Biomaterials 32 (2011) 9494–9503

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Induction of immunotolerance via mPEG grafting to allogeneic leukocytes

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ARTICLE INFO

Article history:
Received 10 June 2011
Accepted 19 August 2011
Available online 1 September 2011

Keywords:
Poly(ethylene glycol)
Tolerance
Immunocamouflage
Lymphocyte
Regulatory T cells
Th17 lymphocytes

ABSTRACT

The induction of anergy or tolerance to prevent allorecognition is of clinical interest. To this end, the effects of methoxypoly(ethylene glycol) [mPEG] grafting to allogeneic lymphocytes on proliferation and phenotype (Th17 and Treg) was examined *in vitro* and *in vivo*. Control studies demonstrated that PEGylation did not affect cells viability or proliferation (mitogen) potential. Conditioned media (1° MLR) collected at 72 h from resting PBMC demonstrated no immunomodulatory effects whereas the control MLR demonstrated significant ($p < 0.001$) pro-proliferative potential and significantly increased in IL-2, TNF-α, and INF-γ. However, 1° media from either resting mPEG-PBMC or the PEGylated MLR resulted in a significant inhibitory effect ($p < 0.001$) in the 2° MLR and no increase in cytokines. PEGylation of donor murine splenocytes resulted in significant *in vivo* immunosuppressive effects in H2-disparate mice. While unmodified allogeneic splenocytes resulted in a significant *in vivo* decrease in Treg and increased Th17 lymphocytes, PEGylated allogeneic splenocytes yielded significantly increased Tregs and baseline levels of Th17 lymphocytes. This effect was persistent to at least 30 days post challenge and was not reversed by unmodified allogeneic cells. These studies demonstrate that PEGylation of allogeneic lymphocytes induced an immunoquiescent state both *in vitro* and *in vivo*.

1. Introduction

Acute and chronic rejection of donor tissues and organs remains a significant clinical problem in transplantation medicine. To minimize or prevent rejection, the administration of immunosuppressive agents is typically required. Acute and chronic rejection are primarily T lymphocyte-mediated events that require allogeneic recognition of the foreign tissue and the subsequent proliferation of alloresponsive T cells. Indeed, because of the central role of the T cell in rejection, it is the primary target of current immunosuppressive drugs (e.g., cyclosporine A, FK506). In general these pharmacologic agents target either the T cell activation (e.g., cyclosporine A that inhibits IL-2 responsiveness) or the proliferation (e.g., methotrexate) of the alloresponsive T cells [1]. However all of today's clinically approved anti-rejection drugs are beset by chronic toxicity; consequently, significant research is underway to identify alternative means of preventing acute and chronic rejection.

A biomaterials approach to the prevention of allorecognition is the immunological camouflage of donor tissues (e.g., pancreatic islets) via microencapsulation within polymer hydrogels [2–6]. However, the hydrogels may impede oxygen and nutrient transport import or the export of cellular proteins (e.g., insulin). In contrast to hydrogels, our laboratory has pioneered direct "immunocamouflage" of donor cells (e.g., erythrocytes, lymphocytes, and pancreatic islets) to prevent allorecognition [7–17]. The immunocamouflage of cells is induced by the direct grafting of low immunogenicity polymers to the cell membrane. These polymers include methoxypoly(ethylene glycol) [mPEG; PEGylation] and hyperbranched polyglycerols (HPG) [7,17–19]. Our previous studies have demonstrated that the immunocamouflage of erythrocytes and lymphocytes resulted in the loss of allorecognition both *in vitro* and *in vivo*. Moreover, in contrast to pharmacologic agents, the grafted polymer exhibited both extremely low toxicity and immunogenicity. Interestingly, our previous studies have also suggested that PEGylation of the donor lymphocytes may gave rise to tolerance or anergy as evidenced by apoptosis of potentially alloresponsive lymphocytes [12,14].

In this study we examined whether the immunocamouflage (via PEGylation) of allogeneic human peripheral blood mononuclear cells (PBMC) or murine splenocytes can give rise to

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doi:10.1016/j.biomaterials.2011.08.061

immunoquiescence (tolerance and/or anergy). To examine this question *in vitro*, mixed lymphocyte reactions (MLR) and conditioned media experiments were conducted and analyzed for lymphocyte proliferation, differentiation and cytokine production. For lymphocyte differentiation, both *in vitro* and *in vivo*, the expansion/contraction of the T regulatory (Treg; favoring tolerance or anergy) and Th17 (pro-inflammatory and allorejection) populations were quantitated [20–24]. To more fully assess the systemic biologic effect of polymer-mediated immunocamouflage, *in vivo* murine studies were conducted to determine both the Treg and Th17 population modulations as well as whether differential effects were noted in the spleen, lymph nodes and blood of control and treated animals.

2. Method and materials

2.1. Human PBMC and dendritic cell preparation

Human whole blood was collected in heparinized vacutainer blood collection tubes (BD, Franklin Lakes, NJ) from healthy volunteer donors following informed consent. PBMC were isolated from diluted whole blood using Ficoll–Paque PREMIUM (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) as per the product instructions. The PBMC layer was washed twice with 1× Hank's Balanced Salt Solution (HBSS; without $CaCl_2$ and $MgSO_4$; Invitrogen by Life Technologies, Carlsbad, CA) and resuspended in the appropriate media as needed for mixed lymphocyte reactions and flow cytometric analysis of Treg and Th17 phenotypes.

Dendritic cells (DC) were prepared from isolated PBMC as described by O'Neill and Bhardwaj [25]. Briefly, freshly isolated PBMC were overlaid on Petri dishes for 3 h of in AIM V serum free culture medium (Invitrogen, Carlsbad, CA). Non-adherent cells were gently washed off the plate. The adherent cells (monocyte rich cells) were treated with IL-4 and GM-CSF (50 and 100 ng/ml respectively; R&D Systems, Minneapolis, MN) in AIM V medium. Cells were again treated with IL-4 and GM-CSF on days 2 and 5. On day 6, cells were centrifuged and resuspended in fresh media supplemented with DC maturation factors (TNF-α, IL-1β, IL-6; R&D Systems, Minneapolis, MN) and prostaglandin E2 (Sigma–Aldrich, St. Louis, MO). The mature DC-like cells were harvested on day 7 and CD80, CD83, CD86 and HLA-DR expressions were determined to confirm DC maturation via flow cytometry (FACSCalibur Flow Cytometer, BD Biosciences, San Jose, CA).

2.2. Murine splenocyte and tissue harvesting

All murine studies were done in accordance with the Canadian Council of Animal Care and the University of British Columbia Animal Care Committee guidelines and were conducted within the Centre for Disease Modeling at the University of British Columbia. Murine donor cells used for the *in vivo* donation and *in vitro* studies were euthanized by CO_2. Three allogeneic strains of mice were utilized for syngeneic and allogeneic *in vitro* and *in vivo* challenge: Balb/c, H-2^d; C57Bl/6, H-2^b; and C3H, H-2^k. Murine spleens, brachial lymph nodes, and peripheral blood were collected at the indicated days. Mouse spleens and brachial lymph nodes were dissected and placed into cold phosphate buffered saline (PBS; 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 154 mM NaCl, pH 7.3) containing 0.2% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO.) and kept on ice until ready to process. Whole blood was collected in heparinized tubes via cardiac puncture. Murine donor splenocytes were prepared from freshly harvested syngeneic or allogeneic spleens via homogenization into a cell suspension in PBS (0.2% BSA) using the frosted end of two microscope slides. The resultant cell suspension was spun down at 500 × g. The splenocyte pellet was resuspended in 1 mL of 1× BD Pharm Lyse lysing buffer (BD Biosciences, San Diego, CA) and incubated for 1 min at room temperature. Lymph node cells were harvested via tissue homogenization as described above, washed twice and resuspended in PBS (0.2% BSA) for flow cytometric analysis of Th17, Treg and murine haplotype. Recipient peripheral blood lymphocytes were prepared via lysis of the red cells (BD Pharm Lyse lysing buffer; BD Biosciences, San Diego, CA) at 1× concentration, followed by washing (1×) and resuspension in PBS (0.2% BSA) for flow analysis of Th17, Treg and murine haplotype.

