

Toward minimal conditioning protocols for allogeneic chimerism in tolerance resistant recipients

David P. Al-Adra¹ and Colin C. Anderson^{1,2,*}

¹Department of Surgery; Alberta Diabetes Institute; University of Alberta; Edmonton, AB Canada; ²Department of Medical Microbiology and Immunology; Alberta Diabetes and Transplant Institutes; University of Alberta; Edmonton, AB Canada

Keywords: chimerism, tolerance, split tolerance, hematopoietic stem cell, transplantation, cyclophosphamide

Abbreviations: anti-CD40L, anti-CD40 ligand; BMC, bone marrow cell; BMT, bone marrow transplantation; CYP, cyclophosphamide; DST, donor specific transfusion; GVHD, graft-versus-host disease; i.v., intravenous; i.p., intraperitoneal; NOD, non-obese diabetic; TBI, total body irradiation

Submitted: 11/04/12

Revised: 12/02/12

Accepted: 12/19/12

<http://dx.doi.org/10.4161/chim.23350>

*Correspondence to: Dr. Colin C. Anderson;
Email: colinand@ualberta.ca

Addendum to: Al-Adra DP, Pawlick R, Shapiro AM, Anderson CC. Targeting Cells Causing Split Tolerance Allows Fully Allogeneic Islet Survival With Minimal Conditioning in NOD Mixed Chimeras. *Am J Transplant* 2012; 12:3235-45; <http://dx.doi.org/10.1111/j.1600-6143.2012.04260.x>; PMID:22974315.

Mixed chimerism is a promising approach toward generating donor-specific immunological tolerance. However, chimerism induction can be toxic; therefore, there is an effort to develop non-myeloablative, minimal intensity protocols that can generate chimerism without the toxic side effects. Recently, with the goal of creating a minimalistic chimerism induction protocol in the tolerance resistant non-obese diabetic (NOD) mouse model, we identified pre-existing T cells as cells that resist fully allogeneic chimerism. With monoclonals targeting NOD T cells, we showed that long-term chimerism and tolerance toward donor islets could be established. However, this promising new protocol relied on the administration of a single dose of anti-CD40 ligand, which is not clinically applicable. In refining protocols to move even closer to clinical utility, we report here initial success at generating fully allogeneic mixed chimerism in NOD mice by adding cyclophosphamide to the conditioning regimen in place of anti-CD40 ligand antibodies.

The generation of mixed hematopoietic chimerism in transplant recipients has the potential to generate donor specific tolerance, thereby eliminating both rejection episodes and the need for immunosuppressive medications.^{1,2} In the clinical setting, generating mixed chimerism is in its infancy and induction protocols are being constantly revised.¹⁻³ As such, chimerism induction protocols developed in experimental animal systems should be

refined based on clinical translatability and scrutinized to ensure donor specific tolerance occurs across fully mismatched barriers in the most challenging donor recipient combinations. Inbred mouse strains are the most widely used models in the development of protocols for allogeneic chimerism generation. The tolerance resistance and autoimmune propensity of non-obese diabetic (NOD) mice has made it the most challenging inbred mouse model for chimerism. Relatively mild non-myeloablative conditioning protocols that generate stable fully allogeneic mixed chimerism in other recipients routinely fail in NOD mice. Recently, with the aim of creating fully tolerant mixed chimeras in the NOD mouse model, we evaluated the role of various tissues (radiosensitive vs. radioresistant) and cell populations (NK cells, B cells and T cells) in the resistance to chimerism induced tolerance. We identified the barrier that pre-existing T cells impose both on the induction of mixed chimerism and the ability of mixed chimerism to generate donor-specific tolerance across full major and minor histocompatibility barriers.⁴ After identifying the pre-existing T cell barrier, we developed a nonmyeloablative (irradiation free) chimerism induction protocol based on antibodies targeting NOD T cells that was able to generate long-term mixed chimerism. In brief, the protocol included preconditioning with antibodies against CD4 and CD8, a single dose of both busulfan (day -1) and anti-CD40L (day 0), bone marrow transplantation (BMT; day 0), and a short course of rapamycin (day 0–28). These chimeric NOD mice

