The role of Presenilin-1 (Psen1) as a scaffold protein in the NF-κB mediated inflammation

A Doctoral Thesis

By

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Dedicated to

my Late Grandpa Mr. Tilak Raj Sabharwal

(1924-2018), my biggest cheerleader.

I miss you…

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Contents

Abstract

Chronic inflammation is critical for the development of various diseases. We previously discovered one mechanism associated with this development and specific to nonimmune cells, such as synovial cells, fibroblasts, and endothelial cells, is an NF-κB activator – the inflammation amplifier (formerly IL-6 amplifier), which is activated by a simultaneous stimulation of NF-κB and STAT3 to express inflammatory mediators including chemokines, cytokines and growth factors, which deregulate local homeostasis via an accumulation of various immune cells and proliferate various regional cells that contribute to the development of various inflammatory diseases. The amplifier activation has been observed in several disease models such as F759 arthritis model and EAE as well as in patient samples. To further understand the detailed molecular mechanism of the inflammation amplifier and its role in human diseases, genome wide screening was performed using 16000 mouse genes and identified 1289 genes that are positive regulators of the synergistic activation of NF-κB. Out of the 1289 genes, I selected Presenilin-I (Psen-1) and investigated its role in detail.

In 2016, we reported that NF-κB–mediated inflammation caused by breakpoint cluster region (BCR) is dependent on the α subunit of casein kinase II (CK2α). BCR is the cause of certain types of leukemia upon fusing to Abl tyrosine kinase resulting

in abnormal cell survival and proliferation. CK2 is a serine/threonine kinase composed of two catalytic α subunits and two regulatory β subunits. CK2 has been known to play a role in various cellular processes such as cell cycle control, DNA repair, regulation of the circadian rhythm. It was reported that CK2 phosphorylates p65, an action critical for NF-κB–mediated transcription.

In the current study, I demonstrate that Psen1, which is a catalytic component of the γ-secretase complex and the mutations of which are known to cause familial Alzheimer disease (AD), acts as a scaffold for the BCR–CK2α–p65 complex to induce NF-κB activation. Psen1 deficiency in mouse endothelial cells showed a significant reduction of NF-κB p65 recruitment to target gene promoters.

By contrast, Psen1 overexpression enhanced reporter activation under NF-κB responsive elements and IL-6 promoter. Furthermore, the transcription of NF-κB target genes was not inhibited by a γ-secretase inhibitor, suggesting that Psen1 regulates NF-κB activation independently of γ-secretase activity. Mechanistically, Psen1 associated with the BCR–CK2α complex, that phosphorylated p65 at serine 529 and created p300 binding site which increased p65-mediated transcription followed by inflammation development. Consistently, TNF-α–induced phosphorylation of p65 at serine 529 as well as p300 binding was significantly decreased in Psen1-deficient cells. Additionally, the BCR–CK2α–p65 complex association was perturbed in the absence of Psen1.

Therefore, these results suggested that Psen1 functions as a scaffold of the BCR–CK2α–p65 complex and that this signaling cascade could be a novel therapeutic target for various chronic inflammatory conditions, including those in AD.

List of Abbreviations

aa	- amino acid
Ab	- antibody
Aβ	- amyloid-β protein
AD	- Alzheimer's disease
APC	- antigen presenting cells
APP	- amyloid precursor protein
BCR	- breakpoint cluster region
CD	- Crohn's disease
CK2α	- α subunit of casein kinase II
CTF	- C-terminal fragment
EAE	- experimental autoimmune encephalomyelitis
GWAS	- Genome-wide association study
IL	- Interleukin
KO	- knock out
L5	- fifth lumbar chord
MOG	- myelin oligodendrocyte glycoprotein
MS	- Multiple sclerosis
NTF	- N-terminal fragment
Psen1	- Presenilin-1
R	- Receptor
RA	- Rheumatoid arthritis
RF	- Rheumatoid factor
RT	- room temperature

SLE	- Systemic lupus erythematosus
T_C	- T cytotoxic cells
T_H	- T helper cells
TM	- Transmembrane
Y759	- Tyrosine 759 residue

Chapter 1: Introduction

CD4+ T- helper cells (T_H) cells are essential regulators of various immune responses and inflammatory diseases. For more than two decades, classic T_H cells paradigm was limited to two subsets - T_H1 and T_H2 cells [1,2]. T_H1 cells are triggered by interleukin-12 (IL-12) and their effector cytokines are Interferon-γ (IFN-γ) and IL-2. The IL-12 production is increased by IFN-γ and IL-12, via positive feedback, stimulates the production of IFN-γ in T_H1 cells, thereby maintaining its profile. The main effector cells are macrophages, T_C cells and B cells. The major T_H1 favoring transcription factors include STAT4 and T-bet. IFN-γ also activates macrophages to phagocytose and eliminate microbial pathogens [2,3]. T_H1 cells are vital for cell-mediated immune responses [2].

T_H2 cells are triggered by IL-2 and IL-4, and their effector cytokines are IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 [1,3]. The main effector cells are mast cells, B cells, eosinophils, basophils, and IL-4/IL-5 CD4+ T cells [3]. The key transcription factors for T_H2 are STAT6 and GATA3 [1]. T_H2 cells differentiation depends on the positive feedback action of the cytokine, IL-4 [3]. T_H2 cells provides humoral immunity by stimulating B-cells to proliferate, inducing B-cell antibody class switching, and increases neutralizing antibody production (IgG, IgM, IgA, IgE) as well as providing

protection against extracellular parasites [1]. In addition to expressing different cytokines, T_H2 cells and T_H1 cells have different cell surface glycans that make them less susceptible to some cell death inducers [2,3].

While the T_H1-T_H2 paradigm provided sufficient basis to further explore the nuances of immune responses to infection and autoimmune diseases, several evidences indicated that T_H1 cells were not the only contributors of the autoimmune disease development and progression. For example, IFN-γ-knock out (KO) mice did not show resistance to autoimmunity [4]. On the contrary, these mice were even more susceptible to autoimmunity, which led to the hypothesis that there might be additional T_H subsets distinct from the classic T_H cells. The additional T_H subsets include T_H17 cells, regulatory T cells (Treg) cells and the most recent, T_H9, T_H22, and T follicular helper cells (Tfh) [2,5,6].

T_H17 cells secrete IL-17A, IL-17F, IL-22, IL-6 and TNF-α [5]. IL-17 is a pleiotropic cytokine with a molecular weight of 20-30 kDa. The IL-17 family comprises of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F [13]. Among them, IL-17A and IL-17F are highly homologues and can be expressed in activated T cells [13]. IL-17A (hereinafter referred to as IL-17) specifically binds to the IL-17 receptor (specifically to the variant complex IL-17RA and IL-17RC) expressed in multiple tissues including fibroblast, vascular endothelial cells, myelomonocytic

cells, peripheral T cells, lung, marrow stromal cells and B cell lineages [13-16]. At homeostasis, T_H17 cells promote gut barrier defense, granulopoiesis, granulocyte chemotaxis, and immunity against extracellular pathogens through neutrophils and macrophages recruitment [6].

The most notable role of IL-17 is that it mediates tissue inflammation by inducing many pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α and chemokines such as CXCL1 [17]. Furthermore, IL-17 KO mice have shown resistance against collagen-induced arthritis (CIA) and EAE development, and that blocking of IL-17 prevents the development of EAE which further highlights its importance in the autoimmune disease development [18,19]. The cytokines most important for T_H17 differentiation are TGF-β, IL-6, and IL-1β, and the phenotype is maintained long term in the presence of IL-21 and IL-23 [5,6]. Along with IL-6, TGF-β induces the key transcription factor— the orphan nuclear receptors: retinoid related orphan receptor (ROR)γt and RORα in naive CD4+ T cells, which in turn drives their differentiation to a T_H17 phenotype [5,6,18,19].

STAT3 regulates IL-6-induced expression of RORγt and RORα and IL-17 production [5,6]. Contrary to STAT3 activation, T_H17 development is inhibited by STAT1 activation. While STAT3 and STAT1 are both activated by IL-6, it's been observed that activation of STAT1 is curbed in T_H17 cells but activation of STAT3 is maintained [20]. TGF-β and IL-6 also induce IL-23 which further activates

STAT3, RORα and RORγt in T_H17 cells to maintain their long-term phenotype. IL-1β also induces alternative splicing of Foxp3, inhibiting Treg differentiation and promoting IL-17 production [21] and IL-21 activates STAT3 downstream that induces T_H17 differentiation [6]. Additionally, transcription factor JunB also supports the T_H17 phenotype while repressing alternate CD4+ T_H1 and Treg phenotypes [6]. As described in detail below IL-6 acts as a potent proinflammatory cytokine to promote T_H17 differentiation, the controlled regulation of IL-6 is vital to maintain the T_H17 balance in order to avoid the alleviation of autoimmune symptoms [5, 22-23].

1.1 IL-6

IL-6 was cloned as B-cell stimulatory factor-2 in 1986 by Dr. Toshio Hirano and Dr. Tadamitsu Kishimoto at Osaka University [24-26]. IL-6 is a 21–28 kDa glycosylated protein composed of four long antiparallel α helices arranged in an up-up-down-down topology, forming three distinct epitopes which act as receptor-binding sites [27-30]. IL-6 is a pleiotropic cytokine that regulates multiple biological processes, including immune regulation, hematopoiesis, acute phase responses, inflammation, and oncogenesis [5,31-33]. IL-6 forms a protein complex with the IL-6R with nanomolar affinity [27,32,34]. IL-6R exists in both transmembrane and soluble form. Both the forms of IL-6R bind IL-6 with similar affinity [35]. The

80 kDa transmembrane IL-6R is limited to very few cell types such as hepatocytes, intestinal epithelial cells, neutrophils, monocytes/macrophages and some lymphocytes [36-38], while the 50-55 kDa soluble form of IL-6R (sIL-6R) is found in human serum generated by either translation from alternatively spliced mRNA or by proteolysis of transmembrane IL-6R [36,38,39]. The complex formed between IL-6 and sIL-6R can initiate autocrine or paracrine IL-6 signaling in any cell type that expresses gp130, thereby enabling IL-6 function in most parts of the body [27,40]. This form of IL-6 signaling is termed IL-6 trans-signaling, while signaling through transmembrane IL-6R is IL-6 classic signaling [38,41,42]. Classic IL-6 signaling plays important role in regenerative or anti-inflammatory activities, such as hepatic acute phase response activation, regeneration of intestinal epithelial cells that are STAT3-dependent and epithelial apoptosis inhibition [38,41,42]. IL-6 trans-signaling drives the pro-inflammatory actions of IL-6, that includes Treg cell differentiation inhibition, mononuclear cells recruitment and T cell apoptosis inhibition [38,41,42]. However, binding of IL-6 to the IL-6R alone does not lead to signaling.

The initiation of the various IL-6 functions happen when the IL-6 and IL-6R complex associates with the ubiquitously expressed 130 kDa transmembrane protein gp130 inducing its homodimerization leading to the formation of hexameric structure comprising of two molecules each of IL-6, IL-6R, and gp130 that triggers

various downstream signaling cascades [43-46]. gp130 has no intrinsic kinase activity but contains Box-motifs in its cytoplasmic domain which upon the dimerization comes in close proximity with Janus kinase family (JAK1, JAK2, TYK), which are tyrosine kinases that results in transactivation of each other and the phosphorylation of the tyrosine residues in the cytoplasmic domain of gp130 [27, 43-48]. Among the JAK kinases, JAK1 serves a major role in the gp130-mediated pathways. The IL-6 induced gp130 triggers the JAK-signal transducer and activator of transcription (STAT1/3) pathway mediated by the YxxQ (Y767, Y814, Y905, and Y915 in human gp130); the JAK—SHP2–Gab-Ras-Erk– mitogen-activated protein kinase (MAPK) pathway, regulated via tyrosine 759 (Y759) residue of gp130 [36,47,49-53] and the Src-YAP-Notch pathway [54,55]. The gp130 also acts as a signal transducer for the other members of the IL-6 family of cytokines, which include the leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor (CNTF), IL-11, cardiotrophin 1, cardiotrophin-like cytokine factor 1 (CLCF1), IL-27, IL-35 and IL-39 [27,45,56-58].

Dysregulation or overproduction of IL-6 is associated with autoimmune diseases such as MS and RA, where the primary cause of pathology is thought to be T_H17 cells [7,8,13]. High concentration of IL-6 has been observed in the blood and synovial fluid samples of RA patients [59]. As IL-6 plays a very critical role in T_H17 regulation, various autoimmune and inflammatory diseases can be treated effectively

by controlling IL-6 activities [59,60]. In fact, IL-6 signal blockade has been a novel approach to treat inflammatory and autoimmune disorders with promising outcomes [61].

1.2 Rheumatoid arthritis and its treatment

RA is a symmetrical inflammatory arthritis in the joints of the whole body that affects nearly 1% of the world's adults mainly between ages 40-60 where women are more susceptible [62]. The underlying mechanism involves the immune system attacking the joints, characterized by symmetric polyarticular inflammation of the synovium, typically of the small joints of the feet, hands and wrists [63,64]. This inflammation results in stiffness and pain and leads to progressive joint, bone and cartilage damage resulting in loss of function and deformities. The major symptoms of RA are joint pain, swelling, stiffness and loss of joint function [62-65]. Additionally, chronic inflammation that occurs secondary to RA leads to an increased risk of cardiovascular disease, organ damage and bone metabolism changes [63,65].

RA is characterized by the expression of rheumatoid factor (RF) which is the antibody against IgG's Fc portion [63]. RF forms immune complex with IgG that may contribute to the disease process. While the cause of RA is not clear, it is believed to involve a combination of genetic and environmental factors such as

smoking, periodontal disease and drugs that might trigger the disease [63]. RA is strongly associated with mutations in HLA-DR4 of MHC class II molecule [62,63,66]. Infact, there are reports suggesting that the cartilage-derived antigen HC gp-39, cyclic citrullinated peptide (CCP), derivatives of fibrillin and collagen are the self-antigen candidates for the T-cell-mediated immune response [62, 67]. However, it is unclear whether these peptides are the cause or the result of joint damage. Despite the evidence for antigen-specific T cell activation in some RA patients, tissue-specific self or non-self-antigens recognized by activated CD4+ T cells in majority of RA cases have not been well-established [67]. This raises the possibility that tissue-specific antigens are not vital for the break-down in CD4+ T cell tolerance to cause the tissue-specific autoimmune disease. Instead, CD4+ T cells may act as a source for variety of inflammatory cytokines such as TNFα, IL-1 and IL-6 that orchestrate synovial inflammation and stimulate cartilage degradation [68,69].

Currently, FDA has approved many biologics for the treatment of RA, including TNF inhibitors (infliximab, etanercept, adalimumab, certolizumab pegol, and golimumab), anakinra (IL-1 receptor antagonist), abatacept (CTLA4-Ig fusion protein), rituximab (anti-CD20 antibody) [64,70], tocilizumab [56,61] and sarilumab (IL-6R blocker) [38,71]. Other IL-6 blockers currently undergoing phase trials are

sirukumab, olokizumab, clazakizumab, situximab (targets both transmembrane IL-6R and sIL-6R) and tofacitinib, ruxolitinib (target JAK) [38].

Tocilizumab, a humanized anti-IL-6R antibody, was jointly developed by Chugai Pharmaceutical and Osaka University, that became a novel therapeutic strategy for other inflammatory and autoimmune diseases besides RA including, systemic-onset juvenile idiopathic arthritis (sJIA), CD, Castleman's disease, giant cell/Takayasu arteritis and SLE [38,56,70,72]. Currently, tocilizumab has been approved for the treatment of RA in more than 100 countries [56]. In RA patients, the symptoms and the disease scores (upto 70% respectively) were significantly improved by tocilizumab, as well as normalized serum amyloid A and C-reactive protein in patients in 6 weeks post the drug administration [70,72]. IL-6 is important for T_H17 differentiation in both humans and mice [56,59,67] which is one possibility that tocilizumab may improve the symptoms of RA.

