

Dendritic cell immunoreceptor: A novel receptor for intravenous immunoglobulin mediates induction of regulatory T cells

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Background: Intravenous immunoglobulin (IVIg) is a polyclonal IgG preparation with potent immunomodulating properties. Our laboratory demonstrated that IVIg significantly increases numbers of forkhead box protein 3-positive regulatory T (Treg) cells through generation of tolerogenic dendritic cells (DCs) in an allergic airways disease model. **Objective:** We sought to investigate potential receptors on DCs mediating these events.

Methods: C57BL/6 mice were either sensitized to ovalbumin (OVA) intraperitoneally or through adoptive transfer of OVA-primed DCs and then challenged with intranasal OVA. IVIg was fractionated into sialic acid-enriched IVIg (SA-IVIg) and sialic acid-depleted IVIg (non-SA-IVIg). Dendritic cell immunoreceptor (DCIR) constructs in CHO cells or on DCs were examined by using fluorescent microscopy and flow cytometry. **Results:** Administration of SA-IVIg, but not non-SA-IVIg, to OVA-sensitized and OVA-challenged mice induced Treg cells and attenuated airway hyperresponsiveness (AHR) and inflammation comparably with IVIg. Bone marrow-derived dendritic cells cultured with SA-IVIg or IVIg adoptively transferred to mice before OVA challenge induced Treg cells and inhibited AHR. IVIg-treated bone marrow-derived dendritic cells from Fcγ receptor knockout mice inhibited AHR, suggesting IVIg's action was not caused by Fcγ receptor-

mediated events. Fluorescently labeled IVIg or SA-IVIg bound DCs and colocalized specifically to the C-type lectin DCIR. IVIg binding to DCIR induced phosphorylation of Src homology domain 2-containing protein tyrosine phosphatase (SHP) 2 and Src homology domain 2-containing inositol phosphatase 1 (SHIP-1) and internalization of IVIg into DCs. Inhibition of IVIg binding to DCIR by small interfering RNA completely blocked induction of Treg cells. Inhibition of SHP-2 or abrogation of IgG internalization through clathrin inhibitors rendered IVIg ineffective.

Conclusions: IVIg alleviates allergic airways disease through interaction of SA-IgG with DCIR. DCIR is a novel receptor for IVIg, mediating interaction of innate and adaptive immunity in tolerogenic responses. (*J Allergy Clin Immunol* 2013;■■■■:■■■-■■■.)

Key words: Intravenous immunoglobulin, dendritic cells, regulatory T cells, immune modulation, asthma

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Intravenous immunoglobulin (IVIg), a preparation of pooled human polyclonal IgG, is widely used as a treatment for primary immune deficiency and increasingly is used as immunomodulatory therapy for patients with autoimmune and inflammatory diseases. IgG molecules are glycosylated with various oligosaccharides, endowing them with distinctive properties, including the ability to bind to innate immune receptors.¹ These properties have led to multiple proposed mechanisms for the immunomodulatory actions of IVIg, including blockade of Fcγ receptors (FcγRs), upregulation of inhibitory FcγR (FcγRIIB) on macrophages, modulation of cytokine secretion, and inhibition of dendritic cell (DC) activity.^{2,3}

Using a robust antigen-driven murine model of allergic airway disease, we demonstrated that IVIg markedly improved ovalbumin (OVA)-induced airway hyperresponsiveness (AHR) associated with induction of regulatory T (Treg) cells from non-Treg cells in pulmonary tissues.⁴ This mechanism was dependent on CD11c⁺ DCs because IVIg infusion could be replaced by adoptive transfer of CD11c⁺ DCs from IVIg-treated mice into OVA-sensitized and OVA-challenged recipients. IVIg can modulate DC maturation and promotes IL-10 production by DCs^{4,6}; however, it is unknown how IVIg interacts with DCs to confer tolerance.

The dose of IVIg required for immune modulation is 3 to 4 times that of immune supplementation, suggesting a minor component of polyclonal IgG is responsible for these effects. Anthony et al⁷ and Kaneko et al⁸ determined that 2-6 sialic acid linkages on the Fc region of IgG mediated immune regulation in murine models of serum-induced arthritis and immune thrombocytopenic purpura. In these models sialylated IgG interacted with SIGN-R1 (the murine ortholog of DC-SIGN), a lectin receptor expressed on splenic marginal zone macrophages.

Abbreviations used

AAD:	Allergic airways disease
AHR:	Airway hyperresponsiveness
BMDDC:	Bone marrow–derived dendritic cell
DC:	Dendritic cell
DCIR:	Dendritic cell immunoreceptor
FcγR:	Fcγ receptor
FcγR-KO mice:	FcγR chain–deficient mice
Foxp3:	Forkhead box protein 3
GFP:	Green fluorescent protein
HSA:	Human serum albumin
ITIM:	Immunoreceptor tyrosine-based inhibition motif
IVIg:	Intravenous immunoglobulin
KD-DC:	Knock-down dendritic cell
MCh:	Methacholine
non-SA-IVIg:	Sialic acid–depleted intravenous immunoglobulin
OVA:	Ovalbumin
SA-IVIg:	Sialic acid–enriched intravenous immunoglobulin
SHIP:	Src homology domain 2-containing inositol phosphatase
SHP:	Src homology domain 2-containing protein tyrosine phosphatase
Treg:	Regulatory T
WT:	Wild-type
WT-DC:	Wild-type bone marrow–derived dendritic cell

Seite et al⁹ also demonstrated that sialic acid–enriched intravenous immunoglobulin (SA-IVIg) binds to a lectin receptor, CD22 (Siglec-2), promoting apoptosis in B cells.

In this study we explored the interaction of IVIg with CD11c⁺ DCs. We demonstrate a requirement for IgG sialylation in induction of tolerogenic DCs and identify a novel receptor for sialylated IgG, dendritic cell immunoreceptor (DCIR), an immunoreceptor tyrosine-based inhibition motif (ITIM)–linked C-type lectin receptor that was required for induction of tolerogenic DCs. Ligation of DCIR by sialylated IgG is a crucial step for induction of Treg cells and abrogation of inflammation by IVIg.

METHODS

Fractionation of IVIg

Fractionation of IVIg was performed by using *Sambucus nigra* agglutinin–lectin affinity columns, according to the manufacturer's protocol. IVIg (Talecris Biotherapeutics, Mississauga, Ontario, Canada) was loaded onto a 2-mL *Sambucus nigra* agglutinin column. The flow-through fraction (sialic acid–depleted intravenous immunoglobulin [non-SA-IVIg]) was collected by washing the column with 10 mL of Tris-buffered saline. The SA-IVIg fraction was eluted by using 4 mL of 0.5 mol/L lactose. The non-SA-IVIg fraction was used as is or further treated with neuraminidase (Sigma-Aldrich, Oakville, Ontario, Canada) to desialylate IgG; both produced similar results.

Induction of allergic airway inflammation

C57BL/6 wild-type (WT) and FcγR chain–deficient (FcγR-KO) mice were purchased from Jackson Laboratories (Bar Harbor, Me). Mice were sensitized intraperitoneally with 100 μg of OVA (Sigma-Aldrich) in 400 μg of AL(OH)₃ on days 0 and 14, followed by intranasal OVA challenges from days 29 to 33 (20 μL × 10 mg/mL OVA). One day before antigen challenge, IVIg (2 g/kg), SA-IVIg (0.1 g/kg), non-SA-IVIg (2 g/kg), or 2 g/kg 3% human serum albumin (HSA; Talecris Biotherapeutics) was administered to mice intraperitoneally.

Alternatively,¹⁰ 1×10^6 bone marrow–derived dendritic cells (BMDDCs) were primed *in vitro* with OVA (1 mg/mL) for 3 hours, and then IVIg (10 mg/mL), SA-IVIg (0.5 mg/mL), non-SA-IVIg (10 mg/mL), or HSA was added for 18 hours. After 3 washes, conditioned DCs were instilled intratracheally into naive mice, followed by 3 days of intranasal OVA challenges (days 5–8). Analysis was performed 24 hours after the final challenge.¹⁰

AHR to methacholine

AHR was measured, as previously described.⁶ Mice were exposed to doubling concentrations of aerosolized methacholine (MCh; 16–256 mg/mL, Sigma-Aldrich) by using a FlexiVent small-animal ventilator (Scireq, Montreal, Quebec, Canada). The relative peak response at each MCh dose compared with the saline baseline was calculated.¹¹

Preparation of cell suspensions from lung tissues

Lungs were removed, minced, and incubated for 45 minutes at 37°C in 3 mL of collagenase (Sigma-Aldrich) in 0.5 mmol/L Ca²⁺ PBS and passed through a 40-μm strainer. Single-cell suspensions were resuspended in complete medium consisting of RPMI-1640 (Invitrogen, Burlington, Ontario, Canada) plus 10% FCS, L-glutamine, penicillin/streptomycin, and HEPES (Invitrogen).

