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CD137-Deficient Mice Have Reduced NK/NKT Cell Numbers and Function, Are Resistant to Lipopolysaccharide-Induced Shock Syndromes, and Have Lower IL-4 Responses

Dass S. Vinay,* Beom K. Choi,† Jun S. Bae,*† Won Y. Kim,*† Bryan M. Gebhardt,* and Byoung S. Kwon2*†

CD137, a member of the TNF superfamily, is involved in T cell and NK cell activation and cytokine production. To establish its in vivo role in systems dependent on NK and NKT cells, we studied the response of CD137−/− mice to LPS-induced shock, tumor killing, and their IL-4-controlled Th2 responses. In both high and low dose shock models, all the CD137-deficient mice, but none of the wild-type BALB/c mice, survived. After injection of LPS/2-amino-2-deoxy-D-galactose (D-gal), CD137−/− mice had reduced serum cytokine levels and substantially impaired liver IFN-γ and TNF-α mRNA levels. Phenotypic analysis of mononuclear cells revealed fewer NK and NKT cells in the CD137−/− mice. The knockout mice did not generate a rapid IL-4 response after systemic T cell activation, or effective Ag-specific Th2 responses. In addition, both in vitro and in vivo NK-specific cytolytic activities were reduced. These findings suggest that CD137-directed NK/NKT cells play an important role in the inflammatory response leading to the production of proinflammatory cytokines, LPS-induced septic shock, and tumor killing, as well as IL-4-dependent Th2 responses. The Journal of Immunology, 2004, 173: 4218–4229.

CD137, a 30-kDa member of the TNF receptor superfamily, exists as both a monomer and a 55-kDa dimer on a major subset of mouse peripheral T lymphocytes, dendritic cells (DCs), and NK cells (1, 2). The expression of CD137 is activation dependent in that CD137 mRNA levels rise a few hours after activation; once it is expressed, CD137 binds CD137 ligand (CD137L), a high affinity ligand present on a variety of APCs (1). In an exception to this, a recent study reported that CD137 is constitutively expressed on CD4+CD25+ regulatory T cells (3). The basis of this constitutive expression and its consequences are not known. The biology of CD137-restricted T cell activation and its role in various clinical conditions are, however, well documented (1, 4, 5). CD137 transduces activation signals, eradicates established tumors, and prevents activation-induced cell death (1, 4–6). Growing evidence also suggests that signals relayed through the CD137 receptor amplify CD8 responses (7). CD137-mediated activation is distinct from and independent of CD28 and is known to involve members of the TNFR-associated factor family leading to activation of various kinases, such as apoptosis signal-regulating kinase, MAPK kinase, MAPK3/MAPK4, p38, and JNK/stress-activated protein kinase, eventually to culminate in the activation and nuclear translocation of several transcription factors, namely ATF-2, Jun, and NF-κB (8).

Although the role of the CD137/CD137L pathway in T cell immunity is well characterized and has been confirmed using CD137 and CD137L gene knockout mice (9, 10), there have been few studies relating CD137 to NK and NKT cell function. The few available reports suggest that CD137 is a signal-transducing molecule in NK cells (11, 12). Thus, Ab cross-linking of NK cell CD137 stimulates secretion of IFN-γ, cellular proliferation, CD25 expression, and responsiveness to IL-2 and is associated with tumor activity, but not cytotoxicity (11, 12). Although the above studies have defined in vitro roles of CD137 in NK cell activation, the nature of CD137-directed NK and NKT cell function in vivo remains largely unclear.

NK cells are large, bone marrow-derived, granular lymphocytes that lyse tumor cells and virally infected cells, bypassing MHC restriction and prior sensitization (13); they are believed to direct the rejection of mismatched bone marrow allografts in lethally irradiated mice (14). There is also evidence that they are not merely cytotoxic effectors, but also regulate the immune response to bacterial (15), protozoan (16), and viral (17) infection. This immunoregulatory capacity is believed to depend in part on the ability to secrete various cytokines, including CSF, IFN-γ, IL-1, IL-2, IL-18, and TNF-α (18–23). NK cells stimulate Ig secretion both in vivo (24) and in vitro (25), possibly by releasing IFN-γ (26), which is a potent stimulator of Ig secretion (27) and inducer of isotype switching to IgG2a (28). Unlike the receptor genes of B and T cells, those of NK cells do not undergo recombination to create a large repertoire of specificities. NK cells exist as two major populations: NKT and NK cells. NKT cells are a unique T cell subset that expresses NK cell markers such as NK1.1 (29). They also express Var14-Jo281 gene segments (29) and are abundant in the liver (30). Upon stimulation, they promptly

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3 Abbreviations used in this paper: DC, dendritic cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATF-2, activating transcription factor-2; CD137L, CD137 ligand; D-gal, 2-amino-2-deoxy-D-galactose; KLH, keyhole limpet hemocyanin; MNC, mononuclear cell.
secrete large amounts of type 1 and 2 cytokines, namely IFN-γ and IL-4 (31).

To probe the in vivo roles of CD137 in the murine immune system, we have generated CD137-deficient mice by homologous recombination (10). We showed previously that these animals have normal T and B cell numbers, but produce less Ag-specific IFN-γ in vitro, and have diminished Ag-driven CD8+ CTL activity (10). In this study, we examined their sensitivity to lethal, LPS-induced, septic shock. We also examined the ability of these mice to mount Th2 responses in systems dependent on IL-4 and their cytotoxic potential to clear tumors. We found that the CD137-deficient mice were completely resistant to death in both low and high dose LPS shock models; they also had impaired NK-restricted cytolytic activity and were defective in generating Ag-specific IL-4 responses.

Materials and Methods

Animals

Homozygous CD137-deficient (CD137<sup>−/−</sup>) mice (10) were backcrossed for at least 12 generations to wild-type (CD137<sup>+/+</sup>) BALB/c or C57BL/6 backgrounds. The latter were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the Louisiana State University Health Sciences Center specific pathogen-free animal facility. All experiments were performed using strain-, age-, and sex-matched mice. Animal experimentation protocols were approved by the Louisiana State University Health Sciences Center institutional animal care and use committee.

