The Journal of Immunology

RESEARCH ARTICLE | MAY 15 2002

# CD40 Ligation in the Presence of Self-Reactive CD8 T Cells Leads to Severe Immunopathology[1]

Evelyn Roth; ... et. al

*J Immunol* (2002) 168 (10): 5124–5129.
https://doi.org/10.4049/jimmunol.168.10.5124

# CD40 Ligation in the Presence of Self-Reactive CD8 T Cells Leads to Severe Immunopathology[1]

**Evelyn Roth, Johannes Schwartzkopff, and Hanspeter Pircher[2]**

**Previous work has shown that stimulation of APCs via CD40 strongly influences the outcome of a CD8 T cell response. In this study, we examined the effect of CD40 ligation on peripheral tolerance induction of self-reactive CD8 T cells in an adoptive transfer model. Naive CD8 T cells from TCR-transgenic (tg) mice specific for the gp33 epitope of lymphocytic choriomeningitis virus were tolerized when transferred into H8-tg mice expressing the gp33 epitope under the control of a MHC class I promoter. However, if the H8 recipient mice were treated with agonistic anti-CD40 Abs, TCR-tg cells vigorously proliferated, and induced destruction of lymphoid organs and hepatitis. Break of peripheral tolerance induction was B cell independent and did not require CD28/B7 interactions. These findings provide further in vivo evidence for the crucial role of the activation state of the APC in peripheral tolerance induction and suggest the need for caution in systemically activating APC via CD40 ligation in the presence of self-reactive T cells.** ***The Journal of Immunology,*** **2002, 168: 5124–5129.**

Induction of potent cytotoxic CD8 T cell (CTL) responses against viruses and tumors are often dependent on CD4 T cells. A few years ago, three studies simultaneously showed that activation of APC by agonistic anti-CD40 Abs can replace the requirement for CD4 T cell help in vivo (1–3). CD40 is constitutively expressed by B cells, dendritic cells (DC),[3] and macrophages, whereas the corresponding ligand, CD40 ligand, is induced on CD4 T cells upon activation. In B cells, CD40 cross-linking results in Ig class switching and growth stimulation, whereas CD40 ligation in DC induces maturation (4, 5). The studies mentioned above indicated that the activation state of the APC has a dramatic effect on the outcome of a CD8 T cell response. In addition, they encouraged attempts to boost weak CD8 T cell responses by stimulation of APC via CD40. Subsequently, several groups have used this approach to improve CD8 T cell responses against tumors or viruses (6–10). However, systemic therapeutic immunostimulation via CD40 also bears potential hazards. It is well known that central T cell tolerance in the thymus is incomplete and that peripheral tolerance mechanisms are also required to prevent pathological reactivity against self. Therefore, activation of APC via CD40 stimulation may interfere with induction of peripheral tolerance and may lead to activation of potentially self-reactive T cells.

We have addressed this issue in a transfer model using H8-transgenic (tg) mice ubiquitously expressing the CD8 T cell epitope gp33 from lymphocytic choriomeningitis virus (LCMV) (11) and TCR-tg mice specific for gp33 (12). Naive T cells from TCR-tg mice transfused into H8-tg mice are rapidly tolerized. In the present study we show that stimulation via anti-CD40 interferes with peripheral tolerance induction and activates self-reactive TCR-tg cells to cause immunopathology in this transfer system. The break of tolerance via anti-CD40 stimulation was B cell independent and did not require CD28/B7 interactions.

## Materials and Methods

### *Mice*

C57BL/6 (B6) mice were obtained from our breeding colony and from Harlan Winkelmann (Borchen, Germany). Thy1.1$^+$ P14 TCR-tg mice, line 327, specific for amino acids 33–41 (GP33 epitope) of the LCMV glycoprotein (12, 13) and H8-tg mice ubiquitously expressing the LCMV gp33 epitope as a transgene (11) have been described previously. H8-tg mice had been generated on a B6 background. TCR-tg mice deficient in CD28 and H8-tg mice deficient in B cells were generated through breeding with B6.CD28$^{-/-}$ mice (14) and B6.$\mu$MT mice (15), respectively. Female or male mice were used at 8–16 wk of age. Mice were bred and kept in a conventional animal house facility.

### *Virus*

The LCMV-WE isolate used in this study was originally obtained from R. Zinkernagel (University Hospital, Zurich, Switzerland). Mice were infected i.v. with 200 PFU and viral titers were determined in a virus plaque assay as described (16).

### *Adoptive cell transfers and anti-CD40 Ab treatment*

Spleen cells containing $10^5$ TCR-tg cells were injected (i.v.) into nonirradiated B6 or H8-tg mice. Anti-CD40 treatment was performed by i.p. injection of 100 $\mu$g of anti-CD40 mAb, clone FGK45 (17). Unless otherwise indicated, Abs were given on the day of cell transfer and 2 days afterward.

