Lymphotoxins and cytomegalovirus cooperatively induce interferon-beta, establishing host-virus détente

Immunity, Vol. 15, 617–626, October, 2001, Copyright ©2001 by Cell Press

Lymphotoxins and Cytomegalovirus Cooperatively Induce Interferon-β, Establishing Host-Virus Détente

Chris A. Benedict,[1,6] Theresa A. Banks,[2,6] Lionel Senderowicz,[1] Mira Ko,[1] William J. Britt,[3] Ana Angulo,[4] Peter Ghazal,[4,7] and Carl F. Ware[1,5]
[1] Division of Molecular Immunology
La Jolla Institute for Allergy and Immunology
[2] Allimmune, Inc.
San Diego, California 92121
[3] Department of Pediatrics
University of Alabama School of Medicine
Birmingham, Alabama 35294
[4] Departments of Immunology and Molecular Biology
Division of Virology
The Scripps Research Institute
La Jolla, California 92037

Summary

Tumor necrosis factor (TNF)-related cytokines regulate cell death and survival and provide strong selective pressures for viruses, such as cytomegalovirus (CMV), to evolve counterstrategies in order to persist in immune-competent hosts. Signaling by the lymphotoxin (LT)-β receptor or TNF receptor-1, but not Fas or TRAIL receptors, inhibits the cytopathicity and replication of human CMV by a nonapoptotic, reversible process that requires nuclear factor κB (NF-κB)-dependent induction of interferon-β (IFN-β). Efficient induction of IFN-β requires virus infection and LT signaling, demonstrating the need for both host and viral factors in the curtailment of viral replication without cellular elimination. LTα-deficient mice and LTβR-Fc transgenic mice were profoundly susceptible to murine CMV infection. Together, these results reveal an essential and conserved role for LTs in establishing host defense to CMV.

Introduction

Herpesviruses establish life-long infections in their natural host with little or no disease manifestations; however, this benign course of infection depends on active resistance by innate and specific immune defenses, as demonstrated by their striking virulence upon immunosuppression. This pattern of infection is characteristic of a viral pathogen that has evolved the means to coexist with its natural host. Herpesviruses, such as cytomegalovirus (CMV), use a variety of strategies that target host defenses, from the disruption of antigen-processing pathways to the modulation of cytokines (Tortorella et al., 2000), all of which may contribute to the success of CMV in establishing coexistence. Several members of the tumor necrosis factor (TNF) superfamily of membrane-anchored cytokines play crucial roles in the innate and adaptive immune responses by regulating cell death and survival pathways. Members of the TNF family that regulate cell viability may provide strong selective pressure for viruses to evolve counterstrategies. In this regard, TNF and lymphotoxin (LT)-α induce antiviral activity to a broad range of viruses in tissue culture by causing premature death of the virus-infected cell, thus limiting the production of new virions (Wong and Goeddel, 1986).

TNF and LTα, along with LTβ and LIGHT, define a core group of ligands within the larger TNF superfamily that bind four cognate cell surface receptors with distinct, but significant, overlapping specificity. TNF and LTα, as homotrimers, bind the same two receptors, TNFR1 (55–60 kDa, CD120a) and TNFR2 (75–80 kDa, CD120b). TNFR1 contains a death domain that couples the receptor to caspase 8 via the adaptors TRADD and FADD leading to apoptosis (reviewed in Wallach et al., 1999). The related death domain receptors, Fas and TRAIL receptor (TRAIL-R) types 1 and 2, induce apoptosis via the death domain adaptor FADD (Bodmer et al., 2000). LTα assembles with LTβ to form the LTα1β2 heterotrimer that binds exclusively to the LTβR. The LTβ-related ligand, LIGHT, engages the herpesvirus entry mediator (HVEM or HveA) and signals through the LTβR. HVEM binds LTα but not to either TNF or LTαβ heterotrimer (Mauri et al., 1998). LTβR, TNFR2, and HVEM use members of the TRAF family of zinc RING finger adaptors to propagate signals that activate transcription factors, including nuclear factor-κB (NF-κB) and AP1 (Wallach et al., 1999), which regulate expression of inflammatory and cell survival genes. The LTβR can also induce a slow apoptotic death of some tumor lines, which appears to involve TRAF3 (Force et al., 1997). Although the complexities of receptor cross-utilization by this group of LT-related ligands suggest functional redundancy, gene deletion studies in mice have revealed unique and cooperating roles for these cytokines in the immune response. The LTαβ-LTβR system is essential for the differentiation of specific progenitor cells involved in the development of lymphoid organs and innate effector cells, NK and NK-T (Fu and Chaplin, 1999; Elewaut et al., 2000). TNF is crucial for inflammatory responses but cooperates with LTαβ in the homeostasis of splenic architecture and formation of germinal centers during response to antigen (reviewed in Fu and Chaplin, 1999). LIGHT and HVEM are currently less well understood, but LIGHT can interfere with herpes simplex virus entry (Mauri et al., 1998) and may play a role in lymphocyte activation and tumor immunity (Tamada et al., 2000).

This group of TNF-related cytokines provides a potentially powerful selective force that may direct evolution toward virus-host coexistence. Recent evidence shows that several herpesviruses specifically target TNF-related cytokine systems. These virus-encoded genes include envelope glycoprotein-D of herpes simplex virus (α-herpesvirus), which acts as a competitive antagonist of the LIGHT-HVEM binding interactions (Mauri et al., 1998); the latent membrane protein-1 of Epstein-Barr virus (γ-herpesvirus), which usurps the signaling adaptors TRAF and TRADD (Farrel, 1998); vFLIP of equine γ-her-

[5] Correspondence: carl_ware@liai.org
[6] These authors contributed equally to this work.
[7] Present address: Scottish Centre for Genomic Technology and Informatics, University of Edinburgh, Summerhall, Edinburgh, EH9 1QH, Scotland.

pesvirus, an antagonist of caspase 8 activation that blocks apoptosis by Fas and TRAIL-R (Tschopp et al., 1998); and the UL144 open reading frame of human CMV (β-herpesvirus; HCMV), an ortholog of HVEM and TRAIL-R2 (Benedict et al., 1999). We reasoned that these diverse molecular links reflect a specific evolutionary history between the LT/TNF cytokine system and different subtypes of herpesviridae. These observations prompted us to ask whether LT/TNF-related cytokines can elicit resistance to herpesvirus infection, perhaps providing selective pressures directing the evolution of viral countermeasures.

Here we show that signaling by the LTβR and TNFR1, but not by Fas or TRAIL-R, initiates a potent nonapoptotic antiviral activity that inhibits cytopathicity and replication of HCMV in diploid fibroblasts. Furthermore, the efficient induction of interferon-β (IFN-β) depends on both HCMV infection and LT receptor signaling, which demonstrates a mutual dependence on both host and virus factors to establish a state of coexistence. Although mouse CMV (MCMV) and HCMV are genetically distinct, LTs also appear to play a fundamental role in host defense against MCMV, as revealed by the profound susceptibility of LTα-deficient mice and LTβR-Fc transgenic mice to MCMV infection. Together, these results reveal a previously undisclosed mechanism for controlling a β-herpesvirus infection.

Results

Inhibition of HCMV by LTs and LIGHT

The replication cycle of HCMV in normal human diploid fibroblasts (NHDF) reaches completion after ~72 hr. At a low multiplicity of infection (MOI ≤ 0.1), HCMV infection appears in focal areas of cytopathicity typified by cell rounding and detachment, which spreads throughout the culture by days 6–7 (Figure 1a). The addition of LTα, LTα1β2, or LIGHT to the cultures completely inhibited the cytopathic effect of HCMV (Figures 1b, 1e, and 1g). The anti-HCMV activity of LTα was neutralized when excess soluble decoy receptor TNFR1-Fc was added (Figure 1c). Furthermore, point mutations in LTα (LTαY108F) (Williams-Abbott et al., 1997) or LIGHT (G119E) (Rooney et al., 2000b), mutations that disrupt binding to their specific receptors TNFR1 and LTβR, abolished the inhibitory action of these cytokines (Figures 1d and 1f). Cytokine-mediated inhibition was equivalent whether added at the time of virus infection or several hours after virus adsorption, indicating that they did not disrupt attachment or entry of HCMV (data not shown).

