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Preferential Induction of CD4+ T Cell Responses through In Vivo Targeting of Antigen to Dendritic Cell-Associated C-Type Lectin-1

Robert W. Carter, Clare Thompson, Delyth M. Reid, Simon Y. C. Wong, and David F. Tough

Targeting of Ags and therapeutics to dendritic cells (DCs) has immense potential for immunotherapy and vaccination. Because DCs are heterogeneous, optimal targeting strategies will require knowledge about functional specialization among DC subpopulations and identification of molecules for targeting appropriate DCs. We characterized the expression of a fungal recognition receptor, DC-associated C-type lectin-1 (Dectin-1), on mouse DC subpopulations and investigated the ability of an anti-Dectin-1 Ab to deliver Ag for the stimulation of immune responses. Dectin-1 was shown to be expressed on CD8α−CD4−CD11b+ DCs found in spleen and lymph nodes and dermal DCs present in skin and s.c. lymph nodes. Injection of Ag-anti-Dectin-1 conjugates induced CD4+ and CD8+ T cell and Ab responses at low doses where free Ag failed to elicit a response. Notably, qualitatively different immune responses were generated by targeting Ag to Dectin-1 vs CD205, a molecule expressed on CD8α−CD4−CD11b+ DCs, dermal DCs, and Langerhans cells. Unlike anti-Dectin-1, anti-CD205 conjugates failed to elicit an Ab response. Moreover, when conjugates were injected i.v., anti-Dectin-1 stimulated a much stronger CD4+ T cell response and a much weaker CD8+ T cell response than anti-CD205. The results reveal Dectin-1 as a potential targeting molecule for immunization and have implications for the specialization of DC subpopulations. The Journal of Immunology, 2006, 177: 2276–2284.

S
ince dendritic cells (DCs) are key APCs for the initiation and regulation of immune responses, strategies for targeting these cells in vivo have potential for use in various clinical settings. One of these is vaccination, the efficiency of which can be enhanced by optimizing Ag delivery to DCs. This can be done by using Abs specific for DC-expressed molecules as in vivo Ag carriers; using such an approach, a lowered requirement for Ag dose in stimulating immune responses in mice has been observed after targeting a variety of molecules, including MHC class II, CD11c, CD205, CD80/86, F4/80-like receptor (FIRE), CIRE, and the undefined Ag recognized by the mAb 33D1 (1–9).

In addition, in vitro studies have shown that Abs specific for the mannose receptor or DC-SIGN can effectively deliver Ag to human DCs, indicating that this strategy will also be applicable to human vaccination (10, 11). Although these reports have illustrated the general effectiveness of DC targeting as a strategy for vaccination, there is currently limited information on whether immune responses may be triggered differently through distinct targeting receptors. In support of this idea, though, it was shown that Abs against FIRE or CIRE triggered (anti-Ig) Ab responses when injected in the absence of adjuvant, while anti-CD205 failed to do so (9).

Responses elicited by targeting distinct DC surface molecules may differ for several reasons. First, if targeting molecules are also expressed on cell types other than DCs, the amount of Ag delivered to the DCs could be reduced, and other targeted cells could influence the immune response. Second, surface molecules may vary in their capacity to mediate internalization of bound Ab and to direct Ag into processing and presentation pathways. Third, binding of some cell surface molecules might directly trigger signals in DCs. Fourth, the molecules targeted might be expressed by only a subset of DCs, which may differ in their ability to present Ag and stimulate immune responses. Therefore, investigation of strategies for targeting Ag in vivo can not only aid in the development of vaccination approaches but can also help to enhance understanding of basic principles relating to the regulation of immune responses.

DCs are phenotypically heterogeneous in vivo, and there is accumulating evidence that DC subpopulations play different roles in the immune response (12). Seemingly distinct subpopulations of DCs identified in mouse lymphoid organs include: plasmacytoid pre-DCs (13), which are specialized in the production of large quantities of type I IFN; TNF-α- and inducible NO synthase-producing DCs (14); and the recently described IFN-producing killer DCs, which share properties of IFN-producing cells, NK cells, and DCs (15, 16). Although these subsets appear to play important roles in innate pathogen defense, their ability to take up and process Ags and act as APCs for naive T cells in vivo is unclear. In addition to these, there are five populations of “conventional” DCs, which are thought to be more directly involved in Ag presentation (17). Three populations of conventional DCs are found in both spleen and lymph node (LN), and these are typically divided on the basis of CD8α and CD4 expression into CD8α+CD4+, CD8α−CD4+, and CD8α−CD4− subsets. Due to their presence in spleen, these subsets have been referred to as “blood-derived” DCs, although whether they enter spleen and LN from the blood as...
precursors or as differentiated DCs is currently unknown (12). Additional DCs, which arrive via afferent lymph from tissues, are present in LN. Two tissue-derived populations, representing dermal DCs and epidermal DCs (or Langerhans cells (LC)) can be distinguished in s.c. LN (17).

