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*J Immunol* 2007; 179:3804-3811; doi: 10.4049/jimmunol.179.6.3804

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The B7 Homolog Butyrophilin BTN2A1 Is a Novel Ligand for DC-SIGN

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The MHC-encoded butyrophilin, BTN2A1, is a cell surface glycoprotein related to the extended family of B7 costimulatory molecules. BTN2A1 mRNA was expressed in most human tissues, but protein expression was significantly lower in leukocytes. An Ig-fusion protein of BTN2A1 bound to immature monocyte-derived dendritic cells. Binding diminished upon MoDC maturation and no binding was detected to Langerhans cells. Induction of the counterreceptor was IL-4 dependent and occurred early during dendritic cell differentiation. The interaction required the presence of Ca2+ and was mediated by high-mannose oligosaccharides. These properties matched DC-SIGN, a DC-specific HIV-1 entry receptor. This was confirmed by binding of soluble BTN2A1 to DC-SIGN-transfectants and its inhibition by a specific Ab. DC-SIGN bound to native BTN2A1 expressed on a range of tissues. However, BTN2A1 was not recognized on some normal cells such as HUVECs despite a similar expression level. The BTN2A1 of tumor cells such as HEK293T have more high-mannose moieties in comparison to HUVECs, and those high-mannose moieties are instrumental for binding to DC-SIGN. The data are consistent with tumor- or tissue-specific glycosylation of BTN2A1 governing recognition by DC-SIGN on immature monocyte-derived dendritic cells. The Journal of Immunology, 2007, 179: 3804–3811.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

The Journal of Immunology

1 This work was supported by the Wellcome Trust.
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3 Current address: Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, U.K.
4 Current address: Max Planck Institute for Molecular Physiology, Department of System Cell Biology, Otto-Hahn-Str. 11, Dortmund, Germany
5 Abbreviations used in this paper: BTN, butyrophilins; BTN2L, butyrophilin-like 2; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; CEA, carcinoembryonic Ag; MoDC, monocyte-derived DCs; LC, Langerhans cells; GNA, Galanthus nivalis agglutinin; IHC, immunohistochemistry; imMoDC, immature MoDCs; Endo H, endo-β-N-acetylhexosaminidase; TRIM, tripartite motif; CI, calcium ionophore; HFF, human foreskin fibroblasts.

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hexamers. Full-length DC-SIGN cDNA was amplified by RT-PCR with BIO-X-ACT DNA polymerase (Bioline) from total human MoDC cDNA, cloned into pcDNA5/Frt/TOPO (Invitrogen Life Technologies), and confirmed by DNA sequencing. Human embryonic kidney HEK293T cells were transfected with pcDNA5/Frt/TOPO-DC-SIGN using Effectene (Qiagen) and analyzed after 48 h.

To create a DC-SIGN-FLAG fusion protein, a second PCR insert of DC-SIGN was generated using sense oligonucleotide 5′-CCCAGCTC CTATAAGTCAGGAA-3′ and antisense primer 5′-AAGTTCTGCTACG CAGGGAGG-3′ corresponding to the C terminus, and ligated in frame into BamHI pFLAG- CMV-3 (Sigma-Aldrich).

Full-length human BTN2A1 cDNAs were cloned into mammalian expression vectors as described (2) and transfected into a panel of primary cells and cell lines using Fugene (Roche Applied Science). BTN2A1 fusion proteins (referred to as BTN2A1-Ig) were prepared by cloning the extracellular domain in-frame with the hinge-CH2-CH3 domain of human IgG1 (14). To produce fusion proteins, HEK293T cells were transfected using Effectene. BTN2A1-Ig, hFc and DC-SIGN-FLAG fusion proteins were purified from culture supernatant using protein A-Sepharose or M-Flag-agarose (Sigma-Aldrich).

