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Brief Definitive Report

TRANCE (Tumor Necrosis Factor [TNF]-related Activation-induced Cytokine), a New TNF Family Member Predominantly Expressed in T cells, Is a Dendritic Cell–specific Survival Factor

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Summary

TRANCE (tumor necrosis factor [TNF]–related activation-induced cytokine) is a new member of the TNF family that is induced upon T cell receptor engagement and activates c-Jun N-terminal kinase (JNK) after interaction with its putative receptor (TRANCE-R). In addition, TRANCE expression is restricted to lymphoid organs and T cells. Here, we show that high levels of TRANCE-R are detected on mature dendritic cells (DCs) but not on freshly isolated B cells, T cells, or macrophages. Signaling by TRANCE-R appears to be dependent on TNF receptor–associated factor 2 (TRAF2), since JNK induction is impaired in cells from transgenic mice overexpressing a dominant negative TRAF2 protein. TRANCE inhibits apoptosis of mouse bone marrow–derived DCs and human monocyte-derived DCs in vitro. The resulting increase in DC survival is accompanied by a proportional increase in DC-mediated T cell proliferation in a mixed leukocyte reaction. TRANCE upregulates Bcl-x_L expression, suggesting a potential mechanism for enhanced DC survival. TRANCE does not induce the proliferation of or increase the survival of T or B cells. Therefore, TRANCE is a new DC-restricted survival factor that mediates T cell–DC communication and may provide a tool to selectively enhance DC activity.

Apoptosis plays a critical role in the development and maintenance of the immune system (1–3). Members of the TNF family can regulate apoptosis in addition to an array of other biological effects such as cell proliferation and differentiation (4). Despite the functional redundancy of this family, specificity may be accomplished by coordinating the spatial and temporal expression of TNF-related ligands and their receptors and by restricting the expression of signal transduction molecules to specific cell types. TNF receptors interact with a family of molecules called TRAFs (TNF receptor–associated factors) that act as adaptors for downstream signaling events (5). For example, TRAF2 activates NF-κB (6) and also c-Jun NH_2-terminal kinase (JNK; references 7–9). The biochemical events leading to apoptosis involve the caspase family of cysteine proteases (10), whereas NF-κB appears to inhibit cell death (11). The TNF receptor family can also regulate apoptosis by modulating the expression of Bcl-2 and Bcl-2–related proteins (12, 13). Recent data indicates that the Bcl-2 family controls apoptosis by altering transmembrane conductance in mitochondria and by preventing the activation of caspases (14–16).

An important role of TNF members in dendritic cell (DC) biology has recently emerged. DCs have several specializations that lead to the stimulation of naive T cells and play a role in the initiation of the immune response (17). TNF-α and CD40 ligand (L) are molecules involved in the differentiation of DC from $CD34^+$ bone marrow or cord blood progenitors (18–20). Moreover, CD40L increases DC survival, upregulates MHC and costimulatory molecule expression, and induces the expression of a variety of cytokines (e.g., IL-12) in DCs (21). Both CD40 and TNFR interact with TRAF2, suggesting that TRAF2 plays a role in DC function.

Recently, TRANCE (TNF-related activation-induced cytokine), a novel ligand of the TNF family, was cloned during a search for apoptosis regulatory genes (22). Remarkably, TRANCE expression is restricted to lymphoid-specific organs and is selectively expressed in T cells (22). In this study, we describe that TRANCE-R signals via TRAF2 in thymocytes and increases DC survival by upregulating Bcl-x_L expression, a property shared with CD40L. However, unlike CD40L, TRANCE selectively acts on mature DCs but not on B cells. In addition, high levels of the TRANCE-R are only detected on DCs, suggesting that a major function of TRANCE in vivo is to modulate DC activity.

Brian R. Wong and Régis Josien contributed equally to this report.

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Materials and Methods

Expression and Purification of Soluble TRANCE. A FLAG epitope-tagged TRANCE molecule (FLAG-TRANCE) was expressed in 293T cells and purified as previously described (22). To create a human CD8-TRANCE recombinant molecule (hCD8-TRANCE), the extracellular domain of murine TRANCE (amino acid 245–316) was fused to human CD8α (amino acid 1–182) and produced in a baculovirus expression system according to the manufacturer's instructions (BaculoGold; PharMingen, San Diego, CA). hCD8-TRANCE was purified on cyanogen bromide (CNBR)-activated Sepharose gel conjugated to OKT8 following the manufacturer's protocol (Pharmacia Biotech, Piscataway, NJ). mCD8-CD40L in insect cell culture supernatant was provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Hanover, NH).

