Research Article

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Alloantigen gene transfer to hepatocytes promotes tolerance to pancreatic islet graft by inducing CD8$^+$ regulatory T cells

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Background & Aims: Induction of donor-specific immune tolerance is a good alternative to chronic life-long immunosuppression for transplant patients. Donor major histocompatibility complex (MHC) molecules represent the main targets of the allogeneic immune response of transplant recipients. Liver targeted gene transfer with viral vectors induces tolerance toward the encoded antigen. The aim of this work was to determine whether alloantigen gene transfer to hepatocytes induces tolerance and promotes graft acceptance.

Methods: C57BL/6 (H-2b) mice were treated with adeno-associated viral (AAV) vector targeting the expression of the MHC class I molecule H-2K^d to hepatocytes, before transplantation with fully allogeneic pancreatic islet from BALB/c mice (H-2d).

Results: AAV *H-2K^d* treated mice were tolerant to the alloantigen, as demonstrated by its long-term expression by the hepatocytes, even after a highly immunogenic challenge with an adenoviral vector. After chemical induction of diabetes, the AAV treated mice had significantly delayed rejection of fully allogeneic pancreatic islet grafts, with more than 40% of recipients tolerant (>100 days). AAV-mediated expression of H-2K^d in the liver induced the local expansion of CD8$^+$ T lymphocytes with allo-specific suppressive properties. The adoptive transfer of these liver-generated CD8$^+$ Tregs into naive diabetic mice promoted the long-term survival of allogeneic pancreatic islet grafts.

Conclusion: AAV-mediated long-term expression of a single MHC class I molecule in the liver induces the generation of a subset of allo-specific CD8$^+$ Treg cells, which promote tolerance toward fully allogeneic graft. Liver gene transfer represents a promising strategy for *in vivo* induction of donor-specific tolerance.

Lay summary: The liver has a special immune system, biased toward tolerance. In this study, we investigated the possibility of harnessing this property of the liver to induce tolerance to an allogeneic transplantation.

We demonstrate for the first time that the *in vivo* gene transfer of an allogeneic antigen with an adeno-associated viral vector to mouse hepatocytes induces the expansion of a population of CD8$^+$ regulatory T lymphocytes. These Tregs are then instrumental in preventing the rejection of allogeneic pancreatic islets transplanted in these animals.

Allogeneic transplantation is the main treatment for the end-stage diseases of a number of organs. Life-long immunosuppressive treatments are still required to limit graft rejection, and these treatments exhibit serious side effects. Our present findings open a new avenue for promoting allo-specific tolerance via *in vivo* induction of CD8$^+$ Treg expansion.

Introduction

Allogeneic transplantation remains the only treatment available for end-stage diseases of various organs, such as kidney, heart, liver, and pancreas. However, the donor-specific allogeneic immune response of the host requires life-long immunosuppression. Despite constant progress, these long-term treatments still carry serious side effects, from toxicity to increased risk of opportunistic infections and of neoplasia [1,2]. An ideal alternative to the need for long-term exposure to immunosuppressors would consist of the induction of a donor-specific immune tolerance, which, despite having been achieved experimentally, remains a challenge in the clinical arena. The first intentional tolerance induction in transplant patients was achieved by induction of donor mixed chimerism. Various methods were used after combined kidney and bone marrow transplantation, with long-term stable allograft survival without maintenance immunosuppression being achieved in a number of patients [3–5]. Another promising approach is to actively establish peripheral tolerance by inducing and increasing *in vitro* immune cells with regulatory functions that can control allo-specific effector T cells [6]. Several different regulatory cell populations are being tested in clinic, and these suggest that donor-specific regulatory cells may be also a promising strategy for achieving immune tolerance [6,7]. Experimentally, the higher suppressive potential of allo-specific CD4$^+$ regulatory cells over their naturally occurring polyclonal

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counterparts has been demonstrated to protect against alloimmunity in a challenging skin graft model [8]. A number of obstacles (e.g., risk of graft *vs.* host disease [GvHD] and/or vascular toxicity in induction of mixed chimerism [9,10]) and difficulties (such as the logistics of *in vitro* preparation of specific regulatory cells [11]) with these cell therapies persist. There is still the need for new preventive strategies of tolerance induction that could alleviate some of these concerns and permit the reduction of allograft loss through immunological injury and long-term exposure to non-specific immunosuppression.

The strategy we outline in this paper is to induce antigen-specific immune tolerance, through harnessing an "immune-privileged" site, the liver. This was carried out by forcing expression of the antigen directly at such a site, in the hope of benefiting from its unique regulatory properties on systemic immune response. The immune response in the liver is biased toward tolerance rather than immunity [12]. Due to its anatomic location, the blood supply from the portal vein constitutively exposes the hepatic immune cells to pathogen associated molecular patterns, toxins and food-derived antigens [13]. Exorbitant inflammation and tissue damage in this unique, vital organ are limited by control mechanisms. These are developed by hepatic immune cells to reduce inflammation, induce a pro-tolerogenic microenvironment, and act as a checkpoint to determine if antigenic presentation will result in immunity or tolerance [14,15]. Several mechanisms have been involved in hepatic tolerance. Among them, the most described are effector $CD8^+$ T cell exhaustion and premature death, which result from persistent T cell receptor (TCR) engagement. These occur in the presence of co-inhibitory signals provided by various liver resident antigen presenting cells including the hepatocytes [16,17], as well as active suppressive mechanisms relying on activation of regulatory T cells [18].

