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MHC class II-independent and -dependent T cell expansion and B cell hyperactivity *in vivo* in mice deficient in CD152 (CTLA-4)

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Abstract

One of the key downregulators of T cell activation is CD152 (CTLA-4). Mice genetically deficient in CD152 (*cd152*$^{-/-}$ mice) develop massive expansion of both CD4+ and CD8+ T cells as well as increased numbers of splenic Ig-secreting cells and serum Ig levels. To determine the dependence of the lymphoproliferation and B cell hyperactivity on MHC class II (MHCII), MHCII-deficient (*mhcii*$^{-/-}$) *cd152*$^{-/-}$ mice were generated. Compared to that in their *mhcii*$^{+/+}$ counterparts, expansion of CD4+ cells in *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice was markedly attenuated. Nonetheless, expansion of CD8+ cells was identical in both sets of mice, demonstrating that the effects of CD152 deficiency on CD4+ cells can quantitatively be dissociated from those on CD8+ cells, and pointing to a critical downregulatory role for CD152 in MHCII-independent CD8+ cell activation *in vivo*. B cell hyperactivity also developed in *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice, albeit in a manner less rapid and less intense than that in their *mhcii*$^{+/+}$ counterparts, demonstrating an underlying MHCII-independent diathesis to B cell dysregulation and pointing to a critical downregulatory role for CD152 in MHCII-independent B cell activation *in vivo*. When human DQ8 was introduced as a transgene into *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice, B cell hyperactivity was restored to levels observed in *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice, pointing to a critical downregulatory role for CD152 in MHCII-dependent B cell activation *in vivo* superimposed upon its downregulatory role on MHCII-independent B cell activation.

Introduction

T cells play a vital and central role in the adaptive immune response. On the one hand, insufficient T cell activation may result in clinically significant immunodeficiency, with the host being incapable of adequately combating microbial pathogens. On the other hand, excessive T cell activation can result in clinically significant autoimmunity, potentially involving any organ system. Accordingly, not only must the host be capable of responding quickly and vigorously to antigenic stimulation, but the host must also be capable of tightly controlling such responses and maintaining T cell activation in check.

One of the critical downregulators of T cell activation is CD152 (CTLA-4). In resting T cells, CD152 is predominantly expressed intracellularly with only limited cell surface expression (1). Following TCR engagement, intracellular and the limited cell surface CD152 focus to the sites of TCR engagement (2), with greater strength of the TCR signal resulting in greater surface accumulation of CD152 (3). CD152 mRNA levels are upregulated within hours (4), and cell surface CD152 expression greatly increases within days (5,6), although intracellular CD152 still predominates (7). At least two independent intracellular signaling pathways mediate CD152 upregulation (8).

CD80 (B7.1) and CD86 (B7.2), expressed by cells capable of antigen presentation (e.g. monocytes, macrophages, dendritic cells, B cells), are the natural ligands for CD152 (9–12). Engagement of CD152 delivers a direct inhibitory signal and also sequesters CD80/CD86, thereby indirectly impeding CD28-mediated activation (13,14). At least part of the inhibition is due to increased indoleamine 2,3-deoxygenase activity by dendritic cells with consequent increased catabolism of tryptophan (15). The net result of CD152 engagement is inhibition of activation-induced upregulation

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of membrane lipid raft expression (16) and arrest of T cells in the G0/G1 phase of the cell cycle (17,18).

Blockade of CD152 engagement has a myriad of biologic consequences. On the one hand, treatment with soluble anti-CD152 mAb results in enhanced *in vitro* T cell proliferation and IL2 production (5, 6, 19). Similar blockade of CD152 *in vivo* enhances expansion of antigen-specific T cells, promotes protective immunity against parasitic infection, blocks 'immunostimulatory' chronic graft-versus-host disease and augments development of anti-tumor immunity (20–23). On the other hand, *in vivo* blockade of CD152, rather than being beneficial, may be deleterious to the host, in that it accelerates and exacerbates onset and severity of experimental autoimmune encephalomyelitis (EAE) and autoimmune diabetes (24–26) and confers susceptibility to EAE in otherwise resistant mice (27). Indeed, engagement of CD152 is crucial to development and/or maintenance of tolerance *in vivo* (28, 29), and the potent suppressor effects of $CD4^+CD25^+$ regulatory T cells are substantially mediated via CD152 (30,31).

Mice genetically deficient in CD152 ($cd152^{-/-}$ mice) spontaneously develop massive systemic T lymphoproliferation with infiltration of numerous vital organs by lymphocytes and inflammatory cells (32–34). Thymocyte development, including negative and positive selection, is normal in $cd152^{-/-}$ mice (35,36), indicating that the physiologic defect is in control of peripheral T cell activation rather than in central T cell development. Since the accelerated T cell activation, lymphoproliferation and mortality are markedly attenuated in TCR-transgenic $cd152^{-/-}$ mice with highly limited T cell repertoires (36–39), it is likely that the proliferating T cells in these mice respond to self antigens and/or to highly prevalent environmental antigens. The fact that the activated T cells in non-transgenic $cd152^{-/-}$ mice manifest a diverse and unbiased TCR repertoire (40) indicates that no individual self or environmental antigen is uniquely driving the pathologic response.

The ramifications of CD152 deficiency for B cells *in vivo* are less well understood. Increased B cell numbers and circulating Ig levels, as well as upregulation of B cell surface CD86, have been reported in $cd152^{-/-}$ mice (32,34). This B cell hyperactivity may be secondary to excessive $CD4^+$ cell-mediated help, inasmuch as treatment of $cd152^{-/-}$ mice with a depleting anti-CD4 mAb blocks the B lymphocytosis (34). Since $CD4^+$ T cell-mediated help is predominantly MHC class II (MHCII)-restricted and antibody responses to T cell-dependent antigens are profoundly blunted in MHCII-deficient ($mhcii^{-/-}$) mice (41,42), one might predict that numbers of Ig-secreting cells (IgSC) and circulating Ig levels would be (near-) normal in MHCII-deficient $cd152^{-/-}$ mice. Moreover, the widespread activation and expansion of $CD8^+$ cells in $cd152^{-/-}$ mice is also greatly attenuated by depletion of $CD4^+$ cells (34), suggesting that dysregulation of $CD4^+$ cells is also vital to dysregulation of $CD8^+$ cells. Accordingly, one might predict that $CD8^+$ cell activation and expansion in MHCII-deficient $cd152^{-/-}$ mice would be, at most, very limited.

