

Antigen Conjugated to Anti-CD23 Antibodies is Rapidly Transported to Splenic Follicles by Recirculating B Cells

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Abstract

IgE–antigen complexes, administered intravenously to mice, induce a several 100-fold higher specific antibody response than antigen alone. Additionally, *in vivo* activation and proliferation of specific CD4⁺ T cells is enhanced. The mechanism behind these effects is thought to be that peripheral B cells capture IgE–antigen complexes via their low-affinity receptor for IgE, CD23, and rapidly transport them to splenic B cell follicles where an immune response is initiated. Here, we demonstrate that ovalbumin, covalently coupled to anti-CD23 antibodies and administered intravenously to mice, is also transported to splenic follicles and induces an enhanced primary antibody response. These effects are absent in CD23-deficient mice. No enhanced induction of immunological memory was observed. These findings extend previous observations regarding the *in vivo* role of CD23 and emphasize that recirculating B cells play an important role in antigen transport to the spleen.

Introduction

Antibodies, passively administered together with their antigen, can feedback regulate the antibody response towards this antigen [1–3]. Depending on the types of antibody and antigen, the response can be either enhanced by several 100-fold or suppressed by more than 99%. For instance, antibody responses to large antigens such as sheep red blood cells (SRBC) can be completely suppressed by IgG anti-SRBC [4, 5]. The mechanism behind this is poorly understood although it is used in the clinic to prevent Rh immunization of RhD-negative women carrying RhD-positive fetuses [6, 7]. Absence of all known FcγRs in mice, including the inhibitory FcγRIIB, does not affect the suppression, indicating that this process is Fc independent [5]. In mice immunized with SRBC together with IgM anti-SRBC, the antibody response will be enhanced [4, 8]. This process requires that IgM can activate complement and that complement receptors 1 and 2 (CR1/2) are present [8, 9]. Most likely, IgM-mediated enhancement is caused by increased transport of IgM–antigen–complement complexes by CR1/2⁺ marginal zone (MZ) B cells into splenic B cell follicles where it is deposited onto follicular dendritic cells (FDCs) [8, 10, 11].

IgE, administered with small proteins such as ovalbumin (OVA) or bovine serum albumin, can also feedback enhance the antibody response [12–14]. In parallel, *in vivo* proliferation and activation of antigen-specific CD4⁺ T

cells is enhanced [15, 16]. The low-affinity receptor for IgE, CD23, is crucial during these processes as demonstrated by the loss of enhancement in CD23 knockout (KO) mice [13, 14]. In mice, the CD23a isoform, expressed constitutively on mature B cells and FDCs [17, 18], is likely to mediate these effects as IgE enhances equally well in wild-type and IL-4-deficient mice (which only express CD23a) [19]. Expression of CD23 on B cells but not on FDCs is required for enhancement of antibody as well as of CD4⁺ T cell responses [15, 20]. *In vitro*, CD23⁺ B cells can endocytose and present IgE–antigen to CD4⁺ T cells [21–24]. Therefore, it was suggested that this mechanism also operates *in vivo* and explains both the enhanced proliferation of CD4⁺ T cells and the increased antibody production. However, an alternative role for CD23⁺ B cells was revealed when it was found that these cells capture IgE–antigen complexes in peripheral blood and deliver them to splenic follicles [25]. Subsequently, it was demonstrated that CD11c⁺, but not CD19⁺, cells (obtained from mice immunized *in vivo*) present IgE-complexed antigen to CD4⁺ T cells *ex vivo* [16]. This argued against the idea that CD23⁺ B cells actually present IgE–antigen and suggested that their function is to transport the complexes to secondary lymphoid organs where they are transferred to CD11c⁺ dendritic cells which present peptides to CD4⁺ T cells. The novel role for B cells as transporters of IgE–antigen complexes is an interesting addition to the relatively few known pathways for delivery of antigen into splenic follicles [26].

