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D-enantiomer peptide of the TCRα transmembrane domain inhibits T-cell activation in vitro and in vivo

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ABSTRACT

T cell activation requires the cross-talk between the CD3-signaling complex and the T cell receptor (TCR). A synthetic peptide coding for the TCRα transmembrane domain (CP) binds CD3 molecules, interferes with the CD3/TCR cross-talk, and inhibits T cell activation. Intermolecular interactions are sterically constrained; accordingly no sequence-specific interactions are thought to occur between D- and L-stereoisomers. This argument was recently challenged when applied to intra-membrane protein assembly. In this paper we studied the ability of a D-stereoisomer of CP (D-CP) to inhibit T cell activation. L-CP and D-CP co-localized with the TCR in the membrane and inhibited T cell activation in a sequence-specific manner. In vivo, both L-CP and D-CP inhibited adjuvant arthritis. In molecular terms, these results suggest the occurrence of structural reorientation that facilitates native-like interactions between D-CP and CD3 within the membrane. In clinical terms, our results demonstrate that D-stereoisomers retain the therapeutic properties of their L-stereoisomers, while they benefit from an increased resistance to degradation.

Key words: transmembrane domain assembly • peptide inhibitor • immunosuppression • protein-protein interaction

The T cell receptor (TCR) complex of the majority of the mature T cells is a TCRαβ heterodimer associated to CD3γ, δ, ε and ζ chains in a TCRα:β:γ:δ:ε:ζ ratio of 1:1:1:2:2 (1). T cell activation is triggered by the interaction of the TCR with a peptide bound to a major histocompatibility complex molecule (MHC) on the surface of an antigen-presenting cell. The TCR-peptide/MHC interaction induces a conformational change in the TCR that triggers the phosphorylation of CD3. Mutagenesis studies revealed a direct interaction between Lysine 244 in the transmembrane domain (TMD) of TCRα and acidic amino acids in the TMD of CD3δ/ε (1–3) and between Arg 239 in the TMD of the TCRα and acidic amino acids in CD3ζ (1). These interactions are required for the correct assembly of a functional TCR complex.

A 9-amino acid (aa) peptide (made up of L-aa) derived from the TMD of the TCRα chain, termed core peptide (CP), has been shown to inhibit T cell antigen specific activation in vitro and

in vivo (4). The CP peptide co-localizes with the TCR to the immune synapse, inhibiting the proper assembly of the TCR-CD3 complex (4–6). The effectiveness of CP in inhibiting adjuvant arthritis (AA) in Lewis rats has opened up new horizons for the therapy of autoimmunity.

Inhibition of the TCR/CD3 cross-talk is probably facilitated by forming salt-bridges between its positive charges and the negative charges within CD3δ or CD3ζ transmembrane domains. Thus, the peptide either competes with the salt-bridge between the TCR and CD3 chains (7) or with the Asp/Asp driven dimerization of CD3 (8).

According to the position of the side chain relative to the α-carbon atom, amino acids (aa) can take two mirror forms, the L-isomer and the D-isomer. The chirality of the aa limits intermolecular interactions. Indeed, mammalian proteins are made up by L isomers only, enzymes can process L substrates only and generally speaking, only complexes of L isomers are found in vivo, probably reflecting the lack of L/D interactions in solution. This was best demonstrated by the total chemical synthesis of a D-protease (9), which could cleave the D-isomer ligand but not the L-isomer. Emil Fischer explained this phenomenon with the "Lock and Key" theory over a century ago (10), which was revised into the "induced fit" theory by Koshland (11). In short, interactions of L-proteins in solution depend on a geometrical fit, which is improved during the docking of two proteins by slight structural changes. In contrast, within the microenvironment of cell membranes L/D interactions can be established. Using glycophorin A (GPA), a well-documented model for studying protein-protein interactions within the membrane (12, 13), we have recently shown the formation of GPA heterodimers with D stereoisomers of the GPA TM in a sequence-specific manner (14).

In this study we evaluated the structural adaptation of CP in the inhibition of T cell activation. To this end, we compared the activity of two stereoisomers of the CP sequence: the native L isomer of CP (L-CP) and its mirror image made of D aa (D-CP) (Fig. 1). We also used a known inactive mutant (2G CP) as a control to ensure that the observed biological effects are sequence specific, as previously reported (4). Our results demonstrate a similar inhibitory activity of T cell proliferation in vitro for both L and D CP. Furthermore, both peptides were equally active in alleviating adjuvant arthritis (AA) in vivo, while the 2G CP was inert. At the molecular level, these results imply a structural reorientation process within the membrane, allowing sequence-specific interactions to be formed between proteins irrespective of their chirality. The inhibition of a disease model in vivo encourages the use of D peptides for control of immune-mediated disorders.

MATERIALS AND METHODS

Peptide synthesis and fluorescent labeling

Peptides were synthesized by solid phase on PAM-amino acid resin (0.15 meq), as described previously (15, 16). The synthetic peptides were purified (>98% homogeneity) by RP-HPLC on a C_4 column using a linear gradient of 20–60% acetonitrile in 0.05% TFA for 60 min. The peptides were subjected to amino acid analysis and mass spectrometry to confirm their composition. Unless stated otherwise, stock solutions of concentrated peptides in DMSO were used to avoid aggregation of the peptides before use. The final concentration of DMSO in each experiment had no effect on the system under investigation. Resin-bound peptides were treated

with 4-chloro-7-nitrobenz-2-oxa- 1,3-diazole fluoride (NBD-F) or 5-carboxytetramethylrhodamine, succinimidyl ester (Rhodamine-SE), respectively. The reaction with NBD-F took place in DMF alone, and the reaction with rhodamine took place in DMF containing 2% diisopropylethylamine. The fluorescent probes were used in excess of 2 equivalents, leading to the formation of resin bound N-terminal NBD or rhodamine peptides. After 1 h, the resins were washed thoroughly with DMF and then with methylene chloride. All purified peptides were shown to be homogeneous (>98%) by analytical RP-HPLC (17).

Circular dichroism (CD) spectroscopy

The CD spectra of the peptides were measured in an Aviv 202 spectropolarimeter. The spectra were scanned with a thermo stated quartz optical cell with a path length of 1 mm. Each spectrum was recorded in 1 nm intervals with an averaging time of 20 s, at a wavelength range of 260 to 190 nm. The peptides were scanned at a 100 μM concentration in 1% LPC micelles.

