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Lack of the Transcriptional Coactivator OBF-1 Prevents the Development of Systemic Lupus Erythematosus-Like Phenotypes in Aiolos Mutant Mice

Jian Sun,* Gabriele Matthias,* Michael J. Mihatsch,† Katia Georgopoulos,‡ and Patrick Matthias*2*

Here we show that mice lacking the zinc finger transcription factor Aiolos develop symptoms of human systemic lupus erythematosus (SLE), which is characterized by the production of anti-dsDNA Ab and immune complex-mediated glomerulonephritis. This finding indicates that normal Aiolos function is necessary to maintain immune homeostasis and suppress the development of systemic autoimmune disease and implicates Aiolos as a possible candidate gene for SLE. Interestingly, Aiolos-null mice can no longer mount autoimmune reactions and completely fail to develop SLE when they are deficient for the B cell-specific transcription coactivator OBF-1. The lack of OBF-1 reverses several Aiolos mutant mouse phenotypes, such as B cell hyperproliferation, high expression of activation marker on B cells, and spontaneous germinal center formation. Unexpectedly, B cell development at the immature B cell stage is severely impaired in the bone marrow of Aiolos/OBF-1 double-deficient mice, demonstrating the key role of these factors in the transition from pre-B to immature B cells. Our results indicate that B cells play a crucial role in the development of SLE in Aiolos mutant mice and might be useful for the strategy of SLE treatment. The Journal of Immunology, 2003, 170: 1699–1706.

OBF-1, alternatively called OCA-B or Bob-1, is a transcription coactivator that is predominantly expressed in B cells and forms a ternary complex with the POU proteins Oct-1 and/or Oct-2 on a subset of octamer sites-containing promoters (1–3). We and others have shown that OBF-1-null mice have normal early B cell development, but B cell maturation in the periphery is impaired, and the number of IgM⁺/IgD⁺ recirculating B cells in the bone marrow is greatly reduced. Furthermore, Ab production in response to T cell-dependent (TD) T cell independent; WT, wild-type.

CD40L, CD40 ligand; GC, germinal center; SLE, systemic lupus erythematosus; TI, T cell independent; WT, wild-type.

Materials and Methods

Mice

Aiolos-deficient mice (129Sv × C57) (14) were crossed with OBF-1−/− mice (also 129Sv × C57) (4) to generate the double heterozygotes. Interbreeding these double heterozygotes was used to generate double-knockout mice as well as the corresponding controls. The genotype of the mice was

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determined by PCR of tail DNA. All the mice were maintained in a conventional facility.

**Immunohistochemistry and histopathology**

Kidney and spleen were embedded in OCT compound (Miles, Elkhart, IN). For detection of immune complexes, frozen sections were stained with anti-IgG-FITC, anti-IgM-FITC (Southern Biotechnology Associates, Birmingham, AL), and anti-C3-FITC (Cappel). For spleen staining, B cell follicles and GCs were revealed by anti-IgM-rhodamine (Southern Biotechnology Associates) and biotinylated peanut agglutinin (Vector Laboratories, Burlingame, CA), respectively. The latter was developed by streptavidin-FITC (Southern Biotechnology Associates). For histological examination, kidneys were embedded in paraffin, and sections were stained with periodic acid-Schiff.

**Table 1. Autoantibody production in mutant and WT mice**

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>WT</th>
<th>Aiolos−/−</th>
<th>Aiolos−/− OBF−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ANA</td>
<td>19 (21)</td>
<td>71 (26)</td>
<td>0 (28)</td>
</tr>
<tr>
<td>Anti-ssDNA</td>
<td>28 (21)</td>
<td>63 (26)</td>
<td>0 (28)</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>9 (21)</td>
<td>82 (49)</td>
<td>0 (31)</td>
</tr>
<tr>
<td>Anti-histone</td>
<td>10 (21)</td>
<td>43 (24)</td>
<td>0 (28)</td>
</tr>
</tbody>
</table>

*The percentage of mice positive for a specific autoantibody is shown. The number of mice analyzed (male and female, 3–7 mo old) is given in parentheses. Serum autoantibodies were detected by ELISA. The mean ± 2 SD of the OD from WT mice was set as the lower limit for a sample to be scored as positive.*

