

Letter to the Editor

Rapamycin preferentially inhibits human IL-5⁺ T_H2-cell proliferation via an mTORC1/S6 kinase-1-dependent pathway

To the Editor:

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that integrates metabolic and activation signals to regulate cell proliferation and differentiation. mTOR plays a major role in T-cell proliferation and differentiation.¹⁻³ The transplant drug rapamycin is the canonical inhibitor of mTOR and in the 5 to 10 nM concentration range used clinically, rapamycin largely exerts its effect via inhibition of mTOR complex 1 (mTORC1).⁴

Recently, several groups have characterized an IL-5⁺ pathogenic effector T_H2 (peTh2) subpopulation with enhanced effector function.⁴ Notably, peTh2 cells demonstrate greater proinflammatory function in murine models of asthma and atopic dermatitis (AD) and are increased in human eosinophilic gastrointestinal diseases (EGID) and AD.⁵⁻⁹ Because of their pathogenic role in eosinophilic inflammation, we sought to examine mTOR function within the human peTh2 subpopulation in samples from subjects with eosinophilic asthma or EGID (see Table E1 in this article's Online Repository at www.jacionline.org).

To investigate the mTOR pathway in human T_H1 and T_H2 cell responses, we used dye dilution proliferation cultures coupled with intracellular cytokine staining (see this article's Methods section in the Online Repository at www.jacionline.org) to examine the dominant T_H1- or T_H2-cell proliferative responses associated with respiratory syncytial virus or house dust mite (HDM) antigen, respectively (see Fig E1 in this article's Online Repository at www.jacionline.org). Although rapamycin inhibited the output of both T_H1 and T_H2 cells in these proliferation cultures, the greatest inhibition was seen in IL-5-expressing cells (Fig 1, A). Accordingly, a dose response using a Boolean gating strategy was performed to examine the differential effect of rapamycin on IL-5⁺ T_H2 (IL-5⁺, IL-13⁺) versus IL-5⁻ T_H2 (IL-5⁻, IL-13⁺) versus T_H1 (IFN-γ⁺) cells. Notably, a consistent and highly significant hierarchy of inhibition was seen: IL-5⁺ T_H2 > IL-5⁻ T_H2 > T_H1 (Fig 1, B, $P < .0001$, 2-way ANOVA). The rapamycin IC₅₀ for IL-5⁺ T_H2, IL-5⁻ T_H2, and T_H1 cells was 0.1, 0.25, and 10 nM, respectively. Torin1, an mTOR catalytic domain inhibitor, reiterated this highly significant hierarchy (Fig 1, C, $P < .0001$, 2-way ANOVA). The Torin1 IC₅₀ for IL-5⁺ T_H2, IL-5⁻ T_H2, and T_H1 cells was 3, 10, and 25 nM, respectively. No inhibition was noted when rapamycin was added during the last day of culture (Fig 1, A, rightmost column), indicating that rapamycin is inhibiting effector T-cell proliferation, but not directly affecting cytokine gene expression.

Next, *in vitro* differentiated T_H1 and T_H2 cells were used to determine whether this T_H2 specificity was generalizable beyond the above antigen-specific cultures. Rapamycin preferentially inhibited T_H2 proliferation significantly more than T_H1 cells (90% vs 61%, $P = .003$; see Fig E2, A-C, in this article's Online Repository at www.jacionline.org). In contrast to its preferential inhibition of T_H2 proliferation, rapamycin did not

affect T_H1/T_H2 differentiation within these cultures, as seen by the cytokine profiles (Fig E2, D).

We next sought to determine whether this same hierarchy of rapamycin inhibition was found in *ex vivo* CD4 cells using phenotypically defined effector T-cell subpopulations. Per the recent report, IL-5⁺ peTh2 and IL-5⁻ conventional T_H2 (cTh2) were defined as CD161^{hi}, CRTH2⁺ and CD161⁻, CRTH2⁺, respectively (see Fig E3, A, in this article's Online Repository at www.jacionline.org).⁹ T_H1 and naive cells were identified using the well-established markers CXCR3 and CD45RA, respectively (Fig E3, B and C).

As expected, rapamycin inhibited overall CD4 T-cell proliferation (Fig E3, D and F). Rapamycin inhibition of anti-CD3-activated phenotypically defined effector CD4 subpopulations reiterated the hierarchy seen using antigen: peTh2 > cTh2 > T_H1 > naive (Fig E3, H). Notably, this rapamycin effect was not due to increased apoptosis in the peTh2 or cTh2 subpopulations (see Fig E4 in this article's Online Repository at www.jacionline.org). In sum, across the 3 T_H2 model systems shown above, rapamycin demonstrated the same hierarchy of effector T-cell inhibition.

The mTORC1 pathway is the dominant target of rapamycin, particularly at the nanomolar and subnanomolar concentrations used in this work.² Accordingly, phosphorylation of the mTORC1 downstream effectors S6 ribosomal protein (S6RP) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) was next measured. Induction of S6RP phosphorylation reiterated the hierarchy shown previously: peTh2 > cTh2 > T_H1 > naive (Fig 2, A-C). pS6RP induction in peTh2 cells was significantly greater than that in other CD4 subpopulations (Fig 2, C). Conversely, rapamycin inhibition of S6RP phosphorylation was significantly greater in T_H2 cells than in T_H1 or naive CD4 cells (Fig 2, D). In addition, rapamycin inhibition was greater in peTh2 than in cTh2 cells, but did not reach statistical significance ($P = .078$). Similar to S6RP, p4E-BP1 induction was significantly greater in T_H2 cells compared with T_H1 or naive CD4 cells (see Fig E5, A-C, in this article's Online Repository at www.jacionline.org). In contrast to S6RP, 4E-BP1 phosphorylation was actually increased by rapamycin and there was no significant difference between the effector T-cell subpopulations (Fig E5, A, D, and E). In sum, these data indicate that rapamycin is inhibiting mTORC1 through the S6 kinase (S6K1) pathway.

Small interfering RNA (siRNA) knockdown was next used to determine whether specific inhibition of the S6K1 pathway recapitulated the hierarchical inhibition found with rapamycin (see this article's Methods section). Individual S6K1 and S6RP siRNA knockdowns each partially blocked phosphorylation of S6RP, whereas the double knockdown was more effective (see Fig E6, A, in this article's Online Repository at www.jacionline.org). Notably, using the double knockdown in both phenotypically defined and cytokine-defined effector subpopulations (Fig E6, B and C, respectively), inhibition was significantly greater in peTh2 cells and reiterated the peTh2 > cTh2 > T_H1 hierarchy. The difference in inhibition, although statistically significant, was of relatively low magnitude, which may be due to the relatively low efficiency of siRNA knockdown in *ex vivo* cells, relative to that in immortalized cell lines.

