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Cutting Edge: 4-1BB Is a Bona Fide CD8 T Cell Survival Signal[1]

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After recognition of Ag/MHC and ligation of a costimulatory molecule, resting T cells will clonally expand and then delete to very low levels. Previously, it was shown that deletion can be prevented by coinjection of cytokines or proinflammatory agents such as adjuvants. Here, we demonstrate that ligation of 4-1BB blocks deletion of superantigen-activated T cells in the absence of adjuvant or additional cytokine treatment. Nearly 10 times as many staphylococcal enterotoxin A-specific T cells were detected in the spleens of mice injected 21 days previously with staphylococcal enterotoxin A and an agonist anti-4-1BB Ab compared with mice given staphylococcal enterotoxin A and a control IgG. Even though both CD4- and CD8-activated T cells expressed 4-1BB, a higher proportion of CD8 T cells were rescued compared CD4 T cells. These data suggest that although 4-1BB provides costimulation, it may also promote long-term T cell survival. ***The Journal of Immunology*****, 1999, 162: 5037–5040.**

For years it has been known that T cells require two signals for full activation (1, 2). Ag presented in the context of MHC is signal one and a costimulatory receptor provides the second signal. CD28 and its ligands CD80 and CD86 have been the most extensively studied. It is documented that CD28 ligation helps T cells to proliferate probably by driving the production of IL-2 (3–5). Thus, it is surprising that ligation of CD28 in vivo on Ag-activated T cells does not block peripheral T cell deletion (6, 7). This is the case even when APC have been activated in vivo by CD40 ligation (6), which potently enhance B7 expression on APC (8). Therefore, the likely role for CD28-mediated costimulation in vivo is the enhancement of clonal expansion and possibly short-term survival.

In many cases, the use of adjuvants are thought to be the difference between the induction of tolerance vs immunity. Specifically, it has been proposed that a "danger" signal can impede tolerance induction and promote long-term immunity (9). Recently, it has been shown that LPS can block superantigen (SAg)[3]-induced deletion consistent with the danger theory (10). These data suggest that proinflammatory factors promote long-term survival of Ag-activated T cells in vivo. Additionally, it is clear that IL-2 and IL-4 therapy can inhibit deletion of activated T cells (11, 12). Therefore, Ag-activated T cells may die in vivo by failing to acquire growth factors.

To examine how T cells avoid death in vivo after exposure to SAg, we examined the possibility that other costimulatory molecules may function differently than CD28. 4-1BB is a member of the TNF receptor family and its ligand 4-1BB ligand is a member of the TNF family (13, 14). 4-1BB has been shown to be a potent costimulatory molecule for CD8 T cells by enhancing cytokine production, and proliferative and cytolytic activity (15–17). Functionally, this receptor/ligand pair can facilitate anti-tumor activity (18). Thus, 4-1BB is a costimulatory molecule that heightens CD8 T cell function.

We report that 4-1BB expression on SAg-activated T cells is rapid and differential between CD4 and CD8 subpopulations. After ligation of 4-1BB on SAg-activated CD8 T cells in vivo, using an agonist mAb, peripheral deletion is almost entirely inhibited. This occurs in the absence of an adjuvant or cytokine treatments. Thus, 4-1BB ligation can inhibit activation-induced cell death in vivo possibly by providing a long-term survival signal to activated T cells.

Materials and Methods

Mice

Female B10.A mice were purchased from the National Cancer Institute (Frederick, MD) and maintained in our animal facility under specific pathogen-free conditions.

Reagents, protocols, and Abs

Staphylococcal enterotoxin A (SEA) was purchased from Toxin Technology (Madison, WI) and administered to mice as i.p. injections of 0.30 μg. The agonist anti-4-1BB mAb 3H3 and biotinylated anti-4-1BB mAb 1D8 were used as a receptor agonist or staining reagent, respectively (17). As a control, rat IgG (Sigma, St. Louis, MO) was injected in doses equal to mAb 3H3.

Anti-TCR Vβ3 was purified from hybridoma supernatants over protein G columns (Pharmacia, Piscataway, NJ) (19). The Ab was FITC-conjugated by us. FITC-conjugated anti-Vβ14, PE-conjugated anti-CD4, PE-conjugated streptavidin, and biotinylated anti-TCR Vβ3 were purchased from PharMingen (San Diego, CA). Red 613-conjugated anti-CD4 and Red

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[3] Abbreviations used in this paper: SAg, superantigen; SEA, staphylococcal enterotoxin A; LN, lymph node.