**FIGURE 1.** Immune complex-mediated glomerulonephritis in Aiolos-deficient mice. a, Immunofluorescence staining of kidney cryosections with FITC-conjugated anti-mouse Abs as indicated on the left. Deposits of IgG, IgM, and C3 in the glomeruli of Aiolos−/− mice were identified, but not in age-matched WT mice or double-mutant mice. b, Periodic acid-Schiff staining of paraffin-embedded sections. A normal glomerulus from a wild-type mouse is shown. Sections from Aiolos−/− mice show an abnormal glomerulus with hypercellularity, lobularity, segmental sclerosis (upper right), or an enlarged glomerulus (lower left), which are characteristic of severe inflammation. No sign of inflammation was visible in the kidney of the double-deficient mouse.
Autoantibodies and Ig detection by ELISA

Autoantibodies were detected by ELISA using plates precoated for ssDNA, histone, ANA, dsDNA (Euroimmun) or ssDNA and dsDNA (Euroimmun and Sigma-Aldrich, St. Louis, MO). The serum dilution used was 1/50. For some double-mutant mice, a 1/5 dilution of serum was used. Anti-mouse IgG labeled with HRP (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary Ab. The mean ± 2 SD of the OD obtained with wild-type (WT) serum was set as the lower limit for a sample to be scored as positive. Igs were anti-ssDNA (anti-IgM, anti-IgA, anti-IgG, and anti-IgG subclasses (Southern Biotechnology Associates)). Relative isotypes Abs conjugated with AP were used as the second Ab.

Flow cytometric analysis

Single-cell suspension was prepared from spleen and bone marrow. The following anti-mouse Abs were used to detect surface markers in direct or indirect immunofluorescence: anti-CD45R (B220) coupled to FITC or allophycocyanin, anti-CD23 (TAC) coupled to biotin, anti-IgM-biotin, anti-IgD-biotin, anti-CD23-PE, and anti-CD40 ligand (CD40L)-PE. Biotinylated Abs were developed with streptavidin-PE. Some 3 × 10^5 events were collected on the lymphocyte gate using a FACSCalibur.

RT-PCR analysis

Bone marrow B cells were isolated by Dynabeads B220 (Dynal, Oslo, Norway) according to the manufacturer’s instruction. Total RNA from B29° bone marrow cells was extracted by TRIzol (Life Technologies, Gaithersburg, MD), and first-stand cDNA was synthesized with RT-PCR kit (Life Technologies). Amplification conditions were as follows: 2 min at 94°C; 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 50 s; and 10 min at 72°C. The primers were designed to amplify sequences across introns to avoid contamination by genomic DNA. The primers for B29, vpreB5, A5 and β-actin were described previously (17). The other primers were as follows: μ, 5'-CACCTCCATTCCCTACATGGAAC and 5'-GGCACGGTCAGAATTCTG; and mb-1, 5'-GATGCCAGGGGGTTCTAG AAGCC and 5'-CCGTCGCTCCTCCACCT.

B cell isolation and proliferation assay

Splenic B cells were purified by negative selection using a B cell isolation kit (Cytovax Biotechnologies). B cells (5 × 10^5/ml) were cultured in the triplicate with the indicated stimuli for 72 h and were pulsed with 1 µCi of [methyl-3H]thymidine for the last 12 h of culture. Incorporation of isotope was measured by liquid scintillation counting.

Results

Aiolos mutant mice develop human SLE-like phenotypes

Anti-dsDNA Abs and immune complex deposition in glomeruli are important pathological features in SLE. We first examined serum autoantibodies and found that a dramatic 82% of the Aiolos mutant mice produced anti-dsDNA Abs (Table I); in some cases, the anti-dsDNA Abs could already be detected in the serum from 3-mo-old Aiolos−/− mice. The percentage of Aiolos mutant mice positive for autoantibodies against ANA, ssDNA, and histones was also significantly higher than that in age-matched, WT mice (Table I). Although autoantibodies, in particular anti-dsDNA Ab, are an important marker for SLE, their presence is not sufficient to diagnose this systemic autoimmune disease. Therefore, we next stained the kidney sections from Aiolos mutant mice for the presence of immune complexes. As shown in Fig. 1a, strong deposits of IgG, IgM, and C3 were identified in the glomeruli from Aiolos−/−, but not from WT mice. Furthermore, we found that the percentage of Aiolos−/− mice positive for autoantibodies was higher in females than in males, except for anti-histones (Table II). This tendency is consistent with earlier observations (18), suggesting that sex hormones play a role in the development of SLE. In addition, histological staining of the kidney demonstrated severe glomerulonephritic changes consisting of hypercellularity, lobularity, segmental sclerosis, and enlarged glomeruli, which were caused by immune complex deposits (Fig. 1b). Severe glomerulonephritis in SLE can lead to chronic renal failure. We therefore measured the serum levels of creatinine and urea, which are indicators for renal failure. The results obtained showed that seven of 24 Aiolos−/− mice 3–5 mo old had significantly increased levels of creatinine (Fig. 2a) and/or urea (Fig. 2b) compared with the age-matched control group. Moreover, five of these seven mice had elevated levels of both markers simultaneously. Proteinuria was also detected in several of these mice (data not shown). These findings could explain the observation that some Aiolos-deficient mice die prematurely.

