Anti-CD2 producing pig xenografts effect localized depletion of human T cells in a huSCID model

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Running Heading

Local depletion by anti-CD2 xenografts

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Abstract

Background: We investigated whether graft produced anti-human CD2, mediated by adenovirus (Adv) transduction of pig neonatal islet cell clusters (pNICC), would protect xenografts in a humanized mouse model from immune attack and whether such immunosuppression would remain local.

Methods: A mouse anti-human CD2 Ab (CD2hb11) previously generated by us was genetically engineered to produce chimeric and humanized versions. The three forms of CD2hb11 were named dilimomab (mouse), diliximab (chimeric) and dilizumab (humanized). All 3 forms of CD2hb11 Ab were tested for their ability to bind $CD3^+$ human T cells and to inhibit a human anti-pig xenogeneic mixed lymphocyte reaction (MLR). They were administered systemically in a humanized mouse model in order to test their ability to deplete human $CD3^+$ T cells and whether they induced a cytokine storm. An adenoviral vector expressing diliximab was generated for transduction of pNICC. Humanized mice were transplanted with either control-transduced pNICC or diliximab-transduced pNICC and human T cells within grafts and spleens were enumerated by flow cytometry.

Results: Dilimomab and diliximab inhibited a human anti-pig xenogeneic response but dilizumab did not. All 3 forms of CD2hb11 Ab bound human T cells *in vitro* though dilimomab and diliximab exhibited 300-fold higher avidity than dilizumab. All 3 anti-CD2 Abs could deplete human $CD3^+$ T cells *in vivo* in a humanized mouse model without inducing upregulation of activation markers or significant release of cytokines. Humanized mice transplanted with dilixumab-transduced pNICC afforded depletion of $CD3^+$ T cells at the graft site leaving the peripheral immune system intact.

Conclusions: Local production of a single Ab against T cells can reduce graft infiltration at the xenograft site and may reduce the need for conventional, systemic immunosuppression.

Key words

Xenotransplantation – local immunosuppression – CD2 – depleting – humanized mice – adenovirus – neonatal islet cell clusters

Abbreviations

Adv, adenovirus; pNICC, pig neonatal islet cell cluster; MLR, mixed lymphocyte reaction; CDR, complementarity determining region; PBMC, peripheral blood mononuclear cell; FCS, fetal calf serum; PBS, phosphate-buffered saline; i.v., intravenous; MOI, multiplicity of infection; IEQ, islet equivalent; PFU, plaque forming unit; BSA, bovine serum albumin

Introduction

Insulin replacement for the treatment of type 1 diabetes prevents death from keto-acidosis but carries a heavy life-style burden. Moreover, long-term complications result in microvascular complications of the heart, kidney, eye and limb. Pancreas or islet transplantation remains the only cure for type 1 diabetes. However, there is a vast shortfall between numbers of such transplants and the number of diabetes sufferers, due predominantly to two factors: supply and systemic immunosuppression. The widening gap between supply (of donor pancreata) and demand (by potential recipients) (www.organdonor.gov/about/graphdescription.html) has led to the search for alternative sources of tissue e.g. stem cells or tissues from another species. Current immunosuppressive regimens used to stop graft rejection result in increased susceptibility to infections and cancer (1, 2) and hence reduce the suitability of transplantation to most patients with type 1 diabetes. Furthermore, the immunosuppressive drugs have deleterious off-target effects; for example, cyclosporine and tacrolimus show toxicity to kidneys and islets, while mTOR inhibitors suppress hematopoiesis (3, 4). Therefore, any regimen that more specifically targets the immune system and reduces the need for systemic immunosuppression would be advantageous. We have previously shown that genetic modification of mouse islet tissue to secrete its own immunosuppressive “drug” (that blocks costimulation or depletes T cells) could indeed effect local immunosuppression against allogeneic rejection (5-8). Hence, we wanted to determine whether a similar strategy for pig xenografts might work against human T cells.

CD2, a type I surface glycoprotein with two external domains is a costimulation/adhesion molecule on T cells. Human CD2 binds CD3 *in cis* (9) and LFA-3 (CD58) *in trans* with low affinity Kd=9-22 μM (10), whereas most antibodies have affinities several logs higher. Human CD2 also binds sheep LFA-3 and is the basis for the E-rosetting method of identifying/purifying T cells (11). LFA-3Ig (Alefacept) has been advocated as an immunosuppressive drug for transplantation (12). It has shown efficacy against islet xeno-responses. As CD2 is elevated in memory T cells, alefacept is one of the few clinical agents that deplete memory cells. Whereas human CD2 is found on T cells and NK cells only, mouse CD2 is also found on B cells. In CD2 knock-out mice, T cells required 3-10-fold more peptide to elicit the same response as wild-type T cells (13). We have chosen anti-CD2 to suppress immune rejection, because it would target all T cells (cf. anti-CD4 or anti-CD8), it would be expected to be less activating than anti-CD3 (see below) and it should mirror the advantages of alefacept.

Recently, we produced a monoclonal Ab recognising CD2 in a broad range of primates including human. We targeted CD2, because a) it is abundantly expressed on all human T cells (both CD4 and CD8), b) it is not down-modulated upon T cell activation (12, 14), c) it acts as a costimulation/adhesion molecule for T cells (hence anti-CD2 Abs can deplete or block costimulation or both) and d) unlike anti-CD3 Abs, anti-CD2 Abs are mostly non-activating and hence unlikely to induce a 'cytokine storm' (15). The monoclonal Ab used here (CD2hb11) is non-activating, depletes T cells and inhibits T cell activation by tetanus antigen or in mixed lymphocyte reactions (16).

As a prelude to generating transgenic pigs, we generated adenoviral vectors expressing CD2hb11 Ab for transduction of pig tissue. We cloned the Ab genes to produce a chimeric Ab with human IgG3 Fc and a humanized Ab (with only complementarity-determining regions (CDR) of the mouse). Human IgG3 binds FcR and complement very efficiently (17). Our aim was to determine whether pig neonatal islet cell clusters genetically modified by adenovirus to secrete anti-human CD2 would afford protection from immune attack without causing immune suppression systemically.