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613-conjugated anti-CD8 were purchased from Life Technologies (Grand Island, NY).

Injection of SEA was at time 0 h, and all other injections were done in relation to SEA. In all experiments, 3H3 mAb was coinjected with SEA, and doses are described in the figure legends. Rat IgG was given to a control group at the same time as 3H3 mAb was given to an experimental group.

Cell processing and flow cytometry

Spleens were removed and teased through nylon mesh (Falcon, Becton Dickinson, Franklin Lakes, NJ) and subjected to ammonium chloride to lyse RBCs. Peripheral lymph nodes (LN) were teased into single-cell suspensions and washed with BSS. T cells from spleen or LN populations were purified on nylon wool columns as previously described (20).

For staining, cells were incubated on ice with a primary mAb in the presence of 5% normal mouse serum, culture supernatant containing anti-mouse Fc receptor mAb (24.G2), and 10 mg/ml human γ-globulin (Sigma) to block nonspecific binding. After a 30 min incubation on ice in staining buffer (BSS, 3% FBS, 0.1% sodium azide), the cells were washed and analyzed by flow cytometry, or if a second step was necessary, the incubation and wash procedures were repeated. Analysis was conducted on an EPICS XL flow cytometer (Coulter Electronics, Miami, FL).

Cell viability counts were completed by trypan blue exclusion. Equal volumes of cells and trypan blue (PBS, 0.4% trypan blue, 0.2% NaN_3) were mixed, and viable cells were counted.

Results and Discussion

In an attempt to examine 4-1BB expression in vivo, mice were immunized with SEA, and activated T cells were evaluated for surface levels of 4-1BB. SEA is a SAg that binds MHC class II molecules on APC and stimulates T cells bearing TCR Vβ3 (21, 22).

At 0, 0.5, 1.5, 3, 12, 24, and 48 h after SEA injection, LN T cells were purified and stained for CD4 or CD8, Vβ3, and 4-1BB expression (Fig. 1). Flow cytometric analysis determined that 3-h activated CD4 Vβ3 T cells do not express detectable levels of 4-1BB compared with the equivalent cell populations from noninjected mice. At 12 h, there is a profound increase in 4-1BB expression, and by 24 and 48 h levels of 4-1BB decrease. In contrast, SEA-activated CD8 Vβ3 T cells began to express 4-1BB by 90 min and further at 3 h (Fig. 1). Peak expression occurred at 12 h followed by a decline to background levels by 48 h. Additionally, at 12 h the amount of 4-1BB appears to be higher on the CD8 over the CD4 T cells. However, it is also possible that the kinetics of peak expression is different between the two populations. We conclude that 4-1BB is rapidly expressed on both CD4 and CD8 SAg-stimulated T cells in vivo and that CD8 T cells express this molecule faster after stimulation than CD4 T cells.

Upon stimulation with SAg, peripheral T cells will clonally expand and then delete (23–25). In the case of SEA, expansion of the Vβ3 T cells is observed 2 days after injection (Fig. 2) (22). Compared with noninjected mice, a threefold increase in the frequency of splenic CD4 Vβ3 T cells and a doubling, of those same cells, in absolute cell number was observed (Fig. 2, *A* and *B*). After expansion, there is a dramatic decrease in the frequency and absolute number of SEA-specific T cells. There is nearly a 10-fold decrease after peak expansion in both frequency and number of Vβ3 T cells. The data show that the levels of SEA-specific T cells are lower

