Improved in vitro and in vivo activity against CD303-expressing targets of the chimeric 122A2 antibody selected for specific glycosylation pattern

Nathalie Fourniera*, Emilie Jacquea, Alexandre Fontayneb, Delphine Deracheb, Gilles Dupontb, Lucie Verhaeghea, Linda Baptistaa, Aurélie Dehennéaa, Anne-Sophie Dezetterb, Aurélie Terriera, Alain Longueb, Virginie Pochet-Beghinb, Cécile Beghin, Sami Chtouroub, and Christophe de Romeuf

aTherapeutic Innovation Department, LFB Biotechnologies, Lille Cedex, Nord Pas de Calais, France; bTherapeutic Innovation Department, LFB Biotechnologies, Lille Cedex, Nord Pas de Calais, France

ABSTRACT

Plasmacytoid dendritic cells (pDCs) play a central role for both innate and adaptive antiviral responses, as they direct immune responses through their unique ability to produce substantial concentrations of type I interferon (IFNs) upon viral encounter while also activating multiple immune cells, including macrophages, DCs, B, natural killer and T cells. Recent evidence clearly indicates that pDCs also play a crucial role in some cancers and several auto-immune diseases. Although treatments are currently available to patients with such pathologies, many are not fully efficient. We are proposing here, as a new targeted-based therapy, a novel chimeric monoclonal antibody (mAb) that mediates a strong cellular cytotoxicity directed against a specific human pDC marker, CD303. This antibody, ch122A2 mAb, is characterized by low fucose content in its human IgG1 constant (Fc) region, which induces strong in vitro and in vivo activity against human pDCs. We demonstrated that this effect relates in part to its specific Fc region glycosylation pattern, which increased affinity for CD16/FcγRIIIa. Importantly, ch122A2 mAb induces the down-modulation of CpG-induced IFN-α secretion by pDCs. Additionally, ch122A2 mAb shows in vitro high pDC depletion mediated by antibody-dependent cell-mediated cytotoxicity and antibody-dependent cellular phagocytosis. Remarkably, in vivo ch122A2 mAb efficacy is also demonstrated in humanized mice, resulting in significant pDC depletion in bloodstream and secondary lymphoid organs such as spleen. Together, our data indicates that ch122A2 mAb could represent a promising cytotoxic mAb candidate for pathologies in which decreasing type I IFNs or pDCs depletion may improve patient prognosis.

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Introduction

Plasmacytoid dendritic cells (pDCs) were originally described in human lymph nodes in the 1950s.1 These cells were later found to secrete high amounts of type I interferons (IFNs) in response to viruses,2,3 thereby corresponding to the enigmatic natural IFN-producing cells that had previously been identified in human peripheral blood.4,5 They exhibit plasma cell morphology, express CD4, CD123, HLA-DR, blood-derived dendritic cell antigen-2 (BDCA-2/CD303) and Toll-like receptor (TLR)7 and TLR9 within endosomal compartments. As key mediators of innate immunity, pDCs sense nucleic acids via TLR7 and TLR9 and, upon TLR7/9 triggering, pDCs become activated, leading to the production of large amounts of type I IFNs that control viral replication.6 Actually, pDCs are the most potent type I IFN producers, secreting up to 1,000 times more IFN-α/IFN-β than other cell types.2,6,7 Type I IFNs are a large group of cytokines that signal through one common receptor (IFN-α/βR), in a complex process that involves different pathways.10,11,12 This signalling usually induces the expression of multiple genes that mainly lead to an antiviral state. pDCs can also secrete other pro-inflammatory cytokines and chemokines, including interleukin-6 (IL-6), tumor necrosis factor (TNF), which, together with type I IFNs, contribute to the regulation of myeloid DC, T, B, and natural killer (NK) cell function.13,14 pDCs have also been described to stimulate plasma cell differentiation through type I IFNs and IL-6.14 They also produce IL-12, CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand (CCL)3 and CCL4.

CD303, also known as blood dendritic cell antigen 2 (BDCA-2), is a C-type lectin exclusively expressed on the surface of human pDCs.15 CD303 consists of a single extracellular carbohydrate recognition domain, a transmembrane region and a short cytoplasmic tail that does not harbour any signalling motif. CD303 transmits intracellular signals through an associated transmembrane adaptor, the FcRγ, and induces a B-cell receptor (BCR)-like signalling cascade. Antibody-mediated ligation of CD303 leads to the recruitment of spleen tyrosine kinase (SYK) to the phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) of FcRγ. SYK activation then leads to the activation of Bruton tyrosine kinase (BTK) and the consequent activation of downstream kinases, including the activation of MAP kinases and the transcription factor NF-κB.
CD303 receptor engagement has been shown to inhibit TLR7- or TLR9-induced IFN-I production and other pDC-derived pro-inflammatory mediators. Based on this observation, one anti-CD303 developed by Biogen, BIIB059/24F4, is currently under evaluation in Phase 2 study (NCT02847598) as a treatment for cutaneous lupus erythematosus. In addition to the inhibition of IFN-I production by pDCs, CD303 ligation with an antibody leads to its rapid internalization by clathrin-mediated endocytosis. Notably, pDCs appear to be involved in the pathogenesis of several inflammatory autoimmune diseases and neoplastic entities. Indeed, similar to other human leukocytes, pDCs can undergo neoplastic transformation (blastic plasmacytoid dendritic cell neoplasm (BPDCN)), and accumulate during some inflammatory pathological conditions (e.g., lupus, systemic sclerosis, Sjogren syndrome). Moreover, studies have shown that breast tumour and ovarian cancer infiltrating pDCs correlate with an adverse clinical outcome, suggesting that pDCs are also involved in the progression of these cancers. A direct contribution of pDCs to tumour progression has also been reported in multiple myeloma (MM). Indeed, pDCs in the bone marrow microenvironment promote MM cell growth, survival and drug resistance.