In this report, we demonstrate that these predictions are each incorrect. $CD8^+$ cell activation and expansion in MHCII-deficient $cd152^{-/-}$ mice are as dramatic as they are in MHCII-intact $cd152^{-/-}$ mice. Furthermore, B cell hyperactivity also develops in MHCII-deficient $cd152^{-/-}$ mice over time, although in a manner less rapid and less intense than that in corresponding MHCII-intact mice. Thus, CD152 plays a vital role in downregulating 'spontaneous' MHCII-independent $CD8^+$ cell expansion and B cell hyperactivity. Moreover, introduction of human MHCII into $mhcii^{-/-}cd152^{-/-}$ mice restores the more rapid kinetics and greater intensity of B cell hyperactivity, demonstrating a vital role for CD152 in downregulating both 'spontaneous' MHCII-dependent as well as 'spontaneous' MHCII-independent B cell hyperactivity.

Methods

General

All reported studies were approved by the University of Southern California (USC) Institutional Animal Care and Use Committee.

Mice

All mice were housed at USC in a single specific pathogen-free room. MHCII-intact ($mhcii^{+/+}$) $cd152^{+/-}$ mice bearing a C57Bl/6 (B6) background (35) were propagated by mating with B6 ($cd152^{+/+}$) mice and screening the progeny for *cd152* heterozygosity (see below). MHCII-deficient ($mhcii^{-/-}$) $cd152^{+/-}$ mice were generated by first crossing $mhcii^{+/+}$ $cd152^{+/-}$ mice with Aβ0 mice ($mhcii^{-/-}cd152^{+/+}$) (41,43) and selecting for *cd152* heterozygosity. The resulting $mhcii^{+/-}$ $cd152^{+/-}$ mice were backcrossed to Aβ0 mice and selected for *cd152* heterozygosity and the absence of MHCII (see below).

To reconstitute $mhcii^{-/-}cd152^{-/-}$ mice with human MHCII, $mhcii^{-/-}cd152^{+/-}$ mice were crossed with Aβ0-DQ8 mice ($mhcii^{-/-}dq8^{+/+}cd152^{+/+}$; deficient for murine endogenous MHCII and homozygous for a transgenic fragment containing both DQA*0301 and DQB*0302 genes) (44). The progeny (all $mhcii^{-/-}dq8^{+/-}$) were selected for *cd152* heterozygosity and were backcrossed to Aβ0-DQ8 mice. Progeny from this backcross that were $cd152^{+/-}$ were screened for *dq8* homozygosity by mating to B6 mice and testing the progeny for *dq8* by PCR (see below). At least 12 pups from a given $cd152^{+/-}$ parent were tested, and $cd152^{+/-}$ parents were deemed to be $dq8^{+/+}$ if all $\geqslant$12 pups bore *dq8*.

Assignment of cd152 genotype

cd152 genotype was determined by PCR. Small clippings of mouse tails were digested for >4 h at 56°C by proteinase K in Tris/EDTA/SDS buffer followed by phenol/chloroform extraction of genomic DNA. Genomic DNA was PCR-amplified for 42–45 cycles each at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. The primer sequences used were: 5′-CCAGAACCATGCCCGGATTCTGACTTC-3′ (*cd152*-intact sense), 5′-CCAAGTGCCCAGAGGGGCTGCTAAA-3′ (*cd152*-deficient sense), 5′-AAACAACCCCAAGCTAACTGCGACA-AGG-3′ (anti-sense). The PCR products were subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide, and bands were visualized under UV light. Band sizes for *cd152*-intact and *cd152*-deficient were ~90 and ~180 bp respectively.

