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ICOS Signaling Controls Induction and Maintenance of Collagen-Induced Arthritis

Vincent Panneton,*,† Sahar Bagherzadeh Yazdchi,*,‡ Mariko Witalis,*,§ Jinsam Chang,*,§ and Woong-Kyung Suh*,†,‡,§

ICOS is a key costimulatory receptor facilitating differentiation and function of follicular helper T cells and inflammatory T cells. Rheumatoid arthritis patients were shown to have elevated levels of ICOS$^+$ T cells in the synovial fluid, suggesting a potential role of ICOS-mediated T cell costimulation in autoimmune joint inflammation. In this study, using ICOS knockout and knockin mouse models, we found that ICOS signaling is required for the induction and maintenance of collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis. For the initiation of CIA, the Tyr^{181}-based SH2-binding motif of ICOS that is known to activate PI3K was critical for Ab production and expansion of inflammatory T cells. Furthermore, we found that Tyr^{181}-dependent ICOS signaling is important for maintenance of CIA in an Ab-independent manner. Importantly, we found that a small molecule inhibitor of glycolysis, 3-bromopyruvate, ameliorates established CIA, suggesting an overlap between ICOS signaling, PI3K signaling, and glucose metabolism. Thus, we identified ICOS as a key costimulatory pathway that controls induction and maintenance of CIA and provide evidence that T cell glycolytic pathways can be potential therapeutic targets for rheumatoid arthritis. ***The Journal of Immunology*****, 2018, 200: 3067–3076.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by immune cell infiltration of the synovial tissues leading to inflammation with potential destruction of cartilage and bone. The pathology of RA involves components of innate and adaptive immunity (1). Although the pathogenesis of RA remains poorly understood, production of autoantibodies along with infiltration of synovial fluid and tissues by inflammatory cells have been identified as key events (2–4). Collagen-induced arthritis (CIA) is a murine model that closely mimics RA (5). Susceptible mouse strains such as DBA/1 develop humoral and cellular immune reactions to bovine type II collagen (bCII) following immunization, which leads to chronic joint inflammation (6). Studies have shown that T cell costimulation is involved in CIA. For example, mice deficient in CD28 are resistant to CIA and blockade of ICOS using an mAb-ameliorated CIA (7, 8). Interestingly, RA patients were shown to have elevated levels of ICOS$^+$ T cells along with ICOS ligand (ICOSL) in their synovial tissues, suggesting potential therapeutic value of ICOS-mediated T cell costimulatory pathways (9).

ICOS is a member of the CD28 superfamily mainly expressed at the surface of activated T cells (10, 11). The major role of ICOS is to support differentiation and function of CD4$^+$ follicular helper T (Tfh) cells during germinal center (GC) reactions (12–15). Consequently, lack of ICOS or ICOSL in humans and mice causes severe defects in class-switched Ab production (16–20). Alternatively, ICOS overexpression caused by the *sanroque* mutation leads to a lupus-like disease with an increase of Tfh cells and spontaneous GC reactions in mice (21, 22). Additionally, ICOS signaling plays important roles in the production of an array of Th1, Th2, and Th17 inflammatory cytokines depending on the context of immune stimuli (23–26).

Despite evidence suggesting that ICOS could be implicated in CIA and RA, little is known about the specific downstream signaling components involved. We have previously described an evolutionarily conserved SH2 binding motif (Y^{181}MFM) in the cytoplasmic tail of ICOS that is critical for the activation of the PI3K–Akt–mTOR signaling cascade (27, 28). Thus, mice carrying the ICOS^{Y181F} mutation cannot signal through this pathway and display reduced numbers of Tfh cells and impaired GC reactions (27). However, the ICOS^{Y181F} mutation does not completely phenocopy ICOS deficiency. For example, the anti-chlamydial Th17 response and graft-versus-host disease severity were only partially reduced in ICOS^{Y181F} mice compared with ICOS$^{-/-}$ mice (26, 29). These findings suggest the existence of PI3K-independent signaling mechanisms of ICOS. In fact, there are two more signaling motifs identified in the cytoplasmic tail of ICOS. The second motif, KKKY^{170}, in the cytoplasmic tail of ICOS can potentiate intracellular calcium release from the endoplasmic reticulum triggered by TCR engagement (30). The third TNFR-associated factor–like IProx motif links ICOS to TANK-binding kinase 1 and serves as an important mechanism promoting the late stage of Tfh cell differentiation (31). However, its downstream signaling mechanisms remain unclear.

In this study, we evaluated the impact of ICOS signaling on CIA initiation and maintenance using ICOS mutant mice. We found

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Abbreviations used in this article: bCII, bovine type II collagen; 3-BrPA, 3-bromopyruvate; CIA, collagen-induced arthritis; cKO, conditional KO; dLN, draining lymph node; ES, embryonic stem; GC, germinal center; ICOSL, ICOS ligand; KO, knockout; Neo, neomycin resistance; RA, rheumatoid arthritis; Tfh, follicular helper T.

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that ICOS is required for both induction and maintenance of CIA. Although these two processes are heavily dependent on Tyr^{181}-mediated ICOS signaling, the downstream effector pathways are distinct. Importantly, we uncovered a potential overlap between ICOS signaling, T cell glycolysis, and joint inflammation.

Materials and Methods

Mice

ICOS germline knockout and $ICOS^{Y181F}$ knockin mice have been previously described (17, 27). For CIA experiments, these two lines have been backcrossed onto DBA/1J background for 12 generations. DBA/1J mice (Jax 000670), UBC-CreERT2 mice (Jax 008085), and the FLPe FRT deleter line (Jax 005703) were purchased from The Jackson Laboratory. All mice were housed in the Institut de Recherches Cliniques de Montréal animal facility under specific pathogen-free conditions. Animal experiments were performed in accordance with animal use protocols approved by the Institut de Recherches Cliniques de Montréal Animal Care Committee.