Lack of OBF-1 prevents the development of SLE in Aiolos-deficient mice

We crossed OBF-1 and Aiolos knockout mice, initially because they have opposite phenotypes with respect to GC formation; this should therefore allow us to establish a genetic hierarchy between these two factors with respect to the GC pathway. However, after our identification of SLE development in Aiolos−/− mice, we first examined the production of autoantibodies in the double-mutant mice and found that it was unexpectedly completely abolished even at the lowest serum dilution tested (1/5). Furthermore, no immune complex deposits were found in the glomeruli of Aiolos−/− OBF-1−/− mice (Fig. 1a), and histological examination of the kidney showed no sign of inflammation in these mice (Fig. 1b). All these results support the conclusion that OBF-1 is essential for the autoimmune responses observed in Aiolos−/− mice and that lack of OBF-1 prevents the development of SLE in these mice.

Table II. Autoantibody production in male and female Aiolos-deficient mice

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Aiolos−/−</td>
</tr>
<tr>
<td>Anti-ANA</td>
<td>30 (10)</td>
<td>53 (17)</td>
</tr>
<tr>
<td>Anti-ssDNA</td>
<td>30 (10)</td>
<td>56 (16)</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>6 (16)</td>
<td>77 (26)</td>
</tr>
<tr>
<td>Anti-histone</td>
<td>10 (15)</td>
<td>53 (17)</td>
</tr>
</tbody>
</table>

The percentage of positive for a specific autoantibody is shown. The number of mice analyzed (3–7 mo old) is given in parentheses. Serum autoantibodies were detected by ELISA. The mean ± 2 SD of the OD from WT mice was set as the lower limit for a sample to be scored as positive.

FIGURE 2. Aiolos−/− mice develop renal failure. Serum creatinine (a) and urea (b) were measured in 24 Aiolos−/− mice, 3–5 mo of age, and in 11 age-matched control mice. The values of creatinine and urea showed a significant difference between Aiolos−/− and WT mice (p < 0.05). The levels of creatinine and urea of seven Aiolos−/− mice are more than the mean ± 2 SD of the WT group. Five of these seven mice had elevated levels of both markers simultaneously.
Transition from pre-B to immature B cell in bone marrow requires either Aiolos or OBF-1

To investigate why the lack of OBF-1 blocks the apparition of Aiolos-induced SLE, we first examined B cell development in the double-mutant mice. The numbers of pro-B (B220^+c-Kit^+) and pre-B (B220^+Tac^+) cells in the bone marrow showed no significant difference between double-mutant and wild-type mice (Fig. 3a). Surprisingly however, B cell development was severely impaired at the immature stage (B220^+IgM^+) in the double-knockout mice, while it was normal in the single-mutant mice (Fig. 3a). This strongly affected

**FIGURE 3.** B cell development in mice of the different genotypes. a, Bone marrow cells were double-stained with the indicated Abs and analyzed by flow cytometry. b, Splenic cells were analyzed with anti-B220 and anti-IgM Abs by flow cytometry. The percentage of B220^+ and B220^+IgM^+ cells is indicated. Genotypes are indicated above the panels. Data are representative of six mice for each genotype, aged 6–10 wk. c, Gene expression in B220^+ bone marrow cells. Total RNA was isolated from equal numbers of B220^+ cells from WT or double-deficient mice (cells from three mice were pooled). The expressions of μ, mb-1, B29, λc, VpreB, and β-actin were measured by RT-PCR. Lanes 1, 3, 5, 7, 9, and 11, WT RNA; lanes 2, 4, 6, 8, 10, and 12, Aiolos/OBF-1 double-mutant RNA.
further maturation to IgD+ B cells, which were largely missing (Fig. 3a). Furthermore, CD23+ B cells, which represent mature B cells in the bone marrow, were also drastically reduced in the double-mutant mice (Fig. 3a). A severe block at the transition from pre-B to immature B cells, as observed here, has not been previously reported. This block is only seen when both factors are missing, indicating that efficient transition from the pre-B to the immature B cell stage requires either Aiolos or OBF-1. Although dramatically impaired, the production of IgM+ B cells was not eliminated, and in the spleen of double-mutant mice B220+ cells were found at ~2- to 4-fold reduced levels (Fig. 3b). Importantly, these cells expressed normal levels of surface IgM (Fig. 3b).

