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CUTTING EDGE

Cutting Edge: CD8^{+} T Cell Clones Possess the Potential to Differentiate into both High- and Low-Avidity Effector Cells[1]

Charles J. Kroger and Martha A. Alexander-Miller[2]

The property of functional avidity is recognized to be of critical importance in determining pathogen clearance. An unresolved question with regard to this property is whether distinct naive subsets exist that display inherent differences in their peptide sensitivity requirements for activation, i.e., functional avidity, or whether differences in peptide sensitivity are induced following peptide encounter. In this study, we demonstrate that naive populations that can give rise to both high- and low-avidity cells do not contain subsets that exhibit differences in the amount of peptide required for activation. Furthermore, we show that an individual T cell clone can generate both high- and low-avidity effectors. The work presented here provides the first formal demonstration that an individual cell can give rise to both high- and low-avidity progeny, suggesting that avidity modulation at the level of an individual cell may play an important role in the CD8^{+} T cell response generated in vivo. The Journal of Immunology, 2007, 179: 748–751.

C ytotoxic T lymphocytes are a critical component of the immune system, responsible for the lysis of infected cells and the secretion of IFN-γ. Within a developing immune response, individual CTL clones exhibit a wide spectrum of functional avidities (1, 2). High-avidity CTLs can be activated at low levels of Ag, whereas low-avidity CTLs require high levels of Ag. High-avidity CTLs appear to be the more important population in vivo because they are more effective at viral clearance and tumor eradication compared with their low-avidity counterparts (for examples, see Refs. 2–4).

Although it is apparent that avidity is an important parameter, it is unclear how CTLs with distinct avidities arise in vivo. Although our previous work suggested that CTLs can modulate their functional avidity as a result of stimulation with different concentrations of peptide Ag (5), a limitation to those studies is that the analyses were performed on cell populations. Thus, the capacity of individual clones to give rise to high- vs low-avidity cells remained to be determined. Furthermore, whether there

was a differential requirement among naive cells for the amount of peptide necessary for T cells to form conjugates and subsequently proliferate was unknown. We have used our TCR-transgenic model to approach these questions. The results from the current study indicate naive CD8^{+} cells do not display differences in the amount of peptide required for conjugate formation or proliferation. In addition, we directly tested the hypothesis that a single cell can give rise to both high- and low-avidity effectors, finding that ~65% of clones possess the capacity to modulate functional avidity.

Materials and Methods

Mice, cell lines, and peptide

C57BL/6 mice were obtained from the Frederick Cancer Research Development Center (Frederick, MD). P14/rag-2 mice (6), which recognize the peptide gp$_{33–41}$ presented by the H-2K^b molecule, were purchased from Taconic Farms. All experiments in this study comply with the institutional guidelines approved by the Wake Forest Animal Care and Use Committee. DC2.4, an immortalized mature dendritic cell line, was a gift from Dr. K. Rock (University of Massachusetts, Medical School, Worcester, MA) (7).

Conjugate assays

DC2.4 were pulsed with 10^{-4} M or 10^{-9} M peptide for 3 h followed by washing. DC2.4 were then stained with CellTracker Orange (CTO)[3] and P14 cells with CellTracker Green (CTG). P14 cells and the APC were mixed at a 1:1 ratio and pelleted for 5 min at 1200 rpm. Samples were placed at 37°C for 0–60 min. Cells were resuspended in ice-cold medium to prevent additional conjugate formation. For the two-step conjugate assay, peptide-pulsed DC2.4 were stained with CTO or CellTrace Far Red DDAO-SE. Cells were mixed at a 1:5 ratio (P14:APC), centrifuged for 5 min, and incubated for 15 min at 37°C. Tubes were then removed and the secondary APC were added (equivalent number to primary APC). Samples were pelleted and placed at 37°C for an additional 5 min (preliminary analyses showed maximal conjugate formation by this time). Samples were kept on ice until acquired on the FACSCalibur (BD Biosciences).

Productive conjugate formation

P14 cells were labeled with CFSE (Molecular Probes) and mixed with CTO-labeled peptide-pulsed DC2.4 (1:1), pelleted, and incubated for 20 min at 37°C to allow for conjugate formation. Conjugates were sorted and cultured for 48 h, after which proliferation was determined.

