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## Ultrastructural changes in peripheral blood leukocytes in $\alpha$ -synuclein knockout mice

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### Abstract

Effects of  $\alpha$ -synuclein deficiency on cellular blood components have not been extensively investigated. This study evaluated ultrastructural changes of leukocytes in  $\alpha$ -synuclein knockout (KO) mice using electron microscopy (EM). The following ultrastructural characteristics were quantified in leukocytes: mitochondria, primary granules, specific granules (SG), Golgi apparatus (GA), inclusions, rough-endoplasmic reticulum (RER), smooth-endoplasmic reticulum (SER), and cellular projections (CP). EM showed increased numbers or amounts of SG, inclusions, and SER in KO group ( $5.3 \pm 4.5$  in WT vs.  $14.1 \pm 10.3$  in KO,  $p=0.02$ ;  $0.4 \pm 0.9$  in WT vs.  $3.2 \pm 2.8$  in KO,  $p=0.007$ ; and  $7.7 \pm 6.7$  in WT vs.  $17.7 \pm 12.2$  in KO,  $p=0.03$ , respectively). Although CP number was not significantly different between the two groups ( $13.4 \pm 5.3$  in WT vs.  $16.3 \pm 7.5$  in KO,  $p=0.32$ ), their size and shapes were altered in KO mice. Notably, findings occurred in the setting of significant lymphopenia.  $\alpha$ -synuclein deficiency leads to changes in size and shape of secretory particles and increases in SER, SG, and inclusions, indicating a potential role for  $\alpha$ -synuclein in vesicular trafficking in leukocytes. Further studies are needed to elucidate functions mediated by  $\alpha$ -synuclein.

### Keywords

$\alpha$ -synuclein; leukocytes; morphology; ultrastructure

### Introduction

$\alpha$ -synuclein is a small protein (14 kDa) that is important to normal brain function and in certain conditions undergoes aberrant protein conformational changes that lead to the pathologic findings seen in Lewy bodies characteristic of Parkinson's disease [1]. The function of this molecule in the central nervous system still remains to be fully elucidated; but it appears to play important roles in promoting the health of the presynaptic terminal,

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neurotransmitter release in response to stimuli, and neuronal plasticity [2]. It can also have inhibitory roles, as seen in dopaminergic neurotransmitter release [3, 4]. This protein is co-localized at or near the plasma membrane and cytoplasmic membranous structures, and this localization may mediate its neuronal functional roles.

It has also become evident that this protein is also well expressed in the hematopoietic system, but its functions in this system remain obscure. Reports have indicated that  $\alpha$ -synuclein expression is highest in megakaryocytes, platelets, red blood cells (RBC), and erythroid precursors [5, 6].  $\alpha$ -synuclein expression has also been reported to be a specific marker of neoplastic changes in the megakaryocytic lineage [7]. Platelets have been shown to have the highest concentration of  $\alpha$ -synuclein per milligram of cellular protein in peripheral blood [8], and time-dependent  $\alpha$ -synuclein concentration increases in plasma supernatant of stored platelets may be indicative of changes that platelets undergo while in storage [9]. Importantly,  $\alpha$ -synuclein in platelets has been shown to inhibit  $\alpha$ -granule release in a calcium dependent manner [10]. In addition,  $\alpha$ -synuclein deficiency leads to production of small circulating platelets and significant anemia [11].

In leukocytes, the function of  $\alpha$ -synuclein appears to be critical since its deficiency leads to severe paucity in the number of mature B and T lymphocytes, lack of immunoglobulin class switching in response to stimuli (with a resulting absence of IgG), and abnormal development of T helper and T regulatory cells [11, 12]. In light of these observations that  $\alpha$ -synuclein leads to quantitative and functional impairment of lymphocytes, we proceeded to look at the ultrastructural effects of  $\alpha$ -synuclein deficiency on leukocytes by electron microscopy analysis of peripheral blood samples from  $\alpha$ -synuclein knockout (KO) mice.

