Blockade of Virus Infection by Human CD4+ T Cells via a Cytokine Relay Network

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CD4+ T cells directly participate in bacterial clearance through secretion of proinflammatory cytokines. Although viral clearance relies heavily on CD8+ T cell functions, we sought to determine whether human CD4+ T cells could also directly influence viral clearance through cytokine secretion. We found that IFN-γ and TNF-α, secreted by IL-12-polarized Th1 cells, displayed potent antiviral effects against a variety of viruses. IFN-γ and TNF-α acted directly to inhibit hepatitis C virus replication in an in vitro replicon system, and neutralization of both cytokines was required to block the antiviral activity that was secreted by Th1 cells. IFN-γ and TNF-α also exerted antiviral effects against vesicular stomatitis virus infection, but in this case, functional type I IFN receptor activity was required. Thus, in cases of vesicular stomatitis virus infection, the combination of IFN-γ and TNF-α secreted by human Th1 cells acted indirectly through the IFN-α/β receptor. These results highlight the importance of CD4+ T cells in directly regulating antiviral responses through proinflammatory cytokines acting in both a direct and indirect manner. The Journal of Immunology, 2008, 180: 6923–6932.

Abbreviations used in this paper: ISG, IFN-sensitive gene; cDMEM, complete DMEM; cIMDM, complete IMDM; HCV, hepatitis C virus; IFNAR, IFN-α/β receptor; LT, lymphotxin; rh, recombinant human; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus.

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infections than has previously been attributed to this subset. Indeed, studies in mice have demonstrated a CD4+ -dependent component to clearance of Sendai virus, influenza A virus, and γ-herpesvirus (40–44). In cases of γ-herpesvirus infections, CD4+ T cells were shown to inhibit reactivation from latency, and neutralization of IFN-γ could inhibit this activity. However, administration of IFN-γ was not sufficient to maintain latency, particularly within infected B cells (44, 45). Based on these observations, it is likely that CD4+ T cells play a significant role in the inhibition of viral replication through the action of a complex mixture of cytokines, the nature of which has not been investigated.

We therefore sought to answer two distinct questions. First, how do innate cytokines present during viral infections shape effector CD4+ T cell responses? Second, can cytokines secreted by effector CD4+ T cells directly impact viral infections? We found that IL-12 is primarily responsible for the generation of antiviral CD4+ T cell effector cytokine responses. IL-12 drives the secretion of IFN-γ and TNF-α, which induce potent antiviral responses against a number of viruses. Furthermore, we found that this antiviral effect on vesicular stomatitis virus (VSV) infection requires IFN-α/β receptor (IFNAR) expression on the target cell, indicating the presence of a novel cytokine relay network.

Materials and Methods

Human subjects

A total of 100–120 ml of peripheral blood was obtained from healthy adult volunteers by venipuncture. Informed consent was obtained from each donor, and all procedures related to this study were approved by the Institutional Review Board (University of Texas Southwestern Medical Center).

Cell lines

THP-1 cells, a human monocytic lymphoma line; CV-1 cells, a green monkey fibroblast line; and HeLa cells, a human cervical carcinoma line, were purchased from American Type Culture Collection. The 2TGH cells, a human fibroblast line, and 2TGH-derived INFAR2-deficient USA cells were a gift from G. Stark (Cleveland Clinic, Cleveland, OH) (46–48). A7 replicon cells, a human hepatoma line carrying a replicating hepatitis C virus (HCV) genome, have been previously described (49).

Cytokines, Abs, and reagents

Human rIL-4 (rHL-4), rIL-12, rIFN-γ, rTNF-α, and rHL-1β, and the anti-human IL-4, anti-human IFN-γ receptor (IFN-γR1), and anti-human LT Abs were purchased from R&D Systems. rIFN-α and rIFN-ω, the anti-human INFAR2 and anti-human IFN-α Abs, and polyclonal antisera against human IFN-α and IFN-β were purchased from PBL Laboratories. rIL-1α was a gift from M. Racke (Ohio State University, Columbus, OH). rIL-2 was a gift from M. Bennett (University of Texas Southwestern Medical Center, Dallas, TX). The anti-human CD3, anti-human CD28, anti-human TNF-α, and allopurinol-conjugated anti-human TNF-α Abs were purchased from BioLegend (San Diego, CA). The PE-conjugated anti-human CD4 and FITC-conjugated anti-human IFN-γ Abs were purchased from Caltag Laboratories. The FITC-conjugated anti-human CD45RA and PE-Cy7-conjugated anti-human IFN-γ Abs were purchased from BioLegend. PBMC isolated from buffy coats were stained with FITC-conjugated anti-human CD45RA and PE-conjugated anti-human CD4 Abs, and CD45RA+CD4+ cells were sorted on a MoFlo cell sorter (DakoCytomation). Cells were activated at 2–2.5 × 10⁶ cells/ml for 3 days in complete IMDM (cIMDM; HyClone) supplemented with 10% FBS (Valley Biomedical) on culture plates coated with 5 μg/ml anti-human CD3 plus 5 μg/ml anti-human CD28 in the presence of 50 U/ml rHL-2. Cytokines and neutralizing Abs were added, as indicated in the figures, at the following concentrations: anti-human IFN-γ (4S.B3), 5 μg/ml; anti-human IL-4, 2 μg/ml; anti-human IL-12 (20C2), 5 μg/ml; anti-human INFAR2, 2 μg/ml; rHL-12, 10 ng/ml; rHL-4, 10 ng/ml; rIFN-α, 1000 U/ml. On day 3, cells were split into fresh medium containing IL-2 and were rested to day 7. On day 7, cells were washed in fresh cIMDM and left unstimulated or restimulated for 24 h on culture plates coated with 5 μg/ml anti-CD3. Conditioned medium from these cells was harvested and assayed for antiviral activity by in vitro infection.

