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CD30 Ligand/CD30 Plays a Critical Role in Th17 Differentiation in Mice

Xun Sun,*,† Hisakata Yamada,* Kensuke Shibata,* Hiromi Muta,‡ Kenzaburo Tani,‡ Eckhard R. Podack,§ and Yasunobu Yoshikai*

A CD30 ligand (CD30L; CD153) and its receptor, CD30, is a membrane-associated glycoprotein belonging to the TNF superfamily and TNFR superfamily. These were expressed preferentially by activated CD4⁺T cells. In this paper, we show that CD44^low^CD62^hi^ CD4⁺ T cells from CD30L^−/−^ or CD30^−/−^ mice exhibited impaired differentiation into Th17 cells but an increased ability to produce IL-2 after in vitro culture under Th17-polarizing conditions. Neutralization with IL-2 by anti–IL-2 mAb partly restored the ability of Th17 differentiation in CD30L^−/−^ or CD30^−/−^ T cells. Stimulation via CD30L by immobilized anti-CD30L mAb suppressed IL-2 production by CD30^−/−^CD4⁺ T cells, indicating that the reverse signal to CD30L is responsible for downregulation of IL-2 production. In vivo Th17 differentiation of CD30L^−/−^CD4⁺CD45RB^high^ T cells was also impaired after transfer into SCID mice, whereas CD30L^+/+^CD4⁺CD45RB^high^ T cells normally differentiated into Th17 cells in CD30L^−/−^SCID mice. The results of these studies demonstrate that CD30L/CD30 signaling executed by the T-T cell interaction plays a critical role in Th17 cell differentiation, at least partly via downregulation of IL-2 production. *The Journal of Immunology*, 2010, 185: 2222–2230.

I nterleukin-17A is a T cell-derived cytokine exerting inflammatory effects, and the helper CD4⁺ T cell producing IL-17A has been identified as a newly defined helper CD4⁺ T cell subset, Th17. Th17 differentiation in mice is potentially induced by synergistic activation by TGF-β and IL-6 through expression of a transcriptional factor, retinoid-related orphan receptor γ t (RORγt) (1, 2). IL-21 further sustains Th17 differentiation in an autocrine manner (3). IL-23 and IL-1β finalize the differentiation program of Th17 cells and help to maintain Th17 cells (4, 5). In contrast, IL-27 and IL-2 inhibit Th17 differentiation (6–8). Thus, a cytokine milieu plays an important role in regulation of Th17 cell differentiation.

A CD30 ligand (CD30L; CD153) is a 40-kDa type II membrane-associated glycoprotein belonging to the TNF superfamily (9) and is expressed on activated CD4⁺ Th cells in addition to macrophages, dendritic cells, B cells, and unique CD4⁺CD3⁻CD11c⁻ accessory cells (10–12). CD30, the receptor for CD30L (and belonging to the TNFR superfamily) was reported to be preferentially expressed by activated or memory Th cells but not by resting B or T cells (13–15). CD30L has the potential to induce TNFR-associated factor (TRAF)-2–mediated NF-κB activation via CD30 that can recruit several TRAFs (16, 17). As in other members of the TNF/TNFR family, a bidirectional, instead of a unidirectional, signal transduction occurs after CD30-CD30L engagement in T cells (18–20). Several lines of evidence show that CD30L/ CD30 signaling is involved in Th1 and Th2 cell responses and their associated diseases (21, 22). Recent studies have demonstrated that naturally occurring regulatory T cells (Tregs) suppressed allograft rejection mediated by memory CD4⁺ T cells via a CD30-dependent mechanism (23, 24). However, the role of CD30L in development of Th17 cells still remains unknown.

We show that CD30L/CD30 signaling plays a vital part in Th17 differentiation of CD4⁺ T cells in vitro and in vivo. This effect was partly mediated by downregulation of IL-2 production via reverse CD30L signaling executed by CD30⁺ T-CD30L⁺–T cell interaction.

Materials and Methods

Mice

Age- and sex-matched BALB/c, C57BL/6 (B6), and C.B.17 scid/scid male mice were purchased from Japan KBT (Shizuoka, Japan). The generation and preliminary characterization of CD30L^−/−^ (BALB/c background) mice were described previously (25, 26), and eight or more generations backcrossed onto C57BL/6 (B6) mice. The progeny of a cross of CD30L^−/−^ mice to SCID mice were intercrossed to generate CD30L^−/−^ SCID mice. CD30^−/−^ (B6.129P2-Tnfrsf8 < tm1Mak > /J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions and were offered food and water ad libitum. All mice were used at 6–8 wk of age. This study was approved by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University, Fukuoka, Japan. Experiments were carried out under the control of the Guidelines for Animal Experiments.

Abs and reagents

Abs for flow cytometry, FcγR-blocking mAb (CD16/32; 2.4G2), anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD62 ligand (MEL-14), anti-CD45RB (C363-16A), anti-CD44 (IM7), anti-CD153 (RM153), anti-Foxp3 (MF-14), anti–IL-17 (TC11-18H10.1), anti–IL-2 (JES6-5H4), anti–IFN-γ (XMG1.2), and Alexa Fluor 647 anti-STAT5 (pY694, 47) were purchased from BD Biosciences (San Diego, CA). Purified anti-CD3, anti-CD28 (37.51), anti–IFN-γ (R4-6A2), anti–IL-2 (JES6-1A12), and anti–IL-4 (11B11) mAbs were obtained from eBioscience (San Diego, CA).

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Armenian hamster IgG (eBio299Arm) was purchased from Wako Pure Chemicals (Osaka, Japan). Purified anti-CD153 (CD30L, RM153) and rat IgG2b (RTK4530) were purchased from BioLegend (San Diego, CA). Recombinant cytokines, including IL-6, TGF-β, IL-12, and IL-4, were purchased from PeproTech (Princeton, NJ).

Cell culture

CD44^{low}CD62^{hi}CD4$^+$ T cells were sorted and cultured as described previously (27). In brief, purity of naive CD4$^+$ T cells was confirmed by flow cytometry and was consistently >98%. A total of 2×10^5 naive CD4$^+$ T cells were cultured in wells of a 96-well plate coated with anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence or absence of IL-12 (5 ng/ml) and anti–IL-4 (10 μg/ml) for Th1 cell differentiation; IL-4 (25 ng/ml) and anti–IFN-γ (20 μg/ml) for Th2 cell differentiation; and TGF-β1 (5 ng/ml), IL-6 (25 ng/ml), anti–IL-4 (10 μg/ml), and anti–IFN-γ (10 μg/ml) for Th17 cell differentiation. After 4 d, supernatants were harvested and used to analyze cytokine production by ELISA, using an ELISA Development Kit (Genzyme Diagnostics, Cambridge, MA). In some experiments, naive CD4$^+$ T cells were cultured in Th17-polarizing conditions or TCR-stimuli conditions in the presence or absence of immobilized anti-CD30L, agonistic anti-CD30 (mCD30.1 25 μg/ml) (15), anti-mouse IL-2 (1 or 10 μg/ml), and respective isotype control Abs, and cytokine secretion in the supernatants was measured by ELISA.