Most evidence for functional specialization among DC subpopulations has come from comparisons between CD8α+ and CD8α− (including both CD4+ and CD4−) DCs. In such studies, CD8α+ DCs have been shown to be much more effective than CD8α− DCs in cross-presenting soluble or cell-associated Ag on MHC class I, and these cells appear to be responsible for both cross-priming and cross-tolerance in vivo (18–20). CD8α+ DCs were also found to play a major role in priming CD8+ T cell responses after infection with various viruses, as well as intracellular bacteria (21–23). In addition to comparisons of CD8α+ and CD8α− DCs, some recent studies have focused on defining a role for skin-derived APCs. Although this work has thus far yielded largely negative data regarding a role for LC in priming specific immune responses, results supporting a role for dermal DC in stimulating CD4+ T cell responses after intradermal or mucosal administration of Ag have been reported (24, 25). Notwithstanding these findings, however, it remains unknown whether many of the phenotypically defined DC subpopulations have specific and unique functions.

One way in which DC heterogeneity could be linked with functional specialization is through differential expression of innate receptors, which might lead to differences in pathogen recognition; there is evidence that this is the case. For instance, DC subsets in humans and mice have been shown to express different combinations of TLR family members (26, 27). In addition, DCs are heterogeneous in their expression of C-type lectin receptors (CLRs), which recognize specific carbohydrate structures and are frequently involved in Ag uptake on APCs (28). Examples of CLRs showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 showing differential expression on DC subpopulations include DEC-205 (CD205), which is expressed by CD8 -Dendritic cell-associated C-type lectin-1 (Dectin-1) is an NK cell receptor-like CLR, originally thought to be DC specific (31) but subsequently demonstrated to be expressed on other cells, including certain macrophage populations, neutrophils, and monocytes (32, 33). Dectin-1 has been shown to be a major receptor for β-glucan on macrophages and bone marrow-derived DCs (34, 35), recognizing β1,3- and β1,6-linked glucans found in yeast cell walls (36). Additionally, Dectin-1 is also thought to recognize an unidentified endogenous ligand expressed on T cells, and bacterially produced soluble Dectin-1 has been shown to costimulate anti-CD3-induced T cell proliferation in vitro (31). Dectin-1 can recognize several fungal species, and has recently been shown to play a role in defense against Aspergillus fumigatus in mice (37). This is associated with an ability of Dectin-1 to mediate internalization of β-glucan-containing ligands, including yeast-derived particles and live yeast (38, 39). In addition, cross-linking Dectin-1 by β-glucan-containing particles triggers intracellular signaling, leading to the production of various cytokines and chemokines, generation of reactive oxygen species, activation of phospholipase A2 and enhancement of cyclo-oxygenase 2 expression (35, 38, 40–44). Dectin-1 is also able to initiate signaling based on the presence of an ITAM in its cytoplasmic domain, which is unusual among CLRs (31, 35). Thus, based on its ability to recognize fungal pathogens and initiate an inflammatory response, Dectin-1 has the characteristics of a pattern recognition receptor that could serve to link innate and adaptive immunity. At present, however, it is unknown whether Dectin-1 might play a direct role in targeting fungal Ags for presentation to T or B cells.

We have investigated the use of Dectin-1 as a targeting molecule for the induction of immune responses. Phenotypic analysis demonstrated that Dectin-1 was expressed by two subpopulations of conventional DCs, namely CD8α+ CD4− DCs and dermal DCs. Low doses of Ag conjugated to anti-Dectin-1 Ab elicited immune responses when injected into mice, indicating that this strategy effectively targeted Ag to APCs in vivo. Notably, the immune response stimulated by Ag-anti-Dectin-1 conjugates differed qualitatively from that induced by Ag-anti-CD205, suggesting specialized roles for APCs expressing these molecules.

Materials and Methods

Mice

B6, B6CD45.1, OT-I, and OT-II mice were bred in the specific pathogen-free facility at the Institute for Animal Health (Compton, U.K.). All animal experimentation was reviewed and approved by the Home Office and the ethical review committee of the Institute for Animal Health.

Antibodies

The following hybridoma cell lines were used in this study: III/10 (IgG2a, isotype control; provided by R. Steinman, Rockefeller University, New York, NY), NLDC145 (IgG2a, anti-CD205; provided by K. Shortman, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia), YTH913 (IgG2b, isotype control; provided by P. Beverley, Edward Jenner Institute for Vaccine Research, Compton, Berkshire, U.K.), and 2A11 (IgG2b, anti-Dectin-1 (32)). All cell lines were cultured in RPMI 1640 plus glutamine (Invitrogen Life Technologies) with 10% FCS (Harlan SeraLab), 100 U/ml penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Invitrogen Life Technologies), and 1 mM sodium pyruvate (Invitrogen Life Technologies). Each Ab was purified from cell culture supernatants using separate protein G columns (Amersham Biosciences) according to the manufacturer’s protocol.