Cytokine detection

Intracellular cytokine staining was performed on mononuclear cells from lung digests stimulated for 6 hours with phorbol-12-13-dibutyrate (5 ng/mL) and ionomycin (50 ng/mL, Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences, San Jose, Calif). Cells were permeabilized and stained with anti-IFN-γ–allophycocyanin, anti-IL-4–phycoerythrin, or anti-IL-17–PerCP5.5 (BD Biosciences) for 30 minutes at 4°C. For detection of Treg cells, cells were first stained with anti-CD4–fluorescein isothiocyanate and anti-CD25–phycoerythrin, followed by permeabilization and staining with anti-forkhead box protein 3 (Foxp3)–allophycocyanin (BD Biosciences) and analyzed on an LSR-II flow cytometer. IL-4 and IL-17 levels were measured by using ELISA (eBioscience, San Diego, Calif).

Differentiation of BMDDCs

BMDDCs were generated from bone marrow cells of WT C57BL/6 or FcγR-KO mice, as previously described.¹² Bone marrow cells from femurs ($2 \times 10^6/100$ mm Petri dish) were cultured in complete medium supplemented with 10 ng/mL recombinant murine GM-CSF with or without IL-4 (2.5 ng/mL; PeproTech, Montreal, Quebec, Canada). On days 3, 5, 7, and 9, 10 mL of medium was changed, and nonadherent DCs were harvested at day 10. Purity of DCs was verified by using flow cytometry and was greater than 90%, as determined by using CD11c⁺F480⁺ staining.

DCIR transfection

The DCIR-expressing pCMV6-AC vector containing a neomycin resistance gene (OriGene, Rockville, Md) was transfected into CHO cells with Lipofectamine (Invitrogen). CHO cells expressing DCIR were screened by adding neomycin to cultured cells 48 hours after transfection.

Colocalization of DCIR and IgG

BMDDCs from WT or FcγR-KO mice were incubated with Alexa Fluor 488–conjugated SA-IVIg or of Alexa Fluor 488–conjugated non-SA-IVIg (10 mg/mL), and Alexa Fluor 555–conjugated anti-DCIR antibody (BioLegend, Burlington, Ontario, Canada) at 4°C or 37°C for the indicated times in the presence of Fc block (BioLegend). Inhibition of clathrin-mediated internalization was performed with Dynasore

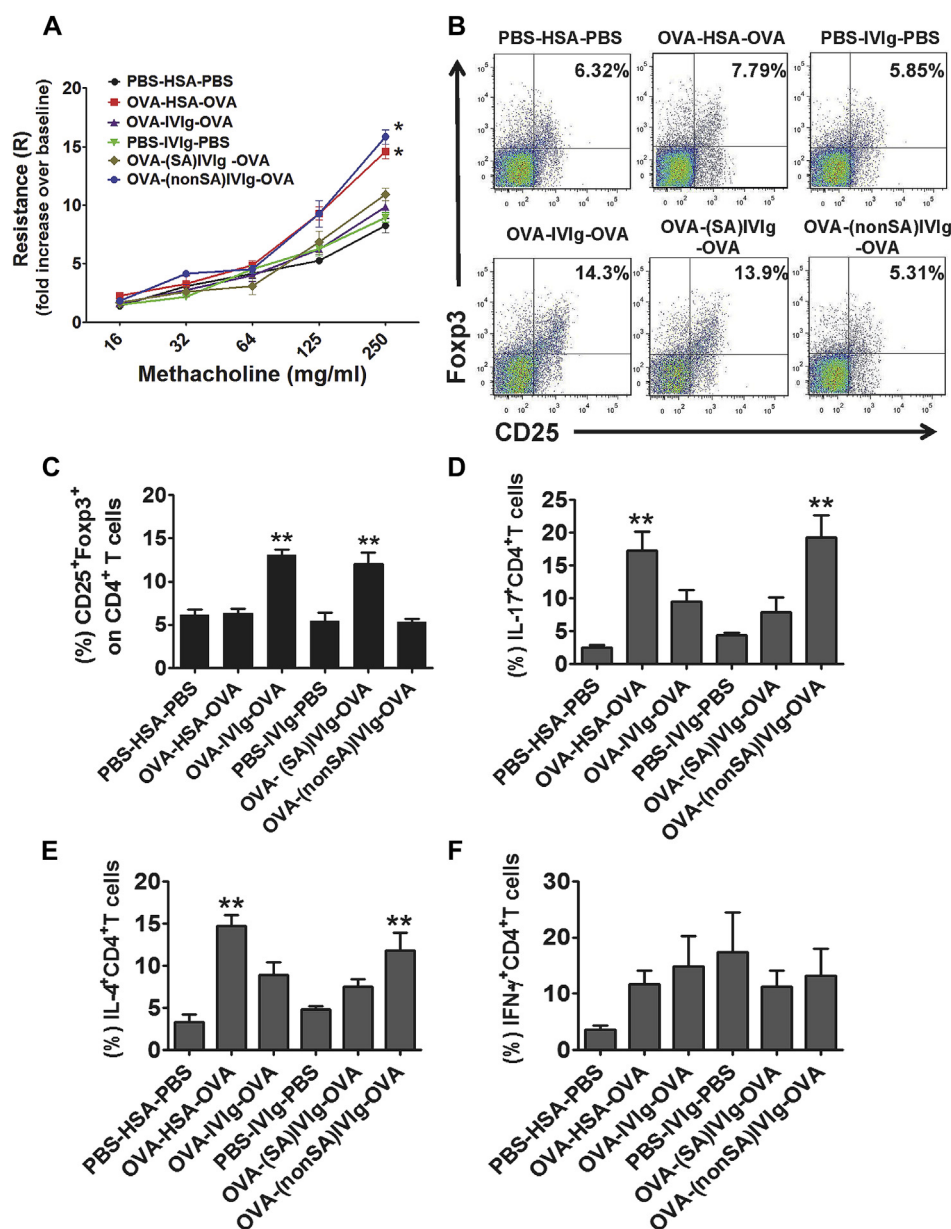


FIG 1. Sialylated IgG abrogates AAD. **A**, IVIg and SA-IVIg, but not non-SA-IVIg, attenuated MCh-induced AHR. **B** and **C**, Representative flow cytometric analysis and cumulative data demonstrating the frequency of pulmonary CD25⁺Foxp3⁺ Treg cells on CD4⁺ T cells. **D-F**, Frequency of pulmonary IL-4, IL-17, and IFN-γ-producing CD4⁺ T cells. Data represent means ± SDs from 3 independent experiments (n = 9). *P < .05 and **P < .01 versus controls.

(100 μmol/L in 0.02 dimethyl sulfoxide per 10⁶ cells, Sigma-Aldrich). Colocalization and internalization of DCIR/IgG was visualized by means of fluorescent microscopy at ×400 magnification or by means of flow cytometry.

DCIR gene silencing by small interfering RNA

The CLEC4a2-Mouse (DCIR) siGENOME small interfering RNA (siRNA; Thermo Scientific, Ottawa, Ontario, Canada) or scrambled siRNA was incubated with BMDDCs (50 nmol/L siRNA/10⁶ cells) at 37°C for 24 hours. A nontargeting green fluorescent protein (GFP)-conjugated siRNA was used to examine delivery of siRNA. DharmaFECT (0.5 nmol/L) was used to increase the efficiency of transfection.

Western blot analysis

After protein extraction, immunoblotting was performed, and blots were probed with anti-phospho-Src homology domain 2-containing protein tyrosine phosphatase (SHP) 1, anti-phospho-SHP-2, or anti-phospho-Src homology domain 2-containing inositol phosphatase (SHIP) 1 antibodies (New England Biolabs, Whitby, Ontario, Canada) and developed by using enhanced chemiluminescence (Amersham, Piscataway, NJ).

