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Jeremy A. Harrop,* Manjula Reddy,* Kimberly Dede,* Michael Brigham-Burke,† Sally Lyn,† Kong B. Tan,§ Carol Silverman,¶ Christopher Eichman,* Rocco DiPrinzio,* Jay Spamanato,* Terence Porter,¶ Stephen Holmes,*‖ Peter R. Young,‡ and Alemseg Truneh*

TR2 (TNFR-related 2) is a recently identified member of the TNFR family with homology to TNFRII. We have demonstrated previously that TR2 mRNA is expressed in resting and activated human T cells and that TR2-Ig partially inhibits an allogeneic mixed leukocyte proliferation response. We now characterize TR2 further by the use of specific mAbs. Flow-cytometry analysis using TR2 mAbs confirmed that resting PBL express high levels of cell surface TR2, and that TR2 is widely expressed on all freshly isolated lymphocyte subpopulations. However, stimulation of purified T cells with either PHA or PHA plus PMA resulted in decreased surface expression within 48 h of activation before returning to resting levels at 72 h. TR2 mAbs inhibited CD4+ T cell proliferation in response to stimulation by immobilized CD3 or CD3 plus CD28 mAbs. Assay of culture supernatants by ELISA showed inhibition of TNFα, IFN-γ, IL-2, and IL-4 production, which, for IL-2 and TNFα was also confirmed by intracellular cytokine staining. Furthermore, expression of activation markers on CD4+ T cells, including CD25, CD30, CD69, CD71, and CD28 (CD134), was inhibited. TR2 mAbs inhibited proliferation in a three-way MLR, and a response to soluble recall Ag, tetanus toxoid. In conclusion, these results suggest that TR2 is involved in the activation cascade of T cell responses and TR2 mAbs prevent optimal T cell proliferation, cytokine production, and expression of activation markers. *The Journal of Immunology, 1998, 161: 1786–1794.

The rapidly expanding nerve growth factor receptor/TNF superfamily now contains more than 13 members, including TNFR1 (1, 2), TNFR2 (3), CD27 (4), CD30 (5), CD40 (6, 7), 4-1BB (CD134) (8), CD19 (9), Fas (10), and nerve growth factor receptor (11), lymphotoxin-(6, 7), 4-1BB (CDw137) (8), OX40 (9), Fas (10), nerve growth factor receptor-associated factor; TT, tetanus toxoid.

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2 Abbreviations used in this paper: OPG, osteoprotegerin; HVEM, herpesvirus entry mediator; TR2, tumor necrosis factor receptor-related 2; TRAF, tumor necrosis factor receptor-associated factor; TT, tetanus toxoid.

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TNF-α secretion and cell surface receptor expression, indicating that TR2 is involved in the control of optimal T lymphocyte activation.

Materials and Methods

Antibodies

Directly conjugated Abs CD27, CD30, CD69, CD71, CD40L (CD154) (PharMingen, San Diego, CA) OX40 (Becton Dickinson Immunocytometry Systems, San Jose, CA), CD54, CD58, and CD11a (Immunotech, Westbrook, ME) were obtained commercially. Control D12 mAb to αβ, was supplied by Dr. Z. Jonak (SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

Production of rTR2-Ig fusion protein

PCR primers were designed to clone the region of the TR2 cDNA encoding the extracellular domain with a 5′ EcoRI and BglII site, and a 3′ factor Xa protease and Asp718 site (5′-cag gaa ttc gca gcc atg gag cct cct gga gac gcg gc-3′ and 5′-cag gaa ttc gca gcc atg gag cct cct gga gac gcg gc-3′). The PCR product was digested with EcoRI and Asp718 and ligated into the COSFclink plasmid (33) to produce TR2Fclink. This vector encodes amino acids 1–192 of TR2, followed by the amino acids RSIEGRGT and 5′-cca tac cca ggt acc cct tcc ctc gat aga tct tgc ctt cgt cac cag cca gc-3′. Surface plasmon resonance (BIAcore, Uppsala, Sweden) was conducted to assess binding of TR2 to the BIAcore sensor chip followed by binding of TR2 mAbs as described in Materials and Methods. Affinity values are expressed as nanomolar concentrations.

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HEPES buffer, 2 mM l-glutamine, and 50 μg/ml gentamicin in the presence of 1/800 dilution of TT (Massachusetts Public Health Biologic Laboratory, Jamaica Plain, MA) in 96-well round-bottom microtiter plates. Proliferation was monitored after 5 days following the addition of 1 μCi of [3H]thymidine for the last 6 h of culture, as described previously.

