Published in final edited form as:

Am J Transplant. 2016 February ; 16(2): 497–508. doi:10.1111/ajt.13509.

Enhanced B-cell alloantigen presentation and its epigenetic dysregulation in liver transplant rejection

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AUTHOR CONTRIBUTIONS

M.N. performed genotyping, epigenetic experiments, review of public databases, and chromatin immunoprecipitation, writing and review of manuscript. C.A. performed epigenetic experiments, B-cell alloantigen presentation in B-cells and flow cytometry, writing and review of manuscript. B.W.H. performed analysis of pyrosequencing, data writing, and review of manuscript. Q.S. performed analysis of genotyping data. R.J. performed re-review of liver transplant biopsies from all subjects, writing, and review of manuscript. G.M. performed subject recruitment, review of B-cell alloantigen presentation data, writing and review of manuscript. D.L. reviewed pyrosequencing data and its analysis. D.E.W. reviewed statistical analyses, results of genotyping and pyrosequencing, overall review of results and writing of the manuscript. S.S. Analysis of data and writing of the manuscript. R.F. provided overall review of results and summary data and edited manuscript. H.H. provided overall review of results and summary data and writing of the manuscript. R.S. designed the study, obtained funding, supervised study and analysis, and provided overall review and writing of the manuscript.

Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. Disclosed conflicts of interest have been managed in accordance with the University of Pittsburgh's policies and procedures. The other authors have no conflicts of interest to disclose.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Supplemental Materials and Methods

Table S1: One hundred three SNPs identified by 454 pyrosequencing of 45 rejectors and 42 non rejectors. Nine SNPs were identified with regulome db scores of $\geq 2b$.

Figure S1: HaploReg evaluation of rs2395304 locus. Linkage disequilibrium for two of 103 significant SNPs rs2395304 and rs6906636 identified by next-generation sequencing (NGS) was evaluated using HaploReg v2 on CEU population from all SNPs in 1KG pilot project. The SNP locus rs2395304 lies 5.5kb upstream of HLA-DOA and is in strong linkage disequilibrium (r^2 threshold of 0.8) with 34 SNPs including rs6906636. rs6906636 is one of nine putative regulatory SNPs among the 103 SNPs identified by NGS. Also the SNP rs2395032 is located in (a) binding sites for the transcription factors CTCF, RFX5 and WHIP, and (b) a region enriched for enhancer-associated Histone Mark in Gm12878 and H1 cell lines.

Figure S2: Comparison of API generated with donor or surrogate donor antigenic lysate resulted in identical rejection-risk assignment in 6 LTx recipients (two rejectors and four non-rejectors). Dotted line indicates API of 1. API >1 indicates increased risk of rejection and API <1 indicates decreased risk. R=Rejector, NR=Non-rejector.

Figure S3: HLA-DOA MO-transfected Raji cells demonstrated enhanced presentation of alloantigenic lysate (Upper panel) and decreased HLA-DOA protein expression (lower panel), compared with untreated or non-transfected Raji cells. Results from three independent replicates of this experiment are summarized as mean and standard error. p-values calculated with student's T-test are shown for comparisons between non-transfected (b) and transfected cells (c). MO=morpholino antisense oligonucleotides.

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Abstract

T-cell suppression prevents acute cellular rejection but causes life-threatening infections and malignancies. Previously, liver transplant (LTx) rejection in children was associated with the SNP rs9296068 upstream of the HLA-DOA gene. HLA-DOA inhibits B-cell presentation of antigen, a potentially novel anti-rejection drug target. Using archived samples from 122 Caucasian pediatric LTx (including 77 described previously), we now confirm the association between rs9296068 and LTx rejection ($p=0.001$, $OR=2.55$). Next-generation sequencing reveals that the putative transcription-factor-(CTCF)-binding SNP locus rs2395304, in linkage disequilibrium with rs9296068 ($D'=0.578$, $r^2=0.4$), also associates with LTx rejection ($p=0.008$, $OR=2.34$). Further, LTx rejection is associated with enhanced B-cell presentation of donor antigen relative to HLA-non-identical antigen in a novel cell-based assay, and with a downregulated HLA-DOA gene in a subset of these children. In lymphoblastoid B-(Raji) cells, rs2395304 co-immunoprecipitates with CTCF, and CTCF knockdown with morpholino antisense oligonucleotides enhances alloantigen presentation and downregulates the HLA-DOA gene, reproducing observations made with HLA-DOA knockdown and clinical rejection. Alloantigen presentation is suppressed by inhibitors of methylation and histone deacetylation, reproducing observations made during resolution of rejection. Enhanced donor antigen presentation by B-cells and its epigenetic dysregulation via the HLA-DOA gene represent novel opportunities for surveillance and treatment of transplant rejection.

Introduction

The clinical management of transplant rejection requires novel solutions for several reasons. Acute cellular rejection can affect 50% of liver transplant recipients (LTx) and limits graft survival, thereby necessitating lifelong prevention, treatment and surveillance (1). Prevention and treatment are predominantly based on drugs that suppress T-cells by inhibiting calcium-dependent signaling in response to antigenic stimulation (2). However, these drugs also cause life-threatening infections or cancers, creating a need for alternative drug targets (3, 4). Personalized surveillance of rejection can be performed by measuring T-cell alloresponses (5). However, ablative immunosuppressive antibodies used in some regimens reduce peripheral T-cell counts and test accuracy, thus arguing for alternatives to T-cell-based mechanistic assays (6).