DC purification

Cells were isolated from spleen and LN by digestion in 1 mg/ml collagenase D (Worthington Biochemical) and 0.5 mg/ml DNase 1 (Sigma-Aldrich) for 30 min at room temperature. EDTA (0.1M) was added for the final 5-min incubation. Single-cell suspensions were then subjected to anti-CD11c-positive selection using mAb-coated MACS beads (Miltenyi Biotec) according to the manufacturer’s protocol.

T cell isolation

OVA-specific CD8+ and CD4+ T cells were isolated from OT-I and OT-II TCR-transgenic mice, respectively (both B6, CD45.2 background). Single-cell suspensions of spleen cells were obtained and incubated with mAbs against B220, class II and CD4 or CD8, followed by anti-rat IgG beads (Dynal) to remove unwanted cells.

Flow cytometry

Isotype control, anti-CD205, and anti-Dectin-1 Abs were conjugated to AlexaFluor 488 and AlexaFluor 647 fluorochromes using Ab labeling kits (Molecular Probes) according to the manufacturer’s protocols. For extracellular staining, cells were washed in wash buffer (PBS/1% FCS/0.1% sodium azide) and surface stained with a range of fluorochrome-conjugated mAbs (BD Biosciences). Cells were then washed and fixed in 1% paraformaldehyde. For staining of intracellular langerin, cells were first stained for surface cell markers followed by fixation and permeabilization using Cytofix/Perm solution (BD Biosciences). Cells were then washed using perm/wash buffer (BD Biosciences). Anti-langerin mAb (clone 306G9.01; AbCys) was incubated with cells in the presence of perm/wash solution. Cells were washed in perm/wash buffer, then wash buffer before fixation in 1% paraformaldehyde. Stained cells were acquired using a FACSCalibur and events were analyzed using WinMDI software.

Immunohistochemistry

Frozen sections of mouse skin (5 μM) were cut using a Leica CM1900 cryostat (Leica Microsystems), mounted on precleaned slides, fixed in ethanol and air dried before use. Sections were rehydrated in PBS and blocked in 10% normal rabbit serum. Sections were washed in PBS and then incubated with biotinylated rabbit anti-rat IgG (Vector Laboratories) for 30
min. Slides were washed in PBS and Ab binding was detected using the avidin-biotin-complex-HRP reagent (Vector Laboratories). Slides were counterstained with hematoxylin (Vector Laboratories), dehydrated with alcohol, cleared with histoclear (Fisher Scientific), and mounted with DPX (Fluka Chemical).

**Ab-OVA conjugation**

Monovalent Ab was conjugated to maleimide-activated OVA as previously described (8). Briefly, monovalent Ab was obtained by reduction using 2-mercaptoethanesulfonic acid sodium salt (Sigma-Aldrich) for 30 min at 37°C and the OVA (Sigma-Aldrich) activated with succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce). Both were then desalted and incubated together overnight at 4°C. OVA-conjugated Ab was then obtained by protein G purification.

**Western blotting**

Ab conjugated to OVA (0.5 μg) was subjected to SDS-PAGE using a 7.5% polyacrylamide gel under nonreducing conditions. Protein was transferred to nitrocellulose (Hybond; C, Amersham Biosciences) using semidry electrophoresis. Membranes were blocked in 10% milk powder/TBS-0.1% 2-mercaptoethanesulfonic acid sodium salt (Sigma-Aldrich) for 30 min at 37°C and the OVA (Sigma-Aldrich) activated with succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce). Both were then desalted and incubated together overnight at 4°C. OVA-conjugated Ab was then obtained by protein G purification.

**Ab injections and immunizations**

Abs were injected s.c. at the base of the tail or i.v. via the tail vein. Normal B6 mice were immunized by injection of 1 μg of OVA-Ab conjugate or unconjugated OVA plus 100 μg of poly I:C as indicated. For adoptive transfer experiments, 2 × 10⁶ CD8⁺ T cells or CD4⁺ T cells from OT-I or OT-II-transgenic mice, respectively, were injected i.v. or s.c. with 1 μg of OVA-Ab conjugates plus 100 μg of poly I:C or with poly I:C alone (naïve control). To evaluate expansion of OVA-specific cells, OT-I or OT-II cells were enumerated by flow cytometry, using Abs to CD45.2 and CD4 or CD8. To assess Ag-specific cytokine production, total splenocytes or inguinal LN cells from mice immunized 3 days previously were cultured in vitro with the CD8⁺ I-OT-I-specific class I-restricted OVA257–264 peptide or the CD4⁺ I-OT-II-specific class II-restricted OVA275–282 peptide. Cytokines were detected in the culture supernatant after 3 days of culture using cytokine bead assay and flow cytometry (see below).

