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S. Giraud, B. Barrou, S Sebillaud, P. Debré, D. Klatzmann, Véronique  
Thomas-Vaslin

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**Transient Depletion of Dividing T Lymphocytes In Mice Induces the Emergence of Regulatory T cells and Dominant Tolerance to Islet Allografts \***

**Running title:** T-cell dynamics and transplantation tolerance

**S. Giraud<sup>a,b,e,g</sup>, B. Barrou<sup>ab,e,f,g</sup>, S. Sebillaud<sup>a,b,e</sup>, P. Debré<sup>a,b,e</sup>, D. Klatzmann<sup>c,d,e</sup>, V. Thomas-Vaslin<sup>c,d,e</sup>**

<sup>a</sup>UPMC Univ Paris 06, U543, Laboratoire d'Immunologie Cellulaire et Tissulaire, Paris F-75013 France

<sup>b</sup>INSERM, U 543, Laboratoire d'Immunologie Cellulaire et Tissulaire, F- 75013 Paris, France

<sup>c</sup>UPMC Univ Paris 06, UMR7087, Biologie et Thérapeutique des Pathologies Immunitaires, F- 75013 Paris, France

<sup>d</sup> CNRS, UMR7087, Biologie et Thérapeutique des Pathologies Immunitaires F- 75013 Paris, France

<sup>e</sup>IFR113, Pitié-Salpêtrière, F- 75013 Paris, France

<sup>f</sup>AP-HP, Hôpital Pitié-Salpêtrière, Service d'Urologie, F- 75013 Paris, France

<sup>g</sup> **These authors contributed equally**

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***Corresponding author:***

Véronique Thomas-Vaslin,  
Biologie et Thérapeutique des Pathologies Immunitaires,  
CNRS-UPMC UMR 7087, CERVI,  
Groupe Hospitalier Pitié-Salpêtrière, 83 Bd de l'Hôpital  
75651 PARIS CEDEX France.  
Phone : 33 (1) 42 17 74 70 Fax: 33 (1) 42 17 74 62  
E-mail: [veronique.thomas-vaslin@chups.jussieu.fr](mailto:veronique.thomas-vaslin@chups.jussieu.fr)

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***Abbreviations:***

B6, C57Bl/6; GCV, Ganciclovir ; LN, lymph nodes; TK, HSV1-Thymidine Kinase transgene ; TK<sup>+</sup>GCV<sup>+</sup>, mouse transgenic for TK treated with GCV ; STZ, streptozotocine ; Treg, regulatory T cells

**Abstract**

We previously showed that transient depletion of dividing T cells at the time of an allogeneic transplantation induces long-term tolerance to the allograft. Here we investigated the role of homeostatic perturbation and regulatory T cells (Treg) in such tolerance. Transient depletion of dividing T cells was induced at the time of an allogeneic pancreatic islets graft, by administration of ganciclovir for 14 days, into diabetic transgenic mice expressing a thymidine kinase (TK) conditional suicide gene in T cells. Allograft tolerance was obtained in 63% of treated mice. It was not due to global immunosuppression, permanent deletion or anergy of donor-alloantigens specific T cells but to a dominant tolerance process since lymphocytes from tolerant mice could transfer tolerance to naïve allografted recipients. The transient depletion of dividing T cells induces a 2-3 folds increase in the proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg, within 3 weeks that persisted only in allograft bearing mice but not in non-grafted mice. Tolerance with similar increased proportion of Treg cells was also obtained after a cytostatic hydroxyurea treatment in normal mice. Thus, the transient depletion of dividing T cells represents a novel means of immuno-intervention based on disturbance of T-cell homeostasis and subsequent increase in Treg proportion.

## **Introduction**

Various mechanisms operate during induction and maintenance of transplantation tolerance. Recessive processes such as T-cell deletion or anergy can reduce effector functions against allogeneic donor cells but are not always sufficient. Consequently, dominant processes mediated by regulatory T cells (Treg) are important to establish natural and transplantation tolerance (1-4). Treg can be selected centrally in the thymus and can also be selected in the periphery. In the thymus, epithelial cells play an essential role in the positive selection of specific CD4<sup>+</sup> regulatory T cells that are able to induce transplantation tolerance (4-6). In the periphery, dominant transplantation tolerance can be obtained through different methods that result in the emergence of Treg. These methods include (i) costimulatory blockade (7-11); (ii) non-depleting anti-CD4 therapy (11-13) associated or not with donor specific blood transfusion (14); and (iii) induction of tolerogenic dendritic cells by various protocols including treatment with vitamin D receptor ligands (15), opsonization of apoptotic cells and capture of antigens by immature or plasmacytoid dendritic cells (16, 17). Induction of peripheral tolerance can also be triggered by experimental protocols that result either in clonal deletion or inactivation of T cells, associated with expansion of Treg (18, 19). Depletion of alloreactive T cells by apoptosis, leading to the emergence of Treg, was suggested to be a requirement for transplantation tolerance induction (20). However, T-cell depletion can also trigger homeostatic proliferation of memory T cells, which can impair the induction of transplantation tolerance (21). Thus, a therapeutic strategy that can destroy alloreactive T cells by apoptosis, including memory T cells, but preserve quiescent T cells and induce the emergence of Treg would be valuable for allograft tolerance induction.

We previously described a strategy by which long-term tolerance to surgically vascularized heart allograft was induced in mice. Our treatment was based on a time-controlled, selective

destruction of dividing T cells for two weeks, starting at the time of transplantation, thus mainly targeting alloreactive T cells. This was achieved by the pharmacogenetic depletion of dividing T cells expressing the therapeutic conditional suicide gene, thymidine kinase of type 1 Herpes Simplex Virus (HSV1-TK). HSV1-TK phosphorylates the inactive prodrug ganciclovir (GCV), changing it into a toxic triphosphorylated-GCV nucleotide analog. This analog, when incorporated into a growing DNA strand, blocks its elongation and leads to apoptosis of the dividing cells. We previously generated transgenic mice specifically expressing the HSV1-TK suicide gene in both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (TK<sup>+</sup> mice). Using these mice, we showed that the transient depletion of dividing T cells by GCV (i) controls T-cell mediated immune diseases involving alloreactive and pathogenic T cells (22-26); (ii) induces long-term, specific, and robust tolerance to vascularized allogeneic heart grafts (27) or delayed rejection of self-vascularized skin and heart (28); (iii) and disrupts immunological memory maintenance (29). Here we show, in allogeneic pancreatic islet transplantation, that tolerance induction is mediated by a progressive expansion of Treg. This expansion is a direct consequence of an altered homeostatic balance following the depletion of dividing T cells by apoptosis.

## **Materials and Methods**

### ***Mice***

FVB/N (H-2<sup>q</sup>), C57Bl/6 (B6, H-2<sup>b</sup>, CD45.2), and BALB/c (H-2<sup>d</sup>) mice were obtained from Charles River, France. The HSV1-TK transgenic mice, FVB EpCD4TK line 40, (referred to as TK<sup>+</sup>) have been described previously (28) and were bred in our animal facility and used at 10-15 weeks of age. In the transgenic animals, the absence of the CD4 silencer (30) led to transgene expression in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (28). Mice were kept under specific pathogen-free conditions and were manipulated according to European council directive 86/609/EEC of 24 November 1986.

