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The C-Type Lectin-Like Receptor CLEC-1, Expressed by Myeloid Cells and Endothelial Cells, Is Up-Regulated by Immunoregulatory Mediators and Moderates T Cell Activation

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C-type lectin receptors have recently been described as playing crucial roles in immunity and homeostasis since these proteins are able to recognize pathogens as well as self-Ags. We identified the C-type lectin-like receptor-1, CLEC-1, as being overexpressed in a model of rat allograft tolerance. We previously described in this model the expression of numerous cytoprotective molecules by graft endothelial cells and their interplay with regulatory CD4+CD25+ T cells. In this study, we demonstrate that CLEC-1 is expressed by myeloid cells and specifically by endothelial cells in tolerated allografts and that CLEC-1 expression can be induced in endothelial cells by alloantigen-specific regulatory CD4+CD25+ T cells. Analysis of CLEC-1 expression in naive rats demonstrates that CLEC-1 is highly expressed by myeloid cells and at a lower level by endothelial cells, and that its expression is down-regulated by inflammatory stimuli but increased by the immunoregulators IL-10 or TGFβ. Interestingly, we demonstrate in vitro that inhibition of CLEC-1 expression in rat dendritic cells increases the subsequent differentiation of allogeneic Th17 T cells and decreases the regulatory Foxp3+ T cell pool. Additionally, in chronically rejected allograft, the decreased expression of CLEC-1 is associated with a higher production of IL-17. Taken together, our data suggest that CLEC-1, expressed by myeloid cells and endothelial cells, is enhanced by regulatory mediators and moderates Th17 differentiation. Therefore, CLEC-1 may represent a new therapeutic agent to modulate the immune response in transplantation, autoimmunity, or cancer settings. The Journal of Immunology, 2009, 183: 3099–3108.

The C-type lectins are a superfamily of proteins containing at least one C-type lectin domain that have been classified into different groups depending on the arrangement of their C-type lectin domains (1). C-type lectin proteins have been shown to act as pattern recognition receptors for pathogens, playing important roles in innate immunity and in the subsequent induction of the adaptive immune response by stimulating cytokine secretion or T cell priming (1, 2). Moreover, C-type lectins have also been described to bind self-Ags or altered self-Ags and to play a direct role in the orchestration of the immune response. Among the vast C-type lectin superfamily, the C-type lectin-like proteins are a subgroup of receptors that possess the C-type lectin domain but lack calcium- or carbohydrate-binding elements (3). Recently, several studies demonstrated that C-type lectin-like receptors are not restricted to NK cells, but are expressed by many other cell types, including myeloid cells and endothelial cells (ECs) (1). These receptors have diverse ligands and, by acting as activatory or inhibitory receptors, govern cellular activation (1). Among the C-type lectin-like receptors, a subgroup of receptors, the “Dectin-1 cluster,” including the DECTIN-1, LOX-1, CLEC-1, CLEC-2, MICL, CLEC12B, and CLEC9A genes, has recently been described to play important roles in immunity and homeostasis (3). Lox-1, expressed by monocytes and ECs, has been shown to recognize a variety of ligands, including bacteria, modified lipoproteins, activated platelets, hsP70, and apoptotic cells; it has also been shown to play a role in cell activation and in specific diseases such as atherosclerosis or diabetes (4–6). Moreover, Dectin-1, which binds zymosan or an endogenous ligand expressed on T cells, enhances the activation of dendritic cells (DCs) and cytokine secretion or T cell priming (2, 7).

We identified C-type lectin-like receptor-1 (CLEC-1), one of the genes from the Dectin-1 cluster, as being overexpressed in tolerated grafts in an experimental model of heart allograft transplantation. We previously demonstrated in this model the specific expression of the cytoprotective molecules heme oxygenase-1, NO synthase, and IDO by graft ECs and their interplay with regulatory CD4+CD25+ T cells to regulate local effector cells and to maintain...
tolerance (8–10). CLEC-1 is an orphan receptor that has previously been described to be expressed by DCs and ECs in humans (3, 11). However, its function has not been described so far, and its ortholog in rodents has not been characterized. CLEC-1, in contrast to most of the members of this family, does not contain ITAM or inhibitory motifs, but rather contains one tyrosine residue in its cytoplasmic portion. Additionally, CLEC-1, like NKG2A or NKG2C, seems to require an additional chain to form a complex at the membrane surface of cells (3, 11).

