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TNF Receptor-Associated Factor 3 Is Required for T Cell-Mediated Immunity and TCR/CD28 Signaling

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We recently reported that TNFR-associated factor (TRAF)3, a ubiquitously expressed adaptor protein, promotes mature B cell apoptosis. However, the specific function of TRAF3 in T cells has remained unclear. In this article, we report the generation and characterization of T cell-specific TRAF3−/− mice, in which the traf3 gene was deleted from thymocytes and T cells. Ablation of TRAF3 in the T cell lineage did not affect CD4 or CD8 T cell populations in secondary lymphoid organs or the numbers or proportions of CD4+CD8−, CD4−CD8+ or double-positive or double-negative thymocytes, except that the T cell-specific TRAF3−/− mice had a 2-fold increase in FoxP3+ T cells. In striking contrast to mice lacking TRAF3 in B cells, the T cell TRAF3-deficient mice exhibited defective IgG1 responses to a T-dependent Ag, as well as impaired T cell-mediated immunity to infection with Listeria monocytogenes. Surprisingly, we found that TRAF3 was recruited to the TCR/CD28 signaling complex upon costimulation and that TCR/CD28-mediated proximal and distal signaling events were compromised by TRAF3 deficiency. These findings provide insights into the roles played by TRAF3 in T cell activation and T cell-mediated immunity. The Journal of Immunology, 2011, 186: 143–155.

The shared usage of TRAF3 by so many receptors predicts its ubiquitous and critical developmental functions of TRAF3 (11). The online version of this article contains supplemental material.

Abbreviations used in this paper: DP, double positive; LAT, linker of activated T cells; LLO, listeriolysin O; LMC, littermate control; LMO-ova, Listeria monocytogenes expressing secreted ovalbumin protein; LN, lymph node; N, purified splenic non-T cells; NS, nonspecific band; PI, propidium iodide; p.i., postinfection; PLC, phospholipase-C; T, T cells; TD, T-dependent; TNP, trinitrophenol; TNP-KLH, trinitrophenol-keyhole limpet hemocyanin; TRAFT, TNFR-associated factor; Treg, regulatory T cell.

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the specific roles of TRAF3 in T cell biology. However, TRAF3 binds to many TNFR family receptors expressed by T cells, and these receptors make important contributions to T cell activation and costimulation (reviewed in Ref. 20). In 2008, Gardam et al. (15) reported the generation of TRAF3floxfloxLck-Cre mice. The investigators found that TRAF3 deletion from T cells did not result in T cell expansion in the spleen and lymph nodes (LNs). In contrast to TRAF3−/− B cells, TRAF3−/− T cells do not exhibit prolonged survival, although high constitutive levels of NF-kB2 processing were observed in both cell types in the absence of TRAF3 (15). However, no further study of TRAF3 in T cell-mediated immunity or T cell signaling was described. In the current study, we generated T cell-specific TRAF3−/− mice (TRAF3floxfloxCD4-Cre, T-TRAF3−/−) to discover the functions of TRAF3 in T cell-mediated immunity.

CD4-Cre–mediated deletion of floxed genes occurs predominantly from double-positive (DP) thymocytes onward (21, 22), hence T-TRAF3−/− mice have TRAF3 deleted in CD4 and CD8 T cells. We report that deletion of TRAF3 in T lymphocytes resulted in defective TD IgG1 responses, as well as impaired Ag-specific CD8 and CD4 T cell responses to infection with the intracellular pathogen Listeria monocytogenes. Furthermore, we found that TRAF3 was unexpectedly recruited to the TCR and CD28 signaling complex upon costimulation and that TCR/CD28-mediated signaling events were affected by TRAF3 deficiency. Taken together, our findings demonstrate essential roles for TRAF3 in TCR/CD28 signaling and T cell-mediated immunity.

Materials and Methods
Generation of T-TRAF3−/− mice
TRAF3floxflox mice were generated as previously described (12). CD4-Cre transgenic mice (21) were purchased from Taconic Farms (Germantown, NY). The Taconic CD4-Cre mice have been bred for nine generations onto C57BL/6 mice. TRAF3floxflox mice were crossed with CD4-Cre mice to generate TRAF3floxfloxCD4-Cre mice, which were subsequently backcrossed with TRAF3floxflox mice to generate TRAF3floxfloxCD4-Cre (T-TRAF3−/−) mice. Mouse tails were screened by genomic PCR using primer sets FC3 + BT6 and Cre-F + Cre-R, as described (12). Deletion of exons 1 and 2 of the TRAF3 gene in thymocytes and splenic T cells was detected by genomic PCR using primers U7 and BT6, as previously described (12). T-TRAF3 mice have TRAF3 deleted in CD4 and CD8 T cells. Mouse tails were screened by genomic PCR using primer sets FC3 + BT6 and Cre-F + Cre-R, as described (12). Deletion of exons 1 and 2 of the TRAF3 gene in thymocytes and splenic T cells was detected by genomic PCR using primers U7 and BT6, as previously described (12). T-TRAF3 mice have TRAF3 deleted in CD4 and CD8 T cells.

Abs and reagents
Polyclonal rabbit Abs to TRAF1 (N19), TRAF3 (H122), and TRAF6 (H274) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit Abs to TRAF2 was from Medical and Biological Laboratories (Nagoya, Japan). FITC-, PE-, or Cy5-labeled Abs against mouse CD3, TCRαβ, TCRγδ, CD4, CD8, CD45R (B220), CD25, CD43, CD44, CD46L, CD127, and FoxP3 were purchased from eBioscience (San Diego, CA). Anti-mouse IFN-γ, TNF-α, IL-4, and IL-2 ELISA Abs were also purchased from eBioscience. FITC- or PE-labeled Abs against mouse TNF-α and IFN-γ were purchased from BioLegend (San Diego, CA). Polyclonal rabbit Abs against total or phosphorylated ERK, PLCγ1, Lck, and ZAP70 were from Cell Signaling Technology (Beverly, MA). Anti-actin Ab was from Chemicon International (Temecula, CA). HRP-labeled secondary Abs were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alkaline phosphatase-conjugated goat Abs specific for mouse IgM and IgG1 were from Southern Biotechnology Associates (Birmingham, AL). Pan T cell, CD4, CD8, and regulatory T cell (Treg) purification kits were from Miltenyi Biotec (Auburn, CA). A mouse anti-hamster IgG Ab (clone MAH1.12) was obtained from R&D Systems (Minneapolis, MN). ELISA kits for detection of TNF-α, IL-2, and IFN-γ in supernatants of PMA and ionomycin-treated TNP-specific splenocytes were from BD Biosciences (San Jose, CA) and in intracellular staining with FITC-labeled anti-FoxP3 Abs. Listmode data were acquired on a FACSCalibur (BD Biosciences) using Cell Quest software. The results were analyzed using FlowJo software (Tree Star, Ashland, OR). Forward light scatter/side scatter gating for single lymphocytes, which excludes cell aggregates, small erythrocytes, and dead cell debris, was used to analyze flow-cytometric data.

