

## Mice lacking all conventional MHC class II genes

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**ABSTRACT** MHC class II (MHC-II) molecules play a central role in the selection of the T cell repertoire, in the establishment and regulation of the adaptive immune response, and in autoimmune deviation. We have generated knockout mice lacking all four of the classical murine MHC-II genes (MHCII<sup>Δ/Δ</sup> mice), via a large (80-kilobase) deletion of the entire class II region that was engineered by homologous recombination and Cre recombinase-mediated excision. These mice feature immune system perturbations like those of  $A\alpha$  and  $A\beta$  knockout animals, notably a dearth of CD4<sup>+</sup> lymphocytes in the thymus and spleen. No new anatomical or physiological abnormalities were observed in MHCII<sup>Δ/Δ</sup> mice. Because these animals are devoid of all classical MHC-II chains, even unpaired chains, they make excellent recipients for MHC-II transgenes from other species, avoiding the problem of interspecies cross-pairing of MHC-II chains. Therefore, they should be invaluable for engineering “humanized” mouse models of human MHC-II-associated autoimmune disorders.

MHC class II (MHC-II) molecules play a crucial role in the development and function of the immune system. They are heterodimeric proteins expressed on the surface of antigen-presenting cells such as B cells, macrophages, and dendritic cells. In mice, they comprise the A and E complexes ( $A\alpha:A\beta$  and  $E\alpha:E\beta$ , respectively). Their primary function is to present peptides processed from extracellular proteins to CD4<sup>+</sup> helper T cells. The recognition of peptide/MHC-II complexes by CD4<sup>+</sup> T cells initiates an efficient immune response via cognate help to B cells or the production of inflammatory cytokines. MHC-II molecules are also expressed on thymic stromal cells, where they direct the processes of positive and negative selection, shaping the repertoire during T cell maturation and lineage commitment. The result is a repertoire of peripheral CD4<sup>+</sup> T cells that are self-tolerant but competent to recognize foreign peptides in the context of self-MHC molecules. Studies with already available MHC-II-deficient mice with targeted  $A\beta$  or  $A\alpha$  gene deletions have confirmed the importance of MHC-II molecules in the immune system (1–3).

Given the multiple central roles played by MHC-II molecules in the immune system, it is not surprising that their genetic variations are linked to various immunological disorders. For example, the absence of MHC-II gene expression leads to severe immunodeficiencies in humans (4). Inherited susceptibility or resistance to autoimmune disorders—such as multiple sclerosis, insulin-dependent diabetes, and rheumatoid arthritis—are associated with particular MHC-II alleles (5). Despite long-term efforts, the molecular mechanism or mechanisms for these MHC/disease associations are still not clear

(6). One way to analyze them in an organismal setting is to generate transgenic mice expressing disease-associated human MHC-II (HLA-D) genes. Such mice already have proven useful in delineating the role of disease-associated MHC-II molecules in pathogenic immune responses and in the development of disease models (7–11). Mutant mouse lines genetically deficient in both the  $E\alpha$  and  $A\beta$  genes—and thus lacking MHC-II complexes—have been used to ensure that phenomena observed derive from the activity of the human class II transgenes themselves. However, the possible pairing between human MHC-II molecules and the remaining murine  $E\beta$  and  $A\alpha$  chains (12–14) complicates the interpretation of such experiments. It would therefore be desirable to express human MHC-II molecules in the complete absence of their murine counterparts. To resolve this problem, we have generated an MHC-II knockout mouse line harboring a deletion of all classical MHC-II genes.

### MATERIALS AND METHODS

**Targeting Vectors.** A 3-kilobase (kb) *SalI*–*HindIII* fragment from pDLox420 carrying a 5' *loxP* site and a thymidine kinase-neomycin (*TK-neo*) resistance fusion gene (15) was blunt-end cloned into the unique *BstEII* site in exon 2 of the  $A\beta$  gene [as a 6.4-kb *EcoRI* genomic fragment in pcEXV (ref. 16; Fig. 1C)]. From this construction, an 8.8-kb *EcoRI*–*HindIII* fragment (Fig. 1C) was purified and used for transfection. A 16.5-kb *NotI* genomic fragment containing the  $E\alpha$  gene (isolated from a D3 ES cell genomic bacteriophage  $\lambda$  library) was cloned into the *NotI* site of pSuperCos 1 (Stratagene). A 2-kb *KpnI*–*HindIII* fragment from pHLox420, containing a 5' *loxP* site and a hygromycin-resistance marker, was cloned into the unique *BspEI* site in exon 3 of the  $E\alpha$  gene (Fig. 1C). A 11.7-kb *SmaI*–*XhoI* fragment from this construct (Fig. 1C) was isolated and used for transfection.

