Novel Protein Transduction Domain Mimics as Nonviral Delivery Vectors for siRNA Targeting *NOTCH1* in Primary Human T cells

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RNA interference technology has recently been highlighted as a powerful research method as well as a potential therapeutic treatment for several diseases. However, the delivery of small interfering RNA (siRNA) into T cell lines and primary blood cells is exceedingly challenging, as they are resistant to transfection by conventional reagents. As a result, there is an unmet need for nonviral, efficient, and easily prepared carriers for siRNA delivery into hard-to-transfect cell types. Here, we report a novel system based on protein transduction domain mimics (PTDMs), generated by ring opening metathesis polymerization, for intracellular delivery of siRNA molecules. PTDM-based siRNA delivery induced efficient *NOTCH1* knockdown in Jurkat T cells and human peripheral blood mononuclear cells without any measured toxicity. Furthermore, delivering siRNA to *NOTCH1* in human peripheral blood cells modulated cell proliferation and differentiation of T cells into T_H1 cells.

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INTRODUCTION

The use of small interfering RNA (siRNA)[1–3] to study gene function in T cell lines and primary blood cells[4–6] has been limited due to lack of safe and effective delivery vehicles. The use of different tools, including electroporation,[7] chitosan based-polymers,[8] carbon-nanotubes,[9] and protein transduction domains (PTDs)[10,11] has been explored to introduce siRNA into hard-to-transfect cells. Each of these systems offers some benefits but generally they produce unsatisfactory results. As a result, there is a great need for easily prepared reagents that efficiently deliver siRNA into hard-to-transfect cells without inducing significant toxicity. In the present work, we report two ring opening metathesis polymerization based PTD mimics (PTDMs), which are inspired by the PTDs, also known as cell-penetrating peptides, used in the delivery of several different cargos into a variety of cell types.[12,13] There are two different approaches in PTD-based cargo delivery, the first one is attaching cargo to PTD with a covalent linkage and the second approach is the formation of stable noncovalent complexes between the PTD and cargo.[14] Especially, for siRNA delivery, the second approach is preferred in terms of simplicity, delivery efficiency, and cargo stability.[15]

PTDs used in siRNA delivery via noncovalent complexation generally have primary or secondary amphiphilic structures such as MPG,[16] CADY,[17] and Pep[18] peptides, to enhance both the stability of the complexes and their internalization properties. For instance, in the case of Pep-2 peptide which was designed to deliver DNA mimics (peptide nucleic acids), the alanine mapping was performed to determine the essential residues required to form stable complexes with nucleic acids and to improve their delivery into cells. The results showed that aromatic residues are required both for binding of the carrier to the cargo and for cellular uptake. In addition, it was highlighted that the cationic residues have more impact on internalization than cargo stabilization.[18] Moreover, in the siRNA delivery via PTDs, arginine sequences have been shown to be more effective than their lysine analogs.[19] Therefore, we have designed and studied two different PTDMs inspired by polyarginines and amphiphilic-peptides to deliver siRNA via noncovalent complexes into two hard-to-transfect cell types, Jurkat T cells and human peripheral blood mononuclear cells (PBMCs). PTDM-1 is a hydrophilic molecule designed as a mimic of the oligoarginine peptide having guanidinium functionalities along a polyoxanorbonene backbone (**Figure 1a**). PTDM-2 is a block-copolymer having both hydrophilic guanidinium and hydrophobic phenyl moieties on the same backbone (**Figure 1b**) and is inspired by the amphiphilic PTDs such as MPG and Pep peptides.

In order to determine the ability of PTDMs to deliver functional siRNA molecules, *NOTCH1* was chosen as a target in Jurkat T cells and PBMCs. *NOTCH1* is a member of the Notch transmembrane receptor family which constitutes important regulators of cell-fate decisions and cell survival in many systems during embryogenesis and postnatal development, including the immune system.[20,21] Moreover, *NOTCH1* has been shown to play an important role in the development and differentiation of peripheral T cells. Delivery efficiency of PTDMs is demonstrated in both serum-containing and serum-free conditions; knockdown of *NOTCH1* was analyzed

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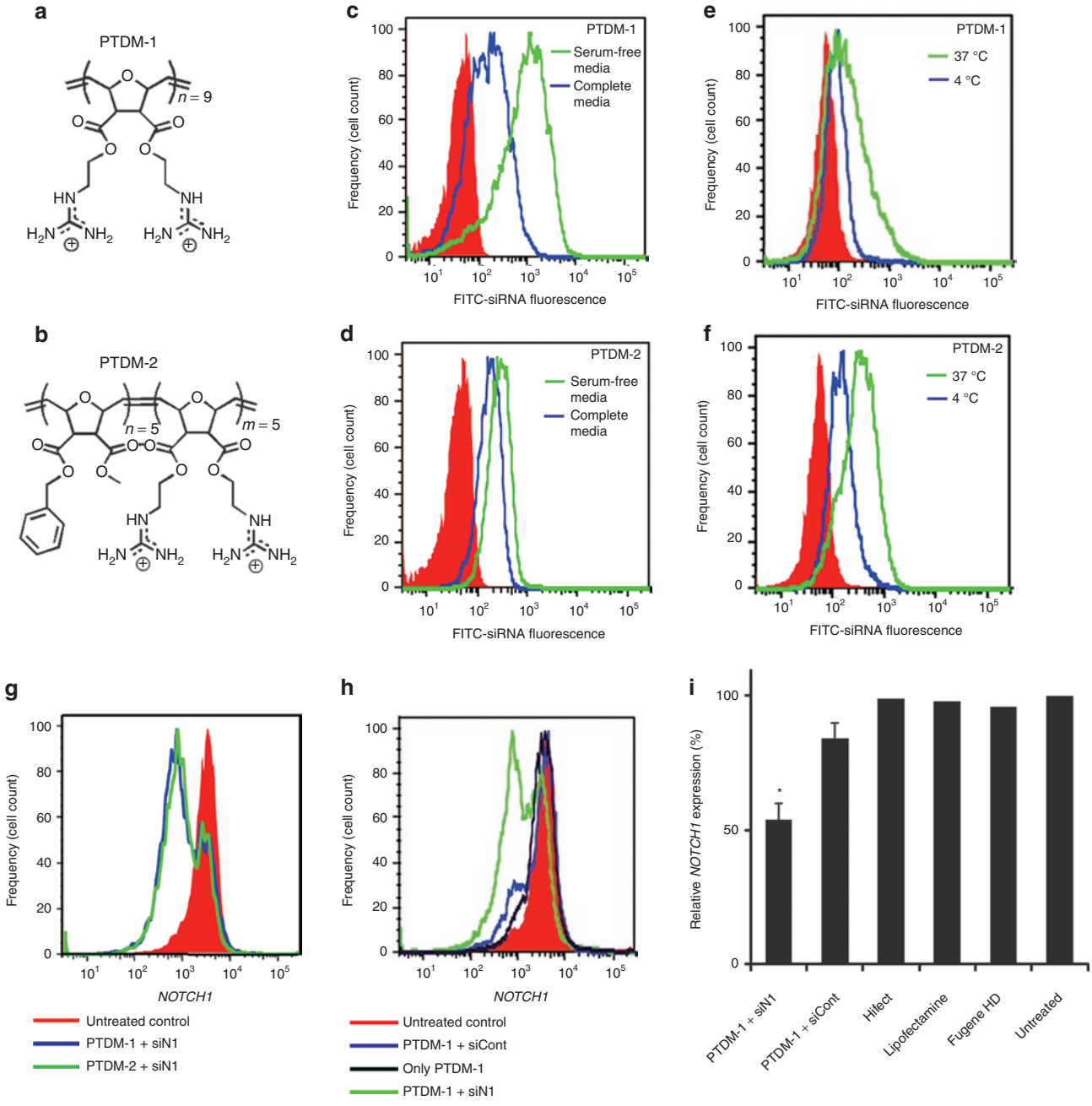

