K14E7 grafts on type 1 NKT cell-deficient Jo18KO recipients, some of which were reconstituted 3 d prior to grafting by transfer of 1 × 10⁷ splenocytes isolated from WT mice, as a source of NKT cells. Spleenocytes transferred from Jo18KO mice were used as controls (n = 8 mice/group). C. K14E7 skin graft survival on CD1dKO recipients following systemic depletion of CD8 T cells, using anti-mouse CD8α (53-5.8) mAb or similar treatment with an isotype-matched rat IgG mAb. Anti-CD8α mAb-treated recipients that failed to reject the primary K14E7 graft received a second K14E7 graft on the contralateral flank on day 42 (n = 12 for rat IgG and anti-CD8α mAb-treated groups; n = 6 for second K14E7 graft group). *p < 0.0001, log-rank test.

FIGURE 2. NKT cells suppress rejection of grafts expressing K14-restricted HSV gpB. Survival of K14 promoter-driven gpB (K14gB) grafts on WT C57BL/6 and CD1dKO recipients. C57BL/6 recipients, 63% rejection; median survival, 90 d. CD1dKO recipients, 88% rejection; median survival, 46 d (n = 8 mice/group). *p = 0.03.
inhibiting the priming and activation of E7-specific CD8 T cells. Subcutaneous immunization with E7 protein generated less IFN-γ-producing, E7-specific CD8 T cells in skin-DLNs in WT mice than in CD1dKO or Jo18KO mice (Fig. 4A). We were unable to detect Ag-specific IFN-γ production by DLN CD8 T cells following grafting with K14E7 skin in mice with or without NKT cells, likely a result of insufficient sensitivity in the assay system. To assess whether NKT cells mediated suppression of Ag-specific CD8 T cell proliferation following grafting, we transferred CFSE-labeled E7-specific CD8 T cells isolated from skin-DLNs of WT, CD1dKO, and Jo18KO mice 7 d after primary stimulation with either s.c. GF001 peptide immunization (E7 Imn) or grafting with K14E7 skin (E7 graft). IFN-γ-producing cells were measured by ELISPOT following overnight restimulation with GF001 peptide and IL-2. ∆ IFN-γ is the corrected spot-forming units (SFU) value after subtracting counts obtained from control wells containing no peptide stimulation. Each symbol represents the mean value from triplicate wells. ND, no spots detected.

NKT cells might inhibit DC maturation and T cell-priming activity. To determine the effect of NKT cells on DC function, as well as to establish whether any effect was dependent on DC activation by grafting, we isolated CD11c+ cells from skin-DLNs of WT mice that had not been grafted. In each case, the stimulation was decreased if the donor was NKT cell deficient (Fig. 5A). This confirms that DCs require activation to effectively present Ag and that NKT cells possess the ability to suppress T cell proliferation whether the DC is activated or not. We next investigated whether the decrease in the T cell-stimulatory capacity of DCs that were conditioned in the presence of NKT cells during grafting was due to inadequate DC expression of costimulatory molecules. CD86 expression was increased on DCs in the DLNs of mice that had received a graft possessed a substantially greater capacity to stimulate E7-specific IFN-γ production from CD8 T cells compared with DCs isolated from the skin-DLNs of mice that had not been grafted. In each case, the stimulation was substantially enhanced if the donor was NKT cell deficient (Fig. 5A). This confirms that DCs require activation to effectively present Ag and that NKT cells possess the ability to suppress T cell proliferation whether the DC is activated or not. We next investigated whether the decrease in the T cell-stimulatory capacity of DCs that were conditioned in the presence of NKT cells during grafting was due to inadequate DC expression of costimulatory molecules. CD86 expression was increased on DCs in the DLNs of WT mice that had been grafted with K14E7 skin and was higher compared with skin-grafted WT mice (Fig. 5B). In contrast, CD40 and CD80 expression on DCs remained constant when comparing ungrafted and grafted WT and CD1dKO mice. Therefore, abrogated stimulation of CD8 T cells by DCs in WT mice does not seem likely to be attributable to NKT cell-mediated suppression of expression of the key DC costimulatory molecules. Notably, at the time when DCs were isolated from the skin-DLNs of grafted mice, no evidence of NKT cell activation was detected.

DCs isolated from skin-DLNs of NKT cell-deficient mice induce greater Ag-specific CD8 T cell activity

To explain the reduction in proliferation of E7-specific T cells in the DLNs of mice harboring E7 grafts, we hypothesized that
observed in this organ. Expression of CD69 (Fig. 6A) and CD25 (Fig. 6B) on NKT cells in WT mice, as a measure of activation in response to grafting, was not increased following grafting with either control C57BL/6 skin or K14E7 skin. Thus, intrinsic or default function of skin-resident NKT cells seems to be regulatory.

