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ORIGINAL ARTICLE

Effect of different induction strategies on effector, regulatory and memory lymphocyte sub-populations in clinical islet transplantation

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Summary

This prospective study assessed lymphocyte subsets in the peripheral blood of 42 islet allograft recipients using flow cytometry from 2 weeks and up to 2 years post-transplantation. Subjects received daclizumab ($n = 16$), Thymoglobulin ($n = 12$) or alemtuzumab ($n = 14$). Alemtuzumab was associated with an early (within 1 month) and transient (up to 6 months) increase in the frequency of $CD3^+$ $CD4^+$ $Foxp3^+$ T cells, while daclizumab induced a near complete loss of these cells ($P \leq 0.001$). The frequency of memory $CD4^+$ T cells was increased following depleting immunosuppression induction with either Thymoglobulin or alemtuzumab ($P \leq 0.05$), but remained unchanged while using daclizumab. Alemtuzumab induction resulted in a significant loss of memory B lymphocytes when compared with the other induction groups ($P \leq 0.001$). While the clinical significance of these findings remains to be fully determined, the observed altered balance between effector, regulatory and memory cells suggests that the immune status of patients will be affected according to the induction strategy chosen.

Over the past decade, the use of antibody-based immunosuppression induction therapy has increased for almost all types of organ transplantation. The most common agents are anti-CD25 (daclizumab and basiliximab), rabbit anti-thymocyte globulin (Thymoglobulin) and anti-CD52 (alemtuzumab) antibodies [1].

The decision to use one therapy or the other has mainly been guided by tolerability and their ability to prevent early acute rejection episodes. However, there is increasing evidence that antibody-mediated induction therapies induce long-term and sometimes permanent changes in the profile of lymphocytes, especially with the marked depletion observed following Thymoglobulin and alemtuzumab therapies (reviewed in [2]).

Daclizumab, Thymoglobulin and alemtuzumab are individually based on different modes of action and are thus expected to exert different effects on various lymphocyte sub-populations. Daclizumab is generally recognized to be a nondepleting humanized monoclonal antibody (mAb) that blocks the IL-2 receptor α chain (CD25) and inhibits lymphocyte activation [3]. Thymoglobulin is a depleting rabbit polyclonal antibody directed against multiple antigens expressed on T lymphocytes. Alemtuzumab is a depleting humanized mAb targeting $CD52^+$ cells, which include T and B lymphocytes, monocytes and dendritic cells [4].

The effect of different induction strategies upon various lymphocyte sub-populations, including effector, regulatory and memory cells remains to be fully defined, especially during the first months after transplantation when most rejection episodes occur. The present prospective study assessed peripheral blood lymphocyte sub-popula-

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tions by flow cytometry, in subjects undergoing clinical islet transplantation and receiving three alternative induction strategies in a single center.

Patients and methods

Study design and inclusion criteria

This prospective study investigated the impact of three alternative induction strategies upon peripheral blood lymphocyte profiles in subjects undergoing clinical islet transplantation alone at a single center. It was performed between April 2003 and September 2007 and has been reviewed and approved by the Health Research Ethics Board at the University of Alberta. Written consent was obtained from all subjects.

Immunosuppression

Subjects were grouped according to the induction therapy as daclizumab, Thymoglobulin and alemtuzumab. In the first group, daclizumab (Zenapax; Hoffman-La-Roche, Mississauga, ON, Canada) was given as described in Table 1 and was followed by a combination of tacrolimus (Prograf; Astellas, Markham, ON, Canada) and sirolimus (Rapamune; Wyeth, Laval, QC, Canada). Thymoglobulin (Genzyme, Mississauga, ON, Canada) was combined with an anti-TNF therapy (etanercept, Enbrel, Wyeth, Laval, QC, Canada) and was followed by a maintenance therapy with tacrolimus (level controlled 5–10 ng/ml) and mycophenolate mofetil (2 g/day or less as tolerated, Hoffman-La-Roche). In the last group, two regimens of alemtuzumab (Campath-1H; Genzyme) were used as shown in Table 1. They were followed either by a combination of tacrolimus (level controlled 8–10 ng/ml) and mycophenolate mofetil (up to 2 g/day as tolerated) or a combination of sirolimus (levels 14–18 ng/ml) and ultra-low-dose tacrolimus (0.5 mg given every second day).

Table 1. Immunosuppression protocols.

Groups	Induction	Maintenance
Daclizumab	Daclizumab 2 mg/kg i.v. days 0 and 5	Sirolimus, tacrolimus
Thymoglobulin	Thymoglobulin 6 mg/kg i.v. over 72 h pretransplant etanercept 50 mg i.v. days 0 and 25 mg s.c. on days 3, 7 and 10	Tacrolimus, MMF
Alemtuzumab	Alemtuzumab 30 mg i.v. day −1	Tacrolimus, MMF
	Alemtuzumab 20 mg i.v. days −2 and −1	Sirolimus, low-dose tacrolimus

Sample collection and flow cytometric analysis

Patients were assessed by flow cytometry of peripheral white blood cells prior to and at various time points after transplantation, during monthly clinical assessments for up to 24 months post-transplantation. Values from treated subjects were compared with untreated, nondiabetic healthy controls ($n = 5$ samples).