**Generation of MHCII<sup>Δ/Δ</sup> Mice.** First, the  $E\alpha$  targeting vector was transfected into H1 ES cells (derived from 129/SvPas mice) by using standard procedures (2). Hygromycin-resistant ES clones were screened for correct targeting of the  $E\alpha$  locus by Southern blotting with the probe GEA1 and a *SpeI* digest on genomic DNA (Fig. 1C). Second, the  $A\beta$  targeting vector was transfected into ES cells bearing a targeted  $E\alpha$  locus. Hygromycin- and neomycin-resistant clones were screened for homologous recombination at the I- $A\beta$  gene by Southern blotting with a *BglII* digest and an external probe ( $A\beta$ 1; Fig. 1C). Third, one of the ES cell clones with targeted  $E\alpha$  and  $A\beta$  loci was transiently transfected with the pIC-CRE plasmid (17). Ganciclovir-resistant clones were screened for the deletion event between the two *loxP* sites with a *BglII* digest

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Abbreviations: kb, kilobase; ES, embryonic stem; RT-PCR, reverse transcription-PCR.

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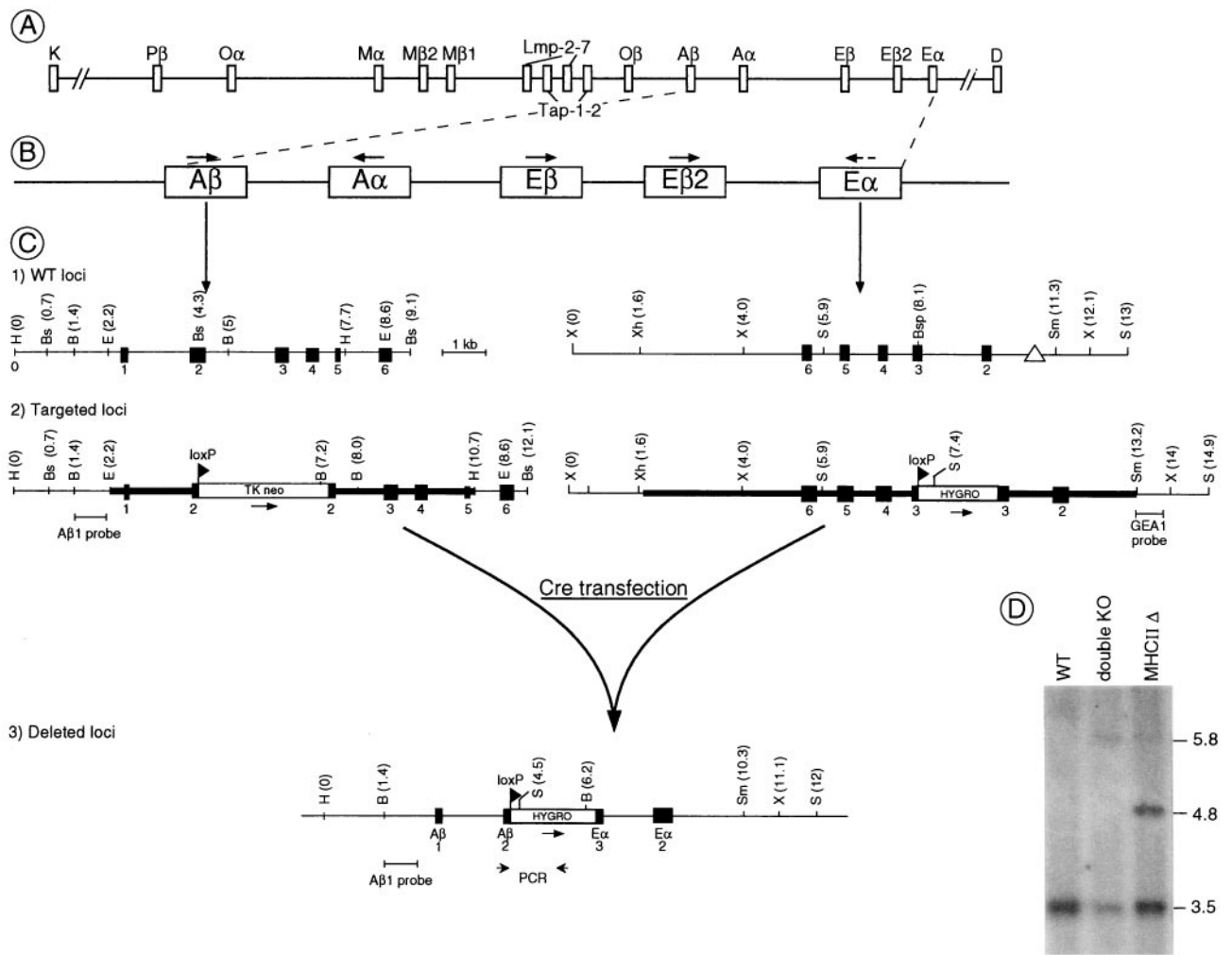


