A Small Molecule Selected from a DNA-Encoded Library of Natural Products That Binds to TNF-α and Attenuates Inflammation In Vivo

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In Memory of Professor Richard A. Lerner

Tumor necrosis factor α (TNF-α) inhibitors have shown great success in the treatment of autoimmune diseases. However, to date, approved drugs targeting TNF-α are restricted to biological macromolecules, largely due to the difficulties in using small molecules for pharmaceutical intervention of protein–protein interactions. Herein the power of a natural product-enriched DNA-encoded library (nDEL) is exploited to identify small molecules that interfere with the protein–protein interaction between TNF-α and the cognate receptor. Initially, to select molecules capable of binding to TNF-α, “late-stage” DNA modification method is applied to construct an nDEL library consisted of 400 sterically diverse natural products and pharmaceutically active chemicals. Several natural products, including kaempferol, identified not only show direct interaction with TNF-α, but also lead to the blockage of TNF-α/TNFR1 interaction. Significantly, kaempferol attenuates the TNF-α signaling in cells and reduces the 12-O-tetradecanoylphorbol-13-acetate induced ear inflammation in mice. Structure-activity-relationship analyses demonstrate the importance of substitution groups at C-3, C-7, and C-4' of kaempferol. The nDEL hit, kaempferol, represents a novel chemical scaffold capable of specifically recognizing TNF-α and blocking its signal transduction, a promising starting point for the development of a small molecule TNF-α inhibitor for use in the clinical setting.

1. Introduction

DNA-encoded chemical libraries (DELs) link the power of chemistry and genetics and have revolutionized drug discovery.[1] DELs allow the simultaneous screening of an enormously large number of chemicals against various targets, thus greatly accelerating the discovery process in the search for new drugs. However, this major advancement does itself bring new challenges in terms of library expansion and DEL selection. The ability to select functional molecules using DEL technology is undoubtedly dependent upon both the total number and the scaffold diversity of members comprising the initial DEL library.[2] Academic laboratories and pharmaceutical corporations are continually developing new DNA-compatible chemical reactions, leading to a significant increase in the number of chemical classes that can now be included in DELs.[3] At the same time, new selection strategies, especially for membrane-bound protein targets, are being developed.[4] Consequently, more
and more DEL-derived new drug candidates are entering clinical development.[5]

Tumor necrosis factor α (TNF-α), a crucial regulator in the immune response, has been the target of multiple approved antibody drugs such asadalimumab, golimumab, and certolizumab.[6] TNF-α antagonism has shown therapeutic efficacy for several autoimmune diseases such as rheumatoid arthritis (RA),[7] psoriatic arthritis, multiple sclerosis (MS), and Crohn’s disease.[8] Nevertheless, only 60–70% of patients achieve a long-term clinical response. In the remaining patients, the poor response to antibody therapies is thought to be at least partially due to the immunogenicity of these biological molecules.[9] This, in combination with other shortcomings of bio-macromolecular drugs, such as high cost (for chronic usage), poor stability,[10] and the requirement for parenteral administration, has prompted increasing interest in the development of orally bioavailable small molecule inhibitors targeting TNF-α.

Over the course of evolution, natural products, including the active ingredients in Traditional Chinese Medicines (TCMs), have developed with highly diverse and complicated chemical scaffolds. These molecules have been extensively studied and used in the clinical treatment of disease for thousands of years.[11] Many highly effective drugs are, in fact, derived from natural products, that is, Taxol[12] and artemisinin.[13] However, due to their polypharmacology, the mechanisms of action for most therapeutic natural products remain largely unclear. Although the polypharmacology of natural products hinders their further pharmaceutical development, it makes them ideal tools for chemogenomic studies.[14] By merging natural products with DEL technology, one is able to fully leverage the power of natural selection in evolution. We developed a late-stage DNA-encoding method that allowed us to generate a natural product-DEL technology, one is able to fully leverage the power of natural selection in evolution. We developed a late-stage DNA-encoding method that allowed us to generate a natural product-DEL library (nDEL), in which molecules with biological activities are DNA-labeled and can be profiled via affinity panning.[15]

In this study, we used the nDEL, an approach that combines the advantages of both structural diversity and polypharmacological function, to probe small molecule binding pockets on TNF-α. A natural product, kaempferol, was selected and shown to not only bind TNF-α but also disrupt the interaction between TNF-α and its cognate receptor TNFR1. It was capable of potent inhibition of both TNF-α-induced cell death and the corresponding signaling pathways in L929 cells. Furthermore, studies using a murine ear model demonstrated that kaempferol reduced inflammation in vivo.

