Functional Detection of TNF Receptor Family Members by Affinity-Labeled Ligands

Yang Xu1, Lingmo Chang1, Anliang Huang2, Xiaojun Liu1, Xinyu Liu4, Hong Zhou1,3, Joshua G. Liang4 & Peng Liang1,2,4

Aberrant expression of TNF family of cytokines has been linked to human diseases, and biologics targeting their signaling have become the best selling drugs globally. However, functional detection with labeled ligands for accurate detection of TNFR family of receptor-expressing target tissues or cell types remains to be developed. Here we show that TNF receptor family members are heat-stable and can be recognized both in vitro and in vivo by their ligands labeled with alkaline phosphatase. Such an approach may be used in lieu of antibodies for the identification of the cell types involved in receptor signaling during disease onset and progression.

The tumor necrosis factor (TNF) superfamily (TNFSF) of cytokines and TNF receptor superfamily (TNFRSF) display diverse physiological functions and play critical roles in the development and homeostasis of the immune-, nervous- and musculoskeletal-systems in mammals1. TNFSF consists of 19 known ligands that contain the extracellular TNF homology domain (THD) and are all initially expressed as type II transmembrane proteins, although most can exist also in soluble form after extracellular domain cleavage by proteolysis1,2. These ligands signal through 29 structurally related type I transmembrane receptor proteins of TNFRSF containing the extracellular cysteine-rich domain (CRD)1,3.

Abnormal expression of TNF family cytokines or their receptors has been linked to a host of major human diseases including arthritis, psoriasis, osteoporosis and cancer. Elevated localized expression of TNFα has been shown to be one of the underlying causes for various autoimmune and inflammatory disorders such as psoriasis and arthritis1,4. While biologic therapies blocking TNFα currently represent the largest-selling class of blockbuster drugs globally5, the underlying causes of TNFα signaling in disease onset and progression as well as resistance to anti-TNFα therapy in some patients remain obscure6,7. Moreover, TNF-related apoptosis-inducing ligand (TRAIL) has been shown to potently induce apoptosis in a tumor-specific fashion against multiple human cancer cell lines from various tissue origins both in vitro and in vivo, and once hailed as a promising magic bullet against cancer8,9. Despite of very encouraging initial data coming of Phase I clinical trials, recombinant TRAIL (Apo2L) as well as its receptor agonist monoclonal antibodies failed to hold up the expectation in later trials10. The failure could be due to the lack of consideration of the expression of death receptors (DR4 and DR5) on tumor cells while selecting disease indications and patients11. Thus a more accurate identification of the tissues and cell types expressing receptors of TNFRSF both in vitro and in vivo, particularly during disease pathogenesis and progression can be enlightening to understanding molecular mechanisms underlying cytokine networks involved in inflammation and cell programmed death. Such knowledge may lead to better diagnosis and more targeted treatment of the diseases involved.

However, current methodologies for detection of TNFRSF are largely limited to antibody-based approaches that without proper controls could be error-prone due to potential non-specificities of primary / secondary antibodies and labels used12.

Alkaline phosphatase (AP)-tagged ligands are a useful tool for receptor detection and have been used to discover a number of important cell surface receptors and ligands, including receptors for leptin13 and IL-2414 as well as ligands for Kit, Mek4 and Sek receptor tyrosine kinases15,16. Although the C-terminal AP-tagged full-length TNFα had been used to track the shedding of soluble TNFα from the type II membrane-bound form on the cell...

1Department of Biochemistry & Molecular Biology, College of Life Sciences, Sichuan University, Chengdu, 610064, China. 2Laboratory for Gene and Cell Therapy, Sichuan University, Chengdu, 610064, China. 3Department of Biochemistry and Molecular Biology, Southwest Medical University, Luzhou, 646000, China. 4Clover Biopharmaceuticals, Chengdu, 610041, China. Yang Xu and Lingmo Chang contributed equally to this work. Correspondence and requests for materials should be addressed to P.L. (email: liang.peng@cloverbiopharma.com)

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membrane\textsuperscript{17,18}, N-terminal labeling of soluble TNF family of cytokines with AP-Tag for receptor detection has not been reported.

