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Type I IFNs and IL-18 Regulate the Antiviral Response of Primary Human γδ T Cells against Dengvirus Cells Infected with Dengue Virus

Chen-Yu Tsai,* Ka Hang Liong,* Matilda Gertrude Gunalan,* Na Li,† Daniel Say Liang Lim,* Dale A. Fisher,‡§ Paul A. MacAry,*¶ Yee Sin Leo,‖ Siew-Cheng Wong,# Kia Joo Puan,# and Soon Boon Justin Wong*†‡¶**

Little is known about the cellular mechanisms of innate immunity against dengue virus (DV) infection. Specifically, the γδ T cell response to DV has not been characterized in detail. In this work, we demonstrate that markers of activation, proliferation, and degranulation are upregulated on γδ T cells in PBMC isolated from individuals with acute dengue fever. Primary γδ T cells responded rapidly in vitro to autologous DV-infected dendritic cells by secreting IFN-γ and upregulating CD107a. The anti-DV IFN-γ response is regulated by type I IFN and IL-18 in a TCR-independent manner, and IFN-γ secreting γδ T cells predominantly expressed IL-18Rα. Antagonizing the ATP-dependent P2X7 receptor pathway of inflammasome activation significantly inhibited the anti-DV IFN-γ response of γδ T cells. Overnight priming with IL-18 produced effector γδ T cells with significantly increased ability to lyse autologous DV-infected dendritic cells. Monocytes were identified as accessory cells that augmented the anti-DV IFN-γ response of γδ T cells. Lack of monocytes in culture is associated with lower IL-18 levels in culture supernatant and diminished production of IFN-γ by γδ T cells, whereas addition of exogenous IL-18 restored the IFN-γ response of γδ T cells in monocyte-depleted cocultures with DV-infected DC. Our results indicate that primary γδ T cells contribute to the immune response during DV infection by providing an early source of IFN-γ, as well as by killing DV-infected cells, and suggest that monocytes participate as accessory cells that sense DV infection and amplify the cellular immune response against this virus in an IL-18–dependent manner. The Journal of Immunology, 2015, 194: 000–000.

The transmission of dengue virus (DV) by the bite of mosquito vectors is a major cause of febrile illness in tropical and subtropical areas of the world (1). The majority of DV-infected patients with clinical symptoms develop dengue fever without attendant complications, but 1–5% of patients develop life-threatening severe dengue infections that are characterized by thrombocytopenia, bleeding tendency, capillary leak, shock, and end-organ damage (2). The immune response to the virus, other host factors, and viral determinants are thought to influence the severity of DV infection (3). Cellular immune responses against DV, αβ T cell responses in particular, were studied carefully by several groups who sought to identify immune correlates of protection or disease severity (4). Although it has been known for some time that CD8+ αβ T cells respond to DV Ags in vitro (5), a seminal date that CD8+ αβ T cells contribute to the immune response during DV infection by providing an early source of IFN-γ, as well as by killing DV-infected cells, and suggest that monocytes participate as accessory cells that sense DV infection and amplify the cellular immune response against this virus in an IL-18–dependent manner. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: BATDA, bis(2-acetoxyethyl)methyl-2,2′,6′,2″-terpyridine-6,6″-dicarboxylate; 15D, 3-[1-(2,2-dichlorophenyl)-4-(5,6,7,8-tetrahydro-3,6-dimethoxy-2-oxopyridine]-1H-tetrazol-1-yl)methyl]pyridine hydrochloride; DC, dendritic cell; DV, dengue virus; IPP, isopentenyl pyrophosphate; MOR, multiplicity of infection; TDA, 2,2′,6,2″-terpyridine-6,6″-dicarboxylic acid.

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well as the activation of non-Vδ2 γδ T cells during CMV infection (15) and HIV-1 infection (16). Alternatively, γδ T cell lines also were used to study the cytotoxic response of Vγ9Vδ2 T cells to HSV-1 (17) to document noncytolytic antiviral activity and IFN-γ production against severe acute respiratory syndrome virus (14), as well as Fas-Fas ligand– and perforin–granzyme B–dependent cytoxicity against cells infected with influenza A virus (18). T cell lines also were used to study the IFN-γ response of Vδ2 γδ T cells to fibroblasts infected with CMV (19). It was reported that the activation marker CD69 is upregulated on fibroblasts infected with CMV (19). It was reported that the activation marker CD69 is upregulated on fibroblasts infected with CMV (19). It was reported that the activation marker CD69 is upregulated on fibroblasts infected with CMV (19). It was reported that the activation marker CD69 is upregulated on fibroblasts infected with CMV (19). It was reported that the activation marker CD69 is upregulated on fibroblasts infected with CMV (19).

Monocyte-derived DC were generated from CD14+ monocytes that had been isolated from PBMC by positive selection (CD14 MicroBeads; Miltenyi Biotec). In brief, CD14+ monocytes were cultured for 7 d in RPMI-10 medium (RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPEs, and 2 mM L-glutamine) supplemented with 800 U/ml GM-CSF (R&D Systems) and 500 U/ml IL-4 (eBioscience), with half of the culture medium refreshed every 2 d.

PBMC were cultured by preparing PBMC within a 37°C CO2 incubator for 2 h in a tissue culture flask to remove adherent cells and further depleting the nonadherent cells of CD14+ cells using CD14 MicroBeads (Miltenyi Biotec). PBMC and PBL were stored in liquid nitrogen until autologous DC were ready for coculture.

