Anti-CD154 mAb treatment but not recipient CD154 deficiency leads to long-term survival of xenogeneic islet grafts

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Abstract

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Anti-CD154 mAb Treatment But Not Recipient CD154 Deficiency Leads to Long-Term Survival of Xenogeneic Islet Grafts

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Key words: Anti-CD154 monoclonal Ab, CD40, CD154, concordant, costimulatory blockade, discordant, islet xenotransplantation

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Introduction

Islet transplantation (Tx) is a promising approach for the treatment of diabetes. Facing the shortage of allogeneic organ donors and the increasing number of patients on waiting lists, xenotransplantation of animal organs or cells to humans could play an important role in the solution of this problem. Currently, organ or cellular xenografts have not achieved long-term survival in pre-clinical or clinical models and rejection mechanisms are still incompletely understood (1).

It has been established that rejection of cellular xenografts is mainly a T-cell-mediated process, in which CD4+ cells have been shown to play a major role (2). Activation of naive xenoreactive T cells by antigens (Ag) requires several signals. The first signal is delivered when T-cell receptors engage with MHC-Ag complex of antigen-presenting cells (APC), whereas second signals use costimulatory pathways that are activated when Ag-stimulated T cells interact with APC (3). Two costimulatory signaling pathways that are important for normal development and maintenance of immunity (4,5) are CD40-CD154 (or CD40L), and the B7/CD28 pathways (6–13).

Costimulatory blockade has been shown to efficiently block allograft rejection in small and large animal models of organ and islet alloTx (14–16). Only a few reports have investigated the ability of costimulatory blockade to prevent xenograft rejection. Anti-CD154 mAb is able to delay T-cell-mediated rejection of porcine skin graft in mice and also to block T-cell-dependent Ab production in the pig-to-baboon model (17). Furthermore, CTLA4Ig has been shown to induce long-term survival of human islets in mice (18). Lehnert et al. reported that administration of CTLA4Ig combined with anti-CD154 mAb allowed indefinite rat islet xenograft survival in mice (19). Consequently, costimulatory blockade is a promising approach to allow long-term survival of cellular xenografts. The purpose of the present study was to determine the role of CD40-CD154 pathway in the rejection process of concordant and discordant islet xenografts, using CD40- and CD154-knockout (KO) mice as recipients.
Materials and Methods

**Animals**

Adult male C57BL/6 mice, CD40-KO, CD154-KO and complement C3 (C3−/−) mice (Centre Medical Universitaire, Geneva, Switzerland), 8–10 weeks old and 25–30 g of body weight were used as recipients of islet xenografts. Sprague-Dawley (SD) rats (Charles River Laboratories, France), approximately 300 g of body weight, were used as islet donors. Animals were maintained in conventional housing facilities and experimental protocols were approved by the ethical committee of the Geneva University Medical School and by the Geneva veterinary authorities.

**Islet isolation**

Rat islets were isolated from adult SD male rats, weighting approximately 300 g. After laparotomy, the proximal bile duct was canulated and distended with collagenase type XI, 2 mg/mL (Sigma, St. Louis, MO) in Hank’s balanced salt solution (HBSS). The pancreas was removed and digested at 37 °C for 19 min. The digested pancreatic tissue was washed and filtered through a 500-μm mesh. Islets were purified by ficoll gradient, washed twice with HBBS solution and kept on ice prior to Tx.

**Human islet isolation**

Human pancreases were obtained from deceased multiorgan donors and islets were isolated using a modified semi-automated method (20). Pancreases were digested with the enzyme Liberase HI (Roche, Basel, Switzerland). Islet tissue was purified through continuous ficoll gradient (Biochrom KG, Berlin, Germany) using the COBE cell processor 2991 (Cobe, Lakewood, CO). Islet number was evaluated by counting islet equivalent number. Only islet layers with a mean purity of more than 80% were used for these experiments.

**Islet xenotransplantation and experimental groups**

Recipient mice were made diabetic by a single intraperitoneal injection of Streptozotocin (Sigma, Buchs, Switzerland), 220 mg/kg. Blood sugar levels were monitored on regular intervals by blood sugar monitor (Precision Q.I.D, St. Louis, MO). Tissue xenografts. Sprague-Dawley (SD) rats (Charles River Laboratories, France), 10 weeks old and 25–30 g of body weight were used in Group 1, 3, 4 and 6, and 30 days after islet Tx for Group 2. As control, non-transplanted naïve C57BL/6 mice were also used as responders.