Animals

Two-month-old female Lewis rats were used. The rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center of the Weizmann Institute of Science. The experiments were performed under the supervision and guidelines of the Animal Welfare Committee.

T cell proliferation

T cell proliferation assays were performed using either lymph node cells (LNC) or the A2b T cell line, which reacts with the Mt176-90 peptide. Popliteal and inguinal LNC were removed 26 days after the injection of *Micobacterium tuberculosis* (Mt) in incomplete Freund's adjuvant (IFA), when strong T cell responses to PPD and Mt176-90 are detectable (18). LNC were cultured at a concentration of 2×10^5 cells per well; 5×10^4 A2b T cells were stimulated in the presence of irradiated 5×10^5 thymic antigen presenting cells (APC) per well, prepared as described previously (19). The cells were plated in quadruplicates in 200 μl round bottom microtiter wells, with or without antigen, in the presence of various concentrations of the peptides under study. Cultures were incubated for 72 h at 37°C in a humidified atmosphere of 7.5% CO_2. T cell responses were detected by the incorporation of [methyl-^{3}H]-thymidine (Amersham, Buckinghamshire, UK; 1 μCi/well), added during the last 18 h of incubation. The results of T cell proliferation experiments are shown as the % of inhibition of the T cell proliferation triggered by the antigen in the absence of peptides.

Induction and assessment of adjuvant arthritis (AA)

To test the effect of CP on T cell activation in vivo, we used AA as a model system. AA was induced in groups of 6 by injecting 50 μl of Mt suspended in IFA (0.5 mg/ml) at the base of the tail. At the time of AA induction, each rat also received 100 μg of L-CP, D-CP, or 2G CP control peptide (or PBS) dissolved in 50 μl of IFA and mixed with Mt/IFA used to induce AA. The day of AA induction was designated as day 0. Disease severity was assessed by direct observation of all four limbs in each animal. A relative score between 0 and 4 was assigned to each limb, based on the degree of joint inflammation, redness, and deformity; thus the maximum possible score

for an individual animal was 16 (18). The mean AA score (± SEM) is shown for each experimental group. Arthritis was also quantified by measuring hind-limb diameter with a caliper. Measurements were taken on the day of the induction of AA and 26 days later (at the peak of AA); the results are presented as the mean ± SEM of the difference between the two values for all the animals in each group. The person who scored the disease was blinded to the identity of the groups.

Delayed type hypersensitivity (DTH)

PPD (20 μl; 0.5 mg/ml in PBS) were injected intradermally into the pinna of the right ear on day 16 after AA induction; 20 μl of sterile PBS was injected in the left ear as control. The thickness of the ear was measured 48 h later using a vernier caliper and expressed as the difference between the right and the left ear.

Fluorescence microscopy

Activated T cells (20) (10^5 cells/sample) were incubated in 100 μl of PBS containing Para-formaldehyde 4% for 15 min on ice. The samples were then washed with cold PBS and centrifuged for 7 min at 1100 rpm. PBS containing BSA 1% was then added at ambient temperature to prevent non-specific binding. After 30 min FITC-labeled antibody against TCR was added at 1:100 dilution and incubated for 2.5 h. For co-localization of TCR with the peptides, L-CP-Rho, D-CP-Rho, or 2D CP-Rho were added at a final concentration of 1 μM (stock in DMSO) and incubated for 5 min. Samples were then washed once with PBS and loaded on a microscope slide.

The cells were then observed under a fluorescent confocal microscope. FITC excitation was set at 488 nm, with the laser set at 20% power to minimize bleaching of the fluorophore. Fluorescence data were collected from 505–525 nm. Rhodamine excitation was set at 543 nm, with the laser set at 5% power. Fluorescence data were collected from 560 nm and up.

FRET between the FITC (donor) and rhodamine (acceptor) was observed as increase in FITC fluorescence in an area where the rhodamine probe was bleached. Bleaching was achieved by point excitation at 543 nm for 6 s with the laser set to 100%. To verify that the increase in FITC fluorescence is not due to auto fluorescence, we bleached using the 488 nm laser and only then at 543 nm. No signal was observed in either 505-525 nm or 560<, eliminating the possibility of auto fluorescence.

Statistical significance

The InStat 2.01 program (Graph Pad Software, San Diego, CA) was used for statistical analysis. The Student's *t*-test and the Mann-Whitney *U*-test were conducted to assay significant differences between the different experimental groups.

RESULTS

D-CP is a structural mirror image of L-CP

To test the role of chirality in the recognition process of the TCRα TMD, we chemically synthesized three CP peptides: wild-type L-CP, which has been shown to inhibit T cell activation by the target antigen; D-CP, which is a mirror image of the first; and an inactive mutated peptide (2G CP). Table 1 shows the peptide sequences and designations, and Fig. 1 visually demonstrates the structural difference between the two stereoisomers, assuming a canonical helical structure. Note that the two bulky positive side chains on D-CP are facing in the opposite direction of those in L-CP.

Circular dichroism experiments were performed to ensure that the secondary structure of the D-CP was indeed a mirror image of the L-CP. The experiments were performed in a zwitterionic detergent (1% LPC in H_2O) to simulate a membrane environment, as described in (21). The spectrum of the D-CP was found to be exactly a mirror image of the L-CP (Fig. 2); both are partially helical. Note that both peptides are 9 aa long, hence their structure is likely to be less stable than that in the context of the full-length protein.

D-CP interferes with T cell activation as does L-CP

We studied the T cell response of LNC from Mt-immunized rats to the Mt antigen PPD or to the Mt176-90 peptide; these antigens are known to induce strong proliferative responses from T cells in the draining LNC of AA rats (18, 22). Figures 3*A* and *B* show that L-CP and D-CP inhibited the T cell proliferative responses to PPD and to Mt176-90 in a dose-dependent manner. Moreover, we found no inhibitory effect for the 2G CP, suggesting that the inhibition is sequence specific and that critical molecular interactions were perturbed by the substitution of two positive aa for Gly residues (see Table 1). L-CP, D-CP, and 2G CP showed no cytotoxicity when incubated with cells, excluding the possibility that inhibitory effects of the L and D-CP peptides on antigen-triggered proliferation were due to cell death. (data not shown). Interestingly, the inhibition of D-CP is consistently higher than that of L-CP at the lower concentrations.

