FcRn regulates antigen presentation downstream of dendritic cell receptor-targeted vaccination.

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Abstract

Dendritic cell (DC)-targeted vaccination is a new model of antigen delivery that relies on the use of monoclonal antibodies (mAb) to target antigen to specific DC subsets. The neonatal Fc receptor (FcRn) is a non-classical Fc receptor that binds to immunoglobulins G (IgG) in acidified endosomes and controls their intracellular transport and recycling. FcRn is known to participate in the antigen presentation of immune complexes, however its contribution to the presentation of DC-targeted vaccination has not previously been examined. Here we have investigated the role of FcRn in antigen presentation using antigen conjugated to IgG mAb which target specific DC receptors, including DEC205 and Clec9A specific for the conventional DC 1 (cDC1) subset. We show that FcRn is expressed at high levels by cDC1, both at steady-state and following activation and plays a significant role in MHC I cross-presentation and MHC II presentation of antigens that are targeted to cDC1 via mAb specific for DEC205. This effect of FcRn is intrinsic to cDC1 and it impacts the efficacy of anti-DEC205-mediated vaccination against lymphoma. In contrast, FcRn does not impact the presentation of antigens targeted to Clec9A and does not regulate the presentation of cell-associated antigen. These data highlight a new and unique role of FcRn in controlling the immunogenicity of anti-DEC205-based vaccination, with consequences for exploiting this pathway to improve DC-targeted vaccine outcomes.

INTRODUCTION

Dendritic cells (DCs) are unique in their ability to capture and present antigen on major histocompatibility complex (MHC) molecules to T cells, making them an attractive cellular platform for vaccine development. DC-targeted vaccination is a strategy that relies on delivering the antigen directly to DCs in situ via conjugation to IgG mAbs specific to DC surface receptors\(^1\)\(^,\)\(^2\). Several receptors have been explored for DC targeting and two of them, DEC205 and Clec9A, are particularly effective at priming immune responses. DEC205 is a C-type lectin receptor that is highly expressed by conventional DC 1 (cDC1) and also expressed by dermal and interstitial DCs, Langerhans cells, B cells and cortical thymic epithelial cells\(^3\). It acts as a receptor for type B oligonucleotide, transporting it to late endosomes for Toll like receptor (TLR)-9 stimulation\(^4\). Clec9A, also known as DC natural killer lectin group receptor 1 (DNGR1), is a C-type lectin displayed at high levels on the surface of cDC1, with low expression by pDCs\(^5\)\(^,\)\(^6\). Clec9A binds to F-actin exposed by necrotic cells and traffics to intracellular locations that enables associated cargo to access the MHC I presentation pathway\(^6\)\(^–\)\(^8\). DC-targeted vaccination using mAbs specific for DEC205 and Clec9A induces cytotoxic T cell immunity\(^5\)\(^,\)\(^9\)\(^–\)\(^11\) that contributes to tumor eradication in prophylactic and therapeutic settings\(^12\)\(^–\)\(^14\). In addition, DEC205 and Clec9A-targeted vaccine antigen is efficiently processed for MHC II presentation, resulting in efficient CD4\(^+\) T cell responses\(^9\)\(^–\)\(^11\)\(^,\)\(^15\). DC-targeted vaccines are now used in clinical treatment\(^16\)\(^,\)\(^17\), and efforts to further boost their efficacy of major interest.

The neonatal Fc receptor (FcRn) is an Fc binding receptor that belongs to the MHC I superfamily, forming a heterodimer encompassing a heavy α chain and a β-2-microglobulin chain\(^18\). FcRn is predominantly
located in endosomal compartments where it interacts with two ligands, IgG and serum albumin via independent binding sites\(^1\). This receptor-ligand interaction is pH dependent, with relatively strong binding affinity below pH 6.5 but low affinity at neutral pH\(^2\). FcRn protects IgG and albumin from intracellular catabolism by transporting ligands in transport carriers for release at the cell surface, and diverting them away from proteases-containing late endosomes/lysosomes\(^3\). Another function of FcRn is to deliver IgG from mother to neonates across the intestinal and/or placental epithelia by transcytosis\(^4,5\). In DCs, FcRn interacts with endocytosed IgG immune complexes and shuttles them to intracellular compartments that enable MHC I and MHC II presentation for priming of CD8\(^+\) and CD4\(^+\) T cell responses\(^6–8\).

Given the role for FcRn in intracellular IgG trafficking and presentation of IgG-associated antigen, we investigated whether FcRn contributes to immune responses downstream of IgG mAb-based DC-targeted vaccines. Here we show that FcRn plays a critical role in the MHC I cross-presentation and MHC II presentation of DEC205-targeted, but not Clec9A-targeted antigen. Our data highlight a critical role for FcRn in settings of DC-targeted vaccination that can be exploited to boost their efficacy.