The ability of IgE to enhance both humoral and T cell immunity makes IgE a potential adjuvant [27]. However, a major limitation in its possible application as an adjuvant is the requirement of antigen-specific IgE to form immune complexes. In addition to the difficulties obtaining specific IgE, this isotype is a potent inducer of allergic reactions and therefore hazardous to administer. One way to solve both problems would be to covalently couple antigen to anti-CD23 antibodies, thus creating complexes that would transport the antigen to B cell follicles and induce antibody responses without the use of IgE. Such studies have been performed and show that OVA or rabbit γ -globulin, conjugated to anti-CD23, enhanced antigen-specific T cell proliferation *in vitro* when co-cultured with B cells [28]. Moreover, anti-CD23-OVA conjugates injected subcutaneously elicited a higher specific IgG1 and IgE response *in vivo* than did OVA alone [28].

In this study, we have investigated the ability of anti-CD23-OVA conjugates to bind to CD23⁺ B cells, be transported into splenic B cell follicles, induce primary antibody responses and to prime for memory responses.

Material and methods

Mice. BALB/c mice were purchased from Bom mice (Ry, Denmark), and CD23 KO mice were obtained from Dr Kishimoto [14] and backcrossed to the BALB/c background for 10 generations [15]. All mice were bred and maintained in the animal facilities at the National Veterinary Institute (Uppsala, Sweden), and experiments were conducted in accordance with approved guidelines, regulations and protocols as determined by the Uppsala Animal Research Ethics Committee.

Antibodies and antigens. Ovalbumin was obtained from Sigma-Aldrich (St Louis, MO, USA). The CD23-specific monoclonal antibody B3B4 (rat IgG2a) [17] and its isotype control C0H2 [28] were derived from culture supernatants and purified over a protein G-Sepharose column (BioVision, Milpitas, CA, USA).

Conjugation of anti-CD23 and isotype control IgG to OVA and biotinylation. Anti-CD23 (clone B3B4) or isotype control (clone C0H2) was covalently cross-linked to OVA using heterobifunctional crosslinkers with a method slightly modified from manufacturer's instruction as described below. 1.7 mg OVA dissolved in 500 μ l PBS with 5 mM EDTA was mixed with 500 μ l Traut's reagent (2 mg/ml in PBS) (Thermo Scientific, Rockford, IL, USA), vortexed and incubated for 1 h at room temperature. 1.4 mg of anti-CD23 or isotype control, dissolved in 500 μ l PBS with 5 mM EDTA, was mixed with 100 μ l Sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Thermo Scientific) (2 mg/ml in PBS with 5 mM EDTA), vortexed and incubated at room temperature for 1 h. After incubation, unreacted Traut's reagent and Sulfo-MBS were removed by Zebra spin desalting columns (1000 $\times$ g,

2 min) (Thermo Scientific). The flow-through fractions, containing modified OVA or modified antibodies, were mixed, put on a gentle shaker, and incubated for 2.5 h at room temperature. A molar excess of OVA was used, and to remove the excess, each mixture was passed over a protein G-Sepharose column (BioVision) which binds IgG-containing conjugates but not OVA. The columns were pre-equilibrated with 50 mM sodium borate buffer (pH 8.0) and eluted with 0.1 M citric acid buffer (pH 2.75). Eluted fractions (0.5 ml/tube) were collected, optical density (OD) at 280 nm was measured (NanoDrop 1000 Spectrophotometer, Thermo Scientific), and the five fractions with the highest OD value were pooled. Analysis by electrophoresis (Fig. 1) revealed that the size of the conjugates was approximately 200 kDa, indicating that the antibody/antigen ratio is 1:1 (IgG has a molecular weight of 150 kDa and OVA 45 kDa). Concentration of the antibody-antigen conjugates was determined by measuring the OD of the purified conjugates and calculated using their molar extinction coefficients as described in [29].

Conjugates to be used for studies of antigen localization were biotinylated using Sulfo-NHS-LC-Biotin (Thermo Scientific) as described [25]. Unreacted biotin was removed with 50 K centrifugal filters (Merck Millipore, Darmstadt, Germany) by washing five times with PBS (1000 $\times$ g, 10 min). Biotinylated conjugate as well as non-biotinylated conjugate was sterile filtered and stored at 4 °C.

