IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Aromatase Controls Sjögren Syndrome—Like Lesions through Monocyte Chemotactic Protein-1 in Target Organ and Adipose Tissue—Associated Macrophages

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Accepted for publication
September 9, 2014.

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Several autoimmune diseases are known to develop in postmenopausal women. However, the mechanism by which estrogen deficiency influences autoimmunity is unknown. Aromatase is an enzyme that converts androgens to estrogens. Herein, we used female aromatase gene knockout (ArKO) mice as a model of estrogen deficiency to investigate the molecular mechanism that underlies the onset and development of autoimmunity. Histological analyses showed that inflammatory lesions in the lacrimal and salivary glands of ArKO mice increased with age. Adoptive transfer of spleen cells or bone marrow cells from ArKO mice into recombination activating gene 2 knockout mice failed to induce the autoimmune lesions. Expression of mRNA encoding proinflammatory cytokines and monocyte chemotactic protein-1 increased in white adipose tissue of ArKO mice and was significantly higher than that in wild-type mice. Moreover, an increased number of inflammatory M1 macrophages was observed in white adipose tissue of ArKO mice. A significantly increased monocyte chemotactic protein-1 mRNA expression of the salivary gland tissue in ArKO was found together with adiposity. Furthermore, the autoimmune lesions in a murine model of Sjögren syndrome were exacerbated by administration of an aromatase inhibitor. These results suggest that aromatase may play a key role in the pathogenesis of Sjögren syndrome—like lesions by controlling the target organ and adipose tissue—associated macrophage. (*Am J Pathol* 2015, 185: 151–161; <http://dx.doi.org/10.1016/j.ajpath.2014.09.006>)

Autoimmune disease is caused by multiple factors. One of them is the disruption of self-tolerance.^{1,2} Several autoimmune diseases, such as rheumatoid arthritis or Sjögren syndrome (SS), occur in postmenopausal women. Sex steroid hormones, such as estrogens, may influence the onset or development of autoimmune diseases through an unknown mechanism.^{3,4}

SS is characterized by a 9:1 ratio of women/men, and almost all SS patients are postmenopausal women.⁵ SS targets exocrine glands, such as the salivary and lacrimal glands, causing patients with SS to experience dryness of the mouth and eyes.⁶ We demonstrated that estrogen deficiency caused by ovariectomy exacerbates autoimmune lesions in a murine model of SS.⁷ Furthermore, we reported that retinoblastoma-associated

protein 48 (RbAp48) induces tissue-specific apoptosis in the salivary and lacrimal glands of mice, depending on the level of estrogen deficiency.⁸ Moreover, we observed that transgenic expression of *RbAp48* in exocrine glands causes the development of autoimmune exocrinopathy, resembling SS.⁹ Although postmenopausal estrogen deficiency triggers the breakdown of immune tolerance and induces autoimmune disease, multiple direct or indirect factors

Supported, in part, by the Funding Program for Next Generation World-Leading Researchers in Japan grant LS090, Ministry of Education, Science, Sport, and Culture of Japan Grants-in-Aid for Scientific Research 25670798, 24659839, and 23390429, and the Uehara Memorial Foundation.

Disclosures: None declared.

that are affected by the change in estrogen level impede efforts to understand the molecular mechanisms involved in autoimmunity.

Estrogens are synthesized from androgens by aromatase, a key step in the biosynthesis of estrogens. Studies on aromatase gene knockout (*ArKO*) mice show that aromatase/estrogens play key roles in controlling sex-specific morphological, neuroendocrinological, and behavioral differences in the brain.^{10–12} Furthermore, *ArKO* mice develop marked abdominal adiposity, suggesting that aromatase/estrogens control the adipose phenotype through the regulation of lipid metabolism.^{13,14} Moreover, *ArKO* mice spontaneously develop an autoimmune disease, resembling age-associated SS lesions through B-cell hyperplasia and autoantibody production.¹⁵ In addition, increased numbers of peripheral blood cells, bone marrow cells, and inflammatory renal lesions are observed in *ArKO* mice.¹⁵ However, the molecular mechanism responsible for the onset of autoimmunity in exocrine glands through aromatase is unknown. In particular, the relationship between aromatase activity and the function of immune cells, in addition to B cells, in the onset or development of autoimmune disease must be determined to understand the dysfunction of the immune system in postmenopausal women.

Herein, we investigated the association of the pathogenesis of autoimmune disease with postmenopausal changes induced by estrogen deficiency. Furthermore, we attempted to define the molecular mechanism that accounts for the breakdown of peripheral immune tolerance with the goal of establishing a new therapeutic strategy to treat sex-specific autoimmune diseases.

Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from the Japan SLC Laboratory (Shizuoka, Japan). *ArKO* mice were obtained from RIKEN BioResource Center (Tsukuba, Japan). Female *ArKO* were used for this study. Female recombination activating gene 2 knockout (*Rag2KO*) mice were obtained from Taconic (Germantown, NY). Female NFS/N mice carrying mutant *sld* were reared in our specific pathogen-free mouse colony and given food and water ad libitum. Thymectomy was performed on day 3 after birth of the NFS/*sld* mice. All mice were bred in the animal facility of the University of Tokushima (Tokushima, Japan) under specific pathogen-free conditions. The experiments were approved by the animal ethics board of the University of Tokushima.

Administration of AI

The aromatase inhibitor (AI) exemestane (Sigma-Aldrich, St. Louis, MO) was prepared as an aqueous suspension in 0.5% carboxymethylcellulose (CMC; Dai-Ichi Chemical Industries, Tokyo, Japan). The i.p. injection of B6 mice

(8 weeks old) with 200 μ L CMC, containing AI (0, 50, and 200 μ g per mouse), was administered. For female thymectomized (Tx)-NFS/*sld* mice, AI in CMC was injected every day until 4 to 8 weeks of age.

Histological Features

All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for histological examination. Formalin-fixed tissue sections were subjected to hematoxylin and eosin (H&E) staining, and three pathologists (N.I., Y.K., A.Y.) independently evaluated the histological features without being informed of the condition of each mouse. Histological grading was performed according to a previously published method.¹⁶ In brief, the inflammatory lesions of lacrimal and salivary glands were evaluated as followed: slight, no change or slight lymphoid cell infiltration; moderate, lymphoid cell infiltration; and severe, marked lymphoid cell infiltration with tissue destruction.

Adoptive Cell Transfer

Spleen cells (5×10^6) from female wild-type (WT) and *ArKO* mice (10 weeks of age) were i.v. transferred into female *Rag2KO* mice (8 weeks of age). In addition, bone marrow cells (5×10^6) from WT and *ArKO* mice were i.v. transferred into irradiated (8-Gy) *Rag2KO* mice. Twelve weeks after transfer, the pathological features of all organs were analyzed.

Flow Cytometry

Antibodies against CD4, CD8, CD11c, F4/80, CCR2, and CD206 were conjugated as required to fluorescein isothiocyanate, phosphatidylethanolamine (PE), allophycocyanin–peridinin chlorophyll protein, PE-Cy5.5, PE-Cy7, or allophycocyanin-Cy7 (eBioscience, San Diego, CA). A FACScanto flow cytometer (BD Biosciences, Franklin Lakes, NJ) was used to determine the cell populations, and the data were analyzed using FlowJo FACS Analysis software version 7.6.3 (Tree Star, Ashland, OR).

ELISAs

The α -fodrin (JS-1),¹⁶ SS-A/Ro (Immunovision, Springdale, AR), SS-B/La (Immunovision), or single strand (ss)-DNA (Immunovision)—specific antibodies in sera from mice were measured using enzyme-linked immunosorbent assays (ELISAs), as previously described.¹⁶ In brief, the antibodies were added to microtiter wells at a concentration of 1 μ g/mL of phosphate-buffered saline. Non-specific sites were absorbed with 1% bovine serum albumin in phosphate-buffered saline. After the plates were washed, diluted sera were added. Horseradish peroxidase–conjugated anti-mouse IgG (heavy and light chains; Vector, Burlingame, CA) was added as the

secondary antibody, and o-phenylenediamine (Sigma-Aldrich) buffer was added. Antibodies were measured with a microplate reader (Bio-Rad Laboratories, Richmond, CA).