Methods

Animals

NOD-SCID IL2rγ^{null} mice were bred under specific pathogen-free conditions at the Walter & Eliza Hall Institute. The Institution's Animal Ethics Committee approved all experimental procedures.

Antibodies

Flow cytometry antibodies were purchased from BD Biosciences, San Jose, CA. Human IgG (IVIG) was a gift from CSL Ltd., Parkville, Australia. Purified anti-CD16/CD32 (Clone 2.4G2) used for blocking of mouse Fc receptors, mouse IgG1 anti-FLAG and OKT3 were produced at the Monoclonal Antibody Facility of the Walter & Eliza Hall Institute. ELISA antibodies were purchased from Southern Biotechnology Associates, Birmingham, AL.

Xenogeneic mixed lymphocyte reactions (MLR)

Assays were performed in triplicate in 96-well round-bottom plates. RPMI-1640 medium (Invitrogen, Carlsbad, CA) was supplemented with 5% pooled male human serum, 25 mM HEPES (Sigma-Aldrich, St Louis, MO), 2 mM L-glutamine (Invitrogen), 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}). 10^5 human PBMCs were used as responders and 10^5 irradiated (20 Gy) porcine splenocytes as stimulator cells. CD2 Abs were added at different concentrations (0.5-50 μg mL^{-1}) from the beginning of the culture. Human IgG was used as negative control at the same concentration. After 6 days, ^{3}H-thymidine (18.5 kBq per well) was added for 16 h, after which the cells were harvested. Incorporated radioactivity was measured by β-scintillation counting. Results are expressed as the mean +/- SEM of triplicate wells.

Cloning of antibody coding regions

CD2hb11

Variable regions of the mouse IgG1 anti-human/baboon CD2 (clone CD2hb11) mAb (16) were cloned as previously described (18), using as sense primer 5'-CGTCGATGAGCTCTAGAATTCGCATGTGCAAGTCCGATGGTCCCCCCCCCC CCCC and antisense primers 5'-CCGAATTCTAACACTCATTCCTGTTG for the κ light chain and 5'-CGTCATGTCGACGGATCCAAGCTTGTCACCATGGAGTTAGTTTGGGC for the IgG1 heavy chain.

Human IgG3 constant region

IgG3 synthesis was induced by pokeweed mitogen (19). Briefly, 10^6 PBMCs were suspended in 1 mL of RPMI-1640 medium (Invitrogen) supplemented with 20 mM L-glutamine (Invitrogen), 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), penicillin (100 U mL^{-1}), streptomycin (100 U mL^{-1}) and 10% fetal calf serum (FCS) (JRH Biosciences Pty Ltd, Brooklyn, Australia) in the presence of 10 μg mL^{-1} pokeweed mitogen (Sigma-Aldrich) and 20% K562 (*ATCC*® CCL-243™) conditioned medium (as a source for IL-6). The cells were cultured for 4 days, after which they were washed three times, plated in fresh medium, and cultured for another 2 days. Total RNA from 10^6 cultured PBMCs was isolated using TRI reagent (Ambion Inc., Austin, TX). RNA was reverse-transcribed with 100 U moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) at 37 °C for 1 h in a 50 μL reaction mixture containing 1 μg of total RNA, 40 U RNasin (Promega), 0.5 mM dNTP (Fisher Biotec, Perth, Australia), 2 μM random hexamers (Sigma-Aldrich), 1 x reaction buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT; Promega). PCR reactions were carried out in a 50 μL reaction mixture containing 5 μL cDNA, 200 μM dNTP (Fisher Biotec), 0.1 μM each primer, 1.5 mM $MgCl_2$, 1 U

KAPAHiFi DNA polymerase (Kapabiosystems, Woburn, MA, USA), 1 x KAPAHiFi Fidelity Buffer with $MgCl_2$; Kapabiosystems). PCR consisted of 35 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 15 s and polymerization at 72 °C for 30 s. Primers used for amplifying were hIgG3CH1.BamF (5'-GGATCCCTGACCAGCGGCGTGCACACC-3') and hIgG3CH2-Hinge4-R (5'-GAGTTCAGGTGCTGGGCACCT-3') for one reaction and hIgG3CH1-Hinge1-F (5'-GACAAGAGAGTTGAGCTCAAA-3') and hIgG3.XbaR (5'-TTCTAGATTCATTTACCCGGAGACAGG-3') for the other reaction. The amplified cDNA was visualized on 2% low-melting agarose (Scientifix, Cheltenham, Vic, Australia), silica purified (20), and cloned into pGEM-T Easy vector (Promega). The hIgG3 construct was generated by cloning together the two PCR products utilizing an *AlwN*I restriction enzyme site located at the beginning of the CH2 region.

Humanized CD2hb11

The CDR sequences from the variable region of a human IgG heavy chain (DNA ID-GI: 194381820; *protein AK303185*) were substituted with the CDR sequences from the CD2hb11 heavy chain. The variable region sequence was then fused to the human IgG3 constant region sequence. The CDR sequences from a human IgG kappa chain (DNA ID-GI: 34526393; *protein AK129779*) were substituted with the CDRs from the CD2hb11 kappa chain. The codon optimized DNA was synthesized by GenScript (GenScript USA Inc., NJ) as a single multi-gene cassette utilizing the furin/F2A 'ribosomal skip' signal to combine the heavy and light chain sequences (21).

CD5L-hIgG3

Leader sequence of bovine CD5 (22) was cloned in frame utilizing a *Sac*I restriction enzyme site at the beginning of the hinge 1 region of human IgG3.

Assembly of expression cassettes

Chimeric CD2hb11

The entire variable region of the CD2hb11 heavy chain gene together with part of the CH1 exon was fused to the corresponding junction of the CH1 exon of human IgG3 (utilizing a *Bam*HI restriction enzyme site) to create a full-length mouse-human chimeric gene. The heavy chain construct was cloned with a CMV promoter and a bovine growth hormone polyadenylation signal. The entire CD2hb11 κ chain gene was cloned with a CMV promoter and SV40 polyadenylation signal. Both the heavy and light chain constructs including their promoters and polyadenylation signals were sub-cloned into a single AdMax shuttle plasmid (Microbix Biosystems Inc., Mississauga, Canada).

