HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2017 April 01.

Published in final edited form as:

J Immunol. 2016 April 1; 196(7): 2986–2994. doi:10.4049/jimmunol.1501056.

Loss of Fancc impairs antibody secreting cell differentiation in mice through deregulating Wnt signaling pathway

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Abstract

Fanconi anemia (FA) is characterized by a progressive bone marrow failure and an increased incidence of cancer. FA patients have high susceptibility to immune-related complications such as infection and post-transplant graft-versus-host-disease. Here we investigated the effect of FA deficiency in B cell function using the *Fancc* mouse model. *Fancc*$^{-/-}$ B cells show a specific defect in IgG2a switch and impaired Antibody-Secreting-Cell (ASC) differentiation. Global transcriptome analysis of naïve B cells by RNAseq demonstrates that FA deficiency deregulates a network of genes involved in immune function. Significantly, many genes implicated in Wnt signaling were aberrantly expressed in *Fancc*$^{-/-}$ B cells. Consistently, *Fancc*$^{-/-}$ B cells accumulate high levels of β-catenin under both resting and stimulated conditions, suggesting hyper-active Wnt signaling. Using an in-vivo Wnt GFP reporter assay, we verified the up-regulation of Wnt signaling as a potential mechanism responsible for the impaired *Fancc*$^{-/-}$ B cell differentiation. Further, we showed that Wnt signaling inhibits ASC differentiation possibly through repression of Blimp1 and that *Fancc*$^{-/-}$ B cells are hypersensitive to Wnt activation during ASC differentiation. Our findings identify Wnt signaling as a physiological regulator of ASC differentiation and establish a role for the Wnt pathway in normal B cell function and FA immune deficiency.

Introduction

B cells are essential for the humoral based immunity. After encountering an antigen, B cells undergo genomic mutation and recombination, proliferation and differentiation. At the genomic level after encountering an antigen, B cells undergo two induced cytidine deaminase (AID) processes called somatic hyper-mutation (SHM) and class switch recombination (CSR). SHM results in introduction of point mutations in the variable regions (V) of the Ig gene in order to enhance Ig affinity for antigens. CSR leads to recombination by non-homologous end joining (NHEJ) DNA repair of the IgM constant region (Cμ) with one of the downstream constant regions to generate different classes of antibody (IgD, IgG, IgE or IgA; 1). After being selected, the high affinity B cells differentiate either into

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Contribution: M.S. designed research, performed research, analyzed data, and wrote the paper; S.A. and A.W. performed research; Q.P. designed research and wrote the paper.

memory B cells, which allow a faster immune response in case of a second encounter with the same antigen, or into antibody secreting cells (ASC; also called plasma cells), which are able to produce a high quantity of Ig. Differentiation into plasma cells is inhibited by Pax5, which is responsible for the expression of genes involved in B cell function and the repression of genes involved in ASC differentiation such as the master regulator of ASC differentiation, Blimp1 (2, 3). After induction, Blimp1 represses Pax5 allowing ASC differentiation while blocking proliferation through repression of c-Myc (4) and by indirect induction of Xbp-1 (5). There are two types of ASCs: a first wave of low affinity and short term ASC producing IgM and a second type of high affinity switched ASCs that can migrate from secondary lymphoid organs to the bone marrow (BM) to become long term non-dividing ASCs (6).

Fanconi anemia (FA) is characterized by a progressive BM failure and a high susceptibility to develop leukemia and solid tumors. The disease is due to a mutation in one of the 19 already identified genes (A to Q) (7). Deficiency in any one of these FA gene-encoding proteins leads to genomic instability and high susceptibility to cancer development (8). FA proteins are mainly involved in DNA repair after DNA damage or replicative stress. Upon activation of the FA pathway, 8 FA proteins (FANCA, −B, −C, −E, −F, −G, −L, and −M) interact to form the FA core complex which activates FANCD2 and FANCI by mono-ubiquitination (8). The activation of FA pathway is thought to favor the homologous recombination while inhibiting the error prone NHEJ DNA repair (9, 10). Aside DNA repair, other specific functions have been described for some FA proteins. For example, *FANCC* is able to interact with HSP70 to inhibit TNF-α induced apoptosis (11, 12), with STAT-1 to allow a normal IFN-γ response (13, 14) and with CtBP1 and β-catenin to modulate the WNT signaling pathway (15, 16).

A lot of effort has been made to understand, improve and try to cure the BM failure of FA patients. Most of the studies on FA proteins are focused on their roles in DNA repair function and hematopoietic stem cell maintenance. So far few studies have addressed the immune function of FA proteins (17). Since high susceptibility to general infection has been reported for a group of FA patients (17), the question of immune function in the context of FA deficiency seems of interest to understand and predict possible complications aside the development of BM failure and cancer. More recently, the study of antigen presenting cells has demonstrated impaired function of *Fancc* deficient macrophages (18). It has also been reported that a sub-group of FA patients has an impaired immunization after pneumococcal vaccination (19); whereas another recent study reported a normal immunization of FA deficient women vaccinated with HPV vaccine (20). In mice, a study has reported an impaired antibody response in *Fancc* deficient animals immunized with only a HPV vaccine formulation containing a TLR4 adjuvant (21). The differences seen in immunization efficiency in FA patients and vaccine formulation in mice raise the question of a specific deficiency of B cells for certain complementation groups and specificity for certain pathogens or adjuvants. In this study, we show that B cells from mice deficient for the *Fancc* gene have a specific defect in Antibody-Secreting-Cell (ASC) differentiation. We further demonstrate that *Fancc* deficiency deregulates a network of genes involved in B cell

activation and ASC differentiation and identify hyper-active Wnt signaling as a potential mechanism responsible for the impaired *Fancc*$^{-/-}$ ASC differentiation.

Materials and Methods

Mice and treatment

Fancc$^{-/-}$ mice (C57bl/6 background) were described elsewhere (22). Heterozygous *Fancc*$^{+/-}$ mice were bred to obtain *Fancc*$^{-/-}$ and *Fancc*$^{+/+}$ (WT) littermate offspring. Animals were maintained in the animal barrier facility at Cincinnati Children's Hospital Medical Center. Six to 8 weeks old mice were used for the entire study. Bone marrow was flushed with PBS from 2 tibias and 2 femurs of each mouse and labelled for flow cytometry analysis of B cells lineage or for LSKs cells sorting. Splenocytes were obtained by smashing mouse spleens on a 70μm strainer placed in a 35mm petri dish. After osmotic lysis of red blood cells by ACK buffer, splenocytes were either used for B cells isolation or Flow cytometry analysis. For in-vivo treatment, 6-8 weeks old mice were injected i.v with 15ug of Lipopolysaccharide (LPS from *Salmonella typhosa*, Sigma-Aldrich). For immunization, mice were injected i.p with 100ug of NP-LPS (BIOSEARCH Technologies) in PBS or with 200ul of PBS only as control. All experiments were approved by the Veterinary Services at Cincinnati Children's Hospital Medical Center and conducted in accordance with the National Institutes of Health guidelines for animal care.

Cell Culture

B cells were purified by negative selection with anti-CD43 magnetic beads (Milteny Biotec, >95%) from RBCs lysed mouse splenocytes using automatic labelling and magnetic separation on an AutoMacs-Pro (Milteny Biotec). B cells were cultured at 5×10^5 cells/ml in complete RPMI1640 medium (10% FBS, 25mM Hepes, 2mM L-glutamine, nonessential amino acid, 50μM 2-mercaptoethanol, 1% penicillin/streptomycin) with 20ug/ml of LPS alone (serotype 0111:B4 from *Escherichia coli*, Sigma-Aldrich) to induce IgG3 CSR or in combination with 5ng/ml of murine rIL-4 (Preprotech) to induce IgG1 CSR or 50ng/ml of murine rIFN-y (Preprotech) to induce IgG2a. For CFSE labelling, B cells were loaded before culture with 1.5μM of CFSE (Life Technology). For culture in presence of Wnt3a, 25% of culture volume were replaced by Wnt3a conditioned medium (generous gift of Dr. Helmrath, CCHMC) or Control medium.

