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The role of Cas-L/NEDD9 as a regulator of collagen-induced arthritis in a murine model

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ABSTRACT

Cas-L/NEDD9 is a cytoplasmic docking protein downstream of β1 integrin-mediated signaling pathway and is essential for cellular migration and β1 integrin-mediated costimulation of T cells. We previously found that increased number of Cas-L positive leukocytes migrated into the inflamed joints of HTLV-I tax transgenic mice which spontaneously develop polyarthritis, suggesting a role of Cas-L in rheumatoid arthritis (RA) pathophysiology. Our current study expanded these findings on the role of Cas-L/NEDD9 in the development of RA by analyzing the pathophysiological changes in a *Nedd9*$^{-/-}$ mouse collagen-induced arthritis (CIA) model. *Nedd9*$^{-/-}$ mice exhibited a decrease in arthritis severity as compared to *Nedd9*$^{+/+}$ mice. In addition, as being conducted bone marrow transplantation experiments with a CIA model, *Nedd9*$^{-/-}$→*Nedd9*$^{+/+}$ transplant showed a decrease in the incidence and severity score of arthritis, compared to those of *Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$ transplant. For analysis of serum levels of various cytokines, IL-1β, IL-6, IL-17, TNF-α, IFN-γ and anti-collagen antibody were decreased, while IL-4 and IL-10 levels were increased, in *Nedd9*$^{-/-}$ mice as compared to those in *Nedd9*$^{+/+}$ mice. Furthermore, collagen-mediated cellular responses of lymphocytes isolated from spleen or affected lymph nodes of *Nedd9*$^{-/-}$ mice were reduced. Our results strongly suggest that Cas-L/NEDD9 plays a pivotal role in the pathophysiology of CIA, and that Cas-L/NEDD9 may be a potential molecular target for the treatment of RA.

Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; CII, chicken type II collagen; Cas-L, Crk-associated substrate lymphocyte type; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; FCS, fetal calf serum; FN, fibronectin; HTLV-I, human T lymphotropic virus type I; IHC, immunohistochemistry; mAb, monoclonal antibody; LN, lymph node; NEDD9, neural precursor cell–expressed, developmentally downregulated 9; PMA, phorbol 12-myristate 13-acetate; RA, rheumatoid arthritis; TBI, total body irradiation; TCR, T cell receptor.

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1. Introduction

β1 integrins exhibit a variety of biological functions through specific interaction with their ligands including fibronectin (FN) [1]. We initially identified pp105 as the major phosphotyrosine-containing protein in H9 T-cell line stimulated with β1-integrins [2,3]. Sequence analysis of isolated cDNA clone revealed homology with p130 Crk-associated substrate (Cas)/breast cancer anti-estrogen resistance 1 (*BCAR1*) which was identified as a tyrosine-phosphorylated protein in v-Crk and v-Src–transformed fibroblasts [4], thus designating pp105 as Crk-associated substrate lymphocyte type (Cas-L). Cas-L is identical to neural precursor cell–expressed, developmentally down-regulated 9 (NEDD9) [5] and human enhancer of filamentation 1 (HEF1) [6]. Cas-L/NEDD9/HEF1 is an integrin signaling adaptor or docking protein that consists of multiple preserved domains common to Cas family members [7]. Cas-L/NEDD9 is phosphorylated at its tyrosine residues by integrins and other stimuli. Ligation of T- and B-cell antigen receptors caused tyrosine phosphorylation of Cas-L/NEDD9, resulting in the association of Crk, Crk-L, and C3G [8]. Integrin- or integrin/T cell receptor (TCR)-elicited tyrosine phosphorylation of Cas-L/NEDD9 was mediated by focal adhesion kinase (FAK) and Src family tyrosine kinases [9]. Ectopic expression of Cas-L/NEDD9 conferred T cells with enhanced motility on the co-engagement of TCR/CD3 complex and β1-integrins [10,11], suggesting a pivotal role of tyrosine-phosphorylated Cas-L/NEDD9 in TCR- and integrin-mediated cell motility.

We previously investigated the *in vivo* role of Cas-L/NEDD9 in the pathophysiology of rheumatoid arthritis (RA) in a mouse model of RA (using human T lymphotropic virus type I (HTLV-I) *tax* transgenic mice) as well as in RA patients [12]. HTLV-I transgenic mice that carry the env-pX gene developed high incidence of inflammatory arthropathy [13]. Our previous study demonstrated that Cas-L/NEDD9 involvement in the pathogenesis of RA-like disease in *tax* transgenic mice was mediated by its overexpression and hyperphosphorylation, resulting in markedly enhanced lymphocyte motility [12]. Moreover, we described the infiltration of Cas-L/NEDD9–positive, CD3-positive T cells into the affected joint lesion of RA patients [12]. These findings strongly suggest that Cas-L/NEDD9 plays a role in the pathophysiology of RA. However, it is unclear whether Cas-L/NEDD9 has a role in arthritis development in the absence of Tax since Cas-L/NEDD9 associates with Tax in HTLV-I-transformed cells [11]. To further characterize the role of Cas-L/NEDD9 in the pathogenesis of RA, our current work utilized a collagen-induced arthritis (CIA) model in *Nedd9*$^{-/-}$ mice to analyze the relevant clinical signs/symptoms, histopathology, serum levels of anti-collagen antibody and various cytokines, and lymphocytes characteristics.

