Supplemental Data

Supplemental Materials and Methods

Generation of Clec1a Knock-out (KO) rats by Zinc Finger Nuclease (ZFN) technology.

In vitro-transcribed mRNA-encoding ZFN-targeted sequences specific for rat C*lec1a* (Sigma-Aldrich, St Louis, MO) were microinjected in fertilized one-cell stage embryos as previously described [1,2]. Mutations in newborn were detected by PCR using the following primers 5′-GCCTGCCTTAATTTTGCATC-3′ forward; 5′-TTGCCTTTAGAAAGATGAAGGG-3′ reverse and were sequenced. One of the founders presented a 7 bp deletion of the following sequence: 5′-ACCACAA-3′ leading to a premature stop-codon at the 114 amino-acid of CLEC-1, and thus, lacked most of the extracellular domain. Heterozygotes were subjected to breeding to generate CLEC-1 deficient Knock-Out (KO) and wild-type (WT) littermates.

Western blot on lungs from CLEC-1 deficient rats.

Lung from Wild-type (WT), Heterozygous (HET) or CLEC-1 deficient (KO) rats were lysed on ice in RIPA solution and nuclei and cellular debris was removed by centrifugation for 20 min at 10,000 rpm supplemented with Protease Inhibitors Cocktail (PIC) (Sigma-Aldrich, St. Louis, MO). Lung extracts (300µg) were treated overnight with PNGase F (Sigma-Aldrich) according to manufacturer's instructions. Protein concentration was determined using the BC assays kit with bovine serum albumin (BSA) as standard (Interchim, San Pedro, CA). PVDF membranes were blocked with TBS (Tween-20-Tris buffered saline), 5% BSA and incubated with polyclonal rabbit anti-rat CLEC-1 (0.8 µg/ml) generated as previously described [3] or mouse anti-β-actin (0.2 µg/ml) (BD Biosciences, Franklin Lakes, NJ) diluted in TBS-1% BSA (1h RT) and then with HRP-goat anti-rabbit (0.1 µg/ml) or anti-mouse IgG (0.3 µg/ml). Detection by chemiluminescence was performed using West Pico chemiluminescence substrate (Thermofisher, Waltham, MA) and protein expression assessed by Las 4000 (Fuji).

Generation of rat CLEC-1 Fc fusion protein.

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The cDNA encoding the extracellular domain of CLEC-1 (ADK94891 amino acids 74-261), was amplified by PCR and the 5' and 3' ends tagged with ECORI BglII restriction sites, respectively. Following digestion, cDNA products were cloned and insert in-frame into pFUSE-mIgG2Ae1-Fc2 v10 [Fab] (Invivogen, San Diego, CA) vector containing IgG2a Fc fragment mutated on 3 amino-acid to prevent FcγRI binding. Plasmids were transfected in eukaryote cells with lipofectamine according to the manufacturers' instructions (ThermoFisher). CLEC-1 Fc was purified from supernatant with HiTrap g affinity column (GE Healthcare Bio-sciences, Pittsburgh, PA), dialysed using a Slide-A-Lyzer dialysis cassette (ThermoFisher) and quantified using BCA Protein Assay Reagent Kit (Pierce). Purity and protein structure was confirmed by SDS-PAGE followed by Coomassie staining and western blot analysis with anti-mouse IgG or anti-rat CLEC-1 antibody as described in western blot section of supplemental Materials and Methods. A control recombinant secreted truncated form of a human embryonic alkaline phosphatase (hSEAP Fc) was generated (pFUSE-SEAP-hFc, Invivogen) and purified under the same conditions than CLEC-1 Fc.

Human cells.

- Human Embryonic Kidney cells (HEK293T) were obtained from American Type Culture Collection.
- Human cells were sorted using the following markers, $SSC^{low}CD45^{+}CD3^{+}$ or $CD19^{+}$ for T and B cells respectively, $SSC^{high}CD45^{+}CD16^{+}$ for neutrophils and $SSC^{low}CD45^{+}CD14^{+}$ for monocytes using mAbs (BD Biosciences) and a FACSAria flow cytometer (BD Biosciences). Purity was >99%.
-Human monocyte-derived DCs (moDCs) were generated as previously described [4] from elutriated monocytes cultured for 7 days in complete RPMI 1640 medium (10% endotoxin-free FCS (ThermoFisher) and with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM Hepes (all reagents from Sigma-Aldrich)), supplemented with IL-4 and GM-CSF (1000 IU/ml; AbCys, Paris, France).
-Human aortic endothelial cells (HAECs) were isolated and cultured as previously described [5].

