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Engineered Th17 cell differentiation using a photo-activatable immune modulator

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.0c07485 • Publication Date (Web): 25 Sep 2020

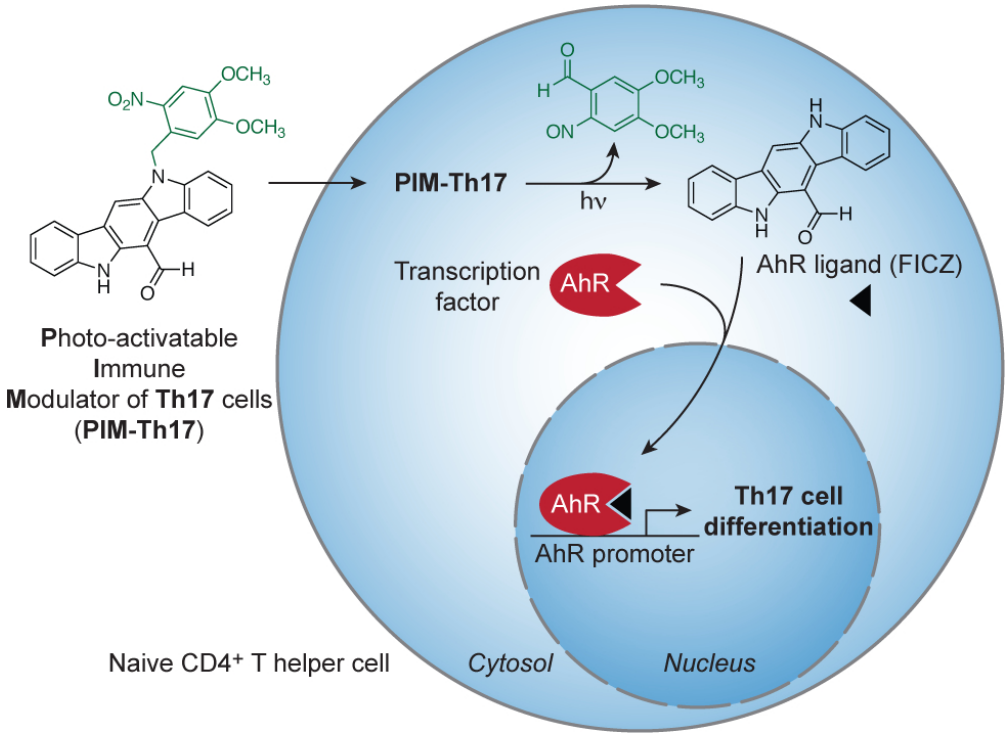


Figure 1. Schematic of synthetic immunology approach for inducing T helper 17 (Th17) cell differentiation by activation of the aryl hydrocarbon receptor (AhR) pathway. UV light illumination of a photocaged AhR ligand, Photo-activatable Immune Modulator of Th17 cells (PIM-Th17), releases the AhR ligand, FICZ, which binds AhR. Ligand binding activates nuclear translocation and induction of Th17 cell differentiation.

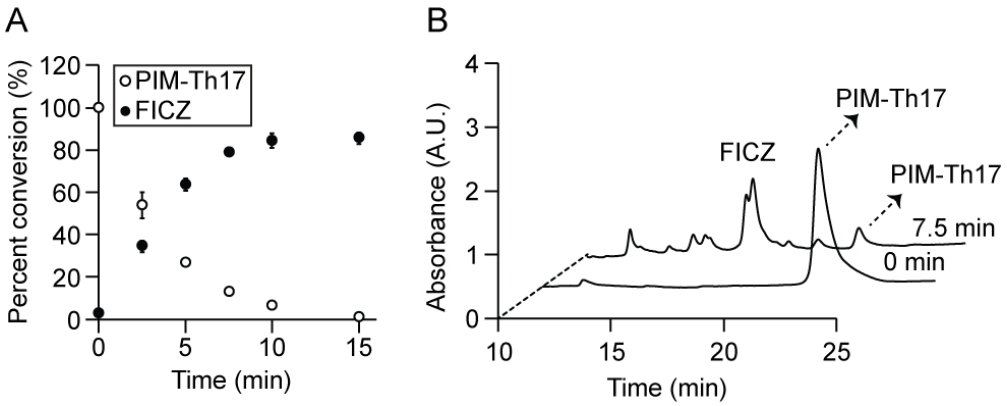


Figure 2. PIM-Th17 is photo-uncaged by UV light. (A) Percent conversion of PIM-Th17 to FICZ after illumination with 365 nm light. (B) HPLC analysis of photo-uncaging reactions. Traces shown were monitored by UV absorbance at 254 nm. A.U. = arbitrary units.