Protein gel electrophoresis. Two to five microgram of the indicated proteins or protein mixtures were mixed with 5 μ l 4 $\times$ lithium dodecyl sulphate sample buffer (Thermo

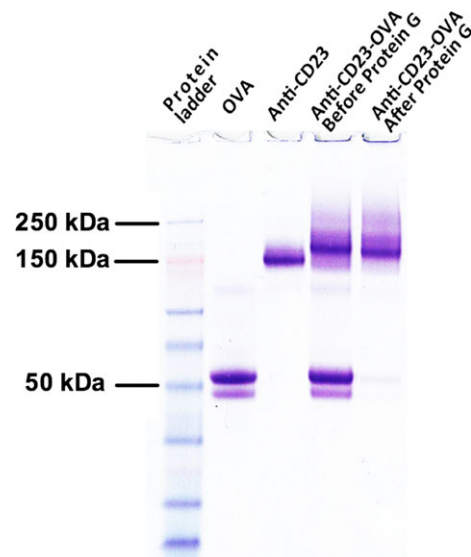


Figure 1 Preparation of anti-CD23-OVA conjugates. Electrophoresis of unconjugated OVA (lane 2), unconjugated anti-CD23 (lane 3) and anti-CD23-OVA after conjugation, still containing free OVA (lane 4) and after removal of free OVA by capture of anti-CD23-OVA conjugates on a protein G-Sepharose column (lane 5). A similar picture is seen when isotype-specific IgG is conjugated to OVA (not shown).

Scientific) and supplemented with de-ionized H₂O to a total volume of 20 μ l. Samples or 5 μ l protein ladder (SeeBlue Pre-Stained Standard; Invitrogen, Carlsbad, CA, USA) was then loaded on a 4–12% precast polyacrylamide gel (Thermo Scientific) buffered with 2-(*N*-morpholino) ethanesulfonic acid running buffer (Thermo Scientific). Gels were run at 120 V for 40 min. Gel was then stained with staining buffer (0.25% coomassie blue (Sigma-Aldrich), 40% methanol, 10% glacial acetic acid and 50% de-ionized H₂O) overnight and then destained with destain buffer (40% methanol, 10% glacial acetic acid and 50% de-ionized H₂O) for 6 h. Images were scanned with a Ricoh Aficio MP C300SR printer (Ricoh, San Jose, CA, USA), and the contrast of the entire image adjusted in Photoshop (Adobe, San Jose, CA, USA).

Immunization and blood sampling. For analysis of antigen localization in the spleen, 120 μ g biotinylated anti-CD23-OVA (B3B4-OVA) or equal amounts of biotinylated iso-OVA (C0H2-OVA) conjugates in 200 μ l PBS were administered in the tail vein. For analysis of antibody responses, 150 μ g of non-biotinylated anti-CD23-OVA or iso-OVA conjugates or 100 μ g of unconjugated OVA in PBS were administered intravenously (i.v.) in 200 μ l. Blood was obtained from tail arteries at the indicated time points, and sera were prepared and stored at -20° .

ELISA. Ovalbumin-specific IgG concentrations in sera were measured by enzyme-linked immunosorbent assay (ELISA) as described before [24]. Briefly, 96-well plates (Costar 96-well enzyme immunoassay/RIA; Sigma-Aldrich) were coated overnight at 4° C with 100 μ l of OVA (50 μ g/ml) in PBS with 0.05% NaN₃. The plates were then blocked with 5% dry milk at room temperature for 4 h. After washing, serum samples and OVA-specific IgG standard were serially diluted and added to the plates. Following washing, alkaline phosphatase-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added and the plates were incubated at room temperature for 3 h. Subsequently, substrate (*p*-nitro-phenylphosphate; Sigma-Aldrich) was added. Absorbance at 405 nm was measured and standard curves constructed with SOFTmax software (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry. Spleen cells were prepared as described [8] and resuspended in FACS buffer (PBS with 2% foetal bovine serum). Rat IgG2bk anti-mouse CD16/CD32 (clone 2.4G2; BD Bio-Sciences, San José, CA, USA) was used as Fc block before staining. Pacific Blue-labelled anti-CD45R (Pacific Blue-B220) (clone RA3-6B2; eBioscience, San Diego, CA, USA) was used to identify B cells, and allophycocyanin-labelled streptavidin (SA-APC; eBioscience) was used to stain biotin-labelled antibody–antigen conjugates. Each sample was stained at 4° C for 30 min, then washed twice in FACS buffer and resuspended in 300 μ l FACS buffer before analysis. For each sample, $4\text{--}8 \times 10^5$ events were acquired on a LSR II cytometer (BD Bio-Sciences). FlowJo software (Tree Star, Ashland, OR, USA) was used to analyse the acquired data.