Since low fucose-content antibodies have been shown to have enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) activity, we used the EMAbling technology to selectively produce a low-fucosylation anti-CD303 with the aim of increasing its ADCC, and consequently its anti-tumor activity.

Given its unique and exclusive surface expression on human pDCs, the depletion of pDCs by a specific anti-CD303 monoclonal antibody (mAb) could be a potential therapeutic strategy in the treatment of diverse pathologies such as cancers or autoimmune and inflammatory disorders, in which infiltrating pDC are involved. In this study, a glyco-engineered anti-CD303 antibody (ch122A2 mAb) has been generated and further characterized. In vitro and in vivo studies demonstrate its ability to efficiently deplete human pDCs cells and inhibit IFN-α secretion.

**Results**

**Murine antibody selection**

Mouse mAbs against human CD303 were derived from several hybridomas and screened by flow cytometry for their ability to bind to human CD303-transfected cells. Antibodies with an absence of cross reactivity with other immune cell types, such as NK (CD56+), T cells (CD3+), B cells (CD20+), monocytes (CD14+), granulocytes (CD15+), purified red blood cells and myeloid dendritic cells (Lin-CD11c+), were selected (Fig. 1).

As CD303 belongs to a conserved C-type lectin receptor family and shares a strong homology in extracellular domain with dendritic cell immuno-receptor (DCIR), Dectin-2, macrophage C-type lectin (MCL), macrophage-inducible C-type lectin (Mincle), we also tested hybridomas for no specific binding on other members of the Dectin-2 C-type lectin family. Finally, we investigated their ability to inhibit TLR-9-induced IFN-α by pDCs. Murine hybridoma 122A2 was selected for its high specificity for pDCs and ability to inhibit IFN-α.

**Variable domain sequence determination, construction and characterization of chimeric 122A2 (ch122A2)**

To generate 122A2 as a chimeric antibody, we determined its variable domain sequences from the murine hybridoma by GeneRacer on total RNA and sequencing. The heavy chain variable domain was homologous to murine genes

![Figure 1](https://example.com/figure1.png)
IGHV1S137*01 and IGHJ2*02 with 94.9% and 92.3% identity (Fig. 2A), and the light chain variable domain was homologous to IGKV10-96*01 and IGKJ1*01 with 98.9% and 100% identity. Synthetic genes optimized in codons for *Rattus norvegicus* and containing in 5’ end a kozak sequence and the artificial signal peptide MB7 were prepared and subcloned into HKgenEFss expression vector, resulting in the fusion backbone of a human IgG1, with \( \kappa \) constant regions. We selected a highly productive clone (8H5) from the YB2/0 cell line transfected with the expression vector encoding IgG1 122A2 for further production in a bioreactor. The culture supernatant (clone 8H5) was concentrated, filtered, and antibodies were purified by affinity chromatography. After purification, we recovered mostly full-size IgG1 122A2 (Fig. 2B) as a monomer (99.5%), and the endotoxin content was low (<0.9 UI/mg). The

Figure 2. Anti-CD303 antibody chimerization (ch122A2) and biochemical characterization. Variable regions of 122A2 mAb were determined by Gene Racer kit, sequenced and compared to IMGT germline sequences (A) After chimerization of the variable regions with human IgG1/\( \kappa \) constant regions the antibody was produced, purified on protein A and run on SDS-PAGE in reducing (R) and non-reducing (NR) conditions (B). Dose response curve for the binding of ch122A2 mAb to CD303-transfected cells (C). Dot plot showing ch122A2 binding to peripheral blood-derived pDCs observed by flow cytometry (D).
glycan structure analysis confirmed the low fucose content (36.87%) of 122A2 produced in YB2/0. When produced in HEK freestyle cells, the fucose content increased to 92.57%.

**Ch122A2 mAb antigen-binding on CD303 transfected CAL-1 cells (CAL-1-CD303)**

To confirm that the chimerization process did not alter binding ability to CD303, we used flow cytometry to evaluate the binding of ch122A2 mAb on CAL-1-CD303 cells (Fig. 2C) and on pDCs in peripheral blood of healthy volunteers (Fig. 2D).