**RESULTS**

**FcRn expression by splenic DCs.**

We first examined FcRn expression in primary mouse cDCs compared to other immune cell types. Total cellular FcRn was detected by western blot for bone marrow-derived macrophages, cDC1, cDC2 and B cells. Low levels of FcRn were detected in CD8\(^+\) T cells whereas CD4\(^+\) T cells expressed very little (Fig. 1A). Activation of cDC1 or cDC2 by exposure to TLR9 agonist CpG did not significantly alter FcRn levels for either subset (Fig. 1B).

We next characterized the phenotype of \(Fcg_r^−/−\) mice. In line with previous reports\(^1\), \(Fcg_r^−/−\) mice develop hypogammaglobulinemia, as evidenced by the absence of IgG1, IgG2c, IgG3 and a strong reduction in IgG2b levels in the serum. In contrast, IgA, IgE and IgM were detected at normal levels (Fig. 1C). Both the cDC1 and cDC2 subsets are present at normal numbers in the absence of FcRn (Fig. 1D).

**FcRn is required for effective tumor immunity in response to DEC205 and Clec9A-targeted vaccination.**

The effectiveness of DC receptor-targeted vaccines in eliminating B cell lymphoma was examined using a mouse model of B cell lymphoma. This model consists of E\(μ\)-myc transformed lymphoma cells that express the model antigen, ovalbumin (OVA)\(^7\). DC vaccines were designed to target whole OVA to DC receptors DEC205 and Clec9A through a genetic fusion of the protein to the Fc region of rat IgG2a specific to the two receptors. Murine FcRn binds rat IgG2a with high affinity\(^8\). WT mice were intravenously immunized with either DEC205 or Clec9A-targeted OVA adjuvanted with LPS. Five days later, mice were inoculated with B cell lymphoma and the number of B cell lymphoma cells in the spleen
evaluated 4 days later (Fig. 2A). Vaccination with either DEC205 and Clec9A-targeted OVA elicited a significant reduction in tumor cell number, indicating successful anti-tumor therapy for this mode of immunization (Fig. 2B). A role for FcRn was evaluated by undertaking the same analysis in Fcgrt<sup>-/-</sup> mice. In the absence of FcRn, the ability to eradicate tumors was lost and there was no significant reduction in tumor cell numbers when mice were immunized with DEC205 or Clec9A-targeted OVA (Fig. 2B). A second measurement of successful tumor immunization was included, whereby evidence of tumor immunoediting in response to vaccination was assessed. In the B cell lymphoma model used here, green fluorescent protein (GFP) is linked by an internal ribosome entry site to the tumor antigen OVA<sup>27</sup>. Therefore, the tumor GFP signal provides a surrogate read out of OVA expression and is a useful marker of tumor antigen expression. In WT mice, DEC205 and Clec9A-targeted OVA immunization results in a significant reduction in tumor GFP. This is likely due to the tumors reducing OVA expression to avoid anti-OVA CD8<sup>+</sup> T cells. In contrast, tumors in Fcgrt<sup>-/-</sup> mice, had a higher GFP signal, suggesting that in the absence of FcRn there was less pressure on the tumor to edit OVA following immunization (Fig. 2C). Taken together, this data suggests that FcRn is required for a robust anti-tumor response elicited by DEC205 and/or Clec9A-directed DC-targeted vaccination.

**FcRn regulated MHC I and MHC II antigen presentation in response to DEC205-targeted DC vaccination.**

We next examined how FcRn participates in DEC205-targeted DC vaccination. Surface DEC205 was unaltered on splenic cDC1 and cDC2 in the absence of FcRn (Fig. 3A). This finding indicates that similar amounts of DEC205-targeted OVA antigen is delivered to cDCs in both WT and Fcgrt<sup>-/-</sup> mice. Next, the capacity of Fcgrt<sup>-/-</sup> mice to present DEC205-targeted antigen by MHC I and MHC II was examined. To do this, Cell Trace Violet (CTV)-labelled OT-I or OT-II T cells were transferred into WT and Fcgrt<sup>-/-</sup> mice. One day later, mice were immunized with DEC205-targeted OVA intravenously and the number of dividing T cells in the spleen was evaluated by flow cytometry after three days. In the absence of FcRn, DEC205-targeted OVA elicited significantly reduced numbers of divided OT-I, and significantly more divided OT-II cells (Fig. 3B). Presentation of OVA-coated splenocytes, used as mAb-independent cell-associated antigen model, to OT-I and OT-II was not impacted by FcRn deficiency (Supplementary Fig. 1A). To determine if the reductions in MHC I and MHC II antigen presentation were intrinsic to FcRn-deficient cDCs, WT and Fcgrt<sup>-/-</sup> mice were intravenously immunized with DEC205-targeted OVA. Twenty hours later cDC1 were sorted to purity by flow cytometry and equal numbers of WT or Fcgrt<sup>-/-</sup> cDC1s incubated in the presence of CTV labelled OT-I or OT-II T cells. Fcgrt<sup>-/-</sup> cDC1 displayed a significantly reduced capacity to stimulate OT-I and OT-II cells in response to <em>in vivo</em> targeting with DEC205-targeted OVA (Fig. 3C). This suggested reduced MHC I presentation, and in contrast to in vivo adoptive transfer experiments, reduced MHC II presentation in response to <em>in vivo</em> targeting with DEC205-targeted OVA.

