Recombinant TRAIL and TRAIL Receptor Analysis

Nicholas Harper* and Marion MacFarlane*

MRC Toxicology Unit,
Hodgkin Building,
University of Leicester,
Lancaster Road,
Leicester,
LE1 9HN

Tel: 44-116 252 5553/7; **Fax:** 44-116 252 5616;
Email (NH): nh19@le.ac.uk; **Email (MM):** mm21@le.ac.uk

*Corresponding Author

Running Title: TRAIL and TRAIL Receptors

Abstract

Death receptors are a subgroup of the Tumour Necrosis Factor Receptor Superfamily (TNFRSF) and mediate activation of what is widely known as the 'extrinsic' apoptosis pathway. TRAIL (Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand) is one of the most recent death receptor ligands identified. The TRAIL receptor family consists of four distinct membrane-bound receptors, named TRAIL-R1 to -R4. TRAIL-R1 and TRAIL-R2 belong to the 'death receptor' subfamily of the Tumour Necrosis Factor Receptor Superfamily (TNFRSF). Unlike other death receptor ligands, such as FasL/CD95L and TNF, TRAIL appears to display selective toxicity by killing tumour and transformed but not normal cells. Importantly, there also appears to be a complete lack of apparent toxicity, specifically hepatotoxicity, when TRAIL is used *in vivo*. Taken together, these observations led to TRAIL being proposed as a potential anti-tumor therapeutic, thus explaining the intense activity surrounding TRAIL and TRAIL receptor research over the past few years.

This chapter describes a number of methods for the production of recombinant TRAIL in E.coli followed by labelling of recombinant TRAIL with either biotin or fluorochromes. These recombinant TRAIL variants are then employed to study various aspects of TRAIL signalling from cell surface receptor levels to the composition of death receptor complexes. This combination of direct binding and functional analysis provides a very powerful approach to aid in further characterization of TRAIL/TRAIL receptor regulation and signalling.

Introduction

The TRAIL receptor family consists of four distinct membrane-bound receptors (reviewed in LeBlanc and Ashkenazi, 2003). TRAIL-R1 and TRAIL-R2 belong to the death receptor subfamily of the Tumour Necrosis Factor Receptor Superfamily (TNFRSF), which also includes TNF-R1 and Fas/CD95.

Like all death receptors, TRAIL-R1/-R2 contain an 80 amino acid motif within their cytoplasmic domain termed the death domain (DD) which is critically required for induction of cell death (Tartaglia et al., 1993). In contrast, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) either completely lack, or contain a truncated intracellular domain, and as a consequence are unable to initiate apoptotic signalling. These TRAIL receptors have therefore been proposed to act as 'decoy' receptors (Pan et al., 1997; Sheridan et al., 1997). Like other members of the TNFRSF, all four TRAIL receptors are also characterised by a series of cysteine-rich repeats within their extracellular domains.

Although there are several reports demonstrating that TRAIL can activate other signalling pathways such as the transcription factors NFkB and AP-1, its main function appears to be the induction of apoptosis (MacFarlane et al., 2000; Harper et al., 2001; MacFarlane, 2003; Varfolomeev et al., 2005).

TRAIL induces apoptosis by binding to either TRAIL-R1 or TRAIL-R2, which then leads to receptor aggregation and the recruitment of the adaptor protein FADD to the cytoplasmic DD motif of the receptors. FADD in turn recruits the initiator procaspase-8 to form the death-inducing signalling complex (DISC)(reviewed in Peter and Krammer, 2003). The "enforced proximity" of procaspase-8 molecules within the DISC leads to autoactivation resulting in its subsequent maturation and release into the cytoplasm. Activated caspase-8 is then able to process the effector caspases -3 and -7 as well as activating the proapoptotic Bcl-2 homolog, Bid. Bid, in turn, acts as a mitochondrial amplification signal, necessary in some cell types for induction of cell death (Li et al., 1998).

TRAIL is of particular interest as a potential therapeutic agent as it appears to display selective toxicity, inducing apoptosis in tumour cells but not the majority of normal cells (Pitti et al., 1996; Wiley et al., 1995). This has led to a number of clinical trials using either TRAIL ligand (Apo2L/TRAIL – Genentech, South San Francisco, CA) or agonistic antibodies directed against either TRAIL-R1 or –R2 (HGS-ETR1 and HGS-ETR2 - Human Genome Sciences, Rockville, MD) for induction of cell death. Of those primary malignancies that have been demonstrated to be resistant to TRAIL and TRAIL receptor agonistic antibodies (MacFarlane et al., 2002), a number can be sensitised when some of these reagents are used in combination with other potential chemotherapeutics such as proteasome inhihitors or histone deacetylase inhibitors (Inoue et al., 2004; Koschny et al., 2007; Dyer et al., 2007).

A fuller understanding of TRAIL signalling in a particular cell type relies on a combination of both functional assays in intact cells and robust TRAIL-TRAIL-Receptor binding assays. This chapter describes complementary methods for the study of TRAIL signalling, all of which rely on the successful generation of recombinant TRAIL. The application of these various methods to evaluate TRAIL-induced apoptosis, TRAIL receptor expression levels, TRAIL DISC composition, and TRAIL-TRAIL-Receptor internalization is discussed.

Generation of Recombinant TRAIL

TRAIL, unlike a number of other TNFSF members, appears to function as a ‘soluble’ protein and as a consequence can be produced relatively easily in large quantities by expression in *E-coli*. CD95L by comparison, is insoluble when expressed in bacteria and appears to require post-translational modification, specifically glycosylation, in order to aid solubility (Peter et al., 1995, and unpublished results). In addition, ‘soluble’ CD95L requires artificial oligomerization for activity. As a result, commercial preparations of CD95L are synthesised as soluble, secretable fusion proteins expressed in mammalian cells. Crosslinking is provided either by an internal oligomerization tag, such as a leucine zipper, or through cross-linking of the

ligand with antibodies directed against an additional common peptide tags such as the Flag peptide.

TRAIL, like other TNFSF members, is a type I transmembrane protein therefore production in E. coli requires removal of both the transmembrane and intracellular domains of the protein in order to aid solubilty. Truncated TRAIL (MacFarlane et al., 1997) or receptor-selective TRAIL mutants (MacFarlane et al., 2005) (residues 95-281), cloned N-terminally in the plasmid expression vector, pet28 (Novagen, Nottingham UK), are fused 'in-frame' with a hexa-histidine tag to allow for a relatively simple purification protocol on nickel or cobalt-charged affinity resins.

Protocol 1: Induction of His-tagged TRAIL in E.coli using IPTG

1. E.coli BL-21 DE3 (pLys) are transformed with pet28-TRAIL using standard laboratory protocols.

2. For induction of recombinant TRAIL protein, a 10 ml culture in LB-medium, containing Kanamycin (25 ug/ml), is inoculated and then grown overnight at 37 °C with constant shaking.

3. The following day this culture is used to initiate a larger (400 ml) culture required for protein induction.

4. The culture is grown to an OD of approximately ~0.6 (log phase), then protein is induced using isopropylthiogalactosidase (IPTG) (1 mM) for 3 h at 27 °C. A sample is taken prior to the addition of IPTG (uninduced) in order to assess the efficiency of protein induction compared to uninduced cultures.

5. Bacterial pellets are then collected by centrifugation at 5000 rpm for 15 min and washed x1 with ice-cold PBS. Pellets can then be further processed or stored at -80 ºC until required.[1]

Protocol 2: Purification of His-TRAIL using a Nickel affinity resin.

1. Previously frozen or freshly prepared, induced bacterial pellets (from 400 ml of culture) are first thawed, then lysed in a Triton lysis buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100) containing Complete™ protease inhibitors without EDTA[2] (Roche, Sussex UK) for 30 min on ice. To ensure full lysis, the bacteria can be sonicated on ice.

2. Lysates are then cleared by centrifugation at 13,000 rpm for 30 min at 4 °C then supplemented with 500 mM imidazole to a final concentration of 20 mM.

3. 6xHis-tagged TRAIL is then purified by batch purification[3] using Ni^{2+}-NTA agarose beads (Qiagen, Sussex UK) for 1.5 h at 4 ºC.

