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Autoimmune Regulator Gene (Aire) is Expressed in Lymph Node Fibroblastic Reticular Cell, BLS4

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Autoimmune regulator gene (Aire) is expressed in the thymus and controls the expression of peripheral self-antigens, known as promiscuous genes. Aire and promiscuous genes are involved in T cell tolerance and autoimmunity in the thymus. Here, we identified Aire-expressing fibroblastic reticular cell (FRC), which was derived from mouse lymph node and also expressed in insulin promiscuous antigen. The expression of insulin was increased in cultured FRC over-expressed with Aire. These data suggest that Aire regulates promiscuous gene expression in FRC, and that this function might be under peripheral selection control.

Key words : Autoimmune regulator gene (Aire), FRC, insulin promiscuous gene, peripheral selection

Introduction

Autoimmune diseases resulted from autoreactive T cells are clearly related to a breach of T cell tolerance. T cell tolerance is acquired by most of the peptides presented with MHC molecules of normal cells of the body. Thymic selection of the T cell progenitors migrated via blood vessels from bone marrow provides the foundation for immunological maturation and tolerance of T cell. Negative selection in the thymus is of importance for the prevention of autoimmunity caused by the absence of the tissue-specific antigens that contribute to negative selection of the developing thymocytes in the thymus [14]. These central tolerance carried out in thymus involves medullary thymic epithelial cells (mTECs), which express endogenously promiscuous genes to remove self-reactive T cells [18]. Recently, a few autoreactive T cells escape negative selection and reach the periphery such as lymph node (LN), where peripheral selection is required to avoid autoimmunity [13,17].

Autoimmune regulator gene (Aire) was identified from positional cloning [16], modulates the expression of promiscuous genes and promotes central tolerance in thymus. The Aire gene is also expressed in LN, thus suggesting that the Aire gene may play a critical role in both the central and peripheral control of immune reaction [3,8]. However, Aire-expressing cell and the exact role of Aire gene still remain uncertain in periphery such as LN.

Fibroblastic reticular cell (FRC) is one of Aire-expressing candidate cells in LN. Because FRC supports and guides T cell within the paracortical region of LN [2] FRC interacts with T cell for immune response.

We herein report that the Aire gene is restrictively expressed in FRC, BALB/c LN stroma (BLS) 4 cells [7,10] derived from peripheral lymph node, thereby suggesting that the Aire gene may play a role in peripheral regulation of immune response.

Materials and Methods

Cells

FRC primary cell (BLS4) was originally a gift from Dr. Tomoya Katakai [10]. Cell maintained in 10% FCS - DMEM medium added with penicillin/streptomycin in CO_2 incubator. When necessary, BLS4 cells were stimulated with 10 ng/ml TNF_{α}, 10 ng/ml IL-1ß, 10 ng/ml IL-4 (Peprotech, London, UK) or 10 μg/ml LPS (Sigma, St Louis, MO, USA) for 48 hr.

Reverse transcription - PCR

Total RNA was extracted from BLS4 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For semi-quantita-

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tive reverse transcription (RT) - PCR analysis, oligo(dT)12 - 18-primed cDNA was synthesized from the total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was amplified by PCR with ExTaq DNA polymerase (Takara, Japan) and the following specific primer pairs : GAPDH, 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′; for the nested PCR about Aire, 1° forward primer, 5′-TCGTGGGACTGAG GTCAGCTTC-3′ 1° reverse primer, 5′-GGCATCCTGC AGTGGGCCATCCAGAG-3′ and 2° forward primer, 5′-TCGTGGGACTGAGGTCAGCTT-3′, 2° reverse primer, 5′-AGACACCACCCTTCTCTTCCTGA-3′. Insulin, 5′-TCTC CAGCTGGTAGAGGG-3′ and 5′-CAGCCCTTAGTGACCAGC-3′ Spt1, 5′-AACTTCTGGAACTGCTGATTCTG-3′ and 5′-GA GGCCTCATTAGCAGTGTTG-3′; Mup1, 5′-TCTGTGACG TATGATGGATTCAA-3′ and 5′-TCTGGTTCTCGGCCAT AGAG-3′; Tff3, 5′-TACGTTGGCCTGTCTCCAAG-3′ and 5′-CAGGGCACATTTGGGATACT-3′; Igf2, 5′-TGGGGGT GGGTAAGGAGAAACCT-3′ and 5′-GGCCCCGGAGAGAC TCTGTGC-3′; Sp2, 5′-GGCTCTGAAACTCAGGCAGA-3′ and 5′-TGCAAACTCATCCACGTTGT-3′; β-actin 5′-TGG AATCCTGTGGCATCCATGAAAC-3′ and 5′-AAAACGC AGCTCAGTAACAGTCCG-3′

