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TRAIL-R as a Negative Regulator of Innate Immune Cell Responses

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Summary

TRAIL receptor (TRAIL-R) signaling has been implicated in inducing apoptosis in tumor cells, but little is understood about its physiological function. Here, we report the generation and characterization of *TRAIL-R*$^{-/-}$ mice, which develop normal lymphocyte populations but possess enhanced innate immune responses. *TRAIL-R*$^{-/-}$ mice exhibited increased clearance of murine cytomegalovirus that correlated with increased levels of IL-12, IFN-α, and IFN-γ. Stimulation of macrophages with *Mycobacterium* and Toll-like receptor (TLR)-2, -3, and -4, but not TLR9, ligands resulted in high levels of TRAIL upregulation and enhanced cytokine production in *TRAIL-R*$^{-/-}$ cells. The immediate-early TLR signaling events in *TRAIL-R*$^{-/-}$ macrophages and dendritic cells are normal, but IκB-α homeostatic regulation and NF-κB activity at later time points is perturbed. These data suggest that TRAIL-R negatively regulates innate immune responses.

Introduction

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), also known as Apo2L, was originally identified through its homology to TNF (tumor necrosis factor), FasL, and other members of the TNF superfamily (Locksley et al., 2001; Nagata, 1999; Wiley et al., 1995). Addition of TRAIL induces apoptosis of many tumor cell lines but, interestingly, has no apoptotic effect on most nontransformed cells (Takeda et al., 2002; Walczak et al., 1999). In humans, four members of the TNF receptor superfamily can bind TRAIL: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1, TRID), and TRAIL-R4 (DcR2, TRUNDD). TRAIL-R1 and -R2 possess an intracellular tail containing a conserved motif known as the death domain. As in TNF-RI and Fas, this domain allows interaction with the downstream adaptor protein(s) to initiate apoptotic signals (Ashkenazi and Dixit, 1999). In contrast, TRAIL-R4 possesses a truncated cytoplasmic tail lacking a death domain and TRAIL-R3 exists as a GPI-linked protein. These latter two receptors are thought to function as decoy proteins that can inhibit signaling through TRAIL-R1 and -R2 (Ashkenazi and Dixit, 1999). The fifth receptor for TRAIL, osteoprotegerin, is a soluble protein that participates in regulation of bone density. Osteoprotegerin can inhibit TRAIL-mediated apoptosis in vitro, but its functional relationship with TRAIL in vivo is not clear. In mice, there is only one full-length receptor, TRAIL-R (mDR5, mTRAILR2, and mKILLER), which is equally homologous to human DR4 and DR5 (Ashkenazi and Dixit, 1999; Wu et al., 1999). Like its human homologs, this receptor is capable of signaling apoptosis in transformed cells after either overexpression or ligation by TRAIL. Two murine decoy receptors (mDcTRAILR1 and mDcTRAILR2) have also been recently reported (Schneider et al., 2003). They lack a death domain and do not induce apoptosis in sensitive cells.

The ability to preferentially signal apoptosis in transformed cells has led to numerous studies of the mechanism of TRAIL-R signaling. We and others have shown that TRAIL-R induces apoptosis in a FADD-dependent manner (Bodmer et al., 2000; Kischkel et al., 2000; Kuang et al., 2000; Sprick et al., 2000). Similar to Fas, only FADD and caspase-8 were found in the TRAIL-R signaling complex in these studies. In contrast, others have reported the recruitment of TRADD and RIP to the signaling complex and showed that TRAIL-R, like TNF-RI, can activate NF-κB and JNK (Hu et al., 1999; Lin et al., 2000; Muhlenbeck et al., 1998). These discrepancies could be due to differences in cell lines or cell type-specific signaling. However, the similarity to TNF receptor signaling has led to speculation that sensitivity to TRAIL induced apoptosis may be regulated by expression of antiapoptotic factors downstream of NF-κB or by more proximal factors such as c-FLIP, which inhibits caspase-8 activation. In addition, mitochondrial factors such as SMAC/Diablo have been implicated in regulating TRAIL-R-induced apoptosis (Deng et al., 2002). Indeed, it may be a combination of different cytoplasmic factors that regulates sensitivity to TRAIL-R-induced apoptosis.

Although the ability to induce apoptosis in transformed cells is well established, the role of TRAIL and its receptor(s) in normal mammalian physiology is not understood. TRAIL has been shown to be expressed on the surface of natural killer (NK) and T cells, macrophages, and dendritic cells in an activation-dependent manner (Almasan and Ashkenazi, 2003; Halaas et al., 2000; Kayagaki et al., 1999; Sato et al., 2001); however, its function remains unclear. Cells normally resistant to TRAIL-induced apoptosis can become sensitive after viral infection (Clarke et al., 2000; Lum et al., 2001; Sato et al., 2001), and treatment of mice with neutralizing anti-TRAIL antibodies enhances their sensitivity to encephalomyocarditis virus (EMCV). TRAIL-deficient animals display increased susceptibility to tumor metastasis (Cretney et al., 2002) and autoimmune disease progression (Lamhamedi-Cherradi et al., 2003) as well as defects in negative selection, although this latter find-

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ing is controversial (Cretney et al., 2003). These data suggest that TRAIL may play roles in tumor surveillance, regulation of autoimmunity, and T cell development.

We have now generated a mouse line deficient for the TRAIL receptor. These mice appear to develop normally with an intact immune system. In contrast to one study using *TRAIL*$^{-/-}$ mice (Lamhamedi-Cherradi et al., 2003) but consistent with another report (Cretney et al., 2003) using the same line of *TRAIL*$^{-/-}$ mice, we found no defects in thymic negative selection in the absence of TRAIL-R. To assess the role of TRAIL-R in immune responses, we challenged *TRAIL-R*$^{-/-}$ mice and cells with a variety of pathogens, including *Listeria monocytogenes*, *Salmonella typhimurium*, encephalomyocarditis virus (EMCV), *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), and murine cytomegalovirus (MCMV). TRAIL-R-deficient cells were also treated ex vivo with several TLR stimuli. We found that loss of TRAIL-R does not affect responses to some challenges but led to enhanced resistance against MCMV challenge and increased cytokine production in BCG-infected macrophages. In addition, macrophages and dendritic cells lacking TRAIL-R had increased cytokine responses after ex vivo treatment with some TLR stimuli. These data suggest that TRAIL-R is an important negative regulator of the cytokine responses of the innate immune system.

Results

Generation of *TRAIL-R*$^{-/-}$ Mice

To assess the normal physiological function of the TRAIL receptor, *TRAIL-R*$^{-/-}$ mice were generated. The knockout construct replaced a portion of exon 5 (containing the transmembrane domain) and exon 6 with a neomycin gene cassette (Figure 1A). Embryonic stem cells containing the desired homologous recombination were identified by Southern blot analysis using 5′ and 3′ probes and were then used to generate TRAIL-R heterozygous mice (Figure 1B and data not shown). *TRAIL-R*$^{+/-}$ mice were crossed to C57BL/6 for several generations and subsequently intercrossed to produce homozygous animals. *TRAIL-R*$^{-/-}$ mice were born at the expected Mendelian ratios and showed no gross developmental abnormalities (data not shown). A rabbit polyclonal antibody generated against a bacterially expressed GST-TRAIL-R (Kuang et al., 2000) protein could detect TRAIL-R in transiently transfected cells, but not the endogenous protein (data not shown), presumably because of extremely low levels of endogenous TRAIL-R. Therefore, RT-PCR analysis was performed to determine if the TRAIL-R was indeed absent in *TRAIL-R*$^{-/-}$ mice. Analysis of transcripts from multiple tissues using oligonucleotides spanning the region between exons 4 and 6 showed a band in TRAIL-R wild-type, but not knockout, animals (Figure 1C). To further ensure we did not have a truncated but functional transcript, we designed primers to amplify the entire TRAIL-R transcript. RT-PCR analysis with these *TRAIL-R*-specific oligonucleotides showed a truncated transcript in all tissues examined, including heart, kidney, and thymus (Figure 1D). Cloning and sequencing of this transcript indicated that the mutation in the *TRAIL-R* locus resulted in mRNA splicing from exon 3 to exon 7, skipping exon 4 and introducing a frame shift and an early stop codon within exon 7 (Figure 1E). The resulting predicted protein contains most of the extracellular domain of the TRAIL-R but lacks a transmembrane domain or any of the cytoplasmic tail. To determine if a stable protein could be encoded by this mutant sequence, constructs containing the coding region from either the wild-type or mutant *TRAIL-R* transcripts were transfected into 293T cells. Consistent with the endogenous *TRAIL-R* transcript levels, Northern blot analysis showed lower mRNA expression of the mutant transcript in transfected cells compared to the level of the wild-type transcript in transfected cells (Figure 1F). Western blot analysis with a *TRAIL-R*-specific polyclonal antibody showed that although the wild-type construct produced TRAIL-R protein at the expected size, no protein was generated by the mutant construct (Figure 1G). These data demonstrated that our targeted allele is indeed a null mutation. To remove neomycin from the gene locus, *TRAIL-R*$^{+/-}$ mice were crossed to CMV-Cre transgenic animals (Nagy et al., 1998). No differences between animals with or without the neomycin cassette were observed, and an identical mutant transcript was produced by the neomycin-deleted locus (data not shown).