4. Beads are washed with several bed volumes of lysis buffer.

5. Bound TRAIL is then eluted from the beads using either 150 mM EDTA or 100 mM Imidazole.

A typical TRAIL purification is shown in Figure 1. After elution, a small sample of each elution fraction is removed for analysis and individual fractions frozen at -80 ºC. After analysis of protein content (using a suitable protein assay reagent) and purity (by SDS-PAGE), those fractions containing the highest purified yield are pooled and then aliquoted. Any precipitate that may have formed following freeze-thawing of the individual fractions can be removed by centrifugation prior to pooling and aliquoting.

The protein content of the final aliquots is again checked, as is the apoptosis-inducing activity of purified TRAIL on a suitable TRAIL-sensitive, tumour cell line (see Protocol 3). Recombinant TRAIL can then be successfully stored at -80 ºC for a number of years with no noticeable loss in activity.

Assessment of the apoptosis-inducing activity of recombinant TRAIL using Annexin V

The apoptosis-inducing activity of recombinant TRAIL can be conveniently assessed by treatment of a TRAIL-sensitive cell line, such as BJAB or Jurkat, with a range of concentrations of recombinant TRAIL and incubation times of up to 6 h at 37^0C. The extent of apoptosis can then be quantified by Annexin V labelling of apoptotic cells followed by FACs analysis. Annexin V is a vascular protein which binds in a Ca^+-dependent manner to the phospholipid, phosphatidylserine, which is present on the external cell membrane of apoptotic cells (Martin et al., 1995). Below, is outlined a method for Annexin V-FITC labelling of a haematopoetic cell line, grown in suspension, using a commercially available form of Annexin V-FITC routinely employed within our laboratory.

Protocol 3: Labelling of Apoptotic cells using Annexin V-FITC.

1. Cells ($1x10^6$ cells), either left untreated, or exposed to TRAIL at 37 ^{0}C for varying times are resuspended in 1 ml Annexin buffer (10mM HEPES/NaOH.pH 7.4, 150mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$) using tubes suitable for flow cytometry (BD Falcon, Cat # 353052).

2. 1.5 μl Annexin V-FITC (eg. Bender Medsystems, cat # BMS306FI) is added to the cell suspension and cells are then incubated for 8 min at room temperature (~22 ^{0}C).

3. At the end of the incubation time, cells are placed on ice. 2 μl propidium iodide (PI; 50 μg/ml PBS) is added, and the cell suspension then incubated on ice for 2 min.

4. Samples are stored on ice and the extent of apoptosis assessed by FACs analysis using a Becton Dickinson FACsCalibur (or equivalent), using excitation/emission wavelengths of 488nm/519nm for Annexin V-FITC (FL1) and 488nm/575nm for PI (FL2). A minimum of 10,000 events are acquired (flow rate ~200 events/sec) and cells are analysed using CellQuest Pro software (Figure 2).

5. Cells are initially analysed by dot-plot analysis of FSC (Forward Scatter) versus SSC (Side Scatter) and the main cell population (Region 1=R1) then further analysed by dot-plot analysis of Propidium Iodide (FL2) versus Annexin V-FITC (FL1). A quadrant marker is then used to quantify the number of cells in the population that are either: non-apoptotic (AnnexinV^-/PI^-: lower left quadrant), apoptotic (Annexin V^+/PI^-: upper left quadrant), or 'secondary' necrotic (Annexin V^+/PI^+:upper right quadrant).

The above method should be carefully optimized for each individual cell line in terms of the amount of Annexin V-FITC used and the optimal incubation time required to obtain differential labelling of Annexin V^+ versus Annexin V^- cells. For example, differential labelling of Jurkat T cells requires only 1.5 μl Annexin V-FITC and an incubation time of 8 min at room temperature, while the cell line, BJAB, requires up to 15 μl Annexin V-FITC and an incubation time of 30 min.

The above Annexin V-labelling method can also be adapted for use on adherent cells (such as MCF-7 cells) exposed to TRAIL (or other apoptotic stimuli). In this case, the adherent cell culture supernatant (containing apoptotic cells that have lost adherence) is carefully retained and the remaining adherent cells collected by gentle trypsinization. These two cell

populations are then combined, and immediately followed by a recovery period of at least 20 min at 37 ^{0}C in complete medium (to allow re-sealing of the outer cell membrane). Cells are then gently pelleted by centrifugation and resuspended in Annexin buffer for staining of apoptotic cells with Annexin V-FITC/ PI as outlined above (Protocol 2; Step 1 onwards).

Labelling of Recombinant TRAIL

One of the benefits of generating ligands, such as TRAIL 'in-house' is that they can be produced in sufficient quantities to allow subsequent modification of the purified ligand depending on the specific application required.

Described below are two such recombinant TRAIL modifications, together with methods that rely on the use of a suitably labelled ligand. Many modifiying reagents that are employed to label proteins, such as TRAIL, do so non-specifically (e.g. N-hydroxysuccinamide esters which label primary amino groups lysine and N-terminal). It should be noted that, we have rarely seen decreased activity following TRAIL modification using these reagents. Care should however be taken to titrate those labelling reagents used in order to ensure that the activity and receptor binding properties of the subsequent TRAIL-conjugates is retained.

In order to retain the biological activity of the subsequent labelled ligand, we have essentially modified the ligand purification protocol to allow the labelling reactions to be performed with recombinant TRAIL still immobilised on agarose beads (see Protocol 2). Although this may not be ideal to accurately control the extent of labelling, its benefit is in obviating the need for extensive dialysis to remove any unincorporated label thereby retaining the biological activity of the labelled ligand. Subsequent to purification and labelling, methods are also available to assess the extent of label-incorporation per mg of purified protein, thus helping to retain consistency between batches of labelled TRAIL protein.

Protocol 4: Preparation of Biotin-labelled TRAIL

Biotin binds with very high affinity to both avidin and streptavidin. This makes it an ideal tag for either capture of biotinylated proteins on avidin/streptavidin conjugated to insoluble supports (agarose or Sepharose^{TM} beads), or for detection/labelling of biotinylated proteins using the many avidin/streptavidin conjugates that are commercially available.

The most common biotin labelling reagents use amine-reactive chemistry to label primary amino groups in proteins (N-terminus and lysines). Importantly, due to its relatively small size (~250 Da), incorporation of biotin in general does not significantly affect the biological activity of the labelled protein.

1. As mentioned above, we label TRAIL as part of the standard purification protocol, primarily to avoid the need for extensive dialysis protocols and in order to retain the maximum amount of biological activity.

2 TRAIL is initially purified as outlined in Protocol 2 (Steps 1-4, only), except that the ligand is then retained immobilized on Ni-NTA-agarose beads in lysis buffer. Due to the presence of primary amines in this buffer (which will interfere with labelling) the agarose beads are then extensively washed with PBS or a bicarbonate buffer at 4 ºC (pH 7.5-9.0). The beads are then resuspended in 1 ml of PBS (or bicarbonate buffer).

3. Immediately prior to labelling, a solution of biotinylaminocaproic acid-N-hydroxysuccinimide ester (Roche, Sussex UK) is dissolved in DMSO (20 mg/ml). 5-20 ul of this solution is added to the beads which are then labelled for 1 h at 4 ºC on an end-to-end shaker[4]. Note, any excess labelling solution should be discarded due to the labile nature of the active group.

4. Following biotin labelling, excess labelling reagent is quenched by the addition of Tris (pH 8.0) to a final concentration of 1M for an additional 15 min. Beads are then washed with lysis buffer and recombinant

biotin-TRAIL eluted as before (Protocol 2, Step 5), using either EDTA or imidazole.

Biotinylated TRAIL is then aliquoted and stored as described above (Protocol 2). Prior to use, biotin-TRAIL must be checked to confirm both its apoptosis-inducing activity (Protocol 3) and the incorporation of biotin. Successful labelling with biotin can be determined by Western blotting. Essentially, a small amount of biotin-labelled TRAIL is subjected to SDS-PAGE followed by Western blotting. Following blocking of the transfer membrane, biotinylated TRAIL can be detected by a short incubation with streptavidin conjugated to horseradish peroxidise, followed by detection by chemiluminescence.

If necessary, the extent of biotin-incorporation can also be assessed using HABA (4´-hydroxyazobenzene-2-carboxylic acid). Free biotin displaces HABA–from avidin causing a decrease in absorbance at 500 nm which is measured and compared to a standard curve in order to determine molar incorporation of biotin following labelling (Janolino, 1996).