Confocal microscopy. Spleens were collected, stored and sectioned as described [8]. Seven- μ m-thick frozen sections were stained with 2 μ g/ml Pacific Blue-B220 (clone RA3-6B2; eBioscience) and 3 μ g/ml SA-APC (eBioscience) to detect B cells and biotin-labelled antibody–antigen conjugates, respectively. To define the border of the splenic follicles, 2 μ g/ml FITC-labelled rat IgG2a anti-mouse CD169 (clone MOMA-1; Bio-Rad, Raleigh, NC) was used [30]. The slides were incubated at room temperature for 1 h, washed with PBS, mounted in Fluoromount G solution (Southern Biotech, Birmingham, AL, USA) and scanned with a LSM 700 confocal microscope from Carl Zeiss (Thornwood, NY, USA). Images were analysed with IMAGEJ (NIH, Bethesda, MD, USA).

Statistical analysis. Statistical differences were determined by two-tailed Student's *t*-test with Prism 5.0 (GraphPad Software, La Jolla, CA, USA). ns = $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Anti-CD23-OVA conjugates are targeted to B220⁺ cells in the blood and spleen and can be detected in splenic B cell follicles

We wanted to investigate whether OVA covalently coupled to anti-CD23, instead of forming an immune complex with specific IgE (Fig. 2), could also be targeted

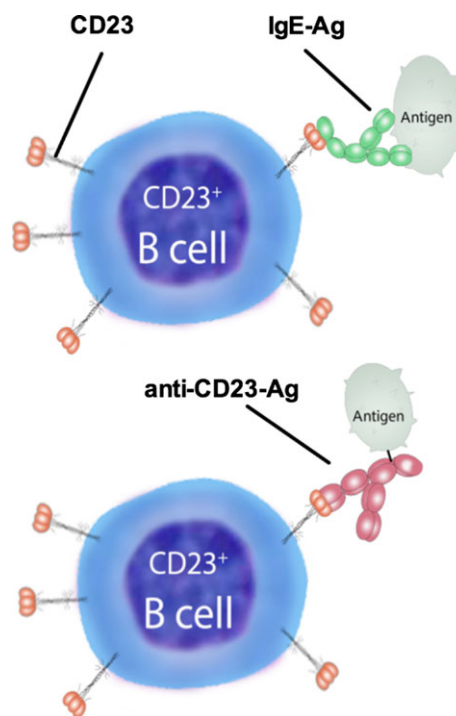


Figure 2 Two principally different ways to target antigen onto CD23. Antigen can be targeted to the low-affinity receptor for IgE, CD23, in the form of IgE–antigen complexes (top) or after covalent coupling to anti-CD23-specific monoclonal antibodies (bottom).