**Immunohistochemistry, immunoprecipitation, and Western blot**

Formalin-fixed sections of normal colon (Imgenex) were stained for BTN2A1 expression using the avidin-biotin-peroxidase system (Vectastain, Vector Laboratories). Sections were blocked with 2% normal rabbit serum and the tissue culture supernatant of a monoclonal rat anti-BTN2A1 Ab was applied followed by biotinylated rabbit anti-rat IgG and the avidine-biotin-HRP complex. Secondary Abs were preabsorbed with 10% human Ab serum before application. HRP activity was developed with diaminobenzidine and sections were counterstained with Carrazzi hematoxylin. Tissue sections of normal colon (Imgenex) were stained for BTN2A1 expression using the avidin-biotin-peroxidase system (Vector Laboratories). Sections were blocked with 2% normal rabbit serum and the tissue culture supernatant of a rat anti BTN2A1 Ab was followed by HRP-rabbit anti rat Ig (DakoCytomation). Western blots were developed using ECL system (Amersham Biosciences).

**Cells, Abs, and reagents**

PBMCs were obtained from buffy coats of healthy donors by Ficoll gradient centrifugation. Monocytes were prepared from plastic adherent PBMC and incubated for 72 h in the presence of IL-4 (500 U/ml) or for 5 days in IL-4 and GM-CSF (500 U/ml and 100 ng/ml, R&D Systems). At day 5, the phenotype of cultured MoDCs was confirmed by flow cytometry and typically was CD14low, HLA-DRhigh, and CD1ahigh with moderate levels of CD86. MoDCs were activated in the presence of 1 μg/ml LPS (L2654; Sigma-Aldrich) for 48 h. Langerhans cells (LCs) were generated as described (15).

HUVECs were grown in Endothelial Growth Medium (PromoCell) and IMR-90 (human lung embryonic fibroblasts) were grown in DMEM with glutamax, glucose 1000 mg/l, and sodium pyruvate. MOLT-4 (human acute lymphoblastic leukemia), MelJuSo (human melanoma cell line), Hela (cervical carcinoma cells), HEK293 (embryonal kidney carcinoma cells), and BAF/3 (mouse B cell lymphoma) were grown in RPMI 1640 supplemented with 2 mM glutamine, penicillin/streptomycin (100 U/ml, Invitrogen Life Technologies) and 10% FCS (Harlan Sprague Dawley). The medium for BAF/3 was also supplemented with mIL-3. T cells were separated by a Dako MoFlo cell sorter by negative selection from PBL stained with MoCD14- (Diatec), MoCD19-FITC and MoCD86-RPE (DakoCytomation), and treated with PHA/IL-2 (5 μg/ml/100 U/ml; R&D Systems), PMA/calciumionophore (10/100 ng/ml), or immobilized UCHT-1 anti CD3 Ab (DakoCytomation).

The following mouse anti-human mAbs were used: NA1/34-HLK (CD1a, Insight Biotechnology); IT2.2 (CD86-R-PE); L243 (HLA-DR); DCN46 (DC-SIGN, BD Biosciences); Mah161 (DC-SIGN, R&D Systems); and IB10 (DC-SIGN, provided by F. Arenzana-Seisdedos, Institute Pasteur, Paris).

The following rat anti-human mAbs were used: RataDC-SIGN-RPE (eBioscience); monoclonal anti-BTN2A1 Ab was raised by immunization of rats with the recombinant B30.2 domain of the BTN2A1 protein and does not cross-react with BTN3 family members, but may also recognize other BTN2 molecules.
Binding of soluble fusion proteins by flow cytometry

