RT-PCR analysis. To our knowledge, c.582+1delG in *NCSTN* is the first loss-of-function mutation in γ-secretase genes identified in the Japanese population. Our data provide further evidence that mutations resulting in haploinsufficiency of *NCSTN* are involved in the pathogenesis of familial HS. Remarkably, familial HS with mutations in the γ-secretase genes has been reported to show complete penetrance with variable disease severity.[2,10] In our analysis of the multi-generational Japanese family, the disease showed complete penetrance, but the proband showed a much more severe phenotype than her uncle. These findings suggest that a loss-of-function mutation in the genes encoding γ-secretase complex causes familial HS but other genetic and/or environmental factors, such as obesity, may influence the disease severity. It has yet to be determined whether mutations in the γ-secretase genes are involved in the onset and/or progression of SCC in patients with HS that preferably affects the prognosis.

Thus, our data indicate that mutations in γ-secretase genes, including *NCSTN,* cause familial HS in the Japanese population. However, it has been unknown whether γ-secretase gene mutations are related to the development of non-familial HS. In the present study, it is noteworthy that none of the nine patients with non-familial HS carry nonsense, frameshift and/or splice site mutations—all of which are considered to induce reduced γ-secretase

activity and therefore to be pathogenic for HS—in any of the γ-secretase genes, and only one of the patients was heterozygous for a p.Thr421Met in *PSEN2*. To clarify whether this missense mutation causes HS, we tried to analyze phenotype/genotype correlations in her family, but we were not able to obtain consent from her family for the DNA analysis. However, taking into account the fact that missense mutations in *PSEN1* and *PSEN2* have been identified in cases of familial Alzheimer's disease but not HS and the fact that familial Alzheimer's disease and HS have been reported to be mutually exclusive, it is most likely that the missense mutation identified in patient 4 was not a cause of her HS phenotype.[2] As for a missense mutation in γ-secretase genes, there is only one report of a heterozygous missense mutation in *NCSTN* causing HS but not Alzheimer's disease, although the authors did not clarify whether the mutation causes protein inactivity of nicastrin.[6] Therefore, their findings will have to be further examined in future studies.

It remains an unresolved question whether missense mutations in *PSEN* induce a gain of function or a loss of function for presenilin.[10] Therefore, the pathomechanism of Alzheimer's disease by a missense mutation in *PSEN* has yet to be elucidated. However, the amino acid p.Thr421 in *PSEN2* is highly conserved among diverse species (Figure 3b) and the missense

mutation p.Thr421Met was not carried by 50 unrelated Japanese control individuals, suggesting that this missense mutation could be pathogenic for the onset of Alzheimer's disease. Although neither patient 4 nor her family members showed any symptoms of Alzheimer's disease or dementia at the time of DNA analysis at our department, we could not exclude the possibility that they might develop Alzheimer's disease in future.

In conclusion, we demonstrated that a novel *NCSTN* mutation underlies familial HS in the Japanese population. Moreover, we revealed for the first time that a mutation in the γ-secretase genes is not linked to the development of non-familial HS. We believe that our findings further pave the way for understanding the contribution of γ-secretase to the pathogenesis of HS and might provide new insight into the pathogenesis of acne vulgaris, as this more common condition shares many clinical features with HS.

Acknowledgments

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Figure legends

Fig 1. Clinical features of the Japanese pedigree with familial hidradenitis suppurativa (HS)

(**a**) The family tree shows the autosomal-dominant inheritance. Solid symbols denote affected individuals; open symbols denote unaffected individuals. '*' denotes individuals who were examined and sequenced in this study.

(**b-d**) The proband, IV-1 **(b, c)**, demonstrated widespread sinuses, skin abscesses and disfiguring scars; her uncle, III-14 **(d)**, showed a milder HS phenotype.

Fig 2. Mutation analysis of familial hidradenitis suppurativa (HS)

(**a**) A heterozygous single-nucleotide deletion in *NCSTN,* c.582+1delG, was identified in the proband, IV-1. Her unaffected sister, IV-2, was wild-type for this mutation.

(**b**) Real-time RT-PCR analysis revealed marked reduction in *NCSTN* mRNA expression in the proband, IV-1, compared with an unaffected normal control.

Fig 3. A missense mutation identified in a patient with non-familial hidradenitis suppurativa (HS)

(a) A heterozygous transition mutation c.1262C→T in *PSEN2* was identified in one of the non-familial HS patients (patient 4) in Table 1, resulting in p.Thr421Met. Normal control sequence from exon 13 of *PSEN2*, corresponding to codons 1254-1271.

(b) p.Thr421 is highly conserved among different species.

Fig 4. Summary of mutations in the γ-secretase genes

Schematic of all the γ-secretase genes including all loss-of-function mutations reported to date.

Table 1. Summary of the clinical and genetic features in non-familial AI patients in this study

Patient Number	Age	Sex	Genotype
1	35	M	WT/WT
2	46	M	WT/WT
3	40	M	WT/WT
4	21	F	p.Thr421Met(*PSEN2*)/WT
5	54	M	WT/WT
6	31	F	WT/WT
7	32	M	WT/WT
8	70	M	WT/WT
9	15	M	WT/WT

