Serial TCR Engagement and Down-Modulation by Peptide:MHC Molecule Ligands: Relationship to the Quality of Individual TCR Signaling Events

Yasushi Itoh, Bernhard Hemmer, Roland Martin and Ronald N. Germain

*J Immunol* 1999; 162:2073-2080;
http://www.jimmunol.org/content/162/4/2073

---

**References**

This article cites 48 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/162/4/2073.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Serial TCR Engagement and Down-Modulation by Peptide:MHC Molecule Ligands: Relationship to the Quality of Individual TCR Signaling Events

Yasushi Itoh, Bernhard Hemmer, Roland Martin, and Ronald N. Germain

In the present study, we examined the relationships among quantitative aspects of TCR engagement as measured by receptor down-modulation, functional responses, and biochemical signaling events using both mouse and human T cell clones. For T cells from both species, ligands that are more potent in inducing functional responses promote TCR down-modulation more efficiently than weaker ligands. At low ligand density, the number of down-modulated TCR exceeds the number of available ligands by as much as 80–100:1 in the optimal human case, confirming the previous description of serial ligand engagement of TCR (Valitutti, et al. 1995. Nature 375:148–151). A previously unappreciated relationship involving TCR down-modulation, the pattern of proximal TCR signaling, and the extent of serial engagement was revealed by analyzing different ligands for the same TCR. Functionally, more potent ligands induce a higher proportion of fully tyrosine phosphorylated ζ-chains and a greater amount of phosphorylated ZAP-70 than less potent ligands, and the number of TCR down-modulated per available ligand is higher with ligands showing this full agonist-like pattern. The large number of receptors showing partial ζ phosphorylation following exposure to weak ligands indicates that the true extent of TCR engagement and signaling, and thus the amount of sequential engagement, is underestimated by measurement of TCR down-modulation alone, which depends on full receptor activation. These data provide new insight into T cell activation by revealing a clear relationship among intrinsic ligand quality, signal amplification by serial engagement, functional T cell responses, and observable TCR clearance from the cell surface. The Journal of Immunology, 1999, 162: 2073–2080.

Antigen-specific activation of peripheral T lymphocytes requires an adequate quantity and quality of intracellular signaling by ligand-engaged TCR complexes that is sustained over a suitable time interval to result in gene transcription (1–3). However, the measured affinity between isolated TCR αβ complexes and their cognate ligands in vitro is generally in the high micromolar range, with fast off-rates being characteristic of these interactions (4–7). Thus, the engagement of a single TCR by a single peptide:MHC molecule complex is not by itself able to evoke a sustained intracellular signal. Prolonged signaling is also unlikely to depend on sequential binding of a large number of TCR to a comparable number of ligands because functional activation often requires only tens to hundreds of specific peptide:MHC molecule complexes per presenting cell (8–13). The problem posed by low affinity of TCR for their ligands may in some degree be ameliorated by recruitment of the CD4 or CD8 coreceptors (14), which stabilize TCR-ligand binding (15, 16) and modify the biochemical characteristics of the signaling event (17, 18). However, available data suggest that coreceptor function can only extend the time of association of the TCR with its ligand a few fold, failing to provide an adequate model of TCR signaling for activation events requiring a substantially longer duration of intracellular messenger generation.

Consideration of these issues led Valitutti et al. (19) to explore the quantitative relationship between ligand density and TCR triggering and to provide evidence for an unexpected mechanism that contributes to sustained TCR signaling in the presence of small numbers of specific ligands on the APC membrane. These investigators used TCR down-modulation (internalization) to estimate the relationship between ligand number and TCR engagement. Their studies suggested that at low ligand densities (<100 per APC), each peptide:MHC molecule complex could serially trigger up to 200 separate TCR, amplifying and extending the duration of intracellular signaling. The required continued availability of peptide:MHC molecule ligands after disengagement from numerous TCR is consistent with the long lifespan of most such peptide:MHC molecule combinations reaching the cell surface (20).

