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Are dendritic cells the missing link in IgE-mediated enhancement of CD4+ T cell proliferation?

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Title (English)

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Abstract

To be able to find better treatments and cures against IgE-mediated allergies and asthma, the mechanism behind these diseases has to be clarified. Mice immunized with IgE-antigen complexes have previously been reported to have an enhanced antigen specific T cell proliferation compared to mice immunized with antigen alone. This study suggests a novel model for this mechanism, where dendritic cells are required for an IgE-mediated enhancement of $CD4^+$ T cell proliferation.

Keywords

IgE, T cell proliferation, dendritic cells

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Populärvetenskaplig sammanfattning

Immunförsvaret använder sig av antikroppar för att hitta främmande substanser i kroppen. En viktig antikropp heter Immunoglobulin E (IgE), som främst har till uppgift att hjälpa till vid försvaret mot parasiter. Dock har det också visat sig att IgE ofta är inblandad vid allergiska reaktioner. För att bättre förstå hur allergiska reaktioner uppkommer och hur dessa ska kunna lindras behöver IgE:s biologiska funktion utredas.

Det är tidigare känt att möss som injiceras med en främmande substans stimuleras att börja producera fler T-celler. T-celler är immunförsvarsceller som behövs för aktivering av antikroppsproduktion. Om mössen i samband med injektion av den främmande substansen också får IgE-antikroppar som kan binda substansen, så blir T-cellsproduktionen mycket större. Dessutom får dessa möss en högre antikroppsproduktion.

Denna studie visar främst att dendritiska celler, som är en viktig immunförsvarscell, verkar vara väsentliga för att IgE ska kunna ge en ökad T-cellsproduktion. Detta kan vara ett viktigt fynd i sökandet efter bättre mediciner mot allergier.

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Introduction

Allergies and asthma have become common states of ill-health in today's society. This has lead to an increasing demand for methods on how to prevent and treat those conditions. Allergies and asthma are caused by a hypersensitive – or actually an inappropriately high – response of the immune system to an antigen. The same immune system, that when working properly, has the objective to defend its multicellular host organism against foreign infectious microbes. There are four different types of hypersensitivity responses, often referred to as type I, II, III and IV. The type I hypersensitivity reaction is mediated by a protein called immunoglobulin (Ig) E. However, IgE does not only cause problems in an individual, it is also involved in the protection against parasites (Gurish et al., 2004). This study will investigate the biological role of IgE.

Introduction to antibodies

Antibodies, or immunoglobulins, are glycoproteins with the purpose to find and bind foreign substances, namely antigens. They are produced by B lymphocytes (referred to as B cells) and can exist in a membrane-bound form or in a secreted form that is circulating the body. The antibodies are divided into five different classes: IgA, IgD, IgE, IgG and IgM. In the murine system the IgG can be subcategorized into the subclasses or isotypes IgG1, IgG2a, IgG2b and IgG3. Each isotype of antibody has distinct properties. However, they all share the basic structure with two identical heavy chains and two identical light chains. These are arranged in an order making the antibody Y-shaped and symmetric. Each antibody has a fragment crystalline (Fc-) domain which determines the isotype, and two antigen binding sites at its so called hypervariable regions (Fig. 1). Due to the complex genetic construction, the antibodies can achieve an astronomic variability of their specificity for antigen.

When an antibody has targeted its specific antigen, other immunological systems are triggered through the Fc-domain for destruction of the foreign substance via e.g. phagocytosis or the complement system (reviewed in Francis et al., 2003). The IgE antibody binds with high affinity to mast cells, which have a heavy granular content. The result of IgE cross-linking on the surface of the mast cells is a release of histamine, heparin and many different peptidases with purpose to activate the immune system.

Antibody production

In order for a B cell to start producing antibodies, it has to be activated. In the chain of events leading to antibody production other types of cells including dendritic cells and T cells are also involved. There are two major ways of antigen presentation to $CD4^+$ T cells (T cells with the surface marker cluster of differentiation (CD) 4, also called T helper cells). (i) B cells can capture antigen on a membrane-bound antibody, also called a B cell receptor. The antigen is endocytosed, processed and presented on major histocompatibility complex 2 (MHC-II) to $CD4^+$ T cells (Fig. 2A). (ii) Dendritic cells can endocytose captured antigen and present processed antigen on MHC-II to $CD4^+$ T cells (Fig. 2B). The presentation of antigen makes the $CD4^+$ T cells active and they start to proliferate. Regardless whether the presentation of processed antigen was performed by a B cell or a dendritic cell, antigen specific B cells present processed antigen on MHC-II to the activated $CD4^+$ T cell. Subsequently the activated $CD4^+$ T cell recognizes the B cell, and with the help of co-stimulatory molecules, the B cell is activated and can start producing antibodies (Fig. 2A and B).

For a naïve B cell to become an active antibody producing cell, costimulatory molecules such as cytokines are also required. Cytokines are responsible for intercellular communication. In this case they are especially important for deciding the outcome of isotype switching from IgM, expressed on non-activated B cells, to for example IgE or IgG. Depending on what cytokines are present, the T helper cells will differentiate into either T helper 1 or T helper 2 cells. T helper 1 differentiation is mainly the result of intracellular microbes, and T helper 2 induction can be a consequence of helminth (parasitic worm) infection or a response to an allergen. The T helper 2 cells can produce the cytokines interleukin-4 (IL-4) and IL-13, which in turn stimulate B cells to produce IgE.

Antibody feedback regulation

When mice are immunized with antigen in connection with the administration of antigen specific antibodies, the antibody response may be very different from when antigen is provided alone. In some cases the antibody response might be more than 1000 times higher, and in others, the response is almost completely suppressed. This phenomenon is recognized as antibody feedback regulation. The most famous example of antibody feedback regulation is

probably the fact that Rhesus D (RhD) antigen specific IgGs are administered to a RhD negative mother, in order to prevent the immune systems from attacking a RhD positive baby's erythrocytes (Bowman, 1988). In this case, there is a virtually complete suppression of the mother's RhD specific Ab production (Clarke et al., 1963). The mechanism behind this is presently not completely understood, though three models have been suggested. The most likely is probably epitope masking (Na et al., 2006). This model implies that IgGs cover the surface of the antigenic epitopes and thus hinder the B cell from finding the antigen. The two other models are dependent on a receptor that binds IgG (FcγR): One where certain $Fc\gamma R^+$ cells phagocytose the IgG-antigen complex before activation of B cells and the other where the B cell receptor is co-crosslinked with the inhibitory FcγRIIB. These two latter models would both result in an inactive B cell. The FcγR dependency is contradicted by knockout experiments where suppression is possible even though all known FcγRs have been removed (Karlsson et al., 2001; Karlsson et al., 1999). Moreover, studies have been done where the Fc domain of the antibody is absent, and these fragments are still able to induce suppression (Karlsson et al., 1999).

There can also be a positive antibody feedback regulation. IgE administered together with for example ovalbumin (OVA) leads to a stronger $CD4^+$ T cell proliferation and a higher antibody production (Getahun et al., 2005). Enhancement via IgE has been proven to be mediated by the B cell's low affinity IgE receptor (FcεRII or CD23). This was shown in CD23 knockout mice, that could not enhance the antibody response via IgE (Fujiwara et al., 1994). CD23 is a member of the C-type lectin family (Kikutani et al., 1986), in contrast to the IgE receptor, where the α-chain belong to the immunoglobulin superfamily. There are two more known murine IgE receptors. One is the high affinity receptor (FcεRI). The other is the newly discovered low affinity FcγRIV (Mancardi et al., 2008). The possibility that the antibody feedback regulation is FcεRI- och FcγRIV-mediated, has been excluded based on knock out experiments (Wernersson et al., 1999).