Flow cytometry analysis and intracellular cytokine synthesis analysis

Cells were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2) for 15 min at 4°C, and then incubated with saturating amounts of FITC-, PE-, allophycocyanin-, and biotin-conjugated mAbs for 30 min at 4°C. To detect biotin-conjugated mAbs, cells were stained with APC-conjugated streptavidin; the stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). For intracellular cytokine staining, cells were washed and then cultured for a further 6 h in the presence of PMA (25 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich, St. Louis, MO); brefeldin A (BFA) (10 μg/ml; Sigma-Aldrich) was added for the last 5 h of incubation. Cells were harvested, washed, and incubated

for 30 min at 4°C with mAbs for surface staining and then subjected to intracellular cytokine staining using the Fast Immune Cytokine System according to the manufacturer's instructions (BD Biosciences). The data were analyzed with CellQuest software (BD Biosciences).

Intracellular staining of phosphorylated proteins

Purified naive CD4$^+$ T cells were incubated with or without Th17-polarizing conditions for 3 h, 24 h, or 48 h. Cells were fixed for 10 min at 37°C with Lyse/Fix Buffer (BD Biosciences Pharmingen) and Perm Buffer III (BD Biosciences Pharmingen) for 30 min on ice and intracellularly stained with anti-STAT5 (pY694; 47) for 30 min at 4°C; then they were analyzed by flow cytometry.

RNA isolation and real-time PCR analysis

For RNA expression, total RNA from the purified naive CD4$^+$ T cells was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) using FACSAria (BD Biosciences) before or after culture with anti-CD3 and anti-CD28. First-stranded cDNA was synthesized with Prime-Script^{TM} Reverse Transcriptase (Takara Bio, Otsu, Shiga, Japan) according to the manufacturer's instructions. cDNA was amplified with IL-2– or GAPDH-specific primers using Gradient PCR (Takara). The following gene-specific primers were used: IL-2, 5′-TCCAGAACATGCCGCAG-AG-3′, 5′-CCTGAGCAGGATGGAGAATTACA-3′; and GAPDH, 5′-CA-ACTCACTCAAGATTGTCAGCAA-3′, 5′-GGCATGGACTGTGGTCAT-GA-3′. Real-time RT-PCR was performed on an ABI PRISM thermal cycler (Applied Biosystems, Foster City, CA), using SYBR Premix Ex Taq (Takara).

T cell-mediated colitis model

SCID or CD30L$^{-/-}$ SCID mice were injected with a sorted CD4$^+$ CD45RB^{hi} T cell subpopulation from BALB/c or CD30L$^{-/-}$ mice with a BALB/c background in PBS (5×10^5 cells/mouse). Body weight, survival rate, and disease activity index were monitored for 5 wk. In some experiments, the lamina propria lymphocytes (LPLs) of colons were collected 4 wk later and examined for the percentage of IFN-γ–, IL-17A–, and IL-2–producing CD4$^+$ T cells or cytokine secretion, as analyzed by ELISA, or histologically after H&E staining.

FIGURE 1. An impaired ability of CD30L$^{-/-}$ or CD30$^{-/-}$ CD4$^+$ T cells to differentiate in Th17 cells in vitro. CD4$^+$CD44^{low}CD62^{hi} T cells were sorted from spleens of BALB/c or CD30L$^{-/-}$ mice with a BALB/c background (*A, D*), C57BL/6 (B6) or CD30L$^{-/-}$ mice with a B6 background (*B*), and B6 or CD30$^{-/-}$ mice with a B6 background (*C*); then cells were cultured with anti-CD3 and anti-CD28 mAbs only (−) or cultured with anti-CD3 and anti-CD28 mAbs under Th1-, Th2-, or Th17-polarizing conditions for 4 d. IFN-γ, IL-4, IL-17A, IL-17F, IL-21, and IL-22 secretions were analyzed by ELISA. Data indicate mean ± SD (five mice per group) from a representative in four separate experiments. Statistically significant differences are shown. ∗$p < 0.05$; ∗∗$p < 0.01$. *E*, The cultured T cells with a B6 background under Th17-polarizing conditions were stimulated with PMA, ionomycin, and BFA for 6 h, and then analyzed by intracellular cytokine staining for the expression of IL-17A and IFN-γ.
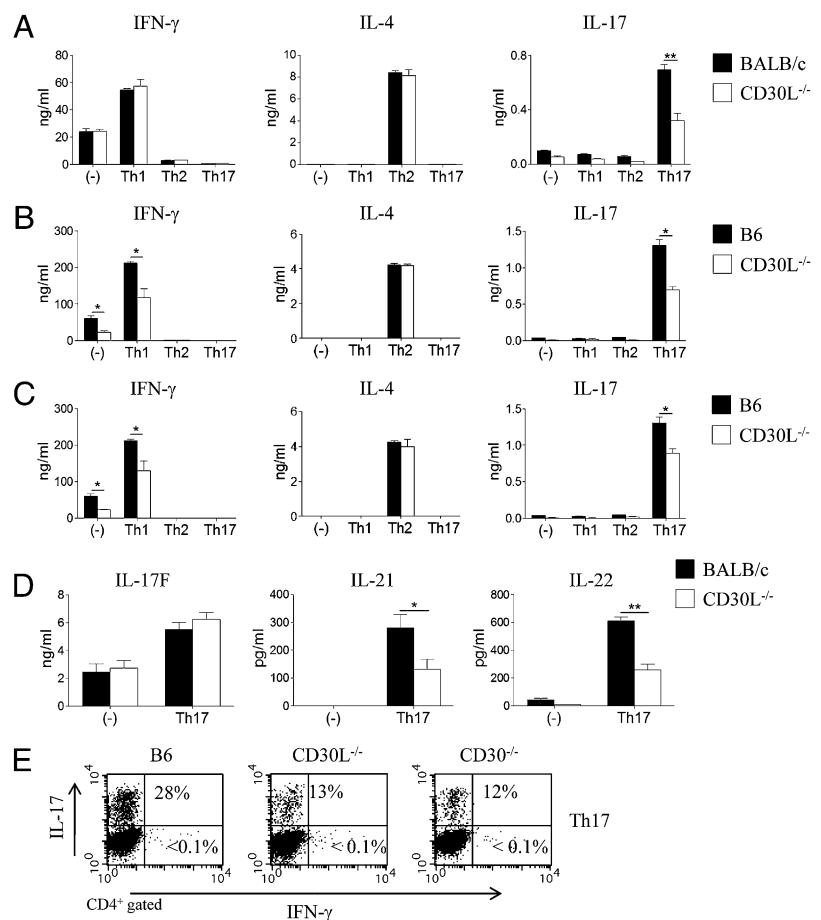
Histological assessment of colitis

The middle parts of colons were removed and fixed with 10% neutral buffered formalin and then embedded in paraffin. After cutting into round slices, the thin tissue sections were stained with H&E. Histology was scored as described previously (28).

