The Journal of Immunology

Cutting Edge: Apoptosis of Superantigen-Activated T Cells Occurs Preferentially After a Discrete Number of Cell Divisions In Vivo

Toufic Renno, Antoine Attinger, Sabrina Locatelli, Talitha Bakker, Sonia Vacheron and H. Robson MacDonald

J Immunol 1999; 162:6312-6315; ;
http://www.jimmunol.org/content/162/11/6312

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cutting Edge: Apoptosis of Superantigen-Activated T Cells Occurs Preferentially After a Discrete Number of Cell Divisions In Vivo[1]

Toufic Renno, Antoine Attinger, Sabrina Locatelli, Talitha Bakker, Sonia Vacheron, and H. Robson MacDonald[2]

Staphylococcal enterotoxins are bacterial products that display superantigen activity in vitro as well as in vivo. For instance, staphylococcal enterotoxin B (SEB) polyclonally activates T cells that bear the Vβ8 gene segment of the TCR. SEB-activated T cells undergo a burst of proliferation that is followed by apoptosis. Using an in vivo adaptation of a fluorescent cell division monitoring technique, we show here that SEB-activated T cells divide asynchronously, and that apoptosis of superantigen-activated T cells is preferentially restricted to cells which have undergone a discrete number of cell divisions. Collectively, our data suggest that superantigen-activated T cells are programmed to undergo a fixed number of cell divisions before undergoing apoptosis. A delayed death program may provide a mechanistic compromise between effector functions and homeostasis of activated T cells. ***The Journal of Immunology,*** **1999, 162: 6312–6315.**

Following activation with staphylococcal enterotoxin B (SEB),[3] T cells proliferate and eventually undergo apoptosis (1–8). Surprisingly, it has been shown that the up-regulation of known apoptosis-mediating molecules such as Fas (reviewed in Ref. 9) or TNF receptor (reviewed in Ref. 10) on the surface of activated T cells does not confer immediate susceptibility to apoptosis (11, 12). One possible explanation of this apparent paradox is that activated T cells are programmed to acquire susceptibility to apoptosis only after a given number of cell divisions. Indeed, a precedent for such "cell division-associated" acquisition of specific function has been established for Ig isotype switch in activated B cells and for activation marker and cytokine expression in activated T cells (13–17). Linking apoptosis to cell division in activated T cells would allow expansion and effector function of the responding cells, while ensuring that these cells will ultimately undergo apoptosis, thus maintaining homeostasis. To address this possibility, we adapted a recently described cell division monitoring technique (18) that, when used in conjunction with cell surface phenotyping, allows the study of the relationship between the number of cell divisions and activation or death markers. Here, we show that SEB-reactive $CD4^+$ T cells divide asynchronously in response to SEB in vivo, and that apoptosis of SEB-reactive T cells occurs preferentially after a discrete number of cell divisions.

Materials and Methods

Mice and treatment

We obtained 4- to 8-wk-old BALB/c mice from Harlan Olac (Bicester, U.K.). A total of 5×10^7 syngeneic splenocytes labeled with 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) (see below) were transferred i.v. into the tail vein. SEB (10 μg, purchased from Toxin Technology, Saratoga, FL) was injected into each of the hind footpads 1 day following cell transfer.

CFSE staining

Single-cell suspensions were made from spleens by homogenization into sterile high glucose DMEM (Life Technologies, Grand Island, NY). Debris was allowed to settle, and splenocytes were subsequently resuspended at 5×10^7/ml in PBS with no protein. CFSE was added to a final concentration of 5 μM, and the suspension was incubated at 37°C for 10 min. At the end of the incubation period, the cells were immediately washed three times in cold DMEM/10% FCS (Irvine Scientific, Santa Ana, CA).

Abs and flow cytometry

For three-color staining, the following mAbs were used: PE-labeled anti-CD4 (Boehringer Mannheim, Mannheim, Germany) and biotinylated anti-Vβ8 (F23.1) (19) (revealed with streptavidin-tricolor (Caltag, San Fransisco, CA). For four-color staining, anti-Vβ8-PE and anti-CD4-APC were purchased from PharMingen (San Diego, CA) and used in conjunction with annexin V staining (see below). Stained cells were analyzed on a FACScan or FACScalibur (Becton Dickinson, San Jose, CA).

Apoptosis assay

Annexin V (Nexins Research, Maastricht, The Netherlands) was used as described previously (20–22). Briefly, cells were washed in 100 μl of

Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Epalinges, Switzerland

Received for publication December 28, 1998. Accepted for publication March 29, 1999.