### ***GCV and hydroxyurea (HU) administration***

The modality of the GCV (Cymevan, Roche, France) administration was previously studied *in vitro* and *in vivo* (28). GCV was diluted in pyrogen-free H<sub>2</sub>O, filtered (0.22µm) and administered by mini-osmotic pumps (Alzet 2001 or 2002, Charles River Laboratories), which delivered GCV at the dose of 50 mg/kg/day for 7 or 14 days allowing a plasma concentration of 7.4 µM. Pumps were implanted s.c. under ketamine (150 mg/kg) and xylazine (10 mg/kg i.p.) anesthesia at the time of transplantation. HU (Hydrea, Bristol-Myers Squibb) was administrated as cycle of 2 ip injections at 1g/kg/day, 7 hours apart; mice received 6 cycles every 2 or 3 days over a 12 days period.

### ***Islet isolation***

Islets were isolated as previously described (31) by stationary digestion with Liberase RI (Roche diagnostics, Meylan, France) after *in situ* distension of the pancreas. Islets were

purified on a discontinuous Euroficoll gradient, handpicked to obtain 100% purity and cultured overnight at 37 °C and 5% CO<sub>2</sub> before transplantation.

### ***Islet transplantation and follow-up***

Recipients were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich, France) and 1000 islets were grafted under the left kidney capsule as previously described (31). The experimental group consisted of TK<sup>+</sup> mice treated with GCV for 14 days (TK<sup>+</sup>GCV<sup>+</sup>). Control groups consisted of TK<sup>+</sup> mice with no GCV treatment (TK<sup>+</sup>GCV<sup>-</sup>) and non-transgenic littermate mice either treated with GCV (TK<sup>-</sup>GCV<sup>+</sup>) or untreated (TK<sup>-</sup>GCV<sup>-</sup>).

Blood glucose concentrations (glycemia) were measured using a glucometer (kindly provided by Bayer Diagnostic, France). Rejection was defined as the observance of two successive non-fasting blood glucose concentrations > 200 mg/dl. Mice that did not become hyperglycemic after removal of the graft-bearing kidney were excluded from the study because regeneration of the native endogenous endocrine pancreatic function had occurred, making clinical assessment of the graft function impossible.

### ***Graft infiltrating cells***

For immunohistological analysis, 8 µm cryostat sections of the islet-bearing kidney were stained as previously described (32). Briefly, we used biotinylated anti-CD4/CD8 mAbs followed by the enzymatic avidin-biotin-peroxidase/ 3-amino -9-ethyl carbazole reaction and H/E staining. We observed the slides on a Nikon DIAPHOT microscope (Nikon, France SA). To obtain cell suspensions for FACS analysis, islets were separated from the kidney and digested with 1.6 mg/ml type IV collagenase (Sigma Aldrich, France) and 200 µg/ml DNase I at 37 °C for 30 minutes.

***Skin grafts***

Skin pieces (1cm<sup>2</sup>) from tail mouse were grafted on the back of recipients under ketamine (150 mg/kg) and xylazine (10 mg/kg i.p.) anesthesia and follow up was done as previously described (28).

***Cell suspensions***

Spleen cell suspensions and pooled axillary, brachial and inguinal lymph node cell suspensions were obtained by mechanical dilacerations. Live cells were counted after trypan blue exclusion. PBL were separated by gradient density on MSL (Eurobio Biotechnology).

***Lymphocyte proliferation and cytotoxicity***

Cells were cultured for 3 days at 37 °C with 5% CO<sub>2</sub> in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 5x10<sup>-5</sup> M 2-ME, penicillin/streptomycin/neomycin (all from Gibco, BRL) in triplicate in 96-well, U-bottom microplates in a total volume of 200 µl. For MLR, responders (4x10<sup>5</sup>/well) were stimulated in the presence of 20 Gy-irradiated stimulators (4x10<sup>5</sup>/well). For mitogenic reactions, Con A (2 µg/ml) was added to 2x10<sup>5</sup> cells/well. Proliferation was assayed on day 3 by a <sup>3</sup>H-dThd pulse (1 µCi/well), for 16 hours. For CTL tests, cells were cultured for 5 days in the presence of allogeneic irradiated spleen cells, then <sup>51</sup>Cr release from EL-4 (H-2<sup>b</sup>) target cells was assayed as described (29).

***Tolerance transfer experiments***

Secondary recipients were made diabetic by STZ injection, then sublethally irradiated (2 Gy). The next day, splenocytes were harvested from primary tolerant recipients. The cell suspension was injected i.v. into the retro-orbital sinus of the secondary recipients at the time of islet allograft transplantation under the kidney capsule.

***In vivo treatment with anti-CD25 mAb***

Mice received one i.p. injection of 100µg in 100µl of PC61 IgG1 anti-CD25 monoclonal antibody.

***Immunostaining and flow cytometry***

Cells ( $2 \times 10^6$ ) were incubated with optimal dilutions of labeled monoclonal antibodies diluted in 50µl PBS/3% newborn calf serum (NCS Gibco, BRL), for 20 minutes at 4 °C and then washed in PBS/3% NCS. Intra-cytoplasmic staining for measurement of IFN-γ was done using the Cytofix/Cytoperm™ Plus Pharmingen kit on cells cultured for 4 hours at 37°C in presence of Golgiplug, with or without cell stimulation with anti-CD3+anti-CD28 (1µg/ml) (Pharmingen, San Diego, CA). The monoclonal antibodies anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD62L (MEL-14), anti-CD44 (IM7), and anti-CD25 (PC61 or 7D4) were obtained from Pharmingen, San Diego, CA. Foxp3 intracellular staining was done with the anti-mouse/rat Foxp3 staining set (clone FJK-16s) from eBioscience (Cliniscience SA, Montrouge, France). A FACSCalibur or a LSR2 (Beckton Dickinson) was used for acquisitions and viable lymphocytes were gated on the basis of forward and side scatter parameters, with Flowjo software (Tree Star, San Carlos, CA).

***Statistical analysis***

Stat view software (SAS Institute Inc.) was used for statistical analysis. Cumulative graft survival was calculated using Kaplan-Meier estimates. Comparisons were made with the Logrank test (Mantel-Cox). Mean graft survival was expressed as mean survival time (MST)± SEM. The Mann-Whitney U test was used to compare values for the different groups. The PSLD Fisher test was used to compare cell numbers over time. The LOWESS smooth (tension 66) was used to draw T cell dynamics.

## Results

### *Transient depletion of dividing T cells induces tolerance to islet allograft*

B6 islets were grafted under the kidney capsule of diabetic FVB mice. Islet allografts were rejected in all control groups (TK<sup>+</sup>GCV<sup>-</sup>, TK<sup>-</sup>GCV<sup>+</sup>; TK<sup>-</sup>GCV<sup>-</sup>) (Fig. 1A to 1C). The mean time of graft survival was  $17.5 \pm 0.9$  days (Figure 1F). In contrast, 14 of 22 (63%) TK<sup>+</sup> grafted mice treated with GCV were normoglycemic for more than 100 days with no additional immunosuppression; hyperglycemia occurred immediately after removal of the graft-bearing kidney (Figure 1D). These results demonstrate that normoglycemia was due to the function of the grafted islets. The remaining nine recipients (37%) experienced delayed graft rejection (Figure. 1E); loss of graft function was less rapid than that of the classical acute rejection observed in controls. The mean graft survival time of these mice was significantly longer ( $35.8 \pm 12.9$  days, Logrank test  $p < 0.0001$ ) than that of the control groups. These results show that a state of long-term tolerance was achieved in at least 60% of the mice.

