The Journal of Immunology

BRIEF REPORT | JANUARY 01 2008

Cutting Edge: Requirement for TRAF6 in the Induction of T Cell Anergy[1] FREE

Carolyn G. King; ... et. al

J Immunol (2008) 180 (1): 34–38.
https://doi.org/10.4049/jimmunol.180.1.34

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Requirement for TRAF6 in the Induction of T Cell Anergy[1]

Carolyn G. King, Jodi L. Buckler,† Takashi Kobayashi,* Jeffrey R. Hannah,* Garrett Bassett,* Taesoo Kim,* Erika L. Pearce,* Gregory G. Kim*, Laurence A. Turka,† and Yongwon Choi[2]**

TRAF6, TNFR-associated factor 6, is a key adaptor downstream from the TNF receptor and TLR superfamily members. T cell-specific deletion of TRAF6 (TRAF6-ΔT) was recently shown to result in the development of multiorgan inflammatory disease and the resistance of responder T cells to suppression by $CD4^+CD25^+$ regulatory T cells. In this study we examined the role of TRAF6 in an additional mechanism of peripheral tolerance, anergy. We have determined that the loss of TRAF6 restores the ability of $CD28^{-/-}$ T cells to proliferate and produce IL-2. Consistent with this, TRAF6-ΔT T cells were resistant to anergizing signals both in vitro and in vivo. Resistance to anergy was correlated with decreased expression of Cbl-b. These findings reveal that in addition to its role in rendering T cells susceptible to control by $CD4^+CD25^+$ regulatory T cells, TRAF6 is essential for the induction of T cell anergy, implicating TRAF6 as a critical mediator of peripheral tolerance. The Journal of Immunology,* 2008, 180: 34–38.

A hallmark of the immune system is its ability to distinguish self from non-self, thereby achieving self-tolerance while retaining the capacity to respond to infectious insult. Peripheral tolerance in $CD4^+$ T cells is maintained by several mechanisms including deletion of autoreactive T cells, suppression by regulatory T cells, and functional inactivation or anergy (1, 2). Although it is clear that breakdown of any of these mechanisms can lead to the development of autoimmune disease, the molecular pathways associated with T cell peripheral tolerance are not well understood. Normal T cell activation requires integrated signaling from both TCR and CD28. CD28 costimulation acts by amplifying TCR-mediated signaling events and is thought to lower the threshold required for a productive T cell response (3, 4). In contrast, TCR ligation in the absence of CD28 costimulation results in anergy characterized by hypoproliferation and impaired IL-2 production in response to subsequent stimulation (3, 5). Recently, E3 ubiquitin ligases including Cbl-b, Itch, and Grail have been implicated in the development and maintenance of T cell anergy (6–10). Anergized T cells express higher levels of these E3 ligases, and T cells deficient in these E3 ligases are resistant to anergy induction. In addition, single deletion of either Cbl-b or Itch results in enhanced susceptibility to or development of autoimmune disease (11–13). Coordinated activity of these E3 ubiquitin ligases is postulated to down-regulate T cell activation by proteasomal degradation or altered subcellular compartmentalization of T cell signaling components (14).

