

A Novel Function of CD40: Induction of Cell Death in Transformed Cells

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Summary

CD40 is known as an important T–B cell interaction molecule which rescues B lymphocytes from undergoing apoptosis. Like other receptors of the tumor necrosis factor (TNF) receptor gene family, CD40 is expressed on cells of different tissue origins including some transformed cells. In contrast to its well-studied effects on B cells, the biological functions of CD40 in non-immune cells remain largely unknown. Here we show that CD40 ligation induces apoptotic cell death in transformed cells of mesenchymal and epithelial origin. This CD40-mediated cell death seems to use a preformed signaling pathway since it occurs even when protein synthesis is blocked. Notably, the CD40 cytoplasmic domain shares a structural homology with the recently defined “death domains” of the 55-kD TNF receptor (p55TNFR) and Fas. Despite these structural similarities, differences are seen in the way phorbol myristate acetate, interleukin 1, TNF, and various metabolic inhibitors influence the cellular responsiveness to CD40, p55TNFR, and Fas-mediated killing. Our study indicates that CD40 induces cell death by a distinct mechanism.

Programmed cell death (apoptosis) serves as a crucial control mechanism not only during embryogenesis for the development of organs but also in the mature organism for the maintenance of tissue homeostasis. In the immune system the development of T and B lymphocytes depends on a selection process involving the controlled triggering of cell death (reviewed in [1]). CTL also eliminate their targets via specific induction of cell death (reviewed in [2]). The broadly expressed receptors Fas/APO-1 (3, 4) and 55-kD TNF receptor (p55TNFR) (5–7), both members of the TNFR gene family, function as membrane triggers for apoptosis in various target cells that are susceptible to CTL, e.g., virus-infected or cancer cells (8).

For both p55TNFR and Fas, a motif spanning 65 amino acids within the cytoplasmic regions is essential and sufficient for their cytotoxic function and has been named the death domain (9, 10). A similar motif was found in the cytoplasmic domain of CD40, which belongs to the same receptor family (4, 11). However, in B lymphocytes CD40 provides signals that rescue them from apoptotic death and therefore has been aptly described as an antiapoptotic molecule (12–14). The ligand of CD40 (CD40L)¹ is a type II transmembrane molecule with a homology to TNF, lymphotoxin- α and β , and the Fas ligand (15–17); it is expressed on activated T lymphocytes. The interaction of CD40 and CD40L is crucial for many other B cell functions, including isotype switching (18), short- and long-

term proliferation (19, 20), homotypic adhesion (21), and the upregulation of B7/BB-1 (CD80) (22). Interestingly the hyper IgM syndrome, a severe immunodeficiency which is characterized by an isotype switch defect, was found to result from a mutated CD40L gene (23–26). A similar defect was seen in CD40- or CD40L-deficient mice (27, 28).

Intracellular signals delivered via CD40 in B cells include the activation of several serine and threonine specific protein kinases (29, 30), the phosphorylation of src type kinases and of the phospholipase C γ 2 and the phosphatidylinositol-3 kinase (31). The first clues of how CD40 is coupled to intracellular signaling pathways were obtained with the yeast two-hybrid technique. A protein, variously named CD40bp, CRAF1, or LAP1, is apparently constitutively associated with CD40 (32–34) and plays a role in CD40-mediated upregulation of CD23 in the Burkitt's lymphoma cell line Ramos (35).

Like the TNFR and Fas, CD40 is not only expressed on hematopoietic cells but also on dendritic cells (36), thymic epithelium (37), basal epithelium as well as on carcinomas and other transformed cells (11, 38–40). However, the functional properties and signaling mechanisms of CD40 in non-B cells are largely unknown. We have recently shown that CD40 induces nuclear factor- κ B (NF- κ B) and IL-6 production in a human fibroblast cell line and in CD40-transfected HeLa cells (41). CD40 shares these functions with the p55TNFR and Fas (42). As the CD40 cytoplasmic domain and the death domains of the p55TNFR and Fas are homologous we were interested to know whether CD40 might also induce signals leading to cell death.

¹Abbreviations used in this paper: BHA, butylated hydroxyanisole; BHK, baby hamster kidney; BHK_{CD40L}, BHK cells transfected with CD40L cDNA; BHK_{wt}, BHK wild-type cells; CD40L, ligand of CD40; CHX, cycloheximide; NF- κ B, nuclear factor κ B; SN, supernatants.

The present study demonstrates that CD40 stimulation induces apoptosis in transformed cells of mesenchymal and epithelial origin. The strongest effect was seen with membrane-bound CD40L. IFN- γ treatment rendered unresponsive cells with low level CD40 expression susceptible to CD40-mediated cytotoxicity. CD40 stimulation seemed to trigger a preformed death program. Despite the structural homology to Fas and the p55TNFR, we found that reagents that modulate p55TNFR or Fas-mediated cell death affected CD40 killing in a unique way. This indicates that CD40 signals cell death through a distinct pathway.

Materials and Methods

Cell Lines. The murine L cell derivative A9 (43) was grown in RPMI 1640; the SV40-transformed human fibroblast cell line SV80 (44), the cervical carcinoma cell line HeLa (CCL 2; American Type Culture Collection, Rockville, MD) and the baby hamster kidney (BHK) cells (ACC 61; German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany) were cultured in DMEM. The culture media were supplemented with 10% heat-inactivated FCS (Biochrom, Berlin, Germany), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-alanyl-L-glutamine. All supplements and culture media were purchased from Gibco BRL (Eggenstein, Germany).

Cloning of CD40 and CD40L cDNA and Transfection. CD40 cDNA was cloned by reverse transcriptase PCR using total RNA isolated from IM-9 cells as previously described (45). For expression, the CD40 cDNA was ligated into the mammalian expression vector pEF-BOS (46) and designated BOS-CD40. SV80, HeLa, and A9 cells were cotransfected with 25 μ g of ApaI-digested BOS-CD40 and 2.5 μ g of HindIII-digested pTCF plasmid encoding a neomycin resistance gene. Transfection was performed with lipofectin (Gibco BRL) according to the manufacturer's instructions. Neomycin-resistant clones were selected in 0.6 mg/ml G418 for A9, 0.8 mg/ml for SV80, and 1 mg/ml for HeLa transfectants. The selected A9, SV80, and HeLa clones were tested for CD40 expression using an mAb against CD40 at 5 μ g/ml (G28-5 [19]) or anti-CD4 mAb (MT413) as an isotype-matched control. After staining with FITC-conjugated goat anti-mouse F(ab')₂ fragment (Dianova, Hamburg, Germany) as secondary antibody, the CD40 expression was assessed by flow cytometry (FACScan[®], Becton Dickinson and Co., Mountain View, CA).

Cloning of the CD40L cDNA and transfection into BHK cells were done as described previously (45).

