Cutting Edge: Contributions of Apoptosis and Anergy to Systemic T Cell Tolerance
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J Immunol 2008; 180:2762-2766; doi: 10.4049/jimmunol.180.5.2762
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Multiple mechanisms contribute to peripheral T cell tolerance, including functional unresponsiveness (anergy), cell death (deletion), and suppression by regulatory T cells. It is unclear whether, or how, these mechanisms interact with one another. Recent work has been devoted to defining the molecular pathways of each of these mechanisms of tolerance and the relationships between them. For instance, it is possible that anergy is a necessary prelude to cell death or the development of peripheral regulatory T cells (Treg). Alternatively, each mechanism may lead to independent fates of cells that encounter self-Ags. The goal of our study was to explore the roles of anergy and apoptosis in a model amenable to detailed cellular analysis.

T cell anergy is associated with an altered and attenuated response to Ag stimulation. In vivo, anergy commonly represses proliferation and cytokine production and is maintained by continuous Ag exposure (1–7). Different mechanisms of anergy have been demonstrated in various experimental systems; these include a block in TCR signaling, the activation of ubiquitin ligases, and the engagement of inhibitory receptors (5, 8). Because anergic T cells cannot produce their own IL-2 and other survival factors, anergy may lead to apoptosis.

Lymphocytes that encounter self-Ags may be deleted through two convergent apoptotic pathways. One is initiated by the engagement of death receptors such as Fas. The other is the mitochondrial pathway, triggered by a family of sensors sharing only the BH3 domain of Bcl-2, and regulated by Bcl-2 and related prosurvival proteins (9, 10). One sensor, Bim, eliminates activated T cells in response to TCR engagement and cytokine deprivation (10). What causes Bim to kill T cells remains a puzzle and may involve changes in Bim expression and phosphorylation, changes in the levels of anti-apoptotic Bcl-2 family members, and the activation of other BH3-only sensors (10–12).

Our goal was to separate apoptosis from anergy and define the contribution of each control mechanism to systemic T cell tolerance. We previously characterized the response of OVA-specific DO11.10 CD4+ T cells transferred into soluble OVA transgenic (sOVA Tg) BALB/c recipient mice, where the OVA protein is encountered as a systemic self-Ag (13). DO11 T cells divide after transfer but rapidly become anergic and are deleted, and the surviving cells are hyporesponsive when restimulated (6). These characteristics of systemic T cell tolerance have also been reported in a different system (14).

We find that if DO11 cells lack Bim they are not deleted in sOVA Tg recipients, indicating that the major pathway of deletion is the mitochondrial apoptotic pathway. However, despite surviving chronic self-Ag exposure, these T cells become anergic. By separating apoptosis and anergy we demonstrate that cell-intrinsic anergy is sufficient to preserve tolerance to a systemic Ag.

**Materials and Methods**

**Mice**

All studies were reviewed and approved by the Committee on Animal Research (University of California, San Francisco, CA). All transgenic and knockout mice were backcrossed more than eight generations to BALB/cAnNCrl and The Jackson Laboratory (BALB/cJ). sOVA transgenic mice (Tg(metallothionein-I-OVA)Akab) have been described (6, 13). DO11.10 TCR transgenic mice (C57Bl/6J Tg[DO11.10]10Dlo/J) producing CD4+ T cells specific for chicken OVA

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Bim-/−Bim-/−sOva Tg

were obtained from Dr. K. Murphy (Washington University, St. Louis, MO) via The Jackson Laboratory. Thy1.1 congenic mice (CBy.PL(B6)-Thy1.2/ScrJ) were provided by Dr. R. Locksley (University of California, San Francisco, CA). B6.129-Bcl2l11tm1.1Ast (Bim−/−) mice (15) were obtained from Dr. A. Strauss (University of Melbourne, Melbourne, Australia).

Abs and flow cytometry

The following Abs were purchased from BD Biosciences unless otherwise noted: anti-B7.2 (clone GL1), anti-Bcl-2 (clone FJK-16s), anti-I-Ad (clone AMS-32.1), and anti-Thy1.1 (clone OX-7). Flow cytometry was done on a FACSCalibur device with CellQuest software (BD Biosciences). To detect Bcl-2 and Bcl-xL, cells were fixed and stained overnight in Perm/Wash buffer (BD Biosciences). Foxp3 was detected using the Foxp3 staining set (eBioscience).

Cell preparation, adoptive transfer, purification, and culture

DO11 donor cells were prepared from lymph nodes and spleens magnetically enriched for CD4+ cells using Dynabead (Dynal-Invitrogen) or EasySep (Stem Cell Technologies) reagents. Donor cells were labeled with 1–2.5 μM CFSE (Invitrogen Life Technologies) for 10 min and suspended in PBS for i.v. adoptive transfer of 3.5–6 × 106 total DO11 cells per recipient. OVA peptide-pulsed dendritic cells were generated as described (13) and 3 × 106 I-Ad+ high B7.2low, CD44high, CD62Llow, and OX-40low CD4+ cells were used to detect BrdU. Thy1.2 cells were provided by Dr. K. Murphy (Washington University, St. Louis, MO) via The Jackson Laboratory. Thy1.1 congenic mice (CBy.PL(B6)-Thy1a−/−/H11002) were obtained from Dr. K. Murphy (Washington University, St. Louis, MO).

