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Intracellular Delivery of Anti-pPKCθ (Thr538) via Protein Transduction Domain Mimics for Immunomodulation

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Targeting cellular proteins with antibodies, to better understand cellular signaling pathways in the context of disease modulation, is a fast-growing area of investigation. Humanized antibodies are increasingly gaining attention for their therapeutic potential, but the collection of cellular targets is limited to those secreted from cells or expressed on the cell surface. This approach leaves a wealth of intracellular proteins unexplored as putative targets for antibody binding. Protein kinase Cθ (PKCθ) is essential to T cell activation, proliferation, and differentiation, and its phosphorylation at specific residues is required for its activity. Here we report on the design, synthesis, and characterization of a protein transduction domain mimic capable of efficiently delivering an antibody against phosphorylated PKCθ (Thr538) into human peripheral mononuclear blood cells and altering expression of downstream indicators of T cell activation and differentiation. We used a humanized, lymphocyte transfer model of graft-versus-host disease, to evaluate the durability of protein transduction domain mimic:Anti-pPKCθ modulation, when delivered into human peripheral mononuclear blood cells *ex vivo*. We demonstrate that protein transduction domain mimic:Antibody complexes can be readily introduced with high efficacy into hard-to-transfect human peripheral mononuclear blood cells, eliciting a biological response sufficient to alter disease progression. Thus, protein transduction domain mimic:Antibody delivery may represent an efficient *ex vivo* approach to manipulating cellular responses by targeting intracellular proteins.

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INTRODUCTION

Protein kinase C enzymes comprise three subfamilies and 10 kinase isoforms that are structurally and functionally related.[1,2] Different isoforms are activated either by proteolysis or translocation to the plasma membrane, where they associate with protein partners to mediate biological functions.[3–5] Among the PKC enzymes, protein kinase C-theta (PKCθ) exhibits a selective pattern of tissue distribution with a predominant expression in T lymphocytes, platelets, and skeletal muscle. It translocates to the center of the immunological synapse (IS) in activated CD4 T cells following the integration of T cell receptor (TCR) and CD28 costimulatory signals.[6] The full activation of PKCθ involves two steps: diacylglycerol (DAG) binding to its C1 domain and subsequent Threonine 538 (Thr538) phosphorylation within its activation loop.[7–10] PKCθ regulates multiple transcription factors including NF-κB, AP-1, and NFAT which, individually and combined, initiate signals that are critical for T cell activation, proliferation, and differentiation.[11–16] Recently, it has been suggested that PKCθ also translocates into the nucleus and associates with a chromatin-bound complex to regulate microRNA and T cell-specific gene expression programs.[17] Immunological studies show that distinct PKC isoforms use unique mechanisms to regulate various different functions and, thus, are attractive therapeutic targets for modulating T cell-mediated adaptive immune responses.[18,19]

Monoclonal antibodies have emerged as potential therapeutics for many diseases such as cancer, infection, and autoimmune disorders due to their unequalled target specificity. Furthermore, advancements in genetic engineering have paved the way for "humanizing" mouse monoclonal antibodies, creating versions for clinical use that are promising due to their greater safety and selectivity. However, targets of these antibody-based biologics are currently limited to cell surface or extracellular proteins because of their inability to pass through the cellular membrane.[20–23]

Intracellular delivery of biologically active molecules remains a significant challenge. In some cases, these therapeutics can be taken up via receptor-mediated endocytosis. However, cellular entry via the endocytic pathway poses its own hurdles, including escape from endosomes and avoiding lysosomal degradation.[24] Therefore, how these macromolecules are designed and delivered

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are quite important. A novel approach to deliver such biologics involves using cell-penetrating peptides, also known as protein transduction domains (PTDs), which are short sequences of peptides capable of translocating across the cell membrane. The first PTD identified was a short sequence of amino acids, consisting of the arginine-rich residues 48–60 of the HIV-1 TAT protein. Since the discovery of TAT, many cationic PTDs have been reported including R9, penetratin, VP22, transportan, pVEC, and Pep-1.[25] Although all are capable of crossing cellular membranes, synthesizing these peptides is challenging due to their structural complexities and most require covalent attachment to their cargoes for delivery. Recent studies demonstrate that incorporating key features of PTDs into simpler, tunable scaffolds improves uptake for a broad range of cell types. Mimics of PTDs within these scaffolds facilitate fine-tuning the chemical composition of novel delivery agents for application-specific needs. For instance, successful design of polymeric mimics of PTDs, also called protein transduction domain mimics (PTDMs), provides an easy, synthetic platform to deliver biological cargo such as siRNA and proteins with superior efficiency.[26,27]

Herein, we describe a PTDM capable of delivering an antibody that recognizes and modulates the activity of the intracellular protein, phosphorylated PKCθ (Thr538), via its delivery into human peripheral mononuclear blood cells (hPBMCs). Successful *ex vivo* transport of antibodies into human immune cells lays the foundation to further develop this platform as a potential clinical modality, especially in the area of immunotherapy.

RESULTS

PTDM design and characterization

For this study, we utilized a single PTDM, $MePh_{13}$-*b*-dG_5 ($P_{13}D_5$), which has 13 repeats of a hydrophobic moiety and five repeats of a guanidinium monomer on a polyoxanorbornene di-ester polymer scaffold (**Figure 1a**,**b**). The choice of $P_{13}D_5$ was based on our previous work that showed that increasing the number of phenyl groups increased the protein delivery efficiency (Tew, Minter, unpublished) .

$P_{13}D_5$ was synthesized using ring-opening metathesis polymerization, allowing for controlled, facile synthesis of the block copolymer (**Supplementary Figure S1a**). The polymer structure was characterized by ^{1}H-NMR and its molecular weight was determined by gel permeation chromatography (see **Supplementary Figure S1b,c**). Later, $P_{13}D_5$ was complexed with anti-pPKCθ and the complex formation was shown both by dynamic light scattering and native gel electrophoresis. The size and polydispersity index (PDI) of $P_{13}D_5$:Anti-pPKCθ complex was measured via dynamic light scattering. The high PDI for $P_{13}D_5$ only and anti-pPKCθ only samples showed that there was an aggregation when present alone in solution. The low PDI and narrow size distribution (narrow peak around 1 μm size, **Supplementary Figure S1d**) for the $P_{13}D_5$-anti-pPKCθ mixture indicated that the two components formed complexes (**Figure 1c**). We confirmed the complex formation using native gel electrophoresis. The $P_{13}D_5$:Anti-pPKCθ complex could be visualized on the native gel with an apparent molecular weight between 1,000–1,200 kDa (**Figure 1d**). Anti-pPKCθ only could be detected as free antibody, migrating at ~150 kDa, and as aggregates with two different sizes of ~480 and 1,200 kDa. These results confirmed that no free antibody could be detected following complexing with $P_{13}D_5$ at the ratios used in this study.

Anti-pPKCθ (Thr538) delivery into hPBMCs

The antibody that we complexed to $P_{13}D_5$ specifically recognizes human phosphorylated PKCθ (Thr538). We incubated the $P_{13}D_5$:Anti-pPKCθ complex with hPBMCs then assessed the uptake efficiency and cellular toxicity. For comparison, we also tested anti-pPKCθ uptake with a commercially available antibody delivery reagent, AbDeliverIN. To determine the amount of antibody delivered intracellularly, we subsequently permeabilized the hPBMCs then stained them with a fluorescently-labeled secondary antibody (**Figure 2a**).

Using flow cytometric analysis, we detected robust fluorescence intensity in cells incubated with $P_{13}D_5$:Anti-pPKCθ complexes and stained with the fluorescent secondary antibody. Approximately 60% of hPBMCs stained positively for anti-pPKCθ (**Figure 2b**,**c**) with an average median fluorescence intensity (MFI) of 4,000 a.u., indicating highly-efficient anti-pPKCθ delivery (**Figure 2d**). This is in stark contrast to hPBMCs that were incubated with $P_{13}D_5$ alone, anti-pPKCθ alone, or AbDeliverIN:Anti-pPKCθ complexes, prior to staining with fluorescent secondary antibody (**Figure 2b–d**). Moreover, when we measured the amount of antibody present in the cells over time, we observed that anti-pPKCθ could be detected for up to 72 hours (see **Supplementary Figure S2a**). Cell viability for $P_{13}D_5$:Anti-pPKCθ-treated PBMCs remained above 80%, suggesting the complex had minimal toxicity (**Figure 2e**).