Cytokines and Reagents. CD40L was used either in its cell surface (membrane-bound CD40L mCD40L) or in a soluble form. BHK cells transfected with CD40L cDNA (BHK_{CD40L}) or BHK wild-type cells (BHK_{wt}) as a control were fixed with 3% paraformaldehyde in PBS for 10 min, followed by six washing steps with PBS. This procedure prevented the attachment of the BHK cells to the microtiter plates, which might interfere with the determination of the target cell viability in cytotoxicity assays.

For the production of CD40L supernatants (SN) or control SN, BHK_{CD40L} cells or BHK_{wt} were grown to subconfluency. After two washes with PBS, serum-free Hybridoma SFM medium (Gibco BRL) was added; 24 h later the culture SN were harvested, centrifuged, and filtered at 0.2 μ m. The SN were pooled, concentrated in a Centriprep-10 or Centricon-10 concentrator (Amicon, Witten, Germany) and stored at -80°C.

Recombinant human TNF was a gift from Dr. G. Adolf

(Bender and Co. GmbH, Wien, Austria). IFN- γ was a present from Dr. W. Wolf (Rentschler-Biotechnologie, Laupheim, Germany). Recombinant human IL-1 β was kindly provided by Dr. C. A. Dinarello (Tufts University School of Medicine, Boston, MA).

Cycloheximide (CHX), PMA, butylated hydroxyanisole (BHA), the calcium ionophore A23187, vanadate, trifluoperazine, and all other reagents were purchased from Sigma Chemical Co. (Deisenhofen, Germany) unless indicated otherwise.

Antibodies. Protein G (Pharmacia, Freiburg, Germany) purified anti-CD40 mAb G28-5 (IgG₁) was used for CD40 stimulation. The anti-p55TNFR mAb htr-9 (IgG₁) (47) was a gift from Dr. M. Brockhaus (Hoffmann LaRoche, Basel, Switzerland), humanized monoclonal mouse antibody against Fas (IgG₃) was a present from Dr. John Grayebh (Centocor, Malvern, PA). Purified murine myeloma immunoglobulins (IgG₁, MOPC 21) (Sigma Chemical Co.) and anti-CD4 mAb (MT413, IgG₁, kindly provided by Dr. E. P. Rieber, Institute for Immunology, Munich, Germany), were used as control antibodies. Polyclonal anti-CD40L antibodies were raised against a fusion protein consisting of the extracellular domain of the murine IL-4 receptor and CD40L as described previously (45).

Cytotoxicity Assay. Target cells were seeded in gelatin-coated flat-bottom microtiter plates (Greiner, Nürtingen, Germany) either untreated or after a 2-d pretreatment with 1,000 U/ml IFN- γ . Cells were seeded at densities of 1×10^4 cells/well for assays performed in the absence of CHX and of 3×10^4 cells/well for assays done in the presence of CHX. The cells were allowed to grow for 24 h (the medium for IFN- γ pretreated cells contained 1,000 U/ml IFN- γ) and then challenged with serial dilutions of the stimulation reagents, either in the presence (50 μ g/ml) or the absence of CHX. Cell viability was assessed 16–18 h later (48 h in assays without CHX) by the neutral red uptake method according to Finter et al. (48) as described previously (45).

Acridine Orange Staining. Acridine orange (Sigma Chemical Co.) was added to the cells at 10 ng/ml in medium for 5 min. The culture plates were briefly centrifuged at 150 g and the dye was carefully replaced by Hank's salt solution (Biochrom, Berlin, Germany). The cellular morphology was analyzed using a fluorescence microscope and photographs were taken at a 400-fold magnification.

Results

CD40L-CD40 Interaction Induces Cell Death in Transformed Cells of Mesenchymal and Epithelial Origin. In many transformed cell lines ligation of Fas or the TNFR leads to apoptotic cell death. Thus far, CD40's main role was seen as that of a molecule with antiapoptotic qualities. This is indeed surprising because its cytoplasmic domain is homologous to the central part of the death domains of Fas and the p55TNFR (Fig. 1). To investigate the functional relevance of this structural similarity we studied CD40 responses in the TNF-sensitive fibroblast cell line SV80, which expresses low levels of CD40. In previous studies it was shown that CD40, the p55TNFR and Fas (41, 42) signal NF- κ B mobilization and IL-6 production in these cells. It was also seen that IFN- γ pretreatment enhanced these CD40-mediated effects. Here we found that the IFN- γ -pretreated SV80 cells died rapidly in response to CD40 ligation when protein synthesis was blocked with CHX (Fig.

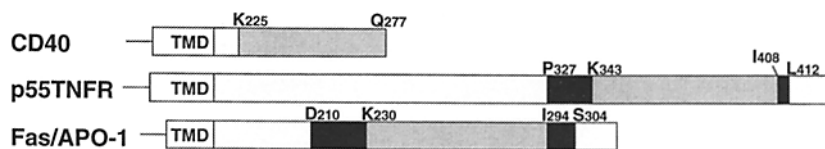


Figure 1. Schematic presentation of the intracellular domains of CD40, p55TNFR, and Fas/APO-1. Homologous regions between CD40 (K₂₂₅-Q₂₇₇), the p55TNFR (K₃₄₃-I₄₀₈), and Fas (K₂₃₀-I₂₉₄) are shown in gray. At the amino acid level these regions show a chemical similarity of 26% for CD40 and p55TNFR, 39% for CD40 and

Fas, and 45% for p55TNFR and Fas. In p55TNFR and Fas the homology regions are found within the "death domains" (P₃₂₇-L₄₁₂ for p55TNFR and D₂₁₀-S₃₀₄ for Fas, shown in black) as defined by Tartaglia et al. (9) and Itoh et al. (10). Similar amino acids were defined as follows: A, G; S, T; E, D; R, K, H; Q, N; V, I, L, M; Y, F; W; P; C. (TMD, transmembrane domain).

2 B). BHK_{CD40L} killed up to 40% of the IFN- γ -pretreated targets while CD40L-negative control effector cells (BHK_w) had only a marginal effect on the viability of the SV80 cells.

IFN- γ pretreatment of the SV80 cells was necessary to induce susceptibility to CD40L. At the molecular level the sensitizing effect of IFN- γ may be explained in two ways: IFN- γ upregulates cell surface CD40 expression (see Fig. 2 A) but it may also induce intracellular changes that increase the sensitivity to CD40-mediated killing. To distinguish between these possibilities we overexpressed CD40 in SV80 cells (SV80_{CD40}) by transfection. In addition, we transfected the CD40 cDNA into the CD40-negative human cervical carcinoma cell line HeLa (HeLa_{CD40}) and the murine L cell derivative A9 (A9_{CD40}) (Fig. 3 A). Both cell lines have been characterized extensively with respect to their TNF responses. Two CD40-expressing clones of each cell line were compared. Membrane CD40L efficiently killed the CD40 transfectants in a dose-dependent manner (Fig. 3 B) demonstrating that CD40-mediated signals are sufficient to induce cell death. In the presence of CHX, SV80_{CD40} and A9_{CD40} responded to a similar extent (40–50% killing) (Fig. 3 B) as IFN- γ -pretreated SV80 wild-type cells (Fig. 2 B). In HeLa_{CD40} killing was almost complete (Fig. 3 B).