In this study, we analyze the expression of CLEC-1 in rodents and evaluate for the first time the function of CLEC-1 in the regulation of an immune response.

Materials and Methods

Animals and transplantation

Rats were purchased from the Centre d’Elevage Janvier and maintained in an animal facility under standard conditions according to our institutional guidelines. The studies were reviewed and approved by the appropriate institutional review committee. LEW.1W (RT1a) or LEW.1A (RT1a) rats served as heart donors, and LEW.1A rats served as recipients. For allograft tolerance models, LF15-0195 (20 days) (Fourier Laboratories) or anti-donor class II Abs were administered to recipients as previously described (12, 13). Chronic allograft rejections were induced by two donor blood transfusions (donor-specific transfusion (DST)) before transplantation or by CD40-Ig administration as previously described (8, 14). Graft function was assessed by scoring pulsations through the abdominal wall for heart or by proteinuria for kidney transplants. For in vivo transfer experiments, a total of 20 × 10^6 CD4^+ T cells from LEW.1A-tolerant LF15-0195-treated recipients or from LEW.1A naive rats were injected i.v. into LEW.1A secondary syngeneic irradiated recipients (4 Gy, whole-body irradiation (Institut Fédératif de Recherche Thérapeutique 26, Nantes, France) 1 day before transplantation) on the day of LEW.1W or LEW.1A (syngeneic) cardiac transplantation.

Antibodies

The following hybridomas for rat mAbs were obtained from the European Collection of Cell Culture (Salisbury, U.K.) and were used in cell depletion, cytfluorometry, cell sorting, or immunohistochemistry after coupling, if necessary, to FITC, biotin, or PE (BioAtlantic): Ox6 (class II MHC), R7/5 (TCRαβ), OX39 (CD25), W3/25 (CD4), OX62 (CD103), and OX41 (CD172a). B7.1-PE (CD80), B7.2-FITC (CD86), and Ox6-allophycocya-nin-Cy7 (MHC class II) were obtained from BD Biosciences, PECAM-1 (CD31) was obtained from Serotec, and Foxp3-allophycocyanin was obtained from CliniSciences. Biotin-anti-mouse IgG, Alexa Fluor 568 anti-mouse, FITC anti-rabbit, HRP-streptavidin, and PE-streptavidin were purchased from CliniSciences. Biotin-anti-mouse IgG, Alexa Fluor 568 anti-mouse, FITC anti-rabbit, HRP-streptavidin, and PE-streptavidin were purchased from Vector Laboratories, and HRP-conjugated goat anti-rabbit or mouse, FITC anti-rabbit, HRP-streptavidin, and PE-streptavidin were purchased from CliniSciences. Biotin-anti-mouse IgG, Alexa Fluor 568 anti-mouse, FITC anti-rabbit, HRP-streptavidin, and PE-streptavidin were purchased from Vector Laboratories, and HRP-conjugated goat anti-rabbit or mouse, FITC anti-rabbit, HRP-streptavidin, and PE-streptavidin were purchased from CliniSciences.

Generation of a polyclonal anti-rat CLEC-1 Ab

Three synthetic peptides, one (MQAKYSSTRDMLDDC-NH2) corresponding to aa 1–14 of the intracellular domain of CLEC-1 and two (53x148) (20
(53x166)HiTrap NHS-activated high-performance columns (GE immunization, serum was collected and subjected to affinity purification on 83–97 and 256–269, respectively, of the extracellular domain of CLEC-1, (DSITEKDERLGNMSR, CERVAGRVVPEELQ) corresponding to aa 3100 CLEC-1 MODERATES T CELL ACTIVATION (TCR (53x343) of three mice. Specificity of the polyclonal anti-CLEC-1 Abs was tested by peptides with HiTrap NHS-activated high-performance columns (GE immunization, serum was collected and subjected to affinity purification on 83–97 and 256–269, respectively, of the extracellular domain of CLEC-1, (DSITEKDERLGNMSR, CERVAGRVVPEELQ) corresponding to aa 3100 CLEC-1 MODERATES T CELL ACTIVATION (TCR (53x343) of three mice. Specificity of the polyclonal anti-CLEC-1 Abs was tested by peptides with HiTrap NHS-activated high-performance columns (GE immunization, serum was collected and subjected to affinity purification on 83–97 and 256–269, respectively, of the extracellular domain of CLEC-1, (DSITEKDERLGNMSR, CERVAGRVVPEELQ) corresponding to aa

ECL (Amersham), exposed to Kodak film, and quantified with Kodak Image Analysis 1D software.

