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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **NC2019/0011688**

Solicitud de patente a nombre de **IMMATICS BIOTECHNOLOGIES GMBH**

Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, obrando como apoderada de la sociedad **IMMATICS BIOTECHNOLOGIES GMBH**, domiciliada en Tübingen, Alemania, solicitante de la patente de la referencia, en respuesta al Oficio No. **14578** notificado mediante notificación electrónica el 15 de septiembre de 2021, relacionado con el primer concepto técnico de fondo, y para el cual solicité oportunamente prórroga el 19 de noviembre de 2021, atentamente me permito presentar la siguiente información:

1. Modificación al capítulo reivindicatorio

Con fundamento en la facultad que confiere el Artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial un nuevo capítulo reivindicatorio, el cual consta de veinticinco (25) reivindicaciones y reemplaza completamente el capítulo presentado anteriormente.

En el nuevo capítulo reivindicatorio se realizaron las siguientes modificaciones:

1.1 Reivindicación 1

1.1.1 La materia objeto de la reivindicación 1 ha sido modificada con el fin de especificar que el constructo reconocedor de antígeno es un constructo reconocedor de antígeno recombinante que se une específicamente a un péptido antigénico PRAME de acuerdo con la SEQ ID NO: 97.

- 1.1.2 La materia objeto de la reivindicación 1 ha sido modificada con el fin de especificar que el constructo reconocedor de antígeno comprende un dominio variable alfa o beta de receptor de células T (TCR) o un dominio variable gamma o delta de TCR y las regiones determinantes complementarias (CDR) comprendidas en dichos dominios variables alfa/beta y gamma/delta de TCR, respectivamente. El soporte para esta modificación se puede encontrar en la página 6, 2.º párrafo y en la página 14, 3.º párrafo de la solicitud tal como fue publicada originalmente (véase la publicación internacional de la solicitud, WO 2018/172533).
- 1.1.3 La materia objeto de la reivindicación 1 ha sido modificada adicionalmente con el fin de especificar que el constructo reconocedor de antígeno comprende los respectivos CDRs con no más de una o dos modificaciones de aminoácidos, y que dicha modificación es una sustitución con un aminoácido conservativo. El soporte para esta modificación se puede encontrar en la página 9, segundo párrafo de la solicitud tal como fue publicada originalmente.
- 1.2** Se presenta una nueva reivindicación 2 dirigida a la modalidad de la invención en donde la sustitución con un aminoácido conservativo ocurre en la primera o en la última posición de aminoácido del respectivo CDR.
- 1.3** Se presenta una nueva reivindicación 4 (i) en donde se especifica que las células T que expresan el constructo reconocedor de antígeno recombinante secretan al menos 200 pg/ml de interferón γ . El soporte para esta modificación se puede encontrar en la página 6, 3er párrafo y en la página 7, 1er párrafo de la solicitud tal como fue publicada originalmente.
- 1.4** Se presenta una nueva reivindicación 4 (ii) en donde se especifica que las células T que expresan el constructo reconocedor de antígeno recombinante secretan el doble de interferón γ que el nivel de fondo no transducido. El soporte para esta modificación se puede encontrar en la página 6, 3er párrafo y en la página 7, 1er párrafo de la solicitud tal como fue publicada originalmente.
- 1.5** La nueva reivindicación 5 depende de la reivindicación 4 y especifica la baja concentración del antígeno objetivo PRAME. El soporte para esta modificación se puede encontrar en la página 6, 3er párrafo y en la página 7, 1er párrafo de la solicitud tal como fue publicada originalmente.

- 1.6** La materia objeto de las reivindicaciones 3 a 7 y 9 ha sido eliminada sin perjuicio de ser presentada en solicitudes divisionales relacionadas.
- 1.7** La materia objeto de la anterior reivindicación 10 (reivindicación 7 modificada) ha sido modificada eliminando las referencias a los porcentajes de identidad de secuencia dirigidas al 50-80 % de identidad de secuencia en la anterior reivindicación alternativa (ii) y con el fin de especificar que se reivindica un constructo reconocedor de antígeno recombinante de acuerdo con la modificación de la reivindicación 1.
- 1.8** En la anterior reivindicación 10 (reivindicación 7 modificada) se añade la alternativa (iii) dirigida a un TCR heterodimérico $\alpha\beta$ o $\gamma\delta$, en el que está presente un enlace disulfuro introducido entre residuos de los dominios constantes. El soporte para esta modificación se puede encontrar en la página 38 en el punto 36 y en la página 14, 3er párrafo de la solicitud como fue publicada originalmente.
- 1.9** Se añaden las nuevas reivindicaciones 9 a 15 dirigidas a TCR recombinantes que comprenden o consisten en las respectivas secuencias de cadena α y β . El soporte para esta modificación se puede encontrar, por ejemplo, en la página 8, 6.º párrafo y en la Tabla 1 que comienza en la página 53 de la solicitud tal como fue publicada originalmente.
- 1.10** La materia objeto de la anterior reivindicación 17 (reivindicación 18 modificada) ha sido modificada con el fin de especificar que el método comprende detectar un péptido PRAME de antígeno asociado a tumor (TAA) y con el fin de especificar además en el paso a) que la muestra se pone en contacto con un TCR recombinante que se une específicamente a dicho péptido PRAME. El soporte para esta modificación se puede encontrar en la página 4, 1er párrafo; Página 31, 3er párrafo y página 32, 2do párrafo de la solicitud como fue publicada originalmente.
- 1.11** El objeto de las anteriores reivindicaciones 18-20 (reivindicaciones 22-24 modificadas) ha sido modificada con el fin de especificar que el péptido/TAA diana es PRAME y con el fin de especificar que el constructo reconocedor de antígeno es recombinante, de acuerdo con la modificación introducida en la reivindicación 1.

1.12 Las demás reivindicaciones han sido modificadas solo para propósitos de reenumeración y para ajustar su dependencia.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la solicitud inicial, por lo que es posible afirmar que el nuevo pliego reivindicatorio se ajusta a las disposiciones del Artículo 34 de la Decisión 486/00.

2. Sobre el título de la solicitud

Con el propósito de adecuar el título de la solicitud de patente al nuevo pliego reivindicatorio, y atendiendo a la mable sugerencia del examinador, ha sido modificada el título de la solicitud como se lee a continuación:

“RECEPTORES DE LINFOCITOS T QUE RECONOCEN ANTÍGENOS PRAME”.

3. Pago de tasas

En relación con el número de reivindicaciones, atentamente nos permitimos decir que se adjunta recibo de pago por cuatro (4) reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio tiene un número de cláusulas menor que el presentado anteriormente.

Asimismo, considerando que el presente memorial se presenta junto con modificaciones a las reivindicaciones, se anexa el comprobante de pago correspondiente a la tasa de modificación a las reivindicaciones, según lo establecido en la Resolución No. 59669 de 2020. En caso de considerar que es necesario realizar un nuevo examen de patentabilidad, rogamos se nos notifique al respecto, a fin de proceder a realizar el pago correspondiente.

4. Claridad y sustento de las nuevas reivindicaciones

El solicitante manifiesta que mediante la presentación del nuevo capítulo reivindicatorio quedan subsanadas la totalidad de las objeciones, de modo tal que las nuevas reivindicaciones proporcionan claridad a la invención descrita, a la vez que cuentan con el debido sustento en la descripción de la solicitud. No obstante, a continuación se desea presentar algunas aclaraciones con respecto a las observaciones realizadas por el examinador:

4.1 Claridad y sustento de la reivindicación 1:

El Examinador objeta que la reivindicación 1 no es clara porque la materia reivindicada se relaciona con constructos reconocedores de antígeno pero no con moléculas de TCR. Además, el Examinador sostiene que la solicitud no divulga constructos reconocedores de antígeno que comprendan únicamente cuatro CDRs, p. CDR1 y CDR2, sino que solo describe moléculas de TCR que comprenden seis CDRs, p. CDR1, CDR2 y CDR3 de un dominio variable alfa y un dominio variable beta.

Al respecto, se hace notar que la materia objeto de la reivindicación ha sido modificada con el fin de especificar que el constructo reconocedor de antígeno es recombinante y que comprende un dominio variable de cadena alfa de TCR y un dominio variable de cadena beta de TCR o un dominio variable de cadena gamma de TCR y un dominio variable de cadena delta de TCR. El solicitante hace notar que estas características, en combinación con la característica funcional de unión específica a un péptido antigénico PRAME de SEQ ID NO: 97 y la característica estructural de las secuencias de los CDR1 y CDR3 definen suficientemente el constructo reconocedor de antígeno recombinante. La persona del oficio normalmente versada en la materia es muy consciente de que la unión específica al péptido antigénico está mediada por los dominios variables del constructo reconocedor de antígeno, en particular los CDRs, y es independiente de si los dominios variables están presentes en el marco de un TCR o un constructo reconocedor de antígeno, o si se usan, por ejemplo, en una molécula multiespecífica (por ejemplo, biespecífica) en la que se combinan con otros dominios variables. En consecuencia, se hace notar que limitar el constructo reconocedor de antígeno a un TCR, como sugiere el examinador, restringiría indebidamente el alcance de la invención divulgada por la presente solicitud.

La materia objeto de la reivindicación 1 ha sido modificada con el fin de especificar que el dominio variable alfa de TCR y el dominio variable beta de TCR o el dominio variable gamma de TCR y el dominio variable delta de TCR comprenden cada secuencia CDR1 y CDR3 especificada. Los CDR1 y CDR3 de un TCR contribuyen principalmente a la unión del TCR al péptido diana y se considera que el CDR2 juega un papel en el reconocimiento del del complejo mayor de histocompatibilidad (MHC) que presenta el péptido diana. Por lo tanto, la materia reivindicada dirigida a un constructo reconocedor de antígeno que comprende los CDR1 y CDR3 de un TCR de las secuencias especificadas comprende todas las

características necesarias para caracterizar la especificidad de unión al antígeno de tal constructo reconocedor de antígeno y cuenta con el debido sustento en la solicitud como fue publicada originalmente. De acuerdo con esto, se describe en la página 9 de la solicitud tal como fue publicada originalmente, que la secuencia de aminoácidos del CDR3 es particularmente relevante para la especificidad y/o selectividad de los constructos reconocedores de antígeno. La presente solicitud divulga en los ejemplos y en la descripción ocho TCRs diferentes que se unen específicamente a la secuencia de aminoácidos de SEQ ID NO: 97. El solicitante señala además la evidencia científica adjunta al presente memorial, es decir, Stadinski *et al.*, que divulga que las secuencias del CDR3 contribuyen fuertemente a regular cómo los TCRs se involucran con los ligandos del complejo péptido-MHC y también se muestra que el CDR1 contribuye a la especificidad peptídica de las células T.

El solicitante hace notar además que especificar combinaciones de CDR3/CDR1 de un TCR es suficiente para definir la especificidad antigénica del constructo reconocedor de antígeno. Stadinski *et al.* enseña que la especificidad de antígeno de las células T se define principalmente por el CDR3:

"También se han utilizado estudios estructurales para desentrañar cómo los TCRs crean especificidad para los complejos pMHC. La mayoría de los TCRs se unen a los ligandos del MHC dentro de una orientación diagonal semiconservada, lo que posiciona en gran medida los bucles del CDR3 encima del péptido unido y posiciona los residuos de los CDR1 y CDR2 del gen V codificados por la línea germinal encima de las hélices alfa del MHC". (véase la página 2, párrafo 3). (énfasis agregado)

"Los residuos del CDR3 a menudo contribuyen con una gran parte del sitio de unión del TCR, y algunos TCRs se involucran con ligandos del pMHC con una contribución mínima de los residuos del CDR1 y CDR2". (véase la página 2, párrafo 5). (énfasis añadido)

Stadinski *et al.* enseña además que ciertos residuos de aminoácidos del CDR1 también contribuyen a la especificidad del péptido:

"Gran parte de la especificidad del TCR se deriva de secuencias de aminoácidos únicas presentes en el sitio de unión del pMHC. Los datos presentados aquí demuestran que el proceso de diversidad combinatoria

del TCR y los reordenamientos de V(D)J también crean una colección de efectos indirectos que regulan la especificidad del ligando de los TCRs. Estos efectos permiten que los residuos del CDR3a modulen la geometría en la que los TCRs interactúan con los ligandos del pMHC, lo que determina si los residuos del TCR individuales interactúan con el MHC y cómo lo hacen. Además, se muestra que un residuo del CDR1a del TCR que se encuentra distal a la interfaz de unión del TCR-pMHC contribuye a la especificidad peptídica de las células T". (página 12, párrafo 1) (énfasis añadido)

A partir de las enseñanzas de Stadinski *et al.*, el solicitante hace notar que la unión del péptido diana depende del CDR3 y CDR1, pero no del CDR2. El solicitante señala además la evidencia científica divulgada por Cole *et al.* en donde se enseña que:

"El reconocimiento del pMHC por parte del TCR está mediado a través de los bucles de la región determinante de la complementariedad (CDR) del TCR. Aunque no es el caso con todos los pares TCR-pMHC, el dogma actual propone que los bucles del CDR2 del TCR codificados en la línea germinal entran en contacto principalmente con la región helicoidal conservada de la superficie del MHC (autointeracción TCR-MHC); los bucles hipervariables del CDR3 reorganizados somáticamente entran en contacto principalmente con el péptido antigénico (interacción no propia del TCR-péptido), y los bucles del CDR1 se encuentran en el medio, entrando en contacto tanto con el péptido como con el MHC". (Énfasis añadido)

Así, también Cole *et al.* enseña que la unión del péptido diana depende del CDR3 y CDR1, mientras que el CDR2 no se requiere para la especificidad del antígeno sino para la unión al HLA. Por lo tanto, la nueva reivindicación 1, que especifica combinaciones específicas del CDR1 y CDR3 de un TCR para ambas regiones variables del constructo reconocedor de antígeno comprende todas las características necesarias para caracterizar la especificidad y selectividad de unión a antígeno de un constructo reconocedor de antígeno que tiene la misma capacidad de unión y especificidad del TCR parental del cual se derivan dichos CDRs. Por lo tanto, la persona del oficio normalmente versada en la materia concluye del contenido de la solicitud tal como fue publicada originalmente que la especificidad de unión de los TCR ejemplificados por la presente solicitud se aplica a todos los constructos reconocedores de antígeno que comprenden las

mismas secuencias CDR1 y CDR3, es decir, a los constructos reconocedores de antígeno recombinantes de la nueva reivindicación 1.

La objeción es por lo tanto disputable y, por lo tanto, debe ser retirada.

Por otra parte, se hace notar que el solicitante ha modificado aún más la materia objeto de la reivindicación 1 con el fin de especificar que las secuencias de los CDRs pueden comprender no más de una o dos modificaciones de aminoácidos que son una sustitución con un aminoácido conservativo. El solicitante hace notar que el número de posibles sustituyentes está limitado al caracterizar la sustitución como una sustitución con un aminoácido conservativo y que la materia objeto de la nueva reivindicación 1 cuenta con el debido sustento en la descripción de la solicitud, al menos las siguientes razones:

Los constructos reconocedores de antígeno recombinante reivindicados se caracterizan por la característica funcional de unirse específicamente a un péptido antigénico PRAME de acuerdo con la SEQ ID NO: 97, con lo cual se excluyen las variantes que no se unen a un péptido antigénico PRAME de acuerdo con la SEQ ID NO: 97 del alcance de la nueva reivindicación 1. La persona del oficio normalmente versada en la materia que lea la solicitud y las reivindicaciones con una mente dispuesta a comprender es consciente de que cualquier sustitución en los CDRs no deberían dar como resultado un TCR que ya no se una al péptido diana PRAME. Si bien el resultado de dicha sustitución en un CDR puede no ser predecible de manera confiable, la característica funcional especificada en la reivindicación limita el número posible de variantes que caen dentro del alcance de las reivindicaciones 1 y 2. El solicitante enfatiza además que la mutación de una secuencia de aminoácidos conocida es tarea de rutina para la persona del oficio normalmente versada en la materia técnica. Los métodos respectivos se conocen en el campo (p. ej., el kit de mutagénesis dirigida al sitio QuikChange de Stratagene desde 2007). El solicitante señala que la limitación introducida que especifica que la modificación del CDR corresponde a una sustitución con un aminoácido conservativo, no requiere ningún cambio en el número de aminoácidos. Sólo existe un número limitado de posibles sustituciones de aminoácidos conservativas. La persona del oficio normalmente versada en la materia es así capaz de introducir una sustitución específica con un aminoácido conservativo en una secuencia de aminoácidos en general y en una secuencia de CDR en particular. La selección de variantes de un CDR que se unen a un blanco específico también es un procedimiento estándar aplicado por la persona del oficio

normalmente versada en el campo de las ciencias de la vida. La solicitud tal como se presentó sugiere que la unión de un TCR al péptido antigénico PRAME se puede evaluar electroporando células T con ácidos nucleicos que codifican el TCR y realizando una tinción de tetrameros MHC/PRAME (Fig. 21 y descripción en la página 48) y también midiendo liberación de interferón γ (por ejemplo, Ejemplo 1, Figura 8, Ejemplo 8, Figura 30). La persona del oficio normalmente versada en la materia que lea la solicitud tal como fue publicada originalmente con una mente dispuesta a comprender, comprende que este método es adecuado para identificar variantes de los constructos reconocedores de antígeno descritos en la solicitud. Por lo tanto, la persona del oficio normalmente versada en la materia consideraría fácilmente estos métodos como adecuados para cribar variantes de los TCRs descritos. Si bien una selección de este tipo puede llevar mucho tiempo, no es una carga indebida para la persona del oficio normalmente versada en la materia probar la unión específica de destino de las variantes de acuerdo con los métodos mencionados anteriormente y, por lo tanto, probar si las variantes estarán dentro del alcance de la reivindicación 1.

En resumen:

Si bien el resultado de una mutación dentro de un CDR puede no ser predecible, los métodos de detección para evaluar los resultados de dichas modificaciones son bien conocidos en el estado de la técnica y la cantidad de posibles modificaciones y combinaciones de las mismas es limitada. Por lo tanto, realizar los experimentos necesarios no es excesivamente oneroso. La persona del oficio normalmente versada en la materia que pretenda sustituir no más de uno o dos aminoácidos en los CDR1, CDR2 y/o CDR3 de la cadena alfa/gamma y/o beta/delta con un aminoácido conservador sin anular la especificidad cuenta con suficiente orientación, apoyo y divulgación en la descripción de la presente solicitud tal como fue publicada originalmente.

4.2 Sustento y concisión de las reivindicaciones 3, 4, 6 y 7

El Examinador objeta que los anticuerpos de las antiguas reivindicaciones 3 y 4 carecen de sustento en la descripción. El Examinador considera además que la materia objeto de las reivindicaciones 6 y 7 es redundante con respecto a la materia de la reivindicación 2.

Al respecto, se hace notar que el solicitante ha eliminado las antiguas reivindicaciones 3 y 4 y 6 y 7, con lo cual el nuevo pliego reivindicatorio no da lugar a la objeción.

4.3 Claridad de las reivindicaciones 8 y 10

El examinador considera que la materia objeto de las antiguas reivindicaciones 8 y 10 no es clara debido a que hace referencia a porcentajes de identidad de secuencia.

El solicitante respetuosamente no está de acuerdo. La nueva reivindicación 6 (anterior reivindicación 8) se refiere a constructos reconocedores de antígeno que comprenden secuencias de dominios variables alfa y beta y secuencias CDR1, CDR2 y CDR3 específicas. La reivindicación permite además que las secuencias de dominio variable tengan al menos un 90% de identidad de secuencia con las secuencias indicadas, mientras que los CDRs se definen específicamente por sus respectivas secuencias de aminoácidos, en otras palabras, la reivindicación no hace referencia a que los CDRs tengan al menos un 90% de identidad de secuencia. De hecho, es la región flanqueante (FR) del dominio variable la que se define por tener al menos un 90% de identidad de secuencia con la FR de la secuencia indicada. El solicitante hace notar que los FRs que permiten una identidad de secuencia del 90% son totalmente claros y corresponden a una razonable generalización de las invenciones ejemplificadas en la descripción de la solicitud.

A continuación, el solicitante explicará en detalle la biología estructural de los dominios variables de TCR y las consecuencias necesarias para la FR de los TCRs resultantes de los mismos. El solicitante adjunta una copia impresa de un artículo de Wikipedia (disponible en https://en.wikipedia.org/wiki/T-cell_receptor#Structural_characteristics) sobre los TCRs. Se basa únicamente en referencias publicadas entre 1982 y abril de 2014 (véanse las páginas 9 a 12 del artículo de Wikipedia) y, por lo tanto, es adecuado para caracterizar el conocimiento general común en el estado de la técnica para la fecha de presentación de la presente solicitud. Con respecto a las características estructurales básicas de los TCRs, enseña lo siguiente:

"El TCR es una proteína heterodimérica unida por enlace disulfuro que se encuentra anclada a la membrana. Normalmente consta de las cadenas

alfa (α) y beta (β) altamente variables expresadas como parte de un complejo con las moléculas de la cadena invariante CD3. Cada cadena está compuesta por dos dominios extracelulares: la región variable (V) y una región constante (C), ambos pertenecientes al dominio de la superfamilia de las inmunoglobulinas (IgSF) que forman láminas β antiparalelas. La región constante está próxima a la membrana celular, seguida de una región transmembranal y una cola citoplásmica corta, mientras que la región variable se une al complejo péptido/MHC. El dominio variable de la cadena α y la cadena β del TCR tiene cada uno tres regiones hipervariables o determinantes de la complementariedad (CDRs). También hay un área adicional de hipervariabilidad en la cadena β (HV4) que normalmente no entra en contacto con el antígeno y, por lo tanto, no se considera una CDR. Los residuos en estos dominios variables están ubicados en dos regiones del TCR, en la interfaz de las cadenas α y β y en la región del almacén de la cadena β que se cree que está cerca del complejo de transducción de señales CD3". (énfasis añadido)

El dominio variable del TCR comprende tres regiones marco, es decir, FR1, FR2 y FR3 y tres CDRs, es decir, CDR1, CDR2 y CDR3 que están situadas entre las respectivas regiones marco. El sitio de unión al antígeno del TCR, es decir, el sitio de interacción del péptido, está conformado por los CDRs, en particular por el CDR3 (ver también la sección anterior). Los CDRs, también llamadas regiones hipervariables, difieren en cada TCR para proporcionar diversidad de unión contra diferentes antígenos. Los aminoácidos de la región marco, de ahí su nombre, posicionan los CDRs dentro del espacio 3D, por ejemplo, funcionan como un andamio y contribuyen solo poco, si es que lo hacen, a la interacción con el péptido unido por los CDRs del TCR. La regiones marco interactúan entre sí y con las regiones marco correspondientes de la cadena beta. Por lo tanto, las modificaciones en las regiones flanqueantes tienen poca o ninguna influencia sobre la especificidad y la afinidad de la unión a un péptido antigénico en complejo con una molécula MHC. Por lo tanto, los porcentajes de identidad de las secuencias reivindicadas son claros y corresponden a una razonable generalización de las invenciones ejemplificadas en la descripción de la solicitud.

Las mismas observaciones aplican para el objeto de la antigua reivindicación 10 (reivindicación 6 modificada) dirigida al TCR recombinante que comprende además una región constante que tiene al menos un 90% de identidad de secuencia con las secuencias indicadas. La región constante puede, por ejemplo,

modificarse mediante la introducción de un puente disulfuro u otras mutaciones puntuales para mejorar los perfiles de expresión, la estabilidad y/o el apareamiento de cadenas de TCR (ver p. 16, último párrafo y p. 26, 4.º párrafo de la solicitud como fue publicada originalmente). Por lo tanto, los porcentajes de identidad de secuencia reivindicados son claras y cuentan con el debido sustento en la descripción de la solicitud.

En consecuencia, se solicita respetuosamente reconsiderar y retirar la objeción.

4.4 Reivindicación 9

El examinador considera que la materia objeto de la antigua reivindicación 9 carece de claridad y sustento en la descripción porque considera que se refiere a los CDRs del fragmento de unión de un TCR pero este comprende entre tres y cinco secuencias como opciones para cada combinación, mientras que todas las TCR divulgadas en la solicitud comprende seis CDRs.

Al respecto, se hace notar que el solicitante ha eliminado la antigua reivindicación 9, con lo cual el nuevo pliego reivindicatorio no da lugar a la objeción.

4.5 Reivindicación 17

El Examinador considera que la materia objeto de la antigua reivindicación 17 carece de claridad y sustento en la descripción porque considera que se refiere a un método *in vitro* de un antígeno asociado a un tumor (TAA) sin mencionar específicamente el antígeno a reconocer y la estructura. del constructo reconocedor de antígeno, es decir, un TCR.

Al respecto, se hace notar que el solicitante ha modificado la antigua reivindicación 17 (reivindicación 18 modificada) de acuerdo con lo mencionado en el numeral 1.10. La objeción es discutible y, por lo tanto, debe retirarse.

4.6 Reivindicaciones 18-20

El Examinador considera que la materia objeto de las antiguas reivindicaciones 18 a 20 carece de claridad porque se refiere a un método que comprende "*un constructo reconocedor antígenos*" y/o un "*TAA*". Sin embargo, el Examinador

sostiene que todas los constructos seleccionadas en la solicitud son TCR y los péptidos TAA tienen una secuencia de aminoácidos específica.

Al respecto, se hace notar que el solicitante ha modificado el objeto de las antiguas reivindicaciones 18-20 (reivindicación modificada 19-21) de acuerdo con lo descrito en la sección 1.11. La objeción es discutible y, por lo tanto, debe retirarse.

Así, en vista de las modificaciones realizadas al pliego reivindicatorio y las aclaraciones aquí presentadas, es posible manifestar que el alcance de la invención es claro y cuenta con el debido sustento en la descripción de la solicitud, cumpliendo así con el requerimiento del Artículo 30 de la Decisión 486.

5. Suficiencia de la descripción

El Examinador sostiene que la descripción no divulga la invención de manera suficientemente completa para que la persona normalmente versada en la materia técnica correspondiente pueda ejecutarla (o reproducirla). En particular, el Examinador sostiene que los constructos reconocedores de antígeno reivindicadas pueden tener cualquier estructura, por ejemplo, pueden ser anticuerpos, y también los antígenos asociados a tumores (TAA) pueden tener cualquier estructura. Sin embargo, considera que la descripción describe únicamente TCRs contra PRAME-004 de acuerdo con la SEQ ID NO: 97.

Al respecto el solicitante hace notar que el objeto reivindicado ahora se dirige específicamente a constructos reconocedores de antígeno recombinante que se unen específicamente a la SEQ ID NO. 97 (PRAME) y comprenden un dominio variable alfa/gamma de TCR con secuencias CDR1 y CDR3 específicas y un dominio variable beta/delta de TCR que comprende secuencias CDR1 y CDR3 específicas (véase la sección 1.1).

El solicitante discrepa respetuosamente y manifiesta que la persona del oficio normalmente versada en la materia es muy capaz de probar si un constructo reconocedor antígenos ejerce la propiedad funcional reclamada, sin que ello represente una carga indebida. La descripción detalla al menos una forma de llevar a cabo la invención. Dado que la solicitud está dirigida a persona del oficio normalmente versada en la materia, no es necesario ni deseable que se den detalles de características auxiliares bien conocidas, pero la descripción debe divulgar cualquier característica

esencial para llevar a cabo la invención con suficiente detalle para que resulte evidente a la persona del oficio normalmente versada en la materia cómo poner en práctica la invención. En el presente caso, el solicitante ha descrito diferentes ejemplos que comprenden ensayos de liberación de IFN γ de ocho moléculas de TCR diferentes, ver los Ejemplos 1-8 en la página 65-69 de la solicitud tal como fue publicada originalmente, en el que las moléculas de TCR se incuban con el péptido diana PRAME o con variantes de sustitución de alanina y treonina. La liberación de IFN γ indica la unión de la molécula de TCR al péptido diana. La persona del oficio normalmente versada en la materia es, por lo tanto, muy consciente de cómo probar la unión de constructos reconocedores de antígeno modificadas al péptido diana o sus variantes sin que esto represente una carga indebida. El solicitante hace notar que se dan varios ejemplos para describir realizaciones alternativas. El solicitante es de la opinión de que la solicitud contiene suficiente información para permitir que la persona del oficio normalmente versada en la materia, utilizando su conocimiento general en el campo técnico, realice la invención en toda el alcance reivindicado sin que ello represente una carga indebida y sin necesidad de llevar a cabo una actividad inventiva.

