Cutting Edge: Trimolecular Interaction of TCR with MHC Class II and Bacterial Superantigen Shows a Similar Affinity to MHC:Peptide Ligands

Stella Redpath, S. Munir Alam, Christina M. Lin, Anne M. O'Rourke and Nicholas R. J. Gascoigne

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Bacterial superantigens, such as Staphylococcus aureus enterotoxin A (SEA), are very potent stimulators of T cells. They bind to the Vβ region of the TCR and to MHC class II, stimulating T cells at nanomolar concentrations. Using surface plasmon resonance measurements, we find that binding between the individual components of the complex (TCR-class II, TCR-SEA, SEA-class II) is very weak, but that the stability of the trimeric complex is considerably enhanced, reaching an affinity similar to that found for TCR interactions with MHC:peptide ligand. Thus, the potency of SEA in stimulation of T cells is not due to particularly strong affinities between the proteins, but to a cooperative effect of interactions in the TCR-SEA-MHC class II trimeric complex that brings the kinetics into a similar range to binding of conventional Ags. This range may be the optimum for T cell activation. The Journal of Immunology, 1999, 163: 6–10.

The αβ TCR initiates activation of T cells by recognition of peptide fragments of Ag bound in the groove of MHC molecules. Bacterial superantigens (b-SAgs), as exemplified by the Staphylococcus aureus enterotoxins, such as SEA and SEB, are powerful T cell mitogens produced by certain bacteria that activate T cells by binding to the Vβ region of the TCR (1, 2). SAgs do not require Ag processing, but efficient presentation requires binding of the SAg to an MHC class II molecule at the surface of an Ag presenting cell (3–5). b-SAgs bind to class II at sites distinct from the conventional peptide binding groove on the MHC class II protein. The precise binding sites between b-SAgs and MHC class II vary for different b-SAgs. SEB binds to a site on the α-chain of class II (6). SEA binds to two sites on the class II molecule: a high affinity site on the β-chain that depends on a Zn2+ ion coordinated between His61 on the β-chain and a Zn2+-binding site on SEA, and a low affinity site on the α-chain similar to the SEB-binding site (7, 8).

The interaction between TCR and SAg is mediated by the Vβ region of the TCR (9–12). The binding sites on the b-SAgs for the TCR Vβ, and on Vβ for the b-SAgs, are well defined from mutagenesis and structural studies (10–16). Residues on one face of the b-SAg contact predominantly main-chain atoms of the Vβ, rather than side chain residues, resulting in the ability of certain b-SAgs to interact with several different Vβ elements (11, 12).

The affinity between a MHC:peptide complex and its TCR ligand is comparatively weak, with dissociation constants in the micromolar range (17, 18). Due to the strong activation of the T cell by b-SAg recognition, it had been assumed that the biochemical interactions between these proteins would show different, and likely stronger, binding kinetics and affinities than for conventional MHC:peptide ligands (1). Data for the binding of b-SAgs to class II show surprisingly low affinity binding, in the micromolar range for SEB, and very fast on and off rates (19, 20). Binding of TCR to b-SAg also shows weak and fast binding kinetics (14, 20–22). A synergistically increased signal was obtained when SEB was premixed with soluble class II protein and allowed to bind to immobilized TCR, although the kinetics of this trimolecular interaction were not determined (20).

In this work we analyze the interaction between soluble, heterodimeric TCR, a soluble b-SAg (SEA), and the mouse MHC class II molecule I-Ek. Kinetic measurements of the interactions between the three components are presented, including binding of the TCR to the MHC class II-b-SAg complex. These measurements demonstrate synergy in the binding of TCR to b-SAg in the presence of class II that result in affinities close to those found for TCR interacting with MHC:peptide complexes.