TNFR-associated factor 6 (TRAF6)[3] is an adaptor protein that can act as an E3 ubiquitin ligase to mediate signaling downstream from both TNFR superfamily members as well as IL-1/TLR family members (15–18). Signaling via TRAF6 results in the activation of transcription factors including NFκB, NFAT, Akt, and MAP kinases (16). Unlike conventional E3 ligases that conjugate K48-linked ubiquitination, TRAF6 mediates K63 ubiquitination resulting in substrate activation as opposed to proteasomal degradation (14, 18). Recently, our group identified a T cell-intrinsic role for TRAF6 in the maintenance of peripheral tolerance (19). T cell-specific deletion of TRAF6 (TRAF6-ΔT) resulted in the resistance of T cells to suppression by control $CD4^+CD25^+$ regulatory T cells and the subsequent development of multiorgan inflammatory disease. TRAF6-ΔT T cells efficiently proliferated in response to stimulation with anti-CD3 alone and exhibited enhanced activation of PI3K and Akt, indicating independence from the normal requirement for CD28 costimulation. Together, these data suggested a negative regulatory role for TRAF6 in costimulation-dependent T cell activation. Given the role of costimulatory signals in preventing the development of anergy both in vitro and in vivo, we investigated the susceptibility of TRAF6-ΔT T cells to anergy induction. We now report that TRAF6 deficiency relieves the requirement for costimulation to elicit productive T cell responses and renders T cells resistant to anergizing signals. Resistance to anergy is correlated with the dysregulation of E3 ubiquitin ligase Cbl-b. Taken together with the previously re-ported role for TRAF6 in rendering T cells susceptible to

*Department of Pathology and Laboratory Medicine, Abramson Family Cancer Research Institute and †Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Received for publication August 27, 2007. Accepted for publication October 29, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by National Institutes of Health Grant AI-43620 (to Y.C. and L.A.T.).

[2] Address correspondence and reprint requests to Dr. Yongwon Choi, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Room 308, Biomedical Research Building II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6144. E-mail address: ychoi3@mail.med.upenn.edu

[3] Abbreviations used in this paper: TRAF6, TNFR-associated factor 6; SEB, staphylococcal enterotoxin B; TRAF6-ΔT, T cell-specific deletion of TRAF6.

suppression by $CD4^+CD25^+$ regulatory T cells, these data place TRAF6 at a central point of convergence for the interpretation of tolerizing signals.

Materials and Methods

Mice

TRAF6-ΔT and TRAF6-ΔT OT-II mice have been previously described (19). B6.PL-*Thy1a*/CyJ (Thy1.1) congenic and $CD28^{-/-}$ mice were obtained from The Jackson Laboratory. Mouse care and experimental procedures were performed in accordance with protocols from Institutional Animal Care and Use Committees of the University of Pennsylvania (Philadelphia, PA).

Reagents and Abs

The following reagents were purchased from BD Pharmingen: purified anti-CD3 and anti-CD28, CTLA4-Ig, anti-CD3-FITC, anti-CD62L-PE, anti-Vβ5-PE, anti-CD4-PerCP, anti-CD44-allophycocyanin anti-Thy1.2-allophycocyanin, biotinylated anti-CD8, anti-B220, anti-IA^b, anti-Gr1, and an OptEIA ELISA kit for IL-2. Anti-biotin magnetic beads were purchased from Miltenyi Biotec. CFSE was purchased from Molecular Probes. Staphylococcal enterotoxin B (SEB) was purchased from Toxin Technologies, and OVA peptide was purchased from AnaSpec. Anti-Cbl-b was purchased from Santa Cruz Biotechnology and anti-actin was purchased from Sigma-Aldrich.

Cell purification

Single cell suspensions from spleen and lymph nodes were incubated with biotin anti-B220, biotin anti-IA^b, biotin-anti-Gr1, and biotin-anti-CD8 followed by streptavidin microbeads. $CD4^+$ T cells were negatively selected after separation from bead-bound cells over a magnetic column. $CD4^+$ T cell purity was checked by FACS and typically ranged between 85 and 95%. Where indicated, naive ($CD62L^{high}CD44^{low}$) $CD4^+$ T cells were isolated by cell sorting on a FACSDiVa flow cytometer (BD Biosciences).

In vitro stimulation

Cells were cultured in RPMI 1640, 5% FCS, 10 mM HEPES, 5×10^{-5} M 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Purified $CD4^+$ T cells were stimulated with the indicated dose of plate-bound anti-CD3. For anergy experiments, purified $CD4^+$ T cells were stimulated with anti-CD3 (1 μg/ml) in the presence of irradiated APCs with or without soluble CTLA4-Ig (15 μg/ml). For Cbl-b expression levels, purified T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble CTLA4-Ig (15 μg/ml).

