Human Hematopoietic Cells and Thymic Epithelial Cells Induce Tolerance via Different Mechanisms in the SCID-hu Mouse Thymus

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Summary

To study the role of thymic education on the development of the human T cell repertoire, SCID-hu mice were constructed with fetal liver and fetal thymus obtained from the same or two different donors. These animals were studied between 7 and 12 mo after transplantation, at which times all thymocytes and peripheral T cells were derived from stem cells of the fetal liver graft. Immunohistology of the thymus grafts demonstrated that thymic epithelial cells were of fetal thymus donor (FTD) origin. Dendritic cells and macrophages of fetal liver donor (FLD) origin were abundantly present in the medullary and cortico-medullary areas. Thymocytes of SCID-hu mice transplanted with liver and thymus of two different donors (FLD_A/FTD_B animals) were nonresponsive to Epstein-Barr virus–transformed B cell lines (B-LCL) established from both the FLD_A and FTD_B, but proliferated vigorously when stimulated with third-party allogeneic B-LCL. Mixing experiments showed that the nonresponsiveness to FTD_B was not due to suppression. Limiting dilution analysis revealed that T cells reacting with the human histocompatibility leukocyte antigens (HLA) of the FLD were undetectable in the $CD8^+$ T cell population and barely measurable in the $CD4^+$ subset. On the other hand, $CD4^+$ and $CD8^+$ T cells reactive to the HLA antigens of the FTD were readily detectable. These results indicate that FLD-reactive cells were clonally deleted, whereas FTD-reactive cells were not. However, the frequencies of FTD-reactive T cells were consistently twofold lower than those of T cells specific for any third-party B-LCL. In addition, the cytotoxic activity and interleukin 2 production by FTD-specific T cells were lower compared with that of third-party-reactive T cell clones, suggesting that FTD-specific cells are anergic. These data demonstrate that T cells become tolerant to autologous and allogeneic HLA antigens expressed in the thymus via two different mechanisms: hematopoietic cells present in the thymus induce tolerance to "self"-antigens by clonal deletion, whereas thymic epithelial cells induce tolerance by clonal anergy and possibly deletion of high affinity clones.

During the last several years, our knowledge of the role of the thymus in the induction of self-tolerance has dramatically expanded primarily due to the availability of transgenic animal models (1) and the discovery of endogenous superantigens (2) in laboratory mice. Immature thymocytes that express TCR recognizing self-peptide-MHC complexes present in the thymus can be physically eliminated by clonal deletion (3–6). Although clonal deletion is believed to be an important mechanism in the acquisition of self-tolerance in the normal thymus, it was demonstrated that thymocytes with self-reactive TCR are not always deleted, but rather functionally inactivated (7–9). This phenomenon of clonal anergy is not yet well understood. In addition, little is known of the factors determining the induction of clonal deletion or clonal anergy. The affinity of the MHC-peptide-TCR interaction and/or the nature of the cell that presents the antigen have been implicated (10, 11). Hematopoietic cells, in particular B cells and dendritic cells (DC),[1] are very potent inducers of clonal deletion (12–15). In contrast, thymic epithelium is incapable or less effective in dictating clonal deletion (7, 10, 16), but equally effective in inducing tolerance (17–19).

Due to the lack of suitable models to study thymic education in humans, it is unclear to what degree the data ob-

[1] *Abbreviations used in this paper:* DC, dendritic cell; FLD, fetal liver donor; FTD, fetal thymus donor; Mϕ, macrophage.

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tained in experimental animals apply to humans. In particular, it remains to be determined whether self-tolerance is achieved in the human thymus by clonal deletion of autoreactive cells. In addition, little is known about the nature of the cells responsible for the induction of tolerance. Information relevant to these questions has been obtained by studying the peripheral T cell repertoire in SCID patients reconstituted with allogenic fetal liver stem cells (20, 21). In these subjects, tolerance can be achieved in vivo to both the donor and host HLA antigens. This is the case even when the transplant results in a split peripheral chimerism where only the T lymphocytes come from the donor and all other hemopoietic lineages are of host origin. We demonstrated that this tolerance is not due to clonal deletion of donor T cells recognizing the host HLA antigens since host-reactive T cell clones were isolated in high frequencies in vitro (22). In contrast, no donor-reactive T cells could be detected, suggesting that these cells were clonally deleted (21). These studies prompted us to hypothesize that after allogenic stem cell transplantation, not only T cells but also macrophages (Mϕ), B cells, and DC differentiate from the donor stem cells in the host "chimeric" thymus (23, 24), whereas the thymic epithelial cells are exclusively of host origin. As shown in experimental models (1–17), the thymic epithelial cells would then be able to induce positive selection but not clonal deletion of host-reactive T cells, whereas the hematopoietic elements would delete the donor-reactive T cells.

To test this hypothesis and to investigate the mechanisms that regulate tolerance induction in the human (chimeric) thymus, we took advantage of the SCID-hu model described by McCune et al. (25). Human fetal liver and human fetal thymus tissue of the same or two different donors were coimplanted under the kidney capsule of SCID mice. In these transplanted mice, stem cells from the fetal liver migrate to the fetal thymus and differentiate into single-positive T lymphocytes (26). These cells finally migrate to the periphery as a mature, functionally competent, and polyclonal T cell population (27, 28). The reactivity of these T cells towards the HLA antigens of the fetal liver donor (FLD) and the fetal thymus donor (FTD) was studied. The HLA phenotype of the various cell populations present in the thymus, determined in parallel, showed that FLD gave rise to the hematopoietic elements, whereas the thymic epithelium derived from the FTD. Results demonstrate that tolerance towards both the FLD and the FTD is achieved. T cells specific for the "self"-HLA antigens expressed by the FLD are clonally deleted, whereas a significant proportion of FTD-reacting T cells is still present, but these cells are anergic.

Materials and Methods

Mice. C.B-17 *scid/scid* mice were transplanted with small pieces of fresh human fetal liver and thymus under the kidney capsule as described (26). Each mouse was transplanted with thymus and liver from the same or from two different fetal donors. Fetal donors between 15 and 21 wk gestational age were used. No attempts were made to deplete the thymus grafts of endogenous thymocytes. However, in most animals endogenous thymocytes are completely replaced by fetal liver derived cells by 3 mo after transplantation (26). SCID-hu mice, with thymocytes exclusively of FLD origin, were studied between 7 and 12 mo after transplantation. The following sets of SCID-hu mice were used: 2868, constructed with liver and thymus of fetal donor L064; 2869, constructed with material from FLD L064 and FTD L065; 1310, constructed with material from FLD K395 and FTD SV031; IM93, constructed with material from FLD F1115 and FTD F1116; C222, constructed with material from FLD F215 and FTD F214; C223, constructed with liver and thymus of fetal donor F215. In all cases a mAb against an HLA class I and HLA class II allotypic determinant was available to discriminate between FTD and FLD.