Figure 1 Small interfering RNA (siRNA) delivery into Jurkat T cells. (**a**) Chemical structure of protein transduction domain mimic (PTDM)-1, $n = 9$. (**b**) Chemical structure of PTDM-2, $n = m = 5$. (**c**) Flow cytometric analysis showing Jurkat T cells treated with PTDM-1(1 μmol/l)/fluorescein isothiocyanate (FITC)-siRNA (50 nmol/l) complexes in complete medium (blue curve) or serum-free medium (green curve) for 4 hours and as compared with untreated cells (red solid curve). (**d**) Flow cytometric analysis showing Jurkat T cells treated with PTDM-2 (1.7 μmol/l)/FITC-siRNA (50 nmol/l) complexes in complete medium (blue curve) or serum free medium (green curve) for 4 hours and as compared with untreated cells (red solid curve). (**e**) Flow cytometric analysis showing Jurkat T cells treated with PTDM-1(1 μmol/l)/FITC-siRNA (50 nmol/l) complexes in serum-free medium for 1 hour at 4 °C (blue curve) or 37 °C (green curve) and as compared with untreated cells (red solid curve). (**f**) Flow cytometric analysis showing Jurkat T cells treated with PTDM-2 (1.7 μmol/l)/FITC-siRNA (50 nmol/l) complexes in serum-free medium for 1 hour at 4 °C (blue curve) or 37 °C (green curve) and as compared with untreated cells (red solid curve). (**g**) Flow cytometric analysis showing Jurkat T cells stained with fluorescent phycoerythrin (PE)-anti Notch1, 72 hours after treatment with siRNA complexes; blue curve: cells treated with PTDM-1(2 μmol/l)/siN1(100 nmol/l) complexes, green curve: cells treated with PTDM-2 (3.5 μmol/l)/siN1 (100 nmol/l) complexes, red solid curve: untreated cells. (**h**) Flow cytometric analysis showing Jurkat T cells stained with fluorescent PE-anti NOTCH1, 72 hours after the siRNA treatment; red curve: untreated cells, blue curve: cells treated with PTDM-1 (1.6 μmol/l) /siCont (80 nmol/l) complexes, black curve: cells treated with only PTDM-1 (1.6 μmol/l), green curve: cells treated with PTDM-1(1.6 μmol/l)/siN1 (80 nmol/l) complexes. (**i**) Relative *NOTCH1* expression level in Jurkat T cells 72 hours after treatment with PTDM-1/siN1 complexes, PTDM-1/siCont complexes, Hifect/siN1, Lipofectamine 2000/siN1 and Fugene HD/siN1 as analyzed by flow cytometry. Cells were treated with siRNA-carrier complexes in serum free medium for 4 hours, then medium was exchanged with fresh complete growth medium (final siRNA concentration is 80 nmol/l). Values and error bars represent the mean ± SD of three independent experiments. *($P < 0.01$) of siN1 versus siCont delivered by PTDM-1.

at protein levels by a flow cytometry. Both PTDM-1 and PTDM-2 successfully delivered functional siRNA molecules into Jurkat T cells and PBMCs, and at least 50% knockdown was observed at or below siRNA concentrations of 100 nmol/l. Moreover, we have investigated the role of *NOTCH1* in cell proliferation and $CD4^+$ T cell differentiation under T_H1 polarization conditions. In activated PBMCs, *NOTCH1* knockdown caused cell growth inhibition and reduction in both T-bet and interferon (IFN)-γ production.

RESULTS AND DISCUSSION

Design and synthesis of PTDMs

We have designed PTDM-1 which is a synthetic mimic of oligoarginine (**Figure 1a**), and PTDM-2 as the mimic of amphiphilic-peptides (**Figure 1b**), such as MPG, and Pep-1. The Boc-protected guanidinium functionalized oxanorbornene monomer and the phenyl-functionalized oxanorbornene monomer were synthesized as described earlier (**Supplementary Figures S1** and **S2** online). PTDM-1 is a homopolymer of the guanidinium functionalized monomer polymerized by Grubb's third generation catalyst (**Supplementary Figures S3** and **S4** online). PTDM-2 is a 1:1 copolymer of both guanidinium and phenyl-functionalized monomers, also polymerized by Grubb's third generation catalyst. Both PTDM-1 and PTDM-2 were characterized by nuclear magnetic resonance and gel permeation chromatography. They have very narrow polydispersity indices, 1.08 and 1.06, respectively (**Supplementary Figure S5** online).

PTDM-1 and PTDM-2 deliver siRNA into Jurkat T cells

Initially, to examine the ability of PTDMs to deliver siRNA into Jurkat T cells, a fluorescein isothiocyanate (FITC)-conjugated siRNA molecule was mixed either with PTDM-1 or PTDM-2 and the resulting complexes were incubated with the cells either in serum-free or complete growth medium with 10% serum at 37 °C. After washing the cells thoroughly with heparin to remove any surface bound complex,[22] fluorescence intensity was measured by flow cytometry (**Figure 1c,d**). As shown in **Figure 1**, both PTDMs are able to deliver siRNA in serum-free medium. Moreover, PTDM-1 showed greater uptake than PTDM-2 in serum-free media; however, its delivery was inhibited in the presence of serum (**Figure 1c** and **Supplementary Figure S6** online). In the case of PTDM-2, there was no significant difference between serum-free and complete medium conditions (**Figure 1d** and **Supplementary Figure S6** online). This demonstrated the importance of aromatic groups on cargo stabilization and delivery properties of PTDM-2. Single populations and narrow peaks indicate that PTDMs target the entire cell population and nearly all cells contain approximately the same amount of siRNA.