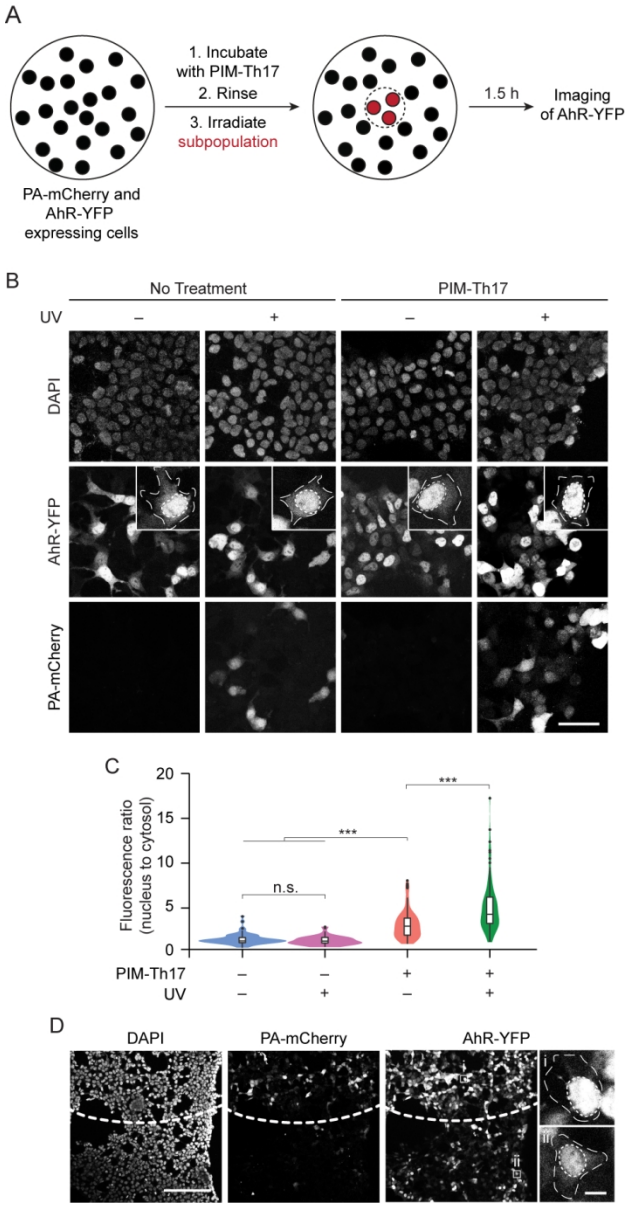


Figure 3. Photo-uncaging of PIM-Th17 is spatially selective and leads to nuclear translocation of AhR. HEK 293T cells co-transfected with AhR-YFP and PA-mCherry were pre-treated with PIM-Th17 (10 μM) or vehicle (DMSO) for 30 min, and the center of the dish was illuminated with UV light (epifluorescence using DAPI filter, 30 s). Cells were subsequently incubated for an additional 1.5 h, fixed, stained with DAPI, and imaged. (A) Schematic of experimental setup. (B) Maximum intensity z-projection images of cells at the periphery (– UV) or center (+ UV) of the same dish, imaged by confocal microscopy. Representative individual cells are shown at higher magnification (3.85X) in inset images. (C) Violin plots of nuclear to cytosolic ratio of the AhR-YFP fluorescence from (B), n = 50–70 cells in each treatment group. Box plot inside the violin plot: Interquartile ranges (IQRs, boxes), median values (line within box), whiskers (lowest and highest values within 1.5 times IQR from the first and third quartiles), and outliers beyond whiskers (dots) are indicated. Statistical significance was assessed using one-way ANOVA followed by post-hoc Tukey's test. n.s. = not significant, ***p < 0.001. (D) Maximum intensity z-projection images of cells at the border of UV illumination. Dashed curve indicates border region of illumination (+ UV, top; – UV, bottom), and representative individual cells are indicated by white squares and shown at higher magnification at

right. Scale bars: 50 μm (B and D, full-size images), 10 μm (D, magnified images).

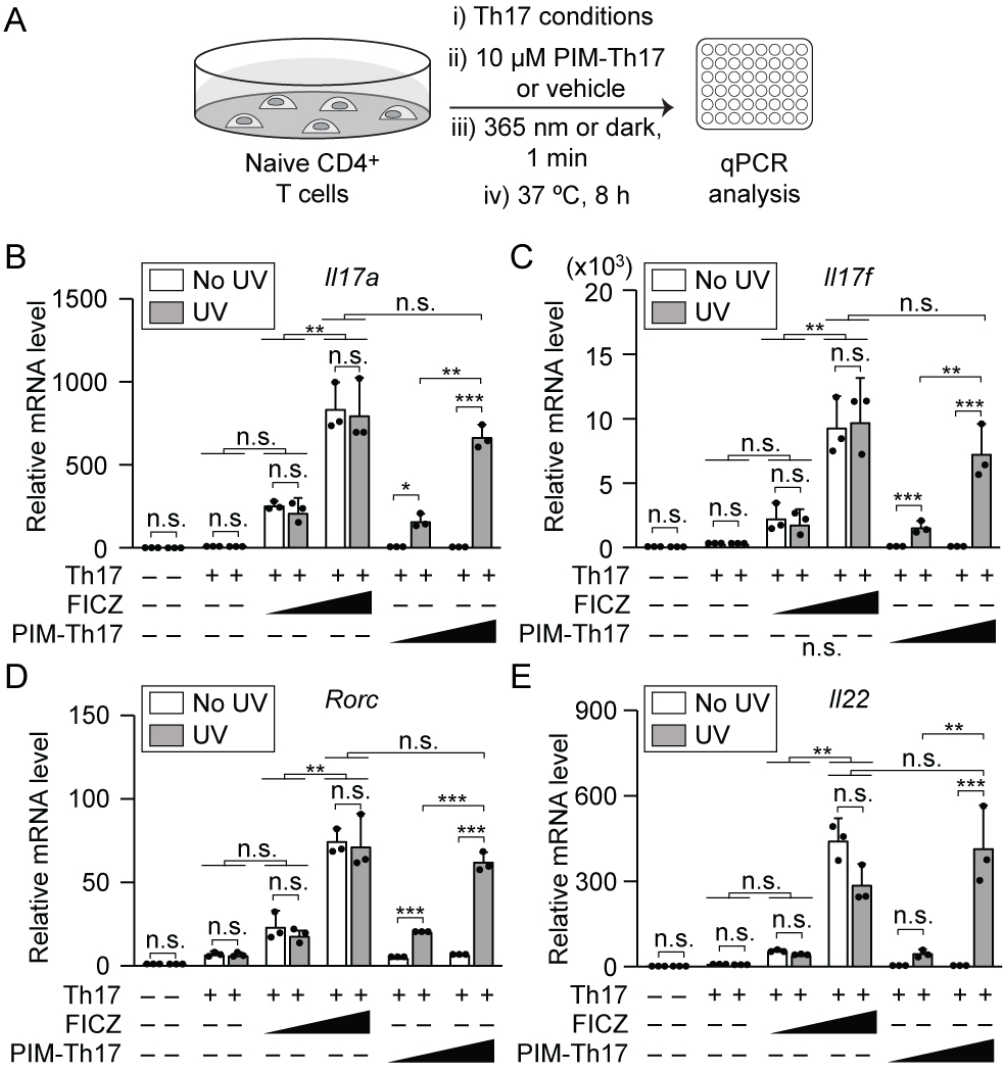
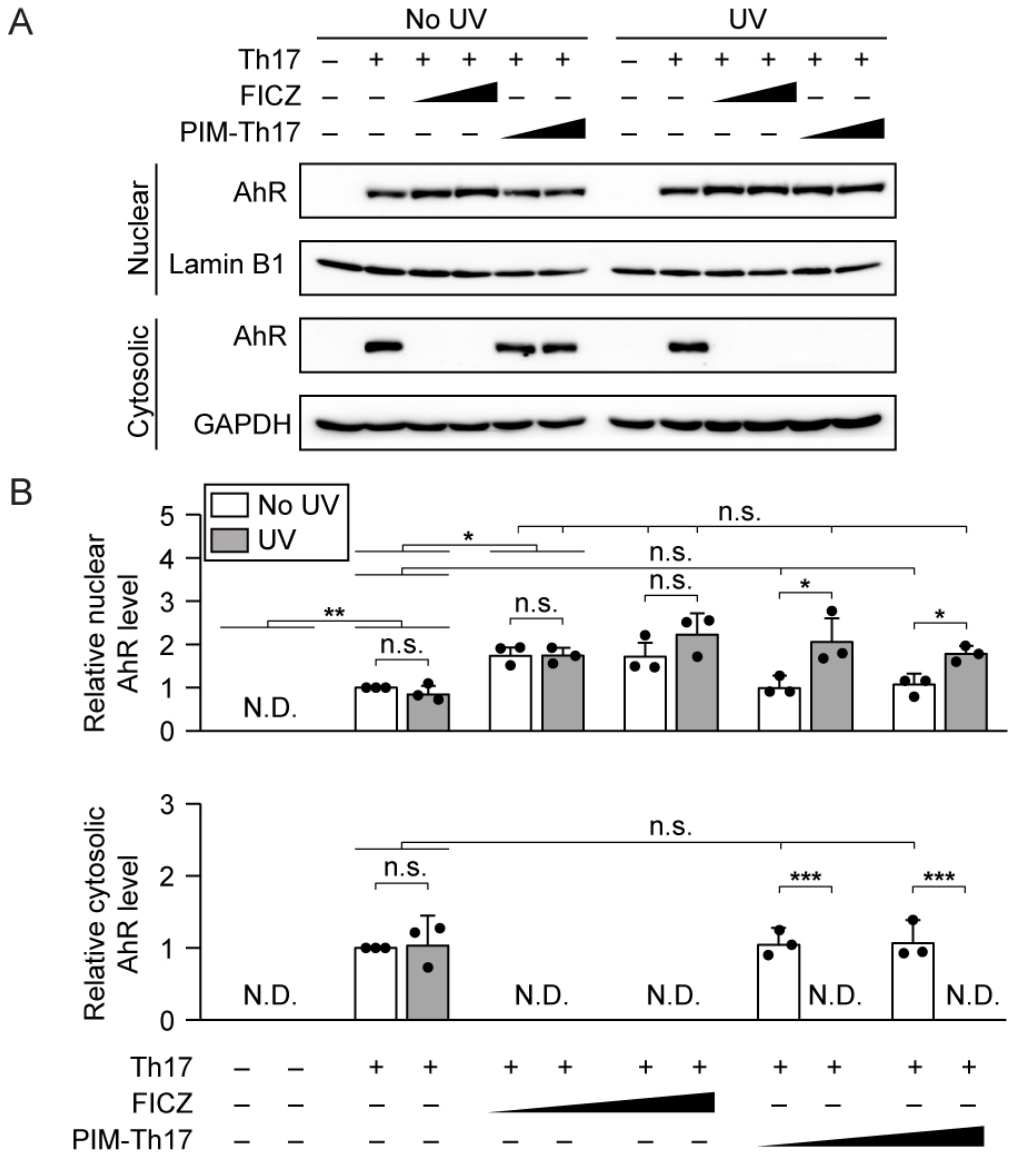