Mice. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were from Taconic Farms (Germantown, NY). Transgenic mice expressing a dominant negative form of TRAF2 (TRAF2.DN) were engineered as previously described (23).

Cells. Bone marrow–derived DCs (BMDC) were generated as previously described (24) and were used on day 8 of culture. Enriched populations of fresh lymph node or splenic DCs were prepared by digesting organs with collagenase then selecting for low density cells via centrifugation on a Nycodenz column (14.5% wt/vol in PBS + 5 mM EDTA; Nycomed Pharmaceuticals, Oslo, Norway) for 15 min at 4°C. Mature spleen DCs were prepared by culturing freshly isolated spleen DCs overnight as previously described (25). The cytokine-induced generation of human DCs from PBMCs was performed as previously described (26). After 2 d in monocyte-conditioned medium, TRANCE or PBS was added to the DCs. Lymph node T cells (99% $CD3^+$ as assessed by flow cytometry) were prepared by magnetic bead depletion (Dynal, Oslo, Norway) of class II, B220, NK1.1, and F4/80 positive cells. B cells were prepared by magnetic depletion of Thy1.2 positive cells (Dynal). Cell viability was assayed by trypan blue exclusion or by propidium iodide uptake.

Flow Cytometry. DC phenotype was assessed by flow cytometry as previously described (27) using the following FITC- or PE-conjugated mAbs: H-$2K^b$, I-A^b, intracellular adhesion molecule (ICAM)-1, CD11b, CD11c, CD80, CD86, CD25, and CD40 (all from PharMingen). Other mAbs used were biotinylated α-Fas, CD3-FITC, B220-FITC (PharMingen), and NLDC-145–FITC. The expression of TRANCE-R was assessed using the hCD8-TRANCE fusion molecule at 10 μg/ml at 4°C followed by biotinylated OKT8 mAb and then streptavidin-PE (BioSource International, Camarillo, CA). Negative controls were performed by omitting hCD8-TRANCE. For analysis of TRANCE-R expression on resting B cells and fresh DCs, low density cells were stained with FITC-B220 or FITC-CD11c, respectively, and analyzed on a FACScan® (Becton Dickinson, Mountain View, CA).

Mixed Leukocyte Reaction. BMDCs treated for 48 h in the presence or absence of recombinant TRANCE were cultured with 10^5 purified allogeneic T cells in flat-bottomed 96-well plates in a final volume of 200 μl for 3 d and then pulsed for 8 h with 0.5 μCi of [^{3}H]thymidine (Dupont-NEN, Boston, MA). The cells were then harvested on glass fiber filters and [^{3}H]thymidine incorporation was measured using a standard scintillation-detection procedure.

JNK Assays. 2–5 × 10^6 cells from TRAF2.DN transgenic mice or from control littermates were incubated for 1–2 h at 37°C on plates coated with OKT8 antibody (10 μg/ml). The cells were treated with either soluble TRANCE or an equal volume of PBS before being harvested at the indicated time points and frozen in a dry ice/ethanol bath. JNK was immunoprecipitated with α-JNK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and kinase activity was assessed as previously described (22).

Western Blot and Reverse Transcriptase–PCR Analysis of Bcl-x_L and Bcl-2. BMDCs (8 × 10^6/well) were cultured in RPMI in 6-well plates and treated with PBS, FLAG-TRANCE (1 μg/ml), or soluble CD40L for 0 or 24 h. The cells were lysed, and 50 μg of protein from each sample was resolved on a 12% SDS-PAGE gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The blots were blocked in 5% skim milk, probed with α-Bcl-2 (4C11) or α-Bcl-x_L (236; both provided by Dr. Gabriel Núñez, University of Michigan, Ann Arbor, MI) and detected with the appropriate horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminesence substrate (ECL; Amersham Corp., Arlington Heights, IL). For reverse transcriptase–PCR analysis of *bcl-x_L* mRNA expression, BMDCs (2 × 10^6 cells/well) were cultured in 24-well plates, treated with the appropriate reagents, and quickly frozen in a dry ice/ethanol bath at the various time points. Total RNA was extracted (RNEasy; QIAGEN Inc., Chatsworth, CA), and cDNA was diluted to allow PCR amplification to occur as a linear function of starting concentrations. PCR was performed using the conditions and primers as previously described (13).

