Delayed expansion of a restricted T cell repertoire by low-density TCR ligands

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Abstract

The role of TCR ligand density (i.e. the number of antigen–MHC complexes) in modulating the diversity of a T cell response selected from a pool of naive precursors remains largely undefined. By measuring early-activation markers up-regulation and proliferation following stimulation with staphylococcal enterotoxin A (SEA), we demonstrate that decreasing the ligand dose below an optimal concentration leads to the delayed activation of a restricted set of TCR\(\beta\)1-bearing T cells, with the specific, non-stochastic exclusion of some TCR\(\beta\)1+ T cells from the activated pool. Our results suggest that the failure of these TCR\(\beta\)1-bearing T cells to reach the activation threshold at sub-optimal ligand concentration is due to the inefficiency of TCR engagement, as measured by TCR internalization, and does not correlate with the relative precursor frequency in the non-immune repertoire. Moreover, even at SEA concentrations that lead to the simultaneous proliferation of all SEA-reactive T cells, we observe marked differences in the ability to secrete cytokines among the different responsive TCR\(\beta\)-bearing T cells. Altogether, our results indicate that the development of a T cell response to a scarce display of ligand significantly narrows TCR repertoire diversity by mechanisms that involve focusing of the repertoire on the expansion of those T cells with the highest avidity of TCR engagement.

Introduction

The recognition of antigens presented by MHC molecules through the TCR is fundamental to the development of an immune response. The diversity of the TCR as generated by somatic molecular rearrangement events is greatest within the distinct complementary determining region 3 which specifically contacts the MHC-bound antigen and, to a much lesser extent, within the gene-encoded variable (V) domain (1, 2). Beyond thymic selection, the diversity of the host’s T cell repertoire is constantly shaped by antigen-driven expansions and differentiation of antigen-specific T cell precursors, followed by the elimination of a large fraction of the pool of expanded cells by apoptosis (3). These processes contribute to the establishment of an immunological memory that is qualitatively dependent on the diversity of the initial antigen-specific response deployed (4–6). As an example, in disease models such as chronic HIV infection, the clonotypic diversity of the primary CD8+ T cell response against viral antigens constitutes one of the earliest and most significant prognostic factors determining the rate of progression to AIDS (7).

Immune responses can be of quite variable repertoire diversity depending on the particular antigen targeted (8, 9). Factors which may affect the diversity of a T cell response include the size of the pool of antigen-responding precursors, as well as the complexity of the antigenic determinant itself (4, 10, 11). Using tetrameric MHC–antigen reagents, investigators have observed that during an antigen-driven immune response, a strong maturation of the repertoire occurs towards T cell clones with the ‘best fit’ for this epitope (12–14). However, this selective focusing of the T cell repertoire was not observed, for example, in H-2L\(^d\)-restricted responses against...
a lymphocytic choriomeningitis virus-derived epitope (5) and the extent to which a repertoire ‘focuses’ on a narrower diversity of cells might very well depend on antigen-related characteristics that remain to be defined (8, 9, 15, 16). These factors and the interplay of early cellular events underlying repertoire selection processes need to be better understood.

Given the increasingly recognized importance of the quality of TCR–MHC/antigen contacts in determining the functional outcome of T cell activation, it is possible that the avidity of this interaction imposes significant restrictions on antigen-driven selection processes despite a remarkable sensitivity of the TCR (12, 13, 17). A role for TCR ligand density in modulating the potential diversity of a selected T cell repertoire has been previously proposed based on circumstantial evidence obtained with distinct endogenously processed peptides (15, 16). However, this has never been directly demonstrated.

One major obstacle is related to difficulties in following the most proximal event of the expansion of individual antigen-specific T cells (23–25), as well as its ability to form an immunological synapse (26). To overcome these limitations, we have developed an approach which employs TCR ligands that are recognized by less variable determinants of the TCR, thereby selecting for a broader frequency of responding precursors. Superantigens (Sags) interact with the V region of the TCR β chain (20, 21), whose primary structure is predictable (e.g. because it is gene-encoded) and against which a wide panel of specific anti-Vβ antibodies are available (22). Moreover, although the interaction of MHC-Sags with the TCR largely depends on the structure of the TCRβ domain, its affinity and kinetics for TCR (23–25), as well as its ability to form an immunological synapse (26), are remarkably similar to MHC-antigen complexes. Also, their binding to MHC class II molecules can be controlled with predictable affinity, avidity and stability (27, 28). The division history of individual precursor T cells was tracked using a specific fluorescent dye (29), in order to determine their distribution and kinetics of expansion within the population. Altogether, our results provide a framework to understand the influence of affinity/avidity differences in TCR–ligand interactions on the dynamics of T cell repertoire selection processes and on the generation of highly diverse versus narrow immune responses.

