

Human C-type Lectin Domain Family 4, Member C (CLEC4C/BDCA-2/CD303) Is a Receptor for Asialo-galactosyl-oligosaccharides^{*[5]}

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Background: The type II C-type lectin CLEC4C inhibits IFN α production by plasmacytoid dendritic cells; its natural ligand is unknown.

Results: Asialo-oligosaccharides with terminal residues of galactose bind CLEC4C and inhibit IFN α production.

Conclusion: CLEC4C-mediated suppression of IFN α production is regulated by the masking/unmasking of galactose moieties.

Significance: This represents a new potential strategy for regulating immune responses in pathological conditions.

Plasmacytoid dendritic cells are specialized in the production of type I interferon (type I IFN), which promotes antiviral and antitumor responses, as well as autoimmune disorders. Activation of type I IFN secretion depends on the pattern recognition receptors TLR7 and TLR9, which sense microbial RNA and DNA, respectively. Type I IFN production is modulated by several receptors, including the type II C-type lectin domain family 4, member C (CLEC4C). The natural ligand of CLEC4C is unknown. To identify it, here we probed a glycan array with a soluble form of the CLEC4C ectodomain. We found that CLEC4C recognizes complex type sugars with terminal galactose. Importantly, soluble CLEC4C bound peripheral blood leukocytes and tumor cells that express glycans with galactose residues at the non-reducing ends. The positive and negative modulation of galactose residues on cell membranes was paralleled by the regulation of type I IFN secretion by plasmacytoid dendritic cells in co-culture experiments *in vitro*. These results

suggest that the modulation in the expression of non-sialylated oligosaccharides by invading pathogens or transformed cells may affect type I IFN response and immune surveillance.

Plasmacytoid dendritic cells (pDC)⁵ are bone marrow-derived cells specialized in the secretion of type I IFN (1, 2). pDC are mainly found in peripheral blood and in primary and secondary lymphoid organs. pDC promptly detect viral nucleic acids, which are endocytosed and delivered to endosomes containing TLR7 and TLR9. Engagement of these receptors results in the immediate release of type I IFN, providing a very early defense against viral infections (3). pDC also secrete type I IFN in response to endogenous nucleic acids that are released during cell necrosis and/or apoptosis or are bound to anti-nuclear autoantibodies. This response may contribute to the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus, psoriasis, and Sjogren syndrome (4). Given that type I IFN activates innate and adaptive immune responses and has antiproliferative and antiangiogenic effects (5), pDC may potentially contribute to antitumor responses. However, the tumor microenvironment often inhibits pDC production of type I IFN, preventing effective antitumor immunity (6–8).

pDC express multiple receptors that inhibit type I IFN secretion, preventing immune surveillance. One of these receptors is CLEC4C, also known as blood dendritic cell antigen-2 (BDCA-2) and CD303 (9). CLEC4C is a type II transmembrane glycoprotein that belongs to the C-type lectin (CTLs) superfamily (10–12). CLEC4C is the most specific marker for human pDC (7, 9) and is only expressed in humans. It is encoded within a gene cluster on chromosome 12, which includes other CTL-encoding genes such as *CLEC4A* and *KLRG1* (13). CLEC4C consists of a single extracellular carbohydrate recognition domain (CRD), a transmembrane region, and a short cytoplasmic domain without an obvious signaling motif (9, 13). CLEC4C transmits intracellular signals through an associated transmembrane adaptor, the Fc ϵ R γ , which recruits the protein tyrosine kinase Syk, inducing protein tyrosine phosphorylation and calcium mobilization (14). Although it promotes cellular activation in other lymphoid and myeloid cells, the Fc ϵ R γ -Syk signaling pathway interferes with TLR9-induced activation of pDC, inhibiting type I IFN secretion (9).

CLEC4C is also involved in other pDC functions, such as the inhibition of soluble TNF-related apoptosis-inducing ligand (TRAIL) secretion, which mediates the killing of target cells that express TRAIL receptor (15). It was shown that pDC rapidly internalize and process a monoclonal antibody bound to CLEC4C, resulting in the generation of antibody-derived pep-

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⁵ The abbreviations used are: pDC, plasmacytoid dendritic cell(s); CLEC4C, C-type lectin domain family 4, member C; NeuAc, N-acetylneuraminic acid; CTL, C-type lectin; CRD, carbohydrate recognition domain; APC, allophycocyanin; PE, phycoerythrin; DC, dendritic cell(s); DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin; ASPG-R, asialo-glycoprotein receptor.

tides that are efficiently loaded onto MHC class II and presented to T cells (9). Thus, CLEC4C may function not only as an inhibitory receptor, but also as an antigen receptor, which pDC require for capturing certain antigens that are processed and presented to T cells.