Fasting blood samples were collected in acid citrate dextrose solution A tubes for flow cytometry and ethylenediaminetetraacetic acid (EDTA) tubes for lymphocyte counts (Becton Dickinson, Franklin Lakes, NJ, USA). Lymphocyte counts were obtained from the clinical hospital laboratory.

Flow cytometry samples were processed within three days on fresh cells. Fluorescent anti-human CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD20 (2H7), CD25 (2A3), CD45RA (HI100), CD45RO (UCHL1), CD62L (DREG-56), Forkhead box P3 (FoxP3, clone PCH101), HLA-DR (G46-6), NKT2 (6B11; all from BD Biosciences, San Jose, CA, USA except Foxp3 from eBiosciences, San Diego, CA, USA) were used as various four-color antibody combinations. After washing, white blood cells were incubated with the antibodies targeting cell surface antigens for 30 min at 4 °C. For Foxp3 staining, the cells were permeabilized using a Fixation-Permeabilization kit (eBiosciences), rat serum was used for 15 min at 4 °C to prevent Fc binding, and the cells were incubated with anti-Foxp3 antibody for 30 min at 4 °C. At the end of the staining, remaining red blood cells were lysed. A FACSCalibur flow cytometer (BD Biosciences) equipped with CELLQUEST PRO software was used for data acquisition and analysis. Over 1500 gated lymphocytes were assessed for each study, even in highly lymphocyte-depleted patients.

In order to rule out a possible interaction between daclizumab and Foxp3 labeling, we performed a triple staining for CD4, CD25 (2A3) and FoxP3 in lymphocytes from healthy untreated nondiabetic controls ($n = 3$, each run in triplicate) with or without preincubation with 10 μg/ml daclizumab for 60 min at 4 °C. While the frequency of $CD4^+$ $CD25^+$ cells decreased from 38 ± 4.6% to 11 ± 2.7% ($P \leq 0.0001$), resulting from fixation of daclizumab, Foxp3 staining remained stable (53 ± 4.6% without and 54 ± 5.3% with daclizumab, P = NS).

Data processing and statistical analysis

Flow cytometry results were expressed as relative numbers. For $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, $CD20^+$ HLA-DR^+ and $CD3^+$ $CD4^+$ $FoxP3^+$ cells, data were also provided as absolute numbers, calculated from the lymphocyte counts and based on the frequency assessed by flow cytometry. Results obtained at 1 month (time of the peak observed

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change in frequency) were compared between patients who were subsequently insulin-free at the 12 months time point and those who were not. For the clinical comparison, only complete procedures (transplantations of ≥10 000 islet equivalent/kg of recipient body weight), with at least 12 months of follow up were taken into account. Panel reactive antibody (PRA) levels were assessed every 3 months after transplantation by a flow-based technique [5].

Categorical variables were studied using Fisher's test. Continuous variables were assessed by nonparametric tests, including Mann–Whitney and Kruskal–Wallis tests. Flow results were compared between groups and according to time with a two-way ANOVA performed on the ranks and using a Bonferroni test as *post hoc* test. Results were reported as mean ± SE. *P* values less than 0.05 were considered significant. Calculations used SPSS 15.0 software (SPSS, Chicago, IL, USA).

Results

Patient characteristics

Forty-two subjects were included in the study (20 females and 22 males, with a mean age of 47 ± 1.6 years). Sixteen subjects were in the daclizumab group, 12 in the Thymoglobulin and 14 in the alemtuzumab (five receiving one dose and nine two doses of alemtuzumab, as described in Table 1). The subjects in the alemtuzumab group were younger than those in the other groups (40 ± 10 vs. 50 ± 10 years in daclizumab and 53 ± 8 years in Thymoglobulin). Flow studies were performed up to 24 months after transplantation, with a mean of 8 ± 0.8 months. In total, 162 samples were analyzed in the various groups with multiple staining combinations. Of note, all data were similar between the two alemtuzumab sub-groups, and results are only presented for the combined group (14 subjects).

Changes in lymphocyte, CD4, CD8 and B-cell numbers

Lymphocyte counts remained stable after daclizumab induction (P = NS, Fig. 1). In contrast, a sharp drop in lymphocytes was observed after Thymoglobulin or alemtuzumab induction, as expected ($P \leq 0.001$ when compared with daclizumab). The decrease in lymphocyte count was greater with alemtuzumab than with Thymoglobulin ($P \leq 0.001$).

