Modified Extracorporeal Photopheresis As An Immunotherapy In Murine Melanoma

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To the love my life, Lauren, 
for her unwavering, support, 
patience, friendship and Love.
Abstract

Extracorporeal Photochemotherapy (ECP) is a widely used immunotherapy for cutaneous T cell lymphoma, as well as an immunomodulatory treatment for graft versus host disease (GVHD) and rejection of allografts. We hypothesized that ECP’s physiologic induction of large-scale monocyte-to-dendritic antigen presenting cell (APC) conversion is mechanistically responsible for both its anti-cancer effect and its tolerogenic impact in the transplant setting. To interrogate this possibility in an experimental system, we developed an ECP device that is scalable from mouse to man and tested its capacity to produce APCs that, when advantageously tuned and tumor antigen-loaded, can limit the growth of otherwise lethal tumors in the engineered Yale University Mouse Melanoma (YUMM) 1.7 model (driven by PTEN loss, BRAFV600E activation and CDKN2A mutations). Untreated control mouse tumors (N=169) were 7 to 10-fold (p<0.001) larger than ECP treated (N=40) tumors at the termination point, as required due to control tumor size. Depletion of ECP-induced APCs from the cellular vaccine preparation prevented the immunoprotective effect (p<0.01), indicating a primary role for ECP-induced APCs. Depletion of platelets from the ECP-processed cellular suspension also prevented the immunizing effect (p<0.01), substantiating prior in vitro evidence that physiologic ECP-induction of functional DC is signaled by transient monocyte adherence to platelets. Addition of 8-MOP-UVA-treated PBMCs to the otherwise immunoprotective APC vaccine completely reversed the immunoprotective vaccine effect (p<0.01), revealing that maturationally truncated APCs are counterproductively tolerogenic. Collectively, these results show that, in ECP, platelet-induced tumor-loaded mature APCs are immunotherapeutic for established murine melanoma, while immature APCs are tolerogenic. These findings verify a pivotal role for ECP-manufactured...
APCs and are the first *in vivo* study to suggest the intriguing possibility that ECP can be modified for immunotherapy of solid tumors.
Acknowledgements

The work presented in this thesis was generated from a joint project with Alessandra Ventura and me. This is collectively our work and she deserves equal recognition for it.

Dr. Richard Edelson was our primary mentor. He guided our experimental design and focused our scientific questions at every point in this process. Without Dr. Edelson, this thesis would not have happened, many times over. I am greatly indebted to him.

Dr. Robert Tigelaar and Dr. Marcus Bosenberg were important mentors, providing experimental insight and guidance, as well as materials and expertise.

Harib Ezaldein, a fellow medical student, was pivotal in the early stages of this project. It was our discussions which provided the impetus to start this project. His engineering insights were extremely helpful in designing our second generation Transimmune box, which for a time, was used for both plate passage and PUVA exposure. Over the course of these experiments, he has continued to be a source of ideas and advice.

Eve Robinson, Dr. Douglas Hanlon, and Katrina Meeth all provided important insight and advice throughout this process. Eve also helped with running experiments. Katrina created and provided us with the YUMM tumor line.

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Introduction

Direct tumor cytotoxicity has been a major focus of anti-cancer therapies for much of the 20th century. However, with the recent clinical successes of immunotherapy, interest has grown in the therapeutic potential of fighting cancer with the body’s immune system (32-35). Traditional dendritic cell (DC) cancer vaccines, although promising, have failed to significantly improve clinical outcomes (36). Efforts to produce clinical remissions by cellular vaccination have been handicapped by two practical limitations: use of antigen presenting DCs which are produced ex vivo under conditions which cannot be reproduced in vivo, thereby limiting their efficacy, and loading of these DCs with antigens not distinctive to the array of patient specific markers. However, one DC-based method, “Extracorporeal Photochemotherapy (ECP)”, developed by Dr. Richard Edelson, and now effectively used worldwide to treat advanced cutaneous T cell lymphoma (CTCL), offers potentially valuable advantages. ECP’s mechanism was recently shown to centrally involve physiologically-induced DCs, efficiently loaded with apoptotic CTCL cells, whose antigens are then used to stimulate clinically potent CD8 anti-CTCL clinical responses. Hence, it is now possible to examine whether ECP can be a treatment for other cancers, including solid tumors.

The ECP mechanism

CTCL describes a group of non-Hodgkin’s lymphoma malignancies, characterized by skin lesions caused by infiltrating malignant T cells (23). Prior to the introduction of ECP, CTCL was almost universally fatal. ECP treatment for CTCL was established in 1987 with a groundbreaking phase I clinical study in the New England Journal of Medicine by Edelson and colleagues. They reported
that 27 of 37 (73%) CTCL treatment resistant patients receiving ECP responded with greater than 25% clearing of skin. Of the responding patients, 33% had greater than 75% skin clearance. All treated patients had no significant side-effects (12). Within a year, it was the first FDA approval of its kind, and far ahead of its time. It would be two decades before another cellular anti-cancer immunotherapy was approved (12, 10).

ECP is a systemic therapy that sequentially involves the separation of leukocytes from whole blood, routing of leukocytes through a chamber with exposure to photochemotherapy via 8-methoxypsoralen (8-MOP or psoralen) and ultraviolet A (UV-a) light, and return of all blood components to the patient. Psoralen irreversibly forms covalent bonds with DNA (ie., photoadducts) in the presence of UV light, inducing cytotoxic DNA damage to exposed cells (23).

For much of the time following its approval, the mechanism of ECP remained largely elusive. Initially, the effectiveness of the treatment was thought to be due to photochemotherapy-induced T cell cytotoxicity. However, subsequent studies by Edelson and colleagues showed that only 10% of circulating malignant T cells are exposed to the psoralen/UVa (PUVA) treatment, which lead them to postulate that the anti-tumor response was immune mediated (10). Additionally, ECP was later shown to produce immunosuppressive responses in graft vs host disease (GVHD) patients. These paradoxical effects of immune activation and suppression in different patient populations clouded the mechanism further. Recently, however, several major elements of this mechanism have come to light: 1) ECP efficiently converts circulating blood monocytes into physiologic antigen presenting DCs; 2) PUVA creates immunogenic apoptotic malignant T cells which are processed and presented by
these DCs to initiate a CD8 anti-tumor response; 3) tolerance, in part, results from maturational truncation of monocytes following PUVA exposure (8, 10, 27).