Immunohistology

Cardiac tissue was snap-frozen in liquid nitrogen after embedding in OCT compound (Tissue Tek. Miles Laboratories). Cryostat sections (7 μm) or cytopsins of cells were fixed in acetone and incubated overnight with FITC-anti-CLEC-1 Abs (4 μg/ml), followed by anti-rat PECAM-1 or CD172a (5 μg/ml) and by Alexa Fluor 568 anti-mouse Abs and 4’,6-diamidino-2-phenylindole (DAPI), mounted in Vectashield mounting medium (Vector Laboratories), and observed by fluorescence microscopy (Axioskop 2 Plus; Zeiss). Images were visualized (×600) and processed using the AxioVision Viewer program (Zeiss). For the quantification of CLEC-1 staining on transduced cells with RNA interference (RNAi), images of cell spot were acquired with Zeiss Apotome and AxioVision software using a 63×/1.4 objective lens. Positive cells were determined by counting 36 fields on each spot. The percentage of CLEC-1 positive cells was calculated by counting the number of cells positive for the CLEC-1 staining divided by the total number of cells (DAPI), for four different counters.

Cell purification, culture, and activation

Rat peritoneal macrophages were isolated by lavage of the peritoneal cavity with 5 ml of PBS. The collected cells were centrifuged and suspended in complete DMEM medium.

Rat bone marrow-derived DCs (BMDCs) were obtained as previously described (15). Briefly, bone marrow cells were cultured in RPMI 1640 complete medium: 10% endotoxin-free FCS (Perbio Science) and 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEpes, and 5 × 10^-5 M 2-ME (all from Sigma-Aldrich), supplemented with rat IL-4 (4 ng/ml) and murine GM-CSF (1.5 ng/ml). At day 8, adherent immature BMDCs were collected, plated (1 million/ml), and stimulated with LPS (1 μg/ml) (Sigma-Aldrich), recombination rat IL-10 (20 ng/ml) (R&D Systems), recombina-nant human TGFβ1 (20 ng/ml) (R&D Systems), poly(LC) (25 μg/ml) (In-vivoGen), or rat rIFN-γ (50 μU/ml) (Serotec). Rat splenic DCs (OX62*, MHC class II^+^), T cells (R73^+^), CD4^+^, CD25^+^ T cells, or regulatory CD4^+^ CD25^+^ T cells (R73^+^ W3/25* OX9^+^) were purified by positive selection using a FACSaria flow cytometer (BD Biosciences) as previously described (16). Purity was >99%. Rat EC lines of LEW.1W and LEW.1A origin were isolated as previously described (17) and plated overnight into 12-well plates (Nunc; Merck/Eurolab France) (1 million cells/well) in complete RPMI medium culture and highly purified CD4^+^ CD25^+^ T cells from naive rats or from tolerant recipients were added (5 × 10^5^ cells/well) to the EC cultures. Alternatively, for transwell assays, 2.5 × 10^5^ LEW.1W ECs/ well were added in the lower compartment of a 24-well plate, and in the upper compartment (separated by a 0.45-μm pore size membrane (BD Biosciences)) we added 2.5 × 10^5^ LEW.1W ECs/well with 10^5^ LEW.1A ECs in 0.2-ml cultures from tolerant recipients. Twenty-four or 48 h later (for IDO or CLEC-1 quantification, respectively), the adherent ECs were washed several times and analyzed by quantitative RT-PCR for hypoxan-thine phosphoribosyltransferase (HPRT), CLEC-1, and IDO expression.