2. Materials and methods

2.1. Mice

Nedd9$^{-/-}$ mice derived of C57BL/6J (B6) were generously provided by Dr. Seo from the Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo (Tokyo, Japan) [14]. Mice were used at 8–12 weeks. Animal experiments were conducted following protocols approved by the Animal Care and Use Committees at the Institute of Medical Science, University of Tokyo. All mice were housed in a specific pathogen-free facility in micro-isolator cages. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Induction of CIA and bone marrow transplantation (BMT)

CIA was induced as previously described with minor modifications [15]. Briefly, mice were injected intradermally at the base of tail with 200 μg of chicken type II collagen (CII) (Sigma, USA) in 10 mM acetic acid, emulsified in complete Freund's adjuvant (CFA) (Difco Laboratories, USA). Twenty-one days after the first immunization, the mice were boosted by the same methods. Day of the first immunization was designated as day 0 for CIA induction. Arthritis clinical scores in CIA mice were evaluated as described in previously published literature [16]. For adoptive transfer experiments, after 6hrs of total body irradiation (TBI) at 950 cGy, *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice were injected intravenously with 1×10^6 CD34$^+$ bone marrow (BM) cells isolated from *Nedd9*$^{-/-}$ (*Nedd9*$^{-/-}$→*Nedd9*$^{+/+}$) or *Nedd9*$^{+/+}$ (*Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$), respectively. After 28 days and 49 days of transplantation, mice were immunized and evaluated by the same methods as described above.

2.3. Histopathology and immunohistochemistry (IHC)

Specimens of paw joints for histopathology and IHC were prepared by the same method as described in our previous report [12]. Antibodies used in IHC assays were anti-CD3 rabbit monoclonal antibody (mAb) (clone SP7) from Nichirei Bioscience (Japan), anti-mouse CD45R, B220 mAb (clone RA3-6B2) from BD Pharmingen (USA), and anti-Ly-6G/GR1 rat mAb (clone RB6-8C5) from Acris Antibodies (Germany).

2.4. Measurement of cytokines and anti-CII antibody

The collected sera were analyzed for various cytokines using Procarta Cytokine Assay (VERITAS, Tokyo, Japan) with Bio-Plex Suspension Array System (Bio-Rad Laboratories, USA). The serum antibody level to CII was evaluated using Mouse Anti-Chick Type II Collagen IgG Antibody Assay Kit (Chondrex, USA). All samples were run in triplicates.

2.5. Flow cytometry

The following antibodies were from BD Biosciences: Fluorescein isothiocyanate conjugated anti-mouse CD4 rat mAb (clone H129.19), phycoerythrin conjugated anti-mouse CD8α rat mAb (clone 53-6.7), peridinin-chlorophyll-a Protein-Cy5.5 conjugated anti-mouse CD19 rat mAb (clone 1D3), and allophycocyanin conjugated anti-mouse TCRβ hamster mAb (clone H57-597). Analysis was performed on 2-laser FACSCalibur (BD Biosciences) and files were analyzed in FlowJo software (Tree Star, USA).

2.6. In vitro costimulation assay

For assay of proliferative responses to FN, CD4$^+$T cells were purified from splenocytes isolated from *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice using mouse CD4$^+$T cell Isolation Kit II (Miltenyi Biotec, Germany). 1×10^5 cells per well were seeded in 96-well flat bottom plate with immobilized 0.5 μg/ml of anti-mouse CD3ε hamster mAb (clone 145-2C11, BD Pharmingen) and/or 1.0 μg/ml of FN (BIOPUR, Switzerland). The cells were cultured for 72hrs, and pulsed with [^{3}H]-thymidine (1μCi/well; Perkin–Elmer, USA) for the last 6hrs. The incorporated radioactivity was measured using a microplate beta counter (Micro Beta Plus; Wallac, USA). For assay of proliferative responses to CII, mononuclear cells were isolated from brachial, axillary, inguinal and popliteal lymph nodes (LNs) of *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice after 6 days of CIA immunization. Single suspension cells were prepared from LN lymphocytes using Spleen

Dissociation Kit, mouse (Miltenyi Biotec). 2×10^5 cells per well were cultured in the presence of 100 or 200 μg/ml of CII. Cell proliferation was evaluated by the same [^{3}H]-thymidine uptake assay as described above.

2.7. Statistical analysis

All experiments were performed in triplicates and repeated at least 3 times. Data were analyzed by two-tailed Student's *t* test for two group comparison or by ANOVA test for multiple comparison testing followed by the Tukey–Kramer *post-hoc* test. *P* values ≤ 0.05 were considered statistically significant. The calculations were conducted using SPSS Statistics software (IBM, USA).

3. Results and discussion

3.1. Reduced severity of CIA in Nedd9$^{-/-}$ mice

We first compared clinical signs/symptoms of arthritis between *Nedd9*$^{+/+}$ and *Nedd9*$^{-/-}$ mice following induction of CIA. The incidence of onset of arthritis in *Nedd9*$^{-/-}$ CIA mice was significantly decreased compared to that in *Nedd9*$^{+/+}$ CIA mice (Fig. 1A). Moreover, *Nedd9*$^{-/-}$ CIA mice exhibited decreased arthritis score compared to *Nedd9*$^{+/+}$ CIA mice (Fig. 1B). In addition, the severity and incidence of arthritis in *Nedd9* hetero knockout *Nedd9*$^{+/-}$ CIA mice were in-between those of *Nedd9*$^{+/+}$ and *Nedd9*$^{-/-}$ CIA mice (Fig. 1A and B). These data suggest that expression levels of Cas-L/NEDD9 affect induction of CIA.

