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Development of an inflammatory tissue-selective chimeric TNF receptor

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ABSTRACT

Background: Inhibiting TNF-α is an effective therapy for inflammatory diseases such as rheumatoid arthritis. However, systemic, nondiscriminatory neutralization of TNF-α is associated with considerable adverse effects.
Methods: Here, we developed a trimeric chimeric TNF receptor by linking an N-terminal mouse Acrp30 trimerization domain and an MMP-2/9 substrate sequence to the mouse extracellular domain of TNF receptor 2 followed by a C-terminal mouse tetranectin coiled-coil domain (mouse Acrp-MMP-TNFR-Tn).
Results: Here, we show that the Acrp30 trimerization domain inhibited the binding activity of TNFR, possibly by closing the binding site of the trimeric receptor. Cleavage of the substrate sequence by MMP-9, an enzyme highly expressed in inflammatory sites, restored the binding activity of the mouse TNF receptor. We also constructed a recombinant human chimeric TNF receptor (human Acrp-MMP-TNFR-Tn) in which an MMP-13 substrate sequence was used to link the human Acrp and the human TNF receptor 2. Human Acrp-MMP-TNFR-Tn showed reduced binding activity, and MMP-13 digestion recovered its binding activity with TNF-α.
Conclusion: Acrp-masked chimeric TNF receptors may be able to be used for inflammatory tissue-selective neutralization of TNF-α to reduce the adverse effects associated with systemic neutralization of TNF-α.

1. Background

Upregulation of tumor necrosis factor-α (TNF-α) is associated with many inflammatory diseases [1–3]. In rheumatoid arthritis, overexpression of TNF-α can directly contribute to disease progression by activating synovial fibroblasts to secrete matrix-degrading enzymes, such as matrix metalloproteinases (MMPs), which leads to cartilage erosion [4–6]. Accordingly, blocking TNF-α with a decoy TNF-α receptor [7] or monoclonal antibodies [8,9] represents a rational treatment for these conditions [10]. Currently, neutralization of TNF-α with

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Abbreviations: TNF, Tumor Necrosis Factor; TNFR, Tumor Necrosis Factor Receptor; MMP, Matrix Metalloproteinase; FDA, Food and Drug Administration; RANKL, Receptor Activator of Nuclear Factor Kappa-B Ligand; BAFF, B-cell Activating Factor

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decoy TNF-α receptors or monoclonal antibodies is approved by the FDA for a variety of inflammatory diseases, including rheumatoid arthritis, plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, juvenile idiopathic arthritis, Crohn's disease and ulcerative colitis [10]. As a result, these decoy TNF-α receptors and monoclonal antibodies have become the most prescribed biologic drugs for rheumatoid arthritis [11].

However, TNF-α also plays important roles in host defense against microbial infections [12,13]. Systemic blockade of TNF-α for the treatment of the aforementioned human diseases often induces generalized immunosuppression that undermines the therapeutic benefits of the drugs. Increased fungal, bacterial and viral infections are among the most commonly observed complications due to immunosuppression [14–16]. Reactivation of herpes simplex virus (HSV) and *Mycobacterium tuberculosis* has also been observed after injections of decoy TNF receptors and monoclonal antibodies [17,18]. In addition, increased incidences of leukemia and other tumors have been reported in patients receiving these drugs [14,19]. These adverse effects are most likely due to systemic, nondiscriminatory neutralization of TNF-α by the drugs. Therefore, future drug development in this area should focus on preventing or reducing the generalized immunosuppression caused by the TNF-α neutralization drugs while maintaining their therapeutic benefits for the treatment of chronic inflammation.

We aimed to address this problem by developing an inflammatory tissue-selective chimeric TNF receptor in which a trimeric cap is linked to the trimeric TNF receptor through an MMP-cleavable sequence. The cap can be removed by MMP digestion to reopen the binding site of the TNF receptor once it is in inflammatory tissues. In this study, we constructed a trimeric, chimeric TNF receptor (C3-TNFR) by linking a trimerization domain of Acrp30 [20] to the N-terminus of the extracellular domain of TNFR2 [21] through an MMP substrate sequence and linking a coiled-coil domain of tetranectin [22] to the C-terminus of the receptor. In this proof-of-concept study, we tested whether additions of trimeric domains at both the N- and C-terminal regions of TNFR2 could block the binding activity of the receptor. Furthermore, we tested whether the binding activity of TNFR2 could be restored upon removal of the Acrp30 trimerization domain. Our data indicate that this strategy may be useful for designing inflammatory tissue-selective TNF-α antagonists.