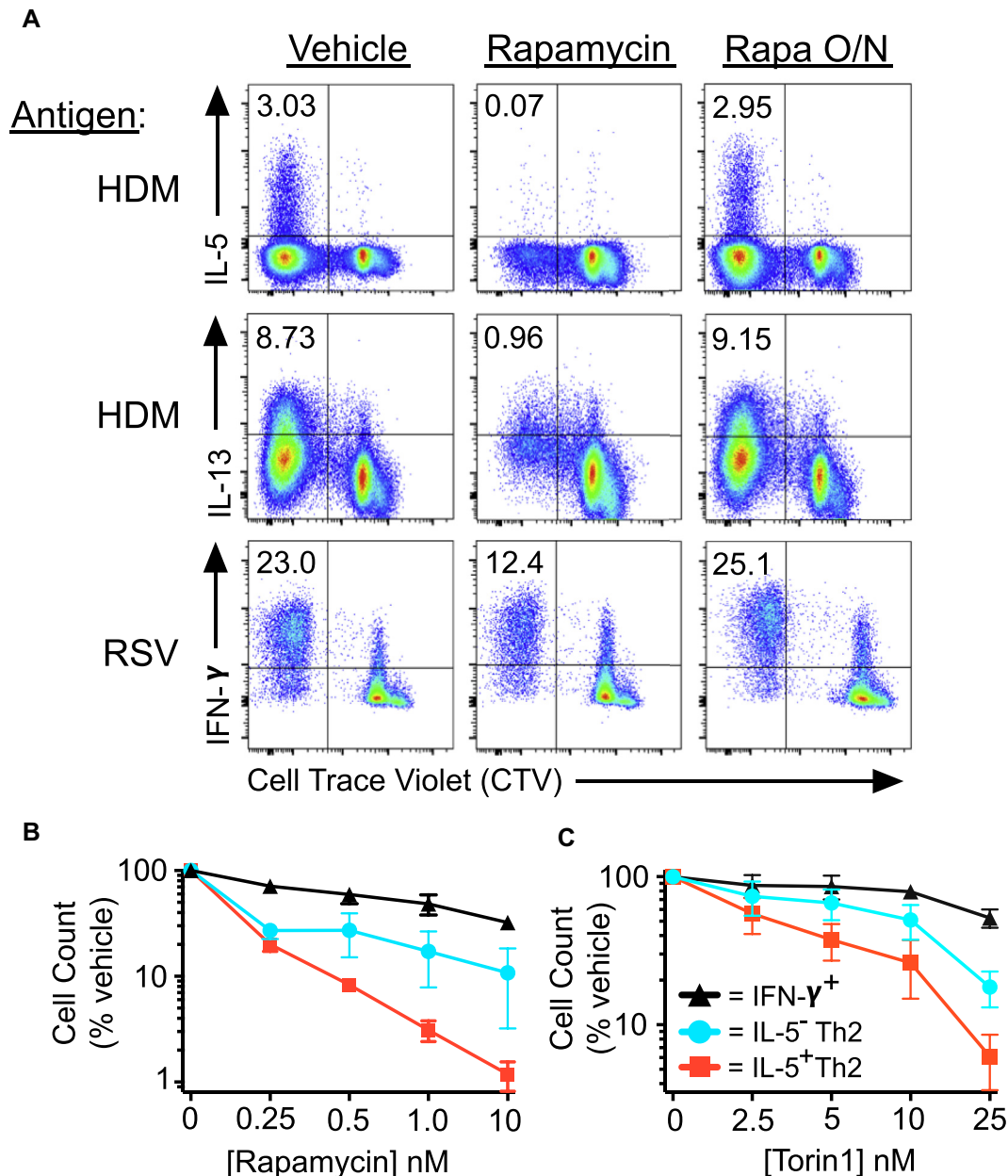


FIG 1. Rapamycin preferentially inhibits allergen-driven T_H2 cell output. **A**, Representative intracellular cytokine staining of CTV-stained CD4 T cells after a 7-day culture with HDM or RSV antigen. Rapamycin 1 nM was added as noted. **B** and **C**, Output cell count of CTV^{low}, cytokine⁺ CD4 subpopulations from HDM (T_H2) and RSV (T_H1) antigen-driven cultures after rapamycin and Torin1 dose response, n = 4 independent cultures, each from a different dust mite allergic subject (subjects 1-4). Fig 1, B and C, $P < .0001$ for the IL-5⁺ T_H2 > IL-5⁻ T_H2 > T_H1 trend, 2-way ANOVA. RSV, Respiratory syncytial virus.

The findings of this work support the mTORC1/S6K1 pathway being the dominant mTOR pathway operating in human IL-5⁺ peT_H2 cells. Correspondingly, peT_H2 cells were the CD4 subpopulation most sensitive to mTORC1 inhibition. These data support a mechanism whereby rapamycin preferentially inhibits human IL-5⁺ peT_H2 cells through the mTORC1/S6K1 pathway.

The initial experiments were performed using T_H1- and T_H2-dominant antigen-specific proliferation assays (Fig 1) and were then confirmed using both *in vitro* differentiated and

phenotypically defined *ex vivo* T_H1 and T_H2 cells (see Figs E2 and E4). Similarly, 3 different methods were used to inhibit mTOR: rapamycin, Torin1, and siRNA knockdown. Across these various assay systems and mTOR inhibitors, a consistent hierarchy of inhibition was observed (IL-5⁺ peT_H2 > IL-5⁻ cT_H2 > T_H1), strongly supporting the veracity and generalizability of these observations.

At concentrations below 5 nM rapamycin acts exclusively by inhibiting the mTORC1 pathway. Thus, the current finding that rapamycin inhibits peT_H2 proliferation at subnanomolar