In vivo anergy induction

For OVA tolerance induction, gender- and age-matched control and TRAF6-ΔT OT-II transgenic mice were injected i.v. with either PBS or 500 μg of OVA peptide on days 0 and 3. One week later, splenic and lymph node $CD4^+V\beta5^+$ T cells were isolated and CFSE labeled. T cells were then subcutaneously injected into congenic B6 Thy1.1 recipients followed by immunization with OVA peptide (50 μg) in CFA. Three days later, lymph nodes were collected and Thy1.2 $V\beta5^+$ positive cells were analyzed for CFSE dye dilution.

For SEB tolerance induction, control and TRAF6-ΔT mice were injected with 75 μg of SEB. On days 0, 3, and 7 after injection, CD4, Vβ8, and Vβ6 expression was monitored by FACS analysis of PBMC. After 7 days, $CD4^+V\beta8^+$ T cells were purified and stimulated in vitro with the indicated dose of SEB and irradiated APCs.

Western blots

Cells were washed with ice-cold PBS and lysed in buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.0), 10% glycerol, 0.75% Triton X-100, and protease and phosphatase inhibitors. SDS gel-loading buffer was added to cell lysates and samples were boiled and subjected to SDS-PAGE and Western blot analysis. Relative expression was determined with a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/.

Results and Discussion

TRAF6 deficiency uncouples the normal requirement for CD28 costimulation Previous work in our laboratory demonstrated that TRAF6-ΔT T cells efficiently proliferate in response to stimulation with anti-CD3 alone (19). Because normal T cell activation requires cooperative signals from both TCR and CD28, these findings suggested removal of the requirement for CD28 costimulation. To definitively test this, we crossed TRAF6-ΔT mice with $CD28^{-/-}$ mice. No abnormalities in T cell development or peripheral T cell populations were observed in TRAF6-ΔT $CD28^{-/-}$ mice. However, similar to TRAF6-ΔT mice, 8- to 10-wk-old mice doubly deficient for TRAF6 and CD28 exhibited the accumulation of activated ($CD69^+$) and effector/memory ($CD44^{high}CD62^{low}$) T cells in vivo (Fig. 1*a*). $CD28^{-/-}$ deficiency in T cells results in profoundly reduced proliferation and a complete defect in IL-2 production following CD3 stimulation (19) and, as expected, sorted naive T cells from control $CD28^{-/-}$ mice responded poorly to stimulation with anti-CD3 in vitro (Fig. 1, *b* and *c*). In contrast, TRAF6-ΔT $CD28^{-/-}$ T cells proliferated and produced IL-2 at levels similar to those of control CD28-sufficient T cells (Fig. 1, *b* and *c*). These data indicate that the absence of TRAF6 restores the ability of $CD28^{-/-}$ T cells to respond efficiently to CD3 stimulation.

TRAF6-deficient $CD4^+$ T cells resist anergy induction both in vitro and in vivo. TRAF6-ΔT T cell independence from

