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Immunology

The absence of IgE antibody-mediated augmentation of immune responses in CD23-deficient mice

(low-affinity IgE receptor/gene targeting)

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ABSTRACT **The CD23 antigen, a low-affinity receptor for IgE (FcεRII), is a type II membrane-bound glycoprotein expressed on various cells, particularly mature B cells. A number of functions have been ascribed to CD23, including specific regulation of IgE production, IgE-mediated cytotoxicity and release of mediators, IgE-dependent antigen focusing, promotion of B-cell growth, prevention of germinal center B cells from apoptosis, proliferation of myeloid precursors, and maturation of early thymocytes. It is not clear whether these activities represent *in vivo* functions. To explore *in vivo* functions of CD23, we have produced CD23-deficient mice. These mice displayed normal lymphocyte differentiation and could mount normal antibody responses, including IgE responses upon immunization with T-dependent antigens and infection with *Nippostrongyrus brasiliensis*. Germinal center formation after immunization and *in vitro* proliferative response of B cells were not affected in mutant mice. However, antigen-specific IgE-mediated enhancement of antibody responses was severely impaired.**

CD23 is a type II membrane-bound glycoprotein expressed mainly on mature B cells (1, 2). The extracellular carboxyl-terminal of this molecule is released from cells as soluble CD23 (3). This molecule has been originally identified as a low-affinity Fc receptor for IgE (FcεRII) expressed on human peripheral blood B cells and some lymphoblastoid cell lines by Spiegelberg and his colleagues (4, 5). Independently, CD23 has been described as a B-cell activation antigen expressed on Epstein–Barr virus (EBV)-transformed B cells (6, 7). Molecular cloning of FcεRII has demonstrated the identity between FcεRII and CD23 (2, 8).

The IgE binding activity of CD23 implies its role in effector functions of IgE and regulation of IgE synthesis (9). In fact, CD23 on monocytes, macrophages, and eosinophils has been reported to be involved in IgE-dependent cytotoxicity and mediator release (10, 11). Some anti-CD23 antibodies and soluble CD23 have been shown to modify *in vitro* IgE secretion of human B cells (12–14). Recently, it has been reported that an interaction of CD23 with CD21, a molecule known as an EBV receptor or a complement receptor 2, is involved in IgE production in humans and rats (15, 16). However, a role of CD23 in regulation of IgE production is still controversial since most of these functions cannot be reproduced in the mouse system (17).

A tight correlation between CD23 expression and EBV transformation of B cells suggests a possible role of CD23 in the transformation and growth of B cells. Affinity-purified soluble CD23 promotes the proliferation of both activated normal B cells and EBV-transformed B cells (18). Gordon *et al.* (19) have reported that purified native and recombinant soluble CD23 induced the proliferation of activated human B cells. However, experiments by other groups, including ours, have failed to reproduce this activity in human and mouse systems (17, 20).

In addition, CD23 has been shown to induce the proliferation of human myeloid precursors (21) and the maturation of human early thymocytes (22). A question may be raised whether all of these activities really represent *in vivo* functions of CD23.

To reevaluate the functions of CD23 *in vivo*, we have produced CD23-deficient mice. These mice displayed normal lymphocyte differentiation and could mount normal antibody responses; however, antigen-specific IgE-mediated enhancement of antibody responses was severely impaired.

MATERIALS AND METHODS

Gene Targeting. The targeting vector contained a 4.1-kb segment of the mouse CD23 gene encompassing exon 2 to exon 4 (23) (Fig. 1*A*). The CD23 mutation was created by inserting a neomycin-resistance gene cassette (*neo*) of the vector pMC1 neo-poly A (24) into the *Pvu* II site within exon 4. The herpes simplex virus thymidine kinase gene (25) was added to the 5′ end of the genomic sequence. Twenty micrograms of the vector was linearized at the 3′ end with *Xho* I, mixed with 10^7 E14-1 embryonic stem (ES) cells (kindly provided by R. Kühn, Institute for Genetics, University of Cologne, Germany) in 0.8 ml of phosphate-buffered saline (PBS; Ca^{2+} and Mg^{2+} free), and electroporated at 500 μF and 230 V (Bio-Rad Gene Pulser). Twenty-four hours after electroporation, the selection was started with the medium containing 300–400 μg of G418 per ml (GIBCO) and 2 μM gancyclovir (gift from Syntex, Tokyo). Individual drug-resistant colonies were picked and expanded for further analysis. Targeted ES clones were injected into blastocysts isolated from C57BL/6 and transferred to foster mothers.

