4-1BB (CD137) Is Required for Rapid Clearance of *Listeria monocytogenes* Infection

Sang-C. Lee,¹ Seong-A. Ju,¹ Ha-N. Pack,¹ Sook-K. Heo,¹ Jae-H. Suh,² Sang-M. Park,¹
Boem-K. Choi,¹ Byoung S. Kwon,^{1,3*} and Byung S. Kim^{1*}

Immunomodulation Research Center, University of Ulsan, Ulsan, Korea, 680-749¹; Department of Pathology,
Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan, Korea, 682-060²;
and LSU Eye Center, Louisiana State University Health Sciences Center
School of Medicine, New Orleans, Louisiana 70112³

Received 27 August 2004/Returned for modification 11 October 2004/Accepted 17 March 2005

4-1BB (CD137), a member of the tumor necrosis factor receptor superfamily, is a T-cell-costimulatory receptor that is expressed on activated T cells, dendritic cells, and NK cells. Little has been reported about its role in early host defense against bacterial infection. In this study, we report that 4-1BB-deficient (4-1BB^{-/-}) mice are much more susceptible to *Listeria monocytogenes* (intracellular bacteria) infections than wild-type mice. Upon *L. monocytogenes* infection, 4-1BB^{-/-} mice showed a lower survival rate, a higher bacterial burden in organs, and larger hepatic microabscesses than 4-1BB^{+/+} mice. 4-1BB^{-/-} mice also had impairment in clearance of bacteria from the bloodstream. Neutrophils from 4-1BB^{+/+} mice constitutively expressed 4-1BB, which could be activated to induce intracellular Ca²⁺ influx by ligation with anti-4-1BB antibody. On the other hand, neutrophils from 4-1BB^{-/-} mice were defective in reactive oxygen species generation, phagocytic activities, and intracellular Ca²⁺ mobilization. In addition, mice pretreated with anti-4-1BB monoclonal antibody were much more resistant to *L. monocytogenes* infection than control antibody-treated mice. Our results support the notion that 4-1BB may play a major role in host defense against intracellular pathogens through neutrophil activation.

Neutrophils constitute a first line of defense and are the first to migrate into tissues in response to invading pathogens. They are capable of chemotaxis in response to mediators present at the infection site. One of their principal roles in inflammatory and immune responses is the phagocytosis and killing of bacteria by generating reactive oxygen species (ROS) and releasing lytic enzymes stored in granules (20). The listeriosis model in mice has proven to be useful for investigating immune responses to bacterial infection (38). *Listeria monocytogenes* is a gram-positive facultative intracellular bacterium that replicates within the cytosol of both phagocytic and nonphagocytic cells (36). Activated macrophages, neutrophils (7), NK cells (12), and $\gamma\delta$ T cells (33) are critical for the initial control of *L. monocytogenes* infection. Upon systemic inoculation, the bulk (>60%) of *L. monocytogenes* cells are cleared rapidly from the bloodstream and can be recovered in the liver 10 min after infection (30). Most *L. monocytogenes* cells taken up in the liver are initially bound extracellularly and subsequently killed by immigrating neutrophils (18). Depletion of neutrophils with antibodies (RB6-8C5) has been reported to cause a sharp decrease in antibacterial activity and even to lead to the death of mice infected with sublethal doses of *L. monocytogenes* (9) or *Salmonella enterica* serovar Dublin (39). Therefore, rapid eradication of *L. monocytogenes* from the bloodstream by neutrophils is an important step in early host defense.

4-1BB (CD137) is a member of the tumor necrosis factor receptor superfamily (TNFRSF 9) that is expressed on activated T cells, dendritic cells (DCs), and NK cells (8, 15, 26, 31, 41) and constitutively expressed on normal human blood neutrophils (23). 4-1BB costimulates T cells to carry out functions such as eradication of established tumors (25, 32, 42, 44, 45), broadening of primary CD8⁺ T-cell responses (5, 28, 37, 43), and enhancement of the memory pool of antigen-specific CD8⁺ T cells (2, 3). However, little is known about its role in early host defense against bacterial infection.

Here, we show that 4-1BB is constitutively expressed on normal mouse blood neutrophils and that 4-1BB-deficient (4-1BB^{-/-}) mice are more susceptible to infection by *L. monocytogenes*. 4-1BB^{-/-} mice have a lower survival rate, higher bacterial loads in livers, and larger hepatic microabscesses than 4-1BB^{+/+} mice upon *L. monocytogenes* infection. 4-1BB^{-/-} neutrophils are defective in generating ROS, in mobilizing Ca²⁺, and in phagocytic activities.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male BALB/c mice were purchased from Hyo Chang Bioscience (Tae-Ku, Korea). 4-1BB-deficient BALB/c mice were established as previously described (27) and backcrossed with BALB/c mice for more than nine generations. All mice were maintained under specific-pathogen-free conditions in the animal facility of the Immunomodulation Research Center, University of Ulsan, Ulsan, Korea, and used at 8 to 10 weeks of age.

Antibodies. Anti-4-1BB monoclonal antibody (MAb) was used for in vivo treatment. The hybridoma cells (3E1 and 3H3) were a kind gift of R. Mittler (Emory University, Atlanta, GA). The MAb was purified from ascites and further purified on a protein G column (Sigma-Aldrich, St. Louis, MO). Anti-4-1BB MAb (3E1) was conjugated with fluorescein isothiocyanate (FITC). The following antibodies were purchased from BD Pharmingen (San Diego, CA): FITC-conjugated rat immunoglobulin G2a (IgG2a), purified anti-CD16/CD32 (Fc γ

* Corresponding author. Mailing address: Immunomodulation Research Center, University of Ulsan, San 29, Mueo-dong, Nam-ku, Ulsan, Republic of Korea, 680-749. Phone for Byoung S. Kwon: 82-52-259-2875. Fax: 82-52-259-2740. E-mail: bskwon@mail.ulsan.ac.kr. Phone for Byung S. Kim: 82-52-259-1541. Fax: 82-52-259-2740. E-mail: sjc513@mail.ulsan.ac.kr.

