anti-FcγRIIB (CD32) antibodies differentially modulate murine FVIII-specific recall response *in vitro*

Running title: Modulation of factor VIII (FVIII) recall response

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Abstract

Fc gamma receptors (FcγRs) for IgG regulate adaptive immune responses by modulating activating and inhibitory signaling pathways within immune cells. Data from a hemophilia A mouse model demonstrate that genetic deletion or blockade of the inhibitory FcγR (CD32) suppresses the formation of antibody secreting cells (ASCs) *in vitro*. Mechanisms preventing the FVIII-specific recall response, however, remain unclear. Here, the potential role of CD32 inhibition was studied by differentially modulating receptor activity with selected anti-CD32 monoclonal antibodies (mAbs). Splenocytes from immunized $FVIII^{-/-}$ mice were re-stimulated with FVIII in the absence or presence of different anti-CD32 mAbs over 6 days. At day 6, cytokine release was quantified from cell culture supernatant and the formation of FVIII-specific ASCs assessed. Binding of FVIII-containing immune complexes (F8-ICs) to bone marrow derived dendritic cells (BMdDCs) was also investigated. The antagonistic CD32 mAb AT128 suppressed the formation of FVIII-specific ASCs and reduced secretion of IFN-γ and IL-10. In contrast, the agonistic mAbs AT130-2 and AT130-5, and their $F(ab')_2$ fragments, allowed the formation of FVIII-specific ASCs, even though the full IgG of AT130-2 reduced binding of F8-ICs to CD32. Data suggest that an inhibitory signal is transmitted when F8-ICs bind to CD32 and that this signal is required during memory B cell (MBC) activation to support formation of FVIII-specific ASCs. If the inhibitory signal is lacking due to CD32 deletion or blockade with antagonistic anti-CD32 mAbs, FVIII-specific T cell stimulation and ASC formation are suppressed, whereas agonistic stimulation of CD32 restores T cell stimulation and ASC formation.

Introduction

Fc gamma receptors for IgG (FcγRs) regulate the adaptive immune response through a series of activating and inhibitory signaling pathways within immune cells. The balance between these signaling pathways sets the threshold for cell activation thereby determining cell fate, e.g. proliferation, differentiation, and/or apoptosis. In turn the signaling pathways are triggered by activatory FcγRs or the single inhibitory FcγR. Indeed, deletion or blockade of the inhibitory FcγR (CD32 in mice, or CD32B in humans; hereafter referred to as CD32) can cause an imbalanced immune response which can result in a loss of tolerance and the development of autoimmune diseases under certain conditions [1-3]. CD32 is a low affinity receptor engaging IgG in the form of immune complexes (ICs) [4]. This engagement leads to the phosphorylation of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain of CD32 which initiates an inhibitory signaling cascade which in the humoral response results in decreased cell proliferation, antibody production and survival [5-7].

Beyond its role in humoral immunity, novel functions of CD32 have been identified including the regulation of antibody immunotherapy. CD32 influences the success of antibody immunotherapy according to the type of monoclonal antibody (mAb) employed and the cellular environment; being either detrimental or beneficial to therapeutic efficacy. In lymphoma, CD32 engagement limits the efficacy of so-called direct targeting mAb such as rituximab by enhancing their internalization, making target cells invisible to effector mechanisms [8-10]. In contrast, agonistic immunomodulatory mAb such as those against tumor necrosis factor receptors (TNFR) require CD32 engagement for their activity [11-15]. The influence of CD32 on the therapeutic efficacy of these antibodies does not rely on the inhibitory signaling cascade deriving from CD32

but rather on its ability to provide additional cross-linking of the TNF receptors to enable efficient signaling [12, 16-18]. These findings underline the potential of CD32 mAb to modulate antibody immunotherapy.

Previous studies showed an unexpected role for CD32 in anti-factor VIII (FVIII) antibody formation in a murine hemophilia A disease model [19]. Hemophilia A patients are treated with human FVIII to control their condition but unfortunately, one out of three patients develop neutralizing antibodies against FVIII [20, 21]. The FVIII-specific immune response can be established and maintained by circulating FVIII-containing immune complexes (F8-ICs) suggesting an interaction of inhibitory or/and activating FcγRs with the Fc portion of the anti-FVIII antibodies [19, 22]. Consistently, CD32 deficiency or blockade with the dual anti-CD16/32-specific mAb suppress the differentiation of murine FVIII-specific memory B cells (MBCs) into antibody secreting cells (ASCs) *in vitro*. This suppression seems to be mediated by two distinct mechanisms: (I) Induction of apoptosis in FVIII-specific MBCs and (II) disturbed FVIII presentation by antigen presenting cells (APCs) [23]. Since anti-CD16/32 mAb blocks IC binding to CD32 but also prevents the phosphorylation of its intracellular ITIM it is unclear whether the suppression of FVIII-specific ASC formation is triggered by the absence of inhibitory signaling or the blockade of F8-ICs binding to CD32. Another issue in analyzing the function of CD32 in the FVIII-specific recall response using anti-CD16/32 mAb is the additional binding to CD16 which may produce unwanted off-target effects by concurrent blockade of this activating FcγRs.

Recently, murine mAb were generated which exclusively bind CD32 [24]. These anti-CD32 mAb either activate (agonists; AT130-2, AT130-5) or inhibit (antagonist; AT128) receptor

function whilst blocking IC engagement to the inhibitory FcγRs. These mAb therefore provide the potential to more specifically probe the role of CD32 in the FVIII-specific recall response.

Materials and Methods

Animals

The following fully inbred, genetically modified mice were used: $F8^{-/-}$ (B6;129S4-*F8*^{tm2Kaz}), $F8^{-/-}CD32^{-/-}$ (B6;129S4-*F8*^{tm2Kaz}/B6;129S4-Fcγr2b^{tm1Ttk}/J), and $F8^{-/-}FcR\gamma^{-/-}$ (B6.129S4-$F8^{tm2Kaz}$/B6.129P2-Fcgr1^{tm1Ttk}) [25-29]. The cross-breeding protocol to generate $F8^{-/-}CD32^{-/-}$ or $F8^{-/-}FcR\gamma^{-/-}$ double knock-out mice has been previously described [25]. Colonies were bred and housed in the animal housing facility at the Hannover Medical School. Mice were used at 10 ± 2 weeks of age. All experimental procedures were performed in accordance with the German animal welfare act and had been approved by the regulatory authority.

Immunization protocol

Mice received 4 doses of 2 IU (~80 IU/kg) albumin-free full length recombinant human FVIII (Kogenate®; Bayer HealthCare, Leverkusen, Germany) pre-diluted in 100 µl sterile Dulbecco phosphate-buffered saline (DPBS; Invitrogen, Darmstadt, Germany) intravenously (i.v.) at weekly intervals.