Proinflammatory cytokine production and Ag-specific cytotoxic activity of CD8 T cells are suppressed following K14E7 skin grafting in the presence of NKT cells

We hypothesized from the observed decrease in the generation of IFN-γ–producing Ag-specific CD8 T cells in K14E7-grafted WT mice compared with NKT-deficient mice (Fig. 4A) that NKT cells might be inhibiting graft rejection by negatively regulating the acquisition of effector functions by CD8 T effector precursor cells. CD8 T cells isolated from skin-DLNs of grafted WT and CD1dKO mice possessed equal capacity to secrete proinflammatory cytokines, including IFN-γ, TNF-α, and IL-2, upon non-specific mitogen stimulation in vitro (Fig. 7A). However, 4 d after grafting with K14E7 skin, CD8 T cells from WT recipients showed significantly reduced capacity for cytokine production compared with CD8 T cells isolated from NKT cell-deficient CD1dKO recipients (Fig. 7A). Intracellular cytokine staining was performed to determine whether the decrease in IFN-γ secretion by CD8 T cells from the DLNs of grafted WT mice, shown by ELISA, was due to a decrease in production by individual CD8 T cells or a reduction in the total number of IFN-γ–producing cells. Expression of IFN-γ in individual CD8 T cells was comparable before and after grafting in WT mice (Fig. 7B); however, the percentage of CD8 T cells with IFN-γ–producing capacity was reduced (Fig. 7C), consistent with the impaired proliferation of these cells in NKT-populated animals (Fig. 4). Minimal cytokine production of IL-4, IL-5, and IL-10 by CD8 T cells was detected over the same period (data not shown), and so it is unlikely that the CD8 T cells were diverting from a Tc1-like to a Tc2-like cytokine profile. Therefore, host NKT cells present during Ag-specific CD8 T cell differentiation in the DLNs following K14E7 skin grafting reduce their proliferation and differentiation toward effector cells.

To determine whether CD8 T cells generated in the presence of NKT cells demonstrated decreased cytotoxic potential, we measured cytotoxic activity of CD8 T cells induced in WT and CD1dKO mice following immunization. WT and CD1dKO mice immunized with E7 peptide and adjuvant, after a period of Ag restimulation in vitro, demonstrated CD8 T cell-mediated and Ag-specific killing of peptide-pulsed targets, and CD8 T cells from CD1dKO animals displayed significantly enhanced killing (Fig. 7D). Enhanced cytotoxicity was not dependent on increased production of cytotoxic granules, because intracellular stores of perforin and granzyme B were comparable between activated CD8 T cells from WT and CD1dKO mice (data not shown). Therefore, NKT cells activated by grafting or local inflammatory stimuli associated with immunization can prevent the acquisition of full cytotoxic functionality of Ag-specific CD8 T cells exposed to HPV16-E7 Ag

FIGURE 6. NKT cell activation is not altered following skin grafting. Expression of CD69 (A) and CD25 (B) on TCRβ+CD1d-tetramer+ NKT cells isolated from skin-DLNs of non-grafted WT C57BL/6 mice or from C57BL/6- and K14E7-grafted WT mice on day 7 postgrafting. Overlay graphs show representative expression of CD69 and CD25 on NKT cells from the indicated mice. Expression of CD69 and CD25 on individual mice is displayed as both percentages (upper graphs) and mean fluorescence intensity (MFI) (lower graphs).
FIGURE 7. Capacity for CD8 T cell proinflammatory cytokine production and cytotoxic activity in skin-DLNs is reduced in the presence of NKT cells. A, Secretion of IFN-γ (top graph), IL-2 (middle graph), and TNF-α (bottom graph) by CD8 T cells isolated from skin-DLNs of K14E7-grafted or nongrafted WT and CD1dKO mice, measured by cytometric bead array following a 4-h period of PMA/ionomycin restimulation in vitro. Each symbol represents the mean value from triplicate wells from one mouse. *p = 0.01, *p = 0.001, /p = 0.0005, unpaired t test. B, Representative flow cytometry dot plots showing intracellular IFN-γ staining in CD8 T cells from nongrafted and grafted WT mice following PMA/ionomycin stimulation. Mean fluorescence intensity (MFI) values shown are of IFN-γ WT mice following PMA/ionomycin stimulation. Mean fluorescence intensity in CD8 T cells isolated from skin-DLNs of K14E7-grafted or nongrafted WT mice producing IFN-γ following PMA/ionomycin stimulation. Mean fluorescence intensity in CD8 T cells isolated from skin-DLNs of K14E7-grafted or nongrafted WT mice producing IFN-γ following PMA/ionomycin stimulation. Means data were pooled from two independent experiments. **p = 0.0001, unpaired t test. D, Percentage lysis of GF001-pulsed EL4 target cells after 24 h of coculture with CD8 T cells (effectors), at three E:T ratios, measured in an in vitro chromium-release assay. CD8 effector T cells were generated in WT and CD1dKO mice by s.c. immunization with GF001 + keyhole limpet hemocyanin + QuilA and isolated from spleen and inguinal lymph nodes (mean + SEM; n = 6). *p < 0.05, /p < 0.005, unpaired t test.

