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Targeting Antigens through Blood Dendritic Cell Antigen 2 on Plasmacytoid Dendritic Cells Promotes Immunologic Tolerance

Craig P. Chappell,* Natia V. Giltiay,*† Kevin E. Draves,* ChangHung Chen,‡ Martha S. Hayden-Ledbetter,* Mark J. Shlomchik,§ Daniel H. Kaplan,§ and Edward A. Clark*†

The C-type lectin receptor blood dendritic cell Ag 2 (BDCA2) is expressed exclusively on human plasmacytoid DCs (pDCs) and plays a role in Ag capture, internalization, and presentation to T cells. We used transgenic mice that express human BDCA2 and anti-BDCA2 mAbs to deliver Ags directly to BDCA2 on pDCs in vivo. Targeting Ag to pDCs in this manner resulted in significant suppression of Ag-specific CD4+ T cell and Ab responses upon secondary exposure to Ag in the presence of adjuvant. Suppression of Ab responses required both a decrease in effector CD4+ T cells and preservation of Foxp3+ regulatory T cells (Tregs). Reduction in Treg numbers following Ag delivery to BDCA2 restored both CD4+ T cell activation and Ab responses, demonstrating that Tregs were required for the observed tolerance. Our results demonstrate that Ag delivery to pDCs through BDCA2 is an effective method to induce immunological tolerance, which may be useful for treating autoimmune diseases or to inhibit unwanted Ab responses. The Journal of Immunology, 2014, 192: 000–000.

A simple approach, Ag targeting, has made it possible to deliver an Ag to a particular dendritic cell (DC) subset and thereby program a characteristic immune response: Ag is coupled to a mAb specific for a receptor expressed only or mainly on a DC subpopulation, and then the Ag/mAb complex is injected alone or with an adjuvant (1, 2). Members of the C-type lectin receptor (CLR) family have been chosen as targets because many CLRs are differentially expressed on DC populations and indeed have been used for defining DC subsets (3–5). Furthermore, most CLRs internalize after crosslinking and thus, after being bound by Ag/mAbs, can deliver Ags to early endosomes and proteosomes (6–10). Crosslinking of CLRs can also induce characteristic signal transduction pathways and programming of DC subsets (11, 12).

Murine plasmacytoid DCs (pDCs) are a distinct subset of DCs that are phenotypically defined as CD11cint, B220+, B220-, and Siglec-H+ (13–16). pDCs were first identified as natural IFN-producing cells (6–10). Crosslinking of CLRs can also induce characteristic signal transduction pathways and programming of DC subsets (11, 12).

The CLR blood dendritic cell Ag 2 (BDCA2; CD303 or CLEC4C) is expressed principally on pDCs in humans (33). Cross-linking BDCA2 with mAbs results in receptor internalization, rapid Ca2+ influx, and signaling via a FcεRI-dependent pathway (34–36). To study pDC-mediated immune responses, we used mice expressing a human BDCA2 construct (B6.BDCA2) specifically in pDCs together with a novel anti-BDCA2 mAb as a model system to deliver Ag to pDCs in vivo. We report that Ag delivered via anti-BDCA2 leads to inhibition of Ag-specific CD4+ T cell and Ab responses upon Ag rechallenge. Inhibition of Ag-specific immune responses was a result of both deletion of effector CD4+ T cells and concomitant preservation of Foxp3+ Tregs. Both CD4+ and Ab responses could be restored by depletion of Tregs in vivo or through administration of a TLR7 agonist at the time of initial priming, which prevented the deletion of effector CD4+ T cells. Our results demonstrate that Ag delivery to BDCA2 expressed on pDCs results in immunological tolerance via a mechanism that requires Tregs.

Materials and Methods

Animals

The human BAC clone RP11-277J24 (Invitrogen) containing the human Clec4C promoter and open reading frame was used to generate transgenic
Biosciences) and analyzed using FlowJo (Tree Star) and GraphPad Prism.