2.3. mPEG modification (PEGylation) of PBMCs and splenocytes

Human PBMC and murine splenocytes were derivitized using methoxypoly(-ethylene glycol) succinimidyl valerate (mPEG-SVA; Laysan Bio Inc. Arab, AL) with a molecular weight of 5 or 20 kDa as previously described [7,8,12,14]. Grafting concentrations ranged from 0 to 5.0 mM per 4×10^6 cells/mL. Cells were incubated with the activated mPEG for 60 min at room temperature in isotonic alkaline phosphate buffer (50 mM K_2HPO_4 and 105 mM NaCl; pH 8.0), then washed twice with 25 mM HEPES/RPMI 1640 containing 0.01% human albumin. Human PBMC were resuspended in AIM V media at a final cell density of 2.0 x 10^6 cells/mL for use in the MLR. Murine splenocytes used for *in vivo* studies were resuspended in sterile saline at a final cell density of 2.0×10^8 cells/ml for *i.v.* injection.

"To determine if the simple presence of the mPEG polymer itself altered the immune response either *in vitro* and *in vivo*, additional studies were done with unactivated polymer incapable of covalent grafting to the cell surface. For these studies, allogeneic human (*in vitro* studies) or syngeneic and allogeneic murine splenocytes (*in vivo* studies) were treated with non-covalently bound mPEG (soluble mPEG) under the same reaction conditions described for the covalent grafting studies. For clarity, "soluble mPEG" refers to cells treated with non-covalently grafted polymer while "mPEG-modified" refers to treatment with activated polymer resulting in the covalent grafting of the mPEG to the cell membrane."

2.4. In vitro and in vivo cell proliferation

Cell proliferation (both *in vitro* and *in vivo*) was assessed via flow cytometry using the CellTrace CFSE (Carboxyfluorescein diacetate, succinimidyl ester) Cell Proliferation Kit (Invitrogen by Life Technologies – Molecular probes, Carlsbad, CA). Human and murine cells labeling was done according to the product insert at a final concentration of 2.5 μM CFSE per 2 x 10^6 cells total. Donor and recipient cell proliferation was differentially determined by haplotype analysis.

In some experiments, cell proliferation was measured by ^{3}H-thymidine incorporation. In these experiments, donor splenocytes (5.12×10^6 cells per well) were co-incubated in triplicate in 96-well plates at 37 °C, 5% CO_2 for 3 days. On day 3, all wells were pulsed with ^{3}H-thymidine and incubated for 24 h at 37 °C, 5% CO_2. Cellular DNA was collected on filter mats using a Skatron cell harvester (Suffolk, U.K.) and cellular proliferation was measured by ^{3}H-thymidine incorporation.

2.5. Mixed lymphocyte reaction (MLR) – control and conditioned media

The effects of polymer grafting (5 kDa SVAmPEG) on allorecognition *in vitro* were assessed using two-way MLR [8,12,14]. PBMC from two MHC-disparate human donors were label with CFSE as described. Each MLR reaction well contained a total of 1×10^6 cells (single donor for resting or mitogen stimulation or equal numbers for disparate donors for MLR). Cells were plated in multiwell flat-bottom 24-well tissue culture plates (BD Biosciences, Discovery Labware, Bedford, MA). PBMC proliferation, cytokine secretion, as well as Treg and Th17 phenotyping was done at days 10 and 14. For flow cytometric analysis, the harvested cells were resuspended in PBS (0.1% BSA). While time course studies (Days 4, 7, 10 and 14) were done, the presented studies show days 10 and 14. These extended studies are, in fact, the most stringent test of the immunomodulatory effects of the grafted polymer as membrane remodeling over this time could have resulted in a latter onset of proliferation.

To investigate *in vitro* whether polymer grafting to allogeneic PBMC gave rise to tolerance or anergy, secondary (2°) MLR studies were conducted using conditioned media. Conditioned media from a primary (1°) 2 way-MLR was collected at 72 h for conducting a secondary (2°) MLR as schematically shown in Fig. 1. Conditioned media from the 1° MLR included: A1) Resting Unmodified PBMC; B2) Resting PEGylated PBMC; C3) two-way MLR; and D4) two-way mPEG-MLR. The 2° MLR utilized freshly prepared MHC-disparate donors (either the same as or different from) the initial plate and plated as described above. As shown in Fig. 1, the 2° MLR samples included: A) Resting PBMC; B) two-way MLR; P) Mitogen Stimulation; D) two-way mPEG-MLR. For these studies, PBMC were derivatized using 1 mM 5 kDa SVAmPEG. Mitogen stimulation (PHA-P; Sigma–Aldrich, St. Louis, MO) of donor PBMC in the secondary plates was done to assess the proliferation potential and viability of cells incubated in the conditioned media. Human PBMC were challenged with 2 μg/ml per 1 x 10^6 cells of PHA-P. All plates were incubated at 37 °C (5% CO_2). Following 13 days of incubation (37 °C, 5% CO_2), the cell culture supernatants were collected and cells were harvested from the 2° MLR plates. Cell proliferation was measured via CSFE-dilution of $CD3^+CD4^+$ lymphocytes by flow cytometry.

2.6. Immunophenotyping by flow cytometry

The T lymphocytes populations (double positive for $CD3^+$ and $CD4^+$) in both the *in vitro* and *in vivo* studies were measured by flow cytometry using fluorescently labeled CD3 and CD4 monoclonal antibodies (BD Pharmingen, San Diego, CA). Human and mouse T regulatory lymphocytes (Treg) were $CD3^+/CD4^+$ and $FoxP3^+$ (transcription factor) while inflammatory Th17 lymphocytes cells were $CD3^+/CD4^+$ and $IL\text{-}17^+$ (cytokine) as measured per the BD Treg/Th17 Phenotyping Kit (BD Pharmingen, San Diego, CA). After the staining, the cells (1×10^6 cells total) were washed and resuspended in PBS (0.1% BSA) prior to flow acquisition. Isotype controls were also used to determine background fluorescence. All samples were acquired using the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and CellQuest Pro software for both acquisition and analysis.

2.7. Cytokine quantitation

Cell culture supernatants were collected from the 1° two-way MLR plate and stored at −80 °C prior to analysis. Conditioned media aliquots from a minimum of four independent experiments were used for quantification of supernatant cytokine levels using the BD Cytometric Bead Array (CBA) system (BD Biosciences, San Diego,

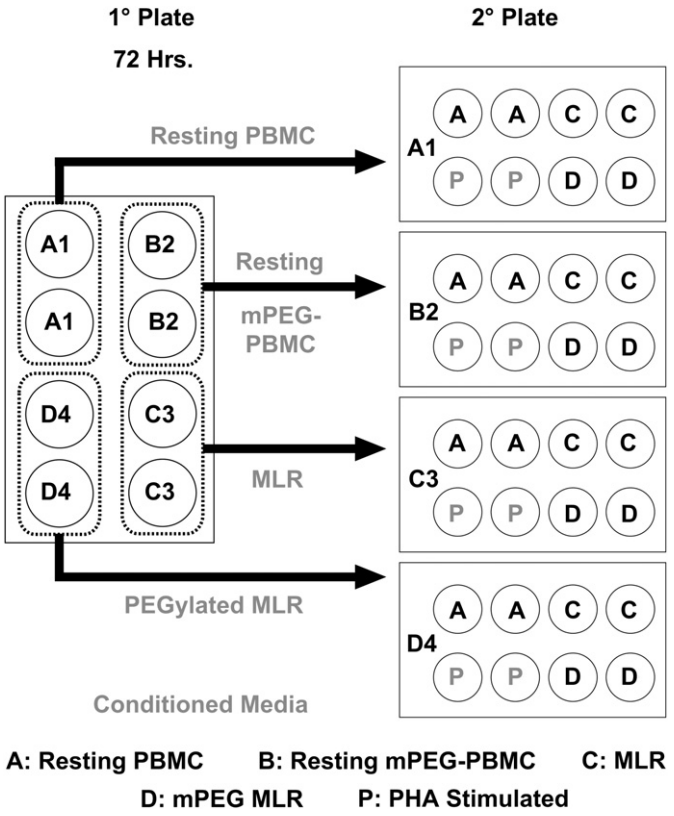

Fig. 1. Shown diagrammatically is the conditioned media protocol. A primary (1°) two way mixed lymphocyte reaction was initiated using two HLA disparate populations consisting of unmodified or polymer grafted (1 mM SVAmPEG; 5 kDa) PBMC. Within the mPEG-MLR, only one donor population was PEGylated. At 72 h, the cell free supernatants (conditioned media) from the wells were collected. Secondary (2°) mixed lymphocyte reactions using control and PEGylated PBMC from the same donors were initiated. A mitogen (PHA) stimulation control was added to assure that the media collected would support proliferation. 1° MLR Conditioned media: 1 Resting unmodified PBMC; 2. Resting mPEG-PBMC; 3. Control MLR; and 4. mPEG-MLR.