VSV infections

Cells were washed and resuspended in cIMDM at 6 × 10⁶ cells/ml (THP-1 cells) or 3 × 10⁶ cells/ml (2TGH and USA cells). Cells were infected with rVSV carrying the GFP transgene (VSV-GFP; a gift from M. Whit, University of Tennessee, Memphis, TN) (51) at 0.05–0.8 PFU/cell for 2 min at room temperature. Cells were then transferred into wells of a 96-well plate containing cytokines or T cell conditioned medium and incubated for 16 h at 37°C, 5% CO₂. Following infection, cells were washed and fixed; analysis for GFP expression was performed on a FACScan or FACSCalibur cytometer (BD Biosciences). Data were processed using FlowJo software (TreeStar). For experiments in which anti-human INFAR2 or anti-human IFN-γR1 neutralizing Abs were used, cells were incubated with 5 μg/ml anti-human INFAR2 or 10 μg/ml anti-human IFN-γR1 for 2 min at room temperature immediately before infection.

Quantitation of VSV-GFP infection by plaque assay

THP-1 cells were cultured for 24 h at 37°C, 5% CO₂ in cIMDM in the absence of 100 U/ml rIFN-α and T cell conditioned medium (10% v/v). Cells were washed and resuspended at 6 × 10⁶ cells/ml in cIMDM. Cells were infected with VSV-GFP at 0.7 PFU/cell for 15 min at room temperature. Cells were then washed in cIMDM, transferred to wells of a 96-well plate, and incubated for 24 h at 37°C, 5% CO₂. Confluent CV-1 cells were infected with supernatants from infected THP-1 cells at dilutions from 10⁻¹ to 10⁻⁵ for 45 min at 37°C, 5% CO₂. CV-1 cells were then washed and overlaid with complete DMEM (cDMEM) containing 0.6% agarose and cultured for 24–72 h at 37°C, 5% CO₂. Cells were stained with crystal violet for quantitation of plaque formation.

A7 replicon cells and Western blotting

The generation and maintenance of the A7 replicon cell line have been previously described (49). A7 replicon cells were maintained in DMEM (Mediatech) supplemented with 10% FBS (cDMEM) and 200 μg/ml G418 (Gemini Bio-Products). Twenty-four hours before treatment, cells were washed with PBS and given cDMEM without antibiotic. The following day, medium was removed, and cells were cultured in cDMEM containing cytokines or 5% (v/v) T cell conditioned medium, as indicated in the figures. Concentrations of cytokines were as follows: rIFN-αA, 100 U/ml; rIFN-γ, 5 ng/ml; rTNF-α, 2.5 ng/ml. Forty-eight hours later, cells were harvested and lysed in radioummunoprecipitation assay buffer, and proteins were separated by SDS-PAGE. Western blotting was performed using Abs against HCV NS5A, human ISG56, or human GAPDH. For experiments in which neutralizing Abs were used, cells were incubated with 5 μg/ml anti-human INFAR2, 10 μg/ml anti-human IFN-γR1, or 5 μg/ml anti-human TNF-α for 1 h immediately before treatment with cytokine or T cell conditioned medium and supplemented with the same Abs 24 h after the initiation of treatment.

Respiratory syncytial virus (RSV) infections

HeLa cells were washed and resuspended in DMEM (Invitrogen) supplemented with 10% FBS (cDMEM) at 10 × 10⁶ cells/ml. Cells were infected with recombinant RSV carrying the GFP transgene (RSV-GFP; a gift from M. Peeples, Columbus Children’s Research Institute, Columbus, OH) (52) at 2–2.5 PFU/cell for 2 min at room temperature. Cells were then transferred into wells of a 96-well plate containing cytokines or T cell conditioned medium and incubated for 72 h at 37°C, 5% CO₂. Following infection, cells were washed and fixed; analysis for GFP expression was performed on a FACScan or FACSCalibur cytometer; and the data were processed using FlowJo software.