Quantitative real-time PCR

RNA was extracted from DCs or dissected lungs by using TRIzol (Invitrogen). cDNA was prepared by means of reverse transcription, and mRNA was quantified by using SYBR Green RT-qPCR (Applied Biosystems, Foster City, Calif). The following primers were used: DCIR, 5'-GATCTAAGAAAGCCTGGTTC-3'

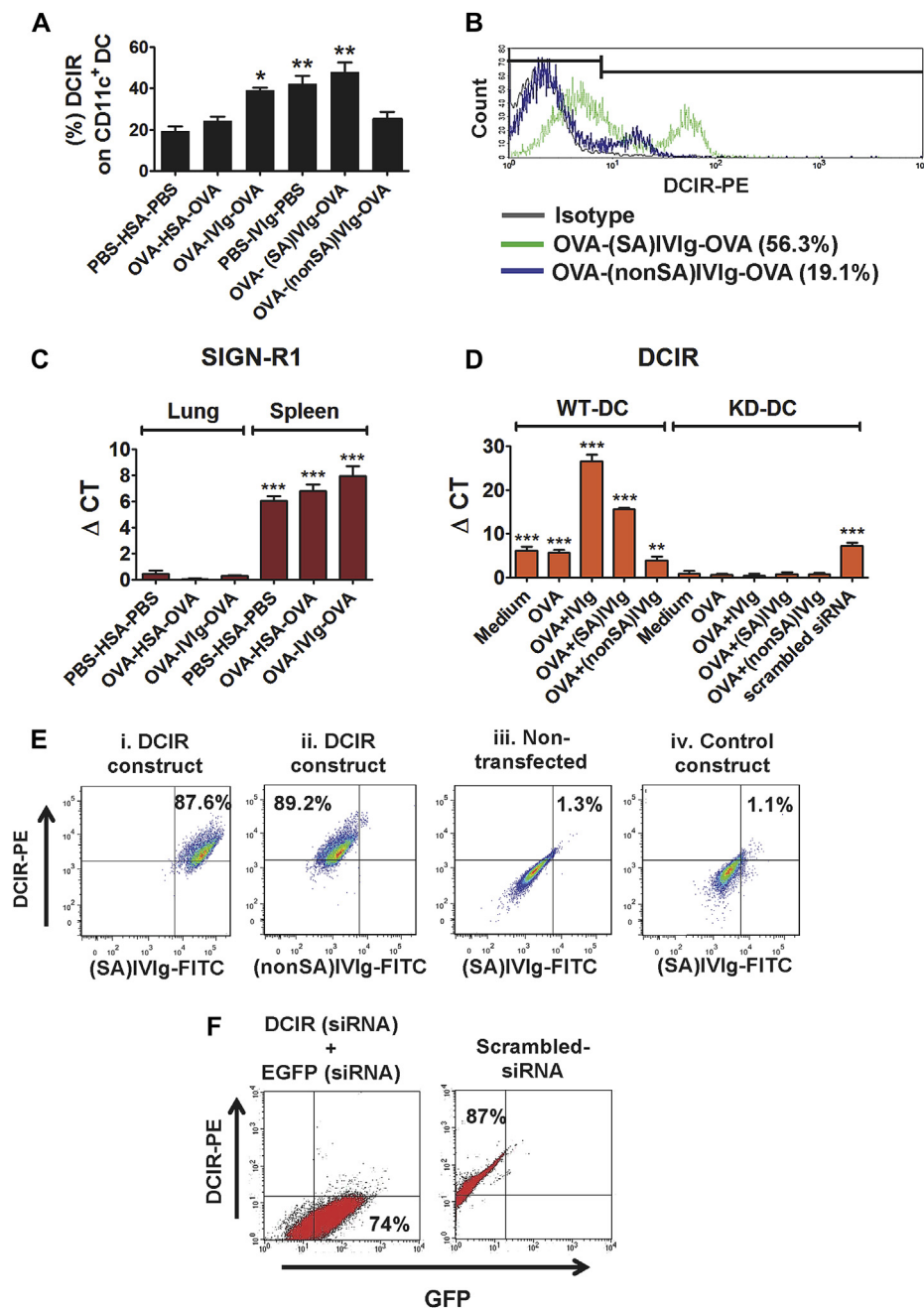


FIG 2. SA-IVIg binds DCIR on CD11c⁺ DCs. **A** and **B**, DCIR expression on pulmonary CD11c⁺ DCs. DCIR is upregulated by IVIg and SA-IVIg. **C**, SIGN-R1 mRNA demonstrating low expression of SIGN-R1 in the lungs. **D**, DCIR mRNA expression with and without siRNA against DCIR in BMDDCs. **E**, DCIR-transfected CHO cells colocalized DCIR and SA-IVIg (*ii*) but neither non-SA-IVIg (*iii*), nontransfected cells (*iii*), nor scrambled construct transfection (*iv*). **F**, BMDDCs incorporated 70% of enhanced green fluorescent protein siRNA. DCIR siRNA, but not scrambled siRNA, blocked expression of DCIR on DCs. Data represent means $\pm$ SDs from 3 independent experiments with 2 replicates in each. * P < .05 and ** P < .01. CT, Cycle threshold; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

(sense) and 5'-GCAAGAGATATCGTTCCAGC-3' (antisense); SIGN-R1, 5'-CTGCAAGAAGTCTGCAACCC-3' (sense) and 5'-TGGCAGAATGGCATGAAGGT-3' (antisense). Glyceraldehyde-3-phosphate dehydrogenase was used for normalization (AGCAATGCCTCTGCACCACC [sense] and GAGGCTGGTAAGGAAGTGG [antisense]).

Statistical analysis

All parameters were analyzed by using GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). ANOVAs were performed with the Bonferroni correction.

RESULTS

Sialylated IgG is required for the anti-inflammatory effect of IVIg

IVIg, SA-IVIg, or non-SA-IVIg were administered to OVA-sensitized mice 1 day before allergen challenge to address whether sialylated IgG attenuates allergic airways disease (AAD). SA-IVIg attenuated MCh-induced AHR in OVA-challenged mice comparable with intact IVIg (Fig 1, A) at a 10-fold lower dose. Inhibition of AHR by IVIg and SA-IVIg

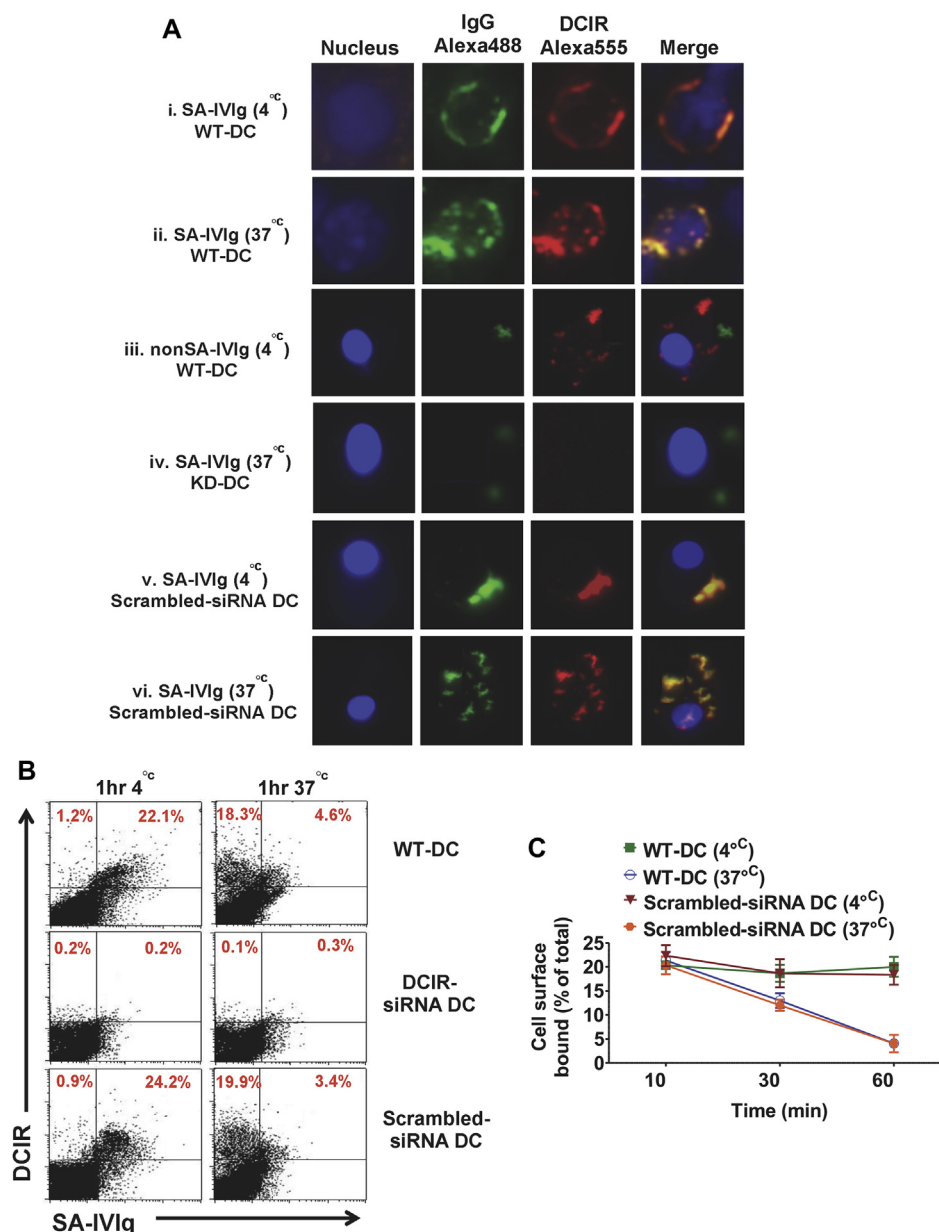


FIG 3. DCIR binds and internalizes IgG into DCs. **A**, Colocalization of DCIR and IgG. FcγR-blocked BMDDCs were stained with Alexa Fluor 555–anti-DCIR (red) and Alexa Fluor 488–SA-IVIg or Alexa Fluor 488–non-SA-IVIg (green). Nuclei were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (blue; original magnification ×400). DCIR specifically colocalized with SA-IVIg at 4°C and internalized SA-IVIg at 37°C. Representative of 3 experiments (n = 50–100 cells per condition). **B** and **C**, Internalization of SA-IVIg (37°C) to WT but not DCIR siRNA–treated BMDDCs. Means ± SDs from 2 experiments are shown.

was accompanied by a significant increase in the frequency of Treg cells within the lungs (Fig 1, B and C). Administration of non-SA-IVIg did not prevent increased AHR nor did it induce Treg cells.