Immunization and triptenophen-specific Ig ELISA
For TD Ab responses, mice were immunized i.p. with 100 μg triptenophen-keyhole limpet hemocyanin (TNP-KLH; Biosource Technologies, Vacaville, CA) precipitated in alum and boosted with 100 μg TNP-KLH/Alum on day 21. Sera were collected on days 7, 14, and 28 after the first immunization. Serum levels of anti-triptenophen (TNP) IgM and IgG1 were measured by ELISA as described previously (12). Standard curves were determined on each plate using serial dilutions of purified TNP-specific IgM or IgG1 standards (BD Pharmingen, San Diego, CA). Plates were read on a Versamax plate reader (Molecular Devices, Sunnyvale, CA), and results were analyzed using SoftMax Pro 4.0 software. Multiplicities of 0.5 or 1.10 of each serial sample were examined. Each standard curve contained 11 dilution points; in all cases, the coefficient of determination for the standard curve (r2) was >0.98. The dilution factor that gave A405 (OD 405 nm) values within the linear range (0.1–1.5) of standard curves of ELISA was used to calculate the concentrations of TNP-specific IgM and IgG1.

L. monocytogenes infection
Recombinant L. monocytogenes expressing secreted OVA protein (LM-OVA) (23) was provided by Dr. John Harty (The University of Iowa). Eight- to twelve-week-old mice were infected i.v. with 0.05 × 108 L. monocytes (for TNP specific IgG1) or 5 × 108 CFU (5 × 108 CFU) virulent LM-OVA. At days 3 and 7 (primary response) post-infection (p.i.), spleens and livers were collected to determine bacterial load, as detailed below. Livers and spleens were homogenized in 10 ml of 0.2% fetal bovine serum in H2O. Organ homogenates were serially diluted and plated on streptomycin agar plates to determine CFU of LM-OVA in liver and spleen. Spleenocytes were also collected during the course of infection and analyzed for cytokine production.}

Enumerating OVA-specific or LLO-specific T lymphocytes by intracellular staining for IFN-γ and TNF-α
Quantification of Ag-specific CD8 and CD4 T cell responses was determined by intracellular cytokine staining, as described (24). Briefly, spleens were harvested from infected mice, and erythrocytes were depleted. Spleenocytes were washed and resuspended in fresh medium. Total splenocytes were counted on a hemacytometer, and 200 μl splenocytes (2 × 107 cells) was transferred to sterile plastic tubes. Two hundred microliters of medium plus 2 μg/ml GolgiPlugs (brefeldin A; BD Pharmingen), with or without 1 μg/ml purified OVA peptide (SIINFEKL, for OVA-specific CD8 T cell analysis) or 5 μM LLO O Ag LLOs122-211 (for LLO-specific CD4 T cell analysis), was added to the tubes. Spleenocytes were subsequently incubated at 37°C for 6 h before staining. Spleenocytes were fixed with aldehydesulfonate-calcium-treated cells were washed with FITC-labeled anti-IFN-γ and PE-labeled anti-TNF-α Abs.
Liver lymphocyte analysis

Littermate control (LMC) and T-TRAF3−/− mice, 7–8 wk old, were infected i.v. with 0.3 LD50 virulent L. monocytogenes. On days 5 and 7 p.i., mice were euthanized, and their livers were perfused with cold Hanks medium, rinsed in PBS, and forced through a 70-μm screen, according to the protocol of Schmidt et al. (25). After centrifugation at 500 × g, 4°C, the pellet was resuspended in 15 ml 35% Percoll in Hanks medium. The cells were pelletted again at 500 × g at 25°C with no brake. The resulting pellet was treated with ACK to lyse RBCs and then suspended in 10 ml RPMI 1640 + 10% FCS and washed three times by centrifugation to yield the mononuclear cell fraction. Total cell counts were determined, and the cells were stained with allophycocyanin-anti-CD4 and FITC–anti-CD8 and analyzed on a FACSCalibur.

Splenic total, CD4, and CD8 T cell purification

Splenic T cell subsets were prepared from naive 2–3-mo-old mice. Splenic T cells and non-T cells were separated using a mouse Pan T Cell Isolation Kit (#130-090-861; Miltenyi Biotec) and a magnetic separator (Miltenyi Biotec), following the manufacturer’s protocols. CD4 Th cells were purified using a mouse CD4 T Cell Isolation Kit by negative selection (#130-090-860; Miltenyi Biotec), and Tregs were depleted using a mouse CD4+ CD25+ Regulatory T Cell Isolation Kit (#130-091-041; Miltenyi Biotec), following the manufacturer’s protocols. CD8 T cells were purified using a mouse CD8 T Cell Isolation Kit by negative selection (#130-090-859; Miltenyi Biotec), following the manufacturer’s protocols. The purity of isolated populations was monitored by FACs analysis using B220–, CD3+, CD4–, CD8–, or CD25–specific Abs, and cell preparations with >90% purity were used for further experiments. Purified splenic T cells were cultured in mouse culture medium (RPMI 1640 medium supplemented with 5% FCS, 10 μg/mL 2-ME, 10 mM HEPES [pH 7.5]), 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mM nonessential amino acids). For detection of TRAF3 expression, thymocytes, splenic T cells, or non-T cells and purified CD4+ or CD8+ splenic T cells were directly lysed as previously described (26). Immunoblot analysis was performed as previously described (26).

