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Late stages of hematopoiesis and B cell lymphopoiesis are regulated by α -synuclein, a key player in Parkinson's disease

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Abstract

α -synuclein plays a crucial role in Parkinson's disease and dementias defined as synucleinopathies. α -synuclein is expressed in hematopoietic and immune cells, but its functions in hematopoiesis and immune responses are unknown. We utilized α -synuclein^{-/-} (KO) mice to investigate its role in hematopoiesis and B cell lymphopoiesis. We demonstrated hematologic abnormalities including mild anemia, smaller platelets, lymphopenia but relatively normal early hematopoiesis in KO mice compared to wildtype (WT) as measured in hematopoietic stem cells and progenitors of the different cell lineages. However, the absolute number of B220⁺IgM⁺ B cells in bone marrow was reduced by 4-fold in KO mice (WT: $104 \pm 23 \times 10^5$ vs. KO: $27 \pm 5 \times 10^5$). B cells were also reduced in KO spleens associated with effacement of splenic and lymph node architecture. KO mice showed reduced total serum IgG but no abnormality in serum IgM was noted. When KO mice were challenged with a T cell-dependent antigen, production of antigen specific IgG1 and IgG2b was abolished, but antigen specific IgM was not different from WT mice. Our study shows hematologic abnormalities including anemia and smaller platelets, reduced B cell lymphopoiesis and defects in IgG production in the absence of α -synuclein. This is the first report to show an important role of α -synuclein late in hematopoiesis, B cell lymphopoiesis and adaptive immune response.

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Authorship Contributions:

WX designed and performed the experiments, analyzed the data and wrote the manuscript. AS performed the experiments, analyzed the data and co-wrote the manuscript. CVH and HJM analyzed the data and reviewed the manuscript. RWM designed and performed experiments, analyzed the data, co-wrote and critically revised the manuscript to its final version.

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Keywords

B cells; lymphopoiesis; α -synuclein; hematopoiesis; anemia

Introduction

Synucleins comprise a family of small proteins (14–17 Kd) that were first identified in normal and neoplastic brain tissues (Fung et al., 2003; George, 2002). It is well established that α -synuclein is a key component of the Lewy body, a large globular protein complex that plays a critical role in the pathogenesis of Parkinson's disease (PD) and other dementias known as synucleinopathies (Beach et al., 2009; Bendor et al., 2013).

The function of α -synuclein in the hematopoietic system is largely unknown. Hematopoietic stem cells (HSCs) are responsible for the generation and regeneration of all blood-forming and immune systems (Reya et al., 2001). As HSCs mature, they differentiate to generate common lymphoid progenitors (CLPs) (Kondo et al., 1997) and common myeloid progenitors (CMPs) (Akashi et al., 2000). CLPs can differentiate into T, B and natural killer (NK) cells but not myeloid cells, while CMPs give rise to all myeloid lineage cells via megakaryocyte/ erythrocyte (MEPs) or granulocyte/macrophage progenitors (GMPs). It was recently shown that α -synuclein is present in megakaryocytes, platelets, erythroid precursors, and erythrocytes, while its expression is reduced in megakaryocytes of myeloproliferative neoplasms (Maitta et al., 2011). Moreover, it was found to be a specific marker of acute megakaryoblastic leukemia subtype M7 (AML M7) suggesting that α -synuclein could play a role in the pathogenesis of some hematological neoplasms.

The role of α -synuclein in immune modulation has recently come to light. Microglia activation in PD may be triggered by aggregates of secreted α -synuclein (Roodveldt et al., 2008). Aggregated forms of α -synuclein have a stronger stimulating effect on microglia than non-aggregated forms (Reynolds et al., 2008). In contrast, macrophages and microglia isolated from α -synuclein transgenic mice, exhibited impaired cytokine release and phagocytosis *in vitro* and *ex vivo*; and activated monocytes from patients with idiopathic or familial PD produced lower levels of cytokines compared to healthy controls (Gardai et al., 2013). Conversely, α -synuclein^{-/-} microglia released elevated levels of pro-inflammatory cytokines upon stimulation (Austin et al., 2011). Collectively, these studies indicate that α -synuclein may inhibit macrophage and microglia activation.

