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A therapeutic DNA vaccination strategy for autoimmunity and transplantation

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ABSTRACT

De novo autoimmunity induced by an allograft may play a significant role in chronic organ rejection, which remains a major barrier to successful transplantation. Accordingly, immunization with non-polymorphic antigens found in both donor allograft and recipient would be an attractive means to prevent long-term graft rejection, because it would rely on recipient mechanisms of immune homeostasis and could minimize the need to identify appropriate donor polymorphic antigens for induction of graft tolerance. Here we show that intradermal injection of plasmid DNA encoding glutamic acid decarboxylase (GAD) polypeptide, which is synthesized in both pancreatic islet and skin tissue, ameliorated new-onset type 1 diabetes in NOD mice and increased skin allograft survival in a BALB/c-C57BL/6 model system in a donor-specific manner. Successful therapy of autoimmune diabetes required CpG-methylation of plasmid DNA and co-delivery of a cDNA coding for the pro-apoptotic BAX protein, which was shown previously to induce Foxp3$^+$ regulatory T cells in NOD mice. In contrast, significantly increased skin allograft survival after immunization of recipient only required CpG-methylation of plasmid DNA coding for GAD alone. Injection of unmethylated plasmid DNA coding for BAX alone near the allograft also promoted graft survival, but induced a pro-inflammatory response to self-antigens. Our results reveal a promising potential for autoimmunity-targeting DNA vaccination to be applied to transplantation.

1. Introduction

Polymorphic antigens carried by an allograft, i.e., major and minor histocompatibility complex molecules, are firmly established as mediators and targets of transplant rejection. The impact of alloimmunity is particularly apparent in acute rejection, where a strong response by recipient CD4$^+$ and CD8$^+$ T lymphocytes activated by both donor and recipient leukocytes that process donor antigens can eliminate the allograft [1]. Nevertheless, evidence indicates that an autoimmune response induced by transplantation can also target allotransplants [2]. *De novo* autoimmunity after organ transplantation could be particularly significant in chronic immune rejection [3,4], which remains a major cause of long-term allograft failure [5].

Evidence for transplant-induced autoimmunity and its possible role in allograft rejection comes from both clinical and animal studies, where different autoantigens have been identified in different transplantation settings. For example, antibodies and T lymphocytes reactive to the intermediate filament vimentin have been detected in human recipients of cardiac and kidney allografts [6,7], and preimmunization of recipient mice with vimentin in complete Freund's adjuvant (CFA) accelerates rejection of heart allografts [8]. Similarly, heart allograft transplantation induces an autoimmune response to cardiac myosin in mice, and preimmunization of recipient animals with the autoantigen in CFA accelerates rejection [9]. In human lung transplant recipients, T lymphocyte-mediated autoimmunity to type V collagen (col(V)) is a significant risk of chronic rejection [10], and patients with higher levels of preformed anti-col(V) antibodies have increased incidence of lung allograft dysfunction [11]. Stress proteins are another example of autoantigens possibly associated with transplant rejection, as indicated by the presence of graft-infiltrating T lymphocytes specific for heat-shock proteins in both a rat cardiac allograft model of chronic rejection and human kidney recipients [12,13].

The strongest evidence for a role of pathological autoimmunity in transplant rejection comes from animal experiments where immunization of recipient with an autoantigen synthesized in the allograft increases its survival. For example, in the rat lung transplant model, oral feeding of human col(V) before transplantation

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prevents both acute and chronic rejection, and up regulates TGF-β systemically [14,15]. In another example, intra-peritoneal injection prior to transplantation of cardiac myosin isolated from recipient prolongs survival of MHC-I-mismatched, allogeneic heart transplant in mice in an IL-4-dependent manner [16].

In the work presented here, we investigated whether therapeutic DNA vaccines coding for GAD, an antigen synthesized in both pancreatic beta cells and skin tissue [17–19], would successfully treat autoimmune (type 1) diabetes and increase allograft survival after skin transplantation which has been reported to induce an autoimmune response in mice [20]. NOD mice with new-onset type 1 diabetes and C57BL/6 mice receiving full-haplotype mismatched skin transplant were immunized with plasmid DNA coding for GAD and the pro-apoptotic protein BAX, which we have shown previously to be necessary for amelioration of diabetes [21]. In addition, we investigated whether CpG-methylation of plasmid DNA would enhance the therapeutic effects of the tolerogenic vaccines. Results indicated that CpG-methylation of GAD-encoding plasmid DNA enhanced therapeutic efficacy in both model systems and confirmed that co-delivery of BAX cDNA is necessary for successful therapy of type 1 diabetes. In contrast, co-delivery of BAX cDNA was not required for increased survival of allografts, although injection of higher amounts of plasmid DNA coding for BAX alone could prolong survival of skin grafts. Our results demonstrate for the first time that the same DNA vaccination strategy can be successfully applied to stringent models of pathological autoimmunity and allotransplantation.

2. Materials and methods

2.1. Plasmid DNA methylation, amplification, and injection

Plasmid pND2-BAX carries a *bax* cDNA under transcriptional control of the CMV promoter, and plasmid pSG5-GAD carries a cDNA construct encoding a secreted form of human GAD65 under transcriptional control of the SV-40 promoter in vector pSG5 (Stratagene, San Diego, CA). Plasmid pSG5-GAD was CpG-methylated in *Escherichia coli* strain ER1821 synthesizing the *Sss*I methylase (New England BioLabs, Ipswich, MA). Methylation of plasmid DNA was confirmed by observing increased resistance to digestion with restriction endonuclease *Hpa*II using agarose gel electrophoresis. Plasmid DNA was isolated after amplification in *E. coli* strain DH5α and ER1821 using the Endofree Plasmid DNA Purification Kit (Qiagen, Valencia, CA). Mice were anesthetized prior to DNA injection with ketamine (66 mg/kg body weight, Phoenix Scientific, St. Joseph, MO) and xylazine (7.5 mg/kg body weight, Lloyd Laboratories, Shenandoah, IA). Animals received intradermal (i.d.) injection of plasmid DNA into skin using a tuberculin syringe fitted with a 27-gauge needle. Mice were cared for in the specific pathogen-free animal facilities of the Loma Linda University Medical Center following NIH guidelines for principles of laboratory animal care.