Results and Discussion

TRANCE-R Is Expressed at High Levels in DCs. To identify cells that express TRANCE-R, hCD8-TRANCE was used as a molecular probe for FACS® analysis. TRANCE-R was detected on mature BMDCs, freshly isolated lymph node DCs, and freshly isolated spleen DCs (Fig. 1). TRANCE-R was greatly upregulated upon the maturation of spleen DCs induced by overnight culture. No expression could be detected on freshly isolated lymph node B cells, lymph node T cells, thymocytes, or peritoneal macrophages. Therefore, the highest levels of TRANCE-R expression are found on mature DCs and suggest that the major role of TRANCE is restricted to DCs.

TRANCE Is a DC Survival Factor. The biological effects of TRANCE were further studied on mature DCs. TRANCE-treated DCs formed densely packed clusters whereas control, untreated cells exhibited relatively sparse aggregates (Fig. 2 *A*). In addition, mature BMDCs treated with FLAG-TRANCE were significantly protected from spontaneous cell death compared to untreated cells. This effect was dependent on the dose of TRANCE (Fig. 2 *B*). hCD8-TRANCE elicited similar results (data not shown). This effect was not due to increased cell proliferation since the total number of cells remained the same over time (data not shown). TRANCE significantly prevented DC cell death until day 6, whereas untreated cells were almost completely dead by day 3 (Fig. 2 *C*). A similar effect on DC survival was observed with human monocyte-derived DC (Fig. 2 *D*). Confirming previous data, CD40L also induced the clustering of DCs (data not shown; 28, 29) and enhanced DC survival comparably to TRANCE (Fig. 2 *C*).

CD40L upregulates the antiapoptotic molecule, Bcl-x_L, in B cells and protects them from Ig receptor–mediated cell death (13). In addition, CD40L upregulates Bcl-2 in hu-

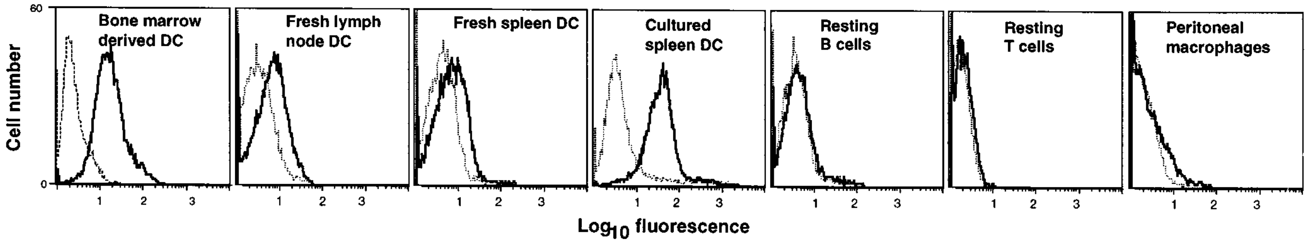

Figure 1. TRANCE-R expression in various cell types. Cells were prepared as described in the Materials and Methods section and stained with 10 μg/ml of the hCD8-TRANCE recombinant protein (*solid lines*) or with secondary reagents alone (*dotted line*). Only viable cells as determined by propidium iodide (PI) exclusion were gated and analyzed for TRANCE-R expression. Fresh DCs were analyzed by two-color staining after gating on CD11c^{high} cells. Each staining was reproduced at least twice.