Generation of ICOS conditional knockout mice

ICOS conditional knockout (cKO) mouse strain was generated using C57BL/6 embryonic stem cell line Bruce-4 at the Institut de Recherches Cliniques de Montréal transgenic core facility. We generated 5.4-kb 5′ and 4.5-kb 3′ homology arms by assembling DNA fragments (1.2–2.3 kb) amplified through high-fidelity PCR (Platinum Pfx DNA polymerase; Thermo Fisher) using C57BL/6 RPCI-23 mouse bacterial artificial chromosome clone DNA as template. Cloned DNA fragments were verified by sequencing and aligning against the C57BL/6 genome sequence available from the National Center for Biotechnology Information. We designed a targeting vector in which exon 2 (encoding extracellular Ig domain) and exon 3 (encoding transmembrane segment) of ICOS gene were flanked by loxP elements, and a neomycin resistance (Neo) cassette was flanked by FRT elements. After electroporation of the targeting vector, G418-resistant embryonic stem (ES) cell clones were screened for successful homologous recombination in the *Icos* locus by Southern blot. Four ES clones with conditional ICOS allele were injected into C57BL/6 blastocysts and three of them gave germline transmission. Heterozygous mice containing ICOS mutant allele were bred with FLPe FRT deleter mice to remove the Neo cassette. Resulting ICOS-floxed, Neo-deleted heterozygous mice were further backcrossed for two generations onto C57BL/6 background and used for experiments.

Collagen-induced arthritis

CIA was induced in mice with a DBA/1J background (for germline KO experiments) or in a 50:50 mix of DBA/1J:C57BL/6J background (for cKO experiments). Eight- to twelve-week-old mice were immunized intradermally at the base of the tail with 50 μg of bCII (2 mg/ml; Chondrex) emulsified in an equal volume of CFA (4 mg/ml *Mycobacterium tuberculosis*; Chondrex). Clinical arthritis was assessed for each paw in a blinded manner based on the following criteria: 0 h,ealthy; 1, mild swelling of one joint; 2, mild swelling of multiple joints; 3, severe swelling of multiple joints; 4, severe swelling and ankylosis. The scores were added up for a maximum of 16 per mouse. For cKO experiments, mice were fed tamoxifen dissolved in corn oil (Sigma-Aldrich) daily at a dose of 200 μg/g body weight by oral gavage for 5 d. For glycolysis inhibition experiments, mice were injected i.p. with 3-bromopyruvate (3-BrPA; Sigma-Aldrich) at a dose of 5 mg/kg per day for 3 d.

Histology

Paws were dissected and fixed in 10% neutral buffered formalin for 12 h at 4°C. Paws were washed in 1× PBS and decalcified in 0.5 M EDTA replaced every 3 d for 2 wk at 4°C. Decalcified paws were embedded in paraffin and 5-μm sections were stained with H&E for assessment of immune infiltration and destruction of joints.

Ab ELISA

Serum was obtained by cheek bleeding at different disease stages. Plates were coated with 5 μg/ml ELISA-grade collagen (Chondrex) overnight at 4°C. A 2-fold serial dilution of serum was performed starting at 1:100. Abs were detected using anti–IgG2a-alkaline phosphatase or anti–IgG2b-alkaline phosphatase along with *p*-nitrophenyl phosphate substrate (SouthernBiotech). The reaction was stopped using 1.5 N NaOH and plates were read at 405 nm.

Proliferation assay and cytokine ELISA

Cells were isolated by mechanical disruption from draining lymph nodes (dLN) of immunized mice and resuspended at 1×10^6/ml in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Wisent), 10 mM HEPES (Thermo Fisher), 100 U/ml penicillin-streptomycin (Thermo Fisher), and 55 μM 2-ME (Thermo Fisher). Cells were restimulated with 100 μg/ml heat-denatured bCII (Chondrex) or 1 μg/ml anti-CD3 (clone 145-2C11; Bio X Cell) for 2 d at 37°C in 96-well plates (1×10^5 per well). Cytokine concentrations in culture supernatant were measured using cytokine Ready-SET-Go! ELISA kits (Thermo Fisher) according to the manufacturer's instructions. For proliferation assays, cells were pulsed with 1 μCi per well [^{3}H]thymidine (PerkinElmer) for 8 h at 37°C, lysed, and harvested using a FilterMate harvester (Packard) and then analyzed using a TopCount NXT scintillation counter (Packard).

Glucose metabolism assay

Lymph node cells from naive WT DBA/1 mice were stimulated with 1 μg/ml anti-CD3 for 2 d at 37°C in 96-well plates (1×10^5 cells per well) in the same manner as for the proliferation assay. During the last 4 h of incubation, cells were exposed to 3-BrPA (3 or 6 μM; Sigma-Aldrich) and D-[3-^{3}H] glucose (1 μCi/well; PerkinElmer). Cells were then lysed, harvested, and incorporated radioactivity was measured as described for the proliferation assay.

Flow cytometry analysis of ICOS cKO

To quantify the efficacy of conditional ICOS deletion, single-cell suspensions were prepared from dLN of ICOS cKO mice treated or not with tamoxifen for 5 d. Cells were incubated for 24 h at 37°C in complete RPMI medium supplemented with 1 μg/ml anti-CD3 (clone 145-2C11; Bio X Cell). Viability was determined by trypan blue staining (Thermo Fisher). Cells were blocked with anti-mouse CD16/CD32, then stained with anti–CD4 FITC (GK1.5) or anti–CD8a-allophycocyanin (53-6.7) along with anti–ICOS-PE/Cy7 (7E.17G9) using 0.3 μl per stain (Thermo Fisher). Data were collected using BD LSRFortessa (BD Biosciences) and analyzed by FlowJo v10.

Statistical analysis

All data are presented as mean ± SEM unless specified otherwise. Differences between experimental groups were tested with two-tailed Student *t* tests in Prism 7 (GraphPad Software). Statistical significance was judged based on *p* values and is denoted as follows: $*p < 0.05$, $**p < 0.02$.

