The Effect of the Autoimmunity-Associated Gene, PTPN22, on the BXSB Model of Lupus

Christian J. Maine[a], **Kristi Marquardt**[a], **John C. Scatizzi**[a], **K. Michael Pollard**[b], **Dwight H. Kono**[a], and **Linda A. Sherman**[a]

[a] Department of Immunity and Microbial Science, The Scripps Research Institute, La Jolla CA 92037

[b] Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla CA 92037

Abstract

A single nucleotide polymorphism in PTPN22 is linked to increased disease susceptibility in a range of autoimmune diseases including systemic lupus erythematosus (SLE). PTPN22 encodes the Lyp phosphatase that dampens TCR signaling and is necessary for signaling downstream of toll-like receptors in myeloid cells. To understand these dual functions in disease, we examined the impact of deficiency in PTPN22 on a spontaneous murine model of SLE. Male PTPN22 KO mice carrying BXSB chromosome 1 and the *Yaa* disease accelerating factor, developed disease at a similar rate and severity as PTPN22 WT. In contrast, although female BXSB mice showed no differences in survival in the absence of PTPN22, autoantibody production was significantly increased and splenic populations associated with pathogenesis in this model were expanded in the PTPN22 KO group. These findings support the notion that when coupled with other predisposing autoimmunity genes, PTPN22 deficiency contributes to a predisposition to lupus pathogenesis.

Keywords

Systemic Lupus Erythematosus; PTPN22; Tolerance; T follicular helper cells; Autoantibodies; BXSB

1. Introduction

The autoimmunity associated allele of PTPN22, R620W (C1858T), has been linked to a number of autoimmune conditions in humans such as type I diabetes (T1D) [1], rheumatoid arthritis (RA) [2] and systemic lupus erythematosus (SLE) [3]. To investigate the role

Corresponding Author: Linda A. Sherman, 10550 N Torrey Pines road, La Jolla, CA, 92037, phone: +1 858 784 8052, lsherman@scripps.edu.

PTPN22 plays in these diseases numerous mouse models of autoimmunity in which PTPN22 has been deleted, overexpressed, knocked down or mutated are beginning to emerge [4-8].

For SLE, multiple studies have shown increased risk associations between the C1858T SNP in PTPN22 [3, 9-11] over a range of ethnic populations with odds ratios (OR) for the T allele varying between 1.32-2.56. Interestingly, one study showed a stronger relationship between the PTPN22 risk variant in pediatric-onset SLE in Mexican populations than in adult-onset SLE in Caucasians [12]. Recent reports have even suggested correlation of this SNP with distinct disease subclasses, for example those patients with anti-cardiolipin antibodies [13].

PTPN22 encodes a phosphatase known as lymphoid tyrosine phosphatase (LYP) in humans or PEST-enriched phosphatase (PEP) in mice. Its function is best characterized in T cells in which it functions to dephosphorylate proximal TCR signaling molecules (Lck, ZAP-70, Src family kinases) that regulate TCR driven activation [4, 14, 15]. Loss of PTPN22 on the B6 background results in accumulation of memory T cells, increased germinal centers and serum IgG although these mice do not exhibit more autoantibodies or autoimmunity, possibly due to increased Treg numbers and function, or the lack of other factors that contribute to autoimmune disease [4, 16, 17]. Studies have suggested a role for PTPN22 in B cell signaling [18-22], although the extent to which this may be a consequence of increased T cell help is unresolved [4, 23]. Recently a novel, nonphosphatase role for PTPN22 in myeloid cell activity has been described downstream of TLR signaling which is necessary for efficient type I IFN production [24].

Two recent reports in which mice were engineered to express a mutation (R619W) analogous to the human R620W variant have described a phenotype similar to that of the PTPN22 KO mouse [5, 19]. The most recent of these papers describes the breaking of tolerance in these mice manifesting in systemic autoimmunity when on a mixed 129/B6 genetic background, a phenotype that is lost with successive backcrosses to B6 [19]. PTPN22 KO or mutation is not sufficient to develop spontaneous autoimmunity so the use of established autoimmune models or multiple gene knock-outs/mutations have been used to study this gene in a disease specific context [6, 7, 17, 23, 25].

The BXSB mouse, a recombinant inbred strain derived from C57BL/6 and SB/Le mice, develops SLE [26, 27]. This disease is characterized by B cell hyperplasia in peripheral lymphoid organs. Male mice develop more rapid and severe disease compared to females due to the presence of a Y chromosome linked accelerating factor (*Yaa*) that is an X chromosome translocation resulting in duplication of at least 16 genes including the *Tlr7* gene [28, 29]. Males typically die around 4-5 months of age and pathology includes immune complex mediated renal disease. Females typically have a 50% survival of approximately 19.4 months of age but develop detectable levels of autoantibodies earlier [26, 27]. BXSB susceptibility regions aside from the *Yaa* locus can be found on chromosomes 1, 3 and 13. Regions on chromosome 1 that have been shown to confer lupus phenotypes include *Bxs1-4* [30, 31]. The *Yaa* locus alone is insufficient to cause disease on non-autoimmune prone backgrounds but accelerates disease on the lupus prone backgrounds through a TLR7/type I

IFN mechanism [28, 29, 32]. Type I IFN is crucial to disease in both mouse models and human lupus [33, 34].