CD16+ monocytes were prepared from PBMC using a CD16+ Monocyte Isolation Kit (Miltenyi Biotec). PBMC were labeled with a depletion mixture containing CD15 and CD56 MicroBeads that was supplied with the kit, supplemented with additional CD3 MicroBeads and CD19 MicroBeads (Miltenyi Biotec) to enrich for monocytes by negative selection. The flow-through from this first step was labeled with CD16 MicroBeads so that CD16+ monocytes could be purified by positive selection. The flow-through from the second step was subsequently labeled with CD14+ MicroBeads to purify CD14+ monocytes by positive selection. Purified monocytes were stored in liquid nitrogen until autologous DV-infected DC or mock-infected DC were ready for coculture.

Virus preparation, titration, and infection of DC

DV serotype 1 strain S3638 (a kind gift from Dr. Eng Eong Ooi, Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore) was propagated in the C3/36 insect cell line. After 7 d, cell-free supernatant from infected cultures was harvested and frozen in aliquots. The viral titer was determined by performing plaque assays on monolayers of BHK cells (23). Immature DC were infected with DV at a multiplicity of infection (MOI) = 1 by incubating cells with virus for 2 h in a 37°C tissue culture incubator. Unabsorbed virus was washed away, and the infected cells were maintained in RPMI-2 medium (RPMI 1640 containing 2% heat-inactivated FCS, 10 mM HEPEs, and 2 mM L-glutamine). Mock-infected DC (DC that had been exposed to heat-inactivated DV) and uninfected DC (DC that had not been exposed to dead virus or live virus) served as experimental controls.

Cocultures of DC with PBMC, PBL, or purified γδ T cells; reconstitution experiments with DC, purified γδ T cells, and purified monocyte subsets

At 24 h postinfection, 1 × 10^8 DV-infected DC or mock-infected DC were seeded into 96-well round-bottom plates (Costar) and cocultured for an additional 4 h with either 2 × 10^6 autologous PBMC or 2 × 10^6 autologous PBL in RPMI-2 with added GolgiStop (BD Biosciences). In separate experiments, 1 × 10^6 DV-infected DC or mock-infected DC were similarly cocultured with 1 × 10^6 purified autologous γδ T cells. In experiments involving purified monocyte subsets, 1 × 10^6 DV-infected DC or mock-infected DC were seeded into 96-well round-bottom plates and cocultured for an additional 4 h with 1 × 10^6 purified autologous γδ T cells in the presence of 2 × 10^6 autologous CD16+ monocytes or 2 × 10^6 autologous CD14+ monocytes.

Cytokines, Abs, and reagents

In some experiments, either recombinant universal type 1 IFN (PBL IFN-α/β Recombinant System; R&D Systems) or 5 µg/ml anti–IFN-γ (clone MAB1; eBioscience), 10 µg/ml anti-IFN-β (clone MAB1; eBioscience), 10 µg/ml anti-IFN-α (clone MAB2471; R&D Systems), and 10 µg/ml mouse IgG1 isotype control (clone MOPC-21; eBioscience) was added to cocultures containing DC and purified γδ T cells. In instances in which cytokine inhibitors or cytokine-neutralizing Abs and their isotype controls were used, they were incubated with DV-infected DC for 30 min prior to coculture with effecter cells. The reagents used and their final concentration in culture are as follows: 5 µg/ml B18R recombinant protein (eBioscience), 10 µg/ml anti–IL-18 (clone 125-2H; R&D Systems), 10 µg/ml anti-IFN-α antibodies (clone MAB1; eBioscience), 10 µg/ml anti-IFN-γ (clone MAB1; eBioscience), 10 µg/ml anti-IL-7 (clone BVD10-406F6; BioLegends), 10 µg/ml anti-IL-12 (clone C8.6; eBioscience), 10 µg/ml mouse anti–IL-15 (clone MAB2471; R&D Systems), and 10 µg/ml mouse IgG1 isotype control (clone MOPC-21; eBioscience). 3-[15-(2,3-Dichlorophenyl)-1-H-tetrazol-1-yl][methyl]pyridine hydrochloride (15D; Tocris) was used at a final concentration of 100 µM as a specific antagonist of the P2X7 receptor (24). DV-infected DC or mock-infected DC were treated with mevastatin (Sigma-Aldrich) at a final concentration of 5 µM for 16 h to block the mevalonate pathway of isopentenyl pyrophosphate (IPP) synthesis (25); they were subsequently cocultured with PBMC for an additional 4 h in the presence of mevastatin.

Materials and Methods

Ethics statement

This research was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Domain Specific Review Board, National Healthcare Group, Singapore, and by the National University of Singapore Institutional Review Board. All volunteers who participated in this study were adults (≥21 y in age) and provided written informed consent.

Samples from patients with dengue fever

Blood from patients suspected of having acute dengue fever was obtained when they presented at the National University Hospital within 8 d of the onset of fever. The diagnosis was confirmed when samples tested positive for either dengue IgM by serology or for dengue viral RNA by real-time PCR. Peripheral blood from an equal number of age- and sex-matched healthy nonfebrile individuals served as negative controls. Paired blood samples also were obtained from 13 laboratory-confirmed cases of dengue fever during the acute phase (between days 4 and 8) and convalescent phase (between days 60 and 120) of illness. All blood samples were lysed with ACK buffer (ammonium-chloride-potassium buffer: 0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA) to remove RBCs, washed with PBS, stained with Abs, and analyzed using flow cytometry.