Results

ICOS is required for the initiation of CIA

To assess the role of ICOS in the induction of CIA, we immunized mice with an emulsion of bCII and CFA at the base of the tail. WT DBA/1J mice developed signs of arthritis 5 wk after immunization (Fig. 1A). This is characterized by paw swelling and erythema along with histological features such as immune cell infiltration of the joint space and erosion of cartilage (Fig. 1B). However, $ICOS^{-/-}$ and $ICOS^{Y181F}$ DBA/1J mice did not develop arthritis for the entire duration of the experiment (Fig. 1A). Next, we analyzed anti-bCII IgG2a and IgG2b serum levels 2 wk post-immunization, as these isotypes are known to activate complement and are required for the induction of arthritis (32). Consistent with disease scores, Ab titers were at least 3-fold lower in $ICOS^{Y181F}$ and $ICOS^{-/-}$ mice compared with WT mice (Fig. 1C). To evaluate the role of ICOS in Ag-specific T cell responses, we immunized mice and harvested dLN 2 wk later. Next, we restimulated a suspension of total dLN cells with bCII for 2 d and measured T cell proliferation and cytokine production. Cells from $ICOS^{Y181F}$ or $ICOS^{-/-}$ mice displayed a 3-fold reduction of proliferation in response to the immunizing Ag when compared with cells from WT mice (Fig. 1D). Levels of IFN-γ, IL-17, TNF-α, and IL-4 were measured in culture media from proliferation assays by ELISA (Fig. 1E). No significant differences were observed for IFN-γ and IL-17. TNF-α was 2-fold lower than WT in $ICOS^{-/-}$ samples. No IL-4 production was detectable in any samples (data not shown). Taken together, these results indicate

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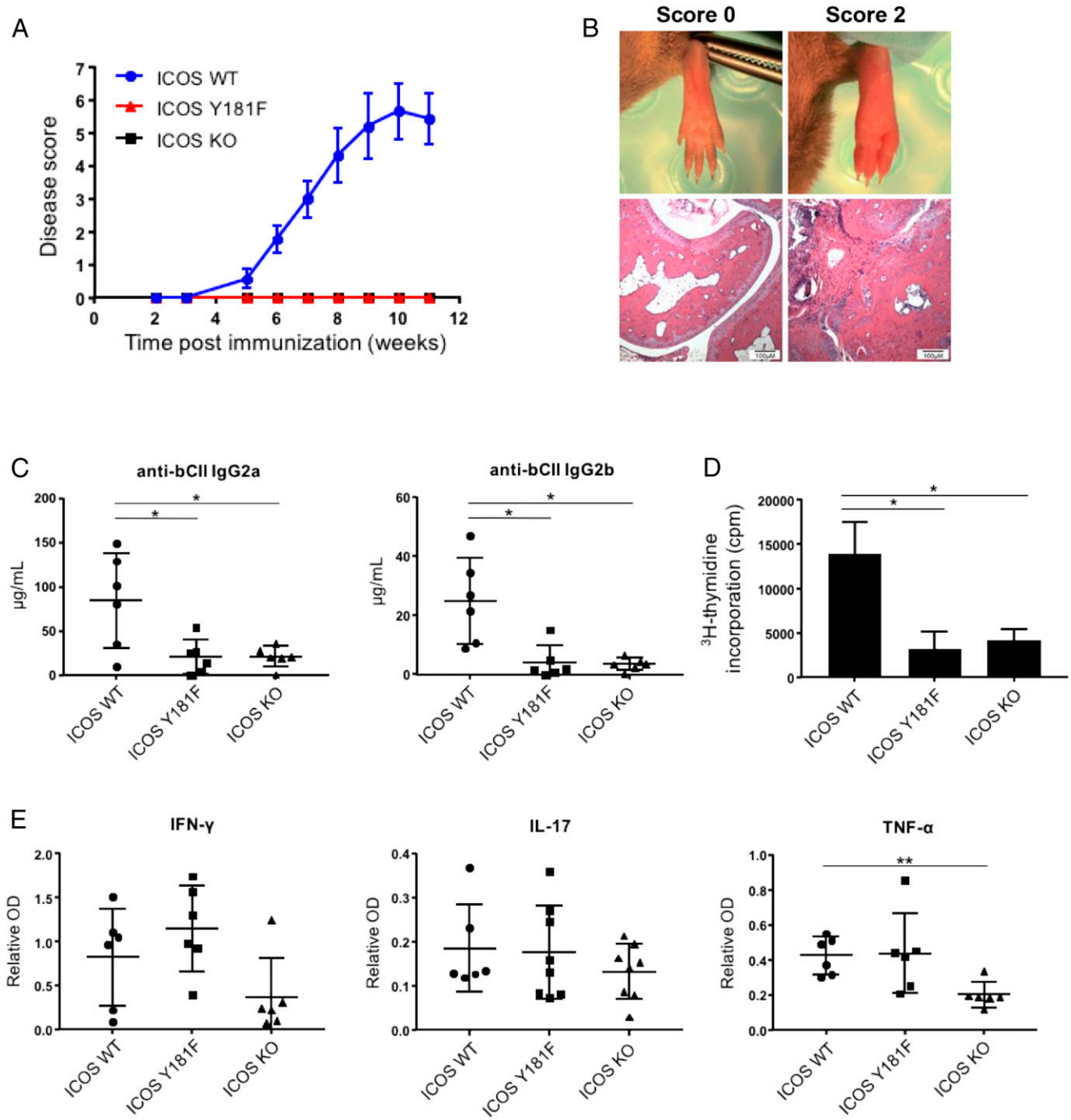

FIGURE 1. ICOS is required for the initiation of collagen-induced arthritis. (**A**) Eight- to twelve-week-old DBA/1 mice were immunized with bCII plus CFA and disease scores were given by visual inspection according to the criteria described in *Materials and Methods*. Data represent two independent experiments with similar results. Mean scores ± SEM of eight mice per group are shown. (**B**) Representative images of normal and inflamed hind paw (disease score 2) along with H&E staining showing normal joint space (left) and immune cell infiltration in arthritic paw (right). Scale bar, 100 μm. (**C**) Serum from mice immunized as in (A) was taken 2 wk postimmunization and anti-bCII IgG2a or IgG2b titers were measured by ELISA. Data represent two independent experiments with similar results ($n = 6$ for each group). (**D**) dLN cells from WT DBA/1 mice were extracted 2 wk postimmunization and restimulated in vitro with 100 μg/ml denatured bCII for 48 h. [^{3}H]thymidine was added to measure incorporation during the last 8 h. Data represent two independent experiments with similar results ($n = 6$ for each group). (**E**) Cytokines (IFN-γ, TNF-α, IL-17) were detected in culture supernatant from proliferation assays by ELISA. $n = 6$ per group for IFN-γ and TNF-α; $n = 6$ ICOS WT, $n = 8$ ICOS Y181F, and $n = 8$ ICOS KO for IL-17. $^*p < 0.05$, $^{**}p < 0.02$.

that ICOS and its ability to signal through PI3K are required for CIA initiation primarily through induction of Ab responses and expansion of inflammatory T cells.

