Anti-CD25 Treatment and FOXP3-Positive Regulatory T Cells in Heart Transplantation

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The interleukin-2 receptor alpha chain (IL-2Ra, CD25) plays a major part in shaping the dynamics of T cell populations following immune activation, due to its role in T cell proliferation and survival. Strategies to blunt the effector responses in transplantation have been developed by devising pharmaceutical agents to block the IL-2 pathways. However, such strategies could adversely affect the CD25+ FOXP3+ Treg populations which also rely on interleukin-2 signaling for survival. The present study shows that a cohort of heart allograft recipients treated with Daclizumab (a humanized anti-CD25 antibody) display FOXP3 expression patterns consistent with functional T regulatory cell populations. High levels of FOXP3 were observed to correlate with lower incidence of and recovery from acute rejection, as well as lower levels of anti-donor HLA antibody production. Therefore, T reg populations appear fully functional in patients treated with Daclizumab, even when
5 doses were administered. By comparison, patients treated with fewer doses or no Daclizumab had a higher incidence of acute rejection, antibody production and graft failure. Therefore, our data indicates that Daclizumab treatment does not interfere with the generation of regulatory T cells and has a beneficial effect on heart allograft survival.

Introduction
The use of calcineurin inhibitors as part of standard immunosuppressive therapy in organ transplantation has resulted in a remarkable increase in the rate of allograft survival. The additional use of various monoclonal (anti-CD3) and polyclonal (antithymocytic/lymphocytic) antibodies (as depletional induction agents) has further contributed to prevention of early episodes of acute rejection (1). However, the risk of infection, malignancy and late rejection remains a looming concern. More recently, a new generation of immunosuppressive agents has been introduced to fine tune T cell responses by specifically targeting activated T cells. The humanized anti-interleukin-2 receptor alpha chain (IL-2R, CD25) monoclonal antibody (Daclizumab, Zenapax®, Hoffmann-La Roche) is such an example. Its efficacy in conjunction with triple immunosuppression has been documented in several studies (1-4).

The underlying hypothesis for the development of this drug has been that blocking of the interleukin-2 receptor may prevent the proliferation and differentiation of effector T cells. However, studies in the field of regulatory T cells have shown that CD25 represents a crucial marker of FOXP3+ T cells with suppressor function (5-12). In the light of this data, the obvious question is whether anti-CD25 treatment results in the blockade or depletion of regulatory T cells and whether quiescence can be attained in their absence. To answer this question we have performed serial determinations of markers deemed to be characteristic of regulatory/suppressor T cells (Treg/Ts), such as FOXP3, CTLA-4, and IL-10, in a cohort of 110 heart recipients transplanted between 2003 and 2006 (Table 1). As putative markers for rejection we used IFN-γ and VEGF. In addition, given the fact that development of anti donor HLA antibodies is detrimental to the graft we monitored such antibodies in patients’ sera. We now report on the positive correlation between quiescence and expression of these Treg markers in peripheral blood CD4+ and CD8+ T cells, demonstrating that the development of regulatory T cells is not impaired in patients treated with Daclizumab. Such regulatory T cells were negatively associated with the occurrence of early acute rejection episodes and development of allo-antibodies. (13-15)

Material and Methods
Patients
110 heart allograft recipients (84 males and 26 females) with a mean age of 49.4 years old (49.4±13.0 SD, range 18-72) were recruited for these studies at the
time of transplantation. The demographic of this study population is shown in Table 1. All patients gave informed consent under the auspices of the appropriate Institutional Review Board. Patients were treated with cyclosporine (CSA), mycophenolate mofetil (MMF), prednisone (MP) and with or without Daclizumab (administered at 1mg/kg body weight).

**Endomyocardial biopsies (EMB)**

Endomyocardial biopsies were performed by the standard transjugular approach weekly for the first month and then at progressively longer intervals to a baseline schedule of every 6 months. The mean number of biopsies per patient was 10 ± 2. A minimum of four biopsy fragments were fixed in 4% buffered formalin, paraffin embedded, and multiple hematoxylin and eosin stained sections from the three levels in the block were examined. An additional fragment was frozen for RT-PCR and Lymphocyte Growth Assays (LGA). Histological grades were assigned according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT): no rejection (grade 0), focal (grade 1A) or diffuse interstitial mononuclear infiltrates without myocyte necrosis (grade 1B), multifocal aggressive infiltrates with myocyte damage (grade 3A), diffuse inflammatory process with necrosis (grade 3B) and diffuse aggressive polymorphous/mononuclear infiltrate with edema, hemorrhage, vasculitis and necrosis (grade 4) (16).

