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_J Immunol_ 1999; 163:6520-6529; 
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Robust B Cell Immunity but Impaired T Cell Proliferation in the Absence of CD134 (OX40)  

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CD134 (OX40) is a member of the TNF receptor family that is expressed on activated T lymphocytes. T cells from mice that lack expression of CD134 made strong responses to a range of challenges, but they showed impaired proliferation in response to direct stimulation through the TCR with monoclonal anti-CD3ε Ab. CD134-deficient mice controlled infection with Leishmania major, Nippostrongylus brasiliensis, and Theller’s murine encephalomyelitis virus, and they made overtly normal Ab responses to a variety of antigens. Thus, CD134 is not essential for many T cell responses in vivo, nor is it required for the provision of help to B cells. Nonetheless, a subtle role in the regulation of T cell reactivity is suggested by the effect of CD134 deficiency on in vitro T cell responses. The Journal of Immunology, 1999, 163: 6520–6529.

Members of the TNF receptor superfamily have prominent roles in the regulation of immune responses and the formation of secondary lymphoid tissue (1–7). Ligands for these receptors also constitute a superfamily whose members share structural similarity to TNF and in many cases are likely to form cell-bound or secreted trimers (8, 9). Lymphocyte activation induces the expression of several TNF receptor-related proteins or their ligands including CD40L, CD95L, 4-1BB, CD30, and CD134 (10). This up-regulation of expression allows for ligand-dependent signals to be relayed from the receptors to the nucleus through signaling molecules that include the TNF receptor-associated factors (TRAFs)6 (4, 11, 12). These signals induce apoptotic or proliferative/differentiative outcomes through the respective activation of caspases and transcriptional regulators such as NF-κB and AP-1 (7, 12). Mutant mice that lack expression of TNF receptors, their ligands, or TRAFs show a range of defects in lymphoid homeostasis and immune responses (3, 5, 6, 13–16).

The CD134 Ag was first identified as a 50-kDa glycoprotein target for the MRC OX40 Ab that was selectively expressed on activated rat CD4+ T cells (17). Mouse and human CD134 show a similar pattern of expression to the rat form, except they are also found on activated CD8+ T cells (18, 19). The ligand for CD134 (CD134L) is a type II trimeric transmembrane protein whose mRNA is markedly induced in human T cell leukemia virus 1-infected cells through the action of the viral tax transactivator (19, 20). Trimeric CD134L binds to cell surface CD134 with high affinity (Kd = 0.2–0.4 nM) and a slow off-rate (koff = 4 × 10−3 s−1) (8).

In several experimental settings, CD134 acts as a costimulator for T cells. For instance, anti-CD134 mAb augments the response of rat T cells activated with allogenic MHC (17) or anti-CD3 (21). Similarly, mouse and human T cells make more robust proliferative and cytokine responses when activated in the presence of transfected cells expressing CD134L (19, 20, 22). This costimulatory activity appears to be synergistic with costimulation through CD28 and is particularly potent in prolonging the responses of differentiated effector T cells (23). Interestingly, a similar type of costimulatory activity has also been described for CD137 (4-1BB), which is a related member of the TNF receptor family that is also induced on activated T lymphocytes (24–26). Costimulatory signals delivered through CD134 have been implicated in selectively promoting the differentiation of Th2 cells (27–29), but other studies suggest they may also regulate Th1 development (23, 30, 31). The cytoplasmic tail of CD134 can associate with TRAF2, TRAF3, and TRAF5 and may well mediate its costimulatory effect through these interactions (12, 32, 33). Finally, recent data indicate that like anti-CD137 (34), anti-CD134 signaling can block activation-induced T cell death in mice treated with superantigens (35).

The physiological significance of costimulatory signaling through CD134 has not yet been resolved. CD134 is expressed on inflammatory T cells in vivo, as in the CNS of rodents with experimental allergic encephalomyelitis (36), in the joints of humans with rheumatoid arthritis (37), in the peripheral blood of rats with
acute graft-vs-host disease (38), or on tumor-infiltrating lymphocytes (39). CD134 is also expressed on T cells in the intestines of mice with inflammatory bowel disease wherein blockade of the CD134-CD134L interaction can lessen the severity of the disease (40). Cumulatively, these expression and functional data raise the possibility that CD134 signaling may help to prolong Ag-specific proliferative responses or otherwise influence the persistence, differentiation, or reactivation of effector/memory populations.