To investigate the effect of PTPN22 on SLE we introduced regions from chromosome 1 of BXSB on PTPN22 KO, this report is the first to describe the effect of PTPN22 on a classical, spontaneous mouse model of lupus.

2. Materials and Methods

2.1 Mice

Experimental procedures were carried out according to the National Institutes of Health Guide for the care and use of laboratory animals and approved by the Scripps Institutional Animal Care and Use Committee. PTPN22 −/− mice were obtained from Dr. Andrew Chan (Genentech, San Francisco, CA) and have previously been described [4]. BXSB/Scr mice were obtained from Scripps breeding colony and bred to PTPN22 −/− mice. Male BXSB-*Yaa* were crossed to female PTPN22 −/− mice and the F1 were then bred to female BXSB mice until all selected microsatellite regions on chromosome 1 were homozygous for BXSB. BXSB PTPN22 +/- mice resulting from this cross were then interbred to yield BXSB PTPN22 +/+, BXSB PTPN22 +/− and PTPN22 −/− and used in subsequent assays. Microsatellite markers used to track BXSB desired regions were *D1mit3*, *D1mit21*, *D1mit387* and *D1mit206* (this includes chromosome 1 regions between 19.8 and 174.9 Mb) as described in [31].

2.2 Flow cytometry

Cells to be stained were resuspended in FACS buffer (HBSS containing 1% FCS) and incubated with the indicated antibodies for 15 minutes on ice. Cells were then washed in FACS buffer before acquisition on an LSR-II flow cytometer (BD Bioscience, Franklin Lakes, NJ) and analysis using Flowjo (Treestar). Antibodies (Biolegend, San Diego, CA unless otherwise stated) used were anti-mouse CD4 PerCP-Cy5.5, CD8 Pacific Blue/APC-cy7, PD-1 FITC, CXCR5-biotin (BD Bioscience), CD44 Pacific Blue, GL-7 FITC, FAS PE, CD138 APC, CD19 APC-cy7, CD23 PE, CD21 PerCP-Cy5.5, CD11b-biotin, CD11c Pacific Blue/APC, B220 PE, PDCA-1 Pacific Blue and streptavidin APC/FITC/PerCP. For intracellular staining of markers, an intracellular staining kit (Fix/Perm, eBioscience, San Diego CA) was used together with anti-mouse Foxp3 PE (eBioscience).

2.3 ELISA

Serum was collected from mice at the stated time points. Maxisorp plates (Nunc, Rochester, NY) were coated with 3.6μg/ml of chromatin overnight at 4°C. Plates were blocked in 1% gelatin (Sigma Aldrich) for an hour at 37°C. Plates were washed three times with wash buffer (HBSS with 0.1% Tween-20 (Sigma Aldrich)). Sera were diluted accordingly following optimization for each experiment in reagent buffer (HBSS containing 1% BSA, 0.1% Tween-20) and incubated on the plate in duplicate for 1 hour at 37°C. Plates were washed three times. Anti-mouse IgG alkaline phosphatase (AP) was then diluted and added to the wells for a further hour at 37°C (Jackson Immunoresearch). Plates were washed and

then incubated with pNPP AP substrate (Sigma Aldrich). Plates were read using a Versamax plate reader (Molecular devices, Sunnyvale, CA) at 405 nm.

2.4 Anti-Nuclear Antibody staining

ANAs were detected on Hep2 slides (MBL Bion, Des Plains, IL) at 1/100 diluted sera and 1/200 diluted Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Invitrogen) as described in [35].

2.5 Proteinuria

Proteinuria was measured by Bio-Rad protein assay (Bio-rad) according to the manufacturers protocol. Urine was diluted 1:100 and BSA serial dilutions were prepared for a standard curve (Sigma-Aldrich).

2.6 Histology

Sections of kidney, lung, liver, heart and spleen were collected from BXSB mice and zinc-formalin fixed. Sections were then stained with Periodic acid-Schiff (PAS) and hematoxylin (TSRI histology core) and scored blindly. For glomerulonephritis a clinical score on the scale of 1-4 was assigned to each [36].

2.7 Immunizations

6-week-old male BXSB-*Yaa* mice were immunized subcutaneously (s.c.) with 100μg of NP-KLH (Biosearch technologies, Novarto, CA) in complete Freund's adjuvant (CFA) (Difco, Detroit, MI). Draining lymph node and spleen were collected at 11 days post immunization.

2.8 Intracellular staining of IFNα

Splenocytes were stimulated in a 96 well plate with either Imiquimod (5μg/ml) or R848 (1μg/ml) in the presence of Brefeldin A (Sigma Aldrich) for 5 hours and 37°C. Following this incubation time, surface markers for pDCs (CD19- PDCA+ B220+) were stained and the cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD bioscience) and stained for IFNα (PBL Assay Science). Cells were then analyzed by flow cytometry.

2.9 Statistics

Graphs were assembled and analyzed using Prism 5 software (Graphpad, San Diego, CA). For multiple group analyses, one-way ANOVA with Tukeys post-test was carried out. For comparison of two-group data sets, a two-tailed Students t-test was used. $P < 0.05$ was considered significant.