CA) for flow cytometry. The CBA system is a multiplexed bead based immunoassay used to quantitate multiple cytokine levels in a single sample simultaneously by fluorescence-based emission and flow cytometry. Cytokine measured included: IFN-γ, TNF-α, IL-10, IL-5, IL-4, and IL-2 using the BD Human Th1/Th2 Cytokine Kit I. The IL-6 and IL-17A levels were measured using the BD CBA Human Soluble Protein Flex Set. Both assays were performed following the manufacturer's product instruction manual. Briefly, cell culture supernatants of resting unmodified PBMC, unmodified MLR, PEGylated (5 kDa SVAmPEG; one donor) resting PBMC, PEGylated MLR, and mitogen (PHA) stimulated PBMC were incubated at room temperature in the dark with a mixture of each cytokine antibody-conjugated capture bead and the PE-conjugated detection antibody. Following the incubation, the samples were washed and acquired using a FACSCalibur flow cytometer and analyzed using CellQuest Pro. Cytokine protein levels were determined using the BD Cytometric Bead Array and FCAP Array analysis software (BD Biosiences, San Diego, CA and Soft Flow Inc, St. Louis Park, MN).

2.8. In vivo murine studies

To investigate whether mPEG grafting to leukocytes would have systemic *in vivo* effects, a murine adoptive transfer system was employed using three genetically different strains: Balb/c, H-2^d; C57Bl/6, H-2^b; and C3H, H-2^k [12,14]. All mice (donors and recipients) were 9–11 weeks old. Donor splenocytes were prepared and CSFE labeled as described. Control and mPEG-grafted (1 mM, 20 kDa SVAmPEG) syngeneic or allogeneic cells (20×10^6 splenocytes) were transfused intravenously (*i.v.*) via the tail vein into recipient animals. BALB/c and C57BL/6 mice injected with sterile saline served as control animals. Animals were euthanized by CO_2 at predetermined intervals (24 h, 48 h, 72 h, 96 h and 120 h) at which time blood, brachial lymph nodes and spleen were collected and processed for Th17/Treg phenotyping analysis and splenocyte proliferation studies by flow cytometry. Donor cell engraftment and proliferation were assessed via flow cytometry using murine haplotype (H-$2K^b$ vs. H-$2K^d$) analysis. To determine the persistence of the immunomodulation, mice were re-challenged (2° challenge) 30 days after the initial transfer of allogeneic or mPEG-allogeneic splenocytes with unmodified allogeneic cells. At 5 days post 2° challenge, Treg and Th17 phenotyping of murine splenocytes isolated from the spleen, lymph node and peripheral blood was again assessed via flow cytometry.

2.9. Statistical analysis

Data analysis was conducted using SPSS (v12) statistical software (Statistical Products and Services Solutions, Chicago, IL, USA). For significance, a minimum p value of <0.05 was used. For comparison of three or more means, a one-way analysis of variance (ANOVA) was performed. When significant differences were found, a post-hoc Tukey test was used for pair-wise comparison of means. When only two means were compared, student-t tests were performed.

3. Results

To determine the effects of immunocamouflage on the immune response, initial *in vitro* experiments examined the cytokine burst characterizing control and polymer modified MLR. The polymer-mediated immunocamouflage of human PBMC resulted in significant changes in the cytokine profile of the conditioned media obtained from the 1° MLR plate (Figs. 1 and 2). As shown in Fig. 2, control MLRs yielded elevated concentrations of IL-2, IFN-γ, IL-17A, TNF-α and IL-6 relative to resting unmodified or PEGylated PBMC. In contrast to the control MLR, the mPEG-MLR (one donor population PEGylated with 1 mM 5 kDa SVAmPEG) resulted in the virtually complete inhibition ($p < 0.001$) of secretion for the pro-inflammatory cytokines examined. However, IL-10 was preferentially elevated in the mPEG-MLR. In the conditioned media, IL-10 levels were 2.01 ± 1.26, 8.90 ± 2.10, 1.69 ± 0.64 and 1.33 ± 0.73 ng/ml for the resting PBMC, mPEG-MLR, resting mPEG-PBMC and Control MLR, respectively. As noted, in the mPEG-MLR, IL-10 levels were significantly ($p < 0.01$) increased suggesting the induction of an immunosuppressive state. Importantly, this cytokine quiescent state was not due to loss of cell viability as evidenced by the very low levels of non-viable cells detected following 72 h incubation (Fig. 2).

The conditioned media produced from the initial 72 h MLR exerted a significant effect on the 2° MLR as demonstrated in Fig. 3. While the 1° media from resting PBMC showed no significant effect on the 2° MLR, the media from the 1° Control MLR demonstrated a significant ($p < 0.01$) pro-proliferative effect in the 2° MLR. As shown, the mean proliferation index of the 2° MLR increased from 26.05 ± 12.47 to 44.72 ± 17.13 in the presence of conditioned media from the 1° Control MLR. The pro-inflammatory effect of the 1° MLR media was noted on even the resting PBMC and PHA-stimulated cells. In contrast, the 1° conditioned media from the mPEG-MLR demonstrated a significant ($p < 0.001$) anti-proliferative effect in not only the 2° MLR but also the PHA-stimulated cells. The differential proliferation response between the control and mPEG-MLR conditions for matching experiments is noted by the lines connecting paired experiments. While not shown, soluble mPEG (5 kDa) had no effect on cytokine levels in the 1° conditioned media nor on the proliferation of PBMC mediated by allorecognition (control MLR) or by mitogen (PHA) stimulation.

Furthermore, as shown in Fig. 4, the proliferation index was positively correlated with an increased population of Th17 T cells and inversely correlated with Treg lymphocytes numbers. As demonstrated, the 1° conditioned media from the control MLR yielded elevated levels of Th17 cells and decreased levels of Treg lymphocytes. In comparison, the 1° media from the mPEG-MLR resulted in significantly elevated ($p < 0.001$) levels of Treg cells and a virtually non-existent population of Th17 lymphocytes. The source of the conditioned media also impacted the efficacy of PHA stimulation. As shown, conditioned media from the mPEG-MLR significantly inhibited mitogen proliferation while the control MLR conditioned media significantly enhanced proliferation

