

# Mice Deficient in CD23 Reveal Its Modulatory Role in IgE Production but No Role in T and B Cell Development

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To assess roles of CD23 in lymphocyte development and immune function in vivo, CD23-deficient mice (CD23<sup>-/-</sup>) were generated. Mice heterozygous with respect to the defective allele (CD23<sup>+/-</sup>) display 50% reduced levels of CD23 expression on CD23<sup>+</sup> cell types. This pattern is consistent with a lack of parental or tissue-specific imprinting of the CD23 gene. Neither a 50% reduced level nor a complete lack of CD23 caused profound changes in lymphocyte compartments (thymocytes, peripheral T cells, and B-1 and B-2 B cells). The lack of CD23 also did not significantly alter in vitro the proliferative response of B cells triggered via the Ag receptor in combination with CD40 ligand, IL-2, and/or IL-4. Effects on polyclonal Ig production were tested in a Th2 cell-driven immune response in vivo after infection with *Nippostrongylus brasiliensis*, a parasite that dramatically enhances CD23 expression on B cells. In both primary and secondary immune responses, heterozygous CD23<sup>+/-</sup> mice developed slightly higher and CD23<sup>-/-</sup> mice similar serum IgE and IgG1 levels as compared with CD23<sup>+/+</sup> (wild-type) mice. The increase in blood eosinophil counts was similar in all three types of mice. These findings show that after *N. brasiliensis* infection 1) a complete lack of CD23 in vivo neither prohibits nor significantly alters quantitatively polyclonal IgE levels in serum, and 2) a 50% reduction in cell-surface CD23 expression (CD23<sup>+/-</sup> mice) correlates with slightly increased serum IgE levels. *Journal of Immunology*, 1994, 152: 3378.

In mice and humans, the expression of the type II membrane glycoprotein CD23 (mCD23)<sup>3</sup> is restricted to a number of, in part, common but also different cell types. In mice, CD23 is expressed on follicular dendritic cells (FDC), thymic asteroid dendritic cells near the corticomedullary junction, and mature IgM<sup>+</sup>IgD<sup>+</sup> B cells. CD23 is absent on B-1 cells including the Ly-1<sup>+</sup> as well as the Ly-1<sup>+</sup>Mac-1<sup>+</sup> "sister" cells, immature B cells, and marginal zone B cells in spleen (5 to 10% of splenic B cells) (1–7). mCD23 can also be detected on certain subsets of T cells including freshly isolated CD4<sup>+</sup>CD8<sup>-</sup>γδ T cells from skin and subsets of CD4<sup>+</sup> and/or CD8<sup>+</sup> T lym-

phocytes after activation (8). Triggering via the CD3/TCR complex, IgE, phorbol ester (tetradecanoyl phorbol acetate), PHA, and IL-4 can up-regulate CD23 expression in some T cells in vitro (7, 9–12). In humans also, monocytes, eosinophils, platelets, and Langerhans' cells can express CD23 (13).

The expression of CD23 on lymphocytes is strikingly altered in a number of diseases. In human, these include allergic disorders, hyper-IgE syndrome, chronic lymphocytic leukemia, and human T cell lymphotropic virus infection (for review see Ref. 13). Mice infected with *Leishmania donovani* show decreased levels of CD23 and sIgM on splenic B cells 24 h after infection. This parasite also provokes a polyclonal B cell deficiency (10, 11). Infection with the extracellular protozoan *Trypanosoma brucei* also causes a marked reduction in B cell CD23 and sIgM (11). In contrast, mice challenged with *Nippostrongylus brasiliensis* (Nb) display high levels of IgE and B cells expressing CD23 (14). Mice that received multiple injections of an anti-CD3 Ab or a superantigen (SEB) have been reported to cause some CD4<sup>+</sup> T cells to express high levels of CD23 and/or FcγRII (11). Large numbers of CD23<sup>+</sup>CD8<sup>+</sup> T cells have been shown to occur in mice

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<sup>3</sup> Abbreviations used in this paper: mCD23, membrane CD23; sCD23, soluble CD23; ADC, asteroid dendritic cells; slg, surface Ig; PE, phycoerythrin; FDC, follicular dendritic cells; ES, embryonic stem; HSA, heat-stable Ag; Nb, *Nippostrongylus brasiliensis*; FCM, flow cytometry; GANC, ganciclovir.

bearing an IgE-secreting hybridoma (for review see Reference 11). Because the expression of CD23 can occur in multiple cell types in both mice and humans ligands of CD23 including IgE (15, 16) and CD21 (17), connect, potentially, to a multiplicity of cells and their specialized functions. How CD23 expression in these different cell types and alterations in CD23 expression relate to disease and specific functions of CD23 in each cell type is largely an enigma. Functions ascribed to CD23 and/or soluble forms derived by proteolytic cleavage of the membrane form include a role in B cell growth (18, 19), germinal center B cell survival (20), prothymocyte maturation (21), myeloid precursor proliferation (22), Ag presentation (23–25), IL-4 induced IgE production (17) and, possibly, a role in protecting cells from oxidative stress (26).

To unravel functions of CD23 related to lymphocyte development and immune function *in vivo*, we are exploiting a dual experimental approach. Transgenic mice expressing either a 38 kDa sCD23 or mCD23 constitutively in most thymocytes and subsets of peripheral T cells lack profound changes in their thymocyte and T and B cell compartments. Upon immunologic challenge with either Nb, anti-IgD, or BPO-keyhole limpet hemocyanin-alum, agents known to elicit a typical Th2, a Th2-like (without an IL-5 component), and a mixed Th1+Th2-type response, respectively, mCD23 but not sCD23 transgenic mice, displayed lower overall polyclonal IgE levels in serum. IgG1 levels were also found below normal after challenge with either Nb or anti-IgD but not when immunized with benzyl-penicilloyl-keyhole limpet hemocyanin-alum. The IL-5-dependent increase in the number of blood eosinophils that accompanies infections with Nb was similar in mCD23 transgenic and control mice (Texido et al., submitted for publication).<sup>4</sup> These down-modulating effects of excess mCD23 expression on polyclonal serum Ig levels led us to further elucidate roles of CD23 *in vivo*. For this purpose we generated mice lacking a functional CD23 gene and studied lymphocyte development and immune function in these mice.

## Materials and Methods

### Construction of the targeting vector

The targeting vector contains a 5.85-kb *XbaI-ScaI* genomic CD23 gene-derived DNA fragment comprising exons 10, 11, and 12 (27). A Neo expression cassette (PyTKNeoA) (28) was used to replace an 800-bp fragment comprising part of exon 10, all of exon 11, and part of exon 12 in the mouse CD23 genomic locus. The replaced region encodes the lectin-homology domain including the sequences that bind the Cε domain of IgE (29). The Neo cassette was flanked 5' and 3', respectively, by 4.1 kb and 0.8 kb of genomic sequences derived from the mouse 129/SvJ CD23 gene. The CD23 gene was cloned from a 129/SvJ genomic DNA library to promote high targeting frequencies that have been reported when using isogenic DNA (30). A Herpesvirus thymidine kinase gene (31) driven by the human CMV promoter (32) was inserted

at the 3' end. Before electroporation, vector DNA was linearized using *XbaI* digestion.

