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Enhanced Dendritic Cell-Induced Immune Responses Mediated by the Novel C-Type Lectin Receptor mDCAR1

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C-type lectins (CLEC) encompass a large family of proteins with varied functions. They contain one or more CLEC domains originally described as mediating carbohydrate-binding activity in a Ca2+-dependent manner (1). After characterization of several members of the family, the definition of the term CLEC has evolved and now also includes proteins containing CLEC-like domains (CTLD). CTLDs do not mediate interactions exclusively with carbohydrates but are able to bind ligands in the absence of calcium ions (2). CTLD-containing proteins, also referred to as CLEC receptors (CLR; Ref. 3), are categorized into 17 groups based on their phylogeny and domain organization (4). Of particular interest in the field of immunology are group II CLRs that are encoded within the Ag-presenting lectin-like receptor gene complex (APLEC) on human chromosome 12 (5, 6). Group II CLRs are type II transmembrane proteins and are frequently expressed by myeloid cells in bone marrow and spleen, whereas the molecule was not detectable on both cell types in lymph nodes and peripheral blood. With respect to the function of CLRs as pattern recognition receptors, Ag delivered via mDCAR1 was internalized, was trafficked to early and late endosomes/lysosomes and, as a consequence, induced cellular and humoral responses in vivo even in the absence of CD40 stimulation. Intriguingly, upon triggering mDCAR1, CD8+ DCs increased the secretion of bioactive IL-12, whereas IL-10 release is markedly reduced, thereby indicating that Ag recognized by mDCAR1 induces enhanced proinflammatory responses. These data indicate that mDCAR1 is a functional receptor on cells of the immune system and provides further insights into the regulation of immune responses by CLRs.

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Materials and Methods

Animals

Wistar-Furth rats and BALB/c and C57BL/6N mice were purchased from Harlan Laboratories. OVA-specific CD8 (OT-I) and CD4 (OT-II) TCR-transgenic mice (C57BL/6N background; CD4+5.2) were derived from our breeding facilities (Miltenyi Biotec). C57BL/6 (CD45.1) mice were obtained from Charles River Laboratories. Animals were used at 4–12 wk of age, and all experiments were performed according to national and institutional animal care and ethical guidelines.

In silico analysis

Nucleotide sequences of BDCA-2 (CD203) and hDCIR were blasted against mouse genome databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification of mouse orthologs. The National Center for Biotechnology Information nucleotide accession number entries (http://www.ncbi.nlm.nih.gov) of the sequences used were: CD203 (AF325460); hDCIR (AJ133532); mDCIR (AJ113533); mDCIR2 (AY397673); mDCIR3 (AY397674); mDCIR4 (AY397675); mDCAR (AY230259); and mDCARI (AY365133). Amino acid sequences of the CLRs were derived from UniProt Knowledgebase (Swiss-Prot and TrEMBL) entries (http://www.expasy.org) according to their primary accession numbers: CD303 (QSWT0); hDCIR (Q9UM7); mDCIR (Q0QZ1); mDCIR2 (Q5YR8); mDCIR3 (Q8XZ0); mDCIR4 (Q8Q17); mDCAR (Q7T558); and mDCARI (Q6TDU). Multiple sequence alignment was performed online by use of DIALIGN-TX (http://dialign-tx.gobics.de/) as described previously (24, 25). Potential protein domains and signaling motifs were predicted by use of PROSITE (http://www.expasy.org/prosite/).

Cell culture

All quoted cell lines and in vitro cultures were kept in RPMI 1640 (Miltenyi Biotec) supplemented with 10% (v/v) heat-inactivated, filtered FCS (Biochrom), 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml 2-ME, and 20 mM HEPES at 37°C in a humidified 5% CO2-incubator. Passage from one flask to another was performed every 3–5 d.

mDCAR1 cloning and transfection

cDNA coding for full-length mDCAR1 was isolated by PCR amplification from whole spleen cDNA using the primers 5'-ggegggtatgATGCGCAAGAAGACCGACC-3' and 5'-tggtggtaataATGTGATCTCATACTATA-3' (gene-specific sequence in capital letters; BamHI and EcoRI restriction sites are underlined). The resulting fragment was cloned into a modified pEP6 vector (Invitrogen) containing a HA tag (gene sequence ATGGGC TACCCATATGACGTCCCAGACTACGCT) between the promoter and the cDNA coding sequence. As a control, HA-encoding pEP6 vectors without mDCAR1 were used. The expression and control vector were transfected into Chinese hamster ovary (CHO) cells and rat basophilic leukemia (RBL-1) cells by electroporation and expression controlled by staining with anti-HA mAb (clone G8-IF3; Miltenyi Biotec). Selection of mDCAR1+ cells was performed by administration of 5 μg/ml blastidcin (Invitrogen) and subsequent cloning by limiting dilution.

