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NMR: An Essential Structural Tool for Integrative Studies of T Cell Development, pMHC Ligand Recognition and TCR Mechanobiology.

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Abstract

Early studies of T cell structural biology using X-ray crystallography, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) focused on a picture of the $\alpha\beta$ T cell receptor ($\alpha\beta$ TCR) component domains and their cognate ligands (peptides bound to MHC molecules, i.e. pMHCs) as static interaction partners. Moving forward requires integrating this corpus of data with dynamic technologies such as NMR, molecular dynamics (MD) simulations and real-time single molecule (SM) studies exemplified by optical tweezers (OT). NMR bridges relevant timescales and provides the potential for an all-atom dynamic description of $\alpha\beta$ TCR components

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prior to and during interactions with binding partners. SM techniques have opened up vistas in understanding the non-equilibrium nature of T cell signaling through the introduction of force-mediated binding measurements into the paradigm for T cell function. In this regard, bioforces consequent to T-lineage cell motility are now perceived as placing piconewton (pN)-level loads on single receptor-pMHC bonds to impact structural change and $\alpha\beta$ T-lineage biology, including peptide discrimination, cellular activation, and developmental progression. We discuss herein essential NMR technologies in illuminating the role of ligand binding in the preT cell receptor (preTCR), the $\alpha\beta$ TCR developmental precursor, and convergence of NMR, SM and MD data in advancing our comprehension of T cell development. More broadly we review the central hypothesis that the $\alpha\beta$ TCR is a mechanosensor, fostered by breakthrough NMR-based structural insights. Collectively, elucidating dynamic aspects through the integrative use of NMR, SM, and MD shall advance fundamental appreciation of the mechanism of T cell signaling as well as inform translational efforts in $\alpha\beta$ TCR and chimeric T cell (CAR-T) immunotherapies and T cell vaccinology.

Keywords

Integrative structural biology; Nuclear magnetic resonance spectroscopy (NMR); Optical tweezers; Single molecule; molecular dynamics (MD); T cell receptor (TCR); preT cell receptor (preTCR)

Introduction-T cell Mediated Immunity

$\alpha\beta$ T lymphocytes form a critical component of the cell-mediated immune system in the mammalian host to protect against viral pathogens or cancerous transformations (Rudolph et al. 2006; Wang and Reinherz 2012). The recognition of cells expressing anomalous gene products is mediated by the $\alpha\beta$ T cell antigen receptor ($\alpha\beta$ TCR) on T lymphocytes. The $\alpha\beta$ TCR detects “foreign” (non-self) peptides displayed on the surface of altered cells bound to host major histocompatibility molecules (MHC) as a foreign peptide-self MHC complex (pMHC). The $\alpha\beta$ TCR is composed of the heterodimeric ligand-recognizing α and β subunits in complex with the CD3 dimers ($\epsilon\delta$, $\epsilon\gamma$, and $\zeta\zeta$), creating the eight subunit surface receptor (Fig. 1a). The $\alpha\beta$ heterodimer is responsible for ligand binding but lacks signaling elements, while conversely, the CD3 subunits lack ligand recognition elements but comprise the entire signaling capacity of the TCR (Fig. 1a). Recognition occurs through the combined α and β complementarity determining region (CDR) loops within each variable (V) immunoglobulin-like domain of which CDR1 and 2 are germline encoded loops and the CDR3 is a hypervariable product of gene segment recombination events [Fig. 1b, (Davis and Bjorkman 1988)]. Each of the eight $\alpha\beta$ TCR subunits is positioned on the T cell membrane by a single spanning transmembrane (TM) region (Fig. 1a). The preT cell receptor (preTCR), the developmental precursor to the $\alpha\beta$ TCR, utilizes CDR loops of the V β subunit in combination with an exposed V β patch region (Fig. 1b) (Zhou et al. 2011; Mallis et al. 2015; Mallis et al. 2018). TCR recognition takes place at the T cell-antigen presenting cell (APC) interface in conjunction with either surface CD4 or CD8 co-receptors and other stimulatory molecules, with TCR-pMHC contact activating the T cell via an orchestrated cascade of intracellular signals starting with phosphorylation of CD3

intracellular tyrosine activation motifs (ITAMs), and culminating in transcriptional activation and subsequent biological functions (Fig. 1c). Depending on current cellular environment, developmental state, prior activation, ligand density and identity, this activation will lead to a variety of T cell responses such as cell proliferation and cytotoxic activity, soluble mediator production, differentiation including formation of memory cells, or apoptosis.

Integrative Structural Biology of the preTCR

In order to mount an effective immune response, a diverse T cell repertoire is generated via a series of ordered stochastic rearrangements of TCR β and TCR α gene segments to ensure a variable ligand binding surface on millions of individual T cells bearing distinct $\alpha\beta$ TCRs. Each T lymphocyte expresses ~20,000–40,000 identical, clonally derived $\alpha\beta$ TCRs specific to that cell. This randomization of receptors ensures that a broad array of potential ligands will be recognized by T cells within the mature $\alpha\beta$ T cell compartment. Prior to maturation of $\alpha\beta$ T cells in the thymus, the organ where T lymphocytes are generated, thymocytes undergo a series of selection steps, removing unwanted TCR reactivities to prevent autoimmunity on the one hand, while retaining useful MHC-compatible reactivities on the other hand, so called negative and positive selection processes, respectively (Fig. 1d). Immediately following rearrangement of the β gene, but prior to rearrangement of the α gene, thymocytes express the preTCR, a heterodimer composed of an invariant preTCR α (pT α) subunit paired with a unique β subunit (Fig. 1b,1d). Given the absence of a V domain, pT α possesses no ligand-binding capacity, in contrast to its disulfide linked β with ligand-binding V β domain shared with that in the mature $\alpha\beta$ TCR, albeit in a configuration lacking the mature V α recognition surface (Fig. 1b). Numerous studies had purported that the preTCR functioned in a ligand-independent manner, based on successful thymocyte development of ectodomain deleted constructs (Irving et al. 1998) and in MHC-deleted host systems (Koller et al. 1990; Crump et al. 1993). However, our structural biological studies have shown prominent roles for ligand recognition in preTCR function including enhanced thymocyte proliferation, developmental progression and repertoire modulation (Mallis et al. 2015; Das et al. 2016).

