

IgE Enhances Antibody and T Cell Responses In Vivo via CD23⁺ B Cells¹

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IgE Abs, passively administered together with their specific Ag, can enhance the production of Abs recognizing this Ag by >100-fold. IgE-mediated feedback enhancement requires the low affinity receptor for IgE, CD23. One possible mechanism is that B cells take up IgE-Ag via CD23 and efficiently present Ag to Th cells, resulting in better Ab responses. To test whether IgE Abs have an effect on Th cells in vivo, mice were adoptively transferred with CD4⁺ T cells expressing a transgenic OVA-specific TCR, before immunization with IgE anti-TNP (2,4,6-trinitrophenyl) plus OVA-TNP or with OVA-TNP alone. IgE induced a 6- to 21-fold increase in the number of OVA-specific T cells. These cells acquired an activated phenotype and were visible in splenic T cell zones. The T cell response peaked 3 days after immunization and preceded the OVA-specific Ab response by a few days. Transfer of CD23⁺ B cells to CD23-deficient mice rescued their ability to respond to IgE-Ag. Interestingly, in this situation also CD23-negative B cells produce enhanced levels of OVA-specific Abs. The data are compatible with the Ag presentation model and suggest that B cells can take up Ag via "unspecific" receptors and activate naive T cells in vivo. *The Journal of Immunology*, 2005, 175: 1473–1482.

Antibodies passively administered to animals together with their specific Ag are known to feedback regulate the Ab response to this Ag (1, 2). The regulatory effects are potent, and small doses of syngeneic Ab can result in 99% suppression or a 1000-fold enhancement. The most well described negative feedback regulation is the ability of all IgG subclasses to completely suppress the response to erythrocytes (3). This effect is also used clinically to prevent rhesus D (RhD)-negative women from becoming immunized against RhD-positive fetal erythrocytes acquired via transplacental hemorrhage (reviewed in Ref. 4). Interestingly, Abs can also enhance Ab responses to erythrocytes, and Henry and Jerne (3) were the first to show that the factor in serum able to enhance SRBC responses was found in the 19S fraction (= IgM) whereas Abs in the 7S fraction (= IgG) suppressed SRBC responses. IgM-mediated enhancement is dependent on the ability of IgM to activate complement (5, 6) and on the presence of complement receptor (CR)⁴ 1 and CR2 (CR1/CR2) (7). CR2 is associated with CD19 on the B cell surface and coligation of CD19 or CR2 with the BCR lowers the threshold for B cell stimulation (8, 9). This result may be one of the explanations for why animals lacking CR1/CR2 or C2, C3, or C4 (all involved

in the generation of the C3 split products that are the ligands to CR2) have poor Ab responses to suboptimal Ag concentrations (reviewed in Ref. 10). In a primary immune response, little specific Ab is available to initiate the classical pathway activation, known to be required for normal Ab responses. A possible explanation to this puzzling observation is that natural IgM in naive animals binds to Ag, albeit with low affinity, activates complement, coligates BCR and CR2/CD19, thus initiating early specific IgM responses which in turn feedback enhance the Ab response. This idea is compatible with observations that mice lacking secretory natural IgM have impaired Ab responses (11). Although IgG suppresses the Ab response to erythrocytes, it can enhance responses to soluble proteins (12). IgG1-, IgG2a-, and IgG2b-mediated enhancement takes place in the absence of complement (7, 13) but seems to require activating FcγR (14) and probably acts by increasing Ag presentation to Th cells. In contrast, IgG3-mediated enhancement is normal in the absence of activating FcγR but reduced in the absence of C or CR1/CR2 (15), suggesting that IgG3, like IgM, operates via the C system.

Apart from IgG3, the most recently discovered feedback regulatory circuit is the one mediated by IgE via the low-affinity receptor for IgE, FcεRII/CD23 (16). The biological role of CD23 is not well understood. Unlike other Fc receptors, it does not belong to the super Ig family but is a type II integral membrane protein with a calcium-dependent lectin domain in the C-terminal end of the extracellular part (17). CD23 is expressed on the cell surface as trimers, which are preassociated in the absence of IgE (18). In its membrane-bound trimerized form, CD23 has an affinity for IgE of $1.45 \times 10^8 \text{ M}^{-1}$ (19). Surface CD23 is cleaved into soluble fragments by an autocatalytic mechanism. Owing to poor trimerization, murine soluble CD23 has an affinity for IgE only in the range from 10^5 to 10^6 M^{-1} (19). Human CD23 exists in two isoforms: CD23a is expressed constitutively on B cells, whereas CD23b requires IL-4 for its expression and is seen on a wide variety of cells such as B cells, eosinophils, and monocytes (20). Murine CD23 has a more restricted cellular expression, and the isoform has been demonstrated on B cells (21) and follicular dendritic cells (FDC) (22), whereas the existence of a murine CD23b isoform has been more controversial. However, cDNA for a new subtype of murine

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⁴ Abbreviations used in this paper: CR, complement receptor; BM, bone marrow; FDC, follicular dendritic cells; KLH, keyhole limpet hemocyanin; TNP, 2,4,6-trinitrophenyl.

CD23 has been cloned (23) and it was recently shown that a CD23b isoform is expressed on murine enterocytes (24).

The most well known *in vivo* function of CD23 is probably its ability to act as a negative regulator of IgE and, to a lesser extent, IgG1 production. This function is evidenced from several experimental systems. Transgenic mice, overexpressing CD23, have reduced production of IgE (25, 26) and, conversely, CD23-deficient mice (CD23^{-/-}) have enhanced IgE production (27). New Zealand Black mice express a variant CD23 allele that cannot form trimers and bind IgE, and these animals have an exaggerated IgE response (28). Negative regulation of IgE responses via CD23 takes place after primary immunization with Ag in alum, and in most systems affects polyclonal IgE production (25, 26, 28). Because only very low initial IgE concentrations are available to ligate CD23 in a primary immune response, it is an open question whether IgE or another CD23 ligand is involved in the pathway leading to negative regulation of IgE production.

The other *in vivo* immunoregulatory role of CD23 is to enhance Ab responses (reviewed in Ref. 2). This effect is dependent on IgE, and is seen when soluble Ag, such as BSA-TNP or OVA-TNP, are administered to mice together with monoclonal IgE anti-TNP. The carrier-specific IgG responses in such animals are frequently >100-fold higher than in mice immunized with the Ag alone, and the effect is strictly dependent on CD23 (16, 29–32). Not only IgG, but also IgM and IgE levels are enhanced (30, 31). The remarkable immunostimulatory effect of IgE takes place without adjuvants (IgE and Ags are always given in PBS) and is Ag-specific, i.e., only responses to antigenic determinants within the IgE-Ag complex are enhanced (16, 30). Immunization with IgE-Ag gives rise to an unusually early IgG response, peaking 6 days after priming (31), and IgE-Ag induces normal Ab responses in mice lacking CR1/CR2 (7). IgE-mediated enhancement is unperturbed in mice lacking IL-4, indicating that the constitutively expressed CD23a isoform is sufficient (33).