Plasmid

Full cDNA fragment encoding mouse Aire gene was amplified from BLS4 total RNA with PCR. Cloning primer pairs for full length of Aire cDNA: EcoRV: 5-CCGATATCATGGGCAGGTGGGGATGGAATGC-3′, NotI: 5′-TTGCGGCCGCTCAGGAAGAGAAGGGTGGTG-3′. The PCR products were sub-cloned into expression vectors (pcDNA3.1, Invitrogen) and evaluated via sequence analysis.

Transfection of BLS4 with mouse Aire cDNA

For transient transfection, cells were transfected by lipofectamine method (Invitrogen). When BLS4 cells were 70-80% confluent, the cell media were 400 µl OptiMEM (Invitrogen, Carlsbad, CA, USA) two hours before transfection. The transfection media were prepared as follows: (a) 0.8 µg plasmid DNA was diluted into 50 µl final volume of OptiMEM; (b) 2µl Lipofectimine 2000 was diluted into 50 µl final volume of OptiMEM; and (c) the Lipofectimine-containing solution was mixed with the plasmid-containing solutions, and incubated at RT for 20 min. Subsequently 25 µl of pcDNA3.1-Aire plasmid/lipofectamine solution was added to each well. The cells were incubated at 37°C, 5% CO_2 for 4 hr. After the incubation the cells were washed in DMEM containing 10% FBS and incubated for 24 hr.

Western blotting

BLS4 cells were washed with PBS and lysed in 1% NP-40, 120 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin. The cell lysates quantified by BCA method were separated by 10% SDS-PAGE and transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Specific bands were detected using polyclonal anti-Aire antibody (Millipore, Bedford, MA, USA) followed by goat anti-rabbit antibody conjugated with HRP (Jackson, Bar Harbor, ME, USA) and chemiluminescent reaction using ECL Plus (Amersham Biosciences).

Immunofluorescence microscopy

Immunofluorescent staining of BLS4 cells with primary and second antibodies was performed by 4% paraformalhdehyde in PBS for 30 min in room temperature. Treated samples were permeabilized with 0.2% Tween-20/PBS for 15 min, and blocked for 1 h at RT in PBS with 5% BSA. Primary antibody was added for 1 hr at RT in blocking solution. After washing phycoerythrin goat anti-rabbit IgG (1/500) in blocking solution was added for 1 hr at RT. Nucleus was visualized with DAPI. Samples were observed using immunofluorescence microscopy (TND330; Nikon).

Results

Expression analysis of Aire in mouse tissue and cells

The Aire mRNA is expressed in a few cells in the thymus medulla [15]. In this study we attempted to evaluate the expression pattern with Aire specific primers for lymph node (LN), lung and liver. Positive signal in LN was detected, whereas mouse lung and liver tissue show no signal (Fig. 1).

Aire mRNA analysis in mouse lymph node derived fibroblastic reticular cell, BLS4

Mouse lymph node derived FRC, BLS4 was established previously [10]. To detect expression of Aire, total RNA of BLS4 was synthesized using oligo (dT) 12 - 18-primed cDNA. Nested PCR was applied to amply mArie expression in BLS4. The first round of PCR amplification revealed Aire specific band (arrow) and non-specific band (Fig. 2A).

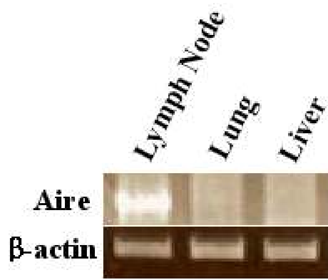

Fig. 1. Aire is expressed in the lymph node. Total RNA was purified from lymph node, lung and liver tissue. cDNA was synthesized by manufacture protocol. RT-PCR was carried out about specific Aire primers.

