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Surface Presentation of Noncanonical Wnt5a Motif to Cytotoxic CD8+ T-Cell Promotes Its Mechanotransduction and Activation

Rui Li,[ab] Walida Ali,[a] Chao Ma,[ab] Apratim Bajpai,[b] Ngoc Luu,[a] Aarushi Varshney,[a] Camden Riley Rowe,[a] and Weiqiang Chen*[abc]

[a]Department of Biomedical Engineering, New York University, New York, USA. E-mail: wchen@nyu.edu

[b]Department of Mechanical and Aerospace Engineering, New York University, New York, USA.

[c]Laura and Isaac Perlmutter Cancer Center, NYU Langone Health, New York, USA.

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1. Supplementary Materials and Methods

PDMS micropillar array substrate fabrication

PDMS Micropillar array substrates were fabricated through a double-molding process. In brief, a silicon master mold of the micropillar array with micropillar of 0.8 μm diameter and 7.57 μm height was first fabricated using standard photolithography and deep reactive ion etching. PDMS negative molds were then fabricated from the silicon master mold by applying a thin layer of uncured PDMS (Sylgard 184, Dow Corning, US; curing agent to prepolymer weight ratio 1:10) to the silicon mold, degassed for 15 minutes in the vacuum desiccator, and baked at 110 ºC for 30 mins. The negative PDMS mold was peeled off from the silicon mold and silanized with tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane (Sigma-Aldrich) overnight in vacuum desiccator. Next, a thin layer of uncured 1:10 ratio PDMS was applied to the surface of negative molds, and the constructs were cast on a 22 mm × 22 mm plasma-etched cover glass (Thermo Fisher Scientific), degassed for 15 minutes in the vacuum desiccator, and were heated at 110°C for 40 hours to fully cure. Then the cured PDMS micropillar array together with cover glass were peeled off from negative mold in 100% ethanol environment (Thermo Fisher Scientific), and dried with liquid CO_2 in a critical point dryer (Samdri-PVT-3D, Tousimis).

Foxy5 peptides synthesis and preparation of ligand coating solution

Foxy5 peptides (N-formyl- MDGCEL), 2-Aminobenzoyl-labeled Foxy5 peptides (Abz-Foxy5), and control scrambled peptides (N-formyl-GELDCM) were synthesized by Genscript. The purity and molecular weight of peptides were confirmed through high-performance liquid chromatography (HPLC) and Mass spectrometry (MS) (**Fig. S1**). Ligand stocking solutions of Foxy5, Abz-Foxy5, and scrambled peptides were prepared in 1 μg/μL with sterilized phosphate buffered saline (PBS) solution containing 0.5 μM Tris-(2-Carboxyethyl) phosphine, Hydrochloride (TCEP) (Thermo Fisher Scientific). The anti-CD3ε antibody OKT3 and anti-CD28 antibody, ICAM-1, Fibrinogen were purchased from Thermo Fisher Scientific. Different coating solutions for PDMS micropillar array functionalization were prepared by separately adding different components as listed in **Table S1** into sterilized PBS solutions containing 0.5 μM TCEP. TCEP in coating solutions helps expose the thiol groups for bioconjugation.

Components	Foxy5 peptides (μg/mL)	Scrambled peptides (μg/mL)	Anti-CD3ε Antibodies (μg/mL)	Anti-CD28 Antibodies (μg/mL)	ICAM-1 (μg/mL)	Fibrinogen (μg/mL)
Foxy5+Abs	15	0	30	30	20	25
Scr+Abs	0	15	30	30	20	25
Abs	0	0	30	30	20	25
Blank	0	0	0	0	20	25

Table S1. Specific components of coating solutions for different PDMS micropillar array functionalization groups.

