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Toll/Interleukin-1 Receptor Domain Derived from TcpC (TIR-TcpC) Ameliorates Experimental Autoimmune Arthritis by Down-modulating Th17 Cell Response*[S]

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Evasion through immunomodulation is one of the several strategies adopted by pathogens to prolong their survival within the host. One such pathogen, *Escherichia coli* CFT073, utilizes an immunomodulatory protein, TcpC, to combat the host's innate immune defense. TcpC abrogates the function of MyD88 in macrophages, thus perturbing all the signaling processes that involve this adaptor protein. Although central to various signaling pathways initiated by IL-1, IL-18, and toll-like receptors, the precise contribution of MyD88 to the development of autoimmunity, particularly rheumatoid arthritis, still needs extensive exploration. Herein, by using the toll/interleukin-1 receptor (TIR) domain homologous C-terminal motif of TcpC, *i.e.* TIR-TcpC, we found MyD88 to be critical for the induction and progression of rheumatoid arthritis through its pivotal role in the development of Th17 cells, the subset of CD4$^+$ T-cells widely implicated in various autoimmune disorders. The TIR-TcpC mediated inhibition of signaling through MyD88, and subsequent amelioration of experimental autoimmune arthritis was observed to be an outcome of perturbations in the NFκB-RORγt (RAR-related orphan receptor γt) axis.

Rheumatoid arthritis (RA),[4] an autoimmune inflammatory disorder primarily of the diarthrodial joints, cripples about 1% of the global population and requires early therapeutic intervention for effective disease management. The clinical picture is characterized by extensive immune cell infiltration, inflammation of the synovial lining, and erosion of the cartilage and underlying bone. The pathogenesis of RA is complex with the involvement of numerous autocrine and paracrine cytokine loops that perpetuate and amplify the vicious cycle of chronic inflammatory pathways (1, 2). Considering the complicated immunopathology of RA and the active involvement of cells of both the innate and adaptive arms of the immune system, inhibition of multiple pathways is highly desirable. Alternatively, identification and targeting of molecules crucially involved in the activation of both arms of the immune system could be an important approach.

Identified as a gene up-regulated during myeloid differentiation in response to IL-6 (3), MyD88 has over the years emerged as a canonical adaptor protein for the toll-like receptors (TLRs) and IL-1 family of receptors including IL-18R (4). Moreover, with the participation of these receptors in the development of both innate and adaptive responses, the relevance of MyD88 as a key player and a promising therapeutic target for RA becomes enormous and demands extensive explorations.

MyD88 is a modular protein (5) that consists of a death domain at the N terminus (6) and a toll/IL-1 receptor (TIR) domain at the C terminus linked by an intermediate domain (5, 7). The cross-talk between MyD88 and its interacting partners is mediated by the TIR domain located at their C termini (8).

In the present study, we utilized the immunomodulatory property of a TIR domain-containing protein, TcpC, derived from uropathogenic *Escherichia coli* CFT073 (9) to study the role of MyD88 in the development of RA. TcpC bears a structural domain closely resembling the mammalian TIR domain in its C-terminal region. This domain aids TcpC in interacting with MyD88, thus preventing the association of MyD88 with its interacting partner (TLR or IL-1 receptor (IL-1R)), leading to abrogation of the signaling cascade (9–11).

We treated mice exhibiting collagen-induced arthritis (CIA) with TIR domain derived from TcpC (TIR-TcpC) and observed a significant remission of the disease activity. The disease-modifying effect of TIR-TcpC could be attributed to limitation of the pathogenic Th17 responses, which turned out to be an outcome of TIR-TcpC-induced inhibition of MyD88 signaling in dendritic cells (DCs) and CD4$^+$ T-cells. Abrogation of the MyD88 signaling cascade by TIR-TcpC arrested the activation of NFκB and subsequent transcription of downstream Th17 signature *RORγt* in naïve CD4$^+$ T-cells cultured under Th17-polarizing conditions.

Our study further demonstrates the significance of MyD88 signaling in the development of RA through its effects on Th17 differentiation and function as observed in complete and cell-specific *MyD88*$^{-/-}$ mice. Cumulatively, these results suggest a novel pathway for regulation of Th17 differentiation by MyD88 and also highlight the superiority of targeting MyD88 for effec-

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[4] The abbreviations used are: RA, rheumatoid arthritis; TLR, toll-like receptor; TIR, toll/interleukin-1 receptor; IL-1R, IL-1 receptor; CIA, collagen-induced arthritis; DC, dendritic cell; EGFP, enhanced GFP; COMP, cartilage oligomeric matrix protein; CTX II, C-terminal telopeptides of collagen type II; MMP, matrix metalloproteinase; PE, phycoerythrin; CII, chicken type II collagen; Treg, regulatory T-cell; RORγt, RAR-related orphan receptor γt.

tive management of Th17 cell-mediated autoimmunities. Furthermore, we put forward TIR-TcpC as a novel and appealing therapeutic molecule for rheumatoid arthritis.

Experimental Procedures

Cloning, Expression, and Purification—Uropathogenic *E. coli* CFT073 strain procured from American Type Culture Collection (ATCC) was cultured and maintained according to the supplier's instructions. Genomic DNA was isolated using a commercially available kit (Qiagen), and the region of interest was amplified using the following gene-specific primers: TIR-TcpC forward primer, 5′-AAGCTTTGTATGATTTTTTCATATCCCATGC-3′; TIR-TcpC reverse primer, 5′-AATTCTTATCTTCTCCTGTATGCTATTTCAGCCAACTC-3′; TIRRAR-TcpC reverse primer, 5′-AATTCTTATCTTGCCCTGTATGCTATTTCAGC-3′. The forward primer for TIRRAR-TcpC was the same as for TIR-TcpC. All the primers were 5′-phosphorylated. The amplified products (TIR-TcpC and TIRRAR-TcpC amplicons) were cloned into pPAL7 expression vector (Bio-Rad) using a restriction endonuclease-free cloning strategy. The identity of recombinants obtained was verified by sequence analysis. Plasmids pPAL7-TIR-TcpC and pPAL7-TIRRAR-TcpC were amplified in *E. coli* DH5α and transformed into *E. coli* BL21(DE3) cells. Protein expression was induced using 1 mM isopropyl β-D-thiogalactopyranoside (Calbiochem) for 4 h at 37 °C. The overexpressed protein was purified from inclusion bodies as follows. The inclusion bodies were harvested by centrifugation at 13,500 rpm for 60 min at 4 °C. The inclusion bodies obtained were solubilized in 0.1 M sodium phosphate buffer, pH 7.0, containing 8 M urea at room temperature (shaking for 1 h). Thereafter solubilized protein was dialyzed against a urea gradient (stepwise) at 4 °C. Refolded proteins TIR-TcpC and TIRRAR-TcpC with an 8-kDa N-terminal Profinity eXact tag (recognized by a mutant subtilisin protease, S189) were purified using Bio-Scale Mini Profinity eXact FPLC columns (Bio-Rad). Identity of the expressed protein was established by Western blotting using polyclonal serum raised against keyhole limpet hemocyanin-conjugated peptides derived from TcpC. Protein concentration was determined spectrophotometrically by measuring absorbance at 280 nm and calculated using the following equation: $A_{280} = \epsilon cl$ where ϵ is molar extinction coefficient (liters/(mol × cm)), A is absorbance at 280 nm, c is concentration (mol/liter), and l is the path length of the cuvette (cm). ϵ for TIR-TcpC and TIRRAR-TcpC is 29,910 liters/(mol × cm). The sequences corresponding to TIR-TcpC and TIRRAR-TcpC were subcloned into mammalian expression vector pEGFP-N1 (Clontech). The sequences of primers used were as follows: TIR-TcpC-EGFP forward primer, 5′-TCAAGCTTCGAATTCTGCCGCCACCATGTATGATTTTTTCATAT-3′; TIR-TcpC-EGFP reverse primer, 5′-GCGGTACCGTCGACTGTTATTATCTTCTCCTGTATGC-3′; TIRRAR-TcpC-EGFP reverse primer, 5′-GCGGTACCGTCGACTGTTATTATCTTGCCCTGTATGC-3′. The forward primer for TIRRAR-TcpC-EGFP was the same as for TIR-TcpC-EGFP. The identity of recombinants was verified by sequence analysis.

