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Research Article

Cancer Immunology Research

CD1d-Restricted Antigen Presentation by Vγ9Vδ2-T Cells Requires Trogocytosis

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Abstract

CD1d-restricted invariant natural killer T cells (iNKT) constitute an important immunoregulatory T-cell subset that can be activated by the synthetic glycolipid α-galactosylceramide (α-GalCer) and play a dominant role in antitumor immunity. Clinical trials with α-GalCer–pulsed monocyte-derived dendritic cells (moDC) have shown anecdotal antitumor activity in advanced cancer. It was reported that phosphoantigen (pAg)-activated Vγ9Vδ2-T cells can acquire characteristics of professional antigen-presenting cells (APC). Considering the clinical immunotherapeutic applications, Vγ9Vδ2-T APC can offer important advantages over moDC, potentially constituting an attractive novel APC platform. Here, we demonstrate that Vγ9Vδ2-T APC can present antigens to iNKT. However, this does not result from *de novo* synthesis of CD1d by Vγ9Vδ2-T, but critically depends on trogocytosis of CD1d-containing membrane fragments from pAg-expressing cells. CD1d-expressing Vγ9Vδ2-T cells were able to activate iNKT in a CD1d-restricted and α-GalCer–dependent fashion. Although α-GalCer–loaded moDC outperformed Vγ9Vδ2-T APC on a per cell basis, Vγ9Vδ2-T APC possess unique features with respect to clinical immunotherapeutic application that make them an interesting platform for consideration in future clinical trials. *Cancer Immunol Res; 2(8); 732–40. ©2014 AACR.*

Introduction

Invariant natural killer T cells (iNKT) are an important immunoregulatory T-cell subset that is restricted by the CD1d antigen-presenting molecule. Their well-conserved T-cell receptor (TCR) repertoire consists of Vα14.Jα18 paired with Vβ2, Vβ7, or Vβ8.2 in mice, and Vα24.Jα18 preferentially paired with Vβ11 in humans (1–3). When activated by the synthetic glycolipid α-galactosylceramide (α-GalCer), iNKT cells produce large amounts of cytokines that play an important role in initiating and orchestrating antitumor immune responses (4). It is well established that activated iNKT promote the development of a long-lasting Th1-biased proinflammatory antitumor immune response in different murine tumor-metastasis models of the liver, lung, and lymph nodes, including colon carcinoma, lymphoma, sarcoma, melanoma, and lung carcinoma, suggesting broad clinical applicability (reviewed in ref. 5). Early clinical trials with α-GalCer–pulsed monocyte-derived dendritic cells (moDC) have shown anecdotal antitumor activity in advanced cancer (6, 7). Although immunologic, biochemical, and clinical responses have been observed in patients treated with α-GalCer, the results are not consistent. Multiple factors might contribute to the heterogeneous antitumor immune responses in the clinical trials that have been performed thus far. However, given that low iNKT numbers predict poor clinical outcome in multiple tumor types (5, 8–10), and activated iNKT can induce clinical responses without causing substantial toxicity, efforts to increase the consistency and predictability of iNKT cell–based cancer immunotherapy should be expanded.

Vγ9Vδ2-T cells are the predominant γδ-T-cell subset in human peripheral blood and account for 1% to 5% of the peripheral blood mononuclear cells (PBMC) in healthy adults. Vγ9Vδ2-T cells have well-established, antimicrobial, and antitumor effector functions; they can be activated and expanded by both natural [i.e., isopentenyl pyrophosphate (IPP)] and synthetic [i.e., bromohydrin pyrophosphate (BrHPP)] phosphoantigens (pAg). Other compounds, such as aminobisphosphonates (NBP), support the intracellular accumulation of endogenous pAg by inhibiting mevalonate metabolism (11–14). Overall, the accumulation of intracellular pAg results in pAg-induced changes in the mobility and conformation of the ubiquitously expressed CD277 that triggers Vγ9Vδ2 T-cell activation and expansion (15).

Previously it was reported that Vγ9Vδ2-T cells can exhibit characteristics of professional antigen-presenting cells (APC), including processing and presentation of antigen and costimulatory signals, allowing the induction of naïve αβ-T-cell proliferation and differentiation (16, 17). It has been suggested that the pAg-responsive Vγ9Vδ2-T cells offer important

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advantages over moDC with respect to clinical immunotherapeutic application, as Vγ9Vδ2-T cells are more numerous compared with DC precursors in the peripheral blood, mature more quickly (<24 hours vs. 7–10 days culture required for moDC) into professional APC, have better lymph node–homing properties, and exhibit a more uniform and consistent proinflammatory functional status (18). Here, we evaluated whether the activated Vγ9Vδ2-T cells could be used as a novel antigen-presenting platform for iNKT cells.

Materials and Methods

Antibodies and flow cytometry

Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll (PerCP)-, or allophycocyanin (AP)-labeled mAb directed against CD3, CD14, CD1a, CD40, CD80, CD86, CD25 (BD Biosciences), CD83, TCR-Vα24, -Vβ11, -Vδ2, (Beckman Coulter, Inc.), -Vγ9, EpCAM (CD326; BioLegend), and CD1d (clone 51.1; eBiosciences, Inc.) were used for flow cytometry analysis. mAb staining was performed in phosphate-buffered saline (PBS) supplemented with 0.1% BSA and 0.02% sodium azide for 30 minutes at 4°C. For intracellular staining of IFNγ, iNKT cells were harvested after 18 hours of coculture with $CD1d^-$ or $CD1d^+$Vγ9Vδ2 T cells or α-GalCer–pulsed HeLa-CD1d cells in the presence of 0.5 μL of Golgi Plug (BD Biosciences), washed, and stained with surface membrane mAb. After fixation with Cytofix/Cytoperm solution and permeabilization with Perm/Wash solution (both from BD Biosciences), cells were stained with PE-conjugated mAbs specific for IFNγ or appropriate isotype control for 30 minutes at 4°C in permeabilization buffer. Cells were washed with permeabilization buffer and FACS buffer before analysis. All stained cells were analyzed on FACSCalibur (BD Biosciences) using CellQuest software.

Generation of moDC

Immature moDC were generated by allowing PBMC to adhere to culture flasks for 2 hours at 37°C. Adherent cells were cultured for 5 to 7 days in the presence of recombinant human (rh)IL4 (10 ng/mL; R&D Systems) and rhGM-CSF (100 ng/mL; Bayer AG) in Iscove's Modified Dulbecco's Medium (IMDM; Lonza) supplemented with 10% fetal bovine serum (Hyclone), 100 IE/mL sodium penicillin (Yamanouchi Pharma), 100 μg/mL streptomycin sulfate (Radiumfarma-Fisiopharma), 2.0 mmol/L L-glutamine (Invitrogen), and 0.05 mmol/L 2-ME (Merck), hereafter referred to as complete medium. Immature moDC were matured with 100 ng/mL lipopolysaccharide (LPS; Sigma) during 24 to 48 hours at 37°C in a humidified atmosphere with 5% CO_2. Mature moDC were harvested by 5 mmol/L ethylenediaminetetraacetic acid (EDTA) in PBS (Braun Melsungen AG) and used for coculturing experiments, or irradiated (5,000 Rad) and used for weekly stimulation of purified cells.