Isolation of lamina propria and flow cytometry analysis

LPLs were isolated as described elsewhere (29). For intracellular cytokine staining, LPLs were stimulated with PMA (25 ng/ml) and ionomycin (1 μg/ml) for 6 h at 37°C and 5% CO_2. BFA (10 μg/ml) was added for the last 5 h of incubation. These cells were harvested, washed, and stained with anti-CD3 and anti-CD4 for 30 min at 4°C. The intracellular expression of IL-17A, IL-2, and IFN-γ in $CD4^+$ T cells was analyzed using a Cytofix/Cytoperm Kit Plus (BD Biosciences) according to the manufacturer's instructions. The data were analyzed using CellQuest software (BD Biosciences).

Culture of LPLs for assay of cytokine secretion

To measure cytokine production by T cells in LPLs, LPLs purified as described above were cultured with anti-CD3 and anti-CD28 for 48 h at 37°C and 5% CO_2. The cultured supernatants were then collected and assayed for cytokine secretion by ELISA.

Statistics

The difference in survival rates was evaluated by the log-rank test (Mantel–Cox). Disease activity index and histological scores were statistically analyzed using the Mann–Whitney U test. Differences in parametric data were evaluated by a Student t test. Differences of $p < 0$ 0.05 were considered statistically significant.

Results

$CD30L^{-/-}$ or $CD30^{-/-}$ T cells exhibited an impaired ability of Th17 differentiation in vitro

Naive $CD4^+CD62ligand^{hi}CD44^{low}$ T cells were sorted from spleens of $CD30L^{-/-}$ mice with a BALB/c or B6 background or $CD30^{-/-}$ mice with a B6 background; they were cultured in vitro for 96 h in a 96-well plate coated with anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) in polarizing conditions for Th1, Th2, or Th17 cells. $CD30L^{-/-}$ T cells with a BALB/c background normally differentiated into Th1 or Th2 cells (Fig. 1A), whereas $CD30L^{-/-}$ or $CD30^{-/-}$ T cells with a B6 background showed impaired abilities to differentiate into Th1 cells (Fig. 1B, 1C). Notably, naive $CD4^+$ T cells from $CD30L^{-/-}$ mice with a BALB/c or B6 background and those from $CD30^{-/-}$ mice with a B6 background exhibited impaired ability to differentiate into Th17 cells after culture in vitro (Fig. 1A–C). Th17-related cytokines, such as IL-21 and IL-22, but not IL-17F, were also lower in $CD30L^{-/-}$ T cells with a BALB/c background cultured under Th17-polarizing conditions (Fig. 1D). Flow cytometry analysis revealed that the frequency of Th17 cells was decreased in $CD30L^{-/-}$ or $CD30^{-/-}$ T cells with a B6 background after culture under Th17-polarizing conditions (Fig. 1E).

Increased production of IL-2 is partly responsible for impaired Th17 differentiation in the absence of CD30L/CD30 signaling

The $CD30L^{-/-}$ T cells with a BALB/c background, which exhibited impaired ability to differentiate into Th17 cells, produced a higher level of IL-2 after culture in Th17-polarizing conditions, as well as in Th1- or Th2-polarizing conditions (Fig. 2A). Similarly, the $CD30L^{-/-}$ or $CD30^{-/-}$ T cells with a B6 background also produced a greater level of IL-2 after culture in Th17-polarizing conditions (Fig. 2B). Intracellular cytokine synthesis analysis showed that the number of IL-17A$^+$ $CD4^+$ cells decreased, but that of IL-2$^+$ $CD4^+$ cells increased in $CD30L^{-/-}$ and $CD30^{-/-}$ Th17 cells (Fig. 2C). Thus, impaired Th17 cell differentiation in $CD30L^{-/-}$ or $CD30^{-/-}$ T cells was inversely correlated with the level of IL-2 production.
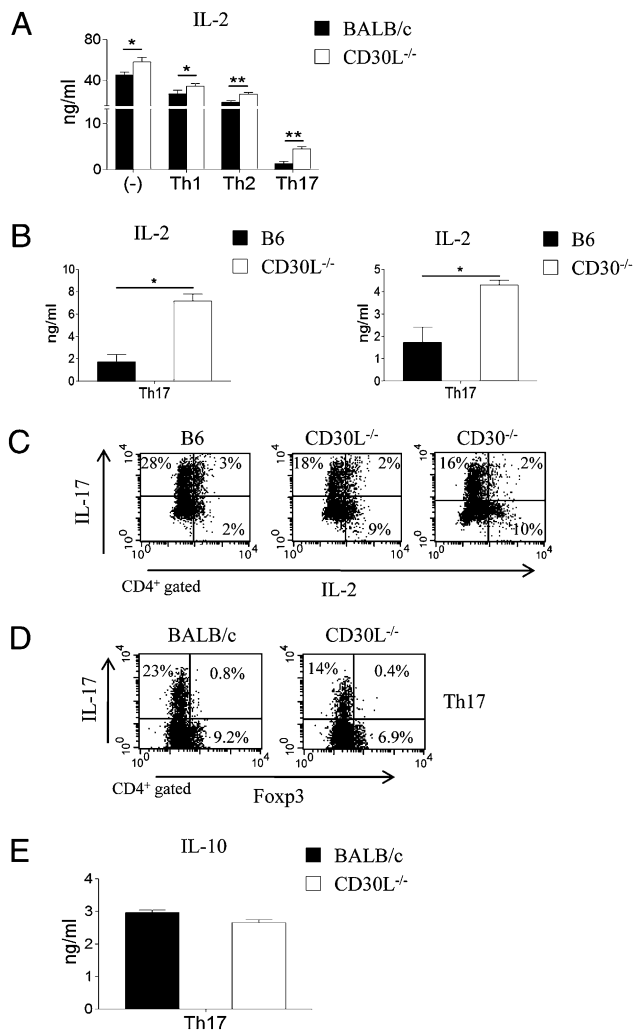

FIGURE 2. Increased IL-2 production by $CD30L^{-/-}$ or $CD30^{-/-}$ $CD4^+$ T cells cultured in vitro under Th17-polarizing conditions. *A*, Naive $CD4^+$ T cells from WT mice and $CD30L^{-/-}$ mice with a BALB/c background were cultured for 4 d under Th1-, Th2-, or Th17-polarizing conditions. *B*, Naive $CD4^+$ T cells from WT mice and $CD30L^{-/-}$ mice or $CD30^{-/-}$ mice with a B6 background were cultured for 4 d under Th17-polarizing conditions. IL-2 production was detected in the culture supernatants with ELISA ($n = 5$). *C*, The cultured T cells with a B6 background were stimulated with PMA, ionomycin, and BFA for 6 h, and analyzed by intracellular cytokine staining for the expression of IL-17A and IL-2. *D*, The cultured T cells with a BALB/c background were stimulated with PMA, ionomycin, and BFA for 6 h, and then were analyzed by intracellular-cytokine staining for the expression of IL-17A and Foxp3. *E*, Naive $CD4^+$ T cells with a BALB/c background were cultured under Th17-polarizing conditions for 4 d. IL-10 secretion was detected in the culture supernatant with ELISA ($n = 5$). Data indicate mean ± SD from a representative in three separate experiments. Statistically significant differences are shown. *$p < 0.05$; **$p < 0.01$.