Blood sampling and anti-FVIII IgG ELISA

Blood samples were obtained one week after immunization by retro-orbital puncture and incubated for 2 hours (h) at room temperature (RT). Serum was prepared by centrifugation for 15 minutes (min) at 13,200 relative centrifugal force (rcf) and subsequently stored at -20°C. Polyclonal anti-FVIII IgG antibody titer was determined from the sera by enzyme-linked immunosorbent assay (ELISA) performed as previously described [19].

In vitro re-stimulation of splenocytes and detection of FVIII-specific ASCs

Spleens of immunized mice were removed one week after immunization and prepared as described previously [19]. The total number of splenocytes was counted by cell counter (ABX Scil ABC Vet Animal Blood Counter; Horiba, Irvine California, CA). Cells were cultured at a density of 1.0 x 10^6 cells/ml in pre-warmed RPMI medium (RPMI Medium 1640 (1x) + GlutaMAX^{TM}; Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (FBS; Biochrom, Merk Millipore, Berlin, Germany) and 1 % penicillin-streptomycin (P-S; Life Technologies) at 37°C and 5 % CO_2 for 6 days in 24 well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). As indicated, splenocytes were pre-incubated with anti-CD32 mAbs at 37°C and 5 % CO_2 for 30 min and/or re-stimulated with different concentrations of FVIII added to the culture at day 0. After 6 days, detection of differentiated FVIII-specific MBCs into newly formed ASCs was performed by enzyme-linked immunospot assay (ELISPOT) as previously described [19].

Generation of bone marrow-derived DCs (BMdDCs)

Naïve $F8^{-/-}$ or $F8^{-/-}CD32^{-/-}$ or $F8^{-/-}FcR\gamma^{-/-}$ mice were killed by cervical dislocation. The bone cavity of the femurs was flushed with DPBS using a 27-G needle (Becton Dickinson (BD) Biosciences GmbH, Heidelberg, Germany) attached to a 10 ml syringe (B. Braun, Melsungen, Germany) until the cavity appeared white. Bone marrow cells were collected in a sterile 50 ml tube (Sarstedt, Nümbrecht, Germany) and washed with DPBS. Hemolysis of erythrocytes was performed using lysis buffer according to the manufacturer's instructions (BD Biosciences). Bone marrow cells were washed twice with DPBS and seeded in pre-warmed RPMI medium supplemented with 10 % FBS and 1 % P-S in the presence of 10 % cell culture medium from the AG 8653 myeloma cell line transfected with murine GM-CSF cDNA (Cell line produced by B. Stockinger; National Institute for Medical Research, Mill Hill, London, U.K.) and 20 ng/ml recombinant mouse IL-4 (R&D Systems, Minneapolis, MN). At day 3 and day 6, 75% of the medium was exchanged. After 9 days, mature BMdDCs were harvested via cell scraper (Greiner Bio-One GmbH, Frickenhausen, Germany) and counted using Luna™ Dual Fluorescence Cell Counter (Logos Biosystems, Annandale, VA). Purity of the BMdDCs was ≥ 96 % as determined by flow cytometry using murine peridinin chlorophyll (PerCP)-labeled anti-CD11c mAb.

Conjugation of polyclonal anti-FVIII IgG antibodies

The sera of 30 immunized $F8^{-/-}$ mice were used to generate a pool of murine polyclonal anti-FVIII IgG antibodies. Purification of polyclonal antibodies was achieved using a Nab™ Protein G Spin Kit according to manufacturer's instructions (Thermo Fisher Scientific, Darmstadt,

Germany). To exchange the buffer and remove salt residues Zeba^{TM} Spin Desalting Columns (Thermo Fisher Scientific) were used. Purified and desalted murine polyclonal anti-FVIII IgG antibodies were finally conjugated with Alexa Fluor 647 (AF647) according to manufacturer's instructions (Alexa Fluor 647 Antibody Labeling Kit; Thermo Fisher Scientific).

Generation of F8-ICs conjugated with AF647

Formation of F8-ICs was performed as previously described [19]. Briefly, purified polyclonal anti-FVIII IgG antibodies were pre-diluted 1:100 in DPBS and incubated with 10 IU (1µg) FVIII for 30 min at 37°C to produce F8-ICs. For visualization two types of F8-ICs were generated: (I) F8-ICs in which the antigen was conjugated with AF647 (F8-AF647-ICs; produced by BioLegend, San Diego, CA) and (II) F8-ICs in which the antibody was conjugated with AF647 (F8-ICs-AF647).

Binding of F8-ICs by C1q

F8-ICs were generated by incubating different concentrations of FVIII (0-40 IU/ml) with sera pooled from 20 immunized F8$^{-/-}$ mice (pre-diluted 1:100 in DPBS; sera contain murine polyclonal anti-FVIII antibodies, titer 1:40,480) for 30 min at 37°C. F8-ICs formation was indicated by binding to C1q via ELISA. Briefly, 10 µg/ml of human C1q (Sigma-Aldrich, Darmstadt, Germany) were immobilized to the solid phase of 96-well flat bottom plates (MaxiSorb; Nunc, Copenhagen, Denmark). After blocking of non-specific binding sites with 5%

bovine serum albumin (BSA; Sigma-Aldrich) in DPBS, plates were incubated with F8-ICs or controls. IC binding was detected by incubating wells with horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (Chemicon, Hampshire, U.K.; Dilution: 1:1,500). Substrate was developed using tetramethylbenzidine substrate (TMB; Sigma Aldrich). Reaction was stopped by 100 µl 1M hydrochloric acid (Sigma-Aldrich). Serial dilution of a master F8-IC preparation (1:100 pre-diluted pooled sera incubated with 10 IU/ml FVIII; defined as 100 arbitrary units [AU/ml]) were used for standardization.

Flow cytometry and antibodies

Flow cytometry was performed using a FACSCanto I (BD Biosciences). Data were analyzed by BD FACSDiva™ Software Version 8.0 (BD Biosciences). For discriminating living and dead cells Fixable Viability Dye eFluor® 780 (eBioscience, Frankfurt am Main, Germany) was used. Peridinin chlorophyll (PerCP)-labeled anti-mouse CD11c (BioLegend) was used as a marker for BMdDCs. anti-CD32 mAbs and corresponding $F(ab')_2$ fragments were produced in-house [24].

