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Profound Enhancement of T Cell Activation Mediated by the Interaction Between the TCR and the D3 Domain of CD4

Dario A. A. Vignali* and Kate M. Vignali

CD4 plays an important role in the activation and development of CD4+ T cells. This is mediated via its bivalent interaction with MHC class II molecules and the TCR:CD3 complex through p56lck. Recent studies have implicated a third site of interaction between the membrane-proximal extracellular domains of CD4 and the TCR. Due to these multiple interactions, direct evidence for the functional importance of this extracellular association has remained elusive. Furthermore, the residues that mediate this interaction are unknown. In this study, we analyzed the function of 61 CD4 mutants. Alanine substitution of just 2 residues, either Q114/F182 or F182/F201, which are partially buried and located close to the D2/D3 interface, completely abrogated CD4 function. Direct evidence for the functional importance of TCR:CD4:D3 interaction was obtained using an anti-CD3:Fc:anti-CD4:antibispecific Ab. Surprisingly, it induced strong T cell activation in hybridomas transfected with cytoplasmic-tailless CD4, despite the lack of association with either p56lck or MHC class II molecules. However, this effect was completely abrogated with the CD4 mutants Q114A/F182A or F182A/F201A. These data demonstrate that TCR:CD4:D3 interaction can have a profound effect on T cell activation and obviates the need for receptor oligomerization.

Materials and Methods

Construction of CD4 mutants and T cell transfectants

The mutant CD4 molecules were made using the Altered Sites site-directed mutagenesis kit (Promega, Madison, WI). The sequence of the oligonucleotides used are available on request (dario.vignali@stjude.org). Following verification of mutations by DNA sequencing, the CD4 mutants were subcloned into one of two eukaryotic expression vectors, pCIneo (Promega), or pHßApr-1neo, which contains the human β-actin promoter, SV40 poly(A), and a neomycin resistance cassette (15).

The 3A9.N49 CD4 loss variant was used as the recipient for all CD4 mutants (12). Transfection and selection protocols have been described elsewhere (9, 12). Briefly, 3A9.N49 was transfected by electroporation

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Flow cytometry

The panel of anti-CD4 Abs and the methodology used in this study have been described previously (9). The mAbs used were all rat IgG unless stated: GK1.5 (16), YTA 191.1 and YTA 3.1 (17), YTS 177 and H129.19 (18), and KT6 and KT9 were provided by Kathryn Wood (Oxford University, Oxford, U.K.); YT4.1 and YT4.2 (19) by Charles Janeway (Yale School of Medicine, New Haven, CT); RL172.4 (20) (IgM) by John Sprent (Scripps Clinic, La Jolla, CA); 2B6 (21) (IgM) by Ethan Shevach (National Institutes of Health, Bethesda, MD). RM4.4 was obtained from PharMingen.