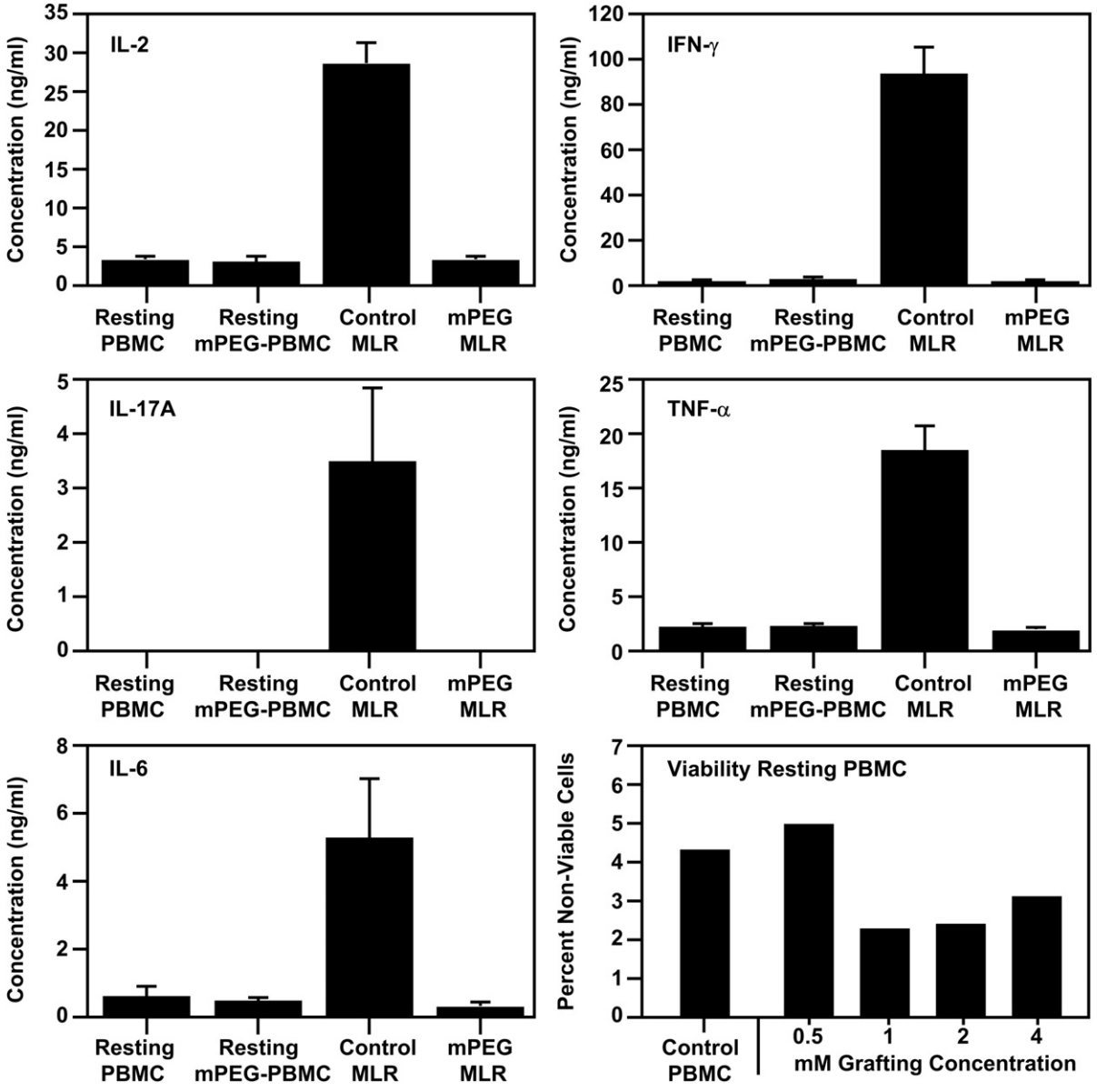

Fig. 2. Primary (1°) media cytokine levels at 72 h. IL-6, IL-17A, IL-2, TNF-α, IFN-γ levels are significantly reduced in the PEGylated two-way MLR utilizing modified and unmodified PBMC populations from HLA disparate individuals. The cytokine profile was analyzed using the BD cytometric bead array. Values shown are the mean ± SD of a minimum of 4 independent experiments. Percent non-viable cells within the control and PEGylated (SVAmPEG; 5 kDa) resting PBMC was assessed by propidium iodine exclusion.

relative to both media from resting PBMC ($p < 0.01$) and resting mPEG-PBMC ($p < 0.001$).

Hence, the *in vitro* experiments demonstrated that covalent grafting mPEG to human PBMC resulted in an immunomodulatory effect governed in part by changes in the Th17 and Treg populations. Moreover, these conditioned media experiments demonstrated that this immunomodulatory effect arises from soluble factors that might be able to induce a systemic effect *in vivo*. To determine if similar effects would be observed *in vivo*, a murine splenocyte adoptive transfer model was utilized. As demonstrated in Fig. 5, PEGylated donor splenocytes resulted in a significant *in vivo* immunomodulatory effect giving rise to elevated levels of Treg lymphocytes within the spleen, brachial lymph node, and peripheral blood. As noted, in all three tissues, a significant ($p < 0.001$ at 120 h) time-dependent increase in Treg lymphocytes over that observed in naïve mice was noted in mice receiving mPEG-modified allogeneic donor cells. In stark contrast, a significant ($p < 0.001$) decrease in Tregs (≥48 h post-injection relative to naïve mice) is noted in mice transfused with unmodified allogeneic splenocytes. The absolute difference between the unmodified (control) and PEGylated splenocytes, shown by the stippled area, demonstrates the true magnitude of the differential impact of donor cell PEGylation. Importantly, as noted at 120 h, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Treg lymphocyte population.

As foreshadowed by our *in vitro* human PBMC findings, murine Th17 lymphocyte levels were influenced by the PEGylation state of the allogeneic donor cells (Fig. 6). While unmodified allogeneic murine donor cells resulted in a significant ($p < 0.001$), time-dependent, increase in the Th17 cell population in the spleen, brachial lymph node and peripheral blood, the covalent grafting of mPEG to the membrane of the donor splenocytes resulted in the complete abrogation of the increase. Indeed, the Th17 population remained at resting levels. The absolute difference between the

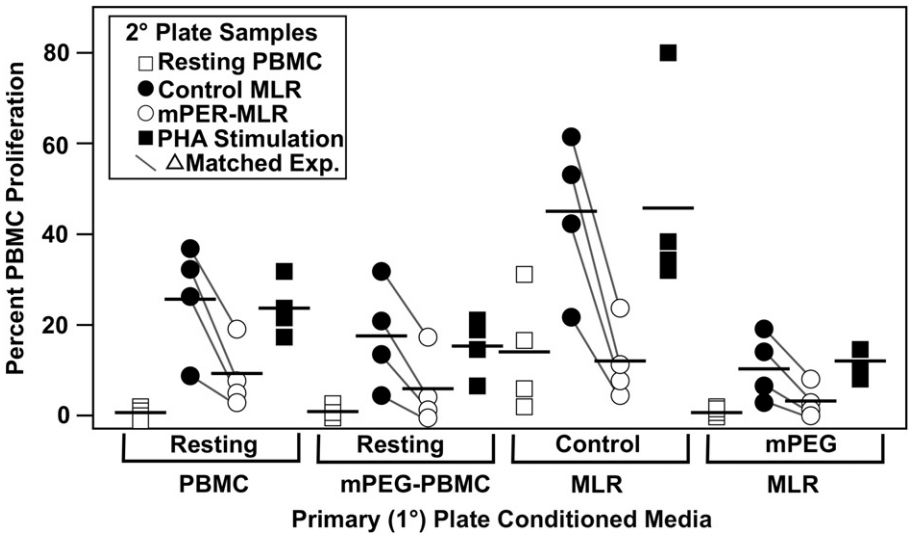

Fig. 3. Shown is the proliferation index of the secondary MLR that were conducted in the indicated (x-axis) conditioned media. As shown, relative to all other conditioned media, the media from the 1° Plate Control MLR demonstrated a significant ($p < 0.01$) pro-proliferative effect in the 2° MLR. This effect was noted on even resting PBMC and PHA-stimulated cells. In contrast, the 1° conditioned media from the mPEG-MLR demonstrated a significant anti-proliferative effect in the 2° MLR. As noted by the lines connecting paired experiments, PEGylation of one donor population resulted in reduced proliferation in all conditioned media experiments. No significant differences were noted between fresh media in a parallel secondary plate and the resting PBMC conditioned media. Shown are the individual results of 4 independent experiments and the mean (line). PEGylated cells were modified with 1 mM SVAmPEG (5 kDa).