In this brief communication, we demonstrate that N-terminal AP-tagged soluble TNF family of cytokines – AP-TNF\textsubscript{α}, AP-TRAIL and AP-RANKL – can be readily produced as secreted proteins from Chinese hamster ovary (CHO) cells, and used as probes based on the AP activity to detect their respective target receptors both \textit{in vitro} and \textit{in vivo}. In particular, we show that TNF receptor family members are extremely heat stable, which makes it possible for their accurate detection under both native and denaturing conditions.

**Results**

Since TNF family of cytokines are initially made as type II membrane proteins with their C-termini protruding on the cell surface, we decided to tag the N-termini of the soluble forms of these cytokines with human placental alkaline phosphatase (AP) using AP-Tag technology. Guided by the signal peptide sequence from the AP, we showed that three members of AP-tagged TNF family of cytokines, AP-TNF\textsubscript{α}, AP-TRAIL and AP-RANKL could be made at high level as secreted proteins from Chinese hamster ovary (CHO) cells, with AP activity ranging from 15–60 U/mL and readily detectable by Western blot analysis (Fig. 1a). These AP-tagged ligands not only manifested excellent receptor binding specificity on an ELISA plate coated with their respective target soluble receptor-Fc fusion proteins (Fig. 1b), but also could be used much as antibodies for the affinity detection of their corresponding soluble receptor-Fc fusion proteins on Western blots under non-reducing condition (Fig. 1c). The fact that these receptor fusion proteins had been heat denatured and separated on an SDS-PAGE and transferred to a PVDF membrane could be still be recognized by their respective AP-tagged ligands suggested that members from TNFRSF are heat-stable with likely linear epitope for ligand recognition.

Next, we examined the ability of AP-tagged ligands to detect their corresponding cell surface receptors from cultured cell lines. Due to the observation that some human cancer cell lines, including AsPC-1 (human pancreas adenocarcinoma ascites metastases), DLD-1 (human colorectal adenocarcinoma) and Capan-2 (human pancreatic ductal adenocarcinoma), exhibit high level of endogenous AP activity which cannot be inactivated by at 65°C usually used for removing background AP activity from host cells (data not shown), we examined the binding of AP-TRAIL to cultured human pancreas adenocarcinoma cell lines after heat treatment of the cells at 100°C for 10 min. We found that the endogenous AP activity was essentially eliminated, while binding of AP-TRAIL to the cell surface death receptors (DRs) on cancer cells was preserved (Fig. 2a, left). Similarly, we found that

![Image](image-url)
WEHI-164 cells – a well established cell line used for bioassays of TNFα19 – were able to bind to AP-TNFα with minimal background AP activity after heat inactivation of endogenous AP in boiling water (Fig. 2a, right). The receptor binding specificities were confirmed by the significant reduction in signals from AP-tagged ligands with excessive unlabeled rhTRAIL and rhTNFα, respectively (Fig. 2a). These findings are consistent with results from receptor binding in vitro (Fig. 1c), indicating that TNFRSF of proteins are heat stable. It has been previously shown that some membrane proteins are thermostable20–22; however, this characteristic which appears to be shared across TNFRSF members has not been previously investigated. Missense mutations have been identified in thermostable mutants of the diacylglycerol kinase and soluble enzyme p-nitrobenzyl esterase20,21, and given our observation that TNFR2-Fc fusion protein under reducing conditions cannot be recognized by its ligand (data not shown), suggesting that primary and secondary protein structures may play a critical role in ligand recognition.

A typical ligand-receptor binding is expected to be saturable with increasing ligand concentration. This was indeed the case for both AP-TRAIL and AP-TNFα, both of which showed saturation receptor binding kinetics to BxPC-3 and WEHI-164 cells with Kd being 18.15 nM and 4.08 nM, respectively (Fig. 2b). In addition, as expected, the AP-tagged TRAIL and TNFα fusion proteins retained significant level of biological activities as determined by their ability to induce apoptosis for BxPC-3 and TNFα-sensitive WEHI-164 cells, respectively as described above.