Methods

Reagents and antibodies
The PE-conjugated anti-Vβ1 (IM2355), anti-Vβ2 (IM2213), anti-Vβ5.3 (IM2002), anti-Vβ9 (IM2003), anti-Vβ16 (IM2294), anti-Vβ21.3 (IM2050), anti-Vβ22 (IM2051) and anti-Vβ23 as well as the FITC-conjugated anti-Vβ1 (IM2406), anti-Vβ2 (IM2407), anti-Vβ3 (IM2372), anti-Vβ5.2 (IM1482), anti-Vβ13.1 (IM1554), anti-Vβ13.6 (IM1330), anti-Vβ17 (IM1234), anti-Vβ20 (IM1562), anti-Vβ21.3 (IM1483) and anti-Vβ22 (IM1484) were purchased from Coulter/ Immunotech (Miami, FL, USA). The PE-conjugated anti-CD69 (Leu-23) and anti-CD25, as well as the peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (Leu-3a) antibodies, were purchased from Becton Dickinson (San Jose, CA, USA). The unconjugated anti-Vβ5 (MH3-2), anti-Vβ5.3 (421C1), anti-Vβ6.7 (OT145), anti-Vβ8 (JR2), anti-Vβ9 (MKB1), anti-Vβ12 (SC511) and anti-Vβ23 (HUT78) were obtained from various commercial sources. Staphylococcal enterotoxin A (SEA) was obtained from Toxin Technology (Sarasota, FL, USA).

Human peripheral blood lymphocytes purification and 3H-thymidine incorporation assays
Peripheral blood obtained from healthy HLA-DR1 blood donors was diluted (1 : 1) in PBS and underlayered with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) at room temperature. After centrifugation, the interface was collected and washed three to four times in PBS/2% FCS and re-suspended in RPMI 1640 (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% FCS to yield PBMCs. CD25- and CD69-positive cells were depleted from total PBMC by magnetic cells sorting using the MACS system (Miltenyi Biotec, Gladbach, Germany). Total PBMC was stained with anti-CD25 (BD347647) and anti-CD69 (BD347823) purchased from Becton Dickinson and washed with degassed PBS/2 mM EDTA (loading buffer). Cells (total = 10^6 cells per column in 500 µl loading buffer) were incubated at 4°C for 30 min with MACS goat anti-mouse IgG microbeads (30 µl per 10^6 cells), washed again and purified on a MACS RS+ separation column and adapter (catalog no. 413-01) using a VarioMACs magnetic field. The purified cells were then checked for purity by immunofluorescence and re-suspended in RPMI 1640 (GIBCO Laboratories) supplemented with 10% FCS. For [3H]thymidine ([3H]TdR) incorporation assays, PBMCs were cultured at 37°C in complete RPMI medium supplemented with 5% FCS in the presence of SEA for 3 days in round-bottomed 96-well plates. Following incubation, 1 µCi [3H]TdR was added for 16 h at 37°C. Cells were harvested and [3H]ThdR incorporation was measured using a β-plate counter (Pharmacia LKB Biotechnology AB).

Intracellular cytokine production
Intracellular IL-2 was measured after 8 h of stimulation using procedures described (30). In brief, PBMCs (2 x 10^6 ml^-1) were incubated with no stimulus, phorbol myristate acetate (25 ng ml^-1) and ionomycin (1 µg ml^-1) or SEA for 8 h. Brefeldin A (10 µg ml^-1) was added for the final 6 h of stimulation. After stimulation, cells were lysed, permeabilized and stained with either anti-IL-2 or anti-IFN-γ, anti-CD69 and either anti-CD4 or anti-TCR. Antibodies, isotype controls and lysing and permeabilizing solutions were purchased from Becton Dickinson. Control antibody for permeabilization was purchased from Medicorp (Montreal, Province of Quebec, Canada).