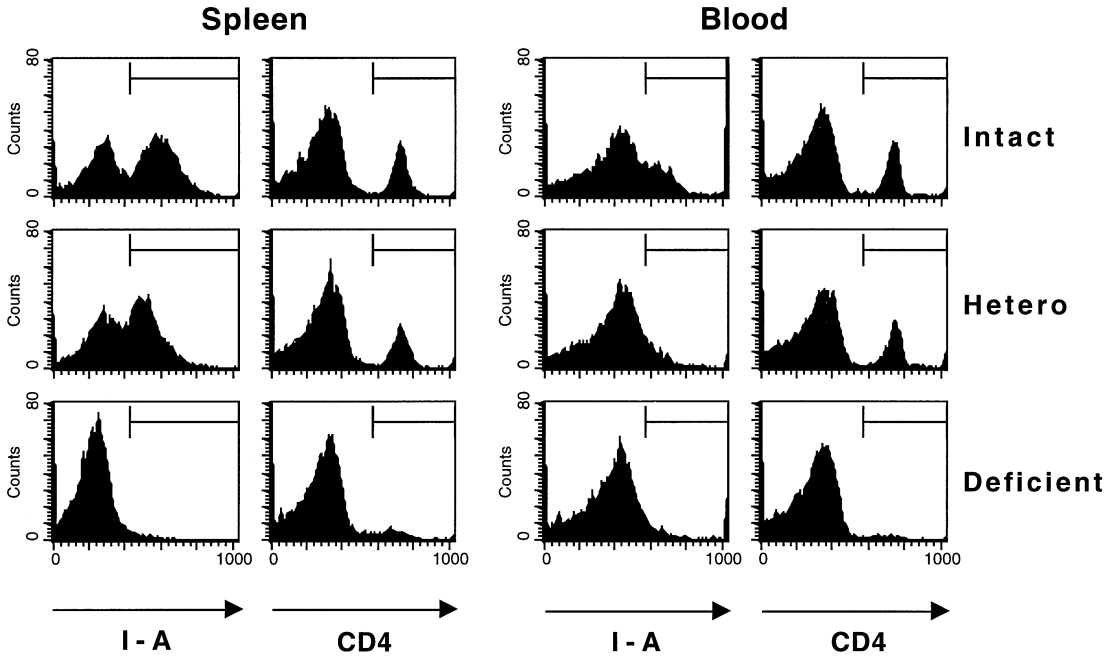

Fig. 1. Surface staining of CD4 and I-A^b in spleen mononuclear cells and PBMC in MHCII-intact, MHCII-intermediate and MHCII-deficient mice. Spleen mononuclear cells and PBMC from mice with the obligatory genotypes of *mhcii*$^{+/+}$ (MHCII-intact), *mhcii*$^{+/-}$ (MHC-intermediate), or *mhcii*$^{-/-}$ (MHCII-deficient) were stained for surface CD4 or surface I-A^b. The bars across the tops of the individual panels indicate positive staining.

Assignment of mhcii genotype

Both copies of the gene coding for I-Aβ^b are disrupted by a *neo* insertion in parental *mhcii*$^{-/-}$ Aβ0 mice (41). Staining splenic B cells for surface I-A^b readily identified by visual inspection the MHCII-intact, MHCII-intermediate and MHCII-deficient phenotypes, which correspond to *mhcii*$^{+/+}$, *mhcii*$^{+/-}$ and *mhcii*$^{-/-}$ genotypes respectively (Fig. 1). Although this procedure was suitable for mice being sacrificed, staining spleen cells was not suitable for mice being used for breeding. Unfortunately, staining of PBMC for surface I-A^b often yielded visually ambiguous results that did not permit discrimination between MHCII-intermediate and MHCII-deficient phenotypes. This was likely due to the lower percentages of MHCII$^+$ cells in blood than those in spleen which, in turn, precluded clear separation of positively staining cells from negative cells. Fortunately, MHCII-deficient mice harbor markedly reduced numbers (and percentages) of CD4$^+$ T cells in the periphery (41, 42), and staining PBMC for surface CD4 readily discriminated visually between the CD4-low phenotype (*mhcii*$^{-/-}$ genotype) and CD4-high phenotype (*mhcii*$^{+/-}$ or *mhcii*$^{+/+}$ genotypes) (Fig. 1).

Accordingly, the first step in assignment of *mhcii* genotype was the staining of PBMC for surface CD4. (Staining of spleen cells for surface I-A^b was always performed in sacrificed mice to confirm the absence of MHCII in CD4-low mice and the presence of MHCII in CD4-high mice.) To distinguish between *mhcii*$^{+/+}$ and *mhcii*$^{+/-}$ genotypes among CD4-high mice, PCR was next performed to detect the *neo* insertion. (PCR alone cannot distinguish among all three *mhcii* genotypes, since a PCR product band indicative of the *neo* insertion would be detected in both *mhcii*$^{+/-}$ and *mhcii*$^{-/-}$ mice.) The primer sequences used were: 5′-GGATCGGCCATTGAACAAG-3′ (sense), 5′-CACCATGATATTCGGCAAGC-3′ (anti-sense). Band size for *neo* was ~600 bp.

Detection of dq8

The presence of both DQ8α and DQ8β were detected by PCR using the following primer sequences: 5′-GAAGACATTGTGGCTGACCATGTTGCC-3′ (DQ8α sense), 5′-AGCACAGCGATGTTTGTCAGTGCAAATTGCGG-3′ (DQ8α anti-sense), 5′-AGGATTTGGTGTWCCAGTTTAAGGGCAT-3′ (DQ8β sense), 5′-TGCAAGGTCGTGCGGAGCTCCAA-3′ (DQ8β anti-sense). Band sizes for *dq8α* and *dq8b* were ~250 and ~350 bp respectively.

Cell surface staining

Murine spleen mononuclear cells were isolated by mechanically teasing the spleens followed by Ficoll density gradient centrifugation. The isolated mononuclear cells were single-, double- or triple-stained with appropriate combinations of FITC-, PE- or Cy-Chrome-conjugated mAb specific for murine CD3, CD4, CD8, CD44, CD45R (B220), CD62L or I-A^b (BD PharMingen, San Diego, CA) and analyzed by flow cytometry. Cell debris, as determined by forward- and side-scatter characteristics, was electronically excluded from the analysis. At least 10 000 events were analyzed for each sample.

Serum Ig and spleen IgSC determinations

Mice were bled and/or sacrificed at the indicated times. Serum was assayed for total IgG and total IgM by ELISA. To do so, 96-well flat-bottomed plates were coated overnight at 4°C with goat anti-mouse IgG+IgA+IgM antibodies (Zymed

Laboratories, South San Francisco, CA) and blocked with 10% FCS for 90 min at 37°C. After washing with 0.05% Tween-20 in PBS, diluted sera were added to the plates for 90 min at 37°C, washed, and detected with HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-mouse IgM (Zymed) for 90 min at 37°C. After washing, color was developed by addition of *o*-phenylenediamine (Sigma-Aldrich, St. Louis, MO). The reaction was terminated by addition of 6N H_2SO_4, and OD_{450} was measured. Purified mouse IgG or IgM (Zymed) were used as standards.

Spleen cells were assayed for total IgSC by the reverse hemolytic plaque assay (45,46). Each plaque-forming cell was taken as an IgSC.