The mRNA for CD134L is expressed in activated T cells, B cells, and also in the mouse brain and kidney (19). In humans, CD134L mRNA is found in the heart, skeletal muscle, pancreas, testes, and ovary (19). Human dendritic cells express CD134L, and its ligation on these cells induces them to differentiate and secrete cytokines (41). CD134L is also expressed on various types of endothelium, including that from umbilical cord, foreskin, and the aorta (42, 43). Thus, the expression of CD134L would allow for a role as a costimulatory ligand for T cells, but it also raises the possibility that CD134L may regulate the extravasation of activated T cells under certain circumstances.

A possible function for CD134L in the regulation of T cell-dependent B cell responses has been proposed based on the finding that a rabbit anti-mouse CD134 antiserum can inhibit Ag-specific B cell responses in mice (44). Treated mice developed germinal centers in response to trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) immunization, but they were impaired in their secretion of TNP-specific IgG Abs and they also lacked TNP-reactive B cell foci in the spleen. Nevertheless, these mice made adequate secondary responses, suggesting that the CD134L signal might regulate differentiation into Ig-secreting plasma cells but not memory B cells. The induction of CD134L expression on activated B cells and the provision of a proliferative signal to B cells through it (45) would be consistent with this hypothesis. Costimulation through CD134L has also been implicated in the up-regulation of the chemokine receptor BLR-1 on T cells (29), which could be important for recruiting Ag-specific T cells into germinal centers and thereby influencing Ab responses. However, a unique role for CD134 in this context has not yet been established.

To study the function of CD134 in more detail, we have generated CD134-deficient mice by gene targeting in embryonic stem (ES) cells. The phenotype of these mice suggests that CD134 is not a primary regulator of many T cell responses in vivo, nor is it essential for B cell differentiation and Ab secretion. Nonetheless, in vitro assays of T cell activation suggest that signals from CD134L may help to promote T cell proliferation under certain circumstances.

Materials and Methods

Generation of CD134-deficient mice

The isolation of 129/Sv genomic phage λ clones that contained the cd134 gene has been described previously (46). These phage clones were further characterized by subcloning, restriction enzyme, and Southern blot analysis. A targeting construct was generated by inserting a 3.5-kb fragment of the cd134 gene into a 3.3-kb Hind III fragment that contained exons 5, 6, and 7 into the plneo-2 vector (modified from the plneo, kind gift from Dr. Hua Gu, National Institutes of Health, Bethesda, MD). The plneo vector contains a neomycin resistance cassette (neo') under the control of the MCI (HSV-thymidine kinase) promoter. Neo' and the CD134 genomic sequence were then reexcised using Hind III and inserted into pBluescript that contained a 4-kb KpnI-HindIII upstream fragment of the cd134 gene. A total of 2 × 10⁵ RF8 ES cells (47) were transfected with 20 µg of the linearized targeting vector by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) set at 500 µF and 250 V. The cells were plated on primary embryonic feeder cells, and G418 selection (200 µg/ml) was applied 36 h after electroporation. Ten days later, individual G418-resistant clones were picked to 96-well plates containing feeder cells. The clones were expanded and then split in half so that a portion of each could be frozen in 96-well plates, and the remaining portion could be expanded for DNA extraction and Southern blot analysis. A probe on the 5' side of the targeted region was used on a BglII digest of the genomic DNA to identify targeted clones. The structure of the mutant allele was subsequently confirmed using a 3' probe on genomic DNA that had been digested with PmlI or BamHI. Approximately 1% of neo' colonies were mutant at the cd134 locus. Chimeric mice were produced by injection of mutant ES cells into 3.5-day-old blastocysts according to standard procedures (48). These chimeras were then bred to C57BL/6 females, and germline transmission of the cd134 mutation was confirmed by Southern blot analysis of tail DNA. CD134+/− mice were interbred to produce the mice that were used in the experiments described below.

Flow cytometric analysis

All flow cytometry was performed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with CellQuest software Becton Dickinson). Fluorescent mAbs were purchased from PharMingen (San Diego, CA) and Caluag (South San Francisco, CA). The OKT8 (18) and OKT9 (D. Shipton, A. Al-Shamkhani, M. Fuklavec, and A. N. Barclay, unpublished data) mAbs were used as a control and a biotinylated Ig from the respective hybridomas. OKT8 and OKT9 are specific for mouse CD134 and CD134L, respectively. Labeling and analysis of cells with 5- and 6-carboxyfluorescein diacetate succinimidyl (CFSE; dieter Molecular Probes, Eugene, OR) was performed as described previously (49, 50).

