Cette thèse intitulée:

«Regulation of T cell response by a new member of the TNF receptor family, DcR3/TR6»

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SUMMARY

TR6, also called DcR3, M68, is a soluble receptor of the TNFR superfamily. It has three identified ligands: FasL, LIGHT and TL1A, all TNF ligand superfamily members. TR6 can interfere with the interaction between FasL versus Fas, LIGHT versus HveA, and TL1 versus DR3. TR6 is over-expressed in some tumours. It has been proposed that TR6-secreting tumours use this molecule to evade immune surveillance and gain survival advantage.

In this project, the function of TR6 in immune regulation was investigated. As we have proven that human TR6 can cross-react with mouse LIGHT, and anti-human LIGHT mAb can bind to mouse LIGHT on the mouse T-cell surface, we use human TR6 and anti-human LIGHT monoclonal antibody to study the function of TR6 in the mouse system.

In the *in vitro* T cell response study, we have found that TR6 on solid phase can costimulate mouse T cell response in terms of proliferation, cytokine production and CTL activity. Blocking experiments indicate that soluble human LIGHT can block TR6-costimulated CTL activity. Anti-LIGHT mAb on solid phase also enhances T-cell proliferation. These effects indicate that LIGHT reverse signaling involves in the solid phase TR6 costimulated T cell response. Based on our findings in LIGHT signaling transduction, it is likely that, during T-cell activation, LIGHT forms capping and quickly moves into clustered TCR. The congregated LIGHT inside the TCR cluster

interacts with other signaling molecules through its association with Grb2 to activate downstream signaling molecules such as ERK1/2 to costimulate T cell activation.

Further, we have explored the TR6 costimulatory function in tumour immunotherapy. As T cells express two TR6 ligands, i.e. FasL and LIGHT, both of which can reversely transduce positive costimulatory signals into T cells, we have developed a tumour vaccine based on surface TR6 expression on tumour cells. *In vitro* studies have shown that TR6 surface expression on tumour cells is able to costimulate both human and syngeneic mouse T-cell proliferation and cytokine production. *In vivo* studies have shown that TR6 surface expression reduces P815 mouse mastocytoma cell tumourigenicity, and induces specific anti-P815 tumour cell immunity. Vaccination with surface TR6 expressing tumour cells is effective in treating existing highly immunogenic as well as lowly immunogenic tumours; the therapeutic effects are enhanced when the vaccine is used in combination with Bacillus of Calmette and Guerin (BCG). These results indicate that expressing TR6 on tumour cell surface might be a very useful strategy in tumour immunotherapy.

We have also found that TR6 in solution inhibits SDF-α–induced T-cell migration both *in vitro* and *in vivo*. TR6-pretreated T cells have impaired actin-polymerization, and T-cell pseudopodium formation; they also have reduced Cdc42 activity. Crosslinking T-cell surface LIGHT with anti-LIGHT monoclonal antibody also impairs the T-cell migration *in vitro*. These results indicate that LIGHT reverse signaling is involved in T-cell migration.

Our study has discovered two important functions of TR6 in the immune regulation, i.e., costimulating T-cell activation and inhibiting T cell migration. For the first time the principle of reverse signaling be applied in tumour vaccine development. Our findings have broadened our knowledge of TR6 in the immune regulation. The surface TR6 based vaccine can be further exploited in immunotherapy of human tumours.

Key words: TR6/DcR3; LIGHT; reverse signaling; costimulation; migration; tumour vaccine.

Résumé

TR6, appelé également DcR3 ou M68, est un récepteur soluble de la grande famille du TNFR. Trois ligands de TR6 ont été identifiés: FasL, LIGHT et TLIA, qui sont tous membres de la famille TNF. Fortement exprimé dans certaines tumeurs, TR6 interfère également dans les interactions entre FasL et Fas, LIGHT et HveA, ainsi qu'entre TL1 et DR3. Il a été donc proposé que les tumeurs secrétant TR6 pourraient utiliser cette molécule afin d'éviter la surveillance immunitaire et s'assurer un avantage de survie.

Le présent projet consiste à élucider les fonctions de TR6 dans la régulation du système immunitaire. Comme nous avons déjà constaté que TR6 humain et LIGHT de souris sont en mesure de réagir ensemble, et qu'un anticorps monoclonal de souris anti-LIGHT humain peut également se lier à LIGHT de souris à la surface des cellules T murines, nous nous permettons donc d'utiliser le TR6 humain et l'anticorps monoclonal de souris anti-LIGHT humain pour étudier la fonction de TR6 dans le système immunitaire de souris.

Par des études *in vitro* de la réponse des cellules T, nous avons constaté que TR6, présenté sur une surface solide, costimule la résponse des cellules T de souris incluant la prolifération, la production de cytokines et l'activité cytotoxique de CTL. Des expériences de competition démontrent que LIGHT humain sous forme soluble, est capable de bloquer l'activité cytotoxique de CTL costimulé par TR6, tandis que l'anticorps monoclonal de souris anti-LIGHT humain, prèsenté sur surface solide, augmente également la prolifération des cellules T. Ces effets indiquent que LIGHT joue un rôle de signalisation inverse sur TR6 en costimulant la réponse des cellules T.

Selon nos résultats sur les voies de signalisation de LIGHT, il semble que pendant l'activation des cellules T, LIGHT change de conformation et interagit rapidement avec le TCR oligomérizé. LIGHT associé aux agglomérats de TCR interagit avec d'autres molécules de signalisation cellulaire en s'associant à Grb2, et active les molécules de signalisation en aval, tels que ERK 1/2, dans l'activation des cellules T.

De plus, nous avons étudié la fonction de TR6 costimulé dans l'immunothérapie des tumeurs. Comme la cellule T exprime deux ligands de TR6, FasL et LIGHT, qui peuvent être impliqués dans une signalisation inverse, provoquant la transduction de signaux positifs dans des cellules T, nous avons développé un vaccin de tumeur basé sur la réaction que TR6 produit à la surface des cellules tumorales. Des études *in vitro* prouvent que cette réaction costimule la prolifération des cellules T humaines ou murines syngénéiques, ainsi que la production de cytokines. En même temps, des études *in vivo* démontrent que cette réaction réduit également la tumorigénicité des cellules de mastocytes de souris P815, et produit une immunité spécifique vis-à-vis les cellules P815. Le susdit vaccin semble être efficace dans le traitement des tumeurs fortement et faiblement immunogèniques. Il a été également remarqué que les effets thérapeutiques deviennent encore plus évidents, lorsque le vaccin est utilisé en combinaison avec le bacille de Calmette-Guerin (BCG). Ces résultats nous indiquent que cette méthode pourrait devenir une stratégie importante dans l'immunothérapie de tumeur.

Nous avons découvert egalement que TR6 sous forme soluble, inhibe la migration des cellules T induite par SDF-1α *in vitro* et *in vivo*. Les cellules T pré-traitées par TR6

démontrent une altération de la polymérisation de l'actine, de la formation de pseudopodes des cellules T, et une réduction de l'activité de Cdc42. La liaison de LIGHT à la surface des cellules T à l'aide de l'anticorps monoclonal anti-LIGHT, a également altéré la migration des cellules T *in vitro*. De ces résultats, on comprend que la signalisation inverse de LIGHT joue un rôle important dans la migration des cellules T.

La présente étude révèle deux rôles importants de TR6 dans la régulation du système immunitaire, soit la co-stimulation/activation des cellules T, ainsi que l'inhibition de la migration des cellules T. Ces principes ont été appliqués pour la première fois dans le dévoleppement d'un vaccin contre les tumeurs. Les résultats obtenus nous permettrons d'élargir notre connaissance sur le rôle de TR6 dans la règulation de la réponse immunitaire. Le vaccin basé sur l'expression de TR6 à la surface des cellules présente une nouvelle avenue thérapeutique contre le cancer.

Mots clé: TR6/DcR3; LIGHT; signalisation inverse; costimulation; migration; vaccin anti-cancer.

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LIST OF ABBREVIATIONS

AA	Amino acid residues
Ag	Antigen
AP-1	Transcription factor activator protein 1
APC	Antigen-presenting cells
Caspase	Cytosolic aspartate-specific protease
CFSE	Carboxyl fluorescein succinimidyl ester
CRD	Cysteine rich domain
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DcR 3	Decoy receptor 3
DD	Death domain
DR	Death receptor
FADD	Fas-associated death domain
FasL	Fas ligand
GPI	Glycosylphosphatidylinositol
GvHD	Graft versus host disease
HVEM/TR2	Herpes virus entry mediator
Ig	immunoglobulin
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IFN-γ	Interferon-γ
KO	Knockout
LC	Langhans cells
LIGHT	Lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D (gD) for HVEM
LN	Lymph nodes
LPS	Lipopolysaccharide
LTα	Lymphotoxin α

LTβ	Lymphotoxin β
LTβR	Lymphotoxin beta receptor
JNK	c-jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MLR	Mixed lymphocyte reaction
MITD	Minimum tumourigenic dose
NF-κB	Transcription factor nuclear factor-κB
NIK	NF-κB-inducing kinase
NK	Natural killer
OPG	Osteoprotegrin
PARP	Poly (ADP-ribose) polymerase
PHA	Phytohemagglutinin
PMA	Phorbol 1,2-myristate 1,3-acetate
PP	Peyer's patch
SDF-1α	Stromal cell-derivedfactor 1α
SFP	Superfamily protein
SODD	Silencer of death domain
TA	Tumour antigen
TAA	Tumour associate antigen
TAA	Tumour specific antigen
Tg	Transgenic
Th1/2	T helper cell 1 and 2
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRAF	TNF receptor associated factor
TRADD	TNFR-associated death domain protein
TRANCE	TNF related activation-induced cytokine

I. INTRODUCTION

I. INTRODUCTION

1. The TNF/TNFR superfamily

Since the first two tumour necrosis factor family members (TNFα and lymphotoxin) were isolated in 1984, researchers have proven the existence of a TNF superfamily and a TNF receptor (TNFR) superfamily. There are 19 members in the TNF superfamily and 30 members in the TNFR superfamily have been identified. The TNF family members are predominantly expressed in the immune system, and play very important roles in immune responses and immune related diseases. The TNF/TNFR superfamily proteins become therapeutic targets in many human diseases. For example, TNFα blockers have already been clinically proven effective in treating rheumatoid arthritis (1) and some other bowel diseases (2). These proteins are also pivotal in embryonic development, as well as in wide rage of organ and tissue functions.

1.1. The TNF ligand superfamily

The first two TNF family members were identified as lymphocyte and macrophage products that caused the lysis of certain types of cells, especially tumour cells, approximately 3 decades ago, and it was believed that they have a therapeutic potential in the treatment of cancer. These products were called lymphotoxin (LT) and tumour necrosis factor (TNF), respectively (3, 4). In 1984 these two proteins were cloned (5, 6) and were identified as two members of a gene superfamily. As of today, 19 different members of the TNF superfamily have been identified in mammalians (7). As listed in table 1 (http://www.gene.ucl.ac.uk/nomenclature/genefamily/tnftop.html), the TNF family members now include TNFα, LTα (lymphotoxin-α), LTβ (lymphotoxin–β),

FasL, OX40l, CD27L, CD30L, CD40L, 4-1BBL, TRAIL, TRANCE/RANKL/OPGL, TWEAK, APRIL/TALL-2, ED1, BAFF/TALL1, LIGHT, TRANCE, VEGI, TL1A and AITRL/TL6/hGITRL (7). All these ligands signal through at least one of TNF receptor family members (fig. 1). Some of the ligands can also work as receptor reversely transducing signals into cells (8).

All the TNF ligand superfamily members, with the exception of LT-α and vascular endothelial cell-growth inhibitor, are type II (intracellular N terminus and extracellular C terminus) transmembrane glycoproteins with limited homology (about 20% overall) to TNF in their extracellular region (7, 9), called "TNF homology domain" (THD). The THD folds into an antiparallel β-sandwich that assembles into trimers, and thus each ligand has three receptor binding sites, forming a groove between adjacent subunits (10). No significant homology exists in other regions of these molecules. Although most ligands are synthesized as membrane-bound proteins, soluble forms can be generated by limited proteolysis (11). The crystal structure of TNF and LTα indicates that these cytokines are biologically active as trimers, and it is generally assumed that the other TNF family members are also trimeric (11, 12, 13, 14, 15, 16, 17).

The expression of almost all of the TNF family members is limited to cells of the immune system, including B cells, T cells, NK cells, monocytes and dendritic cells, the only exception is VEGI and TL1A, which are expressed mainly by endothelial cells (7, 18). The function of TNF family members is as ligands triggering signals through their receptors, meanwhile some TNF members can also work as a receptor reversely trans-

Table 1. Members of the TNF Ligand Superfamily

Approved Symbol	Name	Aliases	References
LTA	lyphotoxin alpha	TNFSF1, TNFB, LT	(5, 19)
TNF	tumour necrosis factor	TNFSF2, TNFA, DIF	(6)
LTB	lyphotoxin beta (TNF superfamily, member 3)	TNFSF3, TNFC, p33	(20)
TNFSF4	tumour necrosis factor superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kD)	OX-40L, gp34, TXGP1	(21)
TNFSF5	tumour necrosis factor superfamily, member 5	CD40LG, IMD3, HIGM1, CD40L, hCD40L, TRAP, CD154, gp39	(22, 23)
TNFSF6	tumour necrosis factor superfamily, member 6	FasL, APT1LG1	(24, 25)
TNFSF7	tumour necrosis factor superfamily, member 7	CD70, CD27L, CD27LG	(26)
TNFSF8	tumour necrosis factor superfamily, member 8	CD30LG	(27)
TNFSF9	tumour necrosis factor (ligand) superfamily, member 9	4-1BB-L	(26)
TNFSF10	tumour necrosis factor (ligand) superfamily, member 10	TRAIL, Apo-2L, TL2	(28)
TNFSF11	tumour necrosis factor (ligand) superfamily, member 11	TRANCE, RANKL, OPGL, ODF	(29)
TNFSF12	tumour necrosis factor (ligand) superfamily, member 12	TWEAK, DR3LG, APO3L	(30, 31)
TNFSF13	tumour necrosis factor (ligand) superfamily, member 13	APRIL	(32)
TNFSF14	tumour necrosis factor (ligand) superfamily, member 14	LIGHT, LTg, HVEM-L	(33, 34)
TNFSF15	tumour necrosis factor (ligand) superfamily, member 15	TL1, VEGI	(18)
TNFSF18	tumour necrosis factor (ligand) superfamily, member 18	AITRL TL6 hGITRL	(35)
ED1	ED1	EDA, EDA1	(36)

ducing signaling after binding with their receptors (detailed in section 1.1.3).

1.2. The TNF receptor superfamily

Currently, the TNF receptor superfamily contains 30 members, as summarized in table 2. The mammalian TNFR superfamily now includes: TNFR-I, TNFR-II, LTβR, Fas, OX40, CD40, CD27, CD30, 4-1BB, DcR1, DcR2, DcR3/TR6, OPG, DR3, DR3L, DR4, DR5, DR6, HVEM (TR2), RANK, TACI, BAFFR, EDAR, BCM, GITR, RELT, NGFR, SOBa, Tnfrh1 and TAJ (TROY) (7, 10, 19, 37, 38, 39, 40). The number of this family is still growing.

Most of the TNFR family members are characterized as type I transmembrane proteins (extracellular N-terminus and intracellular C-terminus). The B-cell maturation antigen (BCMA), transmembrane activator and cyclophilin ligand interactor (TACI), BAFF receptor (BAFFR) and X-linked EDA receptor (XDAR) belong to type III transmembrane protein group, because they lack a signal peptide sequence (7). All the TNFR family members contains one to six conserved motif, called "cysteine-rich repeats" or "cysteine-rich domain (CRD)", in their extracellular portion (7, 10, 17, 23, 41, 42, 43). Unlike TNF ligand family members, which are predominantly expressed by cells of the immune system, the TNFR family members are expressed by a variety of cells (7).

According to the structural and biological features, TNFR family members can be divided into three subgroups:

Table 2. Members of TNF Receptor Superfamily

Approved Symbol	Name	Aliases	References.
TNFRSF1A	tumour necrosis factor receptor superfamily, member 1A	p55-R, CD120a, TNF-R-I p55, TNF-R, TNFR1, TNFAR, TNF-R55, p55TNFR, TNFR60	(44)
TNFRSF1B	tumour necrosis factor receptor superfamily, member 1B	CD120b, p75, TNF-R, TNF-R-II, TNFR80, TNFR2,TNF-R75, TNFBR , p75TNFR	(45, 46)
LTBR	lymphotoxin beta receptor (TNFR superfamily, member 3)	TNFRSF3, TNFR2-RP, CD18, TNFR-RP, TNFCR, TNF-R-III	(39)
TNFRSF4	tumour necrosis factor receptor superfamily, member 4	OX40, ACT35, TXGP1L	(21)
TNFRSF5	tumour necrosis factor receptor superfamily, member 5	p50, Bp50, CD40	(47)
TNFRSF6	tumour necrosis factor receptor superfamily, member 6	FAS, CD95, APO-1, APT1	(48)
TNFRSF6B	tumour necrosis factor receptor superfamily, member 6 b, decoy without transmembrane domain	DcR3	(49, 50)
TNFRSF7	tumour necrosis factor receptor superfamily, member 7	Tp55, S152, CD27	(51, 52)
TNFRSF8	tumour necrosis factor receptor superfamily, member 8	Ki-1, D1S166E, CD30	(27, 43)
TNFRSF9	tumour necrosis factor receptor superfamily, member 9	4-1BB, CD137, ILA	(26, 53)
TNFRSF10A	tumour necrosis factor receptor superfamily, member 10a	DR4, Apo2, TRAILR-1	(54)
TNFRSF10B	tumour necrosis factor receptor superfamily, member 10b	DR5, KILLER, TRICK2A, TRAIL-R2, TRICKB	(55, 56)

TNFRSF10C	tumour necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	DcR1, TRAILR3, LIT, TRID	(56)
TNFRSF10D	tumour necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	DcR2, TRUNDD, TRAILR4	(57)
TNFRSF11A	tumour necrosis factor receptor superfamily, member 11a, activator of NFKB	RANK	(58, 59)
TNFRSF11B	tumour necrosis factor receptor superfamily, member 11b (osteoprotegerin)	OPG, OCIF, TR1	(60)
TNFRSF12	tumour necrosis factor receptor superfamily, member 12 (translocating chain-association membrane protein)	DR3, TRAMP, WSL-1, LARD, WSL-LR, DDR3, TR3, APO-3	(61)
TNFRSF12L	tumour necrosis factor receptor superfamily, member 12-like	DR3L	(62)
TNFRSF13B	tumour necrosis factor receptor superfamily, member 13B	TACI	(63, 64)
TNFRSF13C	tumour necrosis factor receptor superfamily, member 13C	BAFFR	(65)
TNFRSF14	tumour necrosis factor receptor superfamily, member 14 (herpes virus entry mediator)	HVEM, ATAR, TR2, LIGHTR, HVEA	(33, 66)
NGFR	nerve growth factor receptor (TNFR superfamily, member 16)	TNFRSF16	(43)
TNFRSF17	tumour necrosis factor receptor superfamily, member 17	BCM, BCMA	(67)
TNFRSF18	tumour necrosis factor receptor superfamily, member 18	AITR, GITR	(68)
TNFRSF19	tumour necrosis factor receptor superfamily, member 19	TROY, TAJ	(69)
TNFRSF19L	tumour necrosis factor receptor superfamily, member 19-like	FLJ14993, RELT	(70)
TNFRSF21	tumour necrosis factor receptor superfamily, member 21	DR6	(71)
TNFRSF22	tumour necrosis factor receptor superfamily, member 22	SOBa, Tnfrh2, 2810028K06Rik	(72)
TNFRSF23	tumour necrosis factor receptor superfamily, member 23	mSOB, Tnfrh1	(72, 73)
EDAR	EDAR	-	(74)

a. The TRAF (TNFR-associated subgroup). This group includes TNFR1, TNFR2, CD40, CD30, CD27, LTβR, 4-1BB, OX40, NGFR, FN14, HVEM, GITR and RANK. This subgroup of receptors bind to a family of adaptor proteins, TRAFs (75, 76, 77, 78). There are six known TRAFs in mammalian cells; only TRAF2, TRAF5 and TRAF6 are shown to mediate the activation of NFκB and JNK. TRAF2 can bind to TNFR1 through their TNFR-associated death domain (TRADD) protein (7).

b. The death receptor subgroup. This subgroup of TNFRs contains CRDs in their extracellular region and a "death domains" in their intracellular portion. They function as a mediator of apoptotic cell death, and are therefore also called "death receptors" (DRs). Cell killing from such receptors occurs because of recruitment to the receptor of the adaptor protein FADD (Fas-associated protein with death domain), which in turn recruits the pro form of caspase-8. Aggregation of pro-caspase-8 leads to its auto-activation and subsequent activation of effector caspases such as caspase-3 (41, 79). The death receptor subgroup contains eight members: TNFR1 (80, 81), Fas (81, 82), DR3 (61, 79), DR4 (54), DR5 (55), DR6 (71), EDAR (74) and NGFR (83).

c. The decoy receptor subgroup. This subgroup of receptors includes DcR1 (decoy receptor 1, also known as TRID or TRAIL-R3) (54, 56, 84, 85, 86), DcR2 (decoy receptor 2, also known as TRUNDD or TRAIL-P.4) (87, 88), DcR3 (TR6) (50) and OPG (60, 87). DcR1 is a glycosylphosphatidylinositol (GPI)-linked protein with out an intracellular death domain whereas DcR2 is a transmembrane receptor but with a partially deleted death domain. Both of DcR1 and DcR2 are incapable

of transmitting apoptotic signals (40). The product of OPG is a secreted protein that also binds to TRAIL but with much weaker affinity compared with other receptors. DcR3 (TR6), as will be described in details below, is closer to OPG and exists as a secreted protein. This group of receptors has been shown to interfare signaling of other TNFR family members (7).

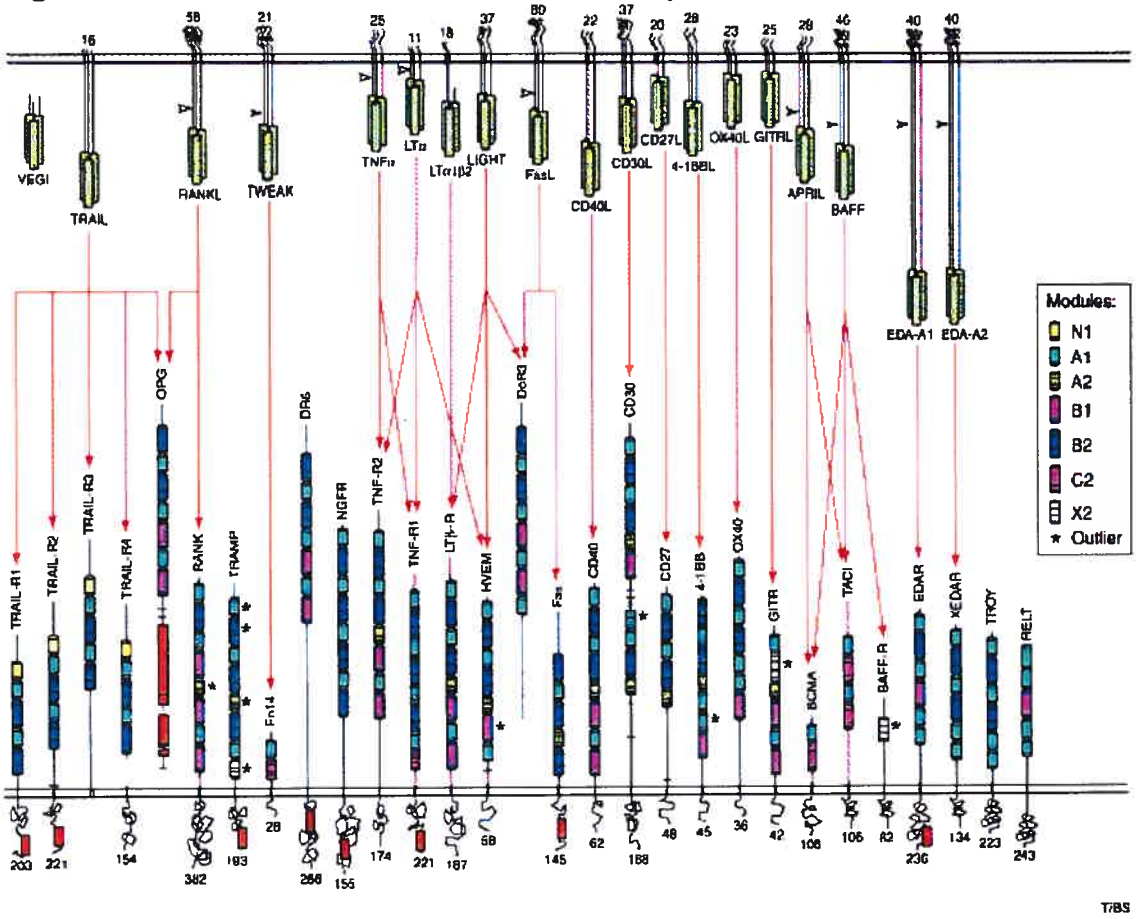
The interaction of TNF-TNFR family members play very important roles in immune response, haematopoiesis and morphogenesis (7, 37, 89, 90, 91, 92).

1.3. The reverse signaling via TNF family members

The interactions between TNF and TNFR superfamily members are summarized in Fig.1. The 19 members of TNF ligand family signal through 29 TNFR family members, and play a very important role in regulating immune response, haematopoiesis and morphogenesis (7). Studies in TNF and TNFR family members also find that some TNF ligand members can function as a receptor reversely transducing signals into cells after engaging with their receptors. This phenomenon is termed "reverse signaling".

The TNF ligand family members capable of reverse signaling are FasL, CD40L, CD30L, OX-40L, TRANCE, TRAIL, DR4 and LIGHT. Studies on FasL have found that Fas-Ig on solid phase augments CD8 T-cell proliferation in the presence of suboptimal anti-CD3; $FasL^+$ CD8 T cell responded better to alloantigen *in vitro* than $FasL^-$ CD8 T cells (93, 94). Moreover naive $CD4^+$ T cells are responsive to FasL-mediated costimulation on encountering with antigen, if Fas-mediated death is prevented (94). These data indicate that FasL can reversely transduce signals into T cells

Fig. 1 The interaction between TNF-TNFR family members



(Adapted from Bodmer et al. Trends in Biochemical Sciences, 2002;27:19)

Fig.1. Interactions between ligands and receptors of the human tumour necrosis factor (TNF) family: TNF ligands (top) and TNF receptors (bottom). The TNF ligands are represented as type II homo- or heterotrimeric transmembrane proteins (with the exception of VEGI, which lacks a predicted transmembrane domain and is therefore drawn as a soluble ligand). TNF homology domains (THDs) are shown as green boxes. Filled black arrowheads indicate processing by furin family members, and open black arrowheads by other types of proteases. The TNF receptors are typically type I or type III transmembrane proteins, but also occur as glycolipid-anchored or soluble proteins. N1, A1, A2, B1, B2, C2 and X2 modules are colour-coded as shown in the inset. The positions of individual cysteines are indicated by horizontal bars, and stars show modules whose cysteine pattern does not conform entirely to that of cannonical A, B, C and N modules. The lengths of intracellular domains are indicated for each ligand and each receptor, and the intracellular homology domains, known as the `death domains', are indicated as red boxes. Red arrows show documented interactions.

Cayabyab et al. found that $CD40^+$ murine transfectants substantially augment anti-CD3-induced T cell proliferation and result in the generation of CTL. $CD4^+$ T cells respond better than $CD8^+$ cells to CD40 costimulation (95). These results suggested that CD40L reversely transduces signals into T cells. Van Essen et al. using CD40 knockout mice, found that CD40L reverse signaling is responsible for germinal centre formation (96). Brenner et al. reported that activation of T-lymphocytes via gp39/CD40L induces strong activation of Jun-N-terminal kinase (JNK) and p38 MAPK, and it also induces tyrosine phosphorylation of cellular proteins including PLC γ (97, 98). Blair et al. found that CD4 T cells costimulated with anti-CD3/CD40L have enhanced production of interleukin (IL)-10, interferon gamma, and tumour necrosis factor alpha but not IL-2 or IL-6. The anti-CD3/CD40L-mediated activation of these cells is followed by apoptosis in a significant fraction of the cells (99). Wiley et al. found that cross-linking of CD30L by a mAb or by CD30-Fc fusion protein induces the production of IL-8 by freshly isolated neutrophils (100). Another study proved that CD30L reverse signaling can inhibit Ig class switch and antibody production in human IgD^+ IgM^+ B cells (101). Chou et al. reported that cross-linking TRAIL by a plate-bound rTRAIL receptor, i.e., death receptor 4-Fc fusion protein, dose dependently enhances T-cell proliferation and IFN-gamma production in conjunction with immobilized suboptimal anti-CD3 stimulation in mouse splenocytes (102). A subset of B-cells proliferates vigorously in response to CD70 mAb but not to CD27 mAb. This result proved that CD27L, which is CD70, can transduce signals reversely into B cells (103). Further, the CD27L ligation-induced B-cell response is synergistically enhanced by ligation of CD40, but inhibited by the presence of IL-4 (103). The cross-linking of

OX40L on CD40L-stimulated B cells, alpha IgD dextran-stimulated B cells, or both, results in a significantly enhanced proliferative response with no change in the cell survival rate. The OX40 stimulation increases immunoglobulin heavy chain mRNA levels and immunoglobulin secretion, and it also down-regulates the transcription factor, B cell lineage-specific activator protein (BSAP) (104, 105). Chen reported that cross-linking TRANCE by its receptor augments IFN-gamma secretion by Th1 cells, and the enhanced secretion of IFN-gamma mediated by TRANCE correlates with the activation of p38 mitogen-activated protein kinase (106). Our group studied another TNF family member, LIGHT. We found that cross-linking of LIGHT with anti-LIGHT mAb or its receptor TR6/DcR3 can dose dependently enhance both proliferation and cytokine production of anti-CD3-stimulated CD4 and CD8 T cells. The cross-linking of LIGHT also enhances mouse spleen CTL activity (8, 107). In general, unlike costimulatory members of the TNFR family, the costimulation through reverse signaling of the TNF ligand family members in various animal disease models is not well investigated.

1.4. TR6/DcR3 and its ligands

TR6, a member of TNFR superfamily, belongs to the decoy receptor subgroup. It has 3 ligands, FasL, LIGHT and TL1A. FasL and LIGHT are expressed on T cells.

1.4.1. TR6

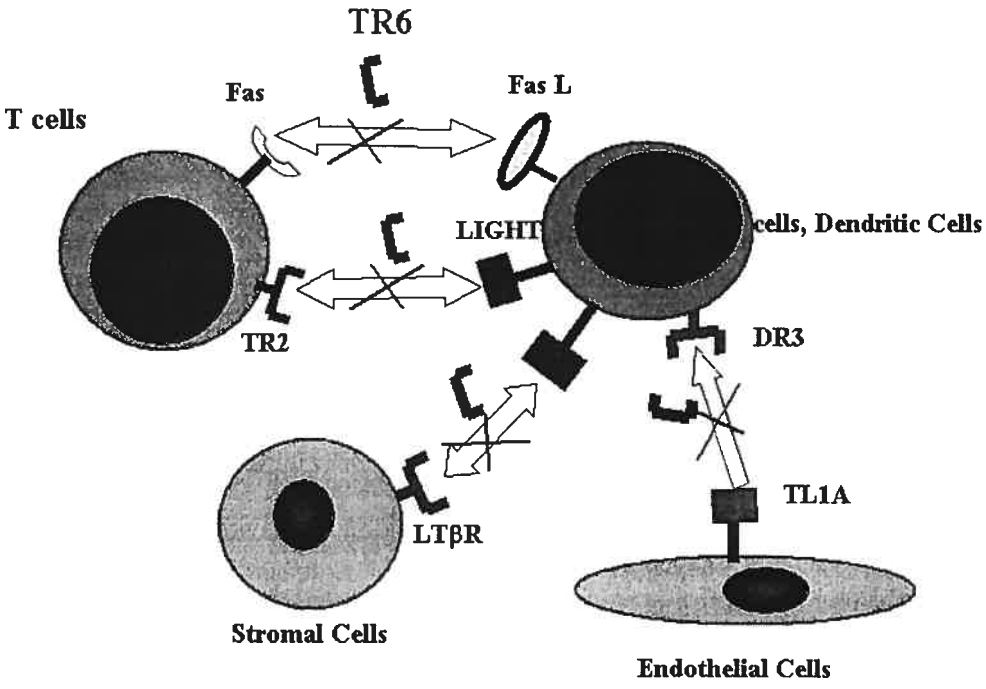
TR6 (TNFRSF6B, tumour necrosis factor receptor superfamily member 6 b), a secreted protein, due to lack of the transmembrane domain in its coding sequence, belongs to the decoy receptor subgroup. The mature form TR6 has 271 amino acid residues. It contains two incomplete cysteine-rich domains (49, 50, 108). TR6 is expressed by many types of

cancers (49, 50, 109, 110, 111, 112, 113). The DcR3 gene is significantly over-expressed in peripheral blood mononuclear cells of silicosis and SLE patients (114). A recent study showed that TR6 protein is overexpressed in monocytes and myeloid-derived dendritic cells (MDC), after stimulation of Toll-like receptor 2 (TLR2) and TLR4 by gram-positive and gram-negative bacterial antigens. It is expressed in plasmacytoid dendritic cells, activated by bacterial antigens via TLR9 or by viral infection (115). In the normal lymphoid organs, TR6 is highly expressed in both lymph nodes and spleen, but its expression in thymus is weak (49, 50).

TR6, as a decoy receptor, can neutralize the biological function of FasL by interfering with the Fas and FasL interaction. As a consequence, it can block FasL-induced apoptosis and FasL dependent CTL killing (50, 116). TR6 can interfere with the interaction between LIGHT and TR2, and between LIGHT and LTβR, and inhibit LIGHT-induced cytotoxicity in HT29 cells (108). TR6 can modulate dendritic cells differentiation and maturation, and such modulation leads to enhanced human T-cell IL-4 production (117). Studies also showed that TR6 modulates monocyte and macrophage differentiation and activation (117, 118, 119). TR6 can block the interaction between DR3 and TLA1; as a result, TR6 inhibits TL1A-costimulated T-cell proliferation and cytokine production, inhibits TL1A-induced apoptosis of DR3-expressing cells (117, 120), and induces angiogenesis by neutralization of TL1A- induced angiostatic action through its receptor DR3 (121). A recent study using transgenically expressed TR6 on mouse islets further proved the function of TR6 in downregulating immune response. The transgenically expressed TR6 protects islets from spontaneous and chemical-

induced diabetes; the transgenic islets have a higher transplantation success rate, and survive longer than wild-type islets (122). Data from our group indicate that TR6 inhibits the anti-alloantigen immune response and prolongs the survival of transplanted heart graft in a mouse model. We have also shown that TR6 prevents islet primary nonfunction after transplantation (123, 124). We have proven that TR6, when placed on solid phase, can costimulate T cell response; TR6 also modulates T-cell migration *in vitro* and *in vivo* (8, 107, 125). These findings will be detailed in Chapter 2 and 3. The signaling pathways, which TR6 interfers with, are summarized in Fig. 2

Figure 2: TR6 interferes with multiple ligand-receptor interactions (adapted from Xiaochun Wan)



The names and major expressing cell types of TR6 ligands and their receptors are indicated. Arrows indicate direction(s) of signalling transduction.

1.4.2. LIGHT

LIGHT is a member of the TNF ligand superfamily, with TNFSF14 as its official symbol. It is also called LTγ or HVEM-L. The term "LIGHT" stands for "homologous to lymphotoxins, showing inducible expression, and competing with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes" (33, 126). LIGHT binds three distinct members of the TNF receptor family, i.e., the herpes virus entry mediator (HVEM) (33, 127), the lymphotoxin receptor (LTβR) (34, 128), and the soluble decoy receptor 3 (DcR3) (108). Among these receptors, TR2/HVEM is consistently expressed on T cells, and the LTβR on stromal cell (33). Northern blot analysis has shown that LIGHT is expressed predominantly in the spleen; it has weak expression in the heart, placenta, liver, lung, appendix, and kidney (33). Cells that express LIGHT include activated lymphocytes, natural killer (NK) cells and immature dendritic cells (33, 34, 129). In T cells, cell surface levels of LIGHT are detectable by 4 h following T cell activation, peak at 24–48 h and gradually decline by day 5 (130, 131). Our group, using fluorescence and confocal microscopy, has studied LIGHT expression on T cells and found that LIGHT was also detectable in resting T cells (8), probably due to higher sensitivity of our methods.

