Regulation of T cell function by interaction between a TNF receptor family member DcR 3/TR6 and a TNF family member LIGHT

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Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophiae Doctor (Ph.D.)

en Sciences Biomédicales

September, 2003

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:

«REGULATION OF T CELL FUNCTION BY INTERACTION BETWEEN A TNF RECEPTOR FAMILY MEMBER DCR3/TR6 AND A TNF FAMILY MEMBER LIGHT»

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SUMMARY

TR6, also named DcR3, M68, is a recently identified soluble receptor belonging to the TNFR superfamily. FasL, LIGHT and TL1 are 3 so-far identified ligands for TR6, and they all belong to the TNF ligand superfamily. TR6 can interfere with FasL and Fas interaction. It can also interfere with the interaction between LIGHT and HveA, and between TL1 and DR3. Some tumors have high TR6 expression. It has been hypothesized that TR6-secreting tumors use this molecule to evade immune surveillance and gain survival advantage.

In this project, the role of TR6 in immune regulation was investigated. We demonstrated that human TR6 could cross-react with mouse LIGHT. As the mouse counterpart of TR6 unlikely exists according to Genebank search, this finding allowed us to use human TR6 in the mouse system. In the mouse system, soluble human TR6 could suppress IL-2, IL-5 and GM-CSF secretion by mitogen-activated T cells, and downregulate cytotoxic T-cell development in vitro. In vivo, soluble TR6 could suppress graft versus host disease and allograft rejection. These effects of TR6 are probably achieved by its interference with the interaction between LIGHT and HveA on T cells.

In human system, we found that solid phase TR6, in the presence of suboptimal solid phase anti-CD3, could significantly costimulate T cells in terms of proliferation. Blocking studies using soluble LIGHT and Fas indicated that LIGHT likely mediated the costimulation. This has revealed a novel mechanism of TR6 triggered reverse signaling

through a ligand, LIGHT. Base on this finding, it is likely that the suppressive effect of soluble TR6 in the mouse system is due to its interference of the two-way costimulation between HveA and LIGHT. Intriguingly, soluble TR6 could augment T-cell proliferation, lymphokine production and cytotoxic T-cell activity in the human system. The opposite effect of soluble TR6 in the human and mouse systems is probably due to different affinity of TR6 to human and mouse LIGHT. The dimeric TR6-Fc might have higher affinity to human LIGHT, hence capable of triggering strong reverse costimulation through LIGHT. Although it might block the two-way costimulation between HveA and LIGHT and abate the immune response, the overall effect is dominated by reverse costimulation through LIGHT. In the mouse system, affinity of human TR6 to mouse LIGHT might be lower, and cannot effectively trigger LIGHT reverse signaling; its overall effect is thus biased to the blocking of the two-way interaction between HveA and LIGHT, hence repressed immune responses.

We also found that human peripheral blood mononuclear cells could secrete TR6 and the secretion was enhanced by T-cell activation. Interestingly, both soluble and solid phase TR6 was able to suppress mitogen-induced T-cell aggregation. T cells pretreated with TR6 had reduced actin polymerization and pseudopodium formation, which are both important for the cell-cell interaction. These results suggest that TR6 might regulate the duration of T-cell interaction with other cells, and allow T cells to disengage from antigen presenting cells or fellow T cells once the interaction becomes unnecessary.

This study has discovered three important functions of TR6 in immune regulation. Some of the findings, such as the suppressive effect of soluble TR6 in immune response and enhancing effect of solid phase TR6 could be exploited for clinical applications. Our findings have also broadened our knowledge on TR6 in immune regulation.

Key words: TR6/DcR3; LIGHT; reverse signaling; costimulation

Résumé

Récemment, TR6 aussi connu sous le nom de DcR3 ou M68, a été identifié comme un récepteur soluble appartenant à la superfamille du récepteur TNF. Les ligands FASL, LIGHT et TL1, qui font partie de la superfamille du ligand TNF, ont la capacité de lier le récepteur TR6. TR6 peut interférer au niveau de l'interaction de Fas et FasL, de LIGHT et HveA ou encore de TL1 et DR3. Puisque certaines tumeurs expriment fortement le récepteur TR6, il a été proposé que les tumeurs sécrétant TR6 pourraient échapper à la surveillance du système immunitaire, par un avantage de croissance.

Ce projet explore le rôle du récepteur TR6 dans la régulation immunitaire. Nous avons démontré que le récepteur TR6 humain peut interagir avec le ligand LIGHT de souris. Cette observation nous a permis d'utiliser le récepteur TR6 humain dans un modèle de souris, puisque l'équivalent murin de TR6 n'existe pas selon les recherches effectuées à partir de Genebank. Chez le modèle murin, le récepteur humain TR6 soluble peut inhiber la sécrétion de IL-2, IL-5 et GM-CSF produite par les cellules T activées et régule négativement le développement des cellules T cytotoxiques in vitro. In vivo, le récepteur TR6 soluble peut supprimer la présence de greffes en favorisant le développement de maladies auto-immunes et le rejet des allogreffes. Ces effets biologiques associés à TR6 sont probablement initiés par l'interférence de TR6 dans l'interaction de LIGHT et HveA dans les cellules T.

Chez un modèle humain, nous avons trouvé qu'en présence d'une concentration suboptimale de la phase solide d'un anti-CD3, la phase solide de TR6 costimule

significativement la prolifération des cellules T. Des études d'inhibition impliquant le ligand LIGHT soluble et Fas indiquent que LIGHT est responsable de la costimulation. Ceci suggère un nouveau mécanisme impliquant une inversion de la signalisation liée à l'activation de TR6 par le ligand LIGHT. Basé sur cette observation, l'effet suppresseur du récepteur TR6 soluble dans le modèle de souris est dû à l'interférence de la costimulation à deux sens entre HveA et LIGHT. Étonnamment, le récepteur TR6 soluble peut augmenter la prolifération des cellules T, la production de lymphokine et l'activité des cellules T cytotoxiques dans le modèle humain. L'effet opposé du récepteur soluble TR6 dans le modèle de souris et humain est probablement le résultat d'une différence d'affinité de TR6 pour le LIGHT humain et le LIGHT murin. Le dimère TR6-Fc pourrait avoir une plus grande affinité pour le LIGHT humain, d'où sa capacité à déclencher une forte costimulation inversée induite par LIGHT. Dans le modèle de souris, l'affinité du TR6 humain pour le LIGHT murin pourrait être plus faible et, par conséquent, incapable de déclencher efficacement la signalisation inversée. Cet effet est alors biaisé par l'inhibition de l'interaction à deux sens de HveA et LIGHT, d'où la répression de la réponse immunitaire.

Nous avons également trouvé que les cellules mononucléaires du sang périphérique peuvent sécréter le récepteur TR6, sécrétion qui serait augmentée après une activation des cellules T. De façon intéressante, les phases soluble et solide de TR6 sont capables d'inhiber l'agrégation des cellules T activées. Les cellules T pré-traitées avec TR6 ont une réduction de la polymérisation de l'actine ainsi qu'une diminution de la formation des pseudopodes, toutes deux importantes pour les interactions cellules-cellules. Ces résultats

suggèrent que TR6 régule la durée des interactions des cellules T avec les autres cellules, conduisant à un désengagement des cellules présentatrices d'antigènes ou des autres cellules T, une fois les interactions devenues non essentielles. Cette étude a permis la découverte de trois fonctions importantes du récepteur TR6 dans la régulation immunitaire. Certaines de ces découvertes, telles que l'effet inhibiteur du récepteur TR6 soluble dans la réponse immunitaire ainsi qu'une augmentation de l'effet du TR6 de la phase solide, pourraient être exploitées pour des applications cliniques. Nos observations ont également permis d'élargir nos connaissances sur le rôle du récepteur TR6 dans la régulation immunitaire.

Mots clé: TR6/DcR3; LIGHT; reverse signaling; costimulation

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LIST OF ABBREVIATIONS

AA	Amino acid residues
ACBP	Acyl-CoA binding protein
AIF	Apoptosis-inducing factor
Ag	Antigen
AP-1	Transcription factor activator protein 1
Apaf-1	apoptosis protease activating factor-1
APC	Antigen-presenting cells
BSAP	B cell lineage-specific activator protein
Caspase	Cytosolic aspartate-specific protease
CRD	Cysteine rich domain
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DcR 3	Decoy receptor 3
DD	Death domain
DR	Death receptor
EST database	Expressed sequence tagged database
FADD	Fas-associated death domain
FasL	Fas ligand
GPI	Glycosylphosphatidylinositol
GvHD	Graft versus host disease
HtrA2	High temperature required A2
IAP	Inhibitor of apoptosis protein
Ig	immunoglobulin
IL-2	Interleukin 2
IFN- γ	Interferon-γ
KO	Knockout
LC	Langerhans cells
LN	Lymph nodes
LPS	Lipopolysaccharide
LTα	Lymphotoxin α
LTβ	Lymphotoxin β
LTβR	Lymphotoxin beta receptor
HVEM/TR2	Herpes virus entry mediator
JNK	c-jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MLR	Mixed lymphocyte reaction
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
SFP	Superfamily protein
Smac	Second mitochond-derived activator of caspase
SODD	Silencer of death domain
Tg	Transgenic
Th1/2	T helper cell 1 and 2
TNF	Tumor 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

Cytokines are important glycoprotein messenger molecules capable of transmitting signals from one cell to another. Most cytokines exist in secreted form but some are either expressed at the cell surface or stored in the extra-cellular space. To date more than 200 cytokines have been identified including interleukins, growth factors, chemokines, interferons, and a host of others (Callard *et al.*, 1999; Locksley *et al*, 2001). Cytokines need to interact with their receptors expressed on the surface of the target cells, thereby triggering complex intercellular signaling cascades, which ultimately control gene expression required for the cellular response. Under normal circumstances, the production of cytokines and the expression of their receptors are under tight and complex biological control, including negative and positive feedback by the cytokines themselves. Cytokines can be divided into several groups such as the hematopoietins, the interferons, the tumor necrosis factor (TNF)-related molecules, the immunoglobulin (Ig) superfamily members, and the chemokines. Among these groups, the TNF superfamily is unique since members of this superfamily are mainly concentrated in the immune system and display crucial functional roles in regulating immune responses (Gruss and Dower, 1995; Gruss *et al.*, 1996).

I.1. *TNF and TNF receptor superfamilies*

TNF ligand superfamily was originated from two proteins, TNF-alpha (TNF α) and lymphotoxin α (LT α). These two structurally and functionally related but distinct proteins, identified by the property of tumor cell lysis, were the prototypic members of

the TNF superfamily (Carswell *et al.*, 1975; Gray *et al.*, 1984, Pennica *et al.*, 1985). The receptors of TNF α are TNFR-I (p55) and TNFR-II (p75) whereas the receptors for LTα are TNFR-I, TNFR-II and HVEM/TR2 (Locksley *et al.*, 2001). These receptors then constituted a new TNF receptor (TNFR) related gene family (Gruss and Dower, 1995; Locksley *et al.*, 2001). Both TNFR and TNF ligand superfamilies have experienced rapid expansion over the past decade and many molecules were identified as TNF or TNFR superfamily proteins (SFPs) (Smith *et al.*, 1994; Idriss and Naismith, 2000; Locksley *et al.*, 2001).

For the past decades there was no well-coordinated, systematic naming system and the nomenclatures for SFPs in these two superfamilies were complicated and somewhat redundant. It was common that some members had multiple names given by different groups (as presented in Table 1 and Table 2). Based on this situation, a standard, official designation system was formally introduced for both TNF ligand and TNFR SFPs while some popular, well-accepted names are still being used in parallel (Locksley *et al.*, 2001). (Details refer to Table 1 for related receptors and Table 2 for related ligands).

I.2. *TNFR family*

Currently, this still growing family has incorporated more than 20 different membrane proteins and several open viral reading frames encoding related molecules (Locksley *et al.*, 2001; Adams *et al.*, 2002). As summarized in Table 1, the mammalian TNFR superfamily now includes: TNFR-I, TNFR-II, Fas, OX40, CD40, CD27, CD30, 4-1BB, DcR1, DcR2, TR6 (DcR3), OPG, DR3, DR4, DR5, DR6, HVEM (TR2), RANK, TACI, BAFFR, EDAR, BCM, RELT, SOBa, Tnfrh1, TAJ (Armitage, 1994; Smith, 1994;

Table 1. TNFR Superfamily Members

Official Symbol	Other Common Names	References
TNFRSF1A	P55-R, CD120a, TNF-R-I p55, TNF-R, TNFAR, TNF-R55, p55TNFR,TNFR60	Van Arsdale and Ware, 1994
TNFRSF1B	CD120b, p75, TNF-R, TNF-R-II, TNFR80, TNFR2, TNF-R75, TNFBR, p75TNFR	Santee and Owen-Schaub, 1996; Smith et al., 1990
LTBR	TNFRSF3, TNFR2-RP, CD18, TNFR-RP, TNFCR, TNF-R-III	Gruss and Dower, 1995
TNFRSF4	OX-40, ACT35, TXGP1L	Godfrey et al., 1994
TNFRSF5	P50, Bp50, CD40	Banchereau et al., 1994
TNFRSF6	FAS, CD95, APO-1, APT-1	Itoh and Nagata, 1991
TNFRSF6B	DcR3, TR6	Pitti et al., 1998; Bai et al., 2000
TNFRSF7	Tp55, S152, CD27	Camerini et al., 1991; Loenen et al., 1992
TNFRSF8	Ki-1, D1S166E, CD30	Smith et al., 1993; Gruss et al., 1995
TNFRSF9	4-1BB, CD137, ILA	Kwon et al., 1994
TNFRSF10A	DR4, Apo-2, TRAILR-1	Pan et al., 1997
TNFRSF10B	DR5, KILLER, TRICK2A, TRAIL-R2, TRICKB	Pan et al., 1997; Sheridan et al., 1997
TNFRSF10C	DcR1, TRAILR3, LIT, TRID	Sheridan et al., 1997;
TNFRSF10D	DcR2, TRUNDD, TRAILR4	Pan et al., 1998; Marsters et al., 1997
TNFRSF11A	RANK	Anderson et al., 1997
TNFRSF11B	OPG, OCIF, TR1	Emery et al., 1998; Simonet et al., 1997
TNFRSF12	DR3, TRAMP, WSL-1, LARD, WSL-LR, DDR3, TR3, APO-3	Chinnaiyan et al., 1996; Masters et al., 1996
TNFRSF12L	DR3L	Grenet et al., 1998
TNFRSF13B	TACI	Xia et al., 2000; Gross et al., 2000
TNFRSF13C	BAFFR	Thompson et al., 2001
TNFRSF14	HVEM, ATAR, TR2, LIGHTR, HVEA	Mauri et al., 1998; Kwon et al., 1997
NGFR	TNFRSF16	Gruss and Dower, 1995
EDAR	EDAR	Srivastava et al., 1997
TNFRSF17	BCM, BCMA	Gross et al., 2000
TNFRSF18	AITR, GITR	Kwon et al., 1999;
TNFRSF19	TAJ, TROY	Hu et al., 1999
TNFRSF19L	RELT	Sica et al., 2001
TNFRSF21	DR6	Pan et al., 1998
TNFRSF22	SOBa, Tnfrh2	Clark et al., 2002
TNFRSF23	MSOB, Tnfrh1	Clark et al., 2002

Table 2. TNF Ligand Superfamily

Symbol	Other names	References
LTA	TNFSF1, TNFB, LT	Gray et al., 1984; Browning et al., 1993
TNF	TNFSF2, TNFA, DIF	Pennica et al., 1984; Takeda et al., 1986
LTB	TNFSF3, TNFC, p33	Browning et al., 1991
ED1	EDA, EDA1	Bayes et al., 1998;Srivastava et al., 1997
TNFSF4	OX-40L, gp34, TXGP1	Godfrey et al., 1994
TNFSF5	CD40LG, CD40L, IMD3, HIGM1, hCD40L, TRAP, CD154, gp39	Armitage et al., 1992; Graf et al., 1992
TNFSF6	FasL, APT1LG1	Tanaka et al., 1995; Suda et al., 1993
TNFSF7	CD70, CD27L, CD27LG	Goodwin et al., 1993; Bowman et al., 1994
TNFSF8	CD30LG	Smith et al., 1993
TNFSF9	4-1BB-L	Goodwin et al., 1993; Alderson et al., 1994
TNFSF10	TRAIL, Apo-2L, TL2	Wiley et al., 1995
TNFSF11	TRANCE, RANKL, OPGL, ODF	Wong et al., 1997
TNFSF12	TWEAK, DR3LG, APO3L	Chicheportiche and Browning, 1997
TNFSF13	APRIL	Hahne et al., 1998
TNFSF13B	Blys, BAFF, THANK,TALL1	Moore et al., 1999
TNFSF14	LIGHT, Ltg, HVEM-L	Mauri et al., 1998; Zhai et al., 1998
TNFSF15	TL1,VEGI	Zhai et al., 1999, Mitogne et al., 2002; Tan et al., 1997
TNFSF18	AITRL, TL6, hGITRL	Kown et al., 1999
TNFSF 20	Withdrawn	

Anderson *et al.*, 1997; Ashkenazi and Dixit, 1998; Gruss and Dower, 1995; Locksley *et al.*, 2001). Some most recent identified members such as BCM, RELT, SOBa, Tnfrh1 and TAJ are currently still not well defined (Locksley *et al.*, 2001). The viral open reading frames encoding soluble TNFRs such as crmB (Hu *et al.*, 1994), Va53 (Smith *et al.*, 1990) G4RG (Howard *et al.*, 1991), and SFV-T2 (Upton *et al.*, 1987) were also identified.

I.2.1. *The structural features of the TNFR superfamily*

The mammalian TNFR family members are type I membrane proteins, whose extracellular N terminal part contains ligand-binding domain. A remarkable feature for TNFR superfamily is the low degree of sequence homology in their extracellular ligand-binding domain (20-25%) (Gruss and Dower, 1995). These SFPs are mostly trimeric (Gruss and Dower, 1995; Armitage *et al.*, 1994; Aggarwal *et al.*, 1996; Bazzoni and Beutler, 1996).

The definition of the TNFR superfamily is mainly based on the conserved motif of "cysteine-rich repeats" in the extracellular N-terminal region. These common conserved cysteine-rich domains are also termed as cysteine-rich motif or cysteine-rich domain (CRD), which consists of multiple cysteine-rich repeats of approximately 30-40 amino acids (Smith, 1994). In general, each member of this family contains varying numbers (2-6) of CRDs as shown in Figure 1. Each CRD is featured by the presence of approximately 6 cysteine residues that are interspersed within CRD domain. The structure of CRD is supported by 3 intrachain disulfide bonds formed by these 6 highly conserved cysteines (Smith *et al.*, 1994). These multiple cysteine-rich domains in the

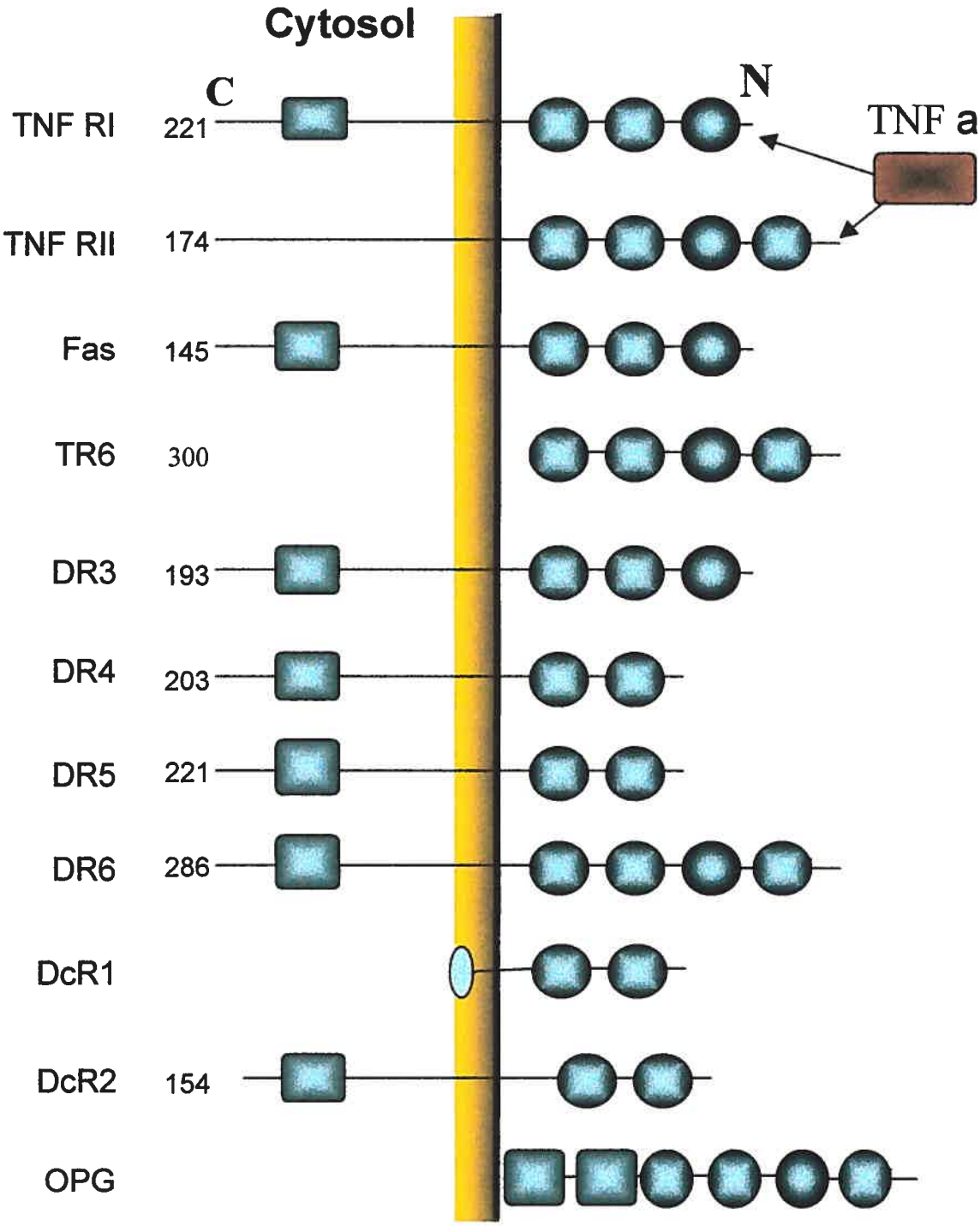

Figure 1. Structural features of TNF receptors. There are two structural domains for common TNF receptors. The cysteine rich domains (CRD) ( ) are located in the extra-cellular N-terminal portion. Several receptors also have a death domain (DD) ( ).

extracellular part have been shown to be involved in ligand binding (Locksley *et al.*, 2001).

The extracellular structure of TNFR SFPs can be best illustrated by TNFR and DR5. These two receptors have been well studied for their crystal structure, which represents common structural features of all TNFR SFPs (Naismith *et al.*, 1996; Hymoowitz *et al.*, 1999). For example, DR5 forms an extended rod-like shape consisting of 3 CRDs which form the interface to bind its cognate ligand(s). DR5 had a total of 7 disulfide bridges: 6 are in CRD 2 and CRD3 (three for each) and 1 in C-terminal part. These disulfide bonds form a structural scaffold and two patches formed are located there for ligand binding. The combination of structural conservation and variable amino acid sequences in the ligand contacting region confer the ligand-binding specificity. Notably, the structure of TNFR is rather flexible and can be optimized for its interaction with ligand through a series of hinging movements (Idriss and Naismith, 2000).

In addition to the membrane-bound form, many TNF receptors also exist in a soluble form. These soluble molecules are, in many cases, generated by proteolytic cleavage of cell surface receptors. Soluble TNFR-I, TNFR-II, CD27, CD30, CD40, and Fas are all generated in this fashion (Gruss and Dower, 1995). The only exception is 4-1BB, whose soluble form is generated by alternative splicing (Gruss and Dower, 1995). The necessity of the soluble forms of these receptors is not fully understood.

I.2.2. *The biological features of TNFR superfamily*

The TNFR SFPs have emerged as prominent regulators of the immune system (Tracey and Cerami, 1994). In the immune system, TNFR SFPs are well known for their critical

roles in regulating immune responses such as cell activation, proliferation, differentiation, apoptosis, immunoglobulin (Ig) class switching, immune evasion, and immune suppression (Smith *et al.*, 1994; Aggarwal *et al.*, 1996; Tewari and Dixit, 1996; Baker *et al.*, 1996; Locksley *et al.*, 2001). Some SFPs are also involved in the generation and development of lymphoid organs (Matsumoto *et al.*, 1997).

The primary feature of TNFR superfamily members is cell death induction including tumor killing and this effect was observed several decades ago (Carswell *et al.*, 1975). Many, if not all, TNFR members are related to cell death induction. Members such as TNF, LTα, CD30L, CD95L, 4-1BBL are all capable of inducing cytotoxic cell death (Gruss *et al.*, 1996; Gruss and Dower, 1995). Probably this ability to induce cell death is one of the unique features with remarkable adaptive value that TNF/TNFR SFPs have developed (Gruss and Dower, 1995; Locksley *et al.*, 2001). As will be detailed in the next section, several TNF receptors contain a structure called "death domain" which is responsible for cell death induction and these receptors are termed "death receptors" (DR). There are 8 receptors containing "death domain" in TNFR superfamily and at least 6 of them can induce apoptosis through activation of caspases (Screaton and Xu, 2000; Raff, 1998). Meanwhile, other TNF/TNFR SFPs lacking death domains can potentially modulate the response to DRs or directly influence cell death/survival. For instance, TNFR-II markedly enhances TNFR-I induced T cell death and CD40 can augment Fas-induced B cell death (Garrone *et al.*, 1995; Chan *et al.*, 2000b).

In contrast to the death induction feature of TNFR SFPs, it is interesting that they are also closely related to lymphocyte survival including T/B cell proliferation and differentiation (Locksley *et al.*, 2001). Indeed, the major documented function of TNFR SFPs is

associated with lymphocyte survival. For instance, Blys (official symbol: TNFSF13B, or THANK, BAFF, see Table 2) expressed on activated dendritic cells can interact with the TACI and BCMA receptors (both are TNFR members, see Table 1) on B cells and promote B cell survival (Laabi and Stresser, 2000, Moore *et al.*, 1999). Similarly, T cell activation is also regulated by TNF/TNFR SFPs. For instance, LTβR enhances T cell activation and promotes T cell clone expansion by engaging with its ligand LIGHT (Tamada *et al.*, 2002). Some other members such as CD40 and Fas may also stimulate T cell survival and proliferation by engaging with their receptors (Cayabyab *et al.*, 1994; Suzuki *et al.*, 1998).

In addition to modulating lymphocyte death/survival, SFPs of this superfamily are also associated with antigen presenting cells (APC) survival and maturation. For instance, dendritic cells (DCs) are potent APCs for antigen presentation but fully differentiated and mature DCs will undergo rapid apoptosis. The life span of mature DCs can be prolonged substantially by TNF SFPs such as CD40L, TNF, TRANCE (TNF related activation-induced cytokine) and recently identified member LIGHT (Wong *et al.*, 1997; Tamada *et al.*, 2002).

Some, if not most, TNFR SFPs are involved in T cell costimulation. For example, both TNFR-I and Fas can co-stimulate T cell activation under different settings (Siegel *et al.*, 2000; Suzuki *et al.*, 2000A, 2000B). In addition, the SFPs such as OX40, CD27, and 4-1BB regulate the activation and expansion of CD4+ and CD8+ T cells responding to dendritic cells bearing their respective ligands. Costimulation effects were also observed for LTβR/LIGHT pair. It was found most likely that this pair provides costimulation for T cell activation and plays a critical role in T cell activation independent of B7/CD28

costimulation pathway (Tamada *et al.*, 2000; Wang *et al.*, 2001). Thus, it is suggested that TNFR SFPs might be critically important in negative selection and determine the activation and/or apoptosis of T cells (Sebzda *et al.*, 1999; Wang *et al.*, 2001).

Another well-documented function of TNFR superfamily is to orchestrate permanent lymphoid organ structure. A remarkable example is LTβR that belongs to TNFR superfamily and plays critical roles in the immune system. Several studies showed that $LT\beta R^{-/-}$ mice lacked lymph nodes (LN) and Peyer's patch (PP) and displayed severe disorganization of splenic architecture, featured by the absence of T/B cell segregation, marginal zones, follicular dendritic cell (FDC) networks, and germinal centers (GC) (De Togni *et al.*, 1994; Banks *et al.,* 1995; Wu *et al.*, 1999). Depletion of RANK or RANKL lead to the disappearance of all peripheral and mesenteric lymph nodes while Peyer's patches remain intact and the splenic architecture is unaffected (Kim *et al.*, 2000; Kong *et al.*, 1999; Dougall *et al.*, 1999). Studies demonstrated that the requirements for RANK/RANKL and $LT\alpha 1\beta 2$ or LTβR do not compensate for each other. All these data suggest that SFPs of TNFR superfamily are necessary for the formation and development of lymphoid immune organs, some of them being indispensable.

In addition to critical functions in the immune system, other functions were also identified for TNFR SFPs. For instance, Edar is a death domain containing protein and recent studies indicate that this protein is important in the development of hair, teeth and other ectodermal derivatives (Headon *et al.*, 2001).

I.2.3. *Subsets of TNFR superfamily*

Based on their structural and biological features, the TNFR superfamily SFPs can be further divided into different groups according to the presence of featured domains in the intracellular portion of the receptor. The SFPs in the first group featured by a TNFR-associated factor (TRAF) binding domain that enables coupling to TRAFs, that in turn activate a signaling cascade that results in the activation of NF-κB and initiation of transcription (Rothe *et al.*, 1995; Wallach *et al.*, 1999). The second group of receptors is featured by a 60- amino acid globular structure named "death domain" (DD) and these death domain-containing receptors are termed as death receptors (DRs) (Tartaglia *et al.*, 1993; Itoh and Nagata 1993). The third group has drawn more attention recently and members of this group are designated as decoy receptors (DcRs) that include DcR1, DcR2, DcR3/TR6 and OPG (Ashkenazi and Dixit, 1999). All members in this group except DcR2 do not have cytoplasmic domain and thus they may act as inhibitors and compete with other signal-transducing receptors for ligand binding (Ashkenazi and Dixit, 1999; Ashkenazi, 2002).

I.2.3.1. *TRAF associated subgroup*

The TRAFs are adaptor proteins belonging to ring and zinc-finger proteins. The members of this group are TNFRII, CD40, CD30, CD27, LTβR, 4-1BB, OX 40, NGFR, HVEM, GITR and RANK (as presented in Table 1). They bind directly to the receptor's cytoplasmic tail. They also bind to one another in homotypic and/or heterotypic interactions. They are believed to convey certain signals in proliferative and pro-inflammatory responses. This is achieved, in large part, by activation of NF-κB, a transcription factor involved in numerous proliferative and pro-inflammatory events

(Jabara *et al.*, 2002; Wallach *et al.*, 1999; Harrop *et al.*, 1998). So far at least 6 TRAFs have been found to associate with non-DD TNF receptors (Locksley *et al.*, 2001). Most TNFR SFPs have no death domains but contain a consensus motif which is capable of binding to the TRAF proteins (Ye *et al.*, 1999).

I.2.3.2. *Death receptor subgroup*

The above mentioned "death domain" was first coined from studies of deletion mutagenesis involving TNFR-I mediated apoptotic cell death (Tartaglia *et al.,* 1993). The proteins harboring "death domains" were thus defined as death receptors (DRs) and they form DR group within the TNFR superfamily (Ashkenazi and Dixit, 1999; Locksley *et al.*, 2001). This death-domain-containing receptor subgroup now includes up to 8 members: TNF-R1, Fas, and recently discovered DR3 (Chinnaiyan *et al.*, 1996; Marsters *et al.*, 1996; Kitson *et al.*, 1996; Bodmer *et al.*, 1997; Screaton *et al.,* 1997), DR4 (Pan *et al.*, 1997), DR5 (Pan *et al.*, 1997) and DR6 (Pan *et al.*, 1998), EDAR (Tucher *et al.*, 2000) and NGFR (Gruss, 1996). The typical conserved death domain (DD) is a 68 amino acid segment located within the cytoplasmic region of the receptor. Upon ligation with either cognate ligands or specific agonistic antibodies, death receptors can activate an apoptotic signaling pathway (Nagata, 1997).

These death receptors have common structural features such as 2-6 CRDs in their extracellular domains and an intracellular death domain. The death domain most likely functions as a protein interaction domain and provides docking sites for signaling molecules, therefore enabling each receptor to couple to the caspase cascades which are critical for the induction of apoptosis.

I.2.3.3. *Decoy receptor subgroup*

Another subgroup of the TNFR superfamily was termed "decoy molecule". This decoy receptor subgroup now includes DcR1 (decoy receptor 1, also known as TRID or TRAIL-R3) (Pan *et al.*, 1997; Degi-Esposti *et al.*, 1997; Sheridan *et al.*, 1997; Pitti *et al.*, 1998), DcR2 (decoy receptor 2, also known as TRUNDD or TRAIL-R4) (Pan *et al.*, 1998; Degli-Esposti *et al.*, 1997), DcR3 (TR6) (Pitti *et al.*, 1999) and osteoprotegerin (OPG) (Simonet *et al.*, 1997; Ashkenazi and Dixit, 1999). DcR1 and DcR2 are cell surface proteins whereas DcR3 and OPG are soluble molecules. Both DcR1 and DcR2 bind to TRAIL with a similar affinity (Pan *et al.*, 1997A; Pan *et al.*, 1997B; Sheridan *et al.*, 1997). DcR1 is a glycosylphosphatidylinositol (GPI)-linked protein without an intracellular death domain whereas DcR2 is a transmembrane receptor but with a partially deleted death domain and accordingly, both are incapable of transmitting apoptotic signals (Ashkenazi and dixit 1999; Deli-Esposti *et al.*, 1999). 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 (Pitti *et al.*, 1998).

Both DcR1 and DcR2 are believed mainly to function as anti-apoptotic decoy receptors that compete with DR4 and DR5 for TRAIL binding and consequently protect those receptor-bearing cells from TRAIL-induced apoptosis (Ashkenazi and dixit, 1998; Deli-Esposti *et al.*, 1999). DcR1 is a GPI-linked protein and its role in active signal transduction has not been fully elucidated. Emerging evidence suggests that DcR2 may be involved in activating signal transduction since it has been shown to activate NF-κB in

some systems (Degli-Esposti *et al.*, 1997). In contrast, there are reports of failure of DcR2 to activate NF-κB in certain systems (Meng, 2000). Thus more studies are necessary to explore the role of DcR1 and DcR2 in anti-apoptosis signal transduction and in apoptosis.

I.2.3.4. *General characteristics of TR6*

TR6 is a novel TNF decoy receptor with the official designation of TNFRSF6B (see Table 1). By screening expressed sequence tagged (EST) database, Pitti (1998) identified a previously unknown full-length cDNA from a human fetal lung library. This cDNA showed homology to other TNFR superfamily members. The protein encoded by this gene was then named decoy receptor 3 (DcR 3) (Pitti *et al.*, 1998). This gene was also independently identified by other groups and named TR6 (Yu *et al.*, 1999) or M68 (Bai *et al.*, 2000), respectively. The human TR6 gene is mapped at position 20q13.3 (Pitti *et al.*, 1998), whilst its mouse counterpart might not exist since a BLAST search of the mouse genome did not reveal sequences with any significant homology. Since the position of 20q 13 is also named "cancer amplicon" where genes responsible for many types of tumors are located, TR6 may be a candidate gene associated with certain types of malignancy (Korn *et al.*, 1999; Medeiros *et al.*, 1999; Stubbs *et al.*, 1999; Savelieva *et al.*, 1997;Sonoda *et al.*, 1997; Sakakura *et al.*, 1999).

The full-length open reading frame of TR6 encodes 300 amino acid residues with the first 29 amino acid residues as signal sequence. The mature form of TR6 has 271 amino acid residues with no transmembrane region. There is only one potential N-linked glycosylation site at Asn-173. Like OPG, TR6 was regarded as a soluble secreted protein.

TR6 shares remarkable amino acid sequence homology with OPG (31%) and TNFR-II (29%) but less homology with Fas (17%) (Pitti *et al.*, 1998). TR6 was reported to have two complete and two incomplete cysteine-rich motifs, which is the hallmark of TNF receptors.

The TR6 mRNA has prominent expression in lung and colon cancers, whereas it is weakly detectable in several hematopoietic cell lines, and is hardly detectable in many tissue samples (Pitti *et al.*, 1998). In the immune system, TR6 is highly expressed in both lymph nodes and spleen, but the expression in thymus is weak (Pitti *et al.,* 1998; Bai *et al.*, 2000). In addition, TR6 is expressed in an endothelial cell line and its expression is inducible by phorbol 12-myristate 13-acetate (PMA) /ionomycin in Jurkat T leukemia cells (Yu *et al.*, 1999). Moreover, TR6 mRNA over-expression was reported in gastrointestinal cancers but without gene amplification (Bai *et al.*, 2000). Also, the expression of TR6 can be detected in malignant glioma cells as well as in human glioblastomas. In addition to human tumors, the TR6 gene is also over-expressed in silicosis or systemic lupus patients (Otsuki *et al.*, 2000).