Several possible explanations for the immunostimulatory effect of IgE can be envisaged. First, IgE-Ag complexes may be captured by CD23 expressed on FDC and presented efficiently to B cells as immune complex-coated bodies, icosomes. However, this is unlikely because chimeric mice, with CD23⁺ FDC and CD23⁻ bone marrow (BM)-derived cells, are unresponsive to IgE-Ag complexes whereas mice with CD23⁻ FDC and CD23⁺ BM-derived cells do respond (32). Because FDC and B cells are the only cell types proven to express the murine CD23a isoform *in vivo* (21, 22), the lack of a role for FDC implies that the B cells are the effector cells. *In vitro*, B cells efficiently take up and present Ag complexed with IgE in a CD23-dependent manner (34–37). Therefore, a second possible mechanism underlying IgE-mediated enhancement of the Ab response *in vivo* is Ag presentation to specific Th cells by CD23⁺ B cells. Arguing against this hypothesis are reports that B cells are unable to present Ag to naive T cells (38–41). Finally, it cannot be ruled out that co-cross-linking of the BCR and CD23 by IgE-Ag complexes increases B cell activation (42) in a way similar to what is seen when the BCR and CR2/CD19 are co-cross-linked (8, 9).

The objectives of the present study were to confirm that B cells, and no other BM-derived cell type, are the effector cells in IgE-mediated enhancement of Ab responses and to test whether IgE-Ag complexes play a role in T cell activation *in vivo*. We demonstrate that IgE-OVA-TNP complexes indeed induce a marked proliferation and activation of OVA-specific CD4⁺ T cells. This response peaks at day 3, precedes the enhanced OVA-specific Ab response by a few days, and is dependent on CD23⁺ B cells. However, once CD23⁺ B cells have initiated the OVA-specific T cell response, CD23-negative OVA-specific B cells are

also activated to produce enhanced levels of Abs. This effect suggests that signaling via co-cross-linking of CD23 and BCR is not required for IgE-mediated enhancement of Ab responses. The model that is most compatible with the experimental data is that in which CD23⁺ B cells capture and endocytose the IgE-Ag complexes and present antigenic peptides to T cells. The activated and expanded T cell population then interacts with Ag-specific B cells and provides T cell help in a similar manner as is seen when the T cells are activated via Ag-presenting dendritic cells in a classical fashion.

Materials and Methods

Mice

CD23-deficient (CD23^{-/-}) mice (29), previously backcrossed to CBA/J for 12 generations (32), were further backcrossed to BALB/c. CD23 deletion was confirmed by PCR as previously described (32). Offspring from the 5th and 10th generations of backcross were intercrossed to generate homozygous CD23^{-/-} BALB/c mice (N5 and N10, respectively). CD23 deletion and MHC haplotype (A^d or A^k) was confirmed by flow cytometry. All experiments done on CD23^{-/-} BALB/c (N5) have been repeated in CD23^{-/-} BALB/c (N10) mice with similar results. DO11.10 mice, carrying a construct containing rearranged TCR α and TCR β genes encoding a TCR specific for OVA_{323–339} bound to I-A^d class II molecules (43), backcrossed to BALB/c for over 15 generations, were obtained from The Jackson Laboratory or from Dr. L. Westerberg (Karolinska Institute, Stockholm, Sweden) with the permission of Prof. K. Murphy (Washington University School of Medicine, St. Louis, MO). Fc γ R^{-/-} (44), backcrossed to BALB/c for 12 generations, were from Taconic Farms and BALB/c mice from Bomblite. C.BK^a-Igh^b/IcrSmmJ (C.B-17) is an Ig congenic strain that has the Igh^b H chain allotype on a BALB/c background and were obtained from The Jackson Laboratory. Animals were bred and maintained in the animal facilities at the Department of Genetics and Pathology, Uppsala University and at the National Veterinary Institute, Uppsala, Sweden and were matched for age and sex within each experiment. All experiments were approved by the local ethical committee.

Antigens

OVA, keyhole limpet hemocyanin (KLH), and TNP (picrylsulfonic acid/hydrate) were obtained from Sigma-Aldrich. TNP was conjugated to OVA in 0.28 M cacodylate buffer, pH 6.9, as described (45). After 45–70 min of incubation at room temperature, the reaction was stopped by an excess of glycyl-glycine (1 mg/ml; Merck). Proteins were dialyzed against PBS, sterile filtered, and stored at 4°C. The number of TNP residues per OVA was determined as described earlier (45). Three batches of OVA-TNP with a TNP to OVA ratio of 1.1, 1.3, or 3 were used with similar results.

Antibodies

Monoclonal Abs were derived from a B cell hybridoma producing murine IgE anti-TNP (IGELb4) (46). The hybridomas were cultured in DMEM with 5% FCS. IgE was purified by affinity chromatography on a Sepharose column conjugated with monoclonal rat anti-mouse κ , 187.1.10 (47). Bound Ab was eluted with 0.1 M glycine-HCl buffer, pH 2.8. Abs were dialyzed against PBS, sterile filtered and stored at –20°C. Protein concentrations were determined by absorbance at 280 nm, assuming that an absorbance of 1.5 equals 1 mg/ml Ab.

For flow cytometry we used PE-labeled anti-CD4 mAbs (KH-CD4 or RM4-5; ImmunoKontakt or BD Pharmingen), PE-labeled anti-CD23 mAb (B3B4; BD Pharmingen), FITC-labeled anti-CD45R/B220 (RA3-6B2; BD Pharmingen), FITC-labeled anti-CD19 (1D3 BD Pharmingen), FITC-labeled anti-mouse I-A^k (11-5.2; BD Pharmingen), biotinylated anti-mouse I-A^d (AMS-32.1; BD Pharmingen), biotinylated anti-IgM (R6-60.2; BD Pharmingen), and PE-labeled streptavidin (BD Pharmingen). The DO11.10 transgenic TCR was detected with a FITC-labeled KJ1-26 mAb (Caltag Laboratories) specific for this particular TCR heterodimer (48).

The cell surface phenotype of DO11.10 T cells was determined by staining with PE-labeled anti-CD4, biotinylated KJ1-26 (Caltag Laboratories), and allophycocyanin-conjugated streptavidin (BD Pharmingen). For triple staining (see Fig. 5), cells were in addition stained with FITC-labeled anti-CD11a/LFA-1 (I21/7; Caltag Laboratories), FITC-labeled anti-CD44 (IM7.8.1; Caltag Laboratories), FITC-labeled anti-CD45RB (16A; Caltag Laboratories), FITC-labeled anti-CD62 ligand (CD62L, MEL-14; Caltag Laboratories), or FITC-labeled anti-CD25 (7D4; BD Pharmingen).

Immunizations

Mice were immunized i.v. in the tail veins with 20 μ g of OVA-TNP in 0.1 ml of PBS alone or 1 h after immunization with 50 μ g of IgELb4 in 0.1 ml of PBS. In experiments in which Ab responses were measured, 10 μ g of KLH was added to the Ag mixture as a specificity control.

Flow cytometry

Spleens were removed at the indicated time points and kept in PBS on ice. Single cell suspensions were prepared by pressing cells through a mesh screen and RBCs removed by hypotonic lysis incubating spleen cells in 5 ml of ACK lysing buffer (0.15 M NH_4Cl (Merck), 1.0 mM KHCO_3 (Sigma-Aldrich), 0.1 mM Na_2EDTA (Sigma-Aldrich), pH 7.3) for 5 min at room temperature. Cells were washed once in PBS, resuspended in 5 ml of PBS and counted. Fluorescence staining was performed at 4°C in 100 μ l of PBS containing 5×10^5 cells and predetermined optimal amounts of PE-labeled anti-CD4 and FITC-labeled KJ1-26. After 30 min, the cells were washed twice in PBS containing 1% BSA (Sigma-Aldrich) and 0.1% NaN_3 (Sigma-Aldrich). Some 40,000 events, acquired by gating on all cells with the forward and side scatter properties of lymphocytes, were collected on a FACSort flow cytometer (BD Biosciences). Triple staining was performed in 200 μ l of PBS containing 4×10^6 cells and predetermined optimal amounts of Abs. From each stained aliquot of cells, 2000 CD4^+ KJ1-26 $^+$ events were collected on a FACSVantage SE flow sorter with DiVa upgrade (BD Biosciences). Data were analyzed using CellQuest version 3.3 software (BD Biosciences). DO11.10 T cells were identified as CD4^+ KJ1-26 $^+$ events, and absolute numbers of these cells calculated by multiplying the percentage of CD4^+ KJ1-26 $^+$ events with the total number of viable spleen cells.

