Pentostatin Plus Cyclophosphamide Safely and Effectively Prevents Immunotoxin Immunogenicity in Murine Hosts

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ABSTRACT

Purpose: The success of immunotoxin therapy of cancer is limited by host production of neutralizing antibodies, which are directed toward the Pseudomonas exotoxin A (PE) component. In this proof-of-principle study using a well-established murine model, we hypothesized that a newly developed immune depletion regimen consisting of pentostatin plus cyclophosphamide would abrogate anti-immunotoxin reactivity.

Experimental Design: BALB/c hosts were injected weekly with recombinant immunotoxin (RIT) SS1P, which is an anti-mesothelin Fv antibody fragment genetically fused to a 38 kDa portion of PE, and has been evaluated in clinical trials. Experimental cohorts received induction chemotherapy consisting of pentostatin (P) plus cyclophosphamide (C) prior to initial RIT exposure; some cohorts received further maintenance PC therapy of varying intensity just prior to each weekly RIT challenge. Cohorts were monitored for T, B, and myeloid cell depletion and for total anti-SS1P antibody (Ab) formation.

Results: Controls uniformly developed anti-SS1P Ab after the third RIT exposure. Induction PC therapy reduced the frequency of hosts with anti-SS1P Ab. Abrogation of antibody generation was improved by maintenance PC therapy: nearly 100% of recipients of intensive PC maintenance were free of anti-SS1P Ab after 9 weekly RIT doses. The most effective PC regimen yielded the greatest degree of host B cell depletion, moderate T cell depletion, and minimal myeloid cell depletion.

Conclusions: Induction and maintenance PC chemotherapy safely prevented anti-immunotoxin antibody formation with uniform efficacy. These data suggest that
immunotoxin therapy might be used in combination with pentostatin plus cyclophosphamide chemotherapy to improve the targeted therapy of cancer.
TRANSLATIONAL RELEVANCE

Clinical trials have demonstrated that Pseudomonas exotoxin (PE)-based immunotoxins mediate effective anti-tumor responses, thereby adding to the arsenal of targeted agents for personalized cancer therapy. However, host immune reactivity to the PE component generates neutralizing antibodies (Ab) that severely limit an ability to deliver repetitive immunotoxin doses required for optimal anti-cancer effects. For example, in clinical trials evaluating the anti-mesothelin PE immunotoxin SS1P, the majority of patients developed anti-SS1P Ab after only one round of therapy. In this proof-of-principle study, we have developed an immune intervention that overcomes this therapeutic obstacle. We demonstrate that a newly developed regimen of pentostatin (P) plus cyclophosphamide (C) safely abrogated murine host capacity to form anti-SS1P Ab with 100% efficacy. An ability to deliver repetitive doses of immunotoxin may enhance anti-cancer effects against a wide range of malignancies.
INTRODUCTION

Immunotoxins (IT) composed of an antibody or antibody fragment linked to various bacterial and plant toxins are being actively developed for the treatment of cancer(1). Our laboratory has focused on the development of recombinant immunotoxins (RITs) in which the Fv portion of an antibody reacting with a tumor cell is directly fused to a 38 kDa portion of Pseudomonas exotoxin A (PE38)(1). We have observed a high complete and partial response rate with immunotoxin BL22 in patients with refractory hairy cell leukemia(2, 3). In these clinical trials, because the immune system of these patients is severely impaired, repeated treatment cycles of RIT can be given without neutralizing antibody (Ab) formation. We are also developing an immunotoxin (SS1P) targeting the mesothelin protein highly expressed in mesothelioma, and ovarian, pancreatic and lung cancers(4-8). However, in patients with mesothelioma, where only a single treatment cycle can be given before antibodies develop, the responses have been much smaller and less frequent than with BL22(9, 10). Specifically, in two recent clinical trials of SS1P therapy, neutralization of immunotoxin activity was observed in 88%(10) and 75%(9) of recipients. Importantly, such neutralizing Ab were typically present after the first cycle of therapy, thereby limiting the ability to provide a potentially effective number of RIT doses. Formation of neutralizing Ab was also observed in a diphtheria toxin-based clinical trial(11) and a Pseudomonas exotoxin (PE40)-based clinical trial(12). Host immunity to immunotoxins may limit the ability of RIT-based therapies to circulate and thus may represent a major therapeutic obstacle.