Fig. 2C showed specific dose response binding to CAL-1-CD303 cells with Bmax values (mean fluorescence intensity (MFI) = 463) and EC50 (0.15 µg/mL). Ch122A2 mAb has a relative binding ability 6-fold higher than that of 24F4 mAb (Bmax: MFI = 419; EC50 = 0.93 µg/mL) produced in CHO in our lab using primary sequence published by Biogen.

We also tested the binding of ch122A2 mAb to pDC in whole blood sample from healthy donor. Fig. 2D showed that ch122A2 mAb binds specifically to pDC (CD3−CD14−CD20−CD123+ cells). Taken together, these results confirmed that ch122A2 recognizes CD303 expressed by transfected cells as well as peripheral pDC.

**Binding to human CD16a and enhanced ch122A2 mAb-mediated ADCC**

Lysis of target cells mediated by mAbs typically involves Fc-receptor binding mechanisms such as ADCC or complement-dependent cytotoxicity (CDC). NK cells and macrophages, both express CD16a and mediate effector cell functions such as ADCC or phagocytosis. We first investigated the binding of ch122A2 mAb to CD16a (F158-polymorphism) transfected in Jurkat cells with a competitive assay using unlabeled anti-CD303 and labelled anti-CD16 antibody (3G8). This data was in agreement with the glycosylation pattern of ch122A2 mAb, confirming that low fucosylated ch122A2 to CD16a would confer better ADCC activity than that mediated by highly fucosylated ch122A2 HEK mAb or 24F4 mAb produced in Chinese hamster ovary (CHO) cells, which had a fucose content of 98.86%.

Fig. 3B indicates that ch122A2 mediates high cytotoxicity toward CAL-1-CD303 cells in the presence of healthy volunteer NK cells with a plateau value corresponding to 100% lysis and EC50 value of 0.69 ng/mL. In comparison, ch122A2 HEK and 24F4 mAbs cytotoxic activity were much lower, with a plateau value of 54.8% lysis and 40.5%, respectively, and EC50 value of 20.2 ng/mL and 38.4 ng/mL, respectively. Thus, based on EC50 values, ADCC activity for ch122A2 mAb is almost 29-fold higher than that of ch122A2 HEK mAb and 55-fold higher than that of 24F4 mAb. Interestingly, target cell lysis occurs even at low ch122A2 mAb concentration, suggesting ADCC as a potential major mechanism of action for anti-CD303 ch122A2 mAb.

**CDC activity on CD303-transfected Jurkat cells (Jurkat-CD303) in vitro**

Antibody effector function also includes CDC, mediated by a series of complement proteins abundantly present in serum. One of these components, C1q, interacts first with the Fc regions of antibodies bound to cell surface, triggering an enzymatic cascade that results in the lysis of the opsonized target cell.

We therefore tested the capacity of ch122A2 mAb to mediate CDC of Jurkat-CD303. Results shown in Fig. 3C indicated
that chimeric anti-CD303 ch122A2 mAb mediated CDC activity on Jurkat-CD303 cells, while the irrelevant antibody did not. Percentage of lysis was 12% at the plateau value with an EC50 of 121 ng/mL, indicating moderate CDC activity supported by ch122A2.

**Enhanced ch122A2 mAb-mediated phagocytosis of CD303-transfected CAL-1 cell lines (CAL-1-CD303) by macrophages**

We also investigated the capacity of ch122A2 mAb to promote phagocytosis mediated by peripheral blood monocytes differentiated in the presence of macrophage colony-stimulating factor (M-CSF) into macrophages expressing the three types of Fc receptors, CD16, CD32 and CD64 (data not shown).

PKH67-labeled CAL-1-CD303 target cells (green) were opsonized with ch122A2 or 24F4 mAb, then incubated with PKH66-labeled macrophages (red). The phagocytosis, corresponding to double-stained cells in yellow (Fig. 3A), was determined by fluorescence microscopy and expressed as the percentage of macrophages that engulfed at least one target cell.

As shown in Fig. 4B, ch122A2 mAb significantly increased phagocytosis (45.5%) compared to 24F4 mAb (31.5%) and the control. This data suggests that enhanced phagocytosis mediated by ch122A2 mAb was related to its ability to engage the low affinity Fc receptor CD16 expressed at the cell surface of effector cells.

Fig. 4C shows that the phagocytosis of CD303+ cells by ch122A2 mAb (46%) was slightly affected by addition of intravenous immunoglobulin (IVIg) (34%) and almost completely abolished in the presence of IVIg and anti-CD16 (~ 3%). This confirms the previous assumption that CD16 is the main Fc receptor implicated in phagocytosis.