Intracellular staining for IFN-γ and TNF-α

Naïve human CD4+ T cells were differentiated for 1 or 2 consecutive wk, as described above. On day 7 or 14, cells were washed and restained overnight in cIMDM. Cells were left unstimulated or were stimulated with 0.8 μg/ml PMA (A.G. Scientific) plus 1 μM ionomycin (Sigma-Aldrich) for 4 h at 37°C, 5% CO₂. In the presence of 1 μg/ml brefeldin A (Epigenetic Technologies). Intracellular staining was performed as previously described (53), using an allopurinol-conjugated anti-human TNF-α Ab and either a FITC-conjugated or PE-Cy7-conjugated anti-human IL-4, anti-human IL-12, and anti-human IFN-γ.
anti-human IFN-γ Ab. Cells were analyzed on a FACSCalibur or LSR II cytometer (BD Biosciences), and the data were processed using FlowJo software.

ELISA for IFN-γ and TNF-α

Concentrations of human IFN-γ and TNF-α in T cell conditioned medium were determined by ELISA using ELISA MAX kits (BioLegend), according to the manufacturer’s instructions.

Listeria innocua infections

THP-1 cells were washed and resuspended in antibiotic-free cIMDM at 2 x 10^6 cells/ml. Cells were activated with 0.8 μg/ml PMA for 48 h at 37°C, 5% CO₂ (54). Cells were washed, and cytokines or T cell conditioned medium were then added for a further 48 h at the concentrations indicated in the figures. Concentrations of cytokines were as follows: rhIFN-αA, 100 U/ml; rhIFN-γ, 10 ng/ml; rhTNF-α, 10 ng/ml. Cells were washed and infected with 3 CFU/cell L. innocua (a gift from L. Hooper, University of Texas Southwestern Medical Center, Dallas, TX) for 45 min at 37°C, 5% CO₂. Gentamicin (Sigma-Aldrich) was added at 50–100 μg/ml, and cytokines or T cell conditioned medium were added at the concentrations indicated in the figures. Cells were incubated for 16 h at 37°C, 5% CO₂. Infected cells were lysed to release intracellular bacteria, and infection was assessed by plating on brain heart infusion agar plates.

Statistical analysis

Significance analysis was performed in Prism software (GraphPad) by one- or two-way ANOVA. Comparisons were considered significant at >95% confidence interval (p < 0.05).

Results

Human CD4^+ T cells secrete an antiviral activity

Th1 cells are known to play a direct role in clearance of bacterial infections by secretion of IFN-γ. Because Th cells are known to secrete a variety of soluble mediators, we hypothesized that these cells may also play a role in viral clearance by direct cytokine signaling to infected cells. To test this hypothesis, we established an in vitro infection model whereby THP-1 cells, a human monocyte line, were infected with VSV carrying a transgene for GFP (VSV-GFP). The percentage of infected cells was monitored by flow cytometry (Fig. 1, A and B), whereas the relative secretion of live virus was quantified by plaque assay (Fig. 1E). With this model, we confirmed that VSV-GFP infection was blocked by treatment of infected cells with type I IFN (Fig. 1, A and B), and this effect was reversed.
by blocking the human type I IFN receptor (IFNAR) by a neutralizing Ab against the IFNAR2 subunit (Fig. 1A), IFN-α significantly reduced the percentage of infected cells, which correlated well with a significant decrease in secretion of live virus (Fig. 1, B and E).

We next examined the effect of CD4⁺ T cell-derived effector cytokines on VSV-GFP infection of THP-1 cells. To isolate the individual contributions of innate cues to the generation of antiviral effector responses, naïve (CD45RA⁺) human CD4⁺ T cells were differentiated with plate-bound anti-CD3 and anti-CD28 in the presence of cytokines or neutralizing Abs for 7–14 days. These cells were then washed extensively in clean medium and restimulated for 24 h with plate-bound anti-CD3, and the conditioned medium from these cells was harvested and used to treat VSV-GFP-infected THP-1 cells. Treatment of THP-1 cells with T cell conditioned medium at the time of infection inhibited VSV-GFP infection as measured by GFP expression, and this effect was dose dependent (Fig. 1C). Furthermore, conditioned medium from resting CD4⁺ T cells did not inhibit VSV-GFP infection, indicating that the secretion of antiviral activity required secondary T cell activation (Fig. 1D, p < 0.05, anti-CD3-restimulated vs unstimulated, all conditions). Pretreatment of THP-1 cells with T cell conditioned medium for 24 h before infection significantly inhibited VSV-GFP virus production from these cells, as measured by plaque assay (Fig. 1E). We also noted that T cell conditioned medium generated from T cells differentiated in the presence of IL-12 or a combination of IL-12 and IFN-α was consistently more effective at reducing VSV-GFP infection in the THP-1 cells than T cell conditioned medium generated from T cells differentiated in the presence of neutralizing Abs or IFN-α alone (Fig. 1C, p < 0.05 vs neutralized). Conversely, T cell conditioned medium generated from T cells differentiated in the presence of IL-4 had no effect on VSV-GFP infection (Fig. 1E). Occasionally, we observed a slight difference in antiviral activity generated from Th cells differentiated in the presence of IL-12 alone vs IL-12 with IFN-α (Fig. 1C); however, this difference was not present in most experiments and was most likely a result of donor variation.