IL-2 is known to promote the differentiation of naturally occurring Foxp3$^+$ CD25$^+$ Tregs (30), raising the possibility that the number of Tregs increased, thereby inhibiting Th17 differentiation of $CD30L^{-/-}$ T cells. However, the number of Foxp3$^+$ Tregs did not increase in $CD30L^{-/-}$ T cells after culture under Th17-polarizing conditions (Fig. 2D). Furthermore, there was no difference in IL-10 production under Th17-polarizing conditions between wild-type (WT) and $CD30L^{-/-}CD4^+$ T cells (Fig. 2E). IL-2 was recently reported to inhibit IL-17A production directly via STAT5 activation (8). Therefore, we next examined the effect of neutralization of IL-2 with anti–IL-2 mAb on Th17 differentiation in vitro. The level of IL-17A production by $CD4^+$ T cells

from CD30L$^{-/-}$ mice with a BALB/c background after culture under Th17-polarizing conditions was restored by addition of IL-2 mAb in a dose-dependent manner and to almost the same level at 10 μg/ml of anti–IL-2 mAb (Fig. 3A). Similarly, the ability of the CD4$^+$ T cells from CD30L$^{-/-}$ or CD30$^{-/-}$ mice with a B6 background to produce IL-17A was restored, albeit partly, by the addition of 10 μg/ml of anti–IL-2 mAb. Intracellular cytokine synthesis analysis revealed that the fraction of IL-17A$^+$ CD4$^+$ T cells also increased by neutralization of IL-2 (Fig. 3B). These results suggest that IL-2 overproduction is at least partly responsible for impaired Th17 differentiation of CD30L$^{-/-}$ or CD30$^{-/-}$ T cells.

Reverse signal via CD30L downregulates IL-2 production by CD4$^+$ T cells

Recent evidence shows that CD30L as a counter receptor induces costimulation through reverse signals, in addition to delivering signals through CD30 as other members of the TNF/TNFR family (18–20, 31–33). To determine which directional signal is involved in Th17 cell differentiation after CD30–CD30L engagement in T cells, sorted naive CD4$^+$ T cells from CD30$^{-/-}$, CD30L$^{-/-}$, or WT mice were cultured under Th17-polarizing conditions with immobilized anti-CD30L or anti-CD30 mAbs. Naive CD4$^+$ T cells from both BALB/c and B6 mice showed increased IL-17A production but decreased IL-2 production by stimulation via CD30L with immobilized anti-CD30L mAb (Fig. 4A). The level of IL-17A

production by CD30$^{-/-}$ T cells was partly restored, and inhibition of IL-2 production by CD30$^{-/-}$ T cells was comparable to that seen in WT T cells after culture with immobilized anti-CD30L mAb (Fig. 4A). Intracellular cytokine synthesis analysis also revealed that the percentage of CD4$^+$ IL-17A$^+$ cells increased but the percentage of CD4$^+$ IL-2$^+$ cells decreased in CD30$^{-/-}$ T cells after culture with immobilized anti-CD30L mAb (Fig. 4B). In contrast, addition of agonistic anti-CD30 mAb in the culture of both WT and CD30L$^{-/-}$ T cells did not affect IL-2 production, but IL-17A production was significantly augmented (Fig. 4C). Intracellular cytokine synthesis analysis confirmed that the fraction of IL-17A$^+$ CD4$^+$ cells increased but the number of IL-2$^+$ CD4$^+$ T cells was not affected by stimulation with an agonistic anti-CD30 mAb both in WT and in CD30L$^{-/-}$ T cells (Fig. 4D). Taken together, these results suggest that a bidirectional signal transduction provided by CD30–CD30L engagement is involved in Th17 differentiation and that CD30L as a counter receptor may induce costimulation for downregulation of IL-2 production through a reverse signal.

We next investigated the kinetics of expression of CD30L on CD4$^+$ T cells after in vitro culture in polarizing conditions for Th17 cells. CD30L expression was detected in CD4$^+$ T cells at the peak level 24 h after culture, and most of the CD30L$^+$ T cells were of IL-2–positive phenotype; then the expression level of CD30L on the CD4$^+$ T cells was reduced to almost an undetectable level by 96 h after culture. Th17 cells were detected 48 h after culture, but they hardly expressed CD30L during in vitro culture under
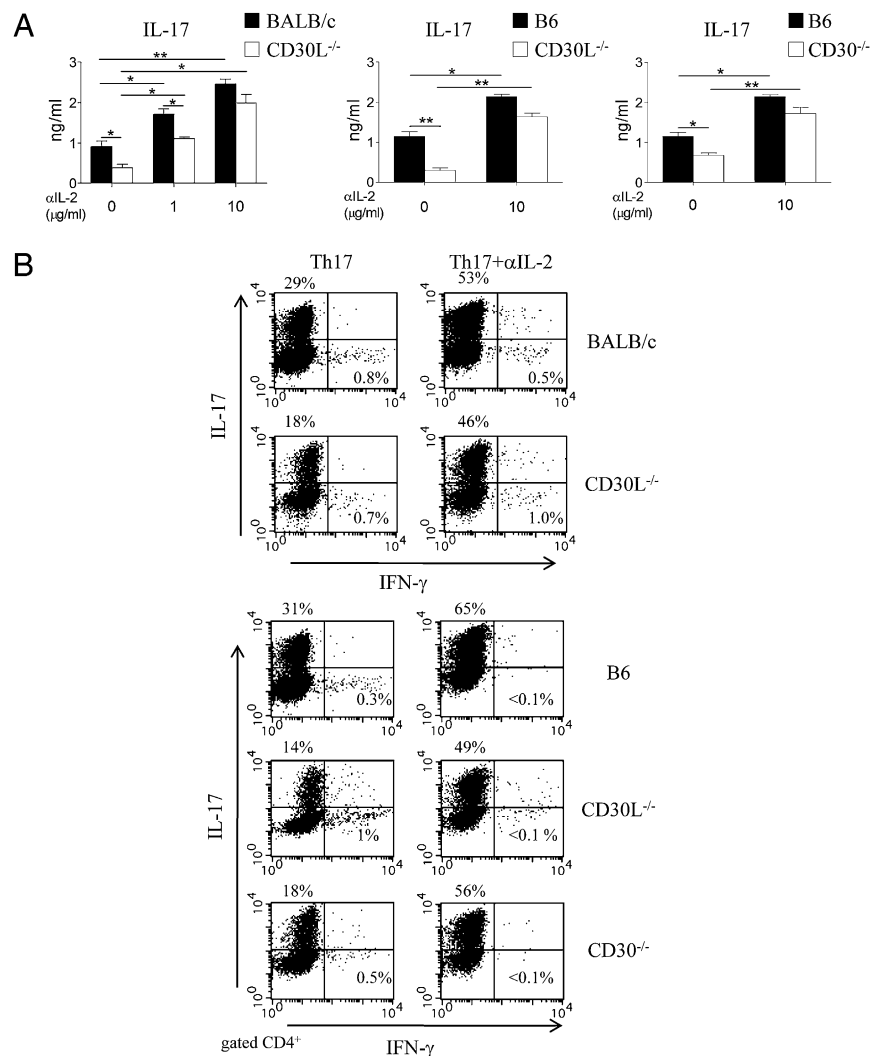