The mechanism behind enhancement via IgE is not clearly understood. Interestingly, it has been shown that $CD23^+$ B cells are involved in the transportation of the IgE-antigen complex to the B cell follicles in the spleen (Getahun et al., 2005; Gustavsson et al., 1994; Hjelm et al., 2008). The next step leading to antibody regulation is vague. However, it is known that the antigen specific $CD4^+$ T cells are activated and proliferate before the antibody response is upregulated (Getahun et al., 2005). The general belief is that when an IgE-antigen complex

has adhered to CD23, the antigen is endocytosed by the B cell, and consequently presented on its MHC-II in order to activate $CD4^+$ T cells (Getahun et al., 2005) (Fig. 2C). However, there are three main aspects that oppose this idea of $CD23^+$ B cells presenting antigen. (i) Neither MHC-II nor any costimulatory molecules are upregulated on the surface of the B cell by IgE-antigen complex. Furthermore, it has not been possible to see the antigenic peptide on the MHC-II on the B cell (Heyman, unpublished data). (ii) The B cells do not present IgE-antigen for $CD4^+$ T cells *ex vivo*. Instead, other spleen cells – probably dendritic cells – seem to have this function (Heyman, unpublished data). (iii) The general view is that dendritic cells rather than B cells present antigen for naïve $CD4^+$ T cells in vivo.

This study had three aims:

(1) The working hypothesis in the present study was that IgE-antigen on $CD23^+$ B cells is transported into the B cell follicles, where the antigen is deposited on dendritic cells (Fig. 2D). The dendritic cells endocytose and process the antigen, and finally present it on MHC-II to the $CD4^+$ T cells. This in turn leads to a proliferation of antigen specific $CD4^+$ T cells. To investigate this, mice lacking dendritic cells were immunized with IgE and antigen. If an up-regulation of antigen specific $CD4^+$ T cells were absent in these mice, while it was present in mice having dendritic cells, the working hypothesis would be strengthened.

(2) To scrutinize the biological function of IgE, this study focused on the transportation of IgE on the B cells' CD23 receptors. Although CD23 has been proven to be imperative for IgE-mediated antibody feedback enhancement, it has not been proven that CD23 is required for transportation of IgE-antigen complexes from the blood to the spleen. This was tested using CD23-deficient mice.

(3) B cells that are specific against a particular antigen are very few. If the CD23 receptors of a B cell in a naïve mouse were saturated with endogenous IgE that is specific against another antigen than its B cell receptor, this would greatly broaden the B cell spectrum of antigen specificity. The B cell would perhaps be able to initiate an immune response upon antigen stimulation to an array of different antigens, either via transportation to the B cell follicles or via antigen presentation to B cells. This might give a larger and possibly faster immune response than if a specific B cell had to find its specific antigen. Therefore the amount of endogenous IgE present on B cells in naïve mice was studied. If naïve B cells have IgE on its

CD23, it would give the B cell and IgE new previously undiscovered roles of the immune system.

Materials and methods

Animals

All experiments involving animals were performed after approval of the Animal Ethics Committee in Uppsala, Sweden. The mice used were bred and maintained at the National Veterinary Institute, Uppsala, Sweden. All mice were on a BALB/c background, including CD23 knockout mice (CD23-/-), FcRγ knockout mice (FcRγ-/-), heterozygous CD11c diphtheria toxin receptor (DTR) mice and wild type mice. The CD11c-DTR mice – obtained from Jackson Laboratories – had a transgenic simian DTR-green fluorescent protein (GFP) fusion protein co-expressed with the CD11c subunit of integrin β2, which is found almost exclusively on dendritic cells (Huleatt and Lefrancois, 1995; Jung et al., 2002). If CD11c-DTR mice are treated with diphtheria toxin, they are transiently depleted of their dendritic cells, suggesting that the DTR is expressed and binds the ligand. This transient deficient condition has been proven to persist for two days, followed by a gradual increment of dendritic cells (Jung et al., 2002). Heterozygous transgenic (CD11c-DTR x BALB/c)F_1 mice were used as test groups in the experiments while their non-transgenic littermates were used as controls.

Genotyping

A small tissue sample (<2 mm) was taken from the tip of the tail of the (CD11c-DTR x BALB/c)F_1 mice. The DNA was extracted using Viogene Blood & Tissue Genomic DNA Extraction Miniprep system (Techtum). The DTR region was specifically amplified using PCR with the primers 5'-gcc acc atg aag ctg ctg ccg-3' and 5'-ggg tgg gga att agt cat gcc-3', which have been previously described (Jung et al., 2002). The reagents for the PCR mixture were from Applied Biosystems. The mixture contained 1 x PCR buffer, 0.1 mM dNTPs, 1 μM of each primer, 0.06 U/ml AmpliTaq DNA Polymerase, 4 mM $MgCl_2$ and one fiftieth of the DNA that had been extracted. The mixture was run in the following PCR program: 94°C for 4

minutes, 35 cycles of 15 seconds at 95°C, 1 minute at 58°C, 15 seconds at 72°C and then finally 5 minutes at 72°C, after which the mixture was cooled down to 4°C. The samples were then analyzed on a 1.5 % agarose gel. A band at 625 bp indicated that the mouse was transgenic, and expressed a DTR protein on its dendritic cells. Absence of a band of length 625 bp implied that the mouse was a non-transgenic littermate.

Purification of IgE anti-TNP antibodies

In order to produce IgE anti-trinitrophenyl (TNP), IGELb4 hybridoma cells were grown in Dulbecco's modified Eagle's medium (Rudolph et al., 1981) with 5 % fetal calf serum. The antibodies were tested with ELISA before purification on a rat anti-mouse κ (187.1.10) Sepharose column, specific for the mouse Ig κ light chain (Ware et al., 1984) that is present on this monoclonal IgE. The purification was done by applying the medium containing the IgE to the column, followed by thorough washing. The IgE was then eluted with an acidic buffer into a pH-neutralizing buffer in fractions (Fig. 3). The IgE was concentrated and the buffer was changed to phosphate buffered saline (PBS) using Amicon Ultra-15, 50 k (Millipore). The concentration was determined using Nanodrop, assuming 1.5 A_{280} units are equivalent to a concentration of 1 mg/ml IgE.

Flow cytometry antibodies

For flow cytometry measurements, the following antibodies were used: biotinylated IgG1 rat anti-mouse IgE (R35-72, BD 553414), Fluorescein isothiocyanate (FITC) -labeled IgG2a rat anti-mouse B220 (RA3-6B2, BD 553087), Phycoerythrin (PE) -labeled IgG2b rat anti-mouse CD4 (L3T4, BD 553730), FITC-labeled IgG2a rat anti-mouse CD4 (L3T4, BD 553651), Allophycocyanin (APC) -labeled Armenian hamster anti-mouse CD11c (HL3, BD 550261), biotinylated IgG2a mouse anti-mouse DO11.10 T cell receptor (TCR) (KJ126, Caltag Laboratories MM7515) and FITC-labeled IgG2a mouse anti-mouse DO11.10 TCR (KJ126, Caltag Laboratories MM7501). PE-labeled Streptavidin (BD 554061) was used to visualize biotinylated antibodies.

Immunizations and blood sampling

Diphtheria toxin (Sigma) was injected intraperitoneally (i.p.) in a dose of 100 ng in 200 µl PBS. Immunizations of 50 µg IgE anti-TNP, 150 µg biotin-OVA-TNP and 20 µg OVA-TNP were performed intravenously (i.v.) into the tail vein. IgE that was injected alone, i.e. not in connection with its antigen, was administered in 200 µl PBS. Antigen administered alone was also injected in 200 µl PBS. IgE anti-TNP injected simultaneously with OVA-TNP was mixed before immunization and given in a total volume of 200 µl PBS (Fig 6C and D). If IgE anti-TNP was injected one hour before OVA-TNP, the IgE and its antigen was administered in 100 µl PBS each (Fig 7B and C).

Blood samples of six drops of blood were withdrawn from the tail artery into three drops of 50 U/ml heparin. If the mouse was to be euthanized in connection to the blood sampling, orbital sinus bleeding was performed.

Flow cytometry

The following flow cytometry protocol was used when analyzing blood unless otherwise is stated. 100 µl of a blood sample – containing 6 drops of blood and 3 drops of 50 U/ml heparin – was transferred to a FACS-tube. To lyse the red blood cells, 2 ml of ACK [0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 1.0 mM EDTA, pH 7.3] was added and the sample was left for 5 min. Centrifugation was performed in a Multifuge 3S-R swing-out rotor at 4°C, 2000 rpm for 5 min. The supernatant was discarded and the pellet was washed by adding 2 ml FACS buffer (PBS with 1 % fetal calf serum) and centrifuged at 4°C, 2000 rpm for 5 min. This washing was performed twice and the supernatant was discarded after each centrifugation. The cells were stained for 30 minutes at 4°C while shaking in a solution of 100 µl FACS buffer containing the following amount of the appropriate antibody (or antibodies): 0.2 µg of anti-CD4-PE or anti-CD11c, 0.1 µg of anti-TCR or 0.5 µg of any other antibody. 0.5 µg Streptavidin-PE was used if needed. The cells were then washed twice as described above. If multiple staining was required, the staining and the washing steps were repeated. When done, the cells were diluted into 500 µl FACS buffer and analyzed with FACScan flow cytometer (BD biosciences).