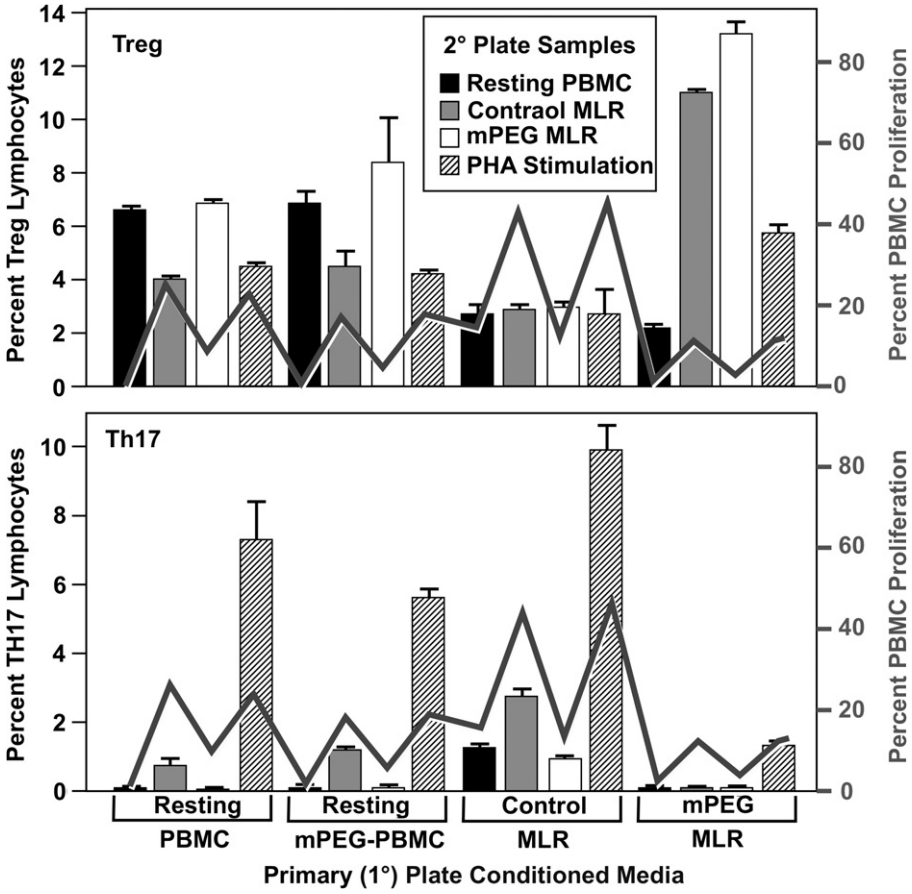

Fig. 4. PEGylation of human lymphocytes resulted in a significant *in vitro* immunomodulatory effects as noted by changes in the percentage of Treg and Th17 T cell populations. As shown, the 1° media from the mPEG-MLR favored production of Treg cells and a decreased population of Th17 lymphocytes. In contrast, the 1° media from the control MLR enhanced Th17 cell production and greatly inhibited Treg levels. The relative ratio of Th17:Treg was highly correlated with lymphocyte proliferation as denoted by the right y-axis and the embedded line graph. The high levels of Tregs in both the resting mPEG-PBMC and in mPEG-MLR correlated with low levels of proliferation. In contrast, an increased level of Th17 cells was associated with the 1° media from the control MLR and PHA stimulation. PEGylated cells were modified with 1 mM SVAmPEG (5 kDa).

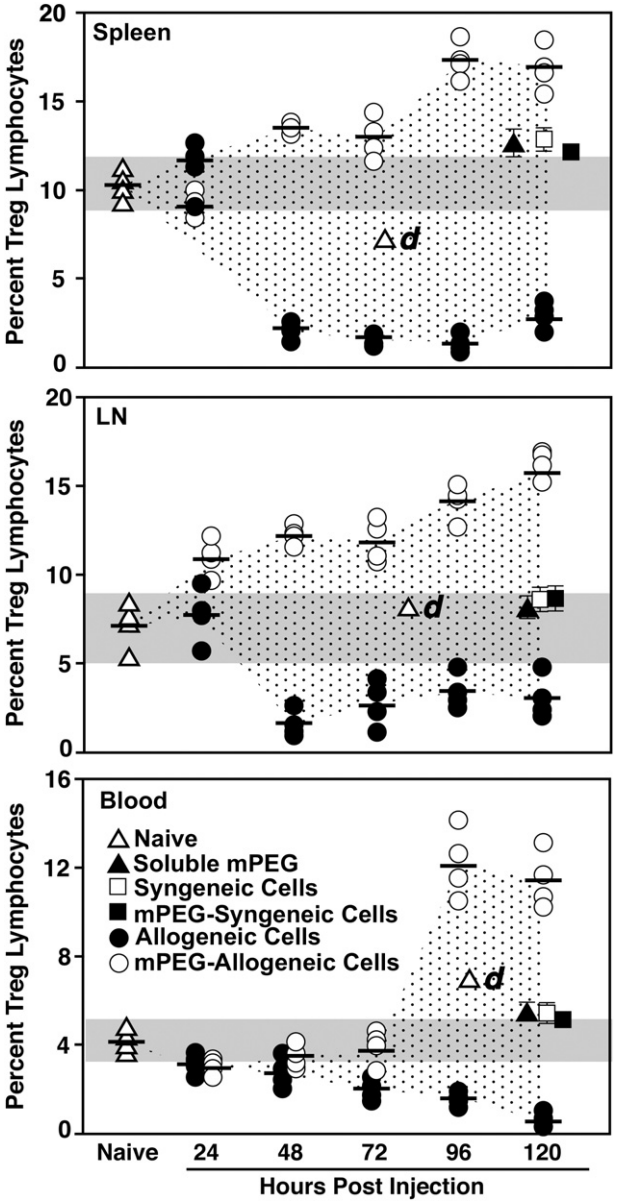

Fig. 5. PEGylation of allogeneic donor murine splenocytes resulted in a significant *in vivo* immunomodulatory effect giving rise to significantly elevated Treg lymphocytes. Shown are the Percent Treg lymphocytes in spleen, "brachial" lymph node, and peripheral blood. As noted, in all three tissues, a significant ($p < 0.001$ at 120 h) increase in Treg lymphocytes over that observed in naïve mice was noted in mice receiving mPEG-modified allogeneic donor cells. In stark contrast, a significant decrease in Tregs (relative to naïve mice) is noted in mice transfused with unmodified allogeneic splenocytes. In comparing the absolute difference between the control PEGylated splenocytes (dotted area) the differential impact of donor cell PEGylation can be fully appreciated. Importantly, as noted at 120 h, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Treg lymphocyte population. The range observed in naïve mice is denoted by the grey bars. PEGylated murine splenocytes were modified with 1 mM SVAmPEG (20 kDa).

