**Supplementary information for Afatinib exerts immunomodulatory effects by targeting the pyrimidine biosynthesis enzyme CAD**

**Supplementary Materials and Methods**

**Reagents and antibodies:** LEAFTM Purified anti-human CD3 Antibody (Biolegend, Cat. #317304). LEAFTM Purified anti-human CD28 Antibody (Biolegend, Cat. #302914). LEAFTM Purified anti-mouse CD3ε (Biolegend, Cat. #100314). LEAFTM Purified anti-mouse CD28 Antibody (Biolegend, Cat. #102112). Afatinib (LC Laboratories, Cat. #A-8644) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM as stock solution, which was stored at −20°C until use. Afatinib dimaleate tablets (GIOTRIF ®,Boehringer Ingelheim, Germany) were stored in a moisture-proof box, and dissolved in pure water at 4°C for animal experiments. *InVivo*Mab anti-mouse PD-1 (Clone: 29F.1A12; BioXCell, Cat. #BE0273). Precision Count Beads™ (Biolegend, Cat. #424902). Recombinant Anti-CD3 antibody [SP162] (Abcam, Cat. #ab135372). Recombinant Anti-CD8a Antibody [4SM15] (Invitrogen, Cat. #14-0808-82). Ficoll-Paque™ PLUS (GE Healthcare, Cat. #17144003). Lipofectamine™ 3000 Transfection Reagent (Invitrogen™, Cat. #L3000001). BD Cytofix/Cytoperm Plus Kit (with BD GolgiStop) (BD Pharmingen™, Cat. #554715).

**Cell culture-** Human Jurkat E6.1 cells (ATCC, VA, USA) and peripheral blood mononuclear cells (PBMCs) were maintained in complete RPMI-1640 media supplemented with 10% FBS and 1% L-glutamine. Since Jurkat T cells are one of the few T cell lines that are commercially available for studying T cell signaling and the molecular mechanisms of drug actions, their use can contribute to an easier and more consistent cell-based study. Although the Jurkat T cell line has been shown to express high levels of mature T cell receptor (CD3) and low levels of CD4 with little CD8 (1,2), it is still considered to be a useful T cell model for T cell-related studies and for characterizing the cellular target profiles of afatinib. LLC cells and MC38 cells (ATCC, VA, USA) were maintained in complete DMEM media supplemented with 10% FBS and 1% L-glutamine. Primary murine lymphocytes were maintained in complete T cell media (CTM, MEM media supplemented with 10% FBS, 5 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 2 mM sodium pyruvate, 2 mM non-essential amino acid, and 2 mM 2-mercaptoethanol). Cells were incubated in a humidified incubator with 5% CO2 at 37°C and all cell lines were sub-cultured every 3 days.

**Antibodies and reagents used in flow cytometry analysis-** APC-H7 Anti-Human CD3 (BD Pharmingen™, Cat. #560176), Alexa Fluor® 700 Anti-Human CD8 (BD Pharmingen™, Cat. #557945), PE anti-human CD25 (Biolegend, Cat. #302606), FITC anti-human CD69 (BD Pharmingen™, Cat. #555530). PE-Cy™7 Anti-Human IFN-γ (BD Pharmingen™, Cat. #557643), Alexa Flour 700 anti-mouse CD8 Antibody (Biolegend, Cat. #344724), APC/Cy7 anti-mouse CD3ε (Biolegend, Cat. #100330), PE anti-mouse CD25 (Biolegend, Cat. #102008), APC anti-mouse CD69 (Biolegend, Cat. #104514), PE anti-mouse CD178 (Biolegend, Cat. #106605), PE-Cy™7 anti-mouse PD-1 (Biolegend, Cat. #109110), and Alexa Fluor® 647 anti-mouse Ki-67 (Biolegend, Cat. #151206).

