Chromogranin A Restricts Drug Penetration and Limits the Ability of NGR-TNF to Enhance Chemotherapeutic Efficacy

Eleonora Dondossola, Anna Maria Gasparri, Barbara Colombo, Angelina Sacchi, Flavio Curnis, and Angelo Corti

Abstract

NGR-TNF is a derivative of TNF-α that targets tumor blood vessels and enhances penetration of chemotherapeutic drugs. Because of this property, NGR-TNF is being tested in combination with chemotherapy in various phase II and III clinical trials. Here we report that chromogranin A (CgA), a protein present in variable amounts in the blood of normal subjects and cancer patients, inhibits the synergism of NGR-TNF with doxorubicin and melphalan in mouse models of lymphoma and melanoma. Pathophysiologically relevant levels of circulating CgA blocked NGR-TNF–induced drug penetration by enhancing endothelial barrier function and reducing drug extravasation in tumors. Mechanistic investigations done in endothelial cell monolayers in vitro showed that CgA inhibited phosphorylation of p38 MAP kinase, disassembly of VE-cadherin–dependent adherence junctions, paracellular macromolecule transport, and NGR-TNF–induced drug permeability. In this system, the N-terminal fragment of CgA known as vasostatin-1 also inhibited drug penetration and NGR-TNF synergism. Together, our results suggest that increased levels of circulating CgA and its fragments, as it may occur in certain cancer patients with nonneuroendocrine tumors, may reduce drug delivery to tumor cells particularly as induced by NGR-TNF. Measuring CgA and its fragments may assist the selection of patients that can respond better to NGR-TNF/chemotherapy combinations in clinical trials. Cancer Res; 71(17); 5881–90. ©2011 AACR.

Introduction

The efficacy of chemotherapy in cancer patients is often limited by inadequate and uneven diffusion of drugs in tumor tissues (1). This phenomenon is related to abnormal vasculature and altered composition of tumor tissues, which may cause irregular blood flow, heterogeneous permeability, increased interstitial fluid pressure and, consequently, uneven drug penetration in tumors (2, 3).

We have previously shown that a neovasculature-homing TNF-α derivative, called NGR-TNF, can be exploited to improve drug penetration in neoplastic tissues (3, 4). NGR-TNF is a cytokine–peptide fusion protein, originally developed by our group, consisting of TNF fused to the CNGLRGCG peptide (3). This peptide is a ligand of a CD13 form overexpressed in the tumor neovasculature (5–7). Experiments in mouse models have shown that the administration of ultra-low doses of NGR-TNF (picograms) increases the penetration of doxorubicin, melphalan, cisplatin, gemcitabine, and paclitaxel into solid tumors (4, 8). NGR-TNF can also induce, at later time points, vascular damage and inflammatory/immune responses (4, 8). Phase I and II clinical studies with low-dose NGR-TNF, alone and in combination with chemotherapy, showed manageable toxicity profile and evidence of disease control, particularly in subpopulations of patients with hepatocellular carcinoma and pleural mesothelioma (9–14). A randomized double-blind phase III study of NGR-TNF (in combination with chemotherapy or supportive care) started in 2010 in patients with malignant pleural mesothelioma (http://www.clinicaltrials.gov/clinical-trials/show/NCT01098266). The identification of factors that promote tumor resistance to targeted-TNF/chemotherapy combination could be, therefore, pharmacologically and clinically relevant.

We have previously shown that chromogranin A (CgA), a protein released in circulation by many endocrine and neuroendocrine cells, neurons and granulocytes, is an important modulator of the endothelial barrier function and a potent inhibitor of TNF-induced vascular leakage in the liver (15, 16). Structure–activity studies have shown that a bioactive site is located in the N-terminal region of CgA (15, 17). A fragment of CgA spanning the N-terminal residues 1–78, called vasostatin-1 (VS-1), can inhibit a series of effects exerted by TNF on endothelial cells, including phosphorylation of p38 MAP kinase [p38-MAPK (mitogen-activated protein kinase)], redistribution of VE-cadherin, formation of gaps, paracellular transport of macromolecules (15, 18), VS-1 can also inhibit VEGF- and thrombin-induced endothelial cell permeability (19). Thus, CgA and its N-terminal fragment VS-1 are efficient inhibitors of endothelial cell activation and permeability caused by TNF and by other stimuli.
Increased levels of circulating CgA have been detected in patients with neuroendocrine tumors, in subpopulations of patients with prostate, breast or non–small cell lung cancer (NSCLC), and in patients with heart failure, renal failure, hypertension, rheumatoid arthritis, sepsis, hepatocellular carcinoma, liver cirrhosis, chronic hepatitis, pancreatitis, inflammatory bowel disease, or atrophic gastritis (20–31). Furthermore, increased levels of CgA have been detected also in patients treated with proton pump inhibitors, a class of drugs commonly used to treat acid peptic disorders (32, 33). Thus, CgA levels higher than normal values can occur not only in patients with neuroendocrine tumors or with tumors that may undergo neuroendocrine differentiation but also in patients with nonneuroendocrine tumors for a variety of reasons (30).