FIGURE 3. Anti–IL-2 mAb partly restores Th17 differentiation of CD30L$^{-/-}$ or CD30$^{-/-}$ CD4$^+$ T cells. A, Naive CD4$^+$ T cells were sorted from CD30L$^{-/-}$ mice with a BALB/c background or CD30L$^{-/-}$ and CD30$^{-/-}$ mice with a B6 background, and cultured with or without anti–IL-2 mAb (1 or 10 μg/ml) for 4 d under Th17-polarizing conditions ($n = 6$). IL-17A secretion was analyzed by ELISA. B, Cells cultured with anti–IL-2 mAb (10 μg/ml) were further cultured with PMA, ionomycin, and BFA for 6 h, and were analyzed by intracellular cytokine staining for the expression of IL-17A and IFN-γ ($n = 6$). Data indicate mean ± SD from a representative in three separate experiments. Statistically significant differences are shown. *$p < 0.05$; **$p < 0.01$.
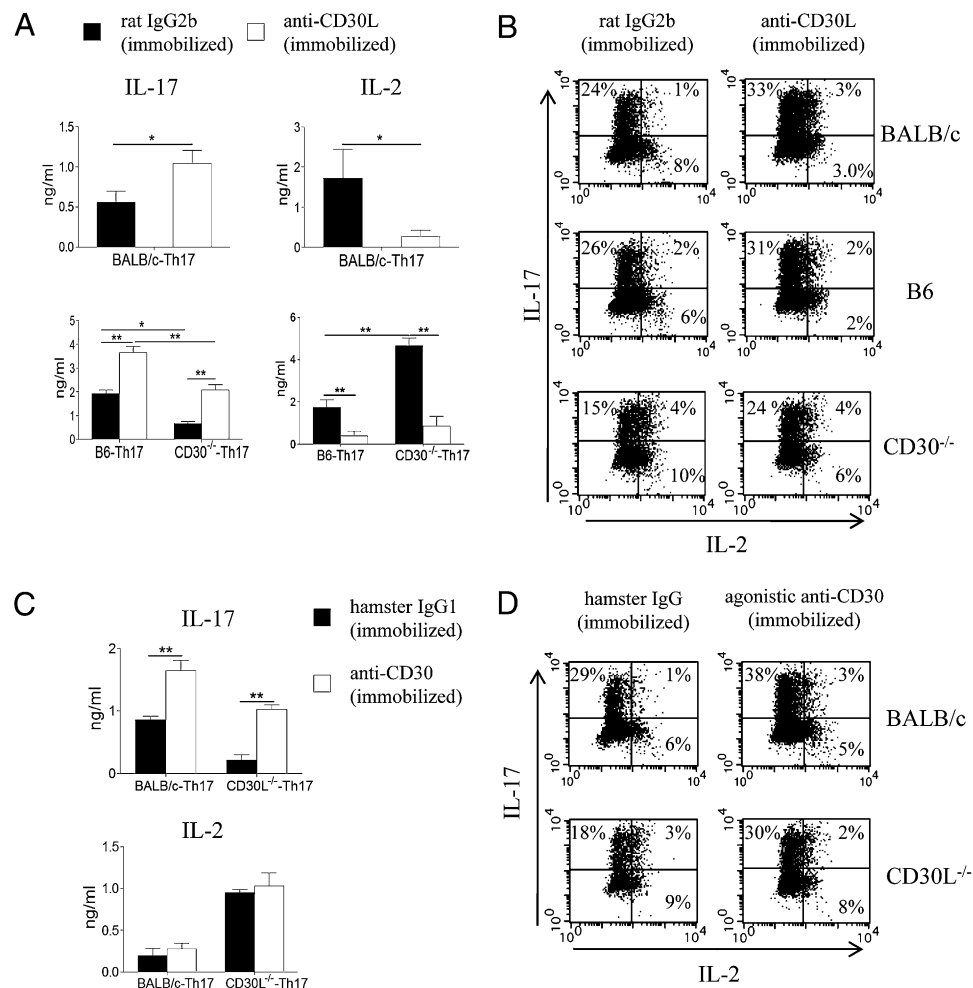
FIGURE 4. Reverse signal via CD30L downregulates IL-2 production by CD4^{+} T cells. Sorted naive CD4^{+} T cells were differentiated into Th17 cells in the presence of immobilized anti-CD30L (25 μg/ml) or control rat IgG2b (*A*, *B*), or immobilized anti-CD30 (25 μg/ml) or control hamster IgG (*C*, *D*). After 4 d, IL-17A and IL-2 production in the culture supernatants was analyzed by ELISA, and the cells were cultured with PMA, ionomycin, and BFA for 6 h, and then were analyzed by intracellular cytokine staining for the expression of IL-17A and IL-2 (*n* = 5). Data indicate mean ± SD from a representative in four separate experiments. Statistically significant differences are shown. $*p < 0.05$; $**p < 0.01$.

Th17-polarizing conditions (Fig. 5*A*). Thus, our results suggest that the reverse signal via CD30L affects IL-2 production by CD4^{+} T cells at an early stage after culture under Th17-polarizing conditions.

To elucidate whether CD30L signaling modulates IL-2 production at the transcriptional level, we examined the kinetics of the IL-2 mRNA expression level in CD4^{+} T cells under Th17-polarizing conditions. The level of IL-2 mRNA expression was significantly higher in CD30L^{−/−} CD4^{+} T cells from 6 to 18 h after stimulation (Fig. 5*B*), and IL-2 protein production significantly increased in CD30L^{−/−} CD4^{+} T cells from 18 h after culture (Fig. 5*C*). These data indicate that CD30L-mediated reverse signaling may suppress IL-2 expression at the transcriptional level. Because IL-2 has been reported to limit Th17 differentiation via STAT5 activation (8), we assessed STAT5 phosphorylation at 3 h, 24 h, or 48 h after culture under Th17-polarizing conditions. Although there was no detectable difference in pSTAT5 level between CD30L^{−/−} T cells and WT T cells after 24 h of culture, we found appreciable enhancement of STAT5 phosphorylation at 48 h (Fig. 5*D*). Hence, our results indicate that a defect of CD30L signaling enhances STAT5 activation during Th17 differentiation in association with increased IL-2 production.

