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Mini Review

Analysis of disease-pathways by susceptibility genes in primary biliary cirrhosis

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High concordance rate in monozygotic twins and familial clustering of patients with primary biliary cirrhosis (PBC) indicate the involvement of strong genetic factors in the development of PBC. To identify susceptibility loci for PBC in Japanese population, a genome-wide association study (GWAS) and subsequent replication study were performed in a total of 1327 PBC cases and 1120 healthy controls. Two significant ($p<5\times10^{-8}$) non-HLA susceptibility loci (*TNFSF15* and *POU2AF1*) for PBC were identified. In addition, 10 loci (*CD80, IKZF3, IL7R, NFKB1, STAT4, CXCR5, TNFAIP2, MAP3K7IP1, rs6974491, DENND1B*) out of 21 non-HLA susceptibility loci for PBC which were recently identified in European descent showed significant associations in Japanese population. These results indicated the importance of two disease-pathways in both European descent and Japanese population, Th1/Th17 differentiation of T cells (CD80, IL12A, IL12RB2, STAT4, TNFSF15) and B cell differentiation to plasma cells (IL7R, CXCR5, POU2AF1, SPIB, IKZF3), although there are some ethnic differences in disease-susceptibility loci for PBC. In addition, the study for systemic and local expression of TNF-like ligand 1A (TL1A), which is encoded by *TNFSF15*, indicated that TL1A may be involved in the pathogenesis of PBC.

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Introduction

Primary biliary cirrhosis (PBC) is a chronic and progressive cholestatic liver disease, presumably caused by autoimmune reactions against biliary epithelial cells, leading to liver cirrhosis and hepatic failure[1, 2]. High concordance rate in monozygotic twins compared to dizygotic twins and familial clustering of PBC patients indicate the involvement of strong genetic factors in the development of PBC[3]. Pre-

Table 1 Clinical Charcteristics of PBC cases

	GWAS (n=487)	Replication (n=808)
cases : M/F	57/430	120/688
age : range years	33-90	24-85
mean + SD years	64.7+11.3	61.1+11.4
clinical stage : 1	320	646
2	110	121
3	57	39
AMA positive (%)	87.3	86.4
Other autoimmune diseases		
Sjogren's syndrome	12.5	14.5
Autoimmune thyroiditis	5.5	10.8
Rheumatoid arthritis	3.7	4
Systemic sclerosis	3.3	3.3
CREST syndrome	1.8	1.7

Clinical stage 1: a stage without any signs indicating portal hypertension or liver cirrhosis
Clinical stage 2: a stage with signs of portal hypertension or liver cirrhosis but without persistent jaundice
Clinical stage 3: a stage with persistent presence of jaundice (total bilirubin>2mg/dL)

vious genome-wide association study (GWAS) and subsequent meta-analyses have identified *HLA* and 21 non-HLA susceptibility loci (*IL12A, IL12RB2, STAT4, IRF5, IKZF3, MMEL1, SPIB, DENND1B, CD80, IL7R, CXCR5, TNFRSF1A, CLEC16A, NFKB, RAD51L1, MAP3K7IP1, PLCL2, RPS6KA4, TNFAIP2*, 7p14 and 16q24) for PBC in European descent[4-7], indicating the involvement of several autoimmune-pathways (i.e. IL12/IL12R signaling, TNF/TLR-NFκB signaling and B cell differentiation) in the development of PBC. To identify host genetic factors related with PBC in Japanese population, we conducted a GWAS and subsequent replication study in a total of 1327 PBC cases and 1120 healthy controls and identified two novel disease-susceptibility genes (*TNFSF15* and *POU2AF1*)[8]. We also validated 10 loci out of 21 non-HLA susceptibility loci for PBC which have been identified in European descent[8]. These results would not only expand our knowledge of disease-pathways in PBC but also lead to develop a rationale for therapies in the future.