The functions of LIGHT have been intensively studied in recent years. It has been proved that LIGHT plays a very important role in regulating immune responses by interacting with its receptors.

LIGHT is able to trigger apoptosis. Zhai et al. reported LIGHT protein triggers apoptosis of various tumour cells expressing both lymphotoxin β receptor (LTβR) and TR2/HVEM

receptors, and its cytotoxicity can be blocked specifically by addition of a LTβR-Fc or a TR2/HVEM-Fc fusion protein. They also found that LIGHT was not cytolytic to the tumour cells that express only the LTβR or the TR2/HVEM (34). Meanwhile, Rooney et al. reported that LTβR is sufficient for LIGHT-mediated apoptosis of tumour cells through both caspase-dependent and caspase-independent pathways (132).

LIGHT was first proven as a costimulation molecule in T-cell response; it can transduce costimulatory signals into T cells through its receptor HVEM. In mixed lymphocyte reactions (MLRs), the introduction of soluble LIGHT enhances T-cell proliferation (126) . Another group observed the same effect which is reduced by the introduction of soluble HVEM–Fc decoy receptor (66). When recombinant human LIGHT is added to T cells in the presence of sub-optimal levels of agonistic anti-CD3 monoclonal antibodies, enhanced proliferation occurs *in vitro*, while the soluble LTβR –Fc blocks this effect (133). Other studies further confirmed that LIGHTand HVEM co-stimulates TCR-mediated T-cell proliferation (133, 134, 135). LIGHT co-stimulation enhances IFN-γ and GM-CSF production; the IL-4 production is slightly increased, and IL-10 expression is not affected (129, 133). Moreover, the LIGHT triggered T cell activation is independent of the B7/CD28 pathway (129, 133).

Our group proved that LIGHT can function as a receptor that reversely transduces costimulatory signals into T cells, it can costimulate TCR stimulated T cell response in terms of proliferation, cytokine production, and CTL activity (8, 107). Further we found that LIGHT reverse signaling can be used in tumour therapeutic vaccine design. The

LIGHT reverse signaling also modulates T cell migration and T cell aggregation (125, 136). These findings will be detailed in Chapter 2 and 3.

Constitutive expression of human or murine LIGHT in transgenic mice results in profound inflammatory phenotypes and enhanced Th1 cytokine activity in mucosal T cells (137, 138). Transgenic expression of LIGHT in T cells leads to abnormalities in both lymphoid tissue architecture and the distribution of lymphocyte subsets. The transgenic mice developed severe autoimmune disease featured by lymphadenopathy, glomerulonephritis, splenomegaly, enhanced levels of autoantibodies and severe lymphocyte infiltration of different tissues (138). These results further prove the two-way costimulatory function of LIGHT and HVEM in T-cell activation and immune response.

In the LIGHT knockout or deficient animal models, one study from LIGHT deficient ($LIGHT^{-/-}$) mice showed that lymphoid organs are intact, and T cells and APCs function normally. But induction of cytokines from CTL (CD8+) were reduced from $LIGHT^{-/-}$ mice (139). Scheu *et al.*, found that LIGHT-deficient T cells displayed reduced proliferation following TCR stimulation with anti-CD3 mAb *in vitro*, and $LIGHT^{-/-}$ mice were deficient in the activation of $CD8^+$ T cells *in vivo*. They also also found that $LIGHT^{-/-}$ splenocytes are impaired in their ability to lyse allogeneic target cells *in vitro* (134). Together, these studies support the importance of LIGHT in the generation and maintenance of $CD8^+$ T cell responses *in vivo*.

In allograft rejection studies, it was found that the mean allograft survival time of LIGHT$^{-/-}$ mice is only slightly prolonged, while in combined with CsA, the survival time is significantly enhanced compared with normal LIGHT$^{+/+}$ mice (140). Scheu *et al.*, found that LIGHT$^{-/-}$CD28$^{-/-}$ showed prolonged skin graft survival than single deficient or WT mice (141). These results suggest that LIGHT plays important role in allo-graft rejection. The reason could be the impired function of cytotoxic T lymphocytes in LIGHT$^{-/-}$ mice (134). It also suggested that LIGHT is necessary for development and activation of CD8^{+} but not CD4^{+} T lymphocytes (139, 142). Together, these results suggest that the two-way signaling between LIGHT and HVEM/TR2 contributes to the organogenesis of secondary lymphoid tissues and the costimulation in T cell mediated immune responses.

1.4.3. Fas ligand

Fas ligand (FasL, CD95L, Apo-1L, CD178, TNFSF6, APT1LG1) is the ligand of Fas, a TNF receptor family member (25). FasL is well conserved at the amino acid level among different species (11). It is expressed on activated T cells (25), NK cells (143), and tumour cells (144, 145, 146, 147, 148). It is also expressed in lung (149), thymocytes (150), CD68^{+}-histiocytes in the dermis (151) intervertebral discs (152), and in immune privileged sites such as the eye (145, 148), testis (153), uterus and placenta (154). FasL expression can be induced on a host of tissues throughout the body (155). FasL induces apoptotic cell death in cells expressing its receptor, Fas (25). Fas is widely expressed in tissues such as the heart, liver, spleen, and thymus (156). The death-inducing function of Fas is best documented in the context of AICD (activation induced

cell death) in T cells (157). Fas expression has been associated with the establishment of immune privilege and tumour survival (148). FasL also binds to TR6/DcR3, a decoy receptor of TNF receptor family. DcR3 competitively binds to FasL, and therefore blocks the Fas/FasL engagement; the blockage of this interaction results in the inhibition of apoptosis (50). Under physiological conditions, FasL is implicated in the control of immune responses (157, 158, 159), erythroid differentiation (160), angiogenesis in the eye (161), skin homeostasis (162) and CTL-mediated killing of virally infected or transformed cells (163). If not controlled properly, this function can cause autoimmune diseases (164) .

FasL can also transduce non-apoptotic signaling, such as cell proliferation or NFkB activation through its receptor Fas (165). As mentioned in section 1.1.3, FasL also has costimulation function by reversely transducing signals into both CD4 and CD8 T cells (93, 94, 166, 166, 167).

2. T-cell costimulation

The two-signals or costimulation model of T cell activation was first proposed by Lafferty and colleagues (168, 169), before the T cell antigen receptor (TCR) and costimulatory molecules were identified (170). They postulated that full activation of naïve T cells requires engagement of an antigen receptor by foreign antigen (signal one) as well as engagement of a "costimulatory" receptor by a soluble or cell surface ligand provided by the antigen-presenting cell (signal two). This two-signals model is consistent with a considerable body of experimental data and is now widely accepted.

Evidence also showed that delivery of signal one alone inactivates T cells by killing them (171) or rendering them unresponsive (anergic) (172).

There is no general agreement on exactly how the term "costimulation" is defined. In some cases it is used broadly to mean nearly any interaction that enhances antigen receptor signaling, while in other cases it is more narrowly construed, meaning only signals that have no stimulatory capacity on their own, but whose synergism with the antigen receptor is required to allow full activation of a naive lymphocyte (173). Because some cytokines, such as IL-1, IL-2, and IL-4, have been found to enhance the activation of B and T cells, it had been considered as costimulation. However, it has become clear that interactions between receptor/ligand pairs of cell surface molecules on the responder lymphocyte and an "accessory" cell represent critical events in the activation process, and it is those events that are generally referred to as costimulation (173). There are two kinds of costimulatory signals, "positive" and "negative" signals. The "positive" signal enhances immune response and the "negative" signal results in physiologic termination of immune responses (174).

2.1. Candidate costimulatory molecules

CD28 is the prototypic T-cell co-stimulatory molecule (170, 175). Since the identification of CD28, the number of proposed costimulatory molecules has grown significantly (173, 176, 177, 178, 179). Based on their sequence homologies, most of costimulatory molecules can be divided into two classes.

The first class is the costimulatory members of the Ig superfamily; this includes CD28 family members and their ligands, which are members of B7 family (180). The CD28

family consists of CD28, CTLA4 (181), ICOS (H4, AILIM) and PD-1. The B7 family consists of B7-1 (CD80), B7-2 (CD86), ICOSL (B7h, B7-H2, GL50, B7RP-1, LICOS, KIAA0653), PD-L1 (B7-H1) and PDL-2 (182) (183).

The second class of costimulatory molecules is some members of the TNF receptor (TNFR) and TNF family. Members of TNFR family belong to this class include OX40, 4-1BB, CD27, CD30, HVEM and BAFFR (183). Members of TNF family belong to this class are the ligands of the above TNF receptors (8, 176, 184, 185).

Beside the above two classes of costimulatory molecules, some other molecules can also costimulate T cells. For example, Yu et al. proved that mouse Ephrin B2 and Ephrin B3 can costimulate T cell proliferation and cytokine production, and costimulation enhances mouse CTL activity (186, 187). Leta et al. found that CD7 has T-cell costimulatory function (188).

2.2. Costimulatory molecules of the Ig superfamily.

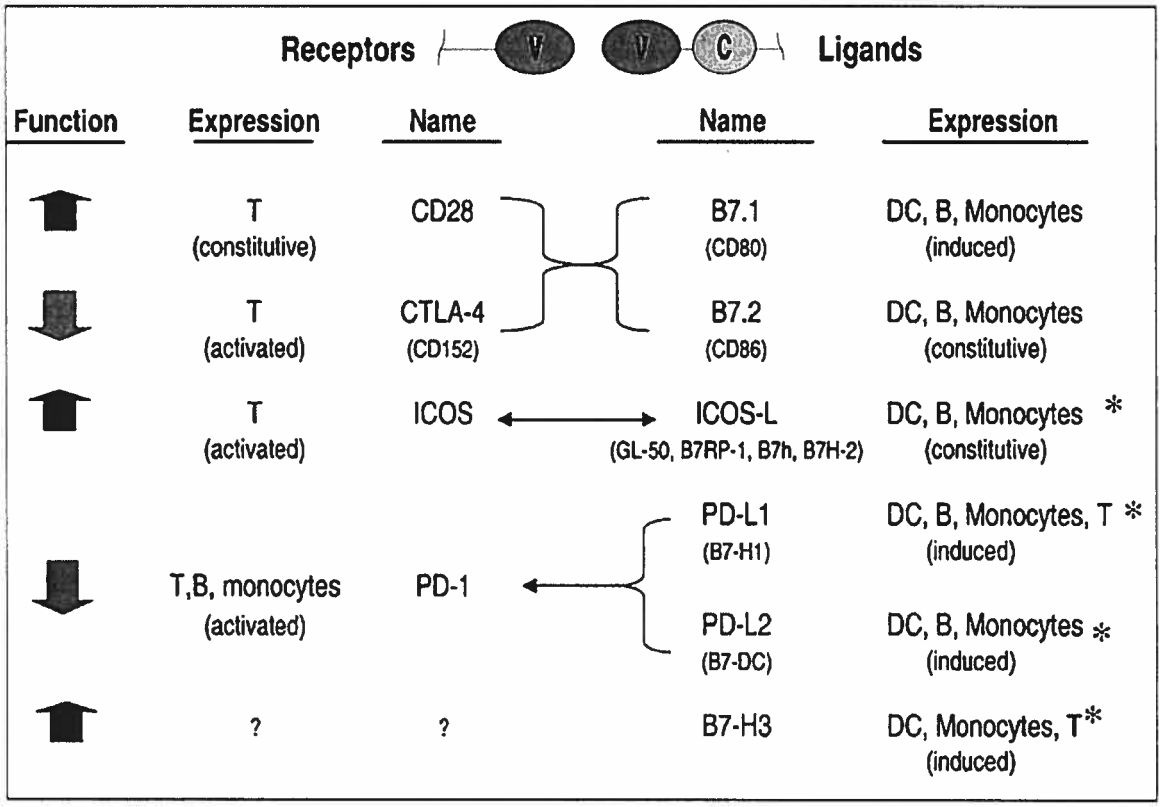
Members of the CD28 family and their ligands are depicted in Figure 3.

2.2.1. The B7/CD28/CTLA-4 pathway

The CD28/CTLA-4/B7-1/B7-2 family provides a paradigm of T cell costimulation. The multiple B7 ligands bind to both activating (CD28) and inhibitory (CTLA-4) receptor (181, 189, 190, 191, 192, 193). These receptors do not stimulate T cell activation independently, but they modify responses delivered by engagement of the antigen-

specific TCR on T cells. The expression of both receptors and ligands is tightly regulated, allowing discrimination between signals that result in activation or inhibition

Figure 3. Summary of CD28 family and their ligands (Adapted from Carreno, B.M et al. Ann Rev Immunol, 2002).



The names of receptors and ligands are indicated, as well as a brief summary of predominant expression patterns for ecch. The conserved structure of a single IgV extracellular domain for receptors and IgV and IgC extracellular domains for ligands is depicted at the top. Function arrows summarize whether the pathway is thought predominantly to costimulate or inhibit the response of the receptor-bearing cell.

of an immune response. CD28 is constitutively expressed on T cells. Ligation of CD28 on naive T cells by either B7-1 or B7-2 ligand on antigen-presenting cells provides a potent costimulatory signal to T cells activated through their T cell receptor (181, 189,190, 191, 192, 193, 194). This leads to IL-2 production, CD25 expression, and entry into the cell cycle. CD28 costimulation is necessary for the initiation of most T cell responses, and blockade of CD28 signaling results in ineffective T cell activation (190, 192). CTLA-4 expression is not detected on naïve T cells, but its expression is transcriptionally induced after T cell activation (191). CTLA-4 plays a critical role in regulating T-cell activation. Its function as an inhibitory receptor of T-cell activation is proven in CTLA-4 knockout mice, which die within 3 to 4 weeks of birth from massive lymphocytic infiltration and tissue destruction in critical organs (194, 195, 196, 197).

Both CD28 and CTLA-4 bind to B7-1 (CD80) and B7-2 (CD86) ligands. The interaction between B7 and CD28 is weaker than the interaction between B7 and CTLA-4. Human B7-1 binds to human CTLA-4 and CD28 with Kd values of 0.42 and 4 μM, respectively (198). The affinity of B7-2:CTLA-4 interaction is similar to that of B7-1:CD28, and the affinity of B7-2:CD28 interaction is the lowest (199). Mice deficient in B7-1 or B7-2 have significant abnormalities in both humoral and cellular immune responses, again illustrating the pivotal role of the CD28 and CTLA-4 pathways (200). Based on the differences of the interaction affinity between CD28 and CTLA-4 with B7, undesired immune responses can be blocked with soluble CTLA-4 Ig (201, 202, 203, 204, 205, 206).

2.2.2. The ICOS/ICOSL pathway

ICOS (AILIM) is an inducible costimulatory receptor homologous to CD28 and CTLA-4 (207, 208). It is expressed on activated, but not resting, T cells (207); it is identical to the H4 T-cell activation antigen (209). ICOS expression is stimulated by both TCR and CD28 signals (183). However, ICOS expression is not absolutely dependent upon CD28 signals, because some T-cell responses in CD28-deficient mice can be modulated with ICOS-Fc (210). The ligand for ICOS (ICOSL), a B7-like molecule, is capable of binding to ICOS and delivering a costimulatory signal to T cells (210, 211, 212, 213, 214).

ICOSL is expressed at low levels on resting B cells, some macrophages and dendritic cells, and can be induced by IFN-γ and TNFα (180, 215). ICOSL is also expressed on other cell types, including fibroblasts (183). A study showed that ICOSL is constitutively expressed on endothelial cells (216). Unlike CD28 signals, which are crucial for the initial co-stimulation of interleukin 2 (IL-2) production, ICOS signals only modestly influence T-cell proliferation and IL-2 production (183, 207, 210, 214, 217). ICOS signals are most important for regulating cytokine production by activated and effector T cells: ICOS can regulate both T_H1 and T_H2 effector cytokine production (180, 218), and it is particularly important in regulating IL-10 production (207, 219). ICOS has a crucial role in T-cell–B-cell collaboration and IgG1 affinity maturation (220).

2.2.3. The PD-1/PD-L pathway

PD-1 (program death-1) is a type I transmembrane receptor that was identified in a T cell line undergoing activation-induced cell death (221). In normal murine tissue, PD-1 mRNA expression is confined to the thymus (221). Under resting conditions, neither T nor B cells express PD-1. However, activation of T or B cells through the antigen receptor or with PMA and ionomycin results in cell surface expression of the PD-1 receptor (222). PD-1 is also expressed in activated macrophages (223). PD-1 has two ligands: PD-L1 and PD-L2 (224). PD-L1, also termed B7-H1 in humans (225), is expressed in a variety of lymphohematopoietic cell types, including a minor proportion of T and B cells in the spleen, a majority of pre-B cells, and myeloid cells in bone marrow and subsets of thymocytes (226). Peripheral tissues such as the heart and lung also express PD-L1. PD-L1 expression is induced by IFNγ on monocytes, dendritic cells (DCs) (227), and human keratinocytes (180). Using PD-L1–Ig fusion proteins, Freeman demonstrated that engagement by PD-L1 inhibits T-cell responses (228). The PD-1 knockout mice have a enhanced immune response to alloantigen (229) and suffer from autoimmune disorders (229). A recent study also showed that endothelial cells expression of PD-L1 and PD-L2 down-regulates CD8+ T-cell activation and cytolysis (230). The second ligand for PD-1, PD-L2 (231) is also termed B7-DC (182); it is predominantly expressed on DCs (227). Although most of the studies showed PD-L1 and PD-L2 as negative regulators (180, 183), there are some report suggesting that PD-L1 and PD-L2 positively stimulate T cell response (182, 232).

2.2.4. B7-H3: A new B7-like ligand

Human B7-H3 was recently identified as a new costimulatory ligand belonging to the B7 family (233). B7-H3 protein is expressed on GM-CSF-stimulated monocytes and IFN-γ activated dendritic cells, as well as on $CD3^+$ T cells activated with PMA and ionomycin (233). The receptor of B7-H3 is currently unknown. B7-H3–Ig fusion protein can costimulate proliferation of CD4 and CD8 T cells through a receptor other than CTLA-4, CD28 or ICOS.

2.3. Costimulatory molecules of the TNF/TNFR family

2.3.1 Costimulation via TNFR family members

Evidence showed that several members of the tumour–necrosis factor receptor superfamily deliver positive costimulatory signals both in early and late stage after T cell encountering with antigen. Six interactions between the TNFR-TNF family members have emerged as positive regulators of the T-cell response. They are: OX40-OX40L, 4-1BB-4-1BBL, CD30-CD30L, CD27-CD70, HVEM-LIGHT (185) and TACI-BAFF (184, 234).

a. The OX40 (CD134) and OX40-ligand pathway

OX40 was originally identified as a cell surface antigen on activated rat CD4 T cells (176, 235, 236). Expression of OX40 is restricted to activated T cells in humans and rodents (237, 238). Expression of OX40L has been documented on activated murine B cells (104, 237), human dendritic cells (239), human vascular endothelial cells (240), and HTLV-1-transformed T cells (241). Studies of OX40- and OX40L-deficient mice showed that CD4 responses to various

antigens are markedly reduced *in vivo*, and *in vitro* (242, 243, 244, 245). These mice have deficiency in numbers of antigen-specific CD4 T cells generated late in the primary response, and additionally after 5 weeks when the T cell memory population is formed (246). Overexpression of OX40L on dendritic cells leads to greater numbers of primed CD4 cells (247). It was proposed that OX40 signals act in a temporal manner after CD28 signals, and allow effector T cells to survive and continue proliferation late in response (176, 185).

b. The CD30-CD30L Pathway

CD30 was originally described as a marker of Reed-Sternberg cells in Hodgkin lymphoma (248). CD30 is expressed on activated but not resting B or T cells (249, 250, 251, 252). T cell expression of CD30 is dependent on the presence of CD28 costimulatory signals or exogenous IL-4 during primary T-cell activation (250). CD30L is a transmembrane protein, expressed on T and B lymphocytes, macrophages, and a variety of hematopoietic cells and tumours (27, 253). On T cells, CD30 is expressed primarily on activated $CD8^+$ T cells and CD30L is expressed primarily on activated $CD4^+$ T cells (249). Although the function of CD30-CD30L interaction is largely unknown, studies have shown that it has effects on both cell activation and cell death (249, 250, 253, 254, 255, 256). Mice deficient in CD30 show a mild impairment in thymic negative selection, and activation-induced death of thymocytes after CD3 crosslinking is impaired both *in vivo* and *in vitro* (257). Costimulation of human peripheral blood T cells with

anti-CD30 antibody results in preferential development of antigen-specific T cell lines and clones with a Th2-like profile of cytokine secretion (258).

C. The CD27 and CD27-ligand (CD70) Pathway

CD27, another TNF-R superfamily member, has been implicated in T cell activation, T cell development, and T cell–dependent antibody production by B cells (259, 260). CD27 is consistently expressed on T cells and its expression on T cells is strongly upregulated by reagents that cross-link TCR or CD3 (261, 262). CD27 is irreversibly lost on a subset of long-term repeatedly stimulated T cells, and this is consistant with $CD27^-$ phenotype of small numbers of memory phenotype cells (261). The expression of CD27L is found on medullary thymic epithelium and is rapidly induced on both T and B cells after activation. The activated B cells synthesized much higher levels of CD27L mRNA than activated T cells. CD27L expression on B cells is enhanced by CD40 signaling and is downregulated by IL-4 (263, 264). The specific interaction of CD27 with its ligand CD27L has been shown to support clonal expansion of both antigen-stimulated CD4 and CD8 T lymphocyte populations and to enhance the generation of cytolytic T cells (265). Results from CD27 knockout mice indicate that CD27 supports antigen-specific expansion (but not effector cell maturation) of naive T cells, and this function is independent of the cell cycle-promoting activities of CD28 and IL-2. Effects of CD27-deficiency are most profound on T-cell memory, particularly in of CD8 T cells (266). Stimulation with immobilized anti-CD27 mAb or CD27L induces proliferation and IFN-γ

production of freshly isolated NK cells and enhances the proliferation and IFN-γ production of anti-NK1.1-sitimulated NK cells, but the NK cytotoxic activity is not enhanced. The enhanced cytotoxic activity of anti-CD27 prestimulated NK cells is IFN-γ-dependent (259).

d. The 4-1BB–4-1BBL Pathway

4-1BB (CD137, ILA), exists as both a 30-kD monomer and a 55-kD homodimers (267). It is primarily expressed on activated $CD4^+$ and $CD8^+$ T cells (267, 268) as well as on activated NK cells (269), B cells, macrophages, dendritic cells and eosinophils (53, 269, 270, 271). 4–1BBL is expressed on mature DC and on activated B cells and macrophages, and its expression can be regulated by LPS, Ig or CD40 signals (26, 53, 272, 273). Stimulation of 4–1BB induces higher levels of $CD8^+$ than $CD4^+$ T-cell proliferation (268, 274) and appears to be critical for $CD8^+$ T cell survival (275, 276). In 4-1BBL-deficient mice, 2–10-fold fewer antigen-reactive CD8 cells are generated in the primary responses to LCMV and influenza viruses, and fewer memory T cells are developed weeks after the priming (277, 278, 279). As compared to wild type mice, anti-4-1BB antibodies or tumour cells transfected with 4-1BBL greatly increase the antigen specific CD8 T cell and CTL response (276, 280, 281, 282). Recent reports showed that anti-4-1BB antibody blocking can reverse autoimmune diseases (283, 284).

e. The HVEM-LIGHT Pathway

As mentioned above, LIGHT, a member of the TNF ligand family, is expressed on T cells and DCs. Its expression is inducible by T-cell activation, and is constitutive on "immature" dendritic cells. It binds three distinct members of the TNF receptor family, i.e. HVEM; LTβR and DcR3. LIGHT exhibits inducible expression on lymphocytes and can block HSV from entering cells via HVEM, a receptor prominently displayed on T cells. Effects of LIGHT–HVEM signaling define LIGHT as a co-stimulatory molecule for T cell activation (129, 135, 285), and a modulator of T-cell responses. T-cells activated by LIGHT–HVEM co-stimulation produce cytokines, such as IFN-γ and GM-CSF, central for T-helper cell type 1 (Th1) immune responses. On the other hand the IL-4 and IL-10 production are only slightly or not increased, respectively (129, 133, 286). LIGHT-deficient mice display defects in T cell activation and effector functions. *In vitro* assays showed that LIGHT-deficient T cells have reduced proliferation following TCR stimulation with anti-CD3 mAb (141, 286). LIGHT-transfected tumour cells evoke antitumour immunity. The blockage of LIGHT prolongs the allograft survival (133, 141). The costimulation between LIGHT and HVEM is in both forward and reverse directions, as described in the section of 1.4.2.

f. The BAFF-TACI Pathway

The B cell-activating factor (BAFF; Blys, TALL-1, zTNF-4, THANK) is a recently identified member of the TNF ligand family (184, 287), and is expressed on monocytes/dendritic cells and T cells. It can bind to three receptors, BCMA, TACI and BAFF-R, which are all TNF receptor family members (65,

184). Among the three receptors, TACI is expressed on activated T cells (234, 288). BAFF has been described as a potent survival factor for B cells (289). Huard and colleagues found that recombinant BAFF protein costimulates T cell proliferation and cytokine production. The costimulation function is obvious on naive, as well as on effector/memory T cells (both $CD4^+$ and $CD8^+$ subsets) (184). Wang (234) reported that TACI-Fc blocks the activation of T cells *in vitro* and inhibits antigen-specific T cell activation and priming *in vivo*. TACI-Fc treatment substantially inhibits inflammation, bone and cartilage destruction and disease development of rheumatoid arthritis in a mouse model (234).

2.3.2. Costimulatory reverse signaling through members of the TNF family

Some TNF family members can transduce signals into cells, and such signals can function as costimulatory signals. This is detailed in section 1.1.3.

3. Cancer vaccines

Using vaccination to treat cancer is not a novel concept and can be traced back to the 1890s when a New York surgeon William B Coley successfully treated some patients with sarcoma using bacterial toxins (290). In 1909, Paul Ehrilich successfully carried out immunization in animals with tumour cells, and suggested that tumours occur at a high frequency in humans but are kept under control by the immune system (291). Although the early attempts in cancer vaccination had some beneficial results, the effect was not significant. Most importantly, at that time, there was no evidence showing that cancers have specific antigens, which can be recognized by the immune system. In

1929, based on the available information concerning cancer immunotherapy, W. H. Woglom wrote, "It would be as difficult to reject the right ear and leave the left ear intact as it is to immunize against cancer" (292). A widely quoted article published in 1976 in the *British Journal of Cancer* reported no evidence of immune response to 27 different spontaneous tumours in mice and concluded that: "transplanted tumour systems entail artifactual immunity associated with viral or chemical induction" (293). In the past 20 years, the field of cancer immunotherapy has changed. Advances in the molecular characterization of human tumours and a better understanding of tumour immunology has led to the identification of tumour-associated antigens (TAAs) (294, 295, 296, 297). More importantly, it was shown that administration of IL-2 to humans with metastatic kidney cancer or melanoma could cause the regression of established cancers (298). Also, a number of therapeutic cancer vaccines have already been tested in animals and subsequently humans, yielding preliminary encouraging results (292, 295, 297, 299). These results have supported the idea that therapeutic vaccines should be useful in restoring immune defenses against cancer.

3.1. Tumour Antigens

The number of tumour antigens has increased rapidly in recent years. They can be categorized in the following groups (292, 300): 1. Cancer testis antigens. This group includes MAGE, BAGE, RAGE, and NY-ESO. These antigens are expressed in melanomas and several other tumours, but not in normal tissues except testis; 2. Differentiation antigens, such as MART-1/Melan-A (MART-1), gp100, gp75, mda-7, tyrosinase and tyrosinase-related protein, the prostate specific membrane antigen, and

prostate-specific antigen. They are expressed in normal and neoplastic cells derived from the same tissue, but in normal tissues they are expressed at relatively low concentration or on a small subset of cells; 3. Tumour-specific antigens, such as immunoglobulin in B-cell NHL (non Hodgkin lymphoma) and myeloma, ACDK4 in melanoma, mutated p21/*ras* and p53. These are unique antigens specific for each individual tumour. They represent normal proteins that contain mutations or gene fusions that result in the generation of unique proteins. 4. Over-expressed "self" antigens. These are normal "self" proteins that are expressed constitutively in tumour cells such as carcinoembryonic antigen and alpha-fetoprotein, normal p53. 5. Viral antigens. This group of antigens are expressed on the virous-associated tumours, such as HPV E6/E7 in cervical and penile cancer, EBV LMP2a in EBV $^{+}$ Hodgkin's disease, HCV or HBV in liver cancer and HHV-8 in Kaposi sarcoma.

3.2. Mechanisms of tumour's escape from immune attack

Two different models for the immune response to tumours have been proposed: the immunosurveillance model and the danger model. According to the immunosurveillance model, tumour antigens expressed by tumours are regarded as "nonself" by the immune system, and a major function of the immune system is to survey the body for the development of malignancy and to eliminate tumour cells as they arise (301). In the "danger" model, the immune system uses professional antigen-presenting cells (APC) as sentinels of tissue damage—the "danger" signal. In the presence of danger signals, APC, such as dendritic cells, activated macrophages, and B cells, stimulate the T cell

responses. The danger model proposes that cancer cells do not appear dangerous to the immune system, so that T cell responses to tumours is not initiated (171, 302, 303).

It had long been suspected that tumours have evolved multiple mechanisms to overcome the immune system (144, 304). This includes the production of soluble immunosuppressive factors, such as TGFβ, IL-10 and prostaglandins. In recent years, the molecular basis for the escape mechanisms, which might impact the efficacy of cancer immunotherapeutics, has been better understood (305).

3.2.1. FasL and tumour escape

Since the finding of FasL overexpression on some tumour cells (146, 306, 307), it was proposed that the FasL on cancer cells plays a role in tumour cell escape from immune attacks (144, 146, 304), because, FasL is physiologically expressed in sites that are "immunoprivileged", such as the eye or the testis. Engagement of Fas by FasL leads to apoptosis of Fas^+ cells (146). The Fas protein is expressed on activated T cells. As a consequence, certain $FasL^+$ tumours could induce apoptosis of activated T cells, which are tumour antigen-specific and migrate to tumour site to kill the tumour cells. This clearly represents a possible mechanism through which tumours evade the immune system, but the situation quickly becomes more complex. It was recognized in murine models that a polymorphism in FasL could dramatically affect its ability to induce apoptosis in target cells (308). Various studies have shown that the overexpression of FasL in murine tumour cells resistant to Fas-mediated apoptosis does not affect tumour cell growth *in vitro*, but causes tumour rejection by neutrophils *in vivo* (309, 310, 311).

This suggests that the killing of T cells by FasL expressing tumour cells might be involved in some, but not necessarily most tumour evasion mechanisms. On the other hand, tumours might indeed manipulate the Fas-FasL interation to gain survival advantage. Soluble Fas is expressed in various malignancies, and elevated levels can be found in the sera of cancer patients (312, 313, 314, 315). High soluble Fas (sFas) serum levels were associated with poor prognosis in melanoma patients. It is conceivable that sFas blocks the death stimuli coming from FasL on CTL and/or NK cells, and thus protects tumour cells from apoptosis. Similarly, DcR3 is secreted by many human cancers, and such secretion may also interfere with tumour cell apoptosis mediated by Fas (49, 50, 109, 110, 111, 112, 113).

3.2.2. MHC Class I molecules and tumour escape

Loss of MHC Class I molecules by a variety of solid tumours may serve as another mechanism for tumours to evade the immune surveillance. Approximately 40-90% of human tumours derived from various MHC class I+ tissues were reported to be MHC class I deficient. It was reported that decreased or absent MHC class I expression is frequently associated with the invasive and metastatic tumour phenotype (316). In a number of cases, this is associated by a down regulation of components of the antigen processing and presentation machinery (316, 317, 318). It is hypothesized to be a mechanism that leads to a deficit of tumour recognition by cytotoxic T cells. Interestingly, this deficit can be at least partially restored by IFN-γ treatment. However, some observations cannot be explained with this hypothesis. 1. According to the NK cell physiology, when a cell loses or decreases its MHC class I antigen, it will activate NK cells and be eliminated; nevertheless, the NK cell adaptive therapy for tumours

seems not as effective as expected (319), also the IFN-recoverable NK activity is not reduced in patients compared to matched controls (320). The cancer patient does not appear to be abnormal in immunity to antigens other than tumour antigen, except in the late stage of disease. 2. Tumours derived from professional antigen presenting cells, which are not have deficient, can still escape the immune attack.

3.2.3. TCR and tumour escape

Some studies reported the alteration in the TCR component expression in peripheral or intratumoural T lymphocytes isolated from cancer patients (321, 322, 323). The signal transduction components, such as $p56^{lck}$ and Zap70 tyrosine kinases, were also reported to be somewhat downregulated in T lymphocytes obtained from cancer patients (321). In addition, the deficits in TCR could be restored by IL-2 therapy (324). These were proposed to be a mechanism of tumour escapes from immunosurveillance. A recent study showed that there is no evidence to suggest that loss of TCR ζ chains is a mechanism for immunocompromise in patients with cervical carcinoma (320).

3.2.4. Tumour associated antigen (TAA) and tumour escape

One obvious mechanism for tumour's resistance to the immune system is the down regulation of tumour-associated antigen (TAA) expression. This phenomenon was clearly demonstrated by monitoring over time the sensitivity of primary tumours versus subsequent metastases to CTL clones (325, 326, 327). Certain tumour cells may transfer antigens from their surface into the cytoplasm, making themselves immunologically invisible. Alternately, tumour cells may stop expressing certain surface antigens (327). Analyses conducted *in vitro* have clearly established that optimal recognition and

activation of tumour specific T cells depends on a minimal level of TAA expression (326). This feature of tumours reveals a disadvantage of tumour antigen-based cancer vaccine; which is based on the premise that it is eliciting immune responses to particular TAA. Due to the diversity of tumour cells in a tumour mass; the effect of the tumour vaccine immunization may only eliminate a fraction of the tumour cells, which are TAA positive on their surface. However, even these initially TAA-positive tumour cells might escape the vaccine-induced immune attack once their TAA is downregulated.

3.2.5. Costimulation molecules and tumour escape

The understanding of the two-signals model in T-cell activation is crucial to the development of novel strategies to enhance antitumour immune responses. The well-organized immune system could effectively eliminate a nearly infinite repertoire of foreign invaders to prevent damage of self. Unfortunately, the immune response to tumours is often ineffective. A major mechanism of immune evasion is the lack of costimulatory ligand expression, which renders tumour-antigen-specific T cells impotent (328, 329, 330, 331). Because cancers usually lack the second signal for full activation of T cell and are short of "danger" signals, tumour antigens need to be presented by other types of cells to stimulate the immune system. This can be achieved by a process known as cross-presentation, which is also involved in responses to viral infection and transplanted organs (332). APCs will pick up tumour antigens released from tumour cells; after processing, APCs will present the tumour antigens on their cell surface restricted by their own MHC class I or II molecules; APC will then present the tumour antigen to T cells, together with the costimulatory molecules expressed on their cell surface; this will be sufficient to stimulate naïve T cell activation. However, this

same process of cross-presentation has been shown in some cases to induce T-cell tolerance, which may play an important role in maintaining tolerance to self antigens (332), as well as in tolerance to tumour antigens.

3.3. Approaches to cancer vaccine

Although approaches to manufacturing cancer vaccines vary and include – but are not limited to – the use of vectors, peptides, or genetically modified cells, they are all geared toward the same end, namely, to stimulate the host's immune system to respond to antigens characteristic of cancer cells.

3.3.1. Peptide and protein vaccine

The development of therapeutic vaccines specifically targeting TAA has the advantage that the immune response would be directed mainly against the tumour cells but few other normal tissues. Also, this kind of vaccines can be used in all patients, whose cancer cell express the targeted TAA. However, one potential major disadvantage of peptide vaccines is the possibility of raising only an irrelevant peptide-specific response, which is not necessarily against intact TAA proteins. For instance, it was reported that patients immunized with her2 peptide developed her2-specific T-cell responses; however, the reactive T cells failed to recognize her2^{+} tumour cells (333, 334). Another potential disadvantage is that the antigen specific immune response may only eliminate a fraction of the cancer cells, because of the diversity and the genetic instability of the cancer cells in terms of TAA expression. Combining antigenic peptides with adjuvants, such as QS21, incomplete Freund's adjuvant, GM-CSF, IL-2 or IL-12 has been proven

to increase efficacy of tumour vaccines, either by improving antigen presentation or by supporting the effector function of vaccine-induced T-cell response (335, 336).