2. Methods

2.1. Antibodies

The following antibodies were used in this research: mouse anti-6X-His tag (1:1000 dilution) (clone: J099B12, BioLegend, San Diego, CA, U.S.A.); Armenian hamster anti-mouse TNFR2 (diluted to 0.1 µg/ml) (clone: TR75-89, BioLegend); mouse anti-human TNFR2 (diluted to 0.1 µg/ml) (clone: 80 M2, GeneTex, Hsin-Chu, Taiwan); HRP-conjugated goat anti-mouse IgG (H + L) (1:20,000 dilution for western blotting, 1:5000 for ELISA) (Jackson ImmunoResearch Labs, West Grove, PA, U.S.A.); HRP-conjugated anti-human IgG Fcγ chain (1:20,000 dilution for western blotting, 1:5,000 for ELISA) (Jackson ImmunoResearch Labs); biotin-conjugated goat anti-Armenian hamster IgG (H + L) (1:5000 dilution) (Jackson ImmunoResearch Labs); and HRP-conjugated streptavidin (1:1000 dilution) (Vector Laboratories, Burlingame, CA, U.S.A.).

2.2. Construction of the C3-TNFR

Mouse TNF-α receptor 2 cDNA and mouse Acrp30 cDNA were prepared from mouse spleens. Mouse tetranectin cDNA was prepared from mouse lung tissues. DNA fragments of the collagen-like domain of Acrp30 (amino acids 1–92), the GPLGVR-TNFR2 extracellular domain (amino acids 1–236) and the coiled-coil domain of tetranectin (amino acids 16–52) were amplified from cDNA by PCR. Amplified DNA fragments were subcloned into the mammalian expression vector pLNCX to derive the Acrp30-TNFR and Acrp30-TNFR-Tn constructs. Each construct contained a histidine tag at the C-terminus for downstream purification of the recombinant protein. All DNA constructs were sequenced to ensure that no mutations were introduced during the cloning steps and to ensure that the correct reading frames were present and the protein domains were correctly organized in the constructs.

To construct the human C3-TNFR, DNA sequences of the collagen-like domain of human Acrp30 (amino acids 1–94) and the coiled-coil domain of human tetranectin (amino acids 16–52) were custom-synthesized (by Genomics, Inc., New Taipei, Taiwan). DNA fragments of the extracellular domain of human TNFR2 (amino acids 1–235, provided by Dr. Tian-Lu Cheng), the collagen-like domain and the coiled-coil domain were amplified by PCR. During PCR, overlapping sequences were added via primer sets. Thus, we constructed the full-length human C3-TNFR through overlapping PCR[47].

2.3. Protein expression and purification

We used the Expi293 expression system (Thermo Fisher Scientific, Waltham, MA, U.S.A.) to produce glycosylated C3-TNFR. Expi293 cells were cultured at 37 °C, 8% CO_2 and 125 rpm. The endotoxin-free plasmid DNA was transfected into Expi293 cells according to the manufacturer's instructions. For each transfection, 30 µg of DNA and transfection reagent were separately diluted with Opti-MEM, mixed together and incubated for 20 min. The DNA complex was added into 30 ml of Expi293 cell culture (7.5×10^7 cells/flask). To prevent degradation of the C3-TNFR, 100 µM EDTA was added to the Expi293 cells 16 h after transfection. The C3-TNFR-containing conditioned media were collected 4 days after transfection.

Supernatants of the cells transfected with each expression vector were collected, and imidazole was added to a final concentration of 20 mM. The C3-TNFR was captured by passing them through a Ni-NTA agarose column (Cube Biotech, Monheim, Germany). We washed off unbound proteins with 2.5 bed volumes (BV) of binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Tween-20 and 20 mM imidazole), 2.5 BV of Tris ion exchange buffer (50 mM Tris pH 7.5, 500 mM NaCl, 1% Tween-20 and 20 mM imidazole), 5 BV of Tris glycerol buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Tween-20, 20 mM imidazole and 10% glycerol), and 22 BV of Tris detergent-free buffer (50 mM Tris pH 7.5, 150 mM NaCl, and 20 mM imidazole), in series. We eluted the C3-TNFR with 200 mM imidazole in 50 mM Tris (pH 7.5). The C3-TNFR was then concentrated with a 10 kDa cut-off Centricon filter (Pall Corporation, Port Washington, NY, U.S.A.) and added to a 1 mM final concentration of EDTA to prevent degradation. The protein concentration was measured with a bicinchoninic acid (BCA) protein quantification kit (Thermo Fisher Scientific).