A baseline angiogram was performed within one month of transplantation to detect unsuspected vascular disease. Angiograms were repeated annually. The diagnosis of CAV was made when contrast angiography demonstrated diffused small vessel involvement, defined as concentric and symmetrical narrowing of terminal branches.

**Molecular HLA typing and testing of anti-HLA antibodies**

HLA genotypes of transplant recipient/donor pairs were determined by PCR with sequence specific primers (SSP) using commercially available kits (One Lambda, Los Angeles, CA). Sequential samples of sera obtained from each patient prior to and following transplantation were tested for anti-HLA alloantibodies by lymphocytotoxicity and solid-phase assays to determine the frequency of panel reactive antibodies (PRA). The specificity of the antibodies for the donor’s HLA antigens was assessed by screening and direct cross-matching procedures as previously described (17).

**Lymphocyte Growth Assay**

A biopsy fragment (1mm piece) was placed in medium supplemented with recombinant IL-2 (5 units/ml) and examined microscopically at 48h. Growth was
scored on a semi quantitative scale from 0 to 3 on the basis of circumferential T cell aggregation. A score of 1 or greater was deemed positive (18).

**Quantitative Real Time (qRT)-PCR**
A total of 508 peripheral blood samples from 110 transplant patients were tested by qRT-PCR for expression of various genes at the transcriptional level. CD4 and CD8 cells were isolated from each of these samples by use of commercially available magnetic isolation kits (Miltenyi Biotec, Gladbach, Germany). Additionally, qRT-PCR studies of were performed on 104 EMB samples. Biopsies were stored overnight at 4°C in 5 volumes of RNAlater tissue collection/RNA stabilization solution (Ambion, Foster City, California). After removal of the RNA stabilization solution biopsies were stored at -80°C for subsequent RNA extraction.

Total RNA was isolated from biopsies with the RNAqueous-4PCR kit (Ambion) following the manufacturer’s recommendations. Complementary DNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Basel, Switzerland). Quantitative real-time PCR was performed using Taqman gene expression assays (Applied Biosystems, Foster City, CA). The gene expression assays used in these studies are identified below, by gene name, manufacturer’s abbreviation and part number: Cytotoxic T Lymphocyte Antigen 4 (CTLA-4, Hs00175480_m1), Forkhead box P3 (FOXP3, Hs00203958_m1), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4326317E), Interleukin-10 (IL-10, Hs00174086_m1), Interferon-gamma (IFN-γ, Hs00174143_m1), and vascular endothelial growth factor (VEGF, Hs00173626_m1) all from Applied Biosystems. Forward and reverse primers are contained in different exons, to prevent amplification of genomic DNA. We used the 7300 RT-PCR instrument (Applied Biosystems), applying the manufacturer’s protocols and recommended amplification conditions, as follows: one cycle at 50°C (2 min), then 95°C (10 min), followed by 50 cycles at 95°C (15 s) and 60°C (1 min). Data were collected and analyzed using the 7300 SDS 1.3.1 software (Applied Biosystems). Each assay plate included “no template” negative controls and a control cDNA. The relative amount of gene expression was calculated according to the formula: \[2^{-\Delta Ct}\], where \(\Delta Ct = [Ct(gene) - Ct(GAPDH)]\) and Ct is the “crossing threshold” value returned by the PCR instrument for every gene amplification.

**Statistical Analysis**
The generalized estimating equation (GEE) approach with working independence was used to study the association between two variables. Two GEE modeling approaches were used, one with constant intercept and the other with different intercept term for each subject. The identity link was used for continuous variables, while the logit link was used for rejection, which is binary.