In vitro T cell assays

Cell preparation and culture were performed in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 50 µM 2-ME, 100 µM nonessential amino acids, 50 µM penicillin, and 50 µg/ml streptomycin. Lympoph node (LN) cells were isolated from CD134+/− and control (littermate) mice using a 40 µM nylon cell strainer (Becton Dickinson). To purify CD4+ T cells, LN cells were incubated at 2 × 10⁵/ml in M5/114 or B10P7 mutant (anti-I-A mAbs) and 0.5 µg/ml purified 2.43 (anti-CD8 mAb). After two washes in medium, the LN cells were cultured at 37°C with a 1/10 dilution of guinea pig complement (Accurate Chemicals, Westbury, NY) at 20 × 10⁶/ml for 30 min. After two additional washes in medium, the cells were rocked at room temperature for 30 min with sheep anti-mouse Ig and sheep anti-rat Ig-coated magnetic beads according to the manufacturer’s instructions (Dynal, Oslo, Norway). Bead-coated cells were then removed from the culture using a magnet. The purity of the final preparation was determined by flow cytometry and was typically >90%. For anti-CD3 proliferation assays, 5 × 10⁴ purified CD4+ LN T cells were incubated in microtiter wells with 5 × 10⁵ irradiated splenocytes (2000 rad) from C57BL/6 × 129/Sv mice in the presence of anti-CD3e mAb (purified 145-2C11) for 1, 2, 3, 4, or 5 days. The plates were then cultured with 1 µCi/ml [3H]thymidine for 18–24 h. For mixed lymphocyte responses, 0.5, 1, 2, or 4 × 10⁴ purified CD4+ LN T cells were incubated with 5 × 10⁴ allogeneic stimulator cells from BALB/c (H-2b) or CBA (H-2d) mice in a volume of 200 µl in flat-bottom 96-well plates for 1, 2, 3, 4, or 5 days.

To confirm the lack of expression of CD134 in the CD134-deficient mice, LN cells from CD134+/− and control mice were stimulated with PMA (1 ng/ml) and ionomycin (500 ng/ml) for 3 days.

Immunizations, ELISAs, and ELISPOT assays

Mice were immunized i.p with T-dependent and T-independent Ags, including 10 µg TNP-KLH, 10 µg TNP-Ficol, and 5 µg nitrophenyl (NP) OVA (all given in alum). For ELISAs, plates were precoated with 0.5 µg/ml TNP-KLH, TNP-Ficol, or NP (13)-BSA (Solid Phase Sciences, San Rafael, CA) in PBS. The plates were blocked with 10% FCS in PBS containing 0.1% (v/v) Tween 20 and then incubated overnight at room temperature with 2-fold serial dilutions of sera from CD134+/− or control mice in PBS containing 2% FCS. NP FP plates were washed and then incubated with biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, or IgE (PharMingen). This was followed by an additional wash before the addition of 1 µg/ml HRP coupled to streptavidin (Jackson Immunoresearch, West Grove, PA). After 30 min at room temperature, the plates were washed and then 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) in a citric acid/phosphate buffer and 0.03% H₂O₂ was added as a substrate. Detection was performed using a Molecular Dynamics plate reader (Molecular Dynamics, Sunnyvale, CA) set at 405 nm and 490 nm. Results for the TNP-KLH assays were quantitated by comparison to standard anti-TNP mAbs (kindly provided by Dr. Robert Coffman, DNAX, Palo Alto, CA). NP ELISAs were similar except alkaline phosphatase-coupled anti-mouse Ig Ab (PharMingen) and Sigma 104 substrate (Sigma, St. Louis, MO). After 30 min at 37°C, the plates were washed and then incubated for 5 min at room temperature with 2-fold serial dilutions of sera from CD134+/− or control mice in PBS containing 2% FCS. NP FP plates were washed and then incubated with biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, or IgE (PharMingen). This was followed by an additional wash before the addition of 1 µg/ml HRP coupled to streptavidin (Jackson Immunoresearch, West Grove, PA). After 30 min at room temperature, the plates were washed and then 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) in a citric acid/phosphate buffer and 0.03% H₂O₂ was added as a substrate. Detection was performed using a Molecular Dynamics plate reader (Molecular Dynamics, Sunnyvale, CA) set at 405 nm and 490 nm. Results for the TNP-KLH assays were quantitated by comparison to standard anti-TNP mAbs (kindly provided by Dr. Robert Coffman, DNAX, Palo Alto, CA). NP ELISAs were similar except alkaline phosphatase-coupled anti-mouse Ig Ab (PharMingen) and Sigma 104 substrate (Sigma, St. Louis, MO) were used.