Tissue Culture, Cell Lines, and Reagents—Cytokines IL-1β, IL-2, IL-4, IL-6, IL-12, IL-23, TNF-α, and TGF-β were purchased from R&D Systems and Peprotech. Cell line RAW-Blue was procured from Invivogen was cultured and maintained according to the suppliers' instructions. Tissue culture media Dulbecco's modified Eagle's medium, heat-inactivated fetal bovine serum (certified), and antibiotic-antimycotic solution were obtained from Gibco. Zeocin, Normocin, Quantiblue, and p65IP were purchased from Invivogen. Methyl-β-cyclodextrin was obtained from Sigma.

Generation of Polyclonal Sera to TIR-TcpC—Antiserum was generated against TIR-TcpC by immunizing a rabbit (New Zealand White strain, 10–12 weeks old, male) subcutaneously with 200 μg of TIR-TcpC-derived peptides EQTLEVGDSLRRNIDL and FLNKKWTQYELDSLIC (Bioconcept) (sequences of the peptides were taken from a previous report (9)) conjugated to keyhole limpet hemocyanin (Sigma) and emulsified in complete Freund's adjuvant (Sigma). TIR-TcpC-derived peptides were coupled to keyhole limpet hemocyanin using the HOOK peptide coupling kit (GBiosciences). At day 14, a booster dosage of 150 μg was given in incomplete Freund's adjuvant (Sigma). Subsequently, on day 23, a 5–10-ml bleed was taken and analyzed for reactivity by Western blotting.

Mice—C57BL6/J, *B6.MyD88*$^{-/-}$, *B6.CD4-cre*, *B6.CD11c-Cre*, and *B6.MyD88*$^{flox/flox}$ mice procured from The Jackson Laboratory were housed and bred under standard conditions with *ad libitum* access to food and water. Animals were allowed to acclimate for at least 2 weeks prior to the experiments. The experimental protocol and animal handling were strictly in accordance with the Institutional Animal Ethics committee of the National Institute of Immunology, New Delhi, India.

Experimental Autoimmune Arthritis Induction and Assessment—Autoimmune experimental arthritis was induced in *C57BL6/J*, *B6.MyD88*$^{-/-}$, *B6.MyD88*ΔM, or *B6.MyD88*ΔT mice (male, 12–14 weeks old) as described previously (12). Briefly, mice were immunized intradermally with 100 μg of chicken type II collagen (Chondrex) emulsified in complete Freund's adjuvant. Chicken type II collagen was dissolved in l0 mM acetic acid with mild stirring at 4 °C. Each paw was scored individually for joint inflammation as described previously (13) on a scale of 0–4. The scoring system used was as follows: 0, no evidence of erythema and swelling; l, mild erythema or swelling (detectable arthritis); 2, moderate erythema and swelling; 3, significant erythema and swelling encompassing entire paw; 4, maximal swelling with or without limb distortion. For radiographic analysis, at the end of the treatment period (~6 weeks), animals were anesthetized, and fore- and hind paws were radiographed using *In Vivo* Imaging System FX Pro (Eastman Kodak Co.). The severity of bone erosion was ranked as described previously (13) using a modified version of the Larsen scoring method: 0, normal; 1, slight abnormality with any one or two of the exterior metatarsal bones showing slight bone erosion; 2, definite early abnormality with any of the metatarsal or tarsal bones showing bone erosion; 3, medium destructive abnormality with any of the metatarsal or any one of the tarsal bones showing definite bone erosion; 4, severe destructive abnormality with all the metatarsal bones showing definite bone erosion and at least one of the tarsometatarsal joints being completely eroded, leaving some bony joint outlines partly preserved; 5,

mutilating abnormality with no bony outlines that can be deciphered.

Assessment of Serological Markers of Disease—Serum samples were collected and assayed for levels of cartilage oligomeric matrix protein (COMP), C-terminal telopeptides of collagen type II (CTX II), and matrix metalloproteinase (MMP)-3 in an ELISA-based assay. Kits for COMP, CTX II, and MMP-3 were purchased from AnaMar, Immunodiagnostics Systems, and R&D Systems. For the cytokine assay, the levels of various cytokines in serum or joint tissue homogenates were quantified using Ready-SET-Go! ELISA kits (eBioscience) following the manufacturer's instructions. Values observed were normalized to control (buffer).

T-cell Isolation, Transfection, Differentiation, and NFκB Activity Estimation—Splenocytes harvested from *C57BL6/J* mice (4–6 weeks old, male) were magnetically sorted for naïve $CD4^+CD62L^+$ T-cells (Miltenyi Biotech). Purified naïve $CD4^+$ T-cells were electroporated with 0.5 μg of TIR-TcpC-EGFP or TIRRAR-TcpC-EGFP using an Amaxa Mouse T-cell Nucleofector kit (Lonza). Transfection efficiency was determined by FACS. Purified naïve T-cells were subjected to Th17 differentiation conditions as described previously (14, 15). Briefly, purified naïve T-cells (0.5 million cells/ml) were treated with anti-CD3 (plate-bound, 1 μg/ml; catalogue number 553057, BD Biosciences), anti-CD28 (1 μg/ml; catalogue number 553294, BD Biosciences), TGF-β (5 ng/ml), IL-6 (40 ng/ml), IL-23 (50 ng/ml), anti-IL-4 (5 μg/ml; catalogue number 554433, BD Biosciences), anti-IFNγ (5 μg/ml; catalogue number 554409, BD Biosciences), anti-IL-2 (10 μg/ml; catalogue number 554375, BD Biosciences), IL-1β (10 ng/ml), and TNF-α (10 ng/ml). For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (Sigma) and ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences) for 4 h prior to staining. To determine levels of NFκB activity in treated or untreated naïve $CD4^+$ T-cells, nuclear fractions were isolated using a Nuclear Extraction kit (Active Motif). NFκB activity was estimated using a TransAM p65 kit (Active Motif). NFκB activity was estimated in RAW-Blue cells according to the supplier's instructions.

Proliferation Assay—To determine proliferative activity, splenocyte cultures at the end of 48 h were pulsed with [^{3}H]thymidine for 10–12h. Thereafter cells were harvested onto Filtermat A (Wallac) and analyzed in a plate scintillation counter (PerkinElmer Life Sciences).

Intracellular Staining and FACS Analysis—Cells were harvested, washed, and resuspended in staining buffer (BD Biosciences). Thereafter cells were treated with Fc Block (1 μg/million cells; catalogue number 553142, BD Biosciences) for 15 min on ice. Subsequently cells were washed and incubated with PE-anti-CD4 (1:100; catalogue number 553730, BD Biosciences), Alexa Fluor 700-anti-CD4 (1:100; catalogue number 557956, BD Biosciences), PE-anti-CD11c (1:100; catalogue number 553802, BD Biosciences), anti-CD121a (1:50; catalogue number Ab8154, Abcam), FITC-anti-CD69 (1:250; catalogue number 553236, BD Biosciences), and Alexa Fluor 488-anti-rat IgG (1:1000; catalogue number 4416, Cell Signaling Technology) for 30 min on ice. Next, for intracellular staining, cells were resuspended in Cytofix/Cytoperm buffer (BD Biosciences) for 15 min at room temperature, washed with Perm/Wash buffer (BD Biosciences), and incubated with PE-anti-IFNγ (1:200; catalogue number 12-7311-41, eBioscience), FITC-anti-IFNγ (1:200; catalogue number 562019, BD Biosciences), Alexa Fluor 488-anti-IL-4 (1:100; catalogue number 557728, BD Biosciences), FITC-anti-IL-17A (1:100; catalogue number 560220, BD Biosciences), Alexa Fluor 488-anti-FoxP3 (1:100; catalogue number 563487, BD Biosciences), and FITC-anti-RORγt (1:100; catalogue number Ab104906, Abcam) for 30 min on ice. Thereafter cells were washed twice with Perm/Wash buffer and resuspended in staining buffer for acquisition. Data were acquired on a BD Biosciences FACSVerse and analyzed using Flowing Software 2.

Co-immunoprecipitation Assay—100 μg of TIR-TcpC carrying an N-terminal affinity tag (Profinity eXact tag) was incubated overnight at 4 °C with agarose coupled to a mutant subtilisin protease, S189, that binds with high specificity to the Profinity eXact tag. The resin bound to TIR-TcpC was washed twice with 0.1 M phosphate buffer (pH 7.2). Then lysates of naïve $CD4^+CD62L^+$ T-cells stimulated with IL-1β for 90 min were incubated with agarose-bound TIR-TcpC for 1 h at 4 °C followed by 30 min at 22 °C. Nonspecifically bound proteins were removed by washing five times with wash buffer (0.1 M phosphate buffer, pH 7.2, 0.1% Nonidet P-40, 1× protease inhibitor mixture (Sigma)). Lysates were prepared using non-denaturing Nonidet P-40 buffer (20 mM Tris HCl, pH 8, 137 mM NaCl, 1% Nonidet P-40, 2 mM EDTA) supplemented with 0.125% (w/v) *N*-octyl glucoside. Bound proteins were recovered from the agarose beads by boiling them in 2× SDS loading buffer followed by 10% SDS-PAGE and Western blotting.