To determine whether the activity secreted by human CD4⁺ T cells represented a general antiviral mechanism, we examined the ability of T cell conditioned medium to inhibit infection with two other viruses: RSV and HCV. HeLa cells were infected for 72 h with RSV carrying a GFP transgene (RSV-GFP). Treatment of HeLa cells with T cell conditioned medium at the time of infection significantly reduced RSV-GFP infection (Fig. 2A). In agreement with our previous results, conditioned medium generated from T cells activated in the presence of IL-12, alone or in combination with IFN-α, contained greater antiviral activity against RSV-GFP than conditioned medium from T cells activated under either neutralizing or IFN-α conditions (p < 0.05 vs neutralized).

Inhibition of HCV infection by T cell conditioned medium was examined using the A7 HCV replicon cell line. These cells carry a full-length, replicating HCV genome and express HCV proteins (49). Addition of 5% (v/v) T cell conditioned medium to these cells reduced HCV NS5A protein synthesis, and this antiviral activity also required restimulation of the T cells by anti-CD3 cross-linking (Fig. 2B). At these concentrations, we did not observe obvious differences between conditioned medium from different T cell conditions, which suggested that NS5A expression was particularly sensitive to very low levels of antiviral factors secreted by human CD4⁺ T cells. Taken together, these results demonstrate for the first time that effector cytokines secreted by human CD4⁺ T cells can directly inhibit viral infection in target cells.

**FIGURE 2.** Human CD4⁺ T cells secrete on antiviral activity. A, HeLa cells were infected for 72 h with RSV-GFP in the absence or presence of 100 U/ml rhIFN-α/α or 10% (v/v) T cell conditioned medium, as indicated. GFP expression was analyzed by flow cytometry. Data are expressed as mean ± SEM of three replicates. B, A7 replicon cells were incubated for 24 h in the absence (lane 1) or presence of 100 U/ml rhIFN-α/α (lane 2) or 5% (v/v) T cell conditioned medium (lanes 3–10), as indicated. Cell lysates were prepared, and Western blotting was performed using Abs against HCV NS5A and human GAPDH.

**IFN-γ and TNF-α secreted by Th cells demonstrate antiviral activity**

The greatest antiviral activity against VSV and RSV was observed in conditioned medium generated from T cells differentiated in the presence of IL-12, suggesting that the secreted factor was a Th1 cytokine. In accordance with previously published work (31), we verified that secretion of IFN-γ and TNF-α from human Th cells depended on IL-12, and IFN-α/β neither enhanced nor inhibited this effect (Fig. 3). In this study, priming with IL-12 significantly enhanced the percentage of IFN-γ-secreting cells (Fig. 3A), and the IFN-γ-secreting cells were found to also secrete TNF-α (Fig. 3, A and B). Furthermore, ~90% of cells were found to secrete TNF-α regardless of whether the cells were primed with neutralizing conditions or with IL-12 (Fig. 3, A and B). However, cells differentiated in the presence of IL-12 produced significantly higher concentrations of IFN-γ (16- to 18-fold) and TNF-α (3- to 5-fold) as compared with cells polarized under neutralizing conditions (Fig. 3, C and D).

IFN-γ and TNF-α are proinflammatory cytokines that markedly inhibit intracellular bacterial infections. These cytokines act in concert to promote the oxidative burst within phagocytic cells. To confirm that the T cell conditioned medium contained functionally relevant levels of these proinflammatory cytokines, we tested these supernatants for their ability to control *Listeria monocytogenes* infection within the THP-1 monocyte cell line. THP-1 cells were differentiated to a macrophage stage with PMA and cultured in the presence or absence of recombinant cytokines (Fig. 4A) or T cell conditioned medium (Fig. 4B) for 48 h. The cells were subsequently infected with *L. innocua*, again in the presence or absence of recombinant cytokines or T cell conditioned medium. As expected, bacterial
replication was markedly inhibited by combined treatment with recombinant IFN-γ and TNF-α (Fig. 4A). rIFN-α also inhibited L. innocua infection (Fig. 4A), as has been previously reported (55).

