in vivo *27*: 29-40 (2013)

Diverging *In Vitro* Antibody Isotype Switching Preference in B-Lymphocytes from C57BL/6 and FVB Mice

ABDUL GAFOOR PUTHIYAVEETIL[1,2], BENJAMIN OKYERE[1,2], CHRISTOPHER M. REILLY[2,3] and DAVID CAUDELL[1,2]

[1]*Laboratory of Molecular Pathology,* [2]*Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA, U.S.A.;* [3]*Edward Via Virginia College of Osteopathic Medicine, Blacksburg, VA, U.S.A.*

Abstract. *Inbred strains of laboratory mice are commonly used in different immunological studies, with varying results. However, these variations are often overlooked and the underlying molecular mechanisms are less explored. In this study, we analyzed the differences in B-lymphocyte response and mechanisms of class switch recombination in two commonly used mouse strains, FVB and C57BL/6. These two strains of mice differ in their major histocompatibility complex (MHC) antigens, C57BL/6 having H-2b and FVB having H-2q, along with multiple cryptic changes. Analysis of in vitro class switch recombination showed that lymphocytes from these two strains of mice had altered preference for immunoglobulin isotype switching, with C57BL/6 producing more IgG1 and IgG3, but less IgG2b compared to FVB. Furthermore, cell-cycle patterns and gene expression studies for critical genes showed moderate differences between the two strains, suggesting that the cells opted for different molecular mechanisms under identical culture conditions.*

Inbred strains of laboratory mice are commonly used in a variety of biomedical research settings to elucidate immunopathogenesis, infectious disease challenge, drug discovery, and cancer biology (1). Many of these inbred strains and transgenic models are also employed in immune system characterization studies. Out of the inbred strains available, FVB and C57BL/6 (C57) are often used to generate knock-out, transgenic, or other conditionally-modified strains of mice mimicing human disease conditions. C57 is the most commonly used mouse strains in biomedical experiments. FVB mice carry homozygous *Fv1*^{b} allele for sensitivity of Friend's leukemia virus (2). These two strains vary in their major histocompatibility complex (MHC) antigens; FVB having H-2q subtype (2) and C57 being H-2b subtype (3). Furthermore, FVB mice carry polymorphisms in *T-cell receptor a* (*Tcra-V*$_{11.1}$) and genomic deletion of some *Tcrb-V* genes, including *Tcrb-V*$_{8.2}$ (4). In addition to these known genetic differences, there can be multiple cryptic genetic variations between these two strains (5). As a result when these mice are immunochallenged, they have variable immune responses, making research findings difficult to translate to the human condition being modeled.

B-lymphocytes are an integral part of the immune system as they produce antibodies, act as antigen-presenting cells and can phagocytize antigens (6). B-lymphocytes develop in the bone marrow from a common lymphoid progenitor (CLP) and proceed through early and late pro- B stages before entering large and small pre- B stages (7). These immature B-lymphocytes exit the bone marrow and populate the spleen and other secondary lymphoid organs, where they undergo further development to form mature naïve B-lymphocytes. Following interaction with antigens and co-stimulatory molecules, B-cells will proliferate and undergo somatic hypermutations (8), class switching (9), and clonal expansion (10) to produce highly specific immunoglobulin molecules against the invading antigens. Finally, they will differentiate into memory B-cells or long-lived plasma cells, the latter of which releases specific antibodies to circulation (11). The specificity and effectiveness of antibodies are attributed to the variable, diversity, and joining (VDJ) rearrangement in the bone marrow and class switch recombination (CSR) in secondary lymphoid organs. VDJ recombination results in enormous diversity in antigen recognition, somatic hypermutation increases the affinity and CSR results in the production of different isotypes of antibodies with varying effector functions. These gene recombination events are initiated by DNA double-strand breaks in the immunoglobulin gene, mediated by recombination activation

Correspondence to: David Caudell, DVM, Ph.D., Laboratory of Molecular Pathology, Department of Biomedical Sciences & Pathobiology, Center for Molecular Medicine & Infectious Diseases, Virginia Tech, 1410 Prices Fork Road, Blacksburg, VA. 24061, U.S.A. Tel: +1 5402317632, Fax: +1 5402313426, e-mail: dcaudell@vt.edu

Key Words: Mouse strain, class switch recombination, antibody, C57BL/6, FVB.

gene (RAG) and activation-induced cytidine deaminase (AID) enzymatic events during VDJ and CSR, respectively (12). These DNA double strand breaks are then repaired through the break repair mechanisms, predominantly by non-homologous end-joining (NHEJ) (12). Although NHEJ mechanisms are conserved across mammalian species, their functional efficiency and preferences varies between individuals, age groups, genetic strains and species. Previous studies have shown that FVB mice produce significant levels of antigen-specific IgE antibody *in vivo* (13, 14). In this study we sought to compare the CSR ability of FVB and C57 mice.

Materials and Methods

Mice. Five 20-week-old FVB and C57 mice were used for each experiment. The mice were bred and maintained at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited core laboratory animal facility at Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech. All experiments were carried out as per National Institute of Health guidelines with the approval from the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Splenic parameters. Spleens were harvested and evaluated grossly for size comparison. Single-cell suspensions were labeled with fluorescently tagged CD4, CD8 and IgM antibodies (eBiosciences, San Diego, CA, USA) and analyzed by flow cytometry. Sections of spleen were fixed in 4% formaldehyde followed by routine processing and paraffin embedding. Four micron-thick tissue sections were stained with hematoxylin and eosin (H&E), and evaluated by a veterinary pathologist using a Nikon Eclipse 50i light microscope. Images of the spleen were acquired using the NIS-Elements D software 3.10 (Nikon, Melville, NY, USA).

CSR assay. Splenic B-lymphocytes were harvested using magnetic beads and a magnetic-assisted cell sorting (MACS) system (Milteny Biotec, Auburn, CA, USA). A total of 2×10^5 cells were treated with 5 μM carboxyfluorescein diacetate succinmidyl ester (CFSE) and cultured in triplicate in 96-well culture plates (BD Biosciences, San Diego, CA) with media containing *Escherichia coli* lipopoly-saccharide (LPS, 25 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) or LPS and interleukin-4 (IL-4, 25ng/ml, Sigma-Aldrich) and incubated at 37˚C for 96 h (15, 16). At 96 h of incubation, cells were washed three times and labeled with fluorescently tagged anti-mouse IgG1, IgE, IgG2b and IgG3 antibodies (e-Biosciences). The percentage of cells undergoing class switch recombination events was assessed by flow cytometry.

Confocal microscopy. Splenic B-lymphocytes were harvested using a MACS system and cultured at 10^6 cells/ml in RPMI 1640 (ATCC, Manassas, VA, USA) containing β-mercaptoethanol (Thermo Scientific, Rockford, IL, USA), LPS and IL-4. Cells were harvested at 0, 24, 48, 72 and 96 h and cytospun onto charged glass slides. Cells were fixed using 4% paraformaldehyde (Thermo Scientific), washed with tris-buffered saline (TBS, Fisher Scientific, Pittsburg, PA, USA) with Tween20 (Fisher Scientific) and blocked with TBS, containing 4% fetal bovine serum (FBS, Fisher Scientific). Cells were incubated with rabbit anti-mouse γH2AX (Cell Signaling, Danvers, MA, USA) at 1:500 dilution in TBS with 1% FBS, followed by incubation with Alexa-fluor 488-conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution in TBS with FBS, as previously published (17). After labeling with 4',6-diamidino-2-phenylindole (DAPI, Cell Signaling), cells were visualized using an LSM700 Carl Zeiss confocal microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) and LSM900® software (Carl Zeiss).