**Ligation of CD303 with ch122A2 mAb inhibits IFN-α production by pDCs**

Previous studies have shown that CD303 ligation with a mAb against CD303 (clone Ac144) suppresses the ability of human pDCs to produce type I IFN in response to TLR7 and TLR9 ligands.16,39 We tested *in vitro* whether the effect of IFN-α-inducing agent, i.e., CpG oligodeoxynucleotides, on plasmacytoid dendritic cells was altered in the presence of

Figure 4. Phagocytosis activity of ch122A2 mAb on CD303-transfected cells. PKH67 labelled CAL-1-CD303 (green) were presensitized with anti-CD303 mAbs and mixed with PKH26 labelled macrophages (red) for 2.5 hours at 37°C in a ratio E/T = 1 (A). Cells were placed onto counting slides (Mallassez) and observed under fluorescence. Phagocytosis (B) was expressed as the percentage of macrophages that engulfed at least one target cell with respect to total macrophages. Results represented mean +/- SEM of two independent experiments. Phagocytosis experiment (C) induced by ch122A2 mAb was performed in presence of IVIg (1 mg/mL) alone or in combination with anti-CD16 mAb 3G8 (10 μg/mL). *: p-values < 0.05.
anti–CD303 mAb ch122A2. Fig. 5 shows that IFN-α was produced by pDCs after stimulation with CpG. However, a dramatic inhibition of IFN-α production was observed in the presence of anti–CD303 ch122A2 mAb as compared to the irrelevant control IgG1 mAb. This inhibition did not appear related to the specific glycosylation profile of ch122A2 since 24F4 mAb produced in CHO cell gave the same extent of inhibition.

**Ch122A2 mAb deplete human pDC in peripheral blood and spleen of mouse humanized model**

To analyze the effects of ch122A2 mAb in vivo, and since CD303 is uniquely and specifically expressed at the surface of human pDCs, we treated 21 BRGS-HIS mice (N = 3–4 mice per time points) with a single dose (30 mg/kg body weight) of either ch122A2 monoclonal antibody or an isotype control (irrelevant) administrated by intravenous route. Graphs showed the frequency of human pDCs (hCD45⁺hCD3⁻hCD19⁻hCD123hihBDCA4⁺) in blood (A), in spleen (B) and in the bone marrow (C) observed at 1 d, 3 d and 7 d after treatment with mAbs. Student’s t-test: *<0.05, ***<0.001.
human pDCs, ‘humanized mice for the immune system’ (BRGS-HIS) mice were used (Fig. 6). BRGS-HIS mice were treated after randomization with a single dose (30 mg/kg body weight) of either ch122A2 mAb or an isotype control administrated by intravenous route. The impact of the treatment on human pDC and other lymphoid cell populations (e.g., B cells, T cells) present in blood, spleen and bone marrow was determined by flow cytometry at different time points: days 1, 3 and 7. As shown in Fig. 6, pDC proportions from BRGS-HIS mice were reduced after treatment with ch122A2 mAb, while the other analyzed cell populations remained unaffected by the treatment (data not shown). Importantly, anti-CD303 antibody treatment differentially affected the pDC population present in the various lymphoid tissues analyzed in this study. The most striking effect was observed in the blood (Fig. 6A), where anti-CD303 antibody injection induced an efficient (~ 90%) and fast pDC depletion (1 d) that was maintained for at least 3 days. In the spleen, the treatment effect was more modest, since only a limited, but statistically significant, reduction was observed in pDC frequency at 3 and 7 days (Fig. 6B). In contrast, no noticeable impact of the treatment was detected in the bone marrow (Fig. 6C). Taken together, these data demonstrated a proof-of-concept of efficient targeting of pDC with the ch122A2 anti-CD303 antibody in the humanized BRGSF-HIS mice.

**Discussion**

Recent studies have shed light on the involvement of pDCs in the pathogenesis of several inflammatory autoimmune diseases, neoplastic entities and solid tumor. Although, depending on the pathology, the role of pDCs seems to be different, in all cases pDCs are pivotal.

Currently, there is no therapeutic monoclonal targeting pDCs on the market and only one anti-CD303 antibody, produced in CHO, is currently in clinical studies.

Our goal was to produce a mAb with very high specificity for pDC and optimized Fc functions, particularly for CD16, in order to mediate ADCC and phagocytosis toward pDC. Previous observations from our group have shown that, using proprietary screening process and cell culture conditions as part of the EMABling technology, mAb production in YB2/0 confers low fucose content, high CD16 binding and greater cytotoxicity than a CHO cell expression system. Interestingly, the use of EMABling mAbs could reduce the antigen amount required for ADCC or phagocytosis induction via efficient recruitment of NK cells and macrophages.

As expected, anti-CD303 ch122A2 mAb produced with EMABling technology has increased affinity for CD16 and displays enhanced ADCC activity and phagocytosis compared to 24F4 produced by a CHO cell line. Moderate in vitro CDC activity was also observed on CD303-transfected Jurkat cells, but was not detected on the pDC cell line (data not shown). Although CDC activity mediated by therapeutic mAbS may represent complementary cytotoxic properties in addition to that supported by effector cells, we do not expect that ch122A2 mAb would mediate CDC activity in vivo.

Ex vivo studies on peripheral blood pDCs have also indicated that ch122A2 mAb strongly reduced the pDC ability to secrete INF-α in response to TLR-9 stimulation.Remarkably, the use of a humanized mouse model, BRGSF-HIS, allowed us to demonstrate a clear depleting activity of ch122A2 mAb in vivo, in particular in the blood stream and in secondary lymphoid organs (such as spleen). In contrast, no depleting activity of ch122A2 mAb was detected in the bone marrow.

