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TITLE:

Novel Protocol for Generating Physiologic Immunogenic Dendritic Cells

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SUMMARY:

The transimmunization (TI) device or plate and related protocols have been developed to replicate the key features of extracorporeal photochemotherapy (ECP), in an experimental setting, allowing for production of physiologically activated, tunable dendritic cells (DCs) for cancer immunotherapy.

ABSTRACT:

Extracorporeal photochemotherapy (ECP) is a widely used cancer immunotherapy for cutaneous T cell lymphoma (CTCL), operative in over 350 university centers worldwide. While ECP's clinical efficacy and exemplary safety profile have driven its widespread use, elucidation of the underlying mechanisms has remained a challenge, partly owing to lack of a laboratory ECP model. To overcome this obstacle and create a simple, user-friendly platform for ECP research, we developed a scaled-down version of the clinical ECP leukocyte-processing device, suitable for work with both mouse models, and small human blood samples. This device is termed the Transimmunization (TI) chamber, or plate. In a series of landmark experiments, the miniaturized device was used to produce a cellular vaccine that regularly initiated therapeutic anti-cancer immunity in several syngeneic mouse tumor models. By removing individual factors from the experimental system and ascertaining their contribution to the *in vivo* anti-tumor response, we then elucidated key mechanistic drivers of ECP immunizing potential. Collectively, our results revealed that anti-tumor effects of ECP are initiated by dendritic cells (DC), physiologically generated through blood monocyte interaction with platelets in the TI plate, and loaded with antigens from tumor cells whose apoptotic cell death is finely titrated by exposure to the photoactivatable DNA cross-linking agent 8-methoxypsoralen and UVA light (8-MOP_A). When returned to the mouse, this cellular vaccine leads to specific and transferable anti-tumor T cell immunity. We verified that the TI chamber is also suitable for human blood processing, producing human DCs fully comparable in activation state and profile to those derived from the clinical ECP chamber. The protocols presented here are intended for ECP studies in mouse and man, controlled generation of apoptotic tumor cells with 8-MOP_A, and rapid production of physiologic human and mouse monocyte-derived DCs for a variety of applications.

INTRODUCTION:

Extracorporeal photochemotherapy (ECP) is an established immunotherapy that is widely operative in university centers worldwide. Its use has been driven by unique selectivity, safety, and bi-directional efficacy of ECP therapy, traits that ECP shares with the physiologic immune system itself with which it appears to partner. ECP is selectively immunizing against the malignant cells in cutaneous T cell lymphoma (CTCL)^{1,2}, and selectively tolerizing for targeted antigens in the transplant, autoimmunity, and graft-versus-host disease (GvHD) settings^{3,4}. The immunogenicity of ECP is highlighted by its dependency on an intact CD8 T cell compartment in CTCL⁵, while specificity is reflected by ECP's very favorable adverse reaction profile, with no off-target immune suppression in transplant or GvHD settings, and no increased opportunistic infection susceptibility in CTCL, where non-pathogenic T cell clones could potentially be lost along with the malignant T cells.

Given the clinical importance of ECP, the hope that better understanding of its mechanisms might expand ECP's therapeutic reach to a wider spectrum of cancers and immunologic disorders has stimulated international interest. Two nationally sanctioned workshops, an National Institutes of Health (NIH) State-of-the-Science symposium⁶ and an American Society for Apheresis consensus conference⁷, were conducted and reported, with the aim of accelerating the identification of the principal cellular contributors to ECP's anti-cancer and tolerogenic effects.

To date, despite numerous published reports attempting to address ECP's mechanism, two primary obstacles have impeded scientific advances. Firstly, investigation of ECP mechanisms in the experimental laboratory setting has been limited by the lack of miniature ECP device that would fully reflect ECP's cellular and in vivo effects, and be applicable to animal models. Secondly, in-depth laboratory analysis of ECP samples in the clinical setting has been restricted by the limited availability of ECP-processed patient immune cells, which require access to treatment centers, and are additionally subject to the timing of the patient's infusions, and the ethical need for uncompromised sets of patient-reinfused leukocytes.

To resolve the obstacles impeding the progress of ECP research, we set out to develop a miniature ECP apparatus that would most closely mimic the key elements of the therapy. Standard ECP treatment involves the passage of a patient's leukapheresis-enriched leukocytes through a 1 mm-thick, ultraviolet A light (UVA)-transparent, plastic plate^{6,8}. In the plate, the leukocytes are exposed to 8-methoxypsoralen (8-MOP), a photo-activatable agent which upon UVA exposure is transiently converted to a reactive form (8-MOP_A), capable of DNA cross-linking via its bivalent binding to pyrimidine bases on sister DNA strands^{9,10}. Processed, 8-MOP_A-treated leukocytes are collected and returned intravenously to the patient.

Since ECP itself is a bi-directional therapy, immunizing in cancer and tolerizing in transplant, autoimmunity and GvHD settings, for clarification we have named model ECP's immunization mode "transimmunization", and the tolerogenic mode "transtolerization". We first focused on the transimmunization modality. Our recently reported miniaturized scalable mouse-to-man ECP device, the transimmunization (TI) chamber or plate, and the corresponding protocol, reproduce both the cellular and in vivo effects of the human ECP device in a cancer setting¹¹.

In order to make the transimmunization in vivo model as clinically relevant as possible, we initiated the immunotherapy after subcutaneously injected syngeneic mouse tumors became palpable, thus testing the protocol's efficacy in established cancer. Similarly to ECP in CTCL, the proof-of-principle transimmunization protocol in the YUMM1.7 model of melanoma uses peripheral blood mononuclear cells (PBMC) from tumor-bearing animals. The cells are passed through the TI plate under flow. While 8-MOP_A treatment of cells in the miniature chamber is possible, it is preferable to conduct it separately, to ensure that only the desired cell type is exposed to 8-MOP_A. Earlier studies indicate that ECP's tolerogenic effect is mediated by 8-MOP_A-injured antigen-presenting cells¹², while the immunizing effect requires 8-MOP_A injury of target tumor cells¹¹. In the transimmunization protocol either the tumor cells, or the immune cells, can be selectively exposed to 8-MOP_A as needed. Since maximizing the time of contact between 8-MOP_A-injured tumor cells and ECP-induced dendritic cells (DC) has been shown to significantly enhance the immunotherapeutic capacity of clinical ECP¹³, we incorporated an overnight co-incubation step into our protocol. After overnight incubation, the treated cells are returned to the tumor-bearing animal.

This system reliably reduced tumor growth and initiated specific anti-tumor immunity in mice with established syngeneic tumors¹¹, and allowed us to dissect the mechanism of ECP's clinical anti-tumor efficacy. In several studies we have demonstrated that ECP immunity is initiated

through ex vivo platelet activation in the TI plate^{14,15}. Activated platelets subsequently signal the monocyte-to-dendritic cell maturation¹⁴, leading to production of physiologic DCs. The newly formed monocyte-derived DCs are able to cross-present internalized antigens from 8-MOP_A-damaged tumor cells to activate antigen-specific T cell responses¹⁶. As suggested previously^{12,17}, however, 8-MOP_A-induced damage of the nascent DCs themselves can counter-act or even reverse the anti-tumor effect¹¹, providing a mechanistic link to ECP tolerance.

The TI plate has also been used to produce physiologically activated DCs from human PBMC. Similarly to the mouse studies, human TI-derived DCs are dependent on plate-passage in the presence of platelets for their generation, appear phenotypically identical to those produced in the clinical ECP plate, and are capable of efficiently processing and cross-presenting human tumor antigens for activation of human T cells^{11,16}.

In uncovering the mechanism of ECP immunotherapy, we have therefore uncovered a method for rapidly generating physiologic mouse and human dendritic cells of desired antigen specificity, that can be functionally modulated. The innovative TI device and protocol have substantial potential importance in the fields of ECP research and therapy and cancer immunotherapy more broadly, and in any other field with interest in physiologic, functional dendritic cells in either the immunizing or the tolerizing modality. We hope that this publication will provide the necessary tools to those with interest in such research areas.

PROTOCOL:

All mouse methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University, in agreement with the National Institutes of Animal Healthcare Guidelines. All human studies were performed with blood donated by healthy volunteers, with written informed consent. Human blood studies were conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule), and were approved by Yale Human Investigational Review Board under protocol number 0301023636.

NOTE: The following is the protocol describing transimmunization for anti-tumor therapy of mouse syngeneic tumors.

1. Syngeneic YUMM1.7 tumor implantation

1.1. Follow established protocols to implant syngeneic tumors into the flanks of mice as appropriate to the tumor cell line of interest.

NOTE: The protocol below describes the YUMM1.7 C57BL/6 mouse melanoma tumor model. YUMM1.7 melanoma cells were kindly provided by Dr. Bosenberg, Yale¹⁸.

1.2. Thaw a frozen aliquot of cells and use cells after one cell passage. Culture freshly-thawed YUMM1.7 cells in Dulbecco's modified Eagle's nutrient mixture F-12 medium (DMEM/F12),

supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% non-essential amino acids, under standard tissue culture conditions (37 °C, 5% CO₂).