Statistical analysis

All analyses were performed using SigmaStat software (SPSS, Chicago, IL). To achieve normality, the results (cell numbers, cell percentages, Ig levels, IgSC) were log-transformed. Parametric testing between two matched or unmatched groups was performed by the paired or unpaired *t*-test respectively. Parametric testing among three or more groups was performed by one-way ANOVA. When log-transformation failed to generate normally distributed data or the equal variance test was not satisfied, non-parametric testing was performed by the Mann–Whitney rank sum test between two groups and by Kruskal–Wallis one-way ANOVA on ranks among three or more groups. Correlations were determined by Pearson product moment correlation for interval data and by Spearman rank order correlation for ordinal data or for interval data which did not follow a normal distribution.

Results

Marked expansion and activation of CD4⁺ and CD8⁺ cells in mhcii$^{+/+}$cd152$^{-/-}$ mice

MHCII-intact (*mhcii*$^{+/+}$) *cd152*$^{+/-}$ male and female mice were mated to each other, and the resulting F2 progeny was genotypically +/+, +/– and –/– for *cd152* at the expected 1:2:1 ratio. Since *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice begin to die by 3–5 weeks of age (32,33,35 and unpublished observations), we studied 3-week-old mice. Consistent with previous reports (32–34), marked (3- to 4-fold) expansion of T (CD3⁺) cells, with similar expansions of both CD4⁺ cells and CD8⁺ cells, developed in *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice compared to *cd152*$^{+/+}$ or *cd152*$^{+/-}$ littermates (Fig. 2A–C; $P < 0.001$ for each cell type). Large percentages of both CD4⁺ and CD8⁺ cells in *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice displayed an activated (CD44⁺62L⁻) phenotype, whereas far fewer activated CD4⁺ and CD8⁺ cells were detected in either *mhcii*$^{+/+}$*cd152*$^{+/+}$ mice (Fig. 3) or *mhcii*$^{+/+}$*cd152*$^{+/-}$ mice (data not shown).

Unmitigated CD8 cell expansion and activation with attenuated CD4 cell expansion in mhcii$^{-/-}$cd152$^{-/-}$ mice

The identity of the antigens to which the activated CD4⁺ and CD8⁺ cells respond in *cd152*$^{-/-}$ mice is unknown. Given that these cells manifest a diverse and unbiased TCR repertoire (40), it is clear that the T cell activation is a polyclonal one. Since *in vivo* depletion of CD4⁺ cells from these mice greatly attenuates the widespread activation and expansion of CD8⁺ cells (34), dysregulation of CD8⁺ cells appears to be linked to dysregulation of CD4⁺ cells. Given the importance of MHCII to CD4⁺ cell responses, MHCII may play a crucial role in both CD4⁺ and CD8⁺ lymphoproliferation.

To formally assess MHCII-dependence of T cell expansion in *cd152*$^{-/-}$ mice, we mated MHCII-deficient (*mhcii*$^{-/-}$) *cd152*$^{+/-}$ male and female mice with each other, and the resulting F2 progeny was genotypically +/+, +/– and –/– for *cd152* at the expected 1:2:1 ratio. There were considerably (~70%) fewer splenic CD4⁺ cells in 3-week-old *mhcii*$^{-/-}$*cd152*$^{+/+}$ and *mhcii*$^{-/-}$*cd152*$^{+/-}$ mice compared to those in their *mhcii*$^{+/+}$ counterparts (compare Fig. 2E with B; $P < 0.001$ for each comparison), likely due to the absence of MHCII-based positive selection of CD4⁺ cells (41,42). In *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice, the number of CD4⁺ cells was modestly greater (<2-fold) than those in *cd152*$^{+/+}$ or *cd152*$^{+/-}$ littermates ($P = 0.012$), but the relative expansion was much more limited than that observed in *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice ($P < 0.001$). Of note, CD4⁺ cells from all MHCII-deficient mice (including those with *cd152*$^{+/+}$ or *cd152*$^{+/-}$ genotype) displayed an activated phenotype (Fig. 3 and data not shown), rendering moot the question of whether CD152 deficiency promotes activation of MHCII-unrestricted CD4⁺ cells.

The attenuated CD4⁺ cell expansion in *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice notwithstanding, expansion CD8⁺ cells in these mice was unimpeded (compare Fig. 2F with C). Moreover, whereas CD8⁺ cells from MHCII-deficient *cd152*$^{+/+}$ or *cd152*$^{+/-}$ mice displayed a non-activated phenotype, CD8⁺ cells from MHCII-deficient *cd152*$^{-/-}$ mice displayed an activated phenotype similar to that of CD8⁺ cells from MHCII-intact *cd152*$^{-/-}$ mice (Fig. 3). This demonstrates that the MHCII-independent increase in CD8⁺ cells in *cd152*$^{-/-}$ mice is due to persistent CD8⁺ cell activation rather than to increased survival of resting or quiescent CD8⁺ cells.

In contrast to the early death of *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice, *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice live into adulthood and can reproduce (unpublished observations). This permitted us to mate *mhcii*$^{-/-}$*cd152*$^{+/-}$ mice to *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice, and the resulting pups (50% being *cd152*$^{+/-}$ and 50% being *cd152*$^{-/-}$) were evaluated at 5 weeks of age. Although CD4⁺ cells were modestly increased (2-fold) in *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice compared to those in *cd152*$^{+/-}$ littermates ($P < 0.001$, Fig. 2H), this increase was overwhelmed by the massive expansion (13-fold) of CD8⁺ cells in the *cd152*$^{-/-}$ mice ($P < 0.001$, Fig. 2I). Thus, CD8⁺ cell activation and expansion is unfettered in the absence of CD152 in MHCII-deficient hosts, demonstrating a vital down-regulatory role for CD152 in MHCII-independent CD8⁺ cell expansion.