Several control experiments demonstrated that the cytotoxic effect of BHK_{CD40L} cells was indeed CD40 mediated: (a) stimulation with mCD40L had no significant effect on the viability of CD40-negative wild-type cells (not shown) or mock transfectants (Fig. 3 B). (b) The cytotoxic effect of BHK_{CD40L} could be blocked by pretreatment with anti-CD40L antibodies (data not shown). (c) SN from BHK_{CD40L} cells containing a soluble form of CD40L were also cytotoxic whereas control SN from BHK_w cells had no effect (data not shown). (d) In addition to CD40L, the

anti-CD40 mAb G28-5, but not an isotype-matched control antibody, elicited a cytotoxic response in the CD40 transfectants, although the effect was weaker than that seen with mCD40L (Fig. 3 B, b, d, and f).

Acridine orange staining was used to assess the form of cell death induced via CD40. Microscopic examination of the stained cells of all three cell types revealed a typical apoptotic morphology after CD40 ligation showing cellular shrinkage, chromatin condensation, and clear nuclear fragmentation (Fig. 3 B, b, d, and f). This demonstrated that CD40 mediated an apoptotic form of cell death.

Influence of Protein Synthesis on CD40 Killing. SV80 and HeLa cells are killed by CD40, p55TNFR, and Fas only when protein synthesis is blocked. In the case of TNFR-mediated cytotoxicity this was explained by the constitutive expression of resistance proteins (49). In contrast, mouse A9 cells are sensitive to TNF and Fas cytotoxicity even in the absence of protein or RNA synthesis inhibitors. Therefore, we tested the response to CD40 ligation in A9_{CD40} cells with intact protein synthesis. Membrane CD40L (Fig. 4, left), a soluble form of CD40L (not shown) and anti-CD40 antibodies had strong cytotoxic effects. CD40-killed cells again displayed a typical apoptotic morphology (Fig. 4, inset). Unexpectedly, CD40-mediated killing was even more effective in A9 cells in the absence of CHX: a dose of mCD40L which induced complete killing of the A9_{CD40} cells in the absence of CHX left 50% of the target cells viable when protein synthesis was blocked (Fig. 4, left). This was in clear contrast to p55TNFR and Fas, which killed A9 cells with intact protein synthesis 100-fold (p55TNFR) and 10-fold (Fas) less efficiently (Fig. 4, middle and right). Thus it appeared that the mechanisms by which CD40 induced cell death were different from both p55TNFR- and Fas-mediated killing.

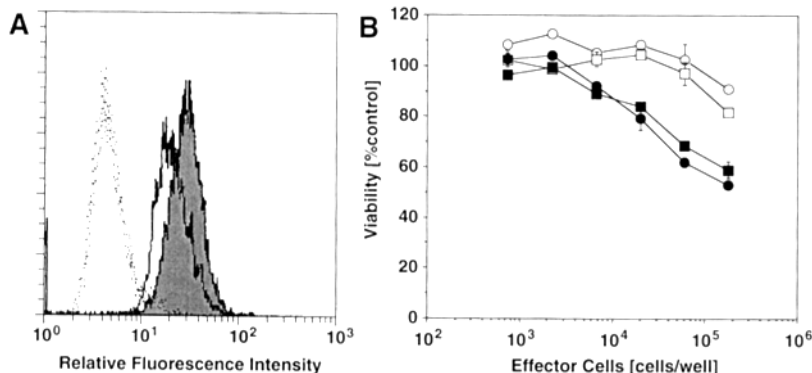


Figure 2. CD40 ligation is cytotoxic for IFN- γ -treated transformed fibroblasts. (A) CD40 expression in untreated SV80 cells (■) or after treatment with 1,000 U/ml IFN- γ for 3 d (●) as determined by immunostaining with the anti-CD40 mAb G28-5 (■, ●) or an isotype-matched control mAb (□, ○), FITC-conjugated goat anti-mouse F(ab')₂ antibodies, and analysis with a FACScan®. (B) Two IFN- γ -treated subclones of SV80 were seeded in microtiter plates at a density of 3×10^4 cells/well. 24 h later paraformaldehyde-fixed BHK_{CD40L} cells (■, ●) or BHK_w cells (□, ○) were added at the indicated effector cell doses in the presence of 50 μ g/ml CHX. Viability of the target cells was determined after 18 h by the neutral red uptake method. Presented are the mean values of duplicate determinations of one representative experiment.