Additionally, treatment with T cell conditioned medium significantly inhibited L. innocua infection, and conditioned medium from cells differentiated in the presence of IL-12 displayed the greatest antibacterial activity in this assay (Fig. 4B). Thus, T cell conditioned medium contains relevant levels of IFN-γ and TNF-α sufficient to inhibit intracellular bacterial replication. These data further indicate that the THP-1 monocyte cell line is sensitive to both IFN-γ and TNF-α signaling.

As noted previously, T cell conditioned medium inhibited HCV infection in A7 replicon cells (Fig. 2B). HCV suppresses antiviral signaling by type I IFN in infected host cells by a variety of mechanisms, including inhibition IFN-α/β synthesis through disruption of the RIG-I pathway (56–58). However, several reports have indicated that HCV is susceptible to the antiviral effects of IFN-γ (59–61). Therefore, we examined the role of IFN-γ and TNF-α secretion by Th cells in inhibition of HCV infection (Fig. 5). Although rTNF-α alone displayed no effect on HCV NS5A expression, rIFN-γ alone inhibited HCV NS5A (Fig. 5A, compare lanes 2 and 3). Furthermore, addition of TNF-α marginally enhanced the antiviral effect of IFN-γ (Fig. 5A, compare lanes 3 and 5).

As was previously observed, addition of T cell conditioned medium to A7 HCV replicon cells reduced HCV NS5A protein synthesis (Fig. 5A). Neutralization of the R1 chain of the IFN-γ receptor (IFN-γR1) on target cells, combined with neutralization of TNF-α in T cell conditioned medium, reversed the previously observed antiviral activity of T cell conditioned medium in this assay system (Fig. 5A, compare lanes 8 and 11), demonstrating an antiviral role for T cell-secreted IFN-γ and TNF-α in HCV infection. As expected, addition of neutralizing anti-human IFNAR2 Ab failed to reverse the antiviral effect of T cell conditioned medium (Fig. 5B, compare lanes 11 and 12).
To elucidate a possible molecular mechanism for the antiviral effects of T cell conditioned medium in the HCV replicon system, we examined the expression of ISG56, an IFN-stimulated gene known to inhibit HCV replication (62). Treatment of A7 replicon cells with rIFN-γ induced ISG56 expression, as expected (Fig. 5A, lane 2). Unexpectedly, rIFN-γ also induced ISG56 expression in these cells, and addition of rTNF-α enhanced this effect (Fig. 5A, lanes 3 and 5). Furthermore, ISG56 was induced by T cell conditioned medium from T cells restimulated with anti-CD3 (Fig. 5A, A and B), and this effect was reversed by blockade of IFN-γ and TNF-α signaling (Fig. 5A, lanes 8 and 11), but not by neutralization of IFNAR2 (Fig. 5B, lane 12).

**IFN-γ and TNF-α signal through a cytokine relay network involving the type I IFN receptor**

Because IFN-γ and TNF-α were found to potently inhibit HCV gene expression, we wished to determine whether these two proinflammatory cytokines were also responsible for the antiviral activity of T cell conditioned medium in VSV infection. rTNF-α alone showed little antiviral activity up to 50 ng/ml, whereas rIFN-γ alone had a modest and dose-dependent effect on VSV infection (Fig. 6A). However, the combination of IFN-γ and TNF-α displayed a very potent and synergistic antiviral activity, comparable to the activity of 100 U/ml rhIFN-αA in this assay (Fig. 6A). Th1 cells also secrete LT, a member of the TNF superfamily (63), and some recent reports have demonstrated that LT secreted by NK cells has noncytopathic antiviral properties (38, 39). However, LT failed to demonstrate antiviral activity, either alone or in combination with IFN-γ (data not shown). Thus, VSV infection is sensitive to the combined effects of IFN-γ and TNF-α.

**FIGURE 5.** IFN-γ and TNF-α secreted by human CD4+ T cells exert antiviral activity against HCV infection. A, A7 replicon cells were incubated for 24 h in the absence (lane 1) or presence (lanes 2–11) of cytokines, neutralizing anti-cytokine Abs, and 5% (v/v) T cell conditioned medium from IL-12 plus IFN-γ-activated T cells, as indicated. Cell lysates were prepared, and Western blotting was performed using Abs against HCV NS5A, human ISG56, and human GAPDH. B, A7 replicon cells were incubated for 24 h in the absence (lane 1) or presence of 100 U/ml rhIFN-αA (lanes 2 and 3) or 5% (v/v) T cell conditioned medium (lanes 4–12), in the absence or presence of 5 μg/ml anti-human IFNAR2 Ab, as indicated. Cell lysates were prepared, and Western blotting was performed using Abs against HCV NS5A, human ISG56, and human GAPDH.