To assess the effect of LTα, LTα1β2, and LIGHT on HCMV protein expression, both the major immediate early protein (IE1/pp72) and the late tegument protein pp28 were analyzed by Western blot (Figure 2a). LTα1β2 and LIGHT showed similar relative antiviral activity (IC50 for inhibition of pp28 expression of ~1 and 0.4 nM, respectively), whereas LTα was ≥40-fold more effective (IC50~0.01 nM; Figure 2b), which is consistent with the higher receptor binding affinity of secreted LTα compared with the normally membrane anchored LTα1β2 and LIGHT (Rooney et al., 2000b). Activation of the LTβR via addition of an agonistic polyclonal IgG elicited the inhibitory effect on HCMV protein expression (Figures 2a and 2b), indicating that this receptor mediated the antiviral effects seen with LTα1β2 and LIGHT. Similarly,

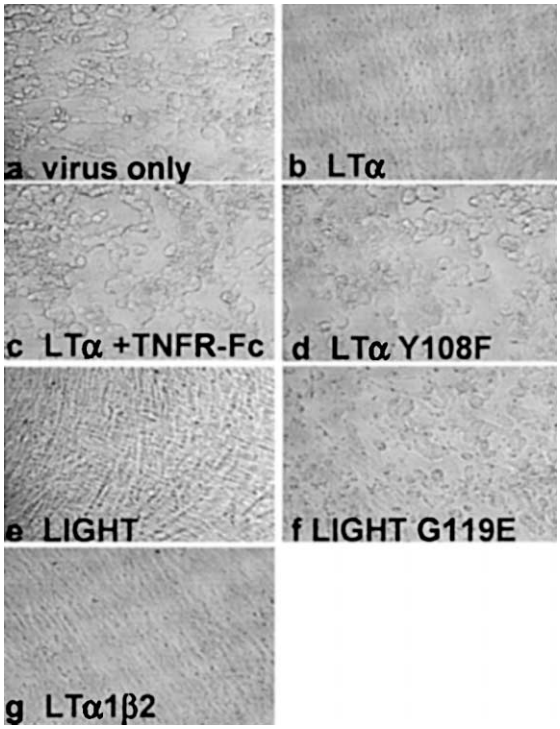


Figure 1. Inhibition of HCMV-Induced Cytopathicity by LTs and LIGHT

NHDFs were infected with HCMV at an MOI of 0.01, and various purified recombinant cytokines were added to medium at a final concentration of 5 nM. After culture for 7 days, the cytopathic effect was visualized by light microscopy (20× magnification).
(a) NHDF infected with virus in medium or in medium with LTα (b), LTα with TNFR1-Fc (25 μg/ml) (c), mutant LTαY108F (d), LIGHT (e), mutant LIGHTG119E (f), and LTα1β2 (g).

the agonistic anti-TNFR1 mAb H398 also inhibited HCMV replication (data not shown). Consistent with the reduction in cytopathicity and viral protein expression mediated by these cytokines, the production of infectious virus was also significantly reduced (Figure 2c).

Obvious cell death was not detected in NHDF treated with LTs whether infected with HCMV or not. Dermal fibroblasts express receptors for FasL and TRAIL and, in the presence of cycloheximide, undergo apoptosis following treatment with pM levels of ligand (C.A.B. and C.F.W., unpublished observations). Surprisingly, FasL and TRAIL were unable to reduce virus production (Figure 2c) or to inhibit virus-protein expression and cytopathicity (data not shown), indicating that the antiviral effect is specific to LIGHT and LTs in this model. Together, these results suggest that the mechanism of blocking virus spread was probably not by inducing death of infected cells.

Nonapoptotic and Reversible Effect of LT and LIGHT on HCMV Replication

Although cell death or cytopathic effects were not observed in HCMV-infected fibroblasts treated with LTs or LIGHT, some expression of IE1 was always detectable 7 days postinfection (Figures 2a and 3a), even at high concentrations of cytokine that completely block infectious virus production (Figure 3b). This suggests that receptor signaling may block viral spread by inhibiting

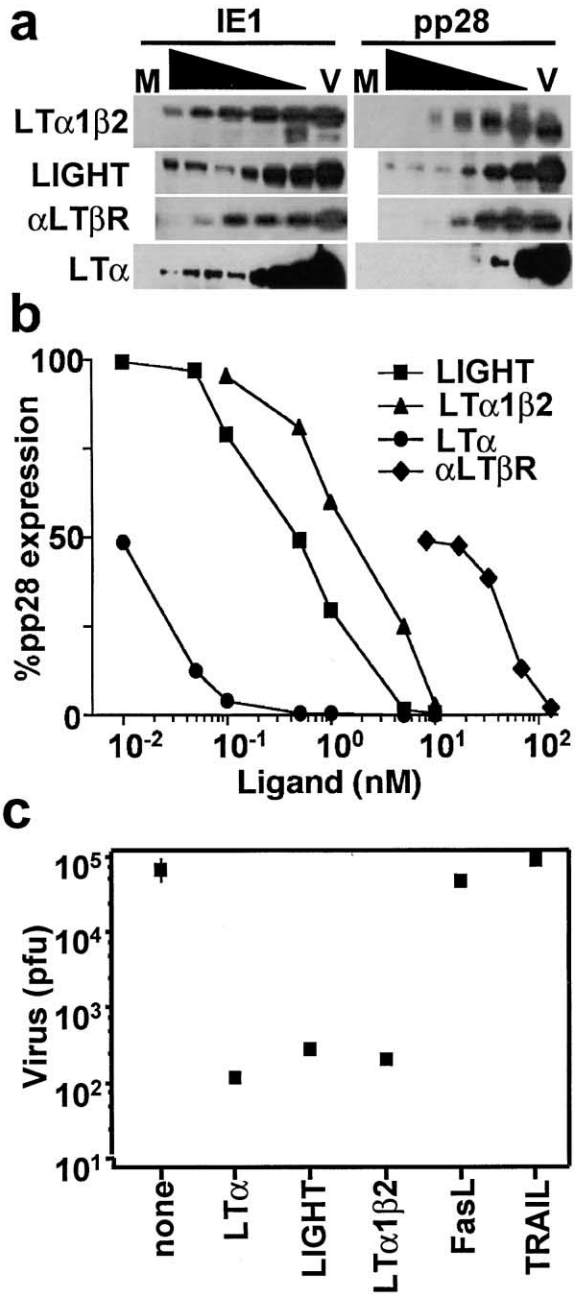


Figure 2. Analysis of HCMV Protein Expression in LT-Treated Cells

(a) HCMV-infected fibroblasts (MOI = 0.01) were treated with indicated purified cytokines, and cells were harvested 7 days later for analysis of the major immediate early I protein (IE1) or late tegument protein (pp28) by Western blot. M, mock; V, virus with no cytokine added.

(b) The percentage of maximal protein expression in the Western blot was calculated as a ratio of the pp28 band density in cytokine treated cells to cells infected with virus in the absence of cytokine.

(c) Infectious virus production (± SD) was measured in supernatants collected from NHDF infected with HCMV alone or infected in the presence of LTα (0.1 nM), LTα1β2 (1 nM) or LIGHT (1 nM),or FasL (12 nM) or TRAIL (12 nM). Cytokine dose response curves and viral titers were performed at least three times; data from a single representative experiment are shown.