2.2. Diabetes study

Female NOD/Tac mice (Taconic Farms, Germantown, NY) received 8 weekly i.d. injections of 50 μg of different plasmid DNA mixes (40 μg SV-40-based plasmid DNA + 10 μg CMV-based plasmid DNA) starting at time of early diabetes onset (fasting blood glucose (FBG) > 140 mg/dL). Fasting blood glucose was monitored bi-weekly using ACCU-CHEK Advantage (Roche Diagnostics, Indianapolis, IN). Mice were killed when FBG > 300 mg/dL and positive for fasting glycosuria, or at the experiment end-point when 40-week-old.

2.3. Skin transplant study

C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H (H-2^k) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and used for transplant when 7–8-week-old. 0.7 × 0.7 cm full-thickness back skin grafts from BALB/c donor or C3H 3rd party were transplanted onto the back of C57BL/6 recipients (8–14 mice/group). ALG (1.6 mg/20 g BW, Fitzgerald Industries Intl, Concord, MA) was given i.p. once on day 0. Plasmid DNA (50 μg) was injected i.d. 0.5 cm from the skin graft on day 0, 3, 7, and then weekly. Rapamycin (1 mg/kg, Wyeth Pharmaceuticals, Madison, NJ) was injected i.p. daily from days 0–27. Bandages were removed on day 10, and skin graft rejection was defined as 85% loss of the graft area, and confirmed by histopathological analysis.

2.4. RNA isolation and qPCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) from freshly taken skin allograft and recipient cervical and axillary lymph nodes (LNs) 2 weeks after transplantation. RNA was also isolated from dispersed LNs cultured for 3 days with mitomycin C-treated splenocytes from C57BL/6, BALB/c, and C3H mice. Quantitative PCR was performed using the iCycler system and SYBR green (BioRad, Hercules, CA) with 200 ng of total RNA as template, and primers specific for the chosen cDNAs, and for the GAPDH cDNA as a housekeeping gene.

2.5. CD4CD25 T cell suppressive assay

Draining LNs from recipient C57BL/6 mice taken 2 weeks after skin transplant were dispersed and stimulated with mitomycin C-treated splenocytes from C57BL/6, BALB/c, and C3H mice for 3 days. $CD4^+CD25^+$ cells were then isolated from culture with immunomagnetic beads (Stemcell Technologies, Vancouver, Canada), and mixed with APCs and 2 μM CFSE labeled $CD4^+CD25^-$ T cells isolated from spleen of untreated C57BL/6 mice, and anti-CD3/CD28 antibodies for an additional 5 days. PE-conjugated Anti-CD4, and PI were used to label cells, and flow cytometry (BD, San Diego, CA) was used to exclude background levels. The suppressive activity of Tregs towards responder cells in coculture with different Tresp–Treg ratio was expressed as the relative inhibition of the percentage of $CFSE^{low}$ cells [100 × (1 − %$CFSE^{low}$ $CD4^+CD25^-$ T cells in coculture/% $CFSE^{low}$ CD4 + $CD25^-$ T cells alone)] for CFSE based measurement of proliferation [22].

2.6. MLR

Draining LNs from recipient C57BL/6 mice taken 2 weeks after skin transplant were labeled with 2 μM CFSE and stimulated with mitomycin C-treated splenocytes from C57BL/6, BALB/c, and C3H mice for 4 days with 2 or 5 μg/ml rhIL-2 (R&D Systems, Minneapolis, MN). Proliferation was quantified based on loss of CFSE fluorescence, and cells were gated by PE-conjugated anti-CD3 and PI, and detected by flow cytometry.

2.7. Adoptive cell transfer

Cells from spleen and LNs of recipient mice were isolated 2 weeks after transplantation, pooled, and suspended in PBS. 5×10^6 total cells were injected i.p. into new recipients at day 2, and 3 Gy TBI irradiation was given on day 3. Donor or third party skin grafts were transplanted on day 0, and rapamycin (1 mg/kg) was given i.p. daily.

2.8. Statistical analysis

Diabetes onset and skin graft survival were analyzed with Kaplan–Meier and Mann–Whitney analyses and other results were analyzed with one-way ANOVA and Mann–Whitney with $P \leq 0.05$ considered significant.

3. Results

3.1. Co-delivery of GAD and BAX plasmid DNA and CpG-methylation act synergistically for successful therapy of type 1 diabetes in NOD mice

We previously reported that i.d. injection of unmethylated plasmid DNA carrying a single transcriptional unit under CMV promoter control and encoding both GAD and BAX ameliorates new-onset diabetes in 60% of NOD mice at 40 weeks of age [23]. Here, we investigated the effects of CpG-methylation of plasmid DNA on vaccine efficacy using a 2-plasmid system, where the *gad* and *bax* cDNAs are under transcriptional control of the SV-40 and CMV promoters, respectively. A 2-plasmid system was used so that CpG-methylation of DNA, which down regulates SV-40 and inactivates the CMV promoters [24,25], would not affect levels of apoptosis induced by BAX. Therefore, only plasmid DNA coding for GAD was CpG-methylated in our experiments (mGAD vaccine). Results showed that neither CpG-methylation of GAD plasmid DNA alone (Fig. 1D), CpG-methylation of vector plasmid DNA and co-delivery of BAX (Fig. 1E), or delivery of unmethylated plasmid DNA coding for GAD and BAX (Fig. 1F) could successfully treat spontaneous diabetes in NOD mice. In sharp contrast, CpG-methylation of plasmid DNA coding for GAD together with co-delivery of unmethylated BAX plasmid DNA (mGAD + BAX vaccine) acted synergistically to ameliorate new-onset diabetes in 80% of mice at 40 weeks of age (Fig. 1G).