Mild *et al.* investigated a large number of colorectal cancers and found nearly 63% (185 out of 294) of patients with DcR3 gene amplification (Mild *et al.*, 2002). In another study, it was found that TR6 was amplified and over expressed in EB virus- and human T cell leukemia virus-I– associated lymphomas (Ohshima *et al.*, 2000). This expression trend is also true at the protein level of TR6. For instance, TR6 protein was found over-expressed in human adenocarcinomas of the esophagus, stomach, colon, and rectum while no gene amplification was detected (Bai *et al.,* 2000). It was reported recently that up to 73% (163 out of 223 patients) of colorectal patients showed up-regulated TR6

protein level (Mild *et al.*, 2002). These data suggested that certain types of tumor as well as EBV and human T cell leukemia virus-1 may use TR6 to escape from immune surveillance during lymphomagenesis, or that virus infected lymphoma cells with TR6 expression might be selected during multi-step tumorigenesis (Ohshima *et al.*, 2000). Recent studies in our laboratory as well as in others suggested that TR6 over-expression correlates with the grade of malignancy and could be used as diagnostic and prognostic parameter (Wu *et al.*, 2003; Roth *et al.*, 2001).

I.2.3.5. *The TR6 Ligands*

The FasL (Fas ligand) was the first ligand identified for TR6 (Pitti *et al.*, 1998). FasL is one of the major effectors of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and a major inducer of apoptosis. It is also involved in the establishment of peripheral tolerance in the activation-induced cell death of lymphocytes (Zhang *et al.*, 2000; Ju *et al.*, 1999). Moreover, the expression of FasL in non-lymphoid and tumor cells contributes to their maintenance (Nagata, 1997). LIGHT (also termed HVEM-L) is another cognate ligand for TR6 (Yu *et al.*, 1999). Very recently, another novel member of TNF ligand superfamily TL1A was found to be a third ligand for TR6 (Migone *et al.*, 2002). TL1A is considered as the longer variant of TL1 and this molecule was designated as VEGI when it was identified in endothelial cell DNA libraries (Zhai *et al.*, 1998; Yue *et al.*, 1999). Studies at the mRNA level show that TL1A is highly expressed in endothelial cells and is inducible by TNF and IL-1. TL1A is the ligand for both DR3 and TR6 (Migone *et al.*, 2002). DR3 is a TNFR member and has the capability of inducing NF-κB activation and apoptosis upon over-expression. DR3 shares another ligand TWEAK (or Apo3L) with a

novel receptor TWEAK (Masters *et al.*, 1998; Wiley *et al.*, 2001). But the relationship between these proteins and the significance of their interactions have yet to be fully studied.

I.2.3.6. *The identified biological functions of TR6*

It has been demonstrated that TR6 could neutralize the biological effects of FasL by inhibiting Fas-FasL interaction (Pitti *et al.,* 1998). This decoy receptor substantially blocks the FasL-induced apoptosis in Jurkat cells as well as FasL-dependant NK cell activity (Connolly *et al.*, 2001). These results suggest that TR6 could interact with FasL under certain conditions and modulate immune responses, especially apoptosis mediated by the Fas/FasL pathway.

TR6 can suppress LIGHT binding to both LTβR and TR2 (Yu *et al.*, 1999; Mauri *et al.*, 1998), and it is conceivable that such interference can inhibit LTβR- and TR2-mediated responses in immune cells. It was demonstrated that TR6 can interact with LIGHT and thus inhibit LIGHT –mediated cytotoxicity of H29 cells. These results suggest that TR6 acts as a natural inhibitor of LIGHT mediated tumor cell killing.

In addition to modulation of immune responses, it was found in a recent study that TR6 profoundly modulates dendritic cells (DCs) differentiation and maturation from $CD14^+$ monocytes (Hsu *et al.*, 2002). Interestingly, it was reported that TR6 enhances CD86/B7.2 expression, whereas CD80/B7.1, CD40, CD54/ICAM-1, CD1.a and HLA-DR were all repressed. In addition, DcR3-treated DCs dramatically enhanced IL4 secretion by naïve T cells ($CD4^+CD45RA^+$), thus favoring Th2 development.

The ligand TL1A can also function as a T cell costimulator. The T cell stimulation induced by TL1A results in an increased responsiveness to IL-2 and other pro-

inflammatory cytokines (Migone *et al.*, 2002). TR6 competes with DR3 for TL1A binding with similar affinity. Therefore, it is possible that TR6 may modulate the duration and magnitude of immune responses mediated by DR3. Moreover, since DR3 can induce apoptosis, blocking the interaction between TL1A and DR3 by TR6 might provide protection from apoptosis to certain types of tumors (Migone *et al.*, 2002).

I.2.4. *The other TNF receptors related with TR6*

Obviously, TR6 may have important functions still to be elucidated in cancer development and in the immune system. In the latter, TR6 might compete with other TNF receptors for core common ligands such as LIGHT and FasL and thus interfere with their biological functions. Among all the possible TNFR SFPs that share LIGHT and FasL with TR6, TR2 and Fas are predominantly expressed in the immune system and are critically important in regulating immune responses. As such, to decipher the nature of TR6, identify with which ligand TR6 interacts and the related receptor(s) such as TR2 and Fas will certainly help to better understand the underlying mechanisms of the regulatory roles of TR6 both *in vitro* and *in vivo*.

I.2.4.1. *HVEM/TR2*

The receptor herpes virus entry mediator (HVEM or TR2) is a recently discovered TNFR superfamily member with broad tissue and cell type expression especially in the immune system (Montgomery *et al.*, 1996; Kwon *et al.*, 1997; Tan *et al.*, 1997). This molecule was also identified by screening expressed sequence tag (EST) cDNA database for sequence homology with cysteine-rich motifs of the TNFR superfamily (Harrop *et al.*,

1998). This molecule was discovered through its ability to mediate HSV infection. The TR2 gene locates at chromosome 1p36, a position close to other TNFR members such as CD30, 4-1BB, OX-40, and TNFR-II (Montgomery *et al.*, 1996). Three ligands so far have been defined for TR2: the HSV surface envelope glycoprotein gD, lymphotoxin α (LT α) and the newly described TNF ligand family member LIGHT (Montgomery *et al.*, 1996; Mauri *et al.*, 1998).

The TR2 mRNA can be readily detected in lung, spleen, thymus, monocytes, dendritic cells, B and T lymphocytes, but not in liver, brain, and skeletal muscle (Morel *et al.*, 2000). Moreover, RNA analysis unveiled that most solid tumor lines do not express TR2. On the other hand, some hematopoietic cell lines do express TR2 (Kwon *et al.*, 1997).

The full-length TR2 encodes a 283 amino acid protein. It is a type I transmembrane protein containing a 50-amino acid cytoplasmic region without a death domain (Harrop *et al.*, 1998). At the protein level, expression studies indicated that TR2 protein has wide distribution and can be readily detected by flow cytometric analysis in peripheral blood T and B lymphocytes, NK cells, and monocytes (Morel *et al.*, 2000). Interestingly, it was reported in a recent study that TR2 and one of its ligands LIGHT display reciprocal expression at T cell surfaces as detected by flow cytometric analysis (Morel *et al.*, 2000). LIGHT is hardly detectable in resting T cells, but its expression was enhanced upon activation (Morel *et al.*, 2000). On the other hand, HVEM showed down-regulation upon T cell activation. Data from confocal microscopy and intracellular staining by flow cytometry showed the existence of intracellular LIGHT in unprimed T cells. After activation, there is a pronounced induction of LIGHT both intracellularly and at the cell surface. This suggests that de novo synthesis and redistribution of LIGHT both contribute

to the enhancement of LIGHT expression at cell surface. The detailed mechanism of down-regulation of TR2 expression by LIGHT is still not well understood.

The biological function of TR2 was an active area of investigation in the past few years. Current evidence suggests that TR2 is closely associated with T cell activation and a number of T cell responses such as proliferation, cytokine production, and expression of cell surface activation molecules (Harrop *et al.,* 1998). LIGHT, as its ligand, stimulates the proliferation of activated T cells expressing TR2 (Harrop *et al.*, 1998), stimulates NF-κB activation, and induces apoptosis in cells expression both TR2 and LTβR (Zhai *et al.*, 1998; Harrop *et al.*, 1998). Recently, studies show that LIGHT can costimulate T cell responses by interacting with TR2 (Kwon *et al.*, 1997; Tamada *et al.*, 2000A; Tamada *et al.*, 2000B) and TR2 is critically important in T cell costimulation. T cell derived LIGHT can readily deliver stimulation to TR2 on dendritic cells, that in turn up-regulate T cell activity (Shaikh *et al.*, 2001). Moreover, TR2 on T cells can also receive LIGHT stimulation directly from LIGHT expressing T cells (Wang *et al.*, 2001).

I.2.4.2. *Fas*

Fas (also named APO-1 or CD95) is the primary receptor for FasL and this molecule is a type I membrane protein belonging to the TNFR family (Suda *et al.*, 1993). Fas is abundantly expressed in various tissues and cell types (Suda *et al.*, 1993), especially thymocytes, activated T cells and virus transformed T cells. Resting B cells do not express Fas, but its expression can be induced by CD40 ligand or endotoxins (Briones *et al.*, 2002; Hahne *et al.*, 1996). Fas has broad tissue distribution, but is most abundantly expressed in the thymus, liver, heart and kidney (Itoh and Nagata, 1993). Cross-linking of

Fas by an agonistic antibody or ligation by FasL will cause Fas clustering which is necessary for receptor activation and death signal initiation (Kischkel, 1995).

I.3. *TNF ligand superfamily*

As listed in Table 2, the mammalian TNF ligand superfamily also exhibited rapid expansion in recent years and it currently includes TNF α, LTA (lymphotoxin-α) or LTB (lymphotoxin–β), FasL, CD27L, CD30L, CD40L, 4-1BBL (Gruss *et al.*, 1995), TRAIL (Wiley *et al.,* 1995), TRANCE/RANKL/OPGL (Lacey *et al.*, 1998), TWEAK (Chicheportiche *et al.*, 1997); APRIL/TALL-2 (Hahne *et al.*, 1998; Shu *et al.*, 1999), AITRL (Kwon *et al.*, 1999), VEGl (Zhai *et al.*, 1999), BAFF/TALL1 (Shu *et al.*, 1999; Schneider *et al.*, 1999), LIGHT (Mauri *et al.*,1998), TRANCE (Wong *et a*l., 1997), TL1/VEGI (Zhai *et al.*, 1999), TL1A(Migone *et al.*, 2002), TL6/hGITRL (Kwon *et al.*, 1999). All these ligands share, to some extent, structural features, which might be important for their functions. Meanwhile, these ligands, in a mechanism similar to other cytokines and growth factors, exert their effects through receptor-ligand interactions that induce downstream signal transduction events (Gruss and Dower, 1995).

I.3.1. *The structural features of TNF ligands*

The members of TNF ligand superfamily are highly diverse in sequence and have an average of 20% (range from 12% to 36%) sequence homology in their extracellular domain. These ligands exist mainly in membrane-bound forms and their biologically active forms are trimeric/multimeric complexes (Gruss and Dower, 1995). Their monomers are composed of two stranded beta pleated sheets (Armitage *et al.*, 1994; Lotz

et al., 1996). TNF ligands are synthesized as type II transmembrane proteins characterized by their extracellular C–terminus. The typical TNF ligand structure consists of a short cytoplasmic segment (10-80 amino acid residues) and a relatively long extracellular region (140-215 amino acid residues). Several members of this superfamily have moderate-sized cytoplasmic regions. The cytoplasmic regions of TNF ligands are not conserved among family members, but highly conserved across species. This cross-species conservation implicates some important biological functions such as signal transduction for the cytoplasmic region (Smith, 1993), as also demonstrated for OX40L and CD40L (Stuber *et al.*, 1995; van Essen *et al.*, 1995).

I.3.2. *The Soluble Form of TNF Ligands*

Although members of the TNF ligand superfamily normally exist as trimeric or multimeric membrane bound proteins, many of them are also expressed and functional in a soluble form. For example, Fas ligand (Suda *et al.*, 1993; Tanaka *et al.*, 1995), TNF α (Kriegler *et al.*, 1988), CD40L (Graf *et al.*, 1995), OX 40L (Stuber and Strober, 1996), CD27L (Lens *et al.*, 1998), 4-1BBL (Salih *et al.*, 2001), LIGHT (Harrop *et al.*, 1998), TRAIL (Wajant *et al.*, 1995) were all found to exist in soluble forms that are biologically active. The release of soluble ligands from the cell surface is mediated by proteolytic cleavage and likely regulates receptor/ligand interactions between cells. Cleavage of membrane-bound ligand to an active soluble form would alter both proximal and distal cellular responses, including cell survival and costimulatory or inflammatory responses. Currently the mechanism of coexistence of both forms is not fully understood. Interestingly, studies on TNFα discovered that while membrane-bound and soluble forms

are both biologically active, soluble TNFα is more potent than the membrane-bound form (Decoster *et al.*, 1995).

Lymphotoxin–beta (LT-β) is an exception to this feature. It only exists as a membrane-bound form (Browning *et al.*, 1996). Human LT-β (also named as p33) is a 33 kDa glycoprotein cloned from T cell hybridoma cell line. On the cell surface, LTβ forms a trimeric complex with TNF β in either LT α2β1 (major form) or LT α1β2 (minor form) ratio (Hochman *et al.*, 1995).

I.3.3. *The biological features of TNF ligands*

The TNF ligand SFPs play multiple roles in both innate and adoptive immune responses (Smith *et al.*, 1994), obviously through their interaction with TNFR SFPs. One fundamental feature of TNF ligand superfamily is the biological activity related to T-cell-mediated immunity (Suda *et al.*, 1993; Armitage *et al.*, 1993)

The ability to induce cell death (either necrosis or apoptosis) is the most thoroughly investigated feature of TNF ligands as established for TNF, LTα, CD30L, 4-1BBL and FasL (Gray *et al.*, 1984; Smith *et al.*, 1993; Alderson *et al.*, 1994; Suda *et al.*, 1993; Liu *et al.*, 1989; Gruss *et al.*, 1994). TNF ligands are also directly involved in lymphoid organ generation and development. For instance, mice genetically deficient for LTα1β2 do not develop secondary organs such as lymph nodes, or Peyer's patches, and have defective spleen structure and humoral immunity (Fu and Chaplin, 1999). Among these TNF ligands, LIGHT and FasL will be discussed in details for their biological functions. Interestingly, these two ligands also have the capability of reverse signaling and receptor function.

I.3.4. *Reverse signaling*

The reverse signaling phenomenon had been observed several years ago and recent studies have provided more evidence for TNFR family members' reverse signaling capability (Hsu *et al.*, 2002; Suzuki *et al.*, 1998). Members such as CD30L, CD40L, TRANCE, TRAIL and DR4 are reported to transmit reverse signals into ligand bearing cells. The reverse signaling of these ligands has important biological functions. For instance, reverse signaling through CD40L is associated with different immune responses such as T cell costimulation and germinal center formation (Rooney *et al.*, 2000). Moreover, CD40L is able to trigger brief CD4 T cell activation as well as regulatory cytokine product secretion and apoptosis (Blair *et al.*, 2000). Cross-linking OX-40L on CD40L-stimulated B cells results in enhanced B cell proliferation and down-regulation of the transcription factor B cell lineage-specific activator protein (BSAP) (Stuber *et al.*, 1995). More studies showed that CD40L reverse signaling leads to T cell costimulation (van Essen *et al.*, 1995). In addition, CD40L reverse signaling was associated with protein tyrosine phosphorylation, Ca^{2+} influx, and activation of Lck, protein kinase C, c-jun N terminal kinase, and p38 mitogen-activated protein kinase activation in EL-4 thymoma cells (Brenner *et al.*, 1997a, Brenner *et al.*, 1997b). In a recent report, it was further demonstrated that maximal proliferation of CTL requires reverse signaling via Fas-L (Suzuki and Fink, 1998). In addition, reverse signaling through CD27/CD70 has been demonstrated to induce pronounced proliferation of a subset of leukemic B cells, an effect that is synergistically enhanced by ligation of CD40 (Lens *et al.*, 1999). In a more recent report, Chen demonstrated that TRANCE enhanced IFN γ production in activated

Th1 cells (Chen *et al.*, 2001). CD30L cross-linking activates neutrophils (Wily *et al.*, 1996) and inhibits Ig class switching in B cells (Cerutti *et al.*, 2000). Crosslinking of TRANCE enhances IFN γ secretion by Th1 cells (Chen *et al.*, 2001). Crosslinking of TRAIL induces p38 mitogen activated protein kinase activation (Chou *et al.*, 2001). Studies on CD40L reveal that reverse signaling associated with TRAIL cross-linking also induces p38 mitogen activated protein kinase activation (Brenner *et al.*, 1997A). Taken together, these findings illustrate the importance of reverse signaling in immune system activation. It would be interesting to know whether bi-directional signaling also exists with other members of the TNF superfamily. For example, we investigated the reverse signaling properties of TR6 via its ligands.

I.3.5. *Fas ligand*

The Fas ligand (FasL or CD95L) belongs to TNF ligand superfamily (Suda *et al.*, 1993). FasL is expressed as a trimeric molecule either in membrane-bound or soluble form. FasL is predominantly expressed in activated T cells and natural killer (NK) cells and also expressed constitutively at immune privileged sites (Nagata, 1997; Oshimi *et al.*, 1996; Suda *et al.*, 1993). It is a type II membrane protein and its extracellular region consists of a 150 amino acid stretch displaying remarkably low homologies (20-25%) with other members of this family. The cytoplasmic region of FasL has 77 amino acid residues.

FasL-triggered apoptosis is the fundamental regulatory factor for cell survival and maintenance of normal immune functions, and dysregulation of this system has been shown to result in many human pathological conditions such as SLE (Wu *et al.*, 1996). Recently it was reported that DcR3 competitively bind to FasL, and therefore block the

Fas/FasL engagement which ultimately resulted in the blockage of apoptosis (Pitti *et al.*, 1998).

The interaction of Fas-L with the extracellular ligand-binding domain of the Fas receptor can induce Fas trimerization and activate of the apoptotic cell death pathway. In addition, the Fas/Fas-L system plays a critical role in CTL activity and regulation of immune response amplitude (Nagata, 1997). This Fas-L induced cell death utilized by CTL system could be involved in the immune response against tumor cells and other cytotoxic activities. The expression of FasL in the plasma membrane of numerous tumor cells allows them to kill Fas bearing immune cells in vitro (Nagata, 1997). These observations have suggested that tumor cells may use FasL to induce a specific immune tolerance. However, in the *in vivo* setting, FasL expression rather induces tumor cell rejection. The quantity and the environment of FasL expressed on tumor cells could determine whether tumor cells are tolerated or rejected (Suzuki *et al.*, 2000)

I.3.6. *LIGHT*

LIGHT is a recently identified and characterized core member of TNF ligand superfamily. 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" (Mauri *et al.*, 1998; Harrop *et al.*, 1998). The standardized symbol of LIGHT is TNFSF14 (refer to Table 2 and Lockslay *et al.*, 2001). Since LIGHT can competitively block the engagement of HSV glycoprotein D to HVEM, it was also named HVEM ligand (HVEM-L) (Harrop *et al.*, 1998).

The LIGHT gene maps on human chromosome 19, adjacent to other TNF ligands such as CD27L, 4-1BBL (Granger *et al.*, 2001). The primary structure of LIGHT as predicted from the cDNA sequence contains 240 amino acid residues, no predicted signal cleavage site, and a stretch of 22 hydrophobic residues as a transmembrane region, characteristic of a type 2 transmembrane protein. The extracellular domain of LIGHT consists of a short membrane extension of 39 amino acid residues close to the receptor-binding domain. There is only one N-linked glycosylation site identified for LIGHT that lies within the major receptor-binding loop (A-A'β strand) (Harrop *et al.*, 1998).

LIGHT is closely related to LTα and LTβ according to amino acid sequence homologies and shares receptor specificity but is genetically different. The similarity of LIGHT to lymphotoxins outside the scaffold regions is reflected in the conservation of tyrosine 173 located in the D-E loop, a contacting region of LTα for TNFR (Banner, 1993). Structural study indicates that LIGHT has a secondary structure of anti-parallel β sandwich conformation that favors the formation of a homotrimer structure.

This is also suggested by sequence homology studies in the TNF ligand family (Rooney *et al.*, 2000B). Species conservation between human and mouse LIGHT is 76% (Mauri *et al.*, 1998). LIGHT exhibits significant sequence homology with the C-terminal receptor-binding domains of LTβ (34% identity), Fas ligand (30%), 4-1BB ligand (29%), TRAIL (28%), LTα (27%), TNF (27%), and CD40L (26%). No sequence homology is found with HSV-1 envelope glycoprotein D. Sequence homology is mainly limited to residues forming the β sandwich structure and assembling as a trimer.

I.3.6.1. *LIGHT expression and distribution*

It was found that LIGHT mRNA was abundantly expressed in spleen and lymph nodes, but weakly in peripheral blood, thymus, appendix, and in bone marrow as well. Visceral organs, heart, colon, small intestine, lung, and liver also exhibit weak expression. Reports about LIGHT expression in the brain are conflicting (Harrop *et al.,* 1998; Mauri *et al.*, 1998). LIGHT expression in T cells requires activation stimuli similar to those for LTα and LTβ. Monocytes and granulocytes may also express LIGHT (Zhai *et al.,* 1998). But these results have not been well confirmed at protein level (Harrop *et al.*, 1998). Mitogen-activated $CD4^+$ and $CD8^+$ T cell subpopulations from peripheral blood have readily detectable LIGHT mRNA. LIGHT can be detected on the surface of activated T cells (Mauri *et al.*, 1998), macrophages and immature dendritic cells (Tamada *et al.*, 2000). Also, activated T cells do not appear to produce soluble LIGHT. However, while LIGHT was expressed by HEK 293 cells, its observed molecular mass was 28-29 kDa while the molecular mass of LIGHT from activated II-23 T cell line was 30 kDa. The analysis of a truncated form of LIGHT (at position G85) suggests that a soluble form of LIGHT protein retains receptor-binding activity (Harrop *et al.*, 1998). In contrast to LTα or LTβ, LIGHT is also expressed by the monocytic cell line THP-1 following activation by phorbol ester (Zhai *et al.,* 1998).

For the II-23 T cell hybridoma ($CD4^+$ T cells), both phorbol ester (PMA) and ionomycin are required for the enhanced expression of LIGHT, while PMA is sufficient for LTαβ complex (Yu *et al.*, 1999; Kuprash *et al.,* 1996; Zhai *et al.,* 1998). Other reagents that activate T cells, such as PHA or anti-CD3, or specific antigens, induce the expression of LIGHT.

These results suggested that LIGHT had a broader tissue expression pattern than LTα and LTβ which are limited to activated T and B lymphocytes and NK cells. Although it is well accepted that activated T cell and dendritic cell have cell surface LIGHT expression, recent findings in our laboratory suggested that LIGHT indeed can be detected at resting T cell surface without activation (Shi *et al.*, 2002).

I.3.6.2. *The receptors of LIGHT*

Three receptors from TNFR superfamily have been identified for LIGHT: LTβ R on stromal cells, HVEM (herpes virus entry mediator, also know as HveA) on T cells (Mauri *et al.*, 1998), and the soluble receptor TR6/DcR3 (Pitti *et al.*, 1998;Yu *et al.,* 1999).

I.3.6.3. *The biological functions of LIGHT*

LIGHT has been intensively studied for its functions. Accumulating evidence suggests that LIGHT plays critical roles in regulating immune responses by interacting with HVEM, LTβR and possibly with TR6 as well.

First, LIGHT is demonstrated as a novel costimulatory molecule for T cell activation, which directly results in increased T cell proliferation, enhanced Th1 type cytokine production, and NF-kB translocation. Moreover, the LIGHT triggered T cell activation was found independent of the B7/CD28 pathway (Tamada *et al.*, 2000A; Tamada *et al.*, 2000B).

In addition to induction of T cell activation, LIGHT also regulates antigen presenting cell (APC) development. For instance, DCs are APCs most potent in initiating primary T cell responses. It was found that blockade of LIGHT can inhibit the optimal induction of

primary T cell responses by allogeneic DCs, suggesting that LIGHT is a pivotal costimulator for DC triggered stimulation of primary T cell responses and the costimulation is most likely through HVEM (TR2) (Tamada *et al.*, 2000) on DCs. In one report, it was found that LIGHT signaling via HVEM was in cooperation with CD40 signaling for DC maturation. The DCs then achieves activation and thus are able to elicit an enhanced anti-tumoral CTL response (Morel *et al.*, 2001).

LTβR is one of the receptor of LIGHT that plays a major role in the formation of secondary lymphoid tissue during embryogenesis (Futterer, 1998). Both $LT\alpha^{-/-}$ and $LT\beta R^{-/-}$ mice showed disappearance of lymph nodes (LN) and Peyer's patches (PP) and a severe disorganization of splenic structure (Fu *et al.*, 1999; De Togni *et al.*, 1994). To study whether T cell derived LIGHT and its interaction with LTβR are sufficient to support the formation of lymphoid tissues, LIGHT transgenic (LIGHT Tg) mice were developed and backcrossed with either $LT\alpha^{-/-}$ or $LT\beta R^{-/-}$ mice (Wang *et al.*, 2002). The results showed that LIGHT–complemented $LT\alpha^{-/-}$ mice (LIGHT Tg/$LT\alpha^{-/-}$) display recovery of secondary lymphoid tissues and restoration of splenic architecture. In addition, blockade of endogenous LIGHT activity in $LT\beta R^{-/-}$ mice give rise to more severe disturbed splenic structure, suggesting the importance of LIGHT in the development and maintenance of the lymphoid organs and tissues (Wang *et al.*, 2002).

LIGHT could trigger apoptosis in some types of tumors both *in vitro* and *in vivo*. One important supporting experiment is that LIGHT gene transfected into a human breast carcinoma line resulted in the complete inhibition of tumor development in T cell deficient athymic nude mice (Zhai, *et al.*, 1998; Harrop *et al.*, 1998). It was found that gene transfection of LIGHT mediates tumor rejection through the generation of tumor

specific CTL. This was supported by data showing that blockade of LIGHT ameliorates acute graft-versus host disease (GVHD) by anergizing host-specific CTL (Tamada *et al.*, 2000). However, the *in vivo* effects of LIGHT, particularly on T cells, can only be well understood by using LIGHT transgenic (LIGHT Tg) and/or knockout (KO) animals.

I.3.6.4. *LIGHT transgenic and knockout mouse models*

Shaikh *et al.*, generated T cell LIGHT transgenic (Tg) mice and found that LIGHT Tg mice displayed abnormal lymphoid organ structure, chaotic lymphocyte distribution in addition to organ inflammation and destruction (Shaikh *et al.*, 2001). T cells from LIGHT Tg mice exhibit an abnormally activated phenotype and elevated Th1 cytokine activity (Wang *et al.*, 2001). Wang *et al.*, also found that LIGHT Tg mice exhibited an unusually enlarged T cell compartment and a hyper-activated peripheral T cell population. In addition, LIGHT Tg mice spontaneously develop severe autoimmune disease characterized by lymphadenopathy, glomerulonephritis, splenomegaly, enhanced levels of autoantibodies and severe lymphocytes infiltration of different peripheral tissues. Blockade of LIGHT activity by HVEM-Ig decreases the severity of T cell-mediated disease. Using the same model, it was found that LIGHT might be one of the important costimulatory molecules functioning in the T-T cells interaction and activation required for the complete expansion of peripheral T cells. The dysregulation or over-expression of LIGHT may play an important role in the pathogenesis of T cell mediated chronic inflammation and autoimmunity. Moreover, the *in vivo* data showed that LIGHT is sufficient to cause the activation and expansion of peripheral T cells that subsequently lead to the breakdown of peripheral tolerance (Wang *et al.*, 2001).

These findings together suggested a profound role for endogenous LIGHT in regulating T cell activation, presumably through costimulation to TR2 on T cells. Moreover, these findings also indicated a critical role for LIGHT as an important T cell costimulatory TNF ligand in T cell activation and expansion, the dysregulation of LIGHT leading to altered T cell homeostasis, breakdown of peripheral tolerance and autoimmune diseases.

A logical question: what are the consequences of LIGHT knockout in animals? One study from LIGHT deficient ($LIGHT^{-/-}$) mice showed that lymphoid organs are largely intact and function normally as T cells and APCs do. But CTL ($CD8^+$) induction and cytokines related to CTL development were reduced from $LIGHT^{-/-}$ mice (Tamada *et al.*, 2002). By using $LIGHT^{-/-}$ mice in an allograft rejection study, it was found that the mean allograft survival time of $LIGHT^{-/-}$ mice is only slightly prolonged whilst in combination with CsA, the survival time is significantly enhanced compared with normal $LIGHT^{+/+}$ mice (Ye *et al.*, 2002). Scheu *et al.*, found that $LIGHT^{-/-}$ mice actually develop intact lymphoid organs whereas $LIGHT^{-/-}$ $LT\beta^{-/-}$ double deficient mice have low percentage of mesenteric lymph nodes. Interestingly, in the $LIGHT^{-/-}$, $CD28^{-/-}$ allogeneic skin graft rejection mice model, it was found that $LIGHT^{-/-}CD28^{-/-}$ showed a skin survival of 19 d, i.e. 6 days (d) longer than single deficient or WT mice. This suggested that LIGHT together with CD28 plays important role in allo-graft rejection. The reason could be the reduced development of cytotoxic T lymphocytes as shown in the followup MLR study on $LIGHT^{-/-}$ mice (Schew *et al.*, 2002).

These results suggested that LIGHT is necessary for activation of $CD8^+$ but not $CD4^+$ T lymphocytes (Tamada *et al.*, 2002). This may be further implicated in the decrease of allogeneic CTL activities and the delay of allogeneic graft rejection. Together, these

results suggested that LIGHT and its receptor TR2 may contribute to the organogenesis of secondary lymphoid tissues and the important involvement of T cell costimulation in T cell mediated immune responses.

I.4. *Interaction of TNF SFP ligands with their receptors*

The biological function of TNFR/TNF ligand SFPs as well as their associated diseases are dependent on an essential signaling stoichiometry. Signaling is assumed to be achieved by ligand-induced trimerization of the monomeric receptor chains. This was demonstrated by crystallography of the extracellular ligand binding domain bound to LT α (TNF β) which showed a three to three symmetry of the ligand-receptor complex (Banner *et al.,* 1993). The X-ray structure for both TNF α and TNF β unveiled that both proteins exist as a "triangular cone" trimer (Eck *et al.*, 1992) and the ligand trimer binds three receptor molecules, one at each of three TNF monomer-monomer interfaces (Banner *et al.*, 1993). The trimeric ligand makes contacts within the CRD 2-CRD 3 region and thus forms a hexameric complex unit containing three receptors and a ligand trimer. Substantial data indicates that other TNF cytokine-receptor interact similarly to this typical, obligatory 3:3 symmetry and lead to receptor activation (Idriss and Naismith, 2000).

I.5. *TNFR SFP signaling*

The last few years have witnessed a proliferation in the knowledge of the proteins participating in the signaling of the TNF system. TNF receptors are activated by interacting with their specific ligands followed by receptor trimerization or

oligomerization and ultimately lead to either apoptosis or survival of receptor-bearing cells according to different stimuli. The general death/survival signaling pathways can be best illustrated by TNF α and LT α which were intensively studied in the past several years for their signaling events.

TNF α and LT α mediate their effects via two receptors: TNFR-I and TNFR-II. These ligands interact with both receptors as homo-trimers and induce receptor trimerization which is pivotal for receptor activation (Nagata, 1997; Ashkenazi and Dixit, 1999). TNFR-II has no intracellular death domain and is generally not implicated in apoptotic signals. Therefore, TNFR-I is the main receptor responsible for mediating the apoptotic signals of TNF receptor-ligand system (Nagata, 1997; Ashkenazi and Dixit, 1999). Upon activation, TNFR-I recruits the intracellular adaptor molecule termed "TNFR-associated death domain protein" (TRADD). TRADD is a death domain containing adaptor molecule which further recruits another death domain containing protein named FADD (Fas –associated death domain protein) (Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1996). The interactions among these adaptor molecules and TNFR-I occur through their respective death domains, leaving the N-terminal death effector domain of FADD free to further interact with the domain of proximal pro-caspase 8 or 10. Recruitment of proximal pro-caspase at the receptor site induces its oligomerization-mediated activation (Ashkenazi and Dixit, 1999). Upon activation, the proximal caspases further activate the downstream caspases such as caspase 3, 6 and 7, also termed the executioner caspases. Once the executioner caspases are activated, the apoptotic signal flows further downstream leading to the cleavage of death substrates and eventually cell demise (as shown in Figure 2).

As presented in Figure 2, in addition to the apoptotic direction, TNFR-I also relays signals that impinge upon cell survival. For instance, TRADD also recruits TRAF1 and TRAF2, the adaptor molecules that lead to activation of JNK (Jun N-terminal kinase) and NF-κB (Ashkenazi and Dixit, 1999). These TRAFs have been shown to bind to TNFR-I and prevent TNFα induced activation of caspase 8 at the receptor site. TRAFs also serve to function in a positive feedback loop to further promote NF-κB activation and generate survival signals. Recruitment of TRADD and FADD at the receptor site also promotes interaction with another death domain containing molecule RIP. RIP has been shown to interact with RAIDD to activate caspase 2 in order to mediate apoptotic signals (Wang *et al.*, 1999). Thus TNFR-I activation involves a complex series of interactions with intracellular molecules to mediate apoptotic and survival signals.

All the other cytokine-receptor pairs of the TNF superfamily have similar signaling events of TNF/TNFR pairs as illustrated in Figure 2 and the signaling is very rapid and highly specific. For instance, Fas is activated by interaction with its ligand Fas-L. Just like any other ligand in this family, FasL binds to Fas as a homotrimer and activates its cognate receptor Fas (Nagata, 1997; Ashkenazi and Dixit, 1999). The basic paradigm of Fas activation is similar to that of TNFR-I except that Fas does not bind with TRADD but directly recruit FADD.

It is worth to mention that mitochondria have an important role in apoptotic-signaling pathway initiated by TNF receptors. The apoptotic signaling can trigger the release of apoptotic factors from the mitochondrial intermembrane space (Gurp *et al.*, 2003). These apoptotic factors include cytochrome C (Li *et al.*, 2000), AIF (Wang *et al.*, 2002), endonuclease G (Li *et al.*, 2001; Ohsato *et al.*, 2002), Smac/DIALO (Verhagen *et al.*,

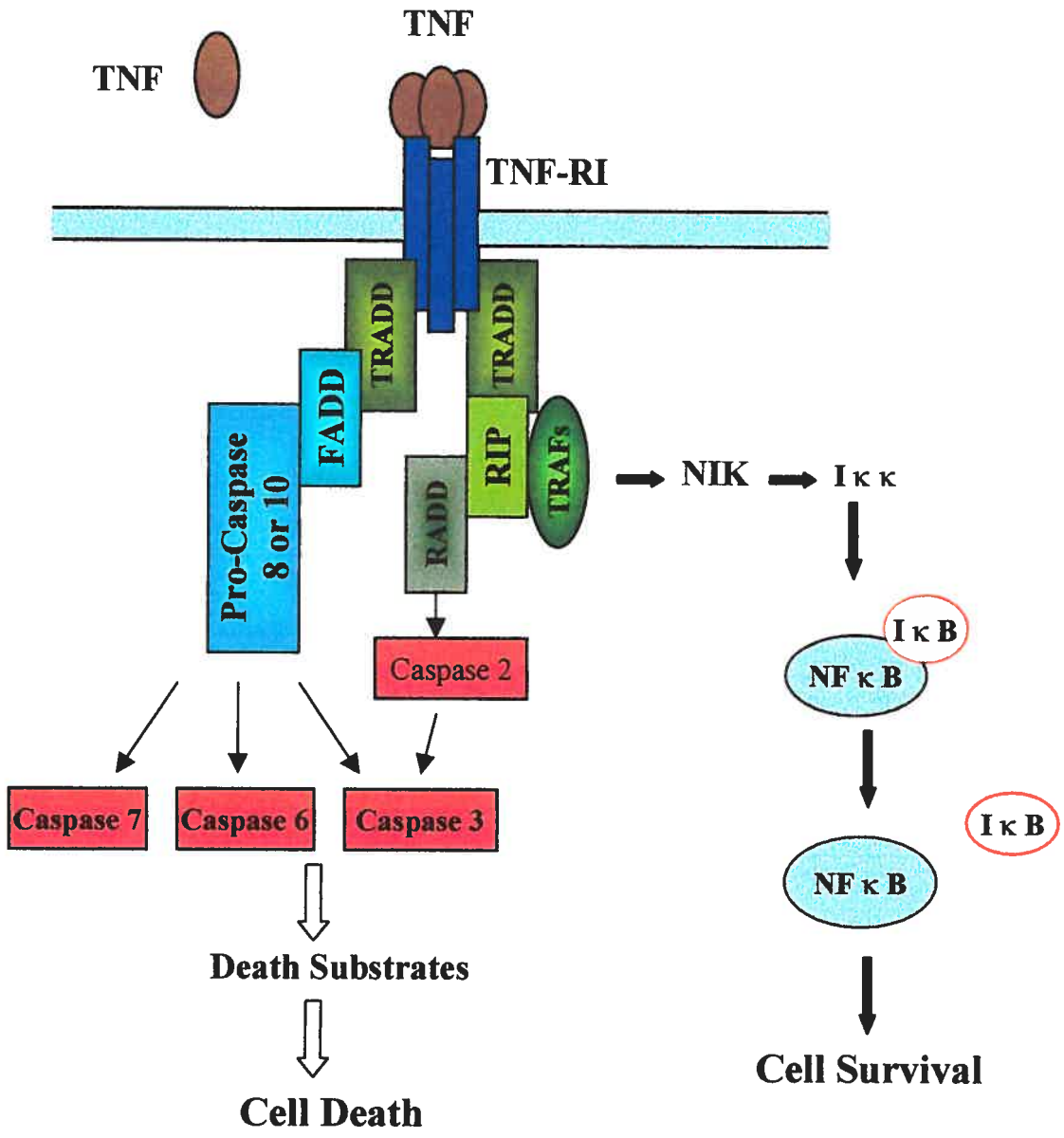

Figure 2. Signaling pathways of TNF and TNF-RI

2000; Du *et al.*, 2000), Omi/HtrA2 (Faccio *et al.*, 2000), and ACBP (Melloni *et al.*, 2000). Cytochrome C induces caspase activation by binding to Apaf-1 whereas Smac/DIABLO and Omi/HtrA can neutralize IAP's inhibition of caspases. AIF and endonuclease G are mainly involved in caspase-independent nuclear DNA degradation (Zamzami *et al.*, 2001).