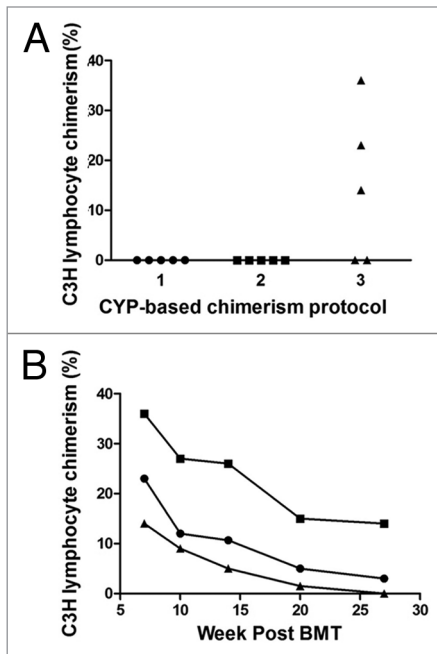


Figure 1. CYP combined with NK and T cell depletion can generate chimerism in NOD mice. Methods, including animals, bone marrow isolation, islet transplantation and flow cytometry are fully described in reference 4. Briefly, induction of allogeneic chimerism by transplantation of bone marrow cells was performed as follows. Recipient NOD mice in all groups were pre-conditioned with NK cell depletion by administering anti-asialo GM1 intraperitoneal (i.p. on day -3 and busulfan (BUS) on day 1 given i.p. at 20 mg/kg. Day 0 intravenous (i.v.) transplantation of 20×10^6 unmodified C3H BMCs into NOD mice was performed as part of one of three different non-myeloablative protocols that included the i.p. administration CYP at 150mg/kg body weight. Protocol 1: CYP and NK depletion. CYP was administered on day +2 relative to BMT and rapamycin at 3 mg/kg was administered i.p. daily from day +4 to +28. Protocol 2: CYP, NK depletion and donor specific transfusion (DST). Recipient NOD mice were given a DST of 20×10^6 unmodified C3H spleen cells i.v. on day -4. CYP was administered on day -2 and rapamycin was given daily from day 0 to +28. Protocol 3: CYP, NK and T cell depletion. On day -3, T cell depletion was performed by administering anti-CD4 (GK1.5) and anti-CD8 (53.6.7). CYP was administered on day +2 and rapamycin was given daily from day +4 to +28. (A) C3H chimerism ($H-2K^b/H-2K^d$) level in peripheral blood assessed by flow cytometry at seven weeks post-BMT ($n = 5$ per group). (B) Peripheral blood in each of the three chimeric mice that received protocol 3 was monitored over time for the presence of allogeneic lymphocytes.

were immunocompetent, diabetes free and could accept donor islet allografts.

Although many aspects of the successful chimerism induction protocols we developed in NOD mice are clinically translatable, they remain protocols that require anti-CD40 ligand (anti-CD40L).⁴ Since anti-CD40L is not available for clinical use,⁵ we began testing whether anti-CD40L can be replaced with cyclophosphamide (CYP). CYP is an alkylating agent used clinically for the treatment of leukemia, lymphoma,⁶ and in some cases as an induction agent for lupus nephritis.⁷ More recently, CYP has been used as an agent to prevent graft-vs.-host disease (GVHD) after BMT.^{3,8} Previously, CYP has been used quite extensively in both solid organ and cellular transplantation experiments in mice.⁹⁻¹² Focusing specifically on BMT, CYP has been used most commonly in combination with total body irradiation (TBI) and T cell depleting antibodies. These studies have shown that CYP can increase the level of chimerism when combined with T cell depleting antibodies and can decrease the amount of TBI required to establish chimerism in B10.BR to B10 chimeras.¹³ The mechanism of CYP action is thought to be mainly through the destruction of donor-reactive T cells.¹⁴

In relation to NOD mice, previous studies have shown that the administration of a high dose of CYP (200 mg/kg) leads to the rapid, synchronous development of diabetes¹⁵ via a mechanism thought to be dependent on reduced regulatory T cell function.¹⁶ However, to our knowledge, CYP has not been tested in the difficult NOD mouse model as an agent for chimerism induction. Therefore, our aim was to determine if inclusion of CYP as an agent to induce chimerism in NOD mice would obviate the need for anti-CD40L.