The role of α -synuclein in the regulation of adaptive immune response is also unclear. α -synuclein expression has been detected by RT-PCR not only in human monocytes and NK cells, but also in B and T lymphocytes (Kim et al., 2004; Shin et al., 2000). It has been reported that PD patients had significantly higher levels of immunoglobulin G (IgG) binding to dopaminergic neurons, whereas IgM binding was not increased (Orr et al., 2005). Autoantibodies against α -synuclein have been detected in 65% of PD patients and their presence strongly correlated with inherited forms of the disease (Papachroni et al., 2007). These results suggest that α -synuclein may regulate both B and T cell mediated immune responses.

Based on the aforementioned studies, we set up to investigate the role of α -synuclein in hematopoiesis and the immune system by utilizing α -synuclein^{-/-} mice as a model. We report an unexpected role of α -synuclein in hematopoiesis, B cell lymphopoiesis and antigen-specific humoral immune response.

Materials and Methods

Mice

α -synuclein^{-/-} mice B6;129X1-Snca^{tm1Ros1} (stock 003692) were purchased from the Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched (8–10 week old) α -synuclein^{+/+} B6;129SF2/J WT mice were selected as controls as recommended (Geng et al., 2011). All animals were housed in pathogen-free conditions at room temperature with a standard light/night cycle, and fed a standard diet with water intake ad libitum. All animal protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Complete blood count

Blood samples (200 μ l) from 8-week old WT and KO mice were collected using heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). Samples were then analyzed using the Hemavet® 950 hematology analyzer according to the manufacturer's protocol (Drew Scientific Inc., Oxford, CT). A full differential for each blood sample was obtained containing absolute numbers and percentages of white blood cells: lymphocytes, monocytes, basophils, eosinophils, neutrophils; red blood cell parameters; and platelet counts including mean platelet volume.

Flow cytometry

The identification and enumeration of hematopoietic progenitors was performed as previously described (Passegue et al., 2004; Xiao et al., 2008). Briefly, to identify hematopoietic stem cells (HSC) and common lymphoid progenitors (CLP), bone marrow (BM) cells were stained with anti-mouse lineage panel (FITC), rat anti-mouse Sca-1 (D7, PerCP), rat anti-mouse c-kit (2B8, APC), and rat anti-mouse IL-7R α (A7R34, PE) (BioLegend, San Diego, CA). To identify the common myeloid progenitors (CMP), granulocyte/monocyte progenitors (GMP), and megakaryoerythroid progenitors (MEP), BM cells were stained with anti-mouse lineage panel (FITC), rat anti-Sca-1 (D7, FITC), rat anti-IL-7R α (A7R34, FITC), rat anti-mouse c-kit (2B8, APC), rat anti-mouse CD34 (HM34, PerCP/Cy5.5), and rat anti-mouse Fc γ R (93, PE) (BioLegend). The HSC phenotype was defined as Lin⁻IL-7R α ⁻Sca-1^{hi}c-Kit^{hi}; CLP as Lin⁻IL-7R α ⁺Sca-1^{int}c-Kit^{int}; CMP as Lin⁻IL-7R α ⁻Sca-1⁻CD34⁺Fc γ R^{lo}; GMP as Lin⁻IL-7R α ⁻Sca-1⁻CD34⁺Fc γ R^{hi}; and MEP as Lin⁻IL-7R α ⁻Sca-1⁻CD34⁻Fc γ R^{lo}. The identification of B cell subsets at different developmental stages was determined by flow cytometry as previously described (Allman et al., 2001; Dingjan et al., 2001; Miller et al., 2007). Briefly, single-cell suspensions were prepared from bone marrow (femurs and tibias), spleen, and lymph nodes; cells were then stained with the following antibodies obtained from eBioscience (San Diego, CA) or BioLegend: B220 (RA3-6B2, APC, FITC), IgD (11–26c, FITC), IgM (II/41, PE-Cy7),

CD93 (AA4.1, APC). Immunostaining was analyzed using the FACSCanto II flow cytometer (BD Biosciences, San Jose, CA).