Normal Lymphocyte Populations and Negative Selection in *TRAIL-R*$^{-/-}$ Mice

Cells from the thymus, spleen, and lymph nodes of 5- to 12-week-old *TRAIL-R*$^{-/-}$ animals and their littermate controls were isolated and analyzed by flow cytometry. Loss of *TRAIL-R* did not result in any changes of the T, B, macrophage, dendritic, or natural killer cell populations (data not shown). TRAIL has been widely reported to function in T cell development and T cell autoimmunity (Lamhamedi-Cherradi et al., 2003; Song et al., 2000). *TRAIL*$^{-/-}$ animals were reported to exhibit defective negative selection by one group, and blocking TRAIL function by soluble DR5 protein was found to enhance anti-CD3 T cell proliferation (Lamhamedi-Cherradi et al., 2003; Song et al., 2000). Detailed examination of T cell development and function in *TRAIL-R*$^{-/-}$ animals revealed no significant alterations in T cell proliferation in the absence of TRAIL-R (Supplemental Figure S1A available online at http://www.immunity.com/cgi/content/full/21/6/877/DC1/). Consistent with these data and in contrast with studies using human T cells (Wendling et al., 2000), semiquantitative RT-PCR analysis of anti-CD3/CD28-treated wild-type T cells did not show any significant increase in TRAIL or TRAIL-R transcript levels after stimulation (Supplemental Figure S1B). To determine if superantigen-induced negative selection was defective in our *TRAIL-R*$^{-/-}$ animals, we backcrossed the *TRAIL-R* null allele onto the Balb/c background for four generations and examined mammary tumor virus (Mtv)-induced negative selection. Balb/c strain of mice are I-E^{d} and carry Mtv-9, which encodes an endogenous superantigen capable of deleting $V_\beta 3$-, $V_\beta 5$-, and $V_\beta 11$-expressing T cells during negative selection (Tomonari et al., 1993; Woodland et al., 1991a, 1991b). No differences were seen in the decrease of the frequencies of $V_\beta 5^+$ and $V_\beta 11^+$ after selection (between DP [$CD4^+CD8^+$] and SP [$CD4^+CD8^-$ or $CD4^-CD8^+$] cells) between *TRAIL-R*$^{-/-}$ animals and their littermate controls (Sup-

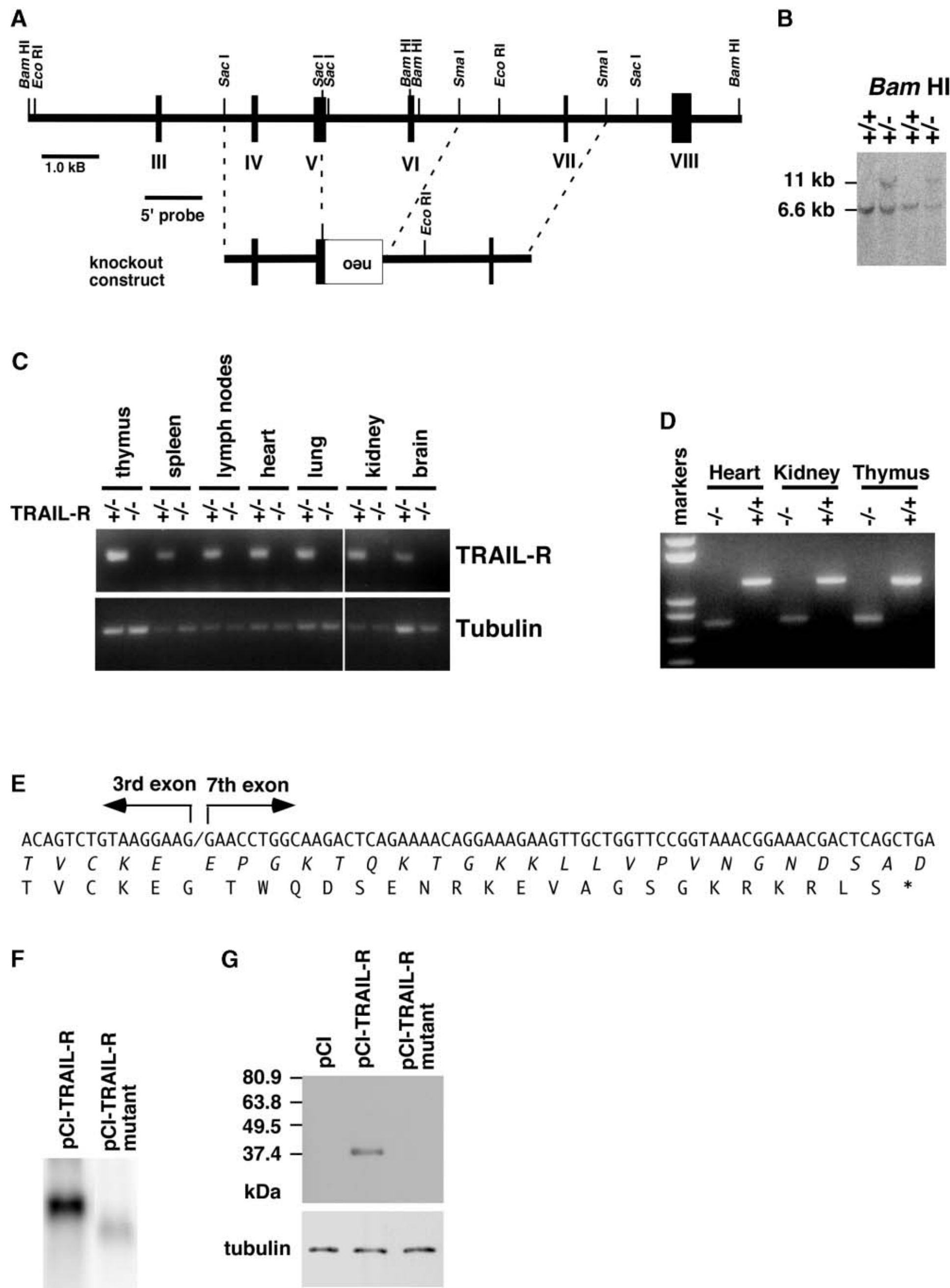

Figure 1. Targeted Disruption of the Mouse TRAIL Receptor Gene Locus

(A) A schematic map of the *TRAIL-R* genomic locus (top) and the targeting vector (bottom). The neo cassette replaces a portion of the transmembrane domain and all of exon 6. The 5′ Southern blot probe (1 kb XbaI fragment) is indicated.

(B) Confirmation of TRAIL-R locus targeting by Southern blot analysis of the transfected embryonic stem (ES) cells. Genomic DNA from ES cells was digested with BamHI, separated by electrophoresis, and hybridized with the radiolabeled 5′ probe shown in (A). Southern blot analysis detected a single 6.6 kb band for the wild-type allele and a 11 kb band for the knockout allele.

(C) PCR amplification of the thymus, spleen, lymph nodes, heart, lung, kidney, and brain TRAIL-R mRNAs from $TRAIL\text{-}R^{-/-}$ and $TRAIL\text{-}R^{+/-}$ mice was done using primers spanning exons 4 to 6 and as described in the Experimental Procedures.