Adoptive transfers

The adoptive transfer system developed in Dr. M. Jenkins group was used (49). Single cell suspensions from DO11.10 spleens were depleted of RBC by hypotonic lysis (see earlier description). Unless otherwise stated, cell suspensions containing $2-3 \times 10^6$ CD4^+ KJ1-26 $^+$ cells, determined by flow cytometry, were adoptively transferred i.v. to unirradiated mice. In experiments with $\text{CD23}^{-/-}$ or $\text{Fc}\gamma\text{R}^{-/-}$ recipients, DO11.10 CD4^+ cells were isolated by labeling spleen cells with anti-CD4-conjugated magnetic beads (MACS anti-mouse CD4; Miltenyi Biotec), collecting labeled cells using LS columns (Miltenyi Biotec) according to the manufacturer's instructions. In some experiments, CD4^+ cells were prepared using Dynabeads Mouse CD4 (L3T4; Dynal Biotech) and DETACHaBEAD Mouse CD4 (Dynal Biotech) according to manufacturer's recommendations. The isolated cell populations were $>96\%$ CD4^+ .

Spleen cell suspensions were depleted of CD19^+ cells with anti-CD19-conjugated magnetic beads (MACS anti-mouse CD19; Miltenyi Biotec) and LD columns (Miltenyi Biotec) according to manufacturer's instructions, resulting in cell populations with $<0.1\%$ CD19^+ cells. In some experiments, B cells were incubated with anti-CD43-conjugated magnetic beads (MACS anti-mouse CD43; Miltenyi Biotec) followed by passage over LS columns (Miltenyi Biotec) with reduced flow rate according to manufacturer's instructions. The CD43^- cell populations were $>97\%$ $\text{CD19}^+\text{B220}^+\text{IgM}^+$.

In some cases, MACS-purified CD4^+ DO11.10 spleen cells were resuspended in PBS containing 0.1% BSA (Sigma-Aldrich) to a final concentration of 5×10^7 cells/ml and incubated with 5 μ M CFSE (Molecular Probes) for 10 min at 37°C. Labeling was stopped by adding 5 volumes of cold PBS containing 10% FCS and incubating 5 min on ice. The labeled cells were washed three times in PBS and $8-9 \times 10^6$ CFSE^+ CD4^+ DO11.10 cells were transferred to each recipient.

Unless otherwise indicated, recipients were immunized the day after the adoptive cell transfer.

ELISA analysis

Mice were bled from the tails and sera were tested in ELISA for OVA- or KLH-specific IgG. Microtiter plates (Immunolon 2 HB; Dynex Technologies) were coated overnight at 4°C with 100 μ l of OVA (50 μ g/ml) or KLH (10 μ g/ml) in PBS with 0.05% NaN_3 . Serial dilutions (50 μ l) of serum samples in PBS-0.05% Tween 20 (Bio-Rad) was added to plates and incubated overnight at 4°C. After washing, 50 μ l of alkaline phosphatase-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories), diluted 1/1000 in PBS-0.05% Tween 20, was added and plates incubated for 1 h at room temperature. After washing, 100 μ l of substrate (*p*-nitro-phenylphosphate; Sigma-Aldrich) diluted in diethanolamine buffer was added and the absorbance at 405 nm was measured after 30 min incubation at room temperature. For OVA-specific ELISA, a polyclonal OVA-specific standard serum with a starting concentration of 3.47 μ g/ml

was used. Absorbance values at 405 nm were used to compare KLH-specific responses. Allotype-specific ELISA were performed as described earlier, but IgG ^{a} was detected with a mixture of biotin-conjugated anti-IgG1 ^{a} (clone 10.9) and anti-IgG2a ^{a} (clone 8.3) (final dilution 1/3000 of both mAbs) and IgG ^{b} with a mixture of biotin-conjugated anti-IgG1 ^{b} (clone B68.2) and anti-IgG2a ^{b} (clone 5.7) (final dilution 1/3000 of both mAbs). All allotype-specific mAbs were from BD Pharmingen. Plates were incubated overnight at 4°C. After washing, alkaline phosphatase-conjugated avidin (Sigma-Aldrich) was added. Construction of standard curves and calculations were made using SOFTmax (Molecular Devices).

Immunohistochemical staining

Spleens were flash frozen in OCT embedding media (Miles) and 7- to 10- μ m sections were cut using a cryostat, air dried overnight, and fixed onto Super frost plus glass slides (Menzel-Gläser) in ice cold acetone for 5 min. Sections were blocked with 0.1% BSA in PBS for 20 min and incubated at room temperature for 1 h with 0.5 μ g/ml biotinylated KJ1-26 mAb (Caltag Laboratories). Bound Ab was detected with preformed avidin-biotin-coupled HRP (Vector elite ABC; Vector Laboratories) and SG substrate kit (Vector Laboratories). The reaction was followed in a microscope until desired staining was obtained, and the staining procedure was repeated using 1.25 μ g/ml biotinylated anti-IgM mAb (R6-60.2; BD Pharmingen) and diaminobenzidine substrate kit for peroxidase (Vector Laboratories).

Statistical analysis

Statistical differences between the control and the experimental groups were determined by Student's *t* test. Values for $p > 0.05$ (not significant), $p < 0.05$, $p < 0.01$, or $p < 0.001$ are represented.

Results

IgE enhances Ag-specific Ab and T cell responses via CD23 in vivo

To investigate whether IgE-Ag complexes could activate Ag-specific CD4^+ T cells in vivo, BALB/c mice were adoptively transferred with transgenic OVA-specific DO11.10 CD4^+ cells and immunized with OVA-TNP alone, IgE anti-TNP and OVA-TNP, IgE alone, or left unimmunized. The number of OVA-specific T cells in the spleens was determined as CD4^+ KJ1-26 $^+$ cells 3 days later (Fig. 1a). Unimmunized BALB/c mice had a small but detectable OVA-specific T cell population (0.33%), mice immunized with OVA-TNP alone had a slight increase in population size (0.46%), whereas mice immunized with IgE-OVA-TNP had 3.2% OVA-specific T cells (Fig. 1a). Controls, immunized with IgE anti-TNP alone had 0.21% (Fig. 1a) and BALB/c mice that had not been transferred with DO11.10 cells $<0.03\%$ OVA-specific T cells (data not shown). Based on nine independent experiments, the number of specific T cells in IgE-Ag-immunized groups were 6- to 21-fold higher than in Ag-immunized groups. When followed over time, OVA-specific T cell numbers were significantly enhanced 2 days after immunization and peaked at day 3 (Fig. 1b, left axis). As expected, IgE also induced an enhanced Ab response: 14 days after immunization, the IgG anti-OVA titers were 75-fold higher in groups given IgE-Ag than in groups given Ag alone (Fig. 1b, right axis). Thus, the enhanced Ab response observed after immunization with IgE-Ag complexes is preceded by an expansion of Ag-specific CD4^+ T cells.