CD30L^{−/−} T cells exhibited an impaired ability for Th17 differentiation in vivo

To examine whether Th17 cell differentiation was impaired in vivo under CD30L-deficient conditions, CD4^{+}CD45RB^{hi} T cells from CD30L^{−/−} mice or WT mice were transferred into SCID or CD30L^{−/−} SCID mice. When CD30L^{−/−} donors were used for

T cell reconstitution in SCID mice, wasting syndrome and colitis were attenuated in SCID mice (Fig. 6*A*). Cytokine secretion by T cells in LPLs from the large intestine in response to immobilized anti-CD3/anti-CD28 mAbs was measured on day 28 after transfer. The levels of IL-17A and IFN-γ were lower, but the level of IL-2 was higher, in SCID mice transferred with CD30L^{−/−} T cells than in those transferred with WT T cells (Fig. 6*B*). The CD4^{+} IL-17A^{+} and CD4^{+} IFN-γ^{+} T cells were significantly decreased, and the CD4^{+} IL-2^{+} T cells were increased in SCID mice transferred with CD30L^{−/−} T cells (Fig. 6*C*). In contrast, when WT donors were used for T cell reconstitution in CD30L^{−/−} SCID mice, the levels of IL-17A, IFN-γ, and IL-2 did not change, and the CD30L^{−/−} SCID mice developed the wasting disease and colitis with comparable severity to that seen in reconstituted SCID mice (Fig. 6*D*, 6*E*). These results suggest that CD30L/CD30 signaling executed by T-T cell interaction is critical to Th17 differentiation in vivo (Fig. 7).

Discussion

In this paper, we show that CD30L/CD30 signaling plays a critical role in Th17 differentiation of CD4^{+} T cells in vitro and in vivo. Naive CD4^{+} T cells from CD30L^{−/−} mice or CD30^{−/−} mice exhibited an impaired ability to differentiate into Th17 cells, whereas they showed a greater ability to produce IL-2 both in vitro after culture in Th17-polarizing conditions and in vivo after transfer into SCID mice. Neutralization with IL-2 by anti–IL-2 mAb partly restored the ability of CD30L^{−/−} T cells and CD30^{−/−} T cells for Th17 differentiation, suggesting that IL-2 overproduction in the absence of CD30L/CD30 signaling is at least partly responsible for impaired Th17 differentiation of CD30L^{−/−} T cells.
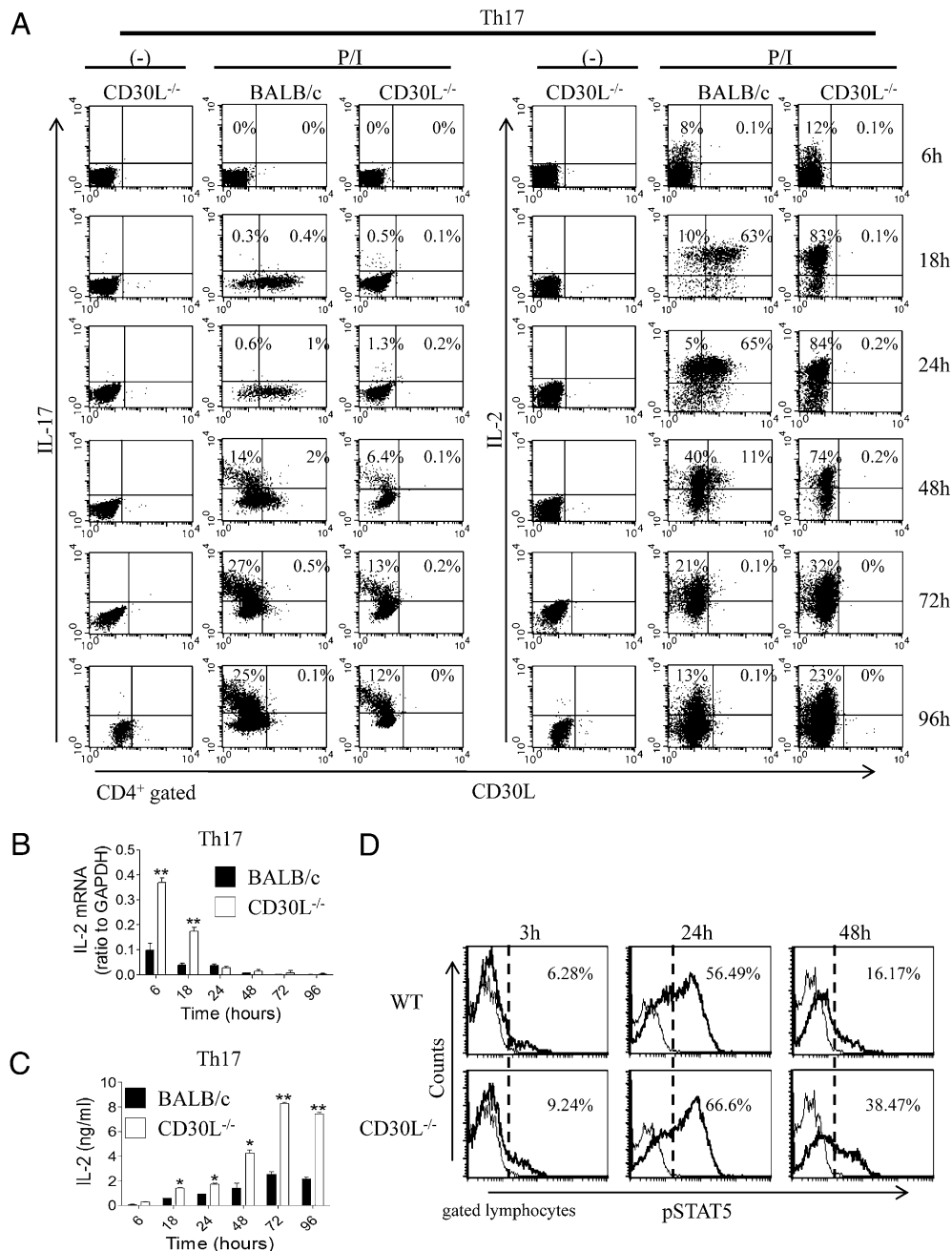
FIGURE 5. Kinetics of CD30L expression and IL-2 production on $CD4^+$ T cells after culture under Th17-polarizing conditions. Naive $CD4^+$ T cells from WT mice or $CD30L^{-/-}$ mice with a BALB/c background were cultured in Th17-polarizing conditions. *A*, Cells were harvested at indicated times (6, 18, 24, 48, 72, and 96 h) and cultured with or without PMA and ionomycin for 6 h, and BFA was added for the last 5 h. After culture, cells were analyzed by surface staining for the expression of CD3, CD4, and CD30L, and then intracellular cytokine staining for the expression of IL-17A or IL-2. *B* and *C*, IL-2 production and the expression of mRNA of IL-2 were quantified by ELISA and real-time PCR, respectively, at indicated times. Results were expressed as the mean ± SD (*n* = 8). *D*, the cells were cultured as in *A* for 3 h, 24 h, or 48 h and assessed for their phosphorylation of STAT5. Thin line plots represent unstimulated cells. Thick line plots represent cells stimulated with Th17-polarizing conditions. The dotted lines intersect at the mean fluorescence intensity of histograms representing cells stimulated in Th17 conditions. Data shown are representative of three independent experiments. Statistically significant differences are shown. *$p < 0.05$; **$p < 0 0.01$.

