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Targeting Antigen to Mouse Dendritic Cells via Clec9A Induces Potent CD4 T Cell Responses Biased toward a Follicular Helper Phenotype

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Three surface molecules of mouse CD8$^+$ dendritic cells (DCs), also found on the equivalent human DC subpopulation, were compared as targets for Ab-mediated delivery of Ags, a developing strategy for vaccination. For the production of cytotoxic T cells, DEC-205 and Clec9A, but not Clec12A, were effective targets, although only in the presence of adjuvants. For Ab production, however, Clec9A excelled as a target, even in the absence of adjuvant. Potent humoral immunity was a result of the highly specific expression of Clec9A on DCs, which allowed longer residence of targeting Abs in the bloodstream, prolonged DC Ag presentation, and extended CD4 T cell proliferation, all of which drove highly efficient development of follicular helper T cells. Because Clec9A shows a similar expression pattern on human DCs, it has particular promise as a target for vaccines of human application. ***The Journal of Immunology*****, 2011, 187: 842–850.**

The potent capacity of dendritic cells (DCs) to capture, process, and present Ags to T cells, and then to control T cell expansion and effector function (1), has made DCs prime targets for immune-modulation strategies. Culture-generated DCs loaded with tumor Ags have been used to vaccinate cancer patients, with limited success (2). An alternative strategy, circum venting the cumbersome propagation of DCs, is to directly deliver Ag to DCs in vivo (3, 4). mAbs recognizing DC surface molecules have been successfully used in mice to shuttle Ags to DCs in vivo and so enhance immune responses (5). This type of approach is now entering clinical trials. In this article, we explore the choice of molecular targets to address their role in dictating immunological outcome.

The DC network consists of various subsets that share many Ag presentation attributes but have distinct functional specializations (6, 7). We have chosen to deliver Ag to the CD8$^+$ DC subset, as these specialize in cross-presentation (6, 7), play a major role in driving immune responses against viruses and tumors (8), and have a human equivalent (9–12).

In this paper, we compare Ag delivery with three different receptors predominantly expressed on CD8$^+$ DCs: (1) DEC-205 (CD205), a well-established and effective target molecule (13–15); (2) Clec9A (also known as DNGR-1), a C-type lectin-like molecule with high specificity for CD8$^+$ DCs and their human equivalent (16–18); and (3) Clec12A, a C-type lectin-like molecule with an intracellular immunoreceptor tyrosine-based inhibition motif and a broad expression pattern (19, 20). We found different receptor and adjuvant requirements for generating cell-mediated or humoral immunity. Of particular importance, we revealed a potent capacity for targeting Clec9A to generate T follicular helper cell (Tfh) responses and, as a consequence, strong humoral immunity. Prolonged and efficient presentation of Ag on MHC class II provided a basis for this response.

Materials and Methods

Mice

C57BL/6J wehi (B6), OT-I, OT-II, OT-I$^{ly5.1+}$, OT-II$^{ly5.1+}$, and C3H-HeJ backcrossed eight times to C57BL/6, maintaining the TLR4 mutation (B6-TLR4 mutant mice), were bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute. Mice were used at 6–12 wk of age and handled according to the guidelines of the National Health and Medical Research Council of Australia. Experimental procedures were approved by the Animal Ethics Committee, Melbourne Health Research Directorate.

Generating genetic fusion mAb–OVA constructs

Total RNA was isolated from hybridomas (anti-Clec9A clone 24/04-10B4; anti-Clec12A clone 1/06-5D3; anti–DEC-205 clone NLDC145; rat IgG2a isotype control clone GL117) using the Qiagen RNeasy Mini Kit (Qiagen) with an on-column DNase digestion, per the manufacturer's recommendations. The 5′ RACE-ready cDNA was prepared using the SMART RACE cDNA Amplification Kit (Clontech). The H and L chain sequences

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M.H.L. designed and generated genetically modified mAbs; I.C., F.A., Y.K., C.N.L., and G.M.D performed experiments; S.K., S.S.W., and D.V. assisted in generating reagents; W.S. and G.S. performed microarray analysis; A.M.L., W.R.H., and K.S. provided crucial intellectual input; B.P. and S.N.M. assisted with statistical and histological analysis, respectively; and I.C. designed and supervised the study and, together with K.S., wrote the manuscript.

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Abbreviations used in this article: DC, dendritic cell; DT, diphtheria toxin; pDC, plasmacytoid DC; PI, propidium iodide; poly-IC, polyinosinic-polycytidylic acid; SA, streptavidin; Tfh, T follicular helper cell.

of the Abs were amplified using the manufacturer's recommended universal primer and gene-specific primers (Supplemental Table I) using HotStar DNA polymerase (Qiagen). PCR products were gel excised, digested with EcoRI and NotI restriction enzymes (Promega), and purified using a Minelute PCR Purification Kit (Qiagen). The κ-chain was subcloned into pcDNA 3.1 (Invitrogen). The H chain was subcloned into a pcDNA 3.1 vector modified to contain an Ala-Ala-Ala linker fused to soluble OVA cDNA inserted in the NotI–XbaI region of pcDNA 3.1 (generated in house). This construct enables the generation of a single fusion protein in which the C-terminal region of the H chain is fused to an alanine linker and OVA. Plasmid DNA was prepared using the EndoFree Plasmid DNA Extraction Kit (Qiagen), and plasmids encoding the κ-chain, as well as the H chain linked to OVA, were transiently cotransfected into Freestyle 293F cells (Invitrogen) per the manufacturer's recommendations. Culture supernatant containing the recombinant Abs was harvested, and the Abs were purified by affinity chromatography using a Sepharose mouse anti-rat κ affinity resin (MAR18-5; generated in house).

Abs and fluorescent staining

The following fluorochrome-conjugated mAbs were used against mouse Ag: CD11c (N418-APC, -Qdot 655, -PE, -Alexa 633, or -FITC); CD4 (GK1.5-Pe. Cy7); CD8 (53-6.7-PerCpCy5.5; YTS 169.4-APC, -Alexa 633, -PE); isotype control IgG2a-biotin (eBioscience); TCR-Vα2 (B20.1-PE; BD Pharmingen); CD49b (DX5-FITC, -biotin); CD3 (KT3-1.1-FITC, biotin); CD19 (1D3-APC, -FITC, -biotin, -PE.Cy7); CD40 (FGK45.5-PE); CD80 (16-10.A1-PE; BD Pharmingen); CD86 (PO3.1-PE; eBioscience); MHC class II (M5/114-PE); Ly5.1 (CD45.1, A20-APC; BD Pharmingen); CD25 (PC-61-FITC); 120G8-Alexa 680; PD-1 (J43-PE; BD Pharmingen); and CXCR5 (2G8-biotin; BD Pharmingen). Bound OVA-conjugated mAb was detected using anti–OVA-biotin sera (Calbiochem). Biotin was detected using streptavidin (SA) conjugated to PE, FITC, or PerCpCy5.5. Fc-mediated binding was blocked by preincubating cells (10 min at 4°C) with rat Ig and anti-FcR mAb (2.4G2). Propidium iodide (PI; 0.5 μg/ml) was added to the final cell wash, and PI-positive dead cells were excluded from analysis.

