The role of G307S mutation of immunoreceptor DNAM-1 in $CD4^+$ T cell-mediated autoimmune inflammation

(CD4 陽性 T 細胞誘導性の自己免疫性炎症における 免疫受容体 DNAM-1 の G307S 変異の役割)

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School of the Integrative and Global Majors in University of Tsukuba

Ph.D. Program in Human Biology

Rikito Murata

筑波大学

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1. Abstract

An increasing number of single nucleotide polymorphisms (SNPs) associated with autoimmune diseases have been identified by genome-wide association studies (GWAS) and case-control studies. Co-stimulatory molecules enhancing the effector functions of $CD4^+$ T cells are promising therapeutic targets for autoimmune diseases. While the functional analysis of autoimmune disease-susceptible SNPs in the genes encoding co-stimulatory molecules has been reported [1-3], little is known about the impact of the SNPs on the pathogenesis of autoimmune diseases.

Here, I show that autoimmune disease-susceptible SNP rs763361 causing non-synonymous mutation from Gly^{307} to Ser^{307} (G307S) of an activating immunoreceptor DNAM-1 is a gain-of-function mutation. G307S DNAM-1 promoted pro-inflammatory cytokine production and cell proliferation of human $CD4^+$ conventional T (Tconv) cells more than wild-type (WT) DNAM-1. Mechanistically, the G307S mutation augmented the phosphorylation of Tyr^{322} of DNAM-1 through the increased interaction with phospho-Tyr kinase Lck. In line with this finding, conversion from Tyr^{322} to Phe^{322} (Y322F) of G307S DNAM-1 canceled the enhanced effector functions of human $CD4^+$ Tconv cells. Moreover, chimeric DNAM-1 (chDNAM-1) was established to examine the impact of G307S mutation in autoimmune disease mouse models. G307S chDNAM-1 promoted the production of pro-inflammatory cytokines of myelin

antigen-specific mouse $CD4^+$ T cells and exacerbated the pathogenesis of experimental autoimmune encephalomyelitis (EAE) more than WT chDNAM-1. These findings demonstrate that rs763361 is a causal polymorphism, and thus, it could be a selection marker of DNAM-1-targeting therapy for patients with autoimmune diseases.

2. Introduction

2.1 Immunity as a host defense system

Immunity is a host defence system to eliminate various foreign and harmful antigens, including microorganisms and cancer cells. This system consists of sequential and coordinated responses called innate immunity and adaptive immunity. The innate immunity is mediated by phagocytosis of macrophages and neutrophils, the cytotoxic activity of natural killer (NK) cells, and pro-inflammatory cytokine production of innate lymphoid cells (ILCs). Activation of the innate immune cells is triggered by the recognition of the molecules which are commonly expressed in microbes and abnormal cells. On the other hand, the acquired immunity is mediated by T cells and B cells, whose activation and effector functions are induced by exposure to specific antigens. However, if the inflammation isn't converged and the activation of immune cells persists, the immune response is induced not only against foreign antigens but also against self-antigens. Therefore, the autoimmune response is required to be tightly regulated.

2.2 Immunologic tolerance and autoimmune disease

The immune responses are not ordinarily induced by self-antigens because autoimmunity is precisely regulated by immunologic tolerance. The immunologic tolerance is divided into two mechanisms. Central tolerance is

induced during the development of T cells and B cells in the thymus and bone marrow, respectively. When T cells and B cells strongly recognize self-antigens during maturation, they undergo apoptosis to eliminate autoreactive immune cells. On the other hand, peripheral tolerance is a mechanism for suppressing autoreactive immune cells that have escaped from central tolerance. In this mechanism, immunosuppressive cells, including regulatory $CD4^+$ T (Treg) cells, impede the activation of the autoreactive immune cells by providing inhibitory cytokines, consuming IL-2, and competing for interaction with antigen-presenting cells (APCs). In addition, the lack of activating receptor signaling and the induction of inhibitory receptor signaling in autoreactive immune cells are also involved in the peripheral tolerance for autoreactive immune cells. The disruption of the immunologic tolerance causes erroneous immune responses to self-antigens, resulting in the development of autoimmune diseases. More than 80 diseases have autoimmune pathogenesis, including type 1 diabetes, multiple sclerosis, and rheumatoid arthritis. It has been estimated that approximately 8.5% of people worldwide suffer from autoimmune diseases, especially in developed countries [4].

Immunosuppressive therapy is widely used as a first-line drug for patients with autoimmune diseases. Still, serious side effects are problems such as the increased risk of infection, pancytopenia, and toxicity for several organs [5, 6]. Accumulated research has led to the developing of molecular targeting therapies for IL-6 and TNF-α, showing high therapeutic efficacy for autoimmune diseases.

However, treatment resistance and adverse effects have been observed even with these biologics [7, 8]. The different therapeutic outcomes among patients could be caused by genetic and environmental factors, which possibly alter the molecules, signaling pathways, and effector immune cells that contribute to the pathogenesis of autoimmune diseases.

2.3 $CD4^+$ T cells in autoimmune diseases

Autoreactive $CD4^+$ T cells play a central role in developing multiple autoimmune diseases. This fact is supported by the evidence that genetic and environmental factors associated with the susceptibility of autoimmune diseases are closely linked to the regulation of $CD4^+$ T cell function. Activation of $CD4^+$ T cells is finely tuned by TCR signals, co-stimulatory signals, and cytokine receptor signals, resulting in cytokine production, proliferation, and differentiation to T helper (Th) cell subsets (Fig. 1A). Among Th cell subsets, IFN-γ-producing Th1 cells and IL-17A-producing Th17 cells exacerbate autoimmune pathogenesis by producing pro-inflammatory cytokines. In contrast, Foxp3-expressing regulatory $CD4^+$ T (Treg) cells inhibit the disease progression by suppressing the effector function of Th1 and Th17 cells through the expression of inhibitory molecules, including IL-10, consumption of the IL-2, and competition for ligands of co-stimulatory molecules. Therefore, the balance between Th1/Th17 and Treg cells is essential for maintaining immune homeostasis [9].

2.4 Environmental and genetic factors in autoimmune diseases

Accumulated evidence has elucidated molecular mechanisms by which environmental factors promote T cell-mediated autoimmune diseases. Excess intake of NaCl and glucose are major risk factors for autoimmune diseases, and these compounds promote Th17 differentiation and inhibit the suppressive function of Treg cells [10-12]. Aging is also associated with the morbidity of autoimmune diseases by augmenting the cell proliferation and pro-inflammatory cytokine expression of $CD4^+$ T cells [13]. Moreover, gut-resident bacteria promote autoimmune inflammation by increasing Th1 and Th17 cells [14, 15].

SNP is a substitution of a single nucleotide in the genome. Some SNPs in a gene-encoding region possibly alter the expression and function of specific proteins. SNPs are closely linked to individual differences in the constitution, disease susceptibility, and responsiveness to therapeutics. Therefore, precision medicine would adapt to patients by examining susceptibility to disease based on the presence of SNPs. GWAS and case-control studies have clarified that SNPs in molecules regulating effector functions of $CD4^+$ T cells are associated with multiple autoimmune diseases [16-18]. Regardless of identifying the autoimmune disease-susceptible SNPs, whether and how they are involved in the pathogenesis is largely unknown. This issue is a rate-limiting factor in establishing precision medicine for patients with autoimmune diseases.

Co-stimulatory receptors promote the TCR-driven activation of $CD4^+$ T cells by augmenting the downstream signaling of TCR, and they are recognized as effective druggable molecules [19]. Although previous reports demonstrated amino acid mutation of co-stimulatory molecules associated with the morbidity of inflammatory diseases [1-3], it is poorly understood their roles in the development of $CD4^+$ T cell-mediated autoimmune diseases.

2.5 Multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)

Multiple sclerosis is an autoimmune disease with the impairment of the central nervous system. Multiple sclerosis is characterized by multifocal lesions in the white matter of the optic nerve, spinal cord, brainstem, cerebrum, and cerebellum, with recurrent clinical symptoms and remissions afflicting approximately 2.5 million people all over the world [20]. This disease is more common in females between the ages of 20 and 40 years. The primary symptoms exhibited during the entire course of multiple sclerosis are visual impairment, cerebellar ataxia, paralysis of the extremities, and sensory disturbance, which vary depending on the site of the lesion. It is reported that not only genetic factors such as SNPs [21], but also environmental factors such as Epstein-Barr virus infection [22, 23] play a complex role in the onset and exacerbation of multiple sclerosis, but the detailed pathogenesis mechanism remains unknown. This is one of the reasons that multiple sclerosis is designated as an incurable disease. The first-line treatment for multiple sclerosis

is steroids. However, if steroids are not effective, immunosuppressive agents, high-dose gamma globulin therapy, and plasma exchange therapy are used even though treatment with molecular-targeting therapy that has fewer side effects than the suppression of the entire immune system is approved. Therefore, it is necessary to elucidate the pathogenesis of multiple sclerosis and search for more effective therapeutic targets and drug-resistance mechanisms.

It has been reported that autoreactive $CD4^+$ T cells play a critical role in the pathogenesis of multiple sclerosis. This knowledge has been confirmed by using a mouse model of multiple sclerosis, EAE. EAE is the most used autoimmune disease mouse model, which can be induced by adoptively transferring myelin-antigen-specific $CD4^+$ T cells into syngeneic mice with pertussis toxin. The encephalitogenic $CD4^+$ T cells migrate into the CNS region and produce pro-inflammatory cytokines upon restimulation by myelin-antigen-presenting cells, including microglia and dendritic cells. The $CD4^+$ T cell-driven inflammation triggers the recruitment and activation of macrophages and neutrophils, resulting in demyelination and neuronal death (Fig. 1B) [24]. Importantly, the findings of EAE were also confirmed in human multiple sclerosis patients [10, 13, 25]. Therefore, it is considered that the EAE model is a useful tool for the investigation of the pathogenesis mechanisms and the screening of therapeutic targets for multiple sclerosis. However, it is important to note that EAE cannot fully reproduce the effects of human SNPs on pathology. Therefore, when examining the role of human SNPs in autoimmune

pathogenesis, it is necessary to devise a method for EAE induction such as the knock-in of human proteins.

2.6 DNAM-1

DNAX accessory molecule-1 (DNAM-1, CD226) is an activating immunoreceptor that is expressed on T cells, NK cells, macrophages, dendritic cells, B cells, ILCs, and megakaryocytes [26-48]. DNAM-1 interacts with CD155 and CD112, which are expressed on most nucleated cells and highly observed on antigen-presenting cells, tumor cells, and inflamed tissues (Fig. 1C) [27, 28]. DNAM-1 shares the functional ligands with TIGIT and CD96 which are known as immune checkpoint molecules for the treatment of cancer patients [29, 30]. Upon binding with the ligands, Tyr^{322} residue of DNAM-1 which is located in the ITT-like activating signaling motif is phosphorylated by Fyn, Src, Lyn, and Lck, and the $pTyr^{322}$ bind to Grb2 to trigger DNAM-1-mediated activating signaling with increased phosphorylation of ERK and AKT (Fig. 1C) [31-33]. The phosphorylated Tyr^{322} residue can be a binding target for not only Grb2 but also Cbl-b which induces the degradation of DNAM-1 in a proteasome-dependent manner after Cbl-b-mediated ubiquitination [34].

DNAM-1 promotes anti-tumor immunity by enhancing the cytotoxicity of NK cells and $CD8^+$ T cells [26, 33-36]. Recent studies also found that DNAM-1 promotes IFN-γ production of ILC1 to limit acute myeloid leukemia and acute liver injury [37, 38]. Moreover, DNAM-1 increases GM-CSF production of

ILC3, activation of megakaryocytes, and maturation of dendritic cells [39-41]. DNAM-1 in $CD4^+$ T cells acts as a co-stimulatory molecule to promote activation, proliferation, pro-inflammatory cytokine production, and Th1 cell differentiation under TCR stimulation [32, 42]. DNAM-1 expression is confirmed in not only Th1 cells but also Th2, Th17, and Treg cells. In line with the expression profiles, DNAM-1 signaling in $CD4^+$ T cells promotes IFN-γ, IL-4, IL-13, IL-17, and GM-CSF [42, 43]. In contrast, DNAM-1 reduces the stable expression of Foxp3 and the suppression activity of Treg cells in inflammatory conditions [44, 45]. Consistent with the findings of the pro-inflammatory functions of DNAM-1 in T cells, several studies have demonstrated that DNAM-1 is involved in exacerbating the pathogenesis of murine autoimmune disease models [46-48].