### Cell culture

CCE embryonic stem (ES) cells (33) were maintained on mitomycin-treated STOne<sup>+</sup> fibroblast feeder cells (34). In addition, the medium was supplemented with 1000 U/ml leukemia inhibitory factor (AmRAD Co. Ltd, New Victoria, Australia). Electroporations were performed as follows. Cells were collected by trypsinization, washed once, and resuspended in ES cell medium at a concentration of  $4.10^7$  cells/0.8 ml. This cell suspension was mixed with 100 μg DNA ( $25 \mu\text{g}/10^7$  cells) of linearized targeting vector DNA. Electroporation was carried out by using a Bio-Rad (Richmond, CA) Gene Pulser apparatus at 250 V and 500 μF. Cells were plated onto 10-cm feeder plates ( $\sim 2.5 \times 10^6$  cells/plate). Selection was applied 24 h after electroporation with medium containing G418 (GIBCO BRL, Grand Island, NY) at 300 μg of active ingredient/ml and  $2 \times 10^{-6}$  M GANC (31). Cells were refed with fresh drug-containing medium every day. Individual colonies were picked 10 to 14 days after the start of the selection and transferred to 24-well trays for expansion. After a brief expansion an aliquot of each well was used to extract DNA and identify homologous recombinant clones.

### Identification of homologous recombinant clones

DNA was isolated from individual ES cell clones. After cell lysis nuclei were pelleted and, after SDS-proteinase K treatment, the DNA was precipitated, solubilized in water, and PCR analysis was performed with Perkin-Elmer Cetus kit (Norwalk, CN) and a Hybaid thermal cycler (annealing temperature: 63°C for 1 min and denaturation at 92°C for 2 s). Oligomers used for the specific amplification of a 1033-bp fragment indicative of the wild-type allele of mouse CD23 were: 5' = GACACCTCCTAATCACATCCTTTGACTGG and 3' = GGTCTGTAGACTGGGCTATGATGTGATGG. The same 3'-oligomer and a Neo gene-specific oligomer as 5'-primer (ATATTGCTGAAGAGCTTGGCGGCGAATGGG) were used to amplify a 1104-bp fragment indicative of a homologous recombination event. The latter was subsequently confirmed using Southern blot analysis. For this purpose we compared the *EcoRI* patterns of DNA isolated from the parental CCE-ES cell line and Neo<sup>+</sup> GANC<sup>+</sup> CCE-ES that yielded a positive signal with the second primer pair including the Neo-specific oligomer. Hybridizations were carried out using a *HindIII-SalI* DNA fragment (Fig. 1) detecting a region present in the endogenous CD23 gene but absent in the targeting vector CD23 homology region.

### Embryo manipulations, chimeras, germ-line transmission of the disrupted CD23 allele, and generating mice for immunization

Approximately 15 cells of CCE-ES clone 26 were injected into C57BL/6 host blastocysts (34). This yielded 14 chimeras (11 males and 3 females). All male chimeras were bred with C57BL/6 females and germ-line transmission of CCE-ES cell genome was scored by counting the number of agouti offspring from such matings. One of the chimeric males yielded one male and two female agouti offspring ( $F_1$ ), all three of which proved to contain a disrupted CD23 allele using the criteria described in the previous section. The three  $F_1$  mice were interbred to generate  $F_2$  animals which, with respect to the CD23 allele, were either wild type (CD23<sup>+/+</sup>), heterozygous carrying one knockout allele (CD23<sup>+/-</sup>), or homozygous (CD23<sup>-/-</sup>). CD23<sup>-/-</sup>, CD23<sup>+/-</sup>, and CD23<sup>+/+</sup> litter mates of the  $F_2$  generation were used for FCM analysis, infection with Nb, and the other analysis described in this report. Homozygous  $F_2$  CD23<sup>-/-</sup> female and male mice have subsequently been used to generate a stock of CD23<sup>-/-</sup> mice.

### Infection with Nb

$F_2$  generation mice comprising 5 CD23<sup>-/-</sup>, 10 CD23<sup>+/-</sup>, and 5 CD23<sup>+/+</sup> mice at age 8 wk were injected s.c. with 750 third stage (L3) larvae of Nb.

### Quantification of blood eosinophilia

Blood obtained by tail vein bleeding was used for blood smears. Smears were fixed and stained with the rapid differential hematology-staining kit

<sup>4</sup> Texido, G., H. Eibel, G. Le Gros, and H. van der Putten. Transgene-mediated expression of CD23 on lymphoid cells modulates IgE and IgG1 responses. Submitted for publication.

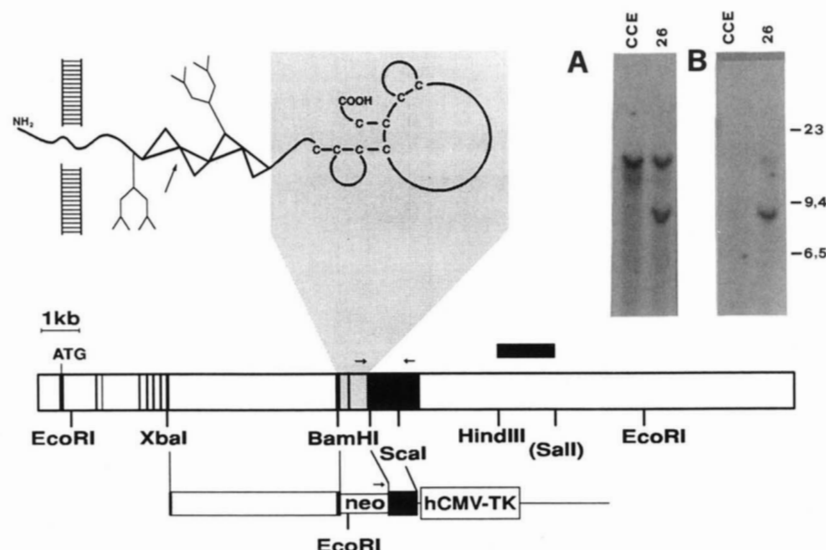


**FIGURE 1.** Targeting construct and homologous recombination at the CD23 locus. The restriction map of the targeting construct and the wild-type CD23 allele (coding exons 2 to 12) (27) is shown. Black boxes in the targeting vector and the CD23 locus represent exons. Part of exon 10, the complete exon 11, and part of exon 12 were replaced by the neo-expression cassette in the targeting vector. The deleted sequences encode the COOH-terminal part of CD23 encompassing the lectin homology indicated by the shaded area. The rest of the CD23 molecule including the position of the two glycosylated residues is shown for reference. The thin line flanking the human CMV-TK cassette represents plasmid vector sequences in the linearized targeting vector. The black box above the CD23 locus map and spanning sequences from an indicated *HindIII* to a *SalI* restriction site represents the probe used to diagnose homologous recombination events by Southern blot analysis. Arrows indicate the position of the PCR primers used for the primary screening of ES cell clones for homologous recombination events. A and B show Southern blot analysis indicative of a representative targeting event. Wild-type CCE and clone 26 DNA were restricted using *EcoRI*. Hybridization probes were the CD23 locus-specific *HindIII*-*SalI* probe (A) and a Neo probe (B).

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 RBC were counted by using the Sysmex Cell-Check-400 system.

#### Quantification of serum IgE and IgG1 levels

Ninety-six-well microtiter plates were coated with rat anti-mouse IgE (mAb 4B3-39; 0.25 µg/well) or anti-mouse IgG1 (Zymed, mAb 04-6100; San Francisco, CA; 0.1 µg/well). To quantify total serum IgE and IgG1, serum and standard dilutions were incubated overnight at 4°C. After washing in PBS, Ig bound to the first Ab was detected using biotinylated anti-mouse IgE (mAb ab-3-11) or anti-mouse IgG1 (Zymed, mAb 04-6140) and subsequent incubation with alkaline phosphatase-avidin conjugate. The enzymatic reaction was performed by adding *p*-nitrophenyl phosphate disodium (159 µg/well) and the OD was measured at 405 nm on a microplate reader (Bio-Rad 405). Titers were determined at the points on the linear part of the titration curve.