Generation of Vγ9Vδ2-T cell lines and Vγ9Vδ2-T APC

Vγ9Vδ2-T cell lines were generated from human PBMC by magnetic-activated cell sorting (MACS) using either the murine anti-human Vδ2 TCR or anti-human Vγ9 TCR mAb, combined with a polyclonal goat-anti-mouse Ab or anti-PE Ab labeled with magnetic beads (Miltenyi Biotec). For culture/expansion of Vγ9Vδ2-T cells, 100 μmol/L of the NBP pamidronate (PCH; Pharmachemie BV) was added to immature moDC during the last 2 hours of maturation with LPS and cocultured with Vγ9Vδ2-T cells with rhIL2 (50 U/mL; BioVision). Purified Vγ9Vδ2-T cells were weekly restimulated with irradiated feeder-mix consisting of allogeneic PBMC and Epstein–Barr Virus (EBV)–transformed B cells in Yssel's medium supplemented with 50 ng/mL of PHA (Murex Biotech) and IL2 at 50 U/mL. Purity of Vγ9Vδ2-T cells used for experiments was >90%.

In experiments in which the acquisition of APC marker expression and function of Vγ9Vδ2-T was studied, both resting and activated freshly isolated Vγ9Vδ2-T cells and Vγ9Vδ2-T cell lines were used. Activated Vγ9Vδ2-T cells were obtained using either soluble anti-CD28 (2 μg/mL) mAb and PHA (3 μg/mL) stimulation, or following coculture with EBV-B cells that were pretreated overnight with 100 μmol/L NBP as indicated (ratio 1:1; both in coculture and in a Transwell setting (0.4-μm pore size; Costar, Corning Incorporated)). After 48 to 72 hours, Vγ9Vδ2-T were harvested (where required after MACS purification) and either analyzed on FACS or used for coculture assays.

Generation of iNKT cell lines and iNKT activation experiments

For iNKT (defined as $Vα24^+Vβ11^+$) cell expansion experiments, iNKT cells were isolated from PBMC by MACS isolation using the 6B11 mAb [kind gift of Mark Exley, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA], or the murine anti-human TCR Vα24-chain mAb, combined with a polyclonal goat-anti-mouse Ab labeled with magnetic beads (Miltenyi Biotec). iNKT cells were expanded by coculturing with moDC, pulsed with α-GalCer (100 ng/mL; Funakoshi Co.) during maturation, and rhIL2 (50 U/mL). Purified iNKT cells were restimulated weekly with irradiated feeder-mix consisting of allogeneic PBMC and EBV-transformed B cells in Yssel's medium supplemented with PHA (50 ng/mL) and IL2 (50 U/mL). Purity of iNKT cells used for experiments was >90%.

For evaluation of iNKT cell activation, different APC (HeLa-CD1d, moDC, freshly isolated Vγ9Vδ2-T cells, Vγ9Vδ2-T cell lines, and sorted Vγ9Vδ2-T APC) were pretreated with 100 ng/mL α-GalCer or vehicle control for 24 hours at 37°C in a humidified atmosphere with 5% CO_2. FACS SORT (BD FACSAria) was performed on the basis of TCR-Vδ2 (FITC- or PerCP-labeled) and TCR-Vγ9 (PE- or AP-labeled) double-positive selection. Cell doublets were excluded using a doublet excluding gate, based on the linear correlation between height and area of a cell. After SORT, all conditions consisted of a >95% pure Vγ9Vδ2-T-cell population. After α-GalCer pretreatment, the different APC were washed thoroughly to remove excessive α-GalCer and cocultured with resting iNKT cells (ratio 1:1). After 18 to 24 hours, supernatants were harvested and analyzed using the Th1/Th2/Th17 BD Cytometric Bead Array (CBA) Kit (BD Biosciences) for the simultaneous flow cytometric detection of IL2, IL4, IL5, IL10, TNFα, and IFNγ, following the manufacturer's instructions and with the use of CBA analysis software (BD Biosciences), and cells

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were used for flow cytometric analysis of CD25 expression or intracellular IFNγ production.

Membrane exchange experiments

EBV-transformed B cells, cultured overnight in the presence or absence of 100 μmol/L NBP, were suspended in PBS, labeled for 10 minutes with 1 μmol/L of the cell-permeable, nonfluorescent pro-dye carboxyfluoresceindiacetatesuccinimidyl ester (CFSE; Molecular Probes/Invitrogen) at 37°C, and washed thoroughly with complete medium to be subsequently labeled with the fluorescent membrane dye PKH26 (Sigma) as per the manufacturers' guidelines. Vγ9Vδ2-T cells were stained with the fluorescent membrane dye CellVue as per the manufacturer's guidelines. CFSE/PKH26-labeled EBV-B and CellVue-labeled Vγ9Vδ2-T cells were cocultured (ratio 1:1) for up to 24 hours at 37°C in a humidified atmosphere with 5% CO_2. Membrane exchange was determined by assessing the amount of PKH26 that was acquired by Vγ9Vδ2-T cells at various time points during this coculture using flow cytometry.

To determine the transfer of CD1d molecules, HeLa or CD1d-transfected HeLa cells, pretreated for 2 hours with 100 μmol/L NBP, were cocultured with Vγ9Vδ2-T cells. At various time points, the activation status and CD1d expression of Vγ9Vδ2-T cells was determined. To evaluate whether the transfer of CD1d resulted from membrane exchange or from the transfer of exosomes, CD1d-transfected HeLa cells were pretreated for 1 hour with either the exosome-transport inhibitor manumycin A (9 μmol/L; Sigma) or NaN_3 (10 mmol/L), which depletes cellular ATP, in the presence or absence of 100 μmol/L NBP. Vγ9Vδ2-T cells, pretreated for 1 hour with the actin filament inhibitor cytochalasin B (10 μmol/L; Sigma), were used to study the role of actin-dependent uptake of membrane fragments. After washing, pretreated Vγ9Vδ2-T cells and NBP pretreated CD1d-transfected HeLa cells were cocultured (ratio 1:1) for flow-cytometric assessment of Vγ9Vδ2-T-cell CD1d expression at various time points.

ImageStream analysis

CD1d-transfected HeLa cells, pretreated with 100 μmol/L NBP, were cocultured overnight with Vγ9Vδ2-T cells, harvested and used for analyses. Anti-TCR-Vδ2 mAb (FITC-labeled) was used to identify Vγ9Vδ2-T cells; anti-CD1d mAb (AP-labeled) was used for determining the CD1d acquisition by Vγ9Vδ2-T cells. Images were collected using an ImageStream ISX imaging flow cytometer (Amnis Corporation). A total of 10,000 to 30,000 events of cell images were collected. IDEAS Image Analysis Software (Amnis Corporation) was used for data analysis.