Human aortic ECs (HAECs) were isolated as previously described (18). HAECs were cultured in cell growth medium supplemented with 10% FCS, endothelial cell growth supplement (0.4%), hydrocortisone (1 μg/ml), human basic fibroblast growth factor (1 ng/ml), human epidermal growth factor (0.1 ng/ml), amphotericin B (50 μg/ml), and gentamicin (50 μg/ml) (PromoCell). HAECs used in experiments were >85% CD14^−^ CD11b^+^ and cultured for 6 days in medium supplemented with IL-4 (40 ng/ml) (AbCys) and GM-CSF (500 IU/ml) (AbCys). Then, DCs were harvested and cultured (1 million cells/ml) in plates coated with poly(2-hydroxyethyl methacrylate) (Sigma–Aldrich) to prevent cells from adhering, and with LPS (1 μg/ml) (Sigma–Aldrich), rHL-10 (20 ng/ml) (R&D Systems) or rTGFβ1 (20 ng/ml) (R&D Systems).

Transfection of BMDCs with Stealth RNAi duplexes, activation, and MLR

Two nonoverlapping Stealth RNAi (Stealth Select RNAi; Invitrogen) duplexes were synthesized commercially by Invitrogen with the help of tools available online (www.invitrogen.com): Stealth RNAi 1 5’-CACCAM
AGCCUGUAUUCUGGAACA-3') and Stealth RNAi 2 (5'-GAAGUGGCAGCCUGUAUUCUGGAACA-3') were designed to target different coding regions of the rat CLEC-1 mRNA sequence (GenBank accession no. NM_001109253 at ncbi.nlm.nih.gov/Genbank/). A BLAST (National Center for Biotechnology Information database) search was conducted to confirm that the only targets of the two Stealth RNAi duplexes were CLEC-1. At day 8 of culture, 2 million adherent LEW.1A BMDCs were transfected with Lipofectamine RNAiMAX (Invitrogen) and with 200 pmol of control RNAi (medium GC content Stealth RNAi negative universal control; Invitrogen) or with RNAi specific for CLEC-1. Cells were treated with LPS (10 μg/ml) (Sigma-Aldrich) for 48 h.

Two days following transfection, LEW.1A BMDCs were harvested and stained using anti-OX6, B7.1, or B7.2 mAbs for flow cytometry analysis or were plated (1.5 x 10⁶ cells/ml) with LEW.1W lymph node-derived T cells in complete RPMI medium. Alternatively, LEW.1W lymph node-derived T cells or highly purified CD4⁺ CD25⁻ or CD4⁺ CD25⁺ T cells were labeled with CFSE (Molecular Probes). 3 days later, cells were pulsed with 0.5 μCi/well [methyl-³H]thymidine (Amersham) and thymidine incorporation was measured using a scintillation counter (TopCount NXT; PerkinElmer). CFSE-labeled cells were stained with anti-TCR, anti-CD4, and intracellularly with anti-Foxp3 Abs as previously described and were analyzed by flow cytometry at day 5 of culture (10). T cells or culture supernatants were harvested at day 5 or 3 of culture for quantitative RT-PCR analysis or cytokine measurement.

RNA extraction and real-time quantitative RT-PCR

Total RNA from tissues or cells was prepared using TRIzol (Invitrogen) according to the manufacturer’s instructions. Real-time quantitative PCR was performed as previously described (20) using a GenAmp 7700 sequence detection system and SYBR Green PCR Master mix (Applied Biosystems). The oligonucleotides used in this study are described in Table I. The oligonucleotides used in this study are described in Table I.

Flow cytometry analysis

Fluorescent labeling was measured using a FACS LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Cytokine assays

 Supernatants from cultures were harvested at 48 h for LPS-stimulated BMDCs transfected with control RNAi or specific CLEC-1 RNAi, or they were harvested at 72 h for MLRs. Cytokines were measured using a LINCoPlex kit (RCYTO-80K; Millipore). Multiplex was performed according to the manufacturer’s instructions on Luminex FIDIS.

Statistical analysis

Statistical evaluation was performed using Student’s t test for unpaired data, and results were considered significant if p values were <0.05. Data are expressed as means ± SEM.