1.2.1. Collect YUMM1.7 cells at 60–70% confluence by adding enough trypsin-EDTA to cover the bottom of the cell culture plate or flask (e.g., 4 mL for a T75 flask).

1.2.2. Rest the cell culture vessels at room temperature for 3–4 min, gently tapping on the bottom or sides of the vessel occasionally to help detach the cells.

1.2.3. Add 1 mL of fetal bovine serum (FBS) per 4 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) to stop the reaction once most of the cells are detached. Collect the cells by pipetting into a 15 mL conical tube. Rinse the flask with phosphate-buffered saline (PBS) and add the rinse to the same collection tube. Fill the tube to 15 mL with PBS.

1.2.4. Take out a small aliquot and count the cells.

1.2.5. Spin down for 10 min at 250 x *g* in a standard tissue culture centrifuge to collect the tumor cells.

1.3. Resuspend YUMM1.7 cells in PBS at density of 1 x 10⁶ cells/mL.

1.4. Inject 1 x 10⁵ YUMM1.7 tumor cells subcutaneously in 100 µL of PBS into the right flanks of recipient 4 to 6-week-old wild type male C57BL/6J mice, anesthetized according to institutional guidelines (e.g., 3–5% isoflurane inhalant gas, anesthesia confirmed by lack of hind paw pinch reflex).

1.5. Monitor tumor volume via bi-weekly measurements of perpendicular tumor diameters and height using a caliper. Calculate YUMM1.7 tumor volume as tumor length x width x height/2.

1.6. Initiate transimmunization therapy when tumors become just palpable; for YUMM1.7 tumors, this is usually day 7–10 post tumor implantation.

2. Collection of peripheral blood mononuclear cells for transimmunization

2.1. Collect 100–150 µL of blood from each tumor-bearing mouse via cheek bleed.

2.2. As blood is being collected, pool blood from each mouse treatment group (each 5 experimental mice + 5 control mice) in a single 15 mL tube containing 5000 U/mL heparin. Use 10 µL of 10% heparin per 100 µL blood collected.

NOTE: Mix the tube frequently during collection to avoid blood coagulation. While control tumor-bearing mice can be bled at the same time as the experimental mice, to ensure equal conditions with the treatment group, the “control” blood can be either discarded, or combined with the

blood of the treatment group to provide additional experimental PBMC for the treatment group, at the investigators' discretion.

2.3. Set up one 15 mL tube with 4 mL of lymphocyte isolation medium (see the **Table of Materials**) per 5–10 mice bled. Slowly layer blood (collected from tumor-bearing mice) on top of the medium. Spin the cells for 20 min at 1000–1500 x *g* to separate the PBMC from red blood cells.

2.4. At the end of centrifugation, collect the top plasma layer, leaving ~0.5 mL remaining above the buffy coat, and store at 4 °C for later use (step 7.1).

2.5. Collect the buffy coat layer into a clean 15 mL tube, fill with PBS to 15 mL, and spin for 10 min at 250 x *g* in a standard tissue culture centrifuge to collect the PBMC.

2.6. Carefully pipet off the supernatant, and resuspend the pellet by flicking the tube. Do not decant, as the pellet is soft and can be lost. Add 2 mL of ACK red blood cell lysis buffer (see the **Table of Materials**) to the tube to remove any remaining red blood cells from the PBMC. Incubate on ice for 10 min.

2.9. Fill the tube with PBS to 15 mL, and spin down for 10 min at 250 x *g* in a standard tissue culture centrifuge to collect the PBMC.

2.10. Carefully pipet off the supernatant, and resuspend the pellet by flicking. Do not decant, as the pellet is soft and can be lost.

NOTE: At this step, the purified PBMC can be modified as best suits the experiment. The various PBMC components (platelets, monocytes, other immune cell types) can be depleted or isolated from the PBMC as needed. Also, PBMC themselves can be exposed to 8-MOP_A, either before or after section 4 (before or after plate passage—after section 2 or before section 5), by following the protocol steps 3.3–3.8 and substituting PBMC for YUMM1.7 cells. This will truncate the immunogenic capacity of the treatment, and instead promote immune tolerance.

2.11. For each treatment group (each 5 experimental mice + 5 control mice), resuspend the PBMC pellet in 300 µL FBS.

3. 8-MOP/UVA treatment of tumor cells

3.1. Culture and collect YUMM1.7 tumor cells as described in steps 1.2–1.3 to prepare an 8-MOP_A-exposed tumor cell antigen source.

3.2. Prepare 2.5 x 10⁶ YUMM1.7 tumor cells per treatment group of 5 mice. For each treatment group, resuspend the tumor cell pellet in FBS at 2.5 x 10⁶ cells/300 µL (~8.33 x 10⁶ cells/mL).

3.3. With tissue culture hood lights off, add 8-MOP (see the **Table of Materials**) to the YUMM1.7 tumor cell suspension for a final 8-MOP concentration of 100 ng/mL.

CAUTION: 8-MOP is a photoactivatable DNA-damaging agent and carcinogen. Use caution when handling and dispensing, avoid contact with exposed skin, and discard appropriately.

3.4. Mix the cells well, wrap the cell container in tin foil, and incubate for 20 min at 37 °C.

3.5. Pre-coat a 12-well tissue culture plate by filling one well for every treatment group of 5 mice (2.5×10^6 YUMM1.7 tumor cells) with 1 mL of FBS, and refrigerate filled plate for 20 min at 4 °C.

3.6. Turn on the UVA light source to pre-warm it.

CAUTION: UVA light is a carcinogen. When working with UVA light sources work quickly and carefully, protect skin from exposure, and use face shields or goggles to protect face and eyes.

3.7. After 20 min, move the refrigerated 12-well plate (step 3.5) to the tissue culture hood, remove FBS from wells, and add 300 μ L (2.5×10^6 cells/well) MOP-exposed tumor cells (from step 3.4) per well.

3.8. Expose the cell-containing plate to the pre-warmed UVA light source for total irradiation of 4 J/cm².

NOTE: The 8-MOP concentration and UVA dose described here are calibrated for the YUMM1.7 tumor cell line. A titration of 8-MOP/UVA dose, sufficient to cause 100% tumor cell death within 1 week of exposure, should be performed for each experimental tumor cell line, in order to determine effective killing dose. This is critically important for mouse in vivo TI experiments, because cells are reinjected retro-orbitally (step 6.1) and thus any undamaged tumor cells may otherwise form eye tumors in the treated animal. As a guideline, 4–8 J/cm² of UVA, 100–200 ng/mL 8-MOP, is the typical effective range for most tumor cell lines.

3.9. Collect 8-MOP_A-treated tumor cells from each well, swirling the plate and pipetting carefully to ensure complete cell recovery.

4. TI plate passage of cells

4.1. For each treatment group of 5 mice, combine in one 1.5 mL conical tube 300 μ L of the appropriate PBMC (from step 2.11) and 300 μ L of 8-MOP_A-treated tumor cells (from step 3.9). Mix the cells.

4.2. Use a 10 mL syringe to fill the “entrance” and “exit” sets of TI tubing (see the **Table of Materials**) with FBS. Open the tubing clamps to fill the tubes, and close clamps once the tubes are filled before disconnecting the syringe, to retain FBS inside the tubing.

4.3. Use a P1000 pipette to add the PBMC and tumor cell mix (from step 4.1) to the TI plate (see the **Table of Materials**). Hold the plate at a 45° angle, place the pipette tip securely into the TI plate intake, and fill the plate slowly, avoiding bubbles.

4.4. Remove the pipette tip from the intake before releasing the pipette plunger. The plate holds 450 µL; return the remaining cells to the 1.5 mL conical tube.

4.5. Incubate the FBS-filled tubing, the cell-filled TI plate, and the 1.5 mL conical tube containing the remaining cells in the tissue culture incubator at 37 °C for 1 h. After incubation, empty the TI tubing by gravity, releasing the clamps.

4.6. Use a P1000 pipette to remove cells from the TI plate by inserting the pipette tip with the plunger depressed into the plate port. Hold the plate at a 45° angle and fill the P1000 pipette tip. Place cells back into their original 1.5 mL conical tube.

4.7. To run the TI plate, connect the exit tube to the plate and secure the TI plate into the plate running system.

4.8. Using a 1 mL syringe, draw up the 600 µL of PBMC and tumor cell mix from the 1.5 mL conical tube, get rid of any bubbles in the syringe, attach the syringe to the entrance tube with the clamp open and slowly fill until fluid reaches the end of the tubing.

4.9. Attach the free end of the entrance tube to the TI plate and continue gently loading the remaining volume. Close the entrance clamp.

4.10. Detach the 1 mL syringe and connect the entrance tubing to the syringe pump. Secure the exit tubing to a clean 1.5 mL conical tube for cell collection.

4.11. Adjust the syringe pump flow rate to 0.09 mL/min, but do not start the pump yet. Tilt the TI plate ~30° towards the syringe pump side, using a TI plate running platform or any other means (e.g., a small flat object under one end of the plate).