When spleen cells were analyzed by flow cytometry the following protocol was performed unless otherwise is stated. The spleens were removed and put into PBS, then they were gently mashed using nylon mesh screens and pistons from syringes. The cells were diluted into 10 ml of PBS and centrifuged in a Multifuge 3S-R swing-out rotor at 4°C, 2000 rpm for 5 min. The supernatant was discarded and 5 ml of ACK was added and the sample was incubated for 5 minutes. 5 ml of PBS was added and the cells were centrifuged again. Then, the pellet was diluted into approximately 0.5 million cells in 50 µl FACS buffer in a FACS tube. The staining and washing steps as described in analyzing blood with FACS were used. After the washing steps, the cells were diluted into 300 µl FACS buffer and analyzed with FACSort flow cytometer (BD biosciences) if APC-labeled antibodies were used. Otherwise the cells were analyzed with a FACScan flow cytometer (BD biosciences)

Adoptive transfers

Splenic cells from DO11.10 mice were extracted by mashing the spleens. The cells were suspended in PBS and centrifuged in a Multifuge 3S-R swing-out rotor at 4°C, 1200 rpm for 10 min. If all cells from the DO11.10 spleen were used, the supernatant was discarded and the pelleted cells were diluted in PBS. The cell suspension was then injected i.v. into the tail in a dose of 0.4 spleens per recipient. In experiments where only $CD4^+$ T cells were used, these were sorted from total spleen cells as follows. 5 ml ACK was added to the pellet for five minutes. Then, 5 ml of PBS was added and the mixture was centrifuged as above. The pelleted cells were resuspended in 900 µl PBS per spleen. The $CD4^+$ T cells were positively selected using a magnetic cell sorting system with a LS column and rat anti-mouse CD4 (L3T4) microbeads (Miltenyi Biotec 5061212036) according to the manufacturer's manual. The cells were diluted in PBS and injected i.v. into the tail of the recipient in a dose of 3 million DO11.10 T cells in 200 µl PBS.

Statistics

Statistical analysis was performed using two-way analysis of variance with Bonferroni's post hoc where applicable. *P*-values below 0.05 were considered significant.

Results

Genotyping of CD11c-DTR mice

In order to determine which mice carried the CD11c-DTR transgene (and therefore would express the DTR protein on the surface of their dendritic cells), DNA was extracted from the tip of the tail of the (CD11c-DTR x BALB/c)F_1 mice. Figure 4 shows a representative electrophoresis run of the PCR-amplified DNA segment, where a band at 625 bp indicate that the individual mouse had the DTR transgene. Absence of that band implied a non-transgenic littermate. Altogether 58 mice were genotyped, 26 of which were transgenic.

Are dendritic cells depleted in diphtheria toxin treated CD11c-DTR mice?

To verify that dendritic cells could be depleted in CD11c-DTR mice, 100 ng of diphtheria toxin was administered i.p. After 24 h the spleens were analyzed for $CD11c^+$ cells by flow cytometry with antibodies against CD11c. An untreated CD11c-DTR mouse and a wild type BALB/c were used as negative controls. Dendritic cells – which are $CD11c^{hi}$ – were depleted in the diphtheria toxin treated CD11c-DTR mice (Fig. 5A), whereas they are present in the untreated CD11c-DTR mouse (Fig. 5B) as well as in the wild type BALB/c mouse (Fig. 5C). It has previously been shown that dendritic cells of wild type mice are unaffected by diphtheria toxin (Jung et al., 2002), and therefore this control was not added.

Does IgE anti-TNP upregulate the proliferation of OVA-specific $CD4^+$ T cells to OVA-TNP?

It has been shown that IgE anti-TNP in complex with OVA-TNP upregulate the $CD4^+$ T cell proliferation *in vivo* compared to administration of OVA-TNP alone (Getahun et al., 2005). This fact was used to assess the functionality of the IgE that was eluted on the rat anti-mouse-κ column, specific for binding IgE. An adoptive transfer of crude splenic cells from DO11.10 mice – which express an anti-OVA T cell receptor on their T cells – was performed to the BALB/c mice. One day later, the mice were immunized i.v. with either IgE, OVA-TNP or with both IgE and OVA-TNP. Three days after immunization, the spleens were removed and analyzed by flow cytometry using anti-TCR-biotin, specific for binding the DO11.10 T cell receptor, and anti-CD4-FITC antibodies. As expected, the OVA-specific $CD4^+$ T cell

proliferation is enhanced 6.3 times, when the antigen was provided with the antibody compared to antigen provided alone (Fig. 6). Hence, the IgE was functional as an up-regulator of the $CD4^+$ T cell response.

Does IgE anti-TNP upregulate the proliferation of OVA-specific $CD4^+$ T cells to OVA-TNP in mice lacking dendritic cells?

Combining *in vivo* $CD4^+$ T cell proliferation with a depletion of dendritic cells would answer the question whether or not dendritic cells are required for IgE-mediated enhancement of the $CD4^+$ T cell response. Day zero, the CD11c-DTR mice were treated with diphtheria toxin to deplete the dendritic cells. Adoptive transfers of enriched $CD4^+$ T cells from DO11.10 mice to the CD11c-DTR mice and to non-transgenic littermates were also performed day zero. The consecutive day, IgE-anti-TNP and OVA-TNP was administered to both the CD11c-DTR mice and to the non-transgenic controls. Another control group received OVA-TNP alone. Since the T-cell proliferation is maximal three days after IgE-antigen complex immunization (Getahun et al., 2005), the spleens were analyzed with flow cytometry at that time point. Anti-TCR-FITC and anti-CD4-PE antibodies were used to assess the proliferation. As expected, the OVA specific $CD4^+$ T cells from non-transgenic littermates that received IgE anti-TNP one hour before OVA-TNP (Fig. 7B) proliferated more than those in mice receiving only OVA-TNP (Fig 7A). Interestingly, $CD4^+$ T cell proliferation in diphtheria toxin treated CD11c-DTR mice, lacking dendritic cells, was abolished since they did not proliferate significantly more than the OVA-TNP-treated non-transgenic littermates (Fig. 7C). This suggests that dendritic cells are important for IgE-antigen mediated up-regulation of $CD4^+$ T cell proliferation.

Is CD23 responsible for transportation of IgE-antigen complexes from the blood to the splenic B cell follicles?

IgE-antigen complexes bind to B cells in the blood and can after 30 minutes be found on B cells in the B cell follicles of the spleen (Hjelm et al., 2008). It has not been formally proven that this transportation of IgE-antigen complexes is CD23 dependent. To test this, CD23-/- mice and wild type BALB/c controls were immunized with IgE anti-TNP and biotinylated

OVA-TNP. Flow cytometry measurements with anti-B220 antibodies, specific for B cells, confirmed that wild type BALB/c mice bind IgE-antigen complexes on B cells in blood after 5 minutes and in the spleen after 30 minutes (Fig. 8). In CD23-/- mice IgE-antigen complexes did not bind to the B cells neither in the blood nor in the spleen. FcRγ-/- mice, which lack FcαR, FcγRI, FcγRII, FcγRIII, FcγRIV and FcεRI (Takai et al., 1994), were also immunized with IgE anti-TNP and OVA-TNP-biotin and used as controls. These mice, which express CD23 receptors, were binding IgE-antigen complexes equally well as wild type mice in the blood. Surprisingly though, the B cells of FcRγ-/- mice bound IgE-antigen complexes better than B cells from wild type mice. To conclude, the transportation of IgE-antigen complexes from the periphery to the splenic B cell follicles is CD23 dependent.

Does endogenous IgE bind to the surface of B cells in naïve mice?