$P_{13}D_5$:Anti-pPKCθ delivery into "unstimulated" hPBMCs greatly reduces their activation potential

To determine whether intracellular anti-pPKCθ delivery neutralizes the actions of pPKCθ, we further analyzed cell proliferation, protein expression levels of signature T cell activation and differentiation molecules via flow cytometry, with and without $P_{13}D_5$:Anti-pPKCθ treatment. We delivered $P_{13}D_5$:Anti-pPKCθ to hPBMCs 4 hours before the cells were stimulated with plate-bound anti-CD3ε plus anti-CD28. Pretreating hPBMCs prior to stimulation did not affect cell viability or cellular proliferation (see **Supplementary Figure S2b,d**), but resulted in a significant impact on biological function. Specifically, expression of the high-affinity IL-2 receptor, CD25, the earliest inducible cell surface glycoprotein, CD69, the intracellular, signaling-competent form of the NOTCH1 transmembrane receptor, NOTCH1^{IC}, and the Th1 transcriptional regulator, T-BET were all significantly reduced compared with levels in dimethylsulfoxide (DMSO)-treated cells (**Figure 3a–f** and **Supplementary Figure S3a,b**). Rottlerin treatment was included as positive control since it is known to prevent Thr538 phosphorylation of PKCθ.[28] As shown in **Figure 3**, pretreating hPBMCs with $P_{13}D_5$:Anti-pPKCθ resulted in similar low levels of expression of all the assayed proteins, as did treatment with rottlerin.

Furthermore, expression of CD25, NOTCH1^{IC}, and T-BET all remained significantly lower in $P_{13}D_5$:Anti-pPKCθ-treated cells, compared with DMSO controls, even at 72 hours after treatment. Compared with DMSO controls, CD69 levels were downregulated

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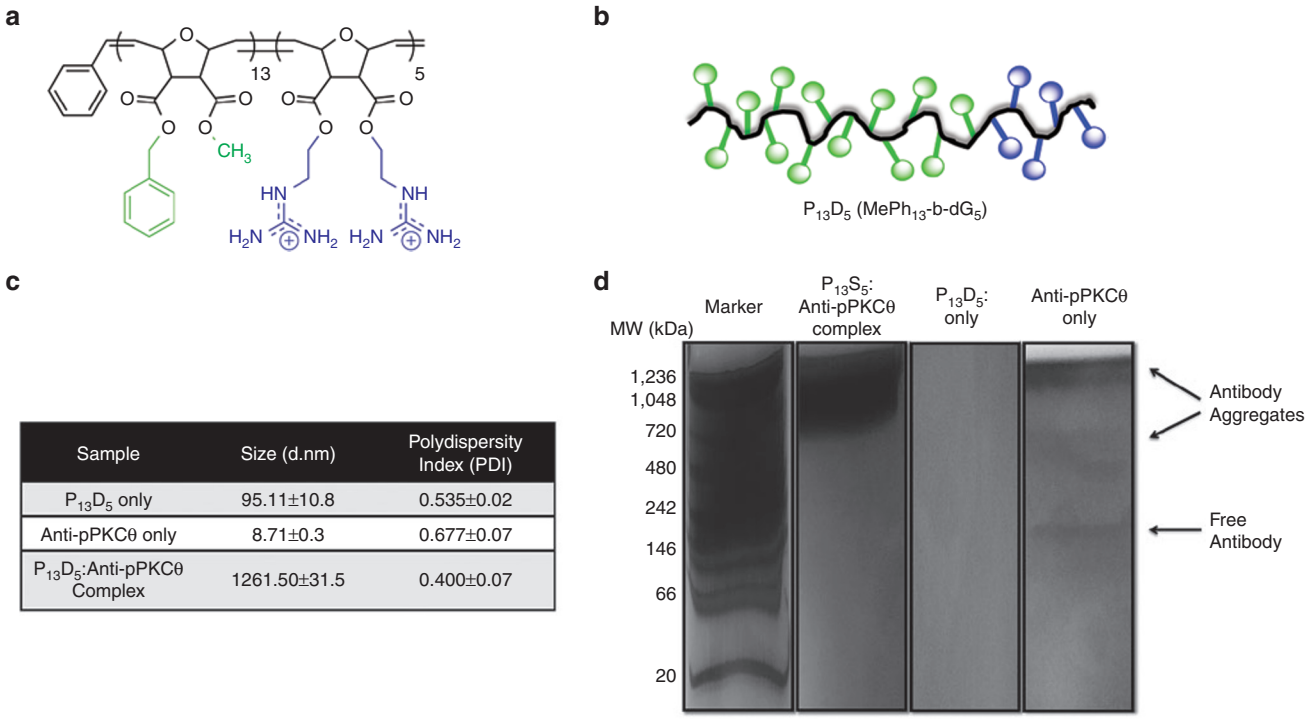

Sample	Size (d.nm)	Polydispersity Index (PDI)
$P_{13}D_5$ only	95.11±10.8	0.535±0.02
Anti-pPKCθ only	8.71±0.3	0.677±0.07
$P_{13}D_5$:Anti-pPKCθ Complex	1261.50±31.5	0.400±0.07

Figure 1 PTDM design and characterization. (**a**) Chemical structure of $MePh_{13}$-*b*-dG_5 ($P_{13}D_5$), with the hydrophobic/aromatic moiety shown in green and cationic/guanidinium moiety in blue. (**b**) Cartoon representation of the $MePh_{13}$-*b*-dG_5 with the same coloring scheme. (**c**) Size and polydispersity index (PDI) measurements for $P_{13}D_5$:Anti-pPKCθ complex by dynamic light scattering. (**d**) Native gel electrophoresis demonstrating complexation between $P_{13}D_5$ and Anti-pPKCθ. PTDM, protein transduction domain mimic; pPKCθ, protein kinase C-theta.

in $P_{13}D_5$:Anti-pPKCθ-treated cells 24 hours after treatment and were not upregulated for 72 hours (see **Supplementary Figure S3a,b**). To assess the functional impact on PKCθ activity of delivered $P_{13}D_5$:Anti-pPKCθ, we also analyzed expression of two important proinflammatory cytokines produced by activated T cells, interferon gamma (IFNγ) and interleukin-2 (IL-2), using standard enzyme-linked immunosorbent assay (ELISA) techniques. We observed that IFNγ and IL-2, both, were significantly lower after $P_{13}D_5$:Anti-pPKCθ treatment during the 24–72 hours cell culture period, showing reduced levels that were comparable to those of rottlerin-treated cells (**Figure 3h,i**). In addition, we asked whether $P_{13}D_5$:Anti-pPKCθ treatment was reversible. To test this, we incubated hPBMCs with $P_{13}D_5$:Anti-pPKCθ for 7 days and measured IFNγ and IL-2 levels via cytometric bead array analyses. We observed that IFNγ levels were similar by day 5 after treatment whereas IL-2 levels were similar by day 6 after treatment suggesting the early inhibitory effects of Anti-pPKCθ treatment were reversible within 5–6 days of treatment (see **Supplementary Figure S4a,b**). Taken together, these results indicated that $P_{13}D_5$:Anti-pPKCθ treatment of T cells prior to delivery of TCR and costimulatory signals greatly reduced their activation potential.

$P_{13}D_5$:Anti-pPKCθ delivery into "activated" hPBMCs diminishes expression of downstream activation markers

In humans, PKCθ in activated T cells contributes to the pathology of various aberrant immune conditions[29] and elevated levels of phosphorylated PKCθ (Thr538) have been observed both in activated $CD4^+$ and $CD8^+$ T cells (Minter, unpublished). Therefore, we asked what effect delivering $P_{13}D_5$:Anti-pPKCθ has on cells which have already been activated and show increased levels of phosphorylated PKCθ (Thr538). In **Figure 4**, we clearly demonstrate that when hPBMCs were stimulated for 24 hours with anti-CD3ε plus anti-CD28, then incubated with $P_{13}D_5$:Anti-pPKCθ, levels of CD25, $NOTCH1^{IC}$, and T-BET were significantly lower following an additional 24 hours of culture (48 hours from time of stimulation), compared with DMSO-treated cells (**Figure 4a–g**). Interestingly, $P_{13}D_5$:Anti-pPKCθ-treated cells did not upregulate these markers, even up to 72 hours after treatment (96 hours after stimulation). This was not due to toxicity of the treatment since cellular viability in $P_{13}D_5$:Anti-pPKCθ-treated cells remained similar to DMSO-treated cells (see **Supplementary Figure S2c**). Additionally, CD69 levels were not upregulated in $P_{13}D_5$:Anti-pPKCθ-treated cells for 72 hours (see **Supplementary Figure S3c,d**). Finally, stimulated cells treated with $P_{13}D_5$:Anti-pPKCθ produced significantly lower amounts of IFNγ and IL-2 compared with DMSO-treated controls and, again, at levels comparable with those of rottlerin-treated samples (**Figure 4h,i**).