**Flow cytometry analysis-** Cells were stained with fluorescence-conjugated antibodies in staining buffer [PBS with 0.2% bovine serum albumin (BSA)], washed, and then resuspended in staining buffer. Data collection was done on a BD LSR II flow cytometer with FACS Diva software (BD Biosciences, San Jose, CA), and analysis on FlowJo 10.4 software (FlowJo LLC). For intranuclear staining to measure cytokine production, mouse lymphocytes or human PBMCs were pretreated with GolgiStop™ (BD) for 6 hours, and cells were then permeabilized using the Cytofix/Cytoperm Kit (BD) and stained for fluorescence-conjugated antibodies.

**Western blot-** Western blot analysis was performed using a standard protocol (3). Antibodies used in this study were as follows: CAD (Cell Signaling, Cat. #11933), pS1859-CAD (Cell Signaling, Cat. #12662), pT456-CAD (Santa Cruz Biotechnology, Cat. #sc-377559), CDK4 (Cell Signaling, Cat. #12790), p18 INK4C (Cell Signaling, Cat. #2896), Cyclin D1 (Cell Signaling, Cat. #2978), CDK6 (Cell Signaling, Cat. #3136), Cyclin D3 (Cell Signaling, Cat. #2936), Caspase 3 (Genetex, Cat. #GTX110543), Caspase 7 (Genetex, Cat. #GTX123679), C-Myc (Santa Cruz, Cat. #sc-40). Anti-afatinib antiserum was homemade and generated as described in the publication (4).

**Propidium iodide cell cycle analysis-** Human PBMCs (1x106 cells/ml) were treated with the indicated concentrations of afatinib for 1 hour and then stimulated with anti-CD3 (1 ug/ml) and anti-CD28 (1 ug/ml) antibodies for 72 hours to induce T cell proliferation. Cells were harvested, washed, and resuspended in PBS supplemented with 0.2% BSA. To examine the CD8+ T lymphocyte population, cells were stained with fluorescent APC-H7 anti-human CD3 and Alexa Fluor® 700 anti-human CD8 antibodies at a dilution of 1:100 for 20 minutes on ice, and then washed twice with staining buffer [PBS with 0.2% bovine serum albumin (FBS)]. After centrifugation, the cell pellets were fixed with 4% paraformaldehyde for 15 minutes at room temperature, and then washed twice. Cell pellets were resuspended in 500 µl PBS containing propidium iodide (PI, 500 µg/ml), RNase A (0.1 mg/ml) and 0.05% Triton X-100 for 15 minutes, and then subjected to cell cycle analysis using a BD LSR II flow cytometer with FACS Diva software (BD Biosciences, San Jose, CA).

**Transfection and co-immunoprecipitation-** HEK293T cells were seeded at a density of 3 × 106 cells per 60-mm dish and cultured in regular media. Next day, the cells were transfected with plasmids encoding wild-type CAD, CAD domain 1 (glutaminase and carbamoyl phosphate synthetase 2), CAD domain 2 (dihydroorotase), and CAD domain 3 (aspartate transcarbamoylase). For transfection, 0.5 ml of transfection solution [a mixture of 4 µg plasmid DNA and 6 µl Lipofectamine 2000 (Cat. #11668019, Thermo Fisher, MA, USA)] in OPTI-MEM (Cat. #31985070, Thermo Fisher, MA, USA) were added to the culture medium. After a 4-hour incubation, the medium was discarded and refreshed with regular culture medium, and then the cells were cultured for 2 days. Cells were lysed with immunoprecipitation buffer [0.1% Triton-X 100 (J.T. Baker™ Triton™ X100, Cat. #10421871), 150 mM NaCl, and 50 mM Tris, pH 7.0], and then equal amounts of lysates were collected and incubated with primary antibodies [anti-c-Myc mAb (9E10, Cat. #sc-40, Santa Cruz, CA, USA)], and anti-afatinib antiserum at 4°C overnight. Pre-washed protein G magnetic beads (Protein G Mag Sepharose Xtra, Cat. #28-9670-70, GE Life Science, CT, USA) were then added and incubated for 1 hour. The beads were precipitated and washed 3 times with PBS. To elude the target proteins, the beads were mixed with SDS sample buffer, followed by boiling. Samples were then subjected to SDS-PAGE and immunoblot analysis.