It is not known if B cells in naïve BALB/c mice, which have not been immunized with IgE-antigen complexes, have endogenous IgE on their CD23 receptors. If they had, it could mean that the response against a specific antigen would be enhanced compared to if B cells with the specific B cell receptor had to find the antigen. To find out if naïve B cells have endogenous IgE bound to its surface, blood samples from untreated CD23-/- and untreated BALB/c mice were analyzed by flow cytometry with anti-IgE and anti-B220 antibodies (Fig. 9). Since CD23 is the only IgE receptor that is present on B cells, CD23-/- mice lack all known IgE receptors. The results showed that BALB/c mice and CD23-/- mice did not have a discernible difference in IgE levels on the surface of their B cells. It is therefore possible to draw the conclusion that B cells of BALB/c mice do not have any detectable IgE antibodies on their CD23 receptors. To ascertain the fact that B cells of BALB/c mice, but not CD23-/- mice, bind monomeric IgE, the mice were immunized with IgE anti-TNP. The IgE could be found on B cells of BALB/c mice in blood after 5 minutes and 30 minutes (Fig. 9A) and in the spleen after 30 minutes (Fig. 9B), but not in the CD23-/- mice. The conclusion that naïve B cells do not have IgE bound to its surface could therefore be drawn.

Discussion

Involvement of dendritic cells in CD4$^+$ T cell proliferation mediated by IgE-antigen complexes

The most important finding in this study was the fact that dendritic cells appear to be required for *in vivo* CD4$^+$ T cell proliferation mediated by presentation of IgE-antigen complexes to CD4$^+$ T cells. In other words, the model we proposed (figure 2D) is the most probable mechanism for IgE-mediated enhancement of CD4$^+$ T cell proliferation. If dendritic cells are involved in the upregulation of CD4$^+$ T cells via IgE-antigen complexes, a hypothesized mechanism is as follows. First the B cell binds the IgE-antigen complex to its CD23. The B cell is transported into the B cell follicles, where the antigen or the immune complex is transferred via an undetermined mechanism to a dendritic cell. The mechanism behind this transfer still remains to be revealed. As shown in knock-out experiments, the transfer cannot be dependent on the high affinity FcεRI (Wernersson et al., 1999), which have been reported on dendritic cells (Maurer et al., 1996). Moreover, the low affinity CD23 receptor cannot work as a receiver, since it is not expressed on DCs. Instead, one possibility is that the antigen is endocytosed by dendritic cell, and then presented to the CD4$^+$ T cell through the usual mechanism on its MHC-II. Here, the supposed mechanism of the B cell is that it acts as a transporter only, in contrast to previous beliefs that it acts as a cell presenting antigen for CD4$^+$ T cells. An analogy to the proposed mechanism is that secreted IgM forms a complex with antigen, which binds to the so called complement receptor 1/2 of splenic marginal zone B cells (Ferguson et al., 2004). The complex is then transported into the follicles of the spleen, where it is deposited on follicular dendritic cells (Ferguson et al., 2004). This in turn initiates an immune response. In this case, the B cells function as transporters, and there exists a mechanism for transferring immune complexes to other cells. If our proposed model is true, the mechanism behind the transfer of antigen from a B cell to a dendritic cell needs to be clarified.

It has been shown that dendritic cells are needed for IgE-mediated enhancement of CD4$^+$ T cell proliferation *ex vivo* (Heyman, unpublished data). This was done by immunizing wild type BALB/c mice with either OVA-TNP alone or simultaneously with IgE anti-TNP. After one hour, the spleens were removed and made into cell suspensions. The mixtures were

divided into three groups each by sorting. The first group contained all cell types, the second only $CD11c^+$ cells (mostly dendritic cells) and the third contained all cells except the $CD11c^+$ cells. The three groups were irradiated with 1000 Gy in order to stop the cells from dividing. Then, $CD4^+$ OVA-specific T cells were exposed to the three populations and the proliferation was studied by analyzing radioactively labeled thymidine incorporation. The *ex vivo* studies revealed that only the groups containing dendritic cells showed an up-regulation of $CD4^+$ T cell proliferation, which is consistent with this *in vivo* study.

The data shown in Figure 7 was a pilot study and experiments with larger groups are necessary to ascertain whether dendritic cells are important for IgE-mediated enhancement of $CD4^+$ T cell proliferation. The possibility that diphtheria toxin has effects that suppresses $CD4^+$ T cell proliferation mediated by something other than the absence of dendritic cells can at the moment not be ruled out. For example, some subsets of cells called macrophages express the *cd11c* gene – consequently they have the DTR on the surface – and are thus sensitive to diphtheria toxin (Probst et al., 2005). The option that proliferation is absent due to another phenomenon than dendritic cell depletion can be investigated by treating wild type BALB/c mice with diphtheria toxin and studying the $CD4^+$ T cell proliferation. If the proliferation remains at the same level as in untreated controls, the involvement of dendritic cells is strengthened. To prove that dendritic cells have a key role in $CD4^+$ T cell proliferation of IgE-antigen complexes, it is necessary to reconstitute dendritic cells into mice that lack dendritic cells. If the $CD4^+$ T cell proliferation is restored, the dendritic cells are imperative for the proliferation.

Since it seems likely that dendritic cells are involved in IgE-mediated enhancement of the $CD4^+$ T cell proliferation, it would also be interesting to investigate the antibody response. BALB/c mice receiving adoptively transferred DO11.10 T cells and that were injected with IgE anti-TNP and OVA-TNP, have been shown to upregulate the production of antigen specific antibodies (Getahun et al., 2005). The specific antibodies that are upregulated belongs to the isotypes IgG1, IgG2a, IgM and IgE (Gustavsson et al., 1994). These antibody concentrations are significantly higher day seven compared to antibody concentrations in mice immunized with antigen alone (Gustavsson et al., 1994). There is however a problem with studying antibody responses in mice with transient deficiency of dendritic cells. The reason for this is that the antibody responses take one to two weeks to develop. Moreover, in CD11c-DTR mice, dendritic cells are progressively restored two days after diphtheria toxin

treatment. This means that after two days, antigen presentation is supposed to function normally. Also, it has been shown that repeated diphtheria toxin treatments are lethal (Jung et al., 2002). The reason for the cause of death is not yet completely established, but is probably due to expression of *cd11c* on vital non-hematopoietic cells. To study the antibody response in transiently depleted mice, it is nonetheless possible to create bone marrow chimeric mice (Zammit, 2005). This is done by irradiating BALB/c mice. Irradiation only depletes bone marrow derived quickly dividing cells (including dendritic cells). Though, since non-hematopoietic cells divide slowly, they survive the irradiation process. A bone marrow transplantation from CD11c-DTR mice to the irradiated BALB/c mice is then performed. This will create a mouse that have endogenous non-hematopoietic cells without a *dtr* expression, and dendritic cells that are susceptible to diphtheria toxin. Repeated administration of diphtheria toxin to DTR bone marrow chimeras will thus only deplete their dendritic cells, while their non-hematopoietic cells are unaffected. Therefore, in this system, dendritic cells can be depleted for a longer period of time and the antibody response can be studied.

The role of the CD23 receptor on B cells

This study showed that CD23 receptors on B cells are imperative for the transportation of IgE-antigen complexes from the blood to the spleen. Unexpectedly, B cells of FcRγ-/- mice were binding IgE-antigen complexes to a larger extent than B cells of wild type BALB/c mice. The cause of this could be due to the absence of Fc receptors other than CD23 on FcRγ-/- mice. Possibly, the CD23 receptor is upregulated on FcRγ-/- mice to compensate for the absence of other receptors. The up-regulation of CD23 receptors could easily be verified by studying the spleen cells from naïve FcRγ-/- mice with anti-CD23 and anti-B220 antibodies. If CD23 is up-regulated, it could be an important step of understanding how the immune system can circumvent problems with nonfunctional pathways – in this case the lack of immunoglobulin receptors.

It was shown that endogenous IgE was not present on naïve B cells in untreated mice. This could depend on the fact that these mice have not been exposed to antigen that stimulates IgE production and that background IgE levels are too low. In an allergic individual, the IgE levels are markedly elevated, and sufficient levels of endogenous IgE to ligate $CD23^+$ B cells might be present. This could be studied by repeatedly exposing BALB/c mice to an antigen,

for example OVA-TNP, together with alum. Alum is an adjuvant that elicits T helper 2 induction (Grun and Maurer, 1989) and consequently stimulates IgE production. After treatment, the IgE levels of B cells can be measured in these mice and compared to unimmunized mice. If the levels risen in OVA-TNP and alum treated mice, this could be of great importance when studying allergies in the future.