Assessment of specific lymphocyte subsets showed that $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ and B lymphocyte ($CD20^+$ HLA-DR^+ cells) counts remained stable after daclizumab induction (Fig. 2a, c and e). In contrast, the other two groups demonstrated decreased counts of $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ and B lymphocytes after induction ($P \leq 0.05$). When looking at the frequency of cells among lymphocytes, the proportion of $CD8^+$ cells remained stable in all groups despite the decrease in the overall lymphocyte counts observed with the depleting induction therapies. However, $CD4^+$ cells decreased proportionally more than the other cells after Thymoglobulin and alemtuzumab treatments ($P \leq 0.001$ versus daclizumab), consistent with the reduced CD4 to CD8 ratio seen in a number of previous studies [2]. Only alemtuzumab decreased the frequency of B cells among circulating lymphocytes ($P \leq 0.001$ when compared with the other two groups, Fig. 2f).

Changes in cells expressing regulatory markers

The frequency of $CD25^+$ cells among $CD3^+$ $CD4^+$ lymphocytes remained stable after Thymoglobulin and

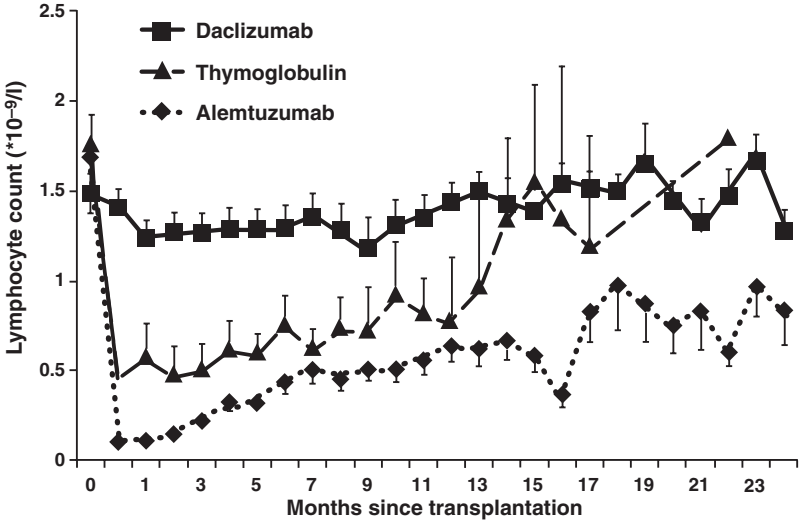

Figure 1 Lymphocyte counts after islet transplantation with daclizumab, Thymoglobulin or alemtuzumab induction ($P \leq 0.001$ between all groups, ANOVA). Induction therapy was started on day 0.

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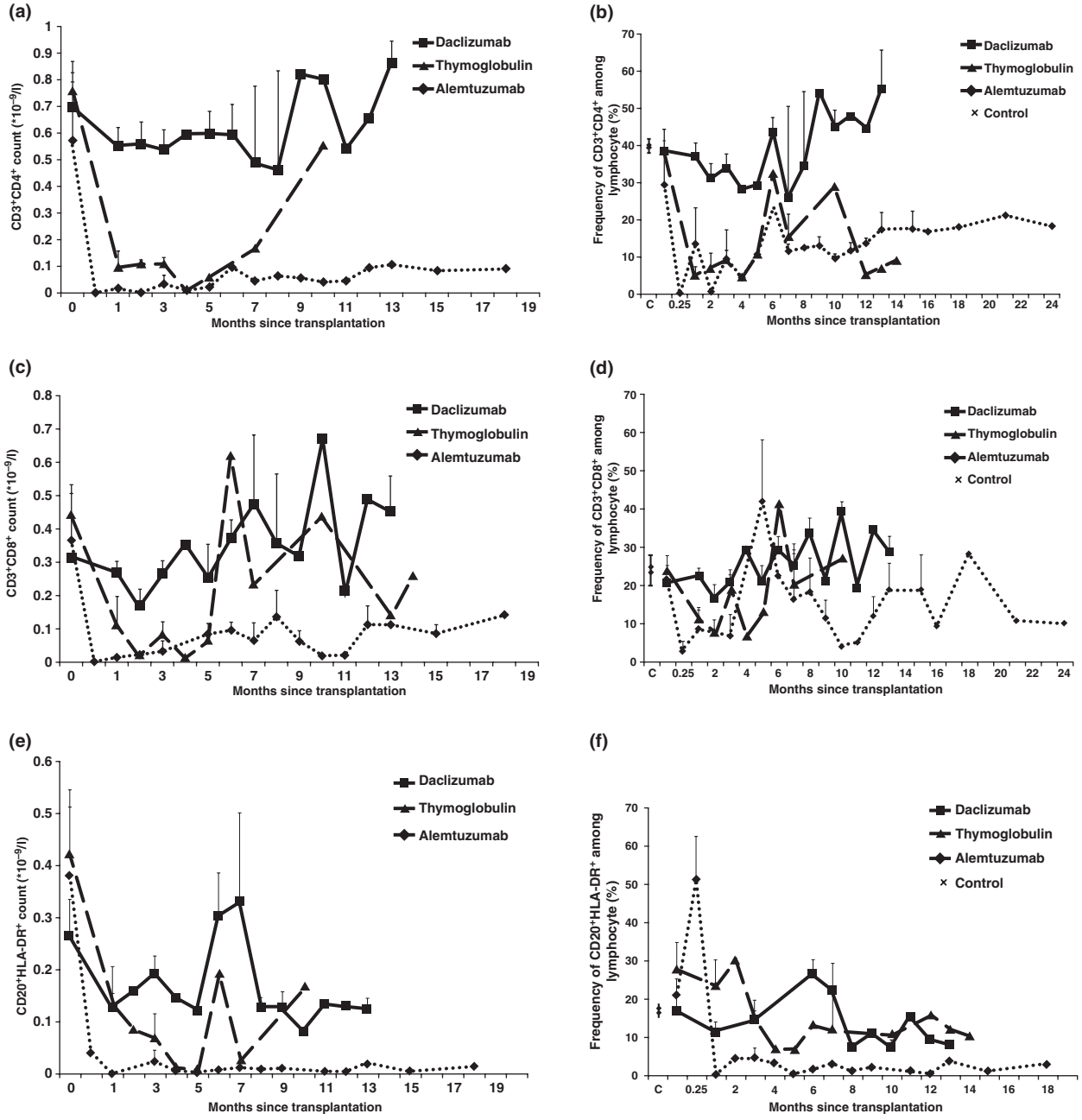