**Results**

**Affinities of TR2 mAbs**

Three murine mAbs to TR2 were generated and their affinities were measured by surface plasmon resonance on a BIAcore instrument. The affinity and kinetic data for 12C5, 18D4, and 20D4 mAbs are as follows:

- **12C5**: High affinity, rapid association, and slow dissociation.
- **18D4**: Moderate affinity, moderate association, and slow dissociation.
- **20D4**: Low affinity, slow association, and fast dissociation.

**FIGURE 1.** Expression of cell surface TR2 on PBMC subpopulations. PBMCs were prepared as described previously, and were stained using biotinylated TR2 mAb 20D4 in combination with mAbs to different CD markers. Specificity of 20D4 binding was determined by preincubating cells with a 10-fold excess (block) of nonbiotinylated 20D4 mAb before the addition of biotinylated 20D4 mAb. Samples were analyzed on a BD FACSsort using CellQuest software. Quadrants were set using isotype controls, and percentage of positive cells is indicated in each quadrant. Data represent one of three experiments.

**FIGURE 2.** Kinetic expression of TR2 on purified CD4+ T cells following activation with PHA or PHA plus PMA. Purified CD4+ T cells were stimulated with PHA (top panel) or PHA plus PMA (bottom panel) for 72 h. Cells were surface stained using TR2 mAb 12C5, followed by goat anti-mouse FITC-conjugated Ab. Data were analyzed on a BD FACSsort and using CellQuest software. Data presented are an example of two similar experiments.

**FIGURE 3.** Effect of TR2 mAbs on CD3-stimulated CD4+ T lymphocyte proliferation. Purified primary CD4+ T lymphocytes were stimulated in 96-well flat-bottom plates precoated with 5 μg/ml of CD3 mAb in the presence of either TR2 (●) or control mAb (▼) for 72 h. Proliferation was monitored by [3H]thymidine incorporation during the last 6 h of culture, and cells were harvested for β-scintillation counting. Data represent one of three similar experiments.
are shown in Table I. Affinities for 12C5, 18D4, and 20D4 were 47, 152, and 1.7 nM, respectively, which is within the affinity range seen for mAbs to other members of this family. TR2 mAbs showed no binding to other members of this superfamily, such as OPG-Ig and DR3-Ig, by surface plasmon resonance. Blocking experiments using these mAbs indicated that 12C5 and 18D4 bound to overlapping epitopes on the TR2 receptor, whereas 20D4 bound to an epitope distinct from 12C5 and 18D4 (data not shown). In addition, the specificity of TR2 mAbs for TR2 and other closely related receptors was tested by ELISA. TR2 mAbs showed no binding to soluble TNFR 1, soluble TNFR 2, CD40-Ig, OPG-Ig, or DR3-Ig (data not shown).

Cell surface expression of TR2 on cell lines and primary T lymphocytes

Previously, we demonstrated that hemopoietic derived cell lines express mRNA for TR2 and that, following activation, TR2 mRNA levels were maintained (29). T and B lymphocyte and monocyte/macrophage-derived cell lines positive for TR2 mRNA were used to demonstrate whether mAbs generated to TR2 were capable of recognizing native cell surface-expressed receptor. Using TR2 mAb 12C5, cell surface TR2 was detected on Jurkat (T cell), U937, THP-1 (myeloid), and Raji cells (B cell), but not MG63 cells (osteosarcoma line), consistent with the mRNA expression data (Table II). Similarly, freshly isolated peripheral blood CD4+ T cells also expressed high levels of surface TR2 and TR2 mRNA (29).

PBMC subpopulations were examined for TR2 expression by two-color flow cytometry using TR2 mAb 20D4 in combination with mAbs to different cell surface markers (Fig. 1). TR2 was expressed on all lymphoid subpopulations, including CD3+ (T), CD4+ and CD8+ (T subpopulations), CD19+ (B cells), and CD57+ (NK cells). This result was expected, as we have shown previously that purified peripheral lymphocyte subpopulations express high levels of TR2 mRNA (29).

