Supplementary Materials for

The TNF Family Member 4-1BBL Sustains Inflammation by Interacting with TLR Signaling Components During Late-Phase Activation

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Fig. S1. Cross-linking of 4-1BBL stimulates TNF-α production by macrophages. (A to C) WT, Trif-deficient, or Tlr4-deficient macrophages were (A) infected with gfp-encoding or 4-1bbl-encoding adenovirus (Adv-gfp or Adv-4-1bbl, respectively), (B) treated with medium, anti-Fc Ab (Ig, 2.5 mg/ml) alone, or mouse 4-1BB-Fc (5 mg/ml) and Ig, or (C) treated with LPS (100 ng/ml) or poly(I:C) (25 mg/ml). Twenty-four hours later, TNF-α production was determined by ELISA. Data are means ± SD from two to three independent experiments. *P < 0.05, **P < 0.01.
Fig. S2. Involvement of TRAF6 in the 4-1BBL-mediated production of TNF-α. (A) RAW264.7 cells were transfected with control siRNA or with two different siRNAs that target different sequences of Traf6, and the extent of TRAF6 knockdown was tested after 48 hours by Western blotting analysis with an anti-TRAF6 antibody. GAPDH was used as a loading control. Forty-eight hours after transfection, cells were treated with medium, anti-Fc antibody (Ig) alone, or mouse 4-1BB-Fc and anti-Fc antibody (4-1BB-Fc + Ig). To test the specificity of TRAF6 knockdown, cells were treated with medium, LPS, poly(I:C), IL-1β, or TNF-α and then culture media were analyzed by ELISA to measure TNF-α and IL-6 production. (B) Association of TRAF6 with 4-1BBL. HEK 293T cells were transfected with the indicated plasmids, and samples were subjected to immunoprecipitation (IP) and Western blotting analysis (IB) with the indicated antibodies. Data are means ± SD from three independent experiments. *P < 0.01, **P < 0.05.
Fig. S3. The 4-1BBL–mediated inflammatory response does not require NF-κB signaling. (A and B) Control cells or Tak1-, Tab1-, or Tab2-knockdown (KD) cells were infected with gfp (control)- or Ikba super repressor (SR)-encoding adenoviruses (10⁴ pfu/10⁶ cells). After 18 hours, cells were (A) further infected with 4-1bbl-encoding adenovirus (Adv-4-1bbl) or (B) stimulated with LPS (0.1 µg/ml). Twenty-four hours later, culture media were analyzed by ELISA to determine the extent of TNF-α production. Data are means ± SD from two to three independent experiments. *P < 0.05, **P < 0.01.
Fig. S4. 4-1BBL–induced TNF-α production is mediated by the activation of protein kinase signaling pathways. Peritoneal macrophages were pre-treated with inhibitors of PI3K (LY 294002), PKC (Go-31-8220), or PKA (H-89), and then were infected with Adv-4-1bbl (10^4 pfu/10^6 cells). Twenty-four hours later, culture supernatants were collected and cell lysates were obtained. The extent of TNF-α production was measured by ELISA. The abundance of 4-1BBL protein in the cell lysates was determined by Western blotting. Data are means ± SD from three to four independent experiments.*P < 0.05, **P < 0.01.
Fig. S5. TIRAP, but not Tollip, interacts with 4-1BBL to stimulate downstream signaling. (A) RAW264.7 cells were transfected with control siRNA or with two different siRNAs that target different sequences of Tollip. Forty-eight hours later, the extent of Tollip knockdown was assessed by semi-quantitative PCR analysis with Tollip-specific primers, with Gapdh used as a control. siRNA-transfected cells were treated with medium, anti-Fc antibody (Ig) alone, or mouse 4-1BB-Fc and anti-Fc antibody (4-1BB-Fc + Ig). Culture media were analyzed by ELISA to determine the extent of TNF-α production. Data are means ± SD from two to three independent experiments. (B) Analysis of the physical interaction between 4-1BBL and TIRAP. HEK 293T cells were cotransfected with plasmid encoding GFP–4-1BBL and together with empty plasmid (control) or plasmids encoding FLAG-tagged TLR4, TIRAP, or p38α. Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibodies and were analyzed by Western blotting (IB) with antibodies against the indicated targets. Blots are representative of three independent experiments.
