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Could post-transplant cyclophosphamide benefit autoimmune disease patients following autologous hematopoietic stem cell transplantation?

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Running title: PTCy following auto-HSCT for AID patients

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Abstract

Autologous hematopoietic stem cell transplantation (auto-HSCT) has emerged as an effective therapeutic option for selected patients with severe autoimmune diseases, particularly relapsing-remitting multiple sclerosis. However, disease relapse remains a significant challenge, suggesting the persistence of autoreactive lymphocytes and incomplete restoration of sustained immune tolerance. Post-transplant cyclophosphamide (PTCy) is widely employed following allogeneic HSCT for graft-versus-host disease prophylaxis. Here, we propose that incorporation of PTCy into auto-HSCT protocols may further improve outcomes in selected autoimmune disease patients by reducing and suppressing residual autoreactive T cells that survive conditioning and subsequently undergo lymphopenia-driven and / or antigen-induced proliferation post-transplant. Drawing on mechanistic and clinical insights from allogeneic transplantation, we discuss how PTCy could augment depletion of pathogenic effector T cells while supporting hematopoietic recovery and immune reconstitution. Because complete removal of autoreactive lymphocyte populations is unlikely, long-term disease control will probably require promoting peripheral immune regulation. Multiple sclerosis is highlighted as a model disease, although the concepts discussed may extend to other autoimmune disorders. Overall, the addition of PTCy to auto-HSCT represents a novel translational strategy with the potential to enhance immune resetting, suppress residual autoreactivity, and improve long-term remission rates in autoimmune disease patients.

Introduction and hypothesis

There are more than 80-100 identified autoimmune diseases (AID) that affect an estimated 5-10% of the human population(1). Psoriasis and autoimmune thyroid disease are the most common; however, others such as systemic sclerosis, rheumatoid arthritis, systemic lupus erythematosus (SLE), type 1 diabetes, inflammatory bowel disease, and multiple sclerosis (MS) affect a significant number of the population, especially women. The immunological challenge to cure such diseases necessitate accomplishing two principal objectives: 1) regulation of the patients existing auto-reactive lymphocyte populations, i.e. T and B effector cells and 2) the re-establishment of immune tolerance to prevent the generation of additional self-reactive cells. Dysregulation of the patient's immune system that can include changes in both adaptive (ex. CD4⁺FoxP3⁺ Treg, TR1 and Bregs) as well as innate (ex. iNKT, MDSC, ILCregs, etc.) regulatory populations have been reported resulting in unwanted effector lymphocyte responses against self-antigens, hence restoring immune regulation in general is essential(2, 3). Based on the studies by a number of laboratories, Treg cells are recognized as a non-redundant and crucial population for preventing unwanted self-reactive responses as their removal results in rapid onset of AID and defects in this population have been proposed to underlie many, if not most autoimmune diseases (4-8). To achieve the objective of regulating the immune system, various treatments have been administered to patients with AID. For example, a number of cytotoxic drugs have been employed to delete and suppress unwanted self-antigen reactive T cells, for example, methotrexate, cyclophosphamide and corticosteroids as well as other strategies involving antibodies and inhibitors to block T and B cell responses including rituximab, ofatumumab and ibrutinib(9).

Multiple sclerosis is a chronic neurological disorder mediated by abnormal T and B cell autoimmune responses resulting in neuroinflammation, demyelination, and axonal degeneration within the CNS. Disease modifying therapies (DMT) based on immune-modulatory regimens have been utilized therapeutically including interferon beta-1a/1b, anti-CD20, anti-CD52 mAbs, natalizumab, sphingosine 1 phosphate (S1P) agonists, and glatiramer acetate(9, 10). Although these treatments have been found to be somewhat effective in reducing immune activation, disease activity, and relapse frequency, unfortunately they do not halt MS progression. One of the more effective treatments for relapse remitting multiple sclerosis (RRMS) has been the application of autologous hematopoietic stem cell transplantation (HSCT). Auto-HSCT has demonstrated effectiveness in achieving long-term remission and neurological improvement in clinical trials (11, 12). Conditioning and auto-HSCT results in reducing effector T cell levels, hematopoietic engraftment and re-establishment of central self-tolerance. Notably, experimental bone marrow transplant studies grounded initially >20 years ago by Fuchs and Luznik at Johns Hopkins University has led to the use of cyclophosphamide administration "post-transplant" (PTCy) following clinical allogeneic HSCT for graft vs host disease (GVHD) prophylaxis (13, 14). Its use has enabled increased numbers of haplo-identical and mismatched transplants(14) and greater application of allo-HSCT to older patients. Based on PTCy's compelling ability to inhibit the powerful alloantigen-reactive Teff responses underlying the development of GVHD, here we propose its use as a novel approach to reduce and suppress self-reactive Teff cells following autologous-HSCT for treatment of some autoimmune disorders. We posit that auto-HSCT with the addition of PTCy may generate a useful platform to enhance the reduction and suppression of self-reactive T cells while enabling effective application of peripheral immune regulation including Tregulatory (Treg) cell therapy (**Fig. 1**). Because auto-HSCT is used to treat RRMS, here we use MS as an example AID to discuss our proposed model.