Figure 2. Cell surface receptor binding of alkaline phosphatase (AP)-tagged TNFSF ligands. (a) Detection of cell surface receptor(s) from either cultured human pancreatic cancer cell lines (BxPC-3, AsPC-1, and Capan-2) with AP-TRAIL (left) or WEHI-164 cells with AP-TNFα (right). AP alone served as a negative control, while 100-fold excess of unlabeled rhTRAIL or rhTNFα served as controls for receptor binding specificity. (b) Saturation binding kinetics of AP-TRAIL to BxPC-3 cells (top) and AP-TNFα to WEHI-164 cells (bottom) were determined with increasing concentration of the AP-tagged ligands. The data presented as Scatchard plots were shown as insets in the bottom right of saturation binding curves. (c) Analysis of the biological activities AP-tagged TRAIL and TNFα in comparison to untagged ligands by bioassays using TRAIL-sensitive BxPC-3 and TNFα-sensitive WEHI-164 cells, respectively as described above.
models where TNFα have been implicated in the pathogenesis. AP-TNFα binding to tissue in the CIA model was localized to what appeared to be immune infiltrate cells, which we later confirmed and pinpointed as macrophages. On the other hand, AP-TNFα binding to tissue sections in the IMQ-induced psoriasis model indicates that its receptors are quite ubiquitously and highly expressed throughout the disease-affected epidermis. AP alone and competition with unlabeled TNFα served as controls for receptor binding specificity. With this method, we further analyzed human biopsy skin sections from both psoriasis patients and normal subjects. The result revealed that certain population of psoriasis patients exhibited striking over-expression of TNFR in keratinocytes, in comparison with the epidermis from normal skins (Fig. 3c). This result is consistent with previous finding in a systematic analysis of TNFR expression from human psoriasis using TNFRI and TNFRII specific antibodies. From these in situ analysis of TNFR expression, it is interesting to note that while TNF antagonists such as soluble TNFRII-Fc fusion protein (Enbrel) and anti-TNFα mAbs have become main stakes in the treatment of autoimmune diseases, the involvement of TNFR expressing cell types seemed to be strikingly different, with immune infiltrates in RA and keratinocytes in psoriasis being the major source of cell types over-expressing TNF receptors.

Discussion

In this study we demonstrated that AP-tagged TNFSF cytokines can be used as probes for accurate functional detection of TNFRSF expression in situ both in vitro and in vivo from disease tissues of animal models and human patients. The simplicity, specificity and functional-binding of AP-tagged TNFSF ligands to TNFRSF may make it a preferable approach compared to standard immunohistochemistry and FACS analysis utilizing antibodies. Such antibody-based approaches without proper controls can be error-prone due to issues of specificities of primary / secondary antibodies and labels used, whereas the use of AP-tagged ligands as probes preserves natural ligand-receptor recognition, requires fewer intermediary steps for receptor detection. Another advantage is that a single AP-tagged TNFSF ligand can detect the presence of all its corresponding receptors; for example, AP-TNFα can bind to both TNFRI and TNFRII, whereas an antibody approach would require two separate primary antibodies recognizing each receptor type. Conceivably, AP-tagged TNFSF ligands could also lead to the discovery of novel receptors or previously unknown cell-types expressing TNFRSF proteins, if used in tandem with standard immunohistochemistry-based approaches.

Importantly, the fundamental involvement of TNF ligand-receptor pathways and their precise coordination with other signaling pathways in human diseases remains to be elucidated. Here we used AP-TNFα to detect the expression of its receptors on what appeared to be immune infiltrate cells in an arthritis animal model; we were subsequently able to identify these specific immune infiltrates as macrophages. Using sequential staining with AP-TNFα and soluble receptor of IL-20R2, we recently discovered that TNFα signaling converges with that of the IL-20 subfamily of cytokines in the pathogenesis of RA. For other more poorly understood TNF ligand-receptor signaling pathways such as the immune- costimulators which are believed to be promising immuno-oncology therapeutic targets – including 4-1BBL/4-1BB, OX-40L/OX-40, GITRL/GITR, CD30L/
CD30, LIGHT/HVEM and CD70/CD27 – AP-tagged TNFSF ligands could similarly be used to help elucidate their differential expression, signaling and functional roles in immune-surveillance of cancer.