To investigate whether CD23 was required for IgE-mediated T cell activation, $\text{CD23}^{-/-}$ and BALB/c mice were adoptively transferred with CD4^+ DO11.10 cells and immunized as described. Although IgE-OVA-TNP induced a 6.3-fold expansion of OVA-specific T cells and an enhanced OVA-specific IgG response in BALB/c mice (Fig. 2a), no effect was seen in $\text{CD23}^{-/-}$ mice (Fig. 2b). T cell expansion in $\text{CD23}^{-/-}$ mice was absent also on days 2, 4, and 7 (data not shown). The lack of a T cell response was not due to an intrinsic defect in the ability of $\text{CD23}^{-/-}$ mice to present Ag, as DO11.10 T cells, transferred to $\text{CD23}^{-/-}$ recipients, proliferated well after immunization with OVA in CFA (Fig. 2c).

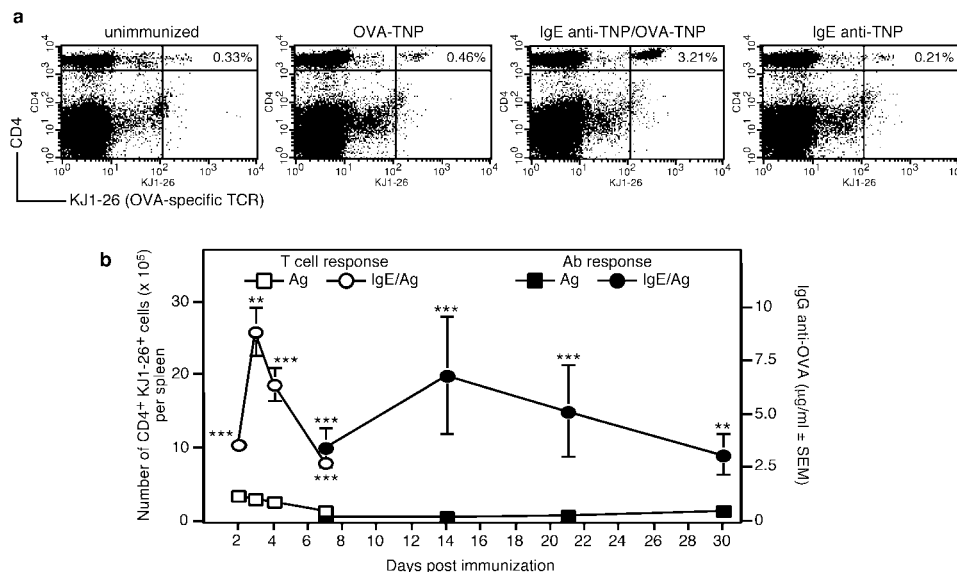


FIGURE 1. IgE induces expansion of the Ag-specific CD4⁺ T cell population as well as the enhancement of Ab responses. *a*, BALB/c mice were adoptively transferred with total spleen cells containing 3×10^6 CD4⁺ KJ1-26⁺ T cells from DO11.10 mice and immunized (five per group) with 20 μg of OVA-TNP alone, 50 μg of IgE anti-TNP 1 h before 20 μg of OVA-TNP, 50 μg of IgE anti-TNP alone, or left unimmunized. The percentage of CD4⁺ KJ1-26⁺ DO11.10 cells per spleen was determined by flow cytometry 3 days after immunization. A representative of nine independent experiments is shown. *b*, BALB/c mice (25 per group) were adoptively transferred and immunized as in *a* with the addition of 10 μg of KLH along with OVA-TNP as a specificity control. On the indicated days, five mice per group were sacrificed and the number of CD4⁺ KJ1-26⁺ cells per spleen was determined by flow cytometry. The remaining five mice per group were saved and tested for serum levels of IgG anti-OVA. Immunization with 50 μg of IgE anti-TNP alone did not result in a T cell response nor an IgG anti-OVA response (data not shown). Statistical differences (*) are shown between mice given Ag alone and mice given IgE-Ag complexes. There was no enhancement of the IgG anti-KLH responses in the IgE-Ag-immunized mice (data not shown).

To visualize the T cell expansion, spleens from BALB/c mice, transferred with CD4⁺ DO11.10 cells and immunized with OVA-TNP or IgE-OVA-TNP, were sectioned and stained for B cells and OVA-specific T cells. The number of specific T cells in the T cell zone remained at a constant low level in mice given Ag alone (Fig. 3, *a-c*) whereas the numbers in BALB/c mice given IgE-Ag, were markedly increased after 72 h (Fig. 3*f*). T cell numbers in CD23^{-/-} recipients of DO11.10 T cells immunized with IgE-Ag did not increase over time (Fig. 3 *g-i*). Thus, IgE-mediated T cell expansion, as well as Ab responses, requires the presence of CD23⁺ cells.

To exclude the possibility that FcεRI played a role in IgE-mediated T cell activation in vivo, FcεRγ^{-/-} (lacking expression of

FcεRI as well as FcγRI and FcγRIII) (44) and BALB/c mice were adoptively transferred with CD4⁺ DO11.10 cells. Immunization with IgE-OVA-TNP complexes enhanced T cell proliferation in both strains to the same degree (6.7- and 6.1-fold, respectively) (data not shown), showing that FcεRI was not required.

Characterization of the T cells induced by IgE-Ag complexes

To verify that the expansion of the OVA-specific T cells was due to proliferation and not merely to increased survival of OVA-specific T cells, we stained CD4⁺ DO11.10 cells with CFSE before adoptive transfer. CFSE is an intracellular fluorescent label, which is divided equally between daughter cells upon cell division (50). Three days after immunization with IgE-OVA-TNP, >95% of the