Humanized CD2hb11

The single multi-gene cassette was sub-cloned into a single AdMax shuttle plasmid containing a CMV promoter and a SV40 polyadenylation signal.

CD5L-hIgG3

CD5L-hIgG3 cassette was sub-cloned into a single AdMax shuttle plasmid containing a CMV promoter and a SV40 polyadenylation signal.

Expression and purification of anti-CD2 Abs

Murine CD2hb11 (dilimomab) was purified from the hybridoma. Chimeric (diliximab) and humanized (dilizumab) CD2hb11 were transiently expressed in Freestyle 293F cells using FreeStyle MAX (Invitrogen). The target protein was captured from the cell culture supernatant by Protein A-Sepharose at pH 6.5. Elution using 0.1 M glycine pH 2.8 and neutralization with 1 M Tris-HCl pH 8.0 was followed by buffer exchange into phosphate-buffered saline (PBS).

Antibody detection by ELISA

Human IgG3 was detected in supernatants of transfected 293F or transduced pNICC by capture ELISA. Microtitre plates were coated with rabbit anti-human IgG, incubated with serially diluted test supernatant, and then with peroxidase-conjugated mouse anti-human IgG3. Detection of bound Abs was with tetramethylbenzidine substrate (Sigma-Aldrich).

Isolation of peripheral blood mononuclear cells (PBMCs)

Human blood was obtained by venepuncture from volunteer donors who gave their informed consent. Buffy coat was obtained from the Australian Red Cross Blood Service. This study was approved by the ethics committee and conformed to the Declaration of Helsinki Principles. PBMCs (fresh or buffy coat) were isolated over Ficoll-Plaque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and washed twice in PBS. After lysis of red blood cells, nucleated cells were again washed twice with PBS.

Hu-SCID, measurement of cytokines and activation markers

NOD-SCID IL2rγ^{null} mice (23) were injected with 2x10^7 PBMCs i.v. Seven days later mice were injected with either 1 μg OKT3, 0.5 mg hIgG, 0.5 mg mIgG1 Ab (anti-FLAG), 0.5 mg dilimomab, 0.5 mg diliximab or 0.5 mg dilizumab. Mice were bled 2, 5 and 24 h later and plasma collected and analysed for cytokines using the Luminex system (Bio-Rad, Hercules, CA) following manufacturer's instructions. Cytokines detected include IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α. Treated hu-SCID mice were sacrificed at 24 h and activation markers analysed by flow cytometry. Human PBMCs from spleens were blocked with 2.4G2 before staining for fluorochrome-conjugated CD69, CD25, CD45 and CD3.

Recombinant adenovirus

Replication-defective recombinant Adv were constructed using the Ad-Max kit (Microbix Biosystems Inc.) (6). Recombinant virus was amplified in HEK293 cells and purified using the Adenopure Adv purification system (Puresyn Inc., Malvern, PA). Viruses were dialysed in 10 mM Tris-HCl, pH 8, containing 10% (v/v) glycerol, before storage at -70 °C. Viral titer was determined by plaque titration in HEK293 cells, and expressed as the number of plaque-forming units per mL (PFU mL^{-1}).

Pig NICC isolation

Neonatal pancrata were removed from euthanized donors (1-3 day-old piglets) and placed into ice-cold Hank's balanced salt solution (Gibco, Mount Waverley, Australia) supplemented with 12.5 mM HEPES (Sigma-Aldrich) and 2% (v/v) penicillin and streptomycin solution (Sigma-Aldrich). Pancreata were cut into 2-mm pieces and digested with 0.5 mg mL^{-1} collagenase type V (Sigma-Aldrich), filtered through 500 μm mesh, and the digest washed with the above solution. Hams F10 (ThermoTrace, Melbourne, Australia) supplemented with 10 mM glucose, 50 μM isobutylmethylxanthine (Sigma-Aldrich), 0.5% (v/v) bovine serum albumin (BSA) (Sigma-Aldrich), 10 mM nicotinamide solution, 1% (v/v) penicillin and streptomycin solution (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen) and 1.6 mM $CaCl_2$ was used as the pNICC culture medium. The resulting pNICC were cultured in bacteriological dishes for 7 days at 37 °C in air containing 5% CO_2, with media changes every second day.

Adenoviral transduction of pNICC

pNICC were transduced with Adv using a multiplicity of infection (MOI) of 10 (24). MOI was defined as the number of infectious virus particles per cell. We estimate there were about 1000 cells per pNICC islet equivalent (IEQ). Transductions were performed in culture medium in the absence of BSA, for 1 h at 37 °C. Unbound virus

was removed by washing twice with pNICC culture medium. pNICC were replated into culture medium for 24 h to facilitate transgene expression and then washed twice again in pNICC culture medium prior to transplantation.

pNICC xenografts

Transduced pNICC (total of 4000 IEQ) were transplanted under the kidney capsule of NOD-SCID IL2rγ^{null} recipients. Graft infiltration was assessed 12 days later by flow cytometry as described below.

Isolation of PBMCs from spleens and grafts

Spleens were made into single-cell suspensions. After lysis of red blood cells, nucleated cells were washed twice with PBS containing 1% FCS (JRH Biosciences Pty Ltd). Grafts were peeled from the kidney and digested for 25 min at room temperature with 1 mg mL^{-1} Type III collagenase (Worthington Biochemicals, Lakewood, NJ) supplemented with 0.14 mg mL^{-1} DNAse I Grade II (Roche Applied Science, Indianapolis, IN), then treated for 5 min with 10 mM EDTA to disrupt cell aggregates. Cells were washed twice with PBS containing 1% FCS.

Measurement of graft infiltration by flow cytometry

Mouse Fc receptors were blocked by incubation with 2.4G2 antibody then cells were stained with fluorochrome-conjugated monoclonal antibodies. Splenocytes were stained for CD45, CD3 and CD19. Grafts were stained for CD45, CD4 and CD8. Cell numbers were quantified by calibration against a known number of Calibrite beads (BD Biosciences). Data were analysed using FlowJo software (version 3.6.1; Tree Star Inc., Ashland, OR).

Statistical analysis

Unpaired one-tailed t test was used to calculate the significance of the difference between groups using Prism (GraphPad Software Inc., San Diego, CA).