We also asked whether anti-pPKCθ or $P_{13}D_5$, individually, exerted any biological effect on T cells. hPBMCs were treated either with anti-pPKCθ alone or with $P_{13}D_5$ alone, and levels of $NOTCH1^{IC}$ and T-BET were measured after 24 hours of stimulation. When compared with DMSO-treated cells, we found that there were no significant differences in $NOTCH1^{IC}$ (see **Supplementary Figure S5a,b**) or T-BET (see **Supplementary Figure S5c,d**) levels in anti-pPKCθ-only-treated or in $P_{13}D_5$-only-treated cells after 24 hours. These observations suggest complex

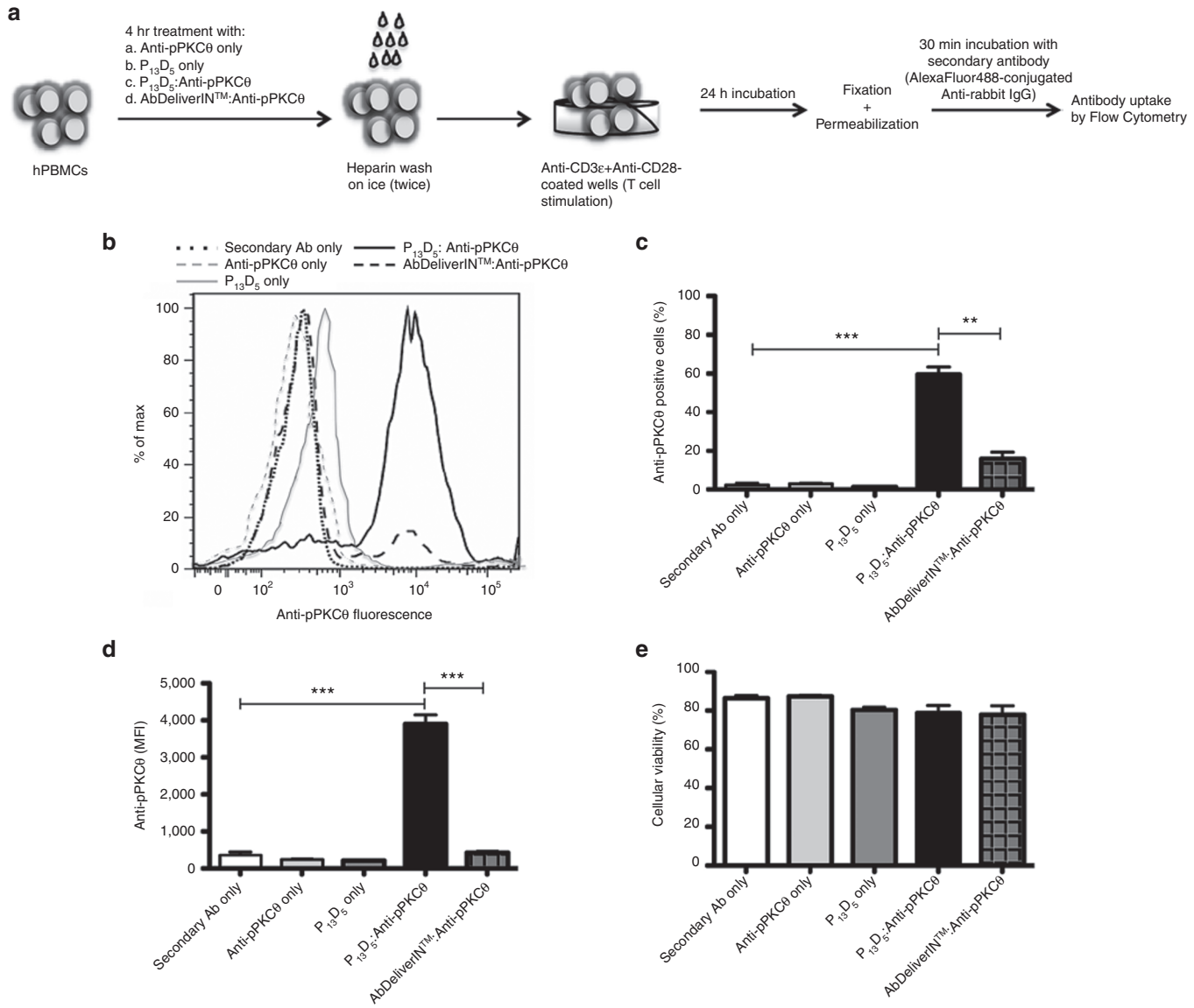

Figure 2 Anti-pPKCθ (Thr538) delivery into hPBMCs. (**a**) Schematic of anti-pPKCθ delivery experiment. Human PBMCs were incubated with anti-pPKCθ only, $P_{13}D_5$ only, $P_{13}D_5$:Anti-pPKCθ, or AbDeliverIN:Anti-pPKCθ for 4 hours. hPBMCs were subjected to a heparin wash to remove surface-bound proteins, stimulated for 24 hours with anti-CD3ε + anti-CD28, fixed and permeabilized, then incubated with AlexaFluor488-conjugated anti-rabbit secondary antibody. (**b**) Histogram showing anti-pPKCθ uptake under different treatment conditions as in **a**, above. (**c**) Percent hPBMCs staining positively for anti-pPKCθ, following treatment as in **a**. (**d**) Median fluorescent intensity (MFI) of AlexaFluor488-conjugated anti-rabbit secondary antibody in hPBMCs following treatment as in **a**, above. (**e**) Viability of hPBMCs following treatment as in **a**. Data represent the mean ± SEM of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, calculated using an unpaired, two-tailed student's *t*-test. hPBMCs, human peripheral mononuclear blood cells; SEM, standard error of mean; pPKCθ, protein kinase C-theta.

formation between antibody and $P_{13}D_5$ is necessary for intracellular anti-pPKCθ delivery (as shown in **Figure 2b–d**) and modulation of PKCθ function.

$P_{13}D_5$:Anti-pPKCθ delivery alters the activity and localization of PKCθ in hPBMCs

The PKCθ signaling pathway coordinates important signaling events to direct Th1 cell functions.[29–31] Following its phosphorylation on Thr538, PKCθ is autophosphorylated at serine residues to become fully activated.[7] pPKCθ will phosphorylate an intracellular scaffold protein, CARMA1, to facilitate formation of the macromolecular signaling aggregate known as the CARMA1/BCL10/MALT1 (CBM) complex. Assembly of the CBM components precedes, and is thought to be necessary for, liberation of NF-κB transcriptional regulators from their cytosolic inhibitory complexes.[1,32,33] Moreover, it has been reported that pPKCθ is also able to translocate into the nucleus where it can associate with a chromatin-bound complex to regulate *IL2* gene expression.[17,31] Having demonstrated that $P_{13}D_5$:Anti-pPKCθ delivery modulated biological functions, we asked whether this might be due to its interfering with specific downstream actions of PKCθ.

To address this question, we evaluated PKCθ autophosphorylation at Ser676 residue, CARMA1 phosphorylation, $NOTCH1^{IC}$ levels, as well as nuclear localization of PKCθ. Compared with

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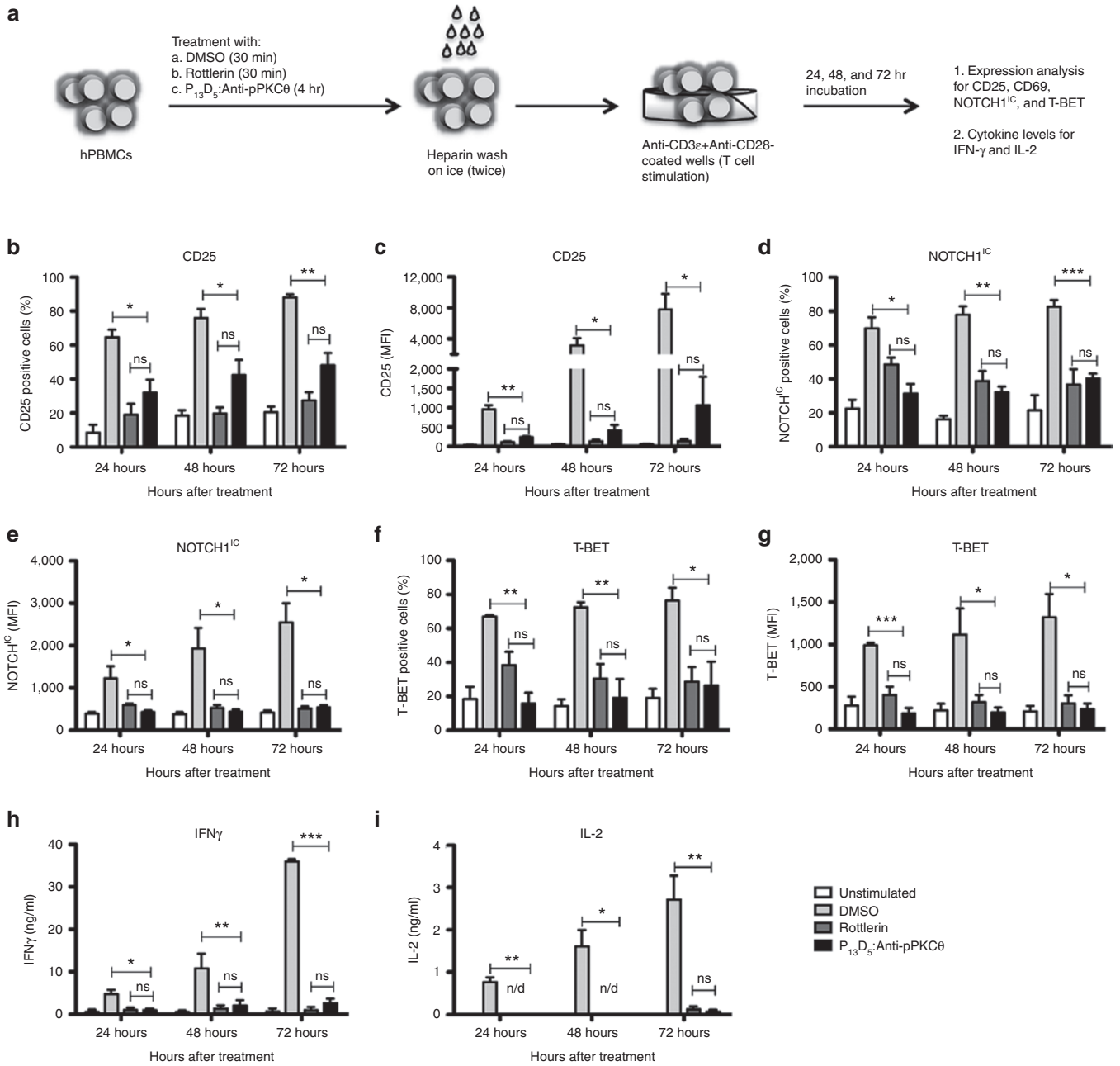

