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A unique unresponsive CD4+ T cell phenotype post TCR antagonism

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ABSTRACT

The functional outcomes of the T cell's interaction with the peptide:MHC complex can be dramatically altered by the introduction of a single amino acid substitution. Previous studies have described the varied effects of these altered peptide ligands (APL) on T cell responses. These outcomes of T cell interaction with an APL include the induction of clonal unresponsiveness (anergy) and inhibition of T cell responses (antagonism). The phenotype of peptide-induced anergy, i.e. low proliferation and low IL-2 production, has been extensively described, and a number of groups have demonstrated antagonism. However, the response of T cells to an agonist ligand after encountering an antagonistic stimulus has not been previously characterized. Here, we show that T cells post-antagonism fail to proliferate but produce large quantities of IL-2 upon stimulation with their wild type ligand. This unique phenotype is not due to differences in IL-2 receptor expression or rates of apoptosis, and cannot be overcome by the addition of recombinant IL-2. The response of CD4 T cells to agonist stimulation after encountering an antagonist is a novel phenotype, and is distinct from previously described forms of anergy.

1. Introduction

A T cell response is elicited following interaction of the T cell receptor (TCR) with its cognate ligand. The outcome of this interaction can be affected by introducing changes in the immunogenic peptide sequence [1,2]. Peptides in which amino acid substitutions have been made at a TCR contact residue are known as altered peptide ligands (APL) [1]. APL have an affinity for MHC similar to that of the wild type peptide and are classified based on the potency of the T cell response they elicit. Agonist peptides stimulate T cells to levels similar to that of the wild type peptide, while weak agonists stimulate suboptimal proliferative and cytokine responses. Partial agonists stimulate only a subset of effector functions [1]. This class of APL has been used to induce anergy, a state of unresponsiveness defined by a lack of proliferation and IL-2 production in response to an immunogenic stimulus [1]. An anergic phenotype can also be induced by lack of costimulation [3–5], a low dose agonist ligand [6], or unstable peptides containing substitutions at their MHC anchor residues [7,8].

In addition to partial agonists, which have been shown to induce T cell anergy, another category of APL that dampens the T cell response is antagonists. Antagonist ligands have been defined *in vitro* by their inability to stimulate T cell responses when presented alone and their ability to inhibit the T cell response to wild type peptide when both the antagonist and agonist are present [2,9,10]. Epitopes behaving as antagonists have been identified in a number of infections, including HIV [11,12], Hepatitis B and C [13,14] and malaria [15]. Additionally, antagonist ligands may play a role in maintaining peripheral tolerance [16].

Although the phenomenon of antagonism has been extensively described in a number of systems, the exact mechanism by which antagonism occurs remains unclear. Studies utilizing dual-receptor expressing T cells suggest that the mechanism is one of active inhibition, rather than a passive effect of competition [17–20]. Further studies have indicated that antagonism impacts the signaling cascades normally activated upon T cell stimulation, activating a negative feedback loop involving the phosphatase SHP-1 [21–23].

The response of a T cell under antagonist conditions is similar to the response of anergic cells to wild type stimulation. In both these conditions, T cells fail to proliferate and secrete little or no IL-2. To define the fate of these cells following rechallenge with agonist ligand has not been considered. We examine the phenotype of previously antagonized T cells in response to wild type stimulation. We have found that the phenotypes of anergy and antagonism differ dramatically when cells are rechallenged with wild type ligand post-antagonism. Both groups fail to proliferate upon stimulation with wild type peptide, but we demonstrate that previously antagonized cells make significant amounts of cytokines including IL-2. This distinct difference in cytokine production is a stark and important contrast between the phenotypes of antagonism and anergy. Thus, we describe a unique response pattern of T cells, a phenotype distinct from previously described forms of anergy.

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2. Materials and methods

2.1. Mice

3.L2 TCR transgenic mice were used at 6–12 weeks of age. The 3.L2 TCR is specific for Hb64-76 presented by I–E^k [24]. Mice were bred and housed in the Emory University Department of Animal Resources facility according to federal guidelines.

2.2. Peptides

The wild type peptide, Hb64-76 (GKKVITAFNEGLK) and the antagonist 72I (GKKVITAFIEGLK) were purchased from Invitrogen (Carlsbad, CA). 72I is a single amino acid substitution, Asn (N) → Ile (I) at position 72.

2.3. Antagonism

Antagonism was performed as previously described by Sette and colleagues [2]. Briefly, for antagonist conditions, cells were pre-pulsed with 1 μM Hb64-76 for 2 h at 37 °C, washed twice in HBSS, then cultured with 10 μM 72I or the indicated concentration of peptide for proliferation assays.

2.4. Cells and reagents

3.L2 spleen cells (2×10^6/well) were cultured *ex vivo* with 1 μM Hb64-76, pre-pulse only (1 μM Hb64-76 for 2 h, at 37 °C) or under antagonist conditions (1 μM Hb64-76 pre-pulse, 10 μM 72I continuously) with 10 pg/mL IL-2 for 7 days in 24-well plates. Live cells were purified over a Ficoll gradient (Mediatech) and were restimulated with irradiated syngeneic splenocytes (2000 rad) and the indicated concentrations of peptide. Culture media consisted of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, .01 M Hepes buffer, 100 μg/mL gentamicin (Mediatech, Herndon, VA) and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO).

