

A Human Minor Histocompatibility Antigen Resulting from Differential Expression due to a Gene Deletion

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Abstract

Minor histocompatibility antigens (minor H antigens) are targets of graft-versus-host disease and graft-versus-leukemia responses after allogeneic human leukocyte antigen identical hematopoietic stem cell transplantation. Only a few human minor H antigens have been molecularly characterized and in all cases, amino acid differences between homologous donor and recipient proteins due to nucleotide polymorphisms in the respective genes were responsible for immunogenicity. Here, we have used cDNA expression cloning to identify a novel human minor H antigen encoded by *UGT2B17*, an autosomal gene in the multigene *UDP-glycosyltransferase 2* family that is selectively expressed in liver, intestine, and antigen-presenting cells. In contrast to previously defined human minor H antigens, *UGT2B17* is immunogenic because of differential expression of the protein in donor and recipient cells as a consequence of a homozygous gene deletion in the donor. Deletion of individual members of large gene families is a common form of genetic variation in the population and our results provide the first evidence that differential protein expression as a consequence of gene deletion is a mechanism for generating minor H antigens in humans.

Key words: hematopoietic stem cell transplantation • graft-versus-host disease • cytotoxic T lymphocyte • UDP glycosyltransferase 2B family • cDNA expression cloning

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT)* from a HLA identical donor is curative for a variety of hematologic malignancies but GVHD mediated by T cells transplanted with the stem cell graft remains a major complication (1). Minor histocompatibility antigens (minor H antigens) are the targets of GVHD and consist of HLA bound peptides, which are derived from cellular proteins that differ in amino acid sequence between donor and recipient due to polymorphisms in the genome (2). Because minor H antigens are also targets of the graft-versus-leukemia (GVL) effect, defining the polymorphisms in the ge-

nome that are responsible for generating minor H antigens and the tissues in which individual determinants are expressed may lead to novel strategies to reduce GVHD and/or augment GVL activity after HSCT (3).

The structure, genetics, and tissue distribution have been characterized for only a few human minor H antigens, although extrapolation from studies in mice suggests a very large number of minor H antigens are likely to be important in human transplantation (4). T cell clones that are specific for minor H antigens have been derived from allogeneic HSCT recipients and provide reagents for discovery of the genes that encode these antigens using either peptide elution and mass spectrometry (5), cDNA expression cloning (6), or genetic linkage analysis (7). Human minor H antigens that have been molecularly characterized include those encoded by the Y chromosome genes *SMCY*, *UTY*, *DFFRY*, and *DBY*, which are polymorphic with homologues on the X chromosome and are recognized by donor T cells after sex mismatched HSCT (5, 8–13). Additionally, four minor H antigens encoded by autosomal genes have been described and designated HA-1, HA-2, HA-8, and HB-1 (6, 14–17). The immunogenicity of all of the previously discovered human minor H antigens results from

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*Abbreviations used in this paper: B-LCL, Epstein Barr virus transformed lymphoblastoid cells; CYP, cytochrome P450; DC, dendritic cells; GST, glutathione S-transferase; HSCT, hematopoietic stem cell transplantation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GVL, graft-versus-leukemia; minor H antigens, minor histocompatibility antigens; SSP-PCR, sequence specific primer-PCR; *UGT2B17*, UDP glycosyltransferase 2 family, polypeptide B17.

polymorphism in the coding sequences of homologous donor and recipient genes that give rise to unique peptides, which are displayed at the surface of recipient cells bound to class I MHC and recognized by donor T cells.

Here, we have used cDNA expression cloning to identify a new autosomal human minor H antigen encoded by the *UDP glycosyltransferase 2* family, polypeptide *B17* (*UGT2B17*) gene. This minor H antigen is presented by HLA-A*2902 and recognized by CD8⁺ T cells that were isolated from a patient with GVHD involving the gastrointestinal tract, liver, and skin. In contrast to previously defined human minor H antigens, the immunogenicity of *UGT2B17* was not due to a polymorphism in the coding sequence between homologous donor and recipient genes but resulted from absent expression of *UGT2B17* in donor cells due to a homozygous deletion of the *UGT2B17* gene. Analysis of 36 unrelated normal donors demonstrated that four (11%) were also deficient in *UGT2B17* demonstrating that this polymorphism is not uncommon in the human population. These results provide the first evidence in humans that minor H antigens can result from discordance in gene expression as a consequence of homozygous deletion of individual members of multigene families.

Materials and Methods

Cell Culture. The CD8⁺ clone PL8 CTL was isolated from a blood sample obtained post-transplant from an allogeneic HLA identical HSCT recipient as described previously (18). HLA typing was performed by standard serologic methods and revealed that both the recipient and his sibling donor were HLA-A2, A29, B44, Cw5, DR3, DR11. PBMCs were obtained from the transplant recipient after engraftment at the onset of GVHD and were stimulated in vitro with aliquots of γ -irradiated PBMCs that had been obtained from the recipient pretransplant and cryopreserved. After three weekly stimulations, the PL8 CTL clone was isolated from the polyclonal T cell culture by limiting dilution cloning. The PL8 CTL clone was propagated by stimulation every 14 d with 30 ng/ml of OKT3 monoclonal antibody (Ortho Biotech), using unrelated allogeneic γ irradiated (35Gy) PBMCs and γ irradiated (50 Gy) Epstein Barr virus transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture media consisted of RPMI-HEPES (GIBCO BRL) containing 10% pooled, heat-inactivated human serum, and IL-2 (50 U/ml; Chiron Corp.). The T cell clone was used in cytotoxicity and epitope reconstitution assays either 12–16 d after stimulation (19), or 1 d after thawing a frozen aliquot. B-LCL were maintained in RPMI-HEPES with 10% FBS (ATLAS Biologicals). COS cells were obtained from the American Type Culture Collection and maintained in DMEM (GIBCO BRL) with 10% FBS.

cDNA Library Construction. A cDNA library consisting of 40,000 clones was constructed using the Superscript Choice System (GIBCO BRL). Total RNA was isolated from B-LCL and poly (A)⁺ mRNA was prepared by Straight A's mRNA Isolation (Novagen). mRNA was converted into cDNA using an oligo-dT primer that contains a NotI site at its 3' end and Thermoscript reverse transcriptase (GIBCO BRL). The cDNA was ligated to BstXI adaptors, digested with NotI, size fractionated by column chromatography, and ligated into BstXI and NotI sites of the pEAK10 expression vector (EdgeBioSystems). *E. coli* DH10B (GIBCO BRL) were electroporated with the recombinant plas-

mids and transformed clones were selected with ampicillin. cDNA pools, each comprising ~50 bacterial clones were amplified in liquid culture for 20 h, following which plasmid DNA was extracted using the MultiScreen filtration system (Millipore) and stored in 96-well plates.

