ウェブサイト公開用

（様式 10）
（海外特別研究員事業）

令和　２年　６月　２５日

海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

採 用 年 度　平成３０年度
受 付 番 号　201860185
氏　　名　福牛 翔一

（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　Boston　（国名：　アメリカ合衆国　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
関節リウマチにおける NETosis の役割

3. 派遣期間：平成３０年６月２６日　～　令和２年６月８日

4. 受入機関名及び部局名
Harvard Medical School

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

We aimed to elucidate the mechanism of how peptidylarginine deiminase 4 (PAD4) attenuates arthritis as shown in a collagen-induced arthritis (CIA) model using a DBA/1 strain previously. However, we needed to establish a better arthritis model using the C57BL/6 strain because our laboratory's PAD4 knockout mice were on the C57BL/6 background. As reported from substantial laboratories, the incidence of arthritis in the CIA model on the C57BL/6 background is relatively poor and unstable. We tried and failed to induce arthritis using C57BL/6 mice. In addition, some of them had severe tail lesions that led to the necessity of euthanasia.

To establish an appropriate arthritis model to evaluate NETosis

We used 3 types of arthritis models in the C57BL/6 strain, fundamentally based on the CIA model (Figure 1). We could not induce arthritis using Model A at all (0%) in the C57BL/6 mouse strain. Meanwhile, we had a 50% incidence rate of arthritis using Model B in the C57BL/6 mouse strain (Figure 2). Complications with this model, including tail lesions, are within tolerance. However, nearly 50% of the mice died of lipopolysaccharide (LPS) injection (Model C). Therefore, we

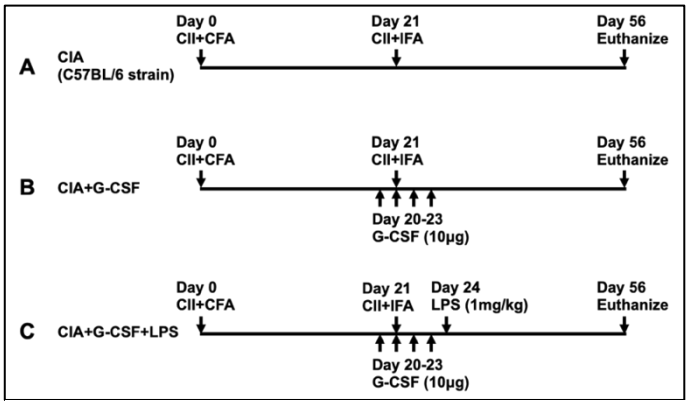

Figure 1. Arthritis models in C57BL/6 mouse strain. CII: type II collagen, CFA: complete Freund's adjuvant, IFA: incomplete Freund's adjuvant, G-CSF: granulocyte colony-stimulating factor, LPS: lipopolysaccharide.

decided to use Model B in the following experiments.

We hypothesized that because PAD4 plays a critical role in neutrophil extracellular traps (NETs) and the associated cell death (NETosis), reduced NETs and NETosis cause the attenuated arthritis in PAD4 knockout mice. To verify our hypothesis, we used Model B in *Padi4*$^{+/+}$ and *Padi4*$^{-/-}$ mice in C57BL/6 background.

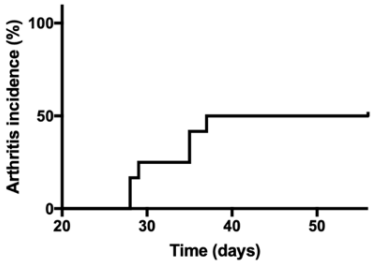

Figure 2. Arthritis incidence of Model B (n=12).

Arthritis incidence in *Padi4*$^{-/-}$ and wild type (*Padi4*$^{+/+}$)

Padi4$^{+/+}$ mice had a 50% incidence of arthritis. Meanwhile *Padi4*$^{-/-}$ mice had no arthritis incidence (Figure 3), which was statistically significant (p= 0.0331). Based on this result, we assumed that PAD4 plays a critical role in arthritis, especially in the association with neutrophil function, including NETosis, or citrullination of proteins.

