

Mechanisms of tolerance to parental parathyroid tissue when combined with human allogeneic thymus transplantation

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Background: The induction of tolerance toward third-party solid organ grafts with allogeneic thymus tissue transplantation has not been previously demonstrated in human subjects.

Objective: Infants with complete DiGeorge anomaly (having neither thymus nor parathyroid function) were studied for conditions and mechanisms required for the development of tolerance to third-party solid organ tissues.

Methods: Four infants who met the criteria received parental parathyroid with allogeneic thymus transplantation and were studied.

Results: Two of 3 survivors showed function of both grafts but subsequently lost parathyroid function. They demonstrated alloreactivity against the parathyroid donor in mixed lymphocyte cultures. For these 2 recipients, parathyroid donor HLA class II alleles were mismatched with the recipient and thymus. MHC class II tetramers confirmed the presence of

recipient CD4⁺ T cells with specificity toward a mismatched parathyroid donor class II allele. The third survivor has persistent graft function and lacks alloreactivity toward the parathyroid donor. All parathyroid donor class II alleles were shared with either the recipient or the thymus graft, with minor differences between the parathyroid (HLA-DRB1*1104) and thymus (HLA-DRB1*1101). Tetramer analyses detected recipient T cells specific for the parathyroid HLA-DRB1*1104 allele. Alloreactivity toward the parathyroid donor was restored with low doses of IL-2.

Conclusion: Tolerance toward parathyroid grafts in combined parental parathyroid and allogeneic thymus transplantation requires matching of thymus tissue to parathyroid HLA class II alleles to promote negative selection and suppression of recipient T cells that have alloreactivity toward the parathyroid grafts. This matching strategy may be applied toward tolerance induction in future combined thymus and solid organ transplantation efforts. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: *Allorecognition, anergy, class II, DiGeorge, parathyroid, tetramers, thymus, tolerance, transplantation*

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Solid organ transplantation offers hope for the treatment of many diseases but continues to face significant challenges in preventing rejection of the graft by the recipient.¹ Alloreactivity by T cells toward foreign HLA molecules presents one of the most significant mechanisms for rejection of transplanted allogeneic tissues.^{2,3} Recipient alloreactivity toward donor tissues can be modulated by positive and negative selection processes within the thymus.

Infants with complete DiGeorge anomaly (cDGA) offer an opportunity to study the role of the thymus in controlling allorecognition responses. DiGeorge anomaly results from abnormal embryonic development, leading to possible defects extending from the first to sixth pharyngeal arches.⁴ Affected children present at birth with a spectrum of malformations involving the heart, parathyroid glands, and thymus.⁵⁻¹⁰ Infants with cDGA lack naive (CD45RA⁺CD62L⁺)¹¹ T cells because of athymia, resulting in a severe primary immunodeficiency that is usually fatal as a result of infection by 2 years of age. Allogeneic thymus transplantation leads to immunoreconstitution and increased survival.^{12,13} The thymus grafts provide an environment in which recipient thymocyte precursors undergo positive and negative selection and emerge in the circulation as functional naive T cells.

Abbreviations used

APC: Allophycocyanin
 cDGA: Complete DiGeorge anomaly
 MLC: Mixed lymphocyte culture
 PTH: Parathyroid hormone

Although the transplanted thymus tissues are not HLA matched to the subjects, the recipients demonstrate tolerance to the grafts.¹⁴ Although thymus transplantation has shown success in correcting the immune defects in subjects with cDGA,¹² hypocalcemia caused by hypoparathyroidism remains an important cause of morbidity and mortality.¹⁵

Because of the importance of the thymus in the development of tolerance, we postulated that congenital athymia would provide a suitable model to assess the induction of tolerance to solid organ grafts when combined with thymus transplantation. We hypothesized that in subjects with cDGA, we could achieve tolerance toward parental parathyroid grafts in transplant protocols using cotransplanted allogeneic thymus tissue.

To assess tolerance in the recipients, we used a combination of traditional and novel methods. Traditionally, mixed lymphocyte cultures (MLCs) have assessed alloreactive T-cell proliferation toward donor cells caused by HLA class II differences,^{16–18} which appear to contribute more to rejection than HLA class I mismatches.¹⁹ However, these and other immune assays that show a lack of alloreactivity toward the donor have at times been questioned as markers for tolerance because of perceived insufficient specificity.^{1,20} Newer technologies now offer the potential to directly visualize the presence of specific alloreactive T cells.^{1,21,22} MHC tetramers consist of fluorescently labeled, multimeric MHC molecules of a defined specificity that can be loaded with oligopeptides.²³ As a result, tetramers of recipient MHC molecules containing donor HLA oligopeptides could identify the presence of recipient donor-specific alloreactive T cells.

Here we discuss these efforts to characterize tolerance and the factors associated with tolerance induction in recipients of allogeneic thymus tissue with solid organ transplantation.

METHODS**Subjects**

All subjects were enrolled in clinical trials under a research protocol approved by the Duke Institutional Review Board. Informed consent for these studies and procedures was obtained from the parents of all thymus donors and transplant recipients, the parathyroid donors, and healthy adult control subjects. All recipients met the clinical and immunologic criteria for cDGA^{4–6,8,10,12} with primary hypoparathyroidism (for more details, see the Methods section in this article's Online Repository at www.jacionline.org). They required the initiation of calcium supplementation shortly after birth and had multiple intact parathyroid hormone (PTH) levels measured near or less than the limit of detection before transplantation.

Donors

All thymus and parental parathyroid donors underwent donor screening, as previously described.^{12,24,25} The parathyroid donors had normal intact PTH levels and were as follows: the mothers for subjects 1, 3, and 4 and the father for subject 2. For subject 3, parathyroid transplantation was delayed by 37 days after thymus transplantation because of postponed collection of the donor parathyroid gland.

Thymus and parathyroid transplantation

Thymus tissue was processed as previously described.^{12,13,26} All recipients were given rabbit antithymocyte globulin and methylprednisolone before transplantation, as used in protocols for subjects with cDGA with immunosuppression.^{12,27}

The cultured allogeneic thymus tissues were transplanted into each recipient's quadriceps muscles.²⁸ At the same time, the parathyroid donor underwent open exploration of the neck after achievement of general anesthesia in an adjacent operating room. After a parathyroid gland was located, the presence of parathyroid tissue was confirmed by means of histology. For further confirmation, a small amount of tissue was suspended in saline, yielding high levels of PTH on rapid testing (Elecsys PTH STAT Test; Roche, Zurich, Switzerland). The gland was then removed and sectioned. The parathyroid tissues were placed into the quadriceps muscle of the subject adjacent to the area used for thymus transplantation.

Routine immune and clinical assessments

Immune phenotyping by means of flow cytometry, lymphocyte proliferative responses to PHA and tetanus toxoid, and MLCs were performed according to standard protocols.^{12,27} All MLCs were performed after 10% of the recipients' circulating T cells had the naive phenotype (CD45RA⁺CD62L⁺). PBMC proliferative responses were assessed by counts per minute of tritiated thymidine incorporation.

Intact PTH levels were measured according to the standard practices of the clinical laboratories at our institution and the referring institutions. The lower limits of normal for intact PTH levels at all facilities ranged from 10 to 15 pg/mL. The limit of detection for samples tested at our institution was 5 pg/mL.