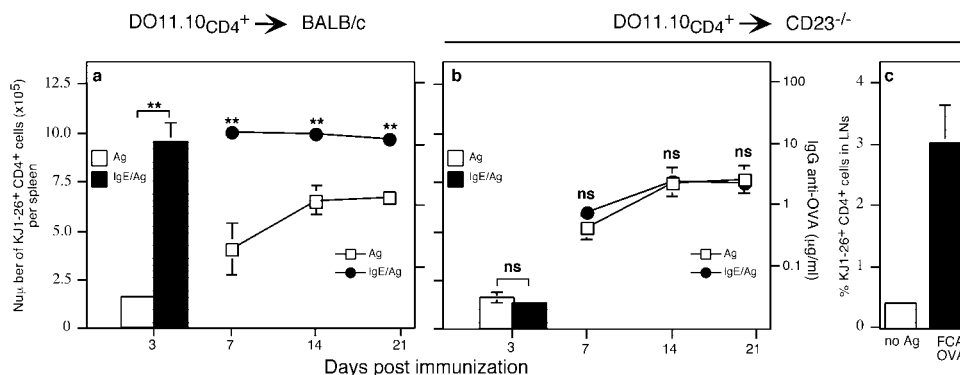
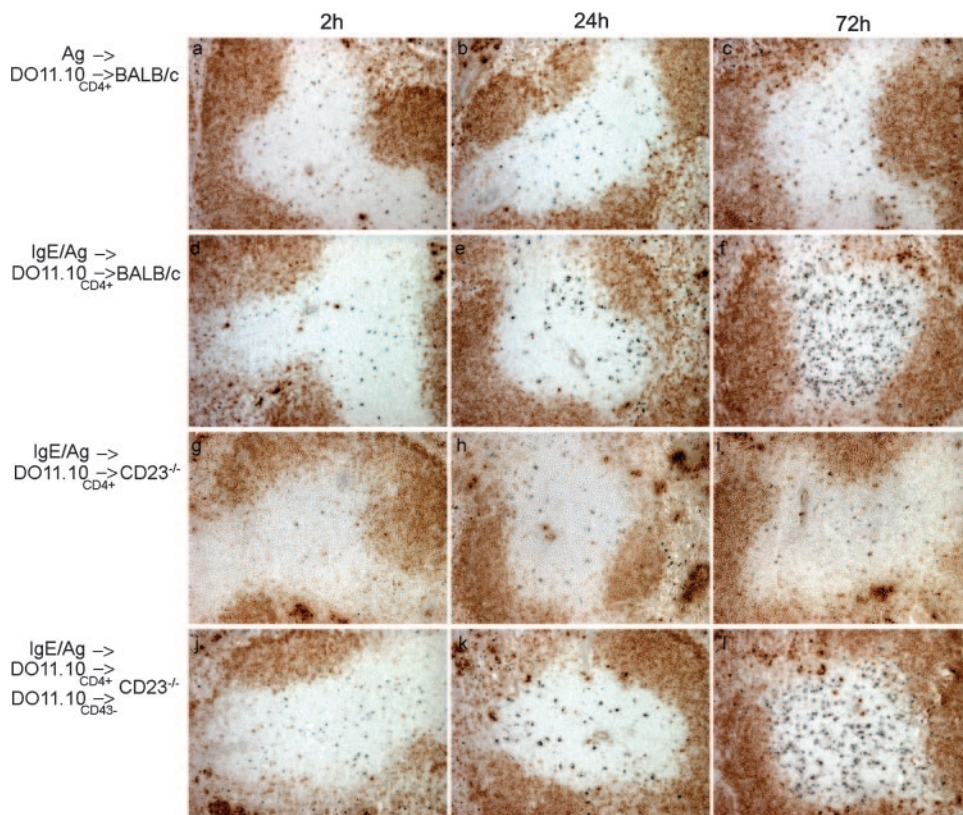


FIGURE 2. IgE-mediated enhancement of Ab and T cell responses is CD23-dependent. A total of 1.6×10^6 purified CD4⁺ KJ1-26⁺ DO11.10 cells were adoptively transferred to BALB/c (*a*) or CD23^{-/-} (*b* and *c*) mice. *a* and *b*, Mice were immunized with 20 μg of OVA-TNP or 50 μg of IgE anti-TNP 1 h before 20 μg of OVA-TNP. Four mice per group were sacrificed after 3 days, and the number of CD4⁺ KJ1-26⁺ cells per spleen was determined. Remaining mice (four per group) were bled at the indicated time points and serum concentrations of IgG anti-OVA were determined. Statistical differences (*) are shown between mice given Ag alone and mice given IgE-Ag complexes. A representative of two experiments is shown. *c*, CD23^{-/-} recipients were immunized s.c. with 20 μg of OVA-TNP in CFA or left unimmunized. Five days later, draining lymph nodes were isolated and the percentage of CD4⁺ KJ1-26⁺ cells determined. A representative of three experiments is shown.

FIGURE 3. Visualization of OVA-specific T cell expansion. *a–f*, A total of 4.2×10^6 purified $CD4^+$ KJ1-26 $^+$ DO11.10 T cells were adoptively transferred to BALB/c mice. *g–i*, A total of 3.8×10^6 purified $CD4^+$ KJ1-26 $^+$ DO11.10 T cells were transferred to $CD23^{-/-}$ mice. *j–l*, A total of 4.2×10^6 purified $CD4^+$ KJ1-26 $^+$ DO11.10 T cells and 6.4×10^6 $CD43^-$ B cells from DO11.10 mice were transferred to $CD23^{-/-}$ mice. *a–l*, Two mice per group were immunized with 20 μ g of OVA-TNP (Ag) or IgE anti-TNP 1 h before 20 μ g of OVA-TNP (IgE/Ag). Spleens were harvested 2, 24, or 72 h after immunization. Results shown are representative of four to seven mice per group obtained from at least two different experiments. All sections were stained with KJ1-26 mAb (blue or black) and anti-IgM mAb (brown).



$CD4^+$ DO11.10 cells had gone through several rounds of division whereas $CD4^+$ DO11.10 cells from unimmunized mice or from mice given OVA-TNP alone did not divide to a significant extent (Fig. 4).

To follow the activation profile of $CD4^+$ DO11.10 cells, BALB/c mice were immunized with OVA-TNP or IgE-OVA-TNP and their spleen cells stained with a panel of markers for T cell activation (LFA-1/CD11a, CD44, CD45RB, CD62L, and IL-2R/CD25). The forward light scatter was used as an indication of morphologic blast formation. By gating separately on $CD4^+$ KJ1-26 $^+$ cells and $CD4^+$ KJ1-26 $^-$ cells, Ag-specific and bystander T cells could be compared. Again, we confirmed that OVA-specific T cells in mice given IgE-Ag expanded and peaked on day 3 (Fig. 5*b*). DO11.10 T cells in unimmunized mice had a naive phenotype (51): small, LFA-1 ^{low} , CD44 ^{low} , CD45RB ^{high} , CD62L ^{high} (Fig. 5, *c–h*, first column). OVA-TNP alone had minor effects on the ex-

pression of these markers (Fig. 5, *c–h*, second column). However, after immunization with IgE-OVA-TNP, the majority of $CD4^+$ KJ1-26 $^+$ cells (Fig. 5, thick histogram), but not $CD4^+$ KJ1-26 $^-$ cells (Fig. 5, thin histogram), acquired an “activated” phenotype (51), (Fig. 5, *c–h*, third and fourth columns): 2 days postimmunization, the majority of the OVA-specific cells were blasts (Fig. 5*c*), LFA-1 (Fig. 5*d*), and CD44 (Fig. 5*e*) were up-regulated and remained high, whereas CD45RB (Fig. 5*f*) was down-regulated. The expression pattern of CD62L (Fig. 5*g*) was two-phased, as observed previously (52). Also in line with these studies (52), the IL-2R (CD25) (Fig. 5*h*) was up-regulated only 1 day postimmunization and had returned to baseline on day 2. Taken together, the results show that the accumulation of OVA-specific $CD4^+$ cells in mouse spleens after immunization with IgE-OVA-TNP is due to proliferation and activation of the transferred T cells.

CD23 $^+$ B cells are the effector cells in IgE-mediated enhancement of Ab and T cell responses

As mentioned, IgE-mediated enhancement of Ab responses requires a $CD23^+$ BM-derived cell (32). This makes the B cell a likely candidate, but because the concept of B cells presenting Ag to naive T cells is controversial (38–41), we established directly that the B cell was the effector cell. The experimental principle was to use $CD23^{-/-}$ mice as recipients of cell populations from $CD23^+$ mice, which contained or did not contain B cells, and to analyze which cells were able to rescue the ability of $CD23^{-/-}$ mice to respond to immunization with IgE-OVA-TNP. When T cell responses were assayed, $CD23^{-/-}$ mice were also transferred with pure $CD4^+$ DO11.10 T cells. As shown, Ab responses were restored by BALB/c total spleen cells (Fig. 6*a*), but not by spleen cells depleted of $CD19^+$ cells (Fig. 6*b*). Similarly, expansion of T cells was restored by total DO11.10 spleen cells but not by $CD19$ -depleted DO11.10 cells (Fig. 6*c*). CD43 is a surface marker that is expressed on most leukocytes except for immature and mature

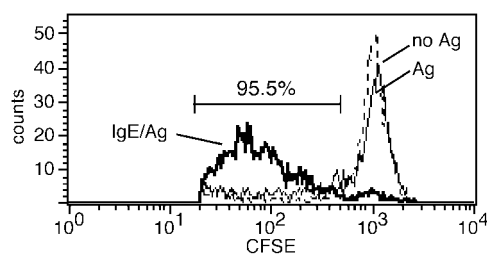


FIGURE 4. IgE induces proliferation of Ag-specific T cells. CFSE-labeled purified $CD4^+$ KJ1-26 $^+$ DO11.10 cells were adoptively transferred to BALB/c mice. Four mice per group were immunized with 20 μ g of OVA-TNP (thin histogram), 50 μ g of IgE anti-TNP 1 h before 20 μ g of OVA-TNP (thick histogram), or left unimmunized (dotted histogram). Mice were sacrificed 3 days after immunization and the CFSE intensity of 3000 $CD4^+$ CFSE $^+$ cells was determined by flow cytometry. A representative of two experiments is shown.