Disease-susceptibility genes for PBC in Japanese population

1)Clinical characteristics of PBC cases used for GWAS

Samples from 2,395 individuals (1,295 cases with PBC and 1,100 healthy controls) were collected in PBC-GWAS Consortium, consisting of 31 hospitals participating in the NHO Study Group for Liver Disease in Japan (NHOSLJ) and 24 University Hospitals participating in gp210 Working Group in Intractable Liver Disease Research Project Team of the Ministry of Health and Welfare in Japan[8]. The patients were diagnosed with PBC if they met at least two of the following internationally accepted criteria: biochemical evidence of cholestasis based mainly on alkaline phosphatase elevation, presence of serum anti-mitochondrial antibodies, histological evidence of non-suppurative destructive cholangitis and destruction of interlobular bile ducts[1,2]. The demographic details of PBC cases were summarized in Table 1.

2)Novel disease-susceptibility genes for PBC in Japanese population

The 1,015 samples (515 Japanese PBC cases and 500 Japanese healthy controls) were genotyped for 600,000 SNPs using the Affymetrix Axiom Genome-Wide ASI 1 Array. After quality check of genotyping data (Dish QC <0.82, overall call rate <97%, outlier in PCA : p<0.05) and SNP filtering (SNP call rate < 95%, MAF < 5%, HWE p-value <0.001), the data of 487 PBC cases and 476 healthy controls for 420,928 SNPs were used for the association analysis[8]. The inflation factor lambda was 1.039 and 1.026, respectively, for all the tested SNPs and SNPs without HLA region. For the GWAS and replication study, a chi-square test was applied to a two-by-two contingency table in an allele frequency model.

Figure 1 shows a genome-wide view of the single-point association data based on allele frequencies (Manhattan plot). The *HLA-DQB1* locus showed the strongest association with susceptibility to PBC (rs9275175, OR = 1.94;

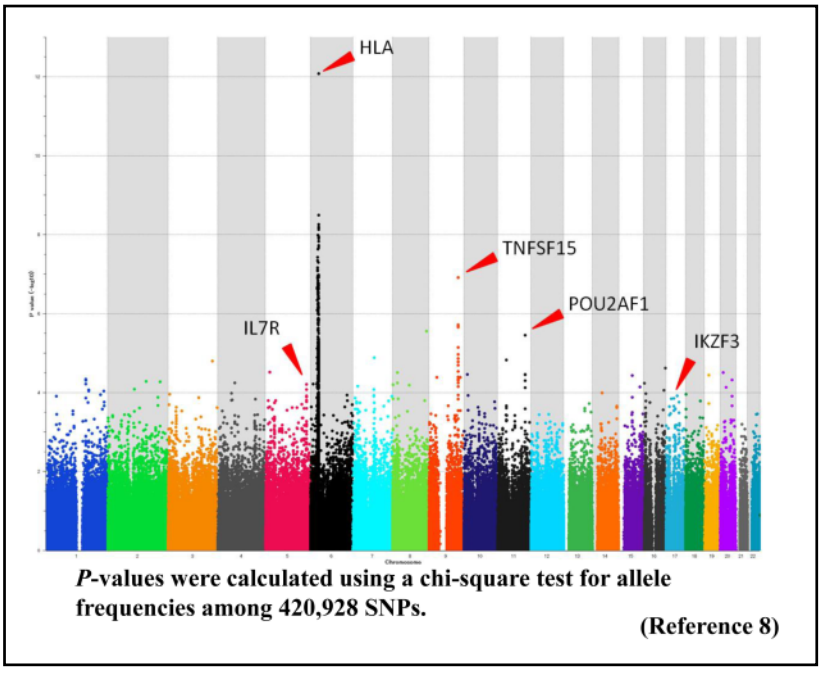

Fig.1 Manhattan plot of 963 samples (487 PBC cases and 476 healthy controls) in Japanese population. In addition to the most significant disease-susceptibility loci of HLA (p=8.3x10^{-13}), the loci of *TNFSF15* and *POU2AF1* showed evidence indicative of association with PBC (p=1.21x 10^{-7} and p= 3.51x10^{-6}, respectively).