### *Flow cytometry*

Lymphocytes were resuspended in PBS containing 2% FCS and 0.1% $NaN_3$ at a concentration of $10^6$–$10^7$ cells/ml, followed by incubation at 4°C for 20 min with 100 $\mu$l of appropriately diluted mAb. For PBL staining 10 U/ml heparin was added to the staining buffer. The following mAb were used: CD4 (clone GK1.5), CD8 (clone 53-6.7), CD25 (clone 7D4), CD44 (clone IM7), CD45R/B220 (clone RA3-6B2), CD62L (clone MEL-14), CD69 (clone H1.2F3), CD90.1 (clone OX-7), TCR V$\alpha$2 (clone B20.1), and TCR V$\beta$8 (clone MR5-2). Abs were purchased from BD PharMingen (San Diego, CA). The mAb were directly labeled with FITC or PE, or were biotinylated. For the latter, PE-streptavidin (both from BD PharMingen) was used as a secondary reagent for detection. Cells were analyzed on a FACSort flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences). Before analysis of PBL, RBCs were lysed using FACS Lysing Solution (BD PharMingen).

Institute for Medical Microbiology and Hygiene, Department of Immunology, University of Freiburg, Freiburg, Germany

Received for publication October 24, 2001. Accepted for publication March 21, 2002.

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 by the Deutsche Forschungsgemeinschaft (PI-295/4-1).

[2] Address correspondence and reprint requests to Dr. Hanspeter Pircher, Institute for Medical Microbiology and Hygiene, Department of Immunology, Hermann-Herder-Strasse 11, University of Freiburg, D-79104 Freiburg, Germany. E-mail address: pircher@UKL.uni-freiburg.de

[3] Abbreviations used in this paper: DC, dendritic cell; tg, transgenic; LCMV, lymphocytic choriomeningitis virus; GLDH, glutamate dehydrogenase.



0022-1767/02/$02.00

*Immunohistochemistry*

Spleen or liver sections (5–7 $\mu$m) were cut on a cryostat microtome, air dried, fixed in acetone, and blocked with TBS containing 5% mouse serum and with the DAKO Biotin Blocking System (DAKO, Hamburg, Germany). Anti-Thy1.1-biotin, anti-CD8-biotin, and anti-B220-biotin (all from BD PharMingen) were used as primary mAb followed by streptavidin-conjugated alkaline phosphatase (StreptAB Complex/AP; DAKO) and alkaline phosphatase substrate kit I (Vector Laboratories, Burlingame, CA). Sections were counterstained with Mayer's hemalum.

*Serum GLDH activity*

Blood (200 $\mu$l) taken from the tail vein was collected in serum separator tubes (MICROTAINER Brand Serum Separator Tube; BD Biosciences) and centrifuged for 20 min at 3300 $\times$ *g*, and sera were analyzed for glutamate dehydrogenase (GLDH; units per liter).

## Results

*Induction of peripheral tolerance in H8 recipient mice of TCR-tg cells*

To study self-reactive T cells in an environment ubiquitously expressing the self-Ag, CD8 T cells ($10^5$) from TCR-tg mice specific for the gp33 epitope of LCMV were adoptively transferred into H8-tg mice (H8 mice) expressing the GP33 epitope as a transgene driven by the H-2K$^b$ promoter. Twelve days after transfer, H8 recipient mice were infected with LCMV to test the responsiveness of the transferred TCR-tg cells. As a control, TCR-tg cells were transferred into non-tg recipient mice. The donor TCR-tg cells (Thy1.1$^+$) were traced in recipient mice (Thy1.2$^+$) by flow cytometry using Thy1.1-specific mAb. Without LCMV infection, Thy1.1$^+$ cells were at detection limit in both types of recipient mice. After LCMV infection, a massive in vivo proliferation of donor TCR-tg cells was found in B6 recipient mice but not in H8 mice (Fig. 1*A*). Lack of in vivo proliferation of TCR-tg in H8 mice was unlikely due to impaired viral replication, because host CD8 T cells proliferated strongly (12 vs 50% CD8$^+$ of PBL) after LCMV infection.

Nevertheless, a small subset of TCR-tg donor cells expanded in H8 mice after LCMV infection. To test whether the time span of "parking" TCR-tg cells in H8 mice determined the extent of tolerance induction, H8 recipients of TCR-tg cells were infected with LCMV at different time points after cell transfer. As shown in Fig. 1*B*, *left panels*, TCR-tg cells "parked" for only 3 days in H8 mice were still capable of undergoing vigorous clonal expansion. Further analysis with mAb specific for the tg TCR (V$\alpha$2), CD44, CD62L, CD25, and CD69 revealed that these cells exhibited an activated phenotype. Parking TCR-tg cells in H8 mice for 6 days strongly impaired their proliferative response to LCMV (Fig. 1*B*, *middle panels*), and TCR-tg cells were no longer detectable in H8 recipient mice that were infected 20 days after cell transfer (Fig. 1*B*, *right panels*). Taken together, these results demonstrate that gp33-specific TCR-tg cells were tolerized when transferred into gp33-expressing H8 mice.