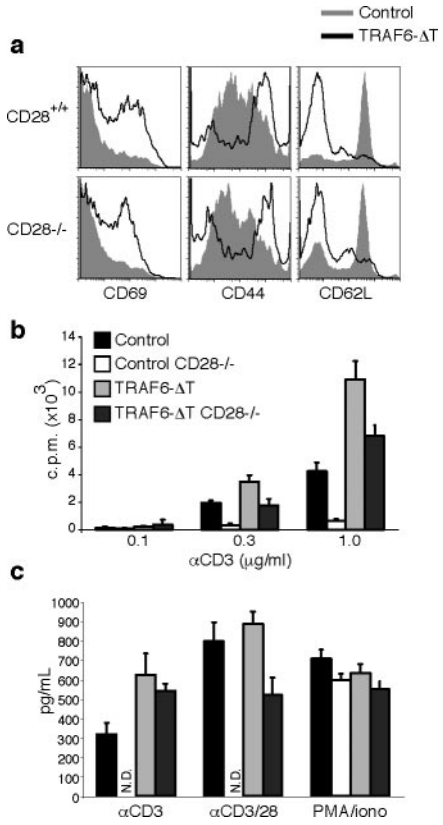

FIGURE 1. TRAF6-ΔT T cells uncouple the requirement for CD28 costimulation. *a*, Expression of CD69, CD62 ligand (CD62L), and CD44 on splenic $CD4^+$ T cells from control $CD28^{-/-}$ and TRAF6-ΔT $CD28^{-/-}$ mice was measured by FACS analysis. Plots are gated on live $CD3^+CD4^+$ T cells and TRAF6-ΔT (bold black line) histogram is overlaid on control (gray-shaded) histogram. *b*, Sorted naive $CD4^+$ T cells from control $CD28^{-/-}$ and TRAF6-ΔT $CD28^{-/-}$ mice were stimulated with the indicated doses of plate-bound anti-CD3 for 72 h. Proliferation was measured after the addition of $[^3H]$thymidine during the final 12 h. Error bars indicate SD of samples run in triplicate, representative of three independently performed experiments. *c*, Sorted naive $CD4^+$ T cells from control and TRAF6-ΔT mice were stimulated with plate-bound anti-CD3 (1 μg/ml) with or without soluble CD28 (2 μg/ml) or PMA (50 ng/ml) and ionomycin (iono) (1 μM). IL-2 was measured from supernatants after 48 h of stimulation. Error bars indicate SD of samples run in triplicate, representative of three independently performed experiments.

CD28 costimulation suggested that TRAF6-ΔT T cells might also be resistant to anergy induction, a normal result of TCR ligation in the absence of costimulation. To test this, we stimulated TRAF6-ΔT and control T cells with anti-CD3 in the presence or absence of soluble CTLA4-Ig and irradiated APCs. T cell responses were measured following secondary stimulation with plate-bound anti-CD3. Although anergy was efficiently induced in control T cells, the costimulatory blockade in TRAF6-ΔT T cells did not impair their ability to proliferate or produce IL-2 (Fig. 2, *a* and *b*). Because CD28 signals are known to promote survival in addition to preventing anergy, we next examined the viability of control and TRAF6-ΔT T cells following in vitro stimulation. No differences in survival were observed between control and TRAF6-ΔT T cells (Fig. 2*c*), indicating that TRAF6-ΔT T cell resistance to anergy induction was not due to enhanced survival.

To examine T cell anergy induction in vivo, we used an adoptive transfer model with OT-II TCR-transgenic mice (19). Administration of high-dose Ag has been shown to induce a hyporesponsive state in Ag-specific T cells (7, 20). Control OT-II and TRAF6-ΔT OT-II mice were injected with OVA peptide or PBS as a control. Ten days after the initial injection, $CD4^+V\beta5^+$ T cells were isolated, CFSE labeled, and injected into congenic recipients followed by immunization with the OVA peptide in CFA. As expected, T cells from both PBS-treated control and TRAF6-ΔT mice proliferated in response to OVA challenge (Fig. 3*a*). This proliferation was abrogated in OVA-tolerized control T cells. In contrast, T cells from OVA-injected TRAF6-ΔT mice maintained their ability to proliferate, indicating the resistance of TRAF6-ΔT T cells in anergy induction in vivo (Fig. 3*a*). Consistent with these data, T cells