*ECP-treated malignant cells can be immunogenic.* Edelson and Berger performed experiments in the mid-90s that involved immunizing mice against an aggressive form of lymphoma (10, 15, 16). They used 2B4.11 hybridoma cells that were the product of pigeon cytochrome C primed T lymphoblasts and BW5147 thymomas. The mice were injected with treated 2B4.11 cells two times per week for four weeks, then challenged with untreated 2B4.11 malignant cells. All 10 of the control mice died within 4 weeks. Two of the 15 treated mice were dead by week 5, while those remaining were free of disease (10, 15,). To further increase the treatment efficacy, they loaded IL4-cultured DCs with PUVA-treated malignant cells followed by co-injection and subsequent challenge as before. All the control mice were dead within 41 days. Mice receiving treatment with DCs and PUVA treated 2B4.11 cells had improved survival, with the best group demonstrating “100% inhibition of tumor growth” (10, 16). Later studies established that PUVA-treated CTCL cells undergo apoptosis and enhance their MHC class I synthesis (3, 20, 10). These results indicated that PUVA-treated malignant cells are sufficiently antigenic.

*ECP passage manufactures physiologic DCs via monocyte-platelet interactions.* Berger and Edelson also developed “table top” ECP using human PBMCs and found that the resulting monocytes began differentiating into DCs within the first 24 hours post-treatment (22, 17). Their first report showed that treated monocytes began to display a DC phenotype consisting of CD83 and CD36 expression in the absence of CD14 staining. These cells were phagocytic, capable of engulfing apoptotic malignant cells, and activating T cells in vitro (22). Subsequent
studies validated these findings, including a genome wide analysis showing that ECP-treated monocytes exhibited a gene expression pattern consistent with DC differentiation (17). Importantly, recent work has shed light on how ECP contributes to this DC differentiation. Durazzo et al. demonstrated that ECP induces monocytes to enter the DC differentiation pathway via their interactions with platelets during extracorporeal passage. They reported a process by which platelets adhere to the device chamber wall, become activated, engaging passing monocytes in a P selectin-dependent interaction that promotes DC differentiation in a manner dependent on platelet density and shear stress (8).

**Tolerance linked to DC maturational truncation.** Given the capacity for ECP to produce both antigenic apoptotic malignant cells and functionally important APCs, it remained an open question how such events might explain its dual nature to both immunize and tolerize. Several reports had generally defined tolerogenic DCs as semi-mature (25), with low expression of co-stimulatory molecules and increased production of immunosuppressive markers (26). Edelson and colleagues observed that during ECP, PUVA exposure was not uniform, instead conforming to a Gaussian distribution. This lead them to hypothesize that ECP might create two different subsets of DCs, one immunizing and the other tolerizing, all based on higher or lower exposure to PUVA. Support for this hypothesis was demonstrated by Futterleib et al by showing PUVA exposure lead to an increasingly immunosuppressive DC phenotype. This included upregulation of the glucocorticoid-induced leucine zipper (GILZ) gene, a distinguishing marker of tolerogenic DCs. In addition, PUVA exposed DCs also down-regulated CD80 and CD86, gained resistance to toll-like receptor maturation, and increased IL-10 production (27). These data illustrated that
the immunosuppressive nature of ECP is tied to PUVA exposure leading to maturationally truncated tolerizing DCs.

*Expanding on ECPs success*

With foundational aspects of the ECP mechanism understood, the possibility emerged of utilizing that knowledge to potentially improve the therapy and test its activity in other cancers, specifically solid tumors. Given the role DCs were understood to have, the question became whether improving their capacity to present antigen would result in a more effective treatment. Girardi et al hypothesized that additional time for antigen processing and presentation following ECP might allow for improved immune activation and better clinical responses in patients. In a phase I trial, they demonstrated that the addition of an overnight incubation, allowing newly formed DCs additional time to process and present CTCL antigens, significantly improved the clinical responses of patients otherwise refractory to ECP (4, 20, 31). This modified version of ECP was termed Transimmunization. However, without additional *in vivo* pre-clinical data, there were limitations on what changes could be reasonably made to the clinical ECP protocol, which had remained largely unchanged since its FDA approval in the late 1980s.

In order to further improve upon ECP and test its potential against solid tumors, a tunable *in vivo* system was needed that could be used in mice for pre-clinical studies. As part of this goal, Edelson and collaborators from Transimmune AG developed a microplate inspired by the original ECP exposure chamber that was directly scalable from mouse to man. Preliminary studies between the two plates demonstrated comparable physical parameters (e.g., sheer
stress, cellular adherence), as well as the capacity to induce DC differentiation of human cells following passage (unpublished data). Additionally, the development of a transgenic (BRAFV600E/PTEN-) inducible melanoma system by Yale’s Marcus Bosenberg offered an ideal murine melanoma model to test (18). From an induced melanoma tumor, Bosenberg and colleagues generated a cell line (YUMM1.7) capable of being grafted into standard C57BL/6 mice (28). This system afforded the opportunity to treat mice using antigens specific to their individual tumors, while conveniently utilizing the institutional expertise amassed by Dr. Bosenberg.

With all the elements of a model system present, we endeavored to design a treatment protocol closely based on clinical ECP that was capable of answering a central question: *Could a modified form of ECP be used as an effective immunotherapy against solid tumors?* Given the complex nature of the ECP mechanism and the possibility that additional elements might yet be undiscovered, our strategy was to make no assumptions about core elements of the procedure. Instead, our protocol was designed by working backwards from the known clinical ECP protocol, transferring as many of those elements as practically possible to the murine system.

