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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐

No. Solicitud Internacional PCT/US2008/002498
Fecha de Presentación Internacional 26/Febrero/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 10 a 159	23/Septiembre/2009
Reivindicaciones:	Folios 160 a 172	23/Septiembre/2009
Dibujos:	Folios 173 a 196	23/Septiembre/2009
Listado de Secuencias:	Folios 197 a 217	23/Septiembre/2009
Prioridad:	US 60/903,693	27/Febrero/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

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3. Objeto de la invención

Título de la solicitud: "Anticuerpos OX40 antagonistas y uso en el tratamiento de enfermedades inflamatorias y autoinmunes".

El problema planteado en esta solicitud consiste en la necesidad de suministrar un anticuerpo antagonista dirigido a la proteína OX40 para el tratamiento de enfermedades inflamatorias y autoinmunes.

Para resolver este problema técnico la solicitud en estudio proporciona un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/395; A61P 37/00; C07K 16/28.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 26 – 34 y 42 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 39 y 40 no es patentable, de acuerdo con el Art 14.

Las reivindicaciones 37, 38 y 41 definen el anticuerpo por referencia al uso, lo cual no es patentable de acuerdo con el Art 14.

6. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque hace referencia a materia no patentable, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 – 12 carecen de concisión porque definen el anticuerpo por una región de cadena pesada o ligera al utilizar el término "y/o". Se recuerda al solicitante que un anticuerpo se define por la secuencia de aminoácidos de las cadenas pesada y ligera, la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera o la secuencia de aminoácidos de las 6 regiones determinantes de complementariedad (CDRs) en su conjunto. Además, no existe soporte en la descripción que un anticuerpo que comprende solamente una CDR comparte la actividad del anticuerpo reclamado.

Las reivindicaciones 20 y 21 no son concisas porque utilizan las expresiones "un marcador", "una citotoxina o inmunotoxina", lo cual dificulta determinar la extensión

del objeto que se busca proteger. Se sugiere definir estos términos por sus características técnicas.

La reivindicación 25 reclama una composición que comprende el anticuerpo. Se recuerda al solicitante que una composición se caracteriza por sus componentes y las proporciones dentro de la misma.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para el hibridoma al que se hace referencia en la reivindicación 36.

Las reivindicaciones 43 y 44 carecen de concisión porque reclaman kits que comprenden el anticuerpo o la composición en "una cantidad predeterminada". Se sugiere definir las proporciones dentro de los mismos.

Las reivindicaciones 45 – 48 carecen de concisión porque reclaman un dispositivo médico. Sin embargo, no se definen las características técnicas que comprenden el dispositivo y no se encuentra soporte en la descripción de las ventajas técnicas del dispositivo, por lo que se sugiere al solicitante eliminar estas reivindicaciones del alcance del capítulo reivindicatorio.

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 4 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

1° Un anticuerpo que se enlaza específicamente a OX40 que comprende i) una CDR1 de VH que tiene la secuencia SEQ ID NO: 17; ii) una CDR2 de VH que tiene la secuencia SEQ ID NO: 18; iii) una CDR3 de VH que tiene la secuencia SEQ ID NO: 19; iv) una CDR1 de VL que tiene la secuencia SEQ ID NO: 12; v) una CDR2 de VL que tiene la secuencia SEQ ID NO: 13; y vi) una CDR3 de VL que tiene la secuencia SEQ ID NO: 14. Reivindicaciones 1 – 48, parcialmente.

2° Un anticuerpo que se enlaza específicamente a OX40 que comprende i) una CDR1 de VH que tiene la secuencia SEQ ID NO: 17; ii) una CDR2 de VH que tiene la secuencia SEQ ID NO: 18; iii) una CDR3 de VH que tiene la secuencia SEQ ID NO: 19; iv) una CDR1 de VL que tiene la secuencia SEQ ID NO: 12; v) una CDR2 de VL que tiene la secuencia SEQ ID NO: 13; y vi) una CDR3 de VL que tiene la secuencia SEQ ID NO: 16. Reivindicaciones 1 – 48, parcialmente.

3° Un anticuerpo que se enlaza específicamente a OX40 que comprende i) una CDR1 de VH que tiene la secuencia SEQ ID NO: 17; ii) una CDR2 de VH que tiene la secuencia SEQ ID NO: 18; iii) una CDR3 de VH que tiene la secuencia SEQ ID NO: 19; iv) una CDR1 de VL que tiene la secuencia SEQ ID NO: 15; v) una CDR2 de VL que tiene la secuencia SEQ ID NO: 13; y vi) una CDR3 de VL que tiene la secuencia SEQ ID NO: 14. Reivindicaciones 1 – 48, parcialmente.

4° Un anticuerpo que se enlaza específicamente a OX40 que comprende i) una CDR1 de VH que tiene la secuencia SEQ ID NO: 17; ii) una CDR2 de VH que tiene la secuencia SEQ ID NO: 18; iii) una CDR3 de VH que tiene la secuencia SEQ ID NO: 19; iv) una CDR1 de VL que tiene la secuencia SEQ ID NO: 15; v) una CDR2 de VL que tiene la secuencia SEQ ID NO: 13; y vi) una CDR3 de VL que tiene la secuencia SEQ ID NO: 16. Reivindicaciones 1 – 48, parcialmente.

No se guarda un concepto inventivo común porque los anticuerpos no comparten una estructura común esencial para la actividad y porque anticuerpos dirigidos a OX40 ya eran conocidos en los documentos D1 y D2.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Febrero 27 de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	<u>WO2004019866</u>	Compositions and methods for treating cardiovascular disease	11/Marzo/2004
D2	<u>WO2006029879</u>	Anti-OX40L antibodies	23/Marzo/2006

D1 <http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2004019866A2&KC=A2&FT=D&ND=&date=20040311&DB=&locale=en> EP divulga un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano (Pág. 20, líneas 31-33).

D2 <http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2006029879A2&KC=A2&FT=D&ND=3&date=20060323&DB=EPODOC&locale=en> EP divulga un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano (Resumen y Pág. 70, reivindicación 15).

9. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La **reivindicación 1** consiste en un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano, el anticuerpo está caracterizado porque comprende:

(a) una región variable de cadena pesada (VH) que comprende:

- i) una CDR1 de VH que tiene la secuencia de aminoácidos de SEQ ID NO:17;
- ii) una CDR2 de VH que tiene la secuencia de aminoácidos de SEQ ID NO:18
y/o
- iii) una CDR3 de VH que tiene la secuencia de aminoácidos de SEQ ID NO:19
y/o

(b) una región variable de cadena ligera (VL) que comprende:

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- i) una CDR1 de VL que tiene la secuencia de aminoácidos de SEQ ID NO:12, 15 o 61;
- ii) una CDR2 de VL que tiene la secuencia de aminoácidos de SEQ ID NO:13 y/o
- iii) una CDR3 de VL que tiene la secuencia de aminoácidos de SEQ ID NO: 14 o 16.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano, el anticuerpo está caracterizado porque comprende: a) una región variable de cadena pesada (VH) que comprende: i) una CDR1 de VH que tiene la secuencia de aminoácidos de SEQ ID NO:17; ii) una CDR2 de VH que tiene la secuencia de aminoácidos de SEQ ID NO:18 y/o iii) una CDR3 de VH que tiene la secuencia de aminoácidos de SEQ ID NO:19 y/o b) una región variable de cadena ligera (VL) que comprende: i) una CDR1 de VL que tiene la secuencia de aminoácidos de SEQ ID NO:12, 15 o 61; ii) una CDR2 de VL que tiene la secuencia de aminoácidos de SEQ ID NO:13 y/o iii) una CDR3 de VL que tiene la secuencia de aminoácidos de SEQ ID NO: 14 o 16.	Un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano (Pág. 20, líneas 31-33)	Un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano (Resumen y Pág. 70, reivindicación 15)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano (Pág. 20, líneas 31-33). D1 también enseña un método para producir el anticuerpo (Pág. 39), una composición que comprende el anticuerpo (Pág. 2) y un dispositivo médico para la liberación del anticuerpo por vía pulmonar (Pág. 54, párrafo 4).

La diferencia entre la invención, definida en la reivindicación 1 y el anticuerpo de D1 consiste en que la solicitud define secuencias específicas para las regiones variables de las cadenas pesada y ligera.

D2 enseña un anticuerpo antagonista aislado que se enlaza específicamente a un epítipo de OX40 humano (Resumen y Pág. 70, reivindicación 15). D2 también divulga un método para producir el anticuerpo que comprende cultivar una célula

hospedera que comprende el vector que a su vez comprende el ácido nucleico que codifica el anticuerpo (Pág. 72, reivindicación 26), una composición farmacéutica que comprende el anticuerpo (Pág. 73, reivindicación 34) y un kit que comprende el anticuerpo (Pág. 73, reivindicación 39).

La diferencia entre la invención, definida en la reivindicación 1 y el anticuerpo de D2 consiste en que la solicitud define secuencias específicas para las regiones variables de las cadenas pesada y ligera.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja al anticuerpo reclamado en esta solicitud porque no parece presentar una mayor actividad moduladora de la actividad de la proteína OX40.

El problema técnico objetivo de esta solicitud consiste en la necesidad de anticuerpos antagonistas de OX40 alternativos a los ya conocidos en los documentos D1 y D2.

Teniendo en cuenta que los documentos D1 y D2 ya divulgaban anticuerpos antagonistas de la proteína OX40, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, obtener líneas celulares alternativas para producir anticuerpos con la misma actividad. La persona del oficio medianamente versada en la materia se vería motivada a obtener anticuerpos antagonistas de OX40 para el tratamiento de enfermedades inflamatorias y autoinmunes porque es bien conocido en el estado del arte que la señalización a través de este receptor resulta en un incremento de la expresión de citoquinas Th1 y Th2^{1,2,3}.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y sus dependientes 2 – 21 y en 22 – 25, 35, 36 y 43 – 48, es obvia y no tiene Nivel Inventivo.

10. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2004019866	1 – 25, 35, 36 y 43 – 48	Nivel Inventivo
D2	WO2006029879	1 – 25, 35, 36 y 43 – 48	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso

¹ K. Sugamura, et al. Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40. Nature Reviews Immunology. Vol. 4, Junio 2004.
² S. Salek, et al. Regulation of CD4 T cell memory by OX40 (CD134). Vaccine, Vol. 24, Pág. 872-883. Publicado 7 de Septiembre de 2005.
³ Y. Satake. Characterization of rat OX40 ligand by monoclonal antibody. Biochemical and Biophysical Research Communications. Vol. 270, Pág. 1041-1048.
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de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

17 8 SET. 2012

Elaborado por: Juan David Moncaleano P. 
Revisado por: Consuelo Leguizamón L. 

THERAPEUTIC TARGETING OF THE EFFECTOR T-CELL CO-STIMULATORY MOLECULE OX40

Kazuo Sugamura*, Naoto Ishii* and Andrew D. Weinberg[‡]

An emerging immunotherapeutic strategy for T-cell-mediated diseases is to directly target antigen-specific T cells that are responsible for the clinical effects, without causing general widespread immunosuppression. A T-cell co-stimulatory molecule, OX40, which is transiently expressed after antigen recognition, fits these criteria in several immune-mediated diseases. *In vivo* blockade of OX40 signalling specifically suppresses the function of recently activated autoantigen-specific T cells, leading to inhibition of autoimmune disease without severe immunosuppression. In addition, deliberate ligation of OX40 in tumour-bearing hosts enhances anticancer immunity. We discuss how targeting OX40 is potentially an ideal strategy for immune-based therapies in several human diseases.

IMMUNOTOXIN

An antibody that is chemically or genetically modified to produce a hybrid molecule that has an antibody-binding domain and toxin that can kill a cell once internalized.

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OX40 (CD134) is a member of the tumour-necrosis factor (TNF) receptor superfamily^{1,2}, and is transiently expressed by T cells after ligation of the T-cell receptor (TCR)¹⁻⁴. Initially identified as a T-cell activation marker in 1987 (REF. 3), it has been shown to have co-stimulatory functions^{2,4,7}. The ligand for OX40 (OX40L) was originally termed glycoprotein 34 kDa (GP34) and was identified on human T-cell leukaemia virus type 1-transformed T cells⁸. Cloning of GP34 revealed that the molecule belonged to the TNF superfamily⁹. Subsequently, GP34 was identified as the ligand for OX40 (REFS 4,5). OX40L is mainly expressed by antigen-presenting cells (APCs), such as activated B cells, dendritic cells, macrophages and Langerhans cells, as well as T cells and endothelial cells¹⁰⁻¹⁷. Studies of primary T-cell responses have revealed that the OX40–OX40L interaction between a T cell and APC contributes to optimal T-cell function^{6,7,12,18,19}. In particular, OX40–OX40L interactions are crucial for the generation of memory T cells, by promoting the survival of effector T cells after antigen priming²⁰⁻²².

It was first shown in 1996 that selective depletion of OX40-expressing effector T cells — by targeting OX40 with a toxin-fused OX40-specific monoclonal antibody

(IMMUNOTOXIN) — was therapeutic for a T-cell-mediated autoimmune disease²³. Since that report, several studies have confirmed the beneficial effects of targeting OX40 in animal disease models^{12,24-43}. However, although immunotherapies using antibodies and recombinant proteins that target T-cell co-stimulatory molecules, such as OX40, 4-1BB (CD137) and CD28 or cytotoxic T lymphocyte antigen 4 (CTLA4), have been successful in several animal disease models^{12,23-45}, clinical trials targeting these molecules are in their infancy. Among these candidate molecules, OX40 is a promising therapeutic target for allergic, inflammatory and autoimmune diseases, as well as a target for enhancing immunity to tumour antigens, through its ability to target antigen-specific effector T cells directly. Here, we discuss the present and future prospects of OX40-specific therapies.

Roles of OX40 and CD28 in co-stimulation

Optimal activation of naive T cells requires not only interaction between the TCR and antigen–MHC complexes, but also co-stimulation provided by co-stimulatory ligands expressed by APCs⁴⁴⁻⁵⁰ (FIG. 1). In the absence of co-stimulatory signals, naive T cells that recognize antigen will become anergic and die.

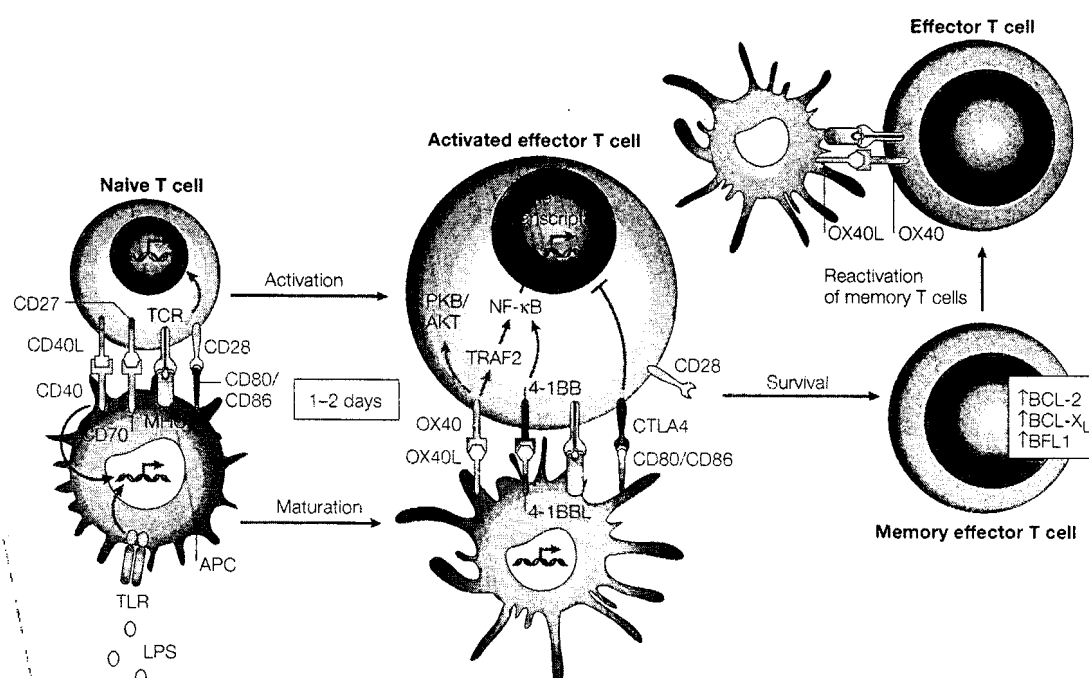


Figure 1 | Roles for OX40–OX40L interactions in T-cell function. Optimal activation of naive T cells after antigen recognition requires signals through early co-stimulatory molecules, such as CD28–CD80/CD86, CD27–CD70 and CD40L–CD40. After antigen stimulation together with signals through CD28, activated T cells express OX40, 4-1BB and cytotoxic T lymphocyte antigen 4 (CTLA4) on their cell surface. Similarly, stimulation of antigen-presenting dendritic cells with CD40L through CD40, or lipopolysaccharide (LPS) through Toll-like receptors (TLRs), induces optimal OX40L and 4-1BBL expression. So, efficient interaction between OX40 and OX40L probably occurs 1–2 days after antigen recognition. During the effector phase, as the affinity of CTLA4 for CD80/CD86 is much higher than that of CD28, CD80/CD86 on antigen-presenting cells (APCs) preferentially binds to CTLA4 on activated T cells, resulting in suppression of T-cell activation. Ligand of OX40 on T cells by OX40L on APCs can induce the activation of nuclear factor- κ B (NF- κ B). It has been shown that crucial OX40-specific signals for memory T-cell generation are transmitted through TRAF2 (tumour-necrosis factor receptor-associated factor 2). Sustained PKB/AKT activation in activated T cells has also been shown to be important for OX40-specific effector T-cell survival. OX40-induced survival of effector T cells might contribute to the generation of memory T cells, in which expression of the anti-apoptotic proteins BCL-2, BCL-X_L and BFL1 is maintained. Furthermore, OX40 signals through T-cell–APC interactions also contribute to the optimal reactivation of memory T cells. PKB, protein kinase B; TCR, T-cell receptor.

