Dlgr2, dendritic cell–derived immunoglobulin receptor 2, is one representative of a family of IgSF inhibitory receptors and mediates negative regulation of dendritic cell–initiated antigen-specific T-cell responses

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Dendritic cells (DCs) play critical roles in the initiation and regulation of immune responses. Maturation and activation of DCs are controlled by a balance of the inhibitory and activating signals transduced through distinct surface receptors. Many inhibitory receptors expressed by DCs have been identified, whereas the new members and their functions need further investigation. In this study, we functionally characterized DC-derived immunoglobulin receptor 2 (Dlgr2) as a novel representative of a family of inhibitory receptors belonging to the immunoglobulin superfamily. We show that Dlgr2 contains 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within its cytoplasmic region and that Dlgr2 associates with Src homology-2 domain–containing protein tyrosine phosphatases-1 (SHP-1). Blockade of Dlgr2 on DCs by pretreatment with Dlgr2-Ig fusion protein or by silencing with specific small interfering RNA enhances DC-initiated T-cell proliferation and antigen-specific T-cell responses both in vitro and in vivo. Furthermore, immunization of mice with antigen-pulsed, Dlgr2-silenced DCs elicits more potent antigen-specific CD4+ and CD8+ T-cell responses, thus protecting the vaccinated mice from tumor challenge more effectively. Our data suggest that Dlgr2 is a functionally inhibitory receptor and can mediate negative signaling to regulate DC-initiated antigen-specific T-cell responses.

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Introduction

Dendritic cells (DCs) play critical roles in the initiation of immune responses and induction of tolerance.1 Balanced signaling transmitted via different activating and inhibitory receptors in DCs can regulate the functional status of DCs, thus determining duration and magnitude of T-cell responses.2 Among the inhibitory immune receptor families known to be expressed by DCs, the immunoglobulin superfamily (IgSF) seems to be unique in terms of structural features and roles in the regulation of DC development and function.1 IgSF receptors are mostly type I membrane proteins, characterized by 1 or more immunoglobulin-like domains in the extracellular regions, which are pivotal in cell-cell recognition and interaction. IgSF receptors are abundant in the immune system and are widely distributed in lymphoid and myeloid cells including T cells, B cells, monocytes, DCs, and natural killer (NK) cells. It is well recognized that a vast quantity of IgSF molecules are involved in shaping the synapse formed between T cells and antigen-presenting cells (APCs), regulating various processes ranging from presentation, T-cell costimulation, and cytokine production by DCs. Up-regulation of ILT3/ILT4 by suppressor T cells is responsible for the tolerogeneity of monocytes and DCs.3 Similarly, paired immunoglobulin-like inhibitory receptor B (Pir-B), a murine homologue of ILT4, has also been defined as a key negative regulator of DC functions, and impaired maturation of DCs and imbalanced T helper 1 (Th1) and Th2 immune responses occurred in Pir-B−/− mice.4–6 Despite the fact that many inhibitory receptors belonging to Ig superfamily have been identified, unidentified members expressed by DCs remain to be isolated and investigated, and the identification of novel inhibitory immunoreceptors should further elucidate the sophisticated interactions between DCs and T cells.

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Human CMRF35-A, previously isolated by recognition with the CMRF-35 monoclonal antibody (mAb), is a surface molecule with an IgV-like domain.12 Chromosome mapping and gene database searches have located CMRF35-A on human chromosome 17,13 where CMRF35-A and at least 6 other genes constitute an Ig family. Members of this family are involved in multiple facets of the immune regulation, such as modulation of mast cell activation and regulation of NK cell and eosinophil activity.14-16 Moreover, single nucleotide polymorphisms in this cluster are linked with psoriasis, atopic dermatitis, and psoriatic arthritis, underscoring the importance of CMRF35-A–like molecules in the regulation of immune response and immunologic pathogenesis.17,18

By searching a murine expressed sequence tags (ESTs) database19 with the human CMRF-35-A sequence, we previously identified several novel receptors, one of which is preferentially expressed on APCs including DCs and designated as DC-derived immunoglobulin receptor 1 (DiGr1).20 Here we report the functional identification of another IgSF receptor, designated as DiGr2. We convincingly demonstrate that DiGr2 acts functionally as an inhibitory receptor and mediates negative regulation of DC-initiated T-cell responses, thus outlining a new pathway for immune regulation and providing a new target for immunotherapy.