Given these premises and considering the protective effects of CgA on TNF-induced alteration of the endothelial barrier function, we have hypothesized that abnormal levels of circulating CgA might inhibit the synergism between NGR-TNF and chemotherapy. To address this hypothesis, we have investigated the effect of CgA and VS-1 on the antitumor activity of NGR-TNF/chemotherapy against murine lymphoma and melanoma, that is, 2 models of nonneuroendocrine tumors. The rationale for this choice relies on the fact that these models have been largely exploited for preclinical studies on NGR-TNF combined with various chemotherapeutic drugs and that this drug combination is currently investigated in patients with nonneuroendocrine tumors (3, 4, 8). We show that both proteins, at levels similar to those found in patients, can markedly reduce the response of tumors to NGR-TNF and chemotherapy. To address this hypothesis, we have investigated the effect of CgA and VS-1 on the antitumor activity of NGR-TNF/chemotherapy against murine lymphoma and melanoma, that is, 2 models of nonneuroendocrine tumors. The rationale for this choice relies on the fact that these models have been largely exploited for preclinical studies on NGR-TNF combined with various chemotherapeutic drugs and that this drug combination is currently investigated in patients with nonneuroendocrine tumors (3, 4, 8).

We show that both proteins, at levels similar to those found in patients, can markedly reduce the response of tumors to NGR-TNF and chemotherapy. To address this hypothesis, we have investigated the effect of CgA and VS-1 on the antitumor activity of NGR-TNF/chemotherapy against murine lymphoma and melanoma, that is, 2 models of nonneuroendocrine tumors. The rationale for this choice relies on the fact that these models have been largely exploited for preclinical studies on NGR-TNF combined with various chemotherapeutic drugs and that this drug combination is currently investigated in patients with nonneuroendocrine tumors (3, 4, 8).

Results

CgA and VS-1 inhibit the synergistic activity of NGR-TNF and chemotherapy in tumor-bearing mice

To investigate the effect of CgA and VS-1 on the synergism between NGR-TNF and chemotherapeutic drugs, we treated RMA lymphoma–bearing mice with CgA or VS-1 (1 or 3 μg, respectively, 3 times in 1 hour), then with NGR-TNF (0.1 ng), and finally with doxorubicin (80 μg). At 2 hours after NGR-TNF: Fig. 1A). The combination of doxorubicin with NGR-TNF, but not doxorubicin alone, delayed tumor growth, as previously shown (4). This effect was completely inhibited by CgA or VS-1 (Fig. 1B). We obtained similar results using a model based on B16-F1 melanoma–bearing mice and melphanal. Also in this case, both CgA and VS-1 inhibited the antitumor effect exerted by NGR-TNF in combination with melphanal (Fig. 1C). These results suggest that CgA can inhibit the synergism between NGR-TNF and chemotherapy and that its N-terminal domain contains a bioactive site.

Notably, CgA and VS-1 reached concentrations of about 5 nmol/L after 1 to 2 hours, that is, at the time at which NGR-TNF is expected to exert its action (Fig. 1D). Notably, these levels of CgA are in the range of those observed in cancer patients with nonneuroendocrine tumors (31, 37).