To measure Abs produced against Thielers murine encephalomyelitis virus (TEMV), serial dilutions of sera were incubated for 45 min at 37°C in commercially available ELISA plates coated with TMEV or control Ags.
The plates were washed and then incubated with alkaline phosphatase coupled anti-IgG Ab (Jackson ImmunoResearch) for 45 min at 37°C. Sigma 104 was used as a substrate as above.

IgE responses were determined as described previously (51). Briefly, ELISA plates were coated with an anti-IgE Ab (B.1E.3) in PBS overnight at 4°C and then blocked with 10% FCS in PBS/Tween 20. After several washes in PBS/Tween 20, serial dilutions of the sera were added and the plates were incubated at room temperature. The plates were washed again and incubated with a secondary biotinylated anti-IgE Ab (EM-95). After another round of washes, the plates were incubated with alkaline phosphatase coupled to streptavidin (The Jackson Laboratory, Bar Harbor, ME). Sigma 104 substrate was added after a final wash.

For enzyme-linked immunospot (ELISPOT) assays, ELISA plates were coated with anti-IL-4 Ab (11B11) in PBS overnight at 4°C. The plates were then blocked with 10% FCS in PBS before incubation at 37°C overnight with cells (2-fold serial dilutions starting at 1 \times 10^5 ). They were then washed again immediately before separate incubations with a secondary anti-IL-4 Ab (BUD6), followed by streptavidin-alkaline phosphatase. After a final round of washes, the plates were treated for 30 min in the dark with 1 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine (Sigma) in 0.1 M alkaline buffer/0.6% agarose. IL-4-secreting cells were detected as blue spots on the bottom of the wells.

Infections and delayed-type hypersensitivity assays

Mice were inoculated with 10^4 PFU intracranially or 10^6 PFU i.p. of the DA strain of TMEV as described (52, 53). Sera were collected on day 7 after i.p. inoculation or on day 90 after intracranial inoculation. Total RNA was prepared from PBS-perfused brain and spinal cord using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Five-fold serial dilutions starting with 10 \mu g RNA were filtered onto Hybond C-extra membranes (Amersham, Arlington Heights, IL) and hybridized with a virus-specific or GAPDH 32P-labeled probe. Positive controls for the detection of viral RNA included brain and spinal cord RNA from a C57BL/6 mouse 7 days after TMEV inoculation and viral cDNA.

Mice were inoculated in the hind footpads with 4 \times 10^5 metacyclic promastigotes of Leishmania major. The progression of the disease was monitored weekly using a metric caliper to measure the hind footpad lesions. Parasite burden was determined as described previously (54).

Infective third stage larvae of Nippostrongylus brasiliensis were isolated from the feces of experimentally infected rats as described (51). The larvae were washed extensively with saline, counted, and injected s.c. at the base of the tail into CD134-deficient and control mice using 500 worms/mouse in 0.2 ml of PBS. After 10 days, the number of adult worms in the intestines was determined by direct visualization. The lungs of infected mice were completely blanched with ice-cold PBS, excised, minced into fine fragments, and dispersed into a single-cell suspension in PBS using a syringe plunger. Cells were passed through a nylon strainer and adjusted to 10^7 cells/ml in culture medium for use in cytokine ELISPOT assays.

For delayed-type hypersensitivity responses, mice were sensitized with 100 \mu l of 100 mg/ml oxazolone (4-ethoxymethylene-2-phenyloxazolone; Sigma) in acetone/olive oil (4:1) applied evenly on a shaved hind flank (55, 56). Five days later, 5 \mu l of the same oxazolone solution was applied to each side of the right ear. Acetone/olive oil without oxazolone was applied to the left ear in a similar fashion. The thickness of the central portion of each ear was then measured at various times using a Dial thickness gauge.

Adoptive transfer experiments with TCR transgenic T cells

These experiments were performed essentially as described (57). In brief, cells from the LN of female H-2d DO11.10 TCR transgenic mice (CD134^+/- or controls, on a B6/129/B10.D2 background) were injected