**Construction of CAD plasmids-** TheDNA fragments encoding full-length CAD (FL-CAD), CAD domain 1, CAD domain 2 and CAD domain 3 were amplified by PCR using each pair of the following primers and a template of the CAD plasmid [Human Tagged ORF Clone (NM\_004341)] obtained from Origene (Cat. #RC209469). PCR-amplified DNA fragments were constructed with pcDNA3.1(+)/myc-His B vectors (Invitrogen, Cat. #V80020) using molecular cloning approaches. For the construction of different CAD plasmids, the primers used in this study were as follows:

FL-CAD: Forward: 5’-CCACCGTGCTGGGCCGTTTC-3’

Reverse: 5’-TAGGGCCCGTGAAACGGCCCAGCACGGTGG-3'

CAD domain 1: Forward: 5'-TGAAGGTGCATGTTGACTGT-3’

Reverse: 5’-TAGGGCCCGTACAGTCAACA TGCACCTTCA-3'

CAD domain 2: Forward: 5'-GCTCTAGAGCATGCTGCACTCATTAGTGGGCCA-3’ Reverse: 5’-TAGGGCCCGTGAAACGGCCCA GCACGGTGG-3'

CAD domain 3: Forward: 5'-GCTCTAGAGCATGACCTCC CAAAAGCTTGTGCG-3’ Reverse: 5’-TAGGGCCCGTCAGGACCACACGGCGGACGG-3'

**Isolation of mouse tumor-infiltrating leukocytes (TILs)-** After the mice were sacrificed, 0.5 g of tumors were dissected from the mice, placed in a 6-mm Petri dish at room temperature (RT), and rinsed with 3 ml HBSS. Scalpels were used to mince the tumors into small fragments (1-2 mm). The suspended tumor tissues were mixed with 6 ml HBSS and 1 ml of 10 x Triple Enzyme Mix in a 10-mm Petri dish (5), and incubated on a shaker at 80 rpm and at RT for 1 hour. After incubation and removing the bigger pellets or undissociated tissue debris in the samples, the tumor cell suspensions were subjected to centrifugation at 50 x g at RT for 10 minutes. The supernatants were then collected and centrifuged at 200 x g for 5 minutes. The pellets were taken and washed twice using HBSS. To avoid contamination of the red blood cells in the samples, the cell pellets were suspended in 2 ml ACK (ammonium-chloride-potassium) lysing buffer for 5 minutes and washed twice with HBSS. The cell pellets were suspended in PBS containing 0.2% BSA and subjected to antibody staining and flow cytometry analysis.

**Immunohistochemistry staining-** LLC and MC38 tumor sections on slides were de-paraffinized with xylene and rehydrated with ethanol. After inactivating the peroxidase using 3% H2O2, the antigen was retrieved by placing the slide in a 100°C hot buffer (10 mM sodium citrate, pH 6.0) for 10 minutes. Samples were then blocked with 10% goat normal serum and incubated with a primary antibody [4SM15: anti-CD8a monoclonal antibody (1:200) and SP162: anti-CD3 monoclonal antibody (1:200)] at 4°C overnight. After 3 rinses with PBS, the secondary antibody conjugated with peroxidase was used to recognize the primary antibody. After 3 rinses with PBS, the samples were incubated with DAB (Dako EnVsion, Agilent Technologies, CA, USA) to reveal the target proteins. Hematoxylin was used as a nuclear counterstain, in accordance with the vendor’s manual.