To further characterize the clinical picture of the arthritis induced in this mouse model, we analyzed affected joints using planar X-ray examination. In *Nedd9*$^{-/-}$ CIA mice, metatarsal and tarsal bones at day 56 following the first immunization showed no significant changes as compared to findings seen in non-immunized mice (cricles in panels b and c of Fig. 1C), while metatarsal and tarsal joints of *Nedd9*$^{+/+}$ CIA mice exhibited narrowing, deformation and synostosis (circle in panel a of Fig. 1C). For additional analysis of the extent of arthritis, we conducted histopathologic examination using specimens of affected joints obtained at day 56 following the first immunization. As shown in Fig. 1D, *Nedd9*$^{+/+}$ CIA mice showed obvious thinning of articular cartilage and hyperplasia of synovium with infiltration of inflammatory cells (panels a and b), in contrast to the normal structure found in non-immunized mice (panel c). Meanwhile, the affected joint in *Nedd9*$^{-/-}$ CIA mice exhibited few signs of inflammation with slight thinning of articular cartilage and synovitis (panel d of Fig. 1D). To characterize the subsets of inflammatory cells found in the affected joints, we performed IHC using specimens of affected joints obtained at day 56 following the first immunization. In the affected joint of *Nedd9*$^{+/+}$ CIA mice, Ly-6G-positive granulocytes were predominantly found (panel a of Fig. 1E), while CD3-poistive T and B220-positive B cells were at undetectable levels (panels b and c of Fig. 1E). On the other hand, in *Nedd9*$^{-/-}$ CIA mice, inflammatory cells were not detected by IHC in the affected joint (panels d–f of Fig. 1E). Taken together, these results suggest that Cas-L/NEDD9 has an important role in the development of inflammatory arthral changes in CIA.

To define the role of Cas-L/NEDD9 in leukocytes in the development of CIA, we next conducted adoptive transfer experiments. For this purpose, BM cells were harvested from *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice, and transplanted into the opposite recipient mice after TBI. Recipients *Nedd9*$^{+/+}$ mice of *Nedd9*$^{-/-}$ BM cells (*Nedd9*$^{-/-}$→*Nedd9*$^{+/+}$) showed a decrease in the incidence and severity score of arthritis, compared to those seen in recipients *Nedd9*$^{-/-}$ mice of *Nedd9*$^{+/+}$ BM cells (*Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$) (Fig. 1F and G). Meanwhile, CIA in *Nedd9*$^{+/+}$→*Nedd9*$^{+/+}$ BMT recipients was induced at a similar level to that seen in *Nedd9*$^{+/+}$ CIA mice as shown in Fig. 1 (data not shown), while CIA was induced in *Nedd9*$^{-/-}$→*Nedd9*$^{-/-}$ BMT recipients at lower levels than *Nedd9*$^{+/+}$→*Nedd9*$^{+/+}$ BMT recipients, which were similar to that seen in *Nedd9*$^{-/-}$ CIA mice as shown in Fig. 1 (data not shown). These data strongly suggest that the presence of Cas-L/NEDD9 in leukocytes, but not in joint organs, is associated with the development of CIA, and that the development of CIA is not affected by BMT with TBI.

3.2. Decreased level of splenic lymphocytes in Nedd9$^{-/-}$ CIA mice

Since Cas-L/NEDD9 is involved in lymphocyte function [14,17], we next analyzed the lymphocytes in spleen and LN of *Nedd9*$^{-/-}$ CIA mice. For this purpose, spleen and LN cells were isolated from *Nedd9*$^{+/+}$ and *Nedd9*$^{-/-}$ mice before and after immunization, and we evaluated the cell numbers of each lymphocyte subset of spleen and LN cells. In spleen cells of *Nedd9*$^{-/-}$ mice, decreased levels of CD4$^+$T, CD8$^+$T and CD19$^+$B cells were observed after immunization (Table 1). On the other hand, in spleen cells of *Nedd9*$^{+/+}$ mice, increased level of CD19$^+$B cells was detected, while the levels of CD4$^+$T and CD8$^+$T cells were almost similar before and after immunization (Table 1). Moreover, among the LN lymphocytes of *Nedd9*$^{-/-}$ mice, increased level of CD19$^+$B cells was observed after immunization, while the levels of CD4$^+$T and CD8$^+$T cells were almost similar before and after immunization (Table 1). Among the LN lymphocytes of *Nedd9*$^{+/+}$ mice, level of CD19$^+$B cells was almost similar before and after immunization, and the levels of CD4$^+$T and CD8$^+$T cells were reduced after immunization (Table 1). Collectively, these data suggest that both T and B cells are involved in CIA of *Nedd9*$^{-/-}$ mice.

3.3. Decreased T and B cell functions in Nedd9$^{-/-}$ CIA mice

Given our previous findings that Cas-L/NEDD9 is involved in costimulatory signaling to CD3/TCR pathway associated with engagement of β1 integrins in T cells [2,9,18,19], we next examined T cell responses in *Nedd9*$^{-/-}$ or *Nedd9*$^{+/+}$ CIA mice. For this purpose, *in vitro* costimulation experiments using CD4$^+$T cells purified from spleen were conducted. As shown in Fig. 2A, CD4$^+$T cells from spleen of immunized or non-immunized *Nedd9*$^{-/-}$ mice exhibited decreased cellular proliferation following TCR plus FN (a ligand for β1 integrin) costimulation, compared to those from *Nedd9*$^{+/+}$ mice. Meanwhile, proliferative responses to PMA plus ionomycin were at similar levels of full activation for CD4$^+$T cells from both *Nedd9*$^{+/+}$ and *Nedd9*$^{-/-}$ CIA mice, with or without immunization (Fig. 2A). These data suggest that Cas-L/NEDD9 is involved in T cell activation through TCR and engagement of β1 integrin. To further characterize the role of Cas-L/NEDD9 in cell proliferation, we examined proliferative response of lymphocytes to CII. As shown in Fig. 2B, LN mononuclear cells isolated from *Nedd9*$^{-/-}$ CIA mice exhibited decreased CII-mediated cell proliferation, compared to those from *Nedd9*$^{+/+}$ CIA mice. Meanwhile, proliferative responses to PMA plus ionomycin were at similar levels of full activation in both *Nedd9*$^{+/+}$ and *Nedd9*$^{-/-}$ CIA mice (Fig. 2B).