Although CLEC4C is a key molecule of the biology of pDC, the nature and identity of CLEC4C ligands are presently unknown. In this study, we have investigated the nature and the biological relevance of the CLEC4C ligands by the use of a recombinant tetrameric form of the CLEC4C CRD domain.

EXPERIMENTAL PROCEDURES

Leukocyte Purification and Stimulation—Human leukocytes and dendritic cells were obtained as described previously (15–17).

Preparation of Recombinant CLEC4C Tetramers—We constructed a chimeric DNA fragment encoding the CLEC4C carbohydrate recognition domain (CLEC4C-CRD; amino acids 83–213) fused at its C terminus with the BirA and His₆ tags. The DNA was cloned into the pET21 vector (EMD Biosciences) and expressed in *Escherichia coli* BL21(DE3)pLysS cells (Promega) to obtain insoluble inclusion bodies. These were dissolved in 6 M guanidine, 10 mM Tris HCl (pH 8.0), and 20 mM β -mercaptoethanol, and the protein was refolded according to standard protocols (18). Following refolding, the CLEC4C preparation was purified by size exclusion chromatography on a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) and *in vitro* biotinylated using BirA biotin-protein ligase (Avidity). Monomeric CLEC4C-CRD was incubated with phycoerythrin-labeled streptavidin (BD Biosciences) at a molar ratio of 4:1 (CLEC4C monomer:streptavidin) to generate tetramers. In some experiments, recombinant human CLEC4C-Fc chimera (R&D Systems, catalog number 1376-DL) was used with similar results (supplemental Fig. 1C).

Binding of CLEC4C-CRD Tetramers to Cells—250,000 cells/sample were incubated with 5 μ g of CLEC4C-CRD tetramers or fluorochrome-labeled mAb (HLA-DR-FITC, CD3-APC, CD40-APC, CD19-APC; Biolegend) for 1 h on ice. For blocking experiments, purified α CLEC4C mAb (Miltenyi) or isotype-matched langerin mAb (Immunotech) was used at a concentration of 10 μ g/ml.

Glycan Array Analysis—To evaluate the carbohydrate binding profile of CLEC4C, we tested the affinity of CLEC4C-CRD-PE for a variety of mammalian-type carbohydrate structures using the printed Glycan Array (version 3.0) developed by the Consortium for Functional Glycomics (CFG) (19). Data were collected and analyzed by the CFG Core H staff and can be viewed in full on the Functional Glycomics Gateway website.

Enzymatic Treatments—Monocytes or B-EBV cell line (1×10^5) were incubated for 1 h at 37 °C with either 0.7 units/ml α -(2–3,6,8,9)-neuraminidase or 0.42 units/ml β -(1–4)-galactosidase (Sigma-Aldrich) to remove terminal residues of sialic acid and galactose, respectively.

Real-time PCR and ELISA—RNA and secreted IFN α were analyzed as described previously (15).

RESULTS

CLEC4C Binds Selected Populations of Leukocytes and Tumor Cells—To identify the ligands of CLEC4C, we generated fluorescent tetramers (CLEC4C-CRD-PE) to be used in flow cytometry assays. CLEC4C-CRD-PE bound purified CD14⁺ monocytes (Fig. 1A) and monocyte-derived dendritic cells with a positivity of about 50% irrespective of the maturation status (data not shown). The interaction of CLEC4C-CRD-PE with monocytes was strongly inhibited by an anti-CLEC4C mAb but was not affected by an isotype-matched antibody (*i.e.* α -langerin/CD207, Fig. 1A). No tetramer binding to CD14⁺ peripheral blood mononuclear cells (which mostly include T and B lymphocytes), natural killer cells, pDC, granulocytes, or an EBV-transformed B cell line (B-EBV) was observed (Fig. 1, B and C, and data not shown), suggesting that CLEC4C-CRD-PE binding is restricted to limited subsets of leukocytes that express CLEC4C ligands.

