Relationships Among TCR Ligand Potency, Thresholds for Effector Function Elicitation, and the Quality of Early Signaling Events in Human T Cells[1]

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Determining how receptor ligand quality and quantity together control the biologic responses of T cells is central to understanding normal and pathologic T cell immunity. Here we have carefully examined how variations in antigenic peptide structure and dose affect multiple functional responses of human T cell clones and have correlated these observations with proximal TCR signaling events induced by the same set of related ligands. As the Ag concentration increases, effector functions are elicited according to a clone-specific hierarchy. The absolute amount of each peptide required to stimulate the entire set of effector functions (potency) differs markedly among ligands for a single TCR, correlating with the efficiency of TCR down-modulation and the extent of ZAP-70 activation. However, distinct patterns of TCR ζ-chain phosphorylation were observed, with the ratios of TCRζ isoforms relating to ligand agonist potency. The appearance of partially phosphorylated TCRζ isoforms was paralleled by relative changes in certain response thresholds within the hierarchy. Thus, a combination of density, potency, and quality of signaling all contribute to the distinct effects of agonist ligands on T cell immunity. ***The Journal of Immunology,*** **1998, 160: 5807–5814.**

T lymphocytes recognize peptides derived from intracellularly processed protein Ags presented in the context of MHC (HLA) molecules (1, 2). Many studies of T cell activation have assumed that recognition of these complex antigenic ligands leads to simultaneous, proportional induction of all functions of a T cell. Experiments using TCR ligands altered in either the peptide or MHC molecule component have recently challenged this concept (3–7). These studies have identified modified peptide/MHC molecule combinations that selectively activate (partial agonists) or inhibit (antagonists) specific effector responses of $CD4^+$ or $CD8^+$ T cells (8–18). Altered TCR ζ-chain phosphorylation patterns and association of ZAP-70 with the ζ-chain without detectable kinase activation were seen in murine T cell clones (TCC)[3] upon exposure to TCR antagonists (19, 20) or partial agonists (21, 22). Because this specific pattern of TCR subunit phosphorylation and associated kinase activation was not seen at any concentration of wild-type ligand, the change in the proximal signaling pattern was considered responsible for the qualitatively altered functional responses. Other data suggest, however, that even for a conventional agonist TCR ligand, the elicitation of different effector/cytokine responses may not be proportional at low and high Ag densities. These more recent observations concerning the effect of Ag concentration on the quality of T cell responses, especially polarization to the Th1 vs Th2 phenotype (23–25), may explain older reports on the humoral vs cell-mediated response bias in animals administered widely different amounts of Ag (26).

Most studies examining these issues have been performed using mouse models and TCC, and only limited data are available concerning the influence of ligand structure and amount on the response phenotype of human T cells (27–31). Even less is known about the TCR signaling events that occur in human T cells when exposed to variant ligands (27). Determining how the quality and the quantity of a TCR ligand together regulate the multiple potential responses of T cells is critical to understanding what controls not only the extent but also the humoral vs inflammatory character of immune responses. Information on human lymphocytes is particularly important to provide a rational basis for understanding the pathogenesis of autoimmune diseases and for designing Ag-dependent interventions in these diseases or for vaccine purposes.

For these reasons, we have systematically examined the relationship between Ag concentration and the induction of a large number of different effector functions characteristic of human $CD4^+$ TCC. These functional studies have been complemented by the measurement of TCR down-modulation as an indicator of TCR engagement and by an analysis of early TCR-associated biochemical signaling events. These parameters have been assessed using for each TCC a series of related receptor ligands. The stimuli include the prototypic agonist as well as ligands with higher (superagonists) or lower (weak agonists) potency or some that function as antagonists (18, 32, 33).

Based on a comparison of Ag dose responses for various effector functions, we demonstrate a hierarchical arrangement of response thresholds for each clone. Alterations in ligand structure result in a general shift in the set of response thresholds (potency change) that correlates with the degree of phosphorylated ZAP-70 kinase and the extent of TCR down-modulation induced by each ligand at

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Received for publication December 4, 1997. Accepted for publication February 13, 1998.

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[1] This work was supported by a postdoctoral fellowship (He 2386/1-2; to B.H.) and a Heisenberg Fellowship from the Deutsche Forschungsgemeinschaft (Ma 965/4-1; to R.M.).

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[3] Abbreviations used in this paper: TCC, T cell clone; MBP, myelin basic protein peptide.

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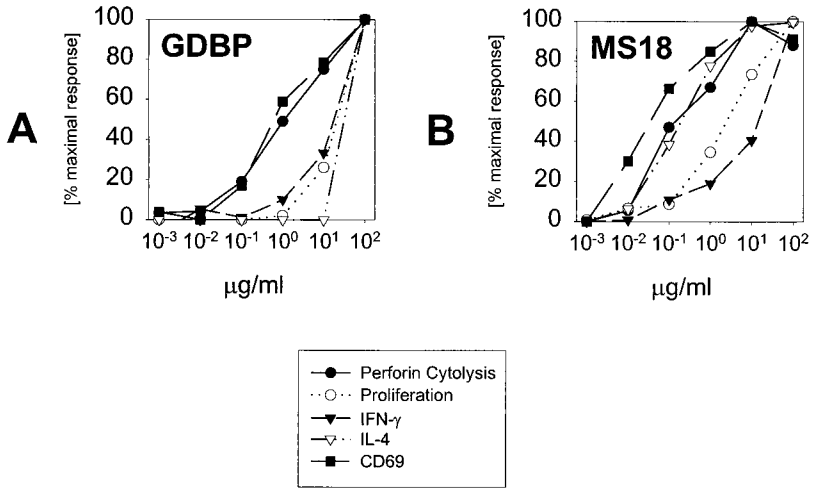