Isolation of DCs

The isolation of DC subpopulations has been described (21, 22). Briefly, tissues were chopped, digested with collagenase (Worthington Biochemical) and DNAse (Boehringer Mannheim) at room temperature, and treated with EDTA. Low-density cells were enriched by density centrifugation (1.077 g/cm^3 Nycodenz, mouse osmolarity). Non–DC-lineage cells were coated with mAb (KT3-1.1, anti-CD3; T24/31.7, anti-Thy1; TER119, anti-erythrocytes; RA36B2, anti-CD45R or 1D3 anti-CD19; RB6-8C5, anti-Ly6C/G or 1A8, Ly6G) and removed using anti-rat Ig magnetic beads (BioMag beads; Qiagen). DCs were stained with various fluorochrome-conjugated mAbs (indicated in figure legends) and sorted into subsets, routinely yielding >95% purity. Depending on the fluorochromes used, sorting was performed on a FACSDiva instrument (Becton Dickinson), a FACSAria instrument (Becton Dickinson), or a MoFlo instrument (Dako Cytomation); the data generated with the sorted populations were consistent among instruments. Analysis was performed on FACSCalibur or LSR II (Becton Dickinson) or on FACScan (Becton Dickinson).

Purification and CFSE labeling of transgenic T cells

Lymph node cell suspensions from transgenic mice (OT-I, OT-I$^{ly5.1+}$, OT-II, and OT-II$^{ly5.1+}$) were stained with mAb against erythrocytes (TER-119), granulocytes (RB6-6C5), MHC class II$^+$ cells and macrophages (M5/114, F4/80), and either CD4 (GK1.5) or CD8 (53-6.7) T cells. These irrelevant cells were removed using anti-rat Ig–coupled magnetic beads at a 1:10 cell-to-bead ratio, and purity was verified to be ~95%. Purified T cells were washed once in 0.1% BSA-PBS, resuspended at 1×10^7 cell/ml, and incubated with CFSE (5 mM) at 37°C for 10 min, then washed twice (2.5% FCS, RPMI 1640) to remove unbound CFSE.

In vitro culture of DCs with transgenic T cells

Purified transgenic T cells (5×10^4 cells per well) were cultured with DCs (numbers indicated in figures) in U-bottomed 96-well plates in modified RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, 10^{-4} M 2-mercaptoethanol). In control experiments DCs and T cells were cultured in the presence of 1 μg/ml MHC class II-restricted OVA peptide (323–339). T cells were enumerated by the addition of 2.5×10^4 calibration beads (BD Biosciences) per well and visualized by staining with mAb against appropriate markers (Ly5.1 and/or TCR-Vα2, Ly5.1). PI$^+$ dead cells were excluded from analysis. Divided T cells were identified by loss of CFSE fluorescence and enumerated relative to the beads, thus calculating cells per well. Analysis was carried out on a FACScan or FACSCalibur (Becton Dickinson).

In vivo proliferation assays of transgenic T cells

C57/BL6 mice were injected with 10^6 CFSE-labeled or unlabeled purified OT-I$^{ly5.1+}$ or OT-II$^{ly5.1+}$ cells. These recipient mice were injected with mAb-OVA, with or without TLR ligands, at the time points specified in the figure legends. Spleens were harvested at various times (indicated in the figure legends), made into cell suspensions, purged of erythrocytes (0.168 M NH_4Cl; 5 min at 4°C), and stained with mAb against Ly5.1, and CD4 or CD8. Proliferating OT-I (Ly5.1$^+$CD8$^+$) or OT-II (Ly5.1$^+$CD4$^+$) cells were visualized by loss of CFSE fluorescence and enumerated by flow cytometry using calibration beads.

Immunization using genetic fusion mAb-OVA constructs

C57/BL6 mice were injected i.v. with specified amounts of genetic fusion rat mAb-OVA constructs (mAb: anti–Clec12A-OVA, 5D3-OVA; anti–Clec9A-OVA, 10B4-OVA; anti–DEC-205-OVA, NLDC145-OVA; isotype control mAb-OVA, GL117-OVA) in the absence or presence of 1 μg LPS (Sigma-Aldrich), 5 nmol synthetic phosphorothioated CpG-1668 (GeneWorks), or 50 μg polyinosinic-polycytidylic acid (poly-IC) (Amersham).

In vivo CTL assay

C57/BL6 mice were immunized with specified amounts of mAb-OVA in the absence or presence of adjuvant. Six days later, immunized mice were injected i.v. with 1×10^7 target cells. OVA-coated target cells and control uncoated cells were prepared as described previously (23). Eighteen hours later, spleens were extracted and cell suspensions analyzed by flow cytometry. The percentage of lysis of OVA-coated target cells was calculated (23).

Depletion of conventional DCs using CD11cDTR chimeric mice and plasmacytoid DCs using mAb

Lethally irradiated (5.5 Gy; 2 doses, 3 h apart) C57/BL6 mice were reconstituted with bone marrow cells (1×10^7) from C57/BL6 mice or CD11cDTR mice and allowed to recover for a minimum of 6 wk. CFSE-labeled OT-II$^{ly5.1+}$ cells (10^6) were adoptively transferred into chimeric mice, which were then injected i.p. with diphtheria toxin (DT; 100 ng). One day later, chimeric mice were injected i.v. with anti–Clec9A-OVA or isotype control OVA-mAb. One day after antigenic challenge, mice received a second i.p. injection of DT (100 ng). One DT injection routinely depleted >80% of conventional DCs. Spleens were harvested 3 d after antigenic challenge, and CFSE-labeled OT-II cells were identified as CD4$^+$ Ly5.1$^+$. To deplete plasmacytoid DCs (pDCs), C57/BL6 mice were injected with 0.7 mg 120G8 mAb 1 d prior to and 1 d after immunization with anti–Clec9A-OVA mAb. This depletion protocol resulted in the ablation of ~70% of pDCs.

Detection of serum Ab by ELISA

ELISA plates (Costar) were coated overnight at 4°C with 1–2 μg/ml rat GL117 mAb, or 10 μg/ml OVA. Unbound protein was washed away (PBS, 0.05% Tween 20). Serially diluted serum samples (PBS, 5% milk powder) were plated and incubated at 4°C overnight. Bound mouse anti-rat or anti-OVA IgG Abs were detected using donkey anti-mouse IgG-HRP (Chemicon International) and visualized using ABTS.

Detection of biotinylated mAb and OVA-mAb in the serum

ELISA plates were coated overnight at 4°C with 1 μg/ml rabbit polyclonal anti-rat Ig (Abcam). Unbound Ab was washed away, and nonspecific protein binding was blocked by preincubating plates for 2 h with 3% BSA-PBS. Serum samples from mice injected with biotinylated mAb or OVA-mAb, were serially diluted and incubated overnight at 4°C. Bound biotinylated mAbs were detected using SA-HRP (GE Healthcare) and ABTS. Bound OVA-mAbs were detected using anti–OVA-biotin sera (4°C; overnight) and detected using SA-HRP and ABTS.