Induction of septic shock

For low dose shock, LPS from *Escherichia coli* (serum type 0111:B4; Sigma-Aldrich, St. Louis, MO) was administered i.p. with LPS (1 µg/mouse) and TH2 responses in systems dependent on IL-4 and their cytotoxic potential to clear tumors. We found that the CD137-deficient mice were completely resistant to death in both low and high dose LPS shock models; they also had impaired NK-restricted cytolytic activity and were defective in generating Ag-specific IL-4 responses.

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Cell isolation and flow cytometry

Liver lymphocytes were prepared as previously described (32) and splenic and thymic lymphocytes were obtained by conventional methods. Phenotypic analysis of lymphocytes (1 × 10<sup>6</sup> cells in 100 µL) was performed at 4°C after an initial blocking step with 1 µL of unlabeled anti-FcYr Ab (eBioscience, San Diego, CA). The mAbs used included NK 1.1, NK2D, NK22A<sup>BD</sup>, CD3, CD1d, DX5, TLR2, and TLR4 (eBioscience). The mAbs used included NK 1.1, NKG2D, NKG2AB6, CD3, CD1d, DX5, TLR2, and TLR4 (eBioscience). For high dose LPS shock, mice were injected i.p. with 25 mg/kg LPS alone. Mortality was recorded for 72 and 168 h, respectively.

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Serum cytokine ELISA

CD137<sup>+/+</sup> and CD137<sup>−/−</sup> mice were injected i.p. with LPS (1 µg/D-gal (8 mg) in saline. Blood was collected at the indicated times, and clarified sera were stored at ~80°C. IFN-γ and TNF-α in sera were determined by ELISA (BD Pharmingen, San Diego, CA).

Serum transaminase

Aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) were assayed in serum samples using a commercial kit (Sigma-Aldrich) adapted to small sample volumes (33).

Analysis of IFN-γ expression in NK and NKT cells

To study the effect of LPS on IFN-γ induction in NK cell subsets, liver mononuclear cells (MNCs) were prepared from wild-type and knockout mice after LPS/D-gal challenge. In some cases, DX5<sup>+</sup> and DX5<sup>−</sup> cells were purified by incubation with biotin-labeled DX5 mAb (eBioscience), followed by streptavidin microbeads. Bound cells were isolated using MACS columns (Miltenyi Biotec, Auburn, CA). Unbound cells were saved and used as a source of DX5<sup>+</sup> cells. Purification of cell subsets was 85–90%, as determined by FACS analysis. The cells were incubated in vitro in complete medium (RPMI 1640 supplemented with 10% FBS, antibiotics, sodium pyruvate, t-glutamine, nonessential amino acids, and 2-ME) containing monensin (3 µM) before staining with ap- propriate fluoresein-labeled Abs. Where indicated, mice were challenged i.p. with LPS alone (25 mg/kg), liver MNCs were obtained 40 h later, and intracellular cytokine levels were measured as described.

Cytolytic responses

In vitro cytolytic activity was assessed against YAC-1 (NK-susceptible target) by a standard 51Cr release assay (16). Briefly, 1 × 10<sup>4</sup> 51Cr-labeled target cells and serial dilutions of effector cells (total spleen cells) that had been stimulated in vitro for 20 h by i.p. injection of 200 µg of poly(I:C) (Sigma-Aldrich) were incubated for 4 h (35). In some cases, targets (YAC-1) were incubated with purified liver DX5<sup>+</sup> cells. Supernatants were harvested and counted with a gamma counter. The percent specific lysis was calculated as: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100. For in vivo tumor killing assay, mice were injected s.c. in the right flank with 1 × 10<sup>6</sup> IL-4 induction in NK cell subsets, liver mononuclear cells (MNCs) were prepared from wild-type and knockout mice after LPS/D-gal challenge. In some cases, DX5<sup>+</sup> and DX5<sup>−</sup> cells were purified by incubation with biotin-labeled DX5 mAb (eBioscience), followed by streptavidin microbeads. Bound cells were isolated using MACS columns (Miltenyi Biotec, Auburn, CA). Unbound cells were saved and used as a source of DX5<sup>+</sup> cells. Purification of cell subsets was 85–90%, as determined by FACS analysis. The cells were incubated in vitro in complete medium (RPMI 1640 supplemented with 10% FBS, antibiotics, sodium pyruvate, t-glutamine, nonessential amino acids, and 2-ME) containing monensin (3 µM) before staining with ap- propriate fluoresein-labeled Abs. Where indicated, mice were challenged i.p. with LPS alone (25 mg/kg), liver MNCs were obtained 40 h later, and intracellular cytokine levels were measured as described.

In vivo NK cell depletion and blockade of NKT-APC interaction

Female wild-type BALB/c mice were treated i.v. (50 µl/mouse) with anti-asialo-GM1 Ab (WAKO, Richmond, VA; final volume raised to 500 µl with saline) to eliminate NK cells. To block NKT-APC interaction, mice were treated i.p. (50 µg/mouse) with anti-CD1d mAb (clone 1B1; eBio- science; final volume raised to 500 µl with saline). Both injections were administered 24 h before LPS challenge.
of individual GAPDH and IL-4 mRNA band intensities was performed using ImageJ (version 1.32j; National Institutes of Health, Bethesda, MD).

**Serum IgE induction by anti-IgD treatment**

Mice were injected i.v. with 50 μl of polyclonal goat anti-mouse IgD anti-serum (γG mAb) diluted to a final volume of 500 μl with PBS. The mice were bled on days 0, 7, and 8 post-treatment, and serum IgE levels were evaluated by ELISA using a commercial kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s instructions.

**Ag-specific responses to keyhole limpet hemocyanin (KLH)**

Mice were immunized in each hind footpad with 75 μg of KLH (Sigma-Aldrich) in CFA (37). Popliteal lymph nodes were removed after 9 days, and single-cell suspensions were prepared. Aliquots of CFSE (5 μM)-labeled cells (2 × 10⁵/ml) were cultured in the presence or the absence of 30 μg/ml KLH. After 96 h, the cultures were washed and rested in medium for an additional 48 h without added KLH to synchronize the cells in G0. After restimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h, intracellular cytokine analysis was performed as described above.