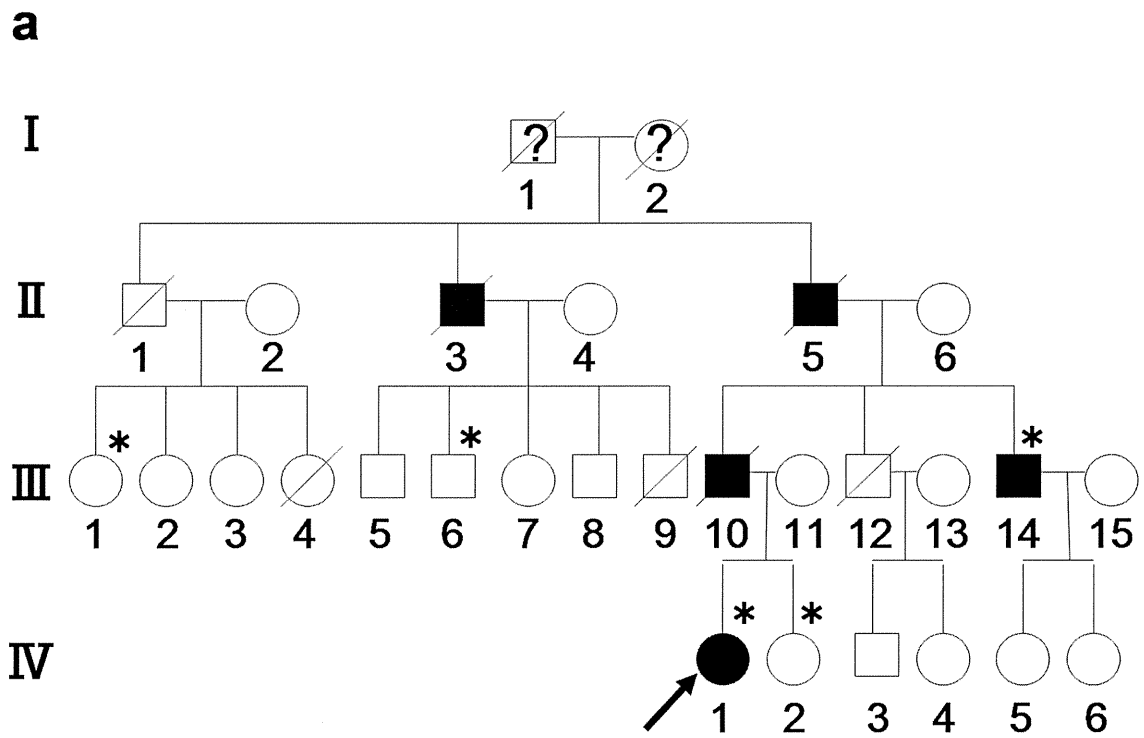
* WT denotes wild-type.

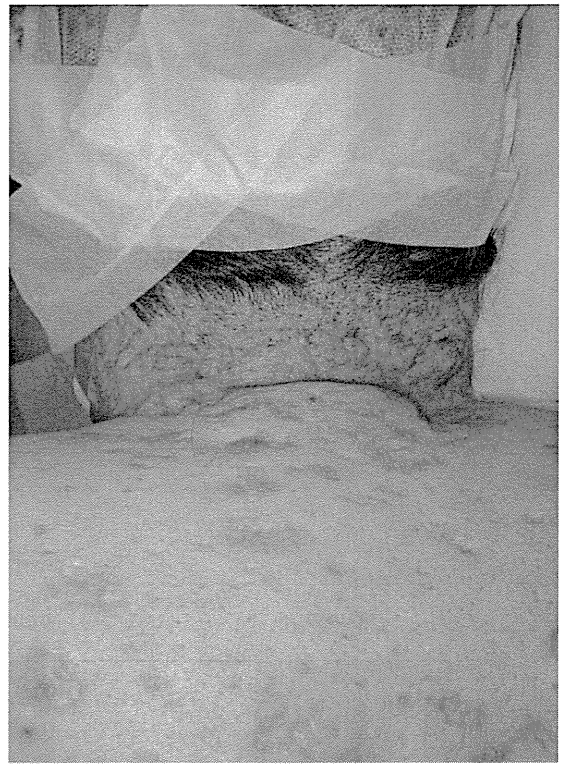
Table 2: Primer sequences for sequencing of the γ-secretase genes

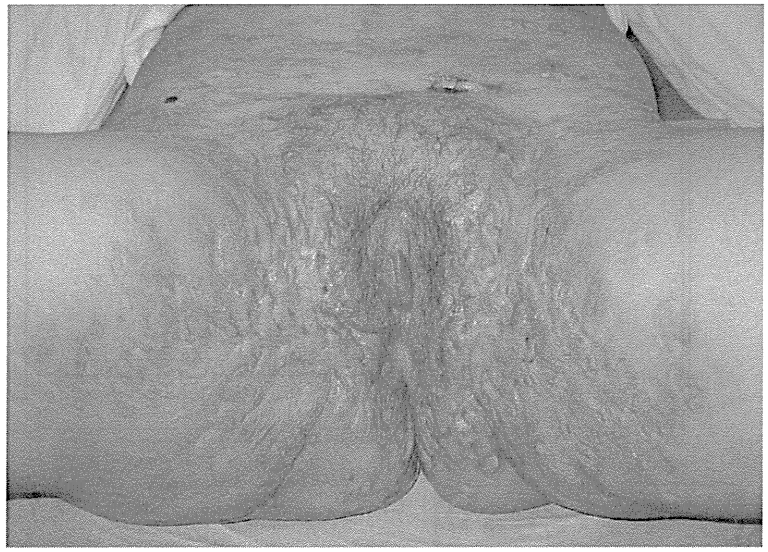
Gene	Exon	Forward Primer (5'-3')	Reverse Primer (5'-3')
PSENEN	1-3	CCTCTCCAGGCGCTTACCAA	CCAGCCTTCCCTTCTCACCC
		GGATTGAGGGCCTGGACTCTT	CCAGTCCCCTCTCTTCTCCT
	4	GAGAGGGGGAAGCGGGGAAT	GCCTCGTACAAAGCTCTCAACA
PSEN1	1	CAGGTGGAGCTCTGGGTTCT	CACTCCAGTTCAGACGGCTACT
	2,3	TTCCTGGTTTACAAATTGGTCTT	ACCTATGTGTTAAACAGCCCTGT
	4	GTTAATCCCAGGTCTAACCGTT	GCTCTCCAGCTAAGTCATGC
	5	CTAGATGGAGCCAGTGTCTG	CCAACCATAAGAAGAACAGGGT
	6	CTTTTTAAGGGTTGTGGGACCT	CTGCCTCTTCGAATTTAAGAGCT
	7	CTTTATTTAAGCATAAACTTTGGGT	GTTATGGGATGTACACGTTACC
	8	TTTCCTTCGTTAATTCCTCCCT	GAAACAAAGAGATCTGCAGGAGTT
	9	GAAATGATGGCTTGTTGTTGTCT	CTGTTAGCTTATAACAGTGACCCTG
	10	TGACAGCTAGTTACTGTTTCCATGT	AAAAAGGTTGATAATGTAGCTACCT
	11	GGGCAGTGATATTTTTGAATTGT	AACTGCCTTAAAGGGACTGTGT
	12	AGACTTGTGATTGAGTTTTGCCT	CATTTGCTGTGGAAGAAGGTCT
PSEN2	1,2	ACTCCTTCTAAGGTCGTCGCTT	TAAACAGGGGTGAGGTCTGC
	3	TGGACCAAATACATAGTCGGGT	GCCTCCACGGAGAAAATCCT
	4	GACTTGTGTCCAAGTCTCCAGGT	TCATCATTACTTCCCTTCTCCCT
	5	CGTGCATTACATGGATAGGCT	GCTGCCTCTGCATGTATTTTACT
	6	CCCTAGCAGGTCCAGAATCACT	TGTTTTTCTAAAGGCGGCTGT
	7,8	CTGGGGGCCTTAGAATTTGT	CACCAGGAGTGTTCCAGAAATAG
	9	CAAGGCATGCTCTGAGAGCT	GCCTGCTTCCTGTCCTAACTT
	10,11	AATGGAGCATGAGCAGATACCT	ATCTTCTACTTTCACAGAGATGCCT
	12	CTGGGCCAGAGTTTCTCTTCTT	ACCTCCTGTGAGCCTTGGTCT
	13	CCCAGGGACTAGACCATGACT	CTGGGCTCACAGCACAAGTT
NCSTN	1	CTCAGGTCTCCGACTCCGAT	TCGAAGTCCAGGAAGAGGCC
	2	TCTGTTACGATAAGTGTGTG	AGACCTATTTTGTGGGGTTG
	3	AGCTTTTCAGTTCAAATCTA	GCTCAAGATCATTTCTTTTG
	4	AGACTCAGAATTTAGAGACT	GAGTCAGTTACTGACACTAC
	5	CTAATCTTACAGCCTAAGAC	TCAAGTGATCCTCCTGTCTC