Figure 4. Photo-uncaging of PIM-Th17 leads to increased tran-scription of AhR target genes in Th17 cells. Splenic naïve CD4+ T cells were harvested from wild-type mice and cultured with or without Th17-inducing factors (Th17). After 4 d of differentiation, PIM-Th17 (1 μM, 10 μM), FICZ (300 nM, 2 μM), or vehicle (DMSO) was added, and the cells were illumi-nated with UV light (365 nm, 1 min, grey bars) or kept in the dark (white bars). RNA was isolated after 8 h, and cDNA was synthesized and analyzed by qPCR (data normalized to Rpl13a). (A) Schematic of experimental setup. Relative mRNA transcript levels of (B) Il17a, (C) Il17f, (D) Rorc, and (E) Il22 are shown. Error bars represent mean ± SD. Statistical signifi-cance was assessed using one-way ANOVA followed by post-hoc Tukey's test. n.s. = not significant, *p<0.05, **p<0.01, ***p < 0.001.



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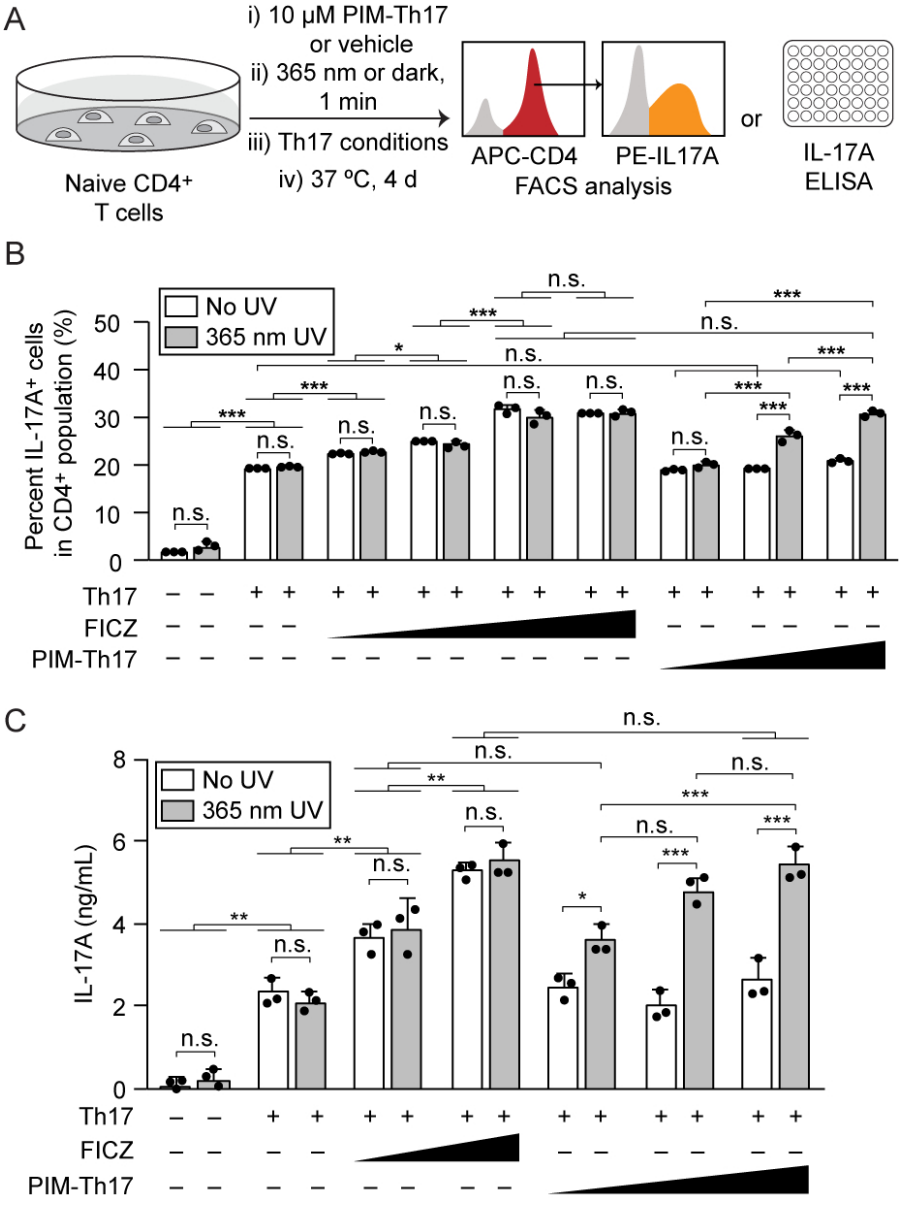