**Ets-1 immunoblotting**

Extracts of 4 × 10⁶ cells were immunoprecipitated with protein A/G (Santa Cruz Biotechnology, Santa Cruz, CA) together with 5 μg of Ets-1-specific rabbit polyclonal antiserum C-20 (Santa Cruz Biotechnology) and analyzed by immunoblotting with 2 μg/ml of the same combination. Immune complexes were detected with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

**Statistical analysis**

Student’s t test was used to determine the significance of cytokine and enzyme data, and the χ² test was used to obtain p values for the mouse survival data.

**Results**

**CD137-deficient mice are resistant to LPS-induced shock**

To investigate whether CD137-deficient mice resist LPS-induced lethal shock, we used two shock models (38, 39). In the LPS/D-gal acute toxic hepatitis model, D-gal depletes hepatocyte uridine triphosphate and blocks RNA synthesis. This increases by 100,000-fold the sensitivity of the mice to the lethal effects of LPS (38); the mice develop severe fulminant hepatitis within 8 h, and 70–90% die in <24 h (38). We found that none of the control BALB/c animals (n = 10) survived beyond 12 h after i.p. injection of low dose LPS (1 μg)/D-gal (8 mg) (Fig. 1a), in agreement with previous data (38). In contrast, no deaths were observed among the CD137-deficient mice (n = 10) up to 72 h after injection (p < 0.001).

The high dose LPS shock model is characterized by massive infiltration of leukocytes and increased serum concentrations that lead to death (39). To determine the effect of high dose treatment, CD137⁻/⁻ mice (n = 6) and BALB/c controls (n = 6) were injected i.p. with LPS, and survival was assessed daily for 7 days (Fig. 1b). Most control mice succumbed to shock between days 3 and 4 of injection, and none survived to day 7. In contrast, all CD137⁻/⁻ animals were still alive on day 7 (p < 0.001). Saline-injected CD137⁻/⁻ (n = 6) and control mice (n = 6) remained healthy throughout the 7-day study (not shown). To further assess the specificity of the immune responses, littermate BALB/c mice, rather than commercially procured mice, were tested and proved to be as sensitive to LPS shock as the commercially procured mice (data not shown).

**Lower cytokine expression and fewer histopathologic lesions in CD137-deficient mice during LPS shock**

Death from LPS/D-gal-induced shock results from elevated cytokine concentrations that cause hepatic failure (38). To understand why the knockout mice are unresponsive to LPS shock, we compared cytokine gene expression in liver and spleen tissues from BALB/c and CD137⁻/⁻ mice by RT-PCR in response to low dose LPS/D-gal. We observed marked increases in several cytokine mRNAs in the livers of BALB/c mice treated with LPS/D-gal for 6 h (Fig. 2a) and much smaller increases in the livers of CD137⁻/⁻ mice (Fig. 2a). Interestingly, cytokine mRNAs levels were much more affected in the livers of knockout mice than in their spleens (Fig. 2a). To address the possibility that the reduced IFN-γ production in the liver is caused by the absence of IFN-γ-inducing factors, we measured IL-12 (p35 and p40) mRNA levels by RT-PCR. There was no significant induction of p35 or p40 IL-12 mRNA at 6 h in the CD137⁻/⁻ mice, whereas increased levels of transcripts were seen in the controls (Fig. 2a).

Studies using IFN-γR-deficient (40) and TNFR type I (TNFRI)-deficient (41) mice have demonstrated the importance of IFN-γ and TNF-α in inducing hepatitis and death in low dose LPS shock (40). We therefore measured serum TNF-α and IFN-γ levels after injection of LPS/D-gal (Fig. 2b). There was a sharp increase in serum TNF-α levels in BALB/c animals 2 h after LPS/D-gal treatment, and these returned to baseline 3 h after treatment (Fig. 2b). In contrast, in CD137-deficient mice there was only a slight rise in TNF-α secretion 1 h after LPS/D-gal treatment, and this rapidly fell back to baseline. Peak TNF-α serum levels in the control animals were 5-fold higher than those in CD137-deficient animals (p < 0.001). Serum IFN-γ levels in the controls peaked 3 h after injection of LPS plus D-gal (Fig. 2c). Importantly, serum IFN-γ remained completely unchanged in CD137-deficient mice throughout the entire 8-h period (p < 0.001). Thus, disruption of the CD137 gene resulted in dramatic inhibition of serum TNF-α and IFN-γ production in the low dose LPS shock model.

Lethal shock in LPS/D-gal-sensitized mice is characterized by fulminant hepatitis (42), and the ensuing hepatic toxicity is a classic manifestation of septic shock (22). To assess the role of CD137 in the regulation of LPS-induced septic shock, we next performed a histopathologic analysis of the livers of treated animals. The
livers of wild-type mice injected with LPS/D-gal showed severe signs of fulminant hepatitis, with disruption of tissue architecture, massive hemorrhage, and hepatocyte death, thus corroborating the observed mortality rates (Fig. 2d). In contrast, the livers of CD137-deficient mice showed only slight signs of nonspecific hepatotoxicity, similar to mice given no injection (Fig. 2d).

Apoptosis is rare in normal livers (two to four apoptotic cells per 10,000 cells) and reflects cell turnover (43). Necrosis and apoptosis are critical pathologic features of liver injury and are seminal features of viral hepatitis. Ischemia and necrosis, when massive, result in fulminant hepatic failure (43). In line with our histopathologic data, in situ TUNEL assays revealed that liver sections of the CD137-deficient mice had markedly fewer apoptotic cells compared with the CD137+/+ mice (Fig. 2e). Additionally, liver injury assessed by 6-h serum transaminase levels revealed ~60% less liver-specific AST and ALT (p < 0.001) in the sera of CD137-deficient mice than in the controls (Fig. 2f). Thus, septic shock-induced hepatic toxicity leading to tissue apoptosis and tissue damage appears to depend on CD137. The LPS/D-gal unresponsiveness of the knockout mice clearly underscores the fact that CD137-CD137L interactions are crucial for LPS-induced shock and the accompanying mortality.