For Foxp3 detection, the mouse Foxp3 staining kit (eBioscience) was used according to the manufacturer’s instructions. Enriched pDCs were cultured in 24-well tissue culture plates at 1 × 10^6/ml in RPMI 1640 with 50 μg/ml polymyxin B (Invivogen) or CpG-A ODN1668 (50 μg/ml rat-anti-mouse IFN-α) (PBL InterferonSource). Plates were blocked with PBS containing 0.05% Tween 20 (PBS-T) plus 1% BSA for 2 h at room temperature (RT), washed with PBS-T, and undiluted or supernatants diluted 1:2 were bound for 2 h at RT. Following three washes, rabbit anti-mouse IFN-α (PBL InterferonSource) was added for 1 h at RT. Following washes, donkey anti–rabbit-HRP (Jackson ImmunoResearch Laboratories) was added for 1 h at RT followed by four washes as above. Concentrations of IFN-α were obtained by comparison with known dilutions of universal type I IFN (PBL Biomedical Laboratories). For detection of IL-12p40, the DuoSet ELISA kit was used according to the manufacturer’s instructions (R&D Systems).

For confirmation of OVA-mAb conjugation, mAb conjugates were captured with anti-mouse IgG (SouthernBiotech) and detected with anti-OVA-biotin (Sigma-Aldrich) followed by streptavidin-HRP (R&D Systems). All plates were developed with tetramethylbenzidine substrate (Sigma-Aldrich) and reactions were stopped with addition of equal volume 2 N H₂SO₄ (Fisher Scientific). OD₄₅₀ values were obtained on a Bio-Rad 550 microplate reader.

**Generation of anti-BDCA2 mAbs**

Hybridomas secreting anti-BDCA2 Abs were generated by the Fred Hutchinson Cancer Research Center’s Ab Development Facility (Seattle, WA) by fusing the Foxp3 fusion partner with splenocytes from RBF/RT1 mice immunized with a BDCA2 mouse Ig fusion protein. Candidate positive wells were identified by screening supernatants on NIH-3T3 transfectants stably expressing BDCA2 under the control of the CMV promoter (NIH3T3.BDCA2), generated using a cDNA encoding human BDCA2 provided by Dr. James Arthos (National Institute of Allergy and Infectious Diseases, Bethesda, MD), followed by testing for binding to human pDCs. We established two clones producing mAbs, UW80.1 and UW80.2 (mouse IgG1), that bound specifically to human pDCs. Anti-BDCA2 mAbs and the mouse IgG1 mAb isotype control G28-1 (specific for human CD37) were prepared from hybridoma supernatants we generated by protein G affinity chromatography columns.

**Flow cytometry**

RBC-lysed mouse splenocytes (1–2 × 10^9) prepared by mechanical disruption of spleens were incubated for 30 min on ice in FACS buffer (1× PBS containing 2% FBS) containing varying combinations of biotin- or fluorochrome-conjugated mAbs against Siglec-H, PDCA-1, B220, CD11c, CD8, CD4, CD3, CD19, IgD, NK1.1, Vε2 TCR, Foxp3, CD25, CD44 (all from eBioscience), and CD62L (BD Biosciences). Detection of BDCA2 was performed using Alexa Fluor 647–conjugated UW80.1 mAb (eBioScience Alexa Fluor 647 conjugation kit). Ab-labeled cells were washed three times with FACS buffer followed by detection of biotinylated mAbs using streptavidin-PerCP-Cy5.5 (eBioscience) or streptavidin-FITC (both from BD Biosciences) for 20 min on ice. For Foxp3 detection, the mouse Foxp3 staining kit (eBioscience) was used according to the manufacturer’s instructions. Apoptotic cells were identified using annexin V (eBioscience) according to the manufacturer’s instructions. Data were acquired using an LSR II or FACScan flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star) and GraphPad Prism software.

**mAb-OVA conjugate preparation**

OVA was conjugated in 3-fold molar excess to mAbs via thioether linkages as described (37). Unconjugated OVA was removed from mAb-OVA conjugates using 100-kDa cut-off spin columns (Millipore). Retained mAb-OVA conjugates were resuspended in PBS, treated with polymyxin B (Sigma-Aldrich) overnight at 4°C to remove endotoxin, sterile filtered (0.2 μM), and stored at −20°C until use. ELISA assays (described below) were used to confirm Ag/mAb conjugation and determine the final concentration of OVA and mAb. The quantities of OVA per milligram mAb were as follows: OVA-DED205, 0.86 mg; OVA-G28-1, 0.85 mg; OVA-UW80.1, 0.84 mg; and OVA–Siglec-H, 0.55 mg.