*Break of tolerance induction in H8 mice by anti-CD40 Ab treatment*

Several recent reports indicate that priming vs tolerance induction of T cells is strongly influenced by the activation state of the APC (18–22). Therefore, we wondered whether stimulation of APC by agonistic anti-CD40 mAb interfered with peripheral tolerance induction of the transferred self-reactive TCR-tg cells in H8 mice. Indeed, this was the case, because TCR-tg cells proliferated vigorously in anti-CD40-treated H8 recipients without LCMV infection (Fig. 2*A*, *left* and *middle panels*). TCR-tg cells adoptively transferred into anti-CD40-treated B6 mice did not expand (Fig. 2*A*, *right panels*). The induced TCR-tg cells in anti-CD40-treated

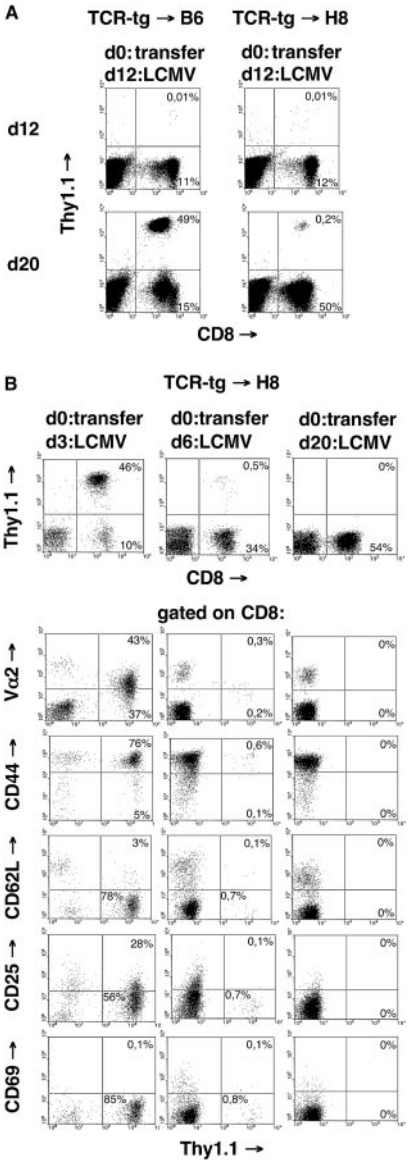


**FIGURE 1.** Induction of peripheral tolerance of TCR-tg cells in H8 mice. *A*, B6 and H8 mice were injected i.v. with $10^5$ Thy1.1$^+$ TCR-tg cells. Twelve days after transfer, recipient mice were infected with LCMV. PBL were analyzed by flow cytometry on the indicated days after cell transfer and infection. *B*, Thy1.1$^+$ TCR-tg cells ($10^5$) were transferred into H8 mice that were infected with LCMV on days 3, 6, or 20 after cell transfer. PBL were analyzed by flow cytometry with the mAb indicated 8 days after infection.

H8 mice exhibited an activated phenotype with up-regulated CD25 and CD44 and down-regulated CD62L expression (Fig. 2*B*). Without Ab treatment, TCR-tg cells remained at detection limit. In absolute numbers, the transferred TCR-tg cells ($10^5$) expanded >100-fold, since within 1 wk ~$10^7$ Thy1.1$^+$ cells were recovered from the spleen of CD40-treated H8 recipient mice (Fig. 3). Titration experiments further revealed that transfer of as few as 1000 TCR-tg cells was sufficient to yield significant (~40% Thy1.1$^+$ of total CD8) expansion of donor cells in the host (Fig. 2*C*). Clonal expansion of TCR-tg cells peaked 1 wk after transfer and treatment, and at this time point all mice showed clinical symptoms including ruffled fur, hunched posture, cachexia, and ataxia. About half of the mice had to be killed due to their moribund stage. The surviving mice recovered rapidly, and TCR-tg cells declined gradually and were no longer detectable 4–5 wk after transfer (Fig.

