

Transgene CD23 Expression on Lymphoid Cells Modulates IgE and IgG1 Responses

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Transmembrane (m) and soluble (s) forms of CD23 perform activities related to various immune functions. Abnormal expression patterns of CD23 on lymphoid cells have been associated with certain pathologic conditions. To explore the effects of CD23 when it is overexpressed, on lymphoid cell development and immune function in vivo, transgenic mice were generated. These mice overexpressed either mCD23 or a 38-kDa molecular form of sCD23. Transgene expression under the control of Thy-1-regulatory sequences and the mouse Ig heavy chain enhancer (E μ) was prominent in thymus, spleen, bone marrow, and lymph nodes. Cells that expressed the transgenes included most thymocytes, peripheral CD4⁺ and CD8⁺ T cells, IgM⁺/high IgD^{-/low} immature B cells, and IgM^{low}IgD^{high} mature B cells. To resolve the expression pattern in the B cell lineage unambiguously, we used mice that carried a transgene and a disrupted endogenous CD23 gene simultaneously. Neither mCD23 nor sCD23 overexpression caused significant alterations in lymphoid cell maturation. In addition, basal serum levels of IgE and IgG1 proved to be normal. In three different experimental immune response paradigms, mCD23 transgenic, but not sCD23 and nontransgenic mice proved to be impaired in increasing serum levels of polyclonal IgE up to expected levels. In addition, mCD23 transgenic mice showed below normal increases of serum IgG1 levels in two of the three immune responses. In the presence of activated T cells and appropriate lymphokines, B cells from mCD23 mice secreted normal amounts of IgE and IgG1 in vitro, which suggests that there was no serious impairment of the T-B cell contact required for Ig production. In addition, there is no evidence for a significant role of mCD23 in IgE clearance. Therefore, we discuss alternative mechanisms by which mCD23⁺ B and/or T cells influence Ig production. *The Journal of Immunology*, 1994, 153: 3028.

CD23, the low affinity receptor for IgE (Fc ϵ RII), is a type II transmembrane (m)⁴ glycoprotein expressed in the mouse predominantly on mature B cells, follicular dendritic cells, and some T cells (1–3). In the B cell lineage, mCD23 is present on mature IgM⁺IgD⁺ B lymphocytes, but absent on cells after isotype switching.

Cells in earlier stages of B cell ontogeny also lack mCD23 (4, 5), although precursor B cells (human) seem to be capable of expressing mCD23 before the acquisition of sIgM by triggering CD40 (6). mCD23 expression seems to be regulated in a tight temporal and developmental fashion, not only in the B cell lineage, but also on the cell surface of certain T cells, where mCD23 expression has been detected occasionally, in particular after activation in vitro or in association with certain pathologic conditions in vivo (3).

Functions proposed for CD23 include roles in T cell-dependent IgE production (7–14), B cell proliferation (15, 16), thymocyte maturation (17), myelopoiesis (18), Ag presentation (19, 20), and rescue of germinal center B cells from apoptosis (21). Some of these roles are thought to involve both mCD23 and proteolytically derived soluble forms (sCD23). A property that has been assigned only to sCD23 in synergy with IL-1 is to act as a differentiation factor for thymocytes in vitro (17, 22).

To address questions regarding the roles of CD23 in vivo, we and others have generated CD23^{-/-} mice that

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⁴ Abbreviations used in this paper: m, membrane; s, soluble; Nb, *Nippostrongylus brasiliensis*; FCM, flow cytometry; BPO-KLH Alum, keyhole limpet hemocyanin-conjugated benzylpenicilloyl adsorbed on aluminum hydroxide; PE, phycoerythrin; TBST, 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20; AP, alkaline phosphatase.

lack a functional CD23 gene (10, 12). CD23^{-/-} mice showed no obvious defects in thymocyte, peripheral T, or B cell compartments, thereby ruling out an essential role for CD23 in T and B cell development (10, 12). These studies also revealed that CD23 is not essential for class-switch, plasma cell differentiation, the development of germinal centers, or the initiation and maintenance of an immune response (10, 12). Immunization with thymus-dependent haptenated-Ags has been shown to provoke twofold increased levels of specific IgG1 and 6- to 12-fold higher levels of specific IgE in CD23^{-/-} mice as compared with control littermates (12). Impaired clearance of serum IgE was shown not to account for an increase in serum IgE in CD23^{-/-} mice (12). In a parasite-induced immune response to *Nippostrongylus brasiliensis* (Nb), CD23^{-/-} mice did not produce significantly more serum polyclonal IgE than did CD23^{+/+} mice (10, 12). The fact that a lack of CD23 in Nb infestation does not alter IgE synthesis is thought to be caused by a dominant activation of Th-2 cells (23) and a strong T cell-independent non-specific activation of B cells (24). Nevertheless, we have found that mice that are heterozygous with respect to the knockout allele (CD23^{+/-}) and that express one-half of the normal levels of mCD23 on the surface of CD23⁺ cells, developed two- to threefold higher polyclonal serum IgE levels after Nb infestation as compared with control and CD23^{-/-} littermates (10). Although most findings support a function for CD23 mainly in negative feedback control of IgE synthesis, some findings favor a positive role in this process. First, CD23 Abs or IgE-containing immune complexes suppress human B cell proliferation (9) and IgE production in vitro (13). Second, anti-CD23 Ab treatment of rats inhibited a specific IgE immune response (11). A net result in the Ig response may depend on CD23 gene dosage, the cell types that express CD23, and the microenvironment in which these cells reside.

To extend the analysis aimed at dissecting roles of CD23 in vivo, we have created transgenic mice that constitutively overexpress CD23, in particular, in cells and tissues in which CD23 has been proposed to play a role in ongoing cellular differentiation or immune regulation. To address putative separate functions of mCD23 and sCD23, mouse lines that overexpressed either mCD23 or a 38-kDa sCD23 molecular form were established. This sCD23 constituted most of the extracellular mCD23 protein, including the lectin-homology IgE-binding domain and the four extracellular repeats that form a stalk structure. Transgene expression was controlled by Thy-1 gene-regulatory sequences and the mouse Ig heavy chain enhancer (E μ) to allow expression in T and B cell compartments (25–29). The transgenic mice were used to study effects on T and B lymphocyte development and immune responses after challenge with anti-mouse IgD Abs (30), Nb (23, 24, 30), or keyhole limpet hemocyanin-conjugated benzylpenicilloyl adsorbed on aluminum hydroxide (BPO-KLH Alum) (31–33). We show that overexpression of mCD23, but not

sCD23, impairs to a significant extent, the increase up to expected levels of polyclonal IgE and IgG1 in serum. This phenomenon is observed after challenge with anti-IgD and after Nb infestation, but not in response to BPO-KLH Alum when IgE, but not IgG1, did not increase up to normal levels in mCD23 mice. Spleen cells from mCD23 mice were shown to secrete normal levels of IgE and IgG1 when cultured on anti-CD3 coated plates in the presence of IL-2, IL-4, IL-5, IL-6, and anti-IFN- γ . Hence, the effect of mCD23 overexpression on lowering IgG1 and IgE synthesis is unlikely to be caused by faulty contact between T and B cells. We discuss several alternative, and not necessarily mutually exclusive, mechanisms that could account for reducing Ig production.

Materials and Methods

DNA constructions

The Thy1-E μ -mCD23 transgene (Fig. 1A) consists of a modified Thy-1.2 genomic clone (8.2-kb *EcoRI-EcoRI* DNA fragment; Ref. 25), in which the Thy-1.2 coding region (including the second and third introns) was removed and replaced by a mouse CD23 cDNA (34). In addition, a 1.1-kb *SsrI-SsrI* DNA fragment in the first intron was replaced by a 1-kb *SsrI-SsrI* DNA fragment, which contained the mouse Ig heavy chain enhancer (E μ ; Fig. 1B). Thy1-E μ -sCD23 is similar to Thy1-E μ -mCD23, but instead of a full-length mouse CD23 cDNA, contains a cDNA that encodes a fusion protein. Sixty base pairs of the insert encode for the mouse CMV signal peptide and are followed by a fragment of the mouse CD23 cDNA that encodes for the extracellular amino acids 67–331 (Fig. 1C).

Generation of transgenic mice

The 8.3- and 8-kb *NotI-PvuI* DNA fragments that contained Thy1-E μ -mCD23 (Fig. 1B) and Thy1-E μ -sCD23 (C), respectively, were used for microinjection into fertilized eggs derived from C57BL/6XCB6 F₁ matings to generate transgenic mice (35, 36). Successful transgene integration was analyzed by Southern blot analysis with use of an α -³²P-radiolabeled DNA probe that spanned the 770-bp *XhoI-BamHI* fragment of the 3'-untranslated region of the Thy-1 gene (Fig. 1A).

RNA analysis

Total RNA from frozen tissues or cells was extracted by using the guanidine isothiocyanate method (37). Standard procedures were used for Northern blotting (38). The 18S and 28S ribosomal RNAs were visualized to control transfer efficiency and to ensure that approximately equal amounts of RNA were analyzed in parallel. The probe used was a radiolabeled mouse CD23 cDNA fragment (a 1.5-kb *EcoRI-TaqI* fragment; Ref. 34).

Tissue staining

Cryostat sections (5- to 10- μ m thick) were fixed in acetone or 0.1% glutaraldehyde and stained for mCD23 by using the horseradish peroxidase technique (10). The B3B4 Ab (rat anti-mouse CD23) was obtained from PharMingen (San Diego, CA). Second Abs, the streptavidine-peroxidase complex, and DAB peroxidase substrate (3–3' diaminobenzidine tetrahydrochloride) were purchased from Amersham (Amersham, UK).