CgA and VS-1 do not inhibit the cytotoxic activity of doxorubicin and melphanal alone and in combination with NGR-TNF

The mechanism of action of CgA and VS-1 was then investigated. Neither CgA nor VS-1 affected the cytotoxic activity of doxorubicin and melphanal, alone and in combination with NGR-TNF, against human umbilical vein endothelial cells (HUVEC) and RMA cells (Fig. 2). These results suggest that the inhibition of the antitumor activity of
Figure 1. CgA and VS-1 inhibit the synergistic antitumor effects between NGR-TNF and chemotherapeutic drugs. 
A, tumor-bearing mice were treated, i.p., with CgA (1 or 0.2 µg) or VS-1 (3 µg), NGR-TNF (0.1 ng) and doxorubicin (80 µg) or melphalan (90 µg) at the indicated time. B and C, effect of CgA or VS-1 on the synergistic activity of NGR-TNF and chemotherapy on tumor growth (n = 5 mice per experiment, mean ± SE). D, serum levels of human CgA and VS-1 as measured by ELISA (mean ± SE, n = 5 mice per experiment). **, P < 0.01; ***, P < 0.001 by t test (2-tailed).
NGR-TNF/chemotherapy by CgA or VS-1 was not related to inhibition of their cytotoxic activity against endothelial or tumor cells. More likely, it was related to a decreased drug delivery to tumor cells.

**CgA and VS-1 inhibit the penetration of doxorubicin in RMA tumors induced by NGR-TNF**

To investigate whether CgA and VS-1 could affect the delivery of chemotherapeutic drugs to tumor cells in vivo, we exploited the fact that doxorubicin is a fluorescent compound and that tumor cell fluorescence is an indication of the amount of doxorubicin that penetrate tumor cells (4). Tumor-bearing mice were treated with doxorubicin and, 2 hours later, tumor were excised, disaggregated, and analysed by fluorescence-activated cell sorting (FACS). Preadministration of NGR-TNF to mice (2 hours before doxorubicin) increased the tumor cell fluorescence (i.e., the penetration of doxorubicin in tumor cells), as observed previously (4). However, pretreatment with CgA and VS-1 completely inhibited this effect (Fig. 3A). Notably, VS-1 significantly reduced also the spontaneous penetration of doxorubicin in tumor cells (Fig. 3A). These results support the hypothesis that CgA and VS-1 can inhibit the delivery of doxorubicin to RMA cells in vivo.

**Induction of endogenous CgA with omeprazole inhibits the penetration of doxorubicin in RMA tumors induced by NGR-TNF**

To assess the role of endogenous CgA, we investigated the effect of omeprazole, a drug known to induce the release of CgA in circulation in patients, on doxorubicin penetration in RMA tumors. Preliminary experiments showed that a single administration of omeprazole to mice (1 mg, i.v.) can increase the circulating levels of murine CgA from 0.9 ± 0.08 to 1.48 nmol/L ± 0.079 (n = 5, mean ± SE) by ELISA, peaking 2 hours after injection (Fig. 3B, left). Furthermore, repeated injection of omeprazole (1 mg/day, for 5 days) induced an increase in circulating murine CgA, reaching levels of 1.75 nmol/L at day 4. The CgA returned to normal values 4 days after discontinuation of omeprazole administration. Repeated administration of omeprazole significantly decreased the penetration of doxorubicin induced by NGR-TNF in RMA tumors (Fig. 3B, right). No significant effects were observed.
when omeprazole was administered in combination with mAb 5A8, a neutralizing anti-CgA antibody (Fig. 3B, right), suggesting that the effect of omeprazole was mediated by CgA.

These results suggest that even a moderate increase of endogenous CgA is sufficient to significantly reduce the penetration of doxorubicin induced by NGR-TNF in tumor cells.

**CgA and VS-1 do not affect RMA cell membrane permeability**

The observation that CgA and VS-1 can reduce the delivery of doxorubicin to RMA tumor cells in vivo prompted us to investigate whether these polypeptides could affect the transport of drugs through RMA cell membranes. To this aim, we...
preincubated RMA cells in vitro with different amounts of NGR-TNF, CgA, or VS-1 (1 hour) and then with different amounts of doxorubicin (1 hour). Finally, we analyzed the fluorescence intensity of cells by FACS. As expected, increasing concentrations of doxorubicin increased the fluorescence intensity of cells, indicating that doxorubicin could penetrate the cells in a dose-dependent manner under these conditions (Fig. 4, left panel). Neither NGR-TNF nor CgA/VS-1 could affect the fluorescence intensity of doxorubicin-treated cells (Fig. 4, right panels). These results argue against the hypothesis that these polypeptides can affect the transport of doxorubicin through cell membranes.