D-CP inhibits T cell immunity in vivo to the same extent as L-CP

To test the inhibitory effects of CP on the activation of specific T cells in vivo, we used the adjuvant arthritis (AA) model. Immunization of Lewis rats with Mt in oil triggers AA, an experimental autoimmune disease driven by Mt-specific T cells cross-reactive with self-antigens (23, 24). Mt176-90-specific T cells are detectable upon induction of AA (25); indeed the A2b T cell clone cross-reacts with cartilage and mediates AA (19). Since L and D-CP inhibited the T cell response of primed LNC and of clone A2b to PPD and Mt17-90 in vitro (Fig. 3), we also investigated the effects of L- or D-CP on the in vivo activation of the T cells that drive AA. D-CP or L-CP administered with the antigen at the time of AA induction led to a significantly milder arthritis, both in terms of clinical score (Fig. 4*A*) and of ankle swelling (Fig. 4*B*). The control peptide 2G CP did not inhibit AA. The mean maximum score was 12 ± 0.3 in the control-treated rats, compared with 6 ± 0.7 in the D-CP-treated rats and 7.3 ± 0.7 in the L-CP-treated rats ($P<0.05$ for the L and D-CP groups compared with the control groups).

The activity of the T cells that mediate AA can also be detected in vivo by studying the delayed type hypersensitivity (DTH) response to PPD (19). We studied the DTH response to PPD 16 days after AA induction in rats treated with the three peptides. Figure 5 shows that the administration of D-CP or L-CP led to a 48 and 39% reduction in the DTH response to PPD, respectively, while the inhibition caused by treatment with the 2G CP peptides was less than 10%.

Taken together, these results indicate that both the D and L-CP can interfere in vivo with T cell activation induced by specific antigens. This interference led to milder AA (Fig. 4*A* and 4*B*) and decreased DTH reactivity (Fig. 5) in response to Mt antigens. Moreover, it seems that in vivo the effect of D-CP is greater than that of L-CP.

Co-localization of L-CP and D-CP with the TCR

The CP peptides function by uncoupling the signal between TCR and CD3, therefore they should co-localize with the receptor complex. To test this hypothesis, we labeled the T cells with FITC-labeled antibodies against TCR and either L-CP or D-CP labeled with rhodamine (Fig. 6). The labeling of the TCR demonstrated the capping phenomenon characteristic of activated T cells. There was almost complete overlap between TCR and either L or D analogs of CP. These results suggest that the CP analogs bind to T cell membrane and co-localize with the TCR within the capping regions. We believe that the labeling does not affect the localization since the same results were obtained with NBD-labeled peptides and PE-labeled TCR (data not shown).

To corroborate the co-localization results, we performed a series of bleaching experiments that demonstrated fluorescence energy transfer between the CP peptides and the TCR. We bleached a point on the membrane of the cells, which exhibited high intensity of both rhodamine and FITC, using the 543 nm laser. Thus, the CP rhodamine-labeled peptides were bleached while the TCRα-FITC was unaffected. This procedure resulted in a significant increase in the fluorescence of the TCRα-FITC, pointed out by the purple arrows in Fig. 6. Thus, we could conclude that fluorescence energy transfer occurs between the TCR and L-CP or D-CP peptides. To eliminate the possibility of autofluorescence as the source of increased signal in the range of 505 to 525 nm, we performed two controls. First, we bleached the same position with the 488 nm laser. This resulted in complete bleach of the signal, suggesting that the fluorescence increase described above is generated by the FITC probe rather than being an artifact of autofluorescence from the sample. Next, we bleached with the 543 nm laser at a position with almost no signal. The cell remained impervious and we observed no increase in the FITC fluorescence, eliminating the possibility of autofluorescence at the same spectral range of FITC.

DISCUSSION

In this work we demonstrated that D-CP can inhibit T cell function in vivo and in vitro, at least as well as L-CP. These CP molecules function by interacting with the transmembrane domains (TMD) of the CD3 complex to uncouple signal transduction between the TCR and CD3 molecules. Thus, a structural adaptation of D-CP probably compensates for the change in aa chirality and allows interactions with L molecules. Nevertheless, the double mutant 2G CP demonstrated that the mechanism of action of the D-CP is sequence specific.

Our current understanding of TMD assembly is largely based on the study of the biophysical properties of the GPA model. The analysis of the GPA NMR structure in micelles revealed two membrane-spanning α-helices crossing at an angle of –40 degrees (26). The dimerization surface of GPA is formed by the common GxxxG motif (13, 27, 28), which creates a groove in the structure. The glycines are believed to allow the two GPA chains to pack close together and establish inter-molecular backbone interactions. These interactions stabilize the homodimer complex in the membrane, while the polar residues control the specificity of the dimerization (26, 29–35). A striking feature of the TCR complex is the presence of two positive charges within the TMD of the TCRα; positive charges within a TMD are rare. Moreover, the bulkiness of the side chains increases the chiral nature of the TCRα TMD (Fig. 1). Based on our knowledge on the assembly of the GPA homodimer, we can assume that the hydrophobic amino acids, comprising most of the sequence, function to pack the helices together, while the two positive charges are responsible for the specificity of the interaction through the formation of salt bridges (1–3).

We propose that the structural adaptation of TMD is a general phenomenon that can be accounted for by a common mechanism; the rotation of the tilt angle may compensate for the replacement of an L chain with a D chain (Fig. 7*A*). The basic TMD homodimer, based on the model of GPA, is built of two L chains crossing at a certain angle. Several amino acids are in contact with the opposite helix; these form the "interaction surface" depicted in pink in Fig. 7*A*. The replacement of one helix with its D enantiomer will result in an interaction surface that has the same amino acid sequence as the wild-type, although in mirror image orientation (Fig. 7*B*). By changing the tilt angle between the two helices, it is possible to bring the interaction surfaces to a similar orientation as in the wild type L-homodimer (Fig. 7*B*). At this point, we have the same amino acid sequence in the D peptide at a position in space similar to the wild-type. Thus, with this relatively straightforward alteration of the tilt angle, we can drive the hetero-assembly of a TMD with its mirror image chain. This model holds true for the more complex TCR-CD3 oligomer (Fig. 7*C*, *D*). Using the same logic, if we start with a homodimer that is in a parallel orientation we would end up with a heterodimer that has a larger tilt angle between the helices. The energy barrier associated with shifting the angle between two TMD may be extensive. However, it is reasonable to assume that once two helices pass this barrier they would remain in contact. Thus, the assembly of TMD with opposite chirality may differ from the wild-type L dimer in kinetics.