γH2AX flow cytometry. Splenic B-lymphocytes cultured in the presence of LPS and IL-4 were harvested at 0, 48, 72 and 96 h, as described above. Cells were fixed with 70% ethanol in PBS at –20˚C for 24 h and permeabilized with 0.1% Triton-X (Sigma-Aldrich) in TBS. Cells were then incubated with rabbit anti-mouse phosphoH2AX antibody (Cell Signaling) at 1:500 dilution in TBS with FBS at 4˚C for 30 min, followed by incubation with Alexa-fluor 488-conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution. Cells were analyzed using a FACScan flow cytometer (BD Biosciences).

Cell cycle analysis. Splenic B-lymphocytes from FVB and C57 mice were harvested and cultured in the presence of LPS and IL-4 as described above. Cells were fixed in 70% ethanol for 48-72 h and incubated with propidium iodide (Sigma-Aldrich) at 5 μg/ml in PBS containing RNAaseA (5 Prime, Gaithersburg, MD, USA), at room temperature for 20 min and kept on ice. Cells were analyzed by flow cytometry.

Gene expression analysis. B-lymphocytes from FVB and C57 mice were cultured in the presence of LPS and IL-4, then harvested at 0, 24, 48, 72 and 96 h to prepare total RNA using RNeasy kit (Qiagen. Valencia, CA, USA). First-strand cDNA was synthesized using Superscript III (Invitrogen, Grand Island, NY, USA). Gene expression was determined in triplicate by reverse quantitative, polymerase chain reaction (RQ-PCR) using an iQ™5 Multicolor Real Time PCR Detection System (BioRad, Hercules, CA, USA) for the following genes *Paired box5* (*Pax5*); 5'AGTCTCCAGTGCCGAATG3', 5'TCCGTGGTGGTGAAGATG3'; *Aid*; 5'GCCAAGGGACGGCATG AGACC3'; 5'CAACAATTCCACGTGGCAGCCAGACTTG3': *DNA ligase 4*; 5'ATTGAAGCCACGAGATTAGGT3', 5'ACTGAATCGGA CACCCAACT3'; *Ku80*; 5'AAT CCTGTTGAAAACTTCCGTT3'; 5'GGAAGCTGTTGAAGCG CTG3'; *Ku70*; 5'CCGCTTCACATACA GGAGCGAC3', 5'GGATTATAACCTGGAGGATAG3'; *DNAPKcs*; 5'GAG AGTGGGCTTTCAGAAGA3', 5'ATTTCCTCTGTCTGTCA GAAAT3'; *p53*; 5'CTCTGAGTAGTGGTTCCTGGCC3'; 5'AAGTA GGCCCTGGAG GATAT3'; *Gapdh* control; 5'GCACAGTCAAGG CCGAGAAT3', 5' CCTTCTCCATGGTGGTG AA3'. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method (18) and results were normalized to that of the corresponding *Gapdh* internal control.

Data and statistical analysis. Flow cytometric data were analyzed using the FlowJo software (FlowJo, Ashland, OR, USA). Data were analyzed with GraphPad Prism 5.0® (Graphpad Software, La Jolla, CA, USA), using either a two-tailed *t*-test or analysis of variance (ANOVA) and Bonferroni post test; a p-value <0.05 was considered significant.

Results

FVB and C57 mice had different spleenic features. In order to better understand the immune system of the two strains of mice (FVB and C57) their splenic parameters were compared. Five mice of the same age group (20 weeks) were euthanized and

the spleen was harvested. Primary evaluation of the size revealed visibly smaller spleens in C57 mice compared to FVB mice (Figure 1A). C57 mice also had a significantly reduced splenic weight (Figure 1B), although histopathological evaluation revealed comparable cellularity of the follicles when compared to those of the FVB mice (Figure 1C). To better understand the B-lymphocyte subsets within the spleen, cells were labeled with fluorescently tagged anti-mouse IgM, CD4 and CD8 antibodies and analyzed by flow cytometry. Our results showed a comparable percentage of total IgM^+ cells in the spleens of both strains. The $CD4^+$ T-lymphocyte population was smaller and the $CD8^+$ T-lymphocyte population was larger (not significantly) in C57 mice compared to FVB mice (Figure 1D and E). The CD4:CD8 ratio is an important parameter for assessing the immune status and regulation of the immune system. Altered CD4:CD8 ratios have been reported in aging (19), viral infections (20), autoimmunity (21), diet (22), cancer metastasis (23) and exposure to sunshine (24). Moreover, strain-specific genetic makeup also plays a critical role in the CD4:CD8 ratio in lymphoid organs and peripheral blood of inbred mice (25, 26). Consistent with previously published reports (27), our analysis showed a significantly lower CD4:CD8 ratio in C57 mice compared to FVB mice (Figure 1F), suggesting altered production of T-lymphocyte subsets in inbred mice with two different genotypes.

FVB and C57 mice have different class switch recombination patterns. Splenic IgM^+ lymphocytes were harvested and cultured in the presence of LPS, or LPS plus IL-4 to induce CSR and produce IgG2b and IgG3, or IgG1 and IgE antibodies, respectively. Cells were harvested 96 h following stimulation and analyzed for their recombination efficiency and proliferation pattern by flow cytometry. Our results showed that C57 mice had increased efficiency for IgG1 and IgG3 antibody recombination ($p<0.05$ and 0.01, respectively). C57 had a reduced efficiency for IgG2b recombination and a slightly higher efficiency for IgE recombination, when compared with cells from FVB mice, although the differences in the levels of the latter were not significant (Figure 2A, B and E). Evaluation of the proliferation pattern of lymphocytes following stimulation using CFSE labeling and flow cytometry showed comparable proliferation in cells from both mouse strains (Figure 2C and D). To better understand the cell proliferation pattern, cell-cycle analysis was determined by labeling cells with propidium iodide and by flow cytometry assessment. Analysis showed comparable cell-cycle phases (apoptotic, G_1, synthetic and G_2/mitotic) between FVB and C57 mice at the time of stimulation (Figure 3A and B). In addition, the cell-cycle pattern for apoptotic, synthetic and G_2/mitotic phases were comparable at 96 h of stimulation with LPS plus IL-4 in cells from both FVB and C57 mice. However, C57 mice had an increased number of cells in the G_1 stage at 96 h following stimulation, suggesting either cell-cycle arrest or a strain-specific prolonged G_1 stage (Figure 3A and B). Because of the prolonged G_1 stage in C57 mice, we analyzed the expression of the pro-apoptotic gene, p53, and found its expression to be elevated at 24, 48 and 72 h in C57 mice (Figure 3C).

FVB and C57 mice had comparable DNA break induction and repair patterns. Naive B-lymphocytes were harvested from FVB and C57 spleens and cultured in the presence of LPS plus IL-4. Stimulation with LPS and IL-4 should result in the expression of *Aid* and lead to DNA double-strand breaks in the immunoglobulin heavy chain gene, a critical step in CSR (28). DNA double-strand break induction results in the phosphorylation of serine139 of histone H2AX (phosphoH2AX or γH2AX), which can be detected by labeling and immunofluorescence assays (29). Flow cytometric analysis for γH2AX showed a comparable percentage of cells with DNA breaks at all the time points tested, viz., 0, 24, 48, 72 and 96 h post-stimulation (Figure 4A and C). To better understand the DNA break induction at the individual cell level, we labeled cells with fluorescently-tagged anti-γH2AX and evaluated the number of breaks by confocal microscopy. Our results showed a comparable number of breaks at the cellular level in both FVB and C57 mice (Figure 4B). Considering the role of Aid in DNA break induction, we analyzed its expression by RQPCR on cDNA isolated at 0, 24, 48, 72 and 96 h post-stimulation. Our results showed significantly higher ($p<0.01$) *Aid* expression in C57 mice than in FVB mice at 24 h; however, the expression was comparable between both strains of mice at other time points tested (Figure 4D).