Statistical analysis

All data were analyzed using paired Student *t* tests, one-way ANOVA or Mann–Whitney tests, as appropriate; $P \leq 0.05$ was considered statistically significant.

Results

α-GalCer–pulsed pAg-activated Vγ9Vδ2-T cells do not trigger iNKT cell activation

To evaluate the APC capacity of Vγ9Vδ2-T cells, we used NBP pretreated (pAg-positive) EBV-transformed B cells (pAg^+ EBV-B) to generate Vγ9Vδ2-T APC according to the protocol described by Brandes and colleagues (16). The Vγ9Vδ2-T APC generated were pulsed with α-GalCer and used to study iNKT cell activation, where α-GalCer–pulsed CD1d-transfected HeLa cells served as a positive control. Notably, after 24 hours of coculture, α-GalCer–pulsed Vγ9Vδ2-T APC were not able to activate iNKT cells (as measured by the upregulation of the activation marker CD25), irrespective of whether Vγ9Vδ2-T APC were derived from freshly isolated Vγ9Vδ2-T cells or from Vγ9Vδ2-T cell lines, and irrespective of the mode of activation that was used (Fig. 1; and data not shown). Phenotypical analysis of Vγ9Vδ2-T APC revealed that coculture of pAg^+ EBV-B cells with Vγ9Vδ2-T leads to appropriate pAg-induced Vγ9Vδ2-T activation and that activated Vγ9Vδ2-T cells expressed higher levels of the costimulatory molecules CD86 and CD40, comparable with activated APC. However, no CD1d expression could be detected on either the resting or the activated Vγ9Vδ2-T APC (Fig. 2).

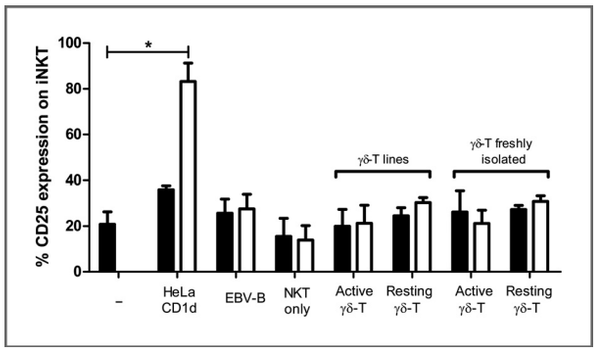

Figure 1. Freshly isolated pAg-activated Vγ9Vδ2-T cells or Vγ9Vδ2-T cell lines cannot support iNKT cell activation. Freshly isolated Vγ9Vδ2-T cells or resting (≥ 6 days after restimulation) Vγ9Vδ2-T cell lines were kept in medium or activated with pAg^+ EBV-B cells, after which they were purified via FACS SORT and cultured with iNKT cells for 24 hours. iNKT cells were then analyzed for CD25 expression. White bars, conditions with α-GalCer; black bars, conditions with vehicle. EBV-B cells, HeLa-CD1d cells, and iNKT cells alone were used as control. Shown are means + SEM (medium vs. HeLa-CD1d/α-GalCer; $P < 0.05$; $n = 3$; others nonsignificant).

Although an earlier report indicated that pAg-induced activation of Vγ9Vδ2-T cells was sufficient to induce APC characteristics in Vγ9Vδ2-T cells, a contribution by additional factors provided by pAg^+B cells was not formally excluded (16). On the basis of the hypothesis that pAg^+ stimulatory cells could be involved in transferring APC characteristics, we considered that although EBV-B cells are known to be capable of presenting antigen via MHC class I and II, their CD1d-restricted antigen-presenting capacities are compromised as EBV-B cells *in vitro* often downregulate CD1d expression on their cell surface and are thus relatively poor in presenting α-GalCer to iNKT cells (19, 20).

pAg-dependent uptake of lipophilic membrane dye and CD1d molecules by Vγ9Vδ2-T cells

Interestingly, when cultures of pAg^+ EBV-B and Vγ9Vδ2-T cells were analyzed by flow cytometry, a small but distinct population of TCR-$Vδ2^+$-T cells also expressing the B-cell

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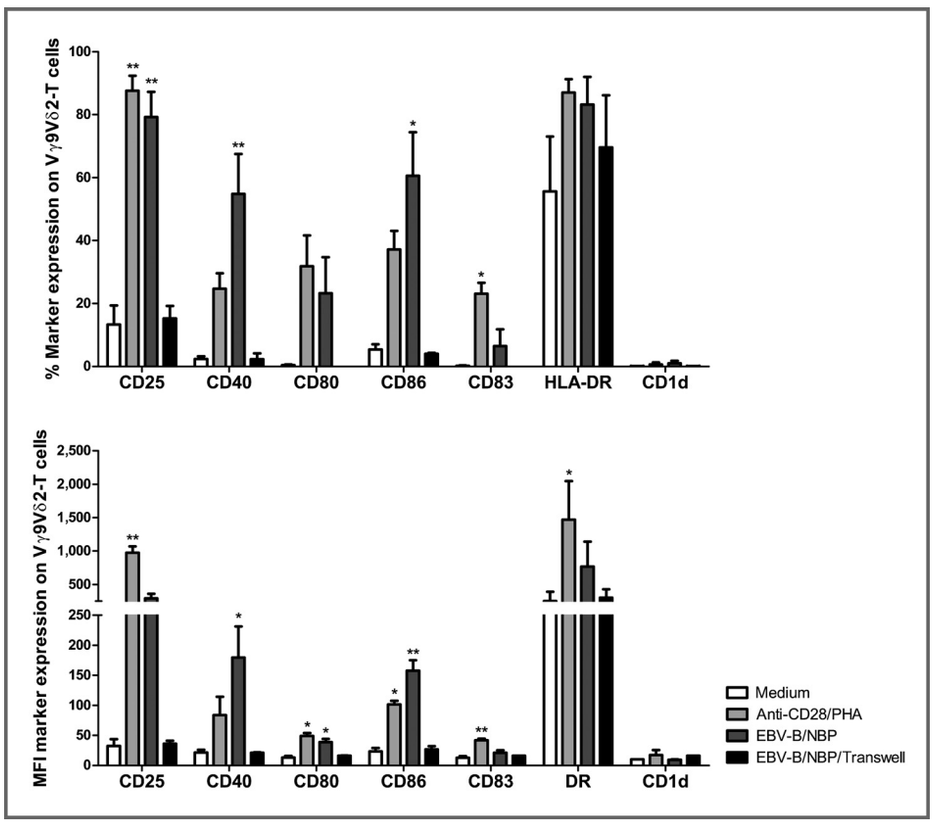