Figure 3 $P_{13}D_5$:Anti-pPKCθ delivery into "unstimulated" hPBMCs greatly reduces their activation potential. (**a**) Schematic of experimental design. hPBMCs were treated with DMSO (vehicle control), Rottlerin, or $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C, then stimulated with plate-bound anti-CD3ε plus anti-CD28. Samples were analyzed at 24, 48, and 72 hours after stimulation, using flow cytometry. Percent positive cells, as well as MFI, were assessed for several markers of activation, as indicated. (**b,c**) CD25, (**d,e**) NOTCH1^{IC}, (**f,g**) T-BET. Cytokine levels of (**h**) IFNγ and (**i**) IL-2 were determined by ELISA. Data represent the mean ± SEM of three independent experiments. nd (not detected); ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, calculated using an unpaired, two-tailed student's *t*-test. DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; hPBMCs, human peripheral mononuclear blood cells; IFNγ, interferon gamma; IL-2, interleukin-2; MFI, median fluorescent intensity; SEM, standard error of mean; pPKCθ, protein kinase C-theta.

control-treated cells, hPBMCs treated with $P_{13}D_5$:Anti-pPKCθ showed reduced Ser676 autophosphorylation (**Figure 5a**) and decreased levels of CARMA1 phosphorylation (**Figure 5b**). In addition, the level of cleaved NOTCH1^{IC} was diminished following anti-pPKCθ delivery (**Figure 5c**). Moreover, when we assessed the accumulation of total PKCθ in the nucleus, we noted that $P_{13}D_5$:Anti-pPKCθ-treated cells exhibited less total PKCθ in the nucleus than did DMSO-treated cells (**Figure 5d**). However, there was no change in cytosolic levels of total PKCθ (see **Supplementary Figure S6**). Although the percent of cells expressing T-BET was diminished following P13D5:Anti-pPKCθ treatment (**Figure 5e,f**), T-Bet expression on a per cell basis remained unchanged (**Figure 5g**). Expression of IFNγ, however, was decreased following treatment with P13D5:Ati-pPKCθ, to

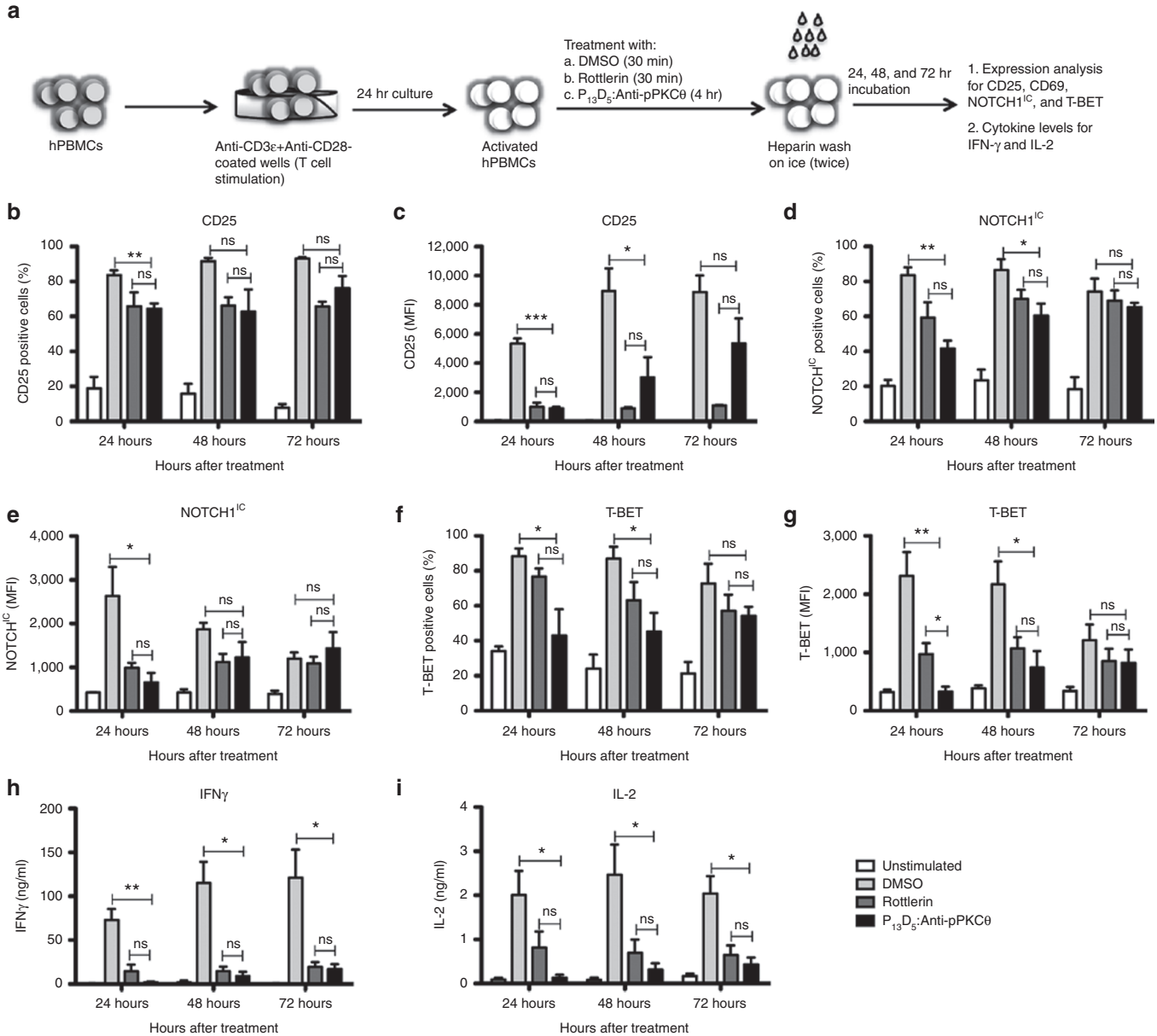

Figure 4 $P_{13}D_5$:Anti-pPKCθ delivery into "activated" hPBMCs diminishes expression of downstream activation markers. (**a**) Schematic of experimental design. hPBMCs were stimulated with plate-bound anti-CD3ε plus anti-CD28. After 24 hours of stimulation, cells were treated with DMSO (vehicle control), Rottlerin, or $P_{13}D_5$:Anti-pPKCθ complex for 4 hours at 37°C. Samples were analyzed 24, 48, and 72 hours after treatment, which corresponded to 48, 72, and 96 hours after stimulation, respectively, using flow cytometry. Percent positive cells, as well as MFI, were assessed for several markers of activation, as indicated. (**b,c**) CD25, (**d,e**) NOTCH1^{IC}, (**f,g**) T-BET. Cytokine levels of (**h**) IFNγ and (**i**) IL-2 were determined by ELISA. Data represent the mean ± SEM of three independent experiments. ns $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ calculated using an unpaired, two-tailed student's *t*-test. DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; hPBMCs, human peripheral mononuclear blood cells; IFNγ, interferon gamma; IL-2, interleukin-2; MFI, median fluorescent intensity; SEM, standard error of mean; pPKCθ, protein kinase C-theta.

levels comparable to those of rottlerin-treated cells (Figure 5h). Thus, not only does $P_{13}D_5$:Anti-pPKCθ delivery into hPBMCs reduce the activation potential of unstimulated cells for up to 72 hours after treatment, it also reduces the ability of stimulated cells to sustain increased expression of various markers of activation. Our mechanistic studies suggest this may result from the combination of reduced CARMA1 phosphorylation and impaired nuclear translocation of PKCθ in $P_{13}D_5$:Anti-pPKCθ-treated cells.