#### Northern blot analysis

Total RNA was extracted from spleen tissue using the guanidinium isothiocyanate procedure. Ten micrograms of total spleen RNA from CD23<sup>+/+</sup>, CD23<sup>+/-</sup>, and CD23<sup>-/-</sup> mice were analyzed by Northern blotting using a mouse CD23 cDNA probe (35).

#### Immunocytochemistry

Cryostat sections (10 µm) were fixed either in acetone (10 min at -20°C) or 2% paraformaldehyde (4 min, 4°C). MCD23 protein expression was monitored using the B3B4 rat anti-mouse CD23 Ab essentially as described previously (36, 37).

#### FCM analysis

FCM analysis were carried out on a Becton Dickinson (Palo Alto, CA) FACScan and LYSYS II software and evaluated with PC-LYSYS on a personal computer. FCM analysis were performed as described previously (38) using the following biotinylated, FITC or PE-labeled mAbs: B3B4 for CD23, RA6-B2 for B220, AF6-120.1 for IA<sup>b</sup>, H1.2F3 for CD69, 7D4 for the IL-2R, IM7 for Pgp-1 (all from PharMingen, San Diego, CA), GK1.5 for CD4, 53-6.7 for CD8 (both from Becton Dickinson), soluble CD40L (39) (donated by Dr. P. Lane) for staining CD40 and goat anti-mouse IgD antibodies to detect IgM/IgD expression (Southern Biotechnology Associates, Birmingham, AL). For two-color fluorescence biotinylated mAbs were counterstained with streptavidin-PE conjugate (Southern Biotechnology). Alternatively, streptavidin-TRI-color (Medac, Hamburg, Germany) was used.

#### Cell proliferation assays

B cells were enriched from spleen cells by eliminating monocytes by adherence to plastic and T cells by one or two rounds of a combined anti-Thy-1.2, anti-CD4 (GK1.5), anti-CD8 (19/178) and rabbit C treatment. Elimination of T cells and enrichment of B cells was controlled by FACS. Viable cells were isolated from the interphase of a 40% to 75% Percoll step gradient. A total of 10<sup>5</sup> cells was plated in quadruplicates into F96-well plates (Nunc, Roskilde, Denmark) and incubated for 3 days with goat anti-mouse IgM (Southern Biotechnology) at a final concentration of 12.5 (optimal) or 2 (suboptimal) µg/ml. The optimal concentration was determined first. Supernatant of a myeloma transfectant producing CD40L (40) was added as a 1/8 dilution. Again, the optimal concentration was determined in a previous assay. During the last 14 h, [<sup>3</sup>H]TdR (Amersham, Braunschweig, Germany) was added. Then cells were harvested and thymidine incorporation was measured using a LKB β-counter (LKB Products, Rockville, MD).

## Results

### Generation of mice with a disrupted *CD23* gene

The targeting vector (Fig. 1) includes a Neo expression cassette, which replaces coding sequences in exons 10, 11, and 12 of the mouse *CD23* gene. The deleted sequences encompass the coding region for the lectin-homology domain of *CD23* (shaded region of the protein in Fig. 1). This region is also responsible for binding to the  $\text{C}\epsilon$  part of mouse IgE (29). The homology regions of *CD23* genomic sequences in the targeting vector and the mouse *CD23* gene include 4.7 kb 5' and 0.9 kb 3' of the Neo cassette. The 3' homology region was flanked by a human CMV-Herpesvirus thymidine kinase expression cassette. After electroporation of the linearized construct into CCE-ES cells, positive/negative selection was applied with G418 and GANC. A total of 3 of 150 colonies (1 in 50) scored positive by a PCR assay. Homologous recombination at the *CD23* locus was confirmed by Southern blot analysis of genomic DNA from all three positive clones. The results of *Eco*RI restriction analysis of clone 26 CCE-ES cells are shown (Fig. 1A) using a probe that detects sequences present in the genomic mouse *CD23* but absent in the targeting vector. Hybridization of the blot with a Neo probe shows the presence of a single Neo copy at the targeted *CD23* locus (Fig. 1B) known to be located on mouse chromosome 8 (41). Both types of analysis reveal an *Eco*RI DNA fragment of approximately 8.5 kb diagnostic for a correct homologous recombination event.

ES cells from clone 26 were injected into C57BL/6 mouse blastocysts and gave rise to 14 (11 male and 3 female) chimeras. The male chimeras were crossed to C57BL/6 females. One male gave rise to 3  $F_1$  mice (1 male and 2 females) on a hybrid background. Each of these 3 mice carried one disrupted *CD23* allele ( $CD23^{+/-}$ ) detected by PCR and confirmed by Southern blot analysis (data not shown). The three  $F_1$   $CD23^{+/-}$  mice were interbred to generate several  $F_2$  generations. Each of these  $F_2$  generations contained mice homozygous, heterozygous, or null with respect to the knock-out *CD23* allele.  $F_2$  littermates representing each of these genotypes were used and compared in FCM analysis of thymocyte, T and B cell populations, the B cell proliferation assay, and the infection with Nb as described below. Neither mice with a  $CD23^{-/-}$  nor those with a  $CD23^{+/-}$  genotype displayed an overt phenotype.

To confirm that the knock-out allele was incapable of producing *CD23* gene products, Northern blot, immunocytochemical and FCM analysis was performed. Northern blot analysis of total spleen RNA of  $CD23^{+/+}$ ,  $CD23^{+/-}$ , and  $CD23^{-/-}$  mice revealed an approximately twofold reduction in *CD23* mRNA levels in  $CD23^{+/-}$  mice as compared with  $CD23^{+/+}$  mice and the complete lack of *CD23* transcripts in  $CD23^{-/-}$  mice (Fig. 2). FCM analysis of B cell populations in spleen (Fig. 3) and other lymphoid organs (data not shown) confirmed the absence of

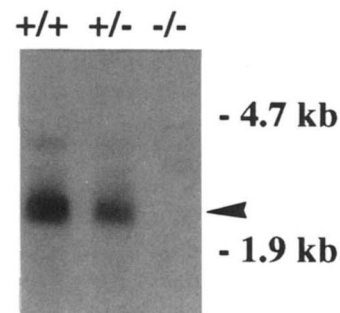


**FIGURE 2.** Expression of *CD23* mRNA in adult mouse spleen. Each lane represents the analysis by Northern blot of 10  $\mu$ g total spleen RNA extracted from either a  $CD23^{+/+}$ ,  $CD23^{+/-}$ , or a  $CD23^{-/-}$  mouse. The arrowhead indicates the expected position of *CD23* mRNA. For hybridization, a mouse *CD23* cDNA was used.

*CD23* protein on the surface of B cells in  $CD23^{-/-}$  mice (Fig. 3; upper). No noticeable differences were observed in  $B220^+$  B cell numbers or surface expression of IgM (Fig. 3, lower). Also in the thymus, in which *CD23* expression normally is found on a subset of asteroid dendritic cells near the corticomedullary junction (3, 5), m*CD23* protein was absent (Fig. 4C). Therefore, in  $CD23^{-/-}$  mice *CD23* expression is completely abolished. FCM analysis (Fig. 3) further show that, on a per cell basis, there is a roughly twofold reduction in the level of *CD23* protein on  $CD23^+$  B cells in  $CD23^{+/-}$  mice. A similar decline in the number of *CD23* molecules on a per cell basis is evident from immunocytochemical analysis of thymic asteroid dendritic cells in  $CD23^{+/-}$  mice (Fig. 4D). In conclusion, both parental *CD23* alleles contribute in an approximately equal fashion to mRNA and protein expression in different m*CD23*<sup>+</sup> cell types. These findings are consistent with a lack of parental and/or tissue-specific imprinting effects.