Time to death was used as the end point in overall graft survival analysis,
and patients who were alive were censored at the date of last clinical contact. The Cox proportional hazards regression model was used for multivariate analysis of Daclizumab treatment. Overall graft survival was computed using Kaplan-Meier product-limit estimators and compared by log rank tests. The influence of Daclizumab treatment on the development of anti-HLA antibodies was evaluated by Kaplan-Meier estimators, with $P$ values obtained using log rank statistics. For categorical variables, a 2x2 table was constructed and compared by either chi-square analysis or Fisher's exact (2-tail) test. A non-parametric Wilcoxon exact test was used for analysis of changes in the level of FOXP3 expression in CD4$^+$ and CD8$^+$ T cells from patients with resolving acute rejection episodes. $P$ values less than 0.05 were considered significant. All statistical tests were 2-sided. Statistical analysis of data was performed using the SAS version 9.1 software package (SAS Institute, Cary, NC) (19, 20).

Results

Expression of FOXP3 in CD4 and CD8 T cells from the peripheral blood of heart allograft recipients

Regulatory T cells are characterized by their capacity to suppress the reactivity of other effector populations of antigen-stimulated T cells. Several distinct populations of regulatory T cells, such as thymus-derived CD4 CD25$^+$ FOXP3$^+$ natural T reg (5, 6), antigen-induced CD4$^+$ CD25$^+$ FOXP3$^+$ peripheral T reg (7-9), IL-10-producing CD4 CD25$^+$ FOXP3$^+$ Tr1 (10, 11), CD8$^+$ FOXP3$^+$ Ts (8, 9, 21-23) and CD8$^+$ IL-10-producing Ts (12) have been shown to play a role in human transplantation and experimental models (8). To assess the presence of T reg in human allotransplantation, we analyzed the expression of FOXP3 in peripheral CD4 and CD8 T cell populations as well as in heart allograft biopsies. EMB grade 3A or higher was considered indicative of acute cellular rejection (16). Analysis of 508 peripheral blood samples showed a negative correlation between the level of FOXP3 expression in CD4$^+$ T cells and EMB (N=1557) grade 3A ($p=0.033$, Figure 1).

Although within the CD8 compartment the level of FOXP3 expression was 5-fold higher during quiescence compared to high-grade rejection, the difference did not reach significance ($p>0.296$).

Similarly, analysis of FOXP3 expression in biopsy tissue (N=103) showed a negative association with the grade of rejection which did not reach statistical significance ($P=0.416$). However, the consistent trend for high expression of FOXP3 in peripheral blood and biopsies during quiescence and low expression during episodes of acute rejection suggests that regulatory T cells play a role in allotransplantation.
The dynamic of FOXP3 expression in peripheral blood T cells during resolving rejection

Resolving rejections were defined as lymphocytic infiltrates grade 3A EMB subsiding to grade 1 or 0 within 1 week. We examined the expression of FOXP3 in peripheral blood T cells from 9 such cases. In one of these 9 cases, the CD4 T cells were not testable for technical reasons. Of particular note was the finding that the level of FOXP3 expression went from low levels to higher values during resolving rejection. Thus, in 7/8 rejection episodes, the level of FOXP3 in peripheral blood CD4 T cells increased 7 fold (P=0.036) during the resolving rejection. Similarly, peripheral CD8 T cells showed a mean fold increase of 13 (P=0.028), in 9/9 cases of resolving rejection (Figure 2).

An exception seemed to occur in a patient who showed low FOXP3 (in both CD4 and CD8 peripheral cells) in conjunction with EMB grade 3A, followed one week later by a 10-fold decrease in the level of FOXP3 as rejection appeared to resolve to grade 1 EMB. This patient, however, showed a second episode of rejection grade 3, two weeks after the first grade 3 episode (Figure 3) consistent with the low level of FOXP3 seen at the time when EMB showed only grade 1B infiltrates. The finding that during recurrent rejection the level of FOXP3 expression in peripheral blood T cells stays at a low value reinforces the concept that FOXP3 is associated with T regulatory cells that may play a role in protecting the graft.