Here, we demonstrate enhanced antitumor activity via large-scale antigen presenting cell (APC) loading with apoptotic tumor cell antigens following murine ECP, termed Transimmune. To our knowledge, this is the first report of an *in vivo* mouse treatment system that establishes a modified form of ECP as a potent immunotherapy against melanoma.
Statement of Purpose

The purpose of this project was to determine if ECP-induced monocyte antigen presenting cells (APCs), armed with tumor antigens distinctive to the melanoma grafted in the same mouse, can generate an anti-tumor immune response.

Specific Aims

- Using ECP as a point of departure, develop a testable, reliable, and well tolerated mouse treatment protocol that approximates conditions typical of the common clinical situation.
- Establish fundamental properties of a potential anti-tumor response including, if it is immune mediated and the nature of that immune response.
- Compare characteristics of the antitumor response with known mechanistic principles associated with ECP.
MATERIALS AND METHODS:

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched mice that were at least 4 weeks of age were used for all experiments. All mice were used according to the labs Yale IACUC approved protocol and in accordance with the National Institutes of Animal Healthcare Guidelines. Murine melanoma xenografts: YUMM 1.7 were injected subcutaneously at $1 \times 10^5$ cells per inoculum into the right flanks of recipient wild type C57BL/6 mice. Tumor formation and growth was assessed every 3 days until the experimental endpoint, at which point untreated mice were euthanized due to tumor volume, as per IACUC protocol. Tumor volume was monitored via biweekly measurement of perpendicular diameters and height using a caliper and tumor volume was calculated as $abc/2$. Relative tumor volumes were calculated based on the average tumor size of untreated mice on the last day of measurements being set at 100%. For example, if the average tumor size of PBS mice at the endpoint was 2000mm$^3$ and TI mice averaged at 200mm$^3$, then they were graphed as 100% and 10%, respectively.

Tumors were harvested in their entirety at each experiments end (approximately 4 weeks post inoculation) for histologic analysis. While the investigators assessing tumor growth were not blinded, all pathologists and laboratory personnel who performed histologic characterizations of tumor specimens were blinded to the group allocation. Sample sizes were chosen to ensure statistical power of detection based on projected outcomes. Differences in tumor volume were statistically assessed using the unpaired Student’s t-test.
Cell line

YUMM 1.7 is a melanoma cell line that is derived from C57BL/6 mouse melanoma with known genetic alterations (BrafV600E PTEN-/- p16-/- p19-/-). Nomenclature for the lines is based from Yale University Mouse Melanoma (YUMM) followed by a number designating the genotype of the cell line.

As follows, Katrina Meeth developed and isolated the YUMM1.7 cell line. Briefly, BRafCA, Tyr::CreER and Ptenlox4-5 mice were treated topically with 1-2 µl of 1.9mg/ml (5mM) 4-HT at 6-8 weeks of age. Tumor growth was monitored with a digital caliper until the tumors ranged from 50mm3 to 100mm3. Melanoma cell lines were derived by physical resection of primary cutaneous tumors. Approximately, a 3 mm³ piece of Induced melanomas was dissociated by finely mincing with sterile scalpel in complete medium followed by collagenase digestion for 3 h and 0.025% trypsin/0.2 mmol/L EDTA for 30 min, triturated through a 20-gauge syringe needle, and filtered by a 100µm nylon cell strainer (BD/Falcon, Bedford, MA) to yield a single-cell suspension. Cell suspensions were plated in media (a 1:1 mix of DMEM and Hams F12) supplemented with fetal calf serum (5%v/v) and penicillin, streptomycin and NEAA. Cells were cultured in DMEM F12 (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated FCS, 1% of NEAA and 1% penicillin/streptomycin (Gibco) under standard conditions. And finally plated into DMEM:F12 with 5% FBS. Media was changed at the 12 hour time point. Once established, cell lines were passaged 2 times and stored in liquid nitrogen stocks.

Transimmune procedure
The overall Transimmune procedure is listed below in parts. An overall schematic is available as Supplemental figure 5.

Isolation of Murine PBMCs:

Peripheral blood was collected from mice twice a week (Monday, Friday) for 3 weeks via non-lethal bleeding of the superficial temporal vein. The mouse was manually restrained with the lateral surface of the head facing the operator. Holding the purpose designed needle (18 gauge) in the other hand, a stab incision is made into the cheek approximately halfway between the ear and the mandible with enough force to produce a small hole. Drops were collected into a 15ml tube prefilled with 0,2ml of 10% heparin per 10 mice.

PBMCs were isolated from peripheral whole blood via Lympholyte gradient separation (LYMPHOLYTE®-M CELL SEPARATION MEDIA) via centrifugation at 2200 RPM for 20 min at RT with no break. Cells underwent 2 washes in PBS followed by ACK buffer (Lonza) to eliminate the residual red blood cells. Lysis involved adding 300ul of ACK buffer to pelleted PBMCs, incubating on ice for 10min, followed by the addition of 700 ul PBS to wash.

PUVA of YUMM1.7 tumor cells:

To induce apoptosis of tumor cells, 2.5X10^6 YUMM1.7 cells were incubated with 100 ng/ml of 8-MOP in 300ul of FBS for 20 minutes, and subsequently exposed to 4J/cm2 UVA light in a 12 well plate. Wells were pre-covered with cold FBS to reduce cell sticking. Coating FBS was removed just prior to the addition of YUMM1.7. Of note, titrating amounts of PUVA
dosages were performed in order to determine the minimum dosage to render YUMM1.7 incapable of cellular division over a ten day long term culture.

**Pre-coating and Plate Passage via Microplate:**

Apoptotic YUMM1.7 tumor cells were combined with isolated murine PBMCS and contaminating platelets. In order to simulate the first five cycles of ECP (which do not utilize PUVA), we pre-coated the microplate with approximately two thirds (400ul) of the PBMC and YUMM mixture for 1hr. Presumably, this allowed for fibrin and platelet adherence to the walls of the microplate. Cells were then removed from the plate and recombined with the remaining 1/3 of cells that remained in the tube at a final volume of 600ul. These cells were subsequently passed through the microplate to simulate ECP plate passage at a rate of 0.09 ml/min using a syringe pump. Of note, during passage, plate was rated at 45 degrees so that cells would “run uphill then downhill.” This was done to reduce bubbles and improve laminar flow. Following passage, cells were collected into an Eppendorf tube. The microplate was washed with 100% FBS at 0.49 ml/min while being physically perturbed by flicking the plate surface. This was done to help remove adherent cells from the plate during the wash step.