Cell Lines. B-LCL autologous to the transplanted material were generated by infection of pretransplant fetal liver or fetal thymus cells with EBV obtained from the marmoset cell line B95.8. T cell lines were derived from fetal liver or thymus by PHA stimulation in the presence of IL-2 (28). Each cell line was typed for HLA antigens by conventional serological techniques. The B-LCL used as targets for cytotoxicity tests were never kept in culture for a prolonged time, since this dramatically increased LAK sensitivity.

mAbs. The following purified mAbs against allotypic HLA determinants were used for FACS® analysis (Becton Dickinson & Co., Mountain View, CA) and histochemical staining: MA2.1 (anti-A2), BB7.1 (anti-B7), BB7.2 (anti-A2), MB40.2 (HLA-B7), GAP-A3 (HLA-A3), and SFR3-DR5 (HLA-DR5) (obtained from the American Type Culture Collection, Rockville, MD). PD3 (polymorphic DR determinant), 7.3.19.1 (DRw52), and IIB3 (polymorphic DQ determinant) were kindly provided by Dr. H. Bruning (University Hospital, Leiden, The Netherlands). XIII and 3E3 (polymorphic DR determinant) and logD6 (DRw53) were kindly provided by Dr. R. Bontrop (TNO, Rijswijk, The Netherlands). Leu-2, Leu-3, Leu-4, Leu-6, Leu-12, Leu-15, Leu-M3, Leu-M5, and HLE were purchased from Becton Dickinson & Co.).

Fluorescence Analysis. 10^5 cells were labeled with mAbs and FITC-labeled goat anti-mouse antibody as described previously (28). For double labeling experiments, after FITC labeling, the cells were washed twice in medium containing 1% normal mouse serum, and a PE-conjugated mAb was added. The cells were incubated for 30 min with the appropriate mAb in PBS with 0.1% BSA and NaN_3 in all staining experiments.

Immunohistologic Staining of Tissue Sections. One-third of the SCID-hu thymus-like structures were embedded in OCT medium and snap frozen in liquid nitrogen. The tissues were stored at −70°C until sectioning. Sections (5 μm) were acetone fixed, washed in PBS, and then subsequently incubated with an HLA class II–specific mAb followed by biotinylated horse anti–mouse IgG (Vector Laboratories, Inc., Burlingame, CA). Alkaline phosphatase (ALP)-conjugated avidin (Caltag Laboratories, South San Francisco, CA) was added and visualized using the substrate naphthol-AS phosphate (Sigma Chemical Co., St. Louis, MO) with fast blue BB salt (Sigma Chemical Co., St. Louis, MO). Endogenous phosphatase activity was blocked by adding levamisole (0.25 mM) in the reaction. Slides were counterstained with hematoxylin and mounted in glycerin-gelatin. Immunofluorescence was used for double staining of sections. For double staining with keratin and HLA class II–specific mAb, the sections were first stained with an HLA class II–specific mAb, then stained with Texas red–conjugated goat anti–mouse IgG (Caltag Laboratories). Next, tissues were stained with rabbit antikeratin (Dakopatts, Copenhagen, Denmark) and FITC-labeled goat $F(ab')_2$ anti–rabbit IgG (Caltag Laboratories). For double staining with CD1 (Leu-6) or CD11c (Leu-M5) mAb and HLA-specific mAbs, tissues were sequentially incubated with HLA class II–specific mAb, biotinylated horse anti–mouse Ig (Vector Labora-

tories, Inc.), and FITC-avidin (Caltag Laboratories). After incubations with 5% normal mouse serum, tissues were stained with either PE-labeled anti-CD1 (Leu-6) or CD11c (Leu-M5).

Proliferation Assay. The proliferation of the total thymocyte populations was tested in a MLR by stimulating 2×10^5 thymocytes with 2×10^4 irradiated B-LCL in round-bottomed microtiter plates. These cultures were incubated for 5 d at 37°C in a humidified atmosphere of 5% CO_2. During the last 16 h, 1 μCi of [^{3}H]thymidine was added. Subsequently, the cultures were harvested onto fiberglass filters, and [^{3}H]thymidine incorporation was determined by liquid scintillation spectroscopy.

Limiting Dilution Analysis for Lymphokine-secreting and Cytotoxic T Cells. Thymocyte suspensions were obtained by squeezing the dissected thymic structure through steel mesh with the plunger of a tuberculin syringe. After washing and counting the cells, 10 μl/10^6 cells of Leu-3-FITC and Leu-2-PE was added to the cell pellet. The cells were placed on ice for 30 min and washed once. Single-positive CD4 and CD8 thymocytes were obtained by cell sorting on a FACStar Plus® (Becton Dickinson & Co.). The $CD4^+$ or $CD8^+$ cells were then seeded manually at 0 (for background determination), 1 (for determination of cloning efficiency), and 20 cells/well in 96-well U-shaped microtiter plates in 50 μl Yssel's medium. A volume of 50 μl of a feeder cell mixture was added consisting of 10^6/ml PBL (irradiated, 4,000 rad), 10^5/ml JY B-LCL (irradiated, 5,000 rad), and 0.1 μg PHA/ml. After 1 wk, 100 μl of culture medium was added which contained 20 IU of IL-2/ml. Hereafter, the procedure differed for helper and cytotoxic precursor frequency determination.

For determination of cytotoxic precursor frequencies, the $CD8^+$ cells were incubated for an additional 5 d. A volume of 130 μl was taken from each well and transferred to V-shaped 96-well plates. After washing the cells in the V-shaped 96-well plate, the contents of each well were split and transferred into two U-shaped 96-well plates for the determination of cytotoxic activity. For each target, five plates seeded with 20 cells/well and one plate seeded with 0 cells/well were tested in duplicate. The cytotoxicity was assayed as described previously (29). In brief, 2×10^3 ^{51}Cr-labeled B-LCL or 5×10^3 PHA blasts were added to the various plates containing the effector cells in a final volume of 0.2 ml of culture medium. The plates were centrifuged and subsequently incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. The supernatants were harvested using a harvesting system (Skatron, Sterling, VA) and counted in a gamma counter. The percentage specific ^{51}Cr-release was calculated as described (29).