Quantitative RT-PCR

cDNA was obtained from purified DO11 cells using TRizol and the SuperScript III kit (Invitrogen Life Technologies) and detected using SYBR Green (Applied Biosystems) and the Opticon 2 system (Bio-Rad Laboratories). Relative gene expression was determined by the critical threshold and normalization to hypoxanthine phosphoribosyltransferase (HPRT). The following PCR primers were used: Bcl-2 (5′-AGTACC-TGAACC-GGCATC-TG/GCT GAG-CAGGGT-CTTCAG-AG-3′), Bcl-x (5′-GCCGTGGG-ACCAT-CTG TGG-AT/AAGAGT-GAGCCC-AGCAGA-AC-3′), and HPRT (5′-GGCGA-GGATTT-GGAAAA-AGTG/CACAGA-GGGCCA-CAATGT-3′).

BrdU labeling and intracellular staining

Mice were given i.p. injections of 1 mg BrdU in PBS on days 0, 1, 2, 4, and 6 beginning day 0 or 14 after DO11 cell transfer. Cells were stained for surface Ag, fixed, and frozen (protocol option 2; BD Biosciences). Thawed cells were incubated for 60 min at 37°C in PBS supplemented with 10 μM HCl, 150 mM NaCl, 4.2 mM MgCl2, and 250 Kunitz units/ml DNase I (Sigma-Aldrich) to digest DNA before staining to detect BrdU.

Results

Deletion of T cells encountering a systemic self-Ag depends on Bim

To define the mechanisms of T cell deletion induced by a systemic self-Ag, we cotransferred DO11 wild-type (WT) Thy1.1+ and Bim−/−/H11002 Thy1.2−/−/H9251 cells into BALB/c or sOVA Tg recipients and followed the fates of the two T cell populations. Both cell populations exhibited a naive phenotype before transfer (not shown). The DO11 WT and Bim−/−/H11002 populations engulfed equally, and by day 4 they displayed similar proliferation in sOVA Tg mice as shown by CFSE profiles (Fig. 1A). WT and Bim−/−/H11002 DO11 cells were also equivalently CD69+ (Fig. 1B), IL-7Rαlow, CD44high, CD62Llow, and OX-40low on day 4 in sOVA Tg mice (not shown). However, compared with their WT counterparts, Bim−/−/H11002 DO11 cells showed much less deletion in sOVA Tg mice and, over time, the ratio of the surviving populations increasingly favored Bim−/−/H11002 cells (Fig. 1, B and C).

In fact, the number of Bim−/−/H11003 DO11 cells remained stable as long as 8 wk after transfer (not shown). We observed similar
enhanced survival of Bim−/− vs WT DO11 cells when the two populations were transferred into separate sOVA Tg recipients (not shown).

**Apoptosis proceeds despite increases in Bcl-2 and Bcl-x**

Death by the mitochondrial pathway may reflect the loss of pro-survival Bcl-2 family members, and failure to induce these proteins following self-Ag recognition is a basis of deletion (16, 17). To test this, we followed the expression of Bcl-2 and Bcl-x following T cell encounter with systemic self-Ag. Relative to naive controls, DO11 cells in sOVA Tg recipients expressed higher levels of Bcl-2 and Bcl-x mRNA (Fig. 2A). Within 1 day of transfer, DO11 cells expressed more Bcl-2 and Bcl-x protein in sOVA Tg than BALB/c recipients (Fig. 2B). Elevated Bcl-2 and Bcl-x expression was sustained through day 7, by which time most WT cells had been deleted. DO11 Bim−/− cells also expressed more Bcl-2 and Bcl-x in sOVA Tg than BALB/c recipients (not shown). Thus, Bim-dependent apoptosis in the sOVA transgenic system is not associated with a failure to express Bcl-2 or Bcl-x.

**Anergy is induced even if deletion is prevented**

It is possible that T cells surviving encounter with self-Ag also retain their functional responsiveness. To test this, we assayed the responses of DO11 T cells transferred into sOVA Tg recipients and, for comparison, into untreated BALB/c mice and BALB/c mice immunized with OVA peptide-pulsed dendritic cells. T cells were purified 5 days after transfer and restimulated in vitro. Both DO11 WT and Bim−/− cells from sOVA transgenic mice were impaired in IL-2 production, failed to make IL-4, IFN-γ, or IL-10, and proliferated no better than naive cells (Fig. 3, and data not shown).

A stringent test of anergy is the proliferation of T cells in vivo after encounter with systemic Ag. We first noticed cell cycle arrest of DO11 cells in sOVA Tg recipients when comparing CFSE dilution beyond 4 days after transfer (Fig. 1A, compare days 4 and 13). To better define cell division, Bim−/− DO11 cells were transferred into BALB/c or sOVA Tg recipients and treated with BrdU. Bim−/− DO11 cells stained BrdU− and therefore had entered the cell cycle during week 1 but not week 3 following transfer into sOVA Tg mice (Fig. 4). Thus, the surviving T cells fail to proliferate in the presence of the systemic Ag.

