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Consecutive low doses of cyclophosphamide preferentially target T_{regs} and potentiate T cell responses induced by DNA PLG microparticle immunization

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ABSTRACT

Cyclophosphamide in combination with immunotherapeutic approaches preferentially impinges on T_{reg} activity and allows for robust generation of T cell effectors. Reduced dosages of cyclophosphamide are necessary to restrict its cytotoxic effects to the negative regulatory cell populations while sparing effector lymphocytes. We investigated cyclophosphamide dosing in combination with ZYC300, a PLG-encapsulated plasmid DNA vaccine which encodes the cytochrome P450 family member, CYP1B1, a known human tumor-associated antigen. In mice, three consecutive, low doses of cyclophosphamide comprised a superior regimen in enhancing the magnitude, diversity of epitopes, and avidity to individual epitopes of specific T cell responses when compared to regimens that used either a single low or a single high dose. Consecutive low doses of cyclophosphamide predominantly targeted T_{regs} while sparing overall T lymphocyte counts. Thus, we report the synergistic activity of pharmacologic T_{reg} depletion with cyclophosphamide on quantitatively and qualitatively increasing T cell responses to a known human tumor-associated antigen.

1. Introduction

Regulatory T cells (T_{regs}) are vital to the prevention of autoimmune disease in vertebrates through the maintenance of immune tolerance to self antigens [1–3]. This function includes mechanisms that are dependent on direct cell contact [4–7], and mediated via secretion of soluble factors [7–12]. T_{regs} are able to suppress autoreactive cellular and humoral immune responses [13,14] and prevent pathological self-reactivity. The importance of avoiding such self-directed immune responses is readily underscored by several disease-causing mutations that are manifested as deficiencies of functional T_{regs}, the IPEX syndrome in man and the spontaneous 'scurfy' mutation in mice; both result in lethal autoimmunity early in life [15,16]. It is now known that there is considerable plasticity in how T_{regs} populations arise. Naturally occurring $CD4^+/CD25^+$/FOX $P3^+$ T_{regs} are produced in the thymus following high avidity TCR interactions with stromal self antigens [17,18]. They can also be generated in the periphery through conversion of $CD4^+/CD25^-$ cells, a process that occurs during the maintenance of normal immune homeostasis, but which also has been implicated in tumor progression and immune evasion [19,20]. Therefore, despite the ability to prevent disease, the suppressive ability of T_{regs} can effectively down-modulate both naturally occurring and induced anti-tumor immune responses [21–25].

Cyclophosphamide monohydrate (Cytoxan™), CY, is a non-myeloablative antineoplastic agent that is administered as a prodrug and undergoes biotransformation by P450 cytochrome oxidases to yield the active alkylating metabolite, 4-hydroxycyclophosphamide [26]. Synthesized and tested as a chemotherapy by Brock and Wilmanns in 1958 [27], other investigators followed with discoveries of cyclophosphamide's immune potentiating effects, including data indicating that CY markedly augmented delayed type hypersensitivity reactions (DTH) and antibody responses to KLH in previously anergic late stage cancer patients [28]. This study and others done in animals suggested a role for the removal of a CY sensitive suppressor cell population for efficacy [29–32]. Later, $CD4^+CD25^+$ regulatory T cells were identified as the suppressor population whose removal by CY was responsible for the reversal in tumor immune tolerance in rodent studies [22]. Encouragingly, depletion or inactivation of T_{regs} by cyclophosphamide has been shown to lead to increased activity of effector T cells, NK cells, and T-dependent innate immune responses when immunotherapeutic vaccines have been used in combination with CY, leading to tumor rejection in multiple animal models

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[22,33–37]. This has also been implicated in clinical studies where marked reversal of tumor induced immune suppression has been observed in patients with late stage disease [38,39]. Additional dose- and schedule-dependent immunomodulatory effects of CY have been reported and may alternately support adaptive immunity by mechanisms that complement T_{reg} manipulation. These include increased tumor antigen cross presentation facilitated by tumor killing, induction of a cytokine storm that initiates homeostatic proliferation, and bone marrow mobilization that aims to equilibrate the void created in cell subset compartments [26,40,41].