Cells

Blood packs were obtained from the Blood Donation Center, National University Hospital, and density gradient centrifugation on a Ficoll-Hypaque PLUS (GE Healthcare) gradient was used to prepare PBMC. γδ T cells were purified by PBMC by positive selection (human anti-TCR γδ MicroBead Kit; Miltenyi Biotec) and stored in liquid nitrogen until autologous dendritic cells (DC) were ready for coculture. The purity of γδ T cells was >97%, as assessed by flow cytometry.

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**TYPE I IFN AND IL-18 REGULATE ANTI-DENGUE γδ T CELLS**
The following reagents and Abs were used for flow cytometry: CountBright absolute counting beads (Invitrogen), LIVE/DEAD fixable yellow stain (Invitrogen), anti-CD3 (Alexa Fluor 700, clone UCHT1; eBioscience), anti-CD3 (clone SK7, PerCP-Cy5.5; eBioscience), anti-pan γδ TCR (allophycocyanin, clone B1; BioLegend), anti-pan γδ TCR (FITC, clone 11F2; BD Biosciences), anti-CD38 (V450, clone HB7; BD Biosciences), anti-CD69 (allophycocyanin-Cy7, clone FN50; BioLegend), anti-CD69 (Pacific Blue, clone FN50; BioLegend), anti-CD69 (Brilliant Violet 605, clone FN50; BioLegend), anti-CD107a (PE-CY7, clone CMSSB; eBioscience), anti-IFN-γ (allophycocyanin, clone 4S.B3; eBioscience), anti-human TCR Vβ2 (FITC, clone B6; BioLegend), anti-human TCR Vβ2 (PerCP, clone B6; BioLegend), anti-CD56 (PE-Cy7; eBioscience), anti-Ki-67 (FITC, clone 20Raj1; eBioscience), and anti-IL-18Rα (PE, clone H44; eBioscience).

Flow cytometry

When staining for cell surface markers, γδ T cells were incubated at 4°C for 30 min with Abs diluted in FACS wash (PBS containing 1% BSA, 0.05% sodium azide), washed to remove excess Abs, and fixed and permeabilized with CytoFix/Cytoperm solution (BD Biosciences). When intracellular staining for IFN-γ was performed, the cells were resuspended in Perm/Wash solution (BD Biosciences), stained with Ab for 30 min at 4°C, washed with Perm/Wash buffer, and stored in FACS wash prior to data acquisition on a LSR Fortessa flow cytometer (BD Biosciences). FACS data analysis was performed using FlowJo software (TreeStar).

DELFIA EuTDA cytotoxicity assay

The DELFIA cytotoxicity assay (PerkinElmer) was used to assess the ability of γδ T cells to lyse DV-infected DC. The assay relies on the ability of bis(acetoxymethyl) 2,2'-6',2'-terpyridine-6,6'-dicarboxylic acid (BATDA) to permeate the membrane of target cells and to become hydrolyzed upon entry into membrane-impermeable 2,2'-6',2'-terpyridine-6,6'-dicarboxylic acid (TDA). During lysis of target cells, TDA that is released into the culture medium forms a highly fluorescent chelate in the presence of added europium salts. This fluorescence signal can be used to quantify target cell lysis.

Culture supernatant that had been conditioned for 24 h by mock-infected or DV-infected DC was aspirated away from pelleted cells and set aside. Mock-infected or DV-infected DC were loaded with BATDA reagent, washed, and resuspended in the conditioned culture medium that had been set aside previously. Resting autologous γδ T cells or γδ T cells that had been primed for 16 h with 100 ng/ml IL-18 (R&D Systems) were used as effector cells. Experimental wells were set up containing 1 × 10^4 labeled DC cocultured with 1 × 10^5 γδ T cells (E:T ratio of 10) in 96-well round-bottom plates. After 2.5 h, 20 μl supernatant was collected and mixed with 200 μl europium solution, and the fluorescent signal was detected using a time-resolved fluorescence counter (PerkinElmer). Maximum release of TDA was determined by treating BATDA-loaded DC with lysis buffer (PerkinElmer). Spontaneous release of TDA was measured by sampling supernatant from wells containing only BATDA-loaded DC growing in culture medium. The percentage specific lysis was calculated using the formula: (maximum release − spontaneous release)/(maximum release − spontaneous release) × 100.

ELISA

The concentration of IL-18 in culture medium was assayed using matched capture and detection Abs purchased from eBioscience (Human IL-18 Matched Ab Pairs), and ELISA was performed according to the instructions provided by the manufacturer.

Statistical analysis

The Student t-test was used to determine whether the observed difference between two experimental groups was statistically significant. One-way ANOVA with the Holm–Sidak posttest was used for statistical analyses that involved three or more experimental groups. Statistical analyses were performed using GraphPad Prism software.