**LC/MS-MS metabolomics profiling-** For analyzing the metabolomics profiles, human PBMCs or mouse lymphocytes were seeded at a density of 1 x 106 cells per ml, treated with or without afatinib for 1 hour, and then T cell activation was stimulated with anti-CD3 and anti-CD28 antibodies for 72 hours. For metabolite extraction, 2 x 106 cells per sample were taken, and the cells were lysed with ddH2O on ice for 5 minutes. In order to properly disrupt the cell membrane to release the cellular metabolites, cell lysates were sonicated at 30 kHz for 10 seconds, followed by intervals of 10 seconds for cooling. Cell lysates were then centrifuged at 18,800 x g for 15 minutes at 4°C. After centrifugation, the supernatant was collected. Small metabolites were extracted with 3 volumes of acetonitrile, and dried in a SpeedVac. For aniline derivatization, each dry sample was dissolved in 28 μl of ultrapure water and mixed with 4 μl of 0.3 M aniline/HCl (molar ratio: 5/1) and 4 μl of 20 mg/ml 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The reaction was carried out at room temperature for 2 hours, and then stopped by adding 4 μl of 10% ammonium hydroxide for a further 30-minute incubation. The aniline-derived sample was centrifuged at 18,800 x g for 10 minutes. The supernatant was subjected to LC-ESI-MS analysis of negative ion mode. The LC-ESI-MS system consisted of an ultra-performance liquid chromatography (UPLC) system (ACQUITY UPLC I-Class, Waters) and an ESI/APCI source of 4kDa quadrupole time-of-flight (TOF) mass spectrometer (Waters VION, Waters). The flow rate was set at 0.2 ml per minute with a column temperature of 35oC. Separation was performed with reversed-phase liquid chromatography (RPLC) on a BEH C18 column (2.1 x 100 mm, Walters) after a 10 μl sample injection. The elution started from a 99% mobile phase A (ultrapure water + 0.1% formic acid) and a 99% mobile phase B

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| --- | --- | --- | --- |
| compound | derivative | EIC±0.02 (ESI-) | RT (minutes) |
| L-Glutamine | L-Glutamine-ANL | 220.1083 | 4.06 |
| N-Carbamoyl-L-aspartic acid | N-Carbamoyl-L-aspartic acid-2ANL | 325.1300 | 5.78 |
| Aspartic acid | Aspartic acid- ANL | 282.1240 | 4.93 |
| Dihydroorotic acid | Dihydroorotic acid-ANL | 232.0800 | 5.78 |
| L-Glutamic acid | L-Glutamic acid-ANL | 221.0926 | 4.09 |
| AMP | AMP-ANL | 412.1028 | 3.97 |
| UMP | UMP-ANL | 398.0755 | 4.48 |
| GMP | GMP-ANL | 437.0980 | 4.09 |
| CMP | CMP-ANL | 397.0916 | 3.92 |

(100% methanol + 0.1% formic acid), was held at a 1% mobile phase B for 0.5 minutes, raised to a 90% mobile phase B for 5.5 minutes, held at a 90% mobile phase B for 1 minute, and then lowered to a 1% mobile phase B in 1 minute. The column was equilibrated by pumping a 1% mobile phase B for 4 minutes. The LC-ESI-MS chromatograms were acquired by ESI+ mode under the following conditions: capillary voltage of 2.5 kV, source temperature of 100oC, desolvation temperature at 250oC, cone gas maintained at 10 L/h, desolvation gas maintained at 600 L/h, and acquisition by MSE mode with a range of m/z 100-1000 and a 0.5 s scan time.The acquired data were processed by UNIFI software (Waters) with an illustrated chromatogram and summarized in an integrated area of signals.

Data process:  Target compounds were elected as in the table below with the indicated mass range for the LC extracted ion chromatogram (EIC) and retention time (RT). ESI: electrospray ionization negative mode; ANL: aniline derivative.

**TCR repertoire sequencing and bioinformatics analysis-** We used PCR amplification and high-throughput next generation sequencing (NGS) for analysis of the junction of V and J segments and the rearranged CDR3s of TCR (6,7). In brief, RNA samples were isolated from the PBMCs of healthy volunteers or NSCLC patients with EGFR mutations who received afatinib as first-line treatment over a 6-month period (*n*=9 in each group). All expressed TCRβ rearrangements were amplified using a panel of TCR primers specifically targeted to the V, D, and J gene regions, and the amplicons were sequenced by Illumina Miseq (8). Repertoire analyses were based on CDR3 amino acid sequences, and the unique molecular identifier-labeled TCR cDNA molecules were extracted using MIGEC (Molecular Identifier Guided Error Correction pipeline) software (9). The averaged TCR repertoire characteristics weighted by clonotype size were analyzed using VDJ tools software (10).