Because tumor-associated pDC have impaired functional activity (7, 8), it is conceivable that certain types of cancer cells may express CLEC4C ligands that engage CLEC4C to inhibit pDC. To test this hypothesis, we analyzed a panel of human tumor cell lines, which included A2780 and OVCAR3 (ovarian carcinoma), HCT116 (colon carcinoma), MiaPaCa2, Panc1 and PT45 (pancreatic carcinoma), MCF7 (breast adenocarcinoma), A2058 (melanoma), H460 (lung carcinoma), and Jurkat (T cell leukemia). CLEC4C-CRD-PE bound to all tested cells, although to different extents (supplemental Fig. 1A). Notably, CLEC4C-CRD-PE bound Jurkat cells, an immortalized T cell line, but not freshly isolated lymphocytes (Fig. 1B). These findings are consistent with the hypothesis that tumor cells express surface molecules that bind CLEC4C on pDC to inhibit their response.

CLEC4C Recognizes Complex Type Asialo-oligosaccharides with Terminal Galactose—To identify molecular structures that are recognized by CLEC4C, CLEC4C-CRD-PE was incubated with 320 oligosaccharides arrayed onto glass slides (19). CLEC4C-CRD-PE consistently bound the glycan Gal β 1-4GlcNAc β 1-2Man α 1-3(Gal β 1-4GlcNAc β 1-2Man α 1-6)-Man β 1-4GlcNAc β 1-4GlcNAc β (glycan 52 in Fig. 1D and supplemental Fig. 2). An additional interaction was observed with a similar oligosaccharide structure, namely Gal β 1-3GlcNAc β 1-2Man α 1-3(Gal β 1-3GlcNAc β 1-2Man α 1-6)-Man β 1-4GlcNAc β 1-4GlcNAc β , that contains terminal β 1-3-galactose (glycan 5 in Fig. 1D and supplemental Fig. 2). Both glycans are biantennary complex type oligosaccharides with terminal non-reducing residues of either β 1-4-galactose or β 1-3-galactose. CLEC4C-CRD-PE binding was lost when the terminal residues of either β 1-4-galactose or β 1-3-galactose were removed (see glycan 51 in Fig. 1D and supplemental Fig. 2). Moreover, these monosaccharides were not recognized by CLEC4C-CRD-PE when attached to structures other than biantennary complex oligosaccharides (data not shown). These results indicate that CLEC4C recognizes with high specificity and selectivity terminal residues of β 1-4- or β 1-3-galactose at the end of biantennary complex sugars. Because tri- and tetra-antennary complex type oligosaccharides were not present in the available arrays, we do not exclude the possibility that