Among several co-stimulatory receptors, signals through CD28, which is constitutively expressed by naive T cells, are indispensable for the T cells to respond to antigen and clonally expand. The ligands for CD28 — CD80 and CD86 — can also bind another receptor, CTLA4, which is transiently expressed following activation of T cells after antigen recognition, and provides a negative signal^{44,45,47,48} that counteracts the pro-inflammatory CD28 signal. As the binding affinity of CTLA4 to CD80/CD86 is much higher than that of CD28, T-cell activation is effectively suppressed after expression of CTLA4 on the T-cell surface^{45,48} (FIG. 1); CTLA4, therefore, is important in downregulating T-cell function.

In addition to the importance of the CD28/CTLA4 pathways during T_H cell priming, further molecular interactions are required to generate memory T cells from activated effector T cells. Several members of the TNF receptor superfamily, such as OX40, CD27 and 4-1BB, are known to function in this capacity. These molecules induce co-stimulatory signals, which prevent antigen-induced cell death and consequently promote the survival of effector T cells, resulting in

the generation of memory T cells^{44,49,50} (FIG. 1). Unlike the CD28/CTLA4 pathways, these co-stimulatory receptors lack additional ligands that can downregulate T-cell function⁴⁹. Among these co-stimulators, the OX40–OX40L interaction is crucial for T-cell activation and survival. OX40 expression by T cells is induced by TCR stimulation, and peaks 48 hours after antigen recognition²⁶. Similarly, OX40L expression by APCs is inducible following CD40 or lipopolysaccharide stimulation, and peaks 48–72 hours after *in vitro* stimulation^{11,19,51,52}. So, the interaction between OX40 and OX40L seems to occur approximately 2 days after antigen recognition by T cells. CD28 and its binding partners CD80/CD86, by contrast, interact early after antigen recognition, due, in part, to constitutive expression of CD28 by T cells^{45,48,49}. The absence of CD28 signals suppresses T-cell division mainly during the first 2–3 days after antigen stimulation^{22,53}, resulting in suppressed effector T-cell generation and a consequent reduction in the number of memory T cells⁵³. OX40-deficient T cells show robust proliferation and cell division 2–3 days after TCR ligation, but reduced proliferation by 5–6 days. By days 12–13, there is also

CONTACT HYPERSENSITIVITY
A delayed-type hypersensitivity in which T cells respond to antigens that are introduced by contact with the skin. In common animal models, hapten treatment of the skin induces this hypersensitivity, which is measured by swelling of the sensitized skin, such as ear thickness.

a marked reduction in the survival of OX40-deficient T cells^{22,54}. These observations are consistent with the different expression kinetics for these two co-stimulatory molecules, and indicate that they have unique roles — early co-stimulation through CD28 and late co-stimulation through OX40 — in the generation of memory T cells (Fig. 1).

To understand fully the impact of the OX40–OX40L co-stimulatory pathway on immune responses, gene-targeted mice for OX40 and OX40L have been extensively studied. As shown in TABLE 1, both OX40- and OX40L-deficient mice have impaired T-cell function, including a reduction in the number of antigen-specific memory CD4⁺ T cells, decreased CD4⁺ T-cell infiltrates in the lungs of mice infected with influenza virus and suppressed CONTACT HYPERSENSITIVITY responses^{18,19,55}. Interestingly, CD8⁺ T-cell functions, cytotoxic T lymphocyte (CTL) activity and cytokine production are normal in these knockout mice^{18,55}.

Furthermore, in contrast to defective T-cell-dependent antibody production in CD28-deficient mice^{45,47}, OX40- or OX40L-deficient mice have normal immunoglobulin production in response to immunization or during viral infections^{15,18,55}. These observations indicate that OX40–OX40L interactions might only affect effector CD4⁺ T-cell function and be dispensable for the CTL response and immunoglobulin production in certain immune responses.

Advantages of targeting OX40

Conventional immunotherapies for autoimmune, allergic and inflammatory diseases, and organ transplantation, have been aimed at the T cell — an important effector cell that is responsible for disease pathogenesis. However, these therapies affect not only the T cells that are responsible for disease but also all the other T cells in the host, which can lead to an immunosuppressive state and the eventual development of opportunistic

Table 1 | **Abnormal immune responses in OX40-deficient and OX40L-deficient mice**

Strain	Immune responses examined	Results or phenotypic features	References
OX40-deficient mice			
(C57BL/6 x 129) hybrid	Influenza virus infection	Impaired CD4 ⁺ T-cell proliferation and IFN- γ production; reduction in CD4 ⁺ T-cell infiltration into the lungs of influenza-virus-infected mice	55
C57BL/6	Ex vivo T-cell proliferation after CD3 stimulation	Reduced T-cell proliferation	15
C57BL/6	T-cell responses to protein antigen	Reduced effector T-cell function and impaired memory T-cell generation	20
C57BL/6	Ex vivo T-cell proliferation and survival after antigen stimulation	Marked reduction in T-cell survival despite almost normal proliferation; reduction in Pkb/Akt activation in effector T cells; reduction in Bcl-2 and Bcl-X _L expression by long-lived T cells	22,54
C57BL/6	Used as donor in experimental GVHD	Reduced GVHD mortality	34
C57BL/6	Airway hyperreactivity using ovalbumin	Diminished lung inflammation; impaired T _H 2-cell responses; impaired reactivation of T _H 2 memory cells	80
C57BL/6	Experimental autoimmune encephalomyelitis	Reduced symptoms; impaired function of infiltrated T cells in the central nervous system	66
C57BL/6	Regulatory T-cell number and function	Reduced numbers of CD4 ⁺ CD25 ⁺ T cells in young OX40-deficient mice; less ex vivo suppressive function of OX40-deficient regulatory T cells	72
OX40L-deficient mice			
BALB/c	CHS in response to haptens	Reduced CHS responses; impaired APC function of splenic dendritic cells and Langerhans cells	13,18
C57BL/6	CD4 ⁺ T-cell responses to protein antigen	Impaired effector CD4 ⁺ T-cell responses; reduced memory CD4 ⁺ T-cell function	19
C57BL/6	Experimental autoimmune encephalomyelitis	Reduced symptoms; impaired function of antigen-specific T cells	65
BALB/c	Airway hyperreactivity using ovalbumin	Suppressed lung inflammation; reduced T _H 2-cell responses	43,81
BALB/c	Infection with <i>Heligmosomoides polygyrus</i>	Reduced T _H 2-cell responses; compromised host defence (increase in parasite eggs)	106
NOD	Spontaneous diabetes development	No disease symptoms	67
C57BL/6	T _H 2-cell responses in B-cell-deficient mice transferred with exogenous B cells	Less T _H 2-cell responses induced in mice transferred with OX40L-deficient B cells compared with those receiving wild-type B cells	52
129/Sv, BALB/c	Used as recipient in experimental GVHD	Reduced GVHD mortality	34
C57BL/6	Experimental inflammatory bowel disease	No disease symptoms in OX40L/Rag2 double-deficient mice transferred with CD4 ⁺ CD25 ⁺ T cells	72

APC, antigen-presenting cell; CHS, contact hypersensitivity; GVHD, graft-versus-host disease; IFN- γ , interferon- γ ; NOD, non-obese diabetic; OX40L, OX40 ligand; Rag2, recombinaase-activating gene 2; T_H, T helper.

Table 2 | Expression of OX40 and OX40L in several disease states

Disease	Cells expressing OX40	Cells expressing OX40L	Tissues	References
Humans				
Rheumatoid arthritis	CD4 ⁺ T cells	N.D.	Synovial fluid	23,107,108
Multiple sclerosis	T cells	N.D.	Perivascular inflammatory lesion	66
Crohn's disease	Mononuclear cells	Endothelial cells	Lamina propria and submucosa of the colon	73,74
Ulcerative colitis	Mononuclear cells	Endothelial cells	Lamina propria of the colon	73,74
GVHD	CD4 ⁺ and CD8 ⁺ T cells	N.D.	Peripheral blood	23,68–70
Polymyositis	Mononuclear cells	N.D.	Perivascular inflammatory sites	109
Breast cancer	CD4 ⁺ T cells	N.D.	Primary tumour and tumour-invaded lymph node	28,92,101
Colorectal cancer	Lymphocytes	N.D.	Primary tumour and tumour-invaded lymph node	110
Mice				
EAE	CD4 ⁺ T cells	Microglia/macrophages	Spinal cord and brain	12,30,65
EAE	N.D.	Vascular endothelial cells	Spinal cord	30
Hapten-induced colitis	Mononuclear cells	N.D.	Peyer's patches and lamina propria of the colon	25
T-cell-transfer-induced colitis	N.D.	CD11c ⁺ dendritic cells	Mesenteric lymph nodes	31
Ovalbumin-induced airway hyperreactivity	CD4 ⁺ CD44 ⁺ T cells	N.D.	Peribronchial and perivascular areas of the lungs	36
Influenza-virus-induced lung inflammation	CD4 ⁺ and CD8 ⁺ T cells	N.D.	Bronchoalveolar lavage and mediastinal lymph nodes	37
Cancer models	CD4 ⁺ and CD8 ⁺ T cells	N.D.	Primary tumour and tumour-draining lymph node	89,90,95
Rats				
EAE	Antigen-specific CD4 ⁺ T cells	N.D.	Spinal cord	23,93
GVHD	CD4 ⁺ T cells	N.D.	Peripheral blood, spleen, lymph nodes and liver	111,112

EAE, experimental autoimmune encephalomyelitis; GVHD, graft-versus-host disease; N.D., not determined; OX40L, OX40 ligand.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS. (EAE). An experimental model for the human disease multiple sclerosis. Autoimmune disease is induced in experimental animals by immunization with myelin antigen or peptides that are derived from myelin. The animals develop a paralytic disease with inflammation and demyelination in the brain and spinal cord.

INFLAMMATORY BOWEL DISEASE (IBD). A chronic condition of the intestine that is characterized by severe inflammation and mucosal destruction. The most common forms in humans are ulcerative colitis and Crohn's disease. Animal models indicate that they result from dysregulation of the local immune response to normally harmless commensal bacteria.

infections and cancer. More recently, second-generation immunotherapies targeting cytokines or their receptors that are responsible for disease pathogenesis have been established, but the consequences of widespread immunosuppression have not yet been resolved. Therefore, an ideal immunotherapeutic strategy for a T-cell-mediated disease would directly target the antigen-specific T cells that are responsible for disease pathogenesis, without disrupting the function of the entire T-cell repertoire.

Targeting the T-cell co-stimulatory molecules CD28 and OX40 is a promising strategy for controlling T-cell-mediated immune disorders, by regulating the T cells that cause disease. In spite of the indispensable role for CD28 during T-cell priming, CD28 blockade is unable to effectively suppress effector T-cell function or reactivation of memory T cells in many models^{56–59}. Therefore, blocking the CD28–CD80/CD86 interaction might be insufficient to prevent ongoing symptoms in certain T-cell-mediated diseases. By contrast, blockade of OX40–OX40L interactions alone will prevent ongoing immune responses being mediated by effector T cells that have recently been generated from either naive or memory T cells after antigen stimulation^{19,25,35–37,60}. OX40L blockade might be a favourable strategy in such clinical settings. In addition to its co-stimulatory roles,

OX40 is thought to contribute to T-cell migration and tissue infiltration through its interaction with OX40L on endothelial cells¹⁴. These OX40-specific functions are not apparent in the CD28 pathway. As cytokines derived from the infiltrating T cells recruit and activate other inflammatory cells, targeting OX40 might have anti-inflammatory effects by preventing the infiltration of effector T cells into the tissues. In fact, *in vivo* blockade of OX40–OX40L interactions results in a marked reduction in infiltration of CD4⁺ T cells into sites of inflammation and therefore in reduced clinical symptoms in mouse models of EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE), INFLAMMATORY BOWEL DISEASE (IBD), allergic asthma and pneumonia induced by influenza virus infection^{12,25,30–32,36,37,43}.

Recently, blocking CD28 signals with a CTLA4–immunoglobulin fusion protein has been shown to be effective in reducing symptoms in patients with rheumatoid arthritis^{61,62}. Based on the differences in function and expression between the CD28 and OX40 pathways, the combination of therapies that target both pathways might be more effective than targeting either pathway alone. For example, in several mouse models of skin transplantation, blockade of both CD28 and OX40 was more effective in preventing graft rejection than either blockade alone⁴⁰. Moreover,

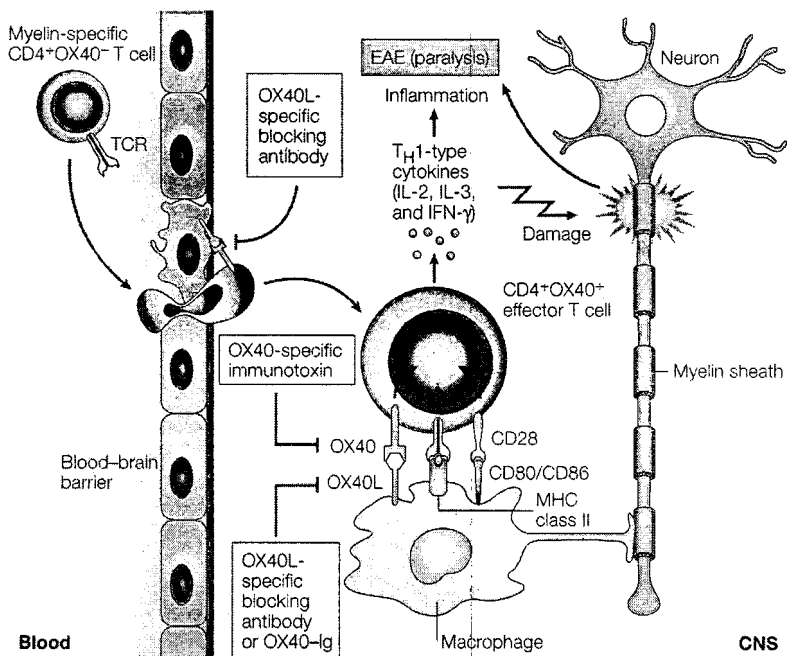


Figure 2 | Therapeutic intervention in animals with experimental autoimmune encephalomyelitis by targeting OX40⁺ T cells at the site of inflammation. Myelin-reactive CD4⁺ T cells isolated from the peripheral blood of animals with experimental autoimmune encephalomyelitis (EAE) do not express OX40, but myelin-reactive T cells isolated from the central nervous system (CNS) are positive for OX40 expression. This scenario allows for target-organ-specific immunotherapy of myelin-reactive T cells. At present, it is not known exactly where and when OX40 expression is upregulated by T cells after they cross the blood-brain barrier and enter the CNS. OX40 ligand (OX40L) is expressed by macrophages and endothelial cells in the CNS. The CNS-isolated macrophages express MHC class II molecules and have been shown to stimulate proliferation of myelin-reactive T cells in an OX40-dependent manner. The current literature indicates that signals from the T-cell receptor (TCR), CD28 and OX40 are all required for effective activation of myelin-reactive effector T cells in the CNS. If all three pathways are intact, T helper 1 (T_H1)-type cytokines are produced, which, in turn, cause demyelination, leading to the clinical signs of EAE (paralysis). Two modes of OX40-specific therapy have been shown to inhibit the clinical signs of EAE: deleting OX40⁺ T cells with an OX40-specific immunotoxin; or blocking OX40–OX40L interactions with an OX40L-specific antibody or an OX40–immunoglobulin fusion protein (OX40-Ig), thereby inhibiting OX40-specific T-cell signalling and/or blocking T-cell entry into the CNS. IFN-γ, interferon-γ; IL, interleukin.

simultaneous blockade of the CD28, CD40 and OX40 pathways completely inhibited allograft rejection, whereas blockade of CD28 and CD40 alone was unsuccessful³⁹.

Targeting OX40/OX40L in autoimmunity
The presence of OX40⁺ lymphocytes and OX40L⁺ cells at the sites of inflammation in T-cell-mediated disorders, such as autoimmunity, allergic and infectious diseases, and GRAFT-VERSUS-HOST DISEASE (GVHD), is well documented (TABLE 2). Targeting OX40 might dampen the autoantigen-specific T-cell responses and therefore be a potential therapy for these diseases. Several reagents that target the OX40-specific pathway have been used. Monoclonal antibodies specific for OX40L and OX40–immunoglobulin fusion proteins bind to OX40L and therefore interfere with OX40–OX40L interactions. Alternatively, toxin-fused OX40-specific antibody can deplete OX40-expressing effector T cells specifically. Here, we discuss how targeting OX40 with these reagents can dampen T-cell-mediated diseases.

GRAFT-VERSUS-HOST DISEASE (GVHD). Tissue damage in a recipient of allogeneic transplanted tissue (usually a bone-marrow transplant) that results from the activity of donor T cells that recognize the recipient's tissue as foreign. GVHD varies markedly in severity, but can be life-threatening in severe cases. Typically, damage to the skin and gut mucosa leads to clinical manifestations.

Experimental autoimmune encephalomyelitis. The first evidence that OX40 could be targeted for therapeutic benefit in an autoimmune disease was obtained in a rat model of EAE⁶³. Autoantigen-specific CD4⁺ T cells that were isolated from the site of inflammation (central nervous system, CNS) expressed high levels of OX40 (REFS 63,64), whereas T cells isolated from non-inflammatory compartments (peripheral blood and spleen) in the same animals had low levels of OX40 expression. At present, it is not known exactly where and when OX40 expression is upregulated by T cells after they cross the BLOOD–BRAIN BARRIER and enter the CNS. Myelin-specific CD4⁺ T cells that were isolated from the CNS one day before disease onset expressed the highest levels of OX40, and as the disease-related signs and symptoms peaked, the number of OX40⁺ T cells declined until EAE resolved, at which time OX40⁺ T cells could no longer be detected⁶³. These observations indicated that OX40-specific reagents might be effective if applied during the early stages of EAE by targeting the autoantigen-specific T cells in inflamed tissue, but leaving the peripheral T-cell repertoire unperturbed.