5.6-Carboxyfluorescein diacetate succinimidyl ester labeling and SEA proliferation assays
5.6-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR, USA). CFSE was dissolved in anhydrous reagent grade dimethyl sulfoxide, sealed under nitrogen and stored desiccated at −20°C. It was determined that each new preparation of CFSE
must be titrated to obtain optimal staining results. Briefly, equal volume of concentrations between 0.5 and 5 μM CFSE was added to 2 × 10^7 PBMCs and incubated with gentle mixing at room temperature for 10 min. The reaction was quenched by the addition of an equal volume of FCS and cells were washed three times with PBS containing 5% FCS. The cells were cultured at 37°C at 5% CO₂ at 1.5 × 10^5 ml⁻¹ in RPMI containing 3% FCS overnight and the fluorescence intensity of cells treated with various concentrations was determined by FACS analysis. The optimal CFSE concentration was defined to be the one at which all cells were stained with fluorescein at a fluorescence intensity between 10^3 and 10^4 log units in flow cytometry. It was determined that the fluorescence intensity decreases significantly between the time of staining and the next day, so all titration results and experimental data were obtained only after 24 h of incubation at the above mentioned conditions. In our experiments, the CFSE concentration used varied between 0.5 and 1.25 μM CFSE.

For cell-division assays, CD69+/CD25– PBMCs (1–2.5 × 10^5) were cultured with SEA or PHA in 1 ml in 24-well plates. Cells were analyzed every 12 h by staining with anti-CD4–PerCP and either anti-Vβ-PE or anti-CD25–PE antibodies on ice and compared with non-stimulated cells. All analyses were gated on live CD4+ T cells. CD69 expression was measured after 24 (at 10 pg ml⁻¹ to 1 ng ml⁻¹) or 48 h (10 pg ml⁻¹ and below) and the levels of CD69 expression were compared with non-stimulated cells stained with anti-CD4–PerCP and anti-Vβ-FITC. Flow cytometry analyses were performed on a FACScan using CellQuest software (Becton Dickinson). For analyses involving anti-Vβ antibodies, at least 150 000 events were acquired for each condition. The average cell-division number (ADN) was calculated by taking the peak number of events (p) within each division peak (n) from n = 0 to n = Nth division, according to the following equation:

\[
\text{ADN} = \frac{\sum_{n=0}^{N} (p_n \cdot n)}{\sum_{n=0}^{N} (p_n)}
\]

**TCR down-regulation**

The human EBV-transformed B cell line LG-2 (DR1/DR1) was provided by Larry Stern (Massachusetts Institute of Technology) and has been described earlier (27). LG-2 cells (2 × 10^5) were pre-incubated with twice the indicated concentration of SEA in 100 μl for 3–4 h in 96-well round-bottom plates to allow the toxin to bind to MHC class II molecules at solution equilibrium. Human peripheral blood lymphocytes (PBLs) were obtained by gently centrifuging the plates (200 g, 30 s). The conjugates were incubated at 37°C for 4 h, after which TCR internalization was stopped by quick incubation on ice. Cells were stained on ice using a PE-conjugated anti-CD4 antibody (Becton Dickinson) and the relevant anti-Vβ antibody, and analyzed by flow cytometry on a FACSScan using the CellQuest software (Becton Dickinson). The percentage of Vβ-specific internalization was determined by calculating the ratio of the mean fluorescence of SEA-stimulated cultures over the mean fluorescence of un-stimulated cultures after 4 h incubation.

**Results**

*Early-activation markers up-regulation following stimulation with different concentrations of SEA*

In our experiments, we have used the Sag SEA produced by *Staphylococcus aureus* and which binds the human MHC class II molecule HLA-DR1 with high affinity (31). T cell activation by SEA can be evidenced by measuring the induction of the CD69 activation marker as well as the up-regulation of the IL-2R α chain (CD25). The details of the kinetics have been described elsewhere (28). CD69 and CD25 expression constitute the earliest and least stringent markers for T cell activation and results, including our own, have shown that their expression on T cells represents a reliable marker of previous cognate antigen exposure (own unpublished data) (32, 33). CD69 and CD25 expression were measured 24 and 48 h following exposure to ligand. These two time points were chosen based on a preliminary determination of the timing of peak marker expression at each of the concentrations tested. Under increasing concentrations of SEA, up-regulation of these markers follows a typical progressive dose–response curve similar to the response to any other typical TCR ligand (Fig. 1A). At the lowest concentration tested (0.01 pg ml⁻¹), we could detect a significant induction of CD25 and CD69 in a small fraction (~5%) of CD4 T cells. Of note, our preliminary observations indicate that most likely the same conclusions apply to CD8+ T cells unless otherwise stated (data not shown). As the dose was increased up to 1 ng ml⁻¹, additional T cells reached the activation threshold. Importantly, higher doses of SEA (e.g. 100 ng ml⁻¹) did not lead to a significant increase in the fraction of CD4 T cells expressing CD25 and CD69 (data not shown).