The release of cytochrome C leads to the formation of apoptosome which include Apaf-1 and caspase 9. Apoptosome is closely associated to the downstream activation of caspases 3, 6 and 7 (Creagh *et al.*, 2003). It is believed that the cytochrome C dependent-apoptosome formation is not an essential trigger for apoptosis but rather an amplifier of the caspase activation cascade (Joza *et al.*, 2001; Green, 2003).

I.6. *Hypotheses*

As described in the above sections, a complex picture of TR6 receptor-ligand interactions emerged as illustrated in Figure 3. TR6 recognizes three confirmed ligands: LIGHT, FasL, and TL1 and each of these has more than one receptor as well. The first ligand FasL has two receptors: Fas and TR6. Another ligand LIGHT has 3 confirmed receptors: LTβR, HVEM (TR2) and TR6. LTβR can interact with its ligands: TNF α and LT α. TL1 is the third ligand of TR6 and it has two receptors: TR6 and DR3. DR3 also binds to TRAIL. Consequently, the resulting complex pattern suggests a highly sophisticated cytokine-receptor system.

The focus of our study is the role of TR6 in immune regulation. Since LTβR and TL1 are not expressed in the immune system, our study focuses on the interaction among TR6, LIGHT, Fas, FasL and HVEM. We formulated the following hypotheses.

(1). *TR6 modulates immune responses such as T cell costimulation by interrupting several pairs of TNFSF and TNFRSF interactions*

It has been confirmed that both LIGHT and FasL are involved in T cell costimulation. For LIGHT, the costimulation is achieved by interaction with TR2 (Tamada *et al.*, 2000); for Fas-L, costimulation is achieved through interaction with Fas (Suzuki *et al.*, 2000). Therefore, we speculate that TR6 (in soluble form) might be able to modulate T cell activation by interfering with these two TNF ligand-receptor pairs.

(2). *TR6 on solid phase might trigger reverse signaling through the ligand(s)*

As mentioned before, some TNF ligands might have reverse signaling properties. Since TR6 binds to FasL and LIGHT, it is possible that solid phase TR6 might act on these ligands to costimulate T cells through reverse signaling.

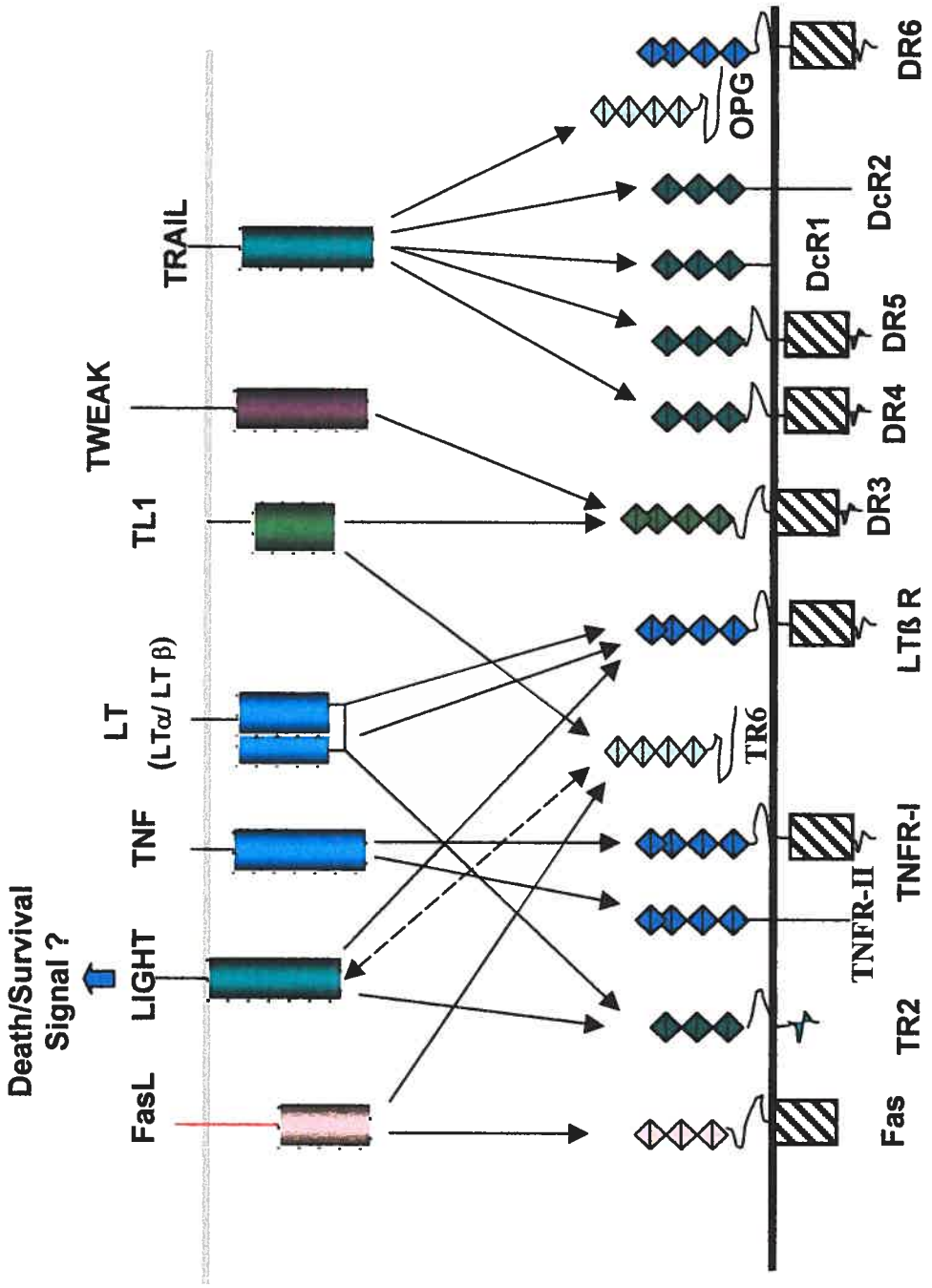

Figure 3. TR6 and related ligands and receptors. (CRD: ◇ , Death domain: ▨)

II. ARTICLES

Article 1:

Modulation of T-cell responses to alloantigens by TR6/DcR3

Xiaochun Wan, Jun Zhang, Theodora W. Salcedo, Stephen Ullrich, Bugen Hu, Theresa Gregorio, Ping Feng, Shijie Qi, Huifang Chen, Yun Hee Cho, Yuling Li, Paul A. Moore and Jiangping Wu

Journal of Clinical Investigation, June 2001, Volume 107, Number 11, 1459-1468

Note: In this paper, as co-first authors, Jun Zhang and Theodora W. Salcedo contributed in recombinant proteins and other related reagents preparation. In addition, they also performed some important experiments as shown Figure 1, 2 and 3 and Table 1 and 2. The remaining works such as results shown in Figure 4, 5 and 6 were performed by Xiaochun Wan.

Modulation of T-cell responses to alloantigens by TR6/DcR3

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Jun Zhang, Theodora Salcedo, and Xiaochun Wan contributed equally to this work.

Received for publication January 8, 2001, and accepted in revised form April 30, 2001.

TR6 (DcR3) is a new member of the TNF receptor (TNFR) family that lacks a transmembrane domain in its sequence, indicating that it is a secreted molecule. TR6 can bind to FasL and prevent FasL-induced apoptosis; it can also associate with LIGHT, another TNF family member. The role of TR6 in immune responses was investigated in this study. According to flow cytometry, recombinant human TR6-Fc binds to human LIGHT expressed on 293 cells or on activated human T cells and competes with the LIGHT receptor TR2 for the binding to LIGHT on these cells. Human TR6 could cross-react with mouse LIGHT in immunoprecipitation. TR6-Fc also downregulates cytotoxic T lymphocyte activity in vitro and graft-versus-host responses in mice. Moreover, TR6-Fc modulates lymphokine production by alloantigen-stimulated mouse T cells. TR6-Fc ameliorated rejection response to mouse heart allograft. These results indicate that TR6 can dampen T-cell responses to alloantigens. Such regulatory effects of TR6 probably occur via interference with interaction between pairs of related TNF and TNFR family members, LIGHT/TR2 being one of the possible candidate pairs.

J. Clin. Invest. **107**:1459–1468 (2001).

Introduction

TR6 (also called DcR3) is a new member of the TNF receptor (TNFR) family. TR6 lacks an apparent transmembrane domain in its sequence and is likely a secreted protein (1). The mRNA of TR6 is expressed at high levels in several normal human tissues such as the stomach, spinal cord, colon, lymph node, and spleen (1, 2), whereas its mRNA expression in the thymus is weak, and in peripheral blood lymphocytes is undetectable. Recombinant TR6 fused with an IgG_1 Fc domain can inhibit the interaction between Fas and FasL and prevent FasL-induced apoptosis in lymphocytes and several tumor cell lines (1). The latter suggests that certain tumors may escape FasL-dependent immunocytotoxic attack by overexpressing TR6.

TR6 can also bind to LIGHT, which is a member of the TNF family (3). LIGHT is a type II transmembrane protein, and its protein is expressed on activated T cells (4) and immature dendritic cells (5). It is a ligand for both TR2/HVEM and LTβR (4). LIGHT was found to induce apoptosis in cells expressing both TR2 and LTβR, but not in cells expressing only TR2 or only LTβR (6). However, a recent report by Rooney et al. (7) raised doubt about this conclusion by showing that LTβR is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. In any case, as LTβR is not expressed on lymphocytes (8), LIGHT has no demonstrated or perceived apoptotic effect on these cells.

Recent studies show that LIGHT can modulate T-cell responses via TR2, which is constitutively expressed at both protein and mRNA levels in most lymphocyte subpopulations including CD4 and CD8 T cells (9, 10). Soluble LIGHT enhances a three-way MLR (11). LIGHT expressed on COS cells or anchored on solid phase augments T-cell proliferation and lymphokine production (5, 12). Molecules that can presumably interfere with the interaction between LIGHT and TR2 were found to downregulate T-cell responses. For example, an antagonistic mAb against TR2 represses proliferation and lymphokine production by CD4 T cells (9); soluble recombinant TR2-Fc inhibits a three-way MLR (9, 10) or dendritic cell–stimulated alloresponse of the T cells (5); soluble LTβR-Fc inhibits solid-phase LIGHT-augmented T-cell proliferation (12); and in vivo administration of LTβR-Fc leads to amelioration of mouse graft-versus-host disease (12).

Article 2:

A TNF Family Member LIGHT Transduces Costimulatory Signals into Human T Cells

Xiaochun Wan, Jun Zhang, HongYu Luo, GuiXiu Shi, Kapnik E, Kim Sonhoo, Kanakaraj P, Jiangping Wu.

Journal of Immunology 2002 Dec 15;169(12):6813-6821

Note: In this paper, as co-first author, Jun Zhang was responsible for experiments involving Th1 and Th2 cell surface staining and cytokine tests as shown in Figure 3 and Figure 4. All the remaining works were performed Xiaochun Wan.

A TNF Family Member LIGHT Transduces Costimulatory Signals into Human T Cells[1]

Xiaochun Wan,[2]* Jun Zhang,[2‡] Hongyu Luo,* Guixiu Shi,* Elena Kapnik,‡ Sunghee Kim,‡ Palanisamy Kanakaraj,‡ and Jiangping Wu[3]*†

DcR3/TR6 is a secreted protein belonging to the TNFR family. It binds to Fas ligand, LIGHT, and TL1A, all of which are TNF family members. LIGHT is expressed on activated T cells. Its known receptors are TR2 and LTβR on the cell surface, and TR6 in solution. In the present study, we report soluble TR6-Fc or solid-phase TR6-Fc costimulated proliferation, lymphokine production, and cytotoxicity of human T cells in the presence of TCR ligation. These costimulating effects were blocked by soluble LIGHT but not by soluble Fas-Fc. TR6-Fc could also effectively costimulate *gld/gld* mouse T cells. We further demonstrated that TR6 bound to both Th1 and Th2 cells, according to flow cytometry, and that the association was inhibited by soluble LIGHT. Cross-linking Th1 and Th2 cells with solid-phase TR6-Fc along with a suboptimal concentration of anti-CD3 enhanced proliferation of both Th1 and Th2 cells, and augmented Th1 but not Th2 lymphokine production. These data suggest that TR6 delivers costimulation through its ligand(s) on the T cell surface, and at least the major part of such costimulation is via LIGHT. *The Journal of Immunology,* 2002, 169: 6813–6821.

Members of the TNF family play important roles in diverse cellular functions, such as proliferation, differentiation, cytokine production, apoptosis, Ig class switching, and T cell costimulation (1–3). Most of them (except lymphotoxin α, which is entirely secreted) are type II membrane proteins, and can exert their effect through cell-cell contact (1). Many members, such as TNF-α (4), CD40 ligand (CD40L)[4] (4), Fas ligand (FasL) (5), and TNF-related activation-induced cytokine (TRANCE) (6) can be cleaved from cell surfaces. The cleaved parts of these members have demonstrated (as in the case of TNF-α) or conceivable biological functions that involve interaction with their respective receptors.

LIGHT/TL5 is a new member of the TNF family (7), with its protein expressed on activated T cells (7) and immature dendritic cells (8). Cell surface LIGHT can be cleaved by matrix metalloproteinase (9). It is a ligand for TR2/Herpesvirus entry mediator, lymphotoxin β receptor (LTβR), and DcR3/TR6, all of which are TNFR family members (7, 10, 11). Recent studies show that LIGHT can costimulate T cell responses via TR2, which is constitutively expressed at both protein and mRNA levels in most lymphocyte subpopulations, including CD4 and CD8 T cells (12, 13). LIGHT, expressed on COS cells or anchored on a solid phase, augments T cell proliferation as well as lymphokine production (3, 8). Molecules that presumably interfere with the interaction between LIGHT and TR2 can down-regulate T cell responses: soluble recombinant TR2-Fc inhibits a three-way MLR (13) or dendritic cell-stimulated alloresponse of T cells (8), an antagonistic mAb against TR2 represses proliferation and lymphokine production by CD4 T cells (12), soluble LTβR-Fc suppresses solid-phase LIGHT-augmented T cell proliferation (3), and in vivo administration of LTβR-Fc leads to amelioration of mouse graft-vs-host disease (3). Taken together, these pieces of evidence show that LIGHT acts on TR2 as a costimulator of T cell activation. Moreover, LIGHT can induce apoptosis in cells expressing both TR2 and LTβR (14), although Rooney et al. (15) reported that LTβR is necessary and sufficient for LIGHT-mediated apoptosis in tumor cells. Because LTβR is not expressed on lymphocytes (16), LIGHT is unlikely to cause apoptosis in these cells.

TR6 is a new member of the TNFR family. Human TR6 lacks an apparent transmembrane domain in its sequence, and is a secreted protein (10, 17). In the immune system, TR6 mRNA is expressed at high levels in lymph nodes and the spleen (17, 18), while its expression in the thymus and PBLs is weak or undetectable, respectively. TR6 has three known ligands, i.e., FasL, LIGHT, and TL1A. TR6 can bind to FasL and inhibit the interaction between Fas and FasL. Consequently, FasL-induced apoptosis of lymphocytes and of several tumor cell lines can be repressed by TR6 (17). TR6 can also bind to LIGHT (10, 11). We have recently reported that human TR6-Fc can compete with TR2 for binding to LIGHT on human T cells, suppress CTL and lymphokine production in mouse lymphocytes, and inhibit mouse heart allograft rejection (10). These findings have raised the possibility that TR6 inhibits LIGHT-triggered costimulation via TR2 in T cells. The third known ligand of TR6 is TL1A, which is a new member of the TNF family, and is predominantly expressed on endothelial cells (19). TR6 can repress TL1A-augmented lymphokine secretion and the graft-vs-host response (19).

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Received for publication May 2, 2002. Accepted for publication October 7, 2002.

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[1] This work was supported by grants from the Canadian Institutes of Health Research (MOP-57697 and PPP-57321), the Canadian Institute of Health Research/Canadian Blood Service, the Kidney Foundation of Canada, the Heart and Stroke Foundation of Quebec, the Roche Organ Transplantation Research Foundation, Switzerland (474950960), the Juvenile Diabetes Research Foundation International, and the J.-Louis Levesque Foundation (to J.W.). J.W. is a National Researcher of the Quebec Health Research Foundation.

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[4] Abbreviations used in this paper: CD40L, CD40 ligand; FasL, Fas ligand; LTβR, lymphotoxin β receptor; TRANCE, TNF-related activation-induced cytokine.

0022-1767/02/$02.00

Our current study has revealed another layer of complexity in the interactions between TR2, TR6, and LIGHT. We present evidence in this study that TR6 ligand on the cell surface actually transduces costimulating signals into T cells, and enhances T cell responses to mitogens and alloantigens. At least a part of such reverse signaling was mediated by LIGHT. Thus, although a ligand, LIGHT can function as a receptor as well. The biological significance of this finding is discussed.

Materials and Methods

Recombinant proteins and mAbs

Recombinant TR6-Fc, TR6, TR11-Fc, LIGHT, and FasL were prepared as described in our previous publications (10, 20).

mAbs (clones 17B07 and SK9E2) against TR6 were prepared as follows. BALB/c mice were immunized i.p. with 50 μg/100 μl of TR6 emulsified in 100 μl of CFA. Three additional s.c. injections of 25 μg of TR6 in IFA were given at 2-wk intervals. The animals were rested for a month before receiving the final i.p. boost of 25 μg of TR6 in PBS. Four days later, splenocytes from one of the immunized mice were fused with 2×10^7 P3 × 63Ag8.653 plasmacytoma cells using polyethylene glycol 1500 (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's instructions. After fusion, the cells were resuspended in 400 ml of hypoxanthine/aminopterin/thymidine medium supplemented with 20% FCS and 4% of hybridoma supplement (Roche Applied Sciences), and distributed into 96-well plates (200 μl/well). Hybridomas were screened for specific Ab production by ELISA using TR6-coated plates. Positive hybridoma supernatants were checked for Ig isotypes using mouse Iso-strip kit (Roche Applied Sciences). mAb affinity was ranked by ELISA according to an approach described earlier (21). Hybridomas producing high-affinity mAbs were cloned by limiting dilution. Cloned hybridoma cells were injected in pristine-primed BALB/c mice (3×10^6cells/mouse) for ascites production. The Abs were purified from the ascites by protein G affinity chromatography using the Acta fast protein liquid chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ).

Preparation and culture of PBMCs, T cells, Th1 cells, Th2 cells, and mouse spleen cells

Adult PBMCs were prepared by Lymphoprep gradient (Nycomed, Olso, Norway), and T cells were prepared from PBMC by sheep RBC rosetting as described elsewhere (22), or by negative selection (deletion of cells positive for CD11b, CD16, CD19, CD36, and CD56) with magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The mouse mononuclear spleen cells were prepared by lysing RBCs in the total spleen cells with 0.84% NH_4Cl. The cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics. RPMI 1640, FCS, penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Burlington, Ontario, Canada). [^{3}H]Thymidine uptake was measured as described previously (22, 23).

For Th1 and Th2 cell generation, cord blood mononuclear cells were isolated by density gradient on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Monocytes from cord blood mononuclear cells were deleted by culture dish adhesion for 1 h at 37°C, and the resulting lymphocyte fraction was cultured with 2 μg/ml PHA (Sigma-Aldrich) in the presence of Th1/Th2 polarizing Abs and cytokines. Th1 differentiation was triggered by addition of 5 ng/ml IL-12 and 5 μg/ml anti-IL-4; Th2 differentiation was initiated by addition of 5 ng/ml IL-4, 5 μg/ml anti-IL-12, and 5 μg/ml anti-IFN-γ. After 72 h, cells were cultured in medium containing 5 ng/ml IL-2. After an additional 11–14 days of culture, >99% of the cells were $CD3^+$ T cells according to flow cytometry analysis; their Th1 and Th2 phenotype was confirmed by their lymphokine production profile. These cells were washed once with serum-free RPMI medium and starved in IL-2-free medium for 3 h. They were then cultured at $1–2 \times 10^5$ cells/well in 96-well plates, which were coated with anti-TCR$\alpha\beta$ (3 μg/ml) in combination with various amounts of solid-phase TR6-Fc. Proliferation of these cells was measured by [^{3}H]thymidine uptake 2–3 days later. The mAbs used in this section were from BD PharMingen (San Diego, CA), and ILs were from R&D Systems (Minneapolis, MN).

Lymphokine assays

IL-2, IL-5, IL-6, IL-10, IFN-γ, TNF-α, and GM-CSF in culture supernatants were measured by commercial ELISA kits from R&D Systems.

Flow cytometry

Th1 or Th2 cells (1×10^6) without further stimulation, or stimulated with solid-phase CD3, were stained with TR6-Fc (15 ng/sample) followed by goat $F(ab')_2$ anti-human IgG-PE (Southern Biotechnology Associates, Birmingham, AL). In some samples, TR6 without the Fc tag, LIGHT, or anti-TR6 mAb was present as an inhibitor (5 μg/sample) during the staining process. The cells were washed and resuspended in buffer containing 0.5 μg/ml propidium iodide; propidium iodide-negative live cells were gated and analyzed by flow cytometry.

Cytotoxic T cell assay

CTL activity of $\gamma\delta$ T cells was assayed as follows. Human PBMC (4×10^5 cells/200 μl/well) were stimulated with mitomycin C-treated Daudi cells (0.7×10^5 cells/well) in round-bottom 96-well plates in the presence of 10 U/ml IL-2 for 6 days. Normal human IgG (20 μg/ml), TR6-Fc (20 μg/ml), or LIGHT (10 μg/ml) was added to the culture in the beginning. On day 6, cells given the same treatment in the 96-well plate were pooled and counted, and their CTL activity was measured by a standard 4-h ^{51}Cr-release assay using ^{51}Cr-labeled Daudi cells (1.5×10^5 cells/well) as targets at different E:T ratios. To test the effect of solid-phase TR6-Fc, the round-bottom wells were precoated with TR6-Fc (0.5 μg/50 μl/well) at 4°C overnight and washed with PBS. PBMC (4×10^5 cells/250 μl/well) and mitomycin C-treated stimulator Daudi cells (0.7×10^5 cells/well) were cultured in these wells with IL-2 (10 U/ml) in the absence or presence of soluble LIGHT (20 μg/ml). On day 3, 70 μl culture supernatant/well was replaced with fresh regular medium. All the other procedures of the CTL assay were the same as described above, except that the ^{51}Cr-release assay was conducted on day 5 instead of day 6.

The lysis percentage of the test samples was calculated as follows:

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

For $\alpha\beta$ T cell CTL activity, PBMCs from donor A were used as responder cells, and mitomycin C-treated PBMCs from donor B were used as stimulators. PBMCs from donor B without mitomycin C treatment were stimulated with solid-phase anti-CD3 and anti-CD28, and used as target cells on day 6 for the 4-h ^{51}Cr-release assay. All the other procedures were the same as described above.

Results

TR6-Fc enhanced proliferation of PBMC stimulated by suboptimal concentrations of mitogens

We made an intriguing discovery in the course of our study on the role of TR6 in immune regulation: soluble TR6-Fc augmented response of human PBMC stimulated with different concentrations of PHA (0.05, 0.1, 0.5, and 1 μg/ml) in the presence of TR6-Fc (20 μg/ml) (Fig. 1*A*). TR6-Fc greatly enhanced the PBMC proliferation when PHA was at suboptimal concentrations (0.1 and 0.5 μg/ml). Using a suboptimal concentration of PHA (0.2 μg/ml), we further demonstrated that soluble TR6-Fc could enhance PBMC proliferation in a dose-dependent manner from 0.3–30 μg/ml (Fig. 1*B*). To ensure that the effect of TR6-Fc plus PHA was not due to a shift in proliferation kinetics compared with that of PHA or TR6-Fc stimulation alone, PBMC stimulated with PHA (0.2 μg/ml), TR6-Fc (10 μg/ml), or both were harvested at 48, 72, and 96 h. The result (Fig. 1*C*) showed that at no time points did TR6-Fc or suboptimal PHA alone lead to significant proliferation, unlike PHA plus TR6-Fc, ruling out a possible kinetic shift. Soluble TR6-Fc (10 μg/ml) also augmented suboptimal soluble anti-CD3 (100 ng/ml)-stimulated proliferation, as shown in Fig. 1*D*. The results of this section demonstrate that TR6-Fc can stimulate PBMC and T cells, likely via its cell surface ligand(s).

TR6 can interact with two ligands on the T cell surface, LIGHT and FasL (11, 17). The third TR6 ligand, TL1A, has little expression in T cells (19), and thus is not relevant in our system. To identify whether LIGHT or FasL was responsible for receiving TR6 stimulation, we added soluble LIGHT (10 μg/ml) or Fas-Fc (10 μg/ml) to T cells stimulated with a suboptimal concentration of PHA (0.2 μg/ml) in combination with solid-phase TR6-Fc (Fig.

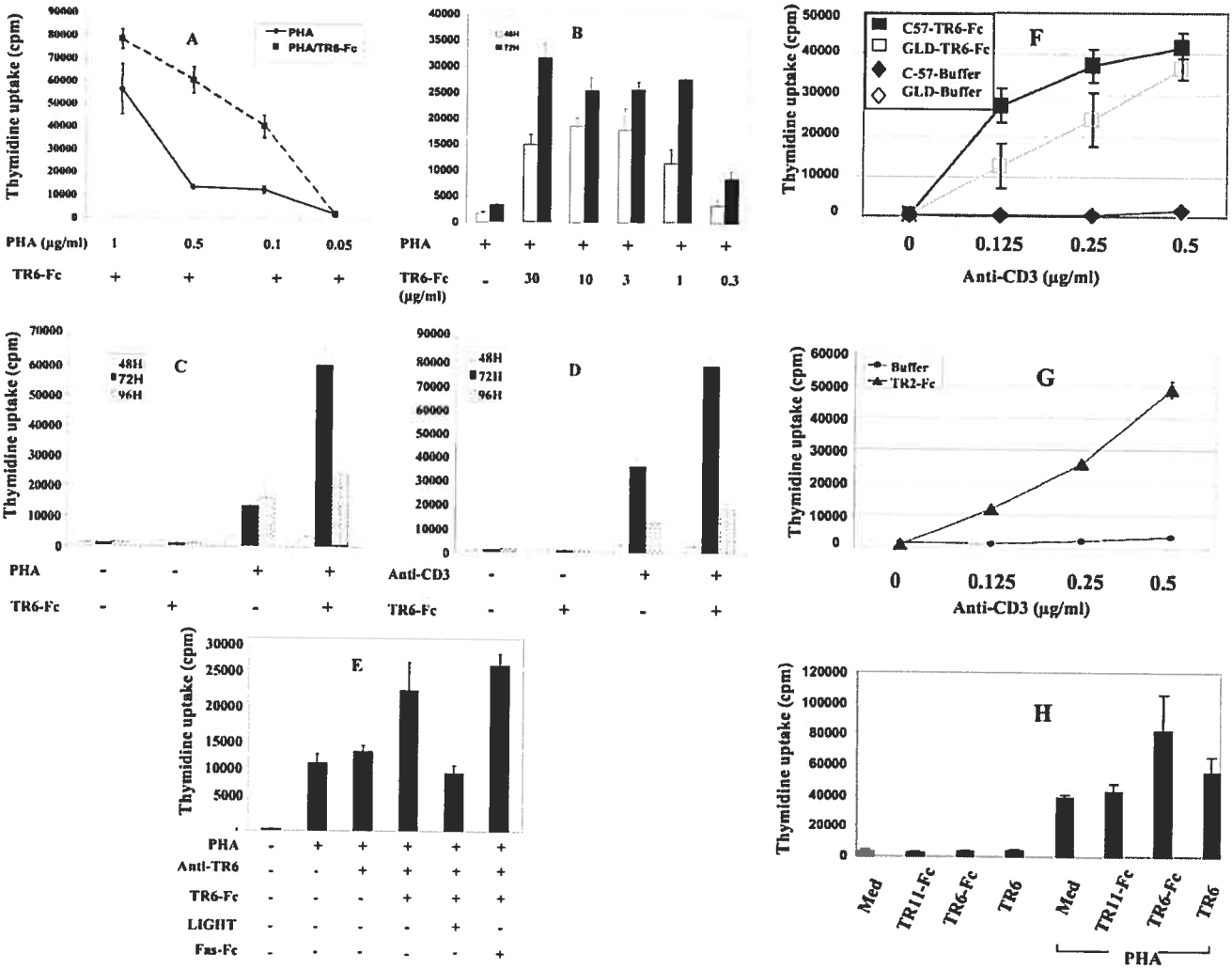

FIGURE 1. TR6-Fc strongly promoted proliferation of PHA- or anti-CD3-stimulated PBMC and T cells. Human PBMC or T cells, or mouse spleen cells, were stimulated with suboptimal concentrations of PHA or anti-CD3. Soluble or solid-phase TR6-Fc was used for costimulation. Cell proliferation was measured by [^{3}H]thymidine uptake at the times indicated. Mean ± SD of the cpm from triplicate samples are shown. The experiments were performed more than three times, and a representative set of data is presented. *A*, Effect of TR6-Fc on PBMC stimulated with various doses of PHA. PHA was used at 0.05, 0.1, 0.5, and 1 μg/ml, and TR6-Fc in solution was used at 10 μg/ml. [^{3}H]Thymidine uptake by the cells between 48 and 64 h after initiation of culture was measured. *B*, Effect of various doses of TR6-Fc on PBMC stimulated with a suboptimal dose of PHA. TR6-Fc in solution was used at 0.3, 1, 3, 10, and 30 μg/ml, while PHA was used at a suboptimal dose of 0.2 μg/ml. [^{3}H]Thymidine uptake by the cells was measured at 48 and 72 h. *C*, Proliferation kinetics of PBMC stimulated by TR6-Fc and a suboptimal dose of PHA. PBMC were stimulated with 10 μg/ml TR6-Fc in solution along with 0.2 μg/ml PHA. The proliferation of these cells was measured at 48, 72, and 96 h. *D*, Effect of TR6-Fc on PBMC stimulated with a suboptimal concentration of anti-CD3. PBMC were stimulated with TR6-Fc (10 μg/ml) in solution and a suboptimal concentration of soluble anti-CD3 (50 ng/ml). Cell proliferation was measured at 48, 72, and 96 h. *E*, Soluble LIGHT inhibited solid-phase TR6-promoted proliferation of PHA-stimulated T cells. Human T cells were cultured in the presence of a suboptimal PHA concentration (0.2 μg/ml), solid-phase TR6, or both as shown. To prepare solid-phase TR6, the wells were precoated overnight at 4°C with nonneutralizing anti-TR6 mAb (clone 17B07, 250 ng/50 μl) in PBS, followed by TR6-Fc (1 μg/50 μl) at room temperature for 6 h. Soluble LIGHT was added as indicated to certain samples at 10 μg/ml. [^{3}H]Thymidine uptake between 48 and 64 h after initiation of culture was measured. *F*, Effect of solid-phase TR6 on anti-CD3 stimulated *gld/gld* mouse spleen cells. Solid-phase TR6-Fc and anti-CD3 were prepared by coating NUNC wells with 5 μg/ml goat anti-human IgG (Southern Biotechnology Associates) along with various concentrations (0.1–0.5 μg/ml) of anti-CD3 in PBS as indicated overnight at 4°C. After washing, the wells were incubated with TR6-Fc (10 μg/ml) or buffer (PBS) at 37°C for 2 h. The coated wells were then used for culture. Spleen cells of C57BL/6 or *gld/gld* mice in B6 background were cultured in the coated wells and [^{3}H]thymidine uptake between 48 and 64 h after initiation of culture was measured. C57-TR6-Fc or GLD-TR6-Fc: C57BL/6 or *gld/gld* spleen cells, respectively, cultured in wells coated with anti-CD3 and TR6-Fc; C57-buffer or GLD-buffer: C57BL/6 or *gld/gld* spleen cells, respectively, cultured in wells coated with anti-CD3 alone followed by incubation of buffer during the second coating period. *G*, Effect of TR2 on anti-CD3-stimulated BALB/c mouse spleen cells. Solid-phase TR2-Fc and anti-CD3 was prepared as described in *F*, except 10 μg/ml TR2-Fc was used in the place of TR6-Fc. Buffer: wells coated with anti-CD3 in the first coating and buffer in the second coating; TR2-Fc: wells coated with anti-CD3 in the first coating and TR2-Fc in the second coating. [^{3}H]Thymidine uptake between 48 and 64 h after initiation of culture was measured. *H*, Comparison of solid-phase TR6-Fc vs TR6 without Fc for their stimulation efficacy of T cell proliferation. TR6-Fc, TR6, or a control fusion protein TR11-Fc was directly coated on wells (1 μg/50 μl/well during coating). PBMC were cultured in these wells in the absence or presence of a suboptimal concentration of PHA (0.2 μg/ml). [^{3}H]thymidine uptake between 56 and 72 h after initiation of culture was measured.

1*E*). The solid-phase TR6-Fc in this experiment was prepared by coating the culture wells with nonneutralizing anti-TR6 mAb (clone 17B07) followed by TR6-Fc. [^{3}H]Thymidine uptake was measured 72 h later. Solid-phase TR6 enhanced T cell proliferation in the presence of suboptimal PHA concentration, indicating that it delivers a costimulation signal through its binding partners on T cells. TR6 has two known ligands on T cell surface, LIGHT and FasL. Ideally, to identify which ligand was involved in the process, soluble LIGHT and FasL should be used as blockers in the culture. The former was used for this purpose. However, because exogenous FasL in solution might lead to apoptosis of activated T cells, it could not be used as a blocker for this purpose. Thus, we used soluble Fas-Fc, which would bind to FasL and block the interaction between FasL and TR6. The result showed that LIGHT but not Fas-Fc inhibited the proliferation, suggesting that LIGHT is a likely molecule through which TR6 induces activation of T cells.

One could argue that soluble LIGHT might block the binding of TR6 to FasL, and soluble Fas might have lower affinity than TR6 in FasL binding (although there is no evidence for this assumption) and thus cannot effectively compete with TR6 for binding to FasL. With such assumptions, whether it is the LIGHT that transduced signals into the cells remains in doubt. To gain additional evidence for LIGHT-mediated reverse signaling, solid-phase TR6 was used to stimulate *gld/gld* mouse spleen cells, which have nonfunctional FasL. Human TR6 can effectively bind to mouse FasL (data not shown) and LIGHT (10). *gld/gld* spleen cells responded well to solid-phase TR6 stimulation (Fig. 1*F*), suggesting that the major part of the reverse signaling is not via FasL. Solid-phase TR2, which binds to LIGHT but not FasL, costimulated mouse T cell proliferation when they were triggered by suboptimal solid-phase anti-CD3 (Fig. 1*G*). Taken together, these data further indicate that LIGHT is the main molecule mediating the TR6-triggered reverse signaling.

To compare the efficacy of dimeric TR6-Fc vs monomeric TR6 without Fc on T cell stimulation, and to test the hypothesis whether the observed stimulatory effect of TR6-Fc was due to blocking of a putative negative regulatory autocrine loop by TR6-Fc or TR6 leaked into solution (see *Discussion* for further elaboration), we coated these molecules directly on wells (1 μg/50 μl/well during coating). PBMCs were cultured in these wells in the presence of a suboptimal concentration of PHA (0.2 μg/ml) for 72 h, and thymidine uptake was measured. As shown in Fig. 1*H*, TR6-Fc but not TR6 could enhance T cell proliferation. This showed that the power of cross-linking is correlated to the efficacy of TR6 stimulation. Moreover, this indicates that the existence of a negative regulatory loop is unlikely, because if so, monomeric TR6 without Fc leaked into solution should more effectively enhance T cell proliferation than dimeric TR6-Fc.

TR6-Fc costimulation led to augmentation of lymphokine production by PBMC

Human PBMCs were cultured in the presence of a suboptimal concentration of PHA (0.2 μg/ml), PHA plus TR6-Fc (20 μg/ml), or PHA plus control recombinant protein TR11-Fc (20 μg/ml). The cytokines secreted into the supernatants at 24, 48, and 72 h were tested with ELISA and the results are presented in Fig. 2*A*. Because PHA was used at a suboptimal concentration, it induced minimal cytokine production. When TR6-Fc was added to the culture, it drastically induced production of cytokines such as IL-2, IL-6, IL-10, GM-CSF, IFN-γ, and TNF-α. In contrast, control protein TR11-Fc in combination with PHA did not augment cytokine production. Some Th2-type cytokines, such as IL-4 and IL-5, were of very low levels in this system, and no changes were detected (data not shown). Thus, costimulation from TR6-Fc led to augmented cytokine production in PBMC.