3. Results

3.1 Disease onset in male BXSB-*Yaa* is unaffected by PTPN22

To study the effect of PTPN22 on SLE we backcrossed defined regions of the BXSB chromosome 1 onto the WT or PTPN22 KO B6 background until homozygosity (see methods 2.1 for more details). In addition, the Y chromosome was fixed for BXSB in all cases so as to include the lupus accelerating *Yaa* locus. Survival was found to be similar in

both the WT and PTPN22 KO cohorts of male mice (median survival was 4.9 vs 5.6 months for WT and KO respectively) (Figure 1A). In addition anti-nuclear antibody and anti-chromatin antibody titers in the sera of both groups of mice was similar (Figure 1B and C). Proteinuria was also similar amongst WT and KO groups of animals (Figure 1D). Autopsies of these animals revealed the major cause of death was kidney disease with minimal lung or heart pathologies. Glomerulonephritis was scored and showed no difference between WT and KO mice (Figure 1E). Figure 4F shows representative images of kidney sections showing typical changes of immune complex-mediated glomerulonephritis including enlarged glomeruli, presence of deposits, increased cellularity, and inflammatory cell infiltrates. Overall, these data suggest that PTPN22 does not influence onset and severity of disease in the male BXSB-*Yaa* model of SLE.

3.2 Loss of PTPN22 on the male BXSB-*Yaa* background enhances T-dependent antigen responses

We have previously reported that PTPN22 KO mice on the B6 background have higher numbers and increased function of T_{FH} cells leading to larger germinal center responses and antibody production. In light of the lack of disease enhancement in PTPN22 KO male BXSB-*Yaa* mice, we asked whether this phenotype was maintained on the BXSB background. At 6-weeks of age, which is prior to the onset of lupus symptoms, mice were immunized with NP-KLH in CFA, and 11 days later the spleen and draining LN (dLN) were collected and analyzed for germinal center activity (Figure 2). T_{FH} numbers were significantly increased in both the spleen and dLN of PTPN22 KO mice compared to WT (Figure 2A and B). GC B cells numbers were increased in the spleens and LN of the KO mice compared to WT although these differences were not statistically significant (Figure 2 C and D). Overall these data show that PTPN22 KO on the BXSB-*Yaa* male background increases GC activity to a foreign antigen.

3.3 PTPN22 KO mice have less IFNα producing plasmacytoid dendritic cells

Type I IFN is a major driver of disease during the initiating stages of BXSB-*Yaa* due to a gene duplication of TLR7 in the *Yaa* locus [28, 29]. It has been reported that PTPN22 deificiency reduces production of IFNα by myeloid cells [24]. To explore the consequence of PTPN22 KO on type I IFN production we stimulated splenocytes *in vitro* with TLR7 agonists, Imiquimod or R848, and measured IFNα by intracellular flow cytometry. Figure 3A and B confirms results previously published showing that in response to TLR7 stimulation, plasmacytoid dendritic cells (pDCs) in B6 PTPN22 KO mice produce significantly less IFNα than WT B6 mice. Similarly on the BXSB-*Yaa* background, PTPN22 KO mice have significantly less IFNα producing pDCs compared to WT (Figure 3C and D). These BXSB-*Yaa* mice were all less than 8 weeks old, prior to the onset of disease. Overall these data show that PTPN22 is required for efficient IFNα production downstream of TLR7, but does not completely abolish its expression.

3.4 PTPN22 KO BXSB females have increased anti-chromatin IgG

Female BXSB mice do not die from systemic autoimmunity until very late, however they do develop auto-antibodies. PTPN22 KO and WT BXSB females showed good survival

throughout the 10-month study period of our experiments, with no symptoms or health concerns (data not shown). However, PTPN22 KO BXSB females exhibit significantly increased and earlier onset anti-chromatin IgG sera titers compared to WT BXSB female mice (Figure 4A). Male BXSB-*Yaa* sera were also included as a comparison and shows higher and earlier onset of anti-chromatin IgG compared to both female genotypes. Proteinuria was also measured and was unaffected by PTPN22 expression (Figure 4B), both female groups have lower levels of proteinuria than male BXSB-*Yaa* mice (Figure 1D). Collectively these data show that PTPN22 can affect auto-antibody production in BXSB females although disease onset is unaffected within the age range observed in this study.

3.5 Spleens of female PTPN22 KO BXSB mice have increased numbers of disease associated lymphocyte populations

Spleens were collected from PTPN22 WT and KO BXSB females and stained for various T cell, B cell and antigen presenting cell populations at 4-months of age (Figure 5), when antibody titers differed significantly (Figure 4). The spleen cellularity overall was similar in KO mice compared to WT (Figure 5A), but T_{FH} cells were significantly increased (Figure 5B). Total B cells as measured by CD19 expression were similar in PTPN22 KO BXSB mice compared to WT, and within this gate, follicular B cells and marginal zone B cells remained constant (Figure 5C-E). Plasma cells and age-associated B cells (ABCs) were both significantly increased in the spleens of PTPN22 KO BXSB mice compared to WT (Figure 5F and G). Dendritic cells were also slightly increased, although not to statistically significant levels in the KO mice compared to WT (Figure 5H). The expression of CD80 (Figure 5I) was marginally higher on KO DCs and CD40 (Figure 5J) was similar on WT and KO DCs. Overall these data suggest that PTPN22 deficiency amongst BXSB mice results in the expansion of lymphocytes that are commonly associated with lupus pathogenesis.