It is well described that SEA can activate T cells bearing different TCRVβ families. We next wanted to determine if the decrease in the proportion of CD25- and CD69-expressing cells at lower SEA doses is due to the specific exclusion of certain Vβ from the activated pool or due to a general failure of T cells bearing different Vβ (i.e. stochastic) to reach the activation threshold. To discriminate between these two non-exclusive possibilities, we examined the TCRVβ expression of SEA-activated T cells using a wide panel of antibodies available. We measured the peak expression (after 24 h) of the CD69 activation marker in response to an optimal dose of SEA (1 ng ml⁻¹; 10⁻¹¹ mol l⁻¹). This analysis allowed us to identify a majority of the SEA-responsive TCRVβ families against which mAbs are available (TCRVβ1, 5.2, 5.3, 9, 16, 21.3, 22, 23) (Fig. 1B). As shown in Fig. 1(C), decreasing the concentration of SEA to 0.5 and 0.01 pg ml⁻¹ leads to the selective exclusion of some Vβ from the activated (i.e. CD69+) population. For example, whereas Vβ22+ T cells express CD69 at all the concentrations tested, Vβ9+ T cells failed to up-regulate the activation marker when the dose is reduced to
and 0.5 pg ml⁻¹ obtained in at least four different blood donors tested. The data were obtained after 48 h of stimulation, corresponding to the time of maximal CD69 expression previously determined for CD4+ T cells. The expression of CD69 was determined at 0.01 pg ml⁻¹ (mean fluorescence value, plain squares) within a large panel of TCRVβ families covering ~60% of the CD4+ T cell repertoire, as determined 24 h post-stimulation. The percentage of each Vβ family within the population of CD4+ cells is also indicated (open circles). (C) The expression of CD69 was determined at 0.01 pg ml⁻¹ (left panel) and 0.5 pg ml⁻¹ (right panel) SEA for different TCRVβ when gating on CD4+ T cells. The data were obtained after 48 h of stimulation, corresponding to the time of maximal CD69 expression previously determined for these two SEA concentrations. Similar data were obtained in at least four different blood donors tested.

0.01 pg ml⁻¹. We obtained similar data by measuring TCRVβ-specific CD25 up-regulation (data not shown).

T cell stimulation by a sub-optimal ligand avidity leads to a slower onset of activation and to the recruitment of lower numbers of precursor T cells in the proliferative pool

We next wanted to determine if the dose-dependent exclusion of T cells from the activated pool was also occurring when assessing T cell proliferation. Similar to CD25 and CD69 expression, T cell proliferation follows a typical dose–response curve. Indeed, SEA concentrations as low as 0.001–0.01 pg ml⁻¹ (10⁻¹⁰–10⁻¹¹ mol l⁻¹) were sufficient for T cell activation, whereas maximal stimulation occurred at an optimal dose of 1 ng ml⁻¹ (10⁻¹⁰ mol l⁻¹) (Fig. 2A). Within this range of concentrations, the number of SEA molecules presented to T cells is linearly related to the concentration of SEA in solution (28).

In the following experiment, human T cells were stimulated, in the presence of autologous APCs, by increasing doses of SEA ranging from a minimal (0.01 pg ml⁻¹) to an optimal (1 ng ml⁻¹) concentration. The kinetic of cell division was measured in activated (CD25+) CD4+ T cells by CFSE dilution. These experiments were performed under conditions where an excess of IL-2 (500 U ml⁻¹) was added to the culture medium, in order to avoid limiting cytokine effects resulting from the insufficient number of IL-2-producing cells at lower SEA concentrations. First, results presented in Fig. 2(B) clearly establish that the onset of cell division, for individual T cell precursors, was slower with decreasing doses of SEA ligand. In order to get a more objective indication of the progression of cell division in activated T cells, a population-based ADN was calculated from the CFSE intensity of each individual flow cytometry event acquired (see Methods for details). Data illustrated in Fig. 2(C) enable us to determine that the average timing of the first division event in cells was significantly delayed (~36 h) when stimulating with a minimal SEA concentration of 0.01 pg ml⁻¹ as compared with stimulation using an optimal dose. The timing of cell cycle entry is thus directly dependent on the amount of ligand encountered by the T cell and correlates with the timing of induction of other T cell activation markers (data not shown). Importantly, Fig. 2(C) shows that only the very first division event was delayed at lower doses, as subsequent division events (n > 1) occurred at a remarkably similar rate regardless of the initial amount of antigen. This conclusion is based on the comparison of the slopes of the ADN plotted as a function of time for each condition. These results suggest that once cycling, activated T cells divide in an ‘antigen-independent’ manner, an interpretation that is consistent with earlier data demonstrating that the rate of T cell division is limited only by the kinetic of the amount of antigen. This conclusion is based on the comparison of the slopes of the ADN plotted as a function of time for each condition. These results suggest that once cycling, activated T cells divide in an ‘antigen-independent’ manner, an interpretation that is consistent with earlier data demonstrating that the rate of T cell division is limited only by the kinetic of the first division event (34, 35). The average doubling time was 18.5 ± 7.8 h on a Gaussian distribution, corresponding to ~1.3 division per day. As such, it is conceivable that a delay of ~36 h, which would correspond to about two divisions when comparing the lowest concentration with the optimal concentration of SEA (Fig. 2C), will have a noticeable effect on the peak amplitude (i.e. at least a 4-fold variation in the total cell number) of the response burst following an exponential division process.