Histology and immunohistochemistry

Tissue specimens were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm , and stained with hematoxylin and eosin (H&E). Expression levels of B220 were examined by immunohistochemistry (IHC). IHC was performed by the Immunohistochemistry Laboratory of University Hospitals Case Medical Center. Briefly, unstained 4 μm -sections of tissue microarrays were prepared from paraffin blocks and baked for 30 minutes at 60° C in a Boekel Lab oven (Boekel Scientific, Feasterville, PA). Slides were then processed using a BenchMark XT (Ventana) automated immunostainer (Ventana Medical Systems, AZ), deparaffinized, antigen retrieved with standard Cell Conditioning 1, a tris-based buffer pH 8.3 solution added for 30 minutes at 100 °C, followed by incubation at 37 °C with the primary antibody anti-B220 rat monoclonal RA3-6B2 (Abcam, Cambridge, MA) for 32 minutes and subsequently counterstained. Membrane immunoreactivity was considered as positive expression. Lymphoid follicles per field were enumerated at 40 $\times$ magnification. At least 5 fields were examined per mouse and the average number of follicles per field was compared.

Immunization regimen and ELISAs

Immunizations were performed as previously described (Dai et al., 2007). Briefly, mice were immunized via intra-peritoneal (i.p.) injection with 100 μg 4-hydroxy-3-nitrophenylacetyl (NP)(36)-chicken γ globulin (CGG)(Biosearch Technologies, Novato, CA) precipitated in alum (200 μl / mouse) (Pierce, Rockford, IL). Animals were bled prior to immunization (day 0) and on days 11 and 21 post-immunization. Total IgM and IgG levels were measured using mouse IgG or IgM total ELISA Ready-SET-Go![®] ELISA Kit according to the manufacturer's protocol (eBioscience, San Diego, CA). NP-specific ELISAs were done as previously described (Rankin et al., 2011). Briefly, plates were coated overnight with 20 $\mu\text{g}/\text{ml}$ NP (18)-BSA (Biosearch Technologies). The following day, serum samples were added, and bound antibody was detected using HRP-conjugated goat antibodies directed against mouse IgM, IgG1, or IgG2b (Southern Biotechnology Associates, Birmingham, AL). Plates were read using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). A standard curve was used to verify that all the readouts were within linear range.

Statistical analysis

All statistics were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA). Results are presented as mean $\pm$ SD. The intergroup data comparisons were performed using the unpaired Student *t* test. All reported *p* values are two-tailed with a type I error rate of 5% and a *p*<0.05 set for significance.

Results

Mild anemia, smaller platelets and lymphopenia in α -synuclein^{-/-} mice

To investigate the effects of α -synuclein on hematopoiesis, peripheral blood was collected for automated complete blood cell count analysis. As shown in Table 1, α -synuclein^{-/-} mice had mild anemia as indicated by lower RBC count (16% decrease, $p=0.0003$), hemoglobin level (14% decrease, $p=0.0005$) and hematocrit (17% decrease, $p=0.0002$) compared to WT mice. All other RBC indices were not statistically different between WT and KO mice (data not shown). Although platelets quantitatively were comparable between WT and α -synuclein^{-/-} mice, mean platelet volume (MPV) of KO platelets was significantly reduced compared to WT mice ($p=0.015$). More interestingly, absolute number of lymphocytes was significantly reduced in KO blood ($p=0.04$).

To determine if the anemia, morphological changes of platelets and lymphopenia translate into more extensive hematologic abnormalities, we enumerated the populations of HSCs, CMPs, CLPs, GMPs, and MEPs as previously described (Passegue et al., 2004; Xiao et al., 2008). This detailed analysis failed to reveal any significant differences among those populations between WT and KO mice (data not shown), suggesting that the hematologic abnormalities present in KO mice take place later in bone marrow hematopoiesis and/or during peripheral maturation.