C57 mice had altered DNA protein kinase expression following stimulation with LPS plus IL-4. As our results showed comparable DNA break induction in both FVB and C57 strains, we considered the possible role of transcription factors and DNA repair mechanism as a reason for the altered preference for isotype switching. Splenic IgM^+ cells were harvested and stimulated with LPS plus IL-4 in culture for 96 h to delineate the NHEJ during CSR. Cells were harvested at 24 h intervals, total RNA was prepared and cDNA was synthesized. Previous studies have shown that *Pax5* is an important regulator for CSR as it serves as master regulator during B-cell differentiation and peripheral development (30); and regulates *Aid* expression (31). Expression of *Pax5* in FVB and C57 cells, determined by RQ-PCR, was comparable at all the time points tested. RQ-PCR analysis for the genes known to be involved in NHEJ mechanism showed comparable expression of *DNA Ligase 4, Ku70* and *Ku80* at 0, 24, 48, 72 and 96 h of culture in both strains of mice (Figure 5). Previous studies have shown that increased expression of *DNAPKcs* correlates with reduced CSR efficiency in the

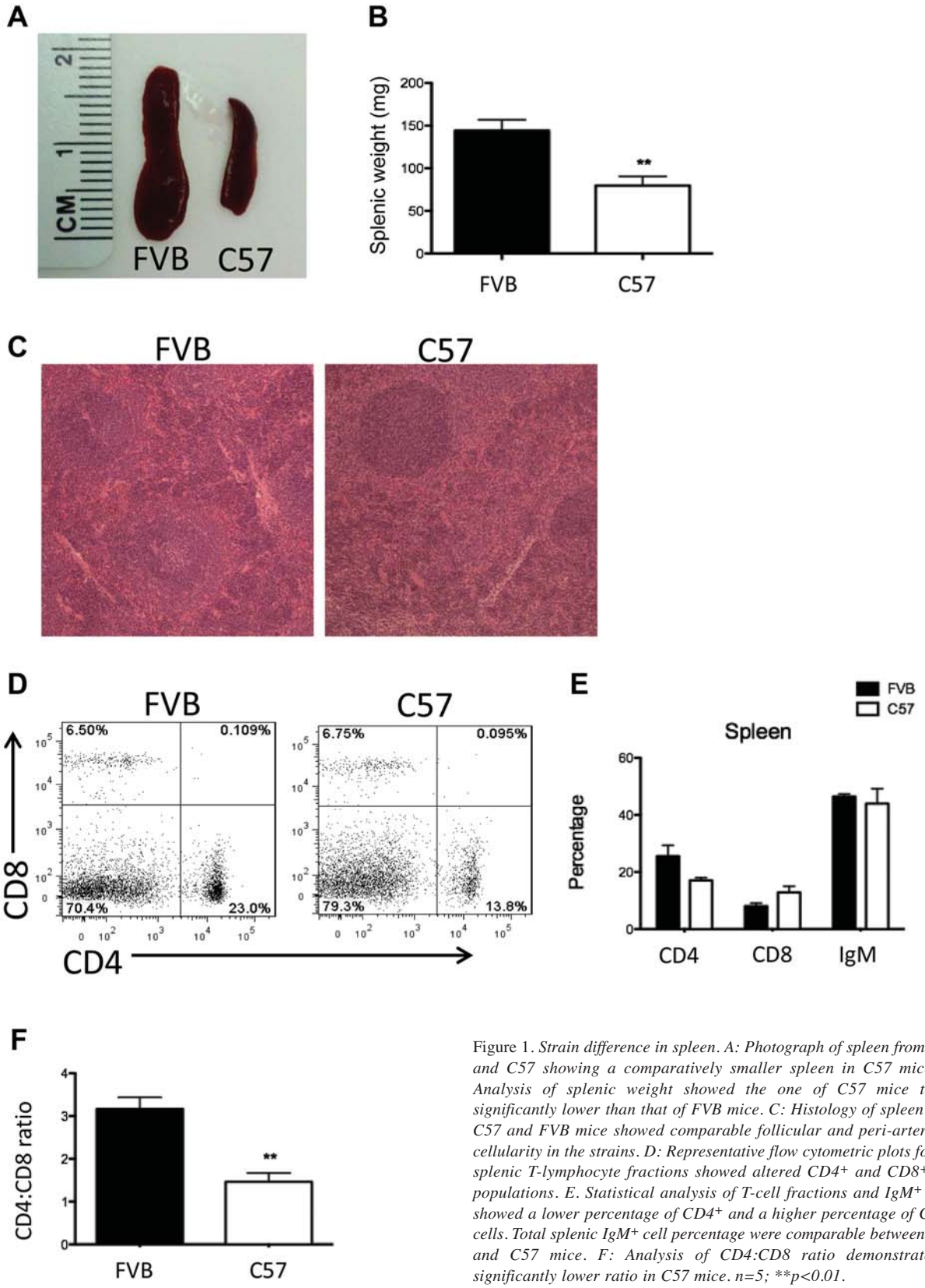

Figure 1. *Strain difference in spleen. A: Photograph of spleen from FVB and C57 showing a comparatively smaller spleen in C57 mice. B: Analysis of splenic weight showed the one of C57 mice to be significantly lower than that of FVB mice. C: Histology of spleen from C57 and FVB mice showed comparable follicular and peri-arteriolar cellularity in the strains. D: Representative flow cytometric plots for the splenic T-lymphocyte fractions showed altered CD4+ and CD8+ cell populations. E. Statistical analysis of T-cell fractions and IgM+ cells showed a lower percentage of CD4+ and a higher percentage of CD8+ cells. Total splenic IgM+ cell percentage were comparable between FVB and C57 mice. F: Analysis of CD4:CD8 ratio demonstrated a significantly lower ratio in C57 mice. n=5; **p<0.01.*