**Supplementary Table**

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Supplementary Table 1 | | | | | | | | |
| No. |  | Gene Name | Score |  | No. |  | Gene Name | Score |
| 01 | • | CAD | 714 |  | 16 |  | Histone H2B | 75 |
| 02 | • | ATP-citrate synthase | 267 |  | 17 |  | Plectin | 67 |
| 03 |  | CCAR2 | 174 |  | 18 |  | PABPC1 | 67 |
| 04 |  | GBF1 | 142 |  | 19 |  | Tubulin beta-4A | 66 |
| 05 |  | PCBP1 | 134 |  | 20 |  | EIF3H | 64 |
| 06 | • | UBA1 | 128 |  | 21 | • | KIF11 | 60 |
| 07 |  | FUBP2 | 119 |  | 22 | • | Gephyrin | 56 |
| 08 | • | HSP 90-alpha | 111 |  | 23 |  | TfR1 | 53 |
| 09 |  | Actin | 107 |  | 24 | • | DNA RLF MCM5 | 49 |
| 10 | • | HSP 90-beta | 105 |  | 25 |  | Fatty acid synthase | 46 |
| 11 | • | NEK9 | 104 |  | 26 |  | SEC24C | 40 |
| 12 |  | EIF3B | 91 |  | 27 | • | DNA RLF MCM3 | 37 |
| 13 |  | EIF3CL | 87 |  | 28 |  | SND1 | 36 |
| 14 |  | Lactotransferrin | 81 |  | 29 |  | Filamin-A | 36 |
| 15 |  | Kinesin-1 heavy chain | 75 |  |  |  |  |  |

• Black circles indicate the proteins containing an ATP-binding cleft.

**Supplementary Table S1. List of protein identities unique in afatinib treatment**

A list of proteins presents only in the gel regions (#2, #4, and #6) of the afatinib-treated group after LC/MS-MS analysis.

**Supplementary Figures and Figure legends**



**Fig. S1. Chemical structures of afatinib and afatinib dimaleate.** (A) Chemical structure of afatinib. The chemical structure of afatinib was adapted from PubChem CID, #10184653. (B) Scheme of afatinib dimaleate (GIOTRIF®). The chemical structure was adapted from the 1995-2021 European Medicines Agency.

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**Fig. S2. Afatinib inhibited the proliferation and activation of mouse CD8+ T lymphocytes.** Mouse lymphocytes were isolated from lymph nodes and used for examination of the effects of afatinib on T lymphocyte proliferation and activation. (A) Analysis of afatinib’s effects on the proliferation of mouse CD8+ T lymphocytes. Mouse lymphocytes (1x106 cells/ml) were labeled with 2.5 μM CFSE at 37°C for 10 minutes, treated with the indicated concentrations of afatinib for 1 hour, stimulated by anti-CD3 and anti-CD28 antibodies for 72 hours, and then were subjected to flow cytometry analysis. (B) Examination of afatinib’s effects on the early-activation marker CD69 of CD8+ T lymphocytes. Mouse lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours, and then were subjected to antibody staining (APC/Cy7 anti-CD3ε, Alexa Flour 700 anti-CD8, and APC anti-CD69 antibodies) and flow cytometry analysis. (C) Examination of afatinib’s effects on the late-activation marker CD25 of CD8+ T lymphocytes. Mouse lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies for 72 hours, and then were subjected to antibody staining (APC/Cy7 anti-mouse CD3ε, Alexa Flour 700 anti-mouse CD8 antibody, and PE anti-mouse CD25) and flow cytometry analysis. A representative of the flow cytometry results from 3 independent experiments are shown in the left panel. The percentages of the CD8+ T lymphocyte populations in proliferation and activation after afatinib treatment were statistically calculated from 3 independent experiments using one-way ANOVA, followed by the Bonferroni post-hoc test. \*, *p*＜0.05; \*\*, *p*＜0.01; \*\*\*, *p*＜0.001.

