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Physical and Functional Bivalency Observed Among TCR/CD3 Complexes Isolated from Primary T Cells

Adam G. Schrum,*†,‡ Diana Gil,*§ Laurence A. Turka,*† and Ed Palmer*§

Unlike BCR and secreted Ig, TCR expression is not thought to occur in a bivalent form. The conventional monovalent model of TCR/CD3 is supported by published studies of complexes solubilized in the detergent digitonin, in which bivalency was not observed. We revisited the issue of TCR valency by examining complexes isolated from primary αβ T cells after solubilization in digitonin. Using immunoprecipitation followed by flow cytometry, we unexpectedly observed TCR/CD3 complexes that contained two TCRs per complex. Standard anti-TCR Abs, being bivalent themselves, tended to bind with double occupancy to bivalent TCRs; this property masked the presence of the second TCR per complex in certain Ab binding assays, which may partially explain why previous data did not reveal these bivalent complexes. We also found that the prevalence of bivalency among fully assembled, mature TCR/CD3 complexes was sufficient to impact the functional performance of immunoprecipitated TCRs in binding antigenic peptide/MHC-Ig fusion proteins. Both TCR positions per bivalent complex required an Ag-specific TCR to effect optimal binding to these soluble ligands. Therefore, we conclude that in primary T cells, TCR/CD3 complexes can be found that are physically and functionally bivalent. The expression of bivalent TCR/CD3 complexes has implications regarding potential mechanisms by which Ag may trigger signaling. It also suggests the possibility that the potential for bivalent expression could represent a general feature of Ag receptors. The Journal of Immunology, 2011, 187: 000–000.

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Abbreviations used in this article: FCM, flow cytometry; gMFI, geometric mean fluorescence intensity; IP, immunoprecipitation; IP-FCM, immunoprecipitation followed by flow cytometry; PE, estimated number of PE molecules; pMHC, peptide/MHC.

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plexes (15–19). Primary T cells provided the source of TCR/CD3 complexes, which were solubilized in digitonin, a condition previously used to define TCR/CD3 valency. The present data support a model wherein a significant proportion of TCR/CD3 complexes display bivalency, their prevalence being sufficient to impact the outcome of a functional Ag binding assay. Additionally, understanding the conditions that govern detection of both TCRs in these bivalent complexes allows a plausible explanation to be suggested as to why they may not have been readily detectable in previous experimental systems. These observations evoke the speculation that the potential for bivalent expression could represent a general feature of the Ag receptors that mediate adaptive immunity.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. DO11.10 (BALB/c) (20), DO11.10/RAG2, 2C, OT1, and 2C × OT1 (F1) mice were bred and maintained in our animal facilities at the Mayo Clinic, University of Pennsylvania, and/or University Hospital-Basel, and all mice were used between 6 and 16 wk of age. Animal procedures were in accordance with Institutional Animal Care and Use Committee regulations at Mayo Clinic, University of Pennsylvania, and University Hospital-Basel.

Abs

Purified mAbs were obtained from hybridoma supernatant: B20.1 (anti-CD28), H57-597 (anti-TCRβ), 1B2 (anti-2C TCR), 145-2C11 (anti-CD3ε), 37.51 (anti-CD28), H57-597 (anti-TCRβ), H146 (anti-CD3ε), and B21.14 (anti-CD8). Purified AF6-120.1 (anti-3-Lε) was purchased from BD Pharmingen. PE-conjugated mAbs purchased from BD Pharmingen included G155-178 (mouse Ig), R35-95 (rat Ig), A19-3 (hamster Ig), 30-F11 (anti-CD45), 53-2.1 (anti-Thy1.2), H129 (anti-CD4), 53.6.7 (anti-CD8α), 53.5.8 (anti-CD8β), AMS-32.1 (anti-H-2Kα), B20.1 (anti-CD2), MR9-4 (anti-Vβ5), 145-2C11 (anti-CD3ε), H57-597 (anti-TCRβ), and B21.14 (anti-CD8). PE-conjugated KJ126 (anti-DO11.10 ε TCR) was purchased from Caltag. H2–Ld–Ig fusion protein and associated reagents, including PE-conjugated secondary mAbs, were purchased from BD Pharmingen. Where indicated, H2–Ld–Ig was loaded with exogenous p2Ca or QL9 peptides following the manufacturer’s instructions.