Given the unique and specific expression of CD303 at the surface of pDCs, we developed an original and potent tool to specifically target, down modulate INF-α secretion by pDC and deplete pDCs in the presence of effector cells.

The first potential clinical application of ch122A2 would be patients with BPDCN, a rare and aggressive hematological disease characterized by a clonal malignant proliferation of blastic pDCs recognized as a distinct disease since the 2008 World Health Organization classification.

These tumors preferentially involve the skin, bone marrow and lymph nodes. It mainly affects elderly patients, but BPDCN have been described in young adults and children. At diagnosis, ~ 90% of patients exhibit cutaneous lesions, which appear as a dermis infiltrate of immature blastic cells with pDC features. Currently, there is no consensus regarding the optimal treatment modality of BPDCN; chemotherapy, allogeneic or autologous stem cell transplantation are used but the overall prognosis of BPDCN is still remarkably poor. Thus, it remains an unmet medical need.

Among new drugs developed for BPDCN, SL-401 is a recombinant human interleukin 3a (IL-3A) protein conjugated with truncated diphtheria a-toxin, an inhibitor of protein synthesis. SL-401 binds to CD123, the alpha chain of IL-3 receptor (IL-3R), causes internalization and, consequently, the translocation of the diphtheria toxin into the cytosol that binds to ADP-ribosylated elongation factor 2, leading to inactivation of protein synthesis and cell death. CD123 is highly expressed in BPDCN and SL-401 is currently undergoing evaluation in a Phase 1/2 clinical study (NCT02113982) of patients with BPDCN. Positive results have been reported, but grade 3 or 4 toxicities such as thrombocytopenia, neutropenia, transaminase elevations, hypo-albuminemia and hyponatremia have been observed.

CD303 is restricted to pDCs whereas CD123 is expressed by various non-malignant cells such as basophils, eosinophils, monocytes/macrophages, and megakaryocytes. Considering efficacy versus side effects, we predict that the use of ch122A2 mAb in BPDCN will result in the specific and exclusive killing of pDCs and subsequently with few adverse effects. Besides BPDCN, anti-CD303 122A2 mAb could be used in other cancers such as breast and ovarian cancers in which the presence of pDC at the tumor site has been associated with a poor prognosis by favoring the expansion of T Reg cells.

Since pDC is the main producer of type I IFNs, growing evidence shows pDC involvement in autoimmune and inflammatory diseases, such as psoriasis, systemic lupus erythematosus (SLE), Sjogren syndrome, systemic sclerosis, lichen planus, vitiligo, and alopecia areata. In psoriasis, a mouse xenograft model showed that activated pDCs infiltrate skin lesions and favor psoriatic skin lesions whereas anti-BDCA-2 antibody inhibits the development of the disease by blocking pDC IFN production. In SLE, mouse models with either pDC...
depletion or pDC inactivation demonstrated the pathogenic role of pDC, and suggested pDCs as a potential therapeutic target.

In addition to their ability to robustly and rapidly produce type I IFNs, as well as type III IFNs (i.e., IFN-λ1–3), human pDCs produce pro-inflammatory cytokines, such as TNF and IL-6, and various chemokines to coordinate the attraction of other immune effectors. Numerous studies have involved pDCs in the trigger and the maintenance of a perpetual inflammatory state in autoimmune context through the direct cell-cell interaction with others immune cells such as T and NK cells. Notably, the pDC activation results in their surface expression of the co-stimulatory molecules CD80, CD86 and CD83 and high levels of MHC molecules allowing antigen presentation and T cell activation. In addition, via direct cell-cell contact, pDCs interact with B cells resulting in B cell growth, differentiation, and immunoglobulin secretion.

As already mentioned, one anti-CD303 is currently developed by Biogen. This antibody 24F4A, currently under evaluation in Phase 2, inhibits type I IFNs production by pDC but does not deplete them since CHO cell expression system essentially produce mAbs with high fucose content. In contrast, our anti-CD303 ch122A2 has been designed to block pDC type I IFNs production via CD303 signaling, but also to specifically deplete pDCs by ADC or phagocytosis. Based on these potential synergic biological activities, we believe that our strategy may bring new therapeutic tools to reduce the progression of various pathologies in the field of cancer and inflammatory diseases.

Materials and methods

Antibodies

A total of 42 hybridomas producing murine anti-CD303 antibodies were obtained from Dendritics (France). CH122A2 mAb is a recombinant and chimeric antibody that targets the ectodomain of CD303. The variable domains of the light chain (VK) and the heavy chain (VH) of its parental antibody were determined from total DNA of murine hybridoma 122A2 by using GeneRacer kit (Invitrogen). Heavy and light chain Fv fragment sequences were used for alignment with the closest murine germline sequences, identified by the IMGT/DomainGapAlign tool, which was also used to calculate percentage of identity.