**Purification of pDCs and in vitro stimulation**

pDCs from single-cell suspensions from spleens obtained from B6.BDCA2 mice were enriched using an anti-mPDCA1 magnetic bead isolation kit via treatment with Liberase B1 and Dynabeads (both from Roche), but otherwise according to the manufacturer’s instructions. Enriched pDCs were cultured in 24-well tissue culture plates at 1 × 10^5/ml in RPMI 1640 with 50 μM 2- ME and 10% FCS with either medium only or the indicated mAbs at 2 μg/ml with or without 20 μg/ml CpG-A (ODN 2216) for 18 h in a 37°C, 5% CO₂ humidified tissue culture incubator. The following day 800 μl supernatant was removed from each well and stored at −80°C until use.

**ELISA assays**

ELISAs were performed as described for measuring OVA- and chicken γ globulin (CGG)–specific IgM or IgG (38), or OVA-conjugated IgG mAbs (39). For detection of IFN-α, high binding capacity 96-well plates (Immobilon) were coated overnight at 4°C with 2.5 μg/ml rat-anti-mouse IFN-α in PBS (PBL InterferonSource). Plates were blocked with PBS containing 0.05% Tween 20 (PBS-T) plus 1% BSA for 2 h at room temperature (RT), washed with PBS-T, and undiluted or supernatants diluted 1:2 were bound for 2 h at RT. Following three washes, rabbit anti-mouse IFN-α (PBL InterferonSource) was added for 1 h at RT. Following washes, donkey anti–rabbit-HRP (Jackson ImmunoResearch Laboratories) was added for 1 h at RT followed by four washes as above. Concentrations of IFN-α were obtained by comparison with known dilutions of universal type I IFN (PBL Biomedical Laboratories). For detection of IL-12p40, the DuoSet ELISA kit was used according to the manufacturer’s instructions (R&D Systems).

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**Inhibition of CD25+ Tregs**

Mice received two i.p. injections consisting of 500 μg rat anti-CD25 (PC61.5.3) or IgG1 isotype control Ab (HRPN) (both from BioXCell) at days 3 and 1 prior to challenge with OVA plus alum. Reduction of Foxp3^+ CD4^+ T cells was assessed using flow cytometry by staining with anti-CD4, anti-Ly5.1, and anti-Foxp3 mAb on day 8 following the final anti-CD25 injection.

**Results**

Characterization of B6.BDCA2 Tg mice and anti-BDCA2 mAbs

We generated mAbs specific for BDCA2 using spleen cells from mice immunized with a BDCA2-Ig fusion protein (see Materials and Methods). A hybridoma producing a mouse IgG1 mAb, designated UW80.1, was established that bound to NIH-3T3 transfectants stably expressing human BDCA2 (Fig. 1A, top panel) and human pDCs (Supplemental Fig. 1). UW80.1 blocked the binding by the commercially available anti-BDCA2 mAb AC144 (Milenyi Biotec), suggesting these mAbs recognized the same or overlapping epitopes (Fig. 1A, bottom panel).

Tg mice expressing BDCA2 derived from a human BAC clone were generated and backcrossed to B6 mice for at least eight generations (B6.BDCA2 Tg mice, see Materials and Methods). Flow cytometry analyses of spleens from B6.BDCA2 mice confirmed BDCA2 expression on CD11c^{int} cells that coexpressed Siglec-H, PDCA-1, and B220 (Fig. 1B). BDCA2 was not detected in either B6 (Fig. 1B) or nontransgenic littermate controls (data not shown), nor was it detected on T cells, B cells, NK cells, or CD11c^{hi} myeloid DCs from B6.BDCA2 mice (Fig. 1B, 1C).