95%CI=1.62-2.33, p=8.30 × 10^{-13}) and the loci *TNFSF15* and *POU2AF1* showed evidence indicative of association with PBC (*TNFSF15* rs4979462-T: OR=1.63; 95%CI=1.36-1.95, p=1.21 × 10^{-7}; *POU2AF1* rs4938534-A, OR=1.53; 95%CI=1.28-1.83, p=3.51 × 10^{-6}). In a subsequent replication analysis, 27 SNPs with p<0.0001 in the initial GWAS were genotyped in addition to high density association mapping at *TNFSF15* and *POU2AF1 loci* using an independent set of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) by the DigiTag2 and custom TaqMan SNP genotyping assays. The strongest associations identified in the initial GWAS were replicated for *TNFSF15* rs4979462-T (OR=1.56; 95% CI=1.39-1.76, p=2.84 × 10^{-14}) and *POU2AF1* rs4938534-A (OR=1.39; 95% CI=1.24-1.56, p=2.38 × 10^{-8}) (Table 2). The other 27 weakly associated SNPs identified in the initial GWAS (p<0.0001) did not show significant association with PBC.

3)Replication study of susceptibility genes identified in European descent

Next, we focused on 21 loci that are reportedly associated with susceptibility to PBC in European descent[4-7]. Three loci (*IL7R, IKZF3, STAT4*) had p values of less than 0.001 and 8 other loci (*RAD51L1, CXCR5, PLCL2, IL12RB2, NFKB1, CD80, DENND1B*, and 7p14) showed evidence of marginal associations (p<0.05) in the initial GWAS in 487 Japanese PBC cases and 476 Japanese healthy controls. Three SNPs (*IL7R* rs6890503, *IKZF3* rs9303277, *STAT4* rs7574865) were genotyped in an independent set of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) using the DigiTag2 and custom TaqMan SNP genotyping assays. The *IL7R* rs6890853 and *IKZF3* rs9303277 showed significant associations (p value = 3.66 × 10^{-8}, OR=1.47 and p value=3.66 × 10^{-9}, OR=1.44, respectively) and *STAT4* rs7574865 showed suggestive association with PBC (p value=1.11 × 10^{-6}, OR=1.35) in 2,365 Japanese samples (1,274 PBC cases and 1,091 healthy controls) (Table 2). Genotyping of additional 16 SNPs, which are the same SNPs as identified in previous studies[4-7], revealed that six SNPs located on *CXCR5, NFKB1, CD80, DENND1B, MAP3K7IP1*, and *TNFAIP2* were replicated (p<0.05) in 2,365 Japanese samples (Table 2). The SNP *CD80* rs2293370 showed a significant association (p value=3.04 × 10^{-9}, OR=1.48) and *NFKB1* rs7665090 showed a suggestive association (p value=1.42 × 10^{-7}, OR=1.35) with PBC in Japanese population. Although further study for determining the primary SNP at each locus is necessary, the remaining 10 loci (*RAD51L1, PLCL2,*