As demonstrated above, T cell conditioned medium markedly inhibited VSV infection, and this activity was partially inhibited by blocking either the IFN-γR1 or TNF-α (Fig. 6B). Furthermore, neutralization of both cytokines resulted in much greater reversal (Fig. 6B, condition 7, p < 0.05 vs T cell conditioned medium alone). As a control, we also preincubated target cells with neutralizing anti-IFNAR2 before VSV-GFP infection in the presence of T cell conditioned medium. Surprisingly, neutralization of IFNAR2 reversed the antiviral effect of T cell conditioned medium as effectively as blockade of IFN-γ and TNF-α (Fig. 6B, condition 4, p < 0.05 vs T cell conditioned medium alone).

We could find no previous reports demonstrating secretion of type I IFN by CD4+ T cells. Thus, we were surprised to find that neutralization of the type I IFN receptor on target cells prevented the antiviral activity of T cell conditioned medium against VSV-GFP. We therefore further pursued the role of type I IFN signaling in the observed antiviral activity secreted by Th cells. We found that the antiviral effect of recombinant IFN-γ and TNF-α could be reversed by neutralization of IFNAR2, indicating that this effect is dependent upon type I IFN signaling (Fig. 7A, p < 0.05, no Ab vs...
absence or presence of 100 U/ml rhIFN-
GFP. GFP expression was analyzed by flow cytometry. Data are expressed
availability of the IFNAR. THP-1 cells were infected for 16 h with VSV-
type I IFN. We also assayed human CD4+ T cells for secretion of IFN-α
and IFN-β by ELISA. We found no detectable IFN-α or IFN-β protein in T cell conditioned medium (data not shown). Additionally,
neutralizing Abs to examine the identity of the type I IFN involved in
neutralization medium, indicating that the secreted activity requires this receptor (Fig. 7B, p < 0.05, no Ab vs anti-IFNAR2, all conditions). These data suggest the existence of a previously
undescribed cytokine relay network whereby IFN-γ and TNF-α synergize to induce type I IFN signaling, which promotes viral clearance.

Either the Th cells or the THP-1 target cells could have been a source of type I IFN. In either case, neutralization of soluble type I IFN would reverse the antiviral activity. We therefore used neutralizing Abs to examine the identity of the type I IFN involved in the observed antiviral activity. As noted previously, pretreatment of THP-1 target cells with anti-IFNAR2 reversed the antiviral activity of T cell conditioned medium (Fig. 8A, condition 4, p < 0.05 vs T cell conditioned medium alone). However, addition of neutralizing anti-IFN-α, anti-IFN-β, or anti-IFN-ω Abs to VSV-GFP infections failed to reverse the antiviral activity of T cell conditioned medium, demonstrating that neither CD4+ T cells nor infected THP-1 cells secrete type I IFNs (Fig. 8A, conditions 5–8). Addition of each Ab was sufficient to block 10–100 U/ml its corresponding type I IFN activity in this assay (Fig. 8B), demonstrating that these Abs possess the capacity to neutralize each specific type I IFN.

We also assayed human CD4+ T cells for secretion of IFN-α and IFN-β by ELISA. We found no detectable IFN-α or IFN-β protein in T cell conditioned medium (data not shown). Additionally, we quantified IFN-β secretion from untreated and T cell conditioned medium-treated uninfected and VSV-GFP-infected THP-1 cells, but we found no detectable secretion of IFN-β from these cells (data not shown). We further examined both human Th cells and THP-1 cells for induction of mRNA transcripts for IFN-ω, IFN-α, IFN-β, IFN-ε, and IFN-κ by quantitative real-time PCR, but no transcripts were detected (data not shown). Taken together, these data demonstrated no detectable type I IFN production from either CD4+ T cells or THP-1 target cells.

Given the lack of detectable type I IFN production in this assay, it was possible that the anti-IFNAR2 Ab was inhibiting the previously observed antiviral activity through pathways not involving the human IFNAR. Therefore, we sought to further verify the role

FIGURE 7. IFN-γ and TNF-α-mediated antiviral activity requires availability of the IFNAR. THP-1 cells were infected for 16 h with VSV-
gFP. Data are expressed as mean ± SEM of three replicates. A, THP-1 cells were infected in the absence or presence of 100 U/ml rhIFN-αA or a combination of 2.5 ng/ml rhIFN-γ and 2.5 ng/ml rhTNF-α in the absence (□) or presence (■) of 5 µg/ml anti-human IFNAR2 Ab, **, p < 0.05, two-way ANOVA. B, THP-1 cells were infected in the absence or presence of 10% (v/v) T cell conditioned medium, as indicated in the absence (□) or presence (■) of 5 µg/ml anti-human IFNAR2 Ab.