	6	CCTTATAGATCCTATGGTGG	TTTAGGAACAAGCTGCCTAA
	7	AGAGAAGCCAACCTCGGA	TATACCTGTCCTGTGGCAAT
	8	GCTCTAGCATATCAGATTTC	TTCTCCAACCAGTAGCCACTCT
	9,10	ACTGTAAGCTGACTAGCAGT	GGTGTTCATCCTTTGGCCAA
	11	AGGAAATTCAGAGAGCCTTGGT	CTGAAGTTCTGGAGGGGGTAGT
	12,13	ATTGTCTCTCACCCCTTTCTTCT	AGACTGGCCAGATAGACTGGAGT
	14	CCCATGACTGGGTCTCCACT	AAAAGCCCAAGATTGGCAGC
	15	TTAGCTGAGGAAAGGGATAG	CCTTCAGATGGTCCATTTTC
	16	CTCAGCAATTGGGTGTTGAA	GCCTCCTAAGGGAACATGCT
	17	AGTCTGTAGGCTGGAGAGATGTT	ATTAGGGTGGGTAACTAGGG
APH1B	1	AGGCCCTAGCGTTTACCC	CGTCTGAAACGCAACAAGGT
	2	TTCCTGCTTTTAAACATTATTAATCC	TCATAACTAAAATCTAACTGGGGAAAA
	3	TTGAAAATTTGCTGGGTTTGTT	GGCCAAGGCAGTAATAAACAACT
	4	CAGGAAGAACATAAGCACCCTT	CCACTGAAATTCCTAAGCCAGT
	5	AAACTCTCTTGCTGAGGTGTGGT	GGTGGTCTTTTTGTGGTAAGACTT
	6	CATGCTTCCCTTTATCTTTGGT	TGAAAAGTGTCAATGACACCCTT
APH1A	1,2	CCTGGAATTGGTGTCTCGACT	CACTTCCCATAGTGCAGTCCTT
	3-5	GGTTTGGAAGAGGAGGCACT	CCTCCTCCTCCCAGGTAAGTT
	6,7	TGGTGCTCTGAAGGGAAAGACT	CCCTCAGCAGAAAATGATACAGTT
		TACCAGGAGCAGCCTGGGTT	

Figure1







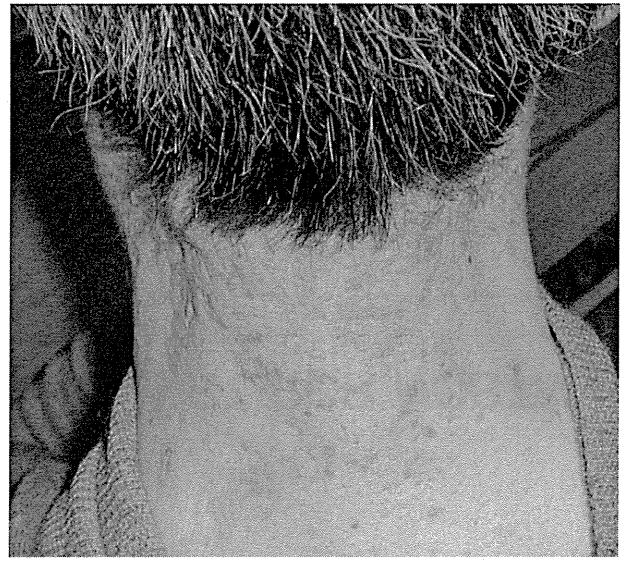
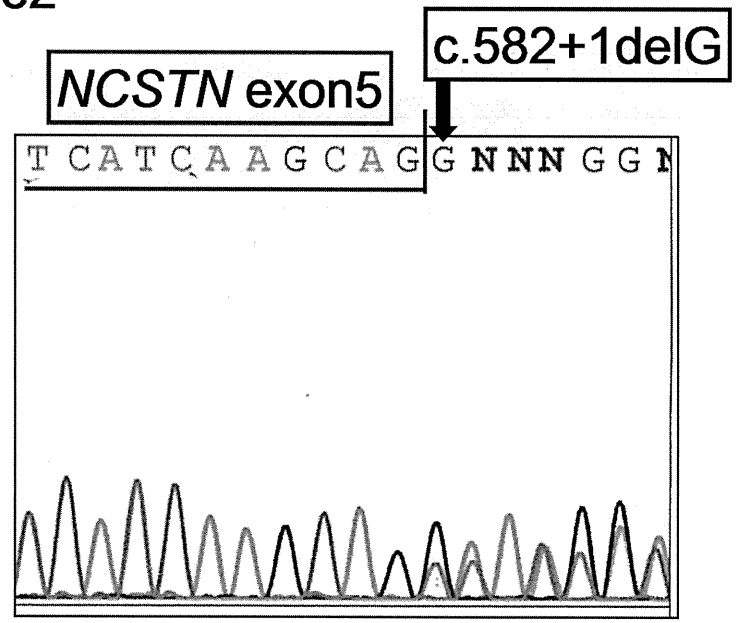