### *Tolerant recipient splenocytes retain normal proliferative and CTL functions in vitro*

To address the mechanisms of tolerance – deletion, anergy or suppression – we assayed the proliferative and cytotoxic capacities of T cells from grafted recipients. Two to three months after transplantation, splenocytes from tolerant TK<sup>+</sup>GCV<sup>+</sup> mice displayed proliferative responses to Con A stimulation (Figure 2A) to donor or third-party alloantigens (Figure 2B) and cytotoxic activities against donor haplotype (Figure 2C) and IFN $\gamma$  secretion (Figure 2D) similar to those of non-manipulated control mice.

TK<sup>+</sup>GCV<sup>+</sup> mice experiencing delayed rejection had significantly lower responses to Con A (Figure 2A), and to both donor and third-party alloantigens (Figure 2B) than tolerant or naive

mice, while IFN $\gamma$  secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 2D) did not differ from that in non-manipulated controls. Thus, following the delayed rejection episode in vitro T cell proliferation is partially affected while IFN $\gamma$  secretion was conserved .

### ***In vivo specificity of the tolerance***

We further assessed the immunocompetence and the antigen specificity of tolerance by the ability of TK<sup>+</sup>GCV<sup>+</sup> tolerant mice to reject a third party skin or islet allograft. When mice tolerant to B6 islets for more than 2 months, received skin grafts without treatment (Figure 3A), the BALB/c third party skin was acutely rejected in 17 days as in naïve recipients ( $p > 0.05$  Logrank test) while the B6 skin rejection was chronic and delayed to 31 days ( $p < 0.05$  Logrank test compared to BALB/c graft and compared to rejection of B6 skin in naïve recipient). A second islet of third-party origin (BALB/c) grafted on day 42 on a tolerant mouse was rejected in less than 29 days (not shown). Thus, tolerance is specific of B6 donor haplotype but not of islet since skin graft survival was prolonged.

### ***In vivo suppression of islet graft rejection by adoptive transfer of splenocytes from tolerant mice***

We analyzed whether splenocytes from TK<sup>+</sup>GCV<sup>+</sup> tolerant mice could suppress rejection of allogeneic islet graft upon transfer to normal immunocompetent mice rendered diabetic. Recipient mice, were sublethally (2Gy) irradiated before cell transfer to favor T-cell engraftment; this conditioning did not alter the capacity of mice to reject islet allografts, which occurred in  $22 \pm 1$  days (Figure 3C). Allograft recipients received  $60 \times 10^6$  total splenocytes from TK<sup>+</sup>GCV<sup>+</sup> tolerant FVB mice. No other treatments were given. Two of the 5 recipients of splenocytes remained fully tolerant to B6 islets, and normoglycemic until graft removal (Figure 3B). The three other recipients experienced significantly delayed rejection of islets (Logrank test  $p < 0.01$ , MST  $32.3 \pm 4.7$  days), as compared with similarly irradiated and

grafted controls that received no cell transfer (MST  $22 \pm 1$  days,) transfer of cells from naïve non tolerant mice (MST  $21.3 \pm 2.0$  days), (Figure 3C). Cells transferred from  $TK^+GCV^-$  mice at the time of islet graft rejection, led to accelerated rejection of islet graft at 15 days in the second recipient (n=1, data not shown), as previously shown in other graft models (33). Noteworthy, another group of recipient mice receiving splenocytes from tolerant donor rejected a B6 skin graft as rapidly as controls receiving cells from naive animals or without cell transfer (MST 17, 16 and 19 days, respectively Logrank test,  $p > 0.2$ ) (Figure 3C). Thus, splenocytes from mice tolerant to B6 islets contain regulatory cells capable to control effector cells responsible for rejection of B6 islets but not B6 skin.

***Depletion of dividing T cells induces the emergence of  $CD4^+$  Treg cells***

In long-term normoglycemic tolerant mice,  $CD4^+$  and  $CD8^+$  T lymphocytes could be detected surrounding the transplanted islets (Figure 4A). High proportions of graft infiltrating  $CD4^+$  T lymphocytes and spleen cells expressed CD25 (Figure 4B). Most graft infiltrating  $CD4^+$  T cells and  $CD4^+CD25^+$  of the spleen have lost CD62L expression corresponding to antigen-experienced T cells, in contrast to  $CD25^{neg}$  T cells that always expressed high levels of CD62L corresponding to naïve cells.

$TK^+GCV^+$  mice either tolerant or experiencing a delayed rejection have a significant reduction in the percentages (Figure 4C) and numbers of  $CD4^+$  splenic T cells (Figure 4D-E) compared to naïve mice. In contrast the numbers and proportion of  $CD4^+CD25^+$  T splenocytes were 2 times higher. Control mice analysed at the time of an acute allogeneic islet graft rejection ( $TK^+GCV^-$  on day 27 or  $TK^-GCV^+$  on day 21) present high numbers of  $CD4^+$  T cells and  $CD4^+CD25^+$  cells (Fig.4E, 6D). This is in accordance with their insensibility to T cell depletion and activation/expansion of effector T cells.

### ***Homeostatic imbalance following transient T cell depletion***

We investigated whether emergence of Treg is related to homeostatic imbalance following the initial depletion of dividing T cells. We compared the T-cell dynamics of TK<sup>+</sup> mice either grafted or not with B6 islets, upon GCV treatment (Figure 4E-H).

In absence of graft (Figure 4F), the total number of CD4<sup>+</sup> T cells significantly decreased in TK<sup>+</sup>GCV<sup>+</sup> mice (PSLD Fisher,  $p < 0.05$  comparing day 0 with days 7, 10 and 28) reaching at nadir, from day 7 to 28, around  $23 \times 10^6$  CD4 T cells. In contrast, the numbers and percentages of CD4<sup>+</sup>CD25<sup>+</sup> and of CD4<sup>+</sup>Foxp3<sup>+</sup> cells increased 2 times (PSLD Fisher,  $p < 0.05$  comparing day 0 with days 3, 7, 10 and 21 for CD4<sup>+</sup>CD25<sup>+</sup> numbers) in spleen and lymph nodes (LN) in 3 weeks (Figure 4G, H). All these alterations are fully reversible, recovery to control values occurring within 2 months (Figure 4F).

In the presence of an islet allograft, the dynamics of T cells from TK<sup>+</sup>GCV<sup>+</sup> mice differs: while a similar decrease of CD4 T cell numbers, and increased proportion of Treg were observed during the first 3 weeks (Figure 4E, G-H) they come back to day 0 values did not occur. A high proportion of Treg cells was maintained in presence of allograft, which may prevent CD4<sup>+</sup> T-cell recovery.