Results

Generation of three forms of CD2hb11

The backbone of CD2hb11 was changed to reduce its immunogenicity in humans. The original heavy and light chain mouse Ab cDNAs were cloned from the hybridoma and the mouse IgG1 Fc was substituted with human IgG3 Fc to produce a chimeric form. Finally, all but the complementarity determining regions were replaced with human sequence to produce a humanized form. The human backbone sequence for the variable region was chosen from published framework sequences using that which most closely aligned with CD2hb11. In accordance with WHO nomenclature, these 3 forms were named dilimomab (mouse), diliximab (chimeric) and dilizumab (humanized); they are schematically depicted in Fig. 1. All three forms of CD2hb11 Ab were purified on immobilized protein A from the supernatants of 293F transient transfectants and shown to bind human $CD3^+$ T cells by flow cytometry (Fig. 2). The binding of diliximab and dilizumab could be directly compared, as the secondary Ab was the same, viz. anti-hIgG3. Dilixumab had a 300-fold higher avidity than dilizumab (Fig. 2).

CD2hb11 mAb inhibits xenogeneic stimulation

We had previously shown that human allogeneic MLR *in vitro* was inhibited by dilimomab (16). Therefore, we wanted to address whether xenogeneic MLR could be inhibited and whether the three forms of our Ab were effective. Both dilimomab and diliximab inhibited proliferation in a dose-dependent manner (Fig. 3A). Dilimomab inhibited proliferation by up to 3.2-fold at 50 μg mL^{-1} and diliximab inhibited proliferation by up to 7-fold at 50 μg mL^{-1}. Dilizumab did not inhibit xeno-MLR at any dose (Fig. 3A).

Systemic CD2hb11 depletes human T cells *in vivo*

Following engraftment of human PBMCs into NOD-SCID IL2rγ^{null} recipients (hu-SCID), we showed depletion of the CD3^{+} population 7 d after injection of 0.5 mg of all three forms of CD2hb11 (Fig. 3B). Compared with the control mice, dilimomab and diliximab depleted 99.8% of T cells and dilizumab depleted 98% of T cells. Both dilimomab and diliximab depleted more T cells than dilizumab when compared to untreated hu-SCID mice or hu-SCID mice treated with control hIgG.

CD2hb11 Ab is much less activating than OKT3 *in vivo*

Some anti-T cell Abs (eg. OKT3 anti-CD3) have been shown to induce cytokine release syndrome and we therefore tested the capacity of the anti-CD2 Abs to activate T cells *in vivo*. Hu-SCID mice were injected with OKT3 or dilimomab with mIgG as a control and diliximab or dilizumab with hIgG as a control. The following cytokines were assayed in plasma of hu-SCID mice: IL1-β, IL-2, IL-6, IL-10, IFN-γ and TNF-α. IL1-β was not detected in any samples at any time point and IL-6 was little changed. All other cytokines were elevated in hu-SCID mice treated with OKT3 peaking at 5 h then subsiding by 24 h; the kinetics of IFN-γ and IL-10 levels are shown in Fig. 4A. Hu-SCID mice treated with dilimomab or dilizumab showed minimal cytokine concentrations that were similar to those in mice treated with isotype Ab. None of the anti-CD2 treatments evoked a significant release of pro-inflammatory cytokines by 2, 5 or 24 h when compared to OKT3 (Fig. 4B showing 5 h). Treatment with diliximab had no effect on circulating TNF-α or IL-10 concentrations at any time, but IFN-γ and IL-2 increased modestly at 5 h. Activation markers (CD25 and CD69) on human T cells were analysed by flow cytometry directly *ex vivo* at 24 h post-treatment. Only

OKT3 treatment of hu-SCID mice upregulated CD25 or CD69 (Fig. 4C); CD2hb11 (dilimomab, diliximab and dilizumab) treatment had no effect above IgG controls.

Transduced pNICC secrete diliximab

As diliximab had good avidity and functional efficacy, we constructed an adenoviral vector expressing diliximab using cre-lox recombination. A control adenoviral vector was also generated expressing human IgG3 Fc (CD5L-hIgG3; the CD5 leader was used to avail secretion of IgG3 Fc). We were able to show expression of diliximab by Adv-diliximab *in vitro*. Antibody in supernatants from transduced pNICC bound $CD3^+$ T cells from human PBMCs by flow cytometry (Fig. 5A) and IgG3 protein was detected by capture ELISA (Fig. 5B). CD5L-hIgG3 expression by Adv-control was confirmed by detecting hIgG3 by capture ELISA (Fig. 5B).

Local deletion of $CD2^+$ cells by diliximab-expressing xenografts

Adv transduction of pNICC cultured for 7 days elicits efficient transgene expression within 24 h (25). To determine whether local depletion of T cells could be effected by the genetically modified graft we transduced pNICC with Adv-control or Adv-diliximab. 4000 IEQ of transduced pNICC were transplanted under the kidney capsule of NOD-SCID IL2rγ^{null} recipients. Two days later mice were injected i.v. with 2×10^7 hPBMCs. Twelve days post-transplant forceps were used to peel the graft from the kidney so as to reduce peripheral lymphocyte contamination giving us an accurate estimation of lymphocyte numbers within the grafts. Compared to the Adv-control grafts there was >60% reduction in the number of human CD45, CD4 and CD8 cells within the graft ($p<0.0001$, $p<0.0001$, $p=0.0290$ respectively; Fig. 6A). There was no conspicuous effect on systemic T cell numbers, as all recipients

(whether receiving Adv-control or Adv-diliximab transduced grafts) had similar numbers of human CD45, CD3 and CD19 cells in the spleen (Fig. 6B). Over 3 independent experiments the number of $CD45^+$ cells infiltrating control transduced grafts ranged from $3x10^4$-$2x10^5$ and the number of $CD45^+$ cells within spleens ranged from $4x10^6$-$2x10^7$.

Discussion

We studied the efficacy of locally produced anti-CD2 antibody, mediated by Adv-diliximab transduction of pNICC, to deplete human T cells. We initially generated three forms of CD2hb11 and determined that dilimomab and diliximab could inhibit a human to pig xenogeneic response but dilizumab could not. We also showed that the less immunogenic versions of CD2hb11 (diliximab and dilizumab) could function *in vivo* by depleting $CD3^+$ human T cells in a humanized mouse model. Adv-diliximab transduction of pNICC afforded depletion of human T cells locally at the graft site after transplantation compared to control transduced grafts.