Synthetic genes encoding for VK and VH, each one completed of a kozak sequence and an artificial signal peptide MB7 (LFB), were then separately obtained (GeneArt) in a codon optimized format for optimal expression in the non-secreting rat myeloma cell line YB2/0 (ATCC CRL1662TM). The VK and VH fragments of 122A2 were then sequentially cloned into the optimized HKGenEFss expression vector (LFB, Switzerland) derived from BPDCN patients, was used. CAL-1 cells were transfected with the plasmid vector pCEP4 vectors, according to manufacturer. For both expressions, CH122A2 IgG were subjected to affinity chromatography, using recombinant protein A-Sepharose fast flow (GE Healthcare). The antibodies were eluted with 25 mM citrate pH3.0 and dialyzed overnight in phosphate-buffered saline (PBS) pH7.4 before being sterilely filtered with 0.2 µm membrane. The level of aggregates and endotoxins were then determined by gel filtration on Superdex HR/200 (GE-Healthcare) and by LAL testing, respectively. Glycan structure analysis and fucose percentage were determined on purified antibodies by high performance capillary electrophoresis laser induced fluorescence (HPCE-Lif). Antibody quality and purity were also monitored by SDS-PAGE and Coomassie staining.

The humanized antibody, 24F4, that specifically recognizes CD303 was prepared from the published sequences described in patent WO2014/093396A1 by Biogen Idec. Briefly, synthetic genes optimized in codons for Cricetulus griseus and coding for the heavy and light chain of 24F4 were sequentially subcloned into the optimized HKgenEFss expression vector as described above. The final construct, HKBIIB059, was used for transient transfection of CHO freestyle (Invitrogen) according to manufacturer. The 24F4 antibody was purified and biochemically characterized as described above.

Primary cells and cell lines

The established cell line CAL-1, (Dr Maeda, Nagasaki University, Japan) derived from BPDCN patients, was used. CAL-1 cells express at their surface a low level of CD303. To obtain higher expression of CD303, CAL-1 cells were transfected with the DNA vector pCDNA3.1 in which the full-length CD303 cDNA was sub-cloned using the Amaxa Human Dendritic cells Nucleofactor kit (Lonza, Switzerland) according to the instructions of the manufacturer. An obtained cell line (CAL-1-CD303) was selected for stably expressing CD303 at a high level (WO2016156449 published on October 6, 2016) and used in all in vitro experiments except when indicated. The same protocol and vector were used to generate the CD303-transfected Jurkat cells (Jurkat-CD303). Peripheral blood mononuclear cells (PBMC) and NK cells were isolated from either healthy volunteers or patients with hematomatosis (EFS Nord de France). PBMC were isolated with density gradient fractionation (Ficoll Hypaque, Sigma) and NK cells by negative depletion using bead technology (Miltenyi) following the instructions of the manufacturer.

Antigen specificity and recognition

CD303+ target cells (1 × 10⁷ cells) were incubated at 4°C for 30 minutes with various concentrations (0 to 40 µg/mL) of antibody (anti-CD303 or negative control) diluted in diluent solution (PBS with 1% fetal calf serum (FCS)). After washing in the diluent, antibodies bound to the cell surface were visualized.
with a goat F(ab')2 anti-human IgG coupled to phyco-erythrin (PE) (Beckman Coulter, PN IM1626) (100 μL of a dilution of 1:100 in diluent) at 4°C for 30 minutes. The cells were then washed and MFI studied with flow cytometer (FC500, Beckman Coulter).

To study the ch122A2 mAb binding on pDCs in whole blood cells, ch122A2 mAb was labelled using Alexa Fluor® 488 (A488) (Invitrogen). For a triple staining, 100 μL of whole blood cells were incubated with a cocktail of PC5-conjugated anti-CD3, anti-CD14, anti-CD20 (all from Beckman Coulter A07749, A07765, A07773, respectively) and PE-conjugated anti-CD123 (Beckman Coulter, B14808) and A488-conjugated ch122A2 mAb anti-CD303.

For in vivo experiments, several combinations of antibodies were used to perform fluorescence-activated cell sorting (FACS) analyses. First, for the quality control screening of 12–14 week old BRGSF-HIS mice, human hematopoietic (hCD45+) cell content in peripheral blood was determined from 50–100 μL blood sample harvested in an EDTA-coated microtube (Microvette® CB300 K2E). Flow cytometry analysis of blood cells was performed after leucocyte purification on a Ficoll density gradient. A cocktail of the following antibodies was then applied on the purified leucocytes after prior cell sample incubation with human and murine Fc-Block reagents: hCD3-PE (UCHT1, ImmunoTools, 21620034); hCD11c-PE-Cy7 (Bu15, BioLegend, 337216); hCD19-FITC (LT19, ImmunoTools, 21270193); hCD45-PerCP (H130, BioLegend, 304026); mCD45-APC-E780 (104, eBioscience, France, 47-0454-82); hCD123-BV711 (9F5, BD Biosciences, 563161); hCD304/BDC-APC (AD5-17F6, Miltenyi, 130-090-900); HLA-DR-E450 (LN3, eBioscience, 48-9996-42).