### ***Persistence of tolerance and Treg cells after depletion of CD25 T cells***

In order to address directly if CD25 T cells are responsible of the tolerant state, we treated tolerant mice (on day 60 after B6 islet graft) with PC61 monoclonal antibody reported to induce depletion of CD25 T cells in vivo. Mice remained normoglycemic until day 74 where removal of the graft induces hyperglycemia (Fig.5A). The kinetics of Treg cells in blood before and after PC61 treatment (Fig.5B-C) revealed efficiency of PC61 to deplete CD25 T cells: the percentage of CD25<sup>+</sup> cells drops from 7% of CD4 cells before treatment to less than 0.2% of CD25 cells on day 7. Nevertheless, Foxp3<sup>+</sup> Tregs (6.8% before treatment)

switched to a  $\text{Foxp3}^+\text{CD25}^-$  cell phenotype representing 4% of CD4 cells on day 7. On day 14 re-expression of CD25 occurred;  $\text{Foxp3}^+$  cells expressing CD25 or not could be observed at high frequencies in the spleen and even more in graft. Thus, although the PC61 treatment was efficient, depletion of Treg could not be achieved and tolerance was maintained.

### ***Transient depletion of dividing T cells by Hydroxyurea induce tolerance***

We also investigated whether the transient depletion of dividing cells obtained by an inhibitor of DNA replication, Hydroxyurea (HU), could also induce tolerance. All FVB mice treated with HU for 12 days at the time of B6 islet transplantation remained tolerant until at least day 54 (Figure 6 A-B). As for  $\text{TK}^+\text{GCV}^+$  mice tolerance was also associated with a persistent decrease of percentages and numbers of CD4 splenic T cells (around 18% vs 30% in naïve mice) and an increased proportion of  $\text{CD25}^+\text{Foxp3}^+$  T cells in the spleen and the grafted islets (Figure 6C), reaching 20-25% compared to 12-13% upon delayed rejection of the graft on day 60.

Finally, we observed that islet grafted mice treated by transient depletion of dividing T cells involving either  $\text{TK}^+\text{GCV}^+$  or HU treated mice, display particular relative numbers of CD4 and CD25 cells, distinct from unmanipulated mice, non-tolerized mice experiencing rejection or  $\text{TK}^+\text{GCV}^+$  non-grafted mice (Fig.6 D).

## Discussion

In this study, the induction of dominant tolerance to islet allografts was obtained through transitory depletion of dividing T cells at the time of transplantation, leading to emergence of Treg cells. Using self-vascularized islet grafts we confirm and extend our previous findings using surgically-vascularized heart allografts (27). The different success rates for tolerance induction differed for self-vascularized heart or skin (0% tolerant, delayed rejection only) (28), islet allografts (63% tolerant), and vascularized heart (84% tolerant) (27, 34). Of interest, percentage of tolerance correlate well with the frequency of T cells triggered to divide, depending on the nature of the graft. Indeed, Jones et al. reported that 14%, 19%, 44%, and of T cells have undergone at least one cell division seven days after skin, islet, and vascularized-heart allografts respectively (35). Thus, allografts that induce a large number of T-cell divisions might be more easily tolerated following a dividing-T-cell depletion protocol, than allografts that induce a smaller number of divisions.

The initial clonal deletion of dividing T cells, including donor-specific T cells is of importance to decrease the size of the cytopathic T-cell compartment (27, 28). Nevertheless, there is no long-term clonal deletion or anergy of effector T cells, nor generalized immunodeficiency and recent thymic emigrants can renew the T-cell compartment and donor-specific T cells. In long-term tolerant mice, although the total number of T lymphocytes was reduced by 30% compared to naïve mice, the immunocompetence was not qualitatively altered in vivo or in vitro. Thus, tolerant mice (i) rejected a third party skin or islet allograft, (ii) maintained proliferative T-cell responses to mitogen, donor and third party antigens, and (iii) maintained donor-specific cytotoxic activities and IFN $\gamma$  production. The fact that in vitro assays of proliferation were done with constant numbers of splenic cells, containing 30% less

T cells in TK<sup>+</sup>GCV<sup>+</sup> mice, underlines that T cell responses from tolerant mice are at least as efficient as those from naïve controls.

The dissociation between *in vitro* reactivity and *in vivo* tolerance, also called “split tolerance”, has been described in various models of tolerance and extensively discussed (36, 37). *In vitro* reactivity is tested against hematopoietic cells of donor haplotype, far from physiological conditions. This differs from the *in vivo* context, where the local concentration and activation of regulatory T cells in graft achieve tissue-specific control of graft rejection, as also previously reported (5, 38). In B6-islet tolerant mice, the B6 skin graft survival was only prolonged (MST 31 days) as compared with third party skin. Of note a similar prolongation of B6 skin graft survival (MST 39 days) was previously observed in TK<sup>+</sup>GCV<sup>+</sup> mice (28). This suggests that the B6 islet allograft could induce a “partial” tolerance to skin of the same H-2<sup>b</sup> haplotype, which is nevertheless per se difficult to tolerize in this model. However, the transfer of splenocytes from B6 islet tolerant mice, while controlling B6 islet rejection in 40% of recipient mice, does not control B6 skin rejection, suggesting that tolerance is dominant, tissue specific and requires a minimal number of tissue specific Treg cells to be achieved (5).

As in other models (7, 39-41), our findings suggest that the induction of tolerance requires two months to be fully and securely established. Mice displaying a delayed rejection, have increased percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells similar to islets tolerant mice and rejected at a slower rate with progressive loss of graft function, suggesting that these cells may exert a partial control of graft-specific effector T cells. Efficient depletion of CD25<sup>+</sup> cells with PC61, did not abrogate tolerance nor induced complete elimination of Foxp3<sup>+</sup> Treg cells that transiently down-regulated CD25<sup>+</sup> expression in accordance with others (42). This suggests that Foxp3<sup>+</sup>CD25<sup>-</sup> and perhaps other regulatory cells play a role in the maintenance of tolerance

We demonstrate that the elimination of dividing T cells for 2 weeks induced a homeostatic imbalance in absolute cell numbers and relative sizes of effector/regulator T cell populations that favor Treg. In absence of graft it resulted in about a 36% decrease in the CD4<sup>+</sup>-cell compartment size within one week (Fig. 4 and (28)), which essentially reflects the natural T-cell renewal rate (43). At the same time, the proportion of CD4<sup>+</sup>CD25<sup>+</sup> cells increased, resulting in Treg percentages and numbers that were two to three times higher. The increased numbers of Treg observed in spleen and LN during GCV treatment could be due to re-circulation of Treg from other compartments including the gut. Also, Treg are resistant to apoptosis (44, 45) which could render them poorly susceptible to depletion.

After cessation of GCV treatment, expansion or de novo generation of Treg may arise, encouraged by the space created by major T-cell depletion. The accumulation of Treg, peaking at three weeks, could result from non-specific or donor-specific Treg selection/expansion because the process also occurs in the absence of an allograft. Of note, a transient T-cell depletion through apoptosis, initiated in normal individuals originally at steady state, is different from the context of pre-existent lymphopenia, where homeostatic proliferation was suspected to induce autoimmunity (46) or to induce rejection via proliferation of memory cells (21). As observed here, homeostatic expansion of regulatory T cells can also take place during the lymphopenic phase of immuno-reconstitution in patients treated for autoimmunity (47), or after T cell depletion via anti-CD52 Ab and rapamycin inducing human renal transplantation tolerance (48)

After the third week, in non-grafted mice thymic export and homeostatic proliferation (Figure 4 and (43)) contributed to return T cell numbers to baseline, in less than two months. In contrast, the presence of the allograft precludes homeostasis recovery and allows for the persistence of elevated proportions of Treg. The presence of alloantigens might stimulates Treg – as revealed by their activated CD62L<sup>lo</sup> CD44<sup>hi</sup> phenotype – allowing their sustained

presence in the spleen, LN and graft. In turn, these activated Treg seem to prevent the CD4<sup>+</sup> T-cell homeostatic recovery in accordance with the role of Treg in the control of T-cell homeostasis (49). Therefore, in long-term tolerant mice the numbers of CD4<sup>+</sup> T cells were maintained at values close to those at the lowest point of depletion.