Although this model has great appeal, even investigators who have used it to explain their functional data on T cell activation (21) have not provided quantitative data confirming this hypothesis. Furthermore, despite progress in understanding some mechanistic aspects of receptor down-modulation following ligand engagement or cross-linking using Ab (14, 22, 23), some more recent studies have questioned whether there is any direct relationship between TCR internalization itself and the elicitation of effector functions (24, 25). These latter experiments have led several investigators to conclude that TCR loss from the cell surface is unrelated to effective T cell activation and that this parameter does not measure a physiologically important aspect of TCR signaling, raising questions about the validity or relevance of the serial engagement phenomenon.
In contrast, other reports suggest a direct correlation between levels of TCR down-modulation and T cell responses (26, 27), though the techniques used do not permit one to argue directly for serial engagement as an underlying mechanism. Recent evidence for a hierarchical organization of effector response thresholds (27–30) and the relationship among ligand quality, response, and TCR down-modulation (27) suggested to us a possible unifying explanation for these apparently divergent results of different laboratories. Therefore, we have reinvestigated the phenomenon of TCR down-modulation in a quantitative manner using a diverse set of mouse and human T cells, various ligands for individual TCR, and parallel biochemical signaling studies. Using highly potent ligands, our studies confirm for human clones the phenomenon of extensive serial TCR engagement reported by Valitutti et al. (19). More importantly, we provide evidence for a clear relationship among the quality of biochemical signaling events involving an engaged TCR complex, the likelihood that this receptor complex will be removed from the cell surface, and the cell’s functional response. These data are discussed from the standpoint of how ligand density, the biochemistry of individual receptor activation, and the phenomenon of serial engagement combine to yield an effective stimulus for the T cell.

**Materials and Methods**

**Cells**

A.E7 and 3C6 are Th1 cell clones specific for pigeon cytochrome c (PCC)\(^3\) 88–104 and 1-E\(^+\) (31). These clones were maintained as previously described (30) and used no earlier than 2 wk after restimulation and cytokine expansion. P13.9 is a supernatant derived derivative of the DAP.3 fibroblast-derived transfectant DCEK H7 that expresses high levels of I-E\(^{k}\), B7.1, and ICAM-1 (32). GDPB is a human T cell clone specific for myelin basic protein (MBP) 87–99 and HLA-DRB1*1302, grown and maintained as described (33). GP-BC is an EBV-transformed B cell expressing DRB1*1302.

**Peptides**

Peptides were synthesized and purified by HPLC in the National Institute of Allergy and Infectious Diseases, Biological Resources Branch, Laboratory of Molecular Structure (Rockville, MD). The sequences of PCC88 – 104, moth cytochrome c (MCC) 88–103, MBP87–99, and MBP87–90 (F88 K94 K95 F98) were KERADLAILQKATAK, ANRADLILAYL QATK, VHHFKNIVTPRT, and VFFKNKIKRPFR, respectively.

**APC preparation and quantitation of peptide:MHC class II complexes**

To create a surface pool of peptide/MHC class II complexes on APCs, P13.9 was cultured overnight with the indicated peptide, or GP-BC was cultured with MBP87–99 for 2 h. In quantitative analyses, N-terminal biotinylated peptides were used rather than unsubstituted peptides. The APC were then washed three times with PBS and used for incubation with T cells (see below) or for quantitation of surface peptide/MHC class II molecule complexes. For the latter, after an additional wash with PBS containing 1% BSA and 0.1% NaN\(_3\), cells were stained with streptavidin-phycocerythrin (PE; Pharmingen, San Diego, CA) or PE avidin D (Vector Laboratories, Burlingame, CA) at a saturating concentration. Dead cells were gated out based on staining with propidium iodide. Cells (1 × 10\(^5\)) were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The number of peptides was calculated from FL2 mean intensity, the PE:protein ratio of the streptavidin reagents and standard curves obtained using beads with known PE content (Flow Cytometry Standards, San Juan, PR), as follows: number of peptides = [(mean intensity of APC with peptides) − (mean intensity of APC without peptide)] × (slope element from standard curve)/(PE:protein ratio).