Histological examination

The method of tissue fixation was adapted from Bajénoff et al. (24). Briefly, spleens were fixed overnight in medium containing 1% paraformaldehyde, 0.075 M L-lysine, and 2.1 mg/ml sodium periodate ($NaIO_4$) in 0.0375 M phosphate buffer (pH 7.4) at 4°C. Tissue was then washed in phosphate buffer and dehydrated in 20% sucrose in phosphate buffer for 6–8 h before being frozen in Tissue-Tek O.C.T. (Sakura Finetek). Next, 10-μm cryostat sections were fixed in ice-cold acetone for 10 min and stained with rat anti-GL7, followed by goat anti-rat Alexa 568 (Invitrogen), and then rat anti-IgD Alexa 647 (BioLegend), and analyzed on a confocal microscope (LSM710, Zeiss).

Statistical analysis

The unpaired two-tailed *t* test was performed on log-transformed data. The significance of differences is as follows: not significant (n/s), $p > 0.05$; $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$. Analysis was performed in Prism (GraphPad Software).

Results

Delivering Ags to CD8$^+$ DC surface molecules

Our aim was to compare CTLs and humoral immunity obtained by targeting Ags to three different molecules (Clec9A, Clec12A, and DEC-205) expressed by CD8$^+$ DCs. Although CD8$^+$ DCs show the highest levels of expression, other cells also show some expression of these molecules. Both DEC-205 and Clec12A are found on CD8$^-$ DCs and B cells. By contrast, Clec9A is found only on CD8$^+$ DCs and pDCs (16, 19, 25) (Supplemental Fig. 1*A*). To deliver Ag to these molecules, we cloned heavy and light chains of specific mAb and genetically fused OVA to their heavy chains. These mAb–OVA complexes all recognized their target molecules on DCs (Supplemental Fig. 1*A*) (16, 19). Others have shown that the anti–DEC-205 mAb does not directly activate DCs (14). Similarly, we found that neither the anti-Clec12A mAb (data not shown) nor the anti-Clec9A mAb activated DCs (Supplemental Fig. 1*B*). The basal level of CD40, CD80, CD86, and MHC class II seen on naive splenic DCs (26) remained unaltered upon injection of isotype control mAb (data not shown) and was not increased upon injection of anti-Clec9A mAb (Supplemental Fig. 1*B*). Therefore, these mAbs simply serve to deliver Ags to DCs.

Proliferative responses of CD8 T cells in vivo after targeting Ags

We set out to analyze the immune responses induced by injecting mice with these mAb-OVA constructs, with or without coadministration of TLR ligands. Because CD8$^+$ DCs are especially equipped to cross-present Ags on MHC class I, we asked whether any of the targeting mAbs were superior at facilitating MHC class I presentation. CFSE-labeled OVA-specific CD8 transgenic T cells (OT-I cells) were transferred into mice; then 1 d later the mice were injected with graded doses of the mAb-OVA constructs in the presence or absence of CpG. Three days later the proliferative responses of the OT-I cells were enumerated by flow cytometry (Fig. 1*A*, 1*B*). Targeting OVA to DEC-205 resulted in the greatest expansion of OT-I cells. However, targeting OVA to Clec9A was nearly as efficient as targeting DEC-205. Targeting OVA to Clec12A resulted in poor OT-I expansion (Fig. 1*A*, 1*B*). The addition of CpG to the immunization mix enhanced overall OT-I proliferation (Fig. 1*B*), but the hierarchy in effectiveness was preserved. In this case, however, the difference between targeting DEC-205 and Clec9A did not reach significance.

CTL generation in vivo after Ag targeting

We next determined whether targeting Ags to the various CD8$^+$ DC surface molecules leads to endogenous production of effector CTLs. Mice were immunized with the mAb-OVA constructs alone or in conjunction with either CpG, poly-IC, or LPS. Six days later, mice were injected with CTL targets (equal mix of syngeneic splenocytes pulsed with the MHC class I-restricted OVA peptide SIINFEKL and labeled with high levels with CFSE, together with unpulsed splenocytes labeled to low levels with CFSE). Robust CTL responses were generated only when the mAb-OVA constructs were coadministered with DC activating agents (Fig. 1*C*). Targeting OVA to DEC-205 or to Clec9A resulted in comparable CTL responses, which were superior to those obtained by targeting Ag to Clec12A. Indeed, when limiting amounts of the targeting mAb-OVA constructs (0.2 μg) were injected in conjunction with optimal amounts of adjuvant, targeting Clec9A and DEC-205 induced similar CTL responses, whereas targeting Clec12A induced significantly weaker responses (Fig. 1*D*).

Ab responses induced by targeting Ags to CD8$^+$ DC surface molecules

We have already demonstrated that targeting Ags in the form of rat Ig to Clec9A (16), but not Clec12A or DEC-205 (19, 27, 28), can induce strong Ab responses in the absence of adjuvants. The mAb-OVA constructs behaved as did the original rat mAb: Targeting Clec9A induced potent anti-rat Ig responses (Fig. 2*A* versus 2*B*) and anti-OVA responses in the absence of adjuvant (Fig. 2*C*). Targeting Clec12A or DEC-205 induced only modest anti-rat Ig (Fig. 2*A* versus 2*B*) or anti-OVA immunity (Fig. 2*C*), and these were enhanced by the coadministration of adjuvant (Fig. 2*A* and data not shown). We have previously shown that targeting Clec9A facilitates potent humoral responses independent of TRIF/MyD88 (16), and in this paper we confirmed that TLR4 mutant mice, which do not respond to endotoxin, also have strong humoral responses when injected with anti–Clec9A-OVA (Supplemental Fig. 2). Thus, the humoral response elicited by anti–Clec9A-OVA was not due to endotoxins acting as adjuvants. Targeting Ag to Clec9A was at least 25-fold more efficient at inducing Ab responses in the absence of adjuvant than targeting Ag to DEC-205 (Fig. 2*B*, 2*C*).

Ag presentation to CD4 T cells after targeting Ags

Because the Ab response induced by targeting Ag to Clec9A was completely dependent on CD4 T cells (16), yet the targeting did not activate DCs, we reasoned that superior MHC class II presentation underpinned the efficient activation of CD4 T cells. Thus we measured the level of Ag presentation after administration of mAb-OVA. Mice were injected with the mAb-OVA constructs; 1 d later the spleens were harvested and the CD8$^+$ DCs were purified. The targeted CD8$^+$ DCs were then assessed for their ability to induce proliferation of CFSE-labeled OVA-specific MHC class II-restricted transgenic CD4 T cells (OT-II cells) in culture (Fig. 3). Contrary to our predictions, OVA targeted to DEC-205 consistently produced greater ex vivo OT-II proliferation, and thus better MHC class II presentation, than targeting Clec9A. This result obtained 1 d after targeting may reflect the higher expression of DEC-205 on the surface of CD8$^+$ DCs. In contrast, targeting Clec12A produced poor OT-II proliferation, despite the relatively high expression on the surface of the CD8$^+$ DCs (Supplemental Fig. 1*A*). This finding did not reflect an inherent inability of the Clec12A-targeted CD8$^+$ DCs to activate CD4 T cells, because pulsing of the isolated CD8$^+$ DCs with class II MHC-restricted OVA peptide induced potent OT-II proliferation (data not shown). However, coadministration of CpG as an adjuvant improved MHC class II presentation on targeting OVA to Clec12A (data not shown).