High-resolution typing for HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 alleles was obtained as a part of routine clinical testing. HLA-DQA1 and HLA-DPB1 analyses were performed by the Duke Clinical Transplantation Immunology Laboratory using a Luminex (Austin, Tex) reverse sequence specific oligonucleotide multiplex bead assay and Invitrogen (Carlsbad, Calif) sequence specific primer kit, respectively. Standard panel-reactive HLA antibody screening was conducted by the Duke Clinical Transplantation Immunology Laboratory for subjects 1, 3, and 4 (at 3.3, 2.5, and 2.2 years after transplantation, respectively).

Assessments for alloantigen-specific T cells

MHC class II tetramers were created to assess for recipient allospecific T cells by means of flow cytometry. "Positive" tetramers consisted of recipient HLA-DR molecules loaded with parental parathyroid donor oligopeptides from the HLA-DRB1 allele not shared with the recipient (see example in [Fig E1](#) in this article's Online Repository at www.jacionline.org). Positive tetramers were constructed (Beckman Coulter Tetramer Synthesis Facility, Fullerton, Calif) for subject 1 (amino acids 62–82 of HLA-DRB1*0701 bound within HLA-DRB1*0101 tetramers) and subject 4 (amino acids 71–90 of HLA-DRB1*1104 loaded into HLA-DRB1*0301 tetramers) (see [Table E1](#) in this article's Online Repository at www.jacionline.org). Tetramers could not be successfully generated for subject 3. For "negative" tetramers, which were used to assess the specificities of the HLA oligopeptides, the tetramer molecules contained an antigenically irrelevant oligopeptide from the human class II-associated invariant chain peptide instead.²⁹ A detailed description of the tetramer synthesis process is provided in the Methods section of this article's Online Repository.

Tetramer staining of subjects' PBMCs was performed according to the manufacturer's (Beckman Coulter) instructions. In brief, 10⁶ PBMCs were incubated with either positive or negative phycoerythrin-conjugated tetramers for 90 minutes at 37°C. Two percent murine serum (Jackson ImmunoResearch Laboratories, West Grove, Pa) was added for another 30 minutes at 37°C to block nonspecific staining of surface antibodies. Surface antibodies to CD3 (allophycocyanin [APC]-Cy7 conjugated; BD Biosciences, San Jose, Calif) and CD4 (fluorescein isothiocyanate conjugated, Beckman Coulter) were applied. An additional mix of APC-conjugated antibodies to CD8, CD13, CD14 (all from BD Biosciences), CD16 (BioLegend, San Diego, Calif), CD19, and CD56 (both from Beckman Coulter) was added to label nonrelevant cell populations. The cells were then further incubated for 20 minutes and washed

before data acquisition in the flow cytometer. The flow cytometric data were analyzed by gating for APC-negative CD3⁺ cells. Then CD4⁺ cells were chosen and plotted versus tetramer-positive cells. For these experiments, PBMCs were obtained from subject 1 at 4.3 years after transplantation, from subject 4 at 3.3 years after transplantation, and from healthy adult control subjects.

Assessments for anergy

To test for anergy, IL-2 (Invitrogen BioSource, Carlsbad, Calif) was added to subject 4's PBMCs in all conditions parallel to a standard MLC assay: to recipient PBMCs cocultured with irradiated autologous PBMCs, to recipient PBMCs cocultured with irradiated parathyroid donor PBMCs, to recipient PBMCs cocultured with irradiated pooled allogeneic PBMCs, and to recipient PBMCs placed in culture medium without irradiated cells. The cells were cultured in triplicate for 6 days. The concentration of IL-2 was optimized at 2 U/mL. This concentration was chosen after titration experiments were performed to identify the concentration that resulted in minimal proliferation when added to recipient PBMCs in culture medium without irradiated stimulator cells.

Statistical analyses

Differences in proliferative responses were tested for statistical significance by using 2-tailed Student *t* tests. The raw proliferation data (in counts per minute) were log-transformed before performing the statistical analyses. Significant statistical differences were defined by *P* values of less than .05. Highly significant differences were demonstrated by *P* values of less than .005. Results were then plotted as bar graphs depicting mean values and SE bars by using GraphPad Prism Software version 5.00 (San Diego, Calif).

RESULTS

Subjects

Four subjects met the criteria for cDGA and primary hypoparathyroidism. Subjects 1, 2, and 4 received combined allogeneic thymus and parental parathyroid transplantation at 3.6, 3.4, and 4.3 months of life, respectively. Subject 3 underwent allogeneic thymus transplantation at 8.7 months of age followed by parental parathyroid transplantation 1.3 months later. All subjects were given rabbit antithymocyte globulin before transplantation. None of the subjects received immunosuppression past 6 months after transplantation.^{12,27,30}

The surgical procedures were tolerated well by the recipients and parathyroid donors, with no immediate adverse events directly related to either thymus or parathyroid transplantation. Subject 2 died at 9.6 months after transplantation from chronic pulmonary disease caused by mechanical ventilation dependence resulting from congenital thoracic anomalies.

Assessments of graft function

All recipients developed circulating naive T cells in similar fashion to other recipients with cDGA after allogeneic thymus transplantation.¹² The 3 surviving subjects also produced normal T-cell proliferative responses to PHA and tetanus toxoid.

All recipients developed normal PTH levels and were able to discontinue calcium supplementation after parathyroid transplantation. Fluctuations in intact PTH levels were observed, which is consistent with the known difficulties in processing serum PTH samples.^{31,32} Subject 4 has maintained normal serum calcium levels and persistent PTH production within the normal range (Fig 1) since calcium supplementation was discontinued, indicating that the parathyroid graft has continued to function more than 4 years after transplantation. Subjects 1 and 3, on the other hand, lost parathyroid graft function at 13 and 9 months after parathyroid transplantation, respectively, and require calcium

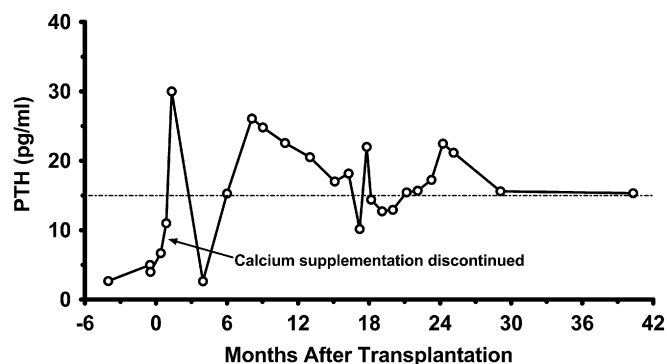


FIG 1. Parathyroid graft function in subject 4 after combined parental parathyroid with allogeneic thymus transplantation. Calcium supplementation was weaned off by 15 days after transplantation. The subject remains off calcium supplementation. Intact PTH levels are shown versus time after parathyroid transplantation. The lower limit of normal (15 pg/mL) is demarcated.

supplementation for hypocalcemia (see Fig E2 in this article's Online Repository at www.jacionline.org).