1.3 Role of IL-6/gp130 signaling in autoimmune disease model

To understand the IL-6 regulated molecular networks better, the laboratory I belong to, investigated IL-6 receptor–related major signaling pathways: STAT3 and SHP2/Gab/MAPK signaling, that involved the gp130 YxxQ and Y759 motives, respectively. In addition to the induction of SHP-2–mediated ERK-MAPK

activation, the Y759 residue provides the binding site for the suppressor of cytokine signaling-3 protein (SOCS-3), which negatively regulates the gp130 signals and attenuates IL-6 signaling [25,73]. To understand the in vivo roles of these gp130-associated signaling cascades, series of knock-in mouse lines were created where gp130-mediated SHP2 or STAT3 signaling was selectively disrupted [25,67,73].

In one knock-in strain, Y759 of gp130 was substituted to a phenylalanine residue. Increased STAT3 activation through gp130 was observed in F759 mice because Y759 required for SOCS3-mediated suppressive mechanisms was specifically defective [47,73]. It was observed that F759 mice, spontaneously developed a RA-like joint disease (F759 arthritis) as they age, which clearly highlighted the critical role for IL-6 signaling in the development of autoimmune disease (Supplementary Fig.1). F759 mice shows a variety of immunological abnormalities, such as autoantibody production, hypergammaglobulinemia and increased memory/activated T cells [31,73]. It was also observed that viral infection, HTLV-1 pX (a product of HTLV1 virus, p40 Tax) enhanced F759 arthritis in a C57BL/6 mice background via NF-κB suggesting that both STAT3 and NF-κB are involved in F759 arthritis [74].

Bone-marrow transplantation studies and experiments with various KO mouse strains revealed that F759 arthritis is CD4+ T cell–dependent, and that the gp130

F759 mutation was present in nonhematopoietic cells. [31,67,75]. Furthermore, the excessive IL-6 signaling in non-hematopoietic cells, particularly in type 1 collagen+ cells lead to an enhanced production of IL-7, a T-cell survival factor, that increased the activation of CD4+ T cells via homeostatic proliferation, which is important for the F759 arthritis development [75]. These findings demonstrated that IL-6 signaling is indispensable for the arthritis development in F759 mice.

It was also demonstrated that as F759 mice aged, there was an increase in the activated T_H17 cells in the spleen and superficial lymph nodes as well as increased serum IL-17 concentration [73-77]. Additionally, deficiency of IL-17 suppressed arthritis in F759 mice, whereas forced expression of IL-17 augmented it [73,76]. Following the forced IL-17 expression in F759 mice, abnormally high concentration of IL-6 and some chemokines were found in the serum, implying a positive feedback mechanism for IL-6 signaling in the presence of IL-17 [73,76,77]. Furthermore, IL-17–induced NF-κB activation strongly augmented IL-6 gene expression in the presence of IL-6 [76,77]. IL-6 also induced T_H17 cells that produced IL-17 which formed a positive feedback loop in the nonimmune cells.

It was also observed that the injection of myelin oligodendrocyte glycoprotein (MOG)-specific T_H17 cells into the wild type mice increased IL-6 expression

followed by encephalomyelitis development [76]. The IL-6 deficiency in the recipient mice attenuated the development of EAE after the injection of T_H17 cells [76]. Also, specific depletion of STAT3 in type I collagen+ fibroblasts attenuated EAE development [76]. Together, these results strongly support the role of IL-17-triggered positive-feedback loop of IL-6 expression in nonhematopoietic cells in the development of EAE [67,76]. Furthermore, the blockade of the IL-6 loop significantly suppressed the development of F759 arthritis and EAE. This positive feedback loop of IL-6 was termed as the "Inflammation amplifier" (Earlier was called "IL-6 amplifier") (Supplementary Fig.2) [67,69,75-77].

It was further observed that local events such as microbleeding in joints along with T_H17 cells accumulation enabled arthritis induction in F759 mice independently of tissue antigen-recognition [69]. Increase in the microbleeding induced T_H17 cells accumulation in the F759 mice with age lead to increased IL-17 presence, that triggers inflammation amplifier activation and corresponding chemokine such as CCL20, which is a target of the amplifier [67,69]. This led to the hypothesis that local events could induce certain MHC class II–associated, tissue-specific autoimmune diseases resulting in an antigen- independent accumulation of effector CD4+ T cells, that activates the inflammation amplifier via cytokines in the affected tissue. Thereby, demonstrating a new concept in explaining the pathogenesis of

autoimmune diseases where the target tissue themselves, determine the specificity of the autoimmune disease via activation of the inflammation amplifier [67,69,76,77]. To explain this hypothesis, a four-step model for MHC class II associated autoimmune diseases was proposed that states:

(1) T cell activation regardless of antigen specificity (2) local events inducing a tissue specific accumulation of activated T cells (3) transient activation of the inflammation amplifier triggered by T cell-derived cytokines such as IL-17 (4) enhanced sensitivity to T cell-derived cytokines and/or IL-6 in type 1 collagen+ cells in the target tissue. This results in chronic activation of the amplifier and subsequent manifestation of autoimmune diseases leading to chronic inflammations. [31,67,77].

In 2012, “gateway reflex” was proposed where an entry site at the dorsal blood vessels of the fifth lumbar cord (L5) for the pathogenic CD4+ T cells into the CNS was described [78]. This location was defined by the inflammation amplifier dependent upregulation of the chemokine CCL20 which attracts T_H17 cells in associated vascular endothelial cells, that depends on gravity-induced sensory neurons activation by the soleus muscle in the leg. This enhanced CCL20 expression in the dorsal blood vessels via the activation of sympathetic neurons indicated that

the neural activation could transform into an inflammatory signal that risks the development of autoimmune disease [78-82]. This study offered a novel location of neuroimmune interactions which could be a valuable therapeutic target for various neuroimmune disorders including autoimmune and inflammatory diseases.

Thus, it led us to conclude that the inflammation amplifier was fundamental for the immune system and nonimmune tissue interaction through the synergistic activation of STAT3 and NF-κB [31,80]. Collectively, our research led us to hypothesize that various events, comprising of viral infection, antigen-independent T cell development, injury, and/or physical stimulation that are capable of activating the inflammation amplifier through NF-κB and/or STAT3 in nonimmune tissues may provide a general etiologic mechanism for various autoimmune diseases.

To further understand the detailed molecular mechanism of the inflammation amplifier and its role in human diseases, genome wide screening was performed where about 65,000 lentivirus lines encoding shRNA corresponding to approximately 16,000 mouse genes were tested and 1,289 candidate genes that were positive regulators of the inflammation amplifier were recognized and many genes (>500) associated with human diseases were highly enriched in these regulators [83] (Supplementary Fig.3), Thereby, offering new clinical targets that could be used to

impair the activation of the inflammation amplifier in affected tissues. For the present study, out of 1,289 candidate genes, I selected Presenilin-1 (Psen-1) for detailed analysis.

1.4 Presenilin-1 (Psen1)

Psen1 is a transmembrane protein that forms the critical catalytic component of the γ-secretase complex along with nicastrin (NCT), the anterior pharynx-defective protein 1 (APH1), and the presenilin enhancer 2 (PEN2) that cleaves many type I membrane proteins [84-87] releasing their corresponding intracellular domains, which are capable of influencing gene expression.

Psen1 possesses a nine transmembrane domain (TM) topology constituting of 467 amino acids (aa), with a cytosolic (1-76 aa) and an extracellular (101-132 aa) C-terminus and a cytosolic N-terminus (271-376 aa). Barring the N and C-terminus, the rest of the TM domains are hydrophobic [88]. Psen1 undergoes endo-proteolytic processing to be cleaved into two stable pieces – a 27-28 kDa N-terminal fragment consisting of 1-6 TM domain (NTF) and a 16-17 kDa C-terminal fragment consisting of 7-9 TM domain (CTF) and remain associated as a heterodimer in cell [85,88]. The interface between the NTF and CTF subunits forms the docking site for the transmembrane domain of the substrate to interact prior to its entry into the internal

catalytic site constituting of aspartate residues – D257 and D385 in the 6th and 7th TM domains, respectively. Thus, harboring the active site of the γ-secretase enzymatic complex [88]. Majority of Psen1 is localized in the plasma membrane, while traces of it are also found in the golgi membranes, endoplasmic reticulum (ER), endosomes and mitochondria [89].

γ-secretase complex is responsible for the generation of amyloid-β peptide from the amyloid precursor protein (APP) which is a type-I transmembrane protein known to play a role in cell adhesion, protein transport, synapse formation, neurite extension, and neuroprotection [86]. Apart from APP, additional substrates have been identified for γ-secretase that have been found to undergo similar proteolysis by the enzyme complex, including Notch and Jagged (cell fate determination), N- and E-cadherins, CD44, and nectin-1α (cell-cell adhesion), β2 subunit of the voltage-gated sodium channel (regulation of ion conductance), ErbB4 (growth factor-dependent receptor tyrosine kinase signaling), and p75 NTR (neurotrophin signaling) [84,86,87,90].
More than 185 mutations have been identified in Psen1 [84]. These mutations cause a subtle but lethal shift in the cleavage of the transmembrane domain of APP resulting in an increase in the ratio of the 42- to 40-residue amyloid-β protein (Aβ), leading to Aβ aggregation that forms plaques in the cortical brain areas and trigger a variety of inflammatory pathways, and inflammation has been suggested to

significantly contribute to the pathogenesis of Alzheimer's Disease (AD) [84-88, 91]. Since some Psen1 mutants of familial AD do not increase the production of neurotoxic Aβ42 or increase the Aβ42/Aβ40 ratio [92], I considered other mechanisms through which Psen1 could promote inflammation.

Many γ-secretase-independent systems have been reported for Psen1 [93]. For example, Psen1 holoprotein forms endoplasmic reticulum (ER) calcium ion leak channels, participates in intracellular protein trafficking regulation, downregulates insulin signaling by inhibiting the transcription of insulin receptor [93,94]. It's also reported that Psen1 modulates the turnover of β-catenin by associating with glycogen synthase kinase 3β and protein kinase A for cell proliferation [95,96]. However, a contribution of Psen1 in the inflammation development has not been established.

In the present study, I investigate the involvement of Psen1 in the inflammation amplifier. We recently reported the role of breakpoint cluster region (BCR) in inflammation development via its association with α subunit of casein kinase II (CK2α) [97]. BCR protein was identified as a fusion protein of Abl tyrosine kinase [97,98]. This fusion forms the Philadelphia chromosome which is a specific genetic abnormality in chromosome 22 in several forms of leukemia such as chronic myeloid

leukemia (CML), acute lymphoblastic leukemia (ALL) [99,100]. The fusion causes dysregulation of Abl tyrosine kinase activity, resulting in abnormal cell survival and proliferation [98]. The BCR gene is ubiquitously expressed and has several functional domains, such as oligomerization and GTPase-activating protein domains [101,102].

CK2 is a multifunctional protein kinase that has crucial roles in cell differentiation, proliferation and survival [103]. It was one of the first serine/threonine kinases having tyrosine kinase activity [104]. CK2 forms a heterocomplex composed of two catalytic α subunits and two regulatory β subunits. CK2 is also known to play a critical role in NF-κB–mediated transcription [105-108].

In this study, I tried to understand the role of Psen1 in inflammation amplifier and inflammation development that could be a novel therapeutic target for diseases that display chronic inflammation such as AD.

Chapter 2: Experimental Materials and Methods

2.1 Experimental materials

2.1.1 Cell lines and culture conditions

- BC-1 (a type 1 collagen+ endothelial cells; provided by Dr. Masayuki Miyasaka, Osaka university) [109]
- H4 neuroglioma cells were obtained from ATCC (Sumitomo Pharma International, Japan).
- HEK293 T cells

DMEM was used as the culture medium supplemented with 10% heat inactivated Fetal bovine serum (FBS) and antibiotics (Penicillin (100 units /ml), Streptomycin (100 µg/ml).

Cells were cultured at 37°C with 5% CO_2. Also, all the equipment used were sterile. All culture procedures were performed in a clean bench in aseptic condition.

2.1.2 Experimental animals

- C57BL / 6 mice (SLC, Shizuoka, Japan)
- F759 mice were back-crossed with C57BL/6 mice for more than 10 generations [69]

All mice were maintained under specific pathogen-free conditions according to the protocols of Osaka University and Hokkaido University.

2.1. 3 Reagents

Human IL-6 (Toray Industries, Tokyo, Japan), human soluble IL-6R (R&D Systems, Tokyo, Japan), mouse IL-17A (R&D Systems), TNF-α (R&D systems, Tokyo, Japan), DMEM, FBS, shRNA's specific for nontarget shRNA (Sigma Mission SHC002V); Psen1 (TRCN0000030520; Sigma-Aldrich), polybrene, siRNAs specific for human Psen1 (s224428, Thermo Fisher Scientific); p65 (Ambion Silencer Select RELA siRNA, Thermo Fisher Scientific) and non-target (Ambion Negative Control #1 siRNA, Thermo Fisher Scientific), Lipofectamine RNAiMAX (Thermo Fisher Scientific), Opti-MEM (Thermo Fisher Scientific), GenElute mammalian total RNA kit and DNase I (Sigma-Aldrich), M-MLV reverse transcriptase (Promega), KAPA SYBR Fast qPCR kit (KAPA BIOSYSTEMS), DNase I (Sigma-Aldrich), ELISA kit specific for mouse IL-6 (BD Biosciences, Tokyo, Japan), thiazolyl blue tetrazolium bromide (sigma-aldrich), Dynabeads protein G (Life Technologies, Tokyo, Japan), Chelex 100 (Bio-Rad, Tokyo, Japan), Cytofix/Cytoperm kit (BD Biosciences)

2.2 Experimental methods

2.2.1 Establishment of Psen1-deficient cells

Mouse endothelial BC1 cells [109] were counted using a cell counter and cultured in a 96-well flat-bottom plate (1,000 cells/ well) in 100 µl of DMEM containing 10% FBS and antibiotics on day 1. The medium was replaced on day 2 with DMEM containing 1 µl of lentivirus carrying candidate shRNA [nontarget shRNA (Sigma Mission SHC002V); Psen1 shRNA (TRCN0000030520; Sigma-Aldrich)] (35 µl diluted 5x), 10% FBS, and 8 µg/ml polybrene. On day 3, 200 µl of DMEM containing 10% FBS and 5 µg/ml puromycin was added to each well. After 24-48 hours, once the cell confluency is around 70-80%, the knockdown cells are transferred to 24 or 6-well plate while being maintained in DMEM, 10% FBS and 5 µg/ml puromycin for further experiments.

siRNAs for human Psen1 (s224428; Thermo Fisher Scientific), p65 (Ambion Silencer Select RELA siRNA; Thermo Fisher Scientific), and nontarget (Ambion Negative Control no. 1 siRNA; Thermo Fisher Scientific) (5 µM ; 0.5 µl/well) using Lipofectamine RNAiMAX (0.28µl/well ; Thermo Fisher Scientific) and opti-MEM (Thermo Fisher Scientific) were transfected in H4 neuroglioma cells that were cultured in a 96-well flat-bottom plate (1 x 10^5 cells/ well) in 70 µl of DMEM containing 10% FBS and incubated at 37°C with 5% CO_2 on day 1. DMEM medium

change is done on day 2. On day 3, the transfected cells are starved for 2 hours in opti-MEM followed by 3 hours of stimulation with TNF-α (50ng/ml) in opti-MEM followed by cell lysis and RNA collection for RT-PCR.

2.2.2 Cytokine-induced arthritis

Non-target shRNA, p65 shRNA or Psen1 shRNA (Sigma Aldrich) lentivirus particles were injected at 20 µl into the ankle joints of F759 mice on days 0, 1 and 2, and then IL-6 and IL-17 (100 ng/ 20 µl each) were injected into the ankle joints on days 6, 7 and 8. Averages for a single point in one leg ankle joint from each mouse were used for clinical assessments. Clinical scores of the arthritis were evaluated using the mobility of the ankle joint of the mouse as an index [69].