Figure 6. Photo-uncaging of PIM-Th17 increases differentia-tion of Th17 cells from naïve CD4+ T cells. (A-C) Splenic na-ïve CD4+ T cells were harvested from wild-type mice and cul-tured with or without Th17-inducing factors (Th17) in the presence of PIM-Th17 (1☐M, 5 ☐M, 10 μM), (B) FICZ (300 nM, 500 nM, 1 ☐M, 2 ☐M), (C) FICZ (300 nM, 2 ☐M), or vehicle (DMSO). The cells were illuminated with UV light (365 nm, 1 min) or kept in the dark, and then incubated at 37 ☐C for 4 d. On day 4, cells were stained with allophycocyanin (APC) anti-CD4 and phycoerythrin (PE) anti-IL17A and analyzed by FACS. Alternatively, the cell supernatants were analyzed by ELISA. (A) Schematic of experimental setup. (B) Quantifica-tion of percent CD4+IL-17+ cells by FACS analysis. (C) Quan-tification of IL-17A by ELISA. Error bars represent mean ± SD. Statistical significance was assessed using one-way ANOVA followed by post-hoc Tukey's test. n.s. = not signifi-cant, *p<0.05, **p<0.01, ***p < 0.001.

Engineered Th17 cell differentiation using a photo-activatable immune modulator

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ABSTRACT: T helper 17 (Th17) cells, an important subset of $CD4^+$ T cells, help to eliminate extracellular infectious pathogens that have invaded our tissues. Despite the critical roles of Th17 cells in immunity, how the immune system regulates the production and maintenance of this cell type remains poorly understood. In particular, the plasticity of these cells, or their dynamic ability to trans-differentiate into other $CD4^+$ T cell subsets, remains mostly uncharacterized. Here, we report a synthetic immunology approach using a photo-activatable immune modulator (PIM) to increase Th17 cell differentiation on demand with spatial and temporal precision to help elucidate this important and dynamic process. In this chemical strategy, we developed a latent agonist that, upon photochemical activation, releases a small-molecule ligand that targets the aryl hydrocarbon receptor (AhR) and ultimately induces Th17 cell differentiation. We used this chemical tool to control AhR activation with spatiotemporal precision within cells and to modulate Th17 cell differentiation on demand by using UV light illumination. We envision that this approach will enable an understanding of the dynamic functions and behaviors of Th17 cells in vivo during immune responses and in mouse models of inflammatory disease.

INTRODUCTION

Synthetic immunology harnesses the ability to manipulate immune cells using rational design of synthetic systems to either understand their functions or treat inflammatory diseases[1–4]. The human immune system represents an incredibly complex consortium of cell types that orchestrates immune responses to foreign invaders, including pathogenic microbes. Upon sensing these microorganisms, the host immune system initiates many signaling pathways to activate different immune cell types to help clear the infection[5,6]. This multifaceted process involves both spatial and temporal positioning of a network of cells to facilitate information exchange. Such cell-cell interactions ensure an effective response because the various cell types communicate with one another through the expression of key molecules that are either secreted or displayed on their cell surfaces. Due to the sheer number of cells and cell types that are involved in the immune response, understanding the precise roles of each cell type in this highly dynamic and interactive process is a challenging task. Thus, new strategies are needed to control the host immune response to help elucidate the functions of various immune cells and the inflammatory pathways that they activate in vivo.

T helper 17 (Th17) cells are a subset of $CD4^+$ T cells whose main roles are to assist in the eradication of extracellular pathogens that are typically associated with mucosal surfaces[7]. When left unchecked by the immune system, these pro-inflammatory $CD4^+$ T helper cells can contribute to allergy and autoimmune diseases due to increased production of pro-inflammatory cytokines such as IL-17. The differentiation of naïve T cells into Th17 cells depends on the presence of specific cytokines secreted by activated antigen-presenting cells, including IL-6 and TGF-β, and key transcription factors that define the Th17 lineage are the retinoic acid receptor-related orphan receptors (ROR)-gamma (RORγ) and RORα[8,9].

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor whose classic roles include activation of the host xenobiotic response to eliminate noxious chemicals from the body[10]. The main target genes of AhR include those that encode cytochrome P450 enzymes, which oxidize these foreign compounds into metabolites that can be more easily excreted by the host[11]. In recent years, immunologists have discovered that AhR also plays a major role in the regulation of numerous cell types, including intestinal epithelial cells, macrophages, and $CD4^+$ T cells[12–14]. Notably, AhR regulates the differentiation of specific $CD4^+$ T cell subsets such as Th17 cells[15,16]. In particular, AhR can enhance Th17 cell differentiation via its activation by specific ligands derived from tryptophan, including 6-formylindolo[3,2-b]carbazole (FICZ), and chronic AhR activation controls the trans-differentiation of Th17 cells into anti-inflammatory T regulatory type 1 cells, which suppress pro-inflammatory T cell responses in vivo[17,18].