In this work we demonstrate that a D peptide corresponding to the TCRα TMD can interfere with the TCR complex in a sequence-specific manner and interfere with T cell activation. The sequence-specific interaction between D and L polypeptides seems to apply to several membrane complexes, including both prokaryotic [the *E. coli* Aspartate receptor (36)] and eukaryotic proteins [GPA (14) and TCRα]. Although the boundaries of this phenomenon need to be further characterized, peptide displacement strategies based on the use of D peptides may allow the formulation of immunotherapeutic peptides with increased resistance to protease degradation useful clinically for inhibiting T cell activation in immune-mediated disorders.

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Table 1

Peptides' designation and sequence.

Peptide Designation	Sequence
L-CP	X-GLRILLLKV-NH_2
D-CP	X-<u>GLRILLLKV</u>-NH_2
2G CP	X-GL**G**ILLL**G**V-NH_2

D-amino acids are underlined and mutations are in bold. X_1 = $–NH_3$, unlabeled peptide. X2 = –NH-Rhodamine, Rhodamine labeled peptide. X3 = –NH-NBD, NBD labeled peptide. The peptides were amidated at their C terminus.

Fig. 1

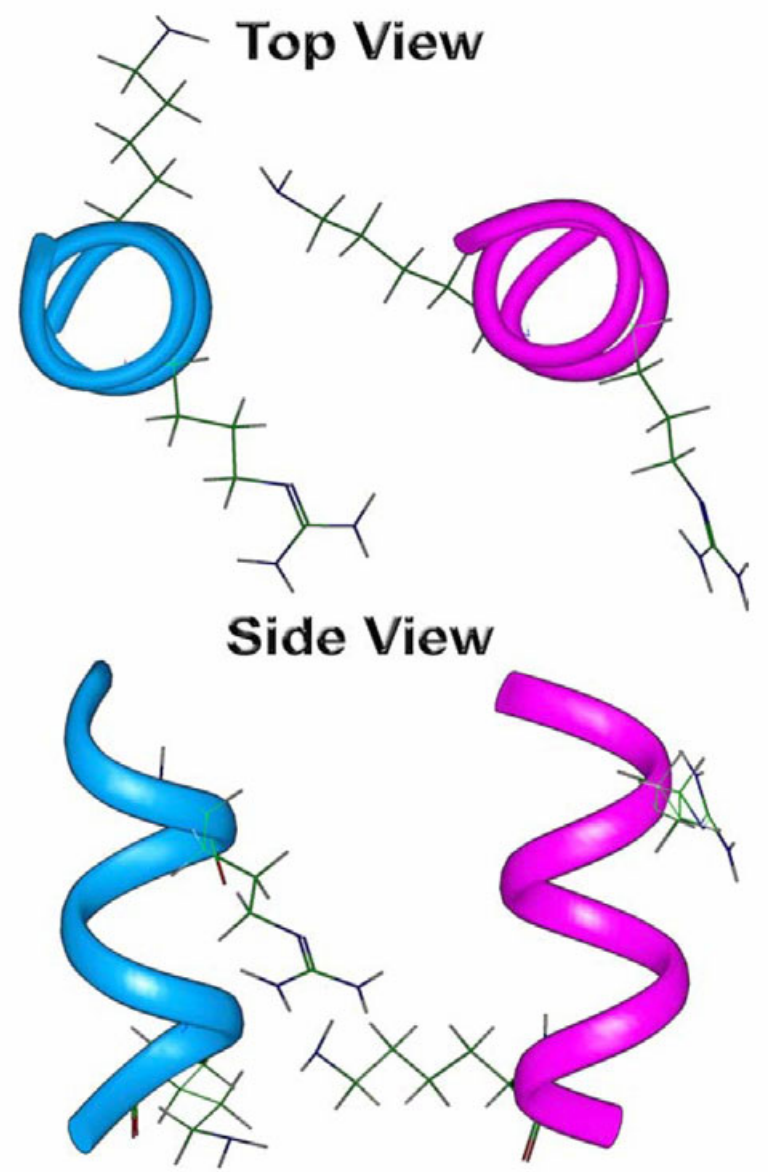

Figure 1. The structural difference between L-CP (pink) and D-CP (blue) is illustrated in the following model. The arginine and lysine side chains are visible in order to demonstrate the effect of the D-amino acid substitutions on side-chain location. We assumed a native α-helical secondary structure in the transmembrane region. The backbone of D-CP turns in the opposite direction than the wild-type analog.