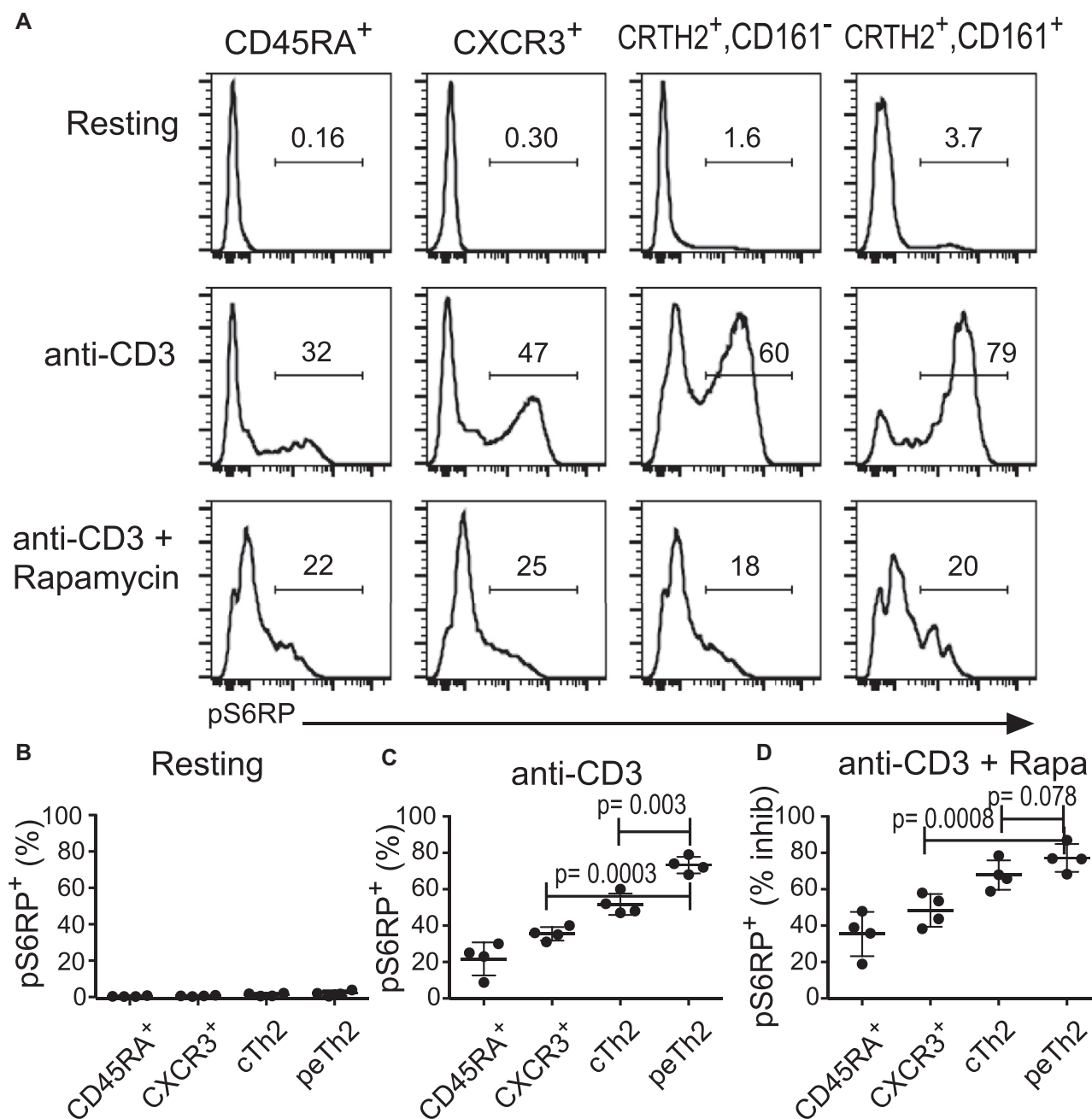


FIG 2. peT_H2 cells have greater S6K1 activity and rapamycin inhibition. **A**, S6RP phosphorylation by flow cytometry in phenotypically defined CD4 subpopulations. Combined data showing pS6RP in resting (**B**) and activated phenotypically defined CD4 subpopulations (**C**). **D**, Combined data showing inhibition of S6RP phosphorylation, 1 nM rapamycin vs vehicle. Fig 2, B-D, n = 4 independent experiments, each from a different subject (subjects 1-4).

concentrations indicate inhibition through the mTORC1 pathway. Dominance of the mTORC1/S6K1 pathway is further indicated by the abundant induction of pS6RP in peT_H2 cells, and conversely, greater inhibition of the S6K1 pathway in T_H2 cells (Fig 2). This mTORC1/S6K1 dominance was confirmed by targeted siRNA knockdown of the S6K1 pathway. In sum, these findings indicate that rapamycin inhibition of peT_H2 proliferation is mediated through the mTORC1/S6K1 pathway.

Powell et al¹⁰ previously examined inhibition of HDM-induced T-cell proliferation and IL-5 expression and using

1 nM rapamycin found minimal inhibition. This difference may be due to the current study's focus on the peT_H2 subpopulation, rather than the entire population of HDM-specific T cells examined in the previous study.

In this work, we demonstrate that nanomolar concentrations of rapamycin preferentially inhibit human IL-5⁺ peT_H2 cells through the mTORC1/S6K1 pathway. This suggests that low concentrations of rapamycin or other mTORC1 inhibitors may be a potential therapeutic strategy in peT_H2 cell-associated diseases, such as eosinophilic asthma, AD, and EGID. Furthermore, these

data suggest that bioenergetic differences specific to the IL-5⁺ pT_H2 subpopulation may allow their selective therapeutic targeting.

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METHODS

Subjects

Clinical and immunological details of the allergic EGID and asthmatic subjects are presented in Table E1. Subjects with eosinophilic gastroenteritis (EGE) were aged 18 to 65 years. The diagnosis of EGE was based on typical gastrointestinal symptoms, tissue eosinophilia (peak of ≥ 40 eosinophils/hpf) in stomach or duodenal biopsy specimens, and the exclusion of other causes of gut eosinophilia, including helminth infection. Subjects with EGE had 3 or more positive allergen skin test results or antigen specific IgE tests out of the following panel (peanut, wheat, soy, shrimp, egg white, milk, walnut, cod, corn). Crohn disease was excluded by lack of typical pathologic findings (ulcerations, granulomata, or crypt architectural distortion) and clinical features (fistula, abdominal mass, and surgical obstructive disease). Subjects with immunodeficiency or a positive Fip1-like 1-platelet-derived growth factor receptor alpha fusion gene PCR were excluded. Allergic asthmatic subjects had a minimum 1-year history of episodic bronchospasm relieved by β -agonist medications and 3 or more positive skin test responses (≥ 3 mm) out of a panel of 10 aeroallergens. All subjects in Fig 1 had positive skin test results to HDM and in pilot experiments demonstrated *in vitro* CD4 T-cell proliferation to both HDM and respiratory syncytial virus. The National Institute of Allergy and Infectious Diseases Institutional Review Board approved the clinical protocol used for this study (10-I-0196). All subjects signed informed consent.

PBMC isolation and storage

Subjects with EGID and asthma underwent lymphapheresis (NIH Clinical Center Department of Transfusion Medicine) and PBMCs were isolated using density gradient separation (Lymphocyte Separation Medium; MP Biomedical, Aurora, Ohio), washed twice in HBSS (Mediatech, Inc, Manassas, Va), cryopreserved in RPMI 1640 (Invitrogen) with 10% HSA (Talecris Biotherapeutics, Research Triangle Park, NC), and stored in liquid nitrogen. Upon thawing, cells were treated with 60 μ g/mL DNase I (Calbiochem, La Jolla, Calif) in RPMI. DNase I is a standard treatment to decrease cell clumping and increase the cell yield from cryopreserved cells.^{E1,E2} Cells were cultured in RPMI complete medium (cRPMI), RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin (Invitrogen, Carlsbad, Calif), nonessential amino acids, 1 mM sodium pyruvate (Mediatech), 50 μ M 2-mercaptoethanol (Sigma-Aldrich, St Louis, Mo) at 37°C in a 5% CO₂ incubator.

Cell culture

T-cell proliferation was measured using the CellTrace Violet (CTV) dye dilution reagent (Invitrogen). A total of 20 to 100 million PBMCs at 10×10^6 cells/mL were labeled with 5 μ mol/L CTV in PBS for 15 minutes at room temperature, quenched by adding 10 $\times$ volume of RPMI/5% human AB serum for 5 minutes, washed once with cRPMI 1640, and resuspended in cRPMI/5% human AB serum (no bovine serum). 2×10^6 PBMCs per well were placed into 24-well plates and cultured with HDM antigen (40 AU/mL; ALK, Round Rock, Tex) or respiratory syncytial virus antigen (400 ng/mL; Biodesign International, Saco, Me) at the indicated concentrations. At the start of culture, rapamycin (Sigma-Aldrich) was added at 1 nM final concentration, unless otherwise noted. An equal volume of dimethyl sulfoxide (DMSO) was added to wells as vehicle control. After 7 days of culture, cells were reactivated with PMA and ionomycin for 6 hours, stained with Aqua LIVE/DEAD fixable viability dye (Invitrogen), and fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, Pa) at room temperature for 5 to 10 minutes. Seven days was found to be an optimal time for allergen-specific proliferation, based on time course experiments performed for a previous report.^{E3} Cells were stored in PBS/10% DMSO (Sigma-Aldrich) at -80°C until analysis.