Materials and methods
Animals and cells
Male C57BL/6 (H-2b) and BALB/c (H-2d) mice were purchased from Shanghai Joint Venture Sippr BK Experimental Animal (Shanghai, China) and maintained in a pathogen-free environment. Ovalbumin (OVA) (323-339)–specific T-cell receptor (TCR)–transgenic mice (DO11.10) were obtained from the Jackson Laboratory (Bar Harbor, ME). CD3- T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells were freshly isolated from mouse splenocytes with CD3, CD4, CD8a, CD19, and NK1.1 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. Peritoneal macrophages were aspirated from peritoneal exudates. Murine bone marrow–derived DCs (BM-DCs) were prepared as described previously.21 The EG7-OV A cell line is derived from the murine lymphoma EL-4 Center for Biotechnology Information (NCBI) mouse EST databases22 to identify homologues to human CMRF-35, 3 mouse ESTs (GenBank accession nos. AA839051, AI391276, and W07987) were found to be full-length cDNA was obtained by contig. Full-length DiGr2 cDNA was obtained from mouse DCs using primers 5'-GGA ATT CTC TCG CTG GCA GCC TC-3' (sense) and 5'-GCT CTA GGA GCC TTC AGA GAC CAA GAT T-3' (antisense), and is available in the GenBank database with accession no. AF211440. The multiple alignments and the phylogenetic analysis were performed with clustalX1.81 software.23

Identification of DiGr2
By Basic Local Alignment Search Tool (BLAST) analysis of National Center for Biotechnology Information (NCBI) mouse EST databases22 to identify homologues to human CMRF-35, 3 mouse ESTs (GenBank accession nos. AA839051, AI391276, and W07987) were found to be highly homologous to human CMRF-35, from which mouse DiGr2 full-length cDNA was obtained by contig. Full-length DiGr2 cDNA was obtained from mouse DCs using primers 5'-GGA ATT CTC TCG CTG GCA GCC TC-3' (sense) and 5'-GCT CTA GGA GCC TTC AGA GAC CAA GAT T-3' (antisense) and is available in the GenBank database with accession no. AF211440. The multiple alignments and the phylogenetic analysis were performed with clustalX1.81 software.23

Analysis of DiGr2 expression pattern
Northern blotting and reverse transcription–polymerase chain reaction (RT-PCR) analysis of DiGr2 expression were performed as described previously.20 Real-time PCR was conducted with the SYBR green I master mix kit (Applied Biosystems, Foster City, CA) as instructed. DiGr2 expression was quantified as a threshold cycle value relative to that of β-actin. Primers used for DiGr2 were 5'-GCA TGT AGC CTG TCG CTG-3' (sense) and 5'-ATC ACC GAC GCC ATC TTC AC-3' (antisense).

Preparation of recombinant GST-DiGr2 fusion protein and generation of anti-DiGr2 polyclonal antibody
The cys region of DiGr2 extracellular region without signal peptide was inserted into pGEX-2T (Pharmacia Biotech, Piscataway, NJ). Purification of the fusion protein and the preparation of polyclonal antibody (Ab) were performed as described previously.20

Eukaryotic expression vector construction and transfection
DiGr2 cDNA was inserted directly, or in frame with Flag tag, into pcDNA3.1/myc-His(-)B vector (Invitrogen, Carlsbad, CA). NIH3T3 cells or L929 cells were transfected with the vectors using PoLyFect transfection reagent (Qiagen, Valencia, CA) as instructed.

Subcellular localization of DiGr2
NIH3T3 cells were transfected with DiGr2 or CD54 (intercellular adhesion molecule [ICAM], positive control) expression vector. Forty-eight hours later, cells were fixed in 4% paraformaldehyde, perforated with 0.1% Triton X-100, incubated with anti-DiGr2 polyclonal Ab or anti-CD54 mAb for 1 hour, and then labeled with fluorescein isothiocyanate (FITC)–conjugated anti-rabbit IgG or anti–mouse IgG. Stained cells were viewed under fluorescence confocal microscopy (Leica, Wetzlar, Germany).

Immunoprecipitation and Western blot assay
L929 cells were transfected with DiGr2-Flag expression vector and total cell lysates were prepared as described.23 For sodium pervanadate stimulation, 1 × 106 cells were pretreated with 0.03% H2O2 and 100 µM Na3VO4 (Sigma, St Louis, MO) for 10 minutes. After preabsorption with protein A agarose (Pierce, Rockford, IL), cell lysates were incubated with anti-Flag M2 agarose beads (Sigma) for 8 hours. Then immunoprecipitated pellets were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and probed with anti–p-Tyr, –SHP-1, or –SHP-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Preparation of DiGr2-Ig fusion protein
The DiGr2-Ig construct was made by fusing the extracellular domain of DiGr2 (residues 1-561), or the extracellular region without the IgV domain (residues 328-561), in-frame with Flag tag, into diethylaminoethanol (DEAE)–dextran, and the DiGr2-Ig fusion protein was purified by chromatography through a protein A column.23 SDS-PAGE and silver staining demonstrated a purity of greater than 90%.