3.2. Plasmid DNA encoding GAD or BAX and CpG-methylation increase BALB/c skin allograft survival in C57BL/6 recipient mice

The DNA vaccines used for therapy of autoimmune diabetes were investigated for efficacy on survival of BALB/c skin allograft in fully mismatched C57BL/6 recipient mice. In addition, recipient mice received minimum immunosuppression to control acute rejection. Because GAD is synthesized in skin, we hypothesized that GAD-encoding DNA vaccines that could successfully treat type 1 diabetes would also increase skin allograft survival by suppressing graft-induced autoimmunity. Fig. 2A shows that injection of unmethylated plasmid DNA coding for GAD alone, BAX alone, or both GAD and BAX into recipient near the allograft increased skin survival, although only injection of plasmid DNA coding for BAX alone was significant compared to non-immunized mice and mice receiving vector alone.

Similarly to the diabetes study, CpG-methylation of plasmid DNA coding for GAD resulted in significantly increased allograft survival compared to mice immunized with the unmethylated GAD vaccines and CpG-methylated vector control (Fig. 2B). However, in contrast with results obtained with NOD mice, co-delivery of 10 μg of unmethylated plasmid DNA encoding BAX in addition to 40 μg of CpG-methylated plasmid DNA coding for GAD (mGAD + BAX) did not improve graft survival.

3.3. The BAX and mGAD + BAX DNA vaccines induce distinct immune responses in C57BL/6 recipient mice

Co-delivery of 10 μg of unmethylated BAX plasmid DNA did not improve efficacy of the mGAD vaccine for skin transplantation, but injection of 50 μg of the same BAX vaccine significantly increased allograft survival, suggesting a dose-dependent effect of BAX. Accordingly, we investigated the effects of DNA vaccines consisting of 50 μg BAX and 40 μg mGAD + 10 μg BAX plasmid DNA on expression of chosen immune genes in skin allografts and LNs freshly taken from recipient C57BL/6 mice. In addition to genes coding for the anti-inflammatory IL-4, IL-10, and TGF-β1 cytokines and the pro-inflammatory TNF-α and IFN-γ cytokines, we quantified the expression of genes coding for the co-stimulatory molecules CD80 and CD86 which are upregulated in immunogenic APCs [26], the transcriptional factor FOXP3 which is synthesized by $CD4^+CD25^+$ regulatory T cells (Tregs) [27], and the inhibitory receptor FcγRIIB and IL-1 receptor antagonist IL-1RA which are up regulated in murine tolerogenic DCs [28–30].

Results indicated that skin allograft from mice immunized with BAX alone showed modest increased expression of the *Tgf-β1*, *Tnf-α*, and *FcγrIIb* genes. In contrast, skin transplants from mice immunized with mGAD + BAX showed marked increased expression of the *Il-4* and *FcγrIIb* genes, and decreased expression of the *Foxp3*, *Tnf-α*, *Ifn-γ*, and *Il-10* genes compared to immunosuppressed controls (Fig. 3A). The different immune responses induced by the two vaccines were also observable in LNs, where mice immunized with BAX alone showed increased expression of the *Foxp3*, *Il-10*, and *Tgf-β1* genes, and drastically increased expression of the *Cd86* gene, while mice immunized with mGAD + BAX showed a marked decreased expression of the *Tnf-α* and *Ifn-γ* genes (Fig. 3B). Notably, the BAX and mGAD + BAX vaccines induced increased expression of *Cd86* that was proportional to the amount of BAX plasmid DNA delivered, with no significant change in the expression of the *Cd80* gene (data not shown).

Analysis of gene expression in recipient LN cells cultured with self, donor, or 3rd party antigens also revealed significantly different immune responses between mice immunized with BAX or mGAD + BAX (Fig. 4). In particular, injection of BAX alone induced increased expression of the pro-inflammatory cytokines TNF-α and IFN-γ in response to self-antigens, and the increased expression of the *Foxp3*, *Il-10*, and *Tgf-β1* genes observed in freshly isolated LNs of mice immunized with BAX alone was recapitulated in cultured LNs stimulated with 3rd party antigens (Fig. 4A). Induction of Tregs by BAX alone was indicated by results from immunosuppressive assays, which showed that $CD4^+CD25^+$ T cells isolated from LNs of recipient mice immunized with BAX alone and stimulated with 3rd party antigens had significantly higher suppressive activity (Fig. 5B).

3.4. Immune responses induced by the BAX and mGAD + BAX DNA vaccines in C57BL/6 recipient mice are donor-specific

Two lines of evidence indicated that the BAX and mGAD + BAX vaccines induced donor-specific immune responses. First, gene expression in LN cells from mice immunized with BAX alone or mGAD + BAX was markedly different when stimulated with donor antigens from that observed after 3rd party antigen stimulation (Fig. 4). Among the 9 genes under study, 8 and 5 genes were differentially expressed in, respectively, BAX- and mGAD + BAX-immunized mice with the two different stimulations, which indicated a donor-specific response. In addition, gene expression after donor antigen stimulation was closer to gene expression after self-antigen stimulation than after 3rd party antigen stimulation.