Many other biotinylation reagents are available from companies such as Roche and Perbio Science UK Ltd, Northumberland UK. Reagents with longer/shorter linkages, thiol/photo-cleavable linkages or containing different reactive moieties e.g. carboxyl or carbohydrate are available depending on the end-user's requirements (Savage et al., 1992).

Protocol 5: Labeling TRAIL using Fluorochromes

The protocol we have developed for labelling recombinant TRAIL with fluorochromes is essentially a further modification of the standard TRAIL purification protocol (Protocol 2). In our laboratory, we have used the Alexa Fluor® 647 protein labelling kit (Molecular Probes, Paisley, UK), which is primarily designed for antibody labelling, but in practice will label any protein. The reactive dye has a succinamidyl ester moiety which reacts with primary amines (essentially using the same chemistry as the biotin-labelling reagent

used previously in Protocol 4). Again, in order to maintain biological activity, TRAIL is labelled while still immobilized on the Ni-NTA-agarose beads during the purification procedure.

1. Recombinant TRAIL is purified as per Protocol 2 (Steps 1-4 only).

2. As before, beads are washed several times with lysis buffer followed by extensive washing with PBS (or bicarbonate buffer) and then resuspended in 1 ml of PBS.

3. The Alexa Dye is warmed to room temperature and dissolved in a small volume of PBS. Beads are then resuspended in 1 ml of PBS and a range of different volumes of dye (eg. 5-20 μl) added to each tube.

4. TRAIL is then labelled for 1 h at 4 ºC on an end-to-end shaker and beads then washed in PBS. Beads are then eluted using either EDTA or imidazole (Protocol 2, Step 5). Due to the lability of the Alexa-647-TRAIL, Alex-labelled TRAIL should be stored in the dark in smaller aliquots than the unlabelled or biotin-labelled TRAIL variants. It should be noted, however, that we have not found any stability issues when Alexa-647-TRAIL is stored aliquoted in black-walled tubes at -80 ºC.

Prior to use, Alexa-Fluor-TRAIL must first be checked to confirm biological activity. Due to the 650 nm emission wavelength of the Alexa-Fluor-647 dye (FL4), there is no direct interference with either FITC-labelled Annexin V (FL1) or propidium iodide (FL2), therefore the Annexin V staining protocol and FACS analysis (Protocol 3) can be again be employed.

Using this particular fluorochrome labelling reagent, we have never observed any reduction in biological activity due to over-labelling. However, significant under-labelling with Alexa-647 will result in either a weak, or no, fluorescent signal. To maintain consistency from batch-to-batch, the degree of labelling can be determined by measuring the absorbance of the TRAIL conjugate at

both 280 nm (protein) and 650 nm (fluorescent dye). Then by correcting for A_{280} of the Alexa dye (A_{280} - (0.03 x A_{650}), and assuming an approximate extinction coefficient for the His-tagged TRAIL of 25,580 $cm^{-1}M^{-1}$, the protein concentration of Alexa-647-TRAIL can be determined. The degree of labelling can then be calculated using the following formula: moles dye/moles of protein = A_{650}/ 239,00 x protein concentration. (239,00 $cm^{-1}M^{-1}$ is the approximate molar coefficient of Alexa 647 at 650 nm)

Flow Cytometric Analysis of TRAIL Receptors

Protocol 6A: Using TRAIL Receptor-Specific Antibodies

What follows is a series of protocols for staining of TRAIL receptors using a number of reagents obtained from eBiosciences (San Diego, USA) which we have found work well in our hands. The protocols can of course be modified for use with alternative receptor-labelling reagents. The following reagents; Phycoerythrin (PE)-conjugated anti-TRAIL-R1 (12-6644), PE-conjugated anti-TRAIL-R2 (12-9908) and a PE-Conjugated isotype-matched control antibody (12-4714) were all obtained from eBiosciences.

At least $1x10^6$ cells/cell line, or specific treatment, is required. For staining, this cell population is then divided equally into 4 reactions: Control - no antibody; Isotype control - isotype-matched antibody; anti-TRAIL-R1; and anti-TRAIL-R2. All subsequent steps are performed with the cells on ice and/or solutions at 4 ºC, unless indicated otherwise.

1. Cells are collected by centrifugation and resuspended in fresh medium at a concentration of 1 $x10^6$/ml.

2. 250 ul of cell suspension is aliquoted into 4 individual flow cytometry tubes. Cells are then pelleted by centrifugation, resuspended in the indicated volume of normal goat serum (Control - 50 ul, Isotype control-

40 ul, anti-TRAIL-R1 - 40 ul, and anti-TRAIL-R2 - 45 ul), and left to recover on ice for 5 min.

3. The following volume of each fluorochrome-conjugated antibody is then added to the appropriate tube: Isotype control - 10 ul; anti-TRAIL-R1 - 10 ul; -anti-TRAIL-R2 - 5ul. Cells are incubated on ice for 1 h in the dark.

4. Cell are then washed 3x with PBS and analysed by flow cytometry (FACSCalibur, Becton Dickinson) using excitation and emission wavelengths of 488 nm and 575 nm, respectively (FL2). A typical analysis of cell surface expression of TRAIL-R1 and TRAIL-R2 in

Protocol 6B: Using Receptor-specific Antibodies to assess 'Cell Surface' Expression of TRAIL Receptors in Adherent Cells

For determination of 'cell surface' expression of TRAIL-R1 or TRAIL-R2 in adherent cells, a modification of the above protocol is required:

1. Adherent cells are first removed from the plastic matrix by gentle agitation in a mild solution of Trypsin and EDTA (essentially following conventional protocols).

2. As before, (Protocol 6A), at least $1x10^6$ cells/cell line, or specific treatment, is required.

3. Cells are then centrifuged, resuspended in fresh ‘complete’ medium, and then left to recover at 37 ºC for 30 minutes (in order to allow for recovery of the cells after gentle trypsinisation).

4. 250 ul of cell suspension is then aliquoted into 4 individual flow cytometry tubes. Following centrifugation cells are resuspended in

normal goat serum, incubated on ice for 5 min, and then stained as described previously in Protocol 6A (Step 3, onwards).

When initial studies were performed on the subcellular localization of TRAIL receptors following TRAIL receptor stimulation it was found that the majority of receptors exhibited an intracellular, specifically perinuclear, localization with re-localization occurring following TRAIL stimulation (Zhang et al., 2000). In view of these findings, it is therefore advisable to also assess total cellular TRAIL receptor expression as well as cell surface levels. By fixing and permeabilizing cells prior to receptor antibody staining, the above protocol can be modified to measure 'total' TRAIL receptor expression i.e. both cell surface and the intracellular pool. Essentially, the difference between the mean fluorescence intensities of the 'cell surface' and 'total' TRAIL receptor expression levels can then provide some indication as to the contribution of the 'intracellular' TRAIL receptor pool. To successfully label the ‘total’ TRAIL receptor pool, cells are first fixed in formalin (10 %) for 20 min at room temperature. Following centrifugation, cells are washed x1 in PBS and then permeabilised by incubating for 5 min at room temperature in 0.5 ml PBS, containing 0.1 % saponin and 1 % BSA. The permeablized cells are then equally divided between 4 flow cytometry tubes (125 ul/tube), centrifuged, then resuspended in normal goat serum and stained for TRAIL-R1 and TRAIL-R2 expression as described in Protocol 6A (Step 2 onwards).

It is important to note that, using receptor-specific antibodies, the relative expression levels of an individual TRAIL receptor can only be reliably compared across various treatments within the same cell line, or across different cell lines. However, due to the potentially different binding affinities of each individual TRAIL receptor-specific antibody, care should be taken when comparing the relative levels/ratios of different TRAIL receptors (even within the same cell line).

The cell surface expression of TRAIL-R1 and TRAIL-R2 in BJAB cells, assessed by flow cytometry using receptor-specific antibodies, is shown in Figure 3A. This data demonstrates that BJAB cells express significant levels

of both TRAIL-R1 and TRAIL-R2 at the cell surface. Based on this data alone, one might be tempted to predict that this particular cell line is sensitive to TRAIL. However, it is now clear that cell surface expression levels of TRAIL receptors does not always correlate with, or indeed reliably predict, sensitivity to TRAIL. Consequently, several other measures, such as DISC analysis and a functional assessment of apoptosis are required in order to fully characterize each individual cell line in terms of TRAIL sensitivity.

