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Cutting Edge: Lack of High Affinity Competition for Peptide in Polyclonal CD4+ Responses Unmasks IL-4 Production

Joshua D. Milner,*† Nicolas Fazilleau,‡,1 Michael McHeyzer-Williams,‡ and William Paul*

Priming of naive monoclonal CD4 T cells via weak agonist permits GATA-3 transcription and Th2 differentiation. To test whether this process can occur in polyclonal naive populations, where a range of TCR affinities exists for any given Ag/MHC complex, we primed naive CD4 cells from 5CC7 Vβ3 transgenic mice, which have a fixed β-chain specific for pigeon cytochrome c peptide I-Ek. Priming populations depleted of higher affinity, moth cytochrome c peptide I-Ek tetramer-binding cells resulted in substantial IL-4 production that did not occur in the presence of higher affinity cells. TCRα-chain sequence analysis showed that clones that possessed TCR features associated with high affinity responses to pigeon cytochrome c made less IL-4 than clones that possessed fewer such motifs. These results indicate that cells bearing TCRs that are weakly stimulated by their cognate Ag preferentially adopt a Th2 phenotype when primed in the absence of competition from cells with higher affinity receptors. The Journal of Immunology, 2010, 184: 000–000.

In priming cultures of CD4 T cells from TCR transgenic mice, low concentrations of agonist peptide strikingly favor Th2 differentiation. Similarly, priming with high concentrations of partial agonists leads to a Th2-biased response, suggesting that low receptor occupancy during priming predisposes to Th2 differentiation, independent of exogenous IL-4 (1–3). The suppression of Th2 responses by TCR stimulations resulting from high receptor occupancy appears in part to be due to increased ERK phosphorylation that in turn suppresses GATA-3 transcription (3). Indeed, at high peptide concentration, T cells from TCR transgenic mice generally differentiate into Th1 cells. Other evidence for an association between weak TCR signaling and Th2 differentiation can be found in multiple studies showing that hypomorphic mutations in TCR signaling molecules can lead to severe atopic or Th2-linked disease (4–7). Furthermore, the Th2-promoting mechanism of the schistosome egg Ag component Ω-1 appears to be attenuation of the Ag-presenting potency of dendritic cells (8).

In polyclonal responses, it is anticipated that there will exist CD4 cells with a hierarchy of affinities to a given peptide–MHC complex. It might be anticipated that the lower affinity members of the population would, as the result of lower receptor occupancy during priming, differentiate to Th2 cells, whereas the higher affinity members would acquire a Th1 phenotype. However, what is generally observed is that in such populations, T cells of high affinity respond robustly, whereas cells whose TCRs provide interactions with the immunizing peptide that is below certain affinity/occupancy thresholds either react minimally or not at all in these mixed populations (9). The failure of these lower affinity cells to respond could be due to competition from higher affinity members of the population or due to cell-intrinsic properties. In the absence of high affinity cells, it would be expected that naive low affinity cells, if they can respond at all, would acquire a Th2 phenotype because they would achieve low receptor occupancy even at high pigeon cytochrome c (PCC) peptide (pPCC) concentrations.

To determine whether such low affinity cells could respond in the absence of high affinity cells and how they would differentiate, we used CD4 T cells from 5CC7 Vβ3 transgenic mice, which have normal endogenous, polyclonal TCRα-chain rearrangement and a fixed β-chain derived from a TCR specific for pPCC 89–104, and tetramer staining to separate TCRs of higher and lower affinity from these polyclonal populations.

Materials and Methods

Mice

Monoclonal 5CC7αβ TCRtg mice on B10.A background were purchased from Taconic Farms (Germantown, NY). The 5CC7 Vβ3 TCRtg and 5CC7 Vβ3 TCRtg × PCCtg mice on B10.A background were provided by N. Singh and R. Schwartz (National Institutes of Health, Bethesda, MD).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: HSA, heat-stable Ag; MFI, mean fluorescence intensity; PCC, pigeon cytochrome c; pMCC, moth cytochrome c peptide; pPCC, PCC peptide.
**Abs and tetramer**

1-Ek–moth cytochrome c peptide (pMCC) tetramer was prepared and used for staining, as described (9). Different tetramer concentrations were used for staining, as indicated in the figures. IL-4 PE, CD44 PE-cy5.5, FITC Abs to CD25, heat-stable Ag (HSA), CD8, NK1.1, B220, DX5, CD16/CD32, and I-Ek were purchased from BD Pharmingen (San Diego, CA).