In order to examine the route of cell entry, in addition to delivery experiments conducted at 37 °C, PTDM/siRNA complexes were incubated on the cells at 4 °C, a temperature at which most energy-dependent pathways are inhibited.[19] Internalization of PTDM/siRNA complexes at 4 °C also increases the likelihood of cytosolic delivery, the proper localization of which is central to their function. When Jurkat T cells were treated with PTDM-1/FITC-siRNA complexes at 4 °C, no significant delivery was observed (**Figure 1e** and **Supplementary Figure S7** online); however, PTDM-2 was able to deliver siRNA efficiently into the cells even at low temperature (**Figure 1f** and **Supplementary Figure S7** online).

NOTCH1 knockdown in Jurkat T cells

Experiments with FITC-tagged siRNA molecules demonstrated that both PTDMs delivered siRNA to the entire cell population with great efficiency; however, they do not show the availability of active siRNA molecules for gene silencing. To demonstrate the ability of PTDMs to deliver functional siRNA molecules, *NOTCH1* was chosen as a target in Jurkat T cells and human PBMCs. In order to evaluate the function of siRNA molecules, Jurkat T cells were treated with complexes of siRNA to *NOTCH1* (siN1) and either PTDM-1 or PTDM-2 in serum-free medium for 4 hours, then Notch1 protein expression was analyzed at 72 hours by flow cytometry after staining the intracellular domain of Notch1 with a fluorescently-labeled antibody specific for its cleaved, active form (**Figure 1g**). Flow cytometry is a powerful technique to determine both the percentages of cells undergoing gene silencing and the extent of protein knockdown in the cells of interest.[25] The cells were also treated with a scrambled, control siRNA (siCont) and/or only PTDM-1 as negative controls, to show that the decrease in *NOTCH1* protein levels is mediated specifically by siRNA to *NOTCH1* (**Figure 1h**). In addition, knockdown efficiencies of the PTDM-based system in Jurkat T cells were compared with the commercially-available cationic lipids, Lipofectamine 2000 (nvitrogen), Hifect (Lonza, Allendale, NJ), and Fugene HD (Promega, Madison, WI) (**Figure 1i**). There was no detectable silencing activity in the cells treated with cationic lipid-based formulations. This agrees with numerous reports in the literature documenting T cells as hard-to-transfect cell types.[4–6,22] By contrast, 50% knockdown of *NOTCH1* was observed in the cells treated with complexes of 80 nmol/l siN1 and 1.6 μmol/l PTDM-1. Efficient knockdown of *NOTCH1* protein was observed in Jurkat T cells treated either with PTDM-1/siN1 or PTDM-2/siN1 complexes. Although PTDM-2 showed better internalization at 4 °C than PTDM-1 (**Figure 1e,f**), both PTDMs performed with similar efficiencies to decrease *NOTCH1* protein levels (**Figure 1g**).

NOTCH1 knockdown in PBMCs

Primary cells present an even greater challenge to transfection using nonviral approaches, making the lack of efficient and sustained siRNA delivery into primary cells a major barrier to use of the RNA interference (RNAi) technique in a therapeutic setting.[4–6] In particular, primary blood cells are resistant to conventional transfection using cationic lipids and polymeric reagents. Therefore, we evaluated *NOTCH1* knockdown in human PBMCs using the PTDM-based siRNA delivery system. PBMCs were cultured the day before treatment in order to separate and work with the T cell-enriched lymphocytes. Stimulated T cells are known to be transfected more efficiently than unstimulated ones. In the experiments reported here, all transfections were performed on unstimulated T cells. These T cells were only stimulated post-transfection as required for *NOTCH1* activation. Initially, unstimulated PBMCs were treated with PTDM/siRNA complexes in serum-free media for 4 hours, after which time it was replaced with complete growth medium and the cells were activated for 72 hours to up-regulate *NOTCH1*. At the indicated time points, cells were harvested and

intracellular *NOTCH1* was stained as before then analyzed using flow cytometry. Both PTDM-1 and PTDM-2 were used to deliver 60 nmol/l siRNA to PBMCs isolated from the same donor and each PTDM demonstrated ~50% knockdown of *NOTCH1* protein levels in activated T cells (**Supplementary Figure S8a,b** online). It has been reported that the transient nature of the RNAi effect is more easily distinguished in activated T cells; maximum knockdown efficiency at the protein level approaches 20–30% and complete recovery of protein expression is seen by 48 hours.[24] In strong contrast, we observed a 50% decrease in *NOTCH1* protein expression, which