FIGURE 1. Hierarchical arrangement of Ag concentration thresholds required for elicitation of perforin-mediated cytolytic activity, proliferation, CD69 up-regulation, IL-4 secretion, and IFN-γ secretion by human $MBP_{87–99}$-specific TCC. TCC GDBP and MS18 were tested for their responses to different concentrations of $MBP_{87–99}$. The measured response curves for different effector functions were compared by normalizing the results as a percentage of the maximal response seen in the assays. To induce a half-maximal response, a difference up to 3 orders of magnitude was found among the different functions. All experiments in Figure 1 and the following figures were performed at least three times.

comparable peptide concentrations. In addition, shifts in the relative positions of some response thresholds and altered patterns of ζ-chain phosphorylation are seen with the less potent ligands. Thus, the selective activation effects reported for the latter peptide-MHC molecule combinations may reflect both the potency change that makes some responses difficult to elicit at achievable Ag densities and the relative changes in response thresholds within the hierarchy.

Materials and Methods

Cells and Ags

Myelin basic protein peptide 87–99 ($MBP_{87–99}$)-specific TCC were generated, characterized, and maintained as previously described (34). The restriction elements used by the TCC are DRB5*0101 for MS18 and DRB1*1302 for GDBP. Clonality was shown by RT-PCR amplification using 21 Vα and 22 Vβ family-specific primers (34). TCR usage is Vα23/Vβ21 for GDBP and Vα3.1/Vβ5.2 for MS18. EBV-transformed B cell lines GP-B (HLA-DR1*0404/1*1302) and AF-B (HLA-DR1*1501/5*0101) were used as APCs in all assays. The peptide $MBP_{87–99}$ (one-letter code, VHFFKNIVTPRTP) and its variants (indicated as the original residue at position X, followed by the substituted residue, e.g., T95-A) were synthesized using Merrifield's solid phase methodology as previously described (35).

Functional and protein expression assays

For functional assays, TCC were rested for 8 days after Ag stimulation. All functional and biochemical experiments were conducted under the same conditions of T cell expansion and rest. For proliferation and immunofluorescence staining assays, irradiated GP-B and AF-B as APC (5000 rad) were incubated for 2 h with different concentrations of peptide ligands and washed twice. B cells (2×10^4) and T cells (5×10^4) were added to each well of 96-well round-bottom plates to a final volume of 200 μl (Iscove's modified Dulbecco's medium, 5% human serum, 1% glutamine, 1% penicillin/streptomycin, and 0.2% gentamicin). For proliferation assays cells were cultured for 48 h at 37°C. Fifty microliters of the supernatant of each well were removed for cytokine measurements and stored at −70°C. One microcurie [^{3}H]thymidine was then added to each culture well. After another 8 h cells were harvested, and incorporated radioactivity was measured by scintillation counting. For the detection of cytokines, supernatants from three wells for each concentration of the ligand were pooled, and the amounts of IL-4, IFN-γ, and TNF-α were determined by ELISA (BioSource, Camarillo, CA). Cells for immunofluorescence staining were harvested after 12 h, washed twice in PBS, and stained with FITC-labeled anti-TCR$\alpha\beta$ Ab, phycoerythrin-labeled anti-CD69 or anti-CD25 Ab (all from Becton Dickinson, San Jose, CA), or FITC- or phycoerythrin-labeled control IgG. Staining was analyzed on a FACScan (Becton Dickinson, Mountain View, CA) for 2000 gated events. For cytotoxicity (CTL) assays, target cells (5×10^5) were labeled overnight at 37°C in 1000 μl of CTL medium (RPMI, 5% FCS, and 1% glutamine) with 50 μCi of ^{51}Cr (DuPont-New England Nuclear, Boston, MA) and then incubated with different concentrations of peptide for 4 h, washed twice (200 × *g*, 20°C, 10 min), and adjusted to 2×10^4 cells/ml. Target cells (2×10^3) were plated into 96-well U-bottom microtiter plates containing 2×10^4 T cells. After 4-h incubation (37°C), supernatants were counted in a gamma counter (ME Plus, ICN Micromedic, Huntsville, AL). Specific lysis was calculated according to the following formula: (test release (cpm) − spontaneous release (cpm))/(total incorporation (cpm) − spontaneous release (cpm)) × 100. The data in Figure 1 are expressed as percentages of the maximum response. The 100% value in multiple experiments ranged from 43 to 62% (GDBP) and from 40 to 65% (MS18) for cytolysis, from 10,505 to 30,054 cpm (GDBP) and from 19,975 to 27,091 cpm (MS18) for proliferation, from 925 to 5,547 pg/ml (GDBP) and from 410 to 4,165 pg/ml (MS18) for IFN-γ, from 34 to 179 pg/ml (GDBP) and from 808 to 2,450 pg/ml (MS18) for IL-4, and from 158 to 234 (GDBP) and from 128 to 510 median fluorescence (MS18) for CD69 up-regulation.