Survival assay and cell cycle analysis

Purified splenic CD4 (Treg depleted) or CD8 T cells (1 × 10^6/ml/well) were cultured in 24-well plates in the absence or presence of 0.5 μg/ml plate-bound anti-CD3 mAb (clone 145–2C11; eBioscience) with or without 2 μg/ml soluble anti-CD28 mAb (clone 37.51; eBioscience) at 37°C. At each time point, an aliquot of cells was removed to determine the number of viable cells by staining with trypan blue. PI staining and CFSE labeling intensity were quantified using a benchtop FACScan flow cytometer (BD Biosciences).

Cytokine ELISA

Purified splenic CD4 (Treg depleted) or CD8 T cells were cultured at 1 × 10^6 cells/well in 24-well plates in the absence or presence of 0.5 μg/mL plate-bound anti-CD3 mAb with or without 2 μg/mL soluble anti-CD28 mAb at 37°C. Culture supernatants were collected at various time points. Concentrations of IL-2, IL-4, IFN-γ, and TNF-α in culture supernatants were determined by ELISA using cytokine-specific coating Abs and biotinylated detection Abs (eBioscience), as previously described (28).

Th1 polarization

Naïve CD4+ T cells were purified from LMC and T-TRAF3−/− mice by negative selection (Miltenyi Biotec CD4+ T cell isolation kit, #130-090-860), followed by positive selection on anti-CD62L mAbs (#130-049-701; Miltenyi Biotec). Cells were all >90% CD4+ by flow cytometry. Cells were suspended in mouse culture medium at 0.5 × 10^6/ml and cultured in 24-well plates that had been coated overnight with 5 μg/mL anti-CD3 agonistic Ab (eBioscience) in PBS and subsequently washed twice with mouse culture medium (RPMI 1640 medium supplemented with 5% FCS) to remove unbound Ab. TCRs were cross-linked with a secondary Ab (mouse anti-hamster IgG, clone MAH1.12; R&D Systems) for various time periods during staining and collection. The sorted cells were serum starved and then stimulated, as described above, for early TCR signaling measurements.

Magnetic immunoprecipitation of the TCR/CD28 signaling complex

Splenic T cells were purified from 2–3-mo-old naive mice using the Pan T cell purification kit (Miltenyi Biotec). Cells (3 × 10^5 cells for each condition) were cultured in serum-free media and incubated with mAbs: 5 μg/ml anti-CD3 alone, 5 μg/ml anti-CD28 alone, or 5 μg/ml anti-CD3 plus 5 μg/ml anti-CD28 on ice for 30 min. Cells were washed once in serum-free medium to remove unbound Ab. Cells were subsequently incubated with protein G-magnetic beads (Dynabeads; Invitrogen) prearmed with mouse anti-hamster IgG (cross-linking Ab for anti-CD3 and anti-CD28) on ice for 30 min to allow the binding of beads with Abs to T cells and then were stimulated at 37°C for 3 or 7 min. Cells were chilled on ice, pelleted by centrifugation at 4°C, and lysed in 400 μl ice-cold lysis buffer (0.5% Triton X-100, 100 mM NaCl, 40 mM Tris [pH 7.5], 1 mM CaCl2, 1 mM MgSO4, complete EDTA-free protease inhibitor mixture [Roche Applied Science, Madison, WI], 2 mM Na3VO4 and 50 μg/ml DNase) for 30 min on ice with repeated mixing. Magnetic beads were pelleted on a magnetic rack without centrifugation (Invitrogen), and unbound materials were removed following the manufacturer’s protocol. The magnetic beads were washed five times with ice-cold lysis buffer (without DNase), left in a final volume of 50 μl, and boiled in SDS sample buffer. Aliquots of lysates and the immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblot analysis.

Statistics

For direct comparison of TNP-specific Ig isotype levels, OVA-specific CD8 T cells, or LLO-specific CD4 T cells between LMC and T-TRAF3−/− mice, statistical significance was determined using the unpaired t test for two-tailed data; p values < 0.05 are considered significant, and p values < 0.01 are considered very significant.

Results

Generation of T-TRAF3−/− mice

We previously generated mice homozygous for the floxed TRAF3 allele (TRAF3lox/lox) (12). To delete the loxP-flanked TRAF3 alleles specifically in T lymphocytes, we used a transgenic mouse strain expressing Cre under the control of the CD4 promoter/enhancer/silencer, providing a T-cell-specific source of Cre (21, 30). It was shown that CD4-Cre mediates deletion of loxP-flanked
gene segments in most thymocytes at the CD4⁺CD8⁺ stage (21, 22). TRAF3<sup>flox/flox</sup>CD4-Cre mice were born at the expected Mendelian frequencies and survive and breed normally. We verified excision of the first two coding exons of the TRAF3 gene in thymocytes by genomic PCR and the elimination of TRAF3 protein expression in thymocytes, as well as in splenic CD4 and CD8 T cells of TRAF3<sup>flox/flox</sup>CD4-Cre mice (T-TRAF3⁻/⁻ mice), by Western blot analysis (Fig. 1A, 1B). Interestingly, we found that TRAF1 and TRAF2 protein levels were modestly upregulated in TRAF3⁻/⁻ T cells. In contrast, the level of TRAF6 was not obviously changed by TRAF3 deletion. Similarly, we also observed modest upregulation of TRAF1 and TRAF2 in TRAF3⁻/⁻ B cells (12). This raises the possibility that upregulated TRAF1 and TRAF2 may partially compensate for the loss of TRAF3 in both cell types. Together, these results validated TRAF3<sup>flox/flox</sup>CD4-Cre mice as T cell-specific TRAF3⁻/⁻ (T-TRAF3⁻/⁻) mice.

**Thymocytes and peripheral T cell populations in T-TRAF3⁻/⁻ mice**

The thymus size of T-TRAF3⁻/⁻ mice was comparable to that of TRAF3<sup>flox/flox</sup> LMC mice. Flow-cytometric analyses revealed that T-TRAF3⁻/⁻ mice exhibited normal frequencies and numbers of thymocyte populations, including double-negative (CD4⁺CD8⁻), DP (CD4⁺CD8⁺), and CD4 (CD4⁺CD8⁻) and CD8 (CD4⁻CD8⁺) single-positive cells (Fig. 1C, 1D). Thus, elimination of TRAF3 from DP thymocytes did not affect CD4/CD8 lineage commitment or survival in the thymus.

