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Title:

A novel recycling mechanism of native IgE-antigen complexes in human B cells facilitates transfer of antigen to dendritic cells for antigen presentation

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Keywords

FcεRII (CD23), IgE, IgE-immune complexes, B cells, moDC, polarization, antigen recycling, T cell activation

Abbreviations used

IgE-IC: IgE-immune complex

MoDC: Monocyte-derived dendritic cells

NIP: 4-Hydroxy-3-iodo-5-nitrophenylacetyl

BSA: Bovine serum albumin

TT: Tetanus Toxoid

GM-CSF: Granulocyte-macrophage colony-stimulating factor

CFSE: Carboxyfluorescein succinimidyl ester

APC: Antigen presenting cell

Key messages :

- IgE-ICs induce CD23 aggregation and internalization in B cells and moDCs
- After CD23 internalization, IgE-IC are recycled in B cells but degraded in moDCs
- IgE-IC-pulsed B cells co-cultured in cell contact with moDCs induce specific T cell proliferation by transfer of recycled IgE-IC to moDCs and by promoting moDC maturation.

Capsule summary

The here described IgE/CD23-dependent antigen recycling and presentation pathway in B cells may be targeted to regulate T cell responses in IgE-mediated diseases.

Abstract**Background:**

IgE-immune complexes have been shown to enhance antibody and T cell responses in mice by targeting CD23 (FcεRII), the low-affinity receptor for IgE on B cells. In humans, the mechanism by which CD23-expressing cells take up IgE-immune complexes (IgE-IC) and process them is not well understood.

Objective:

To investigate this question, we compared the fate of IgE-IC in human B cells and in CD23 expressing monocyte-derived dendritic cells (moDCs) that represent classical APCs and we aimed at studying IgE-dependent antigen presentation in both cell types.

Methods:

B cells and monocytes were isolated from peripheral blood and monocytes were differentiated into moDCs. Both cell types were stimulated with IgE-immune complexes consisting of NIP-specific IgE JW8 and NIP-BSA to assess binding, uptake and degradation dynamics. To assess CD23-dependent T cell proliferation, B cells and moDCs were pulsed with IgE-NIP-Tetanus Toxoid (TT) complexes and cocultured with autologous T cells.

Results:

IgE-IC binding was CD23-dependent in B cells and moDCs and CD23 aggregation, as well as IgE-IC internalization, occurred in both cell types. While IgE-IC was degraded in moDCs, B cells did not degrade the complexes but recycled them in native form to the cell surface enabling IgE-IC uptake by moDCs in cocultures. The resulting proliferation of specific T cells

was dependent on cell-cell contact between B cells and moDC which was explained by increased upregulation of co-stimulatory molecules CD86 and MHC class II on moDCs induced by B cells.

Conclusion:

Our findings argue for a novel model in which human B cells promote specific T cell proliferation upon IgE-IC encounter. On one hand, B cells act as carriers transferring antigen to more efficient APCs such as dendritic cells, on the other hand, B cells can directly promote DC maturation and thereby enhance T cell stimulation.

Introduction

The IgE receptor FcεRII (CD23) is known to play a role in the regulation of IgE synthesis and direct handling of IgE-immune complexes (IgE-ICs) (1–4). More recently, CD23 has gained interest as a mediator of IgE-dependent antigen presentation and activation of T cells in humans as well as in mice (5–8).

CD23 is a type II transmembrane protein that consists of a stalk region and a C-type lectin “head” domain where IgE binding occurs. CD23 was initially described as a low-affinity IgE receptor due to its rather low binding affinity (1.3×10^{-6} M). However, CD23 forms a trimeric molecule through an α -helical coiled coil structure in the stalk region whereby the

interaction with IgE benefits from an avidity effect resulting in an apparent affinity for IgE in the range of 10^{-8} M(9).

There are two splicing variants of the receptor termed CD23a and CD23b isoform that differ only in a few amino acids of the intracellular N-terminal domain. CD23a is the predominant isoform in B cells while expression of CD23b is inducible by IL-4 in various cells of the myeloid lineage as well as in B cells (10,11).

The distinct intracellular domains of the two isoforms of CD23 are likely responsible for their respective functions. CD23a conducts IgE-IC internalization by endocytosis, whereas CD23b is rather involved in phagocytic uptake of IgE-coated particles (12). Accordingly, differences in signaling of the two CD23 isoforms have also been described in comparative studies between B cells and monocytes (13–15). The mechanism by which CD23 induces antigen presentation is still unclear. In mice, CD23⁺ B cells have been claimed to transfer antigen to splenic follicular dendritic cells (8).

In myeloid cells, CD23 cross-linking has been shown to induce inflammatory mediators (16–18) and monocyte-derived dendritic cells (moDCs) express CD23 upon IL-4 and GM-CSF stimulation (19,20) but detailed knowledge about the role of CD23 in moDCs with regards to antigen presentation is lacking.

Unraveling the role of CD23 in antigen presentation and allergic inflammation is also of clinical interest since studies in asthmatic patients have identified CD23 as a potential therapeutic target in allergic asthma exacerbations (21–23).

In the present study, we investigated the mechanisms by which CD23⁺ B cells induce T cell activation upon stimulation with IgE-immune complexes as compared to CD23⁺ moDCs. We used the monoclonal IgE antibody JW8 specific for the hapten NIP (24) and NIP-BSA conjugates to generate IgE-immune complexes. To study proliferation of primary human T

cells, we used NIP-Tetanus Toxoid (TT) conjugates. We demonstrate that binding of IgE-ICs to CD23 on moDCs targets them for degradation while in B cells the IgE-ICs are not degraded but recycled to the surface in an actin-dependent manner. We further show that substantial CD23-dependent T cell proliferation was induced by B cells pulsed with IgE-ICs in contact-dependent coculture with moDC. Moreover, B cells promoted moDC maturation through upregulation of CD86 and HLA-DR. Hence, B cells induce IgE/CD23-dependent antigen presentation in cooperation with moDCs. Our findings show that B cells interact with professional antigen presenters to induce IgE-dependent T cell proliferation and have the potential to act as key regulators of immune responses to IgE-bound antigens.

Material and Methods

Isolation of human B cells, Monocytes, and T cells

Buffly coats were purchased from the blood donation center (Bern, Switzerland). The study was approved by the local ethics committee. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll Paque (GE Healthcare, Chalfont St. Giles, UK). B cells were isolated by negative selection (StemCell Technologies, Grenoble, France) whereas Monocytes were isolated by CD14 positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. T cells were isolated by negative selection (StemCell Technologies).

Cell culture

B cells and T cells were cultured in 48 well plates at 1×10^6 cells/ml in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% FBS (Thermo Scientific, Cramlington, UK), Transferrin 40 $\mu\text{g/ml}$ (Calbiochem, Darmstadt, Germany), mercaptoethanol 50 μM (Sigma, St. Louis, MO, USA), Sodium pyruvate 1 mM (Sigma), bovine insulin 4 $\mu\text{g/ml}$ (Sigma), L-glutamine 2 mM (Gibco) and MEM Non-essential amino acids 1:100 (Gibco). B cells were activated with IL-4 20 ng/ml (Peprotech, Rocky Hill, NJ, USA) and anti-CD40 antibody 1 $\mu\text{g/ml}$ (eBioscience, San Diego, CA, USA) overnight. Monocytes were cultured in 48-well plates at 0.5×10^6 cells/ml in RPMI 1640 (Gibco) containing 10% FBS, L-alanyl, L-glutamine 1 mM (Gibco). Monocytes were activated with IL-4 (Peprotech) 20ng/ml and GM-CSF (Peprotech) 2ng/ml and used for experiments after 3 days.

Real-time PCR

Total mRNA was isolated using Guanidinium thiocyanate-phenol-chloroform extraction (TRI Reagent, MRC, Cincinnati, OH, USA). Isolated mRNA was reverse-transcribed using random hexamer primers (Applied Biosystems, Branchburg, NJ, USA) and Superscript II (Life Technologies, Carlsbad, CA, USA). Real-time qPCR was performed using TaqMan Universal Master Mix (Applied Biosystems) on a 7500 Real-Time System (Applied Biosystems). Expression was normalized to the endogenous control $\beta 2$ -microglobulin and relative expression was determined by 7500 system software (Applied Biosystems). The primers

used were β 2-microglobulin, Hs00187842_m1 (Applied Biosystems), CD23a forward: 5'-TGGAGGAAGGTCAATATTCAGAG-3', CD23b forward: 5'-ATGAATCCTCCAAGCCAGGA-3', CD23 reverse: 5'-GCCACAGGAGAAGCAGAGTC-3' and CD23 probe: 5'-FAM-GGTGTTGCAGGCGTGGGACT-Tamra-3' which were all ordered at Microsynth (Balgach, Switzerland).