Two different OX40-specific therapeutic interventions have been successful in the treatment of animals with EAE (FIG. 2). One method depletes OX40⁺ T cells by administration of an OX40-specific immunotoxin²³, whereas the other uses an OX40L-binding agent to block OX40–OX40L interactions *in vivo*^{12,30}. OX40-specific immunotoxin-mediated depletion of autoreactive T cells was shown to be effective when delivered at the first signs of disease onset²³. This therapy decreased the number of myelin-reactive T cells isolated from the site of inflammation by 10–100-fold. The OX40-depletion therapy was specific, as only the recently activated myelin-specific T cells of the CNS bound the OX40-specific immunotoxin²³.

The second mode of therapy is based on blockade of the OX40–OX40L interaction *in vivo* using agents that bind to OX40L (recombinant OX40–immunoglobulin fusion protein or an OX40L-specific antibody)^{12,24–27,29–31,33–38}. Both OX40 and OX40L have been identified on the surface of cells in the CNS of mice with EAE, and both OX40- and OX40L-deficient mice are less susceptible to EAE^{12,30,65,66}. Two separate studies reported that blocking OX40–OX40L interactions in wild-type mice with EAE decreased clinical signs of the disease^{12,30}, although each study used a different timing scheme and a different reagent to block OX40–OX40L interactions. The first study used an OX40–immunoglobulin fusion protein administered at disease onset¹², whereas the second study used an OX40L-specific antibody that was administered on several consecutive days before disease onset³⁰. The first study showed that OX40L was expressed by CD11b⁺MHC class II⁺ cells in the inflamed CNS of mice with EAE¹² and these cells could co-stimulate autoreactive T cells through OX40–OX40L interactions. Administration of OX40–immunoglobulin fusion protein during the initial stages of disease resulted in a marked reduction in the severity of EAE during the acute phase.

However, the OX40-immunoglobulin-treated mice relapsed with similar frequency when compared with control mice. When OX40-immunoglobulin was delivered during an active relapse, the disease severity was reduced, but as soon as the treatment was discontinued, the mice redeveloped clinical signs of disease¹². These data indicate that blocking OX40–OX40L interactions *in vivo* inhibits the function of autoreactive T cells, but that this effect is short-lived and the autoreactive T cells persist and can reinitiate clinical signs of disease.

The second study showed that treatment with OX40L-specific antibody before clinical signs of disease was effective during the acute phase of EAE and its therapeutic effect was dose dependent. This regimen had little effect on antigen-specific T-cell responses in the draining lymph nodes; however, a decrease in the number of OX40⁺ myelin-specific T cells was observed in the target tissue (spinal cord). These authors detected OX40L expression on endothelial cells in the inflamed CNS, which led them to speculate that the mode of action of OX40L-specific antibody was blockade of T-cell migration through the blood–brain barrier³⁰. Both *in vivo* blocking studies indicate that OX40–OX40L interactions probably have a dual role in this T-cell-mediated disease: OX40-mediated entry into the CNS through endothelial barriers and OX40-specific co-stimulation once the T cells have entered the CNS. FIG. 2 shows the OX40–OX40L interactions that are thought to lead to the development of EAE and illustrates how OX40-specific therapies have been used to ameliorate clinical signs of EAE.

Diabetes. Recently, a crucial role for OX40–OX40L interactions in the development of autoimmune diabetes has been shown. Whereas the mouse models previously discussed were experimentally treated to induce disease, the diabetes model described in this report is a spontaneous form of autoimmune disease, and might be more relevant to human autoimmune diseases. OX40L-deficient mice that were crossed onto the NON-OBESE DIABETIC (NOD) background did not develop diabetes⁶⁷. Interestingly, this report showed comparable numbers of pathogenic antigen-specific memory T cells and infiltrated T cells around islets in wild-type and OX40L-deficient diabetes-prone mice when these mice were old enough to develop diabetes⁶⁷. These results indicate an additional OX40-specific mechanism, other than memory T-cell generation or T-cell trafficking, that might contribute to the development of autoimmune diabetes.

Targeting OX40/OX40L in inflammation

Graft-versus-host disease. Patients with active GVHD have increased numbers of circulating CD4⁺OX40⁺ T cells^{23,68–70}; it has been proposed that quantifying OX40⁺ cells could enable clinicians to predict resistance to therapy as well as the onset of chronic GVHD^{68,70}. In mouse models of GVHD, OX40 signals in effector T cells are crucial for the pathogenesis of this disease; blocking OX40–OX40L interactions by treatment with an OX40L-specific monoclonal antibody or

OX40-immunoglobulin fusion protein prevents lethal GVHD^{24,29,34}. Whereas CD28-deficient or OX40L-deficient donor T cells could still induce GVHD, OX40-deficient T cells failed to induce lethal GVHD in recipient mice³⁴. Importantly, GVHD induced by CD28-deficient T cells could be prevented by OX40L-specific antibodies³⁴. So, reagents that are designed to block OX40–OX40L interactions *in vivo* could be a promising therapy for human GVHD. Donor-derived allo-specific effector T cells during bone-marrow transplantation are also important for GRAFT-VERSUS-LEUKAEMIA (GVL) effects, which contribute to preventing cancer relapse. It is important to note that, depending on timing and dosing schemes, OX40 blockade might increase the risk of cancer relapse after bone-marrow transplantation by suppressing GVL effector T-cell function.

Inflammatory bowel disease. Crohn's disease and ulcerative colitis are forms of idiopathic intestinal inflammation accompanied by inappropriate activation of mucosal effector T cells⁷¹. These IBDs are hypothetically caused by a breakdown in mucosal tolerance to bacterial flora in the intestine⁷¹. OX40 signalling is required for the development of several mouse models of IBD, including hapten-induced colitis^{25,33}, the spontaneous development of IBD in interleukin-2 (IL-2)-deficient²⁵ and OX40L-transgenic mice³², IBD in lymphopaenic mice after transfer of CD4⁺CD25⁺CD45RB^{hi} T cells^{31,35,72}, and IBD induced by treatment with dextran sulphate sodium³⁸. *In vivo* administration of OX40L-specific monoclonal antibody or OX40-immunoglobulin fusion protein ameliorates even ongoing IBD symptoms in these models, and the disease does not develop in OX40L-deficient mice. In these various models of IBD, OX40L expression was found on dendritic cells, whereas OX40⁺ T cells were identified in the mesenteric lymph nodes and in the inflamed colon³¹. An increased number of OX40⁺ T cells has also been identified in the lamina propria of the colon from patients with ulcerative colitis and Crohn's disease^{73,74}. Furthermore, the number of OX40L⁺ blood vessels was also higher in patients with ulcerative colitis and Crohn's disease compared with normal controls⁷³. These observations indicate a possible contribution of OX40–OX40L interactions to the pathogenesis of IBD in both humans and mice.

The development of IBD in some mouse models might be caused by dysfunctional CD4⁺CD25⁺ REGULATORY T (T_{REG}) CELLS, which are thought to have an important role in the control of autoimmune responses in mice⁷⁵. This is based on the finding that co-transfer of CD4⁺CD25⁺ T_{REG} cells and CD4⁺CD25⁺ T cells into lymphopaenic recipient mice completely ameliorates IBD³¹. Mice protected from IBD by the co-transfer of T_{REG} cells had fewer OX40L⁺ dendritic cells than mice with disease. The CD4⁺CD25⁺ T_{REG} cells might limit inflammation in IBD, which in turn might reduce the number of OX40L⁺ dendritic cells found in treated animals. Additional evidence for the involvement of T_{REG} cells in IBD can be found in IL-2-deficient mice, which lack T_{REG} cells in the periphery and spontaneously develop

BLOOD–BRAIN BARRIER

The central nervous system (CNS) is known to be an immune-privileged site such that no lymphocytes can cross from the blood to the brain. In certain diseases, such as multiple sclerosis, the endothelial-cell wall that protects this barrier is broken down and, when this occurs, lymphocytes can pass freely into the CNS.

NON-OBESE DIABETIC MICE (NOD mice). A strain of mice that normally develops idiopathic autoimmune diabetes that closely resembles type 1 diabetes in humans. The target antigen(s) recognized by the pathogenic CD4⁺ T cells that initiate disease is expressed by pancreatic islet cells, but its identity is unknown.

GRAFT VERSUS LEUKAEMIA (GVL). Hosts with leukaemia who receive an allogeneic bone-marrow transplant have far fewer disease relapses than individuals obtaining autologous bone-marrow transplants. This is due to the transplanted T cells recognizing alloantigens expressed by the leukaemia.

CD4⁺CD25⁺ REGULATORY T CELLS (T_{REG} cells). A specialized subset of CD4⁺ T cells that can suppress other T-cell responses. These cells are characterized by expression of the interleukin-2 receptor α -chain (CD25) and some T-cell-activation markers. The transcription factor FOXP3 is specifically expressed by these cells and contributes to the suppressive function.

IBD^{76,77}. Constitutive expression of OX40L rendered recombinase-activating gene 2 (*Rag2*)-knockout mice susceptible to T-cell-transfer-induced IBD, even in the presence of T_{Reg} cells⁷². Furthermore, the use of APCs that constitutively express OX40L or addition of agonistic OX40-specific antibody made CD4⁺CD25⁻ T cells insensitive to T_{Reg}-cell-mediated suppression during CD3-stimulation-induced proliferation in *in vitro* culture⁷². So, enhanced or ectopic expression of OX40L on APCs or other resident cells in colonic tissues might lead to IBD development by interference with T_{Reg}-cell function. Although immunotherapy with TNF-specific monoclonal antibodies and soluble TNF receptor has been established as an effective therapy in Crohn's disease, a quarter of patients are resistant to this therapy⁷⁸, and the efficacy of this therapy for ulcerative colitis is still controversial⁷⁹. In a T-cell transfer model of IBD, treatment with OX40L-specific monoclonal antibody was more effective at ameliorating clinical signs of disease than were TNF-specific antibodies. A combination of monoclonal antibodies specific for both OX40L and TNF was most effective³⁵. Therefore, future clinical trials could test a combination of these two antibodies for refractory Crohn's disease and ulcerative colitis.

Lung inflammation. Following several common respiratory diseases (such as allergic asthma and viral pneumonia), inflammatory lung damage is, in part, caused by an excessive immune response. Cytokines produced by T cells that infiltrate the lung tissue might trigger inflammation by recruiting and activating monocytes, eosinophils and neutrophils. Involvement of OX40–OX40L interactions in the development of respiratory inflammation in a mouse model of allergic asthma has been shown^{36,43,80,81}. Lung inflammation was markedly diminished in OX40-deficient mice (C57BL/6 background) with experimentally induced asthma; there was a marked reduction in the numbers of eosinophils in bronchoalveolar lavage cells, and goblet cells in lung tissues, compared with wild-type controls⁸⁰. This group also showed that treatment of mice with OX40L-specific monoclonal antibody was effective at controlling the memory T-cell reactivation that induces asthma, by inhibiting lung infiltration, goblet-cell hyperplasia and mucus secretion³⁶. Similar results were obtained in BALB/c mice that lack OX40L or those that are treated with OX40L-specific blocking antibody^{43,81}. This treatment, however, did not alter the memory responses of T cells to asthma⁴³. This variation between C57BL/6 and BALB/c strains might indicate that the efficiency of OX40 blockade depends on the genetic background of the mice. Therefore, the efficacy of this therapy in human allergic asthma might also vary from individual to individual.

A recent report using a viral model of lung inflammation provides evidence that OX40L blockade could be successful in modulating the disease process. Administration of OX40–immunoglobulin fusion protein was efficient at reducing influenza-virus-induced lung inflammation even 3 days after the onset of disease symptoms, without any adverse effects on either viral

clearance or memory T-cell responses to viral rechallenge³⁷. Therefore, targeting OX40L might be useful as an anti-inflammatory therapy rather than as an overall immunosuppressive regimen. In a separate study, whereas CTLA4–immunoglobulin fusion protein could also reduce influenza-virus-induced lung inflammation, this therapy concurrently reduced antibody titres and antigen-specific CTL activity, as well as delaying viral clearance⁸². This further shows that OX40L blockade is an alternative option with fewer adverse manifestations.

All these studies in different animal models of autoimmunity and inflammation indicate that blockade of OX40–OX40L interactions, even after the onset of clinical symptoms, is effective in terms of reducing autoimmune, allergic and inflammatory diseases (including tissue inflammation during viral infection) without global immunosuppression. Importantly, the therapeutic effects of OX40–OX40L blockade by treatment with OX40L-specific antibodies or OX40–immunoglobulin fusion proteins are similar to the disease suppression in OX40- or OX40L-deficient mice in most disease settings. This implies that the effects of these regimens are a direct result of the specific blockade of OX40–OX40L interactions. These observations indicate that OX40 blockade is a promising therapeutic target for these diseases.

Targeting OX40 to enhance antitumour responses

As blocking OX40–OX40L interactions decreases autoimmune inflammation and tissue destruction, it could be predicted that augmentation of OX40 signalling might increase T-cell function, leading to enhanced tumour destruction in cancer-bearing hosts. Initially, it was predicted that antibodies specific for OX40 would inhibit autoimmune disease by blocking interaction with its ligand; however, OX40-specific antibodies actually signal through OX40 and caused exacerbation of both autoimmunity and GVHD^{34,83}. Unlike the response to foreign pathogens, which elicit potent danger signals, the immune response to tumour antigens is usually less robust and the tumour environment can be immunosuppressive, leading to antigen-specific T-cell tolerance^{84,85}. As signalling through OX40 *in vivo* leads to a series of pro-inflammatory events that can overcome CD4⁺ T-cell tolerance^{21,72,86–88}, agonist antibodies and a soluble OX40L–immunoglobulin fusion protein were injected *in vivo* to test their ability to enhance immune responses in tumour-bearing mice^{28,89–92}. Ligation of OX40 *in vivo* in tumour-bearing mice enhanced antitumour immunity, leading to tumour-free survival in mouse models of melanoma, sarcoma, colon cancer, breast cancer and glioma^{28,89–92}. In each tumour model, a percentage (20–55%) of mice treated with agonist OX40-specific antibody remained tumour free for an extended period of time (up to 1 year after tumour inoculation). IMMUNOGENIC TUMOURS were more responsive to OX40-specific antibody treatment²⁸; however, even poorly immunogenic tumours were responsive to ligation of OX40 as a sole treatment^{28,92}. *In vivo* administration of OX40-specific antibody was also therapeutic in the presence of brain and

IMMUNOGENIC TUMOURS

The immunogenicity of a tumour is defined by its ability to enhance immune responses after irradiation and subcutaneous vaccination into naive mice. Highly immunogenic tumours will allow 100% of the vaccinated hosts to be protected against live tumour challenge 30 days after immunization, whereas less immunogenic tumours elicit poorer immune responses, resulting in 50% or less of hosts that succumb to live tumour challenge after immunization.

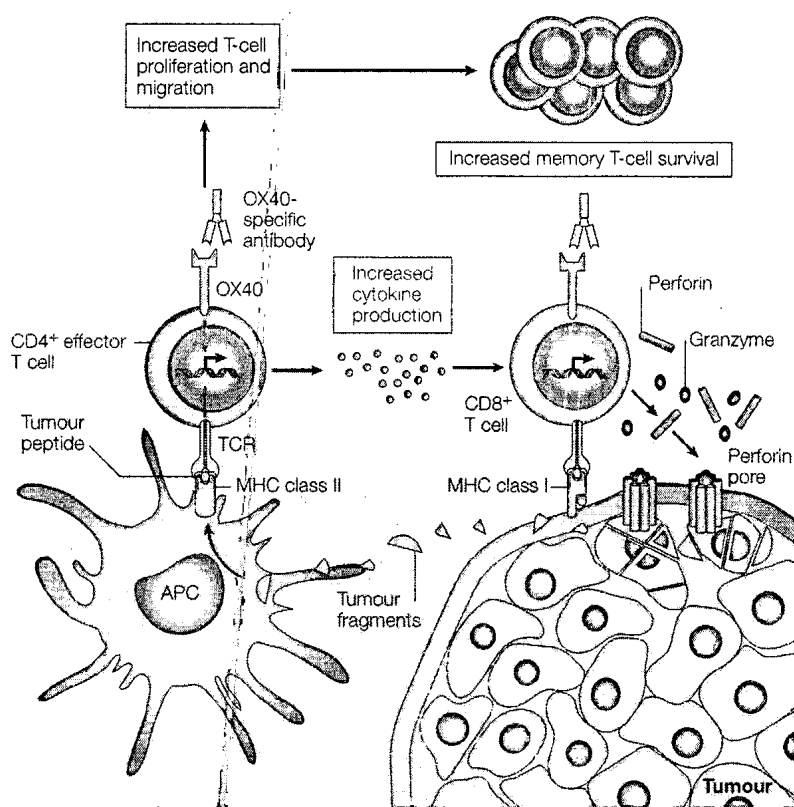


Figure 3 | Ligation of OX40 in tumour-bearing mice enhances antitumour immunity, leading to therapeutic effects. Agonist OX40-specific antibodies or an OX40-ligand-immunoglobulin fusion protein (not shown) can elicit enhanced CD4⁺ and CD8⁺ T-cell responses in tumour-bearing mice. The OX40-accentuated response is mainly attributed to enhanced CD4⁺ T-cell function (through increased cytokine production and increased survival of memory T cells), but CD8⁺ T cells are also essential for OX40-specific antibodies to have a positive impact in tumour-bearing mice. It has been shown both in human tumours and mouse tumour models that OX40⁺ T cells are found in a wide variety of solid malignancies; therefore, the OX40-specific enhancement probably occurs in or near the tumour microenvironment. Potential mechanisms of OX40-enhanced tumour-cell killing (not all shown) include an increase in CD8⁺ T-cell cytotoxicity (through perforin and granzymes), direct lysis of tumour cells through enhanced production of tumour-necrosis factor by CD4⁺ T cells, an increase in CD95/CD95L-mediated tumour-cell killing, or an increase in the production of interferon- γ by CD4⁺ T cells, leading to enhanced tumour-cell lysis mediated by natural killer cells. Most tumour models that have been 'cured' through OX40 ligation are MHC class II negative; therefore, indirect priming of tumour-antigen-specific T cells by professional antigen-presenting cells (APCs) is probably required to initially stimulate CD4⁺ T cells at the tumour site. After mice have been cured by OX40 ligation, they are immune to subsequent rechallenge with the primary tumour, but they are not immune to tumours of a different origin (indicating enhanced tumour-specific T-cell-mediated memory). TCR, T-cell receptor.

liver metastases^{89,91}, and was effective against lung metastases when given in combination with adoptive T-cell immunotherapy⁹⁰. Mice cured through OX40 ligation were resistant to subsequent challenge with the primary tumour; however, they were not resistant to the growth of tumours of a different origin, indicating that tumour-specific T-cell memory had been established²⁸.