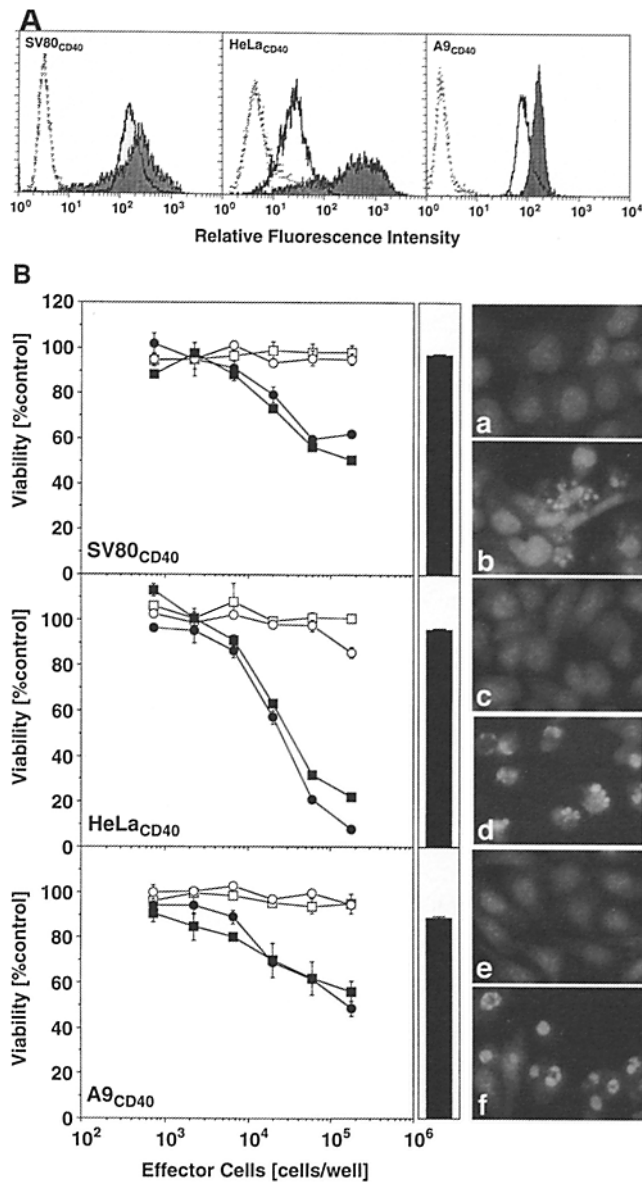


Figure 3. CD40 mediates cell death in SV80, HeLa, and A9 cells transfected with CD40 cDNA. (A) CD40 expression in CD40-transfected SV80, HeLa, and A9 cells as determined by immunostaining with anti-CD40 mAb G28-5 (■, ●) or an isotype-matched control mAb (○, □), FITC-conjugated goat anti-mouse F(ab')₂ antibodies and analysis with a FACScan[®]. Two individual clones (1 ■, 2 ●) of each cell type are shown. (B) CD40-mediated killing was tested in two independent SV80_{CD40}, HeLa_{CD40}, and A9_{CD40} clones (1 ●, 2 ■, left). The cells were challenged with paraformaldehyde-fixed BHK_{CD40L} (●, ■) or BHK_{wt} cells as a control (○, □) at the indicated effector cell doses in the presence of 50 μg/ml CHX. 18 h later the viability was determined by the neutral red uptake method. Presented are the mean values of duplicate determinations of one representative experiment. The black bars indicate the effect of BHK_{CD40L} cells (1.8 × 10⁵ cells/well) on the corresponding mock transfectants. Morphological changes in cells dying after CD40 ligation with 5 μg/ml anti-CD40 mAb G28-5 are shown in the right panels: SV80_{CD40} (a, b), HeLa_{CD40} (c, d), and A9_{CD40} cells (e, f) were stained with acridine orange after an 18-h treatment with anti-CD40 mAb (b, d, f) or an isotype-matched control mAb (a, c, e) in the presence of 50 μg/ml CHX. Cells were then analyzed by fluorescence microscopy at a 400-fold magnification. Cells treated with anti-CD40 mAb are shrunk and show condensed nuclei or nuclear fragmentation.

CD40-, p55TNFR-, and Fas-induced Cell Death Are Regulated Differently. Sensitivity to p55TNFR cytotoxicity can change dramatically with changes in the expression of inducible protective proteins like manganese superoxide dismutase (52), the plasminogen activator inhibitor-2 (53), and the zinc finger protein A20 (54). In some cell types, when protein synthesis is intact, phorbol esters (i.e., PMA), IL-1 and, paradoxically, TNF, upregulate protective proteins. These render the cells resistant to a subsequent TNF challenge in the presence of CHX, as demonstrated for SV80 and HeLa cells (49). Therefore, we tested whether PMA, IL-1, and TNF pretreatment also conferred resistance to CD40-mediated killing in these cells. In the SV80_{CD40} transfectants, p55TNFR-mediated killing was markedly reduced after PMA, IL-1, or TNF prestimulation (Fig. 5, middle); whereas CD40-mediated cytotoxicity was not influenced by PMA or IL-1. TNF pretreatment even enhanced CD40 killing (Fig. 5, top). A dose of mCD40L which induced 40% killing of naive SV80_{CD40} cells killed 75% of the TNF-pretreated targets. Pretreatment with a ligand mimetic mAb against the p55TNFR also sensitized cells for CD40 killing, indicating that this effect was mediated via the p55TNFR (data not shown). Similar results were obtained with HeLa_{CD40} (data not shown). The influence of PMA, IL-1, and TNF distinguished CD40 cytotoxicity not only from p55TNFR but also from Fas-mediated cytotoxicity as this was unaffected by all three reagents (Fig. 5, bottom).

Synergism between CD40 and p55TNFR Cytotoxicity. The observation that target cell prestimulation with TNF resulted in enhanced CD40 cytotoxicity raised the question whether p55TNFR and CD40 synergized when stimulated simultaneously. As shown in Fig. 6 (left), the anti-CD40 mAb G28-5 (1 μg/ml) alone left 90% of the SV80_{CD40} cells viable. In combination with a marginally active TNF dose (0.08 U/ml), G28-5 killed almost 50% of the targets. When applied together with a half-maximal killing dose of TNF (0.4 U/ml), G28-5 induced >90% killing. Inversely, in the presence of 1 μg/ml G28-5 the TNF dose required for 50% killing was 15-fold reduced and the dose yielding 90% cell death was 60-fold reduced (Fig. 6, right). A control experiment with the ligand mimetic anti-p55TNFR mAb, htr-9, revealed that the TNF effect was mediated via the p55TNFR (not shown). Synergism between CD40 and p55TNFR killing was seen also with HeLa_{CD40} and A9_{CD40} (not shown) suggesting that it was not restricted to one particular cell type.

The question whether CD40 synergized with Fas killing could not be definitely answered. Augmentation of Fas cytotoxicity was only seen in IFN-γ-pretreated SV80_{CD40} cells. Here, the dose of anti-Fas antibodies inducing half-maximal or 90% killing could be 7.5-fold reduced when CD40 was simultaneously stimulated with 1 μg/ml G28-5 antibody (not shown). However, this effect was additive rather than synergistic.

CD40 and the p55TNFR not only quantitatively enhanced each other's cytotoxicity but also accelerated each

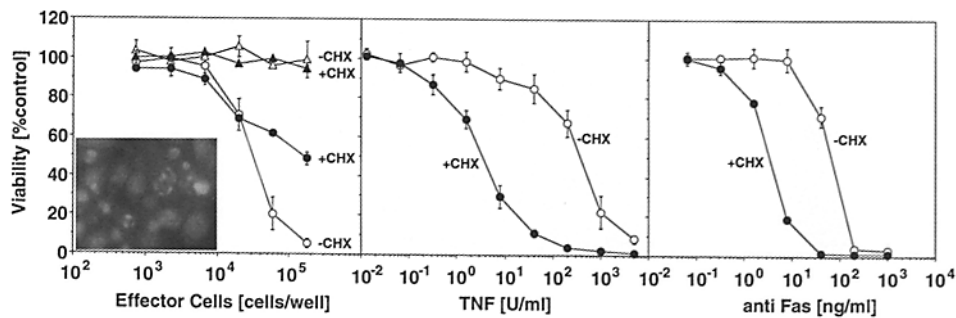


Figure 4. CD40, p55TNFR, and Fas cytotoxicity in murine A9 fibroblasts with intact vs CHX-blocked protein synthesis. Cytotoxicity assays were performed as described in Materials and Methods in the presence (●, ▲) or the absence of CHX (○, △). (Left) Paraformaldehyde-fixed BHK_{CD40L} cells (○, ●) or BHK_{wt} cells (△, ▲) were used to stimulate A9_{CD40} cells at the indicated effector cell doses. The inset shows acridine orange-stained A9 cells after a 48-h treatment with anti-CD40 mAb