In contrast to the severe splenomegaly and lymphadenopathy observed in B-TRAF3⁻/⁻ mice, adult T-TRAF3⁻/⁻ mice exhibited slightly bigger spleens and normal LNs compared with those from LMC mice or wild-type mice (Fig. 1E, P. Xie, unpublished observations). We performed cellularity analysis by immunofluorescence staining and flow cytometry to determine B and T cell populations.
in the spleen and LNs. We found that T-TRAF3\(^{-/-}\) mice have normal proportions and absolute numbers of B cells (B220\(^{+}\)) and T cells (CD3\(^{+}\)) in the spleen and LNs compared with LMC (Fig. 1F). T-TRAF3\(^{-/-}\) mice also display normal proportions of CD4 (CD4\(^{+}\)CD8\(^{-}\)CD25\(^{+}\)) and CD8 (CD4\(^{-}\)CD8\(^{+}\)) T cells in the spleen and LNs (Fig. 2A). Interestingly, however, T-TRAF3\(^{-/-}\) mice show increased frequency and numbers of CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\) Tregs in the spleen and LNs (Fig. 2A, 2B). These results demonstrate that deletion of TRAF3 from thymocytes at the DP stage did not substantially affect CD4 and CD8 T cell development and homeostasis, but it did alter the maturation or homeostasis of Tregs in secondary lymphoid organs.

**Altered expression profile of CD44 and CD43 in T-TRAF3\(^{-/-}\) mice**

As the first step in examining the phenotype of TRAF3\(^{-/-}\) T cells in vivo, we determined the expression profile of a series of cell surface markers on CD8 and CD4 T cells. We found that the expression profiles of CD44, a marker of effector and memory T cells, were considerably altered on CD8 and CD4 T cells in the spleen and LNs compared with LMC (Fig. 2A). The CD4\(^{+}\) population of CD8 T cells was absent in the spleen and LNs of T-TRAF3\(^{-/-}\) mice, suggesting that they may have decreased proportions of effector and memory T cells. Interestingly, the expression level of CD43 was drastically decreased on CD8, but not CD4, T cells in the spleen and LNs of T-TRAF3\(^{-/-}\) mice (Fig. 2A). As the first step in examining the phenotype of TRAF3-deficient T cells, we enumerated Ag-specific T cells in LMC and T-TRAF3\(^{-/-}\) mice using intracellular staining of IFN-\(\gamma\) and TNF-\(\alpha\) following in vitro stimulation with purified peptides OVA\(_{257-264}\) (for CD8 T cell detection) or LLO\(_{190-201}\) (for CD4 T cell detection). On day 7 postinfection, we found significantly lower OVA-specific IFN-\(\gamma\)- or TNF-\(\alpha\)-producing CD8 T cells in T-TRAF3\(^{-/-}\) mice, both in frequency and total numbers (Fig. 4C, 4D). Reductions in the frequency and total numbers of LLO-specific IFN-\(\gamma\)- or TNF-\(\alpha\)-producing CD4 T cells were even more striking in T-TRAF3\(^{-/-}\) mice (Fig. 4C, 4D). However, on day 10 postinfection, the frequency and number of OVA-specific CD8 T cells were nearly normal in T-TRAF3\(^{-/-}\) mice, whereas LLO-specific CD4 T-TRAF3\(^{-/-}\) T cells were still moderately decreased compared with LMC (Fig. 4E). Thus, TRAF3 deficiency seemed to delay the expansion of Ag-specific T cells, with CD4 T cells affected to a greater degree.

**Decreased proliferation of TRAF3\(^{-/-}\) CD4 and CD8 T cells in response to TCR and CD28 stimulation**

Defective TgIgG1 response in T-TRAF3\(^{-/-}\) mice

Following immunization with a TD Ag (TNP-KLH), T-TRAF3\(^{-/-}\) mice showed a modestly decreased TNP-specific IgM response on day 7 but almost normal TNP-specific IgM responses on days 14 and 28 compared with LMC mice (Fig. 3). In contrast, TNP-specific IgG1 responses were defective in T-TRAF3\(^{-/-}\) mice at all time points examined in this study (Fig. 3). Our data extended the initial findings using cells from the neonatally lethal TRAF3\(^{-/-}\) mice (P. Xie and L.L. Stunz, unpublished observations). Considering that CD44 and CD43 are involved in the regulation of T cell migration, adhesion, and activation (31–34), altered expression profiles of these two molecules, together with the increased frequency of Tregs, predict that T-TRAF3\(^{-/-}\) mice might exhibit altered T cell-mediated immunity. Thus, we next sought to investigate immune responses to TD Ag immunization and bacterial infections.

**Defective TD IgG1 response in T-TRAF3\(^{-/-}\) mice**

Following immunization with a TD Ag (TNP-KLH), T-TRAF3\(^{-/-}\) mice showed a modestly decreased TNP-specific IgM response on day 7 but almost normal TNP-specific IgM responses on days 14 and 28 compared with LMC mice (Fig. 3). In contrast, TNP-specific IgG1 responses were defective in T-TRAF3\(^{-/-}\) mice at all time points examined in this study (Fig. 3). Our data extended the initial findings using cells from the neonatally lethal TRAF3\(^{-/-}\) mouse that suggested TRAF3 is required for TD Ab responses (11) and showed that TRAF3 in T cells is especially critical for the production of IgG1. Importantly, our data also suggest that this requirement is intrinsic to T cell function.