Generation of ICOS cKO mice

Although it became clear that ICOS is required for the initiation of CIA in mice, more clinically relevant questions remained unanswered. Is ICOS required to sustain joint inflammation beyond its role in the initiation phase? If so, what are the dominant signaling components involved? To address these questions, we decided to generate ICOS cKO mice. We generated a targeting vector based on C57BL/6 *Icos* genomic sequence and used a C57BL/6-derived ES cell line Bruce-4 to expedite downstream applications without extensive backcrossing. As shown in Fig. 2A, we designed

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the vector to eliminate exons encoding extracellular Ig domain (exon 2) and transmembrane segment (exon 3) by the Cre-loxP system and to remove Neo cassette by the FLPe-FRT system. Through Southern blot analysis of ES cell clones (~400), we identified four clones harboring ICOS-cKO-NEO allele in the *Icos* locus (Fig. 2B). After germline transmission (Fig. 2C), the Neo cassette was deleted in vivo by breeding with FLPe FRT deleter line. We confirmed that T cells harboring the ICOS-cKO allele express normal levels of ICOS upon stimulation and that ICOS expression is completely abrogated in CD4-Cre$^+$ T cells (Supplemental Fig. 1) or when Cre enzyme activity is turned on by tamoxifen treatment in UBC-CreERT2 transgenic mice (Fig. 2D).

ICOS is required for the maintenance of CIA

To determine whether ICOS is required in the maintenance of established CIA, we used our cKO system in which ICOS deletion is induced by tamoxifen (Fig. 3A). Although it has been shown that the C57BL/6 genetic background confers resistance to CIA (33), mice bearing ~50% of DBA/1 background are susceptible to CIA (34). We were able to induce CIA in this mixed genetic background with disease kinetics similar to WT DBA/1 mice (Fig. 3B). At the peak of arthritis symptoms, we induced the deletion of ICOS and observed a marked amelioration of disease severity (Fig. 3B). A similar pattern was observed when we pooled data from paws that were highly inflamed (score 3–4), indicating a critical role for ICOS in the maintenance of severe joint inflammation (Supplemental Fig. 2). The slight reduction in disease scores seen in control mice is a feature of normal CIA progression (35). We also evaluated serum anti-bCII Ab titers at different disease stages but found no significant correlation with disease scores (Fig. 3C). Histological analysis reveals a striking recovery after ICOS deletion, although some mice with severe disease were left with residual cartilage damage and joint inflammation (Fig. 3D).

Next, we modified the cKO system to obtain ICOS$^{fl/Y181F}$ mice (Fig. 4A). Whereas ICOS^{Y181F} mice are resistant to CIA, ICOS$^{fl/Y181F}$ mice develop arthritis symptoms 5 wk post-immunization, indicating that the Y181F mutation does not act as a dominant-negative (Fig. 4B). We performed tamoxifen gavage at the peak of arthritis symptoms, which effectively leaves mice with only the mutant allele (ICOS$^{Y181F/-}$). This caused a substantial reduction in disease scores (Fig. 4B). As before, no correlation between serum anti-bCII titers and disease scores was observed (Fig. 4C). Histological analysis shows partial recovery with persistent cartilage erosion (Fig. 4D). Thus, ICOS is required for the maintenance of CIA, and Tyr^{181}-dependent ICOS signaling mechanisms are needed to sustain the maximal disease process.

Glycolysis inhibitor ameliorates CIA

We have previously shown that ICOS can augment mTOR activities through the PI3K–Akt signaling axis (28). Furthermore, a recent study has shown that ICOS costimulation can augment glycolysis in activated T cells through an mTOR-dependent mechanism (36). Additonally, PI3K–Akt signaling has been implicated in augmentation of glucose uptake in activated T cells (37). Interestingly, increased glucose metabolism in the inflamed joint has been observed in RA patients (38, 39), and inhibition of glycolysis ameliorated arthritis in K/BxN transgenic mice (40). We therefore hypothesized that a glycolysis inhibitor could be used to treat CIA. To test this idea, we immunized WT DBA/1 mice and injected 3-BrPA i.p. for 3 d at the peak of disease (Fig. 5A). This caused a partial but persistent reduction in disease score reminiscent of the improvement seen in ICOS^{Y181F} cKO experiments (Fig. 4B). Histological analysis revealed reduced immune cell infiltration and normal cartilage (Fig. 5B). These results suggest that the amelioration of CIA observed in cKO experiments could be due to reduced glycolysis in ICOS-deleted T cells.

We further examined the impact of 3-BrPA on T cell proliferation and function in vitro (Fig. 5C, 5D). First, T cell viability was heavily compromised when dLN cells from immunized mice were restimulated in the presence of 3-BrPA. Even at a low dose of 3-BrPA (3 μM) where T cell viability is minimally affected, proliferation and cytokine production were greatly reduced (Fig. 5C). Consistent with the notion that activated T cells use glucose as a source of biosynthetic building blocks (41), we found that glucose-derived tritium tracers were readily incorporated into macromolecules (Fig. 5D). Importantly, this process is inhibited by 3-BrPA. Therefore, 3-BrPA effectively inhibits T cell proliferation and cytokine production, at least in part through inhibition of intracellular glucose metabolism. However, we failed to determine whether there is a synergy between 3-BrPA and ICOS mutations because 3-BrPA (3 μM) left no room to detect additional impact of ICOS-Y181F mutation (Supplemental Fig. 3).

Discussion

Our study reveals a critical role of ICOS in the initiation and maintenance of CIA. We show that ICOS is required for the generation of anti-bCII Abs in the early disease phase. Also, ICOS deficiency greatly reduced inflammatory T cell responses during disease progression. ICOS^{Y181F} closely resembled ICOS$^{-/-}$ mice during CIA initiation, highlighting the prominent roles of the Tyr^{181}-dependent ICOS signaling in Tfh cell generation and GC-driven Ab generation at this disease stage. We generated a conditional ICOS KO model and showed that ICOS signaling is also required for CIA maintenance. Signaling mechanisms dependent on Tyr^{181} appear to play an important role in the maintenance stage as well. We also found that inhibition of glycolysis ameliorates CIA, providing a potential link between ICOS costimulation, T cell glycolysis, and sustained joint inflammation.