(D) PCR amplification of full-length TRAIL-R transcript from heart, kidney, and thymus mRNAs from $TRAIL\text{-}R^{-/-}$ and $TRAIL\text{-}R^{+/-}$ mice was done as described in the Experimental Procedures.

(E) The TRAIL-R transcript from knockout mice was cloned and sequenced. Shown here is the abnormal junction between the 3rd and 7th exons formed in the $TRAIL\text{-}R^{-/-}$ heart mRNA, the normal TRAIL-R reading frame, and the predicted truncated protein sequence (the asterisk denotes the termination codon).

(F) Wild-type and mutant transcripts were cloned into the pCI expression vector and transfected into 293T cells. Northern blot analysis (F) showed a truncated TRAIL-R transcript from the knockout transcript construct.

(G) Western blot analysis showed normal TRAIL-R protein from the wild-type construct but no protein from the knockout construct.

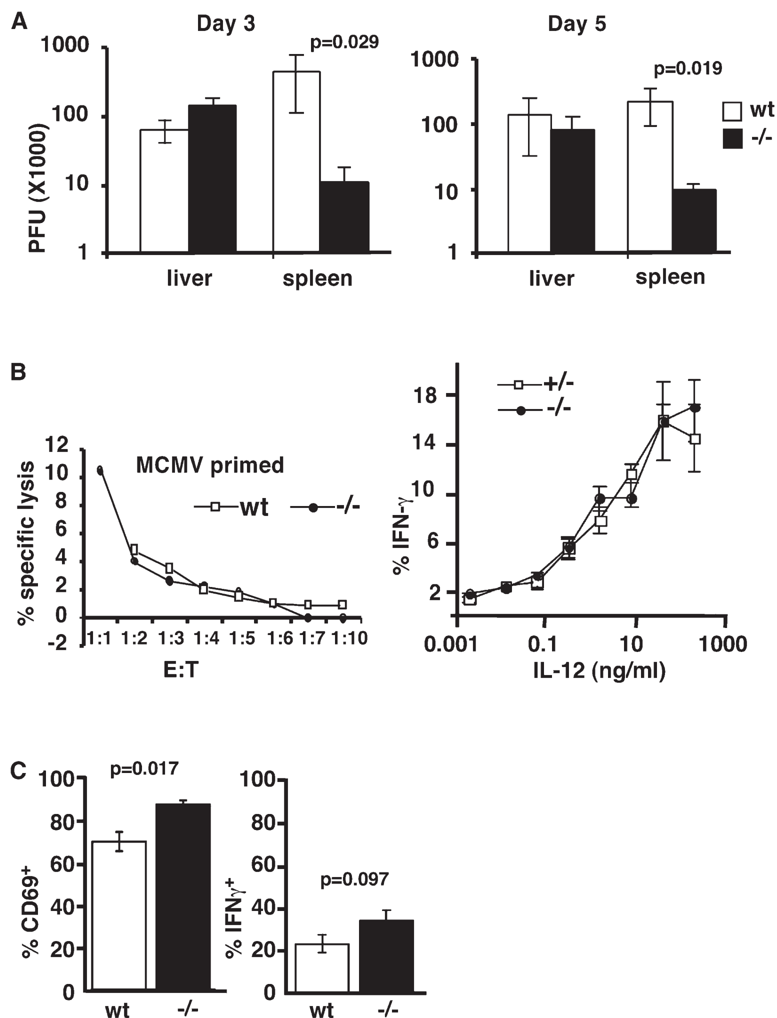

Figure 2. MCMV-Infected *TRAIL-R*$^{-/-}$ Animals Have Decreased Splenic Viral Titers

TRAIL-R$^{-/-}$ (−/−) animals and wild-type (wt) littermates were infected with 3×10^5 plaque forming units (pfu) MCMV via the intraperitoneal (i.p.) route.

(A) Viral titers in the liver and spleen were determined at days 3 and 5 post infection. pfu values are given per 100 mg organ weight. Each time point represents six animals per genotype. The experiment has been repeated three times with similar results.

(B) NK cell lytic activity and IFN-γ production is unaffected by TRAIL-R deficiency. Left: *TRAIL-R*$^{-/-}$ and wild-type littermates were injected with MCMV via the i.p. route, and NK cell lytic activity was assayed 24 hr later against ^{51}Cr labeled YAC-1 targets. The x axis corresponds to decreasing ratios of effector to target cells (E:T). Right: Splenocytes from *TRAIL-R*$^{-/-}$ and wild-type littermates were incubated overnight with increasing concentrations of IL-12 and then stained for DX5 and intracellular IFN-γ.

(C) At 24 hr after MCMV infection, the activation status as determined by CD69 expression and percent IFN-γ^+ of NK cells were determined. ($n = 4$ of each genotype, the experiment has been repeated with similar results.) p is the significance from a Student's t test (values <0.05 are considered significant).

plemental Figure S1C). These data argue against an essential role for TRAIL-R signaling in T cell development.

TRAIL-R$^{-/-}$ Mice Exhibit Enhanced Responses to MCMV

The lack of defects in naive TRAIL-R-deficient animals prompted us to examine the response of these animals to a variety of pathogen challenges. No difference in survival between *TRAIL-R*$^{-/-}$ mice and their wild-type littermate controls was observed after *Salmonella typhimurium* infection; bacterial titers in the liver and spleen after *Listeria monocytogenes* infection were also similar between *TRAIL-R*$^{-/-}$ animals and wild-type littermates (Supplemental Figures S2A and S2B). After encephalomyocarditis virus (EMCV) infection, *TRAIL-R*-deficient animals displayed a slightly enhanced survival rate compared to littermate animals. This increased survival was not observed with a 10-fold higher viral dose (Supplemental Figure S2C and data not shown).

TRAIL-R$^{-/-}$ animals and wild-type littermates in the B6 background were then infected with murine cytomegalovirus (MCMV) (Smith strain) and viral titers were determined for the lungs, spleens, and livers. TRAIL-R-deficient animals had lower viral titers in their spleens compared to control animals (Figure 2A). Differences in viral titers were observed as early as 3 days postinfection with up to a 40-fold difference between *TRAIL-R*$^{-/-}$ animals and their littermate controls (Figure 2A). Liver and lung viral titers were not significantly different between TRAIL-R-deficient and wild-type animals (Figure 2A and data not shown).

It has been shown previously that control of viral titers in the spleen is dependent on NK cell lytic activity (Tay and Welsh, 1997). Similar cell numbers of NK (and NKT) cells were present in wild-type and *TRAIL-R*$^{-/-}$ spleens after MCMV infection (data not shown). The lytic activity of NK cells lacking the TRAIL-R was examined in animals primed with an intraperitoneal injection of poly (I:C). The NK cells from *TRAIL-R* knockout animals and control animals had identical lytic activity against chromium labeled YAC-1 target cells (data not shown). Because poly (I:C) provides a very strong activating signal that might mask subtle differences, we also examined NK cells from *TRAIL-R*$^{-/-}$ and wild-type littermate animals infected with MCMV. The lytic activity of these splenic NK cells was also similar between knockout and wild-type animals 36 hr postinfection (Figure 2B, left). These data indicate that *TRAIL R*$^{-/-}$ NK cells do not have an inherently higher lytic activity than TRAIL-R wild-type NK cells. We next assayed NK cells for their ability to

produce IFN-γ. We isolated splenocytes and treated them with either IL-12 or antibodies to NK1.1 for 24 hr. NK cells were stained for DX5 and intracellular IFN-γ. No difference in IFN-γ-producing NK cells was observed between wild-type and *TRAIL-R*$^{-/-}$ mice after IL-12 and anti-NK1.1 treatment (Figure 2B, right, and data not shown). Interestingly, there was a slight increase in CD69^{+} NK cells directly ex vivo from MCMV-infected animals (Figure 2C), suggesting that although their lytic activity appeared to be normal, NK cells from *TRAIL-R*$^{-/-}$ mice were slightly more activated.