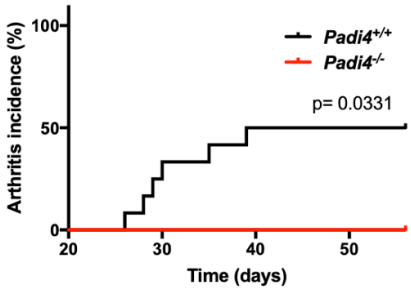

Figure 3. Incidence of arthritis in *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$.

NETs are released in an arthritic joint and peripheral blood

We stained joint tissue with proliferated synovial tissue of Model B in the C57BL/6 mouse strain at day 56 by citrullinated histone 4 (H4cit, the marker of NETs) and Ly-6G (the marker of neutrophils) and found lesions positive for both H4cit and Ly-6G on the joint surface (Figure 4). In addition, we found released DNA with H4cit from peripheral blood (Figure 5). These findings suggested the existence of NETs in joint space and peripheral blood. Thus, we thought Model B is a preferable arthritis model in the C57BL/6 strain to see the contribution of NETs to the pathophysiology of arthritis.

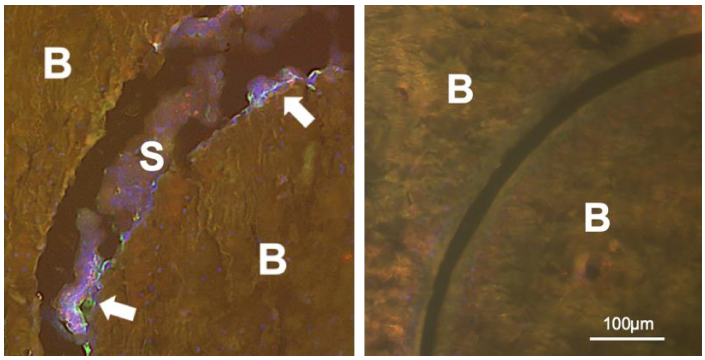

Figure 4. Left panel: NETs in proliferated synovial tissue on joint surface (S and arrows). Arrows indicate proliferated synovial tissue with NETs (citrullinated histone 4: green, Ly-6G: red, DNA: blue). B: bone. Right panel: Normal joint space without arthritis.

No differences of NETosis marker levels in plasma between *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$

To confirm the existence of NETosis in this model, we attempted to detect NETosis markers in serum and plasma. Double-stranded DNA (dsDNA) is known as a marker of NETosis because NETs contain dsDNA released from neutrophils. We measured dsDNA in plasma 12 hours and 48 hours after the injection of type 2 collagen (CII) and complete Fruend's adjuvant (CFA). Levels of dsDNA increased by up to 20% 12 hours after the injection of CII and CFA in both groups. However, there were no significant differences in the two groups (Figure 6). We also compared levels of dsDNA in plasma at days 7, 20, 25, 42, and 56, and there were no differences between the two groups (data are not shown).

Figure 5. Left panel: NETs in peripheral blood of arthritis model mouse (arrow). Right panel: Peripheral blood of untreated mouse. Citrullinated histone 4: green, Ly-6G: red, DNA: blue.

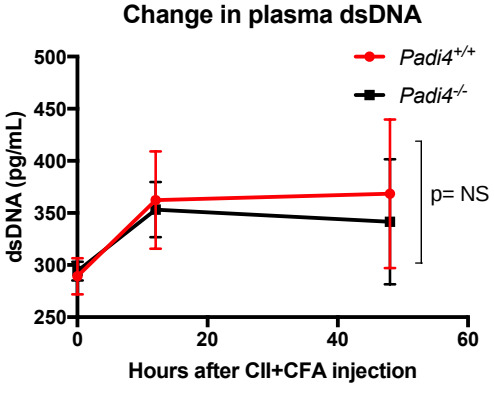

Figure 6. Plasma double-stranded DNA (dsDNA) levels in *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$ 12 hours and 48 hours after the injection of type 2 collagen (CII) and complete Freund's adjuvant (CFA). NS: no significance.