In summary, the use of AP-tagged TNFSF cytokines is a simple, sensitive and accurate approach for functional detection of the expression of corresponding TNF family of receptors in vitro and in vivo. After many years of relying on antibodies as the predominant approach for receptor detection, AP-Tag offers an additional tool that may be used in lieu of antibodies for functional detection of TNFRSF receptors and identification of the cell types involved in receptor signaling during disease onset and progression.

Methods

Ethics Statement. All human tissues were collected from patients at the West China Second University Hospital under approved guidelines by the Institutional Review Board of West China Hospital, Sichuan University and informed consent was obtained from all patients, or their relatives. All animal experiments were approved by the Institutional Animal Care and Use Committee of West China Hospital, Sichuan University, and the experimental procedures were performed in accordance with the approved guidelines.

Construction and expression of AP-Tagged TNF family of cytokines and receptor-Fc fusions. cDNAs encoding the mature human TNFα, TRAIL and RANKL were gene synthesized after codon optimization for CHO cell expression (GeneScript, Nanjing, China). The cDNAs were cloned into pcAP-TAG4-D expression vector (GenHunter Corporation, Nashville, TN) at HindIII-BglII sites for in-frame fusion at the C-terminus of AP. cDNA encoding soluble human TNFRII, DR5 and RANK were gene synthesized after codon optimization for CHO cell expression (GeneScript, Nanjing, China) and cloned into pHG-Fc-D expression vector (GenHunter Corporation, Nashville, TN) at HindIII-BglII sites for in-frame fusion at the N-terminus of human IgG1 Fc. The resulting plasmids were transfected into GH-CHO (dhfr-) cells, selected without hypoxanthine thymine (HT) (Invitrogen), and stepwise gene amplified with increasing concentration of MTX (Sigma) for high titer expression of each fusion proteins under serum free culture using SFM4CHO medium (HyClone, Logan, UT). The conditioned serum free media containing AP alone and AP fusion proteins were collected and used directly for subsequent experimentation. Receptor Fc fusion proteins were purified from the conditioned media via mAbSelect protein A (GE Biosciences) chromatography.

Cell lines and culture. GH-CHO (dhfr-) Chinese hamster ovary (CHO) cell line was obtained from GenHunter Corporation (Nashville, TN), WEHI 164 mouse fibrosarcoma cell line, HCT116 colon cancer cell line and pancreatic cancer cell lines AsPC-1, BxPC-3 and Capan-2 were from the American Type Culture Collection (ATCC). CHO cells were maintained in IMDM (HyClone, Logan, UT) and adapted to serum free culture in SFM4CHO (HyClone, Logan, UT) for production of AP fusion and Fc fusion proteins. WEHI 164 and pancreatic cancer cell lines were maintained in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (HyClone, Logan, UT) at 37 °C with 5% CO₂.

Western blot and ligand affinity blot analysis. Conditioned media of AP or AP-tagged TNF ligands (both at 1 U/mL, about 10 nM according to specific activity of AP) were detected by Western blot on a 10% SDS-PAGE using anti-AP rabbit polyclonal antibody (GenHunter) followed by goat anti-rabbit IgG-HRP (Southern Biotech) to verify each AP-tagged ligands. Affinity blot analysis of TNF receptors was carried out using AP assay reagent S (GenHunter) following the manufacturer’s instructions as previously described. Two μg of each purified recombinant Fc tagged receptor was separated on a 10% SDS-PAGE under non-reducing condition and either visualized by Coomassie Blue staining or transferred to PVDF membranes. After blocking with 5% fat-free milk in PBS, the membranes were incubated for 1 hour with conditioned media of either AP or AP-TNFα, AP-TRAIL and AP-RANKL followed by visualization with AP Assay Reagent S (GenHunter Corporation). The AP activity of all conditioned media were kept equal at 1 U/mL.