Fabs

Fabs were prepared using papain digestion, as previously described (21). Following cleavage, Protein A-Sepharose beads (Pierce) were used to bind and remove Fc fragments from the digest. Fabs were then desalted and passed over a Resource Q anion exchange column (GE Healthcare). Homogeneous eluate fractions were subjected to size-exclusion chromatography using a Superdex 200 10/300 GL column to isolate fractions corresponding to ~50 kDa. Final fractions were coupled to IP-FCM beads or FITC, and those capable of binding specific Ag from lysates were used in experiments.

T cells

Unless otherwise stated, whole splenocyte and/or lymph node cells were used as the source of T cells without further isolation. Where noted, cells were stained with anti-CD8-FITC, anti-CD4-PE, and anti-Thy1.2-allophycocyanin, and specific cell subsets were isolated by FACS sorting using FACS Vantage or FACS Aria cytometers (BD). Sorted cells were cultured in vitro in a 1:1 mixture of AIM V (Life Technologies) and RPMI 1640 (+10% Cosmic Calf serum; HyClone Laboratories), with 1:40 additional tissue-culture supernatant from X63Ag8-653 IL2 cells that secrete recombinant murine IL-2 (22). To remove the mAbs used for FACS
FCM analysis involving digitonin-solubilized complexes allows C
digitonin-solubilized TCR/CD3 complexes (Fig. 1) from extraneous membrane proteins, such as H-2Kd, Thy1.2, contrast to digitonin, the detergent saponin did not fully solubilize extracellular domain of CD4, CD8
samples, and probed with a PE-conjugated mAb specific for the anti-TCR analysis of TCR/CD3 isolated from primary BALB/c T cells after plexes with the primary analyte (Fig. 1) specific for another protein (secondary analyte) in shared com-
beads are probed with a fluorochrome-conjugated mAb that may be
In IP-FCM, immunoprecipitating mAbs are covalently coupled to
method and protocol were described previously in detail (17, 18, 23). Briefly, IP Abs or Fabs were covalently coupled to polystyrene latex beads. These IP beads were incubated with lysates from which protein complexes were captured. Subsequently, beads were washed, probed with PE- or FITC-conjugated probe Abs, and analyzed by flow cytometry (FCM). Fluorescence data were analyzed using FlowJo (TreeStar) or CFlow (Accuri) software and are displayed in raw format without smoothing or scaling, except as noted in Fig. 5. Although the minimum number of acquired events suggested for commercial bead-based flow cytometry applications is 25, we acquired 500–3500 bead events for all samples. The y-axis for all graphs is “bead count”, which is omitted from the figures. In some experiments, the geometric mean fluorescence intensity (gMFI) was computed with fluorescent bead standards to convert semi-quantitative data into estimated number of PE molecules (#PE) per bead, as described previously (17, 18). PE-conjugated mAbs used in these experiments were verified to possess a PE/mAb ratio of 1:1, by at least one of two means: communication with commercial departments of Technical Service or in-house size-exclusion fractionation and purification of the 1:1 conjugation product (data not shown).

Statistics
Where indicated, Student t tests were performed using Microsoft Excel software for duplicate data samples within a representative experiment. For summary fluorescence data, either gMFI or median values are displayed with SE bars.