Progression from the pre-B to the immature B cell stage requires function of the pre-B cell receptor (19, 20). Therefore, we tested whether impaired expression of some of its components might underlie the developmental block observed with double-deficient cells. For this, we isolated RNA from bone marrow B cells and measured gene expression by RT-PCR. As shown in Fig. 3c, no difference in the expression of mb-1 (Igα), B29 (Igβ), Vpexβ, or the control β-actin was seen between WT and double-knockout RNA.
A slight reduction in λ5 expression was found in the compound mice. In addition, the expression of Ig μ-chain was clearly decreased, in good agreement with the reduced number of cells positive for μ expression by FACS analysis.

**B cell activation and proliferation, and Ig production in double-knockout mice**

To further define the role of B cells in the development of SLE, we first analyzed the expression of the activation marker CD23. As shown in Fig. 4a, CD23 expression on splenic B cells in Aiolos−/− mice was elevated compared with that in WT mice. Aiolos−/− splenic B cells also express high levels of activated marker MHC II Ag (14). By contrast, in OBF-1-deficient mice CD23 expression was reduced. In double-mutant mice CD23 expression was close to the level in WT mice, indicating that the lack of OBF-1 antagonizes the defect caused by the absence of Aiolos and inhibits B cell activation in Aiolos−/− mice. Interestingly, up-regulation of CD23, which is a low affinity receptor (FcμRII) for IgE and is regarded as a negative regulator of IgE production (21, 22), did not prevent elevated production of IgE in Aiolos mutant mice (Fig. 4b).

We next examined the ability of purified splenic B cells of the different genotypes to proliferate in vitro in response to mitogens, including LPS, anti-IgM, or anti-CD40 plus IL-4. With the exception of LPS stimulation, Aiolos−/− B cells showed increased [3H]thymidine uptake compared with WT cells (Fig. 4c), in agreement with earlier findings, suggesting that the lack of Aiolos lowers the threshold of the B cell Ag receptor pathway (14). It is noteworthy that Aiolos−/− B cells also showed increased [3H]thymidine uptake in the absence of stimulus (Fig. 4c), indicating that they are hyperproliferative. As shown here, this phenotype is weakened in double-knockout B cells (Fig. 4c), demonstrating that normal OBF-1 function is necessary for the hyperactivity of Aiolos-deficient B cells. In addition, our data show that splenic B cells in OBF-1−/− mice have a poor response to anti-IgM stimulation (Fig. 4c), which, however, can be rescued by the addition of IL-4 (5, 23). Thus, failure of BCR-mediated signaling could contribute to the impaired TI and TD responses in OBF-1−/− mice.

Abnormal Ab production against self-Ags in the Aiolos and double-mutant mice prompted us to examine serum Ig levels. Unlike other lupus models (15), Aiolos-deficient mice do not have significantly increased total IgG levels, but show elevated serum IgG2a, IgA, and IgE and reduced serum IgM and IgG3 (Fig. 4b, 4 day). However, in the double-knockout mice the levels of all Igs were decreased close to those found in OBF-1 mutant mice (Fig. 4, b and d). This result could explain our observation that autoantibodies were not detected in the double-knockout mice (Table I). The IgG2a predominance in Aiolos mutant mice suggests that this subclass could be the main pathological autoantibody observed in these mice. Interestingly, Aiolos−/− mice still show IgM deposition in the glomeruli despite reduced serum IgM.

**OBF-1 is essential for GC formation in Aiolos mutant mice**

GCs play a critical role in affinity maturation, class switching, and somatic hypermutation (24, 25). Mice deficient for Aiolos can form GCs in the absence of Ag challenge (Fig. 5). This persistent GC formation may contribute to the development of SLE in these mice. It is well established that CD40L expression on activated T cells is crucial to trigger B cells to form GCs (25). High expression of CD40L on T and B cells from lupus patients has been reported (26, 27). To understand the mechanism of spontaneous GC formation in Aiolos−/− mice, we therefore examined CD40L expression. However, we failed to observe a significant difference in CD40L expression on unstimulated or PMA/ionomycin-activated T cells or in B cells from Aiolos-deficient or wild-type mice (data not shown). These results suggest that dysregulation of CD40L expression is not responsible for spontaneous and persistent GC formation in Aiolos−/− mice, and that other molecules are implicated in the phenotype. Our previous results showed that OBF-1 is essential for GC formation (4). Here we found that mice lacking both Aiolos and OBF-1 completely fail to form GC (Fig. 5). Thus, the impaired BCR-mediated signaling in the absence of OBF-1 might block B cell hyperproliferation and GC formation in Aiolos−/− mice. Alternatively, OBF-1 and/or its target gene(s) could lie downstream of Aiolos.