Additionally, administration of IVIg or SA-IVIg, but not non-SA-IVIg, to OVA-exposed mice resulted in decreased frequencies of both IL-4⁺ and IL-17⁺CD4⁺ T cells (Fig 1, D and E), whereas the frequency of IFN-γ⁺ T cells was not significantly changed (Fig 1, F). Total and OVA-specific IgE levels significantly increased in OVA-challenged mice, but there was no change from baseline IgE levels in IVIg- or SA-IVIg–treated mice (data not shown).

DCIR acts as a specific receptor for SA-IVIg on DCs

We showed that CD11c⁺ DCs play an integral role in induction of Treg cells and abrogation of AHR by IVIg.⁴ Sialylated IgG is known to interact with lectin receptors, including SIGN-R1, on splenic macrophages.^{2,7} However, we were unable to find significant SIGN-R1 mRNA or receptor expression on CD11c⁺ pulmonary DCs or GM-CSF–cultured BMDDCs (Fig 2, C, and see Fig E1, A, in this article’s Online Repository at www.jacionline.org). To determine potential IVIg receptors on DCs, we focused attention on the recently described DCIR, a C-type lectin ITIM-linked receptor.¹³ DCIR was constitutively expressed on pulmonary DCs (15% to 20%; Fig 2, A). Treatment with either

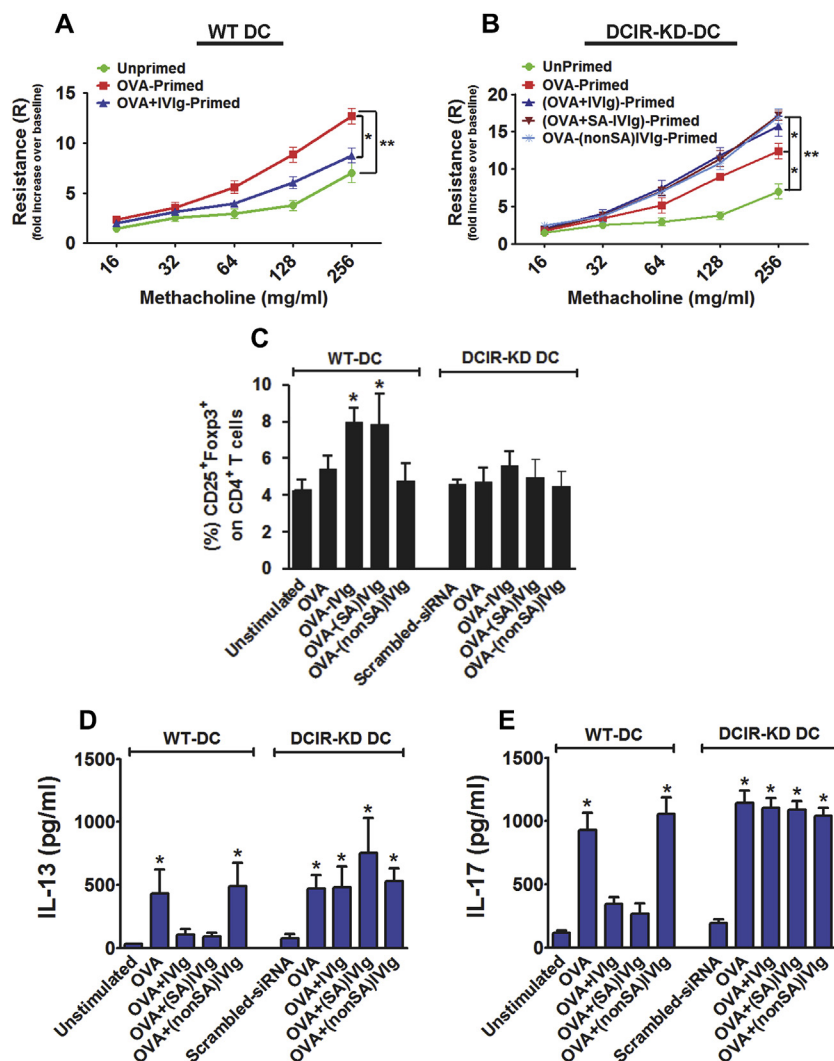


FIG 4. DCIR mediates inhibition of AHR by IVIg/SA-IVIg. **A–C**, BMDDCs were incubated with or without DCIR-siRNA or control and then primed with OVA with or without IVIg and administered intratracheally, followed by OVA challenge. DCIR siRNA-treated BMDDCs did not inhibit AHR (**Fig 4, B**) nor did they induce Treg cells (**Fig 4, C**). **D** and **E**, IL-13 and IL-17 were inhibited by IVIg and SA-IVIg-primed WT-BMDDCs, but not DCIR siRNA-treated BMDDCs (**D** and **E**). * $P < .05$ and ** $P < .01$ ($n = 6$ for each condition).

IVIg or SA-IVIg, but not non-SA-IVIg, enhanced both mRNA and protein expression of DCIR on pulmonary CD11c⁺ DCs (**Fig 2, B** and **D**). We expressed a full-length DCIR construct in CHO cells. Using flow cytometry and fluorescent microscopy, we observed that Alexa Fluor 488-labeled SA-IVIg, but not non-SA-IVIg, consistently colocalized with DCIR on transfected CHO cells (**Fig 2, E**, and see **Fig E2, A**, in this article's Online Repository at www.jacionline.org).

DCIR mediates internalization of SA-IVIg into DCs

Most C-type lectin receptors are involved in rapid cytoplasmic internalization of their cognate ligands.^{14,15} To examine whether DCIR delivered sialylated IgG into the cytoplasm, we used BMDDCs, which abundantly expressed DCIR.¹³ SA-IVIg, but not non-SA-IVIg, colocalized with DCIR on FcγR-blocked WT bone marrow-derived dendritic cells (WT-DCs) when incubated at either 4°C or 37°C (**Fig 3, A**, i–iii). At 37°C, both SA-IVIg and DCIR were internalized into DCs over a 60-minute period

(**Fig 3, A**, ii). To ascertain the specificity of this binding, DCIR expression was diminished by using specific siRNA (**Fig 2, D** and **F**, and see **Fig E2, C**). SA-IVIg could not bind or be internalized after DCIR knockdown (**Fig 3, iv**, and see **Fig E2, D** and **E**). BMDDCs transfected with nonspecific (scrambled) siRNA bound and internalized SA-IVIg similarly to WT-DCs (**Fig 3, A**, v and vi). BMDDCs were also incubated with biotinylated SA-IVIg and anti-DCIR antibodies for 10, 30, and 60 minutes at 37°C, followed by flow cytometric detection of cell-bound SA-IVIg with streptavidin–Alexa Fluor 488. At 37°C, surface-bound SA-IVIg decreased on WT-DCs by more than 80% compared with that seen on WT-DCs incubated at 4°C, whereas SA-IVIg was neither bound nor internalized into DCIR-siRNA-treated BMDDCs (**Fig 3, B** and **C**). In contrast, in DCIR-siRNA-treated BMDDCs grown with IL-4, less than 60% of sialylated immunoglobulin bound to SIGN-R1 was internalized (see **Fig E3, A** and **B**, in this article's Online Repository at www.jacionline.org). This indicates that DCIR serves as a primary portal for internalization of IgG into DCs.