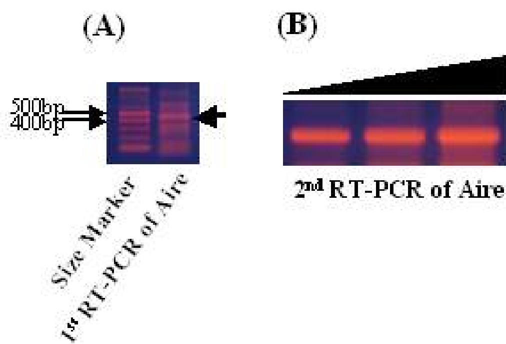

Fig. 2. Aire is expressed BLS4 cell. Nested RT-PCR was carried out. Total RNA was purified from BLS4 and cDNA was synthesized according to the manufacture protocol. Aire was amplified at first round PCR (A, arrow) and specific Aire PCR products were obtained from second round PCR (B, serial five-fold dilution).

However, Aire specific band was amplified by second round PCR using the first PCR products as templates (Fig. 2B).

Analysis of promiscuous gene expression in BLS4

Aire was expressed in BLS4 cells as described in Fig 2. We checked the expression of several promiscuous genes such as insulin, Igf2, Sp2 [1], Tff3 [12], Ins2 [4], Mup1 [12], Spt 1 [12], which were expressed in the thymus for negative selection of thymocytes. Surprisingly, insulin promiscuous gene was expressed in BLS4 cell (Fig. 3), however, no signal was detected in other promiscuous genes described in materials and methods. Although other promiscuous genes were not detected in BLS4 cell, the result suggests the involvement of insulin for peripheral selection.

Involvement of Aire in the expression of insulin

The expression levels of many promiscuous gene expressions are significantly decreased in Aire knockout mice in the thymus [4]. To determine whether the over-expression of Aire has effect on insulin expression we used an over-expression system of Aire (pcDNA3.1-Aire versus pcDNA3.1). Transfection with the pcDNA3.1-Aire resulted in an increased production of Aire protein (Fig. 4A). The pcDNA3.1-Aire transfection also resulted in an increase of insulin transcripts (Fig. 4B). This indicates that Aire controls the ex-

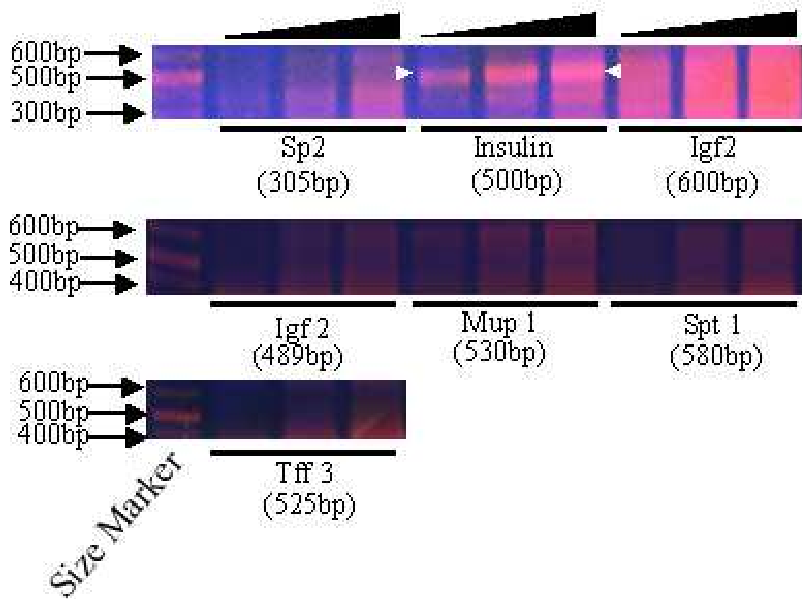

Fig. 3. Insulin transcripts are detected in BLS4 cell. After synthesis of cDNA, RT-PCR was carried out. Insulin was detected from BLS4 cell (arrowhead), however, other genes (Sp2, Igf2 etc) were not amplified from BLS4 (serial five-fold dilution).