**Impaired primary T cell responses to L. monocytogenes infections in T-TRAF3\(^{-/-}\) mice**

*L. monocytogenes*, a facultative intracellular bacterium, was used as a model for T cell-mediated immune responses to bacterial infections. LM-OVA (23) allowed the detection of Ag-specific CD8 T cell responses. Naive T-TRAF3\(^{-/-}\) and LMC mice were infected i.v. with 0.05\(\times\)LD\(_{50}\) (5 \(\times\)10\(^{5}\) CFU) of virulent LM-OVA. Six of 24 (25%) T-TRAF3\(^{-/-}\) mice succumbed to this low, normally sublethal dose of LM-OVA infection between days 7 and 16 p.i. (Fig. 4A). In contrast, all LMC mice (n = 28) survived. Bacterial loads in the spleen on day 3 p.i. were comparable between LMC and T-TRAF3\(^{-/-}\) mice, and both groups were able to clear bacterial infection in the spleen on day 7 (P. Xie, unpublished observations). However, two of six (33%) T-TRAF3\(^{-/-}\) mice, but none of the LMC mice, had high bacterial loads in the liver on day 7 p.i. (Fig. 4B). The slower clearance of bacteria in the liver, but not in the spleen, suggests that TRAF3 \(^{-/-}\) T cells may have defects in migrating to target organs or in trafficking within the target tissues. To test this possibility, we analyzed CD4 and CD8 T cells in the liver on days 5 and 7 p.i. We found that T-TRAF3\(^{-/-}\) mice had decreased numbers of CD4 and CD8 T cells in the liver on day 5 but slightly increased numbers on day 7 p.i. (Supplemental Fig. 1). Thus, TRAF3\(^{-/-}\) CD4 and CD8 T cells exhibited delayed responses in migrating and trafficking to the liver following primary *L. monocytogenes* infection. This is consistent with our observation that peripheral TRAF3\(^{-/-}\) T cells exhibited altered expression profiles of CD44 and CD43, two molecules known to participate in the regulation of T cell migration and trafficking (31–34). Together, these data demonstrate that TRAF3 deficiency in T cells compromised the ability of mice to resist *L. monocytogenes* infection.

To investigate the mechanisms contributing to defective function of TRAF3-deficient T cells, we enumerated Ag-specific T cells in LMC and T-TRAF3\(^{-/-}\) mice using intracellular staining of IFN-\(\gamma\) and TNF-\(\alpha\) following in vitro stimulation with purified peptides OVA\(_{257-264}\) (for CD8 T cell detection) or LLO\(_{190-201}\) (for CD4 T cell detection). On day 7 postinfection, we found markedly lower OVA-specific IFN-\(\gamma\)- or TNF-\(\alpha\)-producing CD8 T cells in T-TRAF3\(^{-/-}\) mice, both in frequency and total numbers (Fig. 4C, 4D). Reductions in the frequency and total numbers of LLO-specific IFN-\(\gamma\)- or TNF-\(\alpha\)-producing CD4 T cells were even more striking in T-TRAF3\(^{-/-}\) mice (Fig. 4C, 4D). However, on day 10 postinfection, the frequency and number of OVA-specific CD8 T cells were nearly normal in T-TRAF3\(^{-/-}\) mice, whereas LLO-specific CD4 T-TRAF3\(^{-/-}\) T cells were still moderately decreased compared with LMC (Fig. 4E). Thus, TRAF3 deficiency seemed to delay the expansion of Ag-specific T cells, with CD4 T cells affected to a greater degree.
FIGURE 2. Increased Tregs and altered CD44 and CD43 expression profiles on peripheral CD8 and CD4 T cells in T-TRAF3−/− mice. A, Representative FACS profiles of splenocytes from LMC and T-TRAF3−/− mice stained for CD4, CD25, and Foxp3 expression. B, Percentages (upper panels) and numbers (lower panels) of CD4 (CD4+CD8−CD25−), CD8 (CD4−CD8+), and Treg (CD4+CD25+Foxp3+) cells in spleens and LN of LMC and T-TRAF3−/− mice. Data shown are results of four independent experiments (mean ± SEM). C, Representative FACS profiles of CD44 expression on CD8+ or CD4+ T cells in spleens, LN, and thymus of LMC and T-TRAF3−/− mice. D, Representative FACS profiles of CD43 expression on CD8+ or CD4+ T cells in spleens, LN, and thymus of LMC and T-TRAF3−/− mice. Mice analyzed were 8–12 wk old.
ionomycin was not decreased in TRAF3−/− mice. However, production of IL-2 and TNF-α was decreased in TRAF3−/− mice, in sharp contrast to the proapoptotic function of TRAF3 in B cells.

The increased apoptosis observed in TRAF3−/− T cells after stimulation with anti-CD3 Ab alone and anti-CD3 + anti-CD28 Abs (Fig. 5) could be due to a decrease in prosurvival factors, such as Bcl-xL and Bcl-2, or an increase in Bim. Thus, we examined the protein levels of Bcl-xL, Bcl-2, and Bim in purified TRAF3−/− and LMC T cells; they were equivalent between TRAF3−/− and LMC T cells in the absence and presence of CD3 + CD28 stimulation (Fig. 5C). We previously found that TRAF3 deficiency results in constitutive processing of NF-κB2 from inactive p100 to the active p52, leading to prolonged B cell survival. We next examined NF-κB2 processing in purified TRAF3−/− and LMC T cells. Consistent with the study by Gardam et al. (15), we observed markedly increased NF-κB2 processing in purified TRAF3−/− T cells compared with those observed in LMC cells (Supplemental Fig. 3). However, in contrast to TRAF3−/− B cells, constitutive NF-κB2 processing did not lead to prolonged survival or decreased apoptosis in TRAF3−/− T cells, indicating that this is a cell type-specific phenomenon. Together, these results indicate that the increased apoptosis observed in TRAF3−/− T cells is not mediated by alterations in Bcl-2 family member expression or constitutive NF-κB2 activation. The specific mechanism responsible for this elevated apoptosis will require additional investigation.

Diminished cytokine production of TRAF3−/− CD8 and CD4 T cells in response to TCR and CD28 stimulation

One potential mechanism contributing to the observed defective Ag-specific IgG1 response is decreased IL-4 secretion by TRAF3−/− CD4 Th cells. Moreover, LLO-specific CD4 and OVA-specific CD8 TRAF3−/− T cells produced less IFN-γ and TNF-α on a per-cell basis as determined by intracellular staining and flow cytometry (Fig. 4C). These observations prompted us to further analyze cytokine production in isolated TRAF3−/− CD4 or CD8 T cells, depleted of Tregs, in response to TCR/CD28 stimulation. We found that following stimulation with anti-CD3 and anti-CD28 mAbs, production of IL-4, IL-2, TNF-α, and IFN-γ was drastically diminished by TRAF3 deficiency in CD4 T cells (Treg depleted) (Fig. 6A) and was modestly reduced in TRAF3−/− CD8 T cells (Fig. 6B). However, production of IL-2 and TNF-α induced by PMA and ionomycin was not decreased in TRAF3−/− CD4 and CD8 T cells (P. Xie, unpublished observations), thereby excluding the possibility that TRAF3 deficiency globally affects the cytokine-production machinery in T cells. Such decreases in cytokine production in the absence of TRAF3 may contribute to the defective TD IgG1 responses (Fig. 3) and impaired T cell immunity to L. monocytogenes infection in T-TRAF3−/− mice (Fig. 4). The decreased IL-2 production may also contribute to the reduced proliferation of TRAF3−/− CD4 T cells following TCR and CD28 stimulation (Fig. 5). The T cell-proliferation response and cytokine production indicate that the intrinsic function of CD4 helper and/or CD8 effector T cells in response to TCR/CD28 engagement is inhibited by TRAF3 deficiency.