To assess whether the effect of TR6 was directly on T cells, TR6-enhanced lymphokine production was tested in purified T cells, which were stimulated with a suboptimal concentration of solid-phase anti-CD3 along with solid-phase TR6-Fc anchored indirectly via anti-human IgG. Solid-phase TR6 significantly enhanced the TNF-α and IFN-γ production at 48 h by T cells (Fig. 2*B*, *top two panels*), as with total PBMC. Soluble LIGHT, but not a control protein, TR11-Fc, prevented the augmentation of lymphokine production. This suggests that costimulation of TR6 is delivered to the T cells through a cell surface TR6 ligand, with LIGHT being a likely candidate. When stimulated with a suboptimal concentration of PHA in solution and solid-phase TR6, these T cells also augmented IL-2 and GM-CSF production, compared with PHA stimulation alone (Fig. 2*B*, *bottom two panels*).

TR6 bound to LIGHT expressed on Th1 and Th2 cells

LIGHT expression is up-regulated on activated T cells (7); we showed previously that TR6 specifically bound to LIGHT expressed on those cells (10). In this study, we examined LIGHT expression and association of TR6 with LIGHT on Th1 and Th2 cells. These cells were stimulated with solid-phase anti-CD3 overnight. As shown in Fig. 3, *row 1*, TR6-Fc bound to anti-CD3-activated Th1 and Th2 cells (shaded areas), but not to ones without activation (solid lines). Unlabeled soluble TR6 (Fig. 3, *row 2* without the Fc tag) and anti-TR6-mAb (Fig. 3, *bottom row*) inhibited the TR6 staining, indicating that the binding was not nonspecific. Soluble LIGHT (Fig. 3, *row 3*) effectively blocked the staining of TR6-Fc. These results suggest that the ligand of TR6 (likely LIGHT) is expressed on activated Th1 and Th2 cells.

TR6 costimulation on Th1 and Th2 cell proliferation and lymphokine production

We next assessed whether TR6 differentially promoted Th1 or Th2 function. In the presence of suboptimal solid-phase anti-TCR$\alpha\beta$, solid-phase TR6 stimulated proliferation of both Th1 and Th2 cells similarly and dose-dependently (Fig. 4, *A* and *B*); TR6-Fc alone without anti-TCR signaling had no effect on these cells (data not shown). As these Th1 and Th2 cells were >99% pure T cells, this experiment excluded the possibility that the effect of TR6 was indirect via dendritic cells or monocytes/macrophages. TR6 neutralizing mAb (clone SK9E2) suppressed TR6-enhanced Th1 and Th2 proliferation in a dose-dependent manner, while control mouse IgG had no effect (Fig. 4, *C* and *D*), indicating that the effect of TR6 is specific.

As TR6 showed no differential effect on proliferation of Th1 and Th2, we next examined its effect on lymphokine production by these cells 48 h after restimulation (Fig. 4, *E* and *F*). As controls, anti-CD28 and suboptimal anti-TCR$\alpha\beta$ on the solid phase resulted in dramatic IFN-γ production by Th1 but not Th2 cells (74,936 $\pm$ 56 vs 72 $\pm$ 23 pg/ml), and marked IL-5 production by Th2 but not Th1 cells (586.9 $\pm$ 16.5 vs 1.1 $\pm$ 0.14 pg/ml), confirming the Th1 and Th2 phenotype of the cells. When solid-phase TR6-Fc was used along with anti-TCR$\alpha\beta$, it significantly increased IFN-γ production by Th1 cells (42,587 $\pm$ 4,535.2 pg/ml), compared with anti-TCR$\alpha\beta$ stimulation alone (8,064.5 $\pm$ 223 pg/ml); this augmentation was blocked by anti-TR6 mAb in a dose-dependent manner, showing the specificity of the TR6 stimulation (Fig. 4*E*). IL-5 production by the Th1 cells was negligible (<5–10 pg/ml) with such stimulation, as expected, since IL-5 is a Th2 lymphokine. Th2 cells stimulated with TR6-Fc and anti-TCR$\alpha\beta$ produced little IFN-γ (<30 pg/ml), as expected, since IFN-γ is a Th1 lymphokine; however, they also failed to produce Th2 lymphokine

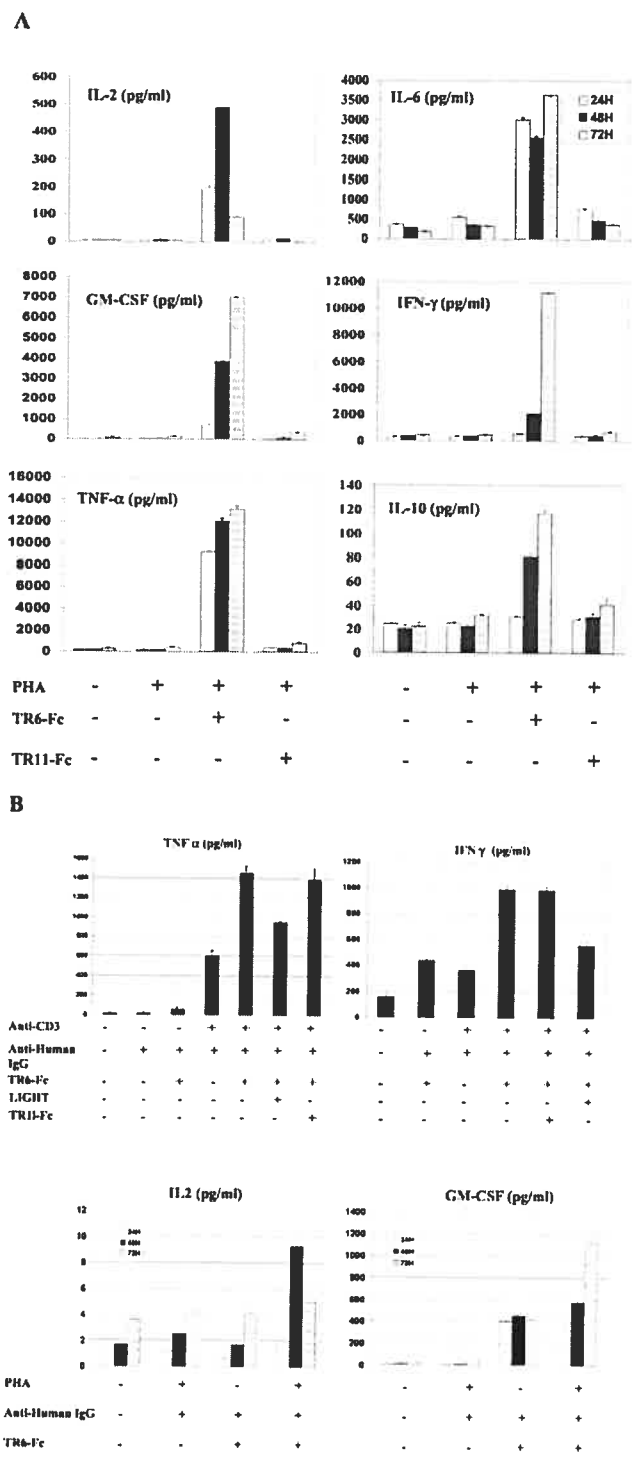

FIGURE 2. TR6-Fc strongly augmented cytokine production by PHA-stimulated PBMC and anti-CD3-stimulated T cells. *A*, Human PBMC were cultured in the presence of a suboptimal concentration of PHA (0.2 μg/ml), PHA plus soluble TR6-Fc (20 μg/ml), or PHA plus control recombinant protein TR11-Fc (20 μg/ml). The cytokines secreted into the supernatants at 24, 48, and 72 h were tested with ELISA. Samples were 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 presented. *B*, Peripheral blood T cells were cultured in wells coated with TR6-Fc and a suboptimal concentration of anti-CD3 (*top panels*). In some culture, the T cells were cultured in the presence of a suboptimal concentration of PHA (0.2 μg/ml) in wells coated with TR6-Fc (*bottom panels*). To prepare solid-phase anti-CD3, the wells of 96-well plates were coated overnight at 4°C with 2.5 ng/50 μl/well anti-CD3 (OKT3) in PBS. To prepare solid-phase TR6, the wells were first coated overnight at 4°C with anti-human IgG (250 ng/50 μl/well) in PBS, followed by TR6-Fc (250 ng/50 μl/well) in PBS at room temperature for 6 h. Soluble LIGHT or control recombinant protein TR11-Fc (10 μg/ml for both) was added to some cultures, as indicated. The culture supernatants were harvested at 48 h and assayed for TNF-α and IFN-γ (*top panels*), or harvested at 24, 48, and 72 h for IL-2 and GM-CSF (*bottom panels*). Samples were in duplicate. The experiments were performed more than twice, and means ± SD of a representative experiment are shown.

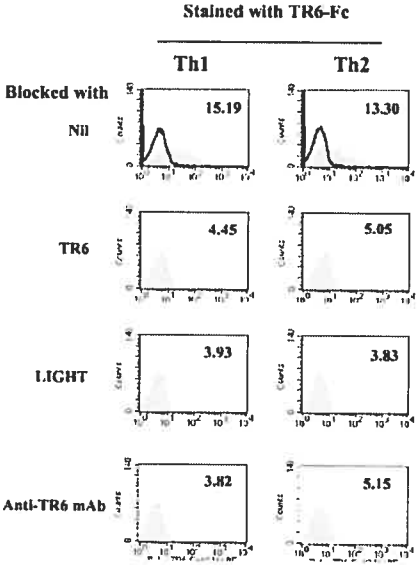

FIGURE 3. LIGHT was the major TR6-Fc ligand on activated Th1 and Th2 cells. Human Th1 and Th2 cells were polarized for 13 days, and reactivated with solid-phase anti-CD3 (clone UCHT1, 1 μg/ml for coating) overnight. Binding of TR6-Fc (15 ng/sample) with these cells (1×10^6 cells/sample) was detected by flow cytometry using PE-conjugated goat anti-human IgG. Soluble TR6 without the Fc tag and LIGHT was used as inhibitors (5 μg/sample) during the staining, as indicated. Solid lines: cells without anti-CD3 reactivation; shaded area: cells reactivated with solid-phase anti-CD3 overnight.

IL-5 (Fig. 4*F*). The results of this section show that TR6 costimulation preferentially augments Th1 but not Th2 cell function in terms of production of certain lymphokines, although it stimulated similar proliferation responses of both cell types.

TR6 and LIGHT on CTL development

To further assess the functional consequence of TR6 costimulation, we examined the CTL development of PBMC in the presence of soluble TR6-Fc. As TR6 expresses in many gastrointestinal tumors (Ref. 17 and our unpublished observations), and ~10% of human intestinal intraepithelial T cells are γδ T cells, we decided to examine the effect of TR6 on CTL activity of γδ T cells. For this purpose, Daudi cells, which are known to elicit massive expansion of Vγ9Vδ2 T cells and are recognized in a TCR-dependent fashion by these T cells (24–26), were used as both stimulators and targets. As shown in Fig. 5*A*, γδ CTL activity was enhanced by soluble TR6-Fc but not normal IgG, in agreement with the proliferation and cytokine studies shown in Figs. 1 and 2. We further demonstrated that soluble LIGHT significantly inhibited unmanipulated (without involvement of TR6) CTL activity (Fig. 5*B*), suggesting the importance of LIGHT reverse signaling in a γδ CTL response.

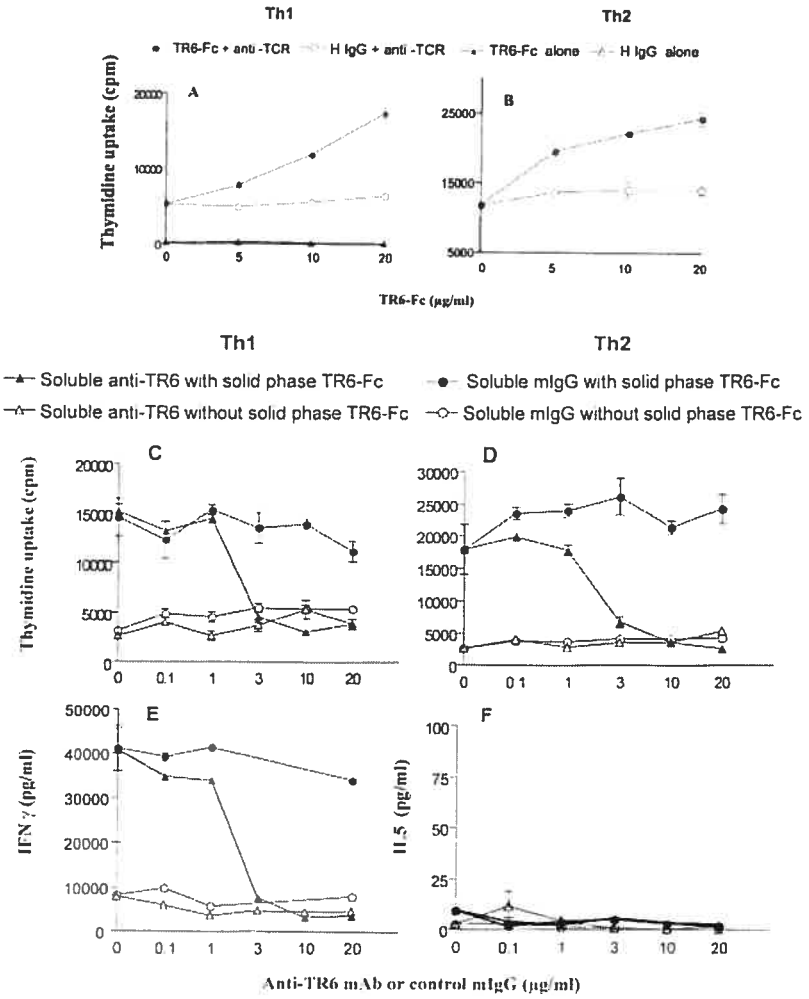

FIGURE 4. Effects of TR6 costimulation on proliferation and lymphokine production of Th1 and Th2 cells. Th1 and Th2 cells were cultured in the presence of suboptimal solid-phase anti-TCR$\alpha\beta$ and various amounts of solid-phase TR6. To prepare solid-phase anti-TCR$\alpha\beta$ and TR6, the wells of 96-well plates were first coated overnight at 4°C with 50 μl/well PBS containing 250 ng goat anti-human IgG and 250 ng goat anti-mouse IgG. After washing, the wells were incubated with 50 μl PBS containing 3 μg/ml anti-TCR$\alpha\beta$ and various concentrations of TR6-Fc, as indicated. In some wells, human IgG was used in place of TR6-Fc as a control. *A* and *B*, Solid-phase TR6 enhanced Th1 and Th2 proliferation dose-dependently. Cell proliferation was measured by [^{3}H]thymidine uptake 2 days after initiation of culture. Means ± SD of triplicate are shown. Similar results were obtained in three experiments. *C* and *D*, Anti-TR6 mAb neutralized the enhancing effect of solid-phase TR6 on Th1 and Th2 proliferation. Th1 and Th2 cells were stimulated with a suboptimal concentration of solid-phase anti-TCR$\alpha\beta$ and an optimal concentration of solid-phase TR6-Fc (20 μg/ml during coating). Anti-TR6 mAb (clone EO_2) of various concentrations, as indicated, was added to the culture; normal mouse IgG1 was used as a control. [^{3}H]Thymidine uptake 2 days after initiation of culture was measured in triplicate, and means ± SD are shown. Similar results were obtained in three experiments. The two bottom curves with hollow symbols in each panel were samples stimulated with anti-TCR$\alpha\beta$ in the absence of solid-phase TR6. *E* and *F*, Differential effects of solid-phase TR6 on Th1 and Th2 cytokine production. Supernatants from samples of *C* and *D* were collected 48 h after initiation of culture, and their IFN-γ (*E*) and IL-5 (*F*) levels were determined by ELISA.

Moreover, solid-phase TR6-Fc similarly enhanced $\gamma\delta$ CTL activity, like soluble TR6-Fc, and such enhancement could be neutralized by soluble LIGHT (Fig. 5*C*), suggesting that LIGHT is the likely molecule that TR6 interacts with, and that cross-linking LIGHT costimulates T cells in $\gamma\delta$ CTL development. We also demonstrated that solid-phase TR6 could augment $\alpha\beta$ T cell CTL, using T cell blasts as target cells (Fig. 5*D*). The revelation of such effect of TR6 under physiological conditions is discussed later.

Discussion

In this study, we report that soluble TR6-Fc enhanced T cell proliferation, cytokine production, and CTL development, which could be blocked by soluble LIGHT. Moreover, TR6-Fc-enhanced proliferation could also be observed in *gld/gld* T cells. Such costimulation preferentially promoted Th1 but not Th2 cells in their lymphokine production. Binding studies showed that the association between TR6 and both Th1 and Th2 cells could be inhibited by LIGHT. Taken together, these results indicate that the ligand of TR6 on T cells actually receives costimulating signals, and LIGHT is likely involved in the process.

We were initially perplexed by the finding that soluble TR6-Fc enhanced proliferation and cytokine production in suboptimally mitogen-stimulated PBMC. Is this because TR6 cross-reacts with other members of the TNF family and thus blocks their putative negative effect on T cells via their receptors? So far, no TNFR members, including Fas and TR2, are known to transduce negative signals into T cells to inhibit their activation. Therefore, no matter how well TR6-Fc cross-reacts with other TNF members, no positive signals will be generated. Thus, we are left with a more plausible mechanism: TR6-Fc cross-links its ligand on the T cell surface, and the ligand reversely transduces costimulating signals into T cells. In this model, soluble TR6, although a TNFR member functions as a ligand, while its ligand functions as a receptor. How plausible is this reverse signaling hypothesis? We established a more clear-cut system by putting TR6 on the solid phase to see whether it could trigger T cell activation. In this system, the possibility that TR6 functioned as a blocker to repress the putative inhibitory interactions between any TNF members and TNFR members was excluded, as TR6 was not in solution, and its total amount was minute. In the presence of TCR cross-linking (e.g., PHA in Fig. 1*E*, anti-CD3 in Fig. 2*B*, anti-TCR$\alpha\beta$ in Fig. 4, or alloantigens in Fig. 5, *C* and *D*), solid-phase TR6-Fc augmented T cell proliferation, lymphokine production, and CTL development, confirming that TR6 can reversely and directly costimulate T cells. Soluble LIGHT inhibited TR6-enhanced proliferation (Fig. 1*E*) and lymphokine production (Fig. 2*B*), and repressed CTL development (Fig. 5, *C* and *D*). Moreover, LIGHT inhibited the binding between TR6 and activated Th1 or Th2 cells (Fig. 3). It is acknowledged that the use of soluble LIGHT as a blocker cannot prove convincingly that LIGHT mediates the reverse signaling, because one could reasonably argue that soluble LIGHT has blocked the binding site of TR6 to FasL, which is also capable of

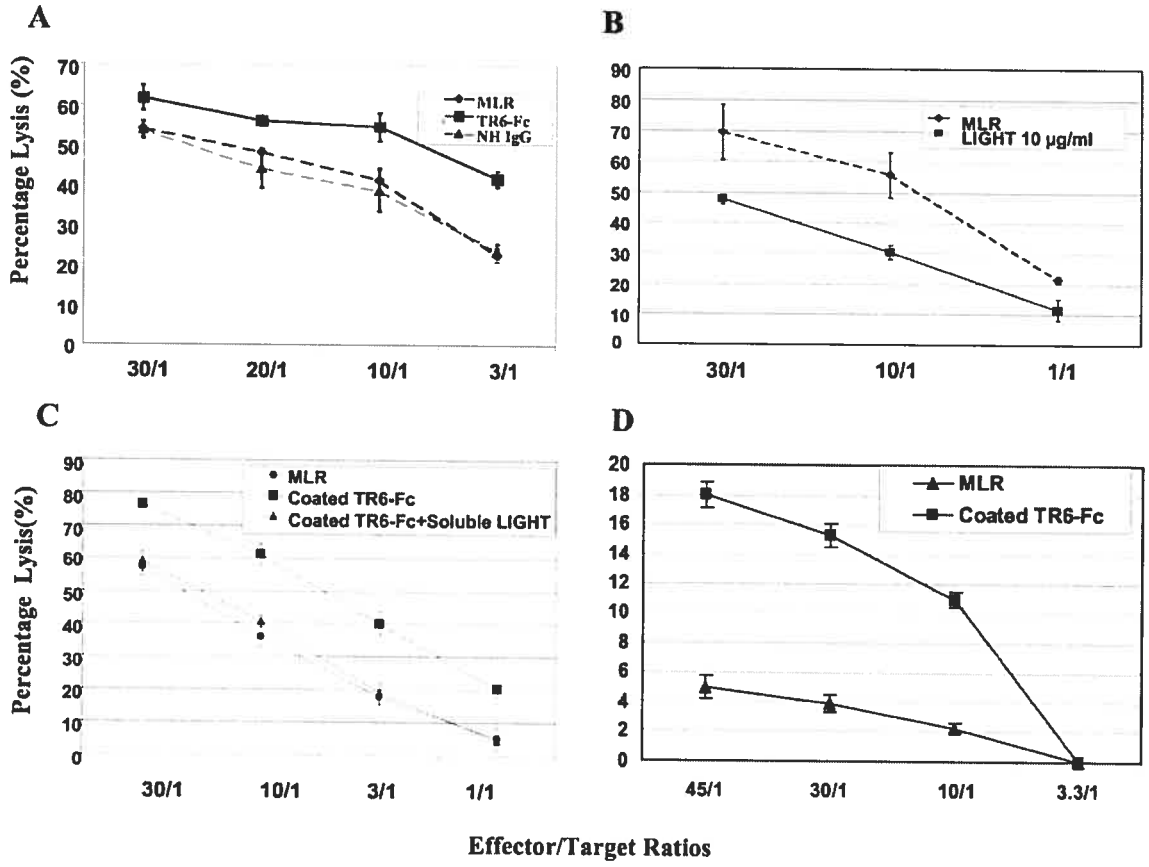

FIGURE 5. Effect of LIGHT reverse signaling on CTL development. *A*, Soluble TR6-Fc enhanced γδ CTL development. Human PBMCs were stimulated with mitomycin C-treated Daudi cells (a B cell lymphoma cell line) for 6 days. TR6-Fc (20 μg/ml) or normal human IgG (NH IgG, as a control, 20 μg/ml) was added to the culture in the beginning. CTL activity in the stimulated cells was measured on day 6 by a standard 4-h ^{51}Cr-release assay, using Daudi cells as targets on day 6. Percentage of target cell lysis is shown. The experiments were performed twice with similar results, and the data of a representative experiment are presented. *B*, Soluble LIGHT inhibited γδ CTL development. The experiment was performed as described above, but in the absence of soluble TR6-Fc. Soluble LIGHT (10 μg/ml) was added in the beginning of the culture. *C*, Solid-phase TR6-enhanced γδ CTL activity and soluble LIGHT neutralized such enhancement. The experiment was performed as described in *A*, with following modifications. The round-bottom wells were precoated with TR6-Fc (0.5 μg/50 μl/well) overnight at 4°C and then washed with PBS. Cells were cultured in these wells containing 250 μl medium in the absence or presence of 20 μg/ml soluble LIGHT. On day 3 of the culture, 70 μl of supernatants per well were replaced with fresh complete medium. The ^{51}Cr-release assay was conducted on day 6. *D*, Solid-phase TR6 enhanced αβ CTL activity. The experiment was performed as described in *A*, except that mitomycin C-treated PBMC from a second individual was used as stimulators. These PBMCs without mitomycin C-treatment were stimulated with solid-phase anti-CD3 and anti-CD28 for 6 days, and were then used as target cells in the standard 4-h ^{51}Cr-release assay.

reverse signaling (27–29). To address this concern, we used solid-phase TR6 to costimulate *gld/gld* mouse T cells, which have mutated nonfunctional FasL, in the presence of solid-phase anti-CD3. At 0.5 μg/ml of anti-CD3, wild-type and *gld/gld* spleen cells similarly augmented proliferation in the presence of TR6; although at lower anti-CD3 concentrations, *gld/gld* spleen cells responded somewhat less well. We are not sure whether such a difference is due to the involvement of FasL, but it is obvious that TR6 could significantly costimulate T cells in the absence of functional FasL. Moreover, TR2, which binds LIGHT but not FasL, could also costimulate mouse T cells in the presence of suboptimal anti-CD3. Our most recent study revealed that the major cell surface ligand that TR6 binds was LIGHT, because TR6 bound well to wild-type T cells but lost most of such binding in LIGHT gene knockout T cells (data not shown). Taken together, these data suggest that a TR6 ligand on the T cell surface can reversely receive costimulation signals from its receptors, and that at least a major portion of such costimulation is via LIGHT. With that said, we cannot rule out the possibility that a small fraction of the reverse costimulation might also be mediated by FasL, or other so far uncharacterized TR6 ligand(s) on the T cell surface. As TL1A, the most recently discovered ligand of TR6, is not expressed on lymphocytes (19), it is thus unlikely to be involved in such reverse signaling.

Reverse signaling through ligands is not a far-fetched concept. Several TNF members on cell surfaces can reversely transduce signals into cells as with LIGHT. Lanier and colleagues (30) and Gray and colleagues (31) showed that CD40L transduces costimulation signals into T cells. Wiley et al. (32) reported that CD30 ligand cross-linking activates neutrophils, and Cerutti et al. (33) showed that such reverse signaling inhibits Ig class switch in B cells. Reverse signaling through membrane TNF-α confers resistance of monocytes and macrophages to LPS (34). Cross-linking of TRANCE enhances IFN-γ secretion by activated Th1 cells (35). Reverse signaling through FasL can promote maximal proliferation of CD8 cytotoxic T cells (27–29). Cross-linking of TRAIL by its solid-phase death receptor 4 increases IFN-γ production and T cell proliferation (36). In the case of CD40L, its ligation results in general protein tyrosine phosphorylation, Ca^{2+} influx, and activation of Lck, protein kinase C, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase in EL4 thyoma cells (37, 38). TRAIL cross-linking also induces p38 mitogen-activated protein kinase activation (36). Therefore, it should not be surprising that

LIGHT can also receive signals from its receptors. The mechanism of signal transduction via LIGHT is unknown at this time. LIGHT has a short and featureless cytoplasmic tail (7). Therefore, the signaling will most likely depend on molecules it associates with.

We recently reported that human TR6-Fc could bind to mouse LIGHT, and that human TR6-Fc inhibited CTL in vitro and allograft rejection in vivo in mice (10). In that paper, the proposed mechanisms of those observations were that TR6 blocked the costimulation from TR6 to TR2, or reversely from TR2 to LIGHT (10), or both, although at that 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 TR6's interference with the bidirectional costimulation between TR2 and LIGHT. However, how can we explain the seemingly opposite effects of soluble human TR6-Fc on human and mouse T cell responses? The Fc portion used in TR6-Fc and TR11-Fc was mutated to eliminate FcγR binding, and TR6-Fc does not bind to either human or mouse FcγR-bearing cells (data not shown). Therefore, the opposite effects of TR6-Fc cannot be explained by its effective anchoring, or the lack of it, on FcγR in the human vs mouse systems, respectively. Rather, this might be a result of different affinity of human TR6 for human and mouse LIGHT. Because TR6-Fc is a dimer (data not shown), in theory it can play dual roles when added into solution: as an inhibitor blocking the bidirectional interaction between TR2 and LIGHT, or as a costimulator cross-linking LIGHT. The former inhibits T cell response while the latter enhances it. Its final effect should be the sum of these two opposite functions, which might have different affinity requirements. In the human system, we believe that higher affinity between TR6 and LIGHT tips the balance toward costimulation; in the mouse system, putative lower affinity between human TR6 and mouse LIGHT does not result in sufficient LIGHT cross-linking, but might be enough for TR6 to interfere with the binding between TR2 and LIGHT. Further studies are needed to examine this hypothesis.

When a stimulatory effect of a molecule is found, we always have to distinguish between two possibilities: the said molecule indeed has a positive effect, or inhibits an existing negative regulatory loop. Does TR6 block an existing negative autocrine loop in which LIGHT acts as a receptor? This possibility is best argued against by the fact that in our model, solid-phase TR6-Fc could stimulate T cells. When TR6-Fc was used to coat the plate, although a concentration of 1–2 μg/100 μl/well was used, only a very small fraction of it actually went onto the plate, and >99.9% of the protein was washed away after the coating process. Thus, not >2 ng of TR6-Fc 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-Fc could interfere with an autocrine loop. Can TR6-Fc 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 three-dimensional entity, solid-phase TR6-Fc can only interfere with a part of the cell surface that has contact with the well. Therefore, the solid-phase TR6-Fc cannot prevent the interaction between a putative soluble suppressive autocrine and LIGHT on most parts of the cell surface that are not in contact with TR6-Fc. Consequently, most LIGHT molecules on a cell surface should still receive negative signals from the putative suppressive autocrine, if there is one. Therefore, it is very difficult to explain the positive effect of solid-phase TR6-Fc. In addition, if solid phase TR6-Fc were to interfere with the negative regulatory loop by leaking itself into solution, TR6 without Fc (likely monomers) should be more efficient to do so, and consequently enhance T cell proliferation better than TR6-Fc. This was obviously not the case, because we showed that TR6 without Fc on solid phase even failed to effectively augment T cell proliferation (Fig. 1*H*). The low efficiency of TR6 without Fc in this experiment might be due to its monomer format, which is less potent in cross-linking LIGHT than the dimeric format of TR6-Fc. Data from literature do not support the negative loop theory. It has been shown that LIGHT transgenic mice overexpressing LIGHT on T cells have enhanced immune response (39, 40, to be detailed in the next paragraph). 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 ~4–5 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.

As mentioned above, LIGHT overexpression in the T cell compartment in LIGHT transgenic mice results in profound inflammation and development of autoimmune syndromes (39, 40); T cells overexpressing LIGHT have an activated phenotype (39). Probably, such up-regulated immune response of T cells is due to stimulation of TR2/Herpesvirus entry mediator 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 T cells (40). However, it is entirely possible that overexpressed LIGHT on the T cells receives stimulation reversely from TR2 expressed on other T cells, and such stimulation augments their responsiveness to TCR ligation.

What is the biological significance of reverse signaling through LIGHT? We found that the reverse signaling through LIGHT preferentially promotes Th1 but not Th2 cell cytokine production. In agreement with this finding, a recent report demonstrated that mucosal T cells overexpressing LIGHT show enhanced Th1 cytokine production (39). Because CTL differentiation depends on Th1 cytokines, it is not surprising that CTL activity was augmented after costimulation through LIGHT by TR6-Fc. It is worth mentioning that soluble LIGHT inhibited CTL activity (without TR6-Fc stimulation) (Fig. 5*C*). This finding underscores the importance of LIGHT costimulation in CTL development. Harrop et al. (41) noticed that at an intermediate concentration LIGHT promotes MLR, but it fails to do so at a higher concentration (10 μg/ml). Such a biphased response probably reflects a shift from costimulation through TR2 by soluble LIGHT to inhibition of TR2-LIGHT bidirectional costimulation, depending on the concentration of LIGHT. This result is consistent with our reverse signaling theory and findings. We speculate that in vivo, the biological function of endogenous soluble LIGHT, which comes from cell surfaces after shedding (9), might be stimulatory or inhibitory, depending on its local concentration and status of aggregation. 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. In this study, we used recombinant TR6-Fc as an artificial binding partner for LIGHT. In vivo, molecules that can trigger LIGHT signaling are probably cell surface TR2 or LTβR. The endogenous TR6 might act as an inhibitor to the bidirectional costimulation between TR2 and LIGHT, or function as a costimulating factor to LIGHT, depending on whether it exists as monomers, or as trimers like other cell surface TNFR family members. This aspect is worth further investigation. If the endogenous TR6 functions as an inhibitor, then gastrointestinal tumors secreting TR6 will certainly gain survival advantage by interfering with $\gamma\delta$ as well as $\alpha\beta$ T cell CTL activities.

Our study revealed a novel phenomenon of reverse signaling through a cell surface TR6 ligand, most likely LIGHT. Increasing cases of such bidirectional signal transduction between receptors and ligands have been found in biological systems. We can take advantage of such a phenomenon by using a soluble ligand (or receptor) to block signaling in both directions and thus modulate biological responses. However, we must be aware that soluble ligands (or receptors) will need to be monomer without aggregation or cell surface anchoring capabilities to be reliable antagonists, or else they will become agonists for one of the directions.

Acknowledgments

We thank Ovid Da Silva, Reviseur-Redacteur, Research Support Office, Research Center, Center Hospitalier de l'Universite de Montreal for the editorial assistance. We also thank Jeff Carrel for his technical assistance, and Drs. Theodora Salcedo and Paul A. Moore for critical reviewing the manuscript.