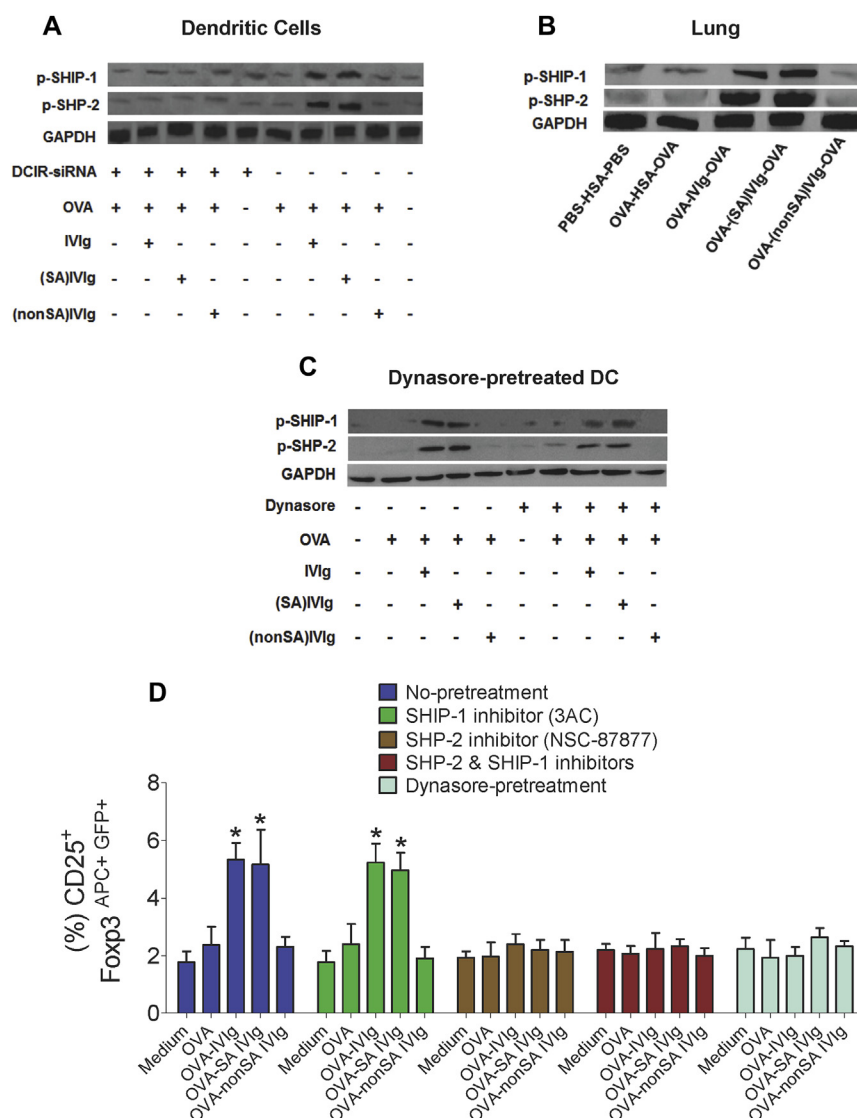


FIG 5. Ligation of DCIR by IVIg/SA-IVIg induces phosphorylation of SHIP-1 and SHP-2. Western blots of phospho-SHIP-1 and phospho-SHP-2 from BMDDCs and lung extracts are shown. **A** and **B**, OVA-IVIg and OVA-SA-IVIg treatment increased phosphorylated SHIP-1 (*pSHIP-1*) and phosphorylated SHP-2 (*pSHP-2*) levels in BMDDCs (**Fig 5, A**) or lung tissue (**Fig 5, B**), which was inhibited by pretreating BMDDCs with DCIR siRNA (**Fig 5, A and B**). **C** and **D**, Dynasore-pretreated BMDDCs showed normal phosphorylation in response to IVIg (**Fig 5, C**), but Dynasore and SHP-2 inhibitors, but not SHIP-1 inhibitors, blocked *in vitro* induction of CD4⁺FoxP3⁺ Treg cells (**Fig 5, D**). **P* < .05. Data are representative of 3 experiments. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Inhibition of AHR by IVIg is dependent on DCIR

To investigate the requirement for SA-IVIg/DCIR interaction in the reversal of AHR, we induced AAD by using conditioned BMDDCs, as in the study by Koya et al.¹⁰ Transferring of OVA-primed BMDDCs markedly enhanced AHR after OVA challenge comparable with that seen after systemic sensitization. BMDDCs conditioned with OVA plus IVIg or OVA plus SA-IVIg did not confer increased AHR and airway inflammation (**Fig 4, A**, and data not shown) and also increased Treg cell numbers in the lungs of OVA-challenged mice (**Fig 4, C**).

We subsequently incubated BMDDCs with siRNA against DCIR before conditioning (KD-DCs). KD-DCs conditioned with OVA plus IVIg or OVA plus SA-IVIg had no protective effect against OVA-induced AHR. In fact, transfer of KD-DCs significantly

enhanced AHR compared with that seen in mice that received OVA-primed WT-DCs (**Fig 4, B**). OVA plus IVIg- and OVA plus SA-IVIg-primed KD-DCs also did not induce Treg cells (**Fig 4, C**). Lung digests from OVA plus IVIg and OVA plus SA-IVIg KD-DC recipients produced higher levels of IL-4 and IL-17 after *ex vivo* OVA restimulation compared with WT-DCs (**Fig 4, D and E**). Delivery of scrambled siRNA-treated DCs behaved similarly to WT-DCs (data not shown). Ligation of DCIR was associated with phosphorylation of the ITIM-linked phosphatases SHIP-1 and SHP-2 (but not SHP-1) in OVA plus IVIg- and OVA plus SA-IVIg-conditioned BMDDCs and pulmonary (**Fig 5, A and B**) but not after OVA plus non-SA-IVIg treatment. Phosphorylation of SHIP-1 and SHP-2 was also not observed in KD-DCs treated with SA-IVIg (**Fig 5**). Furthermore, IVIg and SA-IVIg did not

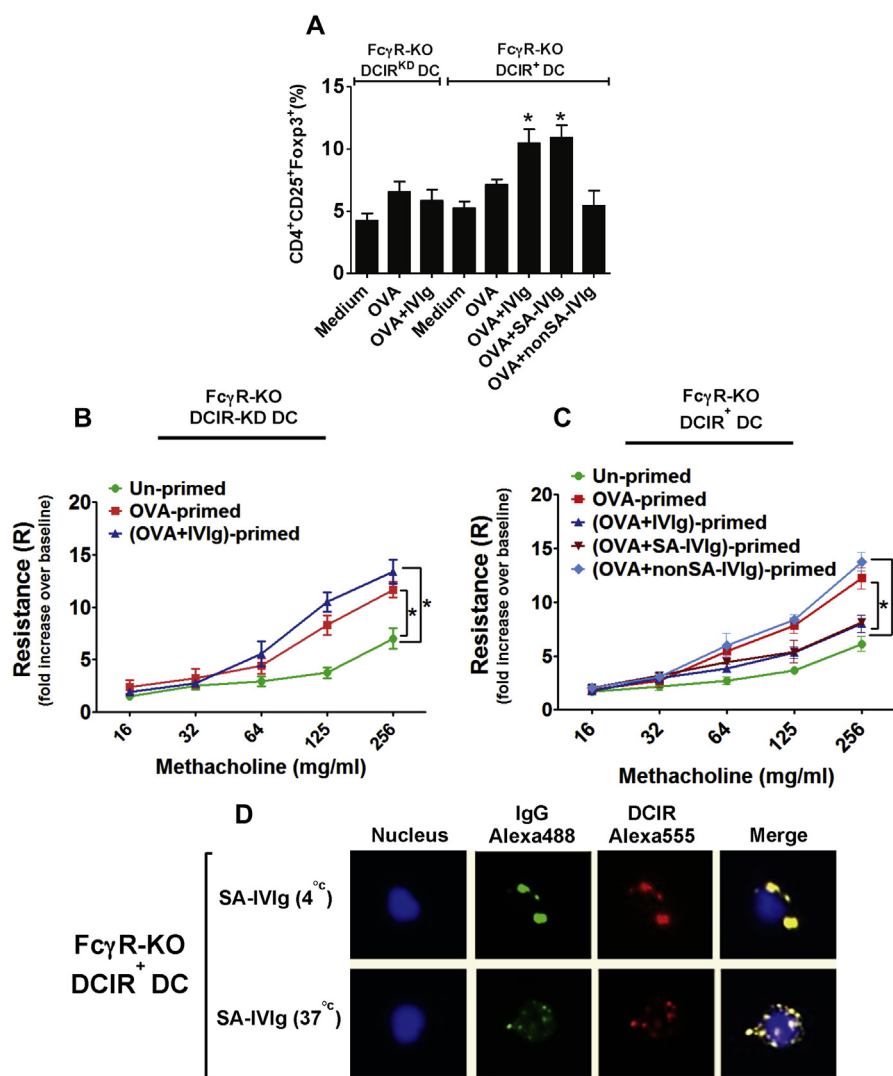


FIG 6. SA-IVIg is not dependent on Fc γ Rs. BMDDCs from Fc γ -KO mice were incubated with or without DCIR siRNA and primed with OVA with or without IVIg *in vitro* and then administered intratracheally to WT mice, followed by OVA challenge. IVIg- or SA-IVIg-primed Fc γ -KO mice without DCIR-siRNA were able to induce pulmonary Treg cells (**A**) and abrogate AHR (**C**). DCIR siRNA abrogated the effects of IVIg, as in WT-DC (**A** and **B**). Colocalization of SA-IVIg to DCIR was identical in Fc γ -KO mice as in WT-BMDDCs. * $P < .05$ ($n = 6$ per condition).

appear to require Fc γ R; Fc γ R-KO BMDDCs bound and internalized SA-IVIg similarly to WT-DCs (**Fig 6, D**). Transfer of Fc γ R-KO BMDDCs conditioned with OVA plus IVIg or OVA plus SA-IVIg fully attenuated AHR and increased Treg cell numbers similarly to WT-DCs (**Fig 6, A-C**). Fc γ R-KO BMDDCs treated with DCIR siRNA before OVA plus IVIg priming were similarly unable to protect against AHR (**Fig 6, B**).