**Coincubation method for inducing TCR down-modulation**

Mouse T cells were mixed with control or peptide-pulsed APC at a ratio of 1 T cell:2 APC in 5-ml polypropylene tubes (FALCON 2058; Becton-Dickinson, Franklin Lakes, NJ), pelleted, and incubated at 37°C for the indicated time. In human cell experiments, T cells and B cells were cultured in 96-well U-bottom plates for 5 h. PBS containing 0.5 mM EDTA was added after incubation to dissociate conjugates. To stain mouse T cells for the TCR complex, 145-2C11 (anti-CD3e (34)) or KJ25 (35) Ab-containing supernatant was added at 4°C and incubated for 30 min. The cells were then washed with PBS containing 1% BSA and 0.1% NaN\(_3\), and FITC anti-hamster IgG Ab (Caltag Laboratories, South San Francisco, CA) was added for another 30 min in the cold. After washing, TCR staining was analyzed by excluding the L cell APC using forward scatter/side scatter parameters and dead cells using propidium iodide.

For quantitative analysis of TCR complex expression, mouse T cells from cultures performed as above were stained directly with FITC-conjugated anti-V\(\beta\)3 or anti-TCR C\(\alpha\)Ab (H57-597; Pharmingen), whereas human T cells were stained with FITC-conjugated anti-human CD3 (Phar- mingen), all at saturating concentrations. Cells (10,000) were then analyzed and the number of TCR on each cell was calculated from the FL1 mean intensity, the fluorescence protein (F/P) ratio of the Abs, and standard curves derived using beads with known FITC content (Flow Cytometry Standards) as follows: number of down-regulated TCR = (mean fluorescence with APC without peptide) − (mean intensity with APC with peptide)] × (slope element from standard curve)/(F/P ratio).

**T cell functional assays**

P13.9 cells were cultured with peptide overnight at 37°C, then treated with mitomycin c (20 μg/ml) at 37°C for 1 h and washed four times with PBS. T cell clones (2.5 × 10\(^5\)/well) were cultured with APC (5 × 10\(^5\) in 96-well flat-bottom plates. For use with human T cells, GP-BC was cultured with peptides for 2 h, and unbound peptides were washed away after 30 Gy irradiation. GDPB (1 × 10\(^5\)/well) was cultured with peptide-pulsed GP-BC (5 × 10\(^5\)/well) in 96-well U-bottom plates. After 48 h of culture, 1 μCi of [\(^3\)H]thymidine was added, and the cells were harvested 16 h later to measure proliferation based on [\(^3\)H]thymidine incorporation into high m.w. DNA.

**Tyrosine phosphorylation analysis**

Peptide-pulsed APC were described as above. Two million A.E7 and 4 × 10\(^5\) P13.9 were mixed and cultured for 5 min at 37°C. In human experiments, GDPB (1 × 10\(^5\)) and GP-BC (2 × 10\(^5\)) were cultured for the indicated time at 37°C. Thereafter, cells were lysed with 1% Nonidet P-40, 140 mM NaCl, 10 mM Tris·HCl (pH 7.2), 2 mM EDTA, 5 mM iodoacetamide, 1 mM NaVO\(_4\), and proteinase inhibitors (Roche Diagnostics, Mannheim, Indianapolis, IN) on ice for 25 min. After centrifugation, the supernatant was incubated with anti-ZAP-70 Ab (36) for 4 h and precipitated with Staphylococcus aureus protein A (Pansorbin; Calbiochem, La Jolla, CA). Precipitated proteins were analyzed using 10% SDS-PAGE with reducing (mouse) or nonreducing (human) conditions and transferred to Immunoblot·P membranes (Millipore, Bedford, MA). Phosphorylated proteins were detected with 4G10 (Upstate Biotechnology, Lake Placid, NY) and horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA). Horseradish peroxidase activity was detected with SuperSignal ULTRA chemiluminescence substrate (Pierce, Rockford, IL).