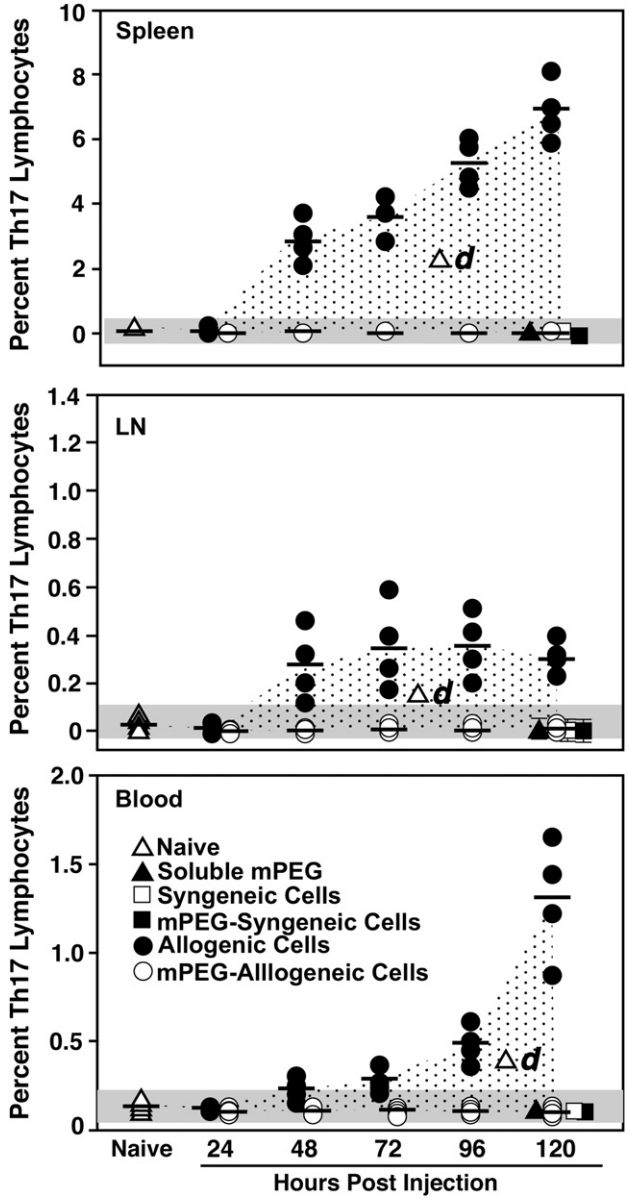

Fig. 6. PEGylation of allogeneic donor murine splenocytes resulted in a significant *in vivo* immunomodulatory effect as evidenced by baseline levels of Th17 lymphocytes. Shown are the Percent Th17 lymphocytes in spleen, "brachial" lymph node, and peripheral blood. As shown, unmodified allogeneic splenocytes resulted in a dramatic increase ($p < 0.001$ at all time points >24 h) in Th17 lymphocytes. However, PEGylation of the allogeneic donor cells completely abrogates this increase and the Th17 levels stay in the range seen in naïve mice (grey zone). In comparing the absolute difference between the control and PEGylated splenocytes (dotted area) the differential impact of donor cell PEGylation can be fully appreciated. Importantly, as noted at 120 h, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Th17 lymphocyte population. The range observed in naïve mice is denoted by the grey bars. PEGylated murine splenocytes were modified with 1 mM SVAmPEG (20 kDa).

unmodified and mPEG-modified donor cells is denoted by the stippled area. As with the Treg population, transfusion of soluble mPEG, syngeneic cells or mPEG-syngeneic cells had no significant effect on the Th17 lymphocyte population at 120 h.

As might be anticipated, the mPEG-allogeneic splenocyte mediated increase in Treg cells in the peripheral blood samples occurred later in the studied time course (96 h) compared to either of the lymphatic tissues (spleen and lymph nodes; 48 h). This clearly suggests that T cell proliferation initially occurred within the lymphatic tissues and secondarily migrated into the peripheral blood. A similar time dependency was noted with the Th17 proliferation induced by the unmodified splenocyte populations. Proliferation initially occurred within lymphatic tissue within ~48 h and only appeared within the peripheral blood after ~96 h.

Of importance was the observation that the immunomodulatory effects of the PEGylated splenocytes were long lived and prevented subsequent changes in Treg and Th17 levels consequent to rechallenge with unmodified allogeneic cells. As shown in Fig. 7, 30 days

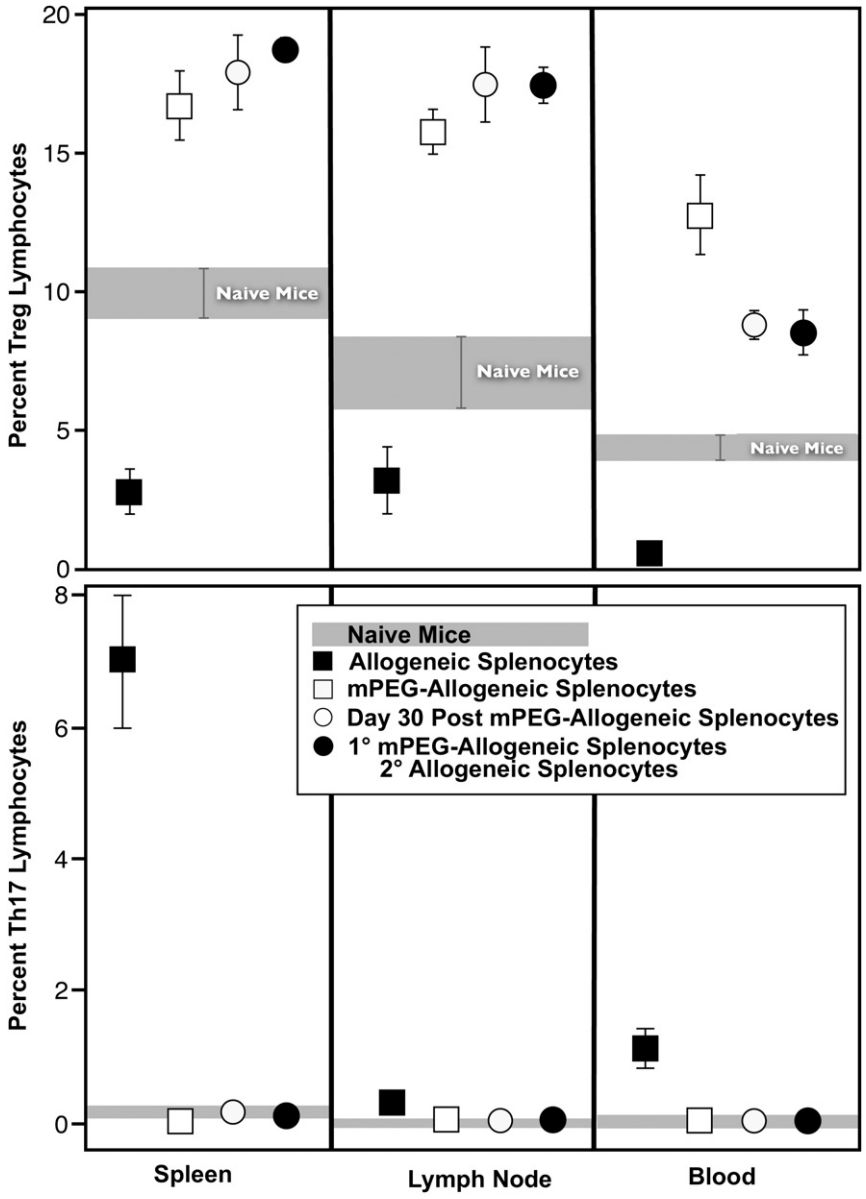

Fig. 7. The immunomodulatory effects of the PEGylated splenocytes is long lived and prevents changes in Treg and Th17 levels consequent to rechallenge with unmodified allogeneic cells. As shown, 30 days post transfusion of the polymer modified splenocytes, Treg levels remain significantly elevated while Th17 levels remain similar to or decreased from that of naïve mice. Furthermore, transfusion of unmodified allogeneic splenocytes to mice previously treated with PEGylated allogeneic cells does not result in decreased Treg or increased Th17 lymphocyte populations. Indeed, Treg levels remain significantly elevated above that seen in naïve mice and very similar to those levels observed at 5 days post PEGylated splenocytes transfusion. The range observed in naïve mice is denoted by the grey bars. PEGylated murine splenocytes were modified with 1 mM SVAmPEG (20 kDa).

post transfusion with polymer modified splenocytes, Treg levels remain significantly elevated and are similar to levels recorded at 120 h post challenge. In contrast, Th17 levels remained similar to or decreased from that observed in naïve mice at day 30. Of even more interest, a secondary adoptive transfer of unmodified allogeneic splenocytes (30 days post 1° challenge; measured at 120 h) to mice previously treated with PEGylated allogeneic showed no significant decrease in Treg cells, or increase in Th17 cells, relative to the day 30 levels. This was in direct contrast to that observed in naïve mice (Fig. 5) injected with unmodified allogeneic cells that demonstrated a dramatic decrease in Treg lymphocytes. Indeed, Treg levels remain significantly elevated above that seen in naïve mice and very similar to those levels observed at 5 days post PEGylated splenocytes transfusion.

To determine if the observed *in vivo* murine findings gave rise to a tolerance to a specific H-2 haplotype or a more generalized anergy to allogeneic tissues, *in vitro* two-way murine MLR studies of three allogeneic splenocyte populations (Balb/c, H-2^d; C57Bl/6, H-2^b; and C3H, H-2^k) were done. As demonstrated in Fig. 8, the immunomodulation arising following exposure to polymer-grafted H-2 disparate splenocytes is not specific to the haplotype of the mPEG-modified spelenocytes thereby suggestive of an anergic state. As shown, PEGylation of stimulator (i.e., irradiated and incapable of proliferation) splenocytes very effectively attenuated allorecognition and proliferation of the responder cell population within a one-way MLR. Moreover, for comparative purposes, the anti-proliferative dose–response effect of cyclosporine A (CSA; which induces a pharmacologically-induced anergy) in a one-way murine MLR under the same experimental condition is shown.