Fig. 2

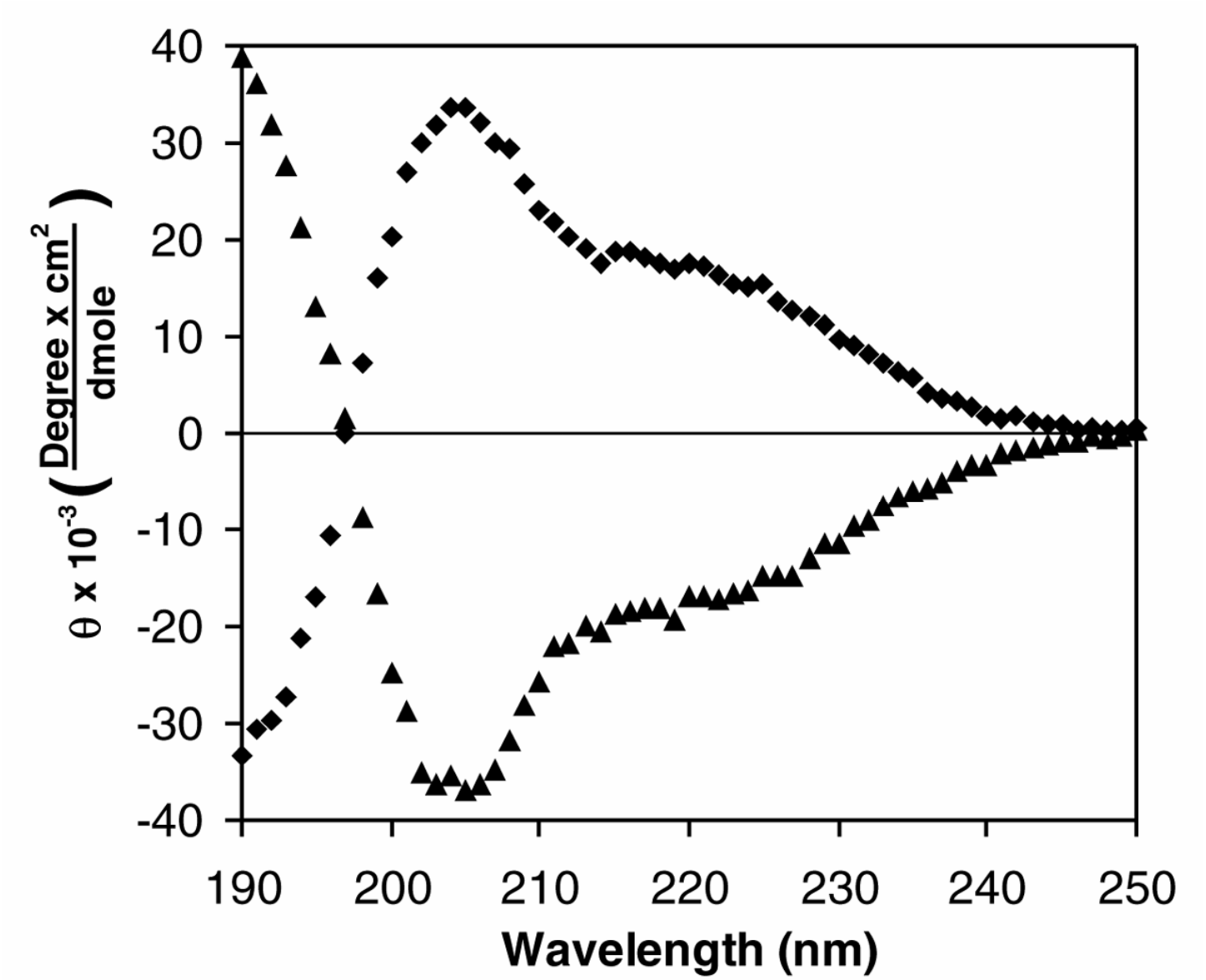

Figure 2. The L and D-CP have mirror image structures. Far-UV circular dichroism spectra of L-CP (triangles) and D-CP (diamonds) were collected in a membrane mimetic environment (1% LPC). Spectra were measured on an Aviv spectropolarimeter at 1-nm intervals with 20 s averaging time, using a 0.1 cm light path. The *y*-axis represents theta (θ) after subtracting the background spectrum of 1% LPC alone. The structures are not canonical α-helices as can be expected for such short peptides (a population with random coil conformation is likely). However, the spectra of the L-CP and D-CP are exactly mirror images.