2.7 Rs763361 is an autoimmune disease-susceptible SNP of DNAM-1

One of the SNPs in *CD226* gene loci rs763361 has been associated with multiple autoimmune diseases, including multiple sclerosis [49, 50], type 1 diabetes [51, 52], rheumatoid arthritis [53, 54], systemic lupus erythematosus [55], neuromyelitis optica [56], primary immune thrombocytopenia [57], juvenile idiopathic arthritis [58], and autoimmune thyroid disease [59]. The rs763361 is a non-synonymous mutation that substitutes Gly^{307} for Ser^{307} in the cytoplasmic region of DNAM-1 (Fig 1D), and its minor allele frequency is over 40% worldwide [49-59]. The percentages of homozygotes and heterozygotes for

rs763361 are approximately 25% and 50%, respectively, with no significant regional differences [50]. Previous studies demonstrated that $CD4^+$ T cells isolated from rs763361-career showed increased phosphorylation of downstream signaling mediators and IL-17 production more than those from non-career [42, 60]. In contrast, Treg cells obtained from rs763361-career showed less suppression activity than those from non-career [61]. However, it cannot be ruled out the possibility that the phenotype is not directly attributed to G307S DNAM-1 because the rs763361 has been reported to show the high linkage disequilibrium with several SNPs, including rs727088, which is existed in 3'-untranslated regions of *CD226* gene loci (Fig 1E) and associated with susceptibility of systemic lupus erythematosus and inflammatory bowel disease by altering DNAM-1 expression [62-64]. Therefore, the precise mechanisms of how G307S DNAM-1 modulates the downstream signaling and pathogenicity of $CD4^+$ T cells have been poorly understood.

3. Purpose

This study aims to elucidate the molecular mechanism by which G307S DNAM-1 promotes $CD4^+$ T cell-mediated autoimmune pathogenesis.

4. Materials and Method

4.1 Mice

WT C57BL/6 mice were bought from CLEA Japan. *Cd226*$^{-/-}$ myelin oligodendrocyte glycoprotein (MOG)-specific 2D2 TCR Tg mice were generated by crossing *Cd226*$^{-/-}$ mice [35] with 2D2 TCR Tg mice [65] bought from Jackson Laboratories. All breeding and in vivo experiments were performed in SFP conditions.

4.2 Plasmid construction and establishment of transfectants

To produce the plasmid for retroviral transduction of DNAM-1, Gly^{307} and Ser^{307} DNAM-1 were isolated from human PBMCs. The N-terminus of each DNAM-1 was tagged with FLAG peptide and inserted into pMXs-IRES-GFP. Phe^{322} of Gly^{307} DNAM-1 and Ser^{307} DNAM-1 was mutated for the Tyr^{322} residue by QuikChange II Site-Directed Mutagenesis Kits to produce Y322F and G307S-Y322F DNAM-1. DNAM-1-BirA* was generated by conjugating FLAG-tagged Gly^{307} and Ser^{307} DNAM-1 with BirA* via GS linker and inserted into pCDH. To produce chDNAM-1, full-length mouse DNAM-1 was restricted by EcoRI. The N-terminal portion of EcoRI-restricted mouse DNAM-1 was ligated with the C-terminal portion of EcoRI-restricted human Gly^{307} and Ser^{307} DNAM-1. The ligation products were inserted into the pMX.

293gp cells were transfected with plasmids for retrovirus production by Lipofectamine 3000. The culture medium was replaced at 16 h post-transfection and harvested at 3- and 5-days post-transfection. The retrovirus was used for gene transduction after 0.45-μm filtration. Jurkat and BW5147 were cultured in a complete RPMI-1640 culture medium. These cell lines were treated with retrovirus encoding FLAG-DNAM-1, FLAG-DNAM-1-BirA*, or chDNAM-1 under 10 μg/mL protamine sulfate.

4.3 Immunoblotting

To examine pTyr of DNAM-1, Jurkat expressing FLAG-DNAM-1 was treated with 10 μg/mL anti-DNAM-1 mAb or human CD155-Fc fusion protein on ice for 30 min, followed by 15 μg/mL anti-mouse IgG polyclonal antibody or anti-human IgG Fc polyclonal antibody at 37°C for indicated times. The cells were lysed with 1% NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. After incubation for 1 h on ice, the lysates were centrifuged and immunoprecipitated with anti-FLAG mAb. To conduct BioID experiments, Jurkat expressing FLAG-DNAM-1-BirA* was cultured for 12 h at 37°C under 0.2-1 mM D-biotin. The cells were lysed with RIPA lysis buffer supplemented with protease inhibitors. After 15 min of incubation on ice, cell lysates were centrifuged and immunoprecipitated with streptavidin. To examine pTyr and Lck binding of chDNAM-1, BW5147 expressing FLAG-WT

chDNAM-1 were treated with 100 μM pervanadate for 5 min at 37°C and lysed with 1% NP-40 or 1% digitonin lysis buffer supplemented with protease and phosphatase inhibitors for 1-2 h at 4°C. The lysates were centrifuged and immunoprecipitated with anti-FLAG Ab. The samples were immunoblotted with anti-pTyr Ab, anti-DNAM-1 mAb, anti-Fyn mAb, anti-Lck mAb, anti-Zap70 mAb, anti-FLAG Ab, anti-β-actin mAb, and anti-DNAM-1 mAb. In some experiments, the cells were treated with 25 μM PP2 for 30 min at 37°C before stimulation.

4.4 Passive EAE

$CD25^{-}CD44^{Lo}CD62L^{Hi}CD4^{+}$ T cells were isolated from *Cd226*$^{-/-}$ 2D2 TCR Tg mice by flow cytometric sorting. The cells were stimulated with 2 μg/mL anti-CD3e mAb, 1 μg/mL anti-CD28 mAb, 1 ng/mL IL-2, and 5 ng/ml IL-12. On day 2, the retrovirus was treated under 10 μg/mL protamine sulfate. On day 5, the cells were injected into WT or *Cd155*$^{-/-}$ C57BL/6 mice with 4 Gy radiation (5×10^5 cells/mouse) via the tail vein. Pertussis toxin 200 ng was intraperitoneally administrated 0 and 2 days after the cell transfer. Mice were monitored every day for EAE clinical scores. For histological analysis, the 4% PFA-fixed sections of spinal cord on day 21 post-cell transfer were stained with H&E, LFB, and immune-histochemistry (IHC).

4.5 Co-culture assay of 2D2 $CD4^+$ T cells and $CD11c^+$ cells

$CD11c^+$ cells were sorted from WT C57BL/6 mice by MACS sorting. 1×10^4 $CD11c^+$ splenocytes were co-cultured with 5×10^4 2D2 Th1 cells under $MOG_{35–55}$ peptides and 12.5-25 ng/mL LPS for 48 h at 37°C.

4.6 Functional analysis of human Tconv cells and Treg cells

PBMCs were from 8 healthy volunteers (average 30.4 years old) after written informed consent had been received. $CD4^+$ $CD25^{Lo}$ $CD127^{Hi}$ Tconv cells and $CD4^+$ $CD25^{Hi}$ $CD127^{Lo}$ Treg cells were sorted by flow cytometry. The sorted cells were stimulated in with anti-CD3, anti-CD28 mAb, and 10 ng/mL human IL-2. On day 1, retrovirus was treated for the cells under 10 μg/mL protamine sulfate. On day 7, GFP^+ cells were sorted and further expanded under the stimulation with anti-CD3, anti-CD28 mAb, and 10 ng/mL human IL-2. On day 14, the cells were used for functional analysis.

To examine the effector function of Tconv cells, Tconv cells expressing FLAG-DNAM-1 were stimulated for 12-24 h with 0.25 μg/mL anti-CD3 and either 3 μg/mL mouse IgG1 isotype control or anti-FLAG mAb with or without 10 μM BrdU. For analysis of the cellular function of Treg cells, Treg cells expressing FLAG-DNAM-1 were stimulated at 37°C for 5 days with 0.2-1 μg/mL anti-CD3 and either 30 μg/mL mouse IgG1 isotype control or anti-FLAG mAb. The cells were stained for BrdU or Foxp3 and analyzed by flow cytometry. The culture supernatants were analyzed by BD Cytometric Bead

Array for cytokine concentrations. To analyze the suppression activity of Treg cells, retrovirally transduced Treg cells were co-cultured with 2×10^4 Tconv cells labeled with CellTrace under anti-CD3 and anti-FLAG mAb stimulation. The CellTrace signals were analyzed at 96 h post-co-culture by flow cytometry.

4.7 Flow cytometry

The cells were stained with the following Abs conjugated with fluorescence dye; anti-CD4, anti-CD25, anti-CD127, anti-DYKDDDDK, anti-DNAM-1, anti-CD4, anti-CD25, anti-CD44, anti-CD62L, anti-TCR Vα3.2, anti-CD45.2, anti-TCR β chain, anti-CD8, anti-B220, anti-NK1.1, anti-CD11b, anti-CD11c, anti-Ly-6G/Ly-6C, anti-IFN-γ, anti-TNF-α, anti-Foxp3 mAb, or anti-T-bet mAb. Propidium iodide or Zombie Fixable Viability Kit was used to exclude dead cells. Samples were measured with flow cytometry and analyzed with FlowJo.

4.8 Statistical analysis

Unpaired t-tests and two-way ANOVA were used to compare the data using Prism 9. $P < 0.05$ was considered statistically significant. Error bars show SEM. Cartoons in Figures 1A-C and 10 were created with BioRender.com.

5. Results

5.1 G307S DNAM-1 promoted the pro-inflammatory cytokine production of human Tconv cells more than WT DNAM-1

To analyze the role of G307S DNAM-1 in the cellular function of $CD4^+$ Tconv cells ($CD4^+$ T cells except for $CD4^+$ $CD25^+$ $CD127^-$ Treg cells), IRES-GFP (Mock), FLAG-WT DNAM-1-IRES-GFP, and FLAG-G307S DNAM-1-IRES-GFP were retrovirally transduced into Tconv cells isolated from human PBMCs (Fig. 2A-D). To elucidate the effect of G307S DNAM-1 on the cytokine production of Tconv cells, FLAG-DNAM-1-expressing Tconv cells were stimulated with anti-CD3 mAb and anti-FLAG mAb to specifically crosslink the transduced FLAG-DNAM-1, but not endogenously expressed DNAM-1. FLAG-G307S DNAM-1-expressing Tconv cells showed higher production of IFN-γ and TNF-α than FLAG-WT DNAM-1-expressing Tconv cells after the stimulation with anti-FLAG mAb, but not control Ig (cIg) (Fig. 2E). In contrast, IL-17A and IL-4 were not detected (Fig. 2E). These results indicated that G307S DNAM-1 enhanced the pro-inflammatory cytokine production of Tconv cells more than WT DNAM-1.

To examine the involvement of G307S DNAM-1 in the suppressive function of Treg cells, Mock, FLAG-WT DNAM-1-IRES-GFP, and FLAG-G307S DNAM-1-IRES-GFP were retrovirally transduced into Treg cells isolated from human PBMCs (Fig. 2A-C, 3A) and their suppression activity was

evaluated. FLAG-DNAM-1-expressing Treg cells were co-cultured with CellTrace-labeled autologous Tconv cells under the stimulation with anti-CD3 mAb and anti-FLAG mAb, and the proliferation of Tconv cells was analyzed. FLAG-DNAM-1-expressing Treg cells showed less suppression activity than Mock Treg cells (Fig. 3B), according to previous research [44, 45]. On the other hand, FLAG-G307S DNAM-1-expressing Treg cells showed comparable inhibition for the proliferation of Tconv cells with FLAG-WT DNAM-1-expressing Treg cells (Fig. 3B). In addition, Foxp3 expression and IL-10 production were equivalent between FLAG-WT DNAM-1- and FLAG-G307S DNAM-1-expressing Treg cells even after the stimulation with anti-CD3 mAb and anti-FLAG mAb (Fig. 3C and D). These findings suggested that G307S DNAM-1 didn't decrease the suppressive function of Treg cells more than WT DNAM-1.