Mouse and rat mononuclear cells were obtained as follows: spleens were harvested and placed on a petri dish with 5 mL of cold complete iscove tissue culture media (IMDM, Iscove’s modified dulbecco’s medium with L-Glutamine and 25mM HEPES, Invitrogen, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Basel, Switzerland), 1% sodium pyruvate (Invitrogen), 1% non-essential amino acid (Invitrogen) and 1% P/S (pencillrin, streptomycin and glutamine, Invitrogen). Spleens were then fractured with a spatula, filtered with a 30-μm mesh, and washed by centrifugation three times. Supernatant was discarded each time, and the remaining pellet was resuspended in complete iscove tissue culture media. Mononuclear cells harvested from non-transplanted C57BL/6 and transplanted mice of Groups 1–4 were designated as responder cells. Mononuclear cells derived from naïve C57BL/6 and Balb male mice, SD and Lewis male rats were designated as stimulator cells, respectively. Stimulator human mononuclear cells were prepared from buffy coat obtained from the blood bank of the University Hospital of Geneva. All cells were purified by Ficoll/Histopaque (Sigma, Buchs, Switzerland) gradient centrifugation. Stimulator cells were irradiated using a γ-irradiator (ORIS Industry, IBL 437C, France) with 3500 rad.

For proliferation assays, stimulator cells were cultured in a 2:1 ratio with responder cells in medium containing IMDM, i.e. 4 x 10^5 responder cells were cultured with 8 x 10^6 irradiated stimulator cells in 96-well round-bottom plates (Nunclon™ Surface, DK-4000, Roskilde, Denmark). Four separate wells were dedicated to each responder-stimulator combination and each experiment was repeated three times. Cells were harvested for 5 days at 37°C in 95% humidified air mixed with 5% carbon dioxide. On day 5, 2 μCi[^3]H-thymidine was added to each well, 12 h later, cells were harvested in paper of Filtermat A (Wallac Oy, Turku, Finland) and dried 4 h. The incorporation of[^3]H-thymidine was assessed and quantified in a liquid scintillation counter (1450 MicroBeta Plus, Wallac, Gaithersburg, MD) as count per minute (CPM). Degree of T-cell proliferation was interpreted using a stimulation index (SI). This SI was calculated as follows:

\[
SI = \frac{CPM\ of\ responder\ lymphocytes\ stimulated\ by\ allo-\ or\ xenogenic\ stimulators}{CPM\ of\ responder\ lymphocytes\ stimulated\ by\ self-isogeneic\ stimulators}
\]

For each responder-stimulator combination, standard deviation (SD) was calculated.

**Histology and immunohistochemistry**

Nephrectomy (graftectomy) was performed on mice at rejection or at 120 days post-islet Tx. Kidneys were embedded in Tissue-Tek (Miles, Elkhart, IN), frozen in liquid methylbutane pre-equilibrated with liquid nitrogen and stored at −80°C. Serial frozen sections were cut at 5 μm intervals using a cryostat (Leica, Glatbrugg, Switzerland). Tissue sections were fixed with absolute ethanol for 1 min and then incubated with phosphate-buffered saline plus 0.5% bovine serum albumin (Sigma, St. Louis, MO) for 15 min to block non-specific binding. Staining was performed for insulin (DAKO, Glostrup, Denmark), Glucagon (DAKO), IgG (Serotec, Oxford, UK), IgM (Sigma) and complement C3 deposition (PC280U, Anawa, Cologne, Germany), infiltration of lymphocytes (CD3, CD4 and CD8, Serotec) and macrophages (mac-1: Immunokontact, Switzerland and F4/80: Serotec). The slides were examined under a fluorescence microscope (Zeiss Axioshot, Göttingen, Germany).

Statistical analysis

Statistical analysis was performed on personal computer using STATISTICA (STATISTICA 6.5 Software for Windows, Statsoft, Inc., Tulsa, OK) and GraphPad InStat (GraphPad InStat version 3.00 for Windows, GraphPad Software, San Diego CA). Survival curves were calculated by Kaplan and Meier method and analyzed with the Cox-Mantel test when appropriate contingency
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Figure 1: Survival of concordant islet xenografts (rat-to-mouse) in untreated C57BL/6 mice (Group 1), C57BL/6 MR1-treated mice (Group 2), CD40-KO mice (Group 3), CD154-knockout mice (Group 4), C3-deficient untreated mice (Group 5) and C3-deficient MR1-treated mice (Group 6). Graft survival is presented as Kaplan-Meier survival curves.