Figure 2. pAg-activated Vγ9Vδ2-T cells express typical APC markers, but not CD1d. Resting Vγ9Vδ2-T cells were cultured with different stimuli for 48 to 72 hours before marker expression was assessed by flow cytometry. The percentage of Vγ9Vδ2-T cells expressing the indicated markers (top) and the mean fluorescence intensity (MFI) of each marker (bottom) are shown and expressed as mean + SEM compared with medium (*, $P < 0.05$; **, $P < 0.01$; $n = 3$).

antigen CD19 could be observed (Fig. 3A). Within this CD19-expressing Vδ2-T-cell population, a $CD19^{bright}$ subset, possibly representing cell doublets, and a $CD19^{dim}$ population were observed, possibly representing Vγ9Vδ2-T cells that had acquired CD19-containing (i.e., EBV-B cell-derived) membrane patches. To evaluate whether membrane exchange between EBV-B and Vγ9Vδ2-T cells occurred, EBV-B cells were pretreated with 0 or 100 μmol/L NBP and stained with a combination of the protein dye CFSE and the membrane dye PKH26 while the Vγ9Vδ2-T cells were stained with the membrane dye CellVue. CFSE/PKH26-labeled EBV-B cells and CellVue-labeled Vγ9Vδ2-T cells were subsequently cocultured. Indeed, during this coculture, we observed an exchange of the lipophilic membrane dye PKH26 from the EBV-B cells to the Vγ9Vδ2-T cells. Notably, this process was pAg-specific and time-dependent (Fig. 3B).

In experiments designed to study the functional relevance of the observed membrane exchange, CD1d-transfected HeLa cells were used as these cells constitute a well-established model for studying iNKT cell activation. Coculture of Vγ9Vδ2-T cells with pAg^+HeLa or pAg^+HeLa-CD1d cells resulted in comparable activation of Vγ9Vδ2-T cells [Vγ9Vδ2-T-cell CD25 expression 90.3% ± 4.6% (mean ± SEM) for pAg^+HeLa cells and 87.6% ± 4.2% for pAg^+HeLa-CD1d cells; $P = 0.37$; $n = 7$; data not shown]. Only the coculture of Vγ9Vδ2-T with pAg^+HeLa-CD1d cells lead to the acquisition of CD1d molecules by Vγ9Vδ2-T cells (mean, 36.4% ± 5.5%). In a coculture with untreated HeLa-CD1d cells, there was minimal acquisition of CD1d by Vγ9Vδ2-T cells (mean, 4.9% ± 1.7%), compared with the expression of CD1d on Vγ9Vδ2-T cells after coculture with pAg^+HeLa cells (mean, 0.4% ± 0.4%), indicating the pAg-specific contact-dependent transfer of CD1d molecules from HeLa-CD1d to Vγ9Vδ2-T cells (Fig. 3C). The acquisition of CD1d by Vγ9Vδ2-T cells was both pAg concentration dependent and time dependent with an optimum between 7 to 21 hours (7 hours mean, 33.1% ± 6.3%; 21 hours mean, 24.8% ± 4.3%; $n = 4$; 0 μmol/L vs. 100 μmol/L; $P = 0.01$; data not shown). Similar levels of CD1d were acquired using effector:target ratios of 1:1 or 1:10. Furthermore, the acquisition of CD1d by Vγ9Vδ2-T cells was also observed after pretreating HeLa-CD1d cells with the agonist anti-CD277 mAb (clone 20.1), which mimics pAg stimulation of Vγ9Vδ2-T cells (data not shown). Of note, transfer of CD1d molecules from donor cells to Vγ9Vδ2-T cells was not restricted to HeLa-CD1d cells as it was also observed using other CD1d-expressing donor cells, including CD1d-transfected C1R cells.

Possible explanations for the transfer of membrane molecules between cells include trogocytosis and exosomal transfer. Trogocytosis is the process whereby lymphocytes intimately interact with other cells to extract surface molecules from these cells and present them on their own surface (21). The formation of an immune synapse through cell surface ligand–receptor interaction enables the rapid transfer of immunomodulatory surface proteins contained in a membrane patch, a process referred to as synaptic transfer (22, 23). To evaluate whether membrane transfer between

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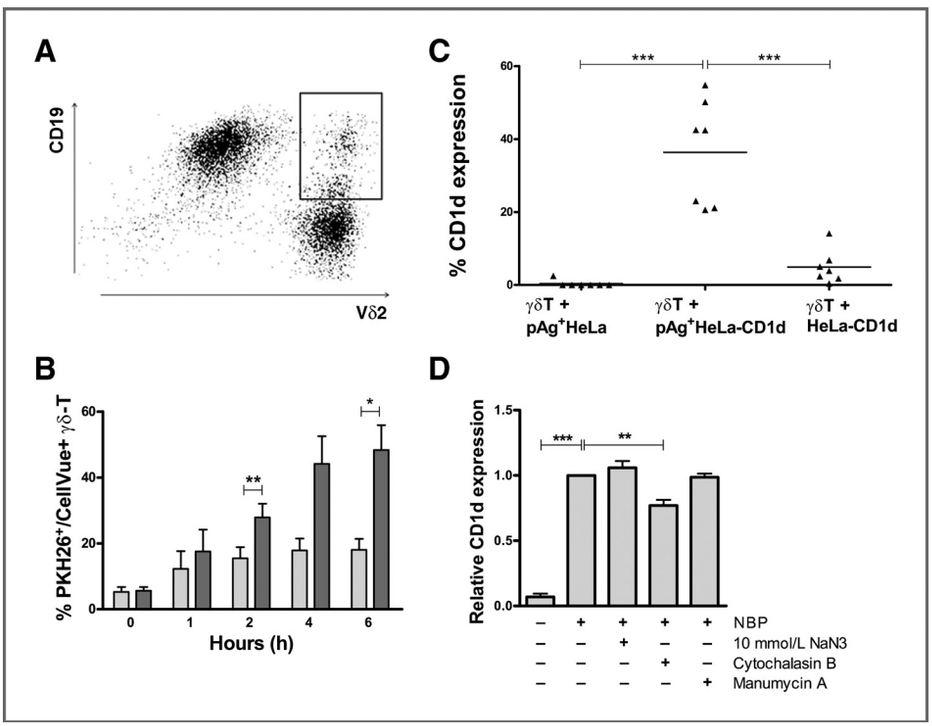