Flow cytometry (FCM)

FCM was performed as described previously (39), with use of the following biotinylated or FITC- or PE-labeled mAbs: B3B4 for CD23, RA6-B2 for B220, 7D4 for the IL-2R, IM7 for Pgp-1, anti-HSA, and anti-TCR- $\alpha\beta$ (all from PharMingen, San Diego, CA); GK1.5 for CD4 and 53–6.7 for CD8 (both from Becton Dickinson, Palo Alto, CA); and goat anti-mouse IgM and goat anti-mouse IgD Abs for IgM/IgD staining (Southern Biotechnology Associates, Birmingham, AL). Biotinylated

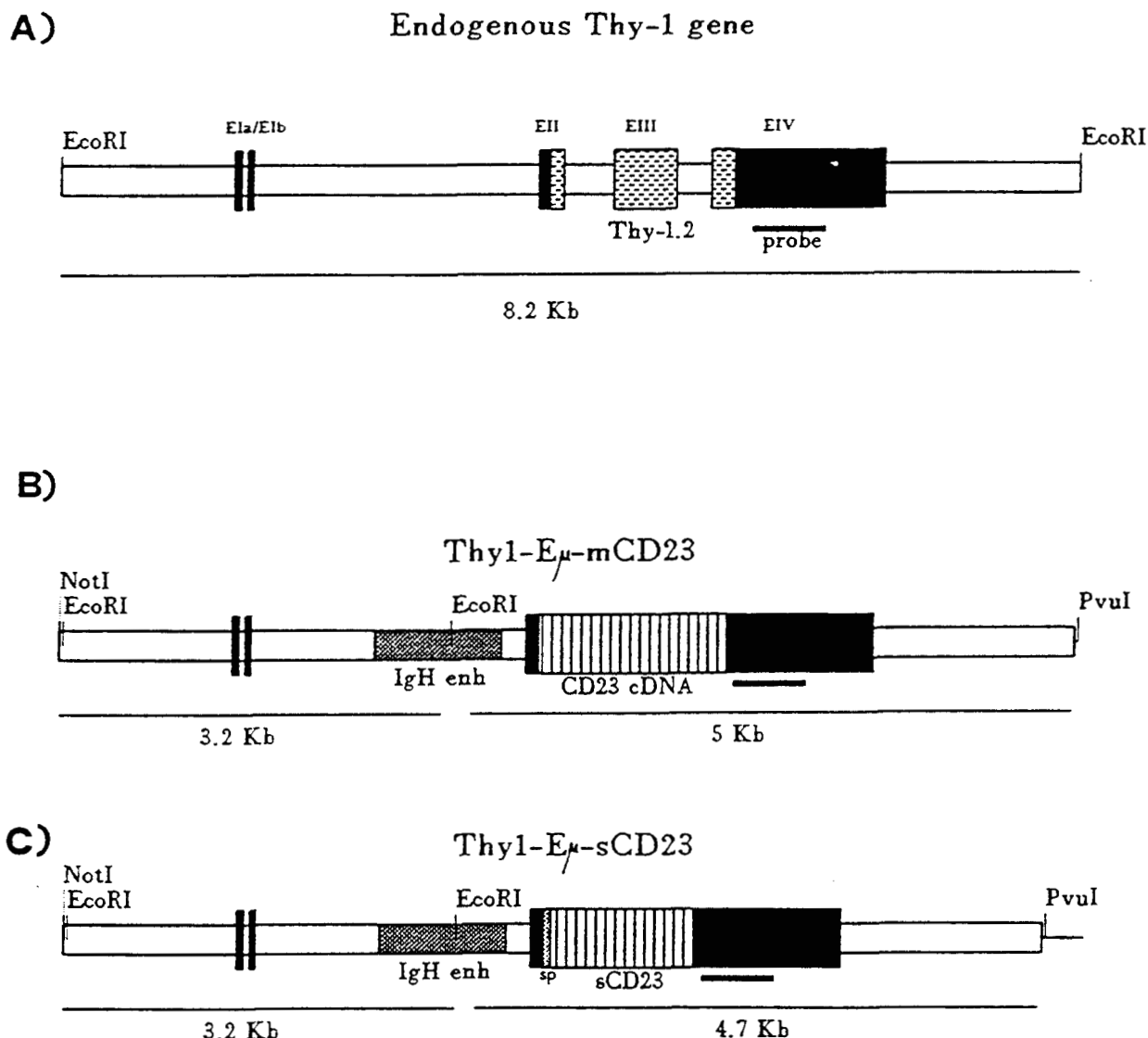


FIGURE 1. A: genomic *EcoRI* DNA fragment encompassing the mouse Thy1.2 gene. Exons 1, 2, 3, and 4 are indicated by *Ela/Elb*, *EII*, *EIII*, and *EIV*, respectively. The coding region is dotted. B and C: line diagrams showing the chimeric Thy1-E μ -mCD23 and the Thy1-E μ -sCD23 transgenes. The cDNA portion encoding the CMV signal peptide is indicated as sp. Also indicated are sizes of fragments obtained after *EcoRI* digestion of genomic DNA and the Thy-1 exon IV probe used for Southern blot analysis of tail DNA.

mAbs were counter-stained with either streptavidin-PE conjugate (Southern Biotechnology Associates) for two-color fluorescence, with streptavidin-TRI-color (Medac, Hamburg, Germany) or RED670 (Life Technologies, Karlsruhe, Germany). The analyses were performed by using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA) and LYSYS II software. Data were evaluated with PC-LYSYS on a personal computer.

Detection of sCD23 in serum and thymocyte supernatants

Serum and thymocyte supernatants were assayed by ELISA to determine the presence of sCD23. Serum was obtained from blood collected by tail bleeding. Thymocyte supernatants were obtained from 48-h cultures at a concentration of 5×10^6 thymocytes/ml in DMEM (4500 mg/ml glucose) containing 1 mM sodium pyruvate, nonessential amino acids, 2 mM glutamine, 50 mM 2-ME, and 100 mg/ml gentamicin. For ELISA,

96-well microtiter plates were coated with the rat mAb anti-mouse CD23 B3B4, (0.5 mg/well), and different dilutions of serum or supernatant were incubated overnight at 4°C. After being washed with PBS, sCD23 captured by B3B4 Ab was detected by a goat pAb anti-mouse CD23. Subsequently, an incubation with alkaline phosphatase (AP)-conjugated rabbit anti-goat Ab was performed. The development reaction was performed with the AP substrate, *p*-nitrophenylphosphate (150 mg/well), and the OD was measured at 405 nm on a microplate reader (Titertek Multiscan MCC/340; BioConcept, Allschwil, Switzerland). The OD₄₀₅ data obtained from the different samples were compared.

Western blot analysis

Thymic tissue was placed in 2 to 3 vol of cell lysis buffer (50 mM Tris (pH 7.8), 5 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 0.5 mM PMSF, 5 μ g/ml leupeptin, and 10 mM DTT) and homogenized by using a polytron; lysis was conducted for 30 min on ice followed by centrifugation

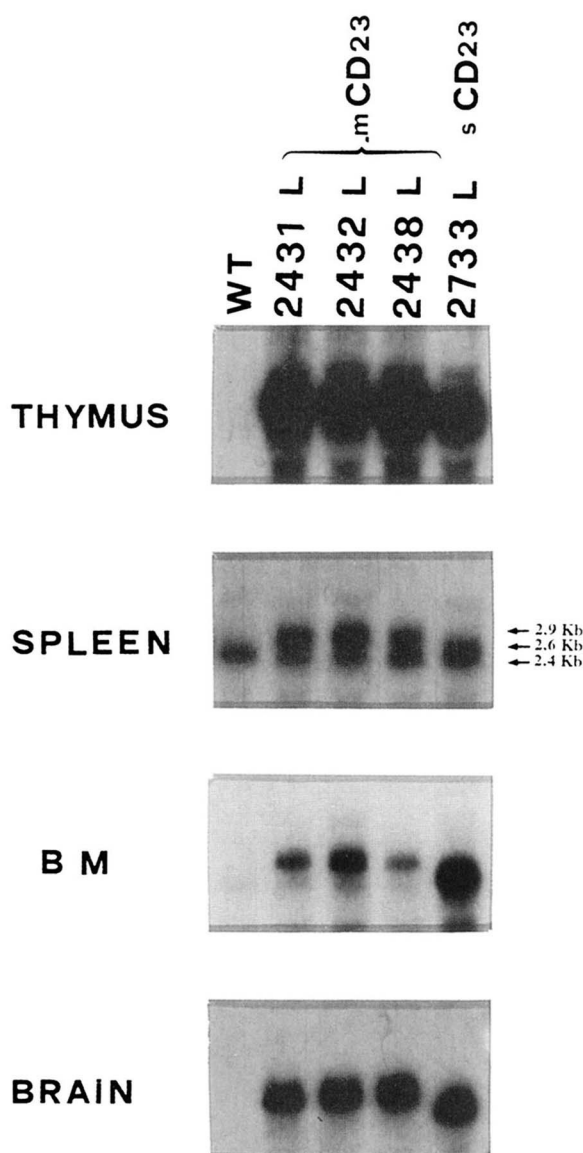


FIGURE 2. Northern blot analysis of total RNA extracted from tissues of wild-type (WT), Thy1-E μ -mCD23 (mCD23), and Thy1-E μ -sCD23 (sCD23) mice. Shown are endogenous CD23 mRNA (2.4 kb) and transgene-derived mRNAs (Thy1-E μ -mCD23, 2.9 kb; Thy1-E μ -sCD23, 2.6 kb).