Results

CLEC-1 is overexpressed in tolerated allografts

As previously described, to identify new molecules that could play a role in allograft tolerance, we applied exhaustive rat DNA chips and compared long-term tolerated cardiac allografts (short-term treatment with LF15-0195) with allografts displaying signs of chronic rejection (DST treated) (8). We previously showed in this model the overexpression of numerous cytoprotective molecules by graft ECs and an accumulation of regulatory CD4⁺ T cells at the graft site (up to 50% of CD4⁺ T cells) (13, 22). We identified the CLEC-1 gene as overexpressed in tolerated allografts. By quantitative RT-PCR, we confirmed the up-regulation of CLEC-1 mRNA expression in tolerated allografts (Fig. 1A). Additionally, we also observed a strong up-regulation of CLEC-1 mRNA expression in kidney tolerated allografts (Fig. 1B) (13, 22). These data demonstrate that CLEC-1 expression is up-regulated in several models of allograft tolerance, suggesting a role for CLEC-1 in regulatory mechanisms.

CLEC-1 is overexpressed by myeloid cells and ECs in tolerated allografts

To determine the cells expressing CLEC-1 in long-term tolerated allografts, we have generated a polyclonal anti-rat CLEC-1 Ab. We observed by immunofluorescence on heart allograft sections that in tolerated allografts, CLEC-1 is expressed by numerous groups of myeloid cells (CD172a⁻) and by ECs in the graft vessels (PECAM-1⁻) (Fig. 2A, a and b, respectively). This staining by myeloid cells and ECs disappears when the Ab was preincubated with an excess of functional CLEC-1 peptide.
with the synthetic peptides used for its generation, demonstrating the specificity of the staining (Fig. 2A). Interestingly, the high expression of CLEC-1 observed in graft ECs is specific to tolerated allografts, as no staining for CLEC-1 in ECs has been observed in cardiac allografts with chronic rejection or in syngeneic grafts (Fig. 2B, a and b, respectively).

We also observed CLEC-1 expression by myeloid cells and ECs (PECAM-1+) in kidney long-term tolerated allografts, whereas no staining by ECs was observed in kidney long-term syngeneic grafts (Fig. 2C, a and b, respectively).

These results demonstrate that CLEC-1 is overexpressed by the APCs, myeloid cells, and ECs in long-term tolerated allografts.

In naive rats, CLEC-1 is expressed by myeloid cells and ECs

To characterize CLEC-1 expression in rats, CLEC-1 mRNAs were assessed by quantitative RT-PCR in various tissues or cells from naive rats. We observed that CLEC-1 mRNA expression is high in the lung, in secondary lymphoid organs (lymph nodes and spleen), and in the aorta (Fig. 3A). A lower CLEC-1 mRNA expression was observed in the heart, and a poor expression was observed in the thymus.

These results were confirmed at the protein level by Western blot on deglycosylated organs with the polyclonal anti-rat CLEC-1 Ab that we have generated and that reveals an expected 32-kDa band (Fig. 3B, representative blot and quantification).

Moreover, in different rat cell populations, we observed a high mRNA expression of CLEC-1 in BMDCs and in peritoneal macrophages and a lower expression in an endothelial cell line and in splenic DCs (Fig. 3C). No expression of CLEC-1 was detected in T cells (Fig. 3C).

At the protein level, we observed by immunohistology that CLEC-1 is indeed more expressed in the myeloid cell BMDCs and macrophages than in the endothelial cell line and that its expression is at the cell surface (representative images in Fig. 3D).

These results demonstrate for the first time that in rodents, CLEC-1 is highly expressed by myeloid cells and, to a lower extent, by ECs and that its expression is at the cell surface.

CLEC-1 expression is down-regulated by inflammatory stimuli and increased by immunoregulatory mediators

To investigate the regulation of CLEC-1 expression, rat BMDCs were cultivated with different stimuli in vitro. As shown in Fig. 4A, expression of CLEC-1 is significantly decreased following LPS, IFN-γ, or poly(I:C) stimulation at 48 h of culture (n = 3; *, p < 0.05 and ***, p < 0.001). In contrast, following stimulation with the immunomodulators IL-10 or TGFβ1, expression of CLEC-1 is significantly increased at 48 h of culture (n = 3; *, p < 0.05 and ***, p < 0.001).