Ex vivo delivery of $P_{13}D_5$:Anti-pPKCθ into hPBMCs provides a survival benefit in a lymphocyte transfer, humanized mouse model of graft-versus-host disease

Our *in vitro* experiments demonstrated that $P_{13}D_5$:Anti-pPKCθ delivered into hPBMCs effectively reduced the functional activity of PKCθ up to 72 hours in culture. However, it was unclear how durable the effects of our *ex vivo* treatment would be if these treated cells were used in proof-of-principle, lymphocyte transfer experiments in a "humanized" model of GvHD, induced when

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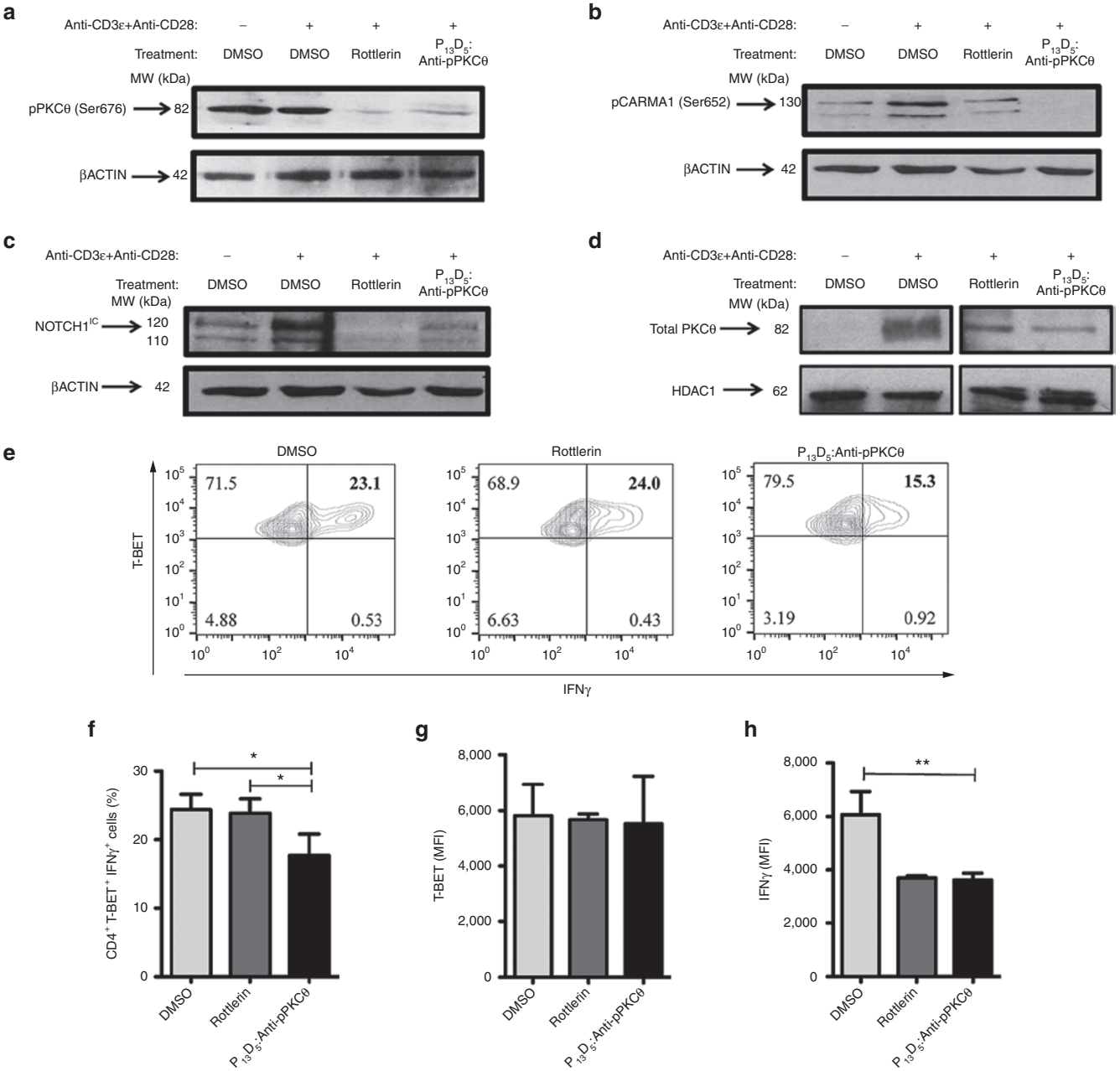
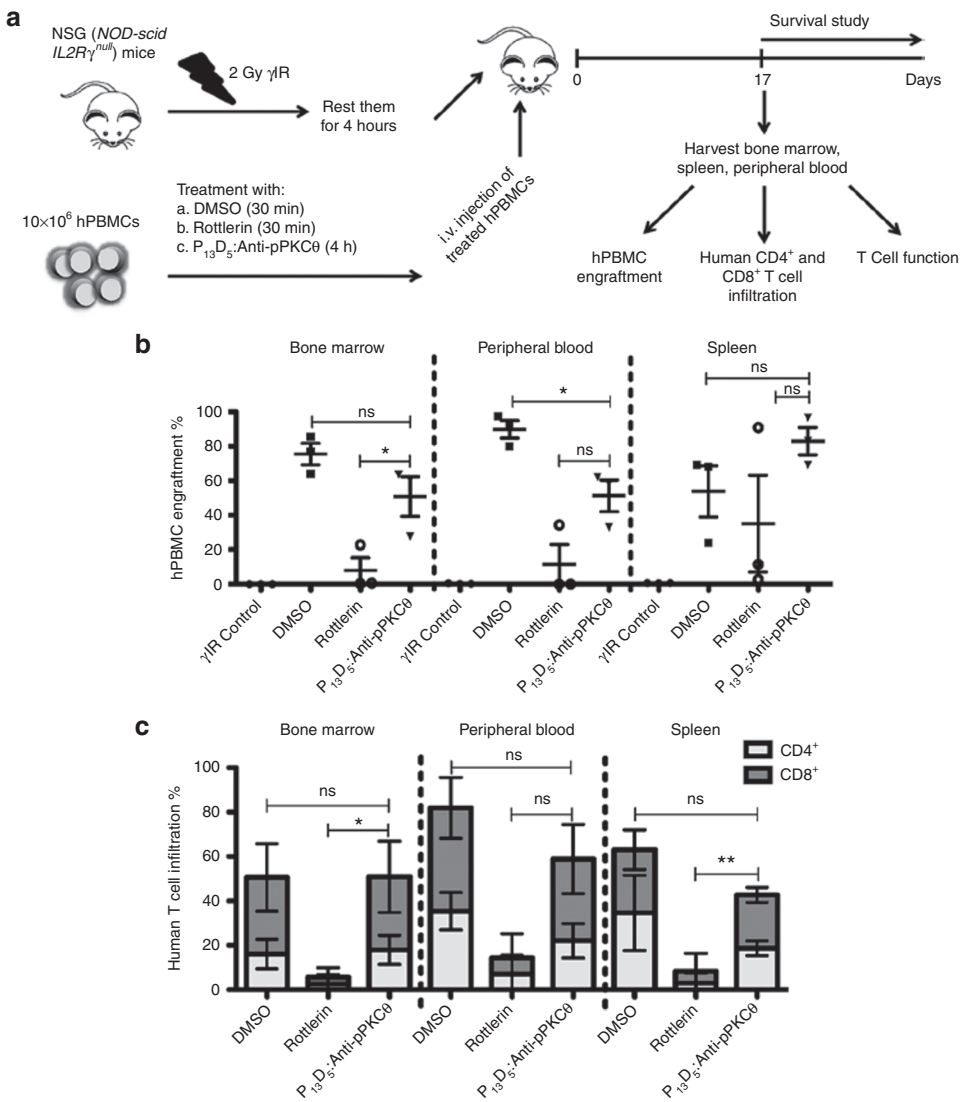


Figure 5 $P_{13}D_5$:Anti-pPKCθ delivery alters the activity and localization of PKCθ in hPBMCs. hPBMCs were treated with DMSO (vehicle control), Rottlerin, or $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24 hours. (**a**) Autophosphorylation of PKCθ (Ser676), (**b**) phosphorylation of CARMA1 (Ser652), and (**c**) expression of NOTCH1^{IC} was determined by immunoblotting proteins isolated from whole cell lysates. (**d**) Nuclear localization of total PKCθ was determined by immunoblotting proteins isolated from nuclear fractions. (**e**) Contour plots for human Th1 cells differentiated *in vitro*, (**f**) Percent of Th1-polarized cells, (**g**) MFI of T-BET expression, and (**h**) MFI of IFNγ in Th1-polarized cells. Data in **a**–**d** are representative of three independent replicates. Data in **f**–**h** represent the mean ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$ calculated using an unpaired, two-tailed student's *t*-test. DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; hPBMCs, human peripheral mononuclear blood cells; IFNγ, interferon gamma; IL-2, interleukin-2; MFI, median fluorescent intensity; SEM, standard error of mean; pPKCθ, protein kinase C-theta.