Development of B cell hyperactivity in mhcii$^{-/-}$cd152$^{-/-}$ mice

Concurrent with the exuberant CD4⁺ and CD8⁺ cell expansion in 3-week-old *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice, these mice, despite no increase in splenic B (B220⁺) cells, displayed markedly increased numbers of splenic IgSC (~15-fold; $P < 0.001$) and circulating levels of IgG (~9-fold; $P < 0.001$) compared to *cd152*$^{+/+}$ or *cd152*$^{+/-}$ littermates (Fig. 4A–C). Circulating IgM levels were also increased ~5-fold ($P < 0.001$, data not shown). In contrast, splenic IgSC and circulating IgG and IgM levels in 3-week-old *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice were not appreciably different from those in their *cd152*$^{+/+}$ or *cd152*$^{+/-}$ littermates (Fig. 4E and F and data not shown). However, by

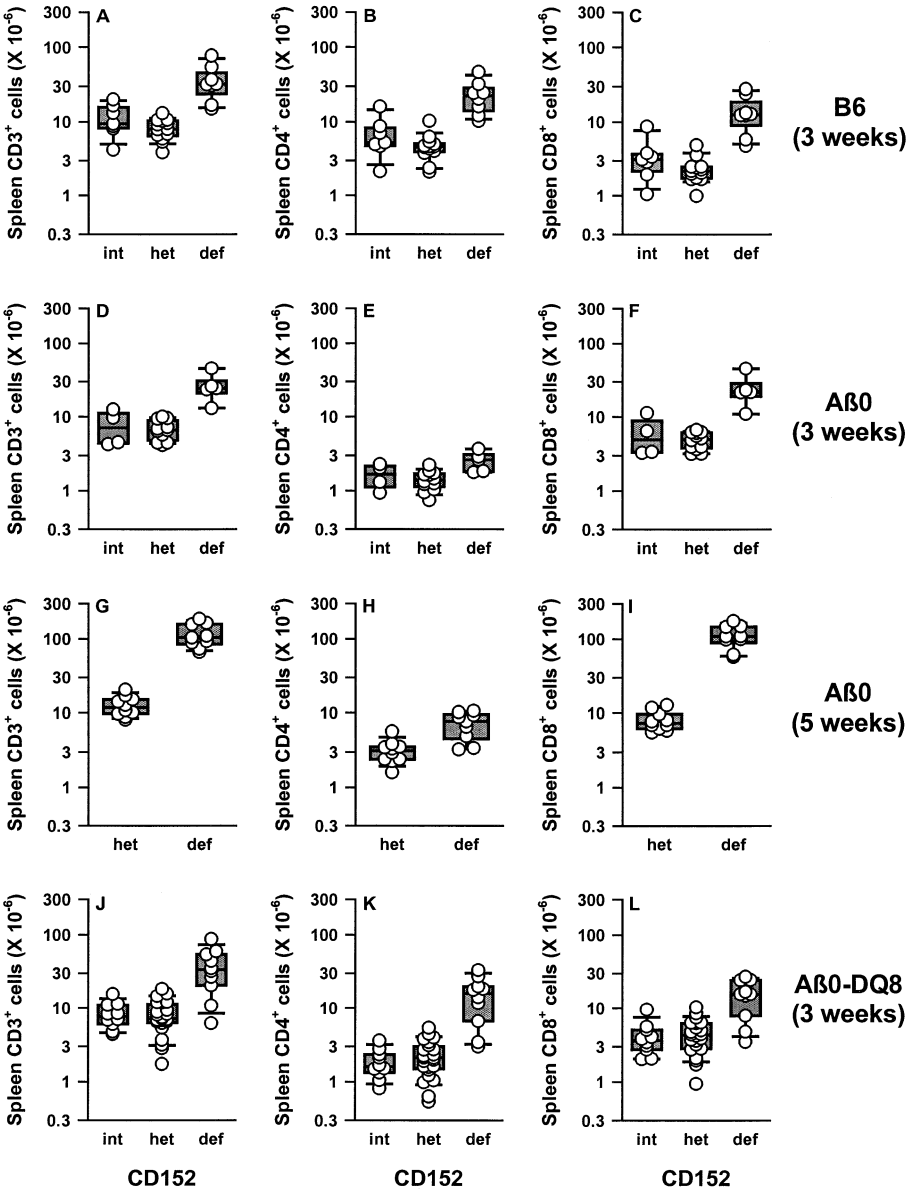

Fig. 2. Spleen T cell populations in *cd152*$^{+/+}$, *cd152*$^{+/-}$ and *cd152*$^{-/-}$ mice. The indicated mice were sacrificed at 3 weeks or 5 weeks of age and assessed for spleen CD3$^+$ cells (panels A, D, G and J), spleen CD4$^+$ cells (panels B, E, H and K) and spleen CD8$^+$ cells (panels C, F, I and L). B6 mice are MHCII-intact; Aβ0 mice are MHCII-deficient; Aβ0-DQ8 mice do not express murine MHCII but do express human MHCII (DQ8). int denotes *cd152*$^{+/+}$ genotype; het denotes *cd152*$^{+/-}$ genotype; def denotes *cd152*$^{-/-}$ genotype. Each circle represents an individual mouse. The composite results are plotted as box plots. The lines inside the boxes indicate the medians; the outer borders of the boxes indicate the 25th and 75th percentiles; and the bars extending from the boxes indicate the 10th and 90th percentiles.

5 weeks of age, B cell hyperactivity in *mhcii*$^{-/-}$*cd152*$^{-/-}$ was apparent, albeit not as dramatic as that appreciated in *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice. Although no expansion of splenic B (B220$^+$) cells in *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice was detected, splenic IgSC numbers had increased ~3-fold ($P = 0.001$), and serum IgG levels were clearly elevated in five of the nine *cd152*$^{-/-}$ mice relative to their *cd152*$^{+/-}$ littermates (Fig. 4G–I). Thus, there is an underlying MHCII-independent diathesis to B cell hyperactivity which CD152 normally controls. Of note, spleen CD8$^+$ cell numbers correlated strongly with serum IgG levels in *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice (Fig. 4J), suggesting that the increase in each was being driven by a common trigger (such as the MHCII-unrestricted CD4$^+$ cells) and/or that the B cell hyperactivity was actually promoted (at least in part) by activated CD8$^+$ cells.