To analyze BTN2A1-counterreceptor expression, cells were incubated on ice with soluble BTN2A1-Ig (10 μg/ml, unless indicated otherwise) in FACS binding buffer, 1% FCS, 0.02% sodium azide in D-PBS with Ca$^{2+}$ and Mg$^{2+}$. After 20 min cells were washed and stained with FITC-conjugated goat F(ab')$_2$ anti-human IgG (CalTag Laboratories) or PE-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories). IgG (Sigma-Aldrich), hFc, and CTLA-4-Ig were used at 10 μg/ml and 1% FCS, 0.02% sodium azide in D-PBS. For inhibition assays, MoDCs or DC-SIGN-transfectants were preincubated on ice for 20 min with increasing concentrations of mannan, Ca$^{2+}$-chelator or rHIV-1SF2 gp120 (from Dr. L. Williams, NIBSC Centralised Facility for AIDS Reagents supported by EU Programme EVA and the U.K. Medical Research Council) before fusion proteins were added. To study the carbohydrate dependency of binding, BTN2A1 was treated with Endoglycosidase H (Endo H, 1 mU/μg; Roche Applied Science) at RT for 15 h. For Ab blocking experiments, DCS and DC-SIGN-transfectants were preincubated at 4°C for 20 min with anti-DC-SIGN 1B10 mAb (35 μg/ml), before directly labeled fusion proteins were added. BTN2A1-Ig was labeled with the Zenon AlexaFluor 488 human IgG labeling kit (Molecular Probes). Cells were analyzed on a FACSscan (BD Biosciences) using CellQuest software. To analyze the binding of DC-SIGN-FLAG to a panel of cells transfected with BTN2A1-GFP, a two-step detection protocol was used. An anti-DC-SIGN Ab (DCN46; BD Biosciences) was used to bridge bound BTN2A1-Ig (5 μg/ml) to a goat anti-mouse Alexa 647 (Molecular Probes). Using Alexa 647 had the advantage that cells could be analyzed using SUMMIT 4.2 software on a Cyan ADP (DakoCytomation) without using compensation. For lectin blocking experiments, HEK293T-DC-SIGN transfectants were preincubated at 4°C for 20 min with 100 μg/ml Galanthus nivalis agglutinin (GNA; Vector Laboratories), before the two-step staining was conducted. Binding of biotinylated GNA was detected using streptavidin-R-PE (Sigma-Aldrich).

To examine internalization, DCS were labeled with soluble BTN2A1-Ig, washed, and subsequently incubated at 37°C. Aliquots were removed at 5 min and 15 min and internalization stopped by metabolic fixation (D-PBS with Ca$^{2+}$ and Mg$^{2+}$ and sodium azide) at 4°C. Bound BTN2A1-Ig was analyzed by a FITC-conjugated goat F(ab')$_2$ anti-human IgG.

**Results**

**Expression of BTN2A1**

Using oligonucleotides specific for BTN2A1, RT-PCR analysis indicated that BTN2A1 mRNA was ubiquitously expressed (Fig. IA). Immunohistochemistry (IHC) using a BTN2A1-specific mAb localized a high level of expression of BTN2A1 to epithelial cells whereas lower levels were also found in leukocytes (Fig. IB). Western blot analysis confirmed BTN2A1 expression on a variety of primary cells and cell lines, such as HUVECs, IMR-90, HEK293T, and Jurkat, whereas expression on freshly isolated leukocytes, such as PBLs and monocytes was low (Fig. 1C, data not shown). This is in contrast to other B7-like molecules, as well as the related set of BTN3 butyrophilins. BTN3 molecules are preferentially expressed on T cells, T cell lines, and at lower levels on other PBMCs and some tumor cell lines (5).

**BTN2A1-Ig binds to MoDCs, not to LCs**

To identify the counterreceptor for BTN2A1, we constructed a fusion protein comprising its ectodomain with the Fc portion of human IgG1. The purified recombinant fusion protein was used to detect the presence of a counterreceptor by flow cytometry. First, we studied lymphocyte subsets including T cells, as BTN3A1 bound these cells (3). PMA/calcium ionophore (CI)-activated and nonactivated MOLT-4 T cells did not bind to BTN2A1, nor did NK-cells or B cells (Fig. 2A). Other tissues tested, including Hela
protein binding is shown as open histogram vs isotype control or hIgG and B7.2 (Fig. 3A). Human monocytes differentiated into a homogenous population of CD1a<sup>high</sup>, CD14<sup>low</sup> imDCs and expressed the BTN2A1 counterreceptor (Fig. 3B). To determine the factors responsible for the induction of the counterreceptor on differentiating DCs, monocytes were isolated and treated with IL-4. CD14 expression was low, as was CD1a expression, in contrast to IL-4/GM-CSF-treated cells (Fig. 3B, data not shown). However, IL-4-stimulated monocytes acquired significant levels of the BTN2A1-counterreceptor. Counterreceptor expression levels were similar on IL-4/GM-CSF treated cells, suggesting that expression was primarily IL-4 mediated. Expression of the counterreceptor was high at 48 h after IL-4 stimulation, indicating that it is acquired early during MoDC differentiation (Fig. 3B).