Transfection of COS Cells and CTL Stimulation Assay. The cDNA expression cloning methodology for identifying genes encoding antigens recognized by CD8⁺ CTL was performed as described by Boon et al. with modifications (20). In brief, 5 × 10³ COS cells were plated in individual wells of 96-well plates, cultured for 24 h, and then transfected with 80 ng of plasmid DNA from each pool of the cDNA library and 40 ng of a plasmid encoding HLA-A*2902 using the FuGENE transfection reagent (Roche). 2 × 10⁴ CD8⁺ CTL were added to each well of COS cells 48 h after transfection, and after a further 24 h of co-culture at 37°C, IFN- γ was measured in the supernatant by ELISA (Endogen).

cDNA Constructs of UGT2B17. Constructs containing either full-length or a defined portion of the *UGT2B17* gene were generated from the 4A2 cDNA by PCR and cloned into pEAK10. Sense and antisense primers contained the recognition sequence (underlined in the primer sequences) for EcoRV and NotI, respectively, to facilitate cloning of the PCR product into pEAK10. The following sequences were used: full-length construct -5'-ATCGGATATCATGTCTCTGAAATGGATGTCAGT-3' (primer A) and 5'-ATCGGCGGCCGCTAATCCCTTTTCTTCTTCTTCTTCT-3'; construct I-primer A and 5'-ATCGGCGGCCGCTTAAACGGCATCTGCCAGAAGGA-3'; construct II-5'-ATCGGATATCATGGTCCTTCTGGCAGATGCCGTAA-3' (primer B) and 5'-ATCGGCGGCCGCTTAGATCATCGACCCAGAGAAAAC-3'; construct III-primer B and 5'-ATCGGCGGCCGCTTAGTACAGAAAGGGTATGTTAAGTAGC-3'. An additional construct UGT₄₉₃₋₅₆₁ was generated using primer B and 5'-ATCGGCGGCCGCTTACAGAAAGGGTATGTTAAGTAGCTC-3'.

Transfection of B-LCL with UGT2B17 cDNA. Donor B-LCL (5 × 10⁶) were electroporated (220 V, 500 μ FD) in 200 μ l K-PBS with 15 μ g of plasmid DNA encoding the full-length *UGT2B17* cDNA and with truncated versions of *UGT2B17*. The transfected B-LCL were placed in culture media, selected with 0.6 μ g/ml of puromycin (EdgeBioSystems) beginning 48 h after transfection, and used as targets in cytotoxicity assays 3 d after selection.

Chromium Release Assay. B-LCL were labeled for 1 h with ⁵¹Cr, washed twice, dispensed at 2 × 10⁵ cells/well into triplicate cultures in 96-well plates, and incubated for 4 h at 37°C with PL8 CTL at various E:T ratios. In peptide recognition assays, B-LCL were preincubated with various concentrations of peptide for 2 h at 37°C before labeling with ⁵¹Cr. These cells were then washed and aliquoted with PL8 CTL as described above.

Peptides. Peptides were synthesized with a free COOH terminus using standard Fmoc chemistry. The synthetic peptides were dissolved in dimethyl sulfoxide and stored at -20°C.

Northern Blot Analysis. Northern blot analysis was performed by standard methodology. In brief, 20 μ g of total RNA was extracted from B-LCL using RNeasy Minikit (QIAGEN), electrophoresed on a 1.2% agarose gel, and transferred to a Nytran SuperCharge Membrane (Schleicher & Schuell). The membrane was hybridized for 2 h at 65°C in ExpressHyb Solution (CLONTECH Laboratories, Inc.) with salmon sperm DNA (GIBCO BRL) and for 16 h at 68°C in the solution with a ³²P-labeled probe from corresponding to nt 400–751 of the *UGT2B17* gene.

After washing, the membrane was exposed for 2 d at room temperature on Storage Phosphor Screen (Molecular Dynamics). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (CLONTECH Laboratories, Inc.) was used as a control for RNA integrity and loading. Expression of *UGT2B17* in various tissues was analyzed using a dot-blot array of poly (A)⁺ RNA (MTE; CLONTECH Laboratories, Inc.).

Sequence-specific Primer (SSP)-PCR for *UGT2B17* in Genomic DNA and cDNA. The sense and antisense sequence-specific primers for PCR to detect exon 1 of the *UGT2B17* gene were (exon 1a) 5'-TGTTGGGAATATTCTGACTATAA-3' and 5'-CCCACTTCTTCAGATCATATGC-3'; (exon 1b) 5'-AAATGACAGAAAGAAACAA-3' and 5'-GCATCTTCACAGAGCTTATAT-3'. The sequence specific primers for exon 6 were 5'-GAATTCATCATGATCAACCG-3' and 5'-ACAGGCAACATTTTGTGATC-3'. The primers for the region 5' to *UGT2B17* were 5'-GGCAGTATCTTGCCAATGT-3' and 5'-AGACTCCAAGTGCCAGTT-3'. 38 cycles of amplification were performed on 0.5 μ l genomic DNA prepared from B-LCL using QIAamp DNA Blood Minikit (QIAGEN), or cDNA synthesized from total RNA using Superscript II (GIBCO BRL). Each reaction contained 0.4 μ l of Advantage 2 Polymerase Mix (CLONTECH Laboratories, Inc.), 0.2 mmol/L of each of the four deoxyribonucleotides, 10 pmol of each primer, and PCR buffer in a volume of 20 μ l. Each cycle consisted of denaturation (94°C; 30 s), annealing (68°C; 20 s), and elongation (72°C; 30 s). 10 μ l of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel. The primers for PCR to detect the GAPDH gene were 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3' and 22 cycles of amplification were used. Expression of *UGT2B17* in various tissues was analyzed using a panel of normalized first strand cDNA prepared from poly (A)⁺ RNA (MTC; CLONTECH Laboratories, Inc.) as a template for PCR with the sequence specific primers for exon 1 of *UGT2B17* and with the primers for *GAPDH* described above.