Crosslinking BDCA2 on human pDCs inhibits both type I IFN and upregulation of costimulatory receptors induced by CpG-A or CpG-B stimulation, respectively (40, 41). To determine whether BDCA2 crosslinking in B6.BDCA2 mice behaved similarly, magnetically enriched pDC-A^− pDCs from B6.BDCA2 mice were treated with CpG-A either in the presence or absence of graded doses of anti-BDCA2 (AC144) or isotype control for 24 h. Treatment with anti-BDCA2 significantly inhibited IFN-α production by pDCs in a dose-dependent manner when compared with untreated or isotype control–treated cells (Fig. 1D, upper panel). CpG-induced IL-12 production, however, was not suppressed by anti-BDCA2 treatment demonstrating that cytokine
responses were not globally affected (Fig. 1D, lower panel). Inhibition of IFN-α production by UW80.1 was also observed in additional experiments, although to a lesser extent than with the AC144 mAb (data not shown). Taken together, we conclude that B6.BDCA2 Tg mice display pDC-restricted expression of BDCA2, and that BDCA2 signaling in B6.BDCA2 mice is intact.
Ag delivery to BDCA2 in vivo does not induce Ag-specific Ab responses but does alter the proportions of Ag-specific effector and Treg subsets

To explore the utility of BDCA2 as a target for Ag delivery, cohorts of B6.BDCA2 mice were injected i.v. with 2 μg Alexa Fluor 647–conjugated UW80.1 anti-BDCA2 (anti–DCA2-647) or a nontargeting mIgG1 isotype control mAb (isotype-647) and sacrificed 1 h later for analysis by flow cytometry. As shown in Fig. 2A, ~85% of PDCA-1+Siglec-H+ splenic pDCs were labeled with anti–BDCA2-647, whereas <2% of pDCs bound to isotype-647. A small percentage of CD11c+PDCA-1+ Siglec-H+ pDCs (~1%) and CD19+IgD+ B cells (~1%) bound anti–BDCA2-647, which was also observed in isotype control–injected mice, suggesting that this minimal binding was FcγR-mediated and not due to expression of BDCA2 on these subsets.

To determine whether pDCs can induce Ab responses when Ag is targeted via BDCA2, we coupled OVA to the UW80.1 anti-BDCA2 mAb (OVA-BDCA2), to a mouse IgG1 isotype control (OVA-isotype), or, as a positive control for inducing Ab production after targeting Ag to a DC subset, to anti-DCIR2 (OVA-DCIR2) (39). We then immunized groups of B6.BDCA2 mice i.v. with 10 μg of each construct with or without 50 μg CpG-B. Serum was collected 10 d later and analyzed for anti-OVA Ab responses by ELISA. Unlike Ag delivery to DCIR2 on CD8+ myeloid DCs (39), neither OVA-BDCA2 nor OVA-isotype induced OVA-specific IgG responses even in the presence of TLR adjuvants CpG-B (Fig. 2B) or R848 (data not shown).

The lack of Ab responses following OVA-BDCA2 immunization could have been due to a failure to generate CD4+ T cell help. Thus, we examined CD4+ T cell responses in vivo following Ag delivery to BDCA2. OVA-specific Ly5.1+ OT-II CD4+ T cells were labeled with CFSE and adoptively transferred to cohorts of B6.BDCA2 mice that were injected 24 h later with 1 μg OVA-BDCA2, OVA-DEC205, OVA-isotype, or PBS as a negative control or OVA-DEC205 as a positive control because delivering Ag to DEC205 on CD8α+ DCs is known to induce CD4+ T cell expansion (42, 43) (Fig. 3A). Four days following immunization, mice were sacrificed and the frequency of dividing Ly5.1+CD4+ OT-II T cells in the spleen was determined by dilution of CFSE using flow cytometry. As expected, administration of OVA-DEC205 induced robust proliferation of OT-II T cells (85.7 ± 8.2%) (44), whereas injection of PBS or OVA-isotype induced little or no proliferation (PBS, 4.5 ± 0.8%; OVA-isotype, 10.9 ± 3.7%) (Fig. 3B, 3C). In contrast, a significant proportion of the transferred OT-II T cells divided in response to OVA-BDCA2 compared with OVA-isotype (28.5 ± 12.2%; p = 0.018) (Fig. 3B, 3C); however, the proliferation index showed that the amount of expansion induced by OVA-BDCA2 was not significantly greater than mice primed with OVA-isotype (Fig. 3D). Despite the induction of modest proliferation, immunization with OVA-BDCA2 resulted in a significant reduction in the frequency (0.29 ± 0.09 versus 0.16 ± 0.05%; p = 0.0012) (Fig. 3E) and total number (Fig. 3F) of transferred OT-II T cells 7 d postimmunization (p.i.) compared with OVA-isotype control mice. The frequency (Fig. 3E) and number (Fig. 3G) of non–Ag-specific Ly5.1+ CD4+ T cells were not affected by immunization with OVA-BDCA2 or OVA-isotype. Further analysis using annexin V revealed that OVA-BDCA2 administration induced a significant fraction of OT-II T cells to undergo apoptosis on days 4 and 7 postimmunization compared with OVA-isotype–injected mice.
controls (Fig. 3H). We conclude that OVA-BDCA2 immunization results in depletion of Ag-specific CD4 T cells.

