A Novel Role of CD30L/CD30 Signaling by T-T Cell Interaction in Th1 Response against Mycobacterial Infection

Ce Tang, Hisakata Yamada, Kensuke Shibata, Hiromi Muta, Worawidh Wajjwalku, Eckhard R. Podack and Yasunobu Yoshikai

J Immunol 2008; 181:6316-6327; doi: 10.4049/jimmunol.181.9.6316
http://www.jimmunol.org/content/181/9/6316

References
This article cites 49 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/181/9/6316.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Novel Role of CD30L/CD30 Signaling by T-T Cell Interaction in Th1 Response against Mycobacterial Infection

Ce Tang,* Hisakata Yamada,* Kensuke Shibata,* Hiromi Muta,† Worawidh Wajjwalku,‡ Eckhard R. Podack,§ and Yasunobu Yoshikai2*

A CD30 ligand (CD30L, CD153) is a type II membrane-associated glycoprotein belonging to the TNF family. To illustrate the potential role of CD30L in CD4⁺ Th1 cell responses, we investigated the fate of Ag-specific CD4⁺ T cells in CD30L-deficient (CD30L⁻/⁻) mice after Mycobacterium bovis bacillus Calmette-Guérin (BCG) infection. The number of bacteria was significantly higher in organs of CD30L⁻/⁻ mice than in wild-type (WT) mice 4 wk postinfection. The numbers of purified protein derivative or Ag85B-specific-IFN-γ-producing-CD4⁺ T cells in spleen, lung, or peritoneal exudate cells were significantly fewer in CD30L⁻/⁻ mice than in WT mice. During the infection, CD30L was expressed mainly by CD4⁺CD3⁺CD4⁺ T cells but not by CD3⁺CD8⁺ T cells, B cells, dendritic cells, or macrophages. Costimulation with agonistic anti-CD30 mAb or coculturing with CD30L-transfected P815 cells restored IFN-γ production by CD4⁺ T cells from BCG-infected CD30L⁻/⁻ mice. Coculturing with CD30L⁺/⁺ CD4⁺ T cells from BCG-infected WT mice also restored the number of IFN-γ⁺CD30L⁺CD4⁺ T cells. When transferred into the CD30L⁺/⁺ mice, Ag-specific donor CD30L⁻/⁻ CD4⁺ T cells capable of producing IFN-γ were restored to the compared level seen in CD30L⁺⁺/⁺ CD4⁺ T cells on day 10 after BCG infection. When naive CD30L⁺⁺/⁺ T cells were transferred into CD30L⁻/⁻ mice, IFN-γ-producing-CD4⁺ T cells of donor origin were normally generated following BCG infection, and IFN-γ-producing-CD30L⁺⁺/⁺ CD4⁺ T cells of host origin were partly restored. These results suggest that CD30L/CD30 signaling executed by CD30⁺ T-CD30⁺⁺ T cell interaction partly play a critical role in augmentation of Th1 response capable of producing IFN-γ against BCG infection. The Journal of Immunology, 2008, 181: 6316–6327.

Several members of the TNFR superfamily (TNFRSF) and TNF superfamily have been shown to regulate the fate of T cell immunity (1–3). Members of the TNFR superfamily such as Fas and TNFRI, which contain a death domain, are the adult equivalent of inducer cells for the development of lymph node and Peyser’s patches in ontogenesis (9–11). CD30, the receptor for members of TNFSF-TNFRSF play important roles in proliferation, differentiation, and survival of T cells. It remained to be elucidated whether CD30L/CD30 signaling is linked to a physiological step for the differentiation of a specific Th cell subset.

It is widely accepted that protection against infection with mycobacteria such as Mycobacterium bovis and Mycobacterium tuberculosis depends mainly on IFN-γ produced by CD4⁺ Th1 cells (25, 26). There are several lines of evidence that the interaction of certain members of TNFSF-TNFRSF play important roles in protection against mycobacterial infection. TNF and lymphotoxin are involved in protection against mycobacterial infection via macrophage activation and granuloma formation (27). CD40 is required for optimal induction of protective immunity to M. tuberculosis through IL-12 production by DCs, whereas CD40 ligand is dispensable for the optimal priming of T cells and control of aerosol M. tuberculosis infection; an alternative ligand for CD40 might function for IL-12 production by DCs (28). Flórido et al. (20) recently reported with neutralizing mAbs that CD30L was important in protection against M. avium infection, whereas CD70 (CD27 ligand), CD134 ligand (OX40 ligand) or CD137 ligand (4-1BBL) were not involved in this protection. Furthermore, they showed that CD30⁻/⁻ mice were susceptible to i.v. infection with M. avium accompanied with decreased Th1 responses. In contrast
to the CD40 ligand/CD40 interaction, both CD30 and CD30L may be indispensable for optimal priming of Th1 cells and protection against i.v. infection with *M. avium*.