5.2 G307S mutation promoted Tyr phosphorylation of DNAM-1

G307S mutation is in the cytoplasmic region of DNAM-1 (Fig. 1D and 2B), and the Tyr^{322} is a potential phosphorylation site involved in the DNAM-1-mediated signaling [31-33]. Because the G307S mutation is closely located with the Tyr residue and G307S DNAM-1 showed more phosphorylation of its downstream molecule VAV-1 and ERK than WT DNAM-1 did [42], I hypothesized that the G307S mutation might modulate the Tyr phosphorylation of DNAM-1. To test this hypothesis, FLAG-WT DNAM-1 and FLAG-G307S

DNAM-1-expressing Jurkat human T cell lines were established, and the equivalent expression of FLAG and DNAM-1 were confirmed (Fig. 4A). These Jurkat transfectants were stimulated with anti-DNAM-1 mAb or CD155-Fc fusion protein and compared phospho-Tyr (pTyr) of WT DNAM-1 to that of G307S DNAM-1. G307S DNAM-1 showed a higher pTyr than WT DNAM-1 after stimulation with anti-DNAM-1 mAb and CD155-Fc fusion protein (Fig. 4B and C). This result suggested that the G307S mutation enhanced DNAM-1-mediated signaling by increasing pTyr.

Phosphorylation of Ser^{329} of DNAM-1 induced by protein kinase C is involved in the binding with the ligand-expressing cells [66]. Therefore, it was assessed whether the G307S mutation increases the ligand binding of DNAM-1. To that end, WT DNAM-1 and G307S DNAM-1-expressing BW5147 mouse T cell lines were established, and the undistinguishable expression of human DNAM-1 was confirmed (Fig. 4D). These BW5147 transfectants were treated with CD155-Fc fusion proteins, followed by various doses of anti-human DNAM-1 blocking mAb. We confirmed that the dissociation of CD155-Fc fusion protein was comparable between WT DNAM-1 transfectant and G307S DNAM-1 transfectant (Fig. 4E). These results suggested that G307S mutation didn’t increase the binding of DNAM-1 to CD155.

5.3 Src-family kinase was required for the augmented pTyr of G307S DNAM-1

To further investigate the molecular mechanism by which G307S mutation enhances pTyr of DNAM-1, cytoplasmic molecules associated with DNAM-1 were analyzed by using proximity-dependent biotin identification (BioID) [67]. To conduct this approach, the C-terminus of FLAG-WT DNAM-1 and FLAG-G307S DNAM-1 were conjugated with R118G-mutant BirA (BirA*) via Gly-Ser linker (Fig. 5A), improving the range of biotinylation [68]. The constructs were transduced into the Jurkat human T cell line at a comparable expression level (Fig. 5B). When FLAG-DNAM-1-BirA*-expressing Jurkat transfectants were cultured in the presence of biotin, the proximal cytosolic molecules of DNAM-1 were biotinylated. It was confirmed that biotinylated molecules weren't specifically present or lacking in G307S DNAM-1 (Fig. 5C). Because the previous reports demonstrated that pTyr of DNAM-1 is induced by Src-family kinases [31, 33, 34], the amount of biotinylated Src-family kinases was compared between WT DNAM-1-BirA* and G307S DNAM-1-BirA* transfectant. The biotinylated Lck was higher in G307S DNAM-1 transfectant than WT DNAM-1 transfectant, but Fyn was not detected (Fig. 5D). To examine whether the Src-family kinases are required for the enhanced pTyr of G307S DNAM-1, pTyr of DNAM-1 was assessed in the presence or absence of a selective Src-family kinase inhibitor PP2. G307S DNAM-1 showed greater pTyr of DNAM-1 in the absence of PP2, while the augmented pTyr of G307S DNAM-1 was not observed in the presence of PP2 (Fig. 5E). These results

suggested that G307S mutation increased the association of DNAM-1 with an Src-family kinase Lck, likely enhancing the pTyr of DNAM-1.

5.4 G307S DNAM-1 enhanced the effector function of human Tconv cells via Tyr^{322}

To investigate whether G307S mutation promotes phosphorylation of the Tyr^{322} residue, FLAG-Y322F DNAM-1 and FLAG-G307S-Y322F DNAM-1-expressing Jurkat transfectants were established (Fig. 2B and 6A). These Jurkat transfectants were stimulated with anti-DNAM-1 mAb and compared pTyr of DNAM-1. G307S DNAM-1 showed a higher pTyr than WT DNAM-1, but Y322F DNAM-1 and G307S-Y322F DNAM-1 didn't show pTyr at all even after the stimulation with anti-DNAM-1 mAb (Fig. 6B). This result indicated that G307S mutation enhanced $pTyr^{322}$ of DNAM-1. This finding prompts me to elucidate whether the Tyr^{322} is required for the gain-of-function effect of G307S DNAM-1 on the effector function of Tconv cells. To address this point, FLAG-WT DNAM-1, FLAG-G307S DNAM-1, FLAG-Y322F DNAM-1, and FLAG-G307S-Y322F DNAM-1 were retrovirally transduced into human Tconv cells (Fig. 2A-C and 6C). FLAG-DNAM-1-expressing Tconv cells were stimulated with anti-CD3 mAb and anti-FLAG mAb, and cytokine production was compared. FLAG-G307S DNAM-1-expressing Tconv cells showed higher production of IFN-γ and TNF-α than FLAG-WT DNAM-1-expressing Tconv cells, but the enhanced cytokine production was not observed in FLAG-G307S-

Y322F DNAM-1-expressing Tconv cells (Fig. 6D). Moreover, FLAG-DNAM-1-expressing Tconv cells were stimulated with anti-CD3 mAb and anti-FLAG mAb in the presence of BrdU to examine the proliferation. FLAG-G307S DNAM-1-expressing Tconv cells showed higher incorporation of BrdU than FLAG-WT DNAM-1-expressing Tconv cells and FLAG-G307S-Y322F DNAM-1-expressing Tconv cells (Fig. 6E). These results suggested that G307S DNAM-1 enhanced the effector functions of Tconv cells more than WT DNAM-1 through Tyr^{322}.

5.5 G307S mutation of DNAM-1 exacerbated $CD4^+$ T cell-mediated autoimmune inflammation in mice

Gly^{307} residue of human DNAM-1 is not conserved in mouse DNAM-1 [69]. To examine the effect of G307S mutation on autoimmune disease mouse models, a part of the intracellular region of mouse DNAM-1 was substituted with human WT DNAM-1 or human G307S DNAM-1 to generate chimeric DNAM-1 (chDNAM-1), respectively (Fig. 7A). Pervanadate stimulation of the BW5147 transfectant expressing WT chDNAM-1 induced physical association of Lck with and Tyr phosphorylation of the chDNAM-1 (Fig. 7B and C), suggesting that the chDNAM-1 mediates an intracellular signal via the intracellular region of human DNAM-1 in mouse T cells. To investigate whether G307S chDNAM-1 promotes cytokine production of autoreactive mouse $CD4^+$ T cells, Mock, WT chDNAM-1, and G307S chDNAM-1 were

retrovirally transduced into DNAM-1-deficient myelin oligodendrocyte glycoprotein (MOG)-specific (2D2) TCR Tg $CD4^+$ T cells under Th1-polarizing condition (Fig. 7D and E). Then, the chDNAM-1-expressing 2D2 Th1 cells were co-cultured with splenic $CD11c^+$ cells in the presence of MOG_{35-55} peptides and LPS. G307S chDNAM-1-expressing 2D2 Th1 cells significantly increased IFN-γ and TNF-α production than WT chDNAM-1-expressing 2D2 Th1 cells (Fig. 7F). These results suggested that G307S chDNAM-1 promoted pro-inflammatory cytokine production of autoreactive mouse $CD4^+$ T cells more than WT chDNAM-1.

To clarify the role of G307S DNAM-1-expressing autoreactive $CD4^+$ T cells in the autoimmune pathogenesis, Mock, WT chDNAM-1, and G307S chDNAM-1-expressing 2D2 Th1 cells were adoptively transferred into 4 Gy-irradiated WT mice to induce EAE (Fig. 8A). The mice transferred with G307S chDNAM-1-expressing 2D2 Th1 cells showed a higher clinical score than those transferred with WT chDNAM-1-expressing 2D2 Th1 cells or Mock-DNAM-1-deficient 2D2 Th1 cells (Fig. 8B). Moreover, histological analysis revealed that the spinal cord from the mice transferred with G307S chDNAM-1-expressing 2D2 Th1 cells showed more severe $CD4^+$ cell infiltration and inflammatory foci than the mice with WT chDNAM-1-expressing 2D2 Th1 cells on day 21 post-adoptive cell transfer (Fig. 8C). Given that DNAM-1 is a co-stimulatory molecule of $CD4^+$ T cells, $MHCII^+CD155^+$ cells are required for the induction of the pathogenicity of G307S chDNAM-1-expressing autoreactive $CD4^+$ T

cells. To analyze the potential stimulators of chDNAM-1-expressing 2D2 Th1 cells *in vivo*, the expression profiles of MHCII and CD155 in the spinal cord from naïve and EAE-induced mice were analyzed. The MHC class II^+CD155^+ population increased after EAE induction and mainly consisted of $CD45^{Mid}CD11b^+$ microglia and $CD45^{Hi}CD11b^+CD11c^+$ monocyte-derived dendritic cells (Fig. 8D), suggesting that these MHC class II^+CD155^+ cell populations are required for chDNAM-1-expressing 2D2 Th1 cells to exert their pathogenicity in the spinal cord. In addition, EAE clinical score was comparable between WT and G307S chDNAM-1 when the cells were adoptively transferred into CD155-deficient mice (Fig. 8E). Altogether, these results indicated that G307S chDNAM-1-expressing autoreactive $CD4^+$ T cells exacerbated the pathogenesis of EAE more than WT chDNAM-1-expressing autoreactive $CD4^+$ T cells.

5.6 G307S chDNAM-1 promoted pathogenicity of autoreactive mouse $CD4^+$ T cells more than WT chDNAM-1

To analyze whether G307S chDNAM-1 augments the effector function of autoreactive $CD4^+$ T cells *in vivo*, the spinal cords were harvested on day 16 post-adoptive cell transfer and analyzed the pro-inflammatory cytokine production of spinal cord-infiltrating $CD4^+$ T cells (Fig. 9A). The numbers of spinal cord-infiltrating $CD45.2^+$ cells, $V\alpha3.2^+$ 2D2 $CD4^+$ T cells, $V\alpha3.2^-$ non-2D2 $CD4^+$ T cells, and $CD8^+$ T cells were comparable between WT and G307S

chDNAM-1 (Fig. 9B). On the other hand, the mice with G307S chDNAM-1-expressing 2D2 $CD4^+$ T cells showed a higher frequency and number of IFN-γ^+ TNF-α^+ 2D2 $CD4^+$ T cells than the mice with WT chDNAM-1-expressing 2D2 $CD4^+$ T cells (Fig. 9C and D). These results suggested that G307S chDNAM-1 augments the pathogenicity of autoreactive $CD4^+$ T cells more than WT chDNAM-1.

6. Discussion

6.1 Rs763361 might assist the HLA-risk alleles-driven autoimmune disease

The morbidity of autoimmune diseases is strongly associated with HLA risk alleles which might preferentially activate autoreactive T cells by presenting self-antigens. However, all carriers don't develop the disorders [70, 71]. This fact strongly suggests that other non-HLA risk SNPs or environmental factors are required to develop autoimmune diseases. Genetic statistics and epidemiological analyses have shown the correlation of SNPs and environmental factors with the incidence of autoimmune diseases, but it is largely unclear how the suggested risk factors cause autoimmune diseases. Given that the co-stimulatory molecules augment TCR signaling to promote T cell activation, autoimmune disease-susceptible SNPs of the genes encoding co-stimulatory molecules may cooperate with HLA-risk alleles and develop autoimmune diseases. In this study, I showed that rs763361 causing G307S mutation of DNAM-1 augmented the effector functions of $CD4^+$ T cells under the TCR stimulation and exacerbated $CD4^+$ T cell-mediated autoimmune disease. This finding highlights the importance of co-stimulatory molecules in the pathogenesis of autoimmune diseases, and the detection of HLA-risk alleles and rs763361 could be a predictive marker of the development of autoimmune diseases.