Finally, apoptosis has been implicated in the induction of transplantation tolerance (20, 50, 51) and may play a role in our model through massive apoptosis of T cells. The capture of apoptotic cells by dendritic cells in the absence of inflammation prevents their maturation (52), favoring the emergence of Treg (53, 54). This is in accordance with the observation that inhibition of dendritic cell maturation by treatment with an active form of vitamin D3 leads to tolerance to both islet and vascularized heart allografts (15).

Depletion of dividing T cells avoids the drawbacks of other models (21, 55) since it decreases the size of the cytopathic donor alloreactive T-cell compartment, but it also targets dividing “memory” T cells turning a typical “2d set” rejection kinetics of a second skin allograft into a primary ones, using GCV in TK<sup>+</sup> recipient or HU treatment (29). Thus, this type of approach could be of interest to control rejection of allograft in naïve but also in allo-sensitized recipients.

Our study is the first to demonstrate that specific and transient depletion of dividing T cells at the time of transplantation is sufficient to alter the homeostatic balance between effector and regulatory T cells and to establish a dominant transferable tolerance to islet allografts. While pharmacogenetic T cell depletion would be difficult to implement in humans, it opens the way for using drugs that induce apoptosis of dividing T cells and mimic the effects of TK/GCV treatment. As shown here, Hydroxyurea is a potent candidate, since tolerance favoring Treg emergence was obtained. Thus, selective depletion of effector and memory cells but preservation of regulatory T cells due to differential division rates or resistance to apoptosis could open a way to manipulate T-cell immune responses *in vivo*.

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## Figure Legends

***Figure 1: Induction of tolerance to B6 islet allograft in diabetic TK<sup>+</sup> mice, treated by GCV for 14 days.***

Glycemia in STZ-induced diabetic recipients (TK<sup>+</sup> or TK<sup>-</sup>) receiving a B6 islet allograft and treated or not treated with GCV. (A-C): control mice which experienced spontaneous rejection; (D): TK<sup>+</sup>GCV<sup>+</sup> tolerant animals (arrows indicate graft removal by nephrectomy) (Mice #327 and #328 were the donors for further cell transfer experiments). (E): TK<sup>+</sup>GCV<sup>+</sup> recipients displaying delayed rejection. (F): Kaplan-Meier estimates of B6 islet allograft survival in TK<sup>+</sup>GCV<sup>+</sup> mice (closed circles, n=22, from D, E) or control mice (open circles, n=12 from A-C) (Logrank test p<0.0001).

***Figure 2: In vitro proliferative, cytotoxic and IFN $\gamma$  responses in TK<sup>+</sup>GCV<sup>+</sup> mice grafted with allogeneic islets.***

(A-D) Splenocyte responses from TK<sup>+</sup>GCV<sup>+</sup> mice grafted with B6 islets. Spleens were harvested from tolerant mice (57 or 100 days after grafts were placed) or from mice that had delayed graft rejection on days 25, 37 and 42 (spleen tested on days 13 to 26 after rejection, normoglycemia in the mice was maintained by insulin administration). Naive mice were non-manipulated FVB mice.

(A): Proliferative response to ConA (on day 3 of culture) tested by <sup>3</sup>H-Tdr uptake; results are expressed as percentage of the response obtained with naïve FVB mice (100%). Each bar represents the response of a separate mouse. (p<0.05 in mice with delayed rejection, p>0.2 in tolerant mice compared to naïve controls, Mann-Whitney U test, )

(B): Proliferative response in MLR (on day 3 of culture) against irradiated splenocytes from FVB recipient (grey bars), B6 donor (closed bars) or BALB/c third party (open bars), (mean  $\pm$  SD from mice either tolerant (n=5), with delayed rejection (n=3) or naïve (n=5)). Responses

against irradiated splenocytes from B6 and BALB/c mice:  $p > 0.2$  (Mann-Whitney U test) comparing tolerant and naive mice,  $p < 0.05$  comparing mice with delayed rejection and tolerant or naïve mice.

(C): CTL activities from separate tolerant mice (closed symbols), 57 or 100 days after grafts were placed. Values are expressed as percentages of chromium release from H-2<sup>b</sup> targets after B6 stimulation (square) or after BALB/c stimulation (circle). Open symbols are responses from control naïve FVB mice.

(D): (Left) IFN $\gamma$  secretion determined by flow cytometry after intracytoplasmic staining. Mean values  $\pm$  SD of % IFN $\gamma$ <sup>+</sup> cells in CD4<sup>+</sup>CD44<sup>+</sup> or CD8<sup>+</sup>CD44<sup>+</sup> T cells, n= 3 mice per group (no significant differences between groups,  $p > 0.05$  Mann-Whitney U test). (Right) Typical dot plot of IFN $\gamma$  staining in gated CD8 T splenocytes from a tolerant mouse with or without in vitro stimulation.

***Figure 3 : In vivo specificity of tolerance and capacities to transfer tolerance***

(A): Kaplan Meier cumulative estimates of survival of skin grafted on TK<sup>+</sup>GCV<sup>+</sup> mice tolerant to B6 islets for more than 60 days (splenectomized and nephrectomized to ascertain tolerance and maintained under insulin), grafted with B6 skin (closed circles, MST 31 $\pm$ 3.5, n=5) and BALB/c skin (closed squares, MST 17 $\pm$ 6.4 days, n=3). Naïve control mice were grafted with B6 skin (open circles, MST 14.4 $\pm$ 0.7 days, n=5) and BALB/c skin (open squares, MST 13.6  $\pm$ 1.1, n=5).

(B-C): Transfer of tolerance to immunocompetent FVB mice: Splenocytes from TK<sup>+</sup>GCV<sup>+</sup> mice tolerant to B6 islet allografts (>80 days) were transferred ( $60 \times 10^6$ /mouse) into 5 FVB mice that had been made diabetic. At the same time, these mice received a B6 islet allograft.

(B) Glycemia of FVB recipients after splenocyte transfer (from the tolerant mice #328 and #327 described in Fig.1D). The arrow indicates islet graft removal. (C) Kaplan-Meier

cumulative survival plot of B6 islets or B6 skin, independently grafted in FVB mice, after cell transfer from tolerant mice (closed square), from non-tolerant mice (open square) or without cell transfer (open circle). Recipient of skin graft were normoglycemic mice.

***Figure 4 : Increased expression of Treg cells following transient depletion of dividing T cells***

(A): Immunohistological analysis of cryostat sections of kidney (K) supporting functional islet allografts (I) on day 104 in a tolerant TK<sup>+</sup>GCV<sup>+</sup> mouse. Lymphocytes (L) were stained with anti-CD4 or anti-CD8 followed by a peroxidase/ AEC reaction and H/E staining. Original magnification x400.

(B): FACS analysis of islet allografts and spleen of tolerant TK<sup>+</sup>GCV<sup>+</sup> mice (day 104). CD25 expression among CD4 gated lymphocytes. CD62L expression in CD4<sup>+</sup>CD25<sup>-</sup> (closed histogram) or CD4<sup>+</sup>CD25<sup>+</sup> (open histogram).

