Molecular Immunology 81 (2017) 143–150

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Antibody-mediated targeting of antigen to C-type lectin-like receptors Clec9A and Clec12A elicits different vaccination outcomes

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ARTICLE INFO

Article history:
Received 27 September 2016
Received in revised form 5 December 2016
Accepted 6 December 2016
Available online 12 December 2016

Keywords:
Dendritic cells
Antigen presentation
Vaccine
C-type lectin

ABSTRACT

Targeting antigen (Ag) to dendritic cell (DC) surface receptors is a potential new mode of vaccination. C-type lectin-like receptors Clec9A and Clec12A are attractive receptor targets however their targeting *in vivo* elicits significantly different outcomes for unknown reasons. To gain insight into the mechanisms responsible, we have examined the intrinsic capacity of Clec9A and Clec12A to elicit MHC I and MHC II Ag presentation following *ex vivo* targeting with primary murine DC. Both receptors exhibited high rates of internalization by $CD8^+$ DCs, while Clec12A delivered a significantly higher Ag owing to its higher expression level. Targeting Ag to immature $CD8^+$ DCs via both Clec9A and Clec12A failed to elicit MHC I cross-presentation above that of controls, while Clec12A was the superior receptor to target following $CD8^+$ DC maturation. $CD8^-$ DCs were unable to elicit MHC I cross-presentation regardless of the receptor targeted. For MHC II presentation, targeting Ag to Clec12A enabled significant responses by both immature $CD8^+$ and $CD8^-$ DCs, whereas Clec9A did not elicit significant MHC II Ag presentation by either DC subset, resting or mature. Therefore, Clec9A and Clec12A exhibit different intrinsic capacities to elicit MHC I and MHC II presentation following direct Ag targeting, though they can only elicit MHC I responses if the DC expressing the receptor is equipped with the capacity to cross-present. Our conclusions have consequences for the exploitation of these receptors for vaccination purposes, in addition to providing insight into their roles as Ag targets *in vivo*.

1. Introduction

Antibody (Ab)-targeted vaccination is a new strategy of vaccine delivery (Cohn and Delamarre, 2014; Kastenmuller et al., 2014; Tacken et al., 2007; Shortman et al., 2009). It consists of inoculating antigen (Ag) fused to Abs specific for a selected dendritic cell (DC) surface receptor. This optimizes the uptake of Ag by DCs, the primary cell type responsible for T cell priming. Moreover, Ab-targeted vaccination enables Ag to be delivered to mature DCs that continue to undertake receptor-mediated endocytosis but shut down other forms of Ag capture (Platt et al., 2010; Moffat et al., 2013; Reuter et al., 2015). Currently, the selection of an optimal DC receptor to target with Ag is largely dictated by its high surface expression, in particular by DC subset/s with known functional specialization/s. It is clear, however, that receptors with similar expression patterns can elicit MHC class I (MHC I) or MHC class II (MHC II) Ag presentation with variable efficacy. This suggests that events downstream of surface receptor binding are more important determinants of a receptor's efficacy than surface expression (reviewed in (Mintern

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http://dx.doi.org/10.1016/j.molimm.2016.12.010
0161-5890/

et al., 2015)). Understanding these events will help design optimal Ab-targeted vaccination strategies and maximize efficacy.

Clec9A (also known as DNGR-1) and Clec12A are C type lectin-like receptors that are of significant interest for Ab-targeted vaccination. Clec9A in particular has demonstrated excellent potential as a receptor for DC targeting and can induce both T cell and humoral immunity *in vivo*. Targeting Clec9A elicits robust $CD8^+$ and $CD4^+$ T cell priming (Caminschi et al., 2008) and in the presence of an adjuvant, can generate cytotoxic T lymphocyte killers (Sancho et al., 2008; Lahoud et al., 2011a). In addition, Clec9A targeting elicits strong humoral immunity that is associated with follicular helper T (T_{FH}) cell responses, even when the targeting Ab is administered in the absence of adjuvant (Caminschi et al., 2008; Lahoud et al., 2011a; Park et al., 2013; Li et al., 2015; Kato et al., 2015). In contrast to Clec9A, Clec12A elicits significantly inferior responses following Ag targeting *in vivo*. Effective T cell and humoral immunity following Ag delivery via Clec12A requires co-administration of an adjuvant and even then targeting Ag to Clec12A is less efficient at generating cytotoxic T cells compared to Clec9A (Lahoud et al., 2011a; Lahoud et al., 2009). Therefore, targeting Clec9A and Clec12A receptors with Ag *in vivo* elicits significantly different immune outcomes. Why this is the case is not well understood.

Immune outcomes downstream of Ab-targeted vaccination depend on numerous parameters *in vivo* that include the expression patterns of the targeted receptor, the location of the cells expressing the receptor, the access of the targeting Ab to those cells, and the affinity and half-life of the Ab. Here we assessed the intrinsic capacity of targeted receptors Clec9A and Clec12A to deliver antigen to the MHC I and MHC II presentation pathways. To exclude other factors that may affect the outcome of targeting *in vivo*, studies were performed *ex vivo* using primary DC. Surprisingly, we observed that targeting antigen via Clec9A elicited poor MHC I and MHC II presentation, while Clec12A was more efficient under specific conditions. Therefore, the intrinsic antigen trafficking properties of these receptors differ to that predicted by their behaviour as antigen targets *in vivo*.