Reduction of Auto-reactive effector cells

Despite diverse strategies implemented for treatment of autoimmune disease, it has been particularly difficult to accomplish complete deletion and / or suppression of autoreactive effector and memory T cells that underlie these disorders. Treatments must also prevent the emergence of new self-reactive naïve T cells that could develop centrally within the thymus and undergo activation in the periphery due to inadvertent presentation of self-antigens. Approaches that could augment Teff deletion and promote self-tolerance in patients with autoimmune disease should lead to improved therapeutic efficacy and further advance treatment.

Over the past several decades, a major advance in GVHD prophylaxis following allogeneic-HSCT was implemented using a brief, two-day administration of cyclophosphamide (Cy) on day 3 and 4 (50mgs/kg/day) after stem cell infusion, i.e. “post” - transplant (PTCy). While Cy has been and continues to be used in conditioning strategies *prior to* HSCT, the use of PTCy initially emerged from findings that hematopoietic stem cells are spared due to the presence of substantial aldehyde dehydrogenase (ALDH) expression. (15, 16). Studies by Luznik and others including our own group, demonstrated several notable effects of PTCy following experimental allo-HSCT. *Dependent on dose*, these effects include: 1) diminution of anti-host alloantigen specific donor T cells, 2) promoting survival and function of a proportion of CD4⁺FoxP3⁺ Treg cells, and 3) suppression of Tconv cells(17-19). These combined effects have led to the prophylaxis of acute and reduction in chronic GVHD levels together with improved steroid responsiveness. These outcomes occurred without apparent change in the recurrence rate of the primary hematological malignancy, while simultaneously achieving hematolymphoid engraftment and establishment of self-tolerance(17-21).

Auto-HSCT has been applied for RRMS patients and as noted, has resulted in disease remission for extended time-periods, up to five years(11). Notably, cyclophosphamide is used together with G-CSF in the mobilization process for these MS patients, in part *to reduce the numbers of effector cells* that could contaminate the graft(12). Nonetheless, relapse does occur indicating that either some self-reactive Teff persist following transplant and / or additional new T cell clones develop. It is very likely that not all self-reactive T cells can be deleted by conditioning and HSCT (**Fig.1**) as susceptibility is, in large measure associated with proliferation. Due to different TcR affinities as well as levels of expression of self-antigens on APCs, it is highly unlikely all self-reactive T cells would be in the process of division at the time of radiation / chemo conditioning thereby decreasing the probability of their elimination. Administration of 50mg/kg cyclophosphamide precisely on Days 3 and 4 post-allogeneic HSCT results in removing some donor T cells because at this time, a significant number in the lymph nodes are undergoing rapid alloantigen induced proliferation thereby diminishing the time-period for effective DNA repair. However, it is apparent that not all alloreactive T cells are deleted or suppressed by D3/4 PTCy which may account for our findings of the subsequent requirement of Tregs to promote optimal PTCy mediated GVHD prophylaxis(22). It is notable that a lymphopenic state – including that induced by conditioning before HSCT – promotes T cell division due to the presence of unbound cytokines (23-25). The availability of IL-7 and IL-15 as a consequence of the low overall numbers of T cells present post-transplant results in binding to persisting T cells and induction of division(23-25). Hence, PTCy’s ability to delete T cells including some alloantigen-reactive T cells post-HSCT is likely enhanced by lymphopenic conditions driving proliferation present post-HSCT(26). Regardless, other T cells, for example, non-alloantigen reactive cells can also undergo deletion by PTCy following conditioning and transplant (**Fig. 2**). By monitoring transplanted T cell receptor transgenic

populations added to the donor inoculum prior to allo-HSCT, our group reported that those populations undergoing a sufficiently rapid rate of proliferation were reduced(26).