To determine lymphokine-secreting cells, the cultures (containing either $CD4^+$ or $CD8^+$ thymocytes) were incubated for an additional 1 wk. The contents of each well were then transferred to a V-shaped 96-well plate and washed three times with PBS. Subsequently, the contents of each well were resuspended in 150 μl, and 50-μl aliquots were transferred to the individual wells of three flat-bottomed microtiter plates. In this way, each culture could be tested for lymphokine secretion after stimulation with three different B-LCL. A total of 10^5 irradiated (5,000 rad) B-LCL was added as stimulators to each well in a final volume of 200 μl. Five plates seeded with 20 cells/well and one plate seeded with 0 cells/well for background were usually analyzed per test. After 20 h of incubation at 37°C for determination of IL-2 content, or 48 h for determination of IFN-γ content, 100 μl of supernatant was harvested from each well. IL-2 activity was assayed in duplicate on the mouse cell line CTLL-2 as described by Gillis et al. (30). IFN-γ was quantified in duplicate with an immunoenzymatic assay as described previously (31).

Calculation of Cytotoxic or Helper Frequencies. A culture was considered positive when the lower value of the duplicate determination was higher than the mean of 96-well background determinations (0 cells/well plate) plus three times the SD. The cloning efficiency (CE) was determined by visual scoring for growth of 96 wells seeded with 1 cell/well at the beginning of the experiment. CE was usually around 20%. The precursor frequency was calculated as follows: 1/frequency = $-\ln$ (number of negative wells/total number of wells)/[(CE/100)×20]. Absolute frequencies measured against a particular B-LCL were reproducible between experiments. Nevertheless, measurements of FLD, FTD, and third-party-specific precursor frequencies were always determined in the same experiment.

Results

Origin of Mature T Cells in SCID-hu Mice. Human liver and thymus were obtained from two different fetal donors, L064 and L065. A limited HLA phenotype performed on B-LCL of both donors with mAbs specific for HLA class I allotypic determinants demonstrated that donor L064 was HLA-$A2^-$,$B7^+$, whereas donor L065 was HLA-$A2^+$,$B7^-$ (Fig. 1). Two sets of SCID-hu mice were constructed with these donors: the 2868 animals, with fetal liver and thymus from donor L064; and the 2869 animals, in which fetal liver of donor L064 was coimplanted with fetal thymus of donor L065. 7 mo after transplantation, the mature human single-positive thymocytes and peripheral T cells present in SCID-hu 2868.1 or 2869.1 were sorted, cultured with PHA in the presence of IL-2, IL-6, and IFN-γ for 5 d to increase HLA expression, and phenotyped. As shown in Fig. 1, >99% of the $CD4^+$ and $CD8^+$ single-positive thymocytes were HLA-A2 negative and HLA-B7 positive in both animals, indicating that virtually all mature human T cells had differentiated from L064 fetal liver stem cells in the L064 or L065 human thymuses. Comparable findings were obtained in all animals used in this study.

Origin of HLA Class II–positive Cells Present in the SCID-hu Thymus. To investigate which cell types other than T cells were present in the SCID-hu thymus at the time of the study and to define their origin, sections of thymus graft were stained with mAbs specific for the HLA class II antigens of either FTD or FLD. As shown for SCID-hu 1310.2 in Fig. 2 *B*, a mAb specific for an HLA class II polymorphic determinant expressed by the FLD intensively stained the medullary and corticomedullary area. In contrast, a mAb specific for an HLA class II polymorphic determinant expressed by FTD cells predominantly stained cortical thymic epithelium, whereas isolated cells were stained in the medulla (Fig. 2 *A*). To determine the nature of the cells expressing HLA class II, sections were double stained with an HLA class II–specific allotypic marker in combination with a mAb specific for either keratin as an epithelial cell marker (Fig. 2, *C* and *D*), or CD1 as a marker for DC in the medulla (32, 33) (Fig. 2, *E* and *F*), or CD11c as a tissue Mϕ and DC marker (34, 35) (Fig. 2, *G*, *H*, *I*, and *J*). Most of the FTD-derived cells with dendritic morphology in the medulla were keratin positive (Fig. 2, *C* and *D*), which indicates that these cells are HLA class II–positive medullary thymic epithelium. No medullary

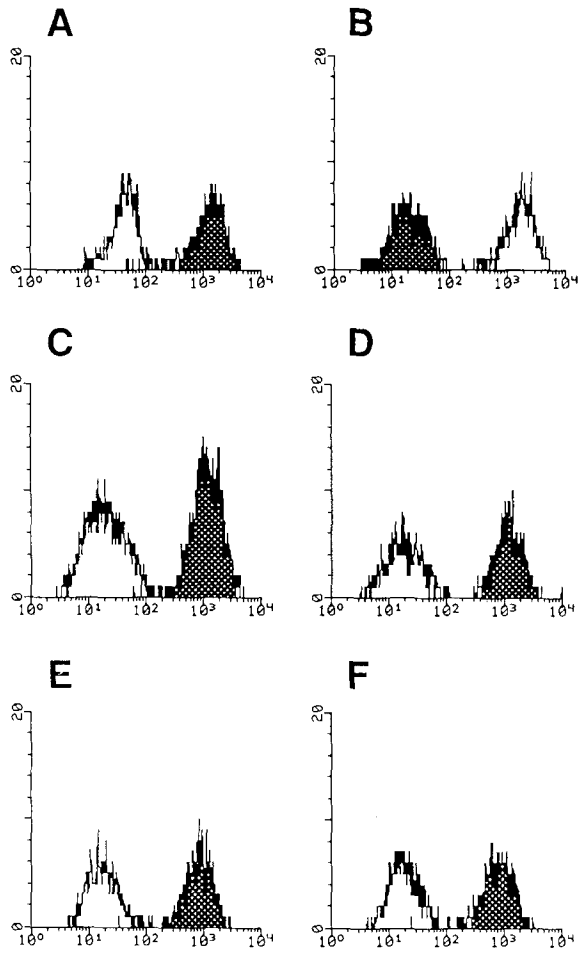

Figure 1. HLA phenotype of fetal donors and SCID-hu single-positive thymocytes. Cultured B-LCL derived from fetal donor L064 (*A*) and L065(*B*) were stained with the HLA-A2-specific mAb MA2.1 (*clear histogram*) or the HLA-B7 specific mAb MB40.2 (*filled histogram*). $CD4^+CD8^-$ (*C* and *D*) and $CD8^+CD4^-$ (*E* and *F*) T cells were purified by FACS® from SCID-hu 2868.1 (*C* and *E*) and 2869.1 (*D* and *F*) thymocytes. Cultured cell lines established from these purified populations were stained with MA2.1 and MB40.2 mAbs. The percentage of MA2.1-positive cells was 0.4% (*C*); 0.0% (*D*); 0.2% (*E*); and 0.1% (*F*). The percentage of MB40.2-positive cells was 98.8% (*C*); 99.9% (*D*); 99.7% (*E*); and 99.8% (*F*).