**Results**

**TCR down-modulation assessed using mouse Th1 CD4\(^+\) T cell clones**

To study TCR down-modulation in a quantitative manner, we established a model system in which measurement of ligand density without cell disruption is possible and in which the APC does not undergo substantial changes in its adhesion or costimulatory molecule repertoire during interaction with the T cell. We first examined numerous T cell clones and hybridomas for TCR down-modulation upon Ag exposure. Even using the same APC displaying a given amount of ligand, the extent of TCR loss from the surface varies for different T cells, including a mouse T hybridoma and a human transfected expressing the identical TCR. In general, immortalized T cells (T hybridomas, Jurkat) show low to intermediate levels of TCR down-modulation, whereas some, though not all nontransformed T cell clones show loss of between 70% and 90% of the total surface pool of TCR at high Ag concentrations (data not shown). Because of the limited down-modulation in the former cells, whose responses to Ag are also attenuated compared with...
nontransformed lymphocytes, we focused our studies on T cell clones that show extensive receptor loss and that are physiologically similar to those studied previously in mouse and human model systems of TCR down-modulation.

To establish the kinetics of TCR clearance, the T cell clone 3C6 was incubated with APC bearing the agonist ligand PCC88–104/I-Ek for various lengths of time, then stained for surface TCR complexes (Fig. 1A). A clear reduction in TCR levels is seen at 15 min. Although the rate of down-modulation decreases after ~1 h of T cell:APC contact, it takes nearly 5 h to reach a plateau level of TCR loss at 10 μM peptide. Therefore, cells were incubated for 5 h in subsequent experiments. T cell clones A.E7 (Fig. 1B) and 3C6 (Fig. 1C) were then cultured for 5 h with APC bearing various numbers of PCC88–104/I-Ek complexes. A total of 70–90% of A.E7 TCRs are down-modulated, but only 50–60% of 3C6 TCRs are lost at 10 μM of PCC88–104. In all cases, the down-modulation appears to involve the entire pool of cells in the population, even though these cloned cells show functional heterogeneity (30).

To explore whether TCR down-modulation shows a direct relationship with functional responses, TCR loss was compared with proliferation upon exposure of the clones to varying ligand densities. A.E7 is derived from a B10.A mouse immunized with PCC, but MCC peptide MCC88–103 stimulates a more potent (heteroclitic) proliferative response than PCC88–104 (Fig. 2A), and in accord with these functional data, MCC88–103 also induces TCR down-regulation slightly more effectively than PCC88–104 (Fig. 2B). On the other hand, the 3C6 clone does not show a heteroclitic functional response to the MCC peptide (Fig. 2A), and in agreement with these results, PCC88–104 induces more TCR down-modulation than MCC88–103 (Fig. 2B). Because the same MCC- and PCC-peptide-bearing APC are used in a single experiment for stimulating the two T cell clones, the inverse nature of these effects cannot be attributed to variations in ligand density, but rather clearly reflects the relative efficiency of the two ligands (PCC/I-Ek and MCC/I-Ek) for triggering both down-modulation and effective intracellular signaling by the slightly different TCR of these cells.

Quantitation of down-regulated TCRs in relationship to the available number of peptide/MHC complexes using mouse clones

A variety of techniques have been used to quantitate the number of specific peptide/MHC molecule complexes on APC. Among those...
permitting the direct analysis of peptide-associated surface molecules on live APC, the most widely used involves biotinylated peptides detected with fluorescent streptavidin conjugates (37). We chose this approach because, compared with methods involving MHC molecule purification, it is less susceptible to underestimating true ligand number due to dissociation of peptide (38). I-Ek-bearing L cells were incubated with biotin-PCC88–104 and washed, and PE-conjugated streptavidin was added at a saturating concentration to promote univalent binding. The number of PE molecules was determined using standard curves generated by analysis of beads conjugated with known numbers of PE fluorochromes. At 100 μM and 10 μM peptide pulsing concentrations, the mean number of PCC88–104 molecules bound is 9.7 \times 10^4 and 3.9 \times 10^4, respectively (Fig. 3A). The background binding to DAP.3 cells lacking expression of class II molecules is negligible. The lowest number of peptides that can be measured directly by this assay is \sim 500 at 1 nM offered peptide. However, a log-log plot of offered peptide concentration vs bound ligand number follows a straight line with an r > 0.99 for values in the detectable range, and it is thus possible to extrapolate with some confidence to substantially lower absolute ligand values (Fig. 3A). This is critical, as the extent of serial engagement is claimed to rise rapidly as ligand density decreases, especially below a few hundred complexes per APC (19).