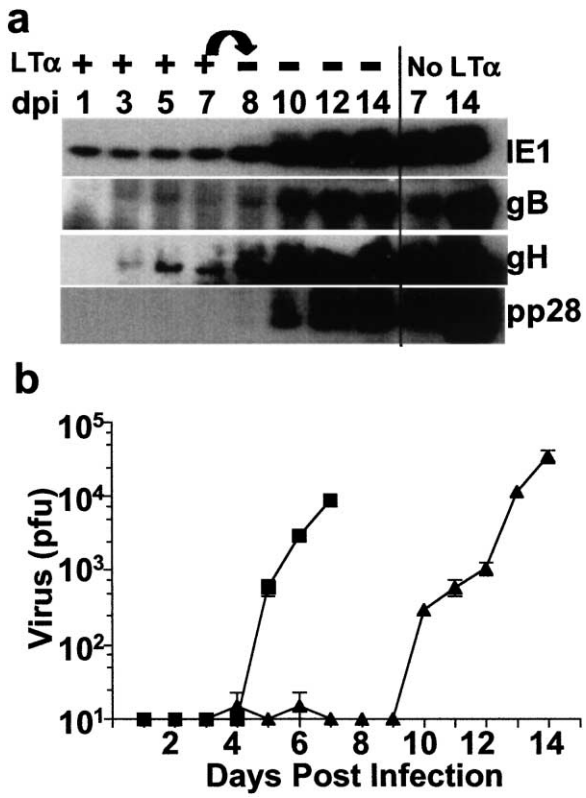
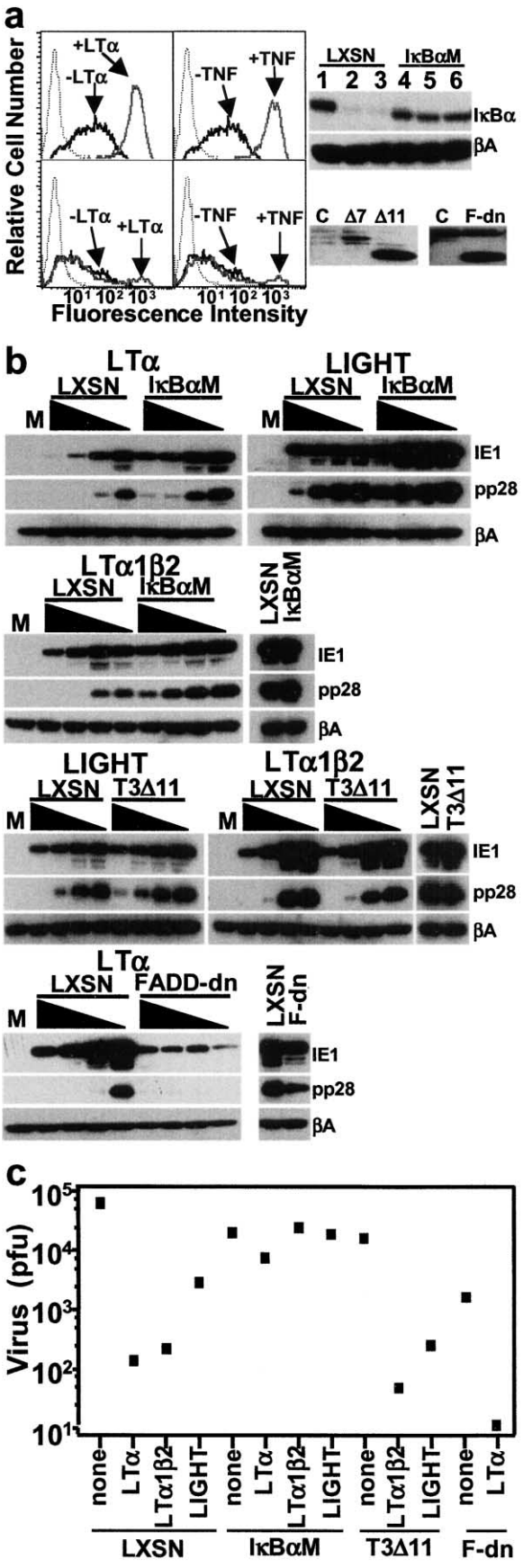


Figure 3. The Antiviral Effect of LTα Is Reversible

(a) NHDFs were infected with HCMV at an MOI of 0.01 and cultured with LTα (1 nM; +). After 7 days, the medium was replaced with fresh medium without LTα⁻, and virus replication was allowed to proceed for an additional 7 days. Cells were harvested at the indicated time postinfection and virus protein expression analyzed by Western blot (IE1, gB, gH, and pp28). The "no LTα" lanes represent HCMV proteins at 7 or 14 days in the absence of LTα.

(b) HCMV titers (± SD) were measured every 24 hr after infection of NHDF (MOI = 0.01) for cells infected in the absence of LTα (square), or with LTα for the initial 7 days and then was removed for days 8–14 (triangle).

gene expression downstream of the immediate early genes. If this is the case, then cytokine-treated cells may harbor HCMV genome in a restricted expression state. To test this hypothesis, NHDFs infected with HCMV and treated with LTα for 7 days were washed and then supplemented with medium without cytokine for an additional 7 days. The immediate early protein 1 (IE1/pp72) and viral glycoproteins gB and gH (early and early-late expressed genes, respectively) (Chambers et al., 1999) could be detected by Western blot during the initial 7 days of infection in the presence of LTα when no cytopathicity or virus production was seen. However, expression of the true late protein pp28 (Kerry et al., 1997) was undetectable (Figure 3a). This level of protein most likely represents expression in cells initially infected at low MOI. A vigorous reemergence of viral protein expression (Figure 3a) with high levels of pp28, concurrent with the release of infectious virions (Figure 3b), occurred after this initial 7 day period when the culture was replaced with fresh medium-lacking cytokine. Thus, the block to HCMV appears to be late in the replication cycle based on Western blot analysis of these representative viral proteins and the short lag time before cytopathicity (~24 hr) and the appearance of virus in the culture supernatant (~48 hr) after removal of cytokine. LTα1β2, anti-LTβR antibodies, and LIGHT similarly



inhibited HCMV (data not shown). Together, these results demonstrate that the antiviral effect of LTs and LIGHT is reversible and does not involve death of virus-infected cells.

NF-κB but Not TRAF3 or FADD Is Necessary for LT/LIGHT-Mediated Anti-HCMV Activity

The LTβR and TNFR1 are capable of activating both apoptotic and nonapoptotic signaling pathways that can be distinguished by introducing dominant-negative (dn) mutants of key signaling molecules. Mutant signaling molecules were introduced into NHDF with retroviral vectors containing dominant-acting forms of IκBα, TRAF3, FADD, or a control empty vector (LXSN). The IκBα mutant (IκBαM) contains two point mutations at serine 32 and 36 to alanine that deletes the critical phosphorylation sites targeted by cytokine-activated serine kinases (Van Antwerp et al., 1996). When introduced into NHDF, IκBαM protein cannot be phosphorylated and degraded by the ubiquitination pathway, thus retaining NF-κB in its latent cytoplasmic state, which in turn prevents nuclear translocation and transcriptional activation of NF-κB target genes such as intercellular adhesion molecule-1 (ICAM-1; Figure 4a). The IκBαM-expressing fibroblasts when compared with control vector transduced cells were refractory to the antiviral effects of LTs and LIGHT, as detected by viral protein expression (Figure 4b) or virus production (Figure 4c). Importantly, the IκBαM did not significantly alter HCMV replication in the absence of cytokine. In contrast, the TRAF3 dn mutant, which can inhibit LTβR-induced death in HT29 carcinoma cells, but not NFκB activation (Force et al., 2000; Rooney et al., 2000b), did not block the antiviral effect of either LTα1β2 or LIGHT. Surprisingly, the FADD dn mutant, which deletes the death effector domain required for caspase 8 recruitment to TNFR1 and Fas, actually enhanced the effect of LTα but also partially diminished virus replication in the absence of cytokine. These results demon-

Figure 4. NFκB, but Not TRAF3 or FADD, Is Critical for Anti-HCMV Signaling by TNFRI and LTβR

(a) NHDF were transduced with retroviral vectors expressing either a dn IκBα mutant (IκBαM), TRAF3.Δ11 and Δ7 mutants, FADD-dn (F-dn), or empty vector (LXSN). NHDF-IκBαM cells were treated with TNF or LTα (1 nM) for 24 hr, and ICAM-1 expression was measured by flow cytometry (LXSN, upper 2 panels; IκBαM, lower 2 panels). Cell lysates were prepared and analyzed by Western blot for expression of IκBα, FLAG-tagged TRAF3.Δ11 and Δ7, and FADD-dn mutants. For the IκBα degradation assay, cells were either mock treated (lanes 1 and 4) or were treated with 1 nM TNF (lanes 2 and 5) or LTα (lanes 3 and 6) in the presence of 10 μg/ml cyclohexmide for 4 hr. C, control LXSN cell lysate.

(b) NHDF-expressing dn mutants were compared with control cells (LXSN) for the ability of LTα, LTα1β2, or LIGHT to inhibit expression of IE1 and pp28 at 7 days by Western blot. Blots were reprobed with an anti-β-actin (βA) antibody. Concentrations of cytokines or antibodies added to the culture medium were: LIGHT and LTα, serial 10-fold dilution starting at 10 nM; LTα1β2, 5 nM,1 nM, 0.1 nM, and 0.01 nM.

(c) Supernatants were collected from HCMV-infected NHDF cell lines treated with cytokine (LTα, 0.1 nM; LTα1β2, 1 nM; LIGHT, 1 nM) for 7 days and then analyzed for plaque-forming units (PFUs). HCMV PFUs were determined in quadruplicate, and SDs are contained within the symbols. Western blots and titers were performed at least three independent times, and representative results are shown.

strate that anti-HCMV signaling mediated by both TNFR1 and LTβR requires activation of NF-κB and further confirm that inhibition of HCMV replication does not involve apoptosis of infected cells.