Quantitative RT-PCR

Total RNA was extracted from white adipose tissue (WAT), spleen, salivary glands, and liver using Isogen (Wako Pure Chemical Industries, Osaka, Japan) and subsequently reverse transcribed. The levels of mRNAs encoding F4/80, monocyte chemotactic protein (MCP)-1, Toll-like receptor (TLR)-4, NF- κ B, IL-6, IL-1 β , interferon (IFN)- γ , tumor necrosis factor (TNF)- α , IL-10, transforming growth factor (TGF)- β , and β -actin were determined using a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories) with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The primer sequences used were as follows: F4/80, 5'-CTTTGGCTATGGGCTTCCAGTC-3' (forward) and 5'-GCAAGGAGGACAGAGTTTATCGTG-3' (reverse); MCP-1, 5'-CTGGATCGGAACCAAATGAG-3' (forward) and 5'-TGAGGTGTTGTGGAAAAGG-3' (reverse); TLR-4, 5'-CAGTGGT-CAGTGTGATTGTGG-3' (forward) and 5'-TTCCTGGA-TGATGATGTTGGCAGC-3' (reverse); NF- κ B, 5'-ATGG-CAGACGATGATCCCTA-3' (forward) and 5'-TAGGCA-AGGTCAGAATGCAC-3' (reverse); IL-1 β , 5'-TGATGA-GAATGACCTGTTCT-3' (forward) and 5'-CTTCTTC-AAAGATGAAGGAAA-3' (reverse); IL-6, 5'-GCTACC-AAACTGGATATAATCAGGA-3' (forward) and 5'-CC-AGGTAGCTATGGTACTCCAGAA-3' (reverse); IFN- γ , 5'-AGCGGCTGACTGAACTCAGATTGTAG-3' (forward) and 5'-GTCACAGTTTTTCAGCTGTATAGGG-3' (reverse); IL-10, 5'-ATCGATTTCTCCCCTGTGAA-3' (forward) and 5'-TGTCAAATTCATTCATGGCCT-3' (reverse); TGF- β , 5'-GACCGCAACAACGCCATCTAT-3' (forward) and 5'-GGCGTATCAGTGGGGGTCAG-3' (reverse); arginase-1, 5'-CAGAAGAATGGAAGAGTCAG-3' (forward) and 5'-CAGATATGCAGGGAGTCACC-3' (reverse); Ym-1, 5'-CAGGTCTGGCAATTCTTCTGAA-3' (forward) and 5'-GT-CTTGCTCATGTGTGTAAGTGA-3' (reverse); Fizz-1, 5'-TCCCAGTGAATACTGATGAGA-3' (forward) and 5'-CC-ACTCTGGATCTCCCAAGA-3' (reverse); and β -actin, 5'-GTGGGCCGCTCTAGGCACCA-3' (forward) and 5'-CGG-TTGGCCTTAGGGTTCAGGGGG-3' (reverse).

Confocal Microscopy

Frozen sections of adipose tissues were fixed using a cold acetone and methanol (1:1) mixture. Whole mounts of WAT were minced into small pieces, fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100 (Thermo Scientific, Waltham, MA) for 1 hour, and blocked with 5% goat serum. The frozen sections or whole adipose tissues were stained with Alexa 488-conjugated anti-mouse F4/80 antibodies (eBioscience). The nuclear DNA was stained with DAPI (Molecular Probe Inc., Eugene, OR). The tissues were counterstained for 1 hour with 5 μ mol/L

BODIPY 558/568 C12[4,4-difluoro-5-(2-thienyl)-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoic acid] (Invitrogen, Carlsbad, CA) to visualize adipocytes. The images were acquired using an LSM 5 PASCAL confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Oil Red O Staining

For staining of lipids, the frozen sections of salivary gland from WT and *Ar*KO mice were fixed with 10% phosphate-buffered formaldehyde, incubated for 1 minute in 60% 2-propanol, stained for 15 minutes with Oil Red O solution (Sigma-Aldrich), rinsed in 60% 2-propanol, and washed with water. The specimens were counterstained with Mayer's hematoxylin solution for 5 minutes.

IHC Analysis

For immunohistochemical (IHC) analysis of MCP-1 in salivary gland, paraffin-embedded sections were deparaffinized and subsequently applied to heat-induced antigen retrieval in 10 mmol/L citrate buffer (pH 6.0). The sections were incubated with rabbit MCP-1 polyclonal antibody (BioSS, Dundee, Scotland) or control rabbit immunoglobulin, subsequently developed using horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Vector Laboratories Ltd, Peterborough, UK), and counterstained with methyl green.

Statistical Analysis

Statistical significance was determined using an unpaired Student's *t*-test.

Results

Inflammatory Lesions of Lacrimal and Salivary Glands in *Ar*KO Mice

*Ar*KO mice spontaneously develop SS-like lesions in their lacrimal and salivary glands.¹⁵ Herein, using another line of *Ar*KO mice,¹⁷ lacrimal and salivary gland tissues were histopathologically analyzed until the mice reached 18 months of age. Severe inflammatory lesions with lymphocyte infiltration and tissue destruction in the lacrimal and salivary glands of *Ar*KO mice were observed during the first 12 months; they resemble those of human SS (Figure 1A). Although slight or mild inflammation of the lacrimal and salivary glands was observed in WT mice older than 12 months, severe inflammatory lesions significantly increased in *Ar*KO mice of the same age (Figure 1B). Many CD4⁺ T cells and B220⁺ cells infiltrated the salivary glands of the *Ar*KO mice (Supplemental Figure S1A). Moreover, significantly higher levels of autoantibodies, such as anti-SSA, anti-SSB, and anti-ssDNA, were detected in the sera of *Ar*KO mice compared with those in the sera of WT mice