Results

Markers of activation, proliferation, and degranulation are upregulated on γδ T cells from patients with acute DV infection

To examine the response of γδ T cells during acute DV infection in vivo, blood samples obtained from patients with a history of acute febrile illness, as well as laboratory evidence of acute dengue fever (either anti-dengue IgM positive or RT-PCR positive for dengue viral RNA), were analyzed by flow cytometry for the expression of activation markers (CD38, CD69), CD107a, and Ki-67 on CD3+ pan-γδ TCR+ cells. The clinical data for each patient is summarized in Table I. All patients made an uneventful recovery from dengue fever. The gating strategy used in this experiment is depicted in Supplemental Fig. 1A. As shown in Fig. 1, γδ T cells in blood taken from patients with acute dengue fever showed significant upregulation of CD38, CD69, CD107a, and Ki-67 compared with γδ T cells from age- and sex-matched healthy nonfebrile donors. Paired blood samples from a separate cohort of DV-infected individuals also were obtained during the acute phase of infection (days 4–8) or convalescence (days 60–120) and analyzed for the expression of these markers, as well as γδ T cell counts (Supplemental Figs. 2, 3). Expression of CD38, CD69, CD107a, and Ki-67 was significantly elevated in both Vδ2+ and non-Vδ2+ γδ T cells during acute dengue fever compared with the convalescent phase (Supplemental Fig. 3A, 3B). Altogether, these results suggest that acute DV infection is associated with the activation, proliferation, and degranulation of γδ T cells in vivo. Absolute numbers of total γδ T cells, Vδ2+ γδ T cells, and non-Vδ2+ γδ T cells in blood were significantly lower during acute dengue infection compared with the convalescent phase (Supplemental Fig. 3C). This likely reflects the transient lymphopenia that is observed during the acute phase of many viral infections, including dengue fever. However, lymphocyte cell counts recovered during convalescence.

Primary γδ T cells in PBMC produce IFN-γ and upregulate CD107a when cocultured with DV-infected DC

PBMC were cocultured with autologous DV-infected DC in vitro to further characterize the response of primary human γδ T cells

<table>
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<th>Gender</th>
<th>Age (y)</th>
<th>Fever Days</th>
<th>RT-PCR</th>
<th>Anti-DV IgM</th>
<th>Anti-DV IgG</th>
<th>CD38 (%)</th>
<th>CD69 (%)</th>
<th>CD107a (MFI)</th>
<th>Ki-67 (%)</th>
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</table>

MFI, mean fluorescence intensity; −, negative reaction; +, positive reaction; X, test not performed.
against DV. Monocyte-derived DC were used in these experiments because they can be readily generated and infected with DV to serve as primary autologous target cells for gd T cells (26). Infection of DC, when performed as described in Materials and Methods, results in an infection rate ~80% at 24 h postinfection, as assessed by intracellular staining for DV pre-M protein followed by flow cytometry (data not shown). Supplemental Fig. 1A depicts the gating strategy used in these experiments.

gd T cells in PBMC rapidly responded to DV-infected DC by producing IFN-γ and upregulating the expression of CD69 and CD107a (Fig. 2A–2C). PBMC only or PBMC cocultured with either mock-infected or uninfected DC served as experimental controls. We compared the ability of Vδ2+ and non-Vδ2+ gd T cells in PBMC to produce IFN-γ in response to DV-infected DC, specifically analyzing individuals with a balanced distribution of these gd T cell subsets in their blood. The gating strategy and distribution of Vδ2+ and non-Vδ2+ gd T cells in study individuals are depicted in Fig. 2D.

We observed that 23.9% of Vδ2+ gd T cells produced IFN-γ in response to DV-infected DC (Fig. 2E), whereas an anti-DV IFN-γ response was elicited in 8.4% of non-Vδ2+ gd T cells (Fig. 2F). Alternatively, by gating on the CD3+ pan-gdTCR+ lymphocytes (gating strategy and representative result are depicted in Supplemental Fig. 1B), it was observed that the Vδ2+ cells constitute the major subset of IFN-γ–producing gd T cells (Fig. 2G). Altogether, these results suggest that Vδ2+ cells are the predominant subset of gd T cells that produce an anti-DV IFN-γ response, even after taking into account the usual predominance of the Vδ2+ subset in blood.

The anti-DV IFN-γ response of primary gd T cells is dependent on type I IFNs and IL-18