(3000 $\times$ g; 4°C). Supernatants were stored at -20°C and, when necessary, were concentrated before storage by centrifugation at 5000 $\times$ g for 1 h in a Centricon-10 microconcentrator (Amicon, Beverly, MA). Proteins were separated on 12 to 15% SDS-polyacrylamide gels and transferred onto Millipore Immobilon (Millipore, Bedford, MA) by using a semidry blotter apparatus (Bio-Rad, Richmond, CA) according to manufacturer procedures. For blocking and Ab dilution, TBST that contained 1 to 3% BSA (Fraction V; Sigma Chemical Co., St. Louis, MO) was used. Incubation of the filter with the rat anti-mouse CD23 (B3B4) Ab (5 $\mu\text{g}/\text{ml}$) occurred overnight at 4°C . After being washed three times with TBST, the membrane was incubated (30 min) with goat anti-rat Ig conjugated to AP (1:3000 diluted). After being washed in TBST, Ag-Ab complexes were visualized by incubation of the membrane in carbonate buffer that contained the AP substrates, *p*-nitroblue tetrazolium chloride (300 $\mu\text{g}/\text{ml}$) and 5-bromo-4-chloro-3-indolyl-phosphate (150 $\mu\text{g}/\text{ml}$).

Experimental immunizations

In all immunization experiments, transgenic and nontransgenic littermate mice at age 8 to 16 wk were used in parallel. Infection with Nb was performed by s.c. injection of 750 third-stage (L3) infective larvae. Anti-mouse IgD immunization was achieved by s.c. injection of 500 μg of rat anti-mouse IgD (LO-M D6 and LO-M D7; University of Louvain, Brussels, Belgium). For the carrier-hapten immunization, mice were injected i.p. with 100 μg BPO-KLH in 0.2 ml $\text{Al}(\text{OH})_3$ (aluminum hydroxide gel) (Serva, Heidelberg, Germany).

Measurement of eosinophilia

Blood was obtained by tail-vein bleeding and used to prepare blood smears, which were fixed and stained with the rapid differential hematology staining kit Diff Quick (Baxter, Düringen, Switzerland). The percentage of eosinophils was measured by counting the different white blood cells under a microscope. Total white blood cells and total RBCs were counted by using the Sysmex 228 Cell-Check-400 system (TOA Medical Electronics Co., Ltd., Kobe, Japan).

Quantification of total serum IgE, IgG1, and IgM by ELISA

First, 96-well microtiter plates were coated with rat mAb anti-mouse IgE (4B3-39) (0.25 mg/well), anti-mouse IgG1 (Zymed 04-6100; Zymed, South San Francisco, CA) (0.1 mg/well), or anti-mouse IgM (b-7-6) (0.25 mg/well) to quantify total IgE, IgG1, and IgM, respectively. Serum and standard dilutions were incubated overnight at 4°C and washed with PBS. To detect the Igs bound to the first Ab, an incubation with the biotinylated second rat mAb anti-mouse IgE (ab-3-11), anti-mouse IgG1 (Zymed 04-6140), or anti-mouse IgM (b-7-6) was performed. After incubation with AP-conjugated avidine, the enzymatic reaction was accomplished by adding *p*-nitrophenyl phosphate disodium (150 mg/well). The OD was measured at 405 nm on a microplate reader (Bio-Rad 405). Titers were determined at any point on the linear part of the titration curve. The indicated IgE and IgG1 concentrations in the serum represented a mean of four mice analyzed.

In vitro IgE and IgG1 production by splenic B cells

Whole spleen cells were washed twice with HBSS, and erythrocytes were lysed with ACT-buffer (140 mM NH_4Cl , 17 mM Tris-base, pH 7.65). Cells were plated at 5×10^4 cells/well in 96-well plates that previously had been coated with anti-CD3 Ab (145-2C11; 20 $\mu\text{g}/\text{ml}$) in 200 μl DMEM that contained 10% FCS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 50 mM 2-ME, 100 mg/ml gentamicin, mL-2 (100 U/ml), mL-4 (650 U/ml), mL-5 (1400 U/ml), mL-6 (50 U/ml), and anti-mIFN- γ (XMG-D6) (10 $\mu\text{g}/\text{ml}$). Supernatants were collected after 6- to 7-days culture and assayed by ELISA for IgE and IgG1 production.

Results

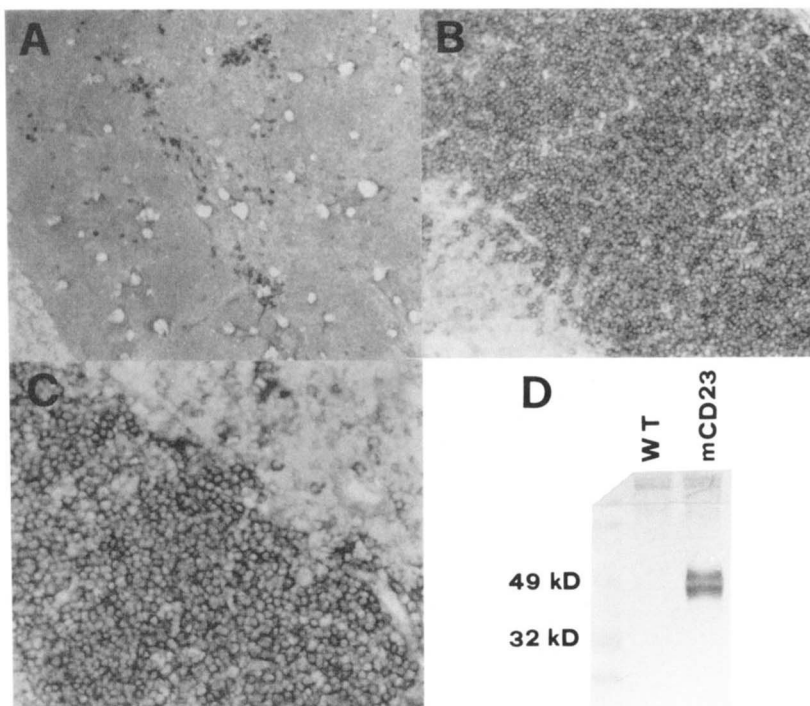
Transgenic mice

Microinjections of the Thy1-E μ -mCD23 (Fig. 1B) and Thy1-E μ -sCD23 (C) chimeric genes into fertilized eggs obtained from CB6XC57BL/6 F₁ mice yielded six and four transgenic mice (F₀), respectively, each carrying at least one full-length copy of the transgene (data not shown). Four Thy1-E μ -mCD23 (2431, 2432, 2435, and 2438) and three Thy1-E μ -sCD23 (2637, 2727, and 2733) F₀ mice successfully were bred to establish lines that were used for additional analysis. Transgenic offspring from all seven lines proved to be healthy and did not suffer from obvious disease.

Transgene expression

By using a mouse CD23 cDNA probe, we have shown that both transgenes mainly were expressed in thymus, spleen,

FIGURE 3. Transgene mCD23 protein expression in thymus. *A*, *B*, and *C*: immunocytochemical staining of cryostat sections of thymi using the anti-CD23 mAb B3B4. *A*: wild-type mouse; only some cells in the cortico-medullary junction express mCD23 ($\times 200$). *B*: Thy1-E μ -mCD23 mouse; high levels of mCD23 expression in cortex ($\times 200$). *C*: Thy1-E μ -mCD23 mouse; higher magnification ($\times 400$) showing membrane staining. *D*: Western blot analysis of thymic lysates; wild-type (WT) and Thy1-E μ -mCD23 (mCD23) mice.



bone marrow, lymph nodes (not shown), and brain (Fig. 2). Transcripts from the endogenous CD23 gene (2.4 kb) and the transgene (2.9 kb for the mCD23, and 2.6 kb for the sCD23 encoding transgene) were distinguished by their difference in size (Fig. 2). Although this difference in the sCD23 transgenic mice is only ~ 0.2 kb, transgene expression was obvious given 1) a dramatic increase in CD23 mRNA in the spleen of transgenic mice and 2) the finding that some organs in transgenic mice showed prominent transgene expression, whereas the same tissues in nontransgenic mice either lacked (e.g., brain) or showed weak expression of endogenous CD23 mRNA (e.g., thymus; Fig. 2).

Proper translation of mCD23 transgene mRNA was shown by immunocytochemical analysis of tissue sections from thymus and by Western blot analysis. In nontransgenic mice, mCD23 in thymus is exclusively expressed by a subset of cells near the cortico-medullary junction (Fig. 3A). In contrast, in mCD23 transgenic mice, thymocytes located throughout the cortex, as well as some in the medulla, expressed mCD23 (Fig. 3, *B* and *C*). Western blot analysis of thymic tissue lysates from mCD23 mice confirmed high levels of expression of mCD23 protein with the expected m.w. (Fig. 3D).