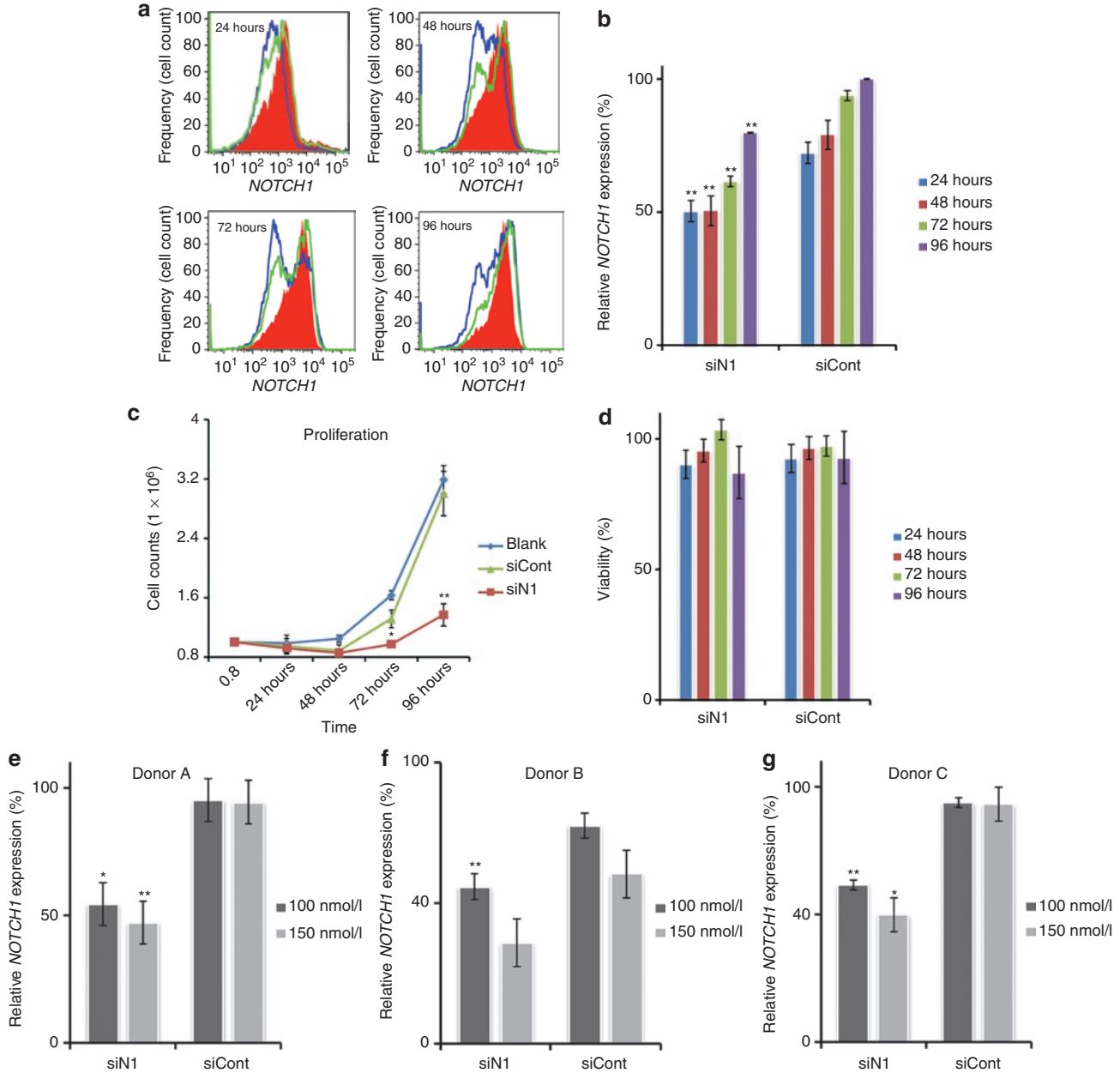

Figure 2 Knockdown of *NOTCH1* by protein transduction domain mimic (PTDM)-2/siN1. (**a**) Flow cytometric analysis showing peripheral blood mononuclear cells (PBMCs) stained with fluorescent phycoerythrin-anti Notch1, 24, 48, 72, or 96 hours after treatment with siRNA complexes; blue curve: cells treated with PTDM-2 (3.5 µmol/l)/siN1(100 nmol/l) complexes, green curve: cells treated with PTDM-2 (3.5 µmol/l)/siCont(100 nmol/l) complexes, red solid curve: untreated cells. (**b**) Relative *NOTCH1* expression levels in PBMCs 24, 48, 72, or 96 hours after treatment with PTDM-2 (3.5 µmol/l)/siN1(100 nmol/l) or PTDM-2 (3.5 µmol/l)/siCont(100 nmol/l) complexes. (**c**) Cell proliferation assay. Equal numbers of PBMCs were seeded and treated either with PTDM-2 (3.5 µmol/l)/siN1 (100 nmol/l) or PTDM-2 (3.5 µmol/l)/siCont(100 nmol/l) complexes. Cell proliferation was measured by cell counting with a hemacytometer at indicated time points. (**d**) 7-Amino-Actinomycin D (7-AAD) viability test. PTDM-2 (3.5 µmol/l)/siN1(100 nmol/l) or PTDM-2 (3.5 µmol/l)/siCont(100 nmol/l) treated cells were stained with 7-AAD at indicated time points after the treatment. (**e**–**g**) Relative *NOTCH1* expression level in PBMCs from three different donors (Donor A, B, and C) 72 hours after treatment with PTDM-2/siN1 and PTDM-2/siCont at a final siRNA concentration of 100 nmol/l or 150 nmol/l. Unstimulated cells were treated with PTDM/siRNA complexes in complete media for 4 hours, and then cells were transferred to anti-CD3/CD28 coated wells for stimulation. Protein level was analyzed by flow cytometry 72 hours after the treatment. Values and error bars represent the mean ± SD of three independent experiments. *($P < 0.05$) of siN1 versus siCont delivered by PTDM-2.**($P < 0.01$) of siN1 versus siCont delivered by PTDM-2.

was sustained even at 72 hours. This highlights the success of the PTDM-based siRNA delivery system in these hard-to-transfect cell types. Further experiments were performed with scrambled siRNA as a negative control and demonstrate a significant difference ($P < 0.01$) between siN1- and siCont-treated cells, confirming that *NOTCH1* knockdown is only mediated by siN1. In order to establish the universality of PTDMs use in human PBMCs, delivery efficiency of PTDM-2 was demonstrated in PBMCs from three different donors (**Supplementary Figure S8c–e** online). PTDM-2 showed similar knockdown efficiencies among the three different donors with a small, but expected, variance.

In addition, to examine the stability of PTDM/siRNA complexes and their ability to deliver functional siRNA molecules in the presence of serum, PBMCs were treated with PTDM/siRNA complexes in complete growth medium without further medium change. While both PTDM-1 and PTDM-2 performed efficiently under serum-free conditions; however, in the presence of serum, only PTDM-2 was able to successfully deliver functional siRNA into cells (**Figure 2**). For further analysis, PBMCs were treated with PTDM-2/siRNA complexes in complete medium to knockdown *NOTCH1*. *NOTCH1* protein levels were monitored for 4 days in cells treated either with PTDM-2/siN1 or with PTDM-2/siCont complexes (**Figure 2**). At 24 hours post-treatment, a 50% or greater decrease in *NOTCH1* protein was observed in cells treated with a complex of 100 nmol/l siRNA and 3.5 µmol/l PTDM-2; the RNAi response showed a slow decay beginning after 48 hours (**Figure 2a,b**). As reported earlier, it is known that *NOTCH1* plays an important role in cell-fate decisions.[21] Therefore, to determine whether knocking down *NOTCH1* expression by PTDM-delivered siRNA impacted PBMC cell growth, cell proliferation was analyzed (**Figure 2c**). We found that knocking down *NOTCH1* expression significantly inhibited cell growth of PBMCs, as compared with nontreated and scrambled siRNA-treated cells which were in their logarithmic growth phase by day 4. One of the major impediments to intracellular delivery into primary cells is the toxicity of the delivery tools. Generally, to achieve efficient knockdown, high concentrations of conventional reagents are required and this invariably results in reduced cell numbers or general cytotoxicity. In order to investigate the potential toxicity of PTDM-2/siRNA treatment, cells were stained with 7-Amino-Actinomycin D and analyzed by flow cytometry. Neither PTDM-2/siN1 nor PTDM-2/siCont treatments affected cell viability at the concentrations used (**Figure 2d**). Knockdown efficiency of PTDM-2/siRNA complexes was also examined using different concentrations of siRNA and among three different donors in the presence of serum (**Figure 2e–g**). Increasing the siRNA concentration from 100 nmol/l to 150 nmol/l did not significantly increase the efficiency of knockdown, and this was consistent among all three donors.