### Thymocytes and T cell compartments in $CD23^{-/-}$ mice

In humans, s*CD23* and IL-1 have been reported to induce synergistically early thymocyte maturation (21). If murine T cell development might be influenced by a similar activity of s*CD23*,  $CD23^{-/-}$  mice might disclose such a role assuming that IL-1 levels and functional activities are normal in these mice. Because profound alterations in early thymocyte maturation are likely to result in detectable changes of individual thymocyte subsets, the latter were analyzed and compared in  $CD23^{-/-}$ ,  $CD23^{+/-}$  and  $CD23^{+/+}$  mice using three-color flow cytometry (Fig. 5). Each of the mice displayed normal proportions of  $CD4^-CD8^-$ ,  $CD4^+CD8^+$  and single positive thymocyte subsets. All the mice also had about 1 to 2% of an early thymocyte precursor population characterized by the expression of the IL-2R and high (hi) levels of the heat stable antigen (HSA) (Fig. 5A, upper right quadrant) show IL-2R<sup>+</sup>HSA<sup>hi</sup> cells) and no significant differences were noted

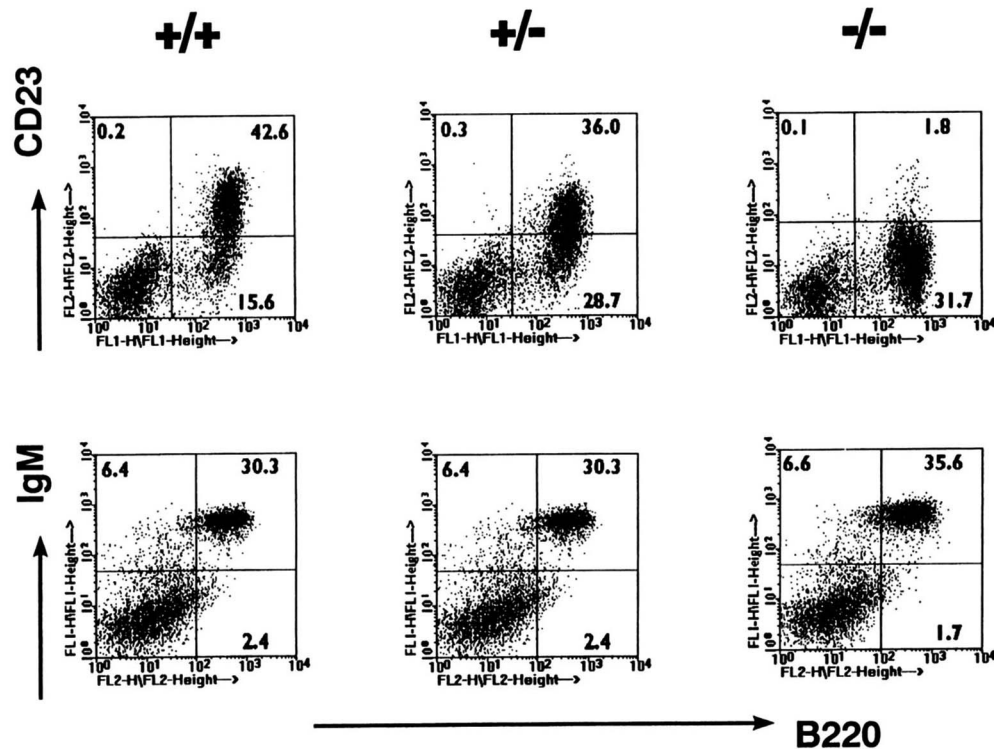


**FIGURE 3.** FCM analysis of lymphocytes in spleen. The *upper* three diagrams show spleen cells of, respectively, CD23<sup>+/+</sup>, CD23<sup>+/-</sup>, and CD23<sup>-/-</sup> mice, stained with anti-B220 (RA3-6B2; FITC) and anti-CD23 (B3B4; PE). The signal in the B220<sup>+</sup>CD23<sup>+</sup> quadrant of the CD23<sup>-/-</sup> mice is caused by background noise. The *lower panels* show the analysis for IgM<sup>+</sup>B220<sup>+</sup> B cell numbers and surface expression of IgM.

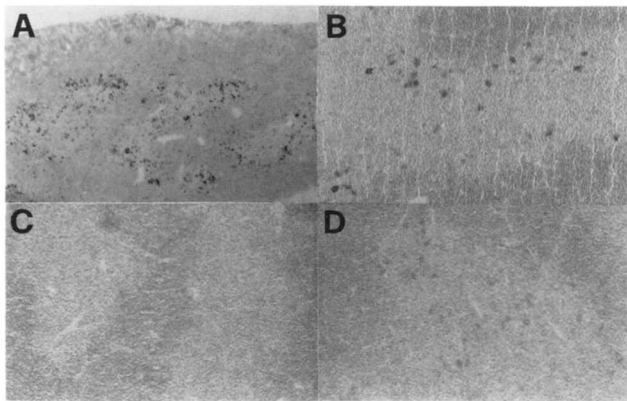


**FIGURE 4.** Immunocytochemical analysis of CD23 expression in thymus. Cryostat sections of thymi from CD23<sup>+/+</sup>, CD23<sup>+/-</sup>, and CD23<sup>-/-</sup> mice were stained with the rat anti-mouse CD23 Ab B3B4 (see *Materials and Methods*). *A*, a low magnification image showing the nonrandom distribution of CD23<sup>+</sup> asteroid dendritic cells (ADC) near the corticomedullary junction in a CD23<sup>+/+</sup> mouse thymus. *B* and *D*, high magnification images showing CD23<sup>+</sup> ADC in CD23<sup>+/+</sup> (*B*) and CD23<sup>+/-</sup> (*D*) mice. *C* shows a complete lack of CD23 staining on ADC in CD23<sup>-/-</sup> mice.

in the proportion of thymocytes displaying the early intra-thymic progenitor phenotypes IL-2R<sup>+</sup> HSA<sup>hi</sup> Pgp-1<sup>-</sup> or Pgp-1<sup>+</sup> IL-2R<sup>-</sup> HSA<sup>+</sup>. A more advanced stage of T cell

maturation was also analyzed using an early activation marker, CD69, which up-regulation on CD4<sup>+</sup>CD8<sup>+</sup> cells has shown to correlate with positive selection for proper recognition of self-MHC (42). Also in this case (Fig. 5B) the three types of mice showed similar numbers of (CD69<sup>+</sup>) cells. Therefore, a lack of CD23 does not provoke alterations in several major aspects of the normal developmental program of murine thymocytes as measured by any of the above parameters. A lack of CD23 also did not cause profound changes in peripheral T cell compartments in spleen and lymph nodes. Neither differences in absolute cell numbers nor the proportion of T cell subsets as defined by expression of CD4 and CD8 were apparent (data not shown).