BDCA2-mediated Ag uptake leads to increased frequencies of Foxp3+ Tregs

pDCs have been implicated in tolerance induction in a number of experimental settings (27, 29, 45, 46). Therefore, following injection of OVA-BDCA2 or OVA-isotype, we quantified the number of OT-II T cells expressing the forkhead box transcription factor Foxp3, a marker for Tregs. In contrast to the total population of transferred OVA-specific CD4 T cells, the frequency of transferred Ly5.1+ Tregs significantly increased in mice primed with OVA-BDCA2 (Fig. 4A, 4B). However, the total number of these cells was not increased compared with OVA-isotype–injected controls (Fig. 4B). We detected no changes in number or frequency of endogenous non–Ag-specific CD4+ T cell populations (Fig. 4A, 4C), suggesting that these changes required OVA-specific Ag presentation. In accord with a lack of Treg expansion, we detected no significant proliferation of Foxp3+ cells by CFSE dilution 4 d following immunization with OVA-BDCA2 (Fig. 4D). The Ly5.1+Foxp3+ Tregs from OVA-BDCA2–treated mice displayed high levels of CD25 and expressed the transcrip-
tion factor Helios, suggesting that these cells were not induced from Foxp3\(^{+}\)CD4\(^{+}\) T cells (Fig. 4E) (47). Finally, OVA-specific Ly5.1\(^{+}\) Tregs from mice that received OVA-BDCA2 displayed higher amounts of CD44 expression on day 7 p.i. compared with endogenous, non–Ag-specific Tregs, suggesting that Ag delivery to BDCA2 resulted in Ag-specific activation of Tregs (Fig. 4F).

These results demonstrate that OVA-specific Foxp3\(^{+}\)CD4\(^{+}\) T cells undergo deletion following immunization with OVA-BDCA2, whereas naturally occurring Foxp3\(^{+}\) Tregs are maintained, thereby increasing their relative frequency.

Although we noted increased cell death and fewer transferred Ly5.1\(^{+}\)CD4\(^{+}\) T cells from mice that received OVA-BDCA2, compared with endogenous, non–Ag-specific Tregs, suggesting that Ag delivery to BDCA2 resulted in Ag-specific activation of Tregs (Fig. 4F). These results demonstrate that OVA-specific Foxp3\(^{+}\)CD4\(^{+}\) T cells undergo deletion following immunization with OVA-BDCA2, whereas naturally occurring Foxp3\(^{+}\) Tregs are maintained, thereby increasing their relative frequency.

OT-II T cells in mice primed with OVA-BDCA2 were again significantly reduced in frequency (Fig. 5B, 5C) and number (Fig. 5C) following secondary Ag exposure compared with B6.BDCA2 mice primed with OVA-isotype. Furthermore, the frequency of naive CD44\(^{hi}\)CD62L\(^{lo}\) OT-II T cells was increased in OVA-BDCA2–primed mice compared with OVA-isotype–injected controls (31.0 \(\pm\) 7.9 versus 10.4 \(\pm\) 4.1%; \(p = 0.0004\)) (Fig. 5B), suggesting that OVA-BDCA2 treatment prevented subsequent Ag-specific T cell activation. In contrast, Tregs from OVA-BDCA2–primed mice displayed increased frequencies of activated CD44\(^{hi}\) CD62L\(^{lo}\) cells compared with mice primed with OVA-isotype. Similar to mice injected once with OVA-BDCA2, primed mice that received a secondary Ag challenge displayed a significantly increased frequency of Tregs due to maintenance of their numbers compared with mice primed with OVA-isotype (Fig. 5D). These results show that the decrease in Ag-specific CD4\(^{+}\) T cells induced by OVA-BDCA2, coupled with the maintenance of Tregs, resulted in a significant increase in the Treg/effectort cell (Teff) ratio in mice primed with OVA-BDCA2 (Fig. 5E).
Ag delivery to BDCA2+ pDCs results in Ag-specific tolerance induction