P14 clone generation

P14 T cells were plated at limiting dilutions (0.3 cells/well). Cultures were maintained in the presence of 10% T-stim (BD Biosciences). On day 7, cultures were

equally divided and plated in two 96-well plates. One replicate was stimulated with the high peptide concentration and one with the low peptide concentration. Thus one plate was maintained on the initial stimulating peptide concentration, while the other was subsequently maintained on the alternative peptide concentration. The number of growth-positive wells was consistent with clonality.

Determination of functional avidity in clones

The functional avidity of the P14 clones was examined by IFN-γ ELISPOT as previously described (8). CD107 analyses were performed as previously described (9).

Results

A similar percentage of naive T cells form conjugates with professional APC, DC2.4, presenting different peptide concentrations

Our previous studies using splenocytes from a TCR-transgenic mouse have shown that, at the population level, CD8$^+$ T cells of high or low avidity could be generated by stimulation with APC bearing low vs high levels of peptide pMHC (10). One possibility to explain this was that precursors with a distinct avidity were selectively activated following stimulation with the appropriate amount of Ag. Alternatively, it was possible that naive precursors were homogeneous in their peptide requirements and differences in functional avidity were induced as a result of stimulation with different levels of peptide. To discriminate between these two hypotheses, we determined whether there were differences in the ability of naive P14 CD8$^+$ T cells (specific for the lymphocytic choriomeningitis virus gp$_{33-41}$ epitope presented in the context of K^b) to form conjugates with an immortalized DC line (DC2.4) pulsed with either a high or low concentration of peptide. Although ~20% of naive P14 CD8$^+$ T cells formed conjugates with the unpulsed DC2.4 at the early time points, these conjugates were relatively unstable (Fig. 1). In contrast, 45–50% of naive P14 CD8$^+$ T cells were in conjugate with peptide-pulsed DC2.4, regardless of the level of presented peptide.

The failure to detect >50% of the cells in conjugate with the conditions tested left open the possibility that distinct naive T cell subsets were interacting with the APC pulsed with the high or low peptide concentrations. We devised a two-step conjugate assay, as described in *Materials and Methods*, to determine
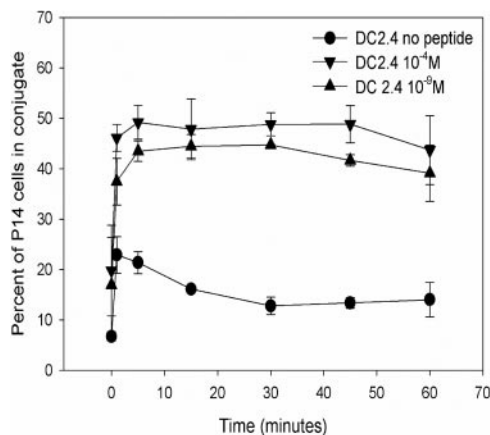

FIGURE 1. A similar percentage of naive T cells form conjugates with DC2.4 presenting a high or low peptide concentration. Naive P14 splenocytes were labeled with CTG and peptide-pulsed DC2.4 (10^{-4} M or 10^{-9} M) or unpulsed DC2.4 cells were labeled with CTO. Naive P14 splenocytes and DC2.4 were added at a 1:1 ratio. Following incubation at 37°C for the indicated time, the percentage of naive P14 splenocytes in conjugate with unpulsed (●), 10^{-4} M (▼), or 10^{-9} M (▲) peptide-pulsed DC2.4 was determined by flow cytometry. Data shown are the average from three independent experiments.
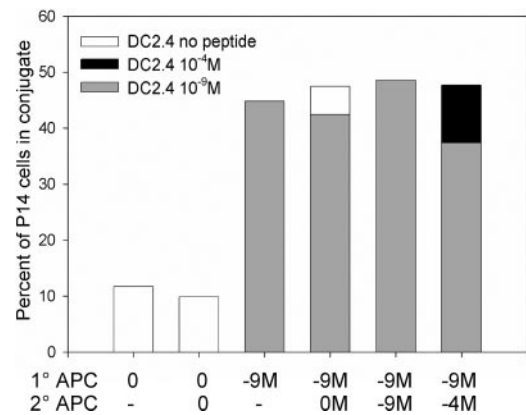

FIGURE 2. A P14 splenocyte subset requiring a high concentration of peptide for conjugate formation was not detected. Naive P14 splenocytes were labeled with CTG. DC2.4 previously pulsed with 10^{-4} M (■) or 10^{-9} M (▦) peptide or left unpulsed (□) were labeled with either CTO or CellTrace FarRed DDAO-SE. Naive P14 splenocytes were incubated with the indicated primary APC for 15 min at 37°C (1:5), followed by addition of the secondary APC (also at a 1:5 ratio), and incubated for an additional 5 min at 37°C. Data shown are the average of three independent experiments.