Figure 1 SS-like lesions in the lacrimal glands (LGs) and salivary glands (SGs) of *ArKO* mice. **A:** Severe inflammatory lesions in the LGs and SGs of *ArKO* mice. Shown are representative images of H&E-stained sections of 12-month-old WT and *ArKO* mice. **B:** Pathological score of inflammatory lesions in LGs and SGs was evaluated using H&E-stained sections of female WT and *ArKO* mice from 3 to 18 months of age (WT, $n = 19$; *ArKO*, $n = 19$). **C:** Spleen cells (5×10^6) from WT and *ArKO* mice were i.v. transferred into female *Rag2KO* mice at 8 weeks of age. Pathological analysis of the LGs and SGs of the recipient *Rag2KO* mice was performed 3 months after transfer. Images are representative of each recipient group ($n = 5$). **D:** Autoantibody production (anti-SSA, anti-SSB, anti-ssDNA, and anti- α -fodrin antibodies) in the sera of recipient *Rag2KO* mice was analyzed using ELISAs. Data are shown as the means $\pm$ SD of five mice of each group. Scale bar = 200 μ m (**A**).

(Supplemental Figure S1B). These findings are consistent with the results of another study that demonstrated that hyperactivation of B cells contributes to the onset of SS-like autoimmune lesions in lacrimal and salivary glands of *ArKO* mice.¹⁵

To determine the contribution of peripheral immune cells, including B cells, to the onset of autoimmunity in *ArKO* mice, 5×10^6 spleen cells from WT or *ArKO* mice were i.v. transferred into *Rag2KO* mice. Twelve weeks after transfer, all organs were pathologically analyzed. Neither WT nor *ArKO* spleen cells induced inflammatory lesions in the lacrimal or salivary glands of the recipient mice (Figure 1C). Furthermore, there were no differences in the serum levels of autoantibodies, including anti-SSA, anti-SSB, anti- α -fodrin, and anti-ssDNA (Figure 1D).⁹ Moreover, transfer of bone marrow cells from *ArKO*

mice into irradiated *Rag2KO* mice could not induce inflammatory lesions in salivary glands (Supplemental Figure S2A). In addition, the serum level of autoantibodies of the recipient mice transplanted with bone marrow cells from *ArKO* mice was similar to that from WT mice (Supplemental Figure S2B). These findings suggest that the transfer of immune cells from *ArKO* mice failed to induce SS-like lesions in the target exocrine glands. Moreover, they suggest that other factors influence the onset or development of inflammatory lesions in *ArKO* mice.

Relationship between Adipose Tissue and Immune Cells of *ArKO* Mice

Aromatase is abundantly present in the adipose tissues,¹⁸ and the adipocytes of *ArKO* mice are significantly larger and more

abundant compared with those of WT mice.¹⁴ Furthermore, monocytes or cytokines in adipose tissue contribute to the pathogenesis of autoimmune disease.^{19,20} Therefore, we focused on adipose tissues to determine whether they were involved in inducing the SS-like lesions in *ArKO* mice. The volume of abdominal WAT in female *ArKO* mice at 12 months of age was greater than that of WT mice (Figure 2A). There was a slight, but insignificant, increase in body weight of *ArKO* mice compared with WT mice. However, the weight of WAT of *ArKO* mice was significantly increased (Figure 2B). Moreover, flow cytometric analysis showed that the proportion of dendritic cells (DCs) and macrophages in the WAT of *ArKO* mice was higher than that of WT mice (Figure 2C), as well as the absolute numbers of DCs and macrophages (Figure 2D). These findings suggest that increased numbers of DCs and macrophages in WAT may influence the onset or development of SS-like lesions.

Expression of mRNAs Encoding Macrophage-Associated Proteins in WAT of *ArKO* Mice

To understand the characteristics of macrophages that accumulated in the adipose tissues of *ArKO* mice, the expression levels of macrophage-associated genes were quantified using real-time RT-PCR. We detected significantly increased expression of mRNAs encoding cell surface glycoprotein F4/80 and MCP-1 in the WAT of *ArKO* mice compared with WT mice (Figure 3A). The expression levels of mRNAs encoding TLR-4 and NF- κ B in *ArKO* mice were significantly higher than in WT mice (Figure 3A).