Several approaches have been investigated to reduce the immunogenicity of protein therapeutics. For example, protein B cell epitopes can be masked by polyethylene glycol
(PEG) or altered through mutagenesis(13-20). Alternatively, modification of protein T cell epitopes represents another approach to reduce immunogenicity(21). Because the clinical safety and success of these protein engineering approaches has not been tested and will require a significant degree of time to evaluate, there exists an immediate need to develop new interventions using FDA-approved reagents to prevent anti-immunotoxin responses.

Host immune suppression has been a successful approach to controlling unwanted immunogenicity towards transgenes introduced through gene therapy approaches(22). However, it is important to note that a relative paucity of studies have investigated the potential benefit of host immune modulation for abrogation of RIT immunogenicity. In a clinical trial, host pretreatment with the anti-B cell monoclonal antibody rituximab did not inhibit the human immune response against the PE-based immunotoxin, LMB-1(23); there exist no reports in the literature of successful host immune modulation interventions in the clinical setting of immunotoxin therapy. Furthermore, there are no publications identifying a role for host immune modulation in experimental animal models of immunotoxin administration. Nonetheless, murine models accurately predict the immunogenicity of clinical immunotoxin products(13, 14). Given this information, we evaluated whether a highly effective immune modulation intervention that we recently developed [the pentostatin plus cyclophosphamide regimen(24)] might prevent host induction of an immune response against SS1P. To evaluate this, we measured total anti-SS1P antibody formation by an immune complex capture (ICC) ELISA, which we have previously shown to correlate with functional neutralization of immunotoxin cytotoxic activity(17, 19).
Specifically, we hypothesized that interventions that abrogate host-versus-graft reactivity (HVGR) to fully MHC-mismatched hematopoietic stem cell (HSC) allografts would also be effective for prevention of host reactivity to immunotoxin, which likely represents a less stringent challenge than allograft reactivity. Host CD4^+ and CD8^+ T cells(25) of a Th1/Tc1 cytokine phenotype(26) are the primary mediators of HVGR; in addition, host B cell production of allospecific antibodies also contributes to allograft rejection, particularly in the setting of pre-sensitized hosts(27). Given the ubiquitous nature of Pseudomonas aeruginosa exposure, it is important to note that delivery of PE-based therapeutics also occurs in the setting of pre-sensitized hosts. Clinical allogeneic HSC transplantation is performed after preparative regimens intended to reduce HVGR, thereby facilitating alloengraftment; increasingly, such transplants are being performed as “mini-transplants”(28, 29) using purine analog-based immune depletion regimens that are safer than myeloablative regimens. Nearly universally, such regimens have focused on use of fludarabine, which is immune depleting, particularly when used in combination with DNA alkylators such as cyclophosphamide(30).

By comparison, relatively few clinical trials have evaluated the ability of pentostatin-based regimens to facilitate alloengraftment(31, 32). Pentostatin inhibits adenosine deaminase (ADA), thereby recapitulating the immune impairment observed in patients with genetic ADA deficiency(33, 34). Nonetheless, pentostatin can be administered with a favorable therapeutic window: pentostatin alone is efficacious for therapy of graft-versus-host disease(35) and hairy cell leukemia(36), and when used in combination with cyclophosphamide, is effective for therapy of chronic lymphocytic leukemia(37). Recently, we found that pentostatin worked synergistically with cyclophosphamide to
deplete host lymphoid cells with relative sparing of myeloid cells; the PC regimen not only depleted CD4+ and CD8+ T cells and B cells, but also inhibited residual immune cell capacity to mediate effector function and recover numerically post-chemotherapy(24). In light of this recent progress in the identification of potent pentostatin-based host conditioning, we applied the PC regimen to address the hypothesis that this regimen will abrogate the immunogenicity of SS1P recombinant immunotoxin.

MATERIALS AND METHODS

Mice

Female BALB/c mice (8-10 weeks old) were purchased from Frederick Cancer Research Facility. Mice were treated according to an approved animal protocol (MB-075) and maintained in a specific pathogen-free facility. Drinking water was supplemented with 100 mg/L of ciprofloxacin (Sigma, St. Louis, MO).