FIG. 1. Neutrophils express 4-1BB constitutively on their surfaces. (A) Peripheral leukocytes were isolated from the blood of wild-type BALB/c mice, and 4-1BB expression on dendritic cells and monocytes was assessed by double staining with PE-conjugated anti-CD11c or anti-F4/80 and FITC-conjugated anti-4-1BB MAbs. Neutrophils (Neu) were isolated from the peripheral blood of wild-type (4-1BB^{+/+}) and 4-1BB-deficient (4-1BB^{-/-}) BALB/c mice and double stained with PE-conjugated anti-Gr-1 MAb and FITC-conjugated anti-4-1BB MAb or rat IgG2a as an isotype control (gray). 4-1BB expression was analyzed by gating CD11c⁺, F4/80⁺, or Gr-1⁺ cells. MΦ, macrophage. (B) 4-1BB mRNA expression in freshly isolated neutrophils was detected by RT-PCR. The data shown are representative of three separate experiments.

III/IR), FITC-conjugated anti-Ly6G (RB6-8C5), and phycoerythrin (PE)-conjugated anti-CD11b (M170).

Bacterial infection and antibody treatment. *L. monocytogenes* was obtained from the Korean Type Culture Collection (ATCC 19111). To maintain virulence, it was passaged through mice, recovered bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C for 18 h, and aliquots were frozen at -80°C. For each experiment, the viability of frozen aliquots was confirmed on brain-heart infusion agar (Difco) and was always more than 90%. To test the effect of anti-4-1BB stimulation, mice were intravenously (i.v.) (tail vein) injected with *L. monocytogenes*. Two hundred micrograms of anti-4-1BB MAb was intraperitoneally injected into mice on the indicated days. To deplete neutrophils, anti-Gr-1 MAb RB6-8C5 (500 µg/mouse) was intraperitoneally injected into mice 2 days before infection.

Cell preparation and flow cytometry. To isolate neutrophils from peripheral blood, we used the Histopaque 1077 and 1119 density gradient method (Histopaque 1077, 1.077 g/ml, and 1119, 1.119 g/ml; Sigma-Aldrich, St. Louis, MO), as previously described (22). In brief, peripheral blood was obtained from the abdominal cavity with a heparin-coated syringe, layered over Histopaque 1077 and 1119, and centrifuged at 700 × g for 30 min at room temperature. Neutrophils were collected from the interface of Histopaque 1077 and 1119 and washed twice with cold Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution. Remaining red blood cells were eliminated with red blood cell lysing buffer (Sigma). Routinely, the isolated cells contained >90% neutrophils, as determined by flow cytometric analysis. To obtain highly pure neutrophils for reverse transcription (RT)-PCR, cells from the Histopaque interface were further incubated with magnetic beads coupled to anti-F4/80, -CD45RB, -CD4, -CD8, and -CD11c MAbs. The neutrophils were negatively selected and used for RNA extraction. Hepatic leukocytes

FIG. 2. Enhanced susceptibility of 4-1BB^{-/-} mice to *L. monocytogenes* infection. Groups of nine mice were intravenously infected with *L. monocytogenes* at 1×10^5 (LD₅₀) or 2×10^5 (LD₁₀₀) CFU/mouse. (A) Survival was monitored daily up to 30 days after infection. For 1×10^5 CFU/mouse, P was <0.05, and for 2×10^5 CFU/mouse, P was <0.001 by the log rank test. (B) Mice were intravenously infected with *L. monocytogenes* at 10^5 CFU/mouse, and the numbers of CFU in liver and spleen were determined on days 1 and 3 postinfection. The data are from one representative of three independent experiments and are expressed as means plus standard deviations. *, P < 0.05; **, P < 0.01.

were prepared as described previously (13, 14). In brief, livers were pressed through a stainless steel mesh after perfusion, and leukocytes were purified on 40% to 70% Percoll gradients (Amersham Bioscience, Uppsala, Sweden). The tubes were centrifuged at 600 × g at room temperature for 30 min, and the leukocyte layers formed between the 40% and 70% layers of Percoll were isolated. The cells (2×10^5) were washed with fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA] and 0.1% sodium azide), incubated with Fcγ receptor blocking antibody (2.4G2) for 10 min on ice, and stained with antibodies for Ly6-G (Gr-1), 4-1BB, and CD11b. All samples were analyzed by FACSCalibur (BD Biosciences, San Jose, CA).

RT-PCR. RNA was extracted from neutrophils isolated from the blood of 4-1BB^{+/+} and 4-1BB^{-/-} mice. It was reverse transcribed into cDNA using a PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA), and PCR was performed with sense/antisense primers. The primer sequences were as follows: mouse 4-1BB forward, 5'-TGTGTGCAGGCTATTTTCAGG-3', and reverse, 5'-GAGCTGCTCCAGTGGTCTTC-3' (expected size, 504 bp); mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward, 5'-TGAAGTCTGGTG TGAACGGATTGGC-3', and reverse, 5'-CACCACCTGGAGTACCGGATG TAC-3' (expected size, 982 bp). PCR products were visualized with ethidium bromide after electrophoresis on 1% agarose gels.