In this study, we found that CD30L−/− mice were susceptible to *M. bovis* bacillus Calmette-Guérin (BCG) infection with impaired Th1 responses, and that CD30L/CD30 signaling executed by CD30+/−T-CD30L−/− T cell interaction is suggest to play a important role in amplification of Th1 responses.

**Materials and Methods**

**Mice**

The generation and preliminary characterization of BALB/c background CD153 (CD30L−/−) mice were previously described (29). Age- and sex-matched BALB/c mice (Charles River Japan) were used as controls. C57BL/6 background CD153+/− mice were backcrossed for >6 generations to C57BL/6 mice, and littermates CD153+/− mice were used as control mice. C57BL/6 Ly5.1 mice were used as donor or host in the cell transfer experiments. All mice were used at 6–8 wk of age, and maintained under specific pathogen-free conditions.

**Micro-organism**

Lyophilized *M. bovis* BCG (Tokyo strain) was purchased from Kyowa Pharmaceuticals, and the generation of Age 85B-expressing r BCG (rBCG-Ag85B) was described previously (30). BCG or rBCG-Ag85B were dissolved in 7H9 medium (Difco) enriched with albumin-dextrose-catalase (Difco). The viable bacterial numbers were determined by a 7H10 (Difco) plate count. Cold PBS. Serial dilutions of the samples were plated on Middlebrook 7H10 medium enriched with oleic acid-albumin-dextrose-catalase, and colony growth was observed in the spleen on days 28, 42, and 63 (p < 0.05). Thus, CD30L−/− mice were susceptible to bacillus Calmette-Gueñas (BCG) infection with impaired Th1 response in CD30L−/− mice from day 14 to day 63 after BCG infection (p < 0.05 or 0.01). A similar tendency in bacterial growth was observed in the spleen on days 28, 42, and 63 (p < 0.05). Thus, CD30L−/− mice were more susceptible to BCG infection than were WT mice.

**Results**

**Flow cytometric analysis and intracellular cytokine staining**

Splenocytes, PECs, or lung MNCs were preincubated with a culture supernatant from 2.4G2 to prevent nonspecific staining. After a washing, cells were stained with various combinations of mAbs. The stained cells were analyzed using a FACS-Calibur flow cytometer (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences). For the intracellular cytokine staining (Cytokine FACS), splenocytes, PECs, or lung MNCs were incubated with 10 μmol/ml peptide 25 or 1 μg/ml PMA + 50 μg/ml ionomycin, and 10 μg/ml brefeldin A (Sigma-Aldrich) for 4 h, or with 5 μg/ml purified protein derivative (PPD, Japan BCG Association) for 6 h at 37°C and 5% CO2 with 10 μg/ml brefeldin A added for the last 2 h in 48-well flat-bottom plates at a concentration of 5 × 105/well in a volume of 500 μl of RPMI 1640 containing 10% FCS. After culture, cells were surface stained with various combinations of mAbs and then were subjected to intracellular cytokine staining using a Fast Immune Cytokine System (BD Biosciences). Samples were acquired in a FACS-Calibur flow cytometer and analyzed by CellQuest software.

**In vitro culture and cytokine ELISA**

Nylon wool-passed T cell-enriched splenocytes were incubated with anti-CD4 mAb microbeads, and CD4− T cells were purified to >90% by positive selection using autoMACS and then resuspended in RPMI 1640 and added to 96-well plates at a concentration of 2 × 103 cells/well. Cells were cultured with 5 μg/ml PPD (Japan BCG Association) in the presence of mitomycin C (MMC)-treated splenocytes (1 × 108) from naive WT or CD30L−/− mice for 48 h at 37°C. In some experiments, purified CD4+ T cells were cocultured with CD30L−/−-transfected P815 cells or isotype control P815 cells or were co-cultured with plate-coated anti-CD30 mAb or hamster IgG, or soluble antagonistic anti-CD30 mAb. In some experiments, steroid IgG plate culture inserts (Millipore) were used to inhibit CD30+/−CD4+ T cells from contacting CD30L−/−CD4+ T cells cocultured in the same wells (by adding CD30+/−CD4+ T cells into the membrane insert to separate them from CD30L−/−CD4+ T cells cultured at the bottom of the well). Supernatants were collected, and concentrations of IFN-γ were measured using ELISA development kits (Genzyme Diagnostics).