Southern Blot and Northern Blot Analysis. Genomic DNA was prepared from ES cells and adult tail tips as described (26, 27). To ascertain targeting of the CD23 allele, 15 μg of DNA was digested with *Sph* I or *Aat* I and probed with a 0.4-kb *Bst*EII/*Xba* I (Probe A, Fig. 1*A*) or a 0.6-kb *Bam*HI/*Xba* I genomic DNA fragment (Probe B, Fig. 1*A*), respectively. Incorporation of the single neomycin-resistance gene

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Abbreviations: ES, embryonic stem; DNP, 2,4-dinitrophenyl; OA, ovalbumin; CFA, complete Freund's adjuvant; Nb, *Nippostrongyrus brasiliensis*; FITC-, fluorescein isothiocyanate-conjugated; PNA, peanut agglutinin; EBV, Epstein–Barr virus; LPS, lipopolysaccharide; IL, interleukin; TNP, 2,4,6-trinitrophenyl.

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was further confirmed using a *neo* probe. For analysis of CD23 mRNA, total RNA was prepared from splenic B cells stimulated with lipopolysaccharide (LPS; 10 μg/ml) in the presence of interleukin 4 (IL-4; 60 units/ml, Genzyme) for 36 hr as described (28), electrophoresed, transferred to Hybond-N (Amersham), and hybridized with a CD23 cDNA fragment or a β-actin probe.

Immunization and ELISAs. Seven-week-old mice were immunized i.p. with 50 μg of 2,4-dinitrophenyl ovalbumin (DNP-OA) in complete Freund's adjuvant (CFA) to induce IgM, IgG, and IgA, or in alum to induce IgE, at day 0 and given booster immunizations on day 14. DNP-specific antibody responses after immunization were measured by ELISA with DNP-bovine serum albumin-coated plates except for the IgE class. Alkaline phosphatase-labeled isotype-specific antibodies (Southern Biotechnology Associates) were used for detection. For the DNP-specific IgE antibody assay, plates were coated with anti-IgE monoclonal antibody 77, diluted sample and standard antibody SPE-7 (29) (anti-DNP monoclonal IgE antibody) (both antibodies were kindly provided by Y. Hattori, Kowa Research Institute, Tsukuba, Japan) were added, and bound DNP-specific IgE was detected by biotinylated DNP-human serum albumin followed by avidin-horseradish peroxidase.

RESULTS

CD23 Gene Targeting. The CD23 mutation was created by inserting a neomycin-resistance gene cassette (*neo*) within exon 4 (Fig. 1*A*). The ES cells were transfected with the linearized vector by electroporation. After selection with G418 and gancyclovir, six of the doubly resistant clones were found to carry the CD23 mutation on a single allele, as examined by PCR and Southern blot analysis. A single integration event was further confirmed in these clones by Southern blot analysis using the *neo* probe (data not shown). Chimeric mice derived from three of six ES clones transmitted the mutated genotype to their offspring. Mice heterozygous for the mutated allele were then intercrossed to produce homozygous mice. Southern blot analysis of the resulting offspring is shown in Fig. 1*B*. Hybridization with probe A yielded a 2.7-kb *Sph* I fragment for the mutated allele and a 5.5-kb fragment for the wild-type allele. In addition, hybridization with probe B of *Aat* I-digested DNA displayed 4.5-kb and 7.3-kb bands for the mutated and wild-type alleles, respectively. To determine the CD23 mRNA expression, RNA from spleen cells stimulated with LPS and IL-4 was prepared and analyzed by Northern blotting. As shown in Fig. 1*C*, a 2.2-kb CD23 transcript was observed in RNA from wild-type mice, whereas no hybridizing RNA band was detectable from CD23-targeted mice, confirming the functional disruption of the CD23 gene. In the following experiments, mice homozygous for the mutated allele or the wild-type allele in the background of (129/Ola × C57BL/6)F_2 were examined. Three independent mutant lines displayed indistinguishable phenotypes.