Citrullinated histone 4 (H4cit) is also known as a marker of NETosis because the citrullination of histone 4 cause chromatin decondensation and enable neutrophils to release DNA-containing NETs into the extracellular space. We tried to detect H4cit in plasma 12 hours after the injection of CII and CFA by Western blot (Figure 7). However, we could not detect H4cit in either *Padi4*$^{-/-}$ or *Padi4*$^{+/+}$. We also examined H4cit in plasma 48 hours after the injection of CII and CFA, at days 7, 20, 25, 42, and 56, and we could not detect H4cit (data are not shown).

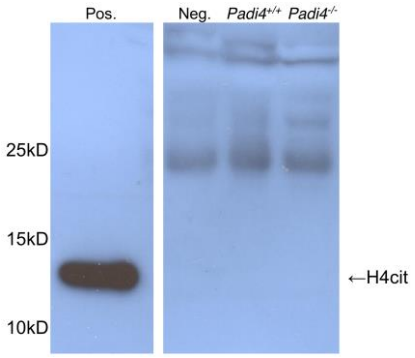

Figure 7. Plasma citrullinated histone 4 (H4cit) in *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$ 12 hours after the injection of type 2 collagen (CII) and complete Freund's adjuvant (CFA). Pos.(positive control): recombinant H4cit; Neg.(negative control): plasma from an untreated mouse.

The different levels of serum anti-collagen antibody between *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$

Anti-collagen antibody is pathogenic in CIA models. We measured serum levels of anti-collagen antibody at day 56. We found a significant difference between *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$ (Figure 8). We assumed that the difference in the incidence of arthritis is partially explained by the different levels of anti-collagen antibody.

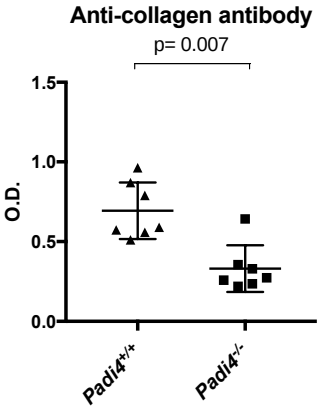

Figure 8. Serum anti-collagen antibody of *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$ at day 56. O.D.: optical density.

The different levels of serum anti-H4cit antibody between *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$

Anti-citrullinated protein antibodies (ACPAs), which are produced in response to citrullinated proteins, are detectable in the serum of two-thirds of RA patients. ACPAs are clinically important to predict subsequent clinical courses and progression of disease. Although the mechanism of ACPAs production is unknown, NETs are reported to be a source of citrullinated autoantigens. Therefore, we measured anti-H4cit antibody, one of the ACPAs, and anti-native H4 antibody in sera. We detected both anti-H4cit antibody and anti-native H4 antibody in the sera of *Padi4*$^{+/+}$. However, we did not detect anti-H4cit antibody in *Padi4*$^{-/-}$ (Figure 9). Therefore, we concluded that *Padi4*$^{-/-}$ had less anti-H4cit antibody in serum. Although anti-native H4 antibody can bind to H4cit, this concern was addressed properly by comparing the anti-H4cit antibody levels with anti-native H4 antibody.

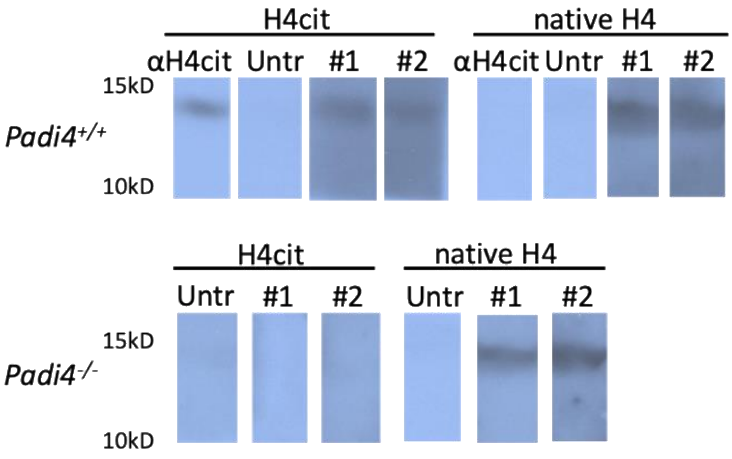

Figure 9. Anti-H4cit antibody and anti-native H4 antibody in *Padi4*$^{+/+}$ (upper panel) and in *Padi4*$^{-/-}$ (lower panel) at day 56. H4cit: citrullinated histone 4, αH4cit: anti-H4cit antibody (Millipore, 1:500 dilution), Untr: Serum of an untreated mouse as negative control (serum at day 0), #: identification number of mouse for each genotype.

