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DcR3/TR6 Modulates Immune Cell Interactions

Xiaochun Wan,[1] Guixiu Shi,[1] Mark Semenuk,[3] Jun Zhang,[3] and Jiangping Wu[1,2]*

[1]Laboratory of Transplantation Immunology, Notre Dame Hospital, University of Montreal, Montreal, Canada
[2]Nephrology Service of Centre hospitalier de l'Universite de Montreal (CHUM), Notre Dame Hospital, University of Montreal, Montreal, Canada
[3]Human Genome Sciences, Inc., Rockville, Maryland

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. © 2003 Wiley-Liss, Inc.

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.

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*Correspondence to: Dr. Jiangping Wu, Laboratory of Transplantation Immunology, Research Center, CHUM, Notre Dame Hospital, DeSeve Pavilion, Room Y-5612, 1560 Sherbrooke Street East, Montreal, Quebec H2L 4M1, Canada. E-mail: jianping.wu@umontreal.ca

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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).

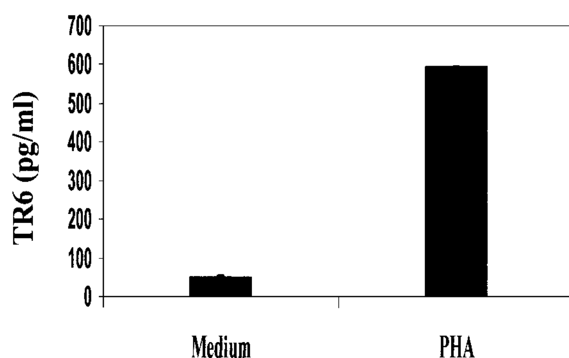

Fig. 1. TR6 is secreted by activated T cells. Human peripheral blood mononuclear cells (PBMC) were cultured for 48 h in 24-well plates at 4×10^6/2 ml/well in the presence of PHA (2 μg/ml). The culture supernatants were harvested and tested for TR6 levels by ELISA. The means ± SD of duplicate samples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

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].