**Adoptive transfer**

Splenocytes were washed and passed though nylon wool columns. CD4− T cells that had been negatively purified using autoMACS by depletion of the cells expressing CD8a, B220, CD11c, NK1.1, γδTCR, or MHC class II were resuspended in PBS and then adoptively transferred i.v. into naive recipient mice. Twenty-four hours after the transfer, the recipient mice were challenged i.p. with 1.5 × 106 CFU/mouse rBCG-Ag85B and were sacrificed 10 or 14 days later. The cells from spleen or PECs were intracellularly stained and were analyzed by identifying the transferred CD4+ T cells by staining with mAbs to Ly5.1/Ly5.2 and CD4.

**Statistical analysis**

The statistical significance of the data was determined by a Student’s *t* test; a value of *p* < 0.05 was considered significant.
To further examine characteristics of the CD4⁺ Th1 population in CD30L⁻/⁻ mice after BCG infection, we harvested the PECs and splenocytes of mice challenged i.p. with BCG 21 days previously, stimulated them with PPD, and then stained for IL-7Rα (CD127), CD62 ligand (CD62L), and intracellular IFN-γ. The CD62L⁺CD127⁺ central memory CD4⁺ T cells scarcely secreted IFN-γ on day 21 after primary infection (data not shown), and IFN-γ was produced mostly by CD127⁻CD44⁺ (effector) and CD127⁺CD44⁺ (effector/memory) CD4⁺ T cells (Ref. 33; Fig. 1C). Both the IFN-γ-secreting effector and effector/memory CD4⁺ T cells in the spleen and PECs were detected from WT mice, whereas numbers of such cells from CD30L⁻/⁻ mice were greatly reduced after BCG infection (p < 0.05 or 0.01).

Expression of CD30L on activated CD4⁺ T cells

We next examined the expression of CD30L on macrophages, DCs, B cells, or T cells after BCG infection. Neither F4/80⁺CD11b⁺CD3⁻ macrophages, nor CD11c⁺CD3⁻ DCs, nor B220⁺CD3⁻ B cells from the spleen of WT mice expressed CD30L on day 10 (Fig. 2A) or day 21 (data not shown) after BCG infection. On the other hand, a significant fraction of CD3⁺CD4⁺ T cells expressed CD30L in WT mice infected with BCG 10 days previously (Fig. 2A) or 21 days previously (data not shown). It was reported that CD30L was constitutively expressed on unique CD4⁺CD3⁻CD11c⁻ accessory cells (11) but that CD4⁺CD3⁺CD11c⁻CD30L⁺ cells were detected in the spleen of the WT mice with very low levels (0.02% of whole splenocytes) after BCG infection (Fig. 2B). CD30L was preferentially expressed on CD3⁺CD4⁺CD44⁺ but not on CD8⁺ T cells (Fig. 2C). Its expression was rapidly up-regulated after in vitro stimulation with PPD for 6 h to 2–4 times more than the expression level before stimulation (Fig. 2C). CD30 expression was also examined on T cells on day 10 or 21 after BCG infection. Although the CD30 expression was not detected on freshly isolated T cells in the spleen of infected mice, an appreciable number of CD30⁺ cells was detected in the CD4⁺ T cell population from BCG-infected mice after 24 h in vitro culture and the number was further increased after 24 h in vitro culture with anti-CD3 mAb stimulation (Fig. 2D).