Induction of CIA depends on the production of autoantibodies (32). This is also seen in collagen Ab-induced arthritis where injection of arthrogenic Abs in mice can directly cause joint inflammation (42). ICOS is known to be critically involved in Tfh cell generation and function, and therefore in GC reactions and production of class-switched high-affinity Abs (17, 18, 20). Importantly, we have shown that ICOS–PI3K signaling plays a crucial role in Tfh cell generation (27). Accordingly, we observed similar defects in Ab production in immunized mice lacking ICOS or selectively defective in ICOS–PI3K signaling (Fig. 1C). The requirement for humoral responses in autoimmune diseases has been described in several studies. Mice deficient in CD28, CXCR5, or SLAM-associated protein are all resistant to CIA and show low levels of anti-collagen Abs after immunization (7, 34, 43). ICOS is also known to be important for T cell proliferation and function (17, 18, 20, 44). We showed that T cells from immunized ICOS^{Y181F} and ICOS$^{-/-}$ mice proliferate less when restimulated in vitro using the immunizing Ag (Fig. 1D). Therefore, Ab production defects and impaired T cell expansion likely explain the resistance to CIA initiation in ICOS$^{-/-}$ and ICOS^{Y181F} mice.

Models such as collagen Ab-induced arthritis have shown that Abs alone are insufficient to maintain disease progression. Consistently, we showed that anti-bCII Ab levels became irrelevant to CIA maintenance in cKO experiments (Figs. 3C, 4C). Also, mice left with ICOS^{Y181F} after tamoxifen gavage did not fully recover, whereas mice that completely lost ICOS expression became free of inflammation in most cases. This suggests that signaling

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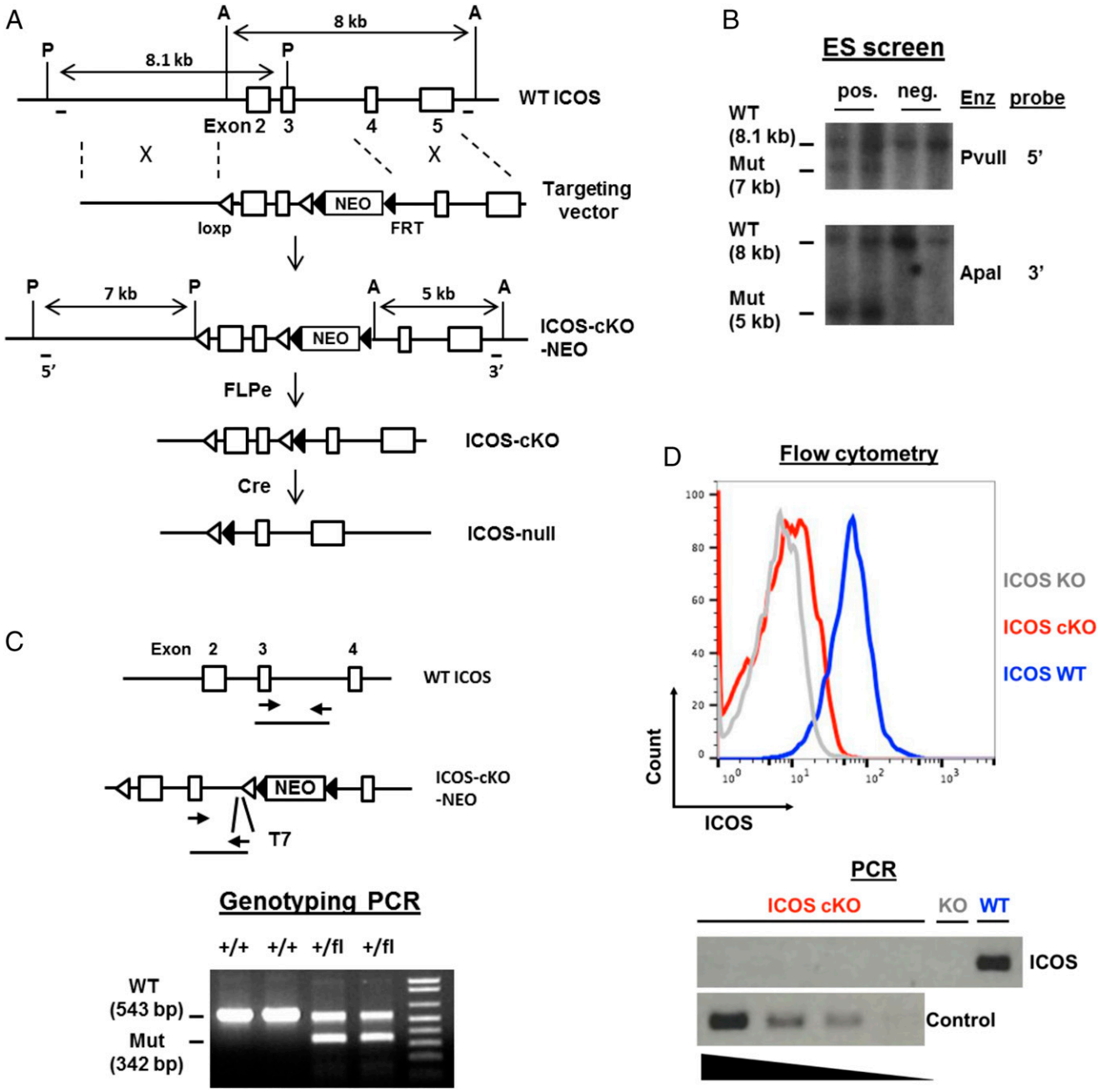

FIGURE 2. Generation of ICOS cKO mice. (**A**) Targeting strategy. Exon 2 and exon 3 of the ICOS gene were flanked with loxP elements and the Neo cassette was flanked by FRT elements. The flanked DNA segments were designed to be removed by Cre and FLPe enzyme, respectively. (**B**) ES cells with targeted ICOS allele were screened by Southern blot using 5′ and 3′ probes as indicated in (A). (**C**) PCR genotyping of ICOS alleles in heterozygous mice. Primers used are represented with arrows. (**D**) Confirmation of tamoxifen-induced ICOS deletion by flow cytometry and PCR. Lymph node cells from UBC-CreERT2^{+}; ICOS$^{fl/-}$ mice treated with tamoxifen were restimulated with 1 μg/ml anti-CD3 for 24 h to induce ICOS expression. Data represent two independent experiments and show ICOS expression in CD4^{+} T cells. For PCR, genomic DNA isolated from CD4^{+} T cell blasts derived from tamoxifen-treated UBC-CreERT2^{+}; ICOS$^{fl/-}$, ICOS WT, or ICOS KO mice were amplified by primers specific for exons 2–3 of ICOS gene or control primers detecting Foxp3 gene (control).