IL-2 is a growth factor for most T cells, but a deficiency of IL-2 results in severe multiorgan autoimmune disease. This is due in part to the role of IL-2 in promoting the differentiation of Tregs, but recently, IL-2 was shown to suppress Th17 development and shift the balance toward expression of Foxp3 (8, 34). Foxp3 induced by TGF-β inhibits the function of RORγt and downregulation of Th17 differentiation (35). However, we found no difference in Foxp3 expression and IL-10 production between WT and $CD30L^{-/-}$ Th17 cells under TCR stimulation or Th17-polarizing conditions.

IL-2 was recently noted to inhibit Th17 differentiation through STAT5 activation (8). In the current study, we found that $CD30L^{-/-}$ T cells showed increased levels of STAT5 activation during Th17 differentiation. Therefore, we speculate that CD30L/CD30 signaling is involved in Th17 differentiation, at least partly directly via downregulation of IL-2 production, but not indirectly via downregulation of the appearance of Tregs.

CD30L/CD30 signaling has been widely examined and discussed over the years, and most in vitro experimental models were
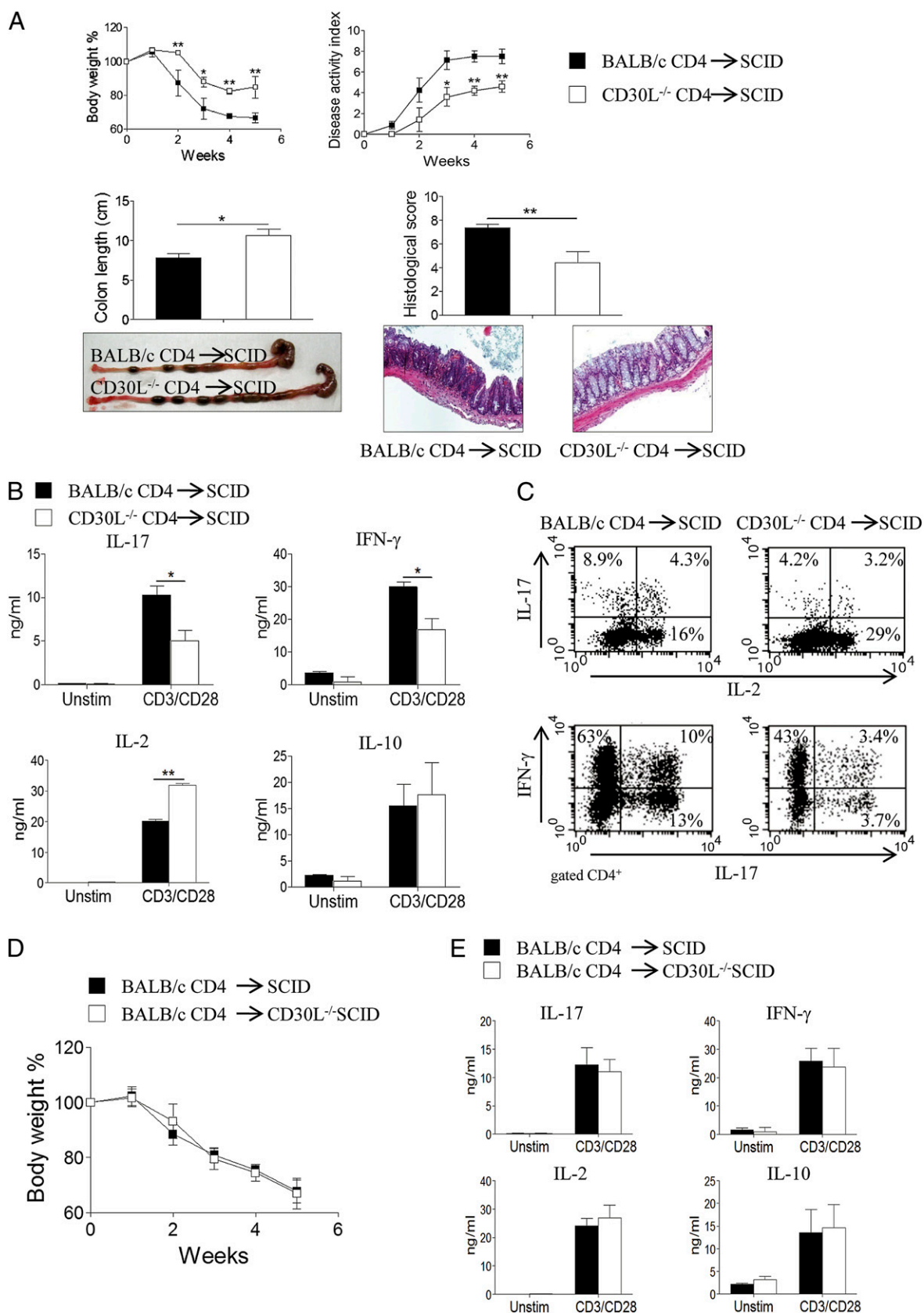
FIGURE 6. An impaired ability of CD30L$^{-/-}$ T cells to differentiate into Th17 cells in vivo. *A*, CD4$^+$CD45RB^{hi} T cells from BALB/c or CD30L$^{-/-}$ mice were adoptively transferred into SCID mice. Body weight and disease activity index were observed every day. Colon length, H&E staining of colon (original magnification ×200), and histological score were analyzed on day 28. Data indicate mean ± SD (n = 10). *B*, LPLs were received from colons of colitis mice on day 28 and were cultured with or without anti-CD3 and anti-CD28 mAbs for 48 h, and secretion of cytokines was analyzed by ELISA (n = 10). *C*, Cells were washed and cultured with PMA, ionomycin, and BFA for 6 h. Intracellular cytokine staining for IL-17A versus IL-2 is shown. *D*, Body weight of SCID or CD30L$^{-/-}$ SCID transferred with naive CD4$^+$ T cells of BALB/c mice (mean ± SD; n = 15). *E*, Cytokine secretion by T cells in LPLs was analyzed by ELISA (mean ± SD; n = 5). Statistically significant differences are shown. *p < 0.05; **p < 0 0.01.