We used two methods to determine expression and release of sCD23 protein. First, we analyzed supernatant from thymocytes, which had been kept in culture for 48 h, for the presence of sCD23 by using ELISA. Second, also by using ELISA, we monitored sCD23 levels in serum. The OD₄₀₅ data of samples from mCD23, sCD23, and nontransgenic mice were compared graphically by using relative OD values. The results show that thymocyte supernatants from con-

trol littermate mice lack sCD23, whereas sCD23 was present at significant levels in thymocyte supernatants of both mCD23 and sCD23 mice (Fig. 4A). In serum, nontransgenic mice contained some, but little, sCD23 (caused by cleavage of endogenously expressed mCD23), whereas both sCD23 and mCD23 mice contained significantly increased levels of sCD23 in serum. As compared with that of sCD23 mice, mCD23 mouse serum contained lower levels of sCD23 protein (Fig. 4B). These results indicate that: 1) sCD23 mice express significantly increased levels of sCD23 protein; and 2) transgene-mediated overexpression of mCD23 also results in increased levels of sCD23. The latter most likely is produced by proteolytic cleavage of transgene-derived mCD23 in a fashion similar to processing of the endogenous mCD23 protein. The data also suggest that there could be less cleavage or faster clearance of mCD23-derived sCD23 in vivo as compared with when cells (in this case, thymocytes) are cultured in vitro.

To characterize cell populations that expressed the mCD23 transgene, we performed FCM with use of anti-mouse CD23 (B3B4) and Abs against various cell surface Ags. In the thymus, most CD4⁺CD8⁺ thymocytes and a considerable proportion of both CD4⁺CD8⁻ and CD8⁺CD4⁻ cells expressed the transgene. In secondary lymphoid organs, sizable proportions of both CD4⁺ and CD8⁺ T cells were mCD23⁺ (Table I).

In the B cell lineage, the detection of transgene mCD23 is complicated by the expression of large numbers of mCD23 molecules that are encoded by the endogenous CD23 gene and are present on mature IgM⁺IgD⁺ lymphocytes. For this reason, transgene expression was analyzed in mice that lacked expression of

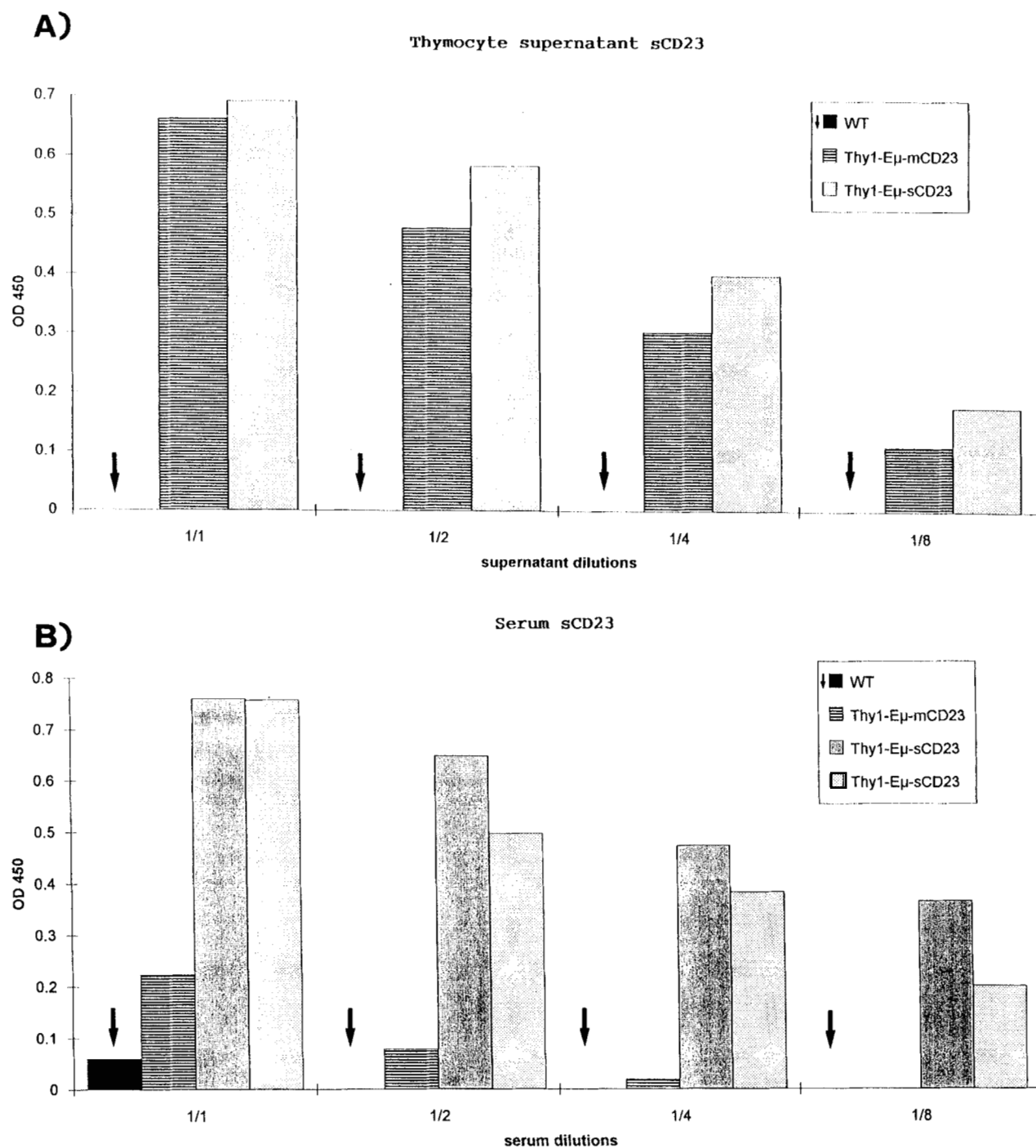


FIGURE 4. ELISA monitoring sCD23. **A:** sCD23 in supernatants of thymocytes after 48-h culture. Levels of sCD23 in thymocyte supernatants from wild-type (WT), Thy1-Eμ-mCD23 (2432L), and Thy1-Eμ-sCD23 (2727L) mice are shown. **B:** sCD23 levels in serum from wild-type (WT), Thy1-Eμ-mCD23 (2432L), and Thy1-Eμ-sCD23 (2727L and 2733L) mice. Arrows indicate samples in which sCD23 was not present or was below the limit for detection.

the endogenous CD23 gene (10). Such mice were generated by crossing mCD23 transgenic with CD23^{-/-} mice. First, mice were generated that were heterozygous with respect to both the transgene (mCD23^{+/-}) and the endogenous CD23 gene (CD23^{+/-}). The genotype of these mice was verified by Southern blot analysis

(*Bst*EII-restricted tail DNA) with use of two probes, one specifically detecting the genotype of the endogenous CD23 locus (10), the second (a Thy-1 gene-derived probe; Fig. 1A), detecting the transgene. Matings of mCD23^{+/-} to CD23^{+/-} mice yielded animals with the genotype mCD23^{+/-}CD23^{-/-}, as assessed by Southern blot analysis (data not

Table 1. Transgene expression in the T cell compartment^a

Cells	WT	Mouse Lines			
		2431L	2432L	2435L	2438L
Thymus					
CD4 ⁺ CD8 ⁺	0%	94%	98%	89%	89%
CD4 ⁺ CD8 ⁻	0%	28%	40%	27%	26%
CD4 ⁻ CD8 ⁺	0%	75%	80%	58%	39%
CD4 ⁻ CD8 ⁻	0%	31%	41%	10%	11%
Lymph nodes					
CD4 ⁺	0%	55%	93%	57%	24%
CD8 ⁺	0%	78%	99%	75%	51%
Spleen					
CD4 ⁺	0%	48%	84%	41%	39%
CD8 ⁺	0%	76%	91%	74%	67%

^a FCM analyses of mCD23 expression on the cell surface in the T cell compartment of the different Thy-1-E μ -mCD23 mouse lines. Control littermates (WT) were analyzed for reference. Each value represents the percentage of cells, within a given T cell population, that express mCD23.

shown). FCM analysis of mCD23^{+/+}CD23^{-/-}, as compared with CD23^{+/+} and CD23^{-/-} mice, revealed that transgene mCD23 expression on bone marrow B cells mainly occurred on subsets of B cells that normally already expressed endogenous mCD23 (Fig. 5, A and B). Immature IgM^{low}B220^{low} IgD⁻ B cells expressed neither endogenous nor transgene-derived mCD23, whereas low levels of transgene mCD23 were detected on the surface of both IgM^{+/high}B220⁺ IgD^{-/low} immature and IgM^{low}IgD^{high} mature B cells (Fig. 5B; and data not shown).

In the spleen, IgM^{low}IgD^{high} B cells normally express high levels of endogenous mCD23, and lower levels of mCD23 are found on IgM^{high}IgD^{high} B cells (Fig. 6A). From the FCM analysis, it is apparent that cell surface expression of transgene-derived mCD23 on these B cell subsets did not reach levels normally attained by endogenous CD23 gene expression. Instead, both B cell subsets expressed similar and lower mCD23 levels, the magnitude of which was comparable to endogenous mCD23 expression on IgM⁺IgD^{low} immature B cells. In the peritoneal cavity, B220^{low} (Ly1/CD5) B cells (40) normally lack expression of endogenous mCD23, whereas conventional B220^{high} B cells express mCD23 levels that are comparable with those of their counterparts in spleen (41). In mCD23^{+/+}CD23^{-/-} mice, the cellular distribution of transgene mCD23 was similar, except that B220^{high} cells had fewer mCD23 molecules on their cell surfaces (Fig. 6B). As compared with CD23^{+/+} mice, coexpression of endogenous and transgene mCD23 in mCD23^{+/+}CD23^{+/+} mice resulted in slightly elevated levels of mCD23 expression on the mCD23⁺ B cells subsets (data not shown).