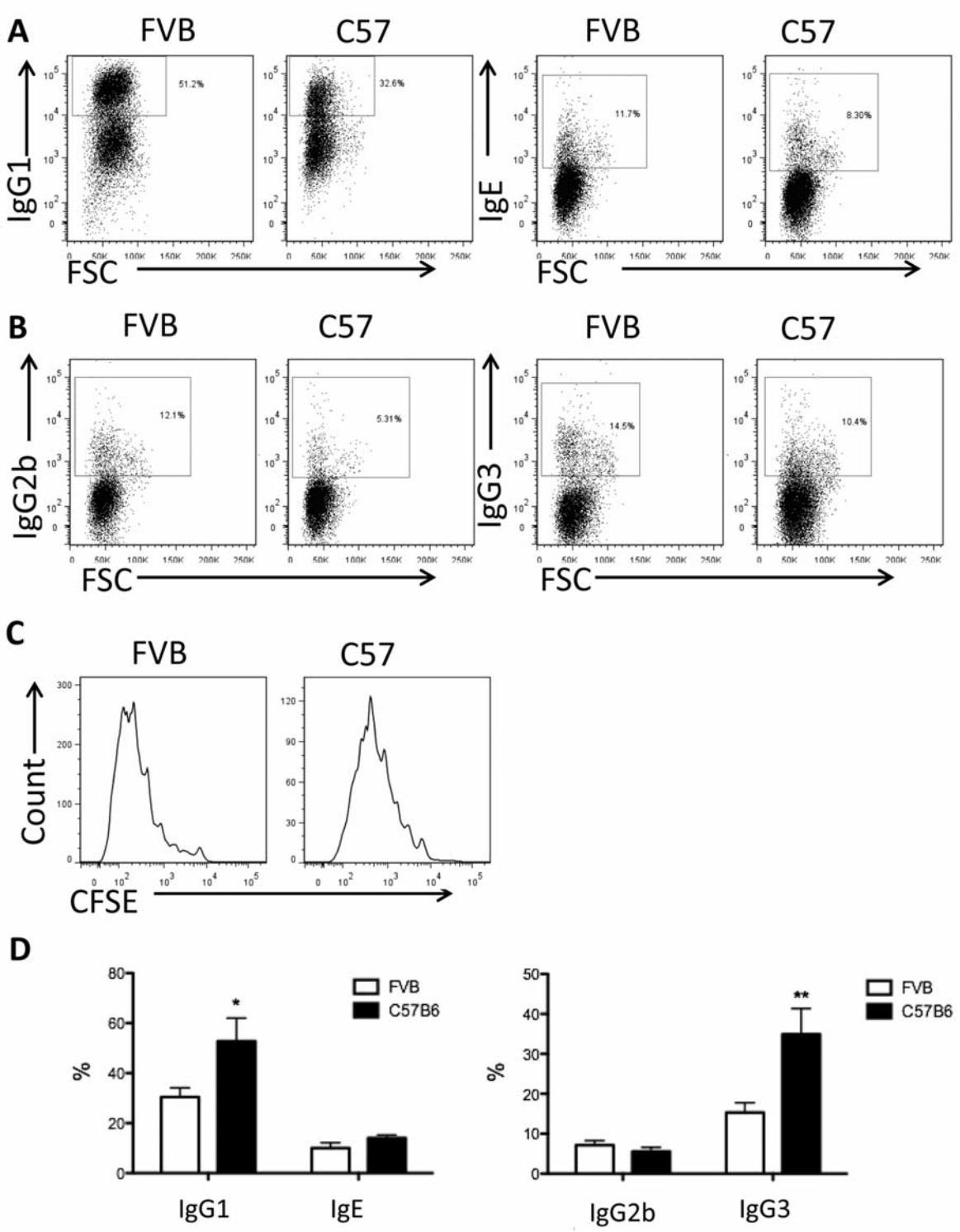

Figure 2. *In vitro class switch recombination assay showed different recombination efficiency in mouse strains under the same conditions. Harvested splenic lymphocytes were cultured in the presence of lipopolysaccharide (LPS) or LPS plus interleukin-4 (IL-4) and analyzed for CSR efficiency by flow cytometry. A: Representative flow cytometric plots of cells stimulated with LPS plus IL-4 and analyzed for IgG1 and IgE class switching. B: Representative flow plots of cells stimulated with LPS and analyzed for IgG2b and IgG3 C: The proliferation pattern of lymphocytes stimulated with LPS plus IL-4 using carboxyfluorescein diacetate succinmidyl ester (CFSE) labeling showed comparable proliferation in FVB and C57 strains. D: Analysis of the CSR efficiency showed higher IgG1 and IgG3 class switching in B-cells from C57 mice. However, IgG2b recombination efficiency was significantly lower in C57 mice compared to cells from FVB mice. n=5; *p<0.05, **p<0.01. FSC: Forward scatter, SSC: side scatter.*

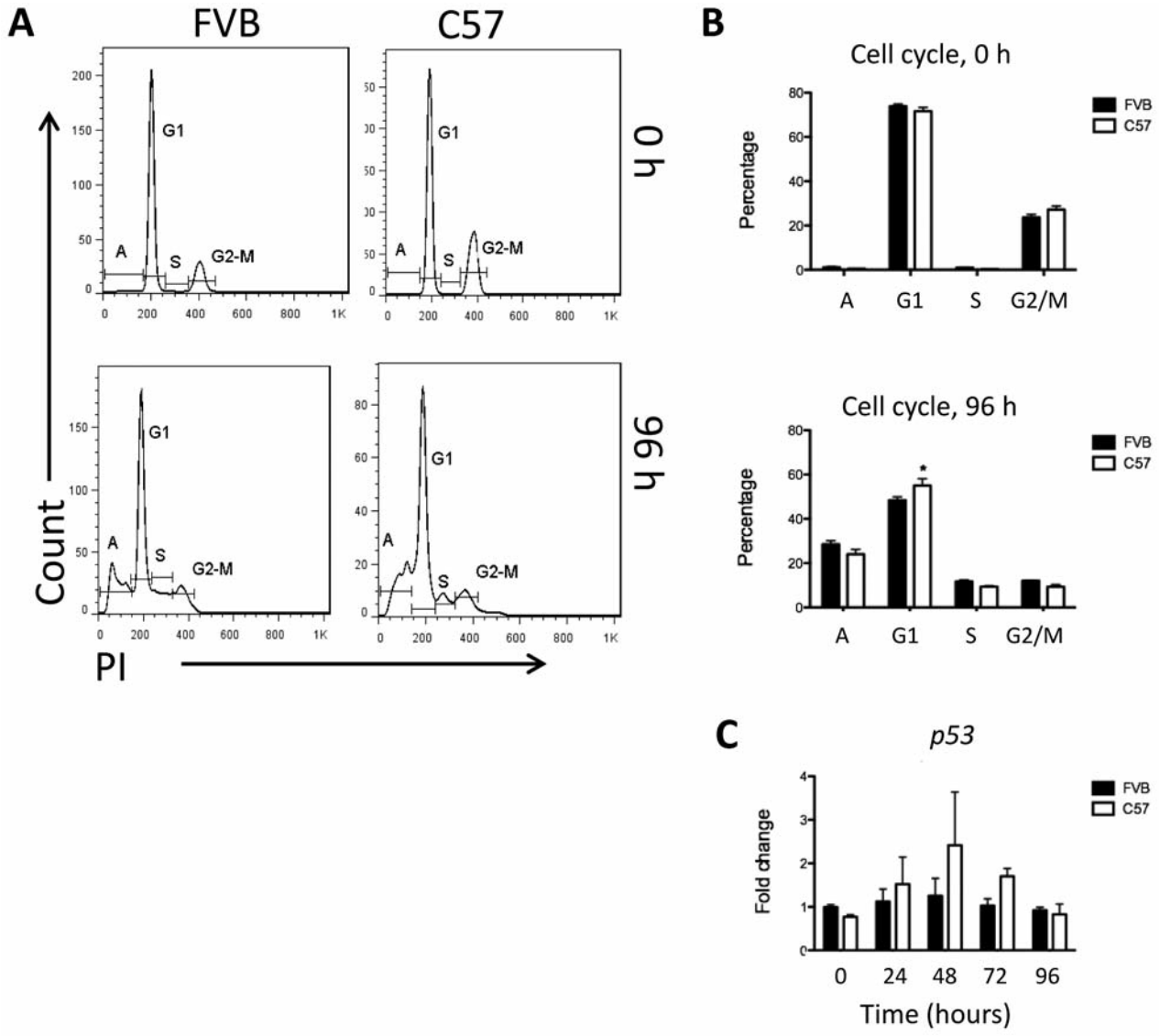

Figure 3. *Cell-cycle kinetics showed growth arrest of B-cells at 96 h post-stimulation. Harvested B-lymphocytes from FVB and C57 mice were cultured in the presence of LPS plus IL-4 and analyzed for cell-cycle pattern using propidium iodide (PI) labeling. Cells were analyzed for DNA content and divided into apoptotic (A), growth phase 1 (G_1), synthetic (S) and growth phase 2/Mitotic (G_2/M) populations. A: Representative flow cytometric plots showing different cell-cycle stages of stimulated B-cells from FVB and C57 mice prior to stimulation (0 h) and 96 h post-stimulation. B: Statistical analysis showed comparable percentages of cells in the apoptotic, synthetic and G2/M stages at 0 and 96 h. However, the percentage of cells in G_1 was higher in C57 mice at 96 h of stimulation suggesting partial cell-cycle arrest. C. Analysis of p53 gene expression showed comparable levels at 0, 24, 48, 72 and 96 h of stimulation between the two strains of mice. n=5; p<0.05.*

chicken cell line DT40 (32). Likewise, our results showed increased expression of *DNAPKcs* in cells from FVB mice at 96 h (Figure 5) and reduced class switch recombination towards IgG1 and IgG3 (Figure 2A, B and E). Expression of *DNAPKcs* was comparable between FVB and C57 mice at the other time points tested (0, 24, 48 and 72 h). These results suggest that under our *in vitro* conditions, gene expressions might also have a role in class switch preference.