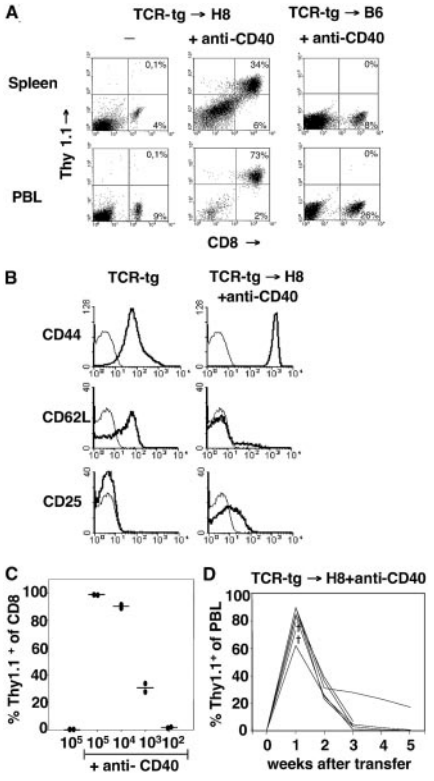


**FIGURE 2.** Break of peripheral tolerance induction in H8 mice by anti-CD40 mAb treatment. *A*, H8 and B6 mice were injected with $10^5$ Thy1.1$^+$ TCR-tg cells and were treated with anti-CD40 mAb (*right panels*) as described in *Material and Methods* or were left untreated (*left panels*). Spleen cells and PBL were analyzed by flow cytometry 7 days after transfer. *B*, Surface phenotype of TCR-tg cells from untreated tg mice (*left panels*) and from anti-CD40-treated H8 recipients of TCR-tg cells (*right panels*). PBL were stained with mAb specific for the cell surface molecules indicated (solid lines) or with isotype controls (dotted lines). Histograms are gated on Thy1.1$^+$ TCR-tg cells. *C*, The indicated numbers of TCR-tg cells were injected into H8 mice that were treated with anti-CD40 mAb 1 day before and 1 day after transfer. PBL were analyzed 7 days after transfer. *D*, Kinetics of TCR-tg cells ($10^5$) in anti-CD40-treated H8 mice. The percentage of TCR-tg cells in PBL from individual mice was determined at the indicated weeks after transfer. †, Death or killing due to moribund stage of the mice.

2*D*). Together, these data show that CD40 triggering of APC in H8 mice abolished induction of peripheral tolerance and induced vigorous proliferation of self-reactive CD8 T cells in vivo.

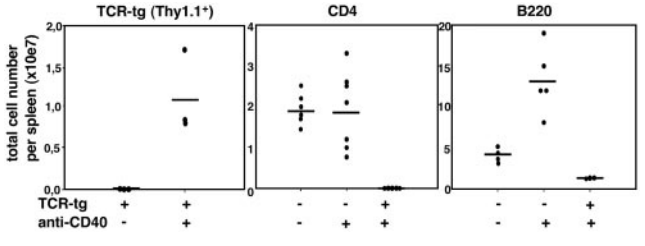


**FIGURE 3.** Absolute numbers of TCR-tg donor cells and host splenocytes in CD40-treated H8 mice. As indicated, groups of H8 mice received TCR-tg cells ($10^5$) and/or anti-CD40 mAb, or were left untreated. Seven days after cell transfer, lymphocytes were collected from the spleen and absolute numbers were calculated by multiplying the total number of viable cells and the percentage of Thy1,1$^+$, CD4$^+$, and B220$^+$ cells determined by flow cytometry. Dots indicate values from individual mice.

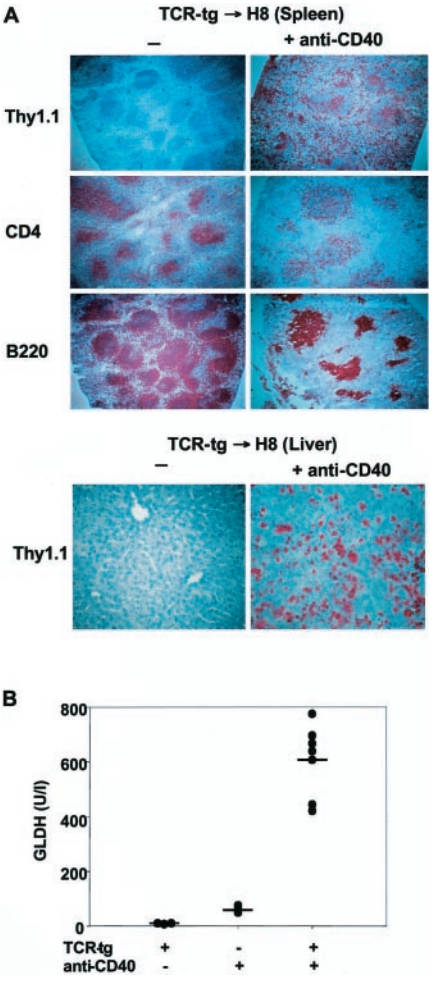


**FIGURE 4.** Destruction of the splenic architecture and induction of hepatitis in H8 recipients of TCR-tg cells after anti-CD40 treatment. *A*, Thy1.1$^+$ TCR-tg cells ($10^5$) were injected into H8 mice with (*right panels*) or without (*left panels*) anti-CD40 treatment. After 7 days, frozen sections of spleen and liver were stained with anti-Thy1.1, anti-CD4, and anti-B220 Abs as indicated. *B*, GLDH levels in the sera of the indicated groups of mice 7 days after transfer. Dots indicate values from individual mice.