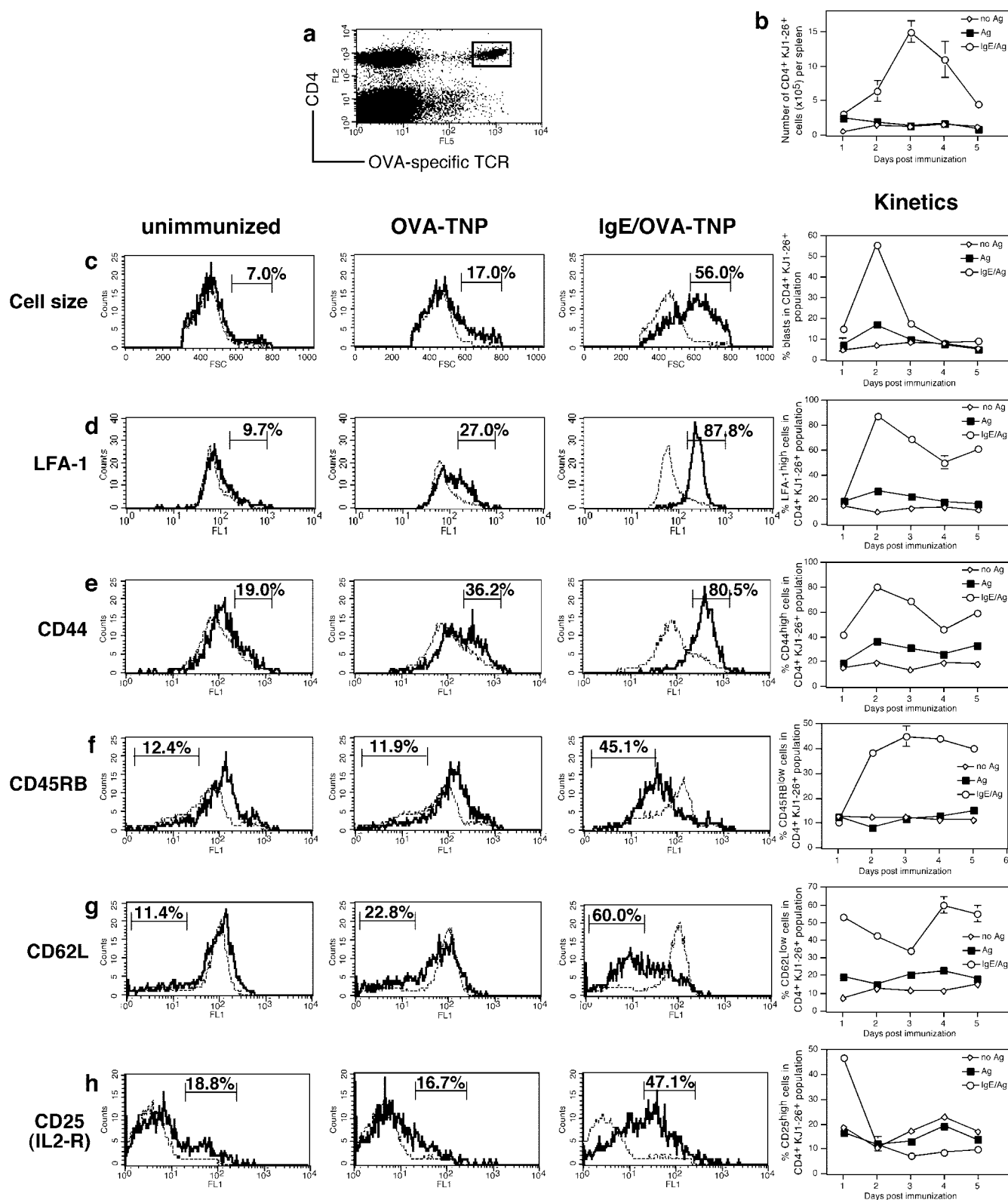


FIGURE 5. Ag-specific T cells exposed to IgE-Ag complexes in vivo show signs of activation. BALB/c mice were adoptively transferred with total spleen cells containing 2×10^6 CD4⁺ KJ1-26⁺ T cells from DO11.10 mice. Recipients were immunized with 20 μ g of OVA-TNP alone, 50 μ g of IgE anti-TNP 1 h before 20 μ g of OVA-TNP, or left unimmunized. Three mice per group were sacrificed and tested during the first 5 days after immunization. *a*, Gated CD4⁺ KJ1-26⁺ population. *b*, Kinetics of CD4⁺ KJ1-26⁺ cell expansion. *c*–*h*, Kinetics of changes in cell size and expression of surface markers of CD4⁺ KJ1-26⁺ cells is plotted in the fourth column. Histograms are shown only from the day of the peak of the expression of the individual surface markers on CD4⁺ KJ1-26⁺ cells (thick histogram). For comparison, expression levels in bystander CD4⁺ KJ1-26[−] cells are also shown (thin histogram). A representative of two experiments is shown.