In addition to NK cells, dendritic cells are critical for the development and maintenance of the early immune response to MCMV (Dalod et al., 2003). The composition of splenic CD11c^{+}/CD8^{+}, CD11c^{+}/CD11b^{+}, and CD11c^{+}/CD11b^{+}/CD4^{+} dendritic cells (Kamath et al., 2000) after MCMV infection was unaltered by TRAIL-R deficiency (Supplemental Figure S3 and data not shown). The Perth strain of MCMV was shown recently to cause functional paralysis of dendritic cells and immune suppression (Andrews et al., 2001) as evidenced by downregulation of class II MHC, CD80, and CD40 levels on activated dendritic cells 4 days postinfection. To determine if the enhanced MCMV immunity in *TRAIL-R*$^{-/-}$ mice could be attributed to alterations in the general activation state of the dendritic cells, we examined surface markers on CD11c^{+}/CD3^{-} spleen cells at days 1, 3, 5, and 7 post-MCMV (Smith strain) infection. No difference in the activation status of dendritic cells during this period was seen between *TRAIL-R*$^{-/-}$ and wild-type littermate mice as assessed by class II MHC I-A^{b}, CD80, CD86, and CD40 expression (Supplemental Figure S3 and data not shown). Expression of activation markers increased on all subsets of dendritic cells from wild-type and knockout mice between days 0 and 1 after infection. Activation markers decreased between days 1 and 3 post infection and remained unchanged through day 7, except for a slight downregulation of CD40 between days 5 and 7 in animals of both genotype (Supplemental Figure S3 and data not shown). These data suggest that TRAIL-R loss has no effect on the expression of cell surface molecules on dendritic cells or the composition of dendritic cells during an MCMV infection.

MCMV-Infected *TRAIL-R*$^{-/-}$ Mice Exhibit Increased Cytokine Production

Cytokine secretion by a variety of cells after viral infection is critical in the establishment of an antiviral state. In the context of an MCMV infection, early IL-12 production by dendritic cells induces NK cell IFN-γ production whose levels in the serum peak 36 hr postinfection (Biron, 1999; Biron et al., 1999; Dalod et al., 2003; Orange and Biron, 1996a, 1996b). NK cells are a major producer of IFN-γ, which is necessary in the liver for MCMV immunity. Examination of serum cytokine levels at 24 hr postinfection revealed a nearly 10-fold increase in levels of the IL-12 p40 subunit in *TRAIL-R*$^{-/-}$ animals compared to wild-type littermates (Figure 3A). Furthermore, serum levels of IFN-γ at 36 hr postinfection were greatly elevated in *TRAIL-R*$^{-/-}$ animals compared to wild-type littermates (Figure 3A). To examine directly the cells that produce IL-12, CD11c^{+} dendritic cells were isolated from the spleens of MCMV-infected animals 24 hr postinfection. Cells were then cultured for 24 hr and IL-12 levels in the culture medium assayed by ELISA. Consistent with our serum IL-12 data, dendritic cells from TRAIL-R-deficient animals produced 8-fold more IL-12 than cells from control animals (Figure 3B). Therefore, enhanced cytokine production by dendritic cells likely accounts for the elevated IL-12 in the serum of knockout animals.

In addition to IL-12, IFN-α and IFN-β are induced early during viral infection and contribute to protection against MCMV (Biron, 1999; Biron et al., 1999; Dalod et al., 2003; Orange and Biron, 1996a, 1996b). Detection of serum IFN-α by ELISA resulted in values close to the limit of detection of the assay. Therefore, we assessed type I interferon levels by quantitative RT-PCR at 4 and 8 hr post-MCMV infection. We found elevated levels of IFN-α (Figure 3C) in the spleen of *TRAIL-R*$^{-/-}$ mice compared to wild-type littermates (the IFN-α level in the liver was below detection). IFN-β levels in the liver and spleen showed the same trend although the difference between TRAIL-R-deficient and wild-type mice was not as dramatic (Figure 3C). These data suggest that TRAIL-R signaling negatively regulates in vivo production of cytokines by innate immune cells. The increased levels of IL-12 by dendritic cells likely leads to a greater number of IFN-γ-secreting NK cells, resulting in the enhanced clearance of MCMV from the spleens of TRAIL-R-deficient animals.

Stimulation through Some, but Not All, TLRs Leads to Increased TRAIL Levels

To explore the mechanism of inhibition of cytokine production by TRAIL-R in vitro, we first examined the expression levels of TRAIL and TRAIL-R in stimulated innate immune cells. Other groups (Griffith et al., 1999; Halaas et al., 2000) have established that human monocytes and macrophages upregulate TRAIL after stimulation with *E. coli*-derived lipopolysaccharide (LPS). We examined TRAIL and TRAIL-R transcript levels in thioglycolate-elicited murine peritoneal macrophages after stimulation with *E. coli* LPS. In contrast to anti-CD3/CD28 stimulated T cells, mouse macrophages significantly upregulated TRAIL transcripts after activation, starting at 3 to 4 hr poststimulation (Figure 4A and Supplemental Figure S4). Exposure of these macrophages to the *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) also led to upregulation of TRAIL transcripts but with slightly delayed kinetics compared to LPS stimulation (Figure 4A). In contrast, the levels of TRAIL-R did not change significantly with either stimulation.

The Toll-like receptors (TLRs) recognize conserved motifs found on many microorganisms and represent the primary means by which macrophages, dendritic cells, and other cells of the innate immune system recognize pathogens. Since LPS and BCG are known to activate macrophages through TLR4 and 2, respectively (Heldwein et al., 2003; Poltorak et al., 1998), we examined changes in TRAIL expression in macrophages in response to other TLR stimuli (Takeda et al., 2003). By using quantitative PCR, we confirmed the kinetics of TRAIL upregulation at 4 hr post-LPS treatment (Figure 4B). Other stimuli like lipoteichoic acid (LTA, TLR2 ligand) and poly (I:C) (TLR3 ligand) also upregulated

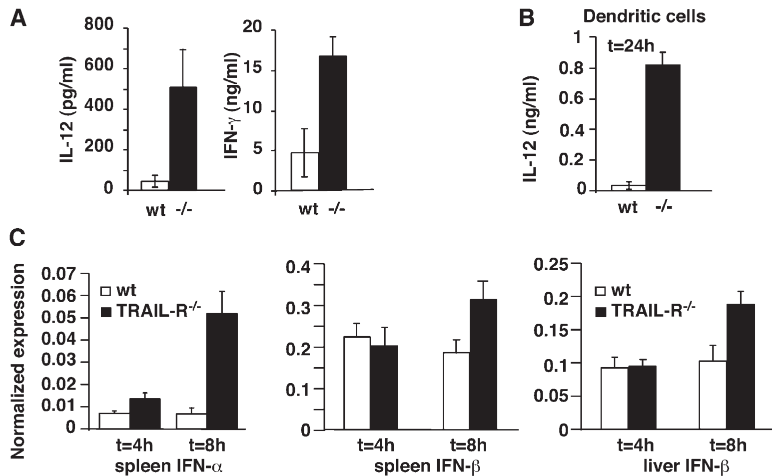

Figure 3. MCMV-Infected *TRAIL-R*$^{-/-}$ Animals Exhibit Increased Cytokine Levels

(A) Serum IL-12(p40) levels were measured by ELISA 24 hr post-MCMV infection, and IFN-γ levels were measured 36 hr post-MCMV infection.

(B) Dendritic cells from MCMV-infected *TRAIL-R*$^{-/-}$ animals display increased IL-12 production. Splenocytes from MCMV-infected animals were isolated 24 hr postinfection and sorted for CD11c expression. Cells were plated and cultured for 24 hr. IL-12(p40) in the culture medium was measured by ELISA.

(C) MCMV-infected *TRAIL-R*$^{-/-}$ animals exhibit increased levels of type I interferons in their spleens and livers. Quantitative RT-PCR (done in triplicate for each sample) was performed on RNA from the spleens and livers of *TRAIL-R*$^{-/-}$ animals and wild-type littermates 4 and 8 hr postinfection (for each genotype, n = 2 for the 4 hr time point and n = 3 for the 8 hr time point). The experiments have been repeated twice with similar results. The levels of interferon-α in the liver were too low to be detected. The interferon expression was normalized to γ-actin.