hPBMCs are transferred into the NOD-*scid-il2rγ*^{null} (NSG) strain of mice. In this graft (hPBMCs)-versus-host (NSG mouse) model, transferred lymphocytes acutely target the bone marrow of recipient mice resulting in lethal immune-mediated bone marrow failure within ~20 days. Th1 cells play an important role in the progression of GvHD.[34] Moreover, PKCθ and NOTCH1 signaling are required for alloreactivity and GvHD induction.[35,36] To evaluate the long-term effects of $P_{13}D_5$:Anti-pPK θ treatment on cells, we induced GvHD in three cohorts of mice. In one cohort, we induced GvHD by transferring DMSO-treated hPBMCs; in a second cohort we induced GvHD with $P_{13}D_5$:Anti-pPKCθ-treated hPBMCs; in the third cohort we induced GvHD using hPBMCs pretreated with rottlerin. We harvested mice on day 17 after GvHD induction to determine how well the transferred cells engrafted and expanded

in target tissues such as the spleen and bone marrow, as well as in peripheral blood (**Figure 6a**). Mice that received $P_{13}D_5$:Anti-pPKCθ-treated cells showed a similar degree of hPBMC expansion as DMSO-treated control cells, indicating $P_{13}D_5$:Anti-pPKCθ treatment did not affect cellular viability *in vivo,* following cell transfer (**Figure 6b**). Additionally, we determined that infiltration of human $CD4^+$ and $CD8^+$ T cells into the target tissues were also similar in mice receiving DMSO-treated or $P_{13}D_5$:Anti-pPKCθ-treated hPBMCs (**Figure 6c**). To determine whether the effects of anti-pPKCθ treatment were consistent with *in vitro* results, we measured levels of pPKCθ, T-BET, $NOTCH1^{IC}$, and CD25 in $CD4^+$ T cells infiltrating the bone marrow, in $P_{13}D_5$:Anti-pPKCθ-treated hPBMCs, although the differences were not significant compared with DMSO or to rottlerin-treated hPBMCs (**Figure 6d–i** and **Supplementary S7a,b**). Furthermore, we did not see any upregulation in CD25 or $NOTCH1^{IC}$ levels, as measured by flow cytometry (see **Supplementary Figure S7c–f**). We also measured IL-2 and IFNγ levels in the plasma of mice from all three cohorts. Although IL-2 levels were similar in all animals evaluated (see **Supplementary Figure S7g**), IFNγ levels were significantly lower in mice receiving $P_{13}D_5$:Anti-pPKCθ-treated hPBMCs, compared with mice receiving DMSO-treated control cells (**Figure 6j**). We next asked whether delivering $P_{13}D_5$:Anti-pPKCθ to hPBMCs prior to transferring them into recipient mice would affect GvHD severity and survival. Remarkably, GvHD clinical scores were significantly reduced (**Figure 6k**) and Kaplan-Maier analysis revealed a significant survival benefit when $P_{13}D_5$:Anti-pPKCθ was delivered to hPBMCs prior to transfer, compared with mice that received DMSO-treated cells (**Figure 6l**).

Two of three mice that received hPBMCs pretreated with rottlerin did not show overt signs of disease. However, no human cells were detected in the peripheral blood of these mice, when they were removed from the study on day +70 (data not shown), suggesting GvHD was not induced in those mice since rottlerin treatment also had cytotoxic effects on hPBMCs. These observations are consistent with other data from our lab supporting the notion that PKCθ plays a critical role in T cell activation, proliferation, and survival. Collectively, our data demonstrate that we can modulate PKCθ activity in hPBMCs, *ex vivo*, using anti-pPKCθ delivery by $P_{13}D_5$. Furthermore, our *in vivo* proof-of-principle experiments suggest the effects of $P_{13}D_5$:anti-pPKCθ delivery are durable and may constitute the basis of a novel therapeutic



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strategy that targets the actions of intracellular proteins to alter disease progression.

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate intracellular delivery of a functional antibody into human PBMCs using PTDMs. The efficiency of anti-pPKCθ delivery by $P_{13}D_5$ was significantly higher than that of a commercial antibody delivery reagent, and did not show cellular toxicity. We observed significant PKCθ neutralization in cells treated with $P_{13}D_5$:Anti-pPKCθ, whether before or after stimulation, and the inhibitory effects persisted up to 72 hours in culture. Activation markers such as CD25 and CD69 were expressed to a much lower extent compared with control samples. Furthermore, expression of NOTCH1^{IC}

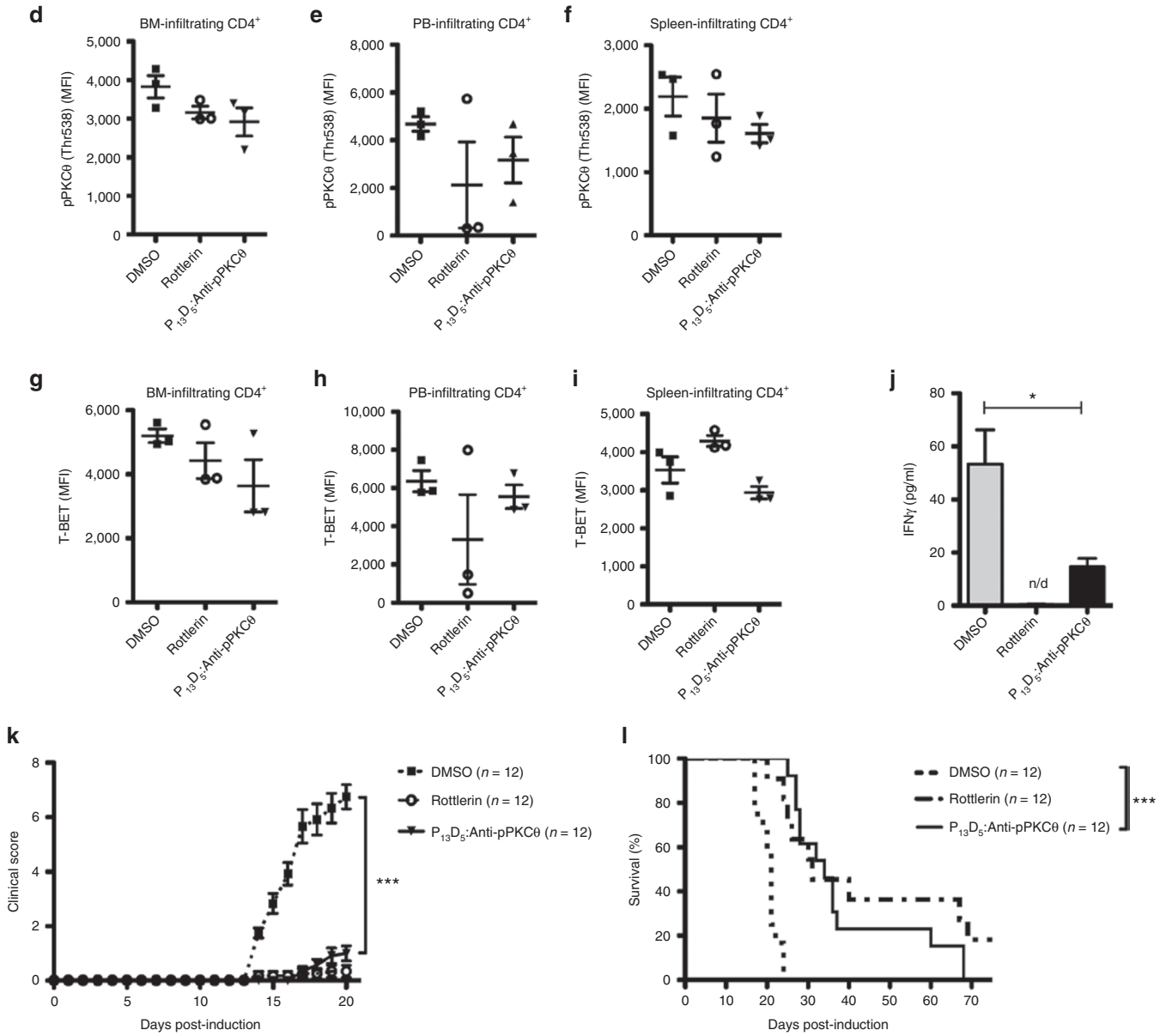

Figure 6 *Ex vivo* delivery of $P_{13}D_5$:Anti-pPKCθ into hPBMCs provides a survival benefit in a lymphocyte transfer, humanized mouse model of graft-versus-host disease (GvHD). (**a**) Schematic of experimental approach. hPBMCs were treated with DMSO (vehicle control), Rottlerin, or $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C. Graft-versus-host responses were induced in NSG mice by transferring 10×10^6 cells treated as above, via tail vein injection. On day +17 after disease induction, mice were humanely killed and (**b**) percent engraftment of human CD45 cells in bone marrow (BM), peripheral blood (PB), and spleen was determined using flow cytometry. (**c**) Infiltration of CD4+ and CD8+ T cells into BM, PB, and spleen was also assessed using flow cytometry. (**d–f**) MFI of pPKCθ (Thr538) expression in CD4+ T cells infiltrating BM, PB, and spleen. (**g–i**) MFI of T-BET expression in CD4+ T cells infiltrating BM, PB, and spleen. (**j**) IFNγ levels in plasma as determined by cytometric bead array analysis. (**k**) GvHD clinical scores were calculated as described in Materials and Methods ($n = 12$). (**l**) Kaplan–Maier analysis was used to determine the survival benefit of *ex vivo* treatment modalities. Mice receiving hPBMCs pretreated with Rottlerin were removed from the study on day +70. $n = 12$ mice for each *ex vivo* treatment of hPBMCs. Data shown in **b–j** represent the mean ± SEM of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ calculated using an unpaired, two-tailed student's *t*-test. DMSO, dimethylsulfoxide; hPBMCs, human peripheral mononuclear blood cells; IFNγ, interferon gamma; MFI, median fluorescent intensity; SEM, standard error of mean; pPKCθ, protein kinase C-theta.