**Overnight Culture:**

To assess effect of an extended incubation period following m-ECP, cells were collected following plate passage and resuspended in RPMI with 15% mouse native serum and incubated overnight. This was identical to how patient samples were treated during the Transimmunization trial (4, 20, 31) The following day, cells were harvested, spun and
resuspended in native mouse serum and administered i.v. by retro-orbital vein to the mice. In early experiments, cell numbers were monitored however not controlled, since during the ECP procedure the number of cells a patient receives is not controlled either. On average, each mouse receives approximately $5 \times 10^5$ PBMC and YUMM each treatment.

**In vitro depletion**

CD11b depletion: Column-free magnetic separation was performed after the ACK buffer and before the incubation with the YUMM cells 1.7 using the EasySep mouse CD11b positive selection kit purchased from STEMCELL Technologies (Catalog # 18770).

CD41 depletion: Column-free magnetic separation was performed after the ACK buffer and before the incubation with the YUMM cells 1.7 using CD41 Biotin and anti-Biotin MicroBeads purchased from Miltenyl Biotec (Catalog # 130-105-869, 130090485 respectively).

**Spleen adoptive transfer**

Treated and untreated mice were sacrificed within 1 week of their 6th and final treatment and splenocytes were collected. The spleen was homogenized by passing the tissue through a nylon mesh (70 μm) using a plunger. Red blood cells were removed using Lympholyte. Splenocytes were resuspended in PBS and injected via the retro-orbital plexus in naive five week old male C57bl6 mice. One donor spleen equivalent per recipient mouse was injected. In a second cohort we repeat the same experiment using CD3 isolated cells from the spleen. A tumor inoculum of $1 \times 10^5$ YUMM 1.7 was injected subcutaneously into the right flank on the same day as the spleen transfers. Tumor volumes were measured along 3 orthogonal
axes (a, b, and c) every 3 to 4 days and tumor volume was calculated as abc/2 every 3 days until the end of the experiment.

**In vivo antibody depletions**

Mice were injected i.p. on day 10 (approximate time of first treatment) with 100μl/mouse of anti-NK1.1 (Biolegend), anti-CD8 (Biolegend), or with anti-CD4 (Biolegend) per the manufactures protocol. Injections were repeated biweekly for the duration of the experiment.

**Treating with PBMCs alone**

To determine the importance of YUMM as an antigen we removed the apoptotic YUMM1.7 from the treatment. Isolated murine PBMCs were subsequently passed the microplate. Cells were collected following plate passage and resuspended in RPMI with 15% mouse native serum and incubated overnight. The following day, cells were harvested, spun and resuspended in native mouse serum and administered i.v. by retro-orbital vein to the mice.

**Treating with YUMM 1.7 Alone**

To assess the effect of YUMM alone we removed the PBMCs from the therapy. 2.5X10^6 YUMM1.7 cells were incubated with 100 ng/ml of 8-MOP in 300μl of FBS for 20 minutes, and subsequently exposed to 4J/cm2 UVA light. Apoptotic YUMM was subsequently passed through the microplate. Cells were collected following plate passage and resuspended in RPMI with 15% mouse native serum and incubated overnight. The following day, cells were harvested,
spun and resuspended in native mouse serum and administered i.v. by retro-orbital vein to the mice.

PKH phagocytic assay

In order to determine the phagocytic potential of plate passaged monocytes, YUMM1.7 cells were labeled with the membrane protein dye PKH-red (Sigma) per the manufactures instructions. Cells then underwent typical PUVA exposure and subsequent plate passage associated with Transimmune, or did not. PBMC and labeled YUMM were incubated overnight as outlined above. The next day, cells were harvested for FACS staining instead of being reinfused into recipient mice. For FACS, cells were washed and resuspended in FACS buffer, blocked with Fcy blocker for 5 min, and stained with fluorescently conjugated antibodies. Anti-CD11b-pacblue (eBiosciences), anti-CD19-FITC (eBiosciences), and anti-MHCII-APC (eBiosciences) were used to identify potential phagocytes. PKH fluorescence was monitored via the PE channel.

Cytokine Analysis

Following the overnight incubation step of Transimmune, 100 ul of supernatant was harvested from individual culture dishes, centrifuged to remove contaminating cells, and stored at -20C for eventual cytokine analysis. Supernatants collected from 3-6 day cultures of purified monocytes and CD4 T cells from splenic transfer mice were also harvested and stored in this way. MHCII blocking was done using anti-MHCII I-A/I-E antibody (Biolegend) 1ug/well for the course of the 3 or 6 day incubation. Every other day, cells were pulsed with an additional
1μg/well. For in vivo serum cytokine analysis, serum was isolated from treated or untreated mice 72 hours after each of the 6 treatments. Aliquots were stored prior to assaying as noted above. Supernatants or serum were thawed and sent to Yale’s Immune Monitoring Core Facility for Luminex testing. Samples were screened for the presence of 23 different cytokines or chemokines. They were as follows: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL12-p70, IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1b, RANTES, and TNF-α.

**Mouse Hematocrit and complete blood counts**

To monitor for anemia or signs of infection, 5 μl aliquots of heparinized blood collected on treatment days were set aside and sent to Yale’s Analytical Core facility for hematocrit and CBC testing.

**Splenic CD4 activation assay**

In order to determine if there was evidence of CD4 activation associated with TI, recipients of spleen transfers from either TI or treated mice were sacrificed after their final measurements (approximately 30 days following tumor inoculations). Spleens from these mice were harvested as noted above. Additionally splenocytes were collected form mice not transferred any splenocytes originally (ie, tumor bearing mice, termed PBS). CD11b+ and CD4+ T cells were negatively selected via STEMCELL isolation kits. These cells were plated overnight either alone or together for 3 or 6 days in RPMI based T cell media at 1x10^5 cells per well in a 96
well flat bottom dish. Supernatants from these cultures were collected and sent for Luminex cytokine analysis, as noted above.