To examine if FcRn impacted presentation of DC-targeted antigens other than OVA, we targeted the self-epitope Ea<sub>46-72</sub> to DEC205 and examined the presence of Ea<sub>52-68</sub> peptide-loaded I-Ab MHC II molecules at the surface of cDC1 using the Yae mAb<sup>29</sup>. WT or Fcgrt<sup>-/-</sup> mice were injected intravenously with DEC205-targeted Ea<sub>46-72</sub> and cDCs isolated from spleen 20 hours later. Significantly reduced MHC II
presentation of DEC205-targeted E$_{a52-68}$ was detected at the surface of Fcgrt$^{-/-}$ compared to WT cDC1 (Fig. 3D). To further confirm this finding, the Fcgrt gene was deleted in the cDC1 cell line MutuDCs using CRISPR/Cas9 (Supplementary Fig. 2A). Following delivery of the E$_{a46-72}$ peptide to DEC205 in vitro, Fcgrt$^{-/-}$ MutuDCs were significantly impaired in the MHC II presentation of E$_{a52-68}$ (Supplementary Fig. 2B). Altogether, this data show that FcRn is an important contributor to the response elicited by anti-DEC205-targeted vaccination by promoting MHC I cross-presentation and MHC II presentation of the delivered antigen.

The role of FcRn in DEC205-targeted DC vaccination is DC-intrinsic.

One possibility for defects in DC antigen presentation following DC-targeted vaccination in vivo, is that FcRn is required to promote circulating DC-targeted vaccine half-life. To address this, we used mixed bone marrow chimeras where WT mice were lethally irradiated and reconstituted with a 1:1 ratio of CD45.1 WT or CD45.2 Fcgrt$^{-/-}$ bone marrow. This created a scenario where CD45.1 WT or Fcgrt$^{-/-}$ CD45.2 DCs are both in a WT environment with similar levels of circulating DC-targeted vaccines. 6–8 weeks after reconstitution, mice were immunized with anti-DEC205-OVA and 20 hours later CD45.1$^+$ WT or Fcgrt$^{-/-}$ cDC1 were isolated from spleens and incubated with CTV labelled OT-I or OT-II. In comparison to WT, Fcgrt$^{-/-}$ cDC1 displayed significantly reduced MHC I and MHC II presentation of DEC205-targeted OVA (Fig. 4). This rules out that differences in vaccine half-life were responsible for the defects in Fcgrt$^{-/-}$ cDC presentation of DEC205-targeted antigen and highlights an intrinsic role of FcRn in DCs during anti-DEC205-targeted vaccination.

FcRn does not regulate MHC I and MHC II antigen presentation in response to Clec9A-targeted DC vaccination.

Next, we examined how FcRn impacts anti-Clec9A-targeted OVA immunization. Similar to DEC205, surface Clec9A on cDC1 was not altered by the deficiency in FcRn (Fig. 5A). In contrast to anti-DEC205-OVA, MHC I and MHC II antigen presentation following immunization with anti-Clec9A-OVA was not significantly impacted by the absence of FcRn. This was the case as measured by T cell responses following adoptive OT-I and OT-II T cell transfer into anti-Clec9A-OVA Fcgrt$^{-/-}$ mice (Fig. 5B), extrinsic isolation and culture of WT or Fcgrt$^{-/-}$ cDC1s from anti-Clec9A-OVA immunized mice (Fig. 5C) and analysis of Fcgrt$^{-/-}$ cDC1 compared to WT cDC1 isolated from WT mice reconstituted with 1:1 ratio of WT and Fcgrt$^{-/-}$ bone marrow (Fig. 5D). In contrast, MHC II presentation of Clec9A-targeted E$_{a46-72}$ peptide on cDC1 was significantly reduced in the absence of FcRn (Fig. 5E). Therefore, in contrast to anti-DEC205-targeted vaccination, FcRn does not have a prominent role in regulating presentation of antigen targeted via Clec9A but can participate under some conditions.

FcRn is required for CTL immunity to DEC205 but not Clec9A-targeted DC vaccination.

Elimination of E$_{µ}$-myc-OVA B cell lymphoma in response to DC-targeted vaccination likely involves the generation of anti-OVA cytotoxic T lymphocytes (CTL). We therefore examined whether FcRn is required
for eliciting CTL in response to anti-DEC205 or Clec9A-OVA. WT or Fcgrt\(^{-/-}\) mice were immunized, intravenously immunized with DC-targeted anti-DEC205 or Clec9A-OVA and six days later the presence of OVA-specific CTL detected by the intravenous injection of OVA\(_{257-264}\) peptide-pulsed, or not, target cells. In the absence of FcRn, immunization with anti-DEC205-OVA elicited a significant reduction in CTL lysis compared to WT mice (Fig. 6A). In contrast, anti-Clec9A-OVA elicited similar CTL in both WT and Fcgrt\(^{-/-}\) mice (Fig. 6B). CTL immunity elicited by OVA-coated splenocytes was not impacted by FcRn-deficiency (Supplementary Fig. 1B). Hence, these results demonstrate that priming a robust CTL response following DC-targeted vaccination requires FcRn when delivering antigen to DEC205, but not Clec9A.