Interferon-β Mediates LT and LIGHT-Dependent Antiviral Activity

Supernatant from LTα-treated HCMV infected fibroblasts, but not from cells treated with LTα or virus alone, was capable of transferring antiviral activity to newly infected cells (Figure 5a). However, neutralization of LTα in these supernatants by TNFR1-Fc failed to block the antiviral activity as measured by IE1 protein expression (Figure 5a). This result suggested that a secondary mediator was responsible for the antiviral activity induced by LTs, implicating type I interferon as a candidate. Neutralizing antibodies to IFN-β added to the medium along with TNFR1-Fc ablated the transfer of antiviral activity (Figure 5a). Inclusion of anti-IFN-β-neutralizing antibodies on initial treatment of HCMV-infected cells with LTα also reversed the block to viral gene expression (Figure 5b) and virion production (Figure 5c). Anti-IFN-α showed very minimal (<5% compared with anti-IFNβ), but consistent, neutralizing activity; anti-IFN-γ was without effect (Figure 5b). Additionally, anti-IFN-β was able to block the antiviral activity of LIGHT or LTβR agonistic antibodies (data not shown), suggesting a similar signaling mechanism is initiated by both LTβR and TNFR1.

A high-level induction of IFNβ mRNA was observed only in NHDF that were both infected with HCMV and treated with LTα (Figure 6a), LIGHT, or LTα1β2 (Figure 6b). The level of IFNβ message increased proportional to the amount of infectious virus, suggesting that the production of IFNβ occurred in virus-infected cells (Figure 6a). The induction of IFNβ was rapid, peaking at 4–6 hr after infection, whereas trace levels of IFNα mRNA were detectable at 8–10 hr after infection (Figure 6c). Treatment of NHDF with polyI:C, a potent IFN inducer, stimulated IFN-α and IFN-β mRNA in the absence of LTs or virus. IFN-β was not detected in uninfected cells treated with LTs but was minimally induced by HCMV alone; however, this level of IFN-β induction with virus alone was not sufficient to restrict CMV replication (Figure 5). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) revealed an induction of IFNβ mRNA 48- to 103-fold (mean = 77 ± 26; n = 4) above the level seen with virus alone (Figure 6d). NHDF-IκBαM had significantly reduced levels of IFN-β mRNA after exposure to HCMV and LTα (8.6-fold induction vs. 103-fold in LSXN control cells; Figures 6d and 6e), consistent with the refractory response to the antiviral effects of LT-related ligands. Importantly, no difference was seen in the ability of recombinant IFN-β to inhibit HCMV replication in NHDF-IκBαM cells (data not shown), indicating that NF-κB activation is critical for the induction of IFN-β and not for subsequent IFN-β-mediated antiviral effects.

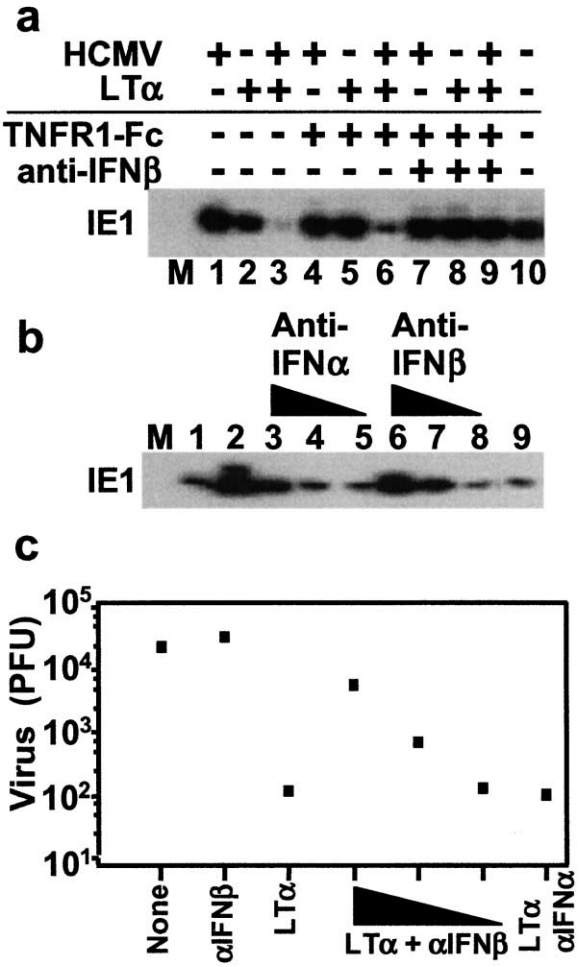


Figure 5. The Anti-HCMV Activity of LTα Is Mediated through the Induction of IFN-β

(a) Medium from NHDF infected with HCMV (MOI = 0.01; lanes 1, 4, and 7), treated with 1 nM LTα (lanes 2, 5, and 8), or infected and treated with LTα (lanes 3, 6, and 9) was collected after 2 days and transferred to NHDF freshly infected with HCMV (MOI = 0.01). The medium from infected cells was untreated (lanes 1–3) or treated with 25 μg/ml TNFR1-Fc (lanes 4–6) or 25 μg/ml TNFR1-Fc and 500 units anti-IFN-β neutralizing antibody (lanes 7–9). Cells were harvested 7 days later and analyzed for IE1 expression by Western blot.

(b) NHDF cells were infected with HCMV (MOI = 0.01) and, at the time of infection, were treated with LTα (1 nM, except for lane 2), anti-IFN-α, or anti-IFN-β neutralizing antibodies. Cells were harvested 7 days after infection and analyzed for expression of IE1 by Western blot. M, mock infected cells; lane 1, LTα alone; lane 2, virus only. Cells were treated with 1 nM LTα (lanes 3–9). Dose titration (500 units serial diluted 10-fold) with anti-IFN-α (lanes 3–5), anti-IFN-β (lanes 6–8), or anti-IFN-γ-neutralizing antibodies (25 μg/ml; lane 9).

(c) Supernatants were collected from NHDF infected with HCMV alone (none) or infected in the presence of anti-IFN-β neutralizing antibody (αIFNβ, 500 U), LTα (1 nM), LTα plus neutralizing anti-IFNβ (500, 50, and 5 U), or anti-IFNα antibody (500 U) as in (b).

LTαβ Is Critical for Host Defense against MCMV

The antiviral activity that LTs exhibit against human CMV suggested that these ligands may be conserved across species. We examined the capacity of LTα-deficient mice ($LT\alpha^{-/-}$) to respond to infection with MCMV. MCMV replicates in most visceral organs and to high levels in spleen, liver, lung, and salivary glands. Depending on virus dose and the strain and age of the mice, MCMV can cause death from acute shock at 2–3 days postinfection. However, the most common course is death at 5–7 days as a result of multiorgan disease and in particular hepatic failure. $LT\alpha^{-/-}$ mice (C57/BL6 background) were profoundly susceptible to lethal infection with MCMV requir-

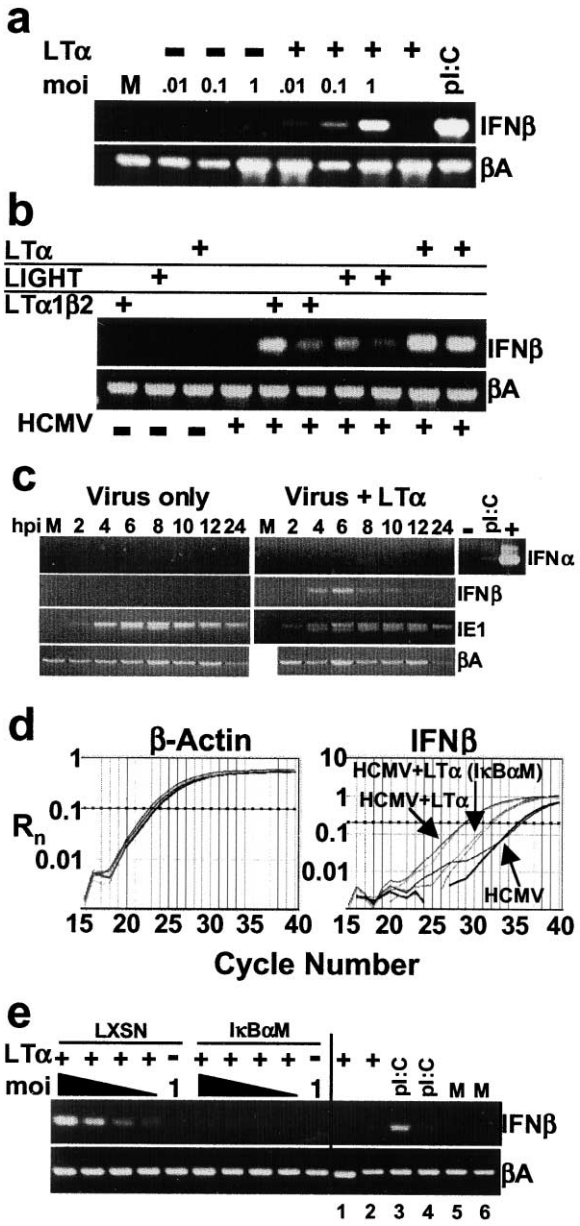


Figure 6. Signaling through TNFR1 and LTβR Induces Transcription of IFN-β in HCMV-Infected Cells

(a) NHDFs were infected with HCMV at indicated MOI in medium with or without LTα (1 nM).