Another potential mechanism contributing to the defects in expansion of Ag-specific CD4 T cells observed in T-TRAF3−/− mice (Fig. 4) is defective development or differentiation of Th cells. To test this, we performed in vitro skewing experiments with naive CD4 T cells purified from LMC and T-TRAF3−/− mice. Because LLO peptide-specific, IFN-γ– and TNF-α–producing CD4 T cells were analyzed in the L. monocytogenes-infection experiments (Fig. 4), our skewing experiment primarily focused on Th1 polarization. Naive CD4 T cells were purified from LMC and T-TRAF3−/− mice and cultured in the presence of 5 µg/ml plate-bound anti-CD3 Ab, 10 µg/ml anti-CD28 Ab, 20 U/ml IL-2, 4 ng/ml IL-12 p70, and 2 µg/ml anti-mouse IL-4 Ab for 4 d to induce Th1 differentiation. We found that in vitro polarized Th1 cells had a partial reduction in IFN-γ and TNF-α production, as well as a marked decrease in IL-2 secretion following stimulation with PMA + ionomycin or CD3 + CD28 (Supplemental Fig. 4). These data suggest that differentiation and maturation of CD4+ Th1 cells, which require stimulation signals from the TCR and costimulatory molecules, may be affected, in part, by TRAF3 deficiency.

Impaired phosphorylation of proximal signaling components of TCR/CD28 in the absence of TRAF3

To understand how TRAF3 deficiency impaired the distal effector functions of TCR and CD28 signaling, including proliferation, cytokine production, and in vitro Th1 polarization, we investigated proximal-signaling events following TCR and CD28 engagement in TRAF3−/− T cells. Splenic T cells were purified from naive mice by negative selection, and Tregs were depleted using magnetic beads. After stimulation with anti-CD3 or anti-CD3 + anti-CD28 mAbs, phosphorylation of proximal-signaling components (ERK, LAT, PLCγ1, and ZAP70) was measured by immunoblot analysis. Phosphorylation of these TCR-signaling components induced by anti-CD3 mAb alone was normal in TRAF3−/− T cells.
In contrast, the synergistic effects induced by anti-CD3 + anti-CD28 mAbs were abolished by TRAF3 deficiency (Fig. 7A, Supplemental Fig. 5). Interestingly, activation of the classical NF-κB pathway was not affected by TRAF3 deletion, as measured by phosphorylation and degradation of IkBα after stimulation with anti-CD3 Ab alone or anti-CD3 + anti-CD28 Abs (Fig. 7A, Supplemental Fig. 5). Considering that T-TRAF3<sup>−/−</sup> mice had a decreased proportion of CD44<sup>hi</sup> memory T cells (Fig. 2), it is possible that the decrease in CD44<sup>hi</sup> memory T cells contributes to the observed decrease in phosphorylation of ERK, LAT, PLCγ<sub>1</sub>, and ZAP70 following stimulation with TCR and CD28. We next sorted Thy1<sup>+</sup>CD25<sup>−</sup>CD44<sup>low</sup> naive T cells and repeated the experiments of stimulation with anti-CD3 + anti-CD28 Abs. We found that sorted TRAF3<sup>−/−</sup> naive T cells also displayed partial impairment in phosphorylation of ERK, LAT, PLCγ<sub>1</sub>, and ZAP70 in response to stimulation through CD3 and CD28 (Fig. 7B, Supplemental Fig. 5). Together, our results indicate that TRAF3 deficiency specifically impairs TCR and CD28 synergy at an early receptor-proximal step, including phosphorylation of ERK, LAT, PLCγ<sub>1</sub>, and ZAP70.

Recruitment of TRAF3 to the TCR/CD28-signaling complex

Previous studies in B lymphocytes demonstrated that ligation of CD40 recruits TRAF3 to CD40 signaling rafts (35). This, together with the receptor-proximal nature of TRAF3 effects in TCR/CD28 signaling, prompted us to assess the possibility that TRAF3 asso-
ciates with the TCR and/or CD28-signaling complex. Splenic total T cells were purified from naive mice by negative selection. We found that upon stimulation with anti-CD3 or anti-CD28 mAbs alone, TRAF3 was not detected in the TCR or CD28-signaling complex in splenic T cells purified from wild-type or LMC mice (Fig. 7C). However, upon coligation of TCR and CD28 by anti-CD3 and anti-CD28 mAbs, TRAF3 was coimmunoprecipitated with the TCR/CD28-signaling complex in splenic T cells purified from LMC mice (Fig. 7C, 7D). It was shown that TRAF6 and Malt1 participate in TCR/CD28 signaling in T cells (36) and that TRAF3 interacts with Malt1 in B cells (37). Thus, we examined the possible involvement of TRAF6 and Malt1 in the TRAF3 association. However, coimmunoprecipitation of TRAF6 or Malt1 with CD3 + CD28 stimulation was not detected in T cells under our experimental conditions (Fig. 7C). Recruitment of ZAP70 to the TCR/CD28-signaling complex was not affected in the absence of TRAF3 (Fig. 7D). Thus, TRAF3 is a component of the TCR/CD28-signaling complex, but the presence of TRAF3 does not affect the association between TCR and ZAP70. To the best of our knowledge, this is the first demonstration of the association of TRAF3 with an Ag-receptor complex.