**DISCUSSION**

DC-targeted vaccination using antigen-conjugated DC-receptor specific IgG mAb allows efficient and precise delivery of vaccine antigen to DC subsets specialized in antigen presentation and T cell priming. The success of this immune therapy relies on the intracellular trafficking pathways inside DCs, that will dictate whether, or not, the vaccine antigen and accompanying targeting IgG can access a compartment that is favourable for antigen presentation. Here we show that mAb-conjugated antigen targeted to DEC205 requires FcRn, a receptor of emerging therapeutic interest\(^{31}\), for efficient MHC I cross-presentation and MHC II presentation. In contrast, Clec9A-targeted antigen is presented via a mechanism that is independent of FcRn.

Our study highlights a role for FcRn in cDC1. Previously, FcRn was identified as critical for MHC I cross-presentation of immune complex-derived antigen in cDC2, but not cDC1\(^{24,25}\). In these systems, the alkaline pH of the cDC1 cross-presentation compartment was not permissive for FcRn binding. In contrast, when antigen is delivered via anti-DEC205 mAb, it accesses late endosomes\(^{32,33}\) with a luminal pH that is sufficiently acidic for FcRn binding to the Fc domain of the targeting mAb. In addition to cDC1, other antigen presenting cell populations that express DEC205 may also use FcRn to effectively present DEC205-targeted antigen. For example, B cells which express FcRn, have high surface expression of DEC205\(^{3}\) and respond to DEC205 mAb-targeting in vivo by presenting the associated antigen in the context of MHC II\(^{34,35}\). Langerhans cells and a subset of dermal DCs also exhibit high surface levels of DEC205. After subcutaneous immunization, these skin DCs capture and transport anti-DEC205 mAbs to the draining lymph nodes for presentation of the conjugated antigen to T cells\(^{36,37}\). It remains to be determined if FcRn regulates the presentation of DEC205-targeted antigen by these cell types.

FcRn impacts immunization with mAb-conjugated antigen to DEC205, but not Clec9A. Why is this the case? Both DEC205 and Clec9A are expressed at high levels by cDC1, an antigen presenting cell type that we observe expresses high levels of FcRn. Therefore, the different roles for FcRn likely stem from differences in the intracellular trafficking properties of the two receptors. DEC205 and Clec9A exhibit similar plasma membrane internalization patterns in cDC1, with rapid and high surface turnover\(^{38}\). Inside the cell, however, their pathways diverge. Anti-DEC205 mAbs traffic to late endosomes in immature and mature bone marrow-derived DCs\(^{32,33}\) and the highly acidic lumen of these compartments may favour
FcRn binding to the Fc-domain of anti-DEC205 IgG. In contrast, Clec9A mAbs transit through early and recycling endosomes\(^8\),\(^3\) compartments without an acidic lumen that may not permit FcRn-IgG binding. Another difference between the two receptors is that mAb binding to Clec9A induces spleen tyrosine kinase (Syk) phosphorylation and endosomal rupture\(^3\). This has not been reported for DEC205. Therefore, endosomes with antigen-conjugated anti-Clec9A mAb may rupture instead of routing to FcRn-containing endosomes. Regardless, the impaired presentation of DEC205-targeted antigen when FcRn is absent is likely due to altered trafficking and processing. In cDC2, FcRn directs captured immune complexes to endosomes that are enriched with components of the cross-presentation machinery. These include the translocon Sec61, which mediates the transfer of antigen from the endosomal lumen to the cytosol, and transporter associated with antigen processing 1 (TAP1), which reimports proteasomal generated peptides into the endosome\(^2\). Interestingly, DEC205-targeted antigen is also cross-presented via a TAP-dependent mechanism\(^9\),\(^4\), suggesting that FcRn may divert anti-DEC205 mAbs to similar cross-presenting compartments in cDC1.

Currently, two anti-DEC205 targeting mAbs are in clinical trials. CDX-1401 is a human IgG1 conjugated to the tumor antigen NY-ESO1 that has been evaluated in six phase I and phase II trials. Another DEC205-specific mAb, DCVax-001 has been conjugated to the human immunodeficiency virus (HIV) antigen Gag p24 and tested in a phase I clinical trial\(^2\),\(^6\),\(^7\). Although these vaccines have shown promising results with robust B cell and T cell immunity, their efficiency may be further enhanced by harnessing FcRn to extend their half-life and activity in vivo. Several therapeutic mAbs have been modified in their Fc domain to introduce mutations near the key FcRn-binding residues in order to increase the IgG-FcRn binding affinity at low pH. For example, inserting the LS (M428L, N434S) mutation into anti-vascular endothelial growth factor (VEGF) IgG1 Bevacizumab greatly extended the mAb half-life in vivo by approximately four fold, resulting in increased anti-tumor activity\(^4\). YTE (M252Y/S254T/T256E) is another mutation introduced into mAbs specific for viral proteins. This modification enhanced the half-life of the anti-respiratory syncytial virus IgG1 Motalizumab up to 100 days post-injection\(^4\). This mAb engineering approach represents a straightforward strategy applicable to DEC205-targeted DC vaccination to improve FcRn affinity and immunogenicity.