$CD1^+$ cells with dendritic morphology stained with the FTD HLA class II marker (data not shown), whereas virtually all $CD1^+$ cells with dendritic morphology were brightly stained with the mAb specific for the HLA class II of the FLD (Fig. 2, *E* and *F*), indicating that by 7 mo after transplantation, all endogenous DC are replaced by FLD-derived DC. In addition, >99% of $CD11c^+$ cells were positive for the FLD-specific marker (Fig. 2, *G*, *H*, *I*, and *J*), however, in one animal of the 2869 series, 5% of the $CD11c^+$ monocyte-like cells of FTD origin were present by 7 mo after transplantation. Collectively, these data show the gradual complete turnover of endogenous medullary and corticomedullary HLA class II–positive hematopoietic cells by FLD-derived cells after transplantation, with conservation of normal histological features.

Reactivity in MLR of FLD_A/FTD_A and FLD_A/FTD_B SCID-hu Thymocytes. To study the role of the thymic environment on the development of T cell tolerance, MLR were performed. A representative example of the results obtained in six experiments performed with thymocytes obtained from different SCID-hu mice is shown in Fig. 3. Unseparated thymocytes obtained from 2868.2 (FLD_A/FTD_A) SCID-hu thymus proliferated vigorously when stimulated with any third-party B-LCL tested, including L065. In contrast, only marginal proliferation was observed in response to the autologous B-LCL L064 (Fig. 3 *A*). Thymocytes of SCID-hu animal 2869.2 (FLD_A/FTD_B) likewise responded to the third-party B-LCL SV031 and K395 but did not proliferate when stimulated with either the FLD L064 or the FTD L065 (Fig. 3 *B*). The observed nonresponsiveness to the donor of the fetal thymus L065 was not due to altered kinetics since time course experiments indicated that thymidine incorporation was never significantly above background (data not shown). To determine whether suppressive activity could account for the nonresponsiveness towards the FTD, we performed a MLR in which unirradiated 2869.3 (FLD_A/FTD_B) "regulator" thymocytes were mixed at various ratios to 2868.3 (FLD_A/FTD_A) "responder" thymocytes and stimulated with L064 (Fig. 4 *A*), L065 (Fig. 4 *B*), or a third-party B-LCL (Fig. 4 *C*). The total cell numbers were kept constant. Responses of the mixture were always intermediate to those of the individual unmixed thymocytes. Significant proliferation after stimulation with the L065 B-LCL occurred even at responder-to-regulator ratios as low as 1:3. Collectively, these data show that T cells become nonresponsive to the HLA antigens expressed by the FTD during development in the thymus and that this nonresponsiveness is not due to the presence of inhibitory interactions between cells.

Frequencies of $CD4^+$ T Cells Specific for the HLA Antigens Expressed by FLD or FTD. To investigate whether the acquired nonresponsiveness of FLD_A/FTD_B SCID-hu thymocytes to the FTD HLA antigens was due to clonal deletion or anergy, a sensitive and quantitative limiting dilution assay was developed. In this assay, limiting numbers of CD4 single-positive thymocytes were stimulated with PHA in the presence of irradiated feeder cells and IL-2. The cells were expanded for 2 wk and were then tested for their ability to produce IL-2 upon stimulation with the FTD, FLD, or third-party-derived B-LCL. After stimulation of individual oligoclonal cultures of 2868.2 (FLD_A/FTD_A) $CD4^+$ thymocytes with the autologous B-LCL (Fig. 5 *A*), only a few cultures secreted IL-2 above background. Significantly more positive cultures and higher IL-2 levels were observed after allogeneic stimulation with L065 and K395 (Fig. 5, *C* and *E*). After stimulation with the allogeneic K395 B-LCL (Fig. 5 *F*), the responses of $CD4^+$ thymocytes from the 2869.2 (FLD_A/FTD_B) SCID-hu mouse were in the normal range. Interestingly, although the MLR responses of 2869.2 thymocytes to L064 or L065 were both negative, clearcut positive IL-2 responses were observed in this assay against FTD L065 (Fig. 5 *D*) but not against FLD L064 (Fig. 5 *B*). A summary of four experiments performed with $CD4^+$ thymocytes from