*Note about performance of individual experiments*

The amount and breadth of work presented in this thesis would not have been possible without the significant contributions of others. In keeping with the spirit of science, this was a highly collaborative effort. This project itself was an equally shared joint project between Alessandra Ventura and me from the outset. The initial idea development, planning and experimental design for every experiment performed in this thesis was conducted together by myself and Alessandra Ventura. Initially, Transimmune experiments were performed by both of us at every level. However, after initial successes, we compartmentalized work to be more efficient, with Alessandra taking on the bleeding of mice, tumor inoculations, i.v. reinfusions, and tumor measurements. This amount of work cannot be understated. Every one of the hundreds of tumors in this thesis was injected and measured by Alessandra.

In consultation with Ale, I took on the task of designing the treatment protocol so that it closely resembled the original ECP procedure. This was based on the original ECP papers describing minute details of the procedure, including the percentage of treated blood volume per patient, the estimated amount of treated cells, the volume ran over the ECP plate, the surface area of the original ECP plate, and the rate of flow through the chamber. Additional insights were gathered by observing several patients undergoing ECP, including how serum, white cells, blood cells layered onto the plate after each cycle. This all heavily informed the Transimmune treatment protocol design. For most of the *in vivo* experiments, I performed the
PBMC isolation, plate coating and passage, and overnight cultures. For later repeat experiments involving *in vivo* depletions, additional help was provided at various points in the TI protocol by Eve Robinson, who took on running plates if Ale or I were unavailable or needed additional assistance.

Later *in vitro* studies, including PKH uptake assays, FACS, cytokine supernatant experiments were conducted by me, with assistance in mouse cell isolation provided by Ale and/or Eve.

At every step of experimental planning, Ale and I benefitted greatly from insight and advice from Doug Hanlon, Eve Robinson, Katrina Meeth, Marcus Bosenberg, Robert Tigelaar and most importantly Richard Edelson.
Results

Transimmune results in slower YUMM 1.7 tumor growth that is immunologically transferrable via splenocytes

To test the therapeutic potential of an ECP-derived protocol against established solid tumors, we utilized the YUMM 1.7 murine melanoma xenograft system developed by Dr. Marcus Bosenberg. Preliminary titrations of increasing dosages of PUVA established 4 J/cm² and 100 ng/ml of 8-MOP as an effective kill dose for YUMM1.7 (data not shown). Two age and sex matched C57BL/6 mouse cohorts were compared, with the treatment arm receiving the Transimmune protocol, while the control group received phosphate-buffer-saline (PBS). Mice were inoculated on day 0 with YUMM 1.7 in the right flank. They received treatment twice a week for three weeks, with the first treatment commencing when tumors were palpable (7-11 days post inoculation). Mice receiving Transimmune experienced a significant delay in tumor growth as compared to untreated mice (Fig. 1, Sup. Fig. 3). By the endpoint, at which untreated mice had to be euthanized due to tumor size, control tumors were >85% larger than Transimmune treated tumors (Fig. 1). Despite an expected transient anemia, mice generally tolerated the Transimmune treatment protocol with no significant complications (Sup. Fig. 2).

Figure 1. Transimmune reproducibly alters YUMM 1.7 melanoma tumor growth kinetics in mice
C57BL/6 mice inoculated s.c. with 1E5 YUMM1.7 tumor cells were treated six times with Transimmune cells i.v. following an overnight incubation. Mean relative tumor volume growth plotted over time. Data is representative of seven independent experiments. (***, p<0.001; ****, p<0.0001)

To determine if the delay in tumor growth was immune mediated, splenocytes were transferred from either Transimmune or PBS mice following their final treatment into untreated tumor-bearing mice. An additional control group received PBS instead of splenocytes. Mice receiving whole splenocytes from Transimmune treated donors demonstrated a reduction in YUMM 1.7 tumor growth compared with controls (Fig. 2a). This pattern of growth was remarkably similar to mice directly receiving Transimmune treatment, indicating the effect was immune mediated and transferrable. To further refine the subsets of immune cells responsible for the TI effect, transfers were repeated with splenocytes enriched for T cells by positively selecting out contaminating B cells and myeloid cells. Mice receiving enriched T cell splenocytes maintained reduced tumor growth compared to those receiving no splenocytes or
those receiving T cells from untreated mice, indicating an important role for T cells in producing the Transimmune anti-tumor response.

**Figure 2.** Transimmune effect is transferrable via splenocytes

![Graph showing transgene expression](image)

*a, b, C57BL/6 recipient mice were injected on day 0 with one spleen equivalent of either whole splenocytes (a) or T-cell enriched splenocytes (b) from untreated tumor-bearing mice or Transimmune treated mice. Recipient mice were challenged with 1E5 YUMM1.7 cells immediately following splenocyte transfer. Mean relative tumor volume growth plotted vs time.

**Transimmune avoids problematic tolerance induction by avoiding PUVA treatment of normal PBMCs**

Given ECP’s history as an immunomodulatory therapy that can both immunize in CTCL and tolerize in GVHD, we hypothesized that Transimmune might share similar mechanistic principles traditionally associated with ECP. If true, these shared principles would aide in helping to further characterize the anti-YUMM1.7 effect associated with Transimmune. Since PUVA-induced maturational truncation of PBMC (specifically, monocytes) has been associated with tolerance induction via ECP, we hypothesized that the addition of PUVA treated PBMCs would prove tolerizing and therefore reduce the TI anti-tumor effect. This was tested by adding...
an equal number of PUVA treated PBMC to TI cells prior to treatment, and comparing tumor growth in mice receiving standard TI treatment or PBS. The addition of PUVA truncated PBMC resulted in complete loss of the TI anti-tumor response (Fig. 3), demonstrating the immunomodulatory potential of this treatment protocol to possibly both immunize and tolerize. Additionally, by separating PUVA treatment of tumor cells from plate passage of PBMCs, Transimmune avoids the potential pitfall of tolerance associated with PBMC truncation.