Migration and adhesion of B cells in *Nedd9*$^{-/-}$ mice have been shown previously to be impaired [14]. To further define the role of Cas-L/NEDD9 on the humoral response in CIA, we analyzed the immunologic response to CII in *Nedd9*$^{-/-}$ CIA mice. For this purpose, serum levels of anti-CII antibody were assayed. As shown in Fig. 2C, serum levels of anti-CII antibody in *Nedd9*$^{-/-}$ CIA mice were significantly decreased as compared to those in *Nedd9*$^{+/+}$ CIA mice, data which are consistent with the findings regarding the incidence and severity of clinical signs/symptoms of arthritis. Taken together, our data strongly suggest that Cas-L/NEDD9 in T cells as well as in B cells plays an important role in the development of arthritis in a CIA model.

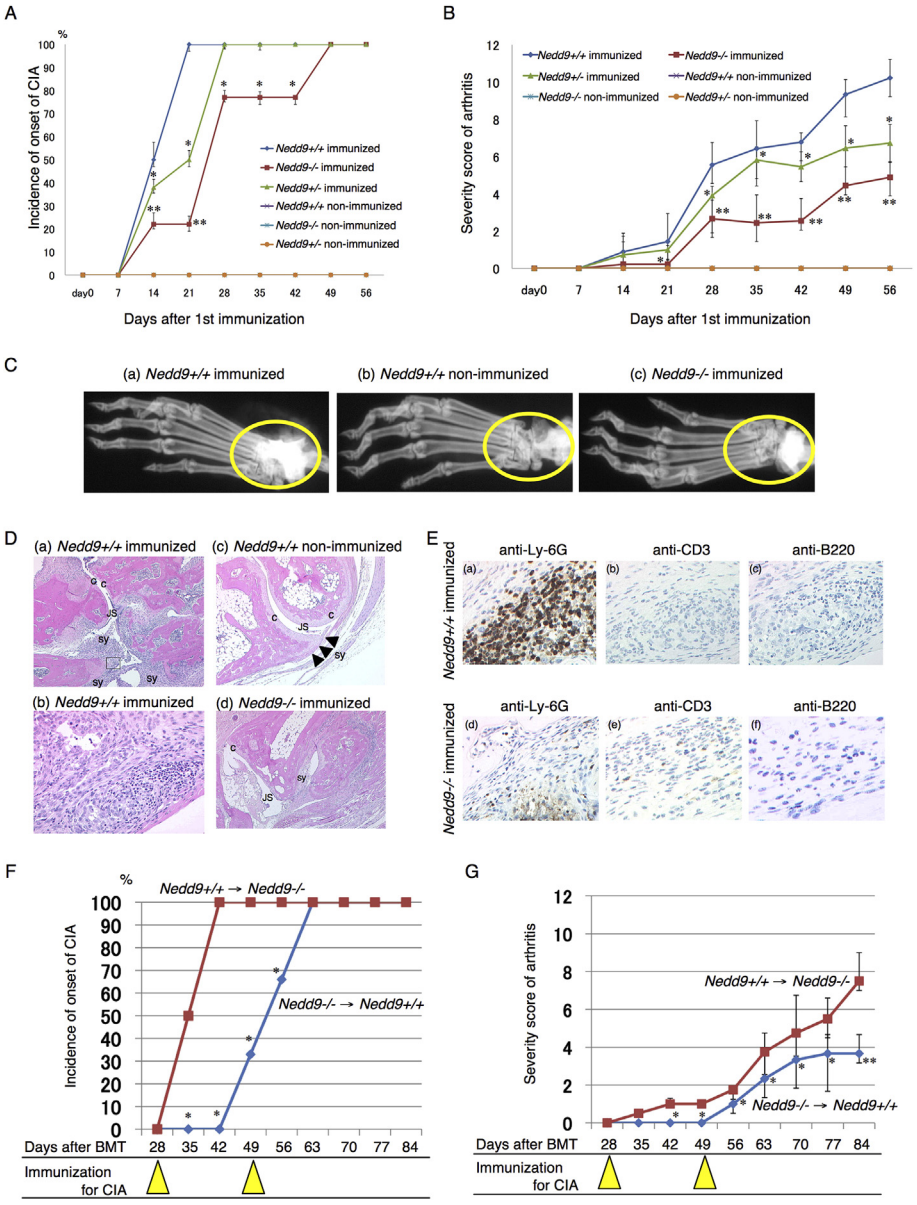

Fig. 1. Reduced incidence and severity of CIA in *Nedd9*$^{-/-}$ mice. A. The incidence of mice developing CIA was calculated as % ratio of mice with severity score ≥1 to total in each cohort (each, n = 9). Incidence of CIA decreased in *Nedd9*$^{-/-}$ cohort, compared to that in *Nedd9*$^{+/+}$ mice. Incidence of CIA in immunized *Nedd9*$^{+/-}$ subset (green line) was in-between that found in *Nedd9*$^{+/+}$ (blue line) and *Nedd9*$^{-/-}$ (red line) cohorts. None of the non-immunized *Nedd9*$^{+/+}$ (purple line), *Nedd9*$^{+/-}$ (orange line) or *Nedd9*$^{-/-}$ (light blue line) mice developed CIA. * indicates $P < 0.05$ *vs.* immunized *Nedd9*$^{+/+}$ CIA cohort, and ** indicates $P < 0.01$ *vs.* immunized *Nedd9*$^{+/+}$ or *Nedd9*$^{+/-}$ CIA cohort. B. The arthritis severity score was evaluated as described in Materials and methods. Severity of CIA decreased in *Nedd9*$^{-/-}$ cohort, compared to that in *Nedd9*$^{+/+}$ mice. Level of arthritis severity score of immunized *Nedd9*$^{+/-}$ CIA cohort (green line) was in-between that of immunized *Nedd9*$^{+/+}$ (blue line) and *Nedd9*$^{-/-}$ (red line) CIA cohorts. None of the non-immunized *Nedd9*$^{+/+}$ (purple line), *Nedd9*$^{+/-}$ (orange line) or *Nedd9*$^{-/-}$ (light blue line) mice developed CIA. *indicates $P < 0.05$ *vs.* immunized *Nedd9*$^{+/+}$ CIA cohort, and ** indicates $P < 0.01$ *vs.* immunized *Nedd9*$^{+/+}$ or *Nedd9*$^{+/-}$ CIA cohort. Data were shown as mean ± standard deviation. C. Representative planar X-ray images of the upper limbs of *Nedd9*$^{+/+}$ mice with or without CIA immunization (panels a or b, respectively), and immunized *Nedd9*$^{-/-}$ CIA mice (panel c) on day 56 after the first immunization. D. H&E staining of the upper limbs of *Nedd9*$^{+/+}$ mice with or without CIA immunization (panels a,b or c, respectively), and immunized *Nedd9*$^{-/-}$ CIA mice (panel d). The specimens were resected on day 56 after the first immunization. Representative histology is shown from 3 independent experiments (each, n = 6). C, JS or Sy denotes articular cartilage, joint space, or