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**Fig. S3. Afatinib suppressed the proliferation of mouse CD8+ T lymphocytes and had no significant effect on cell apoptosis.** Mouse CD8+ T lymphocytes were purified from the splenocytes and lymphocytes of C57BL/6 mice using a CD8+ T cell isolation kit (Biolegend, Cat. #480007). Isolated CD8+ T lymphocytes (1x106 cells/ml) were treated with the indicated concentrations of afatinib for 1 hour, and then were stimulated with anti-CD3 (2.5 ug/ml) and anti-CD28 (0.5 ug/ml) antibodies for 72 hours for T cell proliferation and activation. (A) Percentages of CD8+ T lymphocytes after CD8+ T cell isolation. The CD8+ T lymphocyte populations before and after CD8+ T lymphocyte purification from the splenocytes and lymphocytes of C57BL/6 mice were examined by flow cytometry using the staining of Alexa Flour 700 anti-CD8 antibodies. (B) Examination of afatinib’s effects on the proliferation of mouse CD8+ T lymphocytes. Isolated mouse CD8+ T lymphocytes (1x106 cells/ml) were labeled with 2.5 μM CFSE at 37°C for 10 minutes, treated with the indicated concentrations of afatinib for 1 hour, and stimulated by anti-CD3 (2.5 ug/ml) and anti-CD28 (0.5 ug/ml) antibodies for 72 hours. Cell proliferation was then analyzed using flow cytometry. The results were statistically calculated from 3 independent experiments using one-way ANOVA, followed by the Bonferroni post-hoc test. (C) Examination of afatinib’s effects on CD8+ T cell apoptosis. Isolated CD8+ T lymphocytes were treated with the indicated concentrations of afatinib or 5 μM camptothecin for 1 hour, and then were stimulated by anti-CD3 and anti-CD28 antibodies for 72 hours. Camptothecin (CPT) treatment was used as a positive control to induce cell apoptosis. Cells were then subjected to cell apoptosis analyses using staining with annexin V-APC/propidium iodide (PI) and flow cytometry. The results were statistically calculated from 3 independent experiments by one-way ANOVA, followed by the Bonferroni post-hoc test. \*, *p*＜0.05; \*\*, *p*＜0.01; \*\*\*, *p*＜0.001. n.s., not significant.

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**Fig. S4. Identification of the afatinib-targeted CAD domain.**

1. Maps of different CAD plasmid constructs. The DNA fragments encoding full-length CAD (FL-CAD), Domain 1 [glutaminase and carbamoyl-phosphate synthetase 2 (CPSase 2)], Domain 2 (dihydroorotase, DHOase) and Domain 3 (aspartate transcarbamylase) were constructed into pcDNA 3.1B vectors containing a Myc tag, and schemed with their respective molecular mass. (B) Identification of afatinib-targeted domain(s) of CAD in cells. HEK293T cells were transiently transfected with different CAD plasmids (FL CAD, CAD domain 1, CAD domain 2 and CAD domain 3) and cultured in regular media for 48 hours. Cells were then treated with or without 1 μM afatinib for 24 hours. Lysates were collected for input immunoblots (upper panels), and for immunoprecipitation by an anti-Myc antibody (lower panels) and immunoblot analysis using anti-afatinib antibody and anti-Myc antibody.

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**Fig. S5. Identification of the modification site(s) of afatinib on CAD.** (A) HEK293T cells were transiently transfected with CAD plasmids with a c-Myc tag and cultured in regular media for 48 hours. The cells were then treated with 1 μM afatinib for 24 hours. Lysates were collected for immunoprecipitation of CAD proteins using an anti-c-Myc antibody. After SDS-PAGE and Coomassie blue staining, the gel band was excised and processed for LC/MS-MS analysis to determine afatinib’s modification site(s). The results from MS analysis showed that the amino acid residue of cysteine 758 on CAD protein was identified to be a modification site for afatinib, which was located in the ATP-binding motif of CAD domain 1 (glutaminase and carbamoyl phosphate synthetase 2; CPS2). (B) Relative positions of cysteine 758 with afatinib in the ATP-binding site of human CPS2. Molecular docking to explore the possible interaction mode of afatinib in the ATP-binding site of human CPS2. There is no available crystal structure of human carbamoyl-phosphate synthetase 2 (CPS2) in the public database, due to the difficulty of crystallizing human CAD protein. Hence, we selected homologous protein CPS1 (PDB ID code: 3HNE) as a template to generate the 3D model structure of human CPS2 using a Swiss Model (11). Based on the predicted 3D model structure of human CPS2, we then perform molecular docking to evaluate the potential binding regions of afatinib on CPS2.