Herein we will discuss the impact of solution NMR in coordination with an array of structural and biophysical techniques including X-ray crystallography and the SM biomembrane force probe (BFP) and OT technologies (Fig. 2). Two aspects of T cell biology will be addressed. First, we will delve into recent insights in thymic development wherein the preTCR mediates progression from immature thymic precursors to committed $\alpha\beta$ TCR expressing thymocytes prior to exportation to the peripheral lymphoid tissue as mature T cells. Secondly, we will explore how an iterative cycle of structural, biophysical and biological technologies are illuminating the inner workings of the T cell receptor complex to define the earliest events in T cell signaling. Lastly, we will contextualize some recent findings and suggest avenues of research that appear promising in resolving longstanding mechanistic questions concerning T cell activation.

Our initial observations suggested that the preTCR could retain significant ligand binding function (Wang et al. 2008) based on the retention of overall fold within the β chain of the mature TCR in the absence of α .

(Bentley et al. 1995; Sundberg et al. 2002; Li et al. 2005; Zhou et al. 2011) and confirmed in a crystal structure of a human preTCR (Pang et al. 2010). The N15 β subunit crystal structure (Zhou et al. 2011) retained its overall fold as well as CDR loop conformation when compared with the structure of N15 $\alpha\beta$ (Wang et al. 1998). β chains from three distinct TCRs, N15 and N30, which bind to the VSV8/H-2K^b pMHC ligand (Imarai et al. 1995), and D10, which binds to Conalbumin (CA)/IA^k (Reinherz et al. 1999), exhibit well dispersed ¹H-¹⁵N HSQC spectra (Mallis et al. 2016). The V domain resonances are characteristically distinct, reflective of the variable sequence afforded through recombination events during thymocyte development, while the C domains share most resonances with the exception of those proximal to the V domain (Mallis et al. 2016). Pointedly, the V domain resonances of the D10 β chain are quite similar within the V β C β construct as compared to the V α V β single-chain construct (scD10) previously determined by NMR (Hare et al. 1999), with the exception of those proximal to the V α or C β interface (Mallis et al. 2016). The scD10 solution structure (Hare et al. 1999) was itself found similar to scD10 in complex with its ligand CA/IA^k, as determined by X-ray crystallography (Reinherz et al. 1999). All β domain characterizations provide evidence suggesting a fold capable of ligand binding for the unpaired V β . Notably, the recent publication of resonance assignments for human and murine TCR $\alpha\beta$ s show a general concordance of β chain residue assignments with our observations (He et al. 2015; Natarajan et al. 2017; Rangarajan et al. 2018). Highlighted here is the power of one of the most basic of all NMR experiments, the ¹H-¹⁵N HSQC, for assessing the fold of proteins, assuring consistency of conformation despite construct-specific differences. Standard 3D backbone assignment experiments can then allow sequence-specific evaluation of residue-resolution conformation.

Despite strong functional evidence for the specificity of N15 $\alpha\beta$ for VSV8/H-2K^b and a crystal structure of the complex (Ghendler et al. 1998; Teng et al. 1998) the affinity was determined too weak to be measured precisely by SPR, suggesting that for N15 β alone, measuring this interaction would require a very sensitive assay. Within this apparent weakness of affinity lies a strength of NMR spectroscopy, as we readily detected and measured a specific interaction between N15 β and VSV8/H-2K^b with a K_D of 400 μ M via ¹H-¹⁵N TROSY-HSQC titration experiments (Mallis et al. 2015). Chemical shift perturbation (CSP) analysis together with cross-saturation transfer (CST) experiments (Takahashi et al. 2000) outlined a novel interaction surface, combining canonical CDR interaction surfaces with a V β patch region normally occluded by association with V α in the mature TCR but exposed in the preTCR (Fig. 1b) (Mallis et al. 2015). The use of CST was critical for confirming that the changes resulted from a direct, specific interaction between preTCR and pMHC and not secondary conformational changes transmitted from the CDR region to the V β patch region, for instance, or an artifact caused by self-association in a non-specific manner. We were further able to establish specificity of the interaction by demonstrating site-specific abrogation of chemical shift changes and peak intensity losses from N15 β point mutations within the CDR2 when N15 β was incubated with VSV8/K^b (Mallis et al. 2015). CST was also necessary to define a specific surface for the N30 β -VSV8/H-2K^b interaction, which was much weaker than that for N15 β as measured by CSP and intensity loss parameters. We later showed a binding surface on the NMR-labeled pMHC consistent with the canonical TCR binding surface and thus were able to produce preliminary models of the preTCR-pMHC

interaction through rigid body modeling approaches (Mallis et al. 2018). Most apparent was that in order for the V β patch region to be utilized in addition to the CDR region, there had to be a significant difference in domain orientation relative to that seen in canonical TCR-pMHC interactions (Fig. 3). This suggests potential structural mechanisms for functional biological differences between preTCR and TCR.