Clinical trials using TAA peptide vaccines recently have begun, and have provided substantial information about the type of immune responses that are elicited (336, 337). In general, the therapeutic outcomes in clinical trails to date have been disappointing (338). The results of these trails can be summarized as the following: first, CTL responses can be induced by peptide vaccination (339, 340, 341); second, the presence of an expanded pool of TAA-specific T cells does not lead to tumour regression (342, 343, 344); third, increasing the affinity of the peptide for the MHC can greatly increase the potency of a vaccine by converting a subdominant epitope into a dominant one (340, 345, 346). The modified gp100 peptide vaccine elicited peptide-induced T-cell responses in 91% of the melanoma patients immunized. In contrast, vaccination with the unmodified peptide failed to induce a T-cell response in most patients (340).

Heat shock proteins (HSPs) are involved in protein maturation and functional regulation (347, 348, 349). They have been implicated in numerous processes such as protein folding, transport, assembly, and peptide trafficking in antigen presentation (347, 350). It was observed that certain HSPs (gp96, hsp90, and hsp70) purified from a given tumour could elicit specific immunity against that particular tumour (347, 351, 352). Biochemical and functional studies indicate that the immunogenicity of tumour-derived HSPs is due to the associated antigenic peptides (347, 353, 354). In addition to carrying antigenic peptides for presentation, HSPs also stimulate antigen presenting cells (APC), such as macrophages and dendritic cells (347, 355, 356) to secrete pro-inflammatory

cytokines as well as to promote maturation of dendritic cells (357, 358, 359). Clinical trials using tumour-derived HSP are underway (360, 361, 361).

Most tumour antigens identified so far are poorly immunogenic *in vivo* (362). Studies indicated that using altered agonistic peptide ligands dramatically increases the magnitude of T cell responses and sensitivity to antigen stimulation in both human and mouse models (345, 363, 364). The enhanced T cell responses can retain their specificity to the native antigen, which allows T cells to kill target tumour cells *ex vivo* and, presumably, *in vivo* (362).

3.3.2. DNA vaccine

Direct vaccination with DNA encoding a tumour antigen has the advantage of simplicity and low cost of production. DNA vaccine can induce both humoral and T-cell-mediated immunity (365).

"Naked" DNA-encoding cancer antigens can be injected intradermally or intramuscularly as vaccines. However, the immune responses induced by naked DNA vaccination are generally weak, possibly due to the poor efficacy of *in vivo* transfection of "naked" DNA (336, 365). The efficacy of DNA vaccines can be improved by fusing the coding sequence of TAA with that of "adjuvant-like" elements such as cytokines, the constant region of a foreign immunoglobulin (366), or a known highly immunogenic carrier protein, e.g., fragment C of tetanus toxin (367). A recent study showed that DNA encoding TAA fused with DNA encoding a pan-MHC Class II peptide analogue augments antigen-specific cellular immunity (368).

A variety of gene therapy vectors have been adapted to cancer immunotherapy. The TAA coding sequences can be inserted into viral vectors (335, 369), such as modified vaccinia virus, fowlpox virus, or canarypox virus, and recombinant replication-incompetent viral vectors (adenovirus, retrovirus, lentivirus) (335, 369). Unfortunately, this form of genetic immunization has also resulted in weak immunologic responses in humans (300).

3.3.3. Cellular vaccine

Dendritic cells (DCs) vaccine

Vaccination of cancer patients with DCs presenting TAAs has been believed to be a promising anticancer strategy (370, 370). The use of dendritic cells (DCs) as adjuvants in order to induce tumour-specific killer and helper T cells directly in patients is supported by many animal experiments as well as initial human trials (371, 372, 372, 373, 374). Several strategies have been used to load DCs with tumour antigens, including peptides, proteins, tumour cell lysates or tumour-derived RNA. Some other studies use DCs transfected with viral vectors expressing tumour antigens or fused with the whole tumour cells (291). The demonstration that immunisation of animals with DC-based vaccines can lead to rejection of established tumours, and the fact that functional DCs can be generated *in vitro* in large quantities from cancer patients, has made DCs an attractive vehicle to be used in human clinical trials. Some clinical trials have shown quite promising results (291, 371, 374, 375). However, pulsing of DCs with particular peptides has several disadvantages: i) short duration of antigen-MHC complexes, ii) a

requirement for matching defined peptides with MHC complexes, and iii) exclusive presentation of single antigen epitopes (370). Therefore, DC vaccination still needs to be optimized (337).

A phase I/II clinical trial of DC-based vaccine loaded with PSMA peptides in treating prostate cancer, showed that the vaccination can induce anti-PSMA specific T-cell response, and the numbers of the DCs given correlats to the duration of the immune response, with the majority of the patients (58%) still responding at the end of the follow-up period (374, 376). In a clinical trial of 17 patients with advanced renal carcinoma, the tumour cells were fused with normal allogeneic donor DCs and were given as subcutaneous injections over several months. A 41% response rate (with 4 patients showing a prolonged complete response) was recorded (377).

In several animal models, DCs modified with genes encoding immunostimulatory proteins have been shown to be effective in the induction of antitumour immune responses (375, 378). The genes being used to modify DCs include costimulatory molecules, such as CD40, CD80, or CD86, and cytokines, such as IL-1β, -2, -6, -10, -12, TNF-α, GM-CSF (370, 370, 375, 378).

Tumour cell vaccine

Whole tumour cells have been used to vaccinate cancer patients. The fact that tumour itself is not able to induce an adequate immune response in patients led to attempts to overcome such unresponsiveness by introduction of cytokine genes or co-stimulatory molecules into the genome of the vaccine tumour cells. This should create micro-

environmental conditions that prevent anergy and allows tumour destruction to take place.

a. Cytokine gene modified tumour vaccine

Cytokine gene-modified tumour vaccines are usually composed of autologous tumour cells stably transfected with a cytokine gene. The original hypothesis was that the paracrine expression of cytokines such as IL-2 or IFN-γ allows the tumour cells to directly activate cytotoxic T cells, bypassing the need for host $CD4^+$ T lymphocyte help (379). Some cytokine gene-modified tumour cell vaccines are conducted in clinical trials in different cancers; the cytokines tested are IL-2, -4, -7, -12, TNF, TNFβ, INFα, INFβ, INFγ and GM-CSF (300, 369, 375). The study in preclinical models showed that the GM-CSF gene-modified tumour cells represent the most active vaccine (300). GM-CSF is known to contribute to the maturation of DCs, which in turn enhances antigen presentation *in vivo*. The GM-CSF-transfected autologous tumour cell vaccine has been explored in phase I/II human clinical trials for melanoma (380), renal carcinoma (381), and prostate cancer (382). These trials have shown some success in generating specific immune responses against tumours (291). Some studies indicated that GM-CSF-modified allogenic vaccines also induce immune response towards TAA under the restriction of allogeneic MHC. For example, allogeneic K1735 melanoma cells provides significant protection to mice against B16 syngeneic tumour cell challenge (383).

b. Costimulatory molecular gene-modified tumour cell vaccine

Lack of costimulation is a very important mechanism of tumour escape from immune attack. It is a highly promising approach to evoke an effective anti-tumour immunity by immunization with tumour cells genetically modified to express costimulatory molecules (384), because such a measure will direct-prime anti-tumour T cells, this can also cross-prime T cells when administered with adjuvant, or when the tumour antigens are captured by APCs in the immunization site. Many studies have exploited the various costimulatory pathways to elicit a more potent antitumour immune response (300, 384, 385).

1). B7/CD28

Based on the fact that B7/CD28 interactions enhance, and B7/CTLA-4 interactions inhibit, T-cell activation, many investigators demonstrated that conferring B7 expression on tumour cells augments the antitumour response to tumours of varied origins (386, 387, 388, 389). Chen et al reported that B7 expression on tumour cell is not sufficient to induce immunity against nonimmunogenic tumours (390). However, combining B7 expression with cytokine expression (IFN-γ, IL-12, GM-CSF) leads to enhanced tumour immunity in some models (391, 392, 393).

2). ICOS/ICOSL

ICOSL (B7h) expression on tumour cells was demonstrated to enhance recognition of tumour cells by T cells. B7h expression on Sa1/N fibrosarcoma and J558 plasmocytoma cells enhance tumour rejection in a CD8-dependent manner (394, 395). Zuberek et al. recently studied the *in vivo* efficacy of

CD28/B7 and ICOS/ICOSL costimulatory pathways in murine MethA fibrosarcoma and the B16F1 melanoma tumour models (385).They found that each of these pathways is equally effective in promoting tumour immunity and that the efficacy of both ICOSL and B7.1 vaccines are IFN-γ - but not IL-10-dependent (385). These results suggest that CD28 or ICOS costimulation-based strategies may be equally efficacious in cancer immunotherapy. Meanwhile, another report of human glioma showed that ICOSL gene transfer into glioma cells does not alter their immunogenicity under primary or secondary alloreactive coculture assays *in vitro* (396). These results demonstrated that vaccines based on ICOSL expression might elicit anti-tumour immunity in some, but not all, types of tumours.

3). OX40/OX40-L

The vaccine based on OX40-L expression on tumour cell vaccine was studied as a therapeutic vaccine in the C26 murine colon adenocarcinoma model. Mice injected with C26/OX40L cells displayed only a delay in tumour growth, while the C26/GM (GM-CSF)/OX40L tumours regressed in 85% of mice. Subset-depleted and knockout experiments showed that tumour rejection requires granulocytes, CD4+, CD8+ T cells, and APC-mediated CD40-CD40 ligand cosignaling, but not IFN-γ or IL-12 (397).

4). CD40/CD40L

There is limited evidence suggesting that CD40L directly provides signals to the T cells. The CD40/CD40L interaction initially delivers signals to $CD40^+$ APCs rather than to T cells. However, because such signals significantly augments the ability of APCs to present antigen, it strongly enhances T-cell activation as a result (398). CD40/CD40L costimulation is important for effective antitumour responses. Blocking CD40/CD40L interactions prevents the generation of a protective antitumour response following vaccination with GM-CSF–expressing B16 melanoma cells (399). Several methods using forced CD40L expression in tumour cells as vaccines have significantly enhanced responses to poorly immunogenic tumours (398, 400, 401, 402, 403, 404).

5). 4-1BB/4-1BBL

Immunization with tumour cells transfected with 4-1BBL-expressing vectors can significantly expand $CD8^+$ mediated antitumour responses, and is therapeutically efficacious in mouse models (281). The efficacy of such a vaccine has been shown to increase when it is given together with tumour cells producing IL-12 (405). However, immunization with tumour cells expressing 4-1BBL alone is much less efficacious than administration of anti-4-1BB. Ye et al. expressed the anti-4-1BB antibody single chain on low immunogenic mouse K1735 melanoma cells, and found that these cells induce a strong type 1 T-helper cell response, for which $CD4^+$ but not $CD8^+$ T lymphocytes are necessary. Moreover, such vaccination is NK cell dependent. The vaccinated mice reject established wild-type K1735 tumours growing as subcutaneous nodules or in the lung (406).

6). CD27/CD27L

Kelly et al. reported that CD27L expression on MHC class I-deficient RMA-S tumour cells enhances primary tumour rejection *in vivo* as well as T-cell immunity against secondary challenge of the RMA-S tumour cells. The killing of CD27L tumour cells is mediated by NK cells and is by perforin- and interferon-gamma-dependent mechanisms (407).

7). LIGHT/HVEM

Introduction of LIGHT cDNA into MDA-MB-231 human breast carcinoma causes complete tumour suppression *in vivo*. Histological examination shows marked neutrophil infiltration and necrosis in LIGHT-expressing but not in the parental or the neo-transfected MDA-MB-231 tumours (34). Vaccination with LIGHT gene-transferred P815 tumour cells induces tumour rejection of P815-tumour-bearing mice, accompanied by tumour-specific CTL activity (408). Direct rejected LIGHT-expressing tumour cells into tumour mass leads to eradication of established tumours with a massive infiltration of naïve T lymphocytes (409). The proposed mechanisms are that 1) LIGHT-expressing tumour cells costimulate CD8 cells through HVEM and 2) they stimulate fibrolast stroma cell via LTβR to secrete chemokines, and the secreted chemokine further recruit T cells into the tumour site. (409).

8). Costimulation via reverse signaling

Although reverse costimulatory signals through some TNF ligand family members has been documented, tumour vaccination using such reverse costimulation has not been explored to date.

4. Hypothesis of the study

As illustrated in Figure 1,2. TR6 can bind to three identified ligands: LIGHT, FasL, and TL1A. The aim of this project is to study the function of TR6 in modulating T-cell responses. Since TL1 is not expressed on T cells, our study will focus on the interactions among TR6, FasL and LIGHT. Based on our pilot studies, we hypothesized that:

4.1. TR6 on solid phase might costimulate T cell response by triggering reverse signaling through its ligand, LIGHT.

As mentioned above, several TNF ligand family members including FasL, which is a ligand of TR6, reversely transduces signaling into cells. Our pilot experiments also indicated that TR6 on solid phase can augment T-cell proliferation, and the enhanced T cell proliferation cannot be completely blocked by soluble Fas recombinant protein. So, we hypothesize that the other ligand of TR6, LIGHT, may reversely transduce costimulatory signals into T cells.

4.2. TR6 expressing on tumour cell surface might be useful as therapeutic vaccine.

It is well accepted that full activation of T cells requires two signals; and lack of second signal is a very important mechanism of tumour escape immune attack. If our first hypothesis is proven correct, expressing TR6 on tumour cell surface can trigger

costimulatory signals reversely through both of its ligands, i.e., FasL and LIGHT, and these costimulation signals should enhance the anti-tumour immune responses.

4.3. TR6 might regulate T-cell migration

As reported by our group (136), both soluble and solid-phase TR6 are able to prevent mitogen-induced T-cell aggregation *in vitro*. Further, we found that TR6 pretreatment reduces actin polymerization and pseudopodium formation. Based on these findings, we hypothesized that TR6 might regulate T-cell migration *in vivo*, and it should be one of the mechanisms for TR6-secreting tumour cells to escape immune attack.

II. ARTICLES

Article 1.

Mouse T cells receive costimulatory signals from LIGHT, a TNF family member

Guixiu Shi, Hongyu Luo, Xiaochun Wan, Theodora W. Salcedo, Jun Zhang and Jiangping Wu

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Note: In this paper, Jun Zhang and Theodora W. Salcedo performed cytokine assays as shown in Figures 2, 3 and 4. They also supplied valuable regents, such as TR6, TR2, LIGHT and TR11 proteins, and anti-human LIGHT Abs. Hongyu Luo and Xiaochun Wan contributed in idea development and experimental design. Guixiu Shi carried out the rest of the work.

MOUSE T CELLS RECEIVE COSTIMULATORY SIGNALS FROM LIGHT, A TNF FAMILY MEMBER

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Declaration of conflict of interest: The human Genome Sciences Inc., two employees of which are listed as co-authors of this manuscript, has commercial interest in TR6 and LIGHT.

Running title: T cells receive reverse signaling through LIGHT.

Manu7\shi-mouse-TR6-T-blood-June-5-revision

ABSTRACT

LIGHT is a tumor necrosis factor (TNF) family member and is expressed on activated T cells. Its known receptors are TR2 and LTβR on the cell surface and TR6/DcR3 in solution. TR6/DcR3 is a secreted protein belonging to the TNF receptor family. It binds to Fas ligand (FasL), LIGHT, and TL1A, all of which are TNF family members. In the present study, we report that solid-phase TR6-Fc costimulated proliferation, lymphokine production, and cytotoxicity of mouse T cells upon T-cell receptor (TCR) ligation. A monoclonal antibody against LIGHT similarly costimulated mouse T cells in their proliferation response to TCR ligation. These data suggest LIGHT, although a ligand, can receive costimulation when expressed on the T-cell surface. Mechanistically, when T cells were activated by TCR and CD28 co-cross-linking, TCR and rafts rapidly formed caps where they colocalized. LIGHT rapidly congregated and colocalized with the aggregated rafts. This provided a molecular base for the signaling machinery of LIGHT to interact with that of TCR. Indeed, LIGHT cross-linking enhanced p44/42 mitogen-activated protein kinase activation after TCR ligation. This study reveals a new function and signaling event of LIGHT.

Key words: TR6/DcR3; LIGHT; costimulation; reverse signaling; mouse T cells

INTRODUCTION

LIGHT/TL5 is a new member of the tumor necrosis factor (TNF) family, with its protein expressed on activated T cells (1) and immature dendritic cells (2). It is a ligand for TR2/HVEM, LTβR, and TR6/DcR3, all of which are TNF receptor (TNFR) family members (1,3,4). Recent studies show that LIGHT can costimulate T-cell responses via TR2 (2,5,6) LIGHT can also induce apoptosis in cells expressing both TR2 and LTβR (7), although Rooney et al (8) reported that LTβR is necessary and sufficient for LIGHT-mediated apoptosis in tumor cells. However, LTβR is not expressed on lymphocytes (9).

Several TNF members on cell surfaces can reversely transduce signals into cells. Cayabyab et al (10) and Van Essen et al (11) have shown that CD40L transduces costimulation signals into T cells. Wiley et al (12) have reported that CD30L cross-linking activates neutrophils, and Cerutti et al (13) showed that such reverse signaling inhibits the immunoglobin class switch in B cells. Reverse signaling through membrane TNF-α confers resistance of monocytes and macrophages to lipopolysaccharide. (14) Cross-linking of TRANCE enhances interferon-γ (IFN-γ) secretion by activated Th1 cells (15). Reverse signaling through Fas ligand (FasL) can promote maximal proliferation of CD8 cytotoxic T cells (16-18). Cross-linking of TRAIL by its solid-phase death receptor 4 increases IFN-γ production and T-cell proliferation. (19). Whether LIGHT can reversely transduce signals into T cells has not been assessed.

TR6/DcR3 is a new member of the TNFR family. Human TR6 lacks an apparent transmembrane domain in its sequence and is a secreted protein (3,20). Mouse TR6 has not been cloned. Human TR6 has 3 known ligands, FasL (13), LIGHT (3,4), and TL1A

(21). FasL binding by TR6 interferes with the interaction between Fas and FasL. Consequently, FasL-induced apoptosis of lymphocytes and of several tumor cell lines can be repressed by TR6 (20). We have recently reported that soluble human TR6-Fc can suppress cytotoxic T lymphocyte (CTL) and lymphokine production of mouse lymphocytes and inhibit mouse heart allograft rejection (3). These findings have raised the possibility that TR6 inhibits LIGHT-triggered costimulation via TR2 in T cells.

We have presented evidence in this article that TR6 can trigger LIGHT to transduce signals into T cells and enhance the T-cell response to TCR stimulation in a mouse model. Such signaling is preceded by rapid congregation of LIGHT into the T-cell cap on the cell surface, followed by p42/44 mitogen-activated protein kinase (MAPK) activation. Thus, although a ligand, LIGHT can function as a receptor as well. The biologic significance of this finding is discussed.

MATERIALS AND METHODS

Lymphocyte preparation and culture

Mouse total spleen cells were prepared by lysing red blood cells with 0.84% NH_4Cl, as described elsewhere (22). Spleen T cells were purified by deleting Ig^+ and adhesion cells with T-cell columns according to the manufacturer's instructions (Cedarlane, Hornby, ON, Canada). CD4 and CD8 T cells were prepared from total spleen cells by positive selection, using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), L-glutamine, and antibiotics. RPMI 1640, FCS, penicillin-

streptomycin, and L-glutamine were purchased from Invitrogen (Burlington, ON, Canada). Solid phase TR6-Fc and anti-CD3 were prepared by coating Nunc plates with 5 μg/mL goat anti-human IgG (Southern Biotechnology, Birmingham, AL) along with various concentrations of anti-CD3 in phosphate-buffered saline (PBS) overnight at 4°C. After washing, the plates were incubated with TR6-Fc or a control fusion protein TR11-Fc of desired concentrations in PBS at 37°C for 2 hours. After washing, the coated plates were used for culture. ^{3}H-thymidine uptake was measured as described previously (23,24).

Lymphokine assays

Interleukin 2 (IL-2) and IFN-γ in culture supernatants were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits from R & D Systems (Minneapolis, MN).

Cytotoxic T-cell assay

The assay was performed as detailed previously (3). Briefly, 2C mouse spleen cells (4×10^6 cells/2 mL/well, H-2^b) were stimulated with an equal amount of mitomycin C-treated BALB/c mouse spleen cells (4×10^6 cells/2 mL/well, H-2^d) in flat-bottomed 24-well plates, which were precoated with goat anti-human IgG (5 μg/mL) followed by TR6-Fc (10 μg/mL) or normal human IgG (10 μg/mL), in the presence of 10 U/mL IL-2 for 6 days. Human LIGHT (20 μg/mL) was added to the culture in the beginning of certain cultures. On day 6, cells receiving the same treatment in 24-well plates were pooled and counted, and their CTL activity was measured by a standard 4-hour ^{51}Cr-

release assay, using ^{51}Cr-labeled P815 cells (H-2^d) as targets at different effector-target ratios. The lysis percentage of the test sample was calculated as follows:

$$\% \text{ lysis} = \frac{\text{cpm of the test sample - cpm of spontaneous release}}{\text{cpm of maximal release - cpm of spontaneous release}}$$

Flow cytometry

Mouse spleen cells, cultured in medium or stimulated with concanavalin A (Con-A; 2 μg/mL) for 24 hours, were first cross-linked by anti-CD3 and anti-CD28 hamster monoclonal antibody (mAb) followed by goat anti-hamster IgG, as detailed in the section on confocal microscopy below. After fixing with 3.7% formalin, the cells were stained with a human anti-human LIGHT mAb (clone 1.2.2, 0.1 μg/10^6 cells), followed by phycoerythrin (PE)-F(ab)'$_2$ of goat anti-human IgG (Southern Biotechnology) and fluorescein isothiocyanate (FITC)-anti-Thy-1.2 (clone 53-2.1, Pharmingen, San Diego, CA). In some samples, TR6 without the Fc tag (5 μg/sample) or human LIGHT (5 μg/sample) was present as inhibitors during the staining process. Thy-1.2$^+$ cells were gated and analyzed for anti-LIGHT intensity.

Confocal microscopy

Five million BALB/c T cells were first blocked with 100 μL PBS containing 2% bovine serum albumin (BSA) for 30 minutes. Then, 5 μg anti-CD28 (clone 37.51.1, hamster mAb, Cedarlane) and 2 μg biotinylated anti-CD3 (clone 2C11, hamster mAb) were added to the cell suspension, which was incubated for an additional 30 minutes. After washing with cold PBS, the cells were reacted with goat anti-hamster IgG (5 μg/sample) for 30 minutes. All the procedures above were conducted at 4°C. The cells were washed

with cold PBS and transferred to 200 μL warm PBS to start the 10-minute cross-linking process at 37°C. They were then fixed immediately with 3 mL 3.7% formalin at room temperature for an additional 10 minutes. For T-cell receptor (TCR) and LIGHT staining, the fixed cells were reacted with TR6-Fc (2 $\mu g/10^6$ cells) for 30 minutes on ice. After washing with PBS, the cells were stained with Alexa 488-goat anti-human IgG (1 $\mu g/10^6$cells) and Alexa 594-streptavidin (1 $\mu g/10^6$ cells) on ice for another 30 minutes. For raft and LIGHT staining, the procedure was similar to that described above, but Alexa 594-conjugated cholera toxin (1 μg/sample) was used instead of Alexa 594-streptavidin. The stained cells were then washed with PBS and mounted on slides with Prolong antifade mounting medium (Molecular Probes, Eugene, OR). The slides were examined under a confocal microscope. Digital images were processed with Photoshop (Adobe, Seattle, WA).

Immunoblotting

Plates (24-well) were coated with anti-human IgG (5 μg/mL, 300 μL/well) and a suboptimal concentration of anti-CD3 (0.2 μg/mL, 300 μL/well) overnight at 4°C. After washing, the wells were incubated with TR6-Fc (10 μg/mL, 300 μL/well) or a control fusion protein TR11-Fc (10 μg/mL, 300 μL/well) at 37°C for 2 hours. Mouse spleen T cells were treated with cytochalasin D (15 μM) in complete culture medium or medium containing a similar percentage of vehicle (dimethyl sulfoxide [DMSO]) for 2 hours. After washing, the cells were seeded in precoated plates at 5×10^6 cells/well, and the plates were centrifuged at 228*g* for 5 minutes to achieve rapid contact between the cells and the bottom of the culture wells. The cells were then cultured at 37°C for 90 minutes before being harvested. The remaining procedures were detailed in our previous

publication (22). Briefly, the cells were washed and lysed in lysis buffer for 10 minutes; the cleared lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes, which were sequentially hybridized with rabbit anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibody (New England Biolabs, Mississauga, ON, Canada). Immunoreactive protein bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG followed by enhanced chemiluminescence.

RESULTS

Solid-phase TR6-Fc enhances proliferation of mouse T cells stimulated by suboptimal concentrations of anti-CD3

We investigated whether LIGHT, although a ligand, could transduce stimulating signals reversely into T cells. To this end, we used one of the receptors of LIGHT, TR6, in solid phase to cross-link LIGHT on mouse T cells. Because mouse TR6 has not been cloned, and human TR6 can bind to mouse LIGHT (3), recombinant human TR6 with an Fc tag was used. As shown in Figure 1A, human TR6 in solid phase (10 μg/mL, all the concentrations in this section refer to those used during coating of the wells) could enhance the proliferation of total spleen cells stimulated with anti-CD3 in solid phase, compared with a control fusion protein TR11-Fc. The enhancement was obvious with anti-CD3 at 0.25 μg/mL or 0.5 μg/mL. To assess whether both CD4 and CD8 cells were responsive to the TR6 stimulation, they were purified from total spleen cells with magnetic beads and stimulated with a fixed amount of anti-CD3 (0.2 μg/mL) and various concentrations of TR6-Fc. At 10 μg/mL, TR6-Fc had an obvious stimulatory effect on

the proliferation of both CD4 and CD8 cells (Figure 1B,D). Next, TR6-Fc was tested at a fixed optimal concentration of 10 μg/mL along with different concentrations of anti-CD3. As shown in Figure 1, panels C and E, both CD4 and CD8 T cells were responsive to TR6 costimulation when anti-CD3 was used at several suboptimal concentrations from 0.125 to 0.5 μg/mL. The results of this section show that LIGHT ligation by TR6 costimulates CD4 and CD8 T cells in their proliferation response to TCR cross-linking.

Costimulation through LIGHT enhances lymphokine secretion by T cells

To understand the immunologic consequences of costimulation through LIGHT, we examined lymphokine secretion by total spleen cells and CD4 and CD8 T cells. As shown in Figure 2, solid-phase TR6-Fc (10 μg/mL, the concentrations indicated in this section refer to those used during coating of the wells), compared with control fusion protein TR11-Fc, significantly augmented IFN-γ and IL-2 secretion of total spleen cells stimulated with 0.25 μg/mL or 0.5 μg/mL anti-CD3, which was also in solid phase. Lymphokine secretion by CD4 (Figure 3) and CD8 (Figure 4) cells was evaluated next. TR6-Fc in solid phase dose-dependently enhanced IFN-γ and IL-2 secretion by CD4 cells in the presence of a suboptimal concentration of anti-CD3 (0.125 μg/mL). With an optimal concentration of TR6-Fc (10 μg/mL), an increased amount of anti-CD3 dose-dependently augmented the secretion of these lymphokines (Figure 3), compared with cultures stimulated with TR11-Fc. CD8 T cells produced more IFN-γ than CD4 cells on costimulation through LIGHT, but did not augment IL-2 secretion when different TR6-Fc or anti-CD3 concentrations were used in combination (Figure 4). These data show

that costimulation through LIGHT differentially augments lymphokine production by CD4 and CD8 T cells in mice.

Costimulation through LIGHT augments CTL activity

To further assess the functional consequence of LIGHT costimulation, we examined CTL development in the presence of solid-phase TR6-Fc. We used an established CTL assay system, in which the responders were spleen cells from transgenic 2C mice (in H-2^b background), and the stimulators were mitomycin C-treated BALB/c (H-2^d) spleen cells (3). Almost all T cells in 2C mice carry transgenic L^d-specific TCR, and the majority (about 75%) of their T cells are $CD8^+$ (25). Compared with control human IgG, in the presence of solid phase TR6-Fc, CTL development was significantly enhanced, as shown in Figure 5. Such enhancement could be neutralized by soluble human LIGHT, indicating that the stimulation by TR6-Fc was specific. Therefore, costimulation through LIGHT enhances CTL activity.

Anti-human LIGHT mAb binds to mouse LIGHT and costimulates mouse T cells

In the previous sections, human TR6 was used to engage mouse LIGHT, because human TR6 can bind to mouse LIGHT and because it is a physiologically relevant receptor for LIGHT. However, we could not exclude the possibility that human TR6 exerted its effect through FasL or other so-far unidentified TNF family members other than LIGHT. We, therefore, used a human anti-human LIGHT mAb to further pinpoint LIGHT on mouse T cells as the molecule receiving costimulation. We showed that it could bind to about 30% of resting spleen T cells, and binding increased to 65% after T-cell activation with Con-A overnight (Figure 6A). This binding pattern was consistent with the documented

expression pattern of LIGHT in T cells in that its expression was augmented after T-cell activation (26). The binding could be blocked by soluble human LIGHT, indicating that the mAb was specific. Moreover, the binding could also be blocked by soluble TR6, suggesting that both TR6 and the mAb competitively interacted with the same ligand on the T-cell surface. Taken together, these results indicate that the anti-human LIGHT mAb binds to mouse LIGHT. We next assessed whether the mAb could costimulate T cells, as with TR6-Fc. This human mAb (10 μg/mL) was anchored on plates via goat anti-human IgG (5 μg/mL) in the presence or absence of a suboptimal concentration of anti-CD3 (0.2 μg/mL). As shown in Figure 6B, the anti-LIGHT mAb significantly promoted T-cell proliferation, compared with control normal human IgG. Thus, these data confirm that LIGHT on T cells can indeed receive costimulation.

Signaling events after costimulation through LIGHT

To understand the molecular basis of LIGHT costimulation, we examined the molecular migration of LIGHT and TCR and their relationship with lipid rafts on T-cell membranes immediately after TCR and CD28 co-cross-linking. T cells were preincubated with anti-CD3 and anti-CD28, followed by a second antibody on ice. CD3 and CD28 co-cross-linking started when the cells were transferred to 37°C. The lipid rafts in the T-cell membrane were stained by Alexa 594-cholera toxin in red; TCR was stained by Alexa 594-streptavidin in red; LIGHT was stained in green by TR6-Fc followed by Alexa 488-anti-human IgG. TCR and LIGHT were distributed throughout the surface in the resting T cells, with TCR spreading more evenly, whereas rafts and LIGHT appeared in speckles (first row, Figure 7). After 10 minutes of co-cross-linking

with anti-CD3 and anti-CD28, TCR rapidly polarized and formed a cap in one end of the cell. LIGHT also congregated, and it colocalized with TCR (second row, Figure 7). LIGHT obviously comigrated and colocalized with rafts as shown in row 3 of Figure 7. Taken together, these data indicate that TCR and LIGHT both congregate to a raft cap on the cell surface immediately after TCR and CD28 cross-linking. This provides a molecular base for LIGHT to enhance TCR signaling because now both of them are closely associated and located in aggregated rafts, which are scaffolds accommodating many signaling molecules.

We also investigated signaling events further downstream from the cell membrane. Mouse spleen T cells were plated on wells coated with TR6-Fc alone, a suboptimal concentration of anti-CD3 alone, or both, for 90 minutes, and p44/42 MAPK activation was monitored according to their phosphorylation. As shown in Figure 8A, the level of phosphorylated p42 MAPK was augmented in cells stimulated with both anti-CD3 and TR6-Fc, compared with anti-CD3 or TR6-Fc alone, whereas p44/42 MAPK protein levels remained unchanged in all treatments. When cells were preincubated for 2 hours in cytochalasin D, which prevents actin polymerization, the level of phospho-p42 MAPK no longer increased in the presence of anti-CD3 and TR6-Fc costimulation. This suggests that MAPK activation depends on actin polymerization. MAPK activation in this case was an essential and relevant event in T-cell activation after TCR and LIGHT cross-linking because a p44/42 MAPK-specific inhibitor PD98059, but not its nonfunctional analog SB202474, strongly suppressed the T-cell proliferation triggered by CD3 and LIGHT stimulation (Fig. 8B).

These results indicate that LIGHT can indeed transduce signals into T cells, and such transduction depends on early membrane events, actin polymerization and MAPK activation.

DISCUSSION

In this study, we have reported that costimulation through LIGHT enhances T-cell proliferation, cytokine production, and CTL development. Immediately after T activation by anti-CD3 and anti-CD28, LIGHT congregates to the raft cap where it colocalizes with TCR. This membrane event is followed by MAPK activation.

Because mouse TR6 has not been cloned, we used human TR6 to cross-link LIGHT on mouse T cells. This was a legitimate choice because human TR6 cross-reacts with mouse LIGHT (3). However, the observed stimulatory effect of solid-phase TR6 could operate via molecules other than LIGHT, because TR6 is known to bind FasL, which is also capable of reversely transducing signals into T cells (16-18), and because human TR6 binds to mouse FasL (data not shown). Moreover, TNF and TNFR family members are known to cross-react among themselves, as discussed in the "Introduction" on interactions among TR6, LIGHT, FasL, TL-1A, TR2, and LTβR. To ascertain that LIGHT could receive costimulation, we used mAb against human LIGHT, which shares 67% homology with mouse LIGHT in its amino acid sequence, for costimulation. The binding of this mAb to activated mouse T cells could be inhibited by soluble TR6,

indicating that TR6 and the mAb share a common ligand. Considering the fact that the mAb is specific to human LIGHT, unless there was an extremely rare and unlikely coincidence in which the mAb and TR6 both bind to an unknown molecule X on the T-cell surface, we have to conclude that the anti-human LIGHT mAb is specific to mouse LIGHT as well. Because this mAb could costimulate T cells in the presence of TCR ligation, reverse signaling through LIGHT was thus established. Although we cannot totally exclude the possibility that the costimulating effect of TR6 on T cells might, to a certain extent, be due to its binding to molecules other than LIGHT, we believe that the major effect of TR6 occurs via LIGHT, because the binding of mouse T cells by TR6 was largely eliminated when the LIGHT gene was knocked out (data not shown).

Questions are inevitably raised regarding whether the stimulatory effect of TR6 is due to its positive stimulation to LIGHT or due to its blocking of an existing negative autocrine loop in which LIGHT acts as a receptor. The latter possibility is best argued against by the fact that in our model, solid-phase TR6 could stimulate T cells. When TR6-Fc was used to coat the plate, although a concentration of 1 to 2 μg/100 μL/well was used, only a very small fraction of it actually went onto the plate, and more than 99.9% of the protein was washed away after the coating process. Thus, no more than 2 ng TR6 was actually coated on a well. If we consider how small a fraction of this will leak into solution, it is unlikely such a minute amount of soluble TR6 could interfere with an autocrine loop. Indeed, soluble TR6-Fc up to 10 μg/mL had no effect on anti-CD3-stimulated T cells (data not shown). Can TR6 on the solid phase block an autocrine loop? We are not aware of any example in an experimental system that this can be

achieved. Because a cell is a 3-dimensional entity, solid-phase TR6 can interfere only with a part of the cell surface that has contact with the well. Therefore, the solid-phase TR6 cannot prevent the interaction between a putative soluble suppressive autocrine and LIGHT on most part of the cell surface that is not in contact with TR6. Consequently, most LIGHT molecules on a cell surface should still receive negative signals from the putative suppressive autocrine, if there is one. It is therefore very difficult to explain the positive effect of solid-phase TR6. In addition, recent studies showed that LIGHT transgenic mice overexpressing LIGHT on T cells have enhanced immune response (27,28). This result does not fit to the model in which LIGHT transduces negative signals into T cells, because if so, the LIGHT transgenic mice should have suppressed immune response instead. Lastly, there are about 4 to 5 TNF family members capable of transducing signals into cells, but none of them transduces a negative one. Based on these arguments, it is concluded that TR6 exerts its effect by stimulating T cells via LIGHT, but not by interfering with a putative negative autocrine loop.