Assessments of allorecognition toward the parathyroid graft

HLA typing of the recipients and donors was performed to assess allorecognition-mediated rejection as a potential mechanism for loss of parathyroid graft function. Matching between the recipient, parathyroid donor, and thymus donor at the HLA class I loci was not necessary for long-term parathyroid graft function (see Table E2 in this article's Online Repository at www.jacionline.org). However, HLA class II mismatches between the parathyroid donors and the alleles of the recipients and thymus donors were observed in the 2 surviving subjects who lost parathyroid function (subjects 1 and 3). These mismatched parathyroid donor alleles offer potential targets for allorecognition-mediated rejection (boldfaced in Table I). On the other hand, all of the HLA class II alleles for the parathyroid donor for subject 4 (who has long-term parathyroid graft function) matched alleles in either the recipient or the thymus graft (although DRB1*1101 of the thymus differs from the DRB1*1104 of the parathyroid by 1 substitution in the first 100 amino acids, G to V at position 86). Thus matching of the thymus tissue to the parathyroid graft for HLA class II alleles not shared between the parathyroid and subject 4 was associated with long-term parathyroid graft function.

The subjects were tested for allorecognition toward the parathyroid donors in MLCs. PBMCs from subjects 1 and 3 proliferated strongly against irradiated parathyroid donor PBMCs when tested at 6.7 and 8.5 months after transplantation (Fig 2). In contrast, subject 4 showed no significant proliferative responses toward the parathyroid donor at 15.1 months after transplantation compared with recipient responses toward allogeneic cells. Healthy adult control PBMCs responded strongly to irradiated PBMCs from the parathyroid donor for subject 4. These findings for subject 4 have been replicated in a total of 6 experiments to beyond 40 months after transplantation (data not shown). Thus the presence of allorecognition toward the parathyroid donor observed in the MLCs was associated with the loss of parathyroid graft function, and the absence of allorecognition toward the parathyroid donor was associated with long-term parathyroid graft function. Additional studies after the recipients successfully

TABLE I. HLA class II typing of recipients, their parathyroid donors, and their thymus donors

	HLA-DRB1	HLA-DQB1	HLA-DQA1	HLA-DPB1
Subject 1	1401	0503	01XX*	0301
	0101	0501	01XX*	0402
PT donor	0101	0501	01XX	0402
	0701†	0303	0201	0101
Thymus donor	0101	0501	01XX*	0202
	0301	0201	0501	1101
Subject 2	1301	0603	NT	NT
	0801	0402	NT	NT
PT donor	0801	0402	NT	NT
	0701	0202	NT	NT
Thymus donor	0801	0402	NT	NT
	1001	0501	NT	NT
Subject 3	1303	0301	0505	0601
	0701	0202	0201	1701
PT donor	0701	0202	0201	1701
	0301	0201	0501	0401
Thymus donor	0301	0201	0501	0301
	1301	0603	0103	2001
Subject 4	0301	0201	NT	0401
	1601	0502	NT	0402
PT donor	1601	0502	0102	0402
	1104	0301	0505	0402
Thymus donor	1101	0301	0505	0401
	0405	0303	0302	0201

NT, Not tested; PT, parathyroid.

*Only low-resolution typing is available for this allele.

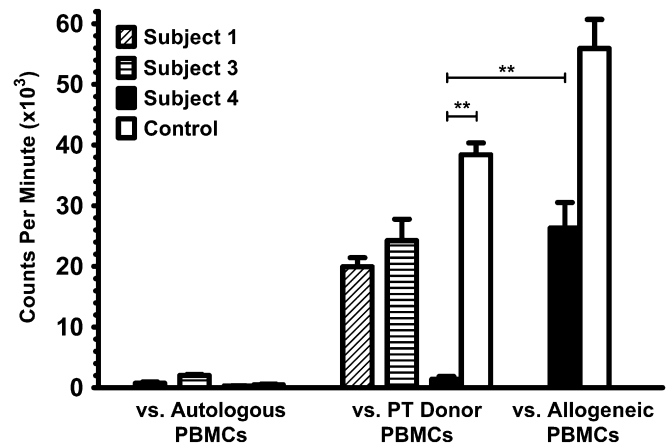
†Parathyroid donor HLA class II alleles not shared with the recipient or the thymus donor are shown in boldface.

discontinued intravenous immunoglobulin replacement showed that no subjects have anti-HLA class I or class II antibodies, arguing against rejection by antibody-mediated allorecognition mechanisms.

We then sought to confirm the presence of recipient donor-specific alloreactive T cells. Parathyroid donor HLA-DRB1 oligopeptides were loaded into recipient-type MHC class II tetramer molecules to test for the presence of alloantigen-specific CD4⁺ T cells. For subject 1, tetramers were generated by using parathyroid donor HLA-DRB1*0701 oligopeptides bound within recipient-type HLA-DRB1*0101 tetramer molecules. The HLA-DRB1*0701 oligopeptides used for subject 1 were previously shown to stimulate alloreactive T cells.³³ Subject 3 could not be assessed because oligopeptides from the only HLA class II mismatch (HLA-DPB1*0401) did not bind stably within recipient-type HLA-DRB1*0701 molecules. For subject 4, HLA-DRB1*1104 oligopeptides (amino acid positions 71-90) were loaded into recipient-type HLA-DRB1*0301 tetramers. We identified the presence of more than 1% of subject 1's CD4⁺ T cells that bound to the tetramer containing the parathyroid donor oligopeptide at 4.3 years after transplantation (Fig 3). In subject 4 at 3.3 years after transplantation, a smaller percentage of CD4⁺ T cells showed affinity for the tetramer loaded with the parathyroid donor HLA-DRB1*1104 peptide. Importantly, binding of the tetramers to T cells from control subjects was not observed (see Fig E3 in this article's Online Repository at www.jacionline.org).

Assessments of peripheral tolerance mechanisms

We studied mechanisms of peripheral tolerance responsible for the suppression of alloreactivity toward the parathyroid donor in

**FIG 2.** MLCs. Nonirradiated responder PBMCs were cocultured with irradiated autologous or parathyroid (PT) donor PBMCs. Results represent counts per minute of tritiated thymidine incorporation. For subject 4, healthy adult control PBMC responses to irradiated PBMCs from the PT donor for subject 4 and responses by subject 4's PBMCs and control PBMCs to pooled allogeneic PBMCs are shown for comparison. ***P* < .005.