Internalization of IVIg is necessary for induction of Treg cells

Internalization of DCIR-ligand complexes is mediated through a clathrin- and dynamin-dependent pathway.¹⁴ Dynasore is a cell-permeable, reversible, noncompetitive dynamin-1 and dynamin-2 GTPase activity inhibitor that disrupts clathrin-mediated endocytosis of surface receptors.¹⁶ Pretreatment of BMDDCs with Dynasore attenuated DCIR-mediated internalization of SA-IVIg into DCs (**Fig 7, A and B**), with the majority of bound IgG remaining on the DC surface. Furthermore, Dynasore-

pretreated conditioned BMDDCs transferred intratracheally did not attenuate AHR after OVA challenge or induce Treg cells (**Fig 7, C, E, and F**). Mice given Dynasore-treated DCs had higher levels of activated effector T cells (CD4⁺CD25⁺Foxp3⁻; **Fig 7, D**), and the frequency of IL-4- and IL-17-producing CD4⁺ T cells was comparable with that of OVA-treated WT-DC recipients (**Fig 7, G and H**).

To determine whether SHIP-1 and SHP-2 phosphorylation were dependent on internalization of IVIg, we pretreated BMDDCs with Dynasore. Although this did not inhibit the effect of IVIg or SA-IVIg on the phosphorylation of SHIP-1 and SHP-2 in DCs (**Fig 5, C**), the ability of BMDDCs to induce Treg cells *in vitro* was attenuated in the presence of the SHP-2 inhibitor NSC-87877, but not 3AC, a SHIP-1 inhibitor (**Fig 5, D**, and see **Fig E4** in this article's Online Repository at www.jacionline.org). Thus internalization of IgG and signaling through SHP-2 might play important roles in producing tolerogenic DCs and Treg cells.

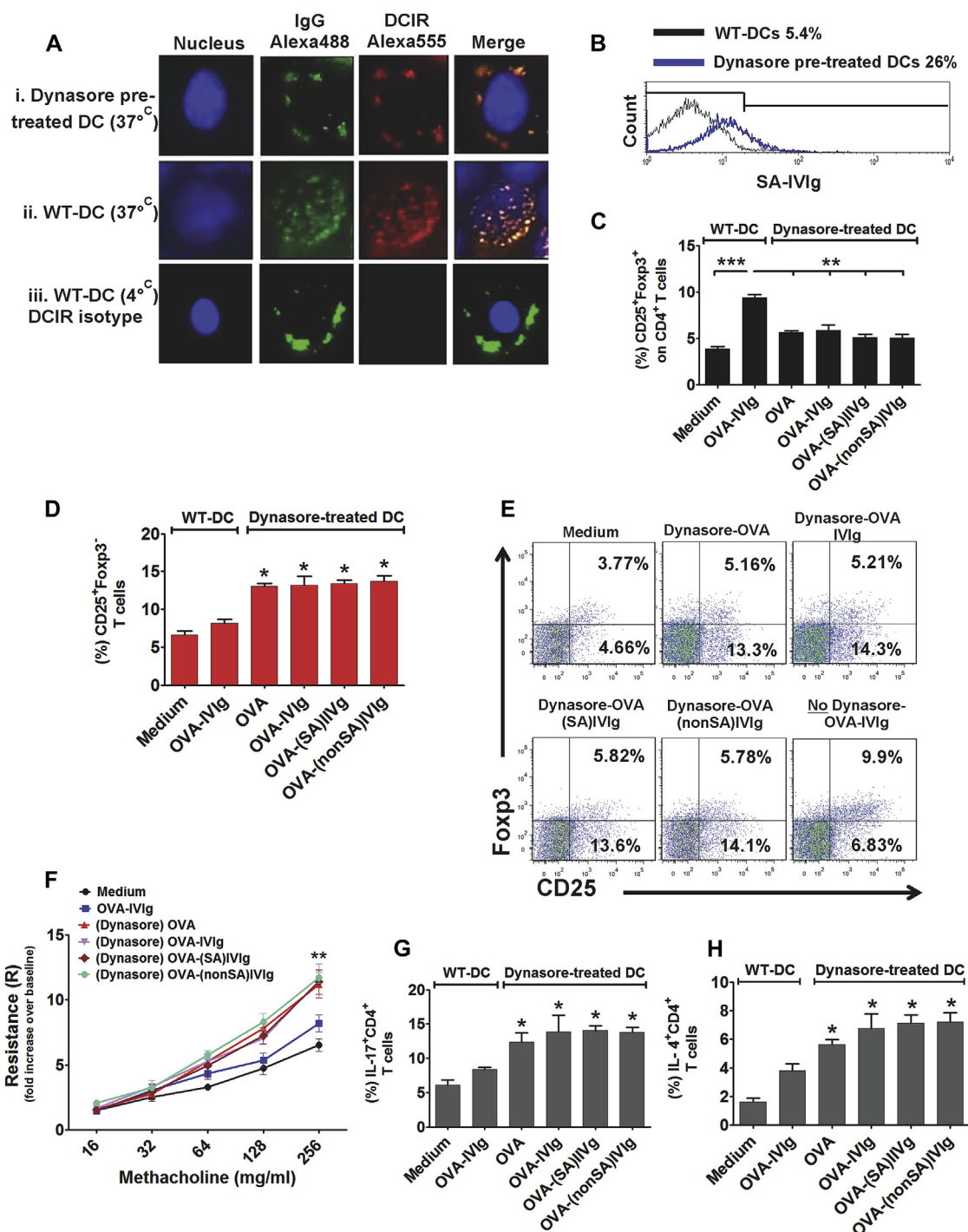


FIG 7. Inhibition of IgG internalization inhibits the effects of IVIg/SA-IVIg on DCs. **A** and **B**, DCIR binding and internalization of IVIg in the presence (i) and absence (ii) of Dynasore by means of fluorescent microscopy (Fig 7, A) and flow cytometry (Fig 7, B). **C-H**, IVIg- or SA-IVIg-treated BMDDCs in the presence of Dynasore do not induce Treg cells (Fig 7, C and E) and have higher effector T-cell numbers (Fig 7, D), increased AHR (Fig 7, F), and significantly increased IL-17-expressing (Fig 7, G) and IL-4-expressing (Fig 7, H) CD4⁺ cells compared with IVIg-treated BMDDCs without Dynasore. **P* < .05 versus medium and OVA-IVIg-DC recipients. All results are representative of 2 experiments (n = 6 mice per group). **P* < .05, ***P* < .01, and ****P* < .001.

DISCUSSION

IgG is a crucial effector molecule in host defense recognized for its role in pathogen elimination and autoimmune diseases.

However, there are many examples of IgG contributing to immune tolerance, and IVIg is frequently used as disease-modifying therapy for autoimmune and inflammatory conditions.¹⁷ Using a

murine model of AAD, we reported that high-dose IVIg induces Foxp3⁺ Treg cells from Foxp3⁻CD4⁺ T cells, attenuating allergen-induced AHR. The effect of IVIg is the result of functional changes in pulmonary CD11c⁺ DCs.⁴ Indeed, adoptive transfer of CD11c⁺ cells exposed to IVIg either *in vivo* or *ex vivo* attenuated AHR and increased Foxp3⁺ Treg cell numbers. We now present convincing evidence that induction of Treg cells by IVIg is mediated through DCIR, a novel receptor on DCs.