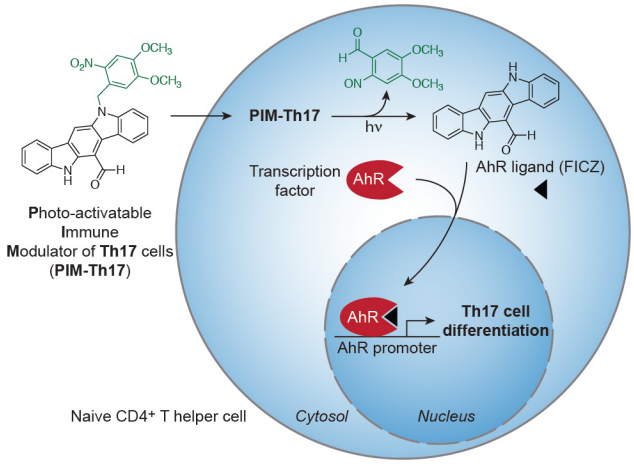


Figure 1. Schematic of synthetic immunology approach for inducing T helper 17 (Th17) cell differentiation by activation of the aryl hydrocarbon receptor (AhR) pathway. UV light illumination of a photocaged AhR ligand, **P**hoto-activatable **I**mmune **M**odulator of **Th17** cells (PIM-Th17), releases the AhR ligand, FICZ, which binds AhR. Ligand binding activates nuclear translocation and induction of Th17 cell differentiation.

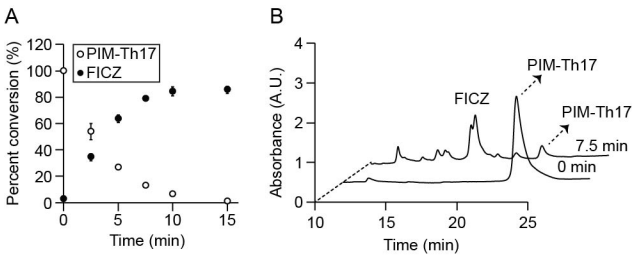


Figure 2. PIM-Th17 is photo-uncaged by UV light. (A) Percent conversion of PIM-Th17 to FICZ after illumination with 365 nm light. (B) HPLC analysis of photo-uncaging reactions. Traces shown were monitored by UV absorbance at 254 nm. A.U. = arbitrary units.

Despite these recent advances, understanding Th17 cell plasticity, i.e., their ability to trans-differentiate into distinct T cell subsets in vivo, has been hindered by a lack of technologies to interrogate or manipulate their activities in vivo under physiologically relevant conditions[19,20]. This need has been partially met by fate mapping using fluorescent reporter mice; however, these genetic approaches suffer from limited spatial and temporal resolution[21]. For instance, the use of cell marking by genetic recombination using tamoxifen-inducible Cre recombinase cannot resolve dynamic processes that occur on a faster timescale than the genetic recombination event, which is required for reporter expression[22]. Here, we have developed a chemical strategy suitable for use in vivo for the precise control of Th17 cell differentiation via activation of AhR. In this approach, we harness the exquisite control afforded by light irradiation to selectively release the AhR ligand FICZ via photochemistry with spatiotemporal precision.

Synthetic immunology approaches exploiting photo-activation have been utilized to modulate additional immune cell types, including dendritic cells and macrophages, by targeting different signaling pathways[23–26]. Alternatively, small-molecule approaches using bioorthogonal chemical deprotection have also been developed to activate cytotoxic $CD8^+$ T cells, a different T cell type[27,28]. However, there exist no reports of chemical strategies to manipulate $CD4^+$ Th17 cells or the AhR pathway with dynamic control. We envision that our strategy to selectively induce Th17 cell differentiation on demand will help elucidate a mechanistic understanding of Th17 cell plasticity in vivo by probing Th17 cell activity during immune responses to pathogens and in mouse inflammatory disease models.

RESULTS AND DISCUSSION

Toward this ultimate goal, we have developed a **P**hoto-activatable **I**mmune **M**odulator of **Th17** cells (PIM-Th17, Figure 1), which releases FICZ upon UV light illumination. To control the release of FICZ in a spatiotemporal manner, we modified the ligand with a well-characterized photo-protecting group, 6-nitroveratryl (NV)[29], to render it inactive to AhR. Upon mild light illumination (365 nm), the NV group is cleaved by photolysis and FICZ is released, allowing its selective delivery to the target cells. We synthesized PIM-Th17 from FICZ in a one-step displacement reaction using 6-NV-bromide (Scheme S1, Figures S1-S3).

We first demonstrated that PIM-Th17 can be photo-uncaged using UV light. In these studies, we illuminated PIM-Th17 with UV light (365 nm) and monitored its conversion to FICZ over time using HPLC, followed by mass spectrometry (Figure 2). We found that photo-uncaging of PIM-Th17 followed first order reaction kinetics, as expected, with a rate constant of 4.85×10^{-3} s^{-1} (Figure S4). Although the major product formed was FICZ, we also observed the expected nitrosoaldehyde and several minor byproducts, which could not be identified by NMR or mass spectrometry (Figure S5A)[29]. We determined that none of these byproducts, including the nitrosoaldehyde, activated AhR using an optimized luciferase reporter assay for this transcription factor (Figure S5B) in which AhR activity is maximal after 2 h (Figure S6A)[30]. We also demonstrated that PIM-Th17 does not activate AhR until it is illuminated with UV light, which releases FICZ (Figure S6B). Furthermore, PIM-Th17 is stable to aqueous hydrolysis for at least 4 days at 37 °C (Figure S7).