Drug treatments and Immunizations

Pentostatin (1 mg/kg/dose; Bedford Laboratories) and cyclophosphamide (50 mg/kg/dose; Bristol Myers Squibb) were injected i.p. according to the schedules shown in Tables I and II. As shown in Table I, a control cohort received immune suppression therapy with rapamycin (Sigma-Aldrich) by i.p. injection at a dose of 3 mg/kg/dose. Mice were injected weekly (i.p.) with SS1P recombinant immunotoxin (RIT; 5 or 10 ug/mouse); SS1P was manufactured in the NCI Laboratory of Molecular Biology according to previous methods(38). Control mice received saline injections in the absence of IT therapy. Between 200-250 µl of blood was drawn into sterile tubes containing 10 µl
of heparin; samples were centrifuged for 10 min at 14k rpm. About 100 µl of plasma supernatants were transferred to a new tube and kept at -80°C until analyzed.

**Determination of Cell Depletion**

Cohorts of mice were euthanized at week 3, 5, or 9 of immunotoxin therapy; spleens were harvested and single cells were quantified. Single cell suspensions were labeled with anti-CD4, -CD8, -B220, -CD11b, or -Gr1 conjugated with FITC, PE, or APC (Becton Dickinson, BD) and analyzed on a FACS Calibur flow cytometer (BD). At least 10,000 live events were acquired; 7-AAD was used to exclude dead cells.

**Immune Complex Captured (ICC)-ELISA**

An ICC-ELISA format was used to measure the native form of PE38-specific antibodies in plasma. Because the antigen-antibody reaction was performed in solution, the antigen was kept in its native form (19). Briefly, microtiter plates (Maxisorp, Nalge Nunc; Rochester, NY) were coated with 100 ng/50 µl/well of mesothelin-rFc in PBS overnight at 4 °C (39). In a separate tube, plasma collected from experimental recipients was diluted in blocking buffer (25% DMEM, 5% FBS, 25 mM HEPES, 0.5% BSA, and 0.1% azide in PBS); plasma was serially diluted, starting from 200-fold. After washing plates with PBS containing 0.05% Tween-20, the SS1P-serum mixtures were transferred to individual wells (50 µl/well). The amount of immune complex captured by the mesothelin-rFc protein was detected by rabbit anti-mouse IgG-HRP (Jackson-Immuno Research Laboratories) and tetramethylbenzidine (TMB substrate kit; Pierce). Each plate contained serial dilutions of anti-PE38 mAb IP30 as a concentration control standard (39). The anti-drug antibody concentrations were expressed as mAb IP30 concentrations.
standard curve of the IP30 reactivity was created with a four-parameter logistic curve model by SoftMaxPro 4.0 (Molecular Devices).

Statistics

Student’s 2-tailed t tests, 2-way ANOVAs, or log-rank tests were used to analyze data. For comparisons between the experimental groups in the immunogenicity study, the Mann-Whitney nonparametric test was used. Values of P<0.05 were considered statistically significant.

RESULTS

PC Therapy (6-day Regimen) Depletes Host Lymphocytes and Prevents anti-SS1P Antibody Formation

In the first experiment, cohorts received three weekly doses of RIT without an immune intervention or in combination with either pentostatin plus cyclophosphamide or rapamycin (experimental schema, Table I); rapamycin was initially evaluated to prepare for the possibility that both immune depletion and immune suppression might be required to prevent anti-immunotoxin Ab responses. In the setting of fully MHC-mismatched murine HSC transplantation, we found that an 8-day regimen of pentostatin plus cyclophosphamide was required to prevent allograft rejection(24); we reasoned that host reactivity to the RIT would be less robust relative to alloreactivity, and therefore we initially evaluated a truncated 6-day PC induction treatment regimen. In addition, because of our goal of administering multiple weekly doses of RIT and in light of the robust capacity of murine hosts to regenerate immunity(40), we built in a weekly maintenance therapy with PC just prior to each RIT dosing. In previous studies(24), we
found that the depth of immune depletion with pentostatin therapy related to the intensity of alkylator co-administration. As such, to assess the degree to which maintenance therapy contributed to abrogation of immunogenicity, we evaluated maintenance therapy that consisted of a single-dose of cyclophosphamide or an intensified, double-dose of cyclophosphamide. Comparison of drug dosing based on body surface area can determine whether doses in experimental animal studies approximate the human equivalence dose(41). We used pentostatin at 1 mg/kg, which translates to approximately 3 mg/m², which is within the recommended range of 2 to 4 mg/m² used in the clinic(31, 37). Similarly, we used cyclophosphamide at 50 mg/kg, which translates to approximately 240 mg/m², which is within the dose range used in the clinic(30, 37).