Cytokine analysis

Murine interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10), interleukin 6 (IL-6), interleukin 4 (IL-4), and interleukin 2 (IL-2) in cell culture supernatant were detected using the BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit

according to the manufacturer's instructions (BD Biosciences). Data were analyzed by FCAP Array™ Software Version 3.0 (BD Biosciences).

Flow cytometric analysis of F8-ICs binding to BMdDCs

0.4 x 10^6 BMdDCs were incubated with F8-AF647-ICs, F8-ICs-AF647, FVIII-AF647 or polyclonal anti-FVIII IgG antibodies-AF647 (pre-diluted 1:100 in DPBS) for 15 min at RT in the dark. After a final washing step, binding was assessed by flow cytometry. Blocking of F8-IC binding was investigated by pre-incubating BMdDCs with 2.5 µg AT130-2 IgG or F(ab')$_2$ fragments for 15 min at 4°C in the dark.

Confocal microscopic analysis of F8-ICs binding on BMdDCs

Mature BMdDCs were seeded on glass slides that have been placed into a 24 well plate and cultured for at least 24 hours to allow cell adherence. 0.4 x 10^6 BMdDCs were incubated with either F8-AF647-ICs, F8-ICs-AF647, FVIII-AF647 or polyclonal anti-FVIII IgG antibodies-AF647 for 15 min at RT in the dark. Blocking of F8-IC binding was investigated by pre-incubating BMdDCs with 2.5 µg Alexa Fluor 488 (AF488) conjugated AT130-2 IgG or F(ab')$_2$ fragments for 15 min at 4°C in the dark. Conjugation of AT130-2 IgG and F(ab')$_2$ fragments with AF488 was achieved using the AF488 Antibody Labeling Kit according to manufacturer's instructions (Thermo Fisher Scientific). After staining, cells were washed, fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set according to manufacturer's instructions

(eBioscience). Finally, cells were mounted in mounting media containing 4',6-Diamidin-2-phenylindol (DAPI) (Immunoselect Antifading Mounting Medium DAPI, Dianova, Hamburg, Germany). Confocal microscopy was performed on a LEICA TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany): Objective: HCX PL APO CS 63.0x1.40 OIL; Numerical aperture: 1.40; Acquisition software: Leica LCS Software; Image processing software: Adobe Photoshop CS2 (Adobe, Berlin, Deutschland).

Statistics

Statistical analysis of data was performed using GraphPadPrism software (Version 6.00; Graph Pad Software, La Jolla, CA). The statistical variance was determined by one-way or two-way ANOVA as indicated. *P* values of statistical significance were indicated as followed: n.s. (not significant) = $p \geq 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Results

Anti-CD32 mAbs modulate FVIII-specific ASC formation

The impact of CD32 activation or inhibition on FVIII-specific ASC formation was investigated using a previously established cell culture system [19, 30]. An efficient differentiation of FVIII-specific MBCs into ASCs was observed upon FVIII (1 IU/ml) re-stimulation of splenocytes obtained from immunized $F8^{-/-}$ mice (Figure 1). The antagonistic anti-CD32mAb AT128 suppressed the formation of FVIII-specific ASCs. In contrast, pre-incubation with agonistic

anti-CD32 mAbs (AT130-2 or AT130-5) or their $F(ab')_2$ fragments did not influence the differentiation of FVIII-specific MBCs. FVIII-specific ASC formation was undetectable upon re-stimulation of splenocytes obtained from naïve $F8^{-/-}$ mice. Data showed that anti-CD32 mAb modulated FVIII-specific MBC differentiation dependent on their functional properties (Table 1): Blockade of CD32 by antagonistic mAb suppressed ASC formation whereas activation of CD32 by agonistic mAbs allowed MBC differentiation despite blocking IC binding. Our results suggest that CD32 signaling is required during re-stimulation of FVIII-specific MBCs, possibly via F8-ICs that form in the cell culture system.

Dose-response of AT128 IgG on the FVIII-specific recall response

Inhibition of FVIII-specific ASC formation by AT128 was dose dependent: the higher the concentration of antibody used, the higher the suppressive effect on FVIII-specific MBC differentiation (Figure 2A). High concentrations of AT128 caused not only an inhibition of FVIII-specific ASC formation but also diminished the secretion of key cytokines in the FVIII-specific recall response, IFN-γ and IL-10 (Figure 2A). Low (5 µg/ml) or intermediate (10 µg/ml) concentrations of the antagonistic mAb were less efficient in suppressing cytokine release, likely reflecting its relatively low affinity for CD32. These data demonstrated that CD32 inhibition by high concentrations of AT128 suppressed both ASC formation and T cell stimulation.

FVIII-specific cytokine secretion is altered by anti-CD32 mAbs

Secretion of IFN-γ and IL-10 was strongly suppressed in the presence of AT128 (50 µg/ml) whereas agonistic mAbs did not suppress the secretion of cytokines, and AT130-2 (50 µg/ml) even enhanced secretion of IFN-γ (Figure 2B). Release of TNF-α remained unchanged in the presence of any mAb and thus, seemed not to be influenced by modulating CD32 activity. Quantity of IL-6 and IL-4 was slightly reduced by blocking CD32 but enhanced when mAb AT130-2 was used compared to 1 IU/ml FVIII alone. Unlike FVIII re-stimulation, the presence of anti-CD32 mAbs caused a slight enhancement of IL-2. $F(ab')_2$ fragments of AT130-2 or AT130-5, allowing IC binding, could also support FVIII-specific cytokine secretion (data not shown). These results showed that both inhibition and activation of CD32 altered the release of T cell dependent cytokines. Blockade of CD32 mainly suppressed cytokine secretion whereas the activation of CD32 facilitated FVIII-specific cytokine release, even though IC binding was prevented. These data also show a direct influence of CD32 on the cytokine secretion observed upon FVIII re-stimulation and indicated that inhibitory signaling triggered by CD32 was required for FVIII-specific T cell stimulation/activation probably mediated by APCs.

F8-IC binding on BMdDCs is dependent on CD32 and can be inhibited by AT130-2

CD32 regulates IC-mediated uptake and internalization of antigen on APCs [1]. Disturbed FVIII-specific cytokine secretion by CD32 blockade indicated that CD32 is engaged during re-stimulation with FVIII, possibly because FVIII is present in ICs. Recently, we demonstrated that FVIII and polyclonal anti-FVIII antibodies form ICs that were efficiently bound by immobilized

CD32 [19]. Formation of F8-ICs was additionally characterized by their binding capacity to C1q (Supplementary Data 1). Results indicate that F8-ICs were potent in binding of Fc binding receptors like C1q or CD32. But the influence of CD32 on F8-IC uptake had not been investigated. Therefore, we analyzed the binding of F8-ICs on murine BMdDCs in the presence of AT130-2. Although AT130-5 has a slightly higher affinity for CD32 (Table 1), FVIII-specific cytokine secretion seemed to be more consistent with AT130-2 (Figure 2), perhaps due to the influence of the differing isotypes, and so it was used to study the binding of F8-ICs on BMdDCs.