2. Results

2.1. Affinity Panning and Competitive Enrichment of nDEL Molecules versus TNF-α

Recombinant human TNF-α (hTNF-α) was purified with Ni-NTA and size-exclusion chromatography (SEC) (Figure S1A–C, Supporting Information). Analytical SEC was carried out using authentic BSA (MW: 66.4 kDa) and lysozyme (MW: 17.9 kDa) as retention volume markers. The recombinant his-tagged hTNF-α appeared to be a trimer (approx. MW: 55.2 kDa) with an SEC retention volume close to that of BSA (MW: 66.4 kDa) (Figure S2, Supporting Information), consistent with previous reports.[16] The purified protein was biotinylated via lysine conjugation and then immobilized on streptavidin beads. An nDEL library containing 400 natural products and pharmaceutical active small molecules in addition to 10^6 combinatorial chemicals was constructed by the late-stage DNA annotation method reported previously (Figure S3 and Experimental Methods, Supporting Information).[16] As shown in Figure 1A, the recombinant hTNF-α-coated beads were mixed with the nDEL library, incubated, and washed to remove any non-specific binders. Bound molecules were then eluted using SPD304, a known small molecule TNF-α inhibitor.[17] The eluent was subjected to DNA-sequencing for structure decoding. The panning fingerprints of the eluted molecules were plotted as enrichment-fold versus normalized sequencing counts (Figure 1B), and compared with that of the negative control (Figure 1C). Seven natural products and one known drug molecule showed significant enrichment (Figure 1D,E) and were selected for further study.

2.2. Affinity Measurement of nDEL Hits Bound to TNF-α

To confirm the binding of the above hit molecules to hTNF-α, surface-plasma-resonance (SPR) was used to measure the affinity of the interaction. A CM5 chip was used with a running buffer consisting of 1% DMSO. Recombinant hTNF-α first flowed through and immobilized on the CM5 chip via amine coupling to form a uniform single molecular layer of hTNF-α on the chip surface. Of the eight nDEL hits, all showed some degree of interaction with the hTNF-α coating surface (Figure 1D). Four of these, three natural products and one known antibiotic displayed measurable binding affinities. The two flavonoid natural products, kaempferol (Kae) and kumatakenin B (KB), showed similar K_d values of 45 ± 5.5 and 15 ± 5.8 μM, respectively; whereas, the natural product, gancaconin I (GCN), and the antibiotic, moxifloxacin hydrochloride (MHCl), showed more potent binding with K_d values of 1.9 ± 0.2 and 7.8 ± 1.5 μM, respectively (Figure 1D,E and Figure S4, Supporting Information).

2.3. Structure–Activity Relationship of Kaempferol Analogues

To further explore the relationship between the structure and affinity of flavonoids to TNF-α, we tested four additional flavonoid analogs of kaempferol that are commercially available and contain different numbers of hydroxyl or methoxyl substitution groups (Figure 2A and Figure S4, Supporting Information).
Figure 1. Library panning and hit identification of nDELs targeting hTNF-α. A) Workflow of competitive panning of nDEL library. SPD304, a known small molecule binder to TNF-α, serves as the competition eluent (red pentagon). B,C) Fingerprint plots for the nDEL screenings enriched by hTNF-α coated or empty magbeads, respectively, in which y-axis represents enrichment-folds, while x-axis represents sequence counts; red dashed lines are the cut-off values for hits selection and red dots represent enriched nDELs. D) Chemical structures (upper panel) and summary table (lower panel) of confirmed nDEL hits, in which four compounds, including kumatakenin B (KB), kaempferol (Kae), gancaonin I (GCN), and moxifloxacin (MHC1), showed measurable $K_{D,app}$ values, and the other four compounds, including flumequine (FMQ), semilicoisoflavone B (SFB), gentisic acid (GA), and glycyrol (GCR), showed weak binding. The “+” sign in the lower panel table represents a weak binding signal in SPR sensorgram that is too weak to quantitate. E) Apparent $K_{D,app}$ values of the four potent nDEL hits. All results are shown as means ± SD.
Figure 2. SAR analysis of flavonoid analogues. A) Chemical structures (upper panel) and $K_{D,app}$ measurements (lower panel) of apigenin (APN), galangin (GLG), kaempferide (KMF), and 4',5-dihydroxyflavone (DHF). B) Competitive affinity measurement of flavonoids to hTNF-α in the presence of hTNFR1-ECD-HRP by ELISA assay ($n = 6$). C) Summary table of apparent binding affinity of flavonoids to hTNF-α in the absence ($K_{D,app}$) and presence ($IC_{50}$) of hTNFR1-ECD-HRP. All results are shown as means ± SD.