subject 4. In assessments for anergy, a low dose of IL-2 (2 U/mL) restored alloreactivity by subject 4's PBMCs (obtained 3.3 years after transplantation) toward the parathyroid donor cells (Fig 4). The response of subject 4's PBMCs to the parathyroid donor was also statistically greater than the autologous response. Proliferation of the recipient PBMCs to irradiated autologous PBMCs and proliferation of the recipient PBMCs in culture medium without irradiated cells remained low after the addition of IL-2. These findings for subject 4 were confirmed by a subsequent analysis. The results suggest that some of subject 4's T cells with allorecognition toward the parathyroid donor have been rendered anergic to maintain tolerance toward the parathyroid graft. In separate experiments we did not observe a role for regulatory T cells in suppressing alloreactivity by subject 4's T cells toward the parathyroid donor when tested at 17 months after transplantation (see Fig E4 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Combined parental parathyroid with allogeneic thymus transplantation in 4 infants with cDGA led to parathyroid and thymus graft function in all recipients. Of the 3 survivors, 2 (subjects 1 and 3) subsequently lost parathyroid graft function. The other survivor (subject 4) continues to demonstrate parathyroid function without long-term immunosuppression. Endogenous PTH production in subject 4 (who had no parathyroid function from birth) remains unlikely. Subject 4 was only 4.8 months of age when calcium supplementation was discontinued. Of 35 infants with cDGA who received calcium supplementation before allogeneic thymus transplantation (excluding the subjects who received parathyroid transplants) and who have survived to at least 6 months of age, only 1 was successfully weaned off of calcium supplementation at less than 6 months of age, as accomplished for subject 4. This infant had a PTH level of 12 pg/mL while calcium supplementation was being given in contrast to the 4 subjects in this report who all had PTH levels of 6 pg/mL or less before receiving the parathyroid grafts. Thus this 1 case that demonstrated almost normal endogenous PTH

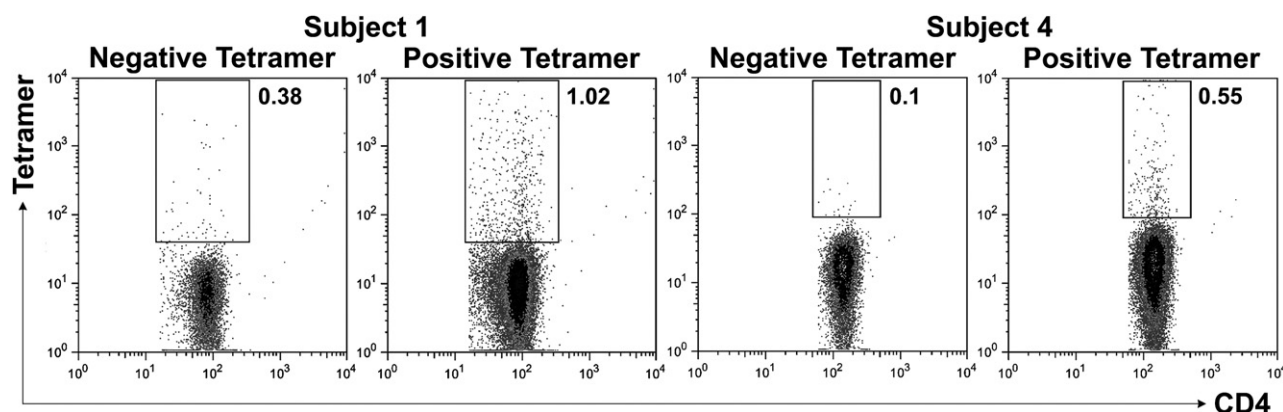


FIG 3. Tetramer analyses. Subjects 1 and 4 were tested for CD4⁺ T cells with allorecognition toward parathyroid donor oligopeptides (from the HLA-DRB1 allele not shared with the recipient) presented within recipient-type MHC class II tetramers ("positive"). "Negative" tetramers contained an antigenically irrelevant peptide. Percentages of tetramer-positive cells are shown gated on CD4⁺ T cells.

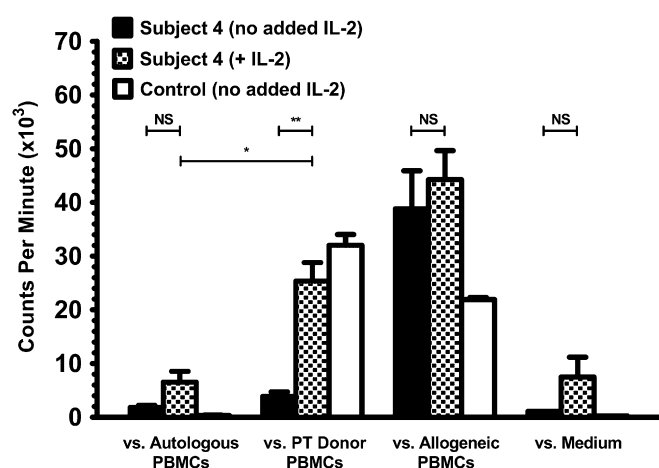


FIG 4. Assessments for anergic alloreactive T cells. PBMCs from subject 4 and control PBMCs were cultured with irradiated PBMCs (autologous, parathyroid [PT] donor, or allogeneic) or in medium without irradiated cells. In parallel, IL-2 was added to subject 4's PBMCs in all 4 conditions. Results (incorporated tritiated thymidine) are shown at 3.3 years after transplantation. NS, $P > .05$, * $P < .05$, and ** $P < .005$.

production before discontinuing calcium supplementation clearly differs from our observations in subject 4.

We hypothesized that loss of parathyroid function occurred because of rejection of the grafts by alloreactive recipient T cells and that long-term function resulted from tolerance. HLA typing revealed potential parathyroid donor HLA class II targets for rejection in the 2 recipients who lost parathyroid graft function but not in the recipient who has continued parathyroid function. Furthermore, the recipients who lost parathyroid function also showed alloreactivity toward their parathyroid donors in MLCs, whereas the subject with persistent function demonstrated tolerance. We were able to observe the presence of recipient T cells with specificity toward parathyroid donor HLA class II alloantigens using MHC class II tetramers. For subject 1, who appears to have rejected the parathyroid graft, the presence of one of many potential populations of recipient T cells with parathyroid donor alloantigen specificity was confirmed. For subject 4, who demonstrates persistent graft function and tolerance toward the

parathyroid donor in MLCs, a small percentage of tetramer-positive cells was observed. These cells might have escaped deletion in the thymus graft because they have specificity for HLA-DRB1*1104 and not the HLA-DRB1*1101 on the thymus graft epithelial cells.

To our knowledge, we report the first use of MHC class II tetramers for direct observation of T cells with alloantigen specificity at the single-cell level. We found that only certain oligopeptides bound within the MHC class II clefts of the designated tetramer molecules. Of note, our empiric method for creating the tetramers also allowed us to map the relevant T-cell epitopes within the first 100 amino acids for HLA-DRB1*0701 (amino acids 62-82) when presented by HLA-DRB1*0101 and HLA-DRB1*1104 (amino acids 71-90) when presented by HLA-DRB1*0301. For subject 3, none of the parathyroid donor's oligopeptides tested bound to HLA-DRB1*0701 tetramers. The peptides within the first 100 amino acids of HLA-DPB1*0401 do not appear to be presented to T cells within HLA-DRB1*0701 molecules of antigen-presenting cells. Instead, more C-terminal peptides from HLA-DPB1*0401 might be presented by HLA-DRB1*0701. Likely, 1 or more of the HLA-DPB1*0401 oligopeptides binds to HLA-DRB1*1303 or other subject 3 HLA class II molecules (HLA-DQ or HLA-DP) for which tetramers are not currently available. This approach for screening the tetramers for binding to the various oligopeptides, although effective, was resource consuming, which might limit its general use at this time. Finally, as exemplified by subject 4, who has detectable tetramer-positive CD4⁺ T cells yet remains tolerant toward the parathyroid graft, tetramer assays performed together with functional assessments for allorecognition (eg, MLCs) enhance evaluation of alloreactivity in allograft recipients.