**Figure 3.** Addition of maturationally truncated PBMCs negates TI effect

![Graph showing relative tumor volume over time](image.png)

**PUVA irradiated PBMC from C57BL/6 tumor bearing mice were mixed with Transimmune treated cells prior to being administered to recipient mice. Remaining protocol was unchanged from traditional Transimmune. Tumor growth over time was compared to mice receiving traditional Transimmune treatment or PBS. Plate passage and the presence of monocytes and platelets are required for both an in vitro proinflammatory cytokine profile and an in vivo anti-tumor response.**

Monocytes have historically been shown to have an important role in clinical ECP, in part through platelet-induced activation during plate passage, leading to downstream tumor
antigen presentation and T cell activation. To determine whether a similar process was involved in Transimmune, we isolated supernatants from plate passaged cells following overnight incubation, and compared their cytokine profile with non-passaged cells and cells depleted of CD11b+ monocytes or CD41+ platelets prior to passage for signs of an inflammatory response. A Luminex panel of 23 cytokines was screened. Transimmune demonstrated a distinctive proinflammatory profile involving monocyte chemoattractant protein-1 (MCP-1), and IL-6 (Fig. 4). In the absence of plate passage, monocytes, or platelets production of both cytokines was significantly diminished (Fig. 4). This indicates that Transimmune acutely induces a proinflammatory milieu in vitro that requires plate passage of monocytes with platelets. Interestingly, there was no evidence of Transimmune-specific cytokine production in the serum of treated mice (Sup. Fig. 4). To determine if plate passage and monocytes also play a role in the in vivo tumor response, mice were treated with Transimmune, un-passaged cells, or cells depleted of monocytes prior to passage. The anti-tumor effect of Transimmune was completely lost without plate passaged treatment cells, resulting in tumor growth matching that of untreated mice (Fig. 5a). Additionally, the removal of CD11b+ monocytes prior to plate passage also resulted in the complete loss of the TI effect (Fig. 5b). Consistent with known elements of ECP, these data illustrate the profound importance of plate passaged monocytes in the anti-tumor response induced by Transimmune.

**Figure 4.** Plate passage, monocytes and platelets are required for Transimmune-associated in vitro proinflammatory cytokine response.
Cytokine concentrations reported in pg/ml. Cytokines were collected from overnight supernatants containing passaged YUMM and PBMC following plate passage, no plate passage, or monocyte or platelet depletion prior to plate passage; *=p<0.05.

In order to determine how the process of plate passage might activate monocytes in Transimmune, we looked to ECP as a potential guide. It was previously shown that during ECP, passaged monocytes interacted with platelets anchored to the plate surface via adhesion molecules. These interactions proved crucial to subsequent monocyte activation and differentiation into immature DCs (8). We hypothesized that removal of platelets from cell mixtures prior to plate passage would reduce monocyte activation and thus the TI in vivo anti-tumor response. Removal of platelets was accomplished through a positive selection CD41 depletion kit following PBMC isolation but before plate passage. Mice were subsequently treated as described previously and compared to TI and PBS mice. Tumors in mice receiving platelet-depleted passaged cells grew at a rate similar to the PBS control mice (Fig. 5c). In addition to their role in producing proinflammatory cytokines (Fig. 4), these results highlight the important role platelets play in the Transimmune anti-tumor response, and are consistent with
previous reports implicating platelet-monocyte interactions as essential for downstream monocyte activation following ECP.

**Figure 5.** Plate passage, monocytes and platelets are required for the Transimmune anti-tumor response

![Graphs showing tumor growth](image)

**a,** In vivo tumor growth following treatment with cells that were not plate passaged, treated with TI, or mice receiving no treatment at all (PBS). **b,** In vivo tumor growth following depletion of monocytes via positive selection of CD11b positive cells prior to plate passage. Tumor growth compared to TI or untreated cells. NS=not statistically significant. **c,** Depletion of platelets via positive selection of CD41 positive cells prior to plate passage.

YUMM are a necessary source of antigen and must be administered in the context of passaged PBMC in order to produce TI effect

Given the role activated monocytes play as APCs in ECP, we hypothesized a similar role in Transimmune. To show plate passaged monocytes as potential APCs, we first established their ability to take up PKH dye-labeled YUMM 1.7 debris (Sup. Fig 1). Next, to establish YUMM as an important source of antigen in vivo, mice were treated with passaged PBMCs alone that were not given a chance to be loaded with YUMM 1.7 debris. Tumor growth in these mice was no different than PBS mice, establishing YUMM as a necessary source of antigen for the TI effect.
effect (Fig. 6). However, when mice were given PUVA-treated YUMM1.7 cells alone, it was not sufficient to reproduce the TI response (Fig. 6), indicating that YUMM antigen in the context of passaged PBMCs is essential for therapeutic efficacy.

**Figure 6.** Passaged YUMM and PBMCs are necessary but insufficient alone for TI response

![Graph showing tumor growth comparison](image)

_YUMM or PBMC passaged separately, and administered separately. Tumor growth of each compared to TI and untreated mice._

_Recipient CD4, CD8, and NK cells are important in efferent arm of Transimmune anti-tumor response, and involve TI-specific IFNg production mediated through MHCII_

In order to help decipher the efferent arm of Transimmune, mice were treated with TI while undergoing _in vivo_ depletions of immune cell subsets. Antibodies against NK1.1, CD4, or CD8 were administered i.p. over the course of treatment, and tumor growth was compared with standard TI and PBS in non-depleted mice. _In vivo_ depletions of cells were confirmed via FACS peripheral blood analysis (data not shown). Tumor growth in mice depleted of CD8, CD4, and NK1.1 cells was no different than untreated controls (Fig. 7). Additional evidence of CD4
involvement came from long term (6 day) incubations of CD4+ T cells and CD11b+ monocytes isolated from recipient mice that had been transferred splenocytes from TI or untreated mice. Supernatants collected from these cultures demonstrated TI specific production of IFN-g when monocytes and CD4+ T cells were co-incubated (Fig. 7b). Low levels of IFNg were produced by mice receiving untreated splenocytes. (Fig. 7b). The addition of an anti-MHCII blocking antibody significantly inhibited the TI-specific IFNg production from co-incubated CD4+ T cells and monocytes (Fig. 7b). These data indicate a potential for a multifaceted effector TI response involving both innate and adaptive arms of the immune system, with CD4+ T cells activated via MHCII antigen presentation likely having a central role to play.