Results
IP-FCM assesses specific inclusion of subunits and exclusion of extraneous proteins in TCR/CD3
In IP-FCM, immunoprecipitating mAbs are covalently coupled to polystyrene latex beads that are used to capture a specific protein (primary analyte) in native conformation from cell lysates. The beads are probed with a fluorochrome-conjugated mAb that may be specific for another protein (secondary analyte) in shared complexes with the primary analyte (Fig. 1A). We performed IP-FCM analysis of TCR/CD3 isolated from primary BALB/c T cells after lysis in digitonin. Complexes were immunoprecipitated with H57 anti-TCRβ mAb-coupled microbeads, separated into parallel samples, and probed with a PE-conjugated mAb specific for the extracellular domain of CD4, CD8β, or CD3ε (see Materials and Methods and associated references). We found that although the known subunit CD3ε was clearly found in complexes together with TCRβ, the functionally relevant coreceptors CD4 and CD8 were not physically associated (Fig. 1B). Conversely, IP of digitonin-solubilized complexes with 2C11 anti-CD3ε mAb confirmed specific inclusion of CD3ε in complexes with TCRβ; however, neither the highly expressed membrane proteins Thy1.2 and CD45 nor the potential TCR ligand H-2Kb was found in digitonin-solubilized TCR/CD3 complexes (Fig. 1C, top panel). In contrast to digitin, the detergent saponin did not fully solubilize cell membranes (24), as observed by failure to fully separate TCR/ CD3 from extraneous membrane proteins, such as H-2Kb, Thy1.2, and CD45 (Fig. 1C, bottom panel). These data confirm that IP-FCM analysis involving digitonin-solubilized complexes allows TCR/CD3 to be isolated and detected with high stringency and specificity.

To estimate TCR/CD3 subunit stoichiometry, we performed IP-FCM analysis on digitonin-solubilized TCR/CD3 from primary BALB/c T cells by capturing complexes with H146, a mAb that binds a C-terminal epitope of the cytoplasmic domain of CD3ζ (25). Captured complexes were probed in parallel with PE-conjugated mAbs (PE/mAb conjugation ratio of 1:1), specific for the extracellular domains of TCRβ or CD3ε, or an N-terminal epitope of the intracellular domain of CD3ζ, to mini-
mize possible steric interference between the paired capture and detection mAbs. Because TCR/CD3 is composed of the associated dimers αεζ/βδζ/δζζ, we found that IP-FCM confirmed the conventionally accepted dimer ratios (Fig. 1B, βεζ = 1:2:2) that were previously observed by experiments involving IP (4) and blue native PAGE (11). Thus, TCR/CD3 complexes analyzed by IP-FCM appeared to contain the expected subunits in their expected relative quantities.

Observation of TCR/CD3 complexes containing two β-chains
By using the same mAb for both IP and probe, it was possible to observe the inclusion of multiple copies of identical subunits within complexes. Thus, when complexes were captured using anti-CD3ε

![FIGURE 2.](http://www.jimmunol.org/) 
Multiple copies of identical TCR/CD3 subunits in shared complexes detected by IP-FCM. A and B, TCR/CD3 complexes were captured from BALB/c T cell lysates that were either undiluted (neat) or diluted in excess lysis buffer by the factor indicated. A, The 2C11 anti-CD3ε mAb was used for both IP and probe. CD3ε subunits that could be probed (and thus did not mediate binding of the complex to the IP bead) are referred to as “hanging” (h), and their relative detection is reported as the ratio of εhβ-chain gMFI (1072340 = 5%), Thy1.2 is a highly expressed surface T cell marker whose absence from the captured complexes indicates adequate membrane solubilization and TCR/CD3 iso-
luration. B, H57 anti-TCRβ mAb was used for both IP and probe in the analysis of captured TCR/CD3 complexes. As in A, β-chains that could be probed under these conditions are “hβ”. The ratio of detectable βh/CD3ε was calculated from gMFI (110/2838 = 4%; 15/1076 = 1%). C, TCR/CD3 complexes from OT1 TCR transgenic T cell lysates were captured with anti-Vβ5 and probed in parallel with PE-conjugated probe mAbs. MR9-4 anti-Vβ5 mAb was used for both IP and probe. Data in all panels represent one of at least two experiments.
mAb and probed with a PE-conjugated version of the same mAb, a positive assay signal was obtained (Fig. 2A, brown histogram). Because each TCR/CD3 complex contains more than one copy of CD3ε (26), one must be required for binding the complex to the anti-CD3ε IP bead, whereas another may be free for detection by a probe mAb. We refer to plural subunits that could be detected under these conditions with the notation “h” (“hanging”, e.g., eh). Because detection of eh was relatively low compared with the total number of complexes captured on beads (Fig. 2A, eh/β = 5%), we reasoned that most ε epitopes in the captured complexes were either occupied by IP mAbs on the beads or otherwise sterically inaccessible to probes.