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] T.R. is supported by a Centennial Fellowship from the Medical Research Council of Canada and by the Ciba Jubilee Foundation. H.R.M. and A.A. are supported in part by a grant (RG-544/95) from the Human Frontier Science Program.

[2] Address correspondence and reprint requests to Dr. H. Robson MacDonald, Ludwig Institute for Cancer Research, Chemin des Boveresses 155, 1066 Epalinges, Switzerland. E-mail address: hughrobson.macdonald@isrec.unil.ch

[3] Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; CFSE, 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester; LN, lymph node.

0022-1767/99/$02.00

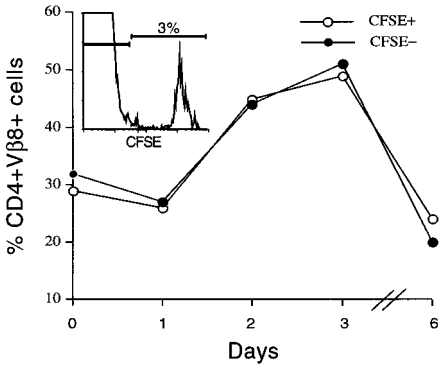

FIGURE 1. Kinetics of response to SEB in host ($CFSE^-$) and donor ($CFSE^+$) T cells. Splenocytes were labeled with CFSE and transferred i.v. into syngeneic BALB/c recipients. At the indicated timepoints following injection with SEB, the percentage of $V\beta8^+$ cells was quantitated in the draining LN after electronically gating on $CD4^+CFSE^+$ or $CD4^+CFSE^-$ cells. Inset: FACS histogram showing $CFSE^+$ donor cells (*right peak*) and $CFSE^-$ host cells (*left peak*) in the absence of SEB stimulation.

binding buffer and incubated in a 1/50 dilution of biotin-coupled annexin V solution for 20 min in the dark. Next, cells were washed, revealed with streptavidin tricolor (Caltag), and analyzed on the FACS.

Quantitation of SEB-reactive undivided cells

At 3 days after SEB administration, the percentage of cells in CFSE peaks was measured after electronically gating on $CD4^+$ $V\beta8^+$ cells. To estimate the initial proportion of cells that gave rise to those in the different division peaks, we divided the percentage of cells in division peaks 1, 2, 3, and 4 by 2, 4, 8, and 16, respectively (because every two cells in a given division peak must have arisen from a single cell from the previous division peak). The corrected percentages thus obtained were used in the following formula: Percent undivided cells = $U/(U + \Sigma Dc)$, where U equals the percentage cells in the undivided CFSE peak and ΣDc equals the sum of the corrected percentages of cells in division peaks 1–4.

It should be noted that cell loss/apoptosis, although not accounted for by this analysis, would not significantly alter the outcome; it occurs mainly in division peak 4 (see Fig. 3), which only contributes a minor fraction of ΣDc.

Results and Discussion

To investigate whether SEB-induced apoptosis of activated T cells is associated with the number of cell divisions in vivo, we used the dye CFSE, which covalently binds to cellular proteins and allows direct measurement by flow cytometry of cell divisions over several cell generations (18). We focused our analysis on the CD4 subset, because SEB-mediated clonal deletion has been shown to be more dramatic in $CD4^+$ than in $CD8^+$ T cells (8, 23, 24). CFSE-labeled spleen cells were injected i.v. into naive syngeneic mice. Typically, 2–3% $CFSE^+$ T cells were detected in the lymphoid organs at 24 h after cell transfer (Fig. 1, inset), and this percentage remained stable over several days (data not shown). To ascertain that CFSE treatment did not compromise the ability of $CD4^+$ T cells to respond normally to SEB, we measured the percentage of $V\beta8^+$ T cells within the CD4 subset in the draining lymph node (LN) in electronically gated CFSE-positive or -negative cells following injection of SEB. As can be seen in Fig. 1, the response to SEB in donor $CFSE^+$ cells was virtually indistinguishable from the typical response of host $CFSE^-$ cells, confirming that neither T cell expansion nor deletion was affected by staining with CFSE.