Protocol 7: Using TRAIL conjugated to Fluorochromes

Many pharmacologic agents have been shown to sensitize cells to TRAIL by upregulating cell surface TRAIL receptor expression (Ganten et al., 2004). Using Alexa Fluor© -647-conjugated TRAIL (TRAIL-AF647), as generated in Protocol 5, the TRAIL binding capacity of a cell can be conveniently measured by flow cytometry. In order to avoid dissociation of the fluorescently labelled ligand from the receptors, or internalization of the ligand-associated receptor complex, cells should first be 'loaded' with Alexa-conjugated TRAIL on ice.

1. To obtain both a convenient and direct measure of the TRAIL binding capacity of BJAB cells, cells (1×10^6 cells) are 'loaded' with TRAIL-AF647 (1 ug/ml), or medium alone, for 1 h on ice.

2. Cells are then washed x2 with ice-cold PBS and the extent of TRAIL-AF647 binding (to cell surface receptors only), is then immediately assessed by flow cytometry (see Step 7).

Alternatively, fluorescently-labelled TRAIL, in conjunction with FACS analysis, can be instead be used to directly assess cellular uptake of recombinant TRAIL, thus providing a quantitative measure of ligand internalization:

3. In this case, BJAB cells (1×10^6 cells) are incubated either at 4 ºC, in the presence or absence of TRAIL-AF647 (to block endocytosis), or at

37 ºC in the presence of ligand (TRAIL-AF647; 1 ug/ml) for the indicated times to assess TRAIL internalization.

4. Samples are then rapidly chilled on ice (to inhibit endocytosis) and pelleted by brief centrifugation at 4 ºC

5. Following x2 washes with pre-chilled 'wash buffer' (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1mM $CaCl_2$, 1 mM $MgCl_2$), cell surface-associated ligand is then efficiently removed by resuspension in pre-chilled 'acid wash' solution (0.2M NaCl, 0.2 M acetic acid) for 5 min on ice.

6. Following x3 wash in 'wash buffer', cells are resuspended in ice-cold PBS containing 2 % (w/v) fetal bovine serum.

7. Either TRAIL-AF647 binding (Steps 1-2 only) or TRAIL-AF647 uptake/internalization (Steps 3-6 only) should then be analysed immediately by flow cytometry, using excitation and emission wavelengths of 635 nM and 668 nM (FL4), respectively.

A typical analysis of TRAIL receptor-mediated internalization of recombinant TRAIL in BJAB cells, assessed by flow cytometry using Alexa-Fluor© 647-conjugated TRAIL, is shown in Figure 3B. Cells were incubated with TRAIL-AF647, either on ice to block endocytosis, or at 37 ºC for increasing times to permit internalization. Increasing the temperature to 37 ºC results in a rapid internalization of TRAIL, with uptake detected at 10 min and proceeding for up to 40 min. Using this method, concentrations of TRAIL as low as 50 ng/ml resulted in uptake of detectable TRAIL (Kohlhaas et al., 2007).

Assessment of Receptor-mediated TRAIL Internalization using Biotinylated TRAIL

The role of receptor internalization in death receptor signalling is controversial with some studies showing that it is required for both TNF-R1 and CD95 -

mediated apoptosis, but not for TRAIL-mediated apoptosis (Schneider-Brachert et al., 2004; Lee et al., 2006; Kohlhaas et al., 2007). Internalization of TRAIL can be conveniently assessed using a combination of biotinylated-TRAIL together with an appropriate Alexa-Fluor© Straptavidin conjugate (Molecular Probes)

Protocol 8: Internalization of Biotinylated TRAIL analysed by Confocal Microscopy

In order to prevent 'inappropriate/premature' internalization, it is imperative that cells are always kept on ice during the initial ligand 'loading' phase. Once 'loaded', cells can then be washed (in pre-warmed medium) and brought up to 37 ºC to permit internalization and start the experiment. After the required time periods at 37 ºC, internalization is then 'stopped' by returning cells to ice.

1. BJAB cells (1 x 10^6 cells) are first chilled on ice for 1 h prior to 'loading' up with ligand.

2. Cells are then 'loaded' with biotinylated TRAIL (500 ug/ml) or medium alone (Control) for 45 min on ice.

3. Following x3 washes with ice-cold PBS (to remove excess TRAIL) cells are stained with Alexa Fluor© 568-conjugated streptavidin (AF568) for 1 h on ice.

4. Cells are then either, immediately adhered to poly-L-lysine-coated slides and fixed (see Step 5) after treatment at 4^0C (0 min, Control), or biotin-TRAIL is allowed to internalize by rapidly switching the cells to 37 ºC (usually by resuspending the cells in pre-warmed medium). Internalization is then ‘stopped’ at the indicated time-points by returning the cells to ice, and excess biotin-TRAIL removed by washing at 4^0C. If required, cell surface bound ligand can be removed using a mild acid wash (0.2 M acetic acid in PBS) prior to analysis.

5. Cells are then adhered to poly-lysine-coated slides and fixed in 4% paraformaldehyde for 10 min at room temperature. After x3 washes in PBS, nuclei are counterstained with the DNA dye, Hoechst 33342 (1 µg/ml) for 5 min.,

6. Biotin-TRAIL internalization is then analysed using a Zeiss LSM510, with Axiovert 200, confocal microscope.

A typical analysis of receptor-mediated internalization of biotinylated TRAIL in BJAB cells, visualised by confocal microscopy, is shown in Figure 3C. Cells loaded at 4^0C show clear labelling of TRAIL on the cell surface, and no biotin-TRAIL appears to be internalized (Fig. 3C, 0 min). Upon warming the cells to 37 ºC, internalization of TRAIL is observed as rapidly as 5 min and is further increased by 15 min, with marked staining of small vesicles (Fig. 3C). More complete internalization has occurred by 30 min, with increased vesicular staining concentrated in larger intracellular compartments.

It should be noted that, in cells that express significant amounts of cell surface TRAIL receptors there is also the possibility that Alexa Fluor-conjugated-TRAIL (as produced in Protocol 5) could be used, in conjunction with confocal microscopy, to directly probe TRAIL/TRAIL receptor internalisation.

Isolation of TRAIL receptor complexes

Upon ligand binding the receptors bind adaptor molecules such as FADD through a death domain motif in their intracellular domain. FADD, a bipartite molecule, in turn binds the apical procaspase-8 through its death-effector domain motifs. Caspase-8 is then thought to be activated through a proximity-induced effect which relies on adjacent procaspase-8 molecules (Muzio et al., 1998). This receptor-bound cell surface complex is known as the death-inducing signalling complex or DISC. In our laboratory we have successfully employed biotin-labelled TRAIL to isolate the TRAIL DISC (Harper et al., 2001; Harper et al., 2003)

Protocol 9: TRAIL DISC Analysis

The number of cells required for DISC analysis is dependent on cell type.

Generally, we use ~50 x 10^6 cells for each DISC isolation.

1. Cells are treated with biotin-labelled TRAIL (500 ng/ml) for the required time periods at 37 ^{0}C (to optimally assess TRAIL DISC formation) then cells are immediately washed x2 with ice-cold PBS.

2. Cells are then immediately lysed (3ml lysis buffer/50 x 10^6 cells), in a Triton lysis buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100) containing Complete™ protease inhibitors (Roche, Sussex UK), for 30 min on ice.

3. Lysates are then clarified by centrifugation at 13,000 rpm for 30 min and the supernatants collected.

4. Biotinylated TRAIL-bound complexes can then be simply precipitated overnight using streptavidin-Sepharose™ beads (beads should be prewashed with lysis buffer) at 4 ^{0}C.

5. Beads are then washed with several volumes of fresh lysis buffer, again containing Complete™ protease inhibitors, and isolated complexes are then solubilised directly in SDS sample buffer.

A typical TRAIL DISC, isolated from BJAB cells and analysed by SDS-PAGE and Western blotting, is shown in Figure 4. Using receptor specific antibodies and FACs analysis we previously demonstrated that BJAB cells express significant cell surface levels of both TRAIL-R1 and TRAIL–R2 (Fig. 3A). Here we show that, exposure of BJAB cells to TRAIL at 37 ^{0}C results in rapid formation of a DISC, with binding of TRAIL-R1 and TRAIL-R2 together with recruitment of FADD and procaspase-8 evident within 5 min (Fig. 4).