Immune complexes

The chimeric human NIP-specific IgE-JW8 (24) was used at 25nM and NIP(15)-BSA (4-hydroxy-3-iodo-5-nitrophenylacetyl conjugated to bovine serum albumin) was used at 2.5nM. Fluorescein-labeled NIP(15)-BSA was also used at the same concentration both compounds were obtained from Biosearch Technologies, Petaluma, CA, USA.. For the internalization studies, IgE JW8 was labeled with the pH-sensitive dye pHrodoTM (IgE_{rodo}) using the pHrodoTM Red Microscale Labeling Kit (Life Technologies). For T cell proliferation experiments, Tetanus Toxoid (TT) (Statens Serum Institut, Copenhagen, Denmark) was coupled with NIP at a molar ratio of TT(1): NIP(15) and mixed with IgE to form complexes (25nM JW8: 7.5nM TTN).

CD23 blocking assay

The generation and characterization of CD23-specific DARPins is published elsewhere (25). For inhibition of IgE-immune complex binding to CD23, anti-CD23 bispecific D89-86 DARPin

molecule at 100nM was mixed with preformed IgE+NIP-BSA complexes (25nM IgE+2.5nM NIP-BSA) and was added to B cells or moDCs for 30min at 4°C. As a control, a non-specific DARPin molecule was used at 100nM.

Antibodies and flow cytometry

To visualize CD23 on B cells, cells were stained using Phycoerythrin (PE)-conjugated mouse anti-human CD23 mIgG1 1:500 (R&D, Minneapolis, MN, USA). Staining for IgE was performed using FITC- or APC-conjugated mouse anti-IgE mIgG1 1:100 (Miltenyi Biotec). Intracellular staining for lysosomal marker LAMP1 was performed using mouse FITC- or APC-anti-LAMP1 mIgG1 1:250 (BD Biosciences, Franklin Lakes, NJ, USA). MoDCs were characterized by mouse APC-anti-DC-sign mIgG2bk (BD Biosciences), PE-anti-CD86 IgG2bk 1:100 (Biolegend, San Diego, CA, USA) and PerCP-anti-HLA-DR mIgG2ak 1:100 (Biolegend). FcεRI expression was monitored with PE-anti-FcεRI mIgG2bk (Biolegend).

For assessing proliferation, T cells were stained with PerCp-Cy5.5-anti-CD3 mIgG1κ and PE-Cy7-anti-CD4 mIgG2bk (Biolegend). MHC class II- was blocked with anti-HLA-DR/DP/DQ mIgG2ak (Biolegend).

Receptor expression or IgE-IC binding was assessed using Guava easyCyte™ (Merck Millipore, Darmstadt, Germany). T cell proliferation assays were analyzed with BD FACSCanto™ (BD Biosciences). All conventional flow cytometry data were analyzed using FLOWJO software (TreeStar Inc., Ashland, OR, USA).

Imaging flow cytometry and analysis using Amnis IDEAS® software

Imaging flow cytometry was performed using Image Stream^x® flow cytometer and the compatible INSPIRE® system software (Amnis Corporation, Seattle, WA, USA). Cells were measured at 40x magnification and a flow speed CV below 0.2% indicating a stable core stream. Single cells were gated based on “area” and “aspect ratio” features of the bright-field channel which was set on Channel 1. Focused cells were selected based on the “gradient root-mean-square (RMS)” feature that measures the resolution of an image, whereby values above 60 were considered for further analysis

At least 5'000 single cell events were acquired for every sample and were analyzed using the IDEAS® software (Amnis Corporation). CD23 aggregation was assessed based on the “circularity” and “area” features of the fluorescence emitted by PE-anti-CD23 using channel 3 as a mask.

Inhibition of actin polymerization

Cytochalasin D (Sigma) was added to IgE+NIP-BSA 8 hours after the pulsing of B cells. Titrated cytochalasin D concentrations (0, 1, 10, 100 and 1000 ng/ml) were added for 16h which is the time point of 24h after IgE+NIP-BSA pulse. The cells were then either stained with anti-IgE (Miltenyi Biotec) or FITC- Annexin V (Biolegend) and propidium iodide (Biolegend).

ELISA

96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with monoclonal mouse anti-human IgE HB235 5 µg/ml (ATCC, Manassas, VA, USA) overnight in PBS. Unspecific binding sites were blocked with PBS-casein (0.15%) for 2 h at 37°C. Cell culture supernatants were incubated for 3 h at 37°C. Anti-human IgE-HRP 1:1000 (Binding Site, Birmingham, UK) was used to detect IgE. The ELISA was developed with TMB (3,3',5,5'-tetramethylbenzidine) and was stopped with 1 M sulphuric acid. IgE JW8+NIP-BSA at 25nM:2.5nM was used for a standard curve and concentrations were interpolated (nonlinear regression) using Prism 6.0 software (GraphPad, La Jolla, CA, USA).

T cell proliferation assay

For T cell proliferation assays, B cells were not pre-treated with CD40L. B cells were pulsed with 7.5nM NIP-TT and 25nM IgE JW8 for 4 hours. Autologous T cells were incubated at 37°C, 5%CO₂ for 3 days during B cell and moDC activation. The T cells were then labeled with 10µM CFSE (Biolegend) according to the manufacturer's protocol.

B cells or moDCs were co-incubated with CFSE labeled T cells at a 1:5 ratio whereas B cells, moDCs and T cells were co-incubated at a 1:1:5 ratio.

Cells were cultured for 6 days at 37°C in RPMI-1640 (Gibco) supplemented with 5% of heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 2 mM L-Glutamine (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin (Bioconcept, Allschwil, Switzerland)..

Transwell experiments were performed with Corning® polyester membrane inserts with a 0.3µm pore size (Sigma) in 24 well plates. T cells and moDCs were incubated below the inserts and untreated or IgE+NIP-TT-pulsed B cells were added on top.

Statistical analysis

Statistical tests were performed with GraphPad PRISM 6.0 (GraphPad Software, Inc.). For all experiments, $\alpha=0.05$ and statistical significance are displayed as $p\leq 0.05$ (*), $p\leq 0.01$ (**), $p\leq 0.001$ (***), $p\leq 0.0001$ (****). Two groups were analyzed by Two-tailed Student's t-test. For time course experiments in Figure 5, two-way ANOVA and Sidak's test as posthoc analysis was performed. All data in graphs are displayed as mean $\pm$ SEM; pictures are always representative of at least 3 independent experiments.

Results

IgE-immune complex binding is CD23-dependent in primary B cells and moDCs

We assessed the expression of surface CD23 by flow cytometry in freshly isolated human B cells and monocytes upon CD23-upregulation by IL-4. In addition to IL-4, human peripheral B cells were stimulated with anti-CD40 antibody whereas monocytes received GM-CSF for maturation into moDCs. CD23 was expressed in 80-90% of B cells after 24 hours of culture

and reached a plateau after 2 days. In moDCs, CD23 was highly expressed in around 95% of the cells after 3 days in culture (Fig 1A).

Since dendritic cells have the potential to express FcεRI upon IL-4 and GM-CSF stimulation, we monitored CD23 and FcεRI expression over time during in vitro maturation. Consistent with previous reports about moDCs (26), FcεRI was not expressed during 6 days of moDC generation indicating that CD23 is the main receptor for IgE-IC binding (Fig 1B) and does not occur via FcεRI.

The two CD23 isoforms differ only in their intracellular N-terminal sequence and cannot be discriminated by flow cytometry; real-time PCR was therefore performed to assess expression of CD23a and CD23b mRNA levels in B cells and moDCs after 2 days and 3 days of stimulation respectively. In line with previous reports (27), CD23a was the predominant isoform in B cells whereas only low levels of CD23b were induced by IL-4/CD40L (Fig 1C, left panel). In moDCs, (Fig 1C, right panel), CD23b was the only isoform expressed upon IL-4 and GM-CSF maturation.

To investigate IgE-immune complex (IgE-ICs) binding, we pre-incubated the NIP-specific IgE antibody JW8 (24) with NIP-BSA before the addition to the cells. Titrations have indicated an optimal IgE+NIP-BSA complex binding at a ratio of 2.5nM NIP-BSA and 25nM IgE-JW8 (data not shown). For comparison, we also incubated the cells with IgE and NIP-BSA alone. Figure 1D shows binding of IgE to B cells and moDCs after 2h at 37° upon IgE, NIP-BSA or IgE+NIP-BSA stimulation whereas Figure 1E shows binding of FITC-labelled NIP-BSA (NIP-BSA_{FITC}) alone or in complex with IgE. As expected, both IgE and NIP-BSA bind better in complex than alone in both cell types.