Direct binding of FITC-labeled anti-TCR Abs was used to quantitate receptor number before and after incubation with APC. Following prolonged rest in culture, A.E7 and 3C6 express about 1.6 \times 10^4 and 2.2 \times 10^4 TCRs, respectively. For both clones, coincubation with APC pulsed with peptide at 10 μM leads to triggering and down-modulation of 1.1–1.2 \times 10^4 TCRs (Fig. 3B), whereas cells pulsed with 0.3 nM peptide show a loss of \sim 700 TCRs. Fig. 3C shows the number of down-regulated TCR per peptide:MHC complex derived from the data in Fig. 3, A and B, while Fig. 3D converts the data into the ratio “TCR down-modulated/ligand” plotted against available ligand density. At high ligand density (equal to or greater than the number of TCR on the T cell), the ratio of TCR down-modulated to the number of available ligands is <1 (Fig. 3D). At 1 nM, which generates ligands at the limit of direct detection (\sim 500 complexes per APC), the ratio rises slightly to \sim 2 for both A.E7 and 3C6. At 10 pM, when extrapolation indicates each APC would have \sim 50 ligands, the ratios rise further to \sim 7 for both cells.

Measurement of serial TCR engagement using human T cell clones and modified TCR ligands

Because the triggered TCR:ligand ratio of mouse T cell clones was substantially lower than expected based on data obtained using human T cell clones, we examined various parameters that differed between the two sets of experiments to search for an explanation. Attempts to use iodinated peptides for quantitation of ligand in the manner of Valitutti et al. (19) were unsuccessful with a large background of precipitated counts even from pulsed cells lacking MHC class II expression (data not shown). However, we have been able to confirm the accuracy of our ligand-counting methodology using Scatchard analysis with Fab fragments of an Ab to a peptide-MHC class II combination (Y-Ae) (39) in comparison to measurements using biotinylated peptides in the same system (data not shown). Another potential difference lies in the species origin of the T cell and APC. Therefore, we applied our method to the combination of a transformed human B cell and a human CD4+ T cell clone, which is specific for the self ligand MBP87–99 bound to DRB1*1302 (Fig. 4A). Experiments were done with both the natural ligand and a modified peptide we had previously identified as “superagonist” for this clone (MBP87–99 (F88 K94 K95 F98)) (40), which in functional assays is up to 1000 times more potent than the wild-type peptide. This comparison was considered important because several studies have indicated a relationship between the overall extent of TCR down-modulation and ligand potency (19, 26, 27). Furthermore, we have found that many peptide: MHC molecule combinations used to propagate human T cell clones are not optimal agonists, as judged by either functional or biochemical signaling criteria (27). Numerous modified peptide ligands are more potent in stimulating these clones than the original immunogen and, as shown in Fig. 2, heteroclitic ligands show an enhanced capacity to promote TCR down-modulation. In agreement with expectations, the modified peptide superagonist was much better at inducing TCR down-modulation than the wild-type peptide (Fig. 4B), even though the latter generated more complexes with DRB1*1302 at equal peptide concentrations (Fig.
5A). At 30 μM offered peptide, about $6 \times 10^4$ MBP87–99 containing complexes are detected per APC, with the lowest detectable number being 900 at 30 nM (Fig. 5A). As with the mouse system, there is a linear log-log relationship between offered peptide and the number of complexes formed, so that we can extrapolate to ligand densities approaching 50 per APC. Using MBP87–99 at 30 μM and 0.3 nM, $1.4 \times 10^3$ and $1.1 \times 10^3$ complexes (which is the smallest difference we could measure compared with the starting number of receptors on resting T cells) were down-modulated, respectively. MBP87–99 (F88 K94 K95 F98) induced a greater amount of TCR loss at any given input peptide concentration, and down-modulation could be seen under conditions in which peptide binding could not be directly measured by our methods (Fig. 5A and B). Thus, at high ligand densities, both peptides form complexes that lead to <1 TCR down-modulation event per available bound peptide (Fig. 5C). With ~900 MBP87–99 complexes on GP-BC, the ratio rises to 3.5, and at an estimated 50 ligands per APC, to 20. This latter ratio is three times greater than that seen with mouse T cell clones (Fig. 5D), but still substantially lower than that claimed for human clones (19). However, using MBP87–99 (F88 K94 K95 F98)-containing complexes at low density, the predicted number of TCR engagements per ligands rises to 100, a number close to that reported by Valitutti et al. (19).