The counterreceptor for BTN2A1 is an endocytic C-type lectin

MoDCs express a variety of cell surface receptors that exhibit dual functions as Ag receptors and as cellular adhesion receptors. To help distinguish between them, we tested whether the BTN2A1 counterreceptor could function as an endocytic receptor. MoDCs were incubated with BTN2A1-Ig under saturating conditions and transferred from 4°C to 37°C. Receptor internalization was stopped at 4°C at various time points. Cell surface binding of BTN2A1-Ig was analyzed by flow cytometry using a FITC-conjugated goat F(ab')<sub>2</sub> anti-human IgG. After 5 min, >80% of the ligand had been removed from the cell surface (Fig. 4A). External loss of bound BTN2A1-Ig correlated with appearance of internalized BTN2-Ig as observed in permeabilized DCs (data not shown). Thus, bound BTN2A1 was rapidly internalized from the cell surface, consistent with endocytosis. Together, the IgV- and the IgC-like domains of the BTN2A1 monomer contain four potential N-linked glycosylation sites. To evaluate the role of sugars in BTN2A1 binding to DCs, we conducted a series of inhibition experiments. Interaction of BTN2A1-proteins with DCs required ligand had been removed from the cell surface (Fig. 4B). Whereas monosaccharides blocked BTN2A1-Ig binding only weakly (data not shown), mannan was a potent, dose-dependent inhibitor (Fig. 4C). The significance of carbohydrates for binding, in particular high-mannose-type oligosaccharides, was further corroborated by Endo H digestion. Endo H-treated BTN2A1-Ig failed to bind to DCs (Fig. 4D). To rule out binding to high-mannose carbohydrate on the Fc-domain, we also tested binding of hFc and hBTN3A3-Ig. hFc and hBTN3A3, expressed in HEK293T like BTN2A1, did not bind (Fig. 2, data not shown). Thus, the counterreceptor recognized mannosie moieties on BTN2A1, most likely branched mannose-structures. Taking these data together, the profile of BTN2A1 binding to MoDCs indicates that its counterreceptor belongs to the C-type lectin family.

**FIGURE 3.** Putative BTN2A1-counterreceptor expression is IL-4-dependent and is down-regulated on mDCs. A. MoDCs were activated with LPS for 48 h and stained for B7.2 expression using MoCD86-RPE. Mo-derived imDC (top) and mDCs (bottom) binding to BTN2A1-Ig or CTLA-4-Ig was analyzed by flow cytometry. Specific binding is shown as open histograms vs binding of hlgG (filled histograms). B. Monocytes, isolated by plastic adherence, were incubated for 72 h in the presence of IL-4 (top) or a combination of IL-4/GM-CSF (bottom). Cells were stained with soluble BTN2A1-Ig, MoCD1a, or RatDC-SIGN-RPE. Specific Ab or fusion protein binding is shown as open histogram vs isotype control or hlgG binding, respectively (filled histogram).

**BTN2A1 counterreceptor expression is up-regulated by IL-4 and down-regulated on mature DCs**

Immature MoDCs (imMoDCs) express low levels of B7 costimulatory molecules such as B7.2 (Fig. 3A). When triggered by microbial stimuli, pattern recognition receptors mediate maturation into immunogenic DCs that express high levels of MHC class II molecules such as HLA-DR and costimulatory molecules such as B7.1 and B7.2 (Fig. 3A, data not shown). To determine how DC activation affects BTN2A1 counterreceptor expression, we compared imMoDCs to LPS-treated mature MoDCs (mMoDCs). After LPS-activation, BTN2A1-counterreceptor expression diminished significantly (Fig. 3A). Down-regulation of the putative BTN2A1 counterreceptor was also observed on DCs activated by TNF-α (data not shown). As expected, a CTLA-4-Ig fusion protein showed a reciprocal staining pattern, bright on mMoDCs and low on imMoDCs, in accordance with the regulation of its ligands B7.1 and B7.2 (Fig. 3A). Human monocytes differentiated into a homogenous population of CD1a<sup>high</sup>, CD14<sup>low</sup> imDCs and expressed the BTN2A1 counterreceptor (Fig. 3B). To determine the factors responsible for the induction of the counterreceptor on differentiating DCs, monocytes were isolated and treated with IL-4. CD14 expression was low, as was CD1a expression, in contrast to IL-4/GM-CSF-treated cells (Fig. 3B, data not shown). However, IL-4-stimulated monocytes acquired significant levels of the BTN2A1-counterreceptor. Counterreceptor expression levels were similar on IL-4/GM-CSF treated cells, suggesting that expression was primarily IL-4 mediated. Expression of the counterreceptor was high at 48 h after IL-4 stimulation, indicating that it is acquired early during MoDC differentiation (Fig. 3B).