Figure2

a



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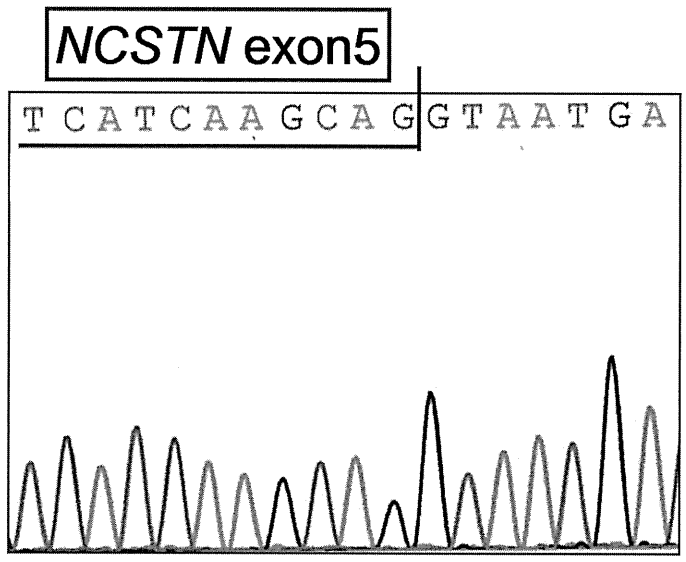

Ⅳ-2: Wild type

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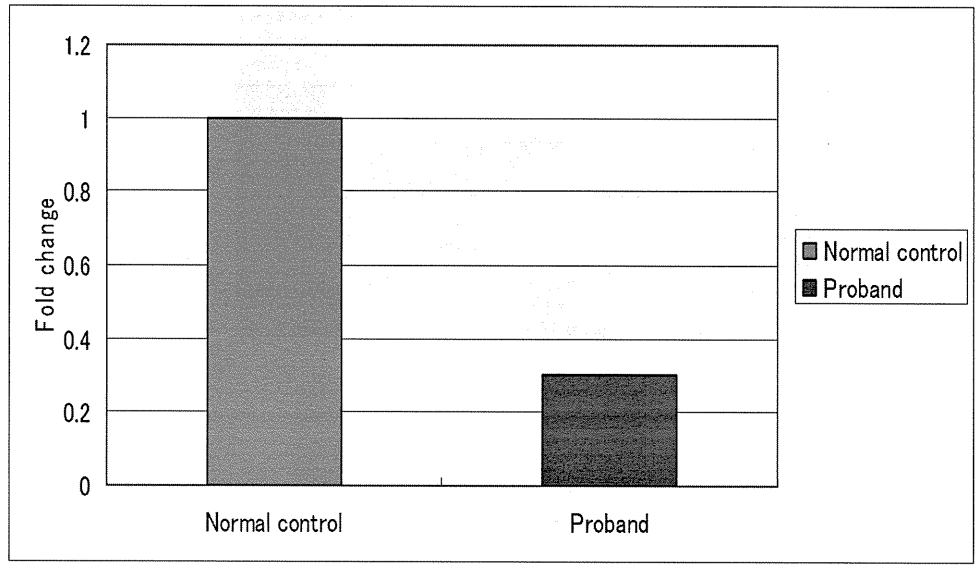
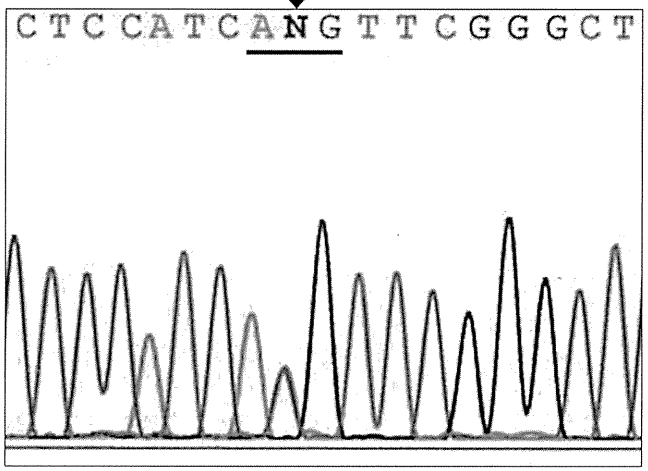