TRAIL with similar kinetics. Interestingly, TRAIL transcription after stimulation with nonmethylated CpG DNA (TLR9 ligand) was not as strong in comparison with other ligands (Figure 4B). TRAIL-R levels were not significantly changed after any of these treatments (Figure 4C and data not shown).

Enhanced Cytokine Production in *TRAIL-R*$^{-/-}$ Cells after Stimulation with TLR Ligands or BCG

We next examined the effects of TRAIL-R deficiency on the macrophage response to LPS or BCG stimulation. After exposure to LPS or BCG, macrophages secrete TNFα. We examined production of TNFα by intracellular

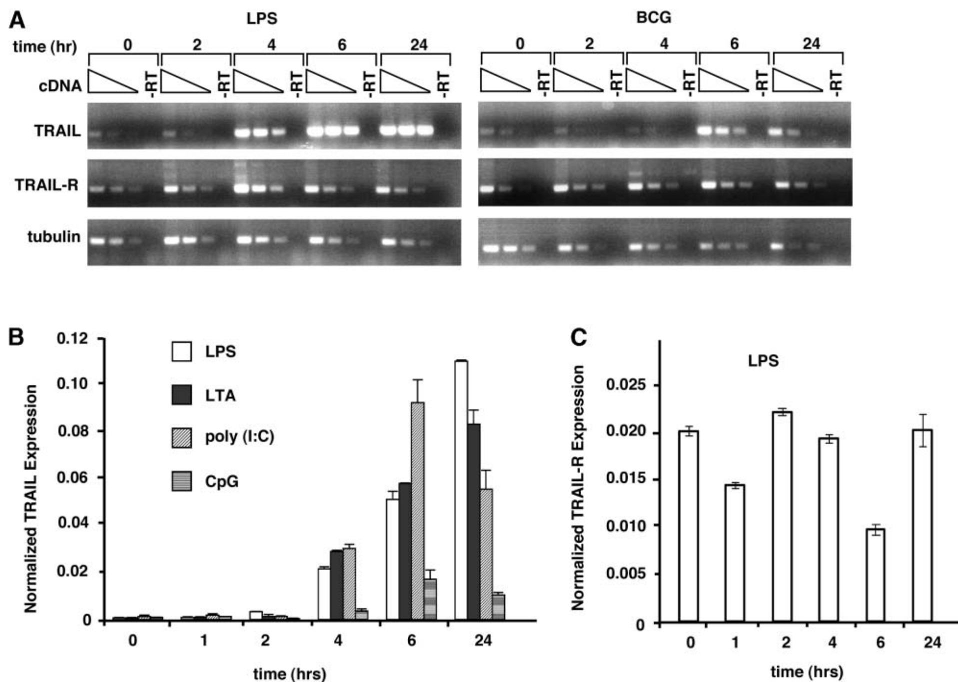

Figure 4. Macrophages Upregulate TRAIL Transcripts in Response to Stimulation with *E. coli* LPS, Bacillus Calmette-Guérin (BCG), LTA, and poly (I:C)

(A) Semiquantitative RT-PCR was used to determine the levels of TRAIL and TRAIL-R transcripts in wild-type thioglycolate elicited peritoneal macrophages after stimulation with *E. coli*-derived LPS or live BCG at the indicated time points. −RT: no reverse transcriptase.

(B) Quantitative RT-PCR analysis of TRAIL transcripts after different TLR stimuli. TRAIL is strongly upregulated by LPS, LTA, and poly(I-C), but not CpG. Normalized TRAIL Expression is the ratio of TRAIL to γ-actin transcripts.

(C) Quantitative RT-PCR analysis of TRAIL-R transcripts after LPS stimulation. TRAIL-R remains unchanged after LPS treatment. TRAIL-R expression was normalized to γ-actin transcripts.

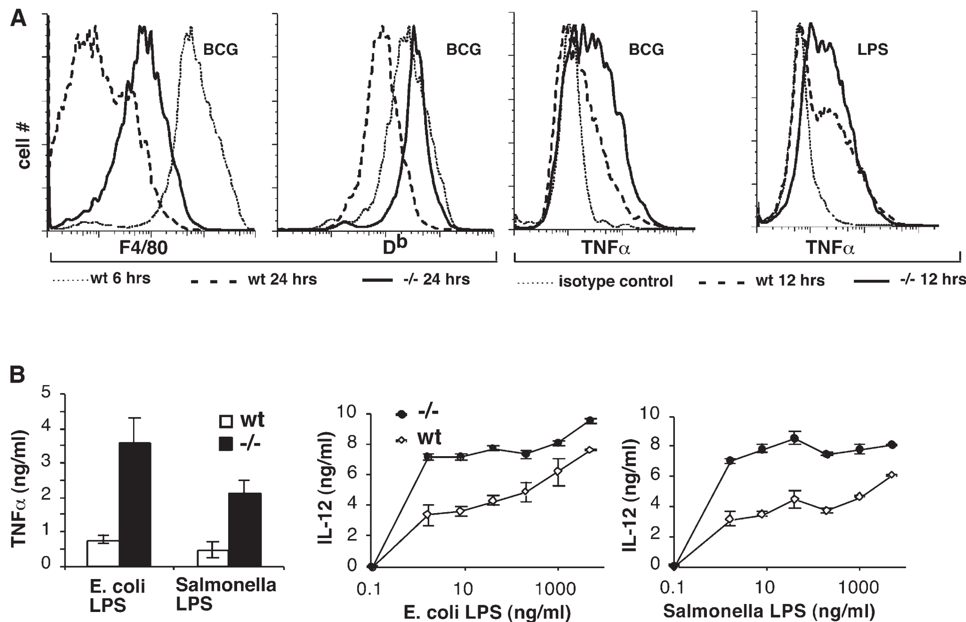

Figure 5. *TRAIL-R*$^{-/-}$ Macrophages Display Increased TNFα Production in Response to LPS and BCG and Abnormal Activation in Response to BCG Infection

(A) Thioglycolate elicited peritoneal macrophages from *TRAIL-R*$^{-/-}$ animals and wild-type littermates were stimulated with live BCG and stained for intracellular TNFα (12 hr) and surface F4/80 and D^b (24 hr). The surface marker staining profiles at 6 hr post infection are equivalent to noninfected controls and are shown here for comparison (left). Cells were also stained for intracellular TNFα after treatment with *E. coli* LPS. The experiments were done three times with similar results.

(B) Thioglycolate elicited peritoneal macrophages from *TRAIL-R*$^{-/-}$ (−/−) and +/− or +/+ (wt) littermates were treated with titrated doses of *E. coli* or *Salmonella* LPS. IL-12(p40) production was measured 24 hr posttreatment. TNFα was measured 6 hr posttreatment by ELISA. Data is representative of at least three different experiments.

cytokine staining in wild-type and TRAIL-R-deficient cells. Intracellular cytokine staining of macrophages isolated from *TRAIL-R*$^{-/-}$ animals consistently revealed higher numbers of TNFα-producing cells compared to macrophages isolated from wild-type littermates after stimulation with BCG or LPS (Figure 5A, right two panels). In addition to TNFα production, BCG, but not LPS, causes downregulation of the surface expression of the F4/80 macrophage specific antigen and class I major histocompatibility complex (MHC) (Ezekowitz and Gordon, 1982). Downregulation of F4/80 and D^b class I MHC cell surface expression was less pronounced in *TRAIL-R*$^{-/-}$ cells after BCG exposure (Figure 5A, left two panels). The function of F4/80 remains unclear, but studies using blocking antibodies suggest F4/80 enhances macrophage cytokine secretion (Warschkau and Kiderlen, 1999), and its downregulation may be a mechanism for shutting off cytokine production. Therefore, increased F4/80 expression in *TRAIL-R*$^{-/-}$ cells is consistent with increased TNFα production. The cause of D^b downregulation by BCG is also unclear but may represent an immunosuppressive effect of BCG. Taken together, these data suggest that TRAIL-R is a regulator of TNFα production as well as the overall macrophage activation state.