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 development of lymph node and Peyer's patch in ontogeny (12). We have recently found that CD30L⁺ T-CD30⁺–T cell interactions quite possibly existed in T cell activation in bacterial infection in mice (36). In the current study, we also found that naive CD4⁺ T cells from CD30L$^{-/-}$ mice exhibited impaired abilities to differentiate into Th17 cells and to induce a serious wasting syndrome and inflammation of the colon in vivo after transfer into SCID mice. In contrast, the naive CD4⁺ T cells from WT mice developed into Th17 cells and induced colitis in CD30L$^{-/-}$ SCID mice at a comparable level to that seen in reconstituted SCID mice. These results suggest that CD30L expression on CD4⁺ T cells but not on non-T cells is indispensable for Th17 differentiation in vivo. CD30L/CD30 signaling executed by T-T cell interaction may play an important role in Th17 differentiation in vivo as well as in vitro.

As in other members of the TNF/TNFR family, it was reported that a bidirectional instead of unidirectional signal transduction occurred after CD30–CD30L engagement in T cells (18–20). Su et al. (37) reported that the reverse signal from CD30 to CD30L inhibited IL-2 production in human Hodgkin's Reed–Sternberg cells. Our results showed that CD30L was expressed on most CD4⁺ IL-2⁺ T cells during in vitro culture under Th17-polarizing conditions. Stimulation via CD30L by immobilized anti-CD30L mAb suppressed IL-2 production by CD30$^{-/-}$ CD4⁺ T cells, whereas the addition of agonistic anti-CD30 mAb in the culture did not affect IL-2 secretion by CD30L$^{-/-}$ CD4⁺ T cells. These results suggest that the reverse signal via CD30L downregulates IL-2 production by activated CD4⁺ T cells. Although our preliminary results show that the reverse signal via CD30L downregulates IL-2 production by activated CD4⁺ T cells at the transcriptional level, the precise mechanism underlying the regulatory role of CD30L-mediated signaling in IL-2 production remains to be fully determined.

Th17 differentiation in mice is potentially induced by synergistic activation by TGF-β and IL-6 through expression of a transcriptional factor, RORγt (1, 2). IL-21 further sustained Th17 differentiation in an autocrine manner (3). IL-23 and IL-1β finalize the differentiation program of Th17 cells and help to maintain Th17 cells (4, 5). TNF-α, similar to IL-1β, was shown to further increase Th17 differentiation in the presence of TGF-β and IL-6 (38). CD30L, a member of the TNF family, has the potential to induce TRAF-2–mediated NF-κB activation via CD30 that can recruit several TRAFs (16, 17). The results in the current study demonstrated that the addition of agonistic anti-CD30 mAb in the in vitro culture of T cells increased IL-17A production by CD30L$^{-/-}$ CD4⁺ T cells without modulation of IL-2 production. Therefore, it is possible that, like TNF-α, CD30 signaling may contribute directly to Th17 differentiation in the presence of TGF-β and IL-6, in addition to downregulation of IL-2 via a reverse signal from CD30 to CD30L (Fig. 7).

Impairment of Th1 differentiation in vitro was also apparent in CD30L$^{-/-}$ or CD30$^{-/-}$ CD4⁺ T cells with a B6 background. Although such an impaired Th1 differentiation was not apparent in CD30L$^{-/-}$ CD4⁺ T cells with a BALB/c background, activation of IFN-γ–producing Th1 cells was impaired in dextran sulfate sodium-induced chronic colitis in CD30L$^{-/-}$ mice with a BALB/c background (data not shown) and colitis in SCID mice transferred with CD30L$^{-/-}$ T cells from BALB/c background mice. We previously reported that CD30L$^{-/-}$ mice were susceptible to Th2 type experimentally oxazolone-induced colitis, but resistant to Th1 type trinitrobenzene sulfonic acid-induced acute colitis. The level of Th1 type cytokine IFN-γ in the T cells of LPLs was significantly lower in oxazolone- or trinitrobenzene sulfonic acid-treated CD30L$^{-/-}$
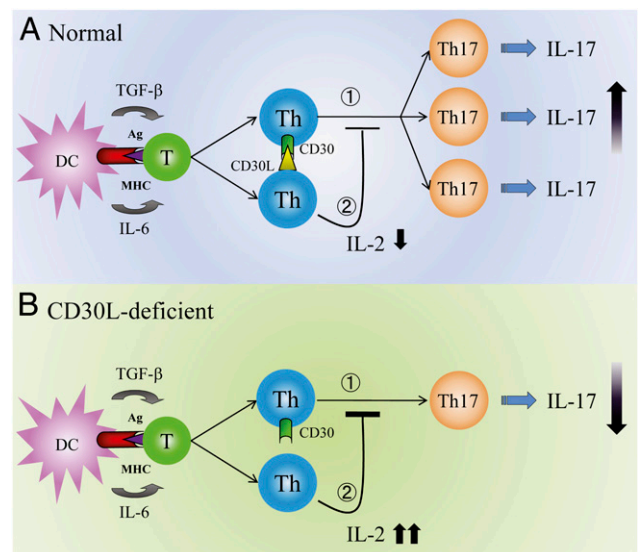

FIGURE 7. CD30L regulates Th17 differentiation. *A*, Reverse signal via CD30L downregulates IL-2 production, and stimulation via CD30 signaling promotes Th17 differentiation in the presence of TGF-β and IL-6. *B*, Loss of reverse signal via CD30L for downregulation of IL-2 and loss of direct CD30 signaling to Th17 cells in CD30L$^{-/-}$ mice may account for impaired Th17 differentiation in CD30L$^{-/-}$ mice.

mice than in WT mice (22). We also found that CD30L$^{-/-}$ mice were susceptible to infection with *Mycobacterium bovis* bacillus Calmette-Guérin in association with reduced Th1 responses (36). These results suggested that CD30L/CD30 signaling is linked to Th1 cell responses and Th1-associated diseases. However, there are several observations that CD30L/CD30 signaling is involved in Th2 cell responses (39, 40) and in naturally occurring Treg responses (23, 24). Thus, CD30 signaling may not be linked to a commitment step for the differentiation of a specific Th cell subset. CD30 possibly contributes to increased T cell survival via induction of TRAF2-mediated NF-κB activation (41, 42). CD30 signaling may be important for amplification of already differentiated effector or memory Th cells, regardless of a specific Th subset. We speculate that CD30 signaling may initially promote cell survival of preactivated specific Th subsets, resulting in acceleration of the Th responses. Because IL-2 promotes Th subset differentiation, including Th2 and Tregs, CD30L$^{-/-}$ T cells would preferentially differentiate into Th2 or Tregs. However, we did not find any increase in Th2 or Treg subset differentiation in CD30L$^{-/-}$ T cells. A defect of CD30 signaling due to absence of CD30L may abolish IL-2–mediated promotion of Th differentiation. An apparent difference in the requirement of CD30L between Th17 and other Th subset differentiation may be explained by different involvement of IL-2–mediated regulation.

In conclusion, we showed important functions of CD30L/CD30 signaling executed by T-T cell interaction in Th17 differentiation both in vitro and in vivo. Our findings indicate not only a novel role for CD30L/CD30 signaling in the IL-17A/IL-2 axis but also a new therapeutic approach to controlling chronic inflammatory diseases associated with Th17 cells.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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