Figure 2 Islet transplantation with daclizumab, Thymoglobulin or alemtuzumab induction: (a) absolute $CD3^+$ $CD4^+$ counts ($P \leq 0.001$ between daclizumab and alemtuzumab or Thymoglobulin); (b) relative frequency of $CD3^+$ $CD4^+$ cells among lymphocytes ($P \leq 0.001$ between daclizumab and Thymoglobulin; $P \leq 0.01$ between alemtuzumab and Thymoglobulin; $P \leq 0.05$ between daclizumab and alemtuzumab); (c) absolute $CD8^+$ counts ($P \leq 0.001$ between daclizumab and alemtuzumab or Thymoglobulin; $P \leq 0.01$ between alemtuzumab and Thymoglobulin); (d) relative frequency of $CD8^+$ cells among lymphocytes (P = NS); (e) absolute $CD20^+$ $HLA\text{-}DR^+$ counts ($P \leq 0.001$ between alemtuzumab and daclizumab or Thymoglobulin); (f) relative frequency of $CD20^+$ $HLA\text{-}DR^+$ cells among lymphocytes ($P \leq 0.001$ between alemtuzumab and daclizumab or Thymoglobulin). 'C' on the *x*-axis refers to values of healthy nondiabetic controls. Induction therapy was started on day 0.

alemtuzumab induction (Fig. 3b). In contrast, the frequency of $CD25^+$ cells appeared to decrease drastically during the first 6 months after daclizumab induction ($P \leq 0.001$ versus alemtuzumab and $P = 0.002$ versus Thymoglobulin, ANOVA, Fig. 3b). As the anti-CD25 antibody used for our study (clone 2A3) is specific for the

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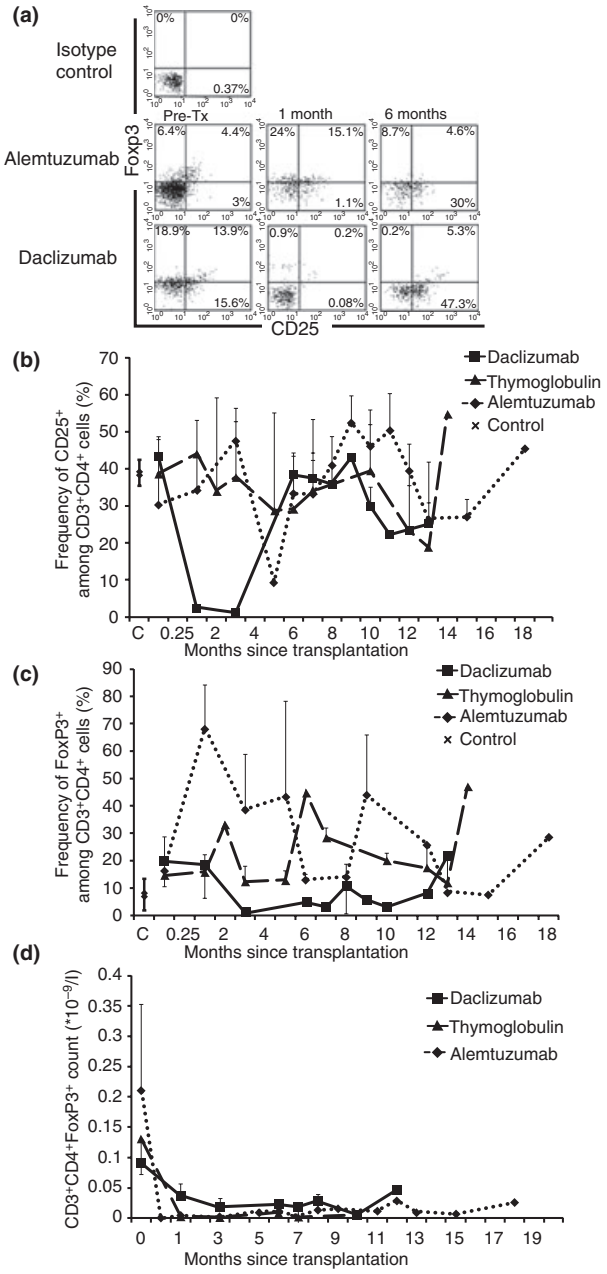