NOTCH1 protein levels were also determined by western blot analysis using Notch2 and Actin as controls (**Figure 3**). PBMCs were treated with either PTDM-2/siN1 or PTDM-2/siCont for 4 hours, and then stimulated on αCD3/αCD28 coated wells for 48 hours in order to activate *NOTCH1*. At 48 hours, cells were lysed and protein levels were analyzed. In all cases, two different antibodies for active NOTCH1 intracellular domain were used. Jurkat T cells (**Figure 3**, lane 1) and stimulated but untreated PBMCs (**Figure 3**, lane 3) were used as positive controls. They show *NOTCH1* expression as expected. Unstimulated and untreated PBMCs which do not contain active *NOTCH1* intracellular domain were used as a negative control (**Figure 3**, lane 2). Comparing these three controls to PBMCs treated with siCont (**Figure 3**, lane 4) or siN1 (**Figure 3**, lane 5) show that the PTDM mediates good knockdown of intracellular *NOTCH1*. This is consistent with the flow cytometric analysis shown in **Figure 2**. Another important factor to consider is specificity of the treatment. Although **Figure 2d** confirmed *NOTCH1* knockdown without cytotocity, it is important to understand whether NOTCH1 is selectively reduced, or in general, if there is a decrease in all protein levels. **Figure 3** also compares *NOTCH1* knockdown in the five experimental conditions to general protein levels (Actin) and to another Notch family protein (Notch2). It is clear from lanes 3–5 that *NOTCH1* can be specifically reduced with little impact on Actin or Notch2 (**Supplementary Figure S17** online).

Effect of *NOTCH1* knockdown on CD4$^+$ T cell differentiation

NOTCH1 has been shown to play an important role in the development and differentiation of peripheral T cells. Activated CD4$^+$ T cells can further differentiate into T helper type-1 (T_H1) or T_H2 cells. T_H1 and T_H2 cells produce specific cytokines during their terminal maturation. For instance, IFN-γ is one of the cytokines which is predominantly expressed by T_H1 cells and T-bet is the signature transcription factor of T_H1 cells.[24] RNAi holds great promise as a tool to analyze protein function within cells. Previously, the ability to study the function of *NOTCH1* in primary human T cells in a gene-specific manner was limited due to the lack of efficient and safe delivery tools. Having demonstrated the efficiency and low toxicity of the PTDM-based delivery system, we next sought to investigate the role of *NOTCH1* on human CD4$^+$ T cell differentiation under T_H1 polarization conditions.

To investigate the effect of *NOTCH1* expression on CD4$^+$ T cell differentiation under T_H1 polarization conditions, PBMCs were treated with PTDM-2/siN1 or PTDM-2/siCont for 4 hours,

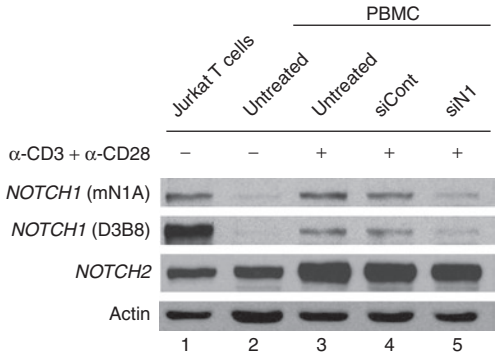

Figure 3 Western blot analysis showing the specific knockdown of *NOTCH1* by protein transduction domain mimics (PTDM)-2/siN1. Unstimulated cells left untreated (lane 3) treated with PTDM-2/siN1 complexes (siN1) (lane 5) or PTDM-2/siCont complexes (siCont) (lane 4) for 4 hours, and then stimulated with plate-bound anti-CD3 plus anti-CD28 for 48 hours. Total protein lysates were immunoblotted with antibodies that detect the active *NOTCH1* intracellular domain, Notch2, and Actin. Jurkat T cells (lane 1), and untreated/unstimulated PBMC (lane 2) were included as positive and negative controls of the active *NOTCH1* intracellular domain, respectively.