Interestingly, the type of polymer-modified cell is important. Human lymphocytes and murine splenocytes express high levels of "self-antigens" (Human Leukocyte Antigens (HLA) and mouse H-2

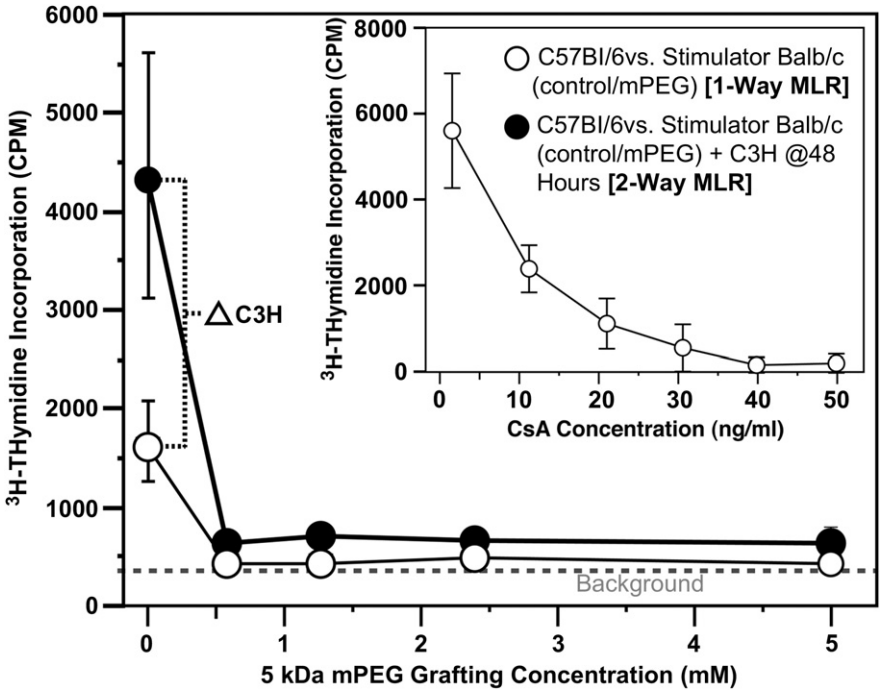

Fig. 8. Initial one-way MLR (open symbol) consisting of C57Bl/6 (H-2b) splenocytes challenged with unmodified or PEGylated irradiated Balb/c (H2-d) splenocytes. Following 48 h of challenge, duplicate samples were challenged with unmodified-nonirradiated C3H (H-2k) splenocytes (two-way MLR). The addition of the fresh responder cells from a third, H2-disparate mouse strain (C3H), at 48 h did not reverse the attenuation of proliferation in responder cells co-incubated with irradiated, cmPEG-modified Babl/c splenocytes. In contrast, the proliferation in the control (0 mM) MLR was significantly ($p < 0.001$) enhanced by the addition of the C3H splenocytes. The data shown represented the co-culturing of 5.12×10^6 C57Bl/6 splenocytes with 5.12×10^6 irradiated, mPEG-derivitized Balb/c splenocytes. After 48 h of incubation, fresh C3H responder cells were added to duplicate wells. The results were expressed as the average mean $\pm$ standard deviation of triplicate samples from a representative experiment. PEGylated murine splenocytes were modified with the indicated concentrations (mM) of activated mPEG (5 kDa).

proteins). If cells devoid of these highly immunogenic antigens are used in the murine model, no changes in either Tregs or Th17 cells are observed. In mice injected with unmodified allogeneic erythrocytes, Treg levels within the spleen, lymph node and peripheral blood were (respectively): 91.7%, 95.0% and 107.0% of control mouse values. Similarly unchanged, Th17 levels were (respectively): 71.2%, 112.1% and 79.2% of control mouse values. Thus, allogeneic murine RBC do not elicit any significant changes in the systemic levels of either Treg or Th17 lymphocytes. This finding was observed despite some antigenic differences between the RBC in H-2 disparate mice. In support of the low immunogenicity of these genetically different RBC, allogeneic RBC exhibit normal *in vivo* circulation nor do they elicit a significant immune response. Hence, polymer coupled to a low-immunogenicity allogeneic cell can not induce the immunomodulation noted with the highly immunogenic splenocytes.

4. Discussion

Bioengineering of donor cells and/or tissues may provide significant opportunities to attenuate both the recognition and rejection of allogeneic tissues. One polymer-mediated approach to this end is the immunocamouflage of donor cells via the covalent grafting of mPEG (or other low immunogenicity polymers) to the exterior face of cell membranes [7,11,17]. Consequent to membrane derivatization, mPEG-modified allogeneic and xenogeneic cells demonstrated a global, multivalent, attenuation of antigenicity and immunogenicity. This effect arouse in part from charge camouflage and significantly diminished cell:cell (e.g., T cell:APC or T cell:islet cell) and ligand:receptor (e.g., antibody:antigen or CD28:CD80) interactions [17,26]. The efficacy of this immunocamouflage was dependent on polymer molecular weight (i.e., size) and grafting density.

However, the inhibition of cell:cell and ligand:receptor interactions are a 'local' immunomodulatory event arising from the steric and charge camouflaging effects of the grafted polymer. For the induction of tolerance, a systemic and persistent immunomodulatory effect would be necessary. Previous studies have been unclear as to whether membrane grafted polymers could result in systemic immunological changes. As demonstrated in this study, covalent grafting of mPEG to allogeneic lymphocytes (human PBMC or murine splenocytes) dramatically reduced allorecognition at both the local (cell:cell; MLR) and systemic (*in vivo* murine models) levels. Importantly, as demonstrated in our *in vivo* studies, it is not the donor cells that differentiate into Treg or Th17 cells, rather it is the recipients immune system that responds to the control or PEGylated splenocytes and upregulates production of either Th17 (upon challenge with unmodified splenocytes) or Treg (upon challenge with mPEG-splenocytes) populations. This was noted by both the absence of CFSE-staining (only donor cells were stained) and H-2 phenotyping of the Th17 and Treg cell populations.

The observed immunosuppressive state induced by PEGylated lymphocytes is surprisingly long lasting *in vivo*. As noted in Fig. 7, the elevated levels of Treg lymphocytes noted at day 5 persist to day 30. Moreover, the presence of these Treg (as well as other probable immunological events) prevents a pro-inflammatory response to unmodified allogeneic splenocytes administered at day 25. Indeed, no increase in Th17 lymphocytes is noted in the immunomodulated mice. Moreover, for the systemic tolerance/anergy to occur, the polymer must be grafted to a highly immunogenic cell type (e.g., lymphocyte and/or antigen presenting cells) as less immunogenic cells, such as H-2 disparate erythrocytes, do not alter the immune (Treg/Th17) response. While allogeneic murine erythrocytes do express antigenic differences at the membrane, these cells are only weakly immunogenic eliciting weak IgG responses and typically