Table 2 Disease-susceptibility genes for PBC identified by GWAS

gene	chromosome	function	reports				OR	p-value[4]	association with other autoimmune diseases PBC[5]
			Canada[1]	Italy・Canada[2]	England[3]	Japan[4]			
HLA-DQB1	6p21.3	antigen presentation	○	○	○	○			many autoimmune diseases
TNFSF15	9q32	costimulation for Th1,Th17 cells				○	1.56	2.84×10^{-14}	CD, UC, AS
POU2AF1	11q23.1	differentiation of B cells to plasma cells				○	1.39	2.38×10^{-8}	none
IL12A	3q25.33-q26	IL12 signaling, Th1 differentiation	○	○	○				celiac disease, MS
IL12RB2/SCHIP1	1p31.2	IL12 signaling, Th1 differentiation	○	○	○				psoriasis, CD, UC, AS, SSc, BD
STAT4	2q32	IL12 signaling, Th1 differentiation			○	○	1.35	1.11×10^{-6}	RA, SLE, SjS, SSc, psoriasis
IRF5/TNPO3	7q32.1	TLR-IFN signaling		○	○				SLE, RA, SSc, SjS, UC
IKZF3-ZPBP2-GSDMB-ORMDL3	17q12-21	B cell/epithelial cell differentiation/apoptosis, regulation of ER stress		○	○	○	1.44	3.66×10^{-9}	asthma, CD, T1D, UC
MMEL1	1p36	membrane metallo-endpeptidase-like 1		○	○				RA, celiac disease, MS
SPIB	19q13	B cell differentiation		○	○				none
DENND1B	1q31	guanine exchange factors(GEFs)for RAB35, phagocytosis			○	○	1.14	4.05×10^{-2}	childhood asthma, CD
CD80	3q13	costimulation for T cells			○	○	1.48	3.04×10^{-9}	celiac disease, JIA, AD
IL7R	5p13	lymphocyte maturation and differentiation			○	○	1.47	3.66×10^{-8}	MS, UC
CXCR5	11q23	receptor for CXCL13, essential for B cell migration			○	○	1.42	4.11×10^{-4}	none
TNFRSF1A	12p13	TNFa receptor、TNFa-NFkB signaling、apoptosis			○				MS
CLEC16A	16p13	C type lectin containing family			○				MS, RA, CD, T1D, celiac disease
NFKB1	4q24	transcriptional factor regulating various genes			○	○	1.35	1.42×10^{-7}	none
RAD51L1	14q24	DNA repair			○				none
MAP3K7IP1(TAB1)	22q13	IL1/TLR-NFkB signaling, TGFb signaling			○	○	1.29	8.59×10^{-4}	CD
rs6974491	7p14	intergenic			○	○	1.33	4.98×10^{-3}	none
rs11117432	16q24	intergenic			○				none
PLCL2	3p24	negative regulation of B cell receptor-signaling			○				none
RPS6KA4	11q13	inhibition of cytokine production induced by TLR signaling			○				none
TNFAIP2	14q32	TNFa induced protein 2			○	○	1.22	6.34×10^{-4}	none

1)reference 4, 2)reference 5, 6, 3)reference 7, 4)reference 8, 5)associated diseases reported from Jan. 2007 to July 2012
○ : disease-susceptibility genes reported in each paper
CD: Crohn's disease, UC: ulcerative colitis, AS: ankylosing spondylitis, SSc: systemic sclerosis, RA :rheumatoid arthritis, SLE: systemic lupus Erythematodes: T1D; type 1 diabetes, AT; autoimmune thyroiditis, SjS; Sjogren syndrome, MS; multiple sclerosis, BD; Behcet's disease, JIA; juvenile idiopathic arthritis, AD; atopic dermatitis

IL12RB2, IRF5, SPIB, RPS6KA4, CLEC16A, TNFRSF1A, IL12A, and *MMEL1*) did not show significant association (p<0.05) with PBC in the Japanese population (Table 2).

Disease-pathways for PBC

1)TNFSF15 and T cell differentiation to Th1/Th17 cells

TNFSF15 (also known as TL1-A) is a cytokine of the TNF superfamily which is mainly produced by dendritic cells (DC) and macrophage by stimulation with TLR ligands. TNFSF15 interacts with death receptor 3 (DR3, also known as TNFRSF25) not only to promote effector T-cell expansion (i.e., Th1 and Th17 cells) and cytokine production (i.e., interferon-γ and IL17) at the site of inflammation, but also to induce apoptosis in cells that over-express DR3[9,10]. As shown in Figure 2, Th1 differentiation pathways include costimulation of T cells with CD80, IL-12/IL12R signaling *via* STAT4, and costimulation of Th1-committed cells with TNFSF15. Th17 differentiation pathways include costimulation of T cells and Th17-committed cells with CD80 and TNFSF15, respectively[9-12]. The variants of *IL12A* and *IL12RB* have been identified as PBC susceptibility loci in European descent but not in Japanese population[4-8]. Variants of *CD80* and *STAT4* have been identified as PBC susceptibility loci in both European descent and Japanese population[4-8], and a variant of *TNFSF15* has been identified as PBC susceptibility loci in Japanese population[8]. These results may indicate that the same Th1/Th17 differentiation pathways are involved in the pathogenesis of PBC in both Japanese populations and European descent, although there are some ethnic differences in susuceptibility genes for PBC.