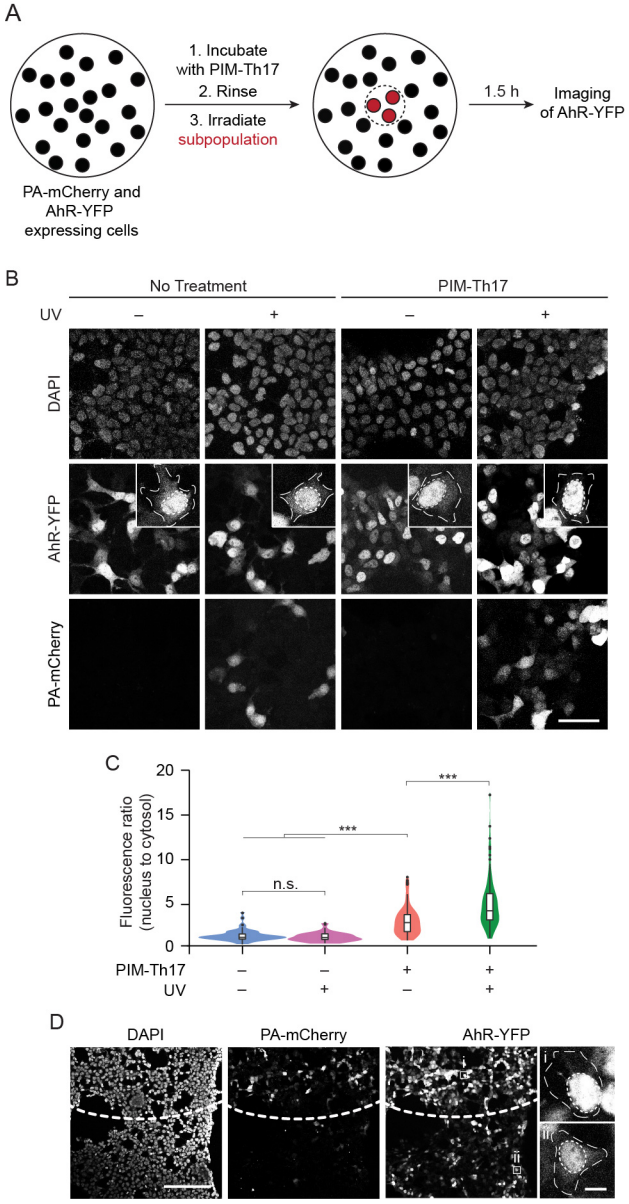


Figure 3. Photo-uncaging of PIM-Th17 is spatially selective and leads to nuclear translocation of AhR. HEK 293T cells co-transfected with AhR-YFP and PA-mCherry were pre-treated with PIM-Th17 (10 μM) or vehicle (DMSO) for 30 min, and the center of the dish was illuminated with UV light (epifluorescence using DAPI filter, 30 s). Cells were subsequently incubated for an additional 1.5 h, fixed, stained with DAPI, and imaged. (A) Schematic of experimental setup. (B) Maximum intensity z-projection images of cells at the periphery (– UV) or center (+ UV) of the same dish, imaged by confocal microscopy. Representative individual cells are shown at higher magnification (3.85X) in inset images. (C) Violin plots of nuclear to cytosolic ratio of the AhR-YFP fluorescence from (B), n = 50–70 cells in each treatment group. Box plot inside the violin plot: Interquartile ranges (IQRs, boxes), median values (line within box), whiskers (lowest and highest values within 1.5 times IQR from the first and third quartiles), and outliers beyond whiskers (dots) are indicated. Statistical significance was assessed using one-way ANOVA followed by post-hoc Tukey's test. n.s. = not significant, ***$p < 0.001$. (D) Maximum intensity z-projection images of cells at the border of UV illumination. Dashed curve indicates border region of illumination (+ UV, top; – UV, bottom), and representative individual cells are indicated by white squares and shown at higher magnification at right. Scale bars: 50 μm (B and D, full-size images), 10 μm (D, magnified images).

Next, we determined whether photo-uncaging of PIM-Th17 leads to AhR nuclear translocation in mammalian cells (Figures 3, S8)[31]. To make AhR reporter cells, we transfected HEK 293T cells with both a yellow fluorescent protein fusion to AhR (AhR-YFP) and photo-activatable mCherry (PA-mCherry), a fluorogenic protein that enables tracking of cells that were illuminated with UV light[32]. To optimize this experiment, we found that FICZ leads to maximal AhR-YFP nuclear translocation after 2 h with no washout (Figure S9). We then incubated the reporter cells with PIM-Th17 for 30 min to allow for cellular uptake, which was optimized in Figure S10, followed by a quick wash and illumination of a population of cells within the center of the dish using the microscope light source (Figure 3A). Using confocal microscopy, we found that photo-uncaging of PIM-Th17 led to increased AhR-YFP nuclear translocation after 1.5 h in the cells that had been illuminated in the center of dish compared to those in the periphery that had not been illuminated (Figure 3B-C). To demonstrate the precise spatial selectivity of this approach, we also imaged adjacent regions of the dish that had been illuminated and non-illuminated (Figure 3D, dashed line denotes border region of illumination). Further, we determined that the effects of photo-uncaging PIM-Th17 on AhR-YFP nuclear translocation are dose dependent (Figure S11). We note that unirradiated PIM-Th17 led to a small increase in nuclear translocation (Figure 3C), which may be due to the probe having some intrinsic ability to activate AhR-YFP, albeit at low levels, that initially leads to AhR-YFP nuclear translocation, followed by the majority relocalizing to the cytosol over 24 h (Figure S12). Importantly, the photo-uncaging byproducts, including the nitrosoaldehyde, did not affect AhR-YFP nuclear translocation (Figure S13). In addition, neither UV irradiation nor the concentrations used of PIM-Th17 were toxic to the cells as determined by propidium iodide staining (Figure S14).

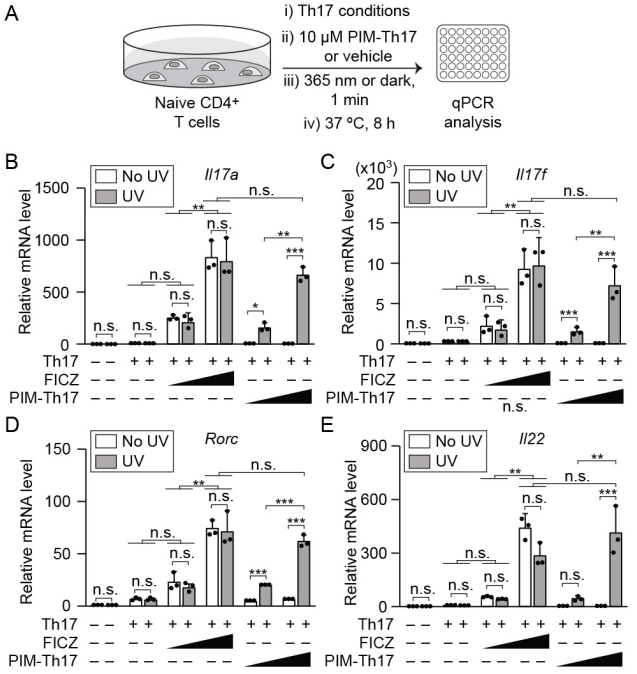
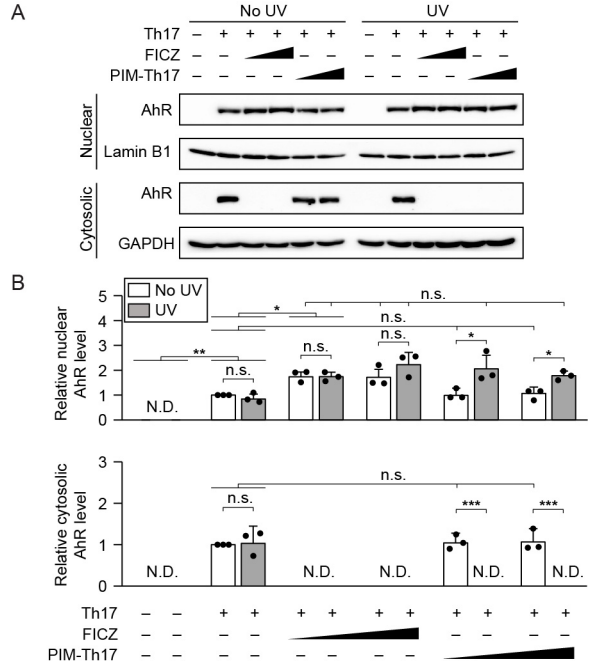