En consecuencia, a la luz de las aclaración aquí presentadas y las modificaciones realizadas al pliego reivindicatorio, es posible manifestar que la descripción de la presente solicitud divulga la invención del actual pliego reivindicatorio de manera suficientemente completa para que la persona normalmente versada en la materia técnica correspondiente pueda ejecutarla (o reproducirla), cumpliendo así con el requerimiento de acuerdo con el artículo 28 de la Decisión 486/00.

6. Unidad de invención

El Examinador considera que la materia reivindicada carece de unidad y categoriza la invención en los siguientes grupos:

Grupo 1: El Grupo 1 comprende la materia dirigida a TCR R11P3D3.

Grupo 2: El grupo 2 comprende la materia dirigida a TCR R16P1C10.

Grupo 3: El grupo 3 comprende la materia dirigida a TCR R16P1E8.

Grupo 4: El grupo 4 comprende el tema dirigido a TCR R17P1A9.

Grupo 5: El grupo 5 comprende el tema dirigido a TCR R17P1D7.

Grupo 6: El grupo 6 comprende el tema dirigido a TCR R17P1G3.

Grupo 7: El grupo 7 comprende la materia dirigida a TCR R17P2B6.

Al respecto, se hace notar que los siete grupos de invenciones, es decir, los siete constructos reconocedores de antígeno recombinantes de la presente solicitud, según lo indicado por el Examinador, forman un solo concepto común inventivo, dado que existe una relación técnica entre las invenciones reivindicadas que implican una o más características técnicas especiales iguales o correspondientes. La característica técnica especial correspondiente de los constructos reconocedores de antígeno recombinante de la reivindicación 1 es que se unen específicamente a un antígeno asociado a tumor (TAA) "PRAME" de acuerdo con la SEQ ID NO: 97 (SLLQHLIGL) y comprenden un dominio variable alfa y beta que comprende CDR1 y CDR3 definidos por cada una de las secuencias de aminoácidos recitadas en la reivindicación 1. Por lo tanto, los constructos reconocedores de antígeno reivindicados no tienen o sustancialmente no tienen reactividad cruzada con péptidos similares, lo que da como resultado un riesgo reducido de producir efectos fuera del objetivo (véase la página 7, segundo párrafo de la solicitud como fue publicada originalmente).

Este efecto se ha demostrado en la presente solicitud de patente para ocho TCRs, es decir, R11P3D3, R11P303_KE, R16P1C10, R16P1E8, R17P1A9, R17P107, R17P1G3 y R17P2B6. Estos TCRs fueron evaluados en cuanto a su especificidad de unión para PRAME-004 y fueron incubados con péptido PRAME-004 presentado por HLA-A*02 y péptidos no relacionados y, posteriormente, se midió la liberación de interferón gamma (IFN γ). Se probó la reactividad cruzada de los TCRs respectivos hacia otros 67 péptidos similares (de los cuales 57 eran idénticos a PRAME-004 en las posiciones 3, 5, 6 y 7) pero no relacionados en el contexto de HLA-A*02. Los resultados se muestran en las Figuras 1-7 y 31 (péptido PRAME-004 y variantes de sustitución de alanina y treonina) y las Figuras 8-14, 22-23 y 26-27 (péptidos muy similares). Los datos obtenidos por el ensayo de liberación de IFN γ muestran una mayor liberación de IFN γ de los TCRs probados cuando se incubaron con el péptido PRAME-004, mientras que la liberación de IFN γ de otras variantes es similar al péptido de control negativo NYSO01-001. Por ejemplo, la liberación de IFN γ de los TCRs disminuye cuando se incubaba con los péptidos TMED9-001, CAT-001 o HDAC5-001, solo por nombrar algunos, y similar para NYESO1-001 utilizado como control negativo. Además, la activación de las células T se evaluó en un ensayo de destrucción usando una línea de células tumorales A375 transducidas con PRAME-004 como células diana en comparación con las células A375 no transducidas. Las células transducidas con los respectivos TCR reconocidos endógenamente presentaron PRAME-004-HLA y condujeron a una disminución del crecimiento tumoral de una manera dependiente de la concentración (véase, por ejemplo, la Figura 32). La reexpresión de TCR en linfocitos T CD8+ primarios humanos conduce al reconocimiento selectivo y la eliminación de líneas celulares tumorales que

presentan HLA-A*02/PRAME-004 (véanse, por ejemplo, las Figuras 24, 25, 30 y 32). El efecto técnico se consigue así en todo el alcance de la reivindicación 1. La materia objeto de la reivindicación 1 representa un concepto común inventivo. Todas las demás reivindicaciones dependen de la reivindicación 1 o comprenden la molécula de TCR recombinante de la reivindicación 1 y, por lo tanto, derivan su unidad de invención de esta dependencia.

Por lo tanto, a la luz de las aclaraciones aquí realizadas, es posible manifestar que las invenciones de la solicitud comprenden un grupo de invenciones relacionadas entre sí, de manera que conformen un único concepto inventivo, cumpliendo así con el requerimiento de acuerdo con el artículo 25 de la Decisión 486/00.

7. Novedad

El examinador establece en su concepto técnico la falta de novedad de la antigua reivindicación 17 a la luz de las enseñanzas técnicas descritas en el documento Zhang, J. Y., *Methods in molecular biology*, titulado "*Identification of tumor-associated antigens as diagnostic and predictive biomarkers in cancer*" (en adelante denominado como **D1**), puesto que considera que las características esenciales del método *in vitro* para detectar un péptido antigénico asociado a tumor (TAA, por sus siglas en ingles), solo o en complejo con una molécula de MHC, en una muestra biológica ya habían sido divulgadas previamente en el documento **D1**.

A este respecto, me permito decir que el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que la invención se considera nueva cuando no está comprendida en el estado de la técnica. Por lo tanto, si de acuerdo con el espíritu de la norma legal indicada, la invención no se considera como nueva si está "*comprendida*" en el estado de la técnica, dicha norma se está refiriendo, naturalmente, a la totalidad del invento y no a alguno o a algunos de sus componentes.

Esto se conoce como NOVEDAD ABSOLUTA, de conformidad con lo cual para desvirtuar el requisito de novedad de una invención será necesario que lo reivindicado por la invención bajo estudio corresponda de forma total con lo divulgado en un documento o anterioridad señalada, conclusión a la que se llega después de un análisis exhaustivo, mediante una comparación entre el capítulo reivindicatorio de la invención en estudio y la divulgación del estado de la técnica.

Ahora bien, si un determinado elemento, condición o circunstancia de la nueva creación ya hace parte del estado de la técnica, no quiere ello decir, desde luego, que la invención deba ser rechazada por carecer del requisito de novedad. No sin razón ha dicho el mencionado Tribunal: *"hay que advertir que toda creación es en cierta forma, producto del estado de la técnica existente, en el sentido de que el inventor ha de tener en cuenta un conjunto de conocimientos técnicos que le permitan crear nuevos procedimientos aplicables a la industria"* (Ibídem, páginas 53 y 54) (el subrayado es nuestro).

Por lo tanto, como mínimo la novedad debe contemplar los siguientes aspectos:

- Que la invención en estudio esté contenida de forma TOTAL y ABSOLUTA en el documento que se menciona como anterioridad o de forma independiente en los documentos que se mencionan, si son varios.
- Que la invención en estudio puede partir de conocimientos conocidos, caso en el cual habrá que hacer la comparación no de lo conocido, sino de lo inédito y novedoso, teniendo que analizarse el valor agregado que aporta la invención con sus nuevas características al campo técnico al cual pertenece la invención en estudio.

Aplicando lo anterior al caso concreto, para que las razones que aduce este despacho para desvirtuar la novedad fuesen ciertas, sería forzoso que se demostrara que la invención de mi mandante está comprendida de forma TOTAL Y ABSOLUTA en el estado de la técnica divulgado en el documento **D1**, lo cual no sucede con la reivindicación 17 (reivindicación 21 modificada) de la presente solicitud.

En particular, se hace notar que solicitante ha modificado la materia objeto de la reivindicación 17 con el fin de especificar que el método *in vitro* detecta un antígeno asociado a tumores (TAA) que es PRAME y que la muestra comprende un constructo reconocedor de antígeno recombinante que se une específicamente a un péptido PRAME o un complejo péptido PRAME/MHC. El documento **D1** divulga un enfoque de identificación de antígenos asociados a tumores (TAA) que comprende el examen de sueros de pacientes con cáncer usando extractos de células de cultivo tisular como fuente de antígenos para ensayos de transferencia por Western y de inmunofluorescencia indirecta en células enteras (ver resumen de **D1**). El documento **D1** describe además que varios TAA nuevos, incluidos HCC1, p62, p90, han sido identificados mediante este enfoque (ver resumen de **D1**, penúltima oración). Sin embargo, el documento **D1** guarda silencio sobre PRAME como TAA. La materia objeto de la antigua reivindicación 17 (reivindicación 20 modificada) es, por lo tanto, novedoso sobre **D1**.

Así, dado que el documento **D1** no divulga de forma TOTAL Y ABSOLUTA las características técnicas esenciales del método reclamado por la presente solicitud, es posible manifestar que la solicitud de las nuevas reivindicaciones 1 a 25 cumple con el requisito de novedad estipulado en el artículo 16 de la Decisión 486.

8. Nivel Inventivo

El examinador establece en su concepto técnico que el TCR recombinante de las antiguas reivindicaciones 1 a 15 carece de nivel inventivo a la luz de las enseñanzas descritas en el documento **US20160263155**, titulado "T cell receptors directed against the preferentially expressed antigen of melanoma and uses thereof" (**D2**).

En particular, el examinador manifiesta que la diferencia entre la invención definida en la reivindicación 1 y el receptor de células T (TCR) divulgado en el documento **D2** consiste en que el constructo presenta secuencias diferentes para cuatro de sus seis CDRs, pero considera que no se evidencia un efecto técnico asociado a dicha diferencia, dado que considera que solo se presentan pruebas técnicas para receptores de células T (TCR) recombinantes y (ii) que dichos TCRs tienen como estructura esencial mínima seis CDRs, por lo que, manifiesta que la reivindicación no suministra todas las características técnicas esenciales de la molécula y, en consecuencia. En consecuencia, el examinador considera que no es posible asociar las pruebas técnicas de los TCR con estructuras específicas del capítulo descriptivo con cualquiera de las moléculas dentro del alcance de la reivindicación.

Al respecto, se reitera sobre el particular que el artículo 18 de la Decisión 486/00 determina que *"una invención tiene nivel inventivo, si para una **persona del oficio normalmente versada en la materia técnica** correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"*. (El subrayado es nuestro).

Lo anterior significa que el análisis de nivel inventivo **NO** es una comparación de los objetos de las anterioridades y la solicitud en estudio. El análisis de nivel inventivo **debe demostrar** la obviedad de la invención y establecer cómo ésta no contiene un salto cualitativo, que demuestre su altura inventiva, teniendo en cuenta que se hace **necesario demostrar** que la invención analizada carece de nivel inventivo y hubiese sido obvia para una persona versada en la materia.

Considerando la definición anterior, a continuación, se proceden a destacar las diferencias entre el receptor divulgado en el documento **D1**, mostrando cómo dichas diferencias le proporcionan un efecto técnico inesperado que no podría haber sido derivado de manera obvia a partir de las enseñanzas del arte previo citado por el examinador, para así evidenciar la no obviedad de la invención reclamada por el solicitante.

8.1 *Enseñanzas del arte previo contenido en D2*

El documento **D2** describe composiciones y métodos para inducir una respuesta inmunitaria contra el antígeno de melanoma expresado preferentemente (PRAME). El documento proporciona métodos para tratar enfermedades hiperproliferativas mediante la inducción de una respuesta inmunitaria contra el antígeno PRAME; la respuesta inmunitaria puede inducirse dirigiéndose específicamente a las células que expresan PRAME usando receptores de células T dirigidos contra PRAME (ver **D2**, página 1, segundo párrafo).

8.2 *La diferencia entre el objeto reivindicado y el estado de la técnica*

La materia objeto de la nueva reivindicación 1, por lo tanto, difiere del estado de la técnica en que los constructos recombinantes que reconocen antígenos de la reivindicación 1 se unen específicamente a un péptido antigénico PRAME que tiene una secuencia de aminoácidos de acuerdo con la SEQ ID NO: 97 y comprende las secuencias de aminoácidos CDR1 y CDR3 de dominio variable α/γ y β/δ como se define en la nueva reivindicación 1.

8.3 *El efecto técnico atribuible a esta diferencia*

El efecto técnico atribuible a esta diferencia es que los constructos reconocedores de antígeno recombinante de la reivindicación 1 se dirigen específicamente y se unen a un antígeno asociado a tumor (TAA) PRAME de acuerdo con la SEQ ID NO: 97 (SLLQHLIGL). En consecuencia, los constructos reconocedores de antígeno recombinantes reivindicadas no tienen o sustancialmente no tienen reactividad cruzada con péptidos similares, lo que da como resultado un riesgo reducido de producir efectos fuera del objetivo en tejidos sanos (ver página 7, segundo párrafo de la solicitud tal como fue publicada originalmente).

Este efecto se ha demostrado en la presente solicitud de patente para ocho constructos reconocedores de antígeno recombinantes, por ejemplo, TCRs, es decir, R11P3D3, R11P303_KE, R16P1C10, R16P1E8, R17P1A9, R17P107, R17P1G3 y R17P2B6. Estos TCRs se examinaron en cuanto a su especificidad de unión para PRAME-004 y se incubaron con péptido PRAME-004 presentado por HLA-A*02 y péptidos no relacionados y, posteriormente, se midió la liberación de interferón gamma (IFN γ). Se probó la reactividad cruzada de los TCRs respectivos hacia otros 67 péptidos similares (de los cuales 57 eran idénticos a PRAME-004 en las posiciones 3, 5, 6 y 7) pero que no estaban relacionados en el contexto de HLA-A*02. Los resultados se muestran en las Figuras 1-7 y 31 (péptido PRAME-004 y variantes de sustitución de alanina y treonina) y las Figuras 8-14, 22-23 y 26-27 (péptidos muy similares). Los datos obtenidos por el ensayo de liberación de IFN γ muestran una mayor liberación de IFN γ de los TCRs probados cuando se incubaron con el péptido PRAME-004, mientras que la liberación de IFN γ de otras variantes es similar al péptido de control negativo NYSO01-001. Por ejemplo, la liberación de IFN γ de los TCRs disminuye cuando se incubaba con los péptidos TMED9-001, CAT-001 o HDAC5-001, solo por nombrar algunos, y similar para NYESO1-001 utilizado como control negativo. Además, la activación de las células T se evaluó en un ensayo de destrucción usando una línea de células tumorales A375 transducidas con PRAME-004 como células diana en comparación con las células A375 no transducidas. Las células transducidas con los respectivos TCR reconocidos endógenamente presentaron PRAME-004-HLA y condujeron a una disminución del crecimiento tumoral de una manera dependiente de la concentración (véase, por ejemplo, la Figura 32). La reexpresión de TCR en linfocitos T CD8+ primarios humanos conduce al reconocimiento selectivo y la eliminación de líneas de células tumorales que presentan HLA-A*02/PRAME-004 (véanse, por ejemplo, las Figuras 24, 25, 30 y 32).

Estos resultados respaldan la unión específica de los TCRs R11P3D3, R11P303_KE, R16P1C10, R16P1E8, R17P1A9, R17P107, R17P1G3 y R17P2B6 al epítipo y la disminución de la reactividad cruzada, propiedades que exhiben todos los TCRs recombinantes de la nueva reivindicación 1 debido a la presencia de CDR 1 y CDR3 ya que estos son los CDRs que están implicadas principalmente en la unión del péptido presentado por HLA y, por lo tanto, en la mediación de la especificidad de unión. Por lo tanto, el efecto técnico se logra en todo el alcance de la reivindicación 1.

8.4 El problema técnico objetivo

El problema técnico objetivo por resolver puede, por lo tanto, formular así:
¿Cómo proveer constructos reconocedores de antígeno recombinante mejoradas?

8.5 *La solución no es obvia cuando se parte del estado de la técnica*

D2 divulga un TCR dirigido a PRAME-004 pero demuestra que el clon específico de PRAME-004, aunque no muestra reactividad contra varias células de tejidos sanos, como las células epiteliales derivadas de la piel, pulmón, colon, tracto biliar, riñón, hígado, así como fibroblastos, células del estroma mesenquimatoso y todos los diferentes linajes hematopoyéticos, incluidas las células madre hematopoyéticas: ejerce una reactividad baja contra las células epiteliales tubulares del riñón y una actividad intermedia contra las células dendríticas maduras (consulte la página 150, líneas 31-37 y Figura 8 de **D2**). Por el contrario, no se prueban ni se presentan en **D2** péptidos similares derivados de tejidos sanos, de manera que la persona del oficio normalmente versada en la materia, a partir de **D2** y con el objetivo de resolver el problema técnico, no habría encontrado ninguna guía en **D2** que enseñara las secuencias de TCR a ser aisladas, y mucho menos que condujera a los constructos reconocedores de antígeno recombinante de la nueva reivindicación 1 con especificidad para PRAME-004 y casi sin reactividad cruzada hacia péptidos similares diana. **D2** no divulga TCR recombinantes que comprendan las secuencias de aminoácidos de CDRs como se define en la reivindicación 1, es decir, los CDR1, CDR2 y CDR3 de los TCRs R11P3D3, R11 P303_KE, R16P1C10, R16P1E8, R17P1A9, R17P107, R17P1G3 y R17P2B6 que son específicos del blanco/objetivo y no muestran reactividad cruzada contra péptidos similares al blanco/objetivo. Por lo tanto, la materia objeto de la nueva reivindicación 1 implica una actividad inventiva. A su vez, las demás reivindicaciones dependen de la reivindicación 1 y, por lo tanto, derivan su actividad inventiva de esta dependencia.

En consecuencia, en vista de las modificaciones realizadas al pliego reivindicatorio en esta oportunidad y los argumentos aquí proporcionados, es posible afirmar que la materia objeto de las nuevas reivindicaciones implica un nivel inventivo con respecto a arte previo citado por el examinador, cumpliendo así con el requerimiento del Artículo 18 de la Decisión 486/00.

9. Conclusiones

Con las modificaciones introducidas y los argumentos presentados puede concluirse que:

- i) Las modificaciones del nuevo pliego reivindicatorio se ajustan a las disposiciones del Artículo 34 de la Decisión 486;
- ii) La descripción de la presente solicitud divulga la invención de manera suficientemente clara y completa para su comprensión y para que una persona capacitada en la materia técnica correspondiente pueda ejecutarla, cumpliendo así con los requerimientos del Artículo 28 de la Decisión 486.
- iii) La invención reclamada por la presente solicitud es clara, concisa y cuenta con el debido sustento en el capítulo descriptivo, cumpliendo así con los requerimientos de los Artículos 30 de la Decisión 486; y
- iv) Las invenciones reclamadas en el nuevo pliego reivindicatorio conforman un único concepto inventivo, cumpliendo así con el requerimiento del Artículo 25 de la Decisión 486.
- v) La invención reclamada no está comprendida en el estado de la técnica, cumpliendo así con los requerimientos del Artículo 16 de la Decisión 486.
- vi) La invención de la presente solicitud no resulta obvia ni se deriva de manera evidente del estado de la técnica, cumpliendo así con los requerimientos del Artículo 18 de la Decisión 486.

Así, respetuosamente se solicita examinar la novedad, el nivel inventivo y la aplicación industrial para la invención titulada *"RECEPTORES DE LINFOCITOS T QUE RECONOCEN ANTÍGENOS PRAME"* a nombre de Immatics Biotechnologies GmbH y, por lo tanto, a la Superintendencia de Industria y Comercio conceder el privilegio de patente, si los cumpliera; o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos comunicarnos los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación del Artículo 42 del Código de Procedimiento Administrativo y de lo Contencioso Administrativo que señala que: *"Habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con*

base en las pruebas e informes disponibles, se tomará la decisión, que será motivada. La decisión resolverá todas las peticiones que hayan sido oportunamente planteadas dentro de la actuación por el peticionario y por los terceros reconocidos”, (el subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el Artículo 29 de la Constitución Nacional.

10. Anexos

10.1 Nuevo capítulo reivindicatorio que incluye veinticinco **(25)** reivindicaciones.

10.2 Stadinski *et al.* Effect of CDR3 sequences and distal V gene residues in regulating TCR-MHC contacts and ligand specificity. J Immunol. 2014 Jun 15;192(12):6071-82. doi: 10.4049/jimmunol.1303209. Epub 2014 May 9. PMID: 24813203; PMCID: PMC4097884. Disponible en: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4097884/pdf/nihms587594.pdf>

10.3 Cole *et al.* T-cell receptor (TCR)-peptide specificity overrides affinity-enhancing TCR-major histocompatibility complex interactions. J Biol Chem. 2014 Jan 10;289(2):628-38. doi: 10.1074/jbc.M113.522110. Epub 2013 Nov 6. PMID: 24196962; PMCID: PMC3887192. Disponible en: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3887192/pdf/zbc628.pdf>

10.4 Artículo de Wikipedia. Disponible en https://en.wikipedia.org/wiki/T-cell_receptor#Structural_characteristics

10.5 Recibo de pago por concepto de modificación al pliego reivindicatorio.

10.6 Recibo de pago por cuatro (4) reivindicaciones adicionales.

Señora Directora,



CAROLINA DAZA MONTALVO

C.C. 66.783.790 de Palmira Valle

TP-141563 CSJ

notificaciones@clarkemodet.com.co

REIVINDICACIONES

1. Un constructo reconocedor de antígeno recombinante que se une específicamente al péptido antigénico denominado Antígeno Expresado Preferencialmente en el Melanoma (PRAME, por sus siglas en inglés), de acuerdo con la SEQ ID NOs: 97, caracterizado porque comprende un dominio variable alfa de TCR o un dominio variable beta TCR o un dominio variable gamma de TCR y un dominio variable delta de TCR, y en el que
 - (i) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende la región determinante complementaria (CDR) 1 según la SEQ ID NO: 1 y CDR3 según la SEQ ID NO: 3; y el dominio variable beta de TCR o el dominio variable delta de TCR comprende CDR1 según la SEQ ID NO: 7 y CDR3 según la SEQ ID NO: 9; o
 - (ii) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende CDR1 según la SEQ ID NO: 13 y CDR3 según la SEQ ID NO: 15; y el dominio variable beta de TCR o el dominio variable delta de TCR comprende CDR1 según la SEQ ID NO: 19 y CDR3 según la SEQ ID NO: 21; o
 - (iii) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende CDR1 según la SEQ ID NO: 25 y CDR3 según la SEQ ID NO: 27; y el dominio variable beta de TCR o el dominio variable delta de TCR comprende CDR1 según la SEQ ID NO: 31 y CDR3 según la SEQ ID NO: 33; o
 - (iv) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende CDR1 según la SEQ ID NO: 37 y CDR3 según la SEQ ID NO: 39; y el dominio variable beta de TCR o el dominio variable delta de TCR comprende CDR1 según la SEQ ID NO: 43 y CDR3 según la SEQ ID NO: 45; o
 - (v) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende CDR1 según la SEQ ID NO: 49 y CDR3 según la SEQ ID NO: 51; y el dominio variable beta de TCR o el dominio

variable delta de TCR comprende CDR1 según la SEQ ID NO: 55 y CDR3 según la SEQ ID NO: 57; o

(vi) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende CDR1 según la SEQ ID NO: 61 y CDR3 según la SEQ ID NO: 63; y el dominio variable beta de TCR o el dominio variable delta de TCR comprende CDR1 según la SEQ ID NO: 67 y CDR3 según la SEQ ID NO: 69; o

(vii) el dominio variable alfa de TCR o el dominio variable gamma de TCR comprende CDR1 según la SEQ ID NO: 73 y CDR3 según la SEQ ID NO: 75; y el dominio variable beta de TCR o el dominio variable delta de TCR comprende CDR1 según la SEQ ID NO: 79 y CDR3 según la SEQ ID NO: 81; y

en el que

- (1) dicho constructo reconocedor de antígeno recombinante comprende las respectivas secuencias de aminoácidos de los CDRs con no más de una o dos modificaciones de aminoácidos; y
- (2) dicha modificación de aminoácido es una sustitución con un aminoácido conservativo.

2. El constructo reconocedor de antígeno recombinante acorde con la reivindicación 1, caracterizado porque la sustitución con un aminoácido conservativo ocurre en la primera o la última posición de aminoácido del respectivo CDR.

3. El constructo reconocedor de antígeno recombinante acorde con la reivindicación 1 o 2, caracterizado porque comprende además una secuencia de CDR2 según la SEQ ID NO: 2, SEQ ID NO: 8, SEQ ID NO: 14, SEQ ID NO: 20, SEQ ID NO: 26, SEQ ID NO: 32, SEQ ID NO: 38, SEQ ID NO: 44, SEQ ID NO: 50, SEQ ID NO: 56, SEQ ID NO: 62, SEQ ID NO: 68, SEQ ID NO: 74, SEQ ID NO: 80, SEQ ID NO: 196, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, y SEQ ID NO: 204.

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4. El constructo reconocedor de antígeno recombinante acorde con cualquiera de las reivindicaciones 1 a 3, en el que las células T que expresan el constructo reconocedor de antígeno recombinante
 - (i) secretan al menos 200 pg/ml o más de interferón γ (IFN- γ) tras el cocultivo con células diana positivas para HLA-A*02 pulsadas con una concentración baja del antígeno PRAME; o
 - (ii) secretan al menos el doble de IFN- γ que el nivel de fondo no transducido de IFN- γ tras el cocultivo con células diana pulsadas con una concentración baja de un antígeno asociado a tumor (TAA).
5. El constructo reconocedor de antígeno recombinante acorde con la reivindicación 4, en el que la baja concentración del péptido PRAME es de alrededor de 10^{-11} mol/l, 10^{-10} mol/l, 10^{-9} mol/l, 10^{-8} mol/l, 10^{-7} mol/l, 10^{-6} mol/l o 10^{-5} mol/l.
6. El constructo reconocedor de antígeno recombinante acorde con cualquiera de las reivindicaciones 1 a 5, caracterizado porque comprende una región del dominio variable de la cadena alfa de TCR y una región del dominio variable de la cadena beta de TCR
 - (i) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 4; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 4 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 1, 2 y 3; y en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 10; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 10 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 7, 8 y 9; o

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- (ii) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 16; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 16 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 13, 14 y 15; y
en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 22; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 22 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 19, 20 y 21; o
- (iii) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 28; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 28 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 25, 26 y 27; y
en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 34; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 34 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 31, 32 y 33; o
- (iv) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 40; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 40 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de

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aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 37, 38 y 39; y

en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 46; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 46 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 43, 44 y 45; o

- (v) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 52; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 52 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 49, 50 y 51; y

en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 58; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 58 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 55, 56 y 57; o

- (vi) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 64; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 64 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 61, 62 y 63; y

en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 70; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 70 y en la que la región del dominio

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variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 67, 68 y 69; o

- (vii) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 76; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 76 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de la SEQ ID NOs: 73, 74 y 65; y

en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 82; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 82 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 79, 80 y 81; o

- (viii) en el que dicha región del dominio variable de la cadena alfa de TCR tiene la secuencia de aminoácidos de la SEQ ID NO: 130; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 130 y en la que la región del dominio variable de la cadena alfa de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de las SEQ ID NOs: 1, 2 y 3; y en el que dicha región del dominio variable de la cadena beta de TCR tiene la secuencia de aminoácidos de SEQ ID NO: 136; o una secuencia de aminoácidos que tiene al menos un 90% de identidad con la SEQ ID NO: 136 y en la que la región del dominio variable de la cadena beta de TCR comprende las secuencias de aminoácidos CDR1, CDR2 y CDR3 de la SEQ ID NOs: 7, 8 y 9.