We subsequently confirmed that CFSE can be used to monitor the specific cell division induced by SEB in vivo. In the representative example shown in Fig. 2, five distinct peaks of CFSE fluorescence intensity (each differing by a factor of two from adjoining peaks) were detected among donor-derived $CD4^+$ $V\beta8^+$ cells in the draining LN 3 days after SEB injection. The brightest peak corresponds to undivided cells, whereas the four remaining peaks identify cells that have undergone 1, 2, 3, and 4 divisions, respectively. As expected, very few $CD4^+$ $V\beta8^-$ cells divided in response to SEB during this time period, resulting in a dominant CFSE peak that corresponded to undivided cells (Fig. 2). Kinetic analysis of CFSE fluorescence among $CD4^+$ $V\beta8^+$ cells following SEB injection indicated a progressive increase in the number of cell divisions between 36 and 72 h, when a fourth (and to a lesser extent fifth) division peak was observed (Fig. 3*A*). No additional cell division was detected between 72 and 96 h. Quantitation of the proportion of $CD4^+$ $V\beta8^+$ cells in each CFSE division peak (Fig. 3*B*) confirmed that SEB-induced proliferation progressed from 36 to 72 h and subsequently ceased. Several points can be made from these data. First, there is a clearly defined population of undivided $CD4^+$ $V\beta8^+$ cells that is detectable even at the latest timepoints. Indeed, we calculate (see *Materials and Methods* for details) that the proportion of undivided cells in the draining LN at 3–4 days after challenge with SEB corresponds to 35–45% ($n = 7$) of the initial $CD4^+$ $V\beta8^+$ population, confirming our previous estimates obtained with continuous 5-bromodeoxyuridine labeling (6). The presence of such a substantial fraction of undivided cells expressing a SEB-specific $V\beta$ domain cannot be attributed simply to a failure to contact SEB, because virtually all (>95%) $V\beta8^+$ T cells underwent CD62 ligand down-regulation and CD69 up-regulation following SEB injection (Ref. 25 and our unpublished observations). Whatever the explanation, it is likely that these undivided cells represent the so-called "anergic" population that remains 7–10 days after SEB challenge, because our previous studies have demonstrated that virtually all dividing cells eventually die after SEB stimulation (6).

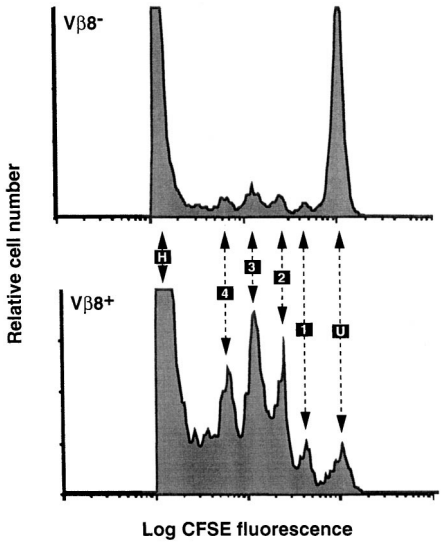

FIGURE 2. Example of CFSE fluorescence in T cells following SEB injection in vivo. Splenocytes were labeled with CFSE and transferred i.v. into syngeneic BALB/c recipients. At 3 days following injection with SEB, CFSE fluorescence in draining LN cells was analyzed after gating on $CD4^+$ $V\beta8^+$ or $CD4^+$ $V\beta8^-$ cells. Boxed numbers refer to respective division peaks; "U" refers to undivided cells; "H" refers to host-derived, CFSE-unlabeled cells. To increase the proportion of $CFSE^+$ cells in the analysis, most cells in peak "H" were electronically eliminated using FL-1 threshold. The mean fluorescence values for the five CFSE peaks from right to left are 1036, 441, 236, 125, and 63, respectively.