In vitro differentiated T_H1 and T_H2 cells

T_H1 and T_H2 differentiation was performed according to published methods.^{E4} Briefly, naive CD4 T cells were isolated from PBMCs using microbeads (naive CD4⁺ T-cell isolation kit, Miltenyi Biotec, Bergisch

Gladbach, Germany), polarized for 4 days in cRPMI in 24-well plates using plate-bound anti-CD3 (5 μ g/mL, clone OKT3, BioLegend, San Diego, Calif), soluble anti-CD28 (1 μ g/mL, clone CK248, in house) supplemented with the following reagents: T_H1 polarization: IL-12 (2.5 ng/mL, Biolegend), IL-2 (40 U/mL, Chiron, Emeryville, Calif), anti-IL-4 (5 μ g/mL, clone MP4-25D2, Biolegend) and anti-IL-10 (5 μ g/mL, clone JES3-9D7, BD Biosciences, San Jose, Calif); T_H2 polarization: IL-2 (100 U/mL), IL-4 (12.5 ng/mL, PeproTech, Rocky Hill, NJ), anti-IFN- γ (5 μ g/mL, clone B133.5, Bio XCell, West Lebanon, NH), anti-IL-10 (5 μ g/mL). Cells were then transferred to fresh 24-well plates and expanded for an additional 3 days in IL-2-containing medium.

Apoptosis

Cryopreserved PBMCs were thawed and recovered overnight and cultured with soluble anti-CD3 (1 μ g/mL) for 24 hours. Rapamycin or vehicle was added for the last 3 or 24 hours of culture. Camptothecin (EMD Millipore, Billerica, Mass), 5 μ M final concentration, was added for the last 24 hours of culture as an apoptosis positive control. Cells were then harvested and stained with Aqua fixable viability live/dead dye, then fixed with 4% PFA labeled with antibodies to CD3, CD4, CD8, CD161, CRTH2, CXCR3, CD45RA, and Asparagine 214 cleaved poly (ADP-ribose) polymerase (clone F21-852, BD Biosciences) for FACS analysis. Apoptotic cells were defined as Aqua⁺, poly-ADP-ribose polymerase⁺.

Flow cytometry

Intracellular cytokine staining was performed as previously described.^{E1,E4,E5} Briefly, cells were activated with 1 μ M ionomycin (EMD Chemicals, Gibbstown, NJ), 20 ng/mL PMA, and 10 μ g/mL brefeldin A (both Sigma) for 6 hours, labeled on ice with Aqua LIVE/DEAD fixable viability dye, washed, and fixed in 4% PFA, resuspended in PBS/10% DMSO, and stored at -80°C . Fixed cells were later thawed and permeabilized in PBS with 0.1% saponin/5% nonfat dry milk buffer. The following antibodies (each clone noted in parentheses) were used: CD3 Qdot 605 (UCHT1), CD8 PE/TR (3B5) (both Invitrogen), CD4 PerCP-Cy5.5 (SK3), IL-5 PE (JES1-39D10), IFN- γ Alexa Fluor 700 (B27) (all BD Biosciences), and IL-13 PE/Cy7 (JES10-5A2, custom conjugate).

For phospho-flow cytometry, 2×10^6 cells per well were activated with plate-bound anti-CD3 (5 μ g/mL) with or without 1 nM rapamycin for 4 hours. Four hours was chosen because it was optimal for detection of S6RP phosphorylation (Fig E7). Detection of phosphorylated mTOR signaling pathway components used the same fixation and permeabilization conditions noted for intracellular cytokine staining.

The following antibodies were used: pS6RP (S235, D57.2.2E), p4E-BP1 (S37/42, 236B4), S6RP, 4E-BP1, S6K1 (Cell Signaling Technology, Beverly, Mass); CRTH2 (BM16), CD161 (HP-3G10), CXCR3 (G025H7), BioLegend; pmTOR (S2448, EPR426(2)) (Abcam, Cambridge, Mass); mTOR (Santa Cruz Biotechnology, Dallas, Tex). For surface staining, after Aqua LIVE/DEAD fixable viability dye staining, cells were stained at room temperature with CXCR3 PE/Cy7 (G025H7, Biolegend) and then fixed as above.

In CTV dye dilution proliferation assays, antigen-specific proliferated CTV^{low} cells were operationally defined as cells that had proliferated at least 2 cell divisions, as evidenced by their violet dye intensity of 4-fold or lower than that of the media control peak representing undivided cells, according to previous publications.^{E3,E6,E7} The proliferative output of specific T-cell subpopulations (cell count) was calculated after gating on cytokine- or phenotypically defined subpopulations and the above CTV criteria. Different culture conditions were compared by acquiring the entire content of each tissue culture well for each experimental condition.

Samples were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashton, Ore). CD4 T cells were identified as viable CD3⁺, CD4⁺, CD8[−] cells with typical lymphocyte forward and side scatter; cell doublets were excluded using forward scatter area versus height. Unimodal and bimodal histograms were quantitated using mean fluorescence intensity (MFI) and percent positive, respectively. MFI ratios were calculated as the MFI of the T-cell population of interest divided

by the MFI of the CD3-negative lymphocyte population in the same culture. Statistical markers were based on the staining of nonactivated media control samples. Numbers shown on histograms and dot plots represent the percentage of cells within the marked statistical gates.

Western blot analysis

Western blotting was performed using *in vitro* differentiated T_H2 cells, which were activated with plate-bound anti-CD3 (5 µg/mL) with or without 1 nM rapamycin for 4 hours. Protein was isolated from cells with RIPA buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 1% SDS, 1 mmol/L sodium orthovanadate, 0.1 mg/mL phenylmethylsulfonyl fluoride, protease inhibitor cocktail, Cell Signaling Technology). Twenty-five microgram of protein per lane was run on a 4% to 12% Nupage gel (Invitrogen). Antibodies used for Western blot were as follows: p4E-BP1 (S37/42), 4E-BP1, pS6RP (S235), and actin (all from Cell Signaling Technology). Secondary antibodies were from LI-COR Biosciences. An Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, Neb) was used for analysis.

siRNA

siRNA knockdown of the S6 pathway was performed as previously described.^{E8} On-TARGETplus siRNA reagents to human S6K1, S6RP, and scrambled controls were obtained from Thermo Scientific (Waltham, Mass). Human PBMC cells were thawed, stained with CTV proliferation dye, then activated with 1 µg/mL soluble anti-CD3 overnight. In pilot experiments it was determined that efficient siRNA knockdown of *ex vivo* human T cells required prior activation and proliferation. The cells were harvested the next day, washed, and resuspended in PBS at a density of 10⁷ cells/mL. Each siRNA sample was resuspended per 20 µM in 250 µL of siRNA suspension buffer (Dharmacon, Lafayette, Colo). Transfections were performed with the Amaxa Nucleofector device (Lonza, Basel, Switzerland) using program V-024.^{E8} Cells were resuspended at 2 × 10⁷ cells/mL in Nucleofector solution, and 100 µL of cells together with 10 to 20 µL of siRNA solution was treated with the appropriate program. Cells were then resuspended in 2 mL of cRPMI and split into 2 wells of a 24-well plate. Transfected cells were incubated at 37°C, 5% CO₂ for 4 days before being harvested. siRNA transfection

efficiencies were more than 90% based on FITC-labeled siRNA controls detected by flow cytometry. Knockdown efficiency was 30% and 40% for S6K1 and S6RP, respectively, as measured by intracellular staining. Cell viability posttransfection was 65% to 70%, using Aqua LIVE/DEAD fixable viability dye (Invitrogen) and flow cytometry.