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Transfection of eukaryotes cells.

2.5X10^5 HEK were transduced using GeneCellin TM (Eurobio, Toulon, France) with plasmids encoding eGFP-T2A or-eGFP-T2A-human-*CLEC1A* DNA constructs (2µg) (Eurofins Genomics GmbH (Ebersberg, Germany)). GFP and ectopic human CLEC-1 expression was evaluated by Flow Cytometry and Fluorescence microscopy at 24h post transfection.

Histology on HEK cells.

Adherent transfected Human Embryonic Kidney cells (HEK293T) (cultured on a coverslip overnight) were fixed in 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) and permeabilized with Triton X100 (0.1%). Cells were stained with anti-hCLEC-1 mAb (D6 clone, Santa Cruz, Dallas, CA) (4 µg/ml) for 1h at room temperature in PBS 1% Fetal Call Serum (Thermofisher), 1% bovine serum albumin and then with secondary Alexa Fluor-568 anti-mouse IgG1 antibodies. After 10 min incubation in PBS containing 1% DAPI, slides were mounted using Prolong Antifade Reagent (Thermofisher) and observed by fluorescence microscopy (Nikon A1 R Si Confocal microscope). Images were obtained (X60 Plan Apo N.A: 1.4, zoom 2) with sequential mode and analyzed by using ImageJ program.

RNA extraction and real-time quantitative RT-PCR

Total RNAs from cells were prepared using Trizol (Thermofisher) according to the manufacturer's instructions. cDNA from human organs (1 to 15 pooled samples) were provided by Human Immune System and MTC Panel I from male or female Caucasians (Clontech Mountain View, CA).

Real-time quantitative PCR was performed using the ViiA 7 Real-Time PCR System and SYBR® Green PCR Master mix (ThermoFisher) as previously described [6]. Oligonucleotides used are human *HPRT* 5'TAGCCCTCTGTGTGCTCAA 3'CTGATAAAATCTACAGTCATAGGAATGG and human *CLEC1A* 5'TCAGAACTACCGTCCCCGAT 3'ACAGGTGCAGATGAAGAGGC. *HPRT* was used as an endogenous control gene for normalization. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [6,7] and expressed in arbitrary units (AU) of specific gene/*HPRT* ratio.

