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Anergy in CD4 memory T lymphocytes. II. Abrogation of TCR-induced formation of membrane signaling complexes

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ABSTRACT

Memory and naive CD4 T cells have unique regulatory pathways for self/non-self discrimination. A memory cell specific regulatory pathway was revealed using superantigens to trigger the TCR. Upon stimulation by bacterial superantigens, like staphylococcal enterotoxin B (SEB), TCR proximal signaling is impaired leading to clonal tolerance (anergy). In the present report, we show that memory cell anergy results from the sequestration of the protein tyrosine kinase ZAP-70 away from the TCR/CD3ζ chain. During SEB-induced signaling, ZAP-70 is excluded from both detergent-resistant membrane microdomains and the immunological synapse, thus blocking downstream signaling. We also show that the mechanism underlying memory cell anergy must involve Fyn kinase, given that the suppression of Fyn activity restores the movement of ZAP-70 to the immunological synapse, TCR proximal signaling, and cell proliferation. Thus, toleragens, including microbial toxins, may modulate memory responses by targeting the organizational structure of memory cell signaling complexes.

1. Introduction

Upon exposure to foreign antigen, T lymphocytes are induced into clonal expansion and differentiation to become "antigen-experienced" cells (e.g. effector and memory T cells) [1,2]. For both naive and memory CD4 T cells, stimulation through the TCR by peptide-MHC complexes involves multiple signal transduction pathways. Further, depending upon the nature of the initial signal (e.g. foreign versus self-antigen, superantigen, anti-TCR Abs), different signaling pathways may be used and different functional outcomes (e.g. cell activation, proliferation, or tolerance) may result [3–8]. Regardless of the stimulus, cell signaling is tightly regulated, in part through the defined organization of signaling molecules into complexes both on the T cell membrane (e.g. lipid raft microdomains) [9–15], and, also, at the T cell-APC interface (e.g. immunological synapses) [16–20]. Studies using primary T cells (e.g. naive, memory) or cloned effector T cell lines (e.g. Th1, Th2) showed that membrane organizational structures may be distinct, depending upon the specific T cell differentiation state [21–24]. However, the relationship of different membrane signaling complexes to specific cell function is unclear. Likewise, stimulatory or tolerogenic signals may lead to different signaling structures. For example, stimulation of resting T cells by agonist peptides promotes the formation of paradigm immune synapses, while tolerogenic peptides lead to incomplete synapses [18,25], where some critical signaling molecules move to the central supramolecular activation cluster (c-SMAC), whereas other critical molecules are excluded from the synapse.

We have previously examined responses of naive and memory CD4 T cells after stimulation by peptide antigens and microbial superantigens. While either stimulus elicited robust proliferation and cytokine secretion by naive cells, only peptide antigen promoted activation of resting memory CD4 cells [26]. In contrast, superantigens, such a Staphylococcal enterotoxin B (SEB)[1] did not stimulate resting memory cells [26]. Indeed, SEB induced memory cells to become anergic, indicated by a failure of the cells to proliferate when subsequently exposed to an agonist peptide [8]. This observation supported the hypothesis that, after naive cells differentiate into memory cells, unique regulatory pathways are utilized. Specific memory cell regulation could facilitate both enhanced responses to recall antigens and, also, prevent untoward responses to self-antigens encountered by high avidity memory cells that traffic through tissues [2]. Exposure of memory cells to SEB revealed an anergy pathway that was characterized by impaired signaling through the TCR/CD3 complex [7]. Normally, the earliest signaling events during

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[1] *Abbreviations used:* SEB, staphylococcal enterotoxin B; SMAC, supramolecular activation cluster; TX-100, Triton X-100; CT-B, cholera toxin-B subunit.

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stimulation through the TCR include tyrosine phosphorylation of the TCR CD3ζ chain by the src kinase Lck, and, recruitment of the protein tyrosine kinase ZAP-70 to the plasma membrane to bind to pCD3ζ, so that ZAP-70 can then be phosphorylated and activated by Lck [27]. However, we found, during co-immunoprecipitation experiments, that when memory CD4 cells were exposed to SEB, there was a failure of association between the activated pCD3ζ molecule and ZAP-70 [7]. Hence, there was an absence of tyrosine phosphorylation of ZAP-70 and further downstream signaling was blocked. Additional studies showed that a specific hyperactivation of the src kinase Fyn was essential to SEB-induced memory cell anergy, as the inhibition or absence of Fyn restored both CD3ζ/ZAP-70 complex formation and cell activation [28].

Given that SEB promoted CD4 memory cell anergy with similar proximal signaling deficiencies as found in other tolerance models (e.g. inactive ZAP-70 [29], Fyn kinase involvement [30]), and given that a failure of ZAP-70 recruitment was a controlling element in SEB-induced anergy, we hypothesized that productive signaling complexes were not formed when SEB was presented to memory T cells. In an earlier study we examined TCR signaling in immune synapses and lipid raft microdomains of memory cells presented with cognate peptide [22]. We identified several features that were distinct from the membrane signaling structures of naive cells responding to the same cognate peptide, suggesting structural bases for alternative activation pathways. In the present study, we investigated whether SEB could mediate a productive interaction between memory T cells and APCs and whether complete immunological synapses were formed. Since our earlier study showed that ZAP-70 did not bind to the TCR/CD3 complex [7], our current study specifically centered upon whether ZAP-70 localized to the same membrane regions as the TCR, when memory cells became exposed to SEB versus peptide antigen. We found that, in contrast to peptide stimulation, exposure of memory, but not naive, CD4 T cells to SEB resulted in the absence of ZAP-70 from both the immune synapse and, also, lipid rafts, suggesting that ZAP-70 and the TCR were physically separated. Given that the absence of Fyn did allow for ZAP-70 to migrate to the immune synapse, we conclude that SEB induces Fyn signaling which in turn leads to sequestration of ZAP-70 from the membrane compartments that contain the TCR, and thus prevents proximal signaling.