Liver targeted gene therapy for inherited diseases has benefited from this property to allow long-term expression of a therapeutic transgene, first in animal models, and now in clinical trial. This is the case for a haemophilia B trial that used a self-complementary adeno-associated viral vector (scAAV8). It permitted a stable expression of human factor IX by hepatocytes for more than 3 years, at a level sufficient to reduce or abrogate the need for prophylactic factor IX concentrate [19]. At a preclinical level, the pro-tolerogenic properties of the liver to induce systemic tolerance to auto-antigens in models of autoimmune diseases have also been investigated. The liver targeted expression of the neural auto-antigen myelin basic protein leads to protection from autoimmune neuroinflammation in a mouse model of multiple sclerosis [20]. More recently, hepatocyte-specific expression of an immunodominant peptide of insulin suppressed the progression of type 1 diabetes in non-obese diabetic (NOD) mice when performed at a late pre-diabetic stage [21]. In both cases, the regulatory mechanism involved the generation of antigen-specific $CD4^+$ Treg cells.

In this paper, we tested whether the long-term stable expression of a donor MHC antigen in the liver with an adeno-associated viral (AAV) vector induces tolerance to allogeneic pancreatic islets. We demonstrate that liver targeted expression of a single donor MHC class I molecule ($H\text{-}2K^d$) prolongs pancreatic islet allograft survival in diabetic H-2b mice. The AAV vector injection results in the long-term expression of the alloantigen in the hepatocytes, and in the generation of a specific population of $CD8^+$ regulatory T cells in the liver. We show that these $CD8^+$ Treg cells display a particular phenotype and allo-specific suppressive function *in vitro* and *in vivo* in transfer experiments.

Materials and methods

Viral vectors design and production

The vector plasmid used for AAV vector production consisted of the *H-2K*^{d} coding sequence, or a non-coding DNA sequence for the AAV null control vector. These were inserted between the liver-specific promoter m*TTR* (mouse transthyretin) sequence, and a bovine growth hormone (BGH) poly-A signal and flanked by 2 AAV2- inverted terminal repeats (ITRs).

Adenoviral vectors used for intramuscular vaccination were produced with a pShuttle vector plasmid containing the *H-2K*^{d} coding sequence inserted behind the phosphoglycerate kinase (*PGK*) promoter and followed by a simian virus 40 (SV40) poly-A signal. Control Ad-*LacZ* and the peptide used for detection of beta galactosidase (βGal)-specific immune response have been described previously [22].

scAAV2/8 and Adenoviral vector productions were performed by the INSERM UMR 1089 Vector Production Centre at the University Hospital of Nantes (France).

Mice

Six to eight-week-old male C57BL/6 (H-2b), BALB/c (H-2d) and C3H (H-2k) mice were purchased from Janvier Laboratory, France. All mice were housed at the Nantes University animal facilities. Procedures were approved by the regional ethical committee for animal care and use and by the French Ministry of Research.

Mice were injected intravenously with $1.2x10^{12}$ $vg.kg^{-1}$ (vg, vector genome) of the scAAV m*TTR H-2K*^{d} or of the AAV null vector for the control group. For the adenoviral vaccination, the mice were injected intramuscularly with 1.6×10^{12} $vg.kg^{-1}$ of Ad-*PGK H-2K*^{d} or Ad-*LacZ*.

Depletion experiments were performed by intraperitoneal (i.p.) injections of anti-CD25 mAb (PC61, 250 μg on day -5, -2, 1 of Ad immunization), and anti-programmed death ligand (PD-L1) mAb (BioXCell, clone 10F.9G2, 200 μg, day -3, 3, 6, 9, 12 of Ad immunization).

Pancreatic islet transplantation

For diabetes induction, mice received an i.p. injection of 200 $mg.kg^{-1}$ of streptozotocin (STZ) and their blood glucose concentration was measured daily with Stat Strip Glucose Xpress Meter (Nova Biomedical). The basal blood glucose level before diabetes induction was 141 ± 25 $mg.dl^{-1}$. There was no difference between AAV control and AAV *H-2K*^{d} mice and blood glucose levels were stable over the time after AAV injections (data not shown). Diabetes was defined as a non-fasting blood glucose greater than 16 $mmol.L^{-1}$ (290 $mg.dl^{-1}$) at two consecutive measurements. Pancreatic islet transplantations were performed when diabetes was confirmed (generally 3 to 5 days after STZ injection). Pancreatic islets were purified from donor mice after digestion by collagenase and isolation by Ficoll gradient centrifugation. 300–500 islets were transplanted under the right renal capsule [23]. When pancreatic islets remained functional for more than 100 days, a total resection of the kidney with the implanted islet graft was performed to control the return to hyperglycemia.

Hepatocyte isolation and immunostaining

Hepatocytes were isolated following an adaptation of the standard 2-step collagenase perfusion protocol [24]. Briefly, a laparotomy was performed and a 30-gauge catheter was inserted into the hepatic portal vein for liver perfusion, first with a buffer consisting of Hank's Balanced Salt Solution, HBSS without Ca^{2+} and Mg^{2+}, 50 mM HEPES, 0.65 mM EDTA pre-warmed at 37 °C (4 $ml.min^{-1}$; 20 ml total), then with the second buffer containing collagenase IV (HBSS without Ca^{2+} and Mg^{2+}, 50 mM HEPES, 6.7 mM $CaCl_2$ and 0.024% collagenase IV) (4 $ml.min^{-1}$; 20 ml total). The liver was dissected and lacerated in William's complete medium (2 mM L-glutamine, 5% fetal bovine serum, 100 nM insulin, 100 nM dexamethasone, 100 $IU.ml^{-1}$ and 100 $mg.ml^{-1}$ streptomycin). Cell suspension was passed through a 250 μm nylon filter and washed 2 times by low speed centrifugation (50 g). Isolated hepatocytes were quickly stained with phycoerythrin (PE) anti-$H\text{-}2K^d$ (SF1-1.1) antibodies and fixed with 4% paraformaldehyde before analysis by flow cytometry.