### *Break of tolerance induction leads to immunopathology*

Lymphocytes in H8 mice may serve as potential target cells for the activated TCR-tg cells. Therefore, spleens of anti-CD40-treated H8 mice were examined by immunohistology. As illustrated in Fig. 4*A*, *top panels*, expansion of TCR-tg cells was accompanied by severe immunopathological alterations of the splenic architecture: TCR-tg cells (Thy1.1$^+$) were distributed all over splenic white and red pulp areas, the few remaining CD4 T cells exhibited a diffuse localization, and the size of B cell follicles was severely reduced. Accordingly, the absolute numbers of CD4 and B cells in the spleen were strongly decreased in H8 mice that received both Abs and TCR-tg cells (Fig. 3). It is likely that this decrease is due to cell elimination by activated TCR-tg cells, because Con A blast spleen cells from H8 mice can serve as target cells for activated TCR-tg cells in vitro (11) and H8 spleen cells have been shown to be rapidly eliminated by gp33-specific CTL in vivo (23).

Similar to the spleen, a massive infiltration of TCR-tg cells was also found in the liver of anti-CD40-treated H8 mice, whereas only a few donor T cells were observed in mice without treatment (Fig. 4*A*, *lower panels*). Furthermore, GLDH levels in the sera of H8 recipients of TCR-tg cells were strongly increased after anti-CD40

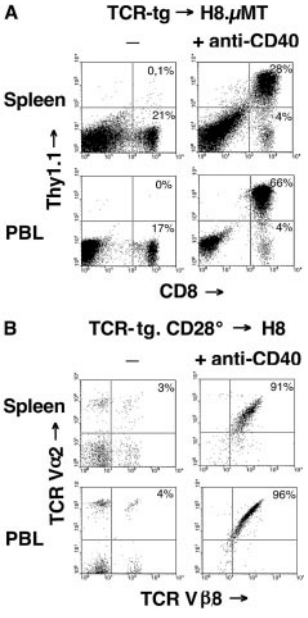


**FIGURE 5.** CD40-triggered break of peripheral tolerance induction is B cell independent and does not require CD28/B7 interactions. *A*, Thy1.1$^+$ TCR-tg cells ($10^5$) were injected in B cell-deficient H8.$\mu$MT mice with (*right panels*) or without (*left panels*) anti-CD40 treatment. *B*, TCR-tg cells ($10^5$) from CD28-deficient mice were injected into H8 mice with (*right panels*) or without (*left panels*) anti-CD40 treatment. Because T cells from TCR-tg.CD28° mice were Thy1.2$^+$, TCR-tg donor cells were traced in H8 mice using Abs (V$\alpha$2, V$\beta$8) specific for the tg TCR. Recipient mice were analyzed 7 days after transfer.

treatment (Fig. 5*B*). Besides liver and lymphoid compartments, massive infiltration of TCR-tg cells was also observed in lung tissue but not in the brain of anti-CD40-treated H8 mice (data not shown). Taken together, these data demonstrate that TCR-tg cells activated by CD40-stimulated APC destroyed lymphoid tissue and induced hepatitis in H8 mice.

### *Break of tolerance induction in H8 mice is B cell independent and does not require CD28/B7 interactions*

Most CD40-expressing cells in lymphoid organs are B cells (4). To examine whether CD40-activated B cells were essential in breaking peripheral tolerance induction of self-reactive T cells, H8 mice lacking B cells (H8.$\mu$MT mice) were used as recipient mice. Nonetheless, TCR-tg cells transferred into anti-CD40-treated H8.$\mu$MT mice proliferated as vigorously as in H8 mice (Fig. 5*A*).

Up-regulation of the costimulatory molecules CD80 and CD86 is thought to be important for the improved stimulating properties of activated APC. To test the role of these costimulatory molecules, transfer experiments with TCR-tg cells from CD28-deficient mice were performed. Because these TCR-tg cells were Thy.1.2$^+$, donor cells were traced in the recipient mice by Abs (V$\beta$8, V$\alpha$2) specific for the tg TCR. Surprisingly, TCR-tg cells lacking CD28 proliferated as vigorously as T cells from wild-type mice in anti-CD40-treated H8 mice (Fig. 5*B*). Moreover, H8 recipients of CD28$^{-/-}$ TCR-tg cells exhibited similar pathology (e.g., destruction of splenic architecture, impaired survival) as recipients of wild type TCR-tg cells (data not shown). Thus, neither B cells nor CD28-mediated costimulation were essential in breaking tolerance induction of self-reactive T cells in H8 mice by anti-CD40 treatment.

## Discussion

Naive CD8 T cells from P14 TCR-tg mice adoptively transferred into H8 mice expressing gp33 on all MHC class I-positive cells are rapidly tolerized. We have previously shown that bacterial and viral infections interfere with tolerance induction in this transfer system (11). In the present report we demonstrate that CD40 ligation of APC mimics these inflammatory processes and induces vigorous proliferation of TCR-tg cells and immunopathology. These results complement previous studies demonstrating conversion of peptide-induced tolerance to CD8 T cell priming through in vivo ligation of CD40 (19, 21). In the CD4 system, CD40 ligation has been shown to prevent tolerance of CD4 T cells induced by tumor cells (20) and induction of transplantation tolerance in neonates (18).