Figure 3. The uptake of membrane fragments by Vγ9Vδ2-T cells is time- and pAg-dependent and is not mediated by exosome transfer. A, representative dot plot of a 24-hour coculture of Vγ9Vδ2-T cells with $CD19^+$ EBV-B cells, illustrating a population of Vδ2-T cells double-positive for CD19. B, pAg^+ and pAg^- EBV-B cells were labeled with both CFSE and PKH26 and subsequently cocultured (ratio, 1:1) with CellVue-labeled Vγ9Vδ2-T cells. Membrane uptake by Vγ9Vδ2-T cells was then determined by assessing the frequency of PKH26 expressing Vγ9Vδ2-T cells (defined as $CellVue^+CFSE^-$). Dark gray bars, Vγ9Vδ2-T cells cocultured with pAg^+ EBV-B cells; light gray bars, Vγ9Vδ2-T cells cocultured with pAg^- EBV-B cells. Shown are means + SEM (0 μmol/L NBP vs. 100 μmol/L NBP at 2 hours; $P = 0.007$; 0 μmol/L NBP vs. 100 μmol/L NBP at 6 hours; $P = 0.048$; $n = 3$). C, HeLa or CD1d-transfected HeLa cells, pretreated with 0 or 100 μmol/L NBP, were cocultured (ratio 1:1) overnight with Vγ9Vδ2-T cells. CD1d expression of Vγ9Vδ2-T cells was determined by flow cytometry. Horizontal lines represent means (Vγ9Vδ2-T/pAg^+HeLa vs. Vγ9Vδ2-T/pAg^+HeLa-CD1d, $P < 0.001$; Vγ9Vδ2-T/pAg^+HeLa-CD1d vs. Vγ9Vδ2-T/HeLa-CD1d, $P < 0.001$; $n = 7$). D, pAg^+HeLa-CD1d cells pretreated with or without inhibitors of nSMase2 (manumycin A) and ATP synthase (NaN_3) were cocultured with resting Vγ9Vδ2-T cells (ratio 1:1) pretreated with or without an inhibitor of clathrin-mediated endocytosis (cytochalasin B). CD1d expression by Vγ9Vδ2-T cells was assessed after 3 hours of incubation by flow cytometry. Shown are relative means + SEM (Vγ9Vδ2-T/HeLa-CD1d vs. Vγ9Vδ2-T/pAg^+HeLa-CD1d, $P < 0.001$; Vγ9Vδ2-T/pAg^+HeLa-CD1d vs. Vγ9Vδ2-T/pAg^+HeLa-CD1d + cytochalasin B, $P = 0.006$; $n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

HeLa-CD1d and Vγ9Vδ2-T cells results from either trogocytosis or transfer of exosomes, a separate set of experiments was performed in which either Vγ9Vδ2-T cells were pretreated with an inhibitor of actin polymerization, cytochalasin B (blocking trogocytosis), or the pAg^+HeLa-CD1d cells were pretreated with manumycin A or the cytochrome oxidase inhibitor NaN_3 (blocking exosome transport/release) before coculture of Vγ9Vδ2-T and HeLa-CD1d cells. As shown in Fig. 3D, pretreatment of pAg^+HeLa-CD1d cells with neither manumycin A nor NaN_3 decreased the acquisition of CD1d by Vγ9Vδ2-T cells at various time points (up to 6 hours tested) after Vγ9Vδ2-TCR engagement, implying that an active role of the CD1d-expressing donor cell is not required for the observed membrane exchange, and that this is therefore not mediated by transfer of exosomes. In contrast, administration of the actin-filament inhibitor cytochalasin B did significantly decrease the acquisition of CD1d by Vγ9Vδ2-T cells without affecting Vγ9Vδ2-T-cell activation as determined using CD25 expression (data not shown), suggesting an active role of this pathway in Vγ9Vδ2-T-cell membrane exchange ($P < 0.01$; $n = 5$).

We then quantified membrane exchange using the ImageStream. This instrument combines microscopy and flow cytometry in one platform. Because it images cells in flow, the system—on a per cell basis—is able to measure the brightness, size, and location of the subcellular components (labeled with fluorochrome-conjugated antibodies) and compile the data into the population statistics of conventional FACS analysis. Hence, after an overnight coculture of Vγ9Vδ2-T cells with either the HeLa-CD1d or pAg^+HeLa-CD1d cells, the Vγ9Vδ2-T cells were analyzed for the expression of CD1d. As observed in previous experiments, most Vγ9Vδ2-T cells cocultured with HeLa-CD1d expressed no CD1d or very low levels of CD1d. However, in a coculture with pAg^+HeLa-CD1d cells, substantially more Vγ9Vδ2-T cells obtained CD1d expression, which could be visualized as trogocytosed CD1d-containing patches on the membrane of Vγ9Vδ2-T cells (Fig. 4A and B). Therefore, both Vγ9Vδ2-T cells cocultured with pAg^+ and pAg^- HeLa-CD1d can acquire CD1d, as is also reflected in Fig. 3C. However, when quantified as a function of area and intensity, the CD1d staining on the plasma membrane of Vγ9Vδ2-T cells cultured with pAg^+HeLa-CD1d was larger and brighter than the CD1d staining on the plasma membrane of Vγ9Vδ2-T cells cultured with HeLa-CD1d (~2-fold increase; data not shown) and the percentage of Vγ9Vδ2-T cells that acquire CD1d after

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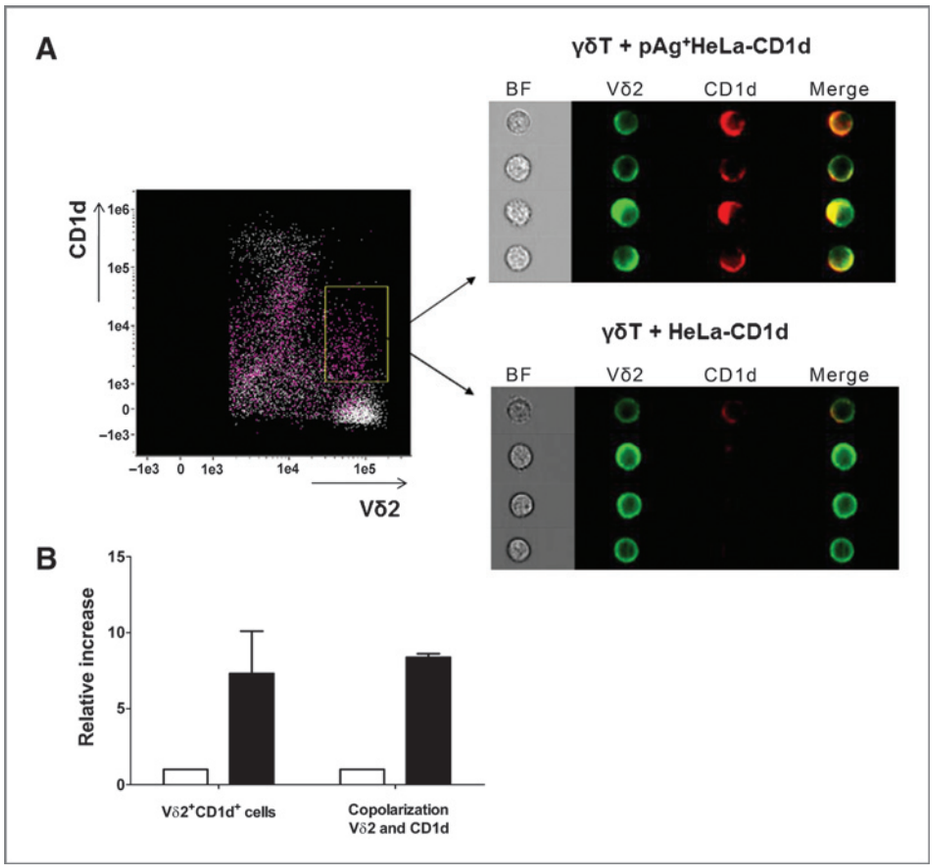