Using this coculture system, we sought to identify soluble factors that regulate the anti-DV response of gd T cells. A significant reduction in IFN-γ production by gd T cells was observed when
FIGURE 2. Primary γδ T cells in PBMC respond to autologous DC infected with DV. DC were infected with DV at MOI = 1 and, at 24 h postinfection, were cocultured in vitro for an additional 4 h with autologous PBMC. The response of CD3+ γδ TCR+ cells was analyzed by flow cytometry. The gating strategy used to define γδ T cells is shown in Supplemental Fig. 1A. DC exposed to heat-inactivated DV (i.e., mock-infected DC) or DC that had not been exposed to any DV (i.e., uninfected DC) served as experimental controls. The response of γδ T cells in PBMC not cocultured with DC (PBMC only) is also shown. (A) The frequency of IFN-γ+ cells among CD3+ γδ TCR+ cells. Contour plots depict representative IFN-γ responses of γδ T cells in PBMC cocultured with DV-infected DC or mock-infected DC. (B) Frequency of CD69+ γδ T cells and representative CD69 expression on γδ T cells in PBMC cocultured with DV-infected DC or mock-infected DC. (C) Summary of CD107a mean fluorescence intensity (MFI) values and representative expression of this marker on γδ T cells in PBMC cocultured with DV-infected DC or mock-infected DC. Data in (A)–(C) are the mean of six independent experiments, each using PBMC from different donors. Error bars denote the SD. *p < 0.05, ****p < 0.0001, one-way ANOVA with Holm–Sidak post hoc test. (D) The Vδ2+ subset represents the majority of IFN-γ-producing γδ T cells in PBMC that respond to DV-infected DC. PBMC were cocultured with autologous DV-infected DC, as described for (A)–(C), using individuals with a balanced distribution of Vδ2+ and non-Vδ2+ γδ T cells in their
The activity of type I IFN was blocked with recombinant vaccinia virus B18R protein (Fig. 3A). A smaller reduction in IFN-γ was observed when IL-18 was neutralized with blocking Ab. Although the ability of type I IFN to regulate CD69 expression, IFN-γ secretion, and the cytotoxic function of human γδ T cells has been well described in the literature (27–29), data regarding the role of IL-18 in activating human γδ T cell function are sparse. For instance, IL-18 is involved in the proliferation of γδ T cells that is stimulated by nitrogen-containing bisphosphonates (30). However, the role of IL-18 in regulating the antiviral function of human γδ T cells has not been documented; therefore, we focused on studying this in subsequent experiments. The importance of IL-18 signaling for the IFN-γ response of these cells to dengue was underscored by the observation that the subset of γδ T cells in PBMC that expressed IL-18Ra was the predominant producer of IFN-γ (Fig. 3B). It was reported that the pathway of IL-18 production following pathogen sensing by sentinel cells involves autocrine signaling triggered by the binding of ATP to the extracellular domain of P2X7 receptors (31). Treating DV-infected DC with an antagonist of the P2X7 receptor significantly reduced the anti-DV IFN-γ response of γδ T cells in cocultured PBMC (Fig. 3C). Although the ATP-P2X7 receptor pathway also can trigger the release of IL-1β, the addition of IL-1 receptor antagonist into our coculture system did not affect the IFN-γ response of γδ T cells to DV-infected DC (Fig. 3D), suggesting that the observed effect of P2X7 receptor blockade was independent of IL-1. Blockade of type I IFN also was associated with a significant reduction in CD69 expression on γδ T cells, but it did not affect CD107a expression, and both markers were unaffected by IL-18 blockade (Supplemental Fig. 4B, 4C). Although previous studies showed that γδ T cell responses can be regulated by additional soluble factors [e.g., TNF-α (32), IL-12 (33), or IL-15 (28, 33)], blocking Abs against these cytokines or IL-7 failed to significantly reduce the IFN-γ, CD69, or CD107a responses of γδ T cells to DV-infected DC (Figs. 3A, 3E, 3F, data not shown). To assess whether interactions between the γδ TCR and endogenous ligands are involved in activating the response to DV-infected DC, mevastatin was used to inhibit the mevalonate pathway so as to reduce intracellular accumulation of IPP. Treatment with mevastatin did not cause a significant reduction in the IFN-γ response of γδ T cells to DV-infected DC (Fig. 3G), indicating that DV-induced γδ T cell activation does not involve TCR signaling.

The IFN-γ response of purified primary γδ T cells to DV-infected DC is reduced compared with the response of γδ T cells when unfractonated PBMC are coculated with DV-infected DC. Interestingly, we observed diminished anti-DV IFN-γ responses when purified primary γδ T cells, instead of unfractonated PBMC, were cocultured with autologous DV-infected DC. Although purified γδ T cells produced IFN-γ in response to DV-infected DC (Fig. 4A), there was a 71% reduction compared with the IFN-γ response of γδ T cells in unfractonated PBMC (contrast Fig. 4A with Fig. 2A; IFN-γ response of purified γδ T cells: 8.0 ± 2.5%; IFN-γ response of γδ T cells in PBMC: 27.8 ± 8.3%). Notably, the IL-18 content of supernatant from cocultures of purified γδ T cells and DV-infected DC was reduced significantly compared with supernatant from PBMC–DC cocultures (Fig. 4B), suggesting that DV-infected DC and/or γδ T cells can only release sufficient IL-18 by themselves to stimulate a low-level γδ T cell IFN-γ response. To determine whether the amount of IL-18 was limiting the γδ T cell response, the coculture experiment was repeated in the presence of exogenous IL-18. Addition of IL-18 was sufficient to raise the IFN-γ response of purified γδ T cells to the levels observed when PBMC were cocultured with DV-infected DC (Fig. 4C).

Data from Fig. 3A indicate that the anti-DV IFN-γ response of γδ T cells is also regulated by type I IFN. However, significant amounts of type I IFN are released by purified cultures of DV-infected DC (26, 34, 35), so that supplementing the culture medium with type I IFN, even at doses as high as 10,000 U/ml, did not increase the anti-DV IFN-γ response of purified γδ T cells (Fig. 4C). This suggests that type I IFN is already present at levels that are not limiting for the IFN-γ response of cocultured γδ T cells.

In summary, our results suggest that IL-18 is found in cocultures of purified γδ T cells with DV-infected DC at levels that limit the IFN-γ response of γδ T cells. We hypothesize that a component within unfractonated PBMC, possibly an accessory cell, releases IL-18 when exposed to DV-infected DC, and this additional IL-18 augments the basal IFN-γ response of γδ T cells that is triggered by type I IFN. The role of IL-18 in activating anti-DV cytotoxic responses also was evaluated. Exposure of purified γδ T cells to IL-18 for 4 h did not increase their ability to kill DV-infected DC compared with unprimed γδ T cells (data not shown), consistent with the minimal reduction in CD107a expression associated with blockade of IL-18 in short-term cocultures (Supplemental Fig. 4C). However, overnight priming with IL-18 was sufficient to convert purified γδ T cells into effector cells with a significantly increased ability to lyse DV-infected DC compared with unprimed γδ T cells (Fig. 4D).

