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# Antigen-specific CD4+ effector T cells: Analysis of factors regulating clonal expansion and cytokine production
# Clonal expansion and cytokine production by CD4+ effector T cells

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ABSTRACT

In order to fully understand T cell-mediated immunity, the mechanisms that regulate clonal expansion and cytokine production by CD4+ antigen-specific effector T cells in response to a wide range of antigenic stimulation needs clarification. For this purpose, panels of antigen-specific CD4+ T cell clones with different thresholds for antigen-induced proliferation were generated by repeated stimulation with high- or low-dose antigen. Differences in antigen sensitivities did not correlate with expression of TCR, CD4, adhesion or costimulatory molecules. There was no significant difference in antigen-dependent cytokine production by TG40 cells transfected with TCR obtained from either high- or low-dose-responding T cell clones, suggesting that the affinity of TCRs for their ligands is not primary determinant of T cell antigen reactivity. The proliferative responses of all T cell clones to both peptide stimulation and to TCRβ crosslinking revealed parallel dose–response curves. These results suggest that the TCR signal strength of effector T cells and threshold of antigen reactivity is determined by an intrinsic property, such as the TCR signalosome and/or intracellular signaling machinery. Finally, the antigen responses of high- and low-peptide-responding T cell clones reveal that clonal expansion and cytokine production of effector T cells occur independently of antigen concentration. Based on these results, the mechanisms underlying selection of high "avidity" effector and memory T cells in response to pathogen are discussed.


The activation and differentiation of effector T cells in response to antigenic stimulation is distinct from that of naïve T cells [1,2]. During the course of the first infection, naïve T cells are primed with pathogen-derived peptides presented in the context of MHC by professional APC. These Ag-primed effector cells proliferate and differentiate into effector cells in regional lymph nodes [3]. Consequently, this process induces a set of pathogen-specific effector T cells that differ in their sensitivity and reactivity, or "avidity", to pathogen-derived peptides. These effector T cells then immigrate to the infection site, where they interact with target cells [4].

At the site of the infection, pathogens are not uniformly distributed; that is, the amount of antigen presented by individual APC to immigrated pathogen-reactive T cells is not uniform. Furthermore, antigenic stimulation by pathogen-presenting APC to T cells is dynamic during the course of infection [5]. Under these circumstances, pathogen-specific effector T cells, whose sensitivities and reactivities to pathogen epitopes are heterogeneous, encounter different strengths of antigen stimulation. In order to fully understand T cell-mediated immunity, analysis of altered T cell effector function in an environment of changing antigen load is important [6].

The goal of the present report is to determine the mechanisms that regulate clonal expansion and cytokine production by CD4+ antigen-specific effector T cells in response to a wide range of antigenic stimulation. To achieve this, we have generated a panel of OVA-specific T cell clones whose optimal proliferative responses occur at peptide concentrations that vary between 3.9 and 1000 nM. The expression levels of TCRβ, CD4 or cell surface adhesion and costimulatory receptors on these T cells does not correlate with their peptide dose-dependent proliferative response. When TCR αβ genes were cloned from high- or low-dose-responding T cell clones and expressed on TCR-negative TG40 T cells, there was no significant difference in their reactivity to various concentrations of OVA-peptide. This result suggests that differences in affinities of TCRs used by individual T cell clones are not responsible for differences in peptide-dose-dependent proliferation. In addition, the proliferative responses of these clones to TCR crosslinking with anti-TCR antibody parallel their response to antigen stimulation. These results strongly suggest that the sensitivity and reactivity of CD4+ effector T cells to their antigenic targets may be largely determined by intrinsic properties of the effector T cell. Furthermore, while high

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"avidity" effector T cell clones reveal high-antigen-dose suppression, resulting in a biphasic proliferation dose–response curve, their cytokine production pattern was monophasic. Thus these results support a model in which clonal expansion and cytokine production by effector T cells is regulated independently at various antigen conditions.