Similar to studies carried out in EAE^{63,93,94}, CD4⁺ OX40⁺ T cells that were isolated from the site of inflammation in tumour-bearing mice (tumour-draining lymph nodes) were enriched for T cells with reactivity to tumour antigens²⁸. So, targeting OX40⁺ T cells in

tumour-bearing mice would preferentially stimulate tumour-specific T cells^{28,89–92,95,96}. Mice that have been cured of their cancer by OX40-based therapy have potent tumour-specific CD4⁺ memory T cells that protect naive mice from challenge with the same tumour after adoptive transfer²⁸. As most of the tumours described in these studies were MHC class II negative, OX40 crosslinking probably enhances the function of CD4⁺ T cells that are activated by APCs presenting tumour antigens in the context of MHC class II molecules. So far, the data indicate that the OX40-enhanced CD4⁺ T cells are not the ultimate effector cells, but provide increased T-cell help to tumour-specific cytotoxic CD8⁺ T cells. Elimination of either CD4⁺ or CD8⁺ T cells before treatment with agonist OX40-specific antibody completely negates the OX40-enhanced antitumour effect⁸⁹. OX40 is preferentially expressed by CD4⁺ T cells³, but in some mouse cancer models, both tumour-infiltrating CD4⁺ and CD8⁺ T cells were shown to express OX40 (REF. 89). Therefore, OX40 ligation might also directly stimulate CD8⁺ T cells to increase their effector function, leading to increased tumour-cell destruction.

Most human tumours have escaped immune surveillance and can inhibit immune function through various mechanisms^{97–100}. In accordance with reports of tumour-induced immune suppression, few OX40⁺ T cells were found in large human tumours after surgical resection¹⁰¹. Therefore, agonist OX40-specific antibody together with other immune-enhancing reagents might be most effective in patients with cancer. Marked antitumour responses have been reported when OX40 ligation was combined with cytokine therapies⁹⁵ and/or ligation of 4-1BB⁹¹ — another co-stimulatory TNF-receptor-family member that mainly functions on CD8⁺ T cells¹⁰². In one study, a colon carcinoma cell line was transfected with OX40L, with or without co-transfection of granulocyte-macrophage colony-stimulating factor (GM-CSF)⁹⁵. Tumours transfected with GM-CSF and OX40L were more immunogenic than untransfected tumour cells or tumour cells transfected with OX40L alone⁹⁵. Furthermore, subcutaneous vaccination with irradiated GM-CSF⁺OX40L⁺ tumour cells eradicated lung metastases with greater efficiency than did vaccination with cells transfected with GM-CSF or OX40L alone⁹⁵. The OX40L/GM-CSF treatment regimen was dependent on both CD4⁺ and CD8⁺ T cells and the functional expression of CD40 (REF. 95). In a second study, a combination of agonist 4-1BB-specific and OX40-specific antibodies, and adenoviral IL-12 (delivered intratumorally), was used in a liver metastases model for colon cancer⁹¹. The triple 'drug' therapy was superior to each individual reagent or double-treatment combinations in this model and generated tumour-specific CTLs with the greatest biological activity⁹¹. As shown in FIG. 3, injection of agonist OX40-specific antibody or soluble OX40L-immunoglobulin fusion protein enhanced both CD4⁺ and CD8⁺ T-cell immunity to tumour cells, leading to the therapeutic effects in tumour-bearing mice described earlier.

Targeting OX40 as a vaccine adjuvant

The potent adjuvant properties that occur after OX40 ligation, which enhance T-cell immunity and increase memory T-cell generation, could be used during vaccinations. A recent report showed that injection of OX40L-immunoglobulin fusion protein into mice that were infected with *Cryptococcus neoformans* increased the number of interferon- γ (IFN- γ)-secreting CD4⁺ T cells and decreased the *C. neoformans* burden in the lungs⁴². An improvement in immune responses to deadly pathogens (such as HIV and malaria) will be an important future arena for OX40-induced augmentation. To this end, our laboratory (A.D.W.) has just completed a project in which rhesus monkeys were immunized with a purified simian immunodeficiency virus (SIV) protein (gp130) and infused with monkey-OX40-specific agonist mouse antibody or control antibody. Monkeys that received the OX40-specific antibody had markedly increased numbers of lymphocytes in vaccine-draining lymph nodes, as well as increased memory T-cell and antibody responses to the SIV protein immunogen (A.D.W., unpublished observations). Although the treatment was not toxic and the immune enhancement was promising and consistent with previous studies in mouse models^{20,21}, purified antibodies might be too expensive for widespread vaccinations (especially in developing countries). Therefore, the development of viruses that produce recombinant OX40L, as described in a recent report¹⁰³, will probably be the most cost-effective and efficient method to enhance immune responses to vaccines through OX40 ligation.

Translation to human clinical studies

Targeting OX40 is a promising therapeutic strategy and has been well documented in many animal disease models. Most importantly, unlike conventional immunotherapies, blockade of OX40–OX40L interactions seems to inhibit disease-responsible effector T-cell function specifically, and therefore does not cause widespread immunosuppression. Although OX40 blockade seems to be an ideal treatment for acute inflammation, some therapeutic strategies that target relapsing EAE and pathogen-induced chronic inflammation indicate that long-term treatment during inflammation might be required to reduce disease in these models^{12,27,41}. For example, in EAE studies, interrupted treatment during the relapse phase induces redevelopment of clinical symptoms¹². These observations indicate that, in relapsing autoimmune/inflammatory diseases, prolonged OX40 blockade might be more effective. However, prolonged blockade might also cause immune suppression of concurrent inflammatory processes that are important to maintain a healthy host. Therefore, timing and dosing will be key issues when planning future clinical trials in relapsing autoimmune diseases.

Mouse monoclonal antibodies specific for human OX40L and human OX40-immunoglobulin fusion proteins have been produced^{8,12,33}. Our laboratory

(K.S. and N.I.) and others have begun to develop a humanized human-OX40L-specific monoclonal antibody for phase I clinical trials. Alternatively, the human OX40-immunoglobulin fusion protein binds mouse OX40L and is effective at dampening clinical signs of autoimmunity in mouse models^{12,25,33}. In these studies, no adverse side effects were evident when using the human OX40 fusion protein. So, the human OX40-immunoglobulin fusion protein has already been tested *in vivo* for efficacy and potential toxicity, and would be a suitable candidate for phase I clinical trials.

For cancer therapy, our group has produced a human-OX40-specific mouse monoclonal antibody that has similar agonist properties to the antibody tested in the SIV monkey studies described above (A.D.W. and W. J. Urba). Phase I safety and immunological testing of the OX40-specific antibody in late-stage cancer patients will be carried out in the coming year. At present, fully humanized antibodies and OX40L molecules are being produced and will probably be used in second-generation clinical trials. Other potentially beneficial OX40-based clinical trials for cancer might involve autologous or allogeneic tumour-cell vaccines transfected with cytokines, such as GM-CSF or IL-12, in combination with OX40-specific antibody treatment.

A potential problem with the clinical trials described above is that excessive OX40 signals might induce autoimmunity and/or unwanted inflammation by breaking *in vivo* T-cell tolerance to self-antigens. This type of response has been seen in clinical trials in which CTLA4-specific antibody was given to patients with cancer¹⁰⁴. Therefore, when treating patients with agonist reagents to OX40, close attention should be paid to clinical signs of autoimmunity and immunological monitoring should be carried out for CD4⁺ T-cell number and function as well as autoantibody production. In accordance with this idea, cancer patients with known autoimmune disorders will be excluded from the cancer clinical trial described above.

Also, a recent paper has shown that OX40 signals might contribute to the deletion and homeostasis of CD4⁺CD25⁺ T_{Reg} cells, which suppress T-cell-mediated autoimmunity in mice. OX40-deficient mice have fewer T_{Reg} cells in the periphery as infants than do wild-type mice, and T_{Reg} cells from OX40-deficient mice have a marked reduction in suppressive effect on CD3-stimulated T-cell proliferation⁷². These observations indicate that OX40 blockade might affect the roles of T_{Reg} cells and lead to autoimmune manifestations under certain conditions. However, OX40 blockade has not yet induced any autoimmune states or enhanced disease symptoms in several disease settings discussed here. Importantly, human T_{Reg} cells in the peripheral blood do not seem to express OX40 (N.I., unpublished observations), whereas mouse T_{Reg} cells constitutively express OX40 (REF. 72). We suggest that there are different roles of OX40 in T_{Reg}-cell function between human and mouse immune systems. Further

studies will be required to understand fully the effect of OX40 blockade on T_{Reg}-cell function, especially how it relates to the human immune system.

Immunological effects of OX40-immunoglobulin fusion proteins are varied depending on the IgG subclass used to produce the fusion protein. The OX40-IgG1 fusion protein activated antibody-dependent cellular cytotoxicity and complement, leading to lysis of OX40L-expressing cells, whereas the OX40-IgG4 fusion protein did not³³. As a TNF-specific monoclonal antibody, which is more effective than other reagents for the treatment of Crohn's disease and rheumatoid arthritis, can also have cytotoxic effects on membrane-bound TNF-expressing cells¹⁰⁵, OX40-IgG1 might be more effective in the clinical setting. On the contrary, possible lysis of OX40L-expressing cells (probably APCs) by OX40-IgG1 might lead to widespread immunosuppression, as the cell lysis might not be specific for antigens that cause disease. Therefore, the appropriate IgG subclass must be selected in accordance with the results of clinical trials.

Concluding remarks

Accumulating evidence now indicates that targeting OX40 and OX40L *in vivo* might lead to new therapeutic strategies for several immunological disorders, cancer and unwanted inflammation from viral infections. Blockade of OX40–OX40L interactions inhibits ongoing antigen-specific immune responses by specifically suppressing the survival of effector T cells. This seems to be an ideal therapeutic approach for T-cell-mediated immune disorders. In certain situations, such as GVHD and organ transplantation, combination therapy that targets both the OX40 and CD28 pathways might be potentially synergistic in controlling disease. *In vivo* ligation of OX40 induces favourable antitumour effects in cancer-bearing mice, and can be augmented with protocols involving other stimulatory molecules, such as GM-CSF, 4-1BB-specific antibody and IL-12. In conclusion, the use of reagents that target human OX40 and OX40L holds great promise for therapeutic disease intervention in several diseases in which current drugs either lack efficacy or are immunosuppressive.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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Review

Regulation of CD4 T cell memory by OX40 (CD134)

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Abstract

CD4 memory T cells play a critical role in protection against repeated exposure to infectious agents such as viruses, bacteria, and helminth parasites, yet can also contribute to the aberrant immune responses associated with autoimmune and allergic reactions. Understanding the mechanisms that control effective memory responses has important ramifications for vaccine design and in the management of adverse immune reactions. Recent advances in studies of T cell memory have implicated the tumor-necrosis-factor receptor (TNFR) family member, OX40 (CD134), as a key co-stimulatory molecule involved in the regulation of CD4 memory T cells. In this review we discuss these new developments in the context of past research and current models for the generation, persistence, and re-activation of memory T cells.

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Keywords: CD4 T cells; Memory; Costimulation; OX40; OX40L; TNFR; Vaccine; Th1; Th2; Asthma

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1. Introduction

Immunological memory is a cardinal feature of the adaptive immune system that protects organisms from recurrent infection by pathogens [1–4]. The protective nature of memory responses is largely attributable to B cells, CD4⁺, and CD8⁺ T lymphocytes. CD4⁺ T cells play an important role in coordinating the host's adaptive immune response to infec-

tious agents. Antigen specific CD4⁺ T cells help phagocytic cells such as macrophages and dendritic cells to destroy vesicular and intracellular pathogens through cell–cell contact and release of immunomodulatory cytokines. Similarly, they provide help to B lymphocytes and CD8⁺ T cells in their response to antigen. CD4⁺ memory T cells develop during a primary infection and, like naïve T cells, are long-lived lymphocytes [5–7]. However, compared with naïve cells, memory cells respond more rapidly and differentiate more quickly into effector cells than their counterparts during the primary response. These attributes of memory T cells, together with an

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increased frequency specific for a given antigen, contribute to the greater magnitude and effectiveness of secondary immune responses in the presence of limiting antigen (Ag) and/or inflammation, as may be the case very early after pathogen encounter. This rapid subsequent effector response would serve to eliminate the pathogen. Central to the question of immunological memory is defining the regulatory mechanisms that govern the effective generation and reactivation of CD4⁺ memory T cells as well as their maintenance throughout the life of an individual. Understanding these regulatory pathways is of great significance for exploiting the immune system to protect against pathogens, immunotherapy of cancer, and to dampen immuno-pathology in allergic responses and in autoimmunity.

Among factors influencing the fate of naive T cells after antigen encounter, functional interaction between co-stimulatory molecules on the surface of T cells and their ligands expressed on APC play an important role. In this review, we will focus on recent studies designed to elucidate the co-stimulatory requirements for effective CD4⁺ memory T cell responses. Particular emphasis will be put on the role of the tumor-necrosis-factor receptor (TNFR) family mem-

ber, OX40 (CD 134) in the regulation of CD4⁺ memory T cell development, reactivation and persistence.

2. Development of CD4 memory T cells

The generation of CD4 memory T cells occurs over two phases after infection or vaccination (Fig. 1). The first phase begins when circulating peripheral naive CD4 T cells enter into secondary lymphoid organs such as LNs, Peyer's patches, and the spleen from the blood and recognize antigenic peptides bound to MHC class II complexes on the surface of mature dendritic cells (DCs) via their TCR. Antigen recognition initiates naive T cell proliferation and differentiation into an expanded population of effector T cells that can be subdivided into at least two functionally distinct populations based on their cytokine secretion profiles. CD4⁺ T helper 1 (Th1) secrete IFN- γ , lymphotoxin, and IL-2 whereas CD4⁺ Th2 cells secrete IL-4, IL-5, IL-9, IL-13, and IL-10 [8]. A number of factors including the duration of TCR-engagement [9–13], the nature and dose of antigen [14–16], the availability and type of APC [13,17,18], co-stimulatory molecules

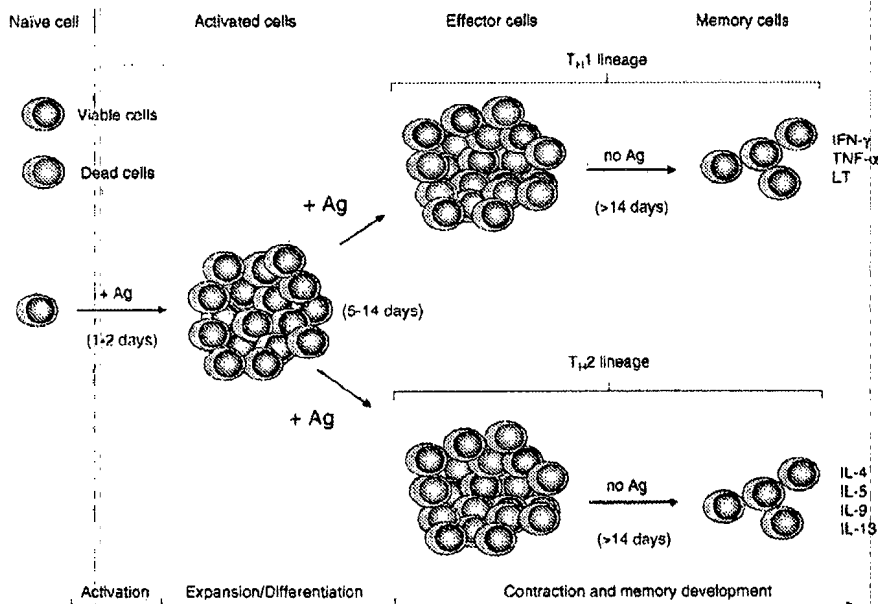


Fig. 1. Sequential differentiation or linear model for effector and memory cell generation. The figure summarizes the known stages in the transition for naive CD4 T cells to effectors and then memory cells. During the primary response, naive T cells recognize MHC class II-peptide complexes on professional antigen presenting cells in spleen and lymph nodes and undergo clonal expansion. Depending on the nature of antigen and the inflammatory milieu, antigen-specific effector T cells are induced to differentiate into T helper type 1 (Th1) or Th2 cells. After eliminating the pathogen, most effector cells die, but some (10–20%) survive to form long-lived memory cells.