Once ligand binding was established at the molecular level, we wanted to extend our observations to the preTCR on the cell surface in a lipid arrayed physiological environment associated with the CD3 components (Fig. 1a). One way to confirm ligand binding specificity in a cellular context is to utilize 2D affinity measurements via biomembrane force probe (BFP) technology (Liu et al. 2014; Ju et al. 2017). BFP can measure ligand binding kinetics of intact receptor complexes on cell surfaces at a single molecule level and has been used to characterize binding affinities and specificities of TCR for pMHC (Liu et al. 2014). In contrast to many structural biology techniques, BFP does not require isolation of target receptor complexes from the expression host, and in fact, the expression host may be identical to the physiological context of the molecule in question. Details of BFP theory and practice have been reviewed previously (Ju et al. 2017). In order to study the ligand binding properties of preTCR N15, i.e. pT α /N15 β in the context of all relevant CD3 components, we utilized the SCID.adh cell line. This line, which expresses a native pT α but no β chain, was established previously as a model system for preTCR function in developing thymocytes (Carleton et al. 1999). The pMHC ligand is oriented on the BFP probe, a pipette-immobilized red blood cell (RBC) which acts as force transducer and regulator, via a C-terminal biotin which binds to RBC surface streptavidin. Following transduction of N15 β into the SCID.adh cells and subsequent confirmation of preTCR complex on the surface, we interrogated these cells with a set of pMHC posited to be specific (VSV8/H-2K^b) and unrelated ligands (OVA/H-2K^b, Hb/IE^k, gp66/IA^b). Consistent with the NMR results, BFP found evidence for the preTCR N15-VSV8/H-2K^b interaction (Mallis et al. 2015). Loss of 2D affinity with mutagenesis of β and when thymocytes were presented with non-canonical pMHC ligands shows preTCR-pMHC specificity, but with less ligand discrimination than seen with the TCR (Mallis et al. 2015). Moreover, in keeping with previous and concurrent work on TCR-pMHC (Liu et al. 2014; Das et al. 2015), we observed first by BFP (Mallis et al. 2015) and then by SM OT assays [(Das et al. 2016), (Fig. 4a,c)], that the preTCR N15 exhibited an increase in bond lifetime with increasing force, up to ~10–12 pN, a phenomenon termed a ‘catch bond’ (Marshall et al. 2003), followed by decreasing lifetime with force, the more conventional ‘slip bond’ (Mallis et al. 2015). Catch bond formation in SM OT experiments utilizing isolated protein domains reveals that the TCR/preTCR ectodomains *per se* possess the essential elements for the catch bond. This binding observation is transformational, as it suggested that contrary to prior supposition, the preTCR is not only capable of pMHC binding, but this ligand interaction is regulated in a physical load-dependent manner.

While it was previously appreciated that antibody-mediated CD3 crosslinking on the surface of thymocytes would trigger Ca²⁺ flux (Shinkai and Alt 1994), because of the lack of recognized preTCR ligands, the effects of preTCR triggering, *per se*, were unknown. Utilizing a multiwell, high-throughput flow cell format, pMHC ligand was immobilized on the flow cell surface and wells were loaded with individual SCID.adh cells identical to those used for the BFP characterization above but carrying a Ca²⁺ sensitive dye. We were thus able to follow

hundreds of individual cells and visualize Ca^{2+} release in response to ligand in real time. As we anticipated, there was a clear Ca^{2+} response to pMHC ligand that was absent in preTCR expressing cells on control surfaces or in untransduced cells that lack the preTCR on pMHC ligand immobilized surfaces (Mallis et al. 2015).

Given biophysical behaviors of pMHC binding to isolated ectodomains and native preTCR complexes on the cell *in situ* as well as confirmation of cell signaling through pMHC ligation of preTCRs, we next tested the role of preTCR-pMHC interaction on developmental progression using differentiation assays. For this purpose we utilized *in vitro* stromal cell culture, a standard method in developmental immunology (Mohtashami et al. 2010). As discussed above, a function of the preTCR is to ensure that the thymocytes mature from the early pT α / β expressing stage (DN3) to the more mature $\alpha\beta$ TCR expressing stage (DP) (Fig. 1d). Thymic progenitors can be transfected with β -chain, such that pT α will pair with the transfected β to form the preTCR. In a background in which genetic recombination of the TCR α and TCR β subunits is prevented, this preTCR will be the only one present in the cultures. If these transfected thymocytes are cultured in the presence of a stromal cell such as the OP9-DL4 cell line (Mohtashami et al. 2010), which provides an array of pMHC as well as the Notch receptor ligand delta-like ligand 4 (DL4), the thymocytes proliferate and progress to the DP stage in a preTCR dependent manner. Paralleling the NMR as well as the BFP and OT observations, we found development depended on both CDR residues as well as V β -patch residues (Mallis et al. 2015). Using a genetically engineered variant of the OP9-DL4 cell line which does not express MHC on the surface, we were able to show a strong dependence on MHC recognition for DN3 thymic expansion and development to the TCR-expressing post-DN3 stages (Das et al. 2016). When a pMHC incorporating VSV8, H-2K^b, and β 2m in a single chain construct (scpMHC) (Hoerter et al. 2013) is expressed on the surface of the otherwise MHC-negative cells, scpMHC stroma are able to rescue the proliferation and development of preTCR-bearing thymocytes. Thus, the essential observation of β chain binding pMHC in the NMR tube has been fully recapitulated as a significant biological receptor function in cellular studies.

Mechanistic Study of preTCR

OT can measure pN-level forces and nm-resolution distances, allowing one to detect structural changes within the molecules measured under preTCR-pMHC bond load. OT SM is able to provide a window on the biophysics of the domains under consideration outside of the cellular context (Fig. 4a), and additionally, can be used in a single molecule/single cell (SMSC) format to observe the behavior of the receptors in their membrane anchored native environment (Fig. 4b). Conformational transitions, potentially representing unfolding and/or domain rearrangements within the $\alpha\beta$ TCR during interactions with pMHC, were observed upon pN force application (Das et al. 2015). Similarly to the TCR, the preTCR exhibits conformational transitions with the application of force [Fig. 4d–e, (Das et al. 2016)]. This suggests that the mechanism of triggering the preTCR is force-dependent, raising the possibility that the preTCR may participate not only in selecting useful β -chains, but also may provide screening in early thymocyte development for their mechanical suitability to subsequent incorporation into $\alpha\beta$ TCRs. How potential differences in preTCR and $\alpha\beta$ TCR β domain orientation relative to pMHC (Fig. 3) may impact this aforementioned physiological

receptor fitness screening remains to be seen. Yet another surprise was the bimodal profile in the preTCR-pMHC force-lifetime curve. Not only is there a catch bond with a force maximum around 12 pN similar to that seen for the $\alpha\beta$ TCR, but there is a second force maximum at about 20 pN (Fig. 4c). This higher-force catch bond may have its origins in the V β patch region of the preTCR, as mutagenesis of three conserved patch residues to Ala results in near complete loss of the high force catch bond (Das et al. 2016). Also unexpected was that not only were non-canonical peptides binding to preTCRs, but that some of these “cross-reactive” peptides show more robust binding than the canonical ligands (Das et al. 2016). For instance, the ovalbumin-derived octamer(SIINFELK)/H-2K^b (OVA/H-2K^b), the ligand for the OT1 TCR, or the Q4H7 variant (the 4th residue substituted with Gln and the 7th substituted with His) showed a catch bond with preTCR N15 (Das et al. 2016). When preTCR N30 was tested, it was found that Q4H7/H-2K^b bound with longer lifetimes than VSV8/K^b, the canonical ligand for N30 $\alpha\beta$ (Fig. 5a). Moreover, the force-lifetime curve for preTCR N30 with Q4H7/K^b showed a more pronounced second force maximum at 20 pN that is absent with the OVA peptide from which it is derived (Fig. 5a). These data demonstrate that despite the inherent cross-reactivity of the preTCR, the multi-modality and kinetics of the catch bond are quite sensitive to changes in the receptor-ligand relationship.