synovium, respectively. Panel b is focused in the inset of panel a. Original magnification × 20 for panels a,c,d, and × 100 for panel b. E. Representative immunohistochemical images of the upper limbs of immunized *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ CIA mice. Representative histology is shown from 3 independent experiments (each, n = 6). Original magnification × 100. F. After total body irradiation, *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice received BM cells of *Nedd9*$^{-/-}$ (*Nedd9*$^{-/-}$→*Nedd9*$^{+/+}$) or *Nedd9*$^{+/+}$ (*Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$) mice, respectively (each, n = 6). Recipient mice were immunized for CIA after 28 and 49 days of BM transplantation (indicated in yellow triangles). The incidence of mice developing CIA was calculated as % ratio of mice with severity score ≥1 to total in each cohort. Incidence of CIA decreased in *Nedd9*$^{-/-}$→*Nedd9*$^{+/+}$ cohort (blue line), compared to that seen in the *Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$ group (red line). * indicates $P < 0.01$ *vs.* *Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$ cohort. G. Mice were treated by the same method as described in F. The arthritis severity score was evaluated as described in Materials and methods. Severity of CIA decreased in *Nedd9*$^{-/-}$→*Nedd9*$^{+/+}$ cohort (blue line), compared to that seen in the *Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$ group (red line). * or ** indicates $P < 0.05$ or $P < 0.001$ *vs.* *Nedd9*$^{+/+}$→*Nedd9*$^{-/-}$ cohort, respectively. Data were shown as mean ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Number of $CD19^+$ B, $CD4^+$ and $CD8^+$ T cells in spleen or lymph nodes of *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice before and after CIA immunization.

(Organ)	(Subset)	*Nedd9*+/+		*Nedd9*−/−	
		Non-immunized	Immunized	Non-immunized	Immunized
Spleen ($\times 10^7$/ml)	$CD19^+$B cell	2.60 ± 0.9	3.40 ± 1.4*	2.20 ± 0.7	1.20 ± 0.4**
	$CD4^+$T cell	0.90 ± 0.3	1.00 ± 0.4	1.00 ± 0.3	0.50 ± 0.1**
	$CD8^+$T cell	1.20 ± 0.4	1.40 ± 0.6	0.60 ± 0.2	0.35 ± 0.1**
Lymph node ($\times 10^7$/ml)	$CD19^+$B cell	0.17 ± 0.1	0.11 ± 0.0	0.10 ± 0.0	0.40 ± 0.1**
	$CD4^+$T cell	0.35 ± 0.2	0.15 ± 0.0**	0.21 ± 0.1	0.20 ± 0.0
	$CD8^+$T cell	0.34 ± 0.2	0.17 ± 0.0*	0.18 ± 0.1	0.19 ± 0.0

CIA was induced in *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice as described in Materials and methods. Spleen and regional lymph nodes of the affected joints were resected on day 56 following the first immunization. Cells were subjected to flow cytometric analysis as shown in Supplemental Figure. Data were shown as mean of absolute cell number ± standard errors. of cumulative results from 3 independent experiments (each, n = 9). * or ** indicates $P < 0.05$ or $P < 0.01$ *vs.* those in each non-immunized cohort, respectively.