7. El constructo reconocedor de antígeno recombinante acorde con cualquiera de las reivindicaciones 1 a 6, caracterizado porque el constructo reconocedor de antígeno es:

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- (i) un TCR que comprende una cadena variable alfa de TCR y una cadena variable beta de TCR o una cadena variable gamma de TCR y una cadena variable delta de TCR;
 - (ii) un TCR recombinante y comprende un dominio alfa de TCR y un dominio beta de TCR, que además comprende una región constante que tiene al menos 90%, 95%, 98%, 99% o 100% de identidad de secuencia con un aminoácido seleccionado entre las SEQ ID NO: 5 y 11; SEQ ID NO: 17 y 23; SEQ ID NO: 29 y 35; SEQ ID NO: 41 y 47; SEQ ID NO: 53 y 59; SEQ ID NO: 65 y 71; SEQ ID NO: 77 y 83; SEQ ID NO: 131 y 137;
 - (iii) un TCR recombinante monocatenario (mcTCR);
 - (iv) un TCR heterodimérico $\alpha\beta$ o $\gamma\delta$ recombinante, en el que un enlace disulfuro está presente entre los residuos de los dominios constantes.
8. Un TCR recombinante caracterizado porque comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 132 y una cadena beta de acuerdo con la SEQ ID NO: 138.
9. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 6 y una cadena beta de acuerdo con la SEQ ID NO: 12.
10. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 18 y una cadena beta de acuerdo con la SEQ ID NO: 24.
11. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 30 y una cadena beta de acuerdo con la SEQ ID NO: 36.
12. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 42 y una cadena beta de acuerdo con la SEQ ID NO: 48.

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13. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 54 y una cadena beta de acuerdo con la SEQ ID NO: 60.
14. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 66 y una cadena beta de acuerdo con la SEQ ID NO: 72.
15. Un TCR recombinante que comprende o consiste en una cadena alfa de acuerdo con la SEQ ID NO: 78 y una cadena beta de acuerdo con la SEQ ID NO: 84.
16. Un ácido nucleico recombinante o dos ácidos nucleicos recombinantes separados, caracterizados porque codifican un constructo reconocedor de antígeno acorde con cualquiera de las reivindicaciones 1 a 7 o un TCR recombinantes acorde con cualquiera de las reivindicaciones 8 a 15.
17. Un vector caracterizado porque comprende el ácido nucleico recombinante o los dos ácidos nucleicos recombinantes separados acordes con la reivindicación 16.
18. Una línea de células hospedadoras caracterizada porque comprende el constructo reconocedor de antígeno recombinante acorde con cualquiera de las reivindicaciones 1 a 7, o el TCR recombinante acorde con cualquiera de las reivindicaciones 7 a 14, o el ácido nucleico recombinante o los dos ácidos nucleicos recombinantes separados acordes con la reivindicación 16, o el vector recombinante acorde con la reivindicación 17.
19. Una línea de células hospedadoras acorde con la reivindicación 18, caracterizada porque la célula hospedadora es un linfocito, un linfocito T, una célula progenitora de linfocitos T, un linfocito T CD4 positivo o un linfocito T CD8 positivo.

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20. Una composición farmacéutica que comprende el constructo reconocedor de antígeno acorde con cualquiera de las reivindicaciones 1 a 7 o el TCR recombinante acorde con cualquiera de las reivindicaciones 8 a 15, o el ácido nucleico recombinante o los dos ácidos nucleicos recombinantes separados acordes con la reivindicación 16, o el vector recombinante acorde con la reivindicación 17, o la línea de células hospedadoras acorde con la reivindicación 18 o 19, y un vehículo, estabilizante y/o excipiente que sean farmacéuticamente aceptables.
21. Un método *in vitro* para detectar un péptido antigénico asociado a tumor (TAA, por sus siglas en inglés) PRAME, solo o en complejo con una molécula de MHC, en una muestra biológica, caracterizado porque el método comprende las etapas de:
 - a. poner en contacto la muestra con un constructo reconocedor de antígeno o un TCR recombinante que se une específicamente a dicho péptido PRAME, o al complejo péptido PRAME/MHC, y
 - b. detectar la unión entre dicho constructo reconocedor de antígeno y dicho péptido PRAME, o al complejo péptido PRAME/MHC.
22. Un método *in vitro* para detectar cáncer en una muestra biológica, caracterizado porque el método comprende las etapas pasos de:
 - a. poner en contacto la muestra biológica con un constructo reconocedor de antígeno recombinante acorde con cualquiera de las reivindicaciones 1 a 7 o el TCR recombinante acorde con cualquiera de las reivindicaciones 8 a 15; y
 - b. detectar la unión del constructo reconocedor de antígeno a la muestra biológica.
23. Un método para producir una línea celular que exprese un constructo reconocedor de antígeno específico de PRAME, caracterizado porque el método comprende:
 - a. proporcionar una célula hospedadora adecuada,

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- b. proporcionar un constructo genético que comprenda una secuencia codificante que codifica un constructo reconocedor de antígeno recombinante acorde con cualquiera de las reivindicaciones 1 a 17 o el TCR recombinante acorde con cualquiera de las reivindicaciones 8 a 15,
 - c. introducir en dicha célula hospedadora adecuada dicho constructo genético, y
 - d. expresar el citado constructo genético por la citada célula hospedadora adecuada.
- 24. El método acorde con la reivindicación 23, caracterizado porque además comprende
 - e. aislar y purificar el constructo reconocedor de antígeno recombinante o el TCR a partir de la célula hospedadora adecuada.
- 25. El método acorde con la reivindicación 24, caracterizado porque además comprende
 - f. reconstituir el constructo reconocedor de antígeno recombinante o el TCR recombinante en un linfocito T.

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Effect of CDR3 Sequences and Distal V gene Residues in Regulating TCR-MHC contacts and ligand specificity

Brian D. Stadinski*, Peter Trenh*, Brian Duke*, Priya G. Huseby*, Guoqi Li*, Lawrence J. Stern*[†], and Eric S. Huseby*

*Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01655, USA

[†]Department of Biochemistry & Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655, USA

Abstract

The mature T cell repertoire has the ability to orchestrate immunity to a wide range of potential pathogen challenges. This ability stems from thymic development producing individual T cell clonotypes that express T cell receptors (TCR) with unique patterns of antigen reactivity. The antigen specificity of TCRs is created from the combinatorial pairing of one of a set of germline encoded TCR variable (V) alpha and V beta gene segments with randomly created CDR3 sequences. How the amalgamation of germline encoded and randomly created TCR sequences results in antigen receptors with unique patterns of ligand specificity is not fully understood. Using cellular, biophysical and structural analyses, we show that CDR3 α residues can modulate the geometry in which TCRs bind pMHC, governing whether and how germline encoded TCR V α and V β residues interact with MHC. In addition, a CDR1 α residue that is positioned distal to the TCR-pMHC binding interface is shown to contribute to the peptide specificity of T cells. These findings demonstrate that the specificity of individual T cell clonotypes arises not only from TCR residues which create direct contacts with the pMHC, but also from a collection of indirect effects which modulate how TCR residues are used to bind pMHC.

Introduction

The ability of a T cell repertoire to target the array of potential pathogen challenges stems from individual T cell clonotypes having unique peptide and host-MHC reactivity patterns (1). Thymocytes are equipped with TCRs during development within the thymus. The sequence of individual TCR clonotypes is created from the pairing of one of a limited set of V α and V β gene segments with highly variable CDR3 α and CDR3 β sequences derived from V(D)J recombination (2). Following the expression of a complete TCR the process of T cell selection then determine the fate of developing thymocytes. Thymocytes expressing TCRs that have weak affinity for self-pMHC complexes, and thus are capable of recognizing

Correspondence should be addressed to E.S.H. (Eric.Huseby@umassmed.edu), Phone: (508) 856-2180, Fax: (508) 856-1094.

Online Supplementary material

There are two Supplementary Figures and two Tables associated with this manuscript.

ligands presented by host-MHC proteins, undergo positive selection and are exported as T cells to the mature repertoire (3–7). However, thymocytes are eliminated during development if they express overtly self-reactive TCRs or TCRs that are unable to bind self-peptide presented by host-MHC with even weak affinity (8, 9). Through the processes of thymic selection, a repertoire of mature T cells is created that express diverse TCR sequences, endowing the T cell repertoire with a vast breadth of antigen specificities (10, 11).

How the merging of germline encoded and randomly created sequences produces TCRs that recognize antigen presented on host-MHC molecules has not been fully defined. Prior to T cell selection, 5–20% of thymocytes expressing randomly generated TCRs react with cells presenting MHC molecules (12–14). These findings demonstrate that TCR-bearing pre-selection thymocytes are biased towards recognizing self-peptides presented by MHC ligands. Though this MHC-bias is undoubtedly helpful in creating a mature T cell repertoire that is reactive to cells presenting pMHC, the underlying mechanisms that drive pre-selection T cells to recognize self-pMHC, and how this repertoire is shaped into a foreign-antigen specific mature T cell repertoire remains controversial (15–18).

Through studying T cells isolated from mice with limited negative selection, we have provided evidence that T cells can have a range of pMHC cross-reactivity patterns (19–21). These and other T cell activation studies suggest that TCRs may have an intrinsic ability to bind pMHC, which is regulated by TCR V gene pairing or CDR3 sequences (12–14, 22–24). Structural studies have also been used to unravel how TCRs create specificity for pMHC complexes. Most TCRs bind MHC ligands within a semi-conserved diagonal orientation, which largely places the CDR3 loops atop the bound peptide and the germline encoded V gene CDR1 and CDR2 residues positioned over the MHC alpha helices (25). Examination of TCRs carrying similar TCR V genes engaged to similar MHC alleles have shown a more limited range of TCR-pMHC docking angles (26, 27). These structural observations have led to a hypothesis that particular germline encoded residues of TCR V genes have been evolutionarily selected to bind MHC in conserved ways and provide TCRs with a built-in specificity for MHC ligands (28, 29).

In contrast to the hypothesis that TCR V genes have evolved to specifically bind MHC in conserved ways, other experiments have suggested that T cell signaling may regulate the ligand specificity of TCRs (30–32). For example, a recent study of T cells that develop in mice devoid of MHC ligands argues that CD4 and CD8 T cell co-receptor signaling has a critical role in selecting T cells that can recognize MHC ligands, and in eliminating T cells that recognize non-MHC ligands (30). Although T cell signaling during positive selection can ensure that mature T cells express TCRs with specificity for ligands presented by host-MHC, these models do not explain why a high-frequency of pre-selection TCRs are preordained to recognize self-pMHC complexes.

Several lines of evidence suggest that CDR3 sequences may strongly contribute to regulating how TCRs engage pMHC ligands. The CDR3 residues often contribute a large portion of the TCR binding site, with some TCRs engaging pMHC ligands with minimal contribution of CDR1 and CDR2 residues (18, 33, 34). Furthermore, MHC proteins are the

most polymorphic genes in humans (35), and differential TCR V gene pairing creates TCRs that express different amino acid residues within CDR1 and CDR2 loops at common sites of contact with the MHC (2, 28). Perhaps as a way to accommodate this variability, a strict bifurcation of CDR1- and CDR2-MHC and CDR3-peptide residue contacts does not occur, with CDR3 residues contributing to MHC as well as peptide contacts and the V-gene encoded CDR1 and CDR2 contributing to both peptide and MHC contacts (18). Thus CDR3 sequences may play a primary role in creating both peptide and MHC specificity.

Studies exploring how TCRs generate specificity for pMHC ligands primarily have focused on identifying how direct contacts between the TCR and the pMHC contribute to the binding reaction. Here, we investigate a variety of indirect effects, whereby residues distal to the binding interface can have profound affects on MHC-TCR binding specificity. We demonstrate that CDR3 α residues can affect the overall geometry in which a TCR engages a pMHC ligand, thereby indirectly affecting how different TCR V α and TCR V β residues interact with pMHC residues. We further demonstrate that a CDR1 residue located outside of the TCR-pMHC binding interface can modulate the peptide specificity of T cells. Thus, in addition to providing direct pMHC contacts, TCR V gene and CDR3 residues create indirect effects that modulate TCR-peptide and TCR-MHC interactions.

Material and Methods

Mice and peptides

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H-2Ab1^{-/-} (MHC class II-deficient) mice were purchased from Taconic (Germantown, NY). Mice expressing the YAe62 TCR β chain (21) were maintained in a pathogen-free environment in accordance with institutional guidelines in the Animal Care Facility at the University of Massachusetts Medical School. The 3K peptide is FEAQKAKANKAVD, numbered P-2 to P11; FMRKA is FEAFMARAKAAVD; QKKLK is FEAQKAKALKAVD; QTKRG is FEAQTAKARGAVD; RCKST is FEARCAKASTAVD; SKKRP is FEASKAKARPAVD; YTRRT is FEAYTARARTAVD.

Viral infection, generation of T cell hybridomas and transfectants, and TCR cloning

Mice were infected with 5×10^6 pfu Vac:IA^b-3K. Responding CD4 T cells were isolated by positive selection using antibody affinity columns (Cederlane) and converted into hybridomas as previously described (36). 13.B1, 13.D5, 14.A6, 14.C6 and T cell hybridomas were constructed from MHC^{wt} YAe TCR β Tg mice (21). To clone TCR chains, RNA was isolated from T cell hybridomas, converted to cDNA, and PCR screened for rearranged TCR α chains using a set of degenerated PCR primers corresponding to all mouse V α gene families. Identified rearranged TCR V α genes were PCR cloned from the IA^b-3K-specific hybridomas and fused to the TCR C α region, using the following PCR primers:

V α 2 – GGGGGCTCGAGAGGAATGGACACGATCCTGACAGCA

C α – CTGGTACACAGCAGGTTCCGGATTCTGGATGT

The J809.B5, J809.G3, J809.H1, 2W1S 20.4, YAe62 and 75–55 hybridomas and cloned TCRs were previously published (19, 21). To generate T cell hybridoma transfectants,

TCR α or TCR β chains were cloned into MSCV based vectors with an IRES element containing resistance genes for puromycin or neomycin, respectively. Retroviral vectors were cotransfected with the pCLEco accessory plasmid into the Phoenix packaging line using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). The TCR α / β -deficient T cell hybridoma 5KC-73.8.20 was transduced with retroviruses expressing the TCR constructs using spinfection ($1000 \times g$, 2hrs) (37). T cell hybridoma transfectants were selected in 1mg/mL neomycin or 0.8 μ g/mL puromycin for 1 week and stained with anti-TCR β antibody (H57-597) to ensure equivalent levels of TCR expression.

T cell hybridoma activation assay

10^5 T cell hybridomas were incubated with 3×10^4 fibroblasts that express MHC, B7.1 and ICAM-1 for 24 hrs, and their supernatants were screened for IL-2 content using an HT-2 cell based bioassay (19). In some experiments, T cell hybridomas were incubated with MHC-expressing fibroblasts presenting titrating amounts of soluble peptides, or with 10^6 spleen cells expressing various MHC alleles. The IL-2 assays were performed three times and had a maximum value of IL-2 release of 1,000Units/mL. EC₅₀ values were calculated by fitting to a log(agonist) vs response variable slope (four parameter) curve (GraphPad, Prism).

Production of soluble and membrane bound MHC-peptide and TCR proteins

Soluble and biotinylated pMHC and TCRs that were used in the MHC display library experiments were produced using the baculovirus expression system and purified as previously described (21, 38). Multivalent fluorescent TCRs were prepared as previously described (20). Briefly, biotinylated anti-C α mAb ADO304 was complexed with AlexaFluor647-streptavidin. The complex was purified by size exclusion chromatography using a superdex200 column. Fluorescently labeled ADO304 was mixed with a concentration of soluble TCRs that saturated the antibody binding sites. The membrane bound IA^b-3K variant baculoviruses have been previously described (20, 21). Soluble TCRs used for SPR experiments and crystallography were expressed in *E. coli* and refolded as previously described (27, 39). The TCRs produced in *E. coli* were murine TCR V α and TCR V β domains fused to the human C α and C β domains carrying an engineered disulfide bond to improve folding yield and protein stability. TCR α and TCR β chains were expressed as inclusion bodies in the Rosetta2 (DE3) strain of *E. coli* (EMD Millipore, Darmstadt, Germany). Soluble TCRs were produced by refolding mixtures of denatured TCR α and TCR β chains isolated from inclusion bodies (21). The refolded TCRs were further purified by FPLC size exclusion and ion exchange chromatography.

SF9 cells expressing IA^b-3K variants with TCR multimers: Staining, analysis and peptide identification

Fluorescently labeled soluble TCR multimers were used to stain SF9 cells expressing IA^b-3K, IA^b-3K variants or IA^b-library peptides as previously described (20, 21). SF9 cells were infected with baculovirus expressing membrane bound IA^b-peptide complexes for 3 days using an MOI of 1–3. Following three days, 10^5 virus infected cells were washed and then stained with 20 μ g/ml TCR staining reagent and anti-IA^b (mAb 17–227) for 2 hours at 27°C. Stained cells were then washed and immediately analyzed by flow cytometry. Each

staining experiment was performed three independent times. To maximize the reproducibility of TCR multimer staining fluorescence, the experiments were repeated on subsequent days using the same prep of TCR multimers and the same flow cytometer and settings. For each TCR:IA^b-3K pair, a standard curve relating the MFI of TCR multimer staining to the SPR measured equilibrium affinity was created using the least squares method of curve fitting with a Boltzmann sigmoidal fit (GraphPad, Prism) (21). The standard curves were then used to generate $\Delta\Delta G$ data for the interaction of TCRs with residues of IA^b-3K. Staining of less than 5% of WT staining was considered non-binding and assigned the maximum $\Delta\Delta G$ value, as determined by SPR.

To identify novel IA^b-peptide complexes recognized by V α 2⁺ Y31A substituted TCRs, SF9 cells were infected with an IA^b-random peptide library at a multiplicity of infection of <1. Fluorescently labeled soluble TCR multimers carrying the V α 2⁺ Y31A substitution were used to stain the infected SF9 cells. SF9 cells that were positively stained with the TCR multimers were FACS cell sorted, and then incubated with additional SF9 cells to expand the virus secreted from the sorted cells. A second round of library enrichment was performed, followed by cloning the baculovirus by limiting dilution. SF9 cells infected with expanded viral clones were tested for I-A^b expression and TCR recognition. The peptide sequence encoded in baculovirus clones that were recognized by the TCRs of interest were determined by PCR sequencing.

Surface Plasmon Resonance

Equilibrium affinity and binding kinetics for TCRs binding to IA^b-3K and APLs were obtained by surface plasmon resonance on a BIAcore 3000 instruments (BIAcore AB, Uppsala, Sweden). For equilibrium affinity experiments, approximately 1,000–8,000 RU of soluble, biotinylated pMHC was captured on the surface of a biosensor flowcell by via streptavidin linker. Soluble TCR was injected for 120 sec. at various concentrations in the range between 0.39 - 100 μ M. All samples reached equilibrium binding within 60 sec. The complex was allowed to dissociate for 5 min between injections, followed by a 60sec pulse of 0.1% SDS to remove any TCR still bound to the flow cell. Data points were collected at 0.5 sec intervals. Raw data were corrected for the bulk signal from buffer and TCR by performing identical injections through a flow cell in which an irrelevant H2-D^b + peptide complex was immobilized. The data were analyzed with BIAcore BIAeval software. Scatchard analyses of the equilibrium data were used to determine the dissociation constant (K_d) for 14.C6, J809.B5, and amino acid substituted TCR and pMHC. No detectable binding was observed for some TCRs binding various IA^b-3K peptide variants. In these cases the minimum possible K_d was estimated as (>250 μ M) assuming a maximum possible equilibrium signal of 50RU with the highest concentration of TCR variants tested (100 μ M) and an Rmax of 5000 RU, i.e. the maximum predicted signal if complete binding of IA^b-3K to TCR occurred.

The kinetics of soluble, monomeric TCRs binding to immobilized pMHC complexes were analyzed by surface plasmon resonance using a BIAcore 3000 instrument and analyzed with BIAcore BIAeval 4.1 software (BIAcore AB, Uppsala, Sweden). Approximately 500 RU of soluble pMHC was captured on the surface of a biosensor flowcell by via streptavidin linker.

TCRs were injected at 20 μ L/min for 60sec through the biosensor flow cell at concentrations that ranged from 3–50 μ M. All samples reached equilibrium binding within 10 sec. The complex was allowed to dissociate for 60 sec between injections. As a control for bulk fluid phase refractive index the TCRs were also injected through a flow cell with an immobilized irrelevant H2-D^b + peptide complex. Data points were collected at 0.1 sec intervals and fit to a 1:1 Langmuir binding model to determine the dissociation rate (k_{off}) and $t_{1/2}$ of the TCR-pMHC complex. The kinetic data were used to determine the dissociation rate and the association rate (k_a) was calculated from the K_D and k_d ($k_a=k_d/K_D$).

Crystallization and Data Collection

TCR:MHC complexes were preformed by mixing the proteins at equimolar concentrations at 12mg/mL. Crystallization trials for all complexes were carried out using the hanging-drop vapor-diffusion method with a 1:1 protein to buffer ratio at room temperature. Single crystals were obtained for the J809.B5-IA^b3K complexes with 100mM sodium cacodylate, 100mM sodium citrate (pH 5.8) and 10% (w/v) PEG4000, conditions similar to our previously reported lower resolution J809.B5-IA^b3K structure. Crystals of the J809.B5 Y31A-IA^b3K complexes were formed in 100mM sodium cacodylate, 100mM sodium citrate (pH 5.4) and 8% (w/v) PEG4000, and crystals of the the 14.C6-IA^b3K complex formed in 100mM sodium cacodylate, 200mM sodium citrate (pH 5.5) 10% (w/v) PEG 4000 and 0.5% (w/v) n-octyl- β -D-glucoside. J809.B5-IA^b3K and 14.C6-IA^b3K crystals generally formed in 2–3 days, while the J809.B5 Y31A-IA^b3K crystals formed within a four weeks. For data collection, crystals were transferred to crystallization buffer containing 25% (w/v) glycerol and were flash-cooled by plunging into liquid nitrogen. X-ray diffraction data were collected from a single crystal at 100°K with 1.10 Å radiation at the National Synchrotron Source X25 undulator beamline at Brookhaven National Laboratory. Diffraction data were indexed, integrated, and scaled with HKL2000 (40). Unit cell parameters and data collection statistics are shown (Table II). A crystal structure for the 809.B5-IA^b3K complex (PDB 3RDT) has been previously reported for lower resolution data (21).

Structure determination

Initial phases were obtained by molecular replacement using Phaser (41) and the lower resolution J809.B5:IA^b3K structure 3RDT as the search model. One MHC and one TCR per asymmetric unit were found with the J809.B5 and the J809.B5Y31A complexes, while 2 TCRs and MHCs per asymmetric unit were found for the 14.C6 complex, consistent with the expected unit cell composition using a Matthews analysis. Initial NCS averaged maps of the 14.C6 complex were generated using RESOLVE. Refinements for 14.C6 constrained the same NCS operators in RESOLVE: pMHC α 1 β 1, MHC α 2 β 2, TCR-V $\alpha\beta$, TCR-C α , and TCR-C β but was relaxed in later cycles of refinement (42).

Density for all MHC and TCR domains and peptide was observed clearly in the composite omit maps using CNS (43), and used as a guide for model building. Cycles of automated rigid body, coordinate position, atomic displacement factor, and TLS refinement using Phenix (44) were alternated with manual model building using Coot (45). Analysis of the J809.B5Y31A-IA^b3K dataset using Xtriage (Phenix) revealed an estimated 10% twin fraction (Britton and H Test) with twin law -h, -k, l. The dataset used for refinement was altered

taking the twin operator into account using Detwin (CCP4i). Molecular replacement and model refinement followed similar steps as outlined for the J809.B5 and 14.C6 IA^b3K complexes. The final models include coordinates for TCR extracellular domains (alpha3-201, beta2-239), MHC IA^b extracellular domains (alpha 1–179, beta 6–191) and all 13 residues of the 3K peptide. No density was observed in any of the crystals for the linker (GGGGSLVPRGSGGGGS) tethering the peptide to the N-terminus of the beta subunit. Final refinement statistics are shown in (Table II).

Structure analysis

The two molecules within the 14.C6 asymmetric unit are highly similar (RMSD of 0.853 or 0.492 if the TCR C-domains are removed), especially within the binding interface including the CDR3 loops. The only observable difference between the two structures within the binding interface occurs at residue R50. In molecule one and molecule two, two different rotomers are found. In molecule one, the R50 side chain interacts with the MHCβ residue E69 (<4.0Å between the two side chains). In molecule two, the R50 side chain does not appear to interact with this side chain, the R50 side chain is >4.0 Å distance away from any E69 side chain atoms. However, alanine scanning experiments show that energetically, the MHCβ E69A substitution coordinates approximately 1.0 kcal/mol free energy (see Fig. S1). This indicates that the TCR R50-MHC E69A interactions are important for binding. Therefore, we used molecule one for generating the figures in the manuscript. Structural alignments were done using LSQMAN (46). The structure of the 14.C6 complex was aligned with the J809.B5 complex using coordinates of the MHC αβ helical domains and the 3K peptide. The angle of engagement was calculated from the p5 Cα atom to the center of mass (Pymol, Delano Scientific) for each TCR. Surface and interaction areas were calculated using the PISA server (47). Coordinate statistics, and atom-atom distances were evaluated using Phenix and NCONT from the CCP4i Suite (48).

Accession Numbers

Coordinates and structure factors for the J809.B5 TCR-IA^b-3K, J809.B5 Y31A TCR-IA^b-3K, and the 14.C6 TCR-IA^b-3K complexes will be available from the Protein DataBank under accession numbers 4P23, 4P46, and 4P5T (www.rcsb.org/).

Results

CDR3α residues can affect the geometry in which TCRs bind pMHC

To examine the influence of CDR3α sequences on TCR-MHC interactions, the IA^b-3K-reactive TCRs J809.B5, J809.H1 and 14.C6 were studied. These three TCRs carry the identical TCRβ and TCR Vα2.8 sequences, differing only in the CDR3α sequence (Table I). Alanine scanning mutagenesis experiments indicate that these TCRs differentially require several MHC residues for binding and for T cell expressing these receptors to undergo activation (Fig 1, Fig. S1 and Table S1, S2). These MHC residues include potential sites of TCRβ-MHC contact (21, 26, 27, 49). For example, the activation threshold (EC₅₀ value) of J809.B5 T cells responding to the 3K peptide is reduced 100-fold and greater than 1,000-fold when APCs express IA^b carrying alanine substitutions at the IA^bαQ57 and αQ61 residues, respectively. In contrast, the response of J809.H1 and 14.C6 T cells was minimally

changed when the IA^b MHC molecules express these alanine substitutions (Fig. 1A–F). Similarly, J809.B5, J809.H1 and 14.C6 T cells differentially require TCR CDR1 β and CDR2 β residues to be activated by APC presenting IA^b -3K (Fig. 1G–L). Importantly, differences in requirements of these TCR V β and MHC residues were not predicated on the TCRs having different equilibrium affinities for IA^b -3K, as the J809.B5 and 14.C6 TCRs bind IA^b -3K with similar affinity ($K_D = 6\mu M$ and $5\mu M$, respectively) (Fig. S1).

To gain insight into how CDR3 α sequences can influence CDR1 and CDR2 interactions with MHC, we determined the 3.2Å co-crystal structure of the 14.C6 TCR bound to IA^b -3K, and compared it to a new 2.3Å structure of the J809.B5 TCR bound to the same ligand. A lower-resolution J809.B5 - IA^b -3K structure has been reported previously (21). The J809.B5 TCR and the 14.C6 TCR engage IA^b -3K similarly but with a marked change in orientation, such that there is a 7.5° difference in the tilt of the TCR relative to pMHC (Fig. 2A, Table II and Fig. S2). This change in TCR-pMHC binding geometry is confirmed using an R-free analysis of the TCR V domains. The 14.C6: IA^b -3K complex has an R-free of 23% whereas if the 14.C6 V domain is forced into the J809.B5 docking geometry, the resulting complex would have an R-free of 42%. This change in tilt allows the 14.C6 TCR to form an increased number of contacts with the IA^b β -chain, as compared to the J809.B5 TCR (Fig. 2B, C). In addition, the total buried surface area (BSA) for the 14.C6 complex (approximately 1970 Å²) was greater than that for the J809.B5 complex (approximately 1540 Å²), primarily stemming from changes to the BSA of the CDR1 α and CDR2 α loops and the IA^b β -chain (Fig. 2D, E). In contrast to these changes, CDR3 β loop conformations and the placement of CDR3 β W94 and β F95 residues within these structures are similar (Fig. 2F).