pDCs express several receptors with sufficiently restricted expression to facilitate Ag delivery specifically to them, including Siglec-H (13, 15, 16). To determine whether the increased frequency of Tregs seen following Ag delivery to BDCA2 is dependent on Ag uptake via BDCA2, or rather is a general property of pDCs, we compared Ag targeting to BDCA2 with targeting to Siglec-H. Although DEC205 is expressed on CD8α+ myeloid DCs (and not pDCs), we also targeted this receptor because it has been shown under some conditions to induce Tregs de novo (48, 49). Priming mice with OVA-DEC205 or OVA-BDCA2, as expected, led to significantly increased frequencies of Tregs (Fig. 5F). In contrast, priming mice with OVA-Siglec-H failed to increase Treg frequencies, which remained similar to OVA-isotype controls as reported previously (46). These results demonstrate that Ag delivery to BDCA2 leads to a different outcome than when Ag is delivered to Siglec-H, and that deletion of Ag-specific CD4+...
T cells is not a universal outcome following Ag delivery to pDCs in B6.BDCA2 mice.

Although Ag delivery to Siglec-H did not increase the frequency of Tregs, it nonetheless has been shown to inhibit Ab responses to subsequent challenge with Ag (46). The decrease in total numbers of OT-II CD4+ cells together with the maintenance of Tregs following OVA-BDCA2 treatment suggested that mice primed with OVA-BDCA2 may be unable to mount Ab responses to specific Ag. To test this possibility, we quantified OVA-specific Ab responses in mice primed with OVA-BDCA2 or OVA-isotype following exposure to Ag plus alum. Groups of B6.BDCA2 mice were primed with OVA-BDCA2, OVA-isotype, PBS, or OVA precipitated in alum, rechallenged 14 d later with OVA plus alum, and monitored weekly for OVA-specific Ab responses (see scheme in Fig. 6A). Mice primed with PBS, OVA-isotype, or OVA plus alum mounted significant anti-OVA Ab responses following the Ag boost (Fig. 6B). In contrast, anti-OVA Ab responses in mice primed with OVA-BDCA2 were significantly inhibited compared with mice primed with OVA-isotype (p < 0.0001 at day 28 p.i.) or PBS (p = 0.037 at day 28 p.i.) (Fig. 6B). The inhibition of Ab responses induced by Ag delivery to BDCA2 was Ag-specific, as mice primed with OVA-BDCA2 remained capable of mounting Ab responses to an unrelated Ag, CGG, when administered in alum (Fig. 6C). These results demonstrate that Ag uptake via BDCA2 in the absence of other costimulation induces a form of Ag-specific tolerance capable of inhibiting Ab responses.

**Tregs are required for BDCA2-mediated inhibition of Ab responses**

The mechanism underlying inhibition of Ab responses following Ag uptake by BDCA2 could be mediated by Tregs because, unlike Foxp3+ CD4+ T cells, the numbers of Tregs are maintained following treatment with OVA-BDCA2. Alternatively, the deletion of Ag-specific Foxp3+ CD4+ T cells we observed may be sufficient to prevent an Ag-specific response from occurring, which would not be expected to require Tregs. To distinguish between these two possibilities, we administered anti-CD25 mAb (PC61) to inhibit the activity of Tregs in mice that had been treated with OVA-BDCA2 or OVA-isotype (50). Mice were primed with OVA-BDCA2 or OVA-isotype, administered anti-CD25 mAb or isotype control on days −3 and −1 prior to rechallenge with OVA plus alum, and bled weekly following the rechallenge or, in some cases, analyzed 7 d after challenge for T cell responses (scheme shown in Fig. 7A).