6.2 Rs763361 might be a causal SNP of autoimmune diseases regardless of the other SNPs showing high linkage disequilibrium

Previous reports demonstrated that the effector function of $CD4^+$ T cells derived from rs763361-career is upregulated compared with non-career, and suppression activity of Treg cells derived from rs763361-career was reduced compared with non-career [42, 60, 61]. However, it has been unclarified whether G307S DNAM-1 directly promotes $CD4^+$ T cell function as a gain-of-function mutation because rs763361 shows high linkage disequilibrium with other SNPs, including rs727088, which is also reported as an autoimmune disease-susceptible SNP [62]. The present study elucidated that G307S DNAM-1 enhanced the production of pro-inflammatory cytokines and cell proliferation of human Tconv cells more than WT DNAM-1 by using a retroviral transduction system. It has been reported that rs727088 decreases DNAM-1 expression [62]. Therefore, it is suggested that G307S DNAM-1 enhances the effector functions of $CD4^+$ T cells and promotes downstream signaling pathways beyond the effect of reduced DNAM-1 expression by rs727088. On the other hand, I found that the suppressive function of human Treg cells was not altered by G307S DNAM-1 (Fig. 3B-D), which might be because the G307S DNAM-1 doesn't modulate AKT phosphorylation in $CD4^+$ T cells [42], which is critical for Treg suppression activity [72, 73]. This phenotype is not consistent with previous research using SNP-typed donor-

derived Treg cells [61]. This discrepancy might be attributed to the contribution of other SNPs showing high linkage disequilibrium with rs763361.

6.3 G307S mutation might affect the protein structure and interaction mode of DNAM-1

I elucidated that the G307S mutation increased the interaction with Lck and enhanced $pTyr^{322}$ of DNAM-1. This fact is consistent with the previous research showing that phosphorylation of DNAM-1 downstream molecule VAV-1 and ERK are increased in rs763361-career compared with non-career because their phosphorylation is promoted by $pTyr^{322}$ of DNAM-1 [42]. However, it is still unclear the mechanism of how G307S mutation increases the interaction between DNAM-1 and Lck. It is speculated that the G307S mutation of DNAM-1 might modulate the binding affinity to Lck because the substitution from Gly to Ser could alter the hydrogen bonding, which possibly affects the three-dimensional structure and protein-protein interaction in the intracellular region of DNAM-1 [74, 75]. To address this point, it is necessary to analyze the three-dimension structure of the intracellular region of WT DNAM-1 and G307S DNAM-1. In addition, given that enhanced pTyr of G307S DNAM-1 was sustained 30 min after stimulation (Figure 4B), it is also possible that the binding of tyrosine phosphatase to DNAM-1 might be attenuated by G307S mutation. However, I didn't address the point because there is no report about tyrosine phosphatase which can interact with DNAM-1, and I could find

enhanced interaction with phospho-Tyrosine kinase to G307S DNAM-1 to explain the phenotype.

6.4 The role of the pTyr^{322}-mediated signal of DNAM-1 in Tconv cells

It is previously revealed that Tyr^{322} of DNAM-1 is required for the enhanced cellular function of CD4^{+} T cells [32]. However, I could not find the difference in cytokine production and proliferation of Tconv cells between WT and Y322F DNAM-1, although both WT and Y322F DNAM-1 significantly promote Tconv cell function as compared with mock and control stimulation (Figure 6D and E). I speculate two possible reasons to explain the phenotype: First, DNAM-1 might transmit an activation signal to promote the effector functions by previously undescribed signaling pathways distinct from the pTyr^{322}-mediated activation signal, e.g., pSer^{329} [66]. Second, DNAM-1 undergoes proteasome-dependent degradation upon Src-family kinase-mediated Tyr phosphorylation and Cbl-b-mediated ubiquitination [34]. These features may make it difficult to confirm the effect of pTyr^{322}-mediated activation signaling of WT DNAM-1. Therefore, further optimization of experimental conditions may need to clarify the significance of Tyr^{322} of WT DNAM-1 in T cell function.

6.5 G307S DNAM-1-expressing 2D2 Th1 cells might indirectly injure the CNS in EAE mice

G307S chDNAM-1-expressing myelin antigen-specific Th1 cells promoted pro-inflammatory cytokine production in the spinal cord and exacerbated the pathogenesis of EAE more than WT chDNAM-1-expressing myelin antigen-specific Th1 cells. However, it is still unclarified how neuronal damage in the spinal cord was promoted by G307S chDNAM-1-expressing 2D2 Th1 cells more than WT chDNAM-1-expressing 2D2 Th1 cells. Several reports demonstrated that myelin-reactive $CD4^+$ T cells induce neuronal damage through the recruitment and activation of spinal cord-infiltrating myeloid cells [25, 76, 77]. Th1 cell-mediated passive EAE showed higher numbers of $CD11b^+$ $MHCII^+$ macrophages and higher expression of *Nos2* in the spinal cord than Th17 cell-mediated passive EAE [76]. Furthermore, $CD4^+$ T cell-derived TNF-α and FASL induce inflammasome-independent IL-1β production by dendritic cells, exacerbating the pathogenesis of EAE [77]. A recent study also showed that myelin-reactive $CD4^+$ T cells migrate into bone marrow and promote myelopoiesis to supply $Ly6C^+$ monocytes and neutrophils and amplify the autoimmune inflammation in the CNS during EAE [25]. Therefore, it is speculated that G307S chDNAM-1 augments autoreactive Th1 cell-mediated recruitment and activation of myeloid cells more strongly than WT chDNAM-1, resulting in worse autoimmune inflammation in the CNS. On the other hand, several reports demonstrated that $CD4^+$ T cells harboring cytotoxic potentials

might induce neuronal damage directly [78, 79]. Therefore, further analysis for the downstream events of autoreactive $CD4^+$ T cell activation would be required to understand G307S DNAM-1-mediated autoimmune pathogenesis.

6.6 Various immune cell subsets expressing G307S DNAM-1 might contribute to the development of autoimmune diseases

DNAM-1 is expressed in NK cells [26, 30, 33], $CD8^+$ T cells [29, 34-36], ILCs [37-39], B cells [80], and dendritic cells (DCs) [41] in addition to $CD4^+$ T cells. DNAM-1 promotes the cytotoxicity of NK cells and $CD8^+$ T cells [26, 30, 33-36], pro-inflammatory cytokine production of ILC1 and ILC3 [37-39], and maturation and activation of DCs [41]. These cell populations also contribute to autoimmune diseases [81], and their cellar functions might be more enhanced by G307S DNAM-1 than WT DNAM-1. Of note, rs763361 is associated with a better outcome of chemotherapy treatment for small-cell lung cancer and overall survival [82], suggesting that the anti-tumor activity of NK cells and $CD8^+$ T cells would be augmented by the G307S mutation of DNAM-1. In addition, the expression of DNAM-1 on $CD4^+$ T cells, $CD8^+$ T cells, and B cells increases in patients with autoimmune diseases compared with healthy controls [83-85]. Therefore, the role of the G307S DNAM-1-expressing immune cells other than $CD4^+$ T cells and their contribution to autoimmune diseases' pathogenesis should be investigated.

6.7 Rs763361 could be a patient selection marker of DNAM-1-targeting therapy

This is the first study to show that the G307S mutation of DNAM-1 exacerbated the autoimmune disease model. The rs763361 is associated with various autoimmune disorders, and its minor allele frequency is over 40% in the analyzed population [49-59]. Preceding studies have shown that the treatment with anti-DNAM-1 blocking mAb ameliorates the pathogenesis of several inflammatory disorders [36, 45, 48]. In addition, this study demonstrated that the gain-of-function effect of G307S mutation in CD155-deficient mice adoptively transferred with chDNAM-1-expressing 2D2 Th1 cells was completely canceled based on the EAE clinical score (Figure 8E). This evidence suggests that anti-DNAM-1 blocking mAb would more effectively inhibit G307S DNAM-1-induced autoimmune disease pathogenesis than the WT DNAM-1, and rs763361 could be a patient selection marker for molecular targeting therapy against DNAM-1. Further analysis confirming the effect of anti-DNAM-1 blocking mAb by using autoimmune disease models and human PBMCs derived from rs763361 SNP-typed career is required for adapting the finding to the development of precision medicine for multiple autoimmune diseases.

7. Conclusion

This study showed that G307S mutation is a gain-of-function mutation of DNAM-1 (Fig. 10). From the retroviral transduction experiments, I directly demonstrated that G307S DNAM-1 promoted the effector function of human Tconv cells but not the suppressive function of human Treg cells. Mechanistically, the G307S mutation increased the association of Src-family kinase Lck and augmented $pTyr^{322}$ of DNAM-1 upon crosslinking with CD155. In line with this finding, G307S-Y322F double mutant DNAM-1 didn't show the enhanced cytokine production and cell proliferation of human Tconv cells. Finally, by establishing chDNAM-1, I showed that the G307S mutation of DNAM-1 enhanced the pathogenicity of encephalitogenic mouse $CD4^+$ T cells and exacerbated EAE pathogenesis. Altogether, this study demonstrated that autoimmune disease-susceptible missense SNP rs763361 is a causal polymorphism that promotes $CD4^+$ T cell-mediated autoimmune inflammation.