We considered potential peripheral mechanisms that could confer tolerance in subject 4. Low-dose IL-2 restored proliferation by subject 4's PBMCs toward the parathyroid donor, indicating a role for anergy³⁴⁻³⁷ as a tolerance mechanism. An attractive unifying theory, incorporating the presence of the tetramer-positive cells, suggests that the cells might have been rendered anergic because the affinity of these cells for HLA-DRB1*1104 could lead to rejection of the similar HLA-DRB1*1101 on the thymus graft. We have not confirmed this hypothesis because of

blood volume constraints in subject 4 that preclude isolating sufficient numbers of the tetramer-positive cells (as in Fig 3) to assess anergy (as in Fig 4). In separate experiments we were unable to observe a role for CD25⁺ regulatory T cells in maintaining tolerance by subject 4 toward the parathyroid graft at 17 months after transplantation.

Our results compare favorably with other human parathyroid allotransplantation efforts. Long-term function of allogeneic parathyroid grafts has been reported in 2 subjects who first received allogeneic renal grafts and were able to discontinue immunosuppression. Their parathyroid grafts were fully or partially matched to the kidney donors (HLA class II typing was not reported in the second case).^{38,39} Other approaches have focused on the use of tissue manipulation and special culture techniques to reduce HLA class II expression (ubiquitous in parathyroid stroma⁴⁰) in the parathyroid grafts rather than to induce tolerance.⁴¹⁻⁴³ Even after these methods, prolonged parathyroid graft function has been poor (55% of grafts retain function beyond 2 months).⁴³ Other attempts forgoing the use of long-term immunosuppression without inducing tolerance toward the parathyroid tissues have not resulted in prolonged graft function or the ability to discontinue calcium supplementation beyond 1 year after transplantation.⁴⁴⁻⁴⁶

Overall, our findings suggest that allogeneic thymus tissue can be used to induce tolerance to solid organ grafts in infants with cDGA. Based on our data in 4 recipients of parental parathyroid and allogeneic thymus grafts, we hypothesize that HLA class II matching between the thymus and solid organ tissues is important for inducing tolerance toward the solid organ graft. We believe that the solid organ graft HLA class II alleles must be expressed in the thymus graft, either by the thymus graft medullary epithelial cells or recipient antigen-presenting cells (eg, dendritic cells). Any T cells that have alloreactivity toward these alleles yet escape negative selection in the thymus graft are peripherally suppressed. Matching of the HLA class I alleles does not appear to be necessary for tolerance. This model suggests the need for additional studies to assess the potential for using this matching strategy to induce tolerance to solid organ grafts in combination with allogeneic thymus transplantation in children with cDGA. Further application of this knowledge toward immunocompetent recipients might be challenging, likely requiring depletion of the recipient T cells and suppression or elimination of the recipient thymic function before transplantation. Our studies might also support the argument³⁹ for the development of pretyped banks of tissues, including both thymus and solid organ tissues, that can be used for transplantation. Tissues of the appropriate HLA types could then be selected to promote thymus graft-mediated induction of tolerance toward the solid organ tissue.

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Key messages

- Allogeneic thymus tissue can be used for tolerance induction to third-party solid organ grafts in athymic subjects.
- Tolerance requires elimination or suppression of recipient allorecognition responses toward solid organ donor HLA class II alleles. HLA class I alleles do not appear to play an important role in tolerance versus rejection.
- Subsets of recipient alloreactive CD4⁺ T cells can be identified by using custom-synthesized MHC class II tetramers.

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METHODS

Case reports

Subject 1 is a white female subject who presented with clinical features of DiGeorge anomaly, including hypoparathyroidism leading to a hypocalcemic seizure on day 11 of life, congenital heart disease, and profoundly low T-cell numbers. At both the referring center and our institution, intact PTH levels were less than the limits of detection (<6 pg/mL and <5 pg/mL, respectively) at times of hypocalcemia. At 4 weeks of life, the subject required surgical repair of 2 large ventriculoseptal defects; no thymus was found during the cardiac surgery. Initial flow cytometric studies on PBMCs performed at 5 weeks of life showed that the infant had no CD3⁺ cells. The subject's PBMCs responded poorly to mitogens (1,591 cpm against PHA with a control response of 268,990 cpm and 1,215 cpm against concanavalin A with a control response of 197,571 cpm). Further testing confirmed athymia with total T cells at 2% of lymphocytes and a naive (CD45RA⁺CD62L⁺) T-cell count of less than 33 cells/mm³. Other problems included poor swallowing with aspiration, a malformed right ear pinna, fusion of the 9th and 10th ribs, and T9-T10 hemi-vertebrae. Several chromosomal analyses showed no deletion at 22q11. The subject was transferred to our institution at 3 months of life for combined thymus and parathyroid transplantation, which occurred when the infant was 3.6 months of age.

Subject 2 was a white male subject given a diagnosis of cDGA because of hypoparathyroidism, type II truncus arteriosus, and the absence of T cells. Other findings included vertebral and rib anomalies, hydrocephalus resulting in ventriculoperitoneal shunt placement, a seizure disorder, and growth retardation. Chromosomal analyses showed a normal karyotype and no deletion at 22q11. The subject was born at 36 weeks' gestation to a mother with type 2 diabetes and was intubated at birth. Several attempts to extubate failed because of the rib anomalies, which prevented adequate chest wall motion for breathing. Long-term ventilation led to the development of bronchopulmonary dysplasia. Hypocalcemia was noted, and the subject was started on calcium supplementation, resulting in nephrolithiasis and a severe skin burn from intravenous infiltration. Immunologically, the absolute lymphocyte count was 949 cells/mm³ on day 2 after birth. At 4 weeks, flow cytometric studies on PBMCs showed that less than 1% of the lymphocytes were T cells, and the subject had no detectable naive T cells. T-cell responses to mitogens were tested at 4 and 11 weeks of life and found to be absent (2,351 cpm against PHA with a control response of 306,520 cpm at 11 weeks of life). The subject was transferred to our institution at 2 months of age for repair of the truncus arteriosus, followed by thymus and parathyroid transplantation at 3.4 months of life. Hypoparathyroidism was confirmed with 3 undetectable intact PTH levels (<5 pg/mL) during episodes of hypocalcemia before transplantation.