FIG. 3. Increased hepatic microabscesses in 4-1BB^{-/-} mice after *L. monocytogenes* infection. Mice were injected systemically with 10^5 CFU/mouse. Two days after infection, the livers of 4-1BB^{-/-} (A and C) and 4-1BB^{+/+} (B and D) mice contained multiple microabscesses (arrowheads). The 4-1BB^{-/-} mice had larger and more necroinflammatory lesions with granulocytic inflammation. Magnification, A and B, $\times 20$; C and D, $\times 400$ (Panels C and D are enlarged). (E) *L. monocytogenes* detected in the liver by Warthin-Starry staining (magnification, $\times 1,000$). (F) Numbers of microabscesses per 10 fields expressed as means plus standard deviations. **, $P < 0.01$. The data are from three mice per group after hematoxylin and eosin staining.

Determination of CFU in liver and spleen. Mice were infected i.v. with 10^5 CFU of *L. monocytogenes*/mouse. On days 1, 3, and 5, mice were anesthetized and their livers were perfused with sterile RPMI 1640 medium containing 10% fetal bovine serum to wash bacteria out of their blood vessels. CFU in livers and spleens were determined by plating serial dilutions of organ homogenates in PBS containing 0.1% BSA and counting the colonies.

Histology. Mice were infected i.v. with 10^5 *L. monocytogenes* cells. The livers were excised 2, 3, and 4 days after inoculation, fixed in 4% paraformaldehyde, and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin (Sigma-Aldrich).

Determination of blood clearance. Mice were anesthetized and infected with 5×10^4 *L. monocytogenes* cells, and blood samples from the tail vein were collected after 1, 3, 6, and 12 min. One hundred-microliter samples were lysed in sterile water containing 0.01% BSA by freezing and thawing, serially diluted, plated in triplicate on brain-heart infusion agar (Difco, Detroit, MI), and incubated for 18 h at 37°C. CFU were counted, and the percentage of surviving bacteria was determined relative to the number found 1 min after infection.

Determination of ROS generation. Blood samples were obtained from the abdominal cavities of 4-1BB^{+/+} and 4-1BB^{-/-} mice, and 2 μ M of the oxidation-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (DCFDA; molecular probe; Sigma-Aldrich, St. Louis, MO) was added and incubated at 37°C for 30 min. Phorbol myristate acetate (PMA) (100 nM) or heat-killed *L. monocytogenes* (10^7 CFU/ml) at a multiplicity of infection of 10 was then added. Samples were withdrawn at the indicated times, and their granulocyte populations were analyzed after gating by FACS following lysis of red blood cells.

Measurement of Ca²⁺ levels. Neutrophils isolated from peripheral blood mononuclear cells were incubated in RPMI 1640 (plus 10% fetal bovine serum) containing 10 μ M flou3-acetoxymethyl ester (Fluo3/AM; Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min and washed with cold PBS. The cell suspensions were allowed to adhere to poly-L-lysine-treated coverslips for 10 min and were placed into a confocal analysis chamber. *L. monocytogenes* (5×10^6), PMA (100 nM), or anti-4-1BB Mab (5 μ g/ml) was added when appropriate to stimulate the neutrophils. $[Ca^{2+}]_i$ was calibrated from the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d (F/F_0) / (K_d / [Ca^{2+}]_{i,rest} + 1 - (F/F_0))$, where F is the fluorescence intensity, F_0 is the resting fluorescence, K_d is the dissociation constant of Fluo 3/AM (1,100 nM), and $[Ca^{2+}]_{i,rest}$ is the resting Ca²⁺ concentration (100 nM). The rate of decay of $[Ca^{2+}]_i$ transients was obtained by fitting the decaying

phases of calibrated fluorescence signals with a standard single exponential function, and intracellular Ca²⁺ levels were monitored in real time by confocal microscopy (FV500; Olympus).

Phagocytosis. Blood from the abdominal cavity was pooled, and neutrophils were isolated using Histopaque 1077 and 1119. Isolated neutrophils were incubated with FITC-labeled dextran (Molecular Probes, Oreg.) at a final concentration of 1 mg/ml. At the indicated times, aliquots were rapidly cooled to 4°C, washed four times with cold PBS, and held on ice for flow cytometric analysis.

Statistical analysis. Statistical evaluation of differences was done with the log rank test for survival curves and with Student's *t* test.

RESULTS

4-1BB is constitutively expressed on neutrophils. Recent studies have shown that 4-1BB is expressed on subpopulations of myeloid cells, including dendritic cells (15), monocytes, and the neutrophils of human blood (23), as well as on subpopulations of activated lymphoid cells. However, there were no reports of 4-1BB expression on murine neutrophils. To investigate whether neutrophils express 4-1BB, we performed a flow cytometric (FACS) analysis. Blood was obtained from the inferior vena cava of mice and analyzed by FACS. We found that 4-1BB was constitutively expressed at a low level on purified neutrophils from peripheral blood, but not on monocytes or DCs (Fig. 1A). We also measured 4-1BB mRNA expression by RT-PCR in freshly isolated blood neutrophils from 4-1BB^{+/+} and 4-1BB^{-/-} mice. Neutrophils isolated from 4-1BB^{+/+} mice, but not those isolated from 4-1BB^{-/-} mice, contained 4-1BB (CD137) mRNA (Fig. 1B). These results indicate that blood neutrophils constitutively express 4-1BB on their surfaces, whereas other myeloid cells, such as monocytes/macrophages and DCs, do not.