8. Abbreviations

BioID: proximity-dependent biotin identification

BirA*: Arg118Gly-mutant BirA

BrdU: bromodeoxyuridine

CBA cytometric bead array

CD: cluster of differentiation

CNS: central nervous system

DC: dendritic cell

DNAM-1: DNAX accessory molecule-1

EAE: experimental autoimmune encephalomyelitis

ERK: extracellular signal-regulated kinase

FASL: FAS ligand

Foxp3: forkhead box protein p3

G307S: mutation to serine from glycine at the 307th residue

GFP: green fluorescent protein

GWAS: genome-wide association study

H&E: hematoxylin and eosin

HLA: Human Leukocyte Antigen

HRP: horseradish peroxidase

IB: immuno-blotting

IFN-γ: interferon-gamma

IHC: immuno-histochemistry

IL: interleukin

ILC: innate lymphoid cells

IP: immuno-precipitation

IRES: internal ribosome entry site

LFA-1: lymphocyte function-associated antigen 1

LFB: luxol fast blue

MHC: major histocompatibility complex

MOG: myelin oligodendrocyte glycoprotein

NK: natural killer

PBMC: peripheral blood mononuclear cell

PCR: polymerase chain reaction

PVDF: polyvinylidene difluoride

SNP: single nucleotide polymorphism

TCR: T cell receptor

TNF-α: tumor necrosis factor-α

Tconv: conventional $CD4^+$ T cell

Tg: transgenic

Th: T helper cell

Treg: regulatory $CD4^+$ T cell

Y322F: mutation to phenylalanine from tyrosine at the 322^{nd} residue

chDNAM-1: chimeric DNAM-1

mAb: monoclonal antibody

pTyr^{322}: phosphorylated 322nd tyrosine residue

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11. Figures

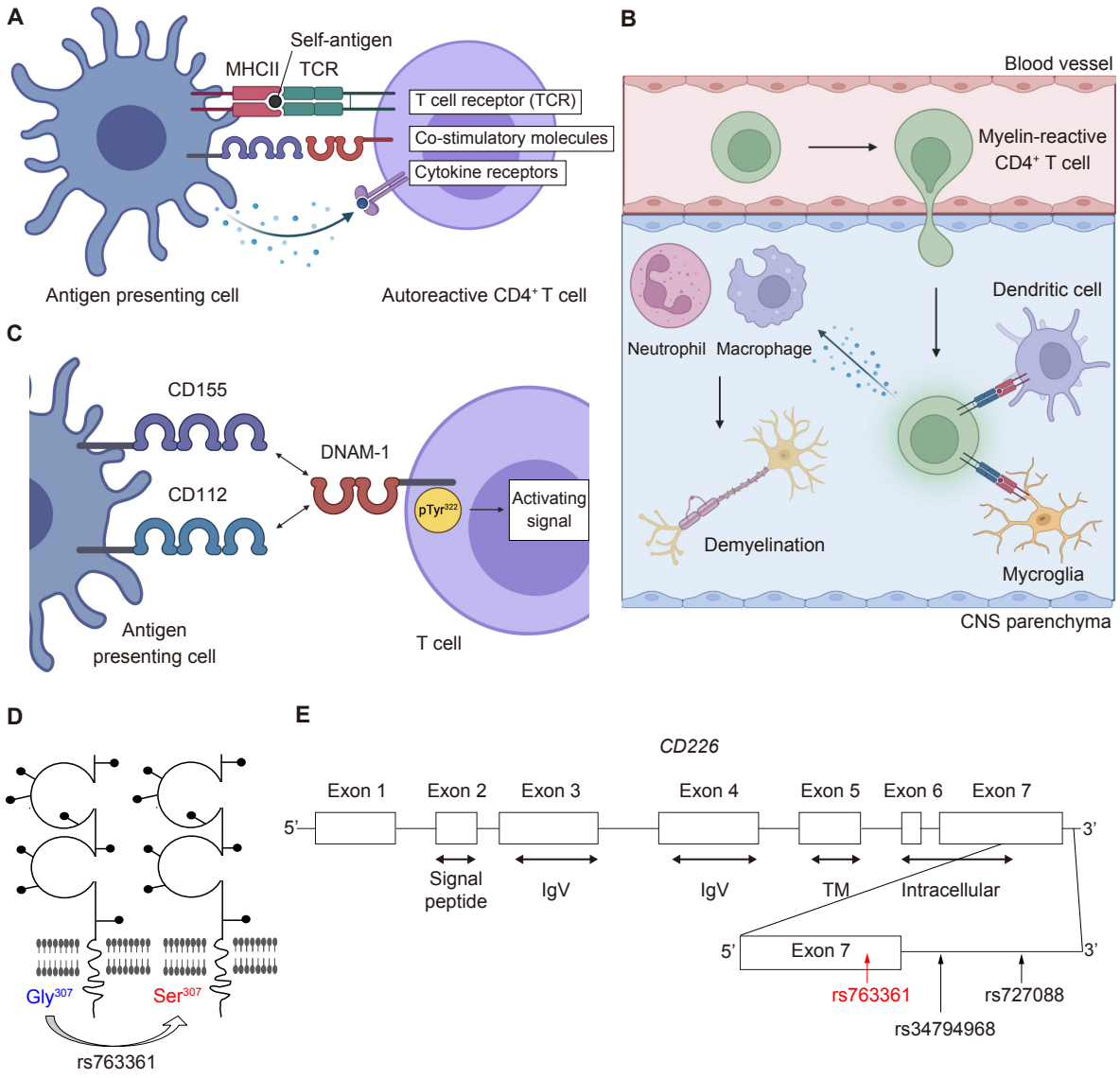

Figure 1. rs763361 is an autoimmune disease susceptible-non-synonymous mutation of DNAM-1

(A) Activation pathways of autoreactive $CD4^+$ T cells. The activation is induced by the recognition of self-antigen presented by MHCII expressed on antigen-presenting cells but TCR signaling is not enough for the full activation. Co-stimulatory receptor signaling and cytokine receptor signaling augments the TCR-mediated $CD4^+$ T cell activation. (B) The pathogenic mechanism of multiple sclerosis mediated by $CD4^+$ T cells. Myelin-antigen-specific $CD4^+$ T cells are recruited to the brain and spinal cords. The migrated cells interact with microglia and dendritic cells presenting myelin-antigen on their MHCII. The restimulated encephalitogenic $CD4^+$ T cells secret pro-inflammatory cytokines to promote the activation of myeloid cells and amplify local inflammation. (C) Interaction with ligands and signal transduction of DNAM-1. DNAM-1 interacts with CD155 and CD112 which are

highly expressed on antigen-presenting cells. The DNAM-1-ligand binding triggers the tyrosine phosphorylation at the 322nd residue which is required for its downstream signaling. (D) The location of G307S mutation in DNAM-1 protein. G307S mutation is in the cytoplasmic region of human DNAM-1 where the signaling event is induced (E) The location of rs763361 and other SNPs on the *CD226* gene. Rs763361 is in exon 7 but rs34794968 and rs727088 are in the intron. Because of the close location of these SNPs, they show a high linkage disequilibrium.

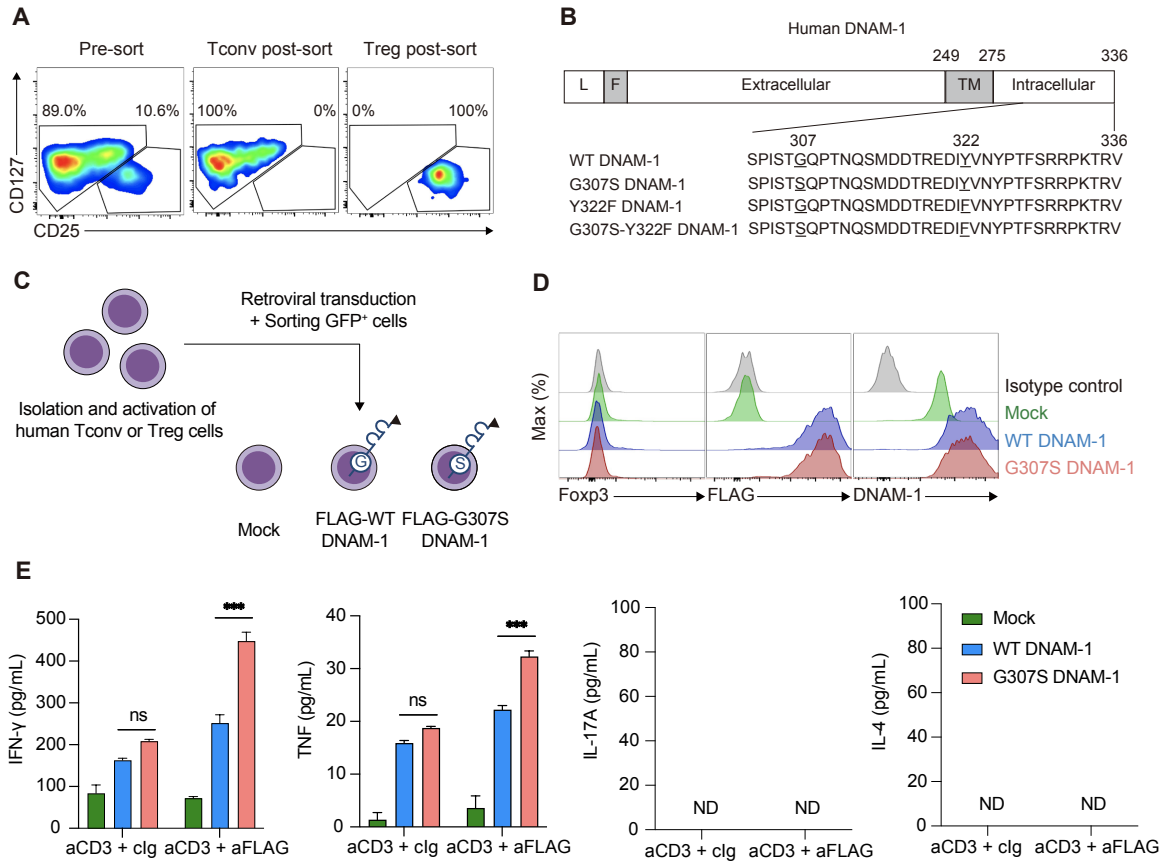

Figure 2. G307S DNAM-1 promoted cytokine production of human Tconv cells.

(A) Human $CD4^+$ $CD25^{Lo}$ $CD127^{Hi}$ Tconv and $CD4^+$ $CD25^{Hi}$ $CD127^{Lo}$ Treg cells were sorted from healthy donor-derived human PBMCs by using flow cytometry. (B) The structure and amino acid sequence of FLAG-tagged human DNAM-1. Gly^{307} and/or Tyr^{322} were mutated for each mutant DNAM-1. (C) The abstract of the method to produce human $CD4^+$ Tconv cells and Treg cells expressing FLAG-DNAM-1. Healthy donor-derived human Tconv cells and Treg cells were retrovirally transduced with Mock, FLAG-WT DNAM-1, or FLAG-G307S DNAM-1. GFP^+ cells were sorted and further functional analysis was performed. (D) The protein expression of Foxp3, FLAG, and DNAM-1 on retrovirally transduced human Tconv cells was analyzed by flow cytometry. (E) Mock, FLAG-WT DNAM-1, or FLAG-G307S DNAM-1-expressing human Tconv cells were stimulated with anti-CD3 mAb and either anti-FLAG mAb or control Ig. The culture supernatant was harvested 12 hrs after stimulation and the concentration of IFN-γ, TNF-α, IL-17A, and IL-4 was analyzed by flow cytometry. The data represents the results of the independent experiments using three different healthy donor-derived Tconv cells. The plasmid construction for retroviral transduction of DNAM-1 was helped by Rei Hirochika (University of Tsukuba).

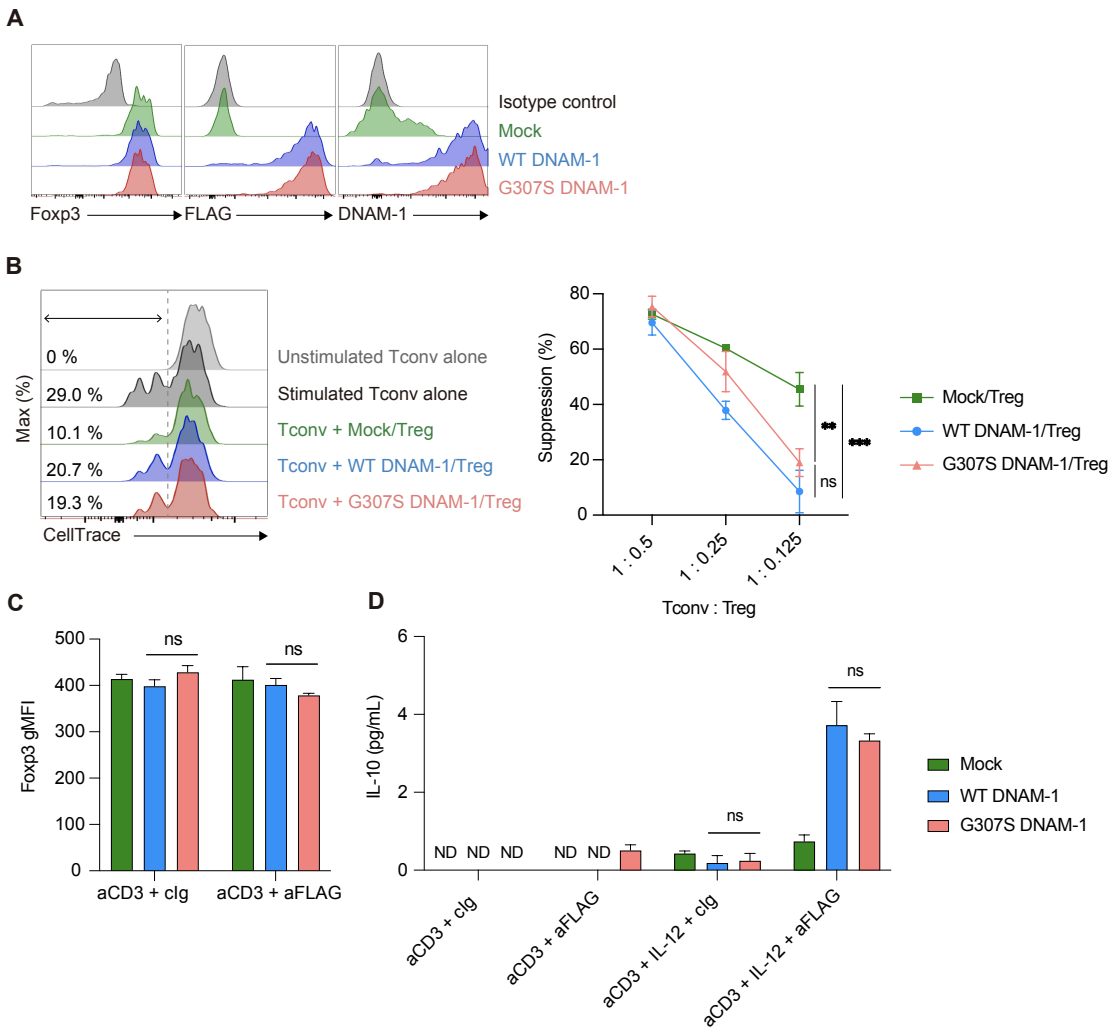

Figure 3. G307S DNAM-1 didn't modulate the suppressive function of human Treg cells. (A) The protein expression of Foxp3, FLAG, and DNAM-1 on retrovirally transduced human Treg cells were analyzed by flow cytometry. (B) Retrovirally transduced human Treg cells were co-cultured with autologous Tconv cells labeled with CellTrace for 96 hrs under the stimulation with anti-CD3 mAb and anti-FLAG mAb. (C and D) The retrovirally transduced human Treg cells after cross-linking with anti-CD3 mAb and either anti-FLAG mAb or control Ig for 120 hrs. The protein expression of Foxp3 was measured by flow cytometry. The culture supernatant was harvested and the concentration of IL-10 was measured by flow cytometry. The data represents the results of two independent experiments (B-D).