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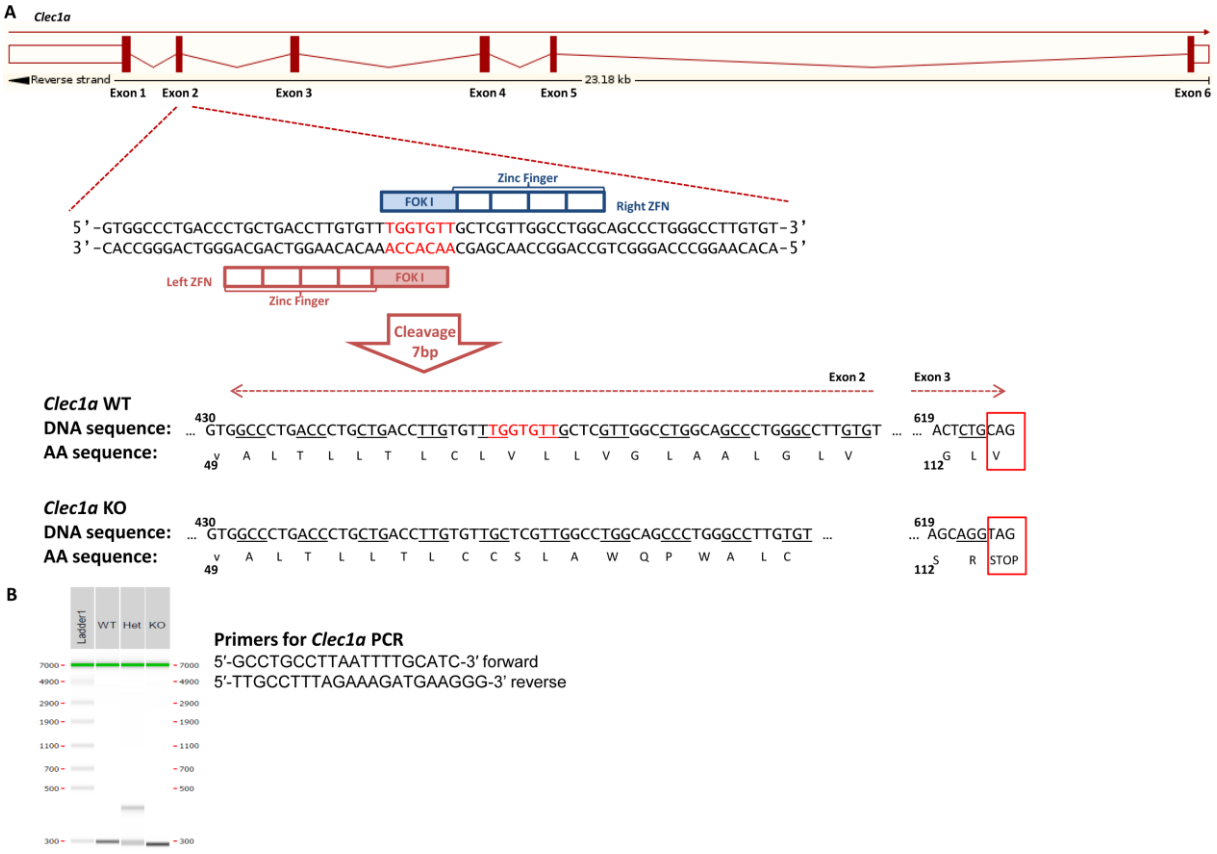
Supplemental Figures

Supplement Figure S1: Strategy of generation and genotyping of Clec1a KO rats.

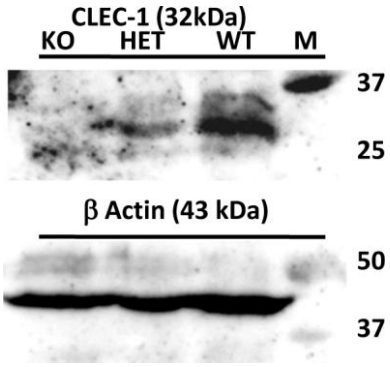
(A) Zinc Finger Nuclease (ZFN) specific of rat *Clec1a* were designed by Sigma and induced a deletion of 7 bp (indicated in red) in exon 2. Amino Acid (AA) of Wild Type (WT) and Knock-Out (KO) rats were represented to demonstrate the generation of a stop codon in the open reading frame at AA 114 in *Clec1a* KO rats. **(B)** DNA was extracted from tails one week after birth and PCR to detect the 7 bp deletion was performed with the forward and reverse indicated primers. Electrophoresis illustrate results from PCR amplifying the bands for WT (one band 304 bp), Heterozygous (HET) (two bands 304 and 297 bp) and KO (one band 297 bp) rats. Additional larger bands represent formation of heteroduplex DNA double strands with mismatches between the two strands resulting in slower migration during electrophoresis.



Supplemental Data

Supplement Figure S2: Absence of CLEC-1 protein in CLEC-1 deficient rats.

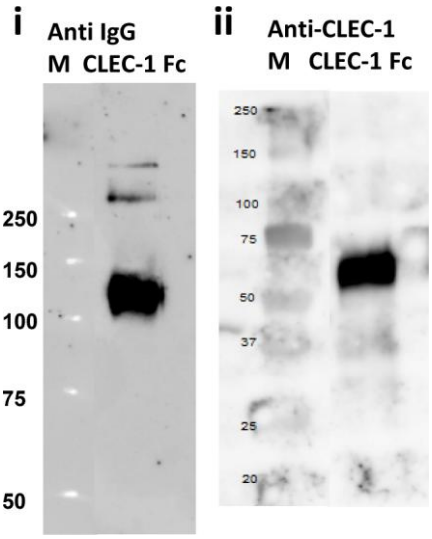
CLEC-1 deficient rats (KO) were generated by ZFN technology and CLEC-1 deficiency was confirmed by western blot as described in Material and Methods in deglycosylated lung extracts from KO, HET and WT rats with anti-rat CLEC-1 polyclonal Ab (expected size 32 kDa) generated as previously described [3] and with anti-ß-actin mAb as control (expected syze 43kDa). Results are representative of three independent experiments with different animals. M line represents Molecular-weight size markers.