Subject 3 is a white female subject who had hypocalcemic seizures at 3 weeks of life. Chromosomal analysis showed a hemizygous deletion at 22q11. No T-cell count was obtained at this time. Other findings included malformed auricles and aspiration from nasopharyngeal reflux that led to a Nissen fundoplication with gastric tube placement. The subject had a reported total T-cell count of 65 cells/mm³ ($<5\%$ of lymphocytes) at 6 weeks of life shortly after the onset of generalized dermatitis, which was treated with emollients. By 6½ months of life, lymphadenopathy had developed. Flow cytometric studies on blood sent to our institution demonstrated 7,325 CD3⁺ cells/mm³ with less than 10 naive T cells/mm³. Subset analyses showed that 40% of the T cells were CD4⁺CD8[−], 38% were CD8⁺, and 21% were CD4⁺. The subject's T-cell proliferative response was 106,106 cpm against PHA (control response of 87,050 cpm). When activation markers were checked, more than 99% of the T cells were CD25⁺. The T-cell receptor repertoire was oligoclonal by means of both flow cytometric and spectratype analysis. Based on the clinical, genetic, and immunologic findings, the subject was given a diagnosis of atypical complete DiGeorge anomaly and started on cyclosporine at 44 days before thymus transplantation. The subject required calcium supplementation and was found to have nephrocalcinosis and renal calculi. The infant was transferred to our institution for treatment at 8 months of age. Calcium supplementation was discontinued for 2 periods of approximately 2 weeks each before parathyroid transplantation because of hypercalcemia, hypercalciuria, or both. One episode was related to dehydration; the cause of the other was unclear. Two intact PTH levels during periods of hypocalcemia were less than

5 pg/mL. The subject was 8.7 and 10 months of age when given allogeneic thymus transplantation and parental parathyroid transplantation, respectively. The immunosuppression was discontinued in subject 3 by 6 months after parathyroid transplantation after naive T cells appeared in the circulation.

Subject 4 is a white male subject who was born with tetralogy of Fallot and hypocalcemia. The subject had hypocalcemic seizures on 2 occasions, and calcium supplementation was initiated. Genetic testing revealed 22q11 hemizygosity. On day 16 of life, the infant had an absolute lymphocyte count of 1,792 cells/mm³, of which less than 1% were T cells, and he was transferred to our institution for further care at 3.6 months of age. Testing after arrival showed that the subject had less than 5 naive T cells/mm³. The subject's T cells proliferated poorly against mitogens (6,154 cpm against PHA with a control response of 166,593 cpm). Three intact PTH levels were obtained. Two were less than 5 pg/mL, and 1 was 5 pg/mL (normal, 15–65 pg/mL). The subject received combined allogeneic thymus and parental parathyroid transplantation at 4.3 months of life.

Tetramer synthesis

Parathyroid donor HLA class II oligopeptides were constructed for assessments of allorecognition by recipient T cells. Because the first 100 amino acids of the HLA class II molecules elicit the most relevant alloreactive T-cell responses,^{E1} the sequences for the first 100 amino acids of the parathyroid donor HLA-DRB1 alleles were aligned with the sequences for the 2 thymus donor HLA-DRB1 and 2 recipient HLA-DRB1 alleles (<http://www.ebi.ac.uk/imgt/hla/>). Sequences for the parathyroid donor HLA-DRB1 allele unshared with the recipient were then chosen to incorporate maximum amino acid differences from the other parathyroid donor HLA-DRB1 allele, the recipient HLA-DRB1 alleles, and the thymus donor HLA-DRB1 alleles (example given in Fig E1). For subject 3, parathyroid donor sequences were selected at HLA-DPB1 by using the same strategy because of observed mismatching between subject 3, the parathyroid donor, and the thymus donor at HLA-DPB1. The parathyroid donor HLA class II oligopeptide sequences are available in Table E1.

The oligopeptide sequences were used to create MHC class II tetramers. To assess subject 1, parathyroid donor HLA-DRB1*0701 oligopeptides were placed into recipient-type HLA-DRB1*0101 tetramers (Beckman-Coulter Tetramer Synthesis Facility). For subject 3, because neither recipient-type HLA-DRB1*0701 nor HLA-DRB1*1303 tetramers were available from Beckman Coulter, synthesis was performed by ProImmune (Oxford, United Kingdom) to place parathyroid donor HLA-DPB1*0401 oligopeptides into HLA-DRB1*0701 "ultimers." Finally, for subject 4, the parathyroid donor HLA-DRB1*1104 oligopeptides were loaded into recipient-type HLA-DRB1*0301 tetramers (Beckman Coulter Tetramer Synthesis Facility). For subject 1, 1 of the 4 parathyroid donor HLA-DRB1*0701 oligopeptides (amino acid positions 62–82, Table E1) bound stably within HLA-DRB1*0101 tetramers. No HLA-DRB1*0701 "ultimers" could be successfully synthesized with the 4 HLA-DPB1*0401 oligopeptides. For subject 4, 1 of the 3 HLA-DRB1*1104 peptides (amino acid positions 71–90, Table E1) demonstrated successful binding to generate HLA-DRB1*0301 tetramers for testing. Subjects 1 and 4's tetramers that bound to the parathyroid donor HLA-DRB1 oligopeptides were designated as "positive" tetramers. For "negative" tetramers, which were used to assess the specificities of the HLA oligopeptides, the tetramer molecules contained an antigenically irrelevant oligopeptide from the human class II-associated invariant chain peptide instead.^{E2}

Assessments for regulatory T cells

All surviving recipients were first tested to confirm the presence of regulatory T cells. Regulatory T cells were assessed by means of intracellular staining of subjects' PBMCs for forkhead box protein 3 (Foxp3) expression with a kit (anti-human Foxp3 PE staining kit, clone PCHI01) from eBioscience (San Diego, Calif). Following the manufacturer's instructions, the cells were first stained with antibodies to surface CD3 and CD4. The cells were treated for 1 hour with the Fixation/Permeabilization Solution and then washed with the Permeabilization Buffer. After incubation with the rat serum provided in the kit, the anti-human Foxp3 antibodies were added. The cells were stained for 30 minutes, washed, and analyzed within 24 hours

in the flow cytometric laboratory. Healthy adult volunteers' PBMCs were tested in parallel as a control. Subjects 1, 3, and 4 were tested at 13.6, 12.5, and 15.1 months after transplantation, respectively, and were determined to have normal percentages of Foxp3⁺CD4⁺ T cells (3.91% of CD4⁺ T cells for subject 1 vs 2.91% for healthy adult control subjects, 5.17% for subject 3 vs 4.01% for control subjects, and 4.24% for subject 4 vs 2.40% for control subjects).

To evaluate regulatory T cell-mediated tolerance, CD25⁺ regulatory T cells were removed from subject 4's PBMCs by means of positive selection in columns by using magnetic bead-conjugated anti-CD25 antibodies (Miltenyi Biotec, Auburn, Calif). Flow cytometry showed efficient removal of CD25⁺ cells (>85% of CD25⁺CD4⁺ T cells and >95% of CD25^{hi}CD4⁺ T cells). The

CD25-depleted cells were then cocultured with irradiated parathyroid donor PBMCs in a standard MLC assay. Nondepleted recipient PBMCs were cocultured with irradiated parathyroid donor PBMCs in a parallel MLC assay.

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- E2. Day CL, Seth NP, Lucas M, Appel H, Gauthier L, Lauer GM, et al. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J Clin Invest* 2003;112:831-42.