4.12. Carefully release the clamp on the entrance tubing. Start the syringe pump, observing carefully how the TI plate fills, and flick tubing or plate as necessary should any air bubbles impede the flow.

4.13. Once the TI plate is completely filled, tilt it ~30° in the opposite direction as it empties. When all the cells and liquid are in the 1.5 mL conical collection tube, stop the pump.

4.14. To wash the TI plate, disconnect the entrance tubing from the syringe pump, connect to a 1 mL syringe filled with 600 µL FBS, and follow the procedure for steps 4.8–4.9.

4.15. Adjust the syringe pump flow rate to 0.49 mL/min, release the entrance clamp, and run the TI plate as described in steps 4.12–4.13, collecting the wash in the same 1.5 mL conical tube. Flick or tap the TI plate gently the entire time, to aid in detaching and eluting any adherent cells.

4.16. Spin the collection 1.5 mL conical tube in the benchtop microfuge at 250 x *g* for 8 min. Discard the supernatant.

5. Preparation of autologous mouse serum for overnight culture medium

5.1. Collect blood from 10–12 week old C57BL/6J mice by any institutionally approved method (e.g., eye bleed, tail vein bleed, cheek bleed, or terminal bleed-out by cardiac puncture), without any anticoagulants. Estimate collecting 1 mL blood for every 300 µL of serum needed.

NOTE: One would need 300 µL of serum for every treatment group of 5 mice in the experiment.

5.2. Allow blood to clot overnight at 4 °C.

5.3. Spin down the coagulated blood at 3000 x *g* for 15 min. Carefully collect the top serum-containing layer

NOTE: Serum can be used immediately to make the overnight cell incubation medium (step 6.1), or preserved for future experiments. To preserve the serum, freeze in aliquots at -20 °C.

6. Overnight co-incubation of PBMC with antigen

6.1. Resuspend the PBMC and tumor cell pellet (step 4.5) in 2 mL of clear RPMI with 15% autologous mouse serum (prepared in step 5). Plate in a 35 mm non-tissue-culture treated sterile dish, and incubate overnight under standard tissue culture conditions (37 °C, 5% CO₂).

NOTE: If PBMC are being used with a non-cellular antigen (peptide, nanoparticle, protein, etc.), omit section 3 of the protocol, and proceed from section 2 directly to section 4, adding the desired antigen to the TI plate-passed PBMC for overnight co-incubation here in step 6.1.

6.2. On the following day, carefully detach any adherent cells from the bottom of the dish with a tissue culture scraper, rotating the dish while scraping to ensure even cell collection. Collect the cells in a 15 mL tube.

6.3. Add 2 mL PBS to the dish and repeat the scraping step, collecting the cells into the same tube. Rinse the 35 mm dish with 1 mL PBS and add the rinse to the same tube.

6.4. Spin for 10 min at 250 x *g* in a standard tissue culture centrifuge to collect the cells. Carefully pipet off the supernatant and resuspend the pellet by flicking the tube. Do not decant, as the pellet is soft and can be lost.

7. Re-injection of TI-treated cells into tumor-bearing mice

7.1. Spin down autologous plasma (prepared in step 2.4) at 750 x *g* for 15 min to sediment any particulates. Resuspend the cell pellet (from step 6.4) in 600 μ L of autologous plasma or PBS.

7.2. Inject cells at 100 μ L per mouse into the retro-orbital plexus of appropriately anesthetized (e.g., 3–5% Isoflurane inhalant gas, anesthesia confirmed by lack of hind paw pinch reflex) tumor-bearing experimental mice. If so desired, re-inject control tumor bearing mice with 100 μ L of autologous plasma alone, or PBS.

7.3. For YUMM1.7 tumor-bearing mice, repeat the treatment (steps 2–7.2) twice a week for 3 weeks, for a total of 6 treatments. Collect and process the PBMC from tumor-bearing mice weekly (protocol sections 2–4) on Mondays and Thursdays, and re-infuse after overnight incubation (protocol sections 5–7) on Tuesdays and Fridays.

NOTE: This treatment regimen was developed for the specific growth kinetics of YUMM1.7 tumors in mice. It may need to be titrated for other tumor systems with slower or faster tumor growth rates—respectively increasing or reducing the number of treatments.

7.4. Monitor tumor volume via bi-weekly measurement—preferably at the time of therapy administration (Tuesday and Friday)—of perpendicular tumor diameters and height using a caliper. Terminate the experiment when control tumor volumes reach maximal size permitted by institutional guidelines and protocols.

8. Protocol adaptation for treating small volumes of human blood

8.1. Adapt the protocol for use with human cells by first isolating PBMC from human blood. Use heparin as an anti-coagulant, with any preferred PBMC isolation protocol.

8.2. Ensure that the isolated PBMC fraction contains a physiologic number of platelets, for best protocol results. Monitor platelet counts pre- and post-PBMC isolation using any standard hematology counter.

8.3. If using a human cellular antigen source, treat the human antigenic cells following the steps described in section 3 for YUMM1.7 cells. Titrate 8-MOP and UVA doses accordingly.

8.4. Perform plate-passage steps described in section 4, using up to 3×10^7 PBMC per TI plate.

8.5. Culture PBMC overnight with cellular/other antigen of choice, as described in section 6, but substitute 15% human AB serum for autologous mouse plasma for the overnight culture medium in step 6.1.

8.6. Use the resulting cells in any desired assay. For instance, co-culture with antigen-reactive T cells to observe antigen-specific T cell responses initiated by the TI-treated cells.

REPRESENTATIVE RESULTS:

We recently developed a mouse-to-man scalable miniature ECP device, the TI plate (**Figure 1A**), and designed corresponding treatment protocols. The device and protocol reproduce key cellular and in vivo features of immunizing ECP, termed “transimmunization”.

The proof-of-principle murine transimmunization protocol¹¹ (**Figure 1B**) consists of extracorporeal TI plate passage of peripheral blood mononuclear cells (PBMC) from tumor-bearing mice together with apoptotic 8-MOP_A-exposed tumor cells. Notably, the TI chamber is transparent and sized to match the standard microscopy slide format, allowing for easy visualization of cell interactions within the TI plate at any point of the protocol (**Video 1**). The TI-plate-activated immune cells are incubated overnight with the 8-MOP_A-exposed apoptotic tumor cells, facilitating tumor cell uptake, processing, and transfer of tumor antigens to the DCs. On the following day, the co-incubated cell mixture is returned into the blood stream of the tumor-bearing animal. Control animals undergo identical blood collection procedures, to normalize for any effects of lymphodepletion on tumor growth, but instead receive PBS re-infusions. Tumor growth in all animals is monitored throughout the experiment.

In studies using the YUMM1.7 syngeneic murine melanoma model¹⁸, the transimmunization protocol was repeated twice weekly over three weeks, for a total of six treatments in each animal (**Figure 1B**). The therapy appeared well tolerated in all animals treated (>500), and consistently showed reduction of YUMM1.7 tumor growth in treated versus control animals, as observed in 10 independent experiments conducted over 2 years (**Figure 2A,B**). The results show cumulative tumor growth data in transimmunization-treated and control animals over all experiments performed (**Figure 2A**), as well as representative tumor growth curves for individual animals within one experiment, to provide a sense of variability in the system (**Figure 2B**).

We found that the protocol’s success critically depends on the presence of monocytes in the treated PBMC, the presence of platelets in the PBMC fraction, and the TI plate passage step. When plate passage is omitted, or when either platelets or monocytes are depleted from the PBMC fraction, the therapeutic effect is no longer observed (**Figure 3A**). The treatment also requires the presence of apoptotic tumor cells. It is ineffective in the absence of either the immune cells or an antigen source, or in the presence of mismatched antigen, for instance when mice bearing YUMM1.7 tumors are treated using MC38 colon carcinoma cells (**Figure 3B**). For an immunizing outcome it is also critical to avoid PBMC exposure to 8-MOP_A. 8-MOP_A-exposed PBMC not only abrogate, or possibly even reverse, anti-tumor immunity (**Figure 3C**), but also inhibit the immunizing potential of unexposed cells, as in the experiment where an equal number of 8-MOP_A-exposed and 8-MOP_A-protected PBMC were used with no observable anti-tumor effect (**Figure 3C**).

With human PBMC, the TI chamber and the transimmunization protocol lead to successful monocyte activation into DC, indistinguishable by cell surface and intracellular activation markers from that achieved by the clinical ECP plate (**Table 1**). As in the mouse studies, DC activation and the ability of transimmunization-generated DCs to process and present antigen critically depend

on the presence of platelets in the PBMC, and on TI plate passage (**Figure 4A**). The Transimmunization-activated human DCs can effectively process and cross-present either peptide antigens (**Figure 4B**), or antigens from whole 8-MOP_A-exposed human tumor cells, to activate human antigen-specific T cell lines in in vitro assays (**Figure 4C**), in a TI and platelet-dependent manner (**Figure 4B,C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Transimmunization (TI) chamber and protocol schematics. (A) Diagram and specifications of the transimmunization treatment chamber (TI plate). (B) Schematic description of transimmunization treatment experimental workflow. Briefly, animals are inoculated subcutaneously (s.c.) with syngeneic tumor cells; animals with palpable tumors are treated twice weekly by blood draw, isolation of PBMC from blood, PBMC flow passage through the autologous platelet-coated TI plate in the presence of 8-MOP/UVA treated tumor cells, PBMC and tumor cell co-incubation overnight, and re-injection of the cells intravenously into the same tumor-bearing animals. Tumor volume is measured throughout the experiment. This figure has been modified from¹¹.