In conclusion, our study demonstrates the crucial role of FcRn in the efficient presentation of antigen targeted to DEC205 by DCs. We have shown that FcRn mediates the presentation of DEC205-targeted antigen, while Clec9A-targeted antigen presentation is independent of FcRn. The differential trafficking and processing of these receptors likely contribute to the distinct roles of FcRn in antigen presentation of DC-targeted mAb. The clinical trials of anti-DEC205 mAbs underscore the potential of utilizing FcRn to enhance the efficacy of DC-targeted vaccines. By engineering the Fc domain of these mAbs to increase their affinity for FcRn, the half-life and activity of the vaccines can be extended, leading to improved immunogenicity. These findings provide valuable insights into the development of DC-targeted vaccination strategies and highlight the importance of FcRn in modulating immune responses for effective antigen presentation by DC subsets.
MATERIALS AND METHODS

Mice

C57BL/6, Fcgrtm1Dcr (Fcgrt−/−), B6.CH-2bm−1 (BM-1), Ly5.1, OT-II x Ly5.1 and OT-I x Ly5.1, mice were used at 6–12 weeks of age. All mice were bred and maintained in specific pathogen–free conditions at the Melbourne Bioresources Platform at Bio21 Molecular Science and Biotechnology Institute. Experimental procedures were approved by the Animal Ethics Committee of the University of Melbourne.

Isolation of immune cells

For purification of DCs, spleens were finely chopped and digested in the presence of DNase I (Roche) and collagenase type 3 (Worthington Biochemicals). Intercellular clusters were disrupted by addition of 10 mM EDTA. Light density cells were isolated by density gradient separation in 1.077 g/cm^3 Nycodenz (Nycomed Pharma). Upper fractions were collected, washed and subject to further enrichment by resuspending in a depletion cocktail containing the following rat anti-mouse mAbs specific for: CD3 (KT3-1.1), CD90 (T24/31.7), red blood cells (Ter119), B220 (RA3-6B2), Ly6G/Ly6C (RB68C5). Cells were incubated with antibodies, washed, and incubated with BioMag anti-rat IgG-coupled magnetic beads (Qiagen). The DC-enriched supernatant was recovered by magnetic separation. Where required, DCs were sorted to purity by flow cytometry on a Becton Dickinson Influx (Murdoch Children’s Research Institute Flow Cytometry and Imaging facility). cDC1 were defined as CD11c^+ CD8^+ CD11b^−, cDC2: CD11c^+ CD8^− CD11b^+. For in vitro activation, cDCs were isolated and incubated overnight in complete RPMI with 0.5 µM CpG 1668 ODN (Bioneer) at 37ºC or 4ºC. Following activation, cDC subsets were sorted by flow cytometry and analysed by immunoblotting.

For T cell isolation, single cell suspensions were generated from lymph nodes. Cells were stained with rat anti-mouse mAbs specific for: CD11b (M1/70), red blood cells (TER119), Ly6G (1A8), MHC II (M5/114), B220 (RA3-6B2) and CD4 (GK1.5) for CD8^+ T cells or CD8 (YTS 169.4) for CD4^+ cells. Cells were washed and incubated with BioMag anti-rat IgG-coupled magnetic beads (Qiagen). After magnetic depletion, the CD4^+ or CD8^+ T cell-enriched supernatant was recovered. Purity, determined by flow cytometry, was > 90%. For B cell isolation, whole-splenocyte suspensions were subjected to gradient centrifugation (at 2237g) with Ficoll-Paque Plus (GE Healthcare) and subsequent negative depletion using FITC-conjugated mAb against CD4 (GK1.5), Ly76 (TER119), CD43 (S7) and magnetic anti-FITC MicroBeads (Miltenyi Biotec). The purity of the B cell preparation, as measured by flow cytometry, was > 95%.

Bone-marrow-derived macrophages

Bone marrow was isolated from the femur and tibia of 6-12-week-old C57Bl/6 mice and non-adherent bone marrow cells cultured for 5–7 days in the presence of conditioned media harvested from L-929 cells as a source of M-CSF. Purity was assessed by flow cytometry as ~ 95% of live cells expressing both CD11b and F4/80.

Immunoblotting
Immune cells were isolated as previously described and were denatured in 1x reducing LDS sample buffer; 25% (v/v) 4x NuPAGE LDS Sample Buffer (Invitrogen) and 10% (v/v) 10x NuPAGE Reducing Agent (Invitrogen) in distilled water, at 100°C and analysed by SDS-PAGE using 4–12% gradient NuPAGE Bis-Tris polyacrylamide gels (Invitrogen). Proteins were transferred onto Immobilon-P PVDF membranes (Merck Millipore) and stained with primary antibodies against FcRn (R&D Systems), GAPDH (6C5, Millipore) followed by HRP-conjugated secondary antibodies.