Antibody humanization is designed to produce a molecule with minimum immunogenicity when applied to humans, while retaining the specificity and affinity of the parental non-human antibody (26). Commonly, affinity decreases after CDR grafting as a consequence of incompatibilities between non-human CDRs and human framework sequences. Accordingly, generating diliximab from dilimomab did not appear to affect Ab affinity but generation of dilizumab appears to have had a significant effect on its affinity. Further molecular modelling may be needed to prevent affinity losses if we are to pursue the least immunogenic version, dilizumab, to the clinic. Human IgG3 binds FcR and complement very efficiently whereas mouse IgG1 is weak in both regards (17). It is possible that diliximab may be a more efficacious immunosuppressant than dilimomab.

We have previously demonstrated proof-of-principle that local expression of anti-CD4 (6, 8) or CTLA4Ig (5) at the graft site affords long-term graft survival in an allograft model. There are numerous reports now showing that local administration of

immunomodulatory genes can protect grafts without demonstrable systemic immunosuppression. Whether this is done by gene delivery (27-30) or coating with recombinant protein (31) this approach leads to gains in graft viability. We have evidence of transient systemic expression of CTLA4Ig in the first 3 days after grafting Adv-CTLA4Ig transduced mouse islets (5). The Adv we used to transduce mouse islets and pNICC is replication defective, such that Adv infection is limited to the graft. Nevertheless, Adv-encoded diliximab is a secreted Ab and may also induce transient systemic immunosuppression. We avoided this possibility by injecting human cells into the transplanted mice several days after grafting so they were not depleted systemically. It should be noted that a short pulse of systemic immunosuppression may actually be beneficial clinically, as this would mimic anti-thymocyte globulin induction therapy (32).

Porcine NICC are an attractive source of insulin-producing tissue for transplantation (33, 34). Although pNICC do not produce sufficient insulin to immediately reverse hyperglycemia they have good proliferative and regenerative capacity and become mature islets after several weeks. We routinely culture pNICC for 6-9 days to enrich for endocrine cells before transduction (25) and transplantation. In order to test graft function the pNICC would need to undergo an *in vivo* maturation period, which is required for immature pNICC to develop physiological insulin secretion (34). Unfortunately, two issues preclude us from these longer-term studies. First, many of our hu-SCID mice develop graft-versus-host disease at about 6 weeks (35). Second, we have previously shown that transgene expression in surviving transduced allografts declines over time (5) so that transient local immunosuppression is then insufficient to overcome the strong xenogeneic response. However, our model does

allow for testing efficacy of potential immunomodulatory agents by enumerating T-cell infiltration within the graft. Thus, viral modification may be used to predict which immunosuppressive molecules should be used to generate transgenic pigs. Viral modification would also allow analysis of synergism of multiple graft-protecting molecules either using multiple viruses (25) or linked transgenes (21).

We have successfully used the furin/2A 'ribosome skip' signal to express the heavy and light chains of dilizumab and previously co-expressed several xenograft-protective proteins using this system (21). Simultaneous expression of multiple graft-protective molecules could not only be used to test synergistic efficacy in our model but represents a powerful method to rapidly generate genetically modified pigs.

The current demonstration that diliximab-expressing xenografts reduce T cell infiltration may represent a major step in overcoming the immunological barrier to islet xenotransplantation. Our study further highlights the potential for local immunosuppression to reduce the requirement for systemic immunosuppression.

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Author Contributions

JLB, RMS, MH, SK, MHL, PMP and WJH participated in data collection. JLB, RMS and AML participated in data analysis and drafting article. LCH, PJC, AJFd'A, PJO'C and AML participated in concept/design, critical revision of article and secured funding. All authors approved article.

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Figure Legends

Figure 1 Schematic diagram illustrating the 3 versions of CD2hb11 generated: the mouse mAb dilimomab, the chimeric diliximab and the humanized dilizumab. The original mouse sequences are shown in grey and human sequences in white.

Figure 2 Titration of Abs binding to $CD3^+$ T cells of human PBMCs. A) Dilimomab binding was detected using goat anti-mouse IgG. B) Diliximab and dilizumab binding was detected using mouse anti-human IgG3 and could be directly compared. Diliximab shows a 300-fold higher avidity when compared to dilizumab.

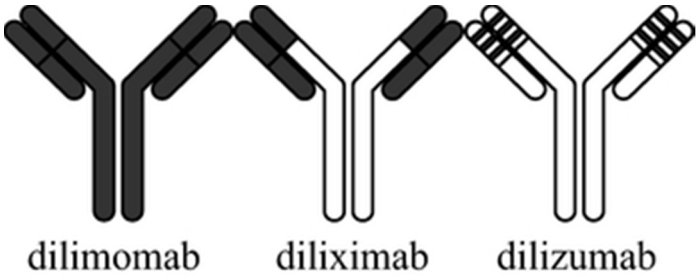
Figure 3 A) Inhibition of xenogeneic MLR by CD2hb11 Abs. Irradiated pig splenocytes were added to human PBMCs. CD2hb11 Abs or IgG control were added at different concentrations (0.5-50 μg mL^{-1}) at the beginning of the 7-day culture. Proliferation was measured by ^{3}H-thymidine incorporation for the last 16 h of culture. Data represent the mean +/- SE of three replicates per group. B) Depletion of lymphocytes by CD2hb11 Abs. Hu-SCID mice were injected with 0.5 mg CD2hb11 Abs or IgG control. Numbers of human T cells in the spleens were quantified 7 d later. Data represent the mean +/- SD of two mice per group.

Figure 4 Measurement of pro-inflammatory cytokines and activation markers. Hu-SCID mice were injected with OKT3, CD2hb11 Abs or IgG controls. Mice were bled at 2, 5 and 24 h. A) kinetics of human plasma IFN-γ and IL-10 release in hu-SCID mice treated with OKT3. B) Release of human plasma cytokines in hu-SCID mice 5 h after antibody injection. Data represent the mean +/- SEM of three mice per group.