Western Blotting—Protein samples electrophoresed on a 10% SDS-polyacrylamide gel were transferred onto nitrocellulose membrane using a semidry Trans-Blot system (Bio-Rad). The blots were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature and then incubated with anti-MyD88 (1:2500; catalogue number 4283, Cell Signaling Technology) and anti-IL-1RI (1:2000; Ab8154, Abcam) antibody overnight at 4 °C. Blots were then washed thrice using 1× TBST (1× Tris-buffered saline, pH 7.6, 0.1% Tween 20). Secondary antibody (anti-rabbit IgG-HRP (1:16,000; catalogue number 7074, Cell Signaling Technology) or anti-rat IgG-HRP (1:10,000; catalogue number 7077, Cell Signaling Technology)) was then added, and blots were incubated for 50 min at room temperature. Following three washes with TBST, blots were visualized using enhanced chemiluminescence reagent (Clarity ECL Western blotting substrate, Bio-Rad).

Cellular Uptake Assay of TIR-TcpC—For the *in vivo* cellular uptake assay, *C57BL6/J* mice (4 weeks old, male) were injected intravenously with FITC-conjugated BSA (FITC-BSA) or TIR-TcpC (FITC-TIR-TcpC) (5 mg/kg of body weight). Four hours later splenocytes were examined by flow cytometry for the FITC-positive population among dendritic cells (DCs) or T-cells ($CD4^+$) using PE-anti-CD11c (1:100) and Alexa Fluor 700-anti-CD4 (1:100) antibodies.

For the *in vitro* assay, $CD11c^+$ dendritic cells or naïve $CD4^+CD62L^+$ T-cells derived magnetically from spleen of *C57BL6/J* mice (4 weeks old, male, 0.1 million cells) were plated in poly-L-lysine-coated cell culture slides and treated with

TABLE 1

Effect of MyD88 deletion, TIR-TcpC treatment, and pretreatment: cumulative arthritic score (total arthritic score recorded over the entire experimental duration) and clinical onset of disease

Experiment	Treatment group	Mean ± SEM
TIR-TcpC: Treatment (cumulative arthritic score)	DC	54.2 ± 3.2
	TIR-TcpC	25.8 ± 1.7
$p < 0.05$, n=5, Student's t-test (unpaired, two tailed)		
TIR-TcpC: Pre-treatment (disease onset)*	DC	33.0 ± 3.5
	TIR-TcpC	40.0 ± 1.6
$p = 0.11$, n=5, Student's t-test (unpaired, two tailed)		
TIR-TcpC: Pre-treatment (cumulative arthritic score)	DC	36.8 ± 12.1
	TIR-TcpC	9.0 ± 4.0
$p = 0.07$, n=5, Student's t-test (unpaired, two tailed)		
Effect of MyD88 deletion (disease onset)*	WT	31.7 ± 3.7
	MyD88$^{-/-}$	44.0 ± 0.0
	MyD88ΔT	32.0 ± 3.8
	MyD88ΔM	34.3 ± 3.6
$p < 0.05$, n=5, Analysis of variance (one factor)		
Effect of MyD88 deletion (cumulative arthritic score)	WT	36.5 ± 11.8
	MyD88$^{-/-}$	0.0 ± 0.0
	MyD88ΔT	13.1 ± 4.4
	MyD88ΔM	6.1 ± 2.6
$p < 0.05$, n=5, Analysis of variance (one factor)		
* Disease onset was considered 44 days (total duration of experiment) in case of disease free animals		

FITC-BSA, FITC-TIRRAR-TcpC, or FITC-TIR-TcpC (0.1 mg/ml) for 90 min. Cells were then washed, fixed with 4% paraformaldehyde, and mounted in Fluoroshield DAPI (Sigma), and images were acquired in a confocal microscope (LSM510 Meta).

Statistical Analysis—Multiple or binary comparisons were analyzed using analysis of variance (single factor) and Student's *t* test (unpaired, two-tailed), respectively. Differences in mean values were considered significant when p was <0.05.

Results

Uropathogenic E. coli-derived TIR-TcpC Ameliorates Collagen-induced Arthritis—TIR homologous domain (residues 1–137) of TcpC, an immunomodulatory protein secreted by uropathogenic *E. coli* CFT073 (9), was expressed recombinantly in a bacterial system (supplemental Fig. S1, *A–F*). Recombinant TIR-TcpC was analyzed for its biological activity in RAW-Blue cells, a mouse macrophage cell line that harbors an NFκB-inducible secreted embryonic alkaline phosphatase reporter construct. The treatment with TIR-TcpC inhibited lipopolysaccharide (TLR-4 ligand)-driven NFκB activation in a dosage-dependent manner with more than 50% reduction at a concentration of 2 μM. Surprisingly, the inhibitory potential of TIR-TcpC was several times greater than that of p65IP (commercially available positive control) (supplemental Fig. S2).To analyze the therapeutic effect of TIR-TcpC, autoimmune arthritis with characteristics similar to RA was induced in *C57BL6/J* mice by immunization with heterologous chicken type II collagen (CII). Nearly 70% of mice immunized with CII displayed joint inflammation on day 28 postimmunization. Upon clinical onset of the disease, mice were randomized into two experimental groups, *viz.* disease control and TIR-TcpC, and treated with vehicle or TIR-TcpC, respectively, for 2 weeks. An approximate 50% reduction in joint inflammation was readily evident (Fig. 1*A* and Table 1). Also, radiographs acquired at the end of the treatment period distinctly showed the absence of early signs of joint erosion in TIR-TcpC-treated animals (modified Larsen score = 0) that had begun in disease control mice (modified Larsen score = 1). Apart from integrity of joint surfaces, radiographs also revealed a drastic decrease in soft tissue volume (translucent regions around bones that essentially signify inflammatory edema) (Fig. 1*B*). An assessment of the serum biomarkers implicated in various disease processes such as inflammation, cartilage damage, and bone erosion was also carried out. The levels of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 were significantly reduced (Fig. 1, *C–E*). These proinflammatory cytokines are known to induce the release of soluble inflammatory mediators such as prostaglandins and leukotrienes. Furthermore, the levels of COMP, a component of the articular cartilage, and fragments of CII (CTX II), which are released into the circulation upon degradative action of MMPs, were found to be significantly low (Fig. 1, *F* and *H*). Low levels of COMP and CTX II were additionally a reflection of reduced activity of MMP-3 in line with its observed serum levels (Fig. 1*G*). The reduced levels of COMP, CTX II, and MMP-3 primarily indicated architectural conservation of the cartilaginous tissue, hence the protective effect of TIR-TcpC treatment. This was also an indication that the synovial lining in TIR-TcpC-treated mice had not been transformed into pannus, a hyperplastic aggressive tissue that is a rich source of matrix-degrading enzymes and cytokines. In another scenario, *i.e.* co-treatment, CII-immunized animals were treated with TIR-TcpC from days 0 to 14 postimmunization. In comparison with treatment, the mean arthritic score in both the experimental groups was lower, which essentially reflects disease-free survival in 25–30% of mice immunized with CII. In the co-treatment experiment, the severity of the arthritic score was found to be reduced by 70% in mice injected with TIR-TcpC. In addition to a reduction in disease severity, clinical onset of disease was delayed by ~1 week (Fig. 1, *I* and *J*, and Table 1).

TIR-TcpC Treatment Impaired CD4$^+$ T-cell Effector Functions—As CIA is a Th17 cell-mediated pathology (16), we examined the splenocytes of treated mice for various CD4$^+$ T-cell subsets. To study the impact of TIR-TcpC treatment on effector T-cell functions, *C57BL6/J* mice were immunized with CII and on clinical onset of the disease were treated with TIR-TcpC. At the end of the treatment period (2 weeks), splenocytes were analyzed by flow cytometry for various CD4$^+$ T-cell phenotypes such as Th1 (CD4$^+$IFNγ$^+$), Th2 (CD4$^+$IL-4$^+$), Th17 (CD4$^+$Th17$^+$), and Treg (CD4$^+$FoxP3$^+$). A significant reduction was noted in the proportion of Th1 and Th17 cells, whereas the proportions of other phenotypes remained unaltered (Fig. 2*A*). The findings of flow cytometric analysis were corroborated by the near-normal levels of IL-17A and IFNγ in the joint tissue homogenates of treated mice vis-à-vis untreated controls (Fig. 2*B*).