FIG. 4. Increase in the number of infiltrating neutrophils in the liver. Hepatic infiltrated leukocytes were isolated from 4-1BB^{-/-} and 4-1BB^{+/+} mice by the Percoll gradient method after *L. monocytogenes* infection (5×10^4 CFU/mouse). The isolated cells were stained with anti-Gr-1-FITC, anti-CD11b-PE MAb and analyzed by flow cytometry. (A) Gr-1⁺ CD11b⁺ cells in the livers of 4-1BB^{+/+} and 4-1BB^{-/-} mice. (B) absolute numbers of neutrophils in the liver at various times. (C) Bacterial titers in the liver were determined by measuring CFU. The results are from one representative of three independent experiments and are expressed as means plus standard deviations. *, $P < 0.05$; **, $P < 0.01$.

4-1BB^{-/-} mice are more susceptible to *L. monocytogenes* infection than 4-1BB^{+/+} mice. To examine the role of 4-1BB in neutrophils, we selected the *L. monocytogenes* infection model that is well established for investigating the role of neutrophils in early host defense (7, 18, 30). We examined the survival of 4-1BB^{-/-} and 4-1BB^{+/+} BALB/c mice following i.v. inoculation with 1×10^5 (the approximate 50% lethal dose [LD₅₀] in wild-type BALB/c mice) or 2×10^5 *L. monocytogenes* cells (a lethal dose for BALB/c mice). Following inoculation with 2×10^5 *L. monocytogenes* cells, 4-1BB^{-/-} mice began to die 3 days after infection, and all mice had succumbed by day 5 p.i., whereas 4-1BB^{+/+} mice started dying only on day 5, and 89% of them had succumbed by day 6 p.i. Moreover, some of the 4-1BB^{+/+} mice (11%) remained alive until day 30 p.i. ($P < 0.001$). When we infected 4-1BB^{-/-} mice with 10^5 *L. monocytogenes* cells, only 37% of the infected mice survived to day 7,

whereas 64% of the 4-1BB^{+/+} mice were still alive at that time ($P < 0.05$) (Fig. 2A). The numbers of bacteria recovered from livers and spleens after i.v. inoculation with a sublethal dose of *L. monocytogenes* were consistent with the survival rates. As expected, the bacterial burdens in both the spleens and livers of *L. monocytogenes*-infected 4-1BB^{-/-} mice were consistently greater than in the 4-1BB^{+/+} mice. On day 1 or 3 p.i., the numbers of *L. monocytogenes* cells in the livers of 4-1BB^{-/-} mice were two- to fourfold higher than in the 4-1BB^{+/+} mice, but there was no significant difference between the bacterial burdens in the spleens of 4-1BB^{-/-} and 4-1BB^{+/+} mice on day 3 p.i. (Fig. 2B).

Microscopic examination at low magnification revealed distinct hepatic microabscesses in both sets of mice on day 2 p.i. (Fig. 3A and B). However, the microabscesses in the 4-1BB^{-/-} mice were generally larger and more numerous than those in the 4-1BB^{+/+} mice (Fig. 3F). We therefore conclude that 4-1BB^{-/-} mice are more susceptible to *L. monocytogenes* infection.

4-1BB^{-/-} mice retain more liver-infiltrated granulocytes than 4-1BB^{+/+} mice. Because granulocytes play a central role in host defense at an early stage of listeriosis (6, 7, 18, 19, 35), we compared the numbers of granulocytes in the livers of 4-1BB^{-/-} and 4-1BB^{+/+} mice by flow cytometry. Unexpectedly, the number of neutrophils was higher in the 4-1BB^{-/-} mice than in the wild-type mice (Fig. 4A), even though neutrophil numbers in the peripheral blood are similar in uninfected 4-1BB^{-/-} and 4-1BB^{+/+} mice. The total number of neutrophils in the liver was 3- to 10-fold higher in the 4-1BB^{-/-} mice than in the 4-1BB^{+/+} mice at 6 h, 12 h (Fig. 4B), and 24 h after infection (data not shown), while the bacterial burden in the liver was greater in the 4-1BB^{-/-} than in the 4-1BB^{+/+} mice (Fig. 4C). The increased number of neutrophils in 4-1BB^{-/-} mice may be due to the larger bacterial burden in their livers, which seems also to be related to their larger number of microabscesses.

Impairment of bacterial clearance from the bloodstream in 4-1BB^{-/-} mice. Although 4-1BB^{-/-} mice had higher numbers of recruited neutrophils, 4-1BB^{-/-} mice had higher bacterial burdens in organs than did 4-1BB^{+/+} mice. This prompted us to investigate whether 4-1BB^{-/-} mice have impaired bacterial clearance from the bloodstream. Mice were injected i.v. with *L. monocytogenes* and bled from the tail vein at indicated times, and the numbers of viable bacteria were determined. After injection of 5×10^4 CFU/mouse, the number of bacteria detected fell more rapidly in the wild-type than in the 4-1BB^{-/-} mice (Fig. 5A). Most bacteria had disappeared by 12 min, and we no longer detected any bacteria after 20 min (data not shown). We also calculated the clearance rate from the numbers of bacteria in blood as described in Materials and Methods. The clearance rate was significantly lower in 4-1BB^{-/-} mice than in 4-1BB^{+/+} mice at 3 and 6 min (Fig. 5B). To investigate the role of neutrophils in bacterial clearance, mice were depleted of neutrophils by injecting them with anti-Gr-1 MAb (RB6-8C5) before *L. monocytogenes* infection. We confirmed neutrophil depletion with a Hemavet 850 blood cell counter and by flow cytometric analysis, and almost all the neutrophils (>99%) were removed (data not shown). Depletion of neutrophils completely abolished the difference in bac-