0: Normal, 1: Small movable limit (the maximum mobility of the joint to form the angle of the shin and instep to 180 degree), 2: Mild movable limit (the angle is from 150 to 180 degree), 3: Medium movable limit (the angle is from 135 to 150 degree), 4: Severe movement limit (the angle is less than 135 degree).

2.2.3 Real-time PCR

The 7300 fast real-time PCR system (Applied Biosystems, Tokyo, Japan) and SYBR Green PCR master mix (Kapa Biosystems, Woburn, MA) were used to quantify levels of target mRNA and hypoxanthine phosphoribosyltransferase (Hprt) mRNA.

Mock (nontarget control) and Psen1 knockdown cells were plated in 12-well plates (1 x 10^5 cells/well) and stimulated with human IL-6 (100 ng/ml; Toray Industries) plus human soluble IL-6R (100 ng/ml ; R&D Systems) and/or mouse IL-17 (50 ng/ml ; R&D Systems) as well as TNF-α (50 ng/ml ; PeproTech) for 3 h at 37°C with 5% CO2 after 2 h serum starvation using plain DMEM. The medium was discarded followed by addition of lysis solution in which 1/100 amount of 2-mercaptoethanol (2-ME) was added to lyse the cells. Total RNA was prepared from cells using a GenElute mammalian total RNA kit and DNase I (Sigma-Aldrich). 2 µg RNA is converted into cDNA in a reaction mixture of 25 µL comprising of 10 mM dNTPs, oligodT, buffer and M-MLV reverse transcriptase (Promega) (42℃ 1 hour, 95℃ 5min). cDNA equivalent to 20 ng was used for PCR reactions. The PCR reactions were performed using KAPA SYBR Green mastermix with respective primers in the final reaction volume of 20 µL. The conditions for real-time PCRs were 40 cycles at 94°C for 15s followed by 40 cycles at 60°C for 60 sec. The relative mRNA expression levels were normalized to the levels of Hprt mRNA expression.

cDNA was amplified by PCR to obtain 10^{-11} to 10^{-17} g/µl, based on a calibration curve prepared from a 10-fold dilution series.

The following primers were used.

Mouse HPRT Forward: 5’-GAAGCGAGAGAACCAGG -3’

Mouse HPRT Reverse: 5’-CCCCCACCCCAGACA-3’

Mouse IL-6 Forward: 5’-GAGGAACCACCCCAACAGACC-3’

Mouse IL-6 Reverse: 5’-AAGGCACACGGCAACA-3’

Mouse LCN2 Forward: 5’- CCACCGGCAGGGAC-3’

Mouse LCN2 Reverse: 5’- GGCCCAACAGGG -3’

Mouse SOCS3 Forward: 5’-GCGGACCGCGGAG-3’

Mouse SOCS3 Reverse: 5’-GAGACGCCGGGACA-3’

Mouse STAT3 Forward: 5’-CACCTTGGATTGAGAGTCAAGAC-3’

Mouse STAT3 Reverse: 5’-AGGAATCGGCTATATTGCTGGT-3’

2.2.4 ELISA

The knockdown BC1 cells were cultured in 96-well plates (1×10^4 cells/well) on day 1. Stimulation with human IL-6 (100 ng/ml ; Toray Industries, Tokyo, Japan) plus human soluble IL-6R (100 ng/ml ; R&D Systems, Tokyo, Japan) and/or mouse IL-17 (50 ng/ml ; R&D systems) as well as TNF-α (50 ng/ml ; PeproTech, Tokyo, Japan) in plain DMEM was done on day 2.

IL-6 levels in culture supernatants were detected by ELISA kits. As human IL-6 acts on mouse cells, detection of IL-6 production from mouse cells [109] stimulated with

human IL-6 can be done using ELISA specific for mouse IL-6 (BD Biosciences, Tokyo, Japan).

2.2.5 MTT assay

To assess the cell growth, the knockdown BC1 cells were cultured in 96-well plates (1×10^4 cells/well) on day 1 and stimulated with human IL-6 (100 ng/ml ; Toray Industries, Tokyo, Japan) plus human soluble IL-6R (100 ng/ml ; R&D Systems, Tokyo, Japan) and/or mouse IL-17A (50 ng/ml ; R&D systems) as well as TNF-α (50 ng/ml ; PeproTech, Tokyo, Japan) in plain DMEM on day 2 for 24 hours. The medium was removed from the cells and 10% MTT solution (thiazolyl blue tetrazolium bromide) diluted in DMEM with 10% FBS was added. The plate was incubated at 5% CO_2 and 37°C for 2 hours. Thereafter, the culture supernatant was removed. 100 µL DMSO/well was added to dissolve the MTT precipitate, the absorbance of color reaction was measured at 550 nm.

2.2.6 Luciferase Assay

Full-length mouse Psen1 cDNA was cloned into pEF-BOS expression vector [110]. pGL4.32 (luc2P/ IL-6-RE/ Hygro), pRL-TK (Promega) and pEF-BOS Psen1 were transiently co-transfected into HEK293T cells (2×10^4/100 µl in DMEM +10% FBS) by using polyethylenimine. 24 hours after transfection, the cells were stimulated

with 50 ng/ml TNF α for 6hours. Luciferase activities of total cell lysates were measured using the Dual-luciferase reporter assay system (Promega).

2.2.7 Chromatin immunoprecipitation (ChIP) assay

Control and Psen1-knockdown cells (5 x 10^5 cells/plate) were stimulated with 50 ng/ml TNFα for 0, 90, 180 min post serum starvation for 2 hours in plain DMEM. These cells were fixed with 1% PFA (10 min RT on shaker) followed by addition of 2.5 M glycine to stop fixing. The cells were lysed with cell lysis buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1% SDS) and 1/100 phosphatase inhibitors (PIs). The collected cells were spin down at 1500 rpm x 5 min at 4^{o}C and 3500 rpm x 5 min at 4^{o}C. The lysed cells were resuspended in the cell lysis buffer (60 µl/10^6 cells) (1/100 PIs), vortexed 10 sec every 5 min and sonicated for 4 cycles of 30 sec ON/30 sec OFF for 5 min twice at 4^{o}C. Post sonication, the samples were centrifuged immediately at 15000 rpm for 10 min at 4^{o}C to collect the chromatin DNA. Dynabeads protein G (Life Technologies, Tokyo, Japan) (30 µl/sample) and anti-p65 (santa cruz biotechnology), anti-p300 (santa cruz biotechnology), anti-acetyl-H3K27 (TaKaRa Bio, Tokyo, Japan) antibodies, or rabbit IgG or mouse IgG (2 hours rotate at 4^{o}C) (5 µg/sample) preparation was done followed by immunoprecipitation (IP) of sonicated samples along with dynabeads + antibody mixture in dilution buffer (10 mM Tris-HCl [pH 7.5],

140 mM NaCl, 1% Triton X-100 and 1 mM EDTA) (1 ml/sample ; 2 h rotate at 4ºC). IP samples were washed 4 times using wash buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 1% Triton X-100, 1 mM EDTA and 0.1% SDS) (700,750,800,850 µl). The input DNA is prepared adding 3 M NaOAc, 20 mg/ml glycogen, 100% ethanol in the lysed sample followed by 30 min incubation on ice, spin at 15000 rpm x 30 min followed by 70% ethanol addition, spin at 15000 rpm x 15 min and pellet collection.

DNA purification was with 10% Chelex 100 (Bio-Rad, Tokyo, Japan) (40 µl) and extracted using proteinase K (1µl of 2 mg/ml) (55ºC, 30 min at 1100 rpm; boil at 100ºC, 10 min). The conditions for real-time PCRs were 40 cycles at 95°C for 15 sec followed by 40 cycles at 60°C for 60 sec. The relative dissociation curve levels of immunoprecipitated samples were normalized to the levels of non-immunoprecipitated input samples (10% of the sample). Real-time PCR was performed with IL-6 or lipocalin 2 (Lcn2) promoter primer that included a p65 binding site.

Relative to the transcription start site, the p65 binding site in IL-6 promoter is -26 to -17 bp, and the forward and reverse primers start at -131 bp and +27 bp, respectively.

The p65 binding site in Lcn2 promoter is -261 to -252 bp, and the forward and reverse primers start at -273 bp and -74 bp, respectively [111].

The following primer sequences were used for the PCR.

Mouse LCN2 Forward: 5'-A CCAAAGCCCGGGAAGC-3'

Mouse LCN2 Reverse: 5'-GGGAGCCACCACCAA-3'

Mouse IL-6 Forward: 5'- CGAGCAAACGACGCAC-3'

Mouse IL-6 Reverse: 5'-GAGCACAGACACCCCAG-3'

2.2.8 Chromatin Accessibility Assay

Control and Psen-1 deficient cells (3 x 10^5 cells/dish) were stimulated with TNFα (50 ng/ml) for 60 min. Then, chromatin DNA was isolated using the Chromatin Accessibility Assay Kit (Epigentek) and amplified with real-time PCR for region-specific analysis of chromatin accessibility. PCR conditions and primers for IL-6 and Lcn2 promoters were the same as those used for the ChIP assay.

2.2.9 Confocal microscopy

Non-target control and Psen1-deficient BC1 (1 x 10^5 cells) were stimulated with TNFα for 0, 15 and 30 min at 37°C and 5% CO_2 post serum starvation for 2 h in plain DMEM. The stimulated cells were fixed in cytofix (BD Biosciences Cytofix/Cytoperm kit) for 10 min at RT, permeabilized with Perm/Wash solution

(BD Biosciences Cytofix/Cytoperm kit) and incubated with rabbit anti-p65 (1/50; Santa Cruz Biotechnology) in 100 µl Perm wash for 1 h at RT. After washing with 1x

PBS (100 µl x 3), the cells were incubated with anti-rabbit Alexa Fluor 488-conjugated secondary Ab (1/200; Life technologies) and Hoechst 33342 nuclear stain (1/10,000; Life technologies) in 100 µl Perm wash for 1 h in dark at RT followed by washing with 1 x PBS (100 µl x 3) and addition of 100 µl of PBS. Cells were then observed by confocal microscopy [112]. The cells were viewed using the LSM 5 Pas confocal microscopy system (Carl Zeiss) using a Plan-Apochromat 63 x/1.4 Oil DIC I lens. Laser lines at 488 nm and 361 nm were used for excitation of Alexa Fluor-488, Hoechest 3342 and emissions wavelengths were separated by band pass (505–530 nm) and (405-450 nm) respectively. The pinhole size was set to 1.2–1.6 Airy Units, and the frame scan rate was 7.86 sec. Images were optimized using LSM 5 Pas software release 3.2 (Carl-Zeiss), and transferred to Photoshop (Adobe Systems) to produce the final figures.

2.2.10 Immunoprecipitation

HEK293T cells (1.5 x 10^6) were cultured in DMEM supplemented with 10% FBS and antibiotics and were co-transfected with pEF-BOS (5 µg/plasmid) containing

full-length WT or mutant Psen1, BCR and/or CK2α cDNA [97]. Mouse Psen1 mutant cDNA lacking amino acids 1-73 (Δ1-73) or 271-376 (Δ271-376), in which a large part of the hydrophilic cytoplasmic loop was deleted, was prepared by an inverse PCR method using full-length Psen1 cDNA. A CK2α mutant cDNA that lacks 35 N-terminal amino acids (ΔN-ter) was also generated by inverse PCR. They were tagged with Flag or HA at the N-terminal. These transfected cells were suspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 500 mM NaCl, 1% NP40, and 3 mM EDTA) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The concentration of protein was measured with a protein assay kit (Promega). They were pre-cleared with 30 μl protein G–Sepharose (Pharmacia, Tokyo). These samples were centrifuged at 9,000 rpm at 4℃ for 3 min, and the supernatants were collected. The samples were mixed with 30 μl anti-FLAG M2 beads slurry (Sigma-Aldrich) or HA beads (Sigma-Aldrich) and incubated for 2 h at 4℃ with gentle agitation. The samples were centrifuged at 9,000 rpm at 4℃ for 3 min, and the supernatants were discarded. Anti-Flag M2 beads/HA beads were washed five times with 800 μl HEPES-buffered saline plus Triton X-100 (HBST). The immunoprecipitates were eluted with 3x flag peptide (Sigma-Aldrich) or 2x SDS-PAGE loading buffer (for HA), separated by SDS–PAGE, and transferred to a PVDF membrane followed by western blotting.

2.2.11 Western blotting

WT or Psen1-knockdown cells were stimulated with indicated cytokines and washed three times with cold PBS, scraped from the bottom of the dish, and lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, and 3 mM EDTA) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The concentration of protein was measured with a protein assay kit (Promega). The cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Tokyo). Blocking to prevent nonspecific binding was done by 5% skim milk with gentle agitation at RT for 1 h. Immunoblotting was performed using Can Get Signal Immunoreaction Enhancer Solution (Toyobo) according to the manufacturer's protocol.

The antibodies used were mouse anti-Flag Ab (1/5,000 ; Sigma-Aldrich), rabbit anti-HA Ab (1/4,000 ; Sigma-Aldrich), rabbit anti-BCR Ab (1/1,000 ; Cell Signaling Technology), rabbit anti-Psen1 (1/2,000 ; Cell Signaling Technology), rabbit anti-Na, K ATP-ase (1/2,000 ; Cell Signaling Technology), goat anti-lamin B (1/4,000 ; Santa Cruz Biotechnology), rabbit anti-phospho CK2α (1/1,000 ; Sigma Aldrich), rabbit anti-CK2α (1/1,000 ; Cell Signaling Technology), rabbit anti-phospho BCR (1/2,000 ; Cell Signaling Technology), mouse anti-tubulin (1/8,000 ; Sigma Aldrich), anti-mouse IgG HRP (1/5,000 ; Southern Biotech) and anti-rabbit IgG HRP (1/10,000 ; Southern Biotech). The proteins were visualized by enhanced

chemiluminescence (Chemi-lumi One L, Nacalai tesque) according to the manufacturer's instructions.

2.2.12 Preparation of cellular fractions

WT and Psen1-deficient BC1 cells (5 x 10^6) were stimulated with TNFα for 5 min and washed three times with cold PBS. The cells were then fractionated into cytosol, membrane and nuclear fractions using EzSubcell Extract (ATTO) kit. Successful separation of these fractions was confirmed by immunoblotting with anti-tubulin (1/10000 ; Sigma Aldrich), anti-Na/K-ATPase (1/3000 ; Cell Signaling technology) and anti-lamin B antibodies (1/3000 ; Santa Cruz Biotechnology).

2.2.13 Statistical analysis

Experimental data represents the mean ± standard deviation (Mean ± SD) or mean ± standard error (Mean ± SEM).

The p values were calculated using student t test (two tailed) or ANOVA tests, and p value less than 0.05 was considered significant.

Chapter 3: Results

3.1. Psen1 is critical for NF-κB pathway in vitro and in vivo

An inflammation-inducing mechanism known as the inflammation amplifier, specific to nonimmune cells, is activated by the simultaneous stimulation of NF-κB and STAT3 and is involved in the pathogenesis of several inflammatory disease models [25,27,31,59,67,69,72-78,82]. Using genome-wide screenings [83], we identified Psen1 as one of the positive regulators of the inflammation amplifier. To confirm this result, I treated mouse BC1 endothelial cells with lentivirus carrying shRNA of Psen1 to establish Psen1-deficient cells which significantly reduced Psen1 expression (Fig. 1A). The protein and mRNA levels of IL-6, a NF-κB target gene, were significantly reduced in Psen1-deficient cells with IL-6 and IL-17 or with TNFα stimulation without affecting the cell viability (Fig. 1B and 1C). The reduced expression of IL-6 was rescued by overexpression of Psen1, excluding the possibility of off-target effects by the shRNA (Fig. 1D).

siRNA mediated knockdown of Psen1 in H4 neuroglioma cells suppressed IL-6 mRNA as well as mRNA levels of other NF-κB dependent genes such as IκBα and Cxcl2, excluding the possibility that the observed effects of Psen-1 were cell type– or knockdown system–specific (supplementary Fig. 4A and 4B). A γ-secretase

inhibitor- Compound E, did not suppress IL-6 production (Supplementary Fig. 4C), suggesting that Psen1 function in the NF-κB pathway does not significantly depend on γ-secretase activity.