One week after the third immunization, recipients of the PC regimen had an approximate two-log reduction in host CD4⁺ and CD8⁺ T cells and B220⁺ B cells (Figure 1A). As anticipated, immune suppressive therapy using rapamycin yielded only modest levels of T and B lymphocyte depletion. The immune interventions appeared to be toxic in this setting, as 3/10 recipients of rapamycin and 4/10 recipients of PC died during the 28-day experimental interval; by comparison, 1/10 recipients of RIT alone died. The control cohort that received RIT without any immune intervention developed an anti-SS1P immune response, as evidenced by positive titers in the ICC-ELISA (Figure 1B; PE-specific Ab concentration, 4.0 ± 1.7 µg/ml). Immune suppressive therapy greatly reduced anti-SS1P antibody production: the concentration of PE-specific antibodies in rapamycin recipients was 0.007 ± 0.02 µg/ml. By comparison, recipients of the PC regimen did not develop antibodies (PE-specific antibody was not detected in any sample; 0/9). The PC regimen was more effective for the prevention of antibody
formation than rapamycin therapy (P<0.05). Because immune depletion therapy with PC was effective in preventing Ab formation, immune suppressive therapy with rapamycin was not further evaluated.

Because the 6-day PC regimen was effective but associated with lethality, we performed an experiment to compare the safety of the 6-day PC regimen and a truncated 4-day PC chemotherapy regimen. We also considered the possibility that the PC regimen and immunotoxin may each contribute to host toxicity; as such, we also reduced the RIT dose from 10 µg to 5 µg. In this experiment, 100% of recipients in each cohort survived the 28-day experimental interval. Moreover, no significant differences in the percentage of baseline weight were found among the cohorts (data not shown). Based on these safety data, further experiments were performed with the truncated 4-day PC regimen and the 5 µg dose of RIT.

**PC Induction and Maintenance Therapy Safely Permits Immunotoxin Dosing Without Antibody Formation Through 5 Weekly Injections**

Using the 4-day PC regimen, experiments were performed to: (1) quantify the balance between PC regimen-associated lymphoid cell depletion versus myeloid cell depletion; (2) determine the specific role of maintenance PC therapy, including an assessment of dose intensity; and (3) test whether such PC regimens could permit weekly RIT administration for 5 weeks or 9 weeks without antibody formation. The experimental schema for these experiments is detailed in Table II.

After 5 weekly doses of RIT, recipients of the induction PC regimen without maintenance PC had reduced absolute numbers of CD4⁺ T cells, CD8⁺ T cells, and B220⁺ B cells relative to recipients of RIT alone (Figure 2A). To test whether weekly
maintenance therapy with pentostatin and cyclophosphamide would preserve the cell depletion effects achieved by the induction PC regimen, we evaluated two regimens consisting of a single dose of pentostatin combined with either one or two doses of cyclophosphamide (Table II). Recipients of PC induction combined with standard dose PC maintenance therapy (Table II, cohort 3) or higher dose PC maintenance therapy (Table II, cohort 4) had further reductions in both T and B cell populations; although these cohorts did not differ in terms of depth of T cell depletion, the higher dose PC maintenance therapy cohort had more severe depletion of B cells. Importantly, the level of myeloid cell depletion, as measured by the absolute number of CD11b+ and Gr-1+ cells, was only moderately reduced in the PC cohorts and was reduced to a similar level independent of whether maintenance PC therapy was delivered (Figure 2A). All mice in each cohort were healthy at this post-5 week time point; survival was 100% (10/10 survival in each cohort).

After 5 weekly doses of RIT, recipients of PC induction therapy alone (without maintenance PC therapy) had a similar magnitude of anti-SS1P formation relative to recipients of RIT alone (Figure 2B). The anti-SS1P concentration was 62 ± 21 µg/ml in recipients of induction PC therapy and 61 ± 10 µg/ml in recipients of RIT alone. Addition of standard dose PC maintenance therapy to PC induction therapy was effective in reducing antibody formation (anti-SS1P concentration of 3.7 ± 2.9 µg/ml); the beneficial effect of standard dose maintenance therapy was statistically significant (PC induction vs. induction plus standard dose maintenance, P=0.002). Remarkably, the higher dose PC maintenance therapy was even more effective at preventing antibody formation (0/10 cases of anti-SS1P positivity).
PC Induction and Maintenance Therapy Safely Permits Immunotoxin Dosing