FIG. 1. Strategy for genomic deletion of all classical MHC-II genes. (A and B) Genomic organization of the mouse MHC around and in the class II region. Arrows indicate the transcriptional orientation of the different genes. (C) Mutation strategy. (C1) The wild-type *Aβ* and *Eα* genes. Exons are shown as filled boxes. The triangle represents the promoter deletion present in the *Eα* gene of the *H2<sup>b</sup>* haplotype. H, *Hind*III; Bs, *Bst*EII; Bsp, *Bsp*EI; E, *Eco*RI; B, *Bgl*II; X, *Xba*I; S, *Spe*I; Sm, *Sma*I; Xh, *Xho*I. (C2) The mutated *Aβ* and *Eα* loci after homologous recombination. The homologous recombination event introduced a TK-neo cassette into exon 2 of the *Aβ* gene and a *loxP*-hygromycin cassette into exon 3 of the *Eα* gene. The targeting DNA fragments used for electroporation are indicated by a bold line, the *loxP* sites as triangles. The probes used for the screening are shown under the targeted loci. (C3) Map of the deleted locus after *Cre*-mediated recombination. The Southern blot probe and PCR primers used to identify the deleted locus are shown under the map. (D) Southern blot analysis of genomic DNA isolated from wild-type embryonic stem (ES) cells and the double-targeted ES clone before and after the deletion event (*Bgl*III digest; *Aβ*1 probe).

and the *Aβ*1 probe (Fig. 1C). Sequencing of a 1.3-kb PCR product spanning from exon 2 of *I-Aβ* to the hygromycin resistance gene at the *Eα* locus confirmed the deletion (Fig. 1C). ES cells bearing the deleted MHC-II locus were injected into C57BL/6 blastocysts by using standard procedures (2). Chimeras were crossed to C57BL/6 mice. Offspring with germ-line transmission of the deleted MHC-II locus were then interbred to generate homozygous knockout *MHCII<sup>Δ/Δ</sup>* mice.

**Immunological Analyses.** The following staining reagents were used as directly conjugated reagents: anti-CD4-PE (Caltag, South San Francisco, CA), anti-CD8-Cy5 (Caltag), Y3P-FITC (anti-A<sup>b</sup>; ref. 18), anti-IgM-PE (Jackson ImmunoResearch), anti-IgD-FITC (Nordic, Lausanne, Switzerland), anti-CD69-FITC (PharMingen), IM7-FITC (anti-CD44; ref. 19), and peanut agglutinin receptor-FITC (Sigma). The following staining reagents were used as hybridoma culture supernatants and were revealed by FITC- or Texas-Red-conjugated anti-rat or anti-mouse IgG second-step antibodies (Jackson ImmunoResearch): M5/114 (anti-A/E; ref. 20), K9-178 (anti-K<sup>b</sup>; ref. 21), KT3 (anti-CD3ε; ref. 22), and RA3-6B2 (anti-CD45RA; ref. 23). Flow-cytometric analyses and immunohistochemistry on cryostat sections were performed as

described (2). Thin sections from thymus and spleen were prepared and stained with Y3P antibody as described (2). The concentrations of Ig isotypes in the serum of naive mice were evaluated by ELISA as described (2).