2)POU2AF1 and B cell differentiation to plasma cells

As shown in Figure 3, POU2AF1 is a B cell-specific transcriptional factor that coactivates octamer-binding transcriptional factors OCT-1 and OCT-2 on B cell-specific promoters[13]. Thus, POU2AF1 is essential for B cell maturation and germinal center formation[13]. The E-twenty six (Ets) transcription factor SPIB is also an essential mediator of B-cell receptor signaling[14]. SPIB was recently identi-

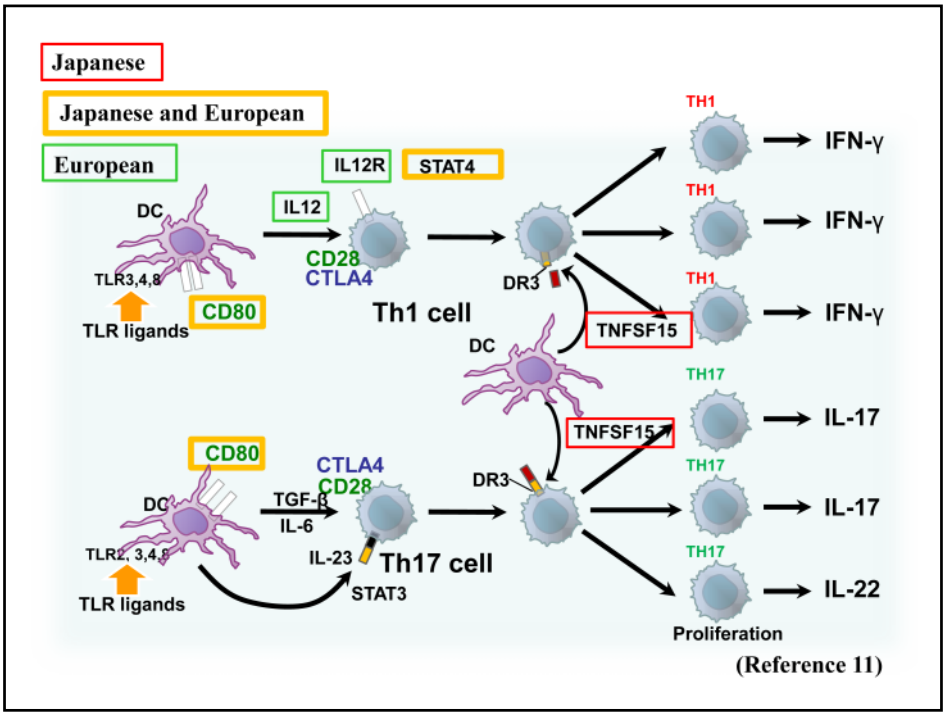

Fig.2 T lymphocyte differentiation and TNFSF15

TNFSF15, the most significant disease-susceptibility gene for PBC in Japanese population, plays a role for Th1/Th17 differentiation. The *IL12A* and *IL12RB2*, the most significant disease-susceptibility gene for PBC in European descent, also plays a role for Th1/Th17 differentiation. The *CD80* and *STAT4*, the significant disease-susceptibility gene for PBC in both European descent and Japanese population, also play a role for Th1/Th17 differentiation.