A similar detection pattern occurred when the H57 anti-TCRβ mAb was used as both capture and probe reagent (Fig. 2B, blue graphs), despite the expectation that only one β-chain would be expressed in each digitonin-solubilized TCR/CD3 complex. The H57 anti-TCRβ mAb binds all mouse TCR β-chains through an epitope in the β constant domain, and the mAb–epitope interaction has been crystallized and shown to involve a unique epitope present in a single copy per β-chain (27). Therefore, we considered the possibility that at least some TCR/CD3 complexes contain more than one β-chain, and we referred to putative plural β-chains that could be probed under these conditions as βh. Detection of βh required the capture of TCR/CD3 and was titratable, because dilution of the lysate during IP reduced βh detection (Fig. 2B). Additionally, observation of βh was not limited to the H57 mAb or its epitope, because TCRs from OT1 TCR transgenic T cells displayed the same pattern when the MR9-4 anti-Vβ5 mAb was used as both capture and probe reagent (Fig. 2C). Therefore, as with CD3ε multiplicity (Fig. 2A), observation of βh suggested that digitonin-solubilized TCR/CD3 complexes can contain more than one β-chain.

To further investigate this possibility, we tested the hypothesis that IP mAbs on the bead influence detection of multiple β-chains per complex. Either intact bivalent H57 anti-TCRβ mAb or monovalent Fab fragments derived therefrom were covalently coupled to beads for use in IP-FCM. Although negative control IP beads failed to capture TCR/CD3 complexes (Fig. 3A), IP with anti-TCRβ mAb captured many complexes with a low degree of detectable free β-chains (Fig. 3B, βh/ε = 1%). In contrast, IP with H57 Fab captured fewer complexes, but these displayed significantly enhanced detection of free β-chains (Fig. 3C, βh/ε = 48%). This result indicated that there were complexes containing a β-chain copy number that was >1, because at least one β-chain mediated capture of a complex onto the Fab beads, whereas at least one additional β-chain remained accessible to subsequent probes.

It remained possible that the β-chain copy number in these complexes was >2, if multiple epitopes within a complex were occupied cooperatively between adjacent IP mAbs on a bead. If correct, then increasing the distance between mAbs on the IP beads would be predicted to diminish adjacent cooperativity and allow the capture of complexes with free, detectable β-chains, in a similar pattern to that observed with Fab beads. However, this proved not to be the case. We tested the idea, increasing the average spacing between IP mAbs on beads by coupling 100-fold less H57 anti-TCRβ mAb. These IP beads captured fewer complexes and lost detection of free β-chains (Fig. 3D) compared with Fab beads (Fig. 3C). Therefore, the copy number of TCR β-chains in complexes that possessed >1 was not >2, meaning it equaled exactly two. Notably, Fig. 3C and 3D display roughly similar quantities of complexes per bead (CD3ε probe, brown histograms), indicating that these samples were “capture matched” (analogous to protein-loading controls that show equal content