We attempted three different protocols that included CYP (150 mg/kg) as part of the conditioning regimen to induce chimerism in pre-diabetic NOD mice using fully allogeneic C3H donor bone marrow (Fig. 1 legend). Protocols 1 and 2 were unsuccessful at chimerism induction, however, protocol 3 was successful at inducing chimerism in 3/5 mice (Fig. 1A). Over time, the level of donor chimerism declined in all three of these

mice, with one mouse losing chimerism completely (Fig. 1B). At approximately 10 weeks post-chimerism induction, we challenged the three chimeric mice with a C3H islet transplant. The mouse with the highest levels of chimerism and the mouse that eventually lost chimerism accepted donor islets for greater than 100 days and returned to hyperglycemia after the islet graft-bearing kidney was removed (data not shown). Despite the maintenance of chimerism, the mouse with an intermediate level of chimerism rejected donor islets at day 23 post-islet transplant. This demonstration of split tolerance in C3H→NOD mixed chimeras is consistent with our previous results.^{4,17} Since higher doses of CYP can be used to induce diabetes in NOD mice,¹⁵ it is important to note none of the mice in these experiments developed spontaneous diabetes (data not shown).

The combination of CYP and rapamycin may be an especially interesting strategy for chimerism induction because it combines the ability of CYP to deplete alloreactive lymphocytes¹⁴ with the anti-proliferative and regulatory T cell promoting effects of rapamycin.^{18,19} Indeed, the combination of these two agents has recently been shown to promote stable chimerism in a BALB/c→C57BL/6 model (personal communication Jonathan D. Powell, manuscript submitted). However, in the current experiments with NOD mice, antibodies targeting T cells were required in addition to CYP and rapamycin in order to generate allogeneic chimerism. The additional T cell targeting required is likely due to the tolerance resistance of NOD mice.²⁰⁻²²

In these preliminary studies, we have found that a lymphocyte depletion protocol that includes CYP (and completely avoids anti-CD40L) is indeed capable of generating fully allogeneic mixed chimeras in NOD mice. While a promising beginning, it will be important to determine if further optimization of this anti-CD40L free protocol will allow stable levels of mixed chimerism. Furthermore, it will be important to examine whether successful CYP-based chimerism induction protocols in pre-diabetic NOD mice are effective in generating chimerism and tolerance after the onset of spontaneous

diabetes. Although the C3H chimerism levels declined in all three chimeric mice, and was completely lost in one case, it is exciting to see that two of these NOD chimeras accepted donor islets. Fully allogeneic chimeric NOD mice have a tendency to develop split tolerance and it will be important in future studies to determine the degree of split tolerance that develops in mice made chimeric with CYP as part of their induction protocol. In addition, mechanistic studies of lymphocyte function in the setting of rapamycin together with anti-CD40L vs. CYP are needed to elucidate the potential of CYP to replace targeting of CD40L.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Jonathan D. Powell for helpful discussions. Support for these studies was from the Canadian Diabetes Association, the Edmonton Civic Employees Charitable Assistance Fund and Alberta Innovates Health Solutions.