Next, to investigate whether Psen1 acts as a positive regulator of the NF-κB pathway in vivo, I utilized cytokine-induced arthritis in F759 mice [69,83,97,1]. Psen1 shRNA or p65 shRNA was injected into the ankle joints of F759 mice followed by injections of IL-6 and IL-17 (co-activation of STAT3 and NF-κB) into the ankle joints to induce NF-κB-mediated arthritis development. The arthritis development was significantly suppressed in the Psen1-knockdown and p65-knockdown (positive control) groups, demonstrating that Psen1 is indeed critical for NF-κB pathway in vitro and in vivo. (Fig. 1E).

3.2. Psen1 regulates NF-κB target genes in nonimmune cells

The simultaneous activation of NF-κB and STAT3 is important for the inflammation amplifier [76,114]. To elucidate which of these signaling pathways is regulated by Psen1, the expression levels of target genes for the NF-κB or STAT3 pathways were examined. In addition to the suppression of IL-6 levels (Fig. 1A-C), the expression of Lcn2, which is another NF-κB target gene, was significantly suppressed in

Psen1-deficient cells, whereas STAT3 and SOCS3 expressions, which are targets of the STAT3 pathway, were unaffected (Fig. 2), suggesting that Psen1 regulates the NF-κB pathway in nonimmune cells. Thus, in the following experiments, we mainly used TNFα for cell stimulation.

3.3 Loss of Psen1 abrogates NF-κB promoter binding ability

NF-κB activation involves multiple stages such as phosphorylation, nuclear translocation, and promoter binding [115]. I first investigated the nuclear translocation of NF-κB p65 using confocal microscopy and found that, the nuclear translocation of p65 before and after TNFα stimulation was not affected in Psen1-deficient cells (Fig. 3A and 3B). I then investigated the nuclear events of NF-κB activation. Chromatin immunoprecipitation (ChIP) revealed that p65 recruitment on NF-κB target promoters such as IL-6 and Lcn2 were significantly impaired in Psen1-deficient cells. Also, the promoter-binding activities of p300 and acetylation of histone H3K27, that establish chromatin accessibility were also significantly reduced in Psen-1 deficient cells. (Fig. 3C, 3D and 3E)

Consistently, chromatin accessibility assessed by DNase I digestion was also reduced at these promoters (Fig. 3F). Moreover, the forced expression of Psen1 significantly increased the reporter activities of IL-6 and NF-κB promoter in the

presence of TNFα (Fig. 3G). These results suggest that Psen1 is involved in a signaling pathway responsible for the binding of NF-κB p65 to target promoter regions.

3.4 Psen1 associates with BCR and CK2α to activate NF-κB pathway

Recently, we reported that BCR gene forms a complex with CK2α that positively regulates NF-κB signaling in nonimmune cells via p65 phosphorylation at serine residue 529, that establishes a binding site with histone acetyltransferase p300 in the nucleus [97].

In the absence of BCR, NF-κB target genes transcription is repressed, as well as chromatin opening and NF-κB binding to the target promoter regions are significantly reduced in nonimmune cells while the nuclear translocation of p65 remains intact [97]. These phenotypes are similar to those observed in Psen1-deficient cells (Fig. 1-3). Therefore, I hypothesized that Psen1 is involved in the BCR-CK2α-p65 pathway. Consistent with this theory, the phosphorylation of p65 at serine 529 was weakened in Psen1-deficient cells (Fig. 4A). In contrast to the unchanged expression of p65 phosphorylation at serine 536 which is mediated by

multiple kinases including IKK [116,117], upon cytokine stimulation (Fig.4A). The phosphorylation states of BCR and CK2α followed by NF-κB activation post TNFα stimulation were reduced in Psen1-deficient cells (Fig. 4B).

Consistently, association of p65 with BCR, CK2α and their phosphorylated forms at endogenous protein levels were also slightly reduced in Psen1-deficient cells (Supplementary Fig.5). Together, these results suggest Psen1's involvement in the BCR-CK2α-p65 pathway.

3.5 Psen1 acts as a scaffold for the formation of BCR, CK2α and p65 complex

As Psen1 is a membrane protein [84,87-89,91] and a probable new entry in the BCR-CK2α-p65 pathway, I checked the interaction of Psen1 with these three subunits of the complex and their cellular localization.

Consistent with supplementary Fig. 6, co-immunoprecipitation assays revealed that Psen1 clearly associated with BCR and CK2α more, as compared to its association with p65 (Fig. 5A), suggesting the possibility that Psen1 directly binds to BCR and CK2α and indirectly to p65.

Next to find the binding regions of Psen1 to CK2α and BCR, two Psen1 deletion mutants were created. Here, the N-terminal region (amino acids 1-73) or a large part of the hydrophilic cytoplasmic loop [125] (amino acids 271-376) of Psen1 was deleted (Supplementary Fig. 6). The binding of Psen1 mutant Δ271-376 to CK2α and BCR was significantly decreased compared to WT Psen1 and Psen1 mutant Δ1-73 (Fig. 5B and C), highlighting the importance of the hydrophilic cytoplasmic loop of Psen1 for the associations.

I, then prepared mutant molecules of CK2α and BCR (Supplementary Fig. 6) to examine their binding regions for Psen1. The N-terminal region of CK2α and the Rho/GEF domain of BCR were important for the association with Psen1 (Fig. 5D and E). These results indicate that the hydrophilic cytoplasmic loop of Psen1, Rho/GEF domain of BCR and N-terminal domain of CK2α are critical regions for association of Psen1 with BCR-CK2α complex, that is required for p65 activation. Also, the association between p65 and BCR became weaker under Psen1 deficiency (Fig. 5F) further confirming the hypothesis.

To examine the cellular localization, I separated and prepared membrane, cytosol and nuclear fractions, and immunoblotted them for p65, BCR and CK2α in control and Psen1-deficient cells. p65, BCR and CK2α were detected in a Psen1-dependent

manner in the membrane fraction even before TNFα stimulation, and their localization was not significantly changed after TNFα stimulation (Fig. 5G). These results suggest that the membrane protein Psen1 can serve as a scaffold for the complex formation and subsequent activation of the BCR-CK2α-p65 axis for NF-κB-mediated inflammation development.

Chapter 4: Discussion

The inflammation amplifier, a local chemokine inducer specific to nonimmune cells such as endothelial cells, fibroblasts is a NF-κB activator, which is activated by the simultaneous stimulation of NF-κB and STAT [25,31,59,69,76,78,83]. In fact, the inflammation amplifier is activated by various chemokines and growth factors including, IL-17, TNF-α, ErbB1 ligands, which act as NF-κB stimulators, and IL-6 which mainly maintains the activation of STAT3, leading to local homeostasis deregulation via accumulation of various immune cells [69,76,78,83,113,118,119]. We have previously studied the role of inflammation amplifier in several disease models and clinical samples [69,76,78,118,120]. To further, understand its role in human diseases, genome-wide screening was performed [83] which revealed around 1289 inflammatory disease associated genes that were found to be positive regulators of the inflammation amplifier including Psen1.

In the present study, I performed a mechanistic study to understand Psen1's contribution to the activation of NF-κB. The data indicates that Psen1 positively regulates NF-κB activation by participating in the BCR-CK2α-p65 pathway, which we recently reported [97]. Indeed, Psen1-deficient cells phenocopied many aspects of BCR-deficient cells including (i) reduced p65 phosphorylation at serine 529,

(ii) impaired histone acetylation at H3K27 due to reduced p300 accumulation at the promoter regions of NF-κB targets, (iii) reduced promoter binding of p65, and (iv) decreased levels of the transcription of NF-κB target genes. Importantly, Psen1 deficiency in joints suppressed cytokine-induced arthritis development in vivo. On the other hand, p65 phosphorylation at serine 536, its nuclear translocation, and the transcription of STAT3 target genes were largely intact in Psen1-deficient cells just like in BCR-deficient cells [97]. Therefore, we concluded that Psen1 is critical for BCR-CK2α-mediated p65 phosphorylation and subsequent inflammation development.

How does Psen1 contribute to the BCR-CK2α-mediated p65 phosphorylation?

I found that Psen1 interacts with BCR and CK2α via its hydrophilic cytoplasmic loop, and that Psen1 deficiency abrogated the phosphorylation of BCR and CK2α, as well as the association of p65 with BCR. Based on these findings, I propose that Psen1 acts as a scaffold protein for the BCR-CK2α complex formation to phosphorylate p65 at serine 529, that allows NF-κB activation through a p300-mediated chromatin opening. Consistent with this notion, the role of Psen1 as a scaffold protein has been described during β-catenin phosphorylation [95,96].

Psen-1 forms a complex with glycogen synthase kinase 3β and protein kinase A to facilitate the phosphorylation of β-catenin, which is required for the rapid turnover

of β-catenin, preventing aberrant cell proliferation and tumorigenesis [95,96]. My study identified BCR, CK2α and p65 as new scaffold partner proteins for Psen1 and that Psen1 is required for NF-κB-induced inflammation development by facilitating the phosphorylation of p65 at serine 529.

Psen1 is known to be the catalytic component of γ-secretase enzyme, which cleaves APP to generate Aβ and has nine transmembrane domains with a large hydrophilic loop [46,48,52,79,80]. I showed that the scaffold role of Psen1 is dependent on the hydrophilic cytoplasmic loop but not on its γ-secretase enzyme activity, as γ-secretase inhibitor, Compound E, did not affect NF-κB activation in nonimmune cells. Many Psen1 mutations found in familial AD are present in the hydrophilic cytoplasmic loop [84,87,121]. Since accumulating evidence indicates that chronic inflammation and proinflammatory cytokines such as IL-6 and TNFα contribute to the pathogenesis of AD [122,123], it is possible that certain familial AD mutation(s) in the hydrophilic cytoplasmic loop of Psen1 might have a gain-of-function effect on the BCR-CK2α complex formation and subsequent NF-κB-driven inflammation, thereby contributing to the pathogenesis of AD.

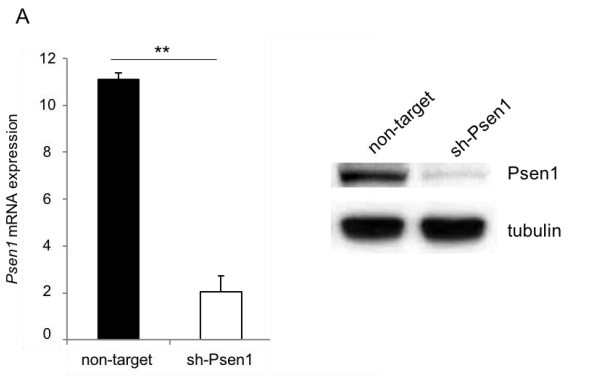
Chapter 5: Conclusion

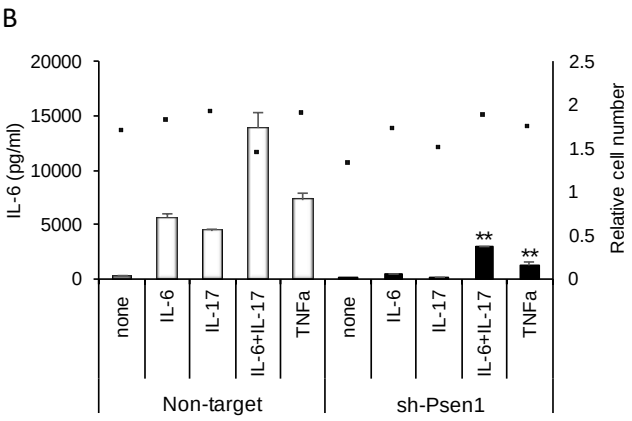
Genome-wide screening identified Psen1 as a positive regulator of the inflammation amplifier, a molecular mechanism that hyperactivates NF-κB signaling in nonimmune cells [83]. In the current study, I performed a mechanistic study on how Psen1 contributes to the activation of NF-κB.

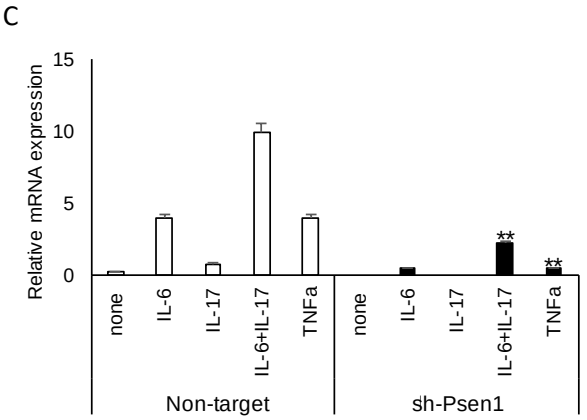
From the above results, I found that Psen1 is involved in BCR–CK2α–p65 complex formation. The chemical inhibitor of γ-secretase did not have an inhibitory effect indicating a γ-secretase independent role of Psen1. The short hairpin RNA (shRNA)–mediated deficiency of Psen1 decreased the phosphorylation of CK2α and BCR at Y177 and the association between BCR and p65 at ser 529. These results suggest that Psen1 acts as a scaffold for BCR, CK2α, and p65, allowing efficient NF-κB activation (supplementary Fig.7).

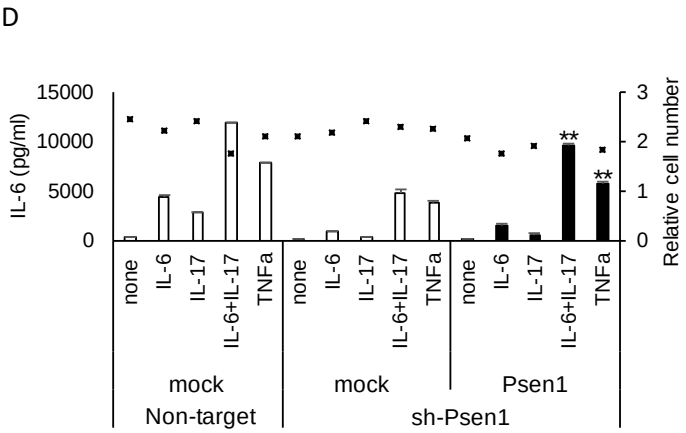
In summary, I identified a novel γ-secretase-independent role for Psen1 in the regulation of the NF-kB pathway and the identification of Psen1–BCR–CK2α–p65 cascade could be a novel therapeutic target for diseases that show chronic inflammation [124].

Chapter 6: Figures









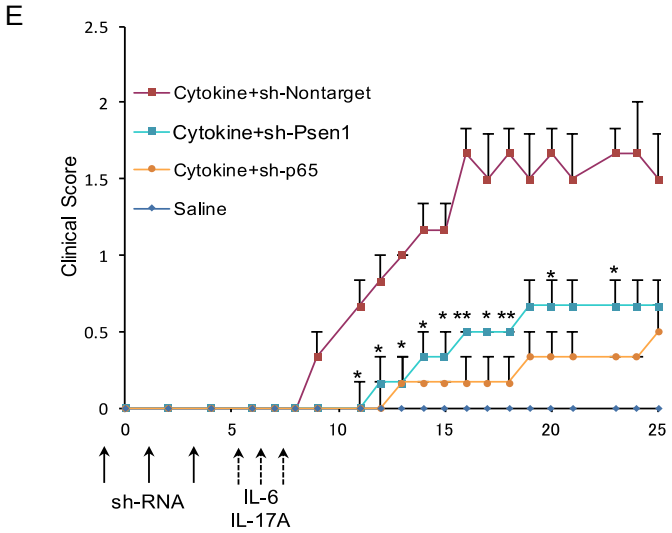


Figure 1. Psen1 regulates the inflammation amplifier in vitro and in vivo.

(A) Psen1 mRNA and protein levels in non-target and Psen1-knockdown cells.