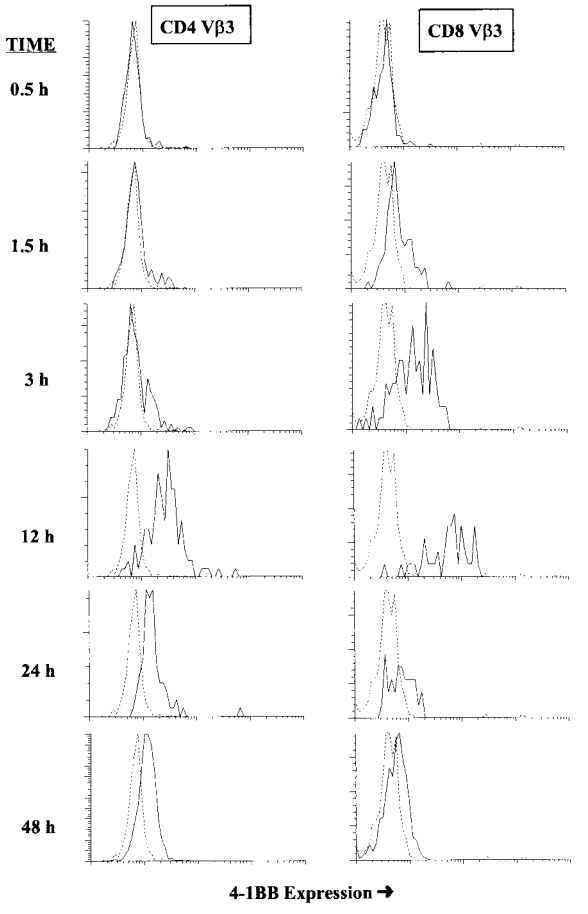

FIGURE 1. Kinetics of 4-1BB expression on in vivo activated T cells. B10.A mice were injected with 0.30 μg of SEA. At various times after SEA injection, LN T cells were purified. These cells were stained for CD4 Vβ3 4-1BB (*left panel*) and CD8 Vβ3 4-1BB (*right panel*) and analyzed by flow cytometry (solid lines). 4-1BB expression on T cells from an uninjected normal mouse (dotted lines) is shown. This is one experiment similar to three others.

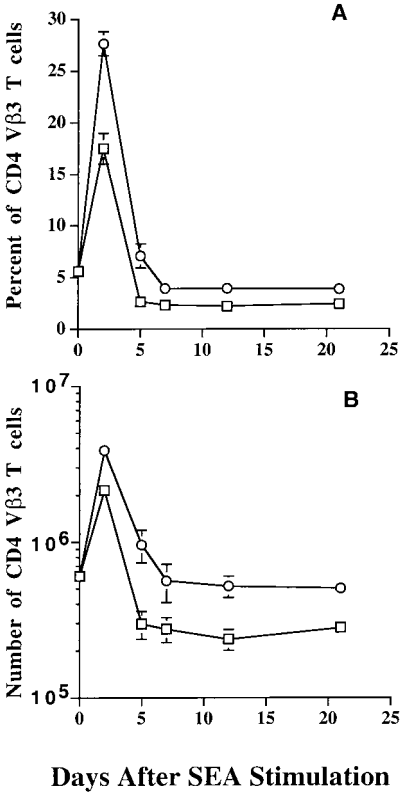

FIGURE 2. Time course of SEA-stimulated CD4 T cell deletion with and without 4-1BB activation. B10.A mice were coinjected with 0.10 mg of anti-4-1BB mAb 3H3 (○) or 0.1 mg rat IgG (□) and 0.30 μg of SEA. Splenic T cells were purified on days 2, 5, 7, 12, and 21 after SEA injection. T cells were stained for CD4 Vβ3 and analyzed by flow cytometry. Each point represents the mean percentages (*A*) and numbers (*B*) ± SEM from six mice over three separate experiments combined.

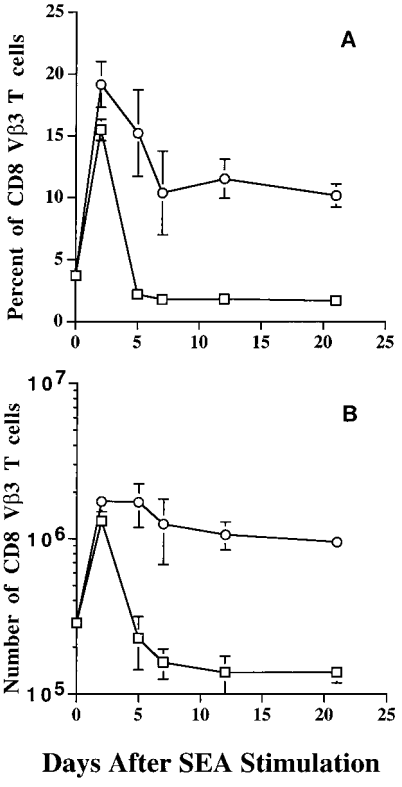

FIGURE 3. Time course of SEA-stimulated CD8 T cell deletion with and without 4-1BB activation. These data are from the experiments described in Fig. 2, except the cells were stained (*A*) and enumerated (*B*) for CD8 Vβ3. Data from LN were similar (not shown).