**Reduced IFN-γ production by NK and NKT cells in CD137−/− mice during LPS shock**

The above data suggested that the reduced IFN-γ production in the mutant mice was responsible for their resistance to lethal shock. NK and NKT cells are known to be key effectors of LPS shock and to be responsible for the increase in IFN-γ production (32, 44, 45). To examine the role of the CD137 pathway in the functioning of NK and NKT cell subsets and to closely replicate the LPS system used in Figs. 1 and 2, we injected mice with LPS/D-gal and IFN-γ expression was measured in the purified NK subsets. As shown in Fig. 3, LPS/D-gal treatment of the knockout mice caused a nearly 3-fold decrease in IFN-γ levels (Fig. 3) in NK and NKT cells, suggesting that the NK- and NKT-CD137 pathway plays a critical role in the septic shock response induced by LPS.

**CD137-deficient mice have reduced numbers of NK and NKT cells**

NK subsets are major producers of IFN-γ and key effectors of lethal shock syndromes, as has been confirmed using NK-deficient mice (32, 44, 45). We therefore looked for a correlation between the observed resistance of CD137−/− mice to LPS shock and their

**FIGURE 2.** Cytokine mRNA levels, histopathologic lesions, serum cytokines, and liver enzymes are decreased in CD137-deficient mice during LPS/D-gal-induced septic shock. a, Total RNA was extracted from livers and spleens of wild-type BALB/c (+/+ ) and CD137-deficient (−/−) mice before and 6 h after injection of LPS (1 μg)/D-gal (8 mg). The mRNA was detected by RT-PCR. Data are from one of three independent experiments. b, Serum TNF-α concentrations after injection of wild-type BALB/c (+/+ ) and CD137-deficient (−/−) mice with LPS (1 μg)/D-gal (8 mg). c, Serum IFN-γ concentrations in the same experiment. Serum cytokine levels were measured by ELISA in one mouse per time point. The data are from one representative experiment of three performed. d, H&E staining of liver paraffin sections before and 6 h after LPS/D-gal challenge. Three mice were studied at each time point and in each group. Sections shown are representative of all tissue samples analyzed (original magnification, ×100). e, TUNEL assay on deparaffinized liver biopsies performed before and 6 h after LPS (1 μg)/D-gal (8 mg) treatment. Note the abundant apoptotic cells, represented by dark brown spots, in livers from wild-type BALB/c mice. Three mice were studied at each time point and in each group. The sections shown are representative of all tissue samples analyzed (original magnification, ×100). f, In vivo LPS-D-gal-induced liver damage in wild-type and CD137-deficient mice. Mice were given LPS (1 μg)/D-gal (8 mg), and blood was taken immediately and after 6 h to measure serum AST and ALT concentrations. AST and ALT concentrations in 0 h groups were <20 U/ml. Results are expressed as means for each of three independent experiments (n = 5 for each experiment). Statistical significance was determined for each time point by combining data from the three experiments. p < 0.001 comparing wild-type BALB/c mice with CD137-deficient mice.
In vivo (LPS/D-gal)  

FIGURE 3. Reduced IFN-γ production by CD137-deficient NK-cell subsets during LPS shock. Wild-type and knockout mice were challenged i.p. with LPS (1 μg)/D-gal (8 mg), and liver MNCs were prepared 1 h after treatment. Liver MNCs were fractionated into DX5⁺ and DX5⁻ subsets using MACS columns. Cells were incubated in the presence of monensin for 5 h and subsequently stained with FITC-conjugated anti-CD3 and PE-conjugated anti-IFN-γ. Saline-injected controls showed a background of <5% IFN-γ-producing cells. The numbers in the contour plots represent the percentages of IFN-γ-expressing cells in the indicated cell populations. Representative results from four to six mice per group are shown.

FIGURE 4. CD137-deficient mice have reduced percentages and absolute numbers of spleen and liver NK (DX5⁺/CD3⁻) and NKT (DX5⁻/CD3⁻) cells, but normal numbers of CD1¹ and TLR2⁺/⁺ cells. a, Flow cytometric analysis of isolated liver and spleen MNCs from BALB/c and CD137-deficient mice was performed as described in Materials and Methods. A minimum of 1 × 10⁶ lymphocyte-gated events were analyzed per sample. a, Percentages (inset) and absolute numbers of DX5⁺/CD3⁻ NKT cells, DX5⁻/CD3⁻ NK cells, and DX5⁻/CD3⁺ T cells from liver samples. b, The percentage of positive MNCs per spleen was determined (inset) and used to calculate the absolute numbers of the indicated cell types. Data in the insets of a and b are representative of at least seven independent experiments with three to five mice pooled per group. +, p < 0.001 comparing wild-type BALB/c with CD137-deficient mice. c, Staining with discrete NK cell markers confirmed reduced NK/NKT cell populations in the livers of CD137⁻/⁻ mice. Liver MNCs from naive female CD137⁻/⁻ mice (C57BL/6) were obtained, and flow cytometry was performed using the indicated NK cell markers. The numbers in the contour plots represent the percentage of positive events. Results from one of two independent experiments with four to six mice per group are shown. d, Vα14-Jα281 mRNA in livers of CD137-deficient and wild-type mice. Input cDNAs were equalized by PCR amplification of several dilutions of each sample using primers for GAPDH until comparable levels of GAPDH amplification products were obtained in the diluted samples. The adjusted cDNAs were then used as templates for amplification of Cα, Vα8-Cα, and Vα14-Jα281, and the PCR products were run on 2% agarose gels. Duplicate experiments gave similar results. e, Ets-1 expression in the livers of CD137-deficient mice. Lysates of various organs (4 × 10⁶ cells/organ) were immunoprecipitated with polyclonal rabbit anti-Ets-1 Ab and fractionated on 12% SDS-PAGE. The 55-kDa Ets-1 protein was detected with anti-Ets-1 Ab by chemiluminescence after transfer to nitrocellulose paper. A representative of three independent experiments is shown. f, Percentages of CD1⁺ cells in wild-type BALB/c and CD137-deficient mice. g, Protection against LPS shock in CD137-deficient mice does not involve reduction of TLR expression. Mice were treated with LPS (1 μg)/D-gal (8 mg). Liver MNCs were prepared 6 h later and analyzed. The bold line in each panel represents staining obtained after cells were stained with the matched isotype control, and the thin lines represent the shift obtained with anti-TLR Ab. The numbers in each panel give the percentage of positive events for the Ab used. Three independent experiments with three to five mice per group yielded identical results.
examined the expression of Vα14-Jα281 (46) mRNA in several organs of CD137−/− and control mice (Fig. 4d). As expected, Vα14 messages were greatly reduced in the livers of the CD137−/− mice, indicating that CD137 may be required for the development and/or expansion of the invariant Vα14+ subset of NKT cells. Vα4-Jα281 expression was relatively normal in the thymus, probably because of the abundance of nonproductive gene rearrangements and unselected TcRs in this organ. The residual Vα14-Jα281 mRNA may derive from cells whose TcRs were selected on other MHC molecules or that contain two rearranged TCR chains. The decrease in Vα14 mRNA in CD137−/− mice is specific, because the expression of Vβ8, a Vα-chain not associated with NKT cells, was not reduced (Fig. 4d). Although many lymphoid-specific genes contain important Ets binding sites (47), studies using Ets-1 knockout mice suggest that Ets-1 is required for the development of mature NK cells (35). The expression of Ets-1 was also reduced in the CD137−/− mice (Fig. 4e), confirming the existence of NK/NKT cell defects in knockout mice.