Glycosylation of IgG Fc determines its relative affinity for inhibitory or activating FcγRs.¹⁸ Recent work has stimulated considerable interest in sialic acid moieties on ASP²⁹⁷ of Fc.⁷ Sialylated IgG molecules have reduced affinity for activating FcγR¹⁹ and are less efficient in antibody-dependent cytotoxicity.²⁰ The lectin receptor SIGN-R1 or its human ortholog DC-SIGN was identified as a binding site for sialylated Fc, initiating regulatory events involving splenic marginal zone macrophages.⁷ In a model of serum-induced arthritis, SIGN-R1-deficient mice do not improve with IVIg therapy,⁷ and ligation of SIGN-R1 by IVIg appears to stimulate basophil-mediated IL-4 production in an IL-33-dependent manner.²¹

In our studies SA-IVIg completely reproduced the action of unfractionated IVIg at a 10-fold lower dose. SA-IgG-induced Treg cells inhibited allergen-induced AHR, decreased inflammatory cytokine levels, and ameliorated pulmonary inflammation. This was not the case for desialylated IVIg, which was ineffective in all measured parameters.

In patients with AAD, T_H2 cytokines, including IL-4 and IL-13, as well as T_H17 cytokines, are integral to the inflammatory response and are inhibited by IVIg and SA-IVIg.⁶ The mechanism of action of IVIg in patients with AAD and potentially in other active immune response models does not mirror the features of passive serum-induced arthritis, where IL-33, IL-4, and potentially other facets of T_H2 immunity might be protective. Additionally, SIGN-R1 has not been reported to be expressed in pulmonary DCs,²² and we did not detect SIGN-R1 mRNA or protein on GM-CSF-induced BMDDCs or pulmonary CD11c⁺ DCs isolated from C57BL/6 mice. Additionally, in GM-CSF plus IL-4-differentiated BMDDCs, SIGN-R1 ligation in the presence of antigen did not increase Treg cell numbers in an *in vitro* differentiation assay (see Fig E3). Therefore we examined other potential receptors with the capacity to bind sialylated IgG.

DCIR is a type II C-type lectin receptor, a member of the Dectin-2 family of receptors with a unique intracellular chain harboring an ITIM.²³ DCIR has a single carbohydrate recognition domain at the COOH terminal.²⁴ DCIR-deficient mice spontaneously have autoimmunity,²⁵ and DCIR2⁺ DCs can differentiate peripheral Foxp3⁺ Treg cells through endogenous production of TGF-β.²⁶ The ligands recognized by DCIR are incompletely elucidated. DCIR is known as a potential receptor and portal for HIV^{23,27} and, as with other lectin receptors, exhibits promiscuous binding of a variety of sugars, including mannose, fucose, N-acetylglucosamine, and α1-acid glycoprotein (AGP),²⁸ which are enriched with sialic acids.²⁹

The primary function of C-type lectin receptors is to trap pathogens through the carbohydrate recognition domain, with subsequent internalization, antigen processing, and presentation by phagocytes. Receptor binding is associated with activation signals or, in the case of ITIM-linked DCIR, inhibitory signals through phosphatases. IVIg binding to DCIR initiated inhibitory signaling involving the phosphatases SHP-2 and SHIP-1. Inhibition of DCIR expression by using siRNAs completely inhibited

binding of sialylated IgG and enhancement of SHP-2/SHIP-1 phosphorylation, suggesting that DCIR ligation was crucial for modification of DCs by SA-IVIg.

We also demonstrated that binding of SA-IVIg to DCIR results in internalization of IgG-DCIR complexes within 1 hour. Internalization appears independent of FcγRs but was crucial for the induction of Treg cells. IVIg can be internalized into antigen-presenting cells independent of FcγRs,^{30,31} leading to diminished T-cell activation, although no specific receptors were identified in the studies of Proulx and colleagues.^{30,31} Tolerogenic DCs play an important role in maintaining peripheral tolerance through induction, expansion, or activation of Treg cells,^{32,33} and IVIg can modulate DCs to a more tolerogenic state.⁵ Induction of Treg cells can explain the protective effects of IVIg in a wide range of autoimmune and inflammatory disorders. In addition to our allergic airway disease model, IVIg has been demonstrated to induce Treg cells in experimental allergic encephalomyelitis,³⁴ systemic lupus erythematosus,³⁵ vasculitis,³⁶ immune thrombocytopenic purpura,³⁷ and Kawasaki syndrome.³⁸

The steps after internalization of IgG in our model remain to be elucidated. Meyer-Wentrup et al¹⁴ demonstrated that antigens targeted to DCs through DCIR are efficiently presented to T cells. De Groot et al³⁹ predicted several Treg cell epitope sequences, named Tregitopes, which are highly conserved in both the Fc and F(ab')₂ regions of IgG. Peptides produced based on these epitopes appear to suppress immune responses in coculture and animal models of inflammatory diseases through expansion of Treg cells.⁴⁰

Furthermore, sialic acid residues are common on both the Fc and F(ab')₂ regions of IgG. Kasermann et al⁴¹ found that the effect of IVIg on monocytes is dependent on sialylated F(ab')₂, rather than the Fc fragment. This suggests that the "handle" for DCIR can be sialic acid residues on either the Fc or Fab portions of IVIg.

In summary, we have clearly identified a novel receptor for IVIg that alters DC activity and bridges the innate and adaptive immune systems to enhance immune regulation. Exploration of DCIR as a modulator of immune tolerance and a novel therapeutic target might lead to improved regulatory cell therapies for patients with inflammatory and autoimmune conditions.

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Key messages

- SA-IVIg induces tolerogenic DCs, which act to generate Treg cells and inhibit allergen-driven airway reactivity.
- Induction of Treg cell numbers by IVIg is mediated through DCIR, a novel receptor on DCs.

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METHODS

Induction of SIGN-R1 on BMDDCs

BMDDCs were generated from bone marrow cells of naive (C57BL/6) mice in the presence of recombinant IL-4 (2.5 ng/mL) plus GM-CSF (10 ng/mL, PeproTech), as in the Methods section. Anti-mouse CD209 biotin (SIGN-R1) was purchased from eBioscience. Streptavidin-phycoerythrin and streptavidin-Alexa Fluor 555 were purchased from Invitrogen. Addition of IL-4 to GM-CSF induced upregulation of SIGN-R1 on BMDDCs by 4% to 60%. In some experiments SIGN-R1^{high} BMDDCs were treated with siRNA against DCIR, as explained in the Methods section, to generate SIGN-R1^{high}DCIR[−] BMDDCs.

In vitro induction of Treg cells

BMDDCs were cultured in complete medium with OVA (1 mg/mL) for 3 hours, and then IVIg (10 mg/mL), SA-IVIg (0.5 mg/mL), non-SA-IVIg (10 mg/mL), or HSA was added for a further 24 hours. CD4⁺ T cells from OT-II FoxP3^{GFP} mice^{E1} were purified from spleens, and CD4⁺FoxP3^{GFP−} cells were selected by means of fluorescence-activated cell sorting. CD4⁺FoxP3^{GFP−}

cells were cultured in the presence of BMDDCs and OVA for 3 days and then analyzed for the induction of CD4⁺FoxP3^{GFP+} cells by means of flow cytometry. NSC-87877 (SHP-2 inhibitor) and 3AC (SHIP-1) inhibitor were purchased from Sigma-Aldrich. In some experiments BMDDCs were pretreated for 12 hours with 20 μmol/L NSC-87877^{E2} or 100 μmol/L 3AC^{E3} before addition of IVIg and OVA. The expression of phosphorylated SHIP-1 and phosphorylated SHP-2 was analyzed by means of Western blotting to ascertain the efficiency of the inhibitors.

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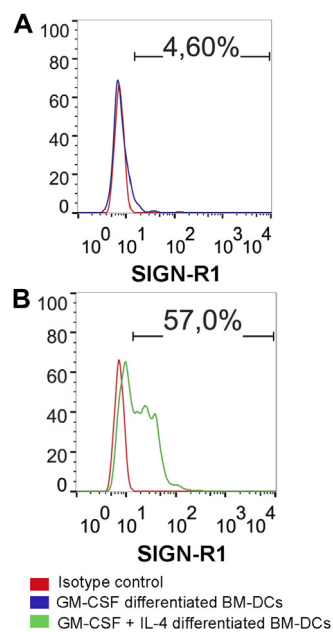


FIG E1. GM-CSF–differentiated (**A**) or GM-CSF plus IL-4–differentiated (**B**) BMDDCs were stained with SIGN-R1–biotin for 30 minutes at 4°C, stained with streptavidin-phycoerythrin, and analyzed on an LSR-II flow cytometer.