Relationships among the biochemistry of TCR signaling events, TCR down-modulation, and the estimation of serial engagement

The above data all support the previous results reported by us (27) and by Valitutti et al. (19) that a functionally more potent ligand induces a greater extent of TCR down-modulation than an equal density of less potent ligand. In our prior study of this phenomenon, we demonstrated that TCR loss from the cell surface parallels the proportion of receptors showing biochemical evidence of optimal signaling events (full TCR ζ phosphorylation/ZAP-70 activation). Furthermore, equal levels of TCR down-modulation and of ZAP-70 phosphorylation produced at different densities of these related ligands result in equivalent functional responses of the T cells. These data indicate that TCR that engage ligand but show a partially phosphorylated status of associated ζ-chains do not undergo efficient internalization, and they do not appear to contribute to elicitation of most effector responses. In the context of the present results, these prior findings suggest that calculation of the number of ligand-TCR engagement events based only on loss of TCR from the cell surface may substantially underestimate the true number of relevant receptor-ligand interactions, in that many such engagements would lead to measurable but incomplete TCR signaling, a failure to internalize and hence, a failure to be counted using flow cytometric approaches.

![FIGURE 4. Functional response and TCR down-modulation of a human T cell clone. A. Human T cell clone GDBP was stimulated with MBP87–99 or "superagonist" MBP87–99 (F88 K94 K95 F98) (FKKF). Proliferation was measured as [3H]thymidine uptake. B. GDBP was incubated with peptide-preloaded EBV-transformed B cells for 5 h. TCR down-modulation was analyzed with flow cytometry.

![FIGURE 5. Quantitative relationship between TCR down-regulation and ligand density using human T cells. A. EBV-transformed B cell GP-BC was incubated with biotinylated MBP87–99 or MBP87–99 (F88 K94 K95 F98) (FKKF) for 2 h. After washing away unbound peptides, surface biotinylated peptides were stained with streptavidin-PE. The number of peptides was calculated as described in Fig. 3. The equations were $y = 2.2030.880^{0.493}$, $r = 0.998$ for MBP87–99; and $y = 1.2936.738^{0.497}$, $r = 0.986$ for MBP87–99 (F88 K94 K95 F98). B. MBP87–99 specific human T cell clone GDBP was cultured with peptide-prepulsed B cells for 5 h. Surface CD3 was stained with FITC-conjugated anti-CD3 Ab, and the numbers of TCR complexes were calculated as described in Fig. 3. The equations were $y = 6.847.957^{0.227}$, $r = 0.993$ for MBP87–99; and $y = 8.967.059^{0.140}$, $r = 0.975$ for MBP87–99 (F88 K94 K95 F98). C. The number of peptides per APC (x-axis) and down-regulated CD3 (y-axis) are from Fig. 5A and B. The lines showed extrapolated curves using the equations $y = 192.834^{0.374}$, $r = 0.951$ for MBP87–99; and $y = 575.364^{0.226}$, $r = 0.994$ for MBP87–99 (F88 K94 K95 F98). D. Comparison of down-modulated human TCR:ligand ratio with mouse TCR:ligand ratio. Ligand density of the two lowest concentrations are from the formula in Fig. 5A. The data for the mouse TCR were derived from Fig. 3.]
This was reevaluated here both as possible explanation for the difference between the mouse and human clone studies and as the basis for the failure of some investigators to see a clear relationship between functional T cell activation and TCR down-modulation. The A.E7 mouse clone and the GDBP human clone were stimulated with either of two ligands differing in their capacity to induce down-modulation when offered at equal density on the APC and thus, showing a difference in the extent of serial engagement estimated using this approach. As seen in Fig. 6A, for A.E7, the heteroclitic ligand MCC induced a higher proportion of the fully phosphorylated p23 form of TCR ζ and a greater amount of phosphorylated ZAP-70 as compared with the less active PCC peptide when offered at an equivalent concentration. The amount of phosphorylated ZAP-70 was similar, however, at concentrations of MCC (1 μM) and PCC (10 μM) that led to a similar decrease in TCR expression and to equal functional responses, consistent with down-modulation reflecting the number of individual TCR complexes undergoing full activation upon TCR engagement. Likewise, for GDBP, the superagonist peptide induced a higher ratio of fully phosphorylated ζ-chain (p38 ζ dimer = p23 ζ monomer analyzed in the mouse system (27)) and more phosphorylated ZAP-70 than the less potent wild-type MBP87–99 peptide (Fig. 6, B and C). At concentrations of the modified peptide (3 μM) that gave TCR down-modulation close to that seen with 30 μM MBP87–99, the total level of phosphorylated ZAP-70 was comparable for the two ligands. Thus, TCR engagements resulting in incomplete receptor activation as assessed by the phosphorylation status of ζ and ZAP-70 do not appear to contribute to TCR down-modulation, leading to a substantial underestimate of the extent of actual TCR-ligand interactions nevertheless able to initiate signaling events.