(b) NHDF were infected with HCMV (MOI = 1) and treated with LTα, LTα1β2, or LIGHT (5 and 1 nM each) or treated with cytokine without virus infection (5 nM).

(c) NHDF infected with HCMV (MOI = 1) plus or minus LTα (1 nM) were harvested at various hours postinfection (hpi) for analysis of IFN-α, IFN-β, and HCMV IE1 expression levels. +, amplification of IFNα from NHDF genomic DNA.

(d) Real-time PCR was performed on NHDF-LXSN and NHDF-IκBαM cells infected with HCMV (MOI = 1) with or without LTα (1 nM) in order to quantify induction of IFNβ mRNA. Quantification of β-actin (βA) mRNA was performed in parallel to allow for normalization and calculation of fold difference in IFN-β induction. Four independent experiments were performed, and the level of IFNβ in infected cells treated with LTα was increased by 48- to 103-fold (mean = 77 ± 26).

(e) NHDF-LXSN or NHDF-IκBαM cells were infected with HCMV at MOI (1, 0.5, 0.25, 0.125 from left to right) and incubated in medium with or without LTα (1 nM). (Right panel) NHDF-LXSN (lanes 1, 3, and 5) and NHDF-IκBαM (lanes 2, 4, and 6) cells were treated with LTα (lanes 1 and 2), polyI:C (lanes 3 and 4) or mock infected (M) (lanes 5 and 6). For all panels except (c), cells were harvested 4 hr postinfection for isolation of total cell RNA; M, mock infected. NHDF were treated with polyI:C (100 μg/ml) with (a and c) or without (e) cycloheximide (10 μg/ml) for 4 or 6 (c) hours. All PCR was performed in the linear range excepting panels (a) and (c) (IFN-α only) to allow for detection of low level induction.

ing ~100-fold less virus than age-matched C57/BL6 mice (Figure 7, upper panel). At viral doses $>4 \times 10^4$ PFU (LD50 for LTα$^{-/-}$ mice = $\sim 3 \times 10^4$ PFU) the LTα$^{-/-}$ mice succumbed at 5–7 days, indicating hepatic failure as the likely cause of death. Similarly, when high doses of MCMV ($\geq 3 \times 10^6$ PFU; LD50 for C57/BL6 = ~ 2–3×10^6 PFU) were used to infect C57/BL6 controls; these mice also died between 5 and 7 days.

LTα$^{-/-}$ mice have defects in the development of peripheral lymphoid organs and also lack most of their NK and NK-T cells (Lizuka et al., 1999; Elewaut et al., 2000), which could account for their susceptibility to MCMV. However, mice expressing the LTβR-Fc decoy as a transgene, which have a normal complement of lymph nodes and NK and NK-T cells, were also susceptible to MCMV compared with transgene negative littermates (Figure 7, lower panel). This result indicates that the developmental abnormalities in the LTα$^{-/-}$ mice are not likely to be responsible for the susceptibility of these mice to MCMV and implicates LTαβ and LIGHT as potentially critical effector molecules in host defense to MCMV.

Discussion

The results presented here reveal a molecular mechanism involved in the establishment of a mutually dependent coexistence between HCMV and its host cell. Signaling mediated by LT-related cytokines activate NF-κB-dependent transcription of IFN-β only in HCMV-infected fibroblasts. IFN-β then acts to confer cellular resistance to HCMV blocking viral spread. The mutually beneficial effect is observed in survival of the infected cell and inhibition of cytopathicity, with maintenance of the HCMV genome. The dependence on virus and LT to induce IFN-β may provide, in part, a molecular basis for the ability of HCMV to establish a state of coexistence, or détente, in immunocompetent hosts.

The HCMV-fibroblast model demonstrates that LTβR and TNFR1 signaling induces a selective antiviral activity by a nonapoptotic mechanism, which is dependent on IFN-β gene expression. This conclusion is based, in part, on the reversible nature of the antiviral effect mediated by LT, neutralization of the antiviral activity with IFN-β antibodies, and the inability of the potent death domain receptors, Fas and TRAIL-R, to confer resistance to HCMV. Conclusive evidence on the nonapoptotic nature of the antiviral activity was demonstrated by dominant-acting mutants of the death pathways for TNFR1 (FADD) and LTβR (TRAF3), which failed to block induction of the anti-HCMV activity. In contrast, cells that express the dominant-acting IκBα mutant were completely unresponsive to LTs or LIGHT signaling both at the level of ICAM-1 induction and antiviral activity. Previous studies

have established that the cell death signaling by TNFR1 or LTβR is independent of the activation of NF-κB, as the two pathways bifurcate at the level of the specific adaptor proteins (Hsu et al., 1996; VanArsdale et al., 1997). NF-κB activation by TNFR1 is achieved by TRADD or RIP and TRAF2 and other pathways (Wallach et al., 1999), whereas LTβR activation of NF-κB is dependent on TRAF2 or TRAF5, but the LTβR also has a TRAF-independent mechanism for activating this transcription factor (Force et al., 2000). Although Fas and TRAIL-R can activate NF-κB in certain contexts (Schneider et al., 1997), they either signal inefficiently in NHDF or are selectively impaired by HCMV in our system. The finding that LTβR and TNFR1 potently activate NF-κB in many cell types including NHDF, but do not induce IFN-β expression in the absence of virus infection, reveals the codependence on host and viral factors for IFN-β induction. The IκBα mutant permits the conclusion that NF-κB is necessary but not sufficient for IFN-β induction and antiviral activity. Interestingly, IFN-β induction by TNF has been observed in fibroblasts, but it requires that the cells must be stressed by aging in culture (Reis et al., 1989) and is further evidence that IFN-β induction requires cooperating signals.

The IFN-β enhanceosome is composed of NF-κB (p50/p65), interferon response factor (IRF)-3 and IRF-7, ATF-2/c-*jun*, HMGI(Y), and p300/CBP (Maniatis et al., 1998). As discussed, activation of TNFR1 and LTβR can induce NF-κB, but interestingly, HCMV infection has also been reported to activate NF-κB to some extent (Sambucetti et al., 1989). Both LTβR and TNFR1 signaling activates Janus kinase-dependent pathways, which could contribute to the AP-1 (ATF-2/c-*jun*) component of the enhanceosome. However, the complete machinery necessary for IFNβ transcription is obviously not activated by LTβR/TNFR1 signaling; thus, HCMV must provide activation of complementary factors, such as IRF-3 (Navarro et al., 1998). Amplification of IFN-β transcription through "priming" of cells with low levels of IFN-α/β has been reported to be important in generating a robust IFN-β response, and IRF-3 and -7 are important mediators (Sato et al., 2000b). However, IFN-β amplification by an IRF-7-dependent mechanism is relatively slow (6 hr to initiate) (Sato et al., 2000a) compared with the response of fibroblasts treated with LT and HCMV (peak response is at 4 hr). This difference suggests that TNFR and/or LTβR signaling can augment the IFN-α/β response in the absence of an autocrine "amplification loop" in HCMV-infected cells and may be important because HCMV can block IFNα signaling pathways (Miller et al., 1999). This suggests that LT signaling may be critical for amplifying the early IFN response to viruses like HCMV. Another possibility is that HCMV has developed a specific strategy to suppress induction of IFN-β, and LTβR/TNFR1 signaling overrides the blockade. This hypothesis is supported by preliminary results indicating that UV-inactivated virus induces IFN-β to a greater extent than replication-competent virus, suggesting that attachment of HCMV to cellular receptors may be sufficient to induce IFN-β. This idea gains support in the observations that purified HCMV envelope glycoproteins gB and gH activate intracellular signaling pathways (Yurochko et al., 1997).

HCMV replication appears to be arrested in a restricted state prior to late gene expression in fibroblasts based on detection of immediate early (IE1) and early/late proteins gB and gH, but not late protein pp28. This restricted pattern of expression induced by LT and IFN-β in fibroblasts is distinct from what is reported for viral latency in cells of monocytic lineage (Kondo et al., 1994). HCMV infects multiple cell types in vivo, and the available evidence indicates cells of monocytic lineage may serve as a reservoir of latent virus (Soderberg-Naucler et al., 1997b). Treatment of monocytes with IFN-γ and TNF may promote virus reactivation, in contrast to the inhibitory effects of LTs in fibroblasts (Soderberg-Naucler et al., 1997a), highlighting the cell type-specific effects of this family of cytokines. Indeed, HVEM, a receptor for LIGHT and LTα expressed predominantly on cells of myeloid/lymphoid origin, could influence viral gene expression in monocytic cells in a fashion distinct from TNF. Clearly, the availability of genome-wide transcriptional profiling for CMV (Chambers et al., 1999) may reveal a clearer definition of genome activity modulated by LTs and IFN, as well as an exact definition of virus latency.