Discussion

Substantial progress has recently been made in understanding the function of TRAF3 in B lymphocytes (1, 12, 13, 15–17, 26, 38). Although initial studies of chimeric mice reconstituted with...
TRAF3−/− fetal liver cells implicated TRAF3 in the TD Ab response (11), no further information about the specific roles of TRAF3 in T cell biology has been forthcoming, including whether TRAF3 plays direct or indirect roles in T cell function. To address this important gap in knowledge, we generated and characterized T cell-specific TRAF3-deficient mice. In addition to leading to defective TD IgG1 responses, ablation of TRAF3 in T cells impaired T cell-mediated immunity to infection with *L. monocytogenes*, indicating that CD8 and CD4 T cells require TRAF3 for optimal function. Thus, TRAF3 plays unique and indispensable roles in T cell immunity, which are not readily compensated for by other members of the TRAF family. Our subsequent evidence revealed that the defective T cell-mediated immunity observed in T-TRAF3−/− mice resulted from decreased intrinsic function of CD4 helper and CD8 effector T cells. Furthermore, TCR/CD28-mediated signaling events were affected by TRAF3 deficiency in CD4 and CD8 T cells, including proliferation, cytokine production, and phosphorylation of ERK, LAT, PLCδ1, and ZAP70. Strikingly, we found that TRAF3 was recruited to the TCR/CD28-signaling complex and coimmunoprecipitated with TCR and CD28 upon costimulation. Together, our findings identify TRAF3 as an essential participant in TCR/CD28 signaling and, thus, as a critical regulator of T cell-mediated immunity.

TRAF3 directly binds to almost all receptors of the TNFR superfamily that do not contain death domains, as well as the EBV-encoded oncogenic protein latent membrane protein 1 (1, 4, 6–8). TRAF3 also regulates signaling by TLRs through interaction with adaptor proteins (5, 9, 10). Our present study expands TRAF3-interacting receptors to include the TCR/CD28 complex. It is intriguing that TRAF3 was not detectably coimmunoprecipitated with CD3 or CD28 alone, although the cytoplasmic tail of mouse CD28 contains a putative TRAF3-binding site PYQPYA (residues 203–208). One possibility is that association between CD28 (or CD3) and TRAF3 is weak and susceptible to disruption by the detergent contained in the lysis buffer but is strengthened by coligation of CD3 and CD28. Alternatively, TRAF3 may be recruited by another signaling component that is present only when CD3 and CD28 are engaged. Consistent with the lack of demonstrable direct binding between CD3 and TRAF3, CD3-induced phosphorylation of ERK, LAT, PLCδ1, and ZAP70 was normal in T-TRAF3−/− T cells. However, CD3-induced proliferation and cytokine production were also affected, in part, in the absence of TRAF3, suggesting that TRAF3 also participates in

![FIGURE 6. Decreased cytokine production by splenic CD4 and CD8 T cells following stimulation through CD3 and CD28. A, Splenic CD4 T cells were purified from 2–3-mo-old LMC and T-TRAF3−/− mice, and Tregs were depleted using anti-CD25 magnetic beads. B, Splenic CD8 T cells were purified from 2–3-mo-old LMC and T-TRAF3−/− mice by negative selection. Cells were cultured ex vivo in the absence or presence of stimulation with 0.5 μg/ml of plate-bound anti-CD3 mAb, alone or in combination with 2 μg/ml of soluble anti-CD28 mAb. Levels of cytokines in the culture supernatants were measured by ELISA. Results shown are representative of at least two independent experiments.](image)
distal CD3-signaling events through indirect mechanisms. Upon coligation of CD3 and CD28, TRAF3 recruitment to the TCR/CD28-signaling complex seems to be required for the synergy between these two receptors, as evidenced by the abolished synergistic effects on phosphorylation of ERK, LAT, PLCγ1, and ZAP70, followed by TRAF3. It was shown that engagement of CD40 or BAFFR induces rapid proteasome-dependent degradation of TRAF3 in B lymphocytes (16, 17, 38–40). Interestingly, a previous study showed that CD3 triggering induced TRAF3 cleavage by caspases in Jurkat T cells (41). However, we did not detect TRAF3 degradation or cleavage upon stimulation through CD3 or CD3 and CD28 in freshly isolated mouse splenic T cells (P. Xie, unpublished observations). Recent evidence suggests that TRAF3 is an E3 ubiquitin ligase that preferentially assembles lysine 63-linked polyubiquitin chains (42). Whether and how TRAF3 exerts its E3 ubiquitin ligase activity in TCR and CD28 signaling await further investigation.

Although our evidence unequivocally demonstrated the direct involvement of TRAF3 in CD3 and CD28-mediated T cell activation, it remains possible that potential roles of TRAF3 in signaling by receptors of the TNFR superfamily also contribute to the defective T cell immunity observed in T-TRAF32/2 mice. In this regard, in vitro studies showed that TRAF3 can directly bind to 4-1BB, CD27, TNFR2, GITR, OX-40, and CD30 expressed on T cells (1). Among these, GITR, CD27, OX-40, and 4-1BB were demonstrated to make substantial contributions to T cell survival, proliferation, and cytokine production in response to stimulation with TCR or TCR/CD28 (43–52). Thus, alterations in signaling by these TNFR family members may also contribute to the defective TD IgG1 response and decreased T cell responses to L. mono-
cytogenes infections observed in T-TRAF3−/− mice. Further studies are needed to delineate specific roles of TRAF3 in signaling by GITR, CD27, OX40, 4-1BB, TNFR2, or CD30. Our T-TRAF3−/− mice provide a valuable model system and useful tools for such future studies.

We found that T-TRAF3−/− mice displayed normal thymocyte populations, as well as normal peripheral B, CD4, and CD8 T cell populations, but an increased frequency of CD4+/CD25−FoxP3+ Tregs in secondary lymphoid organs. This suggests that TRAF3 may regulate Treg maturation or homeostasis. It is very possible that the increased frequency of Tregs also contributes to the defects in T cell immunity observed in T-TRAF3−/− mice. An interesting study recently demonstrated that BAFF-transgenic mice also exhibit increased numbers of Tregs in the spleen (53). In B lymphocytes, BAFFR signals to stimulate NF-κB2 activation and promote peripheral B cell survival by overriding negative regulatory functions of TRAF3 (4). However, BAFF stimulation did not induce NF-κB2 activation in Tregs, and the increased frequency of Treg requires B cells in BAFF-transgenic mice, suggesting an indirect effect of BAFF on Tregs (53). We observed that Tregs purified from T-TRAF3−/− mice did not show prolonged survival in the absence or presence of BAFF (P. Xie, unpublished observations). Thus, although BAFF and TRAF3 regulate B cell survival through the same pathway, they may regulate Tregs through distinct mechanisms. It is noteworthy that TNFR2, GITR, and TLR2 have been implicated in the regulation of Treg proliferation. In resting and activated states, mouse peripheral Tregs express remarkably higher surface levels of GITR than do CD4+CD25− T effector cells (54). TNF-α, in synergy with TCR and even more so with IL-2, markedly promotes the expansion and function of the Treg population (54). Similarly, although GITR is upregulated following activation of CD4+ and CD8+ T cells, a substantially higher level of GITR is constitutively expressed on Tregs (20, 55). GITR costimulates with TCR or IL-2 to induce the proliferation and cytokine production of Tregs, and GITR−/− mice display a modest reduction in Tregs (55–57). In addition, TLR2 also costimulates with the TCR to control the expansion and function of Tregs (58). Thus, it remains to be determined whether TRAF3 participates in signaling by TNFR2, GITR, and TLR2 in Tregs. It would also be interesting to further investigate whether the TRAF3-deficient Tregs have an increased suppressive function on a per-cell basis and whether Tregs express less TRAF3.