FOXP3 associations with the expression of other markers of T cell function

Cytotoxic lymphocyte associated antigen 4 (CTLA-4) negatively regulates the classical costimulation pathways required for efficient T cell activation. This molecule is highly expressed by both CD4 and CD8 regulatory T cells (6, 17, 18). There is also evidence that interleukin-10 mediates the suppressor function of several types of regulatory T cells (8, 10, 11, 12). Because different populations of regulatory T cells may be simultaneously present in the circulation (24, 25), we analyzed the relationship between the level of expression of FOXP3 and that of these suppressor markers in CD4 and CD8 cells from sequential samples of blood. We found a significant correlation between the level of expression of FOXP3 and CTLA-4 as well as FOXP3 and IL-10, irrespective of rejection status (Table 2).

No association between the level of expression of FOXP3 and that of IFN-γ in CD4 and CD8 T cells from the circulation of transplant patients was found during quiescence or rejection (GEE, P-values >0.05, Table 2).

Studies of EMB tissue showed no significant relationships between the level of expression of FOXP3, IL-10, CTLA-4 or IFN-γ (P values >0.05). This most likely reflects the fact that within the EMB tissue, infiltrating T cells represent only a small fraction of the cell sample. Unexpectedly however, there was a significant correlation between the levels of expression of FOXP3 and VEGF (P=0.007, Table 2). This observation was unexpected because VEGF, a growth factor active in angiogenesis, vasculogenesis and endothelial cell growth, was previously shown to be increased in allograft rejection (26).
**FOXP3 is inversely related to the production of anti-donor HLA antibodies**

The generation, differentiation, and maturation of antibody producing B cells are highly dependent on T cell help. Such help can be provided via the production of Th2-type cytokines, or by direct T-B interaction (27). By inhibiting Th (28, 29, and reviewed in ref. 8), T reg can interfere with B cell proliferation and differentiation into Ig secreting plasma cells. To determine whether the presence of T reg may affect the antibody status of the patients, we analyzed the relationship between the level of FOXP3 expression in CD4 and CD8 T cells and the presence of anti-HLA (IgG) antibodies in the patients' circulation. This analysis revealed a negative correlation between high levels of FOXP3 in the CD4 compartment and the development of anti-HLA IgG antibodies (p=0.014, Figure 4A). Patients developing high PRA following transplantation also showed low expression of FOXP3 in CD8 T cells. However, this correlation was not statistically significant (p= 0.976, Figure 4). These findings are consistent with the hypothesis that regulatory T cells suppress the activation and differentiation of alloantibody producing B cells, either directly or acting on allospecific T helper cells.

**Relationship between Daclizumab treatment and immune responses against the graft**

Within this study cohort of 110 patients, 7 were not treated with Daclizumab. All 7 patients showed an early, post-transplantation “spike” of anti-HLA Ab. In three recipients this phenomenon was transient, although the patients remained prone to subsequent sporadic, short-term bouts of anti-HLA antibodies as late as one year after transplantation (Figure 5A, B, C). In the remaining four patients not receiving treatment with Daclizumab anti-HLA antibodies persisted in the circulation over the entire period of observation as illustrated in Figure 5D. The anti-donor-HLA specificity of the antibodies was demonstrated by a direct cross match of the patients’ sera with T cells and B cells from the specific donor spleens.

Study of patients treated with Daclizumab showed that only 42% of the patients that have received 4 or more doses developed anti-HLA antibodies within 24 months post transplantation, compared to 69% and 100% of those who received 1-3 doses or no Daclizumab, respectively (P<0.0001) (Figure 5E, F). Thus, it appears that the activation of the humoral arm of the immune response against the graft was inhibited by Daclizumab in a dose related manner.

We analyzed the frequency of EMB grade 3A or higher in our cohort of patients, after completion of the Daclizumab treatment course. Patients receiving more than 3 doses had a significantly lower incidence of EMB grade 3A rejection compared to patients receiving 0-3 doses (P<0.04, Figure 6). Acute rejection (EMB grade 3+) was strongly associated with positive LGA results which reflect the growth capacity of graft infiltrating cells and their implicitly aggressive nature (P<0.0001, Figure 7). This data indicates that Daclizumab inhibits both the cellular and humoral immune response against the graft.
Effect of Daclizumab treatment on patient survival

Among patients treated with Daclizumab (N= 103), 73.6% received 5 courses of the drug while the remaining 25.4% received lesser amounts. The Cox proportionate hazards model shows that Daclizumab had a protective effect against graft loss, and that this effect was dosage dependent. Thus, receiving three Daclizumab doses or less exposes a patient to 4.3 fold higher risk of graft loss compared to patients receiving four or more treatments (Figure 8A, B). Patients receiving five or more doses of Daclizumab showed the same (about 4-fold) protection from graft loss as those receiving 4 doses, suggesting no further benefit from additional doses. The 12-month actuarial heart allograft survival was 82% among patients receiving 0-3 doses of Daclizumab and 96% among those receiving more (Figure 8C, D).