References

Bowman, J. M. (1988). The prevention of Rh immunization. Transfus Med Rev *2*, 129-150.

Clarke, C. A., Donohoe, W. T., Mc, C. R., Woodrow, J. C., Finn, R., Krevans, J. R., Kulke, W., Lehane, D., and Sheppard, P. M. (1963). Further experimental studies on the prevention of Rh haemolytic disease. Br Med J *1*, 979-984.

Ferguson, A. R., Youd, M. E., and Corley, R. B. (2004). Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. Int Immunol *16*, 1411-1422.

Francis, K., van Beek, J., Canova, C., Neal, J. W., and Gasque, P. (2003). Innate immunity and brain inflammation: the key role of complement. Expert Rev Mol Med *5*, 1-19.

Fujiwara, H., Kikutani, H., Suematsu, S., Naka, T., Yoshida, K., Yoshida, K., Tanaka, T., Suemura, M., Matsumoto, N., Kojima, S.*, et al.* (1994). The absence of IgE antibody-mediated augmentation of immune responses in CD23-deficient mice. Proc Natl Acad Sci U S A *91*, 6835-6839.

Getahun, A., Hjelm, F., and Heyman, B. (2005). IgE enhances antibody and T cell responses in vivo via CD23+ B cells. J Immunol *175*, 1473-1482.

Grun, J. L., and Maurer, P. H. (1989). Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. Cell Immunol *121*, 134-145.

Gurish, M. F., Bryce, P. J., Tao, H., Kisselgof, A. B., Thornton, E. M., Miller, H. R., Friend, D. S., and Oettgen, H. C. (2004). IgE enhances parasite clearance and regulates mast cell responses in mice infected with Trichinella spiralis. J Immunol *172*, 1139-1145.

Gustavsson, S., Hjulstrom, S., Liu, T., and Heyman, B. (1994). CD23/IgE-mediated regulation of the specific antibody response in vivo. J Immunol *152*, 4793-4800.

Hjelm, F., Karlsson, M. C., and Heyman, B. (2008). A novel B cell-mediated transport of IgE-immune complexes to the follicle of the spleen. J Immunol *180*, 6604-6610.

Huleatt, J. W., and Lefrancois, L. (1995). Antigen-driven induction of CD11c on intestinal intraepithelial lymphocytes and CD8+ T cells in vivo. J Immunol *154*, 5684-5693.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Karlsson, M. C., Getahun, A., and Heyman, B. (2001). FcgammaRIIB in IgG-mediated suppression of antibody responses: different impact in vivo and in vitro. J Immunol *167*, 5558-5564.

Karlsson, M. C., Wernersson, S., Diaz de Stahl, T., Gustavsson, S., and Heyman, B. (1999). Efficient IgG-mediated suppression of primary antibody responses in Fcgamma receptor-deficient mice. Proc Natl Acad Sci U S A *96*, 2244-2249.

Kikutani, H., Inui, S., Sato, R., Barsumian, E. L., Owaki, H., Yamasaki, K., Kaisho, T., Uchibayashi, N., Hardy, R. R., Hirano, T.*, et al.* (1986). Molecular structure of human lymphocyte receptor for immunoglobulin E. Cell *47*, 657-665.

Mancardi, D. A., Iannascoli, B., Hoos, S., England, P., Daeron, M., and Bruhns, P. (2008). FcgammaRIV is a mouse IgE receptor that resembles macrophage FcepsilonRI in humans and promotes IgE-induced lung inflammation. J Clin Invest *118*, 3738-3750.

Maurer, D., Fiebiger, S., Ebner, C., Reininger, B., Fischer, G. F., Wichlas, S., Jouvin, M. H., Schmitt-Egenolf, M., Kraft, D., Kinet, J. P., and Stingl, G. (1996). Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. J Immunol *157*, 607-616.

Na, D., Kim, D., and Lee, D. (2006). Mathematical modeling of humoral immune response suppression by passively administered antibodies in mice. J Theor Biol *241*, 830-851.

Probst, H. C., Tschannen, K., Odermatt, B., Schwendener, R., Zinkernagel, R. M., and Van Den Broek, M. (2005). Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. Clin Exp Immunol *141*, 398-404.

Rudolph, A. K., Burrows, P. D., and Wabl, M. R. (1981). Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Iga or Igb heavy chain haplotype. Eur J Immunol *11*, 527-529.

Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J. V. (1994). FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell *76*, 519-529.

Ware, C. F., Reade, J. L., and Der, L. C. (1984). A rat anti-mouse kappa chain specific monoclonal antibody, 187.1.10: purification, immunochemical properties and its utility as a general second-antibody reagent. J Immunol Methods *74*, 93-104.

Wernersson, S., Karlsson, M. C., Dahlstrom, J., Mattsson, R., Verbeek, J. S., and Heyman, B. (1999). IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice. J Immunol *163*, 618-622.

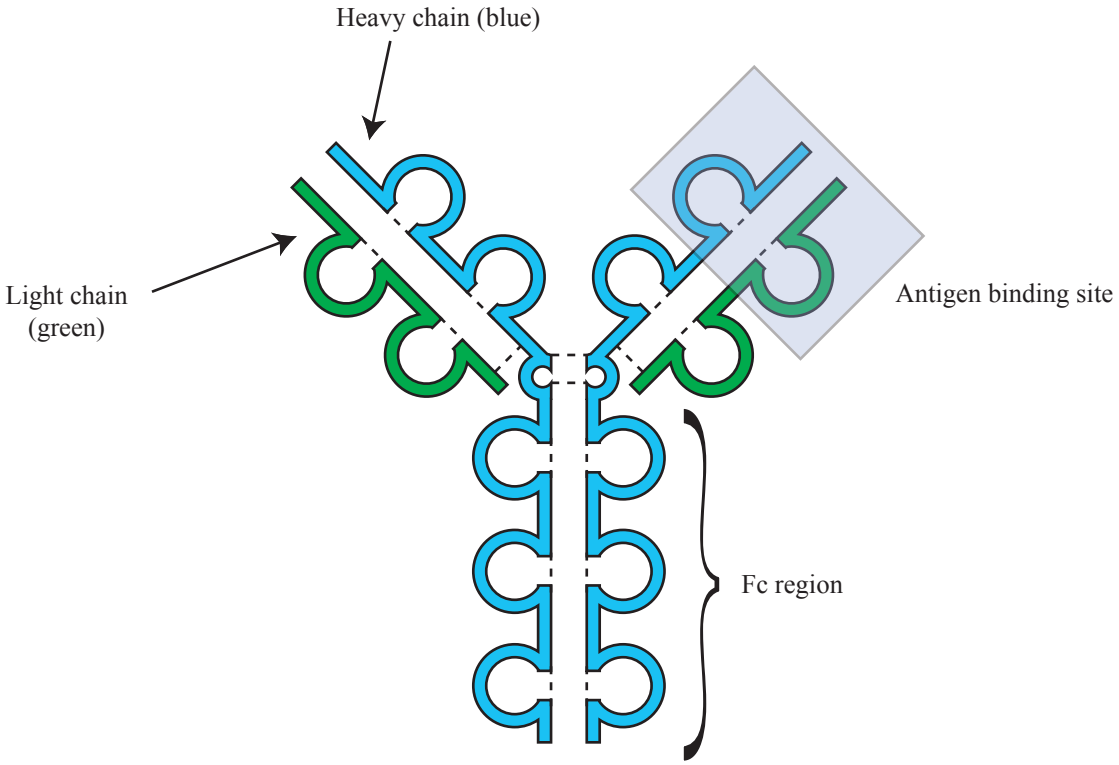
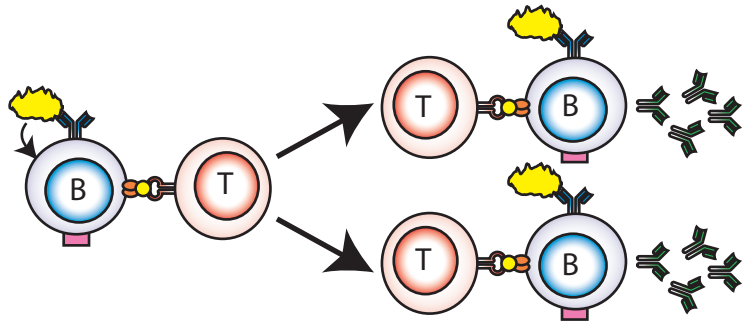
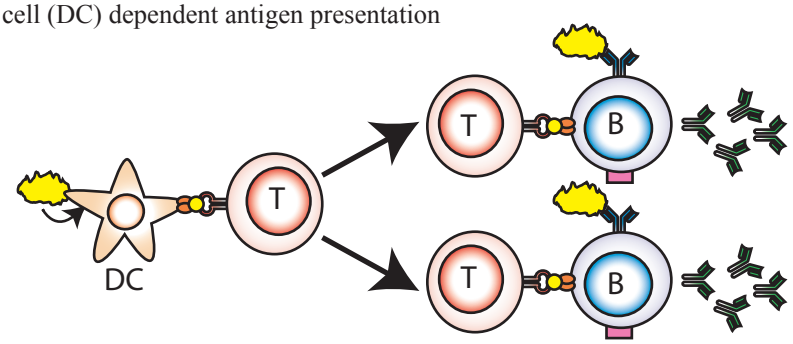