#### The B cell compartment

The homing of B lymphocytes to secondary lymphoid organs like spleen, lymph nodes, or Peyer's patches is carefully regulated (43). Small mature B cells show inducible expression of CD23. The loss of this function might change either the potential to reach a fully activated state or alter their migratory properties that might be reflected by changes in the composition of peripheral B cell subsets in the different lymphoid compartments. To this end we analyzed by FCM the peripheral lymphocyte subsets of spleen (Table I), Peyer's patches (data not shown), and the

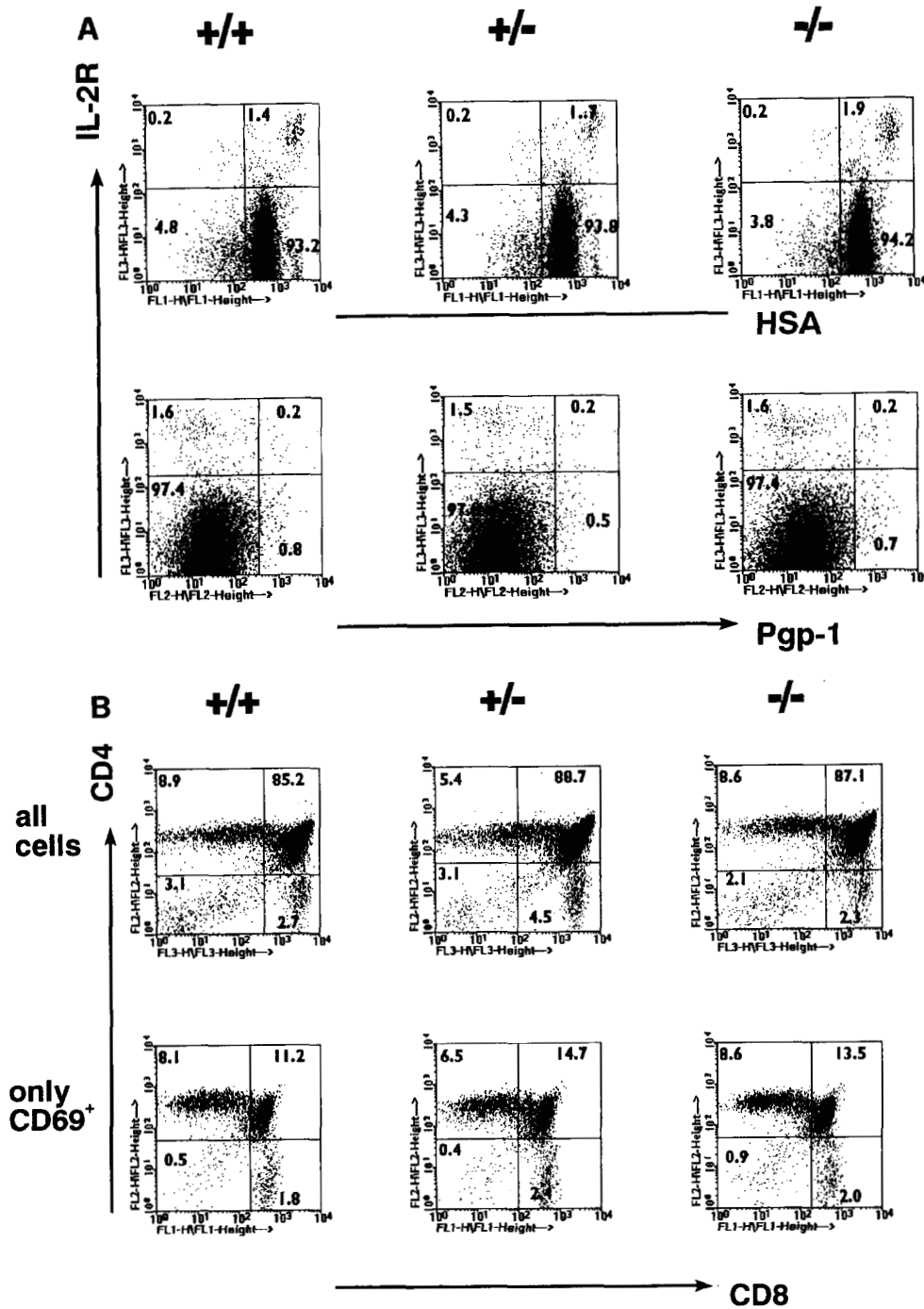


**FIGURE 5.** Thymocyte development in  $CD23^{-/-}$  mice. **A**, analysis of total thymocytes in  $CD23^{+/+}$ ,  $CD23^{+/-}$ , and  $CD23^{-/-}$  mice stained by three-color flow cytometry to detect IL-2R (PE), HSA (bio-SA-Tricolor), and Pgp-1 (FITC). **B**, analysis showing proportions of  $CD4^{-}CD8^{+}$ ,  $CD4^{+}CD8^{+}$ , and single positive thymocyte subsets as well as  $CD69^{+}$  thymocytes in  $CD23^{+/+}$ ,  $CD23^{+/-}$ , and  $CD23^{-/-}$  mice. Stainings were (see *Materials and Methods*) using CD4 (PE), CD8 (bio + SA Tricolor), and CD69 (FITC).

peritoneal cavity (data not shown) in  $CD23^{+/+}$ ,  $CD23^{+/-}$  and  $CD23^{-/-}$  mice. All three types of mice revealed similar expression patterns when monitoring sIgM, sIgD, B220, I-A, CD40, and HSA (Table I) whereas CD23 levels were 50% down on a per cell basis in  $CD23^{+/-}$  mice and

absent in  $CD23^{-/-}$  mice (as shown in Fig. 3). Also cell numbers in the peripheral lymphoid organs were very similar (Table I). Therefore, CD23 expression seems not required for filling peripheral lymphocyte compartments with B cells.

Table 1. Lymphocyte populations in spleen

| Cell Population                     | % of Cells                                    |                                               |                                               |
|-------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|                                     | +/+                                           | +/-                                           | -/-                                           |
| CD4 <sup>+</sup>                    | 16.5                                          | 15.7                                          | 12.7                                          |
| CD8 <sup>+</sup>                    | 10.0 ± 2.4                                    | 4.7; 10.5                                     | 5.0; 9.3                                      |
| IgM <sup>+</sup> B220 <sup>+</sup>  | 44.4 ± 7.5                                    | 55.8; 44.4                                    | 58.3; 48.1                                    |
| IgD <sup>+</sup> B220 <sup>+</sup>  | 47.2 ± 2.0                                    | 47.1                                          | 55.5                                          |
| IgM <sup>+</sup> I-A <sup>+</sup>   | 41.5                                          | 43.9                                          | 46.2                                          |
| CD23 <sup>+</sup> B220 <sup>+</sup> | 33.0 ± 4.2                                    | 42.2                                          | 0.0                                           |
| IgM <sup>+</sup> CD23 <sup>+</sup>  | 35.9                                          | 36.2                                          | 0.0; 0.0                                      |
| CD40 <sup>+</sup> B220 <sup>+</sup> | 54.7 ± 1.3                                    | 59.7                                          | 65.7                                          |
| CD40 <sup>+</sup> IgM <sup>+</sup>  | 47.6 ± 1.2                                    | 50.5                                          | 57.8                                          |
| Cell no.                            | 8.8 × 10 <sup>7</sup> ± 1.3 × 10 <sup>7</sup> | 1.2 × 10 <sup>8</sup> ; 1.8 × 10 <sup>7</sup> | 1.1 × 10 <sup>8</sup> ; 5.6 × 10 <sup>7</sup> |
| No. of animals                      | 1 or 4                                        | 1 or 2                                        | 1 or 2                                        |