Proliferative responses of CD4 T cells in vivo after targeting Ags

As ex vivo presentation did not reveal an advantage of targeting Clec9A, we assessed CD4 T cell proliferation in vivo after targeting OVA to the various molecules. CFSE-labeled OT-II cells were transferred into recipient mice, and 1 d later mice were injected with graded doses of the mAb-OVA, with or without adjuvant. Four days later the proliferative response of the OT-II cells was enumerated (Fig. 4*A*). Targeting 1 μg of mAb-OVA to Clec9A in the absence of adjuvant was significantly more efficient at inducing OT-II proliferation than targeting to DEC-205 ($p < 0.0001$). In fact, targeting Clec9A was at least 5-fold more efficient at inducing proliferation than targeting DEC-205. Targeting OVA to Clec12A without

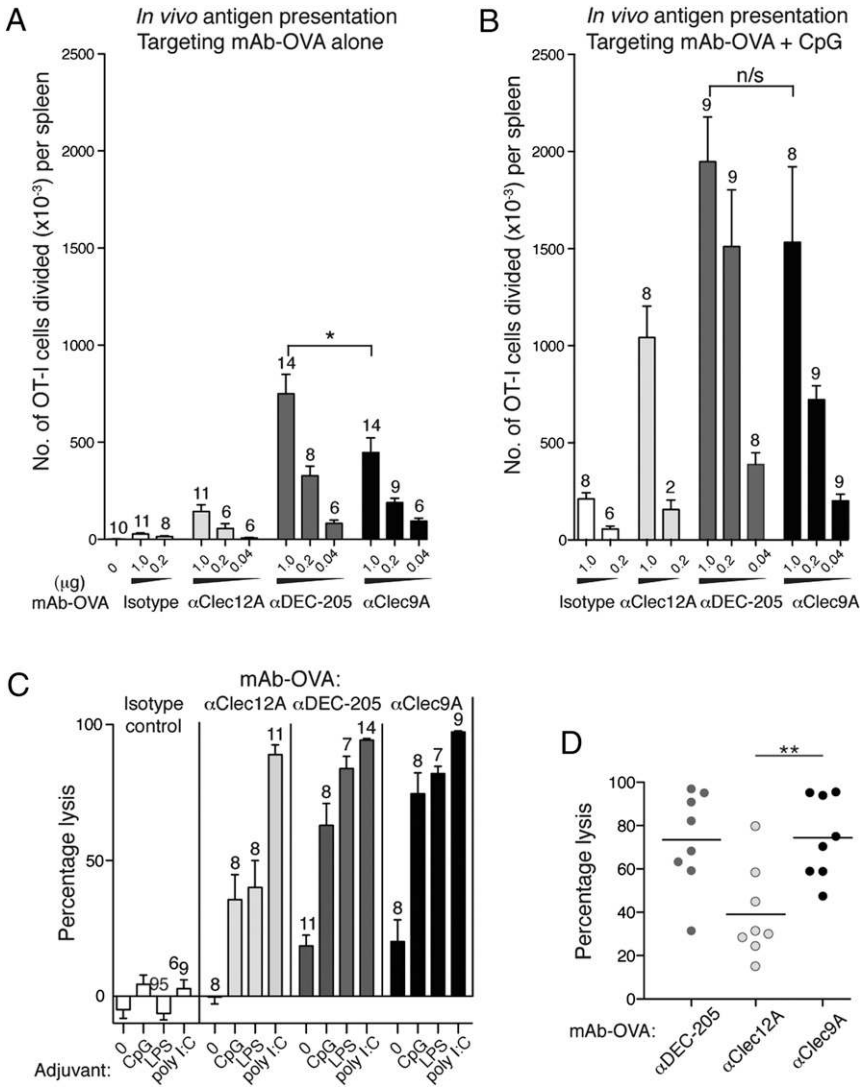

FIGURE 1. CD8 T cell response after targeting OVA to Clec9A, Clec12A, or DEC-205. In vivo proliferation of OT-I cells: CFSE-labeled OT-I$^{ly5.1+}$ cells (10^6) were adoptively transferred into C57/BL6 mice. One day later mice were immunized i.v. with graded doses of mAb-OVA in the absence (*A*) or presence (*B*) of CpG, or were left unimmunized (i.e., "0" mAb-OVA). Three days postimmunization, proliferating OT-I (Ly5.1$^+$CD8$^+$) cells were enumerated by flow cytometry. Cumulative data of five experiments are presented. *$p = 0.0144$. Numerals above bars depict the number of mice per group, and error bars indicate the SEM. In vivo CTL activity of mice immunized with targeting mAb-OVA is represented. *C*, C57/BL6 mice were immunized i.v. with 1 μg of mAb-OVA in the absence or presence of CpG, LPS, or poly-IC. D, C57/BL6 mice were immunized i.v. with 0.2 μg of mAb-OVA and poly-IC. In both *C* and *D*, mice were injected i.v. with target cells 6 d after priming, and the percentage lysis of OVA-coated target cells was determined 18 h later. Cumulative data of nine (*C*) and two (*D*) experiments are presented. *D*, Mice immunized with anti–Clec12A-OVA mAb had significantly less CTL activity than did mice immunized with anti–Clec9A-OVA mAb. **$p = 0.0032$.

adjuvant was only marginally more effective at inducing OT-II proliferation than the untargeted isotype control OVA construct.

When CpG was administered as adjuvant along with mAb-OVA, the proliferative response of OT-II upon targeting DEC-205 showed little increase, whereas the response to targeting Clec9A increased still further (Fig. 4*A*; $p < 0.0001$). The biggest effect of coadministration of CpG was on the response of OT-II cells after targeting OVA to Clec12A; this response now became greater than that elicited when targeting DEC-205 (Fig. 4*A*; $p = 0.0004$). In part this was because on activation by CpG, CD8$^-$ DCs expressing Clec12A made a significant contribution to the presentation of OVA on MHC class II (data not shown).