Information) using SPR. As shown in Figure 2B, similar to $Kae$ and $KB$, apigenin (APN), galangin (GLG), kaempferide (KMF), and 4',5-dihydroxyflavone (DHF) all interacted with the recombinant TNF-α. Compared to $Kae$, removing any of the –OH group at the C3 (e.g., APN), C7 (e.g., DHF), or C4' (e.g., GLG) position resulted in improved binding to hTNF-α. In contrast, methylation at C3 and C7 (e.g., KB) or at C4' (e.g., KMF) had little effect on the binding affinity (Figure 2C).

2.4. Blocking of Protein–Protein Interaction between TNF-α and TNFR1

To further explore if binding to TNF-α by compounds selected from our nDEL inhibited the binding of TNF-α to its cognate receptor, TNF-α receptor 1 (TNFR1), a competitive affinity assay was performed using ELISA. First, the cognate ligand-receptor interaction was established in a 96-well microtiter plate using...
recombinant hTNF-α and HRP-tagged hTNFR1 extracellular domain (hTNFR1-ECD-HRP) (Figure S1D, Supporting Information). The apparent EC_{50} value of hTNFR1-ECD-HRP to hTNF-α was determined to be 4.6 ± 0.3 nM (Figure S5A, Supporting Information). All compounds to be tested were then examined and shown to have a minimal disturbance of absorption at wavelength 450 nm under the assay conditions. The competitive assay was carried out by pre-mixing hTNFR1-ECD-HRP (4.6 nM) with test compounds at various concentrations in a 100 μL assay solution, followed by incubation in a 96-well microtiter plate coated with hTNF-α. In the presence of TNFR1, the non-flavonoid compounds, MHC1 and GCN, showed either no or greatly reduced (>5000-fold less) binding potency, respectively (Figure S6, Supporting Information), which was in line with the lack of efficacy for these molecules seen in later cellular functional assays (Figure 3A,B). In contrast, the flavonoid compounds, Kae and KB, showed nearly three orders of magnitude enhancement in binding potency in the presence of TNFR1, with apparent IC_{50} values of 76 ± 4.3 and 61 ± 12 nM, respectively (Figure 2B,C). Compared to Kae, methylation of the –OH group at C3 and C7 (e.g., KB) did not affect the potency of competitive binding, whereas removal of either the –OH at C3 (e.g., APN) or at C7 (e.g., DHP) resulted in a potency decrease of greater than two orders of magnitude (Figure 2B,C). Moreover, methylation (e.g., KMF) or removal (e.g., GLG) of the –OH substitution at C4 both resulted in a modest 2–5 fold reduction in binding potency (Figure 2B,C). Flavonoids appeared to display a very different structure–activity relationship (SAR) trend of binding to TNF-α with or without the presence of TNFR1.

2.5. Kae Rescued TNF-α-Induced Cell Death by Blockage of TNF-α Cell Signaling

TNF-α is a key molecule in the inflammatory response. It is a multifunctional, pro-inflammatory cytokine involved in various physiological and pathological processes, including cell proliferation, differentiation, and apoptosis, as well as immune modulation and the induction of inflammation.\[18\] In the following experiment, TNF-α-induced apoptosis was measured using a proliferation assay in the L929 cell line that overexpresses TNFR1 (Figure S9B, Supporting Information). Adalimumab, an anti-TNF-α antibody known to inhibit TNF-α-induced apoptosis,\[19\] was used as a positive control. Initially, we showed that the nDEL hit GCN showed apparent intrinsic cytotoxicity to the L929 cell line (Figure S7, Supporting Information) and was excluded from the following rescue experiment. The protective effect of the two flavonoids, Kae and KB, and the antibiotic MHC1 on TNF-α-induced cell death of L929 was examined and compared to that of adalimumab. As expected, MHC1, which only binds to TNF-α in the absence of hTNFR1-ECD, showed no apparent protection at 100 μM; whereas the flavonoids, that bind to TNF-α in both the presence and absence of hTNFR1-ECD, displayed a strong protective effect similar to that of adalimumab (Figure 3A). Furthermore, KB and Kae, the two flavonoids showing similar potency in the previously-described hTNFR1-ECD competition assay, rescued the TNF-α-induced cell death of L929 in a dose-dependent manner, with compatible apparent IC_{50} values of 28 ± 8.3 and 22 ± 1.7 μM, respectively (Figure 3B).