FIG. 5. Blood clearance in 4-1BB^{-/-} or 4-1BB^{+/+} mice. (A) Blood (100 µl) was collected from the tail veins of 4-1BB^{+/+} and 4-1BB^{-/-} mice at the indicated times after inoculation with *L. monocytogenes* (5×10^4 CFU/mouse) and lysed in sterile water. Numbers of surviving bacteria were determined. (B) Mice received 200 mg RB6-8C5 (anti-mGr-1 MAb) or rat IgG the day before *L. monocytogenes* inoculation (5×10^4 CFU/mouse). The percentage of bacterial clearance at a given time was determined by the reduction in CFU in the blood compared to 1 min after infection. (C) Livers were removed from 4-1BB^{+/+} and 4-1BB^{-/-} mice, and hepatic leukocytes were prepared as described in Materials and Methods. Isolated cells were stained with PE-conjugated anti-mF4/80 MAb and incubated with FITC-labeled *L. monocytogenes* for 3 min. After gating of F4/80⁺ cells, we analyzed attachment to Kupffer cells by flow cytometry. The results are from one representative ($n = 3$) of three independent experiments and are expressed as means plus standard deviations. *, $P < 0.05$; **, $P < 0.01$.

terial clearance between 4-1BB^{+/+} and 4-1BB^{-/-} mice (Fig. 5B). In addition, we observed that pretreatment of mice with anti-4-1BB MAb significantly increases the rate of bacterial clearance from the bloodstream (Fig. 5B). These results suggest that neutrophils may be involved in bacterial clearance from the bloodstream and that the absence of 4-1BB signals affects the clearance of *L. monocytogenes* in the early phase of infection.

It has been shown that most of the *L. monocytogenes* cells injected into mice are rapidly cleared from the bloodstream and trapped in the liver, presumably by adhering to Kupffer cell surfaces (30). We determined whether Kupffer cells contribute to the defects in bacterial clearance from the bloodstream in 4-1BB^{-/-} mice by isolating Kupffer cells from mice and incubating them with FITC-conjugated *L. monocytogenes* for 3 min. After incubation, Kupffer cells with adhering bacteria were analyzed by gating F4/80⁺ cells. As shown in Fig. 5C, there were no differences in the proportions of Kupffer cells with adherent bacteria between 4-1BB^{+/+} and 4-1BB^{-/-} mice. Therefore, these results indicated that the defects of 4-1BB^{-/-} mice in bacterial clearance from the bloodstream might be due to neutrophils rather than Kupffer cells.

4-1BB^{-/-} neutrophils are defective in ROS production, phagocytic activities, and Ca²⁺ mobilization. ROS play an important role in microbial killing by activated neutrophils (19, 21, 34). We therefore measured ROS production by 4-1BB^{-/-} and 4-1BB^{+/+} neutrophils using the cell-permeant, oxidation-sensitive dye DCFDA, which becomes fluorescent when oxidized by ROS (11, 24). When freshly isolated neutrophils from peripheral blood mononuclear cells were treated with PMA (100 nM), ROS production was significantly lower at 10, 20,

and 30 min and more delayed in 4-1BB^{-/-} neutrophils than in the 4-1BB^{+/+} neutrophils (Fig. 6A). We also determined the phagocytic activities of neutrophils isolated from 4-1BB^{-/-} and 4-1BB^{+/+} mice. Neutrophils were incubated with FITC-conjugated dextran beads and phagocytosed, or FITC-positive neutrophils were analyzed by flow cytometry at 10, 20, and 30 min after incubation. Although there was no significant difference between 4-1BB^{+/+} and 4-1BB^{-/-} mice, phagocytosis was always slightly reduced in the 4-1BB^{-/-} neutrophils (Fig. 6B).

Since ROS generation in phagocytic cells, such as monocytes and neutrophils, is dependent on an increase in [Ca²⁺]_i signaling (1, 16), we measured Ca²⁺ mobilization in response to PMA (100 nM) or live *L. monocytogenes* (ratio of neutrophils to *L. monocytogenes* cells, 1:50). These treatments caused a greater elevation of [Ca²⁺]_i in the wild-type than in the 4-1BB^{-/-} neutrophils (Fig. 6C). Moreover, exposure of 4-1BB^{+/+} neutrophils to anti-4-1BB MAb elicited a rapid rise in the intracellular Ca²⁺ concentration (Fig. 6C). Therefore, these results indicate that Ca²⁺ influx caused by 4-1BB engagement may be involved in ROS generation and phagocytosis in neutrophils.

Resistance to *L. monocytogenes* is augmented by 4-1BB stimulation, and neutrophil depletion abolishes 4-1BB-mediated protection. To evaluate the effect of 4-1BB stimulation on *L. monocytogenes* infection, we treated mice with agonistic anti-4-1BB MAb the day before *L. monocytogenes* infection. BALB/c mice were intravenously injected with 1×10^5 or 2×10^5 *L. monocytogenes* cells. Following infection, the survival of control antibody-treated mice decreased to 60% and 10%, respectively. In contrast all mice pretreated with anti-4-1BB MAb and injected with 1×10^5 or 2×10^5 *L. monocytogenes*