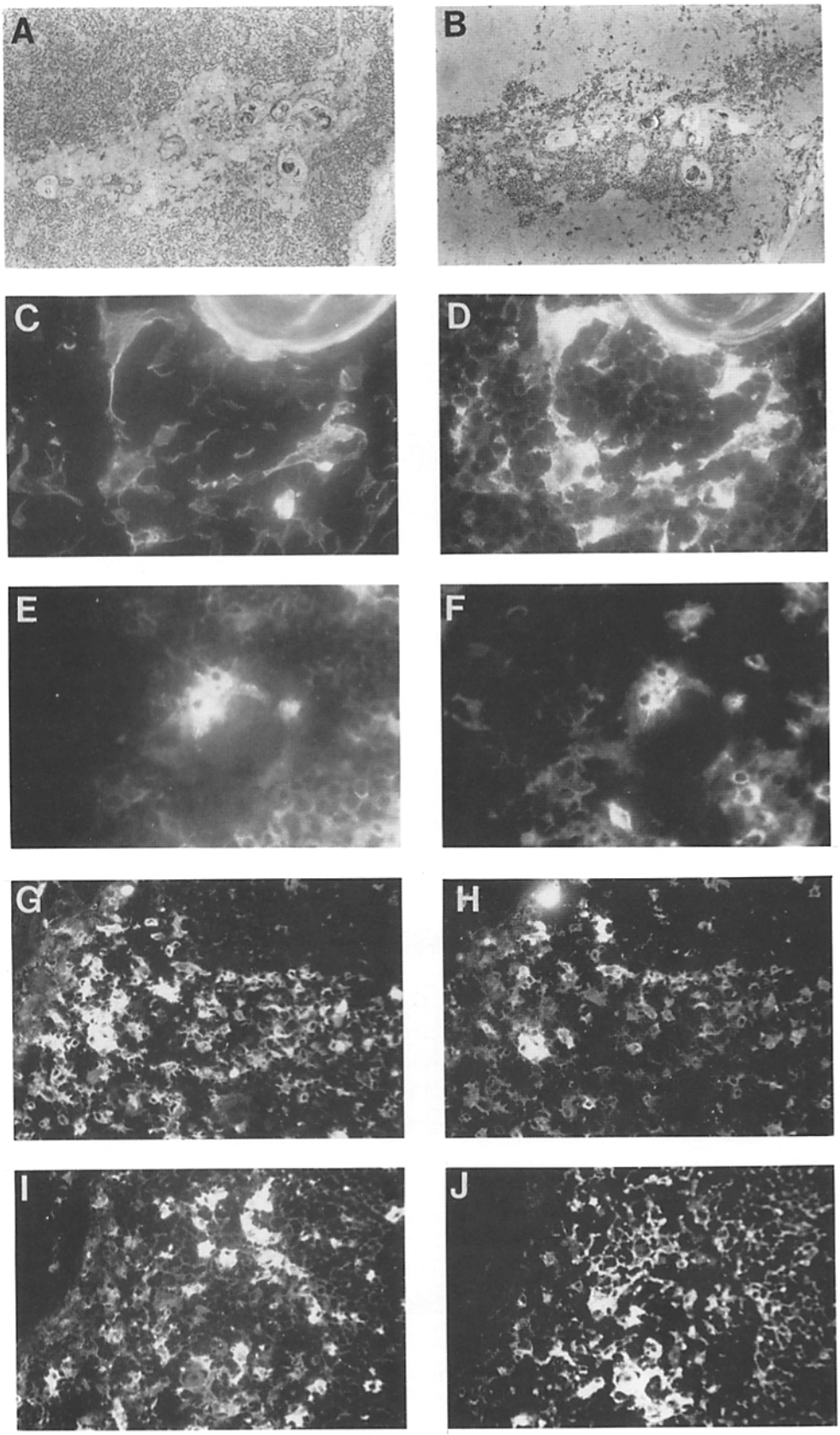

Figure 2. Immunohistology of SCID-hu chimeric thymus: characterization of HLA class II–positive cells in thymus of SCID-hu 1310.2 7 mo after transplantation. The FLD K395 was positive for IIB3 and negative for PD3, the FTD SV031 was positive for PD3 and negative for IIB3. Both mAbs recognize an HLA class II determinant. (*A*) Immuno-enzymic staining with PD3. A fine reticular staining of the cortex contrasts with scantly stained medulla (*center*). The corticomedullary area does not show a concentration of PD3 positive cells. (*B*) Immuno-enzymic staining with IIB3. Isolated cells are positive in the cortical area. Oval monocyte–like cells and cells with dendritic morphology at the corticomedullary junction and medulla are brightly positive. (*C* and *D*) Two-color staining with keratin (*C*) and PD3 (*D*). High-power magnification of a medullary area with bright PD3 staining demonstrates that the HLA class II of FTD origin is expressed by keratin-positive thymic epithelial cells. (*E* and *F*) Two-color staining with CD1 (*E*) and IIB3 (*F*). Irregularly shaped $CD1^+$ cells located in the medulla were considered DC. High magnification of the medullary area shows $CD1^+$ cells that are positive for IIB3. (*G* and *H*) Two-color staining with CD11c (*G*) and IIB3 (*H*). $CD11c^+$ cells express variable levels of HLA class II antigens of the FLD on the membrane. A few scattered Mϕ are seen in the cortex. (*I* and *J*) Two-color staining with CD11c (*I*) and PD3 (*J*). $CD11c^+$ cells are seen at the corticomedullary and medullary area. None of the cells express HLA Ag of the FTD.

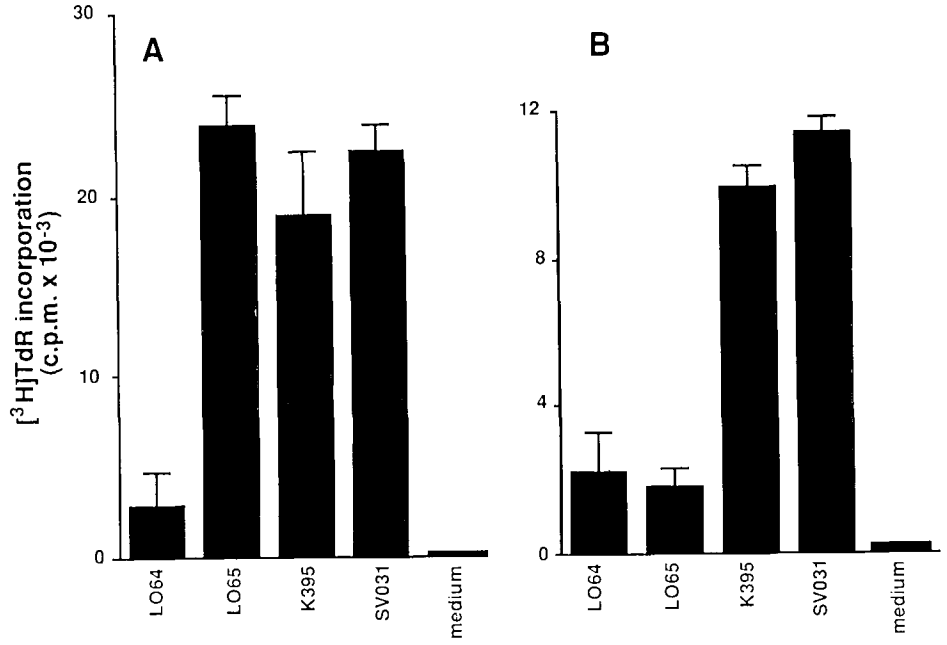

Figure 3. Proliferative responses of SCID-hu 2868.2 and 2869.2 thymocytes upon stimulation with B-LCL of FLD (L064), FTD (L065), and third-party B-LCL K395 and SV031. (*A*) The proliferative responses of thymocytes obtained from SCID-hu animal 2868.2 (FLD_A/FTD_A), constructed with fetal liver and thymus of donor L064; (*B*) proliferative responses of thymocytes from SCID-hu 2869.2 (FLD_A/FTD_B), constructed with material of FLD L064 and FTD L065.