Narrowing of the T cell repertoire diversity selected on a sub-optimal ligand concentration

The expansion of different TCRVβ families was next studied under conditions of sub-optimal doses of SEA, ranging from 0.01 pg ml⁻¹ to 1 ng ml⁻¹. Given the low, but readily detectable, abundance of some of these Vβ families, we took care to obtain a homogenous population of resting T cells by depleting pre-activated (CD69+/CD25+) T cells from the population (see Methods). Indeed, we have observed that ~1% of T cells expressed either of these markers in a typical,
freshly isolated sample of human PBLs (our unpublished data). A typical example is shown in Fig. 3(A) and compares the response of TCRV\textsubscript{b1} with that of TCRV\textsubscript{b22} to different concentrations of ligand. Indeed, selection of the repertoire by low doses of TCR ligand this time led to an asynchronous expansion of the different TCRV\textsubscript{b} populations, as opposed to expansions obtained at an optimal dose (see above and Fig. 3A at 1 ng ml\textsuperscript{-1}). In Fig. 3(A), the time of recruitment of V\textsubscript{b22+} T cells into cell cycle was already maximal at 1 pg ml\textsuperscript{-1}, whereas a 1000-fold more SEA was required to trigger a similarly fast response in V\textsubscript{b1+} T cells. Significant proliferation of TCRV\textsubscript{b1+} cells could not be detected below an SEA concentration of 1 pg ml\textsuperscript{-1} when the population was followed for up to 10 days (data not shown). Most likely, SEA concentrations below 1 pg ml\textsuperscript{-1} were insufficient to break the activation threshold of V\textsubscript{b1+} cells. Consequently, stimulation with SEA concentrations as low as 0.05 pg ml\textsuperscript{-1} led to a prompt cell cycle entry of TCRV\textsubscript{b22}, but no significant division was detected in TCRV\textsubscript{b1} cells.

The behavior of individual TCRV\textsubscript{b} families, in fact, mirrors that of the entire population of responding T cells described in the results of Fig. 2. Indeed, all TCRV\textsubscript{b} families proliferated at the same invariable rate once in the cell cycle. However, the onset of the first division was delayed to an extent that was directly dependent on the amount of ligand encountered. In the next series of experiments, the results of several reproducible kinetics were analyzed using the same methodology described in Fig. 2(C) and the timing of the first division event (i.e. the time when >50% of cells having divided at least once) was determined for each condition within different TCRV\textsubscript{b} subsets. As shown in Fig. 3(B), TCRV\textsubscript{b1} cells did not start to divide until a minimal SEA concentration of 1 pg ml\textsuperscript{-1} was reached. Remarkably, at increasing ligand concentrations, the first division event within V\textsubscript{b1} T cells was still significantly delayed relative to other TCRV\textsubscript{b}s, and this, until the optimal amount of ligand (i.e. 1 ng ml\textsuperscript{-1}) was reached. In contrast, TCRV\textsubscript{b22+} and V\textsubscript{b23+} cells were recruited in the dividing pool at a concentration as low as 0.01 pg ml\textsuperscript{-1} and the onset of division was nearly maximal at concentrations of 0.05 pg ml\textsuperscript{-1}. Similar observations were made within other TCRV\textsubscript{b} subsets tested, albeit each with a different threshold of activation. Importantly, these differences in sensitivity of
Fig. 3. A comparison of the effect of ligand avidity on the kinetics of cell division of different Vβ families. (A) CFSE-stained human PBMCs were stimulated with increasing doses of SEA, up to the optimal 1-ng ml⁻¹ dose. Cells were analyzed by flow cytometry at intervals of 12 h for a period of up to 10 days in order to compare the kinetics of cell division between two different TCRVβ families: TCRVβ1 and TCRVβ22 (gated on CD4+ T cells). Representative dot plots are shown at time points day 4.0, 5.0 and 5.5. The events were analyzed on CD4+ T cells, whereas the histograms (day 5.0) show the corresponding CFSE profile when gating on CD4+/Vβ+ cells. (B) The kinetics of cell division in different SEA-responsive TCRVβ cells were followed as described in Fig. 4, and for each conditions, the time when (sampling at 12-h intervals) a majority (>50%) of CD4+/TCRVβ T cells had gone through their first division event was determined in order to define the timing of recruitment of these cells in the dividing population. White squares (no division) indicate that no significant division could be detected after up to 10 days in culture. The results were consistent in kinetics performed with T cells purified from at least three different blood donors.
different TCRVβ-bearing T cells are not a consequence of differences in levels of TCR expression (data not shown). These results illustrate two distinct effects of ligand avidity on the composition of the immune repertoire. First, as the amount of ligand is limiting, only cells having a lower activation threshold (high functional avidity) are recruited in the response, resulting in a narrowing of the potential diversity of the selected T cell repertoire. Second, as the ligand concentration is increased to break the threshold of activation of individual T cells, non-dividing potential responder cells (i.e. those with the highest activation threshold or low functional avidity) eventually get recruited in the response, but their entry in the dividing pool is delayed. This leads to an ‘expansion drift’ phenomenon by which the relative representation of each responder will change over time until all the potential responders, for that particular concentration, have gone through their first division event. These effects are curtailed when an optimal TCR ligand concentration is reached, giving rise to a broader repertoire expanding synchronously and which includes the potentially reactive T cell clonotypes. This is the first direct demonstration of the existence of a hierarchy in the sensitivity of different TCRVβs for a Sag. This hierarchy most likely reflects different functional avidity of each SEA-reactive human TCRVβ family cells and is in order of decreasing avidity: Vβ22 > Vβ23 > Vβ5.3 > Vβ16 = Vβ9 > Vβ21.3 = Vβ1.