ELISA. Solid phase Sandwich ELISA assays were performed to quantitatively detect recombinant TNF receptor-ligand binding activity under native conditions. Recombinant receptor-Fc proteins at 0.1 μg/mL were added into each well of 96-well ELISA plates (Maxsorp, Corning) which were previously coated with protein A. Following 1 hr of ligand binding with AP or AP-TNFα-TRAIL/-RANKL conditioned media (all at 1 U/mL), the receptor binding activities were detected with AP substrate, AP assay reagent A (GenHunter), following the manufacturer’s instructions as previously described. ELISA plates were washed with PBS three times between each step and once at the final step before adding AP substrate with AP wash buffer (10 mM Tris, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, pH 8.4).

Bioassays. The biological activities of AP labeled TNFα and TRAIL were assessed by a MTT tetrazolium (Sigma) cytotoxicity assay with rhTNFα or rhTRAIL (R&D Systems) as positive controls, respectively. In brief, WEHI-164 cells with 500 ng/mL ActD (Sigma) and TRAIL-sensitive BxPC-3 were seeded to 5000 cells/well in 96-well plates in RPMI 1640. After 24 hr of incubation at 37 °C, 0.5 μg/mL MTT was added and further incubated for 4 hr. After aspirating the media from the wells, 100 μL of DMSO was added to dissolve Formazan crystal. The absorbance at 490 nm was recorded. The specific activity of each cytokine was determined as U/micromole for comparison of each pair of AP-tagged and untagged ligand.

Cell surface receptor binding assay. Quantitative cell surface receptor binding studies were carried out as described previously with some modifications. Briefly, cells were seeded at 5 × 10⁴/well in 6-well plates in duplicates. After cells reached confluence, medium was removed until 100 μL remained in each well, and then plates
were steamed in a boiling water bath for 10 min with open lids. After removing remaining medium, 1 mL of either the AP fusion protein with or without 100 µg/mL unlabeled rhTNFα and rhTRAIL, or AP alone containing media (all at 1 U/mL) was added into each well of cells for 1.5 hr. After removing the AP containing media, the plates were washed 5 times with HBHA wash buffer (1 % Hanks buffer, 0.5 g/L BSA, 1 M HEPES), cells were lysed in cell lysis buffer (1 % Triton X100, 10 mM Tris, pH 8.0) and supernatant were collected for AP activity assay using reagent A (GenHunter Corporation, Nashville, TN) following the manufacturer’s instructions. For saturation binding assays, WEHI-164 and BxPC-3 cells were allowed to bind increasing amount of AP-TNFα or AP-TRAIL ranging from 0 to 40 nM based on the specific activity of AP as described previously32. Receptor specific ligand binding was determined by subtracting the background signals from blank medium incubated cells and analyzed by GraphPad Prism. Each data point was determined in duplicate.

**Histological analysis and tissue in situ ligand staining.** Tissues from human colon, breast, lung and pancreatic cancers, the paws from mouse CIA model and skin samples from the back of mouse IMQ model and psoriasis patient were fixed in 10 % neutral buffered formalin, paraffin-embedded, and sectioned at 5µm and stained with H&E. In situ ligand stained studies were performed essentially as previously described12. The sections were incubated at 65 °C for 90 min to de-wax before being rehydrated and incubated with either 1 U/mL of AP or AP-TRAIL and AP-TNFα, mixture of AP-TRAIL and rhTRAIL at 10 µg/mL or AP-TNFα and rhTNFs at 10 µg/mL for 90 min. After washing 3 times with HBHA wash buffer and fixing with fix reagent (60% acetone, 3 % formaldehyde and 20 mM HEPES, pH 7.5), the sections were stained with AP assay reagent S.

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Author Contributions
P.L. conceived the idea, Y.X., J.G.L. and P.L. wrote the manuscript; Y.X., L.M.C., AL.H., XJ.L., XY.L.and H.Z. performed the experiments; Y.X., J.G.L. and P.L. analyzed data.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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