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**BTN2A1 binds DC-SIGN transfectants and binding is blocked by specific Abs and HIVgp120**

DC-SIGN was first identified as a C-type lectin that binds to HIV envelope glycoprotein gp120 (16). This molecule is predominantly expressed on DCs, including MoDCs, but not LCs (17). Its expression is IL-4 dependent and is negatively regulated by LPS and TNF-α (18). We confirmed that DC-SIGN is IL-4 inducible and its induction peaks at 48 h (Fig. 3). Thus, DC-SIGN has several hallmarks exhibited by the BTN2A1 counterreceptor. To test this, we cloned and expressed the full-length DC-SIGN cDNA in HEK293T cells. Staining with a specific mAb confirmed transient DC-SIGN expression on a majority of the HEK293T transfectants. BTN2A1 bound to DC-SIGN-transfectants, but not to nontransfected cells (Fig. 5A and data not shown). Interaction was specific for the BTN2A1 domain of the chimera because a control FC protein did not bind. We also assayed the interaction in the presence of an inhibitory Ab. As shown in Fig. 5A, binding of BTN2A1 was
inhibited by preincubation with the DC-SIGN-blocking Ab 1B10 but was not affected by an isotype control. We then addressed the question of whether DC-SIGN is the only receptor on DCs or whether there are other C-type lectins interacting with BTN2A1. The 1B10 Ab completely abrogated binding to DCs (Fig. 5B), suggesting that DC-SIGN is the exclusive receptor for BTN2A1 on DCs. Thus, the biochemistry, Ig-fusion protein binding, and Ab blocking studies were all consistent with DC-SIGN as a counter-receptor of BTN2A1.

DC-SIGN is an HIV receptor, and we explored whether BTN2A1 and HIVgp120 compete for binding. Both molecules bind to DC-SIGN via high-mannose oligosaccharides and we found that binding of BTN2A1-Ig to DC-SIGN-transfectants was blocked by recombinant gp120 in a dose-dependent manner (Fig. 5C). Thus, recombinant BTN2A1 is a candidate to inhibit transmission of HIV to DCs.

FIGURE 4. BTN2A1-counterreceptor is a C-type lectin and rapidly internalized. A, BTN2A1 protein is rapidly internalized after ligation. DCs were labeled with BTN2A1-Ig (10 μg/ml) on ice and incubated at 37°C for 0 min (solid line), 5 min (dotted line), and 15 min (scattered line) (see inset). Internalization was stopped at 4°C and bound BTN2A1-Ig detected by flow cytometry. Percent DC-BTN2A1-Ig binding was calculated as follows: [(MFI of sample − MFI of negative control)/MFI of positive control] × 100. B, Binding of BTN2A1 protein to DCs is Ca-dependent. DCs were preincubated on ice for 20 min in the presence of 1 mM Ca²⁺ with increasing concentrations of Ca²⁺-chelator, before soluble BTN2A1-Ig (10 μg/ml) fusion protein was added. Binding without inhibitor (solid line) was compared with binding in the presence of 5 mM or 20 mM EDTA and EGTA (dotted or scattered lines, respectively). C, Binding of BTN2A1-Ig is blocked by mannan and inhibition is dose-dependent. MoDCs were preincubated as described in (B) with increasing doses of mannan and analyzed for binding of BTN2A1-Ig by flow cytometry (see also inset). D, BTN2A1 binding is abrogated by Endo H digestion. BTN2A1-Ig was treated with Endo H (1 mU/μl) at RT overnight and binding to DCs was analyzed by flow cytometry.

FIGURE 5. BTN2A1 binds to DC-SIGN-transfectants with high affinity and binding is blocked by DC-SIGN-specific Ab. A, Binding of soluble BTN2A1-Ig (10 μg/ml) and hFc (10 μg/ml) to HEK293T cells transfected with DC-SIGN and blocking of BTN2A1 binding by a DC-SIGN-blocking Ab (1B10, 35 μl/ml) or an isotype control (IgG2a, 35 μg/ml, BD Biosciences). The number of cells that were positive for ―Zenon Alexa Fluor 488‖-labeled BTN2A1-Ig was analyzed by flow cytometry. The data are representative of three independent experiments. B, BTN2A1 binding and inhibition of binding to DCs by 1B10 as described in (A). C, Binding of BTN2A1 (10 μg/ml) and inhibition of binding to DC-SIGN-transfectants by HIVgp120.