Synchronous T cell expansion but different cytokine secretion ability of the T cell clonotypes at an optimal ligand dose

Under conditions of optimal amount of SEA ligand (i.e. 1 ng ml\(^{-1}\)), the onset of the first division occurred at remarkably similar times in each of the different responsive TCRVβ subsets, regardless of possible differences in TCRVβ affinity for the Sag. This rate was also comparable to the levels of stimulation achieved by PHA (see Fig. 2B) and likely reflects a constant and invariant average doubling time for activated T cells, consistent with previously published data (29). Under these conditions, the diversity of the repertoire selected remained broad and the expansion of individual TCRVβ families occurred synchronously as determined from comparing the CFSE profiles of each different Vβ family after up to 10 days post-stimulation (Fig. 4A and data not shown). In other words, no significant difference in the proliferative rate was observed in expanding SEA-responding TCRVβ family, one relative to another, suggesting a remarkable stability of the repertoire selected following stimulation with an optimal concentration of ligand.

It is well established that the activation threshold to elicit diverse effector functions are different. For example, cytotoxic killing and CD25 and CD69 up-regulation are triggered at much lower antigen concentrations and by weaker ligands as compared with proliferation and cytokine production (32, 33, 36). Moreover, it has been shown that the threshold for IL-2 secretion is higher than for T cell proliferation (37–39). To verify if the different responsive TCRVβs reach similar activation thresholds following stimulation with SEA, we compared their capacity to produce IL-2 by intracellular staining. As illustrated in Fig. 4, a much higher fraction of TCRVβ22+ T cells secrete IL-2 as compared with the other TCRVβ+ T cells. The hierarchy established among the TCRVβ families (see Fig. 3) is also maintained when comparing IL-2 production with Vβ22 > Vβ5.3 > Vβ21.3 > Vβ1. Interestingly, even at the ‘optimal’ (1 ng ml\(^{-1}\)) SEA concentration (i.e. at which all responsive TCRVβ proliferated synchronously), large differences in the frequency of cytokine-producing cells are observed between the different TCRVβ cells. These results suggest that immediate IL-2 secretion requires a stronger TCR stimulus as compared with proliferation and demonstrate that proliferating T cell clones differ in their ability to mediate effector functions.