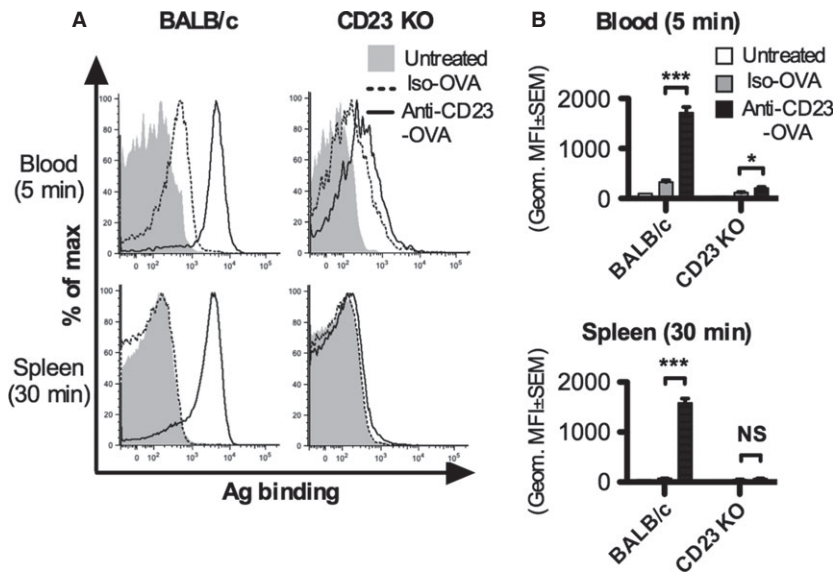


Figure 3 Anti-CD23-OVA conjugates bind to CD23 on B cells in blood and spleen. Representative histograms showing binding of antigen to B220⁺ cells in blood and spleens from untreated mice, mice immunized i.v. with 120 μ g anti-CD23-OVA-biotin or with iso-OVA-biotin (A). Quantification of the data is shown as geometric mean of fluorescence intensity (MFI): untreated ($n = 1$), anti-CD23-OVA ($n = 3$) and iso-OVA-biotin ($n = 3$) (B). Blood samples were obtained 5 min and splenic B cells 30 min after immunization. * $P < 0.05$, *** $P < 0.001$. Representative of two independent experiments.

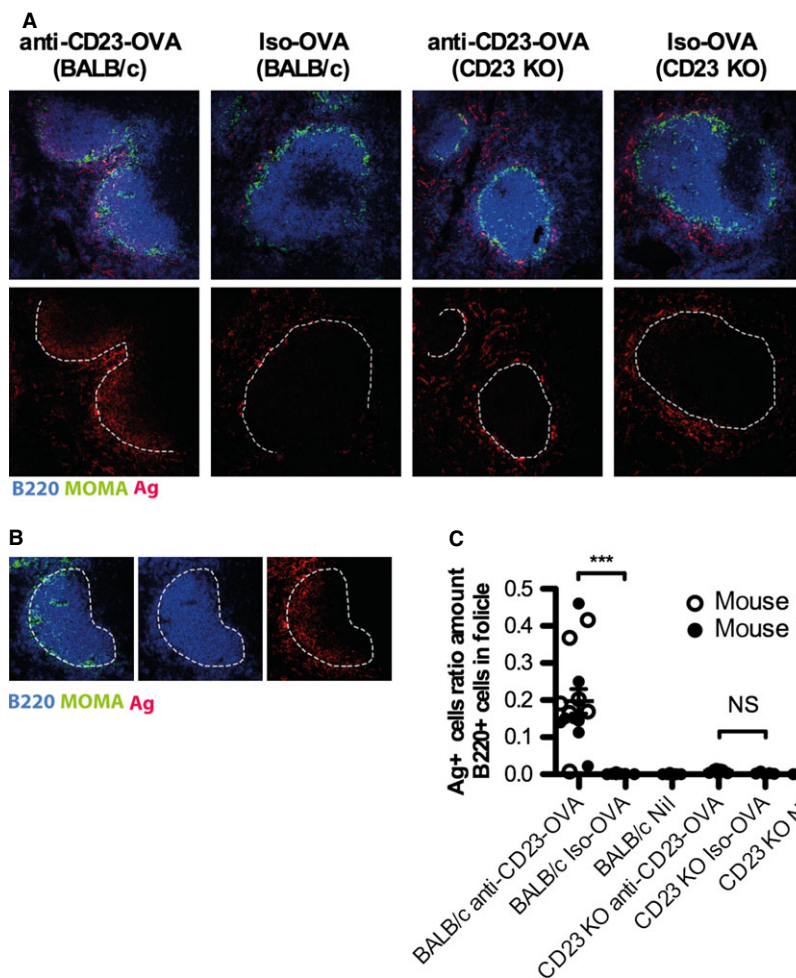


Figure 4 Anti-CD23-OVA conjugates rapidly enter splenic follicles. Spleens from mice immunized as in Fig. 3 were subjected to confocal microscopy. (A) Top row shows representative follicles, stained for B cells (blue), metallophilic macrophages (green) or antigen (red). Bottom row shows the same picture with B cell staining subtracted to better visualize the antigen; the border between marginal zone and follicle is shown in dashed lines. (B) The area staining red (=antigen): area staining blue (B220⁺ cells) within the border formed by metallophilic macrophages was quantified (C). 7–8 follicles from each mouse ($n = 2$ /group) were analysed (solid and open circles represent the individual mice analysed in the experiment shown). *** $P < 0.001$. Representative of two independent experiments.