Besides analyzing the effect of TIR-TcpC treatment on effector T-cell phenotypes, splenocytes of treated mice were also analyzed for activation. T-cell activation was assessed using the activation marker CD69. Surprisingly, the frequencies of CD4$^+$CD69$^+$ cells were comparable between TIR-TcpC treatment and control groups (Fig. 2*A*). Taken together, TIR-TcpC

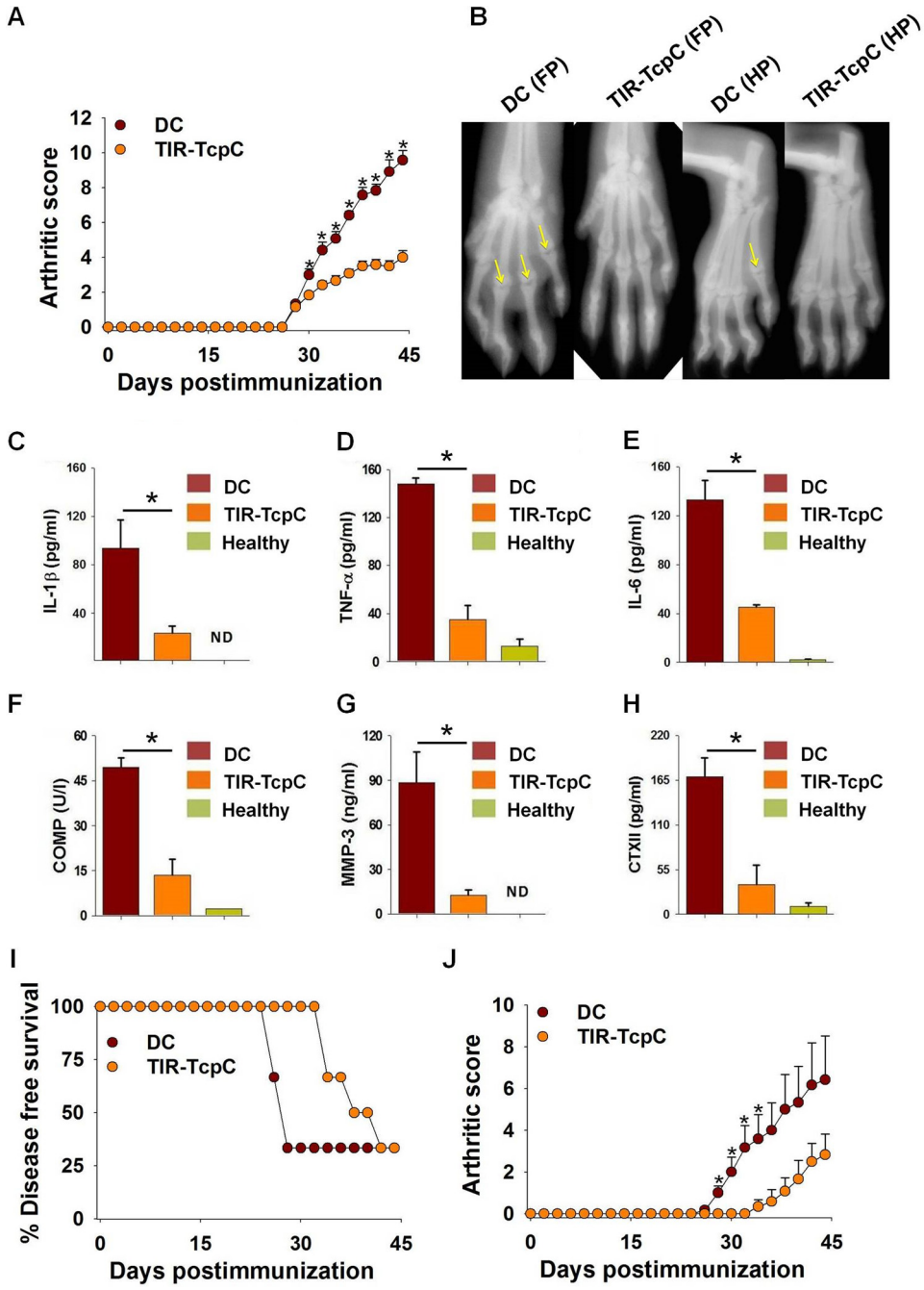

FIGURE 1. **TIR-TcpC treatment alleviates CIA.** *C57BL6/J* mice (12–14 weeks old, male) were immunized intradermally with CII. On clinical onset, diseased mice were treated with TIR-TcpC (5 mg/kg of body weight) or vehicle (PBS) daily for 2 weeks. *A*, arthritic score (mean ± S.E.; $n = 5$; *, $p < 0.05$; data in the figure represent three independent experiments; *error bars* represent S.E.). *B*, radiographs (representative) acquired at the end of treatment period. *C–H*, levels of various disease markers, namely IL-1β (*C*) (mean ± S.D.; $n = 5$; *, $p < 0.05$), TNF-α (mean ± S.D.; $n = 5$; *, $p < 0.05$) (*D*), IL-6 (mean ± S.D.; $n = 5$; *, $p < 0.05$) (*E*), COMP (mean ± S.D.; $n = 5$; *, $p < 0.05$) (*F*), MMP-3 (mean ± S.D.; $n = 5$; *, $p < 0.05$) (*G*), CTX-II (mean ± S.D.; $n = 5$; *, $p < 0.05$) (*H*), in serum samples drawn at the end of the treatment period. *Error bars* in *C–H* represent S.D. *C57BL6/J* mice (12–14 weeks old, male) were immunized with CII and treated with TIR-TcpC (5 mg/kg of body weight) from days 0 to 14 postimmunization, and percent disease-free survival (*I*) and arthritic score (*J*) were determined (mean ± S.E. ($n = 5$); *, $p < 0.05$). *Error bars* represent S.D. Data in the figure represent three independent experiments. *DC*, disease control; *FP*, forepaw; *HP*, hind paw; *ND*, not detected; *U/l*, units per liter.

treatment blocked differentiation of naïve $CD4^+$ T-cells into pathogenic phenotypes, *i.e.* Th1 and Th17. The observation therefore reflects the remarkable immunomodulatory property of TIR-TcpC *in situ*.

Mice with Complete or Cell-specific MyD88 Knockdown Are Resistant to CIA—TcpC has been shown previously to require an interaction with MyD88 for activity (9). To confirm the specificity of this interaction and hence the mechanism of action of TIR-TcpC, we performed pulldown assays in naïve $CD4^+$ T-cells. The cells were activated and prestimulated with IL-1β for 90 min to increase the intracellular expression of MyD88. The pulldown assays revealed a specific interaction between MyD88 and purified TIR-TcpC that was concentration-dependent (Fig. 3). However, TIR-TcpC did not interact with type I IL-1 receptor, another member of the IL-1 signaling cascade. These results corroborate a previous report that highlighted

