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Inhibition of allogeneic cytotoxic T cell ($CD8^+$) proliferation via polymer-induced Treg ($CD4^+$) cells

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ABSTRACT

T cell-mediated immune rejection remains a barrier to successful transplantation. Polymer-based bioengineering of cells may provide an effective means of preventing allorecognition and the proliferation of cytotoxic ($CD8^+$) T lymphocytes (CTL). Using MHC-disparate murine splenocytes modified with succinimidyl valerate activated methoxypoly(ethylene glycol) [SVA-mPEG] polymers, the effects of leukocyte immunocamouflage on $CD8^+$ and $CD4^+$ alloproliferation and T regulatory (Treg) cell induction were assessed in a mixed lymphocyte reaction (MLR) model. Polymer-grafting effectively camouflaged multiple leukocyte markers (MHC class I and II, TCR and CD3) essential for effective allorecognition. Consequent to the polymer-induced immunocamouflage of the cell membrane, both $CD8^+$ and $CD4^+$ T cell alloproliferation were significantly inhibited in a polymer dose-dependent manner. The loss of alloproliferation correlated with the induction of Treg cells ($CD4^+CD25^+Foxp3^+$). The Tregs, surprisingly, arose primarily via differentiation of naive, non-proliferating, $CD4^+$ cells. Of biologic importance, the polymer-induced Treg were functional and exhibited potent immunosuppressive activity on allogeneic CTL proliferation. These results suggest that immunocamouflage-mediated attenuation of alloantigen-TCR recognition can prevent the tissue destructive allogeneic $CD8^+$ T cell response, both directly and indirectly, through the generation/differentiation of functional Tregs. Immunocamouflage induced tolerance could be clinically valuable in attenuating T cell-mediated transplant rejection and in the treatment of autoimmune diseases.

Statement of Significance

While our previous studies have demonstrated that polymer-grafting to MHC disparate leukocytes inhibits $CD4^+$ cell proliferation, the effects of PEGylation on the alloproliferation of $CD8^+$ cytotoxic T cells (CTL) was not examined. As shown here, PEGylation of allogeneic leukocytes prevents the generation of the CTL response responsible for acute rejection. The loss of CTL proliferation is consequent to the polymer-based attenuation of allorecognition and the induction of T regulatory cells (Tregs). Interestingly, the Tregs are primarily generated via the differentiation of non-proliferating naive T cells. Importantly, the Tregs are functional and effectively induce a tolerogenic environment when transferred to an alloresponsive environment. The use of polymer-modified leukocytes provides a unique approach to effectively maximize the biologic production of functional Tregs both *in vitro* and *in vivo*. By using this approach it may be possible to attenuate unwanted alloresponses (*e.g.*, graft rejection) or to treat autoimmune diseases.

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1. Introduction

Transplantation of allogeneic tissues (ranging from discrete cells to intact organs) is an increasingly critical component of modern medicine. However, despite the vast potential of donor tissues to correct tissue/organ failures, immune-mediated rejection

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remains a significant barrier to successful transplantation. Although typically less than 0.1% of an individual's T-cell repertoire is specific to a normal bacterial/viral antigen, a much higher frequency (1–10%) of T cells recognize and react to allogeneic non-self tissues and organs thus posing a significant obstacle to transplantation [1,2]. The increased risk associated with allogeneic tissue is largely due to disparity in major histocompatibility complex (MHC) antigens between donor and recipient. The MHC is a set of highly polymorphic cell-surface proteins essential for recognition of 'self' and 'non-self' by the immune system and, just as the name implies, the MHC determines the histocompatibility of the donor and recipient. Through the antigen peptide-MHC binding complex (pMHC), foreign antigens are presented to the T cell receptor (TCR) on appropriate T lymphocytes initiating the immune response [3]. While the immune response to allogeneic tissue is complex involving both humoral and cellular responses, T lymphocytes ($CD4^+$ and $CD8^+$) play the central role in both effecting and regulating the proinflammatory allogeneic response [4,5].

The effector response is driven by both $CD4^+$ effectors cells and $CD8^+$ cytotoxic T lymphocytes (CTL). While debate exists as to the relative role of $CD4^+$ and $CD8^+$ cells in allograft rejection (with some studies favoring $CD4^+$ effector cells) [6], $CD8^+$ cells are an active participant in acute rejection. Indeed, several studies have reported that cytotoxic CD8 T-cell responses directed against MHC class I alloantigens are one of the principal mediators of allograft rejection [7,8]. Moreover, Ji *et al.* found that CD4 T cell depletion failed to prevent cardiac graft rejection in alloantigen sensitized B cell-deficient mice [9]. In contrast, Ji *et al.* reported that CD8 T cell depletion led to long-term (>100 days) cardiac allograft survival. The authors stated that their results clearly supported *"the paramount role of CD8 T cell primed/memory responses in our model [i.e., Transplant rejection]"* [9]. Regardless of whether $CD4^+$ or $CD8^+$ cells are the primary mediators of injury, an unregulated cytotoxic/proinflammatory response would invariably harm/kill the host. Hence the immune response also has T regulatory (Treg; $CD4^+CD25^+Foxp3^+$) cells to limit both the magnitude and duration of the inflammatory response [10,11].