Fig. 3

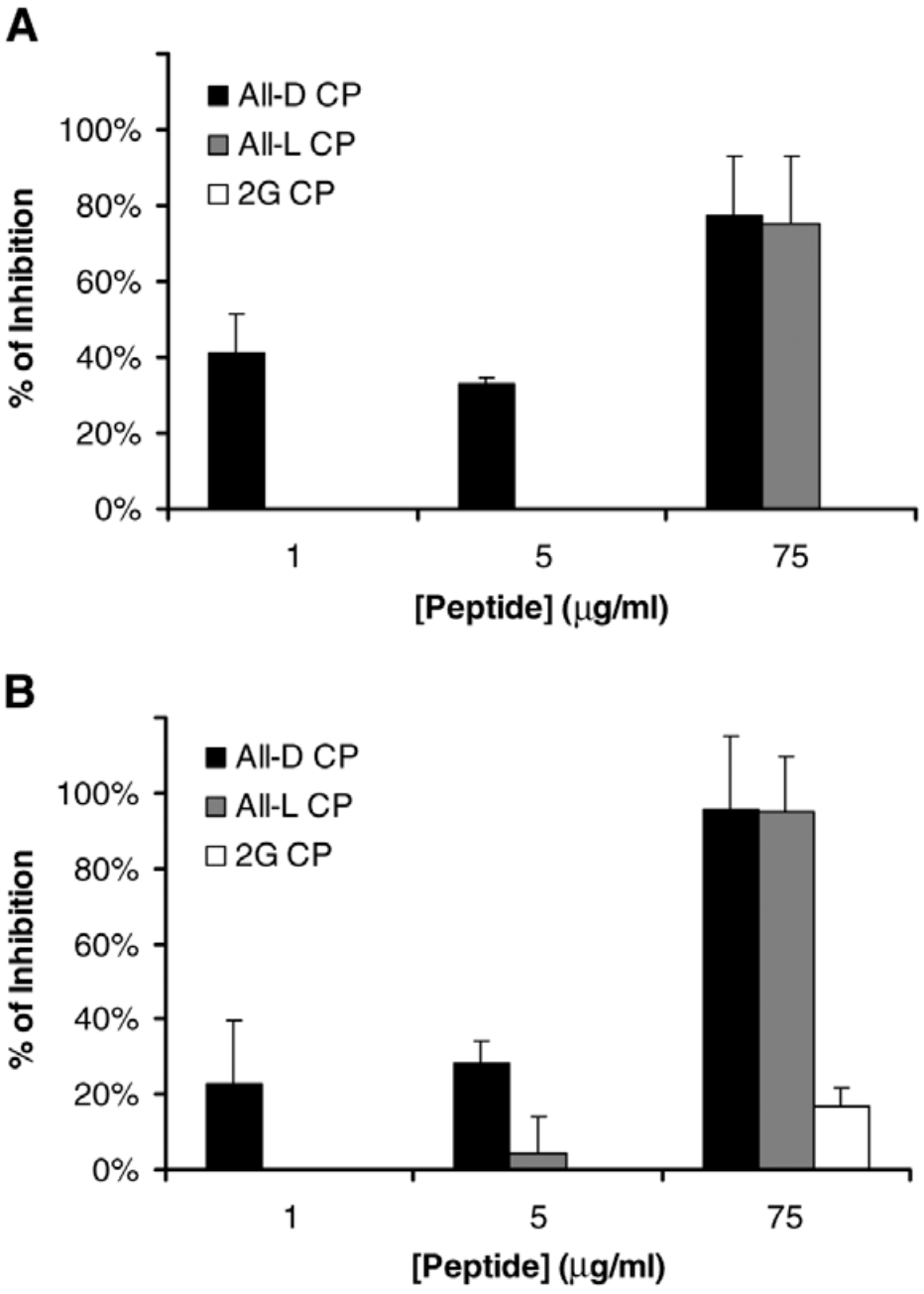

Figure 3. Both L and D-CP inactivate T cells in vitro in a similar fashion. Inhibition of T cell activation was measured by following proliferation after antigen specific activation. T cells were activated with PPD (***A***) or with Mt176-90 (***B***). The activation was in the presence of L-CP (black), D-CP (gray), or 2G CP (white) at concentrations of 1, 5, and 75 μg/ml.

Fig. 4

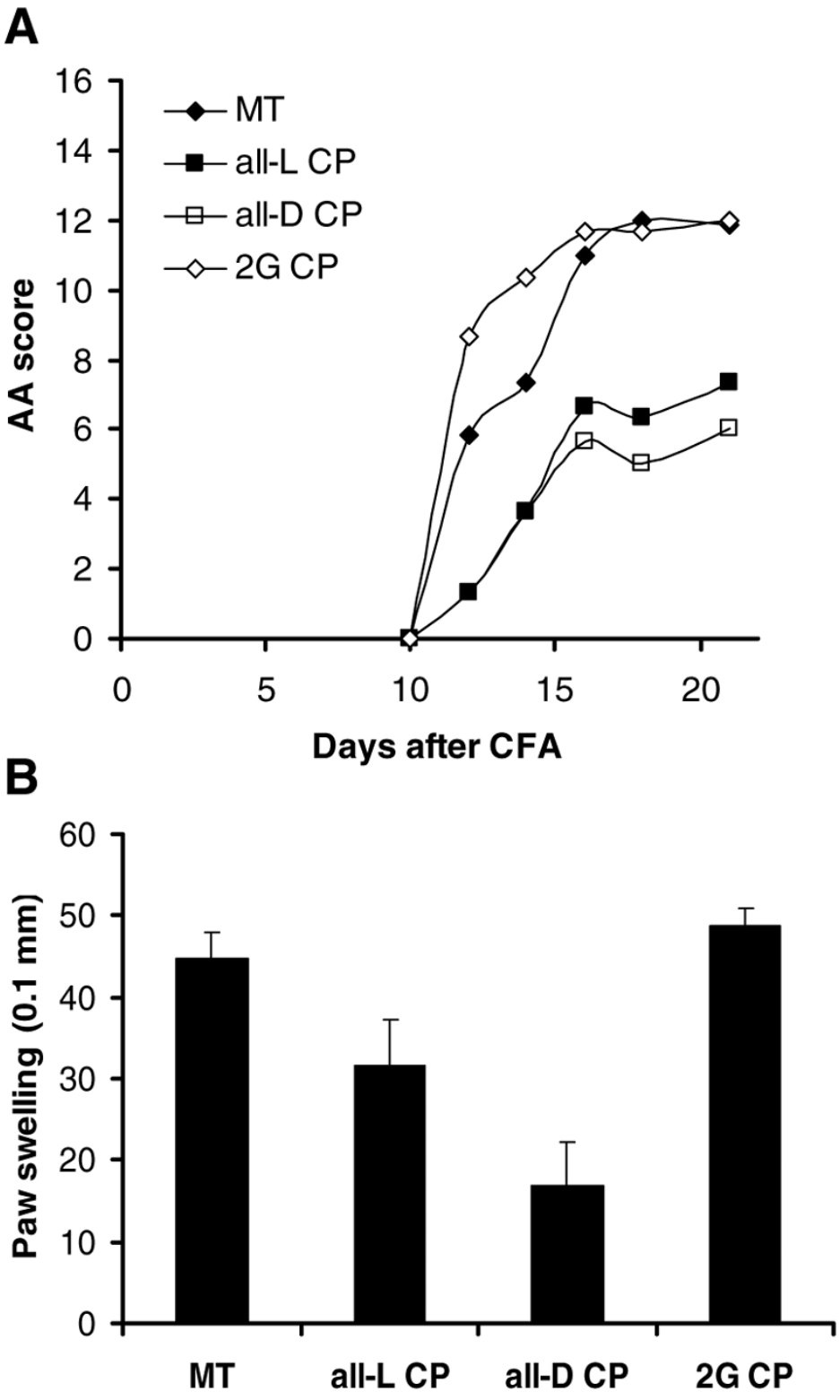

Figure 4. Inhibition of adjuvant arthritis by L and D-CP. AA was induced by immunization to Mt in oil, mixed with L-CP, D-CP, 2G CP, or PBS (6 rats per group). Arthritis was scored every 2–3 days, starting at day 10. ***A***) Demonstrates the time course of the AA disease. ***B***) Presents leg swelling scores measured at day 26 after AA induction. The results are presented as the mean ± SEM of the difference between the values for hind-limb diameter taken at days 0 and 26. The presence of both L and D-CP significantly reduces the severity of AA compared with the control groups ($P<0.05$).