4. Discussion

The presence of the R620W allele of PTPN22 has been associated with multiple autoimmune diseases including SLE [1-3]. This paper is the first report to describe the effects of PTPN22 on a spontaneous mouse model of lupus. By using the BXSB model we were able to investigate the contribution of PTPN22 on both highly and moderately susceptible backgrounds because of the presence of the lupus-promoting *Yaa* in males, but not females.

Surprisingly, PTPN22 deficiency resulted in no change in disease severity or lifespan of male BXSB-*Yaa* mice. Despite the observation that PTPN22 KO BXSB mice still exhibited increased T_{FH} and germinal center responses to foreign antigen as evidenced by the response to NP-KLH, this had no consequence on disease in this model.

A complication in the male BXSB-*Yaa* model is the effect of PTPN22 on myeloid cell TLR7 signaling and type I IFN production. DCs require PTPN22 for efficient TRAF3 auto-ubiquitination downstream of TLR4, 7 and 9 signaling in a non-phosphatase dependent manner [24]. As a result PTPN22 KO DCs are impaired in their type I IFN producing capacity. The male BXSB-*Yaa* model is highly dependent on TLR7 signaling and type I IFN [28, 37]. Early type I IFN blockade is beneficial in attenuating disease in the BXSB male

model, although it is less effective when administered late, suggesting that the subsequent adaptive immune response is sufficient to cause death in this model. In the case of PTPN22 KO BXSB male mice, there are 2 opposing effects; the early type I IFN production by DCs may be lowered but the later germinal center response is amplified. The observation that disease is essentially unchanged by loss of PTPN22 may reflect these two opposing processes.

In the case of human SLE, the behavior of the lupus predisposing PTPN22 R620W variant in leading to reduced type I IFN production in a predominantly type I IFN driven disease is paradoxical [24, 38]. However it has been shown that target tissues of SLE are characterized by pro-inflammatory cytokines that are suppressed by type I IFN e.g. IL-1β and TNF-α [39, 40]. This, together with reported defects in B cell central and peripheral tolerance checkpoints associated with the R620W mutation in humans, could predispose individuals to inflammation and disease [21]. Despite the increased risk associated with the PTPN22 R620W mutation and SLE, the odds ratio is lower than that for RA and T1D where PTPN22 ranks 2nd and 3rd respectively amongst genetic risk variants [41]. As such the effect PTPN22 has on SLE is likely to be smaller than for RA and T1D.

Female BXSB mice live longer than males and all of the female mice in our experiments survived the 10-month study period. As we only bred BXSB chromosome 1 onto the B6 background, this could have resulted in less severe disease than is usually expected in female BXSB mice. PTPN22 KO BXSB females did not exhibit increased severity of disease or the time of onset within the period of observation, but PTPN22 deficiency did increase several factors associated with pathogenesis. Autoantibody production was significantly higher in PTPN22 KO BXSB female mice compared to WT BXSB with detectable anti-chromatin antibodies at 3 months compared to greater than 4 months in WT BXSB mice. In addition female PTPN22 KO BXSB mice had significantly increased numbers of T_{FH}, plasma cells and ABCs at 4 months compared to WT. These results confirm similar reports of ABC accumulation in the PTPN22^{R619W} mouse as well as previous reports of increased T_{FH} activity in the PTPN22 KO mouse [19, 23]. ABCs, as defined in this report as CD19+ CD11b+ CD11c+, have been recently described to accumulate in female strains of autoimmune prone mice at a young age [42]. In the NZB/W F1 model of lupus, ABCs are detectable at 3 months and accumulate to high levels in the spleen at 8-10 months of age. They secrete anti-chromatin antibodies and are dependent on TLR7 for their function. The association between their increased numbers and increased amounts of anti-chromatin antibodies in PTPN22 KO BXSB is an area that will be further studied in this model as well as other autoimmune strains lacking PTPN22.

Despite these differences between WT and PTPN22 KO BXSB females, proteinuria and kidney histology was similar. It is possible that even in PTPN22 KO females, levels of autoantibodies do not reach the threshold level necessary to induce disease within the time frame of our observation. Other pro-autoimmune alleles may be required to push the immune system over the threshold needed for end-organ disease.

6. Conclusion

In summary, the results in this report demonstrate that the disease kinetics of the male BXSB-*Yaa* model was probably too severe to analyze the effect of PTPN22 on spontaneous lupus, while in less susceptible female BXSB mice, PTPN22 deficiency played a role in autoantibody production, but this was not sufficient for progression to kidney pathology. Further investigation of PTPN22 in other spontaneous models of lupus with more favorable kinetics is warranted.