Statistical analysis

In all figures, the mean was used as the measure of central tendency and SD was used as the measure of variation. Statistics were calculated using Prism software (GraphPad, La Jolla, Calif). Paired 2-group comparisons were calculated using the paired Student *t* test. Percent inhibition was calculated as [(normal activity – inhibited activity)/(normal activity)] × 100%. In Fig 1, *B* and *C*, statistical significance of the IL-5⁺ T_H2 > IL-5[–] T_H2 > T_H1 hierarchy of inhibition was tested using 2-way repeated-measures ANOVA.

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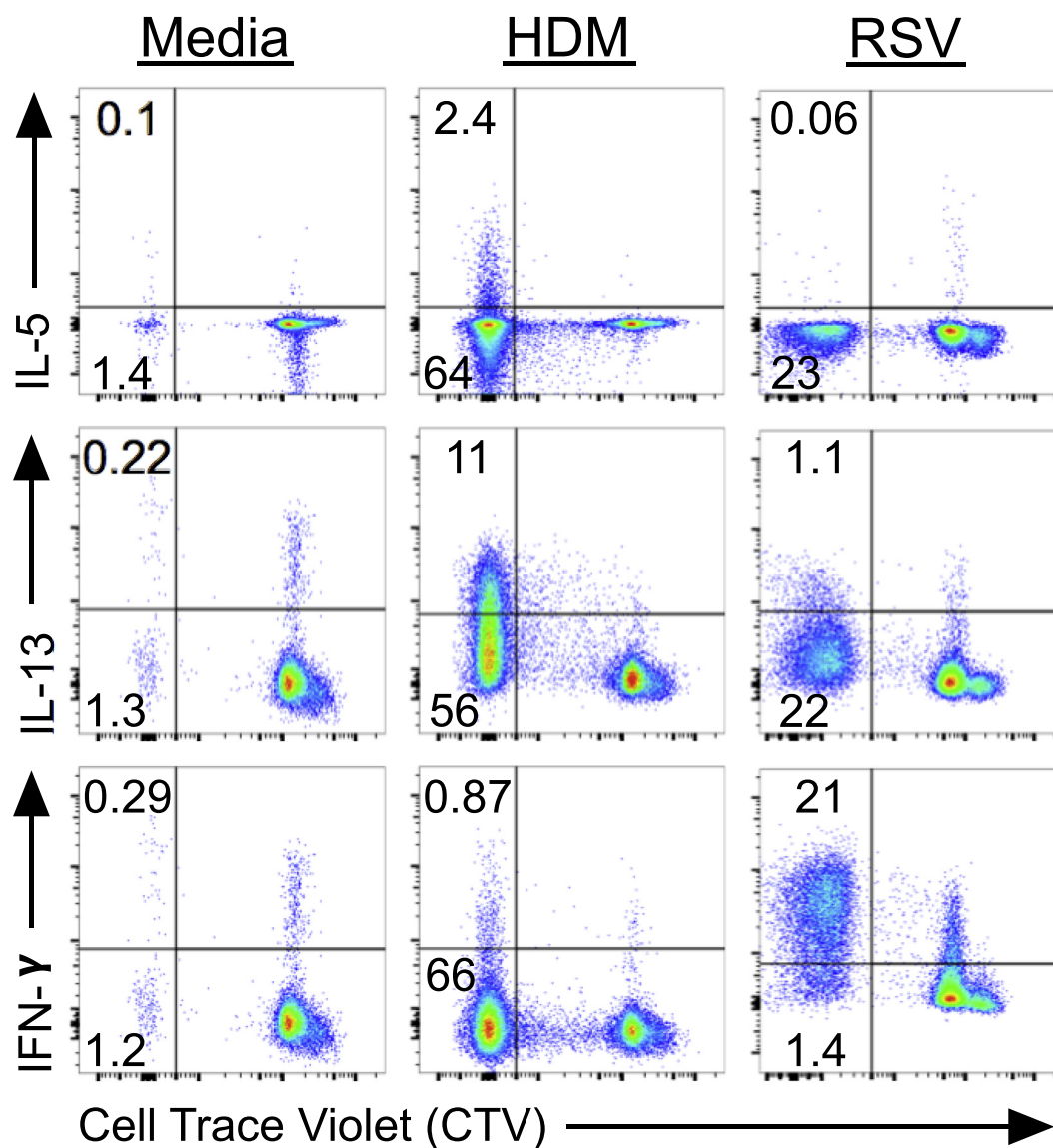


FIG E1. RSV and HDM, respectively, yield dominant T_H1 and T_H2 proliferative responses. Intracellular cytokine staining and CTV staining in CD4 T cells after 7-day culture with the indicated antigen. Results are representative of 4 independent cultures from donors 1 to 4. *RSV*, Respiratory syncytial virus.

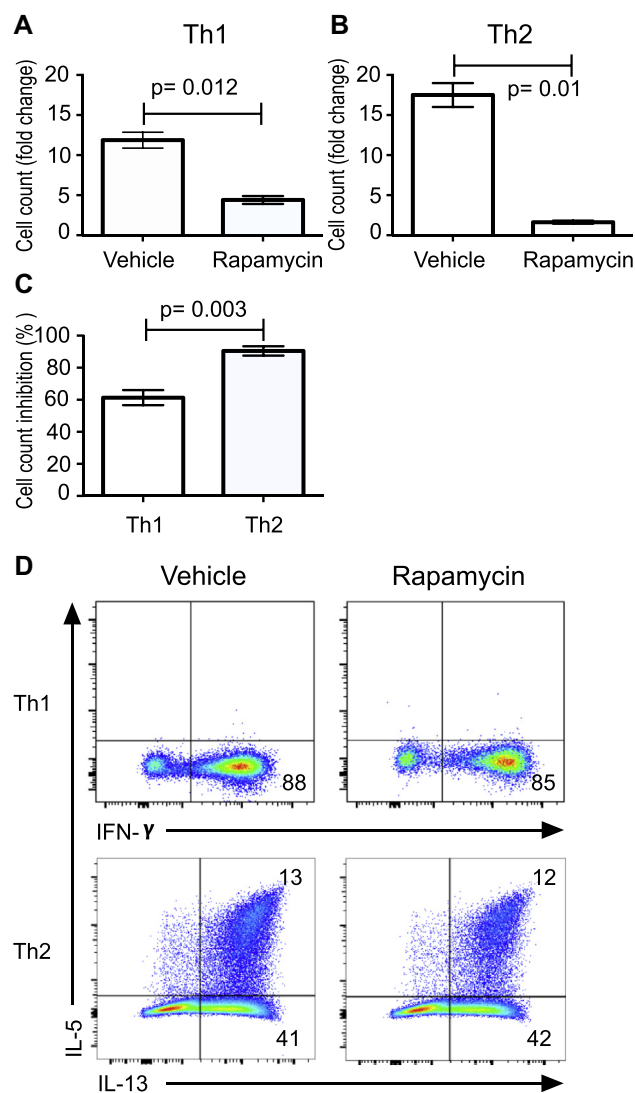


FIG E2. Rapamycin preferentially inhibits *in vitro* differentiated T_H2 cell proliferation. **A** and **B**, Fold change in output cell count in anti-CD3/CD28-activated 7-day cultures of *in vitro* differentiated T_H1 and T_H2 cells. **C**, The % inhibition due to 1 nM rapamycin was calculated from the above. **D**, ICCS profile of *in vitro* differentiated T_H1 and T_H2 cells after stimulation with anti-CD3/CD28 for 7 days, respectively, under T_H1 - or T_H2 -polarizing conditions. Fig E2, A-C, $n = 3$ independent experiments using cells from donors 2 to 4. **D**, Representative of same 3 experiments.