B cell lymphopoiesis defects in α -synuclein^{-/-} mice

We next investigated the effects of α -synuclein on lymphopoiesis and we report our findings on B cell lymphopoiesis. Bone marrow cells from 8-week-old WT and α -synuclein^{-/-} mice were harvested and analyzed by flow cytometry. B cell maturation was examined at developmental stages of immature and mature B cells. As shown in Figure 1A and B, the absolute number of B220⁺IgM⁺ B cells was reduced by 4 fold in KO mice (WT: $104 \pm 23 \times 10^5$ vs. KO: $27 \pm 5 \times 10^5$, $p=0.005$). When anti-IgD and anti-AA4.1 were applied to separate B220⁺IgM⁺ B population into AA4.1⁺IgD⁻, AA4.1⁺IgD⁺, and AA4.1⁻IgD⁺ subsets, the absolute B cell number in all three populations was also significantly decreased ($p=0.017$, $p=0.01$, $p=0.005$ respectively).

We extended our analysis to spleen and lymph nodes to determine if B cell development is affected in peripheral lymphoid organs. B cell populations were subdivided into AA4.1⁺ immature B cells and AA4.1⁻ mature B cells. Interestingly, the absolute number of splenocytes in KO mice was only 50% of that in WT mice (WT: $95 \pm 21 \times 10^6$ vs. KO: $48 \pm 11 \times 10^6$, $p=0.02$). The percentage of AA4.1⁺ immature B cells and AA4.1⁻ mature B cells was comparable between WT and KO spleen (Figure 2A). The development of transitional B cells (T1 and T2), marginal zone B (MZB) cells and mature follicular B cells appeared to be mostly intact, although the absolute number of B cells at each developmental stage was significantly reduced in KO spleen compared to WT (Figure 2B). In contrast, the absolute number of total cells, the percentage and absolute number of B cells in KO lymph nodes (axillary) were not significantly different from WT mice (data not shown).

Abnormal architecture of spleen and lymph nodes from α -synuclein^{-/-} mice

Histologically, splenic white pulp areas were disorganized in KO mice compared to WT counterparts (Figure 3A). WT lymph nodes exhibited normal lymph node architecture with numerous lymphoid follicles in the cortex separated by inter-follicular areas, but this architecture was completely ablated in KO mice (Figure 3B). To further analyze the architectural findings in spleen and lymph nodes, immunohistochemical studies with anti-B220 showed distinct B cell zones in WT spleens which were disrupted in KO spleens (Figure 3C). Although the number of follicles in spleen was not different between WT and KO mice, the size of KO follicles was significantly smaller (Figure 3C and 3D). Compared to WT lymph nodes, B cell distribution in KO lymph nodes was abnormal and random, and a distinct lymphoid follicle was rarely present. Along these lines, the number of follicles in KO mice was 80% lower than in WT mice (Figure 3E; $p=0.001$). Consistent with normal B cell numbers in KO lymph nodes detected by flow cytometric analysis, the total number of B220+ cells in KO lymph nodes did not appear significantly different from that in WT mice, suggesting that B cell localization in lymph nodes might be affected by α -synuclein. Interestingly, B cells uniformly had lower B220 immunohistochemical staining intensity in KO mice although the mean fluorescent intensity of B220 by flow cytometry was comparable between WT and KO B cells (Figure 3C and Figure 2A).

Defects of B cell mediated immune response in α -synuclein^{-/-} mice

To establish whether the abnormal distribution and reduction of mature B cells in α -synuclein^{-/-} mice translate into a functional deficiency, total serum immunoglobulins (Igs) from 8–10 week-old WT and KO mice were assessed. As shown in Figure 4A–B, total serum IgG concentration was 182 ± 23 μ g/ml in WT mice and 62 ± 26 μ g/ml in KO mice ($N=10$ each group, $p<0.0001$). In contrast, total serum IgM concentration was not different between WT and KO mice (203 ± 24 μ g/ml vs. 199 ± 17 μ g/ml, respectively; $p=0.7$). Since follicles are essential for T cell-dependent B cell immune responses, including IgG isotype formation, the loss of follicular architecture in KO lymphoid organs coupled with loss of serum IgG suggested the possibility of deficiency in T cell-dependent B cell responses in KO mice. To test this hypothesis, mice were injected i.p. with a T cell-dependent antigen 4-hydroxy-3-nitrophenylacetyl (NP)(36)-chicken γ globulin (NP36-CGG) and NP-antigen-specific immunoglobulin serum levels were measured on days 0 (before immunization), 11 and 21. In WT mice, the production of NP specific IgG isotypes, IgG2b and IgG1 were induced as expected after antigenic challenge, but production of both NP specific IgG isotypes was nearly abrogated in KO mice (Figure 4C–D and Table 2). While NP specific IgM production was induced after antigenic challenge, no difference was noted between WT and KO mice (Figure 4E and Table 2).