Without Antibody Formation Through 9 Weekly Injections

To further test the stringency of this approach, additional cohorts were continued through 9 weekly administrations of SS1P recombinant immunotoxin. Similar to the results at the 5 week time point, standard dose PC maintenance therapy and higher dose PC maintenance therapy resulted in greater reductions in both T cell and B cell populations relative to recipients of induction PC therapy alone (Figure 3A). Although these maintenance PC cohorts did not differ in terms of depth of T cell depletion, the higher dose PC maintenance therapy cohort had a greater reduction in B cell numbers. The direct correlation between maintenance PC dose intensity and depth of B cell depletion was therefore identified after both 5 weeks and 9 weeks of RIT therapy. Importantly, the level of myeloid cell depletion, as measured by the absolute number of CD11b+ cells, was only moderately reduced in the PC cohorts and was reduced to a similar level independent of whether maintenance PC therapy was delivered (Figure 3A); the level of myeloid cell depletion, as measured by the absolute number of Gr-1+ cells, was more severe with maintenance PC therapy, but did not vary significantly between the standard dose and higher dose maintenance cohorts (Figure 3A).

After 9 weekly doses of RIT, recipients of RIT alone or RIT combined with standard dose maintenance PC therapy developed anti-SS1P antibodies (Figure 3B; anti-SS1P concentrations of 1290 ± 379 µg/ml and 1687 ± 463 µg/ml, respectively). Addition of standard dose PC maintenance therapy to PC induction therapy was not effective in reducing anti-SS1P antibody formation in a statistically significant manner (anti-SS1P concentration, 569 ± 261 µg/ml; P=0.075 relative to RIT therapy alone). In marked
contrast, recipients of the higher dose maintenance therapy were nearly universally protected against the formation of anti-SS1P antibodies (anti-SS1P concentration, 2.7 ± 2.7 µg/ml; P=0.0001 relative to RIT therapy alone).

CONCLUSIONS

Host immunity to immunotoxin represents an obstacle to the further success of this type of targeted anti-cancer therapy. This limitation is particularly formidable in relatively chemotherapy naïve and immune replete patients such as individuals with mesothelioma(9, 10), but is also observed in heavily-treated, immune-depleted patients with hairy cell leukemia(42) and other B cell malignancies(2). Host production of neutralizing antibodies occurs against both Pseudomonas exotoxin-based(2, 3, 42, 43) and diptheria toxin-based agents(11), thereby indicating the broad nature to the therapeutic obstacle. In this manuscript, for the first time, we have utilized an in vivo model to demonstrate that stringent host immune depletion averts immunotoxin immunogenicity during repetitive, prolonged immunotoxin dosing. These results establish a proof-of-principle for safe combination therapy using immunotoxins with immune-depleting pentostatin and cyclophosphamide chemotherapy.

The current results extend our recent identification of the PC regimen as a safe and potent immune depletion strategy for the abrogation of murine host rejection of fully MHC-mismatched allogeneic HSC grafts(24). In our previous murine experiments, we found that pentostatin operated synergistically with cyclophosphamide to ablate immune B and T cells with relative sparing of myeloid cells; most importantly, we demonstrated that the PC regimen induced more severe host immune functional deficits than
fludarabine-based regimens and was more effective in the prevention of allograft rejection. In an ongoing clinical trial (NCT00923845), we are evaluating a pentostatin plus cyclophosphamide regimen to facilitate allogeneic HSC engraftment; in this study, 11/11 patients who received a PC regimen had marked B and T cell depletion without any neutropenia, and each patient achieved allogengraftment (D. Fowler, unpublished data). These observations suggest that it may be possible to development a safe and effective PC chemotherapy regimen for the clinical setting of immunotoxin therapy.

In the current study, we have demonstrated that the PC regimen also has great utility for the abrogation of host neutralizing antibody responses against immunotoxin. It is important to note that the therapeutic effect in the current study was achieved with a 4-day immune depletion regimen, which is truncated relative to the 8-day regimen we established as being required for the abrogation of HSC rejection. It is likely that the ability of a truncated PC regimen to prevent anti-RIT responses relates to reduced immunogenicity of the PE toxin relative to alloantigen. These observations have potential translational significance and might inform the design of next-generation immunotoxin clinical trials: that is, the host immune depletion target might be less stringent for the immunotoxin setting relative to the allogeneic HSC transplant setting.