Second, detection of T cell proliferation from MLR using CSFE-labeled LN cells stimulated with antigens from self, donor, or 3rd party indicated a donor-specific suppression of T cell proliferation induced by the BAX and mGAD + BAX vaccines (Fig. 6). In contrast, T cells from LNs of non-vaccinated mice did not show a significant difference in proliferation when stimulated with donor or 3rd party antigens. These data suggested that allospecific T cells from immunized mice did not undergo significant expansion compared

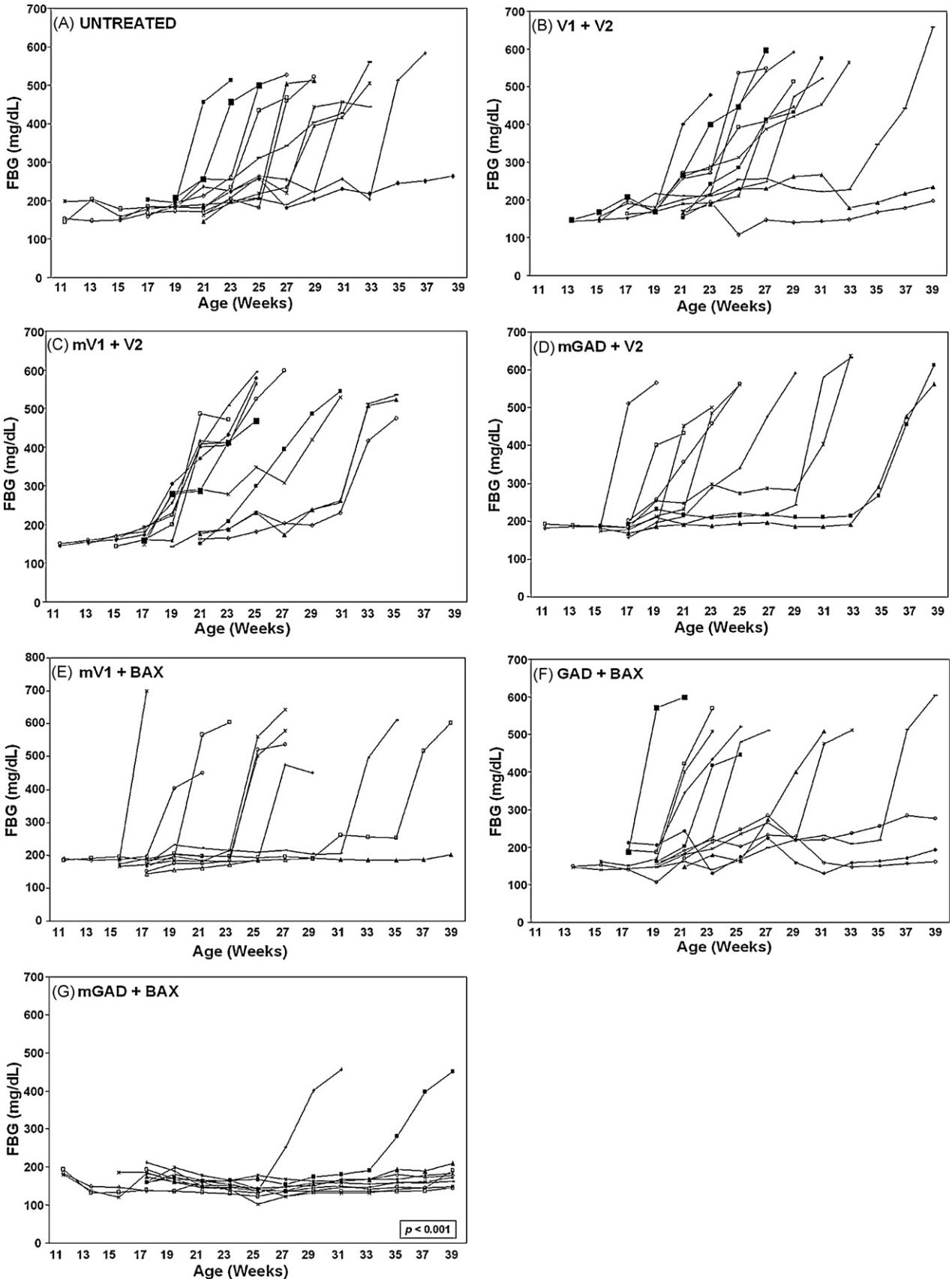

Fig. 1. DNA vaccination suppresses new-onset type 1 diabetes. Female NOD mice ($N = 10–12$/group) were monitored for diabetes onset individually from 8 to 40 weeks of age. Mice received i.d. injection of 40 μg of vector pSG5 unmethylated (V1), CpG-methylated (mV1), unmethylated coding for GAD (GAD), and CpG-methylated coding for GAD (mGAD). In addition, mice received co-delivery of 10 μg of unmethylated vector pND2 (V2) or encoding BAX (BAX). Mice were immunized at time of early new-onset diabetes (fasting blood glucose (FBG) > 140 mg/dL) for 8 weeks (50 μg/week). The first of the data points for each curve corresponds to the time of the first DNA injection for treated animals. For Group G, $P < 0.001$ compared to all other groups.

to non-vaccinated T cell and the same T cell under unrelated antigen stimulation. Since classical anergy of T cell proliferation responding to antigen can be overcome with exogenous IL-2 [31], concentration of rIL-2 was increased from 2 to 5 μg/ml. Results showed that T cell expansion specific to donor antigen could not be restored by increased exogenous IL-2, suggesting that T cell tolerance to donor antigen was unlikely to be the result of IL-2-restorable anergy.