The cross-reactivity of the preTCR N30 highlights a fundamental difference in the recognition of preTCR for its ligand in which peptide restriction is less important than for the TCR. Analysis of TROSY-HSQC titration data, including chemical shift perturbation and resonance intensity losses confirms this interaction between N30 β and Q4H7-H-2K^b (Fig. 5b–d). The combined data show an interaction surface that utilizes both CDR and V β patch regions, in contrast to the surface highlighted for the weak ligand VSV8/H-2K^b illuminated through CST which appeared not to include the lower half of the V β patch region (Mallis et al. 2015). Through comparative analysis of N30 β interactions with these various ligands by NMR and SM we can begin to understand the origins of the catch bond in the preTCR and possibly the complex behavior implicit in the multi-modal force-lifetime curves.

In addition to kinetic measurements of binding events, positional parameters in SM OT can also provide information on the conformational state of the molecule under force. Within the preTCR under force, for instance, stepwise dwells in the probe bead position were found, indicative of a reversible conformational transition [(Fig. 4d–e), (Das et al. 2016)]. The transitions were also found in TCR $\alpha\beta$ (Das et al. 2015) and were correlated with the presence of a catch bond, highlighting the co-dependence of each event on the force applied during interaction (Das et al. 2016). The rate of reversible transitions in the preTCR was approximately ten-fold that of the mature TCR, suggesting that the TCR α subunit adds a substantial stiffening to the molecule relative to pT α , which lacks a V domain (Das et al. 2016). At this time, the site(s) of extension have not been characterized, but given the presence of a common β chain in the TCR and preTCR examined and similar equilibrium force, the β subunit must participate, although this does not rule out a concurrent unfolding in α . In fact, if the α and β transitions are coordinated, then this could explain in part the slower unfolding/refolding rate of the TCR versus preTCR. The transition was observed in cell-surface TCR complexes binding pMHC in SMSC OT experiments (Das et al. 2015) and is possibly linked to triggering, as work derived through repeating the unfolding-refolding

cycle could function to inject energy into the TM regions of CD3 and/or the plasma membrane, disrupting resting-state ITAM conformations and activating the T cell.

Mechanotransduction – Inward from the Ectodomains

Concurrent with work on the preTCR, the mechanoreceptor functions of the $\alpha\beta$ TCR have been a major focus in our laboratories [reviewed in (Brazin et al. 2015; Feng et al. 2018)]. NMR structure determination of the CD3 ectodomains showed a unique side-by-side arrangement of the component ϵ and γ or ϵ and δ subdomains (Sun et al. 2001; Sun et al. 2004). The tightly coupled central β strands with hydrophobic interdigitating interface residues of the two subunits of each CD3 $\epsilon\gamma$ and CD3 $\epsilon\delta$ heterodimer implied a rigidity that would lend itself to transmission of force from the ectodomains into the respective TM domains and thereby into the cell. The membrane-proximal region was also found to possess a constitutively formed disulfide bond in a highly conserved and functionally critical CxxC motif (Touma et al. 2007; Brazin et al. 2014). One could thus propose a mechanistic description of binding within the ectodomains and their relay to intracellular components via the TM domains in a manner consistent with the mechanoreceptor function of the TCR (Kim et al. 2009; Brazin et al. 2015). However, the precise quaternary arrangement of the TCR is as yet unknown, with several models supported by diverse data such as antibody binding, genetic conservation analysis, glycosylation maps, X-ray crystallography, molecular dynamics simulations [reviewed in (Kim et al. 2012)], and more recently, NMR interaction studies (He et al. 2015; Natarajan et al. 2016) and cryoEM (Birnbbaum et al. 2014). For the TM regions, the picture is also unclear, with, until recently, primary structural data only on the CD3 ζ subunits, which are known to form homodimers (Call et al. 2006). All other TM domain associations are implied either by mutagenesis studies (Alcover et al. 1990; Blumberg et al. 1990; Manolios et al. 1990; Call et al. 2002) or molecular modeling (Krshnan et al. 2016).

We thus sought to clarify the TCR assembly and its mechanistic role in signaling through direct structural study, beginning with the TCR α subunit (Brazin et al. 2018). When the NMR structure was solved, we immediately noted that it did not form a straight membrane-spanning helix, but rather formed a kinked structure consisting of two helices with a bend at Asn261 (Fig 6). NMR backbone resonance assignments as well as structural calculations defined two interchanging states, the kinked structure (L state) and a more extended configuration (E state). Sequence analysis ascertained that the residues forming the bend within the helix were highly conserved. Prior studies on TM determinants of assembly focused on several charged residues within the $\alpha\beta$ TCR heterodimer and CD3 TM domains (Alcover et al. 1990; Blumberg et al. 1990), with suggestions that charge-pairing mechanisms dominate subunit assembly (Call et al. 2002). In keeping with this hypothesis, subunit association was perturbed with individual mutation of a Lys (K256) or Arg (R251) residue within TCR α TM. However, in contrast to the prior hypothesis, K256 did not appear to participate in interactions with a charged Asp residue within CD3 δ . The interaction was instead mediated almost entirely by the membrane-proximal connecting peptide (CP) motifs (Bäckström et al. 1996), a long, unstructured region within TCR α and the previously mentioned CxxC motif within CD3 δ (Brazin et al. 2014). Instead, it seemed that the effect of mutating the charged residues on subunit assembly was mediated indirectly through the influence on

membrane positioning, as determined using both NMR and electron paramagnetic resonance spectroscopy (EPR). Importantly, through the use of complementary NMR and EPR methods, we recapitulated essential features of our structural model using two different lipid-like environments, phospholipid micelles and liposomes.