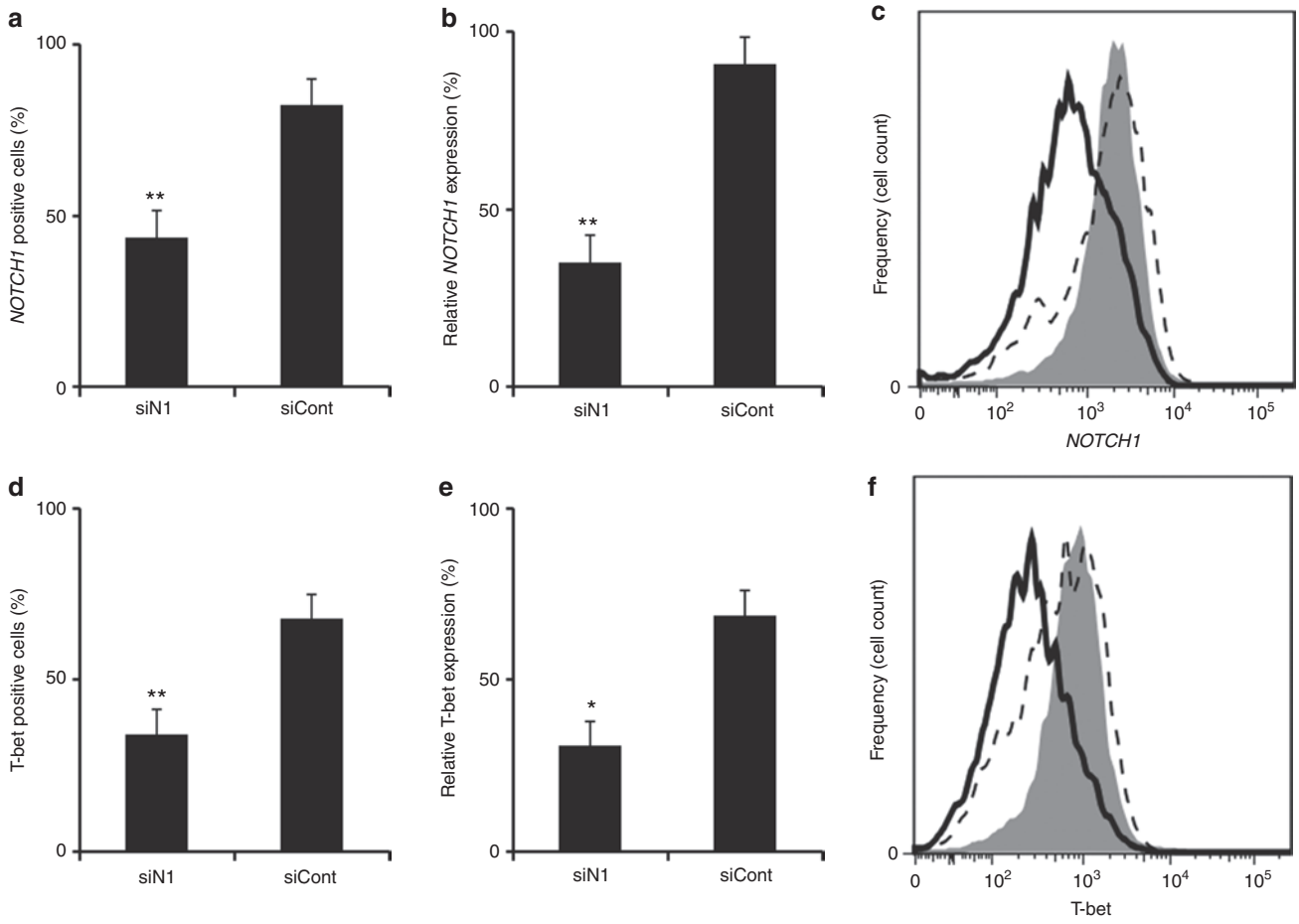

Figure 4 Effect of *NOTCH1* knockdown by Small interfering RNA (siRNA) on CD4+ T cell differentiation at 72 hours. (**a**) Percentage of NOTCH1-expressing, T_H1-polarized $CD4^+$ T cells 72 hours after treatment with protein transduction domain mimics (PTDM)-2/siN1 or PTDM-2/siCont. (**b**) Relative *NOTCH1* expression in T_H1-polarized $CD4^+$ T cells 72 hours after treatment with PTDM-2/siN1 or PTDM-2/siCont. (**c**) Flow cytometric analysis showing T_H1-polarized $CD4^+$ T cells stained with fluorescent phycoerythrin-anti *NOTCH1* 72 hours after treatment with siRNA complexes; blue curve: PTDM-2/siN1 treated cells, green curve: PTDM-2/siCont, red solid curve: untreated cells. (**d**) Percentage of T-bet-expressing T_H1-polarized $CD4^+$ T cells 72 hours after treatment with PTDM-2/siN1 or PTDM-2/siCont. (**e**) Relative T-bet expression in T_H1-polarized $CD4^+$ T cells 72 hours after treatment with PTDM-2/siN1 or PTDM-2/siCont. (**f**) Flow cytometric analysis showing T_H1-polarized $CD4^+$ T cells stained with fluorescent Alexa Fluor 647 anti-T-bet 72 hours after treatment with siRNA complexes; blue curve: PTDM-2/siN1 treated cells, green curve: PTDM-2/siCont, red solid curve: untreated cells. Values and error bars represent the mean ± SD of three independent experiments. *($P < 0.05$) of siN1 versus siCont delivered by PTDM-2. **($P < 0.01$) of siN1 versus siCont delivered by PTDM-2.

then cells were polarized by culturing with interleukin-12 (IL-12) and anti-interleukin-4 (IL-4 mAb), conditions which favor T_H1 differentiation. At 48 (**Supplementary Figure S9–S16** online) or 72 hours after polarization (**Figures 4** and **5**), $CD4^+$T cells were identified according to their reactivity to anti-CD4 monoclonal antibody and, within this subset, *NOTCH1* and T-bet expression were also analyzed. In addition, at 48- or 72-hour time points, the cells were restimulated in the presence of Brefeldin A and stained for IFN-γ, a signature cytokine of T_H1-polarized cells.

At 48 hours, siN1-mediated knockdown of *NOTCH1* concomitantly inhibited the expression of T-bet in $CD4^+$ T cells (**Supplementary Figure S9d–f** online). In addition, *NOTCH1* knockdown reduced IFN-γ production, as compared with untreated and siCont-treated cells (**Supplementary Figure S9j–k** online). However, at the 48-hour time point there was a low level of IFN-γ production in the untreated cells as well (**Supplementary Figure S9g–i** online). In order to analyze the magnitude of IFN-γ reduction more accurately, the cells were harvested at 72 hours, a time at which more IFN-γ production was observed in the control groups (untreated and siCont-treated cells) in accordance with their higher expression of *NOTCH1* (**Figure 4a–c**) and T-bet (**Figure 4d–f**). Even at 72 hours there was a significant and sustained reduction in IFN-γ levels in siN1-treated cells as compared with control groups (**Figure 5**).

Conclusions

One of the major limitations to using RNA interference to study unknown gene function in primary cells is the inefficient delivery of the siRNA. In the current work, we introduced a new system based on novel PTDMs which achieves efficient delivery of siRNA into primary PBMCs with no observable cellular toxicity at the concentrations used. PTDM-1, which is a mimic of polyarginine, successfully delivered functional siRNA molecules into hard-to-transfect cell types, Jurkat T cells and PBMCs, even though it has been reported