Our experiments also point to the importance of maintenance PC chemotherapy to prevent immunogenicity during prolonged immunotoxin dosing. Indeed, we observed that maintenance PC regimens of higher intensity, which can be readily achieved by increasing the cyclophosphamide dose, were required for complete protection against anti-SS1P formation during nine weekly challenges with recombinant immunotoxin. It should be noted that our experiments were performed in a tumor-free model. Further
evaluation of combination RIT plus PC chemotherapy may be warranted in experiments that incorporate tumor cells, such as xenograft models incorporating the mesothelin-expressing human tumor cell line A431/H9(43, 44). It is also interesting to note that thymic function, which is the primary driver of immune reconstitution capacity, is robust in mice of the age used in our experiments(40) and relatively constrained in adult humans(45). As such, it is possible that maintenance immune depletion may be less important in the clinic relative to the experimental model that we used.

It is also important to note that recipients of the more intensive maintenance PC chemotherapy had similar levels of CD4\(^+\) and CD8\(^+\) T cell depletion relative to the other treatment cohorts, but had more exhaustive and persistent depletion of B cells. It has long been established that T cell immunity is required in a cooperative manner to facilitate antibody production(46), and therefore, it is likely that the T cell depletion generated was essential for the abrogation of RIT immunogenicity. However, our data suggest that the depth and/or duration of B cell depletion represent a critical factor in determining whether an anti-immunotoxin neutralizing antibody response is generated. Furthermore, although a previous clinical trial did not identify a beneficial role of rituximab for prevention of neutralizing antibody formation to immunotoxin(23), our data suggest that a rationale may exist to combine PC chemotherapy with rituximab for more exhaustive B cell depletion. Alternatively, because we found that rapamycin acted as a single agent to reduce anti-immunotoxin responses, a rationale may exist to further evaluate the combination of immune depletion with PC chemotherapy plus immune suppression using rapamycin. In such regimens, it is possible that rapamycin may
enhance tumor therapy indirectly (by reducing immunotoxin immunogenicity) and
directly (through tumor cell mTOR inhibition(47)).

Several considerations are important relative to efforts to translate these findings.
Clinical trials should seek to determine the depth of immune B and T cell depletion
required to abrogate host immunity to PE-based immunotoxins. To limit infectious
complications, it will be advantageous to administer prophylactic antibiotics and to
achieve immune depletion with a pentostatin plus cyclophosphamide regimen that
induces minimal neutropenia. Successful translation of this work holds promise as a new
approach in the targeted therapy of cancer using recombinant immunotoxins.
Figure 1: Pentostatin plus cyclophosphamide (PC) chemotherapy severely depletes B and T cells and prevents anti-SS1P antibody production after 3 weekly SS1P exposures. Mice were given three weekly injections of SS1P recombinant immunotoxin (RIT) either without any immune intervention (“RIT” cohort) or with immune suppression consisting of either rapamycin (“R” cohort) or pentostatin plus cyclophosphamide (“PC” cohort) according to the regimens detailed in Table I; a fourth cohort received saline injections (“S” cohort) and did not receive RIT. One week after the third weekly RIT injection, spleens were harvested and the absolute numbers of CD4+ T cells, CD8+ T cells, and B220+ B cells were quantified by flow cytometry (A). Plasma was collected at this time point and analyzed for the presence of anti-SS1P antibodies using the ICC ELISA (B).

Figure 2: PC chemotherapy prevents anti-SS1P antibody production after 5 weekly SS1P exposures: role of intensive maintenance PC chemotherapy. Mice were given five weekly injections of SS1P immunotoxin (RIT) either without any immune intervention (“RIT” cohort) or with immune suppression consisting of either induction PC chemotherapy alone (PC) or in combination with maintenance PC chemotherapy of standard-intensity (PC’ cohort) or higher-intensity (PC” cohort) according to the regimens detailed in Table II; additional control cohorts did not receive RIT and received either saline injections (“S” cohort) or were left untreated (“NT” cohort). Each cohort had n=10 recipients. One week after the fifth weekly RIT injection, spleens were harvested and the absolute numbers of CD4+ and CD8+ T cells, B220+ B cells, CD11b+ myeloid cells, and Gr-1+ granulocytes (A) were quantified by flow cytometry. Plasma was collected at this time point.
point and analyzed for the presence of anti-SS1P antibodies using the ICC ELISA (B). *P<0.05, **P<0.001.