The NMR data led us to assess the consequences on receptor assembly and function by guiding the selection of critical residues to mutate, changing only TCR α TM, within the fully assembled $\alpha\beta$ TCR complexes in T cells. It was found that the R251L weakened the association of predicted binding partner CD3 ζ , although this was only detectable after immunoprecipitation (IP), and not evident by fluorescence activated cell sorting (FACS) analysis or immunofluorescence microscopy (IM). K256L mutation, in contrast, abrogated all CD3 associations ($\epsilon\gamma$, $\epsilon\delta$, and $\zeta\zeta$) as detected by FACS, IM or IP. As one would expect from the loss of CD3 signaling components, standard cellular activation assays showed that the K256L mutation also prevented ligand-triggered activation as measured by IL-2 ELISA, while the R251L mutant showed activation-induced IL-2 production indistinguishable from that of WT TCR-expressing cells. However, the transcriptional profile of each of these cells actually resembled an activated phenotype, with the R251L mutant showing markers of early activation while the more dramatic K256L mutant showing late-stage activation markers, suggesting that TCR $\alpha\beta$ dissociation from CD3 components represents an early step in physiological TCR triggering, consistent with reports of CD3 $\zeta\zeta$ dimer loss in inflammation and cancer [refs in (Brazin et al. 2018)].

SMSC analysis of the mutations targeting the TCR α -CD3 δ CP-based juxtamembrane interaction showed little difference from WT in the force-dependent bond lifetime profile, suggesting that the interaction does not significantly affect the biophysical properties of ligand binding. Surprisingly, mutagenesis of the membrane charged residues did indeed change the force at maximal lifetime for the R251L mutant, shifting the force maximum from almost 15 pN to approximately 10pN, without significantly lowering the maximal lifetime (Brazin et al. 2018). The maximal bond lifetime of the K256L mutant, moreover, decreased to 25% of that of the WT cell surface TCR binding to pMHC (Brazin et al. 2018). Considering the significant distance of the membrane-embedded R251 and K256 from the TCR-pMHC interface, the $\alpha\beta$ TCR is a precisely tuned mechanoreceptor. It is likely that TCR complex quaternary associations and/or the membrane positioning of the TCR α TM buttresses the TCR $\alpha\beta$ subunits in such a way to enable or enhance the intrinsic catch bond. It is clear that the catch bond is coupled or modified through those quaternary subunit associations in the context of the cell membrane since we observe catch bond formation in SM experiments between pMHC ligand and the TCR $\alpha\beta$ heterodimer lacking CD3 (Das et al. 2015). Some quaternary associations may be uncoupled from the force transmission pathways in cells since the TCR α -CD3 δ association-targeting mutants do not affect the force-dependent bond lifetime profiles, despite causing a marked decrease in ligand-dependent IL-2 production and in signaling in single-cell Ca^{2+} triggering assays (Brazin et al. 2018).

To describe the complete signaling system one must understand that load on the TCR $\alpha\beta$ bond occurs externally, from cell movement during immune surveillance at the time foreign pMHC is first detected (Fig. 1c), and prior to a stop movement signal [reviewed in

(Brazin et al. 2015)] as well as internally due to cytoskeletal forces (Comrie and Burkhardt 2016). This mechanical force fosters a structural transition in the TCR $\alpha\beta$ ectodomain to strengthen bond lifetime and energize the TCR, impacting the flanking CD3 heterodimeric ectodomains and inducing TCR complex quaternary change (Das et al. 2015; Das et al. 2016). Force proceeds from subunit ectodomains via their respective connecting peptides to impact the TM segments in the cholesterol-rich lipid rafts where TCRs reside, largely linked to the actin cytoskeleton (Caplan et al. 1995). These changes within the TM likely include a change of the TCR α TM from the L-state conformation shown in Figure 6 to a straightened E-state conformation which affects CD3 subunit association (Brazin et al. 2018). Lipid rafts offer a stiffened platform to suppress mechanical noise while spatially confining force (Anishkin and Kung 2013). These events induce changes in lipid composition vicinal to the $\alpha\beta$ TCR, including loss of negatively charged phosphatidylserine and phosphoinositides (Gagnon et al. 2012). The lipid changes combined with structural coupling between juxtamembrane connectors, TM and cytoplasmic tail segments, result in the release of the CD3 tethered tails from the inner leaflet of the plasma membrane to expose ITAM phosphorylation sites for signaling initiation (Aivazian and Stern 2000; Xu et al. 2008). Similar considerations must be at play in the preTCR ligation by self pMHC with even faster transition rates between compact and extended conformers (Das et al. 2016). Differences as well as similarities in these two receptor systems shall be of great interest to unravel. Such changes in local TCR architecture are akin to a phase transition where an organized crystalline structure melts and fluidizes to accommodate a new geometry. For such a process to occur, it must begin from a stable structure relative to thermal energy $k_B T$. Bioforces acting on the $\alpha\beta$ TCR-pMHC bond have been shown to be on the order of 15 pN with transitions on the length scale of 10 nm. From a perspective of work, each transition represents the exchange of $\sim 150 \text{ pN} \cdot \text{nm}$ of energy which is $\sim 36 k_B T$. Since the transition is reversible (Das et al. 2016) on the time scale of a second one can envision a cell machinery driven 150 zeptowatt heater driving this melting process through sustained reversible single receptor transitioning. Another benefit of a non-equilibrium process such as a phase transition is that the barrier to activation through its exponential dependence on energy can explain the $\sim 10^4$ ligand discrimination fidelity seen in T cell performance.