Because of the resistance of the mutants to LPS, we suspected that expression of the nonpolymorphic class 1B molecule, CD1, might be altered, in that NKT cells recognize glycolipid Ags exhibited by CD1 (29, 30). The expression of CD1, however, was comparable in mutant and wild-type mice (Fig. 4f). Because LPS binds TLR-4 (48), we investigated the possibility that the LPS resistance of the knockout cells is direct and is not the result of altered expression of TLR-4. Analysis of TLR-2 and TLR-4 expression before and after activation with LPS (under the same conditions as those shown in Fig. 1a) revealed no striking differences between wild-type and mutant mice (Fig. 4g). Thus, the observed resistance to LPS in the knockout cells is due to neither reduced TLR2/4 expression nor reduced CD1 levels, but appears to be a result of the lower number of NK and NKT cells and the lack of CD137/CD137L interactions in the remainder of the immune competent cells.

Contribution of NK/NKT cells to LPS shock

Although we showed that resistance to LPS shock in CD137−/− mice is a result of the reduced numbers of NK/NKT cells and/or the lack of CD137-CD137L interaction, it was not clear whether these cells played a role in endotoxic shock. To explore this possibility, we studied the direct role of NK/NKT cells in LPS shock. In view of the early mortality of wild-type mice caused by LPS shock, it was not feasible to study NK or NKT cell number expansion in this model. Thus, we used a high dose LPS model to determine whether NK or NKT cell numbers are affected. We first examined the expansion of NK/NKT numbers in wild-type and knockout mice to determine whether there is a gain/loss over the basal levels during LPS shock. We found that after LPS (25 mg/kg) treatment, wild-type mice exhibited a nearly 2- to 3-fold increase in NK cells, but not NKT cells (Fig. 5a). LPS-challenged CD137−/− mice showed a similar fold increase, but the gross numbers did not reach the levels seen in the wild-type mice. These

FIGURE 5. Contribution of NK/NKT cells to LPS shock. a. Wild-type and CD137−/− mice (BALB/c) were treated with LPS (25 mg/kg) as described in Fig. 1b. Liver MNCs were purified 40 h after treatment, and NK/NKT cell were enumerated by flow cytometry. b. Depletion of NK cells and/or blockade of NKT-APC interaction confer resistance to LPS shock in wild-type mice. NK cell depletion and blockade of NKT-APC interaction were achieved by anti-asialo-GM1 and anti-CD1 Ab treatments, respectively, as described in Materials and Methods. Twenty-four hours after Ab treatment, mice were challenged i.p. with LPS (1 μg/mouse)/D-gal (8 mg/mouse). Control mice received saline by the same route. Mortality was assessed over the following 72 h. c and d. Mice were treated as described in b. Blood was collected every hour by cardiac puncture, and serum TNF-α (c) and IFN-γ (d) levels were analyzed by ELISA. Each time point represents pooled samples from two mice. e–g. Mice were treated as described in b and were killed 1 h after LPS/D-gal challenge. Total liver MNCs were obtained. An aliquot of cells was used to confirm NK cell elimination by flow cytometry (e), and the remainder of the cells were used for enumeration of intracellular TNF-α (f) and IFN-γ (g), as described in Materials and Methods. The numbers in the contour plots indicate the percentage of positive cytokine-expressing events. Two independent experiments yielded a similar trend.
data suggest that NK cell subsets play an important role during LPS shock. Others have shown that mice with depletion of discrete NK cell subsets and NK-deficient mice exhibit differential levels of resistance to LPS shock (32, 44, 45, 49). To confirm a direct role for NK and NKT cells in the LPS shock model, we used wild-type BALB/c mice that were depleted of NK cells, had their NKT-APC interaction blocked, or both during the low dose LPS shock model. NK cell-depleted mice showed no signs of associated shock symptoms and remained viable during the entire 72-h duration of the experiment (Fig. 5b). Also, mice treated with anti-CD1 mAb to block NKT-APC interactions did not show overt signs of LPS shock, as evidenced by the fact that 90% of the mice were alive at the end of 72 h. Similarly, mice that were both depleted of NK cells and had their NKT-APC interactions blocked also remained healthy and free of shock symptoms (100% viability). These data corroborate earlier findings (32, 44, 45, 49) that NK/NKT cells directly participate in the pathway leading to septic shock.

Because IFN-γ and TNF-α were shown to be up-regulated in LPS/D-gal-treated wild-type mice (see Fig. 2, a–c), we determined whether reduced mortality in anti-asialo-GM1- and anti-CD1 Ab-treated mice was also associated with alterations in the expression levels of these cytokines concomitant with blunt NK/NKT responses. The results indicate that NK-depleting and/or NKT-APC-blocking Ab-treated mice showed insignificant elevation of TNF-α (Fig. 5c) and IFN-γ (Fig. 5d). The effectiveness of depletion by anti-asialo-GM1 Ab was evaluated by flow cytometry 1 h after LPS/D-gal challenge (Fig. 5e). To further test whether reduced serum TNF-α and IFN-γ correlate with cytokine levels at the cellular level, we obtained liver MNCs 1 h after LPS/D-gal challenge and analyzed them for total cytokine content. As with the alterations in serum cytokines, intracellular TNF-α (Fig. 5f) and IFN-γ (Fig. 5g) were also significantly reduced in anti-asialo-GM1- and anti-CD1-Ab-treated, LPS/D-gal-challenged mice. Taken together, these data indicate that NK/NKT cells, IFN-γ, and TNF-α play a pivotal role in endotoxic shock and explain in part the basis for the LPS resistance in CD137−/− mice.