Binding assays
Freshly isolated immune cells were either directly stained with 40 µg/mL DiGr2-Ig fusion protein, Flag, DiGr2-Ig fusion protein without the IgV domain, or GST-DiGr2 (400 µg/mL) mixed with DiGr2-Ig, for 30 minutes on ice. After 3 washes, cells were incubated with FITC-conjugated goat anti–human IgG (Sigma). Stained cells were analyzed on a FACSCaliber (Becton Dickinson, Mountain View, CA).

siRNA preparation and transfection
Day 6 BM-DCs were transfected with 21-bp small interfering RNA (siRNA) oligonucleotides specific for DiGr2 (DiGr2-siRNA: 5'-GAUG-GCGCCUGAUUGGTTT-3') or with the mutated control (DiGr2-mut-siRNA: 5'-GAUGCGCGCUUUAGGTTT-3'), using Geneporter (Genlantis, San Diego, CA). Silencing efficiency was confirmed by both RT-PCR and Western blot.

Adenovirus-mediated RNA interference
Recombinant DiGr2 siRNA or DiGr2-mut-siRNA adenoviruses (AV-DiGr2-siRNA and AV-DiGr2-mut-siRNA) were generated using the pSilencer adenov 1.0-CMV System (Ambion, Austin, TX). For infection, day 6 BM-DCs were plated in 24-well plates (2 × 105 cells/well) in 400 µL
serum-free RPMI 1640 and exposed to adenovirus at different multiplicities of infection (MOIs) for 6 hours, then were washed with PBS and further incubated in fresh DC medium for 2 to 3 days.26

**Allogeneic MLR**

T cells purified from BALB/c splenocytes using nylon wool columns were used as responders (2 × 10^5) and were cocultured with mitomycin-pretreated DCs (2 × 10^5 to 20 × 10^5 well) in the presence of DlgR2-Ig or human Ig (50 μg/mL). Alternatively, siRNA-treated DCs were used as stimulators. A 72-hour mixed lymphocyte reaction (MLR) was performed and the cells were pulsed with 1 μCi (0.037 MBq) [³H] thymidine for the last 18 hours. Radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter (Shelton, CT).

**In vitro assays of antigen-specific T-cell responses**

CD4^+ T cells from DO11.10 × C57BL/6 F1 hybrid mice were used as antigen-specific responders and cocultured for 3 days with siRNA-transfected and OVA (323-339) peptide–pulsed DCs (T/DC ratio = 10:1).21 Proliferation of T cells was analyzed via a [³H]-Tdr incorporation assay. Supernatants were collected 48 hours after initiation of the cocultures for detection of IL-2, IFN-γ, and IL-12p70 levels by enzyme-linked immunosorbent assay (ELISA; R&D Systems). For intracellular staining, brefeldin A (Sigma) was added 6 hours before the end of culture. After staining for CD4 or CD11c, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, San Jose, CA), followed by intracellular staining of IFN-γ or IL-12p70.21,25

**In vivo assays of antigen-specific T-cell proliferation**

OVA (323-339)–specific TCR-transgenic splenic CD4^+ T-cells (5 × 10^5) from DO11.10 × C57BL/6 F1 mice were injected intraperitoneally into F1 (BALB/c × C57BL/6) mice. Twenty-four hours later, 5 × 10^6 DlgR2-siRNA, DlgR2-mut-siRNA transfected, or control DCs, loaded with OVA peptide (323-339) and matured with LPS (100 ng/mL) ex vivo, were transferred intraperitoneally into the same recipients. After 5 days, splenocytes were pooled and double-stained with anti-CD4-FITC and KJ1-26 (BD PharMingen, San Diego, CA).21

**DC immunization and assays of antigen-specific CD8^+ T-cell responses**

Day 5 BM-DCs were transduced with AV-DlgR2-siRNA or AV-DlgR2-mut-siRNA at an MOI of 100 and 1 day later pulsed with OVA and stimulated with LPS (100 ng/mL; Sigma) for 24 hours. Then the prepared DCs (1 × 10^6/mouse) were injected subcutaneously into mice. Fourteen days later, splenocytes were pooled and antigen-specific T-cell responses were analyzed.

To detect OVA-specific CD8^+ T cells, H-2-K^b/OVA tetramer assays were performed as described previously.25,27 Splenocytes from the immunized mice were double-stained with anti-CD8a-FITC and H-2-K^b/ovalbumin-PE (Prolimmune, Oxford, United Kingdom). For intracellular staining of IFN-γ–producing CD8^+ cells, splenocytes from the immunized mice were restimulated in vitro with OVA peptide (SIINFEKL) for 2 to 3 days, and analysis was performed as described.25,27

**CD8^+ cytotoxic T-lymphocyte (CTL) responses** were assessed with a standard 4-hour chromium release assay27,28 following restimulation in vitro with OVA peptide for 6 days. Percentage of lysis was calculated as (cpm experimental release − cpm spontaneous release)/cpm maximal release × 100, where cpm indicates counts per minute.