Discussion

Over the last decade, the literature has been dominated by the concept that regulatory T cells which have the CD4^CD25^FOXP3^ phenotype are crucially important to the maintenance of tolerance to self and non-self antigens (5-8). Studies in rodents have demonstrated unambiguously that this population of regulatory T cells is primarily responsible for protecting the animal from autoimmune diseases (5-8, 30, 31). Evidence that both CD4^ and CD8^FOXP3^ T cells mediate allogeneic tolerance has also been provided by numerous studies in rodents (30, 32, 33).

In spite of the large body on information about the capacity of T reg to inhibit T cell responses in malignancy (34, 35) and certain autoimmune diseases, the data regarding their mechanism of action and clinical significance is still controversial. The controversy emerges primarily from the finding that in human CD25, FOXP3 and CTLA-4 are markers which are shared by Treg with a stable phenotype and by ‘transient’ regulatory T cells which represent only a stage in the differentiation of activated T cells (36, 37). The promiscuity of FOXP3 expression in activated human T cells may explain our finding that FOXP3 and IFN-γ showed no negative association either in EMB or circulating T cells. Similarly, the positive correlation between the expression of FOXP3 and a putative rejection marker VEGF (26) may be caused by the presence of activated rather than regulatory T cells within the graft. On the other hand, it is possible that, by analogy to the situation encountered in cancer, VEGF and IL-10 create an immunosuppressive environment within the graft, inhibiting T cell reactivity (38).

Similarly, the finding of FOXP3 and CTLA-4 expression in peripheral blood CD4 and CD8 T cells even during EMB grade 3 rejection episodes, may also indicate ongoing T cell activation rather than presence of Treg. However, the significant rise in the level of expression of these markers after successful treatment of a rejection episode, together with the negative association between high level of FOXP3 and development of alloantibodies indicates that FOXP3^CD4 and CD8 T cell subsets
have a protective role against rejection in humans, similar to animal models (9, 22, 23, 39, 40). Therefore, our data is consistent with a recently proposed model (36, 37), which suggests that in humans, transient expression of FOXP3 is characteristic of all activated T cells. However, stable expression of FOXP3 differentiates T regulatory cells from activated T cells committed to other effector functions (28, 41). The clinical consequences of this observation cannot be underestimated as they warn against the use of these markers for identifying T suppressors for either diagnostic or prognostic purposes.

Although it is difficult to discriminate between the possibility that the cells expressing CD25, FOXP3 and CTLA-4 are precursors of Th or Treg, the quantitative changes which we observed during resolving rejection argue in favor of continuous monitoring of these markers to predict the outcome of a rejection episode. This view is further supported by the finding that declining levels of FOXP3 occurring following treatment of rejection are indicative of recurrence rather than remission of acute rejection.

The fact that CD25 is expressed by all T cells upon activation explains the apparent inconsistency between the clinical efficacy of Daclizumab as an immunosuppressive agent and its potential to block or deplete CD25+ T reg. Our data contribute evidence that Daclizumab, particularly after repeated administration, inhibits the production of allo-antibodies and improves patient survival, consistent with other authors finding that it reduces the rate of acute rejection. (1-4, 42) Furthermore, the data indicate that CD4+ and CD8+ T regulatory cells develop in patients treated with Daclizumab together with standard calcineurin inhibitory therapy arguing against the view that this type of immunosuppression prevents the differentiation of regulatory T cells.

Acknowledgements

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Figures

**Figure 1.** The level of FOXP3 expression (relative to GAPDH) in peripheral CD4 T cells (A), CD8 T cells (B) and EMB (C) is inversely related to acute rejection grade 3A.