Figure 3: PC chemotherapy prevents anti-SS1P antibody production after 9 weekly SS1P exposures: role of intensive maintenance PC chemotherapy. Mice were given nine weekly injections of SS1P recombinant immunotoxin (RIT) either without any immune intervention (“RIT” cohort) or with immune suppression consisting of either induction PC chemotherapy alone (PC) or in combination with maintenance PC chemotherapy of standard-intensity (PC’ cohort) or higher-intensity (PC” cohort) according to the regimens detailed in Table II; additional control cohorts received either saline injections (“S” cohort) or were left untreated (“NT” cohort) and did not receive RIT. Each cohort had n=10 recipients. One week after the ninth weekly RIT injection, spleens were harvested and the absolute numbers of CD4$^+$ and CD8$^+$ T cells, B220$^+$ B cells, CD11b$^+$ myeloid cells, and Gr-1$^+$ granulocytes (A) were quantified by flow cytometry. Plasma was collected at this time point and analyzed for the presence of anti-SS1P antibodies using the ICC ELISA (B). *P<0.05, **P<0.001.
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Anti-SS1P antibody was tested one week after last immunotoxin injection. Mice were immunized with SS1P immunotoxin (“RIT”; 10 µg). Rapamycin (“R”) was administered at a dose of 3 mg/kg/dose (i.p.) Pentostatin (“P”) and cyclophosphamide (“C”) were administered (i.p.) at doses of 1mg/kg/dose and 50 mg/kg/dose, respectively. Control mice received saline injections (“S”; i.p.).

Table I: The PC regimen for abrogation of anti-SS1P antibody formation: experimental design

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<th>Weeks 2 and 3 (Maintenance cycles)</th>
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1 Anti-SS1P antibody was tested one week after last immunotoxin injection.
2 Mice were immunized with SS1P immunotoxin (“RIT”; 10 µg).
3 Rapamycin (“R”) was administered at a dose of 3 mg/kg/dose (i.p.)
4 Pentostatin (“P”) and cyclophosphamide (“C”) were administered (i.p.) at doses of 1mg/kg/dose and 50 mg/kg/dose, respectively.
5 Control mice received saline injections (“S”; i.p.).
Figure 1

A

**CD4**

- # Splenocytes ($\times 10^6$)
- RIT: 20, PC: 15, S: 10
- P<0.01

**CD8**

- # Splenocytes ($\times 10^6$)
- RIT: 10, PC: 5, S: 2
- P<0.05

**B220**

- # Splenocytes ($\times 10^6$)
- RIT: 50, PC: 40, S: 30
- P<0.001

B

**Anti-SS1P antibodies (µg/ml)**

- RIT: 15, PC: 10, S: 5
- P=0.005
- ns
Table II: Long-term administration of the PC regimen\(^1\) for abrogation of anti-SS1P antibody formation\(^2\)

<table>
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1 Cohorts 3 and 4 received weekly PC maintenance dosing, as indicated, through week 9 of the experiment.
2 Anti-SS1P levels in the serum were tested at week 5 and week 9 of the experiment.
3 Mice were immunized with SS1P immunotoxin ("RIT"; 5 µg; i.p. injection).
4 Pentostatin ("P") and cyclophosphamide ("C") were administered (i.p.) at doses of 1mg/kg/dose and 50 mg/kg/dose, respectively.
Figure 2
Figure 3

A

**CD4**

* # Splenocytes (x10^6)

RIT | PC | PC' | PC'' | S | NT

**CD8**

* # Splenocytes (x10^6)

RIT | PC | PC' | PC'' | S | NT

**CD11b**

* # Splenocytes (x10^6)

RIT | PC | PC' | PC'' | S | NT

**B220**

* # Splenocytes (x10^6)

RIT | PC | PC' | PC'' | S | NT

Gr-1

* # Splenocytes (x10^6)

RIT | PC | PC' | PC'' | S | NT

B

P=0.0001

P=0.075

P=0.0002

Anti-SS1P antibodies (µg/ml)

RIT | PC | PC' | PC'' | S

Figure 3
Pentostatin Plus Cyclophosphamide Safely and Effectively Prevents Immunotoxin Immunogenicity in Murine Hosts

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