Flow cytometry analyses of splenocytes 7 d after boost (8 d after depletion) showed that injection of anti-CD25 mAb resulted in an ~38% reduction in the frequency of Foxp3+ CD4+ T cells compared with isotype control–injected animals (Fig. 7B, 7C). Anti-CD25 treatment did not significantly affect the total number of transferred Ly5.1+ (Fig. 7D) or endogenous Ly5.1+ OT-II T cells (Fig. 7B). In OVA-BDCA2–treated mice that did not receive anti-CD25 mAb, the frequency of activated CD44hiCD4+ T cells was significantly decreased compared with OVA-isotype controls (Fig. 7E, 7F). In contrast, treatment with anti-CD25 mAb significantly increased the fraction of activated OT-II T cells in OVA-BDCA2–primed mice, but not in OVA-isotype–immunized controls. (Fig. 7E, 7F). Importantly, inhibition of Treg activity with anti-CD25 restored anti-OVA Ab responses in OVA-BDCA2–primed mice to levels similar to those of isotype control–treated mice primed with OVA-isotype (“untreated” control group) (Fig. 7G). These results demonstrate that Foxp3+ Tregs are required for the inhibition of Ag-specific CD4+ T cell activation and Ab responses induced by delivering Ag to BDCA2 on pDCs.

**Ag uptake under inflammatory conditions prevents BDCA2-mediated tolerance induction**

pDCs can be activated by a number of pathogen-sensing receptors, including TLR7 and TLR9. Administration of the TLR7 agonist R848 to B6.BDCA2 mice induced upregulation of multiple costimulatory molecules on the pDC surface, including CD80, CD86, MHC class II, and CD40. Unlike TLR7 ligation, crosslinking BDCA2 by UW80.1 mAb did not induce pDC activation and did not affect TLR7-mediated upregulation of costimulatory molecules (Supplemental Fig. 2). To determine whether TLR7 stimulation of pDCs at the time of immunization could abrogate BDCA2-mediated tolerance, B6.BDCA2 mice were immunized as in Fig. 3A with or without 50 µg R848 and analyzed for subsequent T and B cell responses. In contrast to mice primed with OVA-BDCA2 alone, the frequency and number of transferred OT-II...
7 d p.i. were not reduced in mice given R848 (Fig. 8A), but were instead maintained and similar to those in mice primed with OVA-isotype (either with or without R848). Mice given OVA-BDCA2 plus R848 had a reduced frequency, but not number, of Tregs compared with mice given OVA-BDCA2 alone (Fig. 8B). This resulted in a Treg/Teff ratio that was similar to OVA-isotype control mice and significantly greater than mice that received OVA-BDCA2 alone (Fig. 8C). Importantly, mice that received R848 together with OVA-BDCA2 mounted significant Ab responses 14 d following rechallenge with OVA plus alum, in contrast to mice primed with OVA-BDCA2 alone (Fig. 8D). We conclude from these results that administration of a TLR7 agonist at the time of initial Ag exposure prevents the deletion of Foxp3$^+$ CD4 T cells and inhibition of Ab responses induced by BDCA2-mediated Ag uptake.

**Discussion**

We found that Ag attached to an Ab against the human CLR, BDCA2, can induce Ag-specific tolerance when injected in vivo via a mechanism requiring Tregs. The data shown in the present study using a mouse model expressing human BDCA2 strongly suggest that Ag targeting to BDCA2 in humans may be useful for inducing
tolerance to prevent adverse Ab responses. Recently, Macauley et al. (51) demonstrated Ag-specific inhibition of Ab responses by induction of B cell tolerance via simultaneous engagement of BCR and the inhibitory BCR coreceptor CD22. The benefit of being able to inhibit Ag-specific Ab production was demonstrated in a model of hemophilia, where anti–factor VIII Abs prohibit replacement therapy with exogenous factor VIII. Our analogous data in the present study show that Ag-specific inhibition of Ab responses can also be achieved by delivering Ag to BDCA2 expressed by pDCs, a cell type known to possess both activating and tolerogenic properties.