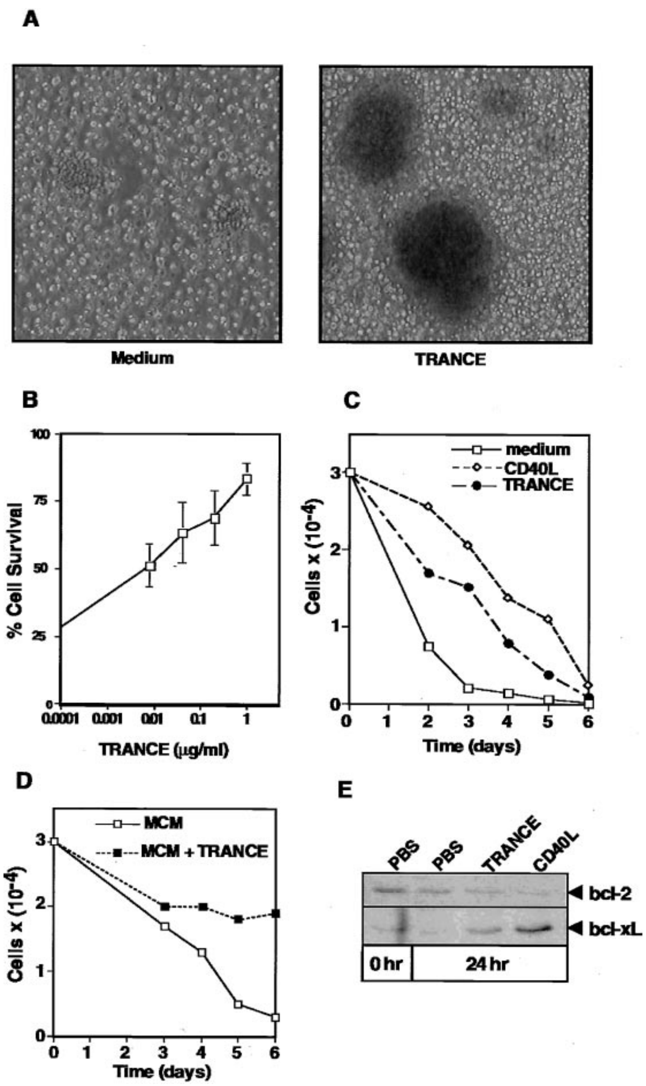

Figure 2. TRANCE is a DC survival factor that upregulates Bcl-x_L. (*A*) BMDCs were cultured in complete medium in the presence or absence of recombinant TRANCE (1 μg/ml) for 48 h and were then visualized under an inverted light microscope. (*B*) Duplicate wells containing 3×10^4 BMDCs were cultured with increasing doses of recombinant TRANCE in complete medium in flat-bottomed 96-well plates. The percentage of cell survival was assessed 48 h later by trypan blue exclusion. The average of three experiments, and the SEMs, are shown. (*C*) 3×10^4 BMDCs were cultured in complete medium in the presence or absence of recombinant TRANCE (1 μg/ml) or mCD8-CD40L (1/1,000 of the culture supernatants). Cell viablity was assessed daily by trypan blue exclusion. Representative data of three independent experiments are shown. (*D*) 3×10^4 GM-CSFs and IL-4 stimulated human monocyte-derived DCs were cultured for 2 d in monocyte conditioned medium to generate mature DCs (26). Thereafter, DCs were cultured in the presence or absence of recombinant TRANCE (1 μg/ml) and cell viability was assessed each day by trypan blue exclusion. (*E*) 50 μg of protein extracted from BMDCs that had been cultured for 24 h as described in Fig. 2 *C* were analyzed for Bcl-2 and Bcl-x_L protein expression by Western blot analysis. Basal levels of Bcl-2 and Bcl-x_L were determined in day 8 BMDCs (*0 h*).

man DC derived from CD34$^+$ progenitor cells, a phenomenon that was correlated with a resistance to Fas-mediated apoptosis (12). To determine whether TRANCE can influence Bcl-2 or Bcl-x_L, we measured their expression in DCs stimulated with TRANCE or CD40L by Western blot analysis. BMDCs expressed relatively high levels of Bcl-2 and relatively low levels of Bcl-x_L after reaching maturity in GM-CSF (Fig. 2 *E*, 0 h). FLAG-TRANCE and CD40L stimulation lead to increased Bcl-x_L expression by 24 h. Bcl-x_L expression was nearly absent in cells treated with medium alone. *bcl-x_L* mRNA was upregulated in TRANCE-treated DCs, suggesting a transcriptional as opposed to posttranscriptional regulation (data not shown). In contrast, Bcl-2 levels were decreased in both the TRANCE-treated and untreated cells (Fig. 2 *E*). These results suggest that TRANCE, in addition to CD40L, upregulates Bcl-x_L in DCs, which enhances their viability in vitro.