Caspase-8 is in turn partially processed to its p43/p41 fragments, and a significant amount of its catalytically active large subunit is also detectable by 15 min. By 60 min, although significant amounts of TRAIL-R1 and -R2 are still bound to biotin-TRAIL, much less FADD and caspase-8 are present, presumably because these proteins have already dissociated from the TRAIL/TRAIL receptor complex.

A number of commonly used cell types express extremely low levels of TRAIL receptors (e.g. Jurkat T cells). In these cells we have often failed to detect TRAIL receptors directly by Western blotting of whole cell lysates. This is either due to poor affinity of some commercially available TRAIL receptor antibodies on Western blots or, more likely, the low level of expression of TRAIL receptors in these cells. In these particular cell types, some form of initial 'concentration' step is therefore required, prior to TRAIL receptor detection by Western blotting.

In our laboratory, we have addressed this issue by routinely using biotin-TRAIL to essentially 'concentrate' all TRAIL receptors from unstimulated cell lysates onto streptavidin beads, prior to SDS-PAGE and Western blot analysis. Importantly, fewer cells are required for this than for 'conventional' DISC analysis, however it does allow for the analysis of TRAIL receptors from many more cells than a crude cell lysate would allow. In this respect, it should also be noted that incubation of live cells at 4 ^{0}C or 37 ^{0}C with biotinylated TRAIL, followed by washing and cell lysis, results in TRAIL binding of only cell surface-associated TRAIL receptors. On the other hand, the addition of biotin-TRAIL to pre-cleared, unstimulated, cell lysates, as described above, results in precipitation of the 'total' cellular TRAIL receptor pool (both cell surface and intracellular TRAIL receptors).

There appears to be a critical requirement for aggregation of death receptors in order to induce signalling and caspase-8 activation (Boldin et al., 1995; Martin et al., 1998). Following TRAIL treatment, TRAIL receptors have been shown to form SDS-stable aggregates (Kischkel et al., 1995). These receptor complexes can also be isolated by DISC precipitation (Protocol 9) and can

then be ‘crudely’ analysed either by conventional SDS-PAGE under non-reducing conditions or by gel filtration column chromatography.

Concluding Remarks

The ability to generate biologically active recombinant TRAIL, as well as biotin or fluorescently -labelled TRAIL variants, has provided our laboratory with tools that have proved invaluable in the assessment of TRAIL/TRAIL receptor signalling in tumour cell lines and primary tumour cells. As described above, an assessment of cell surface TRAIL receptor expression alone, in most cases, gives little/no indication of TRAIL sensitivity. However, by using a combination of the standard protocols listed above, an assessment of cellular sensitivity to TRAIL, TRAIL DISC analysis and TRAIL-TRAIL receptor complex internalization can be routinely assessed, thus providing key information on TRAIL signalling in a particular cell type.

Finally, it is important to note that, many of the protocols presented here can also be applied to study the biological function of other TNF family members.

Acknowledgements:

Work described here was supported by the Medical Research Council at the MRC Toxicology Unit in Leicester. Many thanks go to past and present members of the Apoptosis Laboratory, 416.

In particular, we wish to thank Prof. Gerald M Cohen for his continued support, Drs Andrew Craxton, Susan Kohlhass, Michael Pinkoski, and Laura Hall for permission to use previously published or unpublished data, and Davina Twiddy for help with TRAIL receptor antibody staining protocols.

Footnotes:

[1] IPTG-induced bacterial pellets have successfully been stored at -80 °C for many years without any apparent effect on the stability/activity of the subsequently isolated recombinant TRAIL

[2] EDTA will interfere with binding to the Nickel-containing affinity resin

[3] TRAIL purification can be automated using any of the available prepacked IMAC columns

[4] Although many reagents suggest that the labelling reaction should be carried out at room temperature we have observed that sufficient labelling occurs at 4 ºC.

References

Boldin, M. P., Mett, I. L., Varfolomeev, E. E., Chumakov, I., Shemer-Avni, Y., Camonis, J. H., and Wallach, D. (1995). Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J Biol Chem* **270,** 387-91.

Dyer, M. J., MacFarlane, M., and Cohen, G. M. (2007). Barriers to effective TRAIL-targeted therapy of malignancy. *J Clin Oncol* **25,** 4505-6.

Ganten, T. M., Haas, T. L., Sykora, J., Stahl, H., Sprick, M. R., Fas, S. C., Krueger, A., Weigand, M. A., Grosse-Wilde, A., Stremmel, W., Krammer, P. H., and Walczak, H. (2004). Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs. *Cell Death Differ* **11 Suppl 1,** S86-96.

Harper, N., Farrow, S. N., Kaptein, A., Cohen, G. M., and MacFarlane, M. (2001). Modulation of tumor necrosis factor apoptosis-inducing ligand-induced NF-kappa B activation by inhibition of apical caspases. *J Biol Chem* **276,** 34743-52.

Harper, N., Hughes, M. A., Farrow, S. N., Cohen, G. M., and MacFarlane, M. (2003). Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. *J Biol Chem* **278,** 44338-47.

Inoue, S., MacFarlane, M., Harper, N., Wheat, L. M., Dyer, M. J., and Cohen, G. M. (2004). Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. *Cell Death Differ* **11 Suppl 2,** S193-206.

Janolino, V. G. e. a. (1996). A spectrophotometric assay for biotin-binding sites of immobilized avidin. . *App. Biochem. and Biotech.* **56,** 1-7.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14,** 5579-88.

Kohlhaas, S. L., Craxton, A., Sun, X. M., Pinkoski, M. J., and Cohen, G. M. (2007). Receptor-mediated endocytosis is not required for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *J Biol Chem* **282,** 12831-41.

Koschny, R., Holland, H., Sykora, J., Haas, T. L., Sprick, M. R., Ganten, T. M., Krupp, W., Bauer, M., Ahnert, P., Meixensberger, J., and Walczak, H. (2007). Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Clin Cancer Res* **13,** 3403-12.

LeBlanc, H. N., and Ashkenazi, A. (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* **10,** 66-75.

Lee, K. H., Feig, C., Tchikov, V., Schickel, R., Hallas, C., Schutze, S., Peter, M. E., and Chan, A. C. (2006). The role of receptor internalization in CD95 signaling. *Embo J* **25,** 1009-23.

Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94,** 491-501.

MacFarlane, M. (2003). TRAIL-induced signalling and apoptosis. *Toxicol Lett* **139,** 89-97.

MacFarlane, M., Ahmad, M., Srinivasula, S. M., Fernandes-Alnemri, T., Cohen, G. M., and Alnemri, E. S. (1997). Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* **272,** 25417-20.

MacFarlane, M., Cohen, G. M., and Dickens, M. (2000). JNK (c-Jun N-terminal kinase) and p38 activation in receptor-mediated and chemically-induced apoptosis of T-cells: differential requirements for caspase activation. *Biochem J* **348 Pt 1,** 93-101.

MacFarlane, M., Harper, N., Snowden, R. T., Dyer, M. J., Barnett, G. A., Pringle, J. H., and Cohen, G. M. (2002). Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. *Oncogene* **21,** 6809-18.

MacFarlane, M., Kohlhaas, S. L., Sutcliffe, M. J., Dyer, M. J., and Cohen, G. M. (2005). TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res* **65,** 11265-70.

Martin, D. A., Siegel, R. M., Zheng, L., and Lenardo, M. J. (1998). Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. *J Biol Chem* **273,** 4345-9.

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* **182,** 1545-1556.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An induced proximity model for caspase-8 activation. *J Biol Chem* **273,** 2926-30.

Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. (1997). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277,** 815-8.

Peter, M. E., Hellbardt, S., Schwartz-Albiez, R., Westendorp, M. O., Walczak, H., Moldenhauer, G., Grell, M., and Krammer, P. H. (1995). Cell surface sialylation plays a role in modulating sensitivity towards APO-1-mediated apoptotic cell death. *Cell Death Differ* **2,** 163-171.

Peter, M. E., and Krammer, P. H. (2003). The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* **10,** 26-35.

Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., and Ashkenazi, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* **271,** 12687-90.

Savage, M. D., Mattson, G., Desai, S., Nielander, G. W., Morgensen, S., and Conklin, E. J. (1992). Avidin-Biotin Chemistry: A Handbook. . *Pierce Chemical Co. (Product #15055)*

Schneider-Brachert, W., Tchikov, V., Neumeyer, J., Jakob, M., Winoto-Morbach, S., Held-Feindt, J., Heinrich, M., Merkel, O., Ehrenschwender, M., Adam, D., Mentlein, R., Kabelitz, D., and Schutze, S. (2004). Compartmentalization of TNF receptor 1 signaling:

internalized TNF receptosomes as death signaling vesicles. *Immunity* **21,** 415-28.

Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277,** 818-21.

Tartaglia, L. A., Ayres, T. M., Wong, G. H., and Goeddel, D. V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74,** 845-53.

Varfolomeev, E., Maecker, H., Sharp, D., Lawrence, D., Renz, M., Vucic, D., and Ashkenazi, A. (2005). Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. *J Biol Chem* **280,** 40599-608.

Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and et al. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3,** 673-82.

Zhang, X. D., Franco, A. V., Nguyen, T., Gray, C. P., and Hersey, P. (2000). Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. *J Immunol* **164,** 3961-3970.

Figure Legends

Figure 1. Purification of recombinant His_6-TRAIL.

Recombinant human His_6-TRAIL was purified as described in Protocol 2. Samples of E.coli bacterial supernatant (both pre- and post- purification) and eluted fractions (1-6) were assessed for purity by SDS-PAGE. Fractions 2 and 3 contain the highest levels of TRAIL protein. In this purification, Fractions 2-5 were pooled and protein concentration and apoptosis activity assessed as described in Protocol 3. The post-purification supernatant confirms efficient purification of TRAIL. Molecular Weight standards (Std) are shown in Lane 1 (Precision Plus Protein Standards; Biorad)

Figure 2. Annexin V staining of BJAB cells treated with recombinant His_6-TRAIL.

BJAB cells (1 x 10^6 cells/ml) were either left untreated (Control) or treated with recombinant human TRAIL (500 ng/ml) for 4 h at 37^{o}C and the extent of apoptosis assessed by staining with Annexin V-FITC/PI as described in Protocol 3. Samples were then analysed by flow cytometry (FACSCalibur; Becton Dickinson) and CellQuest Pro software. The right panels show dot-plots of AnnexinV-FITC (FL1) versus PI (FL2) -stained cells. Annexin V-$FITC^{+/}PI^-$ cells are scored as apoptotic (upper left quadrant) and Annexin V-$FITC^+/PI^+$ cells (upper right quadrant) are scored as 'secondary' necrotic. This assay confirms the apoptosis-inducing activity of the purified recombinant human TRAIL preparation from Figure 1. [a] Data kindly provided by Laura Hall.

Figure 3. Cell surface labelling of TRAIL Receptors in BJAB cells.

(A) Cell surface expression of TRAIL-R1 and TRAIL-R2 as assessed by flow cytometry using TRAIL receptor-specific antibodies.

BJAB cells (1 x 10^6 cells/ml) were labelled with PE-labelled IgG1 (grey line, both panels), PE-TRAIL-R1 (solid line; left panel) or PE-TRAIL-R2 (solid line; right panel) as described in Protocol 6A. Surface expression of TRAIL-R1/TRAIL-R2 and IgG1 was measured by flow cytomtery (FACSCalibur; Becton Dickinson) using excitation and emission wavelengths of 488 and 575 nm (FL2), respectively. Data was then analysed using CellQuest Pro

software. The mean-fluorescence intensity (MFI) of TRAIL-R1-stained cells was 28.6 and the MFI of TRAIL-R2-stained cells was 13.5, compared with a MFI value of 3.0 for IgG1-stained cells (Isotype control).

(B) Uptake and Internalization of TRAIL assessed by Flow Cytometry using TRAIL-AF647.

BJAB cells (1 x 10^6 cells/ml) were incubated for the indicated times at 4 ºC or 37 ºC, in the presence or absence of recombinant TRAIL-AF647 (1 μg/ml), to assess uptake and rapid internalization of TRAIL-AF647 as described in Protocol 7. Following acid washes, TRAIL-AF647 fluorescence was detected by flow cytomtery (FACSCalibur; Becton Dickinson) using excitation and emission wavelengths of 635 and 668 nm (FL4), respectively and data analysed using CellQuest Pro software. The histogram shows selected times during a kinetic analysis of TRAIL-AF647 internalization in BJAB cells. Data kindly provided by Andrew Craxton, [b] modified with full copyright permission (Kohlhaas et al., 2007).

(C) Internalization of TRAIL assessed by Confocal Microscopy using Biotinylated-TRAIL and Streptavidin-labelled Alexa-568.

Receptor-mediated internalization of TRAIL was assessed using Biotin-TRAIL as described in Protocol 8. BJAB cells (1 x 10^6 per sample) were chilled to 4^oC for 1 h followed by treatment with biotinylated-TRAIL (500 ng/ml) for 45 min at 4^oC. Cells were then washed three times with ice-cold PBS (to remove excess TRAIL) and treated with Streptavidin labelled Alexa-568 (*red*) for 1 h at 4^oC. Cells were washed and either fixed after 4^oC treatment (0 min) or released up to 37^oC for 5, 15 or 30 min (to allow TRAIL to internalize) and then fixed in 4% paraformaldehyde for 10 min at room temperature. After fixation, cells were counterstained with the DNA dye Hoechst-33342 (*blue*) for up 5 min to stain nuclei and then visualised using a Zeiss LSM510 with Axiovert 200 confocal microscope. Results shown are of one representative cell from each time point. The *white* bar represents 5 μm. [b] modified with full copyright permission (Kohlhaas et al., 2007).

Figure 4. TRAIL induces DISC formation in BJAB cells in a time-dependent manner.

BJAB cells (2.5 x 10^7 cells per sample) were treated with biotinylated-TRAIL (500 ng/ml) at 37^{o}C for the indicated times and then washed and lysed as described in Protocol 9. Analysis of the known DISC proteins, TRAIL-R2/-R2, FADD and Caspase-8, was then carried out by Western blot analysis as described in Protocol 9. Unstimulated cells (u/s), lysed and then treated with 500 ng/ml biotinylated-TRAIL, served as a control and confirmed *in vitro* binding of biotin-TRAIL to TRAIL-R1/-R2. * represents a non-specific band detected by the Caspase-8 antibody. [b] modified with full copyright permission (Kohlhaas et al., 2007).