Incubations were performed in 96-well plates, in the dark at 4°C. All data acquisitions were done with an LSR-II Fortessa flow cytometer interfaced with the FACS-Diva software (BD Bioscience). The analysis of the data was completed using the FlowJo 9.8 software (TreeStar Inc.), and statistical analysis was executed with the GraphPad Prism-5 software (GraphPad Software Inc.). The percentage of hematopoietic cells (hCD45+) was calculated among total leucocytes, using the following formula: (100 x % hCD45+)/(% mCD45+_ + % hCD45+). Only animals with a satisfying frequency of hCD45+ cells among leucocytes (≥ 20%) were used in the study.

To analyze the different human hematopoietic (hCD45+) cell populations after antibody inoculation, the following protocol was used. The cell suspensions were stained with a labelled mAb panel in 96-well plates, in the dark and at 4°C on ice. The different antibodies were the same panel used for the quality control screening (see above). Cells were then incubated with a mixture of mouse and human FcBlock before applying the mAb cocktail prepared in staining buffer (PBS 0.5% bovine serum albumin, 2 mM EDTA). Blood cells were analysed in presence of count beads (CountBright, Life Technologies).

**Anti-CD303 antibodies binding to human CD16a**

MAb binding to CD16a was studied by a competitive assay using the mouse PE-labelled anti-CD16 3G8 (3G8-PE) as previously described, but CD16a-transfected Jurkat cells were used instead of NK cells.79 Briefly, CD16a-transfected Jurkat cells were incubated with variable concentrations (0 to 83 μg/mL) of the anti-CD303 simultaneously with the mouse anti-CD16 mAb 3G8-PE used at a fixed concentration (0.1 μg/mL). After several washes, the binding of 3G8-PE to CD16 expressed by transfected Jurkat cells was evaluated by flow cytometry. The MFI observed were expressed in percentage, 100% being the value obtained with the 3G8-PE alone and 0% the value in the absence of the 3G8-PE. IC50 (antibody concentration required to induce the inhibition of 3G8 binding at 50% of Imax) was calculated using GraphPad PRISM 5.01 software.

**Antibody dependent cell-mediated cytotoxicity assays**

Human NK cells were isolated from PBMC by NK Cell Isolation Kit according to the recommendations of the manufacturer (Miltenyi). 4.5 × 10⁶ cells were incubated over 16 hours in the presence of different concentrations of anti-CD303 mAbs (0 to 5,000 ng/mL) with a ratio of 15/1 (E/T). Cytotoxicity was measured by quantifying supernatant concentration of the intracellular lactate dehydrogenase (LDH) released by lysed cells. Specific lysis results were expressed as the percentage of lysis as a function of antibody concentration, 100% being arbitrarily the percentage of lysis obtained with ch122A2 at 5,000 ng/mL. EC50 values (antibody concentration inducing 50% of maximum lysis) and Emax (percentage of maximum lysis) were calculated using the software GraphPad PRISM 5.01 software.

**Complement dependent activity assay**

Cells were incubated with different concentrations of anti-CD303 ch122A2 mAb (0–5,000 ng/mL) in the presence of 1:10 dilution of baby rabbit serum as a source of complement (Cedarlane, Canada). After 1 hour of incubation at 37°C the quantity of intracellular enzyme LDH released in the supernatant by the lysed target cells was measured chromogenically (Roche, Applied Science Cytotoxicity Detection Kit) to quantify CDC mediated by the antibodies. Maximum lysis (1% Triton X-100) and spontaneous lysis levels (without antibody) served as control. The results were expressed as a percentage of lysis. EC50 (quantity of antibody that induces 50% of maximum lysis) and Emax (percentage of maximum lysis) were calculated using the software GraphPad PRISM 5.01 software.

**Phagocytosis**

CD14+ monocytes were purified from healthy blood donor mononuclear cells by immunomagnetic sorting (Miltenyi) and cultured to 1 × 10⁶ cells/mL in RPMI with 10% FCS, 50 ng/mL final of M-CSF (R&D Systems, 216-MC-500), in 100 × 20 mm cell culture plates (Becton Dickinson) at 10 mL per plate, for 48 hours at 37°C. Macrophages are characterized by specific Fc receptor expression such as CD16, CD32 and CD64 as observed by flow cytometry (data not shown).

CAL-1-CD303 cells and macrophages were stained with PKH67 (green fluorescent) and PKH26 (red fluorescent), respectively, according to manufacturer instructions.
Target cells were incubated with 10 μg/mL of anti-CD303 or control antibodies for 30 minutes at 37°C, before the addition of macrophages (E/T = 1). After 2.5 hours incubation time at 37°C, cells were placed onto counting slides (Mallassez) and observed under fluorescence.

Phagocytosis was expressed as the percentage of macrophages that engulfed at least one target cell with respect to total macrophages. For inhibition studies, macrophages were preincubated for 15 minutes at 37°C with IVIg (1 mg/mL) alone or in combination with anti-CD16 mAb 3G8 (10 μg/mL).

In vitro stimulation and IFN-α measurement

PDCs were obtained from PBMCs by direct magnetic labeling with anti-BDCA-4 mAb (BDCA-4 Cell Isolation Kit; Miltenyi). 5 × 10⁴ purified pDCs were cultured in 24 well plate flat bottom in presence of 1 μM of CPG (ODN 2216, Invivogen) with different concentrations of ch122A2 or irrelevant mAbs. The plate was then incubated overnight at 37°C with 7% CO₂.