Figure 2: Transimmunization controls growth of YUMM1.7 melanoma syngeneic tumors. (A) YUMM1.7 tumor volume over time plotted for C57BL/6 mice inoculated with 1×10^5 YUMM1.7 tumor cells, and receiving either six transimmunization treatments (black line), or six control treatments (gray line). Data are cumulative over nine independent experiments conducted over two years. (B) Data from a single representative YUMM1.7 transimmunization experiment, with each line showing tumor growth for an individual mouse. (A and B) “PBS control” mice in all experiments were bled on the same schedule as the experimental animals, but received six sterile PBS re-infusions. Error bars represent SEM, p-values calculated for each time point using Sidak’s multiple comparisons test; **, $p = 0.0013$; ****, $p < 0.0001$. This figure has been modified from¹¹.

Figure 3: Transimmunization requires monocytes and platelets in TI plate-passed PBMC fraction, as well as 8-MOP_A treatment of antigen-matched tumor cells and 8-MOP_A sparing of PBMC. (A) YUMM1.7 tumor volume over time plotted for C57BL/6 mice inoculated with 1×10^5 YUMM1.7 tumor cells, and receiving either six transimmunization treatments (solid black lines), six control treatments (solid gray lines), or six TI treatments where monocytes or platelets were depleted from PBMC prior to plate passage step (using a-CD11b and a-CD41 depletion kits, respectively), or plate passage was omitted (dotted lines). (B) Tumor volume over time plotted for C57BL/6 mice inoculated with 1×10^5 YUMM1.7 tumor cells, and receiving either six transimmunization treatments (solid black lines), six control treatments (solid gray lines), PBMC alone (dashed line), 8-MOP_A-treated YUMM1.7 cells alone, or TI using 8-MOP_A-treated MC38 tumor cells (dotted lines). (C) Tumor volume over time plotted for C57BL/6 mice inoculated with 1×10^5 YUMM1.7 tumor cells, and receiving either six Transimmunization treatments (solid black lines), six control treatments (solid gray lines), six TI treatments where PBMC were uniformly exposed to 8-MOP_A immediately before plate passage, six TI treatments where PBMC were uniformly exposed to 8-MOP_A immediately after plate passage, or six treatments where TI cells after plate passage were mixed 1:1 with an equal number of PBMC that have been uniformly

exposed to 8-MOP_A irradiation (dotted lines). (A, B and C) “PBS control” mice in all experiments were bled on the same schedule as the experimental animals, but received six sterile PBS re-infusions. Data are provided for representative experiments. Bars represent SEM, P-values calculated for each time point using Sidak’s multiple comparisons test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; NS = differences not significant. This figure has been modified from¹¹.

Figure 4: TI protocol with TI chamber rapidly induces DC maturation, unique activation profile, and T cell activating capacity in human PBMC, dependent on plate passage and platelets.

A. FACS analysis of the indicated markers in CD11c⁺ cells among either freshly isolated human PBMC (“control”), PBMC treated with the TI protocol (“TI”), or TI-treated PBMC where plate passage was omitted, platelets were depleted using the α -CD41 bead kit prior to plate passage, or both of the above were performed. Data summarize six independent experiments with three blood donors. Bars represent mean values, while error bars represent SEM. P-values for each comparison calculated using paired t-test; *, $p < 0.05$; **, $p < 0.01$. Panels have been modified from¹¹. **B.** Platelet-containing or platelet-depleted TI-treated human PBMC were co-incubated overnight with an irrelevant (SIINFEKL) peptide, or with long peptide for head-and-neck squamous cell carcinoma-associated HPV E7 protein. The PBMC were then used to stimulate a human CD8 T cell line specifically reactive to E7 peptide. T cell stimulation was measured by IFN γ production after 5 days of culture. **(C)** Platelet-containing or platelet-depleted TI-treated human PBMC were co-incubated overnight with 8-MOP_A-treated head-and-neck squamous cell carcinoma cell line SCC61, either expressing (SCC61 HPV E6/7) or not expressing (SCC61 no HPV) the antigenic HPV E6 and E7 proteins¹⁹. The PBMC were then used to stimulate a human CD8 T cell line specifically reactive to E7 peptide. T cell stimulation was measured by IFN γ production after 5 days of culture. **(B and C)** Data show representative experiments with three replicates in each. Bars represent mean values, while error bars represent SEM. P-values for each comparison calculated using Sidak’s multiple comparisons test; ***, $p < 0.001$; ****, $p < 0.0001$.

Table 1: TI protocol with TI chamber rapidly induces DC maturation equivalent to that induced by TI protocol with the clinical ECP chamber. FACS analysis of change of the indicated markers from corresponding IgG controls in live human CD11c⁺ cells among either freshly isolated PBMC (“untreated”), PBMC passed through the clinical ECP plate following the TI protocol (“ECP plate with TI protocol”) and analyzed following overnight incubation, or PBMC treated with the TI protocol (“TI plate with protocol”) and analyzed following overnight incubation. For markers, such as HLA-DR, where the entire cell population was altered, data are expressed as change in mean fluorescence intensity (MFI). For markers where only a subset of cells express the marker, such as CCL2, difference in percent marker-positive cells of live CD11c⁺ PBMC is instead represented. Data summarize six independent experiments with three blood donors. Data for each marker are expressed as average \pm standard error of the mean (SEM). P-values for each comparison calculated using paired t test; NS = differences not significant. Table has been modified from¹¹.

Video 1: Live cell imaging of platelet and immune cell interactions within the TI plate.

PBMC were prepared as described in protocol section 2 above and exposed to the transimmunization plate as described in section 4, except the static incubation period in step 4.2.3 was reduced from 1 h to 30 min. During the TI protocol, the TI plate, which is identical in size to a standard microscope slide, was fitted into the slide holding stage of an fluorescence imaging system and the cells within it were imaged at 40x magnification and at 37 °C during the plate filling (4.2.2), plate incubation (4.2.3), and plate flow (4.3.5) stages. Continuous images were acquired and movies produced using the “Automated Scanning Routine” software. The captions below the video indicate the stage of the protocol that the cells are being filmed at. Arrows and circles in the video, with the associated captions, point out the cells and areas of interest. The 10 μ M scale bar is present in the lower right corner throughout the video.

DISCUSSION:

The miniaturized device and protocol described above for the first time allow for efficient laboratory investigation of mechanisms of ECP in mouse experimental systems, and in small human blood samples. This is a great advance; for instance, it allowed us to demonstrate for the first time the efficacy of transimmunization against solid tumors in a mouse model ¹¹, opening the future possibility of a similar application in human oncology.

Prior to the development of the transimmunization device and method described here, it was impossible to fully investigate all aspects of ECP. In mouse models, although the 8-MOP_A aspect of the therapy could be replicated somewhat by treating cells in a Petri dish^{12,20}, there was no capacity to integrate into the method the plate passage, which has been shown to provide dynamic platelet interactions that are critically important for ECP’s physiological DC activation¹⁴. In human studies, alternatively, the flow component was fully present, but the ability to selectively expose specific cellular components to 8-MOP_A, or to protect them from it, was missing^{21, 22}. This prevented the full understanding of ECP mechanism, and its optimization for immunity or tolerance. In addition, the amount of blood required to work with the clinical ECP apparatus is large, hampering scientific inquiry. The miniaturized ECP device and protocol described here for the first time allow for efficient, fully flexible and tunable laboratory ECP modeling. In addition, the TI plate allows for real-time visualization and monitoring of cell interactions within the plate by microscopy.

For the protocol’s success using both in vivo and ex vivo systems, it is critical that the treated PBMC contain monocytes that can be activated into functional DCs. For this activation to proceed, it is also necessary to ensure the PBMC fraction contains a physiologic number of healthy, activatable platelets, and that the TI plate passage protocol is followed closely. In order to direct the newly-activated DCs towards a specific reactivity, they must be provided with antigen. We have found that the most efficient method of antigen delivery for anti-cancer immunity is overnight co-incubation of the newly activated DCs with antigen-containing 8-MOP_A-exposed tumor cells. This has the added advantage of being able to create an immunogenic anti-cancer response without necessitating prior knowledge of the tumor antigens, by allowing the DCs to select them. However, in cases where the antigen is known, we had some success in ex vivo systems when using free peptides as antigens in co-incubation. For immunogenic applications, the DCs themselves should be protected from 8-MOP_A exposure. Finally, in in vivo

experiments, it is important to work with an animal model that is capable of anti-tumor immunity. Transimmunization works by creating activated, antigen-specific DCs, which work in the body to initiate innate and adaptive immune responses. Impaired activity or lack of the NK, CD4, or CD8 T cells in the treated mouse will impact the protocol's efficacy^{5, 11}.