Fig. 1

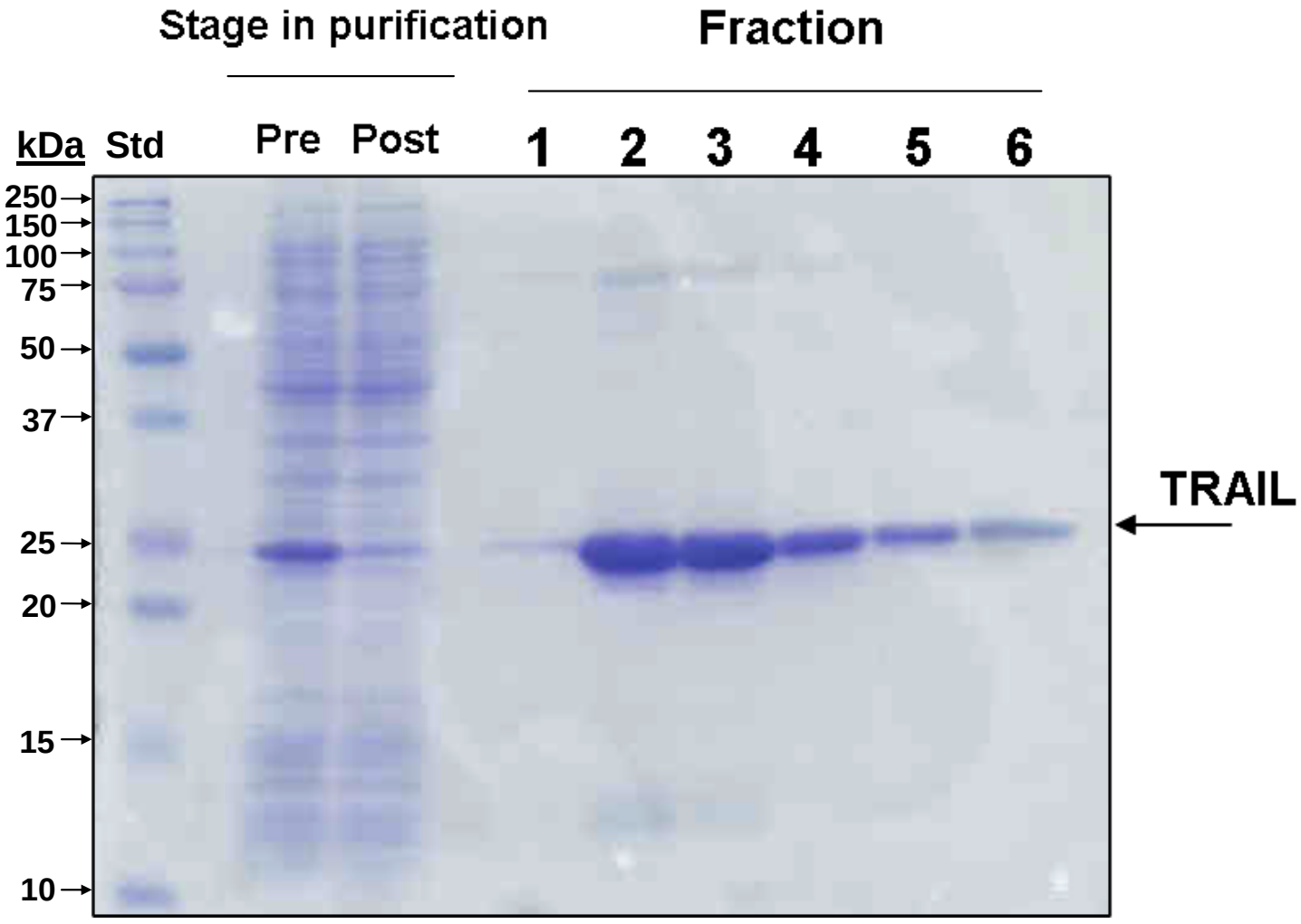

Stage in purification
Fraction
kDa
Std
Pre
Post
1
2
3
4
5
6
250
150
100
75
50
37
25
20
15
10
TRAIL

Fig. 2

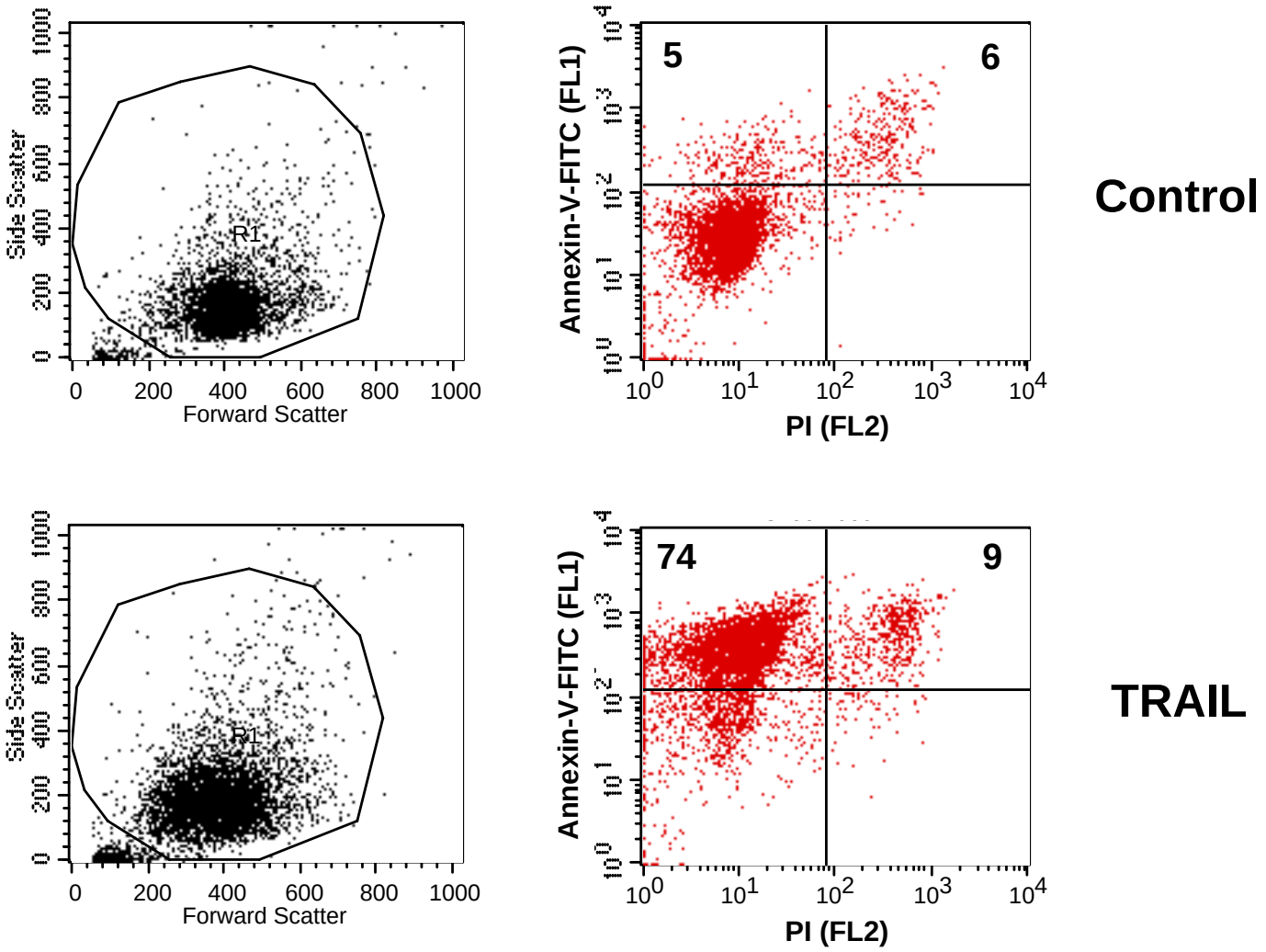
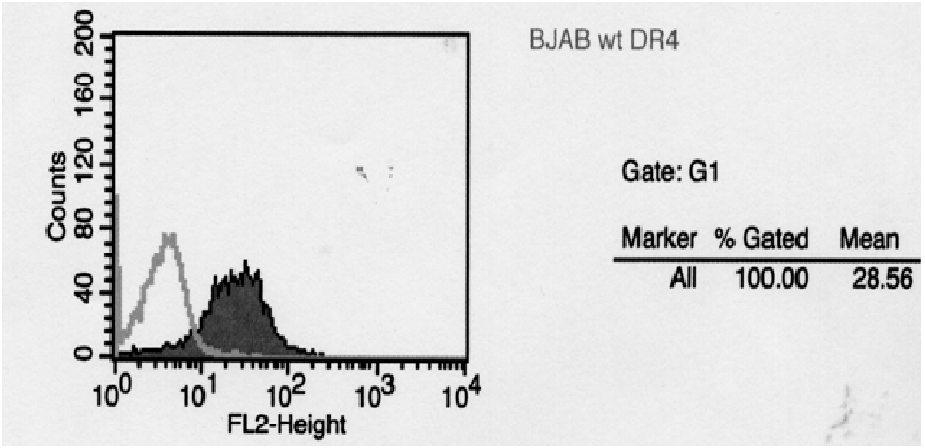

Side Scatter
Forward Scatter
R1
Annexin-V-FITC (FL1)
PI (FL2)
5
6
74
9
Control
TRAIL