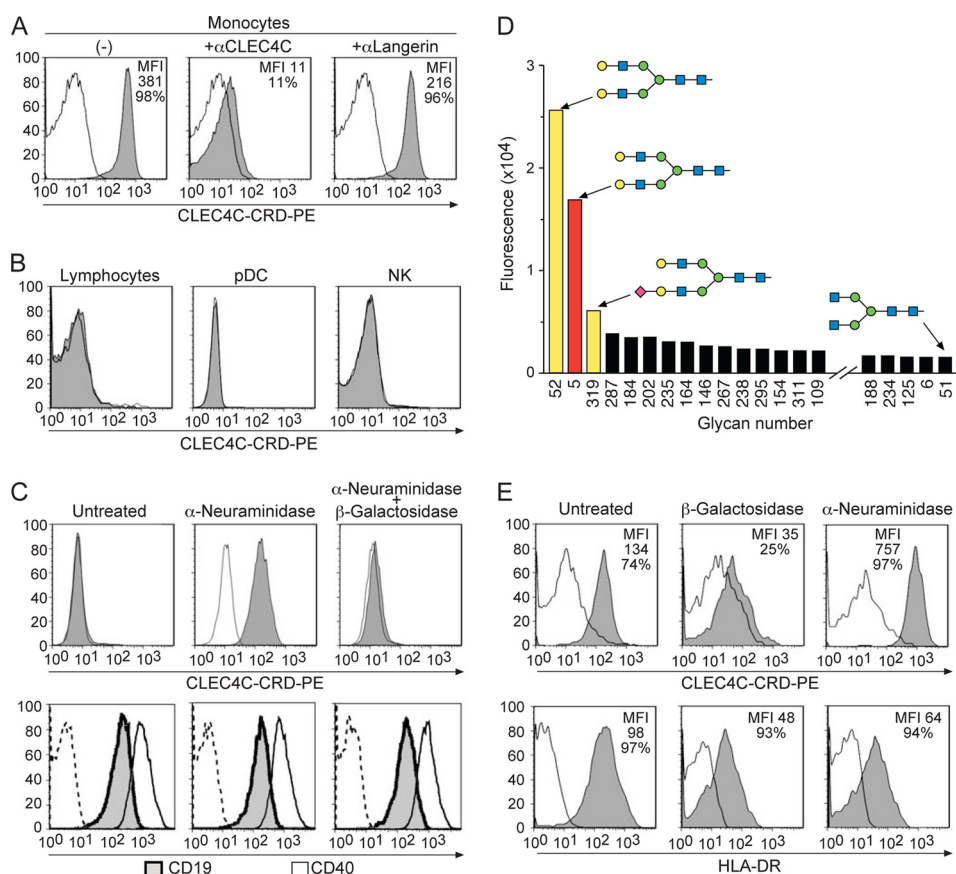


FIGURE 1. CLEC4C-CRD-PE binds to selected leukocyte populations expressing complex type asialo-oligosaccharides with terminal galactose. *A*, freshly isolated monocytes were incubated with CLEC4C-CRD-PE or streptavidin-PE as negative control. In the center and right panels, soluble CLEC4C-CRD-PE was preincubated with an anti-CLEC4C or an irrelevant anti-langerin mAb and then added to monocytes. MFI, mean fluorescence intensity. *B*, freshly isolated lymphocytes, pDC, and natural killer (NK) cells were incubated with fluorescent CLEC4C-CRD-PE. *C*, B-EBV cells were treated with α -(2-3,6,8,9)-neuraminidase or α -(2-3,6,8,9)-neuraminidase + β -(1-4)-galactosidase and then stained with CLEC4C-CRD-PE, anti-CD40-APC, or anti-CD19-APC. *D*, CLEC4C-CRD-PE was incubated with an array of 320 oligosaccharides. Protein binding was revealed by PE fluorescence and expressed as relative fluorescence units. The average intensity detected from all glycans was calculated and set as baseline. Glycans with average intensities that were more than 3-fold of the baseline are shown in the graph. The oligosaccharides giving the highest signal are highlighted in yellow and red, along with other glycans that ranked lower. Schematic structures of relevant oligosaccharides are reported, where shapes and colors indicate monosaccharides (*i.e.* blue square, green and yellow circle, and purple diamonds represent GlcNAc, Man, Gal, and NeuAc residues, respectively). Glycan numbers identify structures shown in supplemental Fig. 2. *E*, monocytes were treated with either β -(1-4)-galactosidase or α -(2-3,6,8,9)-neuraminidase and then stained with CLEC4C-CRD-PE or anti-HLA-DR-FITC monoclonal antibody. Untreated monocytes were used as control. The percentage of positive cells and mean fluorescence intensity obtained by flow cytometric analysis are shown. Data are representative of 2-3 independent experiments.

CLEC4C recognition of galactose might extend to these structures as well.

Residues of β 1-4- or β 1-3-galactose are recurrently found within the oligosaccharides decorating mammalian *N*-glycoproteins. However, *N*-glycoproteins usually terminate with extra residues of sialic acid, which mask galactose units. The attachment of a single unit of either α 2-6-NeuAc or α 2-3-NeuAc (*i.e.* a type of sialic acid) to the terminal monosaccharides of galactose markedly reduced CLEC4C-CRD-PE binding to both glycan 52 and glycan 5, this reduction being more prominent when the sialic acid is linked to the α 6 antenna (see monosialyl glycans number 319 and 295 in Fig. 1D and supplemental Fig. 2). The addition of two sialic acid residues to both antennae completely abolished binding (see disialyl glycan number 6 Fig. 1D and number 6, 53, 54, 143, and 318 in supplemental Fig. 2). Thus, CLEC4C recognizes only asialo-oligosaccharides containing terminal residues of galactose.

Galactose Moieties Are Essential for CLEC4C Binding to Target Cells—To validate that CLEC4C-CRD-PE binding to cells was due to the presence of the complex type asialo-oligosaccha-

rides identified with the glycoarrays, we evaluated the binding of CLEC4C-CRD-PE to target cells that were preincubated with substrate-specific exoglycosidases. Hydrolysis of the β 1-4-glycosidic bond that holds galactose residues at the non-reducing end of glycans and glycoconjugates with β -(1-4)-galactosidase markedly reduced CLEC4C-CRD-PE binding to monocytes (from 74 to 25%; Fig. 1E, upper panels). The residual binding (25%) is likely due to terminal units of β 1-3-galactose, which are not affected by the linkage-specific enzyme and retain binding to CLEC4C. Similar results were obtained with human ovarian A2780 carcinoma cells (data not shown).