Surface Phenotype of Lymphocytes of CD23-Targeted Mice. As shown in Fig. 2*A*, no lymphocytes from CD23-targeted mice could be stained with anti-CD23 antibody, whereas most of the IgM-expressing B cells were positively stained in control wild-type mice. The expression patterns of IgD, IgM, and CD21 on the CD23-deficient B cells were essentially identical to those on wild-type B cells (Fig. 2 *B* and *C*). CD23 has been reported to be involved in regulation of early human T-cell development (22); however, we could not detect any difference in numbers and ratios of $CD4^+/CD8^+$, $CD4^+/CD8^-$, $CD4^-/CD8^+$, and $CD4^-/CD8^-$ thymocytes between age-matched CD23-deficient and wild-type mice (Fig. 1*D*). Furthermore, flow cytometric analysis of spleen, lymph node, bone marrow, and thymus cells using antibodies to

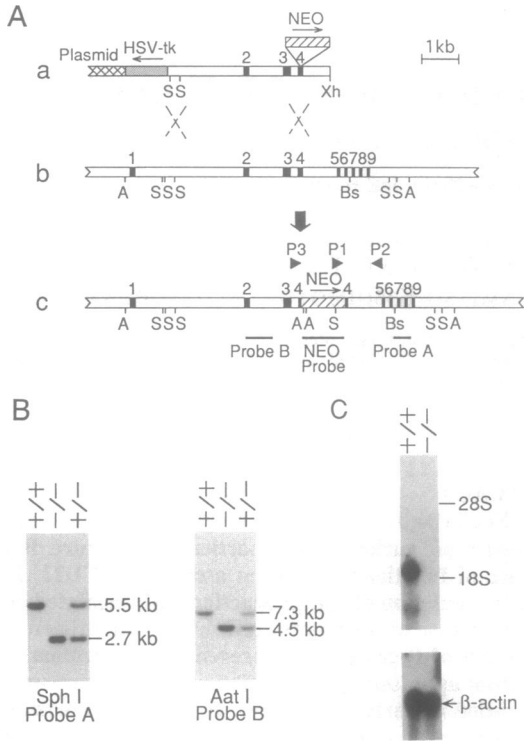

FIG. 1. Targeted disruption of the CD23 gene in the germ line of mice. (*A*) (*a*) Targeting vector. (*b*) Normal CD23 locus. (*c*) CD23 locus after homologous recombination. The CD23 exons are shown as solid boxes, introns as open boxes, and the neomycin-resistance gene (NEO) and herpes simplex virus thymidine kinase (HSV-tk) sequences as hatched boxes. Restriction enzyme sites: S, *Sph* I; Xh, *Xho* I; A, *Aat* I; Bs, *Bst*EII. The positions of the primers used for PCR analysis are shown by the arrowheads, P1–P3. DNA probes used for Southern blotting are indicated. (*B*) Southern blot analysis of offspring from intermatings of mice heterozygous for CD23 disruption. Offspring genotypes are indicated above the lanes: +/+, wild type; −/−, homozygote; +/−, heterozygote. Probes A and B correspond to the probes shown in *A*, *c*. (*C*) CD23 mRNA expression of lymphocytes from mutant and wild-type mice. Northern blot analysis was performed by using RNA from splenic B cells stimulated by LPS and IL-4. The probes used were the 760-bp CD23 cDNA fragment, which included exons 5–12, and β-actin.

heat-stable antigen, T-cell receptor αβ, CD3, CD4, CD8, IL-2 receptor α chain, and Thy1.2 failed to reveal any detectable phenotypic changes in CD23-deficient mice.