Figure 1 | **Schematic illustration of an IgE antibody.** The IgE molecule consists of two identical light chains and two identical heavy chains connected by disulfide bridges (dotted lines). A light chain and a heavy chain together form an antigen binding site. The structure of the Fc region determines the antibody isotype.

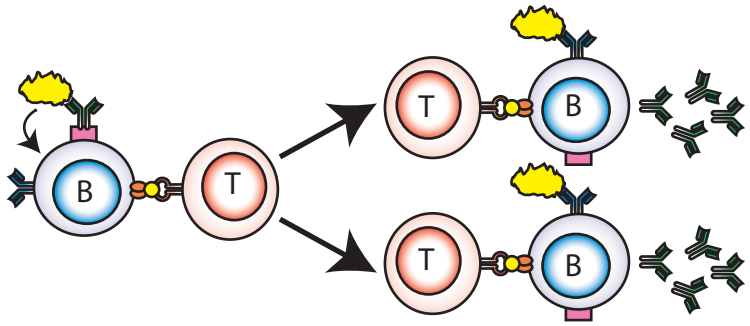
A) B cell receptor dependent antigen presentation



B) Dendritic cell (DC) dependent antigen presentation



C) CD23 dependent antigen endocytosis of IgE-antigen complexes for antigen presentation



D) CD23 dependent transfer of IgE-antigen complexes to dendritic cells for antigen presentation

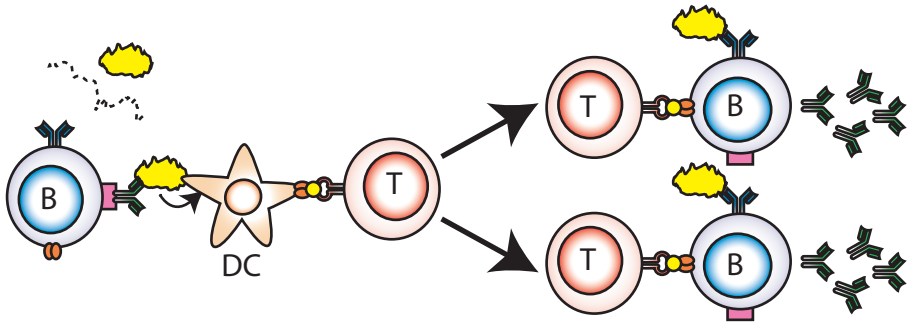


Figure 2 | **Four possible ways for the activation of CD4⁺ T cell proliferation and antibody production.** Antigen has been hypothesized to be presented to naïve CD4⁺ T cells in four ways. **A)** A B cell captures a specific antigen with its B cell receptor, endocytoses and processes it, and presents it on MHC-II to a CD4⁺ T cell. **B)** A dendritic cell (DC) endocytoses the antigen, processes it, and present it on MHC-II to a CD4⁺ T cell. **C)** An IgE molecule captures an antigen and binds the B cell's CD23 receptor. The antigen is endocytosed, processed and presented to a CD4⁺ T cell via MHC-II. **D)** An IgE-antigen complex binds the B cell's CD23 receptor. The antigen is transferred to a dendritic cell, where it becomes endocytosed and processed. Finally the antigen is presented on MHC-II to a CD4⁺ T cell. In all cases (**A**, **B, C** and **D**), the interaction between the antigen presenting cell, which has MHC-II, and the CD4⁺ T cell is dependent on the T cell's CD4 receptors. After interaction, the CD4⁺ T cell is activated and starts proliferating. B cells that have endocytosed and processed the same antigen as presented to the non-activated CD4⁺ T cells present antigen on MHC-II to an activated CD4⁺ T cell. This time, the B cell receives a signal to class switch and produce secretory antibodies with specificity for the same antigen, for example antigen specific IgE.

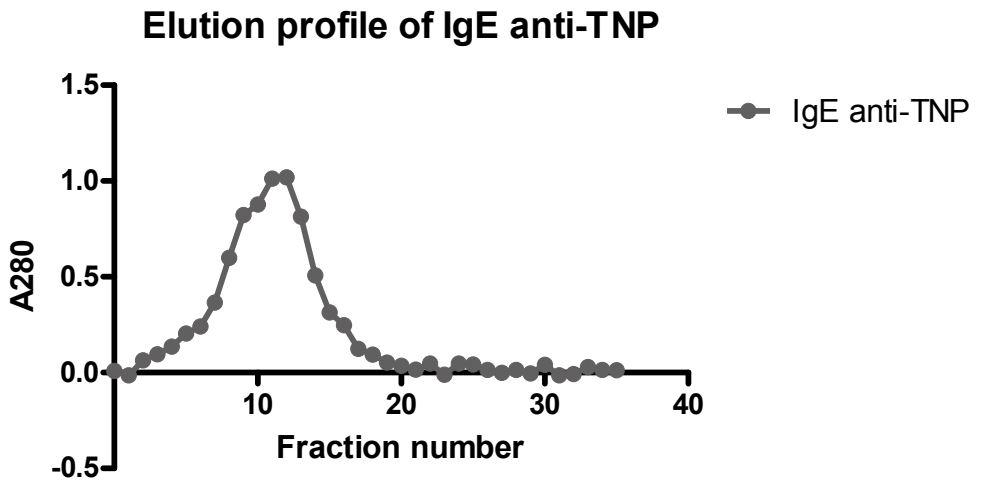


Figure 3 | **Elution profile of IgE anti-TNP purified on a rat anti-mouse κ (187.1.10) Sepharose column.** The absorbance at wavelength 280 nm (A280) of the eluate was measured with nanodrop. 1.5 absorbance units were assumed to equal 1 mg/ml IgE anti-TNP.

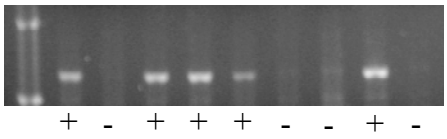
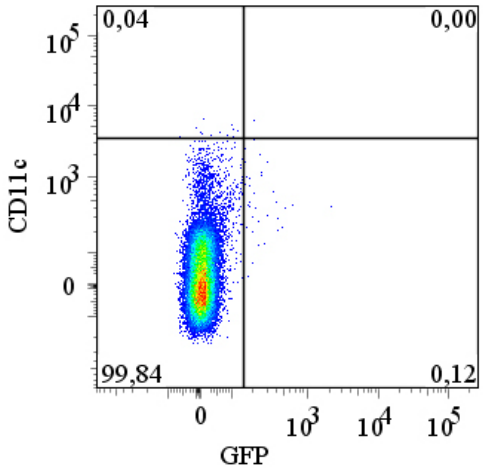


Figure 4 | **Representative electrophoresis run of (CD11c-DTR x BALB/c)F_1 mice.** A band at 625 bp (+) indicate that the mouse has a DTR coding region, and expresses DTRs on the surface of its dendritic cells. Non-transgenic littermates lack the transgene encoded band (-). The first lane to the left is a DNA ladder where two bands are visible, the upper of length 1018 bp and the lower of length 506 and 517 bp.

