Transplant Immunology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Immunological characterization of *de novo* and recall alloantibody suppression by CTLA4Ig in a mouse model of allosensitization

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ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 15 July 2016
Accepted 5 August 2016
Available online xxxx

Keywords:
B cell
CTLA4Ig
Donor specific antibody
HLA-sensitization
Mouse
Plasma cell

ABSTRACT

It is well known that CTLA4Ig inhibits allogenic T-cell activation in transplantation. The immunological features and mechanisms associated with alloantibody suppression by CTLA4Ig, however, are poorly understood. Here, we used a mouse model of allosensitization to evaluate the efficacy of CTLA4Ig (abatacept) in suppression of donor-specific antibody (DSA) during *de novo* and recall alloantibody responses. We found that abatacept inhibited *de novo* DSA IgM and IgG responses to HLA-A2 expressing skin grafts. Abatacept administered during primary T cell priming also reduced recall IgG responses induced by re-immunization. Suppression of *de novo* DSA responses by abatacept is associated with a reduction in splenic expression of the germinal center activation marker GL7 and a reduction of $CD4^+$ $PD1^+$ $CXCR5^+$ follicular T helper (Tfh) subset in splenic lymphocytes detected by flow cytometry. The efficacy of abatacept on recall DSA suppression is moderate. *In vitro* experiments demonstrated that abatacept inhibited DSA IgG secretion by $CD138^+$ plasma cells isolated from allograft recipients. Additional experiments using an IgG1 secreting mouse hybridoma cell line showed that abatacept binds to CD80 expressed on these cells with subsequent inhibition of cell proliferation and reduction in IgG ELISpot formation. In conclusion, CTLA4Ig is a potent suppressor of *de novo* DSA responses and also affects recall responses. The data suggests modification of recall DSA responses is due to a direct suppressive effect on plasma cells.

1. Introduction

CD28 and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) play essential roles in the initial activation and subsequent control of cellular immunity [1]. CD28 is expressed constitutively on T cells, providing a major costimulatory signal upon binding to CD80/CD86 (B7-1/B7-2) on antigen-presenting cells such as dendritic cells, monocytes/macrophages and B cells [2]. Alternatively, CTLA-4, binding to the same ligand CD80/CD86, acts as an "off switch" in balancing CD28 signaling during T cell activation [3,4].

Several fusion proteins composed of the Fc region of a human IgG1 and the extracellular domain of CTLA-4 are formulated for inhibiting the necessary second signal generated from ligation of CD28 with CD80/CD86 during T cell activation [5,6]. The first generation of CTLA4Ig, abatacept, has been used to block CD28-dependent T cell activation for modulating harmful immune responses in autoimmune disorders [7,8]. A second generation of CTLA4Ig, belatacept, differs from abatacept by two point mutations in the binding region of CTLA-4, resulting in stronger binding to CD86 and CD80 in humans [9]. Belatacept (Nulojix®) was approved by FDA for antirejection in kidney transplant patients in June 2011 [10].

Several studies have documented the use of CTLA4Ig in transplant patients, describing its antirejection efficacy, impact on renal functions and adverse side effects [10–13]. A finding in these reports is that CTLA4Ig also possesses some mitigating properties on humoral immunity, suppressing alloantibody responses in transplant patients [14–16]. Reports of animal models have provided further support for suppression of donor-specific antibody (DSA) responses. These studies suggest that CTLA4Ig inhibited both autoantibody and alloantibody formation [7,8,17–19]. Thus, CTLA4Ig may hold promise as a desensitization agent for highly HLA sensitized (PRA >30%) kidney transplant candidates and for prevention of emergence of *de novo* DSA development following graft implantation [20–25]. However, the mechanisms of action responsible for suppression of DSA production are incompletely understood [26].

To address the ability of CTLA4Ig to modify *de novo* and recall DSA responses, we conducted animal experiments in a mouse model of allogeneic sensitization [27–29]. Kinetics of donor-specific antibody productions were monitored by conducting flow antibody binding assay on weekly blood draws. We also extended the studies to explore the mechanism(s) by which CTLA4Ig affects Tfh cells, B-cells and plasma cells. The results of the studies demonstrate that CTLA4Ig is a potent

Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DSA, donor-specific antibody; MFI, mean fluorescence intensity; SG, skin graft; Tfh, follicular T helper.

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http://dx.doi.org/10.1016/j.trim.2016.08.001
0966-3274/

suppressant of *de novo* donor-specific alloantibodies (both IgM and IgG) and can modify recall DSA responses.

2. Materials and methods

2.1. Animal models and drug administration

C57BL/6-Tg(HLA-A2.1)1Enge/J and C57BL/6J mice (male, weighing 25–30 g) were purchased from Jackson Laboratories (JAX® GEMM® Strains). Allogeneic immunization was achieved by transplanting a primary skin graft (0.7–0.8 cm in diameter) from the HLA.A2 transgenic mouse to a C57BL/6J recipient (Fig. 1). Recall alloantibody responses were induced by re-immunizing the pre-sensitized C57BL/6J mouse with a 2nd HLA.A2$^+$ skin allograft at 91 days post-1st skin grafting. Skin grafts were inspected daily to determine graft survival time. A graft rejection was considered when >90% area of a skin patch became dry. Our experience with this mouse model of allosensitization indicates that skin allografts in control and treatment groups are typically rejected within 7 day post-primary skin grafting and 3–4 days post re-sensitization, indicating that administration of immune suppressants have no impact on skin graft survival in the model [28,29]. Instead, skin allograft is effectively used as a sensitization mechanism to study alloantibody responses.