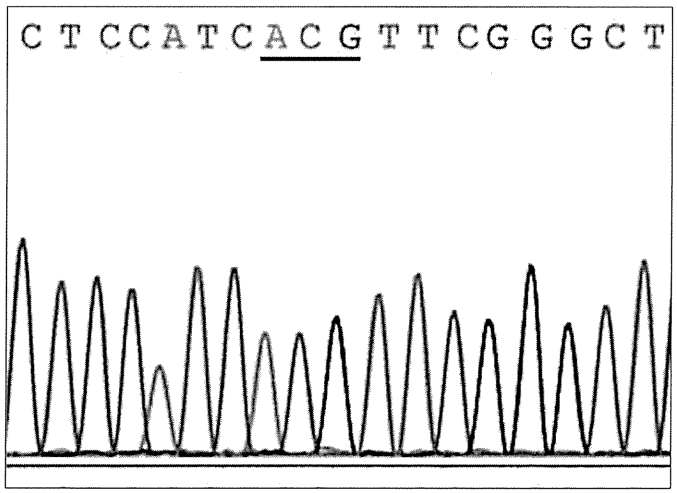
Figure3

a

ACG→ATG
p.Thr421Met



Non-familial AI patient: No. 4



Normal control

b

	418	448
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Pan troglodytes:	ISITFGLIFYFSTDNLVRPFMDTLASHQLYI	
Canis familiaris:	ISITFGLIFYFSTDNLVRPFMDTLASHQLYI	
Bos Taurus:	ISITFGLIFYFSTDNLVRPFMDTLASHQLYI	
Mus musculus:	ISITFGLIFYFSTDNLVRPFMDTLASHQLYI	
Rattus norvegicus:	ISITFGLIFYFSTDNLVRPFMDTLASHQLYI	
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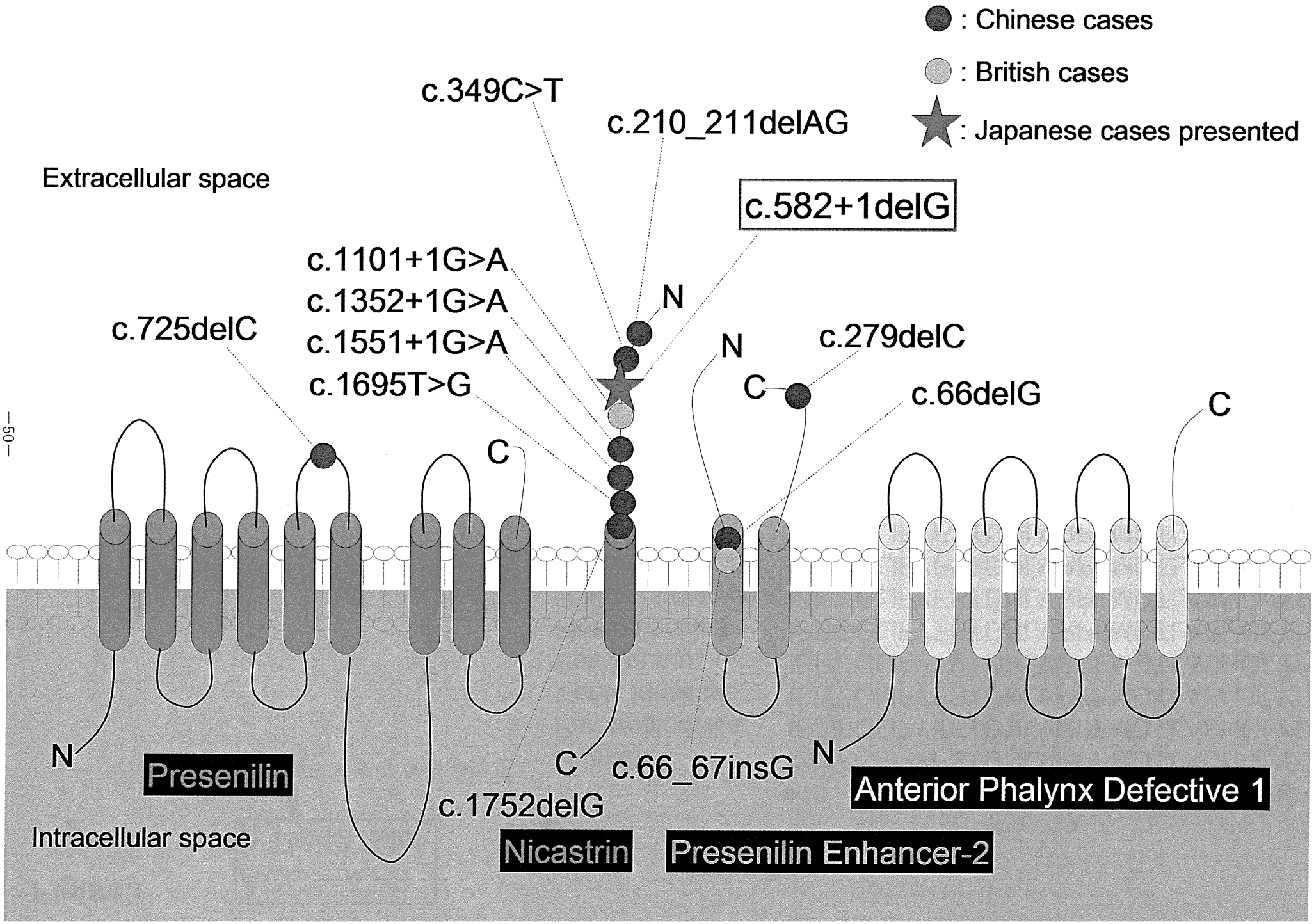
Danio rerio:	ISITFGLIFYFSTDNLVRPFMDTLASHQLYI	

Figure4


: Chinese cases
: British cases
: Japanese cases presented
c.349C>T
c.210_211delAG
Extracellular space
c.582+1delG
c.1101+1G>A
c.1352+1G>A
c.1551+1G>A
c.1695T>G
c.725delC
N
N
c.279delC
C
c.66delG
C
C
N
Presenilin
C
c.66_67insG
N
c.1752delG
Anterior Phalynx Defective 1
Intracellular space
Nicastrin
Presenilin Enhancer-2

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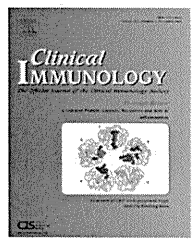

Noncollagenous 16A domain of type XVII collagen-reactive CD4$^+$ T cells play a pivotal role in the development of active disease in experimental bullous pemphigoid model

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KEYWORDS
Autoimmune disease;
Autoreactive T cells;
CD40 ligand;
Pathomechanism