FIG. 6. Defects in ROS generation, phagocytosis, and Ca^{2+} flux in the neutrophils of 4-1BB^{-/-} mice. Blood was collected from the abdominal cavities of 4-1BB^{+/+} and 4-1BB^{-/-} mice. (A) Samples were incubated with 2 μM DCFDA and PMA (100 nM), and Gr-1⁺ cells were analyzed by FACS for ROS production. The results are expressed as means plus standard deviations. (B) Isolated neutrophils collected from the abdominal cavity were pooled and incubated with FITC-dextran. At the indicated times, aliquots were cooled to 4°C, washed, and kept on ice for flow cytometric analysis of FITC-positive neutrophils. The results are expressed as means plus standard deviations, and a representative experiment (10 min) is displayed in the inset. (C) Neutrophils were loaded with 10 μM Flou-3/AM, and $[\text{Ca}^{2+}]_i$ was measured in the cells following stimulation with *L. monocytogenes* (5×10^6 CFU), PMA (100 nM), and anti-4-1BB MAb (5 $\mu\text{g}/\text{ml}$) or rat IgG, as described in Materials and Methods. On the left is the maximal level of $[\text{Ca}^{2+}]_i$ in each experiment (mean plus standard error; $n = 3$). On the right (4-1BB^{-/-}, dotted line; 4-1BB^{+/+}, solid line) are shown representative traces of $[\text{Ca}^{2+}]_i$ obtained in the experiments shown on the left. For the rat IgG and anti-4-1BB MAb experiments, neutrophils from 4-1BB^{+/+} mice were used (rat IgG, dotted line; anti-4-1BB MAb, solid line). The arrows indicate the times (10 s) of exposure to the stimulants. The results are from a representative of three independent experiments and are expressed as means plus standard deviations. *, $P < 0.05$; ***, $P < 0.001$.

cells survived up to 30 days ($P < 0.05$ and $P < 0.001$) (Fig. 7A), and bacterial growth was reduced 5- to 10-fold in the liver on days 1 and 2 (Fig. 7B). To study the role of neutrophils in 4-1BB-mediated protection, we depleted the neutrophils with anti-Gr-1 MAb (RB6-8C5) before anti-4-1BB MAb treatment and found that all the mice succumbed to infection by day 4 p.i. (Fig. 7A). As expected, bacterial growth was significantly increased in the neutrophil-depleted mice, but there was no difference in bacterial titers between the anti-4-1BB MAb-treated and control IgG-treated mice (Fig. 7B). Because mice depleted of neutrophils have a severe defect in their ability to control *L. monocytogenes* infection, and bacterial loads in these mice are several log units greater than in non-neutrophil-depleted mice, we speculated that high doses of *L. monocytogenes* were responsible for the loss of antilisterial activity associated with anti-4-1BB treatment. We inoculated neutrophil-depleted

mice with low doses of 4×10^3 or 4×10^2 *L. monocytogenes* cells. As shown in Fig. 7C, even in this low-dose experiment, the bacterial titers of the livers in 4-1BB MAb-treated and control antibody-treated mice were not different. Therefore, these data suggest that the engagement of 4-1BB on neutrophils is involved in protection against *L. monocytogenes* in the early phase of infection.

DISCUSSION

We have demonstrated that 4-1BB is expressed on naïve mouse neutrophils and is important for early host defense against *L. monocytogenes*. 4-1BB^{-/-} mice showed delayed bacterial clearance from the bloodstream and suffered from augmented listerial titers in liver and spleen. The higher bacterial titers in the organs of 4-1BB^{-/-} mice early in infection (Fig. 2)

FIG. 7. Stimulation of neutrophils with anti-4-1BB MAb increases their resistance to bacterial infection. Mice were administered 200 μ g of anti-Gr-1 MAb on days -4 and -2 p.i., and anti-4-1BB MAb (3H3) or rat IgG on day -1 p.i. The antibody-treated mice were inoculated with 1×10^5 (LD₅₀) or 2×10^5 (LD₁₀₀) CFU *L. monocytogenes*. (A) Survival rates were monitored daily until day 30. The data are from a representative experiment of two performed ($n = 8$ or 9). For 1×10^5 CFU/mouse, P was <0.05 , and for 2×10^5 CFU/mouse, P was <0.001 by the log rank test. (B) Bacterial growth in the liver was determined by measuring CFU. (C) Mice were intravenously infected with *L. monocytogenes* at 4×10^3 CFU/mouse or 4×10^2 CFU/mouse. Livers were homogenized at the indicated times, and viable bacteria were quantified. The mean numbers (plus standard deviations) of CFU from groups of three mice are shown. The data are from a representative of two independent experiments. **, $P < 0.01$.

indicate that the innate immune response against this bacterium is defective in 4-1BB^{-/-} mice and probably results in reduced survival.

Neutrophils have a critical role as effector cells in the host response to microbial invasion (9, 18, 39). They contribute to the early innate immune response by migrating rapidly into inflamed tissue and employing potent effector mechanisms, such as phagocytosis, production of ROS, and release of inflammatory mediators and antimicrobial agents (1, 16, 29). In

this study, we found that there was greater neutrophil infiltration in the livers of 4-1BB-deficient mice than in those of wild-type mice following *L. monocytogenes* infection (Fig. 4A). In spite of high numbers of neutrophils, 4-1BB^{-/-} mice had a lower survival rate and higher bacterial burden in organs than 4-1BB^{+/+} mice. The increased number of neutrophils in 4-1BB^{-/-} mice might be caused by the greater bacterial burden in their livers, which resulted in an increased number of microabscesses in the livers of these mice.

Our results indicate that 4-1BB^{-/-} neutrophils are dysfunctional in activities for removal of bacteria, such as ROS generation and phagocytic activities (Fig. 6). These defects in neutrophils may lead to delay in clearance of infected bacteria from the blood (Fig. 5). Since we suspected functional defects in the neutrophils of 4-1BB^{-/-} mice, we investigated the possibility that 4-1BB signals are involved in phagocytosis and bacterial killing by neutrophils. Neutrophils ingest microorganisms by phagocytosis, and the ingested bacteria are killed by ROS derived from superoxide, which is produced by an activated, phagosome-bound NADPH oxidase (10, 40). Intracellular Ca^{2+} mobilization is required for activation of neutrophil functions, such as degranulation (4), initiation of the respiratory burst, and phagocytosis (17, 40). In this study, we observed that neutrophils derived from 4-1BB^{-/-} mice are dysfunctional in the generation of ROS, phagocytic activities, and intracellular Ca^{2+} mobilization (Fig. 6). In addition, we found that ligation of 4-1BB in neutrophils by anti-4-1BB antibody also produce intracellular Ca^{2+} mobilization (Fig. 6C). These results clearly indicate that 4-1BB ligation induces intracellular Ca^{2+} mobilization, phagocytic activities, and ROS generation, which may lead to the bactericidal activities of neutrophils.