different SCID-hu mice is shown in Table 1. The data are expressed as calculated frequencies (see Materials and Methods). The frequencies of FTD-specific $CD4^+$ T cells were significantly higher than those against autologous B-LCL (FLD), however, they never reached the frequencies measured against third-party B-LCL. The effect of thymic education on FTD-specific T cells can be quantified by taking the ratio of the frequency of T cells specific for a particular B-LCL in the T cell compartment of SCID-hu mice 2868 (FLD_A/FTD_A) to the frequency of the cells with the same specificity in

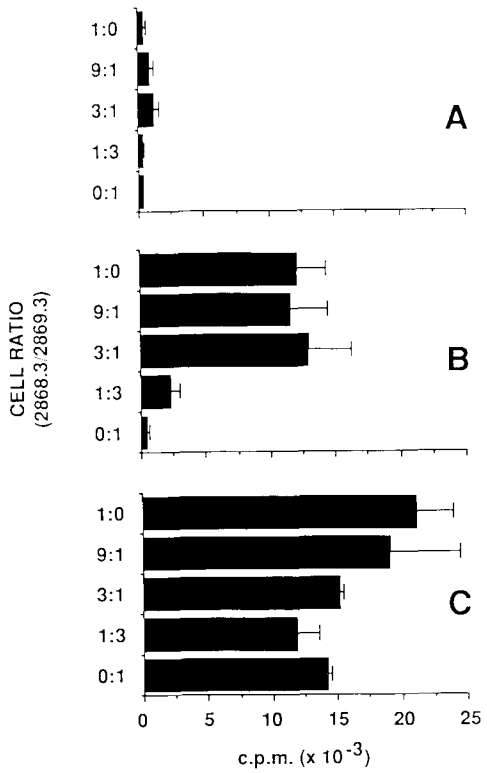

Figure 4. Proliferation of different ratios of thymocytes of SCID-hu 2868.3 (FLD_A/FTD_A), constructed with fetal material of L064, and 2869.3 (FLD_A/FTD_B), constructed with material of FLD L064 and FTD L065, in response to stimulation with L064 (*A*), L065 (*B*), and JY (*C*), a third-party B-LCL.

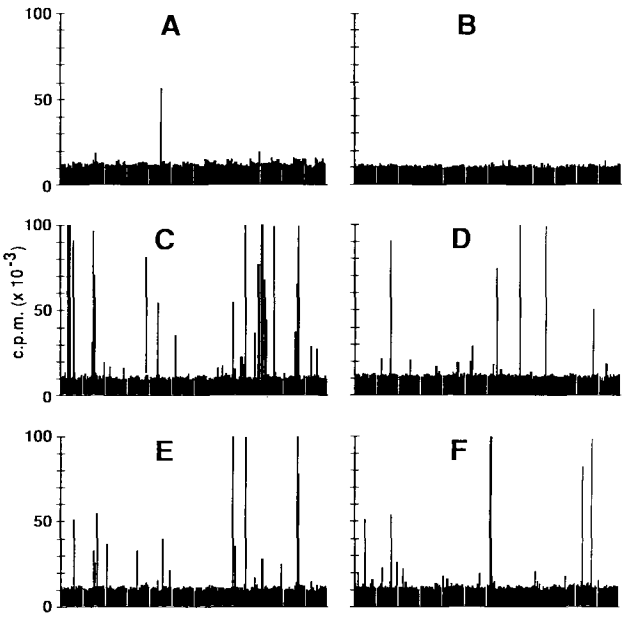

Figure 5. IL-2 production (expressed as cpm of the CTLL-2 cell line) by individual cultures after stimulation with FLD L064 (*A* and *B*), FTD L065 (*C* and *D*), and third-party B-LCL K395 (*E* and *F*). A total of 192 individual cultures of $CD4^+$ thymocytes are shown for each stimulator. $CD4^+$ thymocytes were obtained from SCID-hu animal 2868.2 (FLD_A/FTD_A), constructed with fetal liver and thymus of donor L064 (*A*, *C*, and *E*), and from SCID-hu 2869.2 (FLD_A/FTD_B), constructed with material of FLD L064 and FTD L065 (*B*, *D*, and *F*).

Table 1. *Frequency of IL-2-producing CD4⁺ Thymocytes Specific for FLD, FTD, and Third-party Alloantigens*

Exp.	SCID-hu	FLD	FTD	Frequency of T cells specific for: L064*	K395	L065	SV031
1	1310.2	K395	SV031	1:31	1:180	ND	1:120
2	1310.5	K395	SV031	1:41	1:1,000	ND	1:56
3	2869.1	L064	L065	1:362	1:34	1:72	ND
	2868.1	L064	L064	1:205	1:47	1:32	ND
4	2869.2	L064	L065	1:808	1:114	1:89	ND
	2868.2	L064	L064	1:405	1:89	1:57	ND

* B-LCL.

SCID-hu 2869 (FLD_A/FTD_B). This ratio is 2.3 (Table 1, Exp. 3) and 1.6 (Table 1, Exp. 4) for L065, and ~1 (0.72 and 1.28, respectively) for the third-party B-LCL K395.

Frequencies of CD8⁺ T Cells Specific for the HLA Antigens Expressed by FLD or FTD. Since specific cytotoxic activity could not be generated in MLR when thymocytes were stimulated with allogeneic B-LCL (36), a cytotoxicity assay was developed that bypasses the need for helper cells. CD8 single-positive thymocytes were isolated and stimulated with PHA in the presence of irradiated feeder cells and IL-2 as described for CD4⁺ cells. 12 d later, each culture was assayed for specific killing of FLD, FTD, or third-party B-LCL. One such experiment is shown in Fig. 6, and a summary of all results expressed as frequencies is shown in Table 2. High frequencies of allospecific cytotoxic cells with high lytic activity were obtained in such experiments, whereas the frequencies of cytotoxic cells specific for FLD HLA antigens were near the detection limit. CD8⁺ cells that specifically lysed FTD B-LCL were easily detectable, but the frequencies of these cells were on average twofold lower than those of allospecific B-LCL (Table 2). Comparable results were obtained when PHA blasts were used as target instead of B-LCL, excluding the possibility that EBV-specific lysis restricted by the HLA of the FTD was measured in these assays. Furthermore, it was consistently found in all experiments that the average percentage of lysis observed in individual cultures cyto-