Supplemental Data

Supplement Figure S3: Generation of rat CLEC-1 Fc fusion protein.

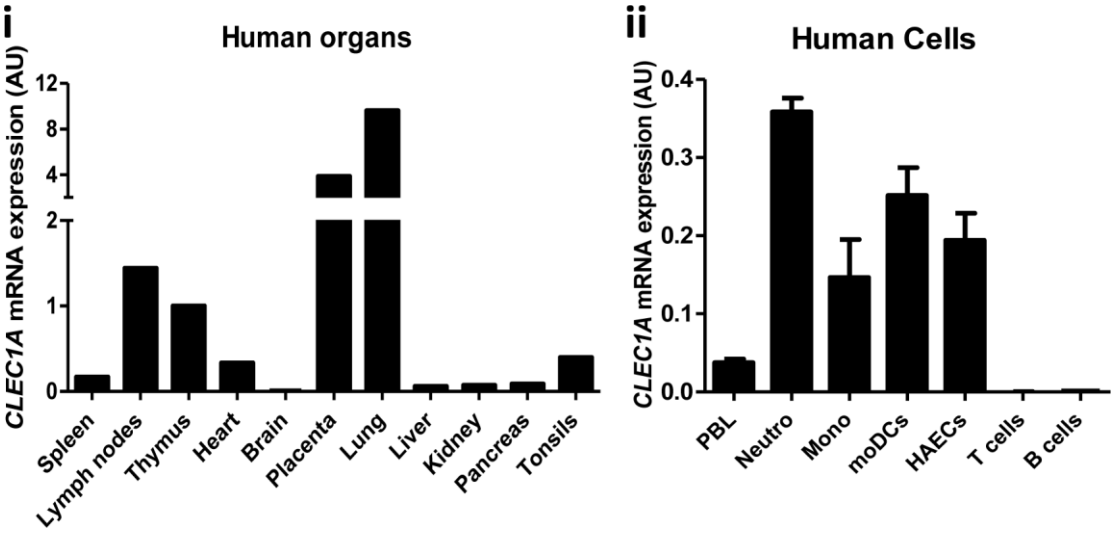
Soluble rat CLEC-1 Fc purified protein was produced in eukaryotes cells transfected with plasmid encoding the extracellular domain of rat CLEC-1 fused to Fc fragment of mouse IgG2a as described in Supplemental Materials and Methods. Western blots analysis of CLEC-1 Fc fusion protein was revealed with **(i)** anti mouse IgG peroxydase in non-denaturing conditions (expected size 130 kDa) and with **(ii)** anti-rat CLEC-1 polyclonal Abs generated as previously described [3] in denaturing conditions (expected syze 65 kDa). M line represents Molecular-weight size markers.



Supplemental Data

Supplement Figure S4: CLEC1A mRNA expression in various human organs and cell-subtypes.