FIGURE 4. Effect of TR2 mAb on CD3- and CD28-stimulated CD4+ T lymphocyte proliferation. Purified primary CD4+ T lymphocytes were stimulated in 96-well flat-bottom plates precoated with 5 µg/ml of CD3 mAb in the presence of CD28 mAb with either TR2 or control mAb for 72 h. Proliferation was monitored by [3H]thymidine incorporation during the last 6 h of culture, and cells were harvested for β-scintillation counting. Data represent one of three similar experiments.

FIGURE 5. Inhibition of CD4+ T lymphocyte IL-2 and TNF-α production by TR2 mAb. Purified primary CD4+ T lymphocytes were stimulated with CD3 and CD28 mAbs in the presence of 25 µg/ml of TR2 mAb 18D4 or control D12 mAb for 14 h with brefeldin A. Conjugated isotype control mAbs were used as controls. Blocks were used to determine the specificity of anticytokine mAbs by preincubating anti-cytokine mAbs with a 10-fold molar excess of human rIL-2 or human rTNF-α before the addition to permeabilized cells. Cells were assessed for intracellular cytokine production, as described in Materials and Methods. Similar results were obtained in a second experiment.
Next we examined the kinetics of TR2 expression on primary CD4$^+$ T lymphocytes following activation. Resting or activated CD4$^+$ T cells were monitored for TR2 expression using 12C5 over a 72-h period (Fig. 2). High levels of TR2 were detected on resting lymphocytes, but after 24 h of PHA stimulation, surface expression of TR2 declined. This was maintained for 72 h, after which TR2 expression increased. A similar pattern was observed following combined PHA and PMA stimulation. Similar data were obtained using TR2 mAbs 12C5 and 18D4 (unpublished data).

Inhibition of CD4$^+$ T lymphocyte proliferation

Since TR2 appeared to be expressed on both resting and activated CD4$^+$ T lymphocytes, this suggested that TR2 may be involved in T cell growth and differentiation, similar to other members of this family, such as CD27, OX40, and 4-1BB. Purified CD4$^+$ T lymphocytes were stimulated with immobilized anti-CD3 and anti-CD28 mAbs in the presence of 25 μg/ml of either mAb 18D4 (●) or control mAb D12 (○). Culture supernatant was removed and frozen at −20°C and assayed using ELISA kits for individual cytokines (R&D Systems).

Inhibition of CD4$^+$ T lymphocyte cytokine production

Since T cell proliferation appeared to be regulated in part by TR2, we determined the effect of TR2 on cytokine production. CD4$^+$ T lymphocyte cytokine production was examined by intracellular cytokine staining using flow cytometry and by ELISA from cell culture supernatants. CD3 and CD28 mAb-stimulated CD4$^+$ T lymphocytes were cultured for 14 h with either 25 μg/ml TR2 mAb 18D4 or control mAb D12 in the presence of the Golgi transport inhibitor, brefeldin A. As shown in Figure 5, TR2 mAbs completely inhibited both TNF-α and IL-2 intracellular production.
Rescent units, and are representative of three similar experiments. CD54, CD58, OX40, and CD40L was monitored from 0 to 72 h by flow D12 mAb (grey). Cell surface expression of CD25, CD71, CD69, CD30, CD27 (unpublished data). Furthermore, the adhesion molecule whereas high levels of both cytokines were detected in cells treated with control D12 mAb. The specificity of anticytokine binding was confirmed by preincubating conjugated anti-cytokine mAbs with 10-fold molar excess of recombinant TNF-α or IL-2.

In a separate experiment, 48-h culture supernatants from CD3 and CD28 mAb-stimulated cells were harvested and assayed for cytokine levels by ELISA (Fig. 6). Treatment of stimulated CD4+ T lymphocytes with TR2 mAb inhibited IL-2, TNF-α, IL-4, and IFN-γ secretion in a dose-dependent manner, whereas cells incubated with a control mAb secreted similar levels of cytokines produced by cells stimulated in medium alone. TR2 mAb 18D4 and control mAb D12 were not found to inhibit the detection of cytokines by ELISA, indicating that TR2 mAb did not inhibit detection of cytokine by ELISA (unpublished data).