A previous study suggests that altered alloantigen presentation by B-cells during rejection is a potential alternative to current T-cell-targeted management of transplant rejection (7). A two-tier family based genetic association test was conducted on 1774 single nucleotide polymorphisms (SNPs) in the major histocompatibility complex region of children with LTx. This test showed that the minor allele of the SNP rs9296068 located upstream of the HLA-DOA gene was associated with rejection. In this study, LTx rejection was also

associated with a repressed first exon of the HLA-DOA gene, which is known to inhibit antigen presentation in B-cells, and with increased infiltration of allografts by B-cells in children with the risk (minor) allele of rs9296068 (8). In public databases, this risk allele is associated with a downregulated HLA-DOA gene (9). Together, these findings suggest that HLA-DOA-dependent B-cell presentation of alloantigen and its regulation may be altered during transplant rejection.

The abovementioned and other previous studies suggest several possibilities. The rs9296068 risk allele could be a surrogate for additional causal variants in linkage disequilibrium (LD). Such variants could modify upstream regulation of the HLA-DOA gene through altered transcription factor binding. Transcription of major histocompatibility genes is silenced by DNA methylation of upstream promoters, and facilitated by upstream assembly of an enhanceosome complex, which also includes the CCCTC-binding transcription factor, CTCF, a transcription regulator of several major histocompatibility genes including HLA-DOA (10–13). In lymphoblastoid B-(Raji)-cells, where these regulatory effects have been well-characterized, the effect of HLA gene regulation on alloantigen presentation was not evaluated. In another previous study, protein antigen uptake by dendritic antigen-presenting cells was suppressed by inhibition of histone deacetylation, although the genes mediating this regulatory effect were not identified (14). Together, these observations support evaluation of the role of CTCF, methylation and histone deacetylation on alloantigen presentation by B-cells, and potentially in LTx rejection. Because increased antigen uptake by antigen presenting cells increases the T-cell response in several studies, the regulation of antigen presentation can be studied by evaluating alloantigen uptake in B-cells (15–18).

To evaluate regulation of B-cell alloantigen presentation in LTx rejection, we now use an expanded cohort of 122 Caucasian children with LTx, which includes 77 previously described recipients treated with identical immunosuppression. We first confirm the primary association between rs9296068 and LTx rejection in this expanded cohort. Using next-generation targeted sequencing, we then search for novel variants in the DNA sequence downstream of this SNP and including the HLA-DOA gene, which may associate with rejection. Among several putative regulatory SNPs, we select one which is closest to a known regulatory sequence previously identified in a well-characterized experimental model of HLA gene regulation, and determine its relationship to rejection (10, 14). Using this experimental model, which is based on lymphoblastoid B- (Raji) cells, subsequent experiments evaluate the effects of regulatory mechanisms associated with this sequence on HLA-DOA gene expression and on a clinically validated assay for alloantigen presentation by B-cells. B-cell alloantigen presentation has not been evaluated in this model previously. The development and clinical validation of B-cell alloantigen presentation in children with LTx yields a cell-based assay, which detects LTx rejection with high sensitivity. The interrogation of regulatory mechanisms in Raji cell experiments confirms that epigenetic regulation of B-cell alloantigen presentation could serve as a novel target for immunosuppressive drugs.

Methods

Human Subjects

All studies were performed after informed consent approved by the University of Pittsburgh's Institutional Review Board (IRB#0405628) in prospectively collected and archived samples from 122 subjects, and in subcohorts of this subject population. All biopsies were re-reviewed by a single pathologist (RJ) using established criteria (19). As in our previous studies, children who experienced biopsy-proven acute cellular rejection within the first sixty days after LTx were termed rejectors (5). Non-rejectors did not experience rejection during this time period.

Genotyping

The previously reported 77 Caucasian LTx recipients had been genotyped at 550000 SNP loci with the HumHap 550.v3 bead array (Illumina, San Diego, CA) (7). DNA was extracted from whole blood of the additional 45 Caucasian children with LTx (Genomic DNA purification kit; Gentra systems, Minneapolis, MN) and genotyped for rs9296068 with Taqman MGB biallelic discrimination assay (Life Technologies ID; C__30105758_10) per manufacturer's instructions. Sixteen of 77 children from our previous report were also re-tested with this assay. Genotyping calls for rs9296068 were identical to those reported with the Illumina 550000 SNP array in these children.

Targeted 454 gene (pyro)sequencing

Detailed methods are provided in the supplement. Briefly, target-specific primers were designed for the 16.6kb region between rs9296068 and the intergenic region including the HLA-DOA gene according to design criteria used by Fluidigm's Assay Design Service Group. Each of 96 subject DNA samples, concentration 50 ng/ml, was combined with a specific Barcode + Sequencer-specific adaptor oligo pair (F+R) and with reagents from the Roche FastStart High Fidelity PCR kit (Roche, PN 04-738-292-001) according to Fluidigm Access Array workflow instructions. Amplicon libraries were generated for each sample with polymerase chain reaction (PCR) using the Fluidigm Access Array IFC per manufacturer's instructions and assessed further for quality. Each sample pool of amplicons was then combined to form the final library for sequencing and subjected to emulsion PCR and enrichment of library capture beads for subsequent pyrosequencing with the Roche GS FLX.