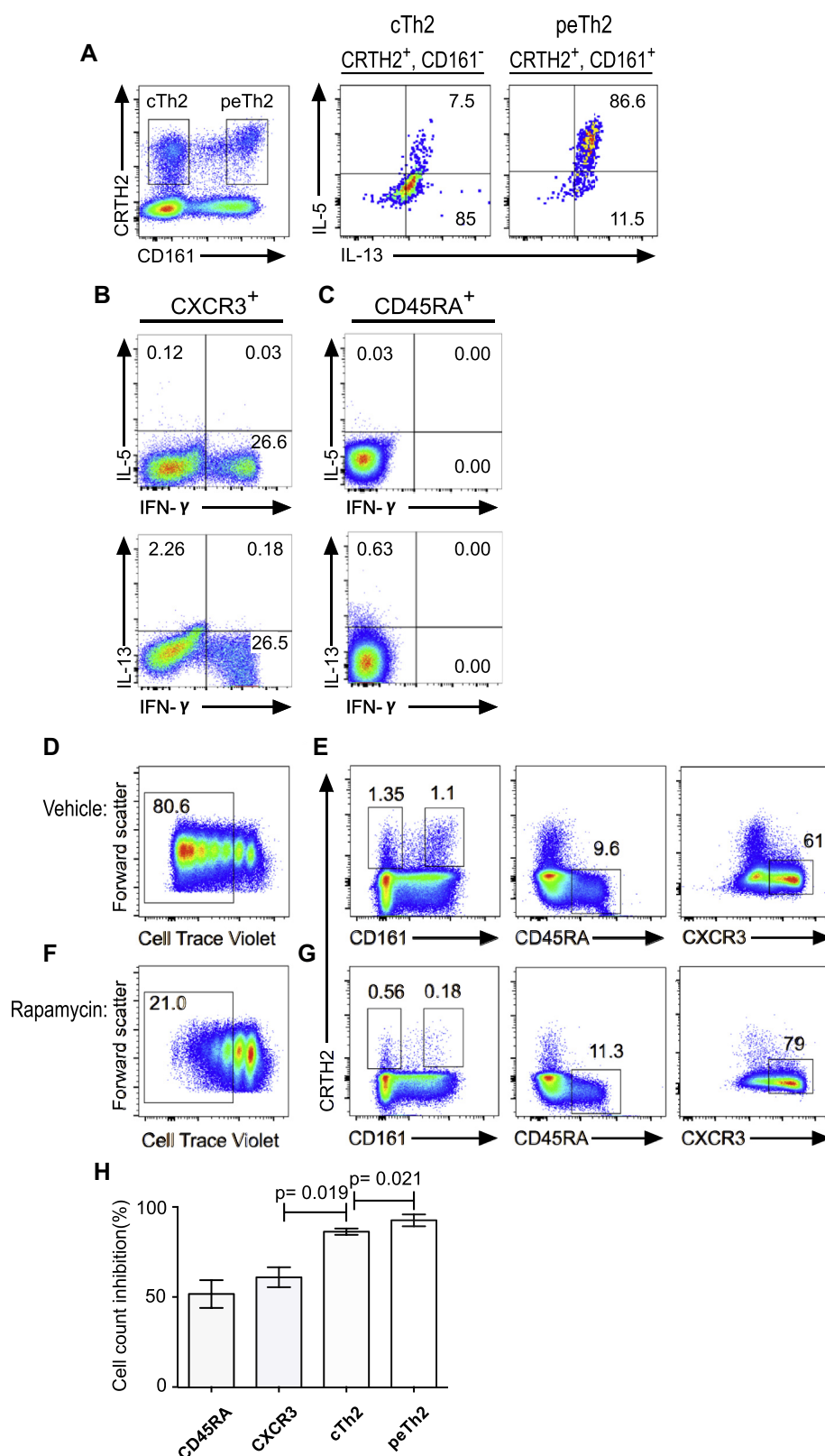


FIG E3. Rapamycin preferentially inhibits peTh₂ cell proliferation. **A-C**, Phenotypic identification and validation of the intracellular cytokine staining profile of peTh₂, cTh₂, Th1, and naive CD4 T cells. PBMCs were activated with PMA/ionomycin for 6 hours, and stained for the noted phenotypic markers and cytokines. Cells were gated on CD3⁺, CD4⁺ cells and the phenotypic markers noted above the plot. **D-H**, Rapamycin inhibition of phenotypically defined effector T-cell subpopulations. PBMCs were stained with CTV, activated with anti-CD3, and cultured for 7 days. Fig E3, **D** and **F**, After first gating on CD4⁺ cells, the population of CTV^{low} cells is noted in the rectangular gate. **E** and **G**, After first gating on proliferated CTV^{low} cells, the frequencies of the above-noted phenotypic subpopulations are noted. **H**, Percent inhibition of output cell count of CTV^{low} phenotypically defined subpopulations (1 nM rapamycin vs vehicle). **A-G**, Results are representative of 4 independent cultures from donors 1 to 4. **H**, Combined data from donors 1 to 4.