Several studies have explored the pathways of reverse signaling. In the case of CD40L, its ligation results in general protein tyrosine phosphorylation, Ca^{++} influx, and activation of Lck, PKC, JNK, and p38 MAPK in EL4 thymoma cells (29,30). TRAIL cross-linking also induces p38 MAPK activation (19). We examined the mechanisms of reverse signaling through LIGHT. A recent study showed that plasma membrane compartmentation plays an essential role in T-cell activation and costimulation. Detergent-insoluble, glycolipid-enriched rafts function as scaffolds and contain many signaling molecules such as Src kinases (31). After cross-linking, TCR translocates to the rafts, where it gains access to the signaling pathways (32,33). We explored the

interaction among TCR, LIGHT, and rafts in this study. Anti-CD3 and anti-CD28 were used to mimic antigen-presenting cells (APCs) for T-cell activation. TCR rapidly formed caps, which colocalized with aggregated rafts, as expected. We found that LIGHT immediately congregated and colocalized with the TCR cap and aggregated rafts. This is a novel finding and is consistent with the costimulatory function of LIGHT. If LIGHT were to costimulate T cells, the best place it should go is aggregated rafts, where its own signaling machinery can interact with that of TCR, and enhance the latter. It is to be noted that in our T-cell activation model, we used TR6 along with suboptimal anti-CD3 to stimulate T cells in the absence of anti-CD28. Under such a condition, the percentage of T suboptimal anti-CD3 alone and the capping was not as drastic and clear-cut as with anti-CD3 plus anti-CD28 treatment (data not shown). It is still possible that a small degree of colocalization of LIGHT, TCR, and raft is present and is sufficient for the costimulation of LIGHT for TCR, but such a small degree is not discernable by our current technology. The anti-CD3 plus anti-CD28 model gave a much stronger stimulation and proves in principle that TCR and LIGHT can lodge together in the raft scaffold. Under the physiologic condition, TCR ligation is normally accompanied by CD28 costimulation. Therefore, LIGHT will be likely recruited to the TCR-raft complex, and our model using anti-CD3 plus anti-CD28 is physiologically relevant.

The activity of p42 MAPK, which is a downstream signaling molecule in the TCR signaling pathway, was enhanced by LIGHT signaling. How this is achieved is not presently clear. LIGHT has a short and featureless cytoplasmic tail incapable of signaling by itself (1); hence, its signaling most likely depends on molecules with which it associates. In our experiment, a MAPK inhibitor was able to inhibit TR6-augmented

T-cell proliferation in the presence of suboptimal anti-CD3. Because anti-CD3-induced T-cell proliferation is also dependent on MAPK, this kinase might be in the common pathway shared by TCR and LIGHT, and its activation is augmented by upstream signals from both TCR and LIGHT. The MAPK inhibitor obviously will not differentiate TCR-derived signals from those that are LIGHT derived.

What is the biologic significance of reverse signaling through LIGHT? LIGHT-expressing T cells can costimulate TR2-expressing T cells. Reverse signaling through LIGHT allows TR2-expressing T cells to stimulate LIGHT-expressing T cells as well, and such 2-way stimulation provides a theoretical base for T cell-to-T cell cooperation, which is not a well-explored topic. It is known that T cells need to reach a certain density in culture to optimally respond to TCR stimulation, and such a phenomenon is often attributed to the need for cytokines in culture. Without discounting the importance of cytokines, bidirectional costimulation via TR2 and LIGHT between T cells may well contribute to the cell density requirement during their activation. Such T cell-to-T cell cooperation can also explain why in vivo T-cell responses tend to occur in regions densely populated with T cells in lymphoid organs. Recently, it has been shown that LIGHT overexpression in the T-cell compartment in LIGHT transgenic mice results in profound inflammation and the development of autoimmune syndromes (27,28); T cells overexpressing LIGHT have an activated phenotype (27). Probably, such an up-regulated immune response of T cells is due to stimulation of TR2/HVEM on dendritic cells by T cell-derived LIGHT, and the dendritic cells in turn augment T-cell activity; TR2 on T cells can also receive LIGHT stimulation directly from their fellow LIGHT-

expressing T cells (28). On the other hand, it is entirely possible that overexpressed LIGHT on T cells receives stimulation reversely, which augments their responsiveness to TCR ligation. In this study, we used recombinant TR6-Fc and anti-LIGHT mAb as artificial binding partners of LIGHT. In vivo, the molecules that can trigger LIGHT signaling are probably cell surface TR2 or LTβR. To support this hypothesis, we found that when TR2 was put on solid phase, it could enhance T-cell response triggered by suboptimal anti-CD3, as with TR6 (data not shown). On the other hand, endogenous TR6 might act as an inhibitor of the bidirectional costimulation between TR2 and LIGHT, or function as a costimulating factor for LIGHT, depending on whether it exists as monomers or trimers like other cell surface TNFR family members. This aspect is worth further investigation. Because dendritic cells also express LIGHT, TR2 on the T-cell surface might activate dendritic cells through LIGHT to modulate their APC function. If so, this will represent a new mechanism for T-cell and dendritic cell interaction and cooperation.

We recently reported that soluble human TR6-Fc inhibited CTL in vitro and allograft rejection in vivo in mice (3). In that paper, we proposed that soluble TR6 blocked the costimulation from LIGHT to TR2, or reversely from TR2 to LIGHT (3), or both, although, at the time, solid evidence of reverse signaling through LIGHT was not available. Our current findings have fulfilled one of our initial predictions that the inhibitory effect of human TR6 in the mouse system should be attributed to the interference of TR6 with the bidirectional costimulation between TR2 and LIGHT. When TR6 was used as a solid-phase molecule instead of in solution, its function

changed from suppression to promotion of the T-cell response, because it no longer served as blocker but rather as a cross-linker of LIGHT. Increasing cases of bidirectional signal transduction between receptors and ligands have been found in biologic systems. We can take advantage of such a phenomenon by exploiting soluble monomer ligands (or receptors) without the cell-anchoring ability to block signaling in both directions and thus modulate biologic responses

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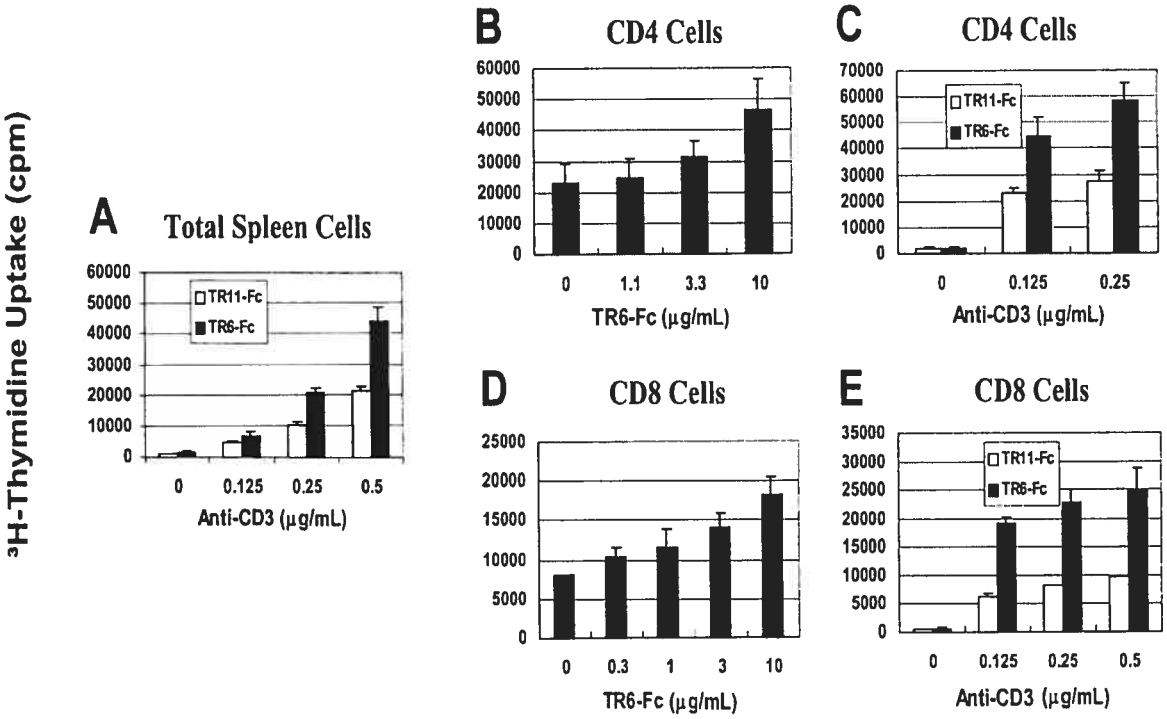
FIGURE LEGENDS

Figure 1. Solid phase TR6-Fc promotes proliferation of anti-CD3-stimulated total spleen cells, and CD4 and CD8 T cells



Mouse spleen cells (A), CD4 cells (B,C) or CD8 cells (D,E) were stimulated with solid-phase anti-CD3 and TR6-Fc. When anti-CD3 was used at different doses, TR6-Fc and its control, TR11-Fc, were tested at an optimal dose of 10 μg/L. When TR6-Fc was used at different doses, anti-CD3 was used at a suboptimal dose of 0.2 μg/mL. These concentrations referred to those used during the well-coating process. Cell proliferation was measured by ^{3}H-thymidine uptake between 48 and 64 hours after the initiation of culture. Means ± SD of the counts per minute from triplicate samples are shown. The experiments were performed more than 3 times, and a representative set of data is presented.

Figure 2. TR6-Fc strongly augments lymphokine production by anti-CD3-stimulated spleen cells.

Mouse total spleen cells were stimulated with solid-phase anti-CD3 and TR6-Fc. The culture conditions were the same as described in Figure 1. The culture supernatants were harvested at 48 hours after the initiation of the culture and were tested for lymphokines with ELISA. Samples were studied in duplicate, and the means ± SD of lymphokine levels are shown. The experiments were conducted at least twice with similar results. A representative set of data is shown.

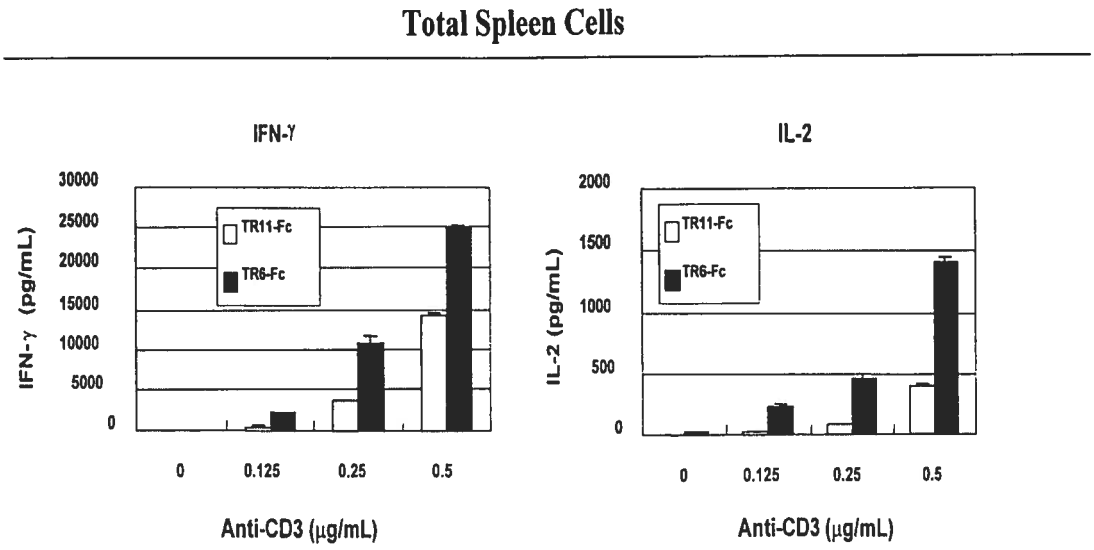


Figure 3. TR6-Fc strongly augments lymphokine production by anti-CD3-stimulated CD4 cells

Mouse spleen CD4 cells were stimulated with solid-phase anti-CD3 and TR6-Fc, and cytokines in the supernatants were measured with ELISA, as described in Figure 2. Data are represented as means ± SD.

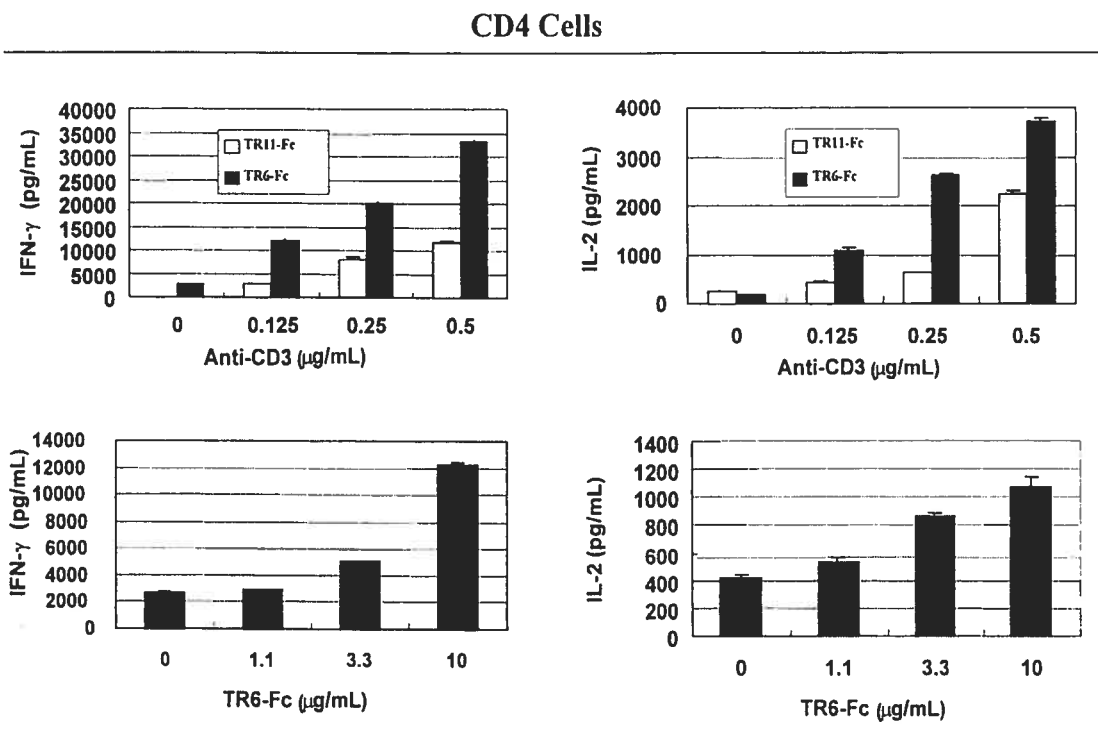
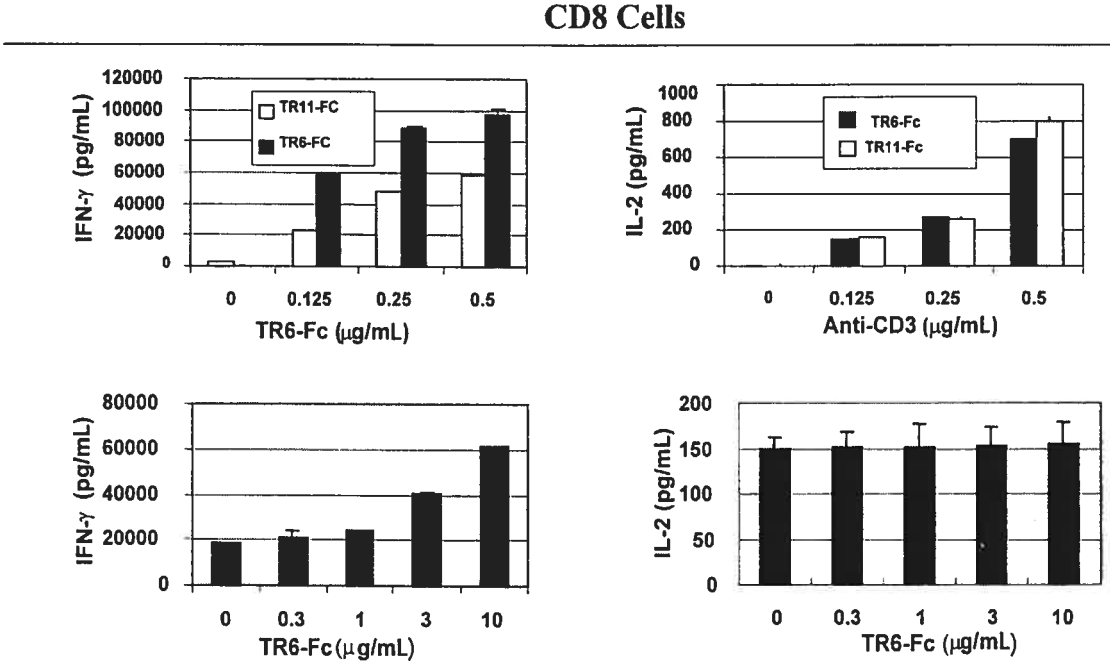
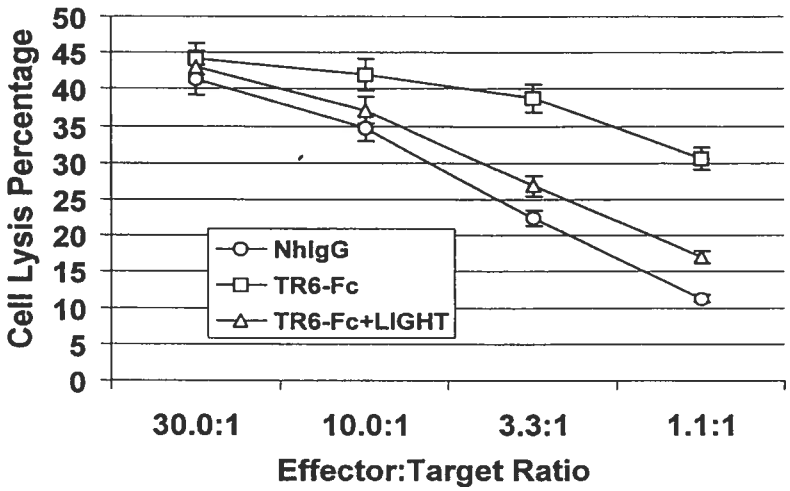


Figure 4. TR6-Fc strongly augments lymphokine production by anti-CD3-stimulated CD8 cells



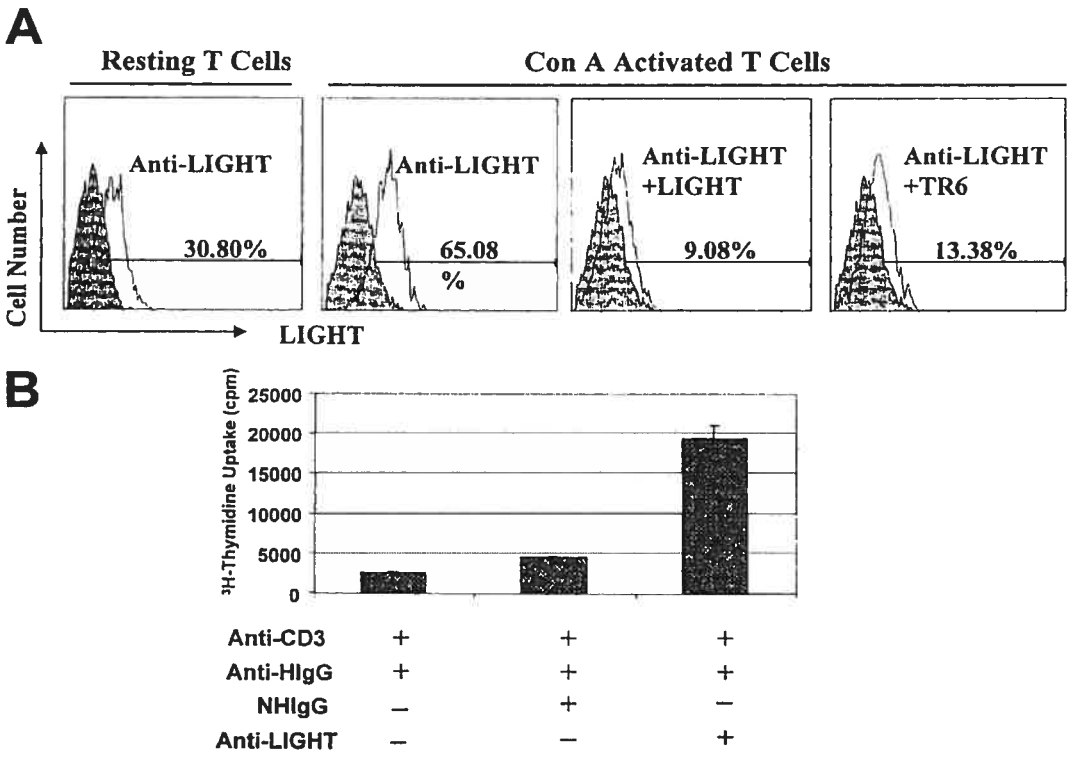
Mouse spleen CD8 cells were stimulated with solid-phase anti-CD3 and TR6-Fc, and cytokines in the supernatants were measured with ELISA, as described in Figure 2. Data are represented as means ± SD.

Figure 5. Effect of LIGHT reverse signaling on CTL development



2C mouse spleen cells (4 x 10^6 cells/2 ml/well) were mixed with an equal amount of mitomycin C- treated BALB/c mouse spleen cells (4 × 10^6 cells/2 mL/well) and seeded in flat-bottomed 24-well plates, which were precoated with TR6-FC (10 μg/mL) or normal human IgG (NhIgG, 10 μg/mL) as a control. Soluble human LIGHT (20 μg/mL) was added at the beginning of the culture in certain samples as indicated. The cells were cultured in the presence of 10 U/mL IL-2 for 6 days. CTL activity in the stimulated cells was then measured by a standard 4-hour ^{51}Cr-release assay, using P815 cells as targets. The samples were tested in triplicate, and means ± SD of percentage of target cell lysis are shown. The experiments were performed twice with similar results, and the data of a representative experiment are presented.

Figure 6. Anti-human LIGHT mAb binds to mouse LIGHT and costimulates mouse T cells



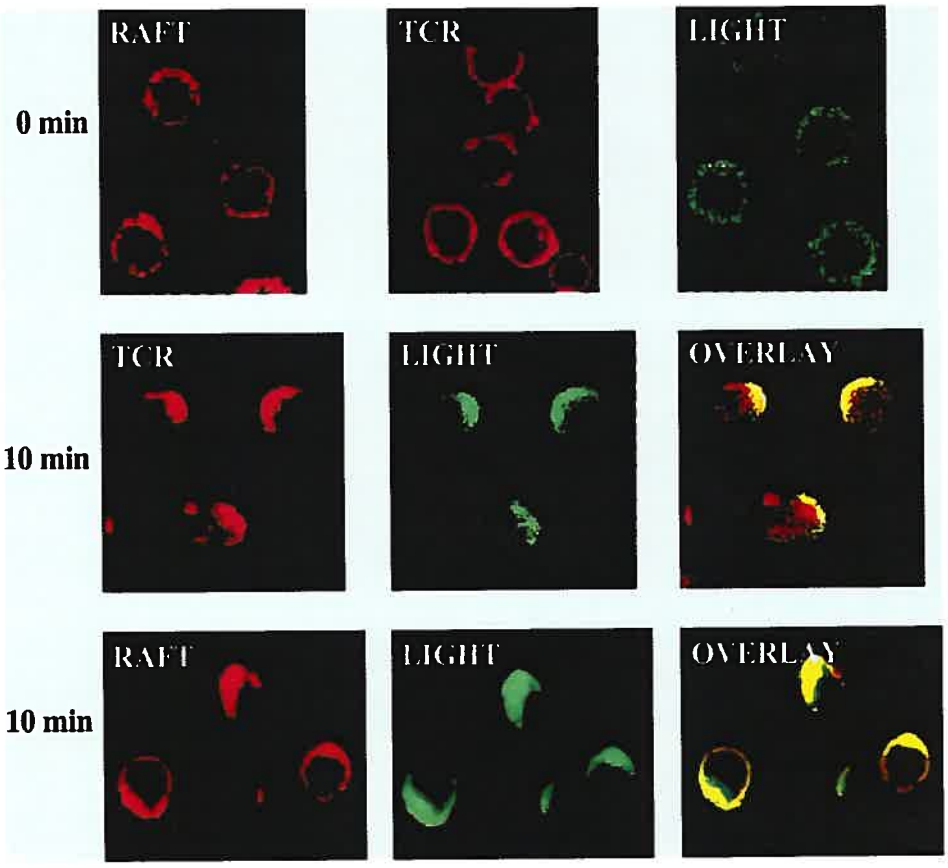
A. Anti-LIGHT mAb binds to mouse T cells according to flow cytometry

Resting BALB/c spleen cells or spleen cells activated by Con-A (2 μg/mL) for 24 hours were cross-linked with anti-CD3 and anti-CD28 hamster mAb followed by goat-anti-hamster IgG. After fixing with 3.7% formalin, the cells were stained with anti-human LIGHT mAb (clone 1.2.2) followed by PE-F(ab)'$_2$ of goat anti-human IgG, along with FITC-anti-Thy1.2. In some samples, soluble human LIGHT (5 μg/sample) or TR6 without the Fc tag (5 μg/sample) was added as inhibitors during the staining process. The cells were analyzed by 2-color flow cytometry, and LIGHT expression on Thy1.2$^+$ cells is shown in the histograms. Shaded areas represent cells stained with an isotypic control mAb, and solid lines represent cells stained with anti-LIGHT mAb

B. Anti-LIGHT mAb costimulates mouse T cell proliferation

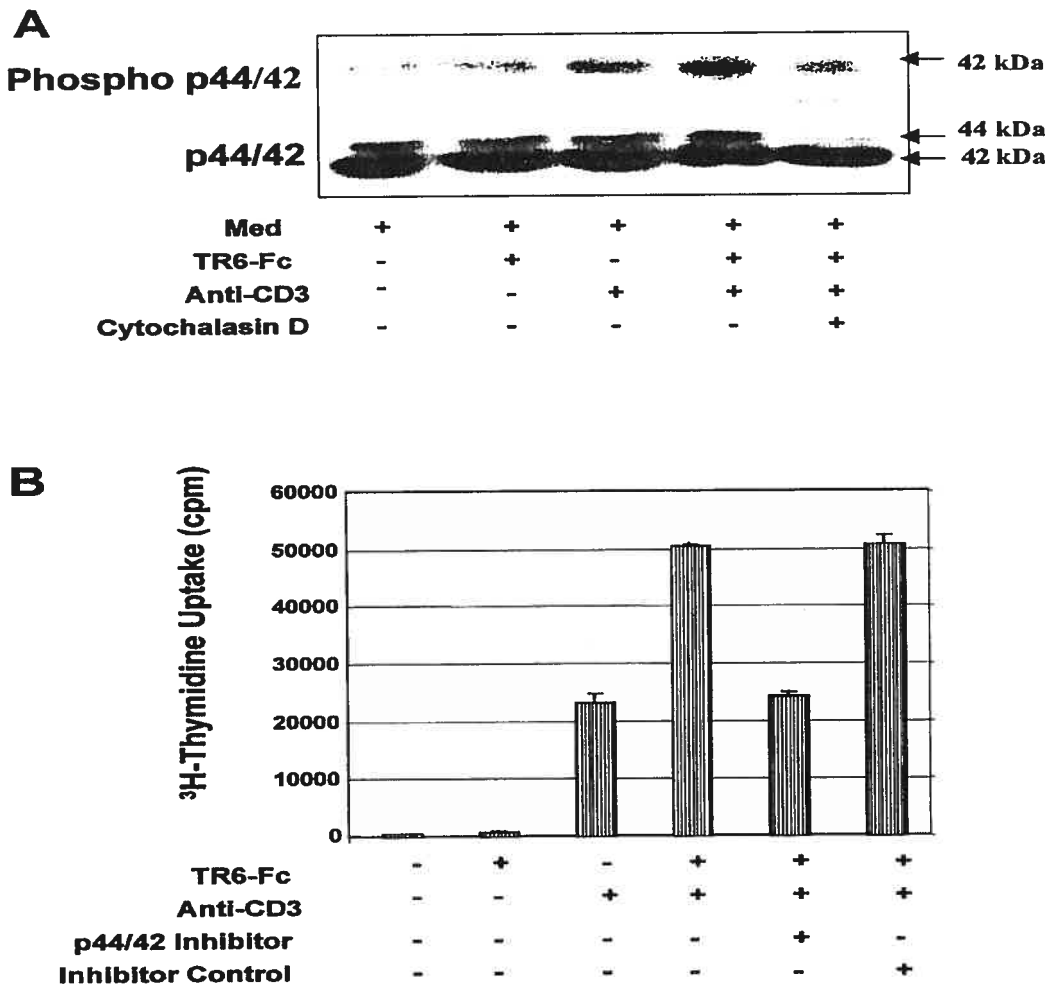
Nunc 96-well plates were coated with a suboptimal concentration of anti-CD3 (0.2 μg/mL) along with goat anti-human IgG (anti-HIgG, 5 μg/mL) overnight at 4°C. After washing, the wells were reacted with normal human IgG (10 μg/mL) or human mAb against human LIGHT (10 μg/mL) at 37°C for 2 hours. The wells were washed and BALB/c T cells were seeded into the wells at 4×10^5 cells/well. ^{3}H-thymidine uptake was measured between 48 and 64 hours. The samples were in triplicate, and the means ± SD of counts per minute are shown. The experiments were performed twice with similar results, and the data of a representative experiment are presented.

Figure 7. LIGHT rapidly translocates into caps of TCR and rafts on the surface of activated T cells



BALB/c spleen T cells were cross-linked with anti-CD3 and anti-CD28 for 0 or 10 minutes. The locations of TCR (stained with biotin-anti-CD3 followed by Alexa 594-streptavidin in red), rafts (stained with Alexa-cholera toxin in red) and LIGHT (stained with TR6-Fc followed by Alexa 488-anti-human IgG in green) were revealed by confocal microscopy. Original magnification, ×600.

Figure 8. Costimulation through LIGHT enhances MAPK activity



A. MAPK activity is enhanced after LIGHT costimulation

BALB/c T cells were precultured in medium for 2 hours in the presence of 0.1% DMSO, or 0.1% DMSO plus 15 μM cytochalasin D. After washing, the cells were cultured in medium (MED), or stimulated with solid phase TR6-Fc, a suboptimal concentration of anti-CD3, or both, for 90 minutes at 37°C. The levels of phosphorylated p44/42 MAPK and total p44/42 MAPK were assessed by immunoblotting with the same membrane. The 42-kDa band representing phosphorylated MAPK and the 44-kDa and 42-kDa bands representing total p44/42 MAPK protein are indicated by arrows.

B. P44/42 MAPK activity is essential for LIGHT-enhanced T-cell proliferation

BALB/c T cells were cultured in wells coated with TR6-Fc (10 μg/mL), a suboptimal concentration of anti-CD3 (0.2 μg/mL), or both. The cells were cultured in medium, or in the presence of a p44/42 MAPK inhibitor PD98059 (15 μM) or its noninhibitory structural homologue SB202474 (15 μM). The ^{3}H-thymidine uptake of these cells was measured between 48 and 64 hours. The samples were tested in triplicate, and means ± SD of counts per minute are shown. The experiments were performed twice with similar results, and the data of a representative experiment are presented.

Article 2.

Death Decoy Receptor TR6/DcR3 Inhibits T-Cell Chemotaxis *in vitro* and *in vivo*[1]

Guixiu Shi, Yulian Wu, Jun Zhang and Jiangping Wu

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Note: In this paper, Jun Zhang supplied valuable reagents such as TR6, TR11, TR2 and LIGHT recombinant proteins, anti-TR6 and anti-LIGHT mAbs. Yulian Wu provided a supporting figure for the Journal of Immunology reviewers, showing lymphocyte infiltration in TR6 positive and negative tumors; the figure was quoted as "data not shown" in the article. Guixiu Shi performed all the remaining experiments.

Death Decoy Receptor TR6/DcR3 Inhibits T-Cell Chemotaxis *in vitro* and *in vivo*[1]

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Key words: DcR3/TR6; LIGHT; T cells; Chemotexis

Running title: TR6/DcR3 inhibits T-cell chemotaxis

FOOTNOTE

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ABSTRACT

TR6/DcR3 is a secreted molecule belonging to the TNF receptor family. Its ligands are LIGHT, FasL and TL1A, all TNF family members. TR6 is expressed in some tumors, and is hypothesized to endow tumor cells with survival advantages by blocking Fas-mediated apoptosis. It can also inhibit T-cell activation by interfering with 2-way T-cell costimulation between LIGHT and HveA. In this study, we discovered a novel function of TR6: inhibition of T-cell chemotaxis. Human T cells pretreated with soluble or solid phase TR6-Fc showed compromised migration towards CXCL12/stromal cell-derived factor 1α in vitro in a Transwell assay. Such an effect could also be observed in T cells pretreated with soluble or solid phase HveA-Fc or anti-LIGHT mAb, suggesting that LIGHT reverse signaling was likely responsible for chemotaxis inhibition. TR6 pretreatment also led to T-cell chemotaxis suppression in vivo in the mice, confirming in vivo relevance of the in vitro observation. Mechanistically, a small GTPase Cdc42 failed to be activated after TR6 pretreatment of human T cells, and further downstream, p38 mitogen-activated protein kinase activation, actin polymerization and pseudopodium formation were all downregulated in the treated T cells. This study revealed a previously unknown function of TR6 in immune regulation, and such an effect could conceivably be explored for therapeutic use in controlling undesirable immune responses.

INTRODUCTION

TR6/DcR3, a TNFR family member, is a secreted protein due to the lack of the transmembrane domain in its sequence (1,2). TR6 has three known ligands, Fas ligand (L) (3), LIGHT (1,4) and TL1A (5), all of which are TNF family members. FasL binding by TR6 interferes with the interaction between Fas and FasL. Consequently, FasL-induced apoptosis of lymphocytes or tumor cells can be repressed by TR6 (2). The consequence of interaction between TR6 and LIGHT is more complex. LIGHT is a ligand of HveA and lymphotoxin β receptor (LTβR) (1,4,6) in addition to TR6. It is expressed on the surface of activated T cells (6) and immature dendritic cells (7). Our recent study has shown that LIGHT is expressed in resting T cells as well according to confocal microscopy (8). LIGHT costimulates T-cell responses via HveA (7,9,10). We have demonstrated that LIGHT, although being a ligand, also reversely transduces costimulatory signals into T cells (8, 11). Moreover, LIGHT induces apoptosis in cells expressing both HveA and LTβR (12), or LTβR alone (13). Soluble TR6 blocks the two-way costimulation between HveA and LIGHT among T cells (1), while TR6 on solid phase costimulates T cells via LIGHT reverse signaling (8, 11). TR6 can modulate dendritic cell maturation (14), whether this effect occurs via LIGHT reverse signaling remains a matter of debate. TR6 also blocks LIGHT-induced apoptosis of LTβR-bearing cells (4). Since LTβR is not expressed on lymphocytes, such an effect does not have significant relevance in the immune system. The third TR6 ligand, TL1A, is predominantly expressed on endothelial cells (5). TL1A is also a ligand of DR3 and can enhance T-cell alloresponses via DR3 in vivo (5). TR6 strongly inhibits TL1A-

augmented T-cell proliferation and lymphokine secretion, and also inhibit TL1A-riggered tumor cell apoptosis (5).

TR6 mRNA and protein are detectable in some malignant tumors (15). The inhibition of apoptosis and repression of T cell costimulation obviously represent plausible mechanisms for TR6-secreting tumors to gain survival advantage by avoiding apoptosis and evading immune surveillance. In this study, we discovered yet another function of TR6 in the immune system. We demonstrated that TR6 inhibited T-cell chemotaxis both in vitro and in vivo, and such an effect was probably mediated by reverse signaling through LIGHT. A cascade of signaling and effector events such as Cdc42 activation, p38 mitogen-activated protein kinase (MAPK) activation, actin polymerization and pseudopodium formation in CXCL12/stromal cell-derived factor 1α (SDF-1α)-stimulated T cells was inhibited by TR6 pretreatment. The physiological significance of these findings is discussed.