(G28-5 at 5 μg/ml) in the absence of CHX. Nuclear condensation and fragmentation are clearly visible. (Middle) TNF was used to activate the murine p55TNFR in A9_{CD40} cells (50) (○, ●). (Right) A9 cells transfected with human Fas (51) were stimulated with anti-Fas mAb at the indicated concentrations (○, ●). Cellular viability was determined 18 h (+CHX) or 48 h (-CHX) later by the neutral red uptake method.

other's killing. Cell death in G28-5 (1 μg/ml)-stimulated A9 fibroblasts was detectable after 6–9 h, increased within the next 16 h in a linear fashion, and reached plateau levels at 60% killing (Table 1). At a TNF dose that was only marginally cytotoxic even after 48 h, addition of the anti-CD40 antibody led to clearly visible cell death as early as 4 h after stimulation (Table 1). At 8 h 42% of the cells were killed and cell death gradually increased to 88% within 48 h (Table 1). In the HeLa transfectants significant p55TNFR- or CD40-mediated killing in the presence of CHX was observed only after 10–12 h; however, the combined activation of both receptors induced cell death after 6–8 h (not shown).

Metabolic Inhibitors Affect CD40-mediated Killing Differently than p55TNFR- and Fas-mediated Cell Death. p55TNFR-mediated killing can be blocked by various inhibitors of signal transduction (55). Previously such inhibitors have been used to compare p55TNFR and Fas cytotoxicity (56). To determine whether or not CD40 cytotoxicity was affected in the same manner as the p55TNFR or Fas cytotoxicity, killing via all three receptors was investigated in the presence and absence of several metabolic inhibitors (Table 2). All experiments were performed in A9 fibroblasts with intact protein synthesis. Under these conditions some reagents that inhibit TNF cytotoxicity when RNA synthesis is blocked, i.e., the phosphatidylcholine-specific phospholipase C inhibitor D609 (56), had no effect at non-toxic concentrations. The oxygen radical scavenger BHA significantly reduced CD40 cytotoxicity suggesting that in A9 cells reactive oxygen intermediates might be involved not only in the p55TNFR- but also in the CD40-activated cell death pathway. A similar result was obtained with the phosphatase inhibitor vanadate which inhibited both CD40 and p55TNFR killing. Under these conditions Fas cytotoxicity was not affected by BHA or vanadate indicating that CD40 cytotoxicity differed from Fas killing. Use of calcium modulators also suggested that CD40 signals were different from p55TNFR-mediated cell death signals. Both the calcium ionophore A23187 and the calmodulin inhibitor trifluoperazine decreased p55TNFR cytotoxicity while CD40 and Fas killing were not inhibited at any concentra-

tion tested. In fact, trifluoperazine enhanced CD40 and Fas cytotoxicity.

Discussion

The discovery of CD40 had an important impact on the understanding of B cell immunity (reviewed in [57]). A preeminent function of CD40 is its ability to rescue germinal center B cells from undergoing apoptosis (12–14). Paradoxically, the intracellular domain of CD40 shares a structural homology with the death domains of the p55TNFR and Fas (9, 10). This prompted us to investigate whether or

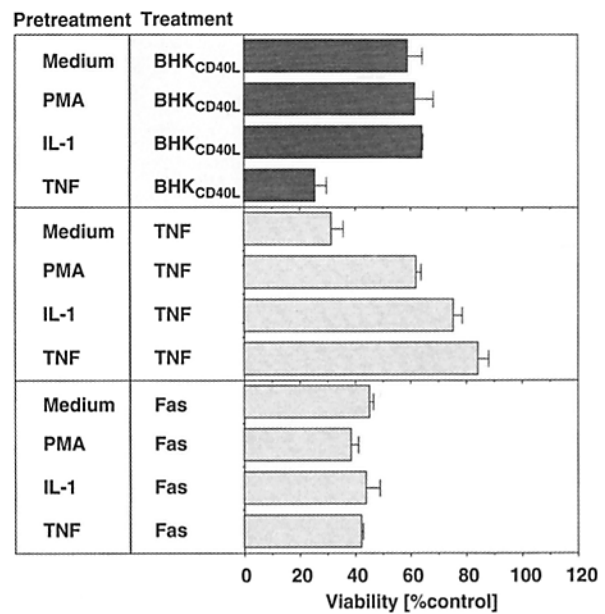


Figure 5. TNF prestimulation induces resistance to p55TNFR cytotoxicity but enhances CD40-mediated cytotoxicity. SV80_{CD40} cells were pretreated with medium, PMA (5 ng/ml), IL-1β (0.15 ng/ml), or TNF (1,000 U/ml) for 3 h. The stimulation reagents were removed by two washing steps and 6 h later the cells were challenged with 6 × 10⁴/well BHK_{CD40L} cells (■, top) (corresponding to an E/T ratio of 1:1), with 200 U/ml TNF (■, middle) or 40 ng/ml anti-Fas mAb (■, bottom), always in the presence of 50 μg/ml CHX. Cellular viability was assessed after 18 h of incubation by the neutral red uptake method.

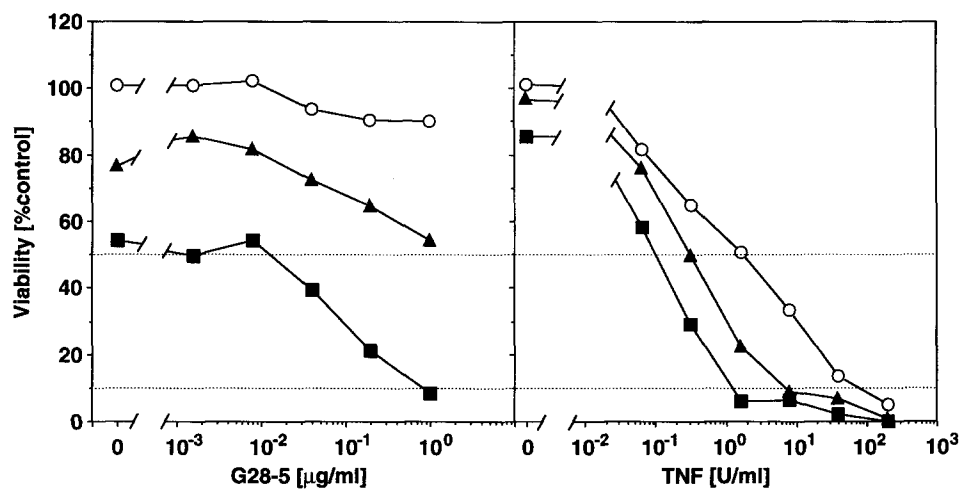


Figure 6. Synergism between CD40 and p55TNFR cytotoxicity. (Left) SV80_{CD40} cells were treated with G28-5 at the indicated concentrations in the presence of TNF at 0.4 U/ml (■) or at 0.08 U/ml (▲) or medium (○). (Right) Titration of TNF on SV80_{CD40} cells in the presence of G28-5 at 1 µg/ml (■) or at 0.2 µg/ml (▲) or a control mAb at 1 µg/ml (○). All assays were done in the presence of 50 µg/ml CHX.

not CD40 could also mediate cytotoxicity. In three different cell lines which were chosen from two different tissue origins (mesenchymal and epithelial) and two different species (human and murine) we observed that CD40 indeed induces cell death. Regardless of the cell type examined the dying cells showed typical morphological features of apoptosis.