**Cell sorting**

Lymph nodes from mice were homogenized into single-cell suspensions and stained with tetramer, CD44, and FITC Abs. Naive CD4+ cells were identified by gating on CD44 low, FITC-negative cells; I-Ek–pMCC tetramer+ cells were removed by sorting on a FACS Aria using FacsDIVA software.

**In vitro priming and cloning**

A total of 30,000 1-Ek–transfected fibroblasts (provided by R. Germain, National Institutes of Health) was treated with 10 μg/ml mitomycin C for 30 min at 37°C, washed, and plated together with sorted T cells and pPCC in 96-well flat-bottom plates for 5 d for bulk priming, or in 96-well round-bottom plates for 14 d, with IL-2 and IL-7 added at days 3 and 10 for cloning. The neutralizing anti–IFN-γ Ab XMG (Harlan Breeders, Indianapolis, IN), 10 μg/ml, was added to some cultures.

**Sequence analysis**

TCR cDNA preparation and PCR amplification were performed, as described (10). Some sequencing was also performed with primer and template combined together in an Applied Biosystems (Foster City, CA) 96-well Optical Reaction Plate (P/N 4306737) following the manufacturer’s recommended concentrations. Sequencing reactions were set up as recommended in the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit. The FINCH data management system (Geospiza, Seattle, WA) was used to store sequence data for all subsequent downstream sequencing analysis. When >1 sequence was found in any sample, that sample was excluded from analysis.

**Intracellular cytokine staining**

For intracellular cytokine staining, cells were restimulated for 4 h with PMA (10 ng/ml) and ionomycin (1 μM) in the presence of monensin (2 μM). Collected samples were fixed with 4% formaldehyde, washed, and permeabilized in 0.5% Triton X-100 and 0.1% BSA in PBS before being stained with anti-CD4 allophycocyanin, PE-conjugated anti–IL-4.

**Results and Discussion**

**IL-4 production by primed polyclonal naive populations depleted of pMCC I-Ek tetramer-binding cells**

Polyclonal lymph node populations from Vβ3Tg mice were stained with various concentrations of pMCC/I-Ek tetramer. Higher tetramer concentrations led to higher peak mean fluorescence intensity (MFI) and more stained cells than did lower tetramer concentrations (Fig. 1A). CD4+CD44low cells that failed to bind tetramer were purified by cell sorting. These sorted cells were then primed for 5 d with pPCC presented on I-Ek–transfected fibroblasts, stimulated with PMA and ionomycin in the presence of monensin, and stained for cytosolic IL-4. These transfected fibroblasts were used as APCs because they fail to produce detectable IL-12 or IFN-γ, and thus do not prime for IFN-γ production. Therefore, we were able to measure the relative inhibition of IL-4 by high doses of peptide independent of suppressive effects of IFN-γ or of Th1 differentiation.

As expected, priming with a low concentration (0.01 μM) of peptide resulted in a much higher percentage of IL-4-producing cells than priming with a high peptide concentration (1 μM) in populations from which tetramer+ cells had not been removed (Fig. 1B, I.C). Cells remaining after removal of those that stained positively with 230 nM pMCC I-Ek tetramer, the highest concentration used, failed to proliferate even to high concentrations of pPCC over the 5-d culture period. This was most likely due to the removal of most peptide-reactive cells. When lower tetramer concentrations were used for depletion of tetramer-binding cells, the remaining cell population was able to expand at the 1 μM concentration of pPCC. The resultant primed cell population produced significantly more IL-4 than did similarly primed naive cells that had not been depleted of tetramer+ cells (23–25% versus 7.5%). When 0.01 μM pPCC was used to prime most tetramer-depleted populations, there was little or no proliferative response, most likely due to the removal of cells of sufficiently high avidity to respond to such low peptide concentrations. When 14 nM tetramer, the lowest concentration tested, was used for depletion, the remaining population was able to respond to low concentrations of peptide (Fig. 1B, 1C), arguing that removing cells stained with lower tetramer concentrations leaves behind cells with TCR of intermediate receptor occupancy that are capable of responding to the lower peptide concentration. Addition of anti–IFN-γ blocking Ab did not change the results (data not shown), indicating that failure to obtain Th2 priming was not due to suppression by IFN-γ. When a small number of 5C7αβ transgenic cells, which have a relatively high avidity for the pPCC–I-Ek complex, was added into the priming culture in the presence of anti–IFN-γ, IL-4 production was strikingly diminished in a dose-dependent fashion (Fig. 1D). Similar results were obtained if 5C7/Vβ3tg pMCC I-Ek tetramer+ cells were added into the cultures (data not shown). The relatively similar amounts of IL-4 produced by populations that had been depleted using a range of tetramer concentrations imply that even the lowest concentration of tetramer can remove those high avidity cells that inhibit priming for IL-4 production. Taken together, these data suggest that a hierarchy of affinities to a given Ag exists within polyclonal populations of T cells, and when cells having the highest avidity for Ag are removed, the resulting cells are capable of developing a Th2 phenotype even when primed at high peptide concentration.