While the protocol described here is a proof-of-principle one that has been optimized for a mouse solid syngeneic tumor model, it nevertheless reveals many opportunities. The mechanisms of ECP in oncology are only just being elucidated, and there is still much room for improved understanding. More broadly, the ability to generate physiologically activated mouse and human DCs, and to selectively direct them towards antigen-specific immunity has many potential applications beyond cancer. The ability to do the same, but instead direct the DCs towards antigen-specific tolerance, as is suggested by the tolerizing efficacy of ECP itself, also has wide-ranging medical implications. With this method, we hope to provide the tools and open a productive avenue of research for anyone with an interest in physiologic DC therapies.

ACKNOWLEDGMENTS:

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DISCLOSURES:

Yale University owns patents deriving from the dendritic cell research of Prof. Richard Edelson that have been licensed to a Yale start-up company, Transimmune AG. Richard Edelson and Michael Girardi are scientific consultants to Transimmune AG, while Olga Sobolev is a Transimmune AG employee. Transimmune AG does not have any current commercial products, however on a collaborative basis it produces the Transimmune plates used in this article. It is possible that these three authors could potentially benefit from commercialization of these discoveries in the future.

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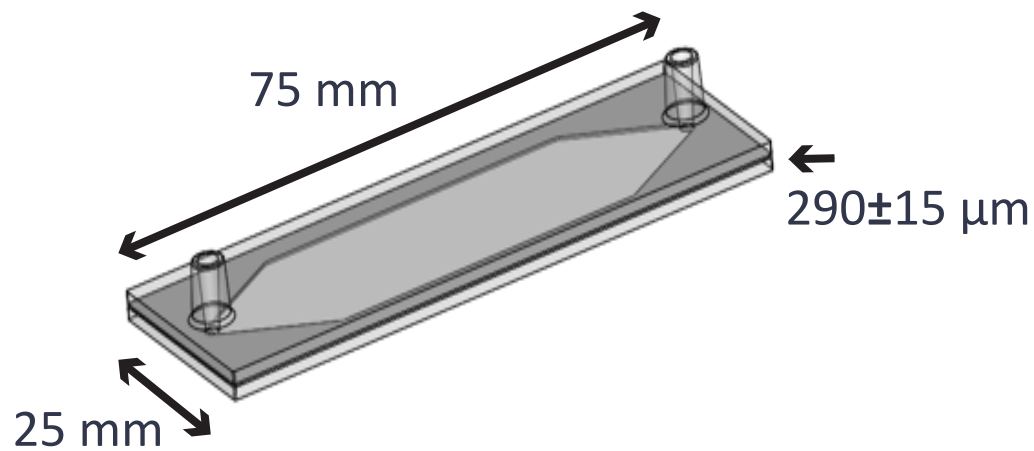
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Figure 1