Position	10	20	30	40	50
Parathyroid Donor					
DRB1*070101 #1	<u>GDTQPRFLWQ</u>	<u>GKYKCHFFNG</u>	TERVQFLERL	FYNQEEFVRF	DSDVGEYRAV
DRB1*070101 #2	GDTQPRFLWQ	GKYKCHFFNG	<u>TERVQFLERL</u>	<u>FYNQEEFVRF</u>	DSDVGEYRAV
DRB1*070101 #3	GDTQPRFLWQ	GKYKCHFFNG	TERVQFLERL	FYNQEEFVRF	DSDVGEYRAV
DRB1*070101 #4	GDTQPRFLWQ	GKYKCHFFNG	TERVQFLERL	FYNQEEFVRF	DSDVGEYRAV
DRB1*010101	---R-----	L-FE-----	---RL---C	I-----S---	-----
Subject 1 (Recipient)					
DRB1*010101	---R-----	L-FE-----	---RL---C	I-----S---	-----
DRB1*140101	---R---EY	STSE-----	---R--D-Y	-H-----	-----
Thymus Donor					
DRB1*010101	---R-----	L-FE-----	---RL---C	I-----S---	-----
DRB1*030101	---R---EY	STSE-----	---RY-D-Y	-H---N---	-----F---
Position	60	70	80	90	100
Parathyroid Donor					
DRB1*070101 #1	TELGRPVAES	WNSQKDILED	RRGQVDTVCR	HNYGVGESFT	VQRRVHPEVT
DRB1*070101 #2	TELGRPVAES	WNSQKDILED	RRGQVDTVCR	HNYGVGESFT	VQRRVHPEVT
DRB1*070101 #3	<u>TELGRPVAES</u>	<u>WNSQKDILED</u>	RRGQVDTVCR	HNYGVGESFT	VQRRVHPEVT
DRB1*070101 #4	TELGRPVAES	<u>WNSQKDILED</u>	<u>RRGQVDTVCR</u>	<u>HNYGVGESFT</u>	VQRRVHPEVT
DRB1*010101	-----D--Y	-----L--Q	--AA---Y--	-----	-----E-K--
Subject 1 (Recipient)					
DRB1*010101	-----D--Y	-----L--Q	--AA---Y--	-----	-----E-K--
DRB1*140101	-----A--H	-----L--R	--AE---Y--	-----V----	-----K--
Thymus Donor					
DRB1*010101	-----D--Y	-----L--Q	--AA---Y--	-----	-----E-K--
DRB1*030101	-----D--Y	-----L--Q	K--R--NY--	-----V----	-----K--

FIG E1. Examples of HLA class II oligopeptide sequences used for testing alloreactivity. The parathyroid donor HLA-DRB1*0701 amino acid sequence is compared with the other parathyroid donor HLA-DRB1 allele (0101) and the HLA-DRB1 alleles of subject 1 and the thymus donor. Amino acid position numbers are shown on the top. *Dashes* indicate identical amino acids between sequences. Sequences for the HLA-DRB1*0701 oligopeptides (1-4) used to assess alloreactivity toward the parathyroid donor in subject 1 are underlined and in boldface.

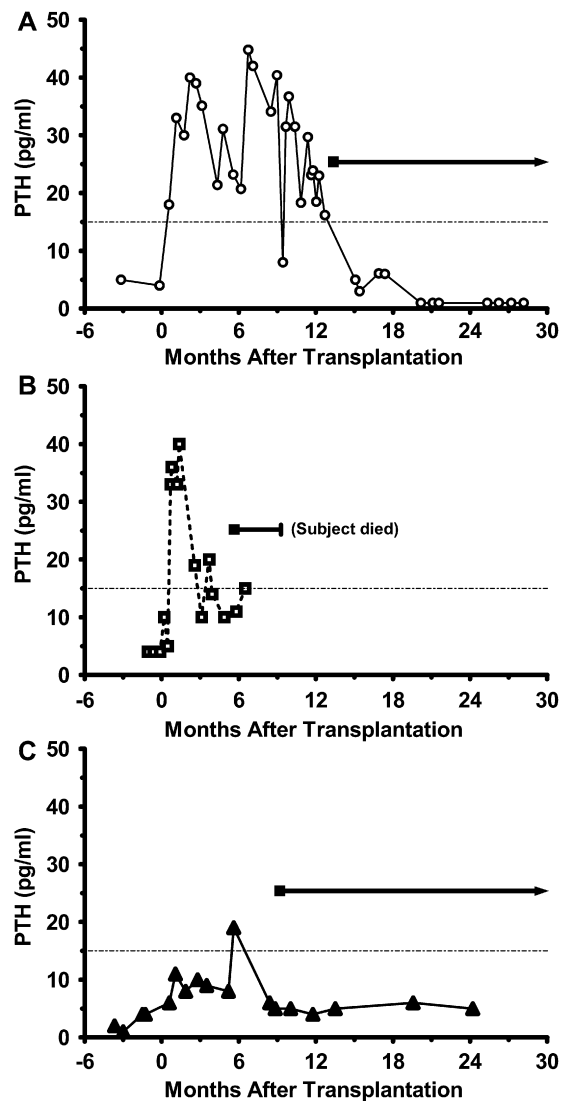


FIG E2. Parathyroid graft function in 3 subjects with cDGA after combined parental parathyroid with allogeneic thymus transplantation. Subjects 1 (**A**), 2 (**B**), and 3 (**C**) were initially able to discontinue calcium supplementation but required restarting the calcium. The use of calcium supplementation after parathyroid transplantation is denoted by the *square followed by the solid bar* in each graph. Intact PTH levels for each subject are shown versus time after parathyroid transplantation. The lower limit of normal (15 pg/mL) is demarcated.