In the context of transplantation, the inhibition of the effector (*i.e.*, cytotoxic/proinflammatory) response has been historically targeted using cytotoxic pharmacologic agents that prevent expansion of the effector T cells. More recently, in both transplantation and autoimmune disease therapy, basic research and clinical efforts have begun shifting to enhancing the Treg response in an effort to prevent the adverse side-effects of the current cytotoxic anti-rejection drugs and/or prevent autoimmune tissue injury [10,11]. These approaches have primarily focused on either the expansion of the endogenous Treg cells via pharmacologic agents [12,13] or by the exogenous expansion of autologous Treg which are then transfused into the patient [14–16]. Showing the potential promise of these approaches, a number of studies have demonstrated that an increased Treg population correlates with positive transplant outcomes [17–19]. However, the methodologies used for both endogenous and exogenous Treg expansion are often complex and time consuming; hence a simple, safe, effective and efficient method for expanding Tregs is much needed.

Nanoscale polymer-based immunocamouflage of cells may provide a simple, safe, inexpensive, but potent means of inducing tolerogenic Tregs both *in vitro* and, more importantly, *in vivo*. Our previous studies in both human and mouse models have shown that the immunocamouflage of the donor allogeneic lymphocytes via membrane grafting of methoxy(poly ethylene) glycol (mPEG; PEGylation) or poly-ethyloxazoline propionic acid (PEOZ; POZylation) effectively attenuated allorecognition [20–33]. Mechanistically the grafted polymer blocks allorecognition by inhibiting cell:cell (*i.e.,* T Cell:Antigen Presenting Cell; T Cell:APC) interaction consequent to both steric interference and charge camouflage [27,32]. Consequent to the attenuated allorecognition a tolerogenic/anergic state is induced. *In vivo*, the immunocamouflage efficacy of membrane PEGylation was demonstrated by the inhibition of Transfusion-Associated Graft versus Host Disease (TA-GVHD) in a murine model [24,25]. The observed immunoquiescent state induced by immunocamouflage was characterized by loss of the proinflammatory $CD4^+$ effector T cell proliferation (Teff; *e.g.*, Th17) and an increase in $CD4^+$ Tregs resulting in a net increase in the Treg:Teff ratio [28,34].

Not addressed in our earlier studies was the efficacy of immunocamouflage on the proliferative response of $CD8^+$ cytotoxic T cells – the cells responsible for the majority of direct cell killing/tissue rejection. Similarly, the complex interplay of polymer size and grafting concentration on the induction of Tregs necessary to regulate the CTL response was not elucidated nor was the origin (donor *vs.* recipient) of the generated $CD4^+$ Treg known. Finally, while indirect evidence of the functional nature of the polymer-generated Tregs was observed, no direct functional studies had been done. In this study, murine mixed lymphocyte reaction (MLR) models were used to assess the effects of polymer-induced immunocamouflage on $CD8^+$ CTL proliferation and to assess the interplay of polymer size and grafting concentrations on the generation of functional $CD4^+$ Treg cells in both the donor and recipient cell populations.

2. Materials and methods

2.1. Murine splenocytes

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. Two MHC (H-2) disparate allogeneic strains of mice were used: BALB/c, H-2d; and C57BL/6, H-2b. Murine splenocytes were prepared from freshly harvested spleen via homogenization into a cell suspension in PBS (0.2% BSA) using the frosted end of two microscope slides. Red blood cells were removed by treating splenocytes with BD Pharm Lyse buffer (BD Pharmingen, San Diego, CA).

2.2. mPEG modification (PEGylation) and CFSE labeling of splenocytes

Murine splenocytes were derivatized using succinimidyl valerate activated methoxypoly(-ethylene glycol) (SVA-mPEG; Laysan Bio Inc. Arab, AL) with a molecular weight of 2, 5, 10, 20 or 30 kDa as previously described [21,27,28,31,32]. Grafting concentrations ranged from 0 to 2.0 mM per 4×10^6 cells/mL. Cells were incubated with SVA-mPEG for 60 min at room temperature in isotonic alkaline phosphate buffer (50 mM K_2HPO_4 and 105 mM NaCl; pH 8.0), washed twice with 25 mM HEPES/RPMI 1640, then resuspended in RPMI 1640 media containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% Pen-Strep, and 0.05 mM β-mercaptoethanol at a final cell density of 2.0×10^6 cells/mL. In all experiments, cell proliferation was assessed via flow cytometry using a dye-dilution assay (CFSE; CellTrace™ Carboxyfluorescein diacetate, succinimidyl ester (CFSE) Cell Proliferation Kit; Molecular probes by Life Technologies, Carlsbad, CA). Murine splenocytes were labeled with 2.5 μM CFSE diluted in PBS containing 0.1% BSA, according to the manufacturer's instructions.

Of note, under the experimental conditions used in this study, PEGylation had no differential effects on cell viability (*e.g.,* propidium iodide exclusion, 7-AAD; mitochondrial depolarization; and caspase-mediated apoptosis) or proliferation potential [21,24,25,29,31,32]. As a standard control for all studies, mitogen stimulation was done to confirm the post-derivatization viability

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