To assess purity and digestion efficiency, the proteins were boiled in reducing sample buffer and separated by 10% SDS-PAGE. To assess purity, total proteins were stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St. Louis, MO, U.S.A.) in SDS-polyacrylamide gels. For western blot analysis, the proteins were transferred onto nitrocellulose membranes (Pall Corporation) in transfer buffer (100 V, 60 min, 4 °C). The membranes were blocked at room temperature with blocking buffer (5% nonfat milk in phosphate-buffered saline, 0.1% Tween-20 (PBST)) for an hour with continuous shaking. The membranes were then washed in PBST and incubated with a primary antibody against a His-tag for an hour. After the appropriate washes in PBST, the membranes were incubated with an HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Labs) for an hour at room temperature with continuous shaking. After the appropriate washes, recombinant proteins bound with antibodies were visualized with chemiluminescent substrates (Thermo Fisher Scientific). Images were acquired with a CCD imaging system.

2.4. Activation of the C3-TNFR

Mouse MMP-9 (0.1 g/l, BioLegend) and recombinant human MMP-13 (0.1 g/l, R&D Systems, Minneapolis, MN, U.S.A.) were mixed with equal volumes of freshly prepared 2X TCNBZ (100 mM Tris, 300 mM NaCl, 20 mM CaCl2, 0.1% Brij35 and 50 µM ZnCl2) buffer with 2 mM APMA (Sigma-Aldrich) and activated in a 37 °C water bath for 4 h as described in the manufacturer's instructions. To digest the mouse C3-TNFR, approximately 300 ng of purified protein was digested with 5 ng of activated mMMP-9 in TCNB buffer in a reaction volume of 40 µl. Typically, reaction mixtures were incubated at 37 °C for an hour. The reactions were stopped by adding 4 µl of 0.5 M EDTA solution or by boiling in reducing sample buffer. We activated the human C3-TNFR under similar reaction conditions, except that approximately 100 ng of human receptors was digested with 5 ng of hMMP-13.

2.5. Binding activity assay

Recombinant mouse TNF-α (40 ng/well) (BioLegend) or recombinant human TNF-α (25 ng/well) (PeproTech, Rocky Hill, NJ, U.S.A.) was coated onto ELISA plates (Thermo Fisher Scientific) in bicarbonate/carbonate buffer at 4 °C overnight. Coated plates were washed extensively in PBST and then blocked with blocking buffer (5% nonfat milk in PBST) at room temperature for 2 h. Nonactivated or MMP-activated C3-TNFR were diluted in PBS and incubated with TNF-α-coated wells at room temperature for 1 h. After extensive washes in PBST, plate-bound TNF receptors were detected with the Armenian hamster anti-mouse TNFR2 or the mouse anti-human TNFR2 antibody for an hour. An HRP- or biotin-conjugated secondary antibody was then added to each well for another hour after extensive washes to remove unbound antibodies. For the human TNF receptor, the plates were washed and incubated with HRP-conjugated streptavidin for 30 min. The color was developed by adding TMB substrate (Thermo Fisher Scientific) to each well. The optical density was measured at 450 nm in a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