Materials and Methods

TCR constructs

The 2B4 TCR recognizes I-Ek with peptide from pigeon cytochrome c residues 81–104. Its TCR is AV11S2J56, BV3S1A1D2J2S5 (23, 24). The α-chain gene was cloned from 2B4 cDNA with the Vα1 primer 5′-CAT GCC TCC GAG AAA AGA GAG GCT GAA GCT AAT TCA AAA GCT AAC T3′ and the Vβ primer was 5′-CGT GGT ACC TGC GCC CGC 3′.
ATC ACA GGG AAC GTC TGA-3'. The β-chain gene was cloned with the Vβ3 primer 5'-CAT CGC TGC GAC AAA AGA GAG GCT GAA GCT GAT CAG GTG GAG AGT CCT-3' and Cβ primer 5'-GTA TCG CGG CCG CAC AGT CTC GCC CCC-3'. TCR was expressed using *Pichia pastoris* expression vectors pPIC9 and pPIC9K (25) (Invitrogen, San Diego, CA), as described (17). The TCR α-chain was cloned into a modified pPIC9K, with a deletion in the His64 gene (612 bp). The TCR β-chain was cloned first into pPIC9, then into pPIC9K.

**Expression in *P. pastoris***

Transfection (by electroporation) of *P. pastoris* strain GS115 and selection for recombinant clones was accomplished using the Invitrogen protocols. Briefly, sequential selection for growth on RDB plates (without histidine), followed by selection for growth on G418 yeast extract/peptone/dextrose (YPD) plates, selected for colonies with both α- and β-chain genes integrated. These were screened for their *Mut'/Mut* phenotype by comparative growth on minimal dextrose (MD) and minimal methanol (MM) plates. Replica MM plates were blotted onto nitrocellulose, and α- and β-chain secreting clones were detected as described using anti-Cα and Cβ mAbs (26). For induction of methanol metabolism, cells were grown to mid-log phase in buffered glycerol-complex medium (BMGY) then resuspended in buffered methanol-complex medium (BMMY) (BMGY with 0.5% methanol, according to Invitrogen protocols), and grown for up to 72 h. In some experiments, *P. pastoris* was grown in a Biostat E fermentation unit (Braun Melsungen, Allentown, PA), in volumes up to 10 L.

**Purification and characterization of TCR protein***

After concentration of the culture supernatant, TCR was purified by ion-exchange chromatography on DE52 anion exchange resin (Whatman, Hatfield, UK) and eluted with a salt gradient (10–500 mM NaCl). Aggregates and monomeric TCR proteins were then removed by secondary purification on Superdex 200 HR and Superdex 75 columns (17). Fractions from the gel filtration column were assayed by surface plasmon resonance using a BIAcore 2000 Biosensor (BIAcore; Pharmacia, Uppsala, Sweden). Anti-Cβ Ab H57-597 was immobilized on a BIAcore chip, and Ab-captured TCR was tested for heterodimer content (TCR αβ) by flowing an anti-Cα Ab, H28-710 over the surface. Integrity and purity of the protein was assessed by nonreducing SDS-PAGE followed by Coomassie blue staining. The presence of the heterodimeric TCR chains were detected by reducing SDS-PAGE followed by immunoblotting. Purified protein was detected with either anti-Cα or anti-Cβ Abs, followed by amplification with biotinylated goat anti-hamster IgG and streptavidin-HRP (Vector Laboratories, Burlingame, CA) and visualization using an ECL detection kit (DuPont, Boston, MA).

**Soluble MHC class II proteins***

1-E8 was purified from Triton X-100 lysates of CH1 (H-2k) B lymphoma cells by affinity chromatography on a 14-4-4S mAb column as previously described (27). 1-E8 preparation was performed for serologic integrity by ELISA and for purity by SDS-PAGE, with protein visualized by silver staining. Proteins were stored at -20°C at 50–200 μM/mL in 0.25% n-octylglucoside, 10 mM Tris (pH 8.0), and 150 mM NaCl.