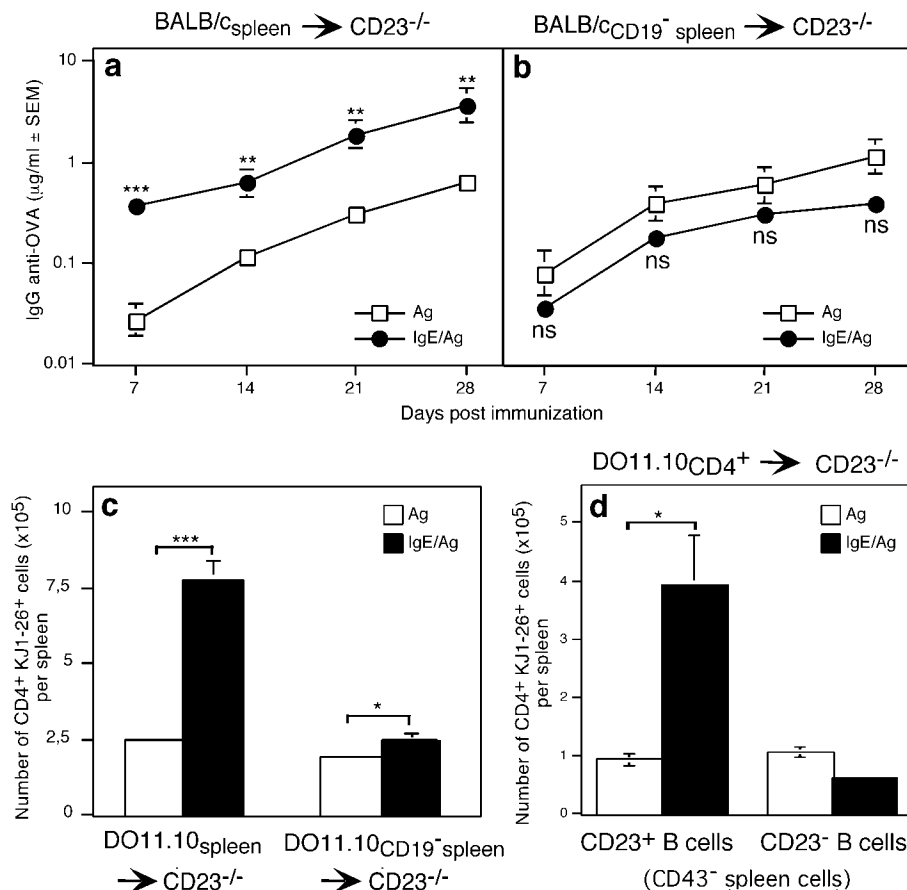


FIGURE 6. IgE-mediated enhancement of Ab and T cell responses is dependent on B cells. *a* and *b*, BALB/c spleen cells were split in two fractions, one of which was depleted of CD19-expressing cells. CD23^{-/-} mice received either 53×10^6 unfractionated spleen cells (*a*) (containing 46% CD19⁺CD23⁺ cells) or the same volume of spleen cells (*b*) depleted of CD19⁺ cells (containing <0.1% CD19⁺CD23⁺ cells). Five mice per group were immunized with 20 μg of OVA-TNP or 50 μg of IgE anti-TNP 1 h before 20 μg of OVA-TNP, and serum concentrations of IgG anti-OVA were determined. Statistical differences (*) are shown between mice given Ag alone and mice given IgE-Ag complexes. A representative of two experiments is shown. *c*, CD23^{-/-} mice (five per group) received CD19-depleted or total DO11.10 spleen cells. Cell suspensions were adjusted so that all mice received 4×10^6 CD4⁺ KJ1-26⁺ cells (undepleted cell populations contained 11×10^6 CD19⁺CD23⁺ cells per mouse). Recipients were immunized as described, and 3 days after immunization, the number of CD4⁺ KJ1-26⁺ cells per spleen was determined. A representative of three experiments is shown. *d*, CD23^{-/-} mice (five per group) received 3×10^6 purified CD4⁺ KJ1-26⁺ cells and 17×10^6 CD43^{-/-} cells from either BALB/c (CD23⁺ B cells) or CD23^{-/-} (CD23⁻ B cells) mice. Eight hours later, the recipients were immunized as described and 3 days after immunization, the number of CD4⁺ KJ1-26⁺ cells per spleen was determined. A representative of two experiments is shown.

naïve B cells and therefore provides a means to obtain negatively selected, untouched B cells for transfer. By depletion of CD43⁺ cells from BALB/c and CD23^{-/-} spleen cell suspensions, we obtained >97% pure B cells (CD19⁺B220⁺IgM⁺). Transfer of such cells, obtained from BALB/c mice, restored the ability of DO11.10 T cells in CD23^{-/-} mice to respond to IgE-OVA-TNP, both when T cells were measured in flow cytometry (Fig. 6d) and when they were visualized in the T cell zones of splenic tissue sections (Fig. 3l). As expected, CD43-depleted cell populations from CD23^{-/-} mice did not reconstitute T cell expansion in CD23^{-/-} recipients (Fig. 6d). Thus, both enhanced Ab and T cell responses are dependent on CD23⁺ B cells.

Transfer of CD23⁺ B cells to CD23^{-/-} mice rescues the ability of CD23^{-/-} B cells to produce Abs after immunization with IgE-OVA-TNP

An interesting question is whether the B cells actually producing the specific Abs after immunization with IgE-Ag have to express CD23 themselves, or whether it is sufficient that CD23⁺ B cells are present in the initial phase of the stimulatory circuit. If the Ab-producing B cell must express CD23, it would indicate that co-

cross-linking of the BCR and CD23 is important for IgE-mediated enhancement of Ab responses. If, on the contrary, B cells were only required to express CD23 at an earlier step in the chain of events leading to Ab production, it would suggest an indirect effect, such as Ag presentation.

To investigate this question, an Ig allotype chimeric system was used. CD23^{-/-} mice on BALB/c background (Ig^a allotype) were reconstituted with spleen cells from CD23-sufficient Ig congenic C.B-17 mice (Ig^b allotype) and immunized. This makes it possible to distinguish between IgG produced by CD23⁺ B cells (Ig^b) and CD23⁻ B cells (Ig^a). As expected from previous experiments (Fig. 6a), CD23⁺ spleen cells rescued the ability of CD23^{-/-} mice to produce enhanced IgG anti-OVA levels when all Ig allotypes were measured (using a conventional anti-mouse IgG Fc antiserum) (Fig. 7a). More interestingly, in the presence of CD23⁺ spleen cells from C.B-17 donors, CD23⁻ B cells in the CD23^{-/-} recipients produced enhanced levels of OVA-specific IgG1^b/IgG2a^a (Fig. 7c). In the absence of CD23⁺ B cells, no response was seen in CD23^{-/-} mice, when neither all allotypes (Fig. 7b) nor the Ig^a allotype alone (Fig. 7d) was measured. We detected little or no production of OVA-specific IgG1^b/IgG2a^b (Fig. 7e). This probably

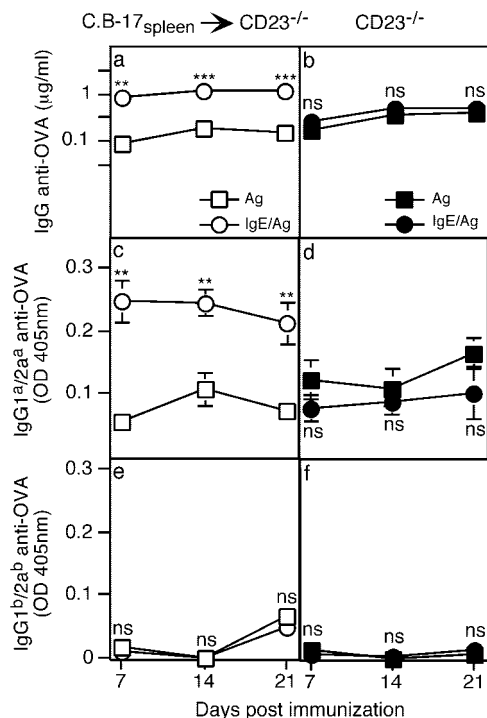


FIGURE 7. Transfer of CD23⁺ cells to CD23^{-/-} mice leads to Ab production by CD23⁻ B cells after immunization with IgE-OVA-TNP. CD23^{-/-} mice were adoptively transferred with 30×10^6 spleen cells (containing 35% CD19⁺CD23⁺ cells) from C.B-17 mice. As controls, untransferred CD23^{-/-} mice were used. Eight hours later, five mice per group were immunized with 20 μ g of OVA-TNP, 50 μ g of IgE anti-TNP 1 h before 20 μ g of OVA-TNP or with 50 μ g of IgE anti-TNP alone. The mice were bled at the indicated time points and the serum concentration of total IgG anti-OVA was determined (a and b). Serum concentration of IgG1 and IgG2 of allotype a (c and d) or allotype b (e and f) were determined to distinguish between Ig production by CD23⁻ B cells and CD23⁺ B cells. Statistical differences (*) are shown between mice given Ag alone and mice given IgE-Ag complexes. IgE anti-OVA alone did not induce a detectable anti-OVA response (data not shown). A representative of two experiments is shown.

reflects the fact that only around 10×10^6 CD23⁺ B cells (Ig^b) were transferred, thus making them a minor Ab-producing population in the recipients. Lack of OVA-specific IgG1^b/IgG2a^b rules out the possibility that cross-reactivity between anti-Ig^b and anti-Ig^a allotype-specific sera explain the enhanced Ig^a response. In addition, no cross-reactivity was observed when the anti-IgG1^a/IgG2^a and anti-IgG1^b/IgG2^b allotype-specific sera were tested in ELISA on hyperimmune OVA-specific serum from BALB/c and C.B-17 mice (data not shown). In conclusion, CD23 does not have to be expressed on the Ab-producing cells per se, but must be present on other B cells in the animals in order for IgE-mediated enhancement of Ab responses to occur.