Similar results were observed in human cells HAECs or human monocyte-derived DCs (HuMoDC); CLEC-1 mRNA expression is decreased following LPS, IFN-γ, or poly(I:C) stimulation at 48 h of culture (n = 3; *, p < 0.05 and ***, p < 0.001). In contrast, following stimulation with the immunomodulators IL-10 or TGFβ1, expression of CLEC-1 is significantly increased at 48 h of culture (n = 3; *, p < 0.05 and ***, p < 0.001).
expression is significantly down-regulated by inflammatory stimuli (IFN-γ in HAECs, LPS in monocyte-derived DCs) (n = 3; **, p < 0.01), whereas IL-10 or TGFβ1 increases the expression of CLEC-1 and renders the cells resistant to the down-regulation of CLEC-1 expression induced by LPS (monocyte-derived DCs) (Fig. 4, B and C, respectively).

These results demonstrate that CLEC-1 expression is down-regulated by inflammatory stimuli and increased by immunoregulatory mediators, suggesting an important role for CLEC-1 in immune response regulation.

**Regulatory CD4⁺ CD25⁺ T cells from tolerant recipients enhance CLEC-1 expression in donor-type ECs**

We previously showed in vitro and in vivo that regulatory CD4⁺ CD25⁺ T cells from tolerant recipients were able to stimu-

late donor-type ECs to express IDO, a molecule required for the establishment of tolerance, demonstrating an interplay between graft ECs and regulatory CD4⁺ CD25⁺ T cells for the maintenance of tolerance (10). Here, we also observed that regulatory CD4⁺ CD25⁺ T cells from tolerant recipients but not regulatory CD4⁺ CD25⁺ T cells from naive rats were able in vitro to increase significantly CLEC-1 mRNA expression in graft donor-type (LEW.1W) ECs (n = 6; **, p < 0.01; Fig. 5Aa). We observed a low induction in recipient-type (LEW.1A) ECs with regulatory CD4⁺ CD25⁺ T cells from tolerant recipients, but this expression is not significantly different with the one observed with regulatory CD4⁺ CD25⁺ T cells from naive rats or with CD4⁺ CD25⁻ T cells from tolerant recipients (n = 6) (Fig. 5Ab). No induction of CLEC-1 expression was observed with CD4⁺ CD25⁻ T cells from either naive rats or tolerant recipients (n = 6) (Fig. 5Aa).

Moreover, we observed that neutralization of IL-10, TGFβ, or both with specific Abs did not further decrease CLEC-1 mRNA expression compared with the irrelevant control Ab (n = 4) (Fig. 5Ac). Indeed, the irrelevant control already decreased by itself CLEC-1 mRNA expression in ECs by a nonspecific mechanism.

Additionally, transwell experiments demonstrated that as for the one of IDO, induction of CLEC-1 expression requires cell contact (Fig. 5A, c and d). These data demonstrate that regulatory CD4⁺ CD25⁺ T cells from tolerant recipients are able in vitro to increase CLEC-1 expression in donor-type ECs by a cell contact-dependent mechanism.

Furthermore, we previously demonstrated in vivo that regulatory CD4⁺ CD25⁺ T cells from tolerant recipients, but not regulatory CD4⁺ CD25⁻ T cells from naive rats, were able to transfer tolerance to a subsequent irradiated host (23). We demonstrated that following transfer, regulatory CD4⁺ CD25⁺ T cells accumulated in the new graft and induced the expression of IDO in graft ECs (10). Here, we also observed a specific expression of CLEC-1 in ECs in the new allogeneic graft following transfer of regulatory CD4⁺ CD25⁺ T cells from tolerant recipients (Fig. 5Ba). No staining of CLEC-1 by ECs in syngeneic grafts was observed following transfer of T cells from tolerant recipients or from naive rats demonstrating donor-specific allorecognition (Fig. 5B, b and c, respectively).

Taken together, these data demonstrate that alloantigen-primed regulatory CD4⁺ CD25⁺ T cells that accumulated in the allografts are able to increase locally the expression of CLEC-1 in donor-specific APCs.

**LEC1 alters T cell activation**

To assess the function of CLEC-1, we used specific CLEC-1 targeted small interfering RNA, or Stealth RNAi, which allows for efficient and long-term inhibition and which can reduce the cytotoxic IFN response unlike conventional small interfering RNA (24). Two nonoverlapping Stealth RNAi specific for CLEC-1 (medium GC content) were tested together with a universal Stealth control RNAi (medium GC content). To evaluate in DCs the effect of CLEC-1 inhibition on subsequent allogeneic T cell activation, we used LPS-stimulated BMDCs, which are able in these mature conditions to stimulate efficiently allogeneic T cells. Indeed, we have not been able with immature BMDCs or an EC line to induce an efficient allogeneic T cell stimulation.