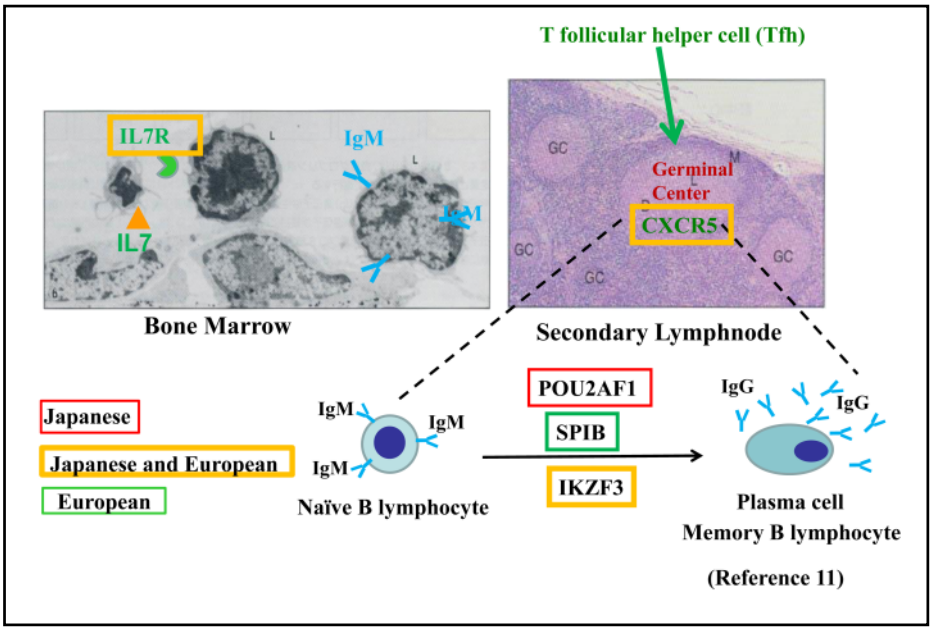

Fig.3 B lymphocyte differentiation and POU2AF1

POU2AF1, a significant disease-susceptibility gene for PBC in Japanese population, plays a role for B cell differentiation to plasma cells. SPIB, a significant disease-susceptibility gene for PBC in European descent, also plays a role for B cell differentiation to plasma cells. IL7R, CXCR5 and IKZF3, significant disease-susceptibility genes for PBC in both European descent and Japanese population, play roles for B cell maturation to naïve B cell in bone marrow, migration of T follicular helper cell to germinal center of secondary lymph node, and B cell maturation to plasma cells, respectively.

fied as a direct target of the coactivator POU2AF1[15], indicating the essential role of SPIB/POU2AF1 for B cell differentiation to plasma cells. IKZF3 functions as a transcription factor that participates in the generation of high-affinity bone marrow plasma cells responsible for long-term immunity[16], and IL7R participates in differentiation and maturation of lymphocytes in bone marrow[17]. CXCR5 is essential for the migration of T follicular helper cells (Tfh) to germinal center, where naïve B cells receive B cell receptor signaling and a help by Tfh for B cell differentiation to plasma cells[18]. Variation of *SPIB* has been identified as a PBC susceptibility gene in European descent[6, 7]. Variation of POU2AF1 has been identified as a PBC susceptibility gene in Japanese population[8]. Variation of IKZF3 has

