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

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The dendritic cell (DC) immunoreceptors (DCIR) and DC-immunoactivating receptors (DCAR) represent a subfamily of cell surface C-type lectin receptors (CLR), whose multifunctional capacities range from classical Ag uptake and immunoregulatory mechanisms to the involvement in DC ontogeny. On the basis of the generation of specific mAbs, we functionally characterized mouse DCAR1 (mDCAR1) as a member of the DCIR/DCAR family. Expression of mDCAR1 was strongly tissue dependent. mDCAR1 expression on DCs was restricted to the CD8+ DC subset in spleen and thymus and on subpopulations of CD11b+ 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. The Journal of Immunology, 2009, 183: 5069–5078.

C-Type lectins (CLEC)2 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 APCs and other phagocytes (7). They have been described as Ag uptake receptors for both pathogen-associated molecular patterns and self-Ags. Depending on the CLR-mediated endocytosis mechanisms, internalized Ags are routed into early endosomal or late endosomal/lysosomal compartments for degradation, loaded on MHC class I and II molecules, respectively, which then leads to the regulation of Ag-specific immune responses (3, 8). In addition, APLEC-encoded CLR genes have been associated with susceptibility to autoimmune diseases, such as arthritis (9–11).

Examples for the APLEC-encoded group II CLRs are the recently identified BDCA-2 (CD303) and dendritic cell (DC) immunoreceptor (human DCIR [hDCIR] or CLECSF6). CD303 is expressed exclusively on the cell surface of plasmacytoid DCs (PDC; Ref. 12), whereas hDCIR expression can be detected on monocytes, macrophages, DCs, neutrophils, and B cells (13–16). Both receptors have been shown to induce receptor-mediated endocytosis, leading to modulation of adaptive immune responses (15–17). Moreover, they regulate DC-induced immunity by inhibition of TLR-induced cytokine profiles (15, 16, 18), which might be a possible mechanism for pathogens to evade immune responses (19).

In mice, APLEC-encoded group II CLRs are located on chromosome 6. Among these are the receptors mDCIR, mDCIR2, and mDCAR, which then leads to the regulation of Ag-specific immune responses (3, 8). In addition, APLEC-encoded CLR genes have been associated with susceptibility to autoimmune diseases, such as arthritis (9–11).

Here, we report a novel group II CLR, mDCAR1, which is expressed exclusively on CD8+ DC and subpopulations of myeloid cells in a tissue-dependent manner. We show that Ag delivered via mDCAR1 is presented on both MHC class I and II molecules and that receptor triggering on DC leads to enhanced proinflammatory cytokine responses. As a consequence, targeting mDCAR1 in vivo induced CD8+- and CD4+-proliferative T cell and humoral responses.

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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 online against mouse genome databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification of mouse orthologs. The National Center for Biotechnology Information Nucleotide information accession number entries (http://www.ncbi.nlm.nih.gov/gov) of the sequences used were: CD203 (AF325460); hDCIR (AJ135932); hDCIR (AJ135933); hDCIR2 (AY397673); mDCIR3 (AY397674); mDCIR4 (AY397675); mDCAR (AY230259); and mDCAR1 (AY365133). Amino acid sequence CLRs of the CLR were derived from UniProt Knowledgebase (Swiss-Prot and TrEMBL) entries (http://www.expasy.org) according to their primary accession numbers: CD303 (Q5WT0); hDCIR (Q9UMR7); mDCIR (Q9QZ15); mDCIR2 (Q5YIR8); and mDCIR3 (Q0JX60); mDCIR4 (Q80U17); mDCAR (Q7TS58); and mDCAR1 (Q67DU8). 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 (<0.2 μm pore size) FCS (PAA Laboratories), 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 (all from Invitrogen) at 37°C in a humidified 5% CO2-containing atmosphere. Ab-producing hybridomas were cultured in DMEM supplemented with 10% (v/v) heat-inactivated, filtered (m pore size) FCS (PAA Laboratories), 2 mM L-glutamine, and 20 mM HEPES at 37°C in a humidified 9% CO2-containing atmosphere.

mDCAR1 cloning and transfection
cDNA coding for full-length mDCAR1 was isolated by PCR amplification from whole spleen cDNA using the primers 5′-ggcggatccATGCAGGA-3′ and 5′-gtctgatcATGCGCGCAGAGCACCAGCC-3′ (gene-specific sequence in capital letters; BamHI and EcoRI restriction sites are underlined). The resulting fragment was cloned into a modified pEF6 vector (Invitrogen) containing an HA tag (gene sequence ATGGGCGTACCATATGCCGCTCCAGTAACTAGTATA-3′) between the promoter and the mDCAR1 coding region. As a control, HA-encoding pEF6 vector without mDCAR1 was 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-IF3.3; Miltenyi Biotec). Selection of mDCAR1 cells was performed by administration of 5 μg/ml blastidixin (Invitrogen) and subsequent cloning by limiting dilution.