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**FIGURE 1.** Inactivation of the murine cd134 locus by gene targeting in ES cells. A, Design of the cd134-targeting vector, structure of the cd134 locus before and after targeting, and the expected sizes of the mutant and wild-type bands on Southern blots. B, Southern blot analysis: ES cell DNA cut with BglII and hybridized with probe A (left) and mouse tail DNA cut with ApaI and hybridized with probe B (right). Mouse and ES cell genotypes are indicated beneath the autoradiographic image.
on CD8 cells gated for CD4 expression. Similar histograms were produced by gating CD4 and anti-CD8 mAbs, and the histograms show relative fluorescence on lowed by streptavidin-tricolor. The cells were costained with conjugated anti-
OX86 and OX89 were detected with a biotinylated anti-rat IgG1 mAb fol-
clude nonactivated cells and cells stained with an isotype-matched mAb. 
CD134L mAbs (OX86 and OX89, respectively). Control cell populations in-
left mAb. Dot plots on the 
FIGURE 2. Cell surface phenotype of CD134+ T lymphocytes. A, FACS analysis of CD134 expression on LN T cells and thymocytes from wild-type and mutant mice after activation with PMA and ionomycin and anti-CD3 mAb. Dot plots on the left show indirect staining with anti-CD134 mAb, whereas histograms on the right show staining with human IgG1 (control, shaded histogram) or human IgG1 fusion proteins containing the extracellular domains of CD134 (solid line) or CD134L (dashed line). PE-conjugated anti-human IgG was used to detect the fusion proteins. Labels next to individual histograms refer to the expression of CD134 (as detected by CD134L-Ig staining), CD134L (as detected by CD134 staining), or background (as detected by staining with control human IgG1). B, FACS analysis of Con A-activated T cells from wild-type and CD134−/− mice stained with anti-CD134 or anti-
CD134L mAbs (OX86 and OX89, respectively). Control cell populations in-
clude nonactivated cells and cells stained with an isotype-matched mAb. 
OX86 and OX89 were detected with a biotinylated anti-rat IgG1 mAb fol-
owed by streptavidin-tricolor. The cells were costained with conjugated anti-
CD4 and anti-CD8 mAbs, and the histograms show relative fluorescence on 
cells gated for CD4 expression. Similar histograms were produced by gating on CD8+ T cells. C, Lymphocyte populations in CD134-deficient mice. Lym-
phocytes from the thymus, spleen, LN, and bone marrow from wild-type and 
mu mutant mice were stained with the indicated Abs and analyzed by flow 
cytometry.

Table I. Lymphocyte populations in CD134-deficient mice

<table>
<thead>
<tr>
<th>Source</th>
<th>Total Cells (\times 10^6)</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD4+/CD8+</th>
<th>CD4−/CD8−</th>
<th>CD3+</th>
<th>B220+/IgM+</th>
<th>B220−/IgM−</th>
</tr>
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<tbody>
<tr>
<td>Thymus</td>
<td>−/−</td>
<td>19 ± 7</td>
<td>11 ± 3</td>
<td>5 ± 3</td>
<td>80 ± 10</td>
<td>5 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>16 ± 8</td>
<td>12 ± 3</td>
<td>5 ± 4</td>
<td>78 ± 10</td>
<td>5 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN (\text{c}, \text{d})</td>
<td>−/−</td>
<td>7 ± 1</td>
<td>66 ± 3</td>
<td>31 ± 1</td>
<td>67 ± 3</td>
<td>28 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>7 ± 1</td>
<td>61 ± 4</td>
<td>33 ± 4</td>
<td>63 ± 7</td>
<td>28 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen(\text{d})</td>
<td>−/−</td>
<td>10 ± 4</td>
<td>64 ± 5</td>
<td>27 ± 4</td>
<td>47 ± 11</td>
<td>38 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>12 ± 5</td>
<td>64 ± 7</td>
<td>26 ± 3</td>
<td>55 ± 3</td>
<td>31 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>−/−</td>
<td>3</td>
<td>5 ± 1</td>
<td>1</td>
<td>3′</td>
<td>12 ± 5</td>
<td>28 ± 4</td>
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</tr>
<tr>
<td></td>
<td>+/+</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>1</td>
<td>2′</td>
<td>12 ± 4</td>
<td>29 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

* All mice were between 6 and 17 wk of age.
* \(n \geq 6\).
* \(n \geq 3\); a blank space indicates that no determination was made.
* Values for CD4+ and CD8+ LN and spleen cells are expressed as percentages of CD3+ cells.
* Cervical, brachial, axillary, superficial inguinal, and mesenteric.
* \(n = 2\).

Activation-induced cell death

A total of \(2 \times 10^6\) LN cells/ml were stimulated with 5 μg/ml Con A for 48 h. The cells were then washed extensively before incubation for 48 h with high (100 U) or low doses (3 U) of IL-2 to predispose for apoptosis as described previously (58). Cells were then stimulated with plate-bound anti-CD3e Ab to induce apoptosis. Viable cells were distinguished from dead cells by size (forward light scatter) and propidium iodide incorporation.

CD134-deficient and control mice were injected i.p. with 100 μg of staphylococcal enterotoxin B (SEB; Sigma). FACS analysis was used to determine the proportions of LN T cells expressing Vβ8.1 or 8.2 and Vβ6 on day 0, 1, 2, 3, 4, and 7.