FIGURE 8. Neutralization of IFN-α, IFN-β, and IFN-ω fails to inhibit the antiviral activity of human CD4+ T cells. THP-1 cells were infected for 16 h with VSV-GFP. GFP expression was analyzed by flow cytometry. Data are expressed as mean ± SEM of three replicates. A, THP-1 cells were infected in the absence (lane 1) or presence (lanes 2–8) of 10% (v/v) T cell conditioned medium from IL-12 plus IFN-α-activated T cells in the absence (lane 2) or presence of 5 µg/ml mouse IgG1 isotype control Ab (lane 3), 5 µg/ml anti-human IFNAR2 (lane 4), 5 µg/ml anti-human IFN-ω (lane 5), 1700 U/ml anti-human IFN-α (lane 6), 5 µg/ml anti-human IFN-ω (lane 7), or a combination of anti-human IFN-α, anti-human IFN-β, and anti-human IFN-ω (lane 8). B, THP-1 cells were infected in the absence or presence of 100 U/ml rhIFN-αA or 100 U/ml rhIFN-β or 10 U/ml rhIFN-ω in the absence (□) or presence of 5 µg/ml mouse IgG1 isotype control Ab (■), 5 µg/ml anti-human IFNAR2 Ab (■), or 5 µg/ml anti-human IFN-α Ab or 1700 U/ml anti-human IFN-β Ab or 5 µg/ml anti-human IFN-ω Ab (■), as indicated.
of type I IFN signaling in the observed antiviral activity. We made use of a genetically modified human fibroblast cell line, U5A, in which the gene for the human IFNAR2 subunit has been ablated (46–48). We compared VSV-GFP infection in these cells to the parent cell line, 2fTGH, which expresses an intact IFNAR. In agreement with our results in THP-1 cells, treatment of VSV-GFP-infected wild-type 2fTGH cells with a combination of recombinant IFN-γ and TNF-α at the time of infection significantly reduced viral infection (33, 34, 36, 37, 67, 68). However, this effect relied upon pretreatment of target cells with cytokines for 16–24 h before in vitro infection. In contrast, we have demonstrated an antiviral activity of IFN-γ and TNF-α which does not require pretreatment of target cells. Thus, secretion of these cytokines by CD4+ T cells at peripheral sites could have beneficial effects even after cells were already infected.

We found that the antiviral activity of T cell-secreted IFN-γ and TNF-α was independent of type I IFN signaling in the case of HCV infection. Surprisingly, this activity was completely dependent upon the presence of a functional IFNAR in the case of VSV infection. It is currently unclear whether this phenomenon is specific to VSV or represents a more general antiviral mechanism. However, we noted during the course of our experiments that Sendai virus, which blocks type I IFN signaling in infected cells, was also completely resistant to the antiviral effects of T cell conditioned medium (K. Hagan and M. Gale, Jr., unpublished observations).

Although the observed antiviral effect of IFN-γ and TNF-α is dependent upon signaling through the IFNAR in the case of VSV, we were unable to detect induction of known type I IFN genes in

Discussion

In the present study, we have demonstrated that secretion of IFN-γ and TNF-α represents a direct, cytokine-mediated antiviral activity of human CD4+ T cells. Elevated secretion of these cytokines was directed by IL-12; we found no significant contribution, positive or negative, of IFN-α/β. A combination of IFN-γ and TNF-α produced by Th1 cells promotes antiviral responses by two distinct mechanisms. First, IFN-γ and TNF-α can transmit an antiviral signal via a type I IFN-independent pathway, as in the case of HCV infection. In this case, the antiviral activity could be mediated by direct effects of IFN-γ and TNF-α or through the induction of another, non-IFN-α/β cytokine. Alternatively, the activity can be mediated through a cytokine relay network, as in the case of VSV infection, in which type I IFN signaling is required for the antiviral effect.

In agreement with our results, several other groups have shown that CD4+ T cells have the capacity to promote viral clearance in vivo in a helper-dependent fashion. For instance, clearance of Sendai virus, γ-herpesvirus (γHV68), or influenza A virus can proceed in a CD4+ T cell-dependent fashion in the absence of B cells and CD8+ T cells (40–44). Additionally, memory Th cells generated against VSV in CTL-nonresponsive mice provide protection in an Ab-independent manner (64). In many cases, a deficiency in IFN-γ in vivo abolished the antiviral capacity of CD4+ T cells (42, 64, 65), and adoptive transfer of an Ag-specific Th1 clone conferred protection from γHV68 infection (45). However, the target of IFN-γ was undetermined in these studies. Therefore, it was possible that viral clearance could have been mediated by a population of innate cells, such as NK cells, which were activated in the presence of IFN-γ. In this study, we definitively demonstrate for the first time that cytokines secreted by Th cells directly impact viral clearance from infected targets.

Furthermore, CD4+ T cell-mediated control of CMV in salivary glands requires IFN-γ, but, paradoxically, treatment of virally infected mice with rIFN-γ failed to clear the virus (66). We have shown that both IFN-γ and TNF-α are required to achieve robust viral inhibition by Th1 cell-secreted factors. Therefore, in vivo treatment of CMV-infected animals with a combination of recombinant IFN-γ and TNF-α could promote viral clearance when neither cytokine alone possessed this activity.