## Methods

### Mice

$\alpha$ -synuclein<sup>-/-</sup> mice B6;129X1-Snca<sup>tm1Ros1</sup> (stock 003692) (KO), and age- and sex-matched (8–10 week old)  $\alpha$ -synuclein<sup>+/+</sup> B6;129SF2/J wild type (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME) [11]. Mice were housed in pathogen-free conditions at room temperature with standard night/light cycle, standard diet and water ad libitum. Animal protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

### Blood sampling and CBC

Mouse blood samples (200  $\mu$ L) were collected from the tail vein in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) and used for peripheral blood smears, manual blood counts, EM, and automated complete blood counts (CBC). The latter was performed as previously described [11].

### Transmission electron microscopy

Preparation of cells for electron microscopy was done as previously described [13]. Blood samples from five WT and five KO mice were fixed by immersion in quarter-strength Karnovsky fixative solution for 2 h at room temperature. Specimens were washed and post-

fixed for 2 additional hours in an unbuffered 1:1 mixture of 2% osmium tetroxide and 3% potassium ferrocyanide. Specimens were subsequently rinsed with distilled water followed by immersion overnight in an acidified solution of 0.25% uranyl acetate. Specimens were washed a second time in distilled water, dehydrated in ascending concentrations of ethanol, passed through propylene oxide, and embedded in a Poly/Bed 812 embedding media (Polysciences, Warrington, PA). Thin sections were obtained using a RMC MT6000-XL ultramicrotome (Boeckeler Instruments, Inc., Tucson, AZ). Sections were mounted on Gilder square 300 mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA) followed by staining with acidified methanolic uranyl acetate and stable lead staining solution. Sections were subsequently coated on a Denton DV-401 carbon coater (Denton Vacuum LLC, Moorestown, NJ), and analyzed in a JEOL 1200EX electron microscope (JEOL Ltd., Tokyo, Japan). The following morphological and ultrastructural characteristics were recorded from all leukocyte subsets [14–16]: cell diameter, cell perimeter, number of mitochondria, primary granules (PG), specific granules (SG), Golgi apparatus, inclusions, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and cellular projections (CP). Separately, peripheral blood smears were obtained and used to derive manual 100-cell differential leukocyte count using light microscopy. Transmission electron microscopy and light microscopy analyses were performed by two pathologists independently.

### Cell measurements

Photoshop CC version 19.1.1 (Adobe Systems, San Jose, CA) was used to obtain measurements. Cell diameter and perimeter were obtained by using the select tool that was customized to micrometer units using the scale bar in the EM images.

### Statistical analysis

All statistics were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA). Results are presented as mean  $\pm$  SD. Intergroup data comparisons were performed using Mann Whitney test and unpaired *t* test. Statistical significance was set at  $p < 0.05$ .

## Results

### Cell differential

Manual 100-cell differential count showed relative lymphopenia ( $88.4 \pm 7.9\%$  in WT vs.  $69.2 \pm 13.5\%$  in KO,  $p=0.03$ ) and neutrophilia ( $5.0 \pm 3.6\%$  in WT vs.  $21.2 \pm 8.0\%$  in KO,  $p=0.006$ ) in KO mice compared to WT controls (Table 1). These data correlated with automated CBC results and those previously published in this mouse strain [11]. KO mice had a marked absolute lymphopenia ( $3.8 \pm 1.0 \times 10^9/L$  in WT vs.  $2.9 \pm 1.0 \times 10^9/L$  in KO,  $p=0.029$ ) and a relative percent neutrophilia, the latter likely secondary to the reduced number of lymphocytes since the absolute neutrophil counts were similar between KO and WT mice. Furthermore, KO animals appeared to have a normocytic anemia phenotype since RBC, hemoglobin (Hgb) and hematocrit (Hct) were lower in KO mice compared to WT ( $9.1 \pm 0.8 \times 10^{12}/L$  vs.  $10.8 \pm 0.8 \times 10^{12}/L$ ,  $p < 0.0001$ ;  $14.3 \pm 0.7$  g/dL vs.  $16.1 \pm 1.2$  g/dL,  $p=0.0005$ ; and  $47.8 \pm 4.4\%$  vs.  $57.8 \pm 4.9\%$ ,  $p < 0.0001$  respectively) in the context of normal mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean

corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) (Table 1B). There were no statistical differences in monocyte counts between the two mouse groups while basophils and eosinophils could not be detected in the majority of animals from both groups (mean of 0 and SD 0, data not shown).