Perspectives and Future Directions

As detailed above, NMR together with SM analyses have defined the mechanical function of the TCR and preTCR through coordinated study. The structural contribution of the C β FG loop (Fig. 1b) in regulating bond lifetime (Das et al. 2015) requires allosteric communication between the C-domain and the CDR loops. One predicts that such communication would be evident in NMR spectra of TCR in complex with pMHC. In that sense, there has been some confirmation from two laboratories (Natarajan et al. 2017; Rangarajan et al. 2018), with some caveats. In neither case are changes measured directly via CSP found in the C β FG loop, although CSP and peak intensity changes are seen within the C domains of both α and β , particularly at the C α -C β interface. The cluster of changes in these studies is proximal to regions previously implicated by NMR studies in the binding of CD3 ectodomains (Birnbbaum et al. 2014; He et al. 2015; Natarajan et al. 2016; Natarajan et al. 2017; Rangarajan et al. 2018). NMR-derived chemical shift propensities as well as X-ray crystallographic B factors have defined

the C β FG loop as a mobile structural element (Natarajan et al. 2017; Rangarajan et al. 2018) despite its role stabilizing the TCR and preTCR during force-dependent binding (Das et al. 2015; Das et al. 2016). MD simulations have suggested that the C β FG loop becomes significantly less mobile during pMHC ligation (Rangarajan et al. 2018), though this observation remains to be confirmed with biophysical measurements.

Two potential avenues exist to expand on the aforementioned observations. First, the force-induced transitions within the TCR and preTCR (Das et al. 2015; Das et al. 2016) may become evident only in the presence of pMHC and force. Thus, MD simulations with an applied force may illuminate allosteric pathways within the involved molecules. To this end, some recent work has proposed mechanistic explanations for catch bond behavior through the use of directional force models (Sibener et al. 2018). Their simulation suggested that transverse rather than longitudinal pulling relative to the TCR-pMHC interface is needed to engage the catch bond (Sibener et al. 2018). Although this is apparently in agreement with the directionality of force required in single cell activation studies with pMHC (Kim et al. 2009; Feng et al. 2017), the 2 ns simulation time was too short to reveal any realistic conformational behavior of the system. Also, since only the variable domains were used, allosteric behavior of TCR could not be addressed (Knapp et al. 2017). In comparison, an extensive simulation study of 172 pMHC complexes indicated that even 100 ns simulation time is not enough to reveal any clear differences in behaviors of peptides with different immunogenicity (Knapp et al. 2014). This indicates a difficulty of using MD simulation, which is currently limited to at most a μ s time scale, in studying second-scale dynamics of the TCR. However, we note that the goal of performing MD is not recapitulating the process in real time. Instead, MD is an effective tool for elucidating physical and dynamic mechanisms. In this regard, careful combination of enhanced sampling, directional load application, and trajectory analysis algorithms may lead to the discovery of structural principles in TCR selection and peptide recognition. Advances in computing power and analysis methods are enabling detailed investigation of functionally significant domain dynamics (Hwang et al. 2017) and organization of surface water molecules (Teng and Hwang 2018). Furthermore, MD can provide an essential linkage between the atomic-level equilibrium NMR data and the mesoscale non-equilibrium OT data.

Second, NMR measurements may yet confirm the existence of dynamically regulated force-dependent pathways through the participating molecules. X-ray crystallographic B factors may be informative but external loops are potentially biased by crystallographic contacts. Direct measurement of dynamics by NMR rather than proxy measurements like chemical shift propensities would likewise be preferable. However, one begins to run up against the large size of the TCR-pMHC complex, approximately 90 kDa, in being able to complete the standard relaxation pulse measurements. This is due to intensity losses from 50 to 100% at the molecular interface for TCR $\alpha\beta$ -pMHC (Natarajan et al. 2017; Rangarajan et al. 2018) or TCR β -pMHC (Mallis et al. 2015). The intensity loss is due to two factors: 1) the overall increase in molecular weight of the complex which results in faster relaxation, and 2) the possibility of chemical exchange at the interface which further broadens the resonances. Relaxation measurements on such systems are further confounded by the challenge that the longitudinal relaxation time (T1) increases with molecular weight and that deuteration is required. This demands longer recycling time especially in the heteronuclear nuclear overhauser effect (HNNOE) measurements. Some of these problems are mitigated by trimming constructs to

critical subdomains, as in removal of distal $\alpha 3$ and $\beta 2m$ on pMHC and/or utilization of β as a proxy for the preTCR (Mallis et al. 2018). The pMHC truncation strategy has been shown to be successful for two MHC gene products to date (Jones et al. 2006; Mallis et al. 2018) and may be more widely applicable to many pMHCs. The use of β as a proxy molecule is only valid for preTCR investigations, but may illuminate essential functions of TCR signaling as well, given the importance of the β subunit and the similarities between TCR and preTCR SM behavior (Das et al. 2016). A single-chain $V\alpha V\beta$ containing construct has been produced as a model for several $\alpha\beta$ TCRs (Novotny et al. 1991; Hare et al. 1999; Maynard et al. 2005) and $\gamma\delta$ TCRs (Xu et al. 2011; Luoma et al. 2013) recapitulating the solution binding properties of the full TCR in each case. However, self-evidently, this will not allow measurement of allosteric changes to the C domains and thus obviates any involvement of the C β FG loop in enhancing binding.

Improvements may also be gained by combining the appropriate labeling strategy with the relaxation optimized NMR methods. Investigation of both structure and dynamics by NMR require resonance assignments to relate spectral changes to structural features at atomic resolution. Relaxation optimized methods such as TROSY combined with labeling techniques including deuteration (Salzmann et al. 1998) and pyruvate labeling (Robson et al. 2018) allow backbone resonance assignment in large molecular weight systems. Incorporating line shapes from C β coupling on the C α resonances can further facilitate backbone assignments without additional relaxation losses (Coote et al. 2018). The methyl-TROSY has allowed the study of very large proteins complexes (Kay 2011; Kerfah et al. 2015a; Huang et al. 2017) including the TCR-pMHC (Natarajan et al. 2017) and has become standard methodology. The relatively fast rotation of methyl residues compared to the overall tumbling of the protein provides favorable relaxation properties for the methyl groups. However assigning the sidechain resonances, including the methyl resonances, has been a major challenge. The out-back-style experiment recently developed leverages the linear ^{13}C -labeling design and the selective protonation of methyl residues to enable side chain resonance assignment of large protein systems (Kerfah et al. 2015b). In cases where there is a high-resolution structure, an alternate strategy is to use 3D/4D methyl-methyl NOESY experiments to obtain the assignment of the methyl resonances (Pritišanac et al. 2017).