MATERIALS AND METHODS

Recombinant proteins and mAb

The recombinant proteins, i.e., TR6 fused with Fc (TR6-Fc), HveA fused with Fc (HveA-Fc), and TR11/GITR fused with Fc (TR11-Fc), was described in our previous publication (1). Because TR11-Fc had no effect on T-cell migration, compared with PBS or normal human IgG, it was used as a control protein for TR6-Fc. The Fc sequence in the recombinant protein was mutated, so that it did not bind to FcR on the cell surface. The endotoxin levels in all the recombinant proteins were below 10 endotoxin units/mg

(1). Human mAb against LIGHT was described in our earlier publication (8).

Preparation and culture of human T cells and mouse spleen cells

Adult PBMC were prepared by Lymphoprep gradient (Nycomed, Oslo, Norway), and T cells were prepared from PBMC by negative selection (deletion of cells positive for CD11b, CD16, CD19, CD36 and CD56) with magnetic beads (Miltenyi, Auburn, CA) according to the manufacturer's instructions. Blood samples from different donors were used to prepare T cells for repeated experiments. Mouse total spleen cells were prepared by lysing RBC with 0.84% NH_4Cl among the cells flushed out from the spleen. The cells were cultured in serum-free medium.

In vitro T-cell migration assay

In vitro migration assays were performed in Transwell chambers (6.5-mm in diameter, 5 μm-pore size; Costar Corp., Cambridge, MA). The lower chamber contained 600 μl serum-free medium in the presence of CXCL12/SDF-1α (80 ng/ml) or CCL21 (400 ng/ml; both from R & D Systems, Minneapolis, MN). The upper chamber held 100 μl serum-free medium containing 0.3 x 10^6 T cells pretreated (16 h preincubation) with TR6-Fc, a control fusion protein, TR11-Fc (both at 20 μg/ml), or a p38 MAPK inhibitor, SB203580, or its nonfunctional structural analog, SB202474 (both at 5 μM). In some experiments, cross-linked human anti-LIGHT mAb was also used: the cells were preincubated with anti-LIGHT mAb (clone 1.2.2, 20 μg/ml) along with goat anti-human IgG (10 μg/ml). The Transwell ensemble was then incubated at 37^0C for 2 h, and T cells

migrating into the lower chamber were counted by flow cytometry. All the samples were in duplicate.

Flow cytometry

For F-actin staining, 1 x 10^6 T cells from PBMC were incubated with CXCL12/SDF-1α for 1 min at 37^0C, and then fixed with 3.7% formalin for 30 min at room temperature. The cells were stained with Alexa Fluor-488-conjugated phalloidin (Molecular Probes, Eugene, OR). For CXCR4 staining, T cells were first stained with biotinylated anti-human CXCR4 (clone 44716.111, R & D Systems), followed by streptavidin-conjugated Alexa Fluor-488 (Molecular Probes). For CD69 staining, T cells were stained with PE-conjugated anti-CD69 mAb (clone FN50, BD PharMingen, Mississauga, Ontario, Canada). Mouse peritoneal exodus cells (PEC), including CFSE (Molecular Probes)-labeled cells, were stained with PE-conjugated anti-CD3 mAb (clone 2C11). The cells were analyzed with a Coulter Epics-XL flow cytometer (Coulter, St.-Laurent, Quebec, Canada).

In vivo T-cell migration assay

BALB/c spleen cells were incubated overnight with serum-free medium containing TR6-Fc or TR11-Fc (both at 20 μg/ml). The cells were then labeled with CFSE (5 μM) for 10 min at room temperature. After washing, 6 x 10^7 labeled cells in 0.2 ml HBSS were injected i.v. into the tail vein of a BALB/c mouse, and 1 μg of CXCL12/SDF-1αin 2 ml PBS was injected i.p. 2 h later. The mice were sacrificed after another 20 h. Their peritoneal cavities were rinsed with 8 ml PBS, and PEC in PBS were analyzed by flow cytometry for total cell number, total $CD3^+$ cell number, and $CD3^+CSFE^+$ cell number.

For each experiment, three mice were used in each group, and three independent assays were performed.

Confocal microscopy

Human T cells were cultured overnight in the presence of TR6-Fc or a control protein-Fc (TR11-Fc, both at 20 μg/ml). These cells were then stimulated with CXCL12/SDF-1α (80 ng/ml) for 1 min at 37^0C, and fixed with 3.7% formalin for 30 min at room temperature. They were stained with Alexa Fluor-488-conjugated phalloidin (Molecular Probes) for 30 min on ice, and mounted on slides with Prolong Anti-fade Mounting Medium (Molecular Probes) for examination by confocal microscopy. Digital images were processed with Photoshop (Adobe, Seattle, WA).

Immunoblotting

Human T cells were cultured overnight in serum-free medium in 12-well plates at 5 x 10^6 cells/well in the presence of TR6-Fc or TR11-Fc (both at 20 μg/ml). After washing, the cells were stimulated with SDF-1α (80 ng/ml) in serum-free medium at 37°C for 10 min. The remainder of the immunoblotting procedure was detailed in our previous publication (16). Briefly, the cells were washed, and lysed in lysis buffer for 10 min; the cleared lysates were resolved by 10% SDS-PAGE. The proteins were then transferred to Immobilon-P (Millipore, Bedford, MA) membranes, which were sequentially hybridized with rabbit anti-phospho-p38 MAPK and anti-p38 MAPK Ab (New England Biolabs, Mississauga, Ontario, Canada). Immunoreactive protein bands were visualized by HRP-conjugated goat anti-rabbit IgG, followed by ECL.

Rac-1 and Cdc42 activity assays

Twenty-million T cells were preincubated overnight with TR6-Fc or TR11-Fc (both at 20 μg/ml). After washing, the cells were stimulated with CXCL12/SDF-1α (80 ng/ml) for 5 min at 37^{0}C. The cells were lysed, Rac-1 and Cdc42 activity in the lysates were measured by PAK-1 pull-down assays (Upstate Biotech, Lake Placid, NY), according to the manufacturer's instructions. Briefly, GTP-loaded active Rac-1 and Cdc42 were precipitated with PAK-1 PBD-conjugated agarose beads, and resolved in 12% SDS-PAGE. They were then detected with mouse mAb anti-Rac-1 and Cdc42, respectively in immunoblotting.

RESULTS

TR6 inhibits T cell chemotaxis in vitro

In the course of our study into TR6's effect on T cells, we noticed that TR6-treated T cells presented an altered morphology upon mitogen stimulation. This prompted us to examine whether T-cell mobility was affected by TR6. T cells from PBMC were pretreated with TR6-Fc or a control fusion protein TR11-Fc overnight in serum-free medium, and then loaded in the upper Transwell chamber. A chemokine CXCL12/SDF-1α, which is known to induce T-cell chemotaxis, was added to the bottom chamber at different concentrations. T cells were allowed to migrate for 2 h at 37^{0}C. As illustrated in Figure 1A, CXCL12/SDF-1α dose-dependently induced T-cell migration into the lower chamber. At 80 ng/ml CXCL12/SDF-1α, T cells precultured in plain medium or in the presence of TR11-Fc showed similar migration rates, but those precultured in the presence of TR6-Fc manifested a significantly reduced rate. Decreased migration of

TR6-precultured T cells was also observed with CXCL12/SDF-1α at 20 ng/ml and 5 ng/ml ($p<0.01$ for all the 3 concentrations, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test).

The reduced migration rate was not due to the toxic effects of TR6 pretreatment. The viability of TR6-Fc or TR11-Fc precultured T cells, according to trypan blue dye exclusion, was similarly between 90% and 95% (data not shown). To further prove this point, T cells precultured in the presence of TR6-Fc or TR11-Fc, or precultured in plain medium for 24 h, were washed, and restimulated with soluble anti-CD3 (0.2 μg/ml). After additional culture for 16 h, all the cells were gated and analyzed for their expression of a T cell activation marker CD69. As seen in Figure 1B, CD69 expression in all anti-CD3-stimulated groups was increased drastically, compared to cells without stimulation (medium (Med) 24-40h), but there was no statistical difference among cells pretreated with plain medium, TR11-Fc or TR6-Fc ($p>0.05$, one-way variance analysis followed by Tukey test for 2 by 2 comparison of all groups). This indicates that preculturing with TR6-Fc did not affect T-cell viability. Was the inhibited migration due to repression of CXCL12/SDF-1α receptor CXCR4 expression on TR6-Fc treated T cells? Approximately 40% of fresh T cells expressed low intensity CXCR4 (Figure 1C). After overnight culture in plain medium, $CXCR4^+$ cells increased to ~90% with a concomitant rise in CXCR4 intensity. T cells treated with either TR6-Fc or TR11-Fc showed similar up-regulation of CXCR4 as T cells cultured in plain medium Fc ($p>0.05$, one way variance analysis followed by Tukey test for 2 by 2 comparison of all groups),

indicating that TR6-Fc treatment did not affect CXCR4 expression. This result also confirms that T cell viability was not impaired by TR6-Fc pretreatment.

The effect of TR6 on T cell migration was likely mediated by LIGHT

TR6 can bind to FasL and LIGHT, both of which are expressed on T cells. Our recent study revealed that TR6 is capable of triggering reverse signaling through LIGHT and such an effect results in T-cell costimulation (8, 11). We investigated here whether the inhibition of T-cell migration by TR6 involved LIGHT. For this purpose, human anti-LIGHT mAb was crosslinked by goat anti-human IgG in solution (human anti-LIGHT mAb at 20 μg/ml and goat anti-human IgG at 10 μg/ml were added to the culture at the same time), and was used in place of TR6-Fc during preincubation. As shown in Figure 2A, crosslinked anti-LIGHT mAb pretreatment also suppressed T-cell migration in response to CXCL12/SDF-1α in the Transwell assay, compared with crosslinked normal human IgG as a control ($p<0.01$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test). This suggests that such an effect involves LIGHT.

To further prove the involvement of LIGHT, HveA, which binds to LIGHT but not to FasL (6), was used to pretreat the T cells. As shown in Figure 2B, when HveA-Fc was used in solution to pretreat the T cells, it could inhibit T-cell migration provoked by CXCL12/SDF-1α ($p<0.001$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test), similar to TR6-Fc. This suggests that LIGHT is implicated in the process.

To understand whether such inhibition was due to TR6-treggered reverse signaling through LIGHT, or due to disruption of existing interaction between LIGHT and HveA, we used solid phase anti-LIGHT mAb, TR6-Fc or HveA-Fc to condition the T cells. As shown in Figure 2C, when T cells were cultured in well coated with these reagents, their subsequent migration towards CXCL12/SDF-1α in the Transwell assay were significantly reduced ($p<0.01$ for all three reagents, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test)

The amount of reagents coated on the wells was minute, and there were unlikely sufficient molecules to leak from solid phase into solution to block the LIGHT and HveA interaction. This was proved as follows. Media were put in wells coated with TR6-Fc, HveA-Fc, αLIGHT mAb or control normal human IgG for 24 h, and these conditioned media were then used to culture T cells overnight. However, as shown in Figure 2D, T cells cultured in these conditioned media had no difference in their migration capability towards CXCL12/SDF-1α ($p>0.001$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test). This confirms that the inhibitory effect seen with solid phase TR6-Fc, HveA-Fc and anti-LIGHT mAb is not due to leaching of these molecules from the solid phase and suggests that that TR6 triggers reverse signaling through LIGHT on the T-cell surface, and results in compromised T-cell migration.

The observed migration inhibition by TR6-Fc and HveA-Fc was not due to contaminating endotoxin in the protein preparation, because their inhibitory effect could

be destroyed by 5-min boiling (Figure 2B; $p>0.05$ for boiled TR6-Fc vs. medium and for boiled HveA-Fc vs medium, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test). To assess whether TR6 affected the general mobility of T cells and the observed effect was only restricted to CXCL12/SDF-1α stimulation, we tested the T-cell response to another chemokine, CCL21. After incubation with TR6-Fc but not with a control fusion protein (TR11-Fc), T cells showed a significantly reduced migration rate towards CCL21 in the Transwell assay (Figure 2E; $p<0.01$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test). Therefore, it seems that TR6 pretreatment inhibited the general mobility of T cells.

TR6 inhibits T-cell chemotaxis in vivo

To confirm that the observed inhibitory effect of TR6 on T-cell migration in vitro had physiological relevance, we established an in vivo T-cell chemotaxis model. BALB/c spleen cells were first precultured overnight in the presence of TR6-Fc or TR11-Fc, and then labeled with CFSE. The labeled cells were injected i.v. into syngeneic BALB/c mice. Two hours later, CXCL12/SDF-1α was injected i.p. After another 20 h, PEC were analyzed for their total number, total T-cell number, total CFSE-labeled T-cell number, and percentage of CFSE-labeled T cells among total T cells. As shown in Fig 3, CXCL12/SDF-1α injection into the peritoneal cavities resulted in a drastic increase of total PEC and T cells in PEC (Figures 3A and 3B). There was no significant difference in these parameters in mice that received TR6-Fc-, TR11-Fc- or plain medium-precultured cells ($p>0.05$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test), reflecting the fact that the majority of the exodus cells were

of host origin (as CFSE-labeled donor cells showed difference, to be elaborated below). When CFSE-labeled exodus T cells were analyzed, there was a significant decrease in their number in mice receiving cells pretreated with TR6-Fc compared with mice receiving cells pretreated with TR11-Fc or plain medium (Figure 3C; $p<0.001$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test). Such a reduction in T-cell number was also reflected in the percentage abatement of CFSE-labeled T cells among total exodus T cells (Figure 3D; $p<0.001$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test), because the number of the latter remained similar in mice receiving spleen cells with the different treatments (Figure 3B). These data showed the in vivo relevance of compromised T-cell chemotaxis after exposure to TR6.

TR6 pretreatment of T cells represses their actin polymerization upon CXCL12/SDF-1a stimulation

T-cell chemotaxis depends on cytoskeleton reorganization, for which actin polymerization plays an essential role. We, therefore, examined actin polymerization upon SDF-1α stimulation in T cells with or without TR6 pretreatment. The F-actin staining of T cells precultured in plain medium without subsequent CXCL12/SDF-1α stimulation was considered as background (0% positive, Figure 4A). When such cells were stimulated by CXCL12/SDF-1α for 1 min, 65.9% of them became F-actin positive. Preculturing the cells in the presence of TR11-Fc overnight had no apparent effect on actin polymerization upon subsequent CXCL12/SDF-1α stimulation, and 65% of the cells were positive for F-actin staining. However, overnight TR6-Fc pretreatment

significantly reduced F-actin-positive cells to 29.4% ($p<0.01$, one way variance analysis followed by Tukey test for 2 by 2 comparison of all groups). This result shows that TR6 conditioning of T cells leads to compromised actin polymerization.

Consistent with the status of actin polymerization, CXCL12/SDF-1α rapidly (with in 1 min) induced pseudopodium formation in T cells (Fig. 4B, comparing upper left panel with upper right panel; quantitative assessment of Fig. 4B is shown as a bar graph in Figure 4C). T cells cultured overnight in medium or TR11-Fc had similar degree of pseudopodium formation upon CXCL12/SDF-1α stimulation (Fig, 4B, upper right panel vs lower left panel), but TR6-Fc pretreatment of the cells significantly inhibited this process (lower left panel versus lower right panel; $p<0.001$, one-way ANOVA variance analysis followed by Tukey multiple-comparisons test). Thus, it appears that T cells receiving TR6 preconditioning have modified signaling pathways that lead to compromised actin polymerization, pseudopodium formation, and, eventually, chemotaxis.

TR6 pretreatment of T cells compromises p38 MAPK activation

p38 MAPK is an upstream signaling molecule essential for actin polymerization (17). Its activity in TR6-treated T cells was investigated. T cells were precultured overnight in the absence (Med) or presence of TR6-Fc or TR11-Fc. The cells were washed and stimulated with 80 ng/ml CXCL12/SDF-1α for 10 min. p38 MAPK tyrosine phosphorylation, which represents p38 MAPK activation, and total p38 MAPK in these cells were assessed by immunoblotting. As shown in Fig. 5A, CXCL12/SDF-1α

stimulation resulted in similar up-regulation of p38 MAPK tyrosine phosphorylation in T cells precultured in plain medium or in the presence of TR11-Fc. However, preculturing in the presence of TR6-Fc prevented such up-regulation.

To ascertain that p38 MAPK activation was a relevant event that controlled T-cell chemotaxis upon CXCL12/SDF-1α stimulation, T cells were treated with a p38 MAPK-specific inhibitor, SB20358 (5 μM), for 2 h and then evaluated for their chemotaxis by the Transwell assay. The p38 MAPK inhibitor but not its nonfunctional structural analog, SB202474, suppressed the T-cell chemotaxis upon CXCL12/SDF-1α stimulation (Fig. 5B), as expected, indicating that the compromised p38 MAPK activation in TR6-treated T cells is relevant and responsible for T-cell chemotaxis inhibition.

Compromised Cdc42 activation in T cells pretreated with TR6-Fc

Small G proteins Rac-1 and Cdc42, which are signaling molecules upstream of p38 MAPK, are important in cytoskeleton reorganization, which in turn controls pseudopodium formation and cell mobility (18). The activity of Rac-1 and Cdc42 of TR6-treated T cells was examined using PAK-1 pull-down assay. T cells were preincubated overnight in the presence of TR6-Fc or TR-11-Fc, and then stimulated with CXCL12/SDF-1α for 5 min. The activated GTP-associated Rac-1 or Cdc42 was pulled down using solid phase (agarose beads) PAK-1, which is a substrate of and binds to GTP-associated Rac-1 and Cdc42. As shown in Fig. 6, Rac-1 activation, which was reflected by the levels of GTP-associated Rac-1, was not modulated by SDF-1

stimulation; TR11-Fc or TR6-Fc pretreatment did not affect its activity. In contrast, Cdc42 was rapidly activated by CXCL12/SDF-1α; TR6-Fc but not control TR11-Fc pretreatment prevented its activation. The selective inhibition of Cdc42 activation, which is important in filopodium formation (18), by TR6 thus represents a plausible mechanism for the repressive effect of TR6 on T-cell chemotaxis.

DISCUSSION

In this study, we showed for the first time that TR6 could inhibit T-cell chemotaxis both in vitro and in vivo. Mechanistically, such an effect was likely mediated by reverse signaling from TR6 to LIGHT on the T-cell surface. T cells treated with TR6 failed to mobilize the signaling pathway leading from Cdc42 to p38 MAPK to actin polymerization, upon encountering CXCL12/SDF-1α.

There are two possible mechanisms to explain the T-cell migration inhibition caused by TR6. In the first possible mechanism, constitutive interaction between LIGHT and HveA on T cells promotes T-cell mobility; soluble TR6-Fc, anti-LIGHT mAb or HveA-Fc interferes with such interaction, and leads to inhibited T-cell mobility. To examine plausibility of this mechanism, we coated TR6-Fc, anti-LIGHT mAb or HveA-Fc on solid phase. In this case, only a minute amount of those molecules were actually adsorbed to the plate surface, and there were unlikely sufficient molecules leaking into the solution to block the LIGHT and HveA interaction (indeed, media conditioned in TR6-Fc-, anti-LIGHT mAb- or HveA-Fc-coated wells had no effect on T-cell migration). Moreover, these molecules fixed on a flat surface would probably not be

able to interfere with the three dimensional interaction between LIGHT and HveA on the cell surface. We showed that those solid phase molecules could still inhibit T-cell chemotaxis, suggesting that this first mechanism does not seem plausible.

The second possible mechanism dictates that TR6 triggers a negative signal reversely transmitted through a TNF family member(s) on the cell surface, most likely through LIGHT, and such signaling results in reduced T-cell mobility. There are a couple of lines of evidence supporting this mechanism. First, we have recently proved that TR6 can transduce costimulation signals reversely via LIGHT into T cells (8, 11); such reverse signaling is not entirely out of norm and has been reported for several other TNF family members, e.g., CD40L (19), CD30L (20), TNF-α (21), TRANCE (22), FasL (23) and TRAIL (24). Second, when TR6-Fc, anti-LIGHT mAb or HveA-Fc was put on solid phase, they could also inhibit T-cell chemotaxis, as described above, likely by crosslinking their binding partners on the T-cell surface. These data strongly suggest that the second mechanism is in operation. If so, how do we explain that TR6-Fc, HveA-Fc and anti-LIGHT mAb in solution could inhibit T-cell chemotaxis? It is possible that a low-degree of crosslinking by the bivalent TR6-Fc or HveA-Fc, or their aggregates, is enough to trigger reverse signaling. For the anti-LIGHT mAb in solution, it was only effective after being crosslinked by anti-human IgG, and without crosslinking, the mAb was not effective in solution (data not shown), suggesting the necessity of LIGHT crosslinking for the T-cell migration inhibition.

TR6 could in theory reversely signal through LIGHT or FasL to achieve its effect in chemotaxis inhibition. Which one mediates such an effect of TR6? Our following observation is worth mentioning in this regard. 1) HveA-Fc, which binds to LIGHT but not FasL, had a similar effect as TR6-Fc (Fig. 2, B and C), suggesting that the effect could be achieved through LIGHT without FasL. 2) Anti-LIGHT mAb could inhibit T-cell migration (Fig. 2, A and C), further suggesting that LIGHT mediated the effect. 3) LIGHT is expressed on resting T cells as we demonstrated before (8), and this provides a basis for TR6 to act on resting T cells. 4) The binding of TR6-Fc to T cells was predominately through LIGHT, because TR6-Fc could associated with > 80 % wild-type T cells, and only with < 20% LIGHT$^{-/-}$ T cells (data not shown). Taking together, these data suggest that LIGHT but not FasL is the main mediator for the observed effect of TR6 in chemotaxis inhibition. With that said, we cannot totally exclude the possibility that reverse signaling through FasL, or maybe some so far unidentified TR6-binding partner on the T-cell surface, contribute to a lesser extent to the observed T-cell chemotaxis inhibition by TR6. In this regard, it is worth mentioning that soluble TR6 have been shown to suppress soluble FasL-induced chemotaxis of microglial cells in Transwell assay (25). In that system, the proposed mechanism is the blockage of forward signaling from FasL to Fas on microglial cell surface by soluble TR6. However, it will be interesting to know whether such inhibition is the result of the general decrease of cell mobility by TR6 treatment.

We have identified signaling alternation in T cells treated with TR6. Selective change was revealed in one of the small GTPases, Cdc42, known to be essential in filopodium

formation (18). This was followed by inability of p38 MAPK activation, which is essential for actin polymerization. Largely remaining unknown is how signals are initiated from LIGHT in the first place. LIGHT has a short featureless cytoplasmic tail (6), and certainly has no signaling capability by itself. It is reasonable to hypothesize that signaling from LIGHT depends on the adaptor molecules it associates with and such a hypothesis is under investigation.

TR6 is secreted by several kinds of tumors (15). In addition to its effect of blocking Fas-mediated apoptosis of tumor cells (15) and interfering with the two-way T cell costimulation mediated by HveA and LIGHT, TR6 now has a newly found function: inhibition of T-cell chemotaxis. Consistent with this finding, in the rat glioma with forced expression of exogenous TR6, tumor-infiltrating T cells are significantly reduced compared with tumors without TR6 expression (25); our additional study discovered that serum TR6 levels of gastric cancer patients were inversely correlated to degree of lymphocyte infiltration in their tumor mass (data not shown). Therefore, T-cell chemotaxis inhibition seems to be an additional strategy TR6-secreting tumors employ to evade immune surveillance by keeping potential tumor-infiltrating T cells at bay.

Although tumors might deploy TR6 to promote their survival, TR6 could also be used for a good cause. We previously demonstrated, in a mouse heart transplantation model, that TR6 administration results in prolonged heart allograft survival (1). Such a beneficial effect was initially explained by inhibition of LIGHT to HveA forward costimulation (1), and later by inhibition of the two-way costimulation between HveA

and LIGHT on T cells by TR6 (1,8). Obviously, our current finding provides an additional mechanism to explain the prolonged graft survival. Under the influence of TR6, alloresponsive T cells have reduced chemotaxis towards the allograft or its draining lymphoid organs and this will repress T-cell activation, which eventually translates into reduced graft rejection force. Such a property of TR6 could be exploited in the future to repress local or systemic immune responses.

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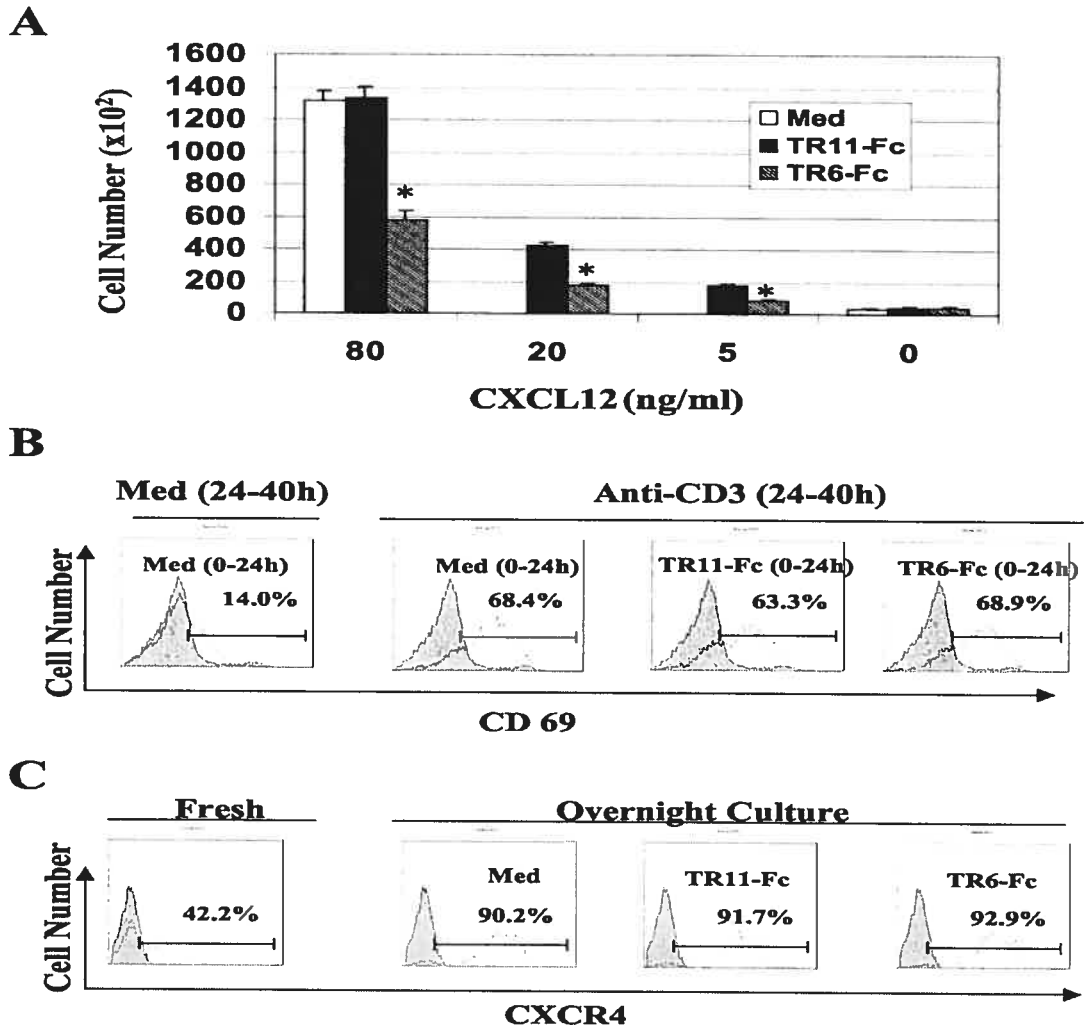
FIGURE LEGENDS

Figure 1. TR6-Fc inhibits human T-cell chemotaxis in vitro



A. T cells pretreated with TR6-Fc showed compromised chemotaxis towards SDF-1α

Human T cells were preincubated overnight in the absence (Med) or presence of soluble TR6-Fc or a control fusion protein TR11-Fc (both at 20 μg/ml) in serum-free medium. The cells were then loaded in the upper Transwell chambers (0.3 x 10^6/well), with the lower chamber containing different concentrations of CXCL12/SDF-1α. After a 2-h

incubation at 37°C, cells in the lower chamber were collected and counted by flow cytometry. Samples were in duplicate in each experiment. Means ± SD of four measurements from two independent experiments are shown. The asterisks indicate highly significant differences ($p < 0.01$, one-way ANOVA followed by Tukey's multiple comparisons test) between TR6-Fc- and control TR11-Fc-treated samples.

B. T cells pretreated with TR6-Fc can be normally activated by mitogens

T cells were pretreated with medium (Med), TR11-Fc, or TR6-Fc for 24 h and then stimulated overnight further (24–40 h) with or without soluble anti-CD3 (0.2 μg/ml), as indicated. At 40 h after the initiation of culture, the expression of a T cell activation marker, CD69, was analyzed with flow cytometry. The experiments were performed three times, and histograms of a representative experiment with percentages of CD69-positive T cells are shown. The percentage of CD69-positive cells in the *second*, *third*, and *fourth panels* has no statistical difference according to pooled data from three independent experiments ($p > 0.05$, one-way ANOVA analysis followed by Tukey's test for 2 x 2 comparison on all groups).

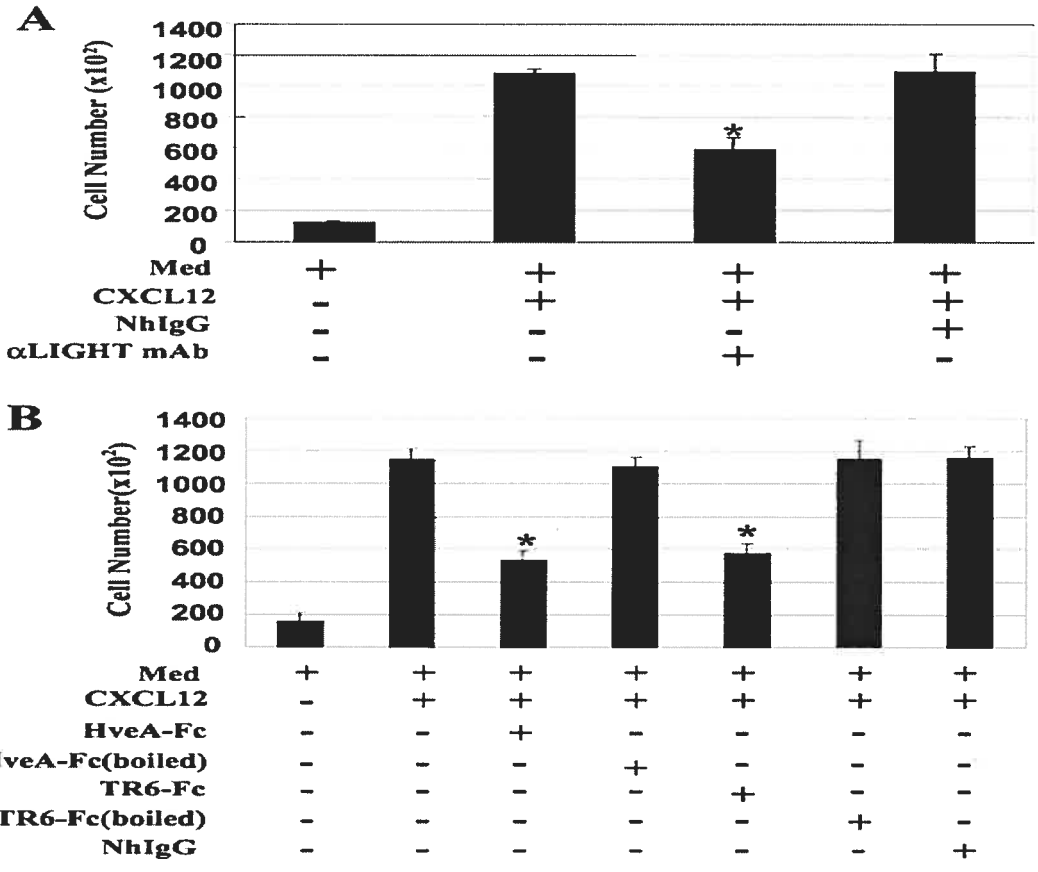
C. TR6 pretreatment does not affect the expression of the CXCL12/SDF-1α receptor CXCR4

Fresh T cells or T cells cultured in the absence (Med) or presence of TR11-Fc or TR6-Fc overnight were stained with FITC-conjugated anti-CXCR4 mAb. Percentages of CXCR4-positive cells are indicated. The experiments were performed three times and a representative set of data is shown. The percentage of CXCR4-positive cells in *second*,

third, and *fourth panels* has no statistical difference according to pooled data from three independent experiments ($p > 0.05$, one-way ANOVA followed by Tukey's test for 2 x 2 comparisons on all groups).

Figure 2. Chemotaxis inhibition by TR6 is mediated by LIGHT and is not specific to CXCL12/SDF-1α

Samples were in duplicate in each experiment and the experiment was performed twice. Means ± SD of four measurements from two independent experiments are shown. The asterisks indicate highly significant differences ($p < 0.01$, one-way ANOVA followed by Tukey's multiple comparisons test) between the test group (treated with anti-LIGHT mAb, HveA-Fc, or TR6-Fc) and control normal human IgG (NhIgG)-treated samples.



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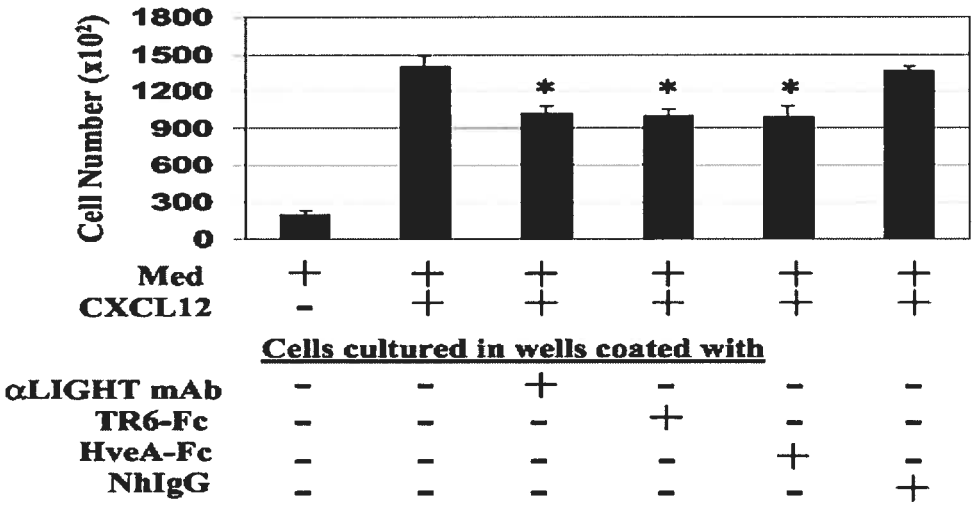
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1200
900
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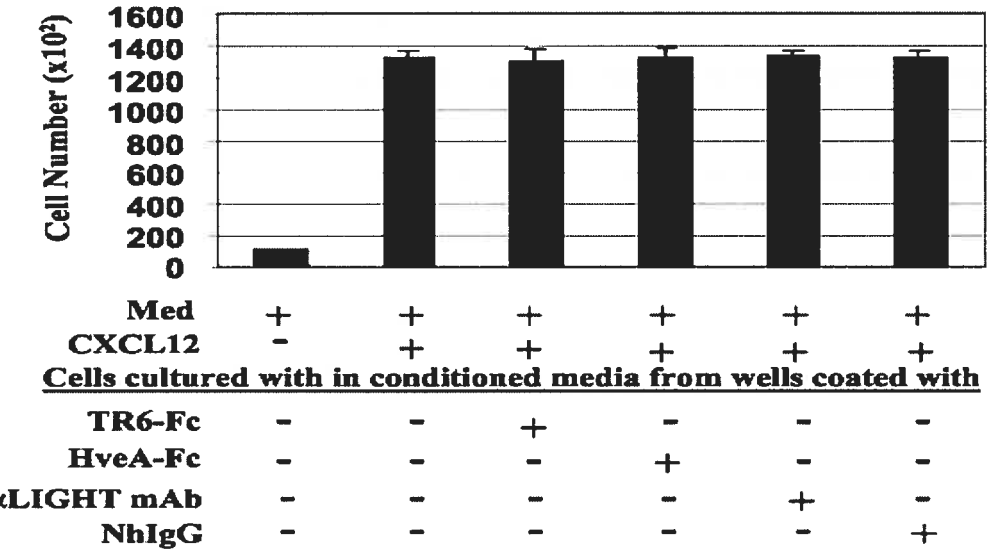
αLIGHT mAb - - + - - -
TR6-Fc - - - + - -
HveA-Fc - - - - + -
NhIgG - - - - - +

D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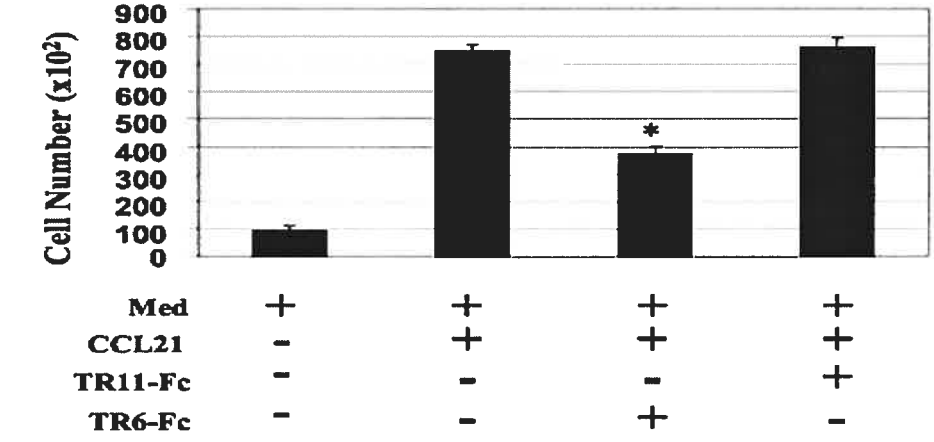
HveA-Fc - - - + - -
αLIGHT mAb - - - - + -
NhIgG - - - - - +

E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TR6-Fc - - + -

A. *Crosslinked anti-LIGHT mAb in solution inhibits T-cell chemotaxis*

T cells were cultured overnight in the absence (Med) or presence of human anti-LIGHT mAb (20 μg/ml) plus goat anti-human IgG (10 μg/ml), and chemotaxis of these cells toward CXCL12/SDF-1α (80 ng/ml) was tested by the Transwell assay as described in Fig. 1*A*. Normal human IgG was similarly cross-linked by goat anti-human IgG and was used as control.