**Reverse Transcription-PCR (RT-PCR).** Total RNA was extracted from the spleen and was reverse-transcribed by using standard procedures. Specific oligonucleotides for the *H2-Oα*, *H2-Oβ*, *H2-Mα*, *Aα*, and *HPRT* genes were used for semi-quantitative PCR amplification. (See <http://biblio-igbmc.u-strasbg.fr/cbdlm> for details.)

## RESULTS

**Generation of *MHCII<sup>Δ/Δ</sup>* Mice.** Because the elimination of the entire MHC-II region required a substantial (80-kb) deletion, we relied on the *Cre* recombinase. *Cre* catalyzes recombination between two *loxP* sequences at high efficiency (24); if they are in the same orientation, the DNA between them is removed from the genome. Given the organization of the murine MHC-II genes (Fig. 1B), we introduced one *loxP* site into the *Eα* gene and another into the *Aβ* gene of the H1 ES cell line (129Sv/Pas background; A.D., unpublished work).

First, a *loxP* site and a hygromycin-resistance cassette were inserted by homologous recombination into the *BspEI* site in exon 3 of the *E $\alpha$*  gene (Fig. 1C). Of 67 hygromycin-resistant ES cell clones, 1 showed the homologous recombination event in Southern blot analysis. This clone (clone TU52) was transfected with a second targeting fragment, bearing another *loxP* site and a TK-neo cassette inserted into the *BstEII* site in exon 2 of the *A $\beta$*  gene. Of 282 hygromycin- and neomycin-resistant ES clones, 27 showed the homologous recombination event; one of the clones with the two targeting events (clone TU52.1) was then transfected with an expression vector encoding Cre to delete the region between the two *loxP* sites. ES cell clones with the 79-kb deletion were enriched by ganciclovir selection, because the deletion should result in the loss of the TK-neo cassette (Fig. 1C). Fig. 1D illustrates the 3.5-kb band corresponding to the wild-type *A $\beta$*  locus, the 5.8-kb band corresponding to the initial homologous recombination event, and the 4.8-kb band corresponding to the deletion event. The deletion was detected in 66 of 290 clones, but in only 10 of these was it complete in every cell (Fig. 1C). This phenomenon is probably linked to a moderate efficacy of ganciclovir selection in ES cells, which has been observed in other contexts (H. Leenders, unpublished results). One of the correct ES cell clones (clone TN430) was injected into blastocysts. Chimeric offspring were bred to C57BL/6 mice, and heterozygous progeny were interbred to produce homozygous mutants. The mutant locus will hereafter be referred to as MHCII $^{\Delta}$  (or H2<sup>dlAb1Ea2</sup>, following the nomenclature of The Jackson Laboratory).

MHCII $^{\Delta/\Delta}$  mice are viable and fertile. They display no obvious anatomical or behavioral defects; histological analysis of a number of tissues showed no alterations in organ structure and composition (not shown).

**Absence of MHC-II Molecules in MHCII $^{\Delta/\Delta}$  Mice.** MHC-II expression in the mutant mice was investigated by flow cytometry of B cells and by immunohistochemistry on tissue sections from thymus and spleen. Expression on B cells was determined by staining splenocytes with an anti-A<sup>b</sup> antibody (Y3P) or with the broadly reactive anti-A/E reagent M5/114. Fig. 2A shows the complete absence of MHC-II expression on gated IgM<sup>+</sup> B cells from MHCII $^{\Delta/\Delta}$  mice. Expression on other cell types was examined by immunofluorescent staining with the Y3P antibody on frozen tissue sections from thymus and spleen (Fig. 2B). No staining whatsoever could be seen in MHCII $^{\Delta/\Delta}$  sections. RT-PCR also failed to detect any RNA transcripts, as expected (Fig. 2C).