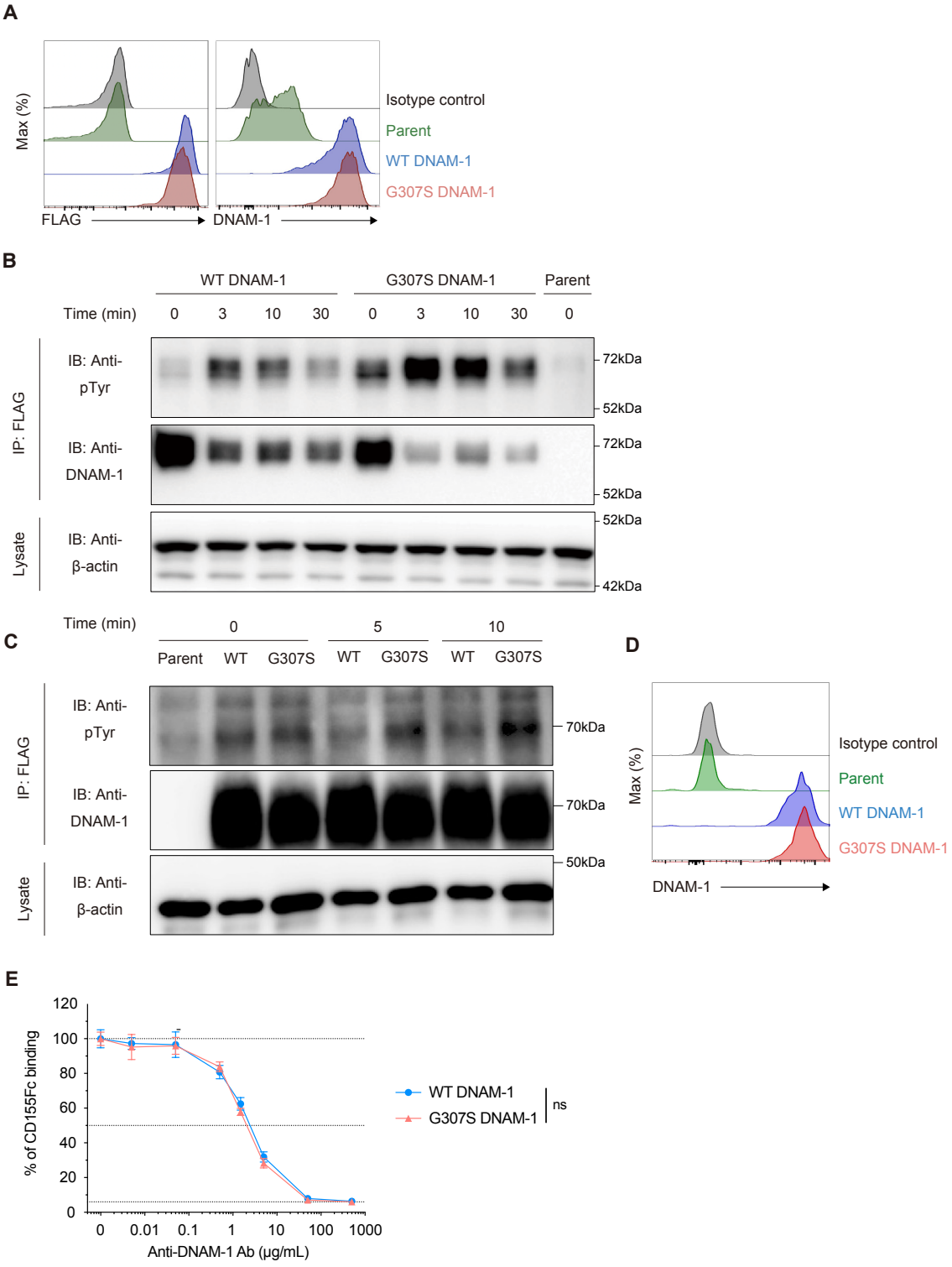

Figure 4. G307S mutation augmented pTyr of DNAM-1 but not ligand binding.

(A) The protein expression of FLAG and DNAM-1 on Jurkat cells was analyzed by flow cytometry. (B) Retrovirally transduced Jurkat cells expressing FLAG-WT DNAM-1 and FLAG-G307S DNAM-1 were crosslinked with anti-DNAM-1 mAb for indicated times to induce Tyrosine phosphorylation followed by the immuno-precipitation with anti-FLAG

mAb. The immunoprecipitates were immunoblotted for pTyr and DNAM-1. (C) Retrovirally transduced Jurkat cells expressing FLAG-WT DNAM-1 and FLAG-G307S DNAM-1 were crosslinked with human CD155 Fc-fusion protein for indicated times to induce Tyrosine phosphorylation followed by the immuno-precipitation with anti-FLAG mAb. The immunoprecipitates were immunoblotted for pTyr and DNAM-1. (D) The protein expression of human DNAM-1 on BW5147 cells was analyzed by flow cytometry. (E) WT DNAM-1 or G307S DNAM-1-expressing BW5147 cells were pre-treated with CD155-Fc fusion proteins (3 μg/2×10^4 cells). Then, the cells were incubated with various doses of anti-human DNAM-1 blocking mAb, and remained CD155 Fc-fusion protein was detected by flow cytometry. The binding of CD155-Fc fusion protein to anti-DNAM-1 mAb-untreated cell was considered 100% binding. The data represents the results of two or three independent experiments (A-E). The anti-human DNAM-1 mAb and human CD155 Fc-fusion proteins used for pTyr induction were generated and provided by Genki Okumura and Tomohei Matsuo (University of Tsukuba).

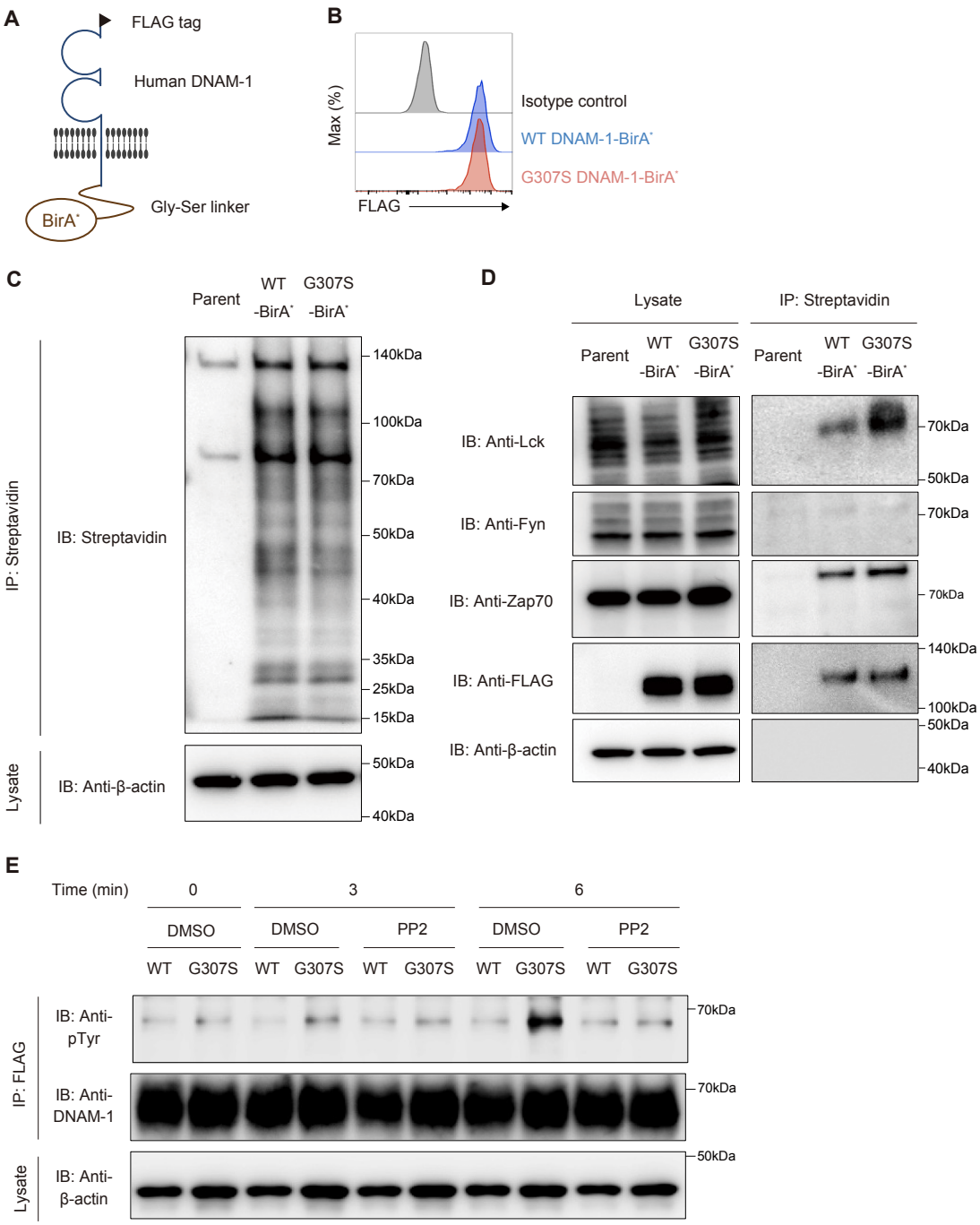

Figure 5. G307S mutation enhanced pTyr of DNAM-1 via the increased association with Lck.

(A) The structure of Jurkat transfectant expressing FLAG-DNAM-1-conjugated with BirA*. FLAG-tagged DNAM-1 was conjugated with BirA* through GS linker to improve the biotinylation efficiency and not to impair the physical interaction of cytosolic molecules to DNAM-1. (B) The protein expression of FLAG on Jurkat transfectants was analyzed by flow cytometry. (C) FLAG-WT or FLAG-G307S DNAM-1 conjugated with BirA*-expressing Jurkat transfectants were incubated with biotin. Whole-cell lysate and streptavidin

immunoprecipitates were immunoblotted by streptavidin-HRP. (D) FLAG-WT or FLAG-G307S DNAM-1 conjugated with BirA*-expressing Jurkat transfectants were cultured with biotin. Whole-cell lysate and streptavidin immunoprecipitates were immunoblotted for Lck, Fyn, FLAG, and β-actin. (E) FLAG-WT DNAM-1 or FLAG-G307S DNAM-1-expressing Jurkat transfectants were incubated with PP2 and crosslinked with anti-DNAM-1 mAb for indicated times, followed by the immuno-precipitation with anti-FLAG mAb. The immunoprecipitates were immunoblotted for pTyr and DNAM-1. The data represents the results of two or three independent experiments (B-E). The BirA* construct was provided by Atsushi Kawaguchi (University of Tsukuba), and the construction of FLAG-DNAM-1-BirA* conjugating protein and establishment of transfectants and the experimental setting was supported by Atsushi Kawaguchi and Takahiro Kuroki (University of Tsukuba).