Generation of specific anti-mDCAR1 mAb

Anti-mDCAR1 mAbs were generated using contralateral footpad immunization. Four-week-old Wistar-Furth rats were immunized four times (days 0, 4, 7, and 10) in the right footpad with 5 × 106 mDCARI-expressing RBL-1 cells and 150 μg of CpG1826 (Metabion) per boost. Vector-control transfected RBL-1 cells (5 × 106) and CpG were applied at days −3, 0, 4, 7, and 10 into the left footpad per boost. Lymph node cells were fused with the mouse myeloma cell line SP2/0 at day 11. Hybridoma cells secreting specific mAbs against mDCAR1 (anti-mDCAR1 SKa5-3DS, -4B2, -16D2, and -12B11, all of rat (r) IgG2b isotype) were identified by flow cytometry analysis of cell culture supernatants using a combination of mDCAR1 and control vector-transfected RBL-1 and CHO cells. Cross-blocking experiments were performed on mDCARI-expressing RBL-1 transfectedants by incubating pure anti-mDCAR1 mAb (50 μg/ml) before labeling with fluorochrome-conjugated anti-mDCAR1 mAb.

Flow cytometric analysis

Single-cell suspensions from mouse spleen, mesenteric, inguinal, and popliteal lymph nodes and thymus were prepared by enzymatic disaggregation with collagenase D (Roche). Bone marrow cells were isolated by flushing femurs with PBS supplemented with 0.5% (w/v) BSA and 5 mM EDTA. Peripheral blood cells were obtained from sacrificed mice and erythrocytes were removed by lysis in iso-osmotic ammonium chloride buffer. Single-cell suspensions were then added to mice FcR Blocking Reagent (Miltenyi Biotec) before addition of fluorochrome-conjugated specific mAbs. For phenotypic analysis, anti-mDCAR1 mAb (clone SKa5-3DS) was labeled in combination with various mAbs (clone names in parentheses) according to manufacturer’s protocols: CD11c (418); CD8α (53.5.7); CD205 (NLDC-145, 53-6-2B2; CD41 (M170.15.12); FcεRI (A3-1); anti-MHCII (M5/14.15.2); anti-Gr-I (R6-8C5); CD40 (FGK45.45) (all from Miltenyi Biotec); anti-mDCIR2 (33D1); CD80 (16-10A1); CD86 (GL1), all from BD Biosciences; BP-1 (6C3; ebioscience), F4/80 (CI-3; Serotec). Propidium iodide (Sigma-Aldrich) was added at a final concentration of 1 μg/ml immediately before flow cytometric acquisition to dead cell discrimination. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). Gates were set according to fluorochrome-conjugated isotype control stainings using mAb hamster IgG1 (A19-3), IgG3 (R3-34), IgG2a (R35-95), IgG2b (A95-1), all from BD Biosciences.

Immunofluorescence microscopy

Frozen splenic tissue sections (5–10 μm; BioChain) derived from BALB/c mice were incubated for 15 h at 4°C with the following mAb conjugates diluted in PBS containing 1% BSA and FcR Blocking Reagent (Miltenyi Biotec): biotinylated anti-mDCAR1 (clone SKa5-3DS) and fluorochrome-conjugated F4/80 (C1A3-1; Serotec); CD8α (53-6-7); CD45R (B220; RA3-6B2), both from Miltenyi Biotec; and anti-mouse SIGNR1 (22D1; ebioscience). Tissue sections were counterstained for 1 h at 4°C with streptavidin-allophycocyanin-eFluor 780 or streptavidin-Alexa Fluor 488 (Invitrogen). Isotype control mAb conjugates (BD Biosciences) were used at the same concentration as primary mAb. Endogenous biotin was blocked with Biotin-Blocking Kit (Invitrogen) before labeling with primary mAb. Sections were mounted in fluorescent mounting medium (Dako) and analyzed on a Leica TCS SP-2 CLSM.

Cell isolation

Splenic CD8+ T cells were isolated using a mouse CD8+ DC Isolation Kit (Miltenyi Biotec). Briefly, single-cell suspensions were depleted of CD90+2, CD45R (B220), and CD49b+ cells, and DCs were subsequently enriched via CD86 MicroBeads. Sorted DCs were always reached a purity of >95%. OVA-specific transgenic CD45.2+ CD8+ (OT-I) and CD4+ (OT-II) T cells were isolated using either a CD8+ or a CD4+ cell isolation Kit (Miltenyi Biotec), respectively. Briefly, single-cell suspensions were depleted of CD8α+ or CD4+, CD45R (B220), CD49b, CD11b, and Ter-119+ cells. Sorted T cells always reached a purity of >98%.