When the proliferative response of OT-II was examined in vivo over an extended time course, the advantage of targeting Clec9A was

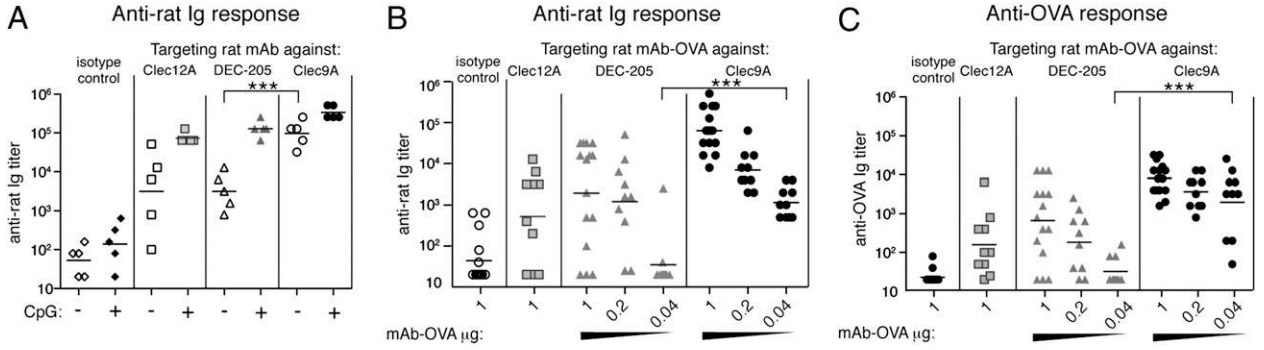

FIGURE 2. Humoral responses elicited by targeting Ag to Clec12A, DEC-205, or Clec9A. C57/BL6 mice were injected i.v. with 10 μg of native mAb against Clec12A, DEC-205, Clec9A, or isotype control mAb in the presence or absence of CpG (5 nmol) (*A*) or, alternatively, with graded doses of the genetic mAb-OVA constructs in the absence of any adjuvants (*B*, *C*). Serum anti-rat (*A*, *B*) and anti-OVA (*C*) Ig reactivity was measured 2 wk later by ELISA. The cumulative data of three independent experiments are depicted, with the geometric mean indicated. ***$p = 0.0005$ (*A*); ***$p < 0.0001$ (*B, C*).

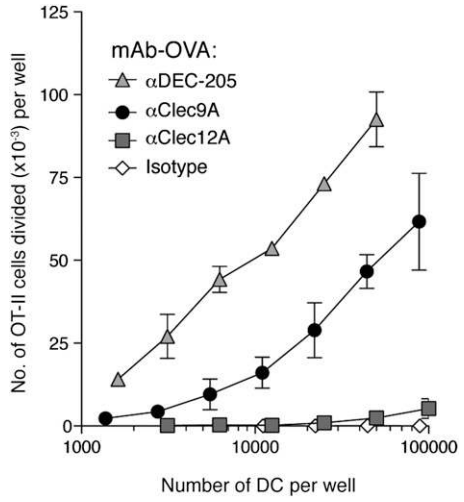

FIGURE 3. Ex vivo MHC class II presentation by $CD8^+$DCs 1 d after in vivo targeting of OVA to Clec9A, Clec12A, or DEC-205. C57/BL6 mice were injected i.v. with 5 μg of either anti–Clec9A-OVA, anti–Clec12A-OVA, anti–DEC-205-OVA, or isotype control-OVA; 22 h later, spleens were harvested and DCs isolated and purified as described in *Materials and Methods*. Graded numbers of $CD8^+$ DCs were incubated with 5×10^4 CFSE-labeled OT-II cells for 4 d, after which time OT-II cells were enumerated by flow cytometry. Data (±SD) presented are from a single experiment and are representative of five independent experiments.

clearly emphasized (Fig. 4*B*). Overall, targeting OVA to Clec9A in the presence or absence of adjuvant was dramatically more effective at expanding OT-II cells than was targeting Clec12A or DEC-205 in the presence of CpG (Fig. 4*B*). This finding suggested that prolonged expansion of CD4 T cell responses upon targeting Clec9A was the basis for the strong humoral response.

Tfh generation in vivo after targeting Ags

It was clear that targeting Ag to Clec9A with our mAb was much more effective at inducing CD4 T cell expansion in vivo than was targeting DEC-205 or Clec12A. As strong humoral immunity was also evident, we examined the production of Tfh, known to promote Ab production. These cells can be recognized by the coexpression of CXCR5 and PD1 (29, 30). In mice in which OVA was targeted to Clec9A, not only was the expansion of OT-II cells vigorous, but a significant proportion of them upregulated CXCR5 and PD1 (Fig. 5*A* and 5*B*). In addition, OT-II cells could be clearly located within the B cell germinal centers by day 6 (Fig. 5*D*). As a consequence of both the greater OT-II expansion and the higher proportion expressing these markers, targeting OVA to Clec9A was dramatically more effective in the total numbers of Tfh produced (Fig. 5*C*). By contrast, we saw no sign of regulatory T cell induction (data not shown). The production of Tfh provided an explanation of why targeting Clec9A was so efficient at inducing Ab production.

We then asked why targeting Ag to Clec9A was so effective at inducing CD4 T cell expansion and Tfh production. Because Clec9A is expressed on pDCs as well as $CD8^+$ DCs, it was conceivable that targeting Ag to pDCs could aid humoral immunity. When conventional DCs were depleted in vivo using diphtheria toxin in CD11cDTR mice, leaving pDCs intact, the pDCs were unable to expand OT-II cells after Clec9A targeting (Supplemental Fig. 3*A*). Conversely, depleting pDCs using the mAb against BST-1 (120G8), but leaving conventional DCs intact, had no effect on the expansion of OT-II cells after Clec9A targeting (Supplemental Fig. 3*B*), even when the dose of targeting Ag was limiting. Thus it seemed unlikely that targeting pDCs was the reason for the efficiency of targeting Clec9A.

Persistence of Ag presentation after targeting

The remarkable effectiveness of inducing CD4 T cell proliferation in vivo by targeting Clec9A (Fig. 4), compared with the more