**Expression of Nonconventional Class II Genes and MHC-I Molecules.** The large deletion of the MHC-II region on chromosome 17 could conceivably influence the activity of neighboring genes through long-range regulatory interference. We therefore verified that their expression was not affected, first by evaluating levels of H2-M $\alpha$ , H2-O $\alpha$ , and H2-O $\beta$  class-II-like molecules involved in antigen processing and presentation. Semiquantitative RT-PCR did not show any significant differences in the amounts of these transcripts when comparing MHCII $^{\Delta/\Delta}$  and control mice (Fig. 3A). Furthermore, as shown in Fig. 3B, there were no differences in the levels of MHC-I K<sup>b</sup> molecules in MHCII $^{\Delta/\Delta}$  mice compared with those of control littermates. This result indicates that the activity of the *TAP* genes, also localized next to the MHC-II locus and important for MHC-I peptide loading and expression, is unaffected by the deletion. These results show that expression of all conventional MHC-II genes was abolished in MHCII $^{\Delta/\Delta}$  mice but that the deletion did not interfere with the activity of either the class I or class II accessory genes. This information was important, because normal H2-M and H2-O function would be required for the activity of any new MHC-II molecules expressed from transgenes in MHCII $^{\Delta/\Delta}$  mice.

**T Cell Populations in MHCII $^{\Delta/\Delta}$  Mice.** We then analyzed the effect of the MHC-II gene deletion on T cell differentiation and on peripheral T cell populations. As expected, there was

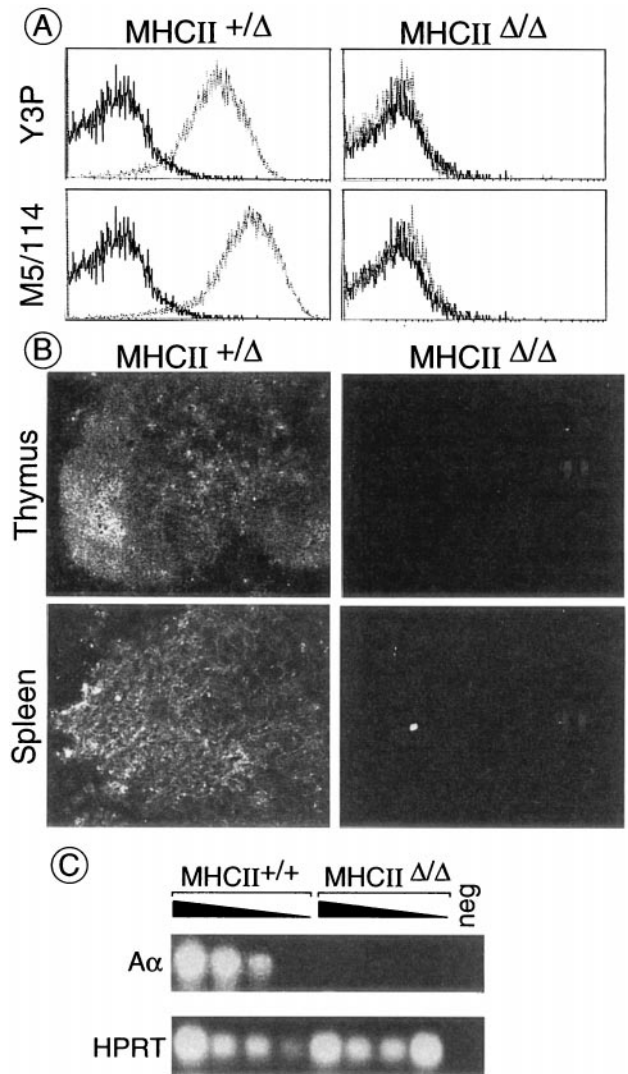


FIG. 2. Expression of MHC-II molecules in MHCII $^{\Delta/\Delta}$  mice. (A) Flow-cytometry profiles of anti-class-II staining on IgM<sup>+</sup> splenocytes in MHCII $^{+/Δ}$  and MHCII $^{Δ/Δ}$  littermates. Upper and Lower panels present staining with Y3P or M5/114 anti-class-II antibodies, respectively (thin lines). Plain lines represent staining with an irrelevant primary antibody. (B) Anti-class-II (Y3P) staining of thin sections from thymus and spleen of MHCII $^{+/Δ}$  and MHCII $^{Δ/Δ}$  littermates. (C) Absence of A $\alpha$  transcripts in MHCII $^{\Delta/\Delta}$  mice. Serial dilutions (1 in 10) of cDNA prepared from spleen RNA were amplified by PCR with A $\alpha$ -specific oligonucleotides; the total reaction run on an agarose gel is visualized by ethidium bromide staining. Amplification with HPRT-specific primers is shown as a control for cDNA quality.