Recovery of IFN-γ production by CD30L⁻/⁻CD4⁺ T cells by costimulation with anti-CD30 mAb or coculture with CD30L-transfected P815 cells

To verify a possibility that APC activity in CD30L⁻/⁻ mice is impaired in transmitting the signaling to CD30⁺ T cells, we purified CD4⁺ T cells in the spleen from WT or CD30L⁻/⁻ mice infected i.p.
with BCG 10 days previously and cultured them with MMC-treated T cell-depleted splenocytes as APCs from naive WT or CD30L−/− mice. After culture with PPD for 48 h, supernatant was harvested, and IFN-γ production was assessed by ELISA. There were no differences in the production of IFN-γ between the two groups composed of the same CD4+ T cells and different APCs (Fig. 3A). We next mixed the

FIGURE 2. Expression of CD30L on APCs and T cells, and expression of CD30 on T cells. Splenocytes from WT (red) or CD30L−/− (blue) mice infected i.p. with BCG 10 days previously were stained with anti-F4/80, -CD11b, -CD11c, -B220, or -CD3 mAbs for APCs (A and C), or anti-CD3, -CD4, or -CD44 mAbs for T cells (A) and anti-CD153 mAb (B). C, Splenocytes or PECs were stimulated with or without PPD and then stained with anti-CD3, -CD4, -CD44, and -CD153 mAbs. D. Splenocytes from WT mice infected with BCG 10 days previously were cultured without any stimulation for 24 h (left), or splenocytes from naive WT mice were stimulated with plate-coated anti-CD3 and soluble anti-CD28 mAbs for 24 h (right), and then were stained with anti-CD3, -CD4, and -CD30 mAbs. Each number in a quadrant indicates the percentage of each quadrant. Each number in parentheses indicates the percentage of total splenocytes or PECs. Representative data are shown from three separate experiments.
purified CD4⁺ T cells from WT mice infected with BCG 10 days previously were purified by nylon wool and autoMACS. A, WT or CD30L⁻/⁻ CD4⁺ T cells were mixed with MMC-treated T cell-depleted splenocytes (by autoMACS) from naive WT or CD30L⁻/⁻ mice, respectively, with PPD stimulation. B, WT CD4⁺ T cells were cocultured with MMC-treated T cell-depleted splenocytes from WT or CD30L⁻/⁻ mice infected with BCG 10 days previously, respectively, without stimulation. CD30L⁻/⁻ or WT CD4⁺ T cells were cultured with plate-coated anti-CD30 mAb or hamster IgG of various doses (C), or CD30L⁻/⁻ CD4⁺ T cells were cocultured with CD30L-transfected P815 or control P815 cells of various doses (D) in the presence of PPD and MMC-treated splenocytes of naive CD30L⁻/⁻ mice. Forty-eight hours later, IFN-γ concentrations of culture supernatants were assayed by ELISA. Representative data are shown from three separate experiments; each column and vertical bar indicates means ± SD of three mice of each group. *, p < 0.05; **, p < 0.01.
FIGURE 4. Recovery of CD30L−/− IFN-γ+CD4+ T cells. A, C57BL/6 background CD30L−/− mice and WT mice were infected with rBCG-Ag85B 21 days previously, and cells were harvested and cultured without stimulation or with PPD, peptide 25, and intracellularly stained for IFN-γ. B, CD4+ T cells of the spleen from Ly5.1 WT or Ly5.2 CD30L−/− mice infected with rBCG-Ag85B 14 days previously were purified. T cell-depleted splenocytes from naïve CD30L−/− Ly5.2 mice as APCs were treated with MMC. WT CD4+ T, CD30L−/− CD4+ T and APCs were mixed together with a ratio of 2:1:3, and then cocultured for 48 h in the presence of PPD. In some cultures, anti-CD30L mAb (50 μg/ml) was added, and sterilized culture plate inserts were used. After 48 h of culture, cells were restimulated with PMA + ionomycin or were not restimulated and were intracellularly stained for IFN-γ. The analysis was set on Ly5.1+ Ly5.1+ CD4+. Each number indicates the percentage of each quadrant. Representative data from three separate experiments are shown (A, as means ± SD of three mice of each group. *, p < 0.05).
previously (data not shown). These results revealed no differences in APC activity between CD30L−/− and WT mice.