On the other hand, unmasking of the galactose residues by removal of capping units of α -(2-3,6,8,9) sialic acid with α -(2-3,6,8,9)-neuraminidase increased the binding of CLEC4C-CRD-PE to monocytes (from 74 to 97%; Fig. 1E, upper panels). Moreover, removal of α -(2-3,6,8,9) sialic acid from B-EBV cells and CD14⁻ peripheral blood mononuclear cells allowed the binding of CLEC4C-CRD-PE (Fig. 1C, upper panels, and supplemental Fig. 1B). Of note, positivity was lost again if B-EBV cells were subsequently treated with β -(1-4)-galactosidase

REPORT: CLEC4C Binds Galactosyl-oligosaccharides

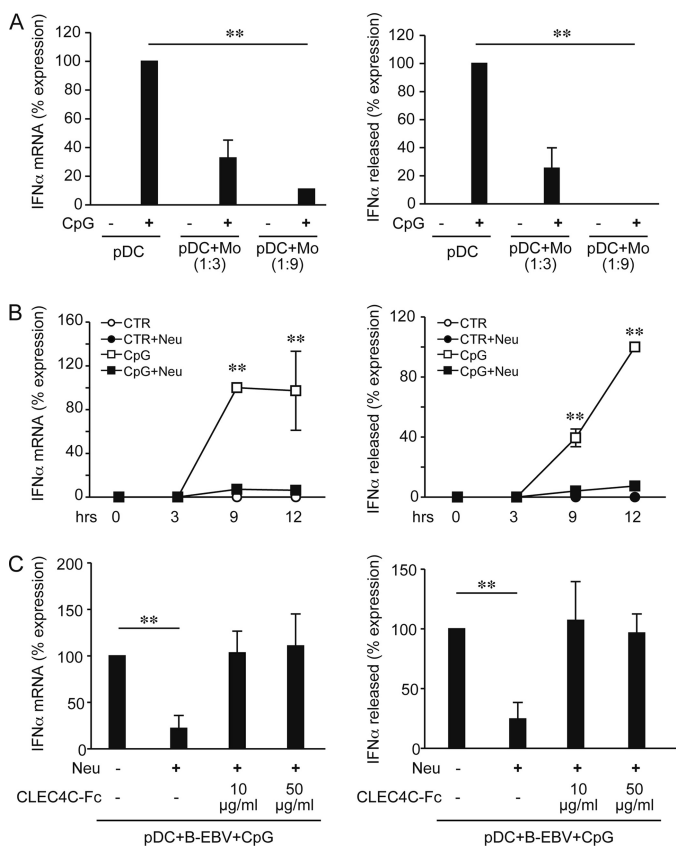


FIGURE 2. CLEC4C ligation suppresses the production of IFN α by CpG-activated pDC. A, pDC were co-cultured with monocytes at a ratio of 1:3 and 1:9 for 6 h. At the end of incubation, IFN α mRNA expression was detected by RT-PCR (left panel), and released protein was quantified in the supernatants by ELISA (right panel). B, pDC were incubated with B-EBV cells (1:3) treated (black symbols) or not (open symbols) with α -(2-3,6,8,9)-neuraminidase and stimulated (squares) or not (circles) with CpG for the indicated times. RNA and supernatants were collected and analyzed as in A. C, pDC and B-EBV cells were co-cultured as in B for 9 h in the presence of different concentrations of CLEC4C-Fc, CpG, and neuraminidase as indicated. RNA and supernatants were collected and analyzed as in A. RT-PCR results (left panels) are expressed as the percentage of expression of IFN α mRNA and represent the mean \pm S.D. of three independent experiments. ELISA results (right panels) are expressed in terms of the percentage of production of IFN α and represent mean \pm S.D. of three independent experiments. The average release of IFN α by CpG-stimulated pDC was 82.7 ± 16.42 ng/ml ($n = 15$). **, $p < 0.01$ by Student's t test.

(Fig. 1C, upper panels). Incubation of cells with galactosidases or neuraminidases did not cause major nonspecific perturbations of the cell surface proteins, as documented by normal expression of MHC class II, CD3, CD40, and CD19 (Fig. 1, C and E, lower panels, and supplemental Fig. 1B). Altogether, these results indicate that at least the terminal moieties of β 1-4-galactose are essential for CLEC4C recognition of cells, consistent with findings from glycan arrays. Sialylation of cell surface glycoconjugates modulates CLEC4C binding.