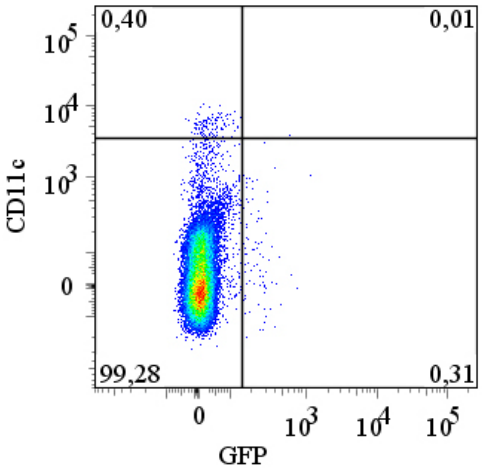
CD11c-DTR mouse
Diphtheria toxin treated

A



CD11c-DTR mouse
Untreated

B



BALB/c mouse
Untreated

C

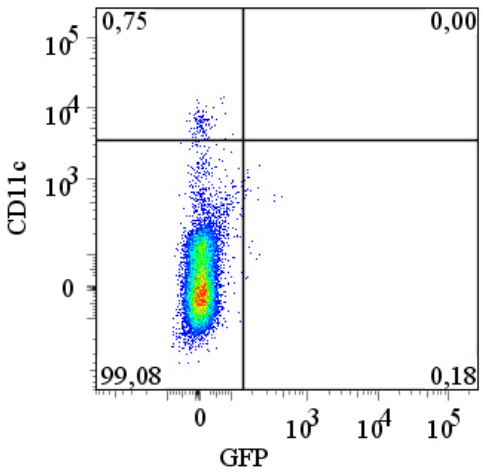


Figure 5 | **Splenic dendritic cells are depleted in diphtheria toxin treated CD11c-DTR mice.** A CD11c-DTR mouse (n=1) was treated i.p. with 100 ng diphtheria toxin (**A**). For controls, a transgenic littermate (**B**, n=1) and a wild type BALB/c mouse (**C**, n=1) were left untreated. Spleens were analyzed by flow cytometry 24 h after diphtheria toxin treatment. The dendritic cells were depleted in the diphtheria toxin treated CD11c-DTR mouse, whereas the dendritic cells of the negative controls still remained. The upper left quadrant represent the dendritic cells.

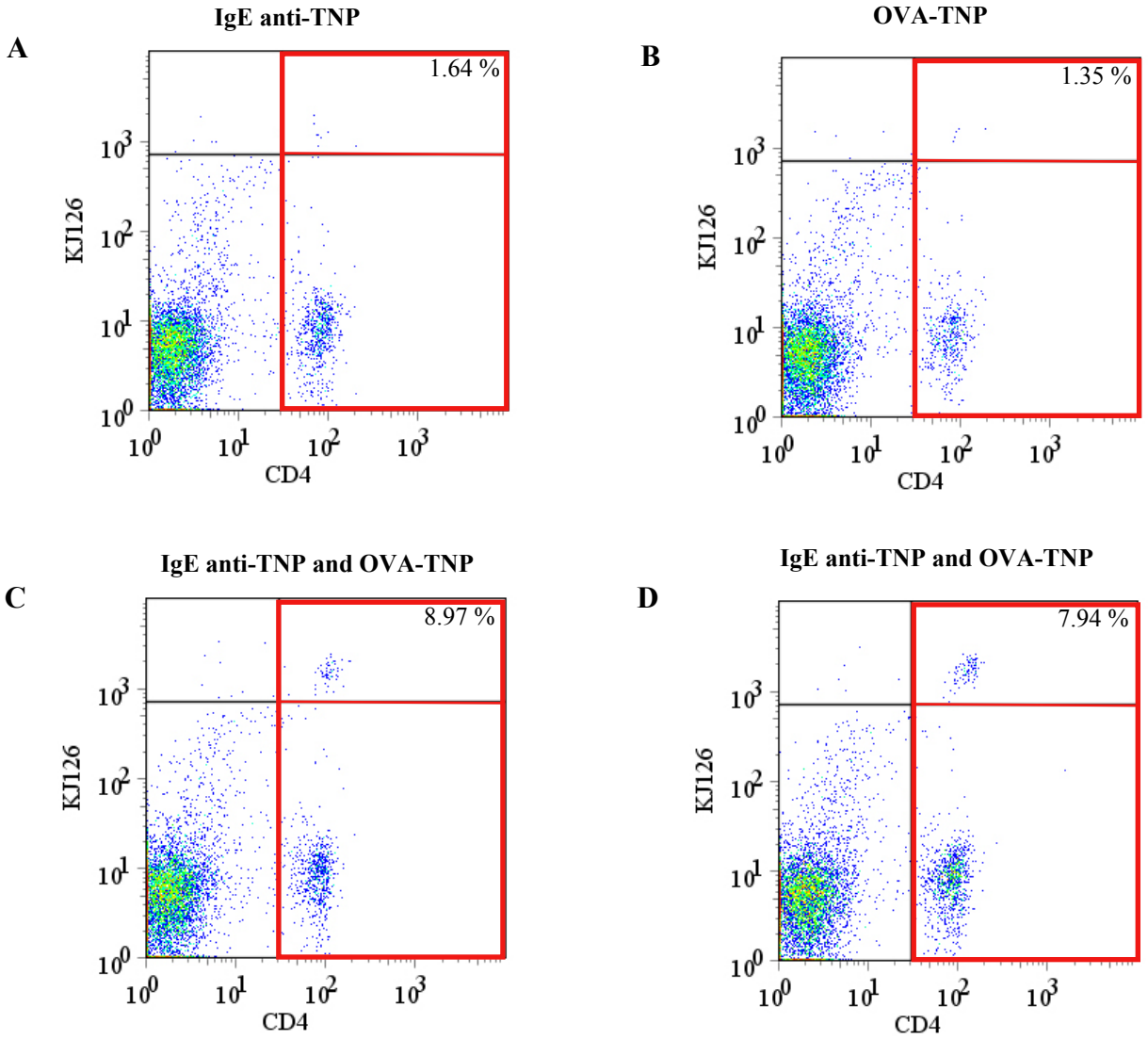
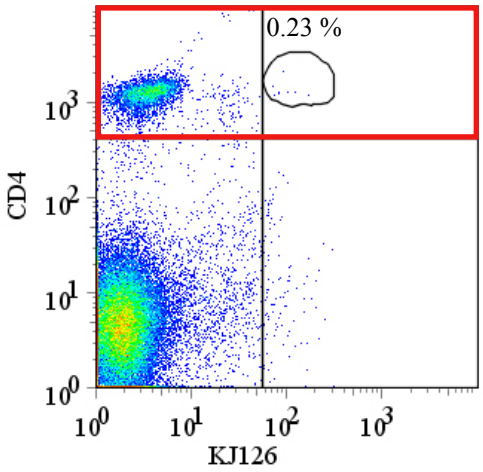


Figure 6 | **TNP-specific IgE upregulate CD4$^+$KJ126$^+$ T cell proliferation to OVA-TNP.** Day 0, an adoptive transfer of crude splenic cells from DO11.10 mice to BALB/c mice was done. The dose was equivalent to 0.4 DO11.10 spleens per BALB/c mouse. Day 1, (**A**) one mouse was immunized with 50 µg IgE anti-TNP, (**B**) one was treated with 20 µg OVA-TNP, and (**C**, **D**) two mice were given both 50 µg IgE anti-TNP and 20 µg OVA-TNP. Three days later, day 4, the spleens were analyzed with flow cytometry. Lymphocytes were gated and are shown in the diagrams. The percentage represents the CD4$^+$KJ126$^+$ cells as a fraction of CD4$^+$ cells. The mean upregulation of CD4$^+$KJ126$^+$ cells was calculated to 6.3 and the IgE anti-TNP thus was functional.