As noted above, autologous HSCT has been used to treat MS patients and patients with other autoimmune diseases including systemic sclerosis(11). We hypothesize that while HSCT conditioning deletes a portion of the auto-reactive T cells present, *the subsequent lymphopenia can induce division of remaining T cells including auto-reactive T cells*(24, 27, 28) (**Fig. 2**). In fact, we have found that mouse anti-MBP specific T cells persisting post-conditioning and HSCT exhibit very high Ki67 levels (DW/RBL personal observation). In addition to the lymphopenia present, as a consequence of conditioning induced lymphocyte death, release of auto-antigens could also induce proliferation of some self-reactive T cells promoting susceptibility to removal following PTCy administration (**Fig. 2**). Hence, in the circumstance of MS, the presence of myelin post-HSCT could drive persisting auto-antigen disease inducing T cells or in its absence, this population would likely undergo lymphopenic expansion, increasing susceptibility to Cy mediated alkylation induced death (**Fig. 2**). Accordingly, we posit administration of post-transplant cyclophosphamide at appropriate doses following conditioning and auto-transplant probably will further diminish the remaining auto-reactive T cell populations. Our own recent studies have shown a marked delay in clinical onset of EAE in mice receiving a two-day injection protocol of PTCy following HSCT compared to animals receiving an HSCT alone without the addition of cyclophosphamide post-transplant (DW, RBL personal observation). The only data reported in the literature to date noted that Cy following auto-HSCT improved experimental autoimmune encephalomyelitis (EAE) outcomes in rats (29). Cyclophosphamide is reportedly better tolerated by the central nervous system (CNS) at low or moderate clinical doses compared to other alkylating agents(30). Regardless, bladder, cardiac and gonadal toxicity are concerns using Cy and common adverse side effects reported with its use include hemorrhagic cystitis, myelosuppression, nausea and others(31-33). Thus, to enable MS or other AID patients to tolerate PTCy without excess toxicities, it will be important to identify the appropriate type and intensity of pretransplant conditioning that will need to be tailored to the individual patient and will depend on the disease characteristics, prior DMT therapy, age, and mobilization strategy. Moreover, the precise dosage of PTCy and time of application will need to be identified.

With regard to MS, conditioning often involves BEAM / ATG or CY / ATG. Based on the global effects of such conditioning, one might consider lowering the dose levels of BEAM (specifically Etoposide, Cytarabine and Melphalan) to reduce toxicities prior to use of post-transplant Cy since the latter will further diminish hematopoietic cell numbers. It may be worth noting that while lower Cy dose in allo-HSCT takes into account the need to maintain immune mediated GVL, such consideration would be absent for autoimmune disease patients. Efficacy in such patients could be evaluated by determining the presence / absence of remission and the length of the time period to disease relapse as assessed by imaging (MRI+/- contrast). Analysis of hemato/lymphoid engraftment following the choice of conditioning regimens will be important with regard to the success of auto-HSCT + PTCy. It would also be important to monitor immune function including viral reactivation (CMV, EBV and adenovirus) for the first 3 months post-HSCT. IgG levels should also be assessed over time. For patients who receive vaccinations post-transplant, specific antibody levels can be determined.

Tolerance and peripheral immune regulation

Key rationale for applying autologous-HSCT to autoimmune disease patients is the notion that transplanted HSC engraft and provide long-term hematopoietic progenitor populations. These progenitors will also generate thymic-derived T and marrow-derived B cells whose differentiation processes re-establishes self-tolerance to prevent subsequent emergence of autoimmune disease (**Fig. 3**). As noted earlier, administration of PTCy following an autologous HSCT – as occurs following allogeneic HSCT – is anticipated to result in similar outcomes regarding: 1) stem cell survival, 2) hematolymphoid engraftment and 3) ensuing self-tolerance of newly derived T cells. For reasons discussed above, conditioning, HSCT and PTCy is unlikely to remove all existing auto-reactive T cells. Moreover, individuals with autoimmune disease could also contain stem-like, progenitor T cells that may be quiescent, undergo minimal proliferation and thus be resistant to deletion by conditioning +/- cyclophosphamide exposure(34). Therefore, patients should be monitored using imaging to detect any new lesions. The presence of peripheral regulatory mechanisms to maintain and enforce self-tolerance will therefore likely be crucial to the maintenance of patient overall health. CD4⁺FoxP3⁺ Tregs provide a critical, non-redundant pathway necessary to control unwanted self-reactivity(35). We posit that as observed following allogeneic-HSCT, autologous-HSCT + PTCy can provide “a platform” that promotes Treg presence and function (**Fig. 3**). Additionally, such treatment would enable the use of other strategies to inhibit self-reactive T cells and suppress inflammation in patients with certain autoimmune disorders.