The ligand avidity activation threshold for each TCR is primarily determined by the efficiency of TCR engagement

In previous studies, it was established that the degree of TCR internalization is a reliable correlate of the avidity of TCR-ligand interactions (40–43). We compared kinetics of TCR down-regulation in CD4+ T cells within different TCRVβ subsets, in order to verify whether the lack of recruitment of some cells in the proliferative pool correlated with the efficiency of TCR engagement. In the following experiments, TCRVβ1, 21.3 and 22 were tested because they are easily detected, as they are more abundant and thus easier to follow, in a typical human CD4+ repertoire (see Fig. 1B). Also, they...
are representative of the continuum of responses to different SEA concentrations (see Fig. 3B). In all SEA-responsive TCRV\(\beta\)s tested, the internalization of TCRs progressed homogeneously in >85% of the cells within the first hour and then remained stable for at least 24 h (data not shown). Remarkably, a smaller dose of SEA was required to induce a very strong and stable internalization in V\(\beta\)22+ T cells (Fig. 5). This TCRV\(\beta\) family indeed has the lowest activation threshold for SEA (see Fig. 3). In contrast, little TCR down-regulation was detected in the subsets having the highest activation threshold, namely V\(\beta\)21.3 and V\(\beta\)1, even at the highest concentration of SEA (~25% TCR internalization with 100 ng ml\(^{-1}\)). No significant TCR internalization was detected in the TCRV\(\beta\)2+ cells that do not proliferate in response to SEA (see Fig. 3B). The correlation between the massive TCR down-regulation in V\(\beta\)22+ T cells and their ability to secrete IL-2 is in agreement with previously published results demonstrating that cytokine production is only triggered in T cells that show substantial TCR internalization (32, 44). Therefore, the efficiency of TCR engagement appears to be a strong correlate of the ability of each TCRV\(\beta\) subset to reach activation and of the hierarchy of response of these cells to increasing doses of ligand.

**Discussion**

In this paper, we dissect the relationships between timing, amplitude and diversity of individual expanding T cell populations evolving within a nascent T cell response triggered by limiting amounts of TCR ligand. The use of Sags has allowed us to target a higher frequency of expanding precursors since the structure of the TCRV\(\beta\) region recognized by Sags is predictable and much less variable, thus avoiding sensitivity problems. But most importantly, we can anticipate T cell populations with predictable specificity, such that they can be probed using available anti-TCRV\(\beta\) antibodies (22).

Using this system as a model has enabled us to make novel interesting observations. Herein, we directly demonstrate that the density of ligands displayed on the surface of APCs strongly impacts the size as well as the potential diversity of the response selected from a pre-established set of antigen-specific T cell precursors. Cells having the highest avidity for that particular ligand dominate the T cell repertoire and the hierarchy of selection is most likely determined by the efficiency of TCR engagement, independent of the distribution of these populations (the percentage of each TCRV\(\beta\) in healthy donors) in a non-immune repertoire. We did not observe any correlation between the frequency of TCRV\(\beta\) cells in a non-immune repertoire and their position in the hierarchy of response to increasing doses of ligand. In fact, populations such as TCRV\(\beta\)1+ cells are abundantly represented in the SEA-responding population, in proportions comparable to TCRV\(\beta\)22+ cells (see Fig. 1B), despite a dramatic difference for their sensitivity of activation. In contrast, TCRV\(\beta\)23+ is one of the least abundant populations and yet is one of the most sensitive to low amounts of SEA (Fig. 3B). Of note, as is the case for TCRV\(\beta\)22+ cells, TCR down-regulation in TCRV\(\beta\)23+ cells was indeed very prominent (data not shown). A competition effect due to the relative abundance of T cell precursors competing for a limited surface of antigen presentation will most likely occur, especially when competing T cells have comparable avidity for the ligand. However, the hierarchy of recruitment of each T cell population in the response pool will mostly depend on the relative efficiency of TCR engagement leading to full T cell activation (42, 45, 46).

Our interpretation is based on the differential kinetics and the extent of TCRV\(\beta\) down-regulation which have been shown to directly correlate with the TCR–ligand affinity and with the biological outcome, both in antigen and Sag models (40–43, 47, 48). Using different murine T cell models, it has been demonstrated that T cells compete for access to MHC–peptide complexes at the surface of APCs (49–52). This competition favors the activation and expansion of high-affinity T cells, especially when the number of complexes is low (17, 49, 53, 54) and may explain the lack of direct correlation between naive T cell precursor frequency and the distribution in the activated pool following T cell proliferation (50, 55). Moreover, the T cell-driven down-modulation of MHC–ligand complexes at the surface of APCs has been shown to contribute to inhibiting the response of lower affinity T cells (45, 56, 57). The massive TCR internalization observed in the V\(\beta\)22+ T cell population (see Fig. 5) might reduce the number of MHC–SEA complexes at the surface of APCs, thereby limiting the availability of ligands for the lower affinity T cells (such as V\(\beta\)1+ T cells). This phenomenon of ligand depletion could delay or even inhibit the response of low-affinity T cells, especially when the number of MHC–SEA complexes at the surface of the APCs is low.
However, depletion of high-affinity Vβ22+ T cells from the cultures does not appear to significantly shift the dose-response of other responsive TCRVβs (A.R.D. and R.P.S., unpublished data), suggesting that the phenomenon of competition does not play a major role in determining the reactivity of lower affinity T cells in our model. According to our observations, the extent to which a response focuses on high-affinity T cells will directly be dependent on the availability of the MHC–ligand; this is in agreement with experiments showing an inverse relationship between the antigen dose and the affinity of the T cell population following a primary response (17).