Blockade of the CD137/CD137L pathway by anti-CD137L mAb reverses LPS-induced septic shock in wild-type mice

The resistance of CD137−/− mice to LPS shock suggested a key role for this Ag in endotoxic shock syndromes. We therefore performed costimulatory blockade experiments with anti-CD137L mAb in wild-type mice. Using the high dose LPS model, we injected BALB/c mice (n = 3) with 25 mg/kg LPS and injected anti-CD137L mAb. Blockade of CD137/CD137L interaction in wild-type mice dramatically increased the level of resistance, as shown by 100% viability over the 7-day experimental period (Fig. 6a). Mice that did not receive anti-CD137L mAb succumbed to LPS shock within 72 h. Complete protection against LPS shock could, however, only be achieved when the blocking mAb was administered 20 h before LPS challenge (Fig. 6a, compare LPS/anti-CD137L vs LPS/anti-CD137L+M). The effectiveness of anti-CD137L mAb was evaluated in CD137−/− mice; the results showed that the Ab had no physiologic effect of its own, but merely functioned as a blocking agent (Fig. 6b).

These data raise the question of whether the protection against LPS shock intrinsic to CD137−/− mice and that conferred by anti-CD137L mAb treatment are similar. To test this, we first evaluated the numbers of cells in NK cell subsets in mice treated with anti-CD137L mAb and challenged with LPS. We chose a 40 h end point in this experiment in view of the increased mortality by 48 h seen in the mice treated with LPS alone. Treatment with anti-CD137L mAb alone or together with LPS did not reduce the proportions of NK and NKT cells (Fig. 6c). Interestingly, LPS stimulation alone led to increased NK (DX5+/CD3−), but decreased liver NKT cell (DX5+/CD3+) numbers. This observation agrees with an earlier report that NK, rather than NKT, cells orchestrate LPS shock (32). We next determined whether treatment with anti-CD137L mAb alters IFN-γ expression during LPS shock. As anticipated from the results in Figs. 2a and 3, anti-CD137L mAb treatment caused a nearly 2.5-fold decrease in IFN-γ in both purified NK and NKT cells after LPS challenge (Fig. 6d). Taken together, these data suggest that the protection against LPS mediated by anti-CD137L mAb is the result of reduced IFN-γ production, as is the case in CD137-deficient mice, but does not involve reduction in NK cell subset numbers.

Role of CD137 in IL-4 responses

Most naive peripheral CD4+ cells cannot secrete IL-4 upon initial activation. However, there exists a population of NK1+ (NKT) thymocytes that produce copious quantities of IL-4 when stimulated by anti-CD3 (50). To determine whether CD137 is crucial for this effect, thymocytes isolated from knockout and control mice were cultured with anti-CD3. Thymocytes from control cells produced large amounts of IL-4, whereas the mutant thymocytes produced negligible IL-4 (Fig. 7a). In normal mice, in vivo administration of anti-CD3 mAb rapidly induces the production of abundant IL-4 from NKT cells (51). To determine whether CD137 is required for IL-4 production in this situation, we injected anti-CD3 into wild-type and knockout mice and compared IL-4 expression by RT-PCR and serum ELISA 90 min after injection of the anti-CD3 mAb. The wild-type mice had high levels of serum IL-4 (Fig. 7b) and IL-4 mRNA (Fig. 7c, upper and lower panels); levels in the mutants were about half those in wild-type mice. In view of the reduced IL-4 expression after systemic TCR stimulation of the mutants, we analyzed their thymuses to determine whether the numbers of NK cell were reduced. Similar to the livers and spleens, the thymuses of the CD137−/− mice had reduced percentages of NK and NKT cells (p < 0.001; Fig. 7d).

Most NKT cells secrete IL-4 rapidly upon ligation of the TCR (51), suggesting that these cells participate in priming Th2 responses. To determine whether IL-4 produced by CD137-stimulated NKT cells is required for Th2 differentiation in vivo, we examined Ag-induced immune responses in CD137−/− mice using two regimens that elicit strong Th2 responses (52, 53). First, we examined the effect of CD137 deficiency on IgE class switching in response to polyclonal stimulation with Gemö, which is dependent on IL-4 (52). Reduced amounts of IgE were induced in CD137−/− mice 7 and 8 days after injection of Gemö (Fig. 7e), indicating that the CD137-dependent NKT cells are an important source of IL-4 under these conditions. There was no difference in basal serum IgE between the knockout and control mice. Therefore, the rapid production of IL-4 by CD137+ NKT cells appears to be needed to generate Th2-type responses in this model.

We next assessed the requirement for CD137 for generating an IL-4-secreting recall response. CD137−/− and control mice were immunized with the particulate Ag, KLH, in adjuvant and restimulated in vitro in the presence or the absence of KLH. This strategy generally results in accumulation of increased quantities of IL-4 and marginal levels of IFN-γ (53). We found significantly lower IL-4 and IFN-γ levels in CD137−/− mice compared with wild-type controls (Fig. 7f), although the Ag-specific proliferative response was comparable in the two groups. In vitro stimulation of nonimmunized mice by KLH did not result in appreciable elevation of these cytokines (Fig. 7f), suggesting the Ag specificity of the results. Thus, the deficiency in CD137 affects both the production of IL-4 in response to Ag and the capacity for IgE switching.
Therefore, as proposed, rapid production of IL-4 by CD137-stimulated NKT cells appears to be essential for Th2-type responses.