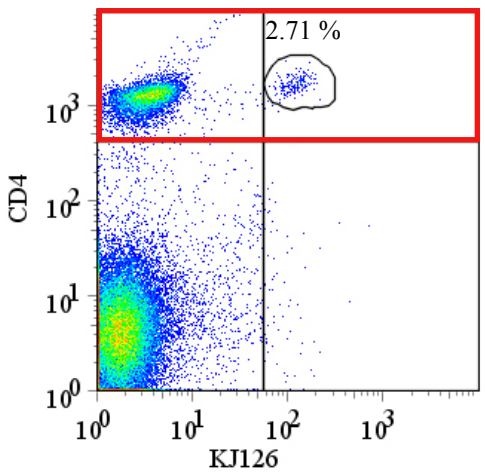
Non-transgenic littermates
OVA-TNP immunized

A



Non-transgenic littermates
IgE anti-TNP and OVA-TNP immunized

B



CD11c-DTR mice
Diphtheria toxin treated
IgE anti-TNP and OVA-TNP immunized

C

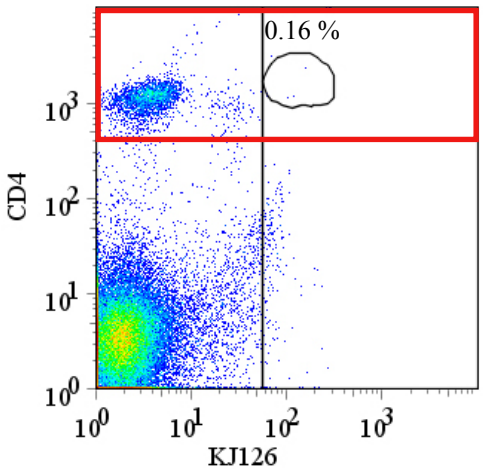


Figure 7 | **Dendritic cell depleted mice cannot up-regulate OVA-specific CD4⁺ T cell proliferation via IgE.** The CD11c-DTR mice (n=2) were treated with 100 ng diphtheria toxin i.p. day zero. Three million CD4⁺ T cells from DO11.10 mice, which express an anti-OVA T cell receptor on most of their CD4⁺ T cells, were adoptively transferred i.v. to all mice the same day. Day one, the non-transgenic littermates were given either 20 µg OVA-TNP i.v. alone (**A**, n=2) or one hour after i.v. administration of 50 µg IgE anti-TNP (**B**, n=2). Diphtheria toxin treated CD11c-DTR mice received 50 µg IgE anti-TNP and 20 µg OVA-TNP (**C**). Flow cytometry analysis of spleen cells was performed day four. Lymphocytes were gated and are shown in the diagrams. The percentages represent the fraction of CD4⁺KJ126⁺ cells (circular gate) of the total number of CD4⁺ cells. The diagrams are representations of two individual mice each.

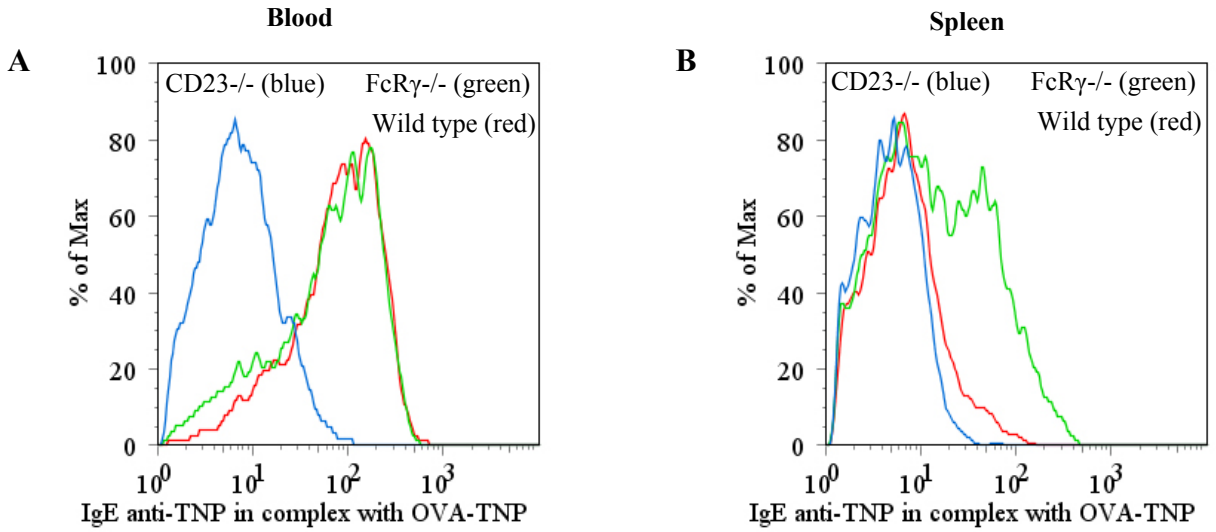


Figure 8 | **B cells of CD23-/- mouse can neither bind nor transport IgE-antigen complexes from blood to the spleen.** BALB/c mice (red, n=2), FcRγ-/- mice (green, n=2) and CD23-/- mice (blue, n=2) were immunized i.v. with 150 µg OVA-TNP-biotin and 50 µg IgE anti-TNP. Cells from the blood five minutes after injection and the spleens 30 minutes after injection were analyzed with flow cytometry, and the lymphocytes were gated. (**A**) shows the IgE-antigen complex on B cells in blood 5 minutes after injection. (**B**) denotes the IgE-antigen complex on splenic B cells 30 minutes after immunization. Each curve in (**A**) and (**B**) is a representation of two mice.

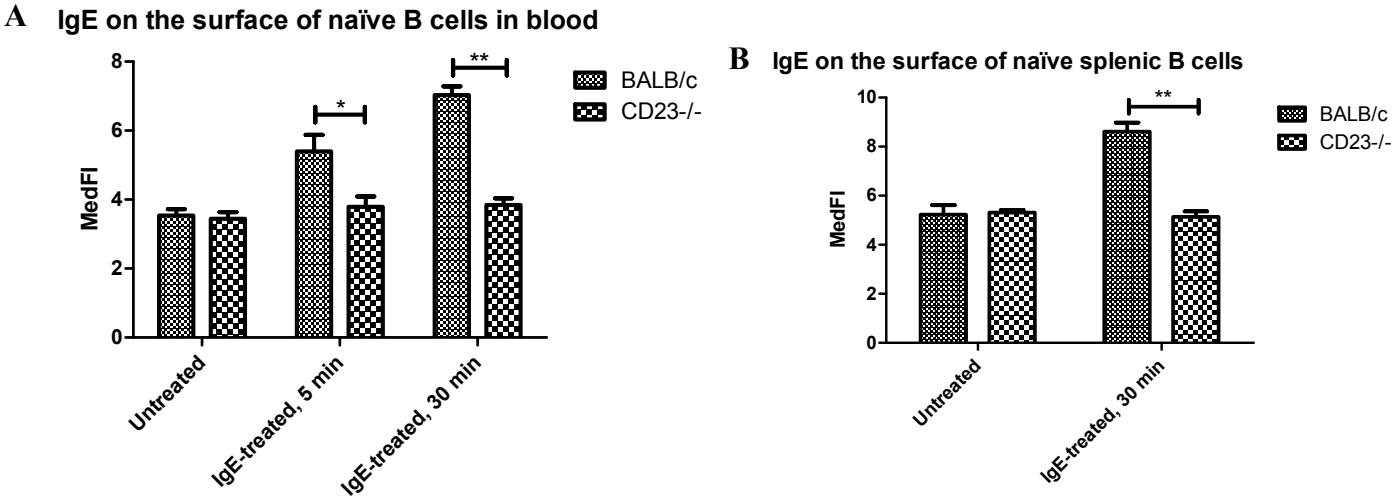


Figure 9 | **Naïve B cells do not have IgE on their CD23 receptors.** One group (n=5) of BALB/c mice and one group of CD23-/- mice were immunized i.v. with 50 µg IgE anti-TNP. Five BALB/c mice and five CD23-/- remained untreated. (**A**) Cells from blood 5 minutes and 30 minutes after injection, and (**B**) cells from the spleens 30 minutes after injection were analyzed with flow cytometry. B cells were gated. Median fluorescent intensity (MedFI) was calculated as a representation of the amount of IgE that bound to the B cells. Bars represent mean±SEM. Statistical differences: * $p<0.01$ ** $p<0.001$.