Antigen presentation assays

Assays were performed essentially as described elsewhere (9, 11). Briefly, T cell hybridomas (5 × 10^5; 100 μl) were cultured with various stimuli in flat-bottom 96-well microtiter plates for 24 h. The following stimuli were used: 1) L3K5.2 as APC (murine B cell lymphoma; H-2A^k, 2.5 × 10^5, 100 μl) with synthetic peptides (Center for Biotechnology core facility at St. Jude Children’s Research Hospital or Chiron Technologies (Mimotopes), Raleigh, NC) at the concentrations indicated. Peptides were purified by reverse phase HPLC (Vidic C-18; The Nest Group, Southborough, MA), verified by mass spectrometry and quantified by amino acid analysis (Center for Biotechnology, Chiron Technologies, or Harvard Microchemistry Unit, Harvard University). 2) A20.J3 APC (murine B cell lymphoma; H-2^A) expressing H-2A^k, 2.5 × 10^5, 100 μl) with antigenic peptides (center for Biotechnology core facility) (11). 3) Plates precoated with GK1.5 for 24 h at 4°C. Eluted proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were blocked with 5% nonfat dry milk in TBS-T (0.2% Tween 20, 10 mM Tris-HCl (pH 8), 150 mM NaCl) at 4°C overnight. p56kx was detected with an N-terminus-specific rabbit polyclonal antiserum (Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature, followed by 1/12,000 dilution of goat anti-rabbit horseradish peroxidase (HRP) (Amersham, Arlington Heights, IL) for 1 h at room temperature, and developed using ECLplus (Amersham). Blots were blocked with 5% BSA (Boehringer-Mannheim, Indianapolis, IN) in TBS-T, and tyrosine phosphorylation was detected with biotinylated 4G10 (1.0 μg/ml; UBI) (90 min at room temperature), followed by 1/12,000 dilution of streptavidin-HRP preformed complexes (Amersham) (60 min at room temperature). Blots were developed using ECLplus (Amersham). To detect the original protein, blots were stripped in 100 mM 2-mercaptoethanol (Bio-Rad), 2% SDS, 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50°C; washed three times; and blocked with 5% nonfat dry milk in TBS-T at 4°C overnight. Blots were probed with the rabbit anti-p56^k-specific antiserum (1/200) (90 min at room temperature), followed by protein A-HRP (1/10,000; Amersham) (60 min at RT). Blots were developed as above.

Results

CD4 binding to MHC class II molecules or p56^k is dispensable

CD4 colocalizes with the TCR:CD3 complex via its association with either MHC class II molecules or p56^k (3, 23). Although these events are known to be important, certain hybridoma systems are independent of one or more of these interactions. We have taken advantage of two unique properties of the 3A9 T cell hybridoma. First, it responds to HEL 48–63 (DGSTDGILQIN SRRW) in the absence of CD4 but is absolutely dependent on CD4 for recognition of HEL 48–61 (DGSTDYGILQINSR) or its peptide analogue 48–61FF (DGSTDYGILQINSRF) (12). Second, 3A9 is not functionally dependent on binding to either MHC class II molecules or p56^k for T cell activation (9). This is demonstrated by the ability of CD4.MM4, which fails to interact with MHC class II molecules (24), and CD4ΔCY, which lacks the cytoplasmic tail, to restore T cell function when expressed in a 3A9.CD4-negative variant (3A9.N49) (12) (Fig. 1A). However, transfectants expressing a CD4 molecule possessing both of these mutations (mCD4.MM4ΔCY) responded poorly, even when MHC:peptide ligand density was substantially increased (Fig. 1). This was achieved by using A20 B cells expressing the HEL 48–63 or 48–61AA (DGSTDYGILQINSRAA) peptide covalently attached to H-2^A (CAP transfectants) (11, 25). This effectively increases the percentage of H-2^A-loaded with this peptide from ~1% on peptide-pulsed APCs to ~30% (26, 27). These results demonstrate that the 3A9 T cell hybridoma used in this study requires one, but not both, of these interactions for T cell function (9). This provided the ideal functional system with which to analyze CD4:TCR interaction.

Identification of CD4 mutations that affect T cell function

We initially made 15 mutants of wild-type murine CD4 containing alanine substitutions at 4–8 residues (10 in the D3 domain and 5 in the D4 domain). Collectively, these mutants covered a substantial proportion of the CD4.D3/D4 domains (Fig. 2A). These mutants were transfected into the CD4-negative T cell hybridoma 3A9.N49. All of the mutants were expressed at comparable levels to the wild-type CD4 transfectant (data not shown). Furthermore, the transfectants responded strongly to immobilized anti-TCR and anti-CD4 Abs in quadruplicate. Cells were transferred into V-well microtiter plates for 24 h. The following stimuli were standard (Genzyme, Cambridge, MA) in 50 μl of medium alone and cultured.
The mutants, M4_10, 24, and 61/62, which had the most dramatic effect on T cell function, were selected for further study, with the production of an additional 27 mutants. Initially, single point mutations were made of the residues substituted in M4_10 (M4_29, 32–35) (Table II). However, these mutants fully restored T cell function. It is conceivable that more than one residue is required, and to test this we mutated only four of the original five residues in M4_10 (M4_36–40). This analysis clearly showed that F201 was the most important residue given that alanine substitution of 202–205 (M4_36) had little effect, whereas the other four mutants, which included F201A, failed to restore T cell responsiveness to HEL 48–61. However, mutants consisting of F201A together with each of the other four mutants individually all restored function (M4_47–50). Thus, F201 appeared to be the most important residue of those altered in the M4_10 mutant (as shown by M4_36), although other residues are clearly required as F201A alone has no effect.