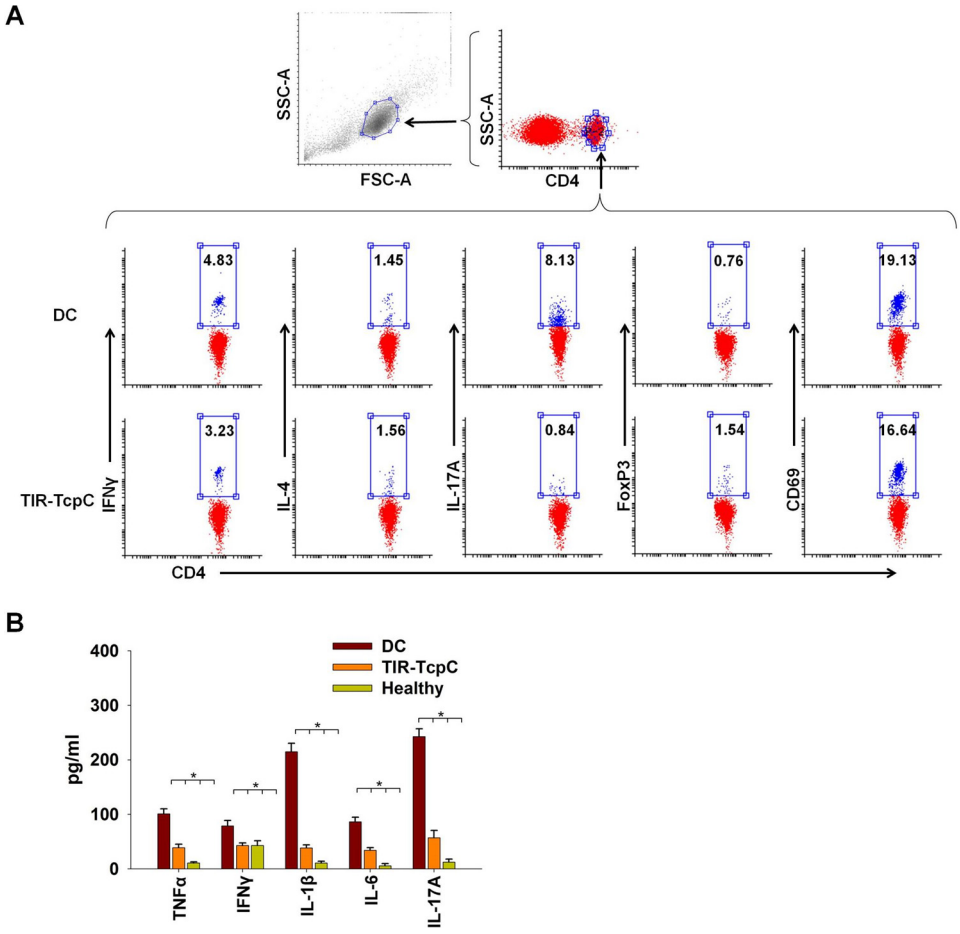

FIGURE 2. **TIR-TcpC impairs CD4$^+$ T-cell effector functions.** Splenocytes derived from *C57BL6/J* mice (12–14 weeks old, male) immunized with CII and treated with TIR-TcpC (5 mg/kg of body weight) from days 28 to 41 were analyzed by flow cytometry for Th1 (CD4$^+$IFNγ$^+$), Th2 (CD4$^+$IL-4$^+$), Th17 (CD4$^+$IL-17A$^+$), Treg (CD4$^+$FoxP3$^+$), and activated (CD4$^+$CD69$^+$) T-cells. *A*, representative FACS dot plots. *B*, levels of proinflammatory cytokines in joint tissue homogenates of TIR-TcpC-treated (5 mg/kg of body weight) or untreated (*DC*) animals (mean ± S.D.; $n = 5$; *, $p < 0.05$). *Error bars* in the figure represent S.D. Data in the figure represent three independent experiments. *DC*, disease control; *FSC*, forward scatter; *SSC*, side scatter.

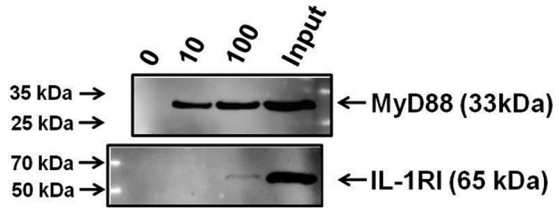

FIGURE 3. **TIR-TcpC interacts specifically with MyD88.** Concentrated lysates of naïve CD4$^+$CD62L$^+$ T-cells (protein concentration, 50 μg/ml) stimulated with IL-1β (10 ng/ml) were subjected to co-immunoprecipitation using the Profinity eXact-tagged TIR-TcpC (total protein, 0, 10, and 100 μg) and S189-agarose bead system (described under "Experimental Procedures"). Proteins co-immunoprecipitated with TIR-TcpC were analyzed for the presence of MyD88 or IL-1RI by Western blotting. Total lysate loaded (*Input*) served as a control for expression of MyD88 and IL-1RI in the lysates. Data in the figure represent three independent experiments.

interactions between the TIR domain of TcpC and MyD88 but not with other components of the TLR signaling pathway in RAW264.7 macrophages (9). Therefore, it was noteworthy to examine the effect of MyD88 deletion on the progression of CIA. For this, *B6.MyD88*$^{-/-}$ mice or their wild type counterparts were immunized with CII and monitored for joint inflammation. Although CIA progressed normally in the control group (wild type), *MyD88*$^{-/-}$ mice did not develop the disease at all (Fig. 4, *A* and *B*). Furthermore, the frequency of Th1 and Th17 cells was negligible in splenocytes when analyzed using flow cytometry at the end of 4 weeks postimmunization with CII (Fig. 4, *C* and *D*). Although the impact of TIR-TcpC treatment or MyD88 deletion on CD4$^+$ T-cell differentiation and consequently on autoimmunity is through macrophages/dendritic cells (antigen-presenting cells) as expected, it was still important to examine the relative contribution of CD4$^+$ T-cell- or CD11c$^+$ dendritic cell-specific MyD88 on CIA and *in situ* T-cell differentiation. Mice with specific MyD88 deletion in CD4$^+$ T-cells (*MyD88ΔT*) or CD11c$^+$ dendritic cells (*MyD88ΔM*) were generated by crossing *B6.MyD88*$^{flox/flox}$ with *B6.CD4-cre* or *B6.CD11c-cre*, respectively. *MyD88ΔT*, *MyD88ΔM*, or wild type mice were immunized with CII. Although the percent incidence and clinical onset remained comparable, the severity of CIA in *MyD88ΔT* or *MyD88ΔM* mice was noted to be 60–65% lower than that of wild type controls (Fig. 4, *A* and *B*, and Table 1). Furthermore, flow cytometric analysis of splenocytes revealed a significant decrease in the frequency of Th17 cells in *MyD88ΔT* mice and *MyD88ΔM* mice, whereas relative proportions of Th1 cells were comparable between *MyD88ΔT* and wild type control (Fig. 4, *C* and *D*). Contrary to CD4$^+$T-cells, CD11c$^+$ dendritic cell-specific MyD88 deletion significantly down-modulated Th1 cell differ-