(B, C) protein (B) and mRNA (C) levels for IL-6 were measured after Psen1-deficient and control (Non-target) BC1 cells were stimulated with IL-6, IL-17, IL-6 + IL-17, or TNFα.

(D) measurement of IL-6 production post the overexpression of Psen1 in Psen1-deficient and non-target cells with (Psen1) or without (mock).

(B) (D) The right Y axis denotes the living cell numbers to assess cytotoxicity by knockdown.

(E) Clinical arthritis scores of F759 mice after ankle joint injections of Psen1 or control shRNA followed by IL-6 + IL-17 (Cytokine) injections. Saline injections without cytokines did not induce arthritis.

Data represent the mean + S.D. (A-D) or S.E.M. (E)

The p values were calculated using ANOVA tests

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

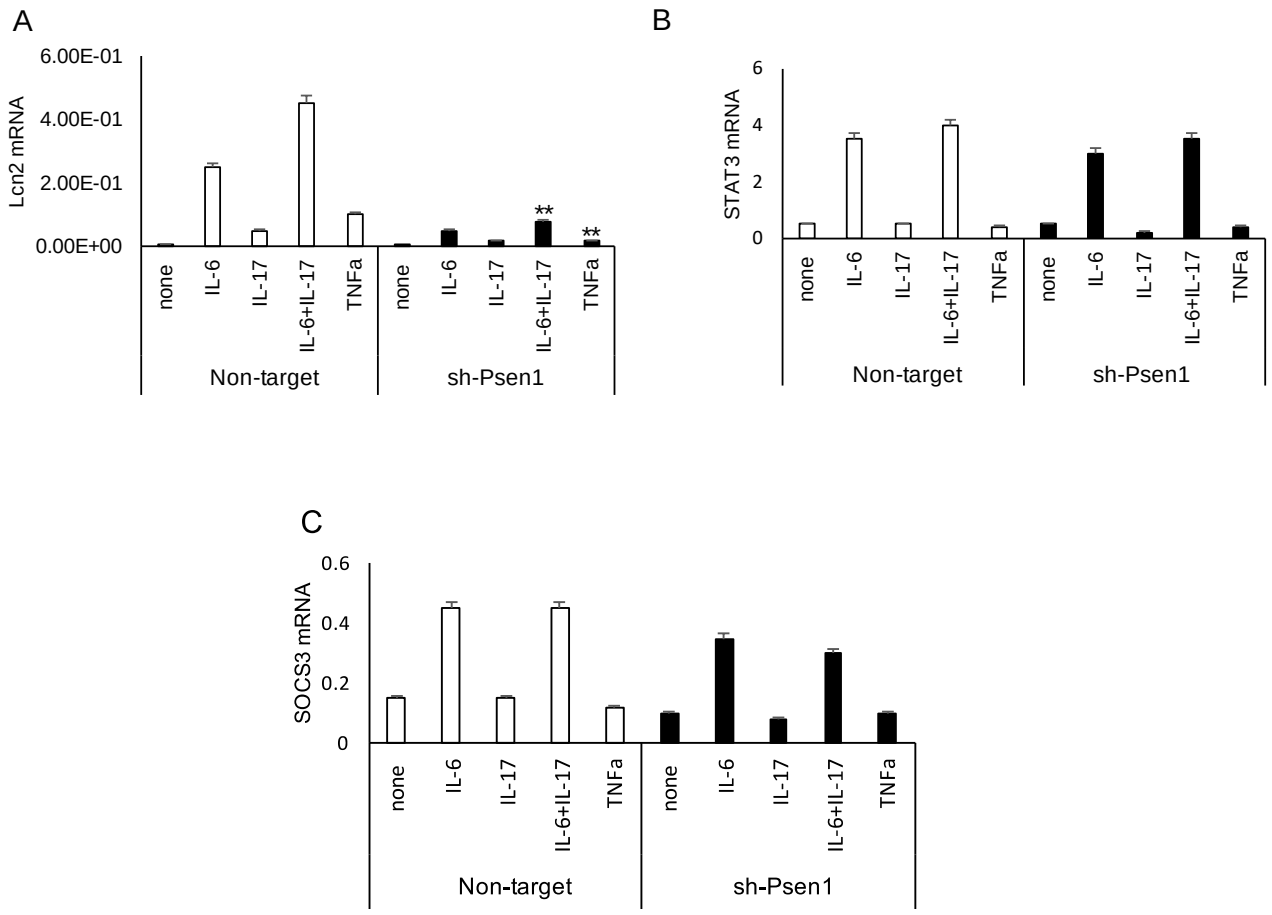


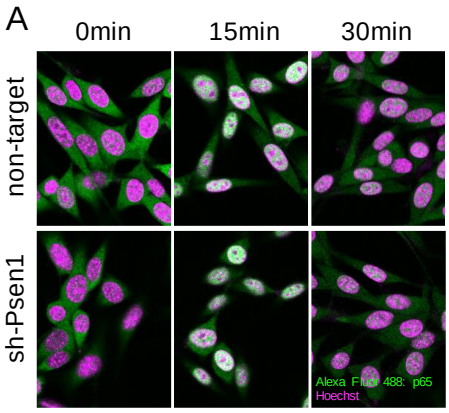
Figure 2. Psen1 mainly regulates the NF-κB pathway.

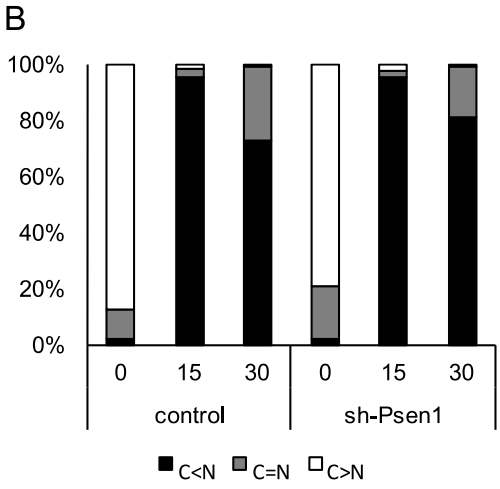
(A-C) mRNA levels for Lcn2 (A), STAT3 (B) and SOCS3 (C) were measured in Psen1-deficient or control (non-target) BC1 cells that were stimulated with IL-6, IL-17, IL-6+IL-17 or TNFα.

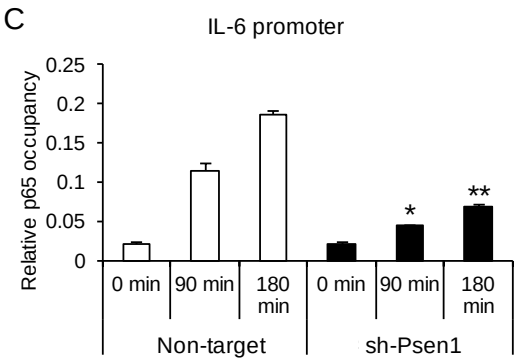
Data represent the mean + S.D.

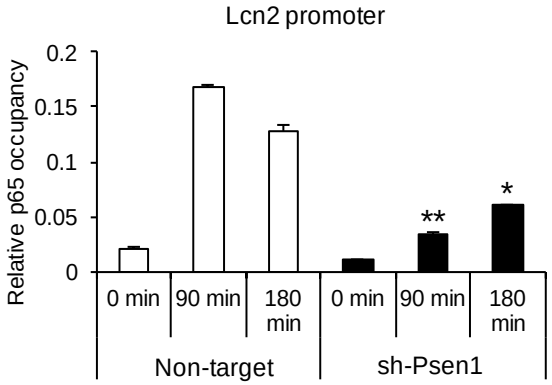
The p values were calculated using ANOVA tests

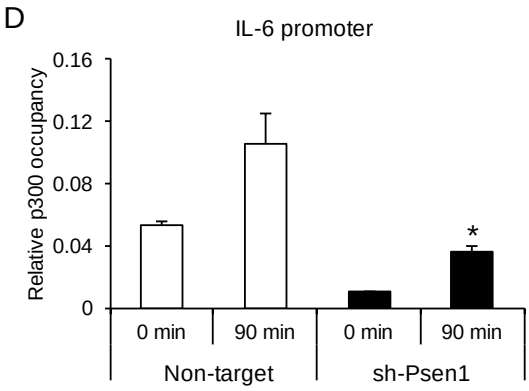
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

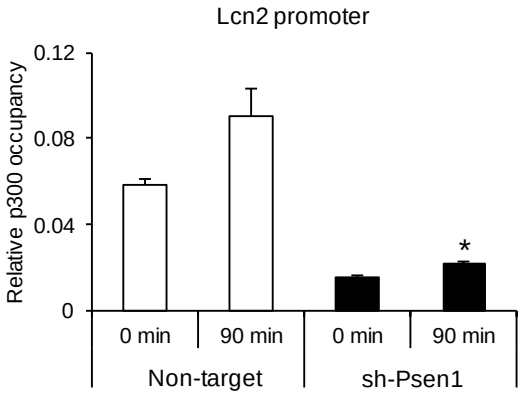

A
0min
15min
30min
non-target
sh-Psen1
Alexa Fluor 488: p65
Hoechst


B
100%
80%
60%
40%
20%
0%
0
15
30
0
15
30
control
sh-Psen1
C<N
C=N
C>N


C
IL-6 promoter
Relative p65 occupancy
0.25
0.2
0.15
0.1
0.05
0
0 min
90 min
180 min
0 min
90 min
180 min
Non-target
sh-Psen1


Lcn2 promoter
Relative p65 occupancy
0.2
0.15
0.1
0.05
0
0 min
90 min
180 min
0 min
90 min
180 min
Non-target
sh-Psen1


D
IL-6 promoter
Relative p300 occupancy
0.16
0.12
0.08
0.04
0
0 min
90 min
0 min
90 min
Non-target
sh-Psen1


Lcn2 promoter
Relative p300 occupancy
0.12
0.08
0.04
0
0 min
90 min
0 min
90 min
Non-target
sh-Psen1

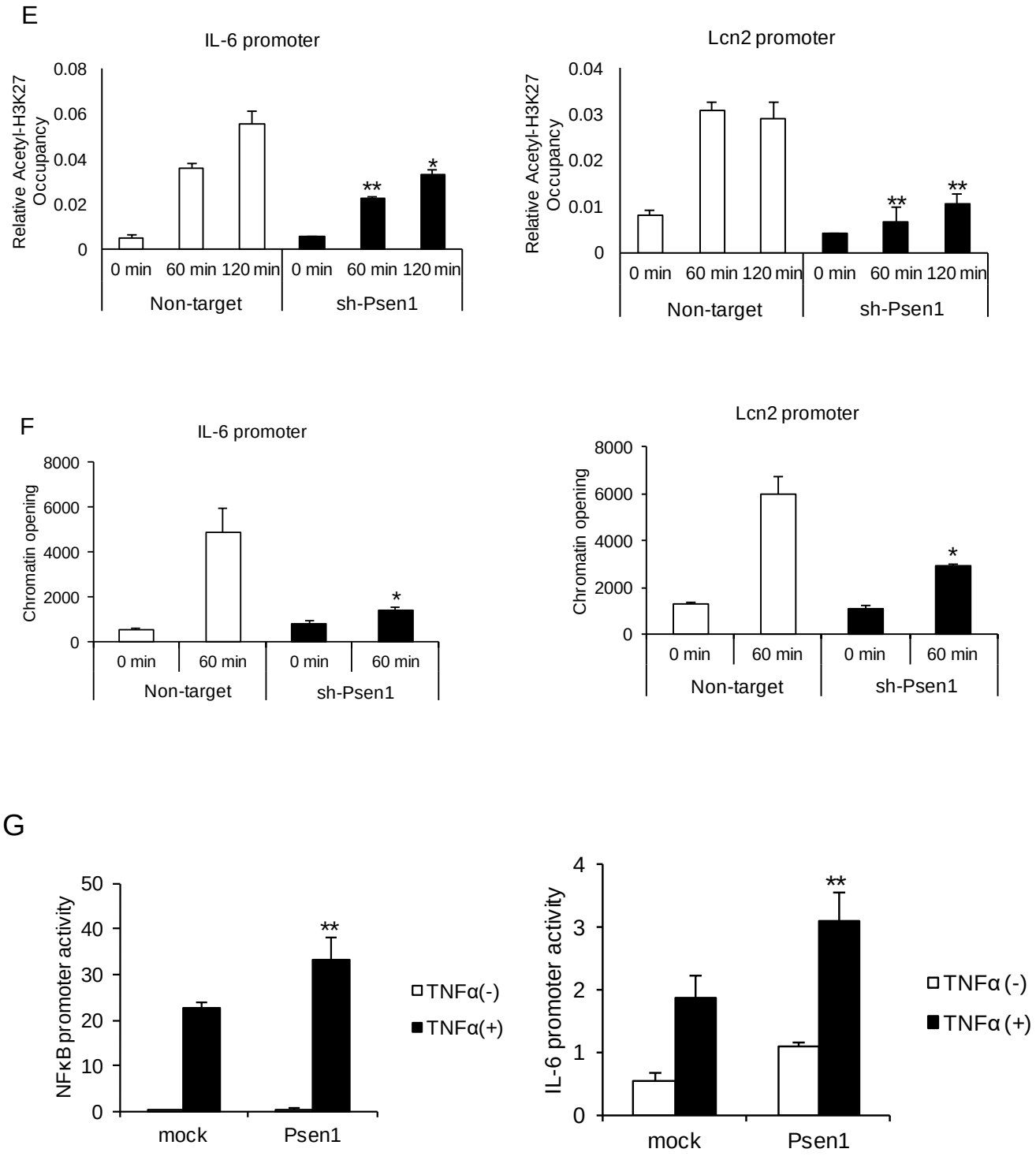

E
IL-6 promoter
Relative Acetyl-H3K27 Occupancy
0.08
0.06
0.04
0.02
0
0 min
60 min
120 min
0 min
60 min
120 min
Non-target
sh-Psen1
**
*
Lcn2 promoter
Relative Acetyl-H3K27 Occupancy
0.04
0.03
0.02
0.01
0
0 min
60 min
120 min
0 min
60 min
120 min
Non-target
sh-Psen1
**
**
F
IL-6 promoter
Chromatin opening
8000
6000
4000
2000
0
0 min
60 min
0 min
60 min
Non-target
sh-Psen1
*
Lcn2 promoter
Chromatin opening
8000
6000
4000
2000
0
0 min
60 min
0 min
60 min
Non-target
sh-Psen1
*
G
NFκB promoter activity
50
40
30
20
10
0
mock
Psen1
**
TNFα(-)
TNFα(+)
IL-6 promoter activity
4
3
2
1
0
mock
Psen1
**
TNFα (-)
TNFα (+)

FIGURE 3. Psen1 deficiency impairs the promoter binding ability of NF-κB p65

(A) Psen1-knockdown or control (non-target) cells were stimulated with TNF-α for 0, 15, and 30 min, and the localization of p65 (green) was observed by confocal microscopy. The nucleus was stained with Hoechst 33342 (blue). Representative images are shown. The images were taken using a 63x lens.

(B) Quantitative analysis of (A) C>N, cells with more p65 localized in the cytoplasm than nucleus; C = N, cells with equal localization of p65 in the cytoplasm and nucleus; C< N, cells with more p65 localized in the nucleus than cytoplasm. (150-200 cells counted)

(C–E) p65 (C), p300 (D), and acetyl-H3K27 (E) recruitment to the IL-6 (left) or Lcn2 (right) promoter were assessed by chromatin immunoprecipitation in nontarget and Psen1-deficient BC1 cells stimulated with TNF-α for the indicated time periods. Chromatin immunoprecipitation values relative to 10% of input are shown.