Expression of preferred CDR3 features and propensity for individual clones to make IL-4 inversely correlate

pMCC/I-Ek tetramer staining appears to differentiate TCRs with high avidity for pPCC from cells with a much lower avidity for that peptide. We therefore wished to determine whether, on an individual cell basis, IL-4 production would be highest among cells with low avidity for pPCC. Using limiting dilution analysis and stimulation with 10 μM pPCC, we found that ~1 of 60 unseparated naive 5C7/Vβ3 CD4 cells could give rise to a pPCC-responsive clone. Among tetramer-negative cells alone, the frequency of cells that could respond to pPCC was ~3-fold less (data not shown). We validated this use of the tetramer to delineate relative TCR avidity for pMHC by sequencing individual clones generated by limiting dilution of pPCC-stimulated CD4+CD44low 5C7/Vβ3 tetramer-negative populations. In parallel, we cloned tetramer-positive populations at 1 cell/well (Supplemental Fig. 1A).

Four characteristics within the rearranging TCR-β chain have been shown to correlate with reactivity and functional avidity to pPCC among 5C7/Vβ3 transgenic T cells (10). Clones derived from tetramer-positive populations initially plated at 1 cell/well were substantially more likely to have
each of the four properties associated with high avidity than clones derived from tetramer-negative populations (average 3.6 ± 0.13 motifs versus 2.25 ± 0.16 motifs, p = 0.0001) (Supplemental Fig. S1A), in agreement with previous work correlating pMCC I-Ek–tetramer staining with binding avidity and reactivity (9).

IL-4 production followed a similar pattern. Clones derived from tetramer-negative cells initially plated at 20 cells/well produced significantly more IL-4 than did clones derived from tetramer+ cells plated at 1 cell/3 wells (58.85% ± 4.55 versus 45.90% ± 2.38, p = 0.0052) (Supplemental Fig. S1A). This pattern was also seen when sorting by tetramer-staining intensity. Using MFI to quantify tetramer binding, cells with higher MFI, cloned at 1 cell/well, made significantly less IL-4 than those cloned from cells with lower MFI (Fig. 2A, Supplemental Fig. S1B,C). Of note, whereas we did not notice any stimulation of T cells by bound tetramer without peptide, it is possible that tetramer binding could interfere with peptide stimulation, in which case one would anticipate even more profound depression of IL-4 production with increased tetramer binding.

To determine how cells with identical TCRs would respond to similar cloning conditions, monoclonal PCC-reactive 5CC7ab transgenic T cells were cloned at the same high concentration of pPCC as the polyclonal Vß3tg (10 μM), as well as a low (0.01 μM) pPCC concentration to mimic weaker agonism/low receptor occupancy. Clones that were obtained from the high concentration priming made substantially less IL-4 than those obtained from low concentration cloning (12.45% ± 1.55% versus 46.80% ± 5.11%, p < 0.0001) (Supplemental Fig. S1D). This argues that this phenomenon is cell autonomous, in that each individual TCR can dictate what the eventual cytokine production profile will be. These results contrast with previous studies (11), and perhaps could be explained by the type of APC used, or other differences in cloning conditions.