Results

Generation of CD134-deficient mice by gene targeting

The gene encoding CD134 was inactivated in murine ES cells according to the strategy depicted in Fig. 1A. Four exons of the gene encoding the first 143 residues of the CD134 protein were deleted and replaced by a neomycin resistance cassette. The expected structure of the resultant mutant allele was verified by Southern blot using probes from the 5′ and 3′ side of the targeted region (Fig. 1B) and also using neomycin and CD134 CDNA probes (data not shown). CD134-mutant mice were generated from the targeted ES cells by standard blastocyst injection procedures (48, 59).

Activated T cells from homozygous mutant animals lacked expression of CD134 as detected by flow cytometry with theOX86 mAb (18) or with a soluble CD134L-Fc fusion protein (Fig. 2A). No other receptor for CD134L could be detected on T cells in the absence of CD134 using soluble CD134L-Fc. The expression of CD134L was significantly increased on activated CD134−/− T cells and thymocytes relative to wild-type cells as detected with either soluble CD134 or the OX89 mAb (Fig. 2A and B). It remains to be established whether this increase in CD134L expression has any impact on the phenotype of the mutant mice.

The absence of CD134 had no detectable effect on the viability or fertility of mice, and there were no obvious indications that the mice were unhealthy. Primary and secondary lymphoid tissues in the mice were normal with respect to cellularity, the expression of...
FIGURE 3. In vitro responses by T cells from CD134-deficient mice. A, Purified CD4\(^+\) T cells were activated by the addition of various doses of anti-CD3\(\varepsilon\) mAb in the presence of irradiated spleen cells. \(^{[3]}\text{H}\)Thymidine incorporation was used as an index of DNA synthesis and proliferation. The graphs show the results of a representative experiment assayed on day 3. A total of 50 U/ml of recombinant mouse IL-2 or 2 \(\mu\)g/ml of anti-CD28 mAb was added to some cultures. In each case, the results are representative of three or more independent experiments. B, Mixed lymphocyte response of CD134\(^{-/-}\) T cells. Purified H-2\(^b\) CD4\(^+\) T cells were stimulated with irradiated CBA (H-2\(^k\)) spleen cells for 1–5 days before performing a \(^{[3]}\text{H}\)thymidine uptake assay. The graph shows the result on day 3. CD134\(^{-/-}\) T cells responded in an equivalent fashion to wild-type cells throughout the time course. This result is representative of more than four independent experiments. C, Relative representation of transgenic T cells in the draining LN of adoptive recipients of DO11.10 T cells. Mice were immunized at day 0 (24 h after adoptive transfer) with the OVA peptide emulsified in CFA as described in Materials and Methods. Each point represents a determination made on an individual recipient mouse (four donor populations and a nonimmunized control were analyzed at each time point). The experiment shown is representative of three others. In two of the experiments, populations of donor cells were labeled with CFSE so that the replicative history of the expanded DO11.10 T cells could be analyzed. Histograms of CFSE fluorescence at day 3 for CD4\(^+\) transgenic T cells from the draining LN of the indicated mice are shown in D. E, T cells from wild-type and CD134\(^{-/-}\) mice were activated in vitro in the presence of 5 \(\mu\)g/ml Con A for 2 days, followed by culture for 2 days in 100 U/ml of recombinant murine IL-2 and activation for 2 days with anti-CD3\(\varepsilon\) mAb. The cells were then labeled with propidium iodide or stained with annexin V before analysis by flow cytometry. The result is representative of three independent experiments.
lymphocyte differentiation markers, and the presence of germinal centers (Fig. 2C; Table I and data not shown). Thus, at first inspection, the absence of CD134 had no detectable impact on lymphocyte development and homeostasis.

T cell responses in the absence of CD134

Several previous reports have implicated CD134 in costimulation during T cell responses (17, 19–23, 35, 40). Therefore, we performed a series of experiments to test for abnormalities in proliferative responses made by CD134-deficient T cells. As shown in Fig. 3A, CD134<sup>−/−</sup> CD4<sup>+</sup> T cells proliferated weakly when stimulated with anti-CD3e mAb in vitro, with the peak response made by CD134<sup>+/+</sup> T cells typically five times lower than that of CD134-expressing control cells. This defect in T cell proliferation could not be corrected either by the addition of recombinant IL-2 to the cultures or by treatment with anti-CD28 mAb (Fig. 3A).