Internalization assay and intracellular routing of internalized mAb

Isolated splenic CD8+ T cells were labeled with 1 μg/ml allophycocyanin-conjugated anti-mDCARI mAb (SKa5-3DS) and isotype-matched control IgG2b (A95-1; BD Biosciences) for 20 min on ice. Cells were washed with ice cold PBS supplemented with 0.5% (w/v) BSA and 5 mM EDTA labeled with biotinylated-anti-allophycocyanin-eFluor 780 or streptavidin-Alexa Fluor 488 (Invitrogen). Isotype control mAb conjugates (BD Biosciences) were used at the same concentration as primary mAb. Endogenous biotin was blocked with Biotin-Blocking Kit (Invitrogen) before labeling with primary mAb. Sections were mounted in fluorescent mounting medium (Dako) and analyzed on a Leica TCS SP-2 CLSM.

Coupling mAb to OVA

DTT-reacted anti-mDCARI mAb (SKa5-3DS), CD205 mAb (NLDC-145), and isotype-matched control mAb IgG2b (JES10-S42) (Miltenyi Biotec) were chemically coupled to maleimide-activated OVA (Pierce Biochemical), as described previously (26). Conjugates were purified using size exclusion chromatography (Superdex 200; Amersham). PAGE and
Western blot analysis were performed to control purity of the conjugates. The amount of OVA conjugated to the mAb was determined by ELISA (not shown).

In vivo T cell proliferation

Enriched CD45.2
CD8
(OT-I) and CD4
(OT-II) T cells were labeled with CFSE (Molecular Probes; 1 μM) and injected i.v. into C57BL/6J (CD45.1) mice (10⁶ cells). After 18 h, increasing concentrations (0.5–5 μg) of OVA-conjugated anti-mDCAR1 mAb (containing 50–500 ng of OVA), 2.5 μg of CD205 mAb (250 ng of OVA), or 0.5–5 μg of rILG2b mAb (50–500 ng of OVA) or 500 ng of soluble OVA (Pierce Biocel) were injected i.v. with or without 25 μg of CD40 mAb (FGK45.5; Miltenyi Biotec). In vivo blocking of mDCAR1 internalization was achieved by i.v. injection of 250 μg of anti-mDCAR1 mAb (SKA5-3DS) together with isolated transgenic CD45.2
T cells 1 day before mDCAR1-OVA conjugate was applied. Three days later, splenocytes were analyzed with fluorochrome-conjugated mAb against CD4 (GK1.5) or CD8 (53-6.7; Miltenyi Biotec) and CD45.2 (10⁴; BD Biosciences). Propidium iodide (Sigma-Aldrich) was added at a final concentration of 1 μg/ml immediately before acquisition to exclude dead cells from analysis. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Proliferating CD45.2
T cells were visualized by loss of CFSE fluorescence.

Detection of anti-OVA serum Ab

Serum samples were obtained after 2 wk from mice immunized i.v. with 1 μg of OVA-conjugated anti-mDCAR1 mAb, 1 μg of rILG2b mAb, or 1 μg of CD205 mAb with or without 25 μg of CD40 mAb. ELISA plates (Greiner Bio-One) were coated with 10 μg/ml OVA (Pierce Biochemical) for 2 h and blocked overnight (PBS, 0.05% Tween, 1% BSA). Diluted serum samples were plated and incubated at 37°C for 2 h. Peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was plated and incubated at 37°C for 1 h. Anti-OVA serum Ab was determined by ELISA. Anti-OVA mAb (Miltenyi Biotec; clone SK1-3C7) was used as standard.