Culture supernatants were collected and dosed by ELISA using the kit Human IFN-α module set (eBiosciences, France).

Generation and in vivo treatment of mice humanized for the immune system

In vivo studies were conducted at AXENIS (Paris, France) in accordance with procedures approved by their Institutional Animal Care and Use Committee. Mice humanized for the immune system (BRGS-HIS) were generated following the procedure previously described by Legrand et al. Briefly, newborn (≤5 days of age) BALB/c Rag2tm1Paw IL-2Rαtm1Cgn SIRPαNOD Flk2tm1Irl (BRGSF) mice were intra-hepatically transplanted with ~1 × 10⁶ human hematopoietic progenitor cells (hHPC; CD34⁺ cord blood cells) ~24 h after full body irradiation conditioning (3.0 Gy; X-ray source). Similarly to BRGS mice, BRGSF mice are immune-deficient mice devoid of murine T, B and NK cells, which are highly permissive to xenograft transplantation in virtue of the SIRPaNOD allele expression. For this study, 21 BRGS-HIS mice were generated with 4 different CD34⁺ cord blood donors. The CD34⁺ cells were stored at AXENIS in a nitrogen storage facility and thawed before use in a 37°C water bath. The cells were placed onto ice before thawing was complete and were transferred to a 50 mL tube. A large volume of ice-cold medium (RPMI GlutaMAX™ supplemented with 10% FCS) is then added drop wise. Cells were next centrifuged and resuspended in fresh medium before counting and inoculation. The animals were bred and kept in specific and opportunistic pathogen-free conditions, in individual-ventilated cages (up to 7 mice per cage) of the ABSL3 facility of AXENIS. Sterile food and water are provided ad libitum. All animal experiments received approval from the local Animal Ethical Committee (CETEA 89, Institut Pasteur Paris).

Prior to antibody inoculation, animals were randomized between two experimental groups and then treatment onset did not occur before 14 weeks post-humanization (i.e., 2 weeks after quality control procedure). Both control and anti-CD303 antibodies were intravenously administered at 30 mg/kg body weight in a final volume of 150–200 μL (7.5 mL/kg), via the retro-orbital vein route. Body weight of the mice was determined 1 day prior to treatment onset to adjust the dose individually. On each of the 3 analysis time points (days 1, 3 and 7), animals were euthanized by lethal retro-orbital exsanguination under deep narcosis (xylazine 12.5 mg/kg and ketamine 125 mg/kg). Blood was collected in the presence of EDTA (~4.55 mM final concentration) and lymphoid organs of interest (bone marrow from 2 femurs and spleen) were also harvested. Blood and spleen leucocyte isolation was performed on a Ficoll density gradient. Splenocytes were obtained by mechanical resuspension of the spleen between frosted glass slides and filtering on 70 μm nylon cell strainers. Bone marrow cells were obtained by flushing the bones with syringes, followed by filtering on 70 μm nylon cell strainers. All of the samples were then analyzed by FACS following the procedure described above.

Abbreviations

ADCC  antibody-dependent cell-mediated cytotoxicity  
BCR  B-cell receptor  
BDCA-2  blood-derived dendritic cell antigen-2  
BPDCN  blastic plasmacytoid dendritic cell neoplasm  
BTK  Bruton tyrosine kinase  
CDC  complement-dependent cytotoxicity  
CHO  Chinese hamster ovary  
CCL  CC-chemokine ligand  
CXCL  CXC-chemokine ligand  
DCIR  dendritic cell immuno-receptor  
PACS  fluorescence-activated cell sorting  
Fc  constant fragment of an antibody  
FCS  fetal calf serum  
HIS  humanized for the immune system  
IFNs  interferon  
IL  interleukin  
ITAM  immunoreceptor tyrosine-based activation motif  
IVIg  intravenous immunoglobulin  
LDH  lactate dehydrogenase  
mAb  monoclonal antibody  
MCL  macrophage C-type lectin  
M-CSF  macrophage colony-stimulating factor  
MFI  mean fluorescence intensity  
Mincl  macrophage-inducible C-type lectin  
NK  natural killer  
PBS  phosphate-buffered saline  
pDCs  plasmacytoid dendritic cells  
PLCγ2  phospholipase Cγ2  
SLE  systemic lupus erythematosus  
SYK  spleen tyrosine kinase  
TLR  toll-like receptor  
TNF  tumor necrosis factor

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.
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Author contributions
Conceptualization and investigation: NF, EF, AF and CdR; methodology: DD, GD, LV, LB, AD, ASD, AT, AL, VB, CB; supervision SC.

Competing interests: All authors have an equity interest in LFB Biotechnology which develops drugs for cancer and autoimmune diseases.

Patents
NF and CdR are inventors on patent application PCT/FR2011/052949 submitted by LFB that covers “the use of antibody directed against the BDCA-2 protein for the prevention or the treatment of the pathologies involving activation of the plasmacytoid dendritic cells”.

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