**Figure 2.** The dynamic of FOXP3 expression in peripheral T cells during resolving rejection.

**Figure 3.** The dynamic of FOXP3 expression in CD8+ T cells from a patient with multiple rejection episodes.

**Figure 4.** FOXP3 expression in peripheral T cells is inversely related to the development of anti-HLA antibodies in heart transplant recipients.
Figure 5. Anti-HLA antibody production. Daclizumab non-treated patients recurrent (A, B, C) or prolonged (D) anti-HLA antibody production. Log rank statistics (E) and cumulative incidence of anti-donor antibody production (F) in patients receiving various Daclizumab treatments.

Figure 6. Incidence of acute rejection after completion of Daclizumab treatment(s).

Figure 7. Relationship between development of acute rejection (EMB grade ≥3A) and LGA results.

Figure 8. Protective effect of Daclizumab on heart graft survival. Daclizumab treatment(s) and hazard ratios of graft loss (A, B) and actuarial survival (C, D).

References


Table 1

**Table 1. Demographics and immunopathological findings**

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<th>Study population transplanted between 06/21/2003 and 05/12/2006</th>
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<tr>
<td>Age (mean ±S.D.)</td>
</tr>
<tr>
<td>Age range:</td>
</tr>
<tr>
<td>Gender: Male</td>
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<tr>
<td>Female</td>
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**Endomyocardial Biopsy Grade Tests**

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<td>57%</td>
</tr>
<tr>
<td>Grade 1A</td>
<td>560</td>
<td>35%</td>
</tr>
<tr>
<td>Grade 1B</td>
<td>73</td>
<td>5%</td>
</tr>
<tr>
<td>Grade 2</td>
<td>9</td>
<td>1%</td>
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<tr>
<td>Grade 3A</td>
<td>36</td>
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**Anti-HLA antibodies screening Tests**

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**Lymphocyte Growth Assay (LGA) Tests**

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Figure 1

A. FoxP3 expression in peripheral CD4 cells

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<th>mean</th>
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<tr>
<td>rejection</td>
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<td>0.0200</td>
</tr>
<tr>
<td>no-rejection</td>
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B. FoxP3 expression in peripheral CD8 cells

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<tbody>
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<tr>
<td>no-rejection</td>
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C. FoxP3 expression in Heart Biopsies

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D. Response and Covariate

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<th>P- value*</th>
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<tr>
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<td>-1.323</td>
<td>(-2.476, -0.169)</td>
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<tr>
<td>CD8 / FOXP3</td>
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<td>-0.030</td>
<td>(-0.085, 0.026)</td>
<td>0.296 (N.S.)</td>
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<td>BX / FOXP3</td>
<td>103</td>
<td>-0.115</td>
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* GEE test
Expression of FoxP3 in CD4+ and CD8+ Peripheral Blood T Cells after Successful Treatment of High Grade (≥3A) Rejections

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<th>CD4+ FoxP3</th>
<th>CD8+ FoxP3</th>
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<td>9/9</td>
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<tr>
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<td>13.52 ± 30.23</td>
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<tr>
<td>Range</td>
<td>4.41 – 27.88</td>
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<tr>
<td>P-value *</td>
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<td>0.028</td>
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* Wilcoxon Exact Test
Figure 3

![Graph showing CD8 T cell counts and FoxP3 expression over biopsy grades.](image)
Table 2. FoxP3 as a marker of suppression in transplant patients: Strong associations with the suppressive markers CTLA4 and IL-10 in T cells (irrespective of rejection status)

<table>
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<td>FOXP3</td>
<td>CTLA4</td>
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<td>0.765</td>
<td>(0.213, 1.317)</td>
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<td>CD8</td>
<td>FOXP3</td>
<td>CTLA4</td>
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<td>0.594</td>
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<td>CD4</td>
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<td>(0.249, 0.452)</td>
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<td>CD8</td>
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<td>IL-10</td>
<td>242</td>
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<td>(0.474, 3.684)</td>
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<tr>
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<td>IFN-G</td>
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<td>CD8</td>
<td>FOXP3</td>
<td>IFN-G</td>
<td>252</td>
<td>-0.070</td>
<td>(-2.257, 2.118)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* GEE tests
Figure 4

Association between anti-HLA Ab and FoxP3 expression (GEE test)