We also monitored the sOVA Tg recipients of Bim−/− DO11 cells up to 8 wk for signs of autoimmune disease. Anti-OVA IgG Abs were essentially undetectable (not shown). DO11 donor cells cause a rapid wasting disease and severe skin inflammation in RAG-deficient sOVA Tg recipients in which tolerance has failed (18). However, Bim-deficient DO11 cells caused neither symptom in WT sOVA Tg mice.
Anergy does not depend on differentiation into regulatory T cells

It is possible that the long-lived unresponsiveness of T cells in the presence of systemic Ag is related to the induction of Treg. To address this, we determined whether WT or Bim-/- DO11 cells differentiated into Foxp3-expressing Tregs after transfer into sOVA Tg mice. Naive KJ1-26+ T cells from conventional DO11 mice are 2–3% Foxp3+, presumably because of the expression of a second, self-reactive TCR (19, 20). At 4 days after transfer there is an increase in the percentage of both WT and Bim-/- DO11 cells expressing Foxp3 (Fig. 5). However, in the surviving Bim-/- population the percentage of Foxp3+ DO11 cells returned to the naive level. Foxp3 was also induced when RAG-deficient DO11 cells were transferred into sOVA Tg mice (not shown). Thus, transient expression of Foxp3 may be a feature of self-Ag recognition, but the development of stable Tregs cannot explain the long-lived unresponsiveness of T cells in the presence of the systemic Ag. 

Discussion

T cell anergy and deletion preserve tolerance to self-Ags. In this study we show that anergy and deletion are separable mechanisms and tolerance can be maintained even if deletion is prevented. When T cells encounter systemic self-Ag in the sOVA Tg recipients, deletion of the cells depends on Bim and occurs despite increased Bcl-2 and Bcl-x. In contrast, Bim-mediated deletion by superantigen is linked to a loss of Bcl-2 (16, 17). This difference may result from different pathways of Bim activation. In sOVA Tg mice, repetitive TCR stimulation could activate Bim by qualitatively changing the protein, perhaps by mRNA splicing or phosphorylation (11), to overcome the increased expression of Bcl-2 and Bcl-x. Superantigen-mediated deletion could instead activate the Bim pathway in the same way as cytokine withdrawal, reducing the expression of Bcl-2 and relieving the inhibition of unmodified Bim (10).

Without Bim, thymic and peripheral T cells resist deletion, lymphocyte homeostasis breaks down, and class-switched autoantibodies are produced (15, 16, 21–24). However, Bim is not required to prevent overt autoimmune disease because Bim-/- C57BL/6 mice do not exhibit increased mortality (24). Thus, Bim is required at the cellular level to delete self-reactive lymphocytes and maintain immune homeostasis, but Bim-mediated deletion is not necessary for immune tolerance if independent mechanisms, such as anergy or suppression by Tregs, compensate for impaired apoptosis.

Besides targeting the Bim pathway, chronic self-Ag exposure induces cell-intrinsic anergy. Repeatedly encountering Ag can desensitize T cells (1, 3–5, 7, 14, 25, 26), and DO11 cells developed a defect in calcium mobilization upon anti-CD3 Ab stimulation within 3 days of transfer into sOVA Tg mice (6). The phenotype of tolerant DO11 Bim-/- cells, namely loss of IL-2 and proliferation, failure to produce effector cytokines, and aberrant calcium response, are commonly shared characteristics of anergy in many systems (5, 25) and could be explained if E3 ubiquitin ligases maintain anergy by degrading key components of the TCR signaling pathway (8).

It is also possible that tolerance is maintained by the generation of Ag-specific Tregs. However, Foxp3 is induced only transiently in DO11 cells following transfer into sOVA Tg recipients, and stable populations of Foxp3+ T cells do not emerge. It may be that T cells encountering systemic self-Ag rapidly become anergic and fail to complete the Treg differentiation program. This hypothesis predicts that some degree of activation is a necessary prelude to peripheral Treg generation. Therefore, anergy and Treg development may be alternative fates of T cells that are exposed chronically to a self-Ag. These results indicate that differentiation into Tregs cannot explain the unresponsiveness of the surviving DO11 Bim-/- population and the absence of autoimmune pathology.

The experimental system of a single T cell population encountering a systemic Ag has allowed us to dissect the requirements for triggering different mechanisms of self-tolerance and the relative contributions of these mechanisms. We find that deletion is mediated by the Bim-dependent mitochondrial pathway of apoptosis and can be significantly reduced by the genetic elimination of Bim. However, prolonged survival alone does not lead to a failure of self-tolerance, because the cells become functionally anergic. Thus, anergy and apoptosis are separable fates of T cells that encounter self-Ag.

Acknowledgments

We thank Dr. A. Strasser for Bim-/- mice. We are indebted to S. Jiang for expert cell sorting and C. Benitez for mice husbandry. We thank Dr. C. Allen for technical advice and members of the Abbas laboratory for helpful discussions.
Disclosures
The authors have no financial conflict of interest.

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