[19–21], and the cytokine milieu [22,23] determine the size of the effector T cell pool and their differentiation into either Th1 or Th2 cells. After antigen removal, a second phase of memory T cell development ensues, whereby most of the antigen-specific CD4 effector T cells die and the surviving effectors go on to persist as long-lived memory T cells [24–26]. The effectiveness of the memory response when antigen is re-encountered is largely dictated by the overall size of the antigen specific CD4 memory T cell pool that is generated after the contraction phase of the primary immune response as well as the particular array of cytokines that these cells are programmed to secrete. The precise factors that govern the transition of effector CD4 T cells into long-lived memory T cells are presently unclear. Several recent reports have suggested that this transition can occur in the absence of further division [26] and antigen [25–28].

3. Phenotypic and functional differences between naïve, effector and CD4 memory T cells

Identification of naïve, effector and memory cells has been based on a combination of phenotypic, biochemical and functional changes that occur upon activation [2,5,29]. Phenotypically, naïve CD4 T cells express high levels of lymph node homing receptor molecule CD62L (MEL-14, 1-selectin), and the CD45 high-molecular-weight isoform (CD45RB/A), CD127 (IL-7R α), and low CD44 expression. Effector CD4 T cells express several activation markers including CD69, IL-2 receptor α (CD25), CD44 high, and have down-regulated the cell surface expression of IL-7R α . Memory cells can be heterogeneous with respect to CD62L expression (so-called central and effector memory subsets), although it can be argued that true memory cells are CD62L high (central memory). Memory cells are generally CD45RB/A low, CD44-high, have regained IL-7R α expression but have lost the expression of activation markers such as CD25 and CD69. Naïve and memory CD4 T cells are characterized as small resting cells whereas effector T cells have a lymphoblastoid morphology characteristic of activated cells. Naïve and memory T cells express activation markers with the same kinetics, but memory T cells proliferate and perform effector functions faster than their naïve counterparts [30]. Functionally, memory and effector T cells produce the same type of effector cytokines [31] but effector T cells can exhibit a more rapid effector function than resting memory T cells [2]. Moreover, effector T cells exhibit a much higher susceptibility to apoptosis in the early stages of activation, whereas the susceptibility to apoptosis in memory T cells is similar to that of a naïve T cell. Consistent with this, expression of the intracellular survival proteins such as Bcl-2 is down-regulated during the effector phase of the immune response, whereas memory cells persisting after clearance of antigen show up-regulation of these proteins again [30].

4. Regulation of primary immune responses and development of CD4 memory T cells by OX40

It is well established that optimal proliferation and acquisition of effector functions by naïve T cells only ensues when costimulatory signals are provided by antigen presenting cells (APC). The Ig superfamily member CD28, a receptor for both B7-1 (CD80) and B7-2 (CD86), is considered to be the primary co-stimulatory receptor that controls initial clonal expansion, and provides early signals to augment expression of anti-apoptotic members of the Bcl-2 family to prevent cell death [32]. CD28 signals also regulate early cytokine production in particular IL-2, and IL-2R signals may provide additional early anti-apoptotic signals. Although, CD28 signals are given within 24 h following encounter with antigen, it is now clear that they are not sufficient for long-term T cell survival. In the absence of additional signals provided 1–2 days later, a majority of T cells undergo apoptosis.

In the case of CD4 T cells, several groups have shown that OX40, a member of the TNFR superfamily, represents a major costimulatory receptor [20,21]. Unlike CD28, OX40 is not constitutively expressed on naïve T cells but is induced 24–48 h after engagement of TCR [33–36], with peak levels *in vivo* generally seen between days 2 and 5 after immunization with antigen [36]. Similarly, OX40 ligand (OX40L), a member of the TNF superfamily, is also induced on APC such as B cells [37,38], macrophages, and DCs [39] after activation. Initially, the relative contribution of CD28- and OX40-mediated co-stimulation in primary CD4 responses was assessed by transfecting MHC class II+ mouse fibroblast cell lines lacking or expressing B7-1 with cDNA encoding murine OX40L [36]. These cells were used to stimulate naïve CD4 T cells isolated from TCR transgenic mice. Although, OX40 was readily expressed 24–48 h after Ag stimulation, APCs transfected with OX40L alone had little ability to induce IL-2 secretion or proliferation of naïve T cells. However, a strong synergy was observed when B7 was co-expressed, resulting in enhanced expansion and survival over a 5 day period. Additional *in vitro* studies using TCR transgenic CD4 T cells demonstrated that in contrast to T cells lacking CD28, those lacking OX40 initially divided normally, were minimally impaired in the production of IL-2, and survived efficiently over a short time frame of 3–5 days [40]. However, OX40-deficient T cells did not maintain high levels of anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Bfl-1, and consequently the majority of T cells did not survive long-term [40,41]. Collectively, these results have implied that a temporal sequence of events is necessary for optimal T cell proliferation and survival, brought about by sequential engagement of CD28 and OX40 [20,21,42]. Consistent with this, optimal expression of OX40 and OX40L require CD28-B7 interactions. Based on these results and others [20,21,42] it has been proposed that OX40 costimulation prolongs T cell survival beyond the effector phase of the immune response and thus serves to increase the number of memory T cells by inhibiting effector T cell death [20,21,42]. Several stud-

ies using OX40 or OX40L-deficient mice, or agonists Abs against OX40, have now provided direct *in vivo* evidence for this proposition [20,21]. In one such study, anti-OX40 Ab treatment enhanced T cell numbers by 34-fold in the draining lymph nodes at day 4, and by 10-fold in the spleen on day 8 compared with mice receiving control Ab. Anti-OX40 treatment also enhanced the secretion of both Th1 and Th2 cytokines by between four- and six-fold [43]. To determine the impact of anti-OX40 treatment on memory development, the frequency of antigen-reactive CD4 T cells and cytokine production in secondary lymphoid organs were assessed 35 days after immunization with antigen in adjuvant [43]. Anti-OX40 resulted in between four- and eight-fold increase in the absolute numbers of memory CD4 T cells in the spleen and draining lymph nodes, correlating with the action of OX40 on promoting enhanced frequencies of primary effector cells. The memory CD4 T cells generated as a result of OX40 engagement were functionally competent as evidenced by their production of cytokines upon re-stimulation with antigen *ex vivo*. Consistent with these results, OX40-deficient mice developed fewer Ag-specific CD4 effector T cells late in the primary response *in vivo* and consequently the frequencies of Ag-specific memory cells were 10- to 20-fold lower 35 days after priming. Interestingly, when LPS was combined with anti-OX40 Ab treatment in another immunization

regime using soluble antigen or superantigen [44], a 60-fold increase in the number of memory T cells was observed two months post immunization as compared with a 15-fold increase in mice that received Ag plus anti-OX40 alone. This observation implies that in addition to TCR- and OX40-engagement, additional signals provided by danger signals (LPS or proinflammatory cytokines) are necessary to promote optimal generation of long-lived CD4 memory *in vivo*. Danger signals may exert their effects both indirectly through activation of APC which can lead to enhanced expression of several co-stimulatory ligands including OX40L [44,45] and induction of pro-inflammatory cytokines, or by acting directly on T cells to enhance their survival [46], cytokine production, and possibly OX40 expression. Interestingly, anti-OX40 Ab treatment also induced a dramatic increase in the number of long-lived Ag-specific CD4 T cells detected in the peripheral blood and other peripheral organs up to 196 days post-immunization [47], suggesting that OX40 engagement can also increase peripheral migration. Collectively, these studies provide strong evidence that OX40-OX40L interactions play a critical role in enhancing naïve CD4 T cell clonal expansion, differentiation, and survival and thus, in determining the number of CD4⁺ memory T cells that are generated at the end of the primary immune response (Fig. 2).

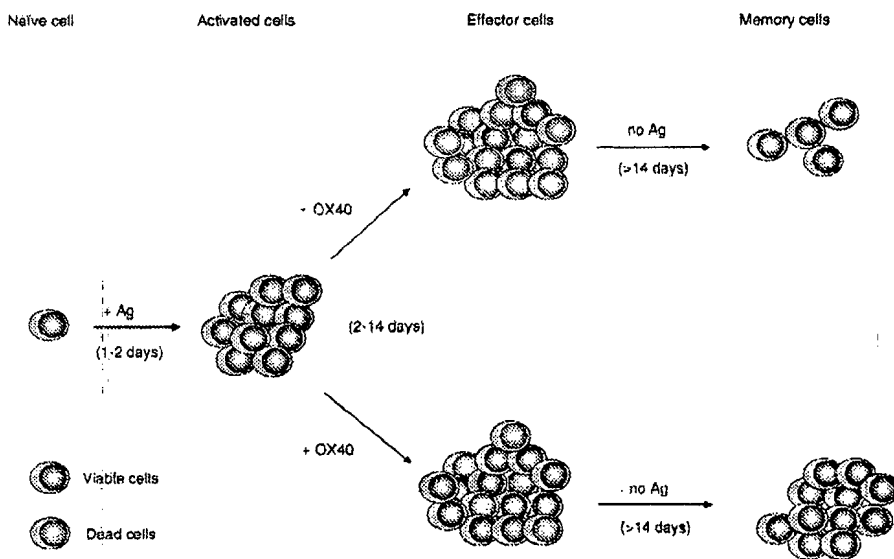


Fig. 2. Role of OX40 in CD4 memory T cell development. OX40 signals provide division and anti-apoptotic signals several days after a naïve T cell encounters antigen. These signals allow continued turnover of cells and promote survival, preventing excessive apoptosis of effector cells at the peak of the primary immune response. Consequently OX40 increases the number of effector cells that enter the memory pool.

5. OX40 and the costimulatory requirements for memory T cell response

The importance of OX40/OX40L interactions in effective priming of naïve CD4 T cells and hence dictating the number of memory T cells has now been well established in a variety of basic and disease-related systems using OX40- and OX40L-deficient or transgenic animals, and in blocking regimens [20,21]. However, studies addressing the specific question of whether OX40 can also influence reactivity or persistence of a CD4 memory T cell once it has been generated have lagged behind.

The requirement of costimulatory signals for reactivation of CD4 memory T cells is controversial at present. In vitro studies carried out a number of years ago using dendritic cells, B cells, or monocytes as APCs suggested that CD4 memory T cells have a reduced requirement for costimulation than their naïve counterparts and that in many cases TCR signaling might be sufficient for their differentiation and effector function [19,48]. More recently, using APCs from B7-deficient mice to stimulate TCR transgenic naïve or memory CD4 T cells, London et al. [49] demonstrated that memory cells are less dependent on B7 molecules than naïve cells with regards to both proliferation and cytokine production. Similarly, in vivo, blocking B7-CD28 interactions during secondary responses to the nematode parasites *Heligmosomoides polygyrus* [50], and *Nippostrongylus brasiliensis* [51], or immunogenic anti-mouse IgD antibody treatment [52], failed to inhibit CD4 memory T cell responses, whereas blocking CD28 at the time of priming was inhibitory. Based largely on these observations it has been thought that while optimal in vivo priming of naïve T cells for proliferation, survival, and effector function requires costimulation, reactivation of CD4 memory T cells might be partly or wholly costimulation independent. The reduced requirement for costimulation has been suggested by some groups to facilitate the more rapid kinetics associated with the secondary immune response, since the lag time (generally 24–72 h) normally required for up-regulation of co-stimulatory receptors on T cells or their cognate ligands on APC is bypassed [53]. However, no or little requirement for costimulatory signals in reactivation of memory cells could pose a potential problem to the host by increasing the likelihood of activation by cross-reacting self-antigens presented by non-professional APCs, as well as when particulate foreign or non-immunogenic air born antigens are repeatedly encountered.

An alternative scenario may be that rather than becoming co-stimulation (B7/CD28) independent, memory T cells may shift to being more reliant on other inducible molecules that are up-regulated on memory T cells with faster kinetics than that observed on their naïve counterparts during the primary immune response. OX40 is down-regulated after the effector phase of primary T cell responses and returns to low or baseline levels within a week after initial antigen encounter [36]. Significantly, however, antigen-primed effector CD4 T cells can up-regulate OX40 more rapidly than naïve T cells and the

majority can re-express OX40 within four hours of antigen stimulation [36]. Early in vitro studies with IE^k expressing OX40L or B7 transfected fibroblasts demonstrated that in contrast to their role in priming of naïve T cells [36], OX40L alone, but not B7, induced high levels of proliferation and cytokine production (including IL-2, IL-4, IL-5, and IFN- γ) from previously activated T cells. This observation is consistent with the notion that in contrast to CD28, the reactivity of an antigen-experienced or memory T cell may be predominantly regulated by inducible costimulatory receptors such as OX40. Similarly, an anergic CD4 T cell, which also represents an antigen-experienced cell, can also re-express OX40 at low levels and be receptive to OX40 engagement resulting in enhanced Ag-specific proliferation and cytokine production [54]. Collectively, these observations imply that, similar to naïve T cells, OX40 may play an important role in a range of CD4 memory T cell activities, including proliferation, survival and effector function.

Recently, this possibility was examined using a well-established murine model of asthma ([55,56]. The allergic response in this model is elicited by initial systemic immunization with ovalbumin (OVA) and the Th2-inducing adjuvant alum [57,58]. Several weeks later, once CD4 memory cells have developed, mice are repeatedly exposed to aerosolized OVA over several days. Within hours after OVA inhalation, a dramatic increase in the number of antigen-specific memory/memory effector T cells (CD4+/CD44^{high}/OX40+) was seen in the draining mediastinal lymph nodes (MLNs) [58]. Notably, these cells were not present in mice that were immunized with alum only, implying that prior exposure to the same antigen is critical in this model, which is consistent with a memory or secondary immune response [58]. Peak numbers of CD4+/OX40+ memory/memory effector T cells were first observed in the MLN at day 1 after recall antigen challenge, after which they remained stable for at least four days. Few CD4+/OX40+ memory/memory effector T cells were detected in the BALF and the perivascular and peribronchial areas of the resting lung before antigen inhalation. However, after challenge, but only in previously primed mice, many CD4+/OX40+ memory effector cells accumulated although with delayed kinetics compared to appearance in the draining lymph nodes. This implied that the initial memory T cell expansion developed in the MLN, and within 24 h the T cells started to migrate to the lung. The appearance of CD4+/CD44^{high}/OX40+ T cells in the lung was closely associated with increases in pulmonary eosinophil numbers, Th2 cytokines (IL-4, IL-5, IL-9, and IL-13 recovered from the BALF, serum OVA-specific IgE levels and the development airway hyperresponsiveness (AHR) to methacholine (MCh).

Studies involving adoptive transfer of labeled antigen-pulsed DCs and/or OVA into the trachea of experimental animals, as well as cell-type specific depletion experiments, have shown that airway DC are the principal APCs responsible for reactivation of memory Th2 cells both within the airway mucosa and the lymph node [59,60]. In this regard, the

kinetics and distribution of OX40+ memory/effector T cells after inhalation of antigen mirror the kinetics and direction of migration of OX40L-expressing CD11c+/CD11b+/MHC-Class II^{high} DCs (Salek-Ardakani and Croft unpublished), implying that OX40/OX40L interactions may be important in the initiation and/or amplification of memory Th2-dependent allergic responses. Consistent with this, an earlier study using OX40-deficient mice had demonstrated a requirement for OX40 signals in the initial development of asthmatic symptoms [57], a finding that was subsequently reproduced in mice with a targeted deletion of OX40L gene [61]. However, as discussed above, because primary immune responses are defective in these mice it is difficult to make any conclusions regarding a requirement for OX40 in the secondary response of a memory Th2 cell that ultimately induces lung inflammation and AHR.

6. Role of OX40 in re-activation, effector function, and survival of CD4 memory T cells

To examine the contribution of OX40/OX40L interactions to reactivation of resting memory Th2 cells a blocking anti-OX40L mAb, RM134L [62], was administered to OVA sensitized mice at the time of re-challenge with aerosolized antigen. Strikingly, specific blockade of OX40-OX40L interactions throughout the challenge period reduced the accumulation of CD4⁺ T cells, and specifically CD4⁺/CD44^{high} OX40+ cells at all days examined, and almost completely suppressed the memory Th2 cell driven pulmonary inflammation. Moreover, MLNs or lung cell cultures from mice where OX40L was blocked showed little reactivity with respect to antigen-specific proliferation and Th2 cytokine production *ex vivo*. This data provided the first direct evidence that OX40-OX40L interactions are required for memory T cell reactivity and for development of a recall immune reaction, and was consistent with the idea that in the absence of OX40 signals few functional antigen-reactive secondary effector T cells developed after memory T cells re-encountered antigen.

To directly show that OX40 expressed on a memory T cell was required for their response and for driving lung inflammation, OVA-specific OX40-deficient CD4 cells were produced by crossing OX40-knockout mice to OT-II TCR transgenic mice, which exclusively bear Va2/V(B5 CD4 cells reactive with the IA^b-restricted epitope of OVA [58]. Purified naïve CD4 cells from wild-type (OX40+/+) or OX40-deficient (OX40-/-) OT-II mice were cultured *in vitro* with anti-CD3 and anti-CD28 under Th2 (IL-4, anti-IL-12, and anti-IFN- γ) polarizing conditions for a time period that allowed primary T cell expansion and contraction to proceed, essentially mimicking an *in vivo* primary T cell response. These *in vitro* rested Th2 cells were then labeled with CFSE, adoptively transferred into naïve recipients, and then these mice were challenged intranasally with OVA. This protocol serves two purposes. One is to bypass the requirement for OX40 in prim-

ing naïve T cells. The second is to bypass the requirement for sensitizing mice *in vivo* before aerosol challenge. Such *in vitro* stimulated and rested cells are phenotypically similar to memory T cells that develop *in vivo*, and they can directly reproduce an inflammatory event that occurs after challenging previously primed animals [25,63,64].