To certify that IFN-γ+CD4+ T cells accounted for the interactions between CD30−/−CD4+ T cells and CD30L+ cells, we first cultured CD4+ T cells from the spleen of CD30L−/− or WT mice infected with BCG 10 days previously with plate-coated agonistic anti-CD30 mAb for 48 h in the presence of PPD and APCs from naive CD30L−/− mice and then performed

FIGURE 5. Recovery of Th1 responses of CD30L−/− CD4+ T cells by transferring them into CD30L+ mice. CD4+ T cells in the spleen of naive CD30L−/− and CD30L+ mice of Ly5.2 C57BL/6 background were purified by nylon wool and autoMACS and were transferred i.v. into Ly5.1 CD30L+ mice with the dose of 2 × 10⁶ cells/mouse. Twenty-four hours after the adoptive transfer, the recipients were infected i.p. with rBCG-Ag85B; 10 days later, splenocytes and PECs from these recipients were harvested, cultured with PPD or peptide 25 or not stimulated, and stained for intracellular IFN-γ. The analysis gate of dot plots was set on Ly5.2+CD4+ T cells. A, Each number indicates the percentage of each quadrant. Representative data are shown from two separate experiments and are expressed as means ± SD of three mice of each group. B, Absolute numbers of donor IFN-γ-producing CD4+ T cells from the spleen or PECs of host Ly5.1 WT mice were calculated by multiplying total cell numbers by the total percentage of these cells in each dot-plot figure. Each column and vertical bar indicates means ± SD of three mice of each group.
ELISA for IFN-γ production. As shown in Fig. 3C, the levels of IFN-γ secreted by both CD30L−/− CD4+ and WT CD4+ T cells in the supernatants were significantly higher when co-cultured with anti-CD30 mAb (p < 0.01 or p < 0.05).

We next cocultured purified CD4+ T cells from the spleen of CD30L−/− mice infected with BCG 10 days previously with CD30L-transfected P815 cells of various doses in the presence of PPD and APCs from naive CD30L−/− mice. After culture for 48 h, we harvested the supernatants and did ELISA analysis for IFN-γ production. As shown in Fig. 3D, the level of IFN-γ secreted by CD30L−/− CD4+ T cells was significantly higher after coculturing with CD30L-transfected P815 cells than coculturing with control P815 cells (p < 0.05). Thus, these results proved that CD30 signaling via CD30L played a role in controlling the Th1 response.

Coculture with CD30L+/+CD4+ T cells could restore the IFN-producing CD30L−/−CD4+ T cells

To confirm the concordance of experiment results from CD30L−/− mice with a BALB/c background and those with a C57BL/6 background, we examined the susceptibility of CD30L−/− mice with a C57BL/6 background against BCG infection. Similar to CD30L−/− mice with a BALB/c background, those with a C57BL/6 background showed an increased susceptibility to rBCG-Ag85B infection as assessed by bacterial growth (data not shown). IFN-γ-producing CD4+ T cells in response to PPD or H-2Aβ-binding peptide 25 were significantly impaired in CD30L−/− mice with a C57BL/6 background on days 14 (data not shown) and 21 after BCG infection (Fig. 4A, p < 0.05). Thus, the susceptibility of CD30L−/− mice to BCG infection with an impaired Th1 response is irrespective of their genetic background.

Because CD30L is mainly expressed on CD4+ T cells but not B cells, macrophages or DCs after BCG infection (Fig. 2, A and B), we assume that CD30L+CD4+ T cells may supply the CD30L to transmit the CD30L/CD30 signaling on CD30+ CD4+ T cells. To verify this, we cocultured CD4+ T cells from the spleen of Ly5.1 CD30L+/+ C57BL/6 mice infected with BCG 14 days previously with CD4+ T cells from Ly5.2 CD30L−/− mice of a C57BL/6 background also infected with BCG 14 days previously in the presence of PPD and T cell-depleted APCs from naive CD30L−/− mice (Ly5.2) for 48 h, then restimulated these cells with PMA + ionomycin for another 4 h, and performed cytokine FACS for intracellular IFN-γ. As shown in Fig. 4B, after coculturing with CD30L+/+ CD4+ T cells, the frequency of CD30L−/− IFN-γ+ CD4+ T cells was markedly restored compared with those cultured solely. To determine whether this restoration was attributed to the CD30L/CD30 signaling, we added anti-CD30 mAb in the coculture group to block the transmission of CD30L/CD30 signaling. As shown in Fig. 4B, IFN-γ-producing CD30L−/− CD4+ T cells were markedly reduced by addition of anti-CD30 mAb, and though after coculture, the frequency of IFN-γ-producing CD30L−/− CD4+ T cells has no difference from those cultured solely by addition of anti-CD30 mAb. To determine whether direct contact between CD30L+ cells and CD30+ cells is indispensable for augmentation of Th1 responses, sterilized culture plate inserts were used to inhibit cell-to-cell contact between CD30L−/− CD4+ T cells and CD30L−/− CD4+ T cells but allow cytokines to penetrate freely. The culture plate inserts totally blocked the restoration of IFN-γ-producing T cells, indicating that direct cell-to-cell interaction was required for restoration of Th1 responses. These results suggested that the transmission of CD30L/CD30 signaling was executed by CD30L+/+ T-CD30− CD4+ T cell interactions in Th1 responses against Mycobacterium infection.