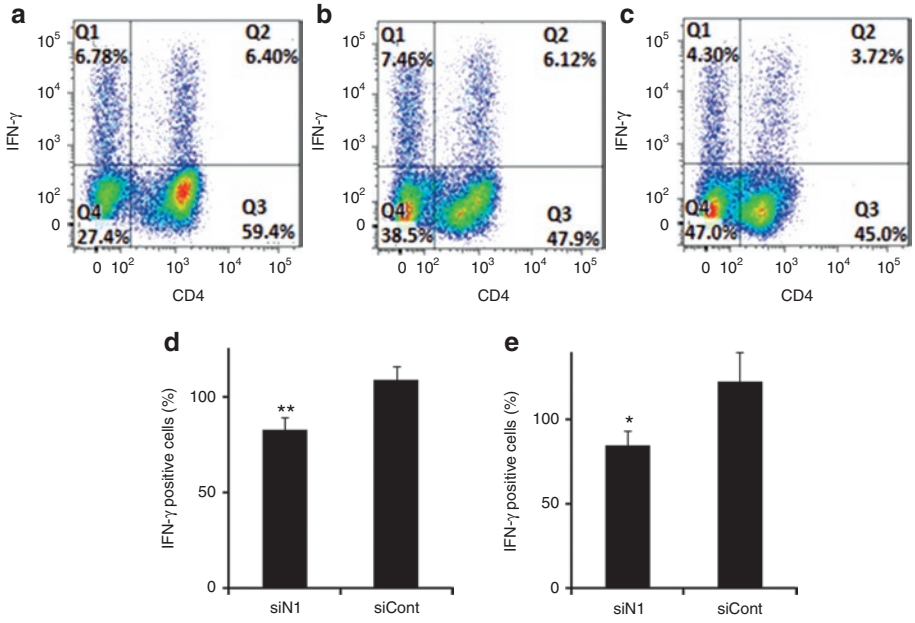

Figure 5 Flow cytometric analysis showing T_H1-polarized CD4+ T cells stained with fluorescent APC-anti interferon (IFN)-γ 72 hours after treatment with siRNA complexes. (**a**) Untreated cells, (**b**) protein transduction domain mimics (PTDM)-2/siCont treated cells, (**c**) PTDM-2/siN1 treated cells. (**d**) Percentage of IFN-γ- expressing T_H1-polarized $CD4^+$ T cells 72 hours after treatment with PTDM-2/siN1 or PTDM-2/siCont. (**e**) Relative IFN-γ expression in T_H1-polarized $CD4^+$ T cells 72 hours after treatment with PTDM-2/siN1 or PTDM-2/siCont. Values and error bars represent the mean ± SD of three independent experiments. *($P < 0.05$) of siN1 versus siCont delivered by PTDM-2. **($P < 0.01$) of siN1 versus siCont delivered by PTDM-2.

that homopolymers of arginines are not able to deliver siRNA via non-covalent complexation.[26,27] Furthermore, in order to test the effect of hydrophobicity in addition to arginine functionalities on the carrier efficiency, PTDM-2 was generated using hydrophobic phenyl and hydrophilic guanidinium functionalities. There was no significant difference on delivery efficiencies of the PTDM-1 and PTDM-2 in the absence of serum. On the other hand, PTDM-2 showed superior efficiency in the presence of serum. This demonstrates that the introduction of hydrophobic groups in the structure of PTDMs improved their delivery ability,[28,29] making them better candidates for delivery. To validate the biological outcomes of this delivery system, knockdown of *NOTCH1* in Jurkat T cells and primary human PBMCs was chosen as a model system. *NOTCH1* is known to contribute to T cell development and differentiation. Therefore, important functions for *NOTCH1* in proliferation and differentiation of human peripheral T cells were successfully confirmed by silencing *NOTCH1* using a novel PTDM-based siRNA delivery system.

MATERIALS AND METHODS

Synthesis of PTDMs. We have designed PTDM-1 which is a synthetic mimic of oligoarginine, and PTDM-2 as the mimic of amphiphilic-peptides. Boc-protected guanidinium functionalized oxanorbornene monomer and phenyl-functionalized oxanorbornene monomer were synthesized as described earlier (**Supplementary Materials and Methods** online). PTDM-1 is a homopolymer of guanidinium functionalized monomer polymerized by Grubb's third generation catalyst (**Supplementary Figures S3** and **S4 online**). PTDM-2 is a 1:1 copolymer of both guanidinium and phenyl-functionalized monomers, also polymerized by Grubb's third generation catalyst. Both PTDM-1 and PTDM-2 characterized by nuclear magnetic resonance and gel permeation chromatography having very narrow polydispersity indices, 1.08 and 1.06, respectively (**Supplementary Materials and Methods** online).

Cell culture conditions. Jurkat T cells (E6.1 clone, ATCC) were cultured in complete RPMI (RPMI 1640 + glutamax I supplemented with 10% fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, non-essential amino acids, sodium pyruvate, and penicillin/streptomycin).

PTDM/FITC-siRNA delivery into Jurkat T cells. 50 nmol/l FITC-labeled siRNA (sc-36869, Santa Cruz Biotechnology, Santa Cruz, CA) and 1.0 μmol/l PTDM-1 or 1.7 μmol/l PTDM-2 were mixed in 100 μl serum-free RPMI 1640 media and incubated for 30 minutes at room temperature for complex formation. Jurkat T cells were counted and plated in 12-well plate at 400,000 cells/well in 900 μl either serum-free RPMI 1640 or RPMI 1640 media with 10% fetal calf serum. Complexes were added onto the cells dropwise and incubated for 4 hours at 37 °C. At the end of 4 hours cells were harvested, and washed three times with heparin (20 U/ml heparin in phosphate buffered saline), and resuspended in 500 μl ice-cold phosphate buffered saline containing 0.2% bovine serum albumin and 1 mmol/l ethylenediaminetetraacetic acid then analyzed by flow cytometry using an LSR-II (BectonDickinson, Franklin Lakes, NJ). Cell-associated fluorophores were excited at 488 nm, and fluorescence was measured at 530 nm. The fluorescence signal was collected for 10,000 cells, and viable cells were gated on to obtain a histogram of fluorescence intensity per cell.

Delivery of siRNA to* NOTCH1 *into Jurkat T cells. 80–100 nmol/l siRNA to *NOTCH1* (sc-36095, Santa Cruz Biotech) or negative control siRNA (Stealth RNAi™ siRNA medium GC, 12935-300, Invitrogen, Carlsbad, CA) and 1.6–2.0 μmol/l PTDM-1 or 3.5 μmol/l PTDM-2 were mixed in 200 μl serum-free RPMI 1640 medium and incubated for 30 minutes at room temperature for complex formation. Jurkat T cells were counted and plated in 12-well plates at 400,000 cells/well in 800 μl of serum-free RPMI 1640. Complexes were added onto the cells dropwise and incubated for 4 hours at 37 °C. At the end of 4 hours, 1 ml of complete medium with 20% fetal calf serum was added into each well and cells were incubated for an additional 20 hours at 37 °C. Next day, cells were spun down and resuspended in fresh growth medium with 10% fetal calf serum, and incubated 72 hours in total before analyzing *NOTCH1* protein expression.

Delivery of siRNA to* NOTCH1 *into human PBMCs. Human PBMCs from healthy donors were purchased from StemCell Technologies (Vancouver, BC). The cells were cultured overnight in complete RPMI (RPMI 1640 + glutamax I supplemented with 10% fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin). On the day of the experiment, only cells in suspension were harvested, counted and used in the experiment. 50–150 nmol/l siRNA to *NOTCH1* (sc-36095, Santa Cruz Biotech) or negative control siRNA (Stealth RNAi siRNA medium GC, 12935-300, Invitrogen) and 1.0–2.0 μmol/l PTDM-1 or 1.9–3.5 μmol/l PTDM-2 were mixed in 100 μl serum-free RPMI 1640 and incubated for 30 minutes at room temperature for complex formation.