Abstract Bullous pemphigoid (BP), the most common autoimmune blistering disease, is caused by autoantibodies against type XVII collagen (COL17). We recently demonstrated that CD4$^+$ T cells were crucial for the production of anti-COL17 IgG and for the development of the BP phenotype by using a novel active BP mouse model by adoptively transferring immunized splenocytes into immunodeficient COL17-humanized mice. Noncollagenous 16A (NC16A) domain of COL17 is considered to contain the main pathogenic epitopes of BP, however, the pathogenicity of COL17 NC16A-reactive CD4$^+$ T cells has never been elucidated. To address this issue, we modulated the immune responses against COL17 in active BP model by using anti-CD40 ligand (CD40L) monoclonal antibody MR1, an inhibitor of the CD40–CD40L interaction, in various ways. First, we show the essential role of CD4$^+$ T cells in the model by showing that CD4$^+$ T cells isolated from wild-type mice immunized with human COL17 enabled naïve B cells to produce anti-COL17 NC16A IgG in vivo. Second, we show that the activation of anti-COL17 NC16A IgG-producing B cells via CD40–CD40L interaction was completed within 5 days after the adoptive transfer of immunized splenocytes. Notably, a single administration of MR1 at day 0 was enough to inhibit the production of anti-COL17 NC16A IgG and to diminish skin lesions despite the presence of restored anti-COL17 IgG at the later stage. In contrast, the delayed administration of MR1 failed to inhibit the production of anti-COL17 NC16A IgG and the development of the BP phenotype. These results

Abbreviations: BP, bullous pemphigoid; COL17, type XVII collagen; BMZ, basement membrane zone; NC16A, noncollagenous 16A domain; WT, wild type; hCOL17, human COL17; Tg, transgenic; CD40L, CD40 ligand; IF, immunofluorescence; OD, optimal density.

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strongly suggest that COL17 NC16A-reactive CD4+ T cells play a pivotal role in the production of pathogenic autoantibodies and in the development of active disease in experimental BP model.

1. Introduction

Bullous pemphigoid (BP) is the most common autoimmune blistering disorder. Clinically, tense blisters, erosions and crusts with itchy urticarial plaques and erythema develop on the entire body. Histologically, subepidermal blisters associated with inflammatory cell infiltration in the dermis are observed. BP is induced by autoantibodies against type XVII collagen (COL17, also called BP180 or BPAG2), a hemidesmosomal protein which spans the lamina lucida and projects into the lamina densa of the epidermal basement membrane zone (BMZ) [1–6]. The juxtamembranous noncollagenous 16A (NC16A) domain is considered to contain the main pathogenic epitopes on COL17, although BP patients' sera can also react with other parts [7–9].

Recently, we developed a novel active BP mouse model by adoptively transferring wild-type (WT) splenocytes immunized by human COL17 (hCOL17)-expressing transgenic (Tg) skin-grafting into *Rag*-$2^{-/-}$/*COL17*$^{m-/-,h+}$ (*Rag*-$2^{-/-}$/COL17-humanized) mice that express hCOL17 in the skin and lack both T and B cells [10]. The recipient mice accepted transferred splenocytes and produced high titers of anti-hCOL17 IgG in vivo for more than 10 weeks after the adoptive transfer, while circulating anti-hCOL17 NC16A IgG titer decreased in a short period for unknown reasons [10]. They developed blisters and erosions corresponding to clinical, histological and immunopathological features of BP [10]. This new active BP model enables us to observe the dynamic immune reactions induced by pathogenic antibodies against hCOL17 molecule.

In BP, the presence of autoreactive CD4+ T cells has been reported [11–13]. Particular MHC class II alleles occur more frequently in BP patients [14]. These findings indicated the contribution of CD4+ T cells to the pathogenesis of BP. Generally, the production of IgG by B cells requires the help of CD4+ T cells [15–17]. Our previous study demonstrated that CD4+ T cells were crucial for the production of anti-hCOL17 IgG and for the development of the BP phenotype because both the depletion of CD4+ T cells from immunized splenocytes, and the administration of cyclosporin A significantly suppressed the pathogenic IgG production and diminished the disease severity [10]. However, the pathogenicity of COL17 NC16A-reactive CD4+ T cells has never been elucidated. To address this issue, we modulated the CD4+ T cell function in active BP model by administering anti-CD40L monoclonal antibody MR1 [18] in various ways, and observed the phenotypic changes of the treated mice.

CD40 ligand (CD40L) is a costimulatory molecule which is transiently expressed on the surface of activated CD4+ T cells and which binds to CD40 on antigen-presenting cells including B cells. CD40–CD40L interaction is crucial for the proliferation and differentiation of B cells into immunoglobulin-secreting plasma cells and for the formation of humoral memory [19]. Immunosuppressive effects of anti-CD40L monoclonal antibody have been shown in some T-cell-mediated antibody-induced autoimmune animal models, such as experimental autoimmune myasthenia gravis [20], and pemphigus vulgaris [21, 22]. In this study, we demonstrate that COL17 NC16A-reactive CD4+ T cells play a pivotal role in the development of BP through the CD40–CD40L interaction at an early stage of the disease in active BP model, which suggests that COL17 NC16A-reactive CD4+ T cell is a promising therapeutic target for BP.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from Clea Japan. *Rag*-$2^{-/-}$/*COL17*$^{m-/-,h+}$ mice which carry the homozygous null mutations of both the *Rag-2* and *mouse Col17* genes and the transgene of *human COL17* were generated by crossing *Rag*-$2^{-/-}$ mice (C57BL/6 background) with *COL17*$^{m-/-,h+}$ (COL17-humanized) mice (C57BL/6 background) as described previously [10]. All animal procedures were conducted according to guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

2.2. Induction of active BP by adoptive transfer of immunized splenocytes

Immunization of WT mice by *hCOL17*-expressing Tg skin graft was performed according to the method reported previously [10, 23]. After the confirmation of anti-hCOL17 IgG production at 5 weeks after skin grafting by indirect immunofluorescence (IF) analysis using normal human skin, splenocytes were isolated and pooled from several Tg skin-grafted immunized WT mice and administered into *Rag*-$2^{-/-}$/COL17-humanized mice by intravenous injection into the tail vain at $1.5–2.0 \times 10^8$ splenocytes in 500 μL PBS per mouse [10, 24].

2.3. Evaluation of active BP model mice

Weekly, the recipient mice were examined for general condition and cutaneous lesions (i.e., erythema, blisters, erosions, crusts and hair loss). Extent of skin disease was scored as follows: 0, no lesions; 1, lesions on less than 10% of the skin surface; 2, lesions on 10–20% of the skin surface; 3, lesions on 20–40% of the skin surface; 4, lesions on 40–60% of the skin surface; 5, lesions on more than 60% of the skin surface, as previously described [10]. Serum samples were also obtained from recipient mice weekly and assayed by indirect IF microscopy and hCOL17 NC16A ELISA as previously described [10]. The ELISA index value was defined by the following formula: index = (OD_{450} of tested serum − OD_{450} of negative control) / (OD_{450} of positive control − OD_{450} of

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negative control)×100 [10]. Biopsies of lesional skin were obtained for light microscopy (H&E), and for direct IF using FITC-conjugated antibody against mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and C3 (Cappel; Valeant Pharmaceuticals, Costa Mesa, CA).