Discussion

Different inbred strains of mice have frequently been employed for immunological studies interchangeably, overlooking the possible variation between these strains (33, 34). Here, we delineate molecular differences occurring in two commonly used strains of mice, FVB and C57, during a typical immunological reaction. To understand the

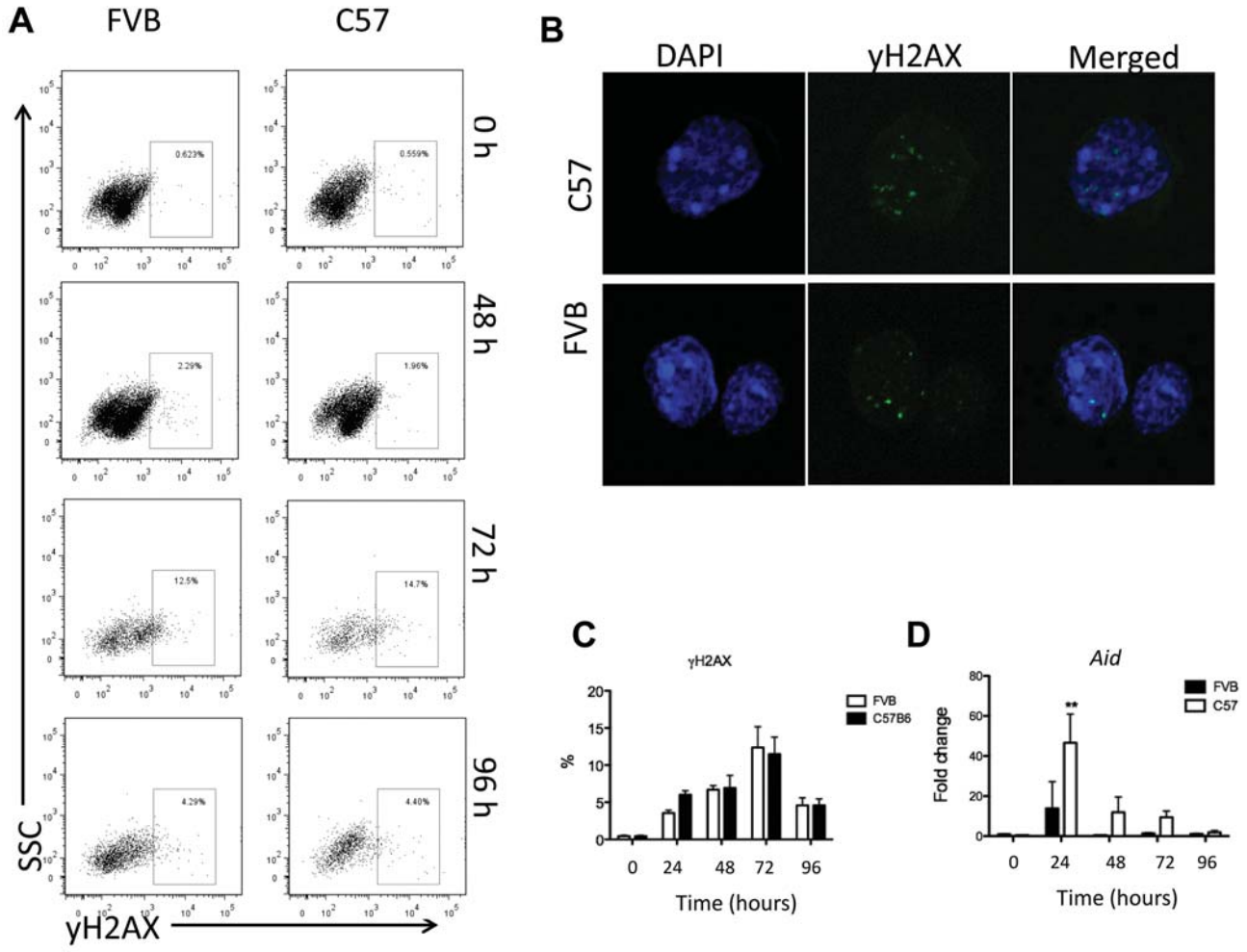

Figure 4. *FVB and C57 mice have comparable DNA break induction and repair patterns. Harvested B-cells were cultured in the presence of LPS plus IL-4 and harvested at 0, 48, 72 and 96 h and labeled with anti-mouse γH2AX antibody. Cells were analyzed by flow cytometry and confocal microscopy. A: Representative flow cytometric plots of B-cells from FVB and C57 mice at different time points showing DNA breaks indicated by H2AX staining. B: Single-cell analysis of the break pattern induced in FVB and C57 mice by confocal microscopy revealed similar patterns. C: Statistical analysis showed there to be a comparable percentage of cells with DNA breaks at all the time points tested. D: Gene expression analysis of Activation induced deaminase (Aid) showed significantly higher levels in C57 mice at 24 h post-stimulation. n=5; *p<0.05, **p<0.01.*

mechanisms at lineage-specific levels and to avoid possible extracellular effects, we performed our experiments on harvested naïve B-lymphocytes. Our results shed additional insight into strain-specific immunoglobulin class switch preferences and patterns under identical culture conditions.

Our preliminary results showed differences in the size of spleen between FVB and C57 mice of the same age group, though the animal weights were comparable (data not shown). Histopathological analysis revealed comparable morphological features, with similar follicular and periarteriolar cellularity. Flow cytometric analysis for total T- and B-cellularity was comparable between the two strains tested; however the CD4:CD8 ratio was skewed in C57 mice, with more $CD8^+$ cells. The ratio of CD4:CD8 is critical in determining the type of immune reaction, as well as regulation of B-lymphocyte activation and proliferation pattern (35). Previous studies have reported low CD4:CD8 ratio in C57 mice thymus, spleen and lymph nodes (27). This could be due to inherent genetic regulations affecting the *Tcrα* locus (26) or strain-specific altered expressions of genes such as *Notch1* (36) and *Bcl-2* (37), which are known to polarize T-cell differentiation. Genetic variation in different strains could result in an altered expression of linkage-related genes and thus alter the CD4:CD8 preference (27, 38). Given that an inherently increased number of $CD8^+$ cells can lead to more of a Th1-type immune reaction,