Treg cell treatment: The use of low dose IL-2 (IL-2_{LD}) selectively drives expansion of Tregs vs Tconv/Teff in part due to different regulation of downstream mediators of the IL-2R/pSTAT5 signaling pathway in Tregs(36, 37). Several studies have demonstrated clinical promise of manipulating Tregs *in vivo* following IL-2_{LD} administration in patients with type 1 diabetes and chronic GVHD – a disorder exhibiting alterations including autoantibody production and fibrosis characteristic of autoimmune diseases(36, 38). Since IL-2 under certain conditions could induce neurotoxicity (Uhthoff phenomenon) in MS and other disorders, other agonists may be useful. Our laboratory and others have expanded and activated Tregs *in vivo* under transplant conditions by introducing TNFRSF25 (DR3) agonists alone and together with IL-2_{LD}(39). Outcomes indicated that allogeneic-HSCT pre-treatment, which included targeting TNFRSF25 using an agonistic fusion protein (TL1A-Ig), resulted in significant benefit compared with untreated recipients(39). We concluded such pre allogeneic-HSCT Treg expansion established tissue environments to inhibit subsequent alloreactive GVHD responses. Here, we posit pre autologous-HSCT treatment to expand Tregs in patient tissues could help suppress residual / emergent autoreactive T cell responses.

Immune checkpoint agonist treatment: Checkpoint agonist treatment activates PD-1 molecules to suppress auto-reactive T cells in autoimmune diseases like MS (40). Genetic variations in the PD-1/PD-L1 pathway often alter immune regulation, directly affecting MS relapse risk and severity(41-43). In the lymphopenic environment following auto-HSCT and PTCy, elevated IL-7 and IL-15 induce PD-1 expression. This enhanced expression provides optimal targeting for PD-1 agonists to effectively regulate persisting self-reactive T cells(44, 45).

CAR-T cell treatment: CAR-T cell treatment shows promise for severe autoimmune diseases like SLE, myositis, and systemic sclerosis(40, 46). While CD19 or dual-target (CD19/BCMA) CAR-T cells can successfully eliminate self-reactive B cells and lower autoantibodies, the high physical toll limits this therapy

to treatment-resistant patients(40, 46, 47). Administering CAR-T cells after autologous-HSCT + PTCy into a lymphopenic environment (**Fig. 2**) may further enhance cell expansion and disease amelioration.

Overall Perspective

Here we discuss how post-transplant cyclophosphamide may be applied to autologous HSCT for autoimmune disease patients. We posit that following conditioning and HSCT, PTCy will promote additional reduction and suppression of auto-reactive, disease inducing T cells. Since complete removal of autoimmune disease mediating lymphocytes is unlikely, peripheral regulatory strategies will probably be required. Together with autologous HSCT + PTCy, administration of treatments targeting Tregs and other cells could lead to new translational strategies for improved outcomes in some autoimmune disease settings. Additional preclinical and clinical studies are needed to define its safety, optimal dosing levels, timing and therapeutic efficacy.