B. *Soluble HveA-Fc inhibits T-cell chemotaxis similar to TR6-Fc, and these proteins lost their function after heat inactivation*

T cells were cultured overnight in the absence or presence of soluble HveA-Fc or TR6-Fc (both at 20 μg/ml). In some samples, HveA-Fc and TR6-Fc were heat inactivated for 5 min at 100°C before use. Chemotaxis of the treated T cells toward CXCL12/SDF-1α (80 ng/ml) was assayed by the Transwell assay..

C. Solid phase TR6-Fc, HveA-Fc and anti-LIGHT mAb inhibit T-cell Chemotaxis

TR6-Fc, HveA-Fc, anti-LIGHT mAb or normal human IgG were coated on wells at 1 μg/100 μl/well overnight at 4°C and the wells were then washed. T cells were cultured in these precoated wells overnight and then used in the Transwell assay using CXCL12/SDF-1α (80 ng/ml) in the lower chamber.

D. *Media conditioned in TR6-Fc-, HveA-Fc, or anti-LIGHT mAb-coated wells had no effect in T cell migration toward CXCL12/SDF-1α.*

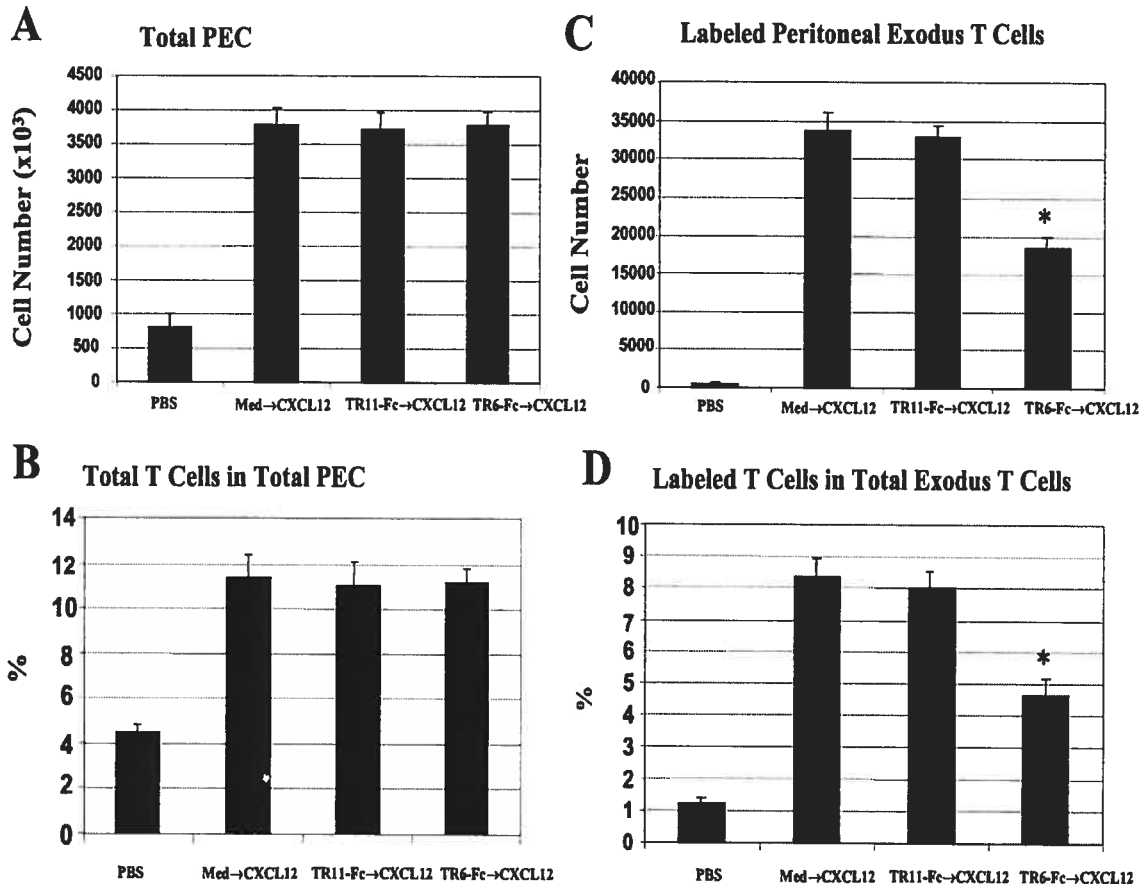
Complete medium was incubated in TR6-Fc-, HveA-Fc-, or anti-LIGHT mAb-coated wells for 24 h, The media were harvested and used to culture T cells in uncoated wells

overnight. The cells were then used in Transwell assay using CXCL12/SDF-1α (80 ng/ml) in the lower chamber.

E, TR6-Fc preculturing inhibits T cell chemotaxis toward CCL21.

T cells were cultured overnight in the absence (Med) or presence of soluble TR6-Fc or TR11-Fc (both at 20 μg/ml) as indicated. Chemotaxis of these cells toward CCL21 (400 ng/ml) in the lower chamber was examined by the Transwell assay as described in Fig. 1*A*.

Figure 3. TR6-Fc inhibits mouse T cell chemotaxis in vivo

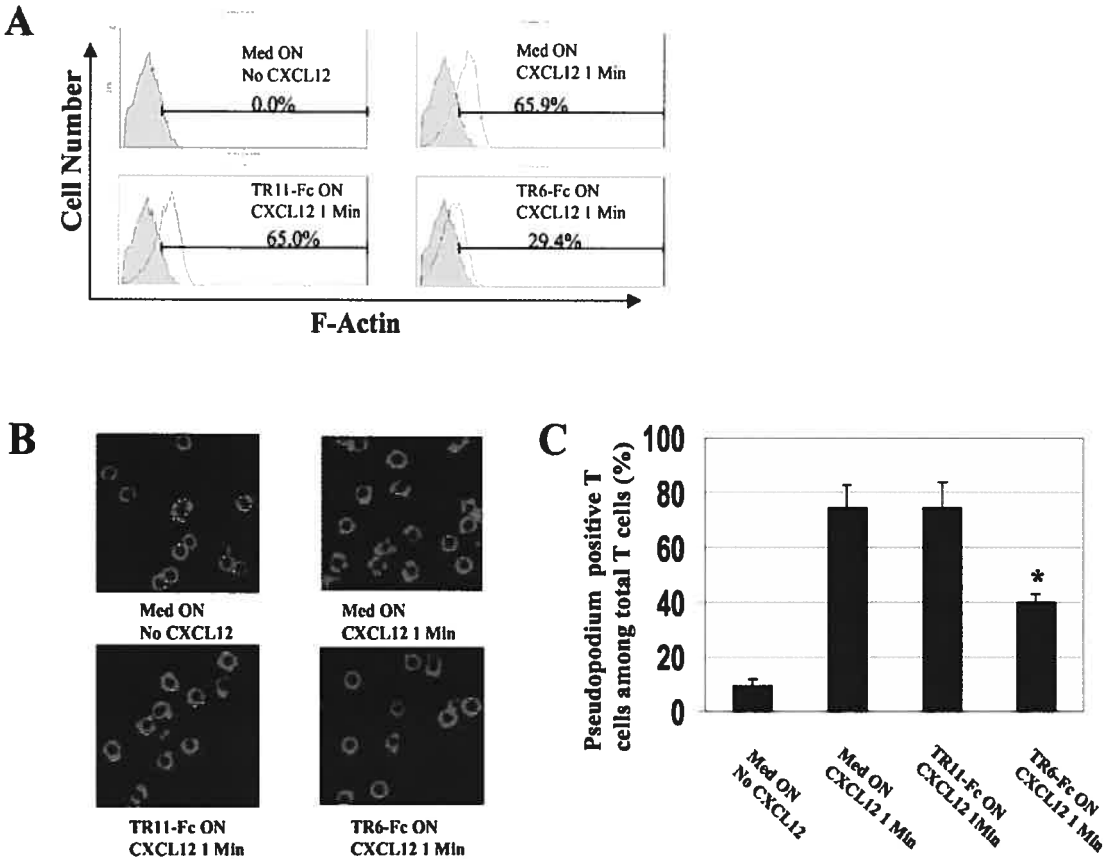


BALB/c spleen cells were cultured overnight in medium (Med) or in the presence of TR6-Fc or TR11-Fc (both at 20 μg/ml). The cells were then labeled with CFSE for 10 min at room temperature and injected i.v. into BALB/c mice. After 2 h, 1 μg of CXCL12/SDF-1α was administered i.p. The mice were sacrificed after another 20 h, and their total PEC (*A*), the percentages of total T cells among total PEC (*B*), CFSE-labeled peritoneal exudate T cells (*C*), and percentages of CFSE-labeled T cells among total peritoneal exudate T cells (*D*) were analyzed. Each group consisted of three mice; similar results were obtained from three independent experiments; and a set of representative data is presented. PBS: mice receiving CSFE-labeled spleen cells cultured in medium alone without subsequent CXCL12/SDF-1α but with PBS administration; Med→CXCL12: mice receiving CFSE-labeled spleen cells precultured in medium, followed by CXCL12 injection (1 μg/mouse) i.p; TR11-Fc→CXCL12: mice receiving CFSE-labeled spleen cells precultured in the presence of soluble TR11-Fc (20 μg/ml), followed by CXCL21 injection i.p.; TR6-Fc→CXCL21: mice receiving CFSE-labeled spleen cells precultured in the presence of TR6-Fc (20 μg/ml), followed by CXCL12 injection i.p.

Figure 4. TR6-Fc inhibits CXCL12/SDF-1α-induced T-cell actin polymerization and pseudopodium formation

Human T cells were cultured overnight in the absence (Med overnight (ON)) or presence of TR11-Fc (TR11-Fc ON) or TR6-Fc (TR6-Fc ON) (both at 20 μg/ml). After washing, the cells were incubated with CXCL21/SDF-1α (80 ng/ml) for 1 min, stained with Alexa

Fluor-488-conjugated phalloidin and analyzed by flow cytometry as well as confocal microscopy.



A. Flow cytometry analysis of F-actin

F-actin staining of T cells cultured overnight in medium without subsequent CXCL12/SDF-1α simulation was used as a negative control, with its F-actin intensity set at 0%. Percentages of F-actin-positive cells among cells with different treatments, as indicated, are shown. The experiments were performed three times and a set of representative histograms are presented. The percentage of F-actin-positive cells in *panel 4* is significantly lower than that of *panels 2* and *3* according to pooled data from the three independent experiments ($p < 0.01$, one-way ANOVA followed by Tukey's test for 2 x 2 comparisons on all groups).

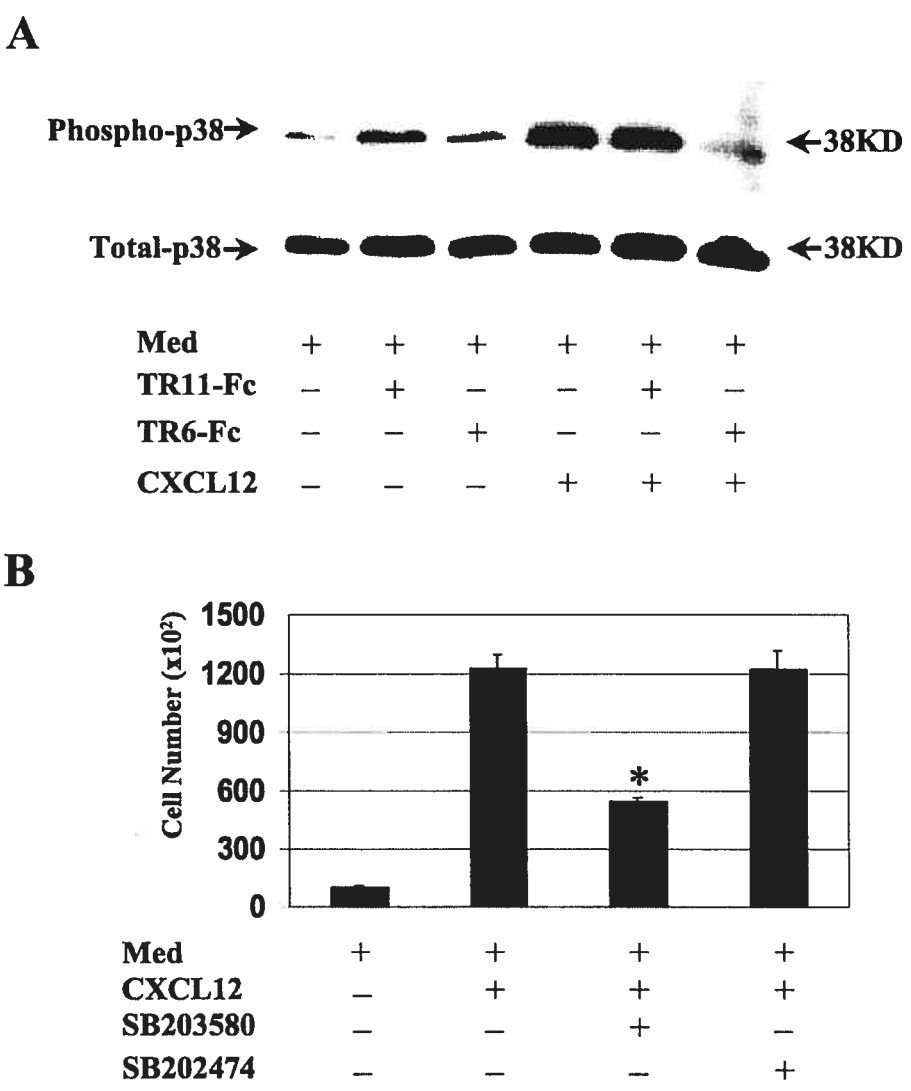
B. Confocal microscopy of T-cell actin polymerization and morphology

The same set of T cells as described in *A* was examined with confocal microscopy. The pseudopodium formation in cells precultured in medium or TR11-Fc was similar upon SDF-1 α stimulation (*upper right panel* vs *lower left panel*, respectively), but preculturing cell in the presence of TR6-Fc reduced pseudopodium formation compared with cell pretreated with TR11-Fc (*lower left panel* vs *lower right panel*). The experiments were performed three times and a set of representative data is presented.

C. Quantitative assessment of T-cell pseudopodium formation

The cells in *B* were quantified for pseudopodium-positive cells. Three view fields (containing ~100 cells/field) per sample were examined, and the means ± SD of the percentages of pseudopodium-positive cells among total cells are indicated. The asterisk indicates a highly significant difference between the test group and the TR11-Fc control ($p < 0.01$, one-way ANOVA followed by Tukey's test for 2 x 2 comparison on all groups). The experiment was performed twice and pooled data from the two independent experiments are shown.

Figure 5. Compromise of p38 MAPK activation by TR6 treatment resulted in chemotaxis inhibition



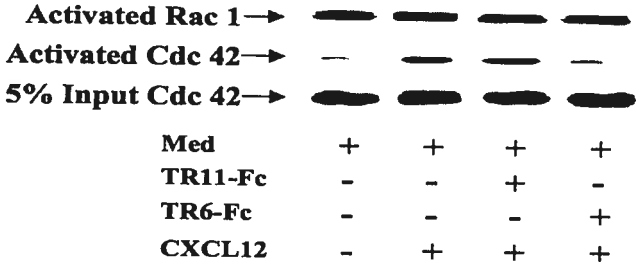
A. Immunoblotting of activated and total p38 MAPK

Human T cells were cultured overnight in the absence (Med) or presence of TR11-Fc or TR6-Fc (both at 20 μg/ml). After washing, the cells were stimulated for 10 min with CXCL12/SDF-1α (80 ng/ml). The cell lysates were resolved in 10% SDS-PAGE and blotted membranes were sequentially hybridized with anti-phospho-p38 MAPK and anti-total p38 MAPK. Arrows indicate 38-kDa phospho-p38 MAPK and total p38 MAPK. The experiments were performed three times and a set of representative data is presented.

B. p38 MAPK inhibitor represses CXCL12/SDF-1α-induced T-cell chemotaxis in Transwell assay

Human T cells were cultured in serum-free medium for 2 h in the presence of 5 μM p38 MAPK inhibitor SB203580 or its nonfunctional structural analog SB202474. After washing, the Transwell assay was performed as described in Fig. 1*A*.

Figure 6. TR6 inhibits Cdc42 activation in T cells



T cells were precultured overnight in serum-free medium or in the presence of TR6-Fc or a control fusion protein TR11-Fc (both at 20 μg/ml) and were then stimulated with CXCL12/SDF-1α (80 ng/ml) for 5 min. The activated GTP-binding Rac-1 and Cdc42 was absorbed by PAK-1 PBD-conjugated agarose beads and resolved in 12% SDS-PAGE. Rac-1 and Cdc42 were then detected with anti-Rac-1 and Cdc42 mAbs, respectively, in immunoblotting. Five percent of the input lysates were examined to confirm similar levels of total Cdc42 in each sample. The experiments were performed three times and a set of representative data is presented.

Article 3.

Tumor vaccine based on cell surface expression of DcR3/TR6

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To be published in the Journal of Immunology; it is undergoing the second revision to address two remaining concerns of one reviewer.

Note: In this paper, Guang Yu performed *in vitro* T cell costimulation assays, which were presented as Figure 2B. Jun Zhang supplied valuable reagents, such as LIGHT and TR6-Fc recombinant protein, anti-TR6 mAb, and $LIGHT^{-/-}$ mice. Guixiu Shi carried out all the remaining experiments.

Tumor vaccine based on cell surface expression of DcR3/TR6[1]

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Running title: Cell surface DcR3/TR6 for tumor vaccine

Footnote

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2. The current address of JZ: Pfizer Inc. 700 Chesterfield Parkway W. Chesterfield, MO.

ABSTRACT

DcR3/TR6, a secreted protein belonging to the TNF receptor superfamily, interacts with LIGHT, FasL and TL1A, all members of the TNF superfamily. Solid phase TR6 can trigger reverse signaling of LIGHT and FasL expressed on T cells, and lead to T-cell costimulation. In this study, we engineered tumor cells to express cell surface TR6, and used these cells as a tumor vaccine. We demonstrated that mastocytoma P815 cells expressing surface TR6 (TR6-P815) effectively augmented the T-cells response in vitro and ex vivo in terms of proliferation, as well as IL-2 and INF-γ secretion. TR6-P815 cells had reduced tumorigenicity compared with parental P815 cells. When inactivated TR6-P815 cells were employed as a vaccine, they protected the mice from challenge with live parental P815 cells, and eliminated established P815 tumors. The cell surface TR6-based tumor vaccine was also effective against low antigenicity tumors, such as B16 melanoma; co-administration of Bacillus Calmette Guerin further enhanced the vaccine's efficacy. Thus, cell surface TR6 expression is a useful addition to our tumor vaccine arsenal.

INTRODUCTION

DcR3/TR6 belongs to the TNF receptor superfamily (1-3). It is a secreted protein due to the lack of transmembrane domain in its coding sequence (3). TR6 has 3 ligands, i.e., FasL, LIGHT and TL1A. As a secreted protein, it does not transduce signals into cells, although it is a member of the TNF receptor family, but is capable of interfering with the function of its ligands: TR6 can inhibit FasL-, LIGHT- and TL1A-induced apoptosis (3-5), and LIGHT-triggered T-cell costimulation via a LIGHT receptor, HveA.

LIGHT, which belongs to the TNF superfamily, It binds to three members of the TNF receptor family, i.e., HveA (6,7), LTβR (8,9) and TR6 (3). In the immune system, LIGHT is expressed on activated T lymphocytes, natural killer cells (10,11), and immature dendritic cells (12). We have demonstrated that resting T cells also express a considerable amount of LIGHT on their surface, but it is better detected by confocal microscopy than by flow cytometry (13). LIGHT can induce apoptosis in cells expressing both HveA and LTβR (3), or LTβR alone (14). Recent studies have shown that LIGHT can costimulate T-cell responses via its receptor HveA in vitro and in vivo (15). Transgenic mice overexpressing LIGHT have augmented immune responses (16), and LIGHT knockout (KO) mice present defects in cytotoxic T-cell activity (15,17,18). Taken together, these lines of evidence indicate that LIGHT acts on HveA for T-cell costimulation.

Certain cell surface ligands can receive stimuli from their receptors, and transduce

signals into the cell. Such a phenomenon is termed "reverse signalling", as in this case the ligands function as receptors, while the receptors function as ligands. Some members of the TNF superfamily are capable of reverse signalling. Lanier (19) and Gray (20) have demonstrated that CD40L transduces costimulation signals into T cells. Wiley (21) reported that CD30L crosslinking activates neutrophils, and Cerutti (22) showed that such reverse signalling inhibits Ig class switch in B cells. Reverse signalling through membrane TNF-α confers resistance of monocytes and macrophages to LPS (23). Crosslinking of TRANCE enhances IFN-γ secretion by activated Th1 cells (24). Reverse signalling through FasL can promote maximal proliferation of CD8 cytotoxic T cells (25-27). Crosslinking of TRAIL by its solid phase death receptor 4 increases IFN-γ production and T-cell proliferation (28). In the case of CD40L, its ligation results in general protein tyrosine phosphorylation, Ca^{++} influx, and Lck, PKC, JNK and p38 MAPK activation in EL4 thyoma cells (29,30). TRAIL crosslinking also induces p38 MAPK activation (28). Our studies proved that LIGHT can reversely transduce signals into T cells when stimulated with solid phase TR6, and such signalling can costimulate T cells (13,31). With these new findings on LIGHT reverse signaling, the results from LIGHT transgenic and knockout mice can be reinterpreted. Increased LIGHT reverse signaling might contribute to the augmented immune responses observed in LIGHT transgenic mice (16); conversely, elimination of such reverse signaling might contribute to the abated immune responses seen in LIGHT knockout mice (15,17,18). Such reinterpretation does not refute the importance of forward LIGHT costimulation mediated by HveA.

Most tumors express unique antigens due to genetic alterations, or express tissue-specific antigens and/or developmental antigens owing to epigenetic effects. However, tumor cells have strategies to evade immune surveillance: down-modulating antigen processing and presentation to T cells; secreting soluble factors (e.g., cytokines and decoy receptors) to dampen or deviate immune responses; inducing T-cell tolerance through multiple pathways (32-38). Several experimental approaches have been employed to enhance the immunogenicity of tumors and to break T-cell tolerance in tumor-bearing animals or humans (38,39). One such approach is to express, on tumor cells, costimulatory molecules, such as B7-1 (40,41), B7H (42), 4-1BBL (43), or anti-4-1BB Ig heavy chain (44). Costimulatory molecules have also been coexpressed on tumor cells with other immune enhancing factors, for example, B7-1 plus IL-2 (45), B7-1 plus CD2 ligand (46), B7-1 plus B7-2 plus 4-1BB (47); this strategy can even work for tumors with low antigenicity (43,48-50). Animals inoculated with such manipulated tumor cells show retarded tumor growth and prolonged survival, compared with those receiving wild type tumor cells (40,42,43,51-53). When such tumor cells are used as vaccines, they can generally protect mice from the challenge of parental wild type tumors. In a limited number of cases, when animals are vaccinated with tumors expressing anti-4-1BB mAb single chain (44), or inoculated with double recombinant adenovirus expressing B7-1 and IL-2 in the tumor mass (45), the existing tumors manifest significant regression. These findings imply that although therapeutic vaccination might not be effective with all costimulating molecules, it does have in certain cases therapeutic effects. This is important in clinical situations, in which tumor burden inevitably already exists.

We have reasoned that it is advantageous to use membrane-bound TR6 to enhance T-cell costimulation and augment tumor antigenicity because, in theory, solid phase TR6 can act on at least two reverse signaling pathways, i.e., via LIGHT and FasL, to achieve T-cell costimulation. In this study, we engineered the surface expression of normally soluble TR6 on tumor cells and explored their usefulness as a tumor vaccine.

MATERIALS AND METHODS

Mice and cell lines

Six- to eight-week-old female DBA/2 and C57BL/6 mice were purchased from Charles River (St-Constant, Montreal, Quebec, Canada). The murine mastocytoma line P815 and the melanoma line B16-F10 were obtained from ATCC. The human 293 primary embryonic kidney cells were procured from Qbiogene (Carlsbad, CA, USA). The cell lines were cultured in complete DMEM medium containing 10% FCS, L-glutamine and antibiotics.

Plasmid construct for cell surface TR6 expression

The coding sequence of human TR6 minus the stop codon was retrieved by PCR and fused with the coding sequence of the human EphB6 transmembrane domain (E591-R621, ref. (54)) followed by a stop codon; the ensemble was cloned into pAdenoVator

(Qbiogene) downstream of the CMV promoter. The insert and junctions were verified by sequencing. The construct was named pCMV-TR6Mem.

Stable transfection of tumor cells with pCMV-TR6Mem

pCMV-TR6Mem and pcDNA III were introduced into P815 cells, 293 cells and B16 cells at a 10:1 ratio (20 μg pCMV-TR6Mem and 2 μg pcDNA per $5x10^6$ cells) with electroporation (350V, 960 μF). The transfected cells were then selected with 800 μg/ml G418 for 2 weeks. The stable transfectants, named TR6-P815, TR6-293 and TR6-B16, were used for experiments after cell surface expression of TR6 was confirmed by flow cytometry. The parental cells were transfected with the empty vector pAdenoVator, and the resulting cells were named vector-293, vector-P815 and vector-B16.

Mouse and human T-cell isolation and culture

Cells were flushed from the mouse spleen, and red blood cells were lysed with 0.84% NH_4Cl, as described elsewhere (13). The resulting cells were referred to as spleen cells. Human PBMC were prepared with density gradients using Lympholyte-H (Cedarlane, Hornby, ON, Canada). Mouse and human T cells were purified from spleen cells and PBMC, respectively, with human and mouse T-cell purification columns according to the manufacturer's instructions (Cedarlane). In some experiments, mouse T cells were further fractionated into CD4 and CD8 cells by magnetic bead positive selection (Miltenyi Biotech, Auburn, CA) according to manufacturer's instructions. The purity of the CD4 and CD8 cells was about 97% according to flow cytometry. In other

experiments, CD4 or CD8 cells were deleted from spleen T cells with the magnetic beads. The cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine and antibiotics. ^{3}H-thymidine uptake was measured as described previously (13,55).

Lymphokine assays

IL-2, IL-4 and IFN-γ in culture supernatants were measured with ELISA kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions.

Confocal microscopy

The binding of TR6-Fc to LIGHT$^{-/-}$ or LIGHT$^{+/+}$ T cells was demonstrated using confocal microscopy, as detailed in our previous publication (13). Briefly, spleen cells were crosslinked with anti-CD3 and anti-CD28 for 2 min, and the cells were stained with Alexa-594-anti-CD3 for the TCR complex, and stained with TR6-Fc followed by Alexa-488-anti-human IgG for TR6 binding. Three view fields from confocal microscopy were randomly selected, and TR6-binding cells in green and TCR positive cells in red were counted. The percentage of TR6^{+} cell among CD3^{+} cells was calculated.

Flow cytometry

Cell surface expression of TR6 on pCMV-TR6Mem-transfected cells was assessed by flow cytometry. The cells were stained with rabbit anti-TR6 polyclonal Ab followed by

PE-conjugated goat anti-rabbit Ab (Cedarlane). Propidine iodine-negative live cells were gated for analysis. Vector-transfected cells served as controls.

Cytotoxic T lymphocyte (CTL) assay

DBA/2 mice (H-2^d) were immunized s.c. with $1x10^6$ mitomycin C-treated TR6-P815 cells (H-2^d) on days 1 and 7. On day 15, spleen cells from these immunized mice were stimulated with mitomycin C-treated wild type P815 cells at a 1:1 ratio in 24-well plates ($8x10^6$/4 ml/well). CTL activity of the cultured cells was assessed 6 days later by standard ^{51}Cr release assay, as described previously (13), using ^{51}Cr-labeled P815 cells (H-2^d) as targets at different effector/target ratios. The lysis percentage of the test sample was calculated as follows:

% lysis = (cpm of the test sample - cpm of spontaneous release)/(cpm of maximal release - cpm of spontaneous release).

Tumorigenicity and tumor challenge assays

For tumorigenicity assay, DBA/2 mice were inoculated s.c. with $5x10^4$ syngeneic parental P815 cells. Tumor size was measured q. 2d until day 30 after inoculation. For tumor challenge assays, DBA/2 mice were vaccinated twice with $1x10^6$ mitomycin C-treated TR6-P815 cells on days 1 and 7. On day 15, the mice were challenged with $5x10^4$ syngeneic parental P815 cells or $5x10^5$ SP A/20 myeloma cells (H-2^d). The product of the longest and shortest tumor diameters was taken as tumor size in this and all other experiments.

Therapeutic vaccination

DBA/2 and C57BL/6 (H-2^b) mice were first inoculated with $5x10^4$ live wild type P815 cells and $1x10^5$ B16 (H-2^b) cells, respectively on day 0. On days 3 and 8, the mice were vaccinated with $5x10^6$ mitomycin C-treated TR6-P815 or TR6-B16 cells.

RESULTS

Cell surface expression of TR6

The coding sequence of human TR6 lacks the transmembrane domain, and consequently, TR6 is a secreted protein. To allow TR6 to anchor on the cell surface, we fused the coding sequence of the human EphB6 transmembrane domain (E591-R621) to the 3' end of the TR6 coding sequence (Figure 1A). The fused sequences were cloned into pAdenoVator downstream of the CMV promoter, and the resulting construct was named pCMV-TR6Mem. This construct was stably transfected into human 293 embryonic kidney cells, high antigenic P815 mouse mastocytoma cells, and low antigenic mouse B16 melanoma cells.

Surface TR6 expression of the transfectants was verified by flow cytometry. As shown in Figure 1B, 55.8% of 293 cells, 70.3% of P815 cells, and 68.7% of B16 cells became cell surface TR6-positive after stable transfection, while the wild type cells or cells

transfected with control vectors remained TR6-negative. These pools of transfected cells without further cloning (to avoid selecting clones with different antigenicity) were used for in vitro of in vivo studies.

T-cell costimulation by cell surface TR6 in vitro

We have previously demonstrated that when TR6 was placed on the solid phase of culture wells, it was able to costimulate T cells activated with a suboptimal concentration of anti-CD3. To assess whether this feature of TR6 could serve as a tumor vaccine, we first tested in vitro whether cell surface TR6 could similarly costimulate T cells. When human T cells were stimulated with mitomycin-C treated parental or control vector-transfected allogeneic 293 kidney cells, little proliferation was detected from days 3 to 5 (Figure 2A, left panel), indicating that 293 cells are not effective in costimulation, even though the antigen on 293 cells was alloantigenic with respect to PBMC. When TR6-293 cells were used as stimulators, they induced drastic T-cell proliferation; this shows that cell surface TR6 expression provides T cells with potent costimulation. Similar findings were made with mouse P815 cells. In the presence of a suboptimal concentration of soluble anti-CD3, T cells from DBA/2 mice failed to proliferate in response to stimulation from syngeneic parental P815 or vector-P815 cells (Figure 2A, right panel). When TR6-P815 cells were tested as stimulators, they triggered significant proliferation from days 3 to 5, suggesting that the cell surface TR6 acts as an effective costimulating molecule.

In figure 2B, we demonstrated in a same experiment that TR6-Fc coated on wells or expressed on cell surface could both costimulate T-cell proliferation. In the left panel, T

cells proliferation was enhanced by TR6 on cells in the presence of suboptimal anti-CD3 (0.8 μg/ml for coating), while anti-CD3, TR6-Fc, or normal human IgG (used as a control for TR6-Fc) alone had minimal effect. In the right panel, surface TR6 expressed by P815 cells in the presence of soluble anti-CD3 (5 ng/ml, suboptimal)was shown to costimulate T cells as well, while soluble anti-CD3 at this concentration, wild type P815 plus anti-CD3, or vector-transfected P815 plus anti-CD3 failed to do so. It is to be noted that the magnitude of T-cell proliferation costimulated by TR6 on wells and by TR6 on cell surface does not reflect the potency of these two ways of costimulation, as the concentration and the route of CD3 administration, which are major factors in determining the strength of proliferation, in these two systems were totally different.

We further showed that highly purified CD4 and CD8 cells similarly responded to TR6-P815 costimulation (Figure 2C) in term of proliferation. This indicates that cell surface TR6 directly costimulates CD4 and CD8 cells without involvement of non-T cells.

Culture supernatants from the above-described experiments were harvested on days 2, 3 and 4 for lymphokine assay. Responding to parental 293 or vector-293 cells, human T cells secreted negligible amounts of IFN-γ, IL-2 and IL-4; they produced high levels of IFN-γ and IL-2 but not IL-4 when stimulated with TR6-293 cells (Figure 2D). The lymphokine production of mouse T cells responding to P815 cells was very similar to that of human T cells: TR6-P815 cells were able to stimulate IFN-γ and IL-2 but not IL-4 secretion, while parental and vector-transfected P815 cells were not effective (Figure 2D). These findings corroborate the T-cell proliferation data, and suggest that cell

surface TR6 is capable of costimulating T cells and inducing Th1-like lymphokine secretion. It is worth mentioning that our ELISA was sufficiently sensitive to detect IL-4 produced by T cells after anti-CD3 and anti-CD28 stimulation (data not shown), indicating that the failed detection of IL-4 in TR6-293- or TR6-P815-stimulated T cells was not due to the lack of assay sensitivity.

We next investigated the nature of cell surface ligand with which TR6 interacted. We showed that soluble human LIGHT (10 μg/ml) effectively blocked TR6-P815-triggered costimulation in this system (Figure 2E). Moreover, using spleen cells from LIGHT gene knockout mice, we demonstrated that TR6 bound to 83% wild type T cells (Figure 2F), but only about 18% $LIGHT^{-/-}$ T cells. Taken these results together, along with our previous studies related to the ligand of TR6 on T cells (13,31), it seems that LIGHT on the T-cell surface is, at least, an important signal recipient of the cell surface TR6.