**Surface plasmon resonance analysis***

This was performed largely as described (17). Before analysis of purified proteins by surface plasmon resonance, both ligand for immobilization and analyte proteins were passed over a Superdex HR200 column to remove aggregates. A flow of PBS (150 mM NaCl and 0.005% surfactant P20 (pH 7.4)) was maintained throughout the immobilization procedure. About 1200–2800 response units (RU) of protein were immobilized. Both TCR proteins and mAbs were injected at a flow rate of 10 μL/min at 25°C. All analyses were done using the nonlinear fit method of O’Shannessy (28) and BIAevaluation 2.0 software (BIAcore). The bimolecular interactions were described by the simple binding model (Langmuir equation [\(k_{on}B + k_{off}A\)]. A noncompetitive analyte binding model was used to derive the rate constants for the TCR-sea-class II interactions (see Fig. 2). This model describes the binding of one analyte molecule (A) to the immobilized ligand (B), which is followed by a second step in which another analyte molecule (A₂) can bind to the existing ligand-analyte complex

\[
\frac{d[B]}{dt} = -k_{d1}[A_1][B] \quad \text{and} \quad \frac{d[A_1B]}{dt} = k_{a1}[A_1][B] + k_{d1}[A_1B] - k_{d2}[A_1B]/[A_2];
\]

**Results and Discussion***

**Bimolecular interactions***

In this study, we have used surface plasmon resonance measurements to determine the kinetics of the binding among soluble 2B4 TCR, the b-SAg SEA, MHC class II (I-Ek), and the kinetics of formation of the complex of these molecules. First, we measured the binding of SEA to immobilized I-Ek (Fig. 1A). SEA bound to I-Ek with a relatively fast association rate, \(k_{on} = 3.2 \times 10^6\) M⁻¹ s⁻¹, and a fairly slow dissociation rate, \(k_{off} = 3.5 \times 10^{-3}\) s⁻¹ (Table I). This gives a \(K_{d} = 1.3 \times 10^{-7}\) M for the SEA-class II interaction which is similar to the \(K_{d}\) determined at steady-state from a Scatchard plot (\(K_{eq} = 3.2 \times 10^{-7}\) M) (Fig. 1A). The affinity of the binding of SEA to class II molecules on, or purified from a cell surface, has previously been found to range between 3 \(\times 10^{-7}\) and 3 \(\times 10^{-8}\) M (5, 7, 10, 29), but the affinity is dependent on the nature of the peptide bound to class II, which can lower the affinity for a particular molecule to 1 \(\times 10^{-6}\) M (8). The higher affinities probably represent the biologically important interactions as far as T cell activation is concerned, and the on rate that we measure is close to the fastest of those measured in the peptide-specific SEA-binding experiments (8). The off rate measured here is intermediate compared with binding to specific class II:peptide complexes, which range between 2 \(\times 10^{-2}\) and 2 \(\times 10^{-3}\) s⁻¹ (8). It is likely that the peptides that lead to weak SEA binding act by interfering with the interaction of histidine 81 of the class II β-chain with the Zn²⁺-dependent binding of SEA, because His³¹β is involved in the interaction with peptide (6). SEB-class II binding appears to have a much lower affinity of \(\sim 10^{-6}\) M (19, 20). This difference is likely to be due to the lack of the high affinity binding site for MHC class II that is present in SEA.

Next, the interaction of soluble 2B4 TCR with immobilized 1-E8 or SEA was determined (Fig. 1B). The Vβ3 element of the 2B4 β-chain recognizes SEA well (1, 2, 9, 10). The 2B4 TCR bound to I-Ek with extremely low affinity, demonstrating very fast binding kinetics for both the association and dissociation phases. This result is not surprising given that the specific cytochrome c peptide is not present. A very small increase in binding signal over that observed on a control surface was observed (Fig. 1B), and as such the association and dissociation constants of this weak interaction could not be measured. We estimate that the \(K_{d} = \sim 2 \times 10^{-4}\) M. In contrast, the affinity of the TCR for the immobilized b-SAg, SEA, although relatively weak, was significantly higher than those for TCR-class II interactions (Figs. 1, B and C). Although the on rate was too fast to be measured, the TCR-SEA binding kinetics revealed a detectable off-rate (\(k_{off} = 0.065\) s⁻¹). \(K_{eq}\) measurement
by Scatchard plot analysis gave an affinity of $6.9 \times 10^{-5}$ M. Scatchard analysis of 2B4 TCR binding to another b-SAg for which the Vβ3 is reactive, SED, gave a $K_d$ of $1.01 \times 10^{-4}$ M (data not shown). These data indicate that the TCR binds to b-SAg with higher affinity than for a class II molecule without the relevant peptide, whereas binding affinity of SEA to I-Ek was more than 200-fold higher than for binding to TCR. As the intrinsic affinity of the TCR for both its ligands is still rather weak, this suggests that binding may be enhanced during the formation of the ternary complex with SEA and class II, to enable T cell activation by b-SAgs to occur at such low concentrations. We believe this to be the first measurement of the binding between TCR and SEA.