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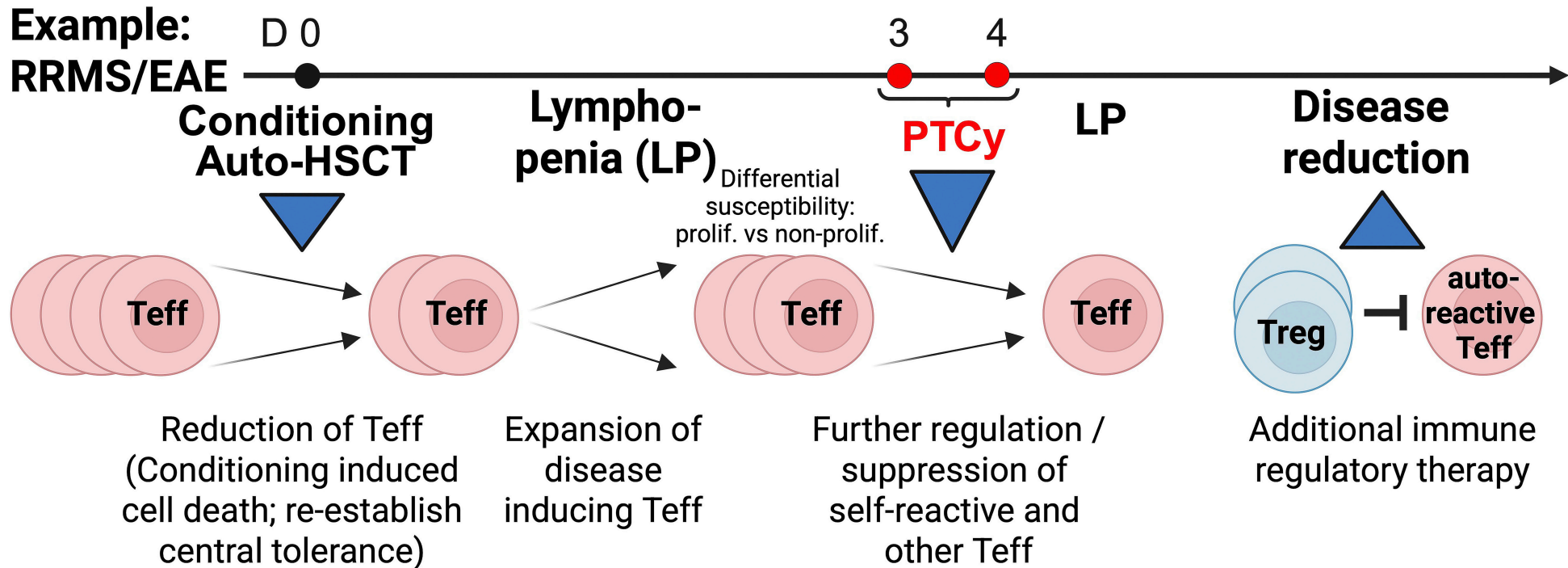
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Figure Legends:

Fig. 1: Hypothesis for effective regulation of autoreactive T cells after autologous-HSC and post-transplant cyclophosphami (PTCy) together with peripheral immune regulatory therapy for preventing disease recurrence. The initial depletion of disease-causing auto-reactive (ex. myelin specific) Teff cells due to conditioning is followed by lymphopenia driven proliferation. Administration of PTCy further reduces this autoreactive Teff population, suppresses Teff and selectively promotes Treg survival and function. Additional peripheral immune regulation is required to inhibit any remaining auto-reactive disease inducing Teff. Here we use RRMS (Relapse remitting multiple sclerosis / EAE (Experimental autoimmune encephalomyelitis) as the prototypic AID. Created in <https://BioRender.com>

Fig. 2: Conditioning and PTCy regulation of T cells present in autoimmune disease patients following autologous-HSCT. The graphic depicts the diminution of several antigen reactive T cell populations subject to conditioning immediately prior to an autologous HSCT (**Days 1-3, Left Panel**). Cells that survive / persist following conditioning and transplant could encounter specific antigen, such as the disease related antigen (here, example is myelin basic protein, MPB) that can drive T cell proliferation. Similarly, other auto (ex. thyroid, reproductive, etc.) or foreign (ex. Influenza, CMV) antigens could also drive division of their respective antigen specific T cells. However, regardless of antigen or in its absence, the lymphopenic conditions resulting in excess IL-7 and IL-15 will promote proliferation of lymphocytes present. We posit cyclophosphamide exposure at this time (**Days 4-5, Right Panel**) resulting in DNA alkylation will lead to both death of cells undergoing division and functional suppression of cells remaining. Created in <https://BioRender.com>

Fig. 3: Proposal to apply autologous HSCT together with post-transplant cyclophosphamide (PTCy) for some autoimmune diseases. The application of HSCT + PTCy is anticipated to promote effective diminishment and suppression of persisting self-reactive T cells together with inducing T cell tolerance. Together with HSCT + PTCy, this platform can accommodate additional peripheral immune regulation. (ex.: DMT = Disease modifying therapies: (ex. β -IFNs, immune-sequestering agents natalizumab, anti-CD49d); Treg expansion (TNFRSF25 agonists, IL-2_{LD}); B cell depletion: (CAR-T, mAbs); Teff inhibition (Immune checkpoint agonists).