In the model used by Garza et al. (21), injection of soluble LCMV peptides together with anti-CD40 Abs induced diabetes in (RIP-LCMV $\times$ LCMV TCR) double-tg mice. These mice expressed LCMV glycoprotein on pancreatic $\beta$-islet cells and the corresponding Ag receptor on almost all T cells. The system described here is distinct from this model because H8 mice were "spiked" with only a few ($10^3$–$10^5$) TCR-tg cells which proliferated vigorously and induced hepatitis and destruction of lymphoid organs after anti-CD40 treatment. Nonetheless, both studies point to potential hazards using systemic CD40 stimulation as a therapeutic approach. In this context, it is noteworthy that transient elevations of serum liver transaminases up to grades 3 and 4 had been observed in patients treated with recombinant human CD40 ligand (9). Furthermore, a recent report has demonstrated that tg overexpression of CD40 ligand in murine epidermis results in chronic skin inflammation and systemic autoimmunity (24). Interestingly, pathology in this model could be transferred by CD8 but not by CD4 T cells from diseased animals.

Clonal expansion of TCR-tg cells in anti-CD40-treated H8 mice was followed by anergy and peripheral deletion. This indicated that the activating properties of the APC were transient only after injection of anti-CD40 Abs. This result fits well with a recent study by den Boer et al. (25) demonstrating that T cell immunity induced by injection of a tolerogenic peptide together with anti-CD40 Ab treatment rapidly declines. Activated CD8 T cells have been shown to trigger maturation of DCs in vitro (26). However, the decline of TCR-tg cells indicates that the large number of the induced TCR-tg effector cells in CD40-treated H8 were unable to sustain the activating properties of DC in vivo when anti-CD40 Abs became limiting.

The results presented here differ considerably from similar adoptive transfer experiments using L$^d$-alloreactive CD8 T cells from 2C TCR-tg mice (27). In this study, agonistic anti-CD40 Abs induced only minimal expansion of 2C TCR-tg cells after transfer into H-2$^{b/d}$ $F_1$ mice. A more sustained expansion of TCR-tg cells in that system required help from CD154-expressing CD4 T cells; therefore, the authors concluded that anti-CD40 agonism cannot completely mimic the physical presence of CD4 T cells. The discrepancy between this finding and our results could be due to 1) the different type of Ag (alloreactive vs MHC-restricted) studied, 2) the particular properties of the anti-CD40 mAb (FGK115 vs FGK45) used, or 3) the different affinities of the two tg TCRs (2C vs P14).

Tolerance induction in the presence of excessive Ag is well documented. Induction of peripheral tolerance by adoptive transfer of TCR-tg cells into Ag-bearing hosts was first shown in the HY model (28) and subsequently also in other Ag systems (29–32). Similarly, T cell tolerance induced by injection of high doses of soluble peptides (12, 33, 34) or by exhaustive differentiation in the

presence of high viral Ag load (35) has been demonstrated. These findings led to the hypothesis that Ag load and distribution primarily determine whether priming or tolerance occurs (36). Transgenic gp33 expression in H8 mice was under the control of promoter and regulatory regions of the H-2K$^b$ MHC class I gene (11). In addition, anti-CD40 Ab treatment did not increase H-2K$^b$ cell surface expression on B cells and DC in these mice (data not shown). Thus, gp33 Ag load and distribution probably did not differ in control and anti-CD40-treated H8 mice. Nevertheless, the fate of the transferred TCR-tg cells differed dramatically. Therefore, our data strongly suggest that the differentiation stage of the APC is considerably more important than Ag load and distribution in determining whether priming or tolerance occurs in the presence of excessive Ag.

The type of APC responsible for the break of T cell tolerance induction by CD40 ligation in vivo has not yet been defined. Our data demonstrate that B cells were not essential for this process. This result agrees with Diehl et al. (19), using soluble peptides to tolerize T cells. Besides B cells, DC, macrophages, parenchymal, microglial, and endothelial/epithelial cells also express CD40 (4, 37–39). In light of their well-established role in T cell activation (40), stimulated DC are the most likely candidates for the conversion of T cell tolerance to T cell priming. CD40 ligation of DC leads to up-regulation of the costimulatory molecules CD80 and CD86 (41), thereby enhancing their stimulating capacities. Interestingly, induction of CD80/86 expression on DC was not crucial in breaking tolerance in H8 mice, because TCR-tg cells from CD28-deficient mice expanded in a similar number to TCR-tg cells from wild-type mice. Therefore, induction of other stimulatory molecules or augmented production of proinflammatory cytokines (42–45) may compensate or may even play the key roles in "licensing" APCs for CD8 T cell activation. Further progress in understanding priming and tolerance induction of T cells will depend on the identification of these factors.