Figure 3 Islet transplantation with daclizumab, Thymoglobulin or alemtuzumab induction: (a) Representative dot plots of CD25/Foxp3 staining on $CD3^+$ $CD4^+$ gated cells and following alemtuzumab or daclizumab induction; (b) frequency of $CD25^+$ cells among $CD3^+$ $CD4^+$ ($P \leq 0.001$ between daclizumab and alemtuzumab, $P \leq 0.01$ between daclizumab and Thymoglobulin within the first 6 months); (c) $FoxP3^+$ cells among $CD3^+$ $CD4^+$ cells ($P \leq 0.01$ between daclizumab and Thymoglobulin; $P \leq 0.001$ between daclizumab and alemtuzumab); (d) $CD3^+$ $CD4^+$ $FoxP3^+$ counts (P = NS). 'C' on the *x*-axis refers to values of healthy nondiabetic controls. Induction therapy was started on day 0.

same epitope as daclizumab (unlike clone M-A251, which targets another epitope on CD25), the observed early low $CD25^+$ levels may be as a result of depletion of these cells, down-regulation of the receptor, or competition with the daclizumab antibody. Therefore, this analysis could not provide information regarding the fate of potential regulatory ($CD25^+$) T cells postinduction therapy.

In order to further explore this question, an independent intracellular marker, the transcription factor $Foxp3^+$, was assessed. In the control group, the frequency of $CD25^+$ $Foxp3^+$ among $CD3^+$ $CD4^+$ cells was $4 \pm 1.8\%$, which is similar to previously reported values and suggests that the gating was appropriate (Fig. 3a) [6].

Further analysis looked at $Foxp3^+$ cells (without CD25, because of the interaction with daclizumab) among $CD3^+$ $CD4^+$ T lymphocytes. These $Foxp3^+$ cells remained stable after Thymoglobulin induction. However, a sharp increase (up to 4.2-fold) was observed starting from the first month after alemtuzumab induction ($P \leq 0.05$ versus pretransplantation, Fig. 3c). Levels returned to baseline after 6 months. In contrast, the frequency of $Foxp3^+$ cells decreased to almost undetectable levels after daclizumab induction ($P \leq 0.001$ versus alemtuzumab and versus Thymoglobulin, ANOVA, Fig. 3c). This reduction was most evident at 3 months, where all six patients analyzed at this time had greatly reduced $Foxp3^+$ cells (Fig. 3c). Despite the contrasting relative frequency of $Foxp3^+$ cells after daclizumab and alemtuzumab, all groups demonstrated similar absolute counts of $CD3^+$ $CD4^+$ $Foxp3^+$ lymphocytes (P = NS), with a sustained drop for at least 12 months ($P \leq 0.001$, Fig. 3d). It should be noted that after alemtuzumab induction, patients demonstrated similar frequencies of $Foxp3^+$ $CD4^+$ T lymphocytes irrespective of whether or not they received sirolimus maintenance therapy (P = NS).

Changes in memory cells

Memory $CD4^+$ T lymphocytes were studied according to the expression of CD45RA and CD62L, as previously described [7]. The frequency of central memory ($CD45RA^-$ $CD62L^+$) cells among $CD4^+$ T lymphocytes remained stable over time in all three groups (Fig. 4a). However, higher frequencies of $CD45RA^-$ $CD62L^-$ (effector memory) and $CD45RA^+$ $CD62L^-$ cells among $CD4^+$ were observed after depleting induction therapies (Thymoglobulin and alemtuzumab) than after daclizumab induction (Fig. 4b and c, Thymoglobulin versus daclizumab, $P \leq 0.05$ and $P \leq 0.001$; alemtuzumab versus daclizumab, $P \leq 0.001$ and $P \leq 0.001$ for the frequencies of $CD45RA^-$ $CD62L^-$ and $CD45RA^+$ $CD62L^-$ cells