Serum antibody analysis

For quantification of total serum antibodies, sandwich ELISA was performed by coating high protein-binding 96-well plates (Coming) with 20 µg/ml of goat mAb specific for mouse IgA (clone MOPC315), IgG1 (MOPC31c), IgG2b (MOPC141), IgG2a/c (UPC10), IgG3 (Y5606), and IgM (MOPC104E) (all Sigma-Aldrich). Coated plates were subsequently incubated with diluted serum or standards, followed by detection with horseradish peroxidase (HRP)-conjugated polyclonal mAbs against mouse IgG1, IgA, IgG1, IgG2b, IgG2a/c, IgG3, and IgM (all SouthernBiotech) and 1-Step Ultra3,3',5,5'-Tetramethylbenzidine ELISA Substrate (Thermo Fisher Scientific). Absorbance at 440 nm was measured with a FLUOstar Omega PlateReader, and 4PL analysis was performed with GraphPad Prism software.

Prophylactic vaccination assay and analysis of killing of Eµ-myc-OVA lymphoma

The Eµ-myc lymphoma expressing GFP-OVA was described previously27. Mice were immunized i.v. with 1 µg of anti-DEC205-OVA or Clec9A-OVA mAb or 20 x 10⁶ OVA-coated splenocytes, all adjuvanted with 1 µg of LPS. Five days later, mice were challenged with 10⁶ Eµ-myc-OVA lymphoma cells and spleens were harvested after 4 days. Single-cell splenocyte suspensions were treated with RBC removal buffer, stained with a mAb specific for B220 (RA3-6B2, BioLegend) and analysed by flow cytometry.

In vivo antigen presentation assay

Single-cell suspensions were generated from lymph nodes of OT-I x Ly5.1 or OT-II x Ly5.1 mice and the CD8⁺ or CD4⁺ T cells were purified as before. Purified OT-I or OT-II cells were washed with PBS supplemented with 0.1% BSA and labelled with Cell Trace Violet (CTV; Thermo Fisher Scientific). To enable accurate quantification of the number of OT-II or OT-I cells, mice also received transfer of a control population of carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific)-labelled splenocytes. Single-cell suspensions were treated with red blood cell (RBC) removal buffer. Cells were washed with PBS, 0.1% BSA, and labelled with CFSE. Equal numbers of CTV-labelled OT-II or OT-I cells and CFSE-labelled splenocytes were pooled and resuspended in RPMI 1640, 2% FCS at 1 x 10⁷ cells/ml, respectively, and injected i.v. Twenty-four hours later, mice received 20 x 10⁶ OVA-coated splenocytes, or 0.2 µg anti-DEC205-OVA or anti-Clec9A-OVA mAb via i.v. injection11. For OVA-coated splenocytes, single-cell splenocyte suspensions were treated with RBC removal buffer, washed, and incubated with 10 mg/ml OVA protein (Sigma-Aldrich) in FCS-free RPMI 1640 at 37 C. Sixty-four hours after immunization, spleens were harvested, single-cell suspensions were generated, and RBCs were lysed. Cells were stained with
mAbs specific for CD8α (53 – 6.7, BioLegend) or CD4 (GK1.5; Walter and Eliza Hall Antibody Facility) and Ly-5.1 (A20; BioLegend). The number of divided OT-II and OT-I was determined as the number of CD4+ or CD8+ Ly-5.1+ cells that had undergone CTV dilution. The number of cells was normalized to the number of CFSE-labelled splenocytes recovered.

**Ex vivo antigen presentation assay**

Mice were immunized i.v. with 1 µg of anti-DEC205-OVA or Clec9A-OVA mAb or anti-DEC205-Edα46-72 peptide. After 22 to 24 hours, splenic cDCs were either examined for Ea52-68-loaded I-Ab surface expression by flow cytometry using biotinylated YAe mAb29 (Thermo Fisher Scientific) and streptavidin-PE (BioLegend) or sorted to purity. Sorted cDC1 or cDC2 were cultured in vitro with 5 x 10⁴ CTV-labelled OT-II or OT-I cells in complete medium. Four or five days later, the number of divided OT cells was determined.

**In vitro targeting of MutuDCs and analysis of antigen presentation**

MuTu DCs stably expressing Cas943 were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM)-GlutaMax™ (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS (University of Melbourne Media Preparation Unit), 100 µM β-mercaptoethanol (Thermo Fisher), 100 µg/mL penicillin and 100 µg/mL streptomycin at 37°C, 10% CO₂. To knockout FcRn, the LV04 vector containing the sgRNA was used (Sigma Aldrich). sgRNA sequences were as follows: non-targeting control: 5’ CGCGATAGCGCGAATATATT 3’; FcRn: 5’ CCGTCGGCCCCTCTCCAGG 3’. To produce lentivirus, HEK293T were transfected using polyethylenimine (Polysciences) with sgRNA vector, pMDL (Addgene #12251), pRSV-REV (Addgene #12253), pMD2.G (Addgene #12259). Lentivirus were subsequently used to transduced Cas9+ MuTu DCs by spinfection in the presence of polybrene (Sigma-Aldrich). Transduced cells were then selected by culturing them for three days with puromycin (Thermo Fisher Scientific).