Depletion of monocytes from PBMC reduced the responses of γδ T cells to DV-infected DC

We next sought to identify accessory cells in PBMC that could augment the IFN-γ response of γδ T cells to DV-infected DC. Because monocytes are a major source of IL-18 (36), and previous studies in murine models of infection suggested that monocytes are important for triggering memory CD8+ T cell and NK cell responses in an IL-15– and IL-18–dependent manner (22), we sought to determine whether monocytes might play an analogous role in augmenting the anti-DV IFN-γ response of human γδ T cells. We compared the ability of primary γδ T cells to secrete IFN-γ when PBMC or PBL (i.e., monocyte-depleted PBMC) were cocultured with DV-infected DC. To control for nonspecific effects associated with the monocyte-depletion process, PBMC used in these experiments also were run through a magnetic separation column but in the absence of selection MicroBeads. γδ T cells in PBL displayed significantly reduced anti-DV IFN-γ responses compared with γδ T cells in PBMC (Fig. 5A). Furthermore, significantly less IL-18 was detected in the supernatant of cocultures

blood. The FACS gating strategy used and the distribution of V62+ and non-V62+ γδ T cells are depicted. Results from four independent experiments using PBMC from different donors are depicted. p = 0.72, paired test. E The frequency of IFN-γ+ cells among CD3+ γδ TCR+ V62+ cells in PBMC. Contour plots depict representative responses to DV-infected DC or mock-infected DC. F The frequency of IFN-γ+ cells among CD3+ γδ TCR+ V62+ cells in PBMC. Contour plots depict representative responses to DV-infected DC or mock-infected DC. G Relative proportion of V62+ versus non-V62+ cells among CD3+ γδ TCR+ cells in PBMC that produced IFN-γ when cocultured with DV-infected DC. The FACS gating strategy and contour plot showing representative response are depicted in Supplemental Fig. 1B. Data in (E)–(G) are the mean of four independent experiments using PBMC from different donors. The error bars denote SD, *p < 0.05, **p < 0.01 paired t test. ns, not significant.
of PBL and autologous DV-infected DC compared with cocultures of PBMC and DV-infected DC (Fig. 5B).

**Reconstitution with CD16+ monocytes augmented the IFN-γ response of purified primary γδ T cells to DV-infected DC**

Human monocytes have been divided into subsets based on the cell surface expression of CD14 and CD16 (37). In particular, CD14dim CD16+ monocytes were reported to produce larger amounts of TNF-α following overnight incubation with HSV-1 and measles virus compared with CD14+ monocytes (38), and CD16+ monocytes infected with DV at high MOI secrete more IL-1β than do CD16+ monocytes (21), suggesting that discrete monocyte subsets express distinct patterns of cytokine secretion in response to viruses. We attempted to identify the monocyte population most likely to be the accessory cell responsible for augmenting the anti-DV IFN-γ response of γδ T cells. Purified γδ T cells and

**FIGURE 3.** The IFN-γ response of γδ T cells to DV-infected DC is dependent on type I IFN, IL-18, and the ATP-P2X7 receptor pathway. DC that were infected with DV (as described in Fig. 2) were pretreated for 30 min with the indicated cytokine inhibitors, blocking Abs, or isotype-control Abs and then cocultured with autologous PBMC for an additional 4 h. For blocking the P2X7 receptor, DC were pretreated with 100 μM of 15D for 30 min prior to coculture with PBMC, and the final concentration of 15D in coculture was maintained at 100 μM. The IFN-γ response of CD3+ γδ TCR+ cells was analyzed by flow cytometry using the gating strategy depicted in Supplemental Fig. 1B, unless otherwise stated. (A) Blockade of type I IFN with recombinant B18R protein derived from vaccinia virus or Ab-mediated neutralization of IL-18 or TNF-α. (B) Expression of IL-18Ra and IFN-γ on γδ T cells in PBMC cocultured with DV-infected DC. (C) Antagonizing the P2X7 receptor with 15D. The IFN-γ response of γδ T cells was analyzed by flow cytometry using the gating strategy depicted in Supplemental Fig. 3A. (D) Blockade of IL-1 with rIL-1R antagonist. (E) Ab-mediated blockade of IL-15. (F) Ab-mediated blockade of IL-12 or IL-15. (G) Inhibition of the mevalonate pathway of IPP synthesis with mevastatin. Data in (A), (B), and (D)–(G) are the mean value obtained from three independent experiments using blood from different donors, whereas data in (C) are from four independent experiments using blood from different donors. Error bars denote the SD. **p < 0.01, ****p < 0.0001 one-way ANOVA with Holm–Sidak post hoc test. ns, not significant.
DV-infected DC were cocultured with equal numbers of purified CD14+ monocytes or purified CD16+ monocytes. Autologous cells were used in all experiments. Coculture with CD16+ monocytes significantly increased the IFN-\(\gamma\) response of purified primary \(\gamma\delta\) T cells against DV-infected DC (Fig. 6A). In contrast, the addition of CD14+ monocytes failed to significantly affect the IFN-\(\gamma\) response of \(\gamma\delta\) T cells. The expression of CD107a on purified \(\gamma\delta\) T cells was not significantly different in the various experimental groups (Fig. 6B).