Mononuclear cells isolated from the bone marrow of naïve $F8^{-/-}$ mice were used to generate BMdDCs, capable of antigen presentation *in vitro* [31]. BMdDCs were characterized by their CD11c expression (Supplementary Data 2). These cells could efficiently bind F8-ICs and this binding was strongly suppressed when BMdDCs were pre-incubated with 2.5 µg unlabeled AT130-2 IgG (Figure 3). $F(ab')_2$ fragments of AT130-2 blocked F8-IC binding less effectively (Figure 3A, B). Soluble AF647-labeled FVIII can also be bound by BMdDCs but considerably less efficiently compared to FVIII-antibody complexes, probably mediated by receptors other than FcγRs (Figure 3) [32]. Pre-incubation with AT130-2 IgG did not alter efficacy of pure FVIII binding to BMdDCs. Similar results were obtained by studying the binding of FVIII complexed to anti-FVIII IgG antibodies conjugated with AF647 (F8-IC-AF647; data not shown). To further evaluate the role of CD32 in F8-IC binding, BMdDCs from naïve $F8^{-/-}CD32^{-/-}$ or $F8^{-/-}CD16/64^{-/-}$ mice were analyzed for their capability to bind F8-ICs. CD32-deficient BMdDCs bound F8-ICs similarly to WT cells in the presence of AT130-2 (Figure 3B). Binding of soluble FVIII was not influenced by the absence of CD32. When activatory Fcγ receptors (CD16 and CD64) were genetically deleted, F8-IC binding was decreased and could be further inhibited by pre-incubation with AT130-2 IgG or

its F(ab')$_2$ fragments. FVIII antigen itself can be bound by BMdDCs independently of CD16, CD64, and blockade of anti-CD32. These data showed that BMdDCs can bind both F8-ICs and soluble FVIII whereas the engagement of FVIII complexed to polyclonal anti-FVIII IgG antibodies was more pronounced compared to the antigen itself. Moreover, FVIII could be bound in an FcγR-independent manner. Engagement of F8-ICs, however, was mediated by CD16, CD64, and CD32 and could be partially reduced by anti-CD32 mAb.

Binding of AF647-conjugated F8-IC and anti-CD32 mAb AT130-2 was subsequently studied by confocal microscopy (Figure 4). F8-ICs were bound in a speckled pattern on the surface of BMdDCs (Figure 4, upper panel). When we added 2.5 µg AF488-labeled AT130-2 IgG, the typical staining of F8-ICs disappeared (figure 4, middle panel). Only some homogenous FVIII staining signal was observed that did not co-localize with AT130-2 staining. When we added the F(ab')$_2$ fragment of AT130-2 that does not prevent Fc binding to CD32 [24], the speckled staining of F8-IC was retained and co-localized with the signal of the F(ab')$_2$ fragment. Similar results were detected using F8-IC-AF647 (data not shown). These results suggest that F8-IC indeed engages CD32 during FVIII uptake by APCs. The full IgG of AT130-2 blocked the binding of F8-ICs to the surface of BMdDCs probably by inhibiting IC engagement to CD32. In contrast, F(ab')$_2$ fragments of AT130-2 co-localized with F8-IC suggesting a simultaneous binding to CD32.

Discussion

Genetic deletion or inhibition of CD32 suppressed the FVIII-specific recall response by inducing apoptosis in FVIII-specific MBCs and reducing T cell stimulation by APCs [23]. The receptor mechanisms preventing the FVIII-specific recall response, however, remained unclear. To approach this issue we studied CD32 function by modulating receptor activity using agonistic and antagonistic anti-CD32 mAbs. Blockade of CD32 using the *antagonistic* mAb AT128 suppressed FVIII-specific ASC formation and reduced secretion of key cytokines, IFN-γ and IL-10. This data confirmed previous result with the antagonistic anti-CD16/32 mAb [23]. In contrast, activation of CD32 by *agonistic* mAbs AT130-2 and AT130-5 allowed the differentiation of FVIII-specific ASCs and enabled normal cytokine release. Of note, these antibodies prevent the binding of F8-ICs to CD32. Hence, we propose that activation of inhibitory signaling, rather than IC binding to CD32, is the critical step in regulating the FVIII-specific recall response.

Formation of FVIII-specific ASCs was suppressed by AT128 in a dose dependent manner, and T cell stimulation was diminished at higher doses (Figure 2A). In comparison to the dual anti-CD16/CD32 mAb, a five times higher concentration of AT128 was needed to suppress the FVIII-specific recall response, probably reflecting its lower affinity for CD32 [23, 24]. Otherwise, results observed with this mAb were similar to those with anti-CD16/CD32 mAb excluding major effects occurring from the additional blockade of CD16 [23]. Taken together, these results confirm the importance of CD32 in the FVIII-specific B cell response.

CD32 is the only FcγR expressed on B cells but it is also expressed on effector cells like macrophages, neutrophils, and mast cells [33, 34]. Crosslinking between high affinity BCRs and

CD32 controls B cell activity and survival [35]. B cells expressing low affinity BCRs receive signals only from CD32, resulting in receptor clustering and induction of apoptosis [6, 36]. By contrast, IC mediated cross-linking between high affinity BCR and CD32 leads to the recruitment of inositol phosphatase SHIP providing an anti-apoptotic signal [6, 37, 38]. Previously, we observed that the blockade of CD32 during FVIII re-stimulation resulted in apoptosis in FVIII-specific MBCs [23]. Pan caspases inhibitor were able to rescue these MBCs allowing for the differentiation into FVIII-specific ASCs. Hence, we suggest that the absence of CD32 caused the induction of apoptosis by high affinity BCRs due to the lack of anti-apoptotic signals mediated through SHIP.