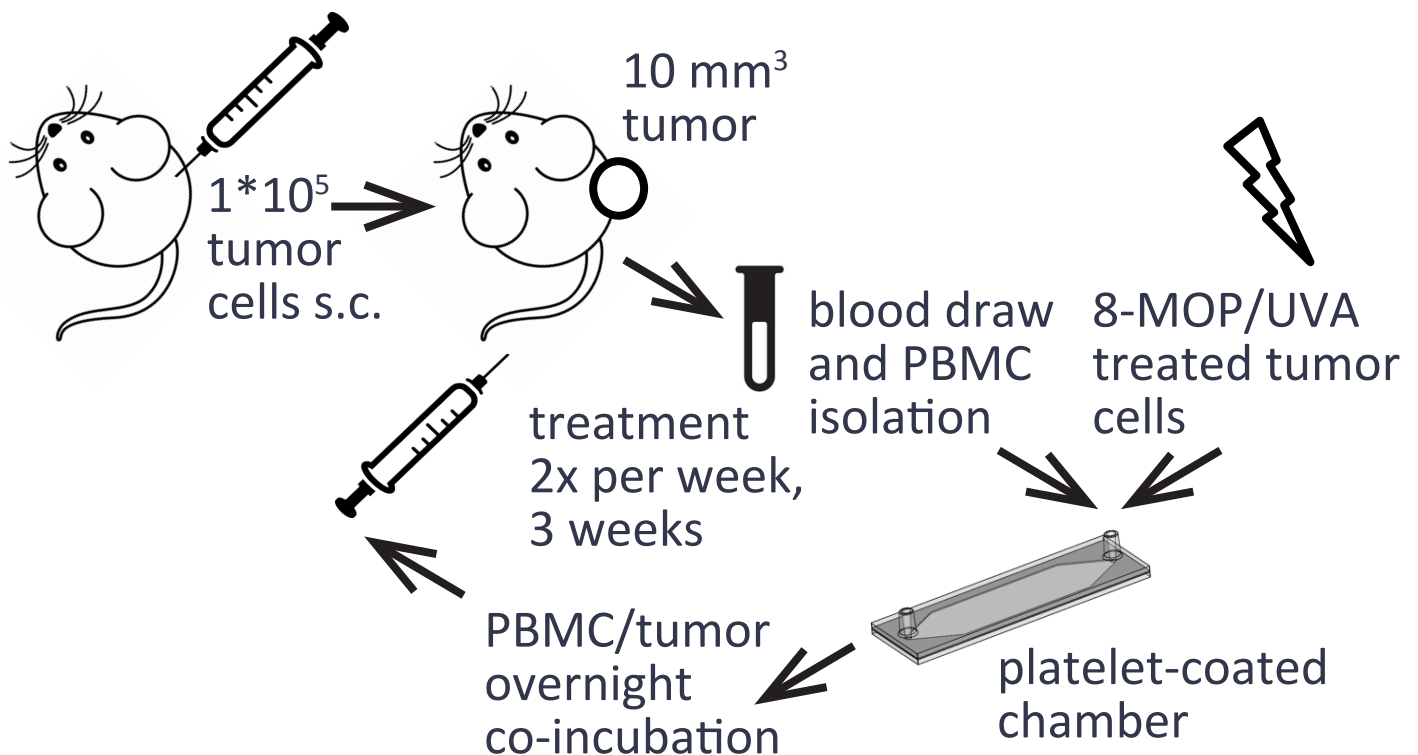
A

polystyrene Transimmunization (TI) plate



B

TI treatment of syngeneic mouse tumors



A

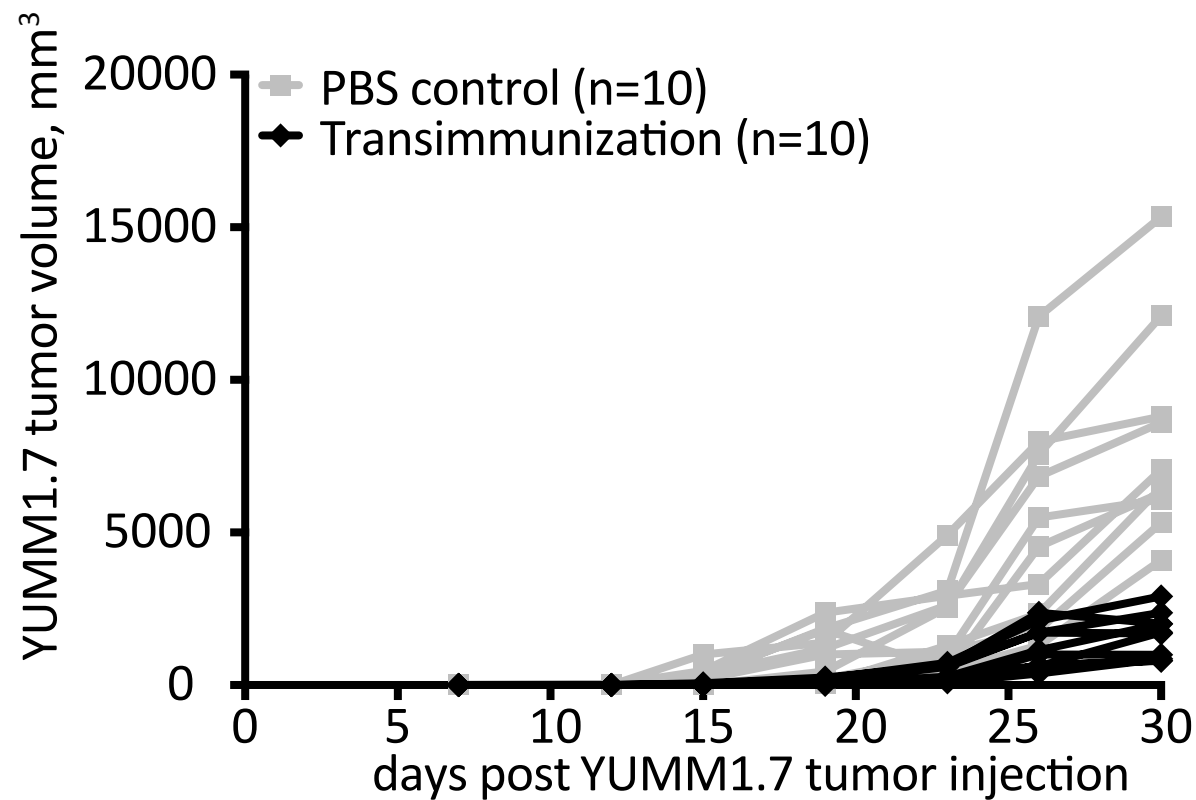


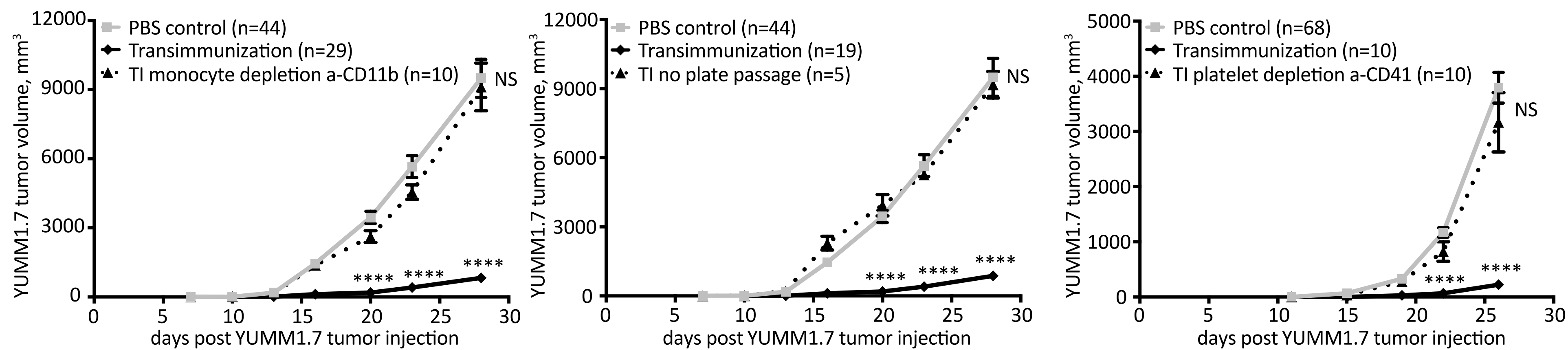
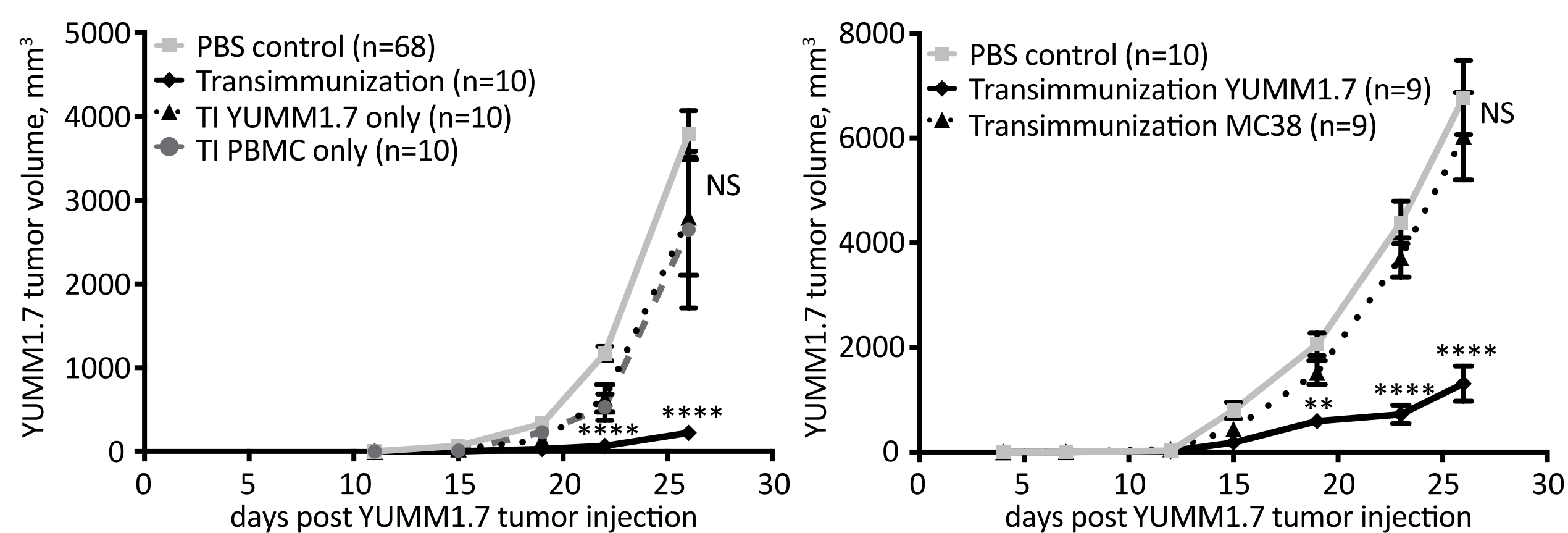
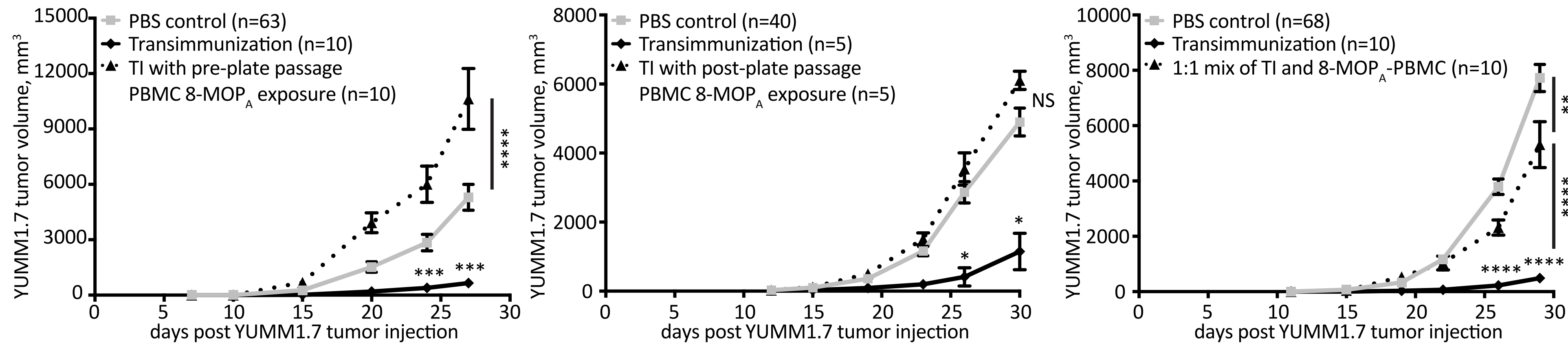
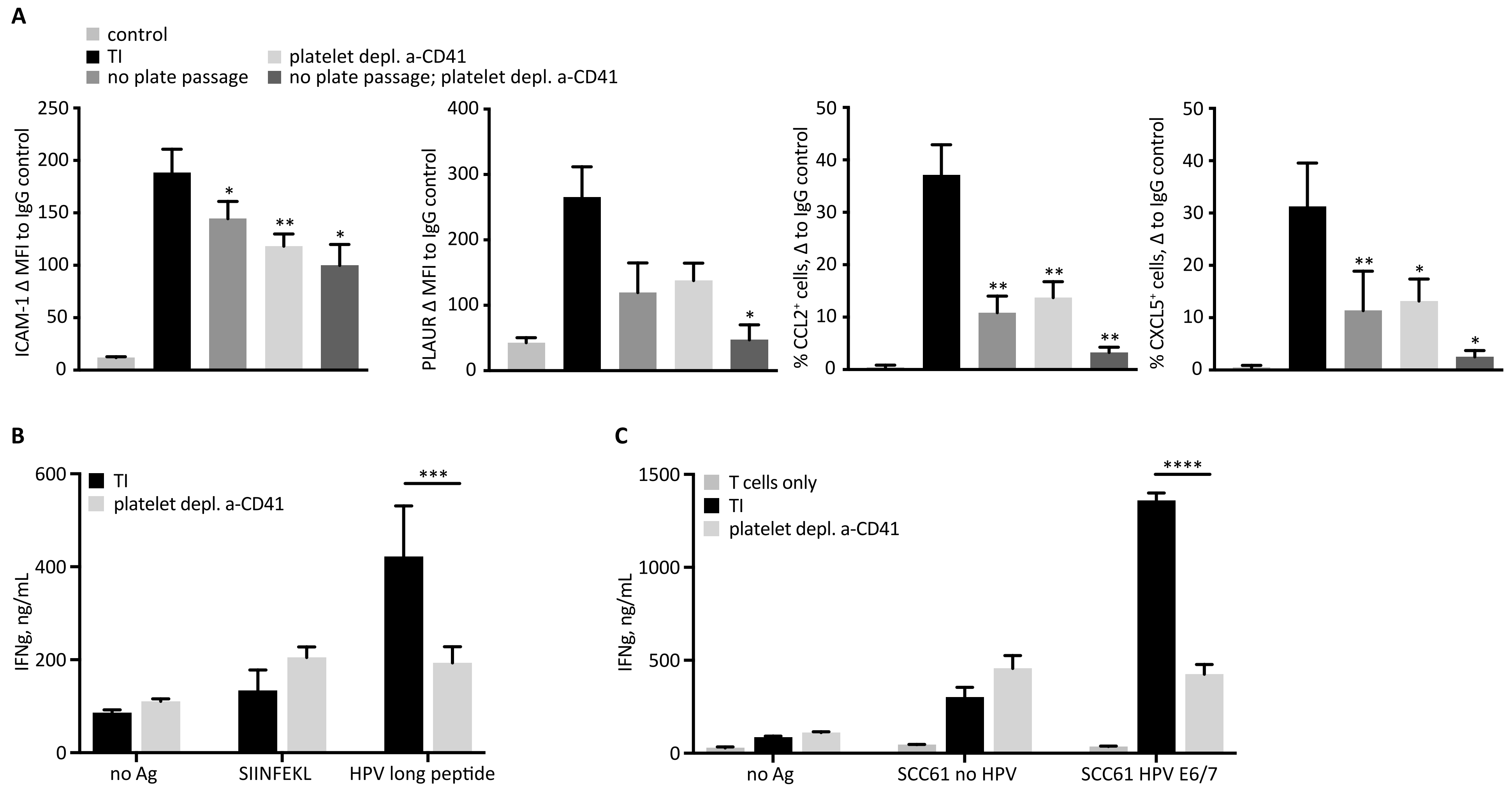
Figure 3**A****B****C**