CDR3 α sequences can modulate CDR1 and CDR2 interactions with pMHC

To evaluate how CDR3 α sequences can affect TCR interactions with pMHC, we first compared the 14.C6 and J809.B5 CDR3 α loop conformations when bound to IA^b -3K. In both structures the 14.C6 and J809.B5 CDR3 α loops make similar types of contacts with the peptide (Fig. 2G, H). Despite the 14.C6 CDR3 α loop being one amino acid longer, the J809.B5 CDR3 α loop extends 2.5Å further towards the peptide, relative to the 14.C6 CDR3 α loop (Fig. 2I). Because of the different CDR3 α loop conformations, a steric clash would occur between the J809.B5 CDR3 α loop and the peptide if this TCR bound IA^b -3K with the same geometry as that of the 14.C6 TCR. Thus, the differences in CDR3 α loop conformation likely underlie why the J809.B5 and 14.C6 TCRs bind IA^b -3K with different tilts. The most prominent differences in how the J809.B5 and 14.C6 TCR α chains engage IA^b -3K involve CDR1 α and CDR2 α residues. The 14.C6 TCR docking geometry allows its CDR1 α loop to make extensive contacts with the P2 and P5 positions of the 3K peptide, as well as the IA^b β chain residue β H81 (Fig. 2J). In contrast, the J809.B5 CDR1 α loop is positioned such that it makes only minimal contacts with the 3K peptide and no contacts with MHC (Fig. 2J). Likewise, the 14.C6 TCR CDR2 α residues interact with IA^b β chain, whereas these contacts are absent in the J809.B5: IA^b -3K structure due to the 4 Å rigid body shift (Fig. 2K).

Difference in the requirements of TCR V β and IA^b α residues for J809.B5 and 14.C6 T cells to undergo activation, shown in Figure 1 and Supplementary Figure S1, arise from less

dramatic structural changes. The main-chain of the J809.B5 and 14.C6 CDR2 β loops are shifted approximately 1 Å at the CDR2 β Y48 position. In addition, some rotamer differences are observed for the CDR2 β residues, β Y46 and β E54; while the IA^b α Q57 and α Q61 side chain rotamers are similarly orientated (Fig. 2L, M). Thus, the requirements of TCR and MHC side chains for T cell activation can be strongly affected by modest shifts in CDR loop placement and side chain rotamer differences.

CDR3 α sequences can regulate the specificity of a TCR V β -MHC interaction

Across different TCR V β gene families, the amino acid expressed at the CDR2 β 48 position is variable (50). Nevertheless, analyses of different TCR-pMHC co-crystal structures have shown that the CDR2 β 48 residue is often located in close proximity to MHC residues (28). Thus, different amino acid residues can be used at the TCR V β CDR2 β 48 position to bind MHC. Because of the variance in how the J809.B5 and 14.C6 TCR CDR2 β loops engage MHC residues, we hypothesized that CDR3 α sequences may influence which amino acids can be expressed at a TCR V β position to facilitate MHC binding.

To test the hypothesis that CDR3 α sequences can modulate the specificity of a TCR V β -MHC interaction, J809.B5, J809.H1 and 14.C6 T cell hybridomas were created that carried different amino acid substitutions at the CDR2 β 48 position. These T cell hybridomas were then tested for the ability to respond to APC presenting the 3K peptide. Consistent with studies of other V β 8.2⁺ T cells (22, 23, 27, 51), the J809.B5, J809.H1 and 14.C6 T cells lost or had a reduced response to 3K peptide when the CDR2 β 48 position carried an alanine (A). In addition, T cells carrying the conservative tyrosine to phenylalanine (F) substitution maintained complete 3K peptide reactivity (Figure 3). When non-conserved side chains were expressed at the CDR2 β 48 position, differing 3K peptide reactivity patterns were observed. J809.B5 T cells retained some response to 3K peptide when a histidine (H) was expressed, but not a tryptophan (W), methionine (M), cysteine (C), serine (S), glutamic acid (E), or lysine (K) (Fig. 3A). In contrast, J809.H1 T cells responded to 3K peptide when the CDR2 β 48 position carried a W, M and H, but not an C, S, E or K, while 14.C6 T cells maintained strong 3K responses when the CDR2 β 48 position carried a W, M, S and H (Figure 3B, C).

Similar studies of the self-reactive and IA^b-3K-reactive T cells, YAe62, 2W1S 20.4 and 75–55 were consistent with the hypothesis that the TCR CDR3 sequences and TCR $\alpha\beta$ chain pairing can modulate the specificity of a TCR V gene-MHC interaction. YAe62 T cells were activated by self-peptides when the CDR2 β 48 position carried most amino acid sequences, although not an alanine (Fig. 4A), while the 2W1S 20.4 and 75–55 T cells required a more limited set of amino acids to be expressed at the CDR2 β 48 position (Fig. 4B, C). In contrast to the anti-self responses, YAe62 T cells required a more restricted set of amino acids at the CDR2 β 48 position to respond to APCs presenting 3K peptide (Fig. 4D). The peptide sequence was directly observed to influence the specificity of this CDR2 β 48-MHC interaction, as YAe62 T cells maintained reactivity to a different peptide ligand, FMRKA, with most amino acids expressed at the CDR2 β 48 position (Fig. 4G). Peptide-dependent effects on the requirements of the V β 8.2 CDR2 β 48 position were also observed for the 2W1S 20.4 T cells, though less so for 75–55 T cells (Fig. 4E, F, H and I).

Collectively, these studies demonstrate that the side chain requirements of a TCR V β -MHC interaction can be distinct for different T cell clonotypes. Furthermore, the resulting change in TCR V gene-MHC specificity can be regulated by the CDR3 α sequence.

A CDR1 residue distal to the TCR-pMHC binding site can contribute to antigen recognition

With the assembly of complete TCRs, interactions occur between the TCR V α and V β domains and between different CDR loops (52, 53). The residues involved in these inter- and intra-chain interactions can be derived from conserved sequences of the TCR framework, or from variable sequences in the CDR loops. The inter- and intra-chain interactions that involve CDR loops can occur distal or proximal to the pMHC binding site. For example, when the J809.B5 TCR binds IA^b-3K, the CDR1 α Y31 forms contacts with the CDR3 β loop at a site that is distal to where the CDR3 loop contacts the peptide, while the CDR2 α R50 residues forms contacts with the CDR3 β loop at a site nearer to the pMHC interface (Fig. 5A, S2) (21). Having observed that CDR3 α residues can create indirect effects that influence the specificity of TCR V gene-MHC interactions, we wondered if TCR V α -V β interactions outside of the binding interface might also create indirect effects that influence ligand specificity.

To determine whether CDR residues that are located outside of the pMHC interface contribute to peptide specificity, J809.B5 T cell hybridomas were created that express alanine substitution at the CDR1 α Y31 residue (α Y31A), or the CDR2 α R50 residues (α R50A). 3K peptide titration experiments show that T cell hybridomas expressing the J809.B5 α Y31A TCR are 260-fold less reactive to 3K peptide presented by IA^b-transfected fibroblasts. In contrast, no change in 3K-reactivity was observed for J809.B5 T cells carrying the α R50A substitution (Fig. 5B). T cell hybridomas expressing the J809.B5 α Y31A TCR also become reactive to C57BL/6 splenocytes presenting IA^b + self-peptides, inferring that the CDR1 α Y31 side chain contributes to the peptide specificity of the J809.B5 TCR (Fig. 5C). Thus, the CDR1 α Y31 side chain of the J809.B5 TCR influences 3K reactivity, as noted by a change in sensitivity to titrating concentrations of 3K peptide, and peptide specificity, as evidenced by the acquisition of self-reactivity even though it is positioned outside of the TCR-pMHC binding site.

The J809.B5 CDR1 α Y31 side chain stabilizes the IA^b-3K binding conformation

To identify how the J809.B5 CDR1 α Y31 side chain contributes to 3K peptide reactivity, we first determined the 2.9Å crystal structure of the J809.B5 α Y31A TCR bound to IA^b-3K and compared it to the 2.3Å structure of the J809.B5 TCR bound to the same ligand. The overall structures of the J809.B5 and J809.B5 α Y31A TCRs bound to IA^b-3K are extremely similar, including the conformation of the CDR loops and the total BSA (approximately 1540 Å²) (Fig. 6, Table II and Fig. S2). To confirm the high degree of similarity between the two models, we calculated an isomorphous difference map from the two x-ray datasets to identify the relative differences in electron density. The largest difference in density was localized to the CDR1 α 31 position and can accommodate a phenol group that is lost with the tyrosine to alanine substitution (Figure 6B, C). Thus, the CDR1 α Y31 side chain is not required for the J809.B5 TCR to adopt its parental IA^b-3K binding conformation.

Despite the similar TCR loop conformations, the reduced 3K reactivity of T cell hybridomas expressing the J809.B5 α Y31A TCR indicated that the TCR-pMHC binding reaction had changed. Therefore, surface plasmon resonance experiments were performed to identify the contribution of the J809.B5 CDR1 α Y31 side chain to the binding of IA^b-3K. Whereas the J809.B5 TCR binds IA^b-3K with a $K_D = 6\mu\text{M}$, the J809.B5 α Y31A TCR binds IA^b-3K with a $K_D = 32\mu\text{M}$ (Fig. 6D, E). The reduced binding affinity for the J809.B5 α Y31A TCR is primarily the result of the interaction having a 7.5-fold faster off-rate (k_{off}) and thus a shorter half-life ($t_{1/2}$) (Fig. 6F). These data indicate that the CDR1 α Y31 side chain contributes to setting the T cell activation threshold by stabilizing the J809.B5 TCR - IA^b-3K binding reaction.

The V α 2 CDR1 α Y31 side chain regulates the peptide specificity of several TCRs

The self-reactivity of T cells carrying the J809.B5 CDR1 α Y31A substitution implies that the CDR1 α Y31 side chain contributes to peptide specificity. To formally test this, an IA^b-random peptide baculovirus library was probed to identify peptides that are recognized by J809.B5 α Y31A T cells (54). Using this method, an IA^b-peptide ligand, IA^b-FMRKA, was identified which binds the J809.B5 α Y31A TCR with a $K_D = 22\mu\text{M}$. In addition, J809.B5 α Y31A T cell hybridomas are activated by APC presenting the FMRKA peptide. The parent J809.B5 TCR, however, does not bind IA^b-FMRK ($K_D > 250\mu\text{M}$) and J809.B5 T cell hybridoma is not activated by the FMRKA peptide (Fig. 7A–C). The data formally demonstrate that the CDR1 α Y31 side chain contributes to the peptide specificity of the J809.B5 TCR.

Six additional V α 2⁺ IA^b-3K-reactive TCRs were studied to assess whether the CDR1 α Y31 residue contributes to cognate peptide recognition and peptide specificity of other receptors. We observed that T cell hybridomas expressing CDR1 α Y31A substituted TCRs could have an increase, decrease or unchanged reactivity to 3K peptide (Fig. 7D–I). In response to additional peptide ligands identified using an IA^b-random peptide baculovirus library, a variety of peptide reactivity patterns were observed (Fig. 7J–O). T cell hybridomas expressing the 14.A6 α Y31A TCR and 14.C6 α Y31A TCR have an increased response to the SKKRP and QKKLK peptide, respectively, even though the response to 3K peptide is unchanged. In contrast, T cell hybridomas expressing the J809.G3 α Y31A TCR have an equivalent response to the YTRRT peptide as compared to the parent J809.G3 TCR, yet lose all reactivity to APC presenting the 3K peptide. In addition, T cell hybridomas expressing the 13.B1 α Y31A TCR have an approximate 80-fold loss in 3K reactivity, even though they show an approximate 140-fold increase in reactivity to APC presenting SKKRP peptide as compared to T cell hybridomas expressing the parent TCR (see Table S2 for EC₅₀ values). Furthermore, T cell hybridomas expressing the 13.D5 α Y31A TCR acquire reactivity to splenocytes presenting self-peptides (not shown). Collectively, these data indicate that the V α 2 CDR1 α Y31 residue contributes to the peptide specificity of multiple MHC class II-restricted T cells.

Discussion

To provide protective immunity, T cell development creates a mature T cell repertoire that has the ability to respond to a broad range of foreign-antigens bound to host-MHC molecules. The T cell repertoire accomplishes this by being comprised of T cell clonotypes that express TCRs that have distinct binding requirements at potential sites of TCR-peptide and TCR-MHC interaction (1, 11). Much of the specificity of the TCR is derived from unique amino acid sequences present within the pMHC binding site. Data presented here demonstrate that the process of TCR combinatorial diversity and V(D)J rearrangements also create a collection of indirect effects that regulate the ligand specificity of TCRs. These effects allow CDR3 α residues to modulate the geometry in which TCRs engage pMHC ligands, governing whether and how individual TCR residues interact with MHC. In addition, a TCR CDR1 α residue that is located distal to the TCR-pMHC binding interface is shown to contribute to the peptide specificity of T cells.

Unlike immunoglobulin genes, TCRs do not undergo somatic hypermutation. Therefore, the only way TCRs can be created with reactivity for antigens presented by host-MHC alleles is through the combinatorial pairing of different TCR V gene segments and the creation of random CDR3 sequences through V(D)J recombination. The major force in creating TCRs with diverse antigen reactivity patterns occurs from V(D)J recombination, as this process determines both the length of the CDR3 loops and the identity of the amino acids present within the central portion of the pMHC binding site. Sequence differences amongst the V α and V β gene segments also contribute to creating TCRs with a diversity of ligand specificities. Differential TCR V gene pairing creates TCRs that express different amino acid sequences at potential sites of pMHC contact. In addition, CDR1 and CDR2 loops can adopt one of a set of unique canonical structures, which allow different TCR V α and V β pairings to create TCRs with a modest range of tertiary structures (55–57).

Several groups have analyzed structures of TCRs carrying the same TCR V α or TCR V β gene sequences or TCR α or TCR β subunit bound to the same MHC ligand. These studies have shown a spectrum of changes in how the identical TCR sequences can engage MHC. Studies of the 2C TCR and a high affinity variant M6 bound to H2-L^d, as well three V β 8.2⁺ TCRs recognizing IA^u, all show minimal differences in TCR V gene-MHC contacts (26, 58). Comparisons of the 2B4 and 226 TCRs bound to I-E^k show similar TCR V gene-MHC interactions, but with some modest shifts and side chain rotamer differences in the TCR V β 3 CDR loop interactions with the IE^k α -chain (59). Modest shifts in TCR β CDR loop placements and differences side chain rotamer usage are also observed for the binding of the YAe62, 2W1S20.4, J809.B5 and 14.C6 V β 8.2 chains with IA^b (21, 27). Although variations in the placements of these TCR β CDR loops atop MHC and side chain rotamer usage can appear subtle, these structural differences were associated with T cells having distinct requirements of TCR V gene residues and MHC residues for activation (see Figures 1, 3 and 4). Relatively subtle changes in MHC structure have also been shown to determine whether a T cell can recognize an allo-MHC ligand (60).

In contrast to the subtle variations outlined above, the comparisons of some TCRs carrying similar TCR V gene segments bound to the identical MHC can show more dramatic

difference in CDR loop placements. Studies of the TCR V β CDR1 and CDR2 loops of the A6 versus B7 TCRs bound HLA-A2 demonstrate that different TCR V gene residues can be involved in the binding reactions (61). Differences in CDR loop placements are also observed when human V α 24⁺ and V α 24⁻ TCRs bind CD1d MHC-like molecules presenting aGalCer (62). A comparison of the 2C TCR and the YAe62 TCR bound to H2-K^b ligands further show that the identical V β 8.2 CDR2 loops can engage different positions atop the MHC (15). For the J809.B5 and 14.C6 TCRs structures bound to IA^b-3K studied here, CDR3 α sequences are shown to regulate the extent in which the CDR1 α and CDR2 α loops contribute to the binding reaction. The range of TCR sequence-dependent changes in TCR V gene-MHC interactions are mirrored by observations that the MHC allele and the bound peptide or lipid sequence can influence how TCR CDR1 and CDR2 loops engage MHC residues (21, 62–69). These findings may explain how very modest changes in TCR sequence, including TCRs with only a single amino acid change in the CDR3 α loop, can distinguish TCRs expressed on CD4⁺ T cells recognizing MHC-II ligands from TCRs on CD8⁺ T cells recognizing MHC-I ligands (70). Collectively, the ability of CDR3 sequences and peptide sequences to modulate how TCR V gene residues engage MHC residues provides a mechanism whereby the TCR-MHC binding properties of the mature T cell repertoire can be tailored during T cell selection to recognize antigens displayed by host-MHC molecules (3, 71–73).

Germline encoded CDR sequence variations extend beyond the positions that most often contact the pMHC and into the core of the TCR V domain (2, 50). Due to the organization of CDR loops within TCRs, some of these variable CDR1 and CDR2 residues, as well as CDR3 residues, often abut other CDR loops (52, 53). By constructing TCRs through combinatorial diversity, TCRs express amino acid residues at potential sites of contact between the CDR loops based on the TCR V gene and CDR3 sequences used. Borg et. al. demonstrated that contacts between a CDR1 α and CDR3 α loop are required for the LC13 TCR to bind its cognate pMHC ligand (33), indicating that TCR intra-chain interactions can contribute to cognate ligand recognition. Through studying a set of MHC class II-restricted V α 2.3⁺, V α 2.8⁺ and V α 2.9⁺ TCRs, as well as re-analyzing MHC class II-restricted TCRs carrying these V α 2 chains bound to their ligand (21, 26, 27) (Figure S2), we observed that the CDR1 α Y31 residue often forms contacts with the CDR3 loops at a site outside of the TCR-pMHC interface. The CDR-CDR contacts made by the CDR1 α Y31 residue in each V α 2⁺ TCR structures are unique. Nevertheless, the alanine substitution of the CDR1 α Y31 residue modulated the ability of a set of V α 2.3⁺, V α 2.8⁺ and V α 2.9⁺ T cells to respond to their cognate ligand. Because in vivo CD4 T cell activation to viral epitopes are highly sensitive to the dwell time of TCR-pMHC interactions, the 100-fold changes in in vitro dose response can determine whether a CD4 T cell productively enters into an immune response (74). A result of the amino acid expressed at the CDR1 α 31 position being variable (50), is that this CDR position may induce a variety of indirect effects based on the particular V gene segment.

To identify how the V α 2 CDR1 α Y31 side chain contributes to the ability of TCRs to bind their cognate ligand, we compared the co-crystal structures of the J809.B5 TCR and J809.B5 α Y31A TCRs bound to IA^b-3K. The CDR1 α Y31A substitution does not change the conformation of the J809.B5 TCR in complex with IA^b-3K. Rather, by slowing the off-rate,

the CDR1 α Y31 side chain stabilizes the TCR conformation that binds IA^b-3K. Through analyzing multiple V α 2⁺ T cells, we observed that the CDR1 α Y31 side chain does not contribute uniform effects on either cognate peptide recognition or the ability of T cells to recognize alternate peptides. The CDR1 α Y31A substitution also caused two of the V α 2⁺ T cells to acquire self-reactivity, indicating that this CDR1 residue can contribute to the ability of T cells to distinguish foreign-antigens from self-antigens. These findings argue that combinatorial diversity allows germline encoded TCR V gene residues to contribute to the specificity of TCRs in multiple ways. CDR1 and CDR2 residues can make direct contacts with the pMHC ligand. In addition, CDR residues that do not contact the pMHC can affect the ability of TCRs to adopt or stabilize different pMHC binding conformations. The indirect effects created by TCR inter- and intra-chain interactions that regulate antigen specificity from positions outside of the binding interface can be seen as analogous to the allosteric regulation of protein function, that is controlled by V(D)J recombination and TCR V gene pairing (75). The common occurrence of interactions between CDR loops suggests that this is a general feature of TCRs, and that one of the functions of combinatorial diversity and T cell development is to equip mature T cells with TCRs that stabilize CDR loop conformations that specifically recognize antigens presented by host MHC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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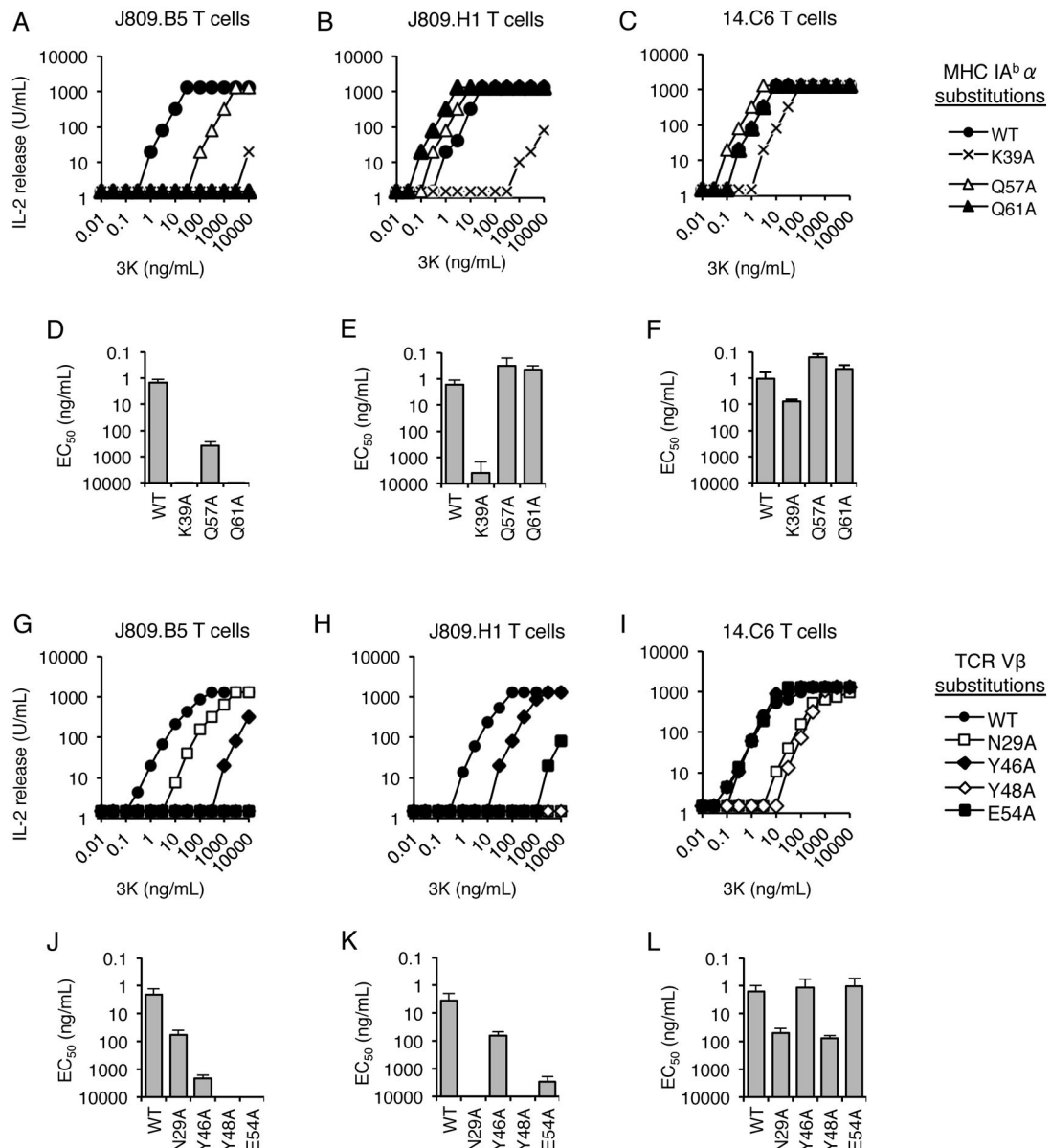
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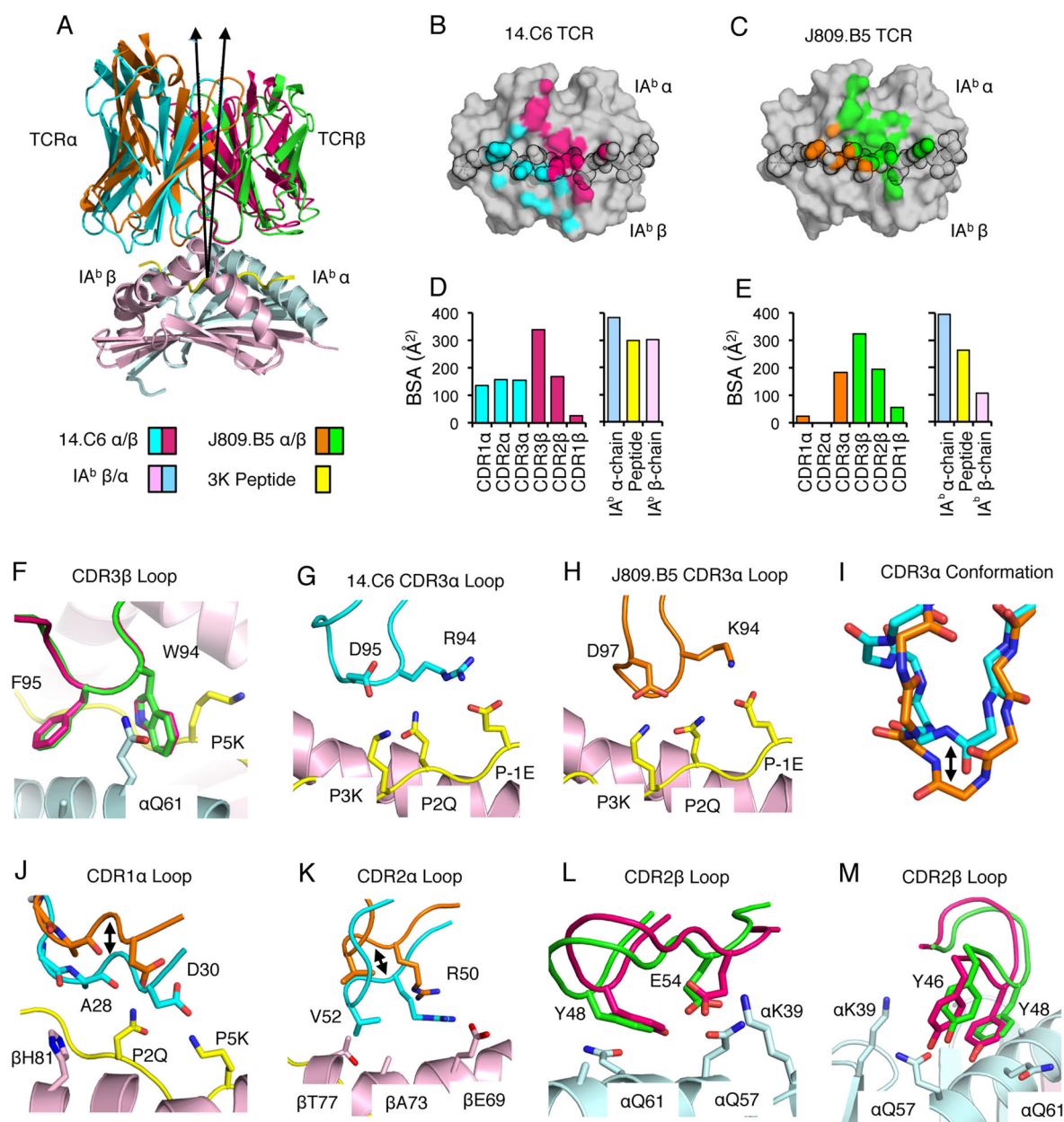
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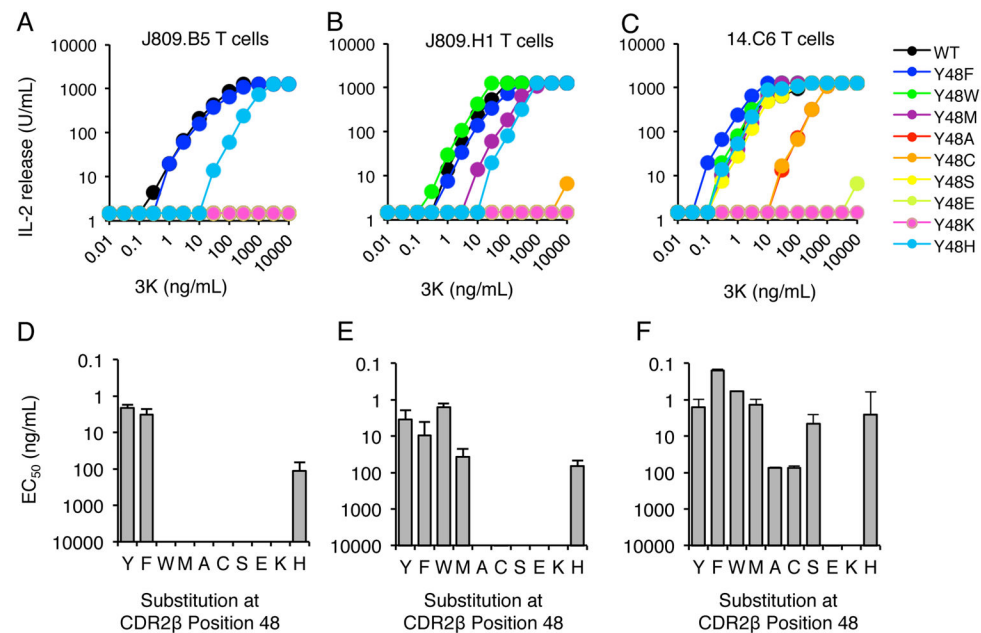
**FIGURE 1.**

CDR3 α Sequences Can Alter CDR1 β and CDR2 β Interactions with MHC. (A–F) The dose response and EC₅₀ values of the (A, D) J809.B5, (B, E) J809.H1 and (C, F) 14.C6 T cell hybridomas responding to titrating concentrations of 3K peptide presented by fibroblasts expressing IA^b, or IA^b in which the IA^bα side chains αK39, αQ57, or αQ61 have been substituted with alanine (labeled WT, K39A, Q57A or Q61A). EC₅₀ values are log₁₀-based half-maximal responses in ng/mL of 3K peptide. The dose response and EC₅₀ value of T cell hybridomas expressing either the (G, J) J809.B5, (H, K) J809.H1 and (I, L) 14.C6 TCR, or each TCR carrying alanine substitutions at the TCR residues CDR1 β N29, CDR2 β Y46, CDR2 β Y48 or CDR2 β E54, responding to titrating concentrations of 3K peptide presented by IA^b expressing fibroblasts. EC₅₀ values are log₁₀-based half-maximal responses in ng/mL of 3K peptide. Error bars represent the SEM of three independent experiments.