Figure 4. Photo-uncaging of PIM-Th17 leads to increased transcription of AhR target genes in Th17 cells. Splenic naïve CD4$^+$ T cells were harvested from wild-type mice and cultured with or without Th17-inducing factors (Th17). After 4 d of differentiation, PIM-Th17 (1 μM, 10 μM), FICZ (300 nM, 2 μM), or vehicle

(DMSO) was added, and the cells were illuminated with UV light (365 nm, 1 min, grey bars) or kept in the dark (white bars). RNA was isolated after 8 h, and cDNA was synthesized and analyzed by qPCR (data normalized to *Rpl13a*). (A) Schematic of experimental setup. Relative mRNA transcript levels of (B) *Il17a*, (C) *Il17f*, (D) *Rorc*, and (E) *Il22* are shown. Error bars represent mean ± SD. Statistical significance was assessed using one-way ANOVA followed by post-hoc Tukey's test. n.s. = not significant, *p<0.05, **p<0.01, ***p < 0.001.



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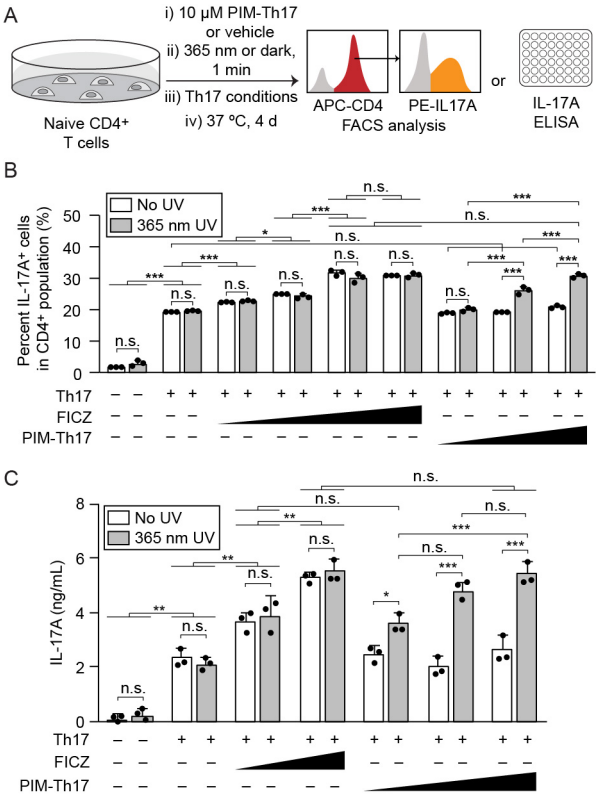
We then determined that photo-uncaging of PIM-Th17 leads to increased transcription of canonical AhR target genes, including those that encode cytochrome P450 enzymes and the aryl hydrocarbon receptor repressor (AhRR)[11]. In these studies, HepG2 cells, which express high levels of AhR, and naïve CD4$^+$ T cells that were treated with Th17 polarizing conditions to induce Th17 differentiation were treated with and without PIM-Th17, followed by UV light illumination. For these and all subsequent experiments, we used a UV transilluminator, which allows broad illumination of the cells, to photo-uncage PIM-Th17. We found by qPCR that photo-uncaging of PIM-Th17 led to increased transcript levels of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, and *Ahrr,* with maximal transcript levels at 8 h (Figures S15 and S16). We also examined the effect of PIM-Th17 on expression of noncanonical AhR genes (e.g., *Il17a*, *Il17f*, *Il22*, *Rorc*) in Th17 cells and found that these mRNA transcripts were increased 8h following PIM-Th17 treatment and UV illumination (Figures 4 and S17A), which suggests that AhR activation by photo-uncaged PIM-Th17 is responsible for increased Th17 cell activity. In these experiments, we determined that photo-uncaging of 10 μM PIM-Th17 had a similar effect as 2 μM FICZ, suggesting a roughly 20% photo-uncaging efficiency in this assay (Figure 4B-E).

We verified that the effects of photo-uncaging PIM-Th17 in Th17 cells lead to AhR activation by Western blot analysis of the cytosolic and nuclear fractions (Figure 5A). We quantified the amount of AhR in each fraction using densitometry (Figure 5B). Interestingly, photo-uncaging of PIM-Th17 at both doses (1 and 10 μM) had similar efficacies on nuclear translocation of endogenous AhR to each other and to both 300 nM and 2 μM of FICZ (Figure 5B). Yet, both photo-uncaged PIM-Th17 and FICZ exhibited dose-dependent effects on downstream transcriptional effects of AhR activation (Figures 4 and S16). One possible explanation for this discrepancy in dose response may be because AhR is subject to additional uncharacterized regulatory steps following its nuclear translocation[33]. Critically, the photo-uncaging byproducts, including the nitrosoaldehyde, did not affect AhR gene transcription of its downstream targets (Figures S17B and S18). Collectively, these results suggest that AhR nuclear translocation caused by photo-uncaged PIM-Th17 results in association of AhR to its transcription factor binding site and increased transcription of downstream target genes. We also verified that the concentrations of PIM-Th17 and UV illumination used in this experiment were not toxic to HepG2, naïve CD4$^+$ T cells, and Th17 cells (Figures S19-S21).