Generation of Dendritic Cells and B Cells. Immature dendritic cells (DCs) were generated from cryopreserved PBMC by culturing for 6 d in AIM-V (GIBCO BRL) with 800 U/ml of recombinant human GM-CSF (Immunex) and 500 U/ml of IL-4 (R&D Systems) as described (21). Mature DCs were generated from immature DCs by culturing for 2 additional days in AIM-V

with recombinant human GM-CSF (800 U/ml), IL-4 (500 U/ml), IL-6 (1,000 U/ml; R&D Systems), IL-1 β (10 ng/ml; R&D Systems), tumor necrosis factor α (10 ng/ml; R&D Systems), and prostaglandin E2 (1 μ g/ml; Sigma-Aldrich) as described previously (22). Activated B cells were generated by culturing PBMCs for 15 d on γ -irradiated (96 Gy) human CD40L-transfected NIH3T3 cell (gift from J. Schultze, Dana-Farber Cancer Institute, Boston, MA) in IMDM (GIBCO BRL) containing IL-4 (100 U/ml) as described previously (23). First strand cDNA was prepared from total RNA extracted from each subset of APCs using RNeasy Minikit and reverse transcribed using Superscript II. PCR to detect *UGT2B17* or *GAPDH* in cDNA was performed as described above. For other subsets of peripheral blood, a cDNA panel (Human blood fractions MTC; CLONTECH Laboratories, Inc.) was used as a template for PCR.

Results

Isolation of a cDNA Encoding the Minor H Antigen Recognized by PL8 CTL. The PL8 CTL clone was isolated from blood obtained from an allogeneic HLA identical HSCT recipient at the onset of acute GVHD involving the gastrointestinal tract, liver, and skin. PL8 CTL lysed recipient B-LCL and 21 of 24 B-LCL lines from unrelated individuals that shared HLA-A29 with the recipient, but failed to lyse B-LCL from the donor and 10 unrelated individuals that shared class I HLA molecules other than HLA-A29 (unpublished data). This data indicated that PL8 CTL recognizes a minor H antigen that is frequent in the population (~88%) and presented by HLA-A29.

To identify the minor H antigen recognized by PL8 CTL, we constructed a cDNA library from B-LCL that expressed the antigen and cotransfected COS cells with plasmid pools containing ~50 cDNAs from the library, and with a plasmid encoding HLA-A*2902. The transfected COS cells were cocultured with PL8 CTL and the production of IFN- γ measured in the supernatant. COS cells transfected with two of 384 cDNA pools stimulated IFN- γ production (Fig. 1 A). These two positive pools were then

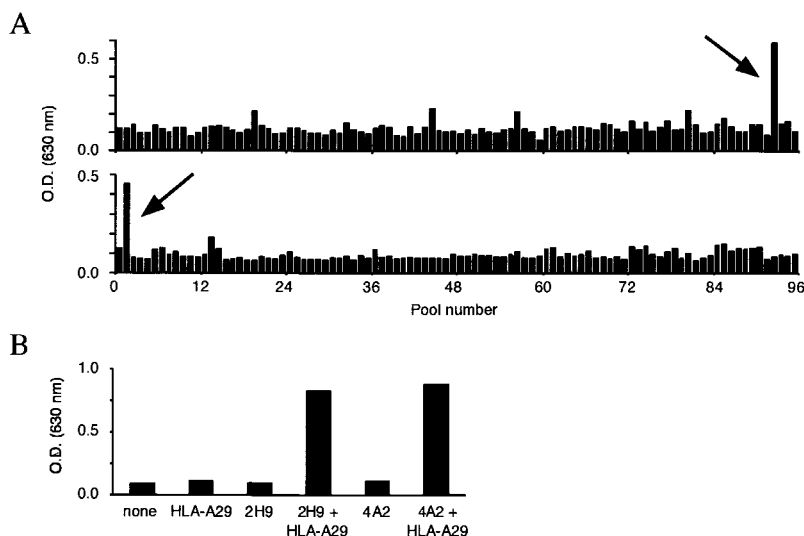


Figure 1. Identification of a cDNA encoding the minor H antigen recognized by PL8 CTL. (A) Pools of a cDNA library containing ~50 cDNA clones were transiently transfected into COS cells in individual wells of a 96-well plate together with a plasmid encoding HLA-A*2902. COS transfectants were cocultured with PL8 CTL and IFN- γ production was measured in supernatant collected after 24 h. Two pools (indicated by arrows) stimulated IFN- γ production by PL8 CTL. (B) Isolation of a cDNA that stimulates IFN- γ production by PL8 CTL. The two pools of cDNA were subcloned in *E. coli*, and individual plasmids were transfected into COS cells alone or with HLA-A*2902. Two clones, designated 2H9 and 4A2 stimulated IFN- γ production by PL8 CTL only when transfected with HLA-A*2902. Data is shown as the mean of triplicate determinations.

subcloned, and individual plasmids were rescreened by COS cell transfection. Two plasmids containing inserts of 2,896 bp and 2,958 bp, respectively, induced HLA-A29-dependent IFN- γ production by PL8 CTL after transfection into COS cells (Fig. 1 B). The cDNA inserts of each plasmid were sequenced and a search of DNA sequence databases revealed that both cDNAs had greater than 99% identity with the *UGT2B17* gene (GI: 4507820).

Identification of the Antigenic Epitope Encoded by *UGT2B17*. The *UGT2B17* sequence that encoded the epitope recognized by PL8 CTL was localized by generating truncated *UGT2B17* constructs containing selected portions of the gene, transfecting each construct into COS cells with the plasmid encoding HLA-A29, and screening the COS transfectants for recognition by PL8 CTL. Construct I included nt 52–513 of the primary open reading frame (ORF) of *UGT2B17* and was generated with a TAA codon at the 3' end to terminate transcription. Constructs II and III included nt 493–993 and nt 493–564 of *UGT2B17*, respectively, and were engineered to have ATG and TAA codons at the 5' and 3' ends of the primary ORF, respectively (Fig. 2 A). COS cells transfected with either construct II or III, but not with construct I, stimulated IFN- γ production by PL8 CTL demonstrating that the antigenic epitope recognized was derived from the 24

amino acid polypeptide encoded by nt 493–564 of *UGT2B17* (Fig. 2 A).