Analysis of TCR-associated phosphorylation events

GP-B or AF-B APC (1×10^6/ml) were incubated for 2 h at 37°C at the indicated concentration of peptide, then washed twice. T cells (1×10^6/ml) were added and, after an 8-s spin in an Eppendorf centrifuge, were incubated for 5 min at 37°C and washed with PBS. The pellet was immediately solubilized for 30 min in ice-cold lysis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, complete protease inhibitor (Boehringer Mannheim, Mannheim, Germany), and 1 mM Na_3VO_4 (Sigma, St. Louis, MO). After removal of nuclear debris by centrifugation, the supernatants were analyzed by immunoprecipitation and immunoblotting. Rabbit antiserum to ZAP-70 (36) was used for immunoprecipitation. Immunoprecipitation and immunoblotting analysis were performed as previously described (37). An anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) was used for detection. Immunoblots were developed by SuperUltraSignal chemiluminescence (Pierce, Rockford, IL). Quantitative data were obtained from film exposures with a Molecular Dynamics laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

T cells activate effector functions according to an Ag dose-related hierarchy

We first explored the activation of different functional responses using two human Th0 $CD4^+$ TCC specific for the native self-peptide $MBP_{87–99}$. To facilitate quantitative comparison among the several measured response parameters, they were all expressed as fractional responses in relationship to the maximum absolute response observed.

As shown in Figure 1, different concentrations of the native peptide were required to activate distinct effector functions as well as to stimulate the expression of surface markers. With the clone GDBP, perforin-mediated cytolysis and CD69 expression were induced at lower peptide concentrations than proliferation, IFN-γ, or IL-4 production (Fig. 1*A*). In contrast, clone MS18 required low

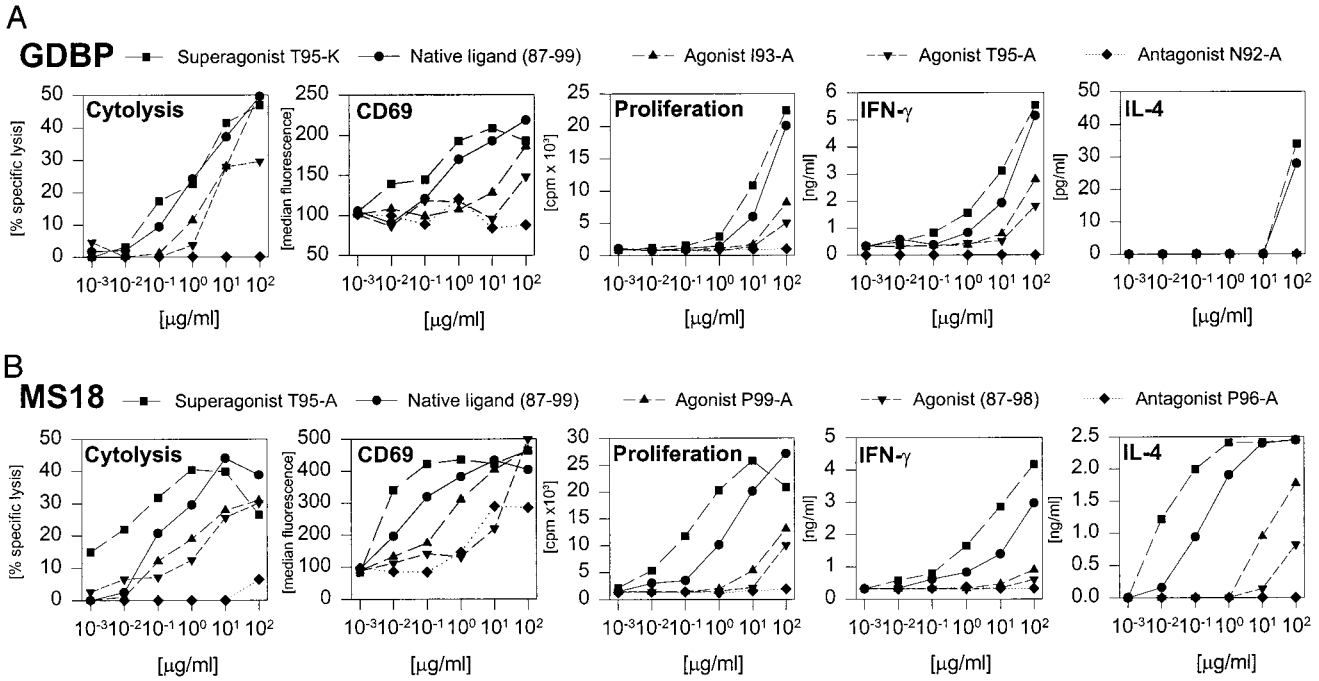

FIGURE 2. Native Ag $MBP_{87–99}$, superagonists, partial agonists, and antagonist ligands each differ in potency, but induce effector functions in a related hierarchical order. The responses of TCC GDBP (*A*) and MS18 (*B*) to different concentrations of the various Ags were measured and compared for different T cell functions.

levels of Ag for CD69 up-regulation, cytolysis, and IL-4 production, but up to 1000-fold more ligand for proliferation and IFN-γ secretion (Fig. 1*B*). Thus, effector functions were activated according to a clone-specific hierarchy and, at certain concentrations, even the native Ag could lead to selective activation of only some T cell functions. Interestingly, in these and other TCC, the order in the hierarchy was similar for certain functions, with perforin-mediated cytolysis and CD69 elicited first, followed by proliferation. In contrast, the relative hierarchical positions for certain cytokines differed among the clones in a stable manner.