Adoptive transfer of wild-type OVA-specific memory Th2 cells followed by repeated intranasal OVA challenge resulted in pronounced division of all OX40+/+ T cells and accumulation in numbers was observed in the lymph nodes and the lung. This was associated with a recruitment of eosinophils and production of Th2 cytokines. Significantly, OX40-/- memory T cells were impaired in expanding in number such that approximately four-fold fewer cells accumulated in MLN and the lung at the end of the antigen challenge period. Consistent with OX40L-blocking experiments, mice receiving OX40-deficient memory T cells, showed little or no increase in the numbers of inflammatory cells recovered from bronchial lavages, relatively normal lung histology, and a virtual absence of Th2 cytokines. These results clearly showed that OX40 expressed on a memory T cell was required for promoting lung inflammation. Furthermore, they suggest that OX40 signals regulate the number of memory effector cells that are generated over time after they re-encounter antigen.

The exact downstream signaling pathways that lead to enhanced division and survival of antigen-responding memory CD4⁺ T cells upon OX40 engagement are currently unclear. Recent data suggest that one target of OX40 are the anti-apoptotic proteins of the Bcl-2 family such as Bcl-xL, Bfl-1, and Bcl-2, which are increased after OX40 ligation and correspondingly decreased when T cells cannot express OX40 [40,41]. Additionally, OX40 might simultaneously exert effects on suppressing expression or activity of pro-apoptotic proteins such as Bad and Bim (J. Song and M. Croft, unpublished observations). The kinase Akt, otherwise known as protein kinase B (PKB), most likely directly or indirectly controls the effects of OX40 whether it is during initial priming of effector cells or during the reactivation of memory T cells. Akt kinase activity was not sustained over time in OX40-deficient T cells, and significantly, a constitutively active form of Akt, introduced into *in vitro* activated OX40-deficient CD4 cells by retroviral transduction, was able to completely compensate for the lack of OX40. Moreover, directly relevant to the data with memory-induced lung inflammation, active Akt restored the ability of OX40-deficient memory T cells to expand in numbers *in vivo* and to promote such inflammation [41]. More recently, another target of OX40 costimulation was elucidated, a molecule termed survivin which belongs to the Inhibitor of Apoptosis (IAP) family (Song and Croft, *in press*). Survivin was shown to be regulated by Akt, not upregulated efficiently in the absence of OX40, and its defective expression was associated with a block in cell cycle progression and coincident apoptosis. Additionally, survivin, retrovirally introduced into *in vitro* primed OX40-deficient T cells, was able to restore division

and clonal expansion of these T cells when encountering recall antigen *in vivo* (Song and Croft, *in press*).

Taken together, these data suggest that OX40 acts at least on some responding memory T cells in an analogous manner to its action on naïve T cells, and strongly controls the absolute number of antigen-specific memory effector T cells that accumulate during the recall response. Regardless of the precise mechanisms, the finding that reactivation of some types of memory cells, like naïve T cells, require co-stimulation through OX40 has important ramifications not only for our understanding of the basic mechanisms of CD4 memory, but also in designing more efficient vaccines to combat infections or autoimmune and allergic disorders.

7. CD4 memory T cell heterogeneity and requirement for OX40

An attractive model of how T cell memory develops depicts a stepwise differentiation of naïve cells through effector cells, directly into memory T cells, and then into secondary effector cells only when recall antigen is encountered (Fig. 1). However, studies carried out in a number of experimental systems suggest that memory CD4 T cell populations are heterogeneous, containing subsets that migrate through lymph nodes (CD62L-high; 'central' memory T cells) and those that migrate to tissues (CD62L-low; 'effector' memory T cells) [65–67]. The relationship between these subsets is still being elucidated, and the effector memory subset might represent an intermediate primed stage that is retained for some time after antigen encounter before a truly quiescent central memory population is produced [4,7]. The 'central' memory cells might be considered to be less ready for action with slower kinetics of effector cytokine production, but with increased proliferative potential. Based on these observations it has been postulated that 'effector' memory T cells that reside or recirculate through non-lymphoid tissues such as the lung participate directly in the initiation of either protective [68,69] or pathogenic [70,71] memory responses by rapidly producing effector cytokines at the site of antigen encounter, whereas activation of 'central' memory CD4 cells in the secondary lymphoid organs is required for the generation of new rounds of 'effector' memory T cells and thus may contribute to the maintenance and/or amplification of the overall response (Fig. 3a).

The existence of at least two phenotypically and perhaps functionally distinct memory subsets raises an interesting question as to whether different memory subsets have different requirements for co-stimulatory signals with respect to their survival and functionality. The aforementioned study of asthmatic lung inflammation demonstrated that blocking OX40/OX40L during recall responses to inhaled antigen almost completely reduced the number of CD4⁺CD44^{high}OX40⁺ cells visualized in the MLN at the height of the response, and prevented the ensuing asthmatic response [58]. At first glance this might suggest that OX40

signals influence antigen presentation by DCs to 'central' memory T cells and dictate their ability to expand/survive over time (Fig. 3b). However, antigen presentation in the MLNs is not the only site of interaction between DCs and memory T cells in this model. Data gathered more recently show that CD44^{high}CD62^{low} 'effector' memory T cells that reside or recirculate through the lung can be stimulated locally by airway submucosal DCs within 2 h of antigen inhalation [70,71]. Perhaps this early interaction between DCs and 'effector' memory T cells in the airways directs migration of DCs into the afferent lymphatics, towards the T cell areas of MLNs where they can interact with resting 'central' memory cells and induce their proliferation and differentiation into effector cells (As shown in Fig. 3a). Certainly it is not known whether DC activation by 'effector' memory T cells in the airways is an absolute prerequisite for DC maturation and migration into LNs and therefore, whether subsequent activation of 'central' memory Th2 cells can occur in its absence, but this is an intriguing possibility. If so this could imply that the reduction in 'central' memory CD4 numbers seen in MLNs in the absence of OX40 signaling may be indirect, i.e. due to reduced activation of 'effector' memory cells in the airways and/or inhibition of DC maturation and migration to LN (through reverse signaling by OX40L). Kinetic blocking experiments where mice received anti-OX40L Ab at different times after the initial antigenic insult showed that although delaying anti-OX40L treatment by 1 or 2 days reduced the overall inflammatory response by 50% and 80% the most dramatic effect was only seen when OX40/OX40L interactions were inhibited at the time of initial encounter with recall antigen [58]. This observation could then be equally consistent with the idea that OX40/OX40L interactions might be crucial for activation and early production of cytokines by 'effector' memory T cells in the airways, and through reverse signaling for optimal DC maturation and migration to LNs (Fig. 3c). Further studies will be required to understand whether there is truly a differential requirement for OX40 signals by central and effector memory T cells.

8. Is there a role for OX40 in persistence of CD4 memory T cells?

It has been known for many years that memory CD4 T cells can survive for a long time at least as a population, and memory appears to be maintained at least in part through periodic slow cell division and continuous replacement of cells. To date, two signals have been considered to play a role in maintenance of CD4 memory T cells, namely MHC-peptide and cytokines. Early reports from mouse models supported the idea that the longevity of effective memory was dependent on periodic exposure to antigen [1,72,73]. However, data gathered subsequently challenged this idea and suggested that memory CD4 T cells can survive for extended periods in the absence of specific antigen and in some cases in the absence of any MHCII molecules [27]. More recently, Kassiotis et

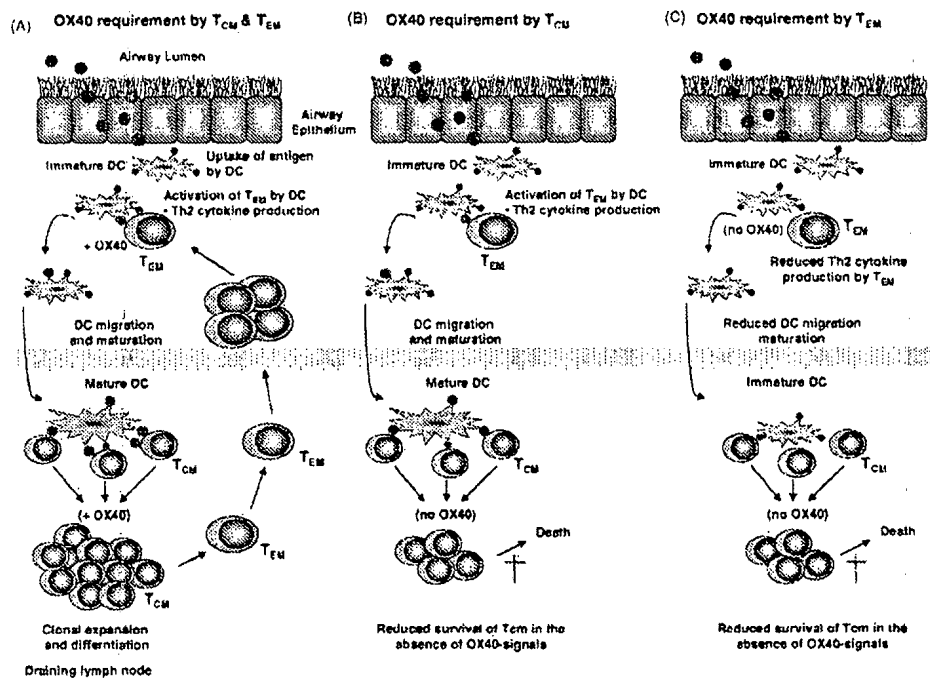


Fig. 3. Different subsets of CD4 memory T cells following immunization/infection and their requirement for OX40 signals. (A) After primary infection or immunization, CD4 T cell memory is established in the lymphoid organs (CD44-high/CD62L-high: central memory T cells (T_{CM})) and the lungs (CD44-high/CD62L-low: Effector memory T cells (T_{EM})). Antigen is taken up across the mucosal impermeable barrier by immature respiratory dendritic cells (DC). Within 2 h after antigen encounter, airway DCs interact with resident long-lived T_{EM} cells in the submucosal areas of the lung. This interaction results in activation of T_{EM} and initiation of their effector function. At the same time DC maturation and migration to the T cell areas of MDLNs via the efferent lymphatics occurs. Within 4 days, this will lead to clonal expansion of resident antigen specific T_{CM} followed by their subsequent differentiation to T_{EM} cells and egress of the T_{EM} cells back to the lung. These effectors (T_{EM}) can then be re-stimulated by lung DCs to then mediate a T helper 2-type (eosinophil-mediated) inflammatory response. (B) OX40 requirement by central memory T cells. In the absence of OX40 signals in the MDLNs, T_{CM} cells initially divide normally but fail to maintain high levels of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, and B6-1, and consequently the majority of T cells do not survive to differentiate to T_{EM} and migrate back to the lung to mediate their effector function. (C) OX40 requirement by effector memory T cells. In the absence of OX40 signals in the lung, T_{EM} are not efficiently activated to produce cytokines in the airways. Additionally, the absence of reverse signaling through OX40L may result in inhibition of DC maturation and migration to MLN, followed by their inability to induce optimal activation and expansion of T_{CM} cells.

al. [28] shed some light on these apparent discrepancies by providing experimental evidence, which indicates that a CD4 memory T cell can persist in the absence of signals accompanying antigen recognition, but such interactions are needed to maintain functional activity of these cells overtime.

Evidence that cytokines play a role in maintenance of memory T cells came initially from studies showing that injection of either type I IFN (IFN- α/β) or inducers of IFN- α/β stimulated TCR-independent proliferation of memory CD8 T cells *in vivo* [74,75]. Subsequently, a number of other cytokines, including IL-12, IL-15, IL-18, and IFN- γ , were found to have similar effects [76–79]. By comparison, it was thought likely that CD4 memory cells also require cytokines for survival. Memory CD4 T cells are found in normal num-

bers in IL-15 $^{-/-}$ and IL15 $\alpha^{-/-}$ mice, and these CD4 T cells are not particularly sensitive to IL-15 induced proliferation suggesting that this cytokine may not play a role in their maintenance [80,81]. Consistent with this, CD4 memory T cells were found to be long-lived in mice deficient for the common γ -chain, indicating that none of the cytokines utilizing this receptor component (IL-2, IL-4, IL-7, IL-9, and IL-15) are essential for their survival [82]. Similarly, unlike memory CD8 cells, homeostatic proliferation of memory CD4 cells is independent of IL-4, IL-7, and IL-15 [83]. In contrast to the reported dispensability of γ -chain cytokines are more recent studies that have suggested a role for IL-7, both in the formation of CD4 memory cells, and also in their maintenance [84–86]. The discrepancies between these studies are

presently unclear but they may suggest that additional classes of survival factors/interactions exist.

One possibility is that memory T cell number/survival and/or functionality is maintained over time through continuous or periodic interaction between costimulatory molecules. We have begun to address this issue in the asthma model described above. Because blocking OX40 signals during the secondary encounter of a memory T cell profoundly inhibited the ability to promote lung inflammation, we tested whether this would result in further impairment if antigen was encountered later in a tertiary response. Accordingly, sensitized mice receiving anti-OX40L at the time of secondary exposure to aerosolized OVA were rested for several months and then re-exposed to a tertiary challenge of aerosolized OVA, but in the absence of anti-OX40L (Salek-Ardakani and Croft unpublished). A robust lung inflammatory response including eosinophilia was found in the lungs of control animals treated with irrelevant Ig in the secondary response. In contrast, much reduced histological evidence of lung inflammation and damage was found in animals that received anti-OX40L at the time of secondary challenge. These data imply that blocking OX40/OX40L interactions can severely limit the persistence of a memory Th2 response and effectively prevent further lung pathology for a significant period of time after antibody treatment.

Exactly how OX40 might control the maintenance of such memory remains to be determined. The simplest idea is that OX40 signals are required for proliferation and survival of memory T cells when antigen is encountered, and as OX40 targets anti-apoptotic proteins, without these signals many of the responding T cells will die rather than live to maintain the high frequency of the memory T cell pool. Another non-mutually exclusive possibility is that OX40 signals impact on the functional activity of the surviving memory T cell population rather than the frequency of antigen-reactive T cells. The latter would be in agreement with the notion put forward by Kassiotis et al. [28] that suggest that signals received during antigen recognition are required for the function of memory T cells but not necessarily for their persistence.

In support of the idea that OX40 signals may play a role in the maintenance of CD4 memory is the recent identification of an accessory cell (CD3-CD4+CD11c-MHCII+) in the B:T cell interface and within B cell follicles of the secondary lymph nodes that constitutively expresses OX40L [87,88]. Interestingly, these cells were visualized to associate with OX40+ but not OX40- memory CD4 T cells several weeks after immunization, and this interaction was suggested to be important for survival of OX40+/+ T cells *in vivo*. Thus, it is conceivable that these accessory cells could influence longevity of 'central' memory CD4 T cells that reside in secondary lymphoid organs by providing survival signals over time. Here the periodically dividing memory population may be more reliant on signals through OX40 or other co-stimulatory molecules [89].

In addition to secondary lymphoid organs, recent studies indicate that memory T cells can also survive for extended

periods of time in non-lymphoid tissues. For example, using tetramer staining and ELISPOT analysis, Hogan et al. [68] showed that high frequencies of antigen-specific CD4 and CD8 T cells remained in the lung airways and parenchyma for several months following resolution of Sendai and influenza virus infections. Similarly, long-lived Th2 memory cells have been detected in the lungs of mice 100–400 days after recovering from acute allergic asthma. These cells have a memory phenotype and respond rapidly by secreting IL-4 and IL-5 upon Ag stimulation [90]. The source and the nature of signals required for longevity of memory T cells in the lung is presently unclear but may result from their interaction with long-lived CD11b+CD11c+IL-4/80+ airway DC population recently described by Julin et al. [91]. The scenario in which airway DCs play a role in maintenance of memory CD4 T cells in the lung is consistent with the findings that airway DCs produce chemokines that selectively attract memory cells to the lung [92]. Whether OX40 and its ligand might be involved in this type of memory longevity is not known, but it is interesting that at least at the peak of a lung inflammatory response, most CD11b+CD11c+DC are expressing OX40L. If this is maintained on these APC for long periods is not clear.

9. Conclusions and future directions

Understanding the regulatory mechanisms involved in CD4 memory cell development has important implications with regards to the important issue of vaccine design. Successful vaccines induce strong and long-term humoral responses, and at least for T cells, depend on strong initial clonal expansion of precursor cells. Further success in vaccine design might rely on developing strategies that reduce cell death at the end of the primary immune response, thereby augmenting the entry of effector cells into the long-lived memory cell pool. Additionally, it might be possible to target molecules or pathways to interfere with homeostasis and offsetting the balance between proliferation and attrition during the persistent phase of memory cells. In this review we have discussed experimental evidence demonstrating that OX40 signals dramatically enhance the survival and functionality of effector T cells at the end of the primary immune response and consequently the number of memory T cells that are developed once antigen is cleared. Moreover, we have discussed recent data obtained in a murine model of asthma demonstrating that OX40 signals are critical for reactivation of resting memory Th2 cells and their persistence/functionality over time. These results strongly imply that targeting OX40 could represent a promising new target for manipulating CD4 memory cell generation, reactivation and persistence. Important questions that remain unanswered are whether OX40 signals are similarly required for re-activation of memory cells (CD4 or CD8) in acute and chronic infection models? In view of recent findings demonstrating that great migratory and functional heterogeneity exists among CD4 memory T cells it will be important to examine whether OX40 signals are

preferentially required by different memory subsets (i.e. central versus effector). In this regard, further characterization of lymphoid and non-lymphoid memory subsets in different organs and systems is needed to dissect the effects of location on memory T cell survival/function and costimulatory requirements. Furthermore, an important but relatively unexplored area of research is the question of whether long-term survival and functionality of memory CD4 T cells requires continuous or periodic signals from costimulatory molecules such as through OX40. Finally, as OX40 is a member of a family of molecules with similar and perhaps overlapping functions, it will be important to examine how additional signals provided by others in the TNFR superfamily such as 4-1BB, LIGHT, HVEM, and CD27 are integrated with OX40 signals to achieve optimal generation, persistence and re-activation of memory T cells.