To examine the cytokine response to other TLR ligands (Takeda et al., 2003), macrophages isolated from *TRAIL-R*$^{-/-}$ animals and wild-type littermates were treated with LPS from *E. coli* and *Salmonella*, zymosan (a TLR2 ligand), lipoteichoic acid (LTA), nonmethylated CpG DNA, and poly (I:C). In response to both forms of LPS, *TRAIL-R*$^{-/-}$ macrophages produced greater TNFα and IL-12 than wild-type macrophages (Figure 5B). The same was true after stimulation with zymosan (Figure 6A). *TRAIL-R*$^{-/-}$ macrophages also produced more IL-12 in response to poly (I:C) and LTA (Figure 6A). Interestingly, no difference in IL-12 production was observed in response to CpG DNA (Figure 6A). This is consistent with lower levels of TRAIL upregulation by CpG DNA (see Figure 4B) and suggests that activation of the TRAIL/TRAIL-R pathway in macrophages provides negative feedback regulation of signaling through TLR2, TLR3, and TLR4, but not TLR9.

To determine if the effects of TRAIL-R deficiency on cytokine production extended to other cells of the innate immune system, we also examined the response of *TRAIL-R*$^{-/-}$ and wild-type bone marrow-derived dendritic cells to *E. coli* derived LPS. Similar to *TRAIL-R*$^{-/-}$ macrophages, dendritic cells lacking TRAIL-R had elevated levels of cytokine production (Figure 6B). Stimulation of Flt3L expanded splenic dendritic cells with LPS also revealed increased IL-12 production (data not shown). These data indicate that TRAIL-R regulation of cytokine production after TLR stimulation is important in both macrophages and dendritic cells.

To examine the effect of exogenously added TRAIL on macrophage cytokine secretion, we stimulated bone marrow and peritoneal macrophages with LPS in the

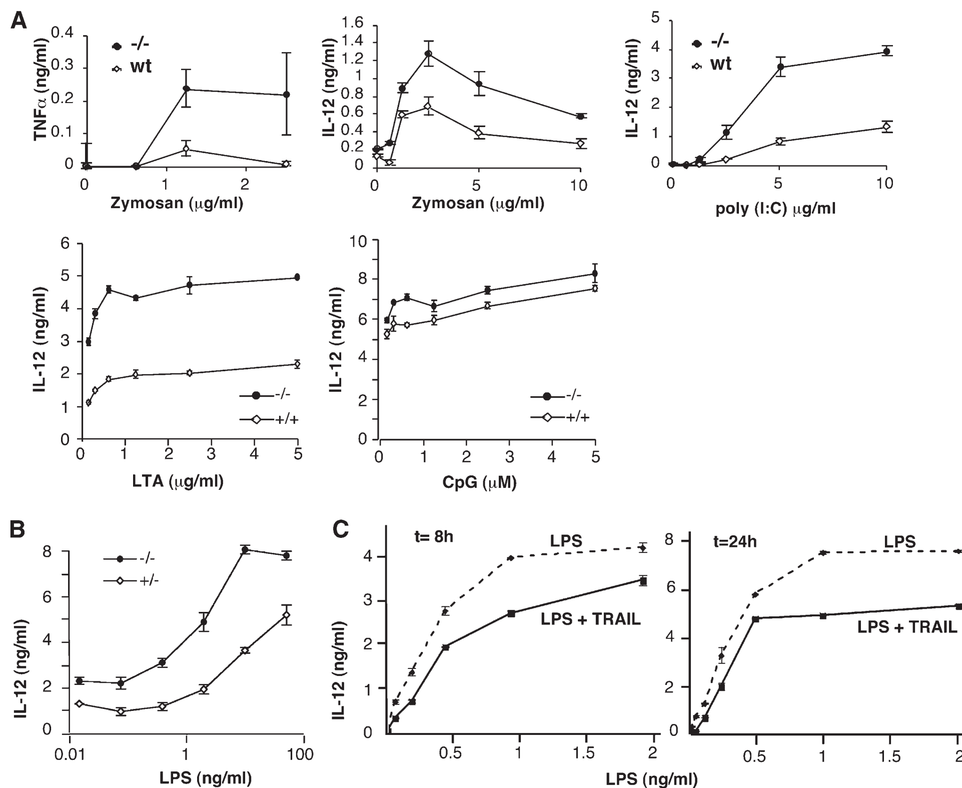

Figure 6. Enhanced Cytokine Production by *TRAIL-R*$^{-/-}$ Cells in Response to Different TLR Stimuli

(A) Thioglycolate elicited peritoneal macrophages from *TRAIL-R*$^{-/-}$ (−/−) and +/− or +/+ (wt) littermates were treated with titrated doses of various TLR specific stimulants. IL-12(p40) production was measured 24 hr posttreatment. TNFα was measured 6 hr posttreatment.

(B) Bone marrow derived dendritic cells from *TRAIL-R*$^{-/-}$ and wild-type littermate controls were treated with serial dilutions of LPS for 6 hr and IL-12 production was measured by ELISA.

(C) TRAIL-R$^{-/-}$ bone marrow-derived macrophages were retrovirally transduced with virus encoding for TRAIL-R. Cells were stimulated with increasing concentrations of LPS in the presence or absence of TRAIL (125 ng/ml).

presence or absence of TRAIL and measured IL-12 and TNFα by ELISA. We observed no difference in IL-12 secretion and only a modest decrease in TNFα production (data not shown). One possible explanation for this finding is that TRAIL-R might be present at such a low level that the amount of endogenously produced TRAIL after LPS stimulation is saturating. Thus, adding more TRAIL would not enhance the endogenous TRAIL-R signaling. To overcome this possible problem, we retrovirally infected *TRAIL-R*$^{-/-}$ bone marrow-derived macrophages with an MSCV based virus encoding the TRAIL-R cDNA. This approach is known to modestly increase the amount of TRAIL-R and accentuate its signal transduction pathway (data not shown). As predicted, IL-12 production after LPS treatment was decreased by the addition of TRAIL in the TRAIL-R reconstituted cells (Figure 6C). No apoptosis was detected in these cells (data not shown). This data further confirmed the notion that TRAIL-R signaling negatively regulates macrophage cytokine production.

TRAIL-R Regulates the Late Phase of TLR Signaling

Recognition of pathogens by Toll-like receptors leads to a rapid defensive response (Barton and Medzhitov, 2003a, 2003b). This response is mediated through activation of MAP kinases as well as NF-κB. We examined these pathways after LPS stimulation to determine if TRAIL-R deficiency affected TLR downstream signaling events. We first assessed the activation of MAP kinases by determining the phosphorylation status of ERK1/2, p38, and JNK. All of them were phosphorylated at similar levels and with similar kinetics in *TRAIL-R*$^{-/-}$ and wild-type macrophages (Figure 7A and Supplemental Figure S4). To examine NF-κB activation, we analyzed the levels of IκB-α, which sequesters NF-κB in the cytoplasm in a transcriptionally inactive complex. Activation of this pathway leads to phosphorylation and degradation of IκB-α, permitting the translocation of NF-κB to the nucleus and activation of its transcriptional targets. Homeostatic regulation of NF-κB occurs through the synthesis of new IκB-α, which sequesters NF-κB back into the cytosol (Hoffmann et al., 2002). Western blot analysis of IκB-α showed that macrophages from *TRAIL-R*$^{-/-}$ animals and wild-type littermates displayed similar kinetics of IκB-α phosphorylation and degradation immediately after LPS stimulation, indicating that TRAIL-R deficiency did not affect the initial activation of NF-κB (Figure 7A). However, at later time points (4 and 8 hr post stimulation), when IκB-α protein was reexpressed in the wild-type cells, it could not be detected in *TRAIL-R*$^{-/-}$ cells (Figure 7A). These data are consistent