mechanisms depending on Tyr^{181} are important for CIA maintenance, but there are ICOS-mediated Tyr^{181}-independent mechanisms still operating to contribute to sustained inflammation. This is also supported by differences seen in the production of TNF-α by restimulated dLN cells from immunized mice (Fig. 1E). When compared with WT samples, cells from ICOS$^{-/-}$ mice showed a 2-fold reduction of TNF-α, whereas ICOS^{Y181F} cells did not show a significant difference. TNF-α is known to be important for the progression of CIA and is involved in human RA (45, 46). We previously reported that ICOS can potentiate TCR-mediated intracellular calcium flux (30). In T cells, calcium signaling is known to regulate a wide array of processes ranging from differentiation to effector functions (47). In fact, inhibiting calcium signaling with cyclosporine has been one of the most widely used immunosuppressive therapies (48, 49). A recent study showed that a T cell–specific deletion of the store-operated calcium entry components Stim1 and Stim2 protected mice from experimental autoimmune encephalomyelitis (50). Therefore, it is possible that calcium signaling potentiation is one of the main mechanisms evoked by ICOS in CIA maintenance. It remains possible that the

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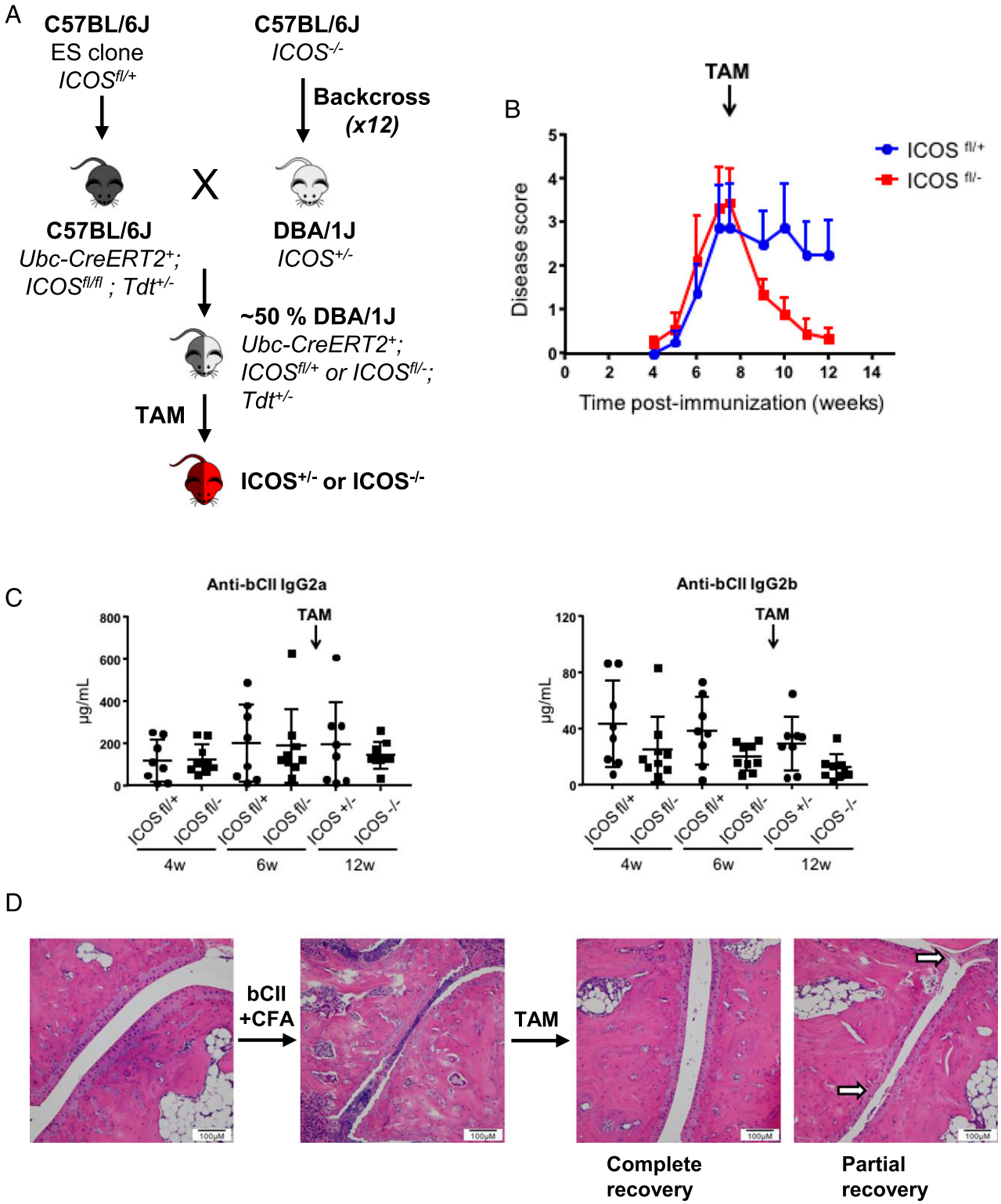

FIGURE 3. ICOS is essential for the maintenance of CIA. (**A**) Experimental design of ICOS cKO experiments. UBC-CreERT2^{+} ICOS$^{fl/+}$ or ICOS$^{fl/-}$ mice are treated with tamoxifen for 5 consecutive days, resulting in deletion of the floxed allele of ICOS. (**B**) Eight- to twelve-week-old mice described in (A) were immunized with bCII plus CFA, then treated for 5 d with tamoxifen at the peak of disease. Disease scores were given by visual inspection according to the criteria described in *Materials and Methods*. Data were pooled from two independent experiments (n = 8 ICOS$^{fl/+}$ and n = 9 ICOS$^{fl/-}$). (**C**) Serum was obtained from mice in (B) at 4, 6, and 12 wk postimmunization and anti-bCII IgG2a or IgG2b titers were measured by ELISA. (**D**) Representative H&E-stained histology images from mice in (B) at different disease stages. From left to right: before immunization, 7 wk postimmunization, 12 wk postimmunization. Images after tamoxifen treatment depict the spectrum of recovery in ICOS$^{fl/-}$ mice. Scale bar, 100 µm.