Statistical analysis

Read QC, mapping, and variant analyses—The 16,630 bp region (32,972,170–32,988,800 using hg19 release) on human chr.6p downstream of rs9296068 and including HLA-DOA was sequenced at an average depth of 13–18X and coverage of 92% (at least one read depth) for each subject. Of the 96 subjects sequenced, 6 failed QC at the sequencing stage and were excluded. The adapter sequences were first removed from the raw reads using FASTX-Toolkit. Next they were trimmed using BRAT at a minimum quality value of 20 (20). The barcodes were then used to separate each trimmed read into a barcode-specific file using FASTX-Toolkit. Three samples failed QC following this stage. Data from 87

subjects were used in downstream analyses. The reads for each subject were then mapped to the UCSC hg19; Feb 2009 release; Genome Reference Consortium GRCh37 genome version using Bowtie (21, 22). SNPs were identified relative to hg19 using VarScan2 v2.3.1 only those with a $p < 0.001$, QPhred > 30 , and mapping quality value > 30 unique to either the NR or R group were retained (103 known SNPs) (23).

Sanger sequencing

PCR was performed on Gene Amp PCR system 9700 using AmpliTaq Gold PCR Master Mix (Life Technologies, Grand Island, NY, USA) with Primers (rs2395304.Forward; 5'-GTTTGCTGGTGCATCGCTCTGTTT-3', Reverse; 5'-CAGCAAGAAATCTGCTGTGCTGGG-3') (IDT, Coralville, IA, USA as described in supplementary materials.

Alloantigen preparation and presentation by B-cells

Lysed cell membranes from peripheral blood leukocytes (PBL), prelabeled with intravital dye Carboxyfluorescein succinimidyl ester (CFSE) or Pacific Blue N-Hydroxysuccinimide ester (PBHSE) (Life Technologies, Grand Island, NY, USA), were used as alloantigen for B-cell alloantigen presentation. Recipient PBL were incubated alone, with donor alloantigen, and with HLA-non-identical or reference alloantigen, for 40–60 minutes at 37 degrees C in 5% CO₂ incubator (5). Thereafter, cells were labeled with fluorochrome-labeled anti-CD19 (BDBiosciences, San Jose, CA) and acquired on BD LSR-II flow cytometer to determine the frequency of B-cells, which express donor and reference alloantigens.

HLA-DOA protein expression

Flow cytometry—Cells were fixed and permeabilized using BD Fix/Perm kit according to manufacturer's protocol (BDBiosciences, San Jose, CA). The permeabilized cells were incubated at 4°C overnight with unconjugated rabbit anti-human HLA- DOA antibody (GeneTex Inc., Cat# GTX114470; Irvine, CA, USA). After incubation cells were washed and incubated with Texas red- conjugated anti-Rabbit IgG (H+L) secondary antibody (TxRd) (GeneTex Inc., Cat# GTX85302; Irvine, CA, USA) and incubated for 30 minutes at room temperature, washed and acquired on BD LSR II flow cytometer.

Western blots

Western blots were performed using 7.5% gels with transfer membranes as described in supplementary materials.

Modeling epigenetic regulation of B-cell alloantigen presentation in Raji cells

The Burkitt's lymphoma B-cell (Raji) line was obtained from American Type Culture Collection (Manassas, VA, USA) and propagated in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) at 37°C and 5% CO₂. Raji cells were grown at 1 million/ ml in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) at 37°C and 5% CO₂ for 48 hours in the absence or presence of 2uM 5-Azacytidine (Sigma-Aldrich, Cat#A2385, Saint Louis, MO, USA) or 150nM Trichostatin A (Sigma-Aldrich, Cat#T1952, Saint Louis, MO, USA). After

incubation the cells were harvested and HLA-DOA protein expression was evaluated by flow cytometry. The cells were also used to evaluate alloantigen presentation.

Knockdown of CTCF and HLA-DOA in Raji cells

Antisense morpholino-oligonucleotides (Gene Tools, LLC, Philomath, OR, USA) were used to knock down CTCF (CTCF-translation target:5'-CTTCGACTGCATCACCTTCCATTTC-3' Fluorescein) and HLA-DOA (HLA-DOA translation target:CCCTGCTCTGAGGGCCATTACACTC-3' Fluorescein). These morpholino antisense oligonucleotides (10uM) were transfected into Raji cells using 6uM Endoportor (Philomath, OR, USA) according to manufacturer's instructions (www.morpholino.com). Following transfection, the cells were cultured at 5 million/ml in RPMI 1640 supplemented with 10% fetal bovine serum (Life technologies, CA) for 48 hr at 37°C and 5% CO₂. CTCF transected cells were then selected for the loss of HLA-DR surface expression using MACS micro beads conjugated to anti-HLA-DR antibody (Miltenyi Biotech Inc., Auburn, CA, USA). HLA-DR surface expression is specifically lost following depletion of CTCF (12). Flow cytometric detection of HLA-DOA expression in these knockdown experiments was also validated with Western blot.