TNF-α cell signaling via TNFR1 orchestrates an intricate cellular network of signal pathways which can lead to two paradoxical effects: a) stimulation of cell survival and expression of pro-inflammatory genes or b) apoptosis and cell death. TNF-α-induced activation of the caspase cascade represents a hallmark of TNF-α signaling leading to programmed cell death (apoptosis).\[20\] We further investigated if KB and Kae rescued cells from TNF-α-induced cell death by inhibiting the caspase cascade activation in L929 cells. The intrinsic effects of the tested compounds on the caspase cascade were first ruled out (Figure S8A, Supporting Information). As shown in Figure 3C, at 50 μM, KB and Kae, like adalimumab, blocked completely the TNF-α-induced cleavage of caspase-3. MHC1, on the other hand, showed only partial inhibition (56% inhibition) of caspase cascade activation at 100 μM.

Induction of mitogenesis is another hallmark of TNF-α signaling and is exerted by the activation of key transcription factors such as NF-κB and AP1. NF-κB activation results in the degradation of IκB protein, a negative regulator of NF-κB, in cells.\[21\] We examined the ability of Kae and KB to inhibit TNF-α-induced NF-κB activation by monitoring IκBα degradation in L929 cells. The intrinsic effects of the tested compounds on IκBα degradation were first ruled out (Figure S8B, Supporting Information). As shown in Figure 3D, both Kae and KB, at 50 μM, inhibited ≈45% TNF-α-induced IκBα degradation in L929 cells. Adalimumab also showed a similar partial inhibition at a saturated concentration (10 nM). Moreover, when compared to the background control, MHC1 showed no inhibition of TNF-α-induced IκBα degradation at 100 μM.

2.6. Kae Attenuated TPA-Induced Ear Inflammation in Mice

To further investigate the anti-inflammatory effect of the flavonoids Kae and KB, an in vivo, phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema model was established in C57BL/6 mice. TNF-α is a key factor involved in TPA-induced dermatitis, especially at the stage where dermal edema is significant.\[21\] Topical application of TPA has been shown to induce TNF-α expression at the application site.\[22\] Exposure of a mouse ear to 2.4 μg TPA (in neat DMSO) for 30 min led to a significant increase in ear thickness and tissue weight (Figure 4A). Indomethacin (Indo), a COX inhibitor, was used as a positive control for anti-inflammation.\[23\] In a typical experiment, Indo (0.5 mg in neat DMSO) or the test compound, Kae (0.1, 0.2, 0.5 mg in neat DMSO), was applied topically to the TPA-treated area at 30 min and 3.5 h after TPA exposure. 3 h following the second application of Indo or Kae, tissue samples were collected for analysis. In experimental mice (four mice/cohort group), the contralateral ear served as an internal background control and was not treated with either TPA or test compounds. As shown in Figure 4A, TPA treatment resulted in a significant swelling of the treated ear, while the contralateral ear showed no effect. This TPA-induced inflammation was greatly attenuated following treatment with either 0.5 mg Indo or 0.5 mg Kae (Figure 4A). Both ear thickness and tissue weight in Kae-treated mice were reduced by levels comparable to those achieved in Indo-treated mice (Figure 4B), indicating a strong anti-inflammatory effect for Kae. Furthermore, Kae was shown
Figure 3. Effects of nDEL hits on cellular TNF-α signaling. A) Morphology images of L929 cells upon treatment with actinomycin D (Control), hTNF-α (TNF-α), hTNF-α and adalimumab (TNF-α+mAb), hTNF-α and KB (TNF-α+KB), hTNF-α and Kae (TNF-α+Kae), and hTNF-α and MHCl (TNF-α+MHCl), respectively. B) Dose-dependent rescue of hTNF-α induced cell death of L929 by KB, Kae, and MHCl (n = 6). C) Western-blot analyses of cellular caspase-3 and cleaved-caspase-3. D) Western-blot analyses of cellular IκBα. Relative intensity of cleaved caspase-3 for each testing compound was quantitated by normalizing against band intensity of the internal β-actin and subtracting band intensity of background. Results are shown as means ± SD (n = 6).
Figure 4. Anti-inflammatory effect of Kae in TPA induced skin edema mouse model. A) Representative images (200 × magnification) of H&E stained mice ear sections from four cohort groups, in which Indo represents the positive control, indomethacin; Blank represents the untreated group (without TPA and compound treatment); TPA+Vehicle represents the group treated with 2.4 μg TPA and neat DMSO (vehicle); TPA+Indo represents the group with 2.4 μg TPA stimulation and 0.5 mg Indo treatment; and TPA+Kae 0.5 represents the group with 2.4 μg TPA stimulation and 0.5 mg Kae treatment. B) Dose-dependent efficacy of Kae against TPA-induced ear edema in mice ear (n = 4). Normalized ear thickness and ear weight of mice in different cohort groups are recorded and compared. For each mouse, normalization was carried out by subtracting the thickness and weight of the other untreated mouse ear from those of the treated ear. Kae 0.5, Kae 0.2, and Kae 0.1 represent different Kae dosages of 0.5, 0.2, and 0.1 mg, respectively. C) Representative FACS histograms. D) Quantitative FACS results showing the comparison of neutrophil amounts in peripheral blood of experimental mice (n = 4). E) mRNA expression of TNF-α, IL-1α, and CXCL2 in experimental mice ear determined by RT-PCR (n = 4). All results were shown as means ± SD. p-values are calculated using one-way ANOVA with Bonferroni correction, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
to reduce mouse ear swelling in a concentration-dependent manner (Figure 4B).