Fig. 4. Insulin gene is controlled by Aire protein in BLS4 cell. (A) Enhanced Aire protein was detected with Aire specific antibody after transfection of pc-DNA3.1-Aire plasmid in BLS4. (B) RT-PCR was carried out for insulin gene after total RNA purification from BLS4 transfected with pc-DNA3.1-Aire plasmid.

pression of insulin in BLS4 cell.

Subcellular localization of Aire was detected with immunofluorescence microscopy

Observed image revealed the protein as concentrated around the nuclear including dot-like spot in nuclear of BLS4 cells (Fig. 5). It seems that Aire localizes in or around nucleus to function as a transcription regulator for Aire dependent genes.

Aire expression on inflammatory agent

The regulatory signaling mechanisms of insulin in BLS4 are unclear. LN is a place of immune responses for inflammation. Several studies revealed that transcription of Aire was promoted by LTβR-TRAF6-NIK-NF-κB pathway [19,20]. To investigate Aire expression levels, BLS4 cells were treated with LPS, IL-1β, TNF-α, IL-4. However, Aire expression levels show similar levels (Fig. 6). This indicates that the expression of Aire controls by other stimulator instead of inflammatory agents.

Discussion

In this study we used mouse lymph node derived FRC, BLS4 cells. At first, this cell was isolated for the cell biological role and mechanism of lymph node about providing

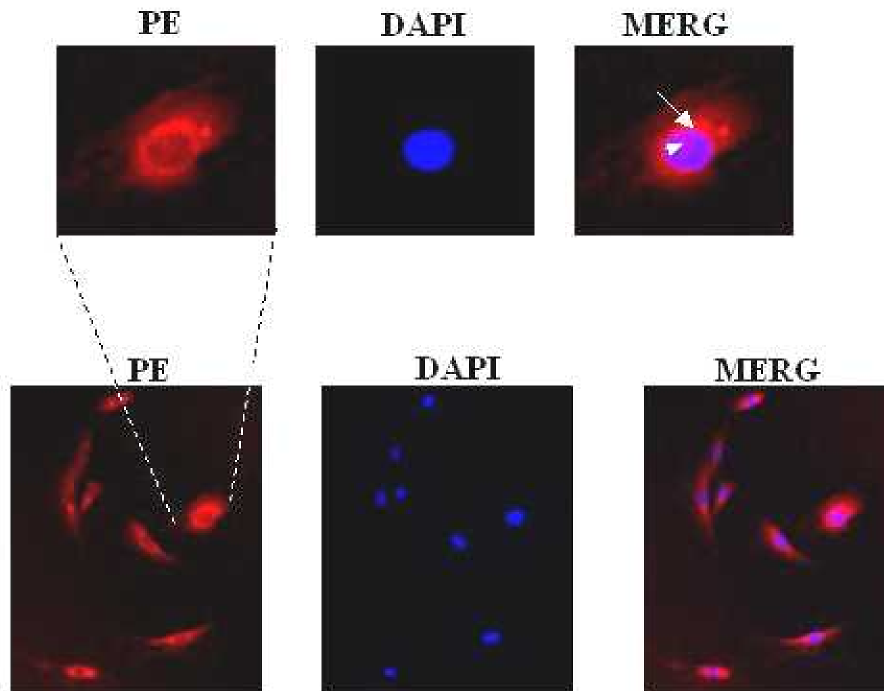

Fig. 5. Aire protein was accumulated around and dot-spotted in nucleus of BLS4. BLS4 was stained with Aire specific antibody interacted with PE-conjugated with secondary antibody.

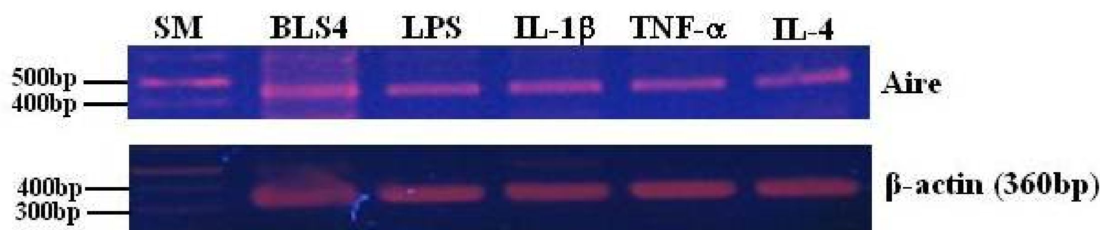

Fig. 6. Relationships between inflammatory agent and Aire expression in BLS4 cell. Aire transcript was amplified from BLS4 treated with indicated inflammatory agents. β-actin was used as a technical control.