ZYC300 is a cancer vaccine targeting the cytochrome P450 member, CYP1B1, a tumor antigen commonly overexpressed in multiple solid tumors and hematological malignancies [42–46]. ZYC300 consists of plasmid DNA encoding CYP1B1 that has been encapsulated in poly lactide-co-glycolide (PLG) microparticles. The PLG encapsulation technology enhances targeting of the plasmid DNA to antigen presenting cells and promotes immune responses to the plasmid encoded antigen(s) [45–48]. In a phase I clinical trial, ZYC300 administration induced immune responses to CYP1B1, and an association was noted between development of CYP1B1 immunity and clinical efficacy by subsequent salvage therapies [49]. These results were encouraging and led to the hypothesis of combining ZYC300 immunization with CY in order to enhance CYP1B1 immune responses. The results of a second phase I trial combining ZYC300 with CY have been reported [39], which suggest that the dose of cyclophosphamide used is critical to maintaining effector cell function while still reducing T_{reg} activity. The studies described here were performed to optimize the dosing schedule and timing intervals of CY and ZYC300 in order to determine if an improved regimen could be identified. We determined that consecutive low doses of CY preceding each cycle of ZYC300 immunization enhanced the overall magnitude, epitope diversity, and avidity of CYP1B1-specific immune responses vs. immunization of mice with ZYC300 alone. Moreover, while a single low dose of CY administered prior to ZYC300 amplified T cell responses to immunodominant CYP1B1 epitopes, increased T cell responses to minor epitopes were elicited when consecutive low doses of CY were used. The use of a single high dose of CY did not improve the generation of CYP1B1 immune responses. Furthermore, in our studies we observed that both $CD25^+$ and $CD25^-$ T_{regs} were sensitive to CY induced decreases, while levels of both non-T_{reg} $CD4^+$ and $CD8^+$ T cells were well preserved during treatment with the consecutive low dose regimens. Finally, while published reports routinely describe enhanced magnitude of T cell responses as a result of transiently abrogating T_{reg} activity, we also observed qualitative enhancements of the avidity of the CYP1B1-specific T cell response.

2. Materials and methods

2.1. Cell lines

The H-2k expressing BW5147 cell line was purchased from ATCC (Manassas, Va).

2.2. Plasmids

A CYP1B1 expression plasmid was generated by PCR amplification of the human CYP1B1 open reading frame and has been described previously [44].

2.3. Encapsulation

Plasmid DNA used for immunization was purified according to the manufacturer's instructions using an endotoxin free mega prep kit (Qiagen Corp, Chatsworth, CA). Plasmids were encapsulated in PLG particles as previously described [48]. ZYC300 is a PLG-encapsulated plasmid DNA immunotherapeutic that encodes a mutated form of full-length human cytochrome P450 CYP1B1 tumor antigen. Where dosages of microparticles are indicated, these are based on the amount of plasmid DNA being dosed as determined by lot specific analytical assays. In general, 7–9 μg of plasmid DNA is encapsulated within each milligram of PLG biodegradable polymer.

2.4. In vivo studies with ZYC300 Immunization and cyclophosphamide dosing

Female 8–10 week old C3HeB/FeJ (H-2k) mice (JAX # 000658) were purchased from Jackson Laboratories, Bar Harbor, Maine. Animals were acclimated for at least one week prior to study enrollment. All studies were conducted in accordance with NIH experimental animal welfare guidelines, and policies and study protocols approved by the corporate Institutional Animal Use and Care Committee (IACUC). Groups of 4–10 mice were used per group, and for microparticle immunizations 50 μg of encapsulated DNA (ZYC300) was injected in a volume of 50 μl of saline into each *tibialis* muscle of mice for a total dose of 100 μg per mouse. Mice were primed and received two booster injections for a total of three ZYC300 injections spaced two weeks apart, unless otherwise specified. In studies examining the generation of high avidity T cells, mice were immunized once with ZYC300 on day 0, boosted at day 21 and then ELISpot assays were performed on day 37. Naïve control mice were injected with saline alone. Spleens were harvested 14–17 days after the final boost. Cyclophosphamide monohydrate (Cat. #C-0768, Sigma–Aldrich, St. Louis, MO) (CY) was resuspended at a 10× concentration in sterile filtered deionized water and then diluted just prior to use. Stocks were freshly prepared and used immediately, or stored for no more than 18 h at 4 °C shielded from light; animals received intraperitoneal CY injections of 20–200 mg/kg in a final volume of ~500 μl, based on body weight. Several in vivo studies were conducted that measured the effects of CY on the generation of ZYC300 specific T cell responses as measured by IFN-γ ELISpot assays. In these studies, mice received a single i.p. dose of CY at 20 or 200 mg/kg 7, 4 or 2 days prior to each *intramuscular* injection of ZYC300. A total of three cycles of ZYC300 and CY were administered. Subsequent studies tested the effects of a single dose versus consecutive daily doses of CY prior to each cycle of ZYC300 immunization. Mice received a single, or three consecutive daily injections of 20 mg/kg of CY, with the last dose occurring 2 days prior to ZYC300 immunization. Finally, several in vivo studies were conducted that measured the effects of CY on various lymphoid cell subsets. In these studies cohorts of mice were injected once daily for 1, 3 or 5 consecutive days with 20 mg/kg or received a single injection of 100 or 200 mg/kg for comparison. Measurements of splenocytes were then made on day 3, 4, 5, 6, 7 or 10 after the last dose of CY.

2.5. Splenocyte processing

Spleens were harvested from mice under sterile surgical conditions. Single cell suspensions of excised spleens were obtained by gently pressing the tissues through sterile 70 μm mesh cell strainers (Falcon, Bridgeport, NJ; Cat. #21008–952) set atop 50 ml conical centrifuge tubes. The resulting cells were washed with RPMI medium supplemented with 10% FBS followed by erythrocyte lysis at ambient temperature by incubation in a 5.0 ml volume of commercially available hypotonic lysing buffer (Sigma, Cat. #R7757). Following two additional media washes, cells were either resuspended in FACS staining buffer or T cell column buffer as described elsewhere in Section 2. Where indicated, spleens from two untreated age-matched control mice were collected on each day of