Fig. 3A

TRAIL-R1



TRAIL-R2

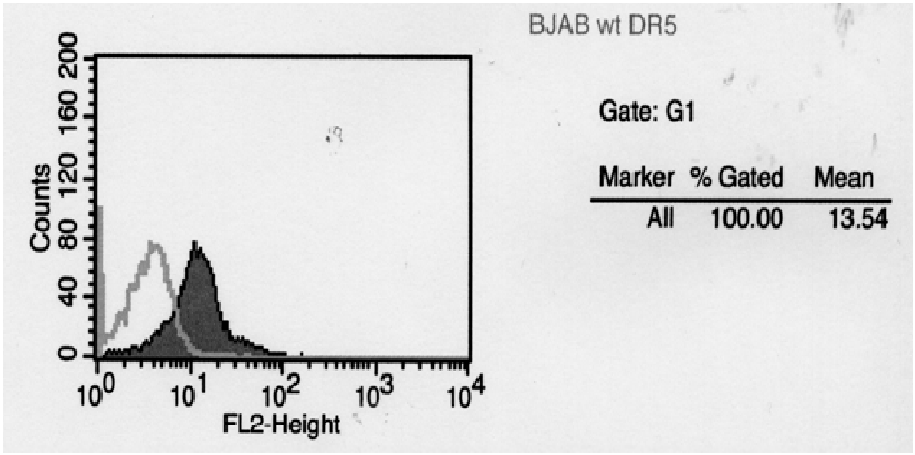
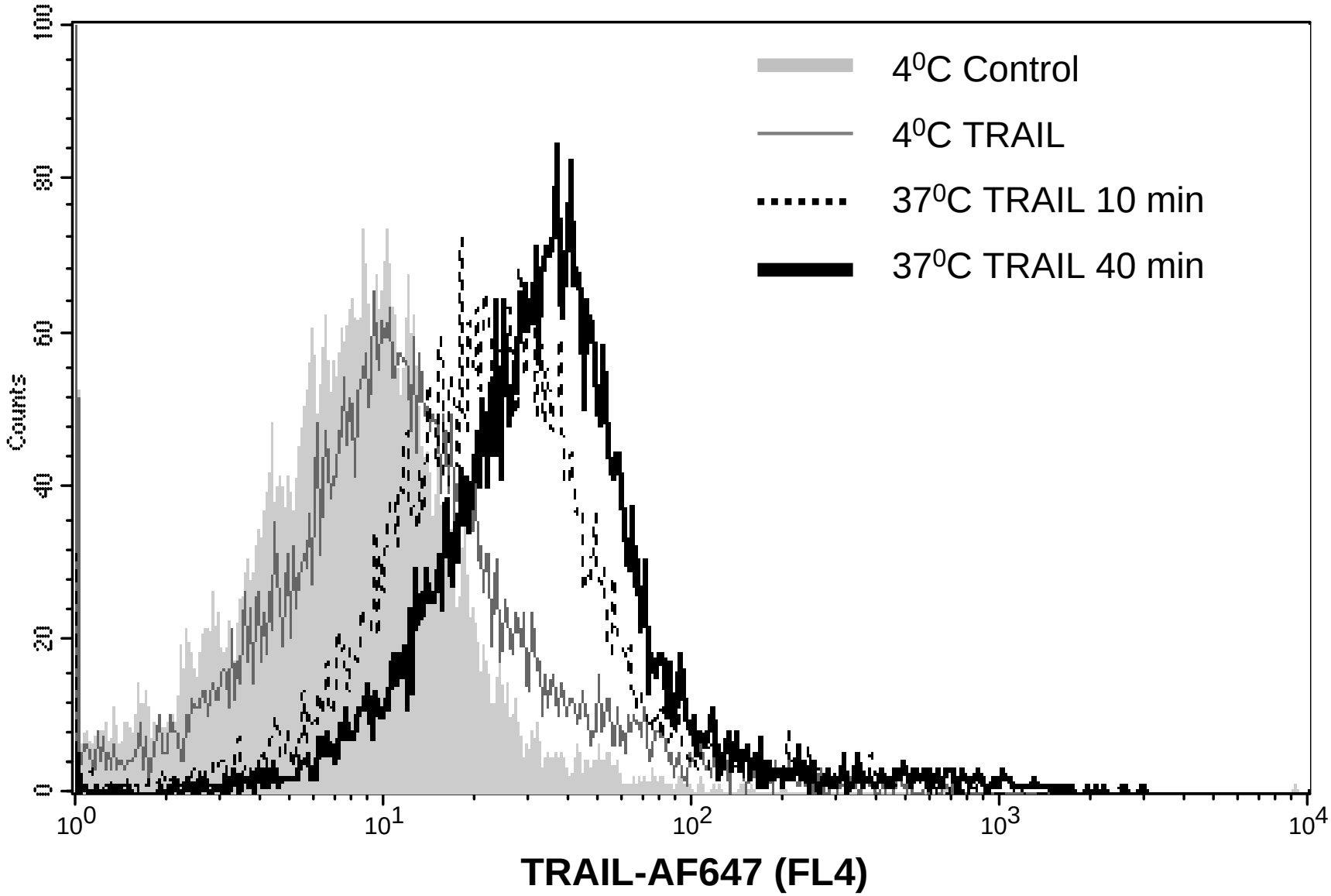
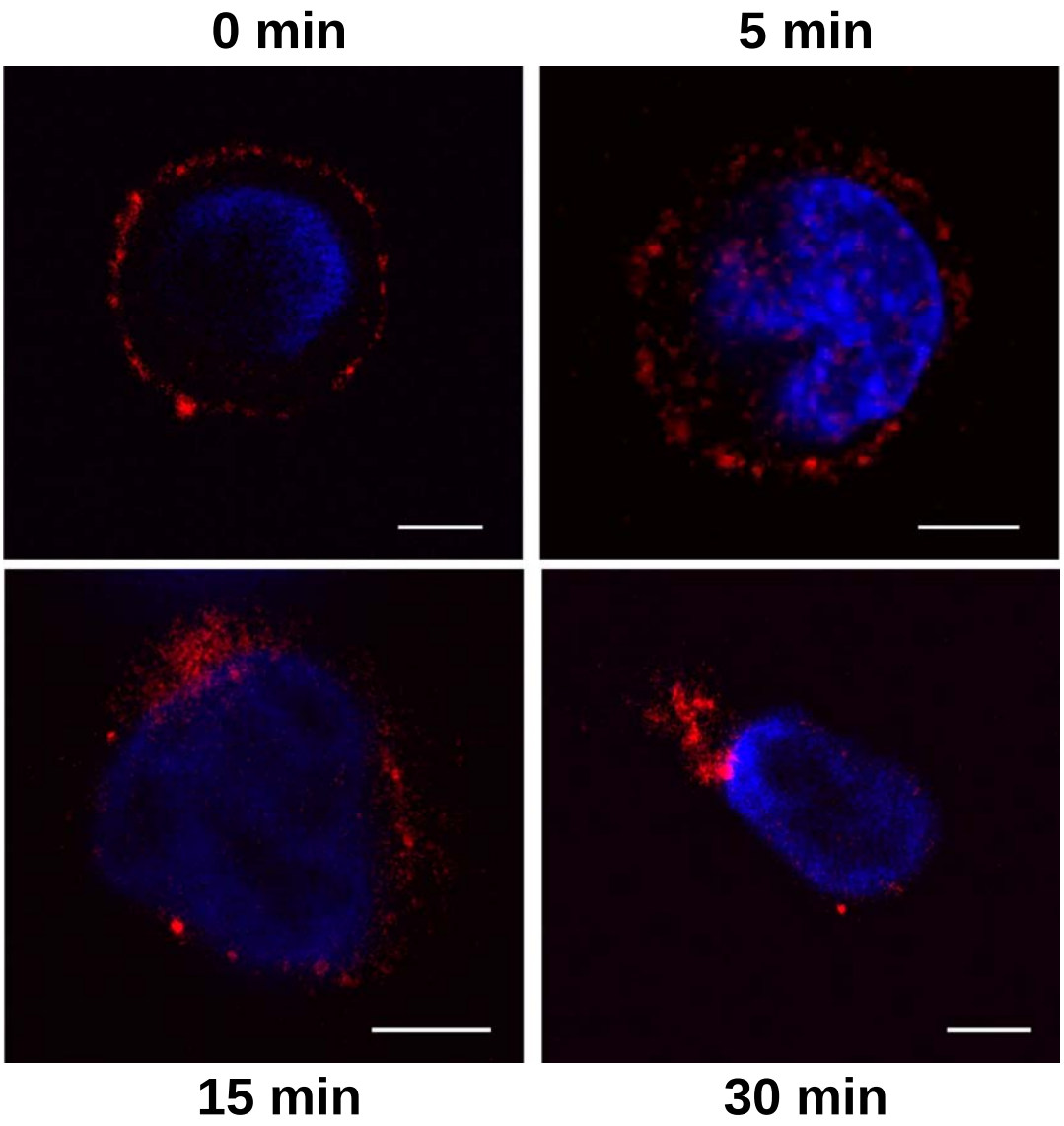


Fig. 3B


4⁰C Control
4⁰C TRAIL
37⁰C TRAIL 10 min
37⁰C TRAIL 40 min
Counts
0
20
40
60
80
100
10⁰
10¹
10²
10³
10⁴
TRAIL-AF647 (FL4)

Harper & MacFarlane

Fig. 3C



Harper & MacFarlane

Fig. 4