3.5. The mGAD + BAX DNA vaccine induces donor-specific allograft tolerance

Adoptive transfer of pooled splenocytes and LN cells from immunized recipient mice into naïve mice receiving donor or 3rd party skin allograft under immunosuppression showed that only cells from mice immunized with the mGAD + BAX vaccine could transfer significantly increased survival of donor allograft compared to 3rd party (Fig. 7). Results from immunosuppressive assays supported the notion of donor-specific immunosuppression after immunization with the mGAD + BAX vaccine, and suggested that at least some of these cells could have been $CD4^+CD25^+$ T cells (Fig. 5). Although not significant, cells from mice receiving rapamycin without immunization also appeared to show donor-specificity (Fig. 7), which may have been the result of alloantigen-specific Tregs induced by rapamycin [32].

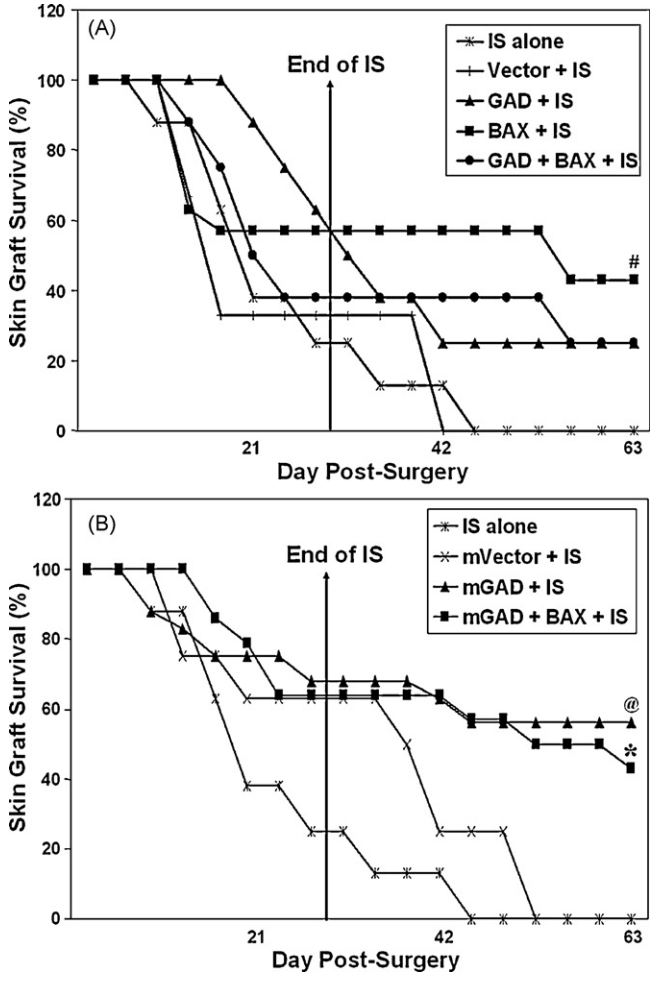

Fig. 2. DNA vaccination prolongs fully mismatched skin allograft survival. 7-week-old, age matched C57BL/6 recipients (N = 8–14/group) received skin grafts from BALB/c donor under minimum immune suppression regimen (IS) that was ended on day 28, and received a weekly i.d. injection of 50 μg of the indicated vaccine. The BAX, GAD, and GAD + BAX vaccines are unmethylated plasmid DNA coding for BAX, GAD, and both GAD and BAX. The mGAD DNA vaccine consists of CpG-methylated plasmid DNA coding for GAD alone. The mGAD + BAX vaccine consists of the 4:1 ratio of mGAD and BAX plasmid DNA that was therapeutic in NOD mice. (A and B) Show allograft survival in mice immunized with the unmethylated and methylated DNA vaccines, respectively. In (A) (#) $P < 0.01$ compared to vector + IS and $P < 0.05$ compared to GAD + BAX + IS. In (B) (@) $P < 0.002$ compared to mVector + IS and $P < 0.05$ compared to GAD + IS; (*) $P < 0.04$ compared to GAD + BAX + IS and mVector + IS. With the exception of Vector + IS and mVector + IS, survival was significant for all groups compared to IS alone.