Figure 2: Survival of discordant islet xenografts (human-to-mouse) in untreated C57BL/6 mice (Group 1), C57BL/6 MR1-treated mice (Group 2), CD40-KO mice (Group 3) and CD154-knockout mice (Group 4). Graft survival is presented as Kaplan-Meier survival curves.

tables were analyzed with Fischer’s exact test. A p-value less than 0.05 was considered statistically significant.

Results

Islet graft survival of rat-to-mouse combination (Figure 1)
In Group 1, C57BL/6 mice receiving no further therapy had a mean graft survival of 17 ± 7 days (SD). In Group 2, C57BL/6 mice receiving MR1 therapy showed a significantly prolonged mean graft survival of >120 ± 37 days (SD), with four of six mice accepting their graft for the length of follow-up (p < 0.001, compared to Group 1). In Group 3, in contrast, all CD40-KO recipients rejected their islet graft promptly (mean graft survival 9 ± 5 days (SD), compared to Group 1, p = n.s.). Notably, CD154-KO mice (Group 4) also rejected their graft without delay (mean graft survival 17 ± 13 days (SD), p = n.s.). C3-deficient untreated mice (Group 5) rejected their graft (mean graft survival 10 ± 2 days) in a similar timing as Group 1 mice. C3-deficient mice treated by anti-CD154 mAb (Group 6) showed prolonged mean graft survival of 25 ± 19 days (p = 0.01, compared to Group 5), but all mice (7/7) rejected their grafts.

Islet graft survival of human-to-mouse combination (Figure 2)
In Group 1, C57BL/6 mice had a mean graft survival of 11 ± 7 days (SD). In Group 2, C57BL/6 mice receiving MR1 therapy showed a significantly prolonged mean graft survival of >120 ± 43 days, with four of six mice retaining their graft (p < 0.005, compared to Group 1). In Group 3, CD40-KO
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mice showed a slightly prolonged graft survival (p = n.s., compared to Group 1, mean graft survival 27 ± 14 days (SD)), but all recipients rejected the islets by day 40. Likewise, all CD154-KO mice (Group 4) rejected their grafts by day 40 (mean graft survival 16 ± 11 days (SD), compared to Group 1, p = n.s.). Thus, while anti-CD154 mAb treatment leads to long-term survival of rat or human islet xenografts, deficiency of recipient CD154 or CD40 does not prolong graft survival.

**Mixed lymphocyte reaction (MLR) (Figure 3)**

T-cell stimulation indices of naive non-transplanted and Group 1 C57BL/6 mice against donor cells and third-party allogeneic, concordant and discordant xenogeneic cells are shown on Figure 3A and B. Stimulation index against donor cells significantly increased in Group 1 mice that had rejected rat islets (Figure 3B), compared to naive and non-transplanted mice (Figure 3A). In Group 2, anti-CD154 mAb-treated mice demonstrated an approximately 50% decrease of T-cell responses against the initial islet donor (SD rat), compared to Group 1 (p = 0.01) (Figure 3C). Responses against concordant third-party stimulators (Lewis rat), were also reduced significantly (p < 0.05), but responses against discordant xenogeneic third-party cells were not modified compared to Group 1 (p > 0.05) (Figure 3C). In Groups 3 and 4, T-cell responses were not modified by the absence of CD40 (D) or CD154 (E) molecules and were similar to naive and Group 1 mice. In Group 6, responses against donor and third-party stimulator cells were similar as in Group 1 (F). (S = Stimulator).

![Figure 3: MLR in Groups 1-4 and 6.](https://example.com/figure3.png)
modified by the absence of CD40 or CD154 molecules and were similar to naïve and Group 1 mice (Figure 3D,E). In Group 6, responses against donor and third-party stimulator cells were similar as in Group 1 (Figure 3F).

**Histopathology and immunohistochemistry (Figures 4–7)**

In Group 1, histological analysis showed for both rat-to-mouse and human-to-mouse xenografts severe graft destruction at rejection. Only rare islet cells stained positive for insulin and glucagon (data not shown) and dense cellular infiltration was detected and composed of CD4+, CD8+ lymphocytes and macrophages (Figure 4A,C,E). Humoral responses were detected for both control groups with IgG, IgM and C3 deposition (Figure 5A,C,E).