**IFN-γ ELISPOT**

A 96-well ELISPOT plate (Millipore) was incubated overnight at 4°C with a rat anti-mouse IFN-γ capture Ab (final concentration 10 μg/ml; BD Biosciences) in sterile PBS. On the following day, the plate was washed with PBS and blocked with cell culture medium (RPMI 1640, 10% FCS, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate) for 2 h at 37°C. Total splenocytes (5 × 10⁶/well) in 100 μl of medium were added to each well containing 2 μM of either OVA257–264 peptide (OVA-specific CD8⁺ T cell responses) or OVA275–282 peptide (OVA-specific CD4⁺ T responses). Medium alone with no added peptide served as a negative control. After 24 h of incubation at 37°C in 5% CO₂, wells were washed with PBS/0.01% Tween 20. Ab binding was detected with a biotinylated rat anti-IFN-γ detection Ab (2 μg/ml; BD Pharmingen), followed by streptavidin-HRP. Spots were developed by incubation with 3-aminophenylborazole HRP substrate (Calbiochem).

**Cytokine bead array**

Cytokine levels were analyzed in the supernatants of cell cultures using the mouse Th1/Th2 and mouse inflammation cytokine bead array kits as according to the manufacturer’s protocols (BD Biosciences).

**Anti-OVA Ab ELISA**

Maxi-sorb ELISA plates (Nunc) were coated overnight with 10 μg/ml OVA. Plates were washed and blocked in 2% milk powder/PBS-0.05% Tween 20. Sera collected from the clotted blood of immunized mice were serially diluted 1/50 to 1/102,400 in 2% milk powder/PBS-0.05% Tween 20 and incubated overnight at 4°C. Diluted sera were then washed off the plates and Ab binding was detected using peroxidase-conjugated anti-mouse IgG (BD Biosciences) followed by tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories).

**Results**

**Dectin-1 is expressed by CD8α⁻CD4⁺CD11c⁺ DC and dermal DC**

To characterize Dectin-1 expression on splenic DCs, CD11c⁺ cells were purified from the spleens of normal B6 mice and labeled with mAbs against various cell surface markers in vitro. A comparison of anti-Dectin-1 and anti-CD205 Ab staining revealed that these two molecules were expressed by completely distinct CD11c⁺ cell populations, with each Ab labeling ~15% of cells (Fig. 1A). Whereas CD205 showed its known pattern of expression as a marker of CD8α⁺ (i.e., CD8α⁺, CD4⁺, CD11b⁻⁻) splenic DC, the Dectin-1⁺ CD205⁻ population was found to be CD8α⁻, CD4⁻, and CD11b⁺⁻. Note that in contrast to a previous report (33), we could not detect Dectin-1 expression on CD4⁺ DCs. Therefore, Dectin-1 appears to be expressed exclusively by CD8α⁻CD4⁺ DCs in the spleen.

Similar in vitro staining of CD11c⁺ DCs isolated from s.c. (inguinal) LN revealed a more complicated expression pattern for Dectin-1 (Fig. 1B). Thus, in addition to populations of Dectin-1⁺ CD205⁻ and Dectin-1⁺ CD205⁺ cells (each ~22% of LN CD11c⁺ cells), a substantial (~30%) proportion of CD11c⁺ cells labeled with both Abs. Dectin-1⁺ CD205⁻ cells were CD8α⁺ and CD4⁻ as seen in the spleen (Fig. 1B and data not shown). However, unlike in the spleen, these cells included both CD11bhigh and CD11blow cells. This suggested that in addition to the blood-derived CD8α⁺CD4⁻ subset common to spleen and LN, Dectin-1 was also expressed on some tissue-derived DCs in the s.c. LN. In support of this idea, staining with anti-I-A⁻ Abs showed that a
The proportion of Dectin-1^+CD205^- cells was MHC class II^{high}, indicative of tissue-derived DCs (17). These cells did not label with anti-langerin Abs, suggesting that they were dermal DC rather than LC.

Dectin-1^+CD205^- cells were similar to Dectin-1^+CD205^- cells in expression of most of the other markers analyzed, but differed in that nearly all expressed high levels of MHC class II (Fig. 1B). This indicated that these double-positive cells largely represented immigrants from tissues, and is consistent with the absence of this population in the spleen. Like Dectin-1^+CD205^- cells, Dectin-1^+CD205^- cells were langerin^- . In agreement with published reports on the expression of CD205 on s.c. LN DCs (17), the Dectin-1^+CD205^- population included both CD8α^+ and CD8α^- cells, as well as a substantial proportion of MHC class II^{high} and langerin^- cells.