Tissue- and/or tumor-specific glycosylation of native BTN2A1 is recognized by DC-SIGN.

We demonstrated that a soluble BTN2A1-fusion protein binds to DC-SIGN on MoDCs. To obtain direct evidence for the interaction
of DC-SIGN to native BTN2A1, we used DC-SIGN-FLAG for immuno precipitation. Lysates of HEK293T expressing BTN2A1 (Fig. 1C) were precipitated and separated by SDS-PAGE. A full-length BTN2A1/2/3 protein at ~69kD was detected in the cell lysate and precipitate, but not in the control. B, PMNs, HEK293 T cells, and PBLs were analyzed for sDC-SIGN binding. SDC-SIGN binding (open histogram) is shown vs binding of a BAP-FLAG protein (black filled histogram). A panel of human cells and cell lines was transiently transfected with BTN2A1- or BTN3A3-GFP. Relative binding of DC-SIGN-FLAG was plotted against the level of BTN2A1-GFP expression (FITC Log). An arbitrary baseline was set to 100% binding for the mean FL 6-fluorescence intensity of DC-SIGN binding to untransfected cells. Percentage of normalized binding was calculated as the mean FL 6 ratio of transfected to untransfected cells.

As we showed in Fig. 1, BTN2A1 is expressed in most tissues and we wondered, therefore, why DC-SIGN has not been reported to exhibit widespread binding. DC-SIGN has been shown to bind selective oligosaccharide residues on various proteins. Therefore, binding of BTN2A1 to DC-SIGN could vary if glycosylation of the butyrophilin molecule is cell-type specific. To address this, we studied binding of DC-SIGN-FLAG to cells by flow cytometry. As a positive control, we first confirmed strong binding of the DC-SIGN fusion protein to MAC-1 and CEACAM-1 on neutrophils (Fig. 6B, (13)). We then showed that HEK293T cells also bound sDC-SIGN, in accordance with our immunoprecipitation data. However, we could not detect PBL binding to DC-SIGN (Fig. 6B).

A potential problem with clearly demonstrating that cell surface BTN2A1 binds DC-SIGN is that the lectin has been assigned a
BTN2A1 is recognized by DC-SIGN

BTN2A1-GFP expression levels (Fig. 6) to study binding of DC-SIGN in the presence of GNA. GNA, a strictly mannose-binding plant lectin, blocked binding of DC-SIGN to HEK293T. Binding was inhibited by ~80% (Fig. 7A). Thus, mannose carbohydrates are mandatory in binding although we cannot rule out other carbohydrates being involved. Next, we used GNA to assess mannose-moieties of BTN2A1. GNA bound strongly to BTN2A1 on HEK293T and not to the related BTN3A3. However, GNA binding to BTN2A1 on HUVECs was low (Fig. 7B). Taking these data together, we demonstrated that BTN2A1 is differentially decorated with high-mannose moieties that determine binding to DC-SIGN.

Discussion

The butyrophilin family member BTN3A1 and mouse BTN2 were recently shown to interact with T cells. The murine BTN2 functioned as an inhibitor of T cell activation (5, 6). Using an Ig-fusion protein, produced in HEK293T cells, we demonstrated that, rather than interacting with T cells, BTN2A1 is recognized by immature MoDCs. Biochemical “fingerprinting” and Ab-blocking studies revealed that DC-SIGN was the receptor expressed on immature MoDCs interacting with BTN2A1. A mAb directed against the DC-SIGN carbohydrate-recognition domain blocked binding of BTN2A1 (20). Other C-type lectins were not involved in the interaction of BTN2A1 with DC-SIGN.