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**Fig. S6. Effects of EGFR-TKIs (afatinib, gefitinib, erlotinib and canertinib) on the CAD-mediated metabolism of mouse CD8+ T lymphocytes.** (A-D) Metabolomics analyses of the effects of EGFR-TKIs [Afa (afatinib), Gef (Gefitinib), Erl (Erlotinib), Can (Canertinib)] on the steady-state levels of N-carbamoyl-aspartate (A), dihydroorotate (B), pyrimidine nucleotides [UMP (C) and CMP (D)] in mouse CD8+ T lymphocytes. Mouse CD8+ T lymphocytes (3 x106 cells) were treated with 500 nM of each of the above EGFR-TKIs for 1 hour, and then were stimulated with anti-CD3 (2.5 ug/ml) and anti-CD28 (0.5 ug/ml) antibodies for 72 hours. After treatment, 2x106 cells of each sample were taken for metabolic analysis. The results were statistically calculated from 3 independent experiments and analyzed by one-way ANOVA, followed by the Bonferroni post-hoc test. \*, *p*< 0.05; \*\*, *p*< 0.01.

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**Fig. S7. Role of CAD in Jurkat T cell proliferation.**

(A) Analysis of afatinib’s effects on CAD-induced Jurkat T cell growth. Jurkat T cells were transiently transfected with 5 ug of pcDNA3.1 vectors and CAD plasmids, and cultured for 48 hours. The expression levels of CAD were examined by western blot analysis using an anti-CAD antibody. α-Tubulin was used as an internal control. For the measurement of cell growth, cells were seeded at a density of 1x104 per 400 ul of regular culture media in 48-well plates and treated with the indicated concentrations of afatinib. Cell counts were measured every 24 hours by trypan blue staining and cell counting. (B) The results on Day 3 were statistically calculated from 3 independent experiments using one-way ANOVA, followed by the Bonferroni post-hoc test. \*\*, p< 0.01; \*\*\*, p< 0.001. n.s., not significant.

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**Fig. S8. Percentage of CD8+ T lymphocytes in the tumor-infiltrating lymphocytes (TILs) of LLC-bearing tumors after afatinib treatment.**

LLC cells were inoculated subcutaneously at a density of 2x105 cells into the right flank of each mouse (n=12 per group). As tumor volumes reached 200 mm3, the mice then received afatinib (10 mg/kg) by oral gavage daily, for 6 days. Vehicle mice received ddH2O. After scarification, the tumor lesions were isolated and digested by collagenase for flow cytometry analysis of the percentages of CD8+ T cells in the tumor-infiltrating leukocytes (TILs). Gating of the CD8+ T lymphocytes within the CD3+ population in the TILs is shown in the left panel. The percentages of CD8+ T lymphocytes in the TILs were statistically calculated using Student’s unpaired t test, and are shown in the right panel (n=12 each group).



**Fig. S9*.* In vivo afatinib targeted therapy modulated the proliferative capability of CD8+ T lymphocytes.**

To mimic in vivo afatinib therapeutic conditions, afatinib was dissolved in water and given to C57BL/6 mice (10 mg/kg/day) by oral gavage. Mice in the control group were given water. After the treatment, mouse lymphocytes were isolated from lymph nodes at the indicated time points and subjected to CFSE staining, stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours, and flow cytometry. (A) Schematic representation of the gating strategy for mouse CD8+ T lymphocyte proliferation using CFSE staining and flow cytometry analysis. (B) Examination of afatinib’s effect on the proliferation of mouse CD8+ T lymphocytes after 1-day, 2-day, 7-day and 14-day oral afatinib treatment. The results were statistically analyzed using Student’s t-test. P values were designated as \*, *p*＜0.05. n.s., not significant.