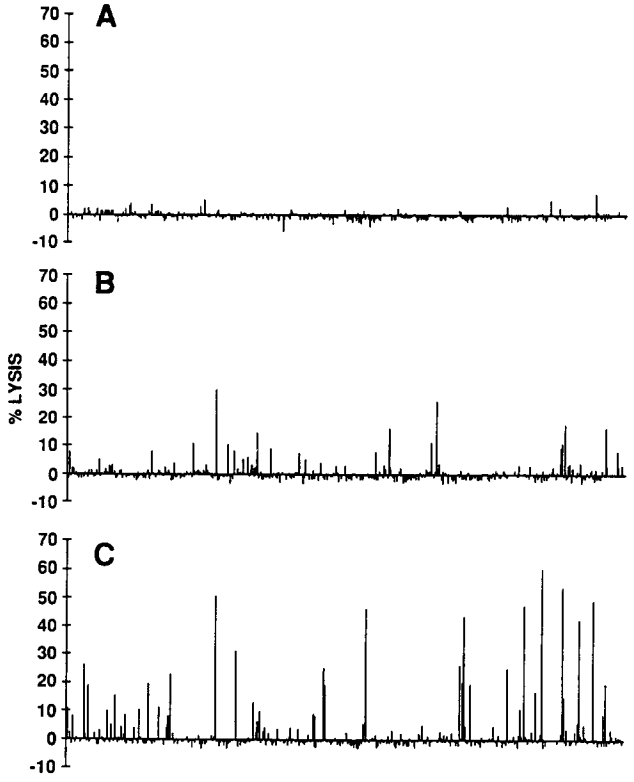

Figure 6. Percentage of lysis of FLD-derived B-LCL K395 (*A*), FTD-derived B-LCL SV031 (*B*), and third-party target F1115 (*C*) by 384 individual cultures of CD8⁺ thymocytes. The effector cells were CD8⁺ thymocytes obtained from SCID-hu 1310.5 (FLD_A/FTD_B), constructed with material of FLD K395 and FTD SV031. The percentage of lysis by the FTD-specific cultures (cultures which gave significant lysis of SV031 targets) was significantly lower than the percentage of lysis by the F1115-specific cultures (Wilcoxon rank test: $p < 0.05$).

Table 2. *Frequency of Cytotoxic CD8⁺ Thymocytes Specific for FLD, FTD, and Third-party Alloantigens*

SCID-hu*	Target	Frequency of T cells specific for: FLD	FTD	Third party
1310.2	PHA blast	1:3,010	1:301	1:251
	B-LCL	1:2,730	1:455	1:152
1310.5	B-LCL	1:1,200	1:150	1:62
IM93	PHA blast	1:2,520	1:420	1:252
	B-LCL	<1:1,600	1:192	1:73

* All SCID-hu mice were constructed with fetal liver and thymus from different donors. 1310.2 and 1310.5 were constructed with material from FLD K395 and FTD SV031; IM93 was constructed with material from FLD F1115 and FTD F1116.

toxic for the FTD was lower than that measured in individual cultures cytotoxic for third-party targets (Fig. 6). To exclude that these lower frequencies of FTD-specific $CD8^+$ cells, as determined by specific cytotoxicity, were due to the presence of noncytotoxic FTD-specific $CD8^+$ cells, large numbers of fresh $CD8^+$ cells were stimulated with PHA in the presence of feeders and IL-2 as described above and subsequently tested for specific IL-2 or IFN-γ production after stimulation with the FTD or third-party B-LCL. $CD8^+$ T cells specific for third party or FTD were reproducibly detected only in the IL-2 assay and are shown in Table 3. The frequencies of IL-2-producing $CD8^+$ T cells were indeed higher than those observed in the cytotoxic assay. This was probably due to the different sensitivity of the two tests. However, $CD8^+$ T cells specific for FTD were again detected at about twofold lower frequencies than third-party-reactive T cells.

Discussion

To better understand the events that regulate T cell reconstitution and induction of tolerance after allogeneic stem cell transplantation in humans, we transplanted SCID mice with human fetal liver and thymus obtained from two different HLA-mismatched donors. In these SCID-hu mice, pluripotent stem cells of fetal liver origin migrate to the human allogeneic thymus and differentiate to mature single-positive thymocytes that give rise to a functional polyclonal T cell repertoire in the periphery (26–28).

By transplanting genetically identical human stem cells in different thymic environments, we showed that the alloreactive T cell repertoire is profoundly altered by the HLA antigens expressed in the thymus. Stem cells that differentiated in an allogeneic thymus gave rise to T cells that did not respond to the HLA antigens of the FTD as well as of the FLD. This tolerance was specific for the alloantigens expressed in the thymus and was not genetically determined but acquired during differentiation in the thymus. Similar results have been obtained in murine models of transplantation. Tolerance, as defined by the absence of GVHD or skin graft rejection, is invariably achieved in radiation bone marrow chimeras (7, 10, 11), thymus-grafted chimeras (17, 19), and transgenic mice in which an MHC transgene is selectively expressed on thymic epithelial cells (37). However, the in vitro proliferative responses against the MHC antigens expressed in the thymus ranged from negative to normal (10, 11, 19). Gao et al. and Sprent et al. (10, 38) attributed the discrepancies among studies to the different sources of T cells tested: in mice transplanted with deoxyguanosine-treated thymus as well as in bone marrow chimeras, nearly complete nonresponsiveness was observed in the MLR when, as was the case in our study, thymocytes were used as responders, but only partial nonresponsiveness when lymph node T cells were tested.

Specific nonresponsiveness can be attributed to clonal deletion, clonal anergy, or suppression. We excluded that the acquired nonresponsiveness to the HLA antigens of the FTD observed in the SCID-hu mice was due to suppression since MLR, in which responder (FLD_A/FTD_A) and nonresponder (FLD_A/FTD_B) thymocytes were mixed, failed to show any inhibition even at the highest nonresponder to responder cell ratio. Limiting dilution analyses were performed to define whether clonal deletion of FTD-specific T cells might account for this nonresponsiveness. In these assays, T cells were activated with mitogens in the presence of IL-2 and in the absence of the tolerogen to revert possible anergy of FTD-specific T cells. Similar assays were successfully used in a previous study to exclude clonal deletion of donor-specific T cells as the cause of the acquired peripheral tolerance observed, both in vitro and in vitro, in a patient with a successful kidney graft (29). In addition, it has been reported in experimental in vivo models that anergy can be reversed when the tolerogen is removed (39, 40). Furthermore, in vitro studies have shown that cells, which do not respond to antigen or TCR-specific mAbs, may proliferate when stimulated with mitogens (41, 42) or when activated in the presence of IL-2 (43, 44).