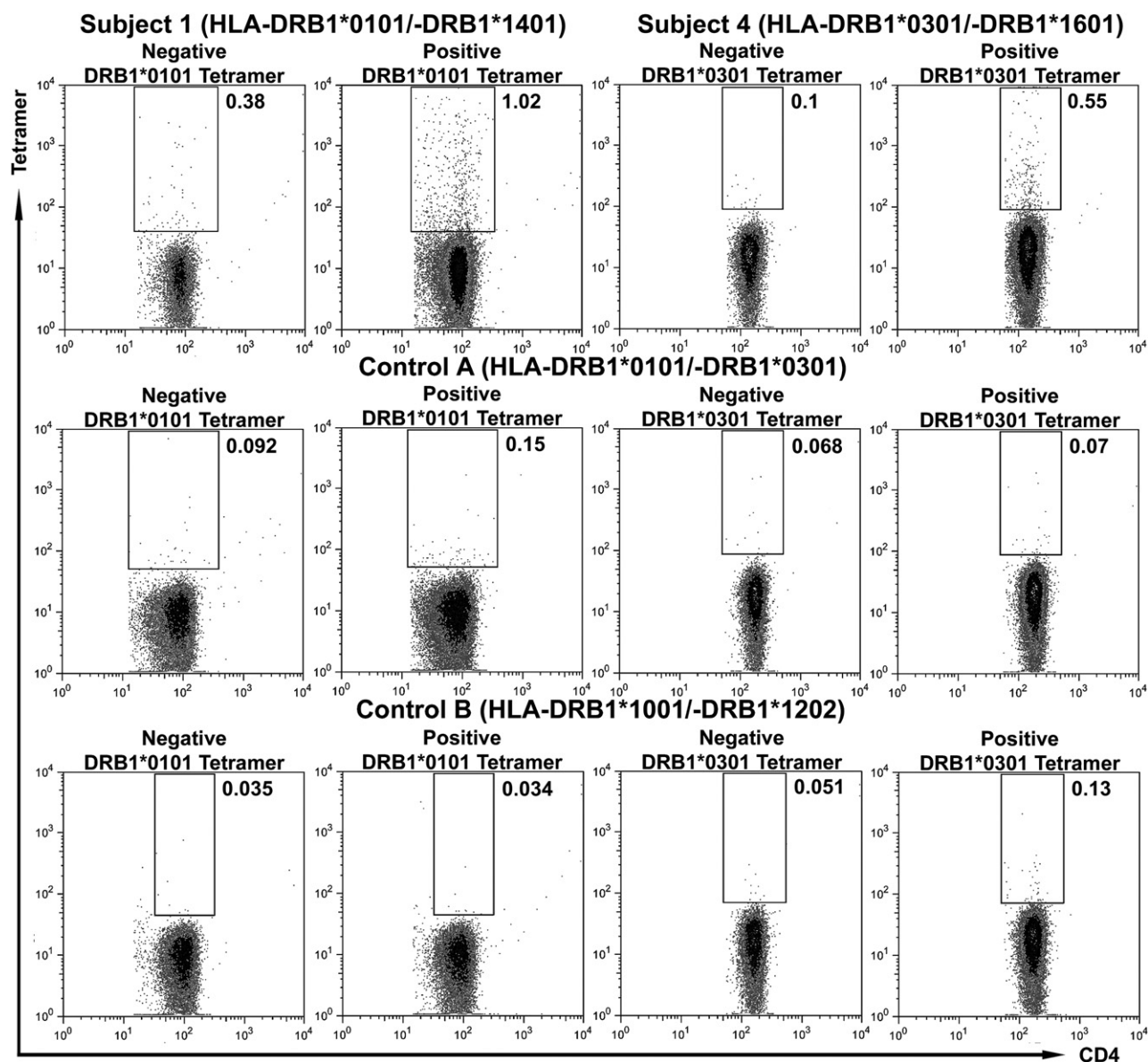


FIG E3. Tetramer analyses, including control assays. Tetramer MHC class II specificities are shown above each of the scatter plots. The *top row* shows the data in Fig 3. The HLA-DRB1 types of the subjects are shown in parentheses. Note that both the positive and negative tetramers were constructed with MHC class II molecules specific for one of each subject's HLA-DRB1 alleles. Positive tetramers were loaded with parathyroid donor HLA-DRB1 oligopeptides. Negative tetramers contain an antigenically irrelevant peptide. In the *second row* a control (control A) was selected with HLA-DRB1 alleles (provided in parentheses), each matching one of the MHC class II specificities of the tetramers used in Fig 3 and the *top row*. These same positive and negative tetramers were tested for binding to control A CD4⁺ T cells. Control A is not known to have been exposed to the HLA-DRB1 types of either parathyroid donor. Evidence of tetramer binding to the control A T cells would suggest nonspecific binding of the tetramer to CD4⁺ T cells because of the oligopeptide loaded within the tetramer molecule. In the *bottom row* another control (control B) was selected with HLA-DRB1 alleles that do not match the MHC class II specificities of any of the tetramers used. Evidence of tetramer binding to control B T cells would suggest a lack of specific binding of the tetramers to CD4⁺ T cells in general. Percentages of tetramer-positive cells are shown in each scatter plot gated on CD4⁺ T cells.

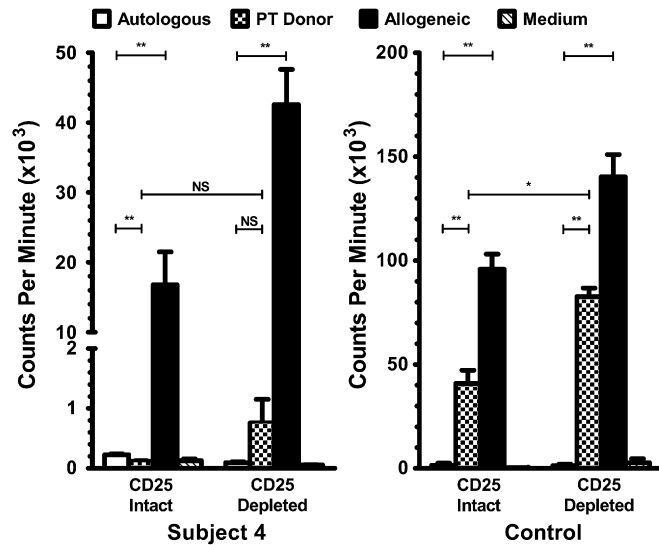


FIG E4. Assessments for regulatory T cell-mediated suppression of alloreactivity. Proliferative responses (in counts per minute) of subject 4's PBMCs (*left panel*) and control PBMCs (*right panel*) were assessed in culture with irradiated autologous parathyroid donor or pooled allogeneic PBMCs or in medium alone. The subject's PBMCs and control PBMCs were placed into parallel cultures after removal of the CD25⁺ regulatory T cells. Note the broken, reduced scale used for the y-axis of subject 4 to allow visualization of the smaller proliferative responses. NS, $P > .05$, * $P < .05$, and ** $P < 0.005$.

TABLE E1. Oligopeptides used to test for recipient T cells with parathyroid donor alloantigen specificity by MHC class II tetramer assays

Recipient	Mismatched parathyroid donor HLA class II allele	Amino acid positions	Parathyroid donor HLA class II oligopeptide amino acid sequence	Tetramer HLA-DRB1 specificity	Oligopeptide binding to tetramer
Subject 1	HLA-DRB1*0701	1-18	GDTQPRFLWQGKYKCHFF	0101	N
		20-41	GTERVQFLERLFYNQEEFVRFD		N
		50-65	VTELGRPVAESWNSQK		N
		62-82	NSQKDILEDRRGQVDTVCRHN		Y
Subject 3	HLA-DPB1*0401	1-20	RATPENYLFQGRQECYAFNG	0701	N
		27-41	RYIYNREEFARFDSD		N
		55-69	AAEYWNSQKDILEEK		N
		74-96	DRMCRHNYELGGPMTLQRRVQPR		N
Subject 4	HLA-DRB1*1104	22-41	ERVRFLLDRYFYNQEEYVRFD	0301	N
		45-64	GEFRAVTELGRPDEEYWNSQ		N
		71-90	RRAAVDTYCRHNYGVVESFT		Y

TABLE E2. HLA class I typing of recipients, their parathyroid donors, and their thymus donors

	HLA-A	HLA-B	HLA-C
Subject 1	0301	3508	0401
	0201	3501	0401
PT donor	0201	3501	0401
	0301	1801	0701
Thymus donor	0101	3501	0401
	1101	1801	0501
Subject 2	0201	1501	0303
	3101	4001	0304
PT donor	3101	4001	0304
	0201	4403	1601
Thymus donor	0101	4002	0202
	3301	7801	1601
Subject 3	6601	4102	1703
	2402	1302	0602
PT donor	2402	1302	0602
	0101	0801	0701
Thymus donor	0101	0801	0701
	0201	5101	1402
Subject 4	2402	0801	0702
	0201	4405	0202
PT donor	0201	4405	0202
	2401	3503	0401
Thymus donor	0201	4403	0202
	3201	4002	1403

PT, Parathyroid.