**Discussion**

Although antiviral activity of human \(\gamma\delta\) T cells was described previously, this usually has been limited in scope to descriptions of immunophenotypic changes in blood samples from virus-infected individuals (11–16, 20) or studies in which previously expanded \(\gamma\delta\) T cell lines or clones were used to demonstrate antiviral responses (14, 17–19). In this study, we analyzed blood samples from DV-infected individuals ex vivo to demonstrate that markers of activation, proliferation, and degranulation are upregulated on \(\gamma\delta\) T cells during acute dengue fever (Fig. 1, Supplemental Figs. 2, 3). In addition, we characterized the response of primary \(\gamma\delta\) T cells toward DV-infected cells in vitro. Primary \(\gamma\delta\) T cells in freshly isolated PBMC rapidly produce IFN-\(\gamma\) and upregulate CD69 and CD107a when cocultured with autologous DV-infected DC (Fig. 2).

Our results suggest that type I IFN and IL-18 regulate the IFN-\(\gamma\) response of \(\gamma\delta\) T cells against DV-infected cells and indicate that this response does not require the recognition of endogenous ligands by the \(\gamma\delta\) TCR (Fig. 3). Previous reports indicated that type I IFN augments IFN-\(\gamma\) production by phosphoantigen-stimulated V\(\gamma\)9V\(\delta\)2 T cells in PBMC isolated from patients with chronic hepatitis C virus infection (27) and is also responsible for...
the production of IFN-γ by γδ T cells cocultured with TLR-stimulated monocyte-derived DC (39). Although we observed that primary γδ T cells can produce IFN-γ in response to levels of type I IFN that is released by DV-infected DC, this response is relatively weak when IL-18 is deficient in culture (e.g., IL-18 blockade [Fig. 3A] or in the absence of monocytes [Fig. 4A]). In contrast, IL-18 has been well characterized as an IFN-γ-inducing factor for unfractionated T cells (40). Type I IFN and IL-18 produced by macrophages infected with influenza A virus cooperate to trigger IFN-γ production by unfractionated T cells (41). Upregulation of mRNA for the IL-18R α- and β-chains by IFN-α was reported as a mechanism that sensitizes unfractionated T cells to lower concentrations of IL-18 (42). We hypothesize that a similar mechanism might explain the ability of type I IFN and IL-18 to enhance the IFN-γ response of γδ T cells to DV-infected cells. Notably, the γδ T cells that secrete IFN-γ in response to DV-infected DC predominantly express IL-18Rα-chain (Fig. 3B). Because elevated levels of type I IFN and IL-18 have been found in the plasma of patients infected with DV (43, 44), IFN-γ production by γδ T cells triggered by this cytokine combination may represent a physiologically relevant source of early IFN-γ that fosters an effective anti-DV Th1 adaptive-immune response. A possible mechanism is by helping to reverse the early defect in IL-12 secretion that was observed in DV-infected DC (26, 35).

We speculate that V82+ γδ T cells might also form a first line of defense against DV-infected cells in the vascular compartment, where blood myeloid DC, as well as human endothelial cells, are potential targets of DV infection (45, 46). Although blockade of type I IFN or IL-18 during 4-h cocultures with DV-infected DC failed to significantly affect γδ T cell degranulation (Supplemental Fig. 4C), priming with IL-18 for 16 h significantly enhanced the ability of γδ T cells to lyse DV-infected cells (Fig. 4D). Because an increased capacity to mount cytotoxic responses toward DV-infected target cells was associated with a decreased likelihood of developing severe dengue disease (7), the ability of IL-18 to enhance the cytotoxic responses of γδ T cells against DV-infected targets might be an important determinant of dengue disease severity. Further studies will be required to address this possibility, particularly because it is unclear whether IL-18 plays a protective role during dengue infection (47) or is associated with adverse outcomes (43).

Although DV-infected DC produce significant amounts of type I IFN (26, 34, 35), at levels that are not limiting for IFN-γ production by γδ T cells (Fig. 4C), the amount of IL-18 that is released by these infected cells is sufficient only for a basal anti-DV response. The presence of monocytes increased the levels of IL-18 in culture supernatant (Figs. 4B, 5B) and enhanced the anti-DV response of γδ T cells against DV-infected DC. DC infected with DV (as outlined in Fig. 2) were cocultured with autologous PBMC or PBL for 4 h. Mock-infected DC cocultured with PBMC served as experimental controls. (A) The IFN-γ response of γδ T cells was analyzed by flow cytometry using the gating strategy depicted in Supplemental Fig. 1B. Data are mean values obtained from four independent experiments, each using cells from different donors. Error bars represent the SD. *p < 0.05 one-way ANOVA with Holm–Sidak post hoc test.
IFN-γ response of γδ T cells (Fig 5A, compare Fig. 2A with Fig. 4A). The anti-DV response was diminished when an antagonist was used to block the P2X7 receptor (Fig. 3C), suggesting that full anti-DV activity by γδ T cells requires activation checkpoints that involve two independent pathways of danger recognition: IRF-dependent pathways that lead to secretion of type I IFN (e.g., sensing of viral RNA by TLR3 and RIG-like receptors) (48, 49) and the detection of extracellular ATP (e.g., released as a result of cell damage) with subsequent inflammasome activation (50). Reconstitution experiments demonstrated that CD16+ monocytes have a greater ability, on a per-cell basis, to augment the IFN-γ response of γδ T cells to DV-infected DC compared with CD14+ monocytes (Fig. 6A), possibly because expression of the P2X7 receptor is higher on CD16+ monocytes than on CD14+ monocytes (data not shown). These results are analogous to the ability of Ly6C+ CCR2+ monocytes to enhance the antimicrobial responses of memory CD8+ T cells and NK cells in a murine model of infection, which occurs via the production of IL-18 and IL-15 that is triggered by inflammasome activation and type I IFN (22). This leads to the Ag-independent activation of murine memory CD8+ T cells. Similarly, recognition of endogenous phosphoantigens is not required for triggering the anti-DV IFN-γ response of γδ T cells. Ongoing experiments are being performed to address the mechanism that triggers monocytes to release IL-18 in the presence of DV-infected DC. The rapid kinetics of monocyte-dependent enhancement of IFN-γ production by γδ T cells suggest that the release and activation of preformed IL-18 may be important (51). Alternatively, it was suggested that TLR ligands can stimulate the release of ATP by monocytes to trigger IL-18 secretion in an autocrine manner (31). Our results do not exclude the involvement of other pathways of inflammasome activation. In murine models of bacterial infection, splenic CD8α+ DC were reported to regulate noncognate IFN-γ secretion by microbe-specific memory CD8+ T cells through an NLRHC4-dependent mechanism of IL-18 release (52). Such alternative pathways may also prove to be relevant during viral infections.