## Materials and methods

*Preparation and assay of $OVA_{323-339}$-speclfic T cell lines and clones.* BALB/c mice were immunized in the footpad with 100 mg (56 nM) $OVA_{323-329}$ peptide in CFA. After two weeks, inguinal and popliteal lymph nodes were harvested from 2 mice and were cultured with serial dilutions of $OVA_{323-329}$ (from 0.97 to 1000 nM). Viable T cells were collected weekly and re-stimulated with irradiated (30 Gy) BALB/c spleen cells and the same concentration of $OVA_{323-329}$ used in the initial culture. Three independent antigen-specific T cell lines were generated and were cloned by limiting dilution after 2, 4, and 8 restimulations, respectively. For their proliferative response to antigen, $2 \times 10^5$ cells were incubated with titrated amounts of OVA peptide and $5 \times 10^5$ irradiated BALB/c splenocytes for 51 h at 37 °C. For anti-TCR mAb crosslinking, $2 \times 10^5$ cells were stimulated in wells pre-coated with 50 ml of serially diluted TCRβ-specific mAb and cultured for 36 h at 37 °C. [$^3$H]TdR was added to the cultures for the last 12 h of incubation. For IFN-γ production, supernatants were removed from cultures 48 h after stimulation; IFN-γ was quantified by ELISA.

*Flow cytometry*. Immunofluorescence staining was carried out as previously described [7]. Fluorescent-conjugated Abs specific for TCRβ, CD4, CD28, LFA-1, ICOS, CTLA4, 4-1BB, and OX40 purchased from PharMingen (San Diego, CA. USA). Cytometric analyses were performed on a FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA. USA).

*Isolation of full-length TCRα and β chains from $CD4^+$ T cell clones and transfection into TCR-negative T cell hybridoma.* cDNA gene encoding the TCRα and TCRβ chains from individual $CD4^+$ T cell clones were cloned, sequenced, and synthesized as previously described [8]. Products were cloned into the multi-cloning site of pMX-IRES-GFP [9]. The TCR-negative T cell hybridoma, TG40 was retrovirally transduced as previously described [10]. Infected cells were stained with PE-labeled TCRβ mAb, and the positive fraction ($TCR^+$, $GFP^+$) was sorted on a FACSVantage (BD Biosciences). For activation of infected TG40 cells, $1 \times 10^5$ cells were incubated with titrated amounts of OVA peptide and $5 \times 10^5$ irradiated T cell-depleted BALB/c splenocytes for 24 h. Supernatants were then collected, and IL-2 production was quantified using the IL-2 indicator cell, CTLL-2.

*Preparation of $OVA_{323-339}$/I-$A^d$ tetramers.* $OVA_{323-339}$/I-$A^d$ tetramers were prepared using baculovirus as previously described [11]. For proliferative responses to $OVA_{323-339}$/I-$A^d$ tetramer, $2 \times 10^4$ cells were added to wells pre-coated with serially diluted $OVA_{323-339}$/I-$A^d$ tetramer. Cells were cultured for 51 h at 37 °C, [$^3$H]TdR was added for the last 12 h of incubation.

## Results and discussions

### *Generation of T cell clones responding to different concentrations of OVA-peptide*

The proliferative responses of lymph-node (LN) cells from OVA-primed mice were assayed by stimulating with titrated OVA-peptide. As shown in Fig. 1A, these cells proliferated to peptide stimu-