Crystal structures of the carbohydrate recognition domain, in combination with binding studies, revealed that DC-SIGN has a dual binding specificity and selectively recognizes endogenous high-mannose oligosaccharides in addition to fucose-containing glycans (21, 22). These studies predicted binding of DC-SIGN to other cell surface or soluble glycoproteins with appropriately displayed high-mannose oligosaccharides. Interaction of DC-SIGN with endogenous glycans and HIVgp120 results from high-affinity binding to a characteristic internal feature of high-mannose oligosaccharides (21). Our studies are consistent with BTN2A1 on some cells, but not all, having appropriate high-mannose moieties for binding to DC-SIGN.

We did not detect binding of DC-SIGN to PBLs. This could be explained by their low level of BTN2A1 expression. However, PBLs have high levels of ICAM-3, an alternative DC-SIGN ligand that was shown to support primary immune responses (17). Bogoevsk et al. (23) showed DC-SIGN binding to ICAM-3 on PMNs but not on T cells. ICAM-3 on T cells is not fucosylated and lacks the appropriate mannose structures for binding, whereas PMNs bind DC-SIGN via fucosylated ICAM-3 (23). Our data are consistent with these observations.

DC-SIGN bound to BTN2A1 expressed on HEK293T, Hela, and MelJuSo cells. In contrast, primary cells such as HUVECs and HFF failed to bind, despite similar BTN2A1 expression levels. However, we found that BTN2A1 of HEK293T have more high-mannose moieties in comparison to HUVECs and those high-mannose moieties are instrumental for binding to DC-SIGN. DC-SIGN did not bind to BTN2A1 expressed on any normal tissues we have studied so far. This is reminiscent of the CEA, a tumor-associated Ag, reported to bind to DC-SIGN via Lewis<sup>a</sup> and Lewis<sup>b</sup> carbohydrates. CEA-glycosylation is dysregulated in a number of malignant tissues and DCs recognize the tumor-specific glycosylation on colorectal cancer cells through DC-SIGN (12). Thus, there is a precedent for binding of DC-SIGN molecules being restricted to tumor tissues. It is also possible that glycosylation of specific tissue-types is responsible for binding to tumor cells.

However, it is too early to conclude that transformed tissues are the target of DC-SIGN. Binding of ligands such as Mac-1, CEACAM-1, and ICAM-3 appears to be due to tissue-specific, rather than tumor-specific, glycosylation patterns (13, 23, 24). The
tumor cell-binding data may be interpreted as being due to tissue-specific glycosylation patterns that happen to be found on tumor cells. Is BTN2A1 binding dependent on tumor-specific or tissue-specific glycosylation? What we know so far is that DC-SIGN binds to BTN2A1 only on cells decorated with oligomannosylated moieties. Further investigations on an extended panel of tissues, ideally, on pairs of normal and tumor tissue, as well as primary tumors are needed to resolve this issue.

We, and others, cloned a molecule related to DC-SIGN called l-SIGN or DC-SIGNR, which is expressed in liver sinusoids, placental capillaries, and the endothelium of lymph node sinuses (25, 26). Both SIGN receptors bind to pathogens such as HIV, CMV, ebola, and mycobacteria, and selectively recognize endogenous high-mannose oligosaccharides. Because DC-SIGN and l-SIGN share the structural basis for selective recognition of high-mannose oligosaccharides it is reasonable to assume that BTN2A1 also interacts with l-SIGN, implying a functional interaction of BTN2A1 beyond DCs (21).

Acknowledgments

We thank Lucy Gardner for help with IHC, Dr. F. Arenzano-Seidesedos (Institute Pasteur, Paris) for DC-SIGN mAb 1B10, Dr. Yoshinaga (Amgen) for CTLA-4-Ig, Dr. M. Douglas (University of Birmingham) for ICAM-3-Ig plasmid, Dr. L. Williams (NIBSC Centralised Facility for AIDS Reagents) for rHIV-1sg gp120, Drs. P. McAry (CIMR, Cambridge) for DC-SIGN mAb 1B10, Dr. Yoshinaga (Amgen) for rHIV-1SF2 gp120, Drs. P. McAry (CIMR, Cambridge) for LCs, Drs. Reija Valtola, Jose Garcia-Bernardo, C. Chang, and Tina Rich (Department of Pathology) for HUVECs, BAF/3, and IMR-90. We also thank Prof. K. Drickamer and Dr. M. Taylor (Imperial College London) for a very fruitful discussion.

Disclosures

The authors have no financial conflict of interest.

References