PDMS micropillar array substrate biofunctionalization

Before surface modification, PDMS substrates were treated with ultraviolet (UV) ozone for 7 min in a UVO cleaner (Jelight) machine to activate the hydroxyl groups for further reactions. Then the activated substrates were silanized with 50 μM Silane-PEG-Mal (MW=600, Biopharma PEG) in methanol (Sigma-Aldrich) to modify the PDMS surface with maleimide groups for four hours at room temperature. Then the micropillar array substrates were thoroughly washed with methanol, and the residual methanol solutions were gradually replaced with 70% methanol, 50% methanol, and sterilized deionized (DI) water. Then the substrates were coated with 30 μL of different coating solutions at 37°C for 2 hours for ligand conjugation. The functionalized PDMS micropillar array substrate was mounted at the bottom of a 60 mm petri dish (Thermo Fisher Scientific) with 16 mm hole with PDMS and finally submerged with 500 μL of T cell culture media for subsequent experiments.

Quantification of ligand conjugation efficiency

To observe the distribution of Foxy5 peptides and T cell activation antibodies on the substrate, fluorescent ligands including Abz-Foxy5, Alexa 488 anti-CD3, and Alexa 555 anti-CD28 antibodies were used instead of the nonlabelled ligands. After the coating, coating solutions were collected to measure the residual protein contents with the Pierce BCA Protein Assay Kits (Thermo Fisher Scientific), and the substrates were thoroughly washed with sterilized PBS. The contents of conjugated ligands were determined by deducting the residual protein contents from the original protein contents in the coating solution, and the ligand conjugation efficiency was determined by dividing the conjugated protein contents to the original protein contents for each group.

Cell culture

Primary CD8+ Cytotoxic T Cells PCS-800-017 were purchased from ATCC and cultured in the ImmunoCult-XF T Cell Expansion Medium (Stemcell Technologies) supplemented with ImmunoCult Human CD3/CD28 T Cell Activator (25uL/ml, Stemcell Technologies). T cells were seeded at 250,000 cells/mm^2 into 24 well plates and incubated in a 37 °C, 5% CO_2 incubator. Cell culture media was split every 24 hours and then added with the same amount of expansion media. After 9 days, T cells were seeded on the elastic substrates for subsequent experiments.

Force dynamics measurement

Before experiments, T cells were first stained with Alexa Fluor 488-labeled anti-CD45 antibody (1:500, 304017, Biolegend) to visualize cell membrane. For force dynamics measurement experiment, T cells were loaded onto the micropillar array substrates and imaged immediately using a Nikon CSU-X1 Spinning Disk Confocal System (Nikon). In each experiment, Brightfield images and fluorescent images of both the pillar tops (Alexa Fluor 647) and the T cells (Alexa Fluor 488) were recorded every 15 seconds for one hour. The acquired images were exported as TIF image stack and analyzed using Cellogram [1] and custom-developed MATLAB programs (Mathworks) to obtain the force dynamics including magnitude and direction. The force directions were presented as the center of gravity (COG) projections of the synaptic force. The COG projections were calculated by projection each force on the deflected pillar along a line connecting the pillar tip and the COG of the T cells at that timepoint[2]. A positive projection represents an outward spreading force, while a negative projection represents an inward contraction force.

Drug treatment for signaling inhibition and activation

To inhibit the RhoA/ROCK or TCR/Lck signaling, T cells seeded on the Foxy5+Abs substrates were supplemented with either 10μM Y27632 (Stemcell Technologies) or 2μM Lck inhibitor RK-24466 (Cayman Chemical), respectively. To activate the RhoA/ROCK signaling, T cells seeded on the Scr+Abs substrates were supplemented with 100nM Narciclasine (R&D Systems). These cell cultures were supplemented with drugs until the samples were collected for flow cytometry, immunostaining and RT-qPCR tests. Cells were treated with drugs for 10 minutes and then seeded on the micropillar array substrates in the culture media containing the representative drugs to consistently inhibit or activate the signaling pathways during the force measurement.