Serum-free medium incubation. Suspension populations of PBMCs were counted and plated in 24-well plates at a density of 10^6 cells/well in 900 μl of serum-free RPMI 1640. Complexes were added onto the cells dropwise and incubated for 4 hours at 37 °C. After 4 hours, serum-free medium was replaced with fresh, complete medium and human PBMCs were stimulated with plate-bound anti-CD3ε (UCHT1, 5 μg/ml) and anti-CD28 (37407, 2.5 μg/ml).

Complete medium incubation. Suspension populations of PBMCs were counted and plated in 24-well plates at a density of 10^6 cells/well in 900 μl of complete RPMI medium with 10% fetal bovine serum. Complexes were added onto the cells dropwise and incubated for 4 hours at 37 °C. After 4 hours, human PBMCs were stimulated with plate-bound anti-CD3ε (UCHT1, 5 μg/ml) and anti-CD28 (37407, 2.5 μg/ml).

Culture of CD4+ T cells under TH1 polarization conditions. Suspension populations of PBMCs were counted and plated in 24-well plates at 10^6 cells/well in 900 μl of complete RPMI medium with 10% fetal bovine serum. Complexes were added onto the cells dropwise and incubated for 4 hours at 37 °C. After 4 hours, human PBMCs were stimulated with plate-bound anti-CD3ε (UCHT1, 5 μg/ml) and anti-CD28 mAb (37407, 2.5 μg/ml) for 48–72 hours in the presence of IL-12 (10 ng/ml) and anti-IL-4 (clone 3007, 10 μg/ml) to polarize cells towards a T_H1 phenotype (all cytokines and antibodies from R&D Systems, Minneapolis, MN).

Intracellular* NOTCH1 *and T-bet staining. Cells were harvested and washed twice with CBE (phosphate buffered saline containing 0.2% bovine serum albumin and 1 mmol/l ethylenediaminetetraacetic acid). Surface staining was performed with PerCP-Cy5.5-conjugated anti-CD4 (RPA-T4, eBioscience, San Diego, CA). The cells were fixed, permeabilized (Foxp3 staining buffer set-eBiosciences) and stained with phycoerythrin-conjugated anti-mouse/human *NOTCH1* (mN1A) and eFlour 660-conjugated anti-mouse/human T-bet (4B10). After staining, the cells were analyzed by flow cytometry using an LSR-II (Becton Dickinson) and the acquisition software BD FACSDiva (Becton Dickinson).

Intracellular cytokine staining. For IFN-γ production, PBMCs were stimulated for 48–72 hours, lifted and counted then restimulated for 6 hours with plate-bound anti-CD3ε(UCHT1, 5 μg/ml) and anti-CD28 (37407, 2.5 μg/ml), with brefeldin A (GolgiPlug, BD Pharmingen, San Diego, CA) being added after 2 hours. Surface staining was performed with FITC- or PerCP-Cy5.5-conjugated anti-CD4 (RPA-T4, eBioscience). Cells were then fixed, permeabilized (Cytofix/Cytoperm, BD Pharmingen) and stained with APC-conjugated anti-IFNγ (clone 45-15, Miltenyi Biotec, Bergisch Glad Bach, Germany). Samples were analyzed using an LSR-II flow cytometer and the acquisition software BD FACSDiva (Becton Dickinson). Additional data analysis was performed using FlowJo software, version 7.6.1 (Tree Star, Ashland, OR).

Western blot analysis. Total protein lysates (40 μg) were resolved on a 8% SDS-PAGE, transferred to nitrocellulose, and probed with an anti-cleaved Notch1 mAb (mN1A, eBiosciences), an anti-cleaved Notch1 Val1744 mAb (D3B8, Cell Signaling Technology, Danvers, MA), an anti-Notch2 mAb (D76A6, Cell Signaling Technology), and an anti-Actin mAb (AC-40, Sigma-Aldrich, St Louis, MO). The primary antibodies were detected with horseradish peroxidase-conjugated Ab (Amhersham, Piscataway, NJ) and enhance chemiluminescence (Pierce, Protein Biology Products, Rockford, IL).

Data Analysis. Relative NOTCH1 expression was calculated by multiplying the percentage of *NOTCH1* positive cells and the geometric mean fluorescence intensity of the gated cells. Then data was normalized to the untreated cells to obtain the relative percent expression of *NOTCH1*.

Statistical analysis. The results are expressed as means ± SD. Unpaired two-tailed Student's *t*-test using GraphPad Prism 5.0 was used to determine *P* values for statistical significance.

SUPPLEMENTARY MATERIAL

Figure S1. Synthesis of monomer 2.
Figure S2. Synthesis of monomer **3b.**
Figure S3. Synthesis of polymer **4b**.
Figure S4. Synthesis of Polymer **5b**.
Figure S5. GPC IR-trace of first (red curve) and second (black curve) blocks of Polymer **5a.**
Figure S6. Dot plots showing Jurkat T cells treated with PTDM/FITC-siRNA complexes in serum-free media (SFM) or complete media (CM).
Figure S7. Dot plots showing Jurkat T cells treated with PTDM/FITC-siRNA complexes at 4 °C or 37 °C.
Figure S8. Relative NOTCH1 expression level in PBMCs.
Figure S9. Effect of *NOTCH1* down-regulation by siRNA on $CD4^+$ T differentiation at 48 hours.
Figure S10. Untreated human PBMCs were polarized under T_H1 conditions for 48 hours.
Figure S11. PTDM-2/siN1-treated human PBMCs were polarized under T_H1 conditions for 48 hours.
Figure S12. PTDM-2/siCont-treated human PBMCs were polarized under T_H1 conditions for 48 hours.
Figure S13. Untreated human PBMCs were polarized under T_H1 conditions for 72 hours.
Figure S14. PTDM-2/siN1-treated human PBMCs were polarized under T_H1 conditions for 72 hours.
Figure S15. PTDM-2/siCont-treated human PBMCs were polarized under T_H1 conditions for 72 hours.
Figure S16. Flow cytometric analysis showing NOTCH1 knockdown in both PBMCs and CD4 T cells.
Figure S17. Flow cytometric analysis showing PTDM-2/siN1 treatment specifically silenced Notch1 and did not affect Notch2 levels.
Materials and Methods.

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