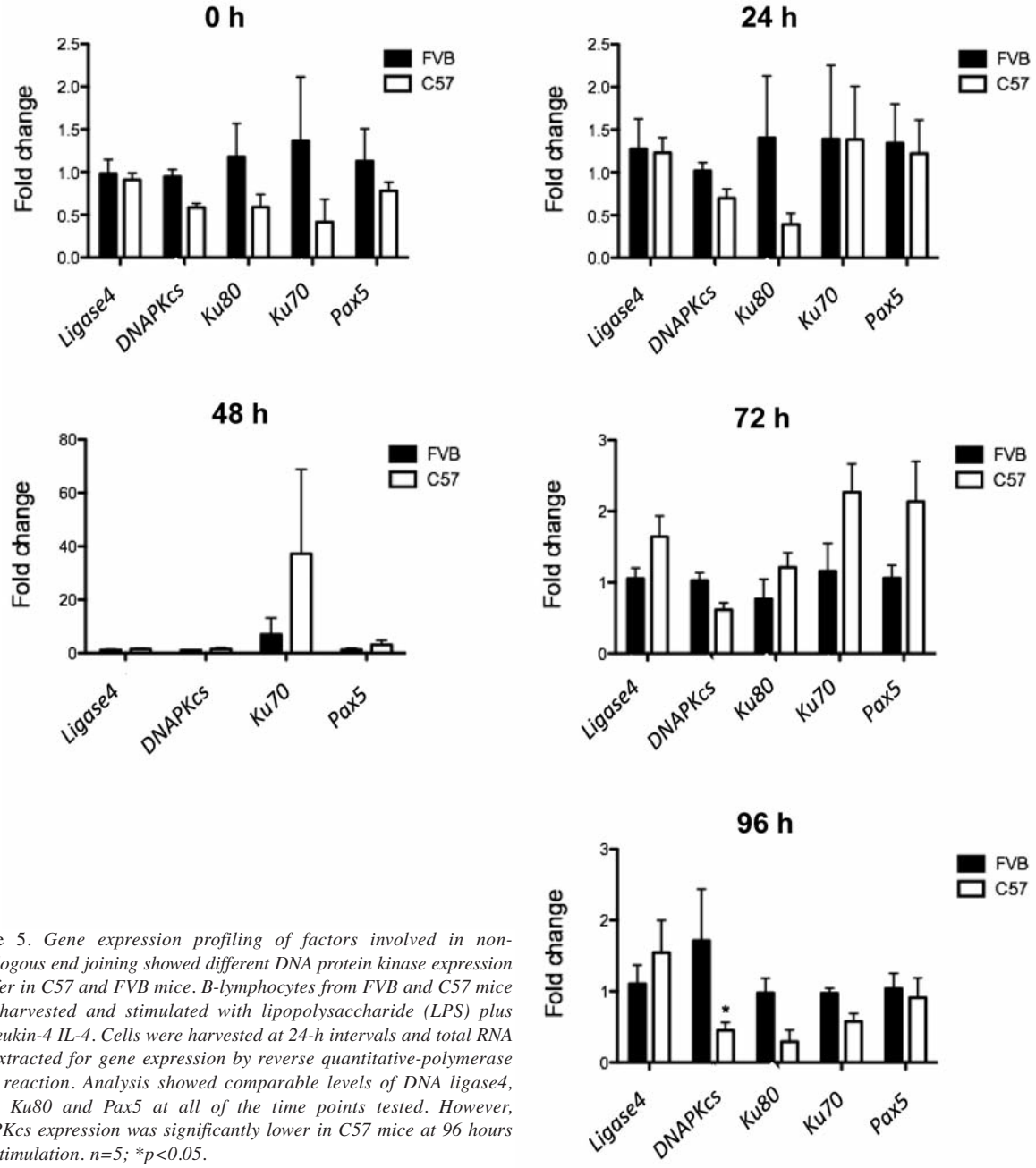

Figure 5. *Gene expression profiling of factors involved in non-homologous end joining showed different DNA protein kinase expression to differ in C57 and FVB mice. B-lymphocytes from FVB and C57 mice were harvested and stimulated with lipopolysaccharide (LPS) plus interleukin-4 IL-4. Cells were harvested at 24-h intervals and total RNA was extracted for gene expression by reverse quantitative-polymerase chain reaction. Analysis showed comparable levels of DNA ligase4, Ku70, Ku80 and Pax5 at all of the time points tested. However, DNAPKcs expression was significantly lower in C57 mice at 96 hours post-stimulation. n=5; *p<0.05.*

resulting in more cytotoxic-type reactions (39), an increased $CD8^+$ population could also be a reason for frequently observed hyper-responsiveness and dermatitis observed in the C57 mouse strain (40).

Antibodies are critical components of the humoral immune response. Different subclasses/isotypes of antibodies are produced under different stimulatory conditions and they have distinct functions. IgG1 is the most abundant IgG subclass, making up 66% of total IgG, and functions mainly by complement fixing. IgG2 subclasses IgG2a and IgG2b are produced in response to bacterial LPS and have low antigenic affinity, although IgG2a is more effective in activating the complement. IgG3 has the highest compliment activation ability and it has high affinity for antigens. IgE antibodies are known to stimulate allergic hypersensitive reactions and parasitic responses. In regards to strain differences in antibody production, FVB mice have been shown to produce

significant amounts of IgE which leads to asthma-like airway responsiveness. This prompted us to explore the antibody isotype switching pattern in these two strains.

Class switch recombination is a process in which IgM^+ B-lymphocytes undergo gene recombination in their immunoglobulin heavy chain gene, resulting in the production of different isotypes of antibodies with the same specificity. To determine the inherent differences in CSR between two different strains, we performed an *in vitro* CSR assay on naïve B-cells. Our results showed that C57 mice had an increased efficiency for IgG1 and IgG3 switching, in contrast to a reduced efficiency for IgG2b production. This difference shows a clear preference towards specific recombinations in the two mouse strains when immune cells are stimulated under identical conditions. Previous studies have shown that CSR efficiency depends on proliferation of stimulated B-cells (41). Our analysis showed comparable proliferation of cells in the two strains, suggesting that proliferation may not be the determining factor for recombination preferences observed in this study.

Recent studies have shown that break induction and fusion during class switching are not entirely random, rather, they are tightly regulated by cellular machinery depending on the type of antigenic stimulation (9, 42). Antigenic stimulation leads to *Aid*-mediated DNA double-strand breaks at switch regions of immunoglobulin heavy chain. Previous studies have suggested that isotype switching preference is regulated mainly by germ-line transcription of immunoglobulin switch regions (43). Specificity of *Aid*-mediated breaks on immunoglobulin gene also depends on binding sites for B-cell signaling downstream mediators, nuclear factor-Kβ (Nf-Kβ) and Stat6 (44, 45). Underlying differences in genetic makeup at switch regions between the two strains of mice or Nf-Kβ or Stat6 binding sites, may contribute to the strain-specific isotype switching preference observed in this study. To better understand the DNA break induction pattern, we analyzed the frequency of DNA breaks in stimulated B-cells from FVB and C57 mice. Our results showed comparable induction and repair patterns of DNA breaks in FVB and C57 mice, suggesting that induction and repair patterns are similar. Expression of Aid was significantly high at 24 h of stimulation in C57 mice; however, these cells did not exhibit a higher number of breaks. This could be due to internal cellular mechanisms preventing the overactivity of DNA damage-inducing signals. Single-cell analysis for breaks using confocal microscopy showed a comparable number of breaks in break-positive cells, thus indicating that the DNA break induction and repair is similar in the two strains of mice tested.