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Characterization of Rat OX40 Ligand by Monoclonal Antibody

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OX40 (CD134) is a member of the tumor necrosis factor (TNF) receptor superfamily first identified as a rat T cell activation marker. We previously identified the rat ligand for OX40 (OX40L) by molecular cloning. In the present study, we newly generated an anti-rat OX40L mAb (ATM-2) that can inhibit the binding of OX40 to rat OX40L and thus efficiently inhibits the T cell costimulatory activity of rat OX40L. Flow cytometric analyses using ATM-2 and an anti-rat OX40 mAb (MRC OX40) indicated that OX40 was inducible on splenic CD4⁺ T cells by stimulation with immobilized anti-CD3 mAb, while OX40L was not expressed on resting or activated T cells. OX40L was expressed on splenic B cells after stimulation with lipopolysaccharide (LPS), but not on peritoneal macrophages. Interestingly, splenic dendritic cells (DC) expressed OX40L constitutively, which was further upregulated by LPS stimulation. The potent costimulatory activities of splenic DC for anti-CD3-stimulated rat CD4⁺ T cell proliferation and cytokine (IL-2, IFN- γ , IL-10, and IL-13) production were substantially inhibited by ATM-2. These results indicated that OX40L is expressed on professional antigen-presenting cells (APC), and may be involved in humoral immune responses via T-B interaction and in cellular immune responses via T-DC interaction in the rat system. © 2000 Academic Press

OX40 (CD134) was originally identified as a cell surface antigen on activated rat CD4⁺ T cells using MRC

Abbreviations used: OX40L, OX40 ligand; HTLV-1, human T cell leukemia virus type I; DC, dendritic cells; mAb, monoclonal antibody; EAE, experimental autoimmune encephalomyelitis; GVHD, acute graft-versus-host disease; RA, rheumatoid arthritis; EAU, experimental autoimmune uveoretinitis; LPS, lipopolysaccharide; BSA, bovine serum albumin.

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OX40 mAb (1). cDNA cloning of rat, mouse, and human OX40 showed that it belongs to the tumor necrosis factor (TNF) receptor superfamily (2–4). We previously identified the rat ligand for OX40 (OX40L) by cDNA cloning (5), which is a type II membrane protein belonging to the TNF superfamily as in mice and humans (6, 7). Members of the TNF/TNF receptor superfamily have been shown to play critical roles in regulating cellular activation, differentiation, and apoptosis (8–10).

Expression and function of the OX40/OX40L molecules have been studied in the mouse and human systems. It has been shown that the expression of OX40 is restricted to activated T cells in rats, humans, and mice (1, 3, 11). The expression of OX40L has been found on activated mouse B cells (3, 12, 13), human dendritic cells (DC) (14), and human vascular endothelial cells (15). *In vitro* studies using mouse and human OX40L cDNA transfectants have shown that OX40L provides a costimulatory signal to T cells, resulting in increased proliferation and cytokine production (1, 6, 7). Very recent studies using OX40- or OX40L-deficient mice have shown that these mutant mice exhibited an impaired T cell proliferation and cytokine production in certain condition, which appeared to be primarily due to a defect in T cell costimulation through interaction with DC (16–19). In addition, OX40L expressed on activated B cells has been suggested to transmit a signal that enhances proliferation and secretion of immunoglobulin upon crosslinking by OX40 (12), and an impaired humoral immune response was observed in OX40L-deficient mice in a certain condition (19). Collectively, these studies suggested that the OX40/OX40L system plays substantial role in regulating cellular and humoral immune responses *in vivo*. Consistent with this notion, we recently demonstrated that administration of a neutralizing anti-mouse OX40L mAb inhibited T cell responses and antibody production in a mouse leishmaniasis model (20). Besides



such physiological functions, OX40 has been suggested to be preferentially expressed on pathogenic T cells in experimental autoimmune encephalomyelitis (EAE) in rats, and acute graft-versus-host disease (GVHD) and rheumatoid arthritis (RA) in humans (21–23). Furthermore, it has been shown that both OX40 and OX40L are expressed at high levels on human T cell leukemia virus type I (HTLV-1)-transformed human T cells possibly due to transactivation by Tax, suggesting that the OX40/OX40L system may be involved in growth and malignant transformation of HTLV-1-infected T cells (24–28). However, the possible contribution of the OX40/OX40L system to these diseases remains to be verified in appropriate animal models.

To further explore the physiological and pathological functions of OX40L, the rat system has some advantages over the human and mouse system. Superior models of autoimmune diseases, such as collagen-induced arthritis, experimental autoimmune uveoretinitis (EAU), and EAE have been developed in rats (29–31). Some models of human diseases, such as HTLV-1-associated pathologies (32), are unique in the rat system. In order to facilitate the studies in the rat system, we now generated a functional blocking mAb against rat OX40L and characterized the expression and function of OX40L in the rat system.

MATERIALS AND METHODS

Animals and cells. Six-week-old female SD rats and Lewis rats were purchased from Charles River Japan (Atsugi, Japan). HTLV-1-transformed rat T cell lines (F344-S1, ACI-S1, WKA-S1, and Lewis-S1) (33) were provided by Dr. T. Yoshiki (Hokkaido University, Sapporo, Japan). A rat thymic lymphoma cell line (FTL13) (34) was provided by Dr. T. Ueda (Hokkaido University). A rat macrophage cell line (WRT7/P2) (35) was provided by Dr. M. Hosokawa (Hokkaido University). A normal rat kidney cell line (NRK-52E) was provided by Dr. T. Otsuka (Institute of Cytosignal Research, Inc., Tokyo, Japan). A murine T lymphoma L5178Y and a murine myeloma P3U1 (P3X63Ag8U.1) were purchased from ATCC (Rockville, MD). These cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mg/ml penicillin and streptomycin, and 50 μ M β -mercaptoethanol.

Antibodies and reagents. PE-conjugated goat anti-human IgG Ab was purchased from Caltag (South San Francisco, CA). Purified anti-CD3 (G4.18), anti-DC (OX62), anti-mouse IgG3 (R40-82), and control mouse IgG1 (107.3) mAbs, FITC-conjugated anti-CD4 (OX35), CD8 (G28), CD11b/c (OX42), CD45R (HIS24), and control mouse IgG2a (G155-178) and IgG2b (49.2) mAbs, PE-conjugated anti-CD25 (OX39) and control mouse IgG1 (A112-2) mAbs, biotinylated anti-rat CD134 (OX40), control mouse IgG1 (107.3) and IgG2b (49.2) mAbs, and PE-labeled streptavidin were purchased from Pharmingen (San Diego, CA). Anti-CD80 (3H5) and CD86 (24F) mAbs were prepared as described previously (36). A fusion protein consisting of the extracellular portion of murine OX40 and the Fc portion of human IgG1 (OX40-Ig) was prepared as described previously (5). Bovine serum albumin (BSA) and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). Collagenase was purchased from Wako (Osaka, Japan).

Preparation of rat OX40L transfectants. Rat OX40L cDNA was subcloned into the pMKITneo expression vector and transfected into NRK-52E and L5178Y cells by electroporation as described previously (5). Mock transfected cells were also produced by electroporation of the pMKITneo vector without an insert. After selection by 1 mg/ml of geneticin, transfectants stably expressing rat OX40L were identified by staining with OX40-Ig.

Generation of anti-rat OX40L mAb. A SD rat was immunized with F344-S1 cells three times at 7-day intervals. Three days after final immunization, the splenocytes were fused to P3U1 mouse myeloma cells. After hypoxanthine-aminopterin-thymidine selection, one hybridoma producing ATM-2 mAb (mouse IgG1, κ) was identified by its strong reactivity with rat OX40L-transfected L5178Y cells, but not with mock-transfected L5178Y cells, and was purified from ascites by standard procedures with caprylic acid, and purity was verified by SDS-PAGE analysis.

Cell preparation and culture. Splenic T cells (2.5×10^6 /ml) were prepared from SD or Lewis rat splenocytes using a nylon wool column (Wako, Osaka, Japan) and stimulated with immobilized anti-CD3 mAb (10 μ g/ml) for 24–48 h. Splenic lymphocytes (2.5×10^6 /ml) were prepared from Lewis rat splenocytes by depleting adherent macrophages and stimulated with LPS (10 μ g/ml) for 24–48 h. Peritoneal macrophages were obtained from Lewis rats by peritoneal lavage with ice-cold PBS and depletion of nonadherent cells after a brief culture on plastic dish, and then stimulated with LPS (10 μ g/ml) for 24–48 h. Splenic DC were prepared according to the previously described methods (37, 38) with slight modifications. Briefly, spleens from Lewis rats were injected with 200 U/ml collagenase in HEPES-buffered saline (pH 7.2) and were chopped into small fragments with scissors in a culture dish. The perfusate from these treatments was collected and stored on ice. The small fragments of spleens were left in the culture dish with 400 U/ml collagenase, incubated for 30 min at 37°C, and then agitated by rapid pipetting. The spleens and the perfusate were disrupted further by passing through a stainless steel screen and grinding with a syringe plunger. All the released cells were pooled and centrifuged at 1,500 rpm for 5 min at 4°C. The cell pellet was resuspended in dense BSA solution ($\rho = 1.080$) at 1×10^6 /ml, overlaid with 1/5 volume of culture medium, and centrifuged at 100,000g for 15 min at 4°C. Low-density cells were collected from the interface, washed twice, resuspended at 8×10^5 /ml in culture medium, and cultured in 6 well-plate for 90 min at 37°C. The non-adherent cells were removed and then the adherent cells were further cultured overnight in culture medium. After this overnight culture, nonadherent cells, which contained 60–80% OX62-positive cells as estimated by FACS analysis, were collected and used for T cell costimulation or stimulated with LPS (10 μ g/ml) for 24–48 h.

Flow cytometric analysis. Cells (5×10^5) were first preincubated with normal rat IgG (1 μ g/ml) to avoid non-specific binding and then incubated with a saturating amount of OX40-Ig, FITC- or PE-labeled mAb, or biotinylated mAb. After washing with PBS twice, the cells were incubated with PE-conjugated goat anti-human IgG Ab for OX40-Ig or PE-labeled streptavidin for biotinylated mAb. After washing with PBS twice, the stained cells (live-gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA), and data were processed using the CellQuest program (Becton Dickinson).

T cell proliferation and cytokine assays. Splenic T cells (5×10^5 /well) were cocultured with 100 Gy irradiated rOX40L/NRK or mock/NRK transfectants (1×10^4 /well) in the presence of 1 μ g/ml anti-CD3 mAb and 0.5 μ g/ml anti-mouse IgG3 mAb in 96-well flat bottomed culture plates at 37°C, 5% CO₂ for 72 h. Various concentrations of ATM-2 were added at the start of culture. Splenic CD4⁺ T cells (5×10^5 /well), which were purified from nylon wool passed splenocytes by using Rat CD4 Cell Recovery Column kit (Cedarlane, Ontario, Canada), were cocultured with 20 Gy irradiated splenic DC

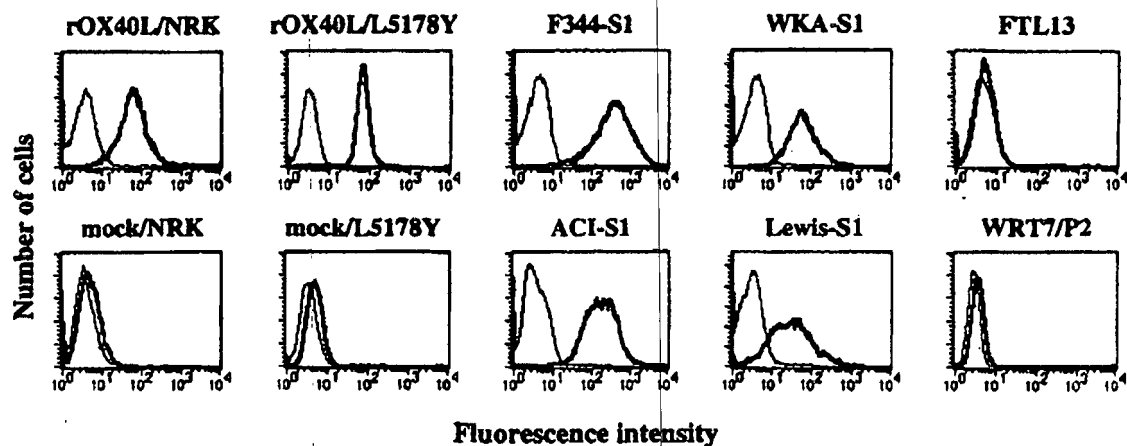


FIG. 1. Reactivity of ATM-2 with rat OX40L transfectants and rat cell lines. NRK-52E- and L5178Y-derived transfectants (rOX40L/NRK, rOX40L/L5178Y, mock/NRK, and mock/L5178Y), HTLV-1-transformed T cell lines (F344-S1, WKA-S1, ACI-S1, and Lewis-S1), a macrophage cell line (WRT7/P2), and a thymic lymphoma cell line (FTL13) were stained with FITC-labeled ATM-2. The bold lines represent staining with FITC-labeled ATM-2 and the plain lines represent background staining with FITC-labeled control mouse IgG.

(1×10^4 /well) in the presence of 0.3 μ g/ml immobilized anti-CD3 mAb in 96-well flat bottomed culture plates at 37°C, 5% CO₂ for 48 h. For estimating proliferative responses, the cultures were pulsed with 0.5 μ Ci/well of [³H]-thymidine (Amersham, Buckinghamshire, UK) for the last 6 h or 12 h and harvested using a Micro 96 Harvester (Skatron, Lier, Norway). Incorporated radioactivity was measured in a micro beta counter (MicroBeta Plus, Wallac, Turku, Finland). To determine cytokine (IL-2, IFN- γ , IL-4, IL-10, and IL-13) production, cell-free supernatants were collected from the replicated cultures at 24, 48, or 72 h and subjected to ELISA using rat IFN- γ or IL-13 Immunoassay kit (BioSource, Camarillo, CA) and OptEIA sets for rat IL-2, IL-4, or IL-10 (PharMingen) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Establishment of Anti-Rat OX40L mAb

We previously demonstrated that an HTLV-1-transformed rat T cell line F344-S1 expressed rat OX40L mRNA abundantly and strongly reacted with OX40-Ig (5). Therefore we immunized BALB/c mice with F344-S1 cells and fused the splenocytes with P3U1 myeloma cells. The hybridomas producing anti-rat OX40L mAb were screened by specific reactivity to rat OX40L cDNA-transfected L5178Y (rOX40L/L5178Y) cells. As shown in Fig. 1, one mAb, designated ATM-2, was obtained, which bound to rat OX40L cDNA-transfected NRK-52E (rOX40L/NRK) and rOX40L/L5178Y cells but not to mock-transfected NRK (mock/NRK) or L5178Y (mock/L5178Y) cells. Some rat T cell lines were also tested for OX40L expression by staining with ATM-2. HTLV-1-transformed rat T cell lines (F344-S1, WKA-S1, ACI-S1, and Lewis-S1), which expressed OX40L mRNA as estimated by Northern blot analysis (5), were reactive with ATM-2, but a rat macrophage cell line (WRT7/P2) and a thymic lymphoma cell line (FTL13), which did not express OX40L

mRNA as estimated by Northern blot analysis (5), were not reactive with ATM-2. These results indicated a strict coincidence of OX40L mRNA expression and ATM-2 reactivity. It has been reported that HTLV-1-transformed human T cell lines expressed OX40L constitutively, possibly due to the transactivation by Tax encoded by the *pX* region of HTLV-1 (7, 25, 27, 39). Tax has been shown to transactivate not only the viral long terminal repeat but also the expression of various host genes, including IL-2 receptor, IL-2, c-fos, c-jun, and OX40, and to play an essential role in HTLV-1-induced cellular transformation (27, 28). It seems likely that the expression of OX40L in the HTLV-1-transformed rat T cell lines also results from the transactivation by Tax.

T Cell Costimulatory Activity of Rat OX40L and Its Blockade by ATM-2

We next examined whether ATM-2 recognizes the binding site of OX40L to OX40. As shown in Fig. 2A, the binding of OX40-Ig to rOX40L/L5178Y cells was almost completely inhibited by preincubation of the cells with ATM-2. This indicated that ATM-2 can interrupt the interaction between OX40L and OX40.

We previously indicated that rat OX40L provided a costimulatory signal for T cell proliferation and cytokine production (5). To examine whether ATM-2 can block the costimulatory activity of rat OX40L, splenic T cells were cocultured with rOX40L/NRK or mock/NRK cells in the presence of anti-CD3 mAb and ATM-2, and the proliferative responses were assessed. As shown in Fig. 2B, rOX40L/NRK cells costimulated the proliferation of anti-CD3-stimulated T cells far more efficiently than mock/NRK cells. This proliferative response costimulated by rOX40/NRK cells was inhibited by

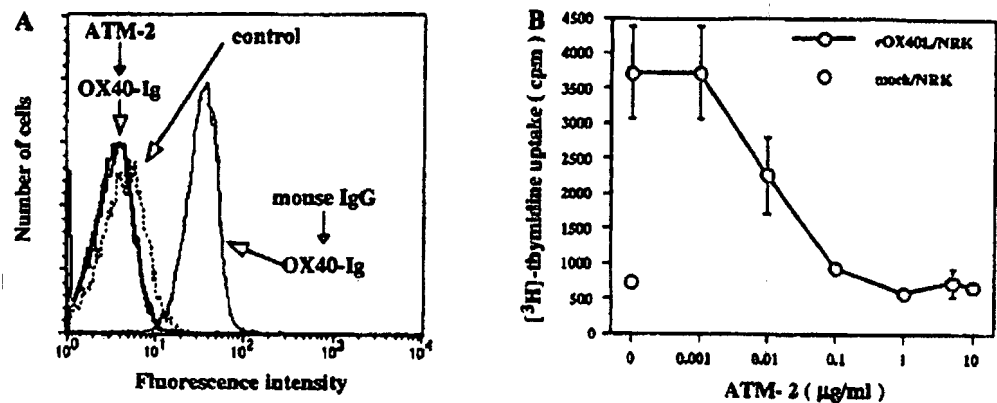


FIG. 2. Functional properties of ATM-2. (A) Blocking of OX40-Ig binding to rat OX40L transfectants. rOX40L/L5178Y cells were pretreated with or without ATM-2 or control mouse IgG and then stained with OX40-Ig followed by PE-labeled goat anti-human IgG Ab. The control histogram represents background staining with ATM-2 and PE-labeled anti-human IgG without OX40-Ig. (B) Blocking of T cell costimulatory activity of rat OX40L by ATM-2. SD rat splenic T cells (5×10^5 /well) were cocultured with irradiated rOX40L/NRK (○) or mock/NRK (□) cells (1×10^4 /well) in the presence of anti-CD3 mAb ($1 \mu\text{g/ml}$) and the indicated concentrations of ATM-2 for 72 h. Proliferative response was assessed by pulsing the cultures with $0.5 \mu\text{Ci/well}$ [^3H]-thymidine for the last 6 h. Data are expressed as the mean $\pm$ SD of triplicate wells.