We observed a strong inhibition of CLEC-1 mRNA expression (>80%) in ECs with the two CLEC-1-specific RNAi as compared with the control RNAi at 48 h following transfection (n = 5; **, p < 0.01; Fig. 6Aa). This strong inhibition of CLEC-1 expression was also confirmed at the protein level with the polyclonal Ab by immunohistology (n = 5; ***, p < 0.001; Fig. 6Ab).
We observed that CLEC-1 inhibition in DC culture does not modulate LPS-induced IL-12p70, IL-6, or IL-10 production or class II MHC, CD86, or CD80 expression \((n=5\), Fig. 6, A and B). Therefore, the differences that will be observed following CLEC-1 triggering with these mature DCs will be not due to a defect in DC generation or maturation.
Interestingly, when these mature DCs were cocultured with allogeneic T cells in a MLR, we observed that CLEC-1 inhibition in DCs enhances significantly allogeneic T cell production of IL-17 (at both the protein and mRNA levels) and mRNA expression of the transcription factor RORγt (n = 5; *, p < 0.05), without modulating T cell proliferation or IL-2 secretion (Fig. 7). Additionally, CLEC-1 inhibition in DCs decreased significantly allogeneic T cell production of IL-13 and IFN-γ and decreased significantly mRNA expression of the transcription factor Foxp3 (n = 5; *, p < 0.05; Fig. 7). The increase in Th17 differentiation and the decrease in Foxp3 expression could have been due to a modulation of IL-6 or TGFβ expression; however, we observed no difference in the production of both cytokines in the MLRs (n = 5) (Fig. 7).

**CLEC-1 modulates regulatory CD4+CD25+ and CD4+CD25− T cell activation**

To investigate whether the decrease in Foxp3 mRNA expression observed at the end of the MLR with CLEC-1 down-regulated DCs was due to a decrease in the number of regulatory Foxp3+ T cells, we analyzed the allogeneic CD4+ and CD8+ T cell proliferation by CFSE and Foxp3 staining in long-term T cell co-cultures (at day 5 of MLR). We observed that inhibition of CLEC-1 in DCs decreases significantly the percentage of Foxp3+CD4+ T cells retrieved at the end of the MLR culture (3% instead of 6%) and increases the percentage of proliferating effector non-Foxp3 CD4+ T cells (36% instead of 23%) (n = 5; *, p < 0.05; Fig. 8, Aa, representative dot plots, and Ab). No difference in effector or regulatory Foxp3+ T cells was observed in the CD8+ T cells (Fig. 8, Ac, representative dot plots, and Ad). These data suggest that CLEC-1 regulates the balance between effector and regulatory T cells.

To determine whether this increase in effector CD4+Th17 differentiation was due to a direct effect of CLEC-1 inhibition on effector T cell differentiation or an effect on regulatory T cell plasticity or function, we performed MLRs with highly purified CD4+CD25− and CD4+CD25+ T cells. We observed that inhibition of CLEC-1 in DCs did not modulate the allogeneic proliferation of purified regulatory CD4+CD25+ or their expression of Foxp3 (maintained at high level only in cells that have proliferated) (Fig. 8Bb, representative dot plots). The allogeneic proliferation of purified CD4+CD25− T cells was also not modified when CLEC-1 expression was down-regulated in DCs (Fig. 8Bb, representative dot plots).

Moreover, we did not observe differences in IFN-γ or IL-17 secretion in stimulated purified regulatory CD25+ or CD25− CD4+ T cells when CLEC-1 expression was down-regulated in DCs (n = 3) (Fig. 8Bc and Bd, respectively). The expression of IL-6 was also not modified in MLRs with purified regulatory
CD25$^+$ or CD25$^-$ CD4$^+$ T cells when CLEC-1 expression was down-regulated in DCs ($n = 3$) (Fig. 8B, c and d, respectively). Therefore, with isolated subpopulations of CD25$^+$ or CD25$^-$ CD4$^+$ T cells, we did not retrieve the increase of IL-17 secretion observed with bulk T cells. These data demonstrate that the up-regulation of IL-17 observed with bulk T cells was not due to a direct effect on differentiation of T cells but rather may be due to an effect on the suppression mediated by regulatory CD4$^+$CD25$^+$ T cells.