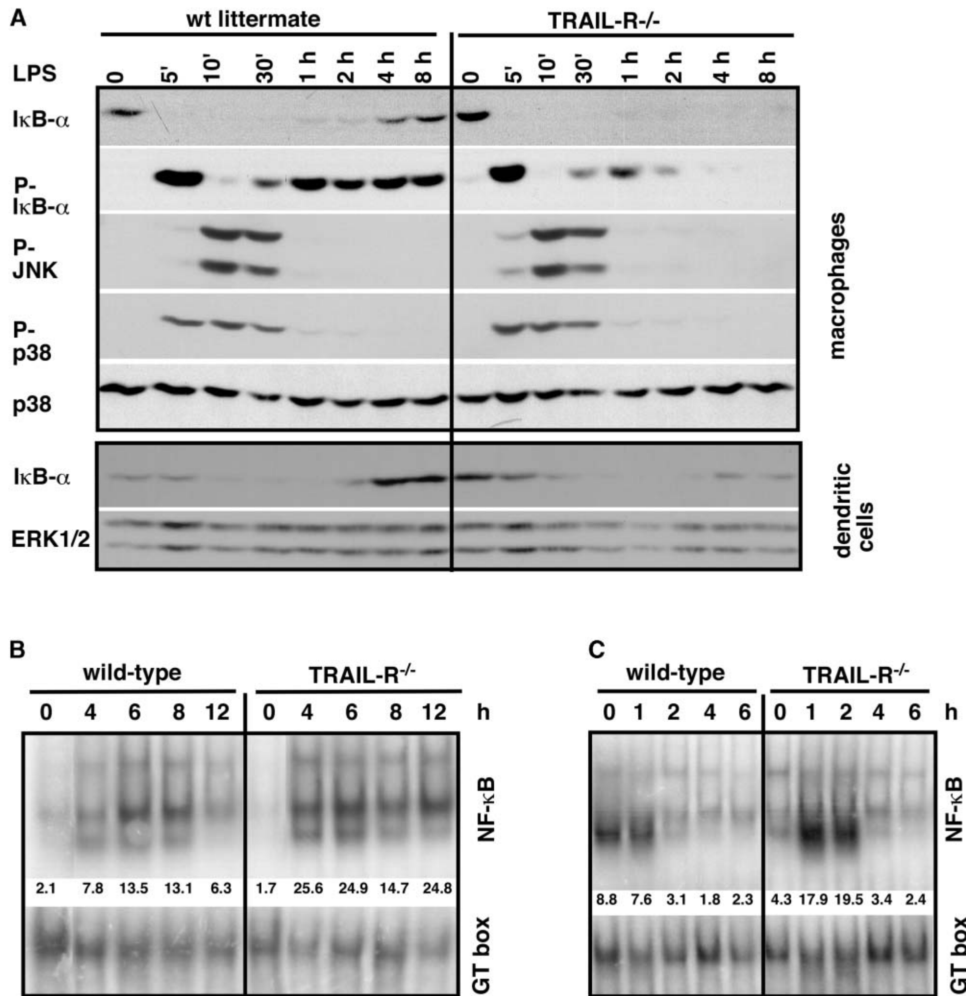

Figure 7. Biochemical Analysis of LPS-Stimulated Macrophages and Dendritic Cells

(A) Bone marrow-derived macrophages (top) and dendritic cells (bottom) from *TRAIL-R*$^{-/-}$ and wild-type littermate controls were treated with 1 μg/ml LPS (macrophages) or 100 ng/ml LPS (dendritic cells) for the indicated time points. Cell lysates were used for Western blot analysis with antibodies specific for total (IκB-α) or phosphorylated IκB-α (P-IκB-α), phosphorylated JNK (P-JNK), or phosphorylated p38 (P-p38). As a loading control, antibodies for total p38 (macrophage) or total ERK1/2 (DC) were used. The experiments have been done more than three times with similar results.

(B) Bone marrow-derived macrophages from *TRAIL-R*$^{-/-}$ and wild-type littermate controls were treated with 50ng/ml LPS and nuclear extracts were generated and analyzed by EMSA for NFκB activity as described in the materials and methods. EMSA for the GT box was performed as a loading control.

(C) LPS pulse experiments. Bone marrow macrophages from *TRAIL-R*$^{-/-}$ and wild-type littermate controls were treated for 1 hr with LPS, washed four times with PBS and incubated for the indicated times with media without LPS. Nuclear extracts were generated and analyzed by EMSA for NF-κB activity. The experiments in (B) and (C) have been done at least twice with similar results. The numbers below the NF-κB gel shift represent phosphoimager values ($\times 10^6$) of the NF-κB binding activities after normalization to the corresponding GT box values.

with the delayed time course of TRAIL upregulation (4 hr post LPS stimulation, see above) and suggest that TRAIL-R primarily impacts late signaling events downstream of Toll-like receptors. Similar findings were found in bone marrow-derived dendritic cells from *TRAIL-R*$^{-/-}$ and wild-type littermates treated with LPS (Figure 7A). No difference in IκB-α protein levels was observed between wild-type and knockout macrophages after TNFα stimulation (Supplemental Figure S4), indicating that the loss of IκB-α is specific to TLR stimuli. Quantitative PCR analysis of IκB-α transcript levels in macrophages at different time points after LPS stimulation showed similar expression levels in wild-type and *TRAIL-R*$^{-/-}$ cells (data not shown). Thus, TRAIL-R signaling most likely regulates IκB-α degradation or stability, but not the transcription of IκB-α after NF-κB activation.

To assess the differences in NF-κB transcriptional activity in TRAIL-R wild-type and knockout macrophages, we performed gel shift assays by using the NF-κB consensus sequence. As a control, the same nuclear extracts were incubated with GT box oligonucleotides, which detect members of the ubiquitously expressed Sp1 transcription factors (Kingsley and Winoto, 1992). Bone marrow macrophages were stimulated with LPS and nuclear extracts were made. Early NF-κB activities were similar between *TRAIL-R*$^{+/-}$ and *TRAIL-R*$^{-/-}$ mac-

rophages (data not shown). At later time points a consistent, albeit modest increase in DNA binding by NF-κB was observed in *TRAIL-R*$^{-/-}$ macrophages (Figure 7B). Others have previously reported that NF-κB binding activities in continuously TNF-α treated IκB-α-deficient cells were barely discernable but the increase was evident in cells treated with a brief pulse of stimulus (Hoffmann et al., 2002). We performed a similar experiment and stimulated wild-type and *TRAIL-R*$^{-/-}$ macrophages for 1 hr with LPS. The cells were washed and assayed at various time points afterward. Quantitative RT-PCR experiment showed that TRAIL is upregulated 2 hr after washing (Supplemental Figure S4). As shown in Figure 7C, *TRAIL-R*$^{-/-}$ macrophages exhibited a more dramatic increase of NF-κB binding activities, especially at 2 hr post wash.

Discussion

The vast majority of studies of TRAIL-R function have examined its abilities to induce apoptosis preferentially in transformed cells. More recently, studies have examined the expression of TRAIL and its receptors after immune cell activating stimuli as well as the role of TRAIL-R in autoimmunity and defense against viral infections. Studies with TRAIL-deficient mice have also suggested a role for TRAIL-R signaling in thymic negative selection, although this conclusion is controversial (Cretney et al., 2003; Lamhamedi-Cherradi et al., 2003). Our data now demonstrate the importance of TRAIL-R signaling in the initial cytokine responses of the immune system to pathogen challenge.

Similar to *TRAIL*$^{-/-}$ mice, examination of unchallenged *TRAIL-R*$^{-/-}$ mice did not reveal any abnormalities in their resting immune cell populations, indicating that TRAIL-R does not play an essential, nonredundant role in the development of the immune system. In addition, lymphocyte homeostasis appears unperturbed, even in aged animals. It is interesting to note that unlike mutations in the related family member Fas, TRAIL-R deficiency does not lead to lymphoproliferative conditions or the development of an abnormal B220^{+}/Thy1^{+} T cell population. It has been shown previously that loss of TNF receptor in the *lpr* background can enhance the lymphoproliferative phenotype even though *TNF-RI*$^{-/-}$ animals display normal peripheral deletion (Zhou et al., 1996). It remains to be seen whether compound deficiency in Fas and TRAIL-R would have any synergistic effects on lymphocyte homeostasis.