Altered peptide ligands differ in their overall potency from the native ligand and alter the agonist-defined response hierarchy

For autoreactive $CD4^+$ TCC, modifications of native antigenic peptides that do not affect binding to the HLA-DR molecule can lead to the production of more potent (superagonist), less potent (weak agonist), or inhibitory (antagonist) ligands, as assessed by examining proliferative responses of the TCC (18, 32, 33). The demonstration of clone-specific response hierarchies, therefore, raised the question of whether the rank order of the entire set of response thresholds would be the same using such altered TCR ligands. Two TCC, GDBP and MS18, were studied in detail because of the availability of a well-characterized set of modified ligands for each. Superagonist (T95-K for GDBP; T95-A for MS18), weak agonist (I93-A and T95-A for GDBP; P99-A and truncated peptide 87–98 for MS18), antagonist/weak agonist peptide (P96-A for MS18) and antagonist peptide (N92-A for GDBP), as well as the native peptide $MBP_{87–99}$ were tested for their abilities to induce different T cell functions (Fig. 2). In general, the potency change seen for proliferative responses was found for all responses to a given ligand. The antagonist peptide N92-A did not elicit any T cell function in GDBP. The antagonist-weak agonist peptide P96-A induced marginal up-regulation of CD69 and some cytolysis in MS18, the two functions requiring the least amount of peptide for their induction by agonist ligands (Fig. 2*B*). The perforin-mediated cytolysis observed with these clones does not require gene transcription, and activation of this effector mechanism might indeed be expected to be less stringent than cytokine production in terms of signal quality, level, and duration.

However, superimposed on this overall potency shift are changes in the relative hierarchical positioning of the different effector response thresholds. For example, only a small (<1 order of magnitude for GDBP and 2 orders of magnitude for MS18) difference was observed in the Ag requirements of the different agonist ligands to induce cytolytic function of either TCC, whereas a much bigger differences in the required dose was seen for the other functions (i.e., CD69 up-regulation or cytokine secretion; Fig. 2). Such changes in the relative ligand requirements for two events evoked by engagement of a single receptor are characteristic of a class of ligands traditionally defined as partial agonists.

The degree of TCR down-modulation reflects ligand potency

TCR/CD3 complexes are down-modulated after engagement with MHC molecule/peptide ligands, providing a single cell measure of receptor engagement and, presumably, effective signaling (27). We therefore evaluated TCR expression after stimulation of the clones by different ligands. In both TCC, TCR down-modulation occurred homogeneously on all T cells rather than on a fraction of the cell population (Fig. 3*A*). The experiments demonstrated a clear relationship between TCR down-modulation and the concentration and nature of the ligand. As expected, for each ligand, down-modulation increased with increasing ligand concentration. More importantly, the hierarchy of ligand potency in functional assays (superagonist $>$ native peptide $>$ partial/weak agonist $>$ antagonist) was reflected in the extent of TCR loss at equivalent Ag concentrations (Fig. 3, *B* and *D*). For each clone, the induction of a particular functional response appeared to require a specific level of TCR down-modulation regardless of the potency of the ligand and thus of the actual Ag concentration needed to achieve this level of