Quantitative RT-PCR

Total RNA was isolated from blood samples collected in PAXgene tubes (PreAnalytiX-Qiagen-BD, Hombrechtikon, Switzerland) using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed and qRT-PCR were performed using Taqman gene expression assay for HLA-DOA (Life Technologies ID; Hs00157946_m1, Grand Island, NY, USA) with Taqman gene expression master mix (Life Technologies, Grand Island, NY, USA). Target mRNA levels of HLA-DOA were expressed relative to the housekeeping gene GAPDH (Life Technologies ID; Hs99999905_m1).

Chromatin Immunoprecipitation (ChIP) qPCR to confirm CTCF binding to the rs2395304 locus

Raji cells were (>90% viability) were cross-linked in 1% formaldehyde for 10 min and ChIP assay was performed with the Chip-IT Express kit (Active Motif; Cat# 53009, Carlsbad, CA, USA) on the MNase-digested chromatin, as per the standard protocol. 5 µg of anti-CTCF (Millipore; cat# 07-729; Billerica, MA, USA); or, isotype IgG (Abcam; cat# ab46540, Cambridge, MA, USA) antibody was used. ChIP-qPCR primers for the rs2395304 site (chr6:32,982,837–32,982,952; 116 bp) were designed and custom-made by Life Technologies (Assay ID: 6457700_CCM5G0D, Grand Island, NY, USA). MNase digested chromatin was divided into three aliquots. Two of three chromatin aliquots were immunoprecipitated with rabbit anti-CTCF antibody or its IgG isotype antibody. The third aliquot was used as total Input DNA control to quantify the CTCF bound to the rs2395304 site. The candidate locus in ChIP samples was then analyzed by real-time PCR. The CTCF-bound to rs2395304 site was expressed as a percentage of input DNA, as described previously (24). ChIP experiments were performed three times from independent preparations of chromatin. The data were averaged with respect to the input chromatin.

Results

Human Subjects

All 122 Caucasian children received primary LTx with rabbit anti-human thymocyte globulin (rATG, Genzyme, Cambridge, MA), and steroid-free Tacrolimus as described previously (7). Rejectors did not differ significantly from non-rejectors in (mean $\pm$ SEM) age, 8.41 $\pm$ 0.86 vs 8.50 $\pm$ 0.87 years, $p=0.94$ (Wilcoxon rank-sum test), or male: female distribution, 29:31 vs. 33:29, $p=0.71$ (Fisher exact test) (Table 1). Samples from the 12 rejectors which were used for clinical evaluation of B-cell alloantigen presentation were collected at a mean (SD) interval of 2.7 (1.5) days before biopsy-proven rejection.

The association between rs9296068 and LTx rejection is validated

Previously, rejectors had demonstrated significantly higher minor allele frequencies (MAF) at rs9296068 compared with non-rejectors among 77 Caucasian children with LTx (44.9% vs 25%, $p=0.012$, OR=2.43, CI95%=1.17–5.15). These findings are now replicated in 45 additional Caucasian children (45.2% vs 22.9%, $p=0.043$, OR=2.746, CI95%=1.027–7.667), and remain significant in the combined cohort of 122 children with LTx (45% vs 24.2%, $p=0.001$, OR=2.553, CI95%=1.435–4.606) (Table 2).

rs2395304 in LD (linkage disequilibrium) with rs9296068 and other potential regulatory loci associates with LTx rejection

The results described above raised the possibility that the rs9296068 risk allele was a surrogate for additional causal variants in LD. To search for additional variants, the 16.6kb sequence region downstream of rs9296068 and including the HLA-DOA gene was pyrosequenced in DNA samples from 96 LTx recipients using the Roche GS FLX at an average depth of 13–18X and minimum one read coverage of 92% per subject. Sequencing data from 87 subjects passed QC (evaluating read counts, quality values, kmer usage, GC-content, and all other relevant parameters).

Compared with the human hg19 reference sequence, the 87 subjects harbored 103 known non-coding SNPs and did not demonstrate a novel variant (Figure 1, Table S1). Nine of 103 SNPs demonstrated low regulome database scores $\leq 2b$, suggesting a high probability of regulatory effects associated with transcription factor or protein binding. In Raji cells, the DNA sequence designated C13 (chr6:32982600–32982742) upstream of the HLA-DOA gene co-immunoprecipitates with CTCF (12). Of the nine potentially regulatory SNPs in our study, rs2395304 at coordinate chr6:32982921 5.5 kb upstream of the HLA-DOA gene is also closest to and located 179 bp downstream of this C13 CTCF binding site. This SNP rs2395304 is also located in enhancer-associated histone marks in GM12878 cell lines and in binding sites for RFX5, also a transcriptional regulator of MHC II genes (<http://www.broadinstitute.org/mammals/haploreg/haploreg.php>), (Figure S1) (11). In our LTx cohort, rs2395304 shows strong LD with rs6906636, another one of the nine putative regulatory SNPs identified by pyrosequencing ($r^2=0.8$, $D'=0.669$ and modest LD with rs9296068, the primary genetic association, which led to this study ($r^2=0.4$, $D'=0.578$) (Figure 2). However, rs2395304 has not been linked to alloantigen presentation or transplant rejection. Confirmatory Sanger sequencing of rs2395304 was performed in 87 DNA

samples, which had been pyrosequenced successfully. Six samples failed. Among 81 remaining samples, the rs2395304 minor allele was present in significantly more rejectors compared with non-rejectors ($p=0.008$, Table 2, Figure 2).