Acknowledgements

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Highlights

- PTPN22 is associated with increased risk to multiple autoimmune diseases including systemic lupus erythematosus

- Crossing of PTPN22 KO to the BXSB model of lupus allowed us to study the effect on disease

- PTPN22 KO does not affect disease onset or severity on the male BXSB-Yaa background

- PTPN22 KO increases autoantibody production in female BXSB mice but does not accelerate disease
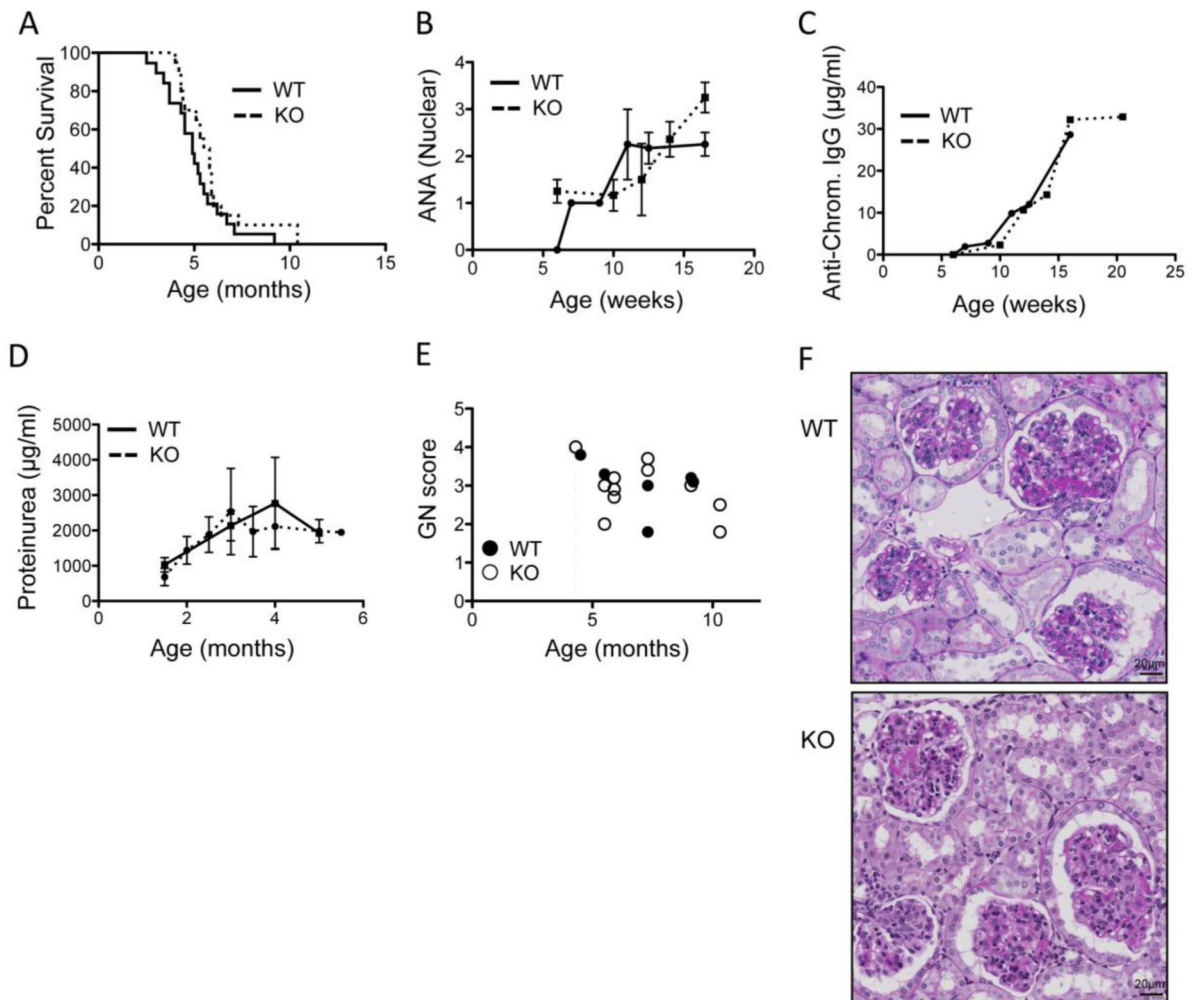
Figure 1.
PTPN22 does not affect the onset of disease on the BXSB.*Yaa* background. A) shows male PTPN22 KO and WT BXSB.*Yaa* survival (WT n=19, KO n=20). B) shows anti-nuclear antibody levels in the serum (WT n=5, KO n=12) C) shows serum anti-chromatin IgG concentration as measured by ELISA for PTPN22 KO and WT BXSB.