Abatacept was supplied by Bristol-Myers Squibb (BMS, New York, www.BMS.com) and was used in all mouse studies described in this paper. Abatacept was diluted in sterile water for injection (SWFI) before administration by intraperitoneal (IP) injection at a dose of 500 μg/mouse. Five doses were given to each recipient at days 0, 2, 7, 14 and 21 post-1st skin grafting and/or post-2nd skin grafting. Control substance (SWFI) was administered by IP injection. In studies of recall DSA suppression, C57BL/6 mice were pre-sensitized with HLA.A2$^+$ skin allografts. At day 91 the pre-sensitized mice were challenged with a second HLA.A2$^+$ skin allograft. The recipients were divided into 4 dosing groups as described in Table 1.

Experimental procedures involving mice were approved by Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center. Animals were handled with humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and published by the National Academies Press (1996 version; http://www.ncbi.nlm.nih.gov/books/NBK44152/) and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Research.

2.2. Measurement of serum donor-specific antibody IgM and IgG

Donor-specific antibodies (anti-HLA.A2) were measured in a flow cytometric antibody-binding assay in which an HLA.A2-expressing T-lymphoblast cell line (CCL-120.1; ATCC, Manassas, VA, USA) serves as target cells for serum anti-HLA.A2 IgM or IgG binding described previously [28].

Table 1
Animal groups and dosing schedule for evaluating the efficacy of CTLA4I treatment in recall DSA responses.

Groups	Dosing schedule post-1st SG	Dosing schedule post-2nd SG
Group-1 (S/S)	Sterile water at days 0, 2, 7, 14, 21.	Sterile water at days 0, 2, 7, 14, 21.
Group-2 (S/A)	Sterile water at days 0, 2, 7, 14, 21.	Abatacept (500 μg/IP injection) at days 0, 2, 7, 14 and 21.
Group-3 (A/A)	Abatacept at days 0, 2, 7, 14, 21.	Abatacept at days 0, 2, 7, 14 and 21.
Group-4 (A/S)	Abatacept at days 0, 2, 7, 14, 21.	Sterile water at days 0, 2, 7, 14, 21.

2.3. Multiparameter flow cytometry

Splenocytes freshly isolated from mice were stained with antibody markers and analyzed in flow cytometry for characterization of cellular subset changes in T, B and plasma cells. Cell-staining procedures, including use of fluorescence-tagged isotype-matched antibody controls and use of 7AAD (7-aminoactinomycin D; Invitrogen, Carlsbad, CA, USA) to exclude dead cells, was described previously [28,29]. Data were acquired in CyAnTM ADP (Dako USA, Carpinteria, CA) and analyzed using a Summit program (v 4.3; Dako USA).

Antibody markers used for identification of mouse lymphocytic subsets include CD3-FITC (clone KT3, Fluorescein Isothiocyanate, AbD Serotec www.abdserotec.com), CD4-FITC (clone RM4-5, eBiosciences) or CD4-APC (clone GK1.5, Allophycocyanin, Biolegend, www.biolegend.com), NK1.1-PE (clone PK13, Phycoerythrin, eBiosciences www.ebiosciences.com), CD11b-APC (clone M1/70, Biolegend), B220-PB (clone RA3-6B2, Pacific blue, Biolegend), CD28-APC (clone 37.51, eBiosciences), CD80-FITC (clone RM80, AbD Serotec), CD38-FITC (clone 90, Biolegend), CD138-PE (clone 281–2, BD Biosciences www.BD.com), CD279 (PD-1)-PE (clone29F-1A12, Biolegend), CD185 (CXCR5)-APC (clone 2G8, BD Biosciences) and CD185-BV (clone L138D7, Brilliant Violet 421, Biolegend).

2.4. CD138$^+$ cell isolation, primary cultures and DSA detection

2.4.1. CD138$^+$ cell isolation

Single cell suspensions were procured from the spleens and bone marrows harvested from allograft recipients at designated day post-transplantation. CD138$^+$ cells were then isolated from single cell suspensions using an EasySep kit (Cat#18554, Stem Cells Technologies) which employs a phycoerythrin (PE) conjugated anti-mouse CD138 mAb (clone 281–2, BD Biosciences) for positive selection through immunomagnetic column-free technique. The CD138 cell isolation experiments were carried out per set of the manual of EasySep kit manufactory.

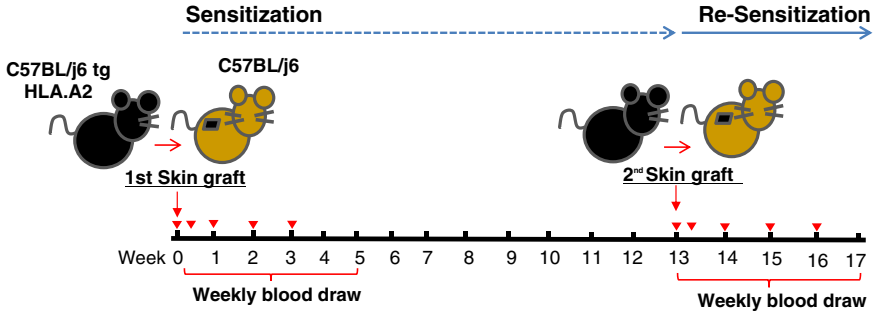

Fig. 1. Schematic presentation of mouse models of allogeneic sensitization to induce *de novo* DSA responses and re-sensitization to induce recall DSA responses. Mice received abatacept (0.5 mg/dose) at days 0, 2, 7, 14 & 21 post-1st skin grafting and with or without treatment post-2nd skin grafting.

Please cite this article as: I. Kim, et al., Immunological characterization of *de novo* and recall alloantibody suppression by CTLA4Ig in a mouse model of allosensitization, Transpl Immunol (2016), http://dx.doi.org/10.1016/j.trim.2016.08.001