to B cells and transported to splenic follicles. Biotinylated anti-CD23-OVA or isotype control-OVA (iso-OVA) conjugates were given i.v. to wild-type BALB/c and CD23 KO mice (Fig. 3). Five minutes after immunization, B cells in

the peripheral blood of wild-type mice bound much more anti-CD23-OVA than iso-OVA (Fig. 3A, upper left). In contrast, B cells from CD23 KO mice bound both types of conjugates poorly (Fig. 3A, upper right). Although the

only strong binding was seen between anti-CD23-OVA conjugates and wild-type B cells, low levels of binding of iso-OVA to B cells from both strains and anti-CD23-OVA to CD23 KO B cells were seen (Fig. 3A, upper panels). The reason for this low-degree binding is not understood, but a similar binding of biotinylated antigen to wild-type peripheral B cells was also observed in a previous study [25]. Thirty minutes after immunization, spleens were harvested and binding of the conjugates to splenic B cells was analysed. As in peripheral blood, high levels of anti-CD23-OVA bound to wild-type splenic B cells (Fig. 3B, bottom left), whereas no binding was seen in CD23 KO mice (Fig. 3B, bottom right). Neither wild-type nor CD23 KO splenic B cells bound to iso-OVA (Fig. 3B, bottom).

Next, spleens from mice immunized as in Fig. 3B were prepared for analysis in confocal microscopy. The amount of anti-CD23-OVA and iso-OVA conjugates that had migrated into B cell follicles, defined by the border made up by metallophilic macrophages, was determined (Fig. 4). Thirty minutes after immunization, anti-CD23-OVA conjugates had passed the border and a massive infiltration of antigen into the follicles was seen in wild-type, but not in CD23 KO mice (Fig 4A, C). Iso-OVA did not enter the follicles (Fig. 4A, C).

In summary, anti-CD23-OVA conjugates bound to peripheral as well as splenic B cells via CD23 and could be detected within splenic B cell follicles 30 min after immunization. These findings are similar to what was previously found in mice immunized with IgE-anti-TNP together with OVA-TNP [25, 31].

Anti-CD23-OVA conjugates induce enhanced primary antibody responses but do not enhance priming for memory responses

To test whether the increased localization of anti-CD23-OVA to follicles resulted in enhanced antibody responses to OVA, wild-type and CD23 KO mice were primed i.v. with anti-CD23-OVA, iso-OVA or unconjugated OVA, bled weekly and boosted with 5 μ g OVA alone 70 days later. Sera obtained throughout this period were analysed for IgG anti-OVA. In wild-type mice, anti-CD23-OVA stimulated a fivefold higher OVA-specific IgG response compared to iso-OVA or OVA alone (Fig. 5, top). The antibody levels peaked as early as day 7 and then gradually decreased. In CD23 KO mice, anti-CD23-OVA did not induce higher responses than iso-OVA or OVA alone (Fig. 5, bottom). Surprisingly, the secondary response was similar in all groups, regardless of whether they were primed with anti-CD23-OVA, iso-OVA or OVA alone (Fig. 5).

Discussion

We here demonstrate that anti-CD23 antibodies, covalently coupled to OVA, target the antigen to peripheral B cells within 5 min after they have been administered i.v. After