Antibody Production of CD23-Deficient Mice. The ability of CD23-deficient mice to mount specific antibody responses to thymus-dependent antigen, DNP-OA was evaluated. As shown in Fig. 3*A* CD23-deficient mice could mount DNP-specific primary and secondary responses of all isotypes, including IgE. The magnitude of the antibody responses was comparable to that observed in wild-type mice, although there was considerable variation among mice. The CD23-deficient mice could also produce specific antibodies to the thymus-independent antigens, such as 2,4,6-trinitrophenyl (TNP)-LPS and TNP-Ficoll as efficiently as wild-type mice did (data not shown).

To further analyze the influence of CD23 mutation on IgE antibody production, 8-wk-old mutant and wild-type mice were infected with third-stage Nb larvae since Nb infection induces a high level of IgE antibody response as well as CD23 expression in normal mice. No significant difference was found in serum IgE antibody levels of CD23-deficient and wild-type mice infected with Nb (Fig. 3*B*).

IgE-Mediated Enhancement of Antibody Responses Is Impaired in CD23-Deficient Mice. Since CD23 has been shown to be involved in IgE antibody-mediated antigen focusing of

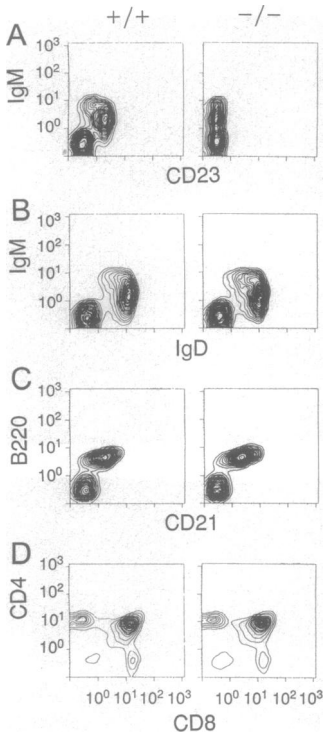

FIG. 2. Flow cytometric analysis of splenocytes and thymocytes from 8-wk-old homozygous mutant and wild-type mice. Single cell suspensions were prepared from spleen (*A–C*) and thymus (*D*) and analyzed by FACStar plus. Splenocytes were stained with fluorescein isothiocyanate-conjugated (FITC) anti-CD23 (B3B4) and biotinylated anti-IgM (R6-60.2) (*A*), FITC-sheep anti-IgD and biotinylated anti-IgM (*B*), and FITC-anti-B220 (RA3-6B2) and biotinylated anti-CD21 (7G6) (*C*). Biotinylated antibodies were counterstained with streptavidin-Texas Red conjugate. Thymocytes were stained with FITC-anti-CD8 (53-6.7) and phycoerythrin-labeled anti-CD4 (GK1.5) (*D*). 20,000 viable cells were collected and analyzed. Fluorescence-activated cell sorting profiles of wild-type (left, +/+) and CD23-deficient (right, −/−) mice are shown.