References

1. Aggarwal, B. B., and K. Natarajan. 1996. Tumor necrosis factors: developments during the last decade. *Eur. Cytokine Netw. 7:93.*
2. Bazzoni, F., and B. Beutler. 1996. The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med. 334:1717.*
3. Tamada, K., K. Shimozaki, A. I. Chapoval, G. Zhu, G. Sica, D. Flies, T. Boone, H. Hsu, Y. X. Fu, S. Nagata, et al. 2000. Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. *Nat. Med. 6:283.*
4. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, et al. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature 385:729.*
5. Kayagaki, N., A. Kawasaki, T. Ebata, H. Ohmoto, S. Ikeda, S. Inoue, K. Yoshino, K. Okumura, and H. Yagita. 1995. Metalloproteinase-mediated release of human Fas ligand. *J. Exp. Med. 182:1777.*
6. Lum, L., B. R. Wong, R. Josien, J. D. Becherer, H. Erdjument-Bromage, J. Schlondorff, P. Tempst, Y. Choi, and C. P. Blobel. 1999. Evidence for a role of a tumor necrosis factor-α (TNF-α)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J. Biol. Chem. 274:13613.*
7. Mauri, D. N., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, et al. 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. *Immunity 8:21.*
8. Tamada, K., K. Shimozaki, A. I. Chapoval, Y. Zhai, J. Su, S. F. Chen, S. L. Hsieh, S. Nagata, J. Ni, and L. Chen. 2000. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J. Immunol. 164:4105.*
9. Morel, Y., J. M. Schiano de Colella, J. Harrop, K. C. Deen, S. D. Holmes, T. A. Wattam, S. S. Khandekar, A. Truneh, R. W. Sweet, J. A. Gastaut, et al. 2000. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. *J. Immunol. 165:4397.*
10. Zhang, J., T. W. Salcedo, X. Wan, S. Ullrich, B. Hu, T. Gregorio, P. Feng, S. Qi, H. Chen, Y. H. Cho, et al. 2001. Modulation of T-cell responses to alloantigens by TR6/DcR3. *J. Clin. Invest. 107:1459.*
11. Yu, K. Y., B. Kwon, J. Ni, Y. Zhai, R. Ebner, and B. S. Kwon. 1999. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J. Biol. Chem. 274:13733.*
12. Harrop, J. A., M. Reddy, K. Dede, M. Brigham-Burke, S. Lyn, K. B. Tan, C. Silverman, C. Eichman, R. DiPrinzio, J. Spampanato, et al. 1998. Antibodies to TR2 (herpesvirus entry mediator), a new member of the TNF receptor superfamily, block T cell proliferation, expression of activation markers, and production of cytokines. *J. Immunol. 161:1786.*
13. Kwon, B. S., K. B. Tan, J. Ni, K. O. Oh, Z. H. Lee, K. K. Kim, Y. J. Kim, S. Wang, R. Gentz, G. L. Yu, et al. 1997. A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. *J. Biol. Chem. 272:14272.*
14. Zhai, Y., R. Guo, T. L. Hsu, G. L. Yu, J. Ni, B. S. Kwon, G. W. Jiang, J. Lu, J. Tan, M. Ugustus, et al. 1998. LIGHT, a novel ligand for lymphotoxin β receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J. Clin. Invest. 102:1142.*
15. Rooney, I. A., K. D. Butrovich, A. A. Glass, S. Borboroglu, C. A. Benedict, J. C. Whitbeck, G. H. Cohen, R. J. Eisenberg, and C. F. Ware. 2000. The lymphotoxin-β receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J. Biol. Chem. 275:14307.*
16. Browning, J. L., I. D. Sizing, P. Lawton, P. R. Bourdon, P. D. Rennert, G. R. Majeau, C. M. Ambrose, C. Hession, K. Miatkowski, D. A. Griffiths, et al. 1997. Characterization of lymphotoxin-$\alpha\beta$ complexes on the surface of mouse lymphocytes. *J. Immunol. 159:3288.*
17. Pitti, R. M., S. A. Marsters, D. A. Lawrence, M. Roy, F. C. Kischkel, P. Dowd, A. Huang, C. J. Donahue, S. W. Sherwood, D. T. Baldwin, et al. 1998. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature 396:699.*
18. Bai, C., B. Connolly, M. L. Metzker, C. A. Hilliard, X. Liu, V. Sandig, A. Soderman, S. M. Galloway, Q. Liu, C. P. Austin, and C. T. Caskey. 2000. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc. Natl. Acad. Sci. USA 97:1230.*
19. Migone, T.-S., J. Zhang, X. Luo, L. Zhuang, C. Chen, B. Hu, J. S. Hong, J. W. Perry, S. F. Chen, J. X. H. Zhou, et al. 2002. TL1A is a novel ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity 16:479.*
20. Connolly, K., Y. H. Cho, R. Duan, J. Fikes, T. Gregorio, D. W. LaFleur, Z. Okoye, T. W. Salcedo, G. Santiago, S. Ullrich, et al. 2001. In vivo inhibition of Fas ligand-mediated killing by TR6, a Fas ligand decoy receptor. *J. Pharmacol. Exp. Ther. 298:25.*
21. Heyningen, V. V., D. J. H. Brock, and S. V. Heyningen. 1983. A simple method for ranking the affinities of monoclonal antibodies. *J. Immunol. Methods 62:147.*
22. Luo, H., H. Chen, P. Daloze, J. Y. Chang, G. St. Louis, and J. Wu. 1992. Inhibition of in vitro immunoglobulin production by rapamycin. *Transplantation 53:1071.*
23. Luo, H., H. Chen, P. Daloze, G. St. Louis, and J. Wu. 1993. Anti-CD28 anti-antibody- and IL-4-induced human T cell proliferation is sensitive to rapamycin. *Clin. Exp. Immunol. 94:371.*
24. Davodeau, F., M. A. Peyrat, M. M. Hallet, J. Gaschet, I. Houde, R. Vivien, H. Vie, and M. Bonneville. 1993. Close correlation between Daudi and mycobacterial antigen recognition by human $\gamma\delta$ T cells and expression of V9JPC1γ/V2DJCδ-encoded T cell receptors. *J. Immunol. 151:1214.*
25. Bukowski, J. F., C. T. Morita, Y. Tanaka, B. R. Bloom, M. B. Brenner, and H. Band. 1995. Vγ2Vδ2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. *J. Immunol. 154:998.*
26. Fisch, P., M. Malkovsky, S. Kovats, E. Sturm, E. Braakman, B. S. Klein, S. D. Voss, L. W. Morrisse, R. DeMars, and W. J. Welch. 1990. Recognition by human Vγ9/Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science 250:1269.*
27. Suzuki, I., and P. J. Fink. 1998. Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through Fas ligand. *J. Exp. Med. 187:123.*
28. Suzuki, I., and P. J. Fink. 2000. The dual functions of Fas ligand in the regulation of peripheral $CD8^+$ and $CD4^+$ T cells. *Proc. Natl. Acad. Sci. USA 97:1707.*
29. Suzuki, I., S. Martin, T. E. Boursalian, C. Beers, and P. J. Fink. 2000. Fas ligand costimulates the in vivo proliferation of $CD8^+$ T cells. *J. Immunol. 165:5537.*
30. Cayabyab, M., J. H. Phillips, and L. L. Lanier. 1994. CD40 preferentially costimulates activation of $CD4^+$ T lymphocytes. *J. Immunol. 152:1523.*
31. van Essen, D., H. Kikutani, and D. Gray. 1995. CD40 ligand-transduced costimulation of T cells in the development of helper function. *Nature 378:620.*
32. Wiley, S. R., R. G. Goodwin, and C. A. Smith. 1996. Reverse signaling via CD30 ligand. *J. Immunol. 157:3635.*
33. Cerutti, A., A. Schaffer, R. G. Goodwin, S. Shah, H. Zan, S. Ely, and P. Casali. 2000. Engagement of CD153 (CD30 ligand) by $CD30^+$ T cells inhibits class switch DNA recombination and antibody production in human IgD^+IgM^+ B cells. *J. Immunol. 165:786.*
34. Eissner, G., S. Kirchner, H. Lindner, W. Kolch, P. Janosch, M. Grell, P. Scheurich, R. Andreesen, and E. Holler. 2000. Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *J. Immunol. 164:6193.*
35. Chen, N. J., M. W. Huang, and S. L. Hsieh. 2001. Enhanced secretion of IFN-γ by activated Th1 cells occurs via reverse signaling through TNF-related activation-induced cytokine. *J. Immunol. 166:270.*
36. Chou, A. H., H. F. Tsai, L. L. Lin, S. L. Hsieh, P. I. Hsu, and P. N. Hsu. 2001. Enhanced proliferation and increased IFN-γ production in T cells by signal transduced through TNF-related apoptosis-inducing ligand. *J. Immunol. 167:1347.*
37. Brenner, B., U. Koppenhoefer, A. Lepple-Wienhues, H. Grassme, C. Muller, C. P. Speer, F. Lang, and E. Gulbins. 1997. The CD40 ligand directly activates T-lymphocytes via tyrosine phosphorylation dependent PKC activation. *Biochem. Biophys. Res. Commun. 239:11.*
38. Brenner, B., U. Koppenhoefer, H. Grassme, J. Kun, F. Lang, and E. Gulbins. 1997. Evidence for a novel function of the CD40 ligand as a signalling molecule in T-lymphocytes. *FEBS Lett. 417:301.*
39. Shaikh, R. B., S. Santee, S. W. Granger, K. Butrovich, T. Cheung, M. Kronenberg, H. Cheroutre, and C. F. Ware. 2001. Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction. *J. Immunol. 167:6330.*
40. Wang, J., J. C. Lo, A. Foster, P. Yu, H. M. Chen, Y. Wang, K. Tamada, L. Chen, and Y. X. Fu. 2001. The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. *J. Clin. Invest. 108:1771.*
41. Harrop, J. A., P. C. McDonnell, M. Brigham-Burke, S. D. Lyn, J. Minton, K. B. Tan, K. Dede, J. Spampanato, C. Silverman, P. Hensley, et al. 1998. Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. *J. Biol. Chem. 273:27548.*

Article 3:

DcR3/TR6 Modulates Immune Cell Interactions

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Journal of Cellular Biochemistry 89 (3): 603-612, 2003

Note: In this paper Guixiu Shi performed experiments as shown in Figure 5. All the remaining works were performed by Xiaochun Wan

Journal of Cellular Biochemistry 89:603–612 (2003)

DcR3/TR6 Modulates Immune Cell Interactions

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Abstract DcR3/TR6, a secreted protein, is a member of TNF receptor family. Its ligands include FasL, LIGHT, and TL1A, all TNF family members. TR6 can interfere with FasL- or LTβR-mediated apoptosis; it can also inhibit T-cell costimulation by blocking the two-way signaling between TR2 and LIGHT, and the one-way signaling from TL1A to DR3. In this study, we discovered that TR6 was secreted by peripheral blood mononuclear cells (PBMC) stimulated by T-cell mitogens. It inhibited actin polymerization of T cells upon mitogen stimulation, and repress T-cell pseudopodium formation, which is known to be important for cell–cell interaction. As a consequence, T-cell aggregation stimulated by alloantigens, anti-CD3 or PHA was suppressed by either soluble or solid phase TR6-Fc. This result suggests that TR6 might regulate T-cell interaction with other cells such as antigen-presenting cells (APC) or their fellow T cells by preventing them from forming inseparable cell clusters, which are undesirable for the progression of immune responses. J. Cell. Biochem. 89: 603–612, 2003.

Key words: TR6/DcR3; LIGHT; cell aggregation; actin polymerization

DcR3/TR6, a soluble factor due to its lack of the transmembrane domain, belongs to the TNFR family. TR6 can bind to TNF family members FasL [Pitti et al., 1998], LIGHT [Yu et al., 1999], and TL1A [Migone et al., 2002]. Binding of TR6 to FasL blocks Fas-mediated apoptosis. Moreover, since LIGHT and its receptor HveA trigger bi-directional costimulation of T cells [Zhang et al., 2001; Shi et al., 2002], LIGHT binding by TR6 can interfere with such costimulation and consequently inhibit T-cell activation [Zhang et al., 2001]. Similarly, the interaction between TR6 and TL1A disrupts T-cell costimulation by TL1A through its receptor DR3 [Migone et al., 2002], and results in abated T-cell responses [Migone et al., 2002]. Due to probably a combination of these mechanisms, in vivo administration of TR6 reduces graft-versus-host diseases, and inhibits heart allograft rejection [Zhang et al., 2001]. It is conceivable that TR6-secreting tumors utilize these mechanisms to avoid apoptosis, and that TR6 plays an importance regulatory role in normal immune responses.

In the course of our study on the immune regulatory role of TR6, we found that TR6 secretion by leukocytes was significantly enhanced during mitogen activation of T cells. In the presence of soluble or solid phase TR6, T cells could no longer form typical clumps upon mitogen stimulation, and their pseudopodium formation was inhibited. These findings revealed a so-far undocumented TR6 function on T cells.

Jiangping Wu is a National Research Scholar of Fonds de la recherche santé du Québec.

Grant sponsor: Canadian Institutes of Health Research; Grant numbers: MOP57697, PPP57321; Grant sponsor: Canadian Institute of Health Research/Canadian Blood Service; Grant sponsor: Heart and Stroke Foundation of Quebec; Grant sponsor: Roche Organ Transplantation Research Foundation (ROTRF), Switzerland; Grant number: 474950960; Grant sponsor: Juvenile Diabetes Research Foundation International; Grant sponsor: Kidney Foundation of Canada; Grant sponsor: J.-Louis Levesque Foundation (to JW).

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Received 19 December 2002; Accepted 20 February 2003
DOI 10.1002/jcb.10523

MATERIALS AND METHODS

Recombinant Proteins

The preparation of recombinant proteins TR6-Fc, TR6, and TR11-Fc was described in our previous publication [Zhang et al., 2001]. TR11 (GITR) [Ronchetti et al., 2002] and Fc fusion protein TR11-Fc had no effect on T-cell aggregation, compared with PBS or normal human IgG, and was thus used as a control protein for TR6-Fc.

Lymphocyte Preparation and Culture

Peripheral blood mononuclear cells (PBMC) were prepared by Lymphoprep gradient (NYCOMED, Oslo, Norway), and T cells were obtained from PBMC by sheep red blood cell rosetting [Luo et al., 1993], or by negative selection (deletion of cells positive for CD11b, CD16, CD19, CD36, and CD56) with magnetic beads according to the manufacturer's instructions (Miltenyi, Auburn, CA).

Mouse spleen cells were prepared by lysing red blood cells flushed out of the spleen [Luo et al., 2001]. Spleen T cells were purified by deleting Ig-positive and adhesion cells with T-cell columns according to the manufacturer's instructions (Cedarlane, Hornby, Ont., Canada).

All cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics. RPMI 1640, FCS, penicillin–streptomycin, and L-glutamine were purchased from Life Technologies, Inc. (Burlington, Ont., Canada). ^{3}H-thymidine uptake was measured as described previously [Luo et al., 1993; Luo et al., 2001].

Mixed Lymphocyte Reaction (MLR)

For human MLR, PBMC were isolated from two healthy volunteer donors (donors A and B). PBMC from donor B were pre-treated with mitomycin C, and were used as stimulators. The cells from donors A and B were then mixed at 1:1 ratio and cultured at a final concentration of 8×10^6 cells/2 ml/well in 24-well plates. Cells from donors A and B were also incubated alone as controls. For mouse MLR, BALB/c spleen cells were pre-treated with mitomycin C, and were used as stimulators. C57BL/6 and BALB/c spleen cell were then mixed at 1:1 ratio, and cultured in 24-well plates at 8×10^6 cells/2 ml/well.

TR6 ELISA

Anti-TR6 mAb (clone 17B07) was described in our previous publication [Zhang et al., 2001]. The TR6 polyclonal antibody was purified from antisera generated from rabbits immunized with four synthetic peptides that spanned the TR6 protein sequence: V30-R46, R64-Q89, E240-R258, and R284-L297 (amino acid positions were relative to the start methionine). Rabbit antisera were purified on a TR6-coupled Affi-Gel10 column. The specificity of the TR6 polyclonal antibody was demonstrated in the ELISA by testing cross-reactivity to recombinant OPG and HveA, the two TNF receptor family members most closely related to TR6. Neither OPG nor HveA was detectable in the TR6 ELISA. The preparation of recombinant TR6 was described in detail previously [Zhang et al., 2001]. The protocol of TR6 ELISA is as follows. Ninety-six-well Nunc Maxisorb plates were coated overnight with anti-TR6 mAb in 0.05 M $NaHCO_3$ buffer (3 μg/ml, 100 μl/well) at 4°C. After washing with buffer A (PBS containing 0.1% Tween-20), the plates were blocked with 3% BSA in PBS (250 μl/well) for 1 h at room temperature. Serum samples were diluted when necessary in buffer B (PBS containing 0.1% Tween-20 and 1% BSA), and incubated overnight in the mAb coated plates at 4°C. The plates were washed and reacted with biotinylated rabbit anti-TR6 Ab (0.125 μg/ml in buffer B, 100 μl/well) at room temperature for 2 h. They were then washed and reacted with streptavidin-peroxidase (1:2,000 v/v in buffer B, Vector Laboratories, Burlingame, CA). After additional washes, a freshly prepared color development mixture (1:1 v/v mixture of tetramethyl benzidine solution and H_2O_2 solution, TMB Microwell Peroxidase Substrate System, Kirkegard & Perry, Gaithersburg, MD) was added to the plates (100 μl/well). The reaction was stopped after 20 min at room temperature with 0.1 N H_2SO_4 (100 μl/well), and $OD_{450\ nm}$ was subsequently measured. Recombinant human TR6 was used as standards. ELISA sensitivity was below 10 pg/ml.

Flow Cytometry and Confocal Microscopy

Human T cells were cultured overnight in the presence of TR6-Fc. They were reacted with 1 μg anti-CD3 in 100 μl cold PBS for 30 min on ice, and after washing, with 0.5 μg of goat anti-human IgG for another 30 min. The cells were

then washed with cold PBS, and transferred to warm PBS at 37°C for 5 min. For F-actin staining, 1×10^6 of the CD3-crosslinked T cells were fixed with 3.7% formalin for 30 min at room temperature and stained with Alexa Fluor-488-conjugated phalloidin (Molecular Probes, Eugene, OR). The cells were analyzed with a Coulter Epics-XL flow cytometer and a confocal microscope. Digital images were processed with Photoshop (Adobe, Seattle, WA).

RESULTS

Human PBMC Secrete TR6 After Mitogen Activation

Although TR6 is produced by some tumors, its possible secretion by leukocytes has not been investigated. As our recent study showed that TR6 could regulate T-cell activation and in vivo immune responses [Zhang et al., 2001], it is logical to ask whether leukocyte could secrete TR6. We developed sensitive ELISA for this purpose. Human PBMC were cultured in the absence or presence of a mitogen, PHA, and TR6 was measured in the supernatants after 48 h. TR6 was detectable in unstimulated culture at about 100 pg/ml. With PHA (2 μg/ml) stimulation, the TR6 level showed a sixfold increment, and reached 620 pg/ml (Fig. 1), suggesting that leukocyte-secreted TR6 might participate in immune regulation.

TR6 Inhibits Leukocyte Aggregations in MLR

During our study of TR6's effect on T-cell activation, we noticed, unexpectedly, that soluble TR6 strongly inhibited cell aggregation in the MLR. As illustrated in Figure 2A, human PBMC from donors A or B alone showed no apparent aggregation in culture. In MLR, the cells formed clumps, starting from 6 h and lasting for up to 6 days. Soluble TR6-Fc (10 μg/ml) completely suppressed clump formation at 16 h. The suppression was obvious at about 6 h after the initiation of culture when untreated MLR or a control recombinant protein TR11-Fc-treated MLR started displaying clumps and lasted for at least 72 h (data not shown).

Human TR6-Fc was also effective in inhibiting clump formation of mouse MLR, as depicted in Figure 2B. This was not surprising because human TR6 binds mouse LIGHT and FasL [Zhang et al., 2001].

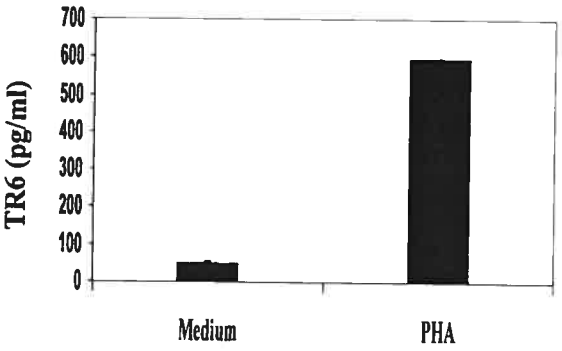

Fig. 1. TR6 is secreted by activated T cells. Human peripheral blood mononuclear cells (PBMC) were cultured for 48 h in 24-well plates at 4×10^6/2 ml/well in the presence of PHA (2 μg/ml). The culture supernatants were harvested and tested for TR6 levels by ELISA. The means ± SD of duplicate samples are shown. The experiments were performed more than twice, and results from a representative one are shown.

TR6 Directly Prevents T Cells From Aggregation Formation

We next tested whether TR6 could inhibit clump formation when T cells were activated by mitogens other than alloantigens. When PBMC were stimulated by PHA (0.2 μg/ml) or anti-CD3 (0.5 μg/ml), they readily formed clumps in 4 h, and the clumps lasted for 3–5 days. Figure 3A documents clumping at 16 h. Clump formation was significantly inhibited in the presence of TR6-Fc (10 μg/ml), but a control fusion protein, TR11-Fc, had no such outcome. To prove that the effect occurred directly on T cells but not via other cell populations in PBMC, purified T cells were similarly treated with PHA in the presence or absence of TR6-Fc (Fig. 3B). Again, TR6-Fc but not TR11-Fc drastically suppressed the cell clustering, suggesting that TR6 acts directly on T cells to prevent their aggregation upon mitogen stimulation.

Anti-Aggregation Effect Can be Achieved by Solid Phase TR6, and is Likely Mediated by LIGHT

TR6 can bind to FasL and LIGHT, both of which are capable of reverse signaling [Suzuki and Fink, 1998; Shi et al., 2002]. To identify which of the two was involved in mediating the inhibitory effect on T-cell aggregation, soluble LIGHT and Fas were tested as blocking reagents. Like soluble TR6-Fc, TR6-Fc on solid phase via plate-bound goat anti-human IgG inhibited T-cell aggregation (Fig. 4). Soluble LIGHT (10 μg/ml) but not Fas (10 μg/ml) potently neutralized the inhibitory effect of solid phase TR6, while LIGHT by itself had no influence on cell aggregation. These data

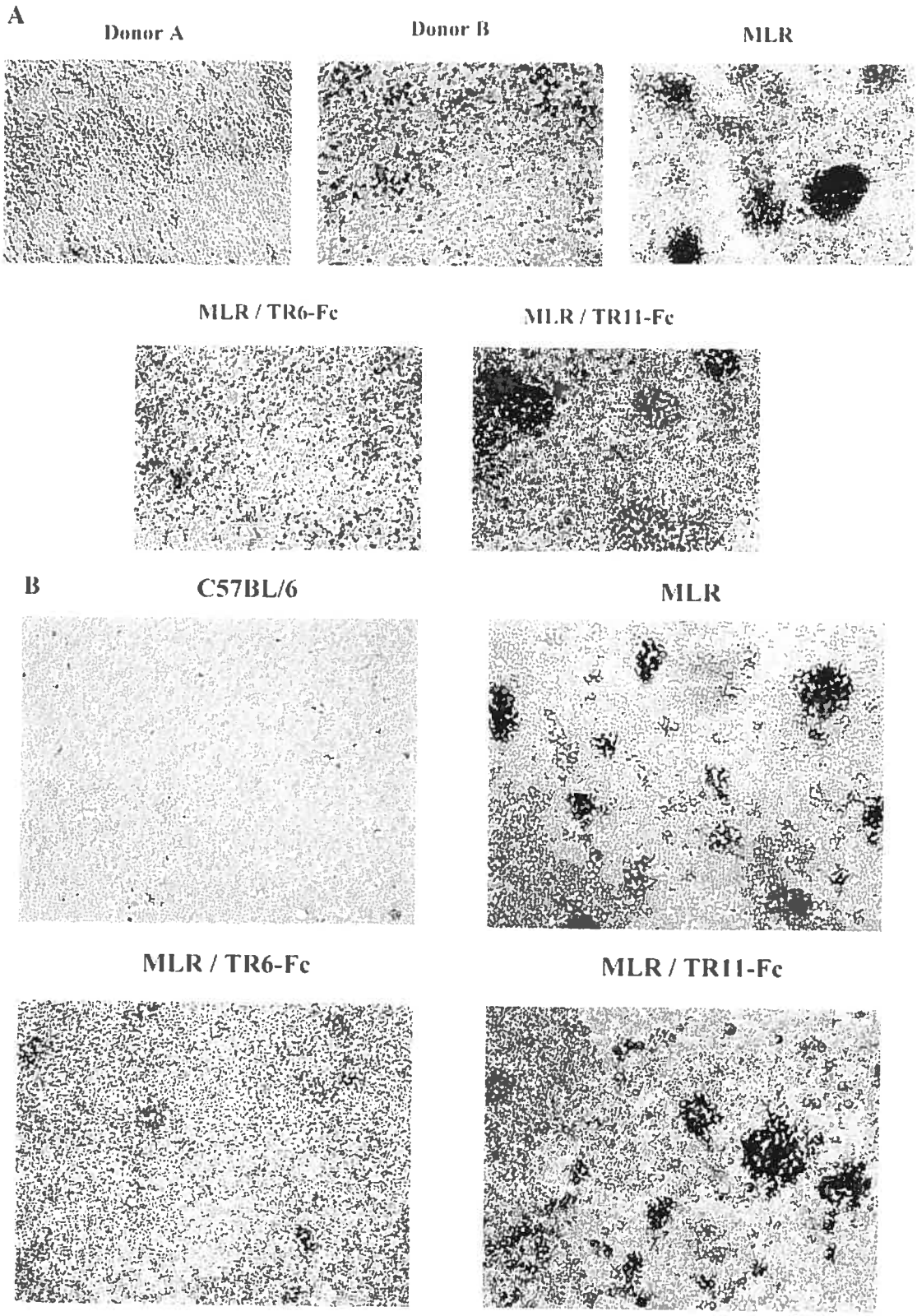

Fig. 2. TR6-Fc inhibits mononuclear cell interaction in the mixed lymphocyte reaction (MLR). Human MLR (**A**) or mouse MLR (**B**) was performed in the presence of TR6-Fc (MLR/TR6-Fc) or a control fusion protein TR11-Fc (MLR/TR11-Fc), both at 10 μg/ml, as indicated. Photos were taken 16 h after culture. For human MLR, PBMC from donors A alone, B alone, or a mixture of both (MLR) at the 1:1 ratio (4×10^6 cells/2 ml/well for each donor) were cultured in 24-well plates. For mouse MLR, spleen cells from C57BL/6 alone, or a mixture of C57BL/6 and BALB/c spleen cells (MLR) at the 1:1 ratio, were cultured in 24-well plates (4×10^6 cells/2 ml/well for each strain). Human donor B PBMC and BALB/c spleen cells were pretreated with mitomycin C before MLR.

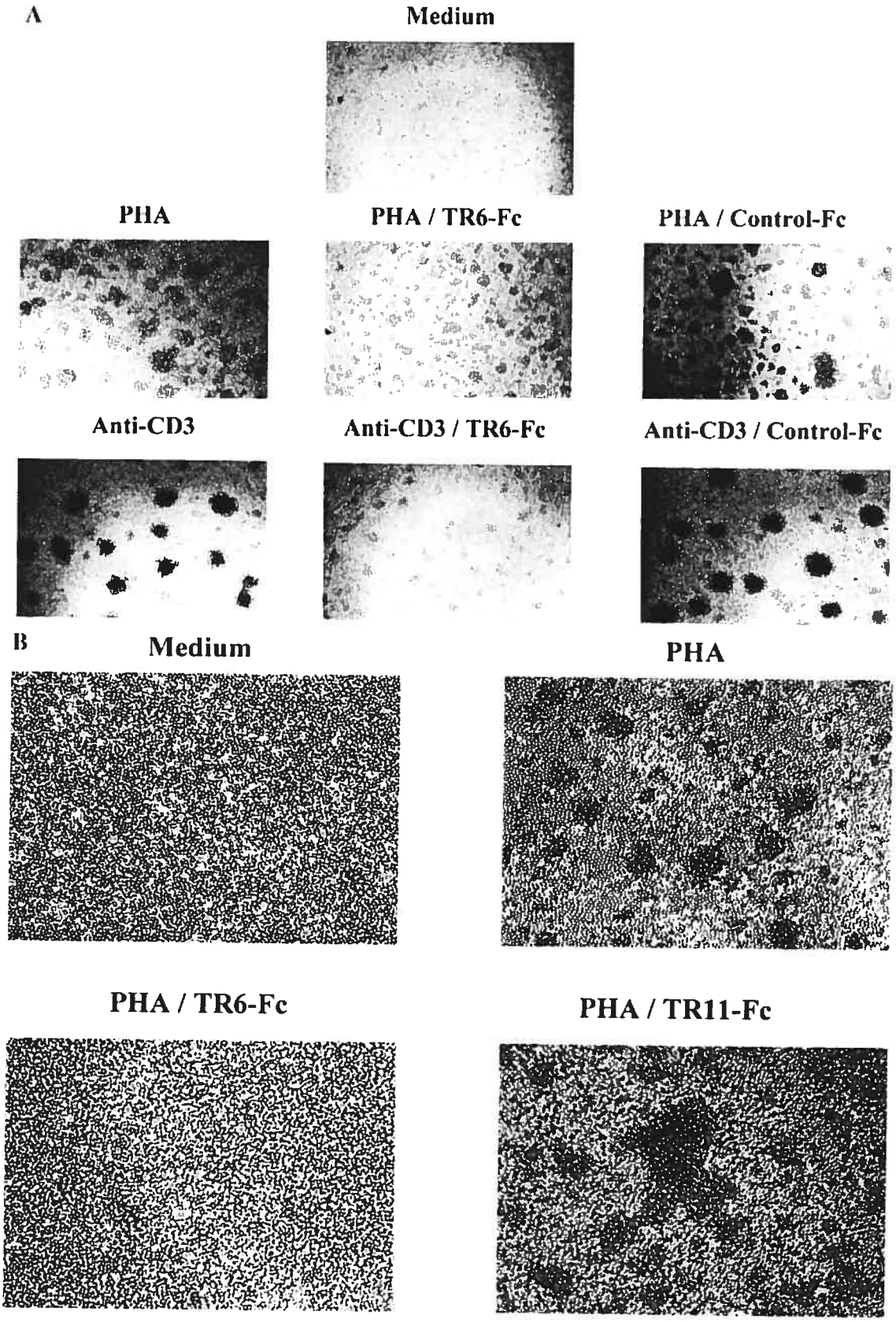

Fig. 3. TR6-Fc inhibits PBMC and T-cell aggregation induced by mitogen stimulation. Human PBMC (**A**) or purified T cells (**B**) were cultured in 96-well flat-bottomed plates at 4×10^5 cells/200 µl/well. PHA (0.2 µg/ml), anti-CD3 Ab (0.5 µg/ml), TR6-Fc (10 µg/ml), and TR11-Fc (10 µg/ml) were present as indicated. Photos were taken 4 h after culture.

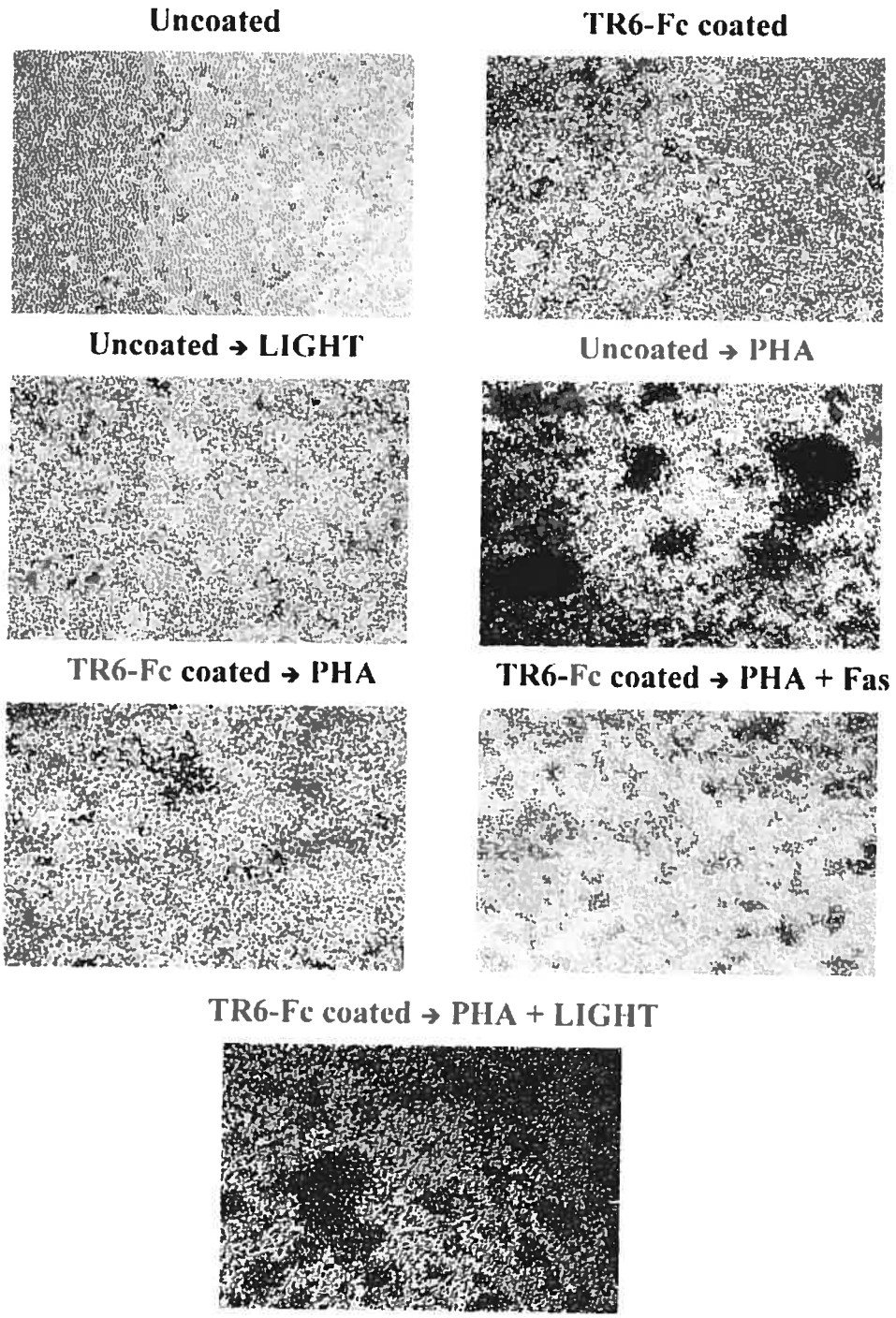

Fig. 4. Effect of solid phase TR6-Fc, soluble Fas and soluble LIGHT on T-cell aggregation. To prepare solid phase TR6-Fc, NUNC 96-well-plates were coated overnight at 4°C with 5 μg/ml goat anti-human IgG (Southern Biotechnology, Birmingham, AL) in PBS at 50 μl/well. After washing, the plates were incubated with TR6-Fc or TR11-Fc (both at 10 μg/ml) in PBS at 37°C for 2 h. T cells were cultured in these wells in the presence of PHA (0.2 μg/ml), soluble Fas (5 μg/ml), or soluble LIGHT (5 μg/ml), as indicated. Photos were taken 48 h after culture. Uncoated: wells were not coated with any reagents; TR6-Fc-coated: wells were coated with goat anti-human IgG followed by TR6-Fc; uncoated → LIGHT: T cells were cultured in uncoated wells in the presence of soluble LIGHT; uncoated → PHA: T cells were cultured in uncoated wells in the presence of PHA; TR6-Fc-coated → PHA: cells were cultured in TR6-Fc-coated wells in the presence of PHA; TR6-Fc-coated → PHA + Fas: cells were cultured in TR6-Fc-coated wells in the presence of PHA and Fas; TR6-Fc-coated → PHA + LIGHT: cells were cultured in TR6-Fc-coated wells in the presence of PHA and LIGHT.

suggest that LIGHT mediates the effect of TR6 in preventing T-cell aggregation.

TR6 Inhibits Actin Polymerization and Pseudopodium Formation of T Cells Upon T-Cell Receptor (TCR) Ligation

T-cell aggregation upon mitogen stimulation is a process requiring cytoskeleton reorganization followed by cellular morphological changes. We examined the actin polymerization of T cells with TR6 pre-treatment. T cells were cultured overnight in the presence of TR6-Fc or TR11-Fc (both at 10 μg/ml) in serum-free medium, and then crosslinked with anti-CD3. The actin polymerization of these cells was analyzed by phalloidin staining using flow cytometry, and cell morphology was examined under a confocal microscope. The actin in T cells activated by CD3 crosslinking rapidly underwent polymerization as evidenced by a significant increase in the intensity of phalloidin staining accompanied by protrusion of pseudopodia within 5 min (Fig. 5). TR6 pretreatment strongly inhibited the increment of phalloidin staining (Fig. 5A,B), and pseudopodium formation also was repressed (Fig. 5B,C). Thus, TR6 pre-treatment likely affected an event upstream of actin polymerization, and this might be responsible for the observed inhibition of T-cell aggregation.

DISCUSSION

In this study, we reported, for the first time, that TR6 was secreted by leukocytes after T-cell activation, and revealed a previously undocumented function of TR6 in regulating T-cell interaction with other leukocytes.

Cell aggregation during T-cell activation was inhibited by soluble and solid phase TR6. However, T-cell proliferation was enhanced in the presence of solid phase TR6 in combination with suboptimal TCR ligation [Zhang et al., 2001], indicating adequate T-cell activation under such conditions. During T-cell activation, essential signals are transduced into cells within several minutes [Gil et al., 2002]. The inhibition of T-cell aggregation several hours after their activation did not interfere with their proliferation, implying that the normally observed T-cell aggregation after mitogen stimulation in in vitro culture is not an essential part of the activation program.

The likely mechanism of TR6's inhibitory effect on T-cell aggregation is reverse signaling through LIGHT. Cell surface LIGHT, and several other TNF family members, such as CD40L [Van Essen et al., 1995], CD30L [Wiley et al., 1996; Cerutti et al., 2000], TNF-α [Eissner et al., 2000], TRANCE [Chen et al., 2001], FasL [Suzuki and Fink, 2000; Suzuki et al., 2000], and TRAIL [Chou et al., 2001], can transduce signals into T cells [Serrador et al., 1998]. We recently found that crosslinking of LIGHT leads to inhibition of p38 MAPK activation and actin polymerization in T cells upon chemokine stimulation (data not shown). This is in keeping with the inhibitory impact of TR6 on T-cell aggregation, since such an effect also depends on actin polymerization. Currently, there are three known TR6 ligands, i.e., LIGHT, FasL, and TL1A. Since TL1A is mainly expressed on endothelial cells [Migone et al., 2002], but not on T cells, it is not relevant to this study. We tested soluble Fas and LIGHT as competitors to solid phase TR6 to assess their involvement. Soluble FasL was not used for this purpose because of its potential apoptosis-inducing effect on activated T cells. LIGHT but not Fas reversed the TR6 effect, suggesting that TR6 inhibits T-cell aggregation via LIGHT reverse signaling. We cannot totally exclude the possibility that a so-far uncharacterized TR6 ligand X on T-cell surface also mediates the anti-aggression effect of TR6, but this ligand X and LIGHT must have an identical binding site to TR6, and consequently LIGHT can competitively inhibit ligand X's binding to TR6. However, evidence for the existence of such a ligand is lacking.

We showed that, like solid phase TR6-Fc, soluble TR6-Fc was also capable of preventing T-cell aggregation. It is possible that only low-degree crosslinking of TR6 ligands by dimeric TR6-Fc or the aggregated form of TR6-Fc in solution is sufficient to trigger such an effect.

T-cell actin polymerization was inhibited downstream of ligand engagement by TR6-Fc. Such inhibition was probably a cause of repressed T-cell pseudopodium formation. The signaling pathway through which TR6 affects actin polymerization is currently under investigation. It has been reported that the formation of uropods, which are rear-end pseudopodia, is essential for T-cell aggregation after mitogen stimulation [Serrador et al., 1998]. Thus, the reduced formation of pseudopodia in TR6-treated T cells likely contributes to poor formation of T-cell aggregation.