The tolerogenic nature of pDCs has been demonstrated in a variety of experimental settings, including graft-versus-host disease, tumors, asthma, and rheumatoid arthritis (52–55). The induction of tolerance by pDCs has been linked in many cases with their ability to induce the differentiation or development of Tregs and/or IL-10–producing CD4+ T cells reviewed in Ref. 32. pDCs use multiple mechanisms to induce Treg differentiation and/or suppression of Teff responses, including pDC-mediated production of IDO, ICOS ligand–mediated induction of IL-10–producing T cells, and secretion of granzyme B (28, 56, 57). Thus far, induction of tolerance by pDCs has been linked in many cases to significantly suppress both CD4+ T cell activation (Fig. 5) and inhibiting their activity via anti-CD25 injection after OVA–BDCA2 priming abrogated the inhibition of Ab responses and the suppression of immune responses induced by Ag delivery to BDCA2.

We found that targeting Ag to BDCA2 in the presence of R848 (a TLR7 agonist) in vivo prevented the deletion of Foxp3+ CD4+ T cells and altered the Treg/Teff ratio such that mice were no longer tolerized and consequently were able to mount Ab responses to a secondary Ag challenge. This change in response correlated with TLR7-induced upregulation of multiple costimulatory molecules on pDCs (Supplemental Fig. 2) and supports the
notion that Ag presentation by pDCs in the absence of costimulation leads to Teff depletion. Thus, tolerance induction via Ag delivery to BDCA2 was prevented when TLR-driven inflammation was induced at the same time.

Although we have been able to demonstrate the effect of Ag targeting to a human pDC receptor in vivo, further studies are required to understand how Ag delivery to BDCA2 induces tolerance compared with Ag delivery to other receptors. Our results thus far suggest BDCA2-mediated tolerance induction may operate via a distinct mechanism compared with what occurs after Ag targeting to other receptors (1). Ag delivery to DEC205 on CD8α+ DCs, when administered in low doses in the absence of adjuvant, induces CD4+ T cell tolerance mediated by de novo induction of Tregs (48). We did not detect de novo induction of Tregs after targeting Ag to BDCA2, yet the frequencies of Foxp3+ Tregs and the Treg/Teff ratio in mice primed with OVA-BDCA2 were similar to those induced by OVA-DEC205 administration (Fig. 5).

Loschko et al. (46) recently showed that Ag delivery to pDC-targeting Ag to BDCA2, yet the frequencies of Foxp3+ Tregs and the Treg/Teff ratio in mice primed with OVA-BDCA2 were similar to those induced by OVA-DEC205 administration (Fig. 5). Loschko et al. (46) recently showed that Ag delivery to pDC-targeting Ag to BDCA2, yet the frequencies of Foxp3+ Tregs and the Treg/Teff ratio in mice primed with OVA-BDCA2 were similar to those induced by OVA-DEC205 administration (Fig. 5).

In conclusion, our data show that Ag delivery to BDCA2 expressed by pDCs has the ability to promote Ag-specific immune tolerance by altering the balance between Teff and Treg populations. Deletion of Ag-specific Foxp3+ CD4+ T cells and simultaneous maintenance of naturally occurring Tregs were both required for BDCA2-mediated tolerance induction, a mechanism that differs from that described for tolerance induction following Ag delivery to Siglec-H and DEC205 (46, 48). It is noteworthy that recently a similar shift toward an increased ratio of CD4+ Tregs/Teffs was found in type 1 diabetes patients treated in a phase 2 clinical trial with a CD2 blocker, alefacept (LFA3-Ig) (66). The authors suggested that “By targeting the most pathogenic T cells, while sparing Tregs, alefacept might contribute to reestablishing a state of immune tolerance.” It will be of interest to determine whether an Ag-specific BDCA2-based therapeutic is beneficial for treating certain autoimmune diseases. BDCA2-mediated tolerance induction could be abrogated by inhibiting the activity of Foxp3+ Tregs, or prevented if deletion of Foxp3+ CD4+ T cells was mitigated by coadministration of a TLR7 agonist at the time of immunization. Thus, Ag uptake by BDCA2 under inflammatory conditions (TLR stimulation) may lead to immune activation versus tolerance. These data contribute to the growing body of evidence demonstrating pDCs as a powerful subset of cells that possess strong immune modulating properties, making them attractive targets for future therapies aimed at manipulating immune responses.

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Disclosures
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References