In the present study we showed that the FVIII-specific recall response could be modified by CD32 activity: CD32 inhibition diminished and CD32 activation allowed ASC formation. This indicates that the differentiation of FVIII-specific MBCs into ASCs is dependent on the CD32 engagement by F8-ICs that form in the cell culture system upon FVIII re-stimulation. This engagement causes the phosphorylation of a tyrosine residue in the ITIM providing inhibitory activity of CD32 [39]. The antagonistic mAb AT128 prevents the phosphorylation of the ITIM and also precludes IC binding to the receptor which leads to the absence of downstream signaling events mediated by CD32 and thus prevents MBC differentiation [24]. $F(ab')_2$ fragments of agonistic anti-CD32 mAbs did not influence the differentiation of FVIII-specific MBCs (Figure 1) probably by allowing F8-IC binding to CD32 (Figure 3, 4). Although $F(ab')_2$ fragments do not activate CD32, residual binding of F8-ICs may induce the phosphorylation of the tyrosine residue in the intracellular domain of CD32 mediating inhibitory signals that are required for the FVIII-specific recall response [24]. By contrast, the full IgG of the agonistic mAb AT130-2 suppressed F8-IC binding but FVIII-specific ASC formation was not disturbed. It was previously shown that

binding of AT130-2 triggers phosphorylation in the intracellular ITIM of CD32. Thus, CD32 signaling but not F8-IC binding seemed to be required to induce MBC differentiation. Further, we support the previous suggestion that $F(ab')_2$ fragments of the agonistic anti-CD32 mAb AT130-2 recognizes an epitope outside of the Fc binding groove of CD32 thereby allowing the engagement of the Fc part of polyclonal anti-FVIII antibodies linked to FVIII [24]. In contrast, the Fc portion of the full IgG probably orientates into the CD32 Fc binding groove and thereby prevents F8-IC binding.

Interestingly, recent data showed that murine BMdDCs were able to internalize FVIII complexed to anti-FVIII IgG antibodies more efficiently than pure FVIII [22]. This internalization was FcγR dependent. The expression of CD32 alone was sufficient to internalize F8-ICs more effectively than soluble antigen. We also demonstrated that the binding of FVIII on murine BMdDCs was enhanced if the antigen was complexed to anti-FVIII IgG antibodies (Figure 3, 4). Further, F8-ICs binding to BMdDCs was also FcγR dependent since genetic deletion of either CD16/CD64 or CD32 or blockade by AT130-2 reduced the binding (Figure 4). These data indicate a critical role of CD32 in F8-IC uptake and internalization by APCs. Additionally, we observed previously that naïve B cells pulsed with F8-ICs in the presence of anti-CD16/CD32 mAb were reduced in their ability to stimulate FVIII primed $CD4^+$ T cells [23]. Taken together, these results suggest that CD32 signaling in APCs due to F8-IC engagement contributes to efficient activation/stimulation of $CD4^+$ T cells.

Our data suggest that signaling through CD32 is the critical step in the FVIII-specific recall response, rather than F8-IC binding itself. Although the agonistic anti-CD32 mAb AT130-2 prevents F8-IC binding, T cell stimulation and FVIII-specific differentiation of MBCs is not

perturbed. This may be attributed to the phosphorylation of the ITIM triggered by AT130-2. These studies underline the central role of CD32 in regulating the adaptive immune response to FVIII and indicate the potential modulatory capacity of anti-CD32 mAb.

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Disclosure of Conflict of Interests

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Figures

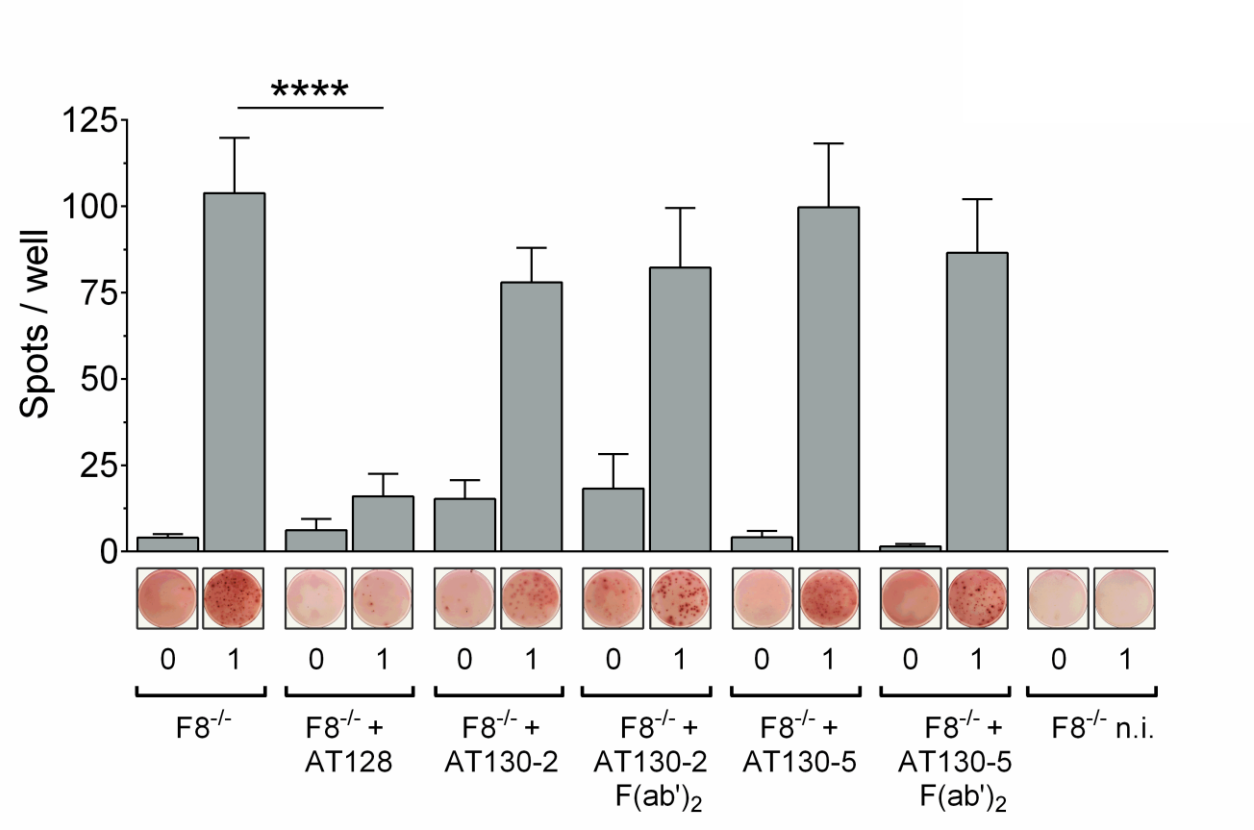


Figure 1: Anti-CD32 mAbs alter FVIII-specific ASC formation. Splenocytes from naïve or immunized $F8^{-/-}$ mice were re-stimulated with (1) or without (0) FVIII [IU/ml] in the absence or presence of (50 µg/ml) anti-CD32 mAb (n=5) or their $F(ab')_2$ fragments (n=2) over 6 days. Formation of FVIII-specific ASCs was detected by ELISPOT. Duplicate or triplicate experiments with pooled cells from at least 3 mice per group were performed. Mean ± SEM is shown. Results obtained by re-stimulating with 1 IU/ml FVIII in the absence or presence of anti-CD32 mAb were analyzed for statistical significance. Results that were not significantly different from each other were not indicated. P-value of statistical significance was indicated as followed: **** = $p < 0.0001$ (two-way ANOVA). n.i.: not immunized.