**FIGURE 3.** TCR/CD3 complexes containing two β-chains. BALB/c T cell lysates were subjected to IP-FCM analysis to ascertain whether 1, >1, or >2 β-chains were present in complexes. Histograms extending beyond the vertical gray line are considered to be above background. A, Negative control IP with anti-I-Aα mAb did not capture TCR/CD3 complexes. B, IP with standard, bivalent anti-TCRβ mAb captured many TCR/CD3 complexes, with a low level of free β-chains when probed with the same mAb clone (H57) that had been used for IP (βh/ε = 28.3/2831 = 1%). C, IP with monovalent Fab fragments of anti-TCRβ (H57) allowed capture of complexes with increased detectable free β-chains when probed with PE-conjugated anti-TCRβ (H57) (βh/ε = 132/280 = 48%). D, Dilution of the IP mAb on the beads (100-fold) resulted in capture of fewer complexes but did not facilitate the detection of free β-chains. **Right panels.** Possible outcomes are illustrated, as TCR/CD3 complexes are captured and probed with the H57 anti-TCRβ Abs. Those outcomes that are compatible with the data are displayed with a peach-colored background. Inclusion of exactly two TCRs in bivalent complexes is consistent with all data considered together (dashed rectangle). Data in all panels represent one of at least two experiments.
between lanes on a gel or Western blot). But free β-chains were only available for detection by probes if the complexes were captured by monovalent Fab fragments instead of bivalent mAbs. Thus, we conclude that when bivalent anti-β-chain mAbs are used to immunoprecipitate digitonin-solubilized complexes bearing two β-chains, both β-chain epitopes tend to be occupied by the two mAb binding sites. IP using Fab fragments allows capture of complexes in which a second TCR remains freely detectable.

**Observation of TCR/CD3 complexes containing two α-chains**

To determine whether digitonin-solubilized complexes could be observed that contained two copies of TCRα, we followed the classic strategy as previously discussed, using T cells that express two different TCR α-chains to determine whether each would coimmunoprecipitate with the other. However, experimental manipulation of TCR expression was avoided by focusing on the small percentage of wild-type T cells that naturally express two different α-chains (28). Rare BALB/c Vo2⁺ Vo8⁺ dual-TCR T cells were FACS purified and expanded, as well as Vo2⁺ Vo8⁺ T cells and Vo2⁺ Vo8⁺ T cells. A single lysate was prepared of Vo2⁺ Vo8⁺ cells and Vo2⁺ Vo8⁺ cells that had been mixed together in equal numbers; separately, a lysate of Vo2⁺ Vo8⁺ dual-TCR cells was prepared for analysis by IP-FCM. We found no evidence for shared complexes containing both Vo2 and Vo8 when each TCR type was expressed in separate cells (Fig. 4A). Thus, the detergent, lysis, and other conditions of these experiments do not induce TCR coassociation per se. In contrast, lysates from dual-TCR T cells revealed that Vo8⁺ TCRs coimmunoprecipitated with anti-Vo2; conversely, Vo2⁺ TCRs coimmunoprecipitated with anti-Vo8 (Fig. 4B).

Compared with complexes captured by the bivalent anti-Vo2 mAb, its Fab fragment permitted detection of greater quantities of both free Vo2⁺ and free Vo8⁺ TCRs (Fig. 5; anti-Vo8 Fab did not function as an IP capture reagent, data not shown). We conclude that primary αβ T cells express TCR/CD3 complexes that contain two α-chains. Because IP with anti-Vo2 Fab improved detection of both Vo2h and Vo8h, we also conclude that in dual-TCR cells, complexes can be expressed as either homo-bivalent (two copies of Vo2) or hetero-bivalent (one copy each of Vo2 and Vo8).