Macrophage phenotypes are defined as M1 and M2, according to their production of certain cytokines.²¹ M1 macrophages produce the proinflammatory cytokines IL-1 β , IL-6, IFN- γ , and TNF- α . In contrast, M2 macrophages produce the anti-inflammatory cytokines IL-10 and TGF- β . The expression levels of mRNAs encoding IL-1 β , IL-6, IFN- γ , and TNF- α mRNA in WAT of *ArKO* mice were significantly increased compared with those in WT mice (Figure 3B). In contrast, expression levels of mRNAs encoding IL-10 and TGF- β mRNA of WAT in *ArKO* mice

were significantly reduced (Figure 3B). In addition, there was no significant difference in mRNA expression of M2-macrophage markers, such as arginase-1, Fizz-1, and Ym-1, in WAT between WT and *ArKO* mice (Figure 3B). These findings suggest that aromatase regulates the migration and differentiation of macrophages in adipose tissues as well as their subsequent activation.

Macrophages in Adipose Tissues of *ArKO* Mice

The distribution of macrophages in WAT was evaluated using immunofluorescence microscopy. H&E staining of adipose tissue sections revealed the accumulation of mononuclear cells around adipocytes in *ArKO* mice (Figure 4A). Moreover, the accumulated macrophages formed a crown-like structure in the WAT of *ArKO* mice; this was detected using DAPI (red) staining and an anti-F4/80 monoclonal antibody (green) (Figure 4A). Confocal microscopy revealed that accumulation of F4/80⁺ macrophages surrounding adipocytes stained with BODIPYR (blue) (Figure 4A). CCR2, a marker of M1 macrophages, was expressed at significantly higher levels by F4/80⁺ cells in the WAT of *ArKO* mice compared with WT mice. In contrast, CD206, a marker of M2 macrophages, was expressed at significantly lower levels in the WAT of *ArKO* mice than in WT mice (Figure 4B). The results suggest that the increased abundance of WAT in *ArKO* mice enhanced the migration and maturation of activated M1 macrophages.

Effect of an AI on WAT and Salivary Glands

MCP-1, produced by adipocytes or macrophages, plays a key role in chemotaxis of monocytes into adipose tissues.²² However, it is unclear whether aromatase/estrogens regulate the expression of MCP-1 in WAT. To address this question, we i.p. administered 0, 50, and 200 μ g of AI into female C57BL/6 mice daily for 1 week. By using real-time RT-PCR, we subsequently determined the expression levels of *Mcp-1* mRNA in various organs. The levels of the mRNA encoding MCP-1 in WAT increased as a function of AI dose (Figure 5). The expression of *Mcp-1* mRNA in the salivary gland, a target organ of SS, was enhanced by AI treatment;

Figure 2 Adipose tissues of *ArKO* mice. **A:** Representative images of WAT of 12-month-old WT and *ArKO* mice (top panels). **Bottom panel:** Formalin-fixed tissues. **B:** Body weight and WAT weight of 12-month-old WT and *ArKO* mice. Data represent the means $\pm$ SD of five mice from each group. **C:** The proportion of CD11c⁺F4/80⁻ DCs or F4/80⁺ macrophages in WAT of WT and *ArKO* mice was analyzed using flow cytometry. Results are representative of five mice from each group. **D:** The number of DCs and macrophages in WAT of 12-month-old WT and *ArKO* mice. Cell number is shown as the means $\pm$ SD of five mice of each group. * $P < 0.05$, ** $P < 0.005$. Scale bar = 1 cm (A).

Figure 3 Real-time RT-PCR of the expression of monocyte-related genes in ArKO (black bars) and WT (white bars) mice. **A:** Levels of mRNAs encoding F4/80, MCP-1, TLR-4, and NF- κ B in WAT of 12-month-old WT and ArKO mice. **B:** The levels of mRNAs encoding IL-1 β , IL-6, IFN- γ , TNF- α , IL-10, TGF- β , arginase-1, Fizz-1, and Ym-1 in WAT of WT and ArKO mice. Data represent the means $\pm$ SD of five mice from each group. * $P < 0.05$, ** $P < 0.005$.

however, it was not enhanced in the liver and spleen (Figure 5). These findings demonstrate that the expression of MCP-1 in the salivary glands and in WAT is regulated by aromatase/estrogens.

Expression of MCP-1 in Salivary Glands and Cervical WAT

Abundant adipose tissue surrounds the salivary gland, and a change in fat levels is often observed in atrophic salivary glands because of chronic inflammation or aging. When the fatty change of the salivary gland in ArKO mice was compared with that in WT mice using Oil Red O staining, more salivary gland cells exhibited a fatty change in ArKO mice (Figure 6A). Moreover, the expression level of *Mcp-1* mRNA of the salivary gland tissue of ArKO mice was significantly increased compared with that of WT mice (Figure 6B). A significantly increased number of F4/80⁺ macrophages in the cervical WAT around the salivary

glands of ArKO mice was detected using immunofluorescence staining (Figure 6C). Furthermore, the expression of MCP-1 in the salivary gland of ArKO mice was significantly increased compared with that of WT mice (Figure 6D). In addition, macrophage markers of salivary gland tissues were analyzed by real-time RT-PCR. TLR-4 mRNA of salivary gland from ArKO mice was significantly increased compared with that of WT mice (Supplemental Figure S3). There was no difference in mRNA expression, such as IFN- γ , TNF- α , IL-10, and TGF- β , in the salivary gland tissues between WT and ArKO mice (Supplemental Figure S3). The findings suggest that MCP-1 may play a key role in the development of SS-like lesions in the salivary glands.