CgA inhibits the NGR-TNF/C0 induced transport of molecules from the vascular compartment to the tumor cell microenvironment

We then investigated whether CgA could inhibit the NGR-TNF/C0 induced transport of drugs from blood to the tumor microenvironment. To this aim, we treated RMA-bearing mice with CgA, alone or in combination with NGR-TNF, followed 2 hours later by Patent Blue VF, a hydrophilic nontoxic dye that does not penetrate into cells and that can be easily quantified. After 5 minutes, the mice were killed and perfused with a saline solution. The amount of Patent Blue VF present in tumor tissue homogenates was then quantified spectrophotometrically. CgA inhibited the NGR-TNF/C0 induced penetration of Patent Blue VF in tumor tissues (Fig. 5). These results support the hypothesis that CgA can indeed affect the transport of molecules from the vascular compartment to the tumor cell microenvironment induced by NGR-TNF.

CgA and VS-1 inhibit NGR-TNF/C0 induced endothelial barrier alteration

The positive effect of TNF on endothelial permeability and vascular leakage is a well-known phenomenon (38). This effect can be efficiently inhibited by CgA and VS-1 (15). Keeping this in mind, we tested the hypothesis that NGR-TNF can promote drug extravasation by affecting the endothelial permeability and that CgA and VS-1 can inhibit this effect by protecting the endothelial barrier integrity. To this aim we analyzed, first, the effect of NGR-TNF on the paracellular transport of FITC-dextran (a macromolecular tracer) through endothelial cell monolayers cultured in transwell systems. As expected, NGR-TNF could increase the flux of FITC-dextran from the upper to the lower chamber of the transwell system. CgA (4–20 nmol/L) efficiently inhibited this effect (Fig. 6A, top panel). This result supports the concept that this protein can inhibit the paracellular transport of molecules induced by NGR-TNF through endothelial cells.
To assess whether CgA and VS-1 could also inhibit the transport of drugs induced by NGR-TNF, we carried out a similar experiment using doxorubicin in place of FITC-dextran. Both CgA and VS-1 could inhibit the flux of doxorubicin from the upper chamber to the lower chamber induced by NGR-TNF (Fig. 6A, bottom panels). These data support the hypothesis that these polypeptides can affect drug delivery to tumor cells in vivo by inhibiting the alteration of the endothelial barrier function induced by NGR-TNF.

**CgA inhibits the NGR-TNF/C0 induced disassembly of VE-cadherin-dependent adherence junctions**

The molecular mechanism underlying the inhibitory effect exerted by CgA on the NGR-TNF/C0-induced permeability was then investigated. First, we analyzed whether NGR-TNF could induce the disassembly of VE-cadherin, a protein critical for adherence junctions, in confluent HUVEC cells. Immunofluorescence staining of VE-cadherin showed a homogeneous distribution in confluent HUVEC cells. VE-cadherin was detected by immunofluorescence analysis with a rabbit anti-VE-cadherin antibody and goat anti-rabbit Alexa Fluor 546 conjugate (red). Nuclei were stained with DAPI (blue; representative picture of 2 experiments each conducted in duplicate). Bar, 10 μm. C, effect of CgA on NGR-TNF/C0-induced phosphorylation of p38 MAPK in endothelial cells. Endothelial cells were incubated with NGR-TNF/C0 and CgA and analyzed by Western blot with anti-p-p38-MAPK and anti-phosphorylated p38-MAPK antibodies after 0, 5, and 20 minutes (representative results of 3 experiments).

**CgA inhibits the phosphorylation of p38-MAPK induced by NGR-TNF in endothelial cells**

TNF is known to induce the phosphorylation of p38-MAPK, a signaling mechanism that can alter VE-cadherin expression and endothelial permeability (39). To assess whether NGR-TNF and CgA regulate this signaling pathway in a positive and negative manner, respectively, we analysed the effect of these proteins on p38-MAPK phosphorylation in HUVEC cells at different time points. Western blot analysis of total and phosphorylated p38-MAPK showed that NGR-TNF can induce the phosphorylation of this enzyme in a time-dependent manner and that CgA can inhibit this effect in a dose-dependent manner (Fig. 6C). CgA alone did not affect p38-MAPK phosphorylation (data not shown).