**Discussion**

Receptor internalization following ligand binding and generation of intracellular signals is a common feature in many biological systems (41). This process is generally considered to be an important component of a homeostatic mechanism that limits the duration of signaling and/or quantity of signals received by the cell. TCR internalization upon either natural (peptide:MHC molecule) or artificial (Ab) engagement has been reported using a wide variety of mouse and human cells (19, 22, 42–44). For T cells, a low density of surface receptors has been shown to limit effective signaling for T cell function in the face of otherwise adequate ligand display, consistent with a role for TCR down-modulation in limiting Ag-induced activation (29, 45). However, although it is thus likely that this process contributes to regulation of signal intensity and duration as with other receptors, the relationship between receptor engagement, signaling, and down-modulation is more complex in the case of the TCR. This is because in contrast to other situations in which ligand binding leads to a stereotypic pattern of signaling, TCR ligands with minor structural differences elicit very distinct patterns of early TCR phosphorylation events (3, 46). We have recently reported that the loss of surface receptor tends to closely track the proportion of TCR generating full signals as defined by the pattern of ζ-chain phosphorylation and ZAP-70 activation, even as a much larger number of TCR in the same cells shows evidence of ligand engagement in the form of partially phosphorylated ζ-chains and recruited but not activated ZAP-70 (27). Our findings relating ligand quality, signaling biochemistry, and TCR down-modulation agree with functional studies of others using altered TCR ligands to induce TCR internalization (26).

These recent observations appeared to us to have clear relevance to the model of serial engagement of TCR by peptide:MHC ligands, which was postulated to permit substantial amplification of signals in the face of low ligand densities on APC (19). This striking observation has not been directly confirmed in a quantitative manner in published work by other laboratories, even though some investigators ascribe their functional observations to this phenomenon (11, 21). Others have failed to observe any relationship between TCR down-modulation and certain measures of T cell activation, leading them to question the physiological relevance of this measurement or the derived serial engagement model (25).

Our own analysis of numerous mouse T cell clones and hybridomas showed that some but not all cells responded to ligand with extensive loss of surface TCR expression. The loss of TCR was similar when measured using anti-CD3e, anti-TCR CB, anti-TCR Vβ, and anti-TCR Va reagents, removing one source of possible discrepancy based on the reagents employed for analysis (47). Nevertheless, hybridomas bearing the same TCR as nontransformed clones showed a different extent of TCR down-modulation upon exposure to the same APC bearing the same ligand. This argues that down-modulation is not controlled simply by the strength of the TCR-ligand binding event, but also depends on properties of the cell expressing the TCR. For many T hybridomas,
the pattern of TCR signaling upon ligand exposure is more like that seen with partial agonists than full agonists, as compared with T cell clones expressing the same TCR (unpublished observations), a phenomenon that may in part relate to the lower level of Lck and/or coreceptor found in these hybridomas as compared with normal T cells (18). These observations fit the data obtained here using mouse and human clones, which show that TCR down-modulation closely tracks the extent of full ς phosphorylation and/or ZAP-70 activation. They are also consistent with the work of La Face et al. (48), who observed a lack of TCR down-modulation with mouse clones exposed to antagonist ligands capable of inducing only partial ς chain phosphorylation. Thus, among a large number of tested cells, the extent of TCR engagement is substantially underestimated by examining down-modulation, as interactions leading to partial signaling events are not translated into internalization events.