SEM imaging of T cell on PDMS micropillar array substrate

$CD8^+$ T cells were cultured on the PDMS micropillar array substrate for one hour. The samples were then gently washed three times with 50 mM sodium cacodylate buffer (pH 7.3; Sigma-Aldrich) and fixed for one hour with 2% glutaraldehyde (Electron Microscopy Sciences) in 50 mM sodium cacodylate buffer. Then the samples were dehydrated by gradually submerging into 30%, 50%, 70%, 80%, and 90% ethanol for 10 minutes in each concentration, and fully dehydrated by submerging into 100% for three times, 20 minutes each. All samples were dried through a critical point dryer (Samdri-PVT-3D, Tousimis) under the standard protocol. The dried samples were coated with gold palladium for 10 seconds and imaged under a Hitachi S-3400N Ultra-High Resolution SEM machine (Hitachi High Technologies America). Pseudo colors were added to the SEM images to exhibit the cells and pillar array.

RT-qPCR examination on the T cell activation related genes

After the activation, T cell samples were collected in the RNA shield solution and lysed with lysis buffer in the Quick-RNA Miniprep Plus Kit (R1057, Zymo Research). Total RNA was then extracted according to the instructions of the manufacturer. The following reverse transcript was conducted using the RevertAid First Strand cDNA Synthesis Kit (K1621, Thermofisher Scientific). The quantitative real-time PCR analysis was performed with Power SYBR™ Green PCR Master Mix (4367659, Thermofisher Scientific) on a BioRad CFX96 qPCR machine. The sequences of all primers were listed in **Table S2**. The expression levels of TCR activation-related genes were normalized to that of housekeeping gene β-Actin, and the relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method.

Genes	Forward	Reverse
β-Actin	CAACCGCGAGAAGATGACCC	AGAGGCGTACAGGGATAGCA
IFN-γ	AGCTCTGCATCGTTTTGGGTT	GTTCCATTATCCGCTACATCTGAA
IL2	CAAGAATCCCAAACTCACCAG	CGTTGATATTGCTGATTAAGTCC
TNF-α	GCCAGAGGGCTGATTAGAG	TCAGCCTCTTCTCCTTCCTG
EOMES	AGGCGCAAATAACAACAACACC	ATTCAAGTCCTCCACGCCATC
GZMB	GGTGGCTTCCTGATACAAGACG	GGTCGGCTCCTGTTCTTTGAT
PRF1	GTGGAGTGCC GCTTCTACAGTT	TGCCGTAGTTGGAGATAAGC CT

Table S2. Forward and reverse primers of the T cell activation related genes.

Flow cytometry

T cells were collected from the substrates and washed with sterilized PBS and fluorescence-activated cell sorting (FACS) staining buffer (Biolegend). Then the cells were stained with FACS staining buffer containing 1:200 LIVE/DEAD fixable aqua dead cell stain (L34965, Thermofisher Scientific), 1:30 PE-conjugated anti-CD69, clone BL-CD6 (Biolegend), and a 1:30 anti-CD25, clone BC96 (Biolegend) antibodies for 30 minutes at 4°C. Samples were then washed with FACS washing buffer (Biolegend) twice and then resuspended in 200 μL of flow running buffer (Biolegend) before reading on a LSRII UV cell analyzer (BD Biosciences).

Immunofluorescent staining against the activation markers and IS components

Cells samples were fixed with 4% weight by volume (w/v) paraformaldehyde for 30 minutes at room temperature, washed with PBS twice, and then permeabilized with 0.1% Triton X-100 in PBS. Then the samples were blocked in the 1% (w/v) bovine serum albumin (BSA) for one hour at room temperature and stained with different antibodies. To exhibit the T cell maturation related surface markers, the samples were stained with Alexa Fluor 488 anti-CD69 (1:50, 310916, Thermofisher Scientific) and Alexa Fluor 532 anti-CD25 (1:50, 58-0251-82, Thermofisher Scientific) antibodies at 4°C overnight, followed by staining with DAPI at room temperature for 30 minutes. To illustrate different SMAC areas during IS formation, samples were stained with a mix of Alexa Fluor 405 anti-CD44 (1:50, NB100-2710AF405, Novus Biologicals), Alexa Fluor 488 anti-LFA-1 (1:30, 363403, Biolegend) and Alexa Fluor 555 anti-CD3 (1:50, ab208514, Abcam) antibodies at 4°C overnight. After thorough washing with phosphate-buffered saline (PBS) with Tween detergent (PBST) buffer, fluorescent images of the samples were taken with a Nikon CSU-X1 Spinning Disk Confocal System (Nikon). The fluorescence intensity of the images was quantified with an open-source software ImageJ (NIH).