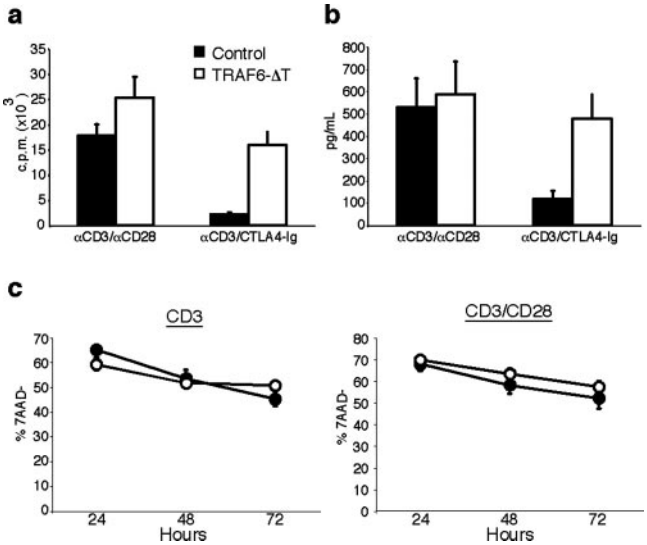

FIGURE 2. TRAF6-ΔT T cells are resistant to anergy induction in vitro. Control or TRAF6-ΔT T cells were stimulated with anti-CD3 (αCD3; 1 μg/ml) and irradiated APCs with or without CTLA4-Ig. After 72 h, cells were washed and cultured for an additional 24 h before restimulation with plate-bound anti-CD3 and soluble anti-CD28. *a*, Proliferation was measured by [^{3}H]thymidine incorporation after 48 h. *b*, IL-2 was measured by ELISA after 24 h. *c*, Control or TRAF6-ΔT T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) or without anti-CD28 (2 μg/ml). $CD4^+$ T cell survival was determined by FACS analysis after staining with the vital dye 7-aminoactinomycin D. Error bars indicate SD of samples run in triplicate, representative of 3–4 independently performed experiments.

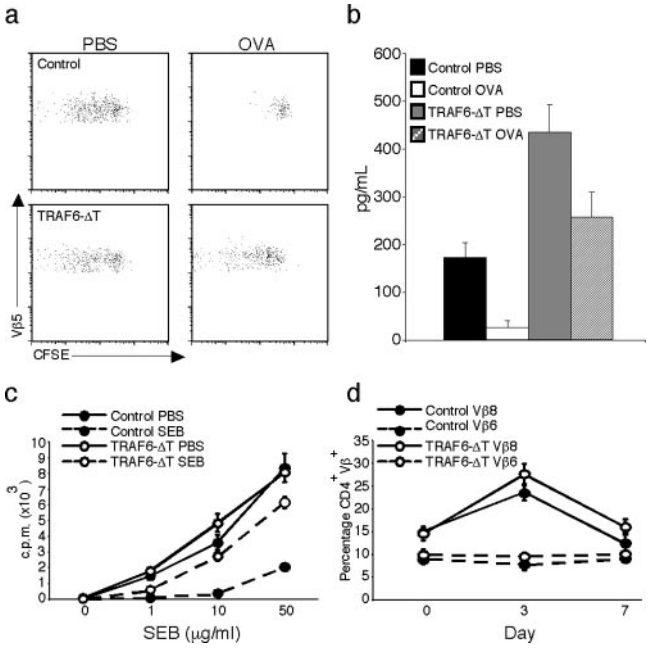

FIGURE 3. TRAF6-ΔT T cells are resistant to anergy induction in vivo. *a* and *b*, Control OT-II and TRAF6-ΔT OT-II mice were injected with either PBS or OVA peptide on days 0 and 3. *a*, On day 10, $V\beta5^+$ T cells were purified, CFSE labeled, and injected into Thy congenic recipients followed by immunization with OVA in CFA. Lymph nodes were harvested 3 days later and analyzed by FACS for CFSE dye dilution. Plots are gated on Thy1.2, CD4, and Vβ5. Data from individual mice are shown and are representative of three mice per treatment group from two independently performed experiments. *b*, ON day 10, $V\beta5^+$ T cells were purified and restimulated in vitro with OVA Ag. IL-2 production was measured by ELISA. *c* and *d*, Control and TRAF6-ΔT mice were injected with SEB (75 μg). *c*, On day 7, $V\beta8^+CD4^+$ T cells were isolated and stimulated in vitro for 72 h with the indicated dose of SEB and irradiated APCs. Proliferation was measured by [^{3}H]thymidine incorporation after 72 h. Error bars indicate SD of samples run in triplicate, representative of three independently performed experiments. *d*, On days 0, 3, and 7 after injection, CD4, Vβ8, and Vβ6 expression was measured on PBMC by FACS analysis. Error bars indicate SD of three individual mice, representative of two independently performed experiments.