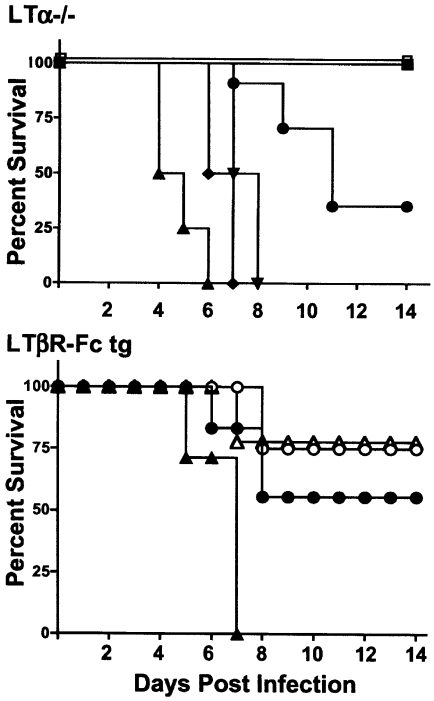


Figure 7. Increased Susceptibility of LTα$^{-/-}$ and LTβR-Fc Tg Mice to MCMV Infection

The upper panel shows groups of LTα-deficient mice (n = 4 to 6) infected with MCMV Smith strain at a dose of MCMV, 5 × 10^3 (square), 4 × 10^4 (circle), 8 × 10^4 (upside down triangle), 2 × 10^5 (diamond), or 5 × 10^5 (triangle) PFU per mouse. Control wild-type mice (C57/BL6; n = 4 per group) were infected with 1 × 10^6 (open square) PFU/mouse and the viability of all mice was monitored daily for 2 weeks.

The lower panel shows groups of LTβR-Fc transgenic mice (filled symbol) or age- or sex-matched littermate control mice (open symbol; n = 4–6) infected with MCMV at 8 × 10^4 (circle) or 2 × 10^5 (triangle) PFU per animal. Both LTβR-Fc$^+$ and control mice infected with doses at or below 4 × 10^4 PFU/mouse demonstrated 100% survival at 14 days postinfection (data not shown). These experiments were repeated on three separate occasions for the LTα$^{-/-}$ and twice for the LTβR-Fc$^+$ mice.

Innate antiviral defenses are critical for resistance to MCMV infection (Biron et al., 1999), and this may be true as well for HCMV infection. The IFN-$\alpha\beta$ system, in addition to its direct antiviral control mechanisms, is a crucial cytokine system produced by the virus-infected cell that activates innate defenses mediated by NK cells (Orange and Biron, 1996). NK cells are critical during the acute phase of MCMV infection, whereas T cells appear to be important for final resolution of the virus from certain organs in some, but not all, strains of mice (Sweet, 1999). NK cell-produced IFN-γ is important for control of MCMV in the liver (Orange et al., 1995). However, even if IFN-γ reaches systemic levels, NK cell recruitment via chemokines to virus-infected tissue is still required for effective defense (Salazar-Mather et al., 1998). This finding is consistent with the involvement of a cell contact-dependent mechanism, such as expression of surface LT$\alpha\beta$ or LIGHT by activated NK cells, for effective defense against MCMV.

Ligands that signal via the LT-β receptor appear to play a fundamental role in protection against MCMV, as demonstrated by the profound susceptibility of LT$\alpha^{-/-}$ mice and mice expressing the decoy receptor for LTα1β2 and LIGHT. The LTβR-Fc Tg is expressed $\sim$3 days following birth of the mice and does not cross into the embryonic circulation or activate complement due to a mutated Fc region, which results in a less severe phenotype compared to LT$\alpha^{-/-}$ mice (Ettinger et al., 1996). Thus, the LTβR-Fc should act in vivo as a strict antagonist of LTα1β2 and LIGHT and not through FcR or complement elimination of effector cells that may display these ligands on the cell surface. The fact that the LTβR-Fc Tg mice have a full complement of lymph nodes, as well as NK and NK-T cells, supports this idea (Elewaut et al., 2000). However, in common with LT$\alpha^{-/-}$ mice, the LTβR-Fc Tg mice exhibit disorganized splenic architecture, which is thought to be a homeostatic process mediated by B cells, and lack Peyer's patches, a late event involved in organogenesis (Fu and Chaplin, 1999). Effector functions such as induction of integrins or chemokines may be absent (Luther et al., 2000). Another observation pointing to a specific role for LT$\alpha\beta$ and LIGHT is the finding that TNFR1-deficient mice are resistant to MCMV but also have a disorganized splenic architecture and lack Peyer's patches (Fleck et al., 1998). This result indicates that signaling by TNFR1 through its two known ligands, LTα and TNF, is insufficient for host resistance to MCMV. The inability of the LIGHT-LTβR pathway to provide protection against MCMV in the LT$\alpha^{-/-}$ mice may be due to the absence of LIGHT and LT$\alpha\beta$-expressing NK cells. Together, these genetic models implicate the LTα1β2/LIGHT-LTβR systems as functionally relevant cytokines for resistance to MCMV. NK cell production of LT or LIGHT could play a role in the induction of IFNs, chemokines, or integrins essential for host defense to CMV. In this regard, early host defense mechanisms must be sufficient to attenuate the initial infection in order for adaptive immunity to develop. LT$\alpha^{-/-}$ mice are capable of resisting some other viruses and thus are not globally impaired in adaptive defenses, although the magnitude of the adaptive immune response in LT$\alpha^{-/-}$ mice can be somewhat reduced. LT$\alpha^{-/-}$ mice cleared mouse γ-herpesvirus-68 with delayed kinetics but were fully competent at preventing reactivation of latent virus, consistent with the control of acute infection by T cells and prevention of reactivation from latency by the redundant action of CD4$^+$T cells and B lymphocytes (Lee et al., 2000). In contrast, a reduced virus-specific CD8$^+$ response was observed in LT$\alpha^{-/-}$ mice infected with lymphocytic choriomeningitis virus (Berger et al., 1999) or human herpes simplex virus (Kumaraguru et al., 2001). These results suggest a possible role for LTs in the adaptive immune control of virus persistence.

The finding that LT$\alpha\beta$/LIGHT is important in controlling both human and MCMV substantiates the idea that CMV and LTs share significant evolutionary history. Although mouse and human CMV have colinear genomes and display similar replicative programs, the molecular mechanisms of immune evasion diverge. This divergence is observed, for example, at the level of viral genes targeted at antigen recognition processes as well as effector mechanisms (see Tortorella et al., 2000). The differences in the details of immune evasion strategies probably reflect the specific adaptations that these viruses and hosts have evolved to achieve coexistence. Nonetheless, the strategies used by CMV are obviously successful as evidenced by the widespread dissemination of CMV in both human and mouse populations (Smith et al., 1993). However, the roles that these elegant evasion mechanisms play in establishing coexistence remain to be fully elucidated. The mechanisms revealed here provide for a clearer understanding of host-virus coexistence and may provide for therapeutic opportunities to reestablish détente with CMV in immune-compromised hosts.

Experimental Procedures

Cells, Virus, Cytokines, and Antibodies

NHDF (Clonetics, San Diego, CA) were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum. HCMV strain AD169 (American Type Culture Collection, Rockville, MD) stocks were prepared and quantified by limiting dilution plaque formation assay on NHDF. Purified recombinant soluble LIGHT (Rooney et al., 2000b), mutant LIGHTG119E (Rooney et al., 2000b), and LTαY108F (Williams-Abbott et al., 1997) were purified as described, and LTα and LTα1β2 were gifts from Jeff Browning (Biogen, Inc., Cambridge, MA) (Browning et al., 1996). Anti-LTβR (goat) antibodies and decoy receptors TNFR1-Fc and LTβR-Fc were purified as described (Rooney et al., 2000a). IFN-α, IFN-β, and their specific antibodies (sheep polyclonal) were from Research Diagnostics (Flanders, NJ), and Fas ligand and TRAIL were from Alexis Biochemicals (San Diego, CA). ICAM-1 was detected with mAB 2146 (Chemicon, Temecula, CA) by flow cytometry (FACS Caliber; Becton-Dickinson, Mountain View, CA) and staining with goat anti-mouse IgG conjugated to R-phycoerythrin. Anti-IκBα (rabbit polyclonal Ab; Upstate Biotech, Lake Placid, NY) and anti-FLAG epitope (Sigma, St. Louis, MO) were used in Western blotting as described (Benedict et al., 1999). Anti-HCMV monoclonal antibodies IE1 (clone 63 and 27), pp28, gB, and gH were prepared as described (Sanchez et al., 1998).