**NK-specific cytolytic activity is reduced in CD137−/− mice**

We next examined the cytolytic activity of NK cells. Unlike wild-type mice, CD137−/− mice were unable to lyse NK-susceptible YAC-1 tumor cells in vitro when total spleen cells were used as effectors (Fig. 8a), but not when purified DX5+ cells were used (Fig. 8b), probably as a result of proportionate E:T cell ratios in the latter case, as opposed to the situation with the unfraccionated spleen cells, where E:T cell ratios were not balanced. Others have shown that CD137 signaling does not affect the cytotoxic potential of NK cells (11, 12). Therefore, the results in Fig. 8a may be interpreted as reduced NK cell numbers in CD137−/− mice causing an imbalance in the E:T cell ratio, thereby affecting cytolytic activity, rather than as a direct role for this Ag in cytotoxicity. The eradication of many class I MHC-deficient tumors is correlated with intact NK cell function in vivo. To determine whether the in vitro defects in NK cell function in CD137−/− mice were reflected in abnormal in vivo NK cell function, we injected mutants and wild-type mice s.c. with 1 × 10^7 RMA-S cells (from an NK cell-dependent class I MHC-deficient tumor) and followed tumor progression for 40 days (Fig. 8c). None of the wild-type mice developed detectable tumors over the course of the experiment, whereas all CD137−/− animals developed s.c. tumors (≥1 cm) between 10 and 26 days after injection (Fig. 8d). These data support the view that the knockout mice have decreased NK cell function and numbers and, therefore, show reduced NK activity.

**Discussion**

We have identified a critical role for the CD137 costimulatory receptor in the regulation of NK and NKT cell function. In addition, we have shown for the first time that CD137-deficient mice, regardless of whether they are sensitized with D-gal, are completely resistant to the lethal effects of LPS, fail to mount normal NK-restricted cytolytic responses, and have defective IL-4 responses. Blocking the CD137-CD137L interaction with Abs to CD137L abolished the sensitivity to LPS shock of wild-type mice. The defect in CD137−/− mice appears to stem from their inability to maintain optimal NK and NKT cell numbers as well as the absence of CD137-CD137L interactions in the residual immune-competent cells and a postreceptor fault in the production of proinflammatory cytokines.
Proinflammatory cytokines are thought to interfere both directly and indirectly with the functions of several tissues and cells, leading to multiorgan failure and eventual death in the LPS models (40–42). The resistance of CD137-deficient mice is associated with reduced levels of TNF-α/R9251 and IFN-γ/R9253 in the serum and of cytokine mRNAs in the liver. IFN-γ/R9253 is considered one of the main agents of LPS-mediated lethality (40), and additional cytokines, such as TNF-α/R9251, IL-12, and IL-6, are believed to add to the pathogenicity (54). This view is supported by previous findings in knockout mice deficient in IFN-γ/R9253R, TNF-α/R9251R, and TNF-α/R9251R, none of which survived either low or high doses of LPS (39). In this regard, the CD137-deficient mice are unique among these

![Graphs and images](image-url)
knockout strains in their resistance to both low and high dose LPS shock, which indicates that the CD137 pathway is critical in both low and high dose LPS shock models. Optimal inflammatory IFN-γ production depends mainly on IL-12 and IL-18 via a positive feedback mechanism (55). Our current data indicate that the reduced levels of IFN-γ in the livers of CD137-deficient mice during LPS challenge are the result of reduced availability of IL-12 (p35 and p40). This view is supported by an experiment in which spleen cells from CD137-deficient mice were cultured in vitro in the presence of graded levels of rIL-12 and rIL-18, or the mutants were injected with rIL-12 (500 ng i.p. for 24 h); the results showed no significant differences in IFN-γ expression compared with wild-type controls (data not shown).

We examined the relationship between the observed resistance to LPS and NK and NKT cell numbers in the CD137-deficient mice. Recent studies have shown that the murine liver contains a unique population of αβ T cells that are positive for TCR NK Ag expression (32), and it has been suggested that these liver T cells are autoreactive (56), major sources of IFN-γ, and key agents in the causation of lethal shock syndromes (32, 45). Our current results suggest that NK cells are a major source of IFN-γ in LPS-induced shock, which is consistent with findings showing a central role for NK cells in IFN-γ production in endotoxemia (45, 49). Although IFN-γ producers were detected among NKT cells in wild-type mice, such cytokine-producing activity was markedly higher in NK cells than in NKT cells. Furthermore, we found that either depletion of NK cells or blockade of NKT-APC interactions reversed LPS sensitivity and significantly curtailed up-regulation of proinflammatory cytokines. Taken together, these findings point to a major role for NK cell subsets and, more importantly, NK cells, because the numbers of NK cells markedly exceed the numbers of NKT cells, and IFN-γ-producing activities are profoundly higher in NK cells than in NKT cells after LPS treatment. We found a marked reduction in the percentage and absolute number of DX5+/CD3− cells in the livers, spleens, and thymuses of naive CD137-deficient mice, and the fact that these mice also have lower Vα14-Jα281 mRNA (29) and Ets-1 (35) expression supports our contention that they are defective in NK and NKT cells. Additionally, staining of liver MNCs with several NK markers confirmed NK/NKT cell defects in the mutants. Taken together, these data strongly suggest that the CD137 molecule plays a critical role, among other things, in the development, function, and/or regulation of liver NK and NKT cells. The decreased numbers of these cells in the livers of CD137-deficient mice lead to a sufficient reduction in cytokine production to protect the mice from both high and low dose LPS-induced shock. Thus, we interpret these results as indicating that CD137-controlled NK and NKT cells make a major contribution to the endotoxic shock elicited by LPS. In addition, this defect in NK cell numbers impairs the ability to mount a cytolytic response to NK-sensitive tumors when total spleen cells were used as effectors. However, when purified NK (DX5+) cells were incubated with targets, cytolytic activities were comparable, suggesting that the disproportionate numbers of NK (DX5+) cells in knockout mice, compared with wild-type mice, were responsible for the reduced cytolytic potential seen.