In conclusion, this study provides further in vivo evidence for the fundamental concept that the activation state of the APC, most likely DC, is crucial in determining whether interaction of T cells with Ag leads to clonal expansion and acquisition of effector T cell function or to tolerance by ignorance, anergy, or deletion. In addition, our data suggest the need for caution in systemically activating APC in vivo by CD40 ligation in the presence of potentially self-reactive CD8 T cells.

## Acknowledgments

We thank Drs. S. Batsford and S. Ehl for comments on the manuscript, Dr. A. Rolink for the FGK-45 hybridoma, and Theresa Treuer, Rainer Bronner, and Thomas Imhof for animal husbandry.

## References

1. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4$^+$ T-helper and a T-killer cell. *Nature 393: 474.*
2. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478.*
3. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature 393:480.*
4. van Kooten, C., and J. Banchereau. 1997. Functions of CD40 on B cells, dendritic cells and other cells. *Curr. Opin. Immunol. 9:330.*
5. Grewal, I. S., and R. A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol. 16:111.*
6. Lode, H. N., R. Xiang, U. Pertl, E. Forster, S. P. Schoenberger, S. D. Gillies, and R. A. Reisfeld. 2000. Melanoma immunotherapy by targeted IL-2 depends on CD4$^+$ T-cell help mediated by CD40/CD40L interaction. *J. Clin. Invest. 105: 1623.*
7. Todryk, S. M., A. L. Tutt, M. H. Green, J. A. Smallwood, N. Halanek, A. G. Dalgleish, and M. J. Glennie. 2001. CD40 ligation for immunotherapy of solid tumours. *J. Immunol. Methods 248:139.*
8. Kedl, R. M., M. Jordan, T. Potter, J. Kappler, P. Marrack, and S. Dow. 2001. CD40 stimulation accelerates deletion of tumor-specific CD8$^+$ T cells in the absence of tumor-antigen vaccination. *Proc. Natl. Acad. Sci. USA 98:10811.*
9. Vonderheide, R. H., J. P. Dutcher, J. E. Anderson, S. G. Eckhardt, K. F. Stephans, B. Razvillas, S. Garl, M. D. Butine, V. P. Perry, R. J. Armitage, et al. 2001. Phase I study of recombinant human CD40 ligand in cancer patients. *J. Clin. Oncol. 19:3280.*
10. Sarawar, S. R., B. J. Lee, S. K. Reiter, and S. P. Schoenberger. 2001. Stimulation via CD40 can substitute for CD4 T cell function in preventing reactivation of a latent herpesvirus. *Proc. Natl. Acad. Sci. USA 98:6325.*
11. Ehl, S., J. Hombach, P. Aichele, T. Rulicke, B. Odermatt, H. Hengartner, R. Zinkernagel, and H. Pircher. 1998. Viral and bacterial infections interfere with peripheral tolerance induction and activate CD8$^+$ T cells to cause immunopathology. *J. Exp. Med. 187:763.*
12. Kyburz, D., P. Aichele, D. E. Speiser, H. Hengartner, R. M. Zinkernagel, and H. Pircher. 1993. T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur. J. Immunol. 23:1956.*
13. Zimmermann, C., M. Rawiel, C. Blaser, M. Kaufmann, and H. Pircher. 1996. Homeostatic regulation of CD8$^+$ T cells after antigen challenge in the absence of Fas (CD95). *Eur. J. Immunol. 26:2903.*
14. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science 261:609.*
15. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin $\mu$ chain gene. *Nature 350:423.*
16. Battegay, M., S. Cooper, A. Althage, J. Banziger, H. Hengartner, and R. M. Zinkernagel. 1991. Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. [Published errata appear in *1991 J. Virol. Methods 35:115* and *1992 J. Virol. Methods 38:263.*] *J. Virol. Methods 33:191.*
17. Rolink, A., F. Melchers, and J. Andersson. 1996. The SCID but not the RAG-2 gene product is required for S$\mu$-S$\epsilon$ heavy chain class switching. *Immunity 5:319.*
18. Flamand, V., V. Donckier, F. X. Demoor, A. Le Moine, P. Matthys, M. L. Vanderhaeghen, Y. Tagawa, Y. Iwakura, A. Billiau, D. Abramowicz, and M. Goldman. 1998. CD40 ligation prevents neonatal induction of transplantation tolerance. *J. Immunol. 160:4666.*
19. Diehl, L., A. T. den Boer, S. P. Schoenberger, E. I. van der Voort, T. N. Schumacher, C. J. Melief, R. Offringa, and R. E. Toes. 1999. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat. Med. 5:774.*
20. Sotomayor, E. M., I. Borrello, E. Tubb, F. M. Rattis, H. Bien, Z. Lu, S. Fein, S. Schoenberger, and H. I. Levitsky. 1999. Conversion of tumor-specific CD4$^+$ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nat. Med. 5:780.*