Sensitivity (limit of detection) of analytes is represented by dashed lines. C) Activation markers on human $CD3^+$ T cells in the spleen of hu-SCID mice 24 h after antibody treatment. Histograms show a representative plot from each treatment group. Only OKT3 treated mice (bold line) show upregulation of CD25 and CD69.

Figure 5 Transduced pNICC secrete diliximab. A) FACS analysis of supernatants from Adv-diliximab or Adv-control transduced pNICC (Multiplicity-of-infection=10) cultured for 24 h *in vitro*. Human PBMCs were incubated in the presence of transduced supernatants. Fluorochrome-conjugated anti-human IgG3 on $CD3^+$ cells detected bound diliximab. B) hIgG3 was detected by ELISA in supernatants from Adv-diliximab or Adv-control transduced pNICC cultured for 24 h *in vitro*.

Figure 6 Deletion of T cells in pNICC grafts is local. A) A significant reduction in the number of human CD45, CD4 and CD8 cells was observed within grafts expressing Adv-diliximab. B) All hu-SCID mice had similar numbers of human T and B cells in the spleen. Data are expressed as mean +/- SEM of three combined experiments; 100% recovery represents the mean of $CD45^+$ cells recovered from control treated mice per experiment. *** $p<0.0001$, *$p=0.0290$ compared to controls.



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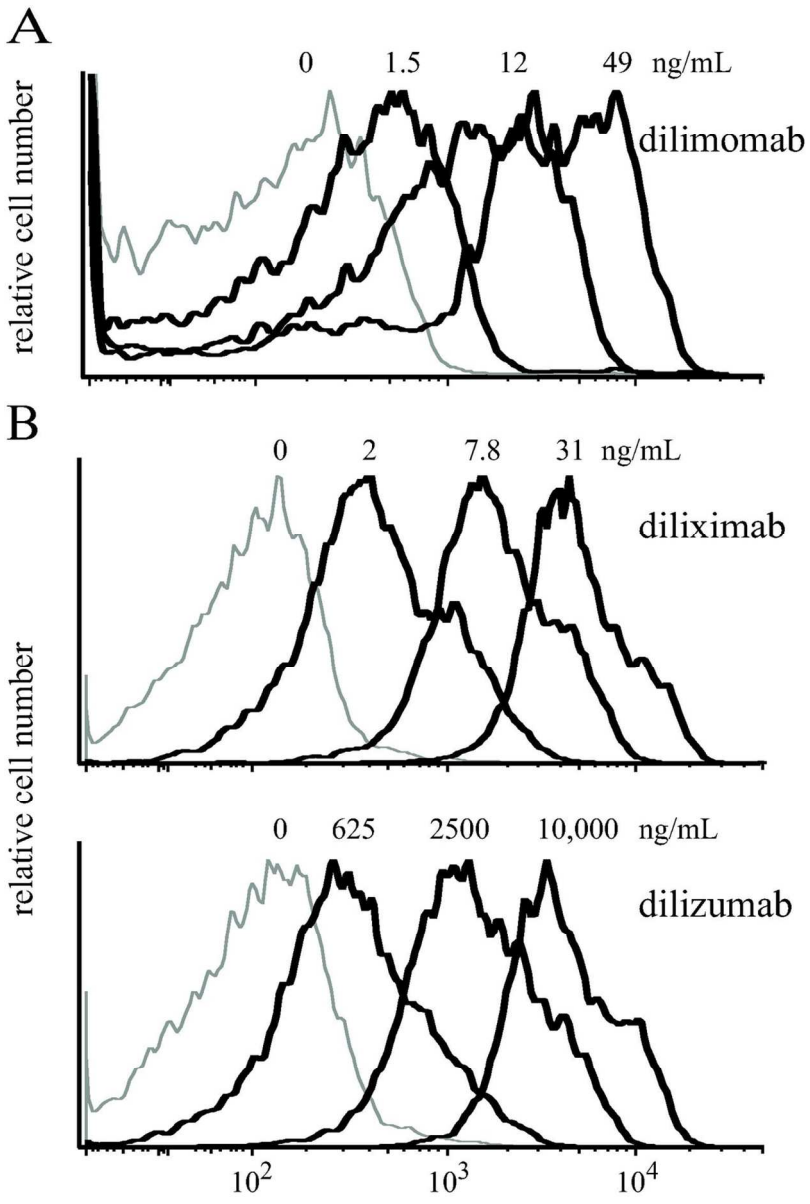