Examination of the lymphocyte populations from TRAIL-R-deficient animals has revealed some differences with previous studies by using either TRAIL-deficient animals or TRAIL-blocking antibodies. It has been reported that TRAIL deficiency leads to defects in negative selection, including defective endogenous superantigen deletion of certain thymocyte subsets (Lamhamedi-Cherradi et al., 2003). However, our *TRAIL-R*$^{-/-}$ animals displayed no such alterations when compared to wild-type littermates using the same model of endogenous superantigen deletion. This is consistent with a recent study by Cretney et al. (2003) reporting normal thymic negative selection in TRAIL deficient animals. Others have reported that TRAIL signaling is important in negatively regulating T cell proliferation with consequences for the development of autoimmune diseases (Hilliard et al., 2001; Song et al., 2000). We have been unable to show significant alterations in T cell proliferation after the addition of exogenous recombinant TRAIL (data not shown). Furthermore, TRAIL-R loss in T cells does not significantly affect proliferation following TCR/CD28 ligation.

The normal lymphocyte homeostasis observed in our TRAIL-R-deficient animals led us to examine TRAIL-R function in the context of an immune response to several bacterial and viral pathogens. Although TRAIL-R loss had no effect on *S. typhimurium* infection, it did have a slight protective effect in the context of EMCV infection and significantly enhanced clearance of MCMV from the spleen. The mechanism of protection from EMCV is unclear but it likely does not involve virally induced apoptosis of infected cells or viral reproduction and spread to the heart, one of the main target organs (data not shown). In response to MCMV infection, *TRAIL-R*$^{-/-}$ animals had enhanced antiviral responses, which led to lower viral titers in the spleens of knockout animals. No increase in lytic activity was found in the *TRAIL-R*$^{-/-}$ NK cells, although slightly more *TRAIL-R*$^{-/-}$ splenic NK cells expressed the CD69 activation marker. Analysis of cytokine production in knockout animals revealed elevated levels of IL-12 and IFN-γ in the serum compared to control animals. In addition, knockout animals displayed higher levels of type I interferons in the spleen at early time points after MCMV challenge. We also examined splenic dendritic cells, which have been identified as the major producer of IL-12 during an MCMV infection (Dalod et al., 2002) and are important for the initiation of NK cell antiviral responses. While surface markers for dendritic cell activation were similar between *TRAIL-R*$^{-/-}$ and wild-type littermates during the course of an MCMV infection, dendritic cells derived from MCMV-infected *TRAIL-R*$^{-/-}$ animals showed elevated production of IL-12 compared to dendritic cells from wild-type littermates. This increased IL-12 could result in increased NK cell lytic activities. Although we did not see an obvious difference in NK activities and activation in vitro, others have shown that in vivo NK cell lytic activities are hard to measure in vitro (Dokun et al., 2001). In these studies, the activities and proliferation state of NK cells between the resistant Ly49H^{+} strain (e.g., C57Bl/6) and the sensitive Ly49H^{-} strain (e.g. Balb/c) were comparable although MCMV titers were already severely attenuated at day 2 of infection in Ly49H^{+} mice. Thus, the decrease of MCMV spleen titer in *TRAIL-R*$^{-/-}$ mice could be due to increased NK lytic activities in vivo although type I interferons might also contribute to the accelerated clearance.

While infection of *TRAIL-R*$^{-/-}$ mice with *Listeria monocytogenes* bacteria did not reveal differences in organ bacterial load, others, using *TRAIL*$^{-/-}$ mice in the Balb/c background, observed faster clearance and increased survival after *Listeria* challenge (Zheng et al., 2004). The difference between these studies may be attributable to the differences in challenge dose as well as differences in the resistant C57/BL6 background versus the more susceptible Balb/c background. However, their results are consistent with our in vitro macrophage data and in vivo MCMV data and support a role for the TRAIL/

TRAIL-R pathway in the negative regulation of innate immunity.

Our results with TRAIL-R-deficient animals differ significantly from other studies that examined the function of TRAIL-R signaling in T cell proliferation (Song et al., 2000) and EMCV immunity (Hilliard et al., 2001) with soluble anti-TRAIL reagents. The ability of membrane bound TRAIL to transduce signals may be one explanation for this discrepancy. Chou et al. (2001) have shown that crosslinking TRAIL by using either plate bound TRAIL-R or a soluble TRAIL-R can lead to increased T cell proliferation in the presence of suboptimal TCR simulation. Signaling through membrane bound TRAIL can lead to increased IFN-γ production in a p38-dependant manner (Chou et al., 2001). These cellular responses to TRAIL- (and not TRAIL-R-) mediated signaling may lead to the phenotypes associated with experiments using TRAIL blocking agents. In particular, it may explain the difference between the results of our EMCV infection of TRAIL-R knockout animals compared to other studies using anti-TRAIL antibodies. It is interesting to speculate that in certain situations, the TRAIL decoy receptors could act as "ligands" to signal through the membrane bound TRAIL "receptor."

Macrophages and dendritic cells are the primary cells, which mediate innate immune responses to pathogens and assist in the shaping of the adaptive response. Stimulation of macrophages and dendritic cells derived from *TRAIL-R*$^{-/-}$ animals demonstrates that TRAIL-R signaling negatively regulates cytokine secretion in both cell types. In the case of macrophages treated with LPS and other TLR stimuli and dendritic cells isolated after an MCMV infection, TRAIL-R deficiency does not appear to affect other aspects of cellular activation (as assessed by surface activation markers) and suggests that TRAIL-R signaling is primarily repressive for cytokine production. However, BCG challenge of macrophages does reveal differences in surface activation markers between *TRAIL-R*$^{-/-}$ and wild-type macrophages. Therefore, it is possible that TRAIL-R signaling may have different effects after different types of pathogen challenges. It is also interesting to note that TRAIL upregulation after TLR stimulation does not extend across all TLR stimulants, nor does TRAIL-R loss affect cytokine production after signaling through all TLRs. This highlights the differences in signaling by different TLRs and suggests that TRAIL-R signaling may be important in the context of specific types of pathogen challenge.

Toll-like receptor ligation results in the activation of MAP kinase pathways as well as the transcription factor NF-κB. Examination of these signaling pathways in TRAIL-R-deficient macrophages after LPS stimulation indicates that the initial IκB-α degradation and activation of MAP kinases JNK, p38, and ERK1/2 are normal. Our observation that TRAIL is not upregulated until 3 to 4 hr after LPS treatment suggests that TRAIL-R primarily impacts the later events after TLR signaling. Consistent with this notion, IκB-α homeostatic regulation after TLR signaling is defective in *TRAIL-R*$^{-/-}$ cells. Lack of IκB-α feedback inhibition and the subsequent NF-κB activity (Hoffmann et al., 2002) likely explains the elevated cytokine production in TRAIL-R-deficient cells. IκB-α transcripts are not affected by the loss of TRAIL-R; thus TRAIL-R signaling most likely stabilizes IκB-α protein to prevent it from degradation. The molecular mechanism by which TRAIL-R signaling stabilizes IκB-α is not clear at present. However, in some cells, FADD and caspase-8 are recruited to the TRAIL-R signaling complex upon ligand stimulation, leading to caspase activation and apoptosis (Kischkel et al., 2000; Kuang et al., 2000; Sprick et al., 2000). In macrophages and dendritic cells, caspase-8 activation does not lead to apoptosis but may result in cleavage of signaling molecules downstream from TLRs. For example, RIP1 is a mediator for IκB phosphorylation and degradation and is a known target of caspase-8 (Lin et al., 1999). More recently, RIP1 has been shown to be an essential signaling protein of NF-κB activation after TLR3 stimulation (Barton and Medzhitov, 2004; Meylan et al., 2004). Thus, TRAIL-R may stabilize IκB and inhibit TLR signaling by inducing cleavage of proteins such as RIP1.

In summary, the data presented here define a role for TRAIL-R signaling independent of its ability to initiate apoptosis in sensitive cells. By using mice deficient for TRAIL-R, we have shown that TRAIL-R signaling contributes to the negative regulation of cytokine production in macrophages and dendritic cells in the context of both ex vivo and in vivo challenges. Furthermore, we showed that the TRAIL/TRAIL-R pathway constitutes a negative feedback loop for TLR2, TLR3, and TLR4, but not TLR9, signaling. Further studies will be necessary to elucidate the exact molecular pathway by which TRAIL-R affects cellular responses to TLR signaling.

Experimental Procedures

Detailed Experimental Procedures are available online as Supplemental Data at http://www.immunity.com/cgi/content/full/21/6/877/DC1/.

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