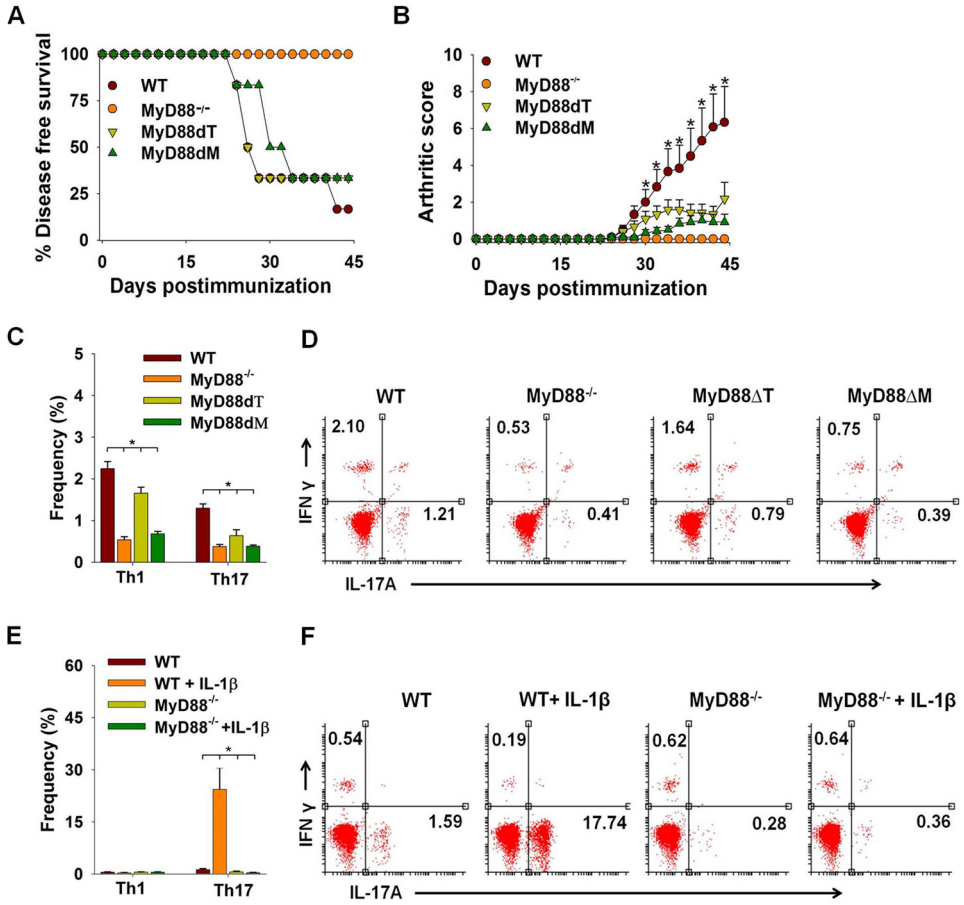

FIGURE 4. **MyD88 deficiency impairs Th17 cell differentiation and development of CIA.** *MyD88*$^{-/-}$, *MyD88ΔT*, *MyD88ΔM*, or wild type *C57BL6/J* mice (12–14 weeks old, male) were immunized with CII, and percent disease-free survival (*A*) and arthritic score (*B*) were determined (mean ± S.E.; $n = 5$; *, $p < 0.05$). *Error bars* represent S.E. Data in the figure represents three independent experiments. *C*, frequencies of Th1 ($CD4^+IFN\gamma^+$)/Th17 ($CD4^+$ $IL\text{-}17A^+$) cells in spleen analyzed by flow cytometry on day 28 postimmunization (mean ± S.D.; $n = 3$; *, $p < 0.05$). *Error bars* represent S.D. *D*, representative FACS dot plots. $CD4^+CD62L^+$ naïve T-cells isolated from *MyD88*$^{-/-}$ or wild type *C57BL6/J* mice (4–6 weeks old, male) were cultured in Th17-polarizing conditions (described under "Experimental Procedures") in the presence or absence of IL-1β (10 ng/ml). *E*, frequencies (mean ± S.D.; $n = 3$; *, $p < 0.05$). *Error bars* represent S.D. *F*, representative FACS dot plots.

entiation also (Fig. 4, *C* and *D*). For *ex situ* analysis, $CD4^+CD62L^+$ naïve T-cells were isolated from *MyD88*$^{-/-}$ or wild type mice and subjected to Th17 differentiation conditions. In line with *in situ* differentiation analysis, naïve T-cells derived from *MyD88*$^{-/-}$ mice showed negligible differentiation into Th17 cells and were unresponsive to IL-1β treatment (Fig. 4, *E* and *F*). To summarize, although MyD88 deletion limited to $CD4^+$ T-cells or $CD11c^+$ dendritic cells significantly impairs Th17 cell differentiation, complete knockdown of MyD88 totally abrogates Th17 development and hence CIA.

TIR-TcpC Blocks IL-1β-dependent Th17 Cell Differentiation—The impaired $CD4^+$ T-cell differentiation and autoimmunity, particularly in *MyD88ΔT* animals, prompted us to examine the direct effect of TIR-TcpC treatment on Th17 cell development. First of all the effect of proinflammatory cytokines, *i.e.* IL-1β, IL-6, and TNF-α, the levels of which were found to be down-modulated in treated joints, was studied on *in vitro* Th17 differentiation. As reported previously (15), the presence of IL-1β in the differentiation milieu significantly enhanced Th17 cell differentiation, whereas TNF-α had no effect (Fig. 5, *A* and *B*). The frequency of Th17 cells was negligible when IL-6 was not included in the differentiation mixture, signifying its indispensable role in Th17 cell differentiation (Fig. 5, *A* and *B*). Surprisingly, TIR-TcpC treatment had an inhibitory effect only when IL-1β was included in the differentiation mixture (Fig. 5, *A* and *B*). In conclusion, TIR-TcpC treatment blocked only the IL-1β-mediated increase in Th17 cell differentiation.

The detrimental effect of TIR-TcpC treatment on IL-1β-mediated Th17 cell differentiation was analyzed in further detail. Expression of IL-1RI is essential for IL-1 activity. Thus, naïve $CD4^+$ T-cells treated with IL-1β in the presence of TIR-TcpC were analyzed for surface expression of CD121a (IL-1RI). Flow cytometric analysis revealed no significant difference in the expression of IL-1RI (Fig. 5*C*). This was also indicative of intact IL-6 signaling even in the presence of TIR-TcpC as the former is known to be a critical factor for IL-1RI up-regulation during polarization (15). Furthermore, TIR-TcpC-treated naïve $CD4^+$ T-cells were noted to express lower levels of RORγt (master transcription factor involved in Th17 differentiation (17)) vis-à-vis the untreated control (Fig. 5*C*).

C-terminal Arg-Arg-Arg Motif Is Critical for Translocation across Cell Membrane—Cirl *et al.* (9) have shown internalization and subsequent blocking activity of TIR-TcpC to be My D88-dependent in uroepithelial cells and macrophages. Because endocytic uptake of protein antigens is relatively

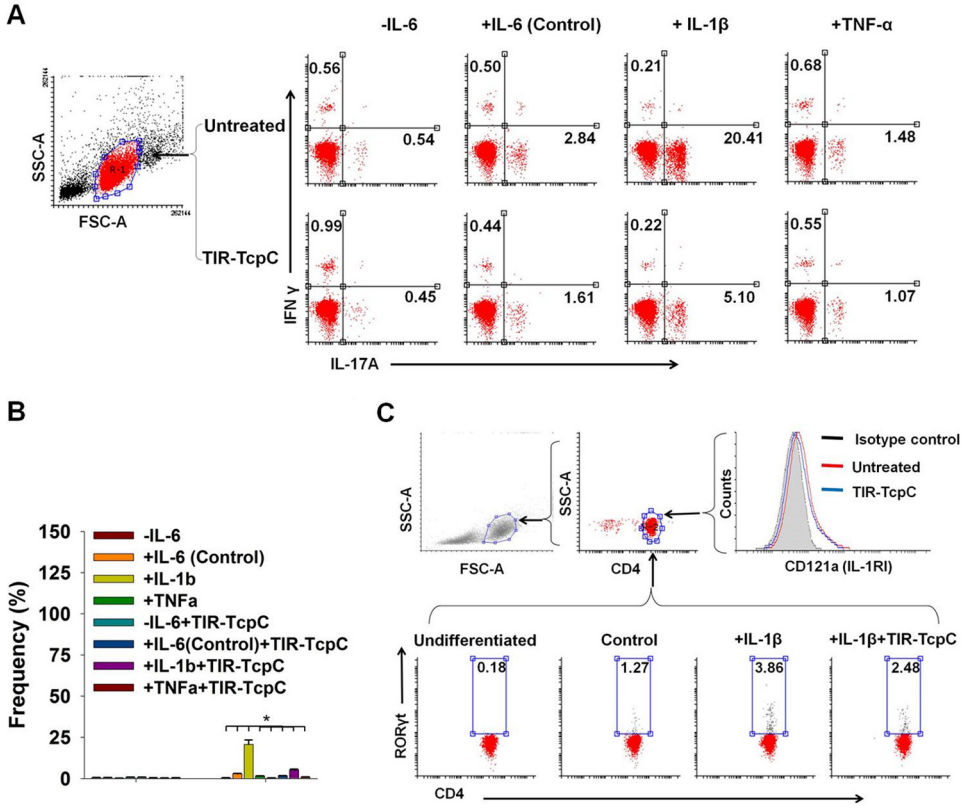

FIGURE 5. **TIR-TcpC treatment blocks IL-1β-dependent RORγt activation and Th17 cell differentiation.** Naïve CD4$^+$CD62L$^+$ T-cells isolated from spleen of *C57BL6/J* mice (4–6 weeks old, male) were subjected to Th17-polarizing conditions (described under "Experimental Procedures") in the presence or absence (control) of IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-6 (40 ng/ml), and/or TIR-TcpC (1 mg/ml) and analyzed by flow cytometry for the expression of IL-17/IFNγ (Th1/Th17). *A*, representative FACS dot plots. *B*, frequencies (mean ± S.D.; $n = 3$; *, $p < 0.05$). *C*, IL-1RI (CD121a) representative histogram and RORγt representative dot plots. *Error bars* in the figure represent S.D. *FSC*, forward scatter; *SSC*, side scatter.