Flow cytometry analysis

Mononuclear BM cells, B cells after culture or splenocytes were treated 10 minutes with FC block solution (BD Bioscience) then labelled 20 minutes on ice with combination of different anti-mouse antibodies (Abs) as indicated in legends of figures. Abs included anti-B220-eFluor660 (Ebioscience, Clone RA3-6B2), anti-IgM-Fitc (Ebioscience, Clone eB121-15F9), anti-IgG1-PE (Ebioscience, Clone m1-14D12), biotin anti-IgG2a^b (BD Bioscience, Clone 5.7), biotin anti-mouse IgG3 (BD Bioscience, Clone R40-82), anti-CD138-BrillantViolet421 (BD Bioscience, Clone 281-2), anti-CD43-PE (BD biosciences, Clone S7), anti-CD45.1-PeCy7 (BD biosciences, Clone A20) and anti-CD45.2-APC-Cy7 (BD biosciences, Clone 104). Biotinylated primary antibodies were detected by incubation of labeled cells with streptavidin-PE (BD Bioscience). For apoptosis analysis, B cells were

first surface labeled for ASC detection, then washed and re-suspended in 1x Binding buffer (Ebioscience) and labeled with anti-AnnexinV-Fitc (Ebioscience). Ten minutes before flow cytometry acquisition, propidium iodide (Ebioscience) was added to all samples for detection and/or exclusion of dead cells. Cells were analyzed with a FACSFortessa or a FACSCanto (BD Bioscience) and Diva (BD Bioscience) or FLowJo (TreeStar) softwares.

RNA isolation, RNA NextGen sequencing (RNAseq) analysis and real-time quantitative PCR (qPCR)

Total RNA was purified with RNeasy plus mini kit from B cells ex-vivo, after culture or after CFSE peak based cell sorting by FACSAria (3 division peak). For RNAseq, Poly-A^+ isolation, library, single-end sequencing and alignment of reads on mouse genome (mm9 version) were done by the CCHMC DNA and Sequencing core according to their standard protocol and QC filters. Bam files provided by the DNA and sequencing core were analyzed using GeneSpring GX v12 (Agilent). Quantification of mRNA expression was done using the RefSeq database. Gene expression and fold change (FC) were evaluated between WT and *Fancc*$^{-/-}$ sample. The RNAseq data have been deposited in NCBI's Gene Expression Omnibus under the accession number GSE76634 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76634). The genes with a significant difference (moderate-T test, $p \leq 0.05$) and a FC≥1.5 were selected to run the pathway analysis module of GeneSpring GX v12 using the curated WikiPathway database (http://www.wikipathways.org).For qPCR experiments, reverse transcription was performed on 500ng RNA with the High-Capacity cDNA Reverse Transcription kit (Life technology) as recommended by the manufacturer. CDNA were used for Real time PCR with the primer listed in Table S1. Relative expression of each mRNA was calculated using the $2^{-\text{delta delta CT}}$ method using ActB and Hprt1 as endogen controls and average of WT mRNA samples expression as reference sample.

ELISA quantification of total IgM and NP specific anti-IgG

Total IgM Abs from B cell cultures supernatant (5 days) and serum of LPS injected mouse (3 days) were measured with the Mouse IgM ELISA Ready-Set-Go kit (Ebioscience) according to the manufacturer protocol using 3,3′,5,5′ tetramethylbenzidine (TMB) as HRP substrate . NP-specific IgG Abs from serum of NP-LPS immunized mice were measured by ELISA. 96 well Elisa plates (Costar) were coated with 10ug/ml NP_{29}-BSA (BIOSEARCH Technologies) overnight. After 1hr blocking with PBS 1% BSA, the wells were incubated with diluted sera for 2h and NP specific IgG were detected by a secondary HRP linked anti-mouse IgG (Biolegend) and TMB. Reaction was stopped by 1M phosphoric acid and absorbance measured at 450nm.

Lentivirus transduction and LSKs transplantation

Mononuclear BM cells from WT or *Fancc*$^{-/-}$ mice (CD45.2) were labeled with the lineage marker (Lin) biotinylated Abs mixture (BD Biosciences) including: anti- CD3ε (Clone 145-2C11), CD11b (Clone M1/70), CD45R/B220 (Clone RA3-6B2), Ly-76 (Clone Ter119), Ly6G, and Ly-6C (Clone RB6-8C5). Cells were then labeled with anti-Sca1-PeCy7 (D7) and anti-cKit-APC (2B8) (BD Biosciences). After washing, biotinylated Abs were revealed by Streptavidin Percp-Cy5.5 (BD Biosciences). $Lin^-Sca1^+Ckit^+$ (LSK) cells were sorted

using FACS Aria (BD Biosciences) from the CCHMC Flow Cytometry Core. Sorted LSKs (≥98%) were activated in StemSpan medium (Stemcell technologies) in presence of 25ng/ml murine rTpo (Preprotech) and 50ng/ml murine rScf (Preprotech). After 12h, LSKs were transduced by the 7TGC-eGFP Wnt reporter lentivirus (23) with 2 hits at MOI=5. Lentiviral particles were produced by the CCHMC Viral Vector core using 293T cells. At 72h, transduction efficiency was evaluated by mCherry detection by Flow cytometry (Transduction efficiency 70-80%). Five thousand LSK cells were transplanted into lethally irradiated Boy/J recipient mice (CD45.1). GFP expression of naïve splenic B cells was detected at 10 weeks post transplantation by Flow cytometry.

Results

Normality of peripheral B cell compartment despite differences of B cell population in the bone marrow of Fancc$^{-/-}$ mice

In FA patients, a previous study reported a decrease of peripheral blood (PB) B cell number (24). We thus first analyzed B cell development in the BM and PB of *Fancc*$^{-/-}$ mice and WT control littermates. In the BM, adult *Fancc*$^{-/-}$ mice exhibited a significant difference in the proportion of B cell lineage subpopulations compared to WT mice. Specifically, *Fancc*$^{-/-}$ mice showed a significant decrease in the frequencies of immature (IgM^{+}B220^{dim}) and mature B cells (IgM^{+}B220^{+}) accompanied by an increase of pre/pro B cells (IgM^{-}B220^{dim}) (Figure 1A, B). However, no significant difference was observed for total BM cellularity or even total B cell count in periphery blood (PB) between *Fancc*$^{-/-}$ and WT mice (Figure 1C; Supplemental Figure 1A). Interestingly, we found that the percentage and the absolute number of total B cell lineage population (B220$^{dim/+}$) were significantly increased in the BM of *Fancc*$^{-/-}$ mice compared to WT mice (Figure 1D, E). We next analyzed the B cell subpopulations in the spleen. As shown in Supplemental Figure 1 B-D, no difference was observed in spleen cellularity and in the percentage of total B (B220^{+}) cells in live splenocytes. We also evaluated the B cell subpopulations in the spleen using IgM, IgD or CD21 and CD23 labeling and observed no significant difference in the proportion of the different B cell subpopulations in *Fancc*$^{-/-}$ mice compared to WT mice (Supplemental Figure 1E-G). These data indicate that despite the aberrant B cell development seen in the BM, *Fancc*$^{-/-}$ mice have normal B cell number in periphery and normal B cell subpopulations in the spleen in steady state. The increase in BM B cells could compensate for the imbalance of B cell development observed in *Fancc*$^{-/-}$ mice and thus maintain B cell number and characteristics in periphery.