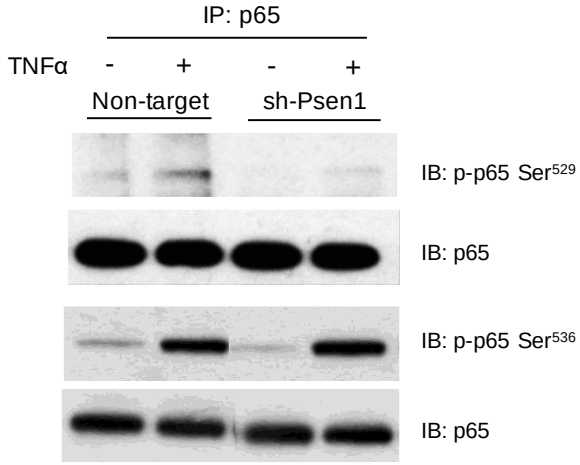
(F) Chromatin accessibility of the IL-6 (left) or Lcn2 (right) promoter was assessed in nontarget and Psen1-deficient BC1 cells with TNF-α stimulation at 0 and 60 min.

(G) Luciferase assay using artificial tandem NF-κB binding elements (left) or IL-6 promoter (right) was performed in HEK293T cells with (Psen1) or without (mock) overexpression of Psen1 in the presence or absence of TNF-α stimulation.

Data represent the mean + S.D. and the p values were calculated using ANOVA tests

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A


IP: p65
TNFα - + - +
Non-target
sh-Psen1
IB: p-p65 Ser529
IB: p65
IB: p-p65 Ser536
IB: p65

B

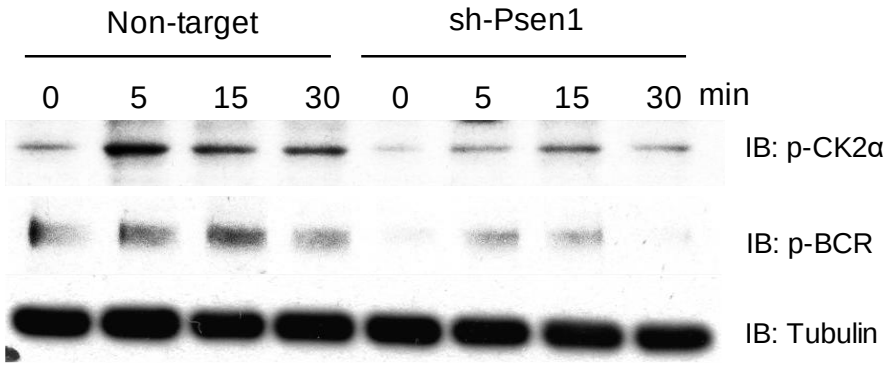
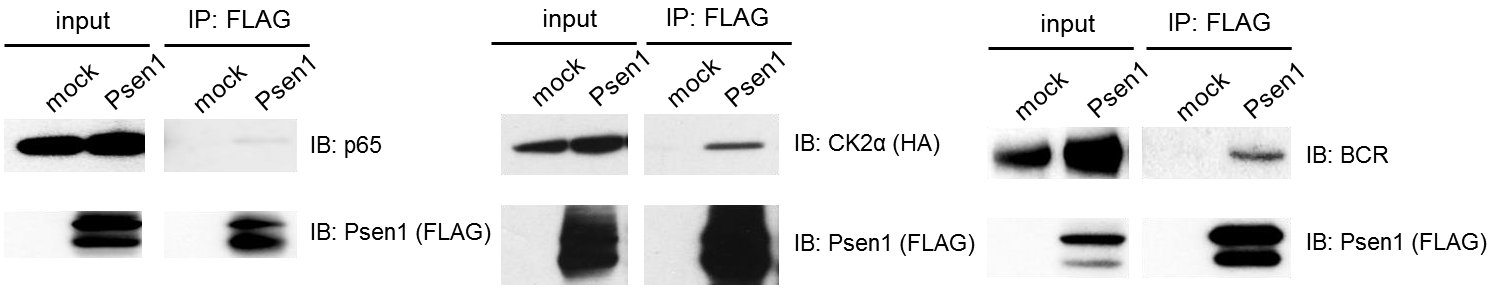

Non-target
sh-Psen1
0 5 15 30 0 5 15 30 min
IB: p-CK2α
IB: p-BCR
IB: Tubulin

FIGURE 4. Psen1 is required for BCR–CK2α-mediated NF-κB p65 activation.

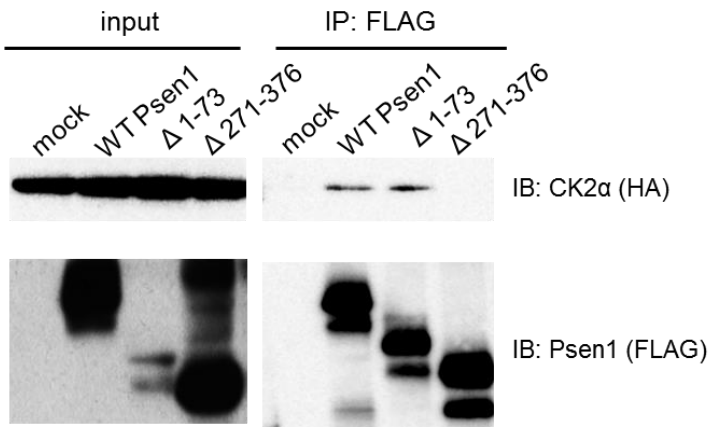
(A) Nontarget control and Psen1-deficient BC1 cells were stimulated with TNF-α for 5 min, and the phosphorylation of p65 at serine 529 or serine 536 was detected by western blotting after immunoprecipitation of p65.

(B) Nontarget and Psen1 knockdown BC1 cells were stimulated with TNF-α for 0, 5, 15, and 30 min, and the phosphorylation of CK2α and BCR at Y177 was detected by western blotting.

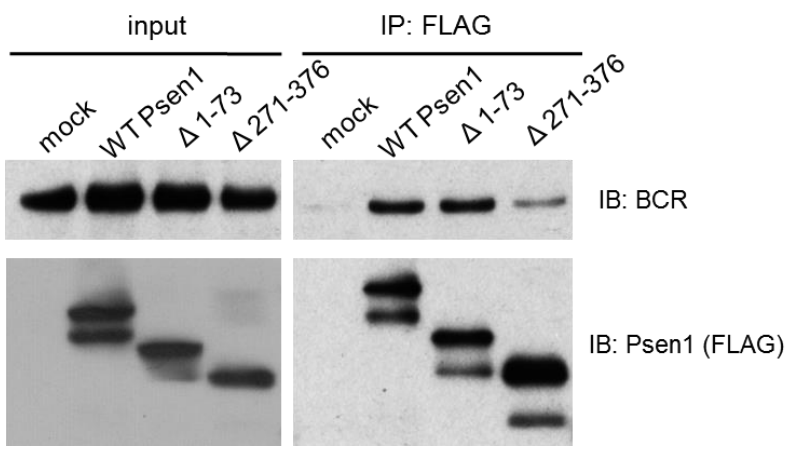
A


input
IP: FLAG
mock
Psen1
IB: p65
IB: Psen1 (FLAG)
IB: CK2α (HA)
IB: BCR

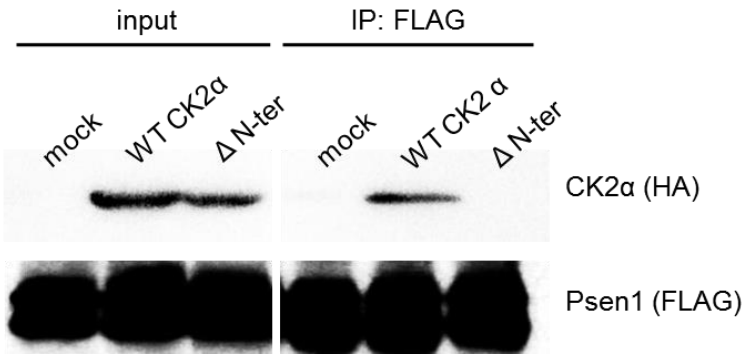
B


input
IP: FLAG
mock
WT Psen1
Δ 1-73
Δ 271-376
IB: CK2α (HA)
IB: Psen1 (FLAG)

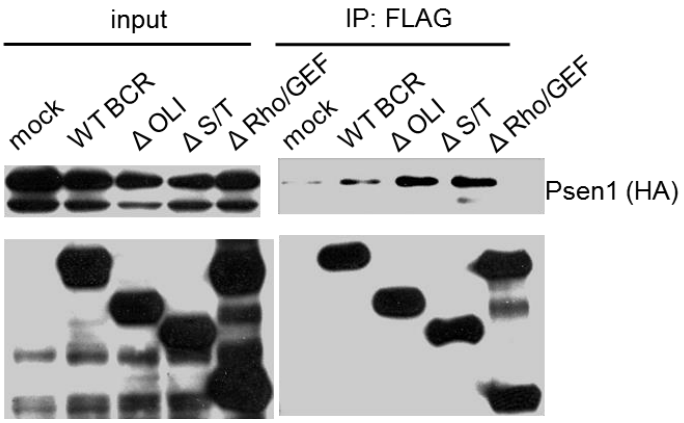
C


input
IP: FLAG
mock
WT Psen1
Δ 1-73
Δ 271-376
IB: BCR
IB: Psen1 (FLAG)

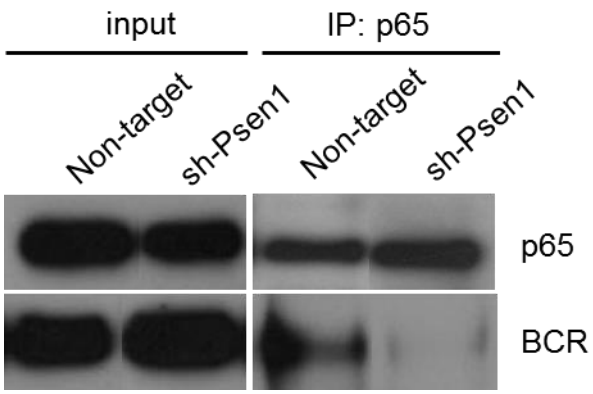
D


input
IP: FLAG
mock
WT CK2α
Δ N-ter
WT CK2 α
CK2α (HA)
Psen1 (FLAG)

E


input
IP: FLAG
mock
WT BCR
Δ OLI
Δ S/T
Δ Rho/GEF
mock
WT BCR
Δ OLI
Δ S/T
Δ Rho/GEF
Psen1 (HA)

F


input
IP: p65
Non-target
sh-Psen1
Non-target
sh-Psen1
p65
BCR

G

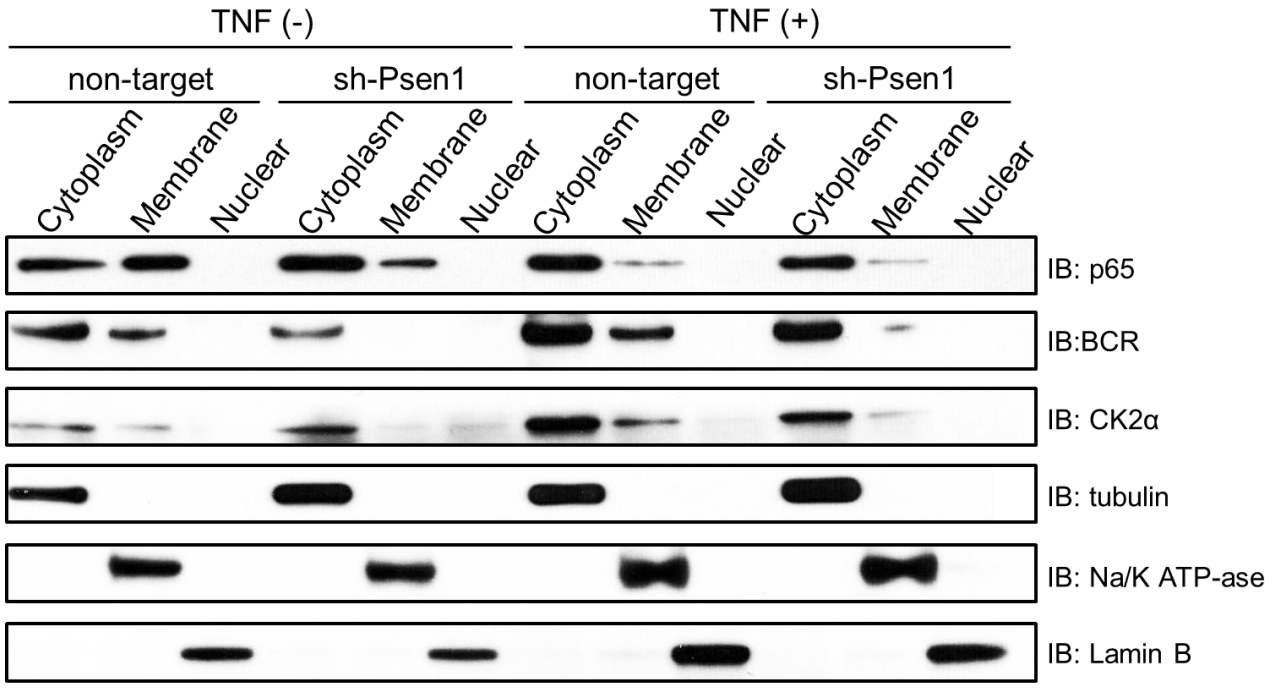

TNF (-)
TNF (+)
non-target
sh-Psen1
non-target
sh-Psen1
Cytoplasm
Membrane
Nuclear
IB: p65
IB:BCR
IB: CK2α
IB: tubulin
IB: Na/K ATP-ase
IB: Lamin B

FIGURE 5 Psen1 associates with BCR and CK2α.

(A) HEK293T cells overexpressing Flag–Psen1 were immunoprecipitated with Flag beads, followed by the detection of p65 (left), CK2α (center), or BCR (right) by western blotting. In the case of CK2α detection, HA-tagged CK2α was co-overexpressed with Flag–Psen1, and anti-HA Ab was used for the immunoblotting. Psen1 expression levels detected by anti-Flag Ab are shown in the bottom.

(B) HEK293T cells co-overexpressing HA-CK2α and Flag-tagged WT Psen1 or its mutants were immunoprecipitated with Flag beads, followed by the detection of CK2α using anti-HA Ab. Psen1 expression levels detected by anti-Flag Ab are shown in the bottom.

(C) HEK293T cells overexpressing Flag tagged WT Psen1 or Psen1 mutants were immunoprecipitated with Flag beads, followed by the detection of BCR using anti-BCR Ab. Psen1 expression levels detected by anti-Flag Ab are shown in the bottom.