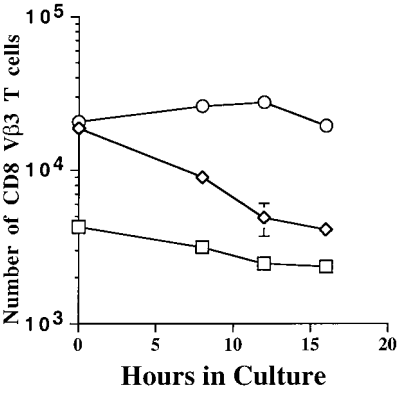

FIGURE 4. Survival of SEA-stimulated and unstimulated CD8 T cells in vitro with and without 4-1BB activation. Three female B10.A mice were set up as follows: one was uninjected (□); one was injected with 0.20 mg of anti-4-1BB mAb 3H3 and 0.30 μg SEA (○); and one was injected with 0.20 mg of rat IgG and 0.30 μg SEA (◇). LN T cells were purified 2 days after injections and cultured at 2×10^6 cells per well in a 96-well plate. T cells were stained and enumerated for CD8 Vβ14 (data not shown) and CD8 Vβ3 expression at 0, 8, 12, and 16 h in culture. Each data point represents the mean numbers ± SEM from triplicate samples for each injection regimen. These data are comparable to three other experiments.

than that found in uninjected mice. When SEA is injected with an agonist mAb specific for 4-1BB, clonal expansion of the CD4 Vβ3 T cells is significantly increased (Fig. 2). Although deletion is inhibited to some degree based on absolute numbers, rescue is not observed based on frequency.

Analysis of SEA/4-1BB mAb-treated splenic CD8 T cells revealed that clonal expansion was not increased on day 2 after SEA injection in comparison to mice that received SEA alone (Fig. 3). Although the frequency of CD8 Vβ3 T cells was slightly elevated (Fig. 3*A*), this effect was not observed when absolute numbers were examined (Fig. 3*B*). One explanation for this difference is the possibility that the CD8 T cells expanded further on day 3 or 4.

When the latter time points were examined, it was observed that deletion was inhibited. There is practically no decrease in the number of CD8 Vβ3 T cells between days 2 and 21 after SEA injection. On day 21, there are nearly 10-fold the number of Ag-specific CD8 T cells in the anti-4-1BB-treated mice in comparison to the control IgG-treated mice (Fig. 3*B*). As a control, Vβ14 T cells were analyzed, and it was determined that the Vβ14 T cells were unaffected by SEA and/or anti-4-1BB treatment (data not shown). Thus, 4-1BB ligation can provide a life signal to SAg-activated CD8 T cells that results in survival.

It is unclear why CD4 T cells were not rescued to the same degree from deletion as CD8 T cells. One possibility is that CD4 T cells are less responsive to 4-1BB ligation compared with CD8 T cells. There is at least one study that supports this notion (17).

These in vivo data demonstrate the effectiveness of 4-1BB ligation in preventing death of SAg-activated CD8 T cells; however, this model does not afford practical mechanistic studies due to the complication of in vivo analyses. To remedy this problem, an ex vivo model was employed. At the peak of SEA-induced clonal expansion, LN T cells were purified and cultured in vitro with no added stimuli. The cells were monitored for survival by counting the remaining number of viable CD8 Vβ3 T cells at 0, 8, 12, and 16 h in culture. As a control, Vβ14 T cells were stained, and the data show that these cells were unaltered regardless of the treatment (data not shown). On the other hand, CD8 Vβ3 T cells from SEA/control IgG-treated mice preferentially died in vitro compared with equivalent cells from normal animals (Fig. 4). In contrast, CD8 Vβ3 T cells from SEA/anti-4-1BB-treated mice preferentially survived compared with all other cell populations tested. This was not the case for SEA/control IgG cells, which decreased by greater than fourfold in absolute numbers over the 16-h incubation. In a separate experiment, we tested the idea that the rescued T cells were surviving due to IL-2 production. Our data indicate that IL-2 was not keeping these cells alive because a neutralizing IL-2 mAb did not block survival but was able to block IL-2-driven proliferation (data not shown). Nevertheless, it is possible that other survival factors were produced by the rescued cells.

Experiments that forced ligation of CD28 by enhancing B7 expression on APC through CD40 ligation potently augmented SEA-specific CD8 T cell clonal expansion (6). Nevertheless, these cells went on to delete after expansion. This is in contrast to the data in these studies using 4-1BB as a costimulus. 4-1BB ligation was extremely potent at rescuing CD8 T cells from deletion after Ag recognition in vivo. These data are consistent with previously published in vitro data showing that 4-1BB ligation can inhibit activation-induced cell death (15).