Discussion

This is the first study, to our knowledge, to show the importance of α -synuclein in hematopoiesis and immune responses manifested by mild anemia, reduced MPV, reduced B cell lymphopoiesis and defects in IgG production. Even though early stages of hematopoiesis appeared largely unaffected by α -synuclein deficiency, our data suggest that α -synuclein may have a more profound effect at relatively later stages (including peripheral

maturation) in hematopoiesis/ megakaryo-erythropoiesis and lymphopoiesis. It has been shown that the number of platelets is inversely correlated with MPV as younger platelets with large MPV are generated in the presence of thrombocytopenia (Corash et al., 1987; Giles, 1981; Ulich et al., 1996). Although we found that MPV was reduced in α -synuclein^{-/-} mice, platelet counts were not different from WT, suggesting that other factors contribute to the reduction in MPV. In this regard, it will be worthwhile to compare the ultra-structural differences between platelets of WT and KO mice.

Likewise, though a previous study showed that erythrocytes secrete α -synuclein (Barbour et al., 2008), the potential mechanisms mediating anemia in α -synuclein^{-/-} mice are unclear but it may involve a reduction in cell lifespan since most KO red cell indices were not different from WT. Further research is needed to address these observations.

The existence of three synuclein isoforms (α -, β -, and γ -synuclein), in many cases expressed by the same cells (George, 2002), raises the possibility that redundancy in expression may account for the normal early hematopoiesis in α -synuclein^{-/-} mice. Expression of all synucleins appears to be vital to murine survival since triple (α -, β -, and γ -synuclein) KO mice show a more severe neurological phenotype than α -synuclein^{-/-} mice and have a significantly shorter lifespan (Gretchen-Harrison et al., 2010). Since β -synuclein expression disappears as α -synuclein begins in megakaryocytic lineage maturation (Hashimoto et al., 1997), and no defect in hematopoiesis at the level of HSCs, early lymphoid progenitors and myeloid progenitors was found in α -synuclein^{-/-} mice, it remains to be determined if β -synuclein expression compensates for α -synuclein deficiency that results in normal early hematopoiesis in α -synuclein^{-/-} mice.

B cells are generated from HSCs via sequential differentiation steps including CLPs, pre-pro-B cells, pro-B cells, and pre-B cells in the bone marrow and enter the circulation as immature, surface IgM-expressing cells (Ramirez et al., 2010). These immature B cells migrate to the spleen where they progress through stages of immature transitional B cell differentiation leading to the development of mature naïve B cells (Bryder and Sigvardsson, 2010; Herzog et al., 2009; Zandi et al., 2010). Our data suggest that B cell number and distribution was dependent on α -synuclein, implicating that α -synuclein may regulate B cell maturation, survival and/or migration. The follicular architecture in lymph node is totally effaced in the absence of α -synuclein. Although lymphoid follicles in spleen are present and comparable in numbers to WT, their size is drastically reduced in KO mice. Therefore, α -synuclein might also be involved in maintaining lymphoid follicles. The lower total IgG concentration and lack of IgG isotype responses to immunization with a T cell-dependent antigen implicate impairment in class switching (Zandi et al., 2010), which requires CD40/CD40L interaction and cytokines from T helper cells (Durandy and Honjo, 2001; Crotty, 2011). In this regard, it remains to be determined if the phenotype caused by α -synuclein deficiency extends to T-helper cells or their function as seen in several mouse models presenting with increased or normal IgM expression (Durandy et al., 2005; Lougaris et al., 2005). A normal IgM level with reduced total B cells in KO mice could be explained by relatively normal number of IgM producing plasma cells in KO mice. Further, our findings in murine B cell development and humoral immune responses need to be assessed in humans and tested in human- α -synuclein transgenic mice or in PD patients.