Peptides that bind to HLA-A*2902 frequently contain a glutamic acid at P2 and tyrosine at the COOH terminus as preferred anchor residues (24). We examined the amino acid sequence encoded by nt 493–564 of *UGT2B17* for candidate peptides that might bind to HLA-A*2902 and identified the 10 mer peptide, AELLNIPFLY, which contained the appropriate anchor residues (Fig. 2 B). Donor B-LCL were pulsed with various concentrations of synthetic AELLNIPFLY and tested as targets for PL8 CTL in a cytotoxicity assay. PL8 CTL lysed donor B-LCL pulsed with AELLNIPFLY and half-maximal lysis occurred at peptide concentrations of 100 fM–1 pM (Fig. 2 C). Synthetic peptides which have 1 amino acid deleted (ELLNIPFLY) or added (LAELLNIPFLY) to the NH₂ terminus, respectively, also sensitized donor B-LCL for lysis by PL8 CTL, but at a one to two log higher concentration. We also synthesized the nonamer peptide AELLNIPFL with the COOH-terminal tyrosine deleted because the computer program PA-ProC (www.paproc.de) that identifies likely COOH-terminal proteosomal cleavage sites predicted AELLNIPFL, but not AELLNIPFLY, as a cleavage product of *UGT2B17*. Leucine also is a preferred residue at the COOH terminus for binding to HLA A*2902 (24). The

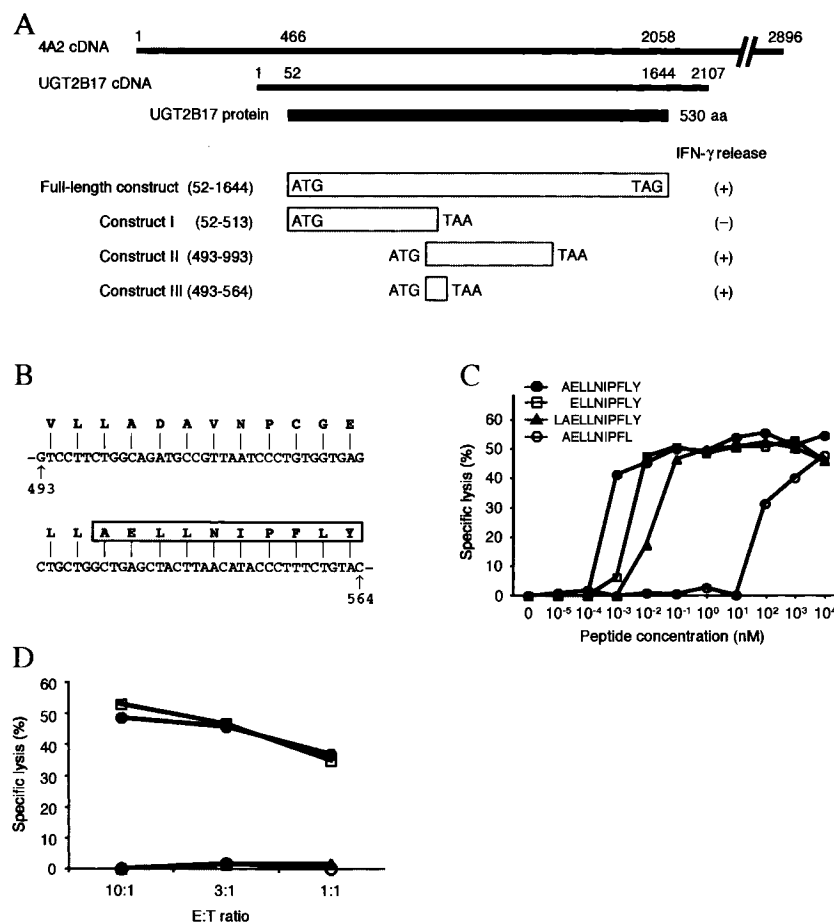


Figure 2. Identification of the antigenic epitope encoded by *UGT2B17*. (A) Location of the epitope-containing region by transfection of truncated *UGT2B17* constructs. Alignment of 4A2 cDNA with the *UGT2B17* cDNA (NM_001077), the nucleotide numbering of the constructs used for transfection corresponds to that provided for the *UGT2B17* GenBank sequence. Constructs I, II, and III contained the indicated *UGT2B17* sequences and were transfected with HLA-A*2902 into COS cells. IFN- γ production by PL8 CTL was measured after coculture with COS transfectants and is indicated by (+) or (-) in the right-hand column. (B) *UGT2B17*₄₉₃₋₅₆₄ encodes a decamer peptide with anchor residues for HLA-A*2902. The amino acid sequence encoded by *UGT2B17*₄₉₃₋₅₆₄ is shown and a putative epitope for PL8 CTL is boxed. (C) CTL recognition of donor B-LCL cultured with synthetic peptides corresponding to *UGT2B17* sequences. The concentration of peptide that elicited half-maximal lysis was ~0.7 pM for AELLNIPFLY, ~6 pM for ELLNIPFLY, ~50 pM for LAELLNIPFLY, and ~80 nM for AELLNIPFL. Specific lysis is shown as the mean of triplicate cultures at an E:T ratio of 5:1. (D) Transfection of minigene constructs define a requirement for tyrosine at the COOH terminus of the naturally processed *UGT2B17* epitope. Donor B-LCL were transfected by electroporation with either *UGT2B17*₄₉₃₋₅₆₄ that encodes the 24 amino acid polypeptide described in B or *UGT2B17*₄₉₃₋₅₆₁ that encodes 23 amino acids with the COOH-terminal tyrosine deleted. Transfected B-LCL were selected for 3 d with puromycin (0.6 μ g/ml). The lysis of *UGT2B17*₄₉₃₋₅₆₄-transfected donor B-LCL (solid circles), *UGT2B17*₄₉₃₋₅₆₁-transfected donor B-LCL (triangles), recipient B-LCL (squares), and untransfected donor B-LCL (open circles) is shown as the mean of triplicate cultures at various E:T ratios.

synthetic AELLNIPFL sensitized donor B-LCL for recognition by PL8 CTL but at a 5 log higher concentration than AELFNIPFLY (Fig. 2 C). Therefore, to further define the COOH terminus of the naturally processed epitope, we transfected donor B-LCL with construct III (UGT2B17₄₉₃₋₅₆₄) encoding a 24 amino acid peptide ending with tyrosine at the COOH terminus, or with a construct (UGT2B17₄₉₃₋₅₆₁) that encoded a 23 amino acid polypeptide with the COOH-terminal tyrosine deleted, and analyzed recognition by PL8 CTL. The donor B-LCL transfected with UGT2B17₄₉₃₋₅₆₄ were lysed as well as recipient B-LCL, but donor B-LCL transfected with UGT2B17₄₉₃₋₅₆₁ were not recognized at all by PL8 CTL (Fig. 2 D). These results indicate that the naturally processed peptide contains tyrosine and not leucine at the COOH terminus and suggest the optimal peptide for PL8 CTL is AELLNIPFLY.