3. Results

3.1. Design of the inflammatory tissue-selective C3-TNFR

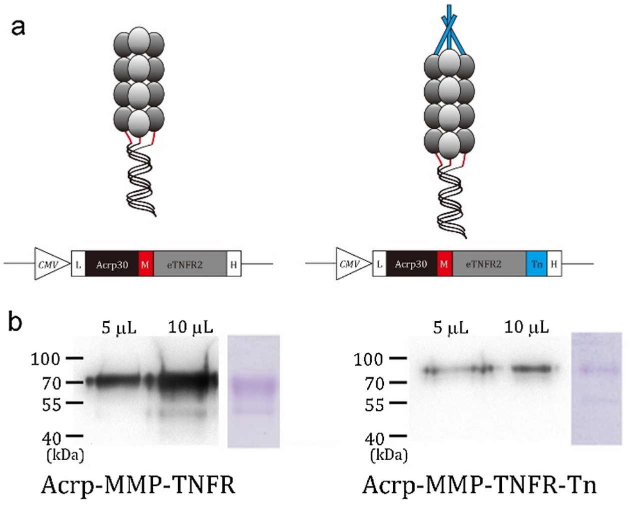

Fig. 2. Design and production of the inflammatory tissue-selective chimeric TNF receptor. (a) In the first design (Acrp-MMP-TNFR, left panel), a collagen-like domain of Acrp30 was linked through an MMP-2/9 substrate to the extracellular domain of TNFR2. In the second design (Acrp-MMP-TNFR-Tn, right panel), a coiled-coil domain of tetranectin was linked to the C-terminal of TNFR2. (b) Chimeric TNF receptors were produced by Expi293 transfectants. Purified proteins were detected by western blotting with a His tag antibody and were assessed by SDS-PAGE/Coomassie blue staining. M, MMP substrate sequence; Tn, coiled-coil domain of tetranectin.

To design an inflammatory tissue-selective C3-TNFR, we explored the crystal structure of soluble TNFR2 complexed with TNF-α from published data (PDB ID: 3ALQ) [21]. The complex of TNFR2 and TNF-α is trimeric, with TNFR2 forming a barrel surrounding the central TNF-α [21] (illustrated in Fig. 1a). This binding model led us to hypothesize that if we could close or block the opening of TNFR2, we may be able to inhibit the entry of TNF-α (Fig. 1b). Furthermore, the binding site of the masked TNFR2 could be opened through digestion by enzymes that are overexpressed in inflammatory tissues, such as matrix metalloproteinases (Fig. 1c).

Based on this reasoning, we linked a trimerization domain of the mouse Acrp30(20) through an MMP-2/9 substrate sequence (GPLGMWSR) to the N-terminus of the extracellular domain of the mouse TNFR2 to form Acrp-MMP-TNFR (left panel, Fig. 2a). Upon translation, folding and trimerization of Acrp30 into a collagen-like helix is expected to close the binding sites of TNFR2. In another construct, we also linked another trimerization domain of tetranectin(25) to the C-terminus of TNFR2 to form Acrp-MMP-TNFR-Tn (right panel, Fig. 2a). We expected the latter construct to close the openings of the soluble trimeric TNFR2 at both ends.

Mouse chimeric TNF receptors (Acrp-MMP-TNFR and Acrp-MMP-TNFR-Tn) were collected and purified from the supernatants of Expi293 cells transiently transfected with the expression vectors (Fig. 2b). We

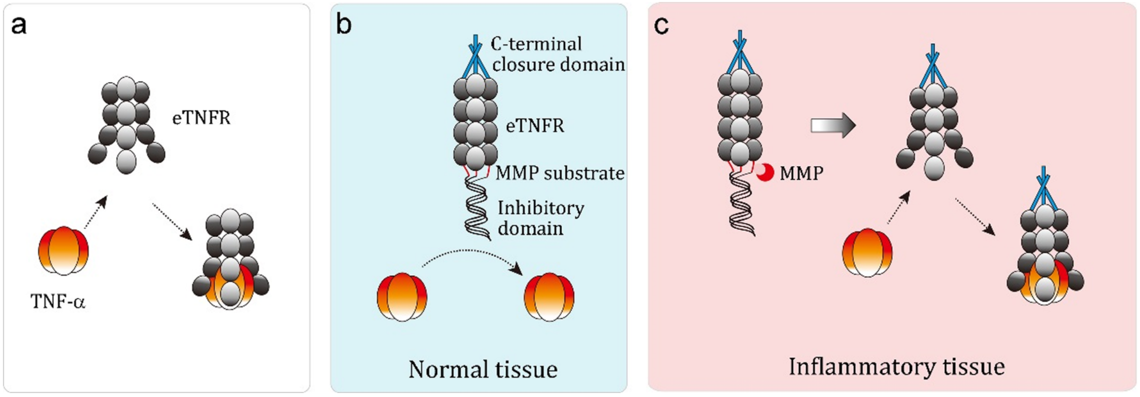

Fig. 1. Schematic representation of the inflammatory tissue-selective chimeric TNF receptor. (a) Natural binding mode of soluble trimeric TNF receptor to TNF-α. (b) In the chimeric TNF receptor, an N-terminal inhibitory domain blocks the opening of the receptor, thus preventing the binding of soluble TNF-α in normal tissues. (c) When the chimeric TNF receptor enters the inflammatory tissue, the substrate sequence in the chimeric TNF receptor is cleaved by MMPs to reopen the binding site; thus, it can bind to TNF-α. eTNFR, extracellular domain of TNF receptor 2; MMP, matrix metalloproteinase.