Finally, we evaluated the ability of PIM-Th17 to increase differentiation of Th17 cells upon photo-uncaging. We isolated naïve CD4$^+$ T cells from the spleens of wild-type mice and cultured these cells under Th17 differentiation conditions in the presence or absence of PIM-Th17[15]. We also optimized the duration of Th17 differentiation, UV irradiation time, and concentrations of PIM-Th17 (Figures 6 and S22) and elected to use 4 d, 1 min, and 10 μM, respectively, for subsequent studies. Gratifyingly, after photo-uncaging of PIM-Th17 to release FICZ, we found that the percentage of Th17 cells within the T cell population increased compared to that in a parallel population of T cells that were treated with PIM-Th17 but were not illuminated (Figure 6). We also found that photo-uncaging of PIM-Th17 led to an increase in IL-17A secreted by these cultures by ELISA (Figure 6C). Importantly, we verified that PIM-Th17 did not induce differentiation of additional CD4+ T cell subsets, including Th1, Th2 and regulatory T (Treg) cells (Figures S23 and S24). However, photo-uncaging of PIM-Th17 led to decreased Treg differentiation under polarizing conditions (Figure S23), which is consistent with reports of FICZ inhibiting Treg induction[34]. Given that AhR is expressed in many different cell types, we also determined that PIM-Th17 activation of AhR in alternative cell types, including intestinal epithelial cells and macrophages, did not affect Th17 cell differentiation (Figure S25), suggesting that its use in vivo to modulate immunity may primarily affect Th17 cells rather than other cell types. Further, we found that the photo-uncaging byproducts, including the nitrosoaldehyde, did not affect Th17 cell differentiation (Figure S5C).

Collectively, these results demonstrate that our PIM-based chemical strategy can be used to selectively differentiate Th17 cells on demand via activation of AhR. We envision that this approach targeting AhR could be used to understand the dynamic roles of Th17 cells in pathological settings driven by the

IL17 pathway, such as psoriasis, whose affected tissues would be easily accessible to light irradiation[35]. The use of photocaging groups that can be removed with near infrared light would enable activation of AhR in deeper tissues beyond the dermis[36,37]. Alternatively, this generalizable strategy could be applied to additional pathways that control Th17 cell differentiation, including RORγ[9].



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CONCLUSION

We have developed a synthetic immunology approach that utilizes a photo-activatable immune modulator (PIM) to control the differentiation of Th17 cells by targeting the AhR pathway with spatial and temporal precision. This chemical strategy exploits the use of photochemistry to selectively deliver an AhR ligand to the target cell type, which will enable manipulation of CD4$^+$ T cell populations on demand to understand their dynamic roles in vivo. Additionally, this approach can be used to activate AhR within alternative cell types, including those involved in xenobiotic metabolism and additional immune cells that are regulated by this pathway[10]. Ultimately, we envision that this technology could be used to control Th17 cells during active immune responses in vivo and in mouse models of inflammatory disease to better understand the plasticity of this enigmatic immune cell type during various physiological and pathological states.

ASSOCIATED CONTENT

Supporting Information

Materials and methods, synthetic procedures, as well as supplementary data (PDF)

AUTHOR INFORMATION

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Notes

No competing financial interests have been declared.

ACKNOWLEDGMENT

This work was supported by the Arnold and Mabel Beckman Foundation (Beckman Young Investigator Award to P.V.C.) and a President's Council of Cornell Women Affinito-Stewart Grant (P.V.C.). We thank the Cornell University NMR facility (NSF MRI: CHE-1531632) and Dr. Ivan Keresztes for technical assistance. We are grateful to Samantha Scott for animal husbandry and Dr. Brittany White for helpful discussions. We also thank the Weill Institute for Cell and Molecular Biology for resources and reagents.

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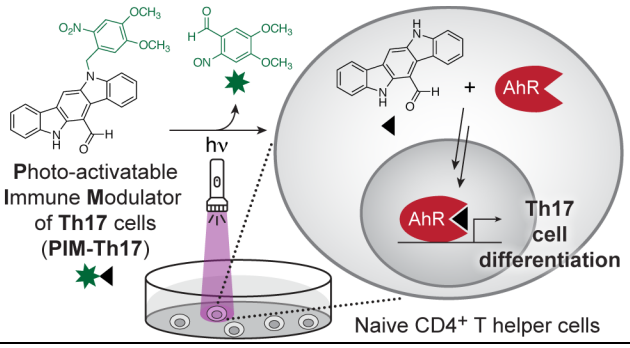
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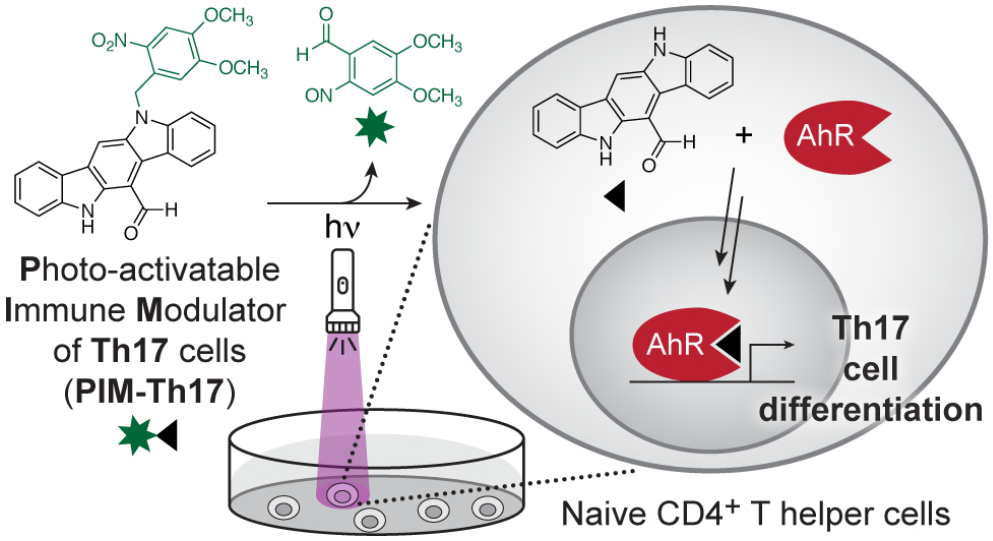
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