Lymphocyte development in transgenic mice

Putative effects on thymocyte development were analyzed by FCM and compared with data obtained from control littermates. Individual thymocyte subsets were stained with

anti-CD4, anti-CD8, anti-TCR- $\alpha\beta$, anti-TCR- $\gamma\delta$, anti-Pgp1, anti-HSA, and anti-IL2R Abs. Because changes in T cell maturation could also affect the proportions of peripheral T cells, these also were scored with use of anti-CD4 and anti-CD8 Abs. The analysis shows that both mCD23 and sCD23 mice have normal absolute numbers and frequencies of the main thymocyte subsets and peripheral T cell populations. The numbers are comparable with those found in control nontransgenic littermates. In the thymus, 1.3% of total thymocytes were Pgp1⁺HSA^{low}IL2R⁻; 1.2%, Pgp1^{low}HSA^{high}IL2R⁺; 42%, CD4⁺CD8⁺TCR- $\alpha\beta$ ⁻; 45%, CD4⁺CD8⁺TCR- $\alpha\beta$ ^{low}; 5% CD4⁺CD8⁻TCR- $\alpha\beta$ ^{high}; and 2%, CD8⁺CD4⁻TCR- $\alpha\beta$ ^{high}. In the spleen, 20% of total lymphocytes were CD4⁺ and 9%, CD8⁺. In lymph nodes, 48% were CD4⁺ and 23%, CD8⁺.

Potential alterations in B cell development were studied by FCM analysis of cell suspensions from bone marrow, lymph nodes, spleen, and peritoneal cavity. By using mainly anti-B220, anti-IgM and anti-IgD Abs, we detected no significant differences in the proportions of individual B cell subsets when comparing mCD23, sCD23, and nontransgenic littermate mice (data not shown). Therefore, overexpression of neither mCD23 nor sCD23 resulted in obvious alterations in T and B lymphocyte development.

Basal levels of IgE and IgG1 levels in the transgenic mice

To test whether overexpression of either mCD23 or sCD23 altered basal IgE levels in serum, these levels were determined in nonimmunized transgenic and control littermate mice kept under standard nonsterile housing conditions. Transgenic mice had basal serum IgE levels (IgG1 levels also are shown for reference) similar to those of control littermates (IgE, approximately 100 to 300 ng/ml and IgG1, approximately 200 to 500 mg/ml; data not shown). Therefore, in response to environmental agents present in conventional animal housing, mCD23 and sCD23 transgenic mice secreted expected basal levels of IgE and IgG1.

Polyclonal serum IgE and IgG1 levels during infection with Nb

Both mCD23 and sCD23 have been postulated to modulate IgE regulation (7–12). Because Th2-like responses stimulate high levels of IgE production in vivo, both primary and secondary Th2 responses were elicited by infecting transgenic and control littermate mice with Nb. This parasite elicits a response that mainly is characterized by a Th2-cell-dependent secretion of IL-4 and IL-5, a production of high levels of IgE and IgG1 Abs, and eosinophilia (48). In three independent series of experiments, four transgenic and four control mice (8 to 16 wk of age) were infected with 750 Nb L3-stage worms each. Mice were bled at days 7, 10, 14, 21, 28, and 35

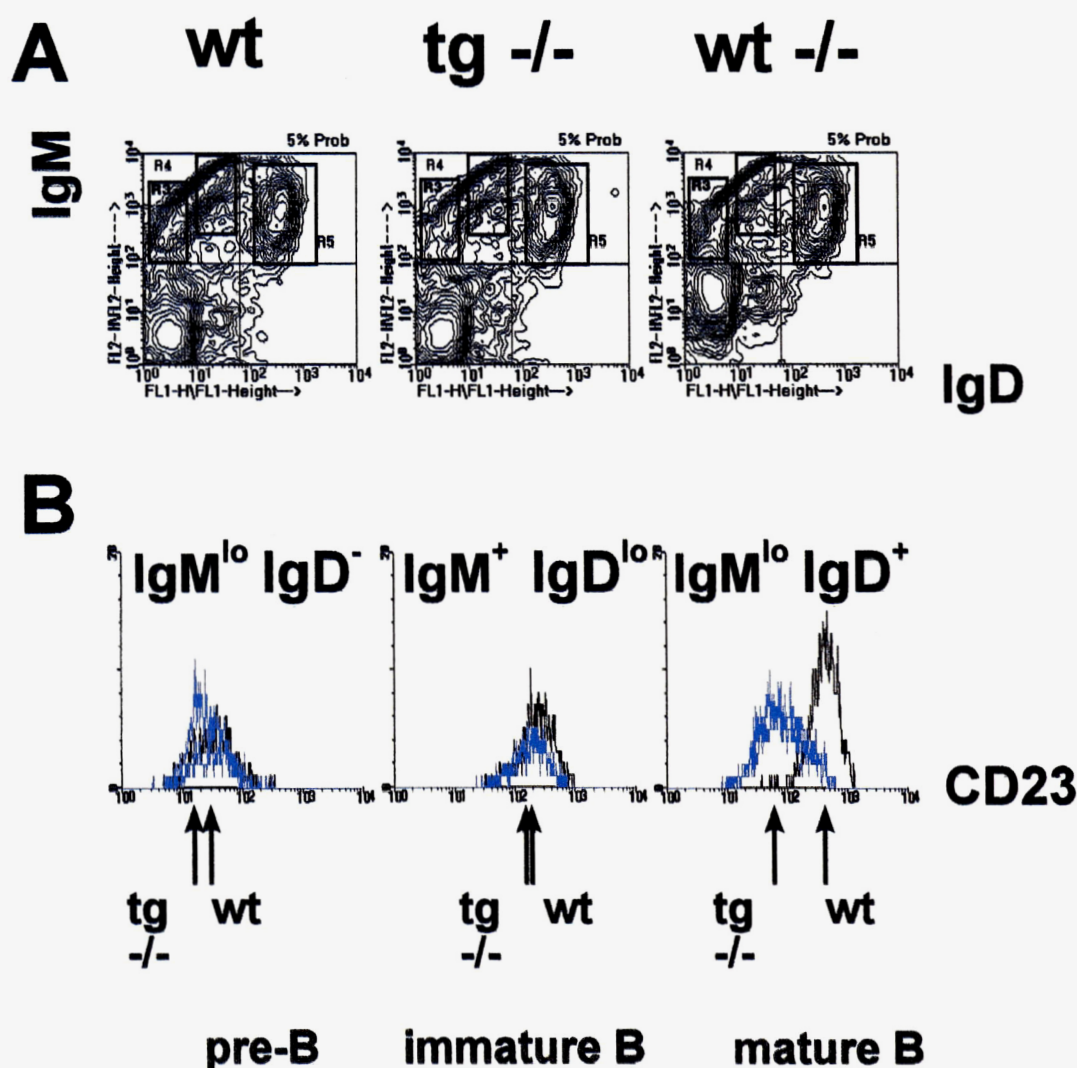
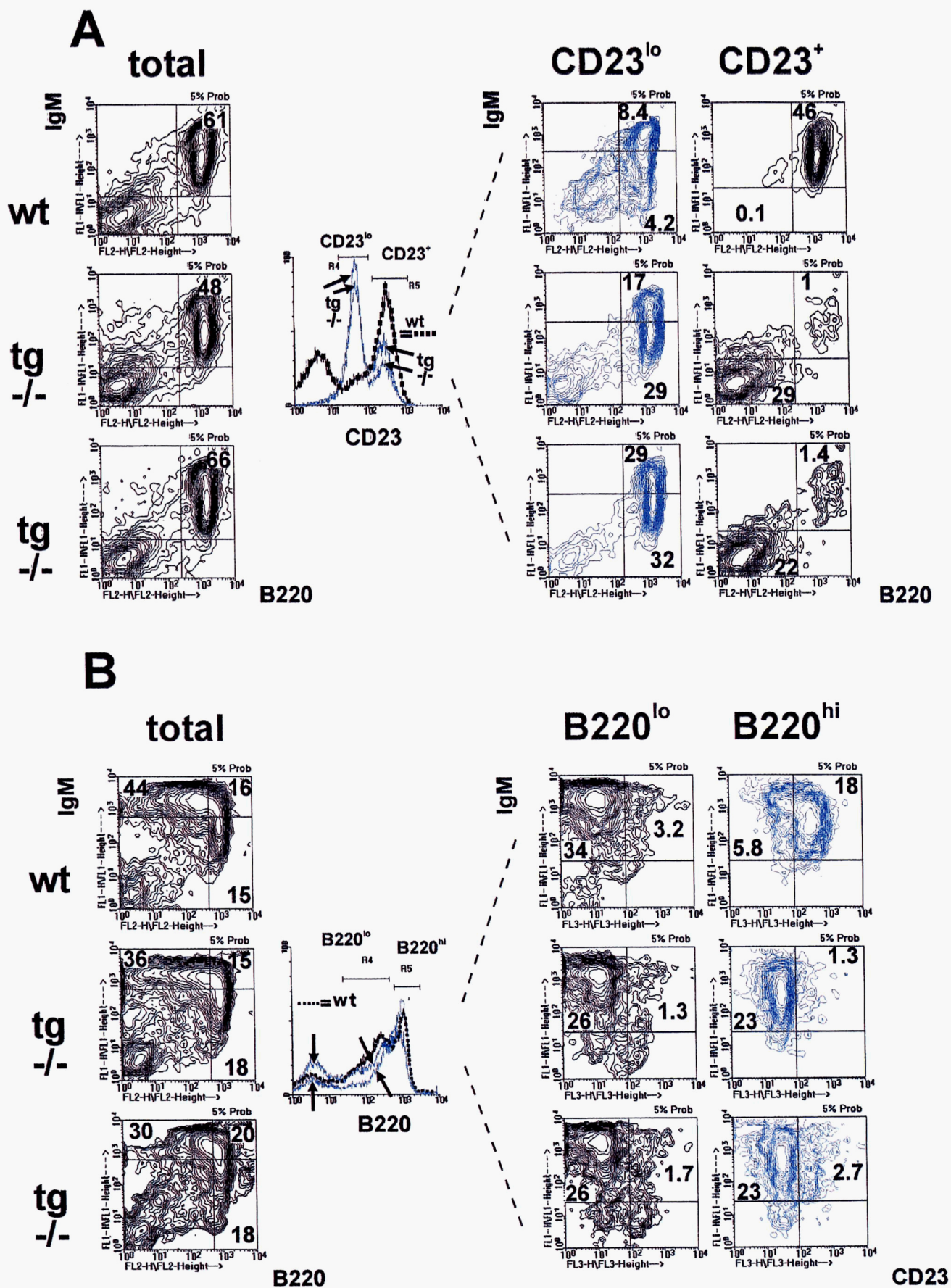