Previous reports observed that CD16-expressing monocytes are increased in patients infected with DV compared with healthy individuals (53). We speculate that CD16+ monocytes may influence the pathogenesis of DV infection in additional ways beyond their role in triggering γδ T cell responses. Immune complexes that contain ribonucleoprotein were reported to induce production of TNF-α by CD16+ monocytes (38), raising the possibility that complexes of DV with nonneutralizing Abs might trigger CD16+ monocytes to release pathological amounts of proinflammatory cytokines during Ag-dependent enhancement of DV pathogenicity. Because CD16+ monocytes crawl and patrol vascular capillaries (38), they may provide an anatomically relevant source of IL-18 that targets local antiviral activity in γδ T cells patrolling the vascular compartment, as well as a source of proinflammatory cytokines that contribute locally to the pathology of capillary leak associated with severe dengue disease. Regulating cytokine production by CD16+ monocytes might very well serve as a novel therapeutic target for the prevention of severe dengue.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary figure S1. Gating strategies used during the acquisition and analyses of flow cytometric data to define CD3+ γδ TCR+ cells, or to identify subsets of CD3+ γδ TCR+ cells that produced IFNγ during coculture of PBMC with DV-infected DC. (A) Gating strategy for defining CD3+ γδ TCR+ cells in PBMC. (B) Gating strategy for defining IFNγ+ cells among CD3+ γδ TCR+ cells in PBMC, and for determining the proportion of Vδ2+ versus non-Vδ2+ cells among these cells that produce an anti-DV IFNγ response.
**Supplementary figure S2. γδ T cells in peripheral blood are activated during the acute phase of dengue fever.** Paired blood samples were obtained from 13 laboratory-confirmed cases of patients with dengue virus infection during the acute phase (between days 4 – 8) and the convalescent phase (between days 60 – 120) of illness. PBMC prepared from these blood samples were stained with appropriate antibodies and analyzed using flow cytometry. (A) Gating strategy used to define Vδ2+ and non-Vδ2+ subsets of γδ T cells. (B) Representative plots depicting the expression of CD38, CD69, Ki-67, and CD107a on Vδ2+ γδ T cells during acute dengue fever, or convalescence. FMO staining controls were used to set the boundary gates between positive and negative populations. (C) Representative plots depicting the expression of CD38, CD69, Ki-67, and CD107a on non-Vδ2+ γδ T cells during acute dengue fever, or convalescence. FMO staining controls were used to set the boundary gates between positive and negative populations.
Supplementary figure S3. Summary charts depicting the activation of γδ T cells in peripheral blood during the acute phase of dengue fever. The expression of CD38, CD69, CD107a, and Ki-67 on (A) Vδ2+ γδ T cells, or (B) non-Vδ2+ γδ T cells, during the acute and convalescent (Conval.) phases of illness for all 13 patients is represented. (C) Counts of total CD3+ pan-γδ TCR+ T cells (left panel), CD3+ pan-γδ TCR+ Vδ2+ T cells (middle panel), or CD3+ pan-γδ TCR+ Vδ2 negative T cells (right panel), in 5 ml of blood obtained from the 13 patients sampled during the acute or convalescent phases of illness. *Paired t-test p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Supplementary figure S4.
(A) Gating strategy used to determine the IFNγ response of CD3+ Vδ2+ cells in PBMC that were co-cultured with DV-infected or mock-infected DC. (B) and (C) The effect of neutralizing type I IFN or IL-18 on the CD69 and CD107α responses of γδ T cells to DV-infected DC. DC and PBMC were prepared as described in figure 3, and the CD69 and CD107α responses of CD3+ γδ TCR+ cells to DV-infected DC in the presence of B18R or neutralizing antibody against IL-18 were analysed by flow cytometry using the gating strategy depicted in supplementary figure S1A. (B) CD69 response, and (C) CD107α response. (B) and (C) Data depicted is the mean of four independent experiments each using PBMC from different donors, and error bars denote the standard deviation. **One-way ANOVA with Holm-Sidak post hoc test $p<0.01$, or not significant (ns).