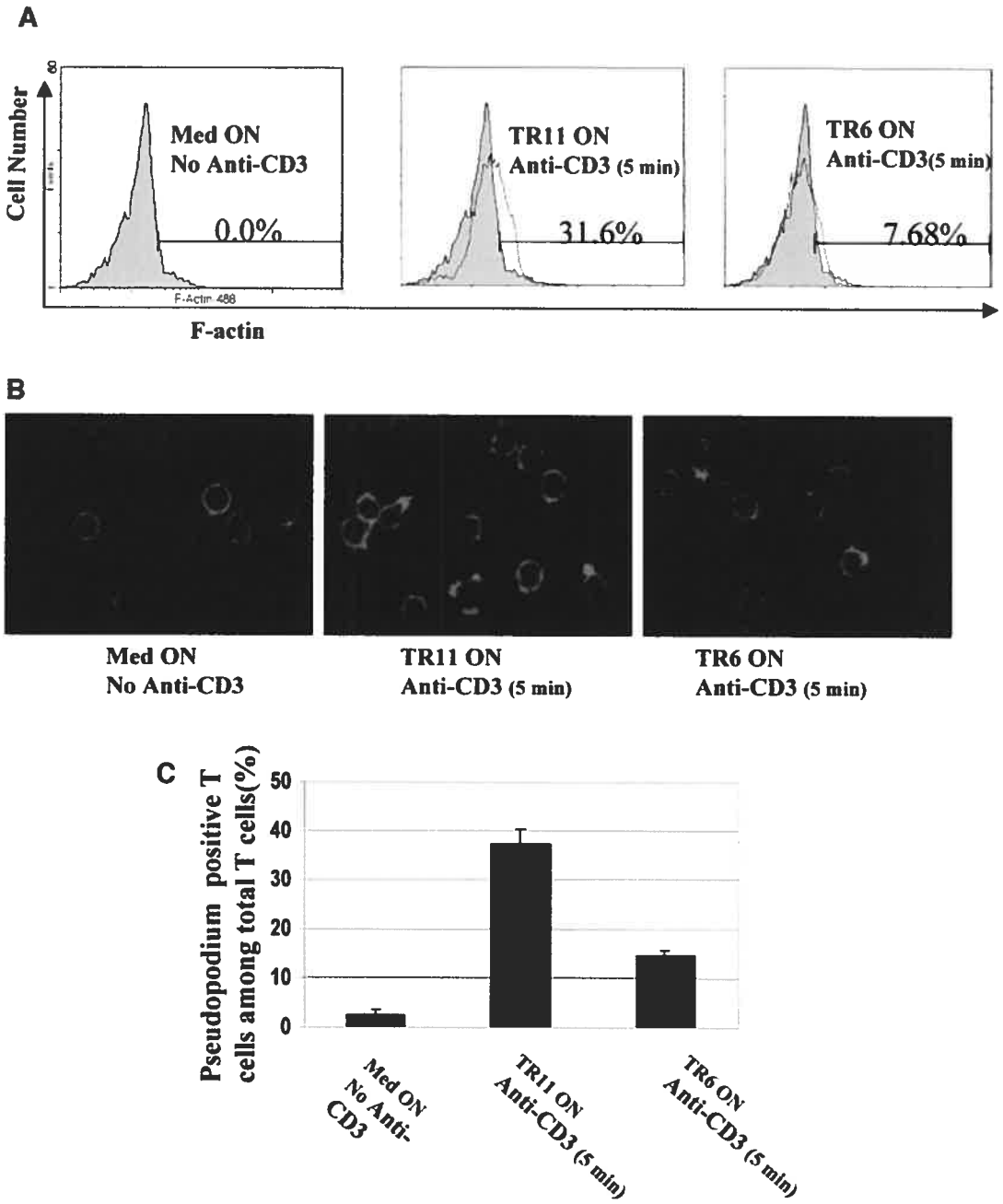

Fig. 5. TR6-Fc inhibits anti-CD3-induced T-cell actin polymerization and pseudopodium formation. T cells were cultured overnight in medium in the absence (Med ON), or presence of TR11-Fc (TR11, ON; 10 μg/ml) or TR6-Fc (TR6, ON; 10 μg/ml). After washing, the cells were crosslinked with anti-CD3 for 5 min, as indicated. All the experiments were performed more than twice, and results from a representative one are shown. **A:** Flow cytometry analysis of F-actin. F-actin staining of T cells cultured overnight in medium without anti-CD3 simulation was used as a negative control, with its F-actin intensity (shaded area) set at 0%. F-actin staining (solid lines) of cells receiving different pre-treatments followed by anti-CD3 crosslinking is shown. Percentage of cells positive for F-actin staining above the control staining is shown. All three panels are in log scale. **B:** Confocal microscopy of T-cell morphology. The same set of T cells, as described in (A), was examined by confocal microscopy. **C:** Quantitative assessment of T-cell pseudopodium formation. The cells in (B) were quantified for pseudopodium formation. Three randomly selected view fields (containing about 80–100 cells per field) per sample were examined, and the means ± SD of the percentages of pseudopodium-positive cells among total cells of the three fields are shown.

What is the physiological significance of TR6 inhibiting T-cell aggregation? When T-cell activation is initiated, T cells in lymphoid organs need to interact with antigen-presenting cells (APC). In addition, close T cell–T cell cooperation is also required for optimal CD4 responses and for CD4 cells to help CD8 cells through local lymphokine secretions. Such T cell–T cell cooperation has been reported in the case of LIGHT and HveA [Tamada et al., 2000], members of TNF and TNFR families, respectively, which are both expressed on T cells, and their interaction leads to optimal T-cell responses. A recent study has shown that T cells recognizing the self-MHC present increased response to foreign antigens [Wulfing et al., 2002], and obviously, the self-MHC could be ones from a fellow T cell. This validates the concept of T cell–T cell collaboration. However, the T cell–APC and T cell–T cell interactions need to be terminated once their purposes are served. Probably, TR6 secreted by T cells helps to dislodge, or prevent T cells from having prolonged engagement with APC and/or other T cells. In in vitro culture, endogenous TR6 in the supernatant reached 620 pg/ml, but cell aggregation was not inhibited. It is possible that a higher TR6 concentration is required, because exogenous TR6 at 10 μg/ml was needed for such an effect. In lymphoid organs in vivo, T cells are tightly packed at a density much higher than in in vitro culture. Therefore, TR6 concentration high enough to dislodge T cells from APC or other T cells is probably achievable locally.

ACKNOWLEDGMENTS

The authors acknowledge the editorial assistance provided by Mr. Ovid Da Silva, Reviseur-Redacteur, Research Support Office, Research Centre, CHUM, Montreal, Que., Canada.

REFERENCES

Cerutti A, Schaffer A, Goodwin RG, Shah S, Zan H, Ely S, Casali P. 2000. Engagement of CD153 (CD30 ligand) by CD30 + T cells inhibits class switch DNA recombination and antibody production in human IgD + IgM + B cells. J Immunol 165:786–794.

Chen NJ, Huang MW, Hsieh SL. 2001. Enhanced secretion of IFN-gamma by activated Th1 cells occurs via reverse signaling through TNF-related activation-induced cytokine. J Immunol 166:270–276.

Chou AH, Tsai HF, Lin LL, Hsieh SL, Hsu PI, Hsu PN. 2001. Enhanced proliferation and increased IFN-gamma production in T cells by signal transduced through TNF-related apoptosis-inducing ligand. J Immunol 167:1347–1352.

Eissner G, Kirchner S, Lindner H, Kolch W, Janosch P, Grell M, Scheurich P, Andreesen R, Holler E. 2000. Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. J Immunol 164:6193–6198.

Gil D, Schamel WW, Montoya M, Sanchez-Madrid F, Alarcon B. 2002. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. Cell 109:901–912.

Luo H, Chen H, Daloze P, St. Louis G, Wu J. 1993. Anti-CD28 anti. Clin Exp Immunol 94:371–376.

Luo H, Wan X, Wu Y, Wu J. 2001. Cross-linking of EphB6 resulting in signal transduction and apoptosis in Jurkat cells. J Immunol 167:1362–1370.

Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P. 2002. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. Immunity 16:479–492.

Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, Huang A, Donahue CJ, Sherwood SW, Baldwin DT, Godowski PJ, Wood WI, Gurney AL, Hillan KJ, Cohen RL, Goddard AD, Botstein D, Ashkenazi A. 1998. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. Nature 396:699–703.

Ronchetti S, Nocentini G, Riccardi C, Pandolfi P. 2002. Role of GITR in activation response of T lymphocytes. Blood 100:350–352.

Serrador JM, Nieto M, Alonso-Lebrero JL, del Pozo MA, Calvo J, Furthmayr H, Schwartz-Albiez R, Lozano F, Gonzalez-Amaro R, Sanchez-Mateos P, Sanchez-Madrid F. 1998. CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell–cell contacts. Blood 91:4632–4644.

Shi G, Luo H, Wan X, Salcedo TW, Zhang J, Wu J. 2002. Mouse T cells receive costimulatory signals from LIGHT, a TNF family member. Blood 100:3279–3286.

Suzuki I, Fink PJ. 1998. Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through Fas ligand. J Exp Med 187:123–128.

Suzuki I, Fink PJ. 2000. The dual functions of fas ligand in the regulation of peripheral CD8+ and CD4 + T cells. Proc Natl Acad Sci USA 97:1707–1712.

Suzuki I, Martin S, Boursalian TE, Beers C, Fink PJ. 2000. Fas ligand costimulates the in vivo proliferation of CD8 + T cells. J Immunol 165:5537–5543.

Tamada K, Shimozaki K, Chapoval AI, Zhu G, Sica G, Flies D, Boone T, Hsu H, Fu YX, Nagata S, Ni J, Chen L. 2000. Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. Nat Med 6:283–289.

Van Essen D, Kikutani H, Gray D. 1995. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. Nature 378:620–623.

Wiley SR, Goodwin RG, Smith CA. 1996. Reverse signaling via CD30 ligand. J Immunol 157:3635–3639.

Wulfing C, Sumen C, Sjaastad MD, Wu LC, Dustin ML, Davis MM. 2002. Costimulation and endogenous MHC ligands contribute to T cell recognition. Nat Immunol 3:42–47.

Yu KY, Kwon B, Ni J, Zhai Y, Ebner R, Kwon BS. 1999. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. J Biol Chem 274:13733–13736.

Zhang J, Salcedo TW, Wan X, Ullrich S, Hu B, Gregorio T, Feng P, Qi S, Chen H, Cho YH, Li Y, Moore PA, Wu J. 2001. Modulation of T-cell responses to alloantigens by TR6/DcR3. J Clin Invest 107:1459–1468.

III. DISCUSSION

III. Discussion

In this project, the role of TR6 in immune regulation was investigated. In the mouse system, soluble TR6 suppressed IL-2, IL-5 and GM-CSF secretion by mitogen-activated T cells, and downregulated cytotoxic T-cell activity in vitro. In vivo, soluble TR6 could suppress the graft versus host disease (GVHD) and allograft rejection. In the human system, solid phase TR6, in the presence of suboptimal solid phase anti-CD3, significantly costimulated T cells in terms of proliferation and cytokine production. Blocking studies using soluble LIGHT and Fas indicated that LIGHT likely mediated the costimulation. This has revealed a novel mechanism of TR6 in triggering reverse signaling through a ligand, LIGHT. Based on this finding, it is likely that the suppressive effect of soluble TR6 in the mouse system is, in part, due to its interference with the two-way costimulation between HveA and LIGHT. Intriguingly, soluble TR6 augmented T-cell proliferation, lymphokine production and cytotoxic T-cell activity in the human system. The opposite effects of soluble TR6 in the human and mouse systems are probably due to different affinity of TR6 to human and mouse LIGHT (to be elaborated below). We also found after T-cell activation, T cells augmented their TR6 secretion. Interestingly, both soluble and solid-phase TR6 were able to suppress mitogen-induced T-cell aggregation. T cells pretreated with TR6 had reduced actin polymerization and pseudopodium formation, which are both important for the cell-cell interaction. These results suggest that TR6 might regulate the duration of T-cell interaction with other cells, and allow T cells to disengage from antigen presenting cells or neighboring T cells once the interaction becomes unnecessary.

III.1. *Monomer TR6 without aggregation is required to inhibit human T-cell responses*

Our findings have confirmed one of our hypotheses that the inhibitory effect of human TR6 in the mouse system should be attributed to TR6's interference with the bi-directional costimulation between HveA and LIGHT. However, how do we explain the seemingly opposite effects of soluble human TR6-Fc on human and mouse T-cell responses? In the mouse system, soluble TR6-Fc inhibited CTL in vitro and allograft rejection in vivo. In contrast, in the human system, soluble TR6-Fc actually enhanced T-cells proliferation, cytokine production, and CTL activity. Theoretically, such a difference might be caused by the Fc tail of the recombinant protein. As the Fc tail used is of human IgG_1 origin, it can bind human FcγR on B cells and monocytes/macrophages. Consequently, the soluble TR6-Fc in the human system might become membrane-bound, and costimulate T cells via the reverse signaling through LIGHT. On the other hand, human IgG_1 does not bind well to mouse FcγR, and remains in solution. This allows it to block the two-way costimulation between LIGHT and HveA. However, the Fc portion of TR6-Fc and other Fc-containing recombinant proteins (including the control recombinant proteins) used in our study was mutated to eliminate its FcγR binding capability. We confirmed that TR6-Fc did not bind to human or mouse FcγR-bearing cells (data not shown). Therefore, there has to be another explanation for the opposite effects of TR6-Fc in the human and mouse system. As TR6-Fc is a dimer, and it might also form aggregates in solution like any other proteins, it may play a dual role in solution: as an inhibitor blocking the bi-directional interaction between HveA and LIGHT, or as a co-stimulator cross-linking LIGHT. The former inhibits T-cell responses and the latter enhances it. The end-result should be the sum of these two opposite effects, which may have different affinity requirements. In the human system, the higher affinity between TR6-Fc (dimers, and

possibly some aggregated form) and LIGHT might result in sufficient LIGHT cross-linking and consequently costimulation, and this effect might overwhelm the possible TR6 effect on the interference of the LIGHT and HveA interaction. In the mouse system, the putative lower affinity between human TR6 and mouse LIGHT might not be sufficient for LIGHT cross-linking, but TR6-Fc can still interfere with the interaction between HveA and LIGHT. In this case, the blocking effect overwhelms the effect of crosslinking, hence downregulating the T-cell response. Further studies are needed to examine this hypothesis. If this is true, we have to use only the soluble monomer form of TR6 without aggregation for the purpose of downregulating human T-cell responses in therapeutic applications.

III.2. *The biological significance of TR6's inhibition on T-cell aggregation*

We have shown that TR6 strongly inhibited T-cell aggregation. This inhibition was accompanied by suppression of actin polymerization, and T-cell pseudopodium formation. Interestingly, we found that while T-cell-T-cell interaction was inhibited, T-cell proliferation was not. This suggests that the T-cell aggregation we observe in vitro during mitogen stimulation is not a necessity, at least not a necessity for the later part of T-cell activation. In vivo, after T-cell activation, which might only require several minutes (Gil *et al.*, 2002), the activated T cells need to dissociate from APC or neighboring T cells to disseminate into circulation for their effector function; TR6 secreted locally by activated T cells might serve this purpose.

III.3. *The in vivo role of TR6 in immune responses*

We have shown that activated lymphocytes secreted TR6. What is the function of such endogenous TR6 in immune responses in addition to terminating T-cell aggregation as discussed above? The answer is highly dependent on the format of the secreted TR6, i.e., monomers, or trimers as other surface TNFR family members, or aggregates. If it is in the form of monomers, then the overall effect of TR6 should be suppressive to immune responses, because it can conceivably inhibit the two-way costimulation between FasL and Fas (Suzuki *et al.*, 2000), and between LIGHT and HveA; it might also repress the stimulatory effect of TL1A to DR3 on T cells. We believe this is the likely scenario, because it fits to our perception that TR6-secreting tumors use this molecule to downregulate tumor surveillance, and it is compatible with our results with the mouse system. Such downregulation by TR6, which is secreted after the activation is achieved, in an immune response might be one of the built-in check-and-balance mechanisms in the immune system to reign in or to terminate on-going immune responses. On the other hand, if the endogenous TR6 is a trimer or easily forms aggregates which are capable of crosslinking LIGHT or FasL and trigger their reverse signaling, it might have an enhancing role in the immune response. Obviously, our next task is to determine the format of endogenous TR6 to fully understand its role in immune regulation.

In the mouse model, in vivo administration of human TR6 led to downregulation of GVHD and allograft rejection. We initially attributed these effects mainly to the blocking of stimulation from LIGHT to HveA. Our subsequent finding of the reverse costimulation through LIGHT suggests that TR6 could block the two-way costimulation between LIGHT and HveA. However, these are not the only mechanisms involved. Recent study by Migone *et al.*, has shown that TL1A, which is mainly produced by endothelial cells, can bind to DR3

and enhance T-cell response to IL-2 (Migone *et al.*, 2002); TR6 can bind to TL1A and repress the enhanced T-cell response. It is possible that during allograft rejection, local inflammation stimulates TL1A secretion by endothelial cells, and the administered TR6 blocks the effect of TL1A in enhancing T-cell responses. This could be an additional mechanism for the observed effect of TR6 in vivo (Shi *et al.*, 2003, in press). Moreover, we recently discovered that TR6 could also inhibit T-cell chemotaxis in vitro and in vivo. Such an effect could also play a role to prevent T cells migration to the alloantigen site in the activation and effector phases. In addition, blocking of FasL and Fas interaction by TR6 could in theory inhibit T-cell costimulation via Fas (Suzuki *et al.*, 1998), or reversely via FasL (Suzuki *et al.*, 2000); it might protect vascular endothelial cells from Fas-mediated apoptosis during graft rejection (Akyurek *et al.*, 1998). Therefore, the in vivo beneficial effects of TR6 during allograft rejection are multifaceted.

In spite of the possible multiple effects of TR6 on T-cell function, prolongation of allograft survival with in vivo administration of TR6 in mice was moderate. Several factors might be responsible for this. First, the half-life of the TR6-Fc used was only about 20 min (data not shown). Such a short half-life is mainly due to the insect cell origin of the recombinant TR6-Fc, with an unusually high content of mannose. According to our calculation, within 4 h (12 half-lives) after TR6 administration, the serum TR6 level is below the effective in vitro concentration (10 μg/ml). Therefore, for most part of the day, the recipient is under no protection from this immune modulator. Secondly, LIGHT and HveA two-way costimulation is only one of the costimulation pathways T cells use to achieve full activation. Many other costimulation pathways such as CD28 (Chen *et al.*, 1992), OX-40 (Godfrey *et al.*, 1994), CD40 (Durie *et al.*, 1994), 4-1BB (Shuford *et al.*, 1997), are not blocked. Moreover, strong

TCR stimulation, such as that triggered by alloantigens is probably less dependent on costimulation (Wang *et al.*, 2000). Conceivably, a combination of these factors led to the moderate prolongation of graft survival. In the future, TR6 with a longer half life will be better in a drug combination rather than as a stand-alone medication to achieve immunosuppression. TR6's effect on inhibiting Fas-mediated apoptosis suggests that it might be more useful in treating chronic rejection, in which vasculopathy caused by Fas-mediated apoptosis of endothelial cells and vascular smooth muscle cells plays a major role (Akyurek *et al.*, 1998).

III.4. *TR6 in tumorigenesis*

Tumor cells might produce many immunosuppressive factors, such as TGF-β, IL-10, DF3/MUC1, in order to obtain survival advantages (Gimmi *et al.*, 1996). TR6 could be another immune modulator, as this gene has increased expression in some malignant tissues (Pitti *et al.*, 1998) and its protein product is over-expressed in human adenocarcinomas of the esophagus, stomach, colon, and rectum (Bai *et al.*, 2000).

Is the overexpression of TR6 in tumors a consequence of malignancy, in which chaotic gene amplification occurs, or causative of tumorigenesis? It is evident that some tumor cells have TR6 gene amplification (Oshima *et al.*, 2000; Mild *et al.*, 2002). But another report has shown that tumors overexpress TR6 without gene amplification (Bai *et al.*, 2000). Recent studies in our laboratory have shown that TR6 gene amplification could be observed in about 40% of liver carcinomas but not in gastric carcinomas (Wu *et al.*, 2003), in spite of TR6 overexpression in 70-80% of these tumors. This indicates that different types of tumors have different mechanisms to upregulate TR6 expression, and in most cases, upregulation is not

the consequence of gene amplification, hence unlikely the consequence of malignancy. Then, does TR6 have a causative role in tumorigenesis?

TR6 interacts with FasL, LIGHT, and TL1A and can block Fas-, LTβR- and/or DR3-mediated apoptosis (Zhang *et al.*, 2001, Shi *et al.*, 2002; Migone *et al.*, 2002). Thus, TR6 might protect tumor cells from apoptosis mediated by these molecules. As DR3 is mainly expressed in lymphoid cells and some other cells of hematopoietic origin (Migone *et al.*, 2002), TR6's effect on DR3 in relation to tumorigenesis might be restricted to some leukemia. In our study, we have demonstrated that TR6 interferes with the interaction between LIGHT and HveA, and inhibits their bi-directional costimulation of T cells. Additionally, Hsu (2002) reported that soluble TR6 regulates dendritic cell differentiation, which, in return, drives T cells into the Th2 phenotype. Moreover, a recent study from our laboratory has demonstrated a novel function for TR6 in inhibiting T-cell chemotaxis in vitro and in vivo in mice (Shi *et al.*, 2003, in press). The relevance of such an effect in tumorigenesis is evidenced by our finding that gastric tumor patients with high serum TR6 levels had lower levels of infiltrating lymphocytes in the tumor mass (Wu *et al.*, 2003). It is conceivable that such an effect will reduce the chance of T cells to interact with tumors both during activation and effector phases. These TR6 effects on T cells might collectively dampen the immune surveillance in vivo. We propose the following model for the role of TR6 in tumorigenesis: fast proliferating cells, such as epithelial cells or hepatocytes in liver cirrhosis, undergo malignant mutation by chance, and at the same time upregulate their TR6 expression by an as yet non-elucidated mechanism; TR6 secreted by these malignant cells protect them from apoptosis and help them to evade immune surveillance; these cells gain survival advantages and eventually develop into tumors. In this scenario, TR6 by itself does

not cause the tumor, but functions as a tumor facilitator. Additional study is warranted to prove this hypothesis.

III.5. *The significance of our study*

We explored the biological function of TR6 and revealed previously undocumented features of this molecule in the immune system. We are the first to demonstrate the reverse signaling property of TR6 through its ligand(s). In this case, its ligand LIGHT functions like a receptor and transduces signals into LIGHT-expressing T cells upon TR6 ligation. Reverse signaling through LIGHT allows HveA-expressing T cells to stimulate LIGHT-expressing T cells, and such two-way stimulation provides a theoretical base for T-cell-T-cell cooperation, which is not a well-studied aspect of T-cell biology. The application value of this finding is to use soluble monomer TR6 to downregulate undesirable immune responses by blocking the two-way costimulation between HveA and LIGHT. We have indeed demonstrated that this can be achieved in the *in vivo* mouse model of GVHD and allograft rejection, although other mechanisms might also contribute to the observed immune downregulation.

III.6. *Future perspectives*

Several points are worth exploring further in TR6 studies. Using blocking studies, we have demonstrated that the reverse signaling triggered by TR6 was mainly mediated by LIGHT, although we cannot absolutely rule out the involvement of FasL, which is also able to reverse-signal, or other so-far unidentified ligands of TR6 in this process. Using LIGHT and FasL knockout mice will convincingly resolve this issue.

The fact that TR6 is upregulated in some types of tumor cells indicates the roles of TR6 in facilitating tumorigenesis. Using the sensitive TR6 ELISA as described in the third article, we have found that serum TR6 is a very reliable parameter for tumor diagnosis, according to a 194-patient clinical study. Currently, additional multicentre trials are under way to further validate this test for routine clinical use. Using TR6 transgenic mice, we are in the process of confirming the facilitator role of TR6 in tumorigenesis.

We have found that soluble TR6 in the mouse system can reduce GVHD and graft rejection, but the effect was moderate. This might be due to the short half-life of the TR6 (produced in insect cells) used, and due to the existence of other costimulating pathways. Stable monomeric TR6 in combination with other immunosuppressants might be more effective in treating undesirable immune responses.

These additional basic and clinical investigations will enhance our understanding of the intricate interactions between TR6, LIGHT, HveA, FasL, Fas, DR3 and TL1A, and make the "bad" molecule TR6 work for a good cause.

IV. REFERENCES

Reference

1. **Adams, A. B., C. P. Larsen, T. C. Pearson, and K. A. Newell.** The role of TNF receptor and TNF superfamily molecules in organ transplantation. *Am. J. Transplant.* 2:12-18, 2002

2. **Aggarwal, B. B. and K. Natarajan.** Tumor necrosis factors: developments during the last decade. *Eur.Cytokine Netw.* 7:93-124, 1996

3. **Akyurek, L. M., C. Johnsson, D. Lange, P. Georgii-Hemming, E. Larsson, B. C. Fellstrom, K. Funa, and G. Tufveson.** Tolerance induction ameliorates allograft vasculopathy in rat aortic transplants. Influence of Fas-mediated apoptosis. *J.Clin.Invest* 101:2889-2899, 1998

4. **Alderson, M. R., C. A. Smith, T. W. Tough, T. Davis-Smith, R. J. Armitage, B. Falk, E. Roux, E. Baker, G. R. Sutherland, and W. S. Din.** Molecular and biological characterization of human 4-1BB and its ligand. *Eur.J.Immunol.* 24:2219-2227, 1994

5. **Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S. A. Nedospasov, and K. Pfeffer.** Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. *Proc.Natl.Acad.Sci.U.S.A* 94:9302-9307, 1997

6. **Allison, J. P.** CD28-B7 interactions in T-cell activation. *Curr.Opin.Immunol.* 6:414-419, 1994

7. **Amiot, F., C. Fitting, K. J. Tracey, J. M. Cavaillon, and F. Dautry.** Lipopolysaccharide-induced cytokine cascade and lethality in LT alpha/TNF alpha-deficient mice. *Mol.Med.* 3:864-875, 1997

8. **Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, and L. Galibert.** A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 390:175-179, 1997

9. **Armitage, R. J.** Tumor necrosis factor receptor superfamily members and their ligands. *Curr.Opin.Immunol.* 6:407-413, 1994

10. **Ashkenazi, A., S. A. Marsters, D. J. Capon, S. M. Chamow, I. S. Figari, D. Pennica, D. V. Goeddel, M. A. Palladino, and D. H. Smith.** Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin. *Proc.Natl.Acad.Sci.U.S.A* 88:10535-10539, 1991

11. **Ashkenazi, A. and V. M. Dixit.** Death receptors: signaling and modulation. *Science* 281:1305-1308, 1998

12. **Ashkenazi, A. and V. M. Dixit.** Apoptosis control by death and decoy receptors. *Curr.Opin.Cell Biol.* 11:255-260, 1999

13. **Ashkenazi, A.** Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat.Rev.Cancer* 2:420-430, 2002

14. **Bai, C., B. Connolly, M. L. Metzker, C. A. Hilliard, X. Liu, V. Sandig, A. Soderman, S. M. Galloway, Q. Liu, C. P. Austin, and C. T. Caskey.** Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc.Natl.Acad.Sci.U.S.A* 97:1230-1235, 2000

15. **Banchereau, J., F. Bazan, D. Blanchard, F. Briere, J. P. Galizzi, C. van Kooten, Y. J. Liu, F. Rousset, and S. Saeland.** The CD40 antigen and its ligand. *Annu.Rev.Immunol.* 12:881-922, 1994

16. **Banks, T. A., B. T. Rouse, M. K. Kerley, P. J. Blair, V. L. Godfrey, N. A. Kuklin, D. M. Bouley, J. Thomas, S. Kanangat, and M. L. Mucenski. Lymphotoxin-alpha-deficient mice.** Effects on secondary lymphoid organ development and humoral immune responsiveness. *J.Immunol.* 155:1685-1693, 1995

17. **Banner, D. W., A. D'Arcy, W. Janes, R. Gentz, H. J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer.** Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell* 73:431-445, 1993

18. **Baum, P. R., R. B. Gayle, III, F. Ramsdell, S. Srinivasan, R. A. Sorensen, M. L. Watson, M. F. Seldin, E. Baker, G. R. Sutherland, K. N. Clifford.** Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J.* 13:3992-4001, 1994

19. **Baum, P. R., R. B. Gayle, III, F. Ramsdell, S. Srinivasan, R. A. Sorensen, M. L. Watson, M. F. Seldin, K. N. Clifford, K. Grabstein, M. R. Alderson, and .** Identification of OX40 ligand and preliminary characterization of its activities on OX40 receptor. *Circ.Shock* 44:30-34, 1994

20. **Bayes, M., A. J. Hartung, S. Ezer, J. Pispa, I. Thesleff, A. K. Srivastava, and J. Kere.** The anhidrotic ectodermal dysplasia gene (EDA) undergoes alternative splicing and encodes ectodysplasin-A with deletion mutations in collagenous repeats. *Hum.Mol.Genet.* 7:1661-1669, 1998

21. **Bazzoni, F. and B. Beutler.** The tumor necrosis factor ligand and receptor families. *N.Engl.J.Med.* 334:1717-1725, 1996

22. **Beg, A. A. and D. Baltimore.** An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 274:782-784, 1996

23. **Bennett, B. L., R. Cruz, R. G. Lacson, and A. M. Manning.** Interleukin-4 suppression of tumor necrosis factor alpha-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-kappaB. *J.Biol.Chem.* 272:10212-10219, 1997

24. **Beutler, B. and A. Cerami.** The biology of cachectin/TNF--a primary mediator of the host response. *Annu.Rev.Immunol.* 7:625-655, 1989

25. **Blair, P. J., J. L. Riley, D. M. Harlan, R. Abe, D. K. Tadaki, S. C. Hoffmann, L. White, T. Francomano, S. J. Perfetto, A. D. Kirk, and C. H. June.** CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. *J.Exp.Med.* 191:651-660, 2000

26. **Bodmer, J. L., K. Burns, P. Schneider, K. Hofmann, V. Steiner, M. Thome, T. Bornand, M. Hahne, M. Schroter, K. Becker, A. Wilson, L. E. French, J. L. Browning, H. R. MacDonald, and J. Tschopp.** TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and Fas(Apo-1/CD95). *Immunity.* 6:79-88, 1997

27. **Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach.** Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 85:803-815, 1996

28. **Bondeson, J., K. A. Browne, F. M. Brennan, B. M. Foxwell, and M. Feldmann.** Selective regulation of cytokine induction by adenoviral gene transfer of IkappaBalpha into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-kappaB independent. *J.Immunol.* 162:2939-2945, 1999

29. **Bowman, M. R., M. A. Crimmins, J. Yetz-Aldape, R. Kriz, K. Kelleher, and S. Herrmann.** The cloning of CD70 and its identification as the ligand for CD27. *J.Immunol.* 152:1756-1761, 1994

30. **Brenner, B., U. Koppenhoefer, H. Grassme, J. Kun, F. Lang, and E. Gulbins.** Evidence for a novel function of the CD40 ligand as a signalling molecule in T-lymphocytes. *FEBS Lett.* 417:301-306, 1997

31. **Brenner, B., U. Koppenhoefer, A. Lepple-Wienhues, H. Grassme, C. Muller, C. P. Speer, F. Lang, and E. Gulbins.** The CD40 ligand directly activates T-lymphocytes via tyrosine phosphorylation dependent PKC activation. *Biochem.Biophys.Res.Commun.* 239:11-17, 1997

32. **Briones, J., J. Timmerman, and R. Levy.** In vivo antitumor effect of CD40L-transduced tumor cells as a vaccine for B-cell lymphoma. *Cancer Res.* 62:3195-3199, 2002

33. **Brockhaus, M., H. J. Schoenfeld, E. J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher.** Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc.Natl.Acad.Sci.U.S.A* 87:3127-3131, 1990

34. **Browning, J. L., M. J. Androlewicz, and C. F. Ware.** Lymphotoxin and an associated 33-kDa glycoprotein are expressed on the surface of an activated human T cell hybridoma. *J.Immunol.* 147:1230-1237, 1991

35. **Browning, J. L., A. Ngam-ek, P. Lawton, J. DeMarinis, R. Tizard, E. P. Chow, C. Hession, B. O'Brine-Greco, S. F. Foley, and C. F. Ware.** Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell* 72:847-856, 1993

36. **Browning, J. L., I. Dougas, A. Ngam-ek, P. R. Bourdon, B. N. Ehrenfels, K. Miatkowski, M. Zafari, A. M. Yampaglia, P. Lawton, W. Meier, and .** Characterization of surface lymphotoxin forms. Use of specific monoclonal antibodies and soluble receptors. *J.Immunol.* 154:33-46, 1995

37. **Browning, J. L., K. Miatkowski, I. Sizing, D. Griffiths, M. Zafari, C. D. Benjamin, W. Meier, and F. Mackay.** Signaling through the lymphotoxin beta receptor induces the death of some adenocarcinoma tumor lines. *J.Exp.Med.* 183:867-878, 1996

38. **Buster, B. L., K. A. Mattes, and W. M. Scheld.** Monoclonal antibody-mediated, complement-independent binding of human tumor necrosis factor-alpha to primate erythrocytes via complement receptor 1. *J.Infect.Dis.* 176:1041-1046, 1997

39. **Callard, R., A. J. George, and J. Stark.** Cytokines, chaos, and complexity. *Immunity.* 11:507-513, 1999

40. **Camerini, D., G. Walz, W. A. Loenen, J. Borst, and B. Seed.** The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J.Immunol.* 147:3165-3169, 1991

41. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** An endotoxin-induced serum factor that causes necrosis of tumors. *Proc.Natl.Acad.Sci.U.S.A* 72:3666-3670, 1975

42. **Cayabyab, M., J. H. Phillips, and L. L. Lanier.** CD40 preferentially costimulates activation of CD4+ T lymphocytes. *J.Immunol.* 152:1523-1531, 1994

43. **Cerutti, A., A. Schaffer, R. G. Goodwin, S. Shah, H. Zan, S. Ely, and P. Casali.** Engagement of CD153 (CD30 ligand) by CD30+ T cells inhibits class switch DNA recombination and antibody production in human IgD+ IgM+ B cells. *J.Immunol.* 165:786-794, 2000

44. **Chan, F. K., H. J. Chun, L. Zheng, R. M. Siegel, K. L. Bui, and M. J. Lenardo.** A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* 288:2351-2354, 2000

45. **Chan, F. K. and M. J. Lenardo.** A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. *Eur.J.Immunol.* 30:652-660, 2000

46. **Chaudhary, P. M., M. Eby, A. Jasmin, A. Bookwalter, J. Murray, and L. Hood.** Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. *Immunity.* 7:821-830, 1997

47. **Chen, L., S. Ashe, W. A. Brady, I. Hellstrom, K. E. Hellstrom, J. A. Ledbetter, P. McGowan, and P. S. Linsley.** 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* 71:1093-1102

48. **Chen, N. and E. H. Field.** Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance. *Transplantation* 59:933-941, 1995

49. **Chen, N. J., M. W. Huang, and S. L. Hsieh.** Enhanced secretion of IFN-gamma by activated Th1 cells occurs via reverse signaling through TNF-related activation-induced cytokine [In Process Citation]. *J.Immunol.* 166:270-276, 2001

50. **Chicheportiche, Y., P. R. Bourdon, H. Xu, Y. M. Hsu, H. Scott, C. Hession, I. Garcia, and J. L. Browning.** TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J.Biol.Chem.* 272:32401-32410, 1997

51. **Chikanza, I. C., P. Roux-Lombard, J. M. Dayer, and G. S. Panayi.** Tumour necrosis factor soluble receptors behave as acute phase reactants following surgery in patients with rheumatoid arthritis, chronic osteomyelitis and osteoarthritis. *Clin.Exp.Immunol.* 92:19-22, 1993

52. **Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit.** FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81:505-512, 1995

53. **Chinnaiyan, A. M., K. O'Rourke, G. L. Yu, R. H. Lyons, M. Garg, D. R. Duan, L. Xing, R. Gentz, J. Ni, and V. M. Dixit.** Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. *Science* 274:990-992, 1996

54. **Chou, A. H., H. F. Tsai, L. L. Lin, S. L. Hsieh, P. I. Hsu, and P. N. Hsu.** Enhanced proliferation and increased IFN-gamma production in T cells by signal transduced through TNF-related apoptosis-inducing ligand. *J.Immunol.* 167:1347-1352, 2001

55. **Clark, L., M. Wei, G. Cattoretti, C. Mendelsohn, and B. Tycko.** The Tnfrh1 (Tnfrsf23) gene is weakly imprinted in several organs and expressed at the trophoblast-decidua interface. *BMC.Genet.* 3:11, 2002

56. **Cobbold, S. and H. Waldmann.** Infectious tolerance. *Curr.Opin.Immunol.* 10:518-524, 1998

57. **Cocks, B. G., M. R. de Waal, J. P. Galizzi, J. E. de Vries, and G. Aversa.** IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int.Immunol.* 5:657-663, 1993

58. **Connolly, K., Y. H. Cho, R. Duan, J. Fikes, T. Gregorio, D. W. LaFleur, Z. Okoye, T. W. Salcedo, G. Santiago, S. Ullrich, P. Wei, K. Windle, E. Wong, X. T. Yao, Y. Q. Zhang, G. Zheng, and P. A. Moore.** In vivo inhibition of Fas ligand-mediated killing by TR6, a Fas ligand decoy receptor. *J. Pharmacol. Exp. Ther.* 298:25-33, 2001

59. **Corcoran, A. E., B. J. Scallon, H. Trinh, Y. Chernajovsky, J. Ghrayeb, and M. Feldmann.** Minimal tumor necrosis factor receptor binding protein: optimum biological activity of a truncated p55 soluble tumor necrosis factor receptor-IgG fusion protein. *Eur.Cytokine Netw.* 9:255-262, 1998

60. **Creagh, E. M., H. Conroy, and S. J. Martin.** Caspase-activation pathways in apoptosis and immunity. *Immunol Rev.* 193:10-21, 2003

61. **Cusson, N., S. Oikemus, E. D. Kilpatrick, L. Cunningham, and M. Kelliher.** The death domain kinase RIP protects thymocytes from tumor necrosis factor receptor type 2-induced cell death. *J.Exp.Med.* 196:15-26, 2002

62. **Dale, D. C., W. C. Liles, C. Llewellyn, and T. H. Price.** Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on neutrophil kinetics and function in normal human volunteers. *Am.J.Hematol.* 57:7-15, 1998

63. **Dallman, M. J., C. P. Larsen, and P. J. Morris.** Cytokine gene transcription in vascularised organ grafts: analysis using semiquantitative polymerase chain reaction. *J.Exp.Med.* 174:493-496, 1991

64. **Daviet, L., M. Erard, D. Dorin, M. Duarte, C. Vaquero, and A. Gatignol.** Analysis of a binding difference between the two dsRNA-binding domains in TRBP reveals the modular function of a KR-helix motif. *Eur.J.Biochem.* 267:2419-2431, 2000