<table>
<thead>
<tr>
<th>Response</th>
<th>Covariate</th>
<th>n</th>
<th>Estimate</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HLA IgG</td>
<td>CD4 / FOXP3</td>
<td>255</td>
<td>-0.068</td>
<td>(-0.123, -0.014)</td>
<td>0.014</td>
</tr>
<tr>
<td>Anti-HLA IgG</td>
<td>CD8 / FOXP3</td>
<td>252</td>
<td>-0.0003</td>
<td>(-0.021, -0.020)</td>
<td>N.S. (0.976)</td>
</tr>
</tbody>
</table>

Anti-HLA (IgG) Antibodies

FOXP3 expression in peripheral CD4 cells

FOXP3 expression in peripheral CD8 cells
Figure 5

A. 
Legend
- Anti-HLA (IgM) ab
- Anti-HLA (IgG) ab

B. 

C. 

D. 

E. 

<table>
<thead>
<tr>
<th>Number of Daclizumab treatments</th>
<th>0</th>
<th>1-3 Doses</th>
<th>&gt;3 Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo.</td>
<td>N=7</td>
<td>N=16</td>
<td>N=87</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>38</td>
<td>23</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>38</td>
<td>31</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
<td>69</td>
<td>39</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
<td>69</td>
<td>42</td>
</tr>
</tbody>
</table>

Log rank statistics: P-value<0.0001

F. 
Cumulative incidence (%) of anti-donor Ab production
### Figure 6

<table>
<thead>
<tr>
<th>Daclizumab Treatment (doses)</th>
<th>Acute Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>0-3</td>
<td>6</td>
</tr>
<tr>
<td>&gt; 3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

Chi square test, \( P < 0.04 \)
**Figure 7**

<table>
<thead>
<tr>
<th>Acute Rej.</th>
<th>LGA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>20</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>-</td>
<td>138</td>
<td>1311</td>
<td>1449</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>1325</td>
<td>1483</td>
</tr>
</tbody>
</table>

Chi square test, $P<0.0001$
Figure 8

A.  
Bar graph showing the comparison of graft loss hazard ratio between different numbers of Daclizumab treatment courses.

B.  
Comparison of # of Daclizumab courses  
<table>
<thead>
<tr>
<th>Comparison of # of Daclizumab courses</th>
<th>Hazard Ratio</th>
<th>95% Hazard Ratio Confidence limits</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0) vs (&gt;0)</td>
<td>0.420</td>
<td>0.051 3.434</td>
<td>0.419</td>
</tr>
<tr>
<td>(≤1) vs (&gt;1)</td>
<td>0.481</td>
<td>0.100 2.321</td>
<td>0.362</td>
</tr>
<tr>
<td>(≤2) vs (&gt;2)</td>
<td>0.342</td>
<td>0.085 1.379</td>
<td>0.132</td>
</tr>
<tr>
<td>(≤3) vs (&gt;3)</td>
<td>0.234</td>
<td>0.062 0.883</td>
<td>0.032</td>
</tr>
<tr>
<td>(≤4) vs (&gt;4)</td>
<td>0.269</td>
<td>0.071 1.016</td>
<td>0.053</td>
</tr>
</tbody>
</table>

* Cox proportional hazard model

C.  
Graph showing survival over months post transplantation for Group A (0-3 Daclizumab Treatments) and Group B (4+ Daclizumab Treatments) with a P-value of 0.028.

D.  
Comparison of patient survival over months post transplantation for Group A (0-3 doses of Daclizumab) N=23 and Group B (4+ doses of Daclizumab) N=87.

<table>
<thead>
<tr>
<th>Mo. Post-Tx</th>
<th>Patient Group A (0-3 doses of Daclizumab) N=23</th>
<th>Patient Group B (4+ doses of Daclizumab) N=87</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>86</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>82</td>
<td>96</td>
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<tr>
<td>18</td>
<td>82</td>
<td>93</td>
</tr>
<tr>
<td>24</td>
<td>82</td>
<td>93</td>
</tr>
<tr>
<td>30</td>
<td>82</td>
<td>89</td>
</tr>
<tr>
<td>36</td>
<td>82</td>
<td>89</td>
</tr>
</tbody>
</table>

Log rank statistics: P-value = 0.028