### Cell ultrastructure

Transmission electron microscopy comparison of the leukocytes of WT and KO mice indicated that the latter had marked increases in the number of SG, inclusions and SER compared to the former ( $5.3 \pm 4.5$  in WT vs.  $14.1 \pm 10.3$  in KO,  $p=0.02$ ;  $0.4 \pm 0.9$  in WT vs.  $3.2 \pm 2.8$  in KO,  $p=0.007$ ; and  $7.7 \pm 6.7$  in WT vs.  $17.7 \pm 12.2$  in KO,  $p=0.03$ , respectively) (Table 2). Analysis of the number of CPs between the two groups showed no significant difference between leukocytes of WT and KO mice ( $13.4 \pm 5.3$  in WT vs.  $16.3 \pm 7.5$  in KO,  $p=0.32$ ). Of note, sizes and shapes of CPs were altered in KO mice (Figure 1). No differences were noted in other organelles between the two groups (data not shown).

### Cell dimensions

Cells from KO and WT mice were measured to determine changes in dimensions caused by  $\alpha$ -synuclein deficiency. KO mice leukocytes had a >14% larger diameter compared to WT leukocytes ( $6.8 \pm 1.0$  vs.  $5.9 \pm 0.8$  respectively,  $p=0.0284$ ) and 10% larger perimeter ( $27.2 \pm 4.5$  vs.  $24.4 \pm 2.3$  respectively,  $p=0.0465$ ) (Table 2).

### Effects of $\alpha$ -synuclein deficiency

To put into context morphological findings, prior results indicating important roles of  $\alpha$ -synuclein from our group and recently from others as well are presented in Table 3. Both B and T cells were markedly reduced in KO mice compared to WT and in particular both mature B cells (B220<sup>hi</sup>) and mature T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) which correlate with the observed absolute lymphopenia. More importantly, lymphocyte reduction occurred in the background of abnormal histological architecture of spleen, thymus and lymph nodes which were also small in size compared to controls. Stimulation of B cells indicated that KO mice were able to mount IgM responses regardless of antigen presented (T-independent or T-dependent) but were unable to class switch upon antigenic challenge with poor to absent IgG responses. In T cells,  $\alpha$ -synuclein deficiency led to increases in numbers of double negative and double positive thymocytes which do not appear to reach full maturation. In addition, the small number of T cells that reach maturity do it in an overactive state since they have higher expression of early activation markers (CD49d, CD69). Activation of CD4<sup>+</sup> cells, favor a Th1 phenotype since Th2 responses are defective as indicated by minimal IL-4 production and markedly increased IL-2. Similarly, T regulatory cells are also decreased in KO mice. In regard to RBC, we showed that KO mice have anemia at baseline and others since have indicated that this is due to the requirement of  $\alpha$ -synuclein expression to allow for enucleation of erythroblasts and to maintain RBC plasma membrane stability. Along the same line, iron deficiency occurs when  $\alpha$ -synuclein is absent. Finally, absence of  $\alpha$ -synuclein leads to small platelets and to hypercoagulability.

## Discussion

$\alpha$ -synuclein is best characterized with regard to its roles in the central nervous system, which can lead to pathophysiological findings as seen in Parkinson's disease when structural changes or misfolding of the protein occur.  $\alpha$ -synuclein is also essential to late stages of hematopoiesis and especially adaptive immunity as shown by the marked reduction in mature B and T cells in  $\alpha$ -synuclein KO mice [11, 12]. Our results complement prior findings from our group indicating that leukocytes in the absence of  $\alpha$ -synuclein undergo morphological changes including alterations in cell size, and the ultrastructural distribution and number of intracellular organelles.

In regard to leukocytes, deficiency of this protein leads to maturation arrest of lymphocytes and this is likely at the core of the abnormal architecture seen in lymphoid organs. We observed that leukocytes from KO mice were larger in size compared to WT. This is an observation that has been reported in microglia from  $\alpha$ -synuclein KO mice, which are significantly larger and have a greater number of cytoplasmic vacuoles [17]. This larger microglial size is associated with decreased phagocytic activity, elevated cellular prostaglandin levels, and accumulation of multiple microglial enzymes needed for responses to signaling events [18]. Since  $\alpha$ -synuclein is closely linked to cellular membranes, including granules, these prior results and our current data suggest its absence may lead to accumulation of material that under normal physiologic conditions is secreted to the extracellular space resulting in larger cells. Likewise, its close plasma membrane and nuclear membrane association hint at one of its functions in RBC since KO mice erythroblasts fail to enucleate leading to impaired RBC maturation and decreased membrane stability [19]. Taken together, these results seem to indicate that of  $\alpha$ -synuclein may be important to proper cellular function.