Impaired Antibody-Secreting-Cell differentiation of *Fancc*$^{-/-}$ B cells

Upon encountering an antigen, B cells go through SHM and CSR and enter differentiation programs to differentiate into memory B cells or antibody secreting cells (ASCs; also called plasma cells) (25). ASCs are the principal source of antibodies and extensive efforts have been made to characterize the process and factors involved in ASC differentiation (6). We evaluated ASC differentiation in vitro, using B220 and CD138 labelling. As shown in Figure 2A-B, 5 days of culture led to a significant decrease in ASC (B220^{dim}CD138^{+}) cells in *Fancc* deficient B cells compared to WT cells for all 3 conditions tested. We next evaluated the expression of Blimp1, which is the major transcription factor controlling ASC

differentiation (6). Consistent with the observed decrease in $B220^{dim}CD138^{+}$ cells, the expression of Blimp1 mRNA was down-regulated in *Fancc*$^{-/-}$ B cells (Figure 2C). To further substantiate these observations, we measured the production of IgM in supernatants of B cells cultured for 5 days. As expected, LPS-stimulated B cells from both genotypes produced an increased amount of IgM compared to resting cells (Figure 2D). However, we observed a significant decrease of IgM production in supernatants from stimulated *Fancc*$^{-/-}$ B cells compared to stimulated WT cells (Figure 2D). We next evaluated the ability of *Fancc*$^{-/-}$ B cells to differentiate into ASCs in vivo. To this end, we treated *Fancc*$^{-/-}$ and WT mice with LPS and determined the proportion of $CD138^{+}B200^{dim/-}$ cells in live splenocytes 3 days post injection. As shown in Figure 2E and 2F, LPS injection induced a burst in the $CD138^{+}B200^{dim/-}$ population in the spleen of both *Fancc*$^{-/-}$ and WT mice. However, stimulated *Fancc*$^{-/-}$ mice exhibited much weaker induction of ASC differentiation than their WT controls (Figure 2E, F). Consistently, *Fancc*$^{-/-}$ mice showed a significant decrease in serum IgM concentration compared to WT mice (Figure 3G). Furthermore, immunization of mice with NP-LPS, a T cell-independent antigen, induced significantly lower levels of NP-specific IgG in the serum of *Fancc*$^{-/-}$ mice compared to WT mice (Figure 2H). Together, these results indicate that *Fancc*$^{-/-}$ B cells have a decreased ability to differentiate into ASCs and to produce antibodies.

Deregulation of Wnt signaling pathway in Fancc$^{-/-}$ B cells

We next investigated the molecular mechanism responsible for the impaired *Fancc*$^{-/-}$ B cell-to-ASC differentiation by conducting a global transcriptome analysis of naïve WT and *Fancc*$^{-/-}$ B cells using mRNA sequencing (RNAseq). We found deregulation of 488 genes (224 up-regulated and 264 down regulated, Moderate T-test, FC≥1.5, Supplemental Figure 2). Pathway analysis showed that among the top 5 pathways, the Prostaglandin and Calcium pathways, two known regulators of B cell activation (26) and ASC differentiation (27), were deregulated in *Fancc*$^{-/-}$ B cells (Figure 4A). Interestingly, we also observed a significant enrichment of genes involved in Wnt signaling (Figure 3A). We decided to evaluate this pathway, as previous studies have suggested a possible interaction of *Fancc* with Wnt signaling (15, 16) and the involvement of Wnt signaling in B cell development (28, 29). Up-regulation and down-regulation of both inhibitors (Camk2b, Ankrd6, Sfrp1, Axin2, Nkd1, Tax1bp3) and activators (Dlg2, Fhl2) of the Wnt pathway and deregulated expression of Wnt receptor genes (Fzd-9, 2 and 4) were detected in *Fancc*$^{-/-}$ B cells (Figure 3B). We confirmed by qPCR up-regulation of Fzd9 and Axin2 and down-regulation of Sfrp1 and Fzd2 in *Fancc*$^{-/-}$ B cells (Figure 3C). To clarify the activation state of Wnt signaling, we first evaluated by western blot the stabilization of β-catenin in B cells in vitro. Stimulation of B cells with LPS led to the accumulation of β-catenin at 2 and 4hrs indicating the activation of Wnt signaling after LPS stimulation (Figure 3D), as previously reported (30). Interestingly, we observed higher levels of β-catenin in *Fancc*$^{-/-}$ B cells than WT cells at both resting and stimulated conditions, suggesting a possible hyper-activation of Wnt signaling in *Fancc*$^{-/-}$ B cells. To verify the activation of Wnt signaling in vivo, we transduced *Fancc*$^{-/-}$ and WT (CD45.2) hematopoietic stem and progenitor cells ($Lin^{-}Sca1^{+}Ckit^{+}$; LSK) with a Wnt GFP reporter lentivirus and transplanted the transduced LSK cells into lethally irradiated Boy/J mice (CD45.1). Strikingly, we observed a decrease in spleen cellularity (Figure 3E) and a profound decrease in B cell number among donor

splenocytes (CD45.2$^+$) of animals transplanted with *Fancc*$^{-/-}$ LSKs at 4 and 10 weeks post-transplant (Figure 3F). Moreover, evaluation of Wnt activity by Flow cytometric analysis of reporter GFP expression showed that WT donor-derived B cells expressed almost no GFP at 10 weeks post-transplant (Figure 3G, H), indicating no or weak activity of Wnt pathway. In contrast, *Fancc*$^{-/-}$ donor-derived B cells exhibited a significant increase in the proportion of GFP-positive cells (Figure 3G, H), indicating an increased activation of Wnt signaling. Taken together, these results identify de-regulation of Wnt signaling as a potential mechanism responsible for the impaired *Fancc*$^{-/-}$ B cell differentiation and functions.

Wnt signaling inhibits ASC differentiation

Because our results demonstrated an up-regulation of Wnt signaling in *Fancc*$^{-/-}$ B cells that showed an impaired ASC differentiation, we next evaluated the effect of Wnt activation on B cell differentiation into ASCs. We used Wnt3a to activate Wnt signaling in WT B cells. As shown in Figure 4A-B, cultures of LPS-stimulated WT B cells in presence of Wnt3a resulted in a significant decrease in the proportion of CD138$^+$B200^{dim} cells compared to control medium (Figure 4A,B), indicating an inhibitory effect of Wnt signaling on ASC differentiation. Consistent with this result, we observed a diminution of IgM production (Figure 4C) and Blimp1 mRNA expression (Figure 4D) in cells cultured in presence of Wnt3a. Interestingly, we also observed an up-regulation of Pax5 (Figure 4D), a known repressor of Blimp1 (31) and a mediator of Wnt signaling (32). These results indicate that Wnt signaling plays an inhibitory effect on ASC differentiation, possibly through repression of Blimp1.

Fancc$^{-/-}$ B cells are hypersensitive to Wnt activation during ASC differentiation

We next evaluated the sensitivity of ASC differentiation to the activation of Wnt signaling by analyzing apoptosis in three B cell differentiation subpopulations (B220$^+$Cd138$^-$ = Non-ASC differentiated B cells, B220$^+$Cd138$^{dim/+}$ = Plasmablasts, B220^{dim}Cd138$^+$ = ASCs). It is noticeable that no difference in apoptosis was observed in the B220$^+$Cd138$^-$ and B220^{dim}Cd138$^+$ subpopulations between LPS-stimulated WT and *Fancc*$^{-/-}$ B cells in the absence of Wnt3a treatment (Figure 5A, B, D). This indicates that the previously observed decrease in the frequencies of *Fancc*$^{-/-}$ B cells and ASCs upon LPS activation was unlikely due to increased apoptosis of mature undifferentiated B cells. We observed a decrease in apoptosis of WT B cells treated with Wnt3a (Figure 5A, B), suggesting an anti-apoptotic function of Wnt activation during B cell stimulation. Unlike the B220$^+$Cd138$^-$ subpopulation, we observed a significant increase in apoptosis in the plasmablast and ASC subpopulations in WT and *Fancc*$^{-/-}$ B cell cultures containing Wnt3a (Figure 5A,C,D). Significantly, Wnt3a induced a greater augmentation of apoptosis in all three subpopulations of *Fancc*$^{-/-}$ cells than in WT cells (Figure 5A-D), indicating hypersensitivity of *Fancc*$^{-/-}$ B cells to Wnt3a. These data demonstrate differential effects of Wnt activation on apoptosis of B cells in these three different differentiation stages and suggest that the inhibitory effect of Wnt3a on ASC differentiation could be due in part to an apoptotic mechanism.