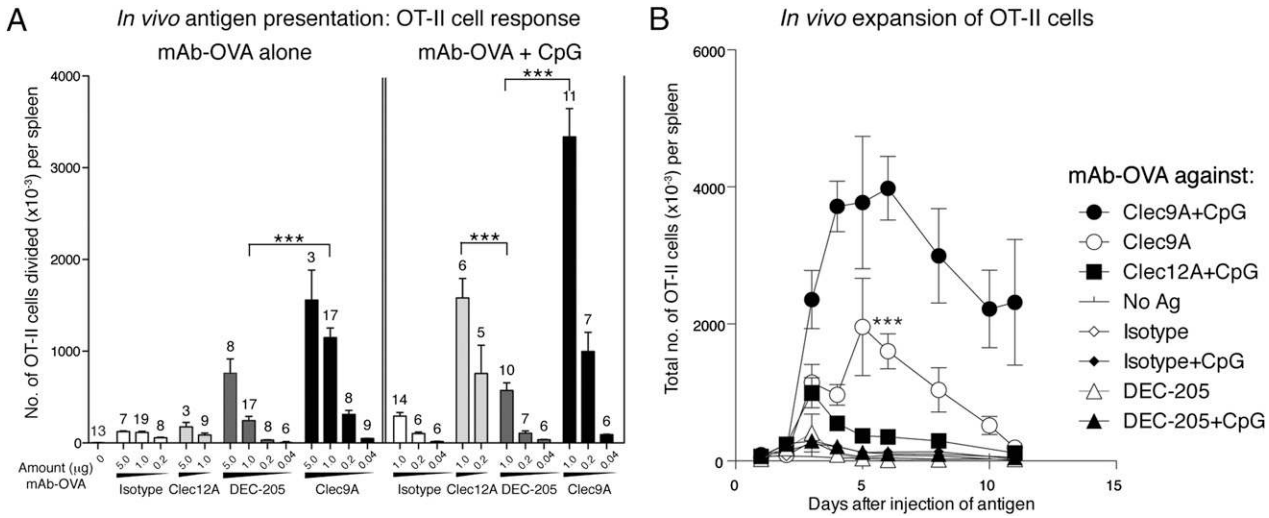

FIGURE 4. In vivo MHC class II presentation and expansion of OT-II cells after targeting OVA to Clec9A, Clec12A, or DEC-205. *A*, Titration of mAb-OVA, measuring in vivo Ag presentation and 4-d OT-II expansion. CFSE-labeled OT-$II^{ly5.1+}$ cells (10^6) were adoptively transferred into naive C57/BL6 mice. One day later, mice were immunized i.v. with graded doses of anti–Clec12A-OVA, anti–Clec9A-OVA, anti–DEC-205-OVA, or isotype control-OVA mAb in the absence or presence of CpG (5 nmol) or were left unimmunized (depicted as "0" mAb-OVA). Four days postimmunization, spleens were harvested and proliferating OT-II ($Ly5.1^+CD4^+$) cells were enumerated by flow cytometry. The cumulative data of seven experiments are presented. Numerals above bars depict the number of mice per group, and error bars indicate the SEM. *B*, Kinetic analysis of OT-II cell expansion. C57/BL6 mice were injected with 10^6 OT-$II^{ly5.1+}$ cells and then, 1 d later, injected with 0.5 μg of anti–Clec12A-OVA, anti–Clec9A-OVA, anti–DEC-205-OVA, or isotype control-OVA mAb, in the absence or presence of CpG (5 nmol). At different time points after immunization, spleens were extracted and the total number of OT-II cells enumerated by flow cytometry. Cumulative data of five experiments are presented, and error bars indicate the SEM. *A*,***$p < 0.0001$ (mAb-OVA alone); ***$p = 0.0004$ (mAb-OVA + CpG: Clec12A versus DEC-205); ***$p < 0.0001$ (DEC-205 versus Clec9A). *B*, ***$p = 0.0002$ (Clec12A+CpG versus Clec9A).

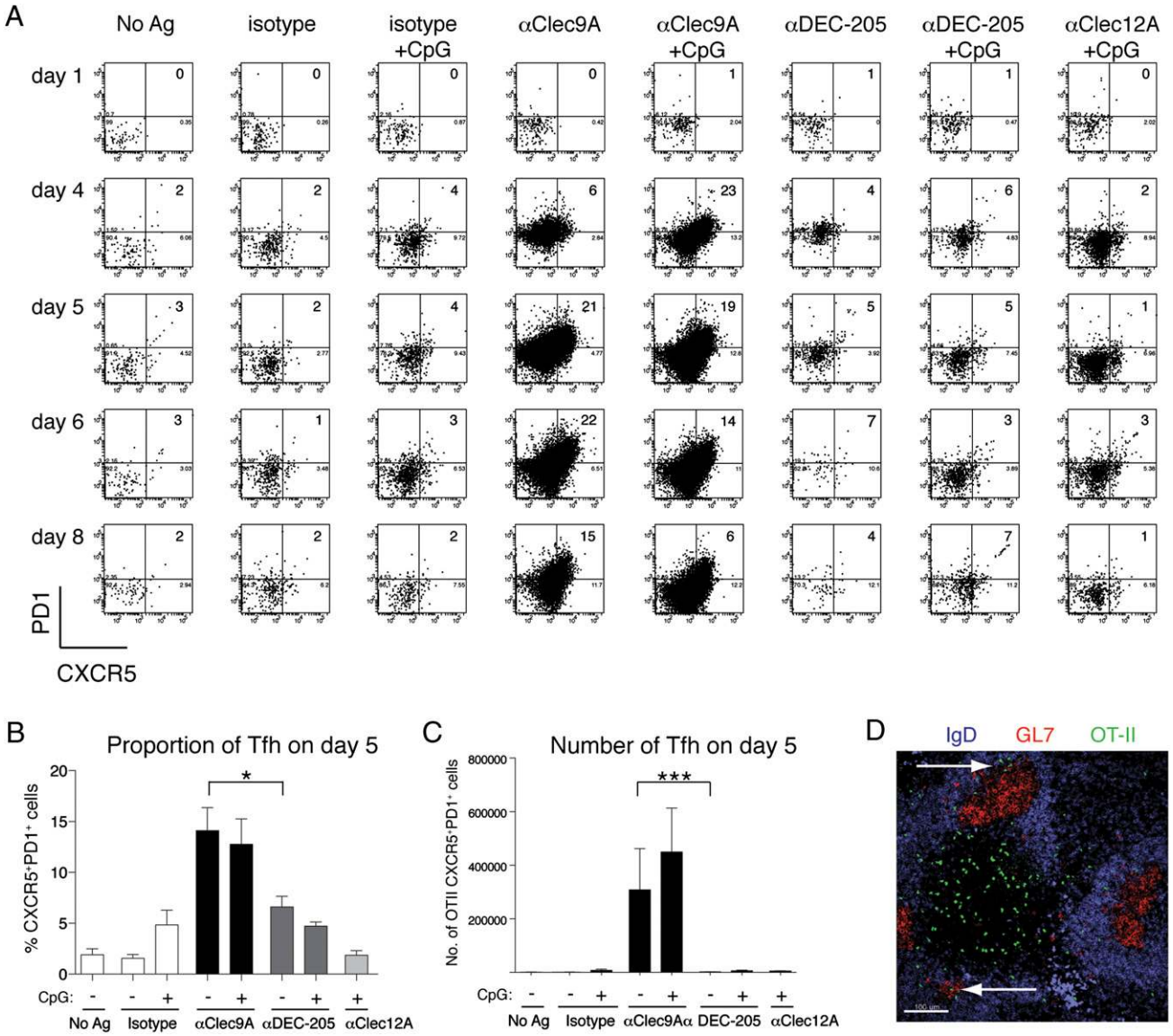

FIGURE 5. Targeting Ag to Clec9A in vivo induces Tfh cells. OT-II$^{ly5.1+}$ cells (10^6) were adoptively transferred into naive C57/BL6 mice. One day later, mice were immunized i.v. with 0.5 μg of anti–Clec12A-OVA, anti–Clec9A-OVA, anti–DEC-205-OVA, or isotype control-OVA mAb in the absence or presence of CpG (5 nmol) or were left unimmunized (no Ag). Five days postimmunization, spleens were harvested and cell suspensions were stained with mAb against Ly5.1 (APC), CD4 (Alexa 680), PD1 (PE), and CXCR5 (biotin). Biotinylated mAb was detected with SA-FITC. In some experiments yielding identical results, B cells were excluded from analysis by staining with anti–CD19-PE.Cy7 mAb. OT-II cells (Ly5.1$^+$CD4$^+$) were identified and the expression of CXCR5 and PD1 assessed. *A*, A kinetic analysis is presented and is representative of three independent experiments. Anti–Clec12A-OVA mAb, in the absence of CpG, gave results similar to those with the isotype control mAb. The cumulative data of three independent experiments were used to determine the mean proportion (*B*) and the absolute numbers (*C*) of Tfh cells on day 5. Error bars indicate the SEM. *D*, eGFP-expressing OT-II cells (5×10^4) (green) were adoptively transferred into naive C57/BL6 mice, which were then injected i.v. with 0.5 μg of anti–Clec9A-OVA mAb. Six days later, spleens were harvested and fixed before sectioning and staining for GL7 (rat anti-GL7, followed by goat anti-rat Alexa 568; red) and IgD (anti-IgD Alexa 647; violet). Germinal centers are identified by their expression of GL7 and lack of expression of IgD (white arrows).

limited Ag presentation 24 h after injection seen in the ex vivo assay (Fig. 3), prompted us to check the persistence of Ag presentation in vivo as a factor determining efficiency.