In tolerated allograft, the increased expression of CLEC-1 is associated with a low expression of IL-17 and a high expression of Foxp3.

To evaluate whether there was a correlation between the increased expression of CLEC-1 and the expression of IL-17 and Foxp3 in long-term tolerated allografts, we compared Foxp3 and IL-17 mRNA expression in tolerated allografts and in chronically rejected allografts. Interestingly, we observed in tolerated allografts where the expression of CLEC-1 was increased ($n = 4$; $**$, $p < 0.01$; Fig. 1), that the mRNA expression of IL-17 was significantly lower and the one of Foxp3 higher than in chronically rejected allografts.
We previously demonstrated also the specific expression of heme oxygenase-1, inducible NO synthase, and IDO by ECs of graft vessels. Interestingly, this expression by ECs is specific to tolerance by a group of myeloid cells and by numerous ECs of graft vessels in tolerated allografts and was not observed in syngeneic grafts or in other factors. Additionally, we found that CLEC-1 expression in tolerated allografts was associated with a low expression of IL-17 and the presence of numerous regulatory Foxp3+CD4+CD25+ T cells that accumulate at the graft site (8, 10).

Taken together, these data suggest that in tolerated allografts, CLEC-1 expressed by myeloid cells and ECs is enhanced by regulatory T cells to, in turn, moderate allogeneic Th17 response. In this regard, the paired immunoreceptors PIR-B (ortholog of ILT3 and ILT4 in human) have clearly been shown to be induced by regulatory CD8+CD28+ T cells in DCs and ECs and to, in turn, generate the expansion of regulatory CD4+CD25+ T cells (28). PIR-B has also been shown to be highly expressed by ECs from tolerated allografts in a rat model of allograft tolerance that involved regulatory T cells (29). The regulation of allogeneic Th17 response mediated in part by overexpression of CLEC-1 by myeloid cells and ECs may contribute to long-term tolerance and prevention of chronic rejection. Indeed, it has recently been shown that human ECs can directly activate allogeneic memory T cells toward production of IL-17, and that IL-17-producing CD4+ Th17 T cells contribute to allograft rejection and vasculopathy in a established experimental model of chronic rejection (30, 31). Interestingly, we also observed that CLEC-1 acts directly on CD4+CD25+ T cells for the expression of IL-13, suggesting also a role for CLEC-1 in Th2 differentiation.

We showed herein for the first time that as for many C-type lectin receptors expressed on myeloid cells, CLEC-1 is involved in the modulation of T cell activation. For example, DCAL-2 triggering has been shown to alter cytokine production by DCs and thereby to regulate the quality of downstream T cell activation (32, 33). DCAL-1 has been described to act as a T cell costimulatory molecule, which skews CD4+ T cells toward a Th2 response by enhancing their secretion of IL-4 (34). Dectin-1, an activating receptor, is shown to promote the activation of DCs to instruct the differentiation of IL-17-producing effector CD4+ T cells and CD8+ CTLs and to convert regulatory CD4+CD25+ T cells into IL-17 producer cells (2, 7, 35). Moreover, AICL receptor expressed on myeloid cells and NKp80 expressed on NK and memory CD8+ T cells are both activating C-type lectin-like receptors interacting with each other, and their mutual engagement promotes reciprocal activation and cytokine release at sites of inflammation (36). CLEC-1 triggering may modulate directly activation of the DCs and ECs and/or give a regulatory signal to T cells.
CLEC-1 MODERATES T CELL ACTIVATION

In conclusion, we demonstrate for the first time that CLEC-1, identified in a model of tolerance and expressed by myeloid cells and ECs, is increased by immunoregulatory mediators and acts as a regulatory receptor able to alter T cell response. Therefore, CLEC-1 may be a useful target to modulate immune responses toward protective immunity or tolerance induction.

Disclosures
The authors have no financial conflicts of interest.

References