To further understand the underlying mechanism of the observed 
Kae effect, peripheral neutrophil levels and tissue mRNA 
levels of pro-inflammatory cytokines (TNF-α, IL-1α, and CXCL2) 
were measured, respectively. TPA-treated mice had an increased 
level of neutrophils in their peripheral blood, as well as increased 
mRNA expression of TNF-α, IL-1α, and CXCL2 in the tissues of the 
treated ear. Application of Kae following TPA exposure completely 
suppressed the increase in peripheral neutrophils and the increase in mRNA expression of all three cytokines (Figure 4C–E). In contrast, although TPA-treated mice receiving subsequent treatment with Indo showed complete suppression of the increase in peripheral neutrophils, only mRNA expression of 
CXCL2 and IL-1α, but not TNF-α, was suppressed in the treated tissue samples (Figure 4C–E). This is consistent with the different anti-inflammatory mechanisms for the observed effects of kaempferol and indomethacin.

3. Discussion

Polypharmacological profiling of multiple targets is an important yet challenging endeavor in drug discovery. In an effort to dis-
sect the underlying mechanisms of diseases and other biological 
phenotypes, the chemogenomic approach has been developed to 
link chemical structures with bioactivity. Current chemogen-
omic research relies on existing knowledge databases and is 
limited to a few disease areas, that is, CNS, drug abuse (DA), 
and cancer.[14a] Natural products-enriched DEL library, acting as 
a molecular probe of polypharmacology, enables the chemogen-
omic approach readily applicable to a wide range of research 
efforts.

DEL approach was successfully applied in an attempt to 
identify novel small molecule inhibitors of TNF-α before.[24] 
In the current study, we took advantage of the polypharma-
cological characteristic of an nDEL library consisting of 400 
biologically active compounds (Table S4, Supporting Informa-
tion) to map potential small molecule binding pockets on TNF-
α. Kaempferol, a flavonoid natural product, was identified as 
one of the compounds capable of direct binding to the hTNF-
α. It was shown to inhibit TNF-α-induced signaling transduc-
sion in L929 cells and attenuate ear inflammation in an acute 
mouse model. Kaempferol, extracted mainly from Kaempferol 
galanga L., is widely found in many edible plants and herbal 
medicines,[25] and is known for its anti-inflammatory and anti-
oxidative effects.[26] Significantly, kaempferol has been shown to 
attempt the severity of arthritis in a collagen-induced arthritis (CIA) mouse model.[17] In addition, it has a high safety profile in 
terms of both liver and renal side effects. Our current study es-
lished that kaempferol can exert its anti-inflammatory effect 
via interruption of TNF-α cell signaling similar to Humira (adal-
imumab).

Polypharmacology associated with chemical drugs is a key factor for their adverse effects in clinical application. MHCl, a 
quinolone antibiotic, is known to potently inhibit the enzymatic 
activities of bacterial DNA gyrase and topoisomerase IV leading 
to strong antimicrobial activity. Its off-target inhibition of mam-
malian topoisomerase II at high concentration (>100 μM), on the 
other hand, is believed as one of the important factors causing 
various adverse events of MHCl such as cardiac, hepatic, and cut-
aneous toxicities.[27] Our observation that MHCl is a potent TNF-
α binder (apparent Kd = 7.8 μM) provides new insight into the 
infamatory nature of this molecule. Interestingly, our study 
showed that the binding of MHCl to TNF-α did not interfere 
with the TNF-α-TNFRI signaling. MHCl showed only partial 
inhibition of TNF-α induced caspase cascade with no inhibition of 
TNF-α induced NFκB activation (Figure 3C–D).