For large molecular weight systems such as the TCR, preTCR and pMHC, structure determination by NMR relies on NOESY based distance restraints obtained from amide and methyl resonances. To date, the only NMR structures are below 20kDa, such as the sc $V\alpha V\beta D10$ TCR (Hare et al. 1999), CD3 $\epsilon\gamma$ and $\epsilon\delta$ (Sun et al. 2001; Sun et al. 2004), and the TM fragments of CD3 $\zeta\zeta$ (Call et al. 2006) and TCR α (Brazin et al. 2018). In order to extend upwards to the larger fragments such as the TCR $\alpha\beta$ ectodomains or full pMHC, each above 40kDa, newer innovative strategies must be employed. Timeshared 3D/4D NOESY experiments leverage the amide and methyl TROSY effect to provide distance restraints between methyl to methyl, methyl to amide and amide to methyl groups (Frueh et al. 2009; Mishra et al. 2014). Site-specific and alternate labeling strategies combined with the aromatic TROSY effect provide distance restraints to aromatic residues (Pervushin et al. 1998; Milbradt et al. 2015). Paramagnetic relaxation enhancement (PRE), pseudo-contact shift (PCS) and residual dipolar coupling (RDC) experiments provide additional structural restraints, especially useful in determining structures of complexes such a TCR-pMHC or preTCR-pMHC (Battiste and Wagner 2000; Hass and Ubbink 2014; Pilla et al. 2016; Nitsche and Otting 2017).

NMR is a powerful method to provide atomistic details of protein dynamics over a wide range of time scales (ps to seconds). In particular, two recent developments in NMR methodology, relaxation dispersion (Rex) (Sprangers and Kay 2007; Loria et al. 2008) and chemical exchange saturation transfer (CEST) (Vallurupalli et al. 2012; Vallurupalli and Kay 2013) provide access to protein dynamics in the millisecond time scale, a regime typically associated with conformational exchange. This sort of chemical exchange has been deduced in the N15 β V domain within the CDR2 and flanking β -strand regions (Mallis et al. 2015) and is likely the first such instance to be found among the diverse TCR sequence space. Catch bond formation could well be engendered by high energy barrier slow exchange events with transitions to tight-binding conformers favored with the addition of force to the nascent TCR-pMHC interaction. Such data would complement the MD approaches discussed above.

As in NMR, recent advances in SM technology as well as further tailoring of current methods can contribute significantly to improving our understanding of initiation of T cell signaling. Higher throughput methods analogous to that used for the preTCR Ca²⁺ assay cited above (Mallis et al. 2015) while incorporating force through fluid flow, magnetic force, obstacles and surface topology or other mechanisms (Ruel-Gariépy and Leroux 2004; van Mameren et al. 2008; Liu et al. 2016; Stockslager et al. 2017; van Mameren et al. 2018)

could aid in evaluation of heterogeneous populations of cells. Additionally, coupling SMSC assays or single-cell triggering with high-throughput next generation sequencing for RNAseq transcriptional analysis would allow precise correlation of biophysical parameters of TCR-pMHC and preTCR-pMHC with signaling outcomes. These studies could inform how different bond lifetimes as well as magnitudes and frequencies of structural transitions under various loads, among other biophysical parameters, may link to differential gene programs altering apoptosis, survival, metabolism, migration, adhesion and the like. Studies *in vitro* involving load-triggered T cells and thymocytes using single cell transcriptomes as well as *in vivo* studies employing structurally and biophysically defined TCRs and preTCRs using retrogenic and transgenic mice shall highlight the translational relevance of structural mechanobiology. We can anticipate implications for T cell effector and regulatory functions as well as T cell memory.

The combined NMR and MD approach could then be leveraged to suggest mutagenesis targets to be tested by SM, with a focus on correlating mechanical parameters with both structural data and biological outcomes. While each method has its unique strengths as well as limitations in size, timescale, nonequilibrium force and other parameters, their integration shall collectively bridge and reveal the emerging picture of TCR mechanobiology. Our experience suggests that we can glean mechanistic insights that feed back into our structural understanding (Fig. 2) by targeted transcriptomic analysis (Brazin et al. 2018), single cell OT (Feng et al. 2017), SM and SMSC (Das et al. 2015; Das et al. 2016) and developmental assays (Mallis et al. 2015; Das et al. 2016). Indeed, perhaps we are approaching the ability to apply changes to components of the TCR signal transduction elements with predictive results, finally understanding the biophysical mechanism of TCR triggering. Different $\alpha\beta$ TCRs directed against the same pMHC may undergo distinct conformational transitions, rates of oscillation between compact versus extended states and/or other bioforce-linked parameters impacting effector or memory functions. Such insights, in principle, could allow for selection of ideal T cell populations for immune protection against pathogens or tumors.

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Fig. 1.

$\alpha\beta$ TCR and preTCR structure and function. **a.** The $\alpha\beta$ TCR is a multimeric cell surface receptor composed of eight membrane-spanning subunits. The TCR α and β , which comprise the ligand-recognition portions of the receptor, are associated as a disulfide-linked heterodimer, with variable regions as well as constant and TM domains but lacking signaling elements. Invariant CD3 $\epsilon\gamma$ and $\epsilon\delta$ subunits without pMHC ligand-binding capacity contain extracellular Ig-like domains which mediate subunit pairing, TM domains, and intracellular domains responsible for signaling. CD3 $\zeta\zeta$ exists as a disulfide-bonded homodimer with TM and intracellular signaling domains, but possesses no extracellular domain. **b.** The preTCR incorporates pT α instead of TCR α prior to the DN to DP transition (see **d.** below) but the receptor complexes are otherwise identical with respect to CD3 components (not shown). The C β FG loop is represented in light blue within each receptor. The preTCR and $\alpha\beta$ TCR recognize pMHC ligand using overlapping but distinct surfaces. Note the V β patch region