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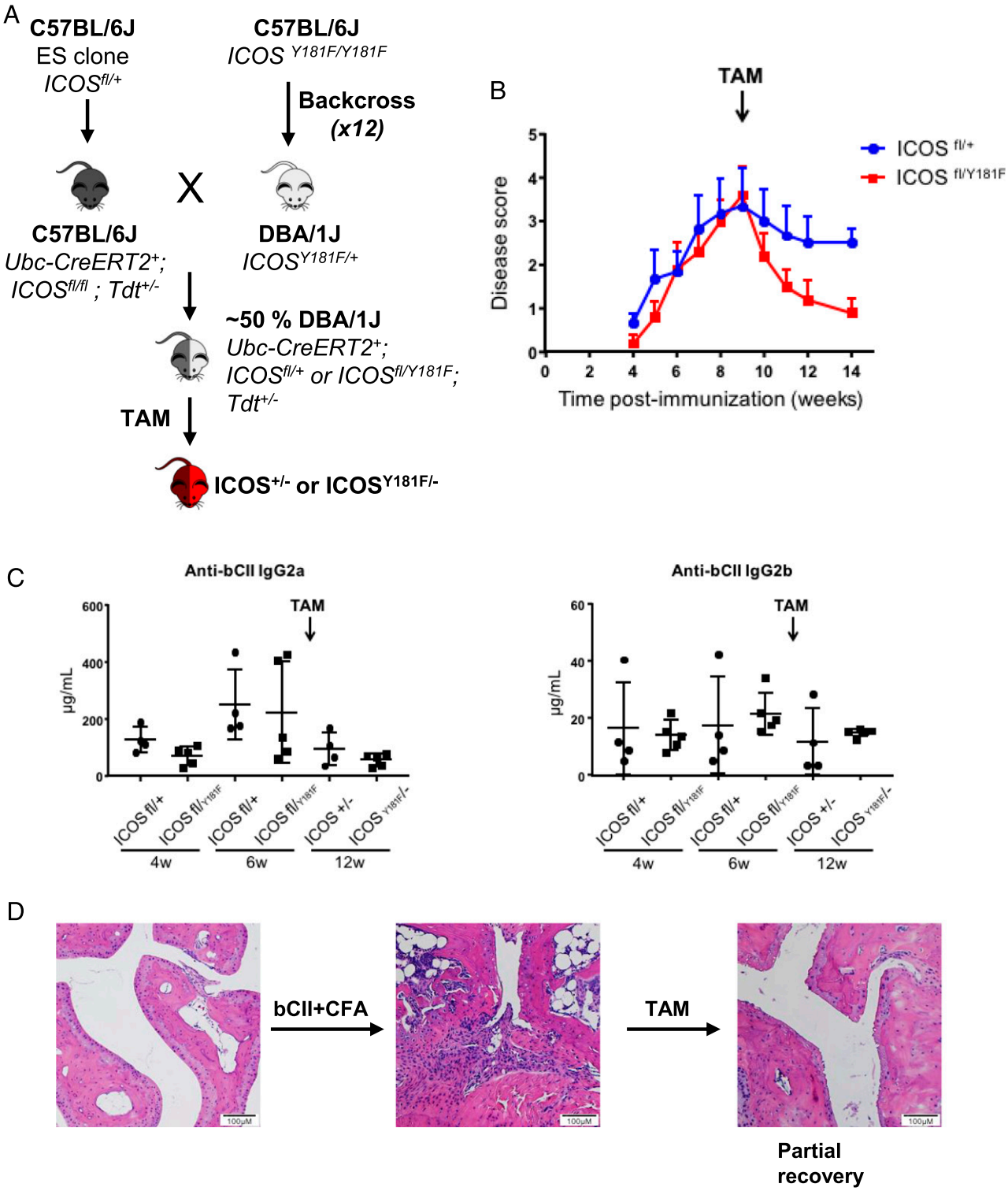

FIGURE 4. ICOS–PI3K signaling is a major contributor to maintenance of CIA. (**A**) Experimental design of conditional ICOS-PI3K experiments. UBC-CreERT2^{+} ICOS$^{fl/+}$ or ICOS$^{fl/Y181F}$ mice are treated with tamoxifen for 5 consecutive days, resulting in deletion of the floxed allele of ICOS. (**B**) Eight- to twelve-week-old mice described in (A) were immunized with bCII plus CFA, then treated for 5 d with tamoxifen at the peak of disease. Disease scores were given by visual inspection according to the criteria described in *Materials and Methods*. Data were pooled from two independent experiments (n = 6 ICOS$^{fl/+}$ and n = 10 ICOS$^{fl/Y181F}$) (**C**) Serum was obtained from mice in (B) at 4, 6, and 12 wk postimmunization and anti-bCII IgG2a or IgG2b titers were measured by ELISA. (**D**) Representative H&E-stained histology images from mice in (B) at different disease stages. From left to right: before immunization, 8 wk post-immunization, 14 wk postimmunization. Scale bar, 100 µm.

recently described ICOS–TANK-binding kinase 1 axis could also play a role in CIA maintenance, although it is more likely involved in CIA initiation considering that it promotes Tfh cell maturation (31).