In the first instance, we sought to determine if Ag targeted to Clec9A preferentially persisted in the bloodstream. To this end, mice were injected with biotinylated mAbs against Clec9A, DEC-205, or Clec12A; then serum concentrations of these mAbs were measured by ELISA at various time points (Fig. 6*A*). Within 2 h of injection, mAbs against Clec12A were no longer detectable in the serum; similarly, mAb against DEC-205 could not be detected 1 d postinjection. By contrast, mAb against Clec9A persisted at high levels in the serum and were readily detected even 4 d after injection. Indeed, clearance kinetics of anti-Clec9A mAb paralleled those of the isotype control mAb, not known to bind mouse Ags. Mice injected with mAb-OVA showed similar differences in clearance rates: Anti–DEC-205-OVA and anti–Clec12A-OVA mAbs rapidly disappeared from the serum, whereas anti–Clec9A-OVA and isotype mAb-OVA mAbs persisted in the serum (Fig. 6*B*). Persistence of anti–Clec9A-OVA was, in fact, greater than that of anti–Clec9A-biotin.

Next, we wished to ascertain whether the prolonged presence of anti-Clec9A mAb in the serum would result in prolonged acquisition of Ag by DCs. Thus, mAbs against Clec9A, DEC-205, or Clec12A were conjugated to Alexa 488 and injected into mice. At different time points, CD8$^+$ DCs from spleen were purified, and their fluorescence was measured by flow cytometry (Fig. 6*C*). Two

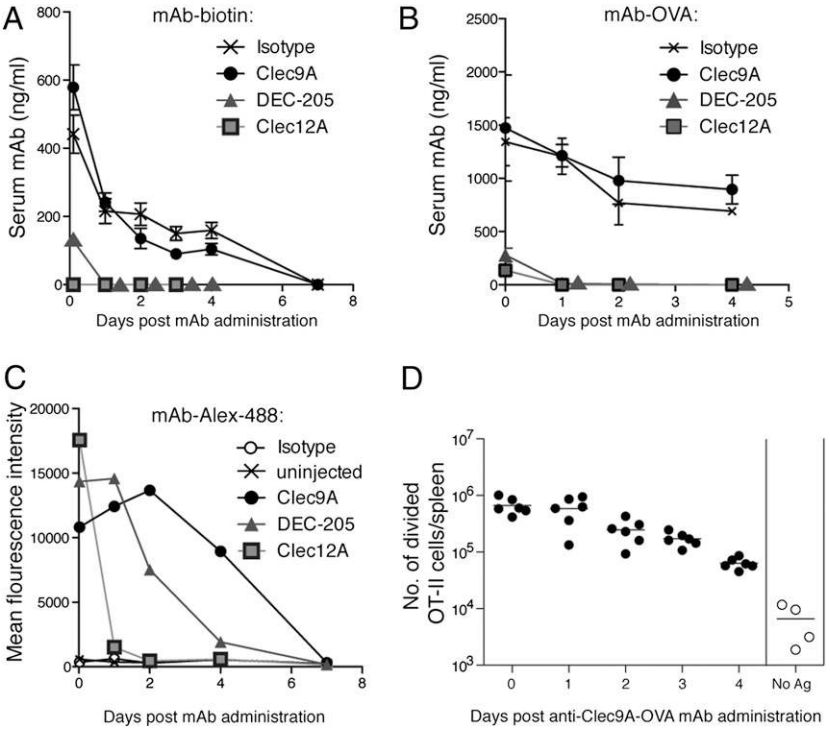

FIGURE 6. Persistence of mAb and Ag after immunization with mAb-OVA constructs. *A* and *B*, Persistence of mAb in serum. Five C57/BL6 mice were injected i.v. with 2 μg of biotinylated mAb (*A*). Alternatively, four mice were injected i.v. with 2 μg of mAb-OVA (*B*). The level of biotinylated-mAb or mAb-OVA in the serum was measured at different time points by ELISA. Data presented in *A* are representative of two independent experiments; those in *B* represent the outcome of one experiment. *C*, Persistence of mAb associated with $CD8^+$ DCs. C57/BL6 mice were injected i.v. with 2 μg Alexa 488 conjugated mAb recognizing Clec12A, Clec9A, DEC-205, or isotype control mAb-Alexa 488. Spleens were extracted at different time points (2 h, days 1, 2, 4, and 7). Low-density cells were enriched by density centrifugation and stained with anti–CD11c-PE, anti–CD8-APC, and 120G8-Alexa 680. The mean fluorescence intensity of Alexa 488 on $CD8^+$ DCs ($CD8^+CD11c^{hi}120G8^-$) after injection of mAb-488 is depicted. Data presented are representative of three independent experiments. *D*, Persistence of Ag presentation results in activation of Ag-specific transgenic CD4 T cells. C57B6 mice were injected with 0.5 μg of anti–Clec9A-OVA. Then, on the same day or 1–4 d later, CFSE-OT-$II^{ly5.1+}$ cells (10^6) were adoptively transferred into the immunized mice. Spleens were harvested 5 d after OT-II cell transfer, and the number of OT-II cells was enumerated by flow cytometry. Cumulative data of two independent experiments are presented.

hours after injection (first data point), all the targeting mAbs were detected at high levels on the $CD8^+$ DCs. However, as early as 24 h after injection, the level of anti-Clec12A dropped markedly and had almost vanished by 48 h. The mAb to DEC-205 persisted longer but had dropped by 48 h and was low by 4 d. By contrast, the $CD8^+$ DCs from mice injected with anti–Clec9A-Alexa 488 retained a high level of fluorescence even 4 d postinjection.

To find out if the persistence of anti-Clec9A in the $CD8^+$ DCs mirrored persistent Ag presentation, mice were injected with anti–Clec9A-OVA, and then on the day of priming, or on various days thereafter, CFSE-labeled OT-II were adoptively transferred into separate mice (Fig. 6*D*). The ability to present OVA and initiate OT-II proliferation persisted well beyond the day of priming. For example, even 4 d after targeting Ag to Clec9A, sufficient Ag was presented to initate proliferation of OT-II cells. By contrast, the nontargeted isotype control mAb-OVA, which also persisted in the serum but did not target DCs, did not induce CD4 T cell proliferation even when T cells were injected as early as day 0 (Fig. 4*B*). It is worth noting that even at the earliest time point, when maximum Ag was available, targeting to Clec12A and DEC-205 induced poor proliferative responses (Fig. 4*A*).