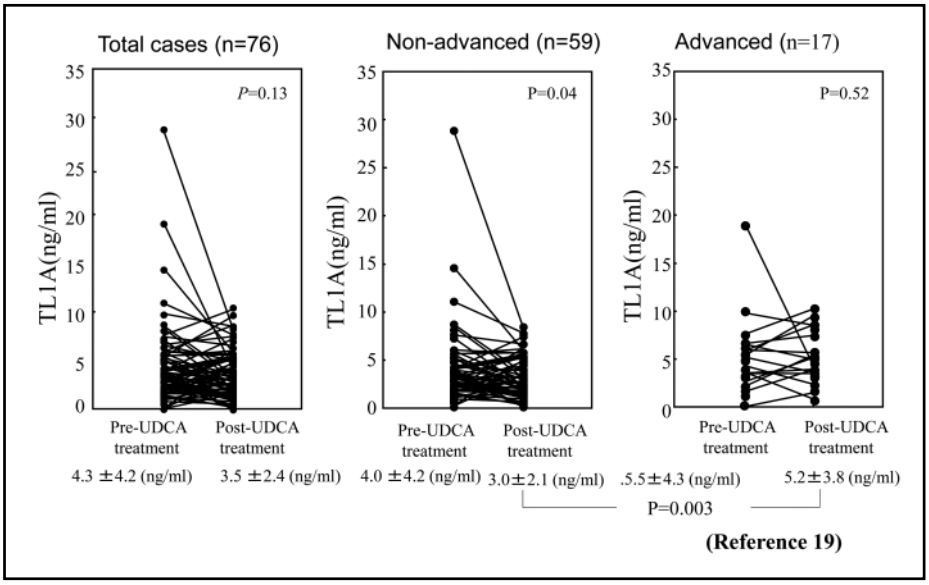

Fig.4 Effect of UDCA treatment on serum TL1A levels in PBC patients

In the PBC patient group as a whole (n=76), serum TL1A levels tended to be decreased by UDCA treatment (pre: 4.5±4.5 ng/ml, post: 3.6±2.5 ng/ml) (A): Serum TL1A levels were significantly decreased in early-stage PBC patients (n=60) after UDCA treatment (pre: 4.0 ± 4.2 ng/ml, post: 3.0 ± 2.1 ng/ml) (B): but not in late-stage PBC patients (n = 16) (pre: 5.5±4.3 ng/ml, post: 5.2 ± 2.3 ng/ml) (C): Statistical analysis was performed using a two-tailed Wilcoxon's single-rank test or Mann-Whitney's *U* test

been identified as a PBC susceptibility gene in both Japanese population and European descent[6-8]. In addition, variations of *IL7R* and *CXCR5* have been identified as PBC susceptibility genes in both Japanese population and European descent[7, 8]. Collectively, these results indicate that the B cell differentiation pathways are involved in the development of PBC.

TL1A in the pathogenesis of PBC

The clinical significance of TL1A for the pathogenesis of PBC was investigated by analyzing the systemic and local expression of TL1A in 110 PBC patients and 46 healthy controls using enzyme-linked immunosorbent assay, quantitative polymerase chain reaction and immune- histochemical staining[19]. Serum TL1A levels were significantly increased in PBC patients at both early and late stages as compared with healthy controls, and its levels were significantly decreased in early-stage PBC patients after ursodeoxycholic acid (UDCA) treatment (Fig.4). TL1A was immunohistochemically localized to biliary epithelial cells, Kupffer cells, blood vessels and infiltrating mononuclear cells in the PBC liver. In addition, TL1A messenger RNA expression was increased in the PBC liver as compared with the non-diseased liver. These results indicate that TL1A may play an important role in the pathogenesis of PBC[19].

Discussion

GWAS in Japanese population identified two novel disease-susceptibility genes for PBC, *TNFSF15* and *POU2AF1*, in addition to 21 disease-susceptibility genes which were identified in European descent[4-8]. Our results expanded our knowledge of disease-pathways of PBC implying that Th1/Th17 differentiation pathways of T cells (CD80-STAT4-TNFSF15 in Japanese and CD80-IL12A-IL12RB2-STAT4 in European descent) and B cell differentiation pathways to plasma cells (IL7R-CXCR5-POU2AF1-IKZF3 in Japanese and IL7R-CXCR5-SPIB-IKZF3 in European descent) are involved in the pathogenesis of PBC, whereas IL12/IL12R signaling pathway for Th1 differentiation has been the main object of attention in European descent[20]. In addition, *SOCS1* (16p13), *SIAE* (11q24), *Tyk2* (19p12), *SH2B3* (12q24), *MAPT* (17q21), and *TNFSF11* were recently identified as disease-susceptibility genes for PBC in European descent[21-23], indicating that various signaling pathways including type 1 interferon and IL2 are also involved in the development of PBC. Very importantly, POU2AF1 was recently reported to promote Th17 differentiation by blocking IL2, a known endogenous repressor of Th17 cells[24]. Pathway-based analysis of PBC-GWAS recently revealed the involvement of phosphatidylinositol signaling pathway in the development of PBC[25]. Recent studies for molecular mechanisms of the Th17-cell development and function revealed the emerging role of PI3K in the differentiation of Th17-cells[26]. Taken together, these results indicate the importance of Th17 differentiation pathway in the pathogenesis of PBC.