Fig. S6. Specific inhibition of TIRAP activity by the TIRAP inhibitory peptide. (A) Wild-type (WT) or 4-1bbl-deficient (4-1bbl KO) macrophages were treated with LPS (0.1 µg/ml), and TNF-α production was measured at the indicated times by ELISA. (B) WT macrophages and (C) 4-1bbl KO macrophages were treated with LPS, and control or TIRAP inhibitory peptides were added after 4 hours later. Culture media were obtained at the indicated times, and TNF-α production was measured by ELISA. Data are means ± SD of two to three independent experiments. *P < 0.05, **P < 0.01. Arrows indicate the times when peptides were added.
Fig. S7. Inhibition of sustained TNF-α production by an anti–4-1BBL antibody. (A to C) Peritoneal mouse macrophages were treated with LPS (0.1 µg/ml) and with either isotype control antibody (Isotype Ab) or anti-4-1BBL antibody (both at 5 µg/ml), or were stimulated with (B) LPS or (C) CpG DNA for 4 hours before the antibodies were added. TNF-α production in culture media was determined by ELISA at the indicated times. Data are means ± SD from two to three independent experiments. *P < 0.05. Arrows indicate the times when peptides were added.
Fig. S8. Formation of a 4-1BBL–TIRAP–TRAF6–TAK1–TAB1 complex. HEK 293T cells were cotransfected with plasmids encoding GFP–4-1BBL and FLAG-TIRAP, together with empty plasmid (Control), plasmid encoding TRAF6-myc, plasmid encoding TAK1-HA, plasmid encoding untagged Tab1, or with a combination of plasmids encoding TRAF6-myc, TAK1-HA, and TAB1. Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibody and were analyzed by Western blotting with antibodies against the indicated proteins. Blots are representative of three independent experiments.
Fig. S9. 4-1BBL physically associates with IRAK2. (A and B) HEK 293T cells were cotransfected with empty plasmid (Control) or plasmid encoding HA–4-1BBL together with (A) empty plasmid (Control) or plasmids encoding FLAG-tagged TLR4, IRAK1, or IRAK4, or with (B) empty plasmid (Control) or plasmids encoding Myc-tagged TLR4 or IRAK2. Whole-cell lysates (WCLs) were subjected to immunoprecipitation (IP) with (A) anti-FLAG or (B) anti-myc antibodies and then were analyzed by Western blotting (IB) with antibodies against the indicated proteins. Blots are representative of two to three experiments.
Fig. S10. IRAK4 physically interacts with IRAK2 but not 4-1BBL. HEK 293T cells were transfected with plasmids encoding HA–4-1BBL, Myc-IRAK2, or FLAG-IRAK4. Whole-cell lysates (WCLs) were subjected to immunoprecipitation (IP) with anti-HA antibodies, and WCLs and immunoprecipitates were analyzed by Western blotting with antibodies against the indicated proteins. Blots are representative of three independent experiments.
Fig. S11. Amelioration of LPS-induced septic shock by inhibition of MyD88 activity. (A to C) Injection of mice with LPS and with either control peptide or MyD88 inhibitory peptide was performed as indicated in Fig. 6A. Mice were injected with control (n = 8 mice) or MyD88 inhibitor (300 µg/mouse) (A) 1 hour before (n = 8 mice), (B) together with (n = 6 mice), or (C) 1 hour after (n = 8 mice) the mice were injected with LPS (350 µg/mouse). Arrows indicate the times of injections of LPS and peptides. Serum TNF-α concentrations at the indicated times were measured by ELISA. *P < 0.05, **P < 0.01.