65. **De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, J. Strauss-Schoenberger, and .** Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264:703-707, 1994

66. **De Wazieres, B., V. Spehner, S. Harraga, F. Laplante, F. Corallo, C. Bloy, J. L. Dupond, D. A. Vuitton, and E. Seilles.** Alteration in the production of free oxygen radicals and proinflammatory cytokines by peritoneal and alveolar macrophages in old mice and immunomodulatory effect of RU 41740 administration. Part I: Effect of short and repetitive noise stress. *Immunopharmacology* 39:51-59, 1998

67. **Decoster, E., B. Vanhaesebroeck, P. Vandenabeele, J. Grooten, and W. Fiers.** Generation and biological characterization of membrane-bound, uncleavable murine tumor necrosis factor. *J.Biol.Chem.* 270:18473-18478, 1995

68. **Degli-Esposti, M. A., W. C. Dougall, P. J. Smolak, J. Y. Waugh, C. A. Smith, and R. G. Goodwin.** The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity.* 7:813-820, 1997

69. **Degli-Esposti, M. A., P. J. Smolak, H. Walczak, J. Waugh, C. P. Huang, R. F. DuBose, R. G. Goodwin, and C. A. Smith.** Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J.Exp.Med.* 186:1165-1170, 1997

70. **Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, and Z. J. Chen.** Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103:351-361, 2000

71. **Desai-Mehta, A., L. Lu, R. Ramsey-Goldman, and S. K. Datta.** Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J.Clin.Invest* 97:2063-2073, 1996

72. **Dick, A. D., P. G. McMenamin, H. Korner, B. J. Scallon, J. Ghrayeb, J. V. Forrester, and J. D. Sedgwick.** Inhibition of tumor necrosis factor activity minimizes target organ damage in experimental autoimmune uveoretinitis despite quantitatively normal activated T cell traffic to the retina. *Eur.J.Immunol.* 26:1018-1025, 1996

73. **Djamali, A. and J. S. Odorico.** Fas-mediated cytotoxicity is not required for rejection of murine nonvascularized heterotopic cardiac allografts. *Transplantation* 66:1793-1801, 1998

74. **Dougall, W. C., M. Glaccum, K. Charrier, K. Rohrbach, K. Brasel, T. De Smedt, E. Daro, J. Smith, M. E. Tometsko, C. R. Maliszewski, A. Armstrong, V. Shen, S. Bain, D. Cosman, D. Anderson, P. J. Morrissey, J. J. Peschon, and J. Schuh.** RANK is essential for osteoclast and lymph node development. *Genes Dev.* 13:2412-2424, 1999

75. **Du, C., M. Fang, Y. Li, L. Li, and X.Wang.** Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33-42, 2000

76. **Duan, H. and V. M. Dixit.** RAIDD is a new 'death' adaptor molecule. *Nature* 385:86-89, 1997

77. **Durie, F. H., T. M. Foy, S. R. Masters, J. D. Laman, and R. J. Noelle.** The role of CD40 in the regulation of humoral and cell-mediated immunity. *Immunol.Today* 15:406-411, 1994

78. **Duval, D., B. Reinhardt, C. Kedinger, and H. Boeuf.** Role of suppressors of cytokine signaling (Socs) in leukemia inhibitory factor (LIF) -dependent embryonic stem cell survival. *FASEB J.* 14:1577-1584, 2000

79. **Eck, M. J., M. Ultsch, E. Rinderknecht, A. M. de Vos, and S. R. Sprang.** The structure of human lymphotoxin (tumor necrosis factor-beta) at 1.9-A resolution. *J.Biol.Chem.* 267:2119-2122, 1992

80. **Edwards, R. H., M. J. Selby, P. D. Garcia, and W. J. Rutter.** Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. *J.Biol.Chem.* 263:6810-6815, 1988

81. **Eissner, G., S. Kirchner, H. Lindner, W. Kolch, P. Janosch, M. Grell, P. Scheurich, R. Andreesen, and E. Holler.** Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *J.Immunol.* 164:6193-6198, 2000

82. **Emery, J. G., P. McDonnell, M. B. Burke, K. C. Deen, S. Lyn, C. Silverman, E. Dul, E. R. Appelbaum, C. Eichman, R. DiPrinzio, R. A. Dodds, I. E. James, M. Rosenberg, J. C. Lee, and P. R. Young.** Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J.Biol.Chem.* 273:14363-14367, 1998

83. **Engelmann, H., H. Holtmann, C. Brakebusch, Y. S. Avni, I. Sarov, Y. Nophar, E. Hadas, O. Leitner, and D. Wallach.** Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J.Biol.Chem.* 265:14497-14504, 1990

84. **Englaro, W., P. Bahadoran, C. Bertolotto, R. Busca, B. Derijard, A. Livolsi, J. F. Peyron, J. P. Ortonne, and R. Ballotti.** Tumor necrosis factor alpha-mediated inhibition of melanogenesis is dependent on nuclear factor kappa B activation. *Oncogene* 18:1553-1559, 1999

85. **Ettinger, R., S. H. Munson, C. C. Chao, M. Vadeboncoeur, J. Toma, and H. O. McDevitt.** A critical role for lymphotoxin-beta receptor in the development of diabetes in nonobese diabetic mice. *J.Exp.Med.* 193:1333-1340, 2001

86. **Faccio, L., C. Fusco, A. Chen, S. Martinotti, J.V. Bonventre, and A. S. Zervos.** Characterization of a novel human serine protease that has extensive homology to bacterial heat shock endoprotease HtrA and is regulated by kidney ischemia. *J Biol.Chem.* 275:2581-2588, 2000

87. **Fouad, A. F.** IL-1 alpha and TNF-alpha expression in early periapical lesions of normal and immunodeficient mice. *J.Dent.Res.* 76:1548-1554, 1997

88. **Frankenberger, M., T. Sternsdorf, H. Pechumer, A. Pforte, and H. W. Ziegler-Heitbrock.** Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood* 87:373-377, 1996

89. **Fu, Y. X. and D. D. Chaplin.** Development and maturation of secondary lymphoid tissues. *Annu.Rev.Immunol.* 17:399-433, 1999

90. **Fujisawa, H., B. Wang, S. Kondo, G. M. Shivji, and D. N. Sauder.** Costimulation with ultraviolet B and interleukin-1 alpha dramatically increase tumor necrosis factor-alpha production in human dermal fibroblasts. *J.Interferon Cytokine Res.* 17:307-313, 1997

91. **Fujita, E., Y. Kouroku, Y. Miho, T. Tsukahara, S. Ishiura, and T. Momoi.** Wortmannin enhances activation of CPP32 (Caspase-3) induced by TNF or anti-Fas. *Cell Death.Differ.* 5:289-297, 1998

92. **Fuss, I. J., W. Strober, J. K. Dale, S. Fritz, G. R. Pearlstein, J. M. Puck, M. J. Lenardo, and S. E. Straus.** Characteristic T helper 2 T cell cytokine abnormalities in autoimmune lymphoproliferative syndrome, a syndrome marked by defective apoptosis and humoral autoimmunity. *J.Immunol.* 158:1912-1918, 1997

93. **Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer.** The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity.* 9:59-70, 1998

94. **Garrone, P., E. M. Neidhardt, E. Garcia, L. Galibert, C. van Kooten, and J. Banchereau.** Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J.Exp.Med.* 182:1265-1273, 1995

95. **Gatanaga, T., C. D. Hwang, M. Gatanaga, F. Cappuccini, R. S. Yamamoto, and G. A. Granger.** The regulation of TNF receptor mRNA synthesis, membrane expression, and release by PMA- and LPS-stimulated human monocytic THP-1 cells in vitro. *Cell Immunol.* 138:1-10, 1991

96. **Gauchat, J. F., S. Henchoz, G. Mazzei, J. P. Aubry, T. Brunner, H. Blasey, P. Life, D. Talabot, L. Flores-Romo, J. Thompson, and .** Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 365:340-343, 1993

97. **Gauchat, J. F., S. Henchoz, D. Fattah, G. Mazzei, J. P. Aubry, T. Jomotte, L. Dash, K. Page, R. Solari, D. Aldebert, and .** CD40 ligand is functionally expressed on human eosinophils. *Eur.J.Immunol.* 25:863-865, 1995

98. **Geraghty, R. J., C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear.** Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* 280:1618-1620, 1998

99. **Gersuk, G. M., C. Beckham, M. R. Loken, P. Kiener, J. E. Anderson, A. Farrand, A. B. Troutt, J. A. Ledbetter, and H. J. Deeg.** A role for tumour necrosis factor-alpha, Fas and Fas-Ligand in marrow failure associated with myelodysplastic syndrome. *Br.J.Haematol.* 103:176-188, 1998

100. **Gil, D., W. W. Schamel, M. Montoya, F. Sanchez-Madrid, and B. Alarcon.** Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* 109:901-912, 2002

101. **Gimmi, C. D., B. W. Morrison, B. A. Mainprice, J. G. Gribben, V. A. Boussiotis, G. J. Freeman, S. Y. Park, M. Watanabe, J. Gong, D. F. Hayes, D. W. Kufe, and L. M. Nadler.** Breast cancer-associated antigen, DF3/MUC1, induces apoptosis of activated human T cells. *Nat.Med.* 2:1367-1370, 1996

102. **Godfrey, W. R., F. F. Fagnoni, M. A. Harara, D. Buck, and E. G. Engleman.** Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. *J.Exp.Med.* 180:757-762, 1994

103. **Goodwin, R. G., W. S. Din, T. Davis-Smith, D. M. Anderson, S. D. Gimpel, T. A. Sato, C. R. Maliszewski, C. I. Brannan, N. G. Copeland, N. A. Jenkins, and .** Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur.J.Immunol.* 23:2631-2641, 1993

104. **Graf, D., U. Korthauer, H. W. Mages, G. Senger, and R. A. Kroczek.** Cloning of TRAP, a ligand for CD40 on human T cells. *Eur.J.Immunol.* 22:3191-3194, 1992

105. **Graf, D., S. Muller, U. Korthauer, C. van Kooten, C. Weise, and R. A. Kroczek.** A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation. *Eur.J.Immunol.* 25:1749-1754, 1995

106. **Granger, S. W. and C. F. Ware.** Turning on LIGHT. *J.Clin.Invest* 108:1741-1742, 2001

107. **Gravestein, L. A., B. Blom, L. A. Nolten, E. de Vries, H. G. van der, F. Ossendorp, J. Borst, and W. A.** Loenen. Cloning and expression of murine CD27: comparison with 4-1BB, another lymphocyte-specific member of the nerve growth factor receptor family. *Eur.J.Immunol.* 23:943-950, 1993

108. **Gray, P. W., B. B. Aggarwal, C. V. Benton, T. S. Bringman, W. J. Henzel, J. A. Jarrett, D. W. Leung, B. Moffat, P. Ng, L. P. Svedersky, and .** Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. *Nature* 312:721-724, 1984

109. **Green, D. R.** Overview: apoptotic signaling pathways in the immune system. *Immunol Rev.* 193:5-9, 2003

110. **Green, D. R. and C. F. Ware.** Fas-ligand: privilege and peril. *Proc. Natl. Acad. Sci. U.S.A* 94:5986-5990, 1997

111. **Green, D. R. and J. C. Reed.** Mitochondria and apoptosis. *Science* 281:1309-1312, 1998

112. **Grenet, J., V. Valentine, J. Kitson, H. Li, S. N. Farrow, and V. J. Kidd.** Duplication of the DR3 gene on human chromosome 1p36 and its deletion in human neuroblastoma. *Genomics* 49:385-393, 1998

113. **Gross, J. A., J. Johnston, S. Mudri, R. Enselman, S. R. Dillon, K. Madden, W. Xu, J. Parrish-Novak, D. Foster, C. Lofton-Day, M. Moore, A. Littau, A. Grossman, H. Haugen, K. Foley, H. Blumberg, K. Harrison, W. Kindsvogel, and C. H. Clegg.** TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature* 404:995-999, 2000

114. **Gruss, H. J. and S. K. Dower.** Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. *Blood* 85:3378-3404, 1995

115. **Gruss, H. J. and S. K. Dower.** The TNF ligand superfamily and its relevance for human diseases. *Cytokines Mol.Ther.* 1:75-105, 1995

116. **Gruss, H. J.** Molecular, structural, and biological characteristics of the tumor necrosis factor ligand superfamily. *Int.J.Clin.Lab Res.* 26:143-159, 1996

117. **Hahne, M., T. Renno, M. Schroeter, M. Irmler, L. French, T. Bornard, H. R. MacDonald, and J. Tschopp.** Activated B cells express functional Fas ligand. *Eur.J.Immunol.* 26:721-724, 1996

118. **Hahne, M., T. Kataoka, M. Schroter, K. Hofmann, M. Irmler, J. L. Bodmer, P. Schneider, T. Bornand, N. Holler, L. E. French, B. Sordat, D. Rimoldi, and J. Tschopp.** APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J.Exp.Med.* 188:1185-1190, 1998

119. **Harakawa, N., M. Sasada, A. Maeda, K. Asagoe, M. Nohgawa, K. Takano, Y. Matsuda, K. Yamamoto, and M. Okuma.** Random migration of polymorphonuclear leukocytes induced by GM-CSF involving a signal transduction pathway different from that of fMLP. *J.Leukoc.Biol.* 61:500-506, 1997

120. **Harrop, J. A., P. C. McDonnell, M. Brigham-Burke, S. D. Lyn, J. Minton, K. B. Tan, K. Dede, J. Spampanato, C. Silverman, P. Hensley, R. DiPrinzio, J. G. Emery, K. Deen, C. Eichman, M. Chabot-Fletcher, A. Truneh, and P. R. Young.** Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. *J.Biol.Chem.* 273:27548-27556, 1998

121. **Harrop, J. A., M. Reddy, K. Dede, M. Brigham-Burke, S. Lyn, K. B. Tan, C. Silverman, C. Eichman, R. DiPrinzio, J. Spampanato, T. Porter, S. Holmes, P. R. Young, and A. Truneh.** Antibodies to TR2 (herpesvirus entry mediator), a new member of the TNF receptor superfamily, block T cell proliferation, expression of activation markers, and production of cytokines. *J.Immunol.* 161:1786-1794, 1998

122. **Headon, D. J., S. A. Emmal, B. M. Ferguson, A. S. Tucker, M. J. Justice, P. T. Sharpe, J. Zonana, and P. A. Overbeek.** Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature* 414:913-916, 2001

123. **Hikichi, Y., H. Matsui, I. Tsuji, K. Nishi, T. Yamada, Y. Shintani, and H. Onda.** LIGHT, a member of the TNF superfamily, induces morphological changes and delays proliferation in the human rhabdomyosarcoma cell line RD. *Biochem.Biophys.Res.Commun.* 289:670-677, 2001

124. **Hirokawa, J., S. Sakaue, Y. Furuya, J. Ishii, A. Hasegawa, S. Tagami, Y. Kawakami, M. Sakai, S. Nishi, and J. Nishihira.** Tumor necrosis factor-alpha regulates the gene expression of macrophage migration inhibitory factor through tyrosine kinase-dependent pathway in 3T3-L1 adipocytes. *J.Biochem.(Tokyo)* 123:733-739, 1998

125. **Hochman, P. S., G. R. Majeau, F. Mackay, and J. L. Browning.** Proinflammatory responses are efficiently induced by homotrimeric but not heterotrimeric lymphotoxin ligands. *J.Inflamm.* 46:220-234, 1995

126. **Hohmann, H. P., R. Remy, M. Brockhaus, and A. P. van Loon.** Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). *J.Biol.Chem.* 264:14927-14934, 1989

127. **Hollenbaugh, D., L. S. Grosmaire, C. D. Kullas, N. J. Chalupny, S. Braesch-Andersen, R. J. Noelle, I. Stamenkovic, J. A. Ledbetter, and A. Aruffo.** The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J.* 11:4313-4321, 1992

128. **Holler, N., R. Zaru, O. Micheau, M. Thome, A. Attinger, S. Valitutti, J. L. Bodmer, P. Schneider, B. Seed, and J. Tschopp.** Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat.Immunol.* 1:489-495, 2000

129. **Howard, S. T., Y. S. Chan, and G. L. Smith.** Vaccinia virus homologues of the Shope fibroma virus inverted terminal repeat proteins and a discontinuous ORF related to the tumor necrosis factor receptor family. *Virology* 180:633-647, 1991

130. **Hsu, H., H. B. Shu, M. G. Pan, and D. V. Goeddel.** TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:299-308, 1996

131. **Hsu, T. L., Y. C. Chang, S. J. Chen, Y. J. Liu, A. W. Chiu, C. C. Chio, L. Chen, and S. L. Hsieh.** Modulation of dendritic cell differentiation and maturation by decoy receptor 3. *J.Immunol.* 168:4846-4853, 2002

132. **Hu, F. Q., C. A. Smith, and D. J. Pickup.** Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. *Virology* 204:343-356, 1994

133. **Hu, S., K. Tamada, J. Ni, C. Vincenz, and L. Chen.** Characterization of TNFRSF19, a novel member of the tumor necrosis factor receptor superfamily. *Genomics* 62:103-107, 1999

134. **Idriss, H. T. and J. H. Naismith.** TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc.Res.Tech.* 50:184-195, 2000

135. **Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata.** The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66:233-243, 1991

136. **Itoh, N. and S. Nagata.** A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J.Biol.Chem.* 268:10932-10937, 1993

137. **Jabara, H., D. Laouini, E. Tsitsikov, E. Mizoguchi, A. Bhan, E. Castigli, F. Dedeoglu, V. Pivniouk, S. Brodeur, and R. Geha.** The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. *Immunity.* 17:265-276, 2002

138. **Jenkins, M. K.** The ups and downs of T cell costimulation. *Immunity.* 1:443-446, 1994

139. **Jiang, Y., J. D. Woronicz, W. Liu, and D. V. Goeddel.** Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science* 283:543-546, 1999

140. **Johnson, R. M. and P. G. Spear.** Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. *J.Virol.* 63:819-827, 1989

141. **Joza, N., S. A. Susin, E. Daugas, W. L. Stanford, S. K. Cho, C. Y. Li, T. Sasaki, A. J. Elia, H. Y. Cheng, L. Ravagnan, K. F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y.Y. Kong, T. W. Mak, J. C. Zuniga-**

Pflucker, G. Kroemer, and J. M. Penninger. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* 410:549-554, 2001

142. **Ju, S. T., K. Matsui, and M. Ozdemirli.** Molecular and cellular mechanisms regulating T and B cell apoptosis through Fas/FasL interaction. *Int.Rev.Immunol.* 18:485-513, 1999

143. **June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson.** The B7 and CD28 receptor families. *Immunol.Today* 15:321-331, 1994

144. **Kayagaki, N., M. Yan, D. Seshasayee, H. Wang, W. Lee, D. M. French, I. S. Grewal, A. G. Cochran, N. C. Gordon, J. Yin, M. A. Starovasnik, and V. M. Dixit.** BAFF/BLyS Receptor 3 Binds the B Cell Survival Factor BAFF Ligand through a Discrete Surface Loop and Promotes Processing of NF-kappaB2. *Immunity.* 17:515, 2002

145. **Kelliher, M. A., S. Grimm, Y. Ishida, F. Kuo, B. Z. Stanger, and P. Leder.** The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity.* 8:297-303, 1998

146. **Kettritz, R., M. L. Gaido, H. Haller, F. C. Luft, C. J. Jennette, and R. J. Falk.** Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. *Kidney Int.* 53:84-91, 1998

147. **Kim, D., R. E. Mebius, J. D. MacMicking, S. Jung, T. Cupedo, Y. Castellanos, J. Rho, B. R. Wong, R. Josien, N. Kim, P. D. Rennert, and Y. Choi.** Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J.Exp.Med.* 192:1467-1478, 2000

148. **Kim, N., P. R. Odgren, D. K. Kim, S. C. Marks, Jr., and Y. Choi.** Diverse roles of the tumor necrosis factor family member TRANCE in skeletal physiology revealed by TRANCE deficiency and partial rescue by a lymphocyte-expressed TRANCE transgene. *Proc.Natl.Acad.Sci.U.S.A* 97:10905-10910, 2000

149. **Kischkel, F. C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P. H. Krammer, and M. E. Peter.** Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* 14:5579-5588, 1995

150. **Kitson, J., T. Raven, Y. P. Jiang, D. V. Goeddel, K. M. Giles, K. T. Pun, C. J. Grinham, R. Brown, and S. N. Farrow.** A death-domain-containing receptor that mediates apoptosis. *Nature* 384:372-375, 1996

151. **Knigge, H., M. M. Simon, S. C. Meuer, M. D. Kramer, and R. Wallich.** The outer surface lipoprotein OspA of Borrelia burgdorferi provides co-stimulatory signals to normal human peripheral CD4+ and CD8+ T lymphocytes. *Eur.J.Immunol.* 26:2299-2303, 1996

152. **Kong, Y. Y., H. Yoshida, I. Sarosi, H. L. Tan, E. Timms, C. Capparelli, S. Morony, A. J. Oliveira-dos-Santos, G. Van, A. Itie, W. Khoo, A. Wakeham, C. R. Dunstan, D. L. Lacey, T. W. Mak, W. J. Boyle, and J. M. Penninger.** OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397:315-323, 1999

153. **Kong, Y. Y., U. Feige, I. Sarosi, B. Bolon, A. Tafuri, S. Morony, C. Capparelli, J. Li, R. Elliott, S. McCabe, T. Wong, G. Campagnuolo, E. Moran, E. R. Bogoch, G. Van, L. T. Nguyen, P. S. Ohashi, D. L. Lacey, E. Fish, W. J. Boyle, and J. M. Penninger.** Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 402:304-309, 1999

154. **Koni, P. A., R. Sacca, P. Lawton, J. L. Browning, N. H. Ruddle, and R. A. Flavell.** Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity.* 6:491-500, 1997

155. **Korn, W. M., T. Yasutake, W. L. Kuo, R. S. Warren, C. Collins, M. Tomita, J. Gray, and F. M. Waldman.** Chromosome arm 20q gains and other genomic alterations in colorectal cancer metastatic to liver, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. *Genes Chromosomes.Cancer* 25:82-90, 1999

156. **Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu.** A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* 53:45-53, 1988

157. **Kuprash, D. V., O. A. Osipovich, D. K. Pokholok, M. B. Alimzhanov, A. Biragyn, R. L. Turetskaya, and S. A. Nedospasov.** Functional analysis of the lymphotoxin-beta promoter. Sequence requirements for PMA activation. *J.Immunol.* 156:2465-2472, 1996

158. **Kwon, B., K. Y. Yu, J. Ni, G. L. Yu, I. K. Jang, Y. J. Kim, L. Xing, D. Liu, S. X. Wang, and B. S. Kwon.** Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand. *J.Biol.Chem.* 274:6056-6061, 1999

159. **Kwon, B. S., C. A. Kozak, K. K. Kim, and R. T. Pickard.** Genomic organization and chromosomal localization of the T-cell antigen 4-1BB. *J.Immunol.* 152:2256-2262, 1994

160. **Kwon, B. S., K. B. Tan, J. Ni, K. O. Oh, Z. H. Lee, K. K. Kim, Y. J. Kim, S. Wang, R. Gentz, G. L. Yu, J. Harrop, S. D. Lyn, C. Silverman, T. G. Porter, A. Truneh, and P. R. Young.** A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. *J.Biol.Chem.* 272:14272-14276, 1997

161. **Laabi, Y. and A. Strasser.** Immunology. Lymphocyte survival--ignorance is BLys. *Science* 289:883-884, 2000

162. **Lacey, D. L., E. Timms, H. L. Tan, M. J. Kelley, C. R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y. X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney, and W. J. Boyle.** Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93:165-176, 1998

163. **Lawton, P., J. Nelson, R. Tizard, and J. L. Browning.** Characterization of the mouse lymphotoxin-beta gene. *J.Immunol.* 154:239-246, 1995

164. **Lazdins, J. K., M. Grell, M. R. Walker, K. Woods-Cook, P. Scheurich, and K. Pfizenmaier.** Membrane tumor necrosis factor (TNF) induced cooperative signaling of TNFR60 and TNFR80 favors induction of cell death rather than virus production in HIV-infected T cells. *J.Exp.Med.* 185:81-90, 1997

165. **Leeuwenberg, J. F., M. A. Dentener, and W. A. Buurman.** Lipopolysaccharide LPS-mediated soluble TNF receptor release and TNF receptor expression by monocytes. Role of CD14, LPS binding protein, and bactericidal/permeability-increasing protein. *J.Immunol.* 152:5070-5076, 1994

166. **Lens, S. M., K. Tesselaar, M. H. van Oers, and R. A. van Lier.** Control of lymphocyte function through CD27-CD70 interactions. *Semin.Immunol.* 10:491-499, 1998

167. **Li, K., Y. Li, J. M. Shelton, J. A. Richardson, E. Spencer, Z. J. Chen, X. Wang, and R. S. Williams.** Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* 101:389-399, 2000

168. **Li, L.Y., X. Luo, and X.Wang.** Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 412:95-99, 2001

169. **Li, X. C., Y. Li, I. Dodge, A. D. Wells, X. X. Zheng, L. A. Turka, and T. B. Strom.** Induction of allograft tolerance in the absence of Fas-mediated apoptosis. *J.Immunol.* 163:2500-2507, 1999

170. **Liebmann, P. M., G. Reibnegger, M. Lehofer, M. Moser, P. Purstner, H. Mangge, and K. Schauenstein.** Circadian rhythm of the soluble p75 tumor necrosis factor (sTNF-R75) receptor in humans--a possible explanation for the circadian kinetics of TNR-alpha effects. *Int.Immunol.* 10:1393-1396, 1998

171. **Linsley, P. S. and J. A. Ledbetter.** The role of the CD28 receptor during T cell responses to antigen. *Annu.Rev.Immunol.* 11:191-212, 1993

172. **Liu, Y., P. Waring, and A. Mullbacher.** Alloreactive cytotoxic T cells induce DNA fragmentation in peritoneal macrophages: evidence for target cell killing by cytotoxic T cells in vivo. *Eur.J.Immunol.* 19:1153-1155, 1989

173. **Locksley, R. M., N. Killeen, and M. J. Lenardo.** The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104:487-501, 2001

174. **Loenen, W. A., L. A. Gravestein, S. Beumer, C. J. Melief, A. Hagemeijer, and J. Borst.** Genomic organization and chromosomal localization of the human CD27 gene. *J.Immunol.* 149:3937-3943, 1992

175. **Loo, D. T., N. J. Chalupny, J. Bajorath, W. W. Shuford, R. S. Mittler, and A. Aruffo.** Analysis of 4-1BBL and laminin binding to murine 4-1BB, a member of the tumor necrosis factor receptor superfamily, and comparison with human 4-1BB. *J.Biol.Chem.* 272:6448-6456, 1997

176. **Lotz, M., M. Setareh, J. von Kempis, and H. Schwarz.** The nerve growth factor/tumor necrosis factor receptor family. *J.Leukoc.Biol.* 60:1-7, 1996

177. **Lynch, C. N., Y. C. Wang, J. K. Lund, Y. W. Chen, J. A. Leal, and S. R. Wiley.** TWEAK induces angiogenesis and proliferation of endothelial cells. *J.Biol.Chem.* 274:8455-8459, 1999

178. **Mackay, F., J. L. Browning, P. Lawton, S. A. Shah, M. Comiskey, A. K. Bhan, E. Mizoguchi, C. Terhorst, and S. J. Simpson.** Both the lymphotoxin and tumor necrosis factor pathways are involved in experimental murine models of colitis. *Gastroenterology* 115:1464-1475, 1998

179. **Marsters, S. A., A. D. Frutkin, N. J. Simpson, B. M. Fendly, and A. Ashkenazi.** Identification of cysteine-rich domains of the type 1 tumor necrosis factor receptor involved in ligand binding. *J.Biol.Chem.* 267:5747-5750, 1992

180. **Marsters, S. A., J. P. Sheridan, C. J. Donahue, R. M. Pitti, C. L. Gray, A. D. Goddard, K. D. Bauer, and A. Ashkenazi.** Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF-kappa B. *Curr.Biol.* 6:1669-1676, 1996A

181. **Marsters, S. A., R. M. Pitti, C. J. Donahue, S. Ruppert, K. D. Bauer, and A. Ashkenazi.** Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. *Curr.Biol.* 6:750-752, 1996B

182. **Marsters, S. A., J. P. Sheridan, R. M. Pitti, A. Huang, M. Skubatch, D. Baldwin, J. Yuan, A. Gurney, A. D. Goddard, P. Godowski, and A. Ashkenazi.** A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr.Biol.* 7:1003-1006, 1997A

183. **Marsters, S. A., T. M. Ayres, M. Skubatch, C. L. Gray, M. Rothe, and A. Ashkenazi.** Herpesvirus entry mediator, a member of the tumor necrosis factor

receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kappaB and AP-1. *J.Biol.Chem.* 272:14029-14032, 1997B

184. **Masihi, K. N., K. Madaj, H. Hintelmann, G. Gast, and Y. Kaneko.** Down-regulation of tumor necrosis factor-alpha, moderate reduction of interleukin-1beta, but not interleukin-6 or interleukin-10, by glucan immunomodulators curdlan sulfate and lentinan. *Int.J.Immunopharmacol.* 19:463-468, 1997

185. **Matsui, H., Y. Hikichi, I. Tsuji, T. Yamada, and Y. Shintani.** LIGHT, a member of the tumor necrosis factor ligand superfamily, prevents tumor necrosis factor-alpha-mediated human primary hepatocyte apoptosis, but not Fas-mediated apoptosis. *J.Biol.Chem.* 277:50054-50061, 2002

186. **Matsumoto, M., S. Mariathasan, M. H. Nahm, F. Baranyay, J. J. Peschon, and D. D. Chaplin.** Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science* 271:1289-1291, 1996

187. **Mauri, D. N., R. Ebner, R. I. Montgomery, K. D. Kochel, T. C. Cheung, G. L. Yu, S. Ruben, M. Murphy, R. J. Eisenberg, G. H. Cohen, P. G. Spear, and C. F. Ware.** LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity.* 8:21-30, 1998

188. **Melloni, E., M. Averna, F. Salamino, B. Sparatore, R. Minafra, and S. Pontremoli.** Acyl-CoA-binding protein is a potent m-calpain activator. *J Biol.Chem.* 275:82-86, 2000

189. **Meng, X. W., M. J. Fraser, J. M. Feller, and J. B. Ziegler.** Caspase-3 activates endo-exonuclease: further evidence for a role of the nuclease in apoptosis. *Apoptosis.* 5:243-254, 2000

190. **Messmer, U. K., V. A. Briner, and J. Pfeilschifter.** Basic fibroblast growth factor selectively enhances TNF-alpha-induced apoptotic cell death in glomerular endothelial cells: effects on apoptotic signaling pathways [In Process Citation]. *J.Am.Soc.Nephrol.* 11:2199-2211, 2000

191. **Migone, T. S., J. Zhang, X. Luo, L. Zhuang, C. Chen, B. Hu, J. S. Hong, J. W. Perry, S. F. Chen, J. X. Zhou, Y. H. Cho, S. Ullrich, P. Kanakaraj, J. Carrell, E. Boyd, H. S. Olsen, G. Hu, L. Pukac, D. Liu, J. Ni, S. Kim, R. Gentz, P. Feng, P. A. Moore, S. M. Ruben, and P. Wei.** TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity.* 16:479-492, 2002

192. **Mild, G., F. Bachmann, J. L. Boulay, K. Glatz, U. Laffer, A. Lowy, U. Metzger, J. Reuter, L. Terracciano, R. Herrmann, and C. Rochlitz.** DCR3 locus is a predictive marker for 5-fluorouracil-based adjuvant chemotherapy in colorectal cancer. *Int.J.Cancer* 102:254-257, 2002

193. **Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, and .** Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* 370:218-220, 1994

194. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87:427-436, 1996

195. **Moore, P. A., O. Belvedere, A. Orr, K. Pieri, D. W. LaFleur, P. Feng, D. Soppet, M. Charters, R. Gentz, D. Parmelee, Y. Li, O. Galperina, J. Giri, V. Roschke, B. Nardelli, J. Carrell, S. Sosnovtseva, W. Greenfield, S. M. Ruben, H. S. Olsen, J. Fikes, and D. M. Hilbert.** BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285:260-263, 1999

196. **Moreau, E., J. Philippe, S. Couvent, and G. Leroux-Roels.** Interference of soluble TNF-alpha receptors in immunological detection of tumor necrosis factor-alpha. *Clin.Chem.* 42:1450-1453, 1996

197. **Morel, Y., J. M. Schiano de Colella, J. Harrop, K. C. Deen, S. D. Holmes, T. A. Wattam, S. S. Khandekar, A. Truneh, R. W. Sweet, J. A. Gastaut, D. Olive, and R. T. Costello.** Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. *J.Immunol.* 165:4397-4404, 2000

198. **Morita, A., M. Grewe, S. Grether-Beck, S. Olaizola-Horn, and J. Krutmann.** Induction of proinflammatory cytokines in human epidermoid carcinoma cells by in vitro ultraviolet A1 irradiation. *Photochem.Photobiol.* 65:630-635, 1997

199. **Murphy, P. T. and R. M. Hutchinson.** Interleukin-4 and tumour necrosis factor-alpha produce non isotype specific partial differentiation of peripheral blood B-cells in myeloma. *Leuk.Lymphoma* 28:377-382, 1998

200. **Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit.** FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* 85:817-827, 1996

201. **Nagahira, K., Y. Fukuda, T. Nasu, H. Kawashima, C. Noguchi, T. Kurihara, S. Oikawa, and T. Nakanishi.** Construction and expression of a mouse-human chimeric antibody against human tumor necrosis factor-alpha. *Immunol.Lett.* 64:139-144, 1998

202. **Nagata, S.** Apoptosis by death factor. *Cell* 88:355-365, 1997

203. **Naismith, J. H., T. Q. Devine, T. Kohno, and S. R. Sprang.** Structures of the extracellular domain of the type I tumor necrosis factor receptor. *Structure.* 4:1251-1262, 1996

204. **Natoli, G., A. Costanzo, A. Ianni, D. J. Templeton, J. R. Woodgett, C. Balsano, and M. Levrero.** Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. *Science* 275:200-203, 1997

205. **Nooijen, P. T., A. M. Eggermont, L. Schalkwijk, S. Henzen-Logmans, R. M. de Waal, and D. J. Ruiter.** Complete response of melanoma-in-transit metastasis after isolated limb perfusion with tumor necrosis factor alpha and melphalan without massive tumor necrosis: a clinical and histopathological study of the delayed-type reaction pattern. *Cancer Res.* 58:4880-4887, 1998

206. **Nophar, Y., O. Kemper, C. Brakebusch, H. Englemann, R. Zwang, D. Aderka, H. Holtmann, and D. Wallach.** Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO J.* 9:3269-3278, 1990

207. **Ohsato, T., N. Ishihara, T. Muta, S.Umeda, S. Ikeda, K. Mihara, N. Hamasaki, and D. Kang.** Mammalian mitochondrial endonuclease G. Digestion of R-loops and localization in intermembrane space. *Eur.J Biochem.* 269:5765-5770, 2002

208. **Ohshima, K., S. Haraoka, M. Sugihara, J. Suzumiya, C. Kawasaki, M. Kanda, and M. Kikuchi.** Amplification and expression of a decoy receptor for fas ligand (DcR3) in virus (EBV or HTLV-I) associated lymphomas. *Cancer Lett.* 160:89-97, 2000

209. **Oshimi, Y., S. Oda, Y. Honda, S. Nagata, and S. Miyazaki.** Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J.Immunol.* 157:2909-2915, 1996

210. **Otsuki, T., A. Tomokuni, H. Sakaguchi, T. Aikoh, T. Matsuki, Y. Isozaki, F. Hyodoh, H. Ueki, M. Kusaka, S. Kita, and A. Ueki.** Over-expression of the decoy receptor 3 (DcR3) gene in peripheral blood mononuclear cells (PBMC) derived from silicosis patients. *Clin.Exp.Immunol.* 119:323-327, 2000

211. **Pan, G., J. Ni, Y. F. Wei, G. Yu, R. Gentz, and V. M. Dixit.** An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 277:815-818, 1997A

212. **Pan, G., K. O'Rourke, A. M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V. M. Dixit.** The receptor for the cytotoxic ligand TRAIL. *Science* 276:111-113, 1997B

213. **Pan, G., J. Ni, G. Yu, Y. F. Wei, and V. M. Dixit.** TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett.* 424:41-45, 1998A

214. **Pan, G., J. H. Bauer, V. Haridas, S. Wang, D. Liu, G. Yu, C.** Vincenz, B. B. Aggarwal, J. Ni, and V. M. Dixit. Identification and functional characterization of DR6, a novel death domain-containing TNF receptor. *FEBS Lett.* 431:351-356, 1998B

215. **Peng, X., D. M. Mosser, M. W. Adler, T. J. Rogers, J. J. Meissler, Jr., and T. K. Eisenstein.** Morphine enhances interleukin-12 and the production of other pro-inflammatory cytokines in mouse peritoneal macrophages [In Process Citation]. *J.Leukoc.Biol.* 68:723-728, 2000

216. **Pennica, D., J. S. Hayflick, T. S. Bringman, M. A. Palladino, and D. V. Goeddel.** Cloning and expression in Escherichia coli of the cDNA for murine tumor necrosis factor. *Proc.Natl.Acad.Sci.U.S.A* 82:6060-6064, 1985