In Group 2, MR1-treated mice showed at day of 120 post-Tx, viable islets staining positive for insulin and glucagon (data not shown) and no cellular infiltration within the grafts (Figure 4B,D,F). Neither immunoglobulin (IgG and IgM) nor complement deposition was observed in MR1-treated mice at day of 120 post-Tx (Figure 5B,D,F).

In CD40- and CD154-KO mice, histological analysis at rejection showed for both rat-to-mouse and human-to-mouse xenografts severe graft destruction. Only rare islet cells, staining positive for insulin and glucagon, still remained detectable in the graft (data not shown). Mixed cellular infiltration was detected within the grafts composed of CD4+, CD8+ lymphocytes and macrophages (Figure 6A–F). Humoral responses were detected with moderate IgM and C3, but no IgG deposition in islet grafts (Figure 7A–F).
**Discussion**

The aim of the present study was to analyze the role of CD40-CD154 pathway in concordant and discordant islet xenotransplantation. The results showed that short-term anti-CD154 mAb therapy significantly prolonged concordant and discordant xenograft survival compared to control groups with graft survivals over 100 days in both species combinations and prevention of cellular and humoral responses.

The potential use of costimulation-blocking reagents to induce transplantation tolerance has recently created considerable excitement. Recent evidence has begun to delineate the mechanisms by which these powerful effects occur. It has become increasingly clear, first, that T-cell costimulation is mediated by a delicate network of signaling pathways and, second, that interference with these systems can lead to numerous different tolerance mechanisms, including immune regulation, anergy and deletion (21).

Although the mechanism by which blocking the interaction of CD40-CD154 at the target site promotes islet xenografts survival remains to be elucidated, it confirms that CD40-CD154 costimulatory pathway can modulate the immune response to islet xenoantigens (22). CD154 is expressed on activated T cells and blockade of CD40/CD154 signal pathway can results in T-cell anergy, induction of suppressor cells or T-cell apoptosis (23). Although it was initially
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Figure 4: Cellular immune responses to discordant islet xenografts in untreated C57BL/6 mice (Group 1) and C57BL/6 MR1-treated mice (Group 2). Sections were stained by anti-mouse CD4, CD8 and macrophage Ab (Mac-1) (Serotec). In Group 1, a mixed cellular infiltrate was detected at rejection with presence of CD4+ (A), CD8+ (C) and macrophages (E). In Group 2, no CD4+ (B), CD8+ (D) or macrophages (F) were detected at 120 days after transplantation (shown at ×100 magnification).

admitted that MR1 is a non-cytolytic neutralizing Ab, recent studies have suggested that the potent immunosuppressive effects of anti-CD154 mAb are not limited to blockade of signal pathways (24) and elimination of CD154 positive cells by complement- or cellular-mediated mechanisms has also been suggested. Monk et al. reported that prolongation of allogeneic skin graft survival by anti-CD154 mAb was dependent on both complement- and Fc-receptor-mediated mechanisms. This result suggested a possible depleting activity of MR1 (25). Moreover, Sanchez-Fueyo et al. treated complement C5-deficient DBA/2 mice with MR1 and could not prevent acute islet allograft rejection, suggesting that the mechanisms of tolerance induction by MR1 are not limited to blockade of CD40/CD154 signals and that a complement-dependent cytotoxic mechanism contributes to MR1-induced immunosuppression (26).

In the present study, we performed islet xenotransplantation using complement C3-deficient mice as recipients to test the effect of anti-CD154 mAb therapy. Interestingly, in the absence of C3, MR1 was not able to induce long-term survival of islet xenografts, confirming that a complement-dependent mechanism is involved in the effect of MR1.

Blair et al. reported that anti-CD3/CD154 costimulation resulted in CD28-independent activation and subsequent deletion of resting T cells. It was suggested that anti-CD154 therapy in vivo might result in part from early and direct effects on CD4+ T cells, including vigorous induction of immunomodulatory cytokines and/or apoptosis of allograft-specific T cells (27). In our study, we evaluated the impact of complete absence of CD40 and CD154 molecules by using CD40- and CD154-KO mice as recipients of xenoislets. Rejection occurred without statistical difference compared to control wild-type C57BL/6 mice, suggesting CD154/CD40-independent mechanisms of T-cell priming.