In the current study, we found that T-TRAF3−/− mice show higher lethality and increased bacterial loads in the liver following infection with L. monocytogenes. In this context, it would be interesting to investigate whether deletion or inactivating mutations of the TRAF3 genes or decreased expression of TRAF3 proteins occurs in T cells of human patients with immunodeficiencies or recurrent infections that result from impaired T cell function. Regulation of lymphocyte homeostasis and functionality is central to the proper functioning of the adaptive immune system in vertebrates. Our findings obtained from B cell-specific TRAF3−/− mice (12) and T cell-specific TRAF3−/− mice presented in this study provide definitive genetic and molecular evidence for the crucial, but distinct, functions of TRAF3 in regulating B cell homeostasis and T cell activation, respectively. These findings define an essential role for TRAF3 in the adaptive-immune system and provide a basis for rational approaches for the development of TRAF3-specific therapeutic drugs to treat autoimmune diseases, immunodeficient disorders, and tumors.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Legends to Supplementary Figures

Supplementary Figure 1. CD4 and CD8 T cells in the liver after primary LM infection. (A) Representative FACS profiles of CD4 and CD8 T cells in the liver of LMC and T-TRAF3−/− mice on d5 and d7 post primary LM infection. (B) Numbers of mononuclear cells, CD4 and CD8 T cells in the liver of LMC and T-TRAF3−/− mice on d5 and d7 post primary LM infection. The graph depicts the results of three pairs of mice on day 5 and two pairs on day 7 (mean ± SD). Mice infected were 2 to 3 months old.

Supplementary Figure 2. Splenic CD4 T cell proliferation analyzed by CFSE-labeling. Splenic CD4+ T cells were purified from 2-3 month-old LMC and T-TRAF3−/− mice, and Treg cells were depleted using anti-CD25 Ab-coupled magnetic beads. Cells were labelled with CFSE, and then cultured ex vivo in the absence or presence of stimulation with 0.5 μg/ml of plate-bound α-CD3 mAb, alone or in combination with 2 μg/ml of soluble α-CD28 mAb or 2 μg/ml of ConA. Each FACS profile shows CFSE signals of cells cultured ex vivo for 4 days in the presence of indicated stimulation. Similar results were observed in an additional experiment. The number of gated population indicates the percentage of proliferating cells.

Supplementary Figure 3. Constitutive NF-κB2 processing in TRAF3−/− T cells. Splenic pan T cells were purified from 2-3 month-old LMC and T-TRAF3−/− mice. Total cellular lysates were immunoblotted for NF-κB2, followed by actin.

Supplementary Figure 4. Partial defects of TRAF3−/− CD4 T cells in in vitro Th1 polarization. Naive CD4 T cells were purified from 2-3 month-old LMC and T-TRAF3−/− mice. Cells were cultured in the presence of 5 μg/ml plate-bound anti-CD3 Ab, 10 μg/ml soluble anti-CD28 Ab, 20 U/ml IL-2, 4 ng/ml IL-12 p70 and 2 μg/ml anti-mouse IL-4 Ab
for 4 days to induce Th1 differentiation. Cells were subsequently counted, re-plated, and
stimulated with 50 ng/ml of PMA and 1 µg/ml ionomycin (Ion.), or 0.5 µg/ml of plate-
bound α-CD3 mAb and 2 µg/ml of soluble α-CD28 mAb. Levels of cytokines in the
culture supernatants were measured by ELISA. Results shown are from 2 pairs of mice,
three cultures per mouse for the CD3+CD28 re-stimulations (Mean± SD) and one culture
per mouse for the PMA + Ion. re-stimulations.

**Supplementary Figure 5. Quantitation analysis of early signaling events of TCR and
CD28.** Quantitation of ERK, LAT, PLCγ1, ZAP70 and IκBα phosphorylation. P- and
total versions of the proteins on immunoblots (Fig. 7A and 7B) were quantitated using a
low-light imaging system, and the results presented graphically. The amount of the
phosphorylated protein in each lane was normalized to the intensity of the corresponding
total version of each signaling protein. Results shown are the summary of 2 independent
experiments (Mean± SD).
Supplementary Figure 1

A

Day 5

Day 7

FL4-H: CD45β

FL4-H: FL4-Height

71.71 20.55

15.42 0.34

74.84 9.40

17.70 1.21

LMC

TRAF3-/-

B

Liver

Mononuclear cells

CD4 T cells

CD8 T cells

Day 5

Day 7

Cell number (x 10^6)

WT T-T3-/-

WT T-T3-/-

WT T-T3-/-

LMC T-T3KO

LMC T-T3KO

LMC T-T3KO
Supplementary Figure 2

No treatment

Anti-CD3

Anti-CD3+anti-CD28

Anti-CD3 + ConA

CFSE

Supplementary Figure 2

No treatment

Anti-CD3

Anti-CD3+anti-CD28

Anti-CD3 + ConA

CFSE
Supplementary Figure 4

In vitro polarized CD4+ Th1 cells

PMA + ion. 24 hr

IFNγ (ng/ml)

TNFα (ng/ml)

IL-2 (ng/ml)

CD3 + CD28 48 hr

IFNγ (ng/ml)

TNFα (ng/ml)

IL-2 (ng/ml)
Supplementary Figure 5

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CD3+CD28

LMC
TRAF3<sup>-/-</sup>

P-ERK1
P-ERK2
P-LAT
P-PLC<sub>1</sub>
P-ZAP70
P-I<sub>B</sub>