TRANCE Enhances DC-mediated T Cell Proliferation. To examine the functional consequences of TRANCE on DCs we measured the MLR-stimulating ability of DCs treated with TRANCE. Increasing doses of FLAG-TRANCE enhanced DC survival at 48 h, which in turn led to a proportional increase in the stimulation of T cell proliferation (Fig. 3 *A*). When equivalent numbers of viable TRANCE-treated or untreated DCs were used in an MLR, there were no differences in T cell proliferation, suggesting that changes in the expression of costimulatory and antigen-presenting molecules did not account for the enhanced T cell proliferation (Fig. 3 *B*). To verify this, the levels of several surface markers were tested by FACS® to evaluate any TRANCE-mediated changes to the DC phenotype. There was a slight but reproducible downregulation of MHC class II expression and a slight upregulation of MHC

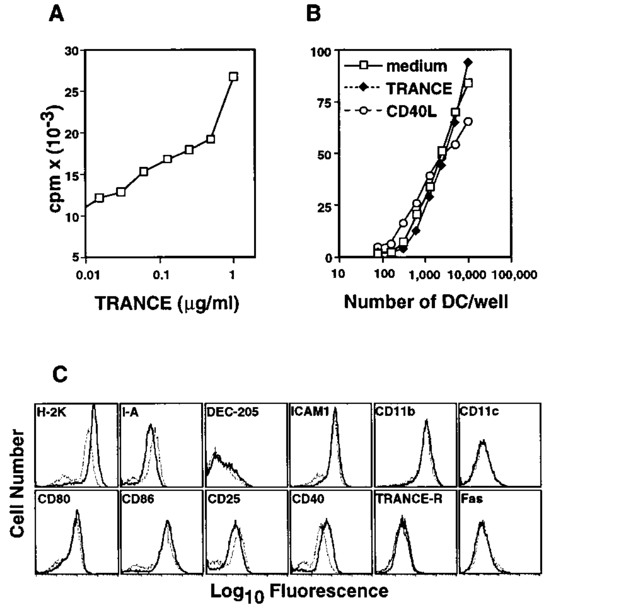

Figure 3. Cell surface marker expression and T cell stimulatory function of TRANCE-treated BMDCs. (*A*) 2.5 × 10^3 BMDCs were cultured with increasing doses of TRANCE in a final volume of 100 µl in triplicate in flat-bottomed 96-well plates. After 48 h, 10^5 purified allogeneic T cells in 100 µl were added in each well and [^{3}H]thymidine incorporation was assessed after 3 d of culture. One experiment out of three is shown. (*B*) 2.5 × 10^4 BMDCs were cultured in the presence or absence of TRANCE or CD40L for 48 h. After washing and counting the cells, dilutions of live cells were cultured with 10^5 purified allogeneic T cells and [^{3}H]thymidine incorporation was assessed after 3 d of culture. (*C*) BMDCs were cultured in complete medium for 24 h in the presence (*solid lines*) or absence (*dotted lines*) of soluble FLAG-TRANCE (1 µg/ml) and analyzed for the indicated surface markers expression by FACS® after gating the live cells. Similar results were obtained after 48 h of culture.

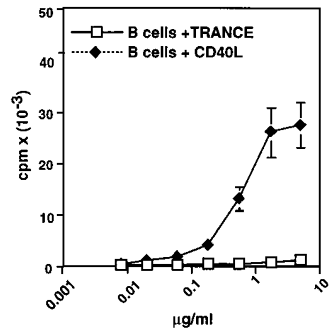

Figure 4. TRANCE does not induce the proliferation of B or T cells. Triplicate wells of 2 × 10^4 purified B cells were cultured in complete medium in the presence of increasing doses of soluble TRANCE or CD40L in flat-bottomed 96-well plates. 10^5 purified T cells were cultured in complete medium containing Con A (2.5 µg/ml) in the presence of increasing doses of soluble TRANCE. [^{3}H]thymidine incorporation was assessed after 2 d of culture.

class I expression (Fig. 3 *C*). There were no TRANCE-mediated perturbations in the expression of the costimulatory molecules CD80 (B7-1) or CD86 (B7-2), and no changes in the expression of the adhesion molecules intracellular adhesion molecule (ICAM)–1, CD11b, and CD11c. Interestingly, CD40 expression increased but Fas and TRANCE-R did not. In sum, TRANCE enhances DC-mediated T cell proliferation by increasing the survival of DCs.