**Mixed bone marrow chimera**

Recipient mice (C57BL/6) were irradiated twice at 550 cGy (rad), three hours apart before being injected i.v. with 50:50 mixed bone marrow cells from Ly5.1 WT mice and Fcgrt--/mice. Recipient mice were intraperitoneally injected with anti-Thy1 (T24) to eliminate radio-resistant host T cells the day after irradiation. Mice were reconstituted for six weeks before analysis.

**In vivo CTL killing assay**

Mice were injected with 1 µg of anti-DEC205-OVA mAb or anti-Clec9A-OVA mAb, or with 2 x 10⁷ OVA-coated splenocytes, all mixed with 1 ug of LPS as adjuvant. Six days later, single-cell suspensions of target cells (splenocytes) were prepared from spleens of WT mice. Half of the suspension (OVA+ splenocytes pulsed with OVA257-264 labelled with a high concentration of CTV [CTVhi]) was pulsed with 1 µg of OVA257-264 (Worthington Biochemical) and labelled with 5 mM CTV (Thermo Fisher Scientific). The other half of the suspension (OVA splenocytes labelled with a low concentration of CTV [CTVlo]) was
labelled with 0.5 mM CTV only. An equal number of OVA$^+$ CTV$^{hi}$ and OVA$^-$ CTV$^{lo}$ target cells were pooled, and 10 x 10$^6$ target cells were injected i.v; 36–42 h later, spleens were harvested and the percentage of OVA-specific lysis was determined as follows: $R = (%CTV^{lo} / %CTV^{hi})$. %OVA-specific lysis = $[1 - (r_{unprimed} / r_{primed})]100$.

**Declarations**

**COMPETING INTERESTS**

The authors declare no competing interest.

**DATA AVAILABILITY**

Data from this study will be made available upon request to the corresponding author (Justine D. Mintern, jmintern@unimelb.edu.au)

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**Supplementary Figure**

Supplementary Figure 1 and 2 are not included with this version

**Figures**
Figure 1

FcRn is expressed by DC subsets. A. Immune cell populations were purified from C57BL/6 mice and analysed by immunoblotting for FcRn expression. Representative of two independent experiments. B. FcRn expression in spleen cDC1 and cDC2 was analysed by immunoblotting either in resting conditions or following in vitro activation with CpG for 18 hours at 37°C. C. Serum levels of the different IgG subtypes, IgA, IgE and IgM in WT and Fcgrt−/− mice. Each symbol represents an individual mouse. **** P <
Fig. 2

**FcRn impacts tumor immunotherapy.** A. WT and *Fcgrt*−/− mice were immunized with anti-DEC205-OVA or anti-Clec9A-OVA mAbs mixed with LPS. After 5 days, mice were challenged with Eμ-myc–GFP-OVA tumor
cells and 4 days later, spleens were collected and analysed by flow cytometry. B. The contour plots show FSC and B220 expression in whole splenocytes for each group. The number of tumor cells per spleen is indicated in the histogram. C. Representative GFP profiles of lymphoma cells for each group. The GFP expression level was quantified and shown in the accompanying histogram. Data are pooled from 5 independent experiments with symbol representing individual mice. *** $P < 0.001$, **** $P < 0.0001$ by two-way ANOVA with Bonferroni test for multiple comparisons. ns, not significant.

Figure 3
Figure 3

**FcRn alters MHC I and MHC II presentation following DEC205-targeted DC vaccination.**

**A.** Flow cytometry analysis of DEC205 expression in WT and *FcgRt*−/− spleen cDCs. Dashed histograms: FMO background. Data are representative of one out of three analyzed mice.

**B.** CTV-labelled OT-I and OT-II T cells and CFSE-labelled BL6 mouse splenocytes were adoptively transferred into WT and *FcgRt*−/− mice. One day later, mice were injected with anti-DEC205-OVA mAb. Spleens were harvested 64 h later and analyzed by flow cytometry. The CTV histograms show OT-I and OT-II proliferation in each group of mice. The number of dividing OT-I and OT-II T cells per spleen was calculated and shown as mean ± SD. Data are pooled from four independent experiments, with 3-4 mice per group. **** *P* < 0.0001, *** *P* < 0.001 by unpaired Student’s t test.

**C.** WT and *FcgRt*−/− mice received anti-DEC205-OVA mAb and 20 hours later, spleens were harvested and cDC1 purified. Increasing number of cDC1 were co-incubated with CTV-labelled OT-I (A) or OT-II at 37°C for 60 hours or 84 hours, respectively. The number of dividing OT-I and OT-II cells was quantified by flow cytometry. Data are pooled from two independent experiments, each one done in triplicate. **** *P* < 0.0001, by two-way ANOVA with Bonferroni test for multiple comparisons. ns, not significant.