Statistics Analysis

Statistica (Statsoft, Tulsa, OK, USA) was used to perform the statistical analyses using two-way ANOVA and Tukey's honest significant difference (HSD) post hoc test of the means. *P<0.05, **P<0.01, ***P<0.001, #P<0.0001 were considered as significant differences. The results, including the error bars in the graphs, were given as the mean ± standard error of the mean (s.e.m.).

2. Supplementary Figures

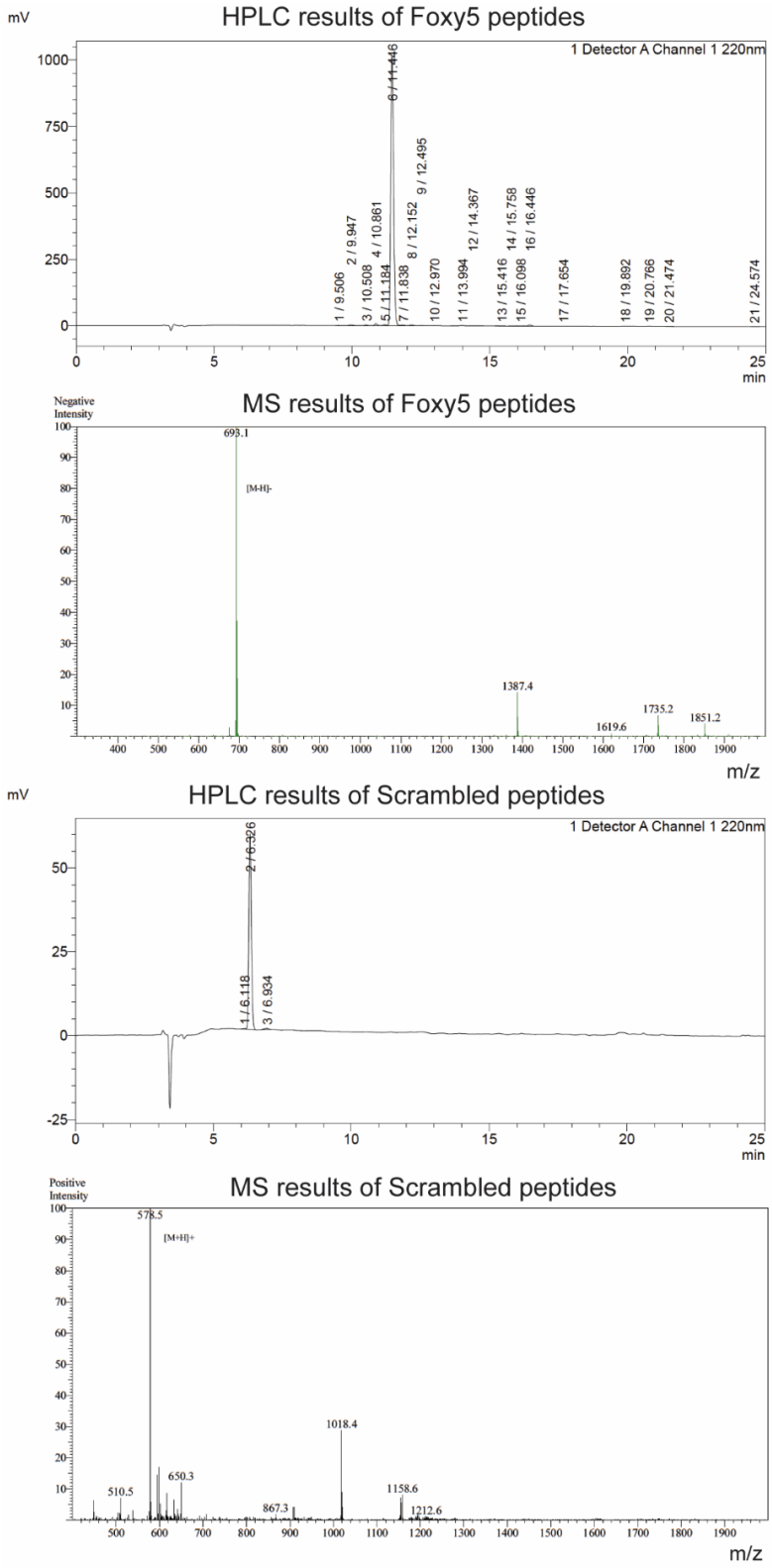

Fig. S1 Confirmation of the purity and molecular weights of peptides through the high-performance liquid chromatography (HPLC) and Mass spectrometry (MS).

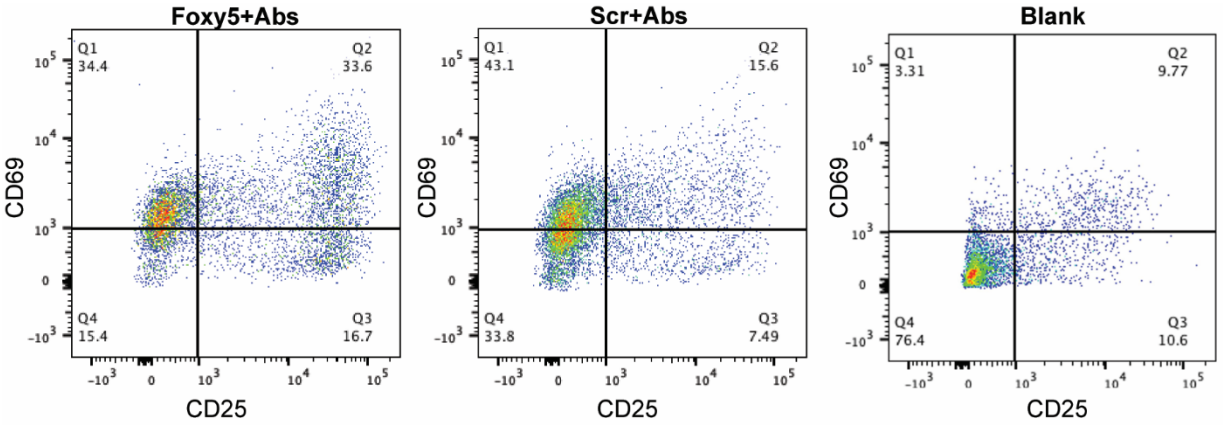

Fig. S2 The flow cytometry analysis results of T cell activation surface markers, CD69 and CD25. The CD25+/CD69+ cells were regarded as the fully activated T cells.