Taken together, this phenotypic analysis showed that Dectin-1 was expressed by at least two distinct subpopulations of DCs: CD8α^-CD4^-CD11b^{high} blood-derived DCs and dermal DCs. Of the latter, some appeared to coexpress CD205, while others were CD205^-.

**Anti-Dectin-1 and anti-CD205 target distinct DCs in vivo**

To investigate whether anti-Dectin-1 Ab could serve as a targeting tool for eliciting immune responses in vivo, we determined whether anti-Dectin-1 reached DCs after injection into mice. Anti-Dectin-1 and anti-CD205 Abs, labeled with distinct fluorochromes, were coinjected s.c. into normal mice, and CD11c^- DCs in the draining LN were analyzed 24 h later for the presence of bound Ab. Because we wished to assess anti-Dectin-1 targeting under immunizing conditions, poly IC was also included in the inoculum as an adjuvant. As with in vitro staining, prominent populations of CD11c^- cells exhibiting mutually exclusive labeling with anti-Dectin-1 (22%) or anti-CD205 (18%) were observed (Fig. 2A). Unlike in vitro staining, however, only a small population of cells (5%) that bound both Abs was detected. This appeared to be due to relatively poor targeting of the Dectin-1^+CD205^- population by the Abs in vivo rather than an effect of poly IC on the presence of the cells, because ex vivo staining of s.c. LN DCs from poly IC-injected mice revealed a prominent double-positive cell population, while coinjection of anti-Dectin-1 and anti-CD205 Abs into non-poly IC-treated mice showed little double labeling of DCs (data not shown). The reason for this is not clear, but it is possible that the anatomical distribution of Dectin-1^+CD205^- DCs is such that s.c.-injected Abs is not efficiently delivered to these cells. Expression of other markers (as in Fig. 1) by Dectin-1^- and CD205^- populations was identical with that observed after in vitro staining (data not shown), indicating that the Abs were targeting the same populations in vivo.

We also examined the localization of Ab in the skin following s.c. injection (Fig. 2B). Clearly distinct patterns of distribution were observed for anti-Dectin-1 and anti-CD205 Abs. After injection of anti-CD205, strong labeling of cells in the epidermis and weaker staining in the dermis was detected. This is in accordance with the reported higher expression of CD205 on LC than dermal DC (17). Conversely, labeling was observed in the dermis but not the epidermis after injection of anti-Dectin-1, which is consistent with our earlier results indicating a lack of Dectin-1 on LC.

Finally, we analyzed labeling of DCs in the spleen after coinjection of fluorescently labeled anti-Dectin-1 and anti-CD205 i.v. As shown in Fig. 2C, anti-Dectin-1 and anti-CD205 bound to distinct populations of CD11c^- cells. Dectin-1^- cells were CD8α^-CD4^-, while CD205^- cells were CD8α^-CD4^- (data not shown). Overall, therefore, the data indicate that anti-Dectin-1 can serve as targeting tool to deliver Ags to DCs in vivo.

**Induction of immune response by injection of anti-Dectin-1-conjugated Ag**

Using a method previously employed to couple Ag to anti-CD205 (8), OVA was conjugated to anti-Dectin-1, anti-CD205, and two different isotype control Abs (IgG2b for anti-Dectin-1; IgG2a for anti-CD205). In this procedure, mild reduction of Abs yields monovalent IgG to which OVA is coupled via a bivalent linker. Abs were purified after conjugation and analyzed by Western blotting with anti-OVA (Fig. 3). Based on molecular weight, the procedure appeared to produce conjugates with two OVA molecules per Ab for the IgG2b isotype control, anti-Dectin-1, and anti-CD205 Abs. Conversely, for the IgG2a isotype control Ab, the molecular weight corresponded to that of a conjugate with a single OVA molecule per Ab. This may be due to the characteristics of the specific control Ab, as similar differences in molecular weight from OVA-anti-CD205 were observed in a previous report (8).

Normal B6 mice were immunized by a single s.c. injection of 1 μg of free OVA or 1 μg of the OVA-Ab conjugates, together with 100 μg of poly I:C. Anti-OVA CD8^- and CD4^- T cell and Ab responses were measured 8 days later (Fig. 4). At this dose, free OVA failed to induce a significant T cell or Ab response. Similarly, no response was detectable after injection of OVA conjugated to either isotype control Ab (Fig. 4), or when free OVA was
simply mixed together with isotype controls, anti-Dectin-1 or anti-CD205 (data not shown). In contrast, both CD8\(/H11001\) and CD4\(/H11001\) T cell responses were induced after injection of OVA-anti-Dectin-1 or OVA-anti-CD205 conjugates. Interestingly, an OVA-specific Ab response was observed after injection of OVA-anti-Dectin-1, but not OVA-anti-CD205. Therefore, the data indicated that directing Ag to Dectin-1 was an effective means of inducing an immune response, and suggested that targeting Dectin-1 and CD205 elicit qualitatively distinct responses.