**Inclusion of properly folded αβ heterodimers and a second TCR in shared complexes**

We sought direct evidence to determine whether a properly folded αβ TCR heterodimer can be present in a shared complex with a second TCR. We used DO11.10 TCR transgenic mice (20), which, on a RAG² background, generate endogenously rearranged Vo2⁺ TCRs coexpressed with the transgenic TCR (29). Importantly, the DO11.10 TCR can be probed with KJ126 mAb, which only binds if the transgenic Vo13 and VB8.2 subunits are both present and properly folded (30). In this system, Vo2⁺ KJ126⁺ dual-TCR T cells represent ~10% of the T cells, with the KJ126⁺ TCR being expressed in considerable excess over the endogenous Vo2⁺ TCR (29). We wished to determine whether capture of the minor TCR (Vo2⁺) would reveal its inclusion in shared complexes with the major, properly folded αβ heterodimer (KJ126⁺). For control samples, Vo2⁺ 3bbm74 TCR transgenic T cells (31) were mixed with a 9-fold excess of DO11.10/RAG2⁻ T cells, which express only the KJ126⁺ TCR. Lysates of the mixed cells or DO11.10 (RAG⁺) cells were prepared and analyzed by IP-FCM. We found that coexpression of Vo2⁺ TCRs in an excess of KJ126⁺ TCRs revealed the presence of shared complexes including the two TCR types (Fig. 6; KJh/ε = 76.9%, where KJh is free KJ126⁺ TCR). We conclude that properly folded αβ heterodimers can be expressed in TCR/CD3 complexes that include a second TCR.
Functional TCR bivalency contributes to optimal binding of antigenic pMHC–Ig fusion protein ligands

We examined whether bivalency in digitonin-solubilized TCR/CD3 complexes can influence binding to pMHC ligands. The strategy was to capture fully assembled TCR/CD3 complexes onto beads and subsequently assess binding to pMHC ligands under varying conditions of TCR bivalency (Fig. 7A). Because assessment of TCR/CD3 complexes required their isolation from digitonin lysates, relatively high-affinity pMHC ligands would be needed because no coreceptors would be present with the isolated TCRs lysates, whereas the alternate hypothesis (HA) was that expression of 2C TCRs in hetero-bivalent complexes has no impact on pMHC–Ig:TCR binding, whereas the alternate hypothesis (HA) was that expression of 2C TCRs in hetero-bivalent complexes has no impact on pMHC–Ig:TCR binding.

2C, OT1, and 2C × OT1 (F1) TCR transgenic mice were used as sources of TCR/CD3 for the following reasons: the F1 mice were previously shown to coexpress both TCRs (35); the 2C αβ heterodimer can be detected by the anti-clonotype mAb 1B2 (36); relatively high-affinity, CD8-independent, allogeneic pMHC complexes (p2Ca/H-2Ld and QL9/H-2Ld) specific for the 2C TCR have been previously characterized (37); in the presence of pMHC ligands specific for 2C, the OT1 TCR could serve as a physically inert pMHC–TCR binding. Also, because it is the last subunit added during assembly (4), ζ-chain was captured as a means of including only fully assembled TCR/CD3 complexes in the experiment.

We found that an equal number of 2C and total TCRs could be captured from each lysate, as confirmed by 1B2 Fab and H57 anti-TCRβ Fab probes, respectively (Fig. 7). Next, because it was previously shown that soluble pMHC ligands must themselves be at least bivalent to stimulate T cell activation (38), we used bi-valent MHC–Ig fusion proteins as probes for the capture-matched, digitonin-solubilized TCR/CD3 complexes. We observed that 2C TCR binding to either p2Ca/H-2Ld or QL9/H-2Ld MHC–Ig ligands was inhibited for 2C TCRs originating from dual-TCR cells compared with single-TCR cells (Fig. 7B). Because the number of captured TCRs was equal between these two expr...
erimental groups, the data indicate that arrangement of TCRs in hetero-bivalent complexes inhibited binding to the MHC–Ig fusion proteins. Thus, data suggest that the property of bivalency is sufficiently prevalent among the population of TCR/CD3 complexes to impact the outcome of this assay of model Ag binding. Additionally, both TCR positions in bivalent complexes must be occupied by Ag-specific TCRs to effect optimal binding to these soluble pMHC–Ig ligands.