Figure 4



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Video or Animated Figure

Olga Sobolev - JoVE Edelson Video1.mp4



marker	parameter	untreated	ECP plate with TI protocol	TI plate with TI protocol
HLA-DR	Δ MFI	100.1 \pm 42.4	439.8 \pm 152.5	417.7 \pm 152.2
CD80	Δ %	3.9 \pm 1.1	22.3 \pm 7.2	24.5 \pm 7.2
CD83	Δ MFI	0.3 \pm 0.2	53.3 \pm 9.7	51.4 \pm 17.4
CD86	Δ MFI	10.8 \pm 1.7	103.9 \pm 23.4	87.1 \pm 17.6
PLAUR	Δ MFI	54.5 \pm 12	721.4 \pm 183.6	528.7 \pm 135.5
ICAM1	Δ MFI	12.2 \pm 1.6	179.6 \pm 28.5	192.5 \pm 25.4
ITGB5	Δ MFI	53.6 \pm 25.7	97.1 \pm 31.6	103.8 \pm 32.8
CCL2 (MCP-1)	Δ %	0.6 \pm 0.5	70.1 \pm 6.7	55.2 \pm 8.9
CXCL5	Δ %	0.7 \pm 0.4	39.1 \pm 7.2	41.5 \pm 4.7
CXCL16	Δ %	0.3 \pm 0.2	28.0 \pm 8.8	35.3 \pm 11.7
CD105 (endoglin)	Δ MFI	3.6 \pm 0.3	124.3 \pm 25.4	141.8 \pm 33.2
CD112 (nectin 2)	Δ MFI	10.9 \pm 1.8	47.3 \pm 5.2	50.9 \pm 9.2
CD120a (TNFR-1)	Δ MFI	10.1 \pm 2.6	2.2 \pm 1.4	1.8 \pm 1.1
CD137L (4-1BBL)	Δ MFI	2.9 \pm 1.5	1.0 \pm 0.4	1.2 \pm 0.6

[illegible]

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
8-MOP (UVADEX 20ug/mL, 10 mL)	Therakos, Inc	Rx only, NDC No. 64067-0216-0	Protocol steps 3 and 8 - mouse and human 8-MOP/UVA treatment of cells
AB serum	Lonza BioWhittaker	14-498E	Protocol step 8.5 - overnight culture of human PBMC
ACK red cell lysis buffer	Lonza BioWhittaker	10-548E	Protocol step 2 - mouse PBMC preparation
Anesthesia Tabletop V1 system with active scavenging	VetEquip	901820	Protocol steps 1 and 7 - mouse sc tumor introduction and TI administration
Autologous mouse plasma	prepare in lab	n/a	Prepare per protocol step 2.4, use in 7.1 - mouse TI treatment administration
Autologous mouse serum	prepare in lab	n/a	Prepare per protocol step 5, use in step 6.1 - overnight culture of mouse PBMC
C57Bl/6J mice	Jackson labs	0000664	Protocol steps 1, 2, 5 and 7 - mouse tumor injection, blood/serum collection, and therapy re
Cheek bleed GoldenRod lancet, 5 um	Medipoint	9891620	Protocol step 2 - mouse PBMC preparation
Clear RPMI	Gibco by LifeTech	11835-030	Protocol steps 6 and 8 - overnight culture of mouse/human PBMC
DMEM/F12	Gibco by LifeTech	11330-032	Protocol steps 1 and 2 - YUMM1.7 cell culture
EasyGrip Petri Dishes, 35mm	Falcon	351008	Protocol steps 5 and 8 - overnight culture of mouse/human PBMC
Eppendorf 1.5mL conical tubes	DOT scientific	1700-GMT	Protocol steps 1-8
FBS (fetal bovine serum, heat-inactivated)	SAFC Biosciences	12306C-500mL	Protocol steps 1-4 - YUMM1.7 cell culture, 8-MOP/UVA treatment of cells, TI plate
Hemavet 950FS hematology counter	Drew Scientific	HV950FS	Protocol step 8.2 - monitoring human platelet numbers
Heparin 5,000U/mL	McKesson Packaging services	949512	Protocol steps 2 and 8 - mouse and human PBMC preparation
Isoflurane	Abbott Laboratories	5260-04-05	Protocol steps 1 and 7 - mouse sc tumor introduction and TI administration
Lympholyte M lymphocyte isolation medium	Cedarlane Labs	CL5035	Protocol step 2 - mouse PBMC preparation
Non-essential amino acids	Gibco by LifeTech	11140-050	Protocol steps 1 and 2 - YUMM1.7 cell culture
PBS (1x DPBS, (-) Ca+2, (-) Mg+2)	Gibco by LifeTech	14190-144	Protocol steps 1-7
Pen/strep	Gibco by LifeTech	15140-122	Protocol steps 1 and 2 - YUMM1.7 cell culture
Polypropylene 15mL conical tubes	Falcon	352097	Protocol steps 1-8
Programmable 2-channel syringe pump	New Era Pump Systems Inc	model NE-4000	Protocol steps 4 and 8 - running the TI plate
Retro-orbital injection needles, 27G x 1/2	BD Biosciences	305109	Protocol step 7 - mouse TI treatment administration
Syringes, 10mL (LUER-LokTip)	BD Biosciences	309604	Protocol steps 4, 8 - running the TI plate
Syringes, 1mL (Slip tip)	BD Biosciences	309659	Protocol steps 1, 4, 7, 8
TI plate and tubing set	Transimmune AG	not commercially available	Please contact Prof. R. Edelson or Transimmune in order to obtain the device on a collaborative basis.
TI plate running platform	Transimmune AG	not commercially available	Please contact Prof. R. Edelson or Transimmune in order to obtain the device on a collaborative basis.
Tissue culture flasks, T75 (75 cm2)	Falcon	353136	Protocol steps 1, 2, 6 and 8 - mouse and human cell culture
Tissue culture plates, 12-well	Falcon	353043	Protocol steps 3 and 8 - mouse and human 8-MOP/UVA treatment of cells
Tissue culture scrapers	Falcon	353085	Protocol steps 6 and 8 - overnight culture of mouse/human PBMC
Trypsin-EDTA 0.25% (1x)	Gibco by LifeTech	25200-056	Protocol steps 1 and 2 - YUMM1.7 cell culture
Tumor injection needles, 25G x 5/8	BD Biosciences	305122	Protocol step 1 - mouse subcutaneous tumor introduction
UVA irradiator	Johnson and Johnson	not commercially available	The apparatus was specifically developed by J&J for Prof. R. Edelson's laboratory. Several machines are available in the laboratory on a collaborative basis; please contact Prof. R. Edelson for use of one. Alternative UVA irradiators are commercially available but have not been tested by us.
Invitrogen EVOS FL Auto 2 Imaging System	Invitrogen		fluorescence imaging instrument

ARTICLE AND VIDEO LICENSE AGREEMENT

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Author(s):	Alessandra Ventura, Aaron Vassall, Alp Yurter, Eve Robinson, Renata Filler, Douglas Hanlon, Katrina Meeth, Harib Ezaldein, Michael Girardi, Olga Sobolev, Marcus W. Bosenberg, Richard L. Edelson

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CORRESPONDING AUTHOR

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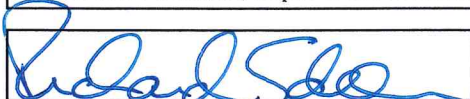
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Chair and Professor, Department of Dermatology, Yale School of Medicine

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Date:

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To the Editor:

We are delighted to hear the very positive response from our reviewers and editor, and to reply to their comments below. Following Dr. Alisha DSouza's suggestions, we have substantially edited the experimental protocol to include more detail, as is reflected in the changes tracked in the main manuscript. We have also implemented the suggested changes to the figures, and removed the video pending further editing, which, unfortunately, will not be possible within our January 3rd resubmission deadline due to the holiday closure of Yale core facilities – with the hope of being permitted to submit the edited version of the video at a later point, as soon as we possibly can. We thank the journal for the opportunity to further refine the protocol and improve its presentation, and hope that the manuscript is acceptable in its present form.