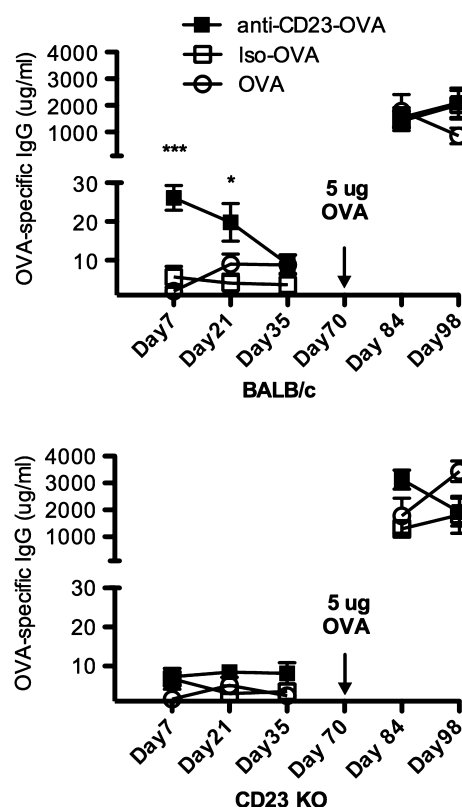


Figure 5 Anti-CD23-OVA induce higher primary antibody responses than isotype control-OVA conjugates. BALB/c and CD23 KO mice were immunized i.v. with 150 μ g anti-CD23-OVA ($n = 6$), 150 μ g iso-OVA ($n = 6$) or 100 μ g OVA ($n = 5$) and bled at the indicated time points. Seventy days after the primary immunization, all mice were boosted i.v. with 5 μ g unconjugated OVA. * $P < 0.05$, *** $P < 0.001$. Representative of two independent experiments with wild-type and CD23 KO mice. In addition, studies of primary antibody responses in wild-type mice have been performed twice with similar results.

30 min, antigen is found in the B cell follicles of the spleen. Following the localization of antigen in splenic follicles, an approximately fivefold higher IgG anti-OVA response is induced in mice given anti-CD23-OVA than in mice given iso-OVA. In CD23 KO mice, these processes are abrogated. The observations are analogous to what is seen in mice immunized with IgE together with specific antigen [25] and show that binding to CD23⁺ B cells, regardless of whether this is mediated by IgE or anti-CD23, suffices for transport and upregulation of primary antibody responses. In a previous study, anti-CD23 covalently coupled to OVA was administered subcutaneously to mice [28]. This resulted in enhanced primary IgG1 and IgE antibody responses as well as in enhanced induction of memory responses. In the present study, i.v. administration of antigen did not result in enhancement of memory, in spite of an enhanced primary antibody response. This was a surprising finding and differs also from previous results in mice immunized i.v. with IgE and antigen which resulted in efficient induction of primary as well as of memory responses [13]. The reason why our

anti-CD23-OVA conjugates failed to induce immunological memory is not clear. A possible explanation is that the antigen/antibody ratio was not optimal for inducing memory B cells, but rather stimulated plasma cell induction. Another possibility is that the induction of memory was already quite strong in controls given iso-OVA or OVA alone, thus not allowing CD23-mediated effects to make a difference.

The most interesting observation in the present study is that anti-CD23-OVA conjugates are rapidly transported to the splenic B cell follicles by CD23⁺ B cells (Figs. 3 and 4). Retention of antigen on FDCs is an essential step for B cell activation and development of an adequate germinal centre reaction resulting in affinity maturation and class switch recombination [32, 33]. In order for this to take place, antigens must gain access to the follicles, a process which only recently is beginning to be understood. The spleen is the most important secondary lymphoid organ for defence against blood-borne pathogens. As it is anatomically located deeper in the body than lymph nodes, and therefore less accessible to intravital microscopy, less is known about how antigens are transported to the spleen than to lymph nodes. Small antigens, administered i.v., can gain access to the splenic follicles via small channels, conduits [34, 35]. For larger antigens, three different B cell-mediated transportation routes have been described (reviewed in [26]). First, specific B cells have been shown to capture particulate antigen in the lung and transport it to the spleen [36]. Second, complement-coated antigens, such as virus-like particles, IgM-antigen or IgG3-antigen immune complexes, can be transported into follicles by MZ B cells expressing high levels of complement receptors 1 and 2 (CR1/2) [10, 11, 31, 37]. MZ B cells continuously shuttle between the MZ and the splenic follicles and are therefore well suited to deliver antigen to FDCs [38, 39]. The third way for B cell-mediated transport of antigen to splenic follicles depends on CD23⁺ recirculating B cells. Whereas MZ B cells express high levels of CR1/2 and are stationary in the spleen, recirculating B cells express high levels of CD23 and migrate between the periphery and the follicles. In a previous report, recirculating B cells were shown to capture IgE-antigen complexes and deliver them to the spleen [25, 31]. As shown here for the first time, recirculating B cells can also capture anti-CD23-antigen conjugates and rapidly transport them to the spleen, thus confirming and extending earlier findings. This transportation route may play a biological role to induce rapid immune responses against antigens bound to IgE. Hypothetically, it may also play a role in induction of immune responses against carbohydrate antigens which may be able to bind directly to CD23, which belongs to the C-type lectin family [40]. Finally, once an optimal conjugation ratio between anti-CD23 and antigen can be determined, such conjugates may serve as efficient inducers both of primary and secondary immune responses.

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