(D) HEK293T cells co-overexpressing Flag-Psen1 and HA-tagged WT or N-terminal–deleted mutant of CK2α (ΔN-ter) were immunoprecipitated with Flag

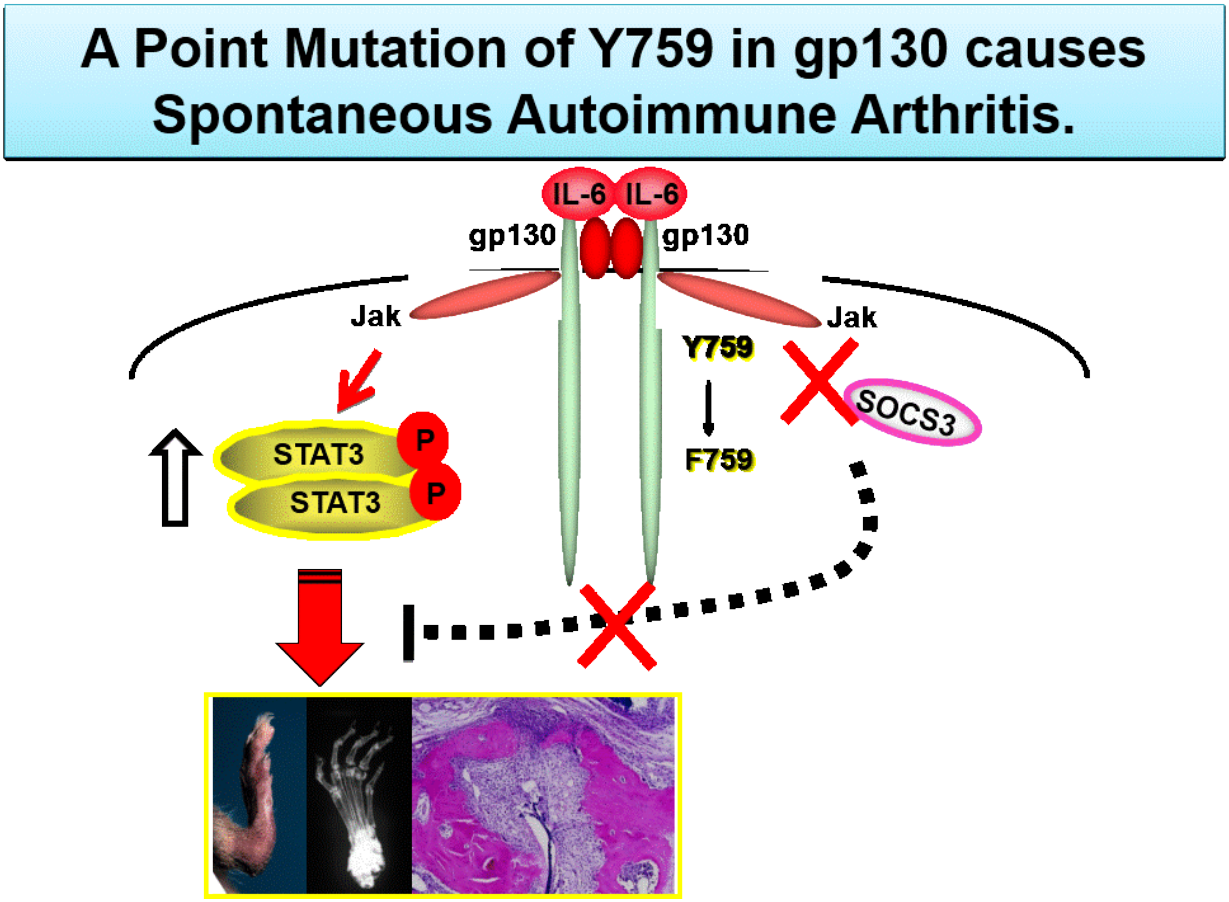
beads, followed by the detection of CK2α using anti-HA Ab. Psen1 expression levels detected by anti-Flag Ab are shown in the bottom.

(E) HEK293T cells co-overexpressing HA-Psen1 and Flag-tagged WT BCR or its mutants (oligomerization domain deletion [ΔOLI], putative serine/threonine kinase domain deletion [ΔS/T], and Rho/GEF domain deletion [ΔRho/GEF]) were immunoprecipitated with Flag beads, followed by the detection of Psen1 using anti-HA Ab. BCR expression levels detected by anti-Flag Ab are shown in the bottom.

(F) Nontarget control and Psen1-deficient BC1 cells stimulated with TNF-α were immunoprecipitated with anti-p65 Ab, followed by immunoblotting for p65 (top) or BCR (bottom).

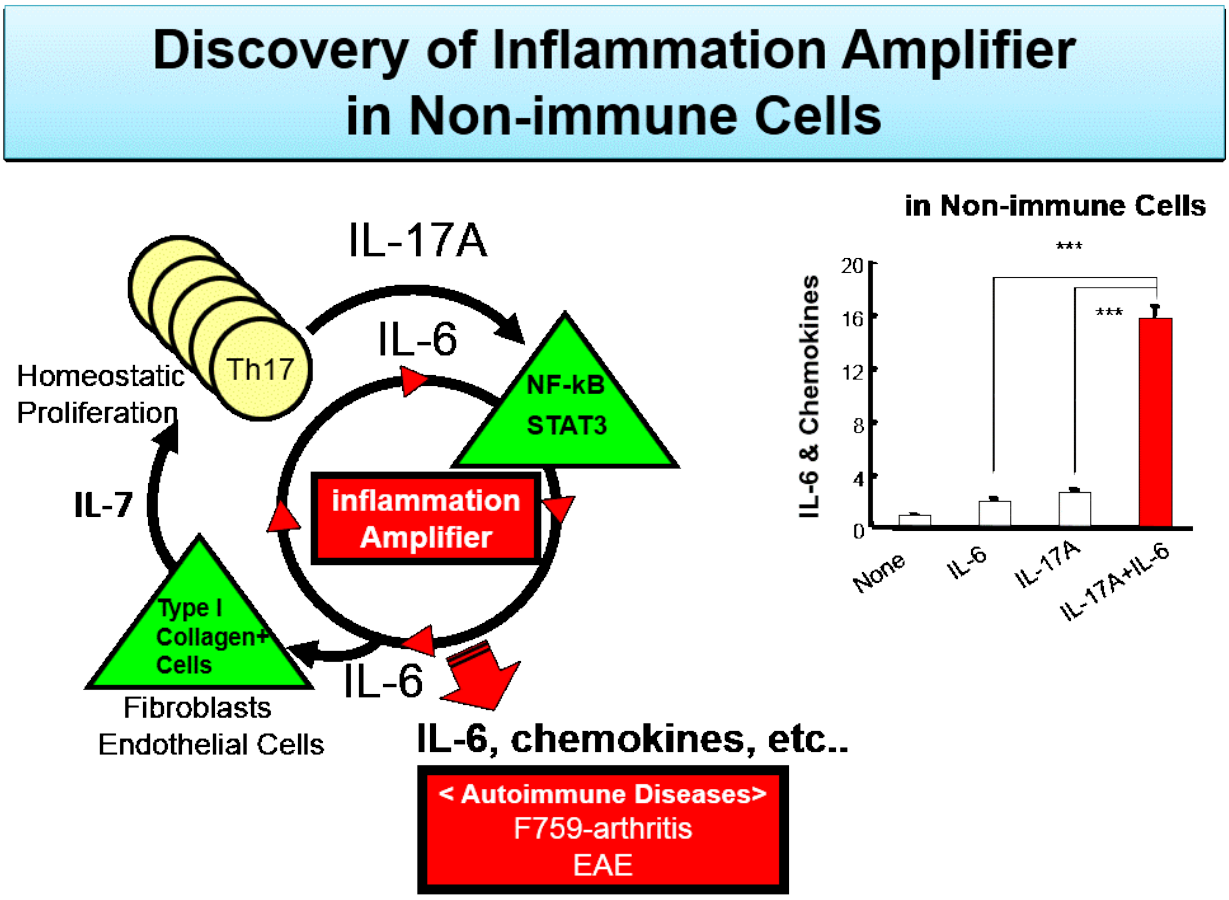
(G) Nontarget control and Psen1-deficient BC1 cells were stimulated with or without TNF-α. Membrane, cytosol, and nuclear fractionations were prepared, and immunoblotting of p65, BCR, CK2α, tubulin (cytosolic marker), Na/K-ATPase (membrane marker), and lamin B (nuclear marker) were performed.

Supplementary Figures



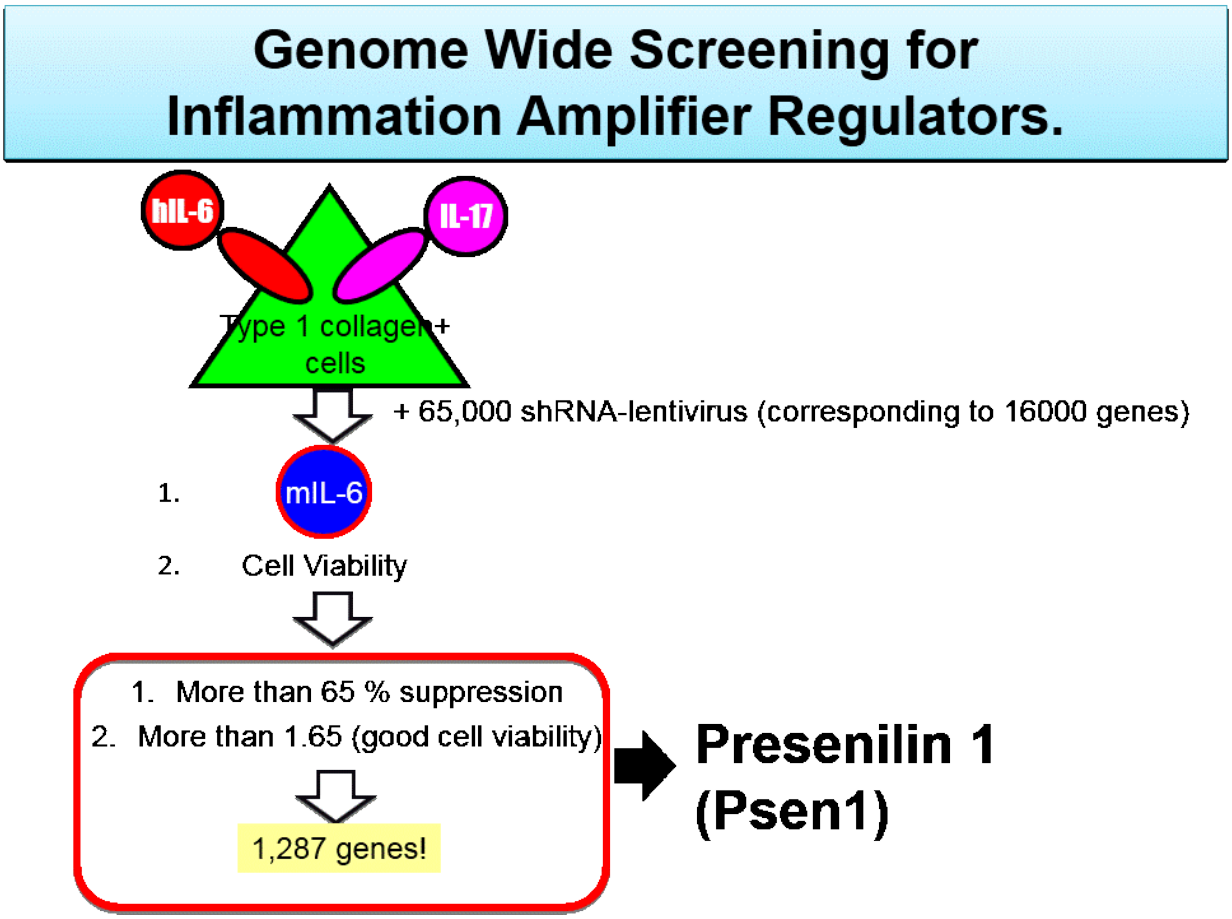
Supplementary Fig.1- Schematic diagram of F759 arthritis

F759 mice show enhanced STAT3 activation in response to IL-6 owing to the Y759F mutation in gp130, which inhibits SOCS3-mediated negative feedback. These mutant mice spontaneously develop a RA-like joint disease (F759 arthritis) in less than a year. [47,73]



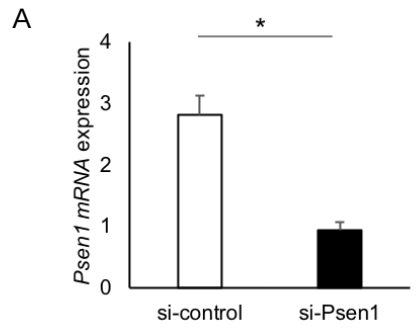
Supplementary Fig 2- Schematic diagram of Inflammation Amplifier

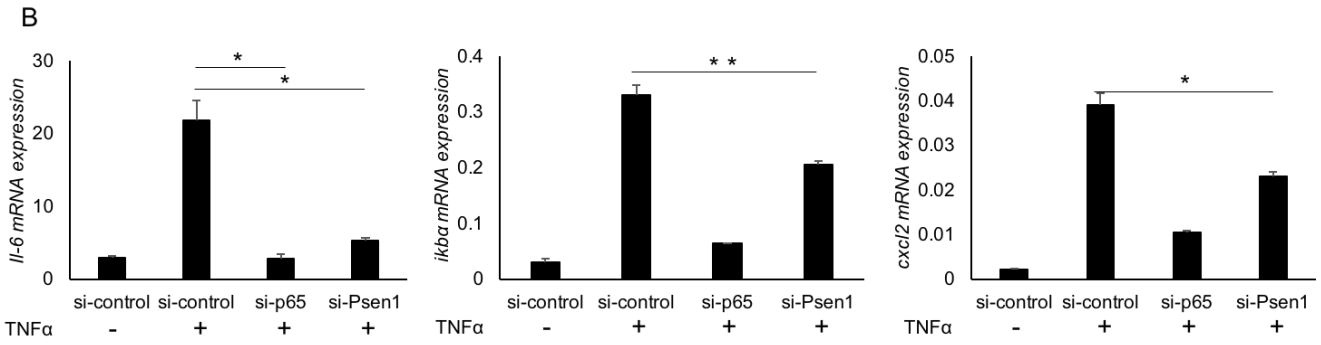
Type1 collagen+ cells in F759 mice oversecrete IL-7 which enable the homeostatic proliferation of T_H17 cells which produce IL-17, together with IL-6 trigger a simultaneous activation of NF-κB and STAT3 that leads to enhanced production of IL-6 and various chemokines that leads to the onset of autoimmune diseases like F759 arthritis and EAE. [47,67,69,77,78,120]

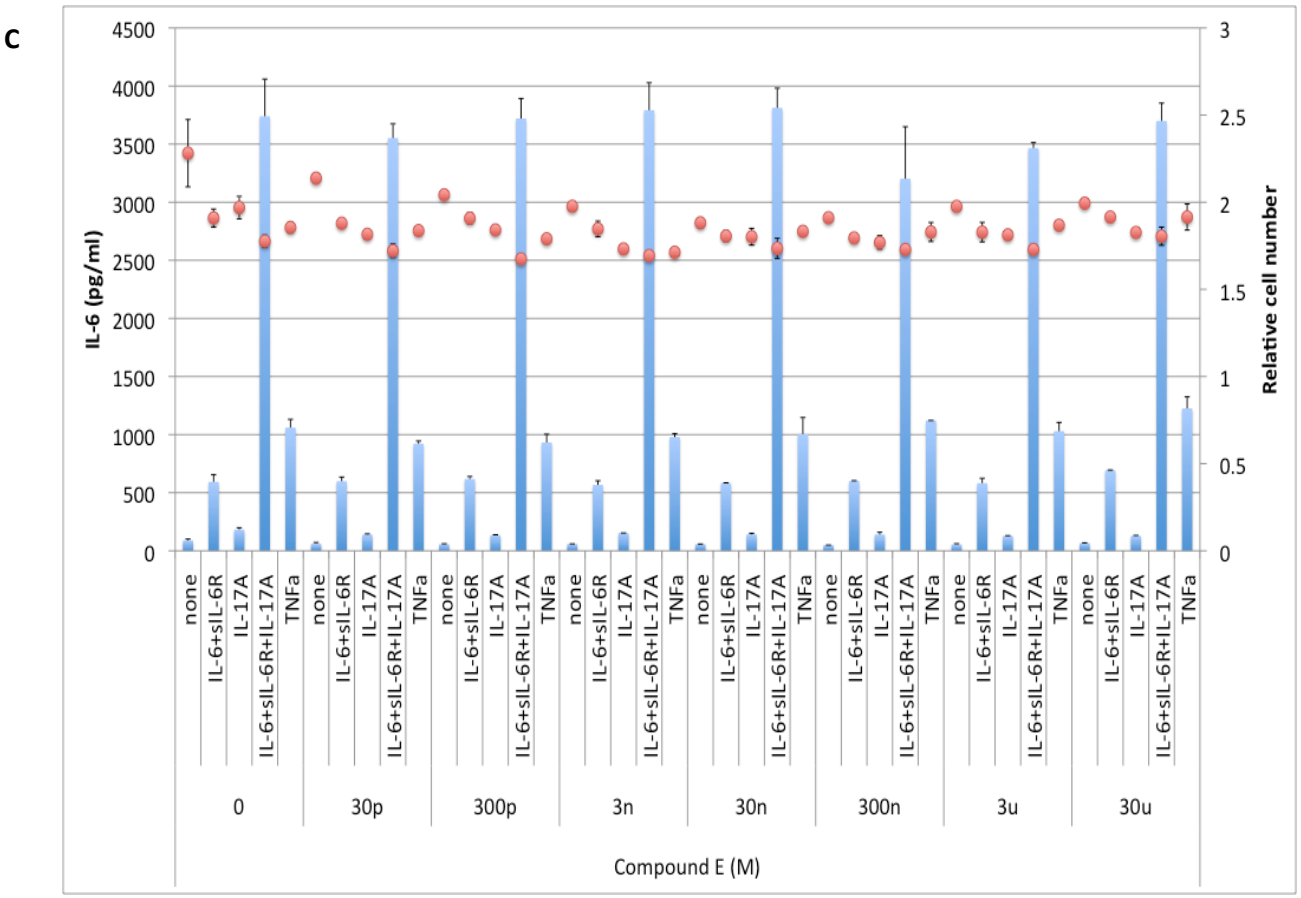


Supplementary Fig.3- Schematic diagram of genome wide screening

In order to elucidate the detailed molecular mechanism of the Inflammation amplifier, 65,000 shRNA libraries (corresponding to 16,000 genes of mouse) were transiently knockdown in BC1 cells. BC1 cells were stimulated with human IL-6, IL-6 receptor and IL-17 and the induced IL-6 expression and cell viability were measured. As a result of 1,289 candidate genes were identified which controlled the inflammation amplifier [83]. Out of these genes, Psen-1 was selected and analyzed in detail.