Fancc-deficient B cells show a specific defect in IgG2a switch

Finally, as *Fancc* is a component of the FA core complex, which is involved in double-strand break (DSB) repair, and since Ig class-switch recombination (CSR) produces DSBs, we evaluated the potency of *Fancc*-deficient B cells to undergo CSR. *Fancc*$^{-/-}$ B cells stimulated with LPS or LPS+IL-4 produced a comparable proportion of CSR as WT cells as evaluated by IgG3 or IgG1 labelling respectively (Figure 6A, B). However, we observed a significant decrease in the percentage of IgG2a-switched *Fancc*$^{-/-}$ B cells after stimulation with LPS+IFN-γ (Figure 6A, B). To examine the observed IgG2a switch defect at the molecular level, we evaluated the expression of different germline and post-switch IgG isotypes mRNA including Iy3-Cy3 and Iu-Cy3 (IgG3 switch), Iy1-Cy1 and Iu-Cy1 (IgG1 switch), and Iy2a-Cy2a and Iu-Cy2a (IgG2 switch). We also evaluated the expression of the major CSR regulator protein Aicda (25). Since CSR is dependent on cell division and it is well known that the FA-deficient lymphocytes have an impaired cell cycle progression (33), we extracted RNA from WT or *Fancc*$^{-/-}$ B cells which underwent 3 divisions (Representative CFSE labeling for Facs sorting, Supplemental Figure 3). We observed no difference in the expression of IgG germline or post-switch mRNA after LPS (Figure 6C) and LPS+IL4 (Figure 6D) stimulations, indicating no defect in IgG3 switch or IgG1 switch. In contrast, we observed a profound decrease in the expression of germline and post-switch IgG2a mRNA in the *Fancc*$^{-/-}$ B cells compared to WT cells stimulated with LPS+IFN-γ (Figure 6E). Interestingly, we observed an increase in *Aicda* mRNA expression in *Fancc*$^{-/-}$ B cells for all three conditions tested (Figure 6C-E). However, this increase in *Aicda* expression had no consequence on the CSR, as we observed similar percentages of IgG1and IgG3 switched cells and equal post-switch mRNA levels in both *Fancc*$^{-/-}$ and WT B cells (Figure 6A-D). Together these data indicate a specific IgG2a switch defect in *Fancc*-deficient B cells and that the impaired plasma cell differentiation is not a consequence of a general defect in CSR.

Discussion

In this study, we have investigated the effect of FA deficiency in B cell function using the *Fancc* mouse model. We have shown that loss of *Fancc* impairs B cell-to-ASC differentiation. We identified hyper-active Wnt signaling as a potential mechanism responsible for the impaired *Fancc*$^{-/-}$ ASC differentiation. There are several findings that highlight the significance of FA deficiency in B cell defects: first, *Fancc*$^{-/-}$ mice produce significantly fewer ASCs and consequently less antibodies; second, *Fancc* loss deregulates genes essential for B cell activation and ASC differentiation; third, hyper-active Wnt signaling is identified as a potential mechanism responsible for the impaired *Fancc*$^{-/-}$ B cell differentiation; fourth,*Fancc*$^{-/-}$ B cells exhibit a specific defect in IgG2a switch; lastly, *Fancc*$^{-/-}$ B cells are hypersensitive to Wnt activation during ASC differentiation. These findings identify Wnt signaling as a physiological regulator of ASC differentiation, establish a role for the Wnt pathway in normal B cell development and FA immune deficiency and suggest that modulation of Wnt activity could be beneficial for improving FA B cells-dependent immunity. In addition, this study extends the understanding of the role of Wnt signaling in primary B cells differentiation into plasma cells.

An important finding of this study is the observation of impaired ASC differentiation in Fancc$^{-/-}$ B cells. The differentiation of B cells to ASC is the main event leading to antibody production. Unlike the specific defect of IgG2a switch CSR observed in Fancc$^{-/-}$ B cells, this deficiency is not specific to the type of co-stimulatory cytokines. This difference is likely responsible for the diminution of IgM production after LPS stimulation and probably contributes to the impaired IgG response after immunization of the mice with NP-LPS. In an effort to understand the mechanism underlying this difference, we identified hyper-active Wnt signaling in Fancc$^{-/-}$ B cells. Wnt signaling pathway has been shown to regulate cell differentiation, migration, polarity and development of organs during embryogenesis (Review in 34). Wnt signaling has also been implicated in B cell development. In fact, impairment of Wnt signaling in Fzd9$^{-/-}$ mice leads to depletion of pre-B cells in BM (28). It has also been described that Wnt signaling is important for pro-B cell proliferation and survival and these effects are mediated by Lef-1 (29). The role of Wnt signaling on mature B cell function is mostly unknown and to our knowledge only one study reported that knock down of β-catenin inhibited ASC differentiation in vitro (35). Our evaluation of Wnt activity by western blot and in vivo Wnt reporter assay demonstrate an increase in constitutive and induced activation of the canonical Wnt signaling in Fancc$^{-/-}$ B cells.

To demonstrate that the effect of Wnt signaling on ASC differentiation is not specific for Fancc$^{-/-}$ B cells, we used Wnt3a to activate WNT canonical pathway in WT B cells. We observed an inhibition of ASC differentiation of WT B cells in the culture exposed to Wnt3a, recapitulating the phenotype of Fancc$^{-/-}$ B cells. These studies also enabled us to identify potential mediators of Wnt signaling on ASC differentiation. Specifically, we found that activation of Wnt signaling by Wnt3a led to decreased expression of Blimp-1 and IgM secretion in LPS-stimulated B cells. Interestingly, we also observed an up-regulation of the expression of Pax5, which has been described as a mediator of Wnt signaling (32) and as an inhibitor of Blimp-1 (3). It is possible that hyper-active Wnt signaling in Fancc$^{-/-}$ B cells leads to an increased activity of Pax5, which in turn represses Blimp1 transcription. As Blimp-1 represses Pax5 transcription (36), the observed up-regulation of Pax-5 could be the result of Blimp1 repression. Moreover, in accordance with an increased activity of Pax-5 we found up-regulation of Aicda, whose expression is positively regulated by Pax-5 (37), in the cultures of Fancc$^{-/-}$ and WT B cells in the presence of Wnt3a (data not shown). Together, we propose that the WNT canonical pathway is an inhibitor of ASC differentiation and that over-activation of the WNT pathway may lead to impaired ASC differentiation seen in Fancc-deficient B cells.

It has recently been proposed that the FA pathway plays a role in the regulation of Wnt signaling. Specifically, two recent studies have shown that *FANCC* is able to interact with Ctbp1 and β-catenin and regulates the expression of the Wnt inhibitor Dkk1 and that the integrity of the FA core complex is indispensable for a normal Wnt activity (15, 16). We observed no alteration in Dkk1 expression in our transcriptome analysis but found deregulation of several genes implicated in the Wnt signaling. Because the previous studies were conducted in human cell line, we cannot rule out a difference between human and mouse and/or between cell types. Nevertheless, our data demonstrate that in mouse primary cells, disruption of *Fancc* could deregulate Wnt signaling. In addition, since oxidative DNA

damage is able to increase Wnt activity by ATM activation (38), and since FA deficient cells have an increased intra-cellular level of reactive-oxygen species (ROS) (39), it would be interesting to evaluate the implication of ROS in hyper-active Wnt signaling observed in *Fancc*$^{-/-}$ B cells.

Our study also points to other defects in Fancc$^{-/-}$ B cells. Our in vitro and in vivo B cell differentiation experiments indicate an augmentation of the pre/pro B cell population and a diminution of the more differentiated B cells in the BM of *Fancc*$^{-/-}$ mice. However, no difference was observed in the periphery of *Fancc*$^{-/-}$ mice compared to WT controls, indicating that in steady state, the hematopoietic system might be able to compensate probably by increasing the B lineage populations in the BM. At the pre- and pro-B cell stages of B cell differentiation, the cells are highly expanding and are going through rearrangement at the locus of the immunoglobulin heavy and light chain (1). It is possible that this high rate of proliferation in combination with genomic DNA breakage and rearrangement cause a replicative stress in *Fancc*$^{-/-}$ B cells in which the FA repair pathway is deficient. We also note that other mechanisms, such as changes in B cell migration/sequestration, may also be responsible for the B cell defects in the Fancc$^{-/-}$ BM. However, our results with transplantation seem to indicate an impairment of B cell differentiation when the hematopoietic system is under stress and might not be able to compensate the B cell compartment without a functional FA pathway. This B cell differentiation defect is in accordance with a previous report showing a diminution of B cell number in a sub-group of FA patients (24).