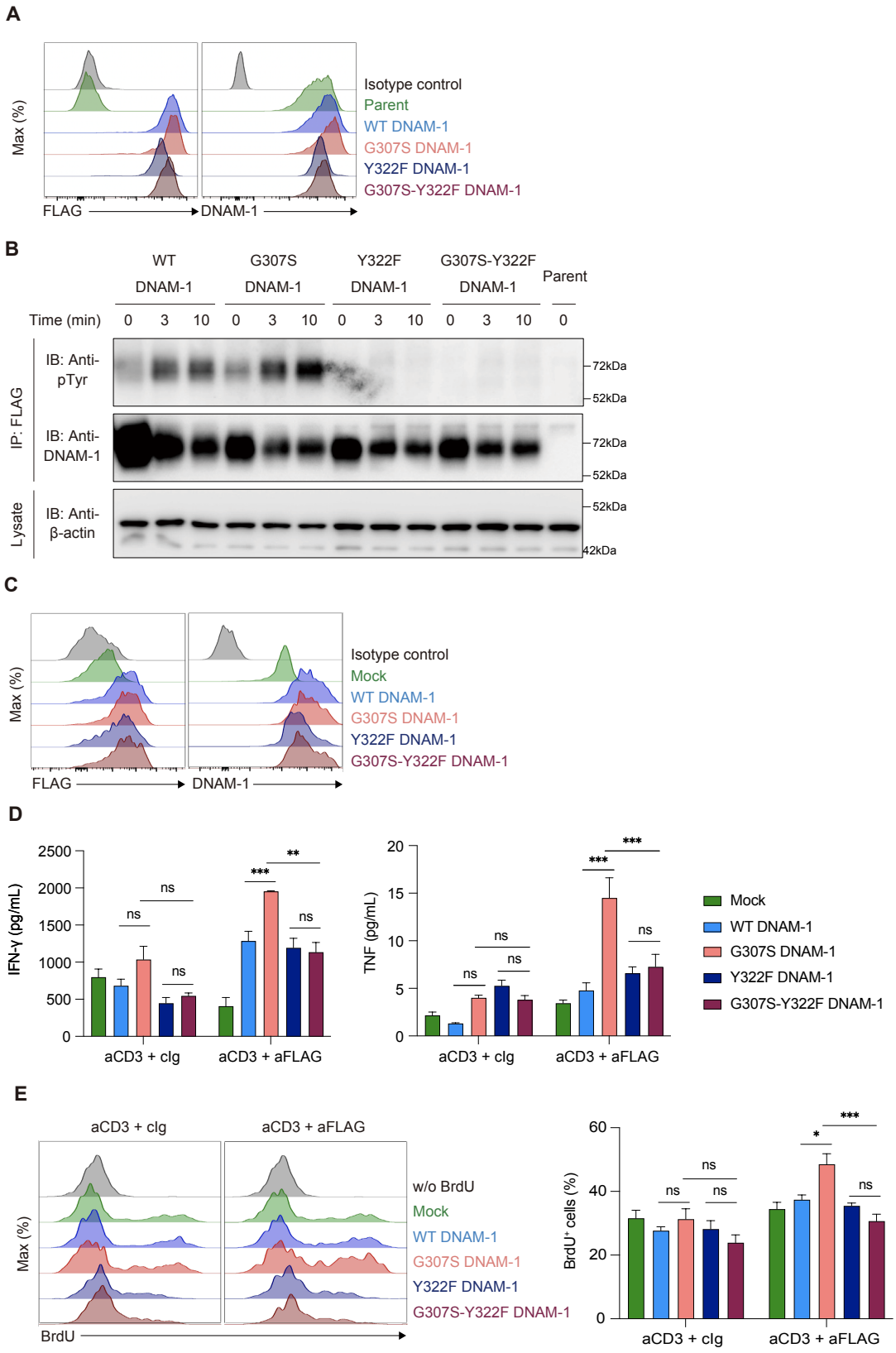

Figure 6. G307S DNAM-1 promoted the effector function of Tconv cells via the Tyr^{322}.

(A) The protein expression of FLAG-DNAM-1 on Jurkat cells was analyzed by flow cytometry. (B) FLAG-DNAM-1-expressing Jurkat transfectants were crosslinked with anti-

DNAM-1 mAb for indicated times to induce Tyrosine phosphorylation followed by the immuno-precipitation with anti-FLAG mAb. The immunoprecipitates were immunoblotted for pTyr and DNAM-1. (C) The protein expression of FLAG-DNAM-1 on human Tconv cells was analyzed by flow cytometry. (D) Human Tconv cells expressing FLAG-DNAM-1 were stimulated with anti-CD3 mAb and either anti-FLAG mAb or control Ig. The culture supernatant was harvested 12 hrs after stimulation and the concentration of IFN-γ and TNF-αwas analyzed by flow cytometry. (E) Human Tconv cells expressing FLAG-DNAM-1 were stimulated with anti-CD3 mAb and either anti-FLAG mAb or control Ig. The BrdU incorporation 24 hrs after stimulation was analyzed by flow cytometry. The data represents the results of the independent experiments (A-E) by using two or three different healthy donor-derived Tconv cells (C-E).

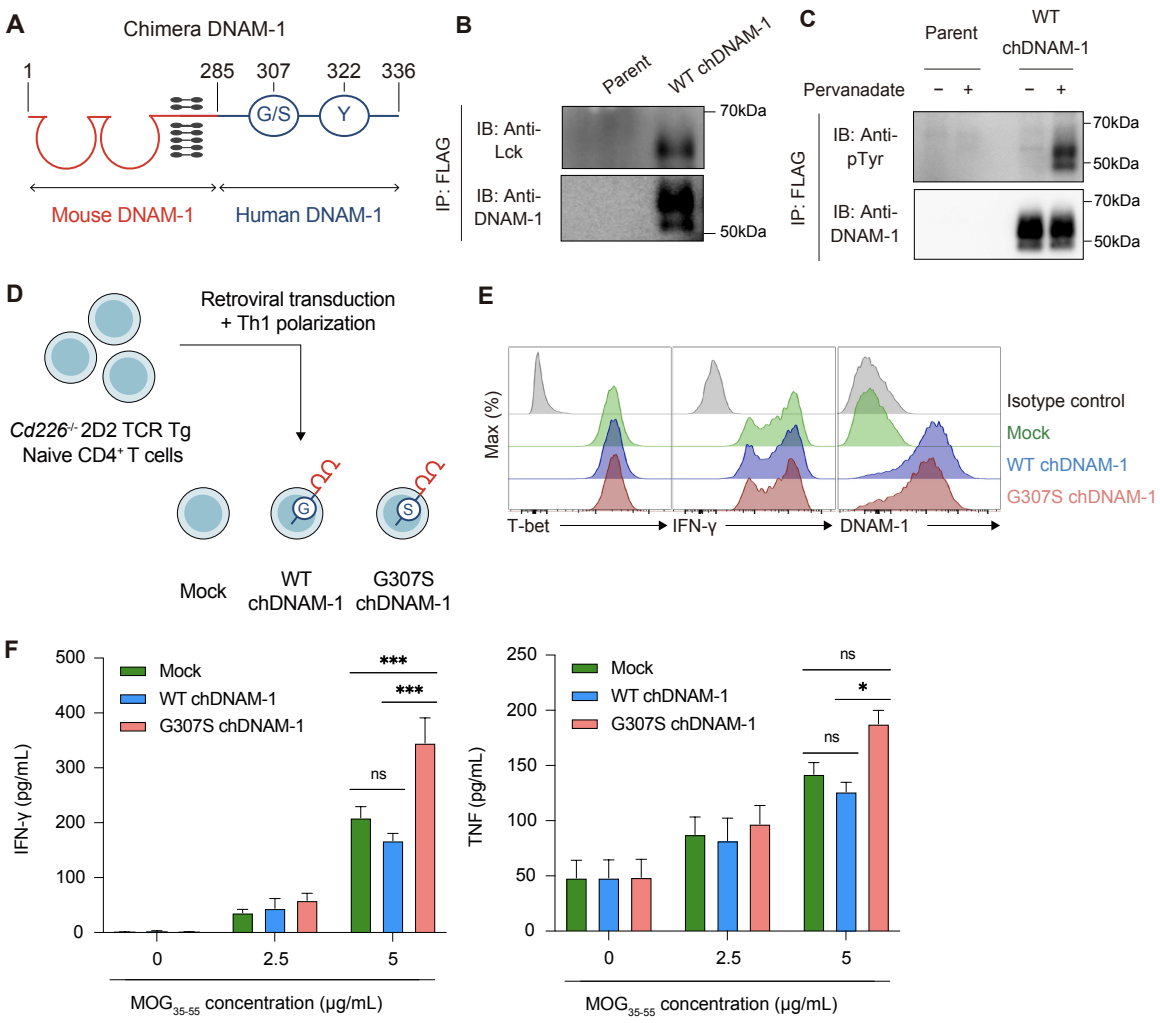

Figure 7. G307S chDNAM-1 promotes cytokine production of mouse CD4$^+$ T cells.

(A) The structure of chDNAM-1. A part of the intracellular region of mouse DNAM-1 was substituted with human Gly^{307} or Ser^{307} DNAM-1 (B) FLAG-WT chDNAM-1-expressing BW5147 transfectants were stimulated with 100 μM pervanadate. The stimulated cells were lysed with 1% digitonin and immunoprecipitated with anti-FLAG mAb, followed by immunoblotting with anti-Lck mAb or anti-DNAM-1. (C) FLAG-WT chDNAM-1-expressing BW5147 transfectants were stimulated or not with 100 μM pervanadate. The stimulated cells were lysed with 1% NP-40 and immunoprecipitated with anti-FLAG mAb, followed by immunoblotting with anti-phosphotyrosine mAb or anti-DNAM-1. (D) The method of generation for encephalitogenic mouse CD4$^+$ T cells- expressing chDNAM-1. CD4$^+$ T cells from *Cd226*-deficient myelin oligodendrocyte glycoprotein (MOG)-specific 2D2 TCR Tg mice were stimulated with anti-CD3 mAb, anti-CD28 mAb, IL-2, and IL-12, followed by the retroviral transduction of WT chDNAM-1 or G307S chDNAM-1. (E) The protein expression of T-bet, IFN-γ, and DNAM-1 on retrovirally transduced 2D2 mouse

$CD4^+$ Th1 cells was analyzed by flow cytometry. (F) Mock, WT chDNAM-1, or G307S chDNAM-1-expressing 2D2 $CD4^+$ Th1 cells were co-cultured with mouse $CD11c^+$ splenocytes in the presence of MOG_{35-55} peptides and LPS. The culture supernatant was harvested 48 hrs after stimulation and the concentration of IFN-γ and TNF-αwas analyzed by flow cytometry. The data were representative of two independent experiments (B and C) or pooled from two independent experiments (F). The plasmid construction for retroviral transduction of chimeric DNAM-1 was helped by Rei Hirochika (University of Tsukuba) and the western blot analysis was helped by Nanami Kamata (University of Tsukuba).