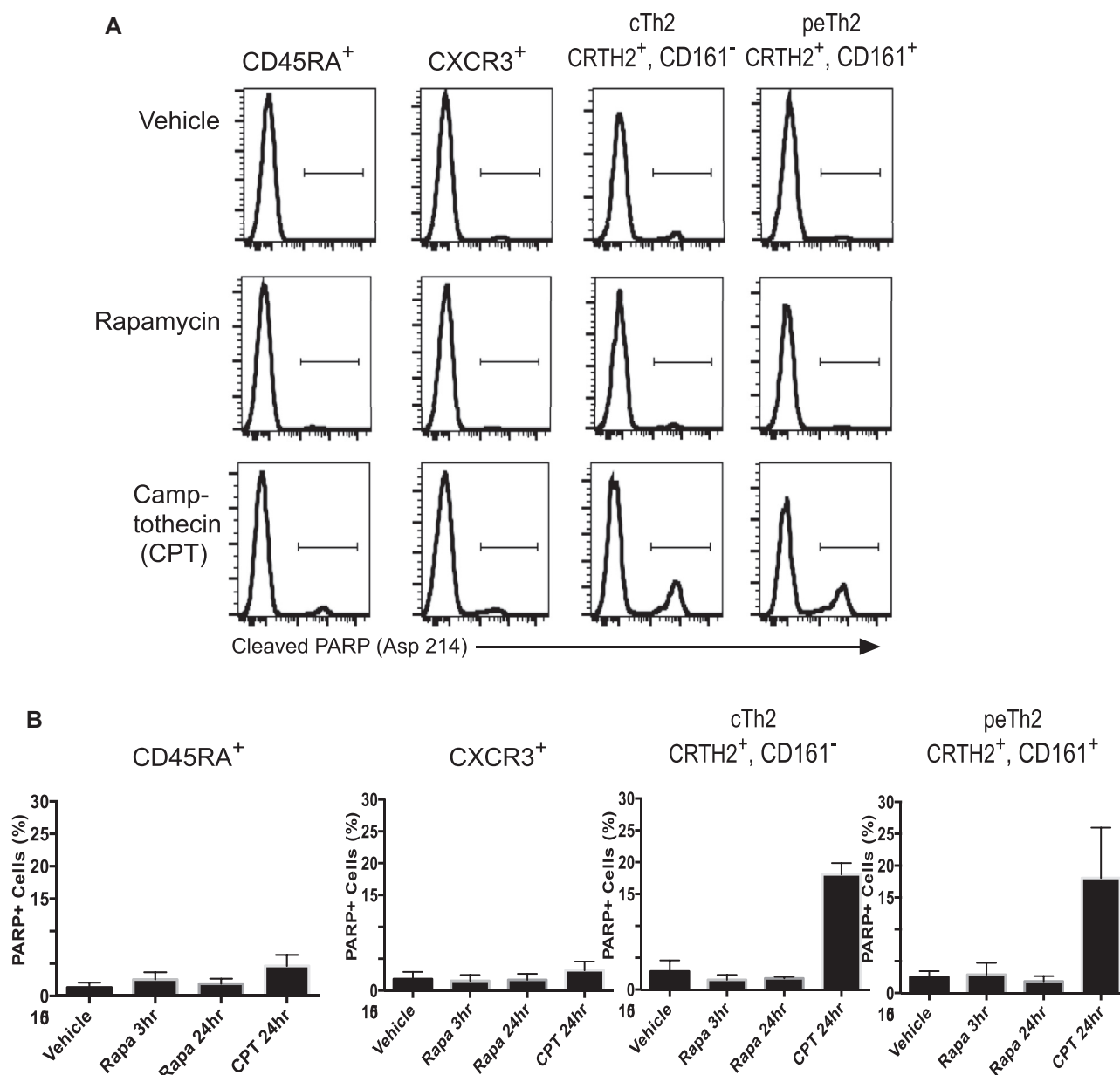


FIG E4. Rapamycin does not preferentially induce apoptosis in T_H2 cells. PBMCs were activated with soluble anti-CD3 (1 μ g/mL) for 24 hours, during which they were treated with the inhibitors noted. **A**, Cleaved PARP staining in phenotypically defined CD4 subpopulations from a representative experiment. **B**, Grouped data of cleaved PARP staining in phenotypically defined CD4 subpopulations. $n = 4$ independent experiments, each from a different subject (subjects 1-4). *PARP*, Poly ADP-ribose polymerase.

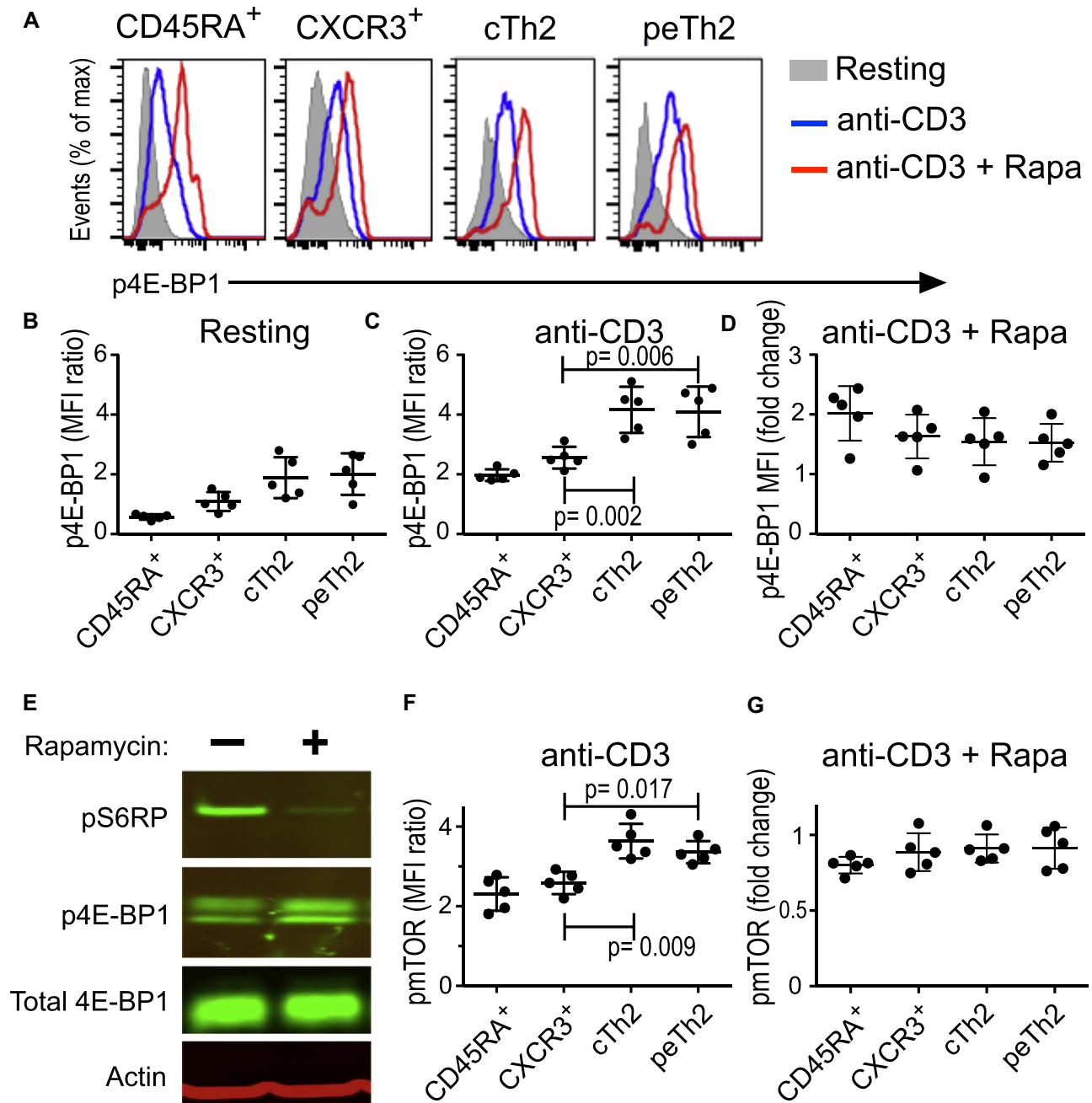


FIG E5. Rapamycin does not inhibit 4E-BP1 phosphorylation. **A**, 4E-BP1 phosphorylation by flow cytometry, after first gating on each phenotypically defined CD4 subpopulation. Combined data showing p4E-BP1 in phenotypically defined CD4 subpopulations (**B** and **C**) and induction of 4E-BP1 phosphorylation by 1 nM rapamycin relative to vehicle (**D**). **E**, Western blotting for the noted mTOR effectors in cell extracts from *in vitro* differentiated T_H2 cells. **F**, Combined data showing pmTOR in phenotypically defined CD4 subpopulations. **G**, Combined data showing pmTOR induction by 1 nM rapamycin relative to vehicle. Fig E5, **B-D**, **F**, and **G**. Results are representative of 5 independent cultures from donors 2 to 6.