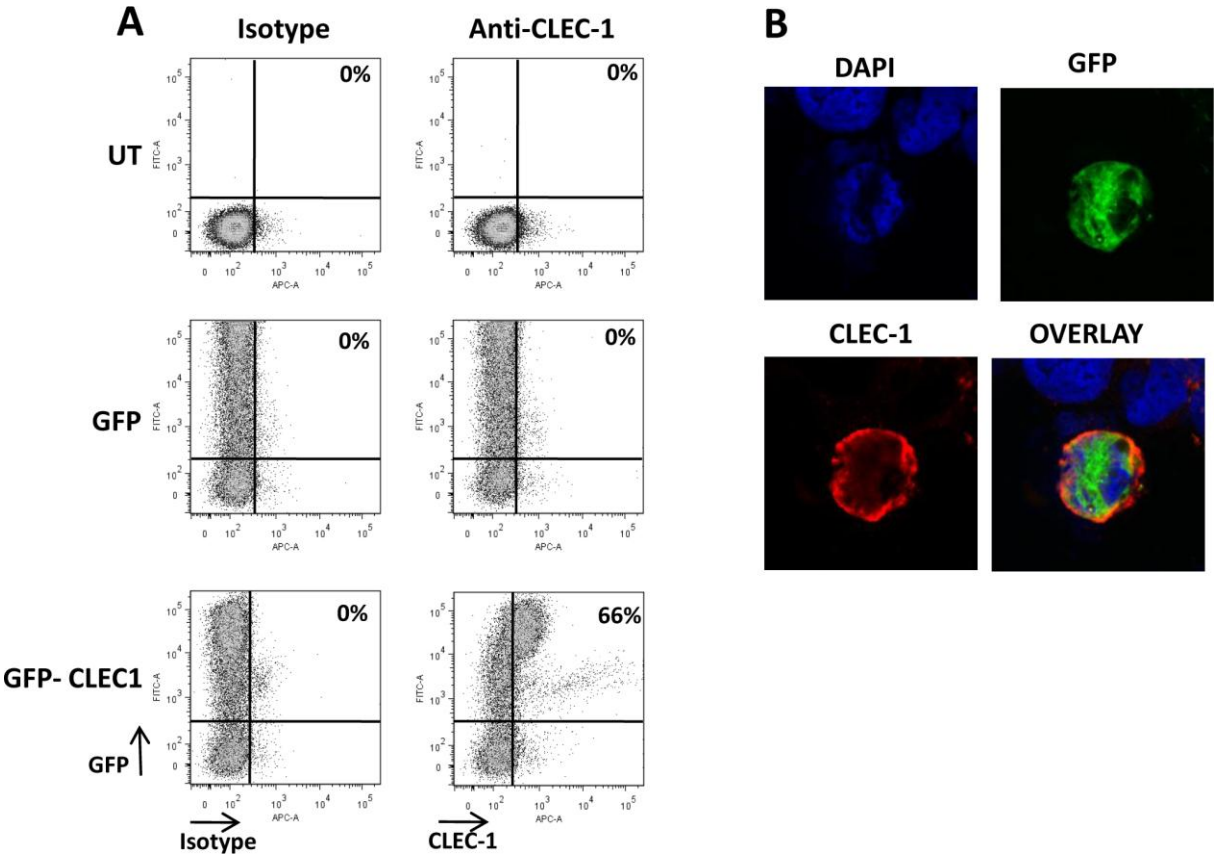
CLEC1A mRNA expression was assessed by quantitative RT-PCR in **(i)** human organs (from 1 to 15 pooled samples) (histogram is representative of three different run experiments and expressed in Arbitrary Unit (AU) of *CLEC1A /HPRT* ratio) and in **(ii)** human peripheral blood leucocytes (PBL), neutrophils (Neutro), monocytes (Mono), monocyte-derived Dendritic Cells (moDCs), Human Aortic Endothelial Cells (HAECs), T and B cells. Results were expressed in histogram as mean ± SEM of 4 samples and are expressed in Arbitrary Unit (AU) of *CLEC1A /HPRT* ratio.



Supplemental Data

Supplement Figure S5: Transfection of eukaryotes cells with plasmids encoding GFP and human CLEC1A.