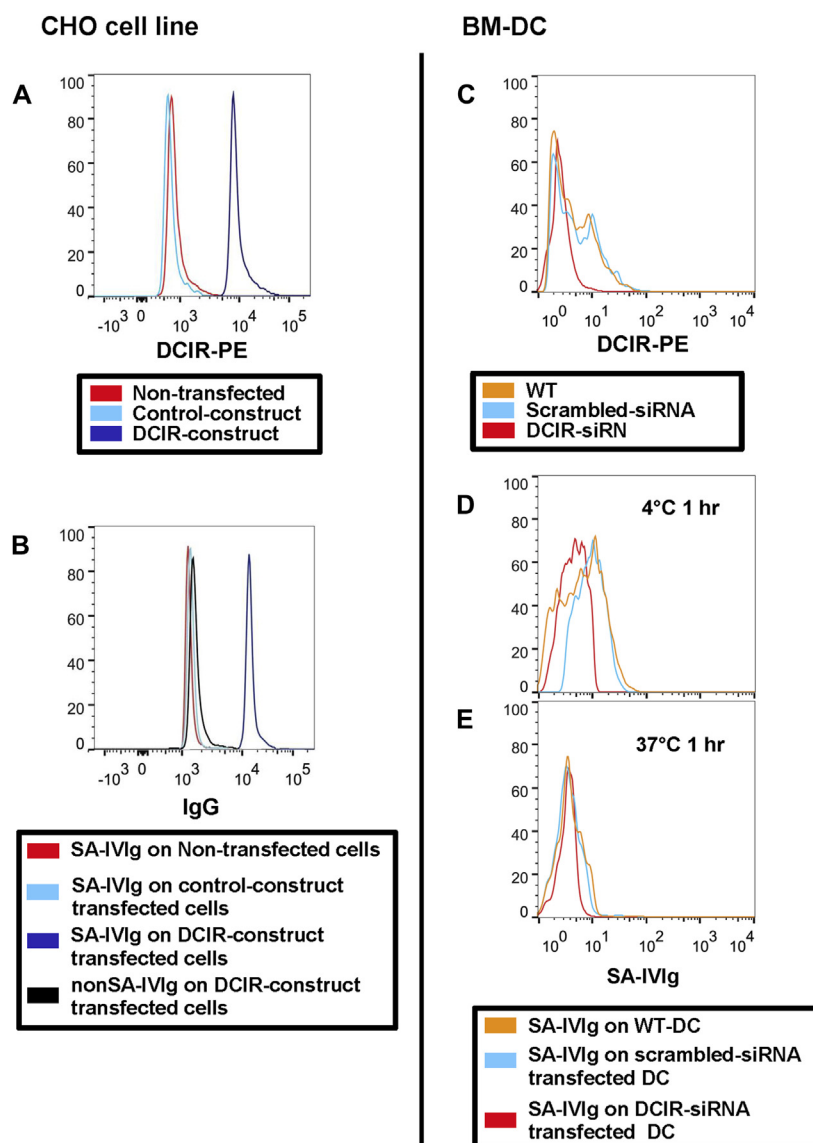


FIG E2. Representative histogram of IgG and DCIR binding to DCIR construct-transfected CHO cells. **A**, DCIR-transfected but not scrambled construct-transfected CHO cells bind to DCIR. **B**, DCIR-expressing CHO cells bound Alexa Fluor 488-conjugated SA-IVlg but not non-SA-IVlg. **C**, DCIR expression on BMDDCs, which is diminished by DCIR siRNA but not scrambled siRNA. **D**, SA-IVlg bound to BMDDCs with normal DCIR expression but not to DCIR siRNA-treated BMDDCs at 4°C. **E**, At 37°C, SA-IVlg is internalized, diminishing surface expression.

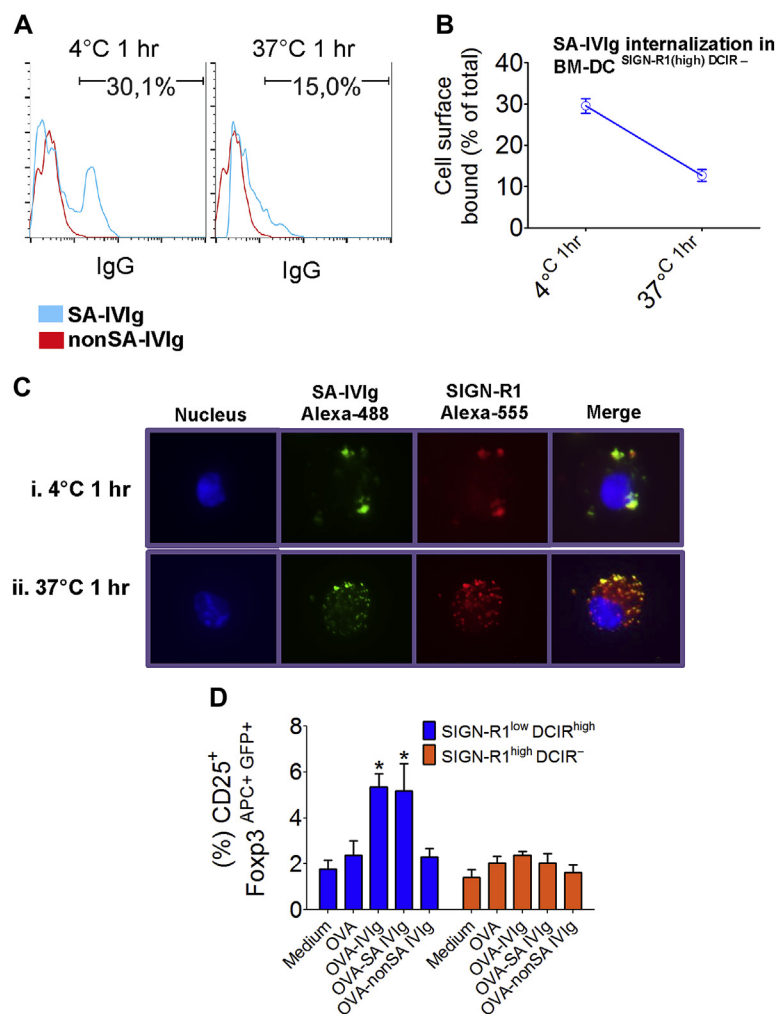


FIG E3. SIGN-R1^{high}DCIR⁻ BMDDCs were used to monitor the IgG internalization and the ability of cells to induce Treg cells *in vitro*. **A-C**, Representative histogram, bar graph, and fluorescent microscopy demonstrating the cell surface-bound SA-IVIg on SIGN-R1^{high}DCIR⁻ BMDDCs at 4°C versus 37°C. **D**, The cells were conditioned as depicted in the bar graph, washed, and cocultured with CD4⁺ T cells purified from OT-II Foxp3-GFP reporter mice in the presence of OVA. OVA plus IVIg- or OVA plus SA-IVIg-pretreated SIGN-R1^{high}DCIR⁻ BMDDCs were not able to induce Treg cells in contrast to OVA plus IVIg- or OVA plus SA-IVIg-pretreated DCIR^{high} BMDDCs. *P < .05.

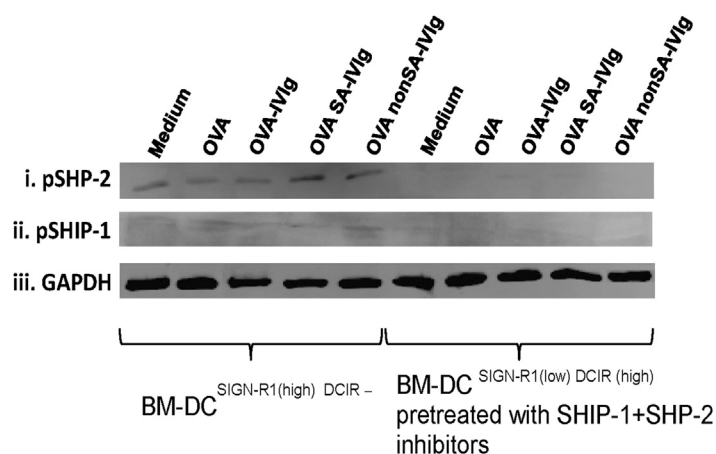


FIG E4. Representative Western blots of phosphorylated SHIP-1 (*pSHIP-1*) and phosphorylated SHP-2 (*pSHP-2*) in protein extracted from conditioned BMDDCs. OVA-IVIg and OVA-SA-IVIg treatment was not able to increase pSHIP-1 and pSHP-2 levels in conditioned SIGN-R1^{high}DCIR⁻ BMDDCs (*i*) and SIGN-R1^{low}DCIR^{high} BMDDCs (*ii*) pretreated with NSC-87877, and 3AC inhibitors did not exhibit pSHIP-1 and pSHP-2 in protein lysate. Incubation with Dynasore did not inhibit phosphorylation of SHIP-1 and SHP-2. All data represent means $\pm$ SDs of 3 replicates.