Decreased proliferation was readily apparent when cells were activated with soluble but not plate-bound anti-CD3 mAb (data not shown). CFSE-labeling experiments showed that proliferating CD134<sup>−/−</sup> cells progressed through successive cell divisions at a rate that was largely indistinguishable from control cells (data not shown and Fig. 3D). Furthermore, while proliferating, the cells up-regulated the expression of activation Ags in a normal fashion. There was no apparent increase in the frequency of apoptotic cells identified by staining with annexin V (data not shown), suggesting that the defect in proliferation was not caused by enhanced apoptosis. Curiously, other in vitro and in vivo assays of T cell proliferation, such as mixed lymphocyte responses, peptide-specific responses of transgenic T cells, or stimulation with SEB, did not reveal the same sort of defect (Fig. 3, B–D, and data not shown).

We also found that CD134<sup>−/−</sup> T cells underwent activation-induced cell death in vitro and in vivo with apparently normal kinetics in response to anti-CD3 or SEB stimulation, respectively (Fig. 3E and data not shown).

H-2<sup>d</sup> CD134<sup>−/−</sup> mice expressing the DO11.10 transgenic TCR (60) were generated so that peptide-specific T cell proliferation could be examined directly in adoptive transfer experiments (56). CD134-expressing or -deficient TCR transgenic LN cells were transferred to groups of nonirradiated mice that were then subsequently injected with OVA peptide in CFA. Mutant transgenic T cells expanded and declined in numbers in the draining LN of recipient mice in a fashion that was indistinguishable from control T cells (Fig. 3C). Furthermore, CFSE-labeling experiments showed that the mutant T cells also progressed through the cell cycle in vivo with apparently normal kinetics (Fig. 3D).

As additional in vivo tests of the responses of CD134<sup>−/−</sup> T cells, we challenged the mutant animals with three kinds of infectious organisms. L. major and N. brasiliensis were used to evaluate the capacity of the mice to mount Th1- and Th2-dependent responses, respectively (61, 62), whereas TMEV was employed as a neuroinflammatory agent whose clearance from the CNS depends primarily on the action of cytotoxic T cells (63). As shown in Fig. 4 and described in more detail below, in all three cases, CD134<sup>−/−</sup> mice made equivalent responses to those of control littermates.

**FIGURE 4.** In vivo assays of T cell function in CD134<sup>−/−</sup> mice. A, Footpad measurements of mice infected with L. major. B, IL-4-secreting cells from the lungs of N. brasiliensis-infected mice.

**FIGURE 5.** Delayed-type hypersensitivity responses by CD134<sup>−/−</sup> mice. Ear swelling after s.c. application of oxazolone to the ears of wild-type or CD134<sup>−/−</sup> mice.
FIGURE 6. Ab responses by CD134<sup>−/−</sup> mice. A, Anti-TNP-KLH-specific Ab titers in the sera of wild-type or CD134<sup>−/−</sup> mice immunized with TNP-KLH. Mice were immunized with TNP-KLH as described in Materials and Methods on day 0. The mice were then reimmunized in the same way on day 20. B, Anti-NP-specific Ab response by wild-type or CD134<sup>−/−</sup> mice 15 days after immunization with NP-chicken γ globulin. C, IgE production by the indicated mice after infection with <i>L. major</i> and <i>N. brasiliensis</i>. D, Anti-TMEV Ab response by the indicated mice 7 days after infection with TMEV. E, T cell independent anti-TNP Ab response by wild-type and CD134<sup>−/−</sup> mice 10 days after immunization with TNP-Ficoll.
Thus, TMEV is effectively eliminated from the CNS of H-2b mice, allowing for an effective cytotoxic T cell response to take place. The significance of cytotoxic T cells in eradicating TMEV is underscored by where it persists in susceptible mice that lack H-2b. The signif-
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cance of cytotoxic T cells, we examined the capacity of CD134−/− mice to clear TMEV from their brains and spinal cords, indicating that they were capable of mounting effective cytotoxic T cell responses in vivo.

TMEV is a neurotropic agent that causes an autoimmune de-
myelinating condition in susceptible mouse strains (63). Mice of the C57BL/6 or 129 backgrounds are not susceptible to chronic disease because of their expression of the H-2Db molecule, which allows for an effective cytotoxic T cell response to take place. Thus, TMEV is effectively eliminated from the CNS of H-2Db mice, whereas it persists in susceptible mice that lack H-2Db. The significance of cytotoxic T cells in eradicating TMEV is underscored by the persistence of the virus in the CNS of β2-microglobulin−/− mice that lack MHC class I expression (65). CD134−/− mice cleared TMEV from their brains and spinal cords, indicating that they were capable of mounting effective cytotoxic T cell responses in vivo (data not shown).