whether a subset of naive P14 CD8$^+$ T cells was present that was incapable of forming a conjugate with a low Ag-pulsed APC, but could conjugate with an APC pulsed with a high Ag concentration added later. The background level of naive P14 CD8$^+$ T cells in conjugate with DC2.4 cells without cognate peptide was ~12% regardless of whether medium or additional unpulsed DC2.4 were added as the secondary APC condition (Fig. 2, first and second bar, respectively). When 10^{-9} M DC2.4 were the primary APC followed by the secondary APC conditions of either medium or unpulsed DC2.4, nearly 40% of the T cells were in conjugate with 10^{-9} M DC2.4 (Fig. 2, third and fourth bar, respectively). The addition of more 10^{-9} M DC2.4 cells (Fig. 2, fifth bar) did not result in an increase in the percentage of naive T cells in conjugate, suggesting the initially added 10^{-9} M pulsed APC were not limiting and all cells capable of forming conjugates with this APC had done so.

If there existed a subset of naive T cells that required high Ag, we would expect a significant percentage of the out-of-conjugate P14 cells to form conjugates upon addition of the 10^{-4} M DC2.4. However, this was not the case. The addition of 10^{-4} M DC2.4 as the secondary APC did not lead to a significant increase in the total percentage of naive T cells in conjugate. There was a small reduction in the percentage of cells in conjugate with the 10^{-9} M DC2.4 concurrent with a similar percentage of cells now in conjugate with 10^{-4} M DC2.4 (Fig. 2, sixth bar). This may reflect a low level of dissociation with the 10^{-9} M DC2.4 with subsequent binding to the 10^{-4} M DC2.4. Using 10^{-4} M DC2.4 as the primary APC led to a similar conclusion (data not shown). Thus, the conjugate data support a model wherein the naive P14 T cell population does not contain subsets with distinct requirements for the amount of peptide necessary for conjugate formation.

Conjugates formed with DC2.4 presenting either a high vs low peptide concentration are productive

Although T cells formed conjugates similarly with APC presenting a high or low concentration of peptide, it was important to determine whether conjugate formation under each circumstance resulted in T cell activation. Thus, CFSE-labeled P14 T

cells in conjugate with peptide-pulsed DC2.4 cells (R2 gate) were sorted as depicted in Fig. 3. A similar percentage of P14 T cells was in conjugate with DC2.4 presenting the high (Fig. 3*C*) and low peptide concentration (Fig. 3*D*). As expected, naive P14 cells that formed conjugates with unpulsed DC2.4 did not undergo division (Fig. 3*E*). However, the naive P14 cells in conjugate with 10^{-4} M DC2.4 and 10^{-9} M DC2.4 did proliferate with nearly every T cell in conjugate proliferating in both cases (Fig. 3*E*). Thus, naive T cells in conjugate with APC bearing the high- or low-Ag density were similarly activated.

A naive T cell can differentiate into a high- or low-avidity CTLs

The data thus far indicated that individual P14 T cells in the naive repertoire do not display a peptide bias. This finding is consistent with the hypothesis that high vs low avidity may be an induced rather than an inherent property in this model. If avidity can be induced following T cell activation, this suggests that an individual naive T cell has the capability to differentiate into either a high- avidity or low-avidity CTLs following its activation, depending on the priming concentration of Ag encountered. To formally test this hypothesis, P14 T cells were plated at limiting dilutions and stimulated with either a high (10^{-5} M) or low (10^{-9} M) concentration of peptide (Fig. 4*A*). The following week, the wells were divided equally, and one well was restimulated with the high Ag concentration and the other with the low Ag concentration. Thus, one-half of the culture was maintained on the initial Ag concentration, whereas the other was switched to the alternative Ag concentration.