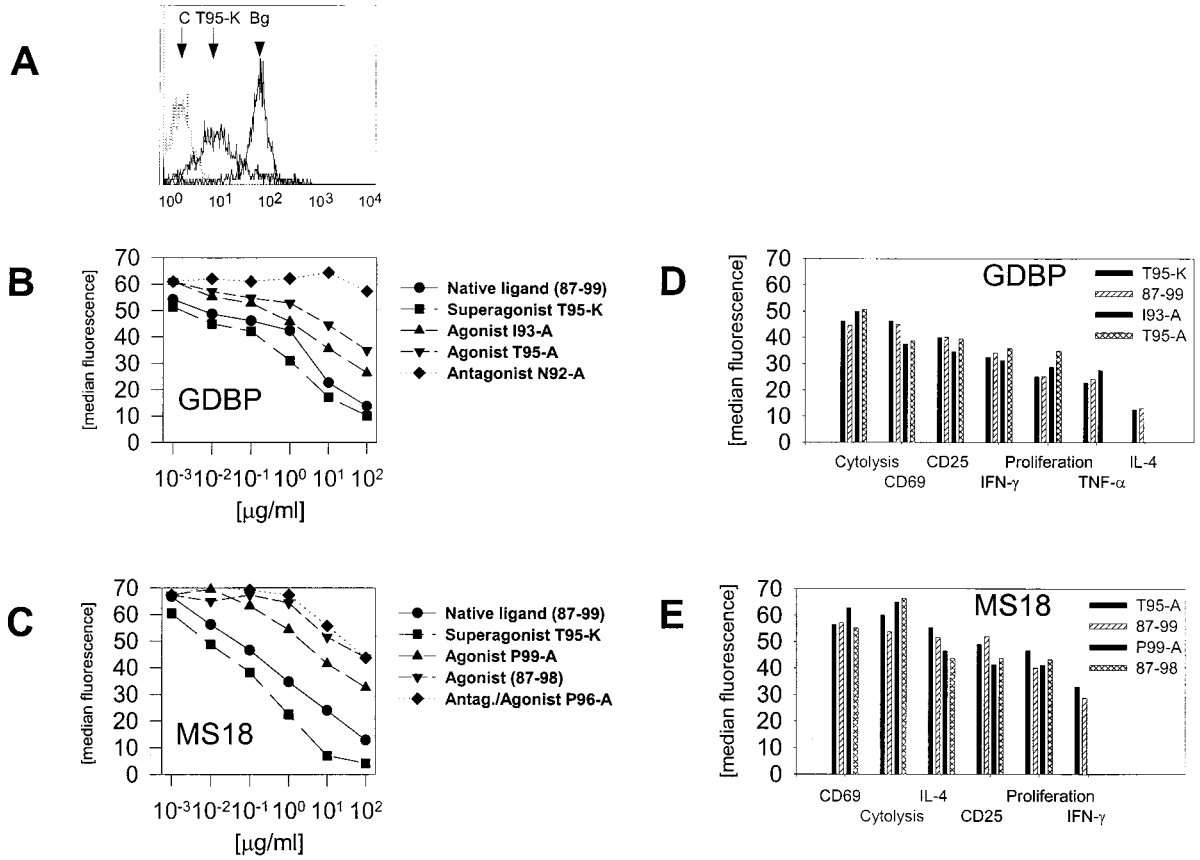

FIGURE 3. Diverse Ag concentrations lead to a similar extent of TCR down-modulation for different ligands, under which conditions similar functional responses are induced. Stimulation of TCC with various ligands results in a decrease in surface TCR expression on all cells in the TCC population (*A*; shown for superagonist T95-K and TCC GDBP). TCR down-modulation of GDBP (*B*) and MS18 (*D*) in response to the various ligands at different concentrations is shown. A comparison of TCR down-modulation and effector functions (obtained in the same experiment) for TCC GDBP (*C*) and MS18 (*E*) was performed. The level of TCR expression required to induce 20% activation of various effector functions is shown for different ligands. Although the ligands differ in potency, the levels of TCR down-modulation required to achieve a certain level of effector functions are comparable.

TCR internalization (Fig. 3, *C* and *E*). The extent of TCR down-modulation needed to elicit the various responses showed the same hierarchical relationship observed by titration of offered peptide. This provided direct evidence that the hierarchy reflects the amount of TCR signaling required for each response to be induced. Interestingly, TCR down-modulation occurred even at concentrations that induced no (GDBP; Fig. 2*A*) or little (MS18; Fig. 2*B*) functional activity, and even the antagonist/weak agonist P96-A (MS18) induced some receptor loss at very high concentrations, indicating that TCR down-modulation may in some cases be a more sensitive measure of engagement by ligand than are traditionally examined functional responses.

Correlation of ligand potency and dose with early signaling events

How do these findings concerning response hierarchies, ligand potency, and TCR down-modulation relate to previous data on qualitatively altered proximal TCR signaling in response to weak/partial agonists and antagonists (7, 20, 21, 38–40)? We have recently begun analyzing proximal TCR signaling events in human T cells in response to peptide/MHC molecule ligands and have demonstrated that several distinguishable phosphorylated isoforms of ZAP-70-associated TCRζ are produced in response to distinct ligands (I. Stefanova et al., manuscript in preparation). To determine the relationship between these patterns of proximal TCR signaling and the functional studies detailed above, we immunoprecipitated ZAP-70 from the lysates of stimulated TCC GDBP and MS18 and analyzed the level of tyrosine phosphorylation of ZAP-70 together with phosphorylation of the associated TCR ζ-chain.

Each individual peptide elicited a distinct pattern of phosphoproteins that was characteristic for a given ligand (Fig. 4), with the intensity, but not the pattern, of phosphorylation changing with the concentration of a particular peptide (Fig. 4*A*, *lower panel*, shown for superagonist T95-K for GDBP). The signaling patterns of the most potent ligands (superagonists; T95-K for GDBP and T95-A for MS18) were characterized by the appearance of substantial amounts of tyrosine-phosphorylated ZAP-70 and three forms of TCR ζ-chain that migrate under nonreduced conditions as dimers with apparent molecular masses of 32, 35, and 38 kDa. Superagonist ligands induced the accumulation of the pp32 and/or pp35 forms and the pp38 form of TCRζ in almost an equal ratio. Native ligand induced pp38 in lesser proportion to pp35 or pp32, along with lower levels of ZAP-70 phosphorylation. The weak agonists stimulated only low levels of phosphorylated ZAP-70 and pp38, and for TCC GDBP, some pp35 but high levels of TCRζ pp32 were obtained. Finally, antagonist ligand N92-A for TCC GDBP induced no detectable phosphorylated ZAP-70 and little or no TCRζ pp38 or pp35, but still yielded significant levels of TCRζ pp32 (Fig. 4*A*). The rank order of the amounts of phosphorylated ZAP-70 and pp38 detected using all the ligands at the same offered peptide concentrations was the same as the potency order seen in