This dichotomy between engagement events leading to at least limited TCR-associated phosphorylation and those resulting in measurable TCR down-modulation can potentially explain the problems faced by many investigators attempting to replicate the results of Valitutti et al. (19). For many human and a smaller number of mouse T cell clones we have studied, the immunizing ligand used to maintain the cells and activate them to effector function are partial, not full agonists, as defined by the pattern of TCR-associated phosphorylation (Ref. 27 and Stefanova et al., unpublished observations). Use of such partial agonist ligands to explore the phenomenon of serial engagement will lead to a low calculated ratio of engaged TCR to ligand, as only a small number of all interactions leading to detectable phosphorylation changes results in full activation of the TCR and internalization.

This biochemical insight into the serial engagement problem does not, however, fully account for the differences we have observed using mouse vs human clones. The mouse clones employed and the ligands for their TCR give patterns of signaling comparable to the best of the ligands tested using GDBP, despite the 10- to 20-fold difference in calculated engagement ratios. Evidence that the mouse ligands fall into the same potently agonist class as those used with human cells also comes from examination of the concentrations of peptide required to generate comparable ligand density (a measure of the efficiency of peptide:MHC class II binding) and the concentration of peptide needed to achieve similar biological responses. The MCC peptide employed with A.E7 achieves half-maximal proliferation at <10 pM, an even lower concentration than required by the superagonist ligand of GDBP. One is thus left with either concluding that murine peptide:MHC ligands and TCR cannot undergo the type of serial triggering seen with their human counterparts, or as we think is more likely, that down-modulation itself is less efficient in mouse cells than human cells, even given comparable phosphorylation signatures at the individual TCR level. Because down-modulation measurements can only place a lower limit on the extent of actual engagement of TCR, it would seem imprudent to conclude that mouse T cells do not use this mechanism as extensively as human cells. We clearly see evidence for some serial triggering with mouse cells, and it is likely that this number is an underestimate even with potent ligands, providing an additional explanation for difficulties in some laboratories in appreciating the relationship between serial TCR:ligand binding and response.

Finally, on the broader issue of the relationship between TCR down-modulation and effector responses, the apparent lack of a direct association between these two parameters in some studies (25) can be explained by combining our observations on TCR signaling and down-modulation with data on the hierarchical organization of thresholds for elicitation of different TCR responses (26–30, 49). Some activation thresholds are reached with very low levels of overall TCR signaling, which can be provided by a very low fraction of all engaged TCR generating the type of full intracellular signal that would lead to internalization. Because signaling in excess of this very low required level does not increase response above the maximum, exposure to potent ligands can give much more down-modulation than weaker ligands for the same functional outcome. Other responses require a much greater number of full signaling events, and their pattern of elicitation by related ligands should and does largely track TCR down-modulation because one is not examining effects at a plateau level of signaling (27). Serial engagement does not improve the quality of signaling at the individual TCR complex level, because the nature of the phosphorylation events show only a change in amount and not pattern as ligand density varies over a range markedly altering the extent of serial triggering (2, 46). What this process does do is reduce the quantitative difference in overall signaling engendered by high and low ligand densities on the APC, as compared with what would be the case if each ligand only activated a single TCR complex. Thus, serial engagement helps to provide a higher absolute level of signal, sustained over a longer time, allowing these response thresholds to be exceeded with only a few ligands present on the APC membrane.

Acknowledgments

We thank Dr. Irena Stefanova for signaling experiments and Dr. J. B. Bolen for providing anti-ZAP-70 Ab.

References