Effect of AI Administration on Autoimmune Lesions in Mouse Model of Primary SS

To confirm the role of aromatase in the SS-like lesions in the salivary and lacrimal glands, AI was administered into a

model for SS, which is Tx-NFS/*sld* mouse strain at 3 days after birth.^{23,24} AI (200 µg per mouse) was i.p. administered into Tx-NFS/*sld* mice daily from 4 to 8 weeks of age. The severity of autoimmune lesions in the lacrimal and salivary glands of Tx-NFS/*sld* mice (8 weeks) was enhanced (Figure 7A). The inflammatory lesions of SS-model mice were markedly more severe compared with those of the control mice (Figure 7B). The proportion of CD11C⁺DCs and F4/80⁺ macrophages in the spleens of SS-model mice treated with AI was significantly higher compared with controls (Figure 7, C and D). Furthermore, the serum levels of anti- α -fodrin and anti-ssDNA antibodies of AI-treated SS-model mice were significantly higher compared with controls (Figure 7E). These findings suggest that imbalance of aromatase/estrogens may contribute to the onset or development of SS-like lesions through accumulated macrophages or DCs.

Discussion

Evidence indicates that the pathogenesis of autoimmune diseases, such as SS, is influenced by multiple factors.²⁵ Most autoimmune diseases occur more frequently in women than men.^{3,4} In particular, various autoimmune diseases occur in postmenopausal women, who are older than middle age around postmenopausal stage. Experimental and clinical evidence suggests that autoimmunity is influenced by several differences in the immune responses between males and females.^{26,27} For example, types 1 and 2 helper T-cell responses are induced by androgens or estrogens, respectively.^{28,29} Among sex hormones, estrogens are the most potent modulator of the immune system, suggesting that a significant change in estrogen levels, such as those that occur during menopause, triggers autoimmunity.³⁰ Aromatase catalyzes critical steps in the synthesis of estrogens.³¹ Several studies demonstrate that *ArKO* mice provide an informative animal model for estrogen insufficiency in humans that facilitates our understanding of how estrogens influence the immune system.^{12,13,15} Therefore, in the present study, we used *ArKO* mice to determine whether there is a link between adipose tissue and the immune system with the hope of establishing the molecular basis of the development of autoimmunity.

ArKO mice spontaneously develop SS-like autoimmune lesions through B-cell hyperplasia and the production of autoantibodies.¹⁵ The report has demonstrated that estrogen

Figure 4 Macrophages infiltrating the WAT of *ArKO* mice. **A:** H&E staining of WAT is shown (top panels). F4/80⁺ macrophages in WAT of WT and *ArKO* mice were detected using immunofluorescence staining. Adipocytes were stained with BODIPYR, and F4/80⁺ macrophages in the WAT were detected using a confocal microscope (bottom panels). Nuclei were stained with DAPI (red). Images are representative of three mice in each group. Arrows show macrophage accumulation. **B:** Flow cytometric analysis of CCR2 and CD206 expression on F4/80⁺ macrophages in the WAT of WT and *ArKO* mice. Results represent three mice from each group. The gray line indicates the data for the isotype control. The number of F4/80⁺ cells/mm² was counted. Data represent the means $\pm$ SD of five mice from each group. **P* < 0.05, ***P* < 0.005. Scale bar = 50 µm (A).

Figure 5 Effect of AI on the expression of MCP-1 mRNA in various tissues. Daily peritoneal injections of AI (0, 50, and 200 μ g per mouse) were administered to 10-week-old female B6 mice for a week. The expression of MCP-1 mRNA in WAT, salivary glands, liver, and spleen was analyzed using real-time RT-PCR. Data represent the means $\pm$ SD of five mice of each group. * $P < 0.05$, ** $P < 0.005$.