References

- Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Millan MT, Shizuru JA, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* 2008; 358:362-8; PMID:18216356; <http://dx.doi.org/10.1056/NEJMoa074191>.
- Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008; 358:353-61; PMID:18216355; <http://dx.doi.org/10.1056/NEJMoa071074>.
- Leventhal J, Abecassis M, Miller J, Gallon L, Ravindra K, Tollerud DJ, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med* 2012; 4:24ra28; PMID:22399264; <http://dx.doi.org/10.1126/scitranslmed.3003509>.
- Al-Adra DP, Pawlick R, Shapiro AM, Anderson CC. Targeting cells causing split tolerance allows fully allogeneic islet survival with minimal conditioning in NOD mixed chimeras. *Am J Transplant* 2012; 12:3235-45; PMID:22974315; <http://dx.doi.org/10.1111/j.1600-6143.2012.04260.x>.
- Sidiropoulos PI, Boumpas DT. Lessons learned from anti-CD40L treatment in systemic lupus erythematosus patients. *Lupus* 2004; 13:391-7; PMID:15230298; <http://dx.doi.org/10.1191/0961203304lu1032oa>.
- Savage KJ. Therapies for peripheral T-cell lymphomas. *Hematology Am Soc Hematol Educ Program* 2011; 2011:515-24; PMID:22160083; <http://dx.doi.org/10.1182/asheducation-2011.1.515>.
- Mina R, von Scheven E, Ardoin SP, Eberhard BA, Punaro M, Ilowite N, et al.; Carra SLE Subcommittee. Consensus treatment plans for induction therapy of newly diagnosed proliferative lupus nephritis in juvenile systemic lupus erythematosus. *Arthritis Care Res (Hoboken)* 2012; 64:375-83; PMID:22162255; <http://dx.doi.org/10.1002/acr.21558>.
- Luznik L, Bolaños-Meade J, Zahurak M, Chen AR, Smith BD, Brodsky R, et al. High-dose cyclophosphamide as single-agent, short-course prophylaxis of graft-versus-host disease. *Blood* 2010; 115:3224-30; PMID:20124511; <http://dx.doi.org/10.1182/blood-2009-11-251595>.
- Tomita Y, Yoshikawa M, Zhang QW, Shimizu I, Okano S, Iwai T, et al. Induction of permanent mixed chimerism and skin allograft tolerance across fully MHC-mismatched barriers by the additional myelosuppressive treatments in mice primed with allogeneic spleen cells followed by cyclophosphamide. *J Immunol* 2000; 165:34-41; PMID:10861032.
- Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood* 2001; 98:3456-64; PMID:11719388; <http://dx.doi.org/10.1182/blood.V98.12.3456>.
- Onzuka T, Tomita Y, Shimizu I, Okano S, Yamada H, Yoshikai Y, et al. Effects of lipopolysaccharide on the induction of mixed chimerism in cyclophosphamide-induced tolerance. *Scand J Immunol* 2009; 70:423-30; PMID:19874546; <http://dx.doi.org/10.1111/j.1365-3083.2009.02314.x>.
- Iwai T, Tomita Y, Zhang QW, Shimizu I, Nomoto K, Yasui H. Requirement of a higher degree of chimerism for skin allograft tolerance in cyclophosphamide-induced tolerance. *Transpl Int* 2005; 17:795-803; PMID:15856178; <http://dx.doi.org/10.1007/s00147-003-0675-2>.
- Xu H, Chilton PM, Huang Y, Schanie CL, Yan J, Ildstad ST. Addition of cyclophosphamide to T-cell depletion-based nonmyeloablative conditioning allows donor T-cell engraftment and clonal deletion of alloreactive host T-cells after bone marrow transplantation. *Transplantation* 2007; 83:954-63; PMID:17460568; <http://dx.doi.org/10.1097/01.tp.0000258679.18684.b0>.
- Maeda T, Eto M, Nishimura Y, Nomoto K, Kong YY, Nomoto K. Direct evidence for clonal destruction of allo-reactive T cells in the mice treated with cyclophosphamide after allo-priming. *Immunology* 1993; 78:113-21; PMID:8436396.
- Harada M, Makino S. Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. *Diabetologia* 1984; 27:604-6; PMID:6530055; <http://dx.doi.org/10.1007/BF00276978>.
- Brode S, Raine T, Zaccane P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol* 2006; 177:6603-12; PMID:17082572.
- Chan WF, Razavy H, Luo B, Shapiro AM, Anderson CC. Development of either split tolerance or robust tolerance along with humoral tolerance to donor and third-party alloantigens in nonmyeloablative mixed chimeras. *J Immunol* 2008; 180:5177-86; PMID:18390699.
- Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 2005; 105:4743-8; PMID:15746082; <http://dx.doi.org/10.1182/blood-2004-10-3932>.
- Qu Y, Zhang B, Zhao L, Liu G, Ma H, Rao E, et al. The effect of immunosuppressive drug rapamycin on regulatory CD4+CD25+Foxp3+ T cells in mice. *Transpl Immunol* 2007; 17:153-61; PMID:17331841; <http://dx.doi.org/10.1016/j.trim.2007.01.002>.
- Pearson T, Markees TG, Serreze DV, Pierce MA, Marron MP, Wicker LS, et al. Genetic disassociation of autoimmunity and resistance to costimulation blockade-induced transplantation tolerance in non-obese diabetic mice. *J Immunol* 2003; 171:185-95; PMID:12816997.
- Pearson T, Markees TG, Serreze DV, Pierce MA, Wicker LS, Peterson LB, et al. Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? *Ann N Y Acad Sci* 2003; 1005:148-56; PMID:14679049; <http://dx.doi.org/10.1196/annals.1288.016>.
- Pearson T, Markees TG, Wicker LS, Serreze DV, Peterson LB, Mordes JP, et al. NOD congenic mice genetically protected from autoimmune diabetes remain resistant to transplantation tolerance induction. *Diabetes* 2003; 52:321-6; PMID:12540603; <http://dx.doi.org/10.2337/diabetes.52.2.321>.