The potency of the rescuing effect may have practical application, such as the design of CD8 T cell-based vaccines. Collectively, these data suggest that 4-1BB may be an integral component of CD8 T cell memory acquisition and may be a link between a putative danger signal and long-term immunity.

References

1. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med. 165:302.*
2. Jenkins, M. K., D. M. Pardoll, J. Mizuguchi, H. Quill, and R. H. Schwartz. 1987. T cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol. Rev. 95:113.*

3. Linsley, P. S., W. Brady, L. Grosmaire, A. Aruffo, N. K. Damle, and J. A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and IL-2 mRNA accumulation. *J. Exp. Med. 173:721.*
4. Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol. 147:2461.*
5. Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science 251:313.*
6. Maxwell, R. J., J. D. Campbell, C. H. Kim, and A. T. Vella. 1999. CD40 activation boosts immunity in vivo by enhancing T cell clonal expansion and delaying peripheral T cell deletion. *J. Immunol. 162:2024.*
7. Vella, A. T., T. Mitchell, B. Groth, P. S. Linsley, J. M. Green, C. B. Thompson, J. W. Kappler, and P. Marrack. 1997. CD28 engagement and proinflammatory cytokines contribute to T cell expansion and long-term survival in vivo. *J. Immunol. 158:4714.*
8. Roy, M., A. Aruffo, J. Ledbetter, P. Linsley, M. Kehry, and R. Noelle. 1995. Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. *Eur. J. Immunol. 25:596.*
9. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol. 12:991.*
10. Vella, A. T., J. E. McCormack, P. S. Linsley, J. W. Kappler, and P. Marrack. 1995. Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity 2:261.*
11. Kuroda, K., J. Yagi, K. Imanishi, X. J. Yan, X. Y. Li, W. Fujimaki, H. Kato, T. Miyoshi-Akiyama, Y. Kumazawa, H. Abe, and T. Uchiyama. 1996. Implantation of IL-2-containing osmotic pump prolongs the survival of SAg-reactive T cells expanded in mice injected with bacterial superantigen. *J. Immunol. 157: 1422.*
12. Vella, A. T., S. Dow, T. A. Potter, J. Kappler, and P. Marrack. 1998. Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc. Natl. Acad. Sci. USA 95:3810.*
13. Vinay, D. S., and B. S. Kwon. 1998. Role of 4-1BB in immune responses. *Semin. Immunol. 10:481.*
14. Kwon, B. S., and S. M. Weissman. 1989. cDNA sequences of two inducible T-cell genes. *Proc. Natl. Acad. Sci. USA 86:1963.*
15. Hurtado, J. C., Y. J. Kim, and B. S. Kwon. 1997. Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J. Immunol. 158:2600.*
16. DeBenedette, M. A., A. Shahinian, T. W. Mak, and T. H. Watts. 1997. Costimulation of $CD28^-$ T lymphocytes by 4-1BB ligand. *J. Immunol. 158:551.*
17. Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, T. C. Pearson, J. A. Ledbetter, A. Aruffo, and R. S. Mittler. 1997. 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J. Exp. Med. 186:47.*
18. Melero, I., W. W. Shuford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellstrom, R. S. Mittler, and L. Chen. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med. 3:682.*
19. Pullen, A. M., P. Marrack, and J. W. Kappler. 1988. The T-cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. *Nature 335:796.*
20. Julius, M. H., E. Simpson, and L. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol. 3:645.*
21. Marrack, P. C., and J. W. Kappler. 1990. The staphylococcal enterotoxins and their relatives. *Science 248:705.*
22. McCormack, J. E., J. E. Callahan, J. Kappler, and P. Marrack. 1993. Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigen. *J. Immunol. 150:3785.*
23. Webb, S., and J. Sprent. 1990. Response of mature unprimed $CD8^+$ T cells to Mls^a determinants. *J. Exp. Med. 171:953.*
24. MacDonald, H. S., S. Baschieri, and R. K. Lees. 1991. Clonal expansion precedes anergy and death of $V\beta8^+$ peripheral T cells responding to staphylococcal enterotoxin B in vivo. *Eur. J. Immunol. 21:1963.*
25. Liblau, R. S., R. Tisch, K. Shokat, X. Yang, N. Dumont, C. C. Goodnow, and H. O. McDevitt. 1996. Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis. *Proc. Natl. Acad. Sci. USA 93:3031.*