from OVA-tolerized wild-type mice failed to produce IL-2 in response to Ag restimulation in vitro, while OVA tolerization failed to anergize TRAF6-ΔT T cells restimulated under the same conditions (Fig. 3*b*).

We next extended our findings to an alternative method of in vivo anergy by examining the role of TRAF6 in superantigen-induced tolerance. Injection of mice with SEB results in the selective expansion of $V\beta8^+$ T cells followed by their contraction (21). Remaining $V\beta8^+$ T cells are unresponsive to subsequent SEB stimulation and are thus rendered functionally anergic (21). As expected, 7 days after injection with SEB, $CD4^+V\beta8^+$ T cells from control mice exhibited impaired in vitro proliferation in response to stimulation with SEB (Fig. 3*c*). In contrast, $CD4^+V\beta8^+$ from SEB-injected TRAF6-ΔT mice proliferated strongly at levels comparable to those of PBS-injected mice (Fig. 3*c*). The enhanced proliferation observed by TRAF6-ΔT T cells was not due to an abnormal deletion of $V\beta8^+$ T cells, as both control and TRAF6-ΔT T cells underwent similar expansion and contraction phases in response to SEB (Fig. 3*d*). Thus, consistent with our in vitro findings, TRAF6 deficiency results in impaired anergy induction in vivo.

TRAF6-deficient T cells exhibit abnormal regulation of E3 ubiquitin ligase Cbl-b

Independence from CD28 costimulation and impaired anergy induction in TRAF6-ΔT T cells suggested that the negative regulation of costimulatory pathways might be defective in TRAF6-ΔT T cells. Consistent with this, previous work in our laboratory showed that TRAF6-ΔT T cells exhibit enhanced activation of PI3K and Akt (22). CD28 ligation results in the recruitment of p85, the regulatory subunit of PI3K, and leads to downstream activation of Akt (3). Cbl-b acts as a negative regulator of this pathway by ubiquitinating p85 and preventing its association with CD28 (23). Because Cbl-b$^{-/-}$ T cells can also respond to CD3 in the absence of CD28 and have recently been shown to resist anergy induction (6, 7), we next examined Cbl-b regulation in TRAF6-ΔT T cells. Surprisingly, while control CD4$^+$ T cells stimulated under anergizing conditions strongly up-regulated Cbl-b mRNA and protein expression, TRAF6-ΔT T cells exhibited delayed and reduced expression of Cbl-b, a difference that was maintained at both early and later time points (Fig. 4). These data indicate that the absence of TRAF6 results in improper regulation of Cbl-b. However, expression of other E3 ubiquitin ligases reported to be important in anergy induction, including Itch and Grail, were not altered in TRAF6-ΔT T cells (data not shown).