The Epitope of UGT2B17 Differs in Amino Acid Sequence with All Other UGT2B Family Members. UGT2B17 is a member of the UGT2B multigene family, which presently consists of 7 genes and 5 pseudogenes and maps to chromosome 4q13. We compared the sequence of the recipient UGT2B17 cDNA with the canonical sequences of other UGT2B family members obtained from the GenBank DNA sequence database (25–31). Although there was a high degree of homology between UGT2B17 and other family members, all family members had one or more amino acid differences in the peptide sequence corresponding to the epitope identified in UGT2B17 (Table I). The most closely related sequence in this region was that encoded by UGT2B15, which contained only a single amino

acid difference, a phenylalanine in place of leucine at P4. The synthetic peptide (AELFNIPFLY) corresponding to the UGT2B15 sequence failed to efficiently sensitize donor B-LCL for recognition by PL8 CTL, although a low level of lysis was observed at μM concentrations of peptide (Fig. 3). The amino acid sequences of UGT2B10 and UGT2B7 in the epitope region were identical with each other but differed from UGT2B17 at P4 with a phenylalanine instead of leucine and at P9 with a valine instead of leucine. The sequences encoded by UGT2B11, UGT2B4, and UGT2B28 also contained the valine substitution at P9 and either an alanine in place of glutamic acid at P2 (UGT2B11 and UGT2B28), an arginine in place of proline at P7 (UGT2B11), or a lysine in place of asparagine at P5 (UGT2B4). Synthetic peptides corresponding to the sequence of each of these other UGT2B17 family members also failed to sensitize donor B-LCL for recognition by PL8 CTL (Fig. 3). Taken together, these data identify an important role for leucine at P4, asparagine at P5, and leucine at P9 in the UGT2B17 epitope for recognition by PL8 CTL, either directly as T cell receptor contact residues or as secondary anchor residues for binding, or indirectly by altering the conformation of T cell receptor contact or MHC binding residues in the epitope (32).

Immunogenicity of UGT2B17 Is Due to Differential Transcription. All previously defined human minor H antigens contain amino acid substitutions that result from nucleotide polymorphism between homologous recipient and donor genes. We initially presumed that the sequence of the donor and recipient UGT2B17 genes would contain a polymorphism in the epitope region. However, a search of the human genome database of single nucleotide polymorphisms failed to uncover any previously described polymorphisms in this region of UGT2B17 encoding the epitope. Therefore, we attempted to isolate UGT2B17 cDNA from donor cells for sequencing to identify a possi-

Table I. Homology between UGT2B17 and Other UGT2B Family Members

Members	GI numbers	Homology ^a	Peptides ^b
UGT2B15	475758	97.6 (1555/1593)	AELFNIPFLY
UGT2B11	4507822	85.9 (1368/1593)	AALLNIRFVY
UGT2B10	4507816	85.2 (1358/1593)	AELFNIPFVY
UGT2B7	4507824	85.6 (1363/1593)	AELFNIPFVY
UGT2B4	10863940	85.2 (1357/1593)	AELLKIPFVY
UGT2B28	16596679	84.2 (1342/1593)	AALLNIPFVY
UGT2B29P ^c	6979424	82.8 (565/682)	Not available
UGT2B27P	6979423	83.9 (559/666)	Not available
UGT2B26P	6979422	82.9 (539/650)	Not available
UGT2B25P	6979421	82.6 (598/724)	Not available
UGT2B24P	6979420	82.3 (596/724)	Not available

^aHomology between recipient UGT2B17 cDNA and canonical sequences of each UGT2B family member is presented as the percentage and actual number (parentheses) of identical nucleotides.

^bAmino acid sequence of the peptides encoded by other family members and corresponding to AELLNIPFLY encoded by UGT2B17. Amino acids that differ from AELLNIPFLY are shown in bold.

^cP indicates a pseudogene.

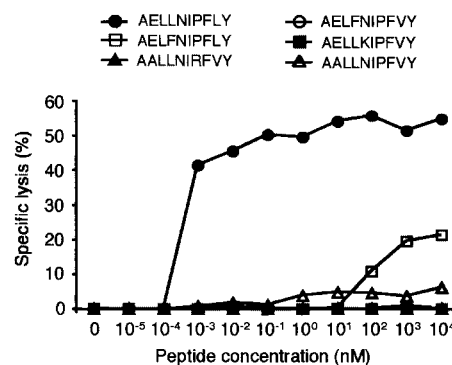


Figure 3. Donor cells pulsed with synthetic peptides corresponding to homologous sequences of other UGT2B family members are not recognized by PL8 CTL. Donor B-LCL were pulsed with various concentrations of the following synthetic peptides: AELFNIPFLY (encoded by UGT2B17), AELFNIPFLY (UGT2B15), AALLNIRFVY (UGT2B11), AELFNIPFVY (UGT2B10 and UGT2B7), AELLKIPFVY (UGT2B4), or AALLNIPFVY (UGT2B28), and tested as targets for PL8 CTL in a cytotoxicity assay. Specific lysis is shown as the mean of triplicate cultures at an E:T ratio of 5:1.

ble polymorphic *UGT2B17* allele. However, a PCR product was not obtained from donor cells despite trying several primer pairs designed specifically to amplify *UGT2B17* (unpublished data). We next determined if the levels of *UGT2B17* gene expression were comparable in total RNA prepared from recipient and donor cells using a probe that overlapped the exon 1 sequence encoding the antigenic peptide and was polymorphic with other UGT family members to minimize crosshybridization. *UGT2B17* transcripts were easily detected in recipient B-LCL and in unrelated HLA-A29⁺ B-LCL that were lysed by PL8 CTL but not in donor B-LCL or unrelated HLA-A29⁺ B-LCL that were not lysed by PL8 CTL (Fig. 4, A and B). To determine if lack of recognition by PL8 CTL was solely attributable to the absence of *UGT2B17* gene expression, the full-length *UGT2B17* cDNA in a plasmid containing the puromycin resistance gene was transfected into donor B-LCL and cells were selected in puromycin. Donor B-LCL transfected with the *UGT2B17* cDNA were lysed equally well as recipient B-LCL, demonstrating the lack of recognition of donor cells by PL8 CTL resulted entirely from

differential transcription of this gene in recipient versus donor cells (Fig. 4 C).