LTx rejection associates with enhanced B-cell presentation of donor antigen and decreased expression of the HLA-DOA gene in children

Although our previously reported genetic association suggests that B-cell presentation of alloantigen and its regulation by HLA-DOA may be altered during LTx rejection, this has not been demonstrated in the clinical setting. To this end, we developed a novel cell-based functional assay, which measures B-cell alloantigen presentation by measuring alloantigen uptake by B-cells. We next confirmed that B-cell presentation of donor alloantigen is increased during LTx rejection in children. This clinically validated test system was used for all subsequent experiments.

Peripheral blood lymphocytes (PBL) from LTx recipients were incubated with donor PBL lysate, which had been pre-labeled with the intravital dye, carboxyfluoresceinsuccinimidylester (CFSE). In parallel, recipient PBL were also incubated with CFSE-labeled lysate from HLA-non-identical or reference PBL from normal human subjects. Reference PBL were dissimilar to donor and recipient at all six alleles at the HLA-A, -B, and -DR loci. Because PBL are not available from deceased donors predominantly used in LTx, CFSE-labeled alloantigenic lysate was prepared from “surrogate” donors. Surrogate donors were normal human subjects matched to donor at a minimum of three of six possible alleles at the abovementioned HLA loci. This general principle has been used previously to elicit donor-specific T-cell alloresponses with surrogate donor cells in a test system, which predicts rejection with high sensitivity and is approved for clinical use in children (5, 25). The frequency of B-cells presenting donor alloantigen was expressed as a fraction of those presenting reference alloantigen. This fraction was termed the antigen presenting index (API). An API > 1 indicates increased risk of rejection, because donor alloantigen presentation exceeds presentation of third-party alloantigen. An API < 1 indicates decreased risk of rejection. In six LTx recipients a comparison of API generated with donor or surrogate donor antigenic lysate resulted in identical rejection-risk assignment (Figure S2). In single samples from children with LTx rejection, B-cell presentation of donor antigen was increased with API > 1 in all 12 rejectors (Figure 3). Samples from 15 of 16 non-rejectors demonstrated decreased antigen presentation with API < 1. Differences between outcome groups were significant ($p=6.574E-8$, Wilcoxon rank-sum test).

Next, we confirmed that enhanced B-cell alloantigen presentation during rejection was associated with decreased HLA-DOA gene expression (Figure 4). For this purpose, B-cell API and gene expression was measured with qRT-PCR in archived serial samples obtained before transplantation, during early rejection in the 1–60-day rejection-prone period and after rejection from four of 12 rejectors and four of 16 non-rejectors. B-cell presentation of donor alloantigen was enhanced among rejectors ($p=0.029$, Wilcoxon rank-sum test) and was associated with decreased HLA-DOA expression ($p=0.029$, Wilcoxon rank-sum test) during the rejection event (Figure 4A), compared with non-rejectors in the same early 1–60-day post-transplant period. The delta Ct of HLA-DOA increased during rejection compared

with that seen before or after rejection ($p=0.029$, Wilcoxon rank-sum test). The increased delta Ct value is consistent with decreased HLA-DOA gene expression during rejection. HLA-DOA expression did not change significantly in serial samples from non-rejectors (Figure 4B). CTCF gene expression did not change significantly in serial samples from either group (Figure 4C).

HLA-DOA knockdown enhances alloantigen presentation in Raji cells

To evaluate the effect of putative epigenetic regulatory mechanisms previously assigned to the rs2395304 locus on B-cell alloantigen presentation and rejection, we modeled enhanced presentation of alloantigen seen during clinical rejection in Raji cells. Raji cells can be obtained in large numbers required for these experiments, and have been used previously to characterize epigenetic regulation of the HLA genes but not alloantigen presentation.

We first confirmed that HLA-DOA knockdown diminished HLA-DOA-mediated negative regulation and resulted in enhanced alloantigen presentation seen during clinical rejection. Raji cells were incubated without and with GFP-labeled antisense morpholino oligonucleotide to HLA-DOA, followed by incubation with HLA-mismatched cell lysate, which had been labeled with the intravital dye Pacific Blue N-Hydroxysuccinimide ester (PBHSE, Invitrogen, Grand Island, NY). Raji cells transfected with morpholino antisense oligonucleotide to HLA-DOA demonstrated enhanced presentation of alloantigenic lysate and decreased HLA-DOA protein expression, compared with untreated cells in three replicates of this experiment (Figure S3).