The similarity in periphery of WT and *Fancc*$^{-/-}$ mice allowed us to evaluate the CSR efficiency in B cells without bias. CSR involves the non-homologous end-joining (NHEJ) DNA repair pathway (1) and the FA pathway has been proposed to inhibit the NHEJ in favor of the homologous recombination (9, 10). As previously reported for *Fanca* (40) and *Fancg* (41) deficient mice, we did not observe a general impairment of CSR in *Fancc*$^{-/-}$ B cells. In contrast, we observed a specific defect of IgG2a CSR in *Fancc*$^{-/-}$ B cells. However, this is unlikely due to the deficiency in DNA recombination process, as the expression of specific Ig germline sequence is required to initiate CSR, and we observed a profound decrease in the germline expression of IgG2a. This result indicates that an impairment of CSR occurs before initiating the DNA recombination process. A possible explanation for the impairment of germline IgG2a mRNA expression could be related to the deficiency in IFN-γ signaling that has been previously reported for *Fancc* deficiency (13, 14). In fact, the downstream effector of IFN-γ signaling T-bet and a proper activation of Stat1 are required for an efficient IgG2a germline mRNA expression (42, 43). The IgG2a switch deficiency likely contributes to the decrease in immunization response observed in *Fancc*$^{-/-}$ mice, as previously reported (17) and shown in this study after NP-LPS immunization. TLR4 activation is known to stimulate the Th1 immune response (44) which is dependent on IFN-γ signaling and secretion. Moreover, as Th1 and IFN- γ are important mediators of intracellular pathogen immunity, it would be important to evaluate this deficiency in FA patients. In effect, it has been reported that a sub-group of FA patients are more sensitive to infections (17) and have an impaired immunization response (19). Therefore, the choice of vaccine formulation without TLR4 adjuvant might be beneficial for FA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Dr. Maxime Mahe from Dr. Helmrath's lab from the CCHMC pediatric surgery department for providing us with the Wnt3a conditioned medium, Dr. Damien Renaud from the CCHMC Experimental hematology department for providing us with antibodies for BM B cell lineage experiments, Ms. Danielle Davis for reviewing the manuscript, the CCHMC DNA Sequencing and Genotyping Core for mRNA sequencing, the CCHMC Research Cytometry core for cell sorting, the CCHMC Viral Vector core for lentivirus production, and the CCHMC Comprehensive Mouse and Cancer core for the mouse transplantation experiments.

Financial Support Information

This work was supported by National Institutes of Health (Heart, Lung and Blood Institute) grant R01 HL076712 and (National Cancer Institute) grant R01 CA157537. Q.P. was supported by a Leukemia and Lymphoma Scholar award.

Abbreviations

FA	Fanconi anemia
ASC	Antibody-Secreting-Cell
SHM	somatic hyper-mutation
CSR	class switch recombination
NHEJ	non-homologous end joining
BM	Bone marrow
PB	Peripheral blood
HR	homologous recombination
LPS	lipopolysaccharide
RNAseq	mRNA sequencing
ROS	reactive-oxygen species
Abs	antibodies
TMB	3,3′,5,5′ tetramethylbenzidine

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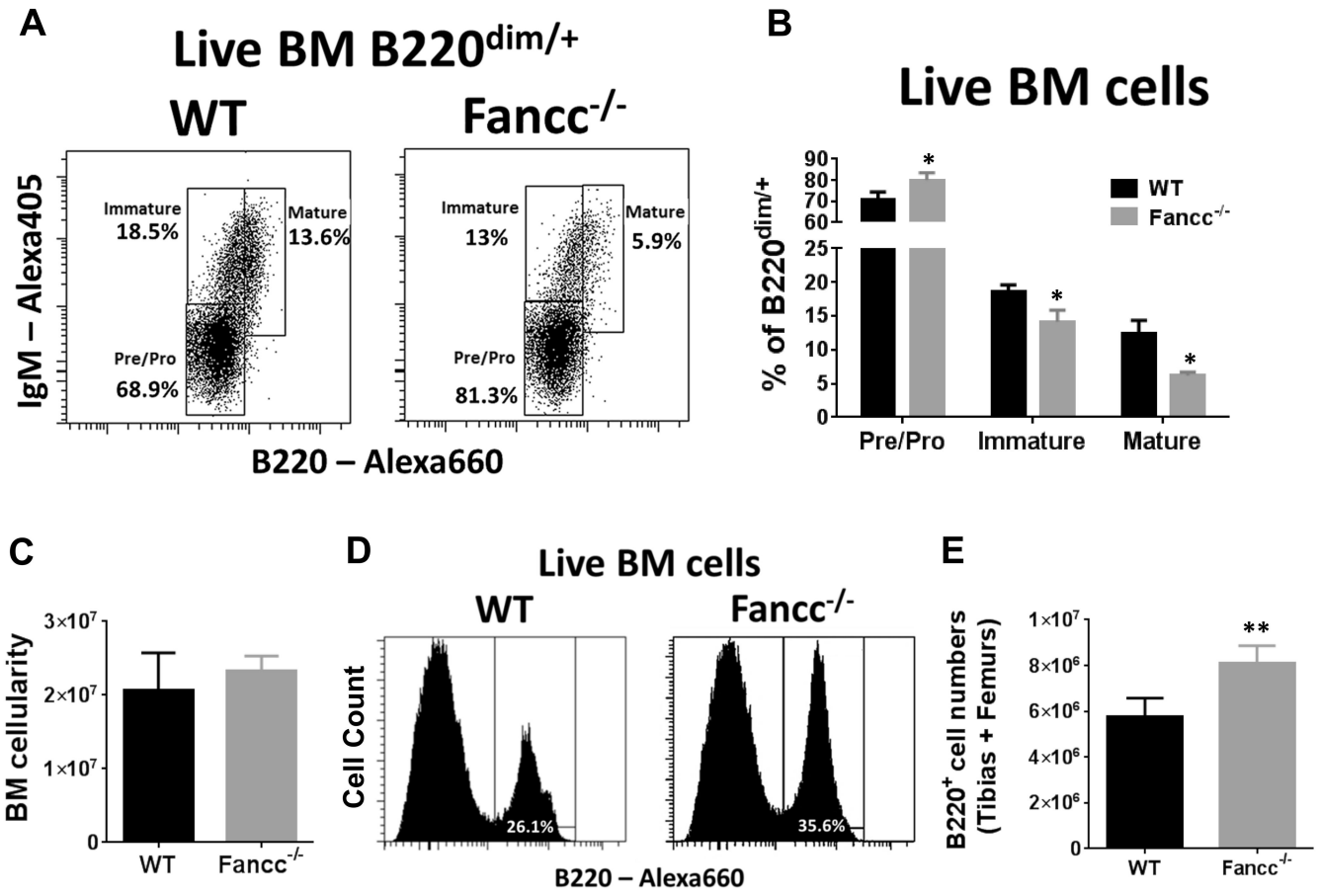

Figure 1. Abnormality of B cells lineage in bone marrow of *Fancc*$^{-/-}$

Bone marrow (BM) was harvested from tibias and femurs of 6-8 weeks old WT or *Fancc*$^{-/-}$ mice. Mononuclear cells were separated by centrifugation on Ficoll and labelled with anti-B220-eFluor660 and anti-IgM-Fitc Abs. Before Facs acquisition, propidium iodide (PI) was added detect and exclude dead cells. (A) Representative FACS plot of the evaluation of Pre/Pro (B220^{dim}IgM^{-}), Immature (B200^{dim}IgM^{+}) and Mature (B220^{+}IgM^{+}) among Live B220$^{dim/+}$ mononuclear BM cells from *Fancc*$^{-/-}$ or WT littermate mice. (B) Quantification of BM B cells populations (n=6 mice/genotype) as described in (A). (C) Bone marrow cellularity from 2 tibias and femurs of WT and Fancc$^{-/-}$ Mice (n=6 mice/genotype). (D) Representative Facs histogram of total B cell lineage percentage in live mononuclear BM cells from Fancc$^{-/-}$ or WT mice. (E) Total B cell (B220^{+}) number from 2 tibias and femurs. (n=6 mice/genotype). For all graphics, Bar represents mean value ± standard deviation (SD). Evaluation of differences between genotype has been conducted using Student's T-test (*=p≤0.05)