2.4. Isolation of CD4+ T cells or CD45R+ B cells from splenocytes in mice

To examine the pathogenic role of CD4+ T cells in active BP model, we isolated CD4+ T cells from splenocytes of Tg skin-grafted WT mice by using a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Galdbach, Germany). 0.5 to 8×10^7 CD4+ T cells were mixed with 2.0×10^8 naïve splenocytes from WT mice and adoptively transferred to *Rag*-$2^{-/-}$/COL17-humanized mice. In another experiment, CD45R+ B cells were isolated from Tg skin-grafted WT mice by using CD45R MicroBeads (Miltenyi Biotec). 0.4×10^8 of CD45R+ B cells were transferred to *Rag*-$2^{-/-}$/COL17-humanized mice. The isolation of CD4+ T cells and CD45R+ B cells was confirmed by flow cytometric analysis on FACSAria (BD Bioscience Pharmingen) using monoclonal antibodies purchased from BD Biosciences Pharmingen: H129.19-FITC (anti-CD4) and RA3-6B2-PE (anti-CD45R/B220).

2.5. In vivo monoclonal antibody treatment

Rag-$2^{-/-}$/COL17-humanized recipients that were adoptively transferred with immunized splenocytes were intraperitoneally injected with 500 μg hamster monoclonal antibody MR1 specific to mouse CD40L (Taconic Farms, Hudson, NY) or an equivalent amount of control hamster IgG (Rockland Immunochemicals, Gilbertsville, PA) at days 0, 2 and 6 after the adoptive transfer of immunized splenocytes as previously described [21], with some minor modifications. In a delayed treatment experiment, MR1 was injected at days 13, 16 and19 after the adoptive transfer. Some recipient mice were injected with 500 μg of MR1 just once on one of days 1 to 5 after the adoptive transfer, respectively. To investigate the immune responses in active BP model modulated by early single administration of MR1, 1000 μg of MR1 was injected into recipient mice at day 0 soon after the adoptive transfer. All treated mice were carefully observed for at least ten weeks after the adoptive transfer.

2.6. ELISPOT assay

ELISPOT assay was performed as previously described [10, 24]. Polyvinylidene-difluoride-bottomed 96-well multiscreen plates (Millipore) were coated with 30 μg/mL of recombinant hCOL17 NC16A protein. Splenocytes isolated from the *Rag*-$2^{-/-}$/COL17-humanized recipients were incubated on the plate at 37 °C in a 5% CO_2 incubator for 4 h. IgG bound to the membrane was visualized as spots, using alkaline-phosphatase-conjugated anti-mouse IgG antibody. The number of spots was counted using the ImmunoSpot S5 Versa Analyzer (Cellular Technology Ltd., Shaker Heights, OH), and the frequency of anti-hCOL17 NC16A IgG-producing B cells was defined as the number of spots in 10^5 mononuclear cells.

2.7. Statistical analysis

Data expressed as mean ± standard error of means were analyzed using Student's *t*-test. We considered *P* values of less than 0.05 as significant.

3. Results

3.1. CD4+ T cells are required for the production of pathogenic antibody in active BP model

We previously reported that CD4+ — but not CD8+ — T cells are crucial for the production of anti-hCOL17 IgG and for the development of the BP phenotype in active BP model [10]. To further analyze the contribution of CD4+ T cells, we additionally conducted two experiments. First, mixed transfer into *Rag*-$2^{-/-}$/COL17-humanized mice of 4 or 8×10^7 CD4+ T cells from WT splenocytes immunized by hCOL17-expressing Tg skin-grafting and 2×10^8 naïve splenocytes from unimmunized WT mice produced high titers of anti-hCOL17 NC16A IgG and severe BP skin changes associated with linear deposition of IgG at the BMZ. In contrast, reducing the number of CD4+ T cells (0.5×10^7) failed to produce such titers and skin changes (n=3, respectively; Fig. 1). Second, we isolated CD45R+ B cells from immunized splenocytes and adoptively transferred 0.4×10^8 of those cells into *Rag*-$2^{-/-}$/COL17-humanized recipients (n=3), which produced quite low levels of anti-hCOL17 NC16A IgG (mean index value of ELISA at day 9: 3.28) and no skin changes (not shown). These results show that the production of anti-hCOL17 NC16A IgG by B cells and the development of BP skin changes in active BP model depend heavily on immunized CD4+ T cells.

3.2. Anti-CD40L monoclonal antibody suppresses the production of anti-hCOL17 IgG and skin changes in active BP model

To investigate the precise mechanism of the activation of B cells by immunized CD4+ T cells in active BP model, we assessed the role of CD40–CD40L interaction. *Rag*-$2^{-/-}$/COL17-humanized recipients were injected intraperitoneally with 500 μg of monoclonal antibody MR1 specific to mouse CD40L or an equivalent dose of hamster IgG as a control on days 0, 2 and 6 after the adoptive transfer of immunized splenocytes (n=6, respectively). All the control *Rag*-$2^{-/-}$/COL17-humanized recipients produced high titers of IgG against BMZ of normal human skin, which reflects the presence of anti-hCOL17 IgG, and those against hCOL17 NC16A, as previously reported [10]. In contrast, the production of those antibodies was almost completely inhibited in all the mice that were injected with MR1, and the inhibitory effect persisted for more than 10 weeks (Figs. 2A, B). The control mice developed patchy hair loss associated with erythema around day 14 after the adoptive transfer. Then, blisters and erosions spontaneously developed in the depilated areas on the trunk (Fig. 3A). Disease severity, scored by the percent of skin surface with the BP phenotype [10, 25], gradually increased, plateauing 7 weeks after the transfer in the control mice (Fig. 3G). In contrast, none of the MR1-