In the SCID-hu mice studied here, FTD-specific $CD4^+$ as well as $CD8^+$ T cells were detected despite the nonresponsiveness of these cells in the MLR. No autologous FLD-specific $CD8^+$ T cells could be detected, and minimal reactivity of FLD specific $CD4^+$ T cells was measured. These results indicate that T cells reacting with the HLA antigens expressed by the FLD are clonally deleted, whereas FTD-specific T cells are not. However, the frequencies of FTD-specific T cells were about twofold lower than those of third-party-reactive T cells. Furthermore, the mean cytotoxic activity of FTD-specific $CD8^+$ T cells was significantly lower compared with that of T cells specific for third-party HLA antigens. A similar tendency, although not statistically significant, was observed for IL-2 production by $CD4^+$ population. In addition, preliminary experiments in which lymphokine production by 10 FTD-specific T cell clones and 10 third-party-reactive clones was compared, showed significantly lower production of IL-2 by Ag-stimulated FTD-specific clones (0.49 ng IL-2/ml vs. 1.98 ng IL-2/ml; Wilcoxon, $p < 0.05$). Collectively, these data indicate that the

Table 3. *Frequency of IL-2-producing $CD8^+$ Thymocytes Specific for FLD, FTD, and Third-Party Alloantigens*

	Frequency of T cells specific for:		
Responder	FLD	FTD	Third party
1310.2*	1:593	1:84	1:36
1310.5*	<1:2,400	1:171	1:80
IM93*	1:1,050	1:202	1:49
CB1†	ND	ND	1:50

* All SCID-hu mice were constructed with fetal liver and thymus from different donors. 1310.2 and 1310.5 animals were constructed with material from FLD K395 and FTD SV031; IM93 was constructed with material from FLD F1115 and FTD F1116.
† CB1, cord blood $CD8^+$ cells were used as control.

$CD4^+$ and $CD8^+$ T cells with low and intermediate functional activity were still present whereas highly cytotoxic or high IL-2-producing T cell clones specific for the HLA of the FTD were undetectable. This could be due to selective clonal deletion of the FTD-specific T cell clones which have high affinity for their antigen. Alternatively, is is possible that FTD-specific T cells are rendered anergic during maturation in the allogeneic thymus and that this anergy was only partially overcome in the limiting dilution assay. In support of the notion that FTD-specific T cells are anergic is the observation that the MLR to FTD HLA antigens was completely negative.

The data obtained so far are comparable with those previously described in SCID children transplanted with fetal allogeneic stem cells. Host-reactive T cells but not donor-reactive T cells are detectable in the blood of these patients despite the nonresponsiveness of these T cells towards both the FLD and the host (21–23). By taking advantage of the SCID-hu model, we are now able to correlate these functional data with the histologic composition of the chimeric thymus.

Hematopoietic cells, in particular the cells expressing HLA class II antigens, are potent inducers of clonal deletion in experimental animals (12). DC and $CD5^+$ B cells, but not Mϕ (13–15), were shown to be the most effective in this respect. In contrast, no or only partial clonal deletion is induced by thymic epithelium (11). In a mouse transgenic model with selective expression of H-2 I-E on thymic epithelium cells, ~20% reduction of I-E-reactive T cells was observed (7). In the SCID-hu mice, thymic cortical and medullary epithelium originated from the FTD. HLA class II–expressing cells of FLD origin were abundantly present in the medulla and corticomedullary area and consisted mainly of $CD11c^+$ cells and $CD1^+$ DC (32, 33). No $CD1^+$ DC of FTD origin were detected in the medulla, indicating that by 7 mo after transplantation endogenous DC are replaced by FLD-derived cells. Virtually all the cells expressing CD11c were of FLD origin. In one SCID-hu mouse, however, a few monocyte-like cells of FTD origin, expressing high levels of HLA class II, were detectable 7 mo after transplantation. The turnover rates for DC and Mϕ previously reported in human and in animal models are compatible with this observation (45). In a rat model, it was demonstrated that DC are completely replaced within 3 wk, whereas Mϕ turnover requires months, and some endogenous Mϕ can still be found 1 yr after transplantation (45). $CD19^+$ B cells were detectable in the thymus of SCID-hu mice by FACS® analysis but not by immunohistology. These cells are CD5 dull and are of FLD origin by 6 mo after transplantation (Vandekerckhove et al., manuscript in preparation).

In summary, we have demonstrated that the SCID-hu thymus is composed of human pre-T cells, T cells, B cells, DC, and Mϕ of FLD origin, whereas the thymic epithelium cells and possibly a few resident Mϕ are derived from the FTD. In these mice, human T cells educated in an allogenic host thymus, are rendered tolerant to autologous FLD as well as host FTD HLA antigens. Tolerance to the FLD is achieved by clonal deletion of FLD-reactive cells, whereas tolerance towards the FTD is mediated by clonal anergy and possibly clonal deletion of clones with high affinity for FTD HLA antigens. These data are comparable with those obtained in murine transplantation models (1–19). We therefore propose a similar sequence of events leading to self-tolerance in the human thymus: during passage through the thymus, FLD-specific cells are negatively selected upon interaction with FLD-derived DC, B cells, or Mϕ. FTD-specific T cells with high affinity may be deleted by FTD-derived thymic epithelial cells. The role in the induction of clonal deletion of few Mϕ present in the thymus of some SCID-hu mice is probably minor since results obtained in these animals and in those where no Mϕ could be detected were comparable. The FTD-specific clones that escape clonal deletion by thymic epithelial cells are rendered anergic upon interaction with these cells.

These findings have important clinical implications for allogeneic stem cell transplantations. They predict that allogeneic HLA-mismatched transplantation of stem cells can lead to a complete reconstitution of the T cell compartment, which will be tolerant to itself as well as to the host, even in situations where the recipient hematopoietic cells are absent due to the preconditioning regimen. Some degree of HLA sharing between peripheral APC and thymic epithelium is needed only to ensure antigen-specific MHC-restricted T cell responses. However, since the tolerance for host antigens is not caused by complete elimination of host-reactive cells, it is possible that these T cells can become functional. In patients who received an allogeneic bone marrow transplantation as part of the treatment for leukemia, it might be advantageous to manipulate these host-reactive cells to initiate a graft-vs.-leukemia effect (46).

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