To demonstrate that IgE-IC binding entirely depends on CD23 in both cell types, B cells and moDCs were incubated with IgE+NIP-BSA in presence or absence of an anti-CD23 DARPIn

molecule. In a previous study (25) this DARPin has been reported to specifically inhibit IgE binding to CD23. As shown in Figure 1F, there was an almost complete inhibition of IgE+NIP-BSA binding when 100nM anti-CD23 DARPin was mixed to the IgE-ICs. In contrast, the presence of a non-specific DARPin molecule had no effect on IgE+NIP-BSA binding to B cells or moDCs. This clearly indicates that binding of IgE-IC is CD23-dependent in both cell types.

Multivalent cross-linking by IgE+NIP-BSA induces polarized CD23 aggregation in B cells and moDCs

In order to assess the ability of IgE-ICs to aggregate CD23 in B cells and moDCs, we stained the cells with anti-CD23 and anti-IgE and analyzed them by imaging flow cytometry. Cross-linking of CD23 with pre-formed IgE+NIP-BSA complexes resulted in visible clustering of surface CD23 in both B cells and moDCs when incubated for 4 hours at 37°C. Aggregation of CD23 was assessed using Amnis® IDEAS software at different time points. Figure 2A shows histograms of “object circularity” and “area” features of anti-CD23 fluorescence. CD23 aggregation caused by IgE + NIP-BSA complexes is shown by the loss of circularity and area of the anti-CD23 fluorescence that can be clearly differentiated from untreated cells (gating strategies are displayed in Fig E1). The percentages of polarized cells from at least 4 donors are summarized in Figure 2B. After IgE+NIP-BSA stimulation for 4h at 37°C, only 30-50% of the cells were fully polarized whereas, in B cells about 80% of the cells were fully polarized indicating that CD23 cross-linking more efficiently leads to polarization in B cells. Representative pictures of B cells and moDCs after 4 hours of IgE+NIP-BSA stimulation showing polarization of CD23 are displayed in Figure 2C.

In a next step, we wanted to demonstrate colocalization of CD23, IgE, and NIP-BSA. To this end, we stimulated B cells and moDCs with IgE+NIP-BSA_{FTC} (Fig 2D, green) for 2h at 37°C. The cells were thereafter stained with anti-CD23 (Fig 2D, yellow) and anti-IgE (Fig 2D, red) and then analyzed by imaging flow cytometry. In B cells, IgE and NIP-BSA colocalized at the site of polarized CD23, this was also the case in moDCs, where CD23 aggregation was clearly visible at the site of the IgE+NIP-BSA complexes.

IgE-ICs are internalized in human B cells and moDCs

Next, we investigated whether aggregation of CD23 precedes IgE-IC internalization in both B cells and moDCs using anti-NIP IgE JW8 covalently conjugated with the pH-sensitive dye pHrodoTM (IgE_{rodo}) that increases light emission upon a drop in pH (28). Since pH decreases along the pathway of endo- or phagocytosis, ranging from pH 6.0-6.5 in early endosomes down to pH 4.5-5.5 in late endosome and lysosome, pHrodoTM dyes provide a valuable tool to assess internalization (28,29).

Thus, B cells and moDCs were incubated with IgE_{rodo} or IgE_{rodo} + NIP-BSA complexes for 4 hours at either 37°C or 4°C. Prior to analysis by imaging flow cytometry, surface IgE was also stained using an anti-IgE antibody in order to visualize non-internalized IgE_{rodo}. The cells that were incubated at 4°C served as a negative control for IgE+NIP-BSA internalization whereas surface IgE_{rodo} binding was still well detected by the anti-IgE staining (Fig 3A+B green).

After 4 hours of incubation, IgE_{rodo} internalization was visible at 37°C in B cells (Fig 3A, orange) and moDCs (Fig 3B, orange) when IgE_{rodo} + NIP-BSA complexes were used to cross-link CD23 and confirm that the pH-sensitive dye is largely inactive on the cell surface and is

clearly localized inside the cells upon internalization of IgE_{rodo}. The images further show that uptake of IgE_{rodo} is more efficient in moDCs. To statistically underline the enhanced fluorescence of pHrodo-labelled IgE + NIP-BSA complexes upon internalization, the pHrodo fluorescence intensity of at least 5'000 acquired cells is shown in Fig 3C for each sample and cell type. The fluorescence intensities were normalized to the 4°C controls since no uptake occurs at this condition.

While both cells showed IgE uptake, moDCs exhibited a higher fluorescence fold change upon IgE_{rodo}+NIP-BSA at 37°C normalized to 4°C than B cells. Without NIP-BSA, lower amounts of IgE_{rodo} were internalized in both cell types (Fig E2). No uptake of IgE_{rodo}+NIP-BSA complexes was observed when the cells were incubated at 37°C for 10 minutes (data not shown) suggesting that internalization and acidification of the endocytic vesicles is a rather slow process.

B cells recycle IgE-immune complexes to the surface while moDCs degrade the complexes upon uptake

After assessing internalization of IgE_{rodo}+NIP-BSA complexes, we next aimed at studying the degradation dynamics of IgE and NIP-BSA in both cell types.

To this end, we first tracked surface IgE after pulsing B cells and moDCs with IgE+NIP-BSA for 2h at 37°C followed by chasing in medium at 37°C. We then measured surface IgE levels over time by anti-IgE staining. In order to compare IgE levels in B cells and moDCs the anti-IgE fluorescence intensity at the time of wash (for absolute values, see Fig 1D) was set as 1 and following time points were normalized accordingly. As seen in Fig 4A, IgE disappeared from

the surface of moDCs within 24h while surface IgE on B cells was reduced for several hours but reappeared after 24h of incubation.

In a next step, we aimed at directly tracking NIP-BSA in B cells and moDCs. Therefore, we used FITC-labelled NIP-BSA to make IgE+NIP-BSA_{FITC} complexes (Fig 4B), pulsed the cells for 2h at 37°C and measured NIP-BSA_{FITC} fluorescence over time by flow cytometry. Again, all values were normalized to NIP_{FITC} intensity levels at the time of wash (absolute gMFI values in Fig 1E). As seen in Figure 4B, NIP-BSA was protected from degradation in B cells, whereas it was degraded in moDCs.

A mechanism that retains antigen and protects it from degradation has been shown in follicular dendritic cells whereby actin-polymerization played an important role (30). To determine whether the IgE-antigen recycling is actin-dependent we added cytochalasin D, an inhibitor of actin polymerization (31) to IgE+NIP-BSA pulsed B cells after 8h of chase in medium and assessed surface IgE after 24h. As seen in Figure 4C, cytochalasin D impaired the resurfacing of IgE in a dose-dependent manner between 8h and 24h. Annexin V/PI staining of treated and non-treated cells showed that cytochalasin D has no effect on cell viability during that time of incubation (Fig 4D).

To exclude that IgE+NIP-BSA is simply released and bound again from B cells, we assessed the presence of IgE in supernatants (Fig 4E). In B cells, IgE was present in supernatants but the amount of free IgE stayed constant after 8 hours of culture indicating that the increase of surface IgE on B cells between 8h and 48h is due to recycling of IgE from the cell interior.

In moDCs, IgE was completely absent in culture thus providing further evidence that IgE is degraded in these cells.

To confirm the differential handling of IgE-IC in moDC and B cells, we visualized IgE by confocal microscopy in B cells and moDCs that were pulsed with IgE+NIP-BSA for 2h and

further incubated for 4 days. Consistent with our flow cytometry data, confocal images showed that B cells still contain IgE after 4 days of incubation while IgE was degraded in moDCs (Fig E3, Method E1).

IgE-IC-pulsed B cells transfer native antigen to moDC in coculture

We next investigated whether the antigen recycling mechanism in B cells makes antigen available to moDCs in coculture. The moDC from cocultures were distinguished from B cells by flow cytometry using Forward Scatter (FSC) and Side Scatter (SSC) in addition to staining of moDC marker DC-sign as shown in Figure 5A.

To assess antigen transfer from B cells to moDCs, we pulsed B cells for 2h with IgE+NIP-BSA_{FITC}, and incubated them in fresh media for 48h before mixing the B cells with unstimulated moDCs for 4h. NIP-BSA_{FITC} binding was then assessed in DC-sign⁺ moDCs by flow cytometry. As seen in Figure 5B, coculture of B cells and moDCs results in the transfer of NIP-BSA_{FITC} to moDCs in an IgE-dependent manner. Figure 5B also shows that supernatants alone can transfer native antigen meaning that B cells do not specifically target the complexes but rather release them into the medium. In order to investigate whether the uptake by moDCs is CD23-dependent, we pre-incubated moDCs with 100nM anti-CD23 DARPIn or with a non-specific DARPIn molecule prior to coculture with IgE+NIP-BSA_{FITC}-pulsed B cells. As seen in Figure 5C, binding of NIP-BSA_{FITC} over 4h at 37°C could be partially inhibited with an anti-CD23 DARPIn but not with a non-specific DARPIn.