(C-D): FACS analysis of splenocytes from naïve non-treated control FVB mice (n=19), or from TK<sup>+</sup>GCV<sup>+</sup> mice allografted with B6 islets, either experiencing delayed rejection (from days 25 to 66, n= 8, spleen tested on days 13 to 26 after rejection with mice maintained under insuline), or tolerant (>day 57, n = 9) or in TK<sup>+</sup>GCV<sup>-</sup> (on day 27, upon rejection n =2) . (C) Representative FACS dot plot staining in lymphocytes. The numbers in bold represent the % of CD4 T cells expressing CD25. (D) Numbers and percentages of T cells in spleen (the line in the middle of the box represents the 50<sup>th</sup> centile of the variable, the box extends to from the 25<sup>th</sup> and 75<sup>th</sup> centile, the lines extending above represent the 10<sup>th</sup> and 90<sup>th</sup> centile and values above are separately represented by points. \* p<0.05, \*\* p<0.01, Mann Withney.

(E): Dynamics of CD4 and CD4<sup>+</sup>CD25<sup>+</sup> splenocytes in individual mice, with LOWESS smooth. TK<sup>+</sup> mice treated with GCV for 14 days (diamonds) at the time of B6 islet grafts. Naïve mice (open circles), grafted controls insensible to depletion treatment (TK<sup>+</sup>GCV<sup>-</sup>, TK<sup>-</sup>

GCV<sup>+</sup>, triangles) are also shown at time of graft rejection. Outcome of the graft: acute rejection (open triangles), delayed rejection (DR) (grey symbols) or tolerance (closed symbols).

(F): Dynamics of CD4 splenocytes recovered from TK<sup>+</sup> mice treated with GCV for 7 days in the absence of any graft. Left panel: Numbers (mean  $\pm$  SEM, n=3 to 8 per point) of CD4<sup>+</sup> cells (closed circle) and CD4<sup>+</sup>CD25<sup>+</sup> cells (open circle) in the spleen. Right panel: Percent of CD4 T splenocytes expressing CD25 (open circles). The grey bar indicates the period of GCV treatment. Similar kinetics was observed in the LN, and also after a 14 days GCV treatment, in spleen and LN. Closed squares represent the percent of Foxp3<sup>+</sup> cells in CD4 upon a 14 days GCV treatment.

(G-H) Differential expression of Foxp3 in CD4 T cells, on day 21 in non-grafted or B6 islet-grafted mice. (G): FACS dot plot of CD4 T cells gated from pooled LN cells (axillary, brachials, inguinal). Upper panels : TK<sup>+</sup> or TK<sup>-</sup> mice grafted with B6 islets, treated with GCV for 14 days and observed on day 21. The TK<sup>+</sup> mice were normoglycemic and tolerant to the graft (TK<sup>+</sup>GCV<sup>+</sup>d21 tol); the TK<sup>-</sup> mice rejected the graft (non-functional graft). Lower panels: naive mouse (control d0 GCV<sup>-</sup>), stained with Foxp3-PE (left) or with the isotype-PE control mAb (right). (H) : Foxp3 percentages (mean  $\pm$  SEM, 3 mice per group) in CD4 gated T cells recovered from pooled LN or spleen. Non-grafted TK<sup>+</sup> or TK<sup>-</sup> mice were either non-treated (day 0 open histogram) or treated for 14 days with GCV and observed on day 21 (closed histogram). Grafted TK<sup>+</sup> or TK<sup>-</sup> mice were treated with GCV for 14 days and observed on day 21 (as in the upper G panel). Bars indicate significant differences (p<0.05 Mann Whitney U test) between groups in both spleen and LN.

***Figure 5: Maintenance of tolerance by Foxp3<sup>+</sup>CD25<sup>-</sup> cells after PC61 treatment***

(A) TK<sup>+</sup>GCV<sup>+</sup> tolerant mice were treated with anti-CD25 PC61 mAb on day 61 post-islet B6 graft and nephrectomized on day 74. (B) Effects of treatment followed by FACS in the blood

and (C) FACS dot plot before or after PC61 treatment in blood and on day 14 in spleen and graft (i.e. on day 74 post graft).

***Figure 6: Induction of tolerance by HU treatment and characteristic patterns of CD4 and CD25 numbers.***

(A-C): FVB diabetic mice grafted with B6 islets and treated with HU for 12 days. (A) Glycemia of 3 mice (one was nephrectomized on day 54, the second had a delayed rejection on day 60 -analysed on day 61- and the third was still tolerant on day 61). (B) Kaplan-Meier cumulative survival plot of B6 islets in HU treated mice (n=3) compared to untreated controls (n=10) (p=0.0051 Logrank test). (C) Representative FACS dot plot staining in lymphocytes. The numbers in bold represent the % of CD4 T cells expressing Foxp3. The non-treated mouse rejected the B6 islets on day 27 and was analyzed on day 61 (maintained under insulin treatment).

(D) Relative numbers of CD4 and CD25 T cells in the spleen of mice, left panel: grafted with B6 islets after tolerance induction protocols with TK/GCV or HU (tolerant mice: black symbols, delayed rejection: grey symbols), or in grafted controls insensible to depletion treatment and that rejected the graft (TK<sup>+</sup>GCV<sup>-</sup> or TK<sup>-</sup>GCV<sup>+</sup>), independantly of time post graft or ungrafted naïve controls; right panel : in TK<sup>+</sup>GCV<sup>+</sup> non-grafted mice (as in Fig. 4F). Linear regression curves are shown. Dotted areas underline the particular pattern of “naïve non-treated”, “grafted tolerized” or “grafted non-tolerized” mice.