A
*
Psen1 mRNA expression
4
3
2
1
0
si-control
si-Psen1


B
Il-6 mRNA expression
30
20
10
0
* *
si-control si-control si-p65 si-Psen1
TNFα - + + +
ikba mRNA expression
0.4
0.3
0.2
0.1
0
* *
si-control si-control si-p65 si-Psen1
TNFα - + + +
cxcl2 mRNA expression
0.05
0.04
0.03
0.02
0.01
0
*
si-control si-control si-p65 si-Psen1
TNFα - + + +

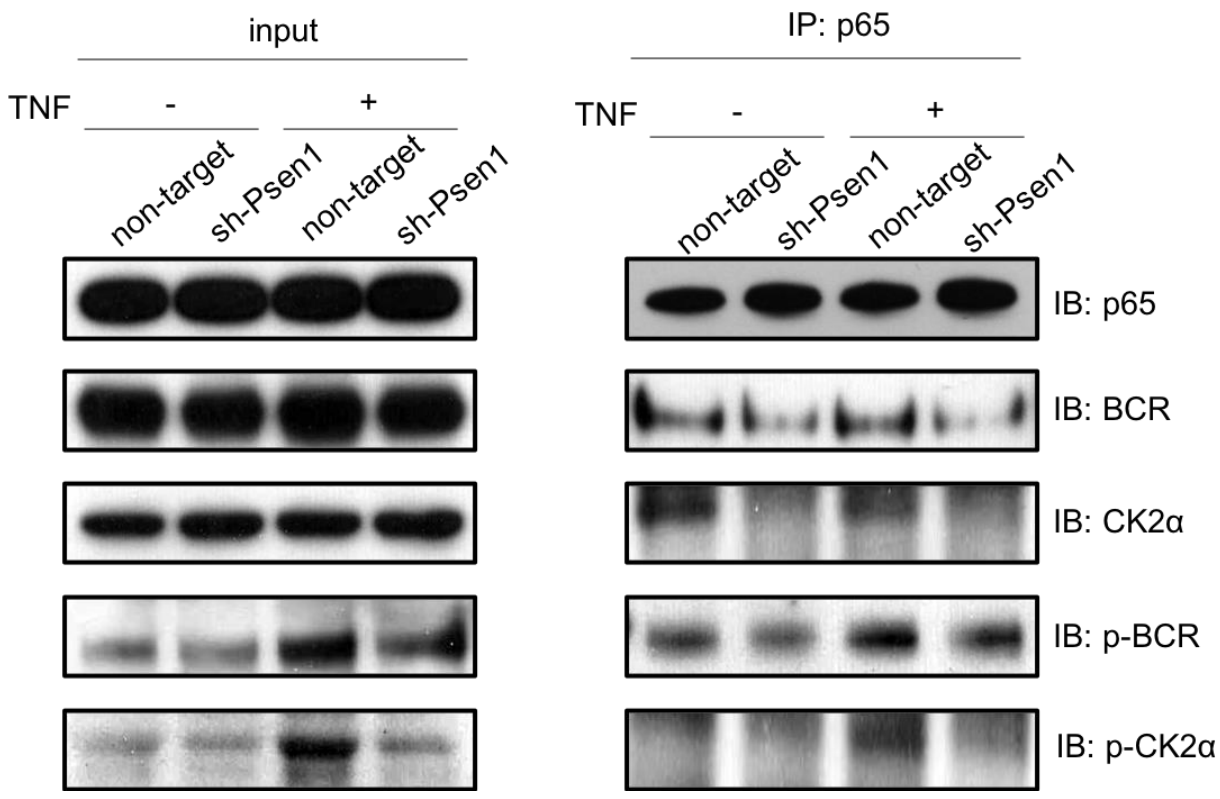

C
IL-6 (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Relative cell number
3
2.5
2
1.5
1
0.5
0
none
IL-6+sIL-6R
IL-17A
IL-6+sIL-6R+IL-17A
TNFa
0
30p
300p
3n
30n
300n
3u
30u
Compound E (M)

Supplementary Figure 4. Psen1 knockdown in H4 neuroglioma cells and the effect of γ-secretase inhibitor on IL-6 production in BC1 cells.

(A, B) Psen1 was knocked down by siRNA in neuroglioma H4 cells. The knockdown efficiency is shown in A. H4 cells were stimulated with TNFα, and mRNA expressions of IL-6, IκBα and Cxcl2 were analyzed. siRNA against p65 was used as a positive control.

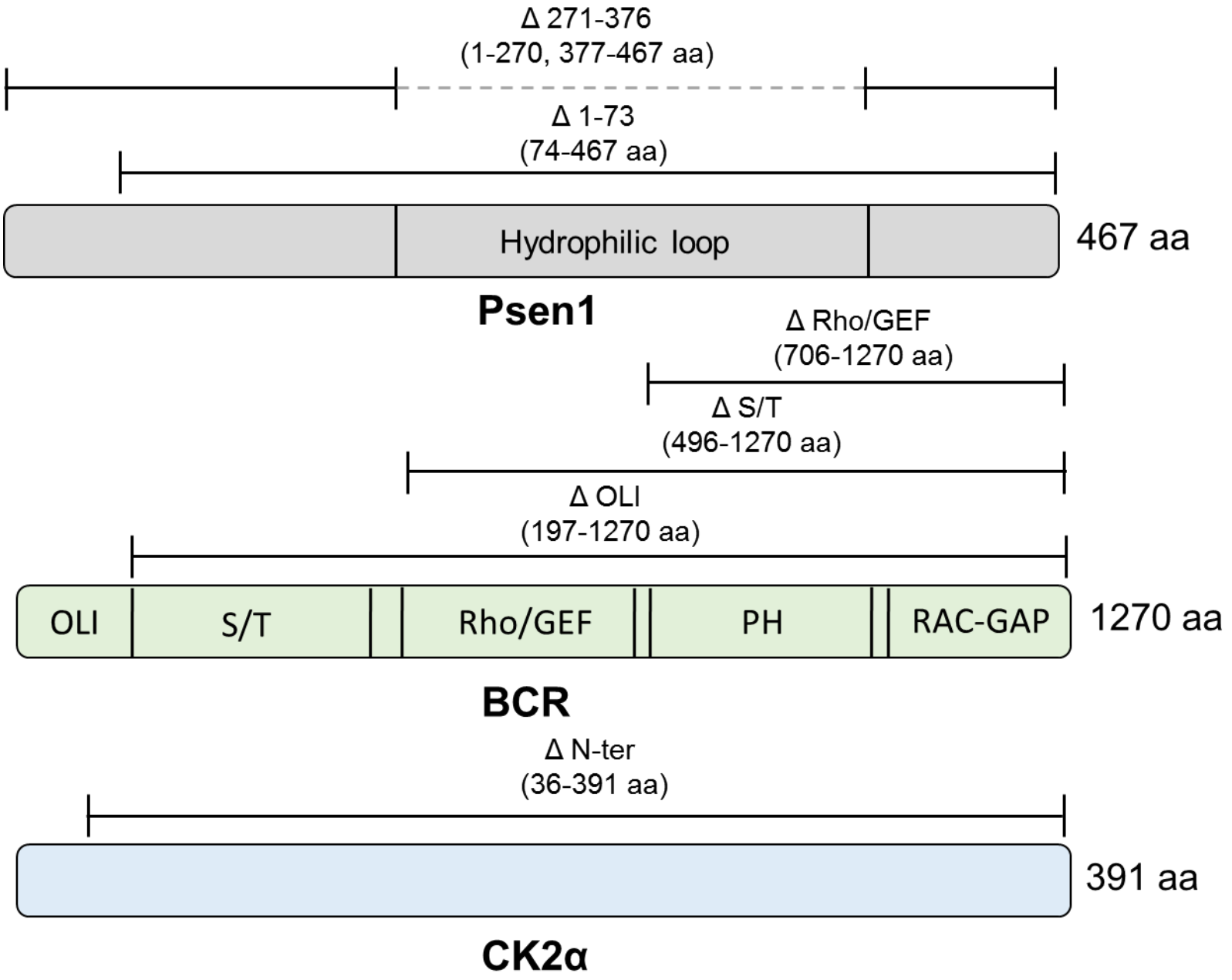
(C) BC1 cells were stimulated with human IL-6 (IL-6+sIL-6R), mouse IL-17, human IL-6 (human IL-6+sIL-6R) plus mouse IL-17, or mouse TNFα in the presence of different concentrations of Compound E. IL-6 levels in the culture supernatant on day 1 were measured by mouse IL-6 ELISA. Red circles indicate relative living number of cells.

Data represent the mean + S.D. *P < 0.05 and **P < 0.01.

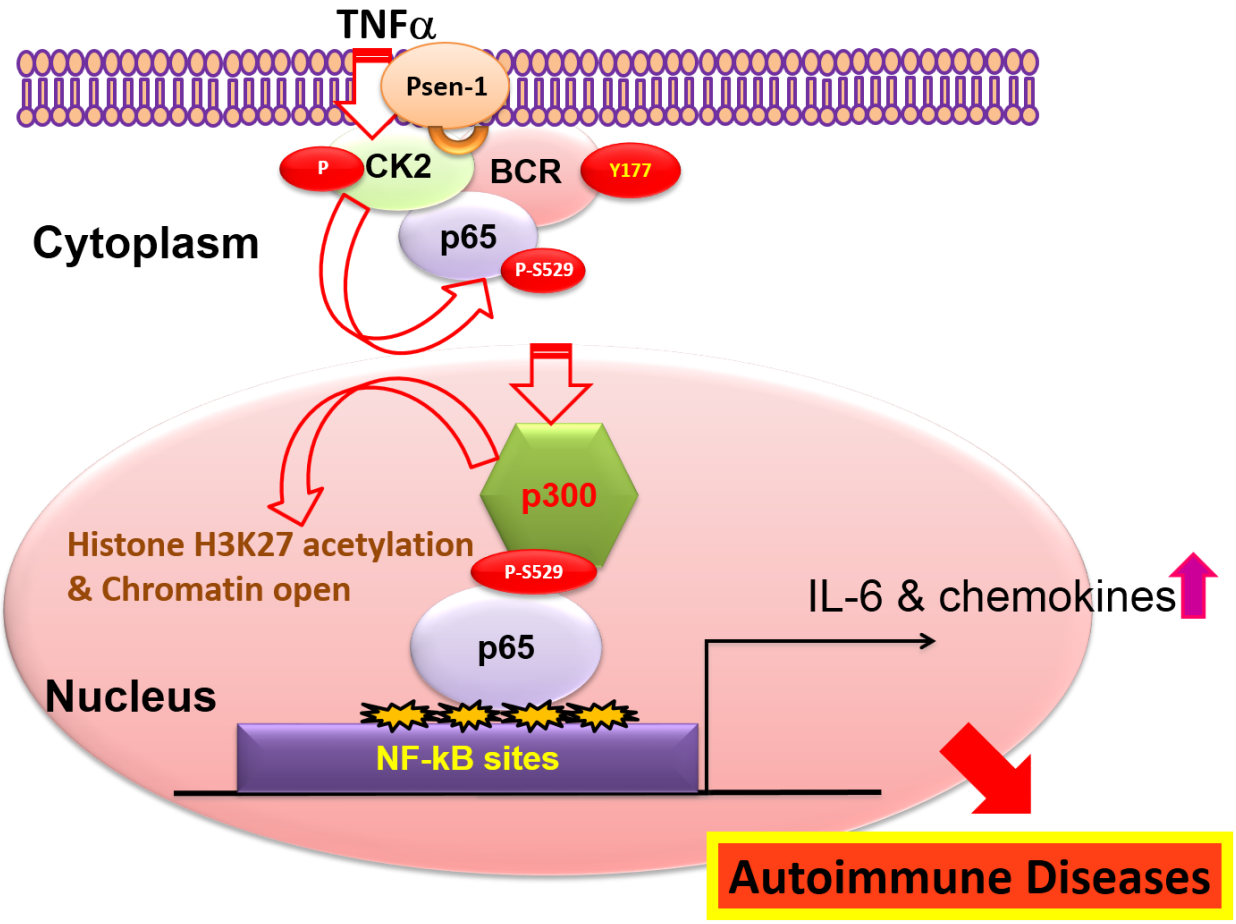


Supplementary Figure 5. Immunoprecipitation at endogenous protein levels.

The immunoprecipitation of p65 was performed in non-target and Psen1-deficient cells with or without TNFα stimulation for 5 min, and then the signals of BCR, CK2α, and their phosphorylated forms were detected by western blotting.



Supplementary Figure 6. The truncation mutant proteins used in this study.



Supplementary Figure 7- Presenilin 1 regulates NF-κB activation via its association with BCR and CK II

Psen1 acts as a scaffold protein and interacts with BCR- CK2α complex via its hydrophilic cytoplasmic loop (271-376) to phosphorylate p65 at serine 529, enabling NF-κB activation through a p300-mediated chromatin opening leading to subsequent inflammation development. [124]

References-

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worlds collide: Th17 and Treg cells in cancer and autoimmunity. Cellular & Molecular Immunology (2018) 15:458–469

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Academic Accomplishments

Publications-

1. Tanaka Yuki*, Sabharwal Lavannya*, Ota Mitsutoshi, Nakagawa Ikuma, Jiang Jing-Jing, Arima Yasunobu, Ogura Hideki, Okochi Masayasu, Ishii Masaru, Kamimura Daisuke, Murakami Masaaki (* equal contribution)

Presenilin 1 Regulates NF-κB Activation via Association with Breakpoint Cluster Region and Casein Kinase II

J Immunol October 2018, 201 (8) 2256-2263

Contribution to this paper-

1) Figure 1-

- Evaluation of IL-6 mRNA and protein levels in Psen-1 knockdown cells
- Rescue experiment with and without Psen-1 overexpression in non-target and shPsen-1 cells.

2) Figure 3-

- Confocal experiment to assess the p65 localization
- ChIP experiment to check the occupancy of p300, p65 and H3K27 on IL-6, Lcn2 promoter

- Chromatin accessibility assay of lL-6, Lcn2 promoter

3) Figure 4-

- IP and western blotting for p65- Ser 529 and p65- Ser 536 in control and shPsen-1 cells.
- Western blotting to assess phosphorylation of CK2a and BCR at Y177 in non-target and shPsen-1 cells.

4) Figure 5-

- IP of Psen1 followed by the detection of p65, CK2a and BCR by Western blotting.
- Membrane, cytosol, and nuclear fractionations.

5) Cloning of Psen-1; Psen-1 Deletion mutants- Δ1-73; Δ271-376

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