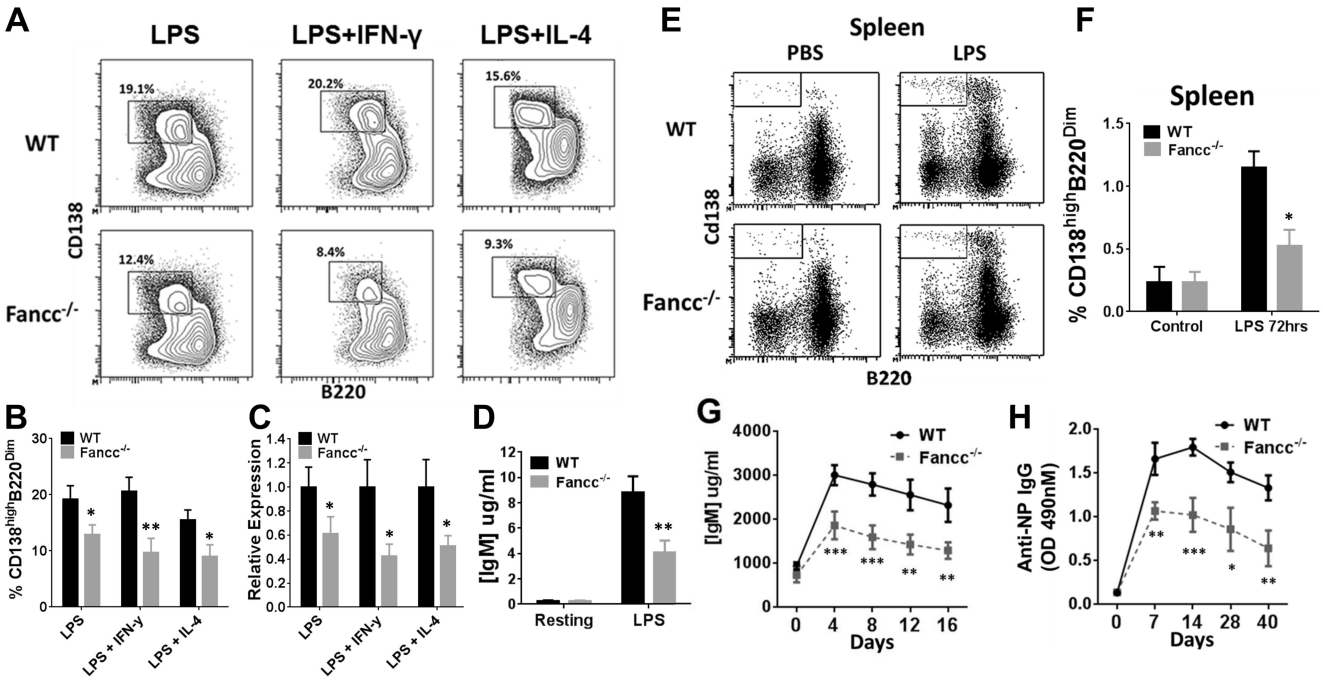

Figure 2. Impaired *Fancc*$^{-/-}$ B cells differentiation into plasma cells

(A-D) Naïve untouched B cells (CD43^{-} cells) were purified from splenocytes of 6-8 weeks old mice and cultured in presence of LPS (20ug/ml) alone or in combination with IL-4 (5ng/ml) or IFN-γ (50ng/ml) for 5 days. (A-B) ASCs (B220^{dim}CD138^{+}) were detected by Facs analysis. (A) Representative Facs plot of ASCs detection in cultures of WT or *Fancc*$^{-/-}$ B cells. (B) Quantification of ASCs percentage as represented in (A) (n=6/genotype, 3 independent experiments). (C) The mRNAs from B cell in culture were extracted for analysis of Blimp1 mRNA expression. Data were normalized on the mean value of WT Blimp1 expression (n=5/genotype, 2 independent experiments). (D) IgM production was measured by ELISA in supernatant of 5 days B cell cultures (n=5/genotypes, 3 independent experiments). (E-H) Mice were injected intra-veinously (i.v) with 15ug of LPS. (E-F) ASC population (B220$^{dim/-}$CD138^{high}) was evaluated in spleen of mice by Facs analysis. (E) Representative plot of ASCs detection in spleen of mice injected with PBS or LPS. (F) Quantification of ASCs percentage as represented in (A) (n=4 mice/condition, 2 independent experiments). (G) IgM serum concentration was measured by ELISA at different times after a single intra-peritoneal (i.p) injection of 100ug of LPS (n=4 mice/group, 2 independent experiments). (H) Anti-NP specific IgG was measured at different times by ELISA in serum of mice immunized by a single i.p injection of 100ug of NP-LPS (n=5 mice/group, 2 independent experiments). For all graphics, Bar represents mean value ± SD. Evaluation of differences between genotype has been conducted using Student's T-test (*=p≤0.05, **= p≤0.01, ***=p≤0.001).