Several groups have reported that TNF-α can induce secretion of IFN-β from target cells and that this IFN-β can synergize with IFN-γ for viral inhibition (33, 34, 36, 37, 67, 68). However, this effect relied upon pretreatment of target cells with cytokines for 16–24 h before in vitro infection. In contrast, we have demonstrated an antiviral activity of IFN-γ and TNF-α which does not require pretreatment of target cells. Thus, secretion of these cytokines by CD4+ T cells at peripheral sites could have beneficial effects even after cells were already infected.

We found that the antiviral activity of T cell-secreted IFN-γ and TNF-α was independent of type I IFN signaling in the case of HCV infection. Surprisingly, this activity was completely dependent upon the presence of a functional IFNAR in the case of VSV infection. It is currently unclear whether this phenomenon is specific to VSV or represents a more general antiviral mechanism. However, we noted during the course of our experiments that Sendai virus, which blocks type I IFN signaling in infected cells, was also completely resistant to the antiviral effects of T cell conditioned medium (K. Hagan and M. Gale, Jr., unpublished observations).

Although the observed antiviral effect of IFN-γ and TNF-α is dependent upon signaling through the IFNAR in the case of VSV, we were unable to detect induction of known type I IFN genes in

Figure 9. IFN-γ and TNF-α signal through a cytokine relay network involving the type I IFN receptor. Wild-type 2fTGH cells and human IFNAR2-deficient U5A cells were infected for 16 h with VSV-GFP. GFP expression was analyzed by flow cytometry. Data are expressed as mean ± SEM of three replicates. A, 2fTGH and U5A cells were infected in the absence (□) or presence of 100 U/ml rhIFN-αA (■), 2.5 ng/ml rhIFN-γ (■), 2.5 ng/ml rhTNF-α (■), or a combination of 2.5 ng/ml rhIFN-γ and 2.5 ng/ml rhTNF-α (■). B, 2fTGH and U5A cells were infected in the absence (□) or presence of 100 U/ml rhIFN-αA (■) or 10% (v/v) T cell conditioned medium generated from IL-12 plus IFN-α-activated hCD4+ T cells (■). *, p < 0.05, one-way ANOVA.
target cells. This further excludes induction of IFN-β by TNF-α as a mechanism for the observed antiviral effect. Many possible explanations exist for this novel antiviral effect of IFN-γ and TNF-α during VSV infection. For instance, IFN-γ and TNF-α may be inducing expression of a novel type I IFN gene in virally infected target cells. Several new type I IFN genes have been described in recent years (69–71); a more extensive search may reveal other, distantly related family members located within or even outside the IFN locus.

Alternatively, IFN-γ and TNF-α may synergize to directly activate IFNAR signaling via a mechanism such as receptor sharing to induce type I IFN-like effects in specialized situations. There are many known cases in which both two or more related receptors are activated by the same ligand. For instance, glial cell-derived neurotrophic factor signals through both the receptor tyrosine kinase RET and the Ig domain-containing receptor neural cell adhesion molecule (72). Alternatively, a single receptor subunit can be shared among multiple distinct receptors, as in the case of the common γ-chain that is used for cytokine signaling (73). Consistent with our in vitro studies, it is interesting to note that Müller et al. (74) demonstrated that the antiviral effects of IFN-γ against VSV were impaired in murine cells lacking IFNAR expression. However, other IFN-γ signaling pathways were unaffected in cells from IFN−/− mice, and IFN-γR−/− mice showed no defect in VSV clearance.

Many viruses encode intracellular or extracellular mechanisms to antagonize antiviral cytokine secretion and signaling by infected host cells. For instance, poxviruses encode soluble, secreted forms of the IFNAR, IFN-γR, and TNFR, which can neutralize host cytokines (75–77). A variety of viruses, including HCV, influenza A virus, and Sendai virus, also inhibit intracellular induction of type I IFN by blockade of the RIG-I pathway (57, 78–80). In such cases, exogenously delivered cytokines from Th cells could provide alternative pathways to overcome these blocks and promote pathogen clearance in a noncytopathic manner.

IFN-α is widely used to treat HCV infections, but many patients fail to respond to this therapy. HCV and other flaviviruses, such as West Nile virus, inhibit IFNAR signal transduction in target cells through inactivation of downstream signaling intermediates (56, 58, 81). In accordance with previous reports, we demonstrated that IFN-γ or TNF-α by blockade of the RIG-I pathway (57, 78–80). In such cases, exogenously delivered cytokines from Th cells could provide alternative pathways to overcome these blocks and promote pathogen clearance in a noncytopathic manner.

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Disclosures

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