**Discussion**

Our results demonstrated that Aiolos-deficient mice produce anti-dsDNA and other autoantibodies. These autoantibodies form immune complexes with complement in the basement membrane of
glomeruli, resulting in tissue injury and glomerulonephritis, eventually leading to chronic renal failure. Thus, these mice represent a novel animal model for human SLE and could be useful to study the interplay between transcriptional control of B cell activation and the development of autoimmunity. Interestingly, we found that the development of these symptoms absolutely requires function of the transcriptional coactivator OBF-1. Furthermore, the B cell hyperactivity associated with the lack of Aiolos is antagonized when OBF-1 is also missing. In agreement with this, GCs that spontaneously develop in Aiolos mutant mice are no longer observed in double-mutant mice. Strikingly, we identified a severe defect in early B cell development in mice lacking both transcription factors, demonstrating that these two factors also play an important role in early B cell development. This result indicates that transition from pre-B to immature B cells may normally proceed through two redundant pathways, each requiring a different transcription factor.

The evidence presented here from the analysis of Aiolos−/− and double-knockout mice indicates that B cell dysfunction is critical to the development of SLE in Aiolos−/− mice. In line with this, the numbers of CD4+ and CD8+ T cells in the thymus and spleen were comparable in mice of all genotypes (data not shown). Aiolos-deficient T cells were shown to have a modestly increased intracellular level of Th1 cytokine (IFN-γ and IL-2) following stimulation by anti-TCR and anti-CD28 (14). Yet although Aiolos−/− T cells have a moderately increased TCR-mediated proliferation in vitro (14), they do not show elevated expression of activation markers such as CD40L, CD25, and CD69 (data not shown). Thus, unlike B cells, Aiolos−/− T cells do not appear to be in an activated state in vivo. It has been shown previously that mice deficient for Aiolos have an increased propensity to develop lymphomas (14). Likewise, the elevated cancer incidence in patients with autoimmune disorders is well established (28). However, the mechanisms linking autoimmune disease to cancer are poorly understood.

Our findings indicate that Aiolos could function as a link between autoimmune disease and cancer. Interestingly, it has been reported that the loss of one allele of the tumor suppressor gene PTEN also leads to autoimmune disorders and cancer development due to impaired apoptosis of lymphocytes (29). Further study of the function of these genes could contribute to our understanding of the relationship between autoimmune diseases and cancers.

In humans, SLE shows severe heterogeneity, and it has been assumed that multiple genes, including MHC and non-MHC genes, contribute to its pathogenesis (30, 31). However, there are spontaneous mouse lupus models in which the mutation of a single gene, such as Ipr or gld, is critical for the development of autoimmunity when present in the proper genetic background (32, 33). In addition, several knockout or transgenic mice have been found to develop SLE-like phenotypes (30, 34, 35). One SLE patient with a Fas ligand mutation has been reported (36), and recently, DNase I mutations have been identified in two SLE patients (37). Therefore, it is possible that in a subgroup of patients, SLE is caused by a single gene defect, and it will be interesting to examine the Aiolos gene in this respect.

The reduced expression of Ig μ-chain observed in RT-PCR analysis of bone marrow cells from double-mutant mice reflects the decreased number of cells expressing this marker. The expression of several molecules that are part of the pre-B cell receptor, such as mb-1, B29, or Vpre-B, was not diminished; only A5 expression appeared to be slightly down-regulated. However, this seems unlikely to lead to a differentiation block, since A5-deficient mice show impairment at an earlier stage of B cell development (38). Several genes, such as Pax-5, EBF, and E2A, have been identified as critical factors for early B cell development. In each case the lack of one of these factors can lead to the block (39). However, our finding that the transition from pre-B to immature B cell requires either Aiolos or OBF-1 suggests that this step may normally proceed through two alternative pathways and reveals a novel regulatory pattern for B cell development in the bone marrow.

Finally, one hypothesis for the mechanism of self-tolerance is that autoreactive B cell clones are deleted or functionally inactivated at the immature B cell stage in the bone marrow (40, 41). Thus, it is conceivable that the loss of a large number of immature B cell clones might prevent the development of autoimmune responses in the double-mutant mice.

Acknowledgments

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