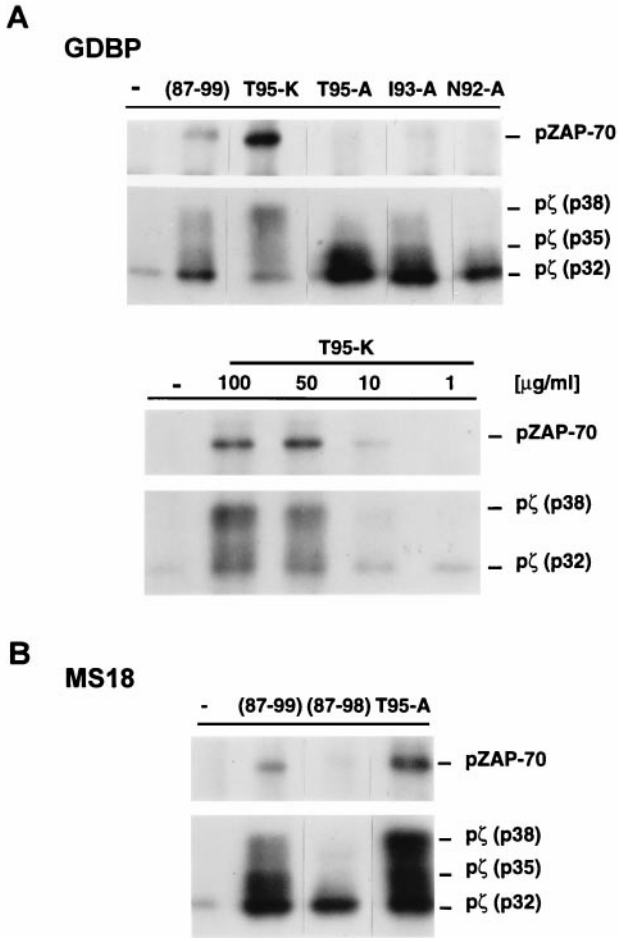

FIGURE 4. Early signaling events induced by various ligands in TCC GDBP (*A*) and MS18 (*B*). TCCs were stimulated with peptides (100 μg/ml or as indicated) using peptide-pulsed EBV-transformed B cells as APCs for 5 min. Tyrosine phosphorylation of ZAP-70 and the ζ-chain in the ZAP-70-immunoprecipitated complex was visualized by anti-phosphotyrosine immunoblotting. The *upper panels* of *A* and *B* display the responses of both TCCs to MBP_{87-99} and various modified ligands. The *lower panel* of *A* shows the responses of TCC GDBP to different concentrations of the superagonist ligand. The three forms of TCR ζ-chain observed under nonreduced conditions with apparent molecular masses of 32, 35, and 38 kDa and ZAP-70 are indicated. The phosphoproteins vary in intensity but not in their pattern when the same ligand is used at different densities (*A, lower panel*). In contrast, the pattern of TCRζ isoforms changes depending on the potency of the ligand (*A, upper panel*, and *B*).

the functional assays and when examining TCR down-modulation (Fig. 5). However, in contrast to TCRζ pp38, the absolute amount of ZAP-70 induced by the different ligands at the highest Ag doses did not directly correlate with the functional activity seen. The relatively high level of ZAP-70 phosphorylation by superagonist ligands that induced little TCRζ pp32 and the relatively low level of ZAP-70 phosphorylation by weak agonists that induced high levels of TCRζ pp32 may indicate that the abundance of pp32 correlates with a biochemical process that interferes with ZAP-70 phosphorylation, providing a possible explanation for the relative shifts in response thresholds by weak agonists.

Discussion

The biologic functions of $CD4^+$ T cells are mediated primarily through their production of cytokines that influence both their own activities and those of other cell types. These mediators participate in complex regulatory circuits, and a single cytokine can exert a positive effect on one limb of the system, while having a negative effect on others (41). Thus, the qualitative nature as well as the magnitude of a T cell-dependent immune response can be influenced by the combination and amounts of the cytokines evoked upon TCR recognition of peptide-MHC molecule ligands. Here we report on studies in which a careful analysis was made of the Ag dose requirements for elicitation of a wide range of different responses from human $CD4^+$ TCC. In addition, the functions and TCR-dependent proximal signals induced by a set of altered peptide ligands were also analyzed in a comprehensive fashion. Our data demonstrate that an array of effector functions of human $CD4^+$ autoreactive TCC are elicited according to a hierarchical order that is intrinsic to each clone. These observations expand the limited available data bearing on this point (13, 31).