and T-BET were significantly diminished in $P_{13}D_5$:Anti-pPKCθ-treated cells, suggesting distal signaling events important for T cell activation[34] were also affected. This was further evidenced by impaired CARMA1 phosphorylation, reduced nuclear translocation of PKCθ, and significantly less production of proinflammatory cytokines, such as IFNγ and IL-2. Moreover, *ex vivo* $P_{13}D_5$:anti-pPKCθ delivery into hPBMCs, showed durable effects on PKCθ signaling in a humanized model of GvHD, and provided a significant survival benefit that was not observed when DMSO-treated cells were used to induce disease.

PTDMs represent a promising platform for cargo delivery into immune cells as a means of modulating their responses. Due to their tunable characteristics, these polymeric structures can be easily designed in order to increase their delivery efficiency, especially for hard-to-transfect cells such as hPBMCs.[26,27] Current strategies for intracellular antibody delivery show low efficiency, high toxicity, nonspecific effects, or reduced endosomal escape. As a result, some biologics which show potential *in vitro* are considerably less effective when used *in vivo*.[21,24,37] Our current strategy, using PTDMs, provides an improved platform for antibody delivery enabling specific targeting of intracellular proteins.

Over the past two decades, the Food and Drug Administration (FDA) has approved an increasing number of therapeutic antibodies for clinical use. For example, antibodies specific for CD20, IL-6, or TNF have been used to treat various aberrant immunological conditions. However, there remain concerns about safety and efficacy when targeting extracellular or cell surface molecules that continue to pose significant drawbacks.[21,38] Depleting healthy as well as abnormal cells or dampening global immune responses can increase patient risk of infection. Additionally, humanized antibodies may be processed as foreign antigens through the endocytic pathway to initiate $CD4^+$ T cell-dependent humoral responses, which can further diminish their clinical efficacy.[21–23] By contrast, our delivery strategy provides very specific targeting of an intracellular molecule, resulting in the precise blockade of a particular cellular response.

Understanding the details of immune function lays the foundation for specifically modulating T cell responses. PKCθ is not necessary for antiviral responses following allogeneic bone marrow transplantation, strengthening the possibility that inhibiting PKCθ may selectively suppress allograft rejection and GvHD without disrupting antiviral or antitumor immunity.[10,35,39–42] We used a known pharmacological inhibitor, rottlerin, as a positive control to inhibit T538 phosphorylation and, thereby, PKC θ actions in T cell signaling. Our intracellular antibody delivery decreased PKCθ signaling as robustly as rottlerin, but with less toxicity. Extensive efforts have been made to develop isozyme-selective PKCθ inhibitors; however, the high degree of homology shared by PKC family members makes this challenging.[43–48] For instance, enzastaurin can inhibit both PKCβ and PKCθ. Therefore, using enzastaurin to target PKCθ, could also shut down PKCβ-related pathways, likely causing undesired off-target effects.[49] Several chemical compounds such as aminopyrimidine, pyridine carbonitrile, and thieno (2,3-b) pyridine-5-carbonitriles (2-alkenyl and 2-phenyl) derivatives are considered to be more selective for PKCθ than for other PKC family members. Preclinical studies have shown that these compounds decreased IL-2 production in activated T cells derived from wild-type mice but had reduced effect on activated T cells from PKCθ knockout mice.[50] Overall, the difficulties encountered creating PKCθ-specific inhibitors highlight the challenges of rational drug design for this target. The PTDM-mediated antibody delivery approach described here may represent a specific and nontoxic means of modulating the function of intracellular targets.

The biological response to neutralizing PKCθ activity supports a novel targeting strategy for PKCθ in the context of T cell activation and differentiation. Cytosolic and nuclear PKCθ may exert differential effects on T cell fate. PKCθ and NOTCH1 physically associate in the cytoplasm and have substantial roles during T cell activation and differentiation.[51,52] Our studies suggest that modulating PKCθ localization, within a narrow window following stimulation, resulted in significantly altered T cell fate. This effect persisted for several weeks, as demonstrated by the attenuated disease severity and prolonged survival in a humanized model of GvHD. The delivery strategy employed here will enable us to further investigate molecular events mediated by PKCθ and will advance our understanding of specific mechanisms of immune response.

In conclusion, this study is the first to describe a successful strategy for routine and effective intracellular antibody delivery by PTDMs and demonstrates its powerful application by targeting PKCθ in the context of T cell immunomodulation. Fine-tuning this approach to specifically inhibit other cellular proteins will lay the foundation for "drugging the undruggable". This may further open the door to modulating intracellular targets with the effectiveness of small molecule inhibitors but coupled with the greater specificity that antibodies provide.

MATERIALS AND METHODS

Materials. Anti-human phospho PKCθ (Thr538, Monoclonal Rabbit IgG, Clone: F4H4L1) was purchased from Invitrogen (Carlsbad, CA). Human PBMCs were obtained from StemCell Technologies (Vancouver, BC, Canada). Antibodies specific for human CD25, CD4, CD45, CD8, $NOTCH1^{IC}$, T-BET, IFNγ, and mouse CD45, and compatible with flow cytometry were purchased from eBioscience (San Diego, CA). Flow cytometric data were acquired using an LSR II Flow Cytometer, LSRFortessa 5 laser (Becton Dickinson, Canaan, CT) and analyzed using BD FACSDiva Software v7.0.1 (Becton Dickinson) or FlowJo (Tree Star, Ashland, OR).

Synthesis of $MePh_{13}$-b-dG_5 ($P_{13}D_5$). We have recently described the synthesis of PTDMs.[27] $P_{13}D_5$ was obtained by ring-opening metathesis polymerization using the Grubbs' third generation catalyst in dichloromethane. The final product was purified by dialysis against RO water and obtained by lyophilization.

Characterization of $MePh_{13}$-b-dG_5 ($P_{13}D_5$). The molecular weight of $P_{13}D_5$ was assessed by gel permeation chromatography. After $P_{13}D_5$ was lyophilized, a sample was analyzed by gel permeation chromatography (GPC) after calibration using a poly (methyl methacrylate; PMMA) standard. The molecular weight was determined and PDI was calculated. To confirm the chemical composition, proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) was performed. The sample was diluted in deuterated acetonitrile (CD_3CN).

Dynamic light scattering. To characterize the complexation of PTDM and antibody, 1 μmol/l of $P_{13}D_5$ and 25 nmol/l of anti-pPKCθ were complexed in phosphate buffered saline (PBS, pH 7.2) at a specific ratio ($P_{13}D_5$:Anti-pPKCθ::40:1). It was incubated for 30 minutes at room temperature (RT). Later, the complex was loaded into a spectrophotometric cuvette. The size of the complex was measured with a Malvern Zetasizer Nano ZSP instrument (Malvern Instruments, West Borough, MA).

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Native polyacrylamide gel electrophoresis and silver staining. About 4–20% Mini-PROTEAN TGX Precast protein gels were used to run $P_{13}D_5$:Anti-pPKCθ complex along with $P_{13}D_5$ only and Anti-pPKCθ only. Each was loaded as 250 µl into individual gels with 7 cm well size and run for 35 minutes at 200 V in 1X running buffer (25 mmol/l Tris, 192 mmol/l Glycine, pH 7.2) without the addition of sodium dodecyl sulfate (SDS) for native conditions. The electrodes were swapped since the charge of the complex is positive. Later, the gels were stained with 0.1% silver nitrate and 0.08% formalin (37%). They were imaged using a Syngene G-box gel documentation system (Syngene USA, Frederick, MD).

$P_{13}D_5$:anti-pPKCθ complex delivery into hPBMCs. About 1 µmol/l of $P_{13}D_5$ and 25 nmol/l of anti-pPKCθ were complexed in PBS (pH 7.2) at a specific ratio ($P_{13}D_5$:Anti-pPKCθ::40:1) and AbDeliverIN:Anti-pPKCθ was complexed in PBS at the ratio recommended by the manufacturer's protocol. The complexes were incubated for 30 minutes at RT. The hPBMCs were treated with the complexes for 4 hours at 37°C. Cells were then harvested and washed with PBS. Later, cells were thoroughly washed twice with 20 U/ml Heparin in PBS for 5 minutes on ice to remove surface-bound complexes outside cellular membrane. Pellets were resuspended in fresh Roswell Park Memorial Institute (RPMI) complete media (10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, 1 mmol/l sodium pyruvate, 2 mmol/l L-Glutamine) seeded into 5 µg/ml anti-CD3ε- plus 2.5 µg/ml anti-CD28-coated tissue culture wells and stimulated for 24 hours at 37°C. After stimulation, cells were harvested and washed with PBS. They were fixed and permeabilized (Foxp3 staining kit, eBioscience, according to manufacturer's recommendation) for 30 minutes at 4°C, then stained with Alexa Fluor488-labeled anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) for 30 minutes at 4°C. Labeled cells were washed and analyzed by flow cytometry to measure delivery efficiency.