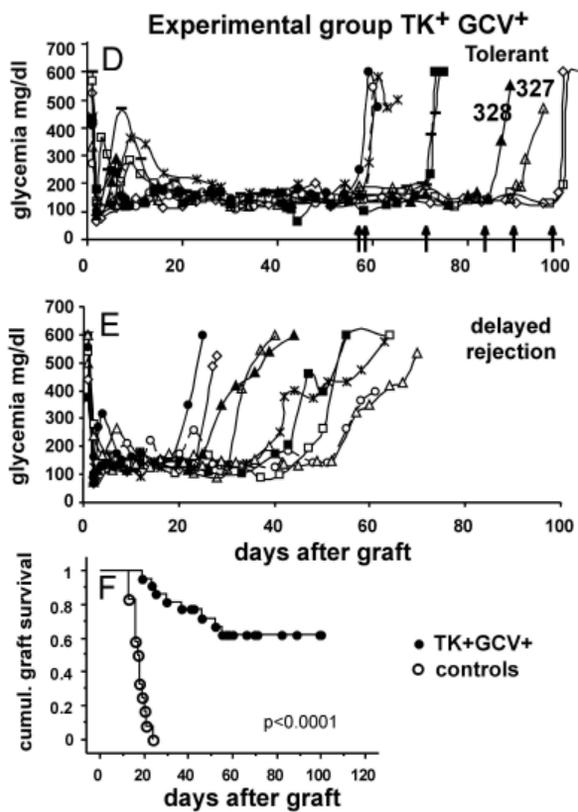
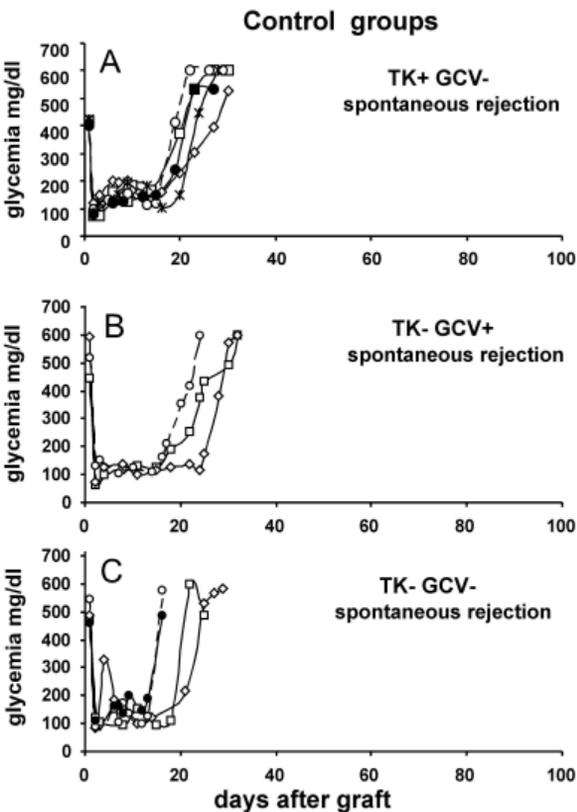
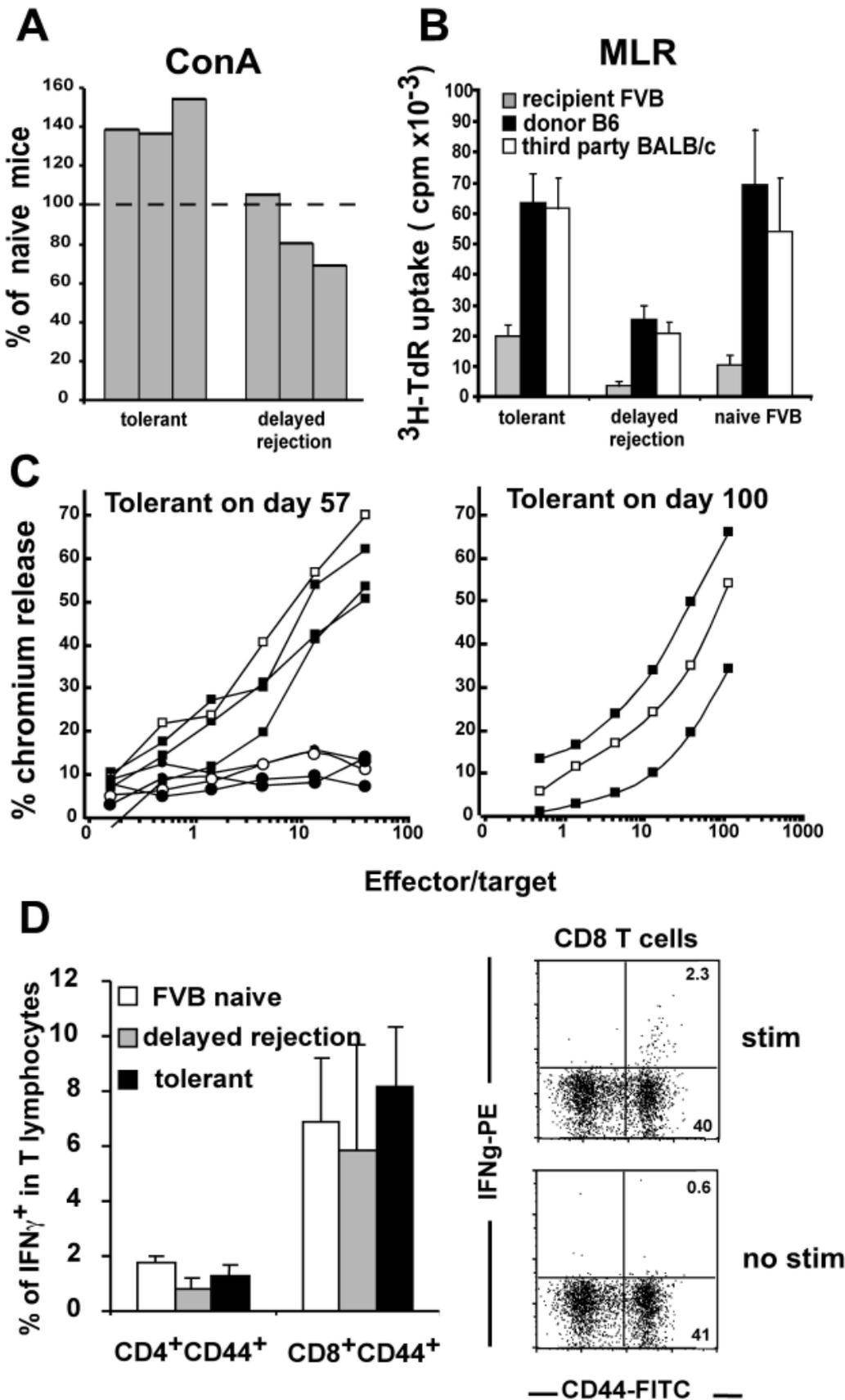