In view of the structural relationship between the three cell death-inducing receptors it was of interest to study whether CD40, p55TNFR, and Fas killed by similar mechanisms. Our initial findings supported this possibility. The cellular responsiveness to CD40 cytotoxicity showed a pattern similar to p55TNFR- and Fas-mediated cytotoxicity: In SV80 and HeLa, CD40 elicited a cytotoxic response only in the presence of a protein synthesis inhibitor, while the murine A9 fibroblasts were efficiently killed with intact or blocked protein synthesis. This demonstrated that CD40 stimulation activates a preformed death program which does not require the synthesis of novel proteins. Moreover,

Table 1. Acceleration of TNF Killing by Simultaneous CD40 Ligation in A9_{CD40} Cells

	Killing after			
	4 h	8 h	12 h	48 h
	<i>Percent cell death</i>			
Medium	<5	<5	<5	<5
TNF	<5	<5	<5	14.2 (±2.0)
Anti-CD40	<5	15.4 (±3.0)	25.5 (±4.1)	59.7 (±3.1)
TNF + anti-CD40	21.6 (±8.2)	42.6 (±6.2)	51.0 (±4.9)	88.6 (±1.0)

A9_{CD40} cells were treated in the absence of CHX with anti-CD40 mAb G28-5 at 1 µg/ml, 200 U/ml TNF, the combination of both, or medium as a control. Cell death was determined after the indicated time intervals. Presented are the mean values of quadruplicate determinations (± SD) of one representative experiment.

in some cell types constitutive expression of resistance proteins seemed to interfere not only with p55TNFR- and Fas-induced cytotoxicity (49, 58) but also with the CD40-induced cell death pathway.

Despite these similarities, further experiments revealed several differences in CD40- and p55TNFR-induced cell death: (a) while TNF killed the murine A9 fibroblasts markedly better in the presence of CHX, CD40 cytotoxicity was much stronger in the absence of CHX. This suggested that in A9 cells CD40 not only triggered a preformed death program but also may have induced proteins that supported the induction of cell death. (b) Treatments that induced resistance to p55TNFR cytotoxicity failed to protect against CD40 and Fas killing. For example, TNF, which paradoxically induces resistance to p55TNFR-mediated cell death, even sensitized cells for CD40 killing. (c) Simultaneous activation of the p55TNFR and CD40 was synergistic resulting both in enhanced and accelerated killing. This synergism did not result from receptor upregulation since it was also observed in the presence of CHX. (d) CD40-, p55TNFR-, and Fas-mediated killing were influenced differently by several metabolic inhibitors. Unlike Fas killing, CD40- and p55TNFR-mediated cytotoxicity were both inhibited by the radical scavenger BHA and the phosphatase inhibitor vanadate in A9 cells with intact protein synthesis. However, similar to Fas but unlike p55TNFR, CD40 killing could not be blocked with modulators of calcium metabolism, such as the calcium ionophore A23187 or the calmodulin inhibitor trifluoperazine. These findings suggest that the cell death pathway of CD40 may partially overlap but is not identical with the signaling pathway of either p55TNFR or Fas.

Molecular evidence for the differences in the cell death pathways triggered by p55TNFR and Fas was recently provided in several studies describing three receptor-associated proteins that can initiate cell killing (59, 62). Although these proteins are also homologous to the death domains of p55TNFR and Fas, they specifically interact only with individual receptors. Thus it emerges that the cell death pathways of the p55TNFR and Fas differ in proximal events, nevertheless they seem to converge in an IL-1-converting

Table 2. Influence of Various Metabolic Inhibitors on CD40, p55TNFR, and Fas-mediated Killing in A9 Transfectants

Inhibitors*		Killing via		
		CD40	p55TNFR	Fas
		<i>Viability (percent control)</i>		
No inhibitor		3.5 (±0.5)	5.7 (±1.3)	1.8 (±0.6)
BHA	50 μM	32.2 (±4.3)	46.5 (±2.7)	1.4 (±1.0)
Vanadate	12.5 μM	20.2 (±1.7)	39.4 (±3.2)	1.3 (±1.0)
A23187	0.1 μM	1.4 (±0.3)	12.3 (±1.6)	1.5 (±0.8)
Trifluoperazine	2.5 μM	2.7 (±0.3)‡	17.4 (±1.9)	1.0 (±1.8)‡

CD40 was stimulated in A9_{CD40} cells with paraformaldehyde-fixed BHK_{CD40L} cells (at 6×10^4 /well), the p55TNFR with 1,000 U/ml TNF. A9 cells transfected with human Fas (57) were activated with 200 ng/ml anti Fas mAb. Results are presented as mean values of eight replicates (±SD).

*Inhibitors were added simultaneously with the indicated treatments and remained in the culture medium during the whole assay period. Cell viability with inhibitors alone was 95.5% (±5.4) for BHA, 93.7% (±5.7) for vanadate, 97.5% (±6.8) for A23187, and 76.5% (±8.7) for trifluoperazine.

‡At lower concentrations of BHK_{CD40L} (2×10^4 /well) and anti-Fas mAb (40 ng/ml) trifluoperazine enhanced CD40- and Fas-mediated cytotoxicity by 55.4 and 66.1%, respectively.

enzyme-dependent route (63, 64). Therefore, it will be interesting to search for CD40-specific associated proteins that are involved in cell death signaling and to determine whether CD40 killing also uses an IL-1-converting enzyme-dependent pathway.