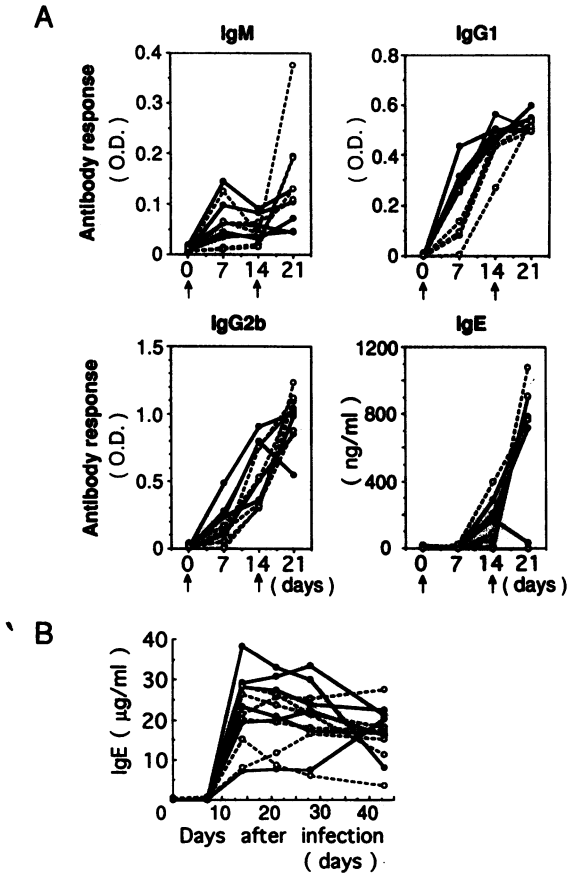

FIG. 3. (*A*) T-cell-dependent antibody responses of mutant and wild-type mice. Seven-week-old mice were immunized i.p. with 50 μg of DNP-OA in CFA to induce IgM and IgG antibody responses or in alum-precipitated forms to induce IgE antibody responses. Animals were given booster immunizations on day 14. At the indicated times before and after immunization, blood was drawn and DNP-specific antibody titers of each isotype were quantified by ELISA. The responses of individual mutant (○) and wild-type (●) mice are shown. (*B*) Serum IgE levels in mutant and wild-type mice after *Nippostrongyrus brasiliensis* (Nb) infection. Eight-week-old homozygous mutant (○) and wild-type (●) mice were infected s.c. with 750 third-stage Nb larvae; at the indicated times, serum IgE levels were determined by ELISA.

human and mouse B cells (30–33), we investigated whether IgE antibody could enhance specific immune responses in CD23-deficient mice (Fig. 4). DNP-OA was injected i.v. into the CD23-deficient and wild-type mice. Prior to injection of antigens, some of the mice received anti-DNP IgE antibody i.v. to examine whether anti-hapten IgE can enhance the carrier-specific antibody response. All five wild-type mice injected with anti-DNP IgE antibody prior to DNP-OA immunization produced significant amounts of OA-specific IgG1 antibody, whereas no antibody response was observed in mice injected with DNP-OA alone. In contrast, all five CD23-deficient mice did not produce any detectable level of anti-OA antibody, suggesting that IgE-dependent antigen focusing is hampered in the CD23-deficient mice.

***In Vitro* Proliferation of CD23-Deficient B Cells.** Soluble CD23 or crosslinking of membrane-bound CD23 has been shown to modify *in vitro* human B-cell proliferation (18, 19, 34). Hence, we examined the capacity of CD23-deficient B cells to proliferate *in vitro* in response to various stimuli, such as LPS or anti-IgM in the presence of IL-4, which is known to induce CD23. These stimuli induced comparable proliferation in B cells from both mice (data not shown).

Immunohistology of Germinal Centers. CD23 is densely expressed on follicular dendritic cells (35). Soluble human CD23 has been suggested to play a role in B-cell maturation in germinal centers (36). Immunohistochemical staining of the spleen sections with antibodies specific for IgM, Thy1.2, and peanut agglutinin (PNA) did not reveal any differences in the appearance of the germinal center (Fig. 5). T- and B-cell areas were clearly seen in both mice (Fig. 5 *A* and *B*). In double staining with anti-IgM and PNA (Fig. 5 *C* and *D*), most T cells weakly stained with PNA (red) were located in the

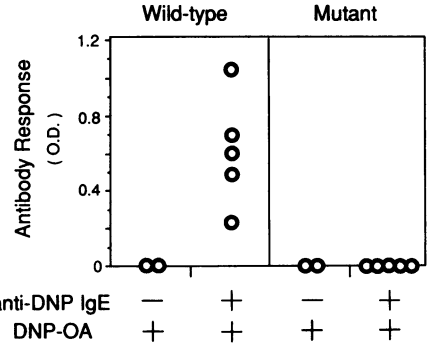

FIG. 4. Anti-hapten IgE antibody-dependent enhancement of carrier-specific immune response. Ten-week-old wild-type and mutant mice were injected i.v. with 40 μg of DNP-specific monoclonal IgE antibody (SPE-7) and injected i.v. with 20 μg of DNP-OA 1 hr later. Control mice received DNP-OA injection alone. OA-specific IgG1 antibody levels were determined by ELISA 3 wk after DNP-OA injection.