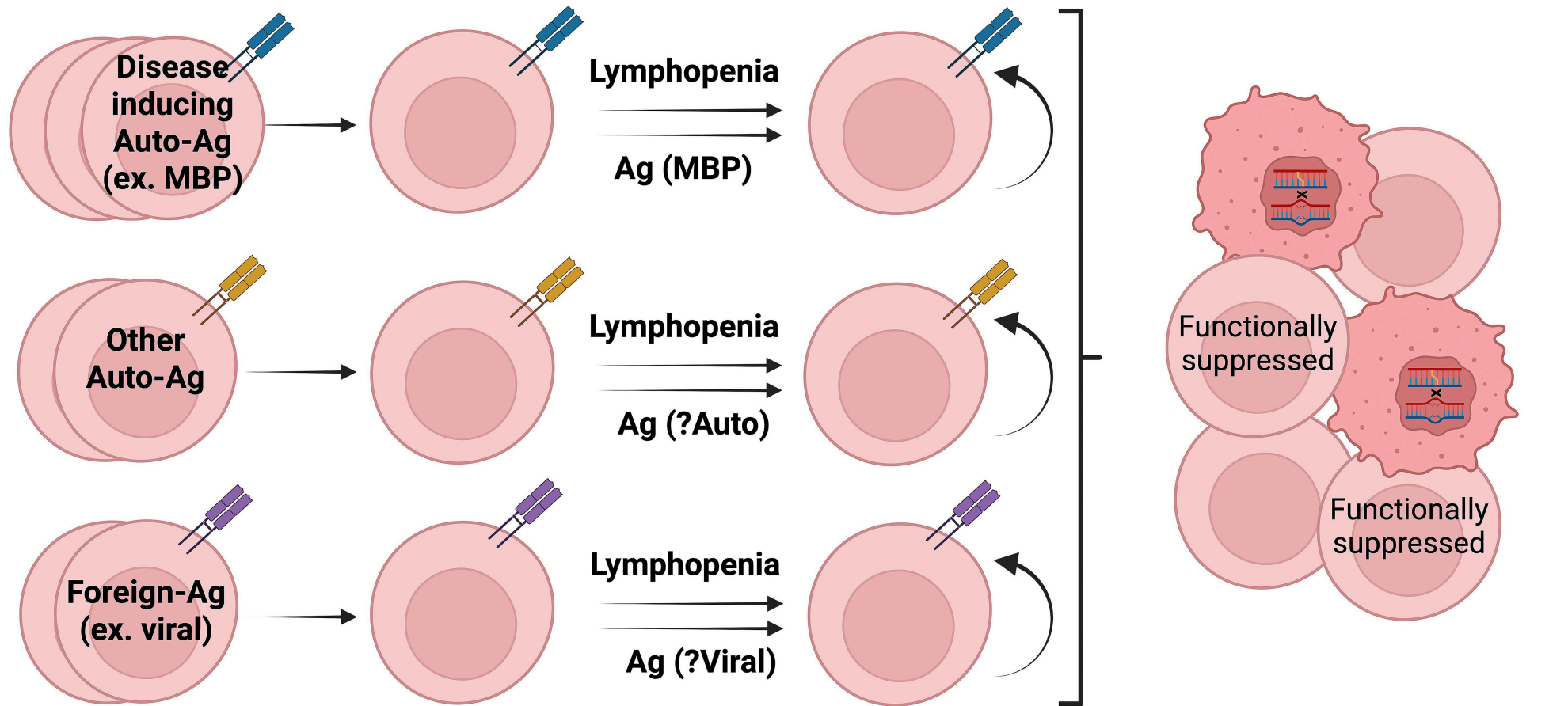


Patient Conditioning & Autologous HSCT

Days 1-3

Post-transplant Cyclophos.

Days 4-5



Conditioning
and auto-HSCT



Persisting CD4 Tconv & CD8
self-reactive T cells



Lymphopenia
(T cell proliferation)



PTCy: 50mgs/kg



Reduction, regulation and suppression
of self-reactive Teff and other Teff

Stem cell engraftment
(de novo poiesis)



T cell tolerance

Additional immune regulatory therapies

Autologous HSCT and

Post-Transplant Cyclophosphamide: A Platform to Treat Autoimmune Diseases?

Teff cell inhibition
(ex. Immune checkpoint agonists)

DMT:
(ex. β -IFNs, immune-sequestering
agents natalizumab, anti-CD49d)

Treg expansion / activation
(ex. TNFRSF25 agonists, IL-2_{LD})

B cell depletion:
(ex. CAR-T, mAbs)