Quantitative restoration of B cell hyperactivity in Aβ0-DQ8.cd152$^{-/-}$ *mice*

To assess whether introduction of MHCII into *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice would restore B cell hyperactivity to the degree observed in *mhcii*$^{+/+}$*cd152*$^{-/-}$ mice, we generated male and female Aβ0-DQ8.*cd152*$^{+/-}$ mice (which do not express endogenous

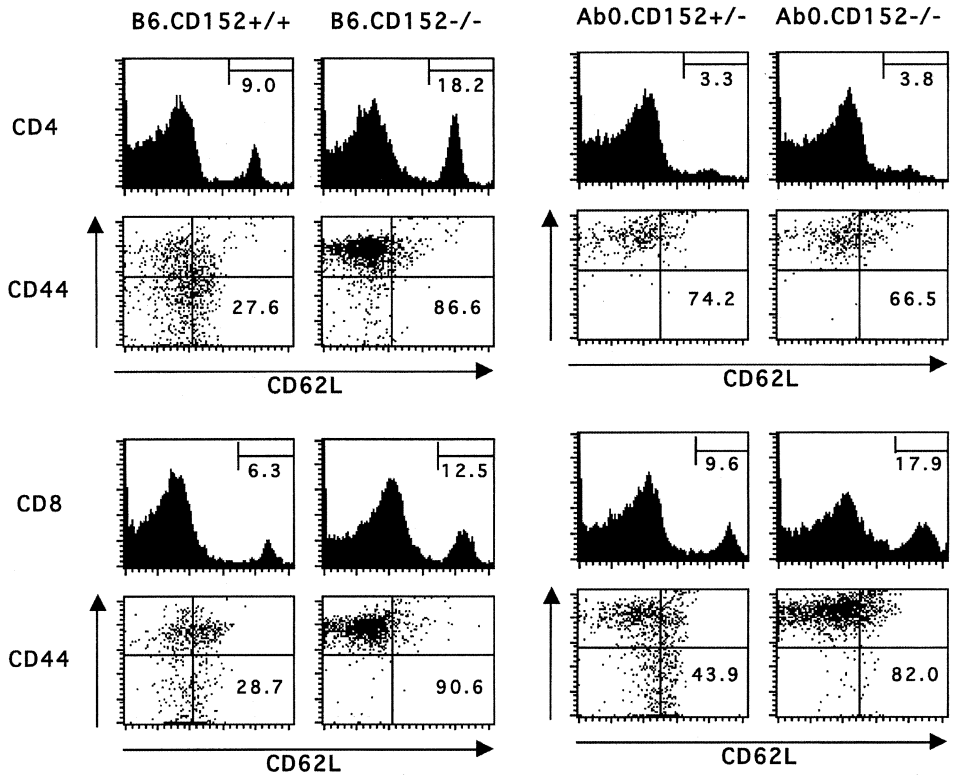

Fig. 3. Activated CD4 and CD8 cells in *cd152*$^{+/+}$ and *cd152*$^{-/-}$ mice. Spleen cells from the indicated mice were triple-stained for surface CD4, CD44 and CD62L (top panels) or for surface CD8, CD44 and CD62L (bottom panels). The histograms illustrate CD4 and CD8 staining respectively, with the corresponding horizontal bars delimiting the CD4$^+$ and CD8$^+$ cells. The numbers across these bars indicate the percentages of cells staining positively for CD4 or CD8. The dot plots illustrate CD44 and CD62L staining for the CD4$^+$ and CD8$^+$ cell populations respectively. The numbers indicate the percentages of activated (CD44$^+$CD62L$^-$) cells within the CD4$^+$ or CD8$^+$ populations.

murine MHCII but do express human DQ8). We mated them to each other, and the resulting F2 progeny was genotypically +/+, +/– and –/– for *cd152* at the expected 1:2:1 ratio. In 3-week-old Aβ0-DQ8.*cd152*$^{-/-}$ mice, CD4$^+$ cell expansion was more than completely restored (6- to 7-fold expansion relative to cd152$^{+/+}$ or cd152$^{+/-}$ littermates; $P < 0.001$), and CD3$^+$ and CD8$^+$ cell expansions were preserved (3- to 4-fold expansion relative to *cd152*$^{+/+}$ or *cd152*$^{+/-}$ littermates; $P < 0.001$ for each cell type) (Fig. 2G–I). Importantly, DQ8.*cd152*$^{-/-}$ mice also manifested increased (~4-fold) splenic IgSC and increased (~4-fold) serum IgG levels in comparison to their CD152-sufficient littermates ($P < 0.001$ for each) (Fig. 4L–M). Taken together, these results demonstrate that B cell hyperactivity in *cd152*$^{-/-}$ mice occurs via both MHCII-dependent and MHCII-independent pathways and that CD152 downregulates both pathways.

Discussion

Both CD4$^+$ and CD8$^+$ cells undergo marked activation and expansion in MHCII-intact *cd152*$^{-/-}$ mice with accompanying infiltration of many vital organs (32–34). Similarly, many (MHCII-intact) humans treated with anti-CD152 mAb develop organ inflammation with infiltration of CD4$^+$ and/or CD8$^+$ cells (47). Moreover, the association of specific human autoimmune diseases with *cd152* genetic polymorphisms (48) suggests that genetically determined differences in CD152 expression may affect susceptibility to clinical autoimmunity.