Recovery of Th1 response in CD30L−/− CD4+ T cells by transferring them into CD30L+/+ mice

To identify whether CD30L−/− CD4+ T cells could normally differentiate into Th1 cells capable of producing IFN-γ in the in vivo environment abounding of CD30+ cells, we transferred naive CD30L−/− or WT purified CD4+ T cells (Ly5.2) into Ly5.1 CD30L+/+ mice and then infected the host mice with rBCG-Ag85B. On day 10 after the infection, we harvested cells of the spleen and PECs from the host mice and performed cytokine FACS for IFN-γ by identifying the transferred cells with staining mAbs to Ly5.2 and CD4. The FACS profile is shown in Fig. 5A, and the absolute numbers of the donor IFN-γ-producing CD4+ T cells from the spleen or PECs of the host mice are shown in Fig. 5B. Both PPD- and peptide 25-specific IFN-γ-producing CD4+ T cells from CD30L−/− mice sufficiently expanded, and the numbers of such cells were restored to the same levels of WT donor CD4+ T cells. Gating on Ly5.2 and CD4+ cell populations showed that there were no differences in numbers of IFN-γ+CD4+ T cells between these two groups of host mice receiving knockout or WT Ly5.2 CD4+ T cells (data not shown).

Recovery of Th1 response in CD30L−/− CD4+ T cells by transferring CD30L−/− T cells into CD30L+/+ mice

Finally, to confirm CD30L+/+CD30 signaling executed by CD30L+/+ T-CD30− T cell interactions in Th1 responses against Mycobacterium infection in vivo, we transferred CD4+ T cells from naive CD30L−/− mice (Ly5.1) into Ly5.2 CD30L+/+ mice, which were then inoculated with rBCG-Ag85B. As controls, we examined PPD-specific IFN-γ-producing CD30L−/− CD4+ T cells in both untransferred Ly5.2 CD30L−/− mice and those transferred with CD4+ T cells from naive CD30L−/− mice (Ly5.2) (although the donor and host cells could not be distinguished). On day 14 after the infection, we harvested cells from the spleen and PECs, and performed cytokine FACS for IFN-γ. As shown in Fig. 6A, PPD-specific IFN-γ-producing CD30L−/− CD4+ T cells of donor

**FIGURE 6.** Recovery of Th1 responses of CD30L−/− CD4+ T cells by transferring CD30L+/+ CD4+ T cells into CD30L−/− mice. CD4+ T cells from the spleen of naive Ly5.1 CD30L−/− mice or Ly5.2 CD30L+/+ mice were purified and transferred i.v. into Ly5.2 CD30L−/− mice with the dose of 1.5 X 107 cells/mouse. The recipients, normal CD30L−/− and untransferred CD30L−/− mice were then infected with rBCG-Ag85B; 14 days later, cells from the spleen and PECs of these infected mice were harvested, cultured with PPD or not stimulated, and then intracellularly stained for IFN-γ. The analysis gate of dot plots was set on Ly5.2+CD4+ or Ly5.2+CD4− T cells. A, FACS profiles of each group. Each number in a quadrant indicates the percentage of each quadrant. Representative data are shown from two separate experiments and are expressed as means ± SD of three mice of each group. *, p < 0.05; **, p < 0.01, showing the significant differences from host + donor: CD30L+/+ groups as control. B, Absolute numbers of host CD30L−/− IFN-γ-producing CD4+ T cells from the spleen or PECs of recipient mice (WT donor) compared with such cells in untransferred CD30L−/− mice. Absolute cell numbers were calculated by multiplying total cell numbers by the total percentage of these cells in each dot-plot figure. *, p < 0.05.
origin were normally generated in the host CD30L−/− mice, suggesting that CD30L+ T-CD30+ T cell interactions of donor origin is enough for induction of Th1 response against BCG infection. The number of IFN-γ-producing CD30L+ CD4+ T cells were restored, albeit partly in the host CD30L−/− mice by being transferred with CD4+ T cells from naive CD30L+/+ mice (Fig. 6, p < 0.05 or 0.01). These results revealed that the transmission of CD30L-CD30 signaling executed by CD30L+ T-CD30+ T cell interactions occurred in vivo for Th1 responses against Mycobacterium infection.