Studies on cell-cycle kinetics during CSR suggested that DNA break induction occurs during the G_1 phase of the cell cycle (46). The DNA break marker γH2AX is detectable during the G_1 and early S phase, but not during the G_2/M phase of the cell cycle (47), suggesting that DNA break repair starts at S phase. The majority of NHEJ-mediated repair occurs during the G_2/M phase (48) and therefore, impaired DNA repair results in cell-cycle arrest at this stage (49). Our analysis of cell-cycle kinetics showed an increased number of G_1 stage cells in stimulated cells from C57 mice at 96 h. As CSR involves DNA breaks and repair, any impairment will result in the expression of pro-apoptotic and apoptotic genes, especially *p53*. Our studies also showed increased *p53* expression in C57 mice at 24, 48 and 72 hours, although the differences were not significant. This slight increase might have resulted in cell-cycle arrest or the prolongation at the G_1 stage at 96 h. Thus, C57 and FVB mice had different cell-cycle kinetics, with there being more cells in the G_1 stage in C57 mice under identical culture conditions than in FVB mice.

As induction of double-strand breaks by *Aid* has been shown to be less specific (50) it might not be the sole reason for isotype preference. We considered the role of NHEJ factors as determining components for class switching preference observed in this study. Previous studies have shown that altered expression or lack of NHEJ factors can impair CSR (51). Our results showed that expression of *Ku70*, a critical component in NHEJ-mediated DNA break repair (52), was higher in C57 mice at 48 and 72 h of stimulation, although the level was not significantly different. Expression of *Ku80* was lower at 24 h of stimulation in C57 mice. Moreover, increased levels of NHEJ factor *DNAPKcs* may reduce the class switching efficiency (32). Consistent with this finding, our gene expression analysis showed increased *DNAPKcs* expression and reduced IgG1 and IgG3 class switch efficiency in FVB mice (Figure 5). Expression of *Pax5*, which is considered a master regulator for the B-cell lineage (30), was comparable at all the time points tested. Taken together, these data suggests that altered expression of critical genes involved in NHEJ might also play a role in the difference in CSR patterns between different inbred strains of mice.

Effective CSR is an important determinant of robust antibody-mediated immune response. Strain-specific differences in the isotype switching pattern might contribute to their different immunity towards various infectious agents and vaccines (53, 54). Our results show that differences in genetic makeup in the background strains can lead to different preferences in isotype switching under identical conditions. These preference changes could be due to changes in DNA break sites in immunoglobulin switch regions and/or altered recombination of switch regions as a result of changes in the expression of NHEJ factors, resulting in preferential recombination in different strains. Further studies are required to elucidate the molecular mechanisms underlying the class switch preferences in different inbred strains of mice.

Conflicts of Interest

The Authors disclose no conflicts of interest.

Acknowledgements

This research was supported by a Virginia Tech Internal Research grant.

We wish to thank our animal care technicians, Melissa Makris for flow cytometry, Bettina Heid for fruitful discussions; and the anonymous reviewers for their constructive comments.

References

1 Dranoff G: Experimental mouse tumour models: what can be learnt about human cancer immunology? Nat Rev Immunol *12(1)*: 61-66, 2012.

2 Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F and Hansen CT: FVB/N: an inbred mouse strain preferable for transgenic analyses. Proc Natl Acad Sci USA *88(6)*: 2065-2069, 1991.

3 Smith PM, Rich RF and Green WR: A shift in the requirement for CD4+ T cells in the generation of AKR/Gross MuLV-specific CTL in AKR.H-2b:Fv-1b mice occurs prior to the onset of age-dependent CTL nonresponsiveness. Cell Immunol *175(2)*: 189-198, 1997.

4 Osman GE, Hannibal MC, Anderson JP, Lasky SR, Ladiges WC and Hood L: FVB/N (H2(q)) mouse is resistant to arthritis induction and exhibits a genomic deletion of T-cell receptor V beta gene segments. Immunogenetics *49(10)*: 851-859, 1999.

5 Davie SA, Maglione JE, Manner CK, Young D, Cardiff RD, MacLeod CL and Ellies LG: Effects of FVB/NJ and C57Bl/6J strain backgrounds on mammary tumor phenotype in inducible nitric oxide synthase deficient mice. Transgenic Res *16(2)*: 193-201, 2007.

6 DiLillo DJ, Horikawa M and Tedder TF: B-lymphocyte effector functions in health and disease. Immunol Res *49(1-3)*: 281-292, 2011.

7 Hardy RR, Carmack CE, Shinton SA, Kemp JD and Hayakawa K: Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med *173(5)*: 1213-1225, 1991.

8 Maul RW and Gearhart PJ: AID and somatic hypermutation. Adv Immunol *105*: 159-191, 2010.

9 Kracker S and Durandy A: Insights into the B cell specific process of immunoglobulin class switch recombination. Immunol Lett *138(2)*: 97-103, 2011.

10 Viau M and Zouali M: B-lymphocytes, innate immunity, and autoimmunity. Clin Immunol *114(1)*: 17-26, 2005.

11 Yoshida T, Mei H, Dorner T, Hiepe F, Radbruch A, Fillatreau S and Hoyer BF: Memory B and memory plasma cells. Immunol Rev *237(1)*: 117-139, 2010.

12 Dudley DD, Chaudhuri J, Bassing CH and Alt FW: Mechanism and control of V(D)J recombination *versus* class switch recombination: similarities and differences. Adv Immunol *86*: 43-112, 2005.

13 Whitehead GS, Walker JK, Berman KG, Foster WM and Schwartz DA: Allergen-induced airway disease is mouse strain dependent. Am J Physiol Lung Cell Mol Physiol *285(1)*: L32-42, 2003.

14 Zhu W and Gilmour MI: Comparison of allergic lung disease in three mouse strains after systemic or mucosal sensitization with ovalbumin antigen. Immunogenetics *61(3)*: 199-207, 2009.

15 McBride KM, Gazumyan A, Woo EM, Schwickert TA, Chait BT and Nussenzweig MC: Regulation of class switch recombination and somatic mutation by AID phosphorylation. J Exp Med *205(11)*: 2585-2594, 2008.

16 Puthiyaveetil AG, Heid B, Reilly CM, Hogenesch H and Caudell DL: A NUP98-HOXD13 leukemic fusion gene leads to impaired class switch recombination and antibody production. Exp Hematol *40(8)*: 622-633, 2012.

17 Puthiyaveetil AG and Caudell DL: Non homologous end joining-mediated DNA break repair is impaired in B lymphocytes of aging mice. Mol Immunol *53(1-2)*: 79-87, 2012.

18 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25(4)*: 402-408, 2001.

19 Callahan JE, Kappler JW and Marrack P: Unexpected expansions of CD8-bearing cells in old mice. J Immunol *151(12)*: 6657-6669, 1993.

20 Tripp RA, Hou S, McMickle A, Houston J and Doherty PC: Recruitment and proliferation of CD8+ T cells in respiratory virus infections. J Immunol *154(11)*: 6013-6021, 1995.

21 Zhang ZL, Constantinou D, Mandel TE and Georgiou HM: Lymphocyte subsets in thymus and peripheral lymphoid tissues of aging and diabetic NOD mice. Autoimmunity *17(1)*: 41-48, 1994.

22 Lee WH and Woodward BD: The CD4/CD8 ratio in the blood does not reflect the response of this index in secondary lymphoid organs of weanling mice in models of protein-energy malnutrition known to depress thymus-dependent immunity. J Nutr *126(4)*: 849-859, 1996.

23 Hernberg M, Muhonen T, Turunen JP, Hahka-Kemppinen M and Pyrhonen S: The CD4+/CD8+ ratio as a prognostic factor in patients with metastatic melanoma receiving chemoimmunotherapy. J Clin Oncol *14(5)*: 1690-1696, 1996.

24 Falkenbach A and Sedlmeyer A: Travel to sunny countries is associated with changes in immunological parameters. Photodermatol Photoimmunol Photomed *13(4)*: 139-142, 1997.