A similar approach was used to determine the residues in M4_61/62 and 24 that were responsible for their failure to restore function. A single residue in each mutant, Q114A in M4_61 and F182A in M4_24, appeared to be responsible for the functional phenotype observed (Table II; M4_79 and M4_41). However, as shown for F201, substitution of either Q114 or F182 alone did not abolish T cell function (M4_84 and M4_53).

This analysis suggested that Q114, F182, and F201 may be functionally important, but single alanine substitutions had little or no effect (Table II; M4_32, 53, 84). Could combinations of these three residues result in a CD4 mutant that completely failed to restore function? Because F182A was the only single point mutation that had any effect on T cell function, we reasoned that in combination with a second point mutation, loss of function might be observed. Indeed, a number of CD4 mutants containing just two single point mutations failed to restore T cell function (Table III). M4_53/84 (Q114A/F182A) and M4_51/53 (F182A/F201A) were selected for further study. These residues are partially buried and therefore unlikely to participate in direct interaction with the TCR (Fig. 2, B–D). These mutations have undoubtedly affected the local conformation/function of residues that directly mediate CD4:TCR interaction.

**Abrogation of CD4:TCR interaction blocks T cell function**

Wild-type CD4 and the mutants, Q114A/F182A and F182A/F201A, were expressed comparably in the 3A9 CD4 loss variant (Fig. 3). Only 2 of 12 mAbs showed different levels of staining between the wild-type and mutant CD4 transfectants; RL172.4, which recognizes an epitope in the D1 domain, showed a twofold increase in staining with the mutants, while staining of the mutants with YT4.2, which recognizes an epitope in the D2 domain, was reduced to one-fourth of the wild-type level. The mCD4-MM4 mutation completely abrogates bind of YT4.2 (9) but has only a small effect on T cell function (Fig. 1A). Taken together, these data suggest that the Q114A/F182A and F182A/F201A mutations have not had a gross effect on structure of CD4.

While the 3A9 CD4 loss variant transfected with wild-type or mutant CD4 produced a similar IL-2 response to immobilized anti-TCR Ab (H57.157) (data not shown), the CD4 mutants completely
fail to restore the ability of transfectants to secrete IL-2 in response to HEL 48–61 or 46–61FF (Table III, Fig. 1A). This defect could be overcome by supertransfection with wild-type CD4, demonstrating that this functional defect was due to the mutant CD4 molecule rather than to another deficiency in the T cell transfectant (data not shown). However, this profound defect was not overcome by substantially increasing ligand density on the APC. This was determined using A20 B cells expressing the HEL 48–63 or 48–61AA peptide covalently attached to H-2A^k (CAP transfectants) (11, 25), which effectively increases ligand density from ~1% to ~30% (27). While the hybridoma expressing wild-type CD4 could respond strongly to both CAP transflectants, hybridomas that lack CD4 or express the CD4 mutants, Q114A/F182A or F182A/F201A, failed to respond to HEL.48–61AA.CAP