Our data raised the question of whether the observed resistance of CD137-deficient mice is merely the result of suboptimal NK and NKT cell numbers or whether the CD137 pathway has a direct role in the pathogenesis of lethal shock. To answer this question, we performed costimulatory blockade experiments in wild-type
mice and showed that CD137-CD137L blockade by Abs to CD137L completely suppressed the lethality and up-regulation of proinflammatory cytokines, strongly suggesting that signaling through the CD137 receptor is critical for LPS shock and that the reduced numbers of NK and NKT cells in the knockout mice are simply an added factor. Nevertheless, complete protection of wild-type mice was only achieved when the anti-CD137L mAb was administered before LPS treatment, perhaps because the lethal effects of LPS are rapid and overwhelming. In addition, these experiments permit the interesting inference that administration of anti-CD137L mAb does not exert any substantial effect of its own in the LPS shock model, in view of the 100% survival of the CD137-deficient mice during the entire experiment. To confirm that NK/NKT cells participate in immune responses leading to LPS shock and to ascertain whether reduced NK/NKT cell numbers coupled with decreased levels of proinflammatory cytokines in mutant mice led to the resistance seen against sepsis, we performed additional experiments to establish the causal relationship. Our results, showing that induction of septic shock in wild-type mice was completely reversed by either depletion of NK cells or blockade of NKT-APC interaction, strongly suggest that optimal NK/NKT cell numbers are required for sensitivity against LPS shock. These data suggest that reduced NK numbers or defective NKT-APC interaction significantly affect the outcome of LPS shock. The fact that the NK cell-depleted mice or mice that have their NKT-APC interaction blocked produce significantly reduced amounts of IFN-γ and TNF-α when challenged with LPS (see Fig. 5, c, d, f, and g) compared with mice treated with LPS alone suggests that NK/NKT cell numbers and IFN-γ and TNF-α are chief perpetuators of LPS shock. Taken together, these results strongly favor our argument that the reduced NK/NKT numbers and diminished IFN-γ levels in the mutants contribute in part to the observed resistance to LPS shock.

Given the impaired IFN-γ production in the CD137-deficient mice during inflammation and the fact that their NKT cell population is clearly defective, we wondered whether the defective NK cell numbers coupled with decreased levels of proinflammatory cytokines in mutant mice are responsible for the observed resistance to LPS shock. In support of this, β2-microglobulin−/− mice, which lack the NK1.1+ T cell population, are unable to produce IgE in response to anti-Id treatment. To test the hypothesis that NKT-dependent Th2 responses are dependent on CD137, we studied two diverse IL-4-dependent in vivo animal models. First, we determined whether the mutant mice were capable of mounting optimal IgE responses to anti-Id antigen (32), because failure to produce IL-4 and IgE in response to anti-Id injection has been linked to defects in IL-4-producing NKT cells (51, 58). This correlation has led to the suggestion that IL-4-producing NKT cells play a critical role in the initiation of Th2 responses (51). In support of this, β2-microglobulin−/− mice, which lack the NK1.1+ T cell population, are unable to produce IFN-γ in response to anti-Id treatment. To test the hypothesis that NKT-dependent Th2 responses are dependent on CD137, we studied two diverse IL-4-dependent in vivo animal models. First, we determined whether the mutant mice were capable of mounting optimal IgE responses to anti-Id antigen (32), because failure to produce IL-4 and IgE in response to anti-Id injection has been linked to defects in IL-4-producing NKT cells (51, 58). Second, we tested the CD137-deficient mice for normal Ag-specific recall responses to the particulate Ag, KLH. Although KLH is not an NKT ligand, it is believed to assess NKT function (37). Immunization with KLH in conjunction with CFA predominantly produces IL-4 responses and minimal induction of IFN-γ in mice (54). Our observation of reduced IgE responses to GmGL in CD137-deficient mice and impaired KLH-specific IL-4 responses indicates that defective NKT cells in the mutant mice are responsible for the observed effects. The observed Th2 responses do reflect the contribution of NKT cell-derived type II cytokines to the overall effect, because IL-4 derived from NKT cells in part controls the acquisition of the Th2 phenotype by the Th precursors (37). Reduced IL-4 production by CD137−/− mice in recall responses to KLH led us to believe that the mutant mice were unable to mount sustained Th2 responses, because the initial burst of IL-4 production by NKT cells shapes the development of naive CD4 precursors into IL-4-secreting Th2 cells (37). It is possible that CD137−/− mice do not generate this early burst due to defective NKT cell function. Our earlier observation that the mice have reduced KLH-specific IgG3 levels (10) also demonstrates that the CD137-CD137L pathway is critical for Ag-specific humoral immunity. Collectively, these results coupled with the observation that CD137-deficient mice have impaired IL-4 responses to systemic in vitro as well as in vivo TCR stimulation and decreased NKT cell numbers indicate that NKT cell-mediated Th2 responses depend on CD137 in the models considered.

Tumor killing assays performed in the present study strongly support our argument that knockout mice have defective NK cell function. Our current study shows that costimulatory blockade with anti-CD137L mAb reverses LPS lethality in wild-type mice; furthermore, ongoing work from our laboratory shows that the resistance of wild-type mice to RMA-S tumors can be reversed when the CD137/CD137L pathway is blocked by anti-CD137L mAb alone or in combination with α-galactosaminidase treatment (unpublished observations). Collectively, these data show that signaling through CD137 is critical not only during LPS shock, but also for tumor killing, and that the reduction in the number of NK cell subsets in the knockout animals is simply an added factor.

In summary, we show that the CD137/CD137L pathway in NK cell subsets is important for LPS-induced septic shock, because the absence of CD137 expression coupled with reduced NK and NKT cell numbers dramatically rescue the mice in the LPS model of toxic fulminant hepatic failure. Levels of inflammatory cytokines, such as IFN-γ and TNF-α, were reduced in the mutant, indicating that CD137 is a promising target for the prevention and treatment of fulminant hepatic failure. Additional understanding of the role of CD137 in LPS shock and in NK and NKT cell maturation and function may lead to new treatments for endotoxic shock syndrome. This is particularly important in view of the fact that bacterial sepsis and its complications following surgery continue to be a major problem in intensive care units, affecting >500,000 patients each year, more than a third of whom die (59, 60). In addition, our finding of reduced IL-4-dependent, NK-restricted, and IL-4 controlled Th2 responses to GmGL and KLH in CD137−/− mice points to a key role for Th2-type immunity controlled by CD137-directed NKT cells.

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