Generation of specific anti-mDCAR1 mAbs

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 × 10^5 mDCAR1-expressing RBL-1 cells and 150 μg of CpG1826 (Metabolon) per boost. Vector-control transfected RBL-1 cells (5 × 10^6) and CpG were applied at days −3, 0, 4, and 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 CD45R, CD107a (LAMP-1)-Alexa Fluor 647 (eBioscience) or rabbit anti-EEA1 (Abcam) or mouse anti-mDCAR1 mAb (SKa5-3D5) or isotype-matched control mAbs (BD Biosciences) and analyzed using FlowJo software (Tree Star). Gates were set according to fluorochrome-conjugated isotype control staining controls using mAb hamster IgG1 (A19-3), IgG1 (R3-34), IgG2a (R3-95), and 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 (C57BL6-2R1; 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 same concentrations as primary mAbs. 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

Spleenic CD8+ T cells were isolated using a mouse CD8+ DC Isolation Kit (Miltenyi Biotec). Briefly, single-cell suspensions were prepared by depleting CD90+ B cells 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 (CD8α-) and CD4+ (CD8+ T) cells were isolated using either a CD8α+ or CD4+ T 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-mDCAR1 mAb (SKa5-3DS) or 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 and labeled with biotinylated allophycocyanin-mAb (Miltenyi Biotec) for 10 min on ice. Cells were washed with medium and then incubated at 37°C for indicated periods of time. Cross-linked mAb complexes remaining on the cell surface were determined by FACS analysis via labeling with PE-conjugated anti-biotin mAb (Miltenyi Biotec). For analysis of intracellular trafficking of internalized mAb, enriched CD8+ DCs were labeled with biotinylated anti-mDCAR1 mAb (SKa5-3DS), CD205 (NLDC-145), or isotype-matched control IgG2b (A95-1; BD Biosciences) for 30 min on ice followed by streptavidin-Alexa Fluor 488 (Invitrogen) for 20 min on ice and incubated in medium for the indicated time points at 37°C. DCs were fixed in PBS containing 1.8% paraformaldehyde for 20 min and permeabilized with 0.1% saponin for 20 min before staining with CD107a (LAMP-1)-Alexa Fluor 647 (eBioscience) or rabbit anti-EEA1 mAb (Cell Signaling) and anti-rabbit IgG (H + L)-Alexa Fluor 647 (Invitrogen). Cells were centrifuged on coverslips, mounted in VectaShield (Vector Laboratories), and analyzed on a Leica TCS SP-2 CLSM.

Coupling mAb to OVA

DTT-reduced anti-mDCAR1 mAb (SKa5-3DS), CD205 mAb (NLDC-145), and isotype-matched control mAb IgG2b (JES51-S422) (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 Biochemical) 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 (SKa5-3D5) 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+CD8+ (OT-I) and CD4+ (OT-II) 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 applied. Three days later, splenocytes were analyzed with fluorochemical mAb against CD4 (GK1.5) or CD8 (53-6.7; Miltenyi Biotec). In vivo blocking of mDCAR1 internalization was achieved by i.v. injection of 250 μg of anti-mDCAR1 mAb (SKa5-3D5) 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+CD8+ (OT-I) and CD4+ (OT-II) T cells were visualized by loss of CFSE fluorescence.

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 rIgG2b (JES10-S42; Miltenyi Biotec). For stimulation, cultures were incubated on a monolayer of CD40L-expressing 3T3 cells supplemented with 5 μg/ml CpG1668 (Metabion). Supernatants were analyzed via ELISA for IL-12p70, TNF-α, and IL-10. Ab pairs used were 18.2 and 17.8 for IL-12p70 and 1F3FD4 and XT3 for TNF-α (Miltenyi Biotec). BD OptEIA Mouse IL-10 ELISA Set (BD Biosciences) was used to determine IL-10.

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 mDCIR 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-CLEC genes GDF3, SLC2A3, 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 CLR, 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.

FIGURE 1. Comparison of the DCIR/CD303/DCAR family. A, Chromosomal comparison of human and mouse APLEC. The human APLEC contains the BDCA-2 (CD303) and hDCIR genes on chromosome 12 (chr12; top diagram). The corresponding mouse region on chromosome 6 (bottom diagram) is characterized by the absence of a CLR gene homolog to CD303 but contains a cluster of four mDCIR and two mDCAR genes. B, Comparative domain structure analysis of DCIR/CD303/DCAR family members. The members of the DCIR/CD303/DCAR family are predicted type II transmembrane (TM) proteins. Their CTLDs are highly conserved. CD303, mDCAR, and mDCAR1 share a short cytoplasmic domain without any known signaling motifs but a positively charged amino acid. R, arginine; K, lysine. hDCIR, mDCIR, and mDCIR2 cytoplasmic domains contain conserved motifs important for cell signaling (ITIM), whereas mDCIR3 and mDCIR4 lack such signal transduction motifs.