217. **Piccotti, J. R., S. Y. Chan, A. M. VanBuskirk, E. J. Eichwald, and D. K. Bishop.** Are Th2 helper T lymphocytes beneficial, deleterious, or irrelevant in promoting allograft survival? *Transplantation* 63:619-624, 1997

218. **Pietravalle, F., S. Lecoanet-Henchoz, H. Blasey, J. P. Aubry, G. Elson, M. D. Edgerton, J. Y. Bonnefoy, and J. F. Gauchat.** Human native soluble CD40L is a biologically active trimer, processed inside microsomes. *J.Biol.Chem.* 271:5965-5967, 1996

219. **Pietravalle, F., S. Lecoanet-Henchoz, J. P. Aubry, G. Elson, J. Y. Bonnefoy, and J. F. Gauchat.** Cleavage of membrane-bound CD40 ligand is not required for inducing B cell proliferation and differentiation. *Eur.J.Immunol.* 26:725-728, 1996

220. **Pinchuk, L. M., S. J. Klaus, D. M. Magaletti, G. V. Pinchuk, J. P. Norsen, and E. A. Clark.** Functional CD40 ligand expressed by human blood dendritic cells is up-regulated by CD40 ligation. *J.Immunol.* 157:4363-4370, 1996

221. **Pitti, R. M., S. A. Marsters, S. Ruppert, C. J. Donahue, A. Moore, and A. Ashkenazi.** Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J.Biol.Chem.* 271:12687-12690, 1996

222. **Pitti, R. M., S. A. Marsters, D. A. Lawrence, M. Roy, F. C. Kischkel, P. Dowd, A. Huang, C. J. Donahue, S. W. Sherwood, D. T. Baldwin, P. J. Godowski, W. I. Wood, A. L. Gurney, K. J. Hillan, R. L. Cohen, A. D. Goddard, D. Botstein, and A. Ashkenazi.** Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396:699-703, 1998

223. **Porter, M. H., M. Arnold, and W. Langhans.** TNF-alpha tolerance blocks LPS-induced hypophagia but LPS tolerance fails to prevent TNF-alpha-induced hypophagia. *Am.J.Physiol* 274:R741-R745, 1998

224. **Porteu, F., M. Brockhaus, D. Wallach, H. Engelmann, and C. F. Nathan.** Human neutrophil elastase releases a ligand-binding fragment from the 75-kDa tumor necrosis factor (TNF) receptor. Comparison with the proteolytic activity responsible for shedding of TNF receptors from stimulated neutrophils. *J.Biol.Chem.* 266:18846-18853, 1991

225. **Posavad, C. M. and K. L. Rosenthal.** Herpes simplex virus-infected human fibroblasts are resistant to and inhibit cytotoxic T-lymphocyte activity. *J.Virol.* 66:6264-6272, 1992

226. **Probert, L. and K. Selmaj.** TNF and related molecules: trends in neuroscience and clinical applications. *J.Neuroimmunol.* 72:113-117, 1997

227. **Probert, L., H. P. Eugster, K. Akassoglou, J. Bauer, K. Frei, H. Lassmann, and A. Fontana.** TNFR1 signalling is critical for the development of demyelination and the limitation of T-cell responses during immune-mediated CNS disease [In Process Citation]. *Brain* 123 (Pt 10):2005-2019, 2000

228. **Raff, M.** Cell suicide for beginners. *Nature* 396:119-122, 1998

229. **Rahman, I.** Regulation of nuclear factor-kappaB, activator protein-1, and glutathione levels by tumor necrosis factor-alpha and dexamethasone in alveolar epithelial cells [In Process Citation]. *Biochem.Pharmacol.* 60:1041-1049, 2000

230. **Regnier, C. H., H. Y. Song, X. Gao, D. V. Goeddel, Z. Cao, and M. Rothe.** Identification and characterization of an IkappaB kinase. *Cell* 90:373-383, 1997

231. **Reiner, S. L. and R. M. Locksley.** The regulation of immunity to Leishmania major. *Annu.Rev.Immunol.* 13:151-177, 1995

232. **Reiter, I., G. Schwamberger, and B. Krammer.** Effect of photodynamic pretreatment on the susceptibility of murine tumor cells to macrophage antitumor mechanisms. *Photochem.Photobiol.* 66:384-388, 1997

233. **Rennert, P. D., J. L. Browning, R. Mebius, F. Mackay, and P. S. Hochman.** Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. *J.Exp.Med.* 184:1999-2006, 1996

234. **Romagnani, S.** The Th1/Th2 paradigm. *Immunol.Today* 18:263-266, 1997

235. **Rooney, I. A., K. D. Butrovich, A. A. Glass, S. Borboroglu, C. A. Benedict, J. C. Whitbeck, G. H. Cohen, R. J. Eisenberg, and C. F. Ware.** The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J.Biol.Chem.* 275:14307-14315, 2000

236. **Roth, W., S. Isenmann, M. Nakamura, M. Platten, W. Wick, P. Kleihues, M. Bahr, H. Ohgaki, A. Ashkenazi, and M. Weller.** Soluble decoy receptor 3 is expressed by malignant gliomas and suppresses CD95 ligand-induced apoptosis and chemotaxis. *Cancer Res.* 61:2759-2765, 2001

237. **Rothe, M., M. G. Pan, W. J. Henzel, T. M. Ayres, and D. V. Goeddel.** The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* 83:1243-1252, 1995

238. **Rothe, M., V. Sarma, V. M. Dixit, and D. V. Goeddel.** TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* 269:1424-1427, 1995

239. **Sakakura, C., T. Mori, T. Sakabe, Y. Ariyama, T. Shinomiya, K. Date, A. Hagiwara, T. Yamaguchi, T. Takahashi, Y. Nakamura, T. Abe, and J. Inazawa.** Gains, losses, and amplifications of genomic materials in primary gastric cancers analyzed by comparative genomic hybridization. *Genes Chromosomes.Cancer* 24:299-305, 1999

240. **Salih, H. R., H. M. Schmetzer, C. Burke, G. C. Starling, R. Dunn, R. Pelka-Fleischer, V. Nuessler, and P. A. Kiener.** Soluble CD137 (4-1BB) ligand is released following leukocyte activation and is found in sera of patients with hematological malignancies. *J.Immunol.* 167:4059-4066, 2001

241. **Santee, S. M. and L. B. Owen-Schaub.** Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization. *J.Biol.Chem.* 271:21151-21159, 1996

242. **Sato, T., K. Asamitsu, J. P. Yang, N. Takahashi, T. Tetsuka, A. Yoneyama, A. Kanagawa, and T. Okamoto.** Inhibition of human immunodeficiency virus type 1 replication by a bioavailable serine/threonine kinase inhibitor, fasudil hydrochloride. *AIDS Res.Hum.Retroviruses* 14:293-298, 1998

243. **Savelieva, E., C. D. Belair, M. A. Newton, S. DeVries, J. W. Gray, F. Waldman, and C. A. Reznikoff.** 20q gain associates with immortalization: 20q13.2 amplification correlates with genome instability in human papillomavirus 16 E7 transformed human uroepithelial cells. *Oncogene* 14:551-560, 1997

244. **Scheu, S., J. Alferink, T. Potzel, W. Barchet, U. Kalinke, and K. Pfeffer.** Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin beta in mesenteric lymph node genesis. *J.Exp.Med.* 195:1613-1624, 2002

245. **Schneider, P., F. Mackay, V. Steiner, K. Hofmann, J. L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, D. Valmori, P. Romero, C. Werner-Favre, R. H. Zubler, J. L. Browning, and J. Tschopp.** BAFF, a

novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J.Exp.Med.* 189:1747-1756, 1999

246. **Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak.** Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 352:621-624, 1991

247. **Schwartz, R. H.** Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 71:1065-1068, 1992

248. **Screaton, G. and X. N. Xu.** T cell life and death signalling via TNF-receptor family members. *Curr.Opin.Immunol.* 12:316-322, 2000

249. **Screaton, G. R., X. N. Xu, A. L. Olsen, A. E. Cowper, R. Tan, A. J. McMichael, and J. I. Bell.** LARD: a new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing. *Proc.Natl.Acad.Sci.U.S.A* 94:4615-4619, 1997

250. **Sebzda, E., S. Mariathasan, T. Ohteki, R. Jones, M. F. Bachmann, and P. S. Ohashi.** Selection of the T cell repertoire. *Annu.Rev.Immunol.* 17:829-874, 1999

251. **Seckinger, P., S. Isaaz, and J. M. Dayer.** Purification and biologic characterization of a specific tumor necrosis factor alpha inhibitor. *J.Biol.Chem.* 264:11966-11973, 1989

252. **Seitz, C. S., R. A. Freiberg, K. Hinata, and P. A. Khavari.** NF-kappaB determines localization and features of cell death in epidermis. *J.Clin.Invest* 105:253-260, 2000

253. **Serrador, J. M., M. Nieto, J. L. Alonso-Lebrero, M. A. del Pozo, J. Calvo, H. Furthmayr, R. Schwartz-Albiez, F. Lozano, R. Gonzalez-Amaro, P. Sanchez-Mateos, and F. Sanchez-Madrid.** CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. *Blood* 91:4632-4644, 1998

254. **Shaikh, R. B., S. Santee, S. W. Granger, K. Butrovich, T. Cheung, M. Kronenberg, H. Cheroutre, and C. F. Ware.** Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction. *J.Immunol.* 167:6330-6337, 2001

255. **Sheikh, M. S. and A. J. Fornace, Jr.** Death and decoy receptors and p53-mediated apoptosis. *Leukemia* 14:1509-1513, 2000

256. **Sheridan, J. P., S. A. Marsters, R. M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C. L. Gray, K. Baker, W. I. Wood, A. D. Goddard, P. Godowski, and A. Ashkenazi.** Control of TRAIL-induced

apoptosis by a family of signaling and decoy receptors. *Science* 277:818-821, 1997

257. **Shi, G., H. Luo, X. Wan, T. W. Salcedo, J. Zhang, and J. Wu.** Mouse T cells receive costimulatory signals from LIGHT, a TNF family member. *Blood* 100:3279-3286, 2002

258. **Shi, G., Y. Wu, J. Zhang, and J. Wu.** Death Decoy Receptor TR6/DcR3 Inhibits T Cell Chemotaxis In Vitro and In Vivo. *J.Immunol. 2003* (In press)

259. **Shirai, T., H. Yamaguchi, H. Ito, C. W. Todd, and R. B. Wallace.** Cloning and expression in Escherichia coli of the gene for human tumour necrosis factor. *Nature* 313:803-806, 1985

260. **Shu, H. B., M. Takeuchi, and D. V. Goeddel.** The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc.Natl.Acad.Sci.U.S.A* 93:13973-13978, 1996

261. **Shu, H. B., W. H. Hu, and H. Johnson.** TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *J.Leukoc.Biol.* 65:680-683, 1999

262. **Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, T. C. Pearson, J. A. Ledbetter, A. Aruffo, and R. S. Mittler.** 1997. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J.Exp.Med.* 186:47-55

263. **Sica, G. L., G. Zhu, K. Tamada, D. Liu, J. Ni, and L. Chen.** RELT, a new member of the tumor necrosis factor receptor superfamily, is selectively expressed in hematopoietic tissues and activates transcription factor NF-kappaB. *Blood* 97:2702-2707, 2001

264. **Siegel, R. M., F. K. Chan, H. J. Chun, and M. J. Lenardo.** The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat.Immunol.* 1:469-474, 2000

265. **Simonet, W. S., D. L. Lacey, C. R. Dunstan, M. Kelley, M. S. Chang, R. Luthy, H. Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H. L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T. M. Hughes, D. Hill, W. Pattison, P. Campbell, W. J. Boyle, and .** Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89:309-319, 1997

266. **Sjoholm, A.** Effects of transforming growth factor beta, tumor necrosis factor alpha and interferon gamma on pancreatic islet beta-cell responsiveness to transforming growth factor alpha. *Biosci.Rep.* 16:415-423, 1996

267. **Smith, C. A., T. Davis, D. Anderson, L. Solam, M. P. Beckmann, R. Jerzy, S. K. Dower, D. Cosman, and R. G. Goodwin.** A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science* 248:1019-1023, 1990

268. **Smith, C. A., H. J. Gruss, T. Davis, D. Anderson, T. Farrah, E. Baker, G. R. Sutherland, C. I. Brannan, N. G. Copeland, N. A. Jenkins, and .** CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell* 73:1349-1360, 1993

269. **Smith, C. A., T. Farrah, and R. G. Goodwin.** The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 76:959-962, 1994

270. **Smith, R. A. and C. Baglioni.** The active form of tumor necrosis factor is a trimer. *J.Biol.Chem.* 262:6951-6954, 1987

271. **Soligo, D., D. G. Lambertenghi, N. Quirici, F. Servida, L. Caneva, and G. Lamorte.** Expansion of dendritic cells derived from human CD34+ cells in static and continuous perfusion cultures. *Br.J.Haematol.* 101:352-363, 1998

272. **Sonoda, G., J. Palazzo, M. S. du, A. K. Godwin, M. Feder, M. Yakushiji, and J. R. Testa.** Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q13 in human ovarian carcinomas. *Genes Chromosomes.Cancer* 20:320-328, 1997

273. **Srivastava, A. K., J. Pispa, A. J. Hartung, Y. Du, S. Ezer, T. Jenks, T. Shimada, M. Pekkanen, M. L. Mikkola, M. S. Ko, I. Thesleff, J. Kere, and D. Schlessinger.** The Tabby phenotype is caused by mutation in a mouse homologue of the EDA gene that reveals novel mouse and human exons and encodes a protein (ectodysplasin-A) with collagenous domains. *Proc.Natl.Acad.Sci.U.S.A* 94:13069-13074, 1997

274. **Stanger, B. Z., P. Leder, T. H. Lee, E. Kim, and B. Seed.** RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* 81:513-523, 1995

275. **Storz, P., H. Doppler, A. Wernig, K. Pfizenmaier, and G. Muller.** Cross-talk mechanisms in the development of insulin resistance of skeletal muscle cells palmitate rather than tumour necrosis factor inhibits insulin-dependent protein kinase B (PKB)/Akt stimulation and glucose uptake. *Eur.J.Biochem.* 266:17-25, 1999

276. **Stubbs, A. P., P. D. Abel, M. Golding, G. Bhangal, Q. Wang, J. Waxman, G. W. Stamp, and E. N. Lalani.** Differentially expressed genes in hormone refractory prostate cancer: association with chromosomal regions involved with genetic aberrations. *Am.J.Pathol.* 154:1335-1343, 1999

277. **Stuber, E., M. Neurath, D. Calderhead, H. P. Fell, and W. Strober.** Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity.* 2:507-521, 1995

278. **Stuber, E. and W. Strober.** The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J.Exp.Med.* 183:979-989, 1996

279. **Suda, T., T. Takahashi, P. Golstein, and S. Nagata.** Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75:1169-1178, 1993

280. **Suzuki, I. and P. J. Fink.** Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through Fas ligand. *J.Exp.Med.* 187:123-128, 1998

281. **Suzuki, I., S. Martin, T. E. Boursalian, C. Beers, and P. J. Fink.** Fas ligand costimulates the in vivo proliferation of CD8+ T cells. *J.Immunol.* 165:5537-5543, 2000

282. **Suzuki, I. and P. J. Fink.** The dual functions of fas ligand in the regulation of peripheral CD8+ and CD4+ T cells. *Proc.Natl.Acad.Sci.U.S.A* 97:1707-1712, 2000

283. **Takeda, K., S. Iwamoto, H. Sugimoto, T. Takuma, N. Kawatani, M. Noda, A. Masaki, H. Morise, H. Arimura, and K. Konno.** Identity of differentiation inducing factor and tumour necrosis factor. *Nature* 323:338-340, 1986

284. **Tamada, K., K. Shimozaki, A. I. Chapoval, G. Zhu, G. Sica, D. Flies, T. Boone, H. Hsu, Y. X. Fu, S. Nagata, J. Ni, and L. Chen.** Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. *Nat.Med.* 6:283-289, 2000A

285. **Tamada, K., K. Shimozaki, A. I. Chapoval, Y. Zhai, J. Su, S. F. Chen, S. L. Hsieh, S. Nagata, J. Ni, and L. Chen.** LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J.Immunol.* 164:4105-4110, 2000B

286. **Tamada, K., J. Ni, G. Zhu, M. Fiscella, B. Teng, J. M. van Deursen, and L. Chen.** Cutting edge: selective impairment of CD8+ T cell function in mice lacking the TNF superfamily member LIGHT. *J.Immunol.* 168:4832-4835, 2002A

287. **Tamada, K., H. Tamura, D. Flies, Y. X. Fu, E. Celis, L. R. Pease, B. R. Blazar, and L. Chen.** Blockade of LIGHT/LTbeta and CD40 signaling induces allospecific T cell anergy, preventing graft-versus-host disease. *J.Clin.Invest* 109:549-557, 2002B

288. **Tan, K. B., J. Harrop, M. Reddy, P. Young, J. Terrett, J. Emery, G. Moore, and A. Truneh.** Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene* 204:35-46, 1997

289. **Tanaka, M., T. Suda, T. Takahashi, and S. Nagata.** Expression of the functional soluble form of human fas ligand in activated lymphocytes. *EMBO J.* 14:1129-1135, 1995

290. **Tanaka, M., T. Itai, M. Adachi, and S. Nagata.** Downregulation of Fas ligand by shedding. *Nat.Med.* 4:31-36, 1998

291. **Tartaglia, L. A. and D. V. Goeddel.** Two TNF receptors. *Immunol.Today* 13:151-153, 1992

292. **Tartaglia, L. A., D. V. Goeddel, C. Reynolds, I. S. Figari, R. F. Weber, B. M. Fendly, and M. A. Palladino, Jr.** Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. *J.Immunol.* 151:4637-4641, 1993

293. **Tartaglia, L. A., T. M. Ayres, G. H. Wong, and D. V. Goeddel.** A novel domain within the 55 kd TNF receptor signals cell death. *Cell* 74:845-853, 1993

294. **Tewari, M. and V. M. Dixit.** Recent advances in tumor necrosis factor and CD40 signaling. *Curr.Opin.Genet.Dev.* 6:39-44, 1996

295. **Thompson, J. S., S. A. Bixler, F. Qian, K. Vora, M. L. Scott, T. G. Cachero, C. Hession, P. Schneider, I. D. Sizing, C. Mullen, K. Strauch, M. Zafari, C. D. Benjamin, J. Tschopp, J. L. Browning, and C. Ambrose.** BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* 293:2108-2111, 2001

296. **Thornberry, N. A. and Y. Lazebnik.** Caspases: enemies within. *Science* 281:1312-1316, 1998

297. **Ting, A. T., F. X. Pimentel-Muinos, and B. Seed.** RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J.* 15:6189-6196, 1996

298. **Tracey, K. J. and A. Cerami.** Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu.Rev.Med.* 45:491-503, 1994

299. **Treton, D., F. Valensi, X. Troussard, G. Gras, G. Flandrin, P. Galanaud, and Y. Richard.** Cytokine response of B lymphocytes from splenic lymphoma with villous lymphocytes: correlation with TNF-RII (p75) and CD11c expression. *Hematol.Cell Ther.* 38:345-352, 1996

300. **Tucker, A. S., D. J. Headon, P. Schneider, B. M. Ferguson, P. Overbeek, J. Tschopp, and P. T. Sharpe.** Edar/Eda interactions regulate enamel knot formation in tooth morphogenesis. *Development* 127:4691-4700, 2000

301. **Ullrich, A., A. Gray, C. Berman, and T. J. Dull.** Human beta-nerve growth factor gene sequence highly homologous to that of mouse. *Nature* 303:821-825, 1983

302. **Upton, C., A. M. DeLange, and G. McFadden.** Tumorigenic poxviruses: genomic organization and DNA sequence of the telomeric region of the Shope fibroma virus genome. *Virology* 160:20-30, 1987

303. **Van Gurp, M., N. Festjens, G. van Loo, X. Saelens, and P. Vandenabeele.** Mitochondrial intermembrane proteins in cell death. *Biochem.Biophys.Res Commun.* 304:487-497, 2003

304. **Van Essen, D., H. Kikutani, and D. Gray.** CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature* 378:620-623, 1995

305. **Van Arsdale, T. L. and C. F. Ware.** TNF receptor signal transduction. Ligand-dependent stimulation of a serine protein kinase activity associated with (CD120a) TNFR60. *J.Immunol.* 153:3043-3050, 1994

306. **Verhagen, A. M., P. G. Ekert, M. Pakusch, J. Silke, L. M. Connolly, G. E. Reid, R. L. Moritz, R. J. Simpson, and D. L. Vaux.** Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43-53, 2000

307. **Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin.** Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu.Rev.Immunol.* 17:331-367, 1999

308. **Wan, X., J. Zhang, H. Luo, G. Shi, E. Kapnik, S. Kim, P. Kanakaraj, and J. Wu.** A TNF Family Member LIGHT Transduces Costimulatory Signals into Human T Cells. *J.Immunol.* 169:6813-6821, 2002

309. **Wang, A. M., A. A. Creasey, M. B. Ladner, L. S. Lin, J. Strickler, J. N. Van Arsdell, R. Yamamoto, and D. F. Mark.** Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 228:149-154, 1985

310. **Wang, C. Y., M. W. Mayo, and A. S. Baldwin, Jr.** TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 274:784-787, 1996

311. **Wang, B., R. Maile, R. Greenwood, E. J. Collins, and J. A. Frelinger. 2000.** Naive CD8+ T cells do not require costimulation for proliferation and differentiation into cytotoxic effector cells. *J.Immunol.* 164:1216-1222.

312. **Wang, J., J. C. Lo, A. Foster, P. Yu, H. M. Chen, Y. Wang, K. Tamada, L. Chen, and Y. X. Fu.** The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. *J.Clin.Invest* 108:1771-1780, 2001A

313. **Wang, J., T. Chun, J. C. Lo, Q. Wu, Y. Wang, A. Foster, K. Roca, M. Chen, K. Tamada, L. Chen, C. R. Wang, and Y. X. Fu.** The critical role of LIGHT, a TNF family member, in T cell development. *J.Immunol.* 167:5099-5105, 2001B

314. **Wang, J., A. Foster, R. Chin, P. Yu, Y. Sun, Y. Wang, K. Pfeffer, and Y. X. Fu.** The complementation of lymphotoxin deficiency with LIGHT, a newly discovered TNF family member, for the restoration of secondary lymphoid structure and function. *Eur.J.Immunol.* 32:1969-1979, 2002

315. **Wang, X., C. Yang, J. Chai, Y. Shi, and D. Xue.** 2002. Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. *Science* 298:1587-1592, 2002

316. **Ware, C. F., P. D. Crowe, M. H. Grayson, M. J. Androlewicz, and J. L. Browning.** Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. *J.Immunol.* 149:3881-3888, 1992

317. **Ware, C. F., S. VanArsdale, and T. L. VanArsdale.** Apoptosis mediated by the TNF-related cytokine and receptor families. *J.Cell Biochem.* 60:47-55, 1996

318. **Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, and .** Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity.* 3:673-682, 1995

319. **Wiley, S. R., R. G. Goodwin, and C. A.** Smith. Reverse signaling via CD30 ligand. *J.Immunol.* 157:3635-3639, 1996

320. **Wiley, S. R., L. Cassiano, T. Lofton, T. Davis-Smith, J. A. Winkles, V. Lindner, H. Liu, T. O. Daniel, C. A. Smith, and W. C. Fanslow.** A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity.* 15:837-846, 2001

321. **Williams-Abbott, L., B. N. Walter, T. C. Cheung, C. R. Goh, A. G. Porter, and C. F. Ware.** The lymphotoxin-alpha (LTalpha) subunit is essential for the assembly, but not for the receptor specificity, of the membrane-anchored LTalpha1beta2 heterotrimeric ligand. *J.Biol.Chem.* 272:19451-19456, 1997

322. **Wong, B. R., J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Chao, S. Kalachikov, E. Cayani, F. S. Bartlett, III, W. N. Frankel, S. Y. Lee, and Y.**

Choi. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J.Biol.Chem.* 272:25190-25194, 1997

323. **Wu, J., J. Wilson, J. He, L. Xiang, P. H. Schur, and J. D. Mountz.** Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J.Clin.Invest* 98:1107-1113, 1996

324. **Wu, Q., Y. Wang, J. Wang, E. O. Hedgeman, J. L. Browning, and Y. X. Fu.** The requirement of membrane lymphotoxin for the presence of dendritic cells in lymphoid tissues. *J.Exp.Med.* 190:629-638, 1999

325. **Wu, Q., B. Salomon, M. Chen, Y. Wang, L. M. Hoffman, J. A. Bluestone, and Y. X. Fu.** Reversal of spontaneous autoimmune insulitis in nonobese diabetic mice by soluble lymphotoxin receptor. *J.Exp.Med.* 193:1327-1332, 2001

326. **Wu, Y., B. Han, H. Shen, M. Lin, P. Moore, J. Zhang, and J. Wu.** The clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. Int. J. Can. 105:724-732, 2003

327. **Wulfing, C., C. Sumen, M. D. Sjaastad, L. C. Wu, M. L. Dustin, and M. M. Davis.** Costimulation and endogenous MHC ligands contribute to T cell recognition. *Nat.Immunol.* 3:42-47, 2002

328. **Xia, X. Z., J. Treanor, G. Senaldi, S. D. Khare, T. Boone, M. Kelley, L. E. Theill, A. Colombero, I. Solovyev, F. Lee, S. McCabe, R. Elliott, K. Miner, N. Hawkins, J. Guo, M. Stolina, G. Yu, J. Wang, J. Delaney, S. Y. Meng, W. J. Boyle, and H. Hsu.** TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. *J.Exp.Med.* 192:137-143, 2000

329. **Yamada, K., M. Yamakawa, Y. Imai, and M. Tsukamoto.** Expression of cytokine receptors on follicular dendritic cells. *Blood* 90:4832-4841, 1997

330. **Ye, Q., C. C. Fraser, W. Gao, L. Wang, S. J. Busfield, C. Wang, Y. Qiu, A. J. Coyle, J. C. Gutierrez-Ramos, and W. W. Hancock.** Modulation of LIGHT-HVEM costimulation prolongs cardiac allograft survival. *J.Exp.Med.* 195:795-800, 2002

331. **Ye, X., P. Mehlen, S. Rabizadeh, T. VanArsdale, H. Zhang, H. Shin, J. J. Wang, E. Leo, J. Zapata, C. A. Hauser, J. C. Reed, and D. E. Bredesen.** TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. *J.Biol.Chem.* 274:30202-30208, 1999

332. **Yeh, W. C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J. L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, P. Ohashi, M. Rothe, D. V. Goeddel, and T. W. Mak.** Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity.* 7:715-725, 1997

333. **Yeh, W. C., J. L. Pompa, M. E. McCurrach, H. B. Shu, A. J. Elia, A. Shahinian, M. Ng, A. Wakeham, W. Khoo, K. Mitchell, W. S. El Deiry, S. W. Lowe, D. V. Goeddel, and T. W. Mak.** FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science* 279:1954-1958, 1998

334. **Yu, K. Y., B. Kwon, J. Ni, Y. Zhai, R. Ebner, and B. S. Kwon.** A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J.Biol.Chem.* 274:13733-13736, 1999

335. **Yuan, J.** Transducing signals of life and death. *Curr.Opin.Cell Biol.* 9:247-251, 1997

336. **Yue, T. L., J. Ni, A. M. Romanic, J. L. Gu, P. Keller, C. Wang, S. Kumar, G. L. Yu, T. K. Hart, X. Wang, Z. Xia, W. E. DeWolf, Jr., and G. Z. Feuerstein.** TL1, a novel tumor necrosis factor-like cytokine, induces apoptosis in endothelial cells. Involvement of activation of stress protein kinases (stress-activated protein kinase and p38 mitogen-activated protein kinase) and caspase-3-like protease. *J.Biol.Chem.* 274:1479-1486, 1999

337. **Zamzami, N. and G. Kroemer.** The mitochondrion in apoptosis: how Pandora's box opens. *Nat.Rev.Mol.Cell Biol.* 2:67-71, 2001

338. **Zhai, Y., R. Guo, T. L. Hsu, G. L. Yu, J. Ni, B. S. Kwon, G. W. Jiang, J. Lu, J. Tan, M. Ugustus, K. Carter, L. Rojas, F. Zhu, C. Lincoln, G. Endress, L. Xing, S. Wang, K. O. Oh, R. Gentz, S. Ruben, M. E. Lippman, S. L. Hsieh, and D. Yang.** LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J.Clin.Invest* 102:1142-1151, 1998

339. **Zhai, Y., J. Ni, G. W. Jiang, J. Lu, L. Xing, C. Lincoln, K. C. Carter, F. Janat, D. Kozak, S. Xu, L. Rojas, B. B. Aggarwal, S. Ruben, L. Y. Li, R. Gentz, and G. L. Yu.** VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas in vivo. *FASEB J.* 13:181-189, 1999

340. **Zhang, J., J. X. Gao, K. Salojin, Q. Shao, M. Grattan, C. Meagher, D. W. Laird, and T. L. Delovitch.** Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase. *J.Exp.Med.* 191:1017-1030, 2000

341. **Zhang, J., T. W. Salcedo, X. Wan, S. Ullrich, B. Hu, T. Gregorio, P. Feng, S. Qi, H. Chen, Y. H. Cho, Y. Li, P. A. Moore, and J. Wu.** Modulation of T-cell responses to alloantigens by TR6/DcR3. *J.Clin.Invest* 107:1459-1468, 2001

342. **Zheng, X. X., A. W. Steele, W. W. Hancock, K. Kawamoto, X. C. Li, P. W. Nickerson, Y. Li, Y. Tian, and T. B. Strom.** IL-2 receptor-targeted cytolytic IL-

2/Fc fusion protein treatment blocks diabetogenic autoimmunity in nonobese diabetic mice. *J.Immunol.* 163:4041-4048, 1999

343. **Zhu, J., H. Huang, L. Guo, T. Stonehouse, C. J. Watson, J. Hu-Li, and W. E. Paul.** Transient inhibition of interleukin 4 signaling by T cell receptor ligation [In Process Citation]. *J.Exp.Med.* 192:1125-1134, 2000

V. Appendix

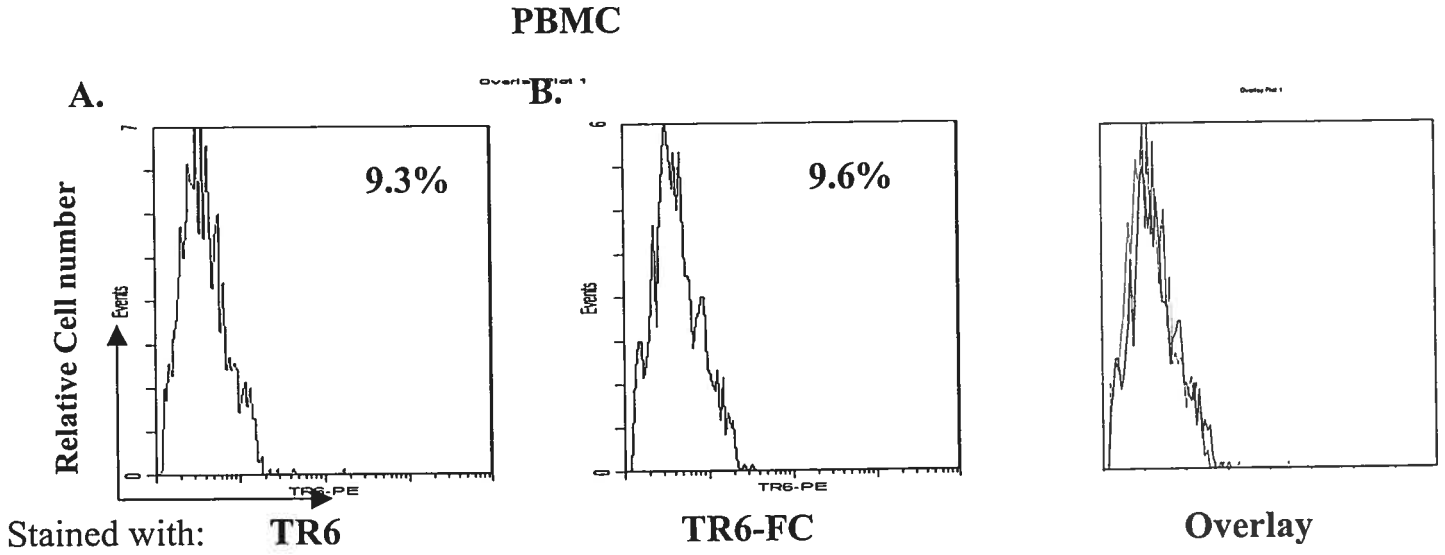

Figure 1. Mutated Fc does not bind to human PBMC. Both TR6 and TR6-Fc were biotinylated before cell surface staining. Human PBMCs were first stained with biotinylated TR6 (panel A) and TR6-Fc (panel B), followed by streptavidin-PE. These results indicate that mutated Fc portion of TR6-Fc has no binding with Fcγ receptor.

Appendix

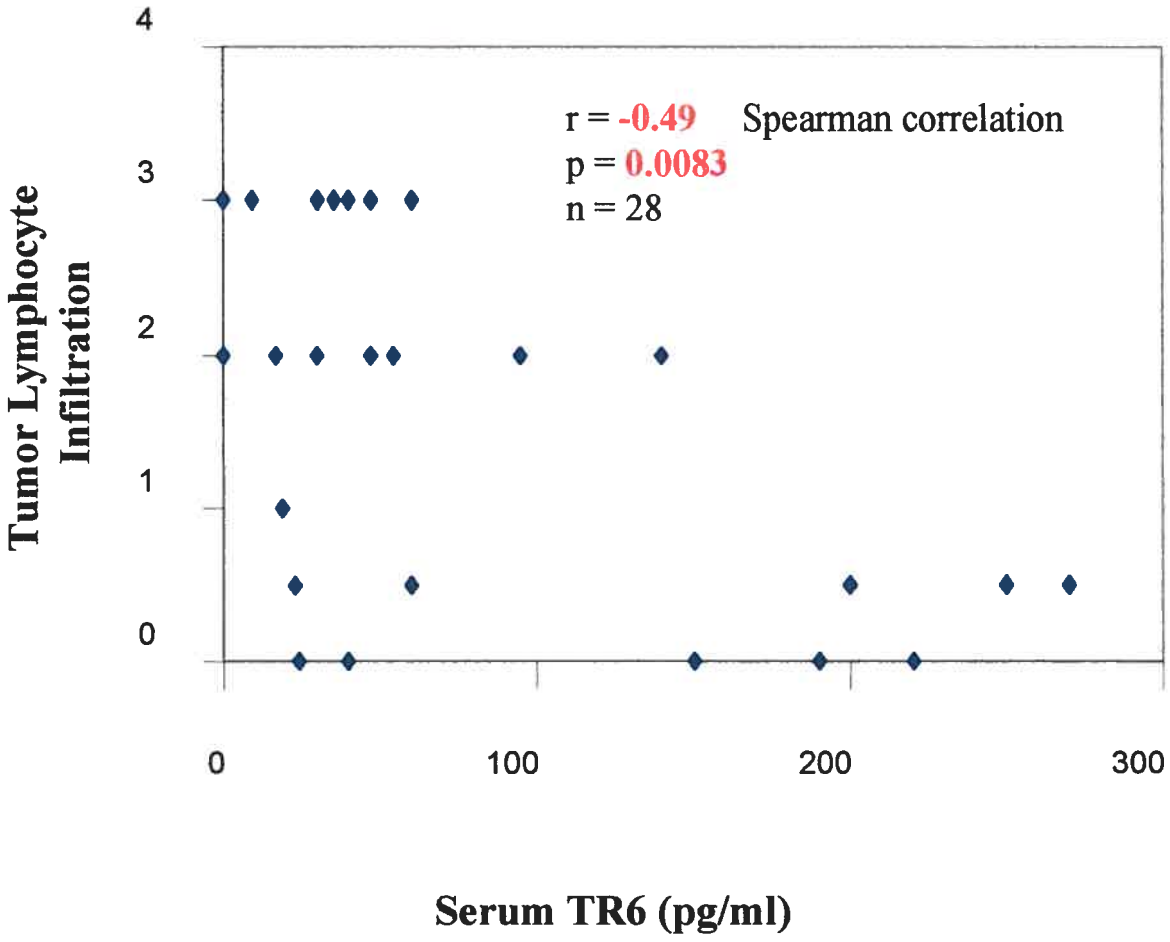

Figure 2. Gastric cancer lymphocyte infiltration is inversely correlated to serum TR6 levels. Human gastric cancer samples were stained with HE, and lymphocyte infiltration was semi-quantified from grade 1 to 4.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Jiangping Wu for his scientific guidance, encouragement and patience throughout this study and during my preparation of this thesis.

I would like to thank all of our collaborators such as Dr. Jun Zhang and coworkers in Human Genome Science Inc. for their support and collaborations.

I would like to thank Dr. Huifang Chan, Dr. Shijie Qi and other coworkers in their group for the assistance in experiments related to transplantation; Dr. Alain Comtois for use of imaging system and fluorescence microscopy.

I would like to thank Dr. Hongyu Luo for her valuable help in many experiments and inspiring discussion.

I would like to thank all people working as a dynamic team in our laboratory for their kindly help and encouragement.

I would like to thank all people who helped me in different ways and contributed to this work.

I would like to thank all my friends and those who always support me with encourage and love.

Finally, I would like to thank all of my family members for their understanding, patience, and their great support and encouragement which are critically important for me to complete this study.