Figure 4. Visualization of trogocytosis of CD1d by Vγ9Vδ2-T cells. CD1d-transfected HeLa cells, pretreated with 0 or 100 μmol/L NBP, were cocultured (ratio, 1:1) with Vγ9Vδ2-T cells. A, the dot plot represents an overlay of two culture conditions: white dots, events from a coculture of Vγ9Vδ2-T and HeLa-CD1d cells; purple dots, events from a coculture of Vγ9Vδ2-T and pAg^+ HeLa-CD1d cells (left). After 11 hours of coculturing, cells were stained with TCR Vδ2 FITC (green) and CD1d AP (red) and analyzed with the ImageStream (right). Shown are representative images of $Vδ2^+/CD1d^+$ cells from a coculture of Vγ9Vδ2-T cells and pAg^+HeLa-CD1d (top right) or HeLa-CD1d, respectively (bottom right), gated in the dot plot. The images are from a single experiment and are representative of two experiments (BF, bright field). B, relative increase in the percentages of $Vδ2^+CD1d^+$ cells and in the percentages of cells that colocalized $Vδ2^+$ and $CD1d^+$. White bars, Vγ9Vδ2-T cells cocultured with HeLa-CD1d cells. Black bars, Vγ9Vδ2-T cells cocultured with pAg^+HeLa-CD1d cells ($n = 2$).

coculture with pAg^+ and pAg^- HeLa-CD1d cells differs greatly (Fig. 4B). Thus, by two different criteria, the ImageStream data indicate a greater abundance of CD1d on the membrane of Vγ9Vδ2-T cells cultured with pAg^+HeLa-CD1d compared with Vγ9Vδ2-T cells cultured with untreated HeLa-CD1d. Polarization of the TCR (Vδ2) was frequently observed at the site of CD1d-positive membrane uptake (Fig. 4A and B), suggesting that the observed trogocytosis took place at an immunologic synapse, as described previously for trogocytosis (21–23).

Trogocytosis of CD1d-containing membrane fragments endows Vγ9Vδ2-T cells with the capacity to act as APC for iNKT

Next, the iNKT cell-activating capability of Vγ9Vδ2-T APC was evaluated after their trogocytosis-mediated acquisition of CD1d. For this purpose, $CD1d^+$ and $CD1d^-$ Vγ9Vδ2-T cells, purified from cocultures with α-GalCer-pulsed pAg^+HeLa-CD1d cells and α-GalCer-pulsed pAg^+HeLa cells, respectively, were cocultured with resting iNKT cells for 24 hours. Purification of Vγ9Vδ2-T cells was performed by FACS SORT using a doublet excluding gate (based on the linear correlation between area and height of cells). After coculturing CD1d-expressing Vγ9Vδ2-T APC and iNKT for 24 hours, iNKT cell activation was determined by CD25 expression, while cytokine production (IL2, IL4, IL6, IL10, TNFα, IFNγ, and IL17) was determined using an inflammatory CBA. As described earlier, Vγ9Vδ2-T cells were equally activated in both conditions, implying that the observed differences resulted from differences in iNKT cell cytokine production.

α-GalCer–loaded $CD1d^+$-sorted Vγ9Vδ2-T APC significantly increased iNKT cell activation when compared with $CD1d^-$ Vγ9Vδ2-T APC [iNKT/$CD1d^-$Vγ9Vδ2-T-cell coculture vs. iNKT/$CD1d^+$Vγ9Vδ2-T-cell coculture for percentage of $CD25^+$ iNKT cells: 31.7 ± 5.4 vs. 38.0 ± 6.2 (mean ± SEM; $P = 0.007$; $n = 13$); for mean fluorescence intensity (MFI), 66.0 ± 8.2 vs. 75.6 ± 6.7 (mean ± SEM; $P = 0.046$; $n = 13$; Fig. 5A]. More prominently, α-GalCer–loaded $CD1d^+$-sorted Vγ9Vδ2-T also stimulated iNKT cells to produce increased levels of IFNγ (iNKT/$CD1d^-$Vγ9Vδ2-T-cell coculture mean concentration of 2,173 ± 780 pg/mL vs. iNKT/$CD1d^+$Vγ9Vδ2-T-cell coculture mean concentration of 4,070 ± 671 pg/mL; $P = 0.004$; $n = 7$; Fig. 5B), which was also confirmed by intracellular IFNγ detection (mean ± SD; iNKT cell IFNγ expression 0.9% ± 0.4% using $CD1d^-$ Vγ9Vδ2-T APC vs. 11.1% ± 1.8% using $CD1d^+$ Vγ9Vδ2-T APC ($n = 2$; Fig. 5C), thereby showing their ability to propagate a Th1-biased immune response. However, the production of IFNγ by iNKT cells that were stimulated by α-GalCer–pulsed moDC was clearly higher (mean concentration of 29,789 ± 10,636 pg/mL; Fig. 5B). For the other cytokines (IL2, IL4, IL6, IL10, and IL17), no significant differences between $CD1d^+$ and $CD1d^-$ Vγ9Vδ2-T APC (IL2, $P = 0.22$; IL4, $P = 0.18$; IL6, $P = 0.62$; IL10, $P = 0.39$; IL17, $P = 0.72$; data not shown) were detected, with the exception of a small but significant