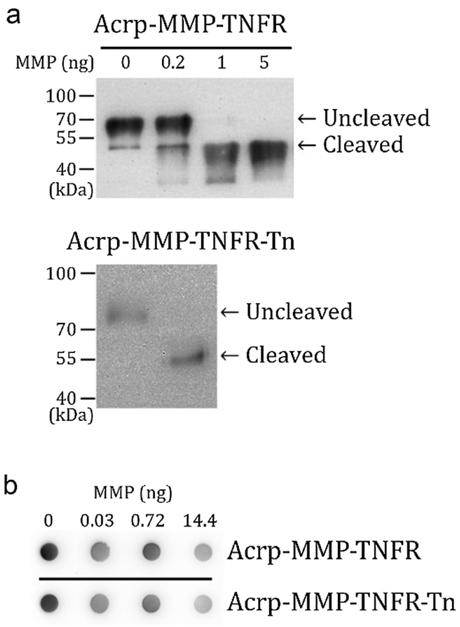

Fig. 3. Digestion of the inhibitory domains of the mouse chimeric TNF receptors by MMP-9. (a) Mouse Acrp-MMP-TNFR (upper panel) and mouse Acrp-MMP-TNFR-Tn (lower panel) were digested with MMP-9. The digested receptors were analyzed by western blotting with a His tag antibody. (b) Untreated and MMP-digested mouse Acrp-MMP-TNFR and mouse Acrp-MMP-TNFR-Tn were analyzed in a dot blot with an anti-mouse TNFR2 antibody (clone: TR75-89).

noticed that the production of Acrp-MMP-TNFR-Tn was lower than that of Acrp-MMP-TNFR. We did not find known ER-retention signals in the C-terminal tetranectin domain using online structure prediction software. The apparent molecular weights of the Acrp-MMP-TNFR (70 kDa) and Acrp-MMP-TNFR-Tn (∼80 kDa) receptors were higher than expected (40 and 45 kDa, respectively). This discrepancy is most likely due to glycosylation on the Acrp30 collagen-like domain and the TNFR extracellular domain [23,24]. A minor band at approximately 55 kDa was also noted in the purified proteins that represented a cleaved form created during production (Fig. 2b).

3.2. Cleavage of the mouse C3-TNFR by MMP-9

We tested whether mouse Acrp-MMP-TNFR and mouse Acrp-MMP-TNFR-Tn could be cleaved by MMP-9, an enzyme that is highly upregulated in rheumatoid arthritis(28–30). As expected, MMP-9 cleaved the mouse chimeric TNF receptors, as indicated by the reduced molecular sizes (by ∼20 kDa) in the western blot analysis (Fig. 3a). Optimal digestion was found when 1–5 ng of MMP-9 was added to 100 ng of Acrp-MMP-TNFR or Acrp-MMP-TNFR-Tn.

The TNFR likely maintained its native structure in the uncleaved recombinant protein, and digestion of the mouse Acrp-MMP-TNFR and mouse Acrp-MMP-TNFR-Tn by MMP-9 did not seem to destroy the conformations of TNFR and TNFR-Tn, as indicated by the detection of a specific antibody against TNFR2 in dot blot analysis (Fig. 3b). This result suggests that TNFR and TNFR-Tn likely maintain their native conformations and remain capable of binding to TNF-α after MMP-9 cleavage of the inhibitory (Acrp) domain. In other words, the inclusion of a self-trimerized inhibitory domain in Acrp-MMP-TNFR and Acrp-MMP-TNFR-Tn does not result in an irreversible structural change in TNFR2.