FIGURE 1. Removal of tetramer-positive cells from polyclonal populations leads to IL-4 production by the remaining cells when primed with high peptide concentrations. Lymph node cells pooled from at least three Vß3 5CC7 mice were stained with tetramer at the concentrations indicated (A), and then sorted for CD4+CD44low tetramer-negative populations. They were then mixed at a density of 25,000 cells/well, along with 35,000 mitomycin C-treated I-Ek–transfected fibroblasts and the indicated pPCC concentration, and placed at 37°C. After 5 d, the cells were counted (B) and restimulated with PMA/ionomycin for 4 h in the presence of monensin, and fixed with 4% paraformaldehyde and stained for CD4 and IL-4. Shown are the percentages of CD4 + cells that are IL-4 + (C). D, CD45.2 Vß3tg lymphocytes were stained with 40 nM pMCC I-Ek tetramer. A total of 5000 CD44low tetramer− CD4 cells was sorted from this population. They were cultured with the indicated number of sorted, monoclonal CD4+CD44low 5CC7ab lymphocytes together with 35,000 I-Ek–transfected fibroblasts and 1 μM pPCC, in the presence of neutralizing anti–IFN-γ Ab. Five days later, cells were then restimulated and stained, as in B. Shown are the percentages of total cells that were IL-4+. These experiments were repeated at least three more times with very similar results.
The residual cells may be more predisposed to differentiate into IL-4 producers after priming with high concentrations of peptide. We therefore used Vβ3tg mice that were bred to PCCtg mice. As expected, CD4+ single-positive thymocytes from the Vβ3tg × PCCtg mice bound substantially less tetramer than did standard Vβ3tg thymocytes (Fig. 3A). Priming of sorted naïve CD4+ single-positive thymocytes from Vβ3Tg PCCtg mice resulted in higher IL-4 production and less expansion across all pPCC priming doses than was seen as a result of priming CD4 T cells from non-PCCtg Vβ3tg mice (Fig. 3B, Supplemental Fig. S2). Therefore, the cells with high avidity to pPCC appear to have been deleted within the thymus; the residual cells can still be primed by pPCC Ag, but this priming is biased toward Th2 differentiation.

Weak TCR signals sufficient to drive proliferation, but not strong enough to suppress GATA-3 production via phosphorylation of ERK during priming, have been shown to lead to Th2 differentiation (3). Our data suggest that such weak TCR signaling can occur within a polyclonal naive setting, and that Th2 cells can emerge independent of exogenous cytokines, but only when T cells that achieve higher receptor occupancy by the stimulating Ag are removed prior to priming. The mechanism by which higher affinity cells prevent lower affinity cells from expanding as Th2 cells does not appear to be IFN-γ dependent. It is possible that feedback inhibitory soluble factors are released, or that competition for resources prevents the survival/expansion of the lower affinity cells. Additionally, the strength of initial TCR signaling correlates with the extent of expansion for individual cells in polyclonal responses (12), providing another mechanism by which higher affinity cells could limit the numbers of lower affinity cells.

Previous work has shown TCRα CDR3 lengths appear to contribute to the intrinsic ability of a T cell to become a Th1 or Th2 cell under polarizing priming conditions (13). As a consequence, the TCRα sequence, presumably through its effect on the avidity/affinity of the cell’s TCR, not only may

**FIGURE 2.** Single-cell cloning reveals propensity for low affinity cells to produce more IL-4. A, The 5CC7 Vβ3 lymphocytes were stained with 230 nm I-Ek tetramer, and naïve CD4 cells were sorted for tetramer high and tetramer low populations. One cell/well was plated with 35,000 mitomycin C-treated fibroblasts with 10 μM pPCC in the presence of 100 U/ml IL-2. On days 3 and 10, 5 ng/ml IL-7 and 100 U/ml IL-2 were added, and clones were restimulated to measure IL-4 production at ~day 14. B, Sorted naïve CD4 lymphocytes were not stained with any tetramer and were plated at 20 cells/well (5CC7 Vβ3), cloned at 10 μM pPCC, and restimulated as above. Clones were sequenced, and preferred CDR3 features were quantified and then correlated to IL-4 production by each clone. Shown is the number of preferred features used for individual clones, organized by tertile of IL-4 production. These experiments were repeated with very similar results.