**Establishment of the tumor model**

C57BL/6 mice were given subcutaneous injections of 1 × 10^6 Ag-loaded, wild-type, or siRNA-treated DCs twice (at weekly intervals). Seven days after the final immunization, mice were given subcutaneous injections of 2 × 10^6 EG7 cells.28,29 Tumor size was monitored daily and measured twice a week. The largest perpendicular diameters of the tumors were measured and the size was recorded as tumor area (mm^2).

**Statistical analysis**

Data are shown as mean ± SD, and statistical significance was determined by the Student’s t test, with P values less than .05 as statistically significant.

**Results**

**DlgR2 is a member of the Ig superfamily containing ITIMs**

By comparing the mouse EST database22 with human CMRF35-A, we identified a mouse homologue of CMRF35-A. A full-length cDNA was isolated from mouse BM-DCs, which contained a single open reading frame encoding a transmembrane protein of 330 amino acids with a predicted molecular mass of 37 kDa (Figure 1A). The amino acid sequence begins with a hydrophobic signal peptide of 21 amino acids followed by an extracellular region composed of one IgV domain. The putative cytoplasmic domain is characterized by the presence of 2 tandem ITIMs spaced by one Tyr-x-x-Met motif, suggesting that an inhibitory signal might be transduced via these motifs. Therefore, this molecule could belong to the expanding Ig-like inhibitory receptor family and was designated as dendritic cell–derived Ig receptor 2 (DlgR2) following previous DIgR1 identification.20 DlgR2 is 96% identical to CLM-150; however, the extracellular region of DlgR2 is 7 residues longer than that of CLM-1. Genomic organization analysis revealed that DlgR2 and CLM-1 are derived from alternatively splicing in 2 different exon/intron junctions, so they may be 2 alternatively splicing variants. DlgR2 shows 48% sequence identity with IgSF13, a novel human inhibitory receptor of the Ig superfamily previously identified by our lab (Sui et al31). It is also closely related to other IgSF members, such as CLM-3 (81% identity) and murine triggering receptors expressed by myeloid cells-1 (TREM-1; 25% identity; Figure 1B-C).

Since DlgR2 contains 2 ITIMs in its cytoplasmic region, we investigated whether DlgR2 could associate with the tyrosine phosphatases SHP-1 and SHP-2. Such association represents a general way for inhibitory receptors to transmit their signals. We treated DlgR2-transfected L929 cells with sodium pervanadate and found that DlgR2 can be phosphorylated on the Tyr residue and could associate with SHP-1 (Figure 1D), indicating that DlgR2 is a structurally inhibitory IgSF receptor with the potential ability to transduce inhibitory signals.

**DlgR2 is a membrane receptor preferentially expressed by APCs**

Northern blot analysis revealed that DlgR2 mRNA was widely distributed as approximately 1.4-kb and/or 3.0-kb transcripts in mouse tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis, with a larger transcript (~4.0 kb) highly regulated by LPS (8-fold increase) and IL-10 (moderate increase) in heart tissues (Figure 2A). RT-PCR demonstrated that DlgR2 mRNA was highly expressed in monocytes (Raw264.7, J774), DCs (XS106), and B cells (A20) but not in T cells (EL-4, CTLL-2, and EG7). This expression pattern was further confirmed by analysis of freshly isolated primary cells (Figure 2B). Moreover, DlgR2 was also abundantly expressed by BM-DCs, and the level increased during culture of DCs. We found that DlgR2 is up-regulated by LPS (8-fold increase) and IL-10 (moderate increase) but is minimally up-regulated following CD40L stimulation (Figure 2C), indicating that DlgR2 might be involved in DC differentiation and activation.
To define the biochemical characteristics of DlgR2, we carried out immunoprecipitations from DlgR2-transfected NIH3T3 cells and detected a prominent band of 60 kDa under both nonreducing and reducing conditions, indicating that the mature protein did not form a disulfide-linked dimer despite several cysteine residues within its extracellular region. The discrepancy between the apparent molecular mass (60 kDa) and deduced mass (37 kDa) could result from glycosylation of the N-linked glycosylation sites in the extracellular region of DlgR2, because the molecular mass was reduced to approximately 37 kDa after PNGase treatment (Figure 2D). Furthermore, we stained DlgR2-transfected NIH3T3 cells with anti-DlgR2 Ab and found DlgR2 expressed as a surface molecule on the cells (Figure 2E), consistent with its predicted protein structure.

DlgR2 binds with its ligand on T cells and inhibits DC-induced T-cell proliferation in vitro

The preferential expression of DlgR2 on APCs, especially inducible expression on DCs, suggested the possible involvement of DlgR2 in APC/T-cell interactions and possibly in immune responses. We examined lymphocyte and myeloid cell subsets for the presence of putative ligand(s) of DlgR2 and demonstrated that the DlgR2-Ig fusion protein bound strongly to CD3+ T cells (Figure 3A), whereas no significant binding was detected to B cells, NK cells, macrophages, or DCs. Further fractionation of CD4+ and CD8+ T cells showed that DlgR2-Ig fusion protein bound strongly to CD4+ T cells and faintly to CD8+ T cells (about 8%; Figure 3B). The specificity of binding to CD3+ T cells was confirmed by the observation that human immunoglobulin (hIg) did not bind to CD3+ T cells and excessive GST-DlgR2 fusion protein could abolish the binding of DlgR2-Ig to T cells (Figure 3A). Additionally, DlgR2-Ig fusion protein lacking the IgV region showed no binding to T cells, indicating that the IgV domain of DlgR2 was necessary for the observed binding (data not shown).