Virus Infection

NHDFs were seeded into 96-well plates at 1×10^4 cells/well or 1.5×10^5 cells in 12-well plates and infected the following day with HCMV (MOI = 0.01). Virus was allowed to adsorb for 2 hr at 37°C; cells were then washed twice and cultured in medium with indicated reagents. For analysis of virus production and protein expression, 12-well cultures were harvested and centrifuged to collect the cell pellet for protein analysis by Western blotting as described (Benedict et al., 1999), and the supernatant was assayed for infectious virus by plaque

assay. To control for equal protein loading, the blots were reprobed with an antibody to human β-actin (MAB810; Chemicon).

Retrovirus Gene Transfer

Stable expression of mutant cDNAs IκBαM(S32,36A), TRAF3.Δ11, and FADD.dn were generated by transduction with replication incompetent retroviral vectors. Retroviral vectors were produced by a three-plasmid $CaPO_4$ transfection method into 293T cells as described (Force et al., 1997). The retroviral vector expression plasmids LXSN, IκBαM (a dn mutant of IκBα cloned into LXSN; gift of I. Verma) (Van Antwerp et al., 1996), and TRAF3.Δ11 dn acting mutants inserted into the pBABE retroviral vector have been described previously (Force et al., 1997). The pBABE-FADD.dn retroviral vector was generated from a plasmid containing full-length FADD (gift of V. Dixit) by PCR amplification of a truncated FADD coding sequence lacking an intact death effector domain (nucleotide 240-627) with the addition of a 5′ SnaB I site and a 3′ EcoR I site. After amplification, the PCR product was digested and ligated into pBABE-FLAG (pBABE-puro containing an N-terminal FLAG epitope tag inserted at the BamH I/SnaB I site) and verified by DNA sequencing (ABI Prism 310; Perkin-Elmer, Foster City, CA). Retroviral vector transduction frequency was >99%, as gauged by resistance of cells to the selection drug.

Analysis of IFN Gene Expression

For analysis of IFN-α/β mRNA induction by RT-PCR, NHDFs (~80% confluent) were harvested 4 hr after infection and the total RNA was isolated (Rneasy mini kit; Qiagen, Chatsworth, CA). RNA was treated with DNase I, and 2 μg was used for reverse transcription. For PCR analysis, volumes of RT reactions and cycles of PCR were determined empirically to ensure analysis was within the linear range. Primer sequences used for PCR were as follows: β-actin, 5′-TGACGGG GTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCG GTGGACGATGGAG-3′; IFN-β, 5′-GTCAGTGTCAGAAGCTCCTGTG GC-3′ and 5′-CTATGGTCCAGGCACAGTGACTG-3′; IFN-α, 5′-GAATCTCTCCTTTCTCCTG-3′ and 5′-CTGACAACCTCCCAGGCAC-3′; and IE1, 5′-GCATAGAATCAAGGAGCACATGC-3′ and 5′-GTGATCA ATGTGCGTGAGCACC-3′. IFN-α primers were designed to hybridize to conserved sequences present in all subtypes. For real-time PCR, (GeneAmp 5700 sequence detection system; PE Biosystems, Foster City, CA) the primers and Taqman probe (PE Biosystems) for detection of IFNβ were 5′-GACATCCCTGAGGAGATTAAGCA-3′, 5′-GGAGCATCTCATAGATGGTCAATG-3′, and probe sequence 5′VIC-CGTCCTCCTTCTGGAACTGCTGCAG-TAMRA3′. For determination of fold differences in message levels, the cycle number (C_t), which the relative fluorescence (R_n) crossed the manually set threshold value, was determined using the analysis software provided with the 5700 SDS. Fold differences were then calculated as follows: $[\log_2 IFN\beta\ C_t(HCMV+LT\alpha) - C_t(HCMV)] \div [\log_2 \beta actin\ C_t(HCMV+LT\alpha) - C_t(HCMV)]$.

Mice and Virus Infections

$LT\alpha^{-/-}$ mice backcrossed to C57BL/6 mice for eight generations (Banks et al., 1995) and wild-type C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The transgenic line expressing a soluble mouse LTβR/human IgG1-Fc fusion protein (LTβR-Fc Tg) on a Balb/c background was provided by Drs. R. Ettinger and H.O. McDevitt (Stanford University, Stanford, CA) (Ettinger et al., 1996). These animals constitutively express the murine LTβR-Fc under the control of the human CMV promoter. The construct is specifically mutated in the CH2 domain to block binding to Fc receptors and complement activation. Serum levels of this soluble chimeric receptor were determined by enzyme-linked immunosorbent assay (Ettinger et al., 1996); experiments used animals with serum levels >1.2 μg/ml. Nontransgenic littermates served as controls. All mice were bred and housed under specific pathogen-free conditions and in accordance with institutional guidelines. In all experiments, mice were age (6–12 weeks) and sex matched.

MCMV (Smith strain) viral stocks were prepared from salivary gland extracts as described (Reddehase et al., 1985). To determine both lethal and sublethal virus doses for acute MCMV infection in the different mouse strains, virus doses ranging from 5×10^3 to 1×10^6 PFU were injected by the intraperitoneal route into groups of four to six mice per virus dose and the mice monitored daily for morbidity and mortality over a period of 14 days.

Acknowledgments

The authors would like to thank J. Goldstein, N. Lurain, P. Spear, and E. Wagner for helpful suggestions and C. Lozano and E. Marino for manuscript preparation. This investigation was supported in part by grants from the Public Health Service; National Institutes of Health, AI33068, CA69381, AI48073 (C.F.W.), AI47644 (T.A.B.), AI44851 (A.A.), AI35602 (W.J.B.), and AI30627 (P.G.); and NIH training grant T32AG00252 (C.A.B.). This is manuscript #416 from the La Jolla Institute for Allergy and Immunology.

Received February 9, 2001; revised August 2, 2001.

References

Banks, T.A., Rouse, B.T., Kerley, M.K., Blair, P.J., Godfrey, V.L., Kuklin, N.A., Bouley, D.M., Thomas, J., Kanangat, S., and Mucenski, M.L. (1995). Lymphotoxin-α-deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. J. Immunol. *155*, 1685–1693.

Benedict, C., Butrovich, K., Lurain, N., Corbeil, J., Rooney, I., Schneider, P., Tschopp, J., and Ware, C. (1999). Cutting edge: a novel viral TNF receptor superfamily member in virulent strains of human cytomegalovirus. J. Immunol. *126*, 6967–6970.

Berger, D.P., Naniche, D., Crowley, M.T., Koni, P.A., Flavell, R.A., and Oldstone, M.B. (1999). Lymphotoxin-beta-deficient mice show defective antiviral immunity. Virology *260*, 136–147.

Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P., and Salazar, T.P. (1999). Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. *17*, 189–220.

Bodmer, J.L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J., and Tschopp, J. (2000). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat. Cell Biol. *24*, 241–243.

Browning, J.L., Miatkowski, K., Griffiths, D.A., Bourdon, P.R., Hession, C., Ambrose, C.M., and Meier, W. (1996). Preparation and characterization of soluble recombinant heterotrimeric complexes of human lymphotoxins α and β. J. Biol. Chem. *271*, 8618–8626.

Chambers, J., Angulo, A., Amaratunga, D., Guo, H., Jiang, Y., Wan, J.S., Bittner, A., Frueh, K., Jackson, M.R., Peterson, P.A., et al. (1999). DNA microarrays of the complex human cytomegalovirus genome: profiling kinetic class with drug sensitivity of viral gene expression. J. Virol. *73*, 5757–5766.

Elewaut, D., Brossay, L., Santee, S.M., Naidenko, O.V., Burdin, N., DeWinter, H., Matsuda, J., Ware, C.F., Cheroutre, H., and Kronenberg, M. (2000). Membrane lymphotoxin is required early in ontogeny for development of different subpopulations of NK T cells. J. Immunol. *165*, 671–679.

Ettinger, R., Browning, J.L., Michie, S.A., van Ewijk, W., and McDevitt, H.O. (1996). Disrupted splenic architecture, but normal lymphnode development in mice expressing a soluble lymphotoxin-β receptor-IgG1 chimeric fusion protein. Proc. Natl. Acad. Sci. USA *93*, 13102–13107.