Differential stimulation of CD4\(/H11001\) and CD8\(/H11001\) T cells by targeting to Dectin-1 vs CD205

To further investigate the immunological consequences of targeting Ags to Dectin-1, we used an adoptive transfer system in which OVA-specific TCR-transgenic T cells were used as responders. CD8\(/H11001\) or CD4\(/H11001\) T cells were purified from OT-I or OT-II mice respectively and injected into CD45 congenic mice. Recipients were left unimmunized or injected either s.c. or i.v. with 1 \(\mu\)g of OVA-anti-Dectin-1 or OVA-anti-CD205 (plus poly I:C) (Figs. 5 and 6). At different times after immunization, the effectiveness of priming was assessed in two ways (1). Transgenic cells in the inguinal LN (after s.c. injection) or spleen (after i.v. injection) were enumerated (2). LN or spleen cells were restimulated in vitro with specific OVA peptides for 3 days and the production of various cytokines measured. Note that by either measurement, responses in mice given OVA-isotype controls were equivalent to that in unimmunized mice (data not shown).

Using the OT-I adoptive transfer model, it was evident that OVA-anti-CD205 elicited a stronger CD8\(/H11001\) T cell response than OVA-anti-Dectin-1 (Fig. 5). After s.c. immunization, OVA-anti-CD205 injection stimulated a somewhat greater expansion of OT-I cells (apparent at day 8), although both Ab conjugates induced similar priming for cytokine production (Fig. 5B). However, a clear disparity in the response induced by the two Ab conjugates was observed when immunization was done by i.v. injection.

**FIGURE 3.** Detection of OVA in OVA-Ab conjugates. OVA conjugation to isotype control, anti-CD205 and anti-Dectin-1 Abs was assessed by Western blotting. OVA-conjugated Abs (0.5 \(\mu\)g) were subjected to SDS-PAGE under nonreducing conditions and transferred to nitrocellulose membranes. OVA was detected by sequential exposure to an anti-OVA polyclonal Ab, peroxidase-conjugated anti-rabbit Ig, and enzyme substrate.

**FIGURE 4.** Stimulation of anti-OVA T cell and B cell responses by OVA-Ab conjugates in vivo. B6 mice were injected s.c. with 1 \(\mu\)g of OVA-Ab conjugates, or unconjugated OVA, plus 100 \(\mu\)g of poly I:C. A and B, IFN-\(\gamma\) ELISPOT analysis of OVA-specific CD8\(/H11001\) (A) and CD4\(/H11001\) (B) T cell responses in the spleen 8 days after immunization. Results are from a total of six mice per group from two independent experiments. C, Endpoint titers of OVA-specific Abs in the serum 8 days after immunization. Results are expressed using a box and whisker plot and data are from a total of 15 mice per group using data from five independent experiments. Boxes represent the interquartile range of data between the 25th and 75th percentile and whiskers represent the upper and lower limits of the data. The dividing line in the box represents the median, means are indicated by a solid circle (\(\bullet\)) and outlier points are represented by an asterisk (\(*\)).
this route, OVA-anti-CD205 stimulated both a much larger expansion of OT-I cells and much greater priming for cytokine production, particularly for IFN-γ and IL-2 (Fig. 5A). Most interestingly, very different results were observed when CD4⁺ T cell priming was examined using the OT-II adoptive transfer model. The two conjugates again elicited similar responses when injected sc., with slightly higher IL-2 production observed after immunization with OVA-anti-Dectin-1 (Fig. 6B). By contrast, OVA-anti-Dectin-1 induced a much stronger CD4⁺ T cell response than OVA-anti-CD205 when the Abs were injected iv. (Fig. 6A). Both OT-II cell expansion and priming for cytokine production was greater with OVA-anti-Dectin-1; cytokines that showed higher production included IFN-γ, IL-2, IL-10, IL-5, IL-6, and TNF-α. Thus, after iv. injection, there was a clear difference in the type of response induced by targeting Dectin-1 vs CD205, which preferentially stimulated CD4⁺ vs CD8⁺ T cells, respectively.

Given the difference in the ability of the two Abs to prime T cells when given iv., we also compared the induction of anti-OVA Ab responses when the conjugates were administered sc. vs iv. to normal B6 mice (Fig. 7). No anti-OVA Ab response was observed after injection of OVA-anti-CD205 by either route. In contrast, OVA-anti-Dectin-1 stimulated an Ab response whether given sc. or iv. and, interestingly, a higher response was induced after i.v. immunization.