To further explore this possibility, we added excess DlgR2-Ig fusion protein into the allogenic MLR to interfere with and block the putative interaction between DCs and T cells via DlgR2. We found that proliferation of T cells was significantly up-regulated or increased by DCs when DlgR2 was blocked with soluble DlgR2-Ig, whereas no increase was seen in the presence of hIg (Figure 3C). To exclude the direct effects of DlgR2-Ig on T cells, a small interfering RNA (siRNA) was also used to specifically block DlgR2 expression on DCs. The levels of DlgR2 in DlgR2-siRNA-transfected DCs were decreased about 60% 48 hours after transfection compared with DlgR2-mut-siRNA-DCs, and an approximate 50% reduction could even be observed 3 days after transfection (Figure 3D). Additionally, DlgR2-Ig fusion protein did not bind to CD3+ T cells and excessive GST-DlgR2 fusion protein could abolish the binding of DlgR2-Ig to T cells (Figure 3A). Additionally, DlgR2-Ig fusion protein lacking the IgV region showed no binding to T cells, indicating that the IgV domain of DlgR2 was necessary for the observed binding (data not shown).

Therefore, it is reasonable to speculate that putative ligand(s) for DlgR2 exists on T cells and that DlgR2 might be involved in DC–T-cell interactions.

DlgR2 is a member of the Ig superfamily containing ITIMs.

(A) Sequences of nucleotides and deduced amino acids of DlgR2. Lines indicate signal and transmembrane sequence. The ITIM is boxed, and circled cysteines in the stalk region may allow homodimerization/heterodimerization through disulfide bridge. (B) Alignment of the amino acid sequence of DlgR2 with the most homologous human or mouse sequences. Identical amino acids are outlined in black and conservative substitutions are shaded. (C) Phylogenetic tree of the IgV of murine DlgR2 and related human and mouse sequences. (D) DlgR2 associates with SHP-1 in pervanadate-treated cells. L929 cells transiently transfected with DlgR2/Flag expression vector were pretreated with or without pervanadate (PV), then digitonin lysates of the cells were incubated with anti-Flag M2-agarose beads, and precipitates were subjected to Western blot (WB) analysis with antiphosphotyrosine (anti-pY; left) or anti–SHP-1 (right). Data represent 1 of 3 independent experiments.
DLgR2 negatively regulates DC-initiated antigen-specific T-cell responses both in vitro and in vivo

To further investigate the role of DLgR2 in the antigen-specific immune response, we used CD4+ T cells purified from DO11.10 x C57BL/6 F1 mice as responders and DLgR2-siRNA-DCs, DLgR2-mut-siRNA-DCs, or control DCs from C57 BL/6xBalb/c F1 mice as stimulators in an MLR assay. After 3 days, DLgR2-silenced DCs showed a more than 50% increase in their ability to promote Ag-specific responses, as determined by the increased proliferation of DC-stimulated DO11.10 T cells (Figure 4A). More importantly, DLgR2-siRNA-DCs stimulated DO11.10 T cells to produce significantly higher levels of IFN-γ (by ELISA assay and intracellular staining, P < .01; Figure 4B-C). IL-2 levels produced by DLgR2-siRNA-DC-stimulated DO11.10 T cells were also significantly increased (P < .05; Figure 4B).

The data described established a critical role of DLgR2 in the fine-tuning of immune responses. It was important to determine how DLgR2 executes its effects on the immune response and if the engagement of DLgR2 with putative ligand(s) on T cells delivers a signal to regulate DC function. When the phenotype and cytokine production of DLgR2-silenced DCs were compared with those of control DCs, we found that the percentage of CD11c+ DCs expressing intracellular IL-12p70 was increased from 14.7% (DCs) and 13.7% (DLgR2-mut-siRNA-DCs) to 32.1% (DLgR2-siRNA-DC; Figure 4D), whereas the percentage of IL-10–positive DCs was only slightly increased in similar conditions (data not shown). A significant increase in IL-12 production by DLgR2-siRNA-DCs was also confirmed by ELISA (Figure 4E). In view of the key role of IL-12 in enhancing DCs’ ability to stimulate T cells to produce IFN-γ, the increased IL-12 production by DLgR2-silenced DCs may be responsible, at least in part, for subsequent increased T-cell proliferation and enhanced IFN-γ production.