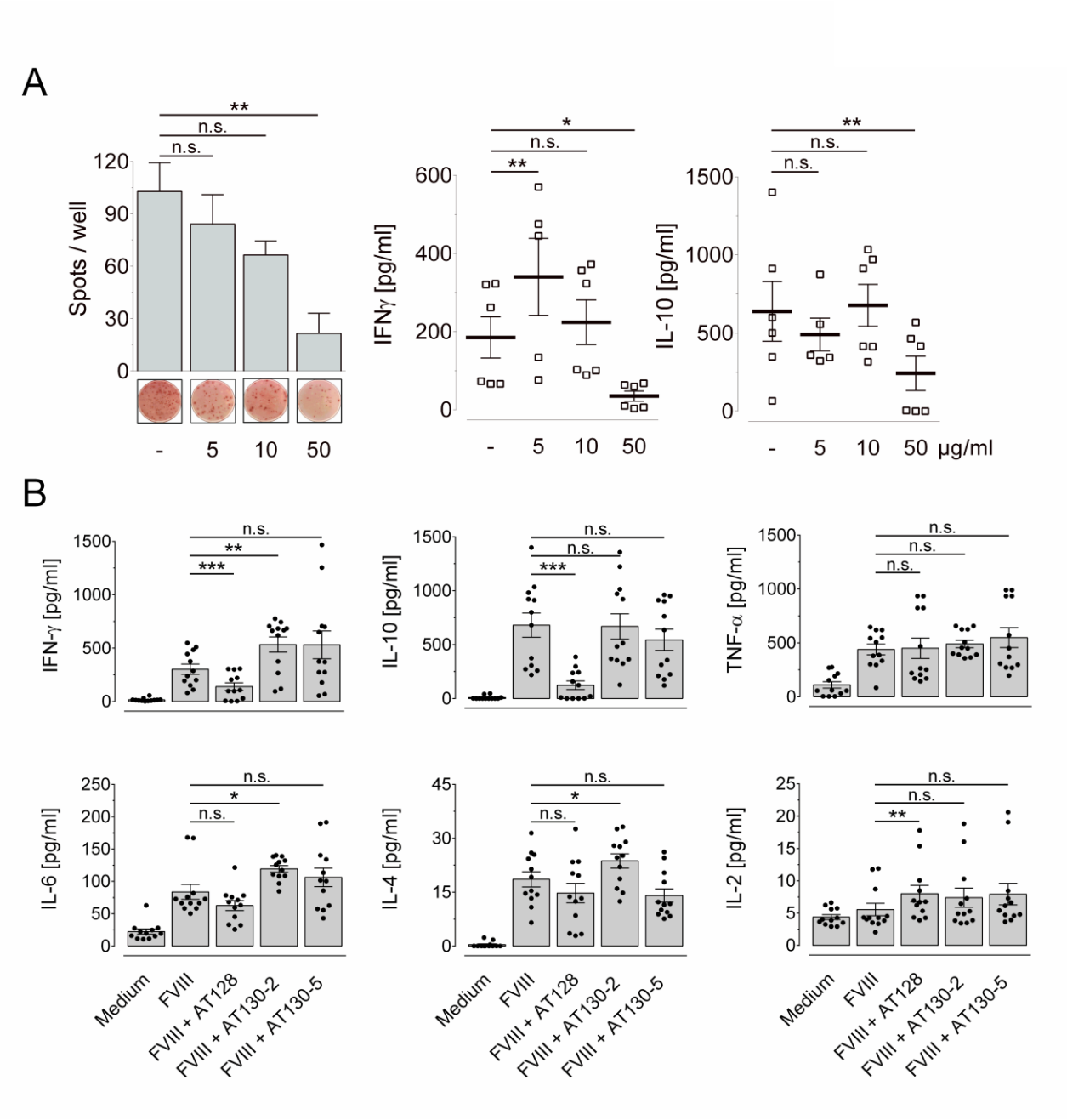


Figure 2: Anti-CD32 mAbs modify cytokine secretion upon FVIII re-stimulation. Splenocytes obtained from immunized $F8^{-/-}$ mice were re-stimulated with 1 IU/ml FVIII in the absence or presence of AT128, AT130-2 or AT130-5. (A) Influence of different concentrations (5 – 50 µg/ml) of antagonistic mAb AT128 on FVIII-specific ASC formation, IFN-γ and IL-10 release at day 6 of cell culture. Data from 2 independent experiments were pooled. Triplicate experiment with

pooled cells from at least 3 mice per group was performed. (B) Influence of (50 µg/ml) anti-CD32 mAb on FVIII-specific cytokines secretion. Cytokines were quantified from the cell culture supernatant at day 6. Data from 5 independent experiments were pooled. Duplicate and triplicate experiments with pooled cells from at least 3 mice per group were performed. Mean ± SEM is shown. Statistical significance is related to values obtained by re-stimulating splenocytes with 1 IU/ml rhFVIII. P-values of statistical significance were indicated as followed: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; (2way ANOVA test, one-way ANOVA test). n.s.: not significant.