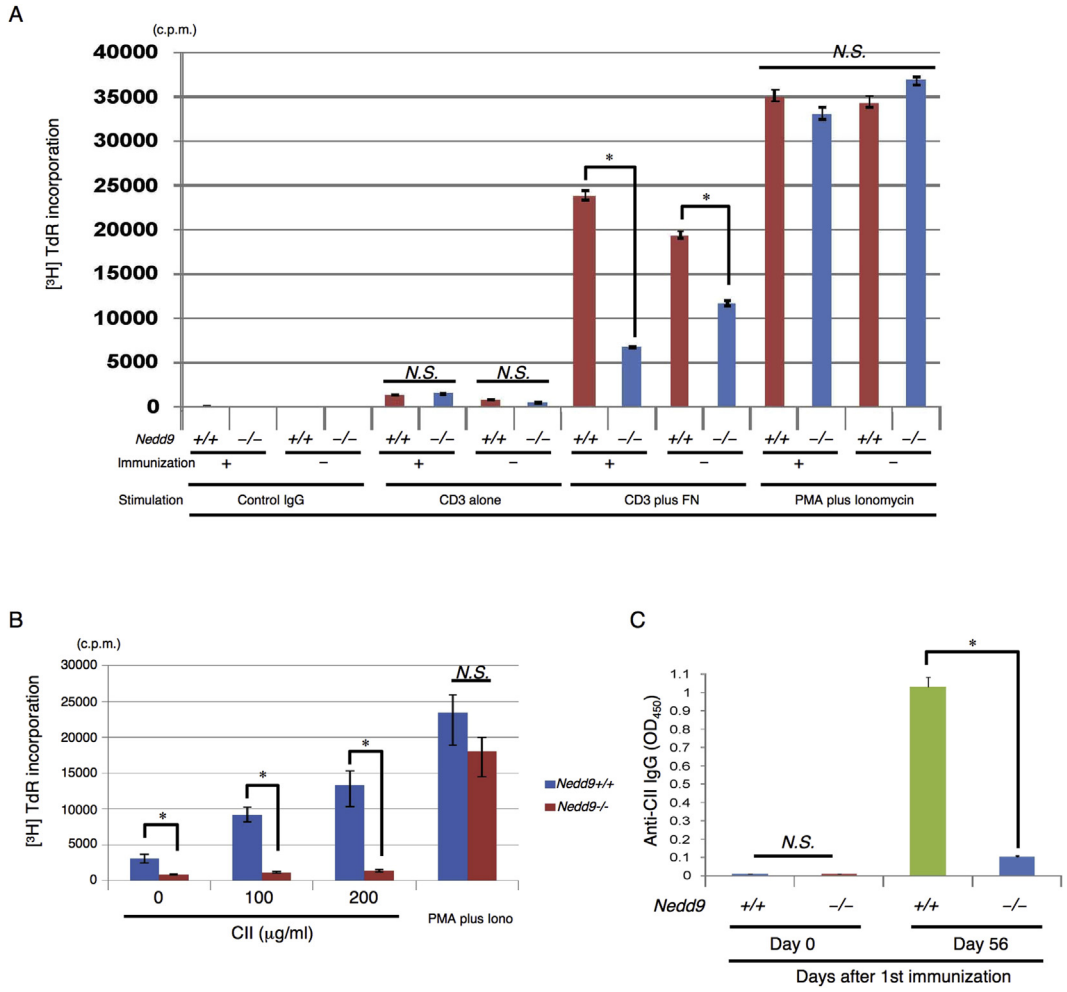

Fig. 2. Decreased proliferation of $CD4^+$T cells and anti-collagen antibody levels in *Nedd9*$^{-/-}$ CIA mice. A. $CD4^+$T cells were purified from spleen cells of *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice before and after immunization. Cells were stimulated with plate-bound anti-CD3 monoclonal antibody (mAb) and/or fibronectin (FN) at indicated concentrations, and assayed for cell proliferation by [^{3}H]-thymidine (TdR) incorporation. Data were shown as mean±standard errors (each, n = 6). * indicates $P < 0.001$, and *N.S.*, not significant. B. Lymph node (LN) mononuclear cells were isolated from the regional LNs of *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice after immunization. Cells were stimulated with type II collagen (CII) at indicated concentrations, and assayed for cell proliferation by [^{3}H]-thymidine (TdR) incorporation. Cell proliferation by CII stimulation was significantly enhanced in *Nedd9*$^{+/+}$ mice (*). Data were shown as mean±standard errors (each, n = 6). * indicates $P < 0.001$, and *N.S.*, not significant. C. Serum levels of anti-CII IgG antibody in *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice on day 0 and day 56 after immunization were measured using ELISA. Data were shown as mean±standard errors with triplicates (each, n = 9). * indicates $P < 0.001$.

3.4. Decrease of proinflammatory cytokines and increase of anti-inflammatory cytokines in Nedd9$^{-/-}$ CIA mice

We previously reported that Cas-L/NEDD9 is involved in TGF-β-related cell regulation [20]. However, it has been unclear whether production of other cytokines is associated with Cas-L/NEDD9. To examine whether Cas-L/NEDD9 has an affect on cytokine production in CIA, we assayed for serum levels of various cytokines in *Nedd9*$^{+/+}$ and *Nedd9*$^{-/-}$ CIA mice. As shown in Fig. 3A, levels of proinflammatory cytokines such as IL-1β (panel a), IL-6 (panel b), IL-17 (panel c), IFN-γ (panel d), and TNF-α (panel e) were significantly decreased in sera of *Nedd9*$^{-/-}$ CIA