FIGURE 5. Expression of transgene mCD23 expression in bone marrow. Bone marrow cells were stained for IgM (goat anti-mouse IgM, PE), IgD (sheep anti-mouse IgD, FITC), and CD23 (B3B4, biotin) and counter-stained with RED670 coupled to streptavidin. **A:** cells pooled from two nontransgenic mice (wt) were compared with cells pooled from two mCD23 $^{+/-}$ CD23 $^{-/-}$ mice (tg $-/-$) and with cells pooled from two nontransgenic CD23 $^{-/-}$ mice (wt $-/-$). In **B**, gated $\text{IgM}^{\text{lo}}\text{IgD}^-$ (R3), $\text{IgM}^+\text{IgD}^{\text{lo}}$ (R4), and $\text{IgM}^{\text{lo}}\text{IgD}^+$ (R5) subsets were analyzed for mCD23 expression. In each case, the *left* arrow indicates the position of the peak obtained for cells from mCD23 $^{+/-}$ CD23 $^{-/-}$ mice (tg $-/-$), and the *right* arrow indicates the position of the peak in nontransgenic CD23 $^{+/+}$ (wt) mice. The *rightmost* panel clearly shows that, in mature $\text{IgM}^{\text{lo}}\text{IgD}^+$ cells from mCD23 $^{+/-}$ CD23 $^{-/-}$ mice (tg $-/-$), transgene-derived mCD23 expression is lower as compared with endogenous CD23 gene-driven mCD23 expression. Staining profiles for cells from CD23 $^{-/-}$ mice yielded, as expected, background signals only (data not shown). Previously, we showed that B cells in CD23 $^{-/-}$ mice are completely devoid of mCD23 expression (10).

after inoculation. Total serum IgE and IgG1 levels were measured and blood eosinophils were counted. Typically, an infection with Nb elicits an increase in serum IgE levels of ~ 100 -fold, and IgG1 levels increase ~ 10 -fold. Interestingly, mCD23 mice displayed two- to ninefold lower serum polyclonal IgE (Fig. 7A) and 1.5- to 4-fold lower IgG1 levels, as compared with control littermates (B). Similar effects were not seen in sCD23 mice (Fig. 7, A and B). Therefore, when overexpressed on T cells and subsets of B cells, mCD23, but not sCD23, can lower serum levels of polyclonal IgE and IgG1. Eosinophilia was as pronounced in mCD23 mice as in control littermates (data not shown).

To monitor whether similar effects occurred in a secondary response, four mCD23 mice and four control littermates were reinoculated with Nb 30 days after a first challenge. Up to 6 days after the second challenge, both groups of mice displayed similar serum IgE levels (Fig. 8A); but, by day 11, IgE levels in mCD23 mice were two- to threefold lower as compared with control littermates (in which IgE levels remained similar to those detected at day 6; Fig. 8A). Serum IgG1 levels in mCD23 mice also proved to be lower at all time points measured (Fig. 8B). Eosinophilia, which was similar in mCD23 and control mice during the primary response, was more pronounced



in the secondary response in mCD23 mice as compared with control littermates (Fig. 8C). Irrespective of a more pronounced eosinophilia, also in the secondary response, constitutive overexpression of mCD23 lowered serum levels of IgE and IgG1.

Polyclonal serum IgE and IgG1 levels in anti-IgD-immunized mice

Although several observations have suggested an involvement of CD23 in IgE regulation (7–12), no evidence for a modulatory role with regard to IgG1 was evident. To challenge the above result, which suggested such a role, an immunization protocol was applied with use of anti-IgD Abs. Typically, this type of challenge induces IL-4 production by Th2 cells, thereby, leading predominantly to increased serum IgE and IgG1 levels. The IgG1 response is more pronounced after an infection with Nb (30). Nontransgenic mice injected with 500 μ g of rat anti-mouse IgD displayed an expected 20- to 50-fold increase in serum IgE and IgG1 levels, whereas mCD23 mice showed only a 5- to 15-fold increase in these Ig isotypes (Fig. 7, C and D). Hence, also in this immune response paradigm, mCD23 mice did not reach expected serum levels of polyclonal IgE and IgG1. Most important, these results confirm an effect of excessive mCD23 expression, reducing not only polyclonal serum IgE, but also IgG1 levels.

Immune responses to a carrier hapten

To analyze the effects of constitutive overexpression of mCD23 on immune responses that provoke increased expression of isotypes other than IgE and IgG1, mice were injected with 100 μ g BPO-KLH alum. Serum samples were collected at days 7, 14, 21, and 28 post-inoculation. To generate a strong Ig response, a second challenge with BPO-KLH alum was given 28 days later, and Ig responses

were studied by measuring total polyclonal IgE, IgG1, and IgM levels in serum. As expected, primary immunization elicited a very weak response in both the mCD23 and the nontransgenic littermate mice, and the second immunization triggered a more significant Ig response. Again, mCD23 mice showed a less significant and subnormal increase in serum IgE (20-fold), whereas the response in the control littermates was as expected (100-fold) (Fig. 9A). Unlike those seen during the immune responses to Nb and anti-IgD, no significant differences were seen when comparing serum IgG1 levels in mCD23 and control littermate mice (Fig. 9B), and IgM levels also were similar (C). These results show that 1) constitutive overexpression of mCD23 can lower serum levels of IgE during a conventional immune response; and 2) parameters that are influenced by mCD23 overexpression and that potentially modulate IgG1 levels are different in this response from those operating in the responses to Nb and anti-IgD, respectively.

In vitro IgE and IgG1 production by splenic B cells

An overexpression of mCD23 on B and T lymphocytes could interfere with several normal functions exerted by these cells including, for example, cell-cell interactions between Th and B cells, which are necessary to trigger IgE and IgG1 Ab responses. To determine whether lymphoid cells from mCD23 transgenic mice respond normally in vitro, spleen cells from control littermate and mCD23 mice were cultured on anti-CD3-coated plates. In the presence of IL-2, IL-4, IL-5, IL-6, and anti-IFN γ , the production of IgE and IgG1 proved to be normal in spleen cell cultures of mCD23 transgenic mice, as compared with nontransgenic littermates (Table II). These results strongly argue against the hypothesis that mCD23 overexpression on either B or T cells impairs cell-cell interactions to trigger IgE and IgG1 responses.

FIGURE 6. Transgene mCD23 expression in spleen and peritoneal B cells. Spleen cells (A) and peritoneal cells (B) were stained for IgM (goat anti-mouse IgM, FITC), B220 (RA3-6B2, PE), and CD23 (B3B4, biotin) and counter-stained with RED670. In both A and B, the *upper* contour plots represent analysis of cells pooled from two nontransgenic CD23^{+/+} mice (wt). Sets of *middle* and *lower* contour plots each represent analysis of cells from one mCD23^{+/+}-CD23^{-/-} mouse (tg -/-). The *middle* panel shows spleen cells gated according to their mCD23 expression levels (mCD23^{low} vs mCD23^{high}), and these respective populations are indicated by horizontal bars. The blocked line shows the distribution of CD23 on cells pooled from two nontransgenic CD23^{+/+} mice (wt). Each of the two other lines (indicated by arrows) represents a CD23 distribution profile on cells of an individual mCD23^{+/+}-CD23^{-/-} mouse (tg -/-); IgM vs B220 contour plots are shown on the *left*. CD23^{low} and CD23⁺ gated subsets were analyzed for IgM vs B220 expression, as shown in the contour plots on the *right*. FCM shows that all B cells that express transgene mCD23 in a CD23^{-/-} background contain low levels of mCD23 on their cell surface. High levels of mCD23 are found only on B220⁻ cells (i.e., T cells). In B, the *middle* panel shows peritoneal cells gated according to their B220 expression levels (low vs high, and the respective populations indicated by horizontal bars). Arrows indicate two separate staining curves, each representing the distribution of B220 on cells of one of two individual mCD23^{+/+}-CD23^{-/-} mice. The blocked line represents cells pooled from two nontransgenic CD23^{+/+} mice. Next, B220^{low} and B220^{high} populations from the two pooled nontransgenic CD23^{+/+} mice and each mCD23^{+/+}-CD23^{-/-} mouse separately were analyzed for IgM and mCD23 expression, as shown in the contour plots on the *right*. B220^{low} cells in neither nontransgenic nor mCD23^{+/+}-CD23^{-/-} mice expressed mCD23.