a strong decrease in the number of CD4 single-positive thymocytes in MHCII $^{\Delta/\Delta}$  mice. This decrease was comparable with that observed in A $\beta^{0/0}$  mice (Fig. 4A). As in A $\beta^{0/0}$  animals, the CD4<sup>+</sup>CD8<sup>-</sup> thymocytes present in low numbers (1–2%) probably represent maturation intermediates dependent on MHC-I molecules (25), as indicated by their high level of surface peanut agglutinin receptors and the fact that most actually displayed low amounts of CD8 (Fig. 4B). We found normal T cell differentiation to the CD8 lineage in MHCII $^{\Delta/\Delta}$  mice (Fig. 4A); as previously observed in A $\beta^{0/0}$  mice, the number of CD8 single-positive thymocytes in MHCII $^{\Delta/\Delta}$  mice was increased, probably because of a compensatory effect in the face of decreased CD4 single-positive cell numbers.

The lack of differentiation to the CD4<sup>+</sup> lineage in MHCII $^{\Delta/\Delta}$  mice was reflected in the periphery by the nearly complete absence of CD4<sup>+</sup> T cells in the spleen and lymph

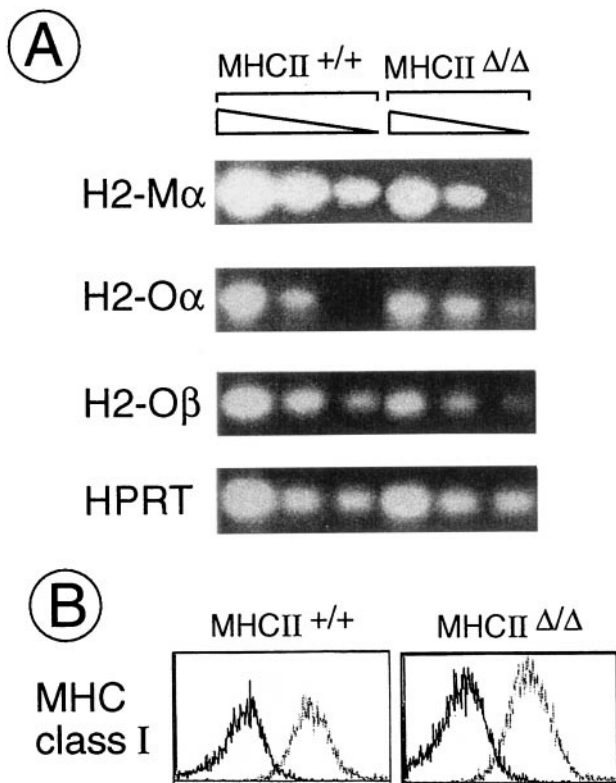


FIG. 3. Expression of nonconventional class II genes and MHC-I molecules in MHCII $\Delta/\Delta$  mice. (A) Semiquantitative RT-PCR for expression of H2-O $\alpha$ , H2-O $\beta$ , and H2-M $\alpha$ . RT-PCR was performed on 10-fold serial dilutions of cDNA from spleen RNA of MHCII $^{+/+}$  and MHCII $\Delta/\Delta$  littermates. (B) Flow-cytometric analysis of MHC-I expression on IgM $^+$  splenocytes. Plain lines represent control staining with an irrelevant primary antibody; dotted lines represent staining with the anti-K $^b$  antibody K9/178.

nodes (Fig. 4C). As in  $A\beta^{o/o}$  mice, only 1–2% of CD4 $^+$  cells were found in the lymph nodes (compared with 40–50% in MHC-II-proficient animals). These CD4 $^+$  T cells had an activated phenotype, as indicated by their high levels of CD44 and low levels of CD62L (data not shown).