**D.** WT and *FcgRt*−/− mice were injected with anti-DEC205-Ea46-72 or PBS. Twenty hours later, spleens were harvested. I-Ab-Ea52-68 peptide complexes (pMHC II) at the surface of cDC1 were measured by flow cytometry. Histograms are representative of one mouse per group. Data are pooled from two independent experiments with symbols representing individual mice. *** *P* < 0.001 by unpaired Student’s t test. ns, not significant.
Figure 4

**A.** WT mice were lethally irradiated and reconstituted with a mix of CD45.1+ WT BM and Fcgrt−/− (CD45.2+) BM. After 6 weeks, mixed BM chimera mice were injected with anti-DEC205-OVA mAb and 20 hours later, spleens were harvested and cDC1 purified. Increasing number of cDC1 were co-incubated with CTV-labelled OT-I or OT-II at 37°C for 60 hours or 84 hours, respectively.

**B.** The number of dividing OT-I and OT-II cells was

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**Figure 4**

*C. FcRn intrinsically regulate the presentation on antigen targeted to DEC205.**

A. WT mice were lethally irradiated and reconstituted with a mix of CD45.1+ WT BM and Fcgrt−/− (CD45.2+) BM. After 6 weeks, mixed BM chimera mice were injected with anti-DEC205-OVA mAb and 20 hours later, spleens were harvested and cDC1 purified. Increasing number of cDC1 were co-incubated with CTV-labelled OT-I or OT-II at 37°C for 60 hours or 84 hours, respectively. **B.** The number of dividing OT-I and OT-II cells was
quantified by flow cytometry. Data are pooled from three independent experiments, each one done in triplicate. ** $P < 0.01$, * $P < 0.05$ by two-way ANOVA with Bonferroni test for multiple comparisons. ns, not significant.

**Figure 5**

A

Clec9A

WT

Fog1<sup>−/−</sup>

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B

OT-I

OT-II

WT

Fog1<sup>−/−</sup>

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C

OT-I

OT-II

H2O2-dependent number of autoreactive OT<sup>I</sup> T cells

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D

OT-I

OT-II

H2O2-dependent number of autoreactive OT<sup>II</sup> T cells

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E

unvaccinated

anti-Clec9A

pMHCI

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Figure 5
FcRn does not impact MHC I and MHC II presentation following Clec9A-targeted DC vaccination. A. Flow cytometry analysis of Clec9A expression in WT and Fcgrt−/− spleen cDCs. Dashed histograms: FMO background. Data are representative of one out of three mice analyzed. B. CTV-labelled OT-I and OT-II T cells and CFSE-labelled BL6 mouse splenocytes were adoptively transferred into WT and Fcgrt−/− mice. One day later, mice were injected with anti-Clec9A-OVA mAb. Spleens were harvested 64 h later and analyzed by flow cytometry. CTV histograms are representative of OT-I and OT-II proliferation in each group and the number of dividing OT-I and OT-II T cells per spleen is shown as mean ± SD. Data are pooled from two independent experiments with 3-4 mice per group. ns, not significant by unpaired Student’s t test. C. WT and Fcgrt−/− mice received anti-Clec9A-OVA mAbs and 20 hours later, spleens were harvested and cDC1 purified. Increasing number of cDC1 were co-incubated with CTV-labelled OT-I or OT-II at 37°C for 60 hours or 84 hours, respectively. The number of dividing OT-I and OT-II cells was quantified by flow cytometry. Data are pooled from two independent experiments, each one done in triplicate. D. BL/6 mice were lethally irradiated and reconstituted with a mix of CD45.1+ BM and Fcgrt−/− (CD45.2+) BM. Mixed BM chimera mice were then treated as in C and the number of dividing OT-I and OT-II cells was measured. Data are pooled from three independent experiments, each one done in triplicate. E. WT and Fcgrt−/− mice were injected with anti-Clec9A-Ea46-72 or PBS. Twenty hours later, spleens were harvested. I-Ab-Ea52-68 peptide complexes (pMHC II) at the surface of cDC1 were measured by flow cytometry. Histograms are representative of one mouse per group. Data are pooled from two independent experiments with symbols representing individual mice. *** P < 0.001 by Student’s t test. ns, not significant.
Figure 6

**Figure 6**

*FcRn is required for CTL in response to DEC205, but not Clec9A-targeted DC vaccination.* WT and *Fcgrt*⁻/⁻ mice were vaccinated with anti-DEC205-OVA mAb (A) or anti-Clec9A-OVA mAb (B), supplemented with LPS as adjuvant. Six days later, mice received with equal number of CTV^{hi} (pulsed with OVA^{257-261} peptide) and CTV^{lo} (unpulsed) target cells. 36-42 h later, spleens were harvested, and the percentage lysis
of CTV\(^\text{hi}\) cells was measured by flow cytometry. Data are pooled from two independent experiments with symbols representing individual mice. **** \(P < 0.0001\) by Student’s t test. ns, not significant.