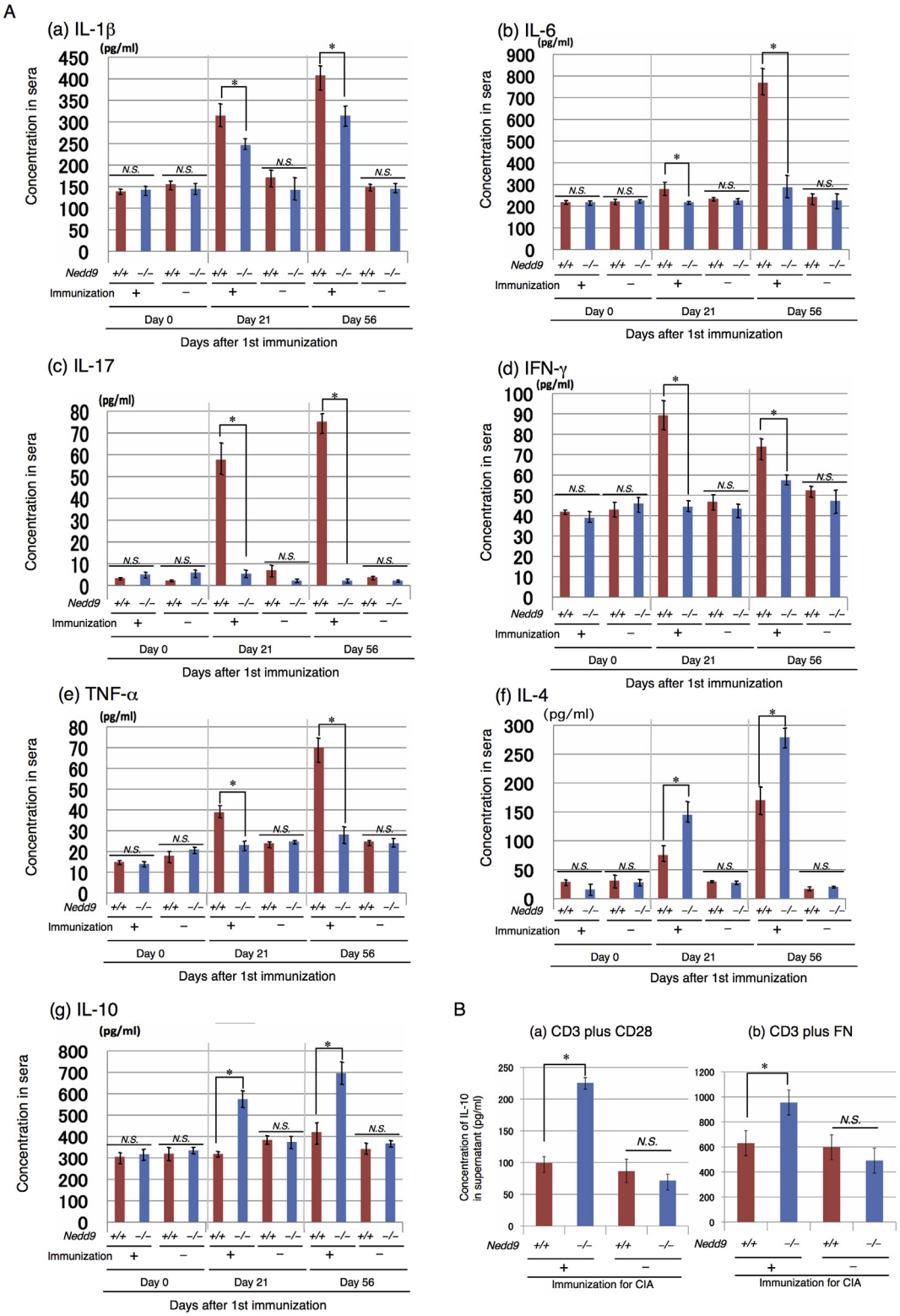

Fig. 3. Increase of IL-10 level in *Nedd9*$^{-/-}$ CIA mice. A. Sera of *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice were collected at the indicated day after CIA immunization, and serum levels of IL-1β (panel a), IL-6 (panel b), IL-17 (panel c), IFN-γ (panel d), TNF-α (panel e), IL-4 (panel f) and IL-10 (panel g) were quantified. Data are shown as mean±standard errors of cumulative results from 3 independent experiments (each, n = 9). * indicates $P < 0.001$, and *N.S.*, not significant. B. CD4^{+}T cells were purified from spleen cells of *Nedd9*$^{+/+}$ or *Nedd9*$^{-/-}$ mice before and after immunization. Cells were stimulated with plate-bound anti-CD3 mAb plus anti-CD28 mAb (panel a), or anti-CD3 mAb plus fibronectin (FN). After 72 h, the supernatants were harvested, and subjected to IL-10 assay. Data are shown as mean±standard errors with triplicates (each, n = 6). * indicates $P < 0.001$, and *N.S.*, not significant.

mice as compared to *Nedd9*$^{+/+}$ CIA mice. Meanwhile, levels of anti-inflammatory cytokines such as IL-4 and IL-10 were significantly increased in sera of *Nedd9*$^{-/-}$CIA mice as compared to *Nedd9*$^{+/+}$ CIA mice (panels f and g of Fig. 3A). These data suggest that the decrease in T cell responses seen in *Nedd9*$^{-/-}$ mice is mediated at least partly by increased production of the anti-inflammatory cytokine IL-10.