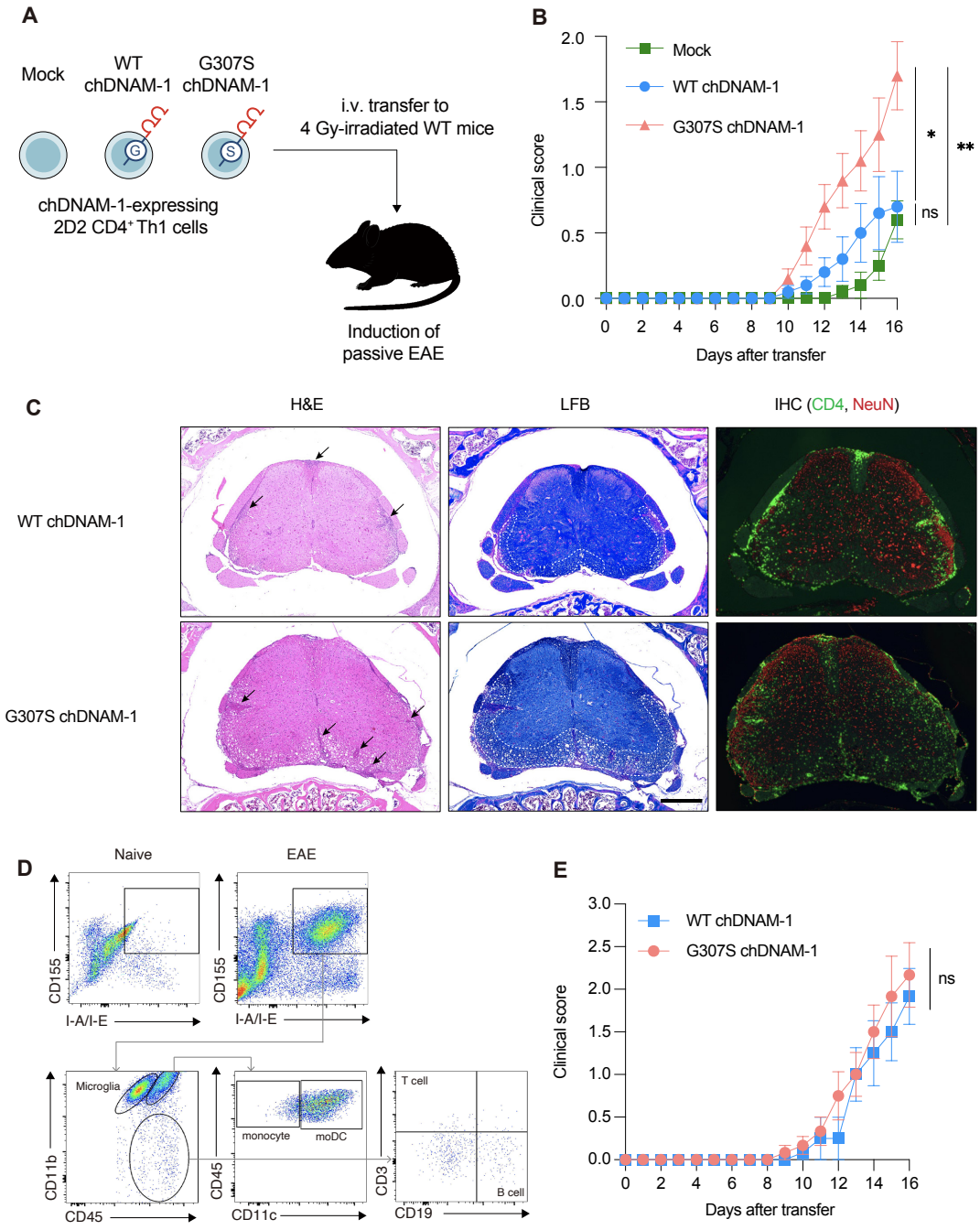

Figure 8. G307S chDNAM-1 promotes CD4⁺ T cell-mediated autoimmune inflammation. (A) The method of induction of passive EAE. Mock, WT, or G307S chDNAM-1-expressing 2D2 CD4⁺ Th1 cells were injected into WT mice. EAE clinical score was monitored daily. (B) Kinetics of EAE clinical score of WT mice that received Mock, WT chDNAM-1, or G307S chDNAM-1- expressing 2D2 CD4⁺ Th1 cells. (n = 10 for each group) (C) Spinal cord sections stained with H&E, LFB, and immune-histochemistry with anti-CD4 (Green) and anti-NeuN (Red) from mice received 2D2 CD4⁺ Th1 cells expressing WT or G307S chDNAM-1 at day 21 post-adoptive cell transfer. Scale bar: 400 μm. (D) Expression of

CD155 and I-A/I-E in the spinal cord of naïve and EAE-induced mice. $CD45^{Mid}CD11b^{+}$ microglia and $CD45^{Hi}CD11b^{+}CD11c^{+}$ monocyte-derived dendritic cells predominantly express CD155 and I-A/I-E after EAE induction. (E) Kinetics of EAE clinical score of *Cd155*$^{-/-}$ mice that received 2D2 $CD4^{+}$ Th1 cells expressing WT or G307S chDNAM-1. (n = 6 for each group) The data were representative of two or three independent experiments (C and D) or pooled from two independent experiments (B and E). The induction of passive EAE was helped by Shota Kinoshita, Kenshiro Matsuda, and Fumie Abe (University of Tsukuba), and the spinal cord isolation and histological analysis of passive EAE was helped by Kenshiro Matsuda (University of Tsukuba).

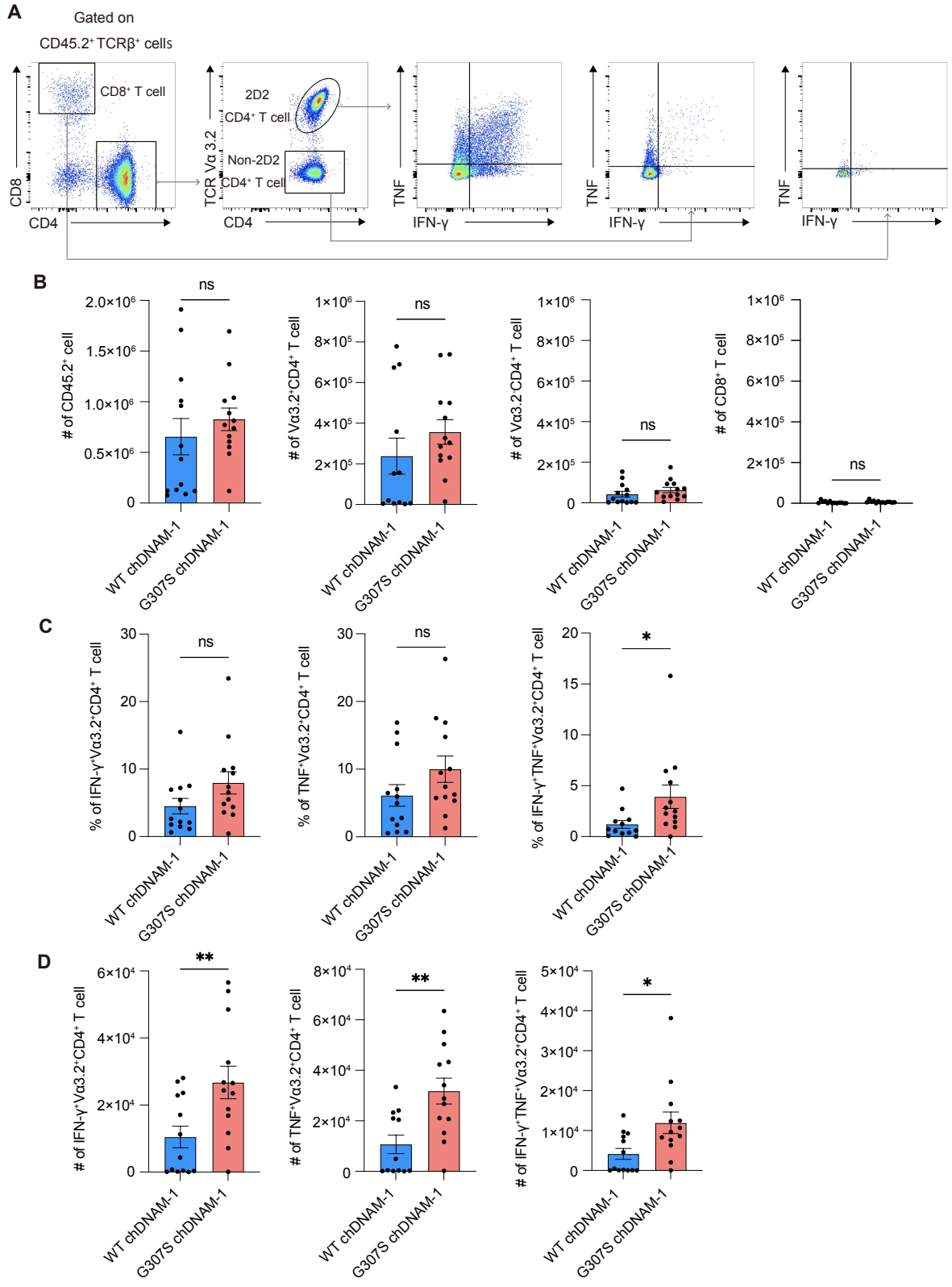

Figure 9. G307S chDNAM-1 promoted the pathogenicity of autoreactive CD4$^+$ T cells *in vivo*.

(**A**) 2D2 CD4$^+$ T cells, non-2D2 CD4$^+$ T cells, and CD8$^+$ T cells in the spinal cord at day 16 post-adoptive cell transfer were detected and their cytokine production was measured by flow

cytometry. (**B**) The absolute number of CD45.2$^+$ cells and each T cell population in the spinal cord at day 16 after disease induction. (n = 13 for each group) (**C and D**) The frequency and absolute number of IFN-γ$^+$ and TNF-α$^+$ 2D2 CD4$^+$ T cells in the spinal cord at day 16 after disease induction. (n = 13 for each group) Data are pooled from three independent experiments (**B-D**). The induction of passive EAE and spinal cord isolation for flow cytometric analysis was helped by Shota Kinoshita, Kenshiro Matsuda, and Fumie Abe (University of Tsukuba)

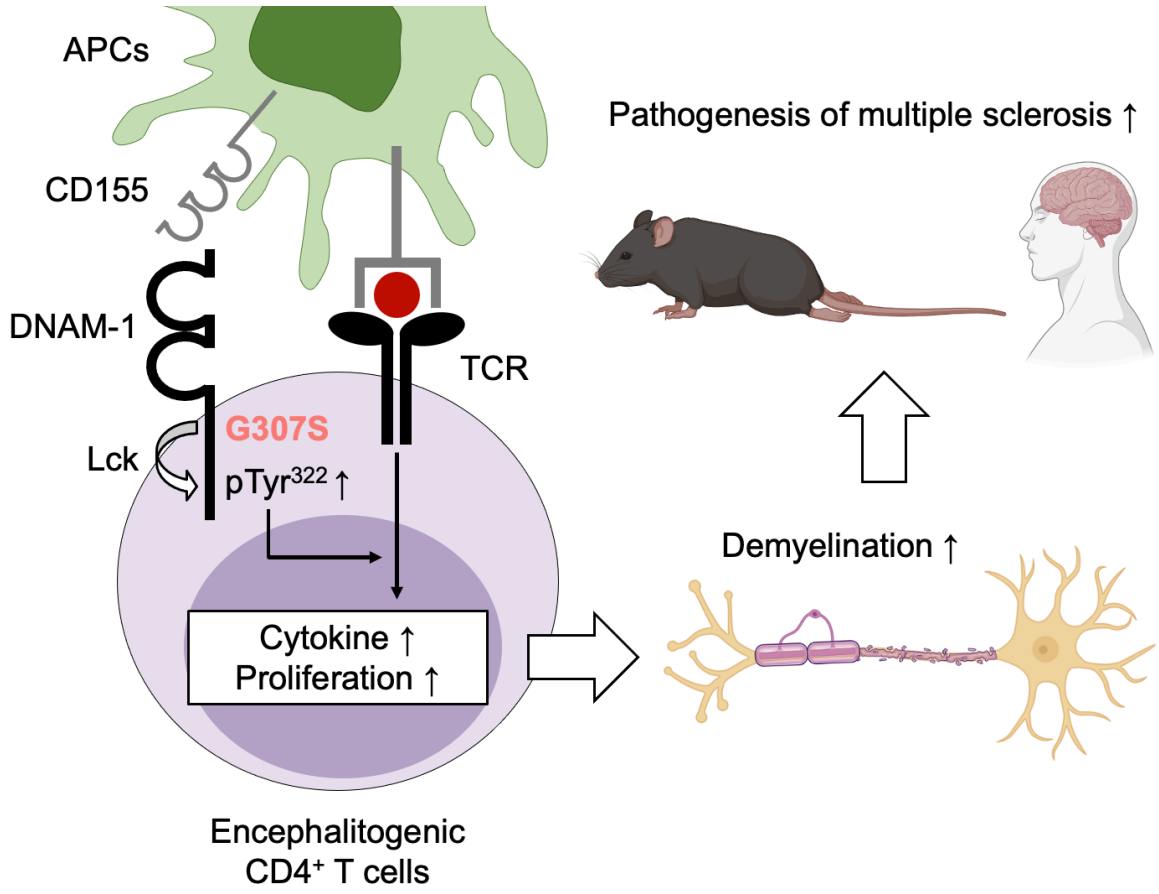

Figure 10. Model of G307S DNAM-1 in the pathogenesis of autoimmune encephalomyelitis

Rs763361 is a polymorphism associated with multiple autoimmune diseases. The SNP causes G307S non-synonymous mutation of DNAM-1. G307S mutation increases the physical association of an Src-family kinase Lck and promotes phosphorylation of Tyr^{322} of DNAM-1 intracellular region which is critical for DNAM-1-mediated activating signaling. The enhanced phosphorylation of Tyr^{322} augmented pro-inflammatory cytokine production and cell proliferation of $CD4^+$ Tconv cells as a co-stimulatory molecule upon the binding with CD155 expressed on antigen-presenting cells, while G307S DNAM-1 didn't reduce the suppression activity of Treg cells compared with WT DNAM-1. The increased effector functions of G307S DNAM-1-expressing encephalitogenic $CD4^+$ T cells promoted demyelination in the spinal cord and exacerbated the pathogenesis of autoimmune encephalomyelitis.