2.5. Proliferation assay

Naïve 3.L2 splenocytes (3×10^5/well) or cultured T cells (5×10^4/well) and irradiated syngeneic splenocytes (2000 rad, 5×10^5/well) were cultured in 96-well plates with the indicated concentration of peptide at 37 °C. Where indicated, recombinant IL-2 (BD Pharmingen, San Diego, CA) was added at 3.5 ng/well. After 48 h in culture, 0.4 μCi/well of [^{3}H] thymidine was added. After an additional 18 h, cells were harvested on a FilterMate harvester (PerkinElmer Life and Analytical Sciences, Wellesly, MA) and analyzed on a Matrix 96 Direct Beta Counter (PerkinElmer).

2.6. Cytokine ELISA

After 1 week in culture under the indicated conditions, 3.L2 T cells (1×10^6/well) were stimulated with irradiated syngeneic splenocytes (2000 rad, 5×10^6/well) and the indicated concentration of peptide for 24 h (IL-2) or 48 h (IFN-γ). Supernatants were incubated in triplicate on microtiter plates that had been coated with 50 μl of purified anti-IL-2 (5 μg/mL, clone JES6-1A12, BD Pharmingen) or anti-IFN-γ (5 μg/mL, clone R4-6A2, BD Pharmingen) overnight at 4 °C. Recombinant IL-2 or IFN-γ (BD Pharmingen) was used as a standard. Captured cytokines were detected using biotinylated anti-IL-2 (JES6-5H4, BD Pharmingen; 100 μg/mL, 100 μl/well) or biotinylated anti-IFN-γ (clone XMG1.2, BD Pharmingen; 100 μg/mL, 100 μl/well) followed by alkaline phosphatase-conjugated avidin (Sigma, St. Louis, MO) and p-nitrophenylphosphate (pNPP) substrate (BioRad, Hercules, CA). Colorimetric change was measured at 405 nm on a Microplate Autoreader (Biotek Instruments, Winooski, VT).

2.7. Flow cytometry

3.L2 T cells were stained with anti-CD25 (IL-2Rα)-FITC (clone PC61), anti-Vβ8.3-PE (clone 1B3.3) and anti-CD4-APC (clone RM4-5; all BD Pharmingen) at 24 and 48 h after restimulation. Alternatively, cells were stained with PE conjugated CD122 (clone TM-b1) or CD132 (clone 4G3). For intracellular cytokine staining, cells were stimulated for 6 h, then fixed and permeabilized with Caltag Fix and Perm cell permeabilization kit per the manufacturers instructions (Caltag, San Diego, CA). Permeabilized cells were stained with anti-CD4-PerCP and anti-IL-2-APC (clone JES6-5H4) or anti-IL-4-APC (clone 11B11; all BD Pharmingen). Annexin V and 7-AAD staining was performed according to manufacturer's instructions (BD Pharmingen). Flow cytometry was performed on a BD FACSCalibur (Franklin Lakes, NJ) and data were analyzed using FlowJo Software (Tree Star, San Carlos, CA). Data are gated on Vβ8.3+ or CD4+ cells as indicated.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism. Statistical significance was determined by *t* test or ANOVA, as indicated in the figure legends.

3. Results

3.1. Characterization of antagonist peptide

The response of 3.L2 T cells to both Hb64-76 and the APL 72I has been previously described [24,25]. As expected, the antagonist peptide alone induced minimal proliferation of 3.L2 T cells, whereas stimulation with the wild type peptide resulted in a dose-dependent proliferative response (Fig. 1A, $p = 0.0184$). Additionally, cells pre-pulsed with the wild type peptide were effectively antagonized (>95%) by addition of 72I (Fig. 1B). Thus the APL 72I acts as an antagonist, as it fails to stimulate proliferation when presented alone, and inhibits the T cell response to wild type peptide in a dose-dependent manner when both wild type and 72I are presented.

3.2. Phenotype post-antagonism

Although T cell antagonism has been extensively described [10,17,19,26–28], the response of these antagonized cells to subsequent stimulation with wild type ligand has not yet been described. Previous studies have shown that under antagonist conditions, T cells exhibit a dose-dependent decrease in proliferation to wild type stimulus [10,17–19,26–28], altered cytokine production [1,29], and altered signaling [10,12,18]. In order to assess the effects of T cell antagonism on subsequent T cell function, we have characterized the proliferative and cytokine responses of previously antagonized T cells to rechallenge with wild type ligand.

After 7d in culture with either wild type peptide, pre-pulse alone or under antagonist conditions, cells were restimulated with wild type peptide. Cells cultured on wild type peptide show characteristic, dose-dependent proliferation upon restimulation with wild type peptide. However, cells cultured on the antagonist condition show significantly blunted proliferation relative to wild type cultured cells (Fig. 2A, $p < 0.05$). Antagonized cells secrete near normal or slightly elevated levels of the effector cytokine IFN-γ after 48 h of wild type stimulation (Fig. 2B, $p = 0.3878$).