Anti-cytokine ELISA

Isolated splenic CD8
DC were cultured at 10⁶ cells/ml for 24 h in a 48-well plate (500 μl/well) in the presence or absence of 5 μg/ml anti-mDCAR1 mAb or isotype-matched control mAb rILG2b (JES10-S42; Miltenyi Biotec). For stimulation, cultures were incubated on a monolayer of CD45.1 mice (10⁶ cells). After 18 h, increasing concentrations (0.5–5 μg) of OVA-conjugated anti-mDCAR1 mAb (containing 50–500 ng of OVA), 2.5 μg of CD205 mAb (250 ng of OVA), or 0.5–5 μg of rILG2b mAb (50–500 ng of OVA) or 500 ng of soluble OVA (Pierce Biocel) were injected i.v. with or without 25 μg of CD40 mAb (FGK45.5; Miltenyi Biotec). In vivo blocking of mDCAR1 internalization was achieved by i.v. injection of 250 μg of anti-mDCAR1 mAb (SKA5-3DS) together with isolated transgenic CD45.2
T cells 1 day before mDCAR1-OVA conjugate was applied. Three days later, splenocytes were analyzed with fluorochrome-conjugated mAb against CD4 (GK1.5) or CD8 (53-6.7; Miltenyi Biotec) and CD45.2 (10⁴; BD Biosciences). Propidium iodide (Sigma-Aldrich) was added at a final concentration of 1 μg/ml immediately before acquisition to exclude dead cells from analysis. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Proliferating CD45.2
T cells were visualized by loss of CFSE fluorescence.

Statistics

Statistical analysis was performed using a one-tailed Student t test for differences among groups. Data are expressed as means ± SEM unless otherwise stated.

Results

Comparative chromosomal and protein analysis of the DCIR/CD303/DCAR family

To identify murine group II CLRs that are related to BDCA-2 (CD303) and hDCIR, we and others (6) conducted an extensive similarity search via Internet-based basic local alignment search tool in protein databases as well as in genomic sequences of mouse genome databases. Six mouse genes were identified forming two subfamilies of CLR. The mDCAR family comprises four genes encoding mDCIR, mDCIR2, mDCIR3, and mDCIR4, as well as the mDCAR family consisting of the genes for mDCAR and mDCAR1. The chromosomal position of the genes was compared with those of CD303 and hDCIR (Fig. 1A). The genes were localized on the APLEC on human chromosome 12 or the corresponding mouse chromosome 6, adjacent to the genes of the CLR Dectin-2, Mcl, and Mincle. The non-CLC genes were GDF3, SLC22A3, and C3AR were found upstream of hDCIR flanking the CD303 gene. The ortholog cluster on the mouse chromosome lacked a CD303-related CLR fragment. The mDCIR/mDCAR family genes are located upstream of C3AR, corresponding to the position of the hDCIR gene.

To examine the homology of the two human and six mouse CLRs, they were compared according to their predicted protein domains (Fig. 1B). All CLRs were characterized by a highly conserved extracellular CTLD, a short neck, and one transmembrane domain. The intracellular domains categorized the receptors into two groups, mDCAR, mDCAR1, and CD303 share a short tail facing the cytosol; whereas the human and mouse DCIR proteins are marked by a long N terminus. The cytoplasmic regions of hDCIR, mDCIR, and mDCIR2 carry an ITIM, whereas mDCIR3 and mDCIR4 lack such signal transduction motifs.
In the following, we focused on the characterization of mDCAR1. cDNA was cloned from splenocytes and stably transfected into the RBL-1 cell line which was then used for controlateral footpad immunization of rats. Generation of mAbs yielded four Ag-specific clones, all recognizing the same epitope as determined by cross-blocking experiments on mDCAR1-transfected CHO cells (data not shown). One representative clone, SKa5-3D5, was used to specifically recognize mDCAR1-transfected RBL-1 cells in a mix with control-transfected RBL-1 cells in flow cytometry (supplemental Fig. 1). 3 All additional experiments were performed with this clone.

mDCAR1 is expressed in a tissue-dependent manner on CD8+ DCs and subpopulations of myeloid cells

To investigate the expression pattern of mDCAR1, we used fluorochrome-conjugated anti-mDCAR1 mAb to analyze murine tissues by flow cytometry. mDCAR1 expression was detected on a subset of CD11c- DC and CD11c- cells in spleen (Fig. 2A). The mDCAR1+CD11c+ subset represented 0.6% of all splenocytes and was further characterized by expression of CD8 and CD205, CD80, and CD86. mDCAR1+ cells expressed high amounts of MHC class II molecules and moderate levels of costimulatory molecules CD40, CD80, and CD86. mDCAR1+ DC lacked the CD4+ DC-specific marker mDCIR2 (33D1). Analysis of gated DC subsets revealed that mDCAR1 was expressed by all CD8+ DCs in spleen (data not shown).