**Discussion**

We have investigated the expression of Dectin-1 by DC subpopulations in mouse lymphoid organs and the use of Dectin-1 as a targeting molecule for the stimulation of immune responses. The data show that Dectin-1 is expressed by two main subsets of DCs: CD8α⁺ CD4⁺ CD11b⁺ high⁺ blood-derived DCs that are present in spleen and LN, and dermal DCs present in s.c. LN. On the latter population, Dectin-1 expression partially overlapped with that of CD205; substantial numbers of Dectin-1⁺ CD205⁺ DCs were detected in s.c. LN, although some Dectin-1⁺ dermal DCs appeared to be CD205⁻. Thus, Dectin-1 appears to have a unique distribution pattern among DC markers so far described, which may aid in the study of DC subpopulations. In particular, its expression on CD8α⁺ CD4⁻ DCs in the spleen might serve as a useful tool for analyzing the function of these cells. Given that Dectin-1 is a receptor for β-glucan, it would be of interest to investigate whether CD8α⁺ CD4⁻ DCs make a particular contribution to antifungal immunity.
Dectin-1 was shown to be an effective targeting molecule for immunization, with conjugation of Ag to anti-Dectin-1 Abs allowing for the stimulation of CD4+ and CD8+ T cell and Ab responses at low doses of Ag. This is the first demonstration that Abs against a fungal recognition receptor can mediate the delivery of Ag for presentation to the immune system. Future studies to determine whether Dectin-1 plays a similar role in Ag presentation following DC encounter with fungal pathogens will be important. In as much as it enhanced the efficiency of immunization, Dectin-1 was similar to a variety of other DC expressed molecules which have been targeted for successful delivery of Ag to APCs. However, this study showed that targeting to different molecules can induce qualitatively different immune responses. Specifically, anti-Dectin-1 favored the stimulation of a CD4+ T cell response whereas anti-CD205 preferentially triggered a CD8+ T cell response. This distinction was not very apparent after s.c. immunization, but was clear when Ab conjugates were injected i.v., which could be related to the distribution of Dectin-1 and CD205 on DCs. Because Dectin-1 and CD205 are both expressed by dermal DCs, these DCs could be involved in T cell priming after s.c. injection of either conjugate. Conversely, i.v. injection of conjugates is likely to target completely separate DC subsets in the spleen. Therefore, a possible explanation for the observed results is that CD8α−CD4− DCs are specialized for initiation of CD4+ T cell responses, CD8α+CD4− DCs selectively trigger CD8+ T cells, while dermal DCs are able to elicit both CD4+ and CD8+ T cell responses. In support of this idea, a previous study showed that CD8α−CD4− and CD8α+CD4− splenic DCs, isolated from mice that had received soluble Ag via i.v. injection, preferentially presented Ag in association with MHC class II or MHC class I, respectively (19). Thus, biased stimulation of CD4+ and CD8+ T cells by these DC subpopulations could reflect a strong tendency for CD8α−DCs to cross-present Ag on MHC class I and an inability of CD8α−CD4− DCs to do so. Whether differences in antigenic peptide presentation could account fully for the biased stimulation of T cells is unclear, because CD8α+ DCs are also capable of presenting Ag on MHC class II (45).

Apart from DC subset specialization, other factors might contribute to the different responses induced by Dectin-1- vs CD205-targeted Ag. First, Abs bound to Dectin-1 and CD205 on the surface of APCs might be targeted to different intracellular compartments and hence different presentation pathways. However, published data indicates that CD205 can deliver Ag to both MHC class I and MHC class II presentation pathways (46). Moreover, the observed induction of CD4+ and CD8+ T cell responses after s.c. injection of either Ab conjugate implies that Dectin-1- and CD205-targeted Ags can enter both presentation pathways in vivo. Therefore, the processing/presentation pathway to which the Ag is delivered may depend more on the specific APC than the CLR targeted. Second, because Dectin-1 contains an ITAM, Abs against this molecule might trigger signals in APCs that modify the immune response. This seems unlikely to take place under the conditions used in these experiments, given that the targeting Abs were used in monovalent form. Consistent with a failure of the Abs to induce signaling, we found no evidence that injection of OVA-anti-Dectin-1 affected cytokine expression by DCs (data not shown). Also, it should be noted that no response was observed when either anti-Dectin-1- or anti-CD205-OVA conjugates were injected in the absence of adjuvant, arguing against overt DC activation by anti-Dectin-1. Nevertheless, it cannot be excluded that monovalent anti-Dectin-1 Abs in some way modify signaling within APCs. Third, because Dectin-1 is expressed by cells other than DCs, targeting of Ag to these cells could modify the immune response. Thus, while presentation of Ag by DCs is likely necessary to initiate the T cell response, subsequent recognition of Ag on other APCs such as macrophages could influence the outcome.