CD28-mediated signaling is vital to development of lymphoproliferation in MHCII-intact *cd152*$^{-/-}$ mice. Treatment of such mice with CTLA4-Ig (which blocks CD28/CD80 and CD28/CD86 interactions) prevents their abnormal T cell activation and expansion for the duration of treatment (49). Moreover, MHCII-intact *cd152*$^{-/-}$ mice that are also deficient in CD80 and CD86 (*cd80*$^{-/-}$*cd86*$^{-/-}$ genotype) are protected from the lymphoproliferative phenotype, but lymphoproliferation can be induced in these mice by artificial ligation of CD28 with an anti-CD28 mAb (50).

CD4$^+$ cells also play a vital role in the lymphoproliferation that develops in *cd152*$^{-/-}$ mice. In contrast to the MHCII-restriction exhibited by most CD4$^+$ T cells, the great majority of CD8$^+$ T cells are MHC class I-restricted. Nevertheless, their antigen-driven activation usually depends critically upon CD4$^+$ cells (51–53). The fact that the massive expansion of CD8$^+$ cells in MHCII-intact *cd152*$^{-/-}$ mice is largely prevented by treatment with a depleting anti-CD4 mAb (34) is consistent with this principle. Therefore, it was unexpected that despite substantially reduced numbers of CD4$^+$ cells, CD8$^+$ cell activation and expansion remained unabated in MHCII-deficient *cd152*$^{-/-}$ mice. The degree of CD8$^+$ cell activation and expansion in these mice was similar to that in their MHCII-intact counterparts and in their counterparts expressing

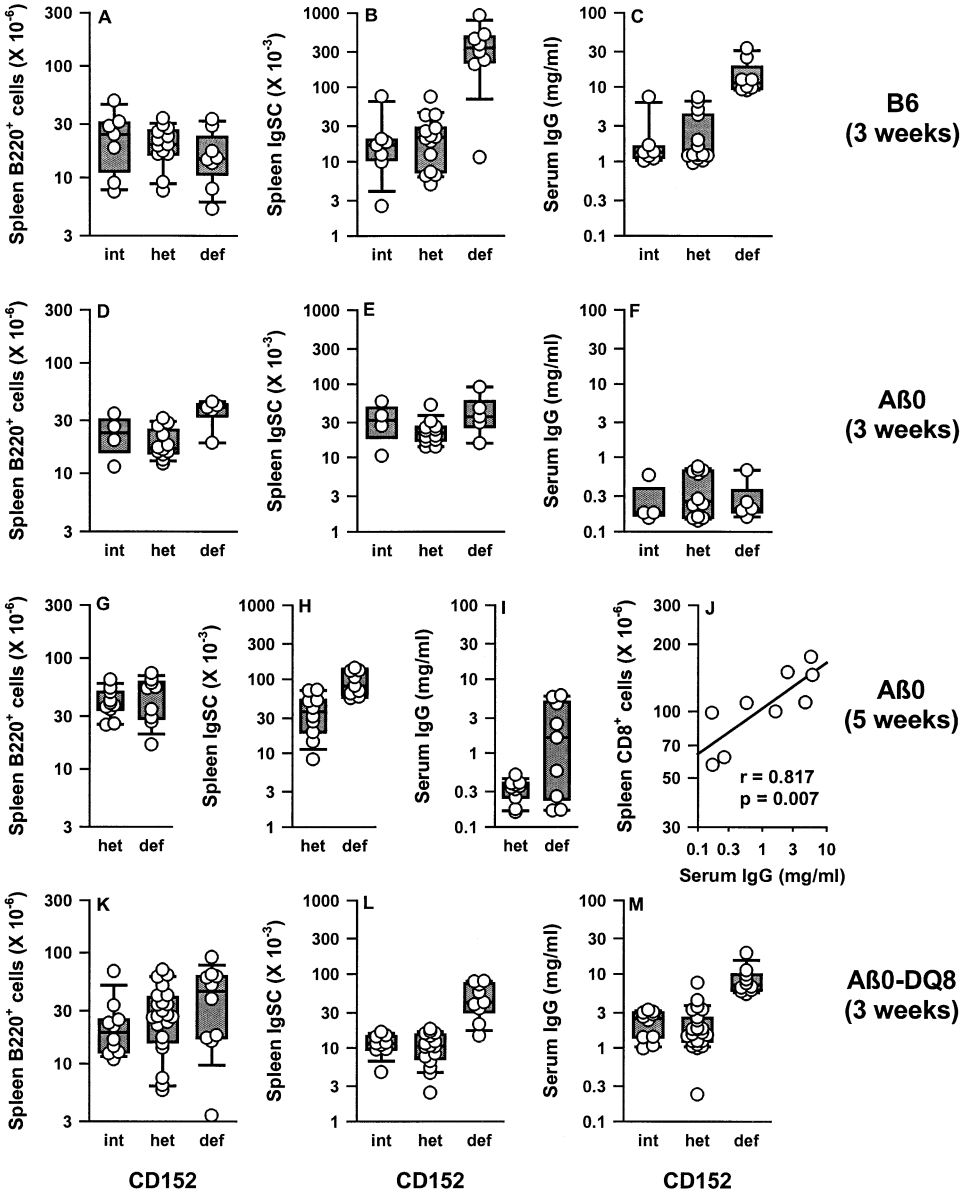

Fig. 4. Spleen B cells, spleen IgSC and serum IgG levels in *cd152*$^{+/+}$, *cd152*$^{+/-}$ and *cd152*$^{-/-}$ mice. The indicated mice were sacrificed at 3 weeks or 5 weeks of age and assessed for spleen B (B220^{+}) cells (panels A, D, G and K), spleen IgSC (panels B, E, H and L) and serum IgG levels (panels C, F, I and M). Presentation of the results is as in Fig. 2. In panel J, serum IgG levels are plotted against spleen CD8^{+} cell numbers for the 9 individual *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice tested. The solid line represents the calculated regression line.

human MHCII rather than murine MHCII (Figs 2 and 3). Taken together, these observations strongly suggest that the helper role played by CD4^{+} cells in CD8^{+} cell activation can be dissociated from the ability of CD4^{+} cells to recognize antigen in the context of MHCII. Whether CD28-mediated signaling plays a role in MHCII-independent activation of CD8^{+} cells remains to be formally established.