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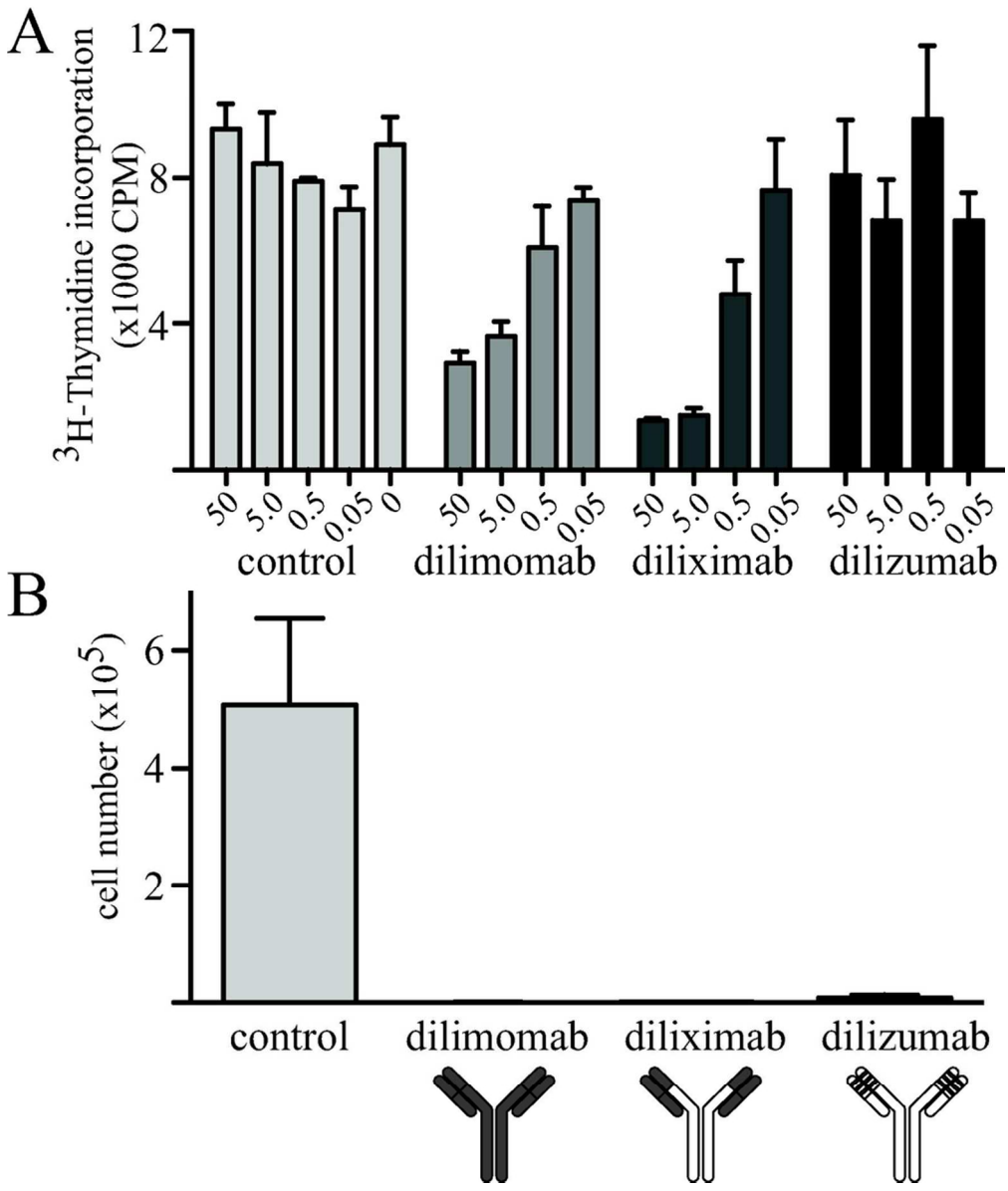

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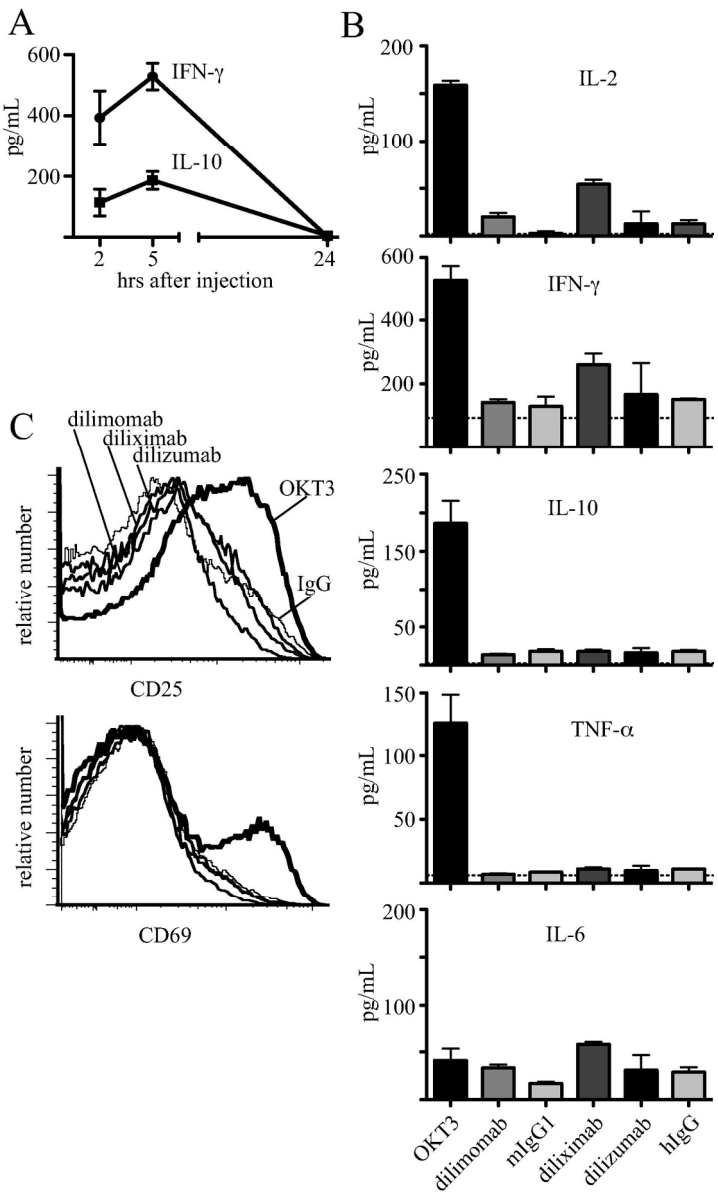