The mDCAR1+CD11c- cells were characterized by expression of the myeloid markers CD11b, CD31, and Gr-1 (Ly6G/C). They were heterogeneous for the expression of F4/80, and the prominent mDCAR1+ compartment expressed CD205. Whereas CD11b was expressed on 20% of all splenocytes, mDCAR1 expression was detected on 10% of the CD11b+ compartment (Fig. 2A and data not shown). mDCAR1+ splenic myeloid cells were found to be negative for marker further defining monocyte/macrophage subsets, such as SIGNR1, MOMA-1, MOMA-2, and MARCO (data not shown). We therefore analyzed mDCAR1 expression pattern on tissue sections of mouse spleen. mDCAR1 was selectively expressed within the white pulp, predominantly in T cell zones, where CD8+ DCs reside, and to a lower extent in B220+B cell-rich follicles (Fig. 2B). SIGNR1+ marginal zone macrophages and F4/80+ red pulp macrophages were distinct from mDCAR1+ myeloid cells, as no coexpression was detectable.

In the thymus, mDCAR1 expression was restricted to the CD8+CD205+CD11b-/-MHC class IIhigh DC compartment (CD4- mPDCA-1-), whereas on CD11b+ myeloid cells the CLR was not detectable (Fig. 2C and data not shown). In contrast to splenic DC, the thymic mDCAR1+ cells showed heterogeneous and increased expression level of CD40, CD80, and CD86. BP-1 confirmed the restriction of mDCAR1 expression to this DC subset (27).

DCs in bone marrow (BM) did not show detectable levels of mDCAR1 (Fig. 2D). However, the phenotype of mDCAR1+ BM cells corresponded to the splenic myeloid cells (CD11b+Gr-1dim/+ F4/80dim/+CD205+CD31dim/+). Whereas CD11b was expressed on 50% of all BM cells, mDCAR1 expression was detected on 10% of the CD11b+ compartment (data not shown).

Unexpectedly, mDCAR1 expression could be detected neither on the cell surface of DC nor on myeloid cells present in mesenteric, inguinal, and popliteal lymph nodes or circulating through peripheral blood (Fig. 2E and data not shown). Additionally, mDCAR1 expression could not be induced on in vitro generated BM-DC (Fit-3L or GM-CSF/IL-4; supplemental Fig. 2). Upon activation with stimulatory agents, i.e., TLR agonists or CD40L, mDCAR1 is down-regulated on both DCs and myeloid cells (data not shown).

As a result, in the lympho-hematopoietic system, mDCAR1 surface expression appeared in a tissue-dependent manner on the CD8+ DC subset in spleen and thymus and on subpopulations of myeloid cells in spleen and BM.

mDCAR1 mediates delivery of bound Ab into the Ag-processing pathway

A prominent function of CLR is the sensing of Ags and their uptake (3). We therefore addressed the question of whether mDCAR1 mediates endocytosis after receptor cross-link by performing internalization experiments. Freshly isolated splenic CD8+ DCs were labeled with anti-mDCAR1 mAb conjugate and cross-linked with secondary conjugates on ice. mDCAR1 on the cell surface was detected after 1 and 2 h of incubation at 4°C and 37°C. mDCAR1-anti-mDCAR1 mAb complexes were stably detectable when incubated at 4°C (Fig. 3A). In contrast, the cell surface level of mDCAR1 was reduced to 50% (compared with mean fluorescence intensity at t=0 min) after 1 h of incubation at 37°C, further decreasing after 2 h (65%). Comparable results were obtained for thymic DCs as well as for myeloid cells in spleen and BM (data not shown).

To follow the fate of internalized mAb, splenic CD8+ DCs were analyzed via confocal laser scanning microscopy. After incubation at the indicated time points, DCs were intracellularly stained for early endosome Ag 1 (EEA1) and LAMP-1 (CD107a), a marker for late endosomes/lysosomes. In the first 30 min, anti-mDCAR1 mAb was internalized, whereas low amounts colocalized with EEA1 in early endosomes (Fig. 3B). During the following hours, anti-mDCAR1 mAb was entirely routed into LAMP-1+ late endosomal/lysosomal compartments. Thus, mDCAR1 can mediate delivery of bound Ab to the endocytotic pathway. The intracellular trafficking of internalized anti-mDCAR1 mAb resembled that of CD205 mAb (Fig. 3B), raising the question whether comparable immune responses are initiated after receptor-mediated Ag uptake.

Targeting Ag via mDCAR1 induces enhanced immune responses in vivo

To assess whether uptake of Ag via mDCAR1 leads to processing and presentation, we investigated the induction of an Ag-specific, proliferative T cell response after internalization of OVA via anti-mDCAR1 mAb in vivo. Therefore, anti-mDCAR1 mAb, CD205 mAb, and isotype-matched control mAb were chemically coupled to OVA protein, resulting in conjugates consisting of 10% OVA (w/w) (supplemental Fig. 3 and data not shown).