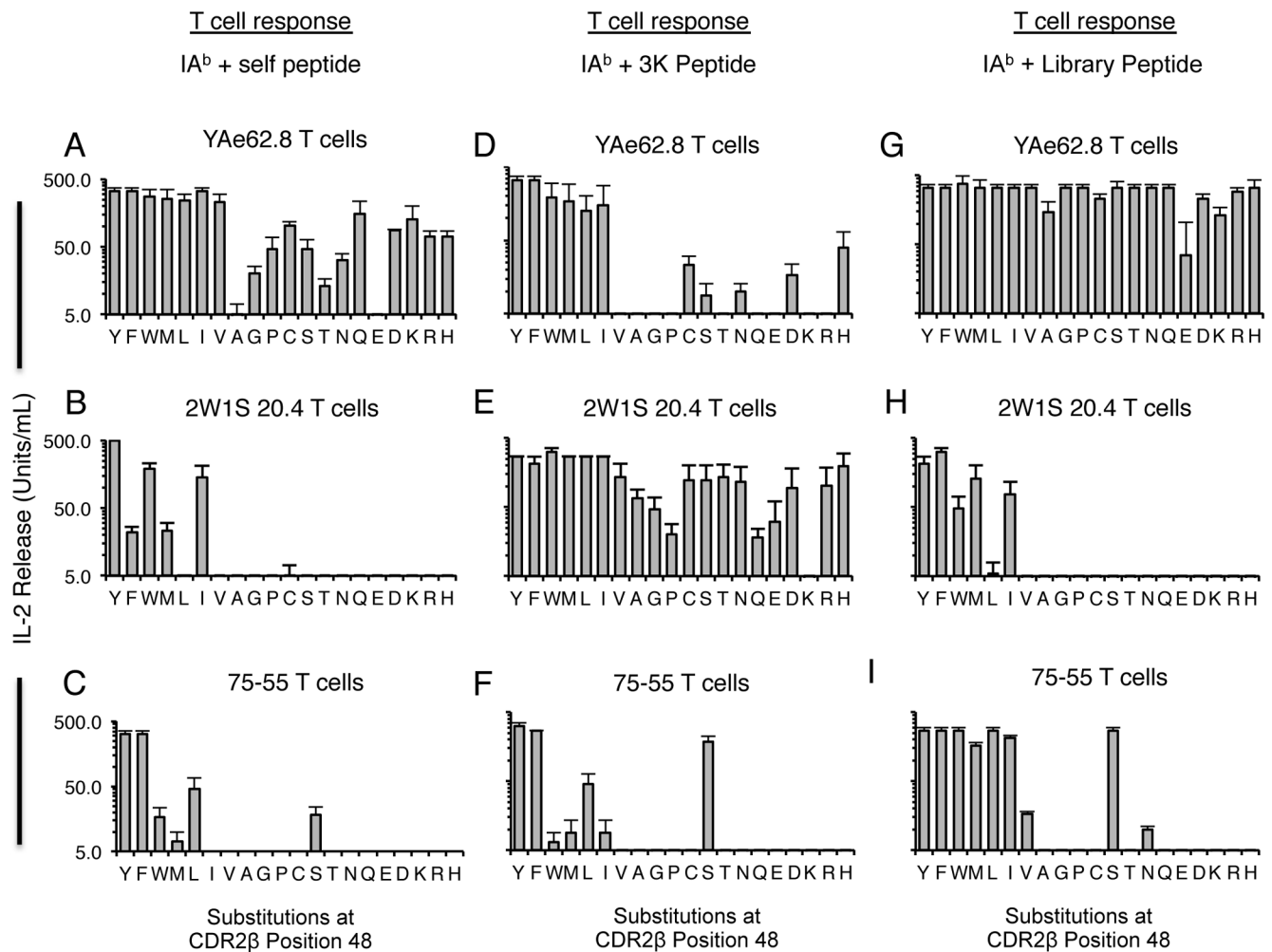
**FIGURE 2.**

CDR3α Sequence Can Alter the Positioning of CDR1 and CDR2 Loop Atop MHC. (A) Overlay of the 14.C6 and J809.B5 TCRs binding IA^b-3K. The 14.C6 TCR is colored red (TCRβ) and blue (TCRα); the J809.B5 TCR is colored green (TCRβ) and orange (TCRα). IA^b-3K is colored cyan (IA^bα chain), yellow (peptide), and magenta (IA^bβ chain). A 7.5% difference in the tilt of the TCR docking geometry atop IA^b-3K is observed based on a vector from the center of mass of each TCR to the α-carbon of the p5 peptide residue. (B) Projection of the 14.C6 TCR or (C) J809.B5 TCR binding onto IA^b-3K. 14.C6 TCRα contacts are colored blue, 14.C6 TCRβ contacts are colored red. The J809.B5 TCRα contacts are colored orange and the TCRβ contacts are colored green. The peptide residues are outlined in black. (D) The amount of buried surface area (BSA) contributed by the 14.C6

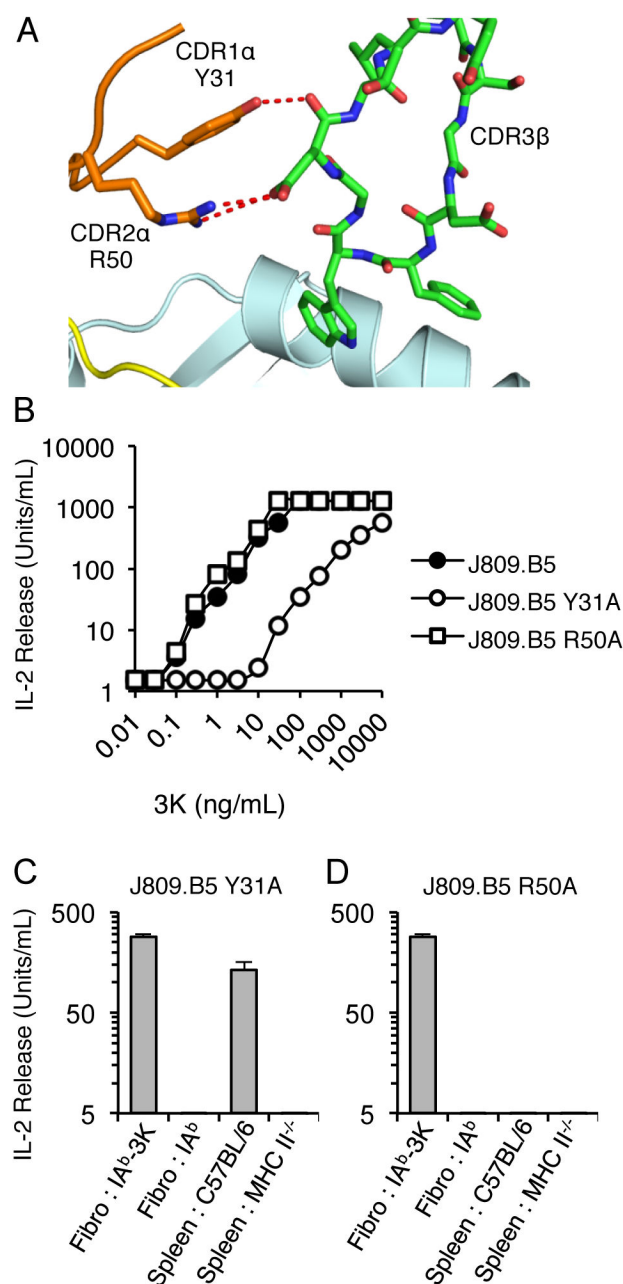
or (E) J809.B5 TCR α , TCR β , peptide or MHC chains for the binding reaction with IA^b-3K. (F) The 14.C6 CDR3 β loop (magenta) and the J809.B5 CDR3 β loop (green) are in a similar conformation and make similar contacts with IA^b-3K. (G) The 14.C6 CDR3 α loop residues R94 and D95 interact with the peptide residues, P-1E, P2Q and P3K. (H) The J809.B5 CDR3 α loop residues K94 and D97 interact with the peptide residues, P-1E, P2Q and P3K. (I) The 14.C6 CDR3 α loop (blue) and the J809.B5 CDR3 α loop (orange) are in a different conformation when bound to IA^b-3K. (J) 14.C6 CDR1 α residues A28 and D30 (blue) make extensive contacts with the P2Q and P5K residues of the peptide, and the IA^b β chain residue H81. The J809.B5 CDR1 α loops residues A28 and D30 (orange) are shifted approximately 4 Å, allowing only the A28 residue to make minimal contacts with peptide, and no contact with MHC. (K) The 14.C6 TCR CDR2 α residues R50 and V52 (blue) interact with IA^b β -chain residues β E69, β A73 and β T77, contacts that are not present in the J809.B5:IA^b-3K structure (orange) due to a 4 Å rigid body shift. (L, M) The main-chain of CDR2 β loop at the β Y48 position in the 14.C6 structure (green) is shifted approximately 1 Å as compared to the J809.B5 structure (magenta), and some rotamer differences are observed for the CDR2 β residues E54 and Y46. Figures were made with PyMol (76).

**FIGURE 3.**

CDR3α Sequences Can Regulate the Specificity of the Vβ8.2 CDR2 β48 Interaction with pMHC. IL-2 production of T cell hybridomas expressing the (A, D) J809.B5, (B, E) J809.H1 or (C, F) 14.C6 TCRs, or TCRs carrying amino acid substitutions at the TCR CDR2β residue Y48, in response to titrating concentrations of 3K peptide presented by IA^b expressing fibroblasts. IL-2 secretion is reported as Units/mL. (D-F) The EC₅₀ values for 3K dose responses shown in panels A-C and error bars represent the SEM of three independent experiments.

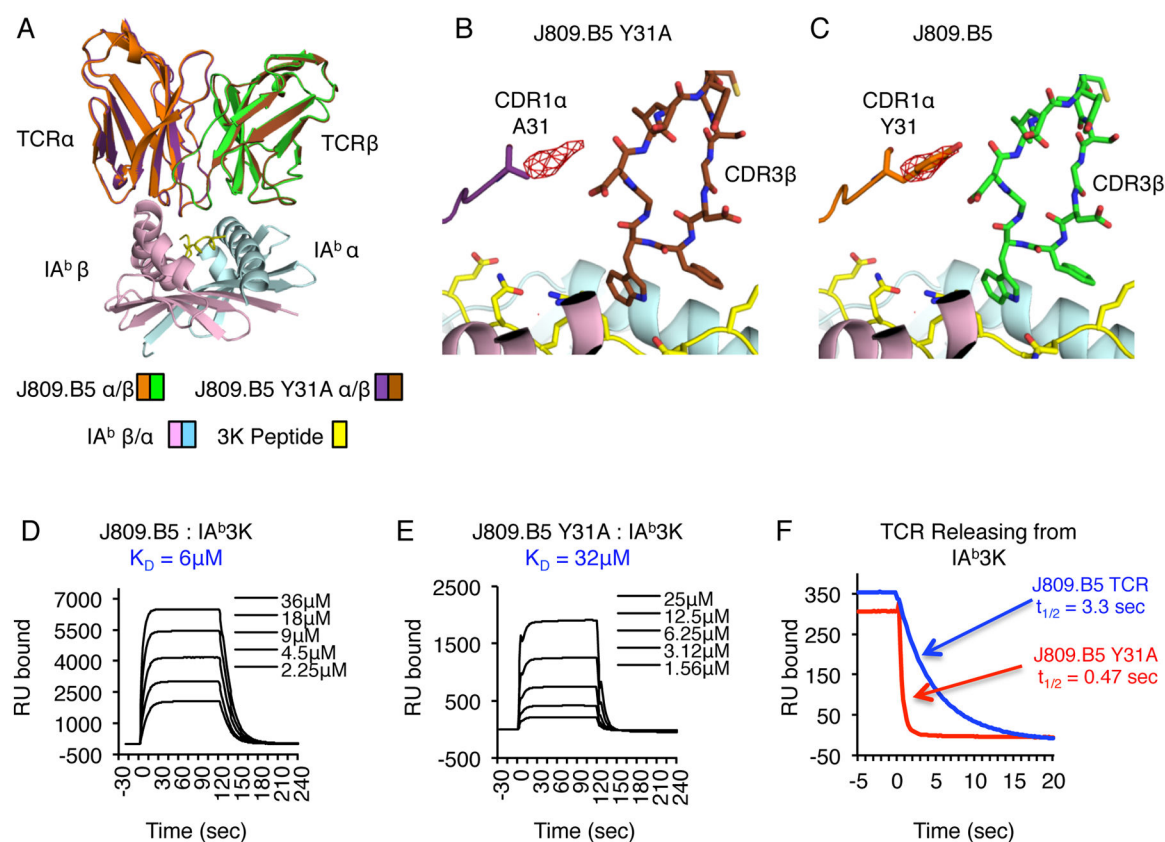
**FIGURE 4.**

Vβ8.2⁺ T cells can have distinct requirements at CDR2 β48 Position when Responding to Self-peptides, and Different Foreign-peptides. IL-2 production of T cell hybridomas expressing the (A, D, G) YAe62.8, (B, E, H) 2W1S 20.4 or the (C, F, I) 75-55 TCRs substituted with all 20 amino acid at the CDR2β 48 position following challenge with (A–C) H2^b expressing splenocytes, (D–F) IA^b-3K expressing fibroblasts, or (G–I) an alternate IA^b-foreign peptide ligand expressed on fibroblasts. For the YAe62 T cells, the alternate peptide is FMRKA. For the 2W1S 20.4 and 75-55 T cells, the alternate foreign peptide is RCKST. IL-2 secretion is reported as Units/mL. Error bars represent the SEM of three independent experiments.

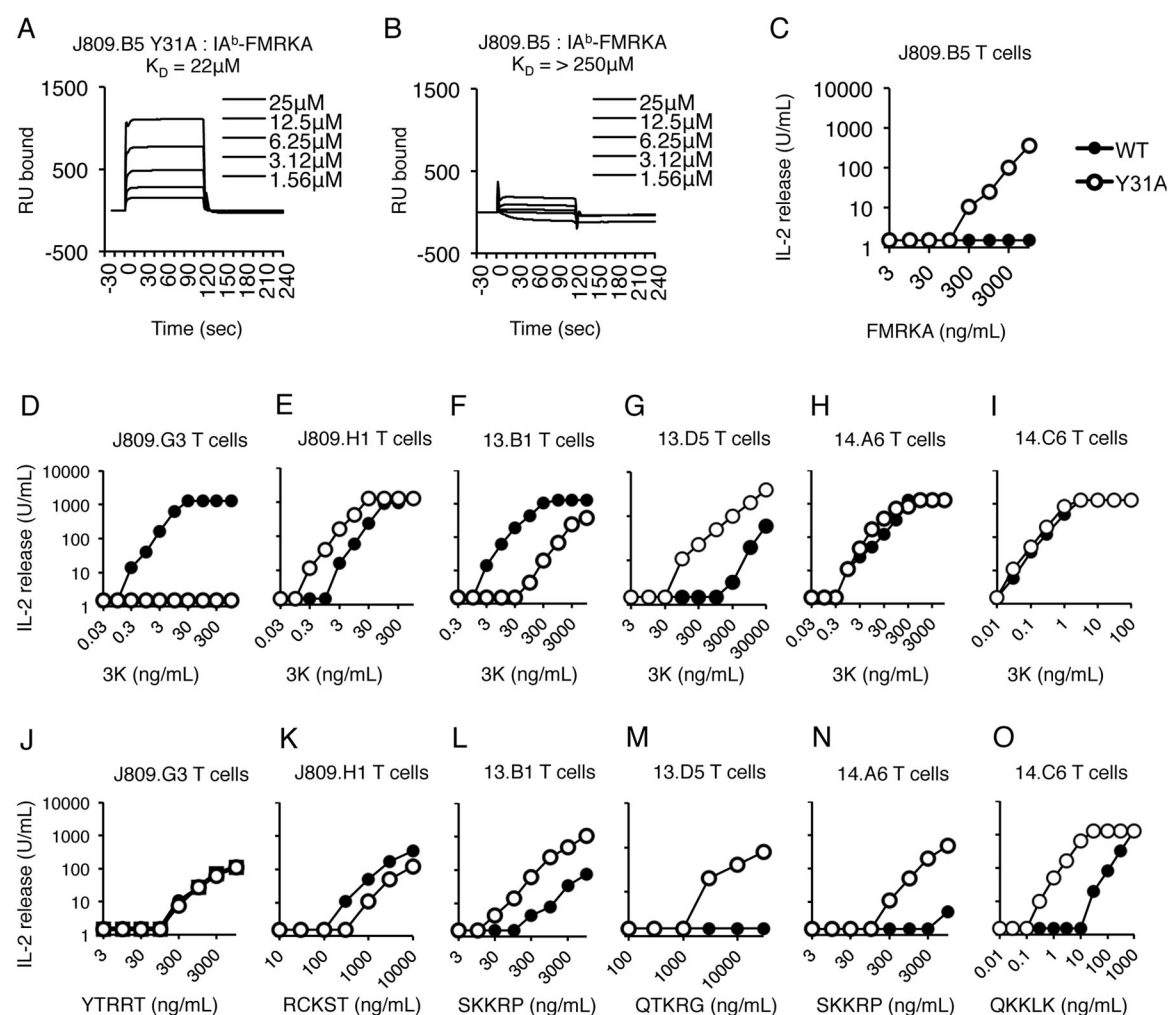
**FIGURE 5.**

The Vα2 CDR1 αY31 side chain contributes to the self-tolerance and IA^b-3K reactivity of J809.B5 T cells. **(A)** The Vα2.8 CDR1α and CDR2α loops interact with the CDR3β loops of the J809.B5 TCR when bound to IA^b-3K. Note the CDR1 αY31 is located 7 Å from the nearest peptide or MHC residue. **(B)** IL-2 production of T cell hybridomas expressing the J809.B5 TCR or J809.B5 TCRs carrying alanine substitutions at the CDR1 residue αY31, or the CDR2 residue αR50, in response to titrating concentrations of 3K peptide (ng/mL) presented by IA^b expressing fibroblasts; or **(C, D)** in response to fibroblasts expressing IA^b-3K, fibroblasts expressing IA^b, C57BL/6 splenocytes expressing H2^b MHC molecules

or splenocytes from C57BL/6 MHC II^{-/-} mice. IL-2 secretion is reported as Units/mL. Error bars represent the SEM of three independent experiments.

**FIGURE 6.**

The V α 2 CDR1 α Y31 side chain stabilizes the J809.B5 TCR binding reaction with IA^b-3K through indirect effects. (A) Overlay of J809.B5 and J809.B5 α Y31A TCRs binding IA^b-3K. The J809.B5 TCR β is colored green, TCR α is orange; the J809.B5 α Y31A TCR β is colored purple, TCR α is brown. IA^b-3K is colored cyan (IA^b α chain), yellow (peptide) and magenta (IA^b β chain). (B, C) Isomorphous difference map indicates that the only major structural changes (5.5 sigma peak) are located in immediate proximity of the V α 2 α Y31 residue, centered on the phenyl ring. Note the next highest peak occurs at a sigma of 3.8. (D) Soluble J809.B5 TCR or (E) J809.B5 α Y31A TCRs were analyzed for equilibrium affinity binding to immobilized IA^b-3K via SPR. Listed K_D are the average of three independent experiments, sensograms are representative analyses. (F) Dissociation of J809.B5 TCR (blue) and J809.B5 α Y31A TCR (red) from immobilized IA^b-3K at a flow rate of 20 $\mu\text{L}/\text{min}$ at 25 °C. Data for multiple TCR concentrations were collected at 0.1-s intervals and fit to a 1:1 Langmuir binding model to determine the dissociation rate (k_{off}) and $t_{1/2}$ of the TCR-pMHC complex. Dissociation plots are examples of three independent experiments.

**FIGURE 7.**

The $V\alpha 2$ CDR1 α Y31 side chain contributes to the antigen specificity of multiple $V\alpha 2^+$ T cells. (A) Soluble J809.B5 α Y31A or (B) J809.B5 TCR TCRs were analyzed for equilibrium affinity binding to immobilized IA^b -FMRKA via SPR. Listed K_D are the average of three independent experiments, sensograms are representative analyses. (C) IL-2 production of T cell hybridomas expressing the J809.B5 (filled squares) or J809.B5 α Y31A (open circles) TCR in response to titrating concentrations of FMRKA peptide (ng/mL) presented by IA^b expressing fibroblasts. IL-2 production of T cell hybridomas expressing the (D, J) J809.G3, (E, K) J809.H1, (F, L) 13.B1, (G, M) 13.D5, (H, N) 14.A6 or (I, O) 14.C6 TCRs (filled squares) or TCRs carrying alanine substitutions at the CDR1 residue α Y31 (open circles), in response to (D–I) titrating concentrations of 3K peptide presented by IA^b expressing fibroblasts, or (J–O) titrating concentrations of alternative peptide identified using baculovirus display library presented by IA^b expressing fibroblasts. IL-2 secretion is reported as Units/mL. Data are the average of three independent experiments. See (Table S2) for EC_{50} and SEM values.

Table I

Sequence of TCRs

TCR	V α	CDR1 α	CDR2 α	CDR3 α	V β	CDR1 β	CDR2 β	CDR3 β
J809.B5	2.8	ENSAFDY	AIRSVSDK	CAASKGADRLTFG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
J809.H1	2.8	ENSAFDY	AIRSVSDK	CAASKSSGQKL ^V FG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
14.C6	2.8	ENSAFDY	AIRSVSDK	CAASRDSGQKL ^V FG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
YAe62	4.12	STTGYPT	QVTTANNK	CAANS ^G TYQRFG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
2W1S20.4	2.9	EDSTFDY	AIRPVSNK	CASSDNRIFFG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDAWG ^E YEQYFG
75-55	4.11	STTWYPT	KVTTANNK	CALAGGSNAKL ^T FG	8.2	TNNHNN	YSYGAGSTEKGD	CASGEAWGGRYFG
13.B1	2.3	ENSAFDY	SILSVSDK	CAASDDNNRIFFG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
13.D5	2.3	ENSAFDY	SILSVSDK	CAASDGNRIFFG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
J809.G3	2.9	EDSTFDY	AIRPVSNK	CAASKDGGADRL ^T F	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG
14.A6	2.9	EDSTFDY	AIRPVSNK	CAASKGGQKL ^V FG	8.2	TNNHNN	YSYGAGSTEKGD	CASGDFWGD ^T LYFG

Table II

Data collection and refinement statistics (molecular replacement)

	14.C6:IA ^b 3K	J809.B5:IA ^b 3K	J809.B5Y31A:IA ^b 3K
Data collection			
Space group	P1 2 ₁ 1	C 1 2 1	C 1 2 1
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	66.007, 74.142, 259.289	239.121, 73.514, 65.73	242.123, 73.429, 65.68
<i>a</i> , <i>b</i> , <i>g</i> (°)	90.00, 92.00, 90.00	90.00, 90.58, 90.00	90, 90.34, 90
Resolution (Å)	50–3.25(3.31–3.25)	41–2.24(2.28–2.24)	42–2.85(2.95–2.85)
<i>R</i> _{merge}	15.5 (53)	8.2(46.2)	13.7 (53)
<i>I</i> / <i>σ</i>	12.9(3.8)	18.14(3.43)	11.6(3.63)
Completeness (%)	99.1(97.9)	97.48(92.10)	100.0(99.7)
Redundancy	6.6(3.8)	5.8(5.3)	6.7(6.6)
Refinement			
Resolution (Å)	47–3.26(3.38–3.26)	39–2.24(2.32–2.24)	42–2.85(2.95–2.85)
No. reflections	39080 (3819)	53984 (5046)	25527 (2335)
<i>R</i> _{work} / <i>R</i> _{free}	18.63/23.62	16.43/21.21	18.13/24.67
No. atoms		6736	
Protein	12366	6377	6273
Ligand/ion			
Water		359	
<i>B</i> -factors		44.2	
Protein	65.4	44.0	60.1
Ligand/ion			
Water		47.4	
R.m.s. deviations			
Bond lengths (Å)	0.003	0.007	0.008
Bond angles (°)	0.666	1.06	1.18
Ramachandran favored (%)	95.55	98	94
Allowed region	4.13	2.0	6.0
Outlier region	0.32	0.0	0.0

X-ray diffraction data were collected from single crystals

T-cell Receptor (TCR)-Peptide Specificity Overrides Affinity-enhancing TCR-Major Histocompatibility Complex Interactions*

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David K. Cole^{†1}, Kim M. Miles[‡], Florian Madura^{‡,2}, Christopher J. Holland[‡], Andrea J. A. Schauenburg[‡], Andrew J. Godkin[‡], Anna M. Bulek[‡], Anna Fuller[‡], Hephzibah J. E. Akpovwa[‡], Phillip G. Pymm^{‡,§}, Nathaniel Liddy[¶], Malkit Sami[¶], Yi Li[¶], Pierre J. Rizkallah[‡], Bent K. Jakobsen[¶], and Andrew K. Sewell^{‡,§}

From [†]Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, the [‡]Medical Research Council Human Immunology Unit, Weatherall Institute for Molecular Medicine, University of Oxford, Oxford OX3 9DS, and [¶]Immunocore Ltd., 57C Milton Park, Abingdon OX14 4RX, United Kingdom

Background: TCR recognition of bipartite ligands composed of self (MHC) and non-self (peptide) maintains T-cell specificity.

Results: Mutation of residues in the cognate peptide override TCR mutations that enhance MHC binding.

Conclusion: TCR-pMHC binding affinity requires specific TCR-peptide interactions.

Significance: Stabilization of TCR-pMHC engagement by TCR-peptide interactions maintains T-cell specificity and prevents recognition of self-pMHC in the periphery.