Vα-chains of clones that make more IL-4 have fewer preferred CDR3 motifs

We then sought to determine the extent to which TCRα features might correlate with IL-4 production. Naïve Vβ3 T cells were cloned by limiting dilution. Twenty sorted naïve CD4+ Vβ3tg T cells/well were primed with 10 μM pPCC presented by I-Ek–transfected fibroblasts. Clones that grew out after 2 wk were then restimulated with PMA/ionomycin to determine the percentage of cells from each individual clonal population that expressed intracellular IL-4. The TCRα-chain of each clone was also sequenced. Clones from the lowest tertile of IL-4 production had more preferred features than clones from the higher IL-4 production terciles (mean 3.9 ± 0.1 motifs for low versus 2.1 ± 0.233 for middle and 2.8 ± 0.389 for high) (Fig. 2B).

Polyclonal populations from 5CC7 Vβ3 mice transgenic for PCC expression show thymic deletion, but increased IL-4 production from residual PCC-reactive cells

Thymic deletion of high avidity cells specific for a given pMHC complex would be anticipated to lead to a residual polyclonal population with lower avidity for that Ag. In turn,

**FIGURE 3.** Polyclonal populations from 5cc7 vβ3 mice transgenic for PCC show thymic deletion, but increased IL-4 production from residual PCC-reactive cells. A, Thymocytes pooled from at least three 5CC7 Vβ3tg or 5CC7 Vβ3tg × PCCtg mice were stained with I-Ek pMCC tetramer at 40 nm, and shown are plots gated on HSA+ CD8+ T cells. B, Naïve CD4+CD8+ CD44dim HSA+ thymocytes were plated at 25,000 cells/well, primed with 10 mM pPCC, and restimulated as in Fig. 1. This experiment was repeated twice with similar results.
be important in differentiation of the precursor, but also for its subsequent survival, particularly when a higher affinity precursor is also present. Further support for the role of TCR signaling itself in Th polarization can be found in recent data that indicate Th17 cells require a high TCR signal strength for differentiation (14).

In response to Ags, Th2 responses may emerge in situations in which cells with higher affinity for the Ag are not available during the priming response. Among the naïve TCR repertoire, the a priori range of affinities that lead to variances in receptor occupancies by a given Ag at a given concentration may explain divergent cytokine responses to pathogens and other Ags. Allergen reactivity, lymphopenia-associated Th2 diseases, and clinical and T cell responses to respiratory syncytial virus (15–18) are all examples of such divergent responses. The data presented in this study could explain the aberrant Th differentiation seen in these cases—namely, that the phenotype is determined by the presence or absence of specific TCRs that normally inhibit the Th2 response.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental figure S1
A) Sorting scheme for tetramer+ and tetramer- cells. Shown is a dot plot of CD4+ lymphocytes from Vb3 transgenic mice stained with 40nm I-Ek pMCC tetramer. For tetramer positive cells, one cell/well was plated and for tetramer negative cells 20 cells/well were plated with 35,000 mitomycin C treated fibroblasts with 10 μM PCC peptide in the presence of 100 u/ml Il-2. On Days 3 and 10, 5 ng/ml IL-7 and 100 u/ml Il-2 were added, and clones were harvested around day 14. The 4 preferred CDR3 features used were: 1) CDR3α length of 8 aa, 2) glutamic acid residue at α93, 3) serine residue at α95, and 4) use of Jα22, Jα34, Jα17, or Jα16 gene segments. Vα11 CDR3 were sequenced from a sample from each clone and ideal motifs quantified while the remainder of each clone was stimulated with PMA/ionomycin in the presence of monensin for 4 hours and stained for IL-4 production as in Fig. 1. (B) I-Ek tetramer was used at 230 nm to identify naïve CD4 tetramer high and tetramer low cells for sorting. (C) Sorted cells were plated at 1 cell/well, cloned and restimulated with IL-4 production measured as above. These experiments were repeated with similar results. (D) Sorted naïve CD4 lymphocytes were plated at 20 cells/well (5CC7 Vβ3), or 1 cell/well (5CC7αβ), cloned at the indicated peptide concentration, and restimulated as above. This experiment was repeated with very similar results.

Supplemental figure S2
Naïve CD4+ CD8- CD44lo HSA- cells sorted from either Vβ3 or Vβ3-PCCtg thymocytes were plated at 25,000 cells/well and primed with the indicated concentration of PCC peptide then restimulated and stained 5 days later for IL-4 as in Fig. 1, and counted for cell recovery.
Supplemental figure S2