Previous analysis of the TCR-SEB interaction showed a relatively fast on-rate of $\sim 10^{4}$ M$^{-1}$s$^{-1}$ (20), in the range of the faster on-rates for TCR binding to MHC:peptide complexes (18). The off-rate for the human TCR binding to SEB ($10^{-2}$ s$^{-1}$) (20) was similar that determined here for TCR-SEA ($6.5 \times 10^{-2}$ s$^{-1}$), and in the range for off-rates for MHC:peptide (18). The affinity of the TCR for SEB ($8 \times 10^{-7}$ M) was thus stronger than the interaction of TCR with class II:peptide but at the top of the range for class I restricted receptors binding MHC:peptide (17, 18). In contrast, it has been reported that murine Vβ8.2 TCR can bind to SEB with low affinity; $1.4 \times 10^{-4}$ M (21), and SEC with higher affinity; 1 or $5 \times 10^{-6}$ M for SEC2 (21, 22) and 4.5–8.5 $\times 10^{-6}$ M for SEC3 (14, 21). Thus the binding of the human TCR to SEB may be particularly high for a TCR-b-SAg interaction (2).

**Trimolecular interactions**

Until now, there has not been a quantitative assessment of the trimolecular complex between TCR, SEA, and MHC class II, although a qualitative experiment using human TCR, MHC class II and the b-SAg SEB demonstrated that this was more than the sum of its parts (20). We decided to determine the affinity and the kinetics of the binding of TCR to the complex of SAg and class II proteins. As shown in Fig. 1A, SEA binding to I-Ek was easily detectable, and while the on-rate was fast, SEA dissociated from I-Ek in a relatively short time (half-life = 2.9 min). This was not long enough to provide a stable surface for TCR to bind. Thus, sequential injection of SEA followed by TCR created a decaying surface for the TCR to bind to the SEA-class II complex that was unsuitable to measure formation of a stable trimolecular complex (Fig. 2A). However, compared with the injection of TCR over a class II surface (Fig. 1B), the sequential injection of TCR after SEA over the class II surface revealed different kinetics, apparently indicating the formation of a transient trimolecular complex (Fig. 2A). A mixed injection of SEA and TCR produced better results: a higher binding response and a more stable complex of TCR-SEA-class II, with different binding kinetics from those of SEA-class II, was observed (Fig. 2B).

Therefore, binding of SEA and TCR to I-Ek was measured by coinjecting SEA and TCR together over immobilized I-Ek (Fig. 2, B and C). With I-Ek immobilized on the sensor surface, coinjected TCR and SEA bound stably to the MHC class II protein surface.

### Table I. Kinetic constants for the binding of 2B4 TCR to SEA and I-Ek

<table>
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<tr>
<th>Analyte</th>
<th>Ligand</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_a$ (μM)</th>
<th>$K_{off}$ (μM)</th>
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<td>SEA</td>
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<td>$3.5 \times 10^{-3}$</td>
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<tr>
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<td>SEA</td>
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<td></td>
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</table>