**Figure 7.** CD4, CD8 and NK cells are central to the TI in vivo response that involves TI-specific IFNg production mediated through MHC Class II.

**a**, in vivo depletions of CD4, CD8 or NK cells in recipient mice over the course of TI treatment. Tumor growth compared to TI and untreated mice. **b**, cytokine production following long term culture (3 and 6 days) of CD4 T cells and monocytes (CD11b+ cells) in combination or separately. Additionally, cells in combination were blocked with
an anti-MHCII antibody. Cell collected from mice that received spleen transfers from TI or PBS mice. For comparison, mice that did not receive spleen are also shown (PBS); *=p<0.05.
Discussion

ECP is widely used in clinical practice, inducing high response rates in patients with advanced CTCL. As a cellular immunotherapy, it activates monocytes to take up and process antigen from apoptotic cells through platelet dependent interactions. Its ability to produce potent APCs without suprapharmocologic concentrations of cytokines has made it particularly attractive as a potential cellular vaccination therapy. A central question for many ECP practitioners has been whether that potential could be realized in fighting solid tumors. (4, 29, 31). Despite progress in understanding the mechanism of ECP, an in vivo pre-clinical system has not existed that would allow proper investigation of its potential to generate antitumor responses outside the realm of CTCL. Here, we establish a tunable, modified ECP melanoma system, scalable from mouse to man. To the best of our knowledge, this is the first known demonstration of an antitumor effect induced by modified ECP (Transimmune) in solid tumors, and has wide potential implications for the treatment of melanoma and other possible malignancies.

A tunable system, optimal for treatment enhancement and gaining mechanistic insights

The Transimmune treatment protocol resulted in reliable slowing of YUMM1.7 melanoma tumor growth, without significant complications or toxicities. The tunable nature of the TI system allowed several areas of the original ECP procedure to be modified in order to take advantage of the recent mechanistic insights by Edelson and others. First, we added an overnight incubation to the clinical procedure given the improvement in antigen uptake and processing demonstrated by the Transimmunization trial (31). Consistent with their findings,
we found an improved antitumor response when the overnight incubation was added (data not shown). The overnight incubation also created the opportunity to assay cells (ie., phagocytosis, cytokines) following Transimmune before they were reinfused into mice, providing additional metrics to further characterize the response.

Second, we compartmentalized PUVA treatment in order to avoid potentially creating maturationally truncated tolerizing DCs (27). Indeed, we established the capacity of PUVA to result in tolerizing APCs that were capable of mitigating a TI anti-tumor response. This was the first *in vivo* evidence to directly support the hypothesis that ECP’s induction of immunity and tolerance is linked with APC exposure to PUVA, as laid out by Futterleib and colleagues (27). This illustrates that limiting PUVA exposure to malignant cells and not APCs, can improve the overall effectiveness of an ECP-derived antitumor therapy. On the other hand, these tolerizing APCs can now be exclusively used to investigate their therapeutic potency in autoimmune models also. In fact, our collaborators at Northwestern University have generated preliminary data indicating that PUVA-exposed passaged splenocytes slow rejection in a murine cardiac transplant model (unpublished data).

The benefits of this tunable system go beyond the ability to modify the original ECP procedure. It can also aid in further exploration of the ECP mechanism. As a result, we were able to establish clear mechanistic parallels with ECP by subtracting components during the treatment process, helping to decipher their relative contributions to the antitumor response.

Initially, we established that the TI effect was immune mediated. This was accomplished by the successful transfer of the TI response to naïve untreated recipient mice via splenocytes. The effect was preserved with the enrichment of T cells and removal of B cells, indicating an
important subset of T cells may provide TI-specific memory. CD4+ T cells being a likely source for such memory, lead us to investigate their cytokine production in spleen transfer recipient mice. We found IFN-g production that was only present when TI-specific CD11b+ APCs and CD4+ T cells were incubated together. This IFN-g production was significantly inhibited by disrupting MHCII presentation with a blocking antibody, indicating that the cytokine production was mediated through MHCII. Moreover, we found through in vivo depletions that CD4+ cells were necessary for the TI response. These data not only support an immune mediated TI effect, but specifically highlight the important role CD4+ T cells have to play. Investigations are underway to determine whether or not antigen-loaded TI passaged monocytes specifically are responsible for this MHCII presentation and CD4+ activation.

Next, we investigated the importance of a major hallmark of ECP--plate passage itself. Removing the microplate, demonstrated that it was necessary for the in vivo antitumor response. This was the first in vivo indication that the microplate retained the functional capacity of its larger ECP sister chamber, and that indeed, the Transimmune response likely shared similarities with traditional ECP effects. On the other hand, we established that plate passaged APCs without PUVA-treated YUMM1.7 cells as a source of antigen, were not sufficient to induce an antitumor response. This illustrated that YUMM were an important source of antigen, and that APCs required plate passage and antigen loading to be effective.