TRANCE Does Not Affect B or T Cell Proliferation. Expression of high levels of the TRANCE-R appeared restricted to DCs by FACS® analysis. However, we found that TRANCE could activate JNK in thymocytes (22), suggesting that FACS® analysis might lack the sensitivity to detect low levels of receptor. To further examine the specificity of TRANCE for DCs, we tested its ability to induce B cell proliferation or survival, two functions mediated by CD40L. Recombinant hCD8-TRANCE, tested for its antiapoptotic function in BMDCs, could not stimulate B cell proliferation (Fig. 4), nor could it activate JNK activation (22). In contrast, CD40L efficiently stimulated B cell proliferation in a dose-dependent manner (Fig. 4). Finally, TRANCE could not prevent the spontaneous apoptosis of B and T cells as assessed by propidium iodide uptake (data not shown). Therefore, functionally, TRANCE appears to exhibit different cellular specificities and functions when compared to CD40L.

TRANCE-mediated JNK Induction Requires Functional TRAF2. Recruitment of TRAF2 to the TNFR complex or the CD40 receptor complex is necessary for JNK activation (7–9, 23). To test the possibility that TRANCE-R also signals via TRAF2, we analyzed TRANCE-mediated JNK activation in thymocytes from transgenic mice overexpressing a dominant negative form of TRAF2 (TRAF2.DN; reference 23). JNK activity peaked 2.5-fold over unstimulated cells at 5 min in control littermates, whereas JNK induction was significantly reduced in TRAF2.DN thymocytes (Fig. 5). These results suggest that signaling from the TRANCE-R requires TRAF2. TRANCE-mediated JNK induction in DCs could not be assayed since TRAF2.DN expression has been restricted to lymphocytes in the TRAF2.DN transgenic mice. In addition, JNK activity was constitutively high in mature DCs (22), which are also known to have high levels of activated NF-κB (30), thus confounding detection of increased JNK activity.

In summary, we have shown that TRANCE, in addition to CD40L, is a regulator of DC function. Similar to CD40L, TRANCE promotes the survival of mature DCs by regulating the expression of Bcl-x_L. However, in contrast to CD40L, TRANCE does not act on other APCs such as B cells. The signal transduction pathways via TRANCE-R in DCs are unknown. TRANCE appears to signal via TRAF2, at least in thymocytes, suggesting that TRAF2

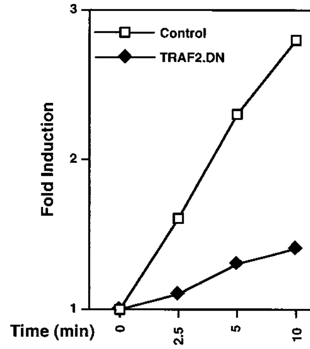

Figure 5. TRANCE-R signaling is dependent on TRAF2. Thymocytes from transgenic mice expressing TRAF2.DN or control littermates were stimulated with hCD8-TRANCE (1 µg/ml) on OKT8-coated (10 µg/ml) plates for the indicated amount of time then assayed for JNK activity. The degree of JNK activation was analyzed on a phosphorimager (Molecular Imager System; Bio-Rad Laboratories, Hercules, CA) and plotted as fold induction over time 0. Representative results of three independent experiments are shown.

may play a critical role in mediating signals for differentiation, activation, and survival in DCs.

These findings complement our previous report describing the selective expression of this new TNF family member in T cells. The high level of expression of TRANCE-R on DCs suggests a specific role for TRANCE in T cell–DC communication during the primary immune response. Rapid upregulation of TRANCE upon TCR engagement on T cells (22) could specifically enhance the survival of DCs during antigen presentation. Both antigen-specific T cells and the antigen-presenting DCs would therefore depend on each other for activation and survival. Mature DCs that fail to present antigen to T cells would not receive T cell help and would therefore die of neglect. This T cell–DC interaction is likely to occur in the T cell area of lymphoid organs that contain DCs of mature phenotypes (31). DCs can only be detected in afferent lymph, not efferent lymph, suggesting that DCs are destined to die when they migrate to the lymph node. TRANCE may be important to maintain DC survival, perhaps acting before CD40L as TRANCE is an immediate early gene (22). Many experiments indicate that DCs pulsed ex vivo with antigen can be used to induce immunity to tumor or viral antigens in vivo (32). TRANCE could provide a tool to specifically enhance DC function by enhancing their survival in vivo. This hypothesis is currently being examined in a variety of viral and tumor models in mice.

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