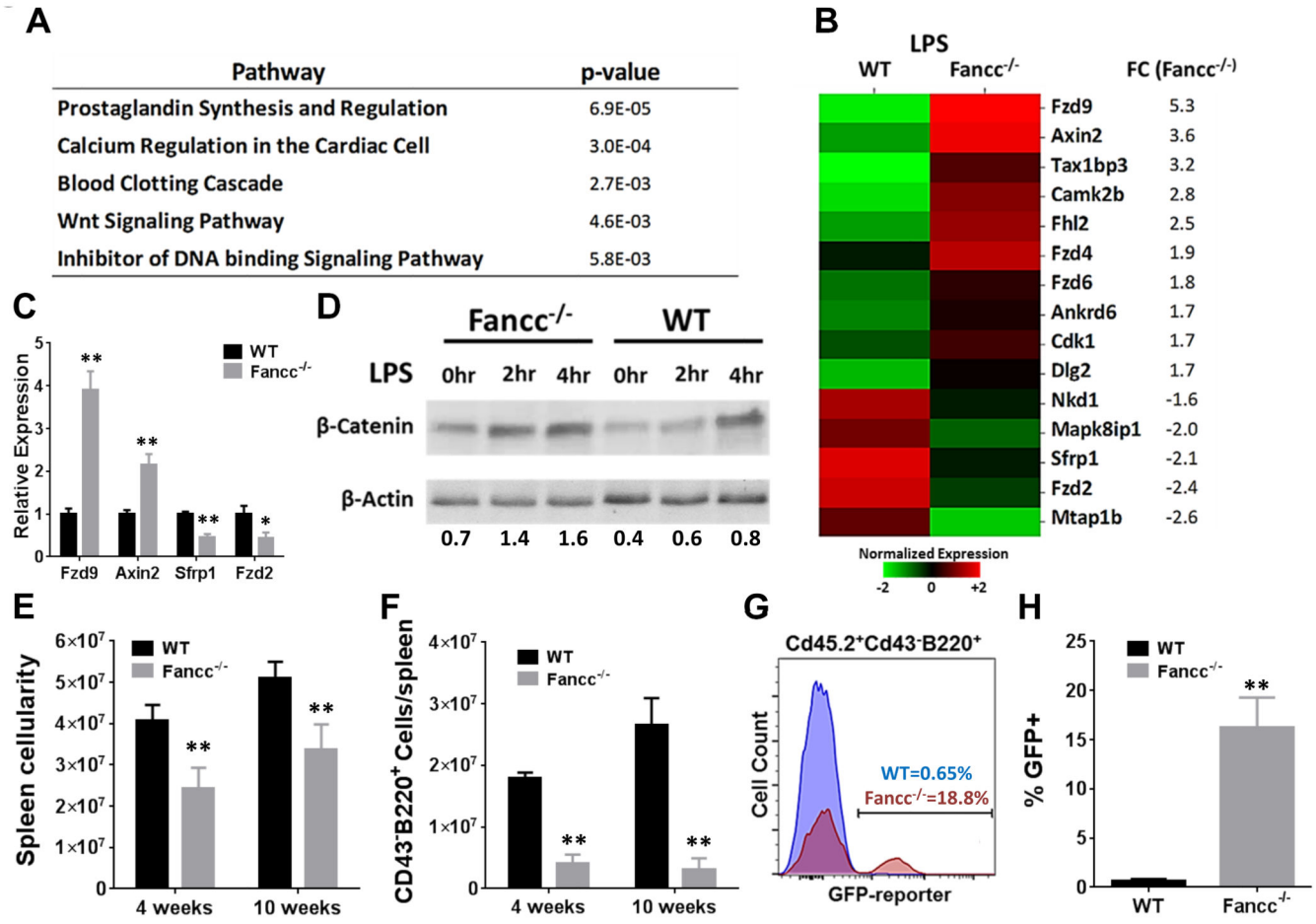

Figure 3. Deregulation of WNT signaling in *Fancc*$^{-/-}$ B cells

(A-B) Total RNA was extracted from naïve B cells of spleen of WT or *Fancc*$^{-/-}$ mice and stimulated 4h *in-vitro* with 20ug/ml LPS. The mRNAs were analyzed by RNAseq to determine transcript abundance and differential expression using GeneSpring GX (FC≥1.5, p≤0.05). (A) Top 5 deregulated pathways determined by Genespring Pathway analysis module using the Wiki Pathway curated database. (B) Heatmap representation (2 independent experiments) of mean normalized expression values of WNT signaling deregulated genes identify by RNAseq. (C) qPCR validation of candidate WNT deregulated genes from WT and *Fancc*$^{-/-}$ B cells culture activated 4h by LPS (n=4 mice/genotype, 2 independent experiments). (D) Western blot analysis of β-catenin protein level in whole B cells lysate for indicated time of culture (Representative of 3 independent experiments with pooled B cells of at least 2 mice for each genotype). Values under the blot represent the density ratio of β-catenin/ β-actin. (E-H) LSKs cells were isolated from WT and *Fancc*$^{-/-}$ BM by Facs sorting, transduced in-vitro with 7TGC reporter lentivirus and transplanted into lethally irradiated Boy/J mice. Donor derived splenocytes (CD45.2^{+}) were analyzed at 4 and 10 weeks post-transplantation. (E) Spleen cellularity of mice at 4 and 10 weeks after BM transplantation with Fancc$^{-/-}$ or WT LSKs. (F) Quantification of proportion of naïve donor B cells percentage at 4 weeks (n=4 mice/genotype, 2 independent experiments) and 10 weeks (n=5 mice/genotype, 2 independent experiments) post-transplantation. (G)

Representative overlay histogram of GFP expression from donor derived naïve B cells at 10 weeks post transplantation. (H) Quantification of GFP^+ donor derived B cells as indicated in (F) (n=5 mice/genotype, 2 independent experiments). For all graphics, Bar represents mean value ± SD. Evaluation of differences between genotype has been conducted using Student's T-test (*=p≤0.05, **= p≤0.01).

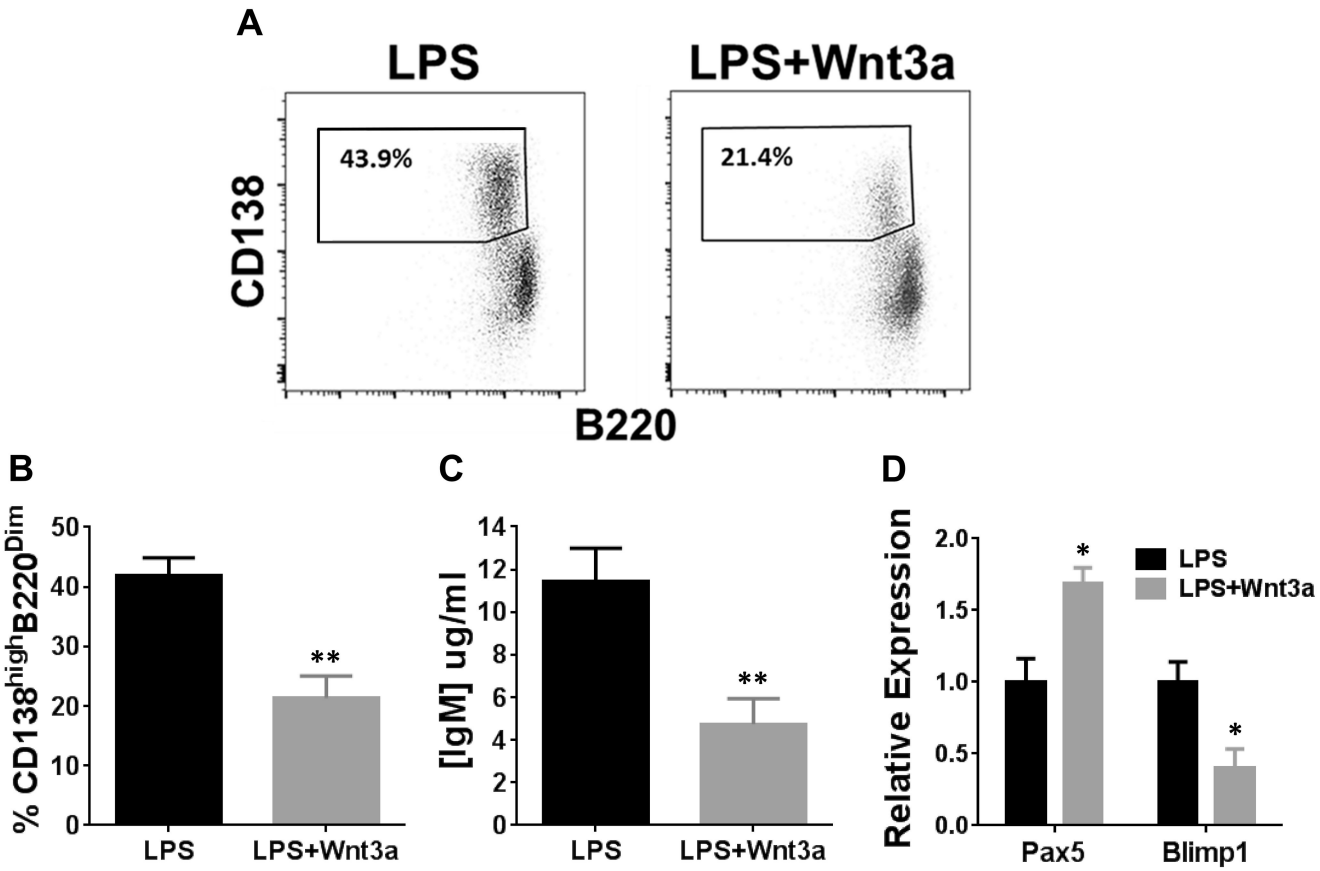

Figure 4. Canonical WNT signaling pathway activation inhibits ASC differentiation in-vitro

Naïve B cells from spleen of 6-8 weeks old WT mice were isolated and cultured in-vitro in presence of 20ug/ml LPS with control medium or Wnt3a conditioned medium (25% of culture volume) for 5 days. (A) Representative Facs plot of B cells in culture labelled with anti-B220-eFluor660, anti-CD138-BV421 and PI. (B) Quantification of ASC percentage in culture as indicated in (A) (n=6/genotypes, 3 independent experiments). (C) ELISA evaluation of IgM concentration in B cells culture supernatants at 6 days of stimulation by LPS with control medium or Wnt3a conditioned medium (n=5/genotypes, 3 independent experiments). (D) qPCR analysis of Pax5 and Blimp1 mRNA expression from 5 days LPS stimulated B cells (n=4, 2 independent analysis). For all graphics, Bar represents mean value ± SD. Evaluation of differences between genotype has been conducted using Student's T-test (*=p≤0.05, **= p≤0.01).