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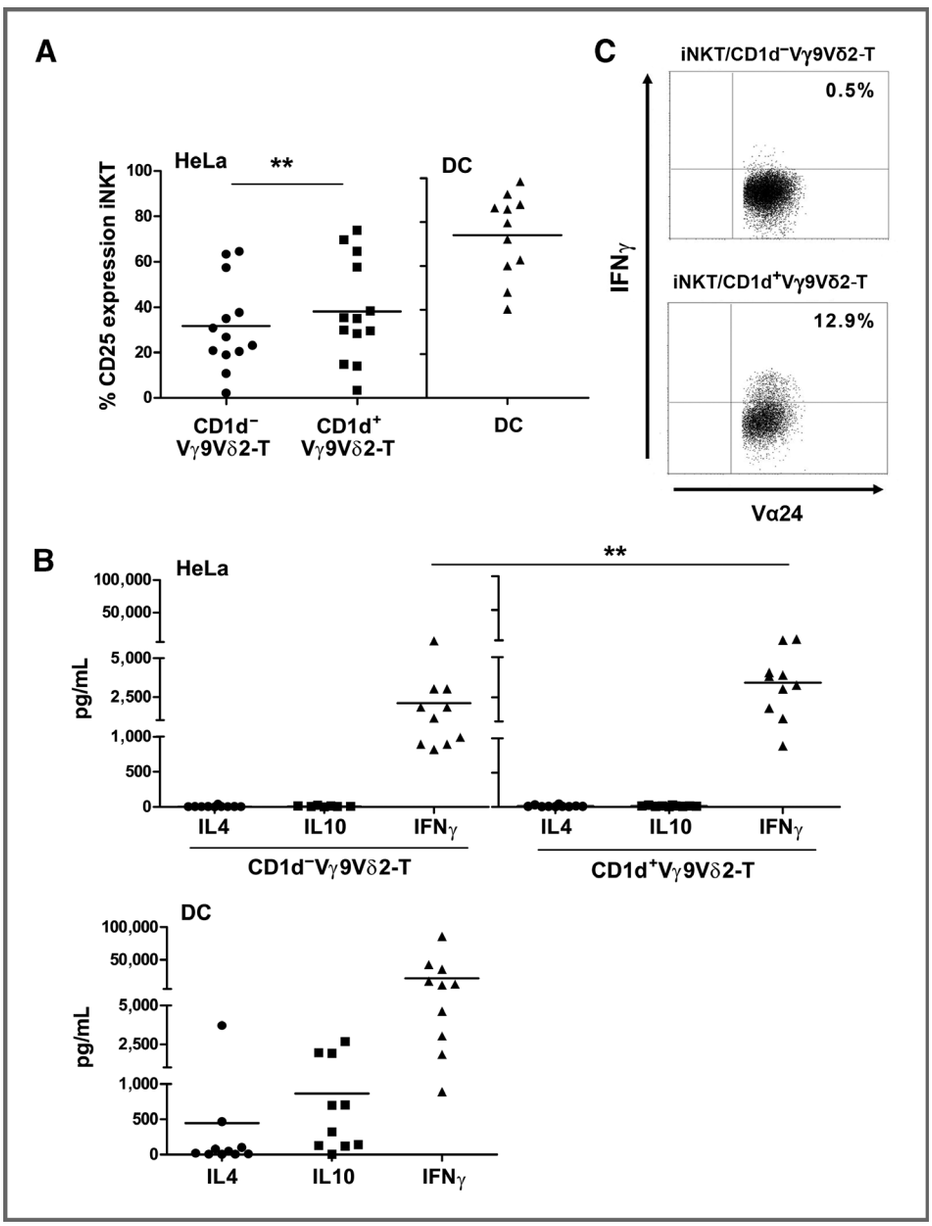

Figure 5. CD1d-expressing Vγ9Vδ2-T cells can act as APC to activate iNKT. Vγ9Vδ2-T cells cocultured with glycolipid-antigen–pulsed pAg$^+$HeLa or glycolipid-antigen–pulsed pAg$^+$ HeLa-CD1d cells were purified using flow cytometric sorting and subsequently cocultured with iNKT cells (ratio, 1:1). After a 24-hour coculture, iNKT cell CD25 expression was assessed by flow cytometry and supernatants were harvested and analyzed using a Th1/Th2/Th17 CBA kit. A, shown are means of CD25 expression (iNKT/CD1d$^-$Vγ9Vδ2-T-cell coculture vs. iNKT/CD1d$^+$Vγ9Vδ2-T-cell coculture; $P = 0.007$; $n = 13$). B, shown are means of IFNγ (iNKT/CD1d$^-$Vγ9Vδ2-T-cell coculture vs. iNKT/CD1d$^+$Vγ9Vδ2-T-cell coculture; $P = 0.004$; $n = 7$), IL4 (iNKT/CD1d$^-$Vγ9Vδ2-T-cell coculture vs. iNKT/CD1d$^+$Vγ9Vδ2-T-cell coculture; $P = 0.18$; $n = 7$), and IL10 production (iNKT/CD1d$^-$Vγ9Vδ2-T-cell coculture vs. iNKT/CD1d$^+$Vγ9Vδ2-T-cell coculture; $P = 0.39$; $n = 7$). Also, means of IFNγ, IL4, and IL10 production by iNKT cells stimulated by moDC are shown ($n = 7$). C, dot plots showing increased intracellular IFNγ expression in iNKT cells cultured for 18 hours with CD1d$^+$Vγ9Vδ2-T cells compared with CD1d$^-$ Vγ9Vδ2-T cells. Representative data shown are from one of two experiments. **, $P < 0.01$.

increase in TNFα (iNKT/CD1d$^-$Vγ9Vδ2-T-cell coculture mean concentration of 8 ± 3 pg/mL vs. iNKT/CD1d$^+$Vγ9Vδ2-T-cell coculture mean concentration of 12 ± 3 pg/mL; $n = 7$; $P = 0.03$; data not shown), in keeping with a Th1-skewed response. Again, the production of TNFα by iNKT was higher when iNKT cells were stimulated by α-GalCer–pulsed moDC (mean concentration of 1,192 ± 739 pg/mL). Interestingly, however, the type-II cytokines, IL4 and IL10, were hardly produced in a coculture of iNKT/CD1d$^+$Vγ9Vδ2-T cells, in contrast with the coculture of iNKT/moDC (Fig. 5B). When considering IFNγ:IL4 ratios, a trend toward a more type I–skewed response is induced by Vγ9Vδ2-T APC compared with moDC (IFNγ:IL4 ratio iNKT/CD1d$^+$Vγ9Vδ2-T cell 687 ± 285 vs. coculture of iNKT/moDC 257 ± 84; $n = 7$; $P = 0.14$; data not shown).

Discussion

iNKT cells represent a conserved immunoregulatory T-cell subset with a well-established role in antitumor immunity based on their capacity to induce long-lasting Th1-biased proinflammatory immune responses upon activation (5, 24, 25). Because preclinical data indicated that i.v. administration of α-GalCer–pulsed DC exerted greater antitumor activity compared with i.v. α-GalCer alone (26), this approach, with variations in the used APC platform, was subsequently evaluated in clinical trials. Of interest, clinical trials with α-GalCer–pulsed moDC have shown anecdotal antitumor activity in advanced cancer (6, 7). Several practical limitations hamper further clinical exploration of treatment with α-GalCer–pulsed DC. In general, DC used for clinical trials are derived from

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monocytes that are differentiated and matured into moDC during an *in vitro* culture period of up to 10 days using combinations of IL4, GM-CSF, and a maturation stimulus. MoDC can acquire different states of function, including tolerogenic (immature DC), proinflammatory (mature DC), and inhibitory (mature exhausted DC; ref. 18). The combination of a relatively long *in vitro* culturing period and functional heterogeneity hampers broad clinical application and could cause unfavorable effects (18). Therefore, alternative APC platforms are the focus of investigation.