The B cell phenotype in KO mice is likely caused by a combination of T helper cell and intrinsic B cell defects (Durandy et al., 2005; Lougaris et al., 2005). The number of CD4 T cells and expression of several activation markers are reduced in thymus and spleen from KO mice (manuscript in preparation, A.S., W.X., R.W.M), implicating a potential role of CD4⁺ T helper cells in the observed phenotype. Alternatively, a cytokine-mediated extrinsic mechanism could also lead to the B cell maturation arrest as shown in *SHIP*^{-/-}, *Lyn/HCK*^{-/-}, and *SHP-1*^{-/-} mice (Nakamura et al., 2004; Xiao et al., 2009). Of interest, erythrocytes could secrete α -synuclein into plasma (Barbour et al., 2008), but there is no information detailing the effect of secreted α -synuclein on immune cells including B cells. In these α -synuclein^{-/-} deficient mice such secretion would not occur and therefore any effect on immune cells is abrogated.

The molecular mechanism of α -synuclein regulation in immune cells is not known. It has been shown that α -synuclein functions as a chaperone of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and mediates the rapid assembly and disassembly of the membrane fusion machinery needed to release neurotransmitters at high frequency in the brain (Bendor et al., 2013; Diao et al., 2013). In this regard, the hydrophilic C terminus of α -synuclein appears to interact with SNARE synaptobrevin-2 and promotes SNARE assembly (Burre et al., 2013), and when α -synuclein oligomerizes it prevents SNARE complex formation via the same synaptobrevin-2 (Choi et al., 2013). By interacting with the SNARE complex, α -synuclein exerts inhibitory effects on neuronal exocytosis, and this inhibitory function is abolished with the A30P α -synuclein mutant found in some hereditary PD patients (Nemani et al., 2010). A similar membrane fusion machinery involving SNARE is also required for granule exocytosis from T cells and NK cells (Chiang et al., 2013; Kasai et al., 2012). In T cells, targeting of the T cell antigen receptor to the immunological synapse is dependent on the SNARE complex (Das et al., 2004); and release of lytic granules from cytotoxic T cells requires SNARE activity (Pattu et al., 2012). In this regard, production of cytokines in T cells could be regulated by α -synuclein. Likewise, in the B cell lineage, it has been shown that another SNARE protein member, SNAP-23 is essential in plasma cells for antibody production and secretion (Reales et al., 2005). Therefore, it is reasonable to hypothesize that α -synuclein might also participate in SNARE-related membrane fusion in B cells and be essential to B cell biology. In regards to erythrocytes, it remains to be determined if SNARE proteins/ complex have a role in these cells or if it can explain the observed anemia which as mentioned above may involve a shorter erythrocyte lifespan. Nevertheless, platelets depend on an intact SNARE complex for function, normal hemostasis and granular release (Ren et al., 2010). Further research to establish the molecular mechanisms by which α -synuclein mediates the observed phenotypes is needed.

In conclusion, we demonstrate that α -synuclein is essential to likely later stages of hematopoiesis, B cell lymphopoiesis, and IgG production. Since murine α -synuclein differs only in 6 amino acids (no difference in amino terminus) from the human homolog (96% identity) (George, 2002), these murine studies may have implications to establish the role of this protein in human hematopoiesis and B cell mediated immune responses.

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Figure 1.

B cell development in bone marrow. Bone marrow cells were harvested and stained with indicated antibodies. Live cells were gated for flow cytometric analysis based on forward scatter and side scatter. A. Anti-B220 and anti-IgM antibodies were utilized to gate B220⁺IgM⁺ B cells that were further separated into three populations: AA4.1⁺IgD⁻, AA4.1⁺IgD⁺, and AA4.1⁻IgD⁺ B cells. B. Absolute counts of B cell subsets in bone marrow. Plots are representative of 10 mice per group from five independent experiments.