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**Fig. S10. Analyses of CD8+ T lymphocyte activation and IFNγ expression in the PBMCs of NSCLC patients who received afatinib-targeted therapy.**

PBMCs were isolated from the blood of lung cancer patients who received afatinib targeted therapy before and at the indicated times after afatinib targeted therapy. The PBMCs were stimulated by anti-CD3 (1 ug/ml) and anti-CD28 (1 ug/ml) antibodies for the indicated times, and subjected to flow cytometry analysis. (A) Flow cytometry examination of the CD69+CD8+ T lymphocyte populations in the patients’ PBMCs after 24-hour stimulation by anti-CD3 (1 ug/ml) and anti-CD28 (1 ug/ml) antibodies. (B) Flow cytometry analysis of the CD25+CD8+ T lymphocyte populations in the patients’ PBMCs after 72-hour stimulation by anti-CD3 (1 ug/ml) and anti-CD28 (1 ug/ml) antibodies. (C) Flow cytometry examination of the IFNγ+CD8+ T lymphocyte populations in the patients’ PBMCs after 24-hour stimulation by anti-CD3 (1 ug/ml) and anti-CD28 (1 ug/ml) antibodies.

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**Fig. S11. Assessment of combined therapy with afatinib and α-PD1 in the LLC-bearing mouse model.**

(A) Schematic time frame for the treatment courses of afatinib and α-PD1 targeting tumor growth in LLC-bearing mice. C57BL/6J mice (n=5 in each group) were each subcutaneously injected with 2 x 105 LLC cells in the right flank. When tumor sizes reached 100 mm3, the mice were randomly assigned to 5 groups (control, afatinib alone, α-PD1 alone, simultaneous combination of afatinib and α-PD1, and sequential combination of afatinib prior to α-PD1 therapy). Afatinib treatment was administered by oral gavage daily for 5 days at a dose of 10 mg/kg per mouse. α-PD1 treatment was administered on Days 0, 3, and 6 or Days 6, 9, and 12 via intraperitoneal injection at a dose of 10 mg/kg per mouse. (B) Effect of afatinib and α-PD1 treatment on tumor growth in LLC-bearing mice. Tumor volumes were measured every 2-3 days beginning on Day 0, and statistically calculated for tumor growth after treatment with afatinib, α-PD1 or both (simultaneous or sequential combination). (C) Effect of afatinib and α-PD1 on the body weight of mice in each treatment group. Body weights were measured every 2-3 days beginning on Day 0 and statistically calculated. (D) Statistical calculation of tumor weight on Day 12 after animal sacrifice. (E) Quantification of tumor-infiltrating CD8+ T lymphocytes in LLC tumors on Day 12 after treatment (n=5 each group). (F) Quantification of Ki67+CD8+ T lymphocytes in LLC tumors on Day 12 after treatment (n=5 each group). (G) Quantification of IFNγ+CD8+ T lymphocytes in LLC tumors on Day 12 after treatment (n=5 each group). The results were statistically analyzed by one-way ANOVA, followed by the Bonferroni post-hoc test. \*, p＜0.05; \*\*, p＜0.01; \*\*\*, p＜0.001.

**Fig. S12. Heat map of T cell receptor beta variable allele profiles of NSCLC patients who were administered afatinib targeted therapy.**

PBMCs were isolated from 10 ml of blood from naïve lung cancer patients or patients who received afatinib treatment for more than 1 year (n=9 per group). RNA was isolated using an RNAspin Mini Kit following the manufacturer’s instructions (Cytiva, Cat. # 25050070), and subjected to deep sequencing by Illumina Miseq. Each column in the heat map represents an individual patient and each row shows a T cell receptor beta variable allele. Orange tiles indicate that an allele was detected, while grey tiles indicate an allele absence. Control and treatment samples were projected onto a 2-dimensional plane based on pairwise similarities (F-statistic metrics).**References**

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