The hallmark of Parkinson's disease and Lewy body dementia is the formation of cytoplasmic inclusions called Lewy bodies, which are mainly aggregates of misfolded  $\alpha$ -synuclein protein, and their accumulation in the neuronal endoplasmic reticulum results in activation of the unfolded protein response [20]. This response helps to resolve protein misfolding or trigger apoptosis in cases of chronic stress [21]. Our results suggest that lack of  $\alpha$ -synuclein leads to a significant increase in cytoplasmic inclusions in leukocytes. Furthermore, leukocytes from the KO group had an increase in SER and SG.  $\alpha$ -synuclein could potentially modulate vesicle/SG trafficking in leukocytes, and the observed inclusions may represent SG that failed to be secreted. Future research in this area will help elucidate these potential mechanisms.

Since  $\alpha$ -synuclein is essential to normal physiological intracellular trafficking, the arrest of lymphocyte development in  $\alpha$ -synuclein KO mice may be caused by a lack of receptor delivery to the cell surface. This is similar to the case seen in the synapse, where absence of  $\alpha$ -synuclein leads to dysregulated vesicle trafficking [22]. This  $\alpha$ -synuclein function may be mediated by its close relationship with membranes and ability to closely associate with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes while promoting their assembly [23]. In T cells, SNARE complex activity is essential to TCR localization to the immunological synapse [24, 25], and SNARE-mediated membrane

fusion is required for granule exocytosis and release from cytotoxic T cells and NK cells [26]. Additionally, in T cells  $\alpha$ -synuclein-deficiency can lead to enhanced localization of TCR to immunological synapses on developing thymocytes [27], resulting in higher affinity interaction and enhanced negative selection of cells during maturation. Similarly, in the B cell lineage, another SNARE protein member, SNAP-23, is essential in plasma cells for antibody production and secretion [28]; and through protein interactions with such SNAREs  $\alpha$ -synuclein may also participate in SNARE-related membrane fusion in B cells and be essential to B cell biology.

In conclusion, our findings suggest that absence of  $\alpha$ -synuclein is associated with ultrastructural changes in leukocytes, including alterations in cell size and the shape of secretory particles, and increases in amount or number of SER, SG, and inclusions. These morphological findings correlate with quantitative and qualitative defects seen in lymphocytes of KO mice. Further studies are needed to elucidate physiological mechanisms mediated by  $\alpha$ -synuclein, and their effects on different leukocyte populations.