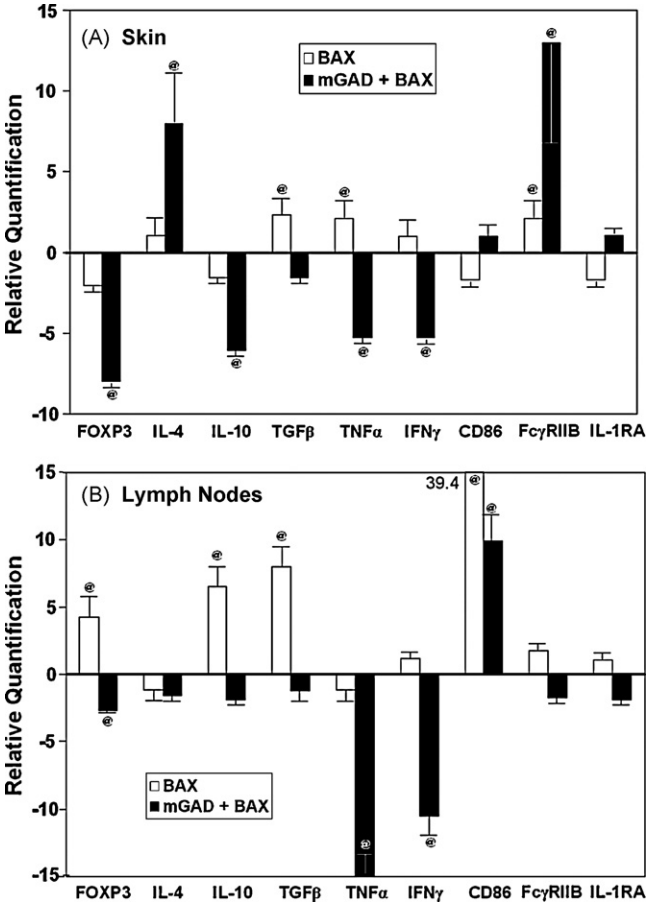

Fig. 3. Different DNA vaccines induce different immune gene expression in fresh skingraft and LNs. C57BL/6 mice (N = 10) under minimum immunosuppression (IS) received BALB/c skingrafts and were immunized with the BAX or mGAD-BAX DNA vaccine. Fresh skin allografts (A) and fresh LNs (B) were taken 2 weeks after transplant for qPCR analysis. Quantification of mRNA levels is relative to IS controls (value of 1, not shown). (@) $P < 0.05$ compared to IS control.

4. Discussion

A body of clinical and experimental evidence indicates that allotransplantation induces an autoimmune response that contributes to graft rejection [2,4]. Transplant-induced, *de novo* pathological autoimmunity is suspected to play a significant role in chronic organ rejection, which is not controlled with current immunosuppressants and remains a major obstacle to lifetime engraftment of allotransplants [5]. Chronic immune rejection is mainly mediated by antigen-presenting cells (APCs) from recipient, which also appear to be crucial for its prevention through induction of Tregs [33,34]. Therefore, immunization to induce long-term allograft tolerance will most likely have to rely on recipient leukocytes that are already attuned to maintaining homeostatic tolerance to self-antigens, which is a context where immunization with autoantigens could be particularly relevant. In the work presented here, we investigated whether a DNA vaccine encoding GAD polypeptide, which is synthesized in both pancreatic islet and skin

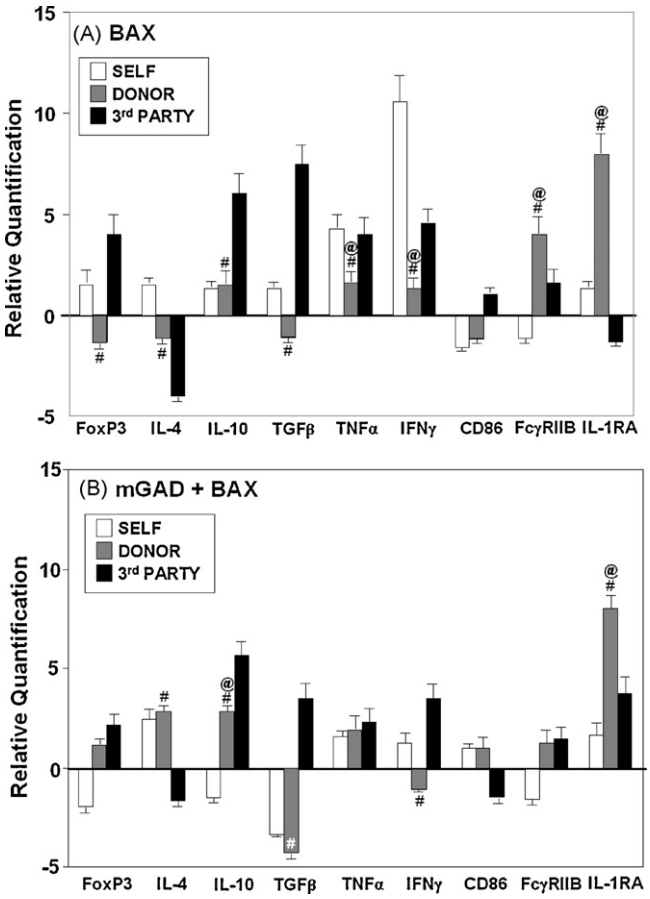

Fig. 4. Different antigen stimulations induce different expression of immune genes in cultured LNs. Draining lymph nodes from 2-week skingrafted and vaccinated recipient C57BL/6 mice ($N = 10$) were stimulated with self, donor (BALB/c), and 3rd party (C3H) antigens, and total RNA was isolated for real time PCR after 3-day culture. Quantification of mRNA levels is relative to IS controls (value of 1, not shown). (@ and #) $P < 0.05$ compared to cells stimulated with self and 3rd party antigens, respectively.

tissue, could both treat new-onset autoimmune diabetes in NOD mice and prevent rejection of a fully mismatched skin allograft.

The DNA vaccination strategy that we have previously developed for type 1 diabetes is based on induction of apoptotic cells, which are constantly processed in steady state by APCs to maintain peripheral tolerance in mammals [35]. Our previous studies indicate that injection into NOD mice of a single plasmid DNA construct carrying a transcriptional unit coding for both GAD antigen and the pro-apoptotic BAX protein recruits dendritic cells (DCs), activates Foxp3$^+$ regulatory T cells, and ameliorates new-onset diabetes [21,23]. Here, we investigated whether CpG-methylation of plasmid DNA would improve efficacy of treatment using a 2-plasmid vaccine, as unmethylated CpG-dinucleotides in bacterial DNA are known to be pro-inflammatory [36]. Results indicated that CpG-methylation of plasmid DNA coding for GAD and co-delivery of unmethylated plasmid DNA coding for BAX were required to ameliorate diabetes. The finding that the unmethylated, 2-plasmid DNA vaccine coding for GAD and BAX did not successfully treat diabetes was in contrast with our previous results. A similar loss of activity for a 2-plasmid DNA vaccine encoding a mutant pro-apoptotic caspase and a chosen antigen compared to its 1-plasmid DNA vaccine equivalent has been reported [37]. Regardless of the reasons for the observed lack of efficacy of the unmethylated 2-plasmid DNA vaccine, the fact that CpG-methylation could restore therapeutic activity for type 1 diabetes was strong evidence of its beneficial effect.