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

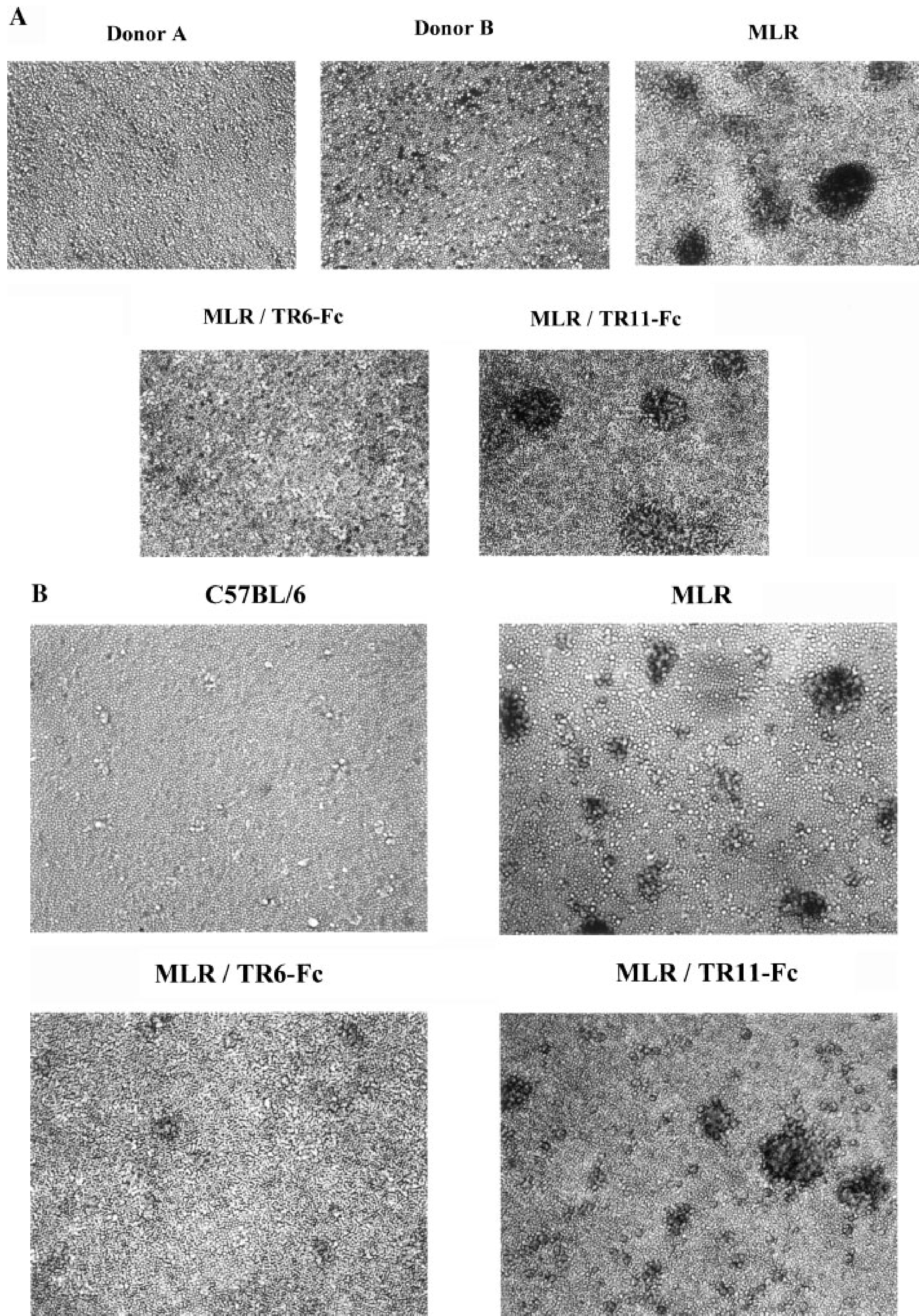

Fig. 2. TR6-Fc inhibits mononuclear cell interaction in the mixed lymphocyte reaction (MLR). Human MLR (**A**) or mouse MLR (**B**) was performed in the presence of TR6-Fc (MLR/TR6-Fc) or a control fusion protein TR11-Fc (MLR/TR11-Fc), both at 10 μg/ml, as indicated. Photos were taken 16 h after culture. 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.