3.3. Binding activity of the mouse C3-TNFR before and after MMP-9 digestion

After showing that the mouse Acrp-MMP-TNFR and mouse Acrp-MMP-TNFR-Tn inhibitory domains could be removed by MMP-9, we next tested the binding activities of the receptors before and after removal of the inhibitory domains by MMP-9. As shown in Fig. 4a, untreated Acrp-MMP-TNFR bound weakly to TNF-α precoated on ELISA plates. However, untreated Acrp-MMP-TNFR-Tn displayed some binding with TNF-α, which may be attributed to TNFR-Tn that was preformed (or precleaved) by MMPs expressed in the Expi293 transfectants. When Acrp-MMP-TNFR-Tn was treated with MMP-9 before being added to the plate coated with TNF-α, the binding activity increased by approximately 5-fold (Fig. 4a). Compared to that of mouse Acrp-MMP-TNFR-Tn, the binding activity of mouse Acrp-MMP-TNFR increased only marginally after MMP-9 digestion. We speculated that the TNFR2 extracellular domain, in the absence of the C-terminal tetranectin, may dissociate after the removal of Acrp. We tested whether prolonged incubation (18 h) at 4 °C could reassemble the TNFR after digestion; however, this treatment did not significantly increase the binding activity of the receptor (Fig. 4b). Furthermore, TNF-α binding activity (OD) was analyzed by a multiple linear regression model that included the type of the C3-TNFR (type C3-TNFR), the dosage of the TNF receptor (dose) and whether the TNF receptor was activated by MMP (MMP cut). The regression model (OD = −0.8946111 + 0.3336667 * (type C3-TNFR) + 0.0717778 * (dose) + 0.308333 * (MMP cut); p = 0.001 for type C3-TNFR, p = 0.024 for dose and p = 0.002 for MMP cut) indicated that changing Acrp-MMP-TNFR to Acrp-MMP-TNFR-Tn

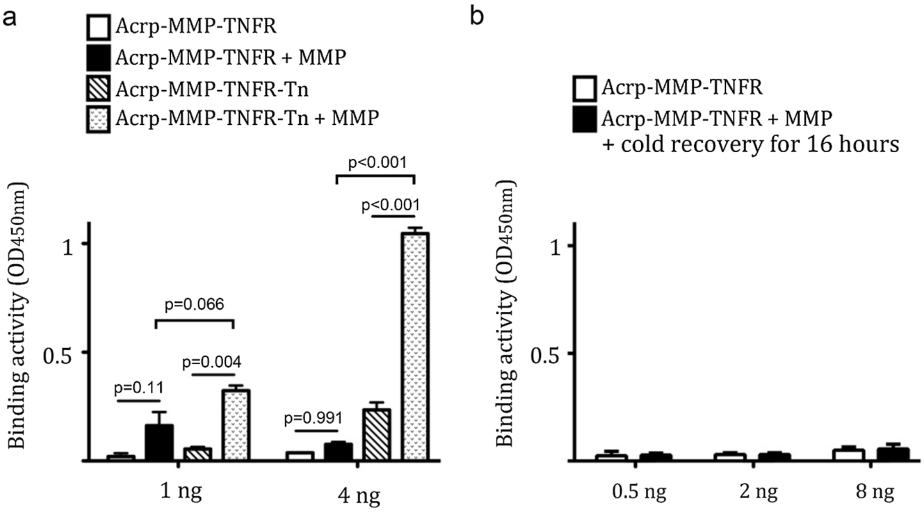

Fig. 4. Binding activity of the chimeric TNF receptors before and after MMP-9 digestion. (a) Acrp-MMP-TNFR and Acrp-MMP-TNFR-Tn were digested with MMP-9. The indicated amounts of untreated and digested receptors were added to ELISA plates precoated with mouse TNF-α. The bound receptors were detected with an anti-TNFR2 antibody. The data are presented as the means ± SEM. One-way ANOVA followed by Scheffe's post hoc comparison was used to analyze the data. (b) Acrp-MMP-TNFR was digested with MMP-9 for 1 h, and the reaction was stopped by EDTA. The digested construct was cold-incubated at 4 °C for 16 h. The indicated amounts of untreated and digested Acrp-MMP-TNFR were added to ELISA plates precoated with mouse TNF-α. The bound receptors were detected with an anti-TNFR2 antibody. The data are presented as the means ± SEM.