The difference in offered peptide concentration required to evoke the most readily elicited vs the least readily elicited responses in the hierarchy can exceed 2 to 3 orders of magnitude. It is thus easy to expose T cells to Ag amounts that allow relatively selective induction of some and not other effector activities. For the Th0 cells tested, IL-4 and IFN-γ show distinct activation thresholds. These differences result in a changing ratio of these counter-regulatory cytokines as the Ag concentration increases, which, in turn, could result in a different degree of polarization of the immune response toward the humoral or inflammatory mode depending on the Ag dose. The early observations of Parish (26) and Bretscher et al. (23) demonstrating that low Ag doses favor cell-mediated immunity, whereas higher doses favor humoral immunity may be explained in part by this hierarchical relationship of cytokine response thresholds. In vivo, when T cell responses to an Ag are heterogeneous, the activation hierarchy of the majority of cells within the bulk population will determine which function(s) will be elicited at a given Ag load. If, for example, clones such as MS18 predominate in a bulk population, stimulation by a weak ligand or a low concentration of native peptide will lead to full IL-4 but little or no IFN-γ secretion. Similarly, in an autoimmune disease or experimental models such as experimental allergic encephalomyelitis, the activation hierarchy and the functional repertoire of disease-mediating cells will determine whether a certain dose of Ag will promote proinflammatory responses and disease or, instead, induce an anti-inflammatory cytokine milieu and be therapeutic (42–45).

The modified peptide ligands tested tend to elicit effector functions in a hierarchical order similar, but not identical, to that seen with the prototypic agonist. They differ, however, in the amount of ligand required to induce any of the responses. Some ligands are more potent than the native ligand and induce each activity at a lower concentration of peptide (superagonists). Other peptides are less potent, and the weakest among these are unable to induce a measurable level of those functions that require the greatest amount of Ag to be stimulated. By itself, the loss of only these most difficult to induce responses upon alteration of a peptide structure classifies such ligands as weak agonists. Some ligands induce little or no functional activity, but efficiently inhibit responses to simultaneously offered native ligand, falling into the category of functional receptor antagonists.

Lanzavecchia and colleagues have introduced flow cytometric analysis of TCR down-modulation as a convenient measure of effective receptor engagement by ligand (27, 31, 40, 46) and have shown that for IFN-γ production, similar levels of cytokine production occur with a strong and weak agonist when the extent of TCR loss they induce is similar (27). We have substantially extended these observations here, showing that for the entire set of

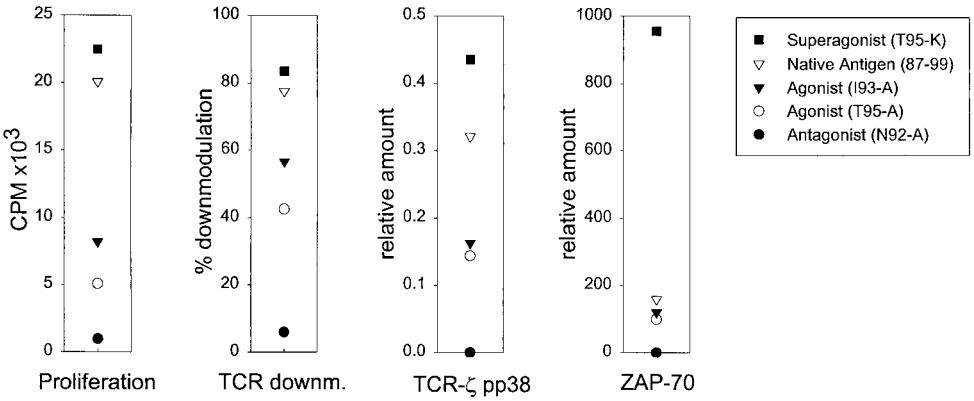

FIGURE 5. Rank order of different ligands for the induction of effector functions (shown for proliferation), TCR down-modulation, induction of TCRζ pp38, and ZAP-70. TCC GDBP was stimulated with 100 μg/ml of the different ligands. Effector functions, TCR down-modulation, and TCR signaling were measured and compared. TCR down-modulation is expressed as % down-modulation {100% − [(TCR expression with ligand/TCR without ligand) × 100]}.

measured responses and for a number of different ligands, the extent of down-modulation for various ligands at the same concentration reflects the potency rank order of the ligands in functional assays. With increasing concentrations of each ligand, the down-modulation increases proportionally and in accord with the increase in functional activation of the TCC. The several peptides tested for each clone form the similar number of complexes with HLA-DR at a given concentration (18), and it is likely that the rates of initial physical interaction of TCR on a TCC with these different ligands are comparable. Although the precise biochemical basis for ligand-induced TCR down-modulation is not well defined (47, 48), these data suggest that the fraction of such TCR-ligand contacts resulting in an effective signal differs according to the structure of the ligand, and it is specifically these effective engagement events that result in T cell responses, at least of the type measured here. This hypothesis is supported by the biochemical data showing a similar relationship among the extent of ZAP-70 phosphorylation, TCR down-modulation, and functional response. Although it is tempting to also suggest from these relationships that ZAP-70 itself is the key proximal signaling molecule whose activity is limiting for all the measured responses, we as yet do not have direct evidence for this contention.