In addition to preferential induction of a CD4+ T cell response, OVA-anti-Dectin-1 conjugates differed from OVA-anti-CD205 in stimulating an OVA-specific Ab response. However, it should be mentioned that injection of anti-CD205 has previously been reported to induce an Ab response (9). The protocol used for immunization in the earlier study differed from ours in several ways. First, a 5-fold higher dose of anti-CD205 was injected. Second, intact bivalent Abs rather than OVA-conjugated monovalent Abs were used for immunization. Third, an anti-rat Ig rather than anti-OVA response was measured. Fourth, oligodeoxynucleotides containing stimulatory CpG motifs rather than poly I/C were used as an adjuvant. Which of these factors was most important in allowing for the detection of an Ab response after injection of anti-CD205 is unknown. With regard to the use of an alternate adjuvant, it is relevant that CD8α− (CD205−) DCs are reported to express the highest amount of TLR3 among splenic DCs, while CD8α+CD4− DCs express only slightly lower levels of this TLR (27). Therefore, the DC populations targeted by anti-CD205 and anti-Dectin-1 are both likely to receive direct activation signals after injection of poly I/C. Nevertheless, it was recently reported that an OVA-anti-CD205 fusion protein elicited T cell help for Ab responses when injected with a combination of anti-CD40 and poly I:C but not with poly I:C alone (47). Hence, the type of immune response triggered by a given DC subset can be influenced by the specific maturation signals it receives. Whatever the reasons, the fact that anti-CD205 can elicit an Ab response under certain conditions indicates that the difference between Dectin-1 and CD205 in targeting for Ab production is relative rather than absolute.

The efficient induction of Ab responses by targeting to Dectin-1 could be linked to its ability to generate strong CD4+ T cell responses. However, since OVA-anti-Dectin-1 and OVA-anti-CD205 stimulated CD4 responses of a similar magnitude after s.c. immunization, this is unlikely to be the full explanation. One possibility is that APCs targeted by anti-CD205 selectively failed to stimulate effective T cell help for Ab production. Similar overall profiles of cytokine production were observed after s.c. injection of OVA-anti-Dectin-1 or OVA-anti-CD205 with the notable exception of IL-2, which was produced in higher amounts after immunization with OVA-anti-Dectin-1. After i.v. injection of conjugates, OVA-anti-Dectin-1 primed for greater production of all
cytokines, including several (IL-6, IL-5, IL-10) that could be relevant for promotion of Ab responses. This probably reflects the quantitative difference in the CD4+ T cell response induced by the two OVA-Ab conjugates. However, we also observed continued high production of IL-2 8 days after i.v. injection of OVA-anti-Dectin-1 (data not shown), a time when OT-II cell numbers in the spleen had already decreased substantially (Fig. 6A). Of interest, cross-linking of Dectin-1 on the surface of bone marrow-derived DCs or Dectin-1-transfected B hybridoma cells by yeast-derived zymosan particles has been shown to stimulate secretion of IL-2 and IL-10 (41). Therefore, it is possible that binding of OVA-anti-Dectin-1 to APCs contributes directly to priming for cytokine production. Whatever the mechanism, the observation that OVA-anti-Dectin-1 evoked a bigger Ab response when given i.v. rather than s.c. suggests that blood-derived CD8α−CD4+ DCs may be the Dectin-1+ APC population responsible for driving the generation of T cell help for B cells.

Differential induction of the Ab response might also be related to the ability of the targeting molecules to make the Ag available to B cells. Importantly, Dectin-1 was not detected on B cells and therefore did not appear to be targeting these cells directly (data not shown). Provision of Ag to B cells could be affected by the anatomical locations where Dectin-1+ and CD205+ APCs reside. Immunohistochemical analysis has shown that Dectin-1+ cells are abundant in the area adjacent to the B cell follicles in the paracortex of LN, and are also detectable within follicles (33). In the spleen, the CD8α−CD4−CD205+ DCs are reported to be found predominantly in the T cell areas, whereas CD8α−CD4− APCs localize in the marginal zones (48, 49). However, Dectin-1+ cells have also been detected in the T cell areas of the spleen by immunohistochemistry (33). In addition, injection of poly I:C would be expected to induce the migration of CD8α−DCs from the marginal zone into T cell areas (50). Therefore, the extent to which CD205+ and Dectin-1+ DCs are anatomically segregated remains uncertain. Alternatively, the rates at which targeted Abs are internalized and perhaps recycled to the surface (51) by the different receptors could affect presentation to B cells. A recent study showed that Ab responses can also be elicited by targeting two other molecules expressed by CD8+ T cells to presentation to B cells. This is publication number 121 from the Edward Jenner Institute for Vaccine Research.

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Disclosures

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References