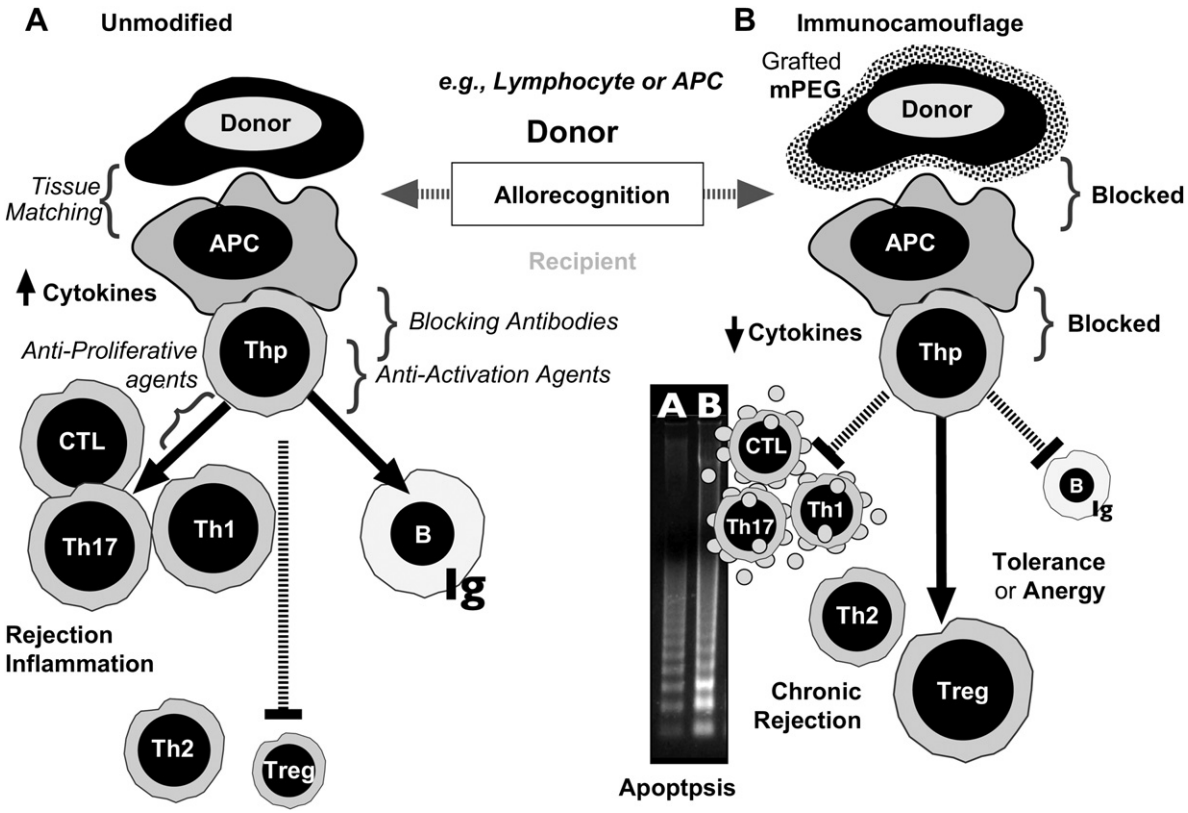

Fig. 9. Immune modulation via pharmacologic and immunocamouflage therapy. Panel A: Current immunomodulation therapy almost exclusively targets the recipient's immune system and does not address the inherent antigenicity and immunogenicity of allogeneic tissues. Response to non-self is in large part mediated by cell–cell interactions between Antigen Presenting Cells (APC; e.g., dendritic cells) and naïve T lymphocytes (Thp). This cell–cell interaction is characterized by adhesion, allorecognition and co-stimulation events. Consequent to allorecognition, cytokine/chemokine burst occurs followed by proliferation of pro-inflammatory T cells (e.g., CTL, Th17, Th1 populations), immunoglobulin production and decreased evidence of regulatory T cells (Treg). Current therapeutic agents are primarily cytotoxic agents preventing T cell activation (e.g., cyclosporine and rapamycin) or T cell proliferation (e.g., methotrexate, corticosteroids, azathiaprine). Additionally some blocking antibodies have been investigated. Panel B: In contrast, PEGylation of donor PBMC results in loss of appropriate cell–cell interaction leading to loss of the cytokine burst, decreased/absent proliferation, evidence of apoptosis of alloresponsive T cells and increased levels of T Regulatory (Treg) cells that, in aggregate, provides a tolerogenic/anergic state both *in vitro* (Figs. 2, 3, and 7) and *in vivo* (Figs. 4–6). Shown with the schematic is a DNA laddering gel of an unmodified MLR (*A*) and a PEGylated MLR (*B*) showing enhanced apoptosis consequent to PEGylation. Size of T cell population denotes increase or decrease in number. Size of B cell indicates antibody response.

remaining in the vascular circulation with a near normal half-life. Also of critical importance, induction of both local and systemic immunomodulation requires the covalent grafting of the polymer to the cell, as soluble mPEG ± allogeneic cells has no effect on the population dynamics of either Treg or Th17 lymphocytes *in vitro* or *in vivo*.

The balance between Treg and Th17 cells has been identified as a key factor that orchestrates the tolerance/inflammation level of human immune system [21,23,24,27]. Regulatory T cells provide suppressor effect and maintain tolerance, while Th17 cells mediate and are indicative of a pro-inflammatory state. Hence, the polymer-mediated modulation of this balance may be clinically useful. Recent findings have shown that cyclosporine, a clinically used immunosuppressive agent, has substantial effects on the Treg/Th17 cell response; though this may be mediated by Th17 cytotoxicity as Treg cells cultured in the presence of rapamycin, but not cyclosporine A, are found to suppress ongoing alloimmune responses [28,29]. Additionally, mycophenolic acid, another immunosuppressive agent, was found to shift the lymphocyte polarization by inhibiting IL-17 expression in activated PBMC *in vitro* [30]. Of clinical importance, all of these pharmacologic agents exert significant systemic toxicity and their ongoing use requires substantial monitoring [31].

As evidenced by these results, induction of tolerance or anergy in transfusion and transplantation medicine by the polymer-mediated immunocamouflage of allogeneic leukocytes may provide a less toxic approach than current conventional pharmacologic agents. Current efforts to prevent and/or regulate the consequences of allorecognition involve phenotype matching (ranging from blood group to HLA matching) and the use of immunosuppressive agents (Fig. 9A) [1,32]. While extensive tissue matching (e.g., Blood groups, HLA) can dramatically enhance transfusion or transplantation success, the necessity of tissue matching dramatically reduces the potential pool of donor tissues. Even in a tissue as plentiful as blood, extensive non-ABO matching for chronically transfused patients (e.g., sickle cell disease), while considered desirable, is costly and often difficult to achieve due to the scarcity of appropriately matched donor cells. This difficulty is greatly exaggerated with less common tissues and organs (e.g., islets and kidneys).

Thus, pharmacological interventions have been employed to enhance the probability of successful donor tissue engraftment (Fig. 9S). The data presented here suggests that polymer encapsulation 'of', or grafting 'to', donor tissue may be further enhanced or replaced by a tolerogeneic or anergic approach. Rather, the pre-challenge of a potential tissue recipient with PEGylated donor specific (or simply allogeneic; see Fig. 8) PBMC several (~5) days prior to tissue transplantation could be used to induce a tolerogenic state within the recipient as shown in Fig. 9B. Elevated levels of Tregs and the down-regulation of Th17 cells would diminish the risks of both hyperacute and acute rejection of the donor tissue. There are several substantial advantages for this approach. Primary amongst

these are the easy collection of donor specific (or simply allogeneic) PBMC, the ease of PEGylation of the PBMC as well as the ease of administration to the transplant recipient. While a potential risk of lymphocyte transfusions is transfusion associated graft versus host disease (TA-GVHD) in immunosuppressed patients, we have previously demonstrated that PEGylation effectively blocks TA-GVHD in a murine model [12,14]. Moreover, this process could be used in conjunction with irradiated PBMC thus obviating any risk of TA-GVHD. Irradiated cells retain their allostimulatory effects and PEGylation similarly inhibits this allorecognition and proliferation.

5. Conclusions

In summary, polymer modification of allogeneic donor lymphocytes prevents allorecognition at the cell:cell level and also gives rise to systemic immunomodulation. The systemic immunomodulation is evidenced by a significant up-regulation of Treg cells and a significant down-regulation of pro-inflammatory Th17 cells. This immunomodulation is persistent ($\geq$30 days) and prevents subsequent pro-inflammatory responses to unmodified allogeneic cells. The polymer effect is dependent upon its covalent grafting to allogeneic cells as soluble PEG itself has no immunomodulatory effects. The clinical use of PEGylated (or other covalently grafted polymers) allogeneic lymphocytes to pre-challenge tissue recipients 5 days or more to transplantation may be useful in inducing a tolerogenic state and preventing acute rejection and/or enhancing tissue engraftment.

Acknowledgments

This work was supported by grants from the Canadian Blood Services, Canadian Blood Services-Canadian Institutes of Health Research (CBS-CIHR) Partnership Fund and Health Canada. The views expressed herein do not necessarily represent the view of the federal government of Canada. We thank the Canada Foundation for Innovation and the Michael Smith Foundation for Health Research for infrastructure funding at the University of British Columbia Centre for Blood Research. The authors would like to thank Ms. Dana Kyluik for her insightful comments.

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