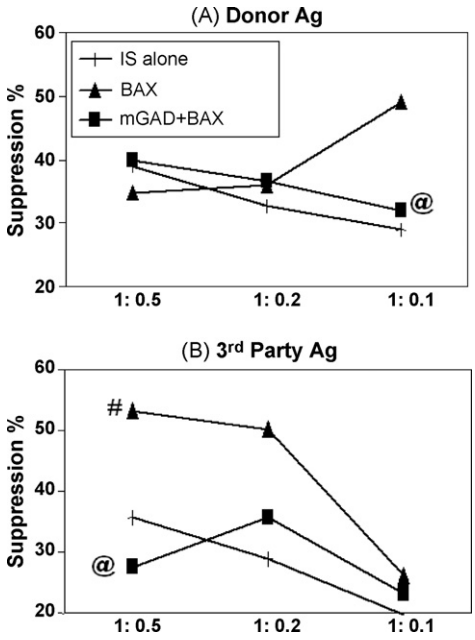

Fig. 5. DNA vaccination induces immunosuppressive CD4$^+$CD25$^+$ T cells. CD4$^+$CD25$^+$ T cells were isolated from draining LNs stimulated with splenocyte antigens from donor and 3rd party and co-cultured with APCs and different ratios of CFSE labeled CD4$^+$CD25$^-$ T cells from untreated C57BL/6 mice in presence of anti-CD3/CD28 antibodies. CFSE-based proliferation was measured and calculated with flow cytometric analysis gated by anti-CD4-PE and PI. (@) $P < 0.03$, (#) $P < 0.03$ for 1:0.5 and 1:0.2 compared to mGAD + BAX and IS alone.

Since allotransplantation may induce autoimmunity and GAD is synthesized in skin, we hypothesized that the DNA vaccine therapeutic for type 1 diabetes would also prevent skin transplant rejection. Translating the vaccination strategy to skin transplantation revealed that it increased skin allograft survival and confirmed

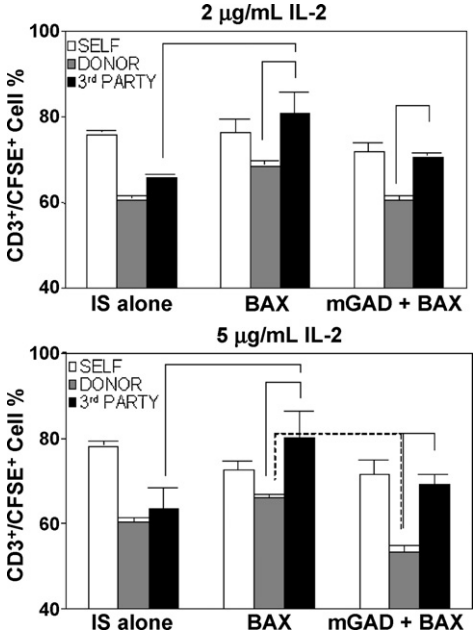

Fig. 6. DNA vaccination induces a donor-specific response and addition of exogenous IL-2 does not restore T cell proliferation. LN cells were isolated from 2-week skingrafted and vaccinated recipients, and 4×10^5 CFSE labeled LN cells were mixed with 4×10^5 splenocyte antigens from self, donor or 3rd party in presence of 2 or 5 μg/ml rhIL-2. Cells were analyzed with flow cytometric analysis gated with anti-CD3-PE, PI, and CFSE. Connecting lines indicate $P < 0.05$.

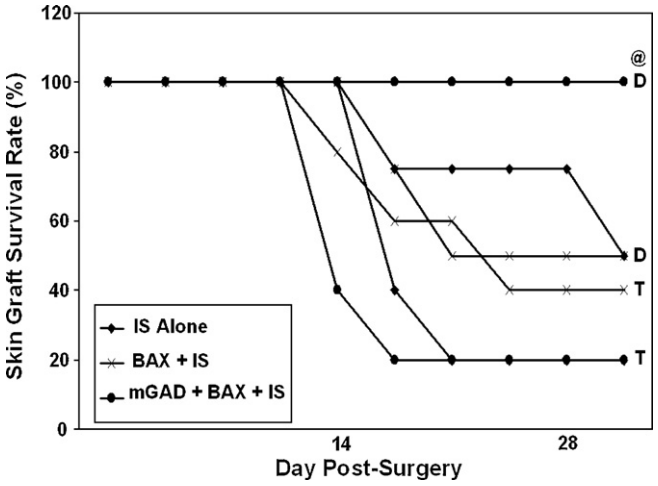

Fig. 7. The mGAD + BAX DNA vaccine induces donor-specific tolerance. Adoptive transfer donor (C57BL/6) received BALB/c skingraft with vaccination and minimum immunosuppression. Splenocytes and draining LN cells were isolated on day 14, and injected i.p. on day 2 into adoptive transfer recipients (C57BL/6, $N = 4–5$) receiving donor (BALB/c, D) or third party (C3H, T) skin grafts at day 0. The recipients also received 3 Gy irradiation at day 3, and daily rapamycin i.p. (@) $P < 0.05$ compared to third party.