Kurtz et al. used CD154-deficient mice instead of anti-CD154 treatment to analyze the establishment of
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Figure 5: Humoral immune responses to discordant islet xenografts in untreated C57BL/6 mice (Group 1) and C57BL/6 MR1-treated mice (Group 2). Sections were stained by anti-mouse IgG, IgM and complement (C3). In Group 1, a humoral response was detected at rejection with presence of IgG (A), IgM (C) and C3 (E). In Group 2, no IgG (B), IgM+ (D) or C3 (F) was detected at 120 days after transplantation (shown at ×100 magnification).

chimerism and allotolerance. Their result showed that anti-CD154 mAb treatment was required only to prevent CD40L/CD40 interactions, and that no signal to the T cell through CD154 was necessary for the induction of CD4+ tolerance (28).

Shimizu et al. performed cardiac allotransplantations using CD154-deficient mice to investigate the mechanisms underlying prolonged allograft survival. Compared to wild-type mice, allograft survival in CD154-KO recipients was prolonged from 2 to 12 weeks. CD154-KO recipients developed allospecific tolerance to the donor and the authors proposed that early alloresponses, without CD40-CD154 costimulation, induce allospecific tolerance (29).

In our study, CD40-KO recipients rejected concordant islet xenograft in a threefold faster timing compared to discordant xenografts (9 ± 5 days vs. 27 ± 14 days); however, the difference did not reach statistical significance (p = 0.1). The absence of CD40 on APC did not prolong survival of concordant xenografts, but delayed the rejection of discordant xenografts (albeit not significantly).

Regarding humoral responses, in our study, anti-CD154 mAb therapy prevented the induced Ab response and complement activation. This result can be explained by inhibition of T-B-cell interaction through anti-CD154 mAb. Furthermore, an induced Ab production was detected at rejection of both concordant and discordant xenografts in control groups, with deposits of IgG, IgM and complement.

In CD40- and CD154-KO recipients, a humoral response was detectable at rejection, with IgM and complement deposits, but with IgG. The results of our study indicate that possible alternate pathways of costimulation, including ICOS and TRANCE, can efficiently be activated leading...
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Figure 6: Cellular immune responses to discordant islet xenografts in untreated CD40-KO mice (Group 3; A,C,E) and CD154-KO mice (Group 4; B,D,F). Sections were stained for anti-mouse CD4 (A,B), CD8 (C,D) and macrophage Ab (Mac-1) (E,F). In CD40- and CD154-KO mice, a mixed cellular infiltrate was detected at rejection with presence of CD4+ (A,B), CD8+ (C,D) and macrophages (E,F) (shown at ×200 magnification).

Previous studies' models have shown over expression of TRANCE in CD40- and CD154-KO recipients with subsequent initiation of cellular immune responses. Bachmann et al. reported that CD40- or CD154-deficient mice challenged with viruses developed protective CD4+ T-cell responses, producing normal levels of interferon γ and TRANCE/TRANCE-R interactions were shown to provide efficient costimulation for CD4+ T-cell priming in the absence of CD40/CD154 (30).

The MLR results correlated with the in vivo findings. Untreated C57BL/6 mice showed increased stimulation indices against donor cells after rejection had occurred, compared to naïve non-transplanted mice (Figure 3A,B). In contrast, MR1-treated mice significantly showed reduced responses to donor Ag (Figure 3C). Interestingly, responses to third-party concordant xenogeneic Ag were also modified by MR1 therapy, but responses to discordant xenogeneic third-party Ag remained unmodified (Figure 3C). A similarity between SD and Lewis rat Ag could explain the species-dependent hyporesponsiveness. In CD40- and CD154-KO mice, stimulation indices against donor and third-party Ag were similar compared to untreated mice, confirming an efficient T-cell activation despite the absence of these costimulatory molecules (Figure 3D,E). In Group 6, MR1-treated complement C3-deficient mice showed responses against donor and third-party stimulator cells were similar as in Group 1 (Figure 3F). This result has confirmed that a complement-dependent mechanism is involved in the effect of MR1.

The current experiments indicate that anti-CD154 mAb therapy led to long-term acceptance of xenografted islets. However, it is not clear from these studies if immunological tolerance was achieved. To further analyze a possible tolerance state, skin grafts should be performed. However, our...
data suggest that clinical applicable therapies are able to induce long-term survival of cellular xenografts and such regimens should be tested in pre-clinical models of islet xenotransplantation.

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