Cell receptor expression and morphology

Expression of receptors on T cells during stimulation has been shown to modulate the capacity of T cells to respond to activation signals. As TR2 mAbs down-regulated T cell proliferation and cytokine production, we also determined whether they were capable of modulating cell surface receptor expression (Fig. 7). At various times, TR2 mAbs suppressed the expression of the proliferation-associated marker CD71 (transferrin receptor) and CD25 (IL-2Rα), the early activation marker CD69, the costimulatory receptors CD30 and OX40, and the B cell stimulatory CD40 ligand. However, no effect was observed on the surface expression of CD27 (unpublished data). Furthermore, the adhesion molecule CD54 (ICAM-1), but not CD58 (LFA-3) or CD11a (LFA-1; unpublished data), was inhibited by TR2 mAb. This was reflected in the reduced size of lymphocyte aggregates in cultures stimulated in the presence of TR2 mAb (Fig. 8) compared with the larger cell aggregates seen in cultures treated with control mAb. Together these data indicate that TR2 is involved in controlling receptor expression on activated CD4+ T cells.

Inhibition of Ag-specific proliferative responses to insoluble and soluble Ags

Having established that TR2 mAbs were capable of inhibiting T cell mitogenesis, cytokines, and surface receptors, we investigated the effect of TR2 mAbs on Ag-specific allogeneic responses in a three-way MLR. TR2 mAb 12C5 inhibited proliferation in a dose-dependent manner from 1.5 to 100 μg/ml, whereas 18D4 only blocked proliferation at the highest concentrations (Fig. 9). In contrast, CD4 mAb inhibited proliferation at all concentrations tested, with maximal inhibition of 94% at 1 μg/ml. Control IL-5 mAb failed to affect MLR proliferation at all concentrations tested.

The effect of TR2 mAbs on Ag-specific proliferative responses to soluble Ag was also determined. Memory recall proliferation responses to TT were inhibited by TR2 mAb 18D4 from 25 to 50 μg/ml, whereas proliferation in the presence of control mAb had no effect (Fig. 10). This indicates that TR2 is involved in optimal Ag-specific responses to both insoluble and soluble Ags, and is involved in memory T cell responses to TT.

Discussion

In the present study, we have further characterized the function of TR2/HVEM using specific mAbs and confirmed its role in T cell biology. We have shown that TR2 mAbs abrogate T cells function by 1) inhibiting cytokine production, 2) inhibiting cytokine receptor expression, 3) decreasing the expression of costimulatory ligands and receptors, and 4) inhibiting the expression of adhesion molecules. This suggests that TR2 signaling is upstream of these events and is involved in the early stages of T cell activation and differentiation.

We first examined the distribution of TR2 on PBL. TR2 is expressed on T cells as well as on the majority of B lymphocytes and NK cells, which is consistent with the distribution of TR2 mRNA (29). This indicates that TR2 is widely expressed on resting PBL and is not restricted to any subpopulations, a distribution that is unique to this superfamily. The wide distribution of TR2 has been confirmed using the recently identified ligand for TR2 (61) (Harrop et al., submitted) in FACS-binding experiments that showed a similar binding pattern to TR2 mAbs (J.A.H., manuscript in preparation). TR2 was also detected on the majority of naive (CD45RA+) and memory (CD45RO+) lymphocytes (data not shown). Activation of CD4+ T lymphocytes with PHA or PHA plus PMA resulted in a temporary reduction in surface TR2 expression. Northern blot data indicated that TR2 mRNA increased slightly following activation with PHA and PMA, suggesting that TR2 expression is not controlled at the mRNA level (29).

Inducible and constitutive expression of TNFR superfamily members has been reported on primary T lymphocytes. Detectable expression of TNFRII (35), CD30 (36), 4-1BB, CD95, and OX40 (37–39) is generally observed after activation of T lymphocytes. However, CD27 is expressed constitutively on resting CD45RO+ memory T cells, but increases after activation (40). Cleavage of CD27 from CD45RO+ memory T cells occurs following repeated stimulation, and has been used as a marker of chronically activated memory cells (41, 42). Interaction of CD27 with its ligand (CD70) results in the down-regulation of cell surface CD27 and an increase in soluble CD27 (43).
Similar mechanisms may be involved in the down-regulation of TR2 on activated T lymphocytes, as TR2-ligand is expressed on activated T cells and soluble TR2 has been demonstrated in patient samples (J.A.H., unpublished observation).