Recent studies suggest that CTL killing relies mainly on the action of perforin and Fas (65); however, the way in which a particular target cell is eliminated depends not only on the mediators expressed by the effector cell but also on the responsiveness of the target cell. Since activated T cells express not only Fas ligand but also TNF and CD40L on their surface it is possible that all three ligands constitute a cytotoxic triad. The potency of this triad is further enhanced by IFN-γ, a secretory product of activated T cells. IFN-γ sensitizes not only for p55TNFR (66) and Fas (67) but also for CD40-mediated killing as shown here. Such an arsenal in CTL would clarify their broad target cell spectrum and efficient rate of target cell elimination. Furthermore, target cell escape from receptor-mediated killing would occur only when all three cytotoxic mechanisms fail. Thus, a CD40-positive tumor cell that became resistant to Fas cytotoxicity, e.g., via downregulation of the receptor or via the upregulation of inhibitory proteins like the recently cloned Fas-associated protein FAP-1 (68), may still be susceptible to TNFR or CD40 killing. Since CD40 mediates cell death in transformed cells, it can even be speculated that tumor-infiltrating lymphocytes may use this mechanism for the elimination of some tumor cells in vivo. In view of our findings, it will be important to determine whether the cytotoxic potential of CD40 can be used in cancer therapy.

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References

- Nossal, G.J.V. 1994. Negative selection of lymphocytes. *Cell*. 76:229–239.
- Henkart, P.A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity*. 1:343–346.
- Oehm, A., I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richards, J. Dhein, B.C. Trauth, et al. 1992. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J. Biol. Chem.* 10709–10715.
- Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*. 66:233–243.
- Loetscher, H., Y.-C.E. Pan, H.-W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer. 1990. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell*. 61:351–359.
- Schall, T.J., M. Lewis, K.J. Koller, A. Lee, G.C. Rice, G.H.W. Wong, T. Gatanaga, G.A. Granger, R. Lentz, H.

- Raab, et al. 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell*. 61:361–370.
7. Nophar, Y., O. Kemper, C. Brakebusch, H. Engelmann, R. Zwang, D. Aderka, H. Holtmann, and D. Wallach. 1990. Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R cloned using amino acid data of its soluble form, encodes for both the cell surface and a soluble form of the receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3269–3278.
 8. Sugarman, B.J., B.B. Aggarwal, P.E. Hass, I.S. Figari, M.A.J. Palladino, and H.M. Shepard. 1985. Recombinant human tumor necrosis factor- α : effects on proliferation of normal and transformed cells in vitro. *Science (Wash. DC)*. 230:943–945.
 9. Tartaglia, L.A., T.M. Ayres, G.H. Wong, and D.V. Goeddel. 1993. A novel domain within the 55 kd TNF receptor signals cell death. *Cell*. 74:845–853.
 10. Itoh, N., and S. Nagata. 1993. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. *J. Biol. Chem.* 268:10932–10937.
 11. Stamenkovic, I., E.A. Clark, and B. Seed. 1989. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1403–1410.
 12. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature (Lond.)*. 342:929–931.
 13. Gregory, C.D., C. Dive, S. Henderson, C.A. Smith, G.T. Williams, J. Gordon, and A.B. Rickinson. 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature (Lond.)*. 349:612–614.
 14. Valentine, M.A., and K.A. Licciardi. 1992. Rescue from anti-IgM-induced programmed cell death by the B cell surface proteins CD20 and CD40. *Eur. J. Immunol.* 22:3141–3148.
 15. Armitage, R.J., W.C. Fanslow, L. Strockbine, T.A. Sato, K.N. Clifford, B.M. Macduff, D.M. Anderson, S.D. Gimpel, T. Davis-Smith, C.R. Maliszewski, et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature (Lond.)*. 357:80–82.
 16. Hollenbaugh, D., L.S. Grosmaire, C.D. Kullas, N.J. Chalupny, A.S. Braesch, R.J. Noelle, I. Stamenkovic, J.A. Ledbetter, and A. Aruffo. 1992. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell costimulatory activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 11: 4313–4321.
 17. Graf, D., U. Korthäuer, H.W. Mages, G. Senger, and R.A. Kroccek. 1992. Cloning of TRAP, a ligand for CD40 on human T cells. *Eur. J. Immunol.* 22:3191–3194.
 18. Jabara, H.H., S.M. Fu, R.S. Geha, and D. Vercelli. 1990. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* 172:1861–1864.
 19. Clark, E.A., and J.A. Ledbetter. 1986. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. *Proc. Natl. Acad. Sci. USA*. 83: 4494–4498.
 20. Banchereau, J., P. de Paoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science (Wash. DC)*. 251:70–72.
 21. Barrett, T.B., G. Shu, and E.A. Clark. 1991. CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. *J. Immunol.* 146:1722–1729.
 22. Ranheim, E.A., and T.J. Kipps. 1993. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J. Exp. Med.* 177:925–935.
 23. Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grosmaire, R. Stenkamp, M. Neubauer, et al. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell*. 72:291–300.
 24. Allen, R.C., R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, C.M. Disteche, D.K. Simoneaux, et al. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science (Wash. DC)*. 259:990–993.
 25. DiSanto, J.P., J.Y. Bonnefoy, J.F. Gauchat, A. Fischer, and G. de Saint Basile. 1993. CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature (Lond.)*. 361:541–543.
 26. Korthäuer, U., D. Graf, H.W. Mages, F. Briere, M. Padayachee, S. Malcolm, A.G. Ugazio, L.D. Notarangelo, R.J. Levinsky, and R.A. Kroccek. 1993. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature (Lond.)*. 361:539–541.
 27. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*. 1:167–178.
 28. Xu, J., T.M. Foy, J.D. Laman, E.A. Elliott, J.J. Dunn, T.J. Waldschmidt, J. Elsemore, R.J. Noelle, and R.A. Flavell. 1994. Mice deficient for the CD40 ligand. *Immunity*. 1:423–431.
 29. Lane, P.J., J.A. Ledbetter, F.M. McConnell, K. Draves, J. Deans, G.L. Schieven, and E.A. Clark. 1991. The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. Inhibition of tyrosine phosphorylation prevents intracellular calcium release. *J. Immunol.* 146:715–722.
 30. Uckun, F.M., G.L. Schieven, I. Dibirdik, L.M. Chandan, A.L. Tuel, and J.A. Ledbetter. 1991. Stimulation of protein tyrosine phosphorylation, phosphoinositide turnover, and multiple previously unidentified serine/threonine-specific protein kinases by the Pan-B-cell receptor CD40/Bp50 at discrete developmental stages of human B-cell ontogeny. *J. Biol. Chem.* 266:17478–17485.
 31. Ren, C.L., T. Morio, S.M. Fu, and R.S. Geha. 1994. Signal transduction via CD40 involves activation of *lyn* kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase C γ 2. *J. Exp. Med.* 179:673–680.
 32. Hu, H.M., K. O'Rourke, M.S. Boguski, and V.M. Dixit. 1994. A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J. Biol. Chem.* 269:30069–30072.
 33. Mosialos, G., M. Birkenbach, R. Yalamanchili, T. VanArsdale, C. Ware, and E. Kieff. 1995. The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell*. 80:389–399.
 34. Sato, T., S. Irie, and J.C. Reed. 1995. A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. *FEBS Lett.* 358:113–118.
 35. Cheng, G., A.M. Cleary, Z.-s. Ye, D.I. Hong, S. Lederman, and D. Baltimore. 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science (Wash. DC)*. 267:1494–