In this study, the role of 4-1BB in anti-listerial activities was also confirmed using agonistic anti-4-1BB MAb. By treatment with anti-4-1BB MAb, the survival of mice after lethal infection with *L. monocytogenes* was greatly increased, from 10% to 100% (Fig. 7A), and this effect was completely abrogated by depletion of neutrophils (Fig. 7B). These results suggest that neutrophils are important for *L. monocytogenes* eradication and that 4-1BB signals are required to activate neutrophils during early host defense.

We have thus shown that 4-1BB is constitutively expressed on neutrophils, although at a low level, as demonstrated by FACS analysis. This expression may contribute to the rapid activation of neutrophils during immunosurveillance against invasion by microorganisms. Signaling via neutrophil 4-1BB appears to be involved in bactericidal mechanisms, such as phagocytosis and ROS production, which might be signaled via Ca^{2+} mobilization. Hence, activation of neutrophils via 4-1BB contributes to the rapid eradication of microorganism from the bloodstream and reduces the bacterial load in the organs of host defense.

ACKNOWLEDGMENTS

This work was supported by the SRC Fund to the IRC at the University of Ulsan from KOSEF and by the Korean Ministry of Science and Technology.

REFERENCES

- Bae, Y. S., S. A. Ju, J. Y. Kim, J. K. Seo, S. H. Baek, J. Y. Kwak, B. S. Kim, P. G. Suh, and S. H. Ryu. 1999. Trp-Lys-Tyr-Met-Val-D-Met stimulates superoxide generation and killing of *Staphylococcus aureus* via phospholipase D activation in human monocytes. *J. Leukoc. Biol.* 65:241-248.