administration into *Ar*KO mice results in preventing SS-like lesions, suggesting that estrogen may play a key role in the pathogenesis of autoimmune lesions of *Ar*KO mice.¹⁵ We also observed SS-like inflammatory lesions in lacrimal and salivary glands and increased autoantibody production by *Ar*KO mice. Moreover, adoptive transfer of both spleen cells and bone marrow cells from *Ar*KO mice into *Rag2*KO mice failed to induce inflammatory lesions, suggesting that in *Ar*KO mice factors other than immune cells contribute to the pathogenesis of SS-like disease. On the other hand, serum concentration of estradiol of *Ar*KO mice at baseline is similar to that of WT mice in normal status.³² Although the estradiol or progesterone level of female WT mice is promoted in the estrus cycle, no change of the level is observed in female *Ar*KO mice.³² It is believed that the hypothalamus-pituitary axis is disrupted in *Ar*KO mice. Therefore, change of estrogen with diestrous/estrous phase of the cycle is not seen in *Ar*KO mice. In addition, it was reported that prolactin and testosterone levels of the sera in female *Ar*KO mice are significantly elevated compared with those of WT mice.³³ There may be any difference in the physiological function for immune response between estrogen and androgen. Previous reports demonstrated that administration of androgen protects autoimmune lesions in murine SS models.^{34,35} However, the function and metabolism of sex hormones are so complicated that the precise molecular mechanism of sex dimorphism in the onset of autoimmunity remains unclear.

It is well known that autoimmunity is a multifactorial disease. Although lack of estrogen is important for the onset or development of autoimmunity, the other factors influence the pathogenesis of the autoimmune disease. For instance, when neonatally thymectomized *NFS/sld* mice, one of the murine SS models, are ovariectomized during childbearing age, the autoimmune lesions are severely exaggerated.⁷ Herein, the incidence of autoimmune lesions in *Ar*KO mice is largely increased after 6 months of age, suggesting that aging or an unknown factor may influence the onset or development of autoimmunity in the model. On the other hand, there is no report as for inflammatory lesions in estrogen receptor α or β knockout. It is believed that autoimmune disease is triggered

Figure 6 Relationship between fatty change and the expression of MCP-1 in the salivary glands of *Ar*KO mice. **A:** Frozen sections of salivary glands in 10-month-old WT and *Ar*KO mice were stained with Oil Red O. Images represent three mice from each group. **B:** The expression of MCP-1 mRNA in salivary glands of WT and *Ar*KO mice was analyzed using real-time RT-PCR. Data represent the means $\pm$ SD of three mice from each group. **C:** F4/80⁺ macrophages in the adipose tissue around salivary glands of WT and *Ar*KO mice were detected using immunofluorescence staining. Nuclei were stained with DAPI. The number of F4/80⁺ cells/mm² was counted. Data represent the means $\pm$ SD of five mice from each group. **D:** MCP-1⁺ cells in the salivary glands of WT and *Ar*KO mice were detected using IHC analysis. Nuclei were stained with methyl green. * $P < 0.05$, ** $P < 0.005$. Scale bars: 100 μ m (A, top panels); 20 μ m (A, bottom panels, and D); 50 μ m (C). Original magnifications: $\times 100$ (A, top panels); $\times 400$ (A, bottom panels).

Figure 7 Effect of AI on the autoimmune lesions in SS-model mice. **A:** AI (200 µg) dissolved in CMC was peritoneally injected daily into female Tx-NFS/*sld* mice 4 to 8 weeks of age. Control Tx-NFS/*sld* mice were injected with CMC. Pathological features of lacrimal glands (LGs) and salivary glands (SGs) were determined using H&E staining. Images are representative of five mice from each group. **B:** Pathological grading was evaluated using H&E-stained sections. Data represent the means ± SD of five mice from each group. **C:** The proportion of monocytes that expressed CD11c and F4/80 was analyzed using flow cytometry. Results are representative of five mice from each group. **D:** CD11c⁺DCs and F4/80⁺ macrophages were quantified using flow cytometric analysis. Data represent the means ± SD of five mice from each group. **E:** Serum levels of anti-SSA, anti-SSB, anti-α-fodrin, and anti-ssDNA antibodies were determined using ELISAs. Data represent the means ± SD of five mice from each group. **P* < 0.05. Scale bar = 100 µm (A). Original magnification, ×200 (A).

by multiple factors. Although deficiency in estrogen/estrogen receptor signaling influences immune responses, only deficiency in the signaling cannot break the immune tolerance to induce autoimmunity. Aromatase may be related with various factors for the pathogenesis of SS-like lesions. However, further research on the basis of sex steroid hormone will be required for understanding the molecular pathogenesis of autoimmunity.