### Activation of B cells in CD23<sup>-/-</sup> and CD23<sup>+/-</sup> mice

Postulated functions for CD23 linking its expression on peripheral B cells and B cell activation and/or proliferation include roles in cellular adhesion (44, 45), autostimulation via CD23-CD23R interactions among B cells (18, 46, 47), autocrine stimulation (48), and signal transduction enhancing B cell stimulation (46, 49). To test whether a loss of CD23 expression severely compromises B cell proliferation we analyzed the *in vitro* proliferative responses of splenic B lymphocytes. After T cell depletion, the remaining cells were stimulated by sIgM cross-linking alone (50, 51) or in combination with costimulatory signals delivered by adding CD40(L) ligand (39, 40, 52–60), IL-2, IL-4, or combinations of these agents. In our assay, the CD40L T cell-surface signal was replaced by a soluble form of CD40L (39). Comparing the proliferative responses of CD23<sup>+/+</sup>, CD23<sup>+/-</sup>, and CD23<sup>-/-</sup> B cells we found slightly less proliferation of CD23<sup>+/-</sup> and CD23<sup>-/-</sup> B cells as compared with CD23<sup>+/+</sup> B cells when engaging the Ag receptor alone (Fig. 6; *left*). Negligible differences were observed, however, when monitoring B cell proliferation in the presence of also CD40L (Fig. 6; *right*). The stimulation index measured in experiments employing, in addition, IL-4 either alone or in combination with IL-2, was two- to threefold higher. Also in these cases no significant differences were observed when comparing CD23<sup>+/+</sup>, CD23<sup>+/-</sup>, and CD23<sup>-/-</sup> B-cells (data not shown). The induction of B7 expression, which correlates well with B cell activation (54) was also found to be similar in CD23<sup>+/+</sup>, CD23<sup>+/-</sup>, and CD23<sup>-/-</sup> mice (data not shown). For reference, we also monitored CD23 and CD40 expression after stimulation. CD40 expression levels proved similar after stimulation of B cells from all three types of mice (Fig. 7A). The addition of CD40L revealed, as compared with anti-IgM mediated stimulation, increased expression of mCD23 on the B cell surface in both CD23<sup>+/+</sup> and CD23<sup>+/-</sup> mice. As compared with stimulation with anti-IgM(Fab)<sub>2</sub> alone, the addition of CD40L further increased CD23 surface expression. In either case, the fluorescent intensity on CD23<sup>+/-</sup> B cells reached

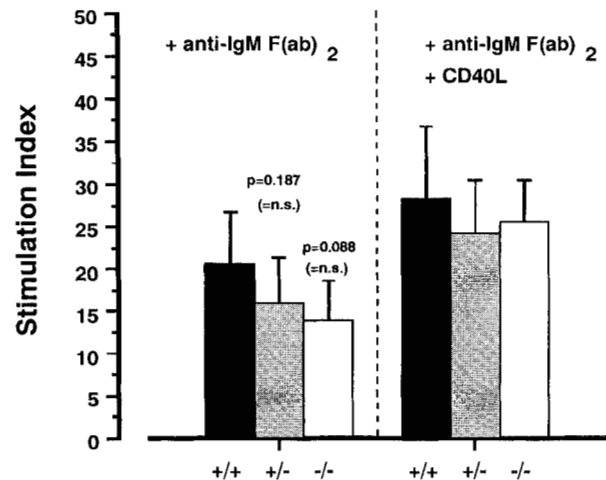


**FIGURE 6.** B cell proliferation assay. B cells from spleen were enriched (see *Materials and Methods*) and 10<sup>5</sup> cells/well were plated and stimulated for 3 days using 2 µg/ml anti-IgM(Fab)<sub>2</sub> without and with CD40L. In parallel, similar experiments were performed adding also IL-2, IL-4, or IL-2+IL-4 (not shown). Control experiments using only IL-2, only IL-4 as well as medium, all without CD40L and anti-IgM(Fab)<sub>2</sub> were also performed (not shown). In each case, during the last 16 h of the incubation 1 µCi [<sup>3</sup>H]TdR was added. The stimulation index was calculated as (cpm of sample plus stimulator/cpm of sample without stimulator) × (% B cells in the sample/% B cells -/-). The percent of B<sup>-/-</sup> cells was taken as 100% equivalent.

~50% as compared with CD23<sup>+/+</sup> B cells. After stimulation CD23<sup>-/-</sup> B cells also showed no CD23 staining (Fig. 7B). Altogether, these findings indicate that a reduced or absent expression of CD23 does not drastically alter the *in vitro* response of B cells when triggered via sIgM and costimulatory signals including CD40L, IL-2, and IL-4.

### Immune responses after infection with Nb

The parasite Nb evokes a typical Th2 cell-mediated immune response characterized by ~100-fold and ~10-fold