**= p≤0.01).

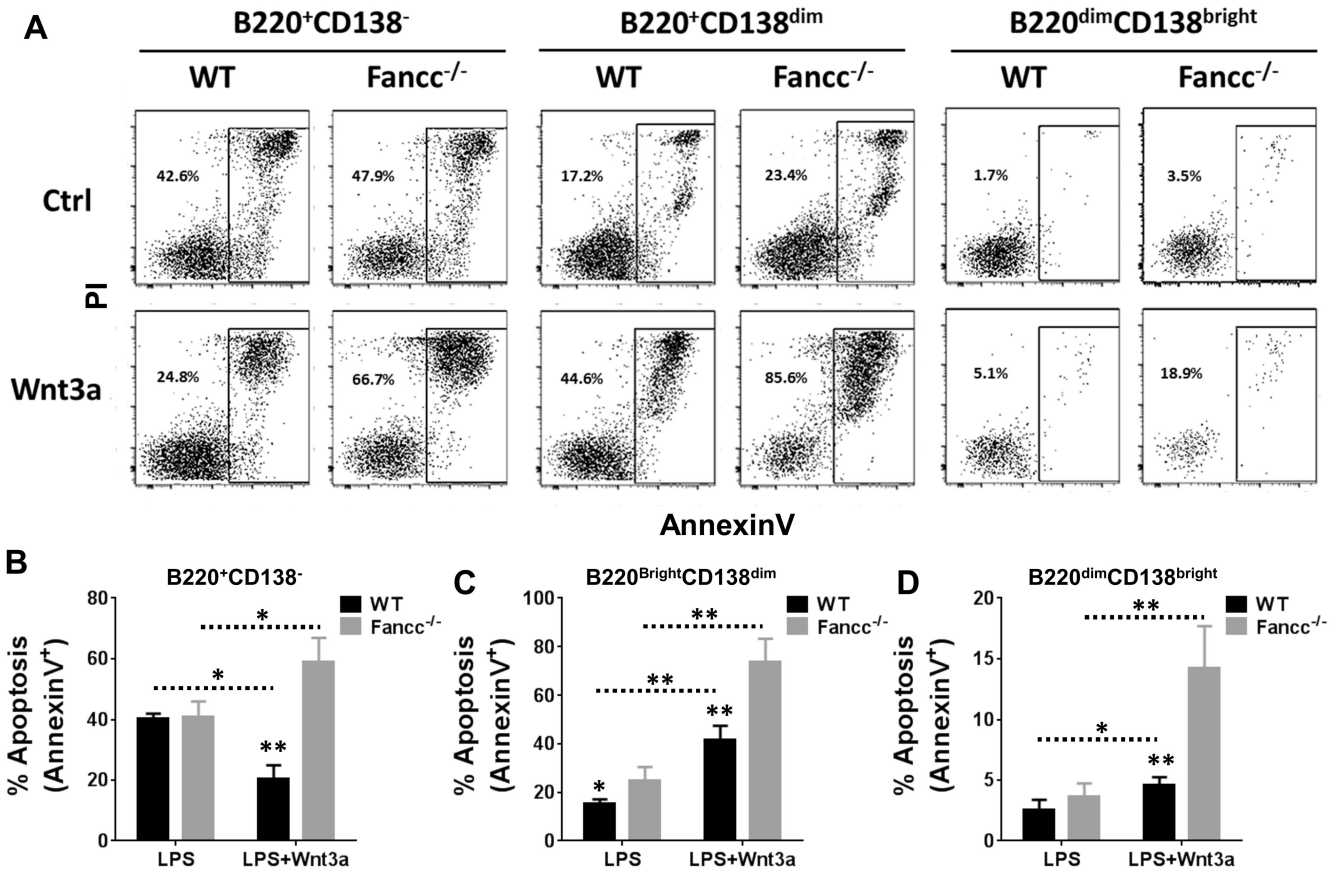

Figure 5. Canonical WNT signaling pathway activation has different effect on apoptosis of WT and *Fancc*$^{-/-}$ B cells in culture

Naïve B cells were isolated from spleen of 6-8 weeks old WT or *Fancc*$^{-/-}$ mice and culture 5 days in presence of LPS (20ug/ml) in control or Wnt3a conditioned medium (25%). Cells in culture were labeled with anti-B220, anti-CD138, anti-AnnexinV and PI. (A-D) Apoptosis was determined by Facs analysis (AnnexinV^{+}PI^{+or-} cells) for non-ASC differentiated B cells (B220^{+}CD138^{-}), Plasmablasts (B220^{+}CD138^{+}) and ASCs (B220^{dim}CD138^{bright}). (A) Representative Facs plot of apoptotic cells percentage in the different cells population after 5 days of B cells culture. (B-C) Apoptotic cells quantification as presented in (A) among (B) non-ASC differentiated-B cells, (C) Plasmablasts cells and (D) ASCs (n=4mice/genotype, 2 independent experiments). For all graphics, Bar represents mean value ± SD. Evaluation of differences between genotype has been conducted using Student's T-test (*=p≤0.05, **= p≤0.01).

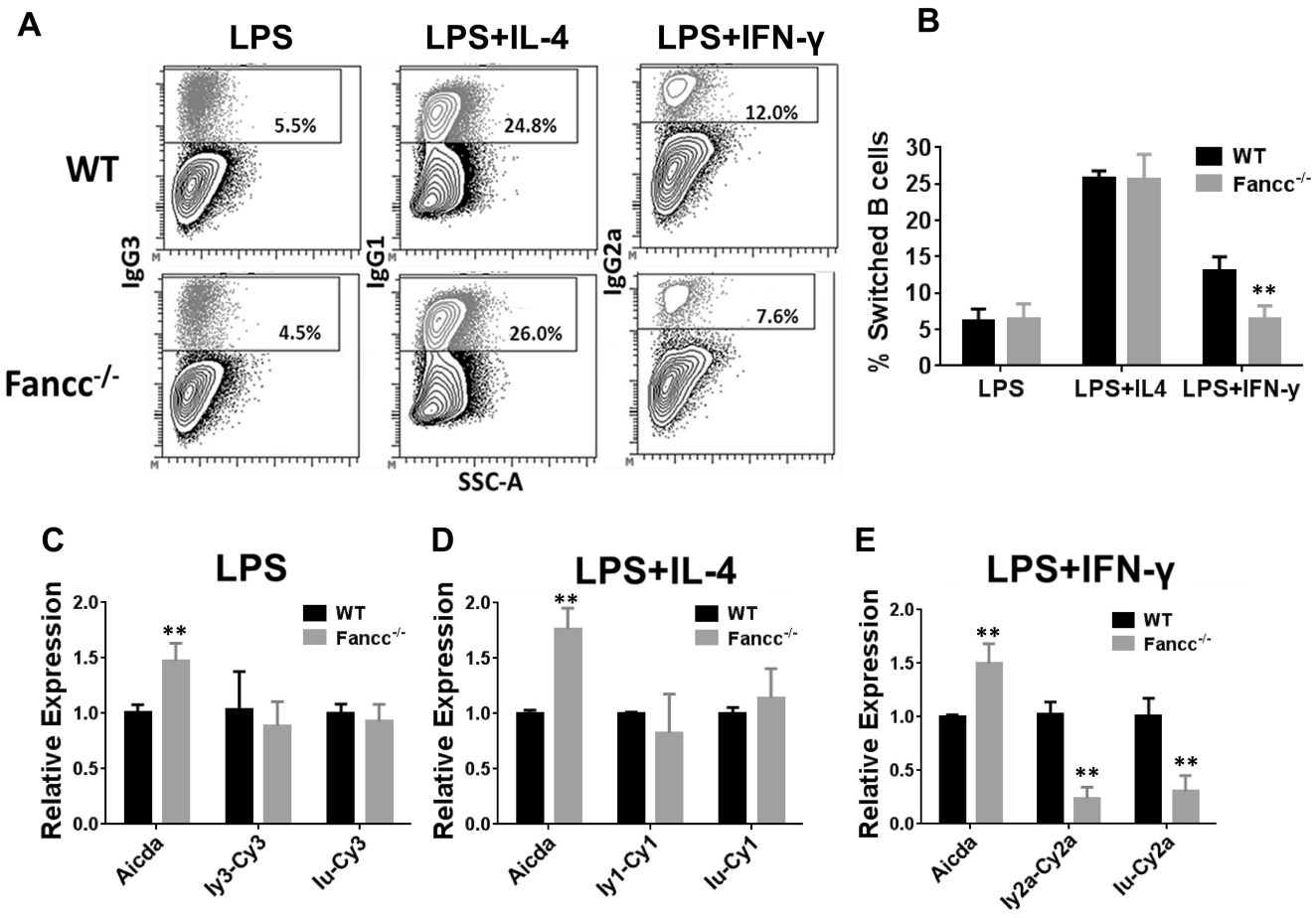

Figure 6. Specific IgG2a class-switch impairment in *Fancc*$^{-/-}$ B cells

(A-E) Naïve untouched B cells (CD43^{-} cells) were purified from splenocytes of 6-8 weeks old mice and cultured 3 days in presence of LPS (20ug/ml) alone or in combination with IL-4 (5ng/ml) or IFN-γ (50ng/ml). (A-B) Isotype specific CSR was evaluated for *Fancc*$^{-/-}$ and WT B cells by Facs analysis. (A) Representative Facs plot of percentage of B cells that undergone CSR (switched). (B) Quantification of switched B cells as represented in (A) (n=5 mice/group, 3 independent experiments). (C-E) qPCR analysis of mRNAs expression of Aicda, isotype specific IgG germline (Iγ3-Cγ3, Iγ1-Cγ1 and Iγ2a-Cγ2a) or post-switched (Iμ-Cγ3, -Cγ1 or Cγ-2a) from 3 division peak (CFSE) sorted B cells (n=4/genotype, 2 independent experiments). For all graphics, Bar represents mean value ± standard deviation (SD). Evaluation of differences between genotype has been conducted using Student's T-test (**= p≤0.01).