Knockdown of CTCF, which binds rs2395304, downregulates HLA-DOA and enhances alloantigen presentation in Raji cells

Next, we confirmed that loss of HLA-DOA-mediated negative regulation of B-cell alloantigen presentation could also be reproduced by knockdown of the transcription factor, CTCF, bound to the rs2395304 locus. Raji cells were transfected as above with GFP-labeled antisense morpholino oligonucleotides to CTCF. B-cell alloantigen presentation increased in Raji cells transfected with morpholino antisense oligonucleotides to CTCF and was accompanied by a decrease in translated HLA-DOA and CTCF proteins in three replicates of this experiment (Figure 5). To confirm that the rs2395304 locus binds CTCF, Raji cell lysates were immunoprecipitated with anti-CTCF. In DNA bound to CTCF, the rs2395304 site with the coordinates chr6:32982837–32982952 was quantified by ChIP-qPCR using Taqman custom assay primers. This site represented 1.98 % of input DNA bound to CTCF and was separated from the previously reported (C13) CTCF-binding site by 95 bp (12). Therefore, CTCF regulates alloantigen presentation via its known effect on HLA-DOA transcription, and also binds to the rs2395304 SNP locus which is in LD with rs9296068.

Inhibitors of histone deacetylation or methylation upregulate HLA-DOA and inhibit alloantigen presentation in Raji cells

Finally, we confirmed that enhanced alloantigen presentation, which is seen during transplant rejection, can be suppressed with inhibitors of histone deacetylation and methylation, which upregulate HLA-DOA gene and/or protein expression. Raji cells were incubated for 48 hours without and with the methylation inhibitor, 5-azacytidine (5-Aza),

and the histone deacetylation inhibitor, trichostatin-A, followed by incubation with antigenic PBHSE-labeled lysate from HLA-mismatched PBL. Raji cells treated with either inhibitor demonstrated increased expression of the HLA-DOA protein, and decreased presentation of PBHSE-labeled alloantigen, compared with untreated cells in three replicates of this experiment (Figure 6).

Discussion

Clinical management of transplant rejection is predominantly based on assessment of T-cell responses for surveillance and targeting of T-cell function for treatment. Our experiments confirm for the first time using a novel cell-based assay that B-cell presentation of donor antigen is enhanced during clinical LTx rejection in children, and is accompanied by loss of negative regulation of antigen presentation by the HLA-DOA gene (Figures 3 and 4). This cellular assay is highly sensitive at identifying rejection-prone recipients, with all 12 rejectors showing enhanced donor alloantigen presentation relative to presentation of HLA-non-identical alloantigen (Figure 3B). Donor alloantigen presentation was decreased in nearly all or 15 of 16 non-rejectors. This test sensitivity has been confirmed in 131 recipients of liver and intestine allografts who have been treated with a variety of immunosuppressants further confirming its usefulness for non-invasive surveillance of rejection (unpublished data).

Among potential mechanisms for our observations during rejection, epigenetic dysregulation of HLA-DOA-dependent antigen presentation has been a prime suspect by virtue of the previously reported association between LTx rejection and rs9296068 upstream of HLA-DOA (7). We now show with targeted sequencing that rs9296068 is in LD with and therefore a surrogate for several other SNPs with putative regulatory function which are also located in the same intergenic region upstream of HLA-DOA. Among these additional SNPs, rs2395304, which is closest to a well-characterized transcription-factor-(CTCF)-binding site in Raji cells, and which also resides within putative enhancer-associated histone marks (Figure S1), was selected for confirmatory Sanger sequencing. The rs2395934 MAF was significantly higher in rejectors compared with non-rejectors ($n=81$, $p=0.008$, $OR=2.34$, Table 2). As predicted by regulome databases, rs2395304 also immunoprecipitates with CTCF in Raji cells in our study. Therefore, mechanisms associated with this locus are highly likely to regulate HLA-DOA-dependent alloantigen presentation.

An evaluation of these regulatory mechanisms shows for the first time that enhanced presentation of alloantigen, which characterizes rejection in children, is reproduced in Raji cells by knockdown of HLA-DOA or by inhibiting translation of the CTCF transcription factor which downregulates HLA-DOA (Figure S3, Figure 5). Additional details about how CTCF contributes to transplant rejection will require further testing. Altered binding of preformed CTCF, which affects chromatin conformation, and not reduced CTCF synthesis is more likely during transplant rejection because CTCF gene expression did not differ ($P=0.114$) between rejectors and non-rejectors (Figure 4C). Higher MAF of the CTCF-binding site, rs2395304, among rejectors also implicates this SNP locus as a potential contributor to variable binding of CTCF.

Unlike knockdown of HLA-DOA or CTCF, which enhances alloantigen presentation, inhibition of methylation and histone deacetylation has the opposite effect of inhibiting presentation of alloantigen in Raji cells and augmenting HLA-DOA expression, a finding associated with resolution of rejection in children with LTx in this study (Figures 6 and 3). DNA methylation is known to reduce CTCF binding (10). Therefore, 5-azacytidine-induced inhibition of B-cell alloantigen presentation, and increased HLA-DOA expression in our experiments, may have also resulted from enhanced affinity of CTCF to its binding site (26). Our study also shows for the first time that histone deacetylase inhibitors can suppress B-cell alloantigen presentation thus presenting a therapeutic alternative to T-cell suppression in the management of transplant rejection. Histone deacetylase inhibitors are being tested in clinical oncology (27). In animal models of transplantation, these agents also promote the development of T-regulatory cells and attenuate cellular and antibody-mediated rejection (28–30).