Spleen T cells from mice immunized with TR6-P815 cells presented an augmented response to in vitro re-stimulation

Next, we assessed whether surface TR6 could augment the in vivo T-cell response to tumor cells. DBA/2 mice were vaccinated with syngeneic mitomycin C-treated TR6-P815 cells on days 1 and 7. Parental and vector-transfected P815 cells served as controls. On day 15, the spleen cells of the vaccinated mice were harvested and re-stimulated with mitomycin C-treated parental P815 cells in vitro. The cell proliferation was measured by ^{3}H-thymidine uptake on days 4, 5 and 6, lymphokine production of the culture was assayed on days 3, 4 and 5 by ELISA, and CTL activity was quantified on

day 6 by ^{51}chromium-release assay. As shown in Figure 3A, parental and vector-P815 cells failed to prime the immune system in vivo, as the spleen T cells from these mice did not respond to secondary in vitro re-stimulation with parental P815 cells in terms of proliferation (Figure 3A), IFN-γ and IL-2 production (Figure 3B), and CTL activity (Figure 3C). However, when the mice were vaccinated with TR6-P815 cells, their spleen T cells became highly responsive to in vitro re-stimulation by parental P815 cells: T cells were strongly proliferative on days 4 and 5 (Figure 3A); IFN-γ and IL-2 but not IL-4 were detected at high levels in the culture supernatants on days 3 and 4 (Figure 3B); they also showed augmented CTL activity against parental P815 cells when stimulated wild type P815 (Figure 3C). We further demonstrated that both CD4 and CD8 cells were essential during re-stimulation, as spleen cells from the immunized mice had significantly reduced CTL against wild type P815 cells, if CD4 or CD8 cells were depleted (Figure 3D). These results revealed that TR6 expression on the tumor cell surface converts the normally ineffective immune response against the tumor cells into an effective one in vivo, and the secondary immune response depends on both CD4 and CD8 cells. This has established the basis of using tumor cells with surface TR6 expression as a tumor vaccine.

Reduced tumorigenicity of P815 cells after surface expression of TR6

Since P815 cells with surface TR6 expression could effectively trigger an in vivo T-cell response, they should have a reduced capability of evading the immune surveillance and, hence, decreased tumorigenicity. This possibility was investigated here. First, different numbers of parental P815 cells were inoculated s.c. into syngeneic DBA/2 mice to

determine the minimal tumorigenic number (MTN) required to achieve 100% solid tumor occurrence at the inoculation site. The MTN for P815 was determined to be $1x10^4$ cells, and 5 times MTN ($5x10^4$) was used in all subsequent experiments. As shown in Figure 4A, all DBA/2 mice inoculated with the 5xMTN of parental or vector-P815 cells developed solid tumors at the injection site, and tumor size reached 400 mm^2 within 20 days. The mice were sacrificed at that time according to Canadian Council on Animal Care guidelines. However, none of the mice inoculated with TR6-P815 cells developed solid tumors at the injection site during the observation period (30 days), and they had no visible tumors in their internal organs upon necropsy on day 30. Differences between the parental P815 versus the TR6-P815 group, and between the vector-P815 versus the TR6-P815 group were both highly significant ($p<0.001$, one-way variance analysis followed by all pair-wise multiple comparison procedures [Tukey test]). Interestingly, 3 out of 6 mice inoculated with TR6-P815 cells had small (less than 20 mm^2) transient tumors at the injection site between days 12 and 15, but these tumors disappeared afterwards; the kinetics of tumor disappearance coincided with that of an effective anti-tumor immune response.

The failed formation of solid tumors by TR6-P815 cells in DBA/2 mice was not due to the reduced growth rate of these cells after stable transfection with pCMV-TR6Mem, as these cells had a similar in vitro proliferation rate compared with parental or vector-P815 cells (Figure 4B).

TR6-P815 vaccination prevented tumor development on subsequent parental P815 cell inoculation and protection was tumor-specific

The failure of solid tumor formation after live TR6-P815 cell inoculation suggested that the recipient mice developed an effective immune response against the tumor, and subsequently eliminated the inoculants. If such is the case, inactivated TR6-P815 cells could be used as vaccine to counter the challenge by live wild type P815 cell inoculation. This possibility was therefore explored. When live wild type P815 cells were inoculated into mice previously vaccinated with mitomycin C-inactivated wild type P815 cells or vector-P815 cells, all them developed tumors at the injection site after 14 days, and the tumors reached 400 mm^2 around day 24 (Figure 5A). In contrast, none of the TR6-P815-vaccinated mice developed tumors upon the live parental P815 cell challenge. The differences between the parental P815- versus the TR6-P815-vaccinated groups, and between vector-P815- versus the TR6-P815-vaccinated groups were highly significant ($p<0.001$, one-way variance analysis followed by all pair-wise multiple comparison [Tukey test]). This indicates that mice vaccinated with inactivated TR6-P815 cells, but not parental P815 or vector-P815 cells, mounted an effective secondary anti-tumor immune response, which eliminated subsequently inoculated live parental P815 tumor cells.

Is this anti-tumor response specific? To answer this question, DBA/2 mice were inoculated with 5xMTN of live syngeneic SP A/20 myeloma cells ($5x10^5$ cells/mouse). These cells rapidly developed into solid tumors in the injection site in 7 days, and by day 14, the tumors reached 400 mm^2 in naïve DBA/2 mice as well as in DBA/2 mice

vaccinated with TR6-P815 cells (Figure 5B). This demonstrates that the anti-tumor immune response by TR6-P815 vaccination is tumor-specific.

TR6-P815 vaccine was effective in eliminating pre-existing P815 tumors

In most clinical cases, tumor vaccines will be administered to patients already diagnosed with tumors. Therefore, a tumor vaccine will only be realistically useful if it can eliminate pre-existing tumors. To this end, we inoculated DBA/2 mice with live parental P815 cells s.c. on day 0, and administered tumor vaccination twice, on days 3 and 8 in the opposite flank. As shown in Figures 6A and 6B, all mice vaccinated with parental P815 or vector-P815 cells developed tumors, and the kinetics were similar to those seen in mice without vaccination, as illustrated in Figure 3A. However, tumor development in 7 out of the 10 mice vaccinated with TR6-P815 cells was totally prevented; 3 mice in this group did develop tumors, but tumor development was delayed for about 4 days (Figure 6C). The differences between the parental P815- versus the TR6-P815-vaccinated groups, and between the vector-P815- versus the TR6-P815-vaccinated groups were highly significant ($p<0.001$, one-way variance analysis followed by all pair-wise multiple comparison [Tukey test]). This result demonstrates that tumor cells engineered to express cell surface-anchored TR6 can be used as a therapeutic vaccine.

TR6-B16 vaccine in combination with adjuvant was effective in eliminating pre-existing low antigenic tumors

P815 mastocytoma is a highly antigenic tumor, while many tumors in humans are of low antigencity. To explore the general utility of cell surface TR6 in tumor vaccines, we

expressed TR6 on the cell surface of B16 (H-2^b), a low antigenic melanoma line. The resulting line was named TR6-B16, and was tested alone or in combination with Bacillus Calmette Guerin (BCG) as a tumor vaccine against parental B16 tumors. C57BL/6 mice (H-2^b) were inoculated s.c. with 5xMTN ($1x10^5$ cells/mouse) of live parental B16 tumors on day 0. These mice were mock vaccinated with PBS (Figure 7A) or vaccinated with vector-B16 (Figure 7B) on days 3 and 8. The two groups had similar tumorigenic kinetics and tumor incidence: all the mice developed palpable solid tumor at the injection site around day 11, and none of the mice were tumor-free on day 17. In mice vaccinated with TR6-B16 cells, the tumorigenic kinetics of parental B16 cells were delayed (Figure 7C); this was demonstrated more clearly when the data were expressed as % of mice with tumors (Figure 7F) and as % of mice with tumors reaching 400 mm^2 at different days after inoculation (Figure 7G). Statistical analysis showed that the differences were highly significant or significant ($p=0.001$ between the mock vaccinated and the TR6-B16 groups; $p=0.024$ between the vector-P815 and the TR6-P815 groups; one-way variance analysis followed by all pair-wise multiple comparison [Tukey test]). When BCG was administered in combination with TR6-B16 cells, the effect became more pronounced: 5 out of 9 mice were tumor-free in this group, compared with 0 out of 8 mice being tumor free in the groups with mock vaccination or with vector-B16 vaccination ($p<0.001$ between the mock vaccinated and the TR6-B16 + BCG groups; $p=0.006$ between the vector-P815 and the TR6-P815 + BCG groups; one-way variance analysis followed by all pair-wise multiple comparison [Tukey test]). BCG is known to moderately enhance immune responses to certain types of tumors (56). However, the significant effect of tumor elimination in the TR6-B16 plus BCG-treated

group could not be attributed to BCG alone, as the BCG-treated group had about only two-day delay in their tumor development (Figure 7E), compared with the mock vaccinated group, and none of the BCG-treated mice was tumor free. This indicates that TR6-B16 and BCG have additive or synergistic effect as a therapeutic tumor vaccine.

DISCUSSION

Low antigenicity and lack of costimulatory molecules are some of the reasons why tumor cells are invisible to immune surveillance. One of the approaches often taken to overcome these problems is to express costimulating molecules, usually from the Ig superfamily or TNF superfamily, on the tumor surface (57-59). We developed a novel strategy in this regard based on costimulation of T cells by the cell surface expression of TR6, which is a soluble protein of the TNF receptor superfamily.

In our previous study, we demonstrated that when TR6 is coated on wells, it enhances the T-cell response to suboptimal stimulation of TCR. Those in vitro assays are subject to criticism that any molecule that can physically increase contact force between the culture well, on which anti-CD3 or anti-TCR is coated, and T-cells has the potential to augment T-cell responses; such a molecule might not fall into a more strict definition of costimulatory molecules. In this study, we proved that when TR6 was anchored on the tumor cell surface, it augmented T-cell responses triggered by tumor antigen in vitro and in vivo. Therefore, TR6 on the cell surface can now be qualified as a genuine

costimulatory molecule, albeit an artificial one, as it is not normally expressed on the cell surface.

Cell surface TR6-based tumor vaccine likely depended on adaptive immunity. First, in vitro study showed enhanced response of naïve T cells to TR6-293 and TR6-P815 costimulation (Figure 2); secondly, T cells from TR6-P815 vaccinated mice had augmented response to wild type P815 cells, and such augmented response was reduced when CD4 or CD8 cells were deleted in vitro; thirdly (Figure 3), TR6-P815 vaccinated mice rejected parental P815 tumors but not a different tumor, i.e., SP A/20 myeloma cells (Figure 5); finally, our data (not shown) showed that NK cell activity was not modulated by either solid phase or soluble TR6.

TR6 employed is of human origin, and mice do not have orthologous counterpart of human TR6. Was the observed tumor immunity in mice due to the xenoantigenic nature of human TR6? Although we used TR6-P815 or TR6-B16 for immunization, the cells used for challenging or inoculation to generate solid tumors (e.g., in the P815 challenging assay (Figure 5), in the P815 therapeutic assay (Figure 6), and in the B16 therapeutic assay (Figure 7)) were all wild type tumor cells carrying no TR6, yet the tumor immunity could curb their growth in vivo, or eliminate them after the tumors were pre-inoculated. If one argues that the TR6 as xenoantigen enhanced the general non-specific immune status, which led to tumor rejection, we have shown in Figure 5B that a difference tumor line SP A/20 was not affected by the vaccine. Therefore, the

enhanced immune response is tumor-specific, but not related to the xenoantigenicity of TR6.

TR6 has three ligands, i.e., LIGHT, FasL and TL-1A, and the former two are capable of reverse signaling and costimulating T cells. According to our genome search, mice do not have an orthologous gene corresponding to human TR6. Therefore, we used human TR6 for both in vitro and in vivo study in a mouse model, as human TR6 can bind to mouse LIGHT and FasL (4,60). We have shown that soluble LIGHT effectively blocks TR6-P815 triggered costimulation, and $LIGHT^{-/-}$ T cells had significantly reduced binding to TR6, compared to $LIGHT^{+/+}$ T cells (a decrease from 83% to 18%). These results along with our previous study (13,31) suggest an important role of LIGHT in the costimulation. With that said, we cannot exclude possible reverse signaling via FasL (25-27) or other so far un-identified TR6 ligands on T cells, as TR6 can still bind to about 18% $LIGHT^{-/-}$ T cells according to confocal microscopy.

Our previous data showed that soluble and solid phase TR6 decreases T-cell chemotaxis (55). This is a possible mechanism to explain that tumors secreting soluble TR6 have less lymphocyte infiltration (61), and the tumor evasion of the immune surveillance, because when T cells encounter soluble TR6 outside the tumor, they will decrease they migration toward the tumor. However, in our current model, TR6 is expressed on the tumor cell surface; T cells would not have chances to interact with the cell surface TR6 until they enter the tumor. Hence, the immune surveillance should not be hampered, but possibly augmented by the cell surface TR6.

We have proved in this mouse model that tumor cells engineered to express surface TR6 could be used as a therapeutic vaccine. In humans, some patients with gastrointestinal tumors have elevated serum TR6; for some tumors, such as gastric, liver, gallbladder and colon tumors, the serum TR6-positive incidence reaches 50-70% (61); serum TR6 is secreted by the tumors (2,61). Conceivably, free serum TR6 might compete with cell surface TR6 in the cell surface TR6-based vaccine, and reduce its efficacy. Therefore, such vaccine might be more effective in tumors that do not secrete soluble TR6. Our study shows that lung and breast cancers have a much lower incidence of serum TR6 positiveness (less than 20%) (61), and patients with these cancers should be optimal candidates for a surface TR6-based therapeutic tumor vaccine. Moreover, we have also shown that in tumors with high serum TR6 levels, tumor resection leads to complete disappearance serum TR6 in 3-4 weeks; this will be the best time window for administration of surface TR6-based tumor vaccine, as tumor-specific T cell immunity can develop in the absence of TR6 interference in such a time window.

In summary, tumor cells engineered to express surface TR6 are a useful addition in our arsenal of therapeutic tumor vaccines.

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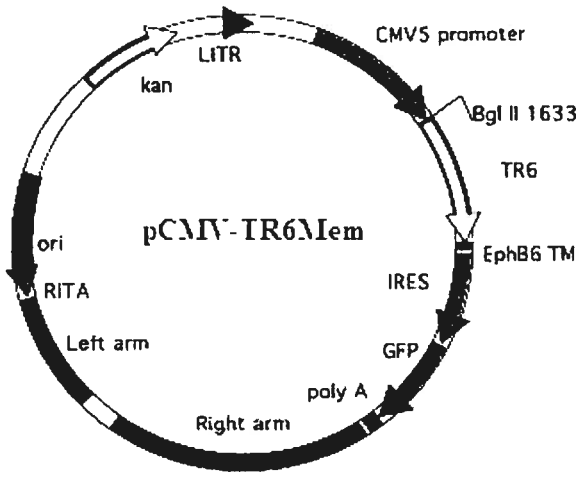
FIGURE LEGENDS

Figure 1. TR6 Expression on the cell surface

Figure 1

A

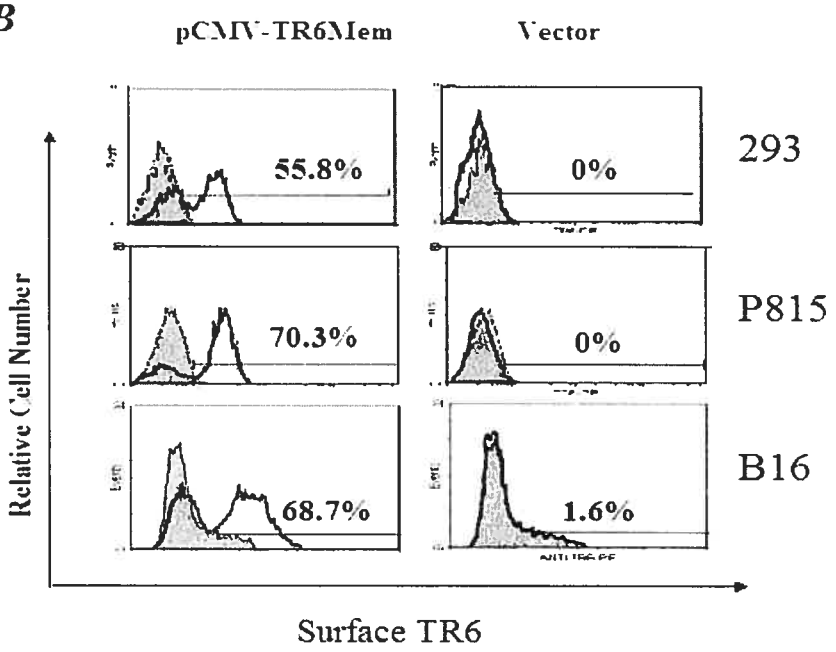


A. Construct expressing membrane-bound TR6

The full-length TR6 cDNA coding sequence minus the stop codon was fused to the coding sequence of the EphB6 transmembrane domain (EphB6-TM) followed by a stop codon. This cassette was cloned into the polylinker of pAdenoVator downstream of the CMV promoter. The resulting construct was named pCMV-TR6Mem.

Figure 1

B

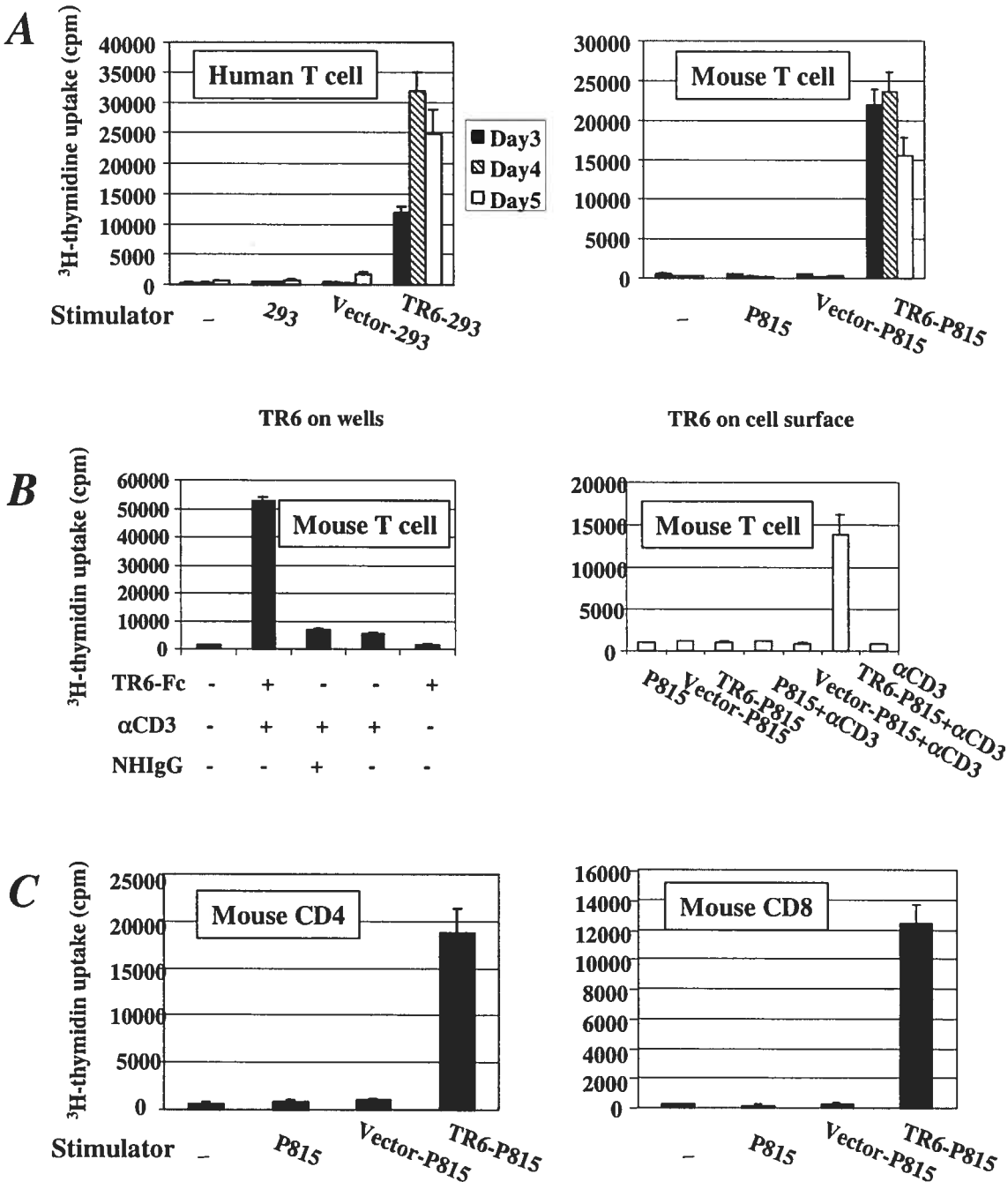


B. TR6 expression on the 293, P815 and B16 cell surface

Human embryo kidney 293 cells, mouse mastocytoma P815 cells, and mouse melanoma B16 cells were stably transfected with pCMV-TR6Men or the parental vector, and surface TR6 expression on these cells was detected by flow cytometry. The fluorescence intensity of untransfected wild type cells (grey area) was used to determine background staining. Solid lines represent cell surface TR6 expression on pCMV-TR6Mem- or vector-transfected cells. The percentage of TR6-positive cells after deduction of background is indicated.

Figure 2. Cell surface TR6 expression costimulates T cell in vitro

Figure 2



A. Cell surface TR6 costimulates T cells.

Mitomycin C-treated surface TR6-293 cells or TR6-P815 cells were used to stimulate human T cells from PBMC or DBA/2 spleen T cells, respectively. Vector-293 cells, vector-P815 cells, parental 293 cells or parental P815 cells served as controls. For mouse T-cell culture, a suboptimal concentration of soluble anti-CD3 (clone 2C11, 20 ng/ml) was present. The cells were pulsed with ^{3}H-thymidine for 16 h before being harvested on days 3, 4 and 5, as indicated.

B. TR6 coated on wells or expressed on cell surface costimulated T cells

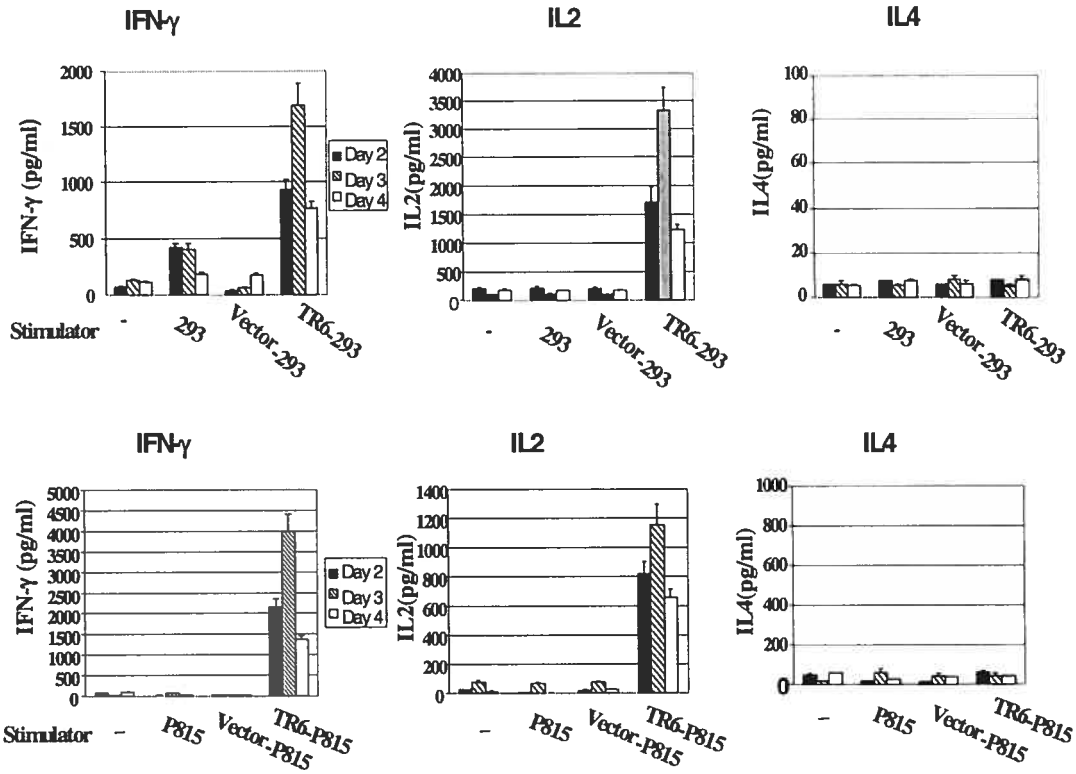
On the left panel, TR6-Fc (10 μg/ml for coating), normal human IgG (NHIgG; as a control for TR6-Fc; 10 μg/ml for coating), and/or suboptimal anti-CD3 (0.8 μg/ml for coating) were coated on the wells as indicated; DBA/2 T cells were added to the wells at $4x10^{5}$/200μl/well. On the right panel, DBA/2 T cells and mitomycin-C treated tumor cells (parental P815 cells, vector-P815 cells, or TR6-P815 cells) were mixed at 3:1 ratio and cultured at a final total density of $4x10^{5}$/200μl/well; the first 3 columns showed proliferation in the absence of soluble anti-CD3, while anti-CD3 was present at 5 ng/ml for the last 4 columns. The cells were pulsed with ^{3}H-thymidine for 16 h before being harvested on days 4.

C. Proliferation of Magnetic beads-purified mouse CD4 and CD8 cells

Magnetic bead-purified mouse CD4 and CD8 cells (about 97% pure) were stimulated with TR6-P815 in the presence of a suboptimal concentration of anti-CD3 (20 ng/ml) as described above, and the cells were pulsed with ^{3}H-thymidine for 16 h before being harvested on days 4.

Figure 2

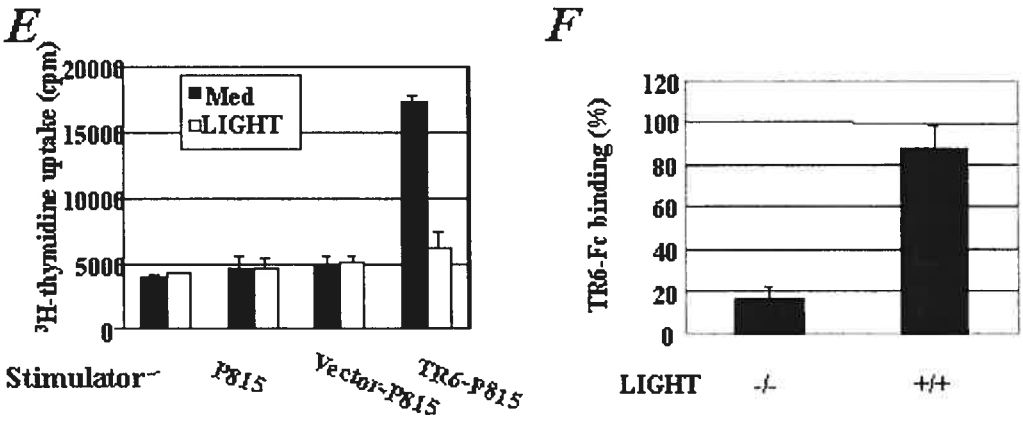
D



D. Lymphokine production.

Supernatants of the cell cultures described in Figure 2A were harvested on days 2, 3 and 4, as indicated, and IFN-γ, IL-2 and IL-4 in the supernatants were measured by ELISA.

Figure 2



E. Soluble LIGHT blocks cell surface TR6-augmente T cell proliferation

The experiment was conducted as described in Figure 2A, using TR6-P815 cells as stimulators, and DBA/2 T cells as responders, in the presence of a suboptimal concentration of soluble anti-CD3 (clone 2C11, 20 ng/ml). Empty columns represent cultures conducted in the presence of 10 μg/ml soluble human LIGHT. Thymidine uptake was measured on day 4.

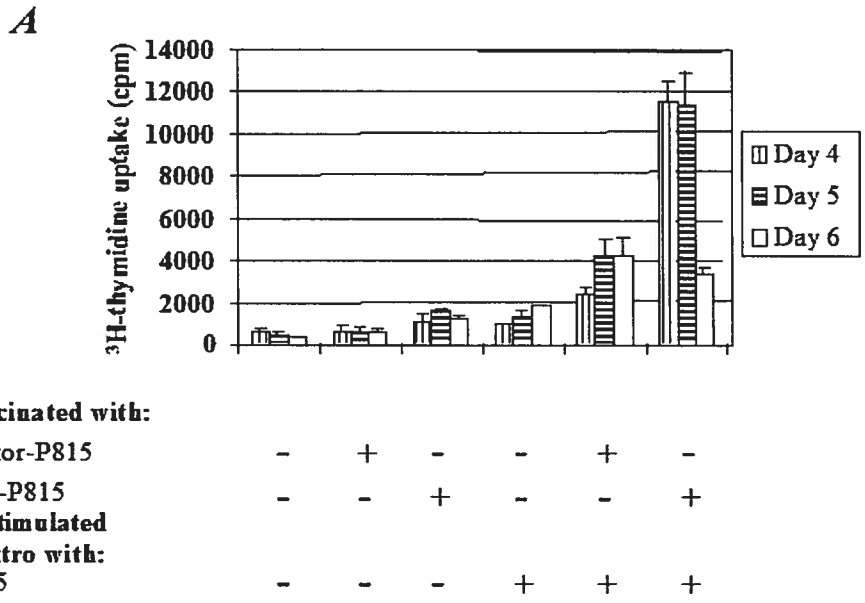
F. LIGHT$^{-/-}$ T cells significantly reduced their binding to TR6

Spleen cells from LIGHT$^{-/-}$ or wild type (LIGHT$^{+/+}$) mice were stained with anti-CD3 and TR6-Fc using 2 color fluorescence, and the percentage of TR6-positive cells among CD3 positive cells according to three randomly chosen fields of confocal microscopy was shown.

Figure 3. Spleen T cells from mice vaccinated with TR6-P815 cells manifest augmented response to in vitro re-stimulation

DBA/2 mice were vaccinated with mitomycin C-treated TR6-P815 cells on days 1 and 7. Vector-P815 cells or parental P815 cells were used as controls. On day 15, spleen T cells (A and B), or total spleen cells (C and D) from the vaccinated mice were harvested and stimulated with mitomycin C-treated parental P815 cells.

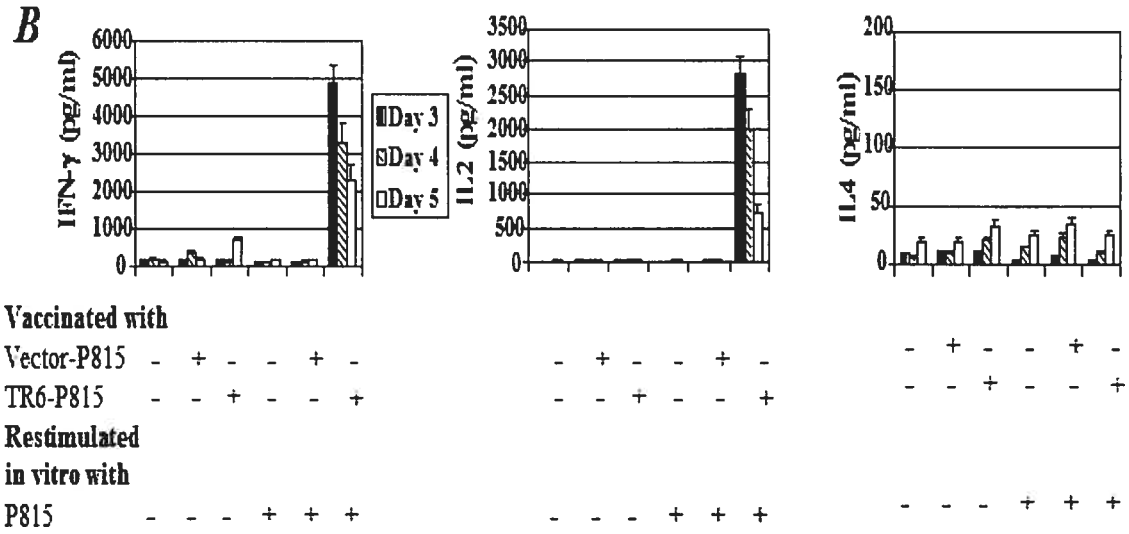
Figure 3



A. T-cell proliferation

The culture as described above was pulsed with ^{3}H-thymidine for 16 h before harvesting, and the cells were harvested on days 4, 5 and 6 for the measurement of thymidine uptake. Samples were in triplicate, and mean ± SD is presented.

Figure 3

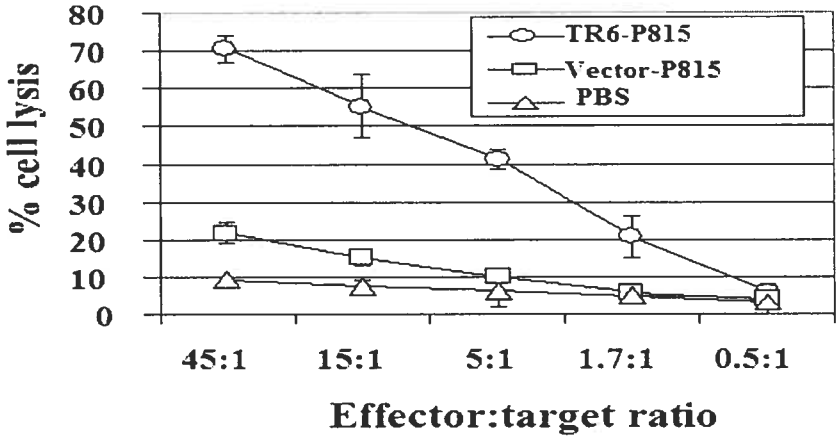


B. Lymphokine production

Supernatants from the culture described above were harvested on days 3, 4, and 5 as indicated, and IFN-γ, IL-2 and IL-4 in the supernatants were measured by ELISA. Samples were in duplicate, and the mean ± SD is presented.

Figure 3

C

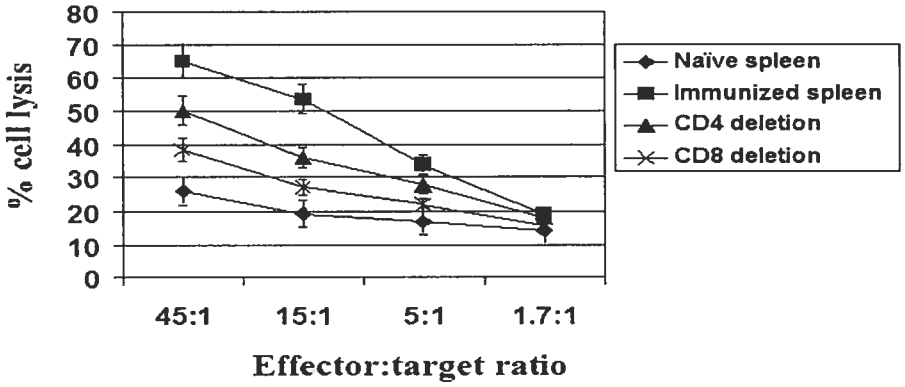


C. Cytotoxic T-lymphocyte activity of TR6-P815-primed spleen cells

Total spleen cells from vaccinated DBA/2 mice (mock vaccination with PBS; vector-P815; TR6-P815) were stimulated with mitomycin C-treated parental P815 cells for 6 days, and the CTL activity of the culture was measured by ^{51}Cr-release assay, using P815 cells as targets.

Figure 3

D



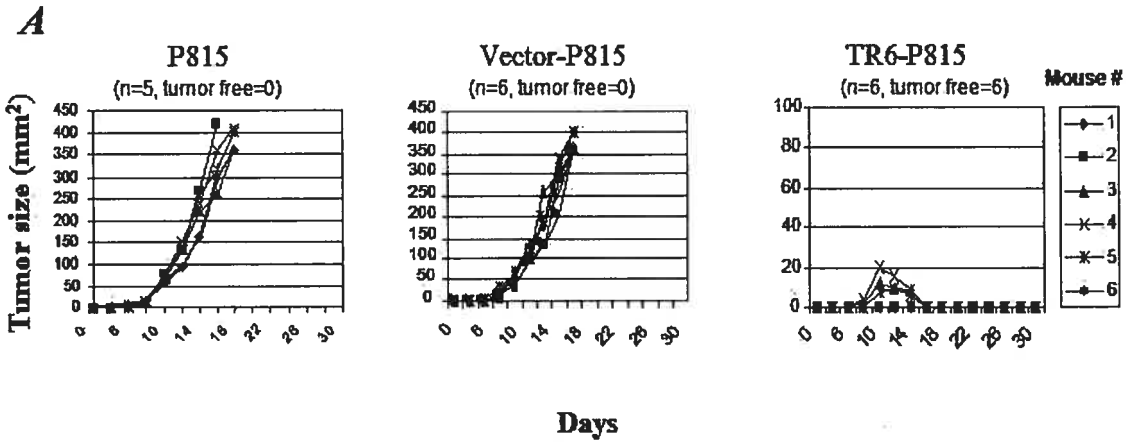
D. CTL activity from TR6-P815-primed mice depends on both CD4 and CD8 cells

Spleen cells from TR6-P815-vaccinated DBA/2 mice were used as a whole, or depleted of CD4 or CD8 cells, as indicated. These cells were stimulated with mitomycin C-treated parental P815 cells for 6 days, and the CTL activity of the culture was measured by ^{51}Cr-release assay, using P815 cells as targets. Naïve spleen: total spleen cells from mice with mock vaccination (PBS); immunized spleen: total spleen cells from mice immunized with TR6-P815; CD4 deletion: CD4-deleted spleen cells from TR6-P815-immunized mice; CD8 deletion: CD8-deleted spleen cells from TR6-P815-immunzed mice.