OVA-conjugates were injected into CD45.1+ mice 1 day after adoptive transfer of CFSE-labeled, OVA-specific CD45.2+CD8+ (OT-I) and CD45.2+CD4+ (OT-II) T cells. After 3 days, the proliferative response of CD45.2+ T cells was determined by flow cytometry as a reduction of CFSE fluorescence. Only mice immunized with OVA-conjugated anti-mDCAR1 mAb were able to induce a proliferative response of both CD8+ and CD4+ T cells (Fig. 4A). Because the application of pure mAb did not result in depletion of mDCAR1+ cells, e.g., CD8+ DCs (supplemental Fig. 4), the specificity of the Ag targeting was verified by blocking the receptor-mediated Ag uptake.

The efficiency of mDCAR1-mediated Ag uptake was analyzed and compared with processing and presentation of soluble
FIGURE 2. mDCAR1 is expressed in a tissue-dependent manner on CD8⁺ DC and subpopulations of myeloid cells. Cell suspensions from mouse tissues and peripheral blood were stained with fluorochrome-conjugated anti-mDCAR1 and CD11c mAb (dot plots) and indicated mouse cell surface markers (histograms) and were analyzed via flow cytometry. Propidium iodide fluorescence and light scatter signals were used for gating of live cells. Numbers in the dot plots indicate percentage of mDCAR1⁺ cells of the CD11c⁺ or CD11c⁻ cell compartment. Histograms display mDCAR1-gated cells, lines reflect staining with fluorochrome-conjugated isotype-matched control mAb of the appropriate cell surface marker (shaded gray histograms). mDCAR1 expression was analyzed in spleen A, thymus C, BM (D), inguinal lymph nodes, and peripheral blood (E). Mesenteric and popliteal lymph nodes were analyzed as well (data not shown). B, mDCAR1 expression in spleen is restricted to white pulp. mDCAR1 expression pattern was analyzed on tissue sections of mouse spleen and counterstained with CD8 (T cell zone), B220 (B cell follicles), SIGNR1 (marginal zone macrophages) and F4/80 (red pulp macrophages). MHCII, MHC class II.
OVA protein and OVA coupled to the multilectin CD205 mAb. The titration of OVA-conjugated anti-mDCAR1 mAb revealed that induction of T cell proliferation was highly efficient and comparable with CD205-mediated uptake (Fig. 4B). No Ag-specific T cell response was observed in mice immunized with soluble OVA protein. When delivering low amounts of OVA (50 ng) conjugated to mAb, presented peptides still initiated a remarkable OT-I response, whereas CD4 T cell proliferation was less efficient. Administration of low doses of fluorochrome-conjugated anti-mDCAR1 mAb (data not shown) preferentially labeled CD8 DCs rather than myeloid cells (supplemental Fig. 5), indicating that anti-mDCAR1 mAb predominantly targets CD8 DC in vivo. Induction of T cell proliferation was observed without CD40 stimulation when targeting mDCAR1, as already shown for CD205 (26). However, in contrast to CD205, mice immunized with anti-mDCAR1-OVA induced enhanced humoral responses in the absence of CD40 mAb, as determined by analyzing the levels of anti-OVA serum Ab (Fig. 4C). Triggering mDCAR1 does not influence the activation state of DC, as expression levels of MHC class II molecules, CD40, CD80, and CD86 were not influenced on the CD8 DC subsets of mice injected with anti-mDCAR1 (Fig. 4D).

In summary, mDCAR1-mediated transfer of OVA into the Ag presentation pathway led to efficient induction of Ag-specific immune responses.