The small size of children enrolled in this study limits the amount of research blood samples that can be obtained safely. This limits the numbers of B-cells available to evaluate regulation of alloantigen presentation by several other enhanceosomal components and transcription factors, e.g., RFX5 or ZNF263, or alternative regulatory loci. Prospective studies are planned to evaluate mechanisms suggested by the several putative regulatory SNPs in other types of adult transplant recipients.

In conclusion, we report a novel finding that B-cell presentation of donor alloantigen is enhanced and is associated with loss of HLA-DOA-mediated regulation of this mechanism during transplant rejection. Because it can be measured rapidly, B-cell presentation of alloantigen is a viable alternative to T-cell-based tests aimed at non-invasive surveillance of rejection. By modulating the regulation of the HLA-gene in human lymphoblastoid B-cells, we also reproduce the changes in B-cell alloantigen presentation seen during clinical rejection and its resolution. These experiments also confirm the therapeutic potential of epigenetic modifiers such as histone deacetylase inhibitors as alternatives to T-cell-based antirejection drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support: 5RO1AI073895-05, Hillman Foundation of Pittsburgh, Szalay Family Foundation, the Herridge and Giventer-Braff families.

Some test systems are based on technology described in Patent application WO 2011/127360, Co-Inventors: Rakesh Sindhi and Chethan Ashokkumar. Assignee: University of Pittsburgh-of the Commonwealth System of Higher Education, Pittsburgh, PA., and licensed to Plexision, Inc., Pittsburgh, PA 15224, in which the University holds equity. Rakesh Sindhi serves as an unpaid consultant and Chethan Ashokkumar as a paid consultant to licensee without other financial relationships.

Abbreviations

CFSE Carboxyfluorescein succinimidyl ester

CTCF	CCCTC binding transcription factor
FITC	fluorescein isothiocyanate
HLA	human leukocyte antigen
LTx	Liver transplantation
MAF	minor allele frequency
MO	morpholino antisense oligonucleotides
PBHSE	Pacific Blue N-Hydroxysuccinimide ester
PBL	peripheral blood leukocytes
PCR	polymerase chain reaction
QC	quality control
SNP	single nucleotide polymorphism
Tx-Red	Texas red

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Figure 1.

Flow chart demonstrates steps to identify SNPs in linkage disequilibrium with rs9296068, which was associated with liver transplant rejection in our previous study. R=Rejector, NR=Non-rejector, MAF=minor allele frequency, dB=database.

Figure 2.

Upper panel shows the HLA-DOA and its upstream SNPs. Arrows denote positions of SNPs and black bars denote transcription factor binding sites from ChIP-seq data of ENCODE.

rs9296068 was associated with rejection previously. rs2305304, located closest to (179 bp) a well-characterized regulatory CTCF binding site termed C13, was selected for confirmatory sequencing and an evaluation of its association with rejection. Also shown is rs6906636, one of the nine putative regulatory SNPs identified with next generation sequencing (NGS).

Lower panel shows chromatogram traces from Sanger sequencing for the reference allele, minor allele and the ambiguous allele at SNP rs2395304.

Figure 3.

Panel A. Three scatterplots each from a rejector (upper three plots) and a non-rejector (lower three plots) show that recipient B-cells present more donor antigen or less donor antigen respectively, compared with HLA-non-identical reference alloantigen. Background=recipient B-cells cultured without antigen.

Panel B. B-cell antigen presentation Index (API) in single samples from Rejectors (R, n=12) and Non-rejectors (NR, n=16). Rejectors show increased antigen presentation or API>1 when compared with Non-rejectors who show API<1. The API expresses B-cells presenting donor alloantigen as a fraction of those presenting reference alloantigen.

Figure 4.

Serial changes in B-cell antigen presentation index (upper bar diagram) dCt of the HLA-DOA gene (middle bar diagram) and dCT of the CTCF gene (lower bar diagram) in four rejectors (black) and four non-rejectors (grey). Serial samples were obtained pre transplant (Pre-Tx) and at 1–60 days and >61 days after transplantation. p-values for R vs NR comparisons are obtained using Wilcoxon rank-sum exact test with continuity correction. p 0.05 is significant. Samples were collected at a median interval of –1 (–13 to 0 days) before transplantation, and at 18 (8 to 60 days) and 209.5 (120 to 730 days) after

transplantation. Among rejectors, samples collected during the 1–60-day period were collected at a median interval of 0 (0 to 41 days) before rejection

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Figure 5.

Scatterplots on left show Raji cells which express HLA-DOA (HLA-DOA+Raji cells, upper three scatterplots) and Raji cells which express alloantigen (alloantigen+Raji cells, lower three scatterplots) among untreated (left and middle columns) and MO-CTCF-FITC-transfected (right) Raji cells. Untreated Raji cells are shown without (left column, negative control) and with (middle column) HLA-DOA staining. Scatterplots depicting HLA-DOA staining after transfection (right column) have been used to obtain frequencies of HLA-DOA +cells and alloantigen+Rajic cells among non-transfected (b) and transfected (c) Raji cells. Bar diagrams on right summarize HLA-DOA+Raji cells (upper) and alloantigen+Raji cells (bar) seen in three replicates of this experiment. P values are shown in figure when non-transfected (b) and transfected cells (c) are compared. Knockdown of CTCF and HLA-DOA is confirmed with western blot (far right) as a second technique. FITC=fluorescein isothiocyanate, MO=morpholino antisense oligonucleotides.