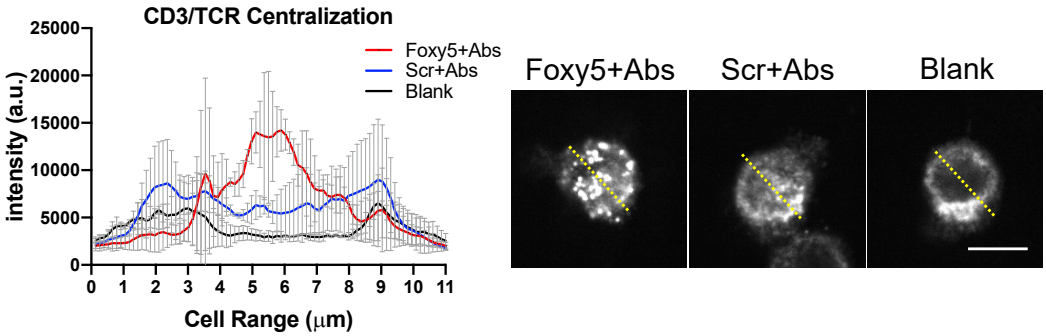

Fig. S3 Representative fluorescent images and quantitative results showing CD3/TCR clustering in T cells activated on different functionalized substrates. Quantified intensities of CD3 fluorescent staining across representative cells (dash lines in the fluorescent staining images) cultured on a Foxy5+Abs, Scr+Abs, or Blank control substrate (n=10). CD8+ T cells on the Foxy5+Abs substrates formed mature IS with obvious CD3/TCR clustering in the cSMAC, while in the Scr+Abs and Blank control groups, CD3/TCR largely located across the IS and in the distal SMAC (dSMAC), respectively. Data in the CD 3 intensity quantification curves are presented as mean value ± s.e.m. The scale bar is 10 μm in the fluorescent staining images.

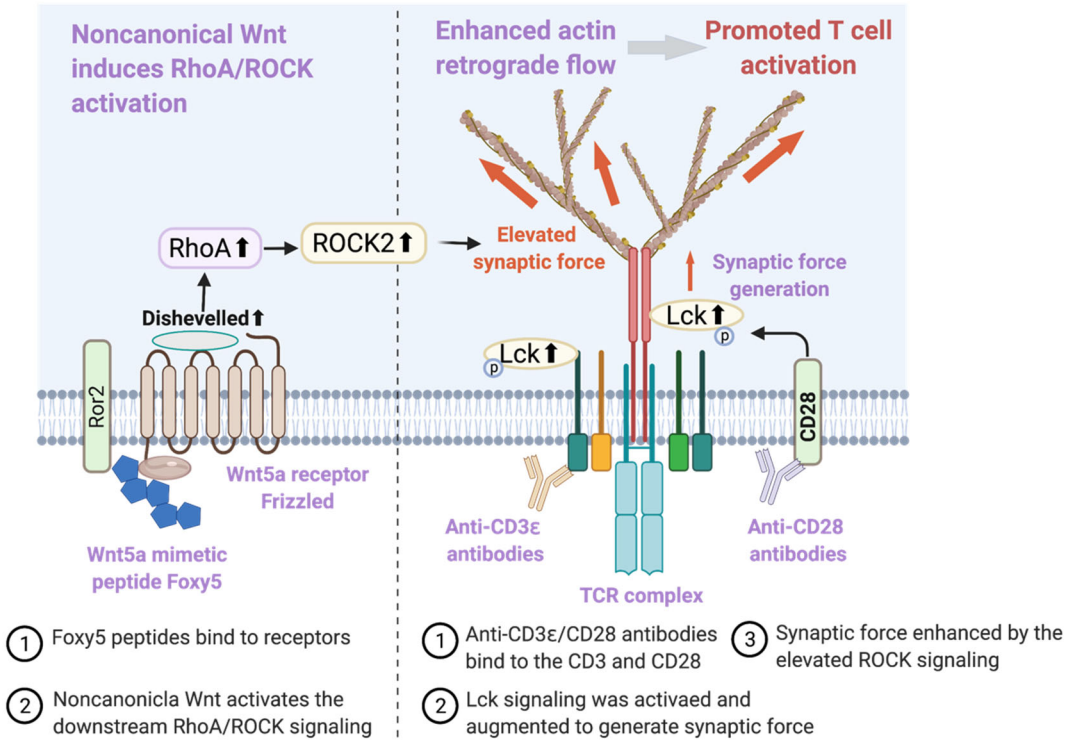

Fig. S4 A schematic of mechanotransductive signaling network showing the molecular mechanisms of how the Foxy5-mediated activation of noncanonical Wnt5a signaling greatly promoted the TCR activation and amplified the synaptic force of activated T cells.