After several weeks of stimulation, the functional avidity of the responding CTL clones was examined. Approximately 64% (21 of 33) and 67% (10 of 15) of the P14 T cell clone pairs
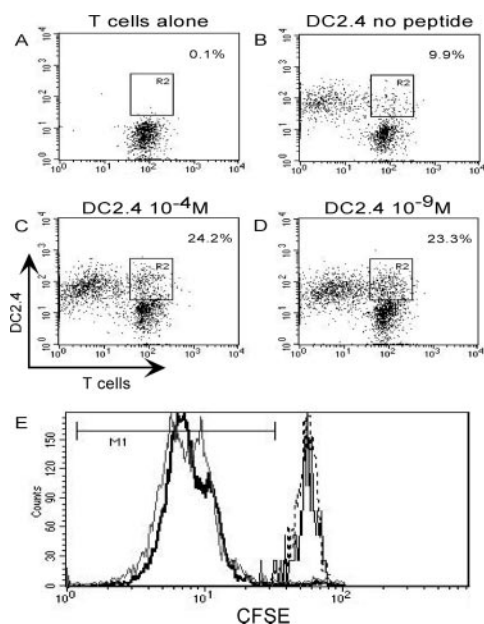

FIGURE 4. Individual naive P14 splenocytes can give rise to both high- and low-avidity CTLs. Clones were generated as in *A* and avidity was assessed on clone pairs by ELISPOT or lytic granule release. Representative clones generated by initial stimulation with 10^{-5} M peptide and maintained on 10^{-5} M (▼) or 10^{-9} M (▽) are shown in *B* and by initial stimulation with10^{-9} M and maintained on 10^{-9} M (▲) or 10^{-5} M (△) in *C*. The average amount of peptide required for IFN-γ production (*D* and *E*) by the panel of paired clones is shown. Clone pairs that exhibited more than a half-log difference in sensitivity were classified as having different functional avidities (*left panel* of both graphs). The clones that exhibited less than a half-log difference were classified as having similar functional avidity (*right panels*). The number of CTL clone pairs that were included in each data set is noted. Avidity in representative high-(▨) and low-(■) avidity clones pairs was also measured by lytic granule release (*F* and *G*). *, $p < 0.05$.

FIGURE 3. Naive P14 splenocytes in conjugate with DC2.4 10^{-4} M or 10^{-9} M proliferate similarly. Naive P14 splenocytes were labeled with CFSE (*A*) and DC2.4 (unpulsed; *B*) or pulsed with 10^{-4} M (*C*) or 10^{-9} M (*D*) peptide were labeled with CTO. Conjugates were allowed to form for 20 min and subsequently sorted (gate R2). Isolated conjugates were cultured for 48 h. The proliferation of naive P14 splenocytes in conjugate with unpulsed DC2.4 (thin line), 10^{-4} M DC2.4 (bold line), 10^{-9} M DC2.4 (gray line). and naive P14 splenocytes alone (dashed line) was examined 48 h later (*E*). The data shown are representative of three independent experiments.
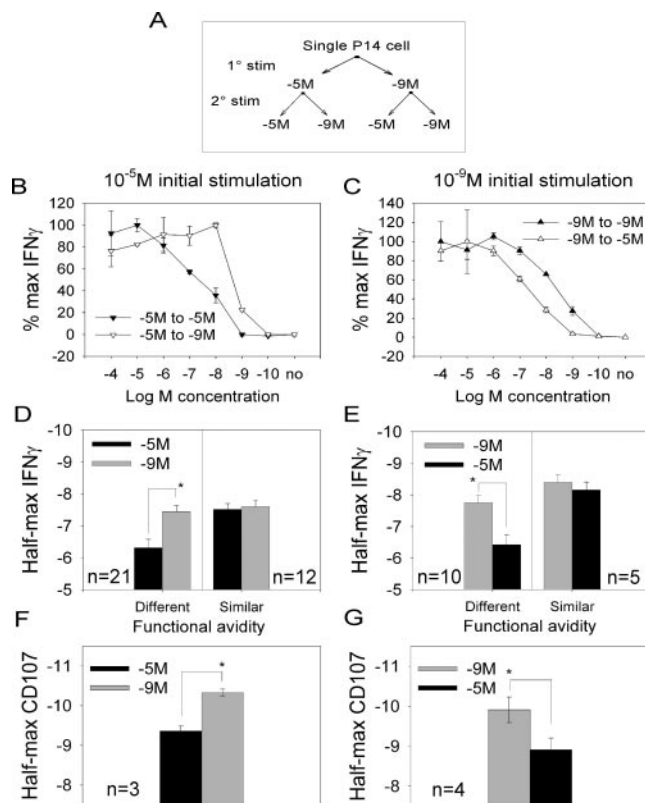
exhibited differences in their peptide dose-response curves when initially stimulated with the high or low Ag concentration, respectively (Fig. 4). Fig. 4, *B* and *C*, show dose-response curves for representative clone pairs generated by initial stimulation with the high or low peptide concentration, respectively. The *left panels* of Fig. 4, *D* and *E*, show the average concentration of peptide required for half-maximal production of IFN-γ in the pairs that exhibited differences in avidity. On average, these pairs demonstrated about a 1.5 log difference in peptide requirement. As would be expected, clones also exhibited differences in the amount of peptide required for TNF-α production (data not shown). We extended these findings to another effector function. Clones were tested for lytic granule release by measuring the acquisition of surface CD107 expression. As shown in Fig. 4, *F* and *G*, high- and low-avidity clones exhibited a 10-fold difference in the amount of peptide required for acquisition of CD107 at the cell surface. Together, these data show that a single P14 CD8^{+} T cell can develop into either a high- or low-avidity CTL depending on the concentration of Ag used to maintain the cells, consistent with the hypothesis that functional avidity can be an induced property.