IgE-IC-pulsed B cells induce antigen presentation in presence of moDCs.

We next determined the impact of antigen transfer from B cells to moDCs on antigen presentation to T cells. To this end, we coupled NIP to Tetanus Toxoid (TT) and measured antigen-specific T cell proliferation since TT-specific T cells should be present in all donors. B cells were pulsed with IgE+ NIP-TT for 4 hours. The B cells were then cocultured with CFSE labeled T cells alone or in presence of moDCs. After 6 days, T cells were stained with anti-CD3 and anti-CD4 and were investigated by flow cytometry. Figure 6A shows representative scatter plots of CD3⁺ T cells when cultured with untreated B cells and B cells pulsed with NIP-TT, IgE+NIP-TT in absence or presence of moDC. When IgE+NIP-TT-pulsed B cells were cocultured in absence of moDCs, T cell proliferation was low (Fig6A, upper panel), however in presence of moDCs, IgE+NIP-TT-pulsed B cells induced substantial proliferation of CD4⁺ T cells (Fig. 6A, lower panel). Figure 6B shows the summarized percentages of proliferated CD4⁺ T cells from at least 4 individual donors.

In order to investigate if direct interaction of IgE-IC-pulsed B cells with moDCs is crucial for T cell proliferation we established a transwell coculture system that allows passage of secreted factors but prohibits interaction of the cells. Our results show that the presence of secreted factors in the supernatant of IgE+NIP-TT-pulsed B cells did not induce T cell proliferation in the presence of moDCs (Fig 6C). This indicates that B cell-mediated antigen presentation via moDCs is contact dependent.

In order to show that NIP-TT-specific T cell proliferation is MHC class II -dependent we incubated IgE+NIP-TT-pulsed B cells with moDCs and CFSE-labelled T cells and added 10µg/ml anti-HLA-DR/DQ/DP blocking antibody over 6 days. Figure 6D shows that T cell proliferation can be blocked in the presence of anti-MHCII antibody. We also investigated the impact of B cells on the capacity of IgE+NIP-TT-pulsed moDCs to induce specific T cell proliferation. Thus, moDCs were first pulsed for 4h with either NIP-TT or IgE+NIP-TT and then

cocultured with CFSE labeled T cells alone or in the presence of B cells. Flow cytometric analysis showed no T cell proliferation in the absence of B cells (Fig E4). In presence of B cells, T cells that were exposed to IgE+NIP-TT-pulsed moDC underwent increased background proliferation. However, specific proliferation upon IgE+NIP-TT was statistically not significant. We next investigated the effect of B cells on the expression of maturation markers in moDCs. As shown in Figure 6E, CD86 and HLA-DR were upregulated in DC-sign⁺ moDCs in presence of B cells. The same was also true for CD23, whereas FcεRI expression remained absent also in presence of B cells. We also assessed the expression of CD86, HLA-DR, and CD23 in B cells upon coculture with moDCs but did not see any striking differences (Fig E5).

Together, these data indicate that antigen transfer from IgE+NIP-TT-pulsed B cells to moDC concomitant with increased moDC maturation in presence of B cells induces proliferation of specific CD4⁺ T cells.

Discussion

Little is known about mechanisms by which CD23 mediates IgE-facilitated allergen uptake, processing and presentation. Especially the functional differences between CD23 expressed in B cells and CD23 expressed in myeloid cells are still poorly understood. Experiments with human cell lines have shown that IgE-IC uptake is mediated by both CD23 isoforms (12). In B cells, CD23 has gained attention as an inducer of T cell activity (5,7) but little is known about allergen processing and presentation. Most of the studies dealing with IgE-IC uptake and antigen presentation have focused on the high-affinity IgE receptor, FcεRI(5,32) while CD23

has been largely ignored. Here, we dissected the role of CD23 on B cells in handling IgE-ICs and driving antigen-specific T cell proliferation in comparison with moDCs. Consistent with previous studies (10,27), CD23a was the predominant isoform in IL-4/CD40L stimulated B cells whereas only CD23b was inducible in moDCs by IL-4 and GM-CSF. Despite differential isoform expression, the initial effects of IgE-IC binding to CD23 were similar in both cell types. CD23 cross-linking induced surface aggregation and subsequent internalization of IgE-ICs in both cell types. However, after antigen uptake, our findings demonstrate a remarkable dichotomy in CD23 function in B cells versus moDCs as the fate of IgE and antigen was entirely different between the two cells types: While moDCs simply degraded the IgE-ICs, B cells recycled the complexes in native form protected from degradation. The B cells released IgE-immune complexes that could subsequently be bound and processed by moDCs. Thus, B cells serve as an IgE-IC depot which release antigen over time for uptake and processing by moDCs. The fact that B cells not simply degrade IgE-ICs and present them in association with MHC class II makes sense from a physiological point of view, as B cells usually only efficiently present antigens which they recognize with their BCR. Non-specific antigen-presentation would cause the risk of B cells receiving T help for antigens they actually do not recognize, fostering induction of non-specific antibody responses, a potential source of autoimmunity. B cells not only fail to efficiently degrade the IgE-ICs but recycle and release them within days. Similar mechanisms have been observed involving FcγRIIb in DCs (33) and complement receptor in FDCs (30) where immune complexes were retained intact with a non-degradative pathway and displayed on the cell surface. Moreover, our results may also support the hypothesis that CD23-ligation can induce the release of exosomes (34) a potential mechanism by which antigen is delivered (35,36).

Our findings might also serve to a better understanding of the role of CD23-dependent antigen presentation pathways in atopic diseases. Considering that allergic asthma has been linked to specific CD23 polymorphisms (21,23), it is plausible that abnormal CD23-dependent antigen shuttling may contribute to asthma severity. Furthermore, our data provide a link between allergy and autoimmunity. IgE autoantibodies have been identified in many autoimmune diseases (37) among them chronic spontaneous urticaria (CSU) whereas the underlying mechanism is still unclear. Clinical trials with Omalizumab, an antibody that binds to the Fc portion of IgE, have shown clinical efficacy in CSU (38) suggesting the pathogenic role of IgE in CSU. Since IgE autoantibodies can trigger CD23-dependent antigen presentation via the here identified mechanism, anti-IgE treatment could prevent inflammation by either neutralizing IgE-ICs or by targeting the complexes to Fcγ receptors followed by degradation.

As previously shown (39), we observed that B cells can impact antigen presentation in moDCs through upregulating CD86 and HLA-DR expression on moDCs.

Thus, B cells may not only provide antigen to moDCs but also enhance their ability to stimulate Th cells due to increased levels of co-stimulation. In a mouse model, a similar interaction of B cells and DCs has already been proposed (40). In order to better understand the interactions of B cells and dendritic cells in humans, further studies are required, especially on the importance of DC subsets and on the B cell-derived signals that are inducers of co-stimulators. Furthermore, the uptake of IgE-ICs by DCs may not always be mediated by CD23 but also by FcεRI which has been shown to be expressed in certain types of DCs. In our system, the moDCs only expressed CD23 and were therefore ideally suited for the study. Interestingly, IgE-IC-pulsed moDCs alone were not capable of inducing substantial T cell proliferation. When untreated B cells were added to pulsed moDCs, T cell proliferation

increased in some cases, however there was no significant TT-specific or IgE-dependent T cell proliferation. Therefore, CD23-dependent antigen degradation has no clear impact on antigen presentation in moDCs and might simply serve as a mechanism of IgE-IC clearance. However, expression patterns and putative function of CD23 and FcεRI in the different DC subsets need further clarification.

Some previous studies have indicated that apart from class-switching and mediating antibody responses, B cells can act as transporters of antigen (8,41). In those studies, however, the mechanism by which B cells process antigen was due to their ability to capture antigen through the B cell receptor or complement receptors (41,42). In our present study, we provide the first evidence in human B cells that CD23 could fulfill such a task with IgE-ICs through internalization and consecutive delayed release.

The type of immune response being elicited by CD23-expressing B cells and whether it has a role in inhibiting or amplifying IgE class-switching or memory formation by inducing Th1 or Th2 responses remains to be elucidated in detail. In case of Th1 polarization, this CD23-dependent mechanism could potentially provide a pathway for allergen tolerance. Therefore, this immunological response initiated by IgE-ICs implicates that CD23 on B cells could be a therapeutic target in IgE-dependent diseases.

In conclusion, we have identified an IgE/CD23-dependent recycling mechanism by which human B cells facilitate antigen transfer to moDCs to induce antigen-specific T cell proliferation in a cell contact-dependent manner. Our data support a role for B cells as regulators of T cell responses to IgE-bound antigens.

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Author contributions

P.E. performed all the experiments, P.E., M.F., D.Y., M.F.B. and M.V. designed the experiments and interpreted data, P.E., M.F.B and M.V. wrote the manuscript.