Studies have shown that PI3K inhibitors can ameliorate CIA, but the mechanisms involved remain ill-defined (51, 52). The substantial recovery seen in mice left with ICOS^{Y181F} after tamoxifen treatment

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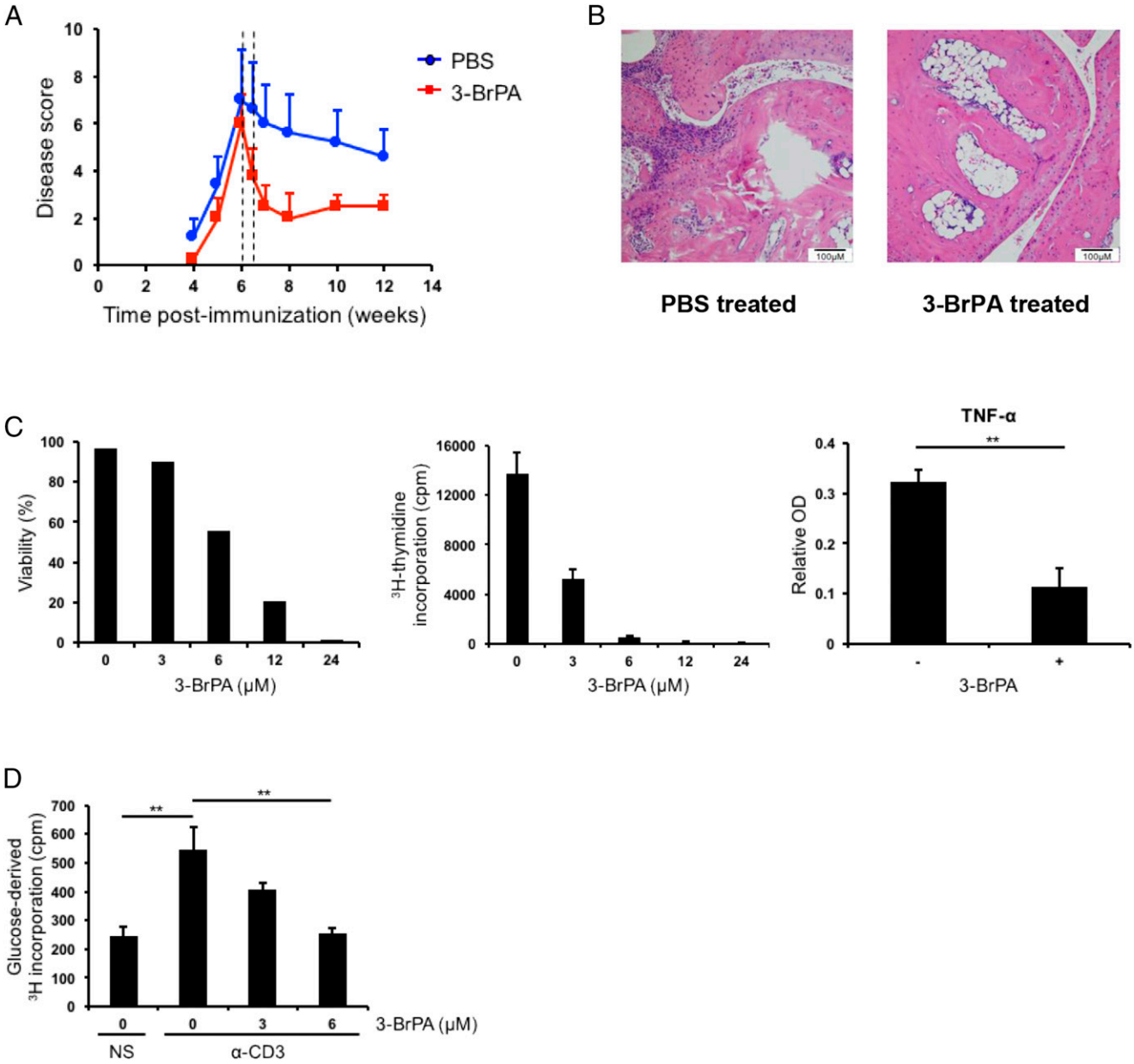

FIGURE 5. Glycolysis inhibitor ameliorates established CIA. (**A**) Eight- to twelve-week-old WT DBA/1 were immunized with bCII plus CFA, then treated with PBS or 5 mg 3-BrPA/kg per day for 3 d at the peak of disease. Disease scores were given by visual inspection according to the criteria described in *Materials and Methods*. Data represent two independent experiments with similar results (n = 5 PBS and n = 4 3-BrPA). (**B**) Representative H&E-stained histology images from mice in (A) at 12 wk postimmunization. Scale bar, 100 μm. (**C**) dLN cells from WT DBA/1 mice were extracted 2 wk postimmunization and restimulated in vitro for 48 h with increasing concentrations of 3-BrPA. Viability was assessed by flow cytometry and a representative sample is shown. Proliferation was measured by a [^{3}H]thymidine incorporation assay. TNF-α was detected by ELISA using culture supernatants taken from culture without (−) or with 3 μM 3-BrPA (+). Data represent two independent experiments with similar results (n = 3 for each group). (**D**) Lymph node cells from naive WT DBA/1 mice were extracted and cultured for 48 h with or without 1 μg/ml anti-CD3. During the last 4 h, 3-BrPA was added at the indicated concentrations along with 1 μCi per well D-[3-^{3}H]glucose to measure glucose metabolism. Data were pooled from two independent experiments. n = 3 for anti-CD3 plus 6 μM 3-BrPA and n = 6 for the other groups. $^{**}p < 0.02$.

prompted us to look for PI3K-dependent roles of ICOS other than Ab production. A recent study reported that ICOS–ICOSL interaction causes an increase of PI3K–Akt signaling that protected inflammatory T cells from apoptosis in lupus-prone mice (53). This could also hold true for joint-infiltrating T cells in CIA. Also, ICOS signaling can affect glycolysis through the PI3K–Akt–mTOR pathway (28, 36). Congruent with this, we found that a small molecule glycolysis inhibitor 3-BrPA can ameliorate established CIA. This confirms similar findings previously reported in the K/BxN arthritis model (40). Glycolysis promotes the proliferation of Th17 cells, which produce IL-17, a key inflammatory cytokine in RA and CIA (54–56).

Therapeutic targeting of glycolysis has been tested in cancer research showing safety and efficacy. For example, administration of 3-BrPA was successfully tested as a cancer treatment in mice (57, 58). It has also shown efficacy in at least one human cancer patient and will soon undergo phase I clinical trials (59). Because glycolysis has been identified as a possible factor in the progression of RA, it would be interesting to see whether 3-BrPA can be used to treat RA as well as cancer (38, 39).

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In conclusion, we demonstrated that ICOS provides important costimulatory signals for the initiation and maintenance of CIA. For the initiation phase, ICOS signaling appears to be critical for Ab production, but its role in the maintenance phase may involve inflammatory cytokines such as TNF-α. We identified a potential overlap between ICOS signaling and T cell glucose metabolism that can be targeted by readily accessible chemical inhibitors.

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Disclosures

The authors have no financial conflicts of interest.

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