Lack of Expression of *UGT2B17* in Donor Cells Is Due to a Gene Deletion. Several possibilities could account for the absence of *UGT2B17* transcripts in donor cells, including a homozygous gene deletion, polymorphism in promoter sequences, or a polymorphism in the 3' portion of the gene that resulted in termination of transcription upstream of the region encoding the epitope and detected by our probe. To determine if all or a portion of the *UGT2B17* gene was deleted in donor cells, we analyzed genomic DNA prepared from donor B-LCL using SSP-PCR with primer pairs to detect exon 1 and exon 6 sequences, which represent the first and last exons of the *UGT2B17* gene (33), and a primer pair that recognizes sequences 5' to the initiation codon for *UGT2B17*. PCR products for exon 1, exon 6, and the region immediately 5' to exon 1 were not detected by amplification of genomic DNA from donor B-LCL or minor H antigen-negative B-LCL from unrelated individuals but were obtained by amplification of genomic DNA from recipient B-LCL and unrelated minor H antigen-positive B-LCL (Fig. 5). These data indicated that the failure to detect a *UGT2B17* transcript in donor cells was a result of homozygous deletion of a large portion of the *UGT2B17* gene including the ATG initiation codon, exon 1, and at least a portion of exon 6. Analysis of B-LCL from 36 randomly selected normal individuals by SSP-PCR identified 4 (11%) that similarly lacked both exon 1 and exon 6 of *UGT2B17* demonstrating that homozygous deletion of the *UGT2B17* gene was not restricted to this particular stem cell donor but occurs as a variant in the human population.

Expression of *UGT2B17* in Tissues and APCs. PL8 CTL were isolated from an HSCT recipient with GVHD involving the liver, gastrointestinal tract, and skin. To determine if *UGT2B17* was expressed in tissues that were targets of GVHD in this patient, first-strand cDNA prepared from different human tissues pooled from multiple donors was evaluated for sequences corresponding to exon 1 using SSP-PCR. Expression of *UGT2B17* was easily detected in liver, colon, small intestine, and pancreas, but absent or barely detectable in other tissues (Fig. 6 A). Similar data was obtained using hybridization of a dot-blot array of poly (A)⁺ RNA from human tissues. A dominant signal for *UGT2B17* mRNA was found in liver, colon, and pituitary gland, with a weaker signal detected in small intestine and spleen (unpublished data).

The expression profile of *UGT2B17* was compatible with it being a target for T cells mediating GVHD in this patient. However, studies in murine models have suggested that expression of minor H antigens by APC is required for initiating CD8⁺ T cell responses in GVHD (34). Thus, we investigated the expression of *UGT2B17* in subsets of hematopoietic cells prepared from cryopreserved pretransplant recipient blood. *UGT2B17* was expressed in immature and mature DC, and resting and activated B cells, but not in resting or activated T cells (Fig. 6 B). To determine whether the level of expression was sufficient for mature

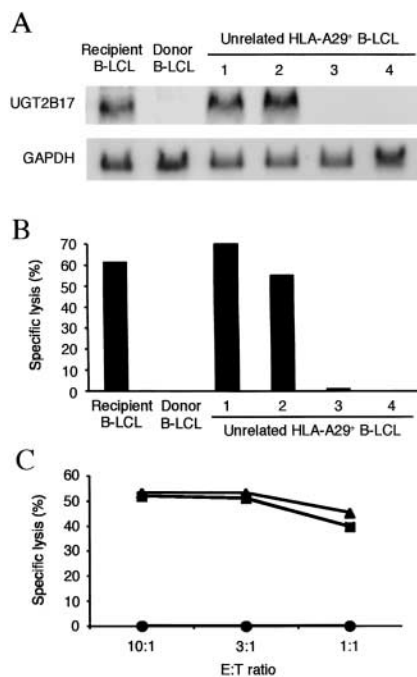


Figure 4. *UGT2B17* mRNA is transcribed in minor H antigen positive but not minor H antigen negative cells. (A) Northern blot analysis of total RNA from recipient and donor B-LCL, and from HLA-A29⁺ B-LCLs from 4 unrelated donors (designated 1–4). The blot was hybridized with a ³²P-labeled *UGT2B17* probe for 16 h at 68°C. Detection of *GAPDH* mRNA was performed as a control. (B) Cytotoxicity assay for B-LCL by PL8 CTL. The B-LCL from unrelated donors (1–4) are the same lines used for analysis of gene expression in Fig. 3 A. Specific lysis is shown as the mean of triplicate cultures at an E:T ratio of 5:1. (C) Transfection of *UGT2B17* cDNA into donor B-LCL restores recognition by PL8 CTL. Donor B-LCL were transfected by electroporation with the full-length construct of *UGT2B17*, selected for 3 d with puromycin (0.6 μg/ml), and assayed as targets for PL8 CTL. The lysis of *UGT2B17*-transfected donor B-LCL (triangles), untransfected donor B-LCL (circles), and recipient B-LCL (squares) is shown at various E:T ratios.