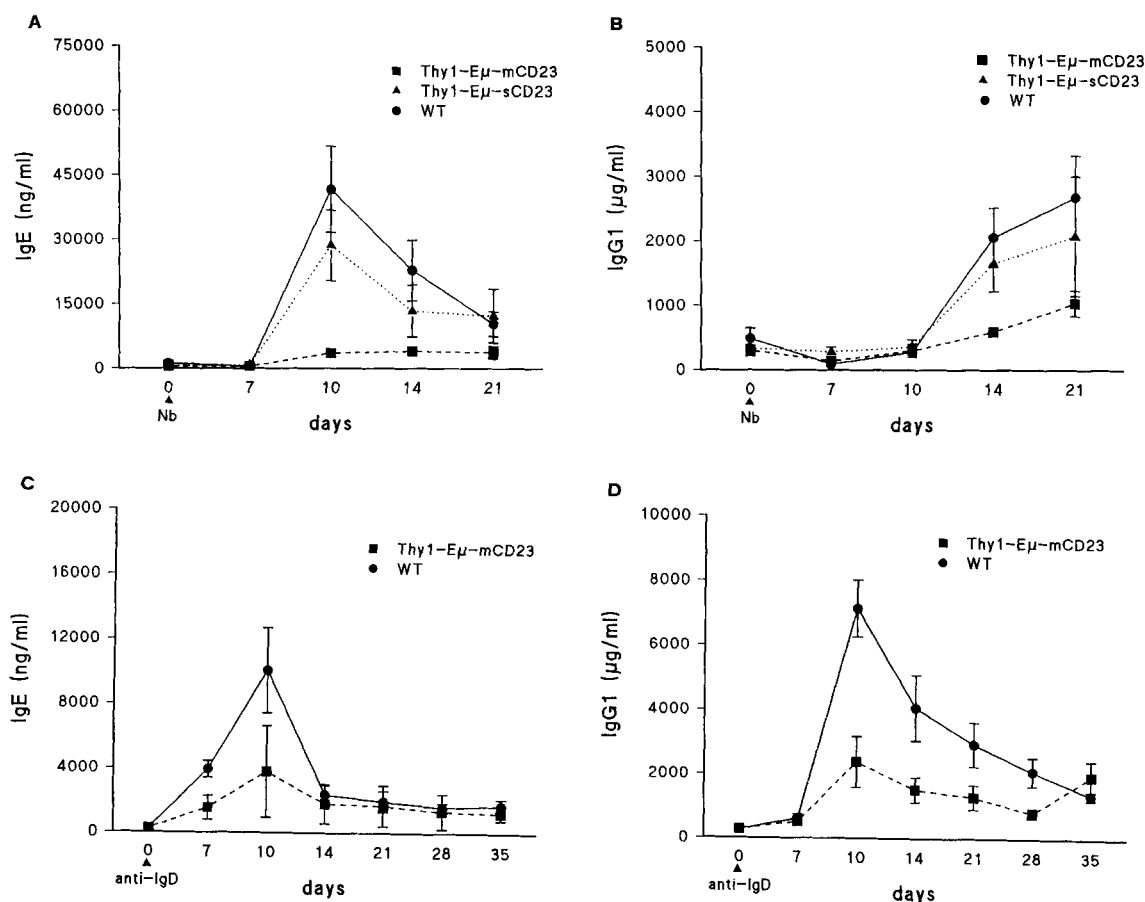


FIGURE 7. A and B: serum levels (measured by ELISA) of IgE and IgG1 in mice after an infection with Nb. The infection time point is indicated by an arrow and Nb. IgE and IgG1 responses in Thy1-Eμ-mCD23 (2431L), Thy1-Eμ-sCD23 (2727L), and control littermate (WT) mice are compared. Each time point corresponds to the mean of four mice $\pm$ SEM, and the data are representative of three series of experiments. In each, four control and four transgenic mice were used. C and D: serum IgE and IgG1 concentrations (ELISA) in anti-IgD immunized mice. The time of challenge is indicated (arrow and anti-IgD). IgE and IgG1 levels in Thy1-Eμ-mCD23 (2431L) and control littermates (WT) are compared. Each time point corresponds to the mean of four mice $\pm$ SEM and the data are representative of two experiments.

Discussion

We have shown that overexpression of neither mCD23 nor a 38-KDa sCD23 form, mediated by Thy-1 regulatory sequences and the Eμ enhancer, alters to the normal composition of thymocyte, peripheral T cell, and B cell compartments to any significant extent. sCD23 has been proposed to act as a differentiation and proliferation factor for human thymocytes (in concert with IL-1 α) and T lymphocytes (17, 42). Our present and previous findings in CD23 $^{-/-}$ mice (10) do not support such a role in mice. In addition, others have been unable to detect an effect of sCD23 on murine thymocyte maturation, even when IL-1 α was added to the system (43). A discrepancy of CD23 function in mouse and human could suggest some species-specific biologic activities of the molecule that possibly are related to differences in structure and cellular distribution in these species. Expression of genetically engineered

human-mouse hybrid CD23 molecules in a CD23 $^{-/-}$ background might answer some of these questions.

The results from three immune response paradigms suggest that overexpression of mCD23, but not sCD23, alters, in a quantitative fashion, the outcome of IgE in all three and of IgG1 in two of the three immune responses. The *in vitro* assay that monitored Ig production suggests that interactions between T and B cells of transgenic mice are not impaired. IgE exhibits a $t_{1/2}$ of only 6 to 12 h, as opposed to weeks for other isotypes (44), and it could be argued that mCD23 overexpression promotes enhanced clearance of IgE. mCD23 binds IgE, albeit with low affinity ($K_a = 10^7$ to 10^8), and can mediate internalization and degradation of IgE (45). Nevertheless, enhanced clearance of IgE in mCD23 mice seems unlikely. First, basal IgE levels in mCD23 and control littermate mice are similar. Second, in two of the three immune response paradigms IgG1 levels also proved below normal,

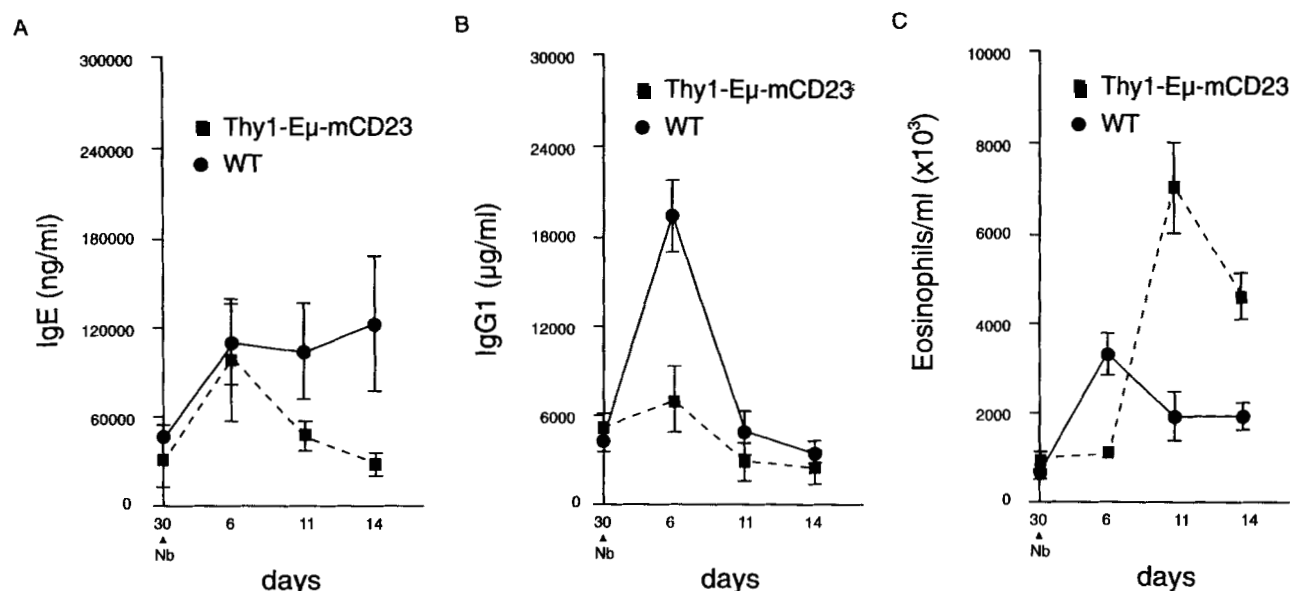


FIGURE 8. Secondary responses showing IgE (A), IgG1 levels (B), and eosinophilia (C) after a second challenge with Nb. Infection time points are indicated (arrows and Nb). Ig levels in Thy1-E μ -mCD23 (2431L) and control littermates (WT) are shown. ($n = 4 \pm$ SEM). These data represent a single experiment.

and IgG1 cannot bind mCD23 (34). Third, mice that lack a functional CD23 gene do not show a significant increase in basal levels of IgE or polyclonal serum IgE after immune challenge with Nb (10, 12). Finally, CD23^{-/-} mice show no measurable alterations in the speed of IgE clearance (12).