The 35% of clone pairs that did not exhibit differences in peptide sensitivity may represent a subset of naive T cells in which peptide sensitivity is an inherent property. Interestingly, these clone pairs required peptide levels similar to high-avidity clones (Fig. 4, *D* and *E*, *right panel*), which may indicate that the P14 repertoire is by default high avidity and that cells are triggered to decrease peptide sensitivity as a result of the conditions encountered during T cell activation.

Discussion

Although many factors have been implicated in the regulation/generation of functional avidity (11–14), it is unclear whether the peptide sensitivity of responding CTLs was the result of the selective activation of a naive precursor population with a "fixed" functional avidity or whether responding cells differentiated into clones of defined avidity. The results from these studies support the latter as a mechanism that can be used. We found no evidence that individual clones within the naive P14 T cell repertoire exhibited differences in the amount of peptide required for activation, even though this population could give rise to clones of distinct avidity. Furthermore, we found that a majority of individual clones could produce daughter cells that differed significantly in the amount of peptide required for activation. To our knowledge, this work provides the first direct evidence that a naive T cell can differentiate into a high- or low-avidity CTL.

The ability to modulate peptide sensitivity during the initial encounters with Ag would provide significant advantages to the developing immune response. This window of "tunability" appears to last through the initial two to three stimulations with Ag, after which avidity begins to become fixed (our unpublished data). Although the differentiation of a single cell into high- and low-avidity cells cannot be tested in vivo, we have found that adoptively transferred P14 cells give rise to an effector population that contains both high- and low-avidity cells, suggesting that the differentiation that occurs in vitro can also occur in vivo (our unpublished data). A period during which avidity can be modulated would allow T cells to tune their sensitivity to peptide such that they were optimally responsive to the levels of peptide presented as a result of infection. It would be a valuable survival mechanism, protecting T cells from death that results from supraoptimal stimulation (15).

The restricted TCR usage in our system may serve as a model for instances where the epitope-specific TCR repertoire has been shown to be highly restricted. For example, in a study by Kedzierska et al. (16), the CD8$^+$ effector cells generated against the NP$_{366}$ epitope following influenza infection exhibited a highly limited TCR. As such, similar TCRs were used by cells of high and low avidity. The authors postulate that under these circumstances, an alternative mechanism must be a prime determinant of avidity. Our data suggest that in such cases, a significant contribution to the control of avidity may be the ability to tune sensitivity at the level of the individual cell.

At the individual cell level, several factors have been implicated in influencing functional avidity, including TCR affinity and the absolute level or isoform of CD8 expressed (3, 5, 10). Although the former is excluded by the model used here, a majority of clones did show evidence of CD8 modulation in the level and/or the isoform of CD8 expressed (data not shown). Given the role of CD8 in facilitating signal transduction (17),

CD8 modulation is an attractive candidate for a contributor to the control of avidity in the clones. However, other mechanisms are also likely at play, i.e., regulation of signal transduction molecules or organization of molecules in the cell membrane.

In summary, the data presented here provide the first demonstration that a single cell can differentiate into a high- or low-avidity CTL. The majority of T cells appear to possess plasticity following their initial encounter(s) with Ag; providing a window of opportunity during which peptide sensitivity can be "tuned" in response to the level of presented peptide. This property may play a critical role in determining the efficacy of the responding population following infection because it would promote optimal sensitivity while at the same time providing protection from death triggered by supraoptimal amounts of peptide.

Acknowledgments

Disclosures
The authors have no financial conflict of interest.

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