**Discussion**

Located next to the NK complex, the APLEC is established as a separate gene complex encoding receptors that belong to the CLR
FIGURE 4. Targeting Ag via mDCAR1 induces enhanced immune responses in vivo. A, OVA-specific transgenic CD45.2^+CD8^+ (OT-I) and CD4^+ (OT-II) T cells were labeled with CFSE and adoptively transferred into CD45.1^+ mice. On day 1 posttransfer, OVA-conjugated anti-mDCAR1 mAb or isotype-matched control IgG2b mAb were injected i.v. with CD40 mAb. On day 4, splenocytes were analyzed by flow cytometry for CD8-or CD4-gated T cells. A proliferative response was determined by loss of CFSE fluorescence on grafted CD45.2^+ T cells. Anti-mDCAR1-OVA targeting was blocked by coinjection of anti-mDCAR1 mAb with T cell transfer. Dot plots are representative of three independent experiments. B, Anti-mDCAR1-OVA induces stronger T cell proliferation than OVA alone even in the absence (w/o) of CD40 stimulation. Titrated amounts of OVA-conjugated anti-mDCAR1 mAb, CD205 mAb, or soluble OVA were injected with or without CD40 mAb. Histograms are representative of three independent experiments gated on CD8^+ (OT-I) or CD4^+ (OT-II) CD45.2^+ T cells. C, Targeting mDCAR1 induced humoral responses even in the absence of CD40 stimulation. Serum samples were derived from mice immunized with OVA-conjugated CD205 mAb, isotype-matched control, or anti-mDCAR1 mAb, with or without CD40 mAb. Concentrations of anti-OVA serum Ab were determined by ELISA. Each circle represents an individual mouse, the line represents the geometric mean. D, Triggering mDCAR1 has no impact on activation status of DC. Mice were injected with anti-mDCAR1 mAb or left untreated. After 18 h, splenic CD8^+ DC were analyzed for their expression of MHC class II and costimulatory molecules by flow cytometry. Data of three independent experiments are shown as the geometric mean fluorescence intensity (MFI) of expressed molecules on CD8^+ DCs.
Several CLR receptors are expressed on APCs and other phagocytes (7). By generating a specific anti-mDCAR1 mAb, we were able to show that the Ag is expressed on DC and myeloid cells in a tissue-dependent manner. mDCAR1 expression is restricted to CD8+ DC in spleen and the corresponding CD8+ BP-1+ DC subset in thymus. BP-1 has been described as a specific marker for this DC subset in mouse thymus (27), which corresponds to the recently described CD8+ Sipr- DC (31). In addition, a heterogeneous non-DC population in BM and spleen was also found to express mDCAR1. According to the expression of CD11b, F4/80, and Gr-1, these cells represent subpopulations of monocytes/macrophages and granulocytes. As flow cytometric analysis revealed, F4/80 expression on mDCAR1+ cells was low compared with classical macrophages, confirming that mDCAR1+ cells are distinct from F4/80+ red pulp macrophages in spleen. Based on the coexpression of CD205, the majority of mDCAR1+ myeloid cells correspond to the DEC205+ Gr-1low population reported by Lamb et al. (32), whereas a minority of mDCAR1+ cells display a different phenotype. Furthermore, we have evidence that mDCAR1+ myeloid cells reflect certain precursor cells in BM (S. A. Kaden et al., manuscript in preparation). Corresponding DC subsets and myeloid cells present in lymph nodes and peripheral blood did not show any detectable levels of mDCAR1. The tissue-specific expression pattern of mDCAR1 could reflect a distinct function that is restricted to these tissues, but identification of potential ligands is required to test this hypothesis. Because single-nucleotide polymorphisms in genes of the DCIR/CD303/DCAR family, particularly DCAR1 in rats (10), are associated with a predisposition to autoimmune diseases such as rheumatoid arthritis (9, 11), potential ligands of mDCAR1 could include not only foreign but also self-Ags.

The expression of mDCIR, mDCIR2, mDCIR3, mDCIR4, mDCAR, and mDCAR1 has thus far been demonstrated only at the mRNA level (6, 20, 21). The mRNA expression of mDCIR2 and mDCAR1 on NK cells, based on analysis of cells derived from spleen (6), contradicts both the protein expression pattern demonstrated here for mDCAR1 using specific mAb and that described for mDCIR2 (22). Therefore, the reported expression patterns of the remaining mouse CLR that are based on mRNA analysis need careful reconsideration and should be verified at the protein level.

CD8+ DCs were reported to have the capacity to cross-present exogenous Ags on MHC class I molecules and, as a consequence, activate CD8+ T cells (33–35). CD8+ DC are also effective inducers of CD4+ T cell responses (36). The expression of CLR on APCs, such as CD8+ DC, is often linked to a prominent function of these receptors, which is the uptake of Ag for further processing and presentation. mDCAR1 serves as an Ag uptake receptor and Ag uptake leads to the initiation of Ag-specific T cell responses as shown by the internalization of mDCAR1-anti-mDCAR1 mAb complexes. OVA delivery via mDCAR1 in vivo resulted in the proliferation of both CD8+ and CD4+ T cells, which was qualitatively and quantitatively comparable with CD205-mediated uptake, indicating that internalized Ag was presented on both MHC class I and MHC class II molecules. Intracellular trafficking of internalized anti-mDCAR1 mAb into EEA1+ early endosomes and late LAMP-1+ endosomes/lysosomes are prerequisites for the observed CD8+ and CD4+ T cell responses, as reported for CD205 (37), as well. Whereas distinctive MHC class I-restricted or MHC class II-restricted Ag presentation were reported for other CLR and non-CLR, as reviewed by Burgdorf and Kurtz (38).