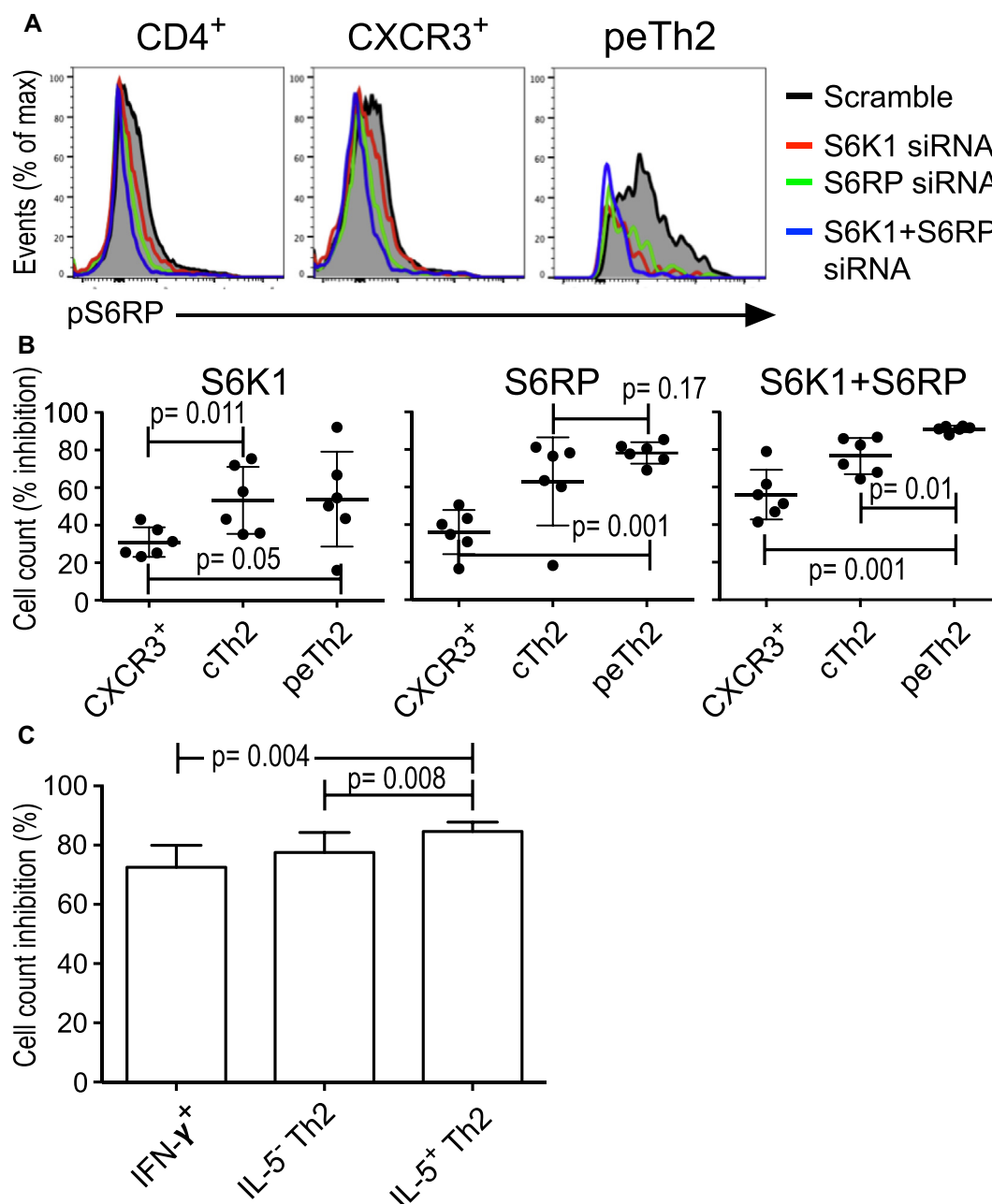


FIG E6. Effect of S6K1 pathway knockdown on IL-5⁺ peTh₂ cell proliferation. **A**, S6RP phosphorylation after siRNA knockdown of S6K1, S6RP, or both, in phenotypically defined CD4 subpopulations. Inhibition of proliferation of **(B)** phenotypically defined or **(C)** cytokine-defined CD4 subpopulations in S6K1/S6RP siRNA-treated PBMCs relative to scramble siRNA. Fig E6, A, Results are representative of 3 independent experiments. B and C, Results are representative of 6 independent cultures from donor 3 and donors 5 to 9.

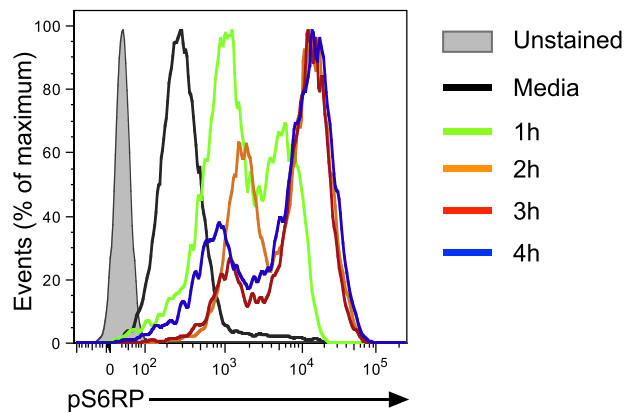


FIG E7. Kinetics of pS6RP induction in human T_H2 cells. *In vitro* differentiated T_H2 cells were activated with plate-bound anti-CD3 (5 μ g/mL) for the times indicated, or not activated (media) and then stained for pS6RP using phospho-flow according to the [Methods](#) section. Data are representative of 3 independent experiments.

TABLE E1. Subjects providing PBMC samples for *in vitro* experiments

Subject #	Diagnoses	Age (y)	Sex	Corticosteroid therapy	AEC ($\times 10^9/L$)	IgE (kIU/L)
1	Asthma, AD	50	M	ICS	0.745	>30,000
2	Asthma	32	F	ICS	0.075	670
3	EGE, asthma	42	M	ICS, prednisone 8 mg qD	4.2	228
4	EoE	23	M	Fluticasone swallowed	0.43	13,700
5	EGE, EoE	56	M	Fluticasone swallowed	1.83	1,805
6	EGE, asthma	53	M	ICS	1.58	5,840
7	EGE, asthma	53	F	ICS, budesonide 4.5 mg qD	0.72	531
8	EGE, asthma	46	M	ICS, prednisone 20 mg QOD	1.4	631
9	EGE	28	M	Budesonide 9 mg qD	1.82	1,504

AEC, Absolute eosinophil count; EoE, eosinophilic esophagitis; F, female; ICS, inhaled corticosteroid; kIU, 1000 international units; M, male; qD, daily; QOD, every other day.