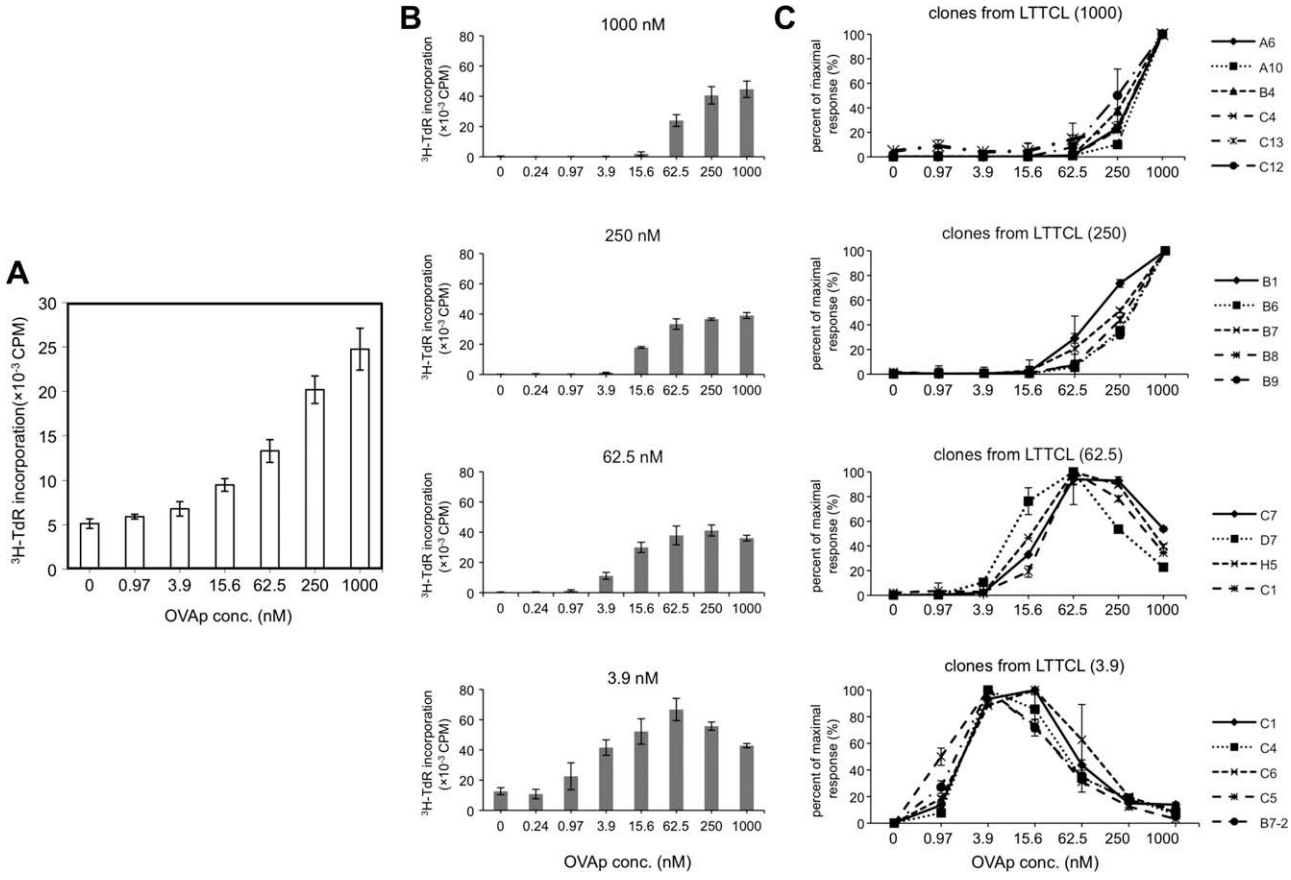


**Fig. 1.** Proliferative responses of OVA-primed LN cells and OVA-specific T cell lines and clones. (A) Proliferative responses of OVA-primed BALB/c lymph node cells to serial titrations of OVA peptide. (B) Proliferative responses of long-term T cell lines, which were generated by restimulation for 4 times *in vitro* with 1000, 250, 62.5, or 3.9 nM OVA peptide. (C) Proliferative responses of T cell clones obtained from long-term T cell lines by limiting dilution. Bars represent group means ± SD. Similar results were obtained for three additional experiments.