*Yaa* mice (WT n=5, KO n=12). D) shows proteinuria concentration measured by Bradford assay (WT n=10, KO n=17). E&F) kidneys were collected, sectioned and scored for glomerulonephritis (each data point represents 1 mouse). Images are representative of the median GN score for each genotype and are PAS and hematoxylin stained (magnification 40x).
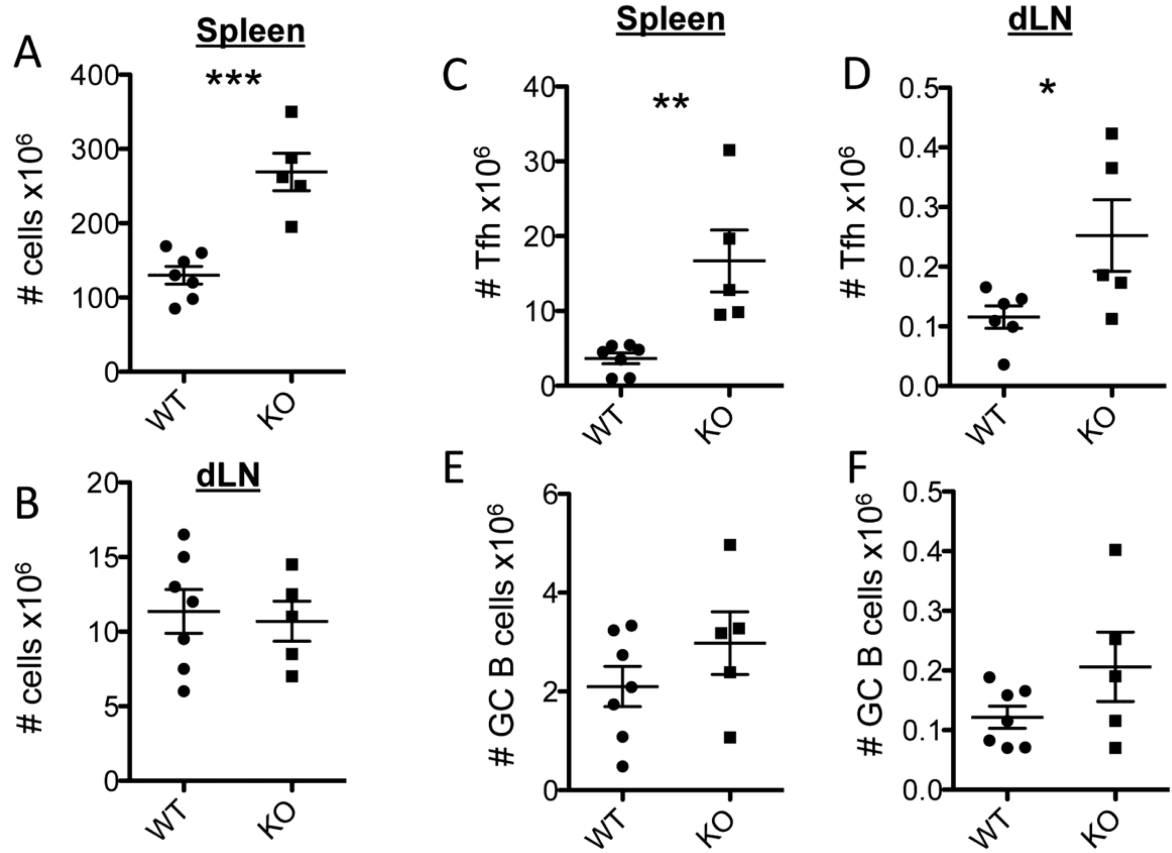
Figure 2.
PTPN22 KO BXSB.*Yaa* mice have increased T-dependent antigen responses. Six-week-old PTPN22 KO and WT BXSB.*Yaa* mice were immunized with NP-KLH in CFA s.c. and 11 days later the spleen and draining LN were harvested and stained for flow cytomteric analysis. Total numbers of cells is shown for the spleen (A) and dLN (B). Absolute numbers of T_{FH} cells (CD4^{+} CD44^{hi} CXCR5^{+} PD-1^{+}) in are shown for the spleen (C) and dLN (D). Absolute numbers of germinal center B cells (CD19^{+} GL-7^{+} FAS^{+}) are shown for the spleen (E) and dLN (F). All data points represent 1 mouse and graphs show pooled data from at least 3 independent experiments. * p<0.05; *** p<0.001.
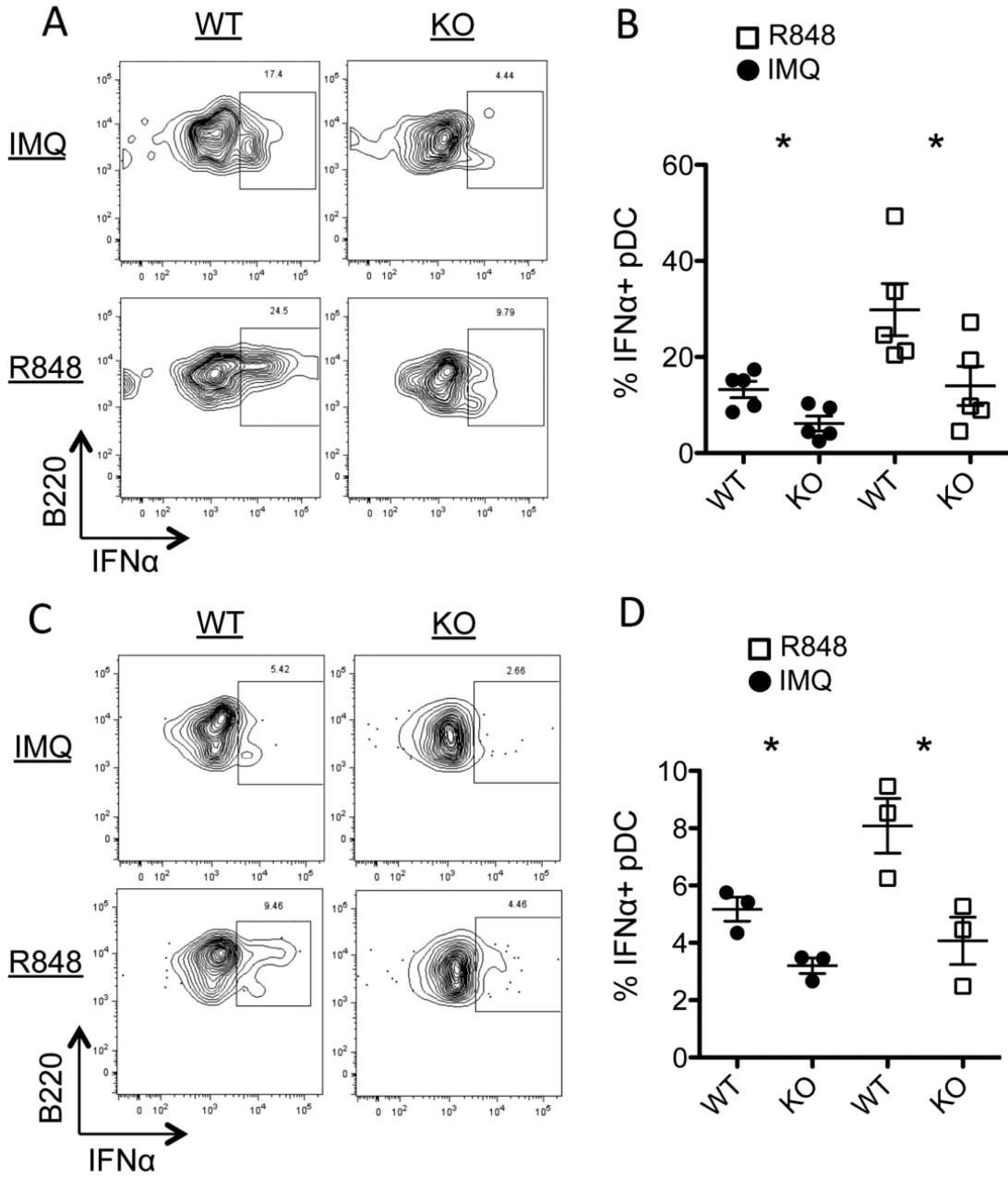
Figure 3.