Although Ag persistence nicely explained the basis for strong humoral immunity upon targeting Clec9A, it remained possible that such targeting activated DCs, especially given the lack of requirement for adjuvant. However, 1 d after injection of anti-Clec9A mAb, no detectable shift was noted in the levels of MHC class II, CD40, CD80, or CD86 on the DC subsets (Supplemental Fig. 1*B*). Furthermore, no cytokine production (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-12p40, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-α, MIP-1β, RANTES, TNF-α, or IFN-α) was induced by targeting DCs in vivo, or in vitro with anti-Clec9A mAbs (data not shown). Finally, no significant difference was observed in the gene expression of $CD8^+$ DCs from mice injected with isotype mAb versus those injected with Clec9A mAb 20 h earlier (data not shown). Together, these data implicated Ag persistence as the driving factor that generates Tfh responses and strong humoral immunity in the absence of adjuvant.

Discussion

In determining the best strategy for enhancing immune responses by delivering Ags to DCs in vivo, we considered two issues. The first was the choice of the DC subset to be targeted. We have focused on $CD8^+$ DCs, the subset of choice for the induction of effective CTL responses, because of their inherent ability to cross-present Ags and prime CD8 T cells (7). Because the $CD141^+$ human DC subset has been identified as the functional equivalent of mouse $CD8^+$ DCs, our findings are likely to be translatable to the human (9–12). The second issue was the particular DC surface molecule targeted. We have compared three different molecules on the surface of $CD8^+$ DCs and shown that the receptors chosen for Ag delivery indeed contribute to the efficiency of Ag presentation.

Targeting Ag to Clec9A, and particularly DEC-205 (31), even in the absence of adjuvant, promoted cross-presentation and proliferation of Ag-specific transgenic CD8 T cells. By contrast, Clec12A was poor at facilitating cross-presentation. However, whereas targeting Ag to DEC-205 and Clec9A was sufficient to activate transgenic CD8 T cells into cell division, the requirement to elicit endogenous CTLs was more stringent: Delivering Ag to all three DC molecules required the coadministration of adjuvant to successfully prime endogenous CTLs, in agreement with the findings of others (14, 15, 32–35). Targeting Ag to Clec12A consistently induced poorer CTL response, in accordance with its inability to promote cross-presentation. Interestingly, in a collaborative study with Steinman's group (36), we have recently shown that when gag-p24 was targeted to DEC-205, Langerin, or Clec9A in the presence of high doses of adjuvants (poly-IC and anti-CD40 mAb), all targeting Abs induced comparable frequency of IFN-γ–producing CD4 and CD8 T cells. Presumably, under such intense stimulatory conditions, even Clec12A would induce robust CTL responses.

In our current study, we show striking differences in the three receptors as targets for promoting CD4 T cell responses and humoral immunity. Delivering Ag to Clec9A, even in the absence of adjuvants, was more efficient at eliciting CD4 T cell proliferation and Ab production in vivo than was targeting Ag to Clec12A or DEC-205, even when these Ags were delivered with adjuvant. Why is targeting Ag to Clec9A with our mAb so effective at inducing Ab responses, even in the absence of adjuvants? One answer is that Ab responses are limited by the availability of helper T cells, and targeting Ag to Clec9A produced not only more extensive CD4 T cell expansion but also a much greater transformation of these cells to the Tfh phenotype, crucial to Ab production.

The mechanism underpinning the striking induction of T helper cells by targeting Clec9A relates to the maintenance of Ag dose. Although targeting to Clec9A did not initially result in more Ag being presented in the MHC class II pathway, injection of anti–Clec9A-OVA mAb led to prolonged Ag presentation. Persistence of Ag presentation by DCs has been shown to be a crucial factor in driving immune responses (37) and particularly the development of Tfh cells (38). The prolonged presentation in our system is most likely a result of the high specificity of Clec9A: The anti-Clec9A mAb in the serum is not "mopped up" by other cells, as occurs for the anti–DEC-205 and anti-Clec12A mAbs. Consequently, there is a prolonged association of anti-Clec9A mAb with the $CD8^+$ DCs, and given that $CD8^+$ DCs have a 3-d lifespan (39), the anti–Clec9A-OVA mAb will be available to newly generated CD8 DCs for at least a 4-d period. In line with this fact, Ab against Clec12A, which is expressed at relatively high levels by a wide range of hematopoetic cells, showed the lowest persistence in the serum or on $CD8^+$ DCs, and was the least effective at inducing Ab production in the absence of adjuvants.

Of importance, we have obtained similar results when targeting OVA to Clec9A with two different mAbs—one the rat IgG2A used in these studies, and the other a rat IgG1. However, we find that rat IgG1 lacks appropriate epitopes for a direct response in C57/BL6 mice; its targeting capacity is apparent only when the epitopes of OVA protein are attached, or when it is used alone in BALB/c mice (data not shown), which presumably can detect appropriate epitopes. This finding is the likely reason why Joffre et al. (40) did not see extensive Ab production without adjuvants when they used rat IgG1 anti-Clec9A (DNGR1) mAb for targeting in C57/BL6 mice. Other factors may also contribute to the effectiveness of targeting Clec9A, including the affinity of the mAb, its persistence in the serum, and its potential for signal transduction.

The induction of an effective T cell-dependent humoral response in the absence of DC activation appears opposed to the general rule that Ag presented by immature, steady-state DCs leads to tolerance and that DC activation is required for a full immune response (13–15, 27). Inadvertent introduction of endotoxins in our constructs is excluded because the comparable results are obtained with TLR4 mutant (Supplemental Fig. 2) and TRIF-MyD88–null mice (16). No overt signs of DC activation after engaging Clec9A with mAb are present, such as upregulation of costimulatory molecules, cytokine production, or gene activation. Perhaps the steady-state levels of MHC class II and costimulator molecules expressed by quiescent DCs provide sufficient costimulation for a Tfh response if efficient and continuous Ag presentation leads to an intense TCR signal.

Overall, our results emphasize the value of Clec9A as a target for delivering Ag and so improving responses to vaccines. Because Clec9A is expressed on $CD8^+$ DCs that specialize in the uptake and processing of Ag from dead cells, introducing an Ag into this route may promote efficient presentation on both MHC class I and MHC class II. Targeting Ags to Clec9A is as efficient as targeting the gold standard DEC-205 for induction of CTL responses, and is far more efficient at inducing Ab responses, even without adjuvants. The prospect of inducing protective Ab responses without the side effects of an adjuvant is very attractive. Because the specificity of targeting Ags and its influence on Ag persistence seems to be an important issue, it is notable that Clec9A appears to be as specific for the $CD141^+$ DC population in humans as it is for the $CD8^+$ mouse DC equivalent. Targeting vaccine Ags to Clec9A may therefore be an effective strategy for human application.

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Disclosures

The authors have no financial conflicts of interest.

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