Experiments were performed more than twice with similar results, and a set of representative data is presented.

Figure 4. TR6-P815 cells were less tumorigenic than parental P815 cells

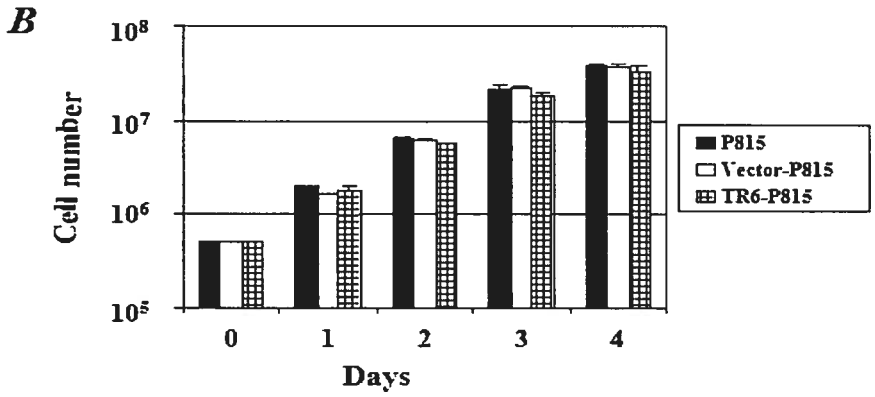
Figure 4



A. In vivo tumor growth

Live TR6-P815 cells, vector-P815 cells or wild type P815 cells were inoculated s.c. into the left flank of DBA/2 mice ($5x10^4$ cells/mouse). Tumor size was recorded q. 2d for 30 days and plotted. The results underwent one-way variance analysis followed by all pair-wise multiple comparison (Tukey test). The TR6-P815-inoculated group was highly significantly different from the vector-P815- or P815-inoculated groups ($p<0.001$ in both comparisons; one-way variance analysis followed by all pair-wise multiple comparison procedures [Tukey test]).

Figure 4



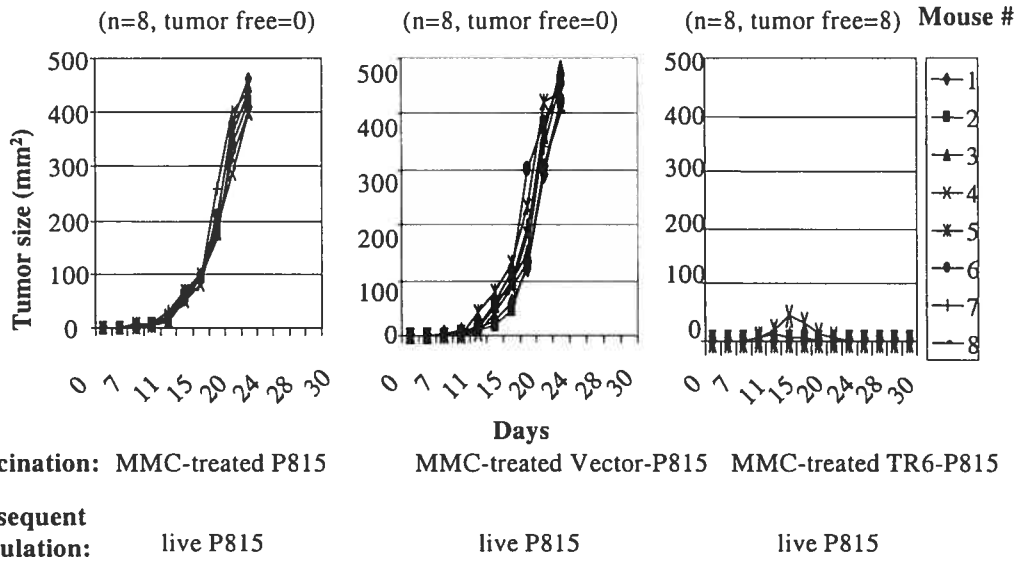
B. TR6-P815 cells, vector-P815 and parental P815 cells had similar growth rates in vitro

The three above-mentioned types of cells were cultured in 6-well plates ($5x10^4$/10 ml medium). The cell numbers in each well were determined in triplicate daily by flow cytometry. Total cell numbers in culture from days 0 to 4 were plotted. The experiments were performed three times with similar results, and a set of representative data is shown.

Figure 5. Protective effect of TR6-P815 vaccine against subsequent tumor cell inoculation

Figure 5

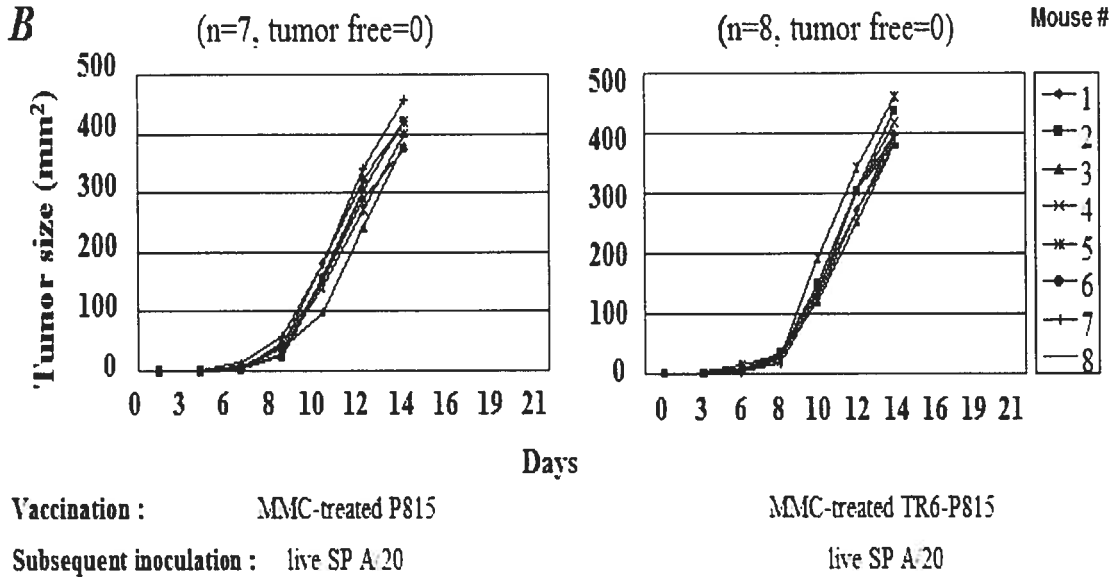
A



A. Mice vaccinated with TR6-P815 cells resisted parental P815 cell challenge

DBA/2 mice were vaccinated with $5x10^6$ mitomycin C (MMC)-treated P815-TR6 cells on days 1 and 7 s.c. on the left flank. On day 15, live parental P815 cells ($5x10^4$ cells) were inoculated s.c. on the right flank. Vector-P815 cells and wild type P815 cells were used as mock vaccines. Tumor size was registered q. 2d for 30 days and plotted. Differences between the TR6-P815 versus the P815, and the TR6-P815 versus the vector-P815 groups were highly significant (one-way variance analysis followed by all pair-wise multiple comparison [Tukey test], $p<0.001$ in both comparisons).

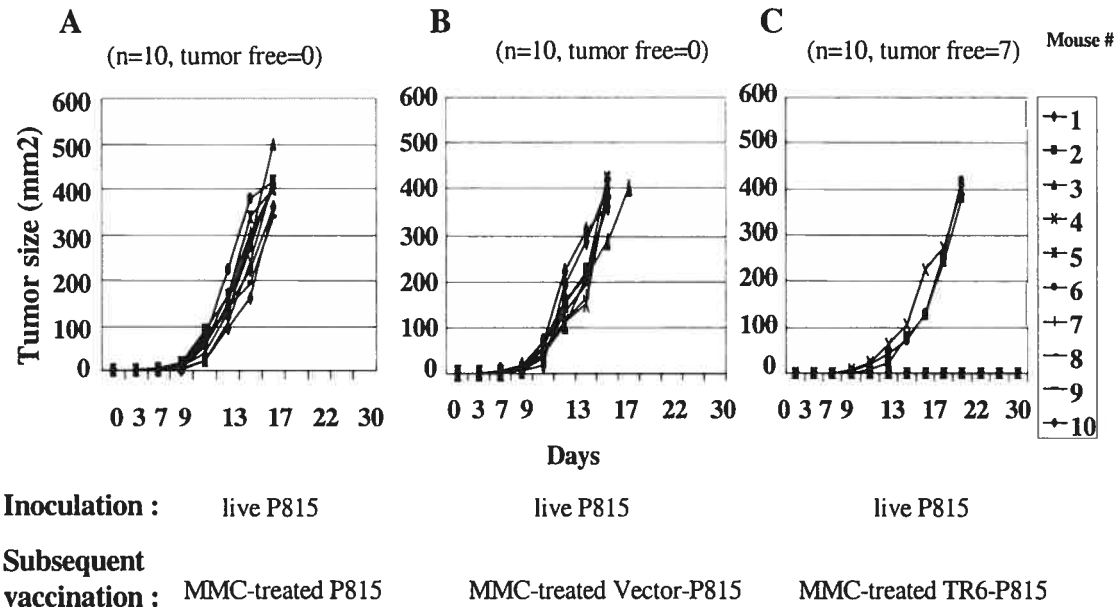
Figure 5



*B. The protective effect of TR6-P815 vaccination was tumor-specific*DBA/2 mice were vaccinated with MMC-treated TR6-P815 cells as described. Naïve DBA/2 mice served as controls. On day 15, live SP A/20 myeloma cells were inoculated s.c. ($5x10^5$/mouse) on the opposite flank. Differences between the TR6-P815-vaccinated and the naïve groups were not significant (one-way variance analysis followed by all pair-wise multiple comparison [Tukey test], $p>0.05$).

Figure 6. TR6-P815 cells were effective as a therapeutic tumor vaccine

Figure 6



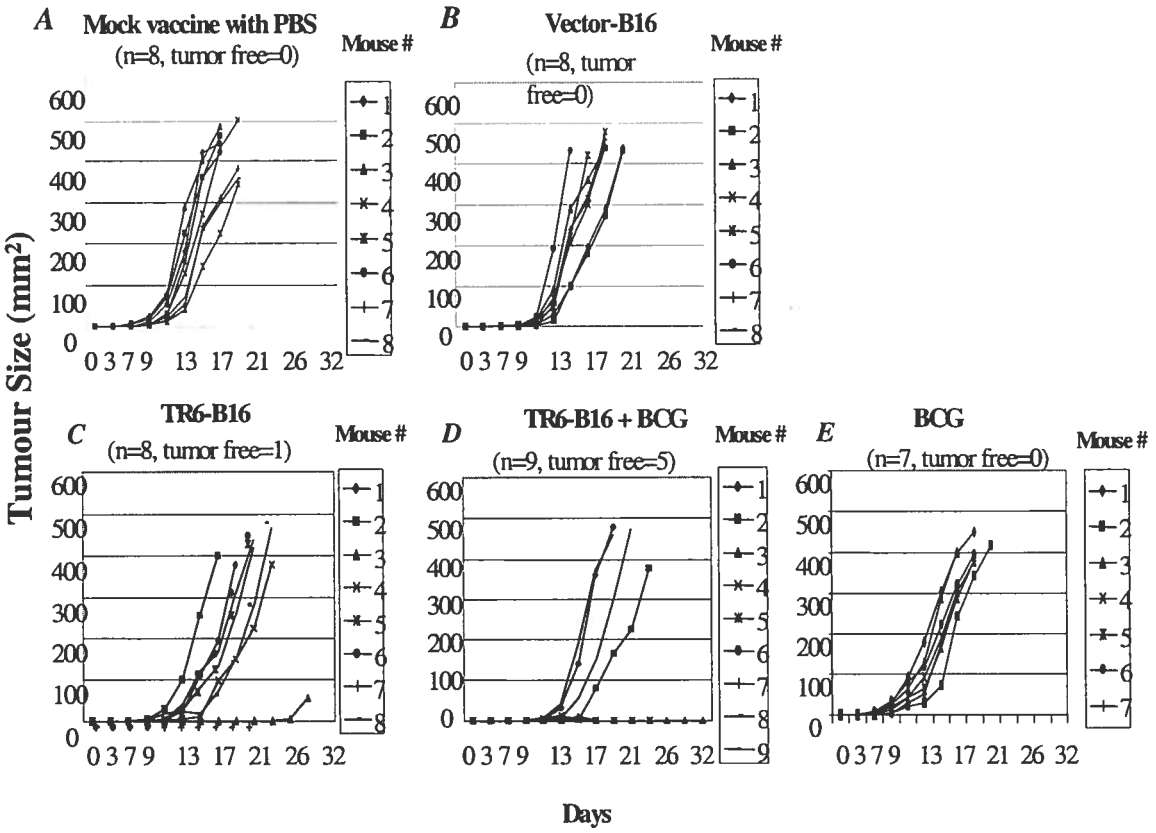
DBA/2 mice were inoculated with $5x10^4$ live parental P815 cells s.c. on the left flank. On days 3 and 8, $5x10^6$ MMC-treated P815-TR6 cells were inoculated on the right flank as a therapeutic vaccine. Parental P815 or vector-P815 cells were used as controls. Differences between the TR6-P815 versus the P815, and the TR6-P815 versus the vector-P815 groups were highly significant (one-way variance analysis followed by all pair-wise multiple comparison [Tukey test], $p<0.001$ in both comparisons).

Figure 7. Low antigenic B16 cells expressing surface TR6 were effective as a therapeutic tumor vaccine

Live parental low antigenic B16 cells were inoculated s.c. on the left flank of syngeneic C57BL/6 mice ($1x10^5$ cells/mouse) on day 0. On days 3 and 8, $5x10^6$ mitomycin C-

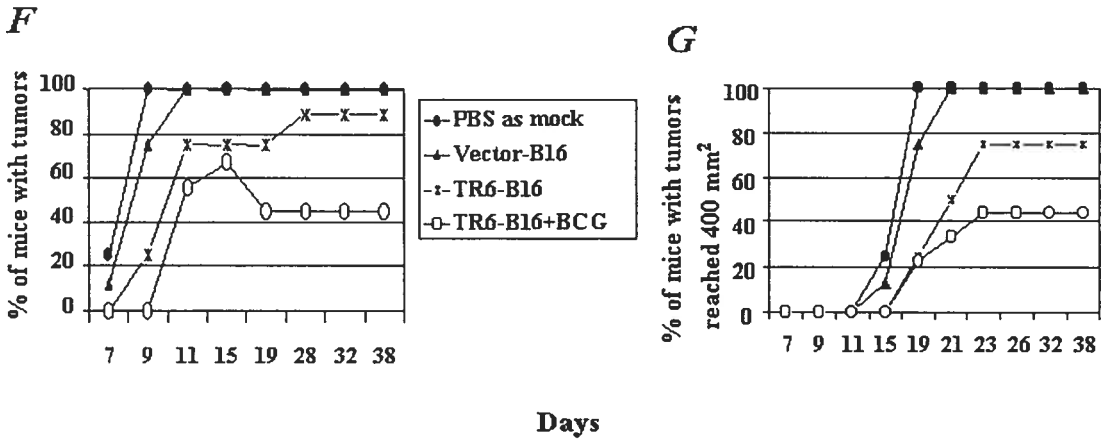
treated TR6-B16 cells (C), BCG (0.5 mg/mouse) alone (E), or both (D) were inoculated on the right flank as therapeutic vaccine. Mice with mock vaccinated (A; with PBS) or vector-B16-vaccination (B) served as additional controls. Tumor size, registered q. 2d until day 30, was plotted.

Figure 7



A-E. Tumor size of individual mice with different treatments

Figure 7



F-G. % of mice developing tumors as examined on day 30 (F), and % of mice with tumors reaching 400 mm^2 in size until day 30 (G).

Differences between the TR6-P815 versus mock vaccination, and between the TR6-P815 versus the vector-P815 groups were highly significant (p=0.001) and significant (p=0.024), respectively; differences between the TR6-P815 + BCG versus the mock vaccinated groups, and the TR6-P815 + BCG versus the vector-P815 groups were highly significant ($p<0.001$ and p=0.006, respectively; one-way variance analysis followed by all pair-wise multiple comparison [Tukey test]).

III. DISCUSSION

III. Discussion

In this project, the role of TR6 in immune regulation was investigated. In the *in vitro* study, TR6 coated on plates or expressed on tumour cell surface enhances TCR stimulated T-cell proliferation, and Th1 cytokine production. TR6 on solid phase can significantly enhance mouse spleen cell CTL activity. *In vivo* study showed that TR6 expressed on tumour cell surface reduces tumourigenicity, increases tumour antigenicity, and elicits specific anti-tumour immunity; the therapeutic effects of TR6-based tumour cell vaccine in treating established tumour is enhanced by BCG adjuvant. We also proved, for the first time, that soluble TR6 inhibits chemokine-induced human T-cell migration *in vitro*, and mouse T-cell migration *in vivo*. TR6 pretreatment reduces SDF1α-stimulated T cell Cdc42 activity, actin polymerization and pseudopodium formation. At the molecular level, we found that LIGHT is co-localized with clustered lipid raft in activated T cells, and that LIGHT reverse costimulatory signaling enhances mouse T cell p44/42 MAPK activity.

1. Reverse signaling through TR6 ligands

Many cell surface ligands can work as receptors to transduce signals reversely into cells. These ligands include: (a) some TNF family members, such as FasL, CD40L, CD30L, OX-40L, TRANCE, TRAIL, DR4 and LIGHT; (b) certain Eph ligands, such as ephrin B1 and ephrin B2 (410, 411); (c) some other ligands, such as cell surface IL-15 (412) and B7 (413).

During our study of TR6 in regulation of immune responses, we found that TR6 can reversely transduce signals into T cell through its ligands. As described in section 1, TR6 has three identified ligands, LIGHT, FasL and TL1A. TL1A is mainly expressed on endothelial cells. So it is not relevant to TR6-regulated T-cell responses. LIGHT and FasL are both expressed on activated T-cell surface and are inducible during T-cell activation (286). Our study proved that LIGHT is also expressed on resting T cells by confocal microscopy (8). The costimulation function of solid phase TR6 from LIGHT or FasL reverse signaling, or from both? We showed that anti-human LIGHT mAb on solid phase enhances mouse T-cell proliferation, and that cross-linked anti-LIGHT antibody pretreatment inhibits T-cell migration. Unlike TR6, which binds to both LIGHT and FasL, the anti-human LIGHT monoclonal antibody can only binds to LIGHT (8). This directly proves that LIGHT reverse signaling is involved in the observed TR6 functions. FasL, another ligand of TR6, expressed on T cell surface, is able to reversely transduce costimulatory signals into T cells when it is engaged with Fas. It was reported that FasL reverse costimulatory signaling occurs immediately on T-cell receptor ligation and correlates with the upregulation of FasL expression on CD8 and naive CD4 cells, both of which are sensitive to the FasL costimulatory signals (94). As the recombinant TR6 protein can bind to FasL (116), it is possible that TR6 on solid phase ligates both LIGHT and FasL on the T cell surface. To address this question, we stained activated mouse lymph node cells from LIGHT knockout mice and wild type mice. Results showed that the binding of TR6 to LIGHT$^{-/-}$ T cells is reduced 80%, using wild type T cells as controls. This indicates that the main binding partner of TR6 on T cells is LIGHT, but it might also bind to other molecules. Using spleen cells from

LIGHT knockout and FasL mutant mice (gld/gld), we found that with solid phase TR6 costimulation, the proliferation rate is reduced in both LIGHT knockout or FasL mutant spleen cells, compared with wild type spleen cells, and the reduction is more significant in LIGHT knockout spleen cells (>50%) (data not shown). These results suggest that both LIGHT and FasL reverse signaling contributes to the enhanced immune responses, But the majority of the reverse signaling is thought LIGHT. Results from other group's studies indicate that TR6 has other undefined ligand(s), which might reversely transduce signals into cells. Hsu TL, et al (117). reported that TR6-Fc binds to freshly isolated $CD14^+$ monocytes and reversely tranduces signals into those cells, but anti-LIGHT, anti-LTα, and anti-FasL Abs do not. Chang YC, et al (119). found that TR6 binds to macrophages and modulates their activation and differentiation, but the known TR6 ligands FasL, LIGHT and TL1A are undetectable in these cells by flow cytometry or by Western blot analysis. Together, these studies suggest that TR6 might have other unidentified ligand(s). It will be interesting in our model to use $LIGHT^{-/-}$ and $FasL^{-/-}$ double null mutant T cells to see whether TR6 can still trigger reverse signaling. Such an experiment will support or refute the existent of the so far unidentified TR6 ligands on T cells.

Our results clearly proved that TR6 can reversely tranduce signals into T cells through its ligands, mainly through LIGHT. The molecular mechanisms of such reverse signaling are not clear. In this study we found that LIGHT is co-localized with TCR in anti-CD3 plus anti-CD28 costimulated T cells in the clustered lipid rafts. Solid phase TR6 costimulation enhances ERK1 activity in the T cells (8). Upon SDF-1α-stimulation, we found reduced Cdc42 and p38 MAPK activities and repressed actin polymerization

in soluble TR6 pretreated T cells (125). These results indicate that the Cdc42, MAPK and cytoskeleton reorganization are involved in LIGHT reverse signaling. Since the cytoplasmic tail of LIGHT is short and incapable of signaling on its own (33), we studied the possible association of adoptor proteins with LIGHT using immunoprecipitation. We found the Grb2, but not Cbl, Vav or PI-3K, is associated with LIGHT. It is possible that Grb2 is a molecule mediating downstream signaling involving Cdc42 and MAPK. Cdc42 is an important Rho GTPase, it regulates T cell polarization towards antigen-presenting cells (APCs) through inducing localized actin polymerization at the site of APC binding (414, 415), and is also essential for chemokine-induced lymphocyte migration (125, 416, 417). The downstream signaling pathways of Cdc42 is still an unresolved question (415). In our study, we found that TR6-pretreated T cells have impaired Cdc42 and p38 MAPK activities after SDF-1α stimulation. This indicates that p38 MAPK might be a downstream signaling molecule of Cdc42 in LIGHT reverse signaling during SDF-1α stimulation. However, in T cells activated by TCR, we found increased p42/44 MAPK activity upon LIGHT reverse signaling. Although it is not known whether the small GTPase are repressed or activated in the presence of TCR activation. It is possible that the effect of LIGHT reverse signaling on GTPase depends on the other signals T cells receive. In the case of SDF-1α, stimulation the GTPase and the downstream actin polymerization are repressed, while in the case of TCR stimulation, it might be the opposite. This hypothesis is worth investigating.

2. The mechanisms of tumour cell-surface TR6-triggered anti-tumour immunity

It is widely recognized that tumour immunity is mainly a cell-mediated immune response. The critical role of tumour antigen-specific T cells in the eradication of cancer has been demonstrated in numerous animal models. Data compiled from both *in vitro* systems and human clinical trials indicate the existence of tumour antigen specific T cells (338). We have demonstrated in this study that tumour cells with forced expression of cell surface TR6 can be used as tumour vaccine. The underlying cellular mechanisms are discussed here.

Our *in vitro* experiments showed that surface TR6 expressing tumour cells could costimulate both human and mouse T-cell proliferation and Th1 cytokine production. This finding is consistent with our data using TR6 coated on cultured wells (8). Furthermore we found that T cells from P815-TR6 immunized mice have significantly enhanced proliferation, Th1 cytokine secretion and CTL activity in response to wild type P815 tumour cell restimulation. This proves that after vaccination, specific anti-P815 tumour T cells are primed. There are two kinds of T cells involved in specific cell-mediated immune response, i.e., CD4 and CD8 cells. Which of the two T cell populations is responsible for the TR6 surface expressing tumour cell vaccination induced anti-tumour immunity? Our *in vitro* data from T cells cultured in TR6 precoated plates or with MMC-treated TR6 surface expression tumour cells, indicate that TR6 directly costimulates both CD4 and CD8 proliferation. Further, we demonstrated that either CD4 or CD8 deletion reduces the anti-P815 CTL activity of spleen cells from P815-TR6 immunized mice. These results proved that both CD4 and CD8 cell are required for the cell-surface TR6-triggered anti-tumour immunity. Our *in vivo* data also showed that P815-TR6 immunized syngenic DBA2 mice specifically resisted the

challenge of wild type P815 tumour cells, but not that of SP A/20 myeloma cells. This proves that P815-TR6 vaccination induces specific anti tumour immunity.

The above evidence clearly demonstrates that cell-surface TR6-based tumour vaccine depends on adaptive immunity. However, we could not exclude the possibility that the innate immunity is also involved during T-cell priming. Indeed, both LIGHT and FasL are expressed on NK cells, and LIGHT is also expressed on DCs. It is possible that P815-TR6 stimulates DCs through their surface LIGHT, or/and stimulates NKs through their surface LIGHT and/or FasL.

It was reported that soluble TR6 modulates DCs differentiation and activation; TR6 pretreated DCs skew naive T-cell differentiation towards a Th2 phenotype (117). It will be interesting to study whether solid phase TR6-treated DCs can drive T cells to differentiate into an opposite phenotype, i.e., Th1, which enhances tumour immunity. We showed that vaccination with B16-TR6 in combination with adjuvant BCG greatly increases the efficacy of the vaccine. It is possible that BCG adjuvant causes inflammation at the injection sites; such inflammation would, in turn recruits APCs and stimulates APCs activation. The activated APCs then, could capture tumour antigens released from the tumour vaccine and indirectly present the captured tumour antigens to tumour-specific T cells. It is also possible that the inflammatory cytokines directly promote the function of tumour-specific T cells.

3. The physiological and pathophysiological role of TR6

TR6 is secreted by activated normal T cells. The peak of TR6 secretion is 24 to 48 hours after T-cell activation (136). We also proved that soluble TR6 inhibits T-cell aggregation (136) and T-cell chemotaxis (125). These results collectively suggest that TR6 plays a very important role in T-cell responses. TR6 secreted by activated T cells can terminate the costimulation by interfering with the interaction between TR2 and LIGHT, LTβR and LIGHT, and between Fas and FasL. Such function of TR6 can also reduce the activation-induced T cell death. As a result of these blocking functions, TR6 might enhance the survival of activated T cells in inflammation sites, and enable these T cells to fight with invaded pathogens for a longer time, or to differentiate into memory T cells.

The chemotaxis inhibition by T cell secreted TR6 might discontinue the flux of T cells to the inflammatory sites once T cell activation is near complete. This seems to be another mechanism to control the scale of an immune response. TR6 also inhibits aggregation of activated T cells. A possible physiological role of this effect is that TR6 secreted by activated T cells will disengage T cells from APC, and disperse the T cells to their destination in the form of effector cells. Taking together, TR6 produced by activated T cells might play a very important role in modulating T-cell responses and memory T-cell development.

TR6 is over-expressed in many malignant tumours, which include tumours of esophagus, stomach, glioma, long, colon, lymphoma and rectum (49, 50, 110, 111, 112). Many cancer patients have high serum TR6 levels, which are correlated to the grade of malignancy, and to TNM (UICC cancer staging system. TNM represent: Primary

tumour (T), Regional lymph nodes (N), Distant metastasis (M)) pathological classification (109). This suggests that TR6 might play a role in tumour progression and immune evasion.

As a decoy TNF receptor, TR6 can neutralize the biological function of FasL, LIGHT and TL1A by interfering with the interaction of them with their respective receptors. Since TR6 can block Fas-, LTβR- and DR3-mediated apoptosis (120, 121, 124), it can protect tumour cells from apoptosis mediated by these receptors. As Fas-mediated apoptosis is involved in immune cell-mediated killing, blocking this pathway will impair the tumour specific CTL to kill tumour cells, and help tumour cells evade the anti tumour immune attack.

TR6 secreted by tumour cells can reduce the anti tumour immune responses by blocking T-cell costimulation. TR6 can block T-cell costimulation through HVEM, (the receptor of LIGHT), which is constitutively expressed on T cells. Blocking this pathway will inhibit T-cell responses. TR6 can also reduce T-cell responses by blocking LIGHT and FasL reverse signaling co-stimulatory signals. Moreover, TR6 can block another T-cell costimulation pathway through the interaction between TL1A and DR3 (418). Such blockage of T-cell costimulation will certainly attenuate anti tumour immunity. Another function of TR6 is to inhibit T-cell chemotaxis. Indeed, we found that high level TR6-secreting tumour have less lymphocyte infiltration in the tumour mass compare with low TR6 secreting tumours (data not shown). This suggests that TR6 will help tumour cell

evade anti tumour immune attack by preventing anti tumour T cells to migrate into tumour sites.

A recent study showed that TR6 can induce angiogenesis by neutralizing TL1A-induced angiostatic action through its receptor DR3 (121). This result indicates that TR6 released by tumours not only prevents tumour cell from apoptosis and immune cell-mediated killing, it also help tumour survival by inducing angiogenesis. Taking together, TR6-secreting tumour can gain survival advantage by using TR6 to block apoptosis, inhibit anti tumour immune responses and induce angiogenesis.

As mentioned in the Introduction, TR6 is a secreted member of the TNF receptor family. There is no solid phase TR6 under physiological conditions. In order to study the reverse signaling of its ligands, we put TR6 on solid phase to crosslink its ligands expressed on the T cell surface. In the first part of this project, we proved that TR6 on the solid phase reversely transduces signals into T cells through its ligand(s), and we proved that LIGHT, one of the TR6 ligands, is mainly responsible for the effect. This has led us to explore the therapeutic applications of the solid phase TR6 by expressing it on tumour cell surface as a therapeutic tumour vaccine, and finding that it does enhance antitumour immunity.

4. Future perspectives

Several translational studies based on the functions of TR6 in the immune system are worth exploring.

We have demonstrated that soluble TR6 can inhibit T-cell chemotaxis, and soluble TR6 can block T-cell costimulation. These functions of TR6 can be used to treat graft rejection or autoimmune diseases by either administering soluble TR6, or expressing TR6 on tumour cell surface by recombinant virus vectors to enhance anti tumour immunity.

We have proved in animal models that surface TR6-expressing tumour cells could be used as a therapeutic vaccine to treat both high and low immunogenic tumours. It is feasible to apply this approach to human tumour therapy. TR6 protein can be anchored on autologous tumour cell surface by bifunctional reagents, which could link TR6 protein on tumour cell surface, or by recombinant virus infection. In order to enhance the therapeutic effects, the vaccine could be used in combination with adjuvant, like BCG. The therapeutic effects can be improved by coexpressing some other costimulating molecules, such as B7-1, ICOS, 4-1BB, LIGHT etc., or coexpressing some cytokine(s), such as GM-CSF, IL-12.

Because LIGHT only has a short intracellular tail, which has no enzymatic activity, it is likely that LIGHT reversely transduces signals into T cells through adaptor proteins it associated with. To understand the mechanism of LIGHT reverse signaling, we have demonstrated that LIGHT is indeed associated with an adaptor protein Grb2; additional LIGHT-associated proteins related to its signaling can be identified by very sensitive mass spectrometry analysis which is currently available.

5. The contribution of this study to science

In this project we have studied the biological functions of TR6 and revealed undocumented features of this protein in regulating T-cell responses. It is the first time that the reverse costimulatory signaling is applied in tumour vaccine development. Our results showed that TR6-based tumour vaccine can treat both high and low immunogenic tumours in mouse models. Since many cell surface ligand molecules can reversely transduce costimulatory signals into T cells, this finding has opened a new area in tumour vaccine development. We have also demonstrated, for the first time, that TR6 inhibits T-cell chemotaxis. This finding could explain why there is less lymphocyte infiltration in TR6-secreting tumour mass, and this property of TR6 may contribute to the evasion of tumours from immune surveillance.

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VI. APPENDIX

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VI. Appendix

Some explanations related to the published articles are provided herewith.

1. *The advantages of using 2C T cell as responder cell in CTL activity assay.* In the first paper of this project, 2C mice spleen cells are used as responder cell in the CTL assay. The 2C TCR transgenic mice, which carry functionally rearranged TCR α- and ß-chain genes from a cytotoxic T cell clone 2C, were bred in our animal facility The TCR is specific for class I MHC Ag L^d (1). The offspring of the founder transgenic mice have been backcrossed with C57BL/6 for more than 25 generations and are now of $H\text{-}2^b$ background. The majority (85%) of their peripheral T cells are $CD8^+$, and almost all the $CD8^+$ cells express clonotypic TCRs recognized by mAb 1B2 (1). The transgenic 2C T cells are functional in that they could develop into L^d-specific cytotoxic T cells in vitro (2, 3). Because of the high frequency of anti-$H2^d$ T cells in the 2C spleen cells, this system provides an enhanced signal/noise ratio and sensitivity in the CTL assay.

2. *The control protein NhIgG and TR11 are interchangeable in our experiments.* In our initial experiments, we used TR11 and NhIgG as control proteins for TR6-Fc. We found that both proteins on solid phase had no function in terms of T cell costimulation; therefore, in the subsequent experiments, these two control proteins were used interchangeably.

3. *The details of LIGHT knockout mice.* $LIGHT^{-/-}$ mice described in the article were generated by Dr Lieping Chen (4), and were provided to us by our collaborator Dr

Jun Zhang (Human Genome Sciences Inc). The description and data related to these mice are now provided in revised article 3.

4. *TR6 based tumor cell vaccine is a promising approach in cancer immunotherapy.* As mentioned in the Introduction and the third article, many costimulatory molecules, such as, B7-1 and B7-2 (5), B7h (6, 7), CD40L (8, 9, 10), OX40L (11), 4-1BBL (12), CD27L (13) and LIGHT (14, 15), can be expressed on the tumor cell surface to initiate and enhance host antitumour immunity. Among these costimulatory molecules, only CD40L, CD27L and LIGHT are reported to induce anti-tumour immunity to tumor cells of low antigenicity. For the other costimulatory molecules, it is necessary to coexpress more than one costimulatory molecules or to coexpress the costimulatory molecule with cytokines to achieve rejection of low antigenic tumours. Our results show that TR6 expression on the tumor cell surface induces strong antitumor immunity against tumors with both high and low antigenicity tumours. It is possible that such antitumour immune responses can be further enhanced by coexpressing TR6 with other costimulatory molecules or cytokines on tumour cell surface.

5. *The anti-human LIGHT mAb 1.2.2 most likely binds to mouse LIGHT.* In the first article of this thesis, we used anti-human LIGHT mAb 1.2.2 to study the LIGHT reverse signaling in mouse T cells. Based the following reasons, we believe that mAb 1.2.2 binds to mouse LIGHT. First, as mentioned in article, mouse LIGHT shares 67% homology with human LIGHT in its amino acid sequence. Data from Dr Wu's group have proved using a Biocore assay that human TR6 recombinant protein

binds to mouse LIGHT (16). Secondly, the binding profile of the anti-human LIGHT mAb is consistent with the LIGHT expression kinetics on the T cells. LIGHT expression is inducible on the T cell surface during T cell activation, similarly, the binding of the anti-LIGHT mAb was only detectable on activated T cells but not resting T cells. Thirdly, we did immunoprecipitation (data not show) using mAb 1.2.2 and detected the protein by Western blot using a rabbit anti-human LIGHT polyclonal Ab. We found that both human LIGHT protein (as positive control) and the protein precipitated by mAb 1.2.2 could be detected by the polyclonal anti-LIGHT Ab, and the size of the precipitated band is the same as that of mouse LIGHT. Taken together, these results indicate that the anti-human LIGHT mAb 1.2.2 binds to mouse LIGHT on the T cell surface. Final proof of the specificity of the anti-human LIGHT mAb against mouse LIGHT will be obviously come from an experiment in which mouse recombinant LIGHT is employed.

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