FIGURE 2. Residues that affect CD4:TCR interaction are located close to the D2-D3 interface. The human CD4 structure has been used to highlight the possible location of important residues in the murine homologue. A. The mutations detailed in Table I are illustrated: M4_61/62 (light blue), M4_10 (pink), M4_24 (light green), M4_9, 11–17, 19–23 (lavender). D2 is at the top (truncated), D4 at the bottom to which the transmembrane (tm) and cytoplasmic domains would be attached. D3 is in gray, D2 and D4 in white. The structure is rotated vertically at 90-degree intervals. B. Residues proposed to bind to MHC class II molecules are indicated in yellow (H27, N30, N32, K35, Q40, F43, K46, S49, K50, R59, N73, T101, N103, S104, D105), and orange (K1, K2, K7, T17, S19, K21, S23, E87, D88, Q89, Q163, Q164, K166) (31–34). The yellow residues in the D2 domain are mutated in the MM4 mutant (Fig. 1) (24). D1 and D3 are in gray, D2 and D4 in white. C and D. These panels show an enlarged view of the D3 domain with CD4 rotated at 0 degrees and 180 degrees to indicate the position of residues affecting CD4:TCR interaction: M4_61/62 (light blue) with Q114 (dark blue), M4_24 (light green) with F182 (dark green), and M4_10 (pink) with F201 (red). Graphics generated using Rasmol (R. Sayle, Glaxo, U.K.) and the coordinates for human CD4 (1WIO.PDB (30)).
been shown to induce strong T cell activation in a CD4-dependent manner (22). APCs are not required and the control anti-CD3/foS homodimer does not stimulate. Consistent with previously published results, 3A9 CD4 loss variants failed to respond to the anti-CD3/foS:anti-CD4jun-bispecific Ab while CD4 wild-type transfectants responded strongly (Fig. 5A). This also demonstrated that activation was not induced by oligomerization of the TCR:CD3 complex by nonspecific Ab aggregation.

It had previously been proposed that the stimulatory effect of this Ab is mediated by recruitment of CD4-associated p56/c (22). However, the cytoplasmic-tailless CD4 transfectants clearly responded strongly to the bispecific Ab. Indeed, they produced 5 times more IL-2 than the wild-type transfectants. This difference could not be the result of the mCD4,ΔCycl transfectants being inherently more sensitive, because their response to HEL 48–61 was comparable to that manifest by the CD4 wild-type transfectants.
II molecules nor p56 by facilitating CD4:TCR interaction, given that neither MHC class anti-CD3 jun fos B (9). These data clearly show that the mechanism by which the 111 5 –2 log 10 reduction; 1111 5 were evaluated as follows: ++++ = response similar to mCD4.WT transfectant; +++ = 1–2 log 10 reduction; ++++ = 2–3 log 10 reduction; + = 3–4 log 10 reduction; +/- = 4–5 log 10 reduction; – = no response. Data represent mean results from two to four experiments with each mutant. a M4.31 has the same amino acid mutations as M4.32 in Table II.

(Fig. 5A). Furthermore, the response by the mCD4.ΔCY transfectants was not due to reexpression of wild-type CD4 as immunoprecipitation of CD4 failed to coimmunoprecipitate p56Lck (Fig. 5B) (9). These data clearly show that the mechanism by which the anti-CD3:anti-CD4/ΔJun-bispecific Ab induces T cell activation is by facilitating CD4:TCCR interaction, given that neither MHC class II molecules nor p56Lck can participate. Importantly, hybridomas expressing the CD4 mutants, Q114A/F182A or F182A/F201A, failed to be stimulated by the bispecific Ab. This did not represent an inability of the bispecific Ab to cross-link CD3 and the CD4 mutants as the bispecific Ab could block the binding of anti-CD3.FITC and anti-CD4.PE (data not shown). Furthermore, the bispecific Ab induced both pp21 and pp23 phospho-CD3ζ in the wild-type and mutant CD4 transfectants but not in the CD4 loss mutant (Fig. 5C). This demonstrates that the ability of the bispecific Ab to cross-link CD3 and either wild-type or mutant CD4 is comparable; inducing CD3ζ phosphorylation without IL-2 secretion in the latter. This confirms that these CD4 mutations have disrupted CD4:TCCR interaction and demonstrates the profound effect this interaction can have on T cell activation.