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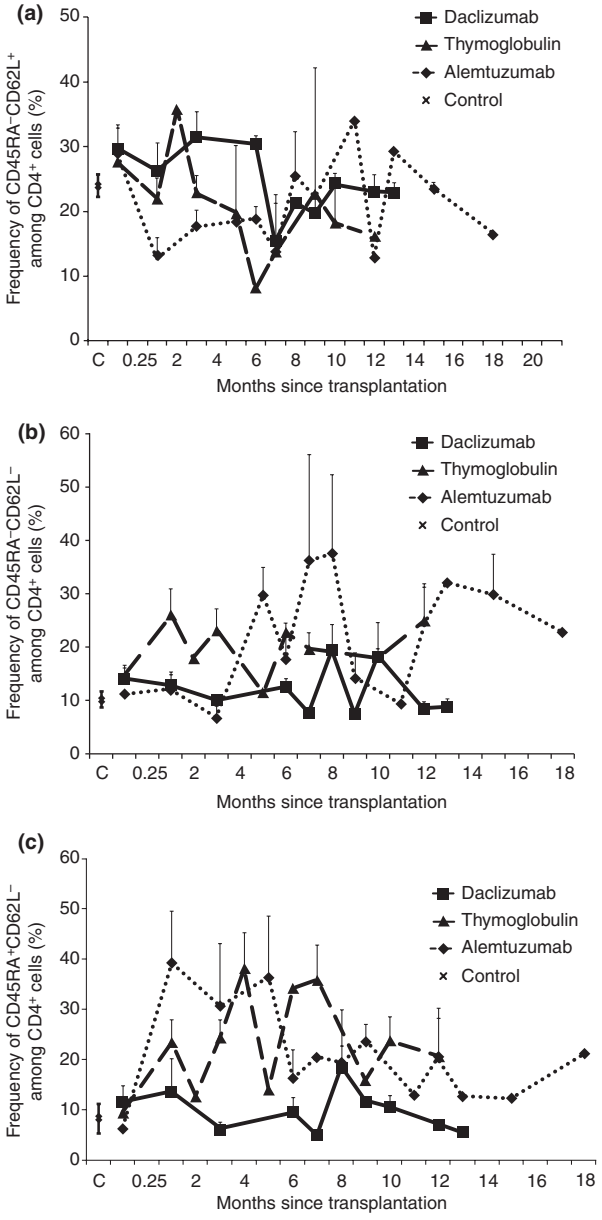

Figure 4 Islet transplantation with daclizumab, Thymoglobulin or alemtuzumab induction: (a) frequency of CD45RA$^-$ CD62L$^+$ (central memory) among CD4$^+$ cells (P = NS); (b) frequency of CD45RA$^-$ CD62L$^-$ (effector memory) among CD4$^+$ cells ($P \leq 0.001$ between daclizumab and Thymoglobulin; $P \leq 0.05$ between daclizumab and alemtuzumab); (c) frequency of CD45RA$^+$ CD62L$^-$ among CD4$^+$ cells ($P \leq 0.001$ daclizumab versus alemtuzumab or versus Thymoglobulin). 'C' on the *x*-axis refers to values of healthy nondiabetic controls. Induction therapy was started on day 0.

respectively, comparing the overall follow up). Of note, the increase in effector memory CD4$^+$ T-cell frequency only became measurable after the first 3 months post-transplant following alemtuzumab induction. Levels of CD45RO within CD4$^+$ T cells remained unchanged in all three groups (P = NS).

Memory B lymphocytes were assessed, using the expression of CD27 [8,9]. This population decreased over time following the use of depleting induction therapies (Fig. 5, $P = 0.01$, ANOVA). This effect was of much higher magnitude following alemtuzumab induction, where a significantly lower frequency of memory B cells was observed when compared with the other two groups (Fig. 5, $P \leq 0.001$, ANOVA, when compared with both daclizumab and Thymoglobulin).

Lack of correlation between the lymphocyte profile and post-transplant insulin requirement or PRA

Flow cytometry results were compared according to transplantation outcomes (insulin dependence versus insulin independence at 12 months). Twenty-nine completed procedures were taken into account for this analysis.

The rates of insulin-independence were similar between the three groups (daclizumab 8/14; Thymoglobulin 3/5; alemtuzumab 5/10; P = NS). Lymphocyte counts; T cells (CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$), B cells (CD20$^+$ HLA-DR$^+$), potential regulatory T cells (CD3$^+$ CD4$^+$ Foxp3$^+$) absolute counts and frequencies, as well as frequencies of CD62L$^+$, CD25$^+$, NKT (NKT2$^+$) and NK (CD56$^+$ and/or CD57$^+$) cells were similar irrespective of whether or not insulin independence was achieved at 12 months.

In order to assess the clinical significance of memory B cells on antibody production, peak post-transplant PRA were determined. PRAs were similar for all three induction therapies that were studied (class I: 10.7 ± 5.2, 18.5 ± 8 and 36.3 ± 14; class II: 0.7 ± 0.6, 14.6 ± 4.6 and 2.8 ± 1.9 for daclizumab, Thymoglobulin and alemtuzumab, P = NS).

Discussion

Daclizumab, Thymoglobulin or alemtuzumab result in major modifications to the profile of peripheral blood lymphocytes following islet transplantation. Alemtuzumab is associated with an early and transient increase in the relative frequency of cells with a potential regulatory phenotype (CD3$^+$ CD4$^+$ Foxp3$^+$), while the opposite picture is observed following daclizumab induction. Depleting induction therapies induce an increase in the frequency of memory effector CD4$^+$ T cells (CD45RA$^-$ CD62L$^-$), while alemtuzumab is associated with a prolonged decrease in the frequency of memory B lymphocytes (CD27$^+$).