$\alpha\beta$ T-cell receptors (TCRs) engage antigens using complementarity-determining region (CDR) loops that are either germ line-encoded (CDR1 and CDR2) or somatically rearranged (CDR3). TCR ligands compose a presentation platform (major histocompatibility complex (MHC)) and a variable antigenic component consisting of a short “foreign” peptide. The sequence of events when the TCR engages its peptide-MHC (pMHC) ligand remains unclear. Some studies suggest that the germ line elements of the TCR engage the MHC prior to peptide scanning, but this order of binding is difficult to reconcile with some TCR-pMHC structures. Here, we used TCRs that exhibited enhanced pMHC binding as a result of mutations in either CDR2 and/or CDR3 loops, that bound to the MHC or peptide, respectively, to dissect the roles of these loops in stabilizing TCR-pMHC interactions. Our data show that TCR-peptide interactions play a strongly dominant energetic role providing a binding mode that is both temporally and energetically complementary with a system requiring positive selection by self-pMHC in the thymus and rapid recognition of non-self-pMHC in the periphery.

$\alpha\beta$ T-cells protect against pathogens and cellular malignancies by recognizing short peptide fragments bound to major histocompatibility complex (pMHC)⁴ molecules (1, 2). The rig-

ors of T-cell immunity require that TCRs bind to self-pMHC during thymic selection but discriminate between self and non-self-pMHC thereafter by the rapid scanning of huge numbers of potential antigens on the target cell surface (3, 4). Here, we examine how TCR interactions with the variable peptide component of the antigen are balanced against contacts with the MHC to enable T-cells to activate if sensing danger, while remaining tolerant to self.

X-ray crystallographic studies have shown that the $\alpha\beta$ TCR docks diagonally across the pMHC class I (pMHCI) peptide binding groove with the TCR α chain contacting the MHCI $\alpha 2$ helix and the TCR β chain contacting the MHCI $\alpha 1$ helix (5). A similar diagonal binding modality has been observed for TCR-pMHC class II interactions with the TCR α chain contacting the MHCII $\beta 1$ helix and the TCR β chain contacting the MHCI $\alpha 1$ helix. This fixed polarity is conserved in all published TCR-pMHCI structures to date, although the binding angle and contacts between individual TCR-pMHCI complexes can vary substantially (5). TCR recognition of pMHC is mediated through the TCR complementarity-determining region (CDR) loops. Although not the case with all TCR-pMHC pairs (6), the current dogma proposes that the germ line-encoded TCR CDR2 loops contact mainly the conserved helical region of the MHC surface (TCR-MHC self-interaction); the somatically rearranged, hypervariable CDR3 loops contact mainly the antigenic peptide (TCR-peptide non-self interaction), and the CDR1 loops lie in between, contacting both the peptide and the MHC (5).

This binding conformation has led to the suggestion that TCRs contact MHC in a genetically conserved manner (7–10). Indeed, a study by Wu *et al.* (11) investigating the role of the TCR CDR loops during pMHC binding concluded that an initial transition state is formed between the TCR and the MHC surface enabling the TCR to scan the antigenic peptide (two-step binding) (12). This two-step binding model is considered to represent an important mechanism of allowing T-cells to sample a diverse array of pMHC antigens (3, 4, 13). In support

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✂ Author's Choice—Final version full access.

The atomic coordinates and structure factors (code 4MNQ) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

¹ Wellcome Trust Research Career Development Fellow supported by Grant WT095767. To whom correspondence may be addressed. Tel.: 442920687006; E-mail: coledk@cf.ac.uk.

² Supported by a Tenovus Ph.D. studentship.

³ To whom correspondence may be addressed. Tel.: 442920687055; E-mail: sewellak@cf.ac.uk.

⁴ The abbreviations used are: pMHC, peptide-major histocompatibility complex; SPR, surface plasmon resonance; TCR, T-cell receptor; CDR loop, complementarity-determining region loop; PDB, Protein Data Bank; MHCI, pMHC class I.

of this notion, combined studies have suggested the existence of so-called “interaction codons” that enable the TCR to contact the MHC surface in a conserved manner (7–10). Furthermore, structural comparison of different TCRs with genetically identical CDR2 β loops, in complex with the same pMHC, lends support to the idea that some TCRs may use genetically fixed pairwise interactions to bind to the MHC surface (9, 14). In combination, these data predict that interactions between the TCR and MHC stabilize the initial “encounter complex.” However, there is also a body of evidence that contradicts this model of TCR binding (15–21). Thus, there is still much controversy over this central question concerning the nature of T-cell antigen recognition.

The binding affinity of natural TCR-pMHC interactions ($K_D \sim 0.1$ – $500 \mu\text{M}$) (22, 23) is near the limits of detection using current biophysical techniques. This restricts the scope for investigating TCR-pMHC interactions by mutating important contacts, because altering this weak interaction often results in the loss of any detectable binding using surface plasmon resonance (SPR). To examine the roles of the TCR CDR loops when binding to pMHC, we designed a range of enhanced affinity soluble TCRs with mutations in either their CDR2 and/or CDR3 loops. These unique enhanced affinity reagents enabled investigation of the effects of altering specific interactions between the TCR and MHC or TCR and peptide to examine how individual components of the interface between the TCR and pMHC contribute to T-cell antigen recognition. These data shed new light on how T-cells might be selected in the thymus to maintain tolerance to self, the mechanism of T-cell cross-reactivity, and the nature of T-cell antigen recognition.

EXPERIMENTAL PROCEDURES

Generation of Expression Plasmids—A number of constructs were prepared that contained wild type and high affinity TCRs to the HLA A*0201-restricted antigens, Melan-A/MART-1(26–35) ELAGIGILTV (24) and hTERT(540–548) ILAKFLHWL (25). The HLA A*0201-ELAGIGILTV- and HLA A*0201-ILAKFLHWL-specific wild type TCRs (MEL5 and ILA1 TCRs, respectively), the high affinity TCR α and β chains, HLA A*0201 heavy chain, and $\beta_2\text{m}$ were generated by PCR mutagenesis (Stratagene) and PCR cloning. All sequences were confirmed by automated DNA sequencing (Lark Technologies). The high affinity HLA A*0201-ELAGIGILTV and HLA A*0201-ILAKFLHWL TCRs were produced using a phage display library as reported previously (26). All of the TCR sequences were constructed implementing a disulfide-linked construct to produce the soluble domains (variable and constant) for both the α (residues 1–207) and β chains (residues 1–247) (27, 28). The HLA A2 heavy chain (residues 1–248) ($\alpha 1$, $\alpha 2$, and $\alpha 3$ domains), tagged with a biotinylation sequence, and $\beta_2\text{m}$ (residues 1–100) were also cloned and used to make the pMHC complexes. The TCR α and β chains, the HLA A2 α chain and $\beta_2\text{m}$ sequences were inserted into separate pGMT7 expression plasmids under the control of the T7 promoter (27).

Protein Expression, Refolding, and Purification—Competent Rosetta DE3 *Escherichia coli* cells were used to produce the TCR α and β chains, HLA A*0201 heavy chain and $\beta_2\text{m}$ in the form of inclusion bodies using 0.5 mM isopropyl 1-thio- β -D-

galactopyranoside to induce expression as described previously (27, 29, 30).

pMHC Biotinylation—Biotinylated pMHC was prepared as described previously (31).

SPR Equilibrium Analysis—The binding analysis was performed using a BIAcore T100™ equipped with a CM5 sensor chip as reported previously (32).

SPR Kinetic Analysis—Experiments were carried out to determine the K_{on} and K_{off} values for the TCRs at 25 °C as reported previously (33). Briefly, for all kinetic experiments, ~ 300 response units of pMHC were coupled to the CM5 sensor chip surface. The TCR was then injected at concentrations ranging from 10 times above and 10 times below the known K_D value of the interaction at 45 $\mu\text{l/min}$. The K_{on} and K_{off} values were calculated assuming 1:1 Langmuir binding ($AB = B \cdot AB_{\text{max}} / (K_D + B)$), and the data were analyzed using a global fit algorithm (BIAevaluation™ 3.1).

SPR Kinetic Titration Analysis—To stringently examine the binding of the TCRs at a greater range of concentrations, we used a new method for analyzing the kinetic parameters of high affinity interactions with long off rates (34). Each TCR was analyzed at five concentrations that represented the greatest range we could accurately achieve around the K_D value of each interaction. During the analysis, ~ 300 response units of pMHC were immobilized onto the CM5 sensor chip surface. Each concentration of TCR was injected at a high flow rate of 45 $\mu\text{l/min}$ for a 240-s association period and a 120-s dissociation period. The final and highest concentration had a longer dissociation period of 600 s. A fast flow rate and a low amount of immobilized pMHC were used to limit association and dissociation mass transfer limitations as recommended by the experts at BIAcore™. The K_{on} and K_{off} values were calculated assuming 1:1 Langmuir binding ($AB = B \cdot AB_{\text{max}} / (K_D + B)$), and the data were analyzed using the kinetic titration analysis algorithm (BIAevaluation™ Version 3.1) (35).

Crystallization and X-ray Data Collection— $\alpha 1\beta 1$ -A2-ILA crystals were grown in 20 mM Tris, pH 7.5, 20% PEG 4000, and 10 mM NaCl. All crystals were soaked in 30% ethylene glycol before cryo-cooling. Data were collected at 100 K at the Diamond Light Source, UK. Reflection intensities were estimated with the XIA2 package (36), and the data were scaled, reduced, and analyzed with SCALA and the CCP4 package (37). Structures were solved with molecular replacement using PHASER (38). Sequences were adjusted with COOT (39), and the models were refined with REFMAC5. Graphical representations were prepared with PyMOL (40). The reflection data and final model coordinates were deposited with the PDB database ($\alpha 1\beta 1$ -A2-ILA, PDB 4MNQ).

RESULTS

Design of a Panel of High Affinity TCRs Specific for Two Different HLA A*0201-restricted Peptides—Investigating the individual roles of the TCR CDR loops when binding to pMHC has been difficult because the weak binding affinities of natural TCR-pMHC interactions (0.1– $500 \mu\text{M}$) (22, 23) are close to the limits of detection by SPR. To overcome this problem, we designed a range of soluble TCRs with up to 18,500-fold enhancement in affinity for cognate antigen using CDR loop

TCR-Peptide Specificity Governs Antigen Recognition

mutations selected by phage display (Table 1) (26). We first measured the binding affinity and kinetics of wild type and enhanced affinity TCRs specific for either the HLA A*0201-restricted peptide antigens, Melan-A/MART-1(26–35) (ELAGIGILTV), or hTERT(540–548) (ILAKFLHWL). The HLA A*0201-ELAGIGILTV-specific TCRs with mutated CDR2 or CDR2 and CDR3 loops bound with considerably stronger affinities (K_D s between 12 and 897 times greater) than the parent wild type TCR (MEL5) (Fig. 1 and Table 2). The HLA A*0201-ILAKFLHWL specific TCRs with mutated CDR2 loops

bound to cognate antigen with a substantially stronger affinity (K_D values between 1423 and 4066 times greater) compared with the parent wild type TCR (ILA1) (Fig. 2 and Table 3). Similarly, when the mutated CDR2 and CDR3 loop mutations were combined, we observed a further increase in binding affinity (K_D values up to 18,500 times greater than ILA1 TCR), indicating that the mutations could be used cooperatively (Fig. 3 and Table 3). In agreement with our previous findings (26, 34, 41–44), enhanced TCR affinity was due to small increases in the on-rate, and vastly extended off-rates (Tables 2 and 3, Figs. 1–3). The stronger affinities of the high affinity TCRs enabled the modulation of individual components of the TCR-peptide interaction through peptide mutations while maintaining enough residual binding to detect using SPR in later experiments.

High Affinity CDR2 Loop Mutated TCRs Do Not Bind to “Null” Peptides—Our recent structure of a high affinity variant of the MEL5 TCR (34) demonstrated that the mutated CDR2 α region of the high affinity MEL5-derived TCRs used in this study was in an identical position to the wild type MEL5 TCR (24), distal from the peptide (Fig. 4, A and B). Thus, we concluded that mutations at residues in the peptide were very unlikely to directly affect the high affinity interactions between the MEL5 derived high affinity TCRs and the MHC surface. We reasoned that, because the general dogma of TCR engagement postulates that TCR-MHC interactions bind before TCR-peptide sampling, the high affinity interaction between the MEL5-derived TCRs and the MHC surface should retain some measurable ability to bind to the surface of HLA A2 irrespectively of the bound peptide, because the interaction between the TCR and the peptide should only account for a small proportion of the overall binding energy (δG). To investigate the role of pep-

TABLE 1
Sequence comparison of high affinity TCRs

TCR	Mutation	CDR2 β_{56-60}	CDR3 $\beta_{104-119}$
MEL5	wildtype	SVGIG	CAWSETGLGTGELFFG
MEL5 β 1	CDR2	YGPF G	
MEL5 β 2	CDR2	WGPF G	
MEL5 β 3	CDR2	FGPY G	
MEL5 β 4	CDR2	YGPY G	
MEL5 β 5	CDR2&3	YGPF G	CAWSETGLG MGWQ FG
MEL5 β 6	CDR2&3	YGPF G	CAWSETGL NLGGW FFG
MEL5 β 7	CDR2&3	WGPF G	CAWSETGLG MGWQ FG
MEL5 β 8	CDR2&3	WGPF G	CAWSETGL NLGGW FFG
TCR	Mutation	CDR2 β_{49-54}	CDR3 $\alpha_{104-119}$
ILA1	wildtype	SVGAGI	CAVDSATSGTYKYIFG
ILA1 β 1	CDR2	SIHPEY	
ILA1 β 2	CDR2	SIWEFE	
ILA1 α 1	CDR3	SVGAGI	CAVDSAT ALPYGY IFG
ILA1 α 1 β 1	CDR2&3	SIHPEY	CAVDSAT ALPYGY IFG
ILA1 α 1 β 2	CDR2&3	SIWEFE	CAVDSAT ALPYGY IFG

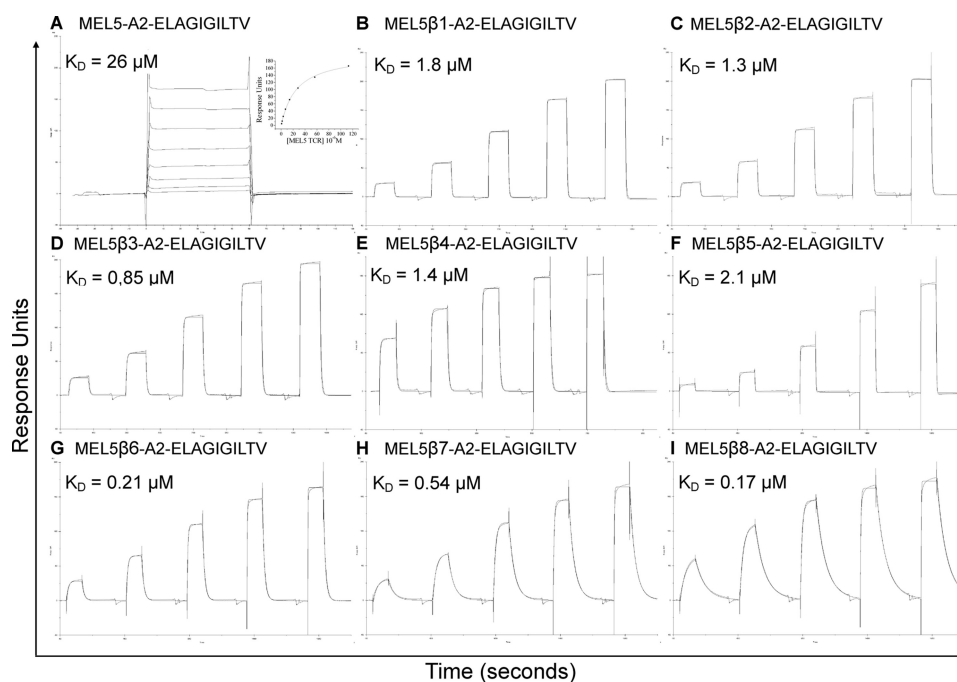


FIGURE 1. Affinity and kinetic analysis of wild type and high affinity MEL5-derived TCRs. A–I, these data were produced using a Biacore T100™ and were then analyzed using equilibrium analysis, kinetic global fit analysis, and kinetic titration analysis. The raw data and the fits are shown in each panel. These data illustrate the improved binding capabilities of the high affinity mutant HLA A2-ELAGIGILTV-specific TCRs compared with the MEL5 TCR. None of the HLA A2-ELAGIGILTV-specific TCRs bound to the HLA A2-ELAGIGILTV with alanine or glycine substitutions.

TABLE 2

Kinetic and affinity analysis of high affinity HLA-A*0201-ELAGIGILTV-specific TCR binding to alanine- and glycine-substituted peptides

TCR	Mutation	$K_{on}M^{-1}s^{-1}$	$K_{off}s^{-1}$	K_D	Half-life Min	ΔG kcal/mol ⁻¹
HLA A*0201-ELAGIGILTV						
MEL5	wildtype	N/M	N/M	26±2.1 μ M	N/M	-5.83
MEL5 β 1	CDR2	1.4×10 ⁵	2.6×10 ⁻¹	1.8±0.4 μ M	0.04	-7.31
MEL5 β 2	CDR2	2×10 ⁵	2.6×10 ⁻¹	1.3±0.3 μ M	0.04	-7.49
MEL5 β 3	CDR2	1.8×10 ⁵	1.6×10 ⁻¹	0.85±0.1 μ M	0.07	-7.72
MEL5 β 4	CDR2	1.6×10 ⁵	2.3×10 ⁻¹	1.4±0.2 μ M	0.05	-7.45
MEL5 β 5	CDR2&3	2.5×10 ⁵	5.2×10 ⁻¹	2.1±0.5 μ M	0.02	-7.22
MEL5 β 6	CDR2&3	2.9×10 ⁵	6.1×10 ⁻²	0.21±0.33 μ M	0.19	-8.49
MEL5 β 7	CDR2&3	4.6×10 ⁴	2.5×10 ⁻²	0.54±0.1 μ M	0.46	-7.97
MEL5 β 8	CDR2&3	8×10 ⁴	1.4×10 ⁻²	0.17±0.1 μ M	0.82	-8.61
HLA A*0201-ALAAAAAAV						
HLA A*0201-GLGGGGGGV						
HLA A*0201-ELAGIGILTV						
HLA A*0201-ELAGIGILTV						
HLA A*0201-ELAGIGILTV						
HLA A*0201-ELAGIGILTV						
HLA A*0201-ELAGIGILTV						
HLA A*0201-ELAGIGILTV						
HLA A*0201-ELAGIGILTV						
MEL5	wildtype	NO BINDING				
MEL5 β 1	CDR2	NO BINDING				
MEL5 β 2	CDR2	NO BINDING				
MEL5 β 3	CDR2	NO BINDING				
MEL5 β 4	CDR2	NO BINDING				
MEL5 β 5	CDR2&3	NO BINDING				
MEL5 β 6	CDR2&3	NO BINDING				
MEL5 β 7	CDR2&3	NO BINDING				
MEL5 β 8	CDR2&3	NO BINDING				

TABLE 3

Kinetic and affinity analysis of high affinity HLA-A*0201-ILAKFLHWL-specific TCRs binding to alanine- and glycine-substituted peptides

TCR	Mutation	$K_{on}M^{-1}s^{-1}$	$K_{off}s^{-1}$	K_D M	Half-life Min	ΔG kcal/mol ⁻¹	* $\Delta\Delta G$ kcal/mol ⁻¹
HLA A*0201-ILAKFLHWL							
ILA1	wildtype	3.5×10 ³	1.3×10 ⁻¹	37±5 μ M	0.09	-5.64	N/A
ILA1 β 1	CDR2	3.0×10 ⁴	2.9×10 ⁻⁴	9.5±1.1 nM	39.66	-10.20	N/A
ILA1 β 2	CDR2	3.1×10 ⁴	2.8×10 ⁻⁴	9.1±1.1 nM	41.07	-10.23	N/A
ILA1 α 1	CDR3	2.4×10 ⁴	6.3×10 ⁻⁴	26±4 nM	18.25	-9.65	N/A
ILA1 α 1 β 1	CDR2&3	8.0×10 ⁴	1.6×10 ⁻⁴	2.0±1 nM	71.88	-11.06	N/A
ILA1 α 1 β 2	CDR2&3	1.4×10 ⁵	4.3×10 ⁻⁴	3.1±1.2 nM	26.74	-10.82	N/A
HLA A*0201-ALAAAAAAV							
HLA A*0201-GLGGGGGGV							
ILA1	wildtype	NO BINDING				N/A	
ILA1 β 1	CDR2	NO BINDING				N/A	
ILA1 β 2	CDR2	NO BINDING				N/A	
ILA1 α 1	CDR3	NO BINDING				N/A	
ILA1 α 1 β 1	CDR2&3	NO BINDING				N/A	
ILA1 α 1 β 2	CDR2&3	NO BINDING				N/A	
HLA A*0201-ILAKFLHWL							
ILA1	wildtype	NO BINDING				N/A	
ILA1 β 1	CDR2	8.2×10 ³	7.5×10 ⁻³	0.92±0.1 μ M	1.53	-7.68	2.53
ILA1 β 2	CDR2	NO BINDING				N/A	
ILA1 α 1	CDR3	3.2×10 ³	8.5×10 ⁻³	2.6±0.2 μ M	1.35	-7.10	2.54
ILA1 α 1 β 1	CDR2&3	3.8×10 ³	3.1×10 ⁻²	8.3±1 μ M	0.37	-6.46	4.60
ILA1 α 1 β 2	CDR2&3	3.4×10 ⁴	2.8×10 ⁻¹	8.4±1.1 μ M	0.04	-6.46	4.37
HLA A*0201-ILAKFLHWL							
ILA1	wildtype	NO BINDING				N/A	
ILA1 β 1	CDR2	4.4×10 ³	4.9×10 ⁻²	11±1 μ M	0.23	-6.31	3.90
ILA1 β 2	CDR2	3.9×10 ³	7.3×10 ⁻²	19±2.2 μ M	0.16	-6.01	4.22
ILA1 α 1	CDR3	1.0×10 ⁴	5.4×10 ⁻²	5.3±0.3 μ M	0.21	-6.71	2.94
ILA1 α 1 β 1	CDR2&3	6.3×10 ³	1.4×10 ⁻²	2.2±0.1 μ M	0.82	-7.20	3.87
ILA1 α 1 β 2	CDR2&3	1.5×10 ⁴	4.9×10 ⁻²	3.3±0.3 μ M	0.23	-6.97	3.85

* $\Delta\Delta G^0 = \Delta G^0$ of each TCR binding to HLA A2-ILAKFLHWL alanine variants minus ΔG^0 of TCR to HLA A2-ILAKFLHWL.

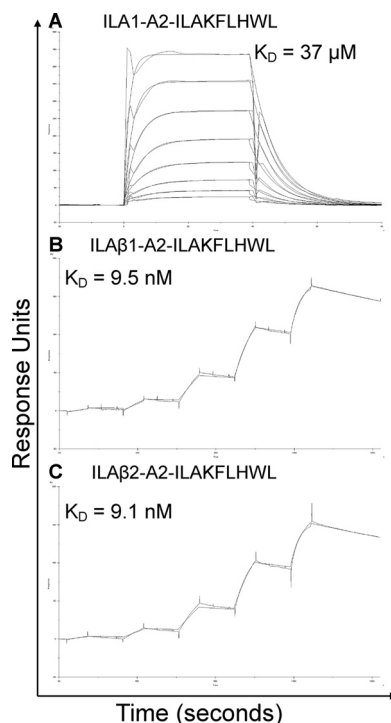


FIGURE 2. Affinity and kinetic analysis of wild type and high affinity ILA1-derived TCRs. A–C, these data were produced using a BIAcore T100™ and were then analyzed using equilibrium analysis, kinetic global fit analysis, and kinetic titration analysis. The raw data and the fits are shown in each panel. These data show that the ILA1 β 1 and ILA1 β 2 TCRs bound to HLA A*0201-ILAKFLHWL with ~4000 times greater affinity than the wild type ILA1 TCR.

tide modifications on TCR-pMHC docking, we manufactured two null HLA A*0201-nonamer peptide complexes where nonprimary MHC anchors were substituted with either glycine or alanine. However, we were unable to detect binding of any of the HLA A*0201-ELAGIGILTV- or HLA A*0201-ILAKFLHWL-specific high affinity TCRs tested against the HLA

A*0201-GLGGGGGGV or HLA A*0201-ALAAAAAAV null antigens (Tables 2 and 3). These observations are remarkable when considering that, for example, the CDR2 loop modified ILA1 β 2 TCR bound to HLA A*0201-ILAKFLHWL with an affinity >4000 times greater than the wild type ILA1 TCR (Table 3 and Fig. 2). These data support the notion that specific interactions between the TCR and peptide are required to allow the TCR to effectively engage MHC and demonstrate that changes to the antigenic peptide override mutations in the TCR that enhance contacts with the MHC molecule.

CDR2-mutated High Affinity HLA A*0201-ELAGIGILTV-specific TCRs Are Extraordinarily Sensitive to Peptide Substitutions—To investigate the role of more conservative peptide modifications on TCR binding affinity, we introduced single alanine substitutions into the HLA A*0201-ELAGIGILTV (MART-1/Melan A-derived) peptide antigen. We substituted residues in the peptide that were very unlikely to directly affect the high affinity regions of the MEL5-derived high affinity TCRs and the MHC surface, according to our structural evidence (Fig. 4, A and B) (24, 34). This enabled the determination of the effect of altering TCR-peptide contacts on TCR-MHC binding. Neither the MEL5 TCR nor any of the high affinity TCRs retained the ability to bind to HLA A*0201-ELAAIGILTV, HLA A*0201-ELAGIAILTV, HLA A*0201-ELAGIGALTIV, or HLA A*0201-ELAGIGILAV (Table 2). This observation that single alanine substitutions in the native peptide can completely abrogate binding of all of the high affinity HLA A*0201-ELAGIGILTV-specific TCRs reaffirms the notion that specific interactions between the TCR and peptide are required in order for optimal docking with the MHC surface to occur, and this is consistent with our previous findings using this system (34).

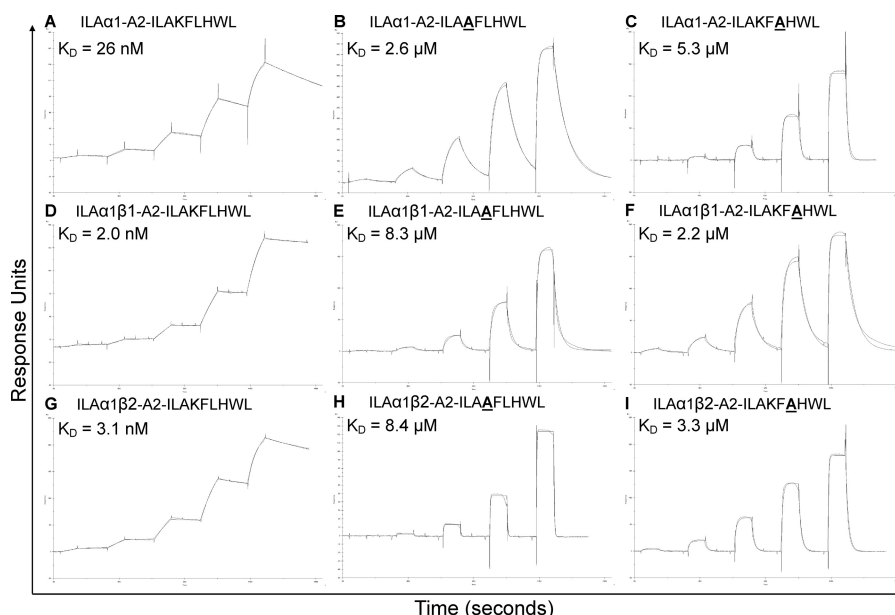


FIGURE 3. Effect of alanine peptide substitutions on high affinity ILA1-derived TCR binding. Binding affinity and kinetic analysis of the HLA A*0201-ILAKFLHWL-specific high affinity ILA1 α 1, ILA1 α 1 β 1, and ILA1 α 1 β 2 TCRs to HLA A2-ILAKFLHWL, HLA A2-ILAAFLHWL, and HLA A2-ILAKFAHWL (A–I). These data were produced using a BIAcore T100™ and were then analyzed using equilibrium analysis, kinetic global fit analysis, and kinetic titration analysis. The raw data and the fits are shown in each panel. These data show the effect of the HLA A2-ILAAFLHWL and HLA A2-ILAKFAHWL peptide modifications on the binding of the high affinity TCRs compared with HLA A2-ILAKFLHWL. These support the notion that TCR-peptide interactions govern TCR-pMHC binding because, although the ILA1 α 1 β 1 TCR with a mutated CDR2 loop did not contact the peptide, the difference in binding between the ILA1 α 1 TCR and the ILA1 α 1 β 1 TCR to HLA A2-ILAAFLHWL and HLA A2-ILAKFAHWL compared with HLA A2-ILAKFLHWL is disproportionately different.