We establish a set of principles that define the execution of cellular events leading to the selection of an immune repertoire. First, under conditions of optimal ligand stimulation, each of the different T cell populations is recruited in the selected repertoire with comparable kinetics despite differences in their avidity for the ligand. Second, all activated T cells divide at a constant and invariant rate that was determined to be ~1.3 division per day, regardless of stimulating ligand concentration or TCR affinity. According to our results, the dominance of a T cell precursor was accounted for only by the timing of recruitment in the dividing pool and not by differences in their rate of expansion.

Lastly, we observed that both the timing and frequency of recruitment of cells in the dividing pool are directly dependent on the amount of ligand exposure, until optimal concentrations are reached. Clearly, the frequency of a T cell clonotype at the peak of the response will depend on the time at which a naive precursor first encountered its cognate antigen. This was proposed in a number of studies, including one which addressed the timing of a response to temporally distinct exposures to a single antigen (55, 58). How can a simultaneous exposure to the same antigen distinctively affect the expansion of two structurally different T cell precursors? We have defined two processes and distinguished them by using the terms ‘expansion drift’ and ‘activation bias’ because they clearly have a different impact on either the distribution or diversity of an immune response. These two processes, which are readily understandable in the context of currently proposed models of T cell activation, are of course clearly functionally related. Indeed, the avidity-related differences in the kinetics at which cells enter the proliferative pool (herein referred to as expansion drift) likely reflect the longer time interval required to sequentially engage a particular number of individual TCR molecules necessary to overcome a given activation threshold or to stabilize the immunological synapse (59, 60).

Essentially, the contribution of this first mechanism alone will influence the temporal distribution of the repertoire but will not have a significant impact on the final diversity of the response as all the potentially responsive cells will eventually get recruited in the dividing pool, albeit at different times. On the other hand, the process referred to as activation bias (selective activation of specific TCRVβ-bearing T cells at different doses of ligand) will have a definite impact on the final diversity of the repertoire as cells of lower avidity are ultimately excluded from the dividing pool. Hence, the relative contribution of each phenomenon will determine the quality of the repertoire generated.

Results from the literature do suggest that a limiting display of ligand can significantly influence the course of an immune response possibly by decreasing both the frequency and the diversity of clones recruited in the immune response, as exemplified in this paper (15, 61–66). Similarly, others have proposed that the survival of a functionally diverse set of T cells might also be limited by clonal exhaustion to persistently supra-optimal levels of antigen exposure (67, 68). Furthermore, in vivo and in vitro studies have demonstrated that T cells stimulated with excessive doses of ligand show impaired functional responses and increased propensity to undergo apoptosis (28, 63, 64, 69–71). This apparent inability of the immune system to either establish or maintain a diversified repertoire outside a relatively narrow range of ligand concentrations may have important functional implications for a rational design of vaccination strategies. In some instances, the structural diversity necessary for induction of a protective immunity may critically depend on the capacity of the delivery vector employed and/or the antigen-processing machinery to set the density of epitopes within a particular window (72, 73).

What is the importance of selecting a structurally diverse repertoire? It may be to the benefit of the immune system to present a repertoire with the broadest spectrum of affinities to a particular epitope (74). The high-avidity T cells which can be triggered by very low amounts of antigen may not be able to persist under conditions of excessive antigen load (69, 71, 75). On the other hand, dilution of a protective ‘high-affinity’ T cell response by less potent, ‘low-affinity’ T cells may adversely impact the total potency of the response and represent a costly trade-off to a broader repertoire diversity (76). Additional studies should allow better understanding of the importance of T cell repertoire affinity and diversity in the context of host–pathogen interactions.

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Abbreviations

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<tr>
<th>Acronym</th>
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<tr>
<td>ADN</td>
<td>average cell-division number</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CFSE</td>
<td>5,6-carboxyfluorescein diacetate succinimidyl ester</td>
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<tr>
<td>[3H]Tdr</td>
<td>[3H]thymidine</td>
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<tr>
<td>PBL</td>
<td>peripheral blood lymphocyte</td>
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<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
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<td>Sag</td>
<td>superantigen</td>
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<td>SEA</td>
<td>staphylococcal enterotoxin A</td>
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References

TCR repertoire selected on low-avidity ligands