- 1498.
36. Hart, D.N.J., and J.L. McKenzie. 1988. Isolation and characterization of human tonsil dendritic cells. *J. Exp. Med.* 168: 157–170.
 37. Galy, A.H., and H. Spits. 1992. CD40 is functionally expressed on human thymic epithelial cells. *J. Immunol.* 149:775–782.
 38. Paulie, S., B. Ehlin-Henricksson, H. Mellstedt, H. Koho, H. Ben-Aissa, and P. Perlmann. 1985. A p50 surface antigen restricted to urinary bladder carcinomas and B-lymphocytes. *Cancer Immunol. Immunother.* 20:23–28.
 39. Ledbetter, J.A., E.A. Clark, N.A. Norris, G. Shu, and I. Hellström. 1987. Expression of a Functional B-Cell Receptor CDw40 (Bp50) on Carcinomas. A.J. MacMichael, P.C.L. Beverly, W. Gilks, M. Horton, D.Y. Mason, S. Cobbold, F.M. Gotch, N. Ling, C. Milstein, H. Waldmann, et al., editors. Oxford University Press, Oxford. 432–435.
 40. Young, L.S., C.W. Dawson, K.W. Brown, and A.B. Rickinson. 1989. Identification of a human epithelial cell surface protein sharing an epitope with the C3d/Epstein-Barr virus receptor molecule of B lymphocytes. *Int. J. Cancer.* 43:786–794.
 41. Hess, S., A. Rensing-Ehl, R. Schwabe, P. Bufler, and H. Engelmann. 1995. CD40 function in nonhematopoietic cells. NF- κ B mobilization and IL-6 production. *J. Immunol.* 55: 4588–4595.
 42. Rensing-Ehl, A., S. Hess, H.W.L. Ziegler-Heitbrock, G. Riethmüller, and H. Engelmann. 1994. Fas/Apo-1 activates NF- κ B and induces IL-6 production. *J. Inflammation.* 45:161–174.
 43. Littlefield, J.W. 1964. Three degrees of guanylic acid-inosinic acid pyrophosphorylase deficiency in mouse fibroblasts. *Nature (Lond.)*. 203:1142.
 44. Todaro, J.G., H. Green, and M.R. Swift. 1966. Susceptibility of human diploid fibroblast strains to transformation by SV40 virus. *Science (Wash. DC)*. 153:1252–1254.
 45. Hess, S., R. Kurrle, L. Lauffer, G. Riethmüller, and H. Engelmann. 1995. A cytotoxic CD40/p55 tumor necrosis factor receptor hybrid detects CD40 ligand on Herpesvirus saimiri-transformed T cells. *Eur. J. Immunol.* 25:80–86.
 46. Mizushima, S., and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18:5322.
 47. Brockhaus, M., H.-J. Schoenfeld, E.J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher. 1990. Identification of two types of tumor necrosis factor receptors on different cell lines by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 87:3127–3131.
 48. Finter, N.B. 1969. Dye uptake methods for assessing viral cytopathogenicity and their application to interferon assays. *J. Gen. Virol.* 5:419–427.
 49. Wallach, D. 1984. Preparations of lymphotoxin induce resistance to their own cytotoxic effect. *J. Immunol.* 132:2464–2469.
 50. Lewis, M., L.A. Tartaglia, A. Lee, G.L. Bennett, G.C. Rice, G.H. Wong, E.Y. Chen, and D.V. Goeddel. 1991. Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc. Natl. Acad. Sci. USA.* 88:2830–2834.
 51. Rensing-Ehl, A., S. Hess, H.W.L. Ziegler-Heitbrock, G. Riethmüller, and H. Engelmann. 1994. Fas and TNF receptor p55 use different signalling pathways for cell death but activate NF- κ B via identical routes. *Eur. Cytokine Netw.* 5:105 (Abstr.).
 52. Wong, G.H., and D.V. Goeddel. 1988. Induction of manganese superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science (Wash. DC)*. 242:941–944.
 53. Kumar, S., and C. Baglioni. 1991. Protection from tumor necrosis factor-mediated cytotoxicity by overexpression of plasminogen activator inhibitor type-2. *J. Biol. Chem.* 266:20960–20964.
 54. Opipari, A.W., H.M. Hu, R. Yabkowitz, and V.M. Dixit. 1992. The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity. *J. Biol. Chem.* 267:12424–12427.
 55. Schulze-Osthoff, K., R. Beyaert, V. Vandevoorde, G. Haegeman, and W. Fiers. 1993. Depletion of the mitochondrial electron transport abolishes the cytotoxic and gene-inductive effects of TNF. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3095–3104.
 56. Schulze-Osthoff, K., P.H. Krammer, and W. Dröge. 1994. Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4587–4596.
 57. Clark, E.A., and J.A. Ledbetter. 1994. How B and T cells talk to each other. *Nature (Lond.)*. 367:425–428.
 58. Wong, G.H.W., and D. Goeddel. 1994. Fas antigen and p55 TNF receptor signal apoptosis through distinct pathways. *J. Immunol.* 152:1751–1755.
 59. Boldin, M.P., E.E. Varfolomeev, Z. Pancer, I.L. Mett, J.H. Camonis, and D. Wallach. 1995. A novel protein that interacts with the death of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.* 270:7795–7798.
 60. Hsu, H., J. Xiong, and D.V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF- κ B activation. *Cell.* 81:495–504.
 61. Chinnaiyan, A.M., K. O'Rourke, M. Tewari, and V.M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell.* 81:505–512.
 62. Stanger, B.Z., P. Leder, T.-H. Lee, E. Kim, and B. Seed. 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell.* 81:513–523.
 63. Enari, M., H. Hug, and S. Nagata. 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature (Lond.)*. 375:78–81.
 64. Los, M., M. Van de Craen, L.C. Penning, H. Schenk, M. Westendorp, P. Baeuerle, W. Dröge, P.H. Krammer, W. Fiers, and K. Schulze-Osthoff. 1995. Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. *Nature (Lond.)*. 375:81–83.
 65. Kägi, D., F. Vignaux, B. Ledermann, K. Birki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science (Wash. DC)*. 265:528–530.
 66. Williamson, B.D., E.A. Carswell, B.Y. Rubin, J.S. Prendergast, and L.J. Old. 1983. Human tumor necrosis factor produced by human B-cell lines: synergistic cytotoxic interaction with human interferon. *Proc. Natl. Acad. Sci. USA.* 80:5397–5401.
 67. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.
 68. Sato, T., S. Irie, S. Kitada, and J.C. Reed. 1995. FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science (Wash. DC)*. 268:411–415.