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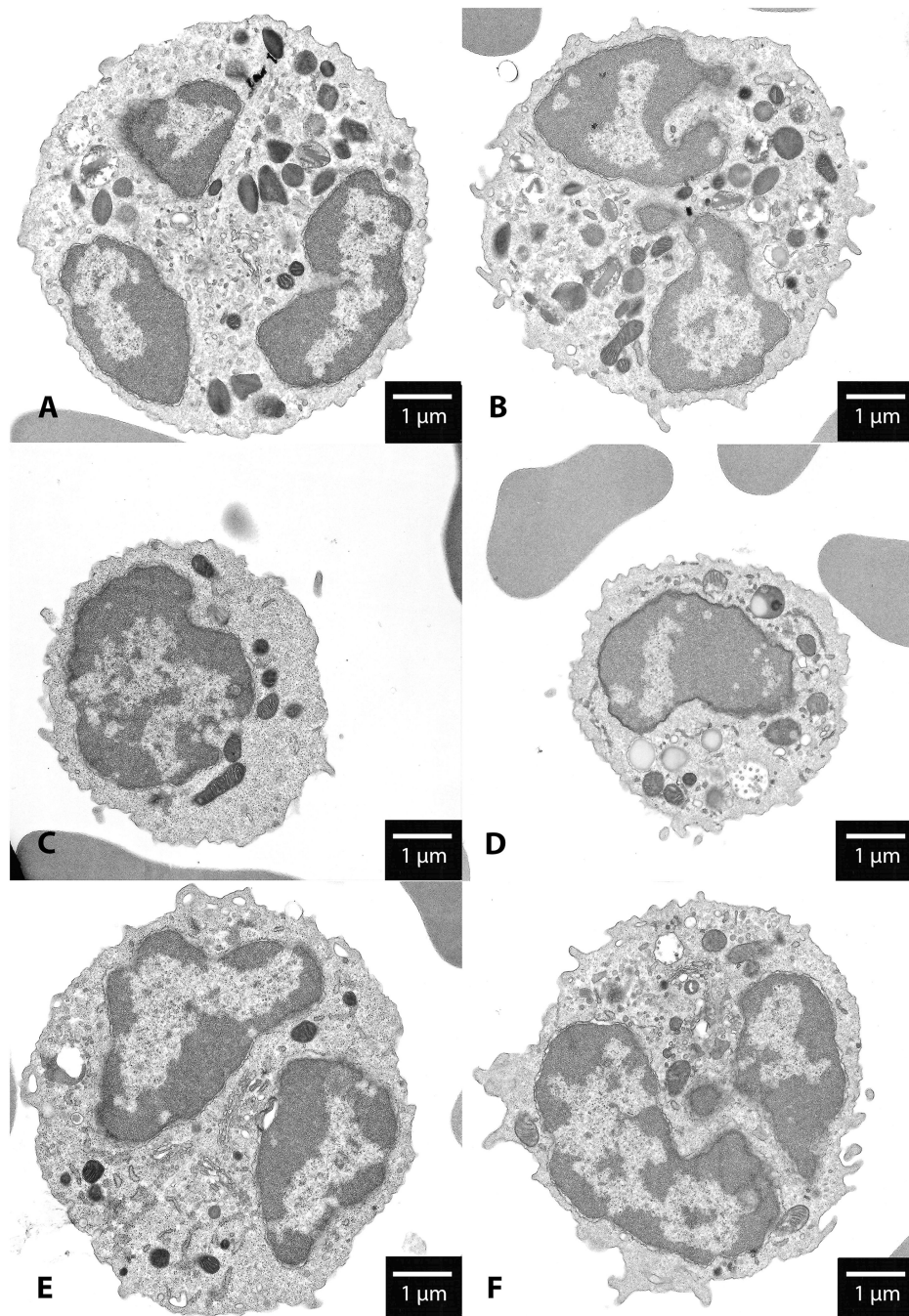
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**Figure 1.** Morphologic changes of leukocytes in  $\alpha$ -synuclein knockout mice by transmission electron microscopy. A, C, and E are representative images of eosinophil, monocyte, and neutrophil respectively in WT mice in the context of surrounding erythrocytes. B, D, and F are representative images of eosinophil, monocyte, and neutrophil respectively in KO mice. Scale bars correspond to 1  $\mu$ m.

**Table 1.**

Cell differentials of peripheral blood samples from KO mice and age-matched WT controls.

<b>A. Manual differential using peripheral blood smears</b>			
	<b>WT</b>	<b>KO</b>	<b>p value</b>
Mean lymph %	88.4 ± 7.9	69.2 ± 13.5	<b>0.03</b>
Eosinophil %	0.6 ± 1.2	0.8 ± 0.75	0.8
Neutrophil %	5.0 ± 3.6	21.2 ± 8.0	<b>0.006</b>
Monocytes %	8.4 ± 3.8	8.8 ± 5.7	0.44
Basophil %	0	0	
Band %	0	0	