Members of the TNFR superfamily have been shown to be involved in activation and differentiation of the immune system, including the regulation of proliferation, cytokine production, receptor expression, and cell survival. TR2 mAbs inhibited both suboptimal and optimal CD4⁺ T lymphocyte proliferation in response to CD3 mAb alone or CD3 plus CD28 mAb, respectively. Inhibition of proliferation during CD3 plus CD28 stimulation also resulted in abrogation of IL-2, IL-4, TNF-α, and IFN-γ production. Since IL-2Rα expression was also inhibited by TR2 mAbs, this suggests that decreased proliferation could in part be attributed to inhibition of the IL-2 pathway. Furthermore, the inhibition of TNF-α and IFN-γ suggests that TR2 contributes to proinflammatory cytokine production by T lymphocytes. Abrogation of IL-2 and TNF-α production was also seen at the single cell level using intracellular cytokine staining, indicating that reduced cytokine production was not due to reduced cell numbers as a result of decreased proliferation following TR2 mAb treatment.

Interestingly, other members of the TNFR superfamily were down-regulated by TR2 mAb treatment, including CD30 at 72-h and OX40 at 24-h stimulation, both of which have been shown to be costimulatory molecules for T cell activation (44, 45), and to provide help for B cell activation and differentiation (46, 36). This indicates that TR2 is upstream of CD30 and OX40 in the T cell activation cascade.

Selective regulation of adhesion molecules was also observed with reduced surface expression of CD54 (ICAM-1), but not its ligand CD11a (LFA-1) (J.A.H., unpublished data) or CD58 (LFA-3). Inhibition of LFA-1/ICAM-1 function has been shown previously to directly inhibit lymphocyte aggregation (47, 48), and can result in suboptimal responses as a result of reduced cell to cell conjugation (49). Consistent with this, homotypic aggregates of activated T lymphocyte blasts were smaller in cultures incubated with 18D4 compared with control mAb, which may result from a combination of reduced adhesion receptor expression such as ICAM-1 expression and decreased proliferation seen following TR2 mAb treatment.
FIGURE 9. Inhibition of three-way MLR proliferation responses by TR2 mAbs. The capacity of TR2 mAbs, 12C5 (●) and 18D4 (■), to inhibit allogeneic proliferation was examined in a three-way MLR. Anti-CD4 mAb (▲) and 2B6 anti-human IL-5 mAb (○) were included as controls. PBMCs from three donors were incubated for 6 days with mAbs, cells were labeled with [3H]thymidine for the last 6 h of culture, and incorporation was assessed using a β-scintillation counter. Experiment represents one of three similar assays.

Ag-specific immune responses were also inhibited by TR2 mAbs, including MLR proliferation and TT recall responses, suggesting that TR2 is involved in Ag-specific responses to both insoluble and soluble Ags. TR2 mAbs may also affect non-T cells. Expression of TR2 mRNA has been demonstrated in cells with Ag-presenting function such as monocytes/macrophages and B lymphocytes (29). Hence, TR2 mAbs may be inhibiting Ag-specific T cell proliferative responses by disrupting APC functions, such as costimulatory molecule expression (CD40, CD80, CD86, HLA-DR) and/or cytokine production. The effect of TR2 mAbs on monocyte and B cell function needs to be addressed to determine whether signals delivered by TR2 ligand(s) are involved in APC function.

T cell stimulation has previously been shown to involve other members of the TNFR superfamily. Blockade of 4-1BB/4-1BBL inhibited murine splenocyte responses to soluble CD3 and allogeneic proliferation, whereas 4-1BB mAbs or cells transfected with 4-1BBL expression cDNA have also been reported (26, 15, 18, 19). The cytoplasmic region of TR2 has a 9-amino-acid region previously found to be crucial in TRAF binding to the cytoplasmic domains of TNFRII, CD30, and CD40 (60). Overexpression of TR2 in HEK 293 cells stimulates TRAF 1, 2, 3, and 5 binding, activation of NF-κB, Jun N-terminal kinase, and AP-1 (31, 32). NF-κB and AP-1 signaling stimulates cellular activation, whereas we show in this study that TR2 mAbs inhibit T cell activation, indicating that TR2 mAbs block the interaction of TR2 with its ligand(s) and prevent activation of T cells. Alternatively, TR2 may exist as a heteromeric receptor with another member of the superfamily, leading to the transduction of signals different from TR2 homotrimers. The mechanism by which TR2 mAbs block T cell activation and their capacity to stimulate NF-κB signaling are currently under investigation in our laboratory. Further characterization of the interaction between LIGHT/ HVEM-L and TR2 will help to further define the importance of this receptor in T lymphocyte activation and differentiation.

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