Figure 6.

Raji cells expressing HLA-DOA increase after treatment with 5-Aza or trichostatin A compared with untreated cells (upper four histograms). Alloantigen presentation by Raji cells decreases with 5-Aza and tricho in the same experiment (lower four scatterplots). Bar diagrams on right summarize results from three replicates of these experiments. P values are shown in figure when treated cells are compared with untreated cells. Aza=5-azacytidine, Tricho=trichostatin-A.

Table 1

Summary of general demographics and the average numbers of HLA antigen mismatches at each of three HLA loci (HLA-A, -B, and -DR, between recipients and donors among Rejectors and Nonrejectors.

	Non rejector	Rejector	p-value
Subjects (n=122)	62	60	0.93 [^] , NS
Age, Y (mean±SEM)	8.50 ± 0.87	8.41 ± 0.86	0.94 ^{**} , NS
Sex, M:F	33:29	29:31	0.71 [*] , NS
Time of biopsy-proven rejection (mean±SEM)	NA	24.15 ± 2	NA
HLA mismatch (mean±SEM)			
A	1.59 ± 0.08	1.55 ± 0.07	0.45 ^{**} , NS
B	1.75 ± 0.06	1.76 ± 0.07	0.74 ^{**} , NS
Dr	1.58 ± 0.08	1.74 ± 0.07	0.07 ^{**} , NS
Cause of liver disease			
Acute liver failure	1	0	0.99 [*] , NS
Alagille	2	2	0.99 [*] , NS
Autoimmune Hepatitis	1	2	0.62 [*] , NS
Biliary atresia	10	18	0.09 [*] , NS
Autosomal recessive polycystic kidney disease	3	2	0.99 [*] , NS
Crigler Najjar	8	5	0.56 [*] , NS
Cystic fibrosis	5	1	0.21 [*] , NS
Glycogen storage disease	1	1	0.99 [*] , NS
Maple syrup disease	11	14	0.51 [*] , NS
Primary sclerosing cholangitis	3	2	0.99 [*] , NS
Tyrosinemia	1	1	0.99 [*] , NS
Urea cycle defects	2	3	0.68 [*] , NS
Wilson's	2	1	0.99 [*] , NS
Alpha-1-antitrypsin	1	0	0.99 [*] , NS
Budd-Chiari	0	1	0.49 [*] , NS
Caroli's	1	1	0.99 [*] , NS
Embryonal sarcoma	2	0	0.50 [*] , NS
Giant cell hepatitis	1	0	0.99 [*] , NS
Hepatoblastoma	2	0	0.50 [*] , NS
Hepatocellular Carcinoma	1	0	0.99 [*] , NS
Intrahepatic cholestasis	2	4	0.44 [*] , NS

	Non rejector	Rejector	p-value
Methylmalonic acidemia	1	0	0.99 [*] , NS
Acyl COA dehydrogenase deficiency	0	1	0.49 [*] , NS
Adenomatous Hyperplastic Nodule	1	0	0.99 [*] , NS
Cryptogeneic cirrhosis	0	1	0.49 [*] , NS

HLA, Human leukocyte antigen; NA, not applicable; NS, not significant.

P-values are derived from

^{*} Fisher exact test,

[^] binomial test, and

^{**} Wilcoxon rank-sum test.

Table 2

Minor allele frequencies (MAF) in rejectors (R) and non-rejectors (NR) of rs9296068 in 77 previously reported, 45 new and 122 combined LTx recipients. Samples from 96 of 122 subjects were pyrosequenced. Eighty seven samples passed quality control, and identified 103 SNPs. Confirmatory Sanger sequencing was performed for the SNP rs2395304 in these 87 samples. In 81 samples that passed quality control, rejectors (R) demonstrated higher MAF of rs2395304 compared with non-rejectors (NR).

SNP	Cohort	Comparison of paired cohorts	Allele count		MAF	95% C.I. of MAF		OR (R:NR)	95% C.I. of OR		p-value
			Ref	Minor		lower	upper		lower	upper	
rs9296068 T>G Minor allele G	Previously reported (n=77)	R (n=39)	43	35	0.449	0.336	0.566	2.427	1.17	5.155	0.012
		NR (n=38)	57	19	0.25	0.158	0.363				
	New subjects (n=45)	R (n=21)	23	19	0.452	0.298	0.613	2.746	1.027	7.667	0.043
		NR (n=24)	37	11	0.229	0.120	0.373				
rs2395304 T>C Minor allele C	Combined cohort (n=122)	R (n=60)	66	54	0.45	0.359	0.543	2.553	1.435	4.606	0.001
		NR (n=62)	94	30	0.242	0.169	0.327				
	NGS and sanger sequencing (n=81)	R (n=41)	34	48	0.585	0.471	0.691	2.34	1.195	4.645	0.008
		NR (n=40)	50	30	0.375	0.271	0.491				

Ref=reference allele, minor=minor allele, MAF=minor allele frequency, OR=odds ratio, C.I.=confidence interval. NGS=next generation sequencing (454 pyrosequencing).