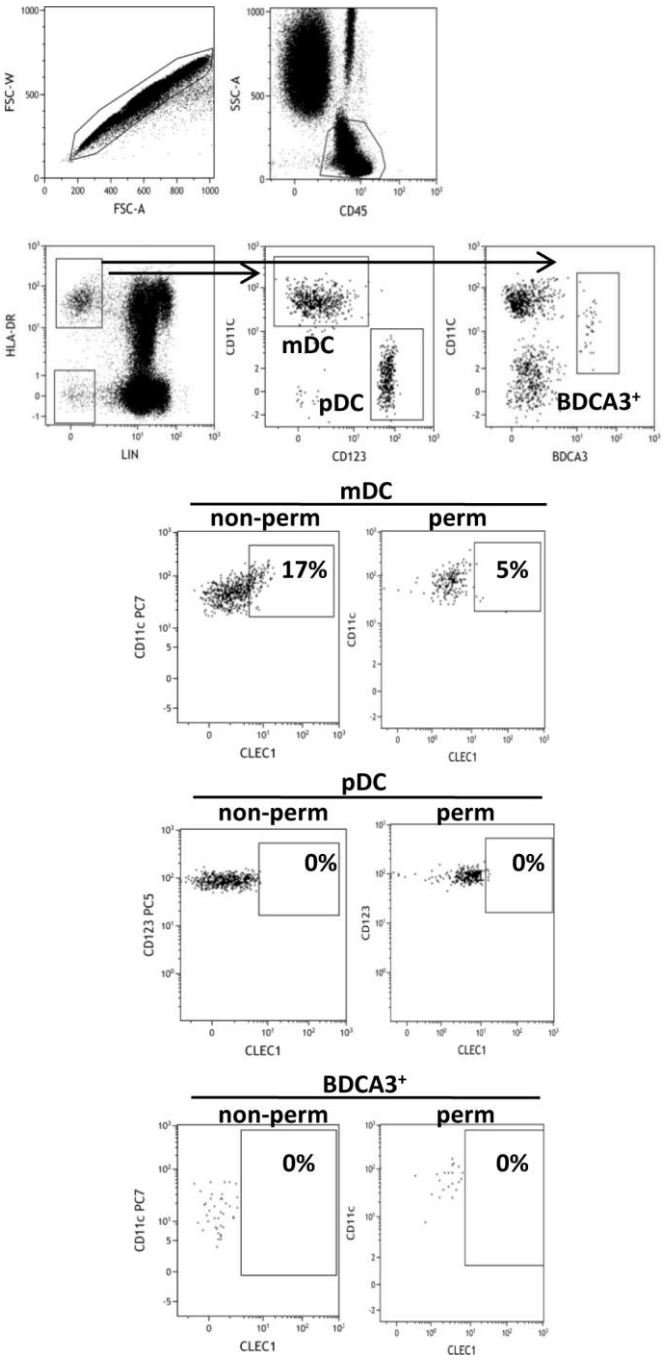
(A) Flow cytometry analysis and **(B)** confocal microscopy images of eukaryote Human Embryonic Kidney (HEK) cells transfected with plasmids encoding *GFP* alone or human *CLEC1A* and *GFP* for 24h. Dot plots exhibit GFP (FITC) and isotype (IgG1) or CLEC-1 (APC) staining revealed by anti-human in house CLEC-1 mAb and evaluated by flow cytometry. Images represent DAPI (blue), GFP (green) and CLEC-1 (red) staining revealed by anti-human CLEC1 mAb (D6 clone) followed by secondary anti-mouse Alexa-568 antibody. Original magnification x600. Data are representative of three independent experiments.



Supplemental Data

Supplement Figure S6: CLEC-1 expression in different subsets of human blood DCs in non-permeabilized and permeabilized conditions.

Representative dot plots of CLEC-1 staining evaluated by flow cytometry in non-permeabilized (non-perm) and permeabilized (perm) conditions in human blood $CD45^{+}LIN^{-}HLADR^{+}$ subpopulation of $CD11c^{+}CD123^{-}$ and $CD11c^{+}BDCA3^{+}$ myeloid DC (mDC) and $CD11c^{-}CD123^{+}$ plasmacytoid DCs (pDC). Data are representative of five independent experiments.



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Supplement Figure S7: Rejection grade in lung transplant biopsies and graft outcome.

Rejection grade was evaluated by anatomical pathology (histopathology) in lung transplant biopsies from the multicentric longitudinal cohort "Cohort in Lung Transplantation" (COLT, NCT00980967) at the indicated time post transplantation. Graft outcome and estimated onset of chronic rejection are indicated for each sample.

No	Time post-transplantation	Grade of rejection	Graft outcome (estimated onset of CR)
1	1 year	0	Stable
2	1 year	0	Stable
3	6 months	0	Stable
4	1 year	0	Stable
5	1 year	0	Stable
6	1 ½ year	0	Stable
7	6 months	0	Stable
8	1 year	0	Stable
9	6 months	1	CR (1 year)
10	1 year	0	CR (3 years)
11	1 year	1	CR (1 year)
12	1 year	0	CR (2 ½ years)
13	6 months	0	CR (1 year)
14	6 months	0	CR (1 year)
15	1 year	0	CR (2 years)

Grade 0 : No interstitial infiltrate, No fibrosis, No endothelial cell injury
Grade 1 : Inflammatory infiltrate
CR (Chronic Rejection)

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Supplemental References

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