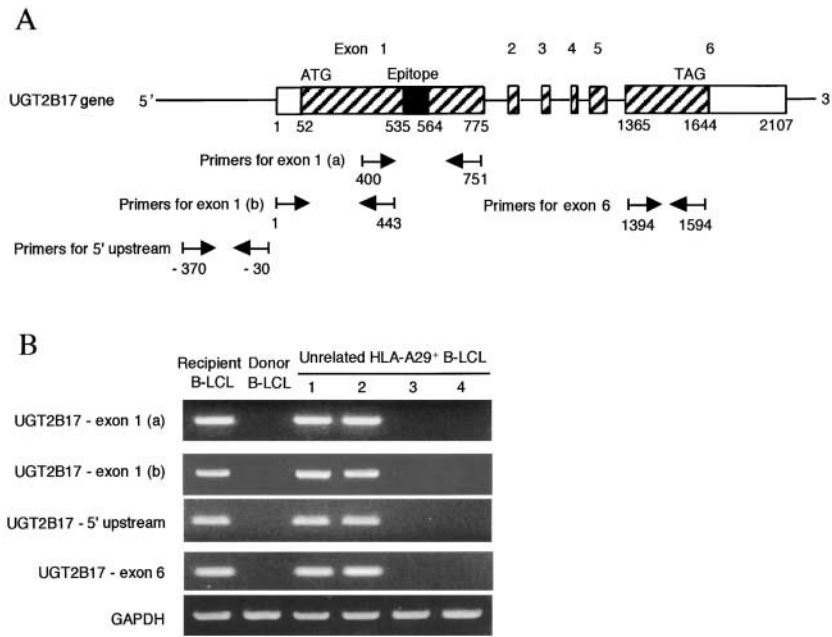


Figure 5. The *UGT2B17* gene is deleted in donor cells and HLA-A*2902 positive cells from unrelated individuals that are not recognized by PL8 CTL. (A) *UGT2B17* consists of six exons encoded over 27 Kb of DNA on chromosome 4. Primer pairs used for PCR to detect exon 1 and exon 6 sequences were selected such that at least one primer of each pair contained nucleotides that were mismatched with all other known UGT family members. The primer pair for the region immediately 5' to the *UGT2B17* start site was selected to amplify nt -370 to -30. (B) SSP-PCR for exon 1 and exon 6 sequences of *UGT2B17* and PCR for 5' sequences upstream of *UGT2B17* on genomic DNA prepared from B-LCL. PCR products for exon 1, exon 6 and the 5' region upstream of *UGT2B17* were detected in the recipient B-LCL and unrelated HLA-A29⁺ B-LCL that were recognized by PL8 CTL. These PCR products were sequenced and found to be identical to *UGT2B17* (unpublished data). No PCR products were detected in donor B-LCL or unrelated HLA-A29⁺ B-LCL that were not lysed by PL8 CTL. The B-LCL from unrelated donors (indicated as 1-4) are the same lines used for analysis of gene expression and cytotoxicity in Fig. 4, A and B. PCR for *GAPDH* was performed as a control.

DCs or activated B cells to present the minor H antigen encoded by *UGT2B17* to PL8 CTL, we measured IFN- γ production after coculture of CTL with these APCs. Both activated B cells and mature DCs stimulated IFN- γ production by PL8 CTL (Fig. 6 C). These data are consistent with participation of APC in the induction of T cell responses to minor H antigens that are expressed by epithelial cells and serve as targets for GVHD.

Discussion

T cell responses to minor H antigens have been implicated in both GVHD and GVL responses after allogeneic HLA-identical HSCT, and a more precise understanding of

the genetics and tissue expression of these determinants could potentially lead to improvements in transplant outcome. However, the polymorphisms in the genome that encode minor H antigens and the basis for antigenicity have been defined for only a few human determinants. In all cases described so far, human minor H antigens have been derived from genes that are expressed in both donor and recipient cells but have polymorphism in their coding sequences. The resulting changes in amino acid sequence may alter the processing of recipient and donor peptides (17), the ability of the peptide to bind MHC (5, 12), or the ability of T cells to recognize the MHC/peptide complex (6). Here, we have identified a new human minor H antigen encoded by the *UGT2B17* gene and demonstrated that

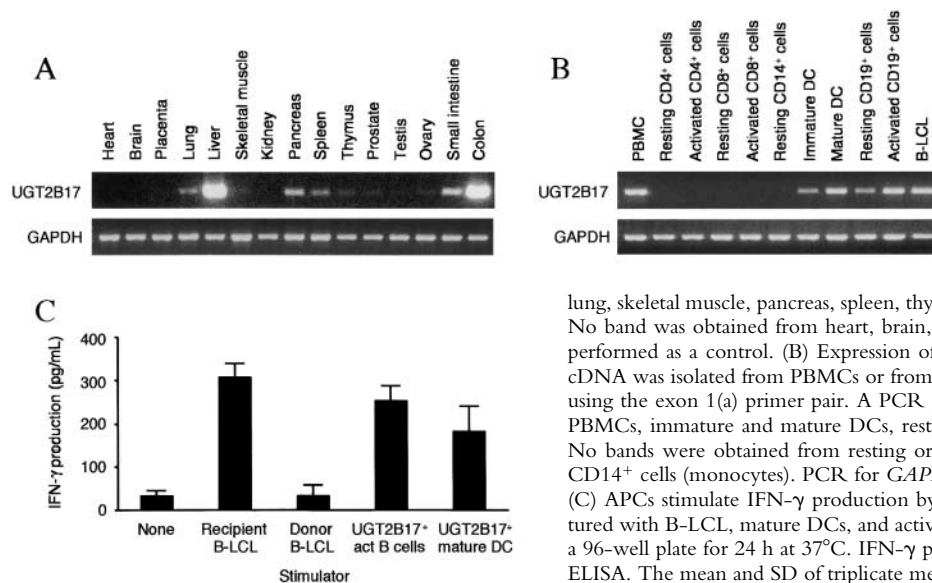


Figure 6. Tissue expression of *UGT2B17*. (A) Expression of *UGT2B17* in tissues by RT-PCR. First-strand cDNA prepared from a series of different human tissues pooled from multiple donors was analyzed by SSP-PCR for *UGT2B17* using the exon 1(a) primer pair. The highest level of expression of *UGT2B17* was observed in cDNA from liver and colon. Detectable bands were also obtained from

lung, skeletal muscle, pancreas, spleen, thymus, prostate, testis, ovary, and small intestine. No band was obtained from heart, brain, placenta, and kidney. PCR for *GAPDH* was performed as a control. (B) Expression of *UGT2B17* in subsets of PBMC. First-strand cDNA was isolated from PBMCs or from subsets of PBMCs and analyzed by SSP-PCR using the exon 1(a) primer pair. A PCR product of the correct size was obtained from PBMCs, immature and mature DCs, resting and activated CD19⁺ B cells and B-LCL. No bands were obtained from resting or activated CD4⁺ and CD8⁺ T cells, or from CD14⁺ cells (monocytes). PCR for *GAPDH* was performed on all samples as a control. (C) APCs stimulate IFN- γ production by PL8 CTL. PL8 CTL (5×10^3) were cocultured with B-LCL, mature DCs, and activated B cells (0.625×10^3) in triplicate wells of a 96-well plate for 24 h at 37°C. IFN- γ production was measured in the supernatant by ELISA. The mean and SD of triplicate measurements are shown.

differential expression in donor and recipient cells as a consequence of a homozygous gene deletion in the donor is responsible for immunogenicity. This is the first example in humans of differential expression of a protein as the basis for generating a minor H antigen. The murine minor H antigens, H60 and H28, that result in rejection of BALB/B skin grafts by C57Bl/6 recipients, are also immunogenic as a result of differential transcription, although the genetic basis for absent expression of these genes in some strains of mice remains to be elucidated (4, 35).