important for preTCR-pMHC interaction is occluded by the presence of the TCR α subunit in the TCR. The surface exposed CDR1–3 region of each ligand binding subunit is shown as denoted by CDR α and CDR β labels. Additionally, the intracellular C-terminal tail of pT α that may possess signaling capacity is depicted. **c.** T cell function is governed by its mobility. The T cell attaches to an APC which presents target pMHC. As the T cell scans the APC (top panel), its surface receptors are exposed to a wide range of forces which engender force-dependent changes (bottom panels) within the TCR ectodomains, TMs, and intracellular ITAM signaling motifs as well as, potentially, proximal membrane lipid environment. For simplicity, CD4 and CD8 co-receptors and CD28 and CD80/86 costimulatory interactions are not shown. **d.** Developmental progression within the thymus. Thymocyte development is a process orchestrated by spatial migration to and within the thymus, somatic genetic rearrangements, pMHC ligand-mediated β -selection by the preTCR and positive and negative selection by the $\alpha\beta$ TCR. Abbreviations used for thymocyte subpopulations: Thymus specific progenitor, TSP; CD4 and CD8 Double Negative (DN) Stage 3 and 4, DN3 and DN4 with preceding stages DN1 and DN2 omitted for clarity; CD4 and CD8 Double positive, DP; CD4 or CD8 Single positive, CD4 SP or CD8 SP.

Fig. 2.

Integrative structural biology utilizes an iterative cycle of technologies.

Fig. 3.

Comparison of docking geometries for $\alpha\beta$ TCR and preTCR with pMHC. N15 $\alpha\beta$ utilizes the CDR loops within V α and V β while preTCR utilizes V β CDR loops and V β patch (see Fig. 1b) resulting in different orientation of the ectodomains relative to pMHC ligand. The C β FG loop is highlighted (FG). Structure of N15 $\alpha\beta$ -VSV8/H-2K^b is from Teng et al. (1998) with distal α 3 and β 2m domains hidden for clarity. Model of preTCR-pMHC is from Mallis et al. (2018).

Fig. 4. OT-based technologies reveal force-dependent binding properties of TCR and preTCR. **a.** Schematic of OT SM assay. TCRαβ-leucine zipper (LZ) is captured by a monoclonal antibody (mAb) specific for paired LZ (2H11) which is covalently attached to a polystyrene bead via digoxigenin-modified DNA linker/anti-digoxigenin mAb. C-terminally biotinylated pMHC (b-pMHC) is immobilized on a movable piezo-stage surface via streptavidin bound to biotin-polyethylene glycol (PEG). Binding is monitored by displacement of bead (Δx) from center of OT which accompanies piezo stage movement. **b.** In OT SMSC assay, cell surface TCR is interrogated by pMHC linked via DNA spacer to bead in OT. **c.** N15αβ (left panel) and preTCR N15 (right panel) SM-measured binding to VSV8/H-2K^b. N15αβ exhibits a single force-lifetime maximum (1) indicative of a catch bond up to approximately 12 pN, while preTCR N15 exhibits two maxima, at 12 pN (1), and at 20 pN (2). **d.** SM OT bead trajectory plots show reversible conformational transitions

within preTCR N15 (top) and TCR N15 $\alpha\beta$ (bottom) while bound to VSV8/H-2K^b at a pulling force of 10 pN. Note the shorter time scale of preTCR transition as compared to TCR. **e.** A schematic diagram illustrates the stages of conformational transition corresponding to the trajectory plots in **d**. The initial state (green) is generally offset a small distance (~3–6 nm) from the post-transition Compact State (blue). The Extended State (also in blue) can be as much as 10 nm offset from the Compact State. Data in **c,d** are from Das et al. (2016).

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Fig. 5.

SM and NMR reveal peptide ligand cross-reactivity in preTCR recognition. **a.** Overlay of force-bond lifetime curves for preTCR N30 interacting with VSV8/H-2K^b (red), OVA/H-2K^b (violet) and Q4H7/H-2K^b (blue) ligands. A single force-maximum is found at 12 pN (1) for VSV8 and OVA while Q4H7 promotes a second at 20 pN (2). Data shown is from Das et al. (2016). **b-d.** Chemical shift perturbation and intensity loss highlight an interaction surface in N30β binding to Q4H7/H-2K^b-t which utilizes both CDR and Vβ patch regions. **b.** Overlay of ¹H-¹⁵N TROSY-HSQC amide resonance spectra of 200μM ¹⁵N N30β-c1 (Mallis et al. 2015) (red) with 200μM ¹⁵N N30β-c1 + 200μM unlabeled Q4H7/K^b-t (Mallis et al. 2018) (blue). **c.** Select spectral regions from **b** with resonance assignments shown, highlighting residues for which chemical shift perturbation and/or peak intensity losses occur. Note residues F50, D52, and E53 from CDR2 and residues F104 and F105 from the Vβ patch show evident changes. **d.** Chemical shift perturbation and intensity loss analysis,

performed as outlined in ^{Mallis et al. (2018)}, is displayed on the N30 β crystal structure (pdb 3Q5T).

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Fig. 6.

TCR α TM structure (PDB 6MF8) illustrating positions of intra-TM helical break and basic amino acids. A dynamic interchange between kinked (L-State) and extended (E State) is present in NMR experiments in which the TM is immersed in lysophosphoglycerol (LPPG) phospholipid micelles. This dynamic interchange in the ms to s timescale is likely enhanced with force application, and could be modulated through intersubunit interactions or post-translational modifications (Brazin et al. 2018). For illustration, the relative position of the segment is placed in the bilayer predicated on both NMR PRE experiments using micelles and EPR spin label depth measurement experiments with liposomes in aforementioned reference. Basic amino acids K256 and R251 are represented as sticks. Gly259 and Asn261 are located within the dynamic hinge region. The light orange color is representative of the lipid acyl chains while the dark orange represents the polar/charged headgroups. The unstructured connecting peptide region is not membrane embedded, but rather, is disordered in structural calculations and dynamic measurements.