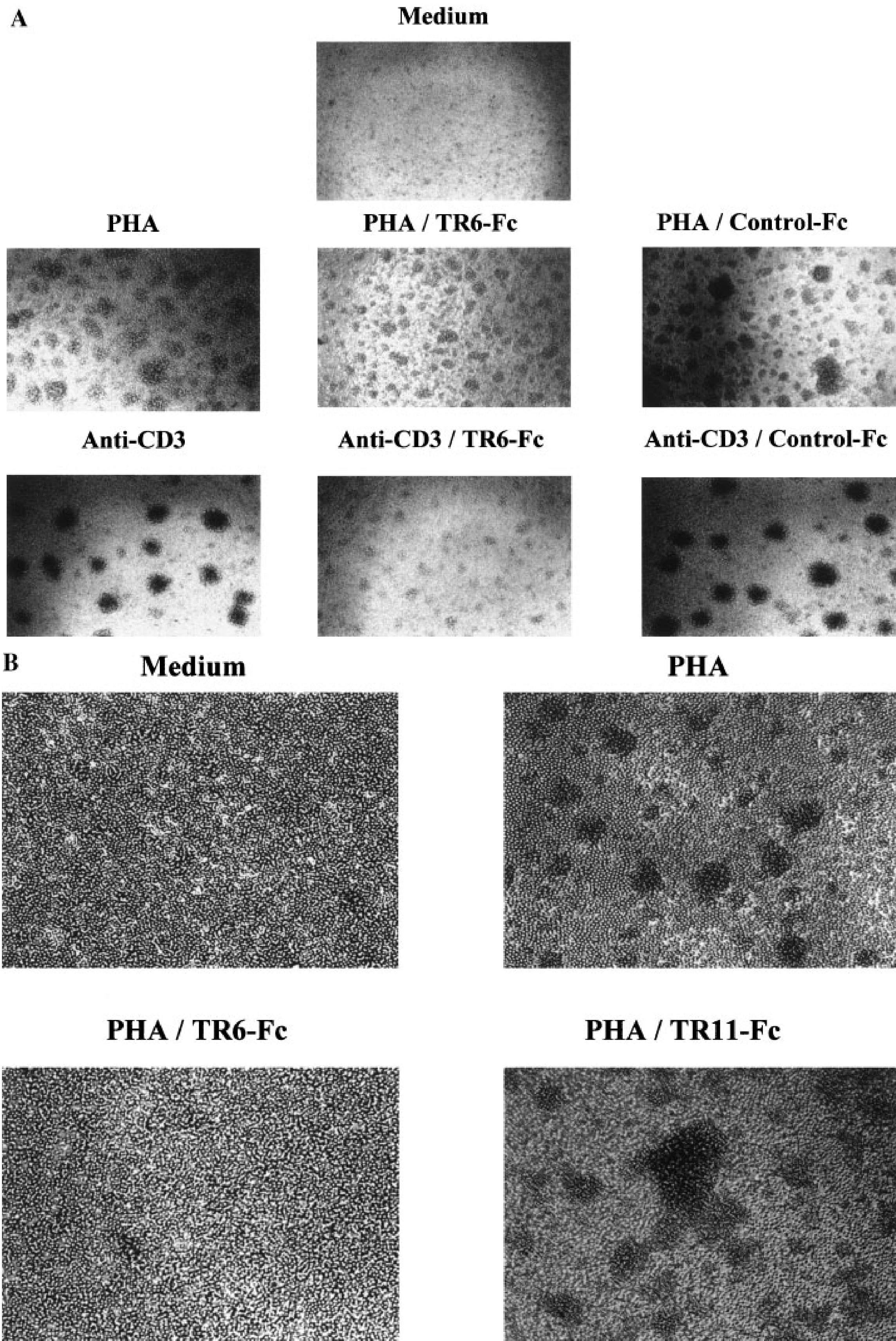

Fig. 3. TR6-Fc inhibits PBMC and T-cell aggregation induced by mitogen stimulation. Human PBMC (**A**) or purified T cells (**B**) were cultured in 96-well flat-bottomed plates at 4×10^5 cells/200 µl/well. PHA (0.2 µg/ml), anti-CD3 Ab (0.5 µg/ml), TR6-Fc (10 µg/ml), and TR11-Fc (10 µg/ml) were present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

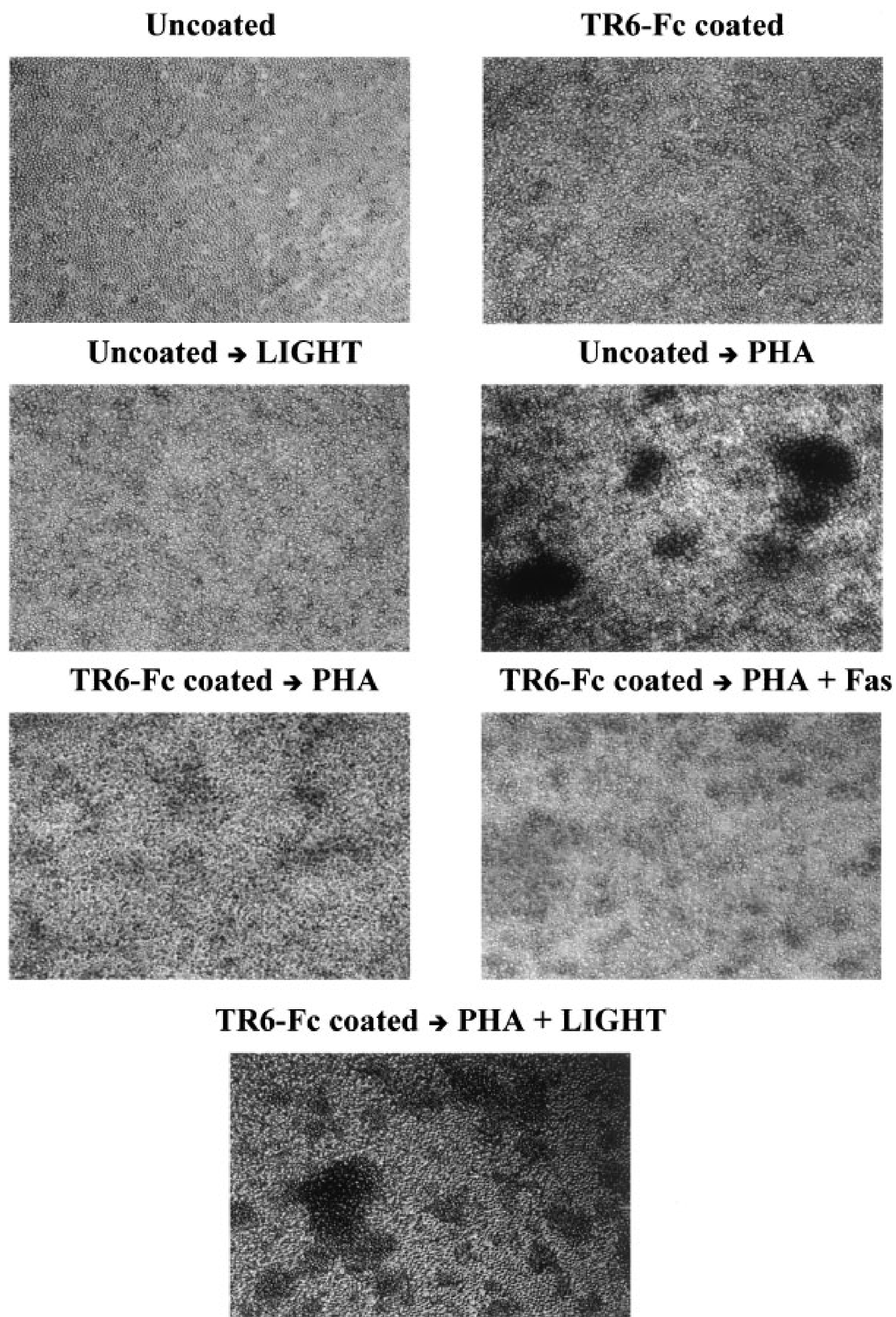

Fig. 4. Effect of solid phase TR6-Fc, soluble Fas and soluble LIGHT on T-cell aggregation. To prepare solid phase TR6-Fc, NUNC 96-well-plates were coated overnight at 4°C with 5 μg/ml goat anti-human IgG (Southern Biotechnology, Birmingham, AL) in PBS at 50 μl/well. After washing, the plates were incubated 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.