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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, low amounts of CD11b, and the absence of CD4 and mPDCA-1. The 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 sub-sets, 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⁺/low MHC class II high 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 (Flt-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

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1 The online version of this article contains supplemental material.
FIGURE 2. mDCAR1 is expressed in a tissue-dependent manner on CD8<sup>+</sup> 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 mDCAR<sup>+</sup> cells of the CD11c<sup>+</sup> or CD11c<sup>-</sup> 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 (1 μg) 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.

**mDCAR1 triggering influences the cytokine profile of activated CD8 DC**

CLR triggering has been shown to polarize effector function by regulating the cytokine secretion of the cell (28). To address the impact of mDCAR1 cross-linking on the cytokine profile of DCs, CD8 DCs were incubated with anti-mDCAR1 mAb or isotype-matched control mAb in the presence or absence of DC stimuli. Because the use of either CD40L or CpG resulted in a negligible cytokine response, both stimuli were used synergistically (data not shown) (29). After 20 h, supernatants were harvested and secreted IL-12p70, TNF-α, IFN-α, and IL-10 were determined by ELISA. mDCAR1 triggering has no impact on cytokine secretion of DC in steady state (Fig. 5). However, the presence of anti-mDCAR1 mAb significantly increased the level of IL-12p70 secreted by activated DC compared with isotype control or without Ab. The amount of TNF-α was not significantly increased, and no IFN-α could be detected (data not shown) after receptor ligation. Strikingly, CD8 DC produced 35% less IL-10 when mDCAR1 was triggered (769 ± 36 pg/ml) compared with isotype mAb cultured DC (1172 ± 42 pg/ml).

In conclusion, upon triggering mDCAR1, the cytokine profile of activated CD8 DC is biased toward a proinflammatory response.

**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 rIgG2b 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 CLRs 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⁺BP-1⁺Sirpα⁻ 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⁺ DCs are also effective inducers of CD4⁺ T cell responses (36). The expression of CLR on APCs, such as CD8⁺ DCs, 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 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 Kurts (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
(32). Furthermore, the administration of low doses of fluoro-
chrome-conjugated anti-mDCAR1 mAb preferentially labeled CD8⁺ DC rather than myeloid cells indicating that anti-mDCAR1 mAb predominantly targets CD8⁺ DC after i.v. application.

How could the observed immune responses be interpreted that occurred in the absence of CD40 stimulation when Ag was delivered via mDCAR1? The overall theory that Ag presented on steady-state DC induces T cell tolerance, whereas presentation on activated APCs leads to immunity, is supported by experiments in which Ag was targeted to the multilectin receptor CD205 (26, 39, 40). Thereby, tolerance induction by CD8⁺ DCs is accompanied by proliferation of T cells and finally results in deletion of autoreactive T cells (41, 42). Recently, another novel CD8⁺ DC-expressed CLRs, CLEC9A and CLEC12A, have been reported to induce humoral and cellular responses in the absence of activating agents (43, 44), which is contrary to the above-mentioned generalization, but is in line with responses induced after mDCAR1-mediated Ag uptake. Determined via expression levels of costimulatory molecules and MHC class II on the surface of mDCAR1, it was assumed that Ag presented on mDCAR1 cells, triggering mDCAR1 does not result in APC activation. However, receptor triggering and the fact that steady-state CD8⁺ DCs already express increased levels of costimulatory molecules and MHC class II might synergize to influence APC function toward T cell stimulatory capacity. It remains elusive whether Ag internalized via certain CLR may gain unhampered access to the Ag processing and presentation pathways independent on the activation state of the DC.

Independent of Ag uptake, interaction of Ags with CLR reg-
ulate DC-induced immune responses (28). This includes the inhibition of cytokine release by activated DCs, as described for CD303 (18) and hDCIR (15, 16), where mAbs were used to mimic specific interactions between CLR and the putative li-
gands. To elucidate the influence of triggering mDCAR1 on the DC-mediated cytokine response, we analyzed cytokines de-
scribed to be secreted by CD8⁺ DC including IL-12, IL-10, TNF-α, and IFN-α (29). Triggering mDCAR1 on activated DC enhanced the secretion of IL-12p70, whereas IL-10 release was inhibited. This agrees with the findings that the presence of IL-10 negatively influences IL-12p70 release in activated CD8⁺ DCs (29). Thus, inhibition of IL-10 upon mDCAR1 triggering 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 transduc-
tion initiated after receptor triggering. As revealed by in silico protein analysis, mDCAR1 was found to lack such signal trans-
duction motifs. Closely related CLRs, such as CD303, mDCAR, and further Dectin-2 and Mincle, have been reported to signal via an associated ITAM-bearing FcR γ-chain (21, 45–
47). 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.

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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 Ig kappa 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.