Discussion

A number of recent studies suggested that CD30L/CD30 signaling is also linked to Th1 cell responses and Th1-associated diseases (19–22). Blocking CD30L/CD30 signaling by treatment with anti-CD153 mAb prevented or delayed the spontaneous development of diabetes in young NOD mice, in which Th1 cells are involved in the induction of diabetes (34). Ab treatment prolonged the survival of mice in a CD4+ T cell-mediated lethal graft-vs-host disease model (29). Florido et al. reported that in vivo treatment with anti-CD153 mAb resulted in exacerbated infection with M. avium as assessed by bacterial growth. Furthermore, they showed that CD30L−/− mice are susceptible to i.v. infection with M. avium associated with decreased T cell expansion and reduced IFN-γ responses as a result of reduced polarization of the Ag-specific IFN-γ-producing T cells (20). We have recently reported that CD30L−/− mice were susceptible to Th2 type experimentally oxazolone-induced colitis, but resistant to Th1 type trinitrobenzenesulfonic acid-induced acute colitis. The levels of Th2 type cytokines such as IL-4 and IL-13 in the lamina propria T cells were significantly higher, but the levels of IFN-γ were lower in oxazolone- or trinitrobenzenesulfonic acid-treated CD30L−/− mice than in WT mice (35). In this study, we found that CD30L−/− mice were also susceptible to i.e. or i.p. infection with BCG in association with decreased T cell expansion and reduced Th1 responses capable of producing IFN-γ.

A previous study with CD30L−/− mice showed no defect of CD30L−/− T cells in in vivo and in vitro responses. Amakawa et al. (36) reported that an in vitro T cell-proliferative response to anti-CD3 mAb or mitogen is comparable with that of controls. Consistently, we found that direct activation of T cells from CD30L−/− mice by anti-CD3-CD28 is not altered (data not shown); furthermore, adaptive transfer experiments showed that CD30L−/− T cells were able to differentiate normally into Th1 cells in CD30L+/+ mice after BCG infection. Thus, impaired induction of effector Th responses may not be due to intrinsic development of T cell defect in CD30L−/− mice.

CD4+ T cells initially stimulated in the presence of IL-12 and IFN-γ tend to develop into Th1 cells (37, 38). CD4+ T cell response to IL-12 is dependent on the expression of high-affinity IL-12R (39, 40), composed of two IL-12R subunits, β1 and β2 (41, 42). IFN-γ activates Jak1 and Jak2, causing the phosphorylation, dimerization, and nuclear translocation of STAT1 which is important for the induction of T-bet (43). T-bet can cause not only chromatin remodeling of the IFN-γ locus and trans activation of the IFN-γ gene but also induce IL-12Rβ2 chain expression, allowing IL-12/STAT4 signaling to optimize IFN-γ production, thereby completing Th1 development commitment process. CD30L/CD30 signaling may be involved in the process for a further differentiation into CD4+ Th1 cells from naive CD4+ T cells upon TCR triggering (22). However, Th1 differentiation of CD30L−/− T cells was reported to normally occur in vitro (36). We previously found that Ag-specific Th1 response was not affected in CD30L−/− mice after acute infection such as Listeria infection (data not shown). Thus, CD30L/CD30 might not be directly linked to a Th1 development commitment process.

We found that CD30L was expressed at low levels in resting naive T cells but that CD30L expression increased during initial T cell activation. Consistent with these expression patterns, Bowen et al. (14) and Harlin et al. (44) found that CD30L stimulated preactivated T cells but not naive resting T cells. The prediction of these expression kinetics is that CD30L-CD30 signaling on T cells would contribute during later stages of initial T cell activation and the reactivation of memory. An initial study shows that CD30L-CD30 signaling promoted T cell death by increasing the susceptibility to TNF-induced apoptosis correlating to decreased amounts of intracellular TNFR-associated factor (TRAF)-2 levels which are rapidly degraded during CD30 signaling. On the contrary, CD30 has the potential to induce TRAF-2-mediated NF-κB activation and can recruit TRAF-1 (3), and possibly also contribute to increased T cell survival (1, 2). Thus, it can be speculated that CD30L-CD30 signaling may initially promote cell survival of preactivated Th1 cells following BCG infection that preferentially induces a Th1 response, resulting in acceleration of Th1 response after BCG infection. CD30L-CD30 signaling may be important for amplification of already differentiated effector or memory Th1 cells. An apparent difference in the adaptive immune responses between BCG and Listeria is the interval required for its development. The adaptive response to BCG required a long interval compared with the response to Listeria. It is possible that CD30L-CD30 signaling for amplification of the Th response may differ between acute and chronic infection.