PTPN22 is necessary for efficient IFNα production by pDCs. Splenocytes were stimulated with either Imiquimod (IMQ) or R848 for 4 hours *in vitro*. The cells were then fixed and permeabilized and stained for IFNα. A) Representative flow cytometry plots of IFNα expression on B6 pDCs (gated on CD19⁻ B220⁺ PDCA-1⁺). B) Shows combined data of B6 PTPN22 WT and KO IFNα+ pDCs. C) PTPN22 KO or WT BXSB-*Yaa* (younger than 2 months) splenocytes were stimulated for 4 hours in vitro and IFNα was stained and analyzed by flow cytometry. D) Shows combined data from PTPN22 WT and KO BXSB-*Yaa* mice. Each data point represents 1 mouse. Figure shows results of 2 independent experiments. * p<0.05.
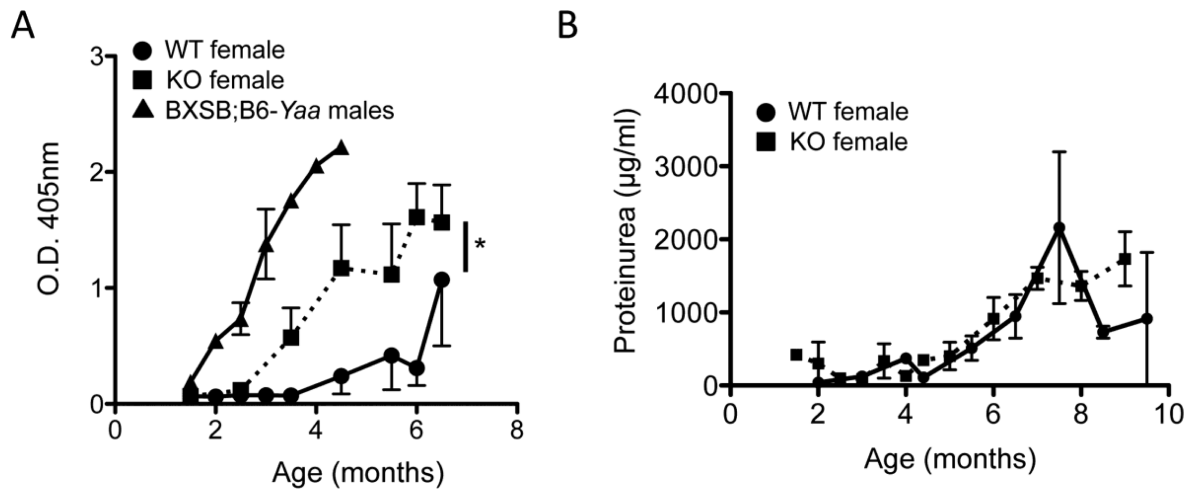
Figure 4.
PTPN22 KO increases autoantibody production in female BXSB mice. A) Sera from PTPN2 KO (squares) and WT (circles) BXSB female mice were collected and anti-chromatin antibody levels were measured by ELISA (WT n=8, KO n=8). Part A also includes male BXSB.*Yaa* as a comparison (triangles). B) shows proteinuria concentration in PTPN22 KO and WT mice (WT n=8, KO n=7). * $p<0.05$.