Aromatase is considerably expressed in adipose tissues, and *Ar*KO mice develop marked abdominal adiposity, which is accompanied by increases in the weight of the gonadal and infrarenal fat pads.^{13,14} Adipose tissues in obesity disrupt the immune system and influence the pathogenesis of autoimmune disease.^{36,37} Therefore, we determined whether the presence of immune cells in adipose tissues plays a role in autoimmunity in *Ar*KO mice. More important, similar to its

effects on adipose tissue, we show that aromatase/estrogens control adiposity and the production of MCP-1 in the salivary gland, a target organ of SS. We speculate that salivary gland cells with adiposity controlled by aromatase may produce MCP-1 to attract macrophages to the target organ. Accumulated M1 macrophages might secrete inflammatory cytokines to enhance SS-like lesions and function as antigen-presenting cells in the target organ to develop SS-like lesions. These findings suggest that the specificity of the salivary gland as a target organ in SS may be explained by the estrogen deficiency—induced phenotypic change of salivary glands.

Excess adiposity increases the risk of developing metabolic syndrome,³⁸ and obesity is a state of chronic systemic inflammation. Macrophages are considered a crucial component of adipose tissues involved in physiological and pathological remodeling.³⁵ Monocytes are recruited to

adipose tissues in obesity to become M1 macrophages and produce MCP-1, IL-1 β , IL-6, IFN- γ , and TNF- α to promote systemic proinflammatory signaling.^{39,40} In contrast, M2 macrophages produce IL-10 and TGF- β to suppress inflammation.^{39,40} Because IL-1 β and TGF- β are enzymatically cleaved, the production of the tissues was measured by ELISA. Although IL-1 β of WAT from *Ar*KO mice was significantly increased compared with that from WT mice (WT versus *Ar*KO, 120.3 $\pm$ 39.2 versus 191.3 $\pm$ 52.3 pg/WAT; $P < 0.05$), TGF- β was undetectable. It seems to be difficult to quantify the concentration of TGF- β in WAT. M1 macrophages contribute to increased insulin resistance in obesity, and M2 macrophages enhance insulin sensitivity through a peroxisome proliferator-activated receptor γ -dependent mechanism.²² The development of obesity in *Ar*KO mice occurs at 3 months of age and is accompanied by a marked increase in the weights of WAT, suggesting that estrogen synthesis regulates lipid metabolism.¹⁴ However, the report did not indicate whether the increased weight of WAT influences the immune system. Herein, we report that the number of M1 macrophages in the WAT of *Ar*KO mice increased compared with WT mice in addition to the increased WAT. Moreover, our findings suggest that the synthesis of MCP-1 by WAT plays a key role in attracting M1 macrophages to WAT, which induces the preferential production of proinflammatory cytokines.

The role of the aromatase-estrogen axis in the onset or development of autoimmune disease is poorly understood. DCs and macrophages, which are present in WAT and salivary glands, may play a key role in disrupting peripheral immune tolerance and triggering the onset or enhancing the development of SS-like lesions. Increased adiposity in the salivary gland, resembling that of the WAT in *Ar*KO mice, suggests that changes occur in the salivary glands in postmenopausal women with SS. Furthermore, fatty change is observed in the salivary glands of women with SS.⁴¹ Furthermore, a clinical study observed that the expression of MCP-1 is significantly up-regulated in salivary gland tissues from patients with SS compared with those from healthy controls.⁴² Our findings suggest that in *Ar*KO mice, adiposity in the salivary gland was due to estrogen deficiency and further suggest the chain of events as follows: MCP-1 produced by the salivary gland and fat tissue attracted macrophages. The production of several proinflammatory cytokines by these macrophages disrupted local immune tolerance in the salivary gland to trigger the onset of SS. Therefore, estrogen deficiency caused by menopause may link the pathogenesis of SS to an imbalance of cytokine production by macrophages in adipose and target tissues. In addition, we previously demonstrated that estrogen deficiency triggers or promotes apoptosis of salivary gland cells.⁸ Our preliminary experiment showed that increased apoptotic cells were found in aged *Ar*KO mice compared with WT mice. Therefore, it is possible that a part of salivary gland cells with adiposity would undergo apoptosis. Aromatase deficiency results in the low estrogen level or estrogen deficiency. Physiological change of estrogen in females is not

observed in *Ar*KO mice. We think that the normal change of estrogen in females through aromatase maintains physiological homeostasis in immune system and fat metabolism. As described, aging is also related with the pathogenesis of autoimmunity in *Ar*KO mice. Although the precise molecular mechanism is not clear, aromatase may play a key role in the onset or development of SS-like lesions through disturbance of immune cells, adipose tissue, and target organs.

In conclusion, aromatase deficiency resulted in the onset and development of SS-like autoimmune lesions through a switch to cytokine production by M1 macrophages in systemic adipose tissues and target organs. Our findings suggest that decrease of estrogens induced by menopause plays a key role in the pathogenesis of autoimmunity.

Acknowledgments

We thank Ai Katayama, Satoko Katada, and Michiko Kino for technical assistance.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.09.006>.

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