mDCAR1+ myeloid cells correspond in their main part to CD205+ myeloid cells that were ascribed to have a very low T cell activation capacity compared with the activation potential of DC.
IL-10 negatively influences IL-12p70 release in activated CD8+ DCs. This agrees with the finding that the presence of TNF-α enhanced the secretion of IL-12p70, whereas IL-10 release was inhibited. This supports the hypothesis that the presence of TNF-α might favor the IL-12p70 release and, as a consequence, CD8+ DCs would be biased toward a proinflammatory response. The regulation of cytokine release by CLR requires signal transduction initiated after receptor triggering. As revealed by in silico protein analysis, mDCAR1 was found to lack such signal transduction motifs. Closely related CLRs, such as CD303, mDCAR1, and further Dectin-2 and Mincle, have been reported to signal via an associated ITAM-bearing FcR γ-chain. Therefore, interaction of mDCAR1 with FcR γ-chain is highly expected but needs further verification.

In conclusion, our data showed that mDCAR1 is a functional receptor on CD8+ DCs and myeloid cells and provided further insights into the regulation of immune responses by CLR.

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Disclosures
The authors have no financial conflict of interest.

References
FUNCTIONAL CHARACTERIZATION OF CLR mDCAR1


Supplementary Figure 1
SKa5-3D5 specifically recognizes an mDCAR1-transfected RBL-1 cell line. mDCAR1/HA-transfected RBL-1 cells were mixed with PKH67-labeled control/HA-transfected RBL-1 cells. The cell mix was stained with fluorochrome conjugated anti-HA mAb and analyzed by flow cytometry (left). The cell mix was also incubated with cell culture supernatants (s/n) derived after fusion of immunized rat lymphocytes with sp2/0 cells, stained with fluorochrome conjugated anti-rat Igkappa and analyzed by flow cytometry (middle, right).

Supplementary Figure 2
mDCAR1 is not expressed on bone marrow-derived DC. Bone marrow cell suspensions were cultured in the presence of GM-CSF and IL-4 (upper row) or Flt-3L (lower row). mFlt-3L-derived cultures were stimulated with recombinant 1000 U/mL mIFN-alpha for one more day. After 9 days with medium change at every third day., cells were analyzed for expression of CD11c, CD8 and mDCAR1 by flow cytometry. Propidium iodide fluorescence and light scatter signals were used for gating of live cells. Histograms display mDCAR1 expression on CD8(-) or CD8(+) CD11c(+) bone marrow derived DC according to the gates shown in the dot plots. The thin line shows the isotype-matched control of anti mDCAR1 staining (bold line). Data shown are representative of four independent experiments.

Supplementary Figure 3
Analysis of Ovalbumin-conjugated mAb via SDS-PAGE. Ovalbumin was chemically coupled to anti-mDCAR1 mAb (Clone 3D5), isotype-matched control mAb and CD205 mAb. 2µg of purified constructs were analysed via SDS-PAGE. As controls 4µg pure anti-mDCAR1 (3D5) and 4µg soluble Ova were applied on gel.

Supplementary Figure 4
DC are not depleted upon injection of anti-mDCAR1 mAb. Mice were injected intravenously with 250µg anti-mDCAR1 mAb (SKa5-3D5) (right dot plot) or left untreated (left dot plot). After two days, splenocytes were stained with fluorochrome-conjugated CD205 mAb and CD11c mAb and analyzed by flow cytometry. Dot plots are representatives of two independent experiments.
Supplementary Figure 5

CD8(+) DC are preferentially targeted by anti-mDCAR1 mAb in vivo. Mice were intravenously injected with graded doses of PE-conjugated anti-mDCAR1 mAb or a single dose of isotype-matched ratIgG2b mAb (5 µg/mouse). Splenocytes were analysed by flow cytometry 15 hours later. Histograms display in vivo labelling of mDCAR1(+) DC (gated on CD8(+)CD11c(+) cells) and mDCAR1(+) myeloid cells (gated on mDCAR1(+)CD11b(+)Gr-1(+) cells). Shaded grey area shows isotype-matched control, while shaded grey with black line indicates ex vivo labelling with PE-conjugated anti-mDCAR1 mAb. Further: black line, 5µg; blue line, 2 µg; red line, 1 µg of injected anti-mDCAR1 conjugate. Histograms are representatives of two independent experiments.