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Conflict of interest

All the authors declare no conflict of interest.

Figure legends

FIG 1. IgE+NIP-BSA binding is CD23-dependent in B cells and moDCs

(A) Surface CD23 expression in B cells and moDCs was assessed by anti-CD23 staining and flow cytometric analysis. Shown are typical histograms with unstained (gray fill), isotype control (dotted line) and anti-CD23 stained samples (solid line). (B) Surface CD23 and FcεRI expression in monocytes stimulated with IL-4/GM-CSF was assessed with anti-CD23, anti-FcεRI and isotype control for anti-FcεRI. Shown are means $\pm$ SEM of fluorescence intensities from at least 4 independent donors. (C) Expression of CD23a and CD23b isoforms was assessed by real-time PCR in B cells and moDCs. Shown are relative expression levels compared to β_2 -microglobulin as mean $\pm$ SEM from at least 3 independent donors. (D) IgE binding to B cells and moDCs upon IgE, NIP-BSA or IgE+NIP-BSA stimulation. Shown are mean $\pm$ SEM fluorescence intensities of anti-IgE staining from 4 independent donors. (E) Binding of NIP-BSA_{FITC} or IgE+NIP-BSA_{FITC} to moDCs and B cells. Shown are mean $\pm$ SEM fluorescence intensities of 3 independent donors. (F) Cells were incubated with a CD23-specific DARPIn or a non-specific DARPIn. Shown are mean $\pm$ SEM fluorescence intensities from at least 3 independent donors. Two-tailed Student's t-test was performed for statistical analysis.

FIG 2. IgE+NIP-BSA induces polarized CD23 aggregation in B cells and moDCs

(A) CD23 aggregation of IgE+NIP-BSA stimulated B cells (upper panel) and moDCs (lower panel) was assessed by imaging flow cytometry. The features used to determine aggregation were "circularity" and "area" of the anti-CD23 fluorescence signal. Blue dots represent CD23

staining of unstimulated cells, red dots represent CD23 staining of IgE+NIP-BSA stimulated cells after 30 minutes, 2 hours and 4 hours respectively. (B) The percentage of B cells and moDCs showing fully polarized CD23 distribution is displayed as mean $\pm$ SEM from at least 4 independent donors. Two-tailed Student's t-test was used to for statistical analysis. (C) B cells and moDCs stimulated with IgE only and IgE+NIP-BSA were stained using anti-CD23 (shown in yellow) after 4 hours of incubation. Representative images of at least 5'000 acquired B cells and moDCs are displayed. (D) B cells and moDCs were stimulated with IgE+NIP-BSA_{FITC} (green) for 2h and subsequently stained with anti-CD23 (yellow), and anti-IgE (red) for showing CD23, NIP-BSA and IgE colocalization. Shown are representative images from at least 5'000 acquired cells.

FIG 3. IgE+NIP-BSA is internalized in B cells and moDCs

B cells (A), and moDCs (B) were cultured with IgE_{rodo}+NIP-BSA for 4 hours at 37°C and 4°C. Internalized IgE_{rodo} was visible due to endo-lysosomal acidity (orange) whereas surface non-internalized IgE_{rodo} was stained using anti-IgE surface staining (green). Internalization and visualization of IgE_{rodo} + NIP-BSA was absent when cells were cultured at 4°C for 4 hours (lower panel of A and B). Shown are representative images from at least 5'000 acquired cells. (C) The enhanced internalization of IgE_{rodo} + NIP-BSA stimulated B cells and moDCs was assessed by measuring the fluorescence intensity of the pHrodo™ dye normalized to IgE_{rodo}+NIP-BSA treated samples at 4°C. Shown are means $\pm$ SEM from at least 3 independent donors. Two-tailed Student's t-test was performed for statistical analysis.

FIG 4. B cells recycle native IgE+NIP-BSA while moDCs degrade both molecules

B cells and moDCs were pulsed with IgE+NIP-BSA (A) or labeled IgE+NIP-BSA_{FITC} complexes (B) for 2h at 37° and chased in fresh medium over time. Fluorescence intensity of anti-IgE staining or NIP-BSA_{FITC} is expressed as fold change normalized to the time of wash. Data are displayed as mean $\pm$ SEM from at least 5 independent donors. Two-way ANOVA followed by Sidak's test was performed for statistical analysis.

C) B cells were pulsed with IgE+NIP-BSA for 2 hours, incubated for 8h, washed and incubated in presence of titrated doses of cytochalasin D for 16h. The cells were stained with anti-IgE (left panel). Shown is the normalized anti-IgE mean fluorescence $\pm$ SEM of 4 independent donors. (D) The cells incubated with cytochalasin D were checked for cell death using Annexin V and PI staining, normalized viable cells $\pm$ SEM from 3 donors are displayed.

E) From the same experiments, IgE was measured in supernatants over time. Shown are IgE concentrations normalized to 25nM IgE/ 2.5nM NIP-BSA as means $\pm$ SEM from 4 individual donors.

FIG 5. IgE+NIP-BSA-pulsed B cells transfer native NIP-BSA to moDCs in coculture

(A) MoDCs from cocultures were discriminated based on side scatter, forward scatter and DC-sign expression. (B) B cells pulsed with either NIP-BSA_{FITC} or IgE+NIP-BSA_{FITC} were mixed in a 1:1 ratio with untreated moDCs. In addition, the moDCs were mixed with supernatants from IgE+NIP-BSA_{FITC} treated B cells. After 4h incubation at 37°C, NIP-BSA_{FITC} fluorescence

was analyzed in DC-sign⁺ moDCs by flow cytometry. Shown are fluorescence intensity means $\pm$ SEM from 4 individual donors. Two-tailed Student's t-test was performed for statistical analysis. C) MoDCs were treated with 100nM anti-CD23 DARPIn or non-specific DARPIn and washed prior to coculture with IgE+NIP-BSA_{FITC} pulsed B cells. DC-sign⁺ moDCs were analyzed by imaging flow cytometry after 4 hours at 37°C. Shown are percentages of NIP-BSA-FITC positive moDC as means $\pm$ SEM from at least 3 individual donors. Two-tailed Student's t-test was performed for statistical analysis.

FIG 6. IgE+NIP-TT-pulsed B cells induce substantial T cell proliferation in presence of moDCs

A) B cells were pulsed with NIP-TT or IgE+NIP-TT and cultured with CFSE-labelled T cells alone or in presence of moDCs. Shown are representative scatter plots of anti-CD4 versus CFSE fluorescence of CD3⁺ T cells. B) Proliferated CD3⁺CD4⁺ T cells upon unstimulated, NIP-TT or IgE+NIP-TT-pulsed B cells in absence or presence of moDC. Shown are means $\pm$ SEM from at least 4 independent donors. Two-tailed Student's t-test was performed for statistical analysis. C) Untreated or IgE+NIP-TT-pulsed B cells were directly mixed with moDCs and T cells or separated from moDCs and T cells by transwell inserts. CFSE low, CD3⁺CD4⁺ T cells from 4 independent donors are displayed as mean $\pm$ SEM.

D) MHC class II molecules were blocked with anti-HLA-DR/DQ/DP during coculture of IgE+NIP-TT-pulsed B cells and moDCs. Shown are percentages of CD3⁺CD4⁺ and CFSE-low cells displayed as means $\pm$ SEM from 3 independent donors. Two-tailed Student's t-test was performed for statistical analysis. (E) The expression of the markers CD86, CD23, Fc ϵ RI and

783 HLA-DR on DC-sign+ moDCs was measured by flow cytometry after overnight culture with B
784 cells at a 1:1 ratio. Shown are the mean fluorescence intensities $\pm$ SEM of 4 individual donors.
785 Two-tailed Student's t-test was performed for statistical analysis.

