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

Xiaochun Wan,2* Jun Zhang,2‡ Hongyu Luo,* Guixiu Shi,* Elena Kapnik,‡ Sunhee Kim,‡ Palanisamy Kanakaraj,‡ and Jiangping Wu3*†

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 but not by soluble Fas-Fc. TR6-Fc could also costimulate proliferation, lymphokine production, and cytotoxicity of human T cells (8). 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 costimulatory signals 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, 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), 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 TNF 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 TNF 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).
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 5K9E2) 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, spleenocytes from one of the immunized mice were fused with 2 × 10^7 P3 X 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 pristane-primed BALB/c mice (3 × 10^5/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, Oslo, 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% NH4Cl. The cells were cultured in RPMI 1640 supplemented with 10% FCS, t-glutamine, and antibiotics. RPMI 1640, FCS, penicillin-streptomycin, and t-glutamine were purchased from Life Technologies (Burlington, Ontario, Canada). [3H]Thymidine uptake was measured as described previously (22, 23).

For Th1 and Th2 cell generation, cord blood mononuclear cells were depleted 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 × 10^5 cells/well in 96-well plates, which were coated with anti-TCRαβ (3 μg/ml) in combination with various amounts of solid-phase TR6-Fc. Proliferation of these cells was measured by [3H]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 × 10^5) without further stimulation, or stimulated with solid-phase CD3, were stained with TR6-Fc (15 ng/sample) followed by goat Fab(‘)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 γδ T cells was assayed as follows. Human PBMC (4 × 10^3 cells/200 μl/well) were stimulated with mitomycin C-treated Daudi cells (0.7 × 10^3 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 51Cr-release assay using 51Cr-labeled Daudi cells (1.5 × 10^3 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 × 10^3 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 51Cr-release assay was conducted on day 5 instead of day 6.

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

% lysis = cpm of the test sample - cpm of spontaneous release 
cpm of maximal release - cpm of spontaneous release

For αβ 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 51Cr-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 responses to mitogens and alloantigens. At least a part of such responsiveness can be explained by our finding that TR6 can interact with two ligands on the T cell surface, LIGHT and TR6 itself.

Results

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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 \(\mu g/ml\), and TR6-Fc in solution was used at 10 \(\mu g/ml\). \[^{3}H\]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 \(\mu g/ml\), while PHA was used at a suboptimal dose of 0.2 \(\mu g/ml\). \[^{3}H\]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 \(\mu g/ml\) TR6-Fc in solution along with 0.2 \(\mu g/ml\) 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 \(\mu g/ml\)) 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 \(\mu g/ml\), 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 \(\mu l\)) in PBS, followed by TR6-Fc (1 \(\mu g/50 \mu l\)) at room temperature for 6 h. Soluble LIGHT was added as indicated to certain samples at 10 \(\mu g/ml\). \[^{3}H\]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 \(\mu g/ml\) goat anti-human IgG (Southern Biotechnology Associates) along with various concentrations (0.1–0.5 \(\mu g/ml\)) of anti-CD3 in PBS as indicated overnight at 4°C. After washing, the wells were incubated with TR6-Fc (10 \(\mu g/ml\)) 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.

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 \(\mu g/ml\) 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 \(\mu g/50 \mu l\)) during coating. PBMC were cultured in these wells in the absence or presence of a suboptimal concentration of PHA (0.2 \(\mu g/ml\)). \[^{3}H\]thymidine uptake between 56 and 72 h after initiation of culture was measured.
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. [3H]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 signalling, 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. 1F), suggesting that the major part of the reverse signalling 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. 1G). 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. 1H, 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. 2A. 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. 2B, 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. 2B, 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 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 binding 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-TCRβ, 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-TCRαβ on the solid phase resulted in dramatic IFN-γ production by Th1 but not Th2 cells (74,936 ± 56 vs 72 ± 23 pg/ml), and marked IL-5 production by Th2 but not Th1 cells (586.9 ± 16.5 vs 1.1 ± 0.14 pg/ml), confirming the Th1 and Th2 phenotype of the cells. When solid-phase TR6-Fc was used along with anti-TCRαβ, it significantly increased IFN-γ production by Th1 cells (42,587 ± 4,535.2 pg/ml), compared with anti-TCRαβ stimulation alone (8,064.5 ± 223 pg/ml); this augmentation was blocked by anti-TR6 mAb in a dose-dependent manner, showing the specificity of the TR6 stimulation (Fig. 4E). 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-TCRαβ produced little IFN-γ (<30 pg/ml), as expected, since IFN-γ is a Th1 lymphokine; however, they also failed to produce Th2 lymphokine
IL-5 (Fig. 4). 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 Vα9Vβ2 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. 5A, 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. 5B), suggesting the importance of LIGHT reverse signaling in a γδ CTL response.

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.
**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-TCRαβ and various amounts of solid-phase TR6. To prepare solid-phase anti-TCRαβ 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-TCRαβ 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 [3H]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-TCRαβ and an optimal concentration of solid-phase TR6-Fc (20 μg/ml during coating). Anti-TR6 mAb (clone EO2) of various concentrations, as indicated, was added to the culture; normal mouse IgG1 was used as a control. [3H]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-TCRαβ 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 γδ CTL activity, like soluble TR6-Fc, and such enhancement could be neutralized by soluble LIGHT (Fig. 5C), suggesting that LIGHT is the likely molecule that TR6 interacts with, and that cross-linking LIGHT costimulates T cells in γδ CTL development. We also demonstrated that solid-phase TR6 could augment αβ T cell CTL, using T cell blasts as target cells (Fig. 5D). 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. 1E, anti-CD3 in Fig. 2B, anti-TCRαβ 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. 1E) and lymphokine production (Fig. 2B), 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
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, Ca2+ influx, and activation of Lck, protein kinase C, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase in EL4 thymoma cells (37, 38). TRAIL cross-linking also induces p38 mitogen-activated protein kinase activation (36). Therefore, it should not be surprising that

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 51Cr-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 51Cr-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 51Cr-release assay.
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. 1H). 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. 5C). 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 to 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 γδ as well as αβ T cell CTL activities.

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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.

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