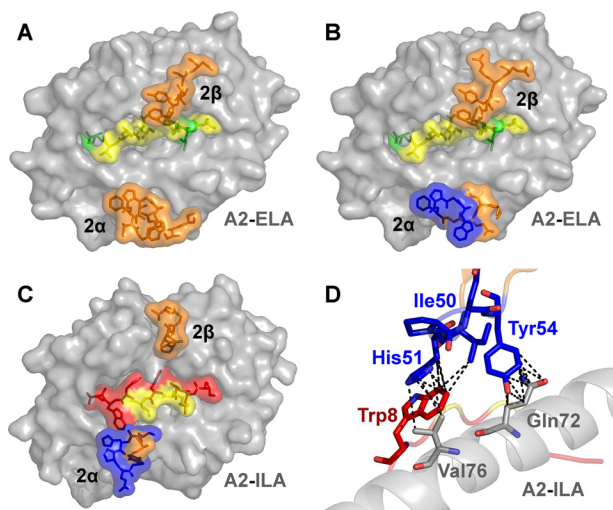


FIGURE 4. Peptide modifications do not directly impinge on the binding of mutated high affinity TCR residues. The complex structures of high affinity MEL5- and ILA1-derived TCRs show that peptide modifications do not directly impinge on the binding of mutated high affinity TCR residues. A, wild type MEL5-A2-ELA (PDB code 3HG1 (24)) complex structure showing the MHC in gray surface, the mutated peptide residues in yellow stick and surface (nonmutated residues in green), and the positions of the TCR CDR2 loops in orange sticks and surface. B, high affinity α 24 β 17-A2-ELA (PDB code 4JFF (34)) complex structure (α 24 β 17 is a high affinity version of the MEL5 TCR) showing the MHC in gray surface, the mutated peptide residues in yellow stick and surface (nonmutated residues in green), and the positions of the TCR CDR2 loops in orange sticks and surface. In this structure, the high affinity mutations in the TCR CDR2 α loop are colored blue and are distal from the peptide. C, high affinity α 1 β 1-A2-ILA (PDB code 4MNQ) complex structure (α 1 β 1 is a high affinity version of the ILA1 TCR) showing the MHC in gray surface, the mutated peptide residues in yellow stick and surface (nonmutated residues in red), and the positions of the TCR CDR2 loops in orange sticks and surface. D, specific contacts between the high affinity mutated residues in the α 1 β 1 TCR CDR2 α loop (blue sticks), the MHC (gray sticks), and the peptide (red). The peptide residues that were mutated to alanine (yellow) were not directly contacted by the high affinity mutated residues in the α 1 β 1 TCR CDR2 α loop. Overall, these structures demonstrate that TCR residues that have been mutated in the CDR2 loops of high affinity TCRs do not directly contact residues that were mutated to alanine in the peptide.

Affinity-enhanced CDR2 α Loops of the ILA1-derived TCRs Do Not Contact Alanine-substituted Peptide Residues—Previous structural comparisons of wild type and high affinity TCRs (34, 41–43) show that these molecules adopt a near identical binding mode (Fig. 5). To confirm that alanine substitutions of these peptide residues would not directly impinge on the binding of the mutated residues in the high affinity CDR2 α loops of the ILA1-derived TCRs, we solved the structure of the ILA1 α 1 β 1 TCR in complex with HLA A*0201-ILAKFLHWL. The complex was solved to a resolution of 2.4 Å in space group C121. The resolution was sufficiently high to show that the interface between the two molecules was well ordered and contained well defined electron density. The crystallographic R/R_{free} factors were 20.1 and 24.6%, within the accepted limits shown in the theoretically expected distribution (Table 4) (45). The structure demonstrated that the mutated CDR2 α region of the high affinity α 1 β 1 TCR could not directly contact the mutated residues in the peptide (Fig. 4, C and D, and Table 5).

HLA A*0201-ILAKFLHWL Peptide Substitutions Disproportionately Affect the Binding of High Affinity CDR2 Loop Mutated TCRs—We then measured the binding of the HLA A*0201-ILAKFLHWL-specific wild type ILA1 TCR and high affinity derivative TCRs to HLA A*0201-ILAAFLHWL and HLA A*0201-ILAKFAHWL (Table 3 and Fig. 3). The ILA1 α 1 high affinity TCR bound to HLA A*0201-ILAAFLHWL and HLA A*0201-ILAKFAHWL with 100 and 200 times weaker affinity, respectively, than to HLA A*0201-ILAKFLHWL. This difference corresponded to a $\Delta\Delta G$ value (difference in binding energy, ΔG , between the ILA1 α 1 high affinity TCR interacting with HLA A*0201-ILAKFLHWL versus HLA A*0201-ILAAFLHWL and HLA A*0201-ILAKFAHWL) of 2.54 and 2.94 kcal/mol $^{-1}$, respectively. We then repeated this analysis using the

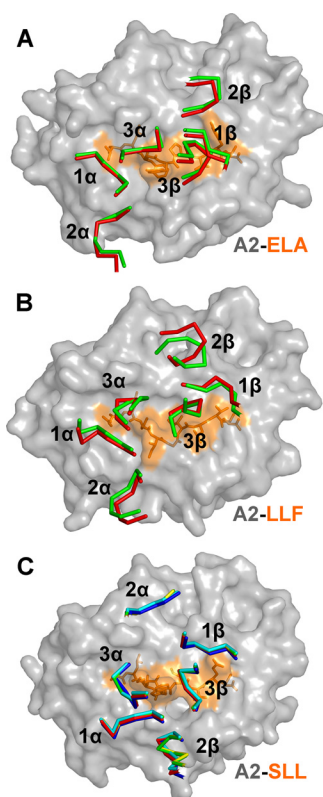


FIGURE 5. Conformation of TCR CDR loops remains very similar between modified high affinity TCRs and their wild type progenitors. Comparison of the CDR loop positions of previously published high affinity and wild type TCRs. *A*, wild type MEL5-A2-ELA complex (24) (CDR loops in orange ribbon) and the high affinity $\alpha 24\beta 17$ -A2-ELA complex (34) (CDR loops in green ribbon). *B*, wild type A6-A2-LLF complex (27) (CDR loops in orange ribbon) and the high affinity c134-A2-LLF complex (41) (CDR loops in green ribbon). *C*, wild type 1G4-A2-SLL complex (64) (CDR loops in orange ribbon) and the high affinity c58c62-A2-SLL (CDR loops in green ribbon), c49c50-A2-SLL (CDR loops in blue ribbon), c549c61-A2-SLL (CDR loops in yellow ribbon), and c5c1-A2-SLL (CDR loops in cyan ribbon) complexes (42, 43). In all cases, the relative positions of the CDR loops over the pMHC for the wild type TCRs and their high affinity TCR derivative are virtually identical.

IL1A1 α 1 β 1 and IL1A1 α 1 β 2 TCRs. These TCRs contained modified CDR2 loops but identical CDR3 loops to the IL1A1 α 1 TCR. According to the assumption that TCR-MHC interactions precede TCR-peptide interactions, we reasoned that each of these TCRs should retain their individual TCR-MHC contacts because only the TCR-peptide interaction should be directly affected (as in Fig. 6A). Therefore, the difference in binding affinity observed for the IL1A1 α 1 TCR between HLA A*0201-ILAKFLHWL compared with HLA A*0201-ILAAFLHWL and HLA A*0201-ILAKFAHWL (100 and 200 times weaker affinity, respectively) should be similar to the IL1A1 α 1 β 1 and IL1A1 α 1 β 2 TCRs. However, the IL1A1 α 1 β 1 TCR bound to the HLA A*0201-ILAAFLHWL and HLA A*0201-ILAKFAHWL with 4150 and 1100 times weaker affinity ($\Delta\Delta G$ value of 4.6 and 3.87 kcal/mol⁻¹), respectively (Table 3 and Figs. 3 and 6B). The IL1A1 α 1 β 2 TCR bound to HLA A*0201-ILAAFLHWL and HLA A*0201-ILAKFAHWL with 2710 and 1065 times weaker affinity ($\Delta\Delta G$ value of 4.37 and 3.85 kcal/mol⁻¹), respectively (Table 3 and Fig. 3). These data demonstrate that TCR interactions with the peptide and MHC are strongly coupled and that modifying the TCR-peptide interaction has a dis-

TABLE 4

Data collection and refinement statistics for $\alpha 1\beta 1$ -A2-ILA complex structure
One crystal was used for solving the structure. Values in parentheses are for the highest resolution shell.

$\alpha 1\beta 1$ -A2-ILA	
PDB code	4MNQ
Data collection	
Space group	P3 ₂ 21
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i>	97.14, 97.14, 123.08 Å
α , β , γ	90, 90, 120°
Resolution (Å)	49.7 to 2.4 Å (10.7 to 2.4 Å)
<i>R</i> _{merge}	19.2%
<i>I</i> / σ <i>I</i>	16.6
Completeness	100%
Redundancy	10.9
Refinement	
Resolution	2.4 Å
No. of reflections	25,403
<i>R</i> _{work} / <i>R</i> _{free}	20.1/24.6
No. of atoms	3694
Protein	3492
Ligand/ion	41
Water	161
<i>B</i> -factors	44.63
Protein	44.60
Ligand/ion	60.79
Water	41.10
Root mean square deviations	
Bond lengths	0.022 Å
Bond angles	1.206°

proportionately strong detrimental energetic effect on TCR-MHC binding.

Effect of Peptide Substitutions on the Binding Kinetics of High Affinity CDR2 Loop Mutated TCRs—Kinetic binding analyses were carried out at 25 °C to measure the on-rate (*K*_{on}) and off-rate (*K*_{off}) for each TCR-pMHC interaction (Figs. 1–3 and Tables 2 and 3). These analyses were important to reveal the kinetic basis for the effect of altering the TCR-peptide interactions by modifying the antigenic peptide. Interestingly, although alanine mutations within the central peptide residues reduced the binding affinity of all of the high affinity TCRs tested to a different extent (97–4150-fold reduction in binding affinity), the on-rate was not substantially affected (average decrease of 10 times) (Tables 2 and 3). Conversely, the stability of the TCR-pMHC complex was affected by a greater extent, as evident by the faster off-rate observed (average increase of 200 times) (Tables 2 and 3). Therefore, a faster off-rate was the major kinetic determinant governing the decrease in binding affinity between the high affinity CDR loop mutated TCRs and the alanine-substituted peptide ligands. These data suggest that in order for the TCR to form a stable long lived interaction with cognate pMHC, the TCR must be able to bind to the peptide to allow optimal MHC docking. Thus, in the systems we have studied, successful TCR-peptide sampling must precede (or occur at the same time as) the stabilizing interaction between the TCR and the MHC surface.

DISCUSSION

Antigen recognition by the TCR usually involves contacts with both self (MHC) and non-self (the antigenic peptide) (5, 16). To avoid autoreactivity, the self-interaction between the TCR and MHC must not be sufficient to activate peripheral T-cells independently of the non-self TCR-peptide interaction. The current database of TCR-pMHC complex structures

TCR-Peptide Specificity Governs Antigen Recognition

TABLE 5

$\alpha 1 \beta 1$ -A2-ILA contacts (residues mutated from wild type shown in red)

TCR	Gene Usage	TCR	Peptide	MHC	vdW (≤ 4 Å)	H-bonds (≤ 3.4 Å)
CDR1 α	<i>TRAV22</i>	Asp27 ^{O82}		Thr163 ^{Oy1}	4	1
	<i>TRAV22</i>	Asp27		Glu166	1	
	<i>TRAV22</i>	Ser28		Ala158	1	
	<i>TRAV22</i>	Val29		Gln155	5	
	<i>TRAV22</i>	Val29	Lys4		2	
	<i>TRAV22</i>	Asn30 ^{O81}		Gln155 ^{Oe1}	3	1
CDR2 α	<i>TRAV22</i>	Tyr48		Glu155	1	
	<i>TRAV22</i>	Pro50		Glu154	1	
	<i>TRAV22</i>	Ser51		Glu154	3	
	<i>TRAV22</i>	Ser51		Arg157	1	
	<i>TRAV22</i>	Gln55 ^{Ne2}		Glu154 ^{Oe2}	1	1
FW α	<i>TRAV22</i>	Thr66		Glu166	1	
	<i>TRAV22</i>	Arg68 ^{NH2}		Glu166 ^{Oe2}	2	1 salt bridge
CDR3 α	<i>TRAJ40</i>	Asp90	Lys4		1	
	<i>TRAJ40</i>	Ser91	Lys4		2	
	<i>TRAJ40</i>	Ala92		Thr163	1	
	<i>TRAJ40</i>	Ala92	Lys4		3	
	<i>TRAJ40</i>	Thr93		Lys66	2	
	<i>TRAJ40</i>	Thr93		Trp167	1	
	<i>TRAJ40</i>	Thr93	Ile1		2	
	<i>n/a</i>	Ala94 ^O	Lys4 ^{Nc}		3	1
	<i>n/a</i>	Leu95		Gly62	1	
	<i>n/a</i>	Leu95	Lys4		2	
	<i>n/a</i>	Pro96 ^O	Lys4 ^{Nc}		1	1
	<i>TRAJ40</i>	Tyr97		Lys66	2	
	<i>TRAJ40</i>	Tyr97		Ala69	1	
	<i>TRAJ40</i>	Tyr97	Lys4		11	
	<i>TRAJ40</i>	Tyr97	Leu6		4	
CDR1 β	<i>TRBV6</i>	Glu30	Trp8		8	
CDR2 β	<i>n/a</i>	Ile50	Trp8		5	
	<i>n/a</i>	His51		Val76	3	
	<i>n/a</i>	His51	Trp8		4	
	<i>n/a</i>	Tyr54		Gln72	12	
	<i>TRBV6</i>	Asp56 ^{O82}		Arg65 ^{Ne}	2	1
CDR3 β	<i>TRBJ1-1</i>	Tyr95		Lys146	3	
	<i>TRBJ1-1</i>	Pro95		Ala150	2	
	<i>TRBJ1-1</i>	Gln96		Lys146	1	
	<i>TRBJ1-1</i>	Gln96		Trp147	5	
	<i>TRBJ1-1</i>	Gln96		Ala150	1	
	<i>TRBJ1-1</i>	Gln96		Val152	1	
	<i>TRBJ1-1</i>	Gln96	Leu6		2	
	<i>TRBJ1-1</i>	Gln96	His7		4	
	<i>TRBJ1-1</i>	Gln96	Trp8		2	
	<i>TRBJ1</i>	Gly97 ^O		Gln155 ^{Oe1}		1
	<i>TRBJ1</i>	Thr98 ^{O81}		Ala150 ^O	5	1

* A 3.4-Å cutoff was used for H-bonds and salt bridges, and a 4-Å cutoff was used for van der Waals (vdW).

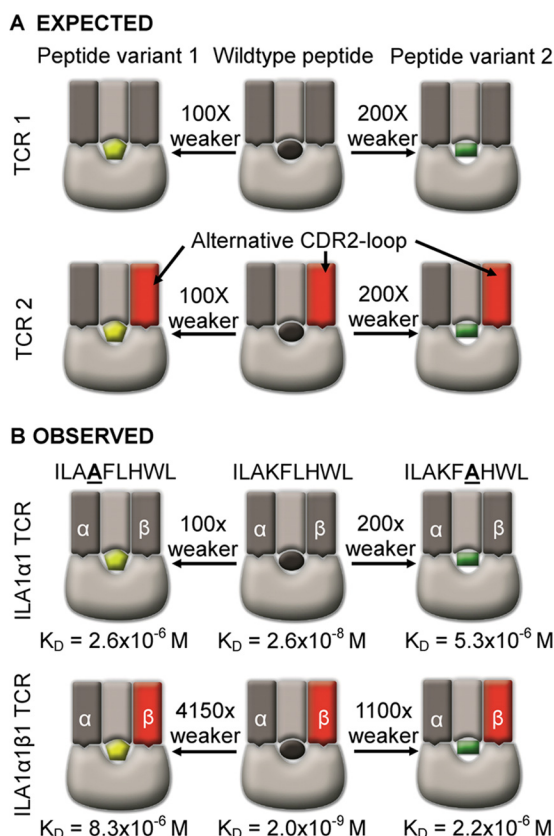


FIGURE 6. Schematic of the effect of alanine peptide substitutions on TCR-pMHC binding affinity. Nonmutated TCR and pMHC components are shown in grayscale. TCRs with high affinity mutations are shown in red. Peptide mutations are shown in yellow or green. *A*, expected difference in the binding of an unmodified TCR (TCR1) compared with a CDR2 loop mutated TCR (TCR2) (mutation shown in red), assuming that the CDR2 loops bind independently of the TCR-peptide interaction. Because the mutated CDR2 loop (shown in red) does not contact the peptide, the theoretical difference in binding between the TCR1 and TCR2 to peptide variants 1 (yellow) and 2 (green) compared with the wild-type peptide (black) should be identical according to the interaction between the CDR3 loops and the peptide. *B*, schematic of the observed difference in the binding of the ILA1 α1β1 TCR, compared with the ILA1 α1 TCR engaging a peptide-MHC complex. These data show that a disproportionate knock-on effect in binding occurs for the ILA1 α1β1 TCR, compared with the ILA1 α1 TCR. These data indicate that TCR-MHC binding does not occur independently of TCR-peptide interactions and that the latter likely governs the former.

shows that the interaction between TCR and antigenic peptide can play a minimal structural role, often being responsible for less than a third of the binding interface relative to contacts between the TCR and MHC (5). Thus, the molecular mechanism by which the TCR maintains peptide-specific recognition is not immediately obvious.

To re-examine how the TCR CDR loops co-operatively act to stabilize TCR-pMHC binding, we designed a range of soluble TCRs that exhibited up to a 18,500-fold enhancement in affinity for cognate antigen using CDR loop mutations selected by phage display (26). Previous studies using high affinity TCRs have shown that these artificial reagents retain a high level of antigen specificity similar to their wild type progenitors (34, 46–48). In all cases, the enhanced affinity observed for the mutated TCRs compared with the wild type TCRs was due to small differences in the on-rate but a vastly extended off-rate. The slower off-rate indicated that any initial “transition state” was less important than the formation of a stable complex dur-

ing high affinity TCR-pMHC binding. These high affinity TCRs enabled modification to the TCR-peptide interaction while retaining a strong enough residual TCR-pMHC affinity to measure using SPR. Furthermore, we were able to incorporate mutations into individual CDR loops to generate a panel of TCRs with an identical sequence except for their CDR2 loops. Structural analyses confirmed that these loops were distal to peptide binding in both wild type and enhanced affinity MEL5 and ILA1 TCRs. Based on some models of TCR engagement (11), we reasoned that HLA A*0201-restricted TCRs with high affinity mutations in their CDR2 loops should retain a residual ability to bind to the surface of HLA A*0201 independently of the peptide because the TCR-peptide interactions should only account for a small proportion of the overall binding energy. In contrast to this prediction, we were unable to show binding to the HLA A*0201-GLGGGGGGV or HLA A*0201-ALAAAAAAV null antigens with any of the CDR2 loop high affinity TCRs tested. These observations support the notion that specific interactions between the TCR and peptide are required to allow the TCR to effectively engage MHC. Using this system, we were also able to examine whether subtle alterations in the interaction between TCR and peptide were independent of TCR CDR2 loop binding to MHC. To investigate this, we tested the binding affinity of a panel of HLA A*0201-ILAKFLHWL-specific CDR2 loop-modified TCRs to peptides that contained alanine substitutions at positions structurally shown to be key TCR contacts. These data revealed that even minimal changes to the TCR-peptide interaction had a substantial impact on the TCR affinity and binding energy (ΔG). These data show that TCR-peptide contacts are strongly “coupled” to TCR-MHC contacts.

We also performed a kinetic investigation of the effect of altering the TCR-peptide interaction. These data showed that the vastly extended off-rates that governed the enhanced affinity of the high affinity mutated TCRs were effectively nullified by altering the TCR interaction with peptide, although the on-rates remained relatively unchanged. Thus, our data indicate that complex formation is not initiated by TCR-MHC binding. Rather, successful TCR-peptide sampling must precede or occur at the same time as the stabilizing interaction between the TCR and the MHC surface. In support of this notion, our data show that altering the TCR interaction with peptide can override the optimal formation of TCR-MHC interactions resulting in a disproportionate knock-on effect on TCR-pMHC affinity.

Mounting evidence from other studies also contests the notion that conserved interactions between the germ line-encoded loops of the TCR and the MHC initiate TCR-pMHC complex formation. First, Burrows *et al.* (16) have demonstrated that disrupting conserved interactions between the TCR and MHC surface resulted in the formation of compensatory interactions. In support of these data, Dyson and co-workers (49) extensively diversified CDR1 and CDR2 loops *in vivo* and demonstrated that the TCR is not genetically hardwired to engage MHC ligands. Second, a number of molecular studies are incompatible with TCR-MHC initiated binding. These include the following: (i) the co-crystal structure of a TCR bound to MHCI complexed with a 13-mer “super-bulged” pep-

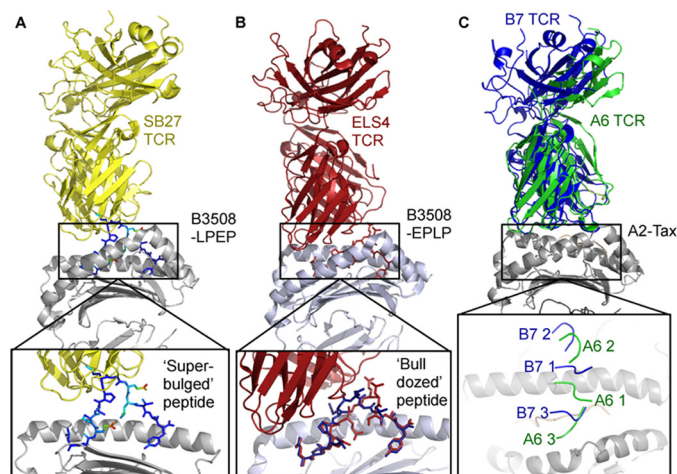


FIGURE 7. Structural evidence demonstrating that TCR-peptide contacts precede TCR-MHC contacts. A, co-crystal structure of the SB27 TCR (shown as yellow schematic) bound to the HLA-B*3508 (shown as gray yellow schematic) LPEP super-bulged 13-mer peptide (shown as sticks, colored using Wilson "B" factor) complex. The expanded panel below illustrates the extended conformation of the peptide, making it highly improbable that the SB27 TCR could contact the MHC surface before the peptide (19). B, co-crystal structure of the ELS4 TCR (shown as red yellow schematic) bound to the HLA-B*3501 (shown as gray yellow schematic) EPLP 11-mer peptide (shown as sticks, colored using red) complexed to the ELS4 TCR and in blue uncomplexed). The expanded panel below illustrates how the EPLP peptide is bulldozed into a different conformation during TCR binding (before TCR binding is shown in blue and after TCR binding is shown in red), allowing the TCR to contact the MHC surface (20). C, co-crystal structure of the A6 TCR (shown as green schematic) superposed with the B7 TCR (shown as blue schematic) which both bind to the HLA A*0201 (shown as gray schematic) Tax (shown as peach schematic) complex (27, 51, 52). These TCRs share the same β -chain germ line-encoded CDR1 and CDR2 loops, and they bind to the same N-terminal region of the A2-Tax complex. The expanded panel below illustrates that the CDR3 loops engage some of the same residues of the peptide, whereas the CDR1 and CDR2 loops bind to distinct regions of the MHC surface.

ptide (19) showing that the extended central peptide bulge physically restricted the TCR from contacting the MHC surface (Fig. 7A) (19); (ii) the co-crystal structure of a TCR bound to MHC I complexed with an 11-mer peptide demonstrating that the peptide was "bulldozed" or flattened by the TCR, allowing the TCR to contact the MHC surface (Fig. 7B) (20); (iii) accumulated studies showing that TCR-MHC interactions can play a minimal energetic role, compared with TCR-peptide interactions, during TCR binding to MHC (15, 18, 21, 50), and (iv) the structures of the A6 and B7 TCRs bound to HLA A*0201-LLFGYPVYV (27, 51, 52), showing that, despite both TCRs sharing a genetically identical germ line-encoded V β -gene (V β 6-5), the TCR-MHC contacts were distinct, although a number of identical TCR-peptide contacts existed (Fig. 7C). Finally, our data, in which we have tested affinity-enhanced TCRs against a range of normal tissue cell samples, show that high affinity CDR2 mutations do not render the TCRs more unspecific than high affinity CDR3 mutations. The examples above are consistent with a model for T-cell antigen recognition in which TCR-peptide binding overrides TCR-MHC engagement.

The idea that TCR-peptide contacts govern T-cell antigen recognition is in accord with several biological requirements of T-cell immunity. First, given that extremely weak TCR binding is required for positive selection of peptide-dependent T-cells in the thymus (53), control of this delicate aspect would repre-

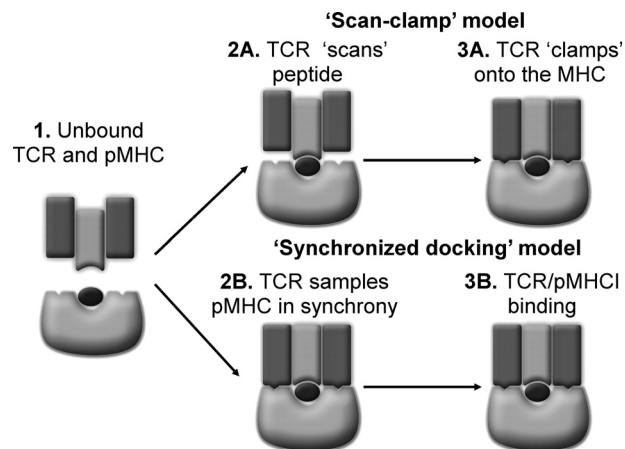


FIGURE 8. New models for TCR engagement of pMHC. 1, schematic of a TCR (dark and light gray) proceeding engagement of peptide (black)-MHC (light gray). A, "Scan-clamp" model. Only specific TCR-peptide contacts (light gray and black) (2A) allow the TCR (shown in dark gray) to clamp-onto the MHC surface and (3A) complete TCR-pMHC docking, which leads to T-cell activation. B, "synchronized docking" model. TCR contacts the peptide and MHC simultaneously (2B), but TCR-peptide interactions are dominant over TCR-MHC interactions (3B). Only the scan clamp and synchronized docking models for T-cell antigen recognition are permissive with our data.

sent a far greater challenge were TCR-MHC contacts to proceed TCR-peptide interactions (11). Second, accumulated studies that have demonstrated that alloreactive TCR recognition is peptide-dependent (54–56) are favored by models where TCR-peptide contacts dominate TCR engagement. Third, if TCR-MHC interactions initiate antigen recognition then the extraordinarily rapid kinetics of CD8 and CD4 coreceptor binding might enable aberrant T-cell signaling, bypassing antigen-specific TCR-peptide sampling (57). Fourth, a system where TCR-MHC contacts dominate TCR binding is difficult to reconcile with the kinetic segregation model of T-cell activation (58, 59). In this model, small molecules such as CD2 and CD28 facilitate contact zones to enable the TCR to scan pMHCs. The proximity of the T-cell and target cell membranes in these contact zones excludes large phosphatase molecules, such as CD45, triggering phosphorylation of the TCR and downstream signaling events. Thus, TCR-MHC binding in these contact zones could enable TCR phosphorylation independently of TCR-peptide binding. Finally, a mode of action that requires that the TCR interacts with MHC prior to peptide scanning wastes both time and energy. This is particularly important for a system that requires an individual TCR to scan a multitude of pMHC molecules to locate a cognate peptide.

We propose two new models of TCR-pMHC binding that are accommodated by our data and are both temporally and energetically complementary with a system requiring recognition of self in the thymus and rapid intolerance of non-self in the periphery. First, the "scan-clamp" model, in which the TCR "scans" the peptide before "clamping" onto the MHC surface (Fig. 8A). Second, the "synchronized docking" model, in which there is no temporal separation between the TCR binding to the peptide or MHC, but TCR-peptide interactions are dominant over TCR-MHC interactions (Fig. 8B). These new models are consistent with the requirement for T-cells to target cells based on their antigenic peptide, allowing them to expeditiously distinguish aberrant cells from healthy cells (60–63).