Farrel, P.J. (1998). Signal transduction from the Epstein-Barr virus LMP-1 transforming protein. Trends Microbiol. *6*, 177–178.

Fleck, M., Kern, E.R., Zhou, T., Podlech, J., Wintersberger, W., Edwards, C.K., and Mountz, J.D. (1998). Apoptosis mediated by Fas but not tumor necrosis factor receptor 1 prevents chronic disease in mice infected with murine cytomegalovirus. J. Clin. Invest. *102*, 1431–1443.

Force, W.R., Cheung, T.C., and Ware, C.F. (1997). Dominant negative mutants of TRAF3 reveal an important role for the coiled coil domains in cell death signaling by the lymphotoxin-β receptor (LTβR). J. Biol. Chem. *272*, 30835–30840.

Force, W.R., Glass, A.A., Benedict, C.A., Cheung, T.C., Lama, J., and Ware, C.F. (2000). Discrete signaling regions in the lymphotoxin-β receptor for TRAF binding, subcellular localization and activation of cell death and NFκB pathways. J. Biol. Chem. *275*, 11121–11129.

Fu, Y.-X., and Chaplin, D. (1999). Development and maturation of secondary lymphoid tissues. Annu. Rev. Immunol. *17*, 399–433.

Hsu, H., Shu, H.B., Pan, M.G., and Goeddel, D.V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299–308.

Kerry, J.A., Priddy, M.A., Kohler, C.P., Staley, T.L., Weber, D., Jones, T.R., and Stenberg, R.M. (1997). Translational regulation of the human cytomegalovirus pp28 (UL99) late gene. J. Virol. *71*, 981–987.

Kondo, K., Kaneshima, H., and Mocarski, E.S. (1994). Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. Proc. Natl. Acad. Sci. USA *91*, 11879–11883.

Kumaraguru, U., Davis, I.A., Deshpande, S., Tevethia, S.S., and Rouse, B.T. (2001). Lymphotoxin alpha(−/−) mice develop functionally impaired CD8(+) T cell responses and fail to contain virus infection of the central nervous system. J. Immunol. *166*, 1066–1074.

Lee, B.J., Santee, S., Von Gesjen, S., Ware, C.F., and Sarawar, S.R. (2000). Lymphotoxin $\alpha^{-/-}$ mice can clear a productive infection with murine gammaherpevirus-68 (MHV-68) but fail to develop splenomegaly or lymphocytosis. J. Virol. *74*, 2786–2792.

Lizuka, K., Chaplin, D.D., Wang, Y., Wu, Q., Pegg, L.E., Yokoyama, W.M., and Fu, Y.X. (1999). Requirement for membrane lymphotoxin in natural killer cell development. Proc. Natl. Acad. Sci. USA *96*, 6336–6340.

Luther, S.A., Lopez, T., Bai, W., Hanahan, D., and Cyster, J.G. (2000). BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. Immunity *12*, 471–481.

Maniatis, T., Falvo, J.V., Kim, T.H., Kim, T.K., Lin, C.H., Parekh, B.S., and Wathelet, M.G. (1998). Structure and function of the interferon-beta enhancesome. Cold Spring Harbor Symp. Quant. Biol. *63*, 609–620.

Mauri, D.N., Ebner, R., Montgomery, R.I., Kochel, K.D., Cheung, T.C., Yu, G.-L., Ruben, S., Murphy, M., Eisenbery, R.J., Cohen, G.H., et al. (1998). LIGHT, a new member of the TNF superfamily and lymphotoxin α are ligands for herpesvirus entry mediator. Immunity *8*, 21–30.

Miller, D.M., Zhang, Y., Rahill, B.M., Waldman, W.J., and Sedmak, D.D. (1999). Human cytomegalovirus inhibits IFN-alpha-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-alpha signal transduction. J. Immunol. *162*, 6107–6113.

Navarro, L., Mowen, K., Rodems, S., Weaver, B., Reich, N., Spector, D., and David, M. (1998). Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex. Mol. Cell. Biol. *18*, 3796–3802.

Orange, J.S., and Biron, C.A. (1996). Characterization of early IL-12, IFN-alpha beta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. J. Immunol. *156*, 4746–4756.

Orange, J.S., Wang, B., Terhorst, C., and Biron, C.A. (1995). Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J. Exp. Med. *182*, 1045–1056.

Reddehase, M.J., Weiland, F., Munch, K., Jonjic, S., Luske, A., and Koszinowski, U.H. (1985). Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J. Virol. *55*, 264–273.

Reis, L.F., Lee, H.T., and Vilcek, J. (1989). Tumor necrosis factor acts synergistically with autrocrine interferon-beta and increases interferon-beta mRNA levels in human fibroblasts. J. Biol. Chem. *264*, 16351–16354.

Rooney, I., Butrovich, K., and Ware, C.F. (2000a). Expression of lymphotoxins and their receptor-Fc fusion proteins by baculovirus. Methods Enzymol. *322*, 345–363.

Rooney, I.A., Butrovich, K.D., Glass, A.A., Borboroglu, S., Benedict, C.A., Whitbeck, J.C., Cohen, G.H., Eisenberg, R.J., and Ware, C.F. (2000b). The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. J. Biol. Chem. *275*, 14307–14315.

Salazar-Mather, T.P., Orange, J.S., and Biron, C.A. (1998). Early murine cytomegalovirus (MCMV) Infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1α (MIP-1α)-dependent pathways. J. Exp. Med. *187*, 1–14.

Sambucetti, L.C., Cherrington, J.M., Wilkinson, G.W., and Mocarski, E.S. (1989). NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. *8*, 4251–4258.

Sanchez, V., Angeletii, P., Engler, J., and Britt, W. (1998). Localization of human cytomegalovirus structural proteins to the nuclear matrix of infected human fibroblasts. J. Virol. *72*, 3321–3329.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., et al. (2000a). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha beta gene induction. Immunity *13*, 539–548.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., et al. (2000b). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity *13*, 539–548.

Schneider, P., Thome, M., Burns, K., Bodmer, J.L., Hofmann, K., Kataoka, T., Holler, N., and Tschopp, J. (1997). TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity *7*, 831–836.

Smith, A.L., Singleton, G.R., Hansen, G.M., and Shellam, G. (1993). A serologic survey for viruses and Mycoplasma pulmonis among wild house mice (Mus domesticus) in southeastern Australia. J. Wildl. Dis. *29*, 219–229.

Soderberg-Naucler, C., Fish, K.N., and Nelson, J.A. (1997a). Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. J. Clin. Invest. *100*, 3154–3163.

Soderberg-Naucler, C., Fish, K.N., and Nelson, J.A. (1997b). Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell *91*, 119–126.

Sweet, C. (1999). The pathogenicity of cytomegalovirus. FEMS Microbiol. Rev. *23*, 457–482.

Tamada, K., Shimozaki, K., Chapoval, A.I., Zhu, G., Sica, G., Flies, D., Boone, T., Hsi, H., Fu, Y.-X., Nagata, S., et al. (2000). Modulation of T-cell mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. Nat. Med. *6*, 283–289.

Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., and Ploegh, H.L. (2000). Viral subversion of the immune system. Annu. Rev. Immunol. *18*, 861–926.

Tschopp, J., Irmler, M., and Thome, M. (1998). Inhibition of Fas death signals by FLIPs. Curr. Opin. Immunol. *10*, 552–558.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R., and Verma, I.M. (1996). Suppression of TNF-α-induced apoptosis by NF-κB. Science *274*, 787–789.

VanArsdale, T.L., VanArsdale, S.L., Force, W.R., Walter, B.N., Mosialos, G., Kieff, E., Reed, J.C., and Ware, C.F. (1997). Lymphotoxin-β receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor κB. Proc. Natl. Acad. Sci. USA *94*, 2460–2465.

Wallach, D., Varfolomeev, E.E., Malinin, N.L., Goltsev, Y.V., Kovalenko, A.V., and Boldin, M.P. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. *17*, 331–367.

Williams-Abbott, L., Walter, B.N., Cheung, T., Goh, C.R., Porter, A.G., and Ware, C.F. (1997). The lymphotoxin-α (LTα) subunit is essential for the assembly, but not receptor specificity, of the membrane-anchored LTα1β2 heterotrimeric ligand. J. Biol. Chem. *272*, 19451–19456.

Wong, G.H., and Goeddel, D.V. (1986). Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. Nature *323*, 819–822.

Yurochko, A.D., Hwang, E.S., Rasmussen, L., Keay, S., Pereira, L., and Huang, E.S. (1997). The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-kappaB during infection. J. Virol. *71*, 5051–5059.