We also tested the ability of DLgR2-silenced DCs to prime an Ag-specific response in vivo. DO11.10 T cells were preinjected into recipient mice, and 1 day later peptide-pulsed and DLgR2-silenced DCs were adoptively transferred into the same recipients. Increased proliferation of antigen-specific CD4+ T cells stimulated by DLgR2-siRNA-DCs was evidenced by the increased frequency of DO11.10 (KJ1-26+) T cells in the spleen of mice that had received DLgR2-siRNA-DCs (Figure 4F). This provided further in vivo evidence that DLgR2 signaling could negatively regulate the ability of DCs to prime Ag-specific T-cell responses.

Blockade of DLgR2 expression in DCs promotes DC-initiated antigen-specific Th1 and CTL responses in vivo

To better understand the contribution of DLgR2 in the negative regulation of DC-initiated T-cell responses in vivo, we also evaluated the immune response elicited by immunization with recombinant DLgR2-siRNA adenovirus-infected DCs (AV-DLgR2-siRNA). As assessed by real-time quantitative PCR, levels of DLgR2 decreased in an adenovirus particle–dependent manner in DCs transduced with AV-DLgR2-siRNA. We chose 100 MOIs in subsequent studies because levels of DLgR2 can be reduced by more than 60% 48 hours after infection, whereas the viability and morphology of DCs were minimally affected (data not shown).

We then immunized C57BL/6 mice with DLgR2-silenced, OVA protein–pulsed, and LPS-matured DCs. Two weeks later, splenocytes from immunized mice were collected and used to assess the Ag-specific immunity. Results showed that more IFN-γ was produced when splenocytes from AV-DLgR2-siRNA mice were restimulated with OVA 17 peptide (323–339) compared with that produced in AV-DLgR2-mut-siRNA mice and control mice (Figure 5A). These data indicate that AV-DLgR2-siRNA-DC immunization induced more potent Th1 responses. Additionally, tetramer staining...
the percentage of IFN-\(\gamma\) was analyzed by fluorescence-activated cell sorting (FACS). CD3

CD6 with 40 ng/mL DIgR2-Ig or hIg (CD3

conventionally. Purity of each cell subset exceeds more than 90%. Cells were stained

isolated from peritoneal exudates, and bone marrow–derived DCs were prepared

immunity in vivo has been seen to be far less satisfactory and

antigen-specific CTLs; however, their efficacy in inducing anti-
tumor effects in vivo

Blockade of DIgR2 expression in DCs promotes DC vaccines to

induce antitumor effects in vivo

Immunization with antigen-pulsed DCs can effectively activate

antigen-specific CTLs; however, their efficacy in inducing antitu-

morrine was not as efficient. A detailed analysis of the data shows

that the percentage of CD8\(^+\) T cells was positive for OVA tetramer in

DCs, whereas only 1.74% and 1.47% of CD8\(^+\) T cells were positive for OVA tetramer in

DCs, indicating a significant difference. Further studies are needed to

understand the mechanisms behind these differences.

**Discussion**

Positive or negative regulation of T-cell immune responses by DCs depends on specialized DC subsets and on their maturation or activation status. Although DC subsets or status may be preprogrammed to direct either tolerance or immunity, accumulating evidence demonstrates that integration of inhibitory and activating signals in DCs can instruct DC differentiation, matura-
tion, and activation and result in complete flexibility of a basic

program. The balance between divergent receptors establishes a

threshold of DC activation and allows for homeostasis between

induction of tolerance or immunity, whereas interference with

unilateral signaling always results in an altered balance of signals

on DCs, which in turn affects the maturation and function of the

DCs themselves. It is worth noting that positive and negative

receptor pairs frequently exist together in the closely linked

clusters in the IgSF, such as activating versus inhibitory IgG Fc

receptors: PIR-A versus PIR-B receptors and signal regulatory

protein-\(\alpha\) (SIRP-\(\alpha\)) versus SIRP-\(\beta\) receptors. Here we have characterized

DIgR2, a novel IgSF receptor containing functional

ITIMs, that is preferentially expressed by DCs and can mediate

negative regulation of DC-initiated T-cell responses both in vitro

and in vivo. Given the similarity in extracellular structure, contrast-

ing with completely different motifs in cytoplasm, between DIgR2,

DIgR1, and other CLMs, it is interesting to speculate that DIgR2,

together with its activating isofrom, is required to enable immuno-

logic equilibrium under physiologic conditions. Our identification

of a novel ITIM-containing IgSF member and the observations that

this molecule can negatively regulate DC function and then

subsequent T-cell responses demonstrate a novel molecular path-

way for the modulation of immune responses.