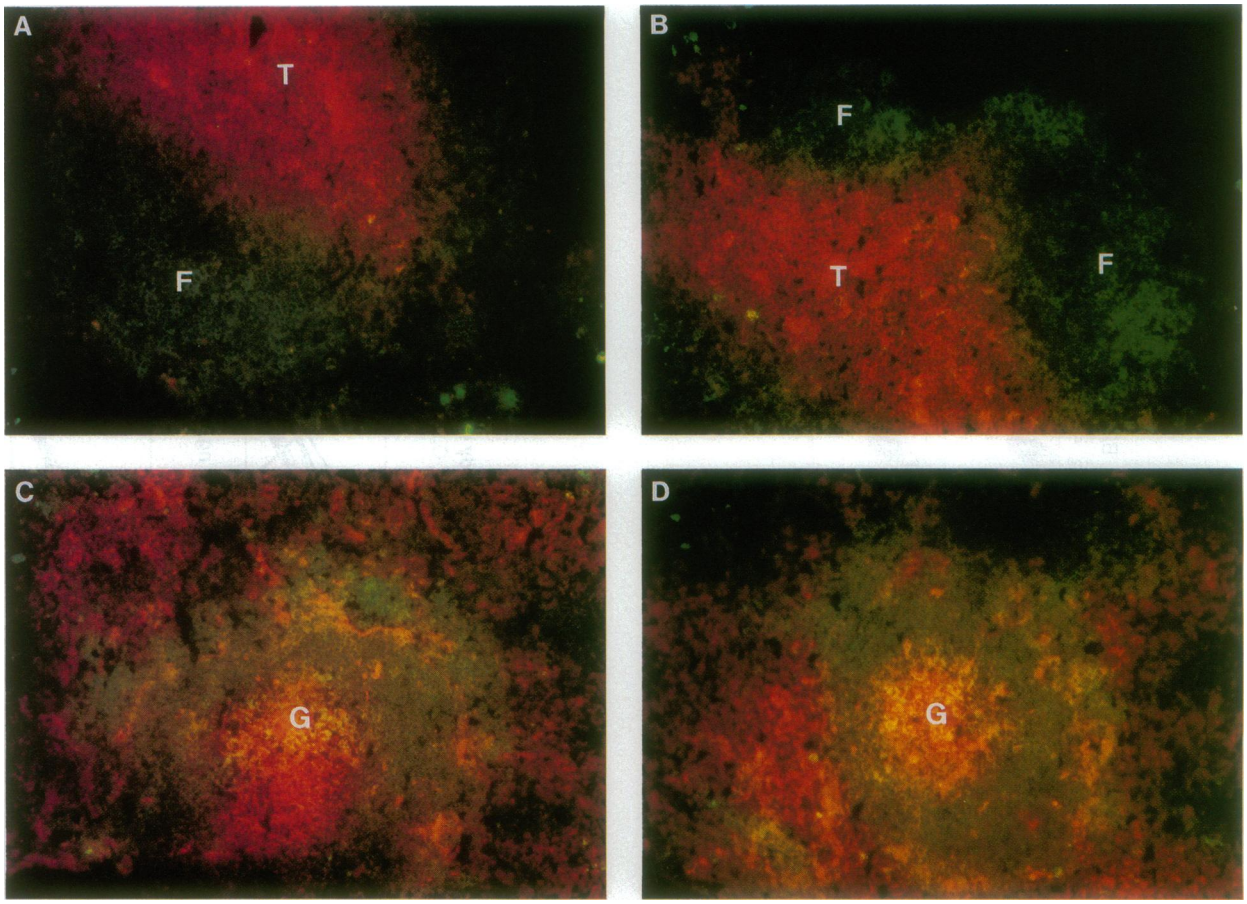

FIG. 5. Immunohistology of lymphoid follicles of spleens. Cryostat sections were prepared from spleens of 8-wk-old wild-type (*A* and *C*) and mutant (*B* and *D*) mice at day 9 after primary immunization with DNP-OA in CFA. (*A* and *B*) Double-stained with FITC-anti-IgM and biotinylated anti-Thy1.2. (*C* and *D*) FITC-anti-IgM and biotinylated PNA. Biotinylated reagents were counterstained with streptavidin-Texas Red conjugate. G, germinal center; F, follicle; T, T-cell zone. [Slides were photographed by a Zeiss Axiophot fluorescence microscope (×120).]