<b>B. Automated cell blood count</b>			
	<b>WT</b>	<b>KO</b>	<b>p value</b>
White blood cells ( $\times 10^9/L$ )	4.7 ± 1.2	4.0 ± 1.4	0.210
Neutrophils (%)	14.7 ± 5.4	19.3 ± 5.0	<b>0.026</b>
Neutrophils ( $\times 10^9/L$ )	0.7 ± 0.3	0.8 ± 0.3	0.525
Lymphocytes (%)	80.0 ± 5.7	72.8 ± 4.1	<b>0.003</b>
Lymphocytes ( $\times 10^9/L$ )	3.8 ± 1.0	2.9 ± 1.0	<b>0.029</b>
Monocytes (%)	5.0 ± 1.2	7.3 ± 2.9	<b>0.030</b>
Monocytes ( $\times 10^9/L$ )	0.2 ± 0.1	0.3 ± 0.2	0.325
Erythrocytes ( $\times 10^{12}/L$ )	10.8 ± 0.8	9.1 ± 0.8	<b>&lt;0.0001</b>
Hgb (g/dL)	16.1 ± 1.2	14.3 ± 0.7	<b>0.0005</b>
Hct (%)	57.8 ± 4.9	47.8 ± 4.4	<b>&lt;0.0001</b>
MCV (fL)	53.4 ± 3.0	52.5 ± 1.5	0.354
MCH (pg)	15.0 ± 1.2	15.7 ± 1.6	0.210
MCHC (g/dL)	28.0 ± 1.9	30.2 ± 3.3	0.083
RDW (%)	17.6 ± 1.1	17.1 ± 0.6	0.234
Platelets ( $\times 10^9/L$ )	624.5 ± 59.3	660.9 ± 91.4	0.282
Mean Platelet Volume (fL)	4.1 ± 0.3	3.9 ± 0.1	<b>0.017</b>

Based on 100 cell counts of smears from 5 mice/ group (Mean ± SD)

Based on counts from 11 mice/group (Mean ± SD)

**Table 2.**

Ultrastructural comparisons between leukocytes from WT and KO mice

	WT	KO	<i>p</i> value
Number of secondary granules	5.3 ± 4.5	14.1 ± 10.3	<b>0.02</b>
Number of inclusions	0.4 ± 0.9	3.2 ± 2.8	<b>0.007</b>
Number of SER	7.7 ± 6.7	17.7 ± 12.2	<b>0.03</b>
Number of cellular projections	13.4 ± 5.3	16.3 ± 7.5	0.32
Cell diameter	5.9 ± 0.8	6.8 ± 1.0	<b>0.0284</b>
Cell perimeter	24.4 ± 2.3	27.2 ± 4.5	<b>0.0465</b>

Results are presented as Mean ± SD

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**Table 3.**Effects of  $\alpha$ -synuclein deficiency in hematopoietic system of KO mice

B Cells [11]	T Cells [12]	RBC [11, 19, 29]	Platelets [11]
Low absolute lymphocyte count	Low absolute lymphocyte count	Normocytic anemia low Hgb, Hct	Small platelets, low MPV
Low B220 <sup>+</sup> IgM <sup>+</sup> cells	Low CD3 <sup>+</sup> T cell absolute count	Decrease in RBC membrane stabilization	
Low AA4.1 <sup>+</sup> IgD <sup>-</sup> , AA4.1 <sup>+</sup> IgD <sup>+</sup> , AA4.1 <sup>-</sup> IgD <sup>+</sup> B cells	Low number of thymocytes/ small thymus	Decrease in erythroblast enucleation	
Low B220 <sup>+</sup> AA4.1 <sup>+</sup> , B220 <sup>+</sup> AA4.1 <sup>-</sup> , mature B cells and marginal zone B cells	Low CD4 <sup>+</sup> and CD8 <sup>+</sup> single positive splenocytes	Iron deficiency (transferrin)	
Normal IgM concentration	High CD4 <sup>-</sup> CD8 <sup>-</sup> thymocytes		
Low IgG concentration	Low CD8 <sup>+</sup> lymph node T cells		
Abnormal B cell zone architecture in lymphoid organs	Increase in thymic cortex and reduction in medulla		
Low number of lymphoid follicles	High number of CD8 <sup>+</sup> cells expressing CD69 and CD49d		
Normal IgM response to T-independent antigen	Low IL-4 production upon CD4 <sup>+</sup> T cell activation		
Absent IgG response to T-independent antigens	High IL-2 production upon CD4 <sup>+</sup> cell activation		
Normal IgM response to T-dependent antigen	Low IFN- $\gamma$ production upon CD4 <sup>+</sup> cell activation		
Absent IgG response to T-dependent antigen	Low Foxp3 <sup>+</sup> CD4 <sup>+</sup> cells in thymus		

Low, absent and high refers to statistically significant differences