Discussion

We provide data strongly suggesting that the scenario behind the ability of IgE to dramatically enhance specific Ab responses in vivo is that CD23⁺ B cells capture, endocytose and present IgE-Ag to specific T cells which then proliferate and subsequently interact in a cognate fashion with Ag-specific B cells. The explanation for the enhanced Ab production would be a much higher likelihood of interactions between the expanded specific Th cell population and Ag-specific B cells, analogous to what takes place when T cells are activated via Ag-presenting DC. This scenario

implies that B cells can present Ag acquired via an "unspecific" Fc receptor instead of via the BCR. However, the idea that B cells can prime naive T cells is controversial and a number of studies claim that B cells do not prime naive T cells (38–41), but can only activate Ag-experienced T cells (38, 40). Despite this claim, we find that the most likely explanation for these existing experimental data is that B cells act as APCs when they encounter IgE-Ag complexes.

That the effector cell is a CD23⁺ B cell was established in two ways. First, the capacity of CD23⁺ spleen cells to restore Ab production and T cell expansion in CD23^{-/-} mice was abrogated when the spleen cells were depleted of CD19⁺ cells. Second, CD23⁺ spleen cells devoid of all CD43⁺ cells, thus consisting of very pure, negatively selected CD43⁻ B cells, restored T cell expansion in CD23^{-/-} mice. The identification of CD23⁺ B cells as effector cells, supports our earlier observation that IgE-mediated enhancement of Ab responses is dependent on a BM-derived cell type and not on FDC (32). Previous workers have unequivocally identified EBV-transformed human B cells as being responsible for presenting IgE-Ag via CD23 to T cells in vitro (35–37, 53, 54). Moreover, only the human CD23a isoform, expressed exclusively on B cells, facilitates IgE-mediated endocytosis (55) and murine CD23a is implied in IgE-mediated enhancement in vivo (33). Given that these various observations all point to the B cell, and that no other murine cell type than B cells and FDC have been shown to express CD23a, we find the possibility of significant involvement of other APCs highly unlikely. It should also be remembered that although the prevailing view in current literature is that B cells cannot present Ag to naive T cells and activate them, other investigators have shown that at least Ag-specific B cells can indeed prime naive T cells in vitro and in vivo (56–59).

The conclusion that the effector mechanism in our system is indeed efficient presentation of Ag to specific T cells is indirect because we have not observed peptides in the peptide-binding groove of the MHC class II molecules. Nevertheless, the experimental findings we report, as well as reports by others, are compatible with this model. The expanding OVA-specific T cells are induced by IgE-Ag to change from a naive phenotype, seen before immunization, into an activated phenotype, seen during the first days after immunization. We cannot definitely state that the DO11.10 T cells are in all aspects naive, but at least judging from the chosen activation markers they seem to be (Fig. 5). The T cell numbers peak 3 days after immunization, thus preceding the Ab response (which notably is markedly enhanced already on day 7) by a few days. In the allotype chimeric model, CD23⁺ cells (Ig^b allotype) transferred to CD23^{-/-} mice, rescue the ability of endogenous CD23⁻ B cells (Ig^a allotype) to produce enhanced levels of IgG anti-OVA after immunization with IgE-Ag. This finding is precisely what would be expected if the CD23⁺ B cells function as APCs, activating specific T cells, which then interact with OVA-specific B cells in a cognate fashion. In contrast, it would not be expected if B cell signaling by co-cross-linking of CD23 and BCR was the mechanism behind the enhanced Ab production. In addition, as mentioned, the ability of IgE-Ag to induce, via CD23⁺ B cells, T cell proliferation in vitro is well documented (34–37, 53, 54) and it has also been shown that IgE-Ag complexes are in fact endocytosed by human CD23⁺ B cells (20, 60).

The possibility that unspecific B cells can present Ag has been considered dangerous because it has been assumed to lead to activation of these B cells to production of Abs of unwanted, maybe even harmful, specificities. Notably however, the unspecific CD23⁺ B cells do not produce Abs after immunization with IgE-Ag, evidenced by the strict Ag-specificity of the enhanced response (16, 30). More than 90% of $\mu^+\delta^+$ B cells express CD23

(61), EBV transformed B cells present Ag for which they are not specific (35–37, 53, 54), and all splenic CD23⁺ B cells bind IgE-Ag complexes ex vivo (62). Therefore, it is likely that all CD23⁺ B cells, regardless of specificity, bind and endocytose IgE-Ag also in vivo. The fact that these B cells are not stimulated to Ab production demonstrates that cognate interaction between a specific B cell, which itself has recognized the Ag, and a specific T cell, is a crucial check point in the immune response, which cannot be overruled even in situations in which unspecific B cells act as APCs.

The biologic role of IgE-mediated enhancement is not clear. The 6- to 21-fold expansion of the T cell population observed after immunization with IgE and 20 μ g of OVA-TNP in PBS is similar in magnitude to what was observed in the DO11.10 adoptive transfer system when 2 mg of OVA, a 100-fold higher dose, was administered s.c. together with LPS (63). This implies that capture of Ag by IgE is a very efficient way of inducing T cell responses, and that it may be important in situations in which Ag is limiting. Although serum levels of IgE are normally very low, it is possible that local concentrations are more substantial. Because IgE binds CD23 even in the absence of Ag (64), B cells in such areas may be preloaded with IgE, ready to capture the Ag. Interestingly, several members of the C-type lectin family, to which CD23 belongs, have been described as pattern recognition receptors binding carbohydrates expressed on various pathogens (reviewed in Ref. 65). Although no such ligand has yet been described for CD23, it is an interesting possibility that CD23 may directly bind to certain pathogens, endocytose and present them to T cells, thus constituting a novel branch of innate immunity.

A situation in which high levels of IgE are present is in atopic disease, and it has been hypothesized that allergen-specific IgE facilitates presentation to T cells of the minute amounts of allergen entering the body (66). Because IgE-Ag complexes can enhance production also of IgE, at least in the murine system (30), this may create a vicious circle. In an autologous in vitro system, in which B and T cells were derived from a patient with atopic dermatitis, patient serum containing IgE anti-Der p II (major house dust mite) facilitated Ag presentation by EBV-immortalized B cells to Th cells (36). Allergen-specific IgG, induced by specific immunotherapy, was shown to compete with allergen-specific IgE from patients undergoing treatment, and lead to reduced IgE/CD23-facilitated allergen presentation (54, 67, 68). This indicates that IgE-mediated allergen presentation may be of clinical relevance.

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Disclosures

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