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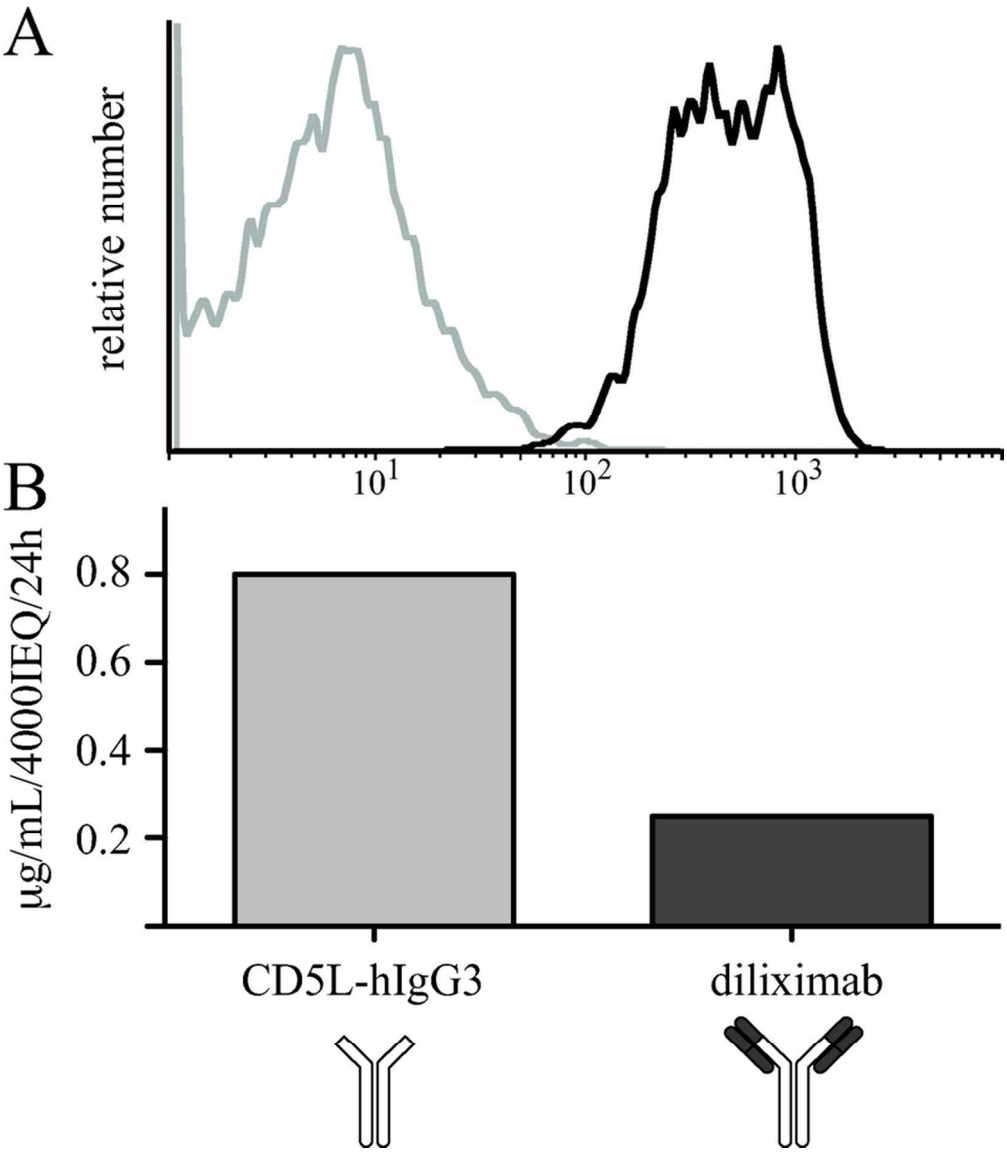

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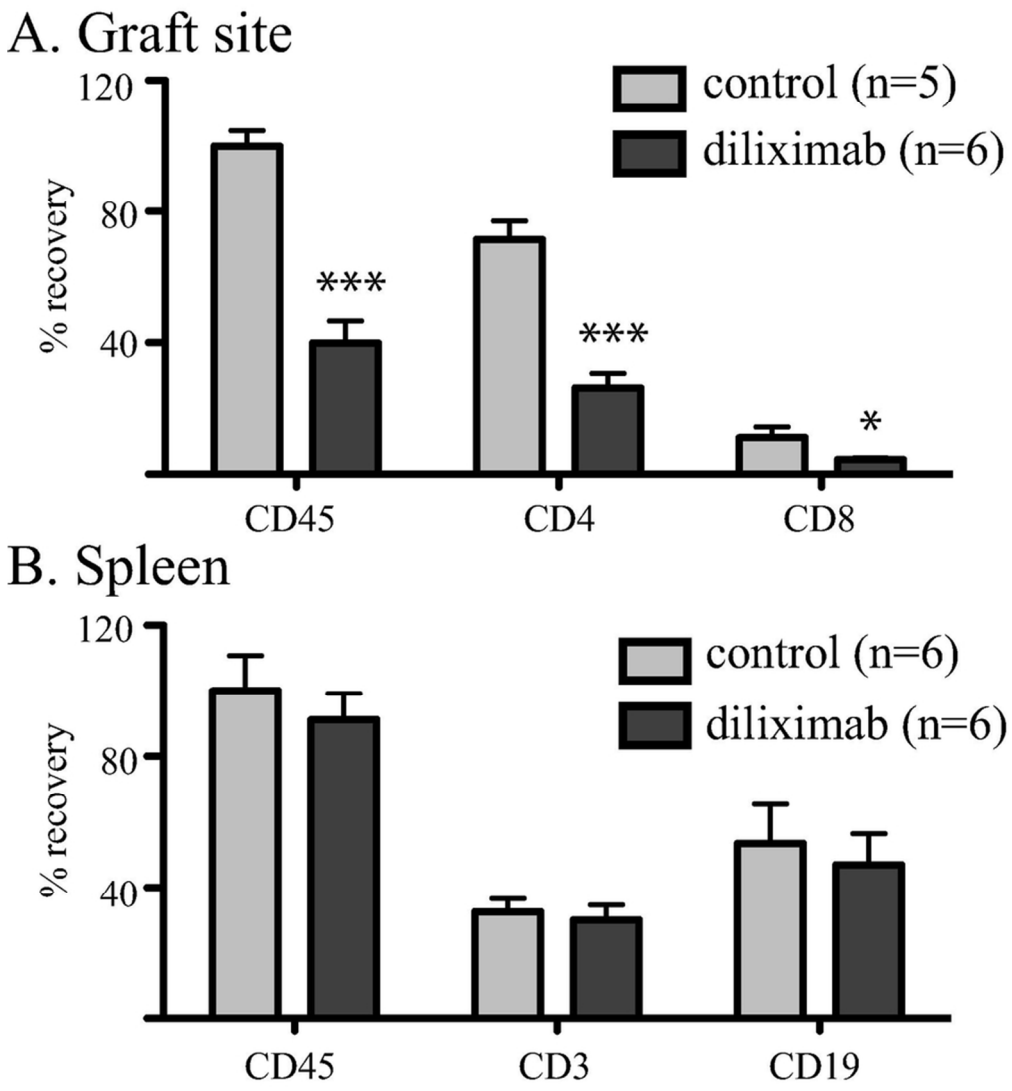

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