Discussion

There is now considerable evidence for a physical and functional interaction between CD4 and the TCR:CD3 complex. While most studies have indicated that this occurs intracellularly, we have previously shown that the membrane-proximal (D3/D4) extracellular domains may also play a role in this interaction (9). Indeed, in the 3A9 T cell hybridoma, our studies have suggested that the primary role of CD4:TCCR interaction via p56Lck or MHC molecules is for anchorage and stabilization, while much of the functional effects are mediated via the D3/D4 domains of CD4. These multiple interactions have confounded attempts to delineate their relative contributions to T cell activation.

We have now extended these studies in an attempt to characterize the residues involved in this interaction and determine their contribution to T cell activation. The production and characterization of 61 CD4 mutants identified three residues that in combination (Q114A/F182A or F182A/F201A), but not individually, abrogated the ability of CD4 to restore T cell responsiveness to HEL 48–61. However, it is evident from the recently published structure of the human CD4 molecule that these residues are partially or completely buried (Fig. 2, C and D) (30). How might these substitutions affect CD4 function? Given that these residues are close to the hinge region between the D2 and D3 domain, these substitutions may affect MHC class II:CD4 interaction. While the 3A9 T cell hybridoma does not require CD4:MHC class II interaction for activation, as demonstrated by using the CD4.MM4 mutant (9, 24), it is difficult to determine whether our mutations have affected CD4:MHC class II binding. It has been suggested that the hinge region of human CD4 may play an important role in maintaining the overall structural integrity of CD4 (31). In their study, alanine substitution of Q180 and K181 affected coreceptor function but not coligand function, suggesting that these residues may mediate CD4:CD3:TCC interaction. However, our mutation of the analogous residues in murine CD4 had no effect on T cell function, highlighting potential differences between human and murine CD4 (Table II, M4.41: Q183/S184). Use of the anti-CD3:anti-CD4/ΔJun-bispecific Ab in our study did provide an MHC-independent analysis of CD4 function. Mutation of either Q114A/F182 or F182/F201 clearly abrogated T cell responses to both HEL 48–61 and the anti-CD3:anti-CD4/ΔJun-bispecific Ab despite the induction.

**Table III. Summary of final alanine-scanning mutants of mCD4 and their effect on T cell function**

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Mutated mCD4 Residues</th>
<th>Response to HEL 48–63</th>
<th>Response to HEL 48–61</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4.84</td>
<td>Q114A</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>M4.53</td>
<td>F182A</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>M4.51b</td>
<td>F201A</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>M4.51/53</td>
<td>F201A, F182A</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>M4.63/64</td>
<td>F201L, F182L</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>M4.53/65</td>
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<td>–</td>
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<td>F201A, F182A, Q114A</td>
<td>+++</td>
<td>–</td>
</tr>
</tbody>
</table>

* Residues highlighted in bold were replaced by alanine using site-directed mutagenesis. The effect of these CD4 mutations on T cell function was assessed by their ability to restore responsiveness of the 3A9.CD4 – hybridoma to HEL 48–61. Data were evaluated as follows: ++++ = response similar to mCD4.WT transfectant; +++ = 1–2 log 10 reduction; ++++ = 2–3 log 10 reduction; + = 3–4 log 10 reduction; +/- = 4–5 log 10 reduction; – = no response. Data represent mean results from two to four experiments with each mutant.

**FIGURE 3.** Gross structural integrity maintained in CD4 mutants. The CD4 loss variant or transfectants expressing wild-type or mutant CD4 were analyzed by flow cytometry using a panel of mAbs. The schematic on the right indicates the domain recognized and which Abs possess overlapping epitopes (the latter has not been determined for YTS177, KT6, and KT9) (deduced from Refs. 9 and 44). Data are presented as mean log fluorescence.
of strong phosphorylation of CD3ζ by the latter. Thus, these mutations directly affect the extracellular interaction of CD4 with the TCR:CD3 complex.