Discussion

We present evidence that complexes containing two TCRs and possessing functional bivalency can be found among digitonin-solubilized αβ TCR/CD3 complexes from primary T cells. IP with anti-TCR Fab fragments significantly increased visualization of a second TCR in shared complexes compared with IP with intact mAbs. Several strategies focused on the critical issue of determining whether the bivalent complexes could be considered mature, properly folded TCR/CD3 complexes, rather than misfolded or incompletely assembled, intermediate complexes. First, much of the data relied on ex vivo, physiologic systems that do not involve overexpression and, thus, should reflect normal subunit interactions in the complex (Figs. 1–5). Second, the Abs used for both IP and probe were conformation dependent and do not bind denatured epitopes, implying that subunits were only detected if they were properly folded. Third, a properly folded and assembled αβ heterodimer was shown to be included in complexes with a second coexpressed endogenous α-chain (Fig. 6). Finally, we showed that the fully assembled TCR/CD3 pool, defined as containing CD3ζ, contains complexes that display functional bivalency (Fig. 7).

The fact that anti-TCR mAbs can bind with double occupancy to bivalent TCR/CD3 suggests a reason why these complexes may not have been apparent in previous studies. A T cell that expresses two different α-chains (e.g., Vα2 and Vα8) and a single β-chain (Vβ*) would express three kinds of bivalent complexes: Vα2:Vβ*/Vα2:Vβ*, Vα2:Vβ*/Vα8:Vβ*, and Vα8:Vβ*/Vα8:Vβ* (Fig. 8). An IP mAb specific for one of the TCRs, such as anti-Vα2, is predicted to preferentially capture homo-bivalent Vα2+:Vα2+ complexes, whereas the hetero-bivalent Vα2+:Vα8+ complexes are at a competitive disadvantage for binding to the IP mAb, because the latter can only bind one site per mAb. The net result is that Vα8+ TCRs are underrepresented in such an IP. We observed that the presence of a second TCR per bivalent complex was uncovered when monovalent Fab fragments were used for IP (Figs. 3, 5). Therefore, we propose that the tendency of bivalent mAbs to bind to bivalent TCRs with double occupancy may have contributed to the previous interpretation of past data in which bivalent TCR/CD3 complexes were not detected.

Although we have shown that bivalent TCR/CD3 are present among digitonin-solubilized complexes, we do not know the proportion of complexes that they represent. One focus of future experiments must be to develop other methods that would permit a quantitative estimate of the prevalence of bivalency among all TCR/CD3 complexes. However, despite this current technical limitation, the data in Fig. 7 imply that the proportion of bivalent complexes is sufficiently high to impact the outcome of the pMHC–Ig fusion protein binding assay performed. Thus, based on the assumption that this assay reflects a true potential for functional impact, we speculate that it is likely that a biologically significant number of TCR/CD3 complexes display bivalency.