Fig. 5

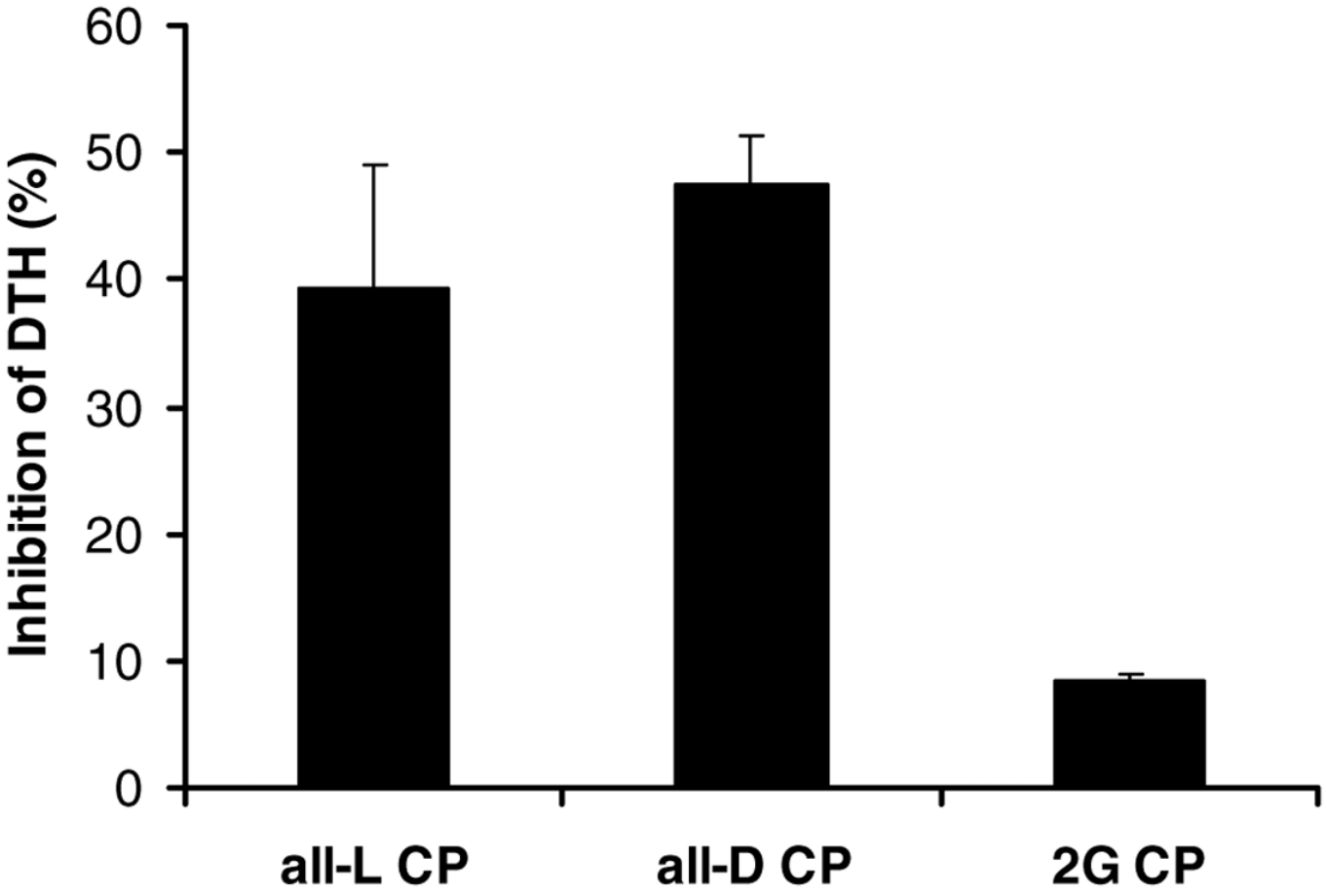

Figure 5. The L-CP and D-CP affect cellular immune responses in vivo. Rats were immunized with Mt to induce AA in the presence of L-CP, D-CP, 2G CP, or PBS. DTH was measured as described in Materials and Methods to quantify the effect of the peptides on the immune response to Mt. The results were normalized as percent inhibition of the DTH response. The values measured for rats co-immunized with PBS were considered as zero inhibition. The results demonstrate a significant effect in vivo for the L and D-CP.