Given the parallels with ECP and the indication that a segment of antigen exposed plate passaged PBMCs were important, we investigated the possibility that these cells were activated monocytes acting as APCs. We chose CD11b as a broad myeloid marker of murine peripheral monocytes, although acknowledging it is also found on B cells and macrophage (38, 39).
Differentiating murine monocytes from macrophage was outside the scope of our study, and so we refer to the larger population as monocytes for simplicity. We first established that there was a CD11b+ segment of plate passed PBMCs that were capable of engulfing PKH-labeled YUMM. Initial uptake studies demonstrated that YUMM-specific CD11b+ phagocytes were not B cells (data not shown). This YUMM uptake occurred regardless of plate passage, which is not unexpected given the reputation peripheral monocytes have as phagocytes. Nonetheless, if plate passaged monocytes were going to act as APCs, they would need to be capable of antigen uptake, which they are. We then established that the presence of these CD11b+ cells prior to plate passage was required for the in vivo TI antitumor response. Given the important role of plate passaged monocytes in vivo, we attempted to find a cytokine in vitro corollary of their potential activation. Elevations in MCP-1 and IL-6 were dependent on the presence of plate passaged monocytes and platelets. MCP-1 may be the most likely monocyte-specific cytokine since it is known to be produced by inflammatory monocytes and is a potent chemokine, attracting monocytes to sites of inflammation (37). Additionally, it has been reported by Weyrich and colleagues that MCP-1 is known to be produced by monocytes following activation via platelet anchoring through P-selectin (40,41). This is remarkably consistent with our data linking MCP-1 production with co-plate passage of monocytes and platelets, as well as Durrazo’s efforts linking human monocyte-to-DC differentiation to P-selectin anchoring of platelets following ECP. Further work is needed to show if MCP-1 is directly produced by plate passaged monocytes and if its production is dependent on P-selectin. Such a marker of TI-specific monocyte activation would be valuable for optimizing the TI procedure without having
to wait for a longer mouse readout, and as a way to monitor the effectiveness of individual plate passage procedures in activating monocytes.

With potential evidence of passaged monocyte activation and their presence being necessary for the TI effect, we moved to the heart of the ECP mechanism—platelet conversion of monocytes into functionally potent physiologic APCs. Although established by Durazzo and colleagues in vitro, evidence requiring platelet-monocyte interactions for a potent antitumor response had not been demonstrated in an in vivo system (8). Here we show that passage of monocytes and platelets together are necessary for producing the Transimmune response. A result consistent with Durazzo’s mechanism involving activated platelets adhering to the device chamber wall and engaging passing monocytes in a P selectin-dependent interaction that promotes DC differentiation (8). This is the first study to demonstrate a murine solid tumor cellular immunotherapy that is potentially dependent on platelet-induced monocyte activation. If born out and applicable to human solid tumors, such a mechanism could open an entirely new field for creating potent physiologic APCs capable of generating significant T cell guided immune responses.

To better characterize the efferent immune response following the reinfusion of the passaged cells, we performed a set of in vivo depletions. These experiments established that CD4 (as noted above), CD8 and NK cells were all required in the recipient mouse in order to develop a TI antitumor response. Given the importance of an intact CD8 T cell compartment in ECP-induced CTCL remissions, it was not surprising to find that cytotoxic and helper T cells were required in Transimmune (8, 10). However, the involvement of NK cells was unexpected. Their specific role is unclear, but it is possible that their influence could come as cytotoxic effectors.
within the intratumoral environment. One possible mechanism might be through antibody mediated cell cytotoxicity (ADCC). Although the role a humoral response might play in TI remains unknown, the potential production of IL-6 following plate passage could point to T follicular helper (Tfh) cell activation of B cells (24). Preliminary intratumoral data from our colleagues has indicated a significant presence of immunoglobulin and NK cells within the tumors of TI treated mice (data not shown). Work is ongoing to further characterize this efferent immune response, including further analysis of the intratumoral environment.

**Study Limitations:** As in any animal model, this is a controlled system that is useful in suggesting potential mechanisms of action and therapeutic benefits. The inability to completely mimic human disease limits its value in necessarily predicting all clinical-level responses. Additionally, it is worth noting that TI mice eventually succumb to their tumors despite an initial delay in tumor growth. We fell short of inducing regressions in any treated mice. Preliminary work by our colleagues has shown that treating with a cell line generated from an escaping tumor instead of YUMM1.7 fails to produce a TI response. This indicates that at least part of the mechanism of escape is likely inherent to the tumor antigen itself, which could involve a clonal selection of a less immunogenic mutant, or a shift in antigen expression by tumor cells over the course of therapy. Both avenues are under ongoing investigation as part of a larger effort to improve the TI antitumor response. Lastly, there is no reason to expect that the current protocol is entirely optimized. Attempts to increase antigen uptake or monocyte activation may help improve antitumor responses. These include but are not limited to: further activation of TI monocytes with adjuvant, use of antibodies for opsinization; the addition of calreticulin or other “eat me” signals for phagocytosis; using mutagenized YUMM for a source of neo
antigens; optimizing platelet layering in the flow chamber or increasing the number of plate passages for monocyte activation.

Despite room for improvement, Transimmune has remarkably demonstrated the potential to create potent physiologic APCs through an ECP-inspired process, and to use those cells to induce a significant immunological response against murine melanoma. We are currently finding similar antitumor responses in colon, ovarian and other melanoma murine systems, expanding the potential application for this therapy (unpublished data). Consequently, there is intriguing hope for ECP-derived cellular immunotherapy to make additional impacts on human disease in the future.
Supplemental Figures

Supplemental Figure 1. PKH labeled YUMM 1.7 uptake by plate passaged and non-passaged monocytes.

a, FACS plot of PKH vs CD11b following overnight incubation with or without plate passage. Data representative of three experiments. b, Phagocytic efficiency defined as a percentage of pkh-labeled CD11b cells in the presence of a constant, non-limiting amount of pkh-labeled YUMM (numerator box / denominator box). Data averaged over 3 experiments.

Supplemental Figure 2. Mouse hematocrit over treatment course.
Hematocrit from treated and untreated mouse over the course of treatment. * Marks the end of the last treatment.

Samples representative of blood from individual mouse.

**Supplemental Figure 3.** Images representative of excised tumors after two and five treatments.

*Images of individual resected tumors after the second or fifth Transimmune treatment. Untreated tumor (PBS) collected on that same day for comparison.*

**Supplemental Figure 4.** Serum cytokine production over treatment course
Serum collected 72 hours following treatment, pooled from five mice in either PBS or TI groups, and assayed via Luminex for cytokine analysis. Cytokines depicted here were the only ones that registered above the limit of detection.

Supplemental Figure 5. Transimmune procedure

Schema of Transimmune protocol. YUMM cells were PUVA treated separately, then incubated with PBMC, applied to microplate, passed, incubated overnight, then reinfused into mice the next morning.
References


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