2. Materials and methods

2.1. Mice

C57BL/6, OT-I and OT-II mice were bred under specific pathogen-free conditions at the Bio21 Institute Animal Facility. All experiments were conducted in accordance with guidelines provided by National Health and Medical Research Council of Australia. Experimental procedures were approved by the Animal Ethics Committees at the University of Melbourne.

2.2. Antibodies

Abs used for murine DC phenotyping were CD11c (clone N418) and CD8 (clone YTS.169) that were purchased from Biolegend. Hybridomas expressing anti-mouse Clec9A clones 24/04-10B4 (rat IgG2a) and 42/04-42D2 (rat IgG1), anti-mouse Clec12A clone 1/06-5D3 (rat IgG2a) or an irrelevant isotype control Ab clone GL117 (rat IgG2a) were cultured in Gibco Hybridoma (serum free) media containing 1% heat inactivated FCS. Abs were purified using protein G. For receptor-targeting with Ag, OVA was genetically fused to monoclonal Abs specific for Clec9A (clone 24/04-10B4) or Clec12A (clone 1/06-5D3) as previously described (Lahoud et al., 2011b). In brief, expression constructs encoding Ab light chains and heavy chains fused to OVA were transfected into Freestyle 293F cells using 293Fectin (Life Technologies). Culture supernatants containing recombinant Ab were harvested at day 6 post-transfection and Ab purified with protein G. Reagents used for detection of OVA were biotin-conjugated anti-OVA Ab (polyclonal, biotinylated in house, Calbiochem) and BV785-conjugated streptavidin (Biolegend).

2.3. Dendritic cell isolation

Spleens were digested with DNAse (Roche Applied Science) and collagenase (Worthington Biochemical) to generate single-cell suspensions. Light-density cells were selected using Nycodenz (Nycomed Pharma) (1.077 g/cm^3). DCs were enriched by depletion of unwanted cells with rat Abs directed against CD3 (clone KT3-1.1), Thy1 (clone TER119), CD45R (clone RA36B2) and Ly6G (clone 1A8) and anti-rat Ig magnetic beads (BioMags from Qiagen). Enrichment yielded preparations of ~70–90% $CD11c^+$ cells. Where indicated, $CD11c^+$ $CD8^+$ and $CD8^-$ DCs were sorted to purity using a BD Influx cell sorter (BD Biosciences) at the MCRI Flow Cytometry Facility (Parkville, Australia). Mature DCs were activated by culture overnight (10% CO_2, 37 °C) in media supplemented with 10 ng/ml GM-CSF (Preprotech).

2.4. Specific hybridization internalization probe (SHIP) assay

Fluorescence internalization probe (FIP)-azide (5′ Cy5-TCAGTTCAG- GACCCTCGGCT-N_3 3′) and quencher (QP_C; 5′ AGCCGAG- GGTCCTGAACTGA-BHQ2 3′) were purchased from Integrated DNA Technologies. Anti-Clec9A clone 42/04-42D2, anti-Clec12A clone 1/06-5D3 and isotype control GL117 Abs were functionalized with Click-IT succinimidyl ester DIBO alkyne (Life Technologies) by incubating with 10-fold molar excess of succinimidyl ester DIBO alkyne for 2 h at 4 °C. Functionalized Abs were purified using a Zeba spin desalting column (Thermo Scientific) and incubated with 2-fold molecular excess of FIP-azide at 4 °C overnight. FIP-labelled Ab was purified using a 30 kDa Amicon filter (Merck Millipore), and the degree of functionalization was measured with a NanoDrop 1000 UV–vis spectrophotometer. Labeling efficiency was determined as the ratio of Cy5-FIP to Ab where Ab concentration was determined by measurement of absorbance at 280 nm following subtraction of the absorbance at 280 nm signal elicited by the Cy5 probe-FIP (0.55 x absorbance at 649 nm) (Liu et al., 2016a).

SHIP internalization assays were performed by staining DCs on ice for 30 min with FIP-Cy5–conjugated Abs in the presence of anti-CD16/32 (BioLegend) to block non-specific binding of Abs to Fc receptors. Staining was followed by two washes and incubation of FIP-Ab-bound cells in complete RPMI 1640 at 37 °C, 10% CO_2. Cells were removed at specific time points and placed on ice. After the time course DCs were washed, phenotyped for surface markers, and resuspended in media containing propidium iodide with or without 1 mM QP_C. DCs were analyzed using a BD Biosciences LSR Fortessa. Data were analyzed with FlowJo (Tree Star).

2.5. Antigen presentation assays

OVA-specific T cells were purified from lymph nodes of OT-I and OT-II mice. Single-cell suspensions were depleted of unwanted cells by Abs specific for erythrocytes (clone TER-119), Ly6C/G (clone RB6-6C5), MHC II (clone M5/114), macrophages (clone F4/80), and either CD4 (clone GK1.5) or CD8 (clone 53-6.7). Labeled cells were removed using anti-rat Ig-coupled magnetic beads (BioMags from Qiagen). Purity of OT-I or OT-II cells was verified to be ~95%. Purified T cells were labelled with Cell Trace Violet (Invitrogen) at 37 °C in 0.1% BSA-PBS. Dividing OT-I or OT-II cells were determined based on the loss of CFSE or Cell Trace Violet fluorescence and enumerated by flow cytometry using calibration beads (BD Biosciences).

In vitro Ag targeting assays were performed with $CD8^+$ and $CD8^-$ DCs sorted to purity by flow cytometry. Cells were targeted with OVA-conjugated Abs specific for Clec9A, Clec12A or an isotype con-