To date, several small molecules that directly interact with 
TNF-α have been identified, including SPD304, a potent but cy-
toxic chemical,[17] C87, a compound blocking TNF-α-induced 
signaling but not TNF-α binding to TNFR1,[28] and benpyrine, 
a natural product molecule with weak potency.[30] He et al. pro-
posed a binding mode of SPD304 in TNF-α regulation, in which 
an SPD304 molecule displaced a subunit from the native trimeric 
TNF-α complex to form a complex with a dimeric TNF-α.[17] 
The binding mode of kaempferol with the TNF-α trimer is cur-
rently under investigation using cryo-EM and X-ray crystallo-
graphy. Our affinity study of nDEL molecules to TNF-α in the pre-
ence and absence of TNFR1 demonstrated a synergistic interac-
tion between flavonoids and TNFR1 that is correlated to the cel-
ular TNF-α/TNFRI signaling pathway. Our preliminary analyses 
of the chemical structure and TNF-α function suggested that the 
erster interactions at C-3 and C-7 and the H-bonding at C-4 of 
kaempferol appeared to be essential. Our current study demonstra-
ted the power of combining natural products with DEL tech-
nology in polypharmacological research, an emerging paradigm 
in next-generation drug discovery and development.

4. Conclusion

In this study, we demonstrated that DEL technology, in com-
bination with natural products and functional small molecules 
(nDEL approach), could serve as an invaluable polypharmacolo-
gical probe in chemogenomic research. As a pilot study for proof-
of-concept, TNF-α, a key cytokine in inflammatory responses, 
was used as the target protein. A natural-product small molecule 
binder of TNF-α, Kae, was identified by nDEL panning and 
showed impressive inhibitory efficacies on TNF-α induced cell 
signaling both in vitro and in vivo similar to that of the known 
anti-inflammatory antibody drug, adalimumab (Humira). Pre-
liminary SAR analysis of kaempferol analogs showed potential 
to develop an “Oral Humira” by further improving the potency 
and efficacy of Kae upon structural modification. Moreover, the 
identification of TNF-α as one of the cellular targets for MHCl, 
a quinolone antibiotic, provides a plausible mechanism account-
ing for some of its adverse effects. The nDEL approach, thus, is 
undoubtedly a powerful tool for next-generation pharmaceutical 
discovery and development.

5. Experimental Section

Cloning, Expression, and Purification: The coding sequences of the 
extracellular domain of the human TNF-α receptor 1 gene (hTNFR1-ECD), 
amino acid 22–211 coding sequence from the N-terminal ROSV to the C-
terminal QIEN, or human TNF-α gene (amino acid 77–233 coding se-
quence from the N-terminal VRSS to the C-terminal IIAL) was PCR am-
plicated from Hela cell cDNA and cloned into a pET-28a expression vector
oxidase using HRP Conjugation Kit (Proteintech, PK20001) aliquoted, and ammonium acetate, pH 6.0, 1 mm DTT, and 300 mm imidazole for hTNF-α.

Supernatant was loaded to a Ni-NTA agarose (GE) column, rinsed with aity purification, insoluble debris was first removed by centrifugation. The hTNF-α was dialyzed twice again with a 4 L buffer (50 mm MES, pH 6.2, 25 mm NaCl) at 4 °C, and dialyzed twice again with a 4 L 50 mm Tris buffer (pH 7.5) at 4 °C. PMSF, and incubated overnight with gentle stirring at 4 °C. Dialysis was performed twice with 20 μg guanidine-HCl, 100 mm Tris, pH 8.5, 4 mm PMSF) for 40 min at room temperature, and kept in the denaturing solution supplemented with 20 μM EDTA for 40 min at 4 °C. The resulting solution was centrifuged at 4000 g for 15 min at 4 °C, and the renatured hTNF-α was dialyzed twice against a 4 L acetate buffer (pH 4.0, 1 mm DTT and 10 mm imidazole for hTNF-α), and eluted with an elution buffer (25 mm MES, pH 6.2, 25 mm NaCl and 300 mm imidazole for hTNF-1-ECD; or 25 mm ammonium acetate, pH 6.0, 1 mm DTT and 300 mm imidazole for hTNF-α).

The supernatant containing recombinant hTNF-α and the renatured hTNF1-ECD were subject to affinity column purification. For typical affinity purification, insoluble debris was first removed by centrifugation. The supernatant was loaded to a Ni-NTA agarose (GE) column, rinsed with a washing buffer (25 mm MES, pH 6.2, 25 mm NaCl and 10 mm imidazole for hTNF1-ECD; or 25 mm ammonium acetate, pH 6.0, 1 mm DTT and 10 mm imidazole for hTNF-α), and eluted with an elution buffer (25 mm MES, pH 6.2, 25 mm NaCl and 300 mm imidazole for hTNF1-ECD; or 25 mm ammonium acetate, pH 6.0, 1 mm DTT and 300 mm imidazole for hTNF-α).