**B Cell Populations in MHCII $\Delta/\Delta$  Mice.** B lymphocytes were studied in the mutant mice to ensure that the deletion did not affect the B cell compartment, a possible problem given reports of abnormal MHC-II expression affecting maturation or cell numbers of B lymphocyte populations (26, 27). Fig. 5A shows that B cell differentiation proceeded normally in the bone marrow of MHCII $\Delta/\Delta$  mice: staining with anti-B220 and anti-IgM antibodies revealed the normal progression from B220 $^{low}$ IgM $^-$ , through B220 $^{high}$ IgM $^{low}$ , to B220 $^{high}$ IgM $^{high}$ . These populations were present in normal proportions compared with wild-type and  $A\beta^{o/o}$  mice (Fig. 5A). The presence of a normal B cell compartment in MHCII $\Delta/\Delta$  mice was confirmed further by analyzing splenic populations (Fig. 5B and C). B cells identified by anti-B220 and anti-IgM expression were present in normal numbers in MHCII $\Delta/\Delta$  mice; the IgD expression pattern was also normal (Fig. 5C). The localization of B cells in the follicular areas of peripheral lymphoid organs (spleen and lymph nodes) was normal, as detected by immunofluorescence on frozen tissue sections (anti-IgM staining; data not shown). These follicles were devoid of germinal centers, as expected in the absence of help from CD4 $^+$  T cells. The B cell compartment was functional with respect to Ig production, as shown in Fig. 5D. MHCII $\Delta/\Delta$  mice produced “natural” antibodies with the normal range of isotypes but with a distinct increase in circulating IgM and somewhat less IgG1 and IgG2a than control littermates, as observed in  $A\beta^{o/o}$  mice

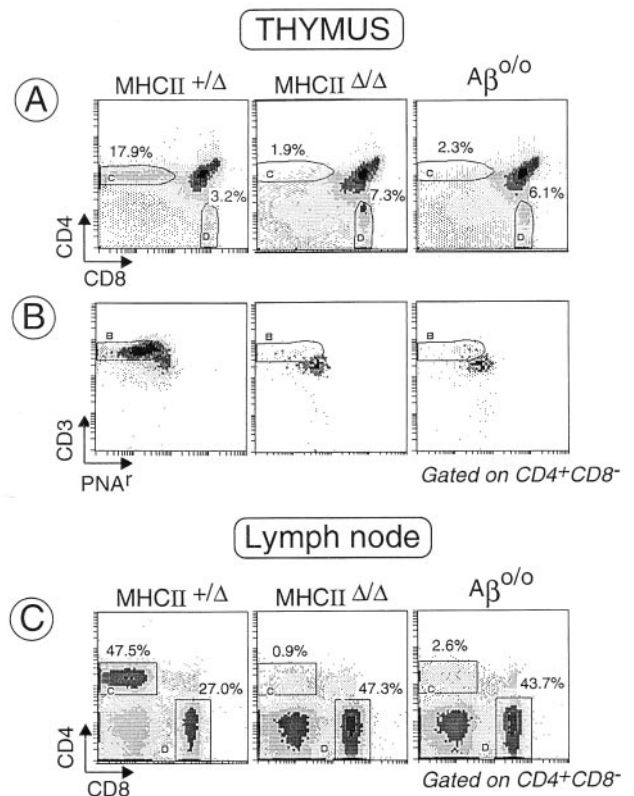


FIG. 4. T lymphocyte populations in MHCII $\Delta/\Delta$  mice. (A) Anti-CD4/CD8 staining of thymocytes from MHCII $^{+/+}$  and MHCII $\Delta/\Delta$  littermates and an  $A\beta^{o/o}$  mouse. The values represent the percentage of thymocytes that fall within the gates in this representative experiment. (B) Profiles of CD3 and peanut agglutinin receptor (PNAr) expression on CD4 $^+$ CD8 $^-$  thymocytes from the same mice described in A. (C) CD4/CD8 profiles of lymph node lymphocytes.

(2, 28)—probably a consequence of the crippled T cell help in such animals.

## DISCUSSION

Existing MHC-II knockout mice suffer from the complication of permitting interspecies  $\alpha:\beta$  complexes to form when used as recipients for MHC-II transgenes (e.g., DR $\alpha$ :E $\beta$ ; refs. 12–14). The study of murine MHC-II transgenes in these mice is also complicated, because interallelic dimers may be generated by pairing of the residual endogenously encoded chains with the transgene-encoded components (29). These problems encouraged us to generate a knockout mouse line in which all four conventional MHC-II genes are deleted; in the line reported here, two ( $A\alpha$  and E $\beta$ ) of the four genes are completely lost, and two ( $A\beta$  and E $\alpha$ ) are irredeemably disrupted. Thus, these mice should be excellent hosts for any class II transgenes, because there is no risk of generating chimeric MHC-II molecules and because all other known genes involved in antigen presentation are properly expressed (M, O, TAP, class I loci). Furthermore, other immune parameters not directly connected with MHC-II genes seem normal in MHCII $\Delta/\Delta$  mice. It should thus be possible to investigate, in “humanized” MHC-II transgenic mice, the T cell repertoires that are selected by particular disease-related MHC-II alleles or their capacity to present autoantigens.