We thank you again for considering this paper for publication in JoVE.

Sincerely,

Richard L. Edelson, MD
Aaron and Marguerite Professor and Chairman
Department of Dermatology
Yale University School of Medicine

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly reviewed the manuscript for any such issues, and hope to have eradicated them.

2. Figure 1B: Please include a space between numbers and their corresponding units (e.g., 10 mm³). Please define s.c. in the figure legend.

This has been done, please see the Figure 1 file.

3. Figure 3: Please define NS in the figure legend.

This has been done, please see the Figure 3 legend, line 588 of main manuscript.

4. Figure 4A: Please convert it to a table, and upload the table to your Editorial Manager account as an .xls or .xlsx file.

This has been done, please see the updated Figure 4 (with the table removed) and the accompanying Table 1 in the Excel format.

5. Video 1: Please include a scale bar to provide context to the magnification used.

Define the scale in the appropriate figure Legend.

The video editing requires assistance from the Yale microscopy core facility, where it was first assembled. Unfortunately, the facility is closed for the holidays, only re-opening on January 2nd, which we feel is too short a time before our January 3rd resubmission deadline.

Therefore, with the editor's permission, if it is possible we'd like to remove the video for now, and add it to the submission at a later point – with the scale bar added to the video and defined in the legend, and the very pertinent changes suggested by Reviewer 3 included.

6. Please revise lines 358-360 to avoid previously published text.

This has been done, please see the re-written lines 489-491 of main manuscript.

7. Please define all abbreviations before use.

We have thoroughly reviewed the manuscript for any such issues, and believe to have defined all abbreviations before first use.

8. Please use the micro symbol μ instead of u. Please abbreviate liters to L to avoid confusion.

This has been done, please see the tracked changes throughout the manuscript.

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such

as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised the protocol to ensure this as best as possible, please see tracked changes throughout the manuscript. Notes have only been left where they are necessary to point out where the protocol may be modified in essential ways by the investigators following it.

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All commercial language has been removed and replaced with generic product descriptions and references to the Table of Materials, as suggested. Please see thracked changes in the manuscript.

11. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We have revised the protocol to ensure this as best as possible, please see tracked changes throughout the manuscript. Additional sub-steps have been added where necessary.

12. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See some examples below.

13. 1.2: Please specify what standard conditions are.

Standard conditions have been defined.

14. 1.5: Please mention how animals are anesthetized (e.g., specify concentration of isoflurane) and how proper anesthetization is confirmed.

Isoflurane concentration and method of confirming anesthesia have been included.

15. 2.2: How many treatments are included? Please specify.

The number of treatments has been specified more clearly.

16. 2.3.1: Please specify the Lympholyte M protocol (centrifugation parameters). We need such details for filming.

The Lympholyte M protocol centrifugation parameters have been specified.

17. 2.4.1: Please provide the composition of ACK buffer. If it is purchased, please cite the Table of Materials.

The ACK buffer is purchased, thus the Table of Materials has been cited.

18. 5.1: What volume of blood is collected?

The volume of blood has been specified.

19. 6.1: Please specify incubation conditions.

Standard incubation conditions have been defined.

20. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

The Table of Materials has been sorted in alphabetical order, please see the revised Table of Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe their protocol for generating physiologic immunogenic dendritic cells for use in either murine or human studies. The detailed explanations of all steps, including tumor preparation, whole blood preparation, TI plate use, and concerns for which to watch are all outlined clearly. Additionally, the figures and videos further enhance the manuscript and support the procedures being presented by the authors. I believe these protocols will be of use to those investigating dendritic cell manipulations for immunization or tolerization.

Major Concerns:

None

Minor Concerns:

Please have copy-editor check use of commas throughout manuscript (there may be too many). Also, please have all materials mentioned include company, location, and model/catalog# so that they can be easily ordered by readers interested in using the protocols described.

Reviewer #2:

Manuscript Summary:

Extracorporeal photopheresis (ECP) is a FDA-approved therapy for cutaneous T cell lymphoma (CTCL), and is also widely used for treatment for many T-cell mediated diseases. Recently, the researches of applications of ECP for cancer therapy draw many attentions. Nevertheless, the mechanisms of ECP action under different diseases are not

fully understood. One of obstacles is lacking a good experimental system/device to study and assess different effects in vitro and in vivo. The authors in this manuscript introduce the Transimmunization (TI) chamber, a scaled-down version of the clinical ECP leukocyte-processing device, suitable for work with both mouse models, and small human blood samples. Overall, the methods and protocols are appropriate for the methods Journal, and researchers/scientists in this field will be interested in this system.

Major Concerns:

1. Because this is a methods article, only one video for the microscopic observation is not enough to introduce a new system/device. Additional videos for some key steps are needed for readers to understand the TI, for examples, collection of peripheral blood mononuclear cells (PBMC); 8-MOP/UVA treatment of tumor cells; TI plate passage of cells; preparation of autologous mouse serum; co-incubation of PBMC with antigen; re-injection of TI-treated cells to mice.

We thank the reviewer very much for their interest in this protocol, and for their desire to see it filmed in as much detail as possible. We wish the same, and have tried our best to select for filming these parts that are not described elsewhere, or have unique features that are essential to the TI method. These sections of the protocol, as the reviewer points out, include the collection of PBMC, the 8-MOP/UVA treatment of tumor cells, the TI plate passage of cells, the co-incubation of PBMC with antigen overnight, and the preparation of cells for re-injection.

We did not select the preparation of autologous mouse serum for filming, because that is a fairly common technique and is not unique to our protocol. For the same reason we did not select for filming the actual re-injection of the cells into mice, only cell preparation for injection, again feeling that retro-orbital cell injection itself is not unique to the TI method, and is a well-described veterinary protocol. However, we will be happy to include these sections for filming, should the editor grant us additional filming time.

2. For the classic ECP therapy for patients, the whole process will be done within the same day, instead of overnight co-incubation as the TI. Is there any data/justification for overnight incubation?

We thank the reviewer for the opportunity to clarify this key point.

As described in the Introduction, the TI protocol differs in several features from standard clinical ECP, the addition of overnight incubation being a very important such feature (lines 125-129). The reason for these alterations is that the Transimmunization protocol aims to not only replicate ECP in the laboratory, but to rationally optimize it. Since it has been demonstrated that antigen-loaded dendritic cell production is the key mechanism of action in ECP, it was next hypothesized that extending the time of contact between the dendritic cells newly produced during cell passage through the ECP device, and the apoptotic tumor cells also produced in the device, will allow for better tumor cell internalization and processing by dendritic cells, and thus improve the clinical impact.

This hypothesis was tested clinically in Cutaneous T Cell Lymphoma (CTCL), where the addition of overnight incubation alone, with no other changes to the ECP protocol, led to

clinical response in 60% of patients previously refractory to standard ECP (reference 13 in the manuscript, Girardi et al, 2006). Furthermore, in the TI mouse model of ECP, the inclusion of the overnight incubation step led to tumor growth reduction with the TI protocol.

We therefore believe that at least in the immunogenic anti-tumor modality of ECP, the co-incubation step is an important improvement. However, it remains to be investigated whether it is equally an improvement in the tolerogenic aspect of ECP, and what length of incubation is optimal in the immunizing and the tolerizing modalities. We hope these questions will be among those studied, as the scientific exploration of ECP continues.

Reviewer #3:

Manuscript Summary:

I think that Edelson and col. present in this article a very important approach to mechanism of action of extracorporeal photopheresis and a derivation of it, that is the treatment of solid neoplasia based in the same mechanism of action of it.

The article shows the knowledge of the author on this topic, and states that the continuous research in the same field may lead to important advances.

Major Concerns:

No.

Minor Concerns:

I think that watching the video does not provide important information. I suggest looking for some way to point out different type of cells that are observed or to make it more instructive.

We thank the reviewer for their observation, and the opportunity to improve on the video. Following the reviewer's and the editor's suggestions, we would like to add to the video a scale bar, to give a better idea of the relative cell sizes in the device, and to also include arrows and labels pointing out the leukocytes, the gradual coating of the plate with platelets, and leukocyte rolling along the plate-bound platelets. We hope that will add to the quality and the instructive value of the video.

However, the video editing requires assistance from the Yale microscopy core facility, where it was first filmed and assembled. Unfortunately, the facility is closed for the holidays, only re-opening on January 2nd, which we feel is too short a time before our January 3rd resubmission deadline.

Therefore, with the editor's permission, if it is possible we'd like to remove the video for now, and add it to the submission at a later point – with the scale bar added to the video and defined in the legend, and the very pertinent changes suggested by Reviewer 3 included. We still feel there is a value to the community visualizing for themselves what happens within the device during this protocol.