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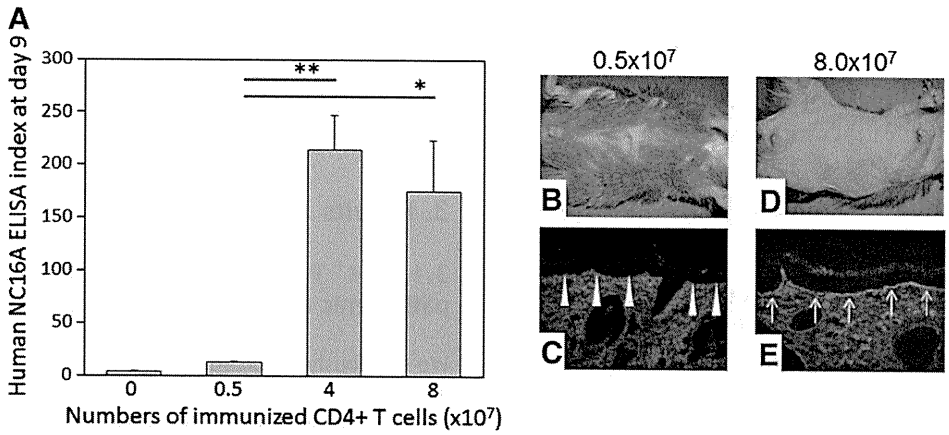

Fig. 1 Immunized $CD4^+$ T cells can activate naïve B cells to produce anti-hCOL17 NC16A IgG in vivo. (A) $CD4^+$ T cells isolated from WT splenocytes immunized by hCOL17-expressing Tg skin-grafting were mixed with naïve splenocytes from untreated WT mice, and were adoptively transferred into *Rag*-$2^{-/-}$/COL17-humanized mice (n=3, respectively). Mice transferred with 4 or 8×10^7 immunized $CD4^+$ T cells mixed with naïve splenocytes produce significantly higher levels of anti-hCOL17 NC16A IgG than with 0.5×10^7 $CD4^+$ T cells mixed with naïve splenocytes (*$P<0.05$, **$P<0.01$). Mice transferred with 0.5×10^7 of immunized $CD4^+$ T cells and naïve splenocytes show no skin changes (B) or deposition of IgG (C). In contrast, mice transferred with 8×10^7 immunized $CD4^+$ T cells and naïve splenocytes develop severe BP skin changes (D) associated with linear deposition of IgG at the BMZ (E).

treated mice developed any skin lesions (Figs. 3D, G). Histopathological analysis of the skin revealed the dermal–epidermal separation that is associated with mild inflammatory cell infiltration in control mice (Fig. 3B), whereas there were no histopathological changes in MR1-treated mice (Fig. 3E). Direct IF analysis of lesional skin revealed linear deposition of IgG (Fig. 3C) at the BMZ in the control mice, whereas IgG deposition was absent or faint in the MR1-treated mice (Fig. 3F). We also examined the number of splenocytes which produced anti-hCOL17 NC16A IgG by enzyme-linked immunospot assay at day 9. In the control, 226.5±25.0 cells in 10^5 splenocytes produced anti-hCOL17 NC16A IgG, whereas only 9.0±3.0 cells in 10^5 splenocytes produced them in the mice treated with MR1 (n=3, respectively; Fig. 3H). Thus, preventive and repetitive administration of MR1 can continuously suppress the production of anti-hCOL17 IgG and skin changes in active BP model.

3.3. Anti-CD40L monoclonal antibody shows no effects in mice with established active BP

To examine the effect of MR1 in mice with producing IgG against hCOL17 and hCOL17 NC16A, 500 μg of MR1 or the equivalent dose of normal hamster IgG were administered into active BP model at days 13, 16 and 19 after the adoptive transfer of splenocytes (n=4, respectively). There were no significant differences in the titers of anti-hCOL17 or anti-hCOL17 NC16A IgG, nor in disease severity in both groups at more than 10 weeks after the adoptive transfer (Fig. 4).

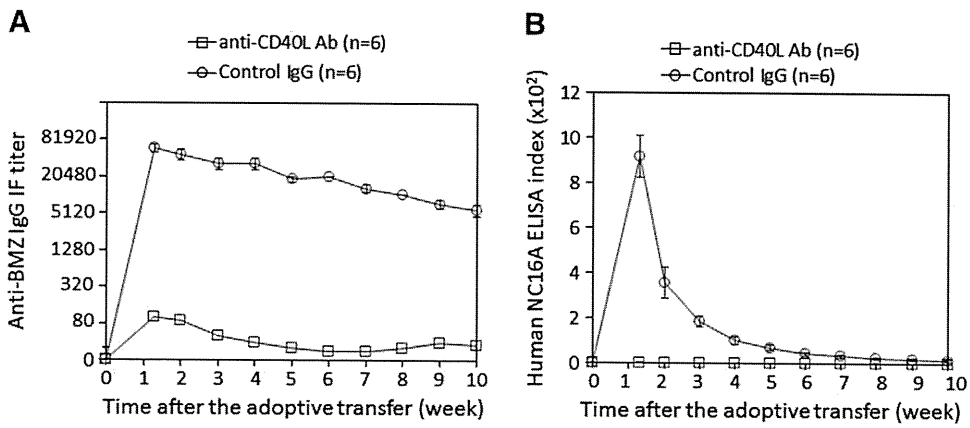

Fig. 2 Anti-CD40L monoclonal antibody strongly suppresses the production of anti-hCOL17 and anti-hCOL17 NC16A IgG and in active BP model. *Rag*-$2^{-/-}$/COL17-humanized recipients were injected intraperitoneally with monoclonal antibody specific to mouse CD40L (MR1) or the equivalent dose of control hamster IgG on day 0, 2 and 6 after the adoptive transfer of immunized splenocytes (n=6, respectively). All the *Rag*-$2^{-/-}$/COL17-humanized recipients that were injected with control IgG produce significantly high titers of IgG against hCOL17 (BMZ of normal human skin) and hCOL17 NC16A, while the production of those antibodies is almost completely inhibited in all mice injected with MR1 (A, B) $P<0.01$ from day 9 to day 70 in both graphs.

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