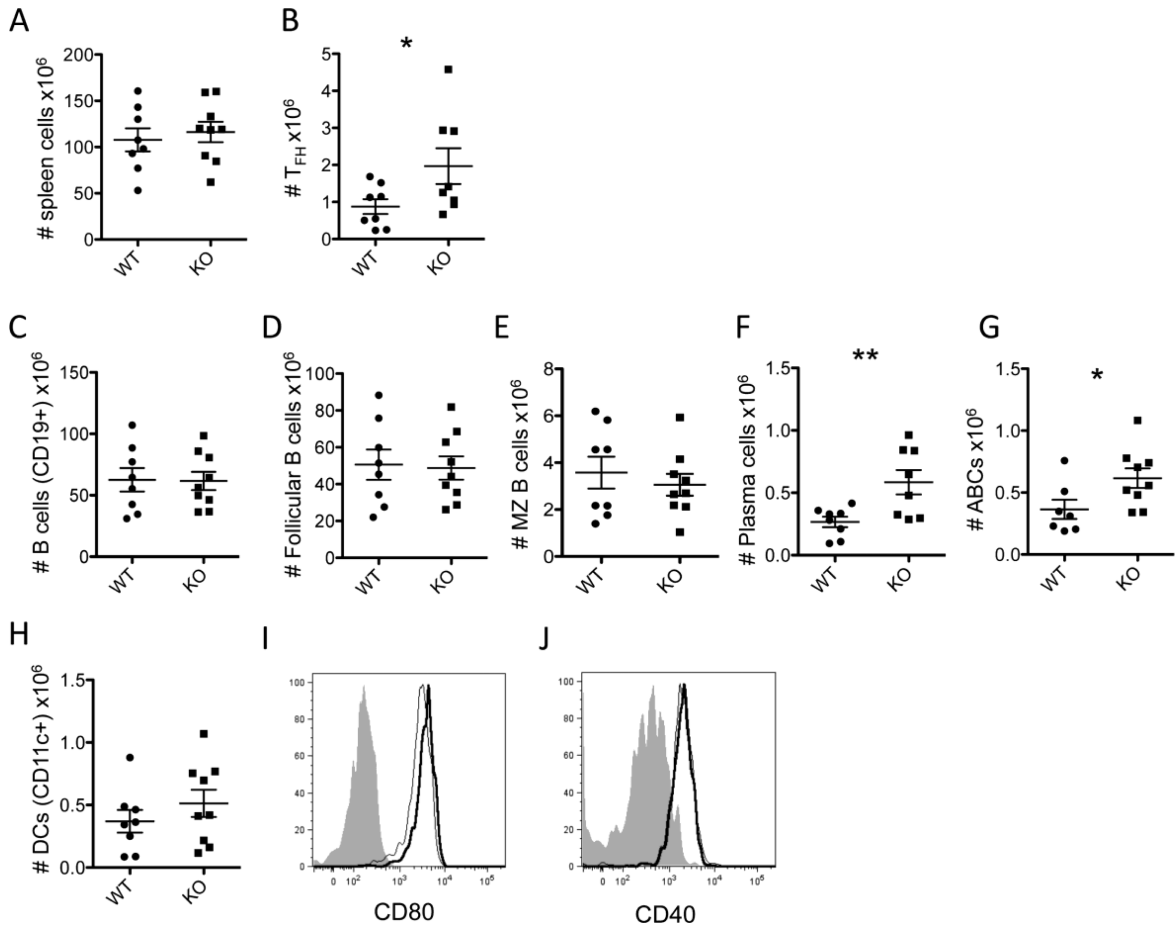
Figure 5.
Analysis of 4-month-old spleen populations of female BXSB mice. Spleens were collected from PTPN22 WT and KO BXSB female mice at 4 months of age and stained for various populations for analysis by flow cytometry. A) total spleen cellularity. Absolute numbers of TFH cells (B). Absolute numbers of CD19+ B cells (C), CD19+ CD21int CD23++ follicular B cells (D), CD19+ CD21+ CD23- marginal zone B cells (E), CD19low CD138+ plasma cells (F) and CD19+ CD11b+ CD11c+ age-associated B cells (G). Absolute numbers of dendritic cells (CD19- CD11c+) are shown in panel H and CD80 (I) and CD40 (J) expression on these cells is shown in the histograms (WT thin line, KO thick line, isotype control filled grey). All data points represent 1 mouse and graphs show pooled data from at least 3 independent experiments.