In conclusion, it is clear that T-cells have evolved to ensure that TCR-pMHC binding is carefully balanced to guarantee that the fidelity of antigen recognition is permissive for the conserved and universal interactions that lead to T-cell activation. Our new data shed light on the mechanisms controlling the seemingly paradoxical observation that a receptor-ligand (TCR-pMHC) interaction with both a self (TCR-MHC) and non-self (TCR-peptide) component can control T-cells by only forming productive interactions when encountering alien antigen.

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T-cell receptor

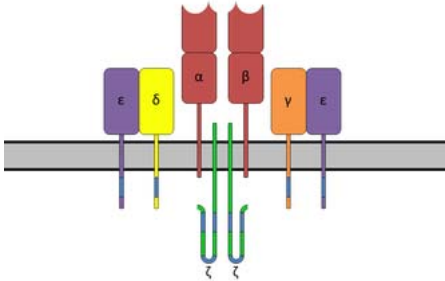
The **T-cell receptor** (**TCR**) is a protein complex found on the surface of T cells, or T lymphocytes,^[1] that is responsible for recognizing fragments of antigen as peptides bound to major histocompatibility complex (MHC) molecules. The binding between TCR and antigen peptides is of relatively low affinity and is degenerate: that is, many TCRs recognize the same antigen peptide and many antigen peptides are recognized by the same TCR.^[2]

The TCR is composed of two different protein chains (that is, it is a hetero dimer). In humans, in 95% of T cells the TCR consists of an alpha (α) chain and a beta (β) chain (encoded by *TRA* and *TRB*, respectively), whereas in 5% of T cells the TCR consists of gamma and delta (γ/δ) chains (encoded by *TRG* and *TRD*, respectively). This ratio changes during ontogeny and in diseased states (such as leukemia). It also differs between species. Orthologues of the 4 loci have been mapped in various species.^{[3][4]} Each locus can produce a variety of polypeptides with constant and variable regions.^[3]

When the TCR engages with antigenic peptide and MHC (peptide/MHC), the T lymphocyte is activated through signal transduction, that is, a series of biochemical events mediated by associated enzymes, co-receptors, specialized adaptor molecules, and activated or released transcription factors. Based on the initial receptor triggering mechanism, the TCR belongs to the family of Non-catalytic tyrosine-phosphorylated receptors (NTRs).^[5]

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<u>NF-κB</u>

CD3 zeta chain and FCER1G family



The **T-cell receptor complex** with TCR-α and TCR-β chains, **CD3** and ζ-chain (CD247) accessory molecules.

Identifiers	
Symbol	TCR_zetazeta
Pfam	PF11628 (http://pfam.xfam.org/family?acc=PF11628)
InterPro	IPR021663 (https://www.ebi.ac.uk/interpro/entry/IPR021663)
OPM superfamily	166 (https://opm.phar.umich.edu/protein_superfamilies/166)
OPM protein	2hac (https://opm.phar.umich.edu/proteins?search=2hac)
Membranome	26 (http://membranome.org/protein_superfamilies/26)

Available protein structures:

Pfam	structures (http://pfam.xfam.org/family/PF11628?tab=pdbBlock) / ECOD (http://prodata.swmed.edu/ecod/complete/search?kw=PF11628)
PDB	RCSB PDB (http://www.rcsb.org/pdb/search/smartSubquery.do?smartSearchSubtype=PfamIdQuery&pfamID=PF11628)

AP1

See also

References

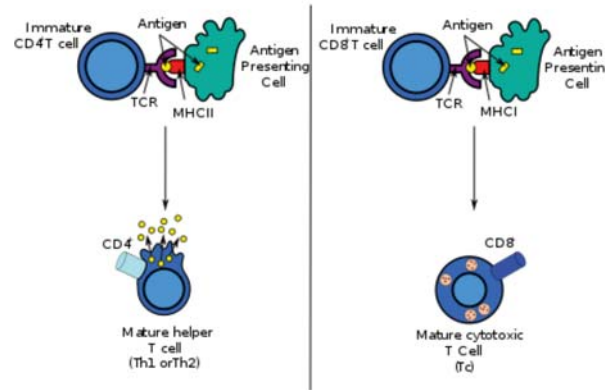
External links

; PDBe (https://www.ebi.ac.uk/pdbe/entry/search/index?pfam_accession:PF11628); PDBj (<https://pdj.org/searchFor?query=PF11628>)

PDBsum structure summary (https://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPfamStr.pl?pfam_id=PF11628)

History

In 1982, Nobel laureate James P. Allison first discovered the T-cell receptor.^[6] Then, Tak Wah Mak^[7] and Mark M. Davis^[8] identified the cDNA clones encoding the human and mouse TCR respectively in 1984. These findings allowed the entity and structure of the elusive TCR, known before as the "Holy Grail of Immunology", to be revealed. This allowed scientists from around the world to carry out studies on the TCR, leading to important studies in the fields of CAR-T, cancer immunotherapy and checkpoint inhibition.



Antigen presentation stimulates T cells to become either "cytotoxic" CD8+ cells or "helper" CD4+ cells.

Structural characteristics

The TCR is a disulfide-linked membrane-anchored heterodimeric protein normally consisting of the highly variable alpha (α) and beta (β) chains expressed as part of a complex with the invariant CD3 chain molecules. T cells expressing this receptor are referred to as α : β (or $\alpha\beta$) T cells, though a minority of T cells express an alternate receptor, formed by variable gamma (γ) and delta (δ) chains, referred as $\gamma\delta$ T cells.^[9]

Each chain is composed of two extracellular domains: Variable (V) region and a Constant (C) region, both of Immunoglobulin superfamily (IgSF) domain forming antiparallel β -sheets. The Constant region is proximal to the cell membrane, followed by a transmembrane region and a short cytoplasmic tail, while the Variable region binds to the peptide/MHC complex.

The variable domain of both the TCR α -chain and β -chain each have three hypervariable or complementarity-determining regions (CDRs). There is also an additional area of hypervariability on the β -chain (HV4) that does not normally contact antigen and, therefore, is not considered a CDR.

The residues in these variable domains are located in two regions of the TCR, at the interface of the α - and β -chains and in the β -chain framework region that is thought to be in proximity to the CD3 signal-transduction complex.^[10] CDR3 is the main CDR responsible for recognizing processed

T-cell receptor alpha locus

Identifiers	
Symbol	TRA
Alt. symbols	TCRA, TRA@
NCBI gene	6955 (https://www.ncbi.nlm.nih.gov/gene?cmd=retrieve&dopt=default&list_uids=6955&rn=1)
HGNC	12027 (https://www.genenames.org/data/gene-symbols-report/#!/hgnc_id/HGNC:12027)
OMIM	186880 (https://omim.org/g/186880)
Other data	
Locus	Chr. 14 <i>q11.2</i> (https://omim.org/search/?index=geneMap&search=14q11.2)

T-cell receptor beta locus

antigen, although CDR1 of the alpha chain has also been shown to interact with the N-terminal part of the antigenic peptide, whereas CDR1 of the β -chain interacts with the C-terminal part of the peptide.

CDR2 is thought to recognize the MHC. CDR4 of the β -chain is not thought to participate in antigen recognition, but has been shown to interact with superantigens.

The constant domain of the TCR consists of short connecting sequences in which a cysteine residue forms disulfide bonds, which form a link between the two chains.

The TCR is a member of the immunoglobulin superfamily, a large group of proteins involved in binding, recognition, and adhesion; the family is named after antibodies (also called immunoglobulins). The TCR is similar to a half-antibody consisting of a single heavy and single light chain, except the heavy chain is without its crystallisable fraction (Fc). The two subunits of TCR are twisted together. Whereas the antibody uses its Fc region to bind to Fc Receptors on leukocytes, TCR is already docked onto the cell membrane. However, it is not able to mediate signal transduction itself due to its short cytoplasmic tail, so TCR still requires CD3 and zeta to carry out the signal transduction in its place, just as antibodies require binding to FcRs to initiate signal transduction. In this way the MHC-TCR-CD3 interaction for T cells is functionally similar to the antigen(Ag)-immunoglobulin(Ig)-FcR interaction for myeloid leukocytes, and Ag-Ig-CD79 interaction for B cells.

Generation of the TCR diversity

The generation of TCR diversity is similar to that for antibodies and B-cell antigen receptors. It arises mainly from genetic recombination of the DNA-encoded segments in individual somatic T cells by somatic V(D)J recombination using RAG1 and RAG2 recombinases. Unlike immunoglobulins, however, TCR genes do not undergo somatic hypermutation, and T cells do not express activation-induced cytidine deaminase(AID). The recombination process that creates diversity in BCR (antibodies) and TCR is unique to lymphocytes (T and B cells) during the early stages of their development in primary lymphoid organs (thymus for T cells, bone marrow for B cells).

Each recombined TCR possess unique antigen specificity, determined by the structure of the antigen-binding site formed by the α and β chains in case of $\alpha\beta$ T cells or γ and δ chains on case of $\gamma\delta$ T cells.^[11]

- The TCR *alpha chain* is generated by VJ recombination, whereas the *beta chain* is generated by VDJ

Identifiers	
Symbol	TRB
Alt. symbols	TCRB, TRB@
NCBI gene	6957 (https://www.ncbi.nlm.nih.gov/gene?cmd=retrieve&dopt=default&list_uids=6957&rn=1)
HGNC	12155 (https://www.genenames.org/data/gene-symbol-report/#!/hgnc_id/HGNC:12155)
OMIM	186930 (https://omim.org/g/186930)
Other data	
Locus	Chr. 7 <i>q34</i> (https://omim.org/search/?index=geneMap&search=7q34)

T-cell receptor delta locus	
Identifiers	
Symbol	TRD
Alt. symbols	TCRD, TRD@, TCRDV1
NCBI gene	6964 (https://www.ncbi.nlm.nih.gov/gene?cmd=retrieve&dopt=default&list_uids=6964&rn=1)
HGNC	12252 (https://www.genenames.org/data/gene-symbol-report/#!/hgnc_id/HGNC:12252)
Other data	
Locus	Chr. 14 <i>q11.2</i> (https://omim.org/search/?index=geneMap&search=14q11.2)

T-cell receptor gamma locus	
Identifiers	
Symbol	TRG
Alt. symbols	TCRG, TRG@

recombination (both involving a random joining of gene segments to generate the complete TCR chain).

- Likewise, generation of the TCR *gamma chain* involves VJ recombination, whereas generation of the TCR *delta chain* occurs by VDJ recombination.

The intersection of these specific regions (V and J for the alpha or gamma chain; V, D, and J for the beta or delta chain) corresponds to the CDR3 region that is important for peptide/MHC recognition (see above).

It is the unique combination of the segments at this region, along with palindromic and random nucleotide additions (respectively termed "P-" and "N-"), which accounts for the even greater diversity of T-cell receptor specificity for processed antigenic peptides.

Later during development, individual CDR loops of TCR can be re-edited in the periphery outside thymus by reactivation of recombinases using a process termed TCR revision (editing) and change its antigenic specificity.

The TCR complex

In the plasma membrane the TCR receptor chains α and β associate with six additional adaptor proteins to form an octameric complex. The complex contains both α and β chains, forming the ligand-binding site, and the signaling modules CD3 δ , CD3 γ , CD3 ϵ and CD3 ζ in the stoichiometry TCR α β - CD3 $\epsilon\gamma$ - CD3 $\epsilon\delta$ - CD3 $\zeta\zeta$. Charged residues in the transmembrane domain of each subunit form polar interactions allowing a correct and stable assembly of the complex.^[12] The cytoplasmic tail of the TCR is extremely short, hence the CD3 adaptor proteins contain the signalling motifs needed for propagating the signal from the triggered TCR into the cell. The signalling motifs involved in TCR signalling are tyrosine residues in the cytoplasmic tail of these adaptor proteins that can be phosphorylated in the event of TCR-pMHC binding. The tyrosine residues reside in a specific amino acid sequence of the signature Yxx(L/I)x6-8Yxx(L/I), where Y, L, I indicate tyrosine, leucine and isoleucine residues, x denotes any amino acids, the subscript 6-8 indicates a sequence of 6 to 8 amino acids in length. This motif is very common in activator receptors of the non-catalytic tyrosine-phosphorylated receptor (NTR) family and is referred to as immunoreceptor tyrosine-based activation motif (ITAM).^[5] CD3 δ , CD3 γ and CD3 ϵ each contain a single ITAM, while CD3 ζ contains three ITAMs. In total the TCR complex contains 10 ITAMs.^[12] Phosphorylated ITAMs act as binding site for SH2-domains of additionally recruited proteins.

Antigen discrimination

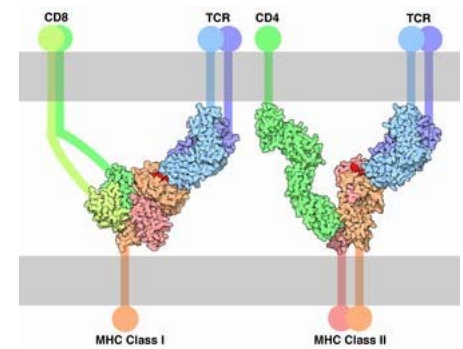
Each T cell expresses clonal TCRs which recognize a specific peptide loaded on a MHC molecule (pMHC), either on MHC class II on the surface of antigen-presenting cells or MHC class I on any other cell type.^[13] A unique feature of T cells is their ability to discriminate between peptides derived from healthy, endogenous cells and peptides from foreign or abnormal (e.g. infected or cancerous) cells in the body.^[14] Antigen presenting cells do not discriminate between self and foreign peptides and typically express a large number of self derived pMHC on their cell surface and only a few copies of any foreign pMHC. For example, it has been shown that cells infected with HIV have only 8-46 HIV specific pMHCs next to 100000 total pMHC per cell.^{[15][16]}

Because T cells undergo positive selection in the thymus there is a non-negligible affinity between

NCBI	6965 (https://www.ncbi.nlm.nih.gov/gene?cmd=retrieve&dopt=default&list_uids=6965&rn=1)
gene	
HGNC	12271 (https://www.genenames.org/data/gene-symbol-report/#!/hgnc_id/HGNC:12271)
Other data	
Locus	Chr. 7 p14 (https://omim.org/search/?index=gene&Map&search=7p14)

self pMHC and the TCR, nevertheless, the T-cell receptor signalling should not be activated by self pMHC such that endogenous, healthy cells are ignored by T cells. However, when these very same cells contain even minute quantities of pathogen derived pMHC, T cells must get activated and initiate immune responses. The ability of T cells to ignore healthy cells but respond when these same cells express a small number of foreign pMHC is known as antigen discrimination.^{[17][18]}

To do so, T cells have a very high degree of antigen specificity, despite of fact that the affinity to the peptide/MHC ligand is rather low in comparison to other receptor types.^[19] The affinity, given as the dissociation constant (K_d), between a TCR and a pMHC was determined by surface plasmon resonance (SPR) to be in the range of 1-100 μM , with an association rate (k_{on}) of 1000 -10000 $\text{M}^{-1} \text{s}^{-1}$ and a dissociation rate (k_{off}) of 0.01 -0.1 s^{-1} .^[20] In comparison, cytokines have an affinity of $K_D = 10\text{-}600 \text{ pM}$ to their receptor.^[21] It has been shown that even a single amino acid change in the presented peptide that affects the affinity of the pMHC to the TCR reduces the T cell response and cannot be compensated by a higher pMHC concentration.^[22] A negative correlation between the dissociation rate of the pMHC-TCR complex and the strength of the T cell response has been observed.^[23] That means, pMHC that bind the TCR for a longer time initiate a stronger activation of the T cell. Furthermore, T cells are very sensitive. Interaction with a single pMHC is enough to trigger activation.^[24] Also, the decision whether a T cell response to an antigen is made fast. T cells rapidly scan pMHC on an antigen presenting cell to increase the change of finding a specific pMHC. On average, T cell encounter 20 APCs per hour.^[25]



T-cell receptor complexed with MHC I and II

Different models for the molecular mechanisms that underlie this highly specific and highly sensitive process of antigen discrimination have been proposed. The occupational model simply suggests that the TCR response is proportional to the number of pMHC bound to the receptor. Given this model, a shorter lifetime of a peptide can be compensated by higher concentration such that the maximum response of the T cell stays the same. However, this cannot be seen in experiments and the model has been widely rejected.^[23] The most accepted view is that the TCR engages in kinetic proofreading. The kinetic proofreading model proposes that a signal is not directly produced upon binding but a series of intermediate steps insure a time delay between binding and signal output. Such intermediate "proofreading" steps can be multiple rounds of tyrosine phosphorylation. These steps require energy and therefore do not happen spontaneously, only when the receptor is bound to its ligand. This way only ligands with high affinity that bind the TCR for a long enough time can initiate a signal. All intermediate steps are reversible, such that upon ligand dissociation the receptor reverts to its original unphosphorylated state before a new ligand binds.^[26] This model predicts that maximum response of T cells decreases for pMHC with shorter lifetime. Experiments have confirmed this model.^[23] However, the basic kinetic proofreading model has a trade-off between sensitivity and specificity. Increasing the number of proofreading steps increases the specificity but lowers the sensitivity of the receptor. The model is therefore not sufficient to explain the high sensitivity and specificity of TCRs that have been observed. (Altan Bonnet2005) Multiple models that extend the kinetic proofreading model have been proposed, but evidence for the models is still controversial.^{[14][27][28]}

The antigen sensitivity is higher in antigen-experienced T cells than in naive T cells. Naive T cells pass through the process of functional avidity maturation with no change in affinity. It is based on the fact that effector and memory (antigen-experienced) T cell are less dependent on costimulatory signals and higher antigen concentration than naive T cell.^[29]

Signaling pathway

The essential function of the TCR complex is to identify specific bound antigen derived from a potentially harmful pathogen and elicit a distinct and critical response. At the same time it has to ignore any self-antigen and tolerate harmless antigens such as food antigens. The signal transduction mechanism by which a T cell elicits this response upon contact with its unique antigen is termed T-cell activation. Upon binding to pMHC, the TCR initiates a signalling cascade, involving transcription factor activation and cytoskeletal remodelling resulting in T cell activation. Active T cells secrete cytokines, undergo rapid proliferation, have cytotoxic activity and differentiate into effector and memory cells. When the TCR is triggered, T cells form an immunological synapse allowing them to stay in contact with the antigen presenting cell for several hours.^[30] On a population level, T cell activation depends on the strength of TCR stimulation, the dose–response curve of ligand to cytokine production is sigmoidal. However, T cell activation on a single cell level can be characterised by a digital switch-like response, meaning the T cell is fully activated if the stimulus is higher than a given threshold, otherwise the T cell stay in its non-activated state. There is no intermediate activation state. The robust sigmoid dose-response curve on population level results from individual T cells having slightly different thresholds.^[22]

T cells need three signals to become fully activated. Signal 1 is provided by the T-cell receptor when recognising a specific antigen on a MHC molecule. Signal 2 comes from co-stimulatory receptors such as CD28, presented on the surface of other immune cells. It is expressed only when an infection was detected by the innate immune system, it is a "Danger indicating signal". This two-signal system makes sure that T cells only respond to harmful pathogens and not to self-antigens. An additional third signal is provided by cytokines, which regulate the differentiation of T cells into different subsets of effector T cells.^[30] There are myriad molecules involved in the complex biochemical process (called trans-membrane signaling) by which T-cell activation occurs. Below, the signalling cascade is described in detail.

Receptor activation

The initial triggering follows the mechanism common for all NTR receptor family members. Once the TCR binds a specific pMHC, the tyrosine residues of the [Immunoreceptor tyrosine-based activation motif]s (ITAMs) in its CD3 adaptor proteins are phosphorylated. The residues serve as docking sites for downstream signalling molecules, which can propagate the signal.^{[31][32]} Phosphorylation of ITAMs is mediate by the Scr kinase Lck. Lck is anchored to the plasma membrane by associating with the co-receptor CD4 or CD8, depending on the T cell subtype. CD4 is expressed on helper T cells and regulatory T cells, and is specific for MHC class II. CD8, on the other hand, specific for MHC class I, is expressed on cytotoxic T cells. Binding of the co-receptor to the MHC bring Lck in close proximity to the CD3 ITAMs. It has been shown that 40% of Lck is active even before the TCR binds pMHC and therefore has the ability to constantly phosphorylate the TCR.^[33] Tonic TCR signalling is avoided by the presence of phosphatase CD45 that removes phosphorylation from tyrosine residues and inhibits signal initiation. Upon binding the balance of kinase activity to phosphatase activity is perturbed, leading to a surplus of phosphorylation and initiation of the signal. How such perturbation is accomplished by TCR binding is still debated. Mechanisms involving conformational change of TCR, TCR aggregation and kinetic segregation have been suggested.^[31] Tyrosine kinase Fyn might be involved in ITAM phosphorylation but is not essential for TCR signalling.^{[34][35]}

Proximal TCR signaling

Phosphorylated ITAMs in the cytoplasmic tails of CD3 recruit protein tyrosine kinase Zap70 that can bind to the phosphorylated tyrosine residues with its SH2 domain. This brings Zap70 into close proximity to Lck which results to its phosphorylation and activation by Lck.^[36] Lck phosphorylates a number of different proteins in the TCR pathway.^[37] Once activated, Zap70 is able to phosphorylate multiple tyrosine residues of the transmembrane protein LAT. LAT is a scaffold protein associated with the membrane. It itself does not have any catalytic activity but it provides binding sites for signalling molecules via phosphorylated tyrosine residues. LAT associates with another scaffolding protein Slp-76 via the Grap2 adaptor protein, which provides additional binding sites. Together LAT and Slp-76 provide a platform for the recruitment of many downstream signalling molecules. By bringing these signalling molecules into close proximity, they can then be activated by Lck, Zap70 and others kinases. Therefore, the LAT/Slp76 complex act as a highly cooperative signalosome.^[36]

Molecules that bind the LAT/Slp76 complex include: Phospholipase C γ 1 (PLC γ 1), SOS via a Grb2 adaptor, Itk, Vav, Nck1 and Fyb.^[36]

Signal transduction to the nucleus

PLC γ is a very important enzyme in the pathway as it generates second messenger molecules. It is activated by the tyrosine kinase Itk which is recruited to the cell membrane by binding to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 is produced by the action of Phosphoinositide 3-kinase(PI-3K), which phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP2) to produce PIP3. It is not known that PI-3K is activated by the T cell receptor itself, but there is evidence that CD28, a co-stimulatory receptor providing the second signal, is able to activate PI-3K. The interaction between PLC γ , Itk and PI-3K could be the point in the pathway where the first and the second signal are integrated. Only if both signals are present, PLC γ is activated.^[30] Once PLC γ is activated by phosphorylation, it hydrolyses PIP2 into two secondary messenger molecules, namely the membrane-bound diacyl glycerol(DAG) and the soluble inositol 1,4,5-trisphosphate (IP3).^[38]

These second messenger molecules amplify the TCR signal and distribute the prior localised activation to the entire cell and activate protein cascades that finally lead to the activation of transcription factors. Transcription factors involved in T cell signalling pathway are the NFAT, NF- κ B and AP1, a heterodimer of proteins Fos and Jun. All three transcription factors are needed to activate the transcription of interleukin-2(IL2) gene.^[30]

NFAT

NFAT activation depends on calcium signaling. IP3 produced by PLC- γ is no longer bound to the membrane and diffuses rapidly in the cell. Binding of IP3 to calcium channel receptors on the endoplasmic reticulum (ER) induces the release of calcium (Ca²⁺) into the cytosol. The resulting low Ca²⁺ concentration in the ER causes STIM1 clustering on the ER membrane, which in turn leads to activation of cell membrane CRAC channels that allows additional calcium to flow into the cytosol from the extracellular space. Therefore, levels of Ca²⁺ are strongly increased in the T cell. This cytosolic calcium binds calmodulin, inducing a conformational change of the protein such that it can then bind and activate calcineurin. Calcineurin, in turn, dephosphorylates NFAT. In its deactivated state, NFAT cannot enter the nucleus as its nuclear localisation sequence (NLS) cannot be recognised by nuclear transporters due to phosphorylation by GSK-3. When dephosphorylated by Calcineurin translocation of NFAT into the nucleus is possible.^[30] Additionally, there is evidence that PI-3K via signal molecules recruits the protein kinase AKT to the cell membrane. AKT is able to deactivate GSK3 and thereby inhibiting the phosphorylation of

NFAT, which could contribute to NFAT activation.^[36]

NF-κB

NF-κB activation is initiated by DAG, the second, membrane bound product of PLCγ hydrolysis of PIP₂. DAG binds and recruits Protein kinase C θ (PKCθ) to the membrane where it can activate the membrane bound scaffold protein CARMA1. CARMA1 then undergoes a conformational change which allows it to oligomerise and bind the adapter proteins BCL10, CARD domain and MALT1. This multisubunit complex binds the Ubiquitin ligase TRAF6. Ubiquitination of TRAF6 serves as scaffold to recruit NEMO, IκB kinase (IKK) and TAK1.^[30] TAK 1 phosphorylates IKK, which in turn phosphorylates the NF-κB inhibitor I-κB, leading to the ubiquitination and subsequent degradation of I-κB. I-κB blocks the NLS of NF-κB therefore preventing its translocation to the nucleus. Once I-κB is degraded, it cannot bind to NF-κB and the NLS of NF-κB becomes accessible for nuclear translocation.^[30]

AP1

Activation of *AP1* involves three MAPK signalling pathways. These pathways use a phosphorylation cascade of three successive acting protein kinases to transmit a signal. The three MAPK pathways in T cells involve kinases of different specificities belonging to each of the MAP3K, MAP2K, MAPK families. Initial activation is done by the GTPase Ras or Rac which phosphorylate the MAP3K.^[30] A cascade involving the enzymes Raf, MEK1, ERK results in the phosphorylation of Jun, conformational change allows Jun to bind to Fos and hence AP-1 to form. AP-1 then acts as transcription factor. Raf is activated via the second messenger DAG, SOS, and Ras. DAG recruits among other proteins the RAS guanyl nucleotide-releasing protein (RasGRP), a guanine nucleotide exchange factor (GEF), to the membrane. RasGRP activates the small GTPase Ras by exchanging Guanosine diphosphate (GDP) bound to Ras against Guanosine triphosphate (GTP). Ras can also be activated by the guanine nucleotide exchange factor SOS which binds to the LAT signalosome. Ras then initiates the MAPK cascade.^[36] The second MAPK cascade with MEKK1, JNKK, JNK induces protein expression of Jun. Another cascade, also involving MEKK1 as MAPK3, but then activating MKK3 /6 and p38 induces Fos transcription. Activation of MEKK1, additionally to being activated by Ras, involves Slp-76 recruiting the GEF Vav to the LAT signalosome, which then activates the GTPase Rac. Rac and Ras activate MEKK1 and thereby initiate the MAPK cascade.^[36]

See also

- Co-stimulation
- ImmTAC
- MHC multimer

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External links

- T-cell Group – Cardiff University (<http://www.tcells.org/scientific/abTCR/>)
- UMich Orientation of Proteins in Membranes *protein/pdbid-2hac* (<http://opm.phar.umich.edu/protein.php?pdbid=2hac>) – Zeta-zeta dimer of T-cell receptor
- T-Cell+Receptor (<https://meshb.nlm.nih.gov/record/ui?name=T-Cell%20Receptor>) at the US National Library of Medicine Medical Subject Headings (MeSH)

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Patente PCT

RECEPTORES DE LINFOCITOS T E INMUNOTERAPIAS BASADAS EN EL USO DE LOS MISMOS CONTRA CÁNCERES POSITIVOS PARA PRAME

Detalle del pago

Id.	Rentístico	Concepto y Servicio	Cant.	Val. Unit.	Subtotal
1	50005-01-03	Modificaciones / Correcciones al registro Patente PCT (DESCUENTO INTERNET)	1	\$ 176,000	\$ 176,000
2	50005-01-02	Pago unitario reivindicacion adicional patente de invencion (A partir de de undí½cima reivindicacion) (DESCUENTO INTERNET)	4	\$ 44,500	\$ 178,000
TOTAL					\$ 354,000