2. Bertram, E. M., P. Lau, and T. H. Watts. 2002. Temporal segregation of CD28 versus 4-1BBL-mediated costimulation: 4-1BBL influences T cell numbers late in the primary response and regulates the size of the memory response following influenza infection. *J. Immunol.* **168**:3777–3785.
3. Bertram, E. M., W. Dawicki, B. Sedgmen, J. L. Bramson, D. H. Lynch, and T. H. Watts. 2004. A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza *in vivo*. *J. Immunol.* **172**:981–988.
4. Borregaard, N., and J. B. Cowland. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**:3503–3521.
5. Cannons, J. L., P. Lau, B. Ghumman, M. A. DeBenedette, H. Yagita, K. Okumura, and T. H. Watts. 2001. 4-1BBL induces cell division, sustains survival and enhances effector function of CD4 and CD8 T cells with similar efficacy. *J. Immunol.* **167**:1313–1324.
6. Conlan, J. W., and R. J. North. 1991. Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. *J. Exp. Med.* **174**:741–744.
7. Conlan, J. W., and R. J. North. 1994. Neutrophils are essential for early anti-*L. monocytogenes* defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. *J. Exp. Med.* **179**:259–268.
8. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T cell immunity? *Nat. Rev. Immunol.* **3**:609–620.
9. Czuprynski, C. J., J. F. Brown, N. Maroushek, R. D. Wagner, and H. Steinberg. 1994. Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to *L. monocytogenes* infection. *J. Immunol.* **152**:1836–1846.
10. DeLeo, F. R., and M. T. Quinn. 1996. Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* **60**:677–691.
11. Devadas, S., L. Zaritskaya, S. G. Rhee, L. Oberley, and M. S. Williams. 2002. Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and Fas ligand expression. *J. Exp. Med.* **195**:59–70.
12. Dunn, P. L., and R. J. North. 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. *Infect. Immun.* **59**:2892–2900.
13. Emoto, M., Y. Emoto, I. B. Buchwalow, and S. H. Kaufmann. 1999. Induction of IFN- γ -producing CD4⁺ natural killer T cells by *Mycobacterium bovis* bacillus Calmette-Guérin. *Eur. J. Immunol.* **29**:650–659.
14. Emoto, M., Y. Emoto, and S. H. Kaufmann. 1995. IL-4 producing CD4⁺TCR $\alpha\beta$ ^{int} liver lymphocytes: influence of thymus, β_2 -microglobulin and NK1.1 expression. *Int. Immunol.* **7**:1729–1739.
15. Futagawa, T., H. Akiba, T. Kodama, K. Takeda, Y. Hosoda, H. Yagita, and K. Okumura. 2002. Expression and function of 4-1BB and 4-1BB ligand on murine dendritic cells. *Int. Immunol.* **14**:275–286.
16. Goodman, E. B., and A. J. Tenner. 1992. Signal transduction mechanisms of C1q-mediated superoxide production. Evidence for the involvement of temporally distinct staurosporine-insensitive and sensitive pathways. *J. Immunol.* **148**:3920–3928.
17. Greenberg, S., and S. Grinstein. 2002. Phagocytosis and innate immunity. *Curr. Opin. Immunol.* **14**:136–145.
18. Gregory, S. H., A. J. Sagnimeni, and E. J. Wing. 1996. Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. *J. Immunol.* **157**:2514–2520.
19. Gregory, S. H., and E. J. Wing. 1998. Neutrophil-Kupffer-cell interaction in host defenses to systemic infections. *Immunol. Today* **19**:507–510.
20. Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1996. Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infect. Immun.* **64**:3512–3517.
21. Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**:3007–3017.
22. Hasegawa, H., K. Suzuki, S. Nakaji, and K. Sugawara. 1997. Analysis and assessment of the capacity of neutrophils to produce reactive oxygen species in a 96-well microplate format using lucigenin- and luminol-dependent chemiluminescence. *J. Immunol. Methods* **210**:1–10.
23. Heinisch, I. V. W. M., I. Daigle, B. Knopfl, and H. U. Simon. 2000. CD137 activation abrogates granulocyte-macrophage colony-stimulating factor-mediated anti-apoptosis in neutrophils. *Eur. J. Immunol.* **30**:3441–3446.
24. Hiroyuki, M., E. Dale, S. David, M. Norikatsu, C. Yang, E. M. Mark, O. Junichi, M. Hiroyuki, and T. Akira. 2003. Generation and function of reactive oxygen species in dendritic cells during antigen presentation. *J. Immunol.* **171**:3010–3018.
25. Kim, J. A., B. J. Averbook, K. Chambers, K. Rothchild, J. Kjaergaard, R. Papay, and S. Shu. 2001. Divergent effects of 4-1BB antibodies on antitumor immunity and on tumor-reactive T-cell generation. *Cancer Res.* **61**:2031–2037.
26. Kwon, B., H. W. Lee, and B. S. Kwon. 2002. New insights into the role of 4-1BB in immune responses: beyond CD8⁺ T cells. *Trends Immunol.* **23**:378–380.
27. Kwon, B. S., J. C. Hurtado, Z. H. Lee, K. B. Kwack, S. K. Seo, B. K. Choi, B. H. Koller, G. Wolisi, H. E. Broxmeyer, and D. S. Vinay. 2002. Immune responses in 4-1BB (CD137)-deficient mice. *J. Immunol.* **168**:5483–5490.
28. Lee, H. W., S. J. Park, B. K. Choi, H. H. Kim, K. O. Nam, and B. S. Kwon. 2002. 4-1BB promotes the survival of CD8⁺ T lymphocytes by increasing expression of Bcl-x(L) and Bfl-1. *J. Immunol.* **169**:4882–4888.
29. Lloyd, A. R., and J. J. Oppenheim. 1992. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol. Today* **13**:169–172.
30. Mackaness, G. B. 1962. Cellular resistance to infection. *J. Exp. Med.* **116**:381–406.
31. Melero, I., J. V. Johnston, W. W. Shufford, R. S. Mittler, and L. Chen. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell Immunol.* **190**:167–172.
32. Melero, I., W. W. Shufford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellstrom, R. S. Mittler, and L. Chen. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med.* **3**:682–685.
33. Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. Kaufmann. 1993. Different roles of $\alpha\beta$ and $\gamma\delta$ T cells in immunity against an intracellular bacterial pathogen. *Nature* **365**:53–56.
34. Naseef, W. M., and R. A. Clark. 2000. Granulocytic phagocytes, p. 89–112. In G. L. Mandel, J. E. Bennett, and R. Dolin (ed.), *Basic principles in the diagnosis and management of infectious diseases*, vol. 1. Churchill Livingstone, New York, N.Y.
35. North, R. J., and J. W. Conlan. 1998. Immunity to *L. monocytogenes*. *Chem. Immunol.* **70**:1–20.
36. Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs. 1988. Role of hemolysin for the intracellular growth of *L. monocytogenes monocytogenes*. *J. Exp. Med.* **167**:1459–1471.
37. Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raech, C. P. Larsen, T. C. Pearson, J. A. Ledbetter, A. Aruffo, and R. S. Mittler. 1997. 4-1BB costimulatory signals preferentially induce CD8⁺ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses. *J. Exp. Med.* **186**:47–55.
38. Unanue, E. R. 1997. Why listeriosis? A perspective on cellular immunity to infection. *Immunol. Rev.* **158**:5–9.
39. Vassiloyanopoulos, A. P., S. Okamoto, and J. Fierer. 1998. The crucial role of polymorphonuclear leukocytes in resistance to *Salmonella dublin* infections in genetically susceptible and resistant mice. *Proc. Natl. Acad. Sci. USA* **95**:7676–7681.
40. Vignais, P. V. 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol. Life Sci.* **59**:1428–1459.
41. Vinay, D. S., and B. S. Kwon. 1998. Role of 4-1BB in immune responses. *Semin. Immunol.* **10**:481–489.
42. Wilcox, R. A., D. B. Flies, G. Zhu, A. J. Johnson, K. Tamada, A. I. Chapoval, S. E. Strome, L. R. Pease, and L. Chen. 2002. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. *J. Clin. Invest.* **109**:651–659.
43. Wu, Z. Q., A. Q. Khan, Y. Shen, K. M. Wolcott, W. Dawicki, T. H. Watts, R. S. Mittler, and C. M. Snapper. 2003. 4-1BB (CD137) differentially regulates murine *in vivo* protein- and polysaccharide-specific immunoglobulin isotype responses to *Streptococcus pneumoniae*. *Infect. Immun.* **71**:196–204.
44. Xu, D., P. Gu, P. Y. Pan, Q. Li, A. I. Sato, and S. H. Chen. 2004. NK and CD8⁺ T cell-mediated eradication of poorly immunogenic B16-F10 melanoma by the combined action of IL-12 gene therapy and 4-1BB costimulation. *Int. J. Cancer* **109**:499–506.
45. Ye, Z., I. Hellstrom, M. Hayden-Ledbetter, A. Dahlin, J. A. Ledbetter, and K. E. Hellstrom. 2002. Gene therapy for cancer using single-chain Fv fragments specific for 4-1BB. *Nat. Med.* **8**:343–348.