The present study was conducted in nonuremic, type 1 diabetic, islet-alone allograft recipients. Islet recipients are perhaps more dependent upon potent induction therapies

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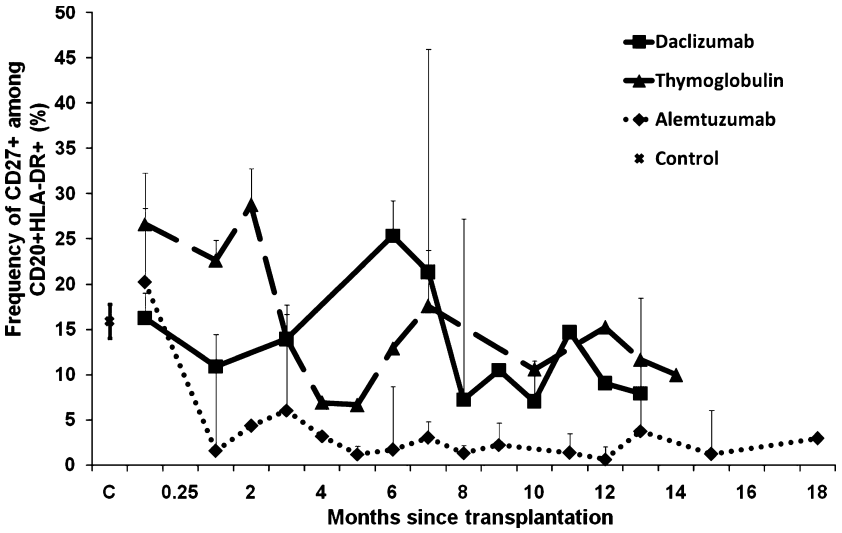

Figure 5 Islet transplantation with daclizumab, Thymoglobulin or alemtuzumab induction: frequency of $CD27^+$ (memory) cells among $CD20^+$ $HLA\text{-}DR^+$ B lymphocytes ($P \leq 0.001$ alemtuzumab versus Thymoglobulin or versus daclizumab).

when compared with other recipients of solid organ grafts, as early rejection cannot be readily identified and treated in this population because of a lack of monitoring modalities. None of the included subjects received additional bolus anti-rejection therapy beyond the induction phase, which could have potentially confounded the flow cytometry results.

Of note, the present study specifically looked at the modification of the various lymphocyte sub-populations after antibody induction. Detailed clinical outcomes and side-effect profiles using these different induction strategies will be provided in a future report. It should also be noted that even though depleting induction agents (alemtuzumab, Thymoglobulin) were used in this study, flow cytometric analysis was only included if a minimum of 1500 gated lymphocyte events were collected, in order to ensure the reproducibility of the readings.

We found that all three alternative induction therapies perturbed the T and B lymphocyte sub-populations in different ways. Daclizumab did not significantly reduce total $CD4^+$, $CD8^+$ or B lymphocytes. However, in accordance with previous data [10], alemtuzumab induced a profound lymphocyte depletion, persisting beyond 24 months. While all cell types were affected, the decrease was proportionally higher on $CD4^+$ cells, consistent with an earlier study [11]. We further found a proportional decrease in B lymphocytes, which may partly explain the protective effect of alemtuzumab against post-transplant lymphoproliferative disorder [12]. Beyond an early decrease (week 2 after transplant), the frequency of $CD8^+$ cells remained stable following alemtuzumab induction.

Thymoglobulin also induced lymphocyte depletion, but to a lesser extent than alemtuzumab, and counts returned to baseline levels around 18 months after transplantation. This depletion affected $CD4^+$ cells preferentially, as expected from the binding properties of Thymoglobulin and its regeneration characteristics promoting an earlier homeostatic response of $CD8^+$ when compared with $CD4^+$ T cells [2].

A striking difference between the different induction strategies was their effect upon potential regulatory T-cell populations. The frequency of $CD25^+$ $Foxp3^+$ cells among $CD3^+$ $CD4^+$ in the control group ($4 \pm 1.8\%$) was similar to data in previous studies [6]. Similarly, the mean pre transplant frequency of $CD25^+$ cells within the $CD3^+$ $CD4^+$ population was $37.6 \pm 4\%$, which is in keeping with the range of 30–40% reported by other groups [13,14].