The optimal peptide of UGT2B17 for recognition by PL8 CTL was AELLNIPFLY, which contains preferred amino acid residues at both of the anchor positions for binding to HLA-A*2902, and sensitized donor B-LCL for lysis at pM concentrations. Peptides with one amino acid deleted or added to the NH₂ terminus of AELLNIPFLY, respectively, or with one amino acid deleted from the COOH terminus also sensitized donor B-LCL for recognition by PL8 CTL, although a much higher concentration of peptide was required. Donor B-LCL that were transfected with UGT2B17₄₉₃₋₅₆₄ encoding AELLNIPFLY but not UGT2B17₄₉₃₋₅₆₁ encoding AELLNIPFL, were recognized by PL8 CTL suggesting tyrosine is the anchor residue at the COOH terminus of the naturally processed UGT2B17 epitope presented by HLA-A*2902. The elution of peptides from HLA-A29 molecules of recipient cells that reconstitute recognition by PL8 CTL will be required to conclusively define the NH₂ terminus of the naturally processed peptide, since the synthetic peptides LAELLNIPFLY and ELLNIPFLY were both able to sensitize target cells for recognition by PL8 CTL, albeit at 1–2 log₁₀ higher concentration than AELLNIPFLY.

The UDP glycosyl transferases are comprised of two subfamilies, UGT1 and UGT2 (36), and serve a major role in the conjugation and subsequent elimination of endogenous compounds including steroid hormones and bilirubin, and potentially toxic exogenous compounds (37). A single gene located on chromosome 2 encodes several UGT1 isoforms that arise by differential splicing of the encoded mRNA. Mutations of UGT1 family members have been described and are responsible for the Crigler-Najjar and Gilbert syndromes (38–40). Individual genes located on chromosome 4 encode the UGT2 family members, but clinical syndromes resulting from mutation or deletion of UGT2 members have not been described. The lack of UGT2B17 in the adult donor in this study was not clinically significant since this individual had a normal physical exam, complete blood count, and serum chemistry at the time of evaluation for donation of hematopoietic stem cells. UGT2B17 has been shown to catalyze the conjugation of the 17 β -hydroxy position of dihydrotestosterone (DHT), testosterone, and androstane-3 α 17 β -diol (3 α -Diol), and glucuronidates androsterone (33, 41). Other UGT family members, such as UGT2B7 and UGT2B15 also conjugate DHT, testosterone, and 3 α -Diol, and UGT2B7 can glucuronidate androsterone (25, 28, 42–44). Thus, redundancy of other UGT2B family members for these substrates may compensate for the deficiency of

UGT2B17 in normal individuals and be responsible for the absence of a clinical phenotype associated with homozygous *UGT2B17* deletion.

Despite the redundancy in substrate specificity, there is significant variation in the nucleotide and amino acid sequences of *UGT2B17* compared with other UGT2B family members. The region of *UGT2B17* that encodes the epitope recognized by PL8 CTL contains one or more unique amino acids compared with all other family members and synthetic peptides corresponding to the sequences of other family members failed to sensitize donor cells for recognition by PL8 CTL. We have analyzed other regions of *UGT2B17* that also differ in amino acid sequence with all other family members using computer algorithms that identify peptides predicted to bind to class I HLA molecules based on preferred anchor and secondary anchor residues (45). Several peptides were identified that are predicted to bind to common HLA alleles such as HLA-A2 and -B44. Thus, studies in donor/recipient pairs that express these HLA molecules and are discordant for *UGT2B17* gene expression are warranted to determine if *UGT2B17* encodes additional minor H antigens.

Establishing a causative role for individual human minor H antigens in GVHD after allogeneic HSCT has proven difficult for several reasons. Until recently, few minor H antigens were molecularly characterized and reagents and methods to detect T cells of selected antigen specificities at tissue sites of GVHD were not available. Additionally, other factors such as the intensity of the conditioning regimen, polymorphism in genes that encode cytokines or cytokine receptors, the intensity of posttransplant immunosuppression, and the relative immunodominance of individual minor H antigens may influence the development of clinical GVHD even when disparity for defined minor H antigens is known to exist (46). Studies in an *in situ* model of human skin GVHD have demonstrated that T cells specific for minor H antigens expressed by both epithelial cells and APC induced the histologic features of GVHD whereas T cells specific for minor H antigens that are selectively expressed in APC did not cause GVHD (47). The requirement that the target antigen be expressed in APC for the induction of GVHD by CD8⁺ CTL is consistent with prior results obtained *in vivo* in murine models (34). The patient from whom the PL8 CTL clone was isolated had acute GVHD involving the liver and gastrointestinal tract and we found UGT2B17 was highly and preferentially expressed in both of these target organs. An *in situ* model is not available to address the potential for PL8 CTL to induce GVHD in the liver or intestine, but based on the known function of UGT2B17, it seemed unlikely this gene would fulfill the requirement that it be expressed in APCs. Surprisingly, our data showed that both dendritic cells and activated B cells express sufficient levels of UGT2B17 to stimulate PL8 CTL *in vitro*. While these results are consistent with the principles suggested by murine studies and *in situ* models of human GVHD involving the skin, they do not definitively establish UGT2B17 as a target of GVHD. However, the development of the SSP-PCR assay used to

demonstrate the homozygous deletion of UGT2B17 in the donor in this study will permit prospective genotyping of HSCT donors and recipients and analysis of the occurrence of GVHD in UGT2B17 positive recipients who undergo HSCT from donors that lack this gene.

The demonstration in this study that differential expression of proteins in donor and recipient cells provides a mechanism for generating minor H antigens in humans has potentially broad implications for understanding T cell responses that mediate GVHD after allogeneic HSCT. Deficiencies of members of other enzyme families have been described in humans. These include deletion or lack of expression of the cytochrome P450 (CYP) family genes *CYP2A6*4*, *CYP2D6*5*, *CYP2D6*8*, and *CYP2C19*4*, which metabolize foreign chemicals as well as endogenous steroids (48–51), and deletion of the *glutathione S-transferase (GST) T1* and *GSTM1* genes, which belong to the *GST* gene family that detoxify mutagenic hydrophobic and electrophilic compounds (52, 53). Deletions of *GSTT1* and *GSTM1* are especially common, occurring in 38 and 50% of individuals, respectively, and these proteins share only 55% amino acid identity. Both *GSTT1* and *GSTM1* are expressed in the gastrointestinal tract and liver and could potentially be targets of GVHD involving these organs after HSCT from a donor with a deficiency of one of these enzymes into a recipient who expresses the protein (54). Thus, the novel mechanism responsible for antigenicity of UGT2B17 may apply to other minor H antigens involved in allogeneic reactions after HSCT.

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