The elution fractions containing the desired protein were combined, dialyzed to remove imidazole, concentrated by ultrafiltration, aliquoted, and stored at −80 °C.

The purified hTNF1-ECD was further conjugated to horse-radish peroxidase using HRP Conjugation Kit (Proteintech, PK20001) aliquoted, and stored at −20 °C.

**SPR Assay**

Binding affinity of small molecule to protein was measured on a Biacore BK instrument (GE Healthcare) by SPR assay. For a typical assay, 20 μl of protein solution in HEPES buffer (pH 4.5), and coupled to a CMS chip (GE Healthcare, 29-1049-88) following the manufacturer’s instruction. The running buffer (PBS-P, pH 7.4) contained 1% instead of 5% DMSO in order to keep the activity of hTNF-α (Table S2, Supporting Information). Chemicals were 1:2 serial diluted from 50 μM to 1.56 μM (final concentrations) and injected at a flow rate of 30 μl/min for 90 s for the association step followed by dissociation for an additional 90 s using the LWM multi-cycle kinetics/affinity method provided by GE Healthcare. Solvent correction was carried out before and after each analysis with eight different concentrations of DMSO solution per cycle. The Kd value was calculated using Evaluation Software (GE Healthcare).

**Competitive Binding Assay**

Microtiter plates (Nunc 96F Maxisorp) were coated overnight with 100 μL/well of 10 μg/mL avidin (Pierce, 21121) in a 50 mm sodium carbonate buffer (pH 9.0) at 4 °C. After removal of the coating solution, the plates were incubated at 275 μL/well with a blocking solution containing 5% milk v/v in a PBST buffer (pH 7.4) at 37 °C for 1 h. The resulting plates were rinsed once with PBST and incubated at 100 μL/well with a 7.5 nm sparsely biotinylated hTNF-α solution (PBS, pH 7.4) for 1 h with shaking. After incubation, plates were rinsed four times with PBST washing buffer (PBST, pH 7.4).

Testing compounds were 1:10 serial diluted from 100 μM to 1 nm using a solution containing 25 mm MES (pH = 6.2), 25 mm NaCl, 1% DMSO, and 4.6 nm TNFR-HRP. The final dilution mixture was added to each well of the above plates (100 μL/well) and incubated with shaking for 90 min. Then the plates were washed three times with PBST followed by the addition of 50 μL TMB peroxidase substrate of HRP (Beyotime, P0209-500 mL) into each well. After incubation at room temperature for 30 min, the reaction was quenched with 2 μl sulfuric acid, and absorbance at 450 nm was measured on a microplate reader (Enspire, PerkinElmer).

**Cell Proliferation and Cytotoxicity Assay**

The Cell Counting Kit-8 (CCK8, Meilunbio, MA0218) was applied to measure cell proliferation and survival. For TNF-α induced cell apoptosis measurement, L929 cells that overexpress TNFR1 receptor were seeded at a density of 10^4 cells/well in a 96-well microtiter plate and incubated for 24 h in a DMEM media with 10% FBS at 37 °C. The overnight L929 cells were switched into a fresh media containing 0.3 μg/mL-1 actinomycin D, 1 ng/mL-1 hTNF-α, and/or 10 nm adilmamab (positive control), and/or indicated concentrations of various compounds (in 1% DMSO), and incubated at 37 °C for an additional 20 h. Cell survival was measured by the CCK8 assay (Meilunbio, MA0218). Cells without hTNF-α or any testing compound were used as blank, while cells with only hTNF-α were used as the negative control.

For cytotoxicity assay of compounds, 10^4 cells/well L929 cells were seeded in a 96-well microtiter plate and incubated in a DMEM media with 10% FBS at 37 °C for 24 h. Compounds at different concentrations were next added and incubated for an additional 20 h at 37 °C. Cell survival was measured by CCK8.

**TNF-α-Induced Signal Transduction in L929**

L929 cells were seeded at a density of 10^4 cells/well in a 96-well plate and incubated in a DMEM media with 10% FBS at 37 °C for 24 h. The overnight L929 cells were incubated into a fresh DMEM media containing 0.3 μg/mL-1 actinomycin D, 1 ng/mL-1 hTNF-α, and 50 μM KB or Kae (in 1% DMSO), or 100 μM MHC1 (in 1% DMSO), or 20 μM GCN (in 1% DMSO), or 10 nm adilmamab (in PBS, pH 7.4), and incubated at 37 °C for an additional 14 h. Cells in each well were lysed, and the cellular proteins were extracted and subject to analysis using BCA Assay Kit (Thermo Scientific, 23227).