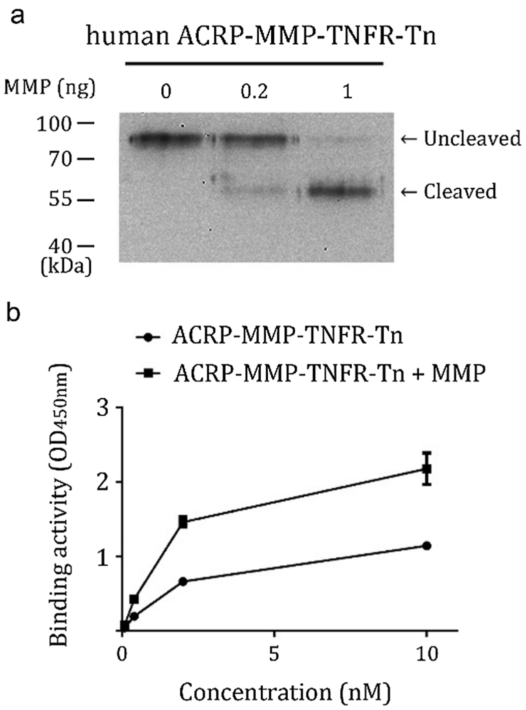

Fig. 5. Binding activity of the human chimeric TNF receptors before and after MMP-13 digestion. (a) Human ACRP-MMP-TNFR-Tn was digested with MMP-13. Digested human ACRP-MMP-TNFR-Tn was detected by western blotting with a His tag antibody. (b) The indicated amounts of untreated and MMP-13-digested receptors were added to ELISA plates precoated with human TNF-α. The bound receptors were detected with an anti-human TNFR2 antibody (clone: 80M2).

increased the OD by 0.3336667 units. Thus, we conclude that a C-terminal trimerization domain is necessary to maintain the trimeric conformation and the activity of the C3-TNFR.

3.4. Binding activity of the human Acrp-MMP-TNFR-Tn

To test whether this strategy could be used for other proteases associated with human inflammatory diseases, we constructed a human ACRP-MMP-TNFR-Tn. We then tested whether the collagen-like domain of human ACRP30 could mask the binding activity of human TNFR2. In the human construct, we replaced the MMP-2/9 substrate linker with an MMP-13 substrate sequence (GPQGLAGQ), as MMP-13 is another protease that is often upregulated in inflammatory diseases such as rheumatoid arthritis. Recombinant human ACRP-MMP-TNFR-Tn was produced and purified from Expi293 cells transfected with the expression vector (Fig. 5a). Similar to the mouse recombinant proteins, recombinant human ACRP-MMP-TNFR-Tn displayed a higher molecular weight than expected due to glycosylation. A minor band at approximately 50 kDa was often noted and most likely represented a cleaved form created during the production of the recombinant proteins.

Similar to the mouse recombinant proteins, the human ACRP-MMP-TNFR-Tn protein was successfully digested by MMP-13 (Fig. 5a). Untreated or MMP-13-digested human ACRP-MMP-TNFR-Tn was serially diluted and added to ELISA plates precoated with human TNF-α to test the binding activity of the proteins. MMP-13-digested human ACRP-MMP-TNFR-TN displayed an increasing signal with increased amounts of digested receptor added to the wells. Although the untreated ACRP-MMP-TNFR-Tn also showed a dose-dependent signal intensity, the binding activity was lower than that of the MMP-13-digested receptors (Fig. 5b). The binding activity of untreated ACRP-MMP-TNFR-Tn most likely originated from a small amount of precleaved TNFR-Tn formed during the production of the protein in the Expi293 transfectants.

4. Discussion

To reduce the adverse effects caused by the systemic neutralization of TNF-α, we constructed an inflammatory tissue-selective TNF-α neutralizing receptor. We demonstrated that trimerization by an Acrp30 collagen-like domain in the N-terminal can block the binding activity of the TNF receptors. We also showed that the masking effect in both mouse and human chimeric TNF receptors is readily removed by two proteases, MMP-9 and MMP-13, that are often overexpressed in inflammatory tissues. Considering that serum and normal tissues contain many protease inhibitors, such as serpins and tissue inhibitors of metalloproteinase (TIMPs) [25,26], the Acrp-masked chimeric TNF receptors are expected to remain inactive until they enter inflammatory tissues. This strategy may represent an effective way to direct the neutralization of TNF-α only in inflammatory tissues.

The TNF/TNFR family is a large ligand/receptor family that plays important pathophysiological roles in the body. Elevated levels of many of the TNF superfamily members have been linked to various inflammatory diseases. For example, RANKL, a TNF member that activates osteoclasts, is highly expressed in osteoporosis [27,28], whereas BAFF, known for its role in B cell maturation and survival [29], is associated with systemic lupus erythematosus [30,31]. Thus, it is conceivable that targeting TNF superfamily members may be an effective treatment for these diseases. Indeed, several biologics (monoclonal antibodies and receptor-Fc fusion proteins) that neutralize RANKL and BAFF have been approved for clinical use, and neutralizing biologics for other TNF superfamily members have been developed. These new developments may lead to better treatments for diseases caused by dysregulated expression of proteins in the TNF superfamily.