786

FIG 1

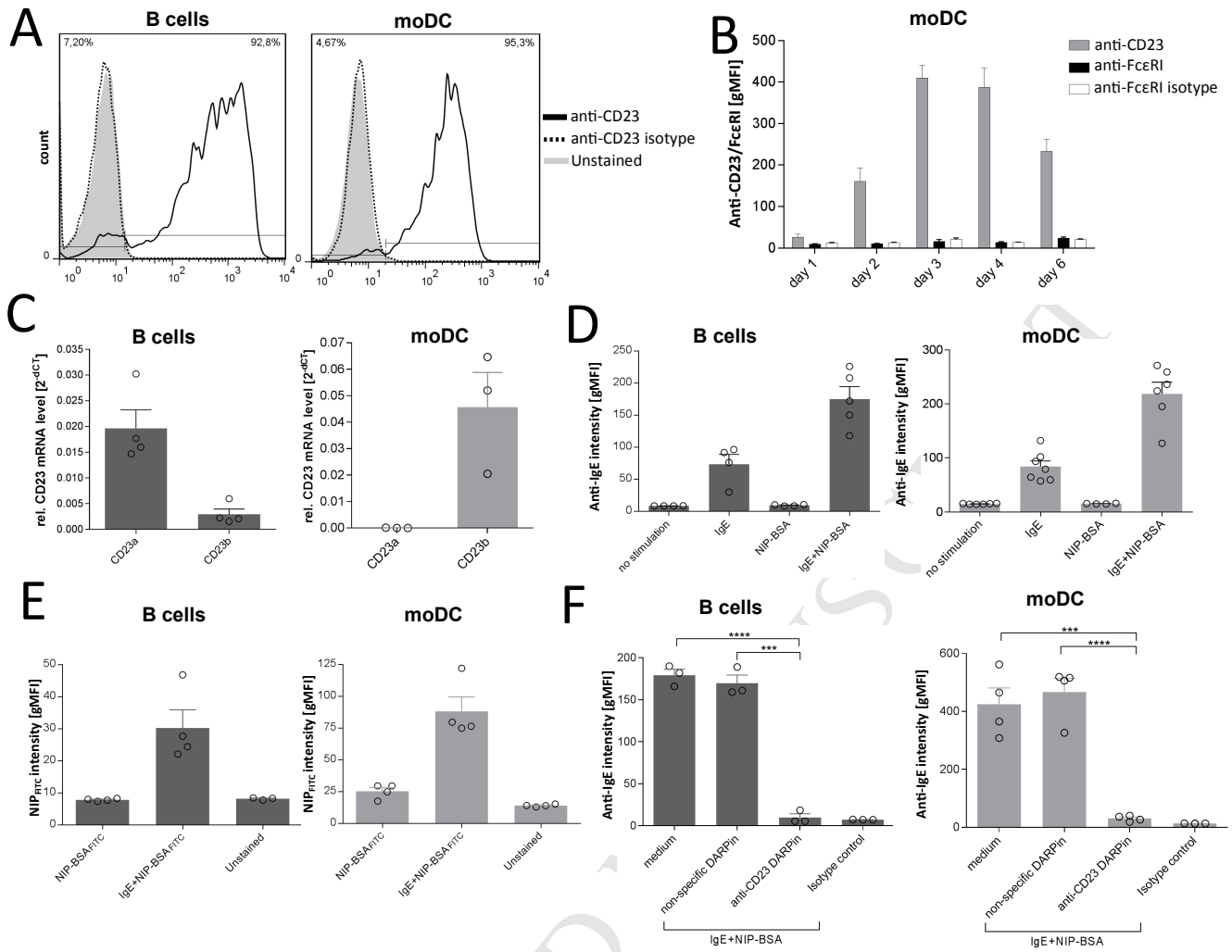


FIG 2

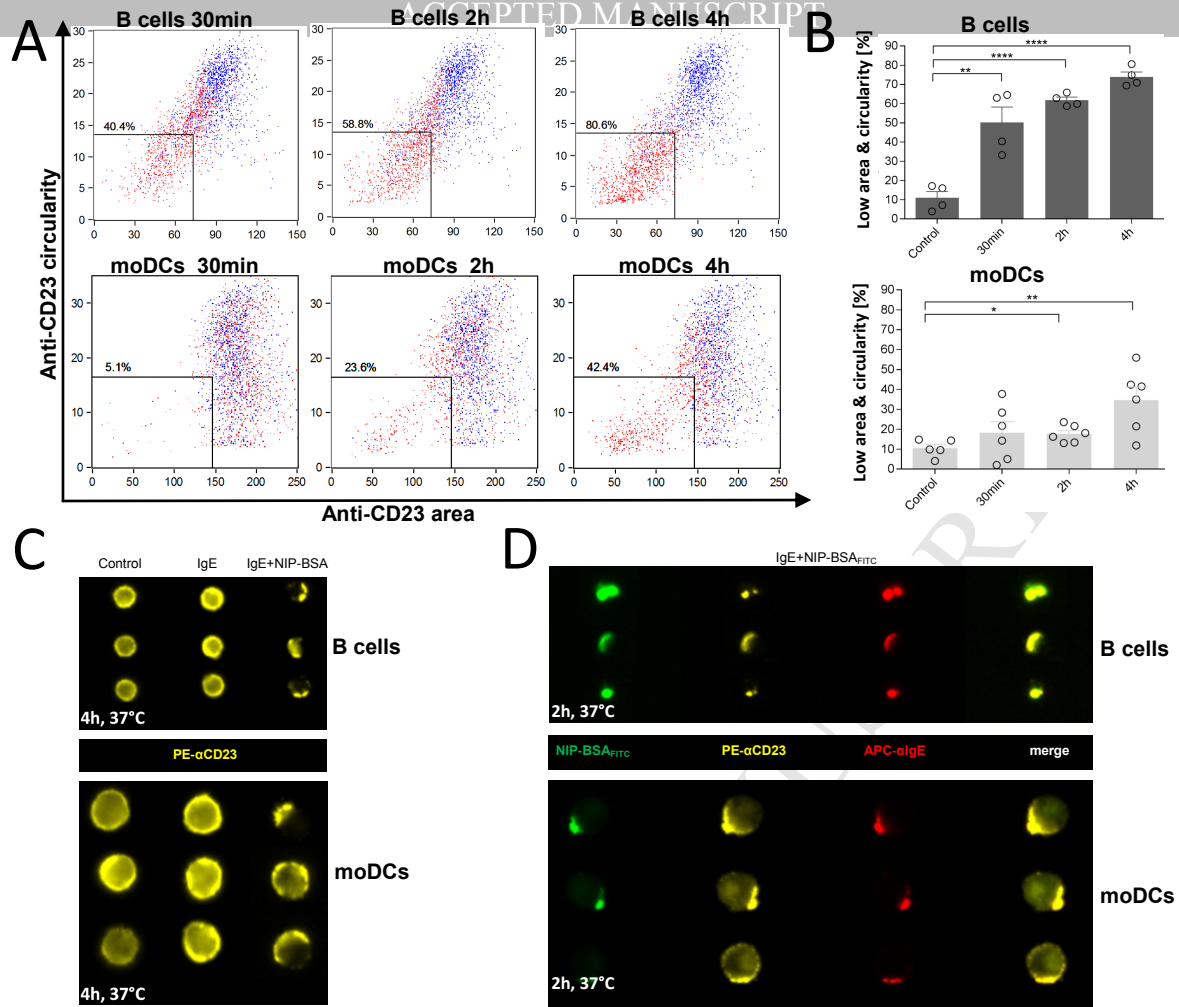


FIG 3

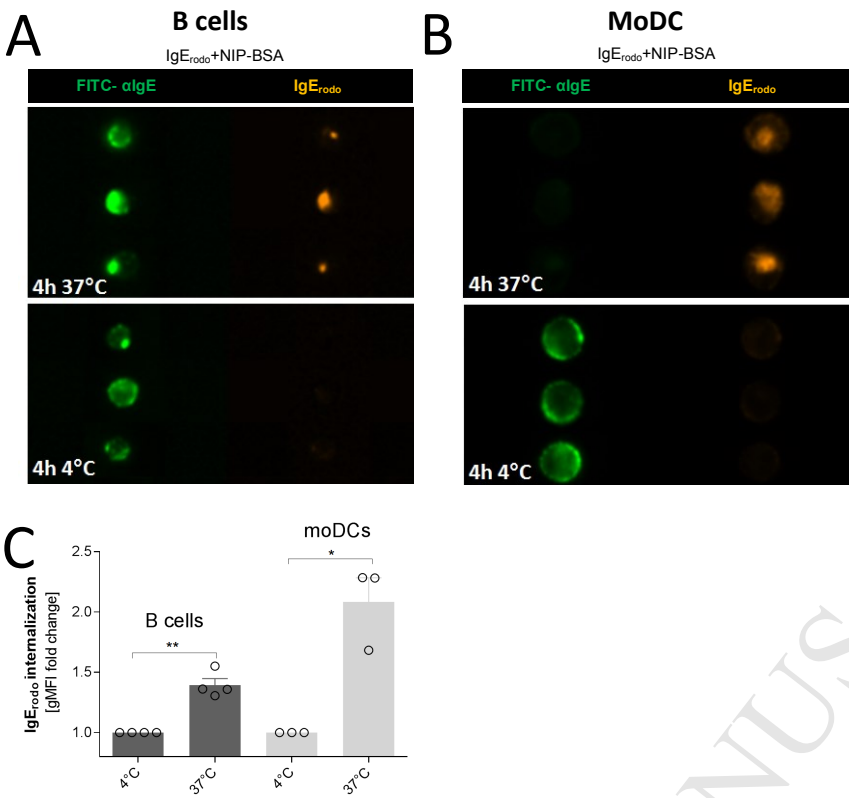


FIG 4

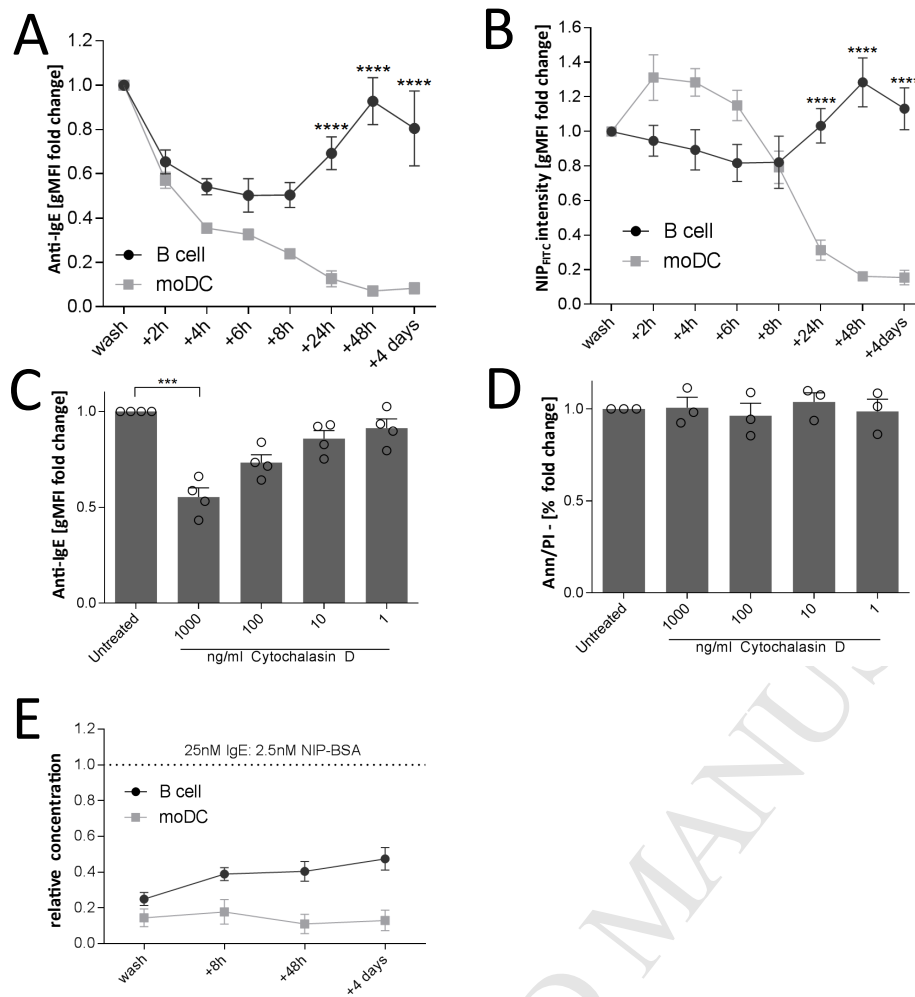


FIG 5

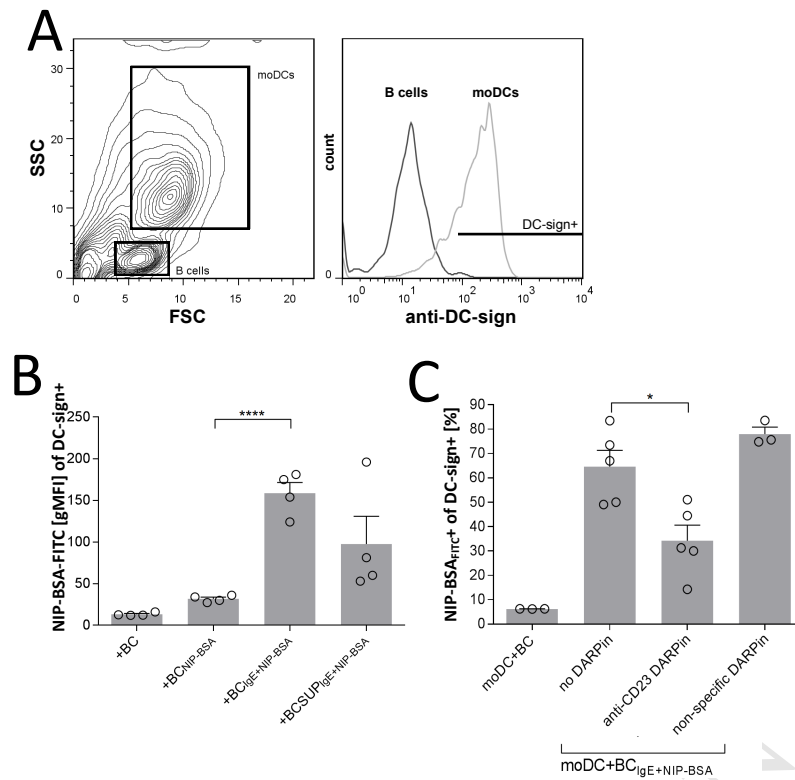
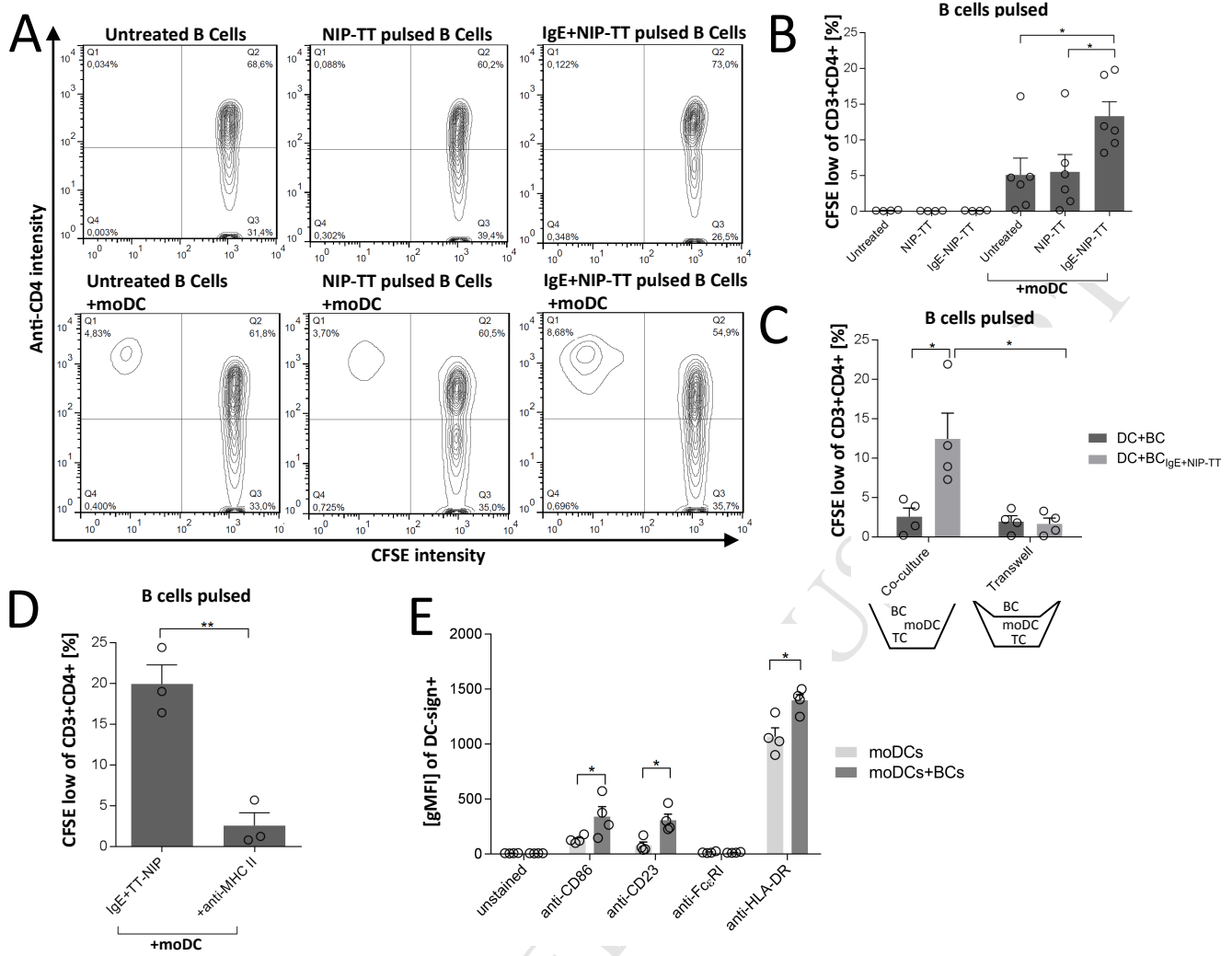


FIG 6



Supplementary Material

Confocal microscopy

For visualization by confocal microscopy, 200'000 cells were prepared using FIX & PERM[®] cell fixation kit (Life Technologies, Carlsbad, CA, USA). Staining was performed with antibodies anti-LAMP1 and anti-IgE that was diluted 1:100 in PBS containing 2%FCS and 5%BSA for 1 hour at 4°C. DAPI was added at 1µg/ml for 2 minutes and cells were then washed several times with PBS. The cells were transferred to superfrost[®] glass slides (Thermo Scientific, Cramlington, UK) by cytopspin at 1000rpm for 3 minutes and the cells were thereafter covered with Dako fluorescent mounting medium (Agilent Technologies, Santa Clara, CA, USA). The slides were then stored overnight in the dark prior to visualization by LSM 700 confocal microscope (Carl Zeiss, Oberkochen, DE).