ATM-2 in a dose-dependent manner. These results indicated that ATM-2 can efficiently block the T cell costimulatory activity of rat OX40L.

Expression of OX40 and OX40L on Rat T Cells

We next examined the expression of OX40 and OX40L on rat splenic T cells by flow cytometric analysis using an anti-rat OX40 mAb (MRC OX40) and ATM-2. An anti-rat CD25 mAb was also included as an activation marker. Splenic T cells from Lewis rats were

stimulated for 24–48 h with immobilized anti-CD3 mAb. As shown in Fig. 3, the expression of OX40 was detected on CD4⁺ T cells after stimulation with anti-CD3 mAb, but not on CD8⁺ T cells. This preferential expression of rat OX40 on CD4⁺ T cells in the rat system was consistent with previous reports by others (1, 40). In contrast, we previously reported that OX40 was expressed on both CD4⁺ and CD8⁺ T cells in the mouse system (13), which represents a species difference in OX40 expression on T cell subsets. On the other

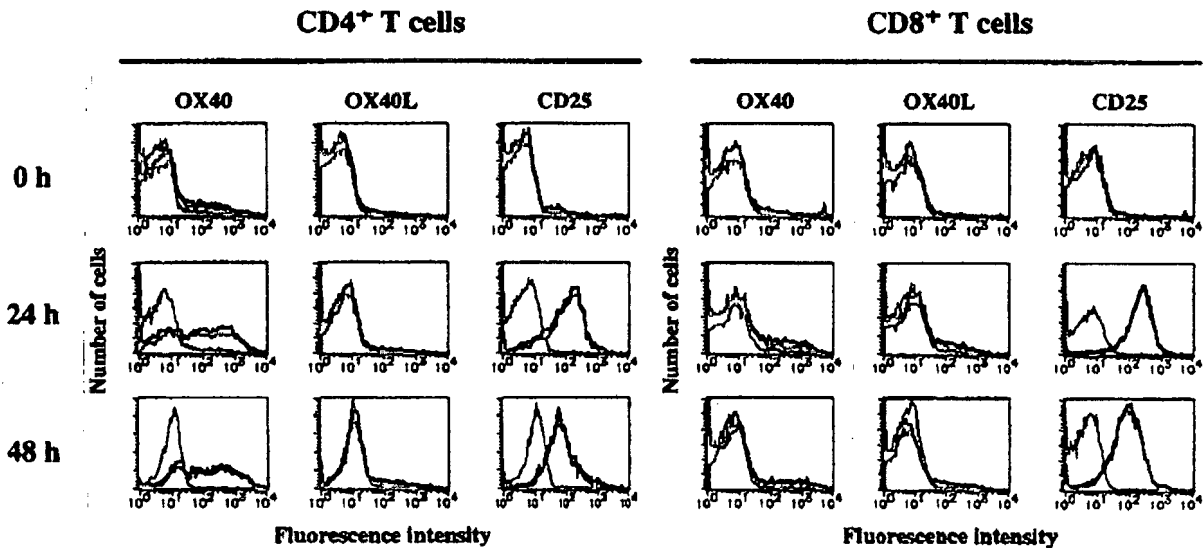


FIG. 3. Expression of OX40 and OX40L on rat T cells. Splenic T cells from Lewis rats were stimulated with immobilized anti-CD3 mAb and harvested at the indicated periods. Cells were stained with FITC-labeled anti-CD4 or anti-CD8 mAb and biotinylated ATM-2, OX40, or control IgG followed by PE-labeled streptavidin or PE-labeled anti-CD25 mAb. Histograms shown are staining of electronically gated CD4⁺ or CD8⁺ cells. The bold lines represent staining with mAb against the indicated molecules and the plain lines represent background staining with control IgG.

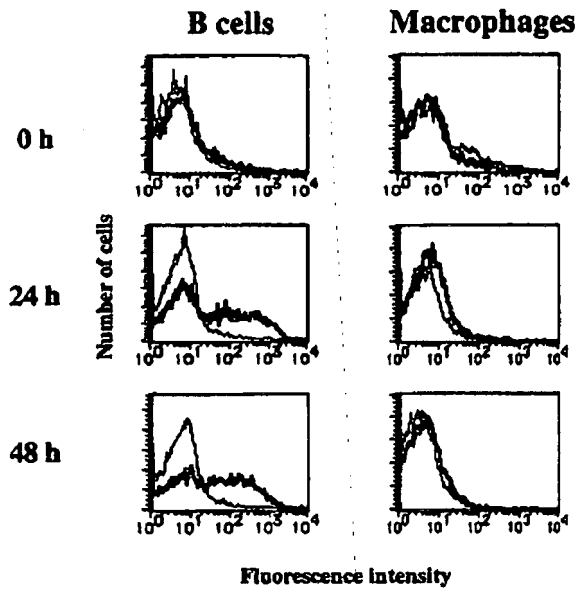


FIG. 4. Expression of OX40L on rat B cells and macrophages. Splenic B cells and peritoneal macrophages from Lewis rats were stimulated with LPS and harvested at the indicated periods. Cells were stained with FITC-labeled anti-CD45R or anti-CD11b/c mAb and biotinylated ATM-2 or control IgG followed by PE-labeled streptavidin. Histograms shown are staining of electronically gated CD45R⁺ or CD11b/c⁺ cells. The bold lines represent staining with ATM-2 and the plain lines represent background staining with control IgG.

hand, OX40L was not found on either CD4⁺ or CD8⁺ T cells even after stimulation with anti-CD3 mAb.

Expression of OX40L on Rat B Cells, Macrophages, and DC

We next examined the expression of OX40L on rat B cells, macrophages, and DC. These cells were purified from the spleen or peritoneum, stimulated with LPS for 24–48 h, and then stained with ATM-2. As shown in Fig. 4, the expression of OX40L was inducible on splenic B cells after stimulation with LPS, which appeared at 24 h, but was not found on resting B cells. In contrast, peritoneal macrophages did not express OX40L significantly even after stimulation with LPS. Most interestingly, splenic DC expressed OX40L constitutively, which was a further upregulated by LPS stimulation, along with the other T cell costimulatory molecules CD80 and CD86 (Fig. 5). These results indicated the expression of rat OX40L on professional antigen-presenting cells (APC) including activated B cells and DC, suggesting the possible contribution of OX40L to humoral immune responses via CD4⁺ T–B interaction and to cellular immune responses via CD4⁺ T–DC interaction in the rat system. It has been reported that CD40 signal regulated the expression of OX40L on mouse and human DC (14, 41), and mouse B cells (12, 13). Although it is interesting to examine

whether the OX40L expression can be regulated by the CD40-CD40L interaction also in the rat system, we presently could not address this issue because an mAb against rat CD40 has not been established yet and CD40L has not been identified in rats. Therefore, the regulation by CD40 remains to be determined in the future study.

Contribution of OX40L and CD80/CD86 to Costimulation of CD4⁺ T Cells by Splenic DC

The above observations of a potent T cell costimulatory activity of OX40L (Fig. 2B) and the expression of OX40 on CD4⁺ T cells (Fig. 3) and OX40L on splenic DC (Fig. 5) suggested that OX40L might play a role in CD4⁺ T cell activation by splenic DC, which also express CD80 and CD86 as the other costimulatory molecules. We then examined the relative contribution of OX40L and CD80/86 to T cell costimulation by splenic DC. Purified splenic CD4⁺ T cells were cocultured with irradiated splenic DC in the presence of anti-CD3 mAb and the combination of anti-OX40L mAb (ATM-2) and/or anti-CD80/86 mAbs for 24–48 h, and then the proliferative response and cytokine (IL-2, IFN- γ , IL-4, IL-10, and IL-13) production were assessed. As shown in Fig. 6, splenic DC exhibited a potent costimulatory activity for anti-CD3-stimulated CD4⁺ T cell proliferation and IL-2, IFN- γ , IL-10, and IL-13 production, whereas IL-4 was not detectable (data not shown).

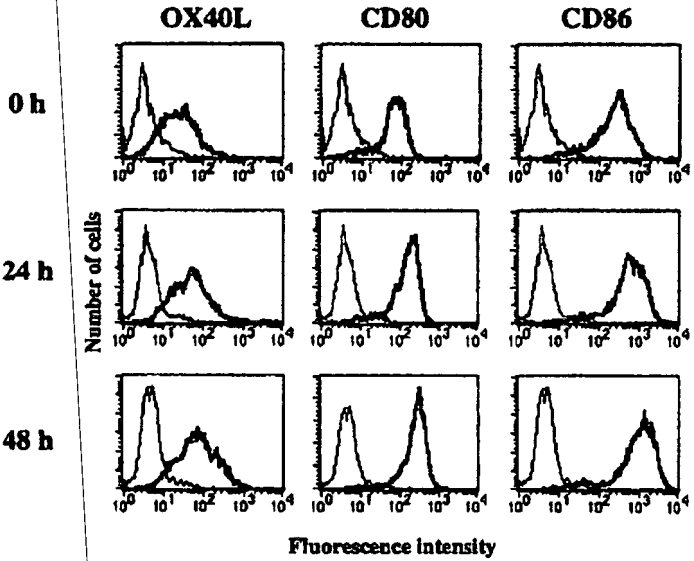


FIG. 5. Expression of OX40L on rat DC. Splenic DC from Lewis rats were stimulated with LPS and harvested at the indicated periods. Cells were stained with biotinylated OX62 mAb and FITC-labeled ATM-2, anti-CD80 mAb, anti-CD86 mAb, or control IgG followed by PE-labeled streptavidin. Histograms shown are staining of electronically gated OX62⁺ cells. The bold lines represent staining with mAb against the indicated molecules and the plain lines represent background staining with control IgG.

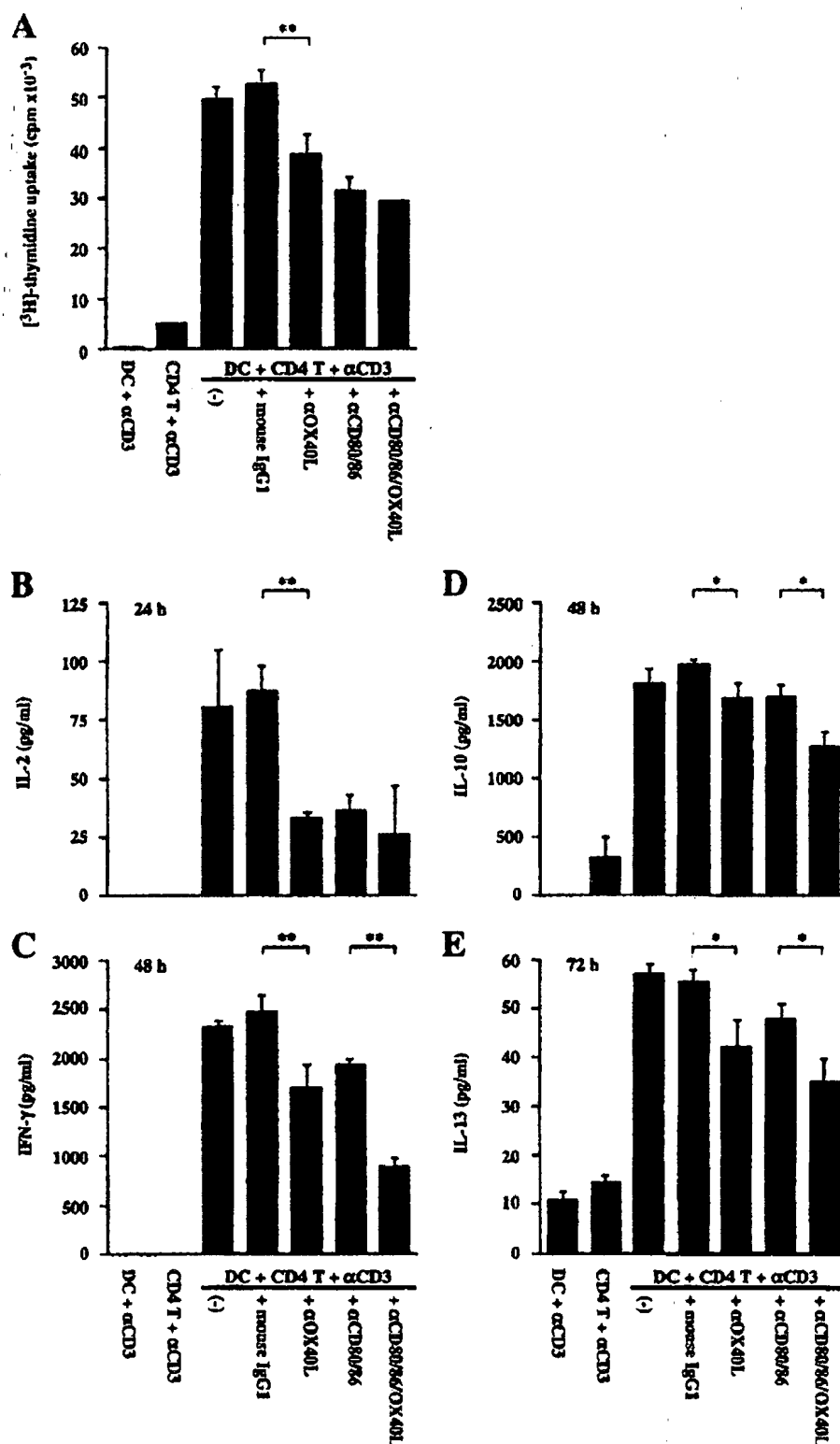


FIG. 6. Contribution of OX40L and CD80/86 to costimulation of CD4⁺ T cells by splenic DC. (A) Inhibitory effects of anti-OX40L and anti-CD80/86 mAbs on CD4⁺ T cell proliferation costimulated by splenic DC. Splenic CD4⁺ T cells from Lewis rats (5×10^5 /well) were cocultured with irradiated DC (1×10^5 /well) in the presence of anti-CD3 mAb ($0.3 \mu\text{g/ml}$) and the indicated mAbs ($5 \mu\text{g/ml}$ each; total concentration of mAbs was adjusted to $15 \mu\text{g/ml}$ with control mouse IgG1) for 48 h. Proliferative response was assessed by pulsing the cultures with $0.5 \mu\text{Ci/well}$ [^3H]-thymidine for the last 12 h. (B-E) Inhibitory effects on cytokine production. Cell-free supernatants were collected from the replicated cultures set as in A at the indicated periods. IL-2 (B), IFN- γ (C), IL-10 (D), and IL-13 (E) contents were measured by ELISA specific for each cytokine. Data in A-E are expressed as the mean $\pm$ SD of triplicate wells. Similar results were obtained from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Proliferative response was partially inhibited by either anti-OX40L mAb or anti-CD80/86 mAbs alone or by the combination of these mAbs (Fig. 6A). IL-2 production was strongly inhibited by either anti-OX40L mAb or anti-CD80/86 mAbs alone (Fig. 6B). Production of IFN- γ , IL-10, and IL-13 was significantly inhibited by either anti-OX40L mAb or anti-CD80/86 mAbs alone and additionally inhibited by the combination of these mAbs (Figs. 6C–6E). In general, the inhibitory effects of anti-OX40L mAb were almost comparable to those of anti-CD80/86 mAbs. These results indicated a substantial contribution of OX40L to the costimulatory activity of splenic DC that enhances T cell proliferation and production of both T helper type I (Th1) cytokines (IL-2 and IFN- γ) and Th2 cytokines (IL-10 and IL-13). This is consistent with a recent observation in mice that OX40L-deficient DC showed an impaired ability to induce IL-2, IFN- γ , and IL-4 production by CD4⁺ T cells (18). The only partial inhibition even by the combination of anti-OX40L mAb and anti-CD80/86 mAbs suggest a contribution of the other costimulatory molecules such as CD70, CD30L, and 4-1BBL, which were found on mouse DC by us and others (20, 42). Further studies are needed to address the contribution of these molecules.

In the present study, we generated a mAb specific for rat OX40L and determined the expression of OX40L on LPS-activated B cells and splenic DC. We also substantiated the costimulatory function of OX40L expressed on splenic DC for CD4⁺ T cells. OX40/OX40L have been implicated in the pathogenesis of HTLV-1-associated diseases, such as adult T cell leukemia (ATL) (43, 44), myelopathy, uveitis, arthropathy, and Sjögren's syndrome (45). OX40/OX40L have been also implicated in the pathogenesis of autoimmune diseases such as EAE (a model of multiple sclerosis) (23, 46) and RA (23), and inflammatory bowel disease (47). The present establishment of a neutralizing anti-rat OX40L mAb enables us to define the pathophysiological roles of the OX40/OX40L system in rat models of inflammatory diseases and virus-associated diseases.

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