Figure 2.

Flow cytometric analysis of B cells in spleen. Single cell suspension was obtained from spleen, stained with indicated antibodies, and live cells were gated for flow cytometric analysis based on forward scatter and side scatter. A. Live splenocytes were gated by flow cytometry and analyzed with anti-B220 and anti-AA4.1. B220⁺ AA4.1⁺ and B220⁺ AA4.1⁻ subsets were further separated into IgM^{hi} and IgD^{hi} expressing populations. The IgM⁻IgD⁻ populations were reported previously (Miller et al., 2007). B. Absolute counts of B cells

subsets in spleen. Results are representative of five independent experiments (N=10 mice/group).

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Figure 3.

Histological analysis of B cells in spleen and axillary lymph nodes. A. Histology of spleen was examined by H&E staining at 20× and 100 × magnifications for WT and KO mice. B. Histology of axillary lymph nodes was examined by H&E staining at 20× and 100× magnifications. Dashed lines highlight follicles. C. B cell density and distribution in spleen and axillary lymph nodes were evaluated by immunohistochemical staining with anti-B220. Images are presented at 40× magnification. D–E. The follicle counts of WT and KO spleen (D) and axillary lymph nodes (E) per high power field (×40). Results are representative of five independent experiments (N=10 mice/group). At least 5 high power fields were counted per mouse.

Figure 4.

Immunoglobulin production in vivo. A–B. Total serum IgG (A) and IgM (B) levels of KO compared to WT mice (N=10 mice/group, * $p < 0.0001$). C–E. NP-specific IgG1, IgG2a and IgM concentration after antigenic challenge prior to immunization (day 0), day 11 and 21 post-immunization (N=4 mice/group, * $p < 0.0001$). Each line represents a mouse (solid: WT, dashed: KO).

Table 1Summary of complete blood cell count in α -synuclein^{-/-} mice

	WT (N=10)	KO (N=10)	p value
White blood cells ($\times 10^9/L$)	4.7 $\pm$ 1.2	4.0 $\pm$ 1.5	0.32
Neutrophils (%)	14 $\pm$ 6	20 $\pm$ 5	0.03
Neutrophils ($\times 10^9/L$)	0.7 $\pm$ 0.3	0.8 $\pm$ 0.3	0.31
Lymphocytes (%)	80 $\pm$ 6	72 $\pm$ 4	0.001
Lymphocytes ($\times 10^9/L$)	3.8 $\pm$ 0.7	2.9 $\pm$ 0.6	0.04
Red blood cells ($\times 10^{12}/L$)	10.8 $\pm$ 0.9	9.1 $\pm$ 0.8	0.0003
Hemoglobin (g/dL)	16.2 $\pm$ 1.2	14 $\pm$ 0.6	0.0005
Hematocrit (%)	57.8 $\pm$ 4.9	47.8 $\pm$ 4.6	0.0002
Platelets ($\times 10^9/L$)	617 $\pm$ 57	648 $\pm$ 86	0.35
Mean platelet volume (fL)	4.2 $\pm$ 0.3	3.9 $\pm$ 0.1	0.015

Note: Peripheral blood was obtained by retro-orbital bleeding

Table 2

Production of serum immunoglobulins in α -synuclein^{-/-} mice after immunization with NP36-CGG

	IgG1			IgG2b			IgM		
	D0	D11	D21	D0	D11	D21	D0	D11	D21
WT (N=4)	0.39±0.22	2.91±0.12	2.92±0.14	0.33±0.07	2.93±0.05	2.98±0.08	0.97±0.36	2.92±0.03	2.86±0.16
KO (N=4)	0.20±0.06	0.32±0.24	0.42±0.16	0.24±0.02	0.29±0.14	0.38±0.30	0.98±0.33	2.93±0.01	2.85±0.07
p value	0.15	<0.0001	<0.0001	0.05	<0.0001	<0.0001	0.54	0.59	0.86