There are several lines of evidence showing that the CD30L-CD30 signaling is involved in Th2 cell responses and Th2-associated diseases (15–18). Treating OVA-immunized WT mice with anti-CD153 mAb resulted in significantly reduced airway inflammation, serum IgE and Th2 cytokine levels. Furthermore, CD30L-deficient mice showed impaired Th2 response against OVA (18). We have also found that CD30L−/− mice showed impaired Th2 response to OVA (data not shown). Therefore, CD30L-CD30 signaling might not be linked to a physiological step for a specific Th cell subset. Lane et al. (11, 45) have reported that both CD30L and OX40L are highly and constitutively expressed by CD4+CD3−CD11c− cells, which are located in B cell follicles and germinal center in the spleen and control the development of memory Th2 cells. They showed, by using OT-II-transgenic mice, that the transgenic Th2 cells lacking CD30 fail to survive when cocultured with CD4+CD3− cells compared with normal T cells, whereas the survival of transgenic Th1 cells was not affected in the absence of CD30 signaling. This raises another possibility that CD30L-CD30 signaling might affect Th response in a different manner between Th1 and Th2 cells. The normal functioning of CD30L-CD30 signaling may initially promote cell survival but at the same time set in motion a signal-dependent mechanism, leaving the cell more susceptible to subsequent apoptosis and thus regulating the physiological phenotypic selection of Th1-Th2 balance during the immune response. Additional experiments are needed to clarify the molecular mechanism for augmentation of the Th1 response in a CD30L-dependent manner.

CD30L/CD30 signaling was wildly examined and discussed over the years, and most of the in vitro experimental models were set to describe the interactions between CD30−/− T cells and CD30L+ APCs including unique CD4+CD3−CD11c− accessory cells, which are the adult equivalent of inducer cells for the
development of the lymph node and Peyer’s patches in ontogeny (46–49). In this study, CD30L was exclusively expressed on activated CD4^+ CD45RO^+ T cells but not on B cells, macrophages, or DCs during BCG infection, and in vitro experiments using T cell-depleted splenocytes from WT or CD30L−/− mice as APCs revealed no differences in APC activity between WT and knockout mice. Stimulation with CD30 signaling by agonistic anti-CD30 mAb increased Th1 response by CD4^+ T cells from CD30L−/− mice. These results proved that CD30 signaling via CD30L played a role in controlling Th1 response.

Coculturing with P815 cells transfected with CD30L gene or CD30L/CD4^+ T cells induced the restoration of IFN-γ^+ CD30L−/−CD4^+ T cells, which required direct cell-to-cell interaction and was inhibited by adding anti-CD153 mAb. Although the existence of interactions between CD30^+ T cells and CD30L^+CD3^−CD11c^− accessory cells could not be completely excluded, with the adoptive transfer experiment we supposed that CD30L^+ T-CD30^+ T cell interactions quite possibly existed after the infection of BCG and that CD30L−/−CD30^+CD4^+ Th1 cells could be amplified in a paracrine manner.

Modulation of CD30L/CD30 signaling by mAb could be useful for enhancing the efficacy of BCG vaccination to tuberculosis. CD30 signaling may have not only prolonged effector responses but also increased memory cells, because CD30L−/− mice showed a greater decrease in the number of not only the IL-7Ra^+ effector cells but also the IL-7Ra^+I1 memory subsets and resulted in a gradual decrease in the number of Ag-specific CD4^+ T cells over a period of several months. Additional experiments on M. tuberculosis infection may enable us to determine conclusively whether CD30 is useful for the development of new immunoprotective approaches against chronic infection. Taken together, our study suggests that agonistic Abs against CD30L/CD30 could be a novel adjuvant for BCG vaccination.

Acknowledgments
We thank Kazue Hirawatari for excellent technical assistance.

Disclosures
The authors have no financial conflict of interest.

References