Concluding remarks

The data presented here conclusively demonstrate a role for TRAF6 in the induction of T cell anergy. Loss of TRAF6 uncouples the normal requirement for costimulation to elicit functional T cell responses and results in dysregulation of Cbl-b, an E3 ubiquitin ligase required for anergy induction. Cbl-b has been previously shown to negatively regulate costimulatory signals by preventing PI3K association with CD28 (23). Indeed, previous work in our laboratory showed that TRAF6-ΔT T cells exhibit enhanced activation of PI3K and Akt, both signaling mediators downstream from CD28 ligation (19). In addition, T cells deficient for PTEN (phosphatase and tensin homologue deleted on chromosome 10), a negative regulator of PI3K activation, are also resistant to anergy induction, and mice with T cell-specific deletion of PTEN develop lymphoproliferative and autoimmune disease (24, 25). Thus, it is possible that decreased Cbl-b expression in TRAF6-ΔT T cells is responsible for the hyperactivation of PI3K/Akt, which in turn renders TRAF6-ΔT T cells resistant to anergy induction. However, as Cbl-b has also been implicated in phospholipase Cγ and protein kinase Cθ ubiquitination and degradation following anergy induction (6, 7), it will be important to examine the expression patterns of these and other known Cbl-b targets in TRAF6-ΔT T cells. Taken together with our previous finding that TRAF6-ΔT T cells are resistant to suppression by CD4$^+$CD25$^+$ regulatory T cells, the data presented here suggest that signals initiated by distinct T cell extrinsic factors, such as tolerizing APCs or CD4$^+$CD25$^+$ regulatory T cells, result in the activation of common molecular signaling components, and place TRAF6 at a central point of convergence for the interpretation of these tolerizing signals.

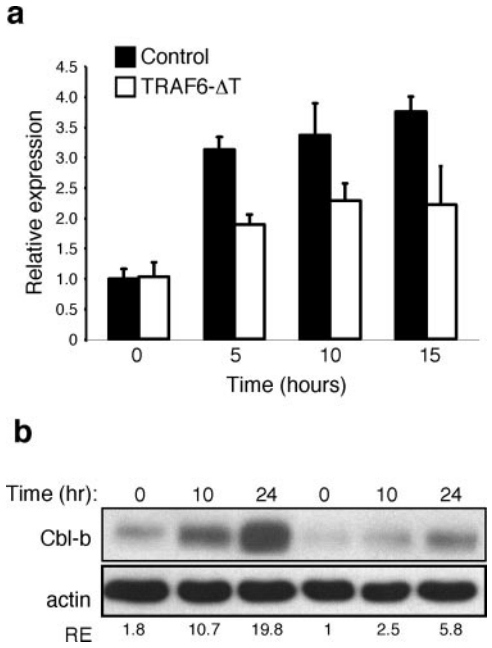

FIGURE 4. TRAF6 deficiency results in dysregulation of Cbl-b under anergizing conditions. Control and TRAF6-ΔT CD4$^+$ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and CTLA-4-Ig (15 μg/ml). *a*, Cbl-b mRNA expression was measured by quantitative real-time PCR normalized to endogenous 18S ribosomal RNA. Error bars indicate SD of samples run in triplicate, representative of four independently performed experiments. *b*, Cbl-b protein expression was measured from whole cell lysates and represents four independently performed experiments. Relative expression (RE) of Cbl-b is indicated below. Anergy induction was verified by secondary stimulation with anti-CD3 and anti-CD28 (data not shown).

Acknowledgments

We thank the University of Pennsylvania Cancer Center Flow Cytometry Core for expert assistance with FACS.

Disclosures

The authors have no financial conflict of interest.