Supplementary Figure Legends**FIG E1. Gating strategy for polarized cells**

B cells and moDCs were cultured with IgE or with IgE+ NIP-BSA for various time points and stained with anti-CD23 on ice before analysis by imaging flow cytometry. The scatterplot displays “area” versus “circularity” of the anti-CD23 fluorescence signal. Representative cells for gates are displayed whereas gate 1 and 2 were considered polarized cells while 3 and 4 showed no full CD23 polarity.

FIG E2. NIP-BSA enhances IgE_{rodo} internalization

The enhanced internalization of IgE_{rodo} only and IgE_{rodo}+NIP-BSA stimulated B cells and moDCs was assessed by measuring geometric mean fluorescence intensity of the pHrodo™ dye normalized to IgE_{rodo} only treated samples at 4°C. Shown are means means ± SEM from at least 3 independent donors.

FIG E3. IgE+NIP-BSA is recycled and present outside LAMP1⁺ compartments in B cells

B cells or moDCs were cultured with IgE + NIP-BSA for 2h, washed and further incubated in fresh medium for 24h and 4 days at 37°C. Thereafter, intracellular staining for LAMP1 and IgE was performed while cells were kept on ice. The cells were then prepared on coverslips by cytospin and were analyzed by confocal microscopy, representative pictures show DAPI (blue), anti-LAMP1 staining (red), anti-IgE staining (green).

FIG E4. B cells enhance moDC-elicited background T cell proliferation

MoDCs (right panel) were pulsed for 4h with NIP-TT or IgE + NIP-TT and incubated with CFSE-labelled T cells in presence or absence of unstimulated B cells for 6 days at 37°C. Proliferated T cells were assessed by gating CD3⁺CD4⁺ and CFSE-low cells. Shown are means $\pm$ SEM from 6 independent donors. Two-tailed Student's t-test was performed for statistical analysis.

FIG E5. B cells do not upregulate CD86 in coculture with moDCs

The expression of the markers CD86, CD23 and HLA-DR was measured by flow cytometry gating on B cells after overnight culture alone or in presence of moDCs at a 1:1 ratio. Shown are the mean fluorescence intensities $\pm$ SEM of 4 individual donors.

FIG E1

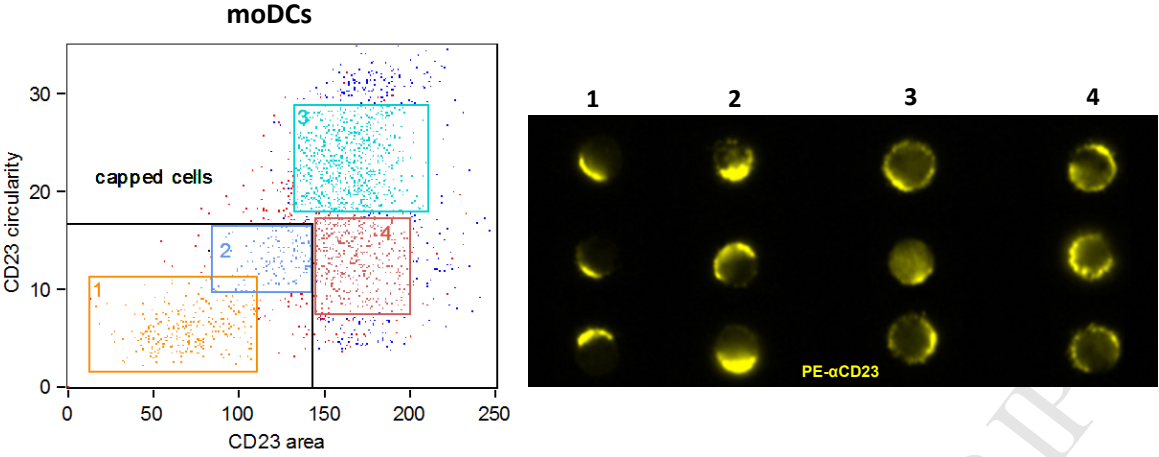


FIG E2

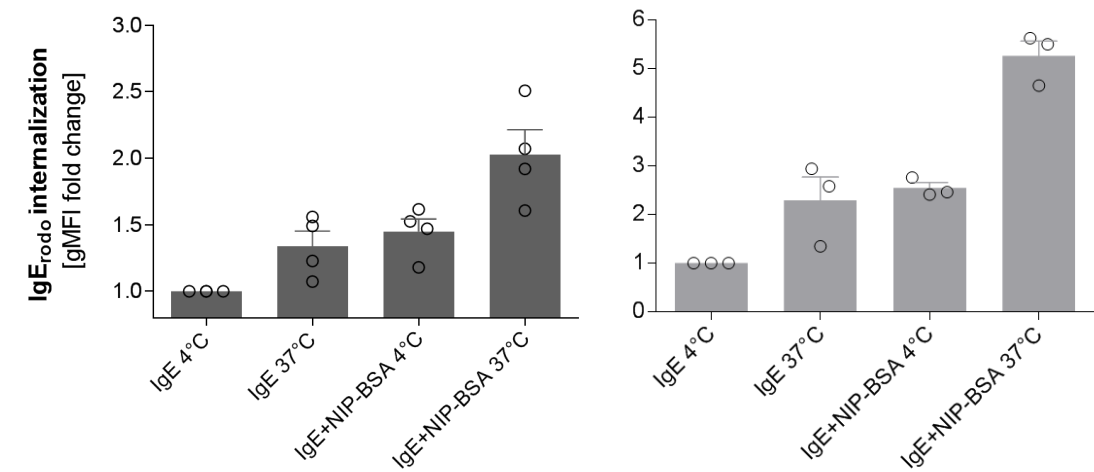


FIG E3

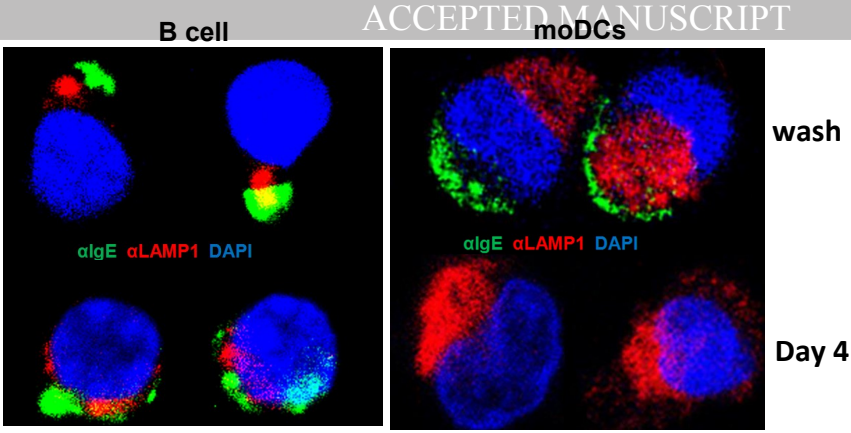


FIG E4

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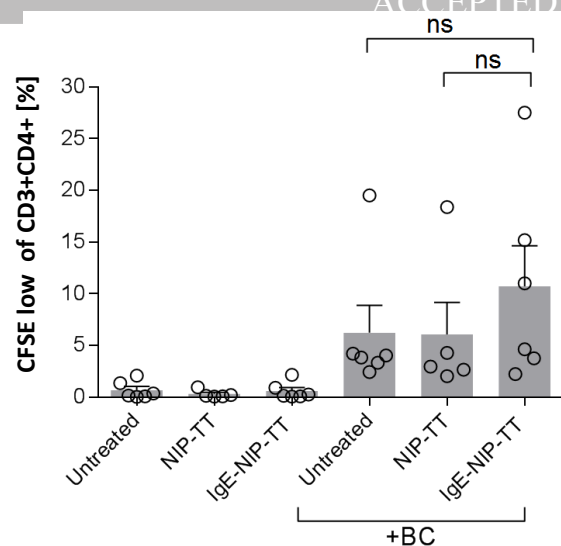


FIG E5

NUSCRIPT