**Discussion**

The results of this study show that pretreatment of mice bearing RMA lymphomas or B16-F1 melanomas with doses of
CgA that enhance its circulating levels 4 to 5 times above the normal values completely inhibits the synergism of NGR-TNF with doxorubicin and melphalan. This inhibitory activity is unlikely related to inhibition of the cytotoxic activity of this drug combination, as no effect was observed in cytotoxicity assays with cultured endothelial or tumor cells. More likely, the inhibitory activity of CgA was related to a reduction of the delivery of chemotherapeutic drugs to tumors. This hypothesis is supported by the results of FACs analysis of tumors recovered from tumor-bearing mice treated with doxorubicin (which is a fluorescent compound) showing that the penetration of drugs into neoplastic cells induced by NGR-TNF in vivo can be almost completely inhibited by CgA.

How does NGR-TNF and CgA increase and decrease, respectively, the penetration of drugs into tumor cells? Drug delivery to neoplastic cells in solid tumors requires, in principle, at least 4 steps: the drug must (a) enter the tumor blood vessels (step 1), (b) cross the endothelial barrier (step 2), (c) migrate through the tumor interstitium (step 3), and (d) cross the membrane of the target cells (step 4; see Fig. 7 for a schematic representation). The results obtained with cultured cells show that neither NGR-TNF nor CgA can affect the penetration of doxorubicin into RNA cells, arguing against a crucial role of these molecules on step 4. The effect on other steps are likely more important. Interestingly, the results of in vivo assays done with RMA tumor–bearing mice injected with Patent Blue VF (a hydrophilic dye that does not cross the cell membrane) show that NGR-TNF can increase the accumulation of dye in the tumor microenvironment and that CgA can inhibit this effect. Because tumors were perfused before excision (to remove the dye present in the intravascular compartment), these findings suggest that the dye was located in the extravascular compartment of the tumor microenvironment. Thus, NGR-TNF enhances the drug extravasation steps (steps 2 and 3), whereas CgA can inhibit this effect. Accordingly, the results of endothelial permeability assays showed that CgA can inhibit the NGR-TNF–induced flux of FITC-dextran through endothelial cell monolayers. Given that FITC-dextran is a good tracer for the paracellular transport of soluble compounds through the endothelial barrier, these findings suggest that NGR-TNF can reduce the barrier function of endothelial cells whereas CgA inhibits this effect (step 2). Notably, CgA could also inhibit the NGR-TNF–enhanced flux of doxorubicin through endothelial cell monolayers. Thus, whereas the alteration of the endothelial barrier function by NGR-TNF might account for the improved delivery of chemotherapeutic drugs to tumor cells, the protective activity exerted by CgA on the endothelial barrier integrity might account for the inhibition of drug penetration and therapeutic activity observed in vivo.

Notably, all the in vitro and in vivo effects observed with CgA were observed also with its N-terminal fragment (VS-1). This observation suggests that the biologically active site of CgA is located in its N-terminal domain. A schematic representation of these concepts is shown in Figure 7.

How do NGR-TNF, CgA, and VS-1 affect endothelial cell permeability? The results of in vitro studies show that NGR-TNF, similar to TNF, can alter the barrier function of endothelial cell monolayers by causing a redistribution of VE-cadherin, a component of adherence junctions critical for the maintenance of endothelial barrier integrity (40) and that both CgA and VS-1 can inhibit this effect. The changes of VE-cadherin expression induced by TNF involve p38-MAPK phosphorylation (39). We observed that also NGR-TNF, similar to TNF, can induce p38-MAPK phosphorylation in endothelial cells. Again, CgA could inhibit this effect. Notably, CgA did not inhibit the cytotoxic activity of NGR-TNF in assays done in the presence of actinomycin D, a transcription inhibitor (data not shown). This observation suggests that CgA does not inhibit the interaction of NGR-TNF with TNF membrane receptors. More likely, CgA inhibits the NGR-TNF–induced phosphorylation of p38-MAPK by interfering with downstream signaling pathways triggered by TNF receptor activation.