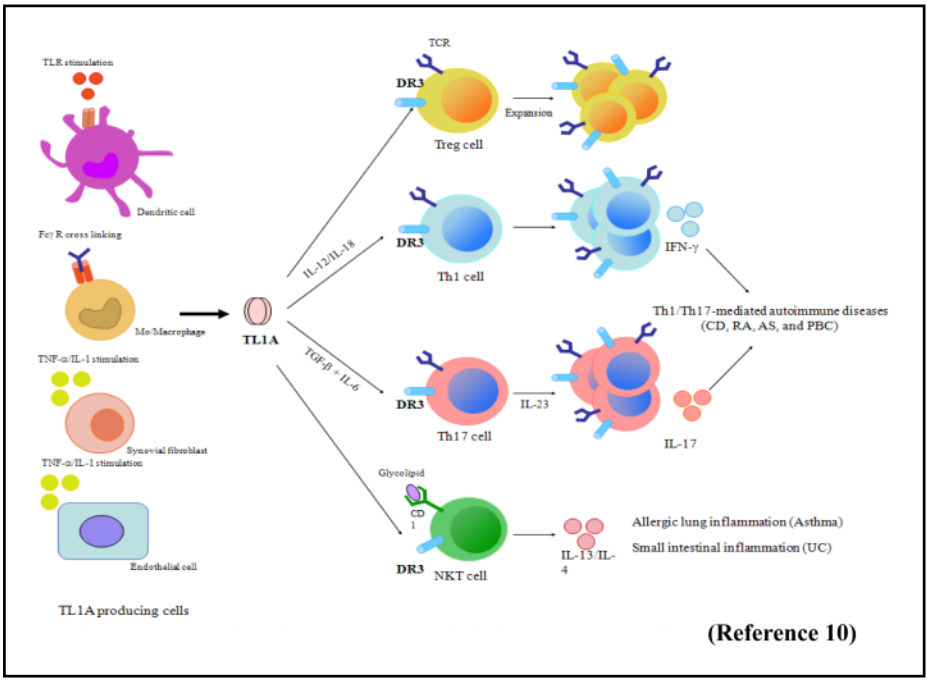

Fig.5 The role of TL1A (TNFSF15) in inflammation and autoimmune diseases

TL1A plays an important role in maintenance of local inflammation by connecting innate to adaptive immune response for differentiation and expansion of Th1/Th17 cells.

Genetic polymorphisms in *TNFSF15* are also associated with susceptibility to other inflammatory diseases including CD, UC, ankylosing spondylitis, and leprosy (Table 2)[27-31]. Strong association of five SNPs (rs3810936, rs6478108, rs6478109, rs7848647, and rs7869487) in the *TNFSF15* region with CD was first reported for a Japanese population[27] and the finding was replicated in an independent Japanese population and in European descent[28, 29]. In addition, the risk alleles of the SNPs were significantly associated with TNFSF15 mRNA expression in peripheral blood[32]. Since there exists strong LD among SNPs in *TNFSF15*, including those in the promoter region (rs6478109 and rs7848647) and introns (rs4263839 and rs4979462), it is very likely that the PBC susceptibility haplotype containing rs4979462 also influences TNFSF15 mRNA expression. The *in vitro* functional assay is now underway to study the transcriptional and post-transcriptional regulatory mechanisms for TNFSF15 production.

Conclusion

Recent GWAS revealed that the most significant disease-susceptibility genes for PBC are *IL12A/IL12RB2* and *TNFSF15* in European descent and Japanese population, respectively. This indicates that the Th1/Th17 mediated autoimmune-pathways are involved in the pathogenesis of PBC as well as Crohn's disease, rheumatoid arthritis, and ankylosing spondylitis (Fig.5)[19, 33-35]. Several evidences including mice models indicated that TL1A plays an important role in maintenance of local inflammation by connecting innate to adaptive immune response, indicating that TL1A-DR3 interaction could be an effective therapeutic target for ameliorating local inflammation in affected organs of autoimmune diseases. The comparative analysis of disease-susceptibility genes in multiple ethnicities, in which there may exist different target-genes as a result of recent positive selection via different environmental factors[36], may further provide an important clue for the dissection of critical disease-pathways in various autoimmune diseases including PBC. In addition, a new approach for investigating "environmental factors" and "gene-environmental interactions" would also be essential to dissect the disease-pathways which are specific for PBC[37].

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Conflict of Interest

None

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