Discussion
In a recent study, we reported a critical role for skin-resident NKT cells and IFN-γ in establishing a local immune-suppressive environment in skin grafts. Immune-competent host animals simultaneously challenged with an NKT-populated and an adjacent NKT-deplete graft, each expressing the E7 protein of HPV16 from a keratin 14 promoter, rejected only the NKT-deplete graft (3). These data demonstrated that immune effector cells, generated by cross-priming of graft-derived Ag in the DLNs, cannot acquire effector functions necessary for graft rejection in the presence of skin donor-derived NKT cells. The present study was undertaken to investigate the effects of systemic NKT cells on the generation and differentiation of Ag-specific CD8 effector T cells following cross-priming from Ag expressed in grafted skin. We demonstrated in this study that systemically derived type I NKT cells also inhibit generation of CD8 effector T cells capable of mediating rejection of grafts. We showed further that this is attributable to suppression, by NKT cells in the DLNs, of generation and differentiation of Ag-specific CD8 T cells in response to Ag cross-presented by DLN APCs.

NKT cells in K14E7-transgenic skin, although sufficient to prevent local CD8 T cell-mediated graft rejection in a graft-primed animal (3), did not inhibit Ag-specific priming and, hence, rejection of K14E7 grafts by CD1dKO or Jo18KO recipients, indicating a further distinct role for host NKT cells in the regulation of local priming to cross-presented Ag. Reconstitution of Jo18KO recipients with NKT cells was sufficient to restore immune suppression, supporting a direct contribution of type I invariant NKT cells. This observation supports findings in a skin transplant model by Oh et al. (14), who reported that, in CD1dKO mice transplanted with sex-mismatched grafts, rejection occurred at an accelerated rate compared with WT recipients. We previously demonstrated that donor- and then host-derived NKT cells and IFN-γ locally suppress CD8 T cell-mediated graft rejection (3). Matsumoto et al. (11) observed that immune suppression to HPV16-E7 can be overcome by transferring an enriched population of E7-specific CD8 T cells, activated in situ by simultaneous immunization with E7 protein. In addition, we showed in this study that CD8 T cells, although not an absolute requirement for rejection in some mice, maximized the rate and probability of rejection of K14E7 grafts in the absence of NKT cell-mediated suppression in CD1dKO mice (Fig. 1C). Based on this evidence for the requirement of CD8 T cells in the rejection of K14E7 grafts, we hypothesized that host NKT cells may also be acting to inhibit graft rejection by suppressing the priming of E7-specific CD8 T cells in the skin-DLNs.

Generally, NKT cells have been associated with enhanced priming of Ag-specific T cells, particularly when NKT cells and CD8 T cells are simultaneously activated by the same DCs (16, 17). In fact, we showed, in a skin-grafting model incorporating OVA protein expressed by a transgene in epithelial keratinocytes (K5mOVA mouse), that NKT cells can enhance CD8 T cell-dependent rejection of OVA-expressing grafts (6). In contrast, we demonstrated that, in response to skin-derived viral Ags that require additional CD4 T cell help for graft rejection, NKT cells inhibited efficient...
priming of endogenous Ag-specific T cells, leading to inhibition of graft rejection. Therefore, the resulting NKT cell effect on CD8 T cell priming (activating versus inhibitory) may be dependent on the type of Ag and linked to the requirement for CD4 helper epitopes. In addition, skin grafts that express both E7 and OVA proteins were rejected rapidly in WT mice, and time to rejection was not altered in NKT-deficient mice (Supplemental Fig. 1), further suggesting that Ag dominance and T cell requirements for graft rejection alter the role of NKT cell regulation.