The amount of β-actin, total Caspase-3, Cleaved Caspase-3, and IκBα were determined by western-blot analysis using an anti-β-actin antibody (Abcam, ab49900), anti-Caspase-3 antibody (CST, 9662), anti-Cleaved Caspase-3 antibody (CST, 9664), and anti-IκBα antibody (CST, 4814), respectively.

**Acute Irritant Contact Dermatitis Murine Model**

All animal experiments were approved by the Institutional Animal Care and Use Committee of ShanghaiTech University. Female C57BL/6 mice (6- to 8-week-old) were purchased from Shanghai Model Organisms Center. All animals were maintained in a pathogen-free, temperature-controlled environment with a 12 h light/dark cycle and provided chow and water ad libitum. All animals were allowed to acclimate for 1 week in the animal facility before handling.

Mice were randomly divided into different cohort study groups. TPA, Kae, and indomethacin were dissolved in neat DMSO (vehicle). Atopic treatment was carried out on the inner and outer surfaces of one ear for each mouse. Acute irritant contact dermatitis was induced by 2.4 μg TPA. After 30 min, vehicle, Indo (0.5 mg), and Kae (0.5 mg) were applied to the TPA treated mouse ear of each cohort group, respectively. After an additional 3 h, a second atopic treatment was carried out to the corresponding mouse ear of each cohort group. After an additional 3 h, mice were sacrificed. Peripheral blood was collected from the canthus vein of mice. 6 mm ear punch biopsies were taken for ear thickness measured with a thickness gauge and for weight. Ear biopsies were stored at −80 °C for the following gene expressions.

Biopsies from negative control and treated mice ears in each group were collected and fixed in 4% paraformaldehyde solution overnight at 4 °C. The tissues were embedded in paraffin and then sectioned at a thickness of 6 μm. Tissue sections were stained with hematoxylin and eosin prior to observation with a light microscope.

Peripheral blood was collected from each mouse and treated with red blood cell (RBC) lysis buffer (Biolegend, 420301). Whole cells except erythrocytes were collected by centrifuged at 500 g for 10 min and incubated with anti-Mo Ly6C/Ly6G (Invitrogen, 11-5931-82) antibody for 20 min. Cells were washed several times and analyzed on a flow cytometer (CytOFLex, Beckman Coulter).

The above ear biopsies were lysed by TRIzol Reagent (Invitrogen) reagent and homogenized by a homogenizer (JXSTFTPRP-48, Shanghai Jing Xin). Total RNA was obtained from lysed cells and then reverse-transcribed into
cDNA using the Vazyme kit (R212-02) following the manufacturer’s protocol. RT-qPCR was performed in triplicates with 2 × SYBR Green qPCR Master Mix (Bimake, B21202) on CFX384 Real-Time System (BIO-RAD). Messenger RNA expression levels of TNF-α, IL-1β, and CXCL2 were measured and normalized to the internal control genes (β-actin). The primer sequences are listed in Table S2, Supporting Information.

Statistical Analysis: Experiments were repeated at least three times, and the results were expressed as means ± standard deviation (S.D.) unless otherwise indicated. Sample numbers are indicated in figure legends. Data analysis was performed with GraphPad Prism software. Four parameter non-linear regression was used for fitting and IC50 calculation. Significance was assumed at a p-value <0.05 by using a two-tailed Student’s t-test or one-way ANOVA for comparisons of two groups or more than two groups, respectively, in the software for normally distributed data sets with equal variances, (* p < 0.05, very significant for ** p < 0.01, and the most significant for *** p < 0.001).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
S.W., X.S., and J.L. contributed equally to this work. Prof. Richard A. Lerner directed and provided insight and method to this study, a work he viewed highly for DEL application before his passing away. S.W. performed the selection, the biochemical experiments, in vivo experiments, and analyzed the data. J.L. synthesized the DEL library and contributed to the SPR experiments. Q.H., X.S., Q.J., L.L., Y.Y., and Y.D. helped in the experiments. X.S. and T.W. helped in the in vivo experiments. M.Y. provided the GCN Master Mix (Bimake, B21202) on CFX384 Real-Time System (BIO-RAD). Messenger RNA expression levels of TNF-α, IL-1β, and CXCL2 were measured and normalized to the internal control genes (β-actin). The primer sequences are listed in Table S2, Supporting Information. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
DNA-encoded library, flavonoid, inflammation, kaempferol, tumor necrosis factor α

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