T cell microenviroment [10]. So far, the function of Aire has been focused in T cell selection in thymus. Especially, Aire was expressed in medullar epithelial cell. Aire was involved in Aire dependent self-antigen expressions and functioned central negative selection of thymocytes [14,18]. Aire regulates a number of promiscuous genes such as insulin, insulin-like growth factor2 (Igf2), salivary protein2 (SP2) in the thymus. Recent report also suggests that Aire is detected among CD45-negative secondary lymphoid stroma [5] and the secondary lymphoid organs may also be a place of promiscuous expression for tissue-specific antigens, though the relationship between Aire and peripheral selection remains contentious [6]. The detection of Aire at low levels in various tissues including secondary lymphoid organs of mice and humans may be due to the existence of rare migrating cells such as monocyte/dendritic cell lineages [9,11]. However, the debate about Aire expression in secondary lymphoid organs continues. Here we demonstrate that the murine Aire and insulin promiscuous gene are first identified in lymph node derived FRC, BLS4 cells. Transfection of Aire plasmid revealed that the expression levels of insulin increased in BLS4 cells. This suggests that Aire specifically promotes insulin gene expression in BLS4 cell. So, this research suggests the possibility about peripheral selection between FRC and peripheral T cells. Further studies to clarify the role of the Aire gene about interaction mechanisms between peripheral immune cell and lymph node stroma about controlling autoimmunity in secondary lymphoid tissue are thus called for.

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References

1. Anderson, M. S, E. S., Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1348-1349.
2. Bajénoff, M., J. G. Egen, L. Y. Koo, J. P. Laugier, F. Brau, N. Glaichenhaus, and R. N. Germain. 2006. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity* **25**, 989-1001.
3. Björses, P., M. Pelto-Huikko, J. Kaukonen, J. Aaltonen, L. Peltonen, and I. Ulmanen. 1999. Localization of the APECED protein in distinct nuclear structures. *Hum. Mol. Genet.* **8**, 259-266.
4. Chen, J., W. Yang, C. Yu, and Y. Li. 2008. Autoimmune regulator initiates the expression of promiscuous genes in thymic epithelial cells. *Immunol. Invest.* **37**, 203-214.
5. Gardner, J. M., J. J. Devoss, R. S. Friedman, D. J. Wong, Y. X. Tan, X. Zhou, K. P. Johannes, M. A. Su, H. Y. Chang, M. F. Krummel, and M. S. Anderson. 2008. Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science* **321**, 843-847.
6. Gardner, J. M., A. L. Fletcher, M. S. Anderson, and S. J. Turley. 2009. AIRE in the thymus and beyond. *Curr. Opin. Immunol.* **21**, 582-589.
7. Hara, T., T. Katakai, J. H. Lee, Y. Nambu, N. Nakajima-Nagata, H. Gonda, M. Sugai, and A. Shimizu. 2006. A transmembrane chemokine, CXC chemokine ligand 16, expressed by lymph node fibroblastic reticular cells has the potential to regulate T cell migration and adhesion. *Int. Immunol.* **18**, 301-311.
8. Heino, M., P. Peterson, J. Kudoh, K. Nagamine, A. Lagerstedt, V. Ovod, A. Ranki, I. Rantala, M. Nieminen, J. Tuukkanen, H. S. Scott, S. E. Antonarakis, N. Shimizu, and K. Krohn. 1999. Autoimmune regulator is expressed in the cells regulating immune tolerance in thymus medulla. *Biochem. Biophys. Res. Commun.* **257**, 821-825.
9. Heino, M., P. Peterson, N. Sillanpää, S. Guérin, L. Wu, G. Anderson, H. S. Scott, S. E. Antonarakis, J. Kudoh, N. Shimizu, E. J. Jenkinson, P. Naquet, and K. J. Krohn. 2000. RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD

mouse. *Eur. J. Immunol.* **30**, 1884-1893.
10. Katakai, T., T. Hara, M. Sugai, H. Gonda, and A. Shimizu. 2004. Lymph node fibroblastic reticular cells construct the stromal reticulum via contact with lymphocytes. *J. Exp. Med.* **200**, 783-795.
11. Kogawa, K., S. Nagafuchi, H. Katsuta, J. Kudoh, S. Tamiya, Y. Sakai, N. Shimizu, and M. Harada. 2002. Expression of AIRE gene in peripheral monocyte/dendritic cell lineage. *Immunol. Lett.* **80**, 195-198.
12. Kont, V., M. Laan, K. Kisand, A. Merits, H. S. Scott, and P. Peterson. 2008. Modulation of Aire regulates the expression of tissue-restricted antigens. *Mol. Immunol.* **45**, 25-33.
13. Lee, J. W., M. Epardaud, J. Sun, J. E. Becker, A. C. Cheng, A. R. Yonekura, J. K. Heath, and S. J. Turley. 2007. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat. Immunol.* **8**, 181-190.
14. Liston, A., S. Lesage, J. Wilson, L. Peltonen, and C. C. Goodnow. 2003. Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* **4**, 350-354.
15. Mittaz, L., C. Rossier, M. Heino, P. Peterson, K. J. Krohn, Gos A, M. A. Morris, J. Kudoh, N. Shimizu, S. E. Antonarakis, and H. S. Scott.1999. Isolation and characterization of the mouse Aire gene. *Biochem. Biophys. Res. Commun.* **255**, 483-490.
16. Nagamine, K., P. Peterson, H. S. Scott, J. Kudoh, S. Minoshima, M. Heino, K. J. Krohn, M. D. Lalioti, P. E. Mullis, S. E. Antonarakis, K. Kawasaki, S. Asakawa, F. Ito, and N. Shimizu.1997. Positional cloning of the APECED gene. *Nat. Genet.* **17**, 393-398.
17. Poliani, P. L., K. Kisand, V. Marrella, M. Ravanini, L. D. Notarangelo, A. Villa, P. Peterson, and F. Facchetti. 2010. Human Peripheral Lymphoid Tissues Contain Autoimmune Regulator-Expressing Dendritic Cells. *Am. J. Pathol.* [Epub ahead of print]
18. Ruan, Q. G., K. Tung, D. Eisenman, Y. Setiady, S. Eckenrode, B. Yi. S. Purohit, W. P. Zheng, Y. Zhang, L. Peltonen, and J. X. She. 2007. The autoimmune regulator directly controls the expression of genes critical for thymic epithelial function. *J. Immunol.* **178**, 7173-7180.
19. Xiao, W. 2004. Advances in NF-κB signaling transduction and transcription. *Cell Mol. Immunol.* **1**, 425-435.
20. Zhu, M., R. K. Chin, P. A. Christiansen, J. C. Lo, X. Liu, C. Ware, U. Siebenlist, Y. X. Fu. 2006. NF-κB2 is required for the establishment of central tolerance through an Aire-dependent pathway. *J. Clin. Invest.* **116**, 2964-2971.

초록 : Autoimmune regulator gene (Aire)의 마우스 림프절 FRC세포, BLS4에서 발현

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Autoimmune regulator gene (Aire)는 흉선에서 발현되며 promiscuous genes으로 알려진 흉선에서 자가항원 발현을 조절한다. Aire 와 promiscuous genes은 흉선에서 T세포 tolerance와 자가면역에 관여한다. 말초 조직 즉 림프절에서 Aire의 역할을 알아보고자 림프절 구성 세포중 하나인 fibroblastic reticular cell (FRC)을 분리 확립하였다. 마우스 림프절로부터 분리된 FRC에서 Aire의 발현을 확인하였고 또한 promiscuous antigen인 insulin의 발현도 확인하였다. Aire 과발현 플라스미드로 형질전환 후 배양 FRC에서 Insulin의 발현이 증가하였다. 이것은 Aire가 FRC에서 promiscuous gene의 발현을 조절한다는 것을 보여주며 peripheral selection과 연관되어 있을 수 있다는 것을 제시한다.