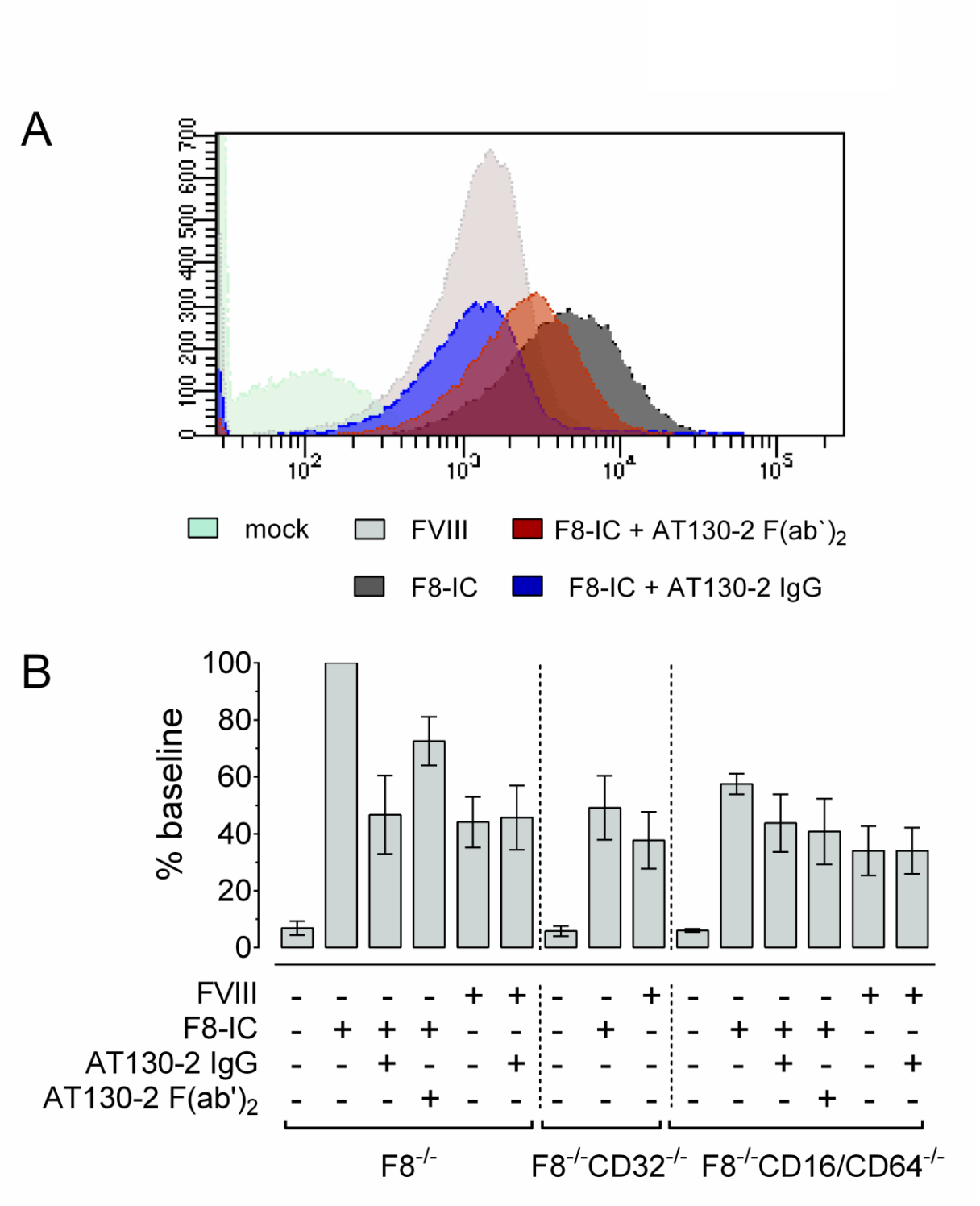


Figure 3: The agonistic anti-CD32 mAb, AT130-2, suppresses F8-IC binding on BMdDCs. Data in (A) demonstrate an representative example of an overlay of flow cytometry histograms showing

the binding of FVIII-AF647 or F8-AF647-ICs on BMdDCs from $F8^{-/-}$ mice in the presence or absence of 2.5 µg unlabeled AT130-2 IgG or its $F(ab')_2$ fragments. Data in (B) represent the percentage ± SEM of F8-AF647-IC or FVIII-AF647 binding on BMdDCs from $F8^{-/-}$ or $F8^{-/-}CD32^{-/-}$ or $F8^{-/-}CD16/CD32^{-/-}$ mice with or without 2.5 µg unlabeled AT130-2 IgG or its $F(ab')_2$ fragments. F8-AF647-ICs were formed by mouse polyclonal anti-FVIII IgG antibodies (1:100) and 10 µg of FVIII-AF647. From each experiment, mean fluorescent intensity (MFI) of F8-AF647-IC binding on BMdDCs from $F8^{-/-}$ mice was normalized to 100 percent and served as positive control. Three independent experiments were performed, where single samples were assessed per condition.