**FIGURE 1.** Binding of SEA to MHC class II, and TCR to MHC class II. A, Dose-dependent binding of SEA to immobilized I-Ek surface. SEA was injected at concentrations of 0.5, 1.0, 2.0, 10, and 100 μg/ml at 5 μl/min. B, 2B4 TCR at 1.2 μM was injected in series over flow cells immobilized with SEA, I-Ek and blank control. TCR binds to class II with a very low, barely detectable affinity, whereas higher affinity binding is observed to SEA. C, 2B4 TCR was injected at 2.0, 0.8, 0.6, and 0.4 μM over an SEA immobilized surface. Non-specific bulk responses (dotted lines) for the highest and the lowest TCR concentrations are overlaid with the specific responses generated with TCR binding to I-Ek. SEA was immobilized at 1800 RU and I-Ek at 2500 RU using the amine coupling protocol.
Although SEA binds to I-E\(k\) to form a binary complex, a subsequent injection produces no stable formation of a TCR-SEA-class II ternary complex. Arrows point to the start of injection for each of the proteins.

Due to the biphasic nature of the binding curves, the simple Langmuir model of binding \((A + B = AB)\) failed to provide a good fit, and Scatchard analysis showed a nonlinear relationship (Fig. 2C). The analysis of two-step interactions is complex, and thus the equilibrium dissociation and association constants were measured for the trimolecular complex using a model that describes "noncompetitive binding of two analytes to the same ligand" (see Materials and Methods). We found that the curves gave a better fit to this model (\(\chi^2 = 0.037\)). The first step of the interactions apparently describes the binding of SEA to class II, whereas the second step describes the binding of TCR to this complex. As shown in Table I, the \(k_{on}\) and \(k_{off}\) of the first step are virtually identical with those calculated for SEA binding to class II. The formation of the ternary complex, described in the second step, gave a \(k_{on}\) of \(4.83 \times 10^5\) M\(^{-1}\)s\(^{-1}\) and a \(k_{off}\) of 0.011 s\(^{-1}\). The half-life of the TCR bound to the SEA-class II complex is therefore 36.5 s, compared with 10.7 s for binding to the SEA alone. Thus, during formation of the trimolecular complex of TCR-SEA-class II, two different complexes were being formed sequentially. It is likely that the first complex formed is that of SEA and class II because of their higher affinity compared with TCR-SEA and TCR-class II, and because of the similarity of the kinetics of the first stage of the reaction to the bimolecular interaction between these molecules (Table I). The SEA-class II complex then serves as a ligand to which the TCR can bind, resulting in the formation of the ternary complex TCR-SEA-class II. In addition, the difference between TCR-SEA-class II binding and TCR-SEA binding probably represents the stability which is added by the interaction between TCR and class II. This may be due to the influence from the TCR \(\alpha\)-chain interaction with class II (12). Crystal structures of TCR-\(\beta\)-complexed to b-SAgs, of b-SAgs complexed to class II, and of TCR-\(\alpha\)-chain, have been used to assemble models for the TCR-\(\beta\)\(\alpha\) interacting with the b-Sag-class II complex (11, 12). These findings show that the VB domain has no contact with class II, but indicate that the VA domain interacts with the class II \(\beta\)-chain, providing an explanation for the influence of V\(\alpha\) on certain SAg responses (12). Our results suggest that the VA interaction with class II provides extra stability in the binding of TCR to b-Sag-class II over binding to b-Sag alone.

The dissociation constant \((K_d)\) of the trimolecular complex of TCR bound to SEA-class II is calculated as 2.27 \(\times 10^{-6}\) M. It is both surprising and interesting that this affinity is close to what has been reported for the higher range of TCR-peptide:MHC interactions with agonist ligands (17, 18). It has been reported that a small number of MHC:peptide complexes are able to cause activation of a large number of TCRs through serial triggering (30). MHC:peptide Ags and b-SAgs are both very effective at T cell activation. In contrast, monovalent anti-CD3 Abs are much less efficient at triggering TCR, perhaps because the high affinity of binding prevents them from dissociating and therefore from serially triggering TCRs. This finding implies that there is a middle ground for TCR affinity, which is optimal for T cell activation (30). MHC:peptide complexes are able to cause activation of a large number of TCRs through serial triggering (30).

### References


CUTTING EDGE