the important role of CpG-methylation in promoting therapeutic efficacy. However, in contrast with diabetes in NOD mice, co-delivery of 10 μg of unmethylated BAX plasmid DNA was not required for efficacy. A possible explanation for this difference is that the site of DNA vaccination (skin) relative to the site of inflammation (islets for diabetes and skin for transplantation) was distal for diabetes and superimposed for transplantation. Co-delivery of plasmid DNA encoding a pro-apoptotic protein induces apoptotic cells that recruit DCs to draining lymph nodes, which is likely an important factor for improved efficacy of immunization [21,38]. Co-injecting 10 μg of plasmid DNA coding for BAX directly into the site of inflammation may have been superfluous, because inflamed tissues are known to recruit DCs [39]. Nevertheless, injection of 50 μg of plasmid DNA encoding BAX alone did result in increased graft survival, suggesting a dose-dependent effect of BAX. Accordingly, we investigated the immune responses induced by the mGAD + BAX and BAX DNA vaccines which contained 10 and 50 μg, respectively, of the same plasmid DNA coding for BAX.

Gene expression analysis of freshly isolated skin allograft and LNs from recipient mice indicated that the two vaccines induced immune responses that were distinct both qualitatively and quantitatively. In particular, the vaccines induced high expression levels of different tolerance-associated genes in the different tissues. In skin allografts, the mGAD + BAX vaccine induced expression of the anti-inflammatory *Il-4* gene and of the gene coding for inhibitory FcγRIIB receptor, which is up regulated in immature and tolerogenic DCs [29,30]. Increased levels of IL-4 have been previously observed in sera of mice receiving heart transplant after immunization with cardiac myosin [16], and suggested a Th-2 like activity that could have resulted in the downregulation of the *Tnf-α* and *Ifn-γ* genes which we observed in both skin allograft and LNs of recipient C57BL/6 mice immunized with mGAD + BAX.

In contrast, the effects of BAX plasmid DNA alone where most apparent in freshly isolated LNs, where the vaccine induced high expression levels of the *Foxp3*, *Il-10*, and *Tgf-β1* genes which are associated with Treg activity [27]. These results were in line with our previous studies, which showed that injection of the BAX cDNA induces Foxp3$^+$ immunosuppressive Tregs in LNs of NOD mice [23]. Our previous results also indicated that injection of the BAX cDNA recruits DCs to LNs [21], which could have explained the increased expression of *Cd86* observed in LNs of C57BL/6 recipient mice immunized with vaccines carrying BAX. However, a concomitant increased expression of *Cd80*, which is also expressed by DCs, was not detected, indicating possible differential expression of the two genes. Increased expression of the *Cd86* gene is generally associated with pro-inflammatory responses [26], and could have been the result of both recruitment of DCs and injection of unmethylated plasmid DNA into the inflammatory milieu of the allograft. Indeed, LNs from mice immunized with 50 μg BAX plasmid DNA showed significantly increased expression of the *Tnf-α* and *Ifn-γ* genes when cultured with self-antigens, indicating that induction of recipient apoptotic cells after injection of BAX DNA made apparent an autoimmune response possibly induced by skin transplantation [20].

Nevertheless, when stimulated with 3rd party antigens, the same LN cells showed increased expression of the *Foxp3*, *Il-10*, and *Tgf-β1* genes similar to that observed in freshly isolated LNs of recipient mice. Furthermore, CD4$^+$CD25$^+$ cells isolated from 3rd party-stimulated LN cell cultures had increased suppressive activity. Altogether, these data indicated that injection of the BAX DNA vaccine alone induced Treg activity in response to alloantigens that could have been responsible for allograft survival. These results are in accordance with other studies, which have shown that infusion of apoptotic cells induce Foxp3$^+$ Tregs and increase allograft survival in different transplant model systems [40–42].

With regard to donor-specificity of immune responses induced by the vaccines, gene expression analysis of LN cells indicated that both the BAX and mGAD + BAX vaccines induced responses after stimulation with donor antigens that were significantly distinct from 3rd party antigen stimulation and closer to self-antigen stimulation. These results indicated a donor-specific immune response and suggested induction of tolerance to donor antigens. In addition, results from MLRs indicated that donor antigen-specific T cell unresponsiveness was induced by both the BAX and mGAD + BAX vaccines and that unresponsiveness could not be overcome using higher amounts of exogenous IL-2, which suggested a mechanism of suppression other than IL-2-restorable anergy.

Donor antigen-specificity induced by DNA vaccination was further underscored with results from adoptive transfer experiments, which revealed that cells from recipient mice immunized with the mGAD + BAX vaccine could prevent rejection of skin allograft in a donor-specific manner. Hypothetically, donor antigen-specific immune responses could be explained by interactions between self-specific regulatory T cells and APCs processing donor antigens [42]. However, splenocytes and LN cells from mice immunized with the BAX vaccine alone did not transfer significant graft survival, which could have been the result of co-transfer of autoimmune cells targeting the allograft. Future work using adoptive transfer of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cell populations should permit us to investigate this question in more details.

In conclusion, our data indicated that a therapeutic DNA vaccine for an autoimmune disease could be directly translated to prevention of allograft rejection in a most stringent model of transplantation. Results also indicated that CpG-methylation of plasmid DNA is an important component for successful therapeutic DNA vaccination to induce tolerance in both autoimmune disease and transplant rejection. The finding that injection of unmethylated plasmid DNA coding for BAX alone near the allograft could promote skin allograft survival in spite of a co-induced pro-inflammatory response suggests that CpG-methylation of BAX plasmid DNA and different sites of vaccine injection may significantly improve efficacy of pro-apoptotic DNA vaccination. Plasmid DNA-mediated induction of apoptotic cells alone could be a promising means to prevent graft rejection without having to identify and deliver specific antigens for induction of tolerance.

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