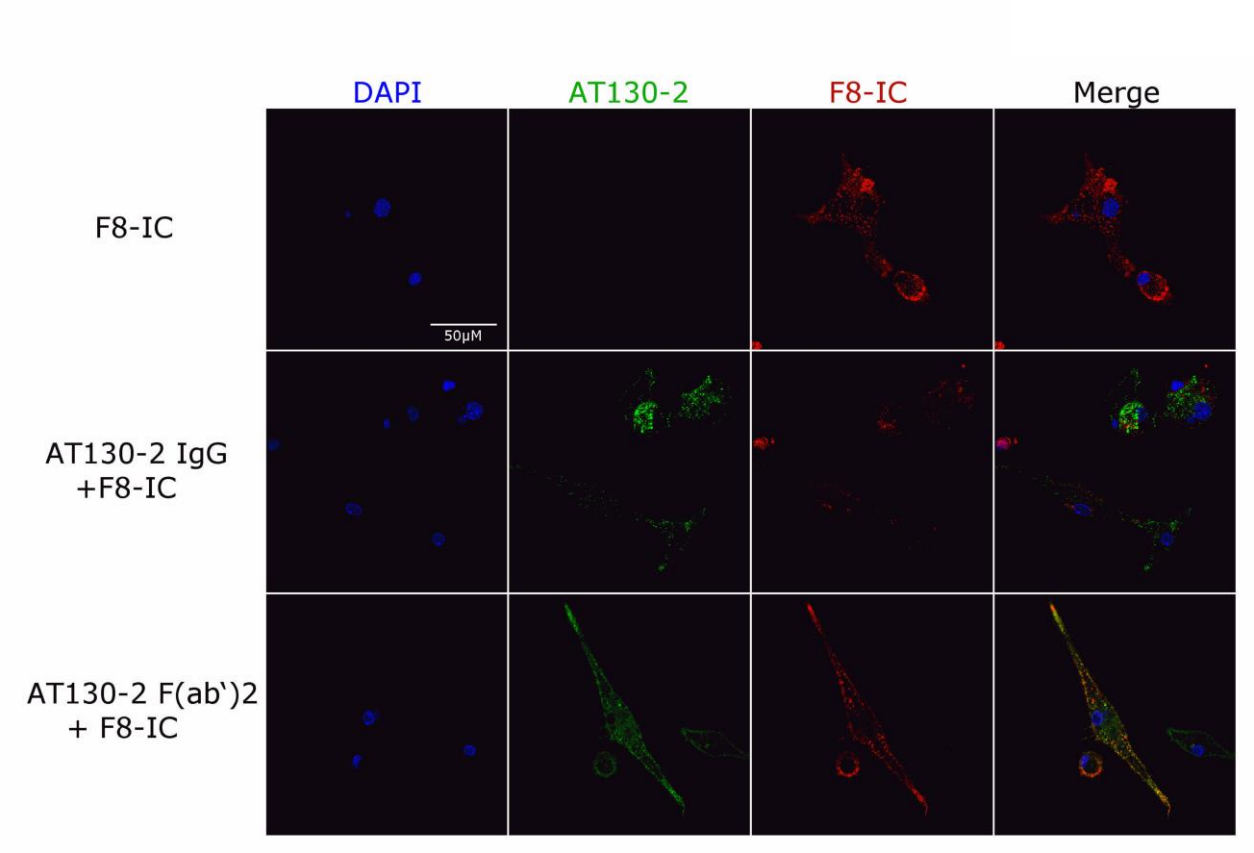


Figure 4: F8-IC binding on BMdDCs by CD32. BMdDCs from $F8^{-/-}$ mice were incubated with F8-AF647-ICs (red) in the presence or absence of (2.5 µg) AF488-conjugated AT130-2 IgG or $F(ab')_2$ fragments (green). F8-ICs were formed by mouse polyclonal anti-FVIII IgG antibodies (1:100) and 10 µg of FVIII-AF647. Cells were fixed and permeabilized. DNA was labeled with DAPI (blue). Cells were analyzed by confocal microscopy. Scale bar = 50 µm.

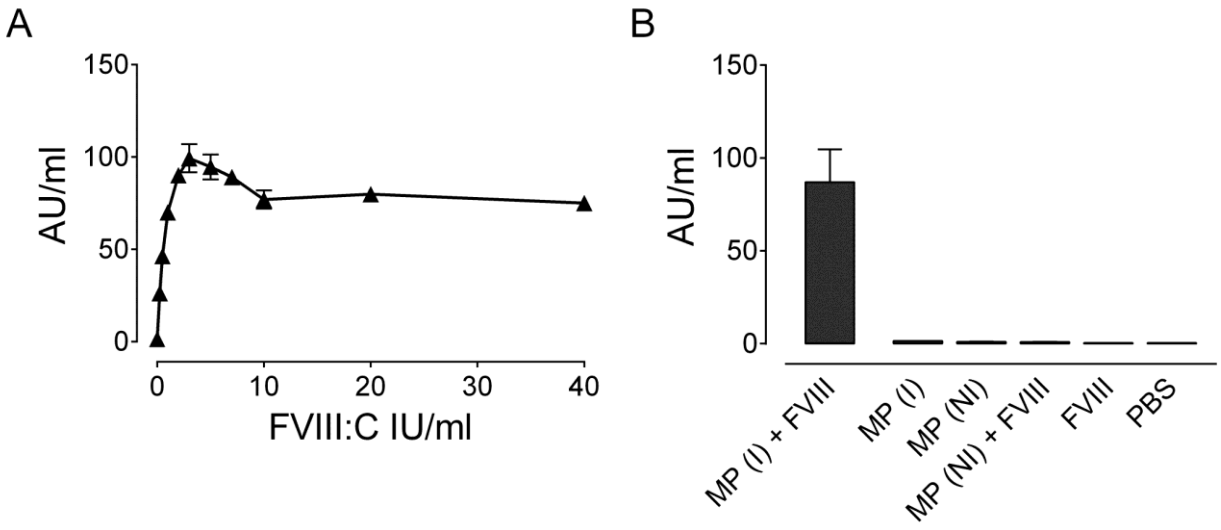
Tables

Table 1: Properties of anti-CD32 mAbs.

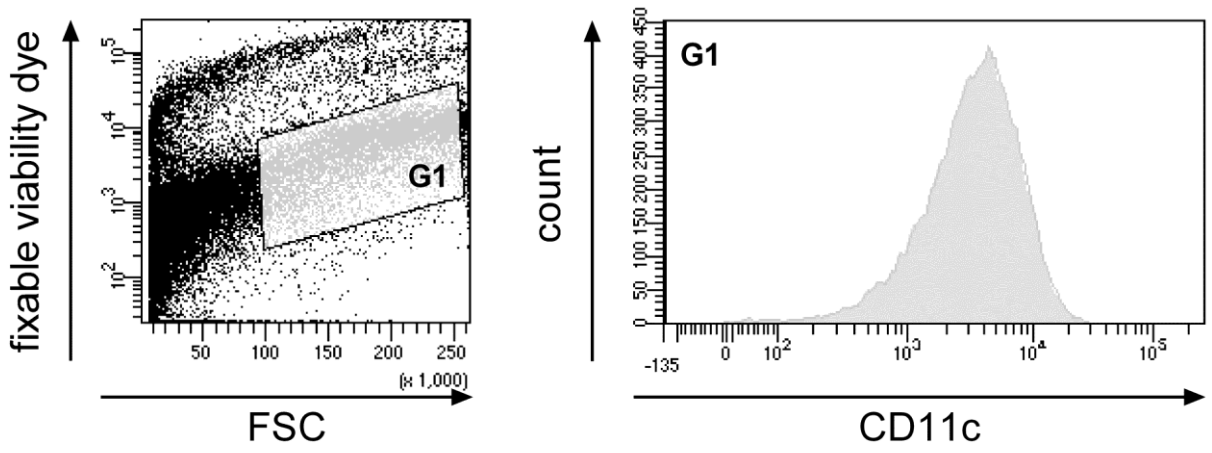
mAB	Subclass	K_D [M]	Classification
AT130-2	mIgG2a	1.9×10^{-8}	Agonist
AT130-5	mIgG1	1.3×10^{-8}	Agonist
AT128	mIgG1	1.4×10^{-7}	Antagonist

Table adapted from [24].

Supplementary data



Supplementary Data 1: Characterization of F8-ICs by their binding capacity to C1q. (A) F8-ICs bind to immobilized C1q. Different concentrations of FVIII (0-40 IU/ml) were incubated with sera pooled from 20 immunized $F8^{-/-}$ mice (MP (I), contains polyclonal anti-FVIII antibodies) to form F8-ICs. (B) Binding of F8-ICs (MP (I) + 10 IU/ml FVIII) to C1q. Neither MP (I), nor sera pooled from 10 naïve $F8^{-/-}$ mice (MP (NI)), nor 10 IU/ml FVIII incubated with MP (NI), nor 10 IU/ml FVIII were bound by C1q. Mean ± SD is shown.



Supplementary Data 2: Characterization of BMdDCs. Purity of the BMdDCs was analyzed by flow cytometry. (Dot plot) Dead cells were excluded from analysis by positive staining using fixable viability dye. (Histogram) Living cells (G1) were stained for murine peridinin chlorophyll (PerCP)-labeled anti-CD11c mAb.