25 Kraal G, Weissman IL and Butcher EC: Genetic control of T-cell subset representation in inbred mice. Immunogenetics *18(6)*: 585-592, 1983.

26 Sim BC, Aftahi N, Reilly C, Bogen B, Schwartz RH, Gascoigne NR and Lo D: Thymic skewing of the CD4/CD8 ratio maps with the T-cell receptor alpha-chain locus. Curr Biol *8(12)*: 701-704, 1998.

27 Myrick C, DiGuisto R, DeWolfe J, Bowen E, Kappler J, Marrack P and Wakeland EK: Linkage analysis of variations in CD4:CD8 T cell subsets between C57BL/6 and DBA/2. Genes Immun *3(3)*: 144-150, 2002.

28 Mai T, Zan H, Zhang J, Hawkins JS, Xu Z and Casali P: Estrogen receptors bind to and activate the HOXC4/HoxC4 promoter to potentiate HoxC4-mediated activation-induced cytosine deaminase induction, immunoglobulin class switch DNA recombination, and somatic hypermutation. J Biol Chem *285(48)*: 37797-37810, 2010.

29 Yin B, Savic V, Juntilla MM, Bredemeyer AL, Yang-Iott KS, Helmink BA, Koretzky GA, Sleckman BP and Bassing CH: Histone H2AX stabilizes broken DNA strands to suppress chromosome breaks and translocations during V(D)J recombination. J Exp Med *206(12)*: 2625-2639, 2009.

30 Medvedovic J, Ebert A, Tagoh H and Busslinger M: Pax5: a master regulator of B cell development and leukemogenesis. Adv Immunol *111*: 179-206, 2011.
31 Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, Yokota Y and Shimizu A: The balance between Pax5 and Id2 activities is the key to AID gene expression. J Exp Med *198(9)*: 1427-1437, 2003.
32 Cook AJ, Raftery JM, Lau KK, Jessup A, Harris RS, Takeda S and Jolly CJ: DNA-dependent protein kinase inhibits AID-induced antibody gene conversion. PLoS Biol *5(4)*: e80, 2007.
33 Schulte-Herbruggen O, Klehmet J, Quarcoo D, Meisel C and Meisel A: Mouse strains differ in their susceptibility to poststroke infections. Neuroimmunomodulation *13(1)*: 13-18, 2006.
34 Darville T, Andrews CW Jr., Sikes JD, Fraley PL, Braswell L and Rank RG: Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. Infect Immun *69(12)*: 7419-7424, 2001.
35 Salgame P, Abrams JS, Clayberger C, Goldstein H, Convit J, Modlin RL and Bloom BR: Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science *254(5029)*: 279-282, 1991.
36 Deftos ML, He YW, Ojala EW and Bevan MJ: Correlating notch signaling with thymocyte maturation. Immunity *9(6)*: 777-786, 1998.
37 Linette GP, Grusby MJ, Hedrick SM, Hansen TH, Glimcher LH and Korsmeyer SJ: Bcl-2 is upregulated at the $CD4^+$ $CD8^+$ stage during positive selection and promotes thymocyte differentiation at several control points. Immunity *1(3)*: 197-205, 1994.
38 Vocanson M, Hennino A, Chavagnac C, Saint-Mezard P, Dubois B, Kaiserlian D and Nicolas JF: Contribution of CD4(+)and CD8(+) T-cells in contact hypersensitivity and allergic contact dermatitis. Expert Rev Clin Immunol *1(1)*: 75-86, 2005.
39 Vocanson M, Hennino A, Cluzel-Tailhardat M, Saint-Mezard P, Benetiere J, Chavagnac C, Berard F, Kaiserlian D and Nicolas JF: CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. J Invest Dermatol *126(4)*: 815-820, 2006.
40 Sundberg JP, Taylor D, Lorch G, Miller J, Silva KA, Sundberg BA, Roopenian D, Sperling L, Ong D, King LE and Everts H: Primary follicular dystrophy with scarring dermatitis in C57BL/6 mouse substrains resembles central centrifugal cicatricial alopecia in humans. Vet Pathol *48(2)*: 513-524, 2011.
41 Hodgkin PD, Lee JH and Lyons AB: B cell differentiation and isotype switching is related to division cycle number. J Exp Med *184(1)*: 277-281, 1996.
42 Stavnezer J: Complex regulation and function of activation-induced cytidine deaminase. Trends Immunol *32(5)*: 194-201, 2011.
43 Stavnezer J: Immunoglobulin class switching. Curr Opin Immunol *8(2)*: 199-205, 1996.
44 Kashiwada M, Levy DM, McKeag L, Murray K, Schroder AJ, Canfield SM, Traver G and Rothman PB: IL-4-induced transcription factor NFIL3/E4BP4 controls IgE class switching. Proc Natl Acad Sci USA *107(2)*: 821-826, 2010.
45 Pone EJ, Zhang J, Mai T, White CA, Li G, Sakakura JK, Patel PJ, Al-Qahtani A, Zan H, Xu Z and Casali P: BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-kappaB pathway. Nat Commun *3*: 767, 2012.
46 Schrader CE, Guikema JE, Linehan EK, Selsing E and Stavnezer J: Activation-induced cytidine deaminase-dependent DNA breaks in class switch recombination occur during G_1 phase of the cell cycle and depend upon mismatch repair. J Immunol *179(9)*: 6064-6071, 2007.
47 Petersen S, Casellas R, Reina-San-Martin B, Chen HT, Difilippantonio MJ, Wilson PC, Hanitsch L, Celeste A, Muramatsu M, Pilch DR, Redon C, Ried T, Bonner WM, Honjo T, Nussenzweig MC and Nussenzweig A: AID is required to initiate Nbs1/gamma-H2AX focus formation and mutations at sites of class switching. Nature *414(6864)*: 660-665, 2001.
48 Mao Z, Bozzella M, Seluanov A and Gorbunova V: DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle *7(18)*: 2902-2906, 2008.
49 Shi M, Vivian CJ, Lee KJ, Ge C, Morotomi-Yano K, Manzl C, Bock F, Sato S, Tomomori-Sato C, Zhu R, Haug JS, Swanson SK, Washburn MP, Chen DJ, Chen BP, Villunger A, Florens L and Du C: DNA-PKcs-PIDDosome: a nuclear caspase-2-activating complex with role in G2/M checkpoint maintenance. Cell *136(3)*: 508-520, 2009.
50 Staszewski O, Baker RE, Ucher AJ, Martier R, Stavnezer J and Guikema JE: Activation-induced cytidine deaminase induces reproducible DNA breaks at many non-Ig Loci in activated B cells. Mol Cell *41(2)*: 232-242, 2011.
51 Stavnezer J, Guikema JE and Schrader CE: Mechanism and regulation of class switch recombination. Annu Rev Immunol *26*: 261-292, 2008.
52 Manis JP, Gu Y, Lansford R, Sonoda E, Ferrini R, Davidson L, Rajewsky K and Alt FW: Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. J Exp Med *187(12)*: 2081-2089, 1998.
53 Eisenstein TK, Killar LM and Sultzer BM: Immunity to infection with Salmonella typhimurium: mouse-strain differences in vaccine- and serum-mediated protection. J Infect Dis *150(3)*: 425-435, 1984.
54 Lyons CR, Lovchik J, Hutt J, Lipscomb MF, Wang E, Heninger S, Berliba L and Garrison K: Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. Infect Immun *72(8)*: 4801-4809, 2004.

Received October 24, 2012
Revised November 10, 2012
Accepted November 12, 2012