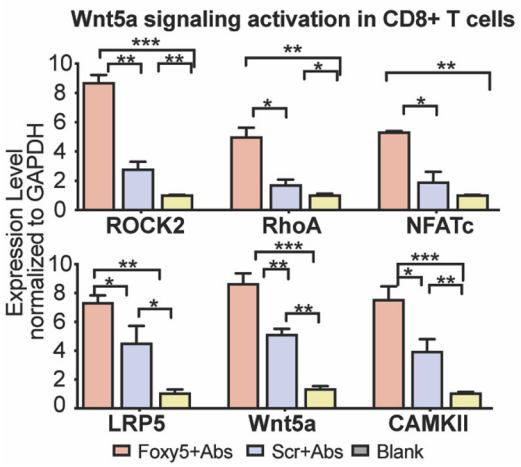

Fig. S5 The RT-qPCR results of Wnt5a activation-related genes (ROCK2, RhoA, NFATc, LRP5, Wnt5a, CAMK II) in T cells cultured on a Foxy5+Abs, Scr+Abs, and Blank control substrates. Results were normalized to the expression levels in the Blank group. Data are presented as average ± s.e.m. (n=4). The significance was calculated via one-way ANOVA with a Tukey post hoc test. *P<0.05, **P<0.01, ***P<0.001.

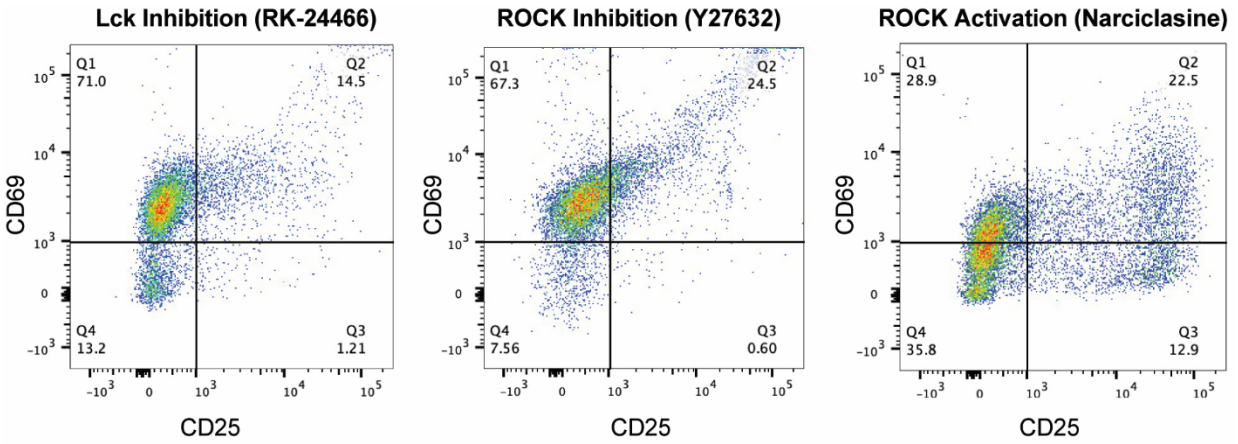

Fig. S6 The flow cytometry analysis of T cell activation related surface markers, CD69 and CD25, in the T cells treated with ROCK inhibitor Y27632, Lck inhibitor, or ROCK agonist Narciclasine on the Foxy5+Abs group.

3. Supplementary Reference

[1] T. Lendenmann, T. Schneider, J. Dumas, M. Tarini, C. Giampietro, A. Bajpai, W. Chen, J. Gerber, D. Poulikakos, A. Ferrari, Nano letters 2019, 19, 6742.

[2] R. Basu, B. M. Whitlock, J. Husson, A. Le Floc'h, W. Jin, A. Oyler-Yaniv, F. Dotiwala, G. Giannone, C. Hivroz, N. Biais, Cell 2016, 165, 100.