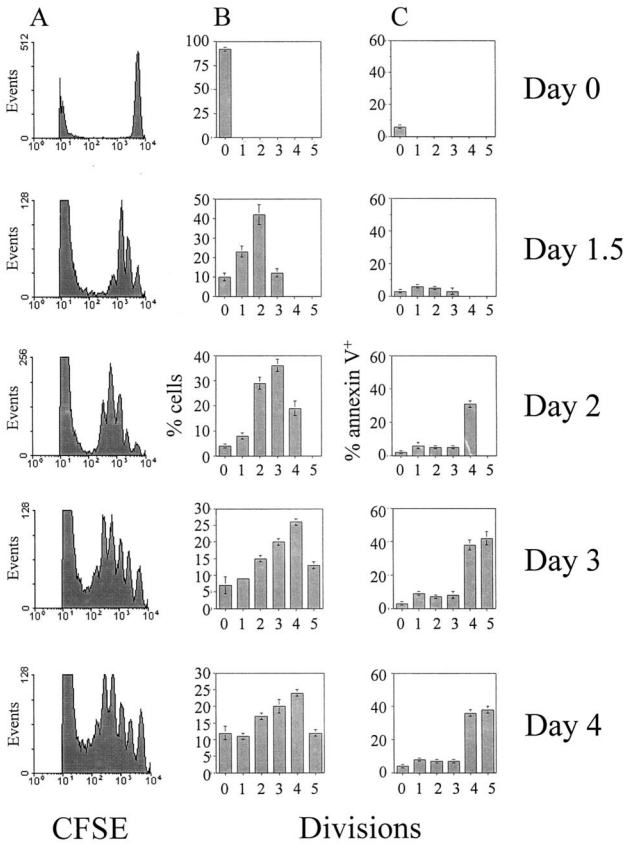

FIGURE 3. Correlation of cell division with apoptosis in SEB-activated $CD4^+$ $V\beta8^+$ T cells. Splenocytes were labeled with CFSE and transferred i.v. into syngeneic BALB/c recipients. At the indicated timepoints following injection with SEB, draining LN cells were analyzed for CFSE fluorescence (*A*), the percentage of total viable cells in each CFSE division peak (*B*), or the annexin V^+ cells in each CFSE division peak (*C*) after gating on $CD4^+$ $V\beta8^+$ cells. The data in *A* are a representative example, whereas *B* and *C* represent the mean ± SD of three individual mice per group.

A second important point arising from the CFSE distribution patterns in Figs. 2 and 3 is that $CD4^+$ $V\beta8^+$ cells are widely distributed among the division peaks at all timepoints after SEB injection. This in turn implies that there is considerable heterogeneity at the single-cell level in the SEB response in vivo. This heterogeneity in cell division among SEB-reactive cells could arise in any of the following ways: 1) asynchrony in contacting SEB and initiating the response, 2) asynchrony in reaching the first mitosis, 3) variations in cell cycle time among dividing cells, 4) variations in cell loss/apoptosis during different division cycles. Because cell cycle parameters are difficult to assess in asynchronous and nonhomogeneous cell populations, we decided to concentrate our attention on apoptosis.

Annexin V is a ligand of phosphatidylserine that, in living cells, is stringently located in the membrane leaflets that face the cytosol. During the early stages of apoptosis, the cell membrane loses asymmetry and phosphatidylserine becomes exposed on the cell surface, where it functions as a tag to signal phagocytes, which then clear the dying cell (20–22). To directly assess the relationship between apoptosis and cell division in SEB-activated T cells, we evaluated annexin V binding to the surface of dividing $CD4^+$ $V\beta8^+$ T cells in the draining LN at various times. For these experiments, activated T cells were incubated in vitro (1 h at 37°C) as described previously to allow apoptosis to occur (6, 26). As shown in Fig. 3*C*, there was a relatively low frequency of apoptotic cells associated with the first three rounds of division irrespective of the time after SEB injection. In contrast, there was a striking increase in the number of apoptotic cells in CFSE peaks corresponding to $CD4^+$ $V\beta8^+$ cells that had divided four or five times. Importantly, the association of apoptosis with division peaks 4 and 5 was observed independently of the time after SEB injection, demonstrating that the number of cell divisions (rather than kinetic parameters) was responsible for this phenomenon. It should be noted that the actual number of cell divisions correlating with the induction of apoptosis was constant within a given experiment but varied slightly (usually by no more than one cycle) from one experiment to another.

In conclusion, our data suggest that upon activation by superantigens, T cells are programmed to undergo a fixed number of cell divisions before undergoing apoptosis. The molecular mechanism underlying such a delayed death program in activated T cells remains to be established. One possibility would be that some long-lived inhibitor of apoptosis is induced early after activation and gradually diluted out during subsequent cell divisions, leading eventually to apoptosis. Alternatively, a critical death effector molecule may be induced (or up-regulated) specifically after a fixed number of cell divisions. Finally, it is possible that apoptosis in this model is not causally linked to cell division but rather to some other parameter (such as activation state) that correlates with division status. Whatever the mechanism, it is tempting to speculate that apoptosis is regulated in this way so as to allow T cells to expand and perform their effector functions, while at the same time ensuring homeostasis.