A differential effect of mCD23 overexpression on IgE and IgG1 responses is in agreement with the existence of different pathways and factors to regulate IgE and IgG1 responses (46, 47). Nb elicits an immune response with Th2-cell characteristics, which is accompanied by a strong T cell-independent nonspecific activation of B cells (48–50). Injection of anti-IgD also increases IL-4 production, thus, eliciting a Th2-like response. The hapten-carrier adjuvant BPO-KLH (Alum) induces expression of a cytokine pattern that is characteristic of a mixed Th1 and Th2 response (51). IL-4 is critical in IgE, but dispensable for IgG1 production (52, 53), and the production of IgG1 can be regulated by both Th2 and Th1 clones (54). In regard to the distinct effects on IgG1 levels in the three immune responses, our findings seem to concur best with the hypothesis that mCD23 overexpression mainly modulates the Th2 cell-driven pathway. Whether this relates to an altered production of cytokines, such as IL-4 and IL-13 *in vivo*, is not clear as yet. Both cytokines up-regulate CD23 expression (55), and excess mCD23 could have a negative feedback on their synthesis and impair Th2 function (55, 56). In any event, a general impairment of Th2 function in mCD23 mice seems to be unlikely. IL-5 levels, as indirectly reflected by the extent of eosinophilia, seemed to be normal in the primary response to Nb and even increased twofold during the secondary response.

In mCD23 mice, alterations in IgE and IgG1 production could be a result of: 1) increased expression of mCD23 on B cells; 2) a constitutive, as opposed to an otherwise rare and mostly transient, expression on T cells; or 3) a combination of these expression patterns. mCD23 has been shown to participate in Ag processing and presentation in both murine (19) and human B lymphocytes (20). Although enhanced expression of mCD23 on mature B cells might increase the effectiveness of this process, we believe that this hardly can account for our observations, in particular, those regarding the findings in CD23^{-/-} mice (10, 12). Alternatively, and in agreement with most findings, the negative effect of mCD23 overexpression on Ig production could be accounted for by CD23 being a negative feedback regulator for IgE synthesis, a process that has been proposed to be mediated specifically by IgE-containing immune complexes (12). Cross-linking of mCD23 with IgE or anti-CD23 Abs down-regulates IgE secretion (8, 9, 13), and CD23^{-/-} mice showed increased Ag-specific IgE levels (12). However, it also has been hypothesized that IgE-Ag complexes that bind to mCD23 on B cells could promote cellular activation, polyclonal B cell proliferation, and a production of hypergammaglobulinemia (3). In parasitic infections, the latter would include parasite-specific IgG that could compete effectively with IgE for Ag and prevent anaphylaxis. CD23 is a ligand for CD21, and anti-CD21 Ab induces IgE production (14). Finally, co-cross-linking of sIg and mCD23 on B cells can generate either an activating or an inhibitory signal (57, 58). The outcome seems to depend on the cross-linking system used and is governed, most likely, by the level of

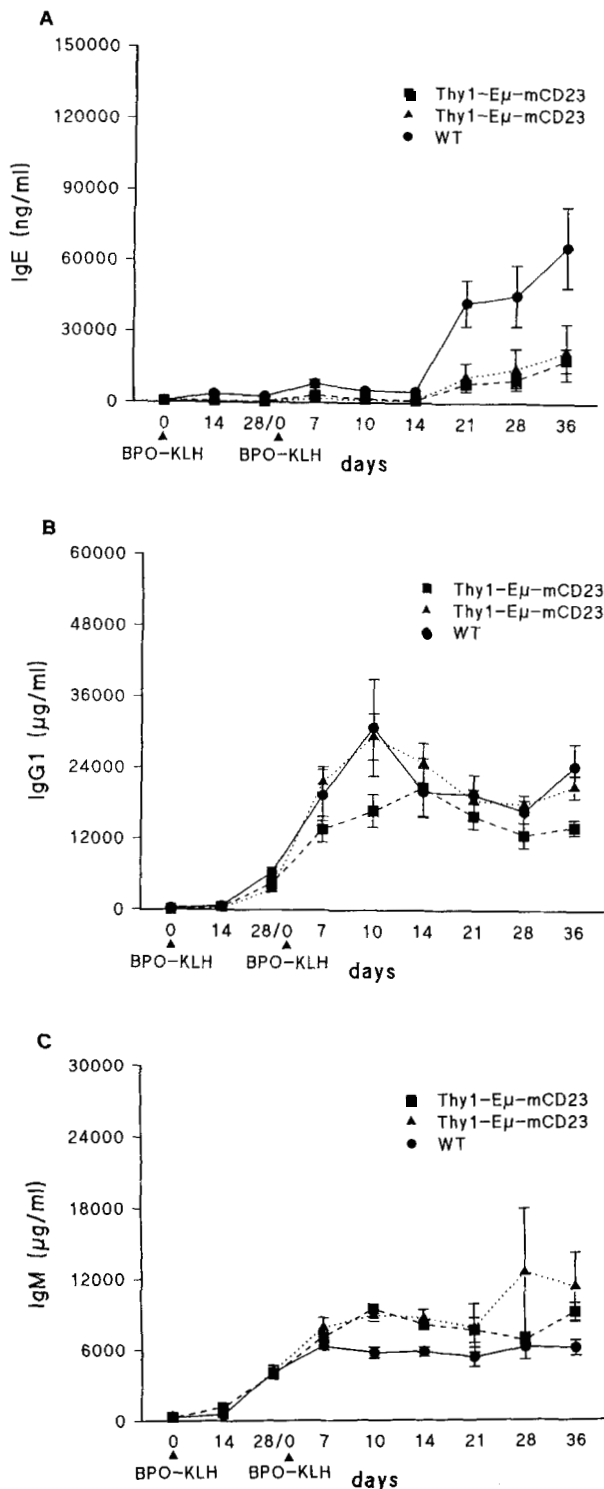


FIGURE 9. Serum levels of IgE (A), IgG1 (B), and IgM (C) in BPO-KLH (Alum)-immunized mice. Ab concentrations were measured by ELISA, and the values obtained in both a primary and the secondary response are shown. The two immunization time points are indicated (arrows and BPO-KLH (Alum)). The response of Thy1-E μ -mCD23 (2431L and 2435L) and control littermate (WT) mice is compared. Each time point corresponds to the mean of four mice $\pm$ SEM, and these data are from a single experiment.

Table II. *In vitro* IgE and IgG1 production^a

Mice	IgE (ng/ml)	IgG1 (μ g/ml)
Thy-1-E μ -mCD23	4437	1872
WT	3276	1404

^a *In vitro* IgE and IgG1 production by spleen cells from Thy-1-E μ -mCD23 and wild-type (WT) mice. The data are representative of three experiments.

co-cross-linking. Low levels would either not influence nor, alternatively, enhance B cell activation, whereas extensive cross-linking would decrease B cell activation. The latter could be favored in mCD23 mice inasmuch as B cells display a higher density of mCD23 molecules on their cell surface. Findings after Nb infestation and that could concur with this model are 1) a reduced hypergammaglobulinemia in mCD23 mice as compared with control littermates, and 2) slightly increased levels of polyclonal serum IgE in CD23^{+/−} mice (10).

Other findings (5) also could prove to be relevant with respect to mCD23 overexpression reducing IgE and IgG1 levels in serum. mCD23 expression is low or absent on virtually all B cells that express mIgG1, IgG2a, and IgE. After injection of a goat anti-mouse IgD Ab, up to 50% of mIgG1⁺ B cells have been found to express mCD23 (5). These mCD23⁺ cells were found to secrete little or no IgG1 *in vitro* in contrast with their mCD23[−] sIgG1⁺ counterparts. Hence, mCD23 expression on γ 1-switched B cells seems to correlate with a lack of IgG1 production. Thus, transgene expression on γ 1 B cells in mCD23 mice (provided it occurs) could provide another explanation for lowering IgG1 levels. It remains to be seen whether mCD23 expression on ϵ -switched B cells could cause similar effects on IgE production. If so, two findings could be relevant: 1) IL-4 can induce Thy-1 expression on B cells (59); and 2) Thy-1⁺ B cells selectively secrete IgE (41, 60). Although we cannot exclude the possibility that such mechanisms play a role in reducing increased IgE levels in mCD23 mice after immune challenge, the *in vitro* Ig production results (Table II) do not seem to favor such a hypothesis. Less specific effects of transgene expression on IgE production also seem to be unlikely, inasmuch as overexpression of sCD23 had no effect on serum IgE.

Finally, mCD23 mice contained significant numbers of mCD23⁺ peripheral CD4⁺ and CD8⁺ T cells. It is tempting to speculate that these cells also might contribute to reducing the magnitude of IgE responses *in vivo*. A regulatory mechanism has been identified that depends on CD8⁺CD23⁺ T cells that occur in mice bearing the IgE-secreting hybridoma B53 (61). These cells can induce specific repression of the Ig heavy chain enhancer, thereby resulting in a suppression of ϵ heavy chain gene transcription (62–64). As for other FcRs, a transient expression of mCD23 on activated T cells seems to be able to regulate Ig-isotype expression (3). With regard to such mechanisms, it is certainly surprising that a constitutive overexpression of mCD23 on

T cells in mCD23 mice does not compromise the immune system in any serious and obvious fashion. In any case, the transgenic mice described in this paper will serve to further elucidate functions of CD23 in vivo, and mCD23⁺ T and B cells from these mice will serve as invaluable tools to study mechanisms by which mCD23 expression on each of these cell types can influence Ig synthesis.

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