periphery of lymphoid follicles while B cells stained with FITC-anti-IgM (green) were within the follicles. Germinal center cells were stained both by anti-IgM (green) and by PNA (red), exhibiting yellow to orange color. Clusters of germinal center cells stained by both markers appeared the same between the wild-type and mutant mice.

DISCUSSION

The expression of CD23 is restricted to a certain differentiation stage of B-cell development, such as mature circulating IgD^+/IgM^+ B cells (37), suggesting a possible role of CD23 in differentiation or growth of B cells. In fact, human soluble CD23 has been shown to have a B-cell growth-promoting activity (18, 19). Furthermore, it has been reported that IL-1α and soluble CD23 rescued centrocytes from apoptosis in germinal centers and induced these cells to adopt a plasmablastic form producing IgG (36). Since most of these experiments were performed *in vitro* with isolated B cells, the mice carrying a null mutation in the CD23 locus allowed us to evaluate *in vivo* functions of CD23. However, remarkable changes were not noticed in lymphocyte development and immune responses of CD23-deficient mice, except for an absence of IgE-mediated enhancement of specific immune responses. One simple explanation for this discrepancy is that such activities obtained in *in vitro* studies may not represent real *in vivo* functions of CD23. Alternatively, some of these functions may be performed by other unknown molecules in the mutant mice or the CD23 mouse may not share some of these functions with its human counterpart.

Human soluble CD23 has been reported to augment *in vitro* IgE production (12, 14), although Bartlett and Conrad (17) failed to detect such an activity in the mouse counterpart. Aubry *et al.* (15) showed that the CD23–CD21 interaction is involved in *in vitro* IgE production of human lymphocytes. Recently, the same group reported that injection of polyclonal anti-human CD23 antibodies, which are also crossreactive to rat CD23, inhibited the *in vivo* antigen-specific IgE response in rat (16). If most functions of rat CD23 are shared by its mouse counterpart, their results are not in agreement with ours. These investigators suggested that their anti-CD23 antibodies might inhibit *in vivo* IgE production by interfering with the CD23–CD21 interaction that leads to an increase of IgE synthesis. If this is the case, IgE production should have been impaired in CD23-deficient mice. Such a mechanism may not operate *in vivo*.

One of the most consistently observed functions of CD23 in humans and mice is IgE-dependent antigen focusing and presentation to T cells. Kehry and Yamashita (30) showed that mouse B cells could present TNP-antigens to T cells via CD23 in the presence of anti-TNP IgE as 100-fold efficiently as in the absence of IgE. The involvement of CD23 in IgE-dependent antigen presentation was repeated in the human system (31, 32). Recently, Heyman *et al.* (33) showed that injection of anti-hapten IgE antibody prior to immunization of haptenated antigen enhanced specific antibody responses of mice and that this enhancement was blocked by anti-CD23. In the present study, we demonstrate that CD23 is essential for IgE antibody-mediated enhancement of specific immune responses. In the physiological state, extremely small amounts of allergen or pathogen may not be able to

induce a humoral immune response. In the presence of high local concentrations of specific IgE antibodies in the airway, gastrointestinal tract, or skin, antigens can be captured or focused by CD23 on antigen-presenting cells and efficiently presented to T cells, resulting in augmentation of specific immune responses. In the allergic status, IL-4 derived from Th2 helper cells may induce increases of CD23 expression as well as IgE production, which may exacerbate the diseases by further augmenting T-cell responses and IgE production.

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