Based on these results, we assumed that NETosis could become the source of H4cit, and released H4cit could induce anti-H4cit antibody, one of the ACPAs, although we could not detect markers of NETosis. We could not confirm that the absence of NETosis in *Padi4*$^{-/-}$ induced attenuated arthritis directly. Therefore, we tried to examine the functions of *Padi4* except for inducing NETosis. Previous reports have shown that *Padi4*$^{-/-}$ have lower levels of serum inflammatory cytokines, including tumor necrosis

factor and interleukin 6 (IL-6) in arthritis models. Because macrophage-lineage cells are the main producer of inflammatory cytokines, we hypothesized that macrophage-lineage cells of *Padi4*$^{-/-}$ have a reduced potential to invoke inflammation. This time, we focused on the ability of bone marrow–derived macrophages (BMDM) to produce IL-6.

The different levels of IL-6 in supernatant of BMDM between *Padi4*$^{-/-}$ and *Padi4*$^{+/+}$

We cultured BMDM of *Padi4*$^{+/+}$ and *Padi4*$^{-/-}$ in vitro with macrophage colony-stimulating factor (MCSF, 25ng/mL). We stimulated them with lipopolysaccharide (1ug/mL) for 4 hours and detected IL-6 in supernatant by an enzyme-linked immunosorbent assay (ELISA). In addition, we used chloramidine (Cl-amidine), a pan-PAD inhibitor, simultaneously. *Padi4*$^{+/+}$ had higher levels of IL-6 in supernatant than those of *Padi4*$^{-/-}$. Furthermore, Cl-amidine reduced IL-6 production not only in *Padi4*$^{+/+}$ but also in *Padi4*$^{-/-}$ (Figure 10). These results suggested an additional function of *Padi4* other than the induction of NETosis. In addition, it is suggested that Cl-amidine had additional effects to reduce IL-6 production in *Padi4*$^{-/-}$ and these effects assumed to be induced by other kinds of PAD, especially by PAD2. We sought to elucidate the mechanism of how PAD4 induced IL-6 and investigated the contributions of PAD4 to NF-κB and Jak2; both are known as the signal pathways to induce IL-6. However, we could not have any evidence of the citrullination of these proteins and could not elucidate the mechanism of how PAD4 and other PAD regulate the IL-6 production within the term of the fellowship.

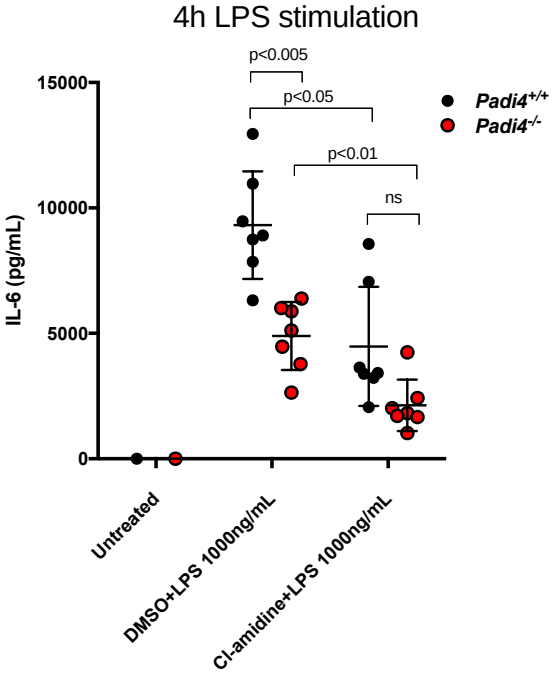

Figure 10. IL-6 production of BMDM from *Padi4*$^{+/+}$ and *Padi4*$^{-/-}$ in vitro. DMSO: dimethyl sulfoxide (solvent control), ns: no significance.

These results were presented at The Program in Cellular and Molecular Medicine (PCMM) Scientific Retreat in 2018 and 2019.