The biochemistry of TCR signaling in the human TCC does not, however, fit completely with a simple model in which recognition of the less potent agonist ligands by the TCR generates the same set of signals, but only less frequently. If this were the case, then the pattern of receptor-associated phosphorylation events should be identical when using either a given amount of the less potent ligand or a lower amount of strong agonist chosen to yield an equivalent functional response, extent of ZAP-70 phosphorylation, or TCR down-modulation. In contrast to this expectation, reproducible and significant differences in the ratios of phosphorylated TCRζ isoforms (pp32, pp35, and pp38) were seen upon receptor engagement with distinct peptide-MHC class II complexes. The combination of high levels of pp32 with little or no pp35 or pp38, as seen with weak agonists and antagonists, could not be reproduced by titration of agonist.

This argues for a more complex view of these early signaling events. Given past data with mouse T cell clones (19, 20, 21) and the present results, it appears likely that any individual TCR on a cell can signal in either of at least two modes: one giving rise to the full phosphorylation pattern including pp38 ζ and phosphorylation of ZAP-70 and TCR down-modulation, and the other producing only pp32 ζ and associated, but not phosphorylated, ZAP-70 and no TCR down-modulation. The appearance of both types of signals in varying ratios when using different ligands with the same clone presumably reflects the relative proportions of engaged TCR signaling in each of the two modes, dictated by such parameters as dissociation rate of TCR-ligand pairs (39, 49) and rate of coreceptor (CD4 or CD8) recruitment to the engaged receptors (50, 51). The overall potency of a ligand would reflect the number of effective signaling events obtained per available ligand on the APC. In addition to the potency change, alterations in the relative positioning of specific responses within the hierarchy may be due to variations in the pattern of intracellular signaling that accompany the changing ratios of fully and incompletely signaling TCR elicited by distinct ligands. Such changes presumably affect the balance of activated transcription factors within the cell, which, in turn, may impact the regulation of individual target genes in distinct ways.

Given 1) the simultaneous generation of two distinct types of proximal TCR signaling complexes in a single T cell and 2) the correlation of pp32 phospho-ζ generation with the inhibitory activity of antagonists, it is tempting to propose that those TCR showing the pp32/pp35 ζ-associated signaling pattern may in some manner contribute to an active inhibition of the effective signaling that would typically occur upon engagement of other TCR. Recent data obtained using human $CD4^+$ as well as $CD8^+$ TCC indicate that the phosphatase SHP-1 can be recruited by TCR-associated pp32/pp35 phospho-ζ (I. Stefanova et al., manuscript in preparation). The presence of this phosphatase in clusters of engaged TCR may modify the level and the nature of downstream signaling, contributing to both the potency shifts and the changes in hierarchical relationships seen using distinct ligands of the TCR. Clearly, additional investigation is needed to examine the roles of such putative active inhibitory processes in TCR-dependent events evoked by ligands of distinct structures.

The modified ratios of phosphorylated TCRζ isoforms elicited in human TCC by TCR antagonists resemble those previously reported for mouse T cells (19–21). The major difference from the mouse data is that with weak agonist ligands for the human TCC, phosphorylated ZAP-70 associated with the TCR complex is detected at the same time as an altered ratio of ζ isoforms is seen. Application to mouse Th1 clones of the same methods able to detect low level ZAP-70 phosphorylation signals in human TCC in this study have not revealed ZAP-70 phosphorylation upon stimulation with the altered ligands (I. Stefanova and R. N. Germain, unpublished observations), and in contrast to the ligands employed

in this report, the stimulatory activities of the native ligand and the altered peptides in the previous mouse studies differed by several orders of magnitude (7, 19, 21). It is therefore difficult to assess whether the patterns observed with our TCC using weak agonist ligands are unique to human T cells or a result of the widely different stimulatory potencies of the ligands examined in the two species.

Taken as a whole, the data presented here indicate that the amounts of the cytokines produced by a $CD4^+$ TCC in response to a given dose of Ag depend on ligand potency, a factor that correlates with the efficacy of inducing adequate signaling upon ligand interaction with the TCR. At the same time, the ratio of the cytokines produced depends on the hierarchical arrangement shown by that T cell in the signaling thresholds for the individual responses. Finally, altered TCR ligands differ from the parental ligand mainly in potency and also in the relative positionings of individual thresholds within the hierarchy. These latter two effects result in changes in the cytokine response to Ag offered at various concentrations 1) as a consequence of a failure of attainable Ag levels to engender enough signaling for the highest response thresholds to be reached, and 2) as a result of the altered positioning of individual thresholds within the overall hierarchy. Although the activation of cloned cells may not necessarily reflect the activation of T cells in vivo, the data could explain reports of a polarization of immunity toward a humoral or cell-mediated response at different levels of Ag exposure, of selective Th1 vs Th2 cytokine production at distinct Ag levels (23–26), and of the ability of TCR partial agonists to modulate immune response quality or the balance between thymocyte-positive and -negative selections (52–54).

Acknowledgments

We thank H. F. McFarland and B. Gran, Neuroimmunology Branch, National Institutes of Health, and A. B. Vogt, German Cancer Research Center (Heidelberg, Germany), for helpful comments, and N. Ling, Neurocrine Biosciences (San Diego, CA), for synthesizing peptides.

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