Interestingly, the Ig domain of DIgR2 is related to another

recently identified Ig family, TREM, that includes several activat-

ing and inhibitory molecules encoded by a group of clustered

gens. Members of TREM have been identified as key regulators

in the immune system and beyond. For example, TREM-1 appears

to be an amplifier of acute inflammation, as engagement of

TREM-1 with agonist mAbs in granulocytes and monocytes can

stimulate the production of proinflammatory chemokines and

cytokines. TREM-2 may play important roles in development and

function of DCs, as suggested by the observation that ligation of

TREM-2 on immature DCs can induce partial maturation and that

inefficient differentiation of myeloid precursors of DCs was

showed that 4.96% of total CD8\(^+\) T cells were positive for OVA
tetramer in AV-DlgR2-siRNA-DCs mice, whereas only 1.74% and 1.47% of CD8\(^+\) T cells were positive for OVA tetramer in AV-DlgR2-
mut-siRNA mice or mock mice, respectively (Figure 5B). These

data indicate that immunization with DIgR2-silenced DCs resulted

in augmented specific CD8\(^+\) CTL responses. The functional status

of CD8\(^+\) T cells was further evaluated by intracellular staining of

IFN-\(\gamma\) production and cytotoxicity against OVA\(^+\) target cells. Both

the percentage of IFN-\(\gamma\)-producing CD8\(^+\) cells (Figure 5C) and the cytotoxicity

of splenocytes against OVA-specific target EQ7.OVA cells

(Figure 5D) were significantly increased in mice immunized

with DIgR2-silenced DCs compared with those of the mice immunized

with control DCs or AV-DlgR2-mut-siRNA-DCs.

**Figure 3.** DIgR2 binds with its unknown ligand expressed by T cells and inhibits

DC-induced T-cell proliferation in vitro. The binding of DIgR2-Ig fusion protein to

CD3\(^+\) T cells (A), CD4\(^+\) T cells, CD8\(^+\) T cells, B cells, NK cells, monocytes, and DCs

(B) was analyzed by fluorescence-activated cell sorting (FACS). CD3\(^+\), CD4\(^+\), and

CD6\(^+\) T cells, B cells, and NK cells were freshly isolated from mouse splenocytes with

CD3, CD4, CD8a, CD19, and NK1.1 MicroBeads, respectively. Macrophages were

isolated from peritoneal exudates, and bone marrow–derived DCs were prepared

conventionally. Purity of each cell subset exceeds more than 90%. Cells were stained

with 40 ng/mL DIgR2-Ig or hIg (CD3\(^+\) T cells were also stained with DIgR2-Ig mixed

with 400 ng/mL GST-DlgR2) and then stained with FITC-conjugated sheep anti-

human IgG (Sigma). Data shown are representative of 3 independent experiments.

(C) Mixed lymphocyte reaction (MLR) of DCs from C57BL/6 mice incubated with

splenocytes from Balb/c mice, with 50 \(\mu\)g/mL DIgR2-Ig or hlg added into the

coculture. Data represent the mean (\(\pm\) SE) of [\(\text{H}\)] thymidine uptake in 3 independent

experiments. (D) Analysis of DIgR2 levels in siRNA-transfected DCs. Day 6 BM-DCs

were transfected with synthetic DIgR2 siRNA, siRNA mutant duplexes, or mock.

Expression of DIgR2 was determined in triplicate after stimulation with LPS (100

ng/mL) by Western blotting 48 hours after transfection (bottom panel), and DIgR2

mRNA was assessed by RT-PCR on different days after transfection (top panel). Data

shown are representative of 3 independent experiments. (E) MLR was conducted as

described. DCs were transfected with DIgR2-siRNA, DIgR2-mut-siRNA, or mock

before coculturing with splenocytes. Data represent the mean (\(\pm\) SE) of [\(\text{H}\)]

thymidine uptake in 3 independent experiments.

needs further improvement. We investigated whether blockade of

DIgR2-mediated inhibitory signals in DCs could promote Ag-
specific immunity and protect mice from tumor challenge. C57BL/6 mice

preimmunized twice with OVA-pulsed, DIgR2-silenced DC

vaccines were inoculated with EG7.OVA lymphoma cells one week after the

final immunization. Both the nontransduced DC vaccines and the

DlgR2-mut-siRNA-DC vaccines could inhibit tumor growth, as demonstrated by

smaller tumor size 24 days after tumor inoculation (14.1 \(\pm\) 2.3 mm and 12.8 \(\pm\) 2.8 mm, respectively) compared with mice treated with PBS (21.7 \(\pm\) 4.1 mm); DIgR2-

siRNA-DC vaccine was significantly more potent, resulting in

superior suppression of tumor growth (3.6 \(\pm\) 0.3 mm; Figure 6A). Moreover, 60% of the mice immunized with DIgR2-siRNA-DCs remained tumor free 60 days after tumor inoculation, whereas only

20% of the mice immunized with DIgR2-mut-siRNA-DCs or

control DCs remained tumor free (Figure 6B).
found in TREM-2–deficient people. In view of the close relationship between DIgR2 and related Ig families (CMRF35, TREM, etc), it is reasonable to speculate that DIgR2, like other related Ig family receptors, can regulate DC-initiated T-cell responses, which is convincingly supported by data presented here.