References

1. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell 63:1249.*
2. MacDonald, H. R., S. Baschieri, and R 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.*
3. Wahl, C., T. Miethke, K. Heeg, and H. Wagner. 1993. Clonal deletion as a direct consequence of an in vivo T cell response to bacterial superantigen. *Eur. J. Immunol. 23:1197.*
4. 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.*
5. Huang, L., G. Soldevila, M. Leeker, R. Flavell, and I. N. Crispe. 1995. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. *Immunity 1:741.*
6. Renno, T., M. Hahne, and H. R. MacDonald. 1995. Proliferation is a prerequisite for bacterial superantigen-induced T cell apoptosis in vivo. *J. Exp. Med. 181: 2283.*
7. Kawabe, Y., and A. Ochi. 1991. Programmed cell death and extrathymic reduction of $V_\beta8^+$ $CD4^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. *Nature 349:245.*
8. Gonzalo, J. A., C. Martinez, T. A. Springer, and J. C. Gutierrez-Ramos. 1995. ICAM-1 is required for T cell proliferation but not for anergy or apoptosis induced by *Staphylococcus aureus* enterotoxin B in vivo. *Int. Immunol. 7:1691.*
9. Nagata, S. 1994. Fas and Fas ligand: a death factor and its receptor. *Adv. Immunol.57:129.*
10. Tartaglia, L. A., and D. V. Goeddel. 1992. Two TNF receptors. *Immunol. Today 13:151.*
11. Boussiotis, V. A., B. J. Lee, G. J. Freeman, J. G. Gribben, and L. M. Nadler. 1997. Induction of T cell clonal anergy results in resistance, whereas CD28-mediated costimulation primes for susceptibility to Fas- and Bax-mediated programmed cell death. *J. Immunol. 159:3156.*
12. Hornung, F., L. Zheng, and M. J. Lenardo. 1997. Maintenance of clonotype specificity in CD95/Apo-1/Fas-mediated apoptosis of mature T lymphocytes. *J. Immunol. 159:3816.*
13. Hodgkin, P. D., J. H. Lee, and A. B. Lyons. 1996. B cell differentiation and isotype switching is related to division cycle number. *J. Exp. Med. 184:277.*
14. Gett, A. V., and P. D. Hodgkin. 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc. Natl. Acad. Sci. USA 95:9488.*
15. Lee, W. T., and W. J. Pelletier. 1998. Visualizing memory phenotype development after in vitro stimulation of $CD4^+$ T cells. *Cell. Immunol. 188:1.*

16. Oehen, S., and K. Brduscha-Rhiem. 1998. Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. *J. Immunol. 161:5338.*
17. Bird, J. J., D. R. Brown, A. C. Mullen, N. H. Moskowitz, M. A. Mahowald, J. R. Sider, T. F. Gajewski, C. R. Wang, and S. L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity 9:229.*
18. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods 171:131.*
19. Staerz, U. D., H. Rammensee, J. Bendetto, and M. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. *J. Immunol. 134:3994.*
20. Martin, S. J., C. P. M. Reutelinsperger, A. J. Mcgahon, J. A. Rader, R. C. A. A. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of bcl-2 and abl. *J. Exp. Med. 182:1545.*
21. Verhoven, B., R. A. Schlegel, and P. Williamson. 1995. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med. 182:1597.*
22. Thiagarajan, P., and J. F. Tait. 1990. Binding of annexin V/placental anticoagulant protein I to platelets: evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J. Biol. Chem. 256:17420.*
23. MacDonald, H. R., R. K. Lees, S. Baschieri, T. Herrmann, and A. T. Lussow. 1993. Peripheral T-cell reactivity to bacterial superantigens in vivo: the response/anergy paradox. *Immunol. Rev. 133:105.*
24. Scott, D. E., W. J. Kisch, and A. D. Steinberg. 1993. Studies of T cell deletion and T cell anergy following in vivo administration of SEB to normal and lupus-prone mice. *J. Immunol. 150:664.*
25. Miethke, T., C. Wahl, B. Holzmann, K. Heeg, and H. Wagner. 1993. Bacterial superantigens induce rapid T cell receptor Vβ-selective down-regulation of L-selectin (gp90^{Mel-14}) in vivo. *J. Immunol. 151:6777.*
26. Renno, T., M. Hahne, J. Tschopp, and H. R. MacDonald. 1996. Peripheral T cells undergoing superantigen-induced apoptosis in vivo express B220 and upregulate Fas and Fas ligand. *J. Exp. Med. 183:431.*