Despite extensive mutagenesis of the CD4 D3 domain, no surface-exposed residues that might be responsible for direct CD4:TCR interaction were identified. It is possible that none of the mutants produced contain all the key residues involved in this interaction (Fig. 4). For instance, several residues in noncontiguous stretches of the linear sequence may be involved, and multiple mutations may be required to observe a functional effect. Furthermore, residues on several faces of the D1/D2 domains have been implicated in MHC class II interaction, raising the possibility that CD4:TCR interaction may also involve residues on two sides of the D3 domain (Fig. 4B) (31–34). The D3 domain of CD4 is unusual in lacking a hallmark of IgSF domains, an intrachain disulfide bond. Interestingly, structural studies have shown that the D1 domain of CD2, which also lacks this disulfide bond, undergoes partial unfolding during dimerization to form a metastable folded state (35). It is conceivable that the lack of an intrachain disulfide bond in the CD4.D3 domain may be of functional importance.

Taken together, it is likely that a large number of surface residues would probably have to be mutated to observe a functional effect. The use of the anti-CD3flos:anti-CD4jun-bispecific Ab provided an opportunity to analyze CD4 function in the absence of MHC class II molecules. It had previously been suggested that this bispecific Ab may induce T cell activation by bringing CD4-associated p56lck into close proximity to the TCR:CD3 complex (22, 36). However, our data clearly show that cytoplasmic tailless CD4 transfectants respond strongly to the bispecific Ab, secreting over 5 times more IL-2 than the wild-type CD4 transfectants. There are two possible explanations for this finding. First, under certain circumstances p56lck can mediate a negative signal which could reduce signaling through the TCR:CD3 complex (37, 38). Second, the mutant CD4 molecule, lacking the cytoplasmic tail and its attachment to p56lck, may have greater mobility in the membrane, thus facilitating optimal extracellular CD4:TCR interaction.

We can only speculate about the mechanism by which CD4:TCR interaction leads to/promotes T cell activation. It is possible...
that this interaction induces a conformational change in the TCR:CD3 complex that improves the accessibility of the ITAM (immune-receptor tyrosine-based activation motif) motifs for tyrosine phosphorylation and binding by SH2-containing signaling molecules. Indeed, it is not clear how ligation signals are transduced through the complex. It seems reasonable to suggest CD4:TCR interaction enhances this process. It also remains unclear how important this interaction is for normal T cell development and function. Expression of these CD4 mutants in transgenic mice should address this issue.

In summary, these data suggest that an interaction between the extracellular domains of CD4 and the TCR is required to facilitate signaling through the TCR:CD3 complex. It is likely that this interaction is of relatively low affinity because only 5% of the TCR:CD3 complexes are associated with CD4 on resting T cells (39). While removing the ability of CD4 to interact with either MHC class II molecules or p56\(^{lck}\) alone has no effect, the combination of these mutations almost completely abrogates function. These data suggest that CD4 needs to be anchored to the TCR:MHC complex for the functional effect of extracellular CD4:TCR association to be manifest. This can be achieved either by p56\(^{lck}\), the interaction of CD4 with MHC class II molecules or the anti-CD3fos:anti-CD4jun-bispecific Ab. The ability of this Ab to induce T cell activation may also provide fresh insight into whether T cell activation is induced by TCR:CD3 oligomerization and/or conformational change (40, 41). While recent studies have highlighted the
potential importance of receptor oligomerization (42), the ability of the bispecific Ab to activate T cells suggests that TCR:CD4 interaction can potentiate signal transduction through the TCR: CD3 complex in the absence of oligomerization. Finally, we report that the physical association between the D3 domain of CD4 and the TCR has a profound effect on T cell activation. Indeed, previous studies attributing the functional importance of CD4 to its association with p56\textsuperscript{lck} may require reevaluation. This view is consistent with the finding that T helper cell development proceeds normally when cytotoxic-tailless CD4 is overexpressed in CD4-deficient mice (43). Our data also emphasize the importance of extracellular association between transmembrane molecules in modulating signal transduction.

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