Unavailability of the natural ligands often makes it difficult to directly explore functions of “orphan receptors.” However, emerging approaches of silencing genes provide promising alternatives for investigations into the functions of novel receptors. Soluble recombinant Fc-tagged fusion proteins or specific mAbs have been widely used as antagonists to block surface molecules to study function of CD200, TREM-1, PD-1, and so forth. siRNA technology is another powerful approach to investigate the function of the gene of interest in specific cells, including primary cells such as DCs.

Figure 4. DIgR2 negatively regulates the ability of DCs to prime Ag-specific T-cell responses. siRNA-transduced DCs were pulsed with OVA-II peptide (323-329), matured with LPS stimulation, and then cocultured with DO11.10 T cells. (A) Proliferation of T cells. (B) Cytokine production by T cells. (C) Percentages of IFN-γ+ T cells in the gated CD4+ T cells quantitated by double-staining of IFN-γ and CD4, with Brefeldin A present in the coculture. (D) Percentages of IL-12p70+ DCs in the gated CD11c+ DCs quantitated by double-staining of IL-12 and CD11c. (E) IL-12p70 production quantitated by ELISA analysis. (F) Reduced ability of DIgR2 siRNA-DCs to prime Ag-specific T-cell responses in vivo. DO11.10 T cells were transferred, together with OVA-pulsed DCs one day later, into recipient mice; after 5 days, collected splenocytes were harvested and double-stained with CD4-FITC and KJ1-26–PE for flow cytometry. The numbers in CD4-gated plots indicate percentage of DO11.10 cells (KJ1-26+) among total CD4+ T cells. Data represent 1 of at least 3 experiments with similar results. *P < .01 and #P < .05 versus DIgR2-mut-siRNA-DCs. Error bars indicate SE.

Figure 5. Induction of more potent Th1 and CTL responses by immunization with DIgR2 siRNA-DCs. C57BL/6 mice were immunized once with DIgR2-siRNA adenovirus-infected DCs that were pulsed with ovalbumin proteins and ex vivo matured with LPS (100 ng/mL) for 24 hours before immunization (1 x 10^6 DCs per mouse). Two weeks later, splenocytes were pooled from 3 mice from each group. (A) IFN-γ production by CD4+ T cells after pooled splenocytes were restimulated with OVA-II peptide (1 mg/mL) for 48 hours. (B) Percentages of H2-Kb/OVA tetramer+ in the total gated CD8+ T-cell population. (C) Percentages of IFN-γ+ T cells in the total gated CD8+ T-cell population when pooled splenocytes were restimulated with OVA-I peptide (10 mg/mL) for 3 days. (D) Cytotoxicity against OVA+ EG7 (left) or OVA+ EL4 (right) target cells by splenocytes restimulated in vitro with OVA-I peptide (10 mg/mL) for 6 days in the presence of 50 U/mL IL-2. E/T indicates effector:target ratio. Data shown are representative of 3 independent experiments. *P < .01 versus AV-DIgR2-mut-siRNA-DCs. Error bars indicate SE.
Despite the data showing that DIgR2 contains functional ITIMs that can associate with the tyrosine phosphatase SHP-1 and that interference with DIgR2 on DCs has a negative influence on T-cell responses, we do not yet fully understand the mechanisms of inhibitory signaling transduced through DIgR2 on DCs. To clarify some of the underlying mechanisms of DIgR2 signaling in the DC–T-cell interaction, we explored if interference with DIgR2 on DCs can affect the maturation and function of DCs. The most pronounced change after blocking DIgR2 expression was a remarkable increase in IL-12 production. Considering that IL-12 produced by DCs plays a crucial role in triggering IFN-γ production and in Th1 polarization of T cells, it seems that restriction of IL-12 production by DIgR2 signaling in DCs might be an important mechanism for immune regulation. As uncontrolled IL-12 production and responsiveness can result in some organ-specific autoimmune diseases, it is urgent that potent negative regulatory feedback mechanisms for IL-12 production are investigated. Because IL-12 is significantly down-regulated by DIgR2 signaling in DCs and DIgR2 is itself rapidly induced upon LPS stimulation, it is conceivable that up-regulation of DIgR2 by LPS is a part of a feedback circuit of LPS signaling, thus providing an explanation to the puzzling observation that DCs can produce IL-12 upon LPS stimulation only transiently. Further studies are needed to define the cross-talk between DIgR2 signaling and other signaling, such as TLR signaling and CD40L signaling in DCs, in order to better understand the precise mechanisms of DIgR2 in immune regulation and immunologic pathogenesis.

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References

DLgR2, dendritic cell-derived immunoglobulin receptor 2, is one representative of a family of IgSF inhibitory receptors and mediates negative regulation of dendritic cell-initiated antigen-specific T-cell responses

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