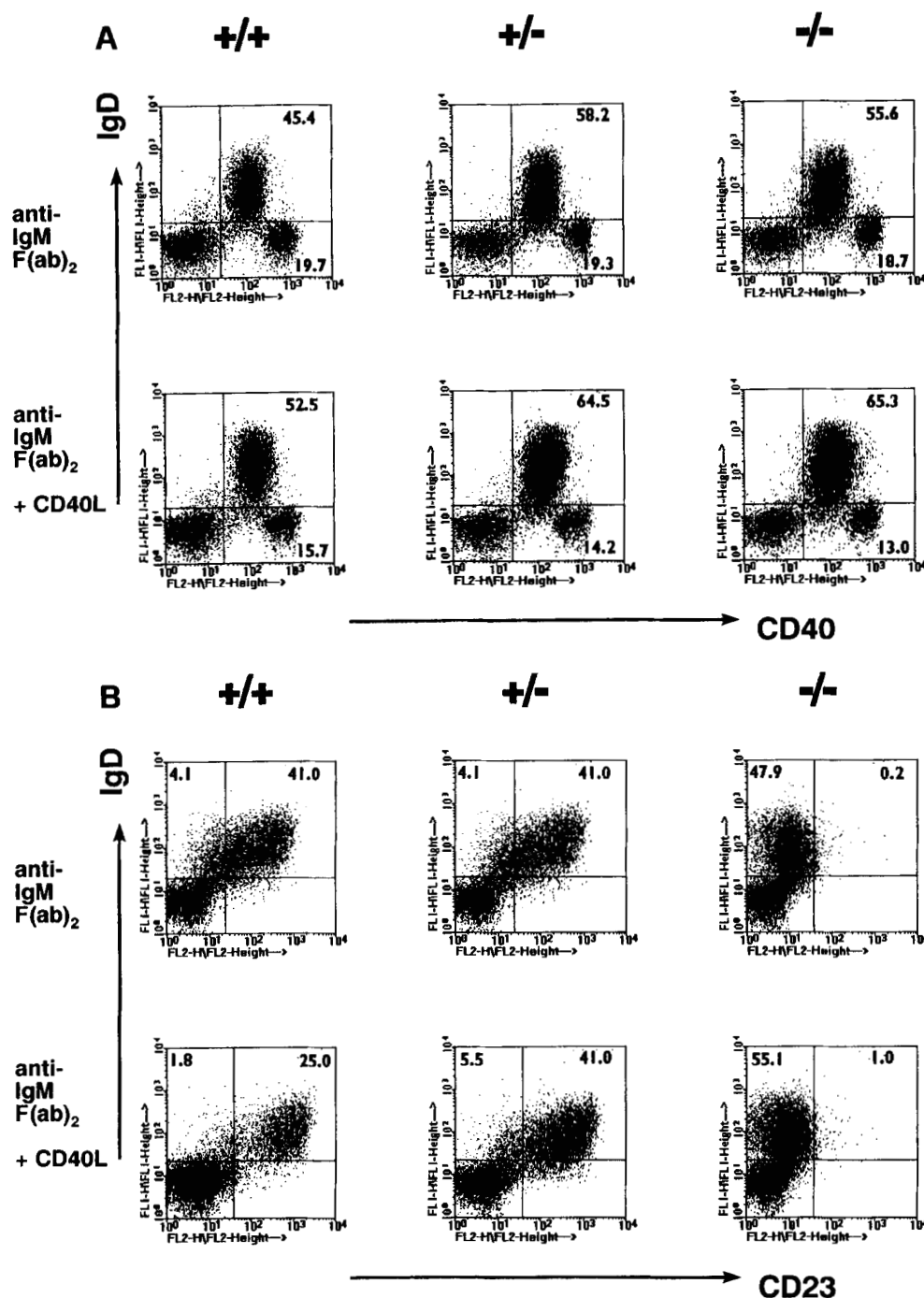
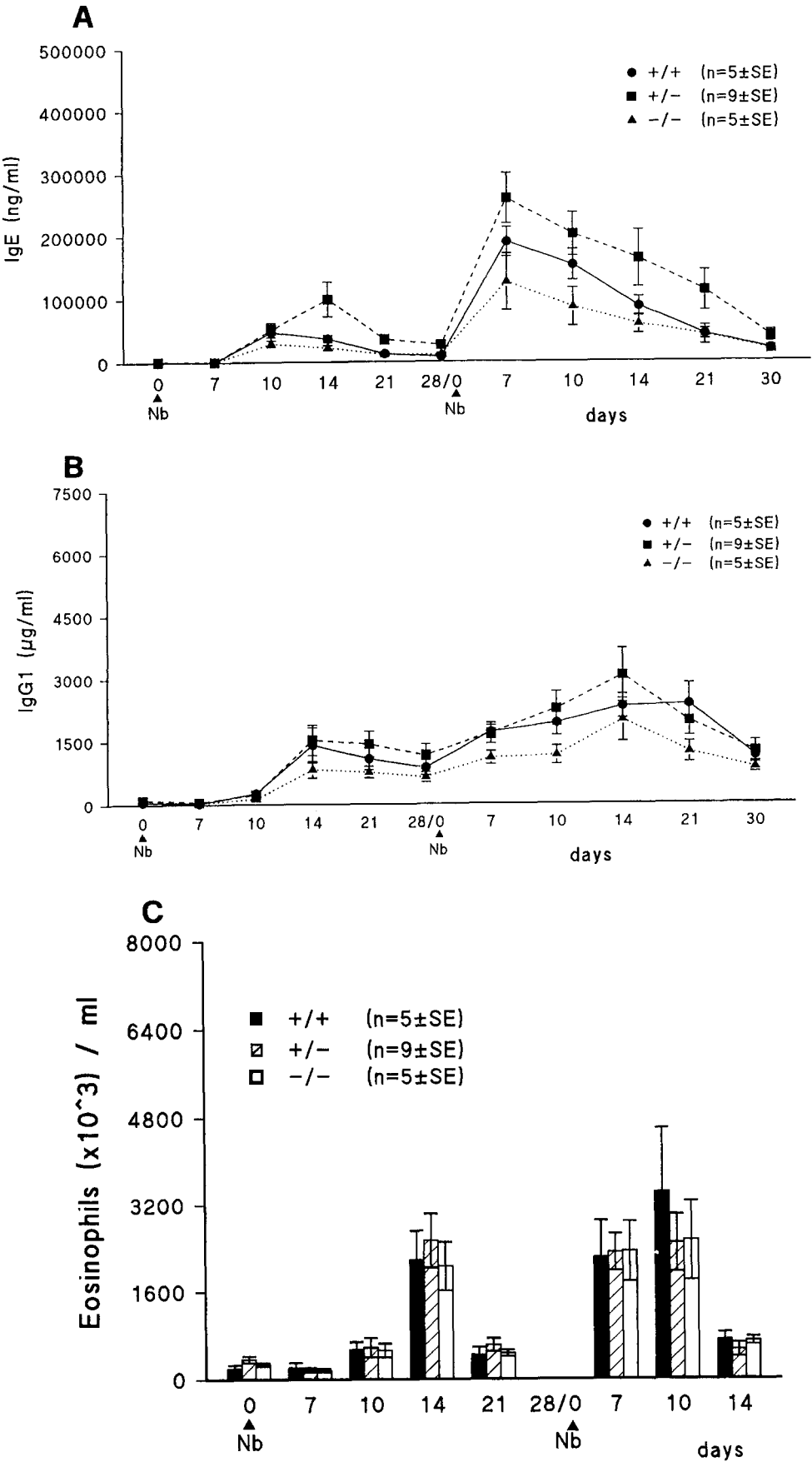


**FIGURE 7.** Expression of CD23 and CD40 after B cell stimulation. *A*, panels represent FCM of spleen cells incubated with either 12.5  $\mu$ g/ml anti-mouse IgM(Fab)2 (optimal concentration) or 2  $\mu$ g/ml anti-mouse IgM(Fab)2 (suboptimal dose) plus CD40L. Cells were stained with CD40L (CD40L-CD8 $\alpha$  fusion protein) and visualized using anti-CD8 $\alpha$  (see *Materials and Methods*). Note that the *lower right quadrants* represent CD8<sup>+</sup> splenic T cells (caused by anti-CD8 $\alpha$  staining procedure). *B*, cells were treated as in *A* and CD23 expression on B cells was tested by staining with sheep anti-mouse IgD (FITC) and rat anti-mouse CD23 B3B4 (Bio SA) Ab.

increased serum levels of IgE and IgG1, respectively, and a pronounced IL-5-mediated eosinophilia. IL-4 is required to mount Th2 cell-mediated IgE responses (61) and the production of Th2 cell-derived cytokines (62). IL-4 in the

mouse (63) and IL-4 and IL-13 in human (64) independently induce mCD23 expression on B cells. These cytokines also up-regulate class II MHC Ag, sIgM and CD72 (64, 65), the ligand for CD5 (66) and induce germ-line  $\epsilon$



transcription in B cells preceding switching to  $\epsilon$  (64, 67–69). Despite a dramatic up-regulation of mCD23 expression after an infection with Nb (14), the role CD23 exerts in this immune response and in the regulation of IgE synthesis, in particular, has remained obscure. We therefore tested whether a 50% reduction (CD23<sup>+/-</sup> mice) or a complete lack (CD23<sup>-/-</sup> mice) of CD23 gene expression has an effect on i) the typically observed increase in serum total polyclonal IgE and IgG1 levels and ii) on the eosinophilia associated with the response elicited by Nb. To this end F2 CD23<sup>-/-</sup>, CD23<sup>+/-</sup>, and CD23<sup>+/+</sup> littermates were infected with Nb. Serum IgE and IgG1, as well as blood eosinophil counts, were monitored during the course of both the primary and secondary immune response. Unexpectedly, CD23<sup>-/-</sup> mice developed serum polyclonal total IgE levels that were similar or slightly below those in wild-type littermates. Serum polyclonal IgE levels consistently proved somewhat higher in CD23<sup>+/-</sup> mice as compared with CD23<sup>+/+</sup> and CD23<sup>-/-</sup> mice during the course of both the primary and secondary infection (Fig. 8A). Less pronounced differences were observed for IgG1 (Fig. 8B). During the course of the infection eosinophilia appeared similar in all three types of mice (Fig. 8C). In conclusion, a complete lack of CD23 expression *in vivo* does not compromise the capacity of the animals to increase polyclonal IgE and IgG1 levels after an infection with Nb. A lack of CD23 also does not affect the increase in eosinophilia accompanying an infection with Nb. Fifty percent reduced CD23 expression *in vivo* in CD23<sup>+/-</sup> mice, which was shown to result in twofold fewer CD23 molecules on the cell surface of each CD23<sup>+</sup> cell in these mice, promotes a two- to threefold increase in total polyclonal serum IgE levels. These findings are consistent with a modulatory but no obligatory role for CD23 in polyclonal IgE production *in vivo*.