Cellular viability assay. hPBMCs were stained with 7-Aminoactinomycin D (7-AAD, eBioscience, San Diego, CA) for 15 minutes at RT, then pelleted by centrifugation for 5 minutes. Cells were resuspended in 0.2% bovine serum albumin (BSA) in PBS and analyzed by flow cytometry.

Flow cytometric analyses of marker expression. hPBMCs were treated with DMSO or 3 µmol/l Rottlerin for 30 minutes or with $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C. Following treatment, cells were washed with PBS and resuspended in fresh RPMI complete media. Later, $P_{13}D_5$:Anti-pPKCθ-treated cells were thoroughly washed twice with 20 U/ml Heparin in PBS for 5 minutes on ice to remove noninternalized complexes. Cells were stimulated by seeding into anti-CD3ε- plus anti-CD28-coated wells and incubated at 37°C. Some cells were stimulated for 24 hours prior to treatment. At 24, 48, and 72 hours after treatment, cells were harvested and washed with PBS. Then, hPBMCs were stained with antibodies specific for surface (CD25 and CD69) or intracellular ($NOTCH1^{IC}$ and T-BET) proteins and analyzed by flow cytometry.

Cell proliferation assay. Now, hPBMCs were treated with DMSO or 3 µmol/l Rottlerin for 30 minutes or with $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C. Following treatment, cells were washed with PBS and resuspended in fresh RPMI complete media. Later, $P_{13}D_5$:Anti-pPKCθ-treated cells were thoroughly washed twice with 20 U/ml Heparin in PBS for 5 minutes on ice. After washing with Heparin, cells were resuspended in prewarmed PBS + 0.1% BSA and cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) dye using the Cell Trace CFSE Cell Proliferation Kit (Thermo Fisher Scientific, Agawam, MA) following the manufacturer's protocol. Cells were stimulated by seeding into anti-CD3ε- plus anti-CD28-coated wells and incubated at 37°C for 7 days. Cell proliferation was measured daily by flow cytometric analysis.

ELISA for cytokine determination. Next, hPBMC culture supernatants were collected at designated timepoints and analyzed for cytokine secretion. 96-well Maxisorp plates were coated overnight at 4°C with the appropriate capture antibody (anti-human IFNγ or anti-human IL-2; BD Biosciences, San Diego, CA). Nonspecific protein binding was prevented by blocking wells with 10% FBS in PBS for 3 hours at RT. Culture supernatants and standards were diluted appropriately and added to wells. The plate was incubated overnight at 4°C, with continuous rocking. Biotinylated detection antibodies were added to wells followed by TMB substrate reagents (BD Biosciences) at a 1:1 ratio. Color development was monitored and the reaction was terminated by the addition of stop solution (2 N H_2SO_4). Absorbance was read at 450 nm using a microplate reader. Cytokine concentrations were determined relative to the standard curves generated.

Nuclear versus cytosolic protein extraction. 3×10^6 cells/ml hPBMCs were used for each sample. Cells were harvested 24 hours after anti-CD3ε plus anti-CD28 stimulation. Nuclear and cytosolic protein extracts were generated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Protein concentration was determined by BCA Assay and 40 µg (cytoplasmic extract) or 50 µg (nuclear extract) of protein was separated by 8% SDS-PAGE. Total PKCθ was detected using rabbit polyclonal anti-PKCθ (C-18) (Santa Cruz Biotechnology, Dallas, TX).

Immunoblotting. About 2×10^6 cells/ml hPBMCs were used for each sample. Cells were harvested 24 hours after anti-CD3ε plus anti-CD28 stimulation, pelleted, and resuspended in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors (25 mmol/l Tris-HCl pH 7.6, 150 mmol/l NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Halt Protease and Phosphatase Inhibitor Cocktail). The suspension was centrifuged for 10 minutes at 4°C. The supernatants were collected as protein lysates, BCA assay was performed to determine protein concentration, and 30 µg of protein was loaded for immunoblotting. CARMA1 Ser652 phosphorylation was detected with anti-phospho-CARD11 (Ser652; Cell Signaling Technology). PKCθ Ser676 phosphorylation was detected with anti-phospho-PRKCQ (Ser676; LifeSpan Biosciences, Burlington, NC), and intracellular NOTCH1 levels were detected with anti-cleaved NOTCH1 (Val1744; Cell Signaling Technology).

In vitro *human Th1 cell differentiation assay.* hPBMCs were sorted for $CD4^+$ T cells using a FACSAria II cell sorter. Then, the cells were treated with DMSO or 3 µmol/l Rottlerin for 30 minutes or with $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C. Following treatment, cells were washed once with PBS and twice with 20 U/ml Heparin in PBS for 5 minutes on ice. For Th1 differentiation, they were resuspended in human Th1 differentiation media (provided in CellXVivo human Th1 cell differentiation kit, R&D systems, Minneapolis, MN). The cells were plated on human anti-CD3-coated wells. Th1-polarized cells were incubated for 5 days. After 5 days of incubation for Th1 cells, the cells were washed with RPMI complete media and restimulated for 1 hour in the media with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 1µg/ml Ionomycin. Following the restimulation, they were incubated with Monensin for 3 hours before cytokine analysis via flow cytometry.

Ex vivo *delivery of $P_{13}D_5$:anti-pPKCθ complex into hPBMCs subsequently transferred into NSG mice.* Animal protocols were approved by the Institutional Animal Care and use Committee of the University of Massachusetts Amherst. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were conditioned with 2 Gy of total body irradiation using a ^{137}Cs source then rested for 4–6 hours. hPBMCs were treated with DMSO or 3 µmol/l Rottlerin for 30 minutes or with $P_{13}D_5$:Anti-pPKCθ for 4 hours at 37°C. 10×10^6 treated hPBMCs in 150 µl of PBS were injected into mice via the tail vein. Body weight and disease symptoms were observed daily. Bone marrow, spleen, and peripheral blood were collected on day 17 to assess percent engraftment of hPBMCs (% positive human CD45 / (% positive human CD45 cells + % positive mouse CD45 cells)) and infiltration of human $CD4^+$ and $CD8^+$ T cells. Also, the human $CD4^+$ and $CD8^+$ T cells were analyzed for CD25, pPKCθ (Thr538), $NOTCH1^{IC}$, and T-BET expression.

Assessment of GvHD. Some mice were assigned to survival studies to assess whether treating hPBMCs, *ex vivo*, with $P_{13}D_5$:Anti-pPKCθ had a durable effect on the function of the transferred hPBMCs, *in vivo*. The severity of GvHD was assessed using a standardized scoring system, as previously described,[53] and which included five different criteria (weight loss, posture, activity, fur texture, and skin integrity). Mice were evaluated on daily basis and graded from zero (the least severe) to two (the most severe) for each criterion. Clinical score was generated by adding grades for five criteria. When a clinical score of eight was reached, mice were removed from the study and humanely euthanized. The day of removal from the study was recorded as the day of lethal GvHD induction.

Cytometric bead array. IL-2 and IFNγ cytokine levels in plasma collected from NSG mice were determined by using human IFNγ and IL-2 Flex Sets (BD Biosciences). Data were acquired using a BD LSR Fortessa Flow Cytometer (Becton Dickinson) and analyzed using FCAP Array Software v3 (Soft Flow, St. Louis Park, MN).

SUPPLEMENTARY MATERIAL

Figure S1. Synthesis and characterization of $P_{13}D_5$.
Figure S2. The presence of anti-pPKCθ and its effect on cellular viability.
Figure S3. CD69 expression in $P_{13}D_5$:Anti-pPKCθ-treated hPBMCs.
Figure S4. Reversibility of $P_{13}D_5$:Anti-pPKCθ treatment.
Figure S5. Effects of $P_{13}D_5$-only or Anti-pPKCθ-only treatment on hPBMCs.
Figure S6. Effects of $P_{13}D_5$:Anti-pPKCθ on total PKCθ expression.
Figure S7. Characterization of $CD4^+$T cells, treated ex vivo, and used to induce GvHD.

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AUTHOR CONTRIBUTIONS:

E.I.O., G.G.P., J.A.T., J.V., R.L., H.L.S., D.T.G., and A.S.B. performed experiments, collected and analyzed data. L.M.M. designed and supervised the experiments with contributions from GNT. EIO co-wrote the manuscript with critical input from G.G.P., J.A.T., and B.A.O., together with G.N.T. and L.M.M.

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