21. Garza, K. M., S. M. Chan, R. Suri, L. T. Nguyen, B. Odermatt, S. P. Schoenberger, and P. S. Ohashi. 2000. Role of antigen-presenting cells in mediating tolerance and autoimmunity. *J. Exp. Med. 191:2021.*
22. Grohmann, U., F. Fallarino, S. Silla, R. Bianchi, M. L. Belladonna, C. Vacca, A. Micheletti, M. C. Fioretti, and P. Puccetti. 2001. CD40 ligation ablates the tolerogenic potential of lymphoid dendritic cells. *J. Immunol. 166:277.*
23. Barchet, W., S. Oehen, P. Klenerman, D. Wodarz, G. Bocharov, A. L. Lloyd, M. A. Nowak, H. Hengartner, R. M. Zinkernagel, and S. Ehl. 2000. Direct quantitation of rapid elimination of viral antigen-positive lymphocytes by antiviral CD8$^+$ T cells in vivo. *Eur. J. Immunol. 30:1356.*
24. Mehling, A., K. Loser, G. Varga, D. Metze, T. A. Luger, T. Schwarz, S. Grabbe, and S. Beissert. 2001. Overexpression of CD40 ligand in murine epidermis results in chronic skin inflammation and systemic autoimmunity. *J. Exp. Med. 194:615.*
25. den Boer, A. T., L. Diehl, G. J. van Mierlo, E. I. van der Voort, M. F. Fransen, P. Krimpenfort, C. J. Melief, R. Offringa, and R. E. Toes. 2001. Longevity of antigen presentation and activation status of APC are decisive factors in the balance between CTL immunity versus tolerance. *J. Immunol. 167:2522.*
26. Ruedl, C., M. Kopf, and M. F. Bachmann. 1999. CD8$^+$ T cells mediate CD40-independent maturation of dendritic cells in vivo. *J. Exp. Med. 189:1875.*
27. Buhlmann, J. E., M. Gonzalez, B. Ginther, A. Panoskaltsis-Mortari, B. R. Blazar, D. L. Greiner, A. A. Rossini, R. Flavell, and R. J. Noelle. 1999. Cutting edge: sustained expansion of CD8$^+$ T cells requires CD154 expression by Th cells in acute graft versus host disease. *J. Immunol. 162:4373.*
28. Rocha, B., and H. von Boehmer. 1991. Peripheral selection of the T cell repertoire. *Science 251:1225.*
29. Bertolino, P., W. R. Heath, C. L. Hardy, G. Morahan, and J. F. Miller. 1995. Peripheral deletion of autoreactive CD8$^+$ T cells in transgenic mice expressing H-2K$^b$ in the liver. *Eur. J. Immunol. 25:1932.*
30. Zhang, L. 1996. The fate of adoptively transferred antigen-specific T cells in vivo. *Eur. J. Immunol. 26:2208.*
31. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med. 184:923.*
32. Morgan, D. J., C. Kurts, H. T. Kreuwel, K. L. Holst, W. R. Heath, and L. A. Sherman. 1999. Ontogeny of T cell tolerance to peripherally expressed antigens. *Proc. Natl. Acad. Sci. USA 96:3854.*
33. Oki, A., and E. Sercarz. 1985. T cell tolerance studied at the level of antigenic determinants. I. Latent reactivity to lysozyme peptides that lack suppressogenic epitopes can be revealed in lysozyme-tolerant mice. *J. Exp. Med. 161:897.*
34. Ria, F., B. M. Chan, M. T. Scherer, J. A. Smith, and M. L. Gefter. 1990. Immunological activity of covalently linked T-cell epitopes. *Nature 343:381.*

35. Moskophidis, D., F. Lechner, H. Pircher, and R. M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature 362:758.*
36. Zinkernagel, R. M. 2000. Localization dose and time of antigens determine immune reactivity. *Semin. Immunol. 12:163.*
37. Alderson, M. R., R. J. Armitage, T. W. Tough, L. Strockbine, W. C. Fanslow, and M. K. Spriggs. 1993. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. *J. Exp. Med. 178: 669.*
38. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med. 180:1263.*
39. Karmann, K., C. C. Hughes, J. Schechner, W. C. Fanslow, and J. S. Pober. 1995. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc. Natl. Acad. Sci. USA 92:4342.*
40. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell 106:255.*
41. Yang, Y., and J. M. Wilson. 1996. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science 273:1862.*
42. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med. 184:747.*
43. Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heufler, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med. 184:741.*
44. Schulz, O., D. A. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher, and C. Reis e Sousa. 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity 13:453.*
45. Bleharski, J. R., K. R. Niazi, P. A. Sieling, G. Cheng, and R. L. Modlin. 2001. Signaling lymphocytic activation molecule is expressed on CD40 ligand- activated dendritic cells and directly augments production of inflammatory cytokines. *J. Immunol. 167:3174.*