## Discussion

We have shown that a complete lack of CD23 *in vivo* does not seriously alter parameters including the cellular composition of lymphocyte compartments *in vivo*, the proliferative capacity of B cells *in vitro*, and the capacity to mount a polyclonal IgE and IgG1 response to Nb *in vivo*. Our findings seem surprising given the many different functions that have been postulated for either mCD23 and/or sCD23 albeit mostly using *in vitro* experimental systems. In humans, sCD23 + IL-1 were reported to induce synergistically early thymocyte maturation *in vitro* (21). In CD23<sup>-/-</sup> mice we were unable to detect major changes even in thymocyte compartments known to con-

tain some of the earliest intrathymic T cell precursors. Our findings indicate that CD23 *in vivo* in the mouse is not required to maintain processes that comprise some of the major steps in thymocyte development. It remains to be seen whether under particular circumstances (e.g., stress-related conditions) CD23 might prove important for thymocyte development.

Peripheral B cell populations in CD23<sup>-/-</sup> are essentially normal with respect to subsets and numbers. The migratory properties of naive B cells, which make up the largest fraction of peripheral B lymphocytes, appear to correlate largely with their stage of maturation (43). Although small mature B cells show inducible expression of CD23, the loss of this function apparently does not interfere with their capacity to reach a fully activated state or alter their migratory properties. Therefore, unlike the expression of a CD45 variant encoding exon 6 (70), CD23 expression seems not required for filling peripheral B lymphocyte compartments.

It may also seem surprising that a complete lack of CD23 does not abrogate the capacity of the animals to mount a typical Th2-cell mediated polyclonal IgE response. Activation of mature IgM<sup>+</sup>IgD<sup>+</sup> virgin B cells results in a transient elevation of mCD23 expression. *In vivo*, this increase is pronounced after an infection with Nb (14). mCD23-derived sCD23 has been proposed to serve as an autocrine B cell growth factor (46) that, in concert with IL-1 $\alpha$ , also encourages the survival of germinal center human B cells and promotes their differentiation toward a plasmacytoid morphology (20, 71). sCD23 can act via CD21 (17) and potentiate IL-4-induced IgE production *in vitro* (17, 72). *In vivo*, administration of a rabbit polyclonal Ab to recombinant human-truncated CD23 resulted in up to 90% inhibition of OVA-specific IgE synthesis in rats. This effect correlated with an inhibition of CD23 expression but neither cross-linking of CD23 nor negative signaling through CD23 seemed involved in inhibiting IgE synthesis (73). In contrast, induction of a Th2-clone-mediated, IL-4-dependent, polyclonal IgE synthesis was not inhibited by the anti-mouse CD23 mAb B3B4 (74). Our results also indicate that CD23 function is not obligatory to mount a polyclonal IgE response *in vivo*. A future qualification of these results is needed. We have measured, so far, only total polyclonal IgE levels in serum. After Nb infection, however, most of the IgE represents bystander and not parasite-specific IgE. Whether a lack of CD23 specifically alters Ag-specific IgE responses, in particular, when less bystander IgE is produced, remains to be analyzed. It was certainly interesting to note that a 50%

**FIGURE 8.** IgE, IgG1, and eosinophilia after infection with Nb. F3 generation littermates comprising 5 CD23<sup>-/-</sup>, 10 CD23<sup>+/-</sup>, and 5 CD23<sup>+/+</sup> mice at the age of 8 wk were injected s.c. with 750 third-stage larvae of Nb. On the various days indicated, blood samples were taken and serum total IgE (A) and IgG1 (B) levels, as well as the number of eosinophils (C), were determined and plotted. The mice received a secondary challenge 28 days after the primary infection.

reduction in the level of cell-surface CD23 expression (CD23<sup>+/-</sup> mice) seems to alter slightly (two- to threefold increase) in a quantitative fashion the outcome of, in particular, the polyclonal IgE response after an infection with Nb. The underlying molecular mechanism remains to be elucidated but several hypothesis need to be envisaged. At the B cell level, cross-linking CD23 with IgE or anti-CD23 Abs down-regulates IgE secretion (75, 76) whereas co-cross-linking of sIg and CD23 on B lymphocytes generates either an activation or an inhibitory signal (2, 77, 78). Low levels of co-cross-linking would either not influence or enhance B cell activation, whereas high levels of co-cross-linking would decrease B cell activation. In line with this hypothesis, a 50% reduction of CD23 levels on the B cell surface in CD23<sup>+/-</sup> mice might increase the threshold levels determining B cell inactivation.

CD23 might serve not only in T-B but also other cellular interactions such as those between B cells and follicular dendritic cells (FDC) (for review see Reference 79). FDCs express high levels of CD23 as well as other FcRs but lack MHC class II. Unlike a role for CD23 in Ag presentation by B cells (24, 25), the expression of CD23 on the FDC cell surface might serve to deposit IgE-immune complexes formed during a primary immune response. Memory B cells are thought to recognize these Ags and, unlike virgin B cells, respond well to Ag captured by FDC (for review see Reference 71). Accordingly, a lack of one Ag source normally captured as IgE-immune complexes by CD23 on FDC might specifically alter events in a Th2 cell-driven immune response after a second challenge when recruiting memory B cells generated in a primary response. Although this question remains to be answered a clearcut effect might not be expected given the fact that FDCs express FcRs for virtually all classes of Ig and that each Ag is probably held by several types of FcRs. In any case, neither a putative Ag-capturing role for FDC-CD23 nor a proposed interactive role for FDC-CD23 with CD21 on B cells (79) seems to provide a clearcut explanation for allowing a slight increase in serum total polyclonal IgE levels after infection with Nb.

Other mechanisms will need to be envisaged including reduced clearance of IgE from serum and above-mentioned effects caused by changes in CD23 levels on the B cell surface. It remains to be seen, however, whether lower levels of FDC-CD23 might favor a more stringent selection of B cells with high affinity receptors by forcing increased competition for binding to Ag-Ig complexes on FDC. Hence, affinity maturation of Ag-specific B cell clones in CD23<sup>+/+</sup> and CD23<sup>+/-</sup> should be compared. It also remains to be seen whether the lack of CD23 compromises T cell help. Follicular B cells can take up Ag from FDCs and process this into a form that activates CD4<sup>+</sup> T cells (80). Because eosinophilia was similar in CD23<sup>-/-</sup>, CD23<sup>+/-</sup>, and CD23<sup>+/+</sup> mice, at least the overall levels of this cytokine and this aspect of Th2-function seems not affected by a lack of CD23. Finally, by trigger-

ing CD40, human precursor B cells can express CD23 before acquisition of sIgM (81). With respect to memory B cell formation, two alternative models can be proposed differing at the point where commitment to the memory or Ab-forming cell lineage occurs (82). The more widely accepted model assumes commitment after Ag stimulation. If commitment can occur before Ag encounter, IgE-Ag complexes might alter the fate of B cell precursors expressing CD23. The generation of CD23<sup>-/-</sup> mice provides us with an important novel biologic system to test many of the above hypotheses.

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