As a final specific test of the functional properties of CD134-deficient T cells, we examined the capacity of CD134−/− mice to mount a delayed-type hypersensitivity response after topical application of oxazolone (55, 56). As shown in Fig. 5, CD134−/− mice showed normal ear swelling after s.c. injection of oxazolone. This result indicated that the absence of CD134 did not obviously impair the capacity of T cells to initiate an inflammatory response.

B cell responses in the absence of CD134
Antagonizing the CD134 receptor/ligand system.

The finding that B cell responses are apparently unaffected by the absence of CD134 is consistent with previous work suggesting that CD134 provides a costimulatory function that can promote T cell proliferation (19, 20, 22). Whereas this costimulatory function is apparently not essential for T cell responses to several model challenges, it may act to sustain the duration of T cell immunity in vivo and perhaps enhance the formation or persistence of memory T cells. These possibilities are currently under analysis.

In vivo, CD134 expression is a characteristic of T cells involved in chronic inflammatory syndromes such as experimental allergic encephalomyelitis (31), rheumatoid arthritis (37) and inflammatory bowel disease (40). It seems feasible therefore that costimulatory signals from CD134 may help to sustain T cell reactivity under these types of chronic inflammatory circumstances. Indeed, recent studies employing soluble CD134 in vivo lend experimental sup-
port to this notion (30, 40). It remains unclear, however, whether the impact of CD134 signaling in such cases is most significant for the T cell while in the LN draining an inflamed site, or, alternatively, within the inflamed tissues themselves. In this regard, CD30 (also a member of the TNF receptor family) has recently been implicated in the regulation of T lymphocyte proliferation in situ in the pancreatic islets (66). Although the reported effect of CD30 in this case is to suppress T lymphocyte responses, it is possible that CD134 may have the opposite effect and instead drive the localized expansion of Ag-reactive T cells. A chronic inflammatory response may therefore depend on the sustained inhibition and potentiation of signals through CD30 and CD134, respectively.

The finding that B cell responses are apparently unaffected by the loss of CD134 is superficially at variance with predictions from
a study employing a rabbit antisera specific for mouse CD134 (44). In this study, the blockade of the CD134 receptor-ligand interaction interfered with the secretion of IgG, a result that was suggestive of an important role for CD134 in T:B cell collaboration and B cell differentiation in vivo. Interestingly, the blockade did not impair recall responses to the priming Ag, indicating that the differentiation of Ag-primed B cells into memory cells was unaffected. Why the absence of CD134 would have a different impact on B cell responses than a blocking Ab is not immediately clear, but it is possible that the anti-OX40 antisera had unanticipated or indirect effects on the survival of T cells or on their capacity to interact with B cells. Given that CD134 is expressed only on activated peripheral T cells, it seems unlikely that its absence would have a significant effect on the composition of the naïve T cell population. Thus, the capacity of CD134−/− T cells to provide adequate help to B cells is probably not due to the selection of an atypical precursor population that is abnormally CD134 independent in its function. Nonetheless, it is possible that the loss of CD134 may have a subtle effect on the capacity of T cells to promote certain B cell responses, and this effect may not be obvious in the settings that we have observed, perhaps because they involve powerful antigenic stimulation. We have observed germinal centers in the spleens of unimmunized mice and in the LN of immunized mice, but further work is required to determine whether these germinal centers are different from those present in normal mice, either in their rate of formation, their constituents, or their function. Other assays of T:B cell interaction, such as sensitive adoptive transfer systems, may be useful in determining whether the absence of CD134 has an effect on B cell function beyond that which might be due solely to diminished T cell expansion (67, 68).

The identity of cell surface molecules that might perform similar functions to CD134 and thereby ameliorate the impact of its absence is an issue of significance in terms of defining the physiologic function of CD134. Candidates in this respect would include the CD137 molecule as well as other members of the TNF receptor family. CD137 is attractive because of its similar pattern of expression and because it can activate NF-κB through TRAF2, as can CD134 (24, 33, 69). Thus, it will be of interest to examine the phenotype of mice that lack expression of both CD134 and as can CD134 (24, 33, 69). Thus, it will be of interest to examine whether these germinal centers are different from those present in normal mice, either in their rate of formation, their constituents, or their function. Other assays of T:B cell interaction, such as sensitive adoptive transfer systems, may be useful in determining whether the absence of CD134 has an effect on B cell function beyond that which might be due solely to diminished T cell expansion (67, 68).

Acknowledgments
We are indebted to Robert Coffman for kindly providing reagents and cells for TNP-KLH ELISAs, Robert Farese for the RF8 ES cells, Thomas Ted-Wilson for helpful discussions, and Marwan Harara for technical assistance.

References