Figure 1



**Figure 2**

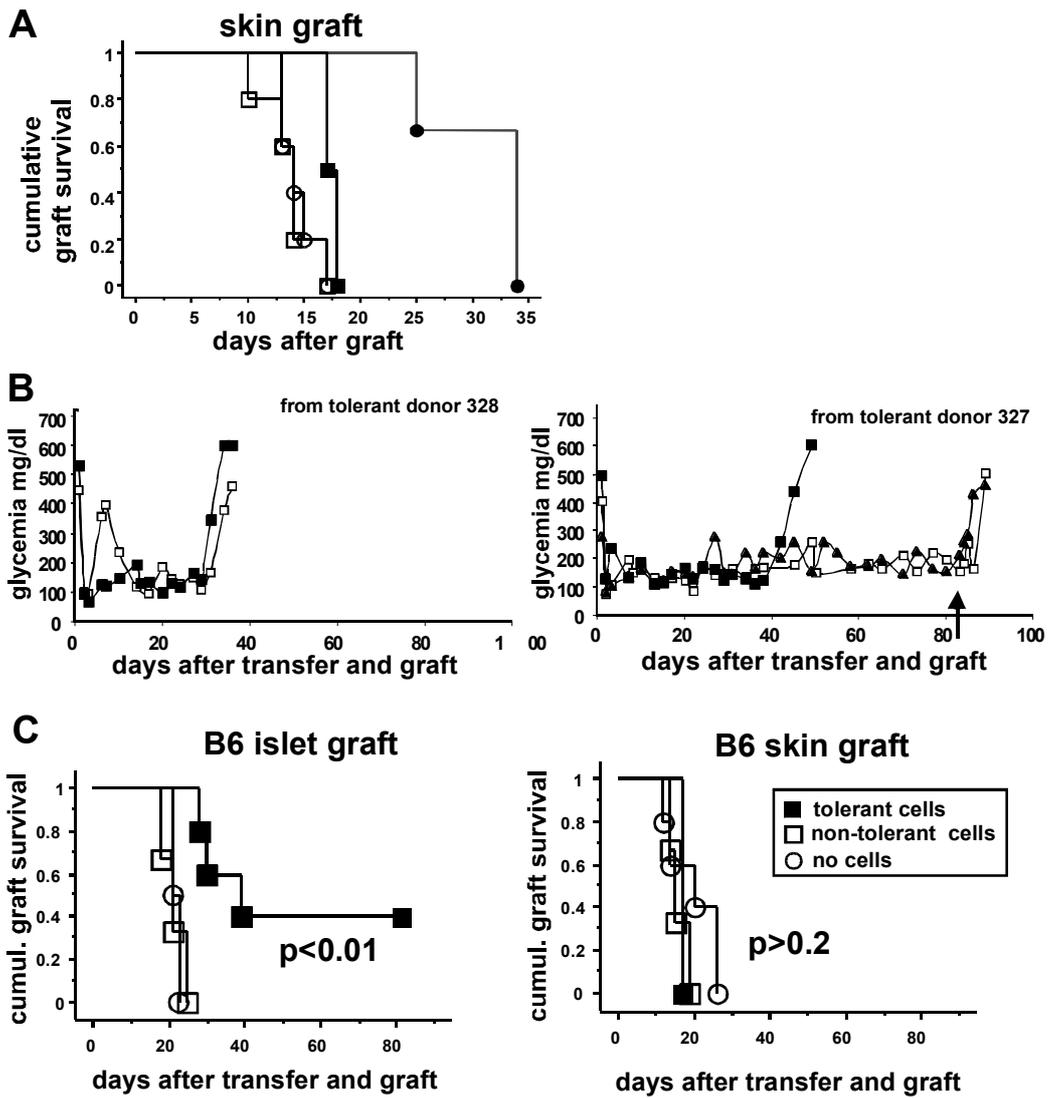
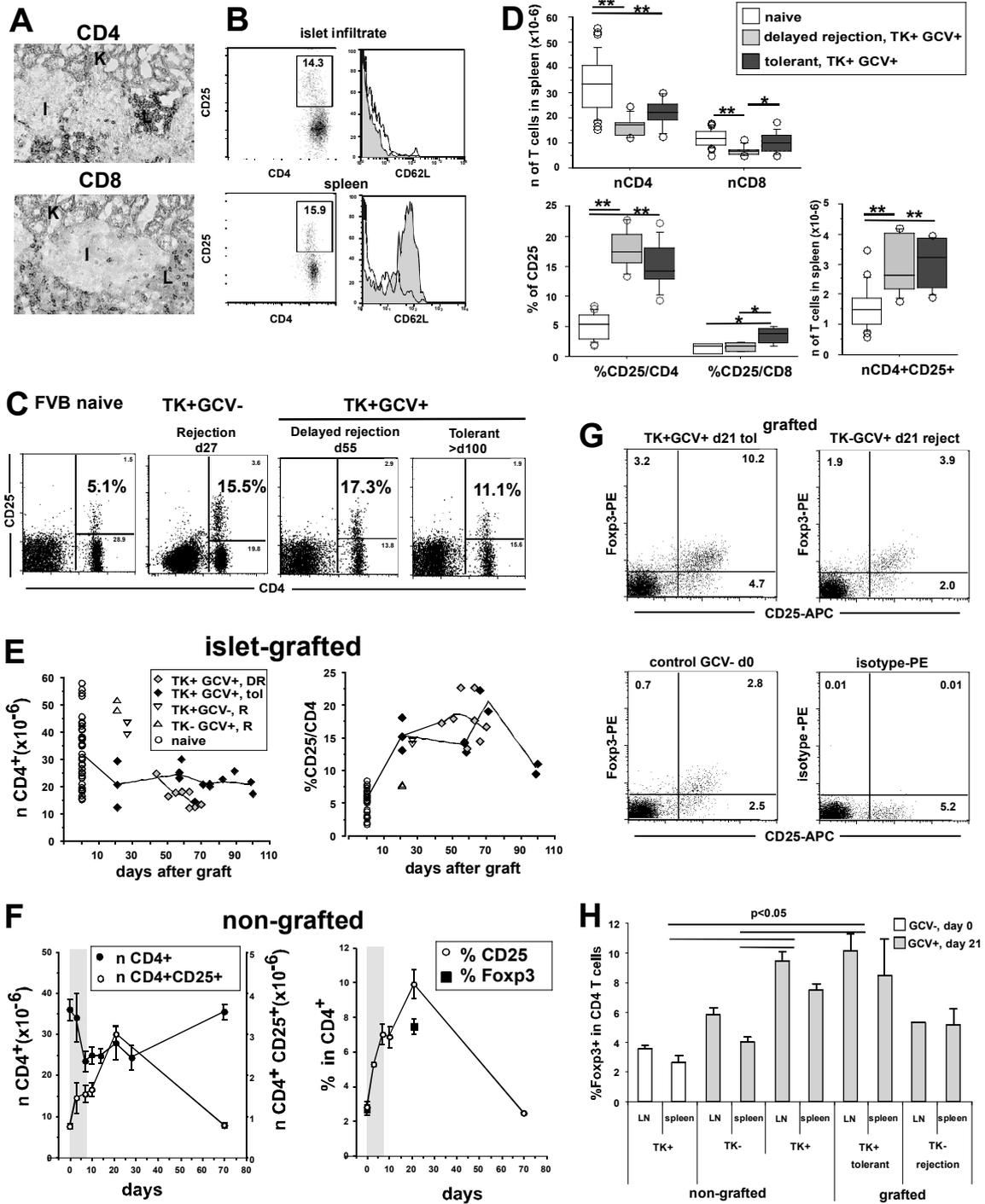
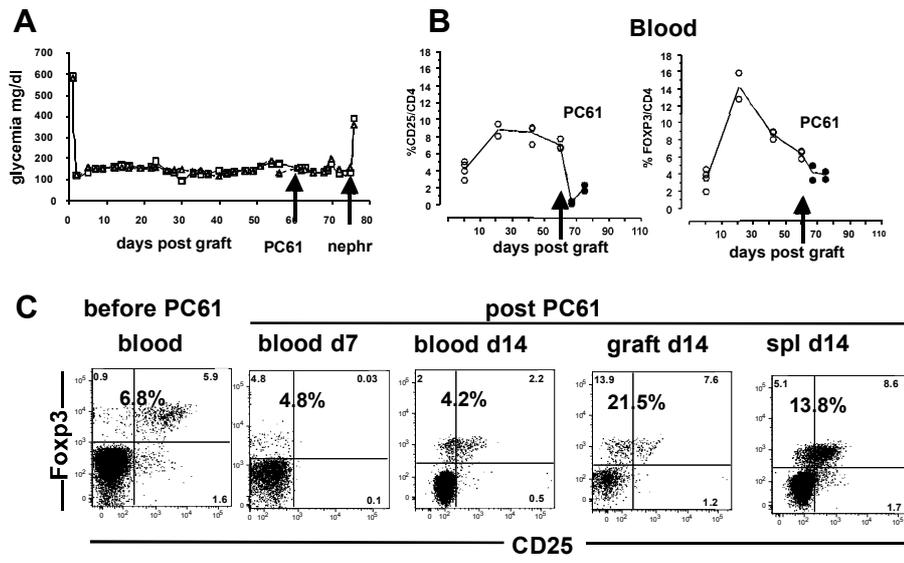


Figure 3

**Figure 4**



**Figure 5**



**Figure 6**