2. Materials and methods

2.1. Animals

The BALB/c ByJ, DO11.10 [31], and DO11.10 x $Fyn^{-/-}$ mice used in these experiments were bred and maintained at the Wadsworth Center Animal Core Facility under specific pathogen-free conditions. The majority of T cells in the DO11.10 and DO11.10 × $Fyn^{-/-}$ mice are $CD4^+$ cells, which bear a TCR that recognizes a chicken ovalbumin-derived peptide, $OVA_{323-339}$ (hereafter referred to as OVA), presented by $I\text{-}A^d$ [31]. This TCR is encoded by transgenes encoding Vβ8.2/Vα13.1 chains and can be identified by the anti-clonotypic mAb, KJ1-26 [32]. We have previously characterized the DO11.10 memory cells and their responses to peptide antigen and superantigen [26,33]. $Fyn^{-/-}$ mice [34] were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and backcrossed over 11 generations onto a BALB/c background, before they were bred to DO11.10 mice, to generate DO11.10 × $Fyn^{-/-}$ mice. For all experiments, cells were obtained from mice that were 10–12 weeks old. Cells from either male or female mice were used, in different experiments, with no discernible differences in the results. All mice used in these studies were bred and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Washington, DC). All experiments were approved by the Wadsworth Center IACUC.

2.2. Reagents and antibodies

Mabs KJ1-26 (anti-DO11.10 clonotype) [32] and 23G2 (anti-CD45RB) [35] were prepared from the supernatants of hybridoma cell lines, as previously described. Additional antibodies and probes used for confocal microscopy were cholera toxin-B-rhodamine (CT-B-rhodamine) conjugate (List Biological Laboratories, Campbell, CA), anti-phospho-ZAP-70 (Tyr319) (Cell Signaling Technology, Beverly, MA), and anti-ZAP-70, goat anti-mouse IgG-HRPO, and goat anti-rabbit IgG-HRPO (Transduction Laboratories, San Diego, CA). The rabbit polyclonal Ab directed against phosphorylated Lck (pY394) [19] was obtained from A. Shaw (Washington University, St. Louis, MO). OVA peptide was synthesized and supplied by the Wadsworth Center Peptide Synthesis Core Facility. SEB (Toxin Technology, Sarasota, FL) was purchased.

2.3. Preparation of cells

In all experiments, enriched populations of $CD4^+$ T cells were prepared by negative selection procedures as previously described [36], and were 90–95% $CD4^+$ and <3% sIg^+, as determined by flow-cytometric analyses. Naive and memory cells were separated as previously described [8] based upon CD45RB expression using mAb 23G2 supernatant and MACS (Miltenyi Biotec, Auburn, CA). There were no discernible differences between DO11.10 and DO11.10 × $Fyn^{-/-}$ mice, in percentage of splenic cell populations, in number of total $CD4^+$ cells, or in populations of naive and memory cells. OVA and SEB were presented to T cells using APCs prepared by T cell depletion of splenocytes using anti-Thy1-1.2 and complement, followed by anti-CD4 (mAb 2B6) and anti-CD8 plus complement [26].

2.4. Cell labeling and culture

For measurement of cell division using CFSE [37], DO11.10 and DO11.10 × $Fyn^{-/-}$ CD4 cells were labeled with CFSE (5 μM) prior to separation into naive and memory populations. DO11.10 naive and memory cells (1×10^5/well) were cultured in 96-well flat-bottom plates with APCs (2×10^5/well) in 0.2 ml RPMI-1640 medium supplemented with 10% FBS, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Where indicated, SEB (20 μg/ml) or $OVA_{323-339}$ (0.2 μg/ml) was added to the cultures. After 66 h, the T cells were analyzed by flow cytometry, after staining with mAb KJ1-26, to identify the DO11.10 clonotype-bearing cells.

2.5. Confocal microscopy

DO11.10 and DO11.10 × $Fyn^{-/-}$ CD4 naive and memory cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 μM 2-Me, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Where indicated, the TCR within conjugates was identified by staining with mAb KJ1-26, to identify the DO11.10 clonotype-bearing cells. Conjugates between T cells and APCs were formed by mixing of T cell and SEB-pulsed (20 μg/ml) or OVA-pulsed (1.0 μg/ml) APCs at a 1:2 ratio, with a brief centrifugation at 400*g* to initiate cell–cell contact [19]. Cells were incubated at 37 °C, under 5% CO_2, for various time intervals. The cells were fixed with freshly prepared 4% paraformaldehyde for 20 min at room temperature and allowed to adhere to poly-L-lysine coated slides at 4 °C overnight or for 2 h at 37 °C [19]. Cells were permeabilized with 0.2% TX-100, blocked with 1% BSA/PBS, and stained with appropriate antibodies for 1 h diluted in 1% BSA/