unknown in the case of helper T-cells, it was intriguing to explore the mechanism of its entry. A careful analysis of the TIR-TcpC sequence revealed the presence of three consecutive arginine residues (arginines 135–137) at the C terminus (supplemental Fig. S1*G*). Numerous studies in the past have highlighted the remarkable property of cationic peptides especially polyarginine to penetrate cell membranes. We, therefore, hypothesized the arginine triplet (arginines 135–137) to mediate cell penetration of TIR-TcpC. To test this, we generated a variant of TIR-TcpC in which the arginine triplet (Arg-Arg-Arg) was mutated to Arg-Ala-Arg, designated as TIRRAR-TcpC, to see whether the consecutive triple arginine is necessary for internalization (supplemental Fig. S1*G*). Spleen-derived CD11c$^+$ dendritic cells or CD4$^+$CD62L$^+$ naïve T-cells were treated with fluorescently labeled TIR-TcpC, TIRRAR-TcpC, or BSA (control) and analyzed by confocal microscopy. TIR-TcpC could readily be observed more or less evenly distributed in the cytosol of dendritic cells and naïve T-cells, whereas TIRRAR-TcpC could not enter the cytosol (Fig. 6*A*). Furthermore, to study the uptake of TIR-TcpC *in vivo*, *C57BL6/J* mice (4–6 weeks old, male) were injected with FITC-TIR-TcpC, and 4 h later CD11c$^+$ DCs and CD4$^+$ T-cells in spleen were analyzed for the FITC$^+$ population by FACS. Significant proportions among DCs and T-cells were noted to be positive for FITC (Fig. 6*B*). To analyze its impact on NFκB activation, naïve CD4$^+$CD62L$^+$ T-cells were stimulated with IL-1β in the presence of TIR-TcpC or TIRRAR-TcpC. NFκB activation was quantified as described under "Experimental Procedures." As expected, the IL-1/MyD88-mediated NFκB activation was persistent in the presence of TIRRAR-TcpC, also suggesting its failure to translocate across the plasma membrane and subsequently prevent IL-1/MyD88 interaction through their TIR domains (Fig. 6*C*). However, it was also important to see that the observed abrogation of activity is not an outcome of mutation-induced structural changes in TIRRAR-TcpC. For this, the tyrosine fluorescence spectrum was recorded. TIR-TcpC and TIRRAR-TcpC showed exact spectral overlap, signifying minimal structural perturbations in TIRRAR-TcpC (Fig. 6*D*). Furthermore, TIR-TcpC and TIRRAR-TcpC sequences were cloned into a mammalian expression vector, electroporated into murine CD4$^+$CD62L$^+$ T-cells, and probed for their ability to block IL-1 signaling-dependent NFκB activation. Both were found to be comparable in inhibiting NFκB activation (Fig. 6*E*). Thus, from these experiments, we concluded that the Arg-Arg-Arg motif is critical for its translocation through the cell membrane.

Several virulence factors, polycationic peptides (polyarginines), have been shown to be internalized into living cells via endocytosis and/or through lipid rafts (18). To explore the role of lipid rafts and/or endocytosis in the entry of TIR-TcpC, naïve T-cells treated with methyl-β-cyclodextrin (a cholesterol-extracting reagent that disrupts lipid rafts) were examined for IL-1β-dependent activation of NFκB. Methyl-β-cyclodextrin

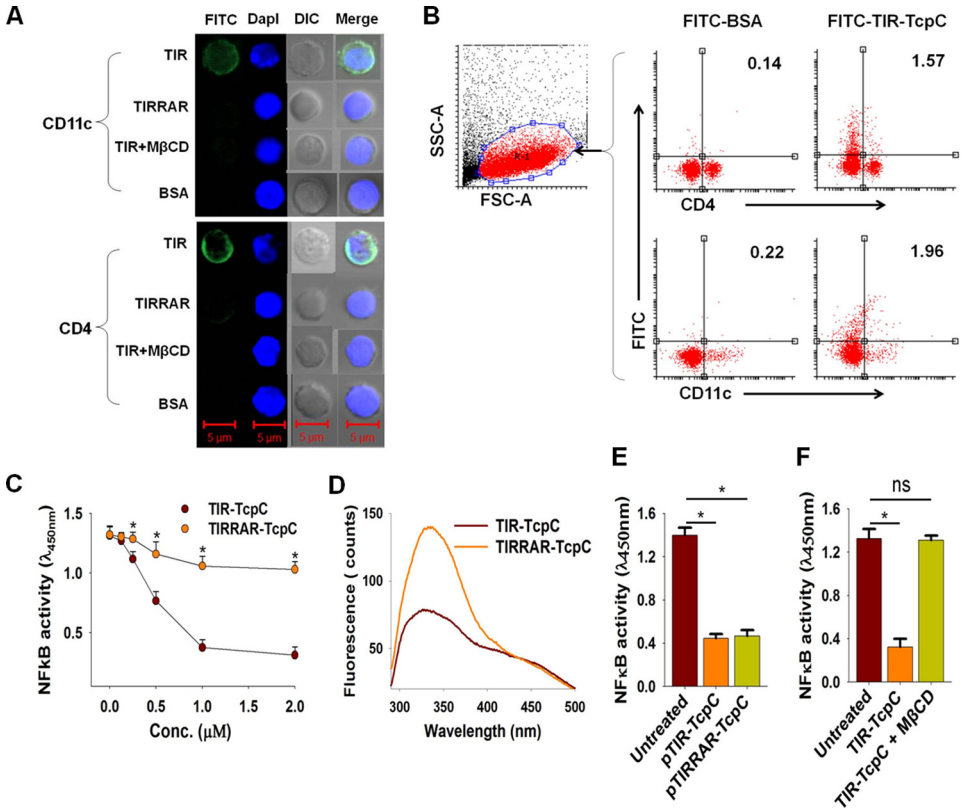

FIGURE 6. **C-terminal Arg-Arg-Arg motif is critical for translocation across cell membrane.** CD11c$^+$ dendritic cells or CD4$^+$CD62L$^+$ (naïve) T-cells isolated from spleen of *C57BL6/J* mice (male, 4–6 weeks old) were treated with FITC-TIR-TcpC (*TIR*), FITC-TIRRAR-TcpC (*TIRRAR*), FITC-BSA (*BSA*) (0.1 mg/ml), and/or methyl-β-cyclodextrin (*MβCD*) (10 mM). *A*, representative confocal photomicrographs acquired 90 min post-treatment. *Scale bars*, 5 μm.*C57BL6/J* mice (male, 4–6 weeks old) were injected intravenously with FITC-BSA or FITC-TIR-TcpC (5 mg/kg of body weight), and 4 h later dendritic cells (CD11c$^+$) or T-cells (CD4$^+$) in spleen were analyzed by flow cytometry for the presence of fluorescent protein. *B*, representative FACS dot plots. *C*, NFκB activity in nuclear fractions of naïve T-cells stimulated with IL-1β (10 ng/ml) in the presence of TIR-TcpC (1 mg/ml) (mean ± S.D.; $n = 3$; *, $p < 0.05$). *D*, tyrosine fluorescence (excitation wavelength, 275 nm) spectra (290–500 nm) of TIR-TcpC and TIRRAR-TcpC. *E*, NFκB activity in nuclear fractions of IL-1β-stimulated CD4$^+$CD62L$^+$ T-cells transfected with TIR-TcpC or TIRRAR-TcpC constructs (mean ± S.D.; $n = 3$; *, $p < 0.05$). *F*, NFκB activity in nuclear fractions of methyl-β-cyclodextrin (10 mM)-treated naïve T-cells upon stimulation with IL-1β (10 ng/ml) in the presence of TIR-TcpC (1 mg/ml) (mean ± S.D.; $n = 3$; *, $p < 0.05$). *Error bars* in the figure represent S.D. *FSC*, forward scatter; *SSC*, side scatter; *ns*, not significant; *DIC*, differential interference contrast.

prevented TIR-TcpC-mediated suppression of NFκB activation (IL-1-driven) (Fig. 6*F*).

TIR-TcpC Treatment Does Not Generate Neutralizing Immune Response—To determine whether TIR-TcpC treatment generates a neutralizing immune response, serum samples drawn at the end of the treatment period were analyzed for antibody titer. Also, splenocytes isolated at the end of the treatment period were examined for proliferative activity in response to TIR-TcpC stimulation. Both anti-TIR-TcpC titer and TIR-TcpC-specific proliferative response were noted to be close to untreated controls, whereas the proliferative response to CII stimulation was high (Fig. 7, *A* and *B*).