Are the doses of CgA used in our experimental models physiologically relevant? Administration of 1 μg of CgA, i.p., generated circulating levels of about 3 to 5 nmol/L at the time of NGR-TNF administration in our murine models. This dose was selected to achieve circulating levels similar to those observed in subpopulation of patients with nonneuroendocrine tumors (11, 12, 31, 37). For example, although serum CgA in the blood of normal subjects is about 1 nmol/L (21), increased levels of CgA, up to 4 to 8 nmol/L, have been detected in the sera of a subgroup of patients with NSCLC, despite the lack of neuroendocrine differentiation, with important prognostic implication (37). Considering that the combination of NGR-TNF and chemotherapy is currently under investigation in patients with NSCLC, hepatocellular carcinoma, or other nonneuroendocrine tumors (9–14), the results showing inhibitory activity of CgA in animal models of nonneuroendocrine tumors might be clinically relevant.
A growing body of evidence suggests that administration of proton pump inhibitors, which are drugs commonly used in the treatment of acid peptic disorders, can enhance 2 to 3 times, and in certain patients even up to 10 times, the circulating levels of CgA, depending on the time of administration (32, 33). These drugs, by decreasing gastric acidity, induce the release of gastrin that, in turn, can induce enterochromaffin cell-like cell hyperplasia and CgA secretion (41, 42). Accordingly, we found that daily treatment of mice with omeprazole can increase 2-fold the circulating levels of CgA and that 4 day discontinuation of therapy is necessary to return to base line. Interestingly, we observed that administration of omeprazole to mice can inhibit the penetration of doxorubicin in tumors and that this effect can be inhibited by administration of a CgA-neutralizing antibody. Thus, a 2-fold enhancement of CgA levels was sufficient to inhibit the penetration of doxorubicin induced by NGR-TNF in tumors. Considering the wide use of proton pump inhibitors in patients, also these findings may have important clinical implications.

This study has a limitation that must be highlighted: although our models are suitable to investigate the role of circulating CgA in nonneuroendocrine tumors, they are not necessarily suitable to address the role of tumor derived/locally produced CgA that may occur in patients with neuroendocrine tumors. Indeed, in the latter case the tumor vasculature is exposed to much higher levels of CgA (possibly >1 μmol/l) compared with those of nonneuroendocrine tumors, owing to the fact that the source of CgA is the tumor itself. Because the dose-response curves of CgA and VS-1 in various biological assays are “bell shaped” with loss of activity at micromolar concentrations (36, 43, 44), higher levels of CgA in the tumor microenvironment could be, paradoxically, less active than low levels of circulating CgA. Furthermore, different proteolytic processing may occur to CgA released by neuroendocrine tumor cells and by normal cells (45). Thus, further studies with suitable neuroendocrine tumor models are necessary to address this point.

Although many studies showed that CgA is a good marker for diagnosis and prognosis of neuroendocrine tumors, even for monitoring their responses to chemotherapy (29, 30, 46), only few works have investigated the capability of baseline circulating CgA to predict the therapeutic response. Interestingly, in 3 studies carried out on SCLC, NSCLC, and prostate cancer, the response rates to chemotherapy inversely correlated with baseline CgA (37, 47, 48). However, another study on prostate cancer, a tumor that may undergo neuroendocrine differentiation, showed higher response rates in patients with high baseline CgA (49). Moreover, in a recent study, a large proportion of patients with metastatic pancreatic endocrine carcinomas experienced a major biochemical response despite an elevated serum CgA before therapy (50). These conflicting results further underline the need of new studies to elucidate the role of CgA in the response to chemotherapy of neuroendocrine tumors or of tumors that may undergo neuroendocrine differentiation.

In conclusion, the results of this study suggest that CgA can inhibit the NGFR-TNF/chemotherapy synergism in murine models of nonneuroendocrine tumors by inhibiting NGR-TNF-induced changes of vascular permeability and drug penetration in neoplastic tissues. These results could stimulate further work aimed at assessing whether circulating CgA and VS-1 levels can predict the response to NGR-TNF/chemotherapy in patients. Furthermore, the finding that omeprazole-induced CgA can inhibit the penetration of doxorubicin in tumor tissues may suggest that discontinuation of administration of proton pump inhibitors to patients and/or its replacement with H2 receptor antagonists (a class of drugs that do not increase CgA levels; ref. 33) might increase the response rate to this therapy in patients with documented elevation of circulating CgA or VS-1.

Disclosure of Potential Conflicts of Interest

Angelo Corti is the inventor of a patent on NGR-TNF.

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