References

1. Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21: 305–334.
2. Walker, L. S., and A. K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* 2: 11–19.
3. Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* 3: 939–951.
4. Alegre, M. L., K. A. Frauwirth, and C. B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. *Nat. Rev. Immunol.* 1: 220–228.
5. Chambers, C. A., and J. P. Allison. 1999. Costimulatory regulation of T cell function. *Curr. Opin. Cell Biol.* 11: 203–210.
6. Heissmeyer, V., F. Macian, S. H. Im, R. Varma, S. Feske, K. Venuprasad, H. Gu, Y. C. Liu, M. L. Dustin, and A. Rao. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* 5: 255–265.
7. Jeon, M. S., A. Atfield, K. Venuprasad, C. Krawczyk, R. Sarao, C. Elly, C. Yang, S. Arya, K. Bachmaier, L. Su, et al. 2004. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity* 21: 167–177.
8. Seroogy, C. M., L. Soares, E. A. Ranheim, L. Su, C. Holness, D. Bloom, and C. G. Fathman. 2004. The gene related to anergy in lymphocytes, an E3 ubiquitin ligase, is necessary for anergy induction in CD4 T cells. *J. Immunol.* 173: 79–85.
9. Venuprasad, K., C. Elly, M. Gao, S. Salek-Ardakani, Y. Harada, J. L. Luo, C. Yang, M. Croft, K. Inoue, M. Karin, and Y. C. Liu. 2006. Convergence of Itch-induced ubiquitination with MEKK1-JNK signaling in Th2 tolerance and airway inflammation. *J. Clin. Invest.* 116: 1117–1126.
10. Mueller, D. L. 2004. E3 ubiquitin ligases as T cell anergy factors. *Nat. Immunol.* 5: 883–890.
11. Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y. Y. Kong, T. Sasaki, A. Oliveira-dos-Santos, S. Mariathasan, D. Bouchard, A. Wakeham, A. Itie, et al. 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* 403: 211–216.
12. Chiang, Y. J., H. K. Kole, K. Brown, M. Naramura, S. Fukuhara, R. J. Hu, I. K. Jang, J. S. Gutkind, E. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* 403: 216–220.
13. Fang, D., C. Elly, B. Gao, N. Fang, Y. Altman, C. Joazeiro, T. Hunter, N. Copeland, N. Jenkins, and Y. C. Liu. 2002. Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. *Nat. Immunol.* 3: 281–287.
14. Liu, Y. C., J. Penninger, and M. Karin. 2005. Immunity by ubiquitylation: a reversible process of modification. *Nat. Rev. Immunol.* 5: 941–952.

15. Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D. V. Goeddel. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature* 383: 443–446.
16. Wu, H., and J. R. Arron. 2003. TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology. *Bioessays.* 25: 1096–1105.
17. Ye, H., J. R. Arron, B. Lamothe, M. Cirilli, T. Kobayashi, N. K. Shevde, D. Segal, O. K. Dzivenu, M. Vologodskaia, M. Yim, et al. 2002. Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* 418: 443–447.
18. Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, and Z. J. Chen. 2000. Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103: 351–361.
19. King, C. G., T. Kobayashi, P. J. Cejas, T. Kim, K. Yoon, G. K. Kim, E. Chiffoleau, S. P. Hickman, P. T. Walsh, L. A. Turka, and Y. Choi. 2006. TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis. *Nat. Med.* 12: 1088–1092.
20. Bansal-Pakala, P., A. G. Jember, and M. Croft. 2001. Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nat. Med.* 7: 907–912.
21. Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Matis. 1990. In vivo induction of anergy in peripheral Vβ8$^+$ T cells by staphylococcal enterotoxin B. *J. Exp. Med.* 172: 1091–1100.
22. King, C., T. Kobayashi, P. Cejas, T. Kim, K. Yoon, G. Kim, E. Chiffoleau, S. Hickman, P. Walsh, L. Turka, and Y. Choi. 2006. TRAF6 is a T cell intrinsic negative regulator required for the maintenance of immune homeostasis. *Nat. Med.* 12: 1000–1002.
23. Fang, D., and Y. C. Liu. 2001. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat. Immunol.* 2: 870–875.
24. Buckler, J. L., P. T. Walsh, P. M. Porrett, Y. Choi, and L. A. Turka. 2006. Cutting edge: T cell requirement for CD28 costimulation is due to negative regulation of TCR signals by PTEN. *J. Immunol.* 177: 4262–4266.
25. Suzuki, A., M. T. Yamaguchi, T. Ohteki, T. Sasaki, T. Kaisho, Y. Kimura, R. Yoshida, A. Wakeham, T. Higuchi, M. Fukumoto, et al. 2001. T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* 14: 523–534.