To further evaluate this possibility, we conducted *in vitro* cytokine production assay using CD4^{+}T cells isolated from *Nedd9*$^{-/-}$ and *Nedd9*$^{+/+}$ CIA mice. As shown in Fig. 3B, production of IL-10 by CD3 plus CD28 costimulation was significantly enhanced in CD4^{+}T cells from *Nedd9*$^{-/-}$ CIA mice, compared to that of non-immunized *Nedd9*$^{-/-}$ or *Nedd9*$^{+/+}$ mice and *Nedd9*$^{+/+}$ CIA mice (panel a). Similarly, production of IL-10 by CD3 plus FN costimulation was significantly enhanced in CD4^{+}T cells from *Nedd9*$^{-/-}$ CIA mice, compared to that of non-immunized *Nedd9*$^{-/-}$ or *Nedd9*$^{+/+}$ mice and *Nedd9*$^{+/+}$ CIA mice (panel b of Fig. 3B). These results suggest that the decreased incidence and severity of CIA seen in *Nedd9*$^{-/-}$ mice are partly associated with an increase in IL-10 production by T cells as well as a decrease in inflammatory T cell responses.

Collectively, our present results strongly suggest that Cas-L/NEDD9 in lymphocytes plays a pivotal role in the pathogenesis of CIA, and that targeting Cas-L/NEDD9 may be an effective novel therapeutic approach for autoimmune diseases including RA.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.156.

Transparency document

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References

[1] R.O. Hynes, Integrins: versatility, modulation, and signaling in cell adhesion, Cell 69 (1992) 11–25.
[2] Y. Nojima, D.M. Rothstein, K. Sugita, et al., Ligation of VLA-4 on T cells stimulates tyrosine phosphorylation of a 105-kD protein, J. Exp. Med. 175 (1992) 1045–1053.
[3] M. Minegishi, K. Tachibana, T. Sato, et al., Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in β1 integrin-mediated signaling in lymphocytes, J. Exp. Med. 184 (1996) 1365–1375.
[4] R. Sakai, A. Iwamatsu, N. Hirano, et al., A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner, EMBO J. 13 (1994) 3748–3756.
[5] S. Kumar, Y. Tomooka, M. Noda, Identification of a set of genes with developmentally down-regulated expression in the mouse brain, Biochem. Biophys. Res. Commun. 185 (1992) 1155–1161.
[6] S.F. Law, J. Estojak, B. Wang, et al., Human enhancer of filamentation 1, a novel p130^{cas}-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in Saccharomyces cerevisiae, Mol. Cell. Biol. 16 (1996) 3327–3337.
[7] G.M. O'Neill, S. Seo, I.G. Serebriiskii, et al., A new central scaffold for metastasis: parsing HEF1/Cas-L/NEDD9, Cancer Res. 67 (2007) 8975–8979.
[8] Y. Ohashi, K. Tachibana, K. Kamiguchi, et al., T cell receptor-mediated tyrosine phosphorylation of Cas-L, a 105-kDa Crk-associated substrate-related protein, and its association of Crk and C3G, J. Biol. Chem. 273 (1998) 6446–6451.
[9] K. Tachibana, T. Urano, H. Fujita, et al., Tyrosine phosphorylation of Crk-associated substrates by focal adhesion kinase. A putative mechanism for the integrin-mediated tyrosine phosphorylation of Crk-associated substrates, J. Biol. Chem. 272 (1997) 29083–29090.
[10] Y. Ohashi, S. Iwata, K. Kamiguchi, C. Morimoto, Tyrosine phosphorylation of Crk-associated substrate lymphocyte-type is a critical element in TCR- and β1 integrin-induced T lymphocyte migration, J. Immunol. 163 (1999) 3727–3734.
[11] S. Iwata, A. Souta-Kuribara, A. Yamakawa, et al., HTLV-I Tax induces and associates with Crk-associated substrate lymphocyte type (Cas-L), Oncogene 24 (2005) 1262–1271.
[12] R. Miyake-Nishijima, S. Iwata, S. Saijo, et al., Role of Crk-associated substrate lymphocyte type in the pathophysiology of rheumatoid arthritis in tax transgenic mice and in humans, Arthritis Rheum. 48 (2003) 1890–1900.
[13] Y. Iwakura, M. Tosu, E. Yoshida, et al., Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I, Science 253 (1991) 1026–1028.
[14] S. Seo, T. Asai, T. Saito, et al., Crk-associated substrate lymphocyte type is required for lymphocyte trafficking and marginal zone B cell maintenance, J. Immunol. 175 (2005) 3492–3501.
[15] E.F. Rosloniec, M. Cremer, A. Kang, L.K. Myers, Collagen-induced arthritis, Chapter 15, Curr. Protoc. Immunol. (2001). Unit 15 15.
[16] D.D. Brand, K.A. Latham, E.F. Rosloniec, Collagen-induced arthritis, Nat. Protoc. 2 (2007) 1269–1275.
[17] S. Iwata, Y. Ohashi, K. Kamiguchi, C. Morimoto, β1-integrin-mediated cell signaling in T lymphocytes, J. Dermatol. Sci. 23 (2000) 75–86.
[18] K. Kamiguchi, K. Tachibana, S. Iwata, et al., Cas-L is required for β1 integrin-mediated costimulation in human T cells, J. Immunol. 163 (1999) 563–568.
[19] T. Matsuyama, A. Yamada, J. Kay, et al., Activation of CD4 cells by fibronectin and anti-CD3 antibody. A synergistic effect mediated by the VLA-5 fibronectin receptor complex, J. Exp. Med. 170 (1989) 1133–1148.
[20] S. Inamoto, S. Iwata, T. Inamoto, et al., Crk-associated substrate lymphocyte type regulates transforming growth factor-β signaling by inhibiting Smad6 and Smad7, Oncogene 26 (2007) 893–904.