Fig. 6

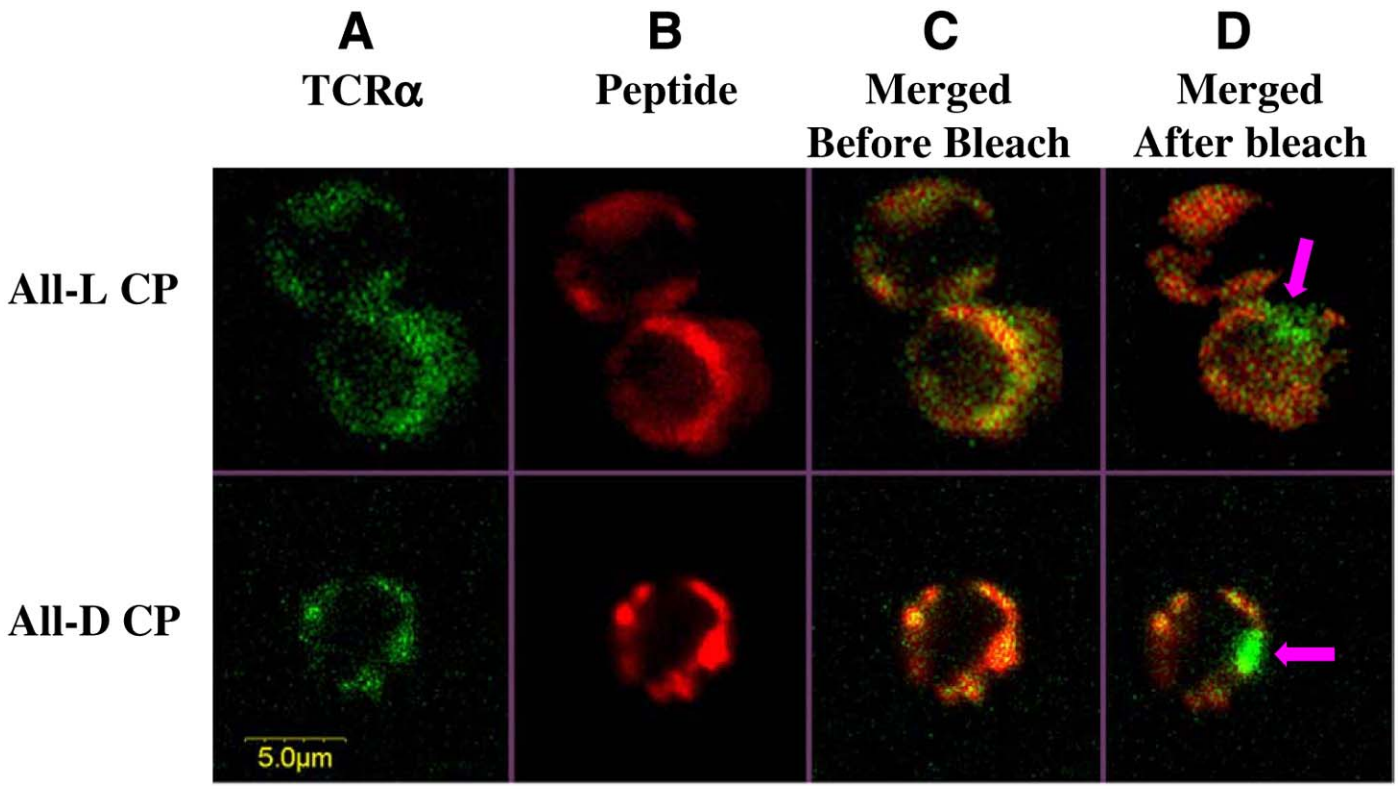

Figure 6. The L and D-CP peptides colocalizes with the TCR receptor in the membrane. ***A*)** The TCR is visualized using αTCR-FITC. Excitation was at 488 nm and the emission was collected between 505 and 525 nm. ***B*)** The peptides are visualized using a Rhodamine probe attached to their N terminus. Excitation was at 543 nm, and emission was collected from 560 nm and up. ***C*)** Merging of **(*A*)** and **(*B*)** demonstrates that all the CP peptides co-localize with TCR. ***D*)** Point bleach at 543 nm, with the laser at 100% for 6 s, demonstrates an energy transfer between the CP-Rho peptides and the TCR FITC-labeled antibody. The arrows points to the area that underwent the bleaching procedure.

Fig. 7

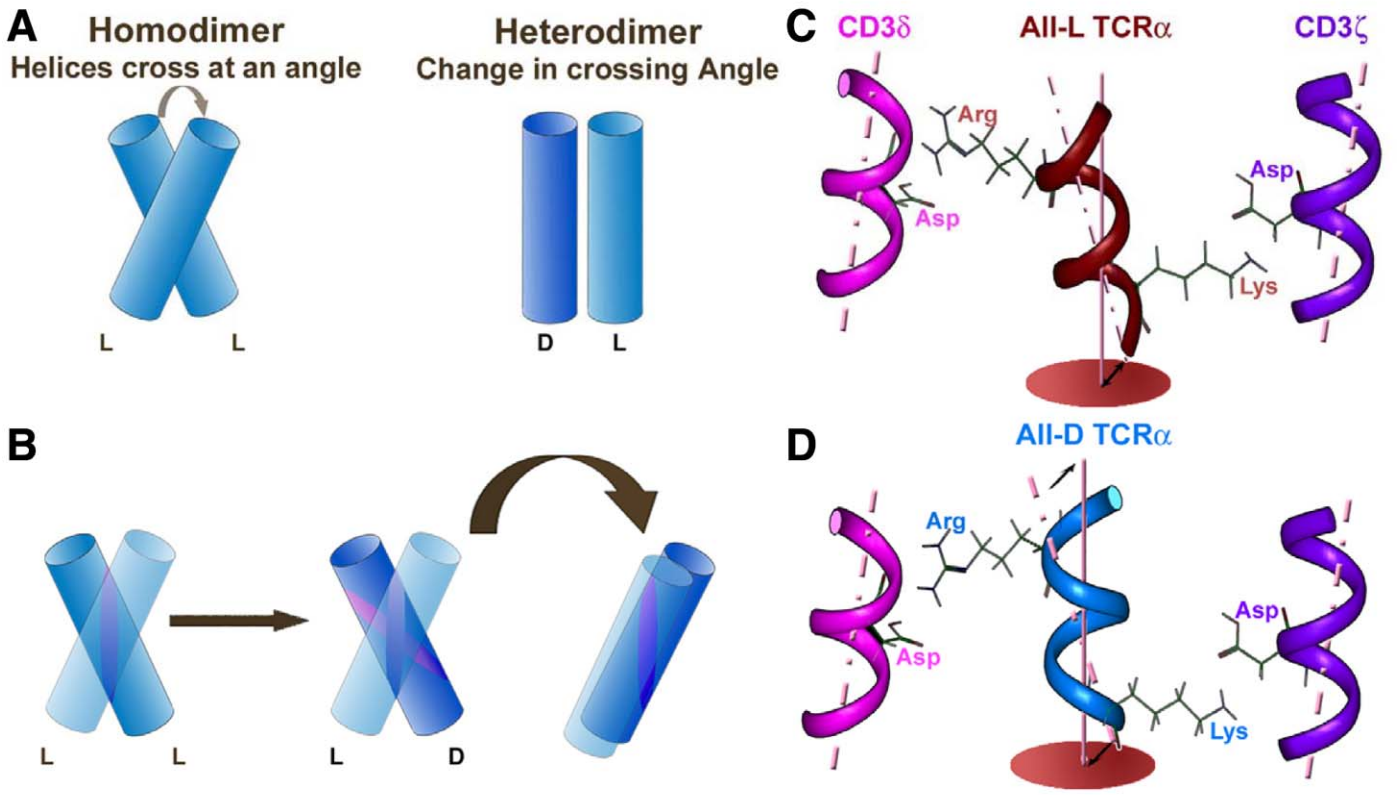

Figure 7. A general model for the assembly of an L helix with its D enantiomer. ***A*)** The orientation of the homodimer TMDs is at a tilt, which is common to dimers, for example, the GPA. Heterodimer assembly of an L (light blue) with a D (dark blue) helix can be facilitated by changing the crossing angle. ***B*)** The surface of interaction between the two all-L helices is seen in pink. When one of the chains is replaced with a D enantiomer TMD, the interaction surfaces cross each other and assembly is impossible. However, by changing the tilt angle between the helices, we can easily re-orient the interaction surfaces to face each other. ***C*)** The TCR complex consists of several TMDs, nevertheless it follows the same rules. The TCRα is known to interact with CD3δ and CD3ζ through salt bridges. We prepared a model of the 3 α-helices and oriented them in such a manner to allow the salt bridges to form. The L TCRα helix is tilted with respect to the CD3 helices. ***D*)** To test our hypothesis, we then replaced the L TCRα with D TCRα. We reoriented the D TCRα helix by changing the tilt angle, until the Arg and Lys side chains were placed at similar positions in space as the original L TCRα side chains.