Vγ9Vδ2-T cells have been shown to be able to acquire professional antigen-presenting capacities, including the uptake and processing of exogenous antigen, and the subsequent presentation of the peptide–antigen to $CD4^+$ and $CD8^+$ T cells (via MHC class II and class I, respectively) upon their activation by pAg (16, 17). When compared with moDC, the use of Vγ9Vδ2-T-APC could provide advantages with respect to clinical application (antitumor immunotherapy), as Vγ9Vδ2-T cells are more numerous compared with DC precursors and can be matured into consistent proinflammatory APC within 24 hours using pAg (16, 18). We therefore evaluated whether Vγ9Vδ2-T APC might constitute an attractive alternative APC platform for glycolipid antigen presentation to iNKT cells.

Here, first we confirmed the results from previous studies demonstrating that activation of Vγ9Vδ2-T cells results in an upregulation of costimulatory molecules and APC maturation markers. Activated Vγ9Vδ2-T cells were, however, not able to activate iNKT cells when pulsed with α-GalCer. Importantly, Vγ9Vδ2-T APC generated using previously reported culture systems were found not to express CD1d molecules. Using an alternative platform for the generation of Vγ9Vδ2-T APC, CD1d expression was induced on the cell surface of Vγ9Vδ2-T cells. Yet, this expression did not result from *de novo* synthesis of CD1d, but was critically dependent on the expression of CD1d on the pAg^+ cells that were used to activate the Vγ9Vδ2-T cells. Using a variety of techniques, we show that the presence of CD1d antigen-presenting molecules on the surface of the activated Vγ9Vδ2-T cells was mediated by the transfer of CD1d molecules from CD1d-positive NBP pretreated cells that were used to stimulate Vγ9Vδ2-T APC. Notably, this acquisition of CD1d occurred in a pAg-specific and time-dependent manner, which is typical for trogocytosis and occurs rapidly after TCR engagement. Membrane transfer was not mediated by exosome transport, as the well-established exosome-transport inhibitor manumycin A and the ATP synthase inhibitor NaN_3 did not diminish the acquisition of CD1d by Vγ9Vδ2-T cells triggered in the first few hours after TCR engagement. In contrast, the actin filament inhibitor, cytochalasin B, decreased the acquisition of CD1d by activated Vγ9Vδ2-T cells, suggesting that actin cytoskeleton rearrangement processes contribute to the observed exchange of membrane fragments. Polarization of the TCR (Vδ2) at the site of CD1d-positive membrane exchange suggests that trogocytosis took place at the immunologic synapse, as reported previously (22, 23).

Upon activation, Vγ9Vδ2-T cells can potentially lyse the pAg^+HeLa-CD1d cells used for their stimulation. To study the potential contribution of phagocytosis (27) of HeLa-CD1d cell fragments by Vγ9Vδ2-T APC, we analyzed whether CD1d expression levels could be enhanced when (EpCAM-expressing) HeLa-CD1d cells were coated with anti-EpCAM antibodies. Importantly, antibody coating of HeLa-CD1d cells did not result in an enhancement of CD1d expression by Vγ9Vδ2-T cells, implicating a limited/absent role for phagocytosis in our experimental system, possibly related to the low CD16 (FcγRIIIa/b) expression levels on Vγ9Vδ2-T cells used in our study (data not shown).

Importantly, glycolipid-loaded $CD1d^+$Vγ9Vδ2-T APC were found to be able to selectively activate and induce the production of IFNγ, and less prominently TNFα in iNKT cells, indicative of Th1 skewing. Although glycolipid-loaded moDC outperformed Vγ9Vδ2-T APC on a per cell basis, and induced higher iNKT cell proliferation as compared with $CD1d^+$ Vγ9Vδ2-T cells in preliminary experiments (data not shown), Vγ9Vδ2-T APC possess several uniquely favorable features with respect to clinical immunotherapeutic application, as Vγ9Vδ2-T cells are more numerous compared with DC precursors, mature quickly into professional APC, have better lymph node–homing characteristics, and have a more consistent proinflammatory phenotype (16, 18). These characteristics make them an interesting platform for further consideration in future clinical trials. In addition, the more limited iNKT cell-activating properties of Vγ9Vδ2-T APC could also be advantageous as they might result in the diminished induction of iNKT cell anergy, which was a limiting factor in earlier studies (28). Apart from their role as APC, Vγ9Vδ2-T cells have well-established direct antitumor effector functions, leading to tumor cytotoxicity via the secretion of perforin, granzyme B, and granulysin. In addition to their direct antitumor effects, locally activated Vγ9Vδ2-T cells could also play a role in influencing the development of a Th1-biased antitumor response in the tumor microenvironment by acting as APC via trogocytosis of tumor-derived immunogenic membrane fragments. Our current findings about the role of trogocytosis in the acquisition of CD1d-restricted antigen presentation characteristics fuel the hypothesis that although *de novo* synthesis of APC molecules was implicated for MHC class I and II molecules, trogocytosis can certainly contribute.

To achieve a more effective APC platform for iNKT cell–based immunotherapy one could envision using retroviral CD1d transduction of Vγ9Vδ2-T cells, hypothesizing that the limited CD1d-restricted APC function of Vγ9Vδ2-T cells could be related to the relatively limited surface density of CD1d when compared with HeLa-CD1d or moDC. Furthermore, as repeated i.v. administration of α-GalCer induces iNKT cell anergy and repeated administration of CD1d-targeted constructs results in sustained iNKT cell activation (29), tumor-targeting of iNKT cells or α-GalCer–pulsed APC might alternatively constitute a powerful yet unexplored clinical approach (5).

Our findings provide evidence that Vγ9Vδ2-T cells can acquire the capacity to act as APC and present glycolipid antigen to iNKT cells. However, we show that this does not result from the *de novo* synthesis of CD1d glycolipid antigen-presenting molecules by Vγ9Vδ2-T cells, but critically depends on the uptake of CD1d-containing membrane fragments from pAg^+ target cells with which they interact.

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Disclosure of Potential Conflicts of Interest

M. Bonneville is a consultant/advisory board member for Innate Pharma SA and has provided expert testimony for Institut Merieux. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: F.L. Schneiders, T. O'Toole, T.D. de Gruijl, H.J. van der Vliet

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F.L. Schneiders, J. Prodöhl, T. O'Toole, M. Bonneville, H.M.W. Verheul

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F.L. Schneiders, J. Prodöhl, T. O'Toole, H.M.W. Verheul, T.D. de Gruijl, H.J. van der Vliet

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F.L. Schneiders, T. O'Toole

Study supervision: T.D. de Gruijl, H.J. van der Vliet

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CD1d-Restricted Antigen Presentation by Vγ9Vδ2-T Cells Requires Trogocytosis

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