The E $\beta$ 2 gene has a structure identical to that of conventional MHC-II genes but is expressed at far lower levels (30). The gene is also located in the murine MHC-II region and was removed by the deletion. It has long been a puzzle what role this gene might play. The MHCII $\Delta/\Delta$  mouse does not show any

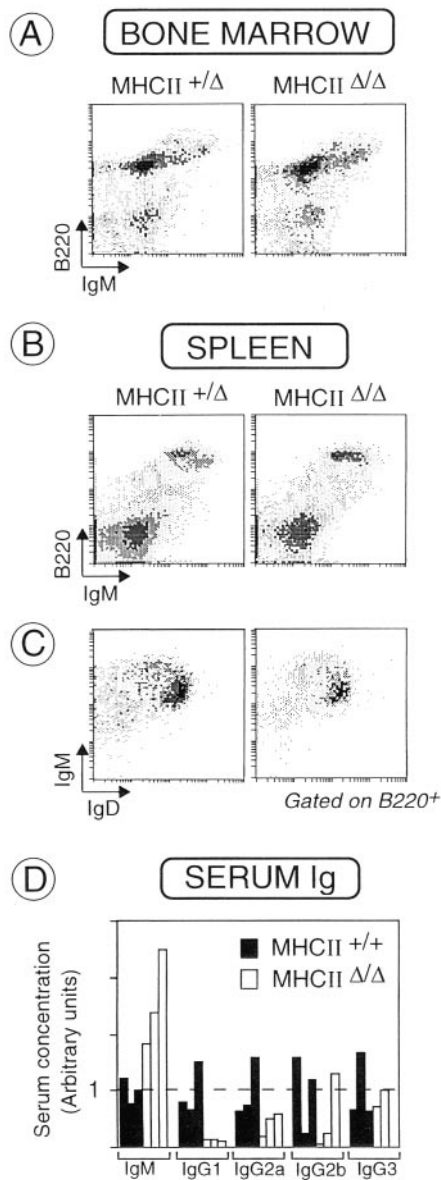


FIG. 5. B lymphocyte lineage in MHCII $\Delta/\Delta$  mice. (A) B lymphocyte differentiation in the bone marrow; anti-B220 and anti-IgM staining of bone marrow cells from MHCII $^{+/+}$  and MHCII $\Delta/\Delta$  littermates (gated on lymphocytes by scatter). (B and C) B cell populations in the spleen: B220/IgM profiles on whole splenocytes and IgM/IgD profiles gated on B220 $^{+}$  B cells. (D) Serum Ig levels in naive MHCII $^{+/+}$  and MHCII $\Delta/\Delta$  mice. Serum concentration for various Ig isotypes determined by ELISA (arbitrary units; 1 is defined as the average of control sera in the experiment). Each bar represents an individual mouse.

particular defects that could be related to the absence of E $\beta$ 2. Its function, if any, must thus be within the context of classical MHC-II activity. More generally, the absence of unexpected phenotypes in MHCII $\Delta/\Delta$  mice suggests that there are as yet unrecognized genes of other function in the MHC-II region, at least ones with a critical role in a function whose absence results in an obvious phenotype. This observation is in accordance with the recently generated sequence of this genomic stretch, in which gene detection algorithms have failed to uncover other coding regions (GenBank accession nos. AF027865 and AF050157).

The ES cell clones with *loxP* sites at the E $\alpha$  and A $\beta$  loci may also be useful as starting points for further genomic engineering of the MHC and of chromosome 17 more generally (i.e.,

deletions in the region or more extensive chromosomal modifications; for examples, see refs. 31–34). MHCII $\Delta/\Delta$  mice could also be used for the recombinational insertion of *loxP*-flanked transgenes into the MHC-II region, placing them under the control of the A $\beta$  gene transcriptional promoter and in a chromosomal context that should ensure faithful and reliable expression patterns.

We have generated a mouse strain that can be used (i) as starting tool for further chromosome engineering and (ii) as an excellent background strain for studying MHC-II transgenes. These possibilities compensate for the slight disappointment of generating a phenotype that does not depart from the predictable.

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