Structurally, proteins of the TNFR superfamily typically contain 2–4 cysteine-rich domains (CRDs). In TNFR2, CRD1 (also known as the preligand assembly domain) is involved in trimer formation on the cell surface and may aid in binding to TNF-α, while CRD2 and CRD3 contain contact surfaces for TNF-α [21]. Overall, the TNFR superfamily proteins exhibit trimer formations. Given the conserved trimeric structures and similar binding modes of the ligand/receptor pairs in this superfamily (for example, see the structures of BAFF/BAFF-R [32] (PDB ID: 1OTZ), RANKL/OPG [33] (PDB ID: 3URF), and APRIL/TACI [34] (PDB ID: 1XU1)), our ACRP-MMP-TNFR-TN may serve as a prototype for designing inflammatory tissue-selective neutralizing receptors for TNF superfamily members. The TNFR entity in the construct can be replaced with a TNFR superfamily member for a specific neutralization need, whereas the ACRP trimerization domain is used to prevent the premature binding of the ligand until it is cleaved and the receptor is activated by proteases in the inflammatory tissues.

Proteases are often overexpressed during inflammation, thus making them an attractive molecular target for designing inflammatory tissue-selective drugs [35–37]. In this study, we used different substrate sequences for two MMPs to test the flexibility of protease selection. We found that both MMPs could efficiently cleave and restore the binding activity of TNFR. This result indicates that the protease substrate linker in our ACRP-MMP-TNFR-Tn construct can be replaced for different protease specificities. This characteristic is advantageous considering that different proteases may be specifically upregulated in various inflammatory diseases. In this manner, disease-specific neutralization of TNF ligands can be additionally improved by the inclusion of a substrate sequence in the construct that is recognized by protease specific to the disease.

In our study, we found that mouse Acrp-MMP-TNFR-Tn, but not mouse Acrp-MMP-TNFR, maintained ligand binding activity after MMP-9 digestion. We suspect that TNFR in the Acrp-MMP-TNFR construct may dissociate once the trimerization domain is cleaved. An overnight incubation at 4 °C did not seem to restore trimer formation, since very little binding to TNF-α was noted. This result indicates that a C-terminal trimerization domain is required in the design of this protease-activated TNF-neutralizing construct. This result is also consistent with the results of a prior publication indicating that a trimeric TNF receptor showed a much higher binding activity than a monomeric TNF receptor [38]. Thus, it appears beneficial to include a C-terminal trimerization domain in the design of chimeric TNF receptors to maintain the trimeric conformation of TNFR.

We consistently noted a precleaved TNF receptor (TNFR-Tn) in our protein purification. This precleaved protein was most likely derived from digestion by endogenous MMPs expressed by the HEK 293 cells [39]. Consequently, the true difference in the binding activity (e.g., the fold change in the EC_{50}) between untreated and MMP-digested human ACRP-MMP-TNFR-TN was difficult to decipher, since the precleaved receptor was expected to bind to TNF-α in the ELISA. We attempted to remove this precleaved form by HPLC with a nickel column followed by an anti-HA affinity column and by preadsorption of the precleaved TNFR to plate-bound TNF-α, but these methods had little effect. However, the addition of EDTA (100 μM) to the Expi293 cells increased the yields of the chimeric TNF receptors (data not shown) and did not completely prevent MMP cleavage of the chimeric TNF receptors. As EDTA interferes with nickel-chelating chromatography, a final solution to these technical difficulties may be the coexpression of tissue inhibitors of metalloproteinases (TIMPs) in the producer cells.

In conclusion, our strategy of linking an inhibitory cap to temporarily block the binding activity of TNF receptors seems successful. This inhibitory cap can be removed by inflammatory tissue-associated MMPs to activate the chimeric TNF receptor. By selectively binding and neutralizing TNF-α in inflammatory tissues, the constructs can achieve therapeutic effects while potentially reducing or preventing undesired adverse effects. Thus, this strategy may be useful for designing inflammatory tissue-selective neutralizing receptors for TNF-α.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

C. J. L., C. C. W., M. C. and T. Y. J. carried out experiments and collected data. K. H. C., T. L. C., Y. W. M. and T. H. H. participated in study design. K. W. L. and S. C. T. designed the study, analyzed the data and wrote the manuscript. All authors gave final approval for publication.

Competing interests

The authors declare no conflicts of interest.

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