Recently published data have suggested that alemtuzumab can promote the induction of regulatory cells [15], while other studies have suggested the converse [11]. The positive effect on regulatory T cells was observed *in vitro* by exposing peripheral blood mononuclear cells to alemtuzumab [15,16], and *in vivo*, as assessed by PCR for Foxp3 mRNA and flow cytometry for $CD4^+$ $CD25^+$ $Foxp3^+$ T cells after kidney transplantation [15,17]. Regulatory cells obtained from alemtuzumab-treated patients demonstrated intact inhibitory properties on effector cell proliferation *in vitro* [15]. The present results confirm these previous observations and add additional information about the impact of alemtuzumab. We demonstrate that the peak increase (up to 4.2-fold) in the frequency of $CD3^+$ $CD4^+$ $Foxp3^+$ T cells appears as early as 1 month after transplantation. In addition, while others have observed a sustained increase up to 3 years [15], this was not the case in the setting of islet transplantation, where the frequency of $CD3^+$ $CD4^+$ $Foxp3^+$ T cells was normalized within 6 months. The mechanisms underlying this difference remain unknown, but may be related to a lower alloanti-

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gen exposure following islet transplantation when compared with solid organ transplantation or differences in the immunosuppressive protocol.

While the frequency of potential regulatory T cells was increased with alemtuzumab, the converse was observed with daclizumab, with a potent depletion after transplantation. These observations clarify previously controversial data about daclizumab [15,17], but their clinical relevance is unknown at the present time. We could hypothesize that the decreased ratio between T cells with a regulatory versus effector phenotype may lead to a lower potency of daclizumab. However, the absolute number of $CD3^+$ $CD4^+$ $Foxp3^+$ T cells was similar to those observed after alemtuzumab (Fig. 3d) and the importance of the relative versus the absolute numbers of regulatory T cells remains unknown in the clinical setting.

While *in vitro* experiments have demonstrated an induction of functional regulatory T cells using Thymoglobulin with a conversion of $CD4^+$ $CD25^-$ to $CD4^+$ $CD25^+$ [18], the present results are consistent with previous *in vivo* data [17] showing that Thymoglobulin does not modify the frequency of regulatory T cells.

The induction therapies appear to have a more dominant effect on the frequency of regulatory T cells than the maintenance immunosuppression. While sirolimus has been described to promote the generation of regulatory T cells [19–21], patients receiving daclizumab in this study demonstrated lower levels of $CD3^+$ $CD4^+$ $Foxp3^+$ cells, despite combination with sirolimus. In addition, patients treated with alemtuzumab demonstrated similar profiles irrespective of whether or not they received sirolimus. This said, and in order to minimize the risk of misinterpretation, all results included in this study should be instead viewed as changes over a period of time because of a specific induction/maintenance combination, rather than a pure comparison between induction therapies.

Regulatory T cells have been implicated in the control of autoimmune disease states and the induction of transplantation tolerance [16,22–26]. In the present population of islet recipients, no difference of outcome could be observed between the various treatment groups and no correlation could be made with the level of $CD3^+$ $CD4^+$ $Foxp3^+$ T cells. While many factors can impact on the outcome of islet transplantation [27], our study may have been of insufficient power to observe the true impact of regulatory T cells. This question merits further investigation.

A potential further limitation of this report is that while changes in the proportion of cells with regulatory phenotype have been observed, we have not formally confirmed the regulatory properties of these populations by further *ex vivo* testing. However, previous studies have consistently demonstrated such properties [15].

While daclizumab had virtually no effect on the frequency of memory T and B cells, both depleting therapies altered these sub-populations. As suggested by a previous study assessing kidney recipients, effector memory $CD4^+$ T cells demonstrated relatively higher levels than other types of CD4 T cells after Thymoglobulin or alemtuzumab induction, as shown by a higher frequency of $CD4^+$ $CD45RA^+$ $CD62L^-$ T lymphocytes [7]. This observation may be related to a higher resistance of these memory T cells or to other mechanisms like homeostatic proliferation. In contrast, the frequency of memory $CD27^+$ B cells decreased more than 'naïve' B cells after depleting therapy. This effect was even more pronounced after alemtuzumab therapy, with significantly reduced memory B-cell frequency when compared with induction using either Thymoglobulin or daclizumab. This result may be related to varying expressions of CD52 within the B-cell sub-populations. While previous studies have suggested lower levels of CD52 on plasma cells than on other B lymphocytes, the present data suggests that memory B cells either express higher levels of CD52 or are more sensitive to depletion using alemtuzumab [28]. Peak class I and class II post-transplant PRA levels remained similar in all three induction groups included in this study, and the clinical significance of memory B cells remains to be fully characterized.

Overall, our data suggest that changes in the lymphocyte sub-populations are primarily driven by the degree of cellular depletion and response to homeostatic repopulation, characterized by a conversion in the CD4/CD8 ratio, expansion of effector memory $CD4^+$ cells and $Foxp3^+$ T cells. Alemtuzumab is associated with an early (within 1 month) and reversible increase in the frequency of $CD3^+$ $CD4^+$ $Foxp3^+$ T cells, while the converse is observed after daclizumab induction. The frequency of memory B cells decreased more than other B-lymphocyte sub-populations following depleting strategies, particularly following alemtuzumab induction. However, the full clinical impact of these outcomes still remains to be determined.

Authorship

CT, TFM, AMJS, CCA: study design. CT, RD, RP, JE, SM, PD, TFM, AMJS, CCA: data collection and analysis. CT, JE, SM, TFM, AMJS, CCA: writing of the paper.

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