Discussion

The present study utilized an immunomodulatory protein of bacterial origin, TIR-TcpC, known for active subversion of MyD88-dependent TLR signaling pathway, to underscore the functional relevance of MyD88 in the pathogenesis of rheumatoid arthritis (9, 11). Surprisingly, TIR-TcpC was not only instrumental in delineating the precise contribution of MyD88 to RA pathology, but it also emerged as a promising therapeutic molecule targeting multiple pathways simultaneously. It drastically suppressed several aspects of arthritis in terms of inflammation, cartilage damage, and bone erosion. Although treatment with TIR-TcpC reduced the articular score by 50–60%, it is likely that prolonged treatment or higher dosages would have further suppressed the inflammatory disease processes. As TIR-TcpC has been shown to be as effective as full-length protein, TIR-TcpC was used in our study (9). Th17 cells, a subset of the CD4$^+$ helper T-cell lineage, have emerged as key contributors to the disease processes in RA as well as in CIA (19–22). In humans a strong correlation between systemic disease activity and the circulating levels of IL-17 and Th17 cells has been reported in RA patients wherein IL-17 promotes joint destruction and bone resorption; in CIA neutralization of IL-17 has been found to be protective against disease development, and its overexpression is associated with a spontaneous and more severe form of autoimmune arthritis (23–29). Consistent with this, abrogation of Th17 responses by TIR-TcpC led to a significant limitation of the arthritic processes.

The observed defect in Th17 function induced by TIR-TcpC was determined to be an outcome of inhibition of various pro-inflammatory signaling cascades involving MyD88. Furthermore, TIR-TcpC affected the differentiation of Th17 cells in a drastic manner with modest effects on Th1 cells. This is in agreement with recent reports that highlight increased expres-

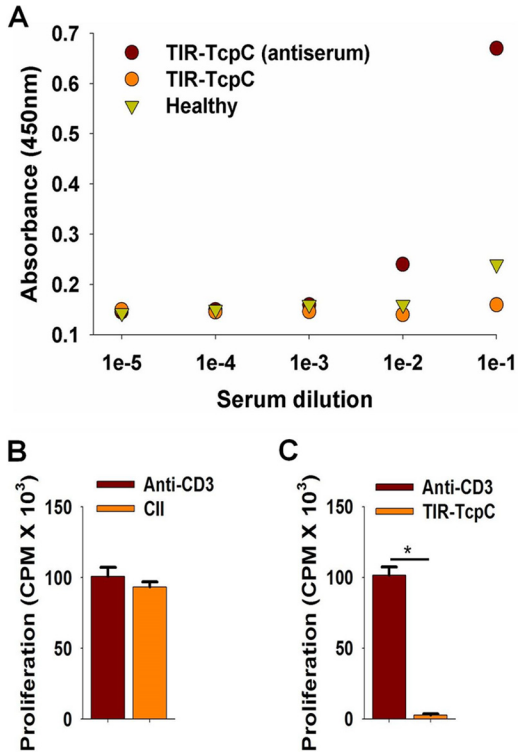

FIGURE 7. **TIR-TcpC treatment does not generate neutralizing immune response.** *C57BL6/J* mice (12–14 weeks old, male) were immunized with CII and treated with TIR-TcpC (5 mg/kg of body weight) or vehicle from days 0 to 14. *A*, anti-TIR-TcpC antibody titer. *B* and *C*, proliferative activity of splenocytes in response to *in vitro* CII (50 μg/ml) (*B*) or TIR-TcpC (50 μg/ml) (*C*). Proliferation in response to anti-CD3 (100 ng/ml) was used as a positive control (mean ± S.D.; $n = 3$; *, $p < 0.05$). *Error bars* in the figure represent S.D.

sion of MyD88-dependent receptors IL-1R and TLRs on Th17 cells vis-à-vis Th1 cells (15, 30). Moreover, these receptors are intricately involved in generation of Th17 cells in an indirect (31) as well as direct manner (15, 30, 32). Acting in an indirect manner, these pathways activate innate immune cells, particularly antigen-presenting cells, which in turn potently stimulate adaptive immune responses (32–34). On the other hand, evidence also suggests a direct action of MyD88-dependent (IL-1 and TLR) pathways on $CD4^+$ T-cells and promotion of their differentiation into Th17 cells (15, 30). It is noteworthy that the MyD88-dependent IL-1 and TLR pathways are not only important for Th17 differentiation but also have a significant contribution in the onset and progression of RA through other mechanisms. Elevated levels of various TLRs such as TLR-2, -3, -4, and -7 and their ligands such as bacterial DNA, peptidoglycans, heat shock proteins, fibronectin fragments, etc. have been observed in rheumatoid synovium in RA patients; these in addition to viral infections are implicated in disease initiation and progression (35–42). Conversely, IL-1 is a well established therapeutic target in RA (43–45). Elevated levels of IL-1, with a positive correlation with disease activity, have been detected in the plasma and synovial fluid of RA patients (46, 47). In animal models, IL-1 appears to be the main contributor toward disease pathogenesis (13, 48–50).

Thus, TIR-TcpC targeted MyD88-dependent signaling in both innate and adaptive immune cells to provide a comprehensive inhibition of Th17 differentiation *in vivo*.

TIR-TcpC-induced inhibition of the IL-1/MyD88 pathway resulted in impairment of NFκB activation, expression of RORγt, and subsequent Th17 differentiation, which is in line with a recent report that highlights the importance of p65 activity within $CD4^+$ T-cells for induction of optimal Th17 responses through the up-regulation of RORγt (51). Notably, TIR-TcpC was added during early stages of lineage commitment, which indicated that early MyD88 signaling is essential for Th17 generation and is in agreement with recent reports that highlight the importance of IL-1 signaling during early stages of Th17 differentiation (15). It could also be inferred that NFκB activation during early stages of differentiation is important for optimal Th17 differentiation.

Although a few sporadic reports had pointed out an indirect involvement of MyD88 (based on the studies in mice deficient in MyD88-dependent TLRs) in the pathogenesis of RA, direct evidence and/or underlying mechanisms have so far remained elusive (52–54). Addressing this ambiguity, the present study attempts to emphasize the unforeseen role of MyD88 as a valuable therapeutic target in RA. The absence of CIA in *MyD88*$^{-/-}$ mice was a clear indicator of the absolute necessity of this molecule for the induction of arthritic processes. MyD88 deletion (complete or cell-specific) was associated with deficient or defective Th17 development both *in vivo* and *in vitro*. In the case of $CD4^+$ cell-specific MyD88 deletion, the defect could be the result of impaired IL-1-induced NFκB activation during early stages of development as apparent from experiments with TIR-TcpC. However, this needs further investigation. Other possible explanations of the observed defect could be impaired IL-23R expression and inability of naïve $CD4^+$ T-cells to overcome Treg-mediated suppression as highlighted in studies by Chang *et al.* (14) and Schenten *et al.* (55), respectively. The present study, therefore, brings into light the importance of targeting $CD4^+$ T-cell-specific MyD88 in controlling the differentiation of autoreactive Th17 cells and subsequently RA pathogenesis.

It could be argued that TIR-TcpC is less efficient than gene knock-outs. Several factors may account for the observed difference. For example, limited biological half-life may affect the complete blockade of MyD88 in target cells. Consequently, resurgence of proinflammatory responses during drug-free intervals between two consecutive dosages could also contribute to the limited *in vivo* efficacy of TIR-TcpC. It could also be a matter of timing of treatment as disease processes in arthritis are continuous. Hence, early, sustained, and prolonged treatment regimens are likely to be more effective at near-complete blockade of MyD88 signaling, which may require the use of suitable delivery vehicles such as osmotic pumps, nanoparticles, etc. and are beyond the scope of the present study. It is also likely that use of high initial dosages could result in an improvement in MyD88 blockade.

Interestingly, we did not find any compromise in the entry of TIR-TcpC into target cells as revealed through *in vitro* cellular internalization experiments in dendritic and $CD4^+$ T-cells. Furthermore, the interaction of TIR-TcpC was very specific for MyD88 as we did not find any interaction with IL-1R.

Notably, the inhibitory activity of TIR-TcpC was dependent on its cellular entry. TIR-TcpC crossed the plasma membrane

through cholesterol-rich lipid rafts and its entry was similar to the internalization of arginine rich polycationic peptides which is in corroboration with a previously published report (9).

Taking into account the central role of Th17 lymphocytes in RA, disruption of pathogenic processes leading to their generation is highly desirable. TIR-TcpC, herein, emerged as a potent anti-arthritic molecule capable of arresting disease processes in terms of initiation and progression in an effective manner, which it did so by controlling Th17 cell responses both *in vivo* and *in vitro*, through regulation of the common adaptor molecule MyD88.

Author Contributions—S. P. and R. K. contributed equally to the study. S. P., R. K., and A. S. conceived and designed the study. A. S. provided the entire infrastructure and supported the research. S. P. and R. K. performed experiments, analyzed data, and wrote the manuscript. A. S. reviewed the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Toll/Interleukin-1 Receptor Domain Derived from TcpC (TIR-TcpC) Ameliorates Experimental Autoimmune Arthritis by Down-modulating Th17 Cell Response

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