CLEC4C-mediated Interaction between pDC and Ligand-expressing Cells Blocks the Production of IFN α —To gain insight into the biological outcome of CLEC4C interaction with its ligands, pDC were co-cultured with CLEC4C-CRD-PE-positive and -negative cells. Fig. 2A shows that the presence of autologous monocytes, used as a prototype of positive cells, at different ratios significantly reduced the production of IFN α mRNA (left panel) and protein (right panel) in CpG-activated pDC. In contrast, when pDC were co-cultured with B-EBV cells

(CLEC4C-CRD-PE-negative), the release of type I IFN was not affected (not shown). As expected based on the previous findings, the unmasking of CLEC4C-CRD-PE binding sites on B-EBV cells by neuraminidase treatment caused the complete block of IFN α production by pDC (Fig. 2B). The involvement of CLEC4C was proven by the lack of inhibition of IFN α production observed using neuraminidase-treated B-EBV cells preincubated with 10 μ g/ml recombinant CLEC4C, a concentration that optimally stains monocytes and A2780 cells (supplemental Fig. 1C).

DISCUSSION

This study identifies CLEC4C as a receptor for a group of asialo-oligosaccharides with terminal residues of β 1-4- or β 1-3-galactose. CLEC4C is a member of the CLEC-DC-SIGN-like subfamily of CTLs, which includes dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), the DC-SIGN receptor, the human hepatic asialo-glycoprotein receptor (ASPG-R), and langerin (20). DC-SIGN, DC-SIGN receptor, and langerin recognize mannose ligands, with DC-SIGN and langerin also recognizing fucosylated Lewis antigens. Conversely, ASPG-R binds terminal residues of galactose or *N*-acetylgalactosamine. Thus, CLEC4C specificity is more similar to that of ASPG-R than DC-SIGN or langerin.

Cell surface glycoproteins are normally decorated by sialic acid residues, which mask terminal residues of β 1-4- or β 1-3-galactose, preventing CLEC4C recognition. Accordingly, we found no binding of CLEC4C to normal leukocytes with the only exception of monocytes. However, changes in the glycosylation status of glycoproteins and/or other glycoconjugates (for example, naturally occurring glycans) are frequently associated with pathological conditions, such as cancer (21). Tumors exploit modifications of cell surface carbohydrates to influence cell-cell adhesion and cell migration, promoting invasiveness and metastasization. Additionally, tumors can use modifications of cell surface carbohydrates to avoid immune responses. Because of carbohydrate changes, the carcino-embryonic antigen expressed by colorectal cancer cells binds DC-SIGN on dendritic cells, inhibiting the maturation of DC and capacity to elicit antitumor T cell responses (22). Remarkably, all tumor cell lines we analyzed expressed the CLEC4C ligands. Cross-linking of CLEC4C by a specific monoclonal antibody inhibits pDC secretion of type I IFN (9). Consistent with this, we show that CLEC4C-mediated interaction with monocytes or neuraminidase-treated B-EBV dramatically decreases type I IFN production by pDC. Therefore, it is conceivable that the expression of CLEC4C ligands by tumor cells may provide an efficient strategy to evade type I IFN responses. In addition, the interaction between activated pDC and CLEC4C-bearing monocytes may represent a strategy to prevent excessive release of type I IFN by activated pDC in pathological tissues.

A similar immune evasion mechanism could be used by viruses. It has been shown that Ebola virus, hepatitis C virus, Dengue virus, and cytomegalovirus overexpress glycoproteins that are recognized by DC-SIGN on DC, preventing DC capability to process and present peptides and to activate the adaptive immune response. Additionally, HIV-1 exploits the gp120-

DC-SIGN and gp120-CLEC4C interactions to bind DC, which act as a vehicle to spread viral infection to T cells (23, 24). Thus, viruses may display CLEC4C ligands to prevent type I IFN responses and facilitate spreading. Accordingly, it has been recently reported that plasma-derived Hepatitis B surface antigen inhibits TLR9-mediated IFN α secretion through CLEC4C engagement (25). We are in the process of analyzing whether Hepatitis B surface antigen expresses terminal galactose residues that can explain this process. Future effort will be aimed at the identification of CLEC4C ligand-bearing microorganisms. The characterization of CLEC4C ligands reported in our study may contribute to the development of therapeutic strategies to augment type I IFN secretion in response to viruses and tumors, as well as to reduce excessive type I IFN responses that may lead to autoimmune diseases.

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