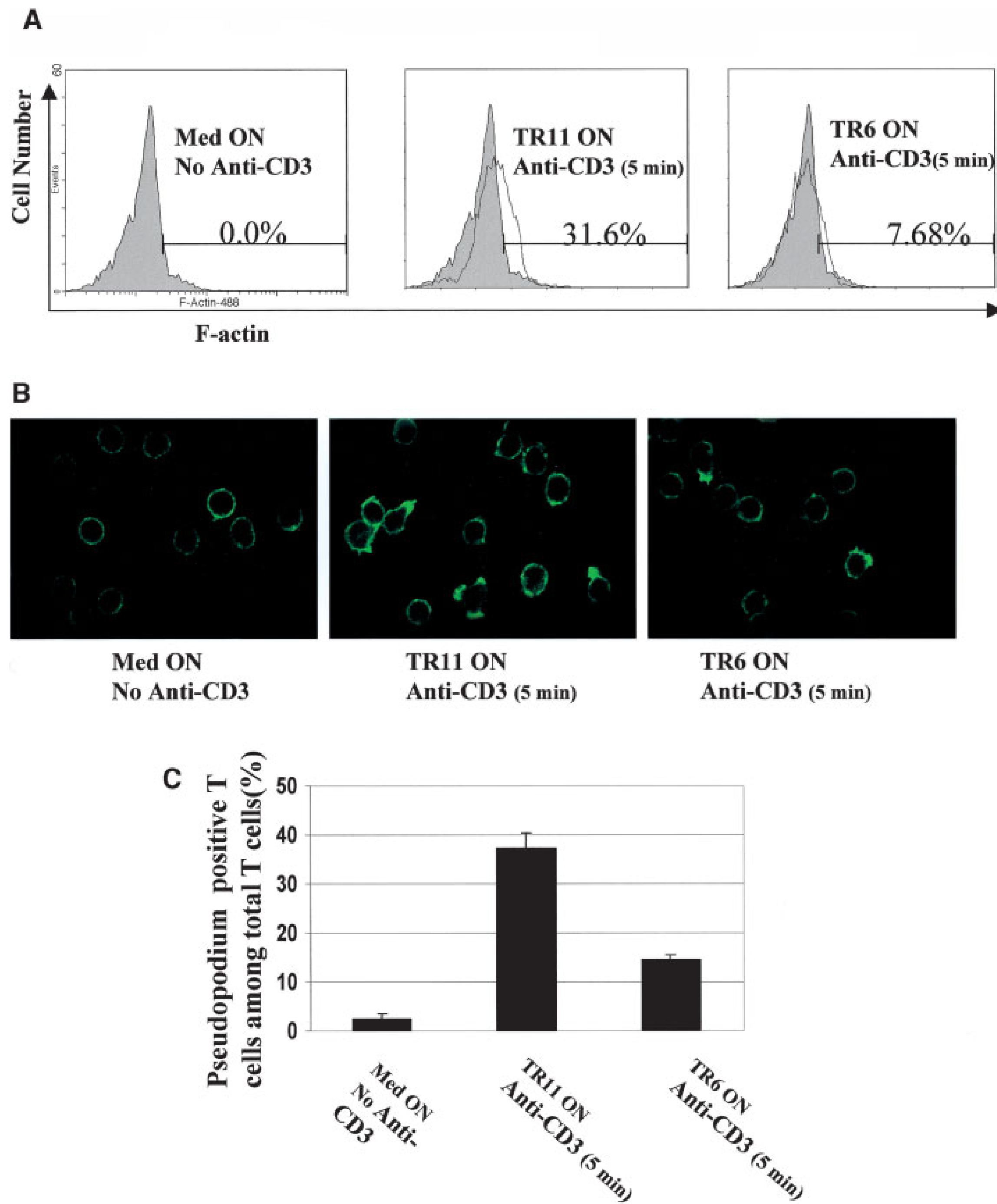

Fig. 5. TR6-Fc inhibits anti-CD3-induced T-cell actin polymerization and pseudopodium formation. T cells were cultured overnight in medium in the absence (Med ON), or presence of TR11-Fc (TR11, ON; 10 µg/ml) or TR6-Fc (TR6, ON; 10 µg/ml). After washing, the cells were crosslinked with anti-CD3 for 5 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 pretreatments 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.

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