FIGURE 8. Model of bivalent IP mAbs binding with double occupancy to bivalent TCRs. Assay bias can occur when using bivalent mAbs to assess inclusion of two different coexpressed TCRs in shared complexes (such as those containing Vα2 and Vα8; all other TCR/CD3 subunits not depicted). For this example, two assumptions are made: expression of the two TCR species is equal, and because two different α-chains can be detected in single complexes, then the sequence(s) mediating bivalency involve constant, nonvariable domains. A, Lysates of T cells expressing both Vα2+ and Vα8+ TCRs could contain three populations of bivalent receptors that might freely associate with the following ratios: Vα2+Vα2+ (25%), Vα2+Vα8+ (50%), or Vα8+ Vα8+ (25%). B, IP mAbs specific for one TCR (Vα2) would capture large numbers of Vα2+:Vα2+ homo-bivalent complexes, because this TCR species would bind with the highest affinity to the bivalent mAbs with double occupancy on the beads. At a low level, some Vα2+:Vα2+ complexes would bind with single occupancy, allowing their detection (mAb-bound complexes, far right). Because of competitive disadvantage, hetero-bivalent Vα2+:Vα8+ complexes would rarely be captured and detected on beads, although this occurs to detectable levels by IP-FCM (mAb-bound complexes, left and middle). The net result is that bivalent IP mAbs selectively capture homo-bivalent TCRs that match the specificity of the IP mAb, underestimating the levels of the second TCR species in bivalent complexes, and resulting in the previously held interpretation that bivalent complexes are not expressed. C, Capturing TCRs with anti-TCR Fab fragments would minimize the assay effects described in B, although some double-occupancy binding would still be possible if two Fabs attached to a bead can bind TCR cooperatively or if the Fabs have any natural tendency to spontaneously dimerize noncovalently. Nevertheless, Fabs will allow single-occupancy binding to a greater extent than expected for bivalent mAbs, and this property allowed increased visualization of complexes containing two TCRs in the present work.
It is not known which motifs might specifically interact to compose a bivalent complex. Even in the standard monovalent model of TCR/CD3, the interactions between subunit dimers (αβ/εγ/8ζ/ξ) that compose the multiprotein complex are not fully characterized; as a consequence, it is not known where the various dimers are situated relative to each other. Most schematics of TCR/CD3 follow the model integrated by Sun et al. (39), with TCR in the middle of CD3 subunits, compatible with the proposed interaction of ionizable amino acids between subunit transmembrane domains (7). However, other data support the possibility that the relative subunit positions may be arranged differently, with ε closer to one extreme instead of in the middle of the complex (40). The inability to observe binding between engineered extracellular domains of subunit dimer pairs has left the issue empirically unresolved by crystallography. Thus, how the various subunit dimers interact to compose the complex remains a significant outstanding question, which is not simplified by the proposal that a proportion of complexes may be bivalent. However, our observations argue that bivalency in these complexes is “closed”, mediated by interaction(s) without an open end, because concatemerization is not observed in the digitonin-solubilized complex.

Higher-scale oligomerization or concentration is possible beyond the digitonin-solubilized complexes on which we have focused (14, 41). Using size-exclusion chromatography, sedimentation-velocity ultracentrifugation, surface plasmon resonance, and other methods, several groups have shown that some CD3-free TCR εβ ectodomains can oligomerize when bound by agonist pMHCs (42–44). In the context of full TCR/CD3 expression, a recent study identified sequences in the extracellular constant domain of TCRs that are required for optimal TCR accumulation at the principal site of pMHC contact (40). Therefore, it is an interesting possibility that antigenic engagement might induce association of formerly separate complexes into higher-order catemers/oligomers through TCR motifs.

Bivalent complexes might impact T cell function by enhancing the strength with which these receptors could bind to pMHC ligands (Fig. 7). At least some pMHC ligands have been described to be expressed as a “dimer of dimers” (45–48), meaning that two pMHCs are expressed in coassociation. In such complexes, if both peptides are identical, then a bivalent TCR/pMHC interaction could occur as 2:2, with predicted enhanced binding strength over 1:1 or 1:2 interactions. Other data have shown that two coassociated pMHCs can enhance T cell activation, even if only one of the two peptides is antigenic (49–51). Thus, a pseudo-dimer model of T cell activation was proposed that requires engagement of one TCR by antigenic pMHCs to recruit a second TCR that would somehow contribute to signaling upon interaction with the second nonantigenic pMHC. A model of TCR expression that includes catemers/oligomers of TCR/CD3 complexes by flow cytometry.

In conclusion, bivalent TCRs can be observed among the TCR/CD3 complexes isolated from primary T cells. The glycoprotein sequences mediating this bivalency and its precise functional consequences to T cell signaling are a focus of future work.

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