These findings support observations in a recent report by Guillonneau et al. (18), who showed diminished effector T cell generation in response to an influenza virus following NKT cell activation by α-GalCer. In our model, NKT cell-mediated suppression of effector CD8 T cell priming and graft rejection occurred in the absence of exogenous NKT ligand, was not altered by local or systemic administration of α-GalCer (data not shown), and did not seem to be associated with expression of the conventional NKT cell-activation markets. It likely reflects NKT cell-mediated modulation of DCs in the skin-DLNs. Activated CD11c+ DCs isolated from skin-DLNs of WT mice grafted with K14E7 skin were impaired in their capacity to induce E7-specific CD8 T cell responses in vitro compared with DCs isolated from NKT-deficient CD1dKO recipients. Apart from CD86, which was elevated in grafted CD1dKO recipients, the expression of costimulatory markers on DCs isolated from WT and CD1dKO recipients, the expression of costimulatory molecules was comparable on DCs isolated from WT mice grafted with K14E7 skin, indicating that NKT cells were not suppressive DC activity by directly inhibiting costimulation. In NOD mice, activation of NKT cells by injection of α-GalCer was associated with the appearance of tolerogenic DCs that prevented the development of diabetes by suppressing pathogenic T cell responses (4, 19). In addition, a study of human NKT cells revealed that APCs instructed by NKT cells acquired a tolerogenic phenotype and silenced downstream T cell responses, including IFN-γ production and proliferation (20). NKT cell licensing of DCs toward a suppressive or tolerogenic phenotype in skin-DLNs of K14E7-grafted mice, in the absence of exogenous ligand stimulation, might be acting via similar mechanisms; this is currently under investigation.

CD8 T cells from skin-DLNs of K14E7-grafted mice, generated in the presence of NKT cells, possessed a reduced capacity to produce the proinflammatory cytokines IFN-γ, TNF-α, and IL-2. Reduced secretion levels of these cytokines was not due to lower cytokine production on a per cell basis, because intracellular expression was comparable in CD8 T cells from NKT-sufficient and NKT-deficient mice. Instead, lower secretion was associated with a reduced proportion of CD8 T cells with the capacity for proinflammatory cytokine production. The absence of anti-inflammatory cytokine secretion (IL-4, IL-5, IL-10) indicated that these NKT cell-instructed CD8 T cells were not being diverted to a Tc2-like phenotype. It is possible that differentiation of CD8 T cells into proinflammatory effector cells, in response to grafting stimulus, is being suppressed by NKT cells. Inhibition of CD4 T cell differentiation by NKT cells was reported in a type 1 diabetes mouse model (21, 22). Vv14-transgenic NOD mice, containing enhanced numbers of NKT cells, are resistant to the development of diabetes. This was attributed to NKT cell-mediated inhibition of the expansion and differentiation of pathogenic anti-β cell CD4 T cells (21). In addition, or alternatively, to a defect in differentiation, CD8 T cells might be rendered anergic or dysfunctional upon receiving inhibitory signals from NKT cells. This may explain why cytokine production by CD8 T cells from grafted WT mice was refractory, even to in vitro mitogen stimulation. In association with a defect in proinflammatory cytokine production, Ag-specific CD8 T cells generated to E7 protein in the presence of NKT cells also possessed impaired cytotoxic activity.

The mechanism by which NKT cells inhibit DC-mediated priming of E7-specific CD8 T cells and differentiation into cytotoxic effector cells is unknown. We showed that NKT cell-mediated suppression in skin-DLNs is independent of Foxp3+ Tregs and does not involve production of IFN-γ, IL-10, or IL-17 from NKT cells, in contrast to the local suppression of effector T cell function in skin, which depends on IFN-γ (3). Alternatively, there may be redundancy in the NKT cell-regulatory mechanisms contributing to APC signaling. Other cytokines produced by NKT cells may be implicated in the immunosuppression (e.g., IL-4, IL-13, and TGF-β) (23, 24); however, cell contacts are likely to be critical (22). Future work will address the relative contribution of cell contacts versus soluble mediators in NKT cell-mediated immune suppression.

Collectively, the results from this study, along with our recently published work (3), demonstrated that NKT cells possess diverse immune-suppressive functions in response to skin-derived viral Ag. They infiltrate into inflamed skin to inhibit CD8 effector T cell function locally and suppress the generation of Ag-specific CD8 T cells in the associated skin-DLNs. Therefore, NKT cells are capable of suppressing CD8 T cell immunity both in the priming phase in secondary lymphoid organs and in the effector response at the local site. NKT cell-mediated suppression of rejection of skin grafts expressing viral Ag is potentially mediated by IFN-γ production by NKT cells may be a potential means of virus evasion of the immune system, leading to chronic viral persistence and development of precancers and malignancy. Future studies investigating the molecular signals released by epithelial cells harboring viral proteins that induce immunosuppressive activity by NKT cells will be imperative in understanding the host–pathogen interactions leading to immune escape, associated chronic viral infection, and cancer development.

Acknowledgments
We thank Kon Kyparissoudis for generating the α-GalCer–loaded mouse CD1d tetramer, Shanu Sinha for genotyping of mice, and the staff of the Biological Research Facility at Princess Alexandra Hospital for excellent technical assistance and animal care.

Disclosures
The authors have no financial conflicts of interest.

References