Our results of unabated CD8^{+} cell expansion in MHCII-deficient *cd152*$^{-/-}$ mice can readily be reconciled with the prevention of CD8^{+} expansion in anti-CD4-treated *cd152*$^{-/-}$ mice (34). In the latter study, repeated injections of a depleting anti-CD4 mAb, beginning within 2 days after birth, had to be administered to *cd152*$^{-/-}$ mice over a 3-week period to effect continuous depletion of CD4^{+} cells. Without continuous CD4^{+} cell depletion, low numbers of activated CD4^{+} cells developed, and CD8^{+} cell expansion was not effectively blocked. Unfortunately, we could not similarly treat MHCII-deficient *cd152*$^{-/-}$ mice with a depleting anti-CD4 mAb, since all MHCII-deficient mice in our colony, regardless of *cd152* genotype, do not tolerate repeated i.p. injections and succumb prematurely. Whether this intolerance to repeated i.p. injections is due to undue susceptibility to occult infections in MHCII-deficient hosts or due to some other cause is uncertain.

Regardless, the great majority of the (MHCII-unrestricted) CD4^{+} cells in MHCII-deficient mice bear an activated phenotype (Fig. 3). Indeed, it may be that activated MHCII-

unrestricted CD4+ cells are crucial helpers of CD8+ cell expansion even in MHCII-intact hosts. The vast majority of MHCII-unrestricted CD4+ cells are restricted by CD1d, so the requisite CD4+ cell-mediated help for antigen-driven CD8+ cell activation may arise from recognition by CD4+ cells of CD1d-restricted antigens. Future experiments utilizing CD1-deficient mice should help resolve this issue.

Alternatively, CD8+ cell activation and expansion in *mhcii*$^{-/-}$ *cd152*$^{-/-}$ mice may be occurring by a pathway that altogether circumvents the requirement for antigen recognition by CD4+ cells. APC can be activated via engagement of their surface CD40 even in the absence of CD4+ cells (51–53). CD154 (CD40 ligand) can be expressed by both CD4+ and double-negative (DN; CD4$^-$CD8$^-$) CD1d-restricted T cells (54, 55), so direct engagement of APC by CD154+ cells (even in an antigen-non-specific manner) could result in conditions permissive to activation and expansion of CD8+ cells. The reason that continuous depletion of CD4+ cells blocks CD8+ cell expansion in *cd152*$^{-/-}$ hosts may have nothing to do with MHCII/CD4+ cell interactions but may be consequent to the elimination of most CD154+ cells. MHCII-deficient mice may have sufficient numbers of CD154+ cells to activate APC and permit unabated CD8+ cell activation and expansion. Experiments utilizing CD154-deficient mice should help clarify this point. In any case, our findings clearly point to a vital downregulatory role for CD152 in MHCII-independent activation and expansion of CD8+ cells.

In vivo deficiency of CD152 leads not only to marked activation and expansion of T cells, but it also leads to marked increases in IgSC and circulating Ig levels (Fig. 4). Of note, numbers of splenic B (B220+) cells are not increased in *cd152*$^{-/-}$ mice despite the increased *in vivo* Ig production in such mice. This may reflect a preferential downregulatory effect of CD152 on differentiation, rather than on proliferation, of B cells. Alternatively, B cell numbers may truly increase in *cd152*$^{-/-}$ mice, but the expanded B cells may preferentially home to extrasplenic sites. Increased numbers of lymph node B cells have been described in *cd152*$^{-/-}$ mice (34), and other sites, such as the intestine or bone marrow, may also harbor increased numbers of B cells.

In either case, the observed B cell hyperactivity in *cd152*$^{-/-}$ mice is effected via both MHCII-dependent and MHCII-independent pathways. In *mhcii*$^{-/-}$*cd152*$^{-/-}$ mice, B cell hyperactivity is not apparent at 3 weeks of age but is readily apparent at 5 weeks of age (Fig. 4), demonstrating MHCII-independent B cell hyperactivity in the absence of CD152. Introduction of a transgene for human DQ8 into *cd152*$^{-/-}$ mice deficient for murine MHCII (but now expressing human MHCII) restores the vigorous B cell hyperactivity (Fig. 4), demonstrating superimposed MHCII-dependent B cell hyperactivity in the absence of CD152.

Of note, splenic IgSC numbers were essentially identical in MHCII-deficient *cd152*$^{+/+}$ or *cd152*$^{+/-}$ mice compared to those in the corresponding MHC-intact mice (compare Fig. 4E with B). This strongly suggests that B cell maturation to the IgSC state can occur normally in the absence of MHCII. A component of this B cell maturation may be T cell-independent, but it is likely that there is also a T cell-dependent component. Although serum IgG levels were lower in MHCII-deficient *cd152*$^{+/+}$ or *cd152*$^{+/-}$ mice compared to the respective levels in the corresponding MHC-intact mice (compare Fig. 4F with C), serum IgG levels had dramatically increased in the majority of MHCII-deficient *cd152*$^{-/-}$ mice by 5 weeks of age (Fig. 4I). Importantly, serum IgG levels in these mice correlated strongly with the degree of CD8+ cell expansion (Fig. 4J), raising the possibility that a component of the MHCII-independent B cell hyperactivity in *mhcii*$^{-/-}$ *cd152*$^{-/-}$ mice is driven by CD8+ cells. It may be that optimal treatment of autoimmune disorders associated with B cell hyperactivity may require targeting not just B cells and CD4+ cells but CD8+ cells as well.

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Abbreviations

DN	double-negative
EAE	experimental autoimmune encephalomyelitis
IgSC	Ig-secreting cells
MHC II	MHC class II

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