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Invariant Natural Killer T Cells Regulate Conventional Dendritic Cell Maturation to Re-establish Immune Tolerance to Rheumatoid Arthritis in DBA/1 Mice

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ABSTRACT

Rheumatoid arthritis (RA) is a type of autoimmune disease that results in immune disorder and excessive inflammatory response due to a reduction of self-tolerance. Invariant natural killer T (iNKT) cells can effectively alleviate clinical symptoms and hyper-inflammation in RA, but their mechanism of action is not well-defined. This study aims to investigate the mechanism of iNKT cell therapy for RA.

We established a DBA/1 mouse model for RA and treated it with specific iNKT cells. A cytometric bead array was used to measure the amounts of cytokines in the serum. Flow cytometry was then employed to identify different subsets of helper T cells (Th), the frequency of conventional dendritic cells (cDC), the expression of CD80, CD86, programmed cell death ligand 1 (PD-L1), and PD-L2 on cDC surfaces, and associated pathway proteins.

iNKT cell treatment reduced Th1/Th2 and Th17/ regulatory T (Treg) cell ratios while increasing interleukin-4 (IL-4) and IL-10. It enhanced the generation of immature cDCs, and it upregulated the level of PD-L2 by stimulating the signal transducer and activator of transcription 3 (STAT3) signaling pathway. Meanwhile, it activated the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and inhibited the nuclear factor kappa B (NF-$\varkappa$B) pathway.

According to our findings, iNKT cell treatment increased the expression of phosphates STAT3 in lymph node cDC, causing them to upregulate PD-L2 molecules. While activating the ERK1/2 pathway and inhibiting the NF-$\varkappa$B pathway, tolerogenic cDC was produced, restoring immune homeostasis and correcting excessive inflammation. These results deliver new insights into the treatment of RA by iNKT cells.

Keywords: Dendritic cells; Extracellular signal-regulated kinase; Invariant natural killer T cells; Nuclear factor kappa B; Rheumatoid arthritis

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The first, second and third authors contributed equally to this study

INTRODUCTION

Rheumatoid arthritis (RA) is marked by a systemic inflammatory response, abnormal antibody production, and persistent synovitis.[1-3] The lack of immune tolerance caused by autoantigen presentation leads to an imbalance of helper T cell (Th) subsets and an excessive inflammatory response.[4] When Th1 and Th17 subsets increase and Th2 and regulatory T (Treg) cells are inhibited, the inflammatory cytokine interferon-gamma (IFN-γ) is produced, while the anti-inflammatory cytokine interleukin-4 (IL-4) is suppressed. The maintenance of tolerance, suppression of inflammation, and restoration of immune homeostasis are crucial elements in the treatment of RA.[5]

Invariant natural killer T (iNKT) cells are precise immunomodulatory agents.[6] In the context of RA, immune dysregulation and excessive inflammatory responses are linked to diminished frequency and anomalous function of iNKT cells.[7,8] Our previous research has demonstrated that RA mice exhibit reduced iNKT cell numbers, imbalanced Th subset rates, and elevated levels of inflammatory cytokines.[8] Following adoptive infusion, specific phenotypic and functional iNKT cells prove to be highly efficacious in mitigating clinical symptoms in RA mice. Consequently, reinstating iNKT cell levels in RA patients could provide a new therapeutic approach for RA.

The only antigen-presenting cells (APCs) that may stimulate naive T cells are dendritic cells (DCs). By triggering the immunological response and fostering immune tolerance, they contribute to the establishment of immune homeostasis.[9,10] DCs are a diverse group and can be classified into 2 functionally different subpopulations: conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC).[11] In the context of RA, DCs play a critical role. When mature cDC presents autoantigens to autoreactive T cells, it promotes disease progression.[12-14] On the other hand, immature cDC present their antigens to unsensitized T cells, causing apoptosis and thus establishing immune tolerance.[15,16] Depending on the maturation status of the DC, iNKT cells interact with DC via direct cell contact or cytokine release and induce immune responses or immune tolerance upon activation.[17-19]

The programmed cell death protein 1 (PD-1) and programmed cell death ligand 1/2 (PD-L1/2) pathways, responsible for the activation, expansion, and activity of negatively regulated effector T cells, are crucial for establishing peripheral immune tolerance.[20] Research has revealed that alpha-galactosylceramide (α-GalCer)–activated iNKT cells can prompt immature DCs to become resistant DCs through the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the absence of toll-like receptor 4 (TLR4) stimulation. However, when TLR4 stimulation is present, these same NKT cells can induce pro-inflammatory DC maturation through CD40:CD40 ligand (CD40L) interactions and nuclear factor kappa B (NF-κB) pathway stimulation.[21] This study investigates the mechanism of iNKT cell therapy for RA by examining the regulation of DC maturation by iNKT cells. The findings offer a fresh understanding of the etiology of RA and suggest possible treatment avenues.

MATERIALS AND METHODS

Experimental Animals

One hundred and twenty 7- to 8-week-old healthy male DBA/1 mice (20.0±1.0 g) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK (Beijing), 2016-0006). The mice were given free food and water, and the experiments were conducted after 1 week of acclimatization in an SPF-class animal housing.

Establishment of the Mouse Rheumatoid Arthritis Model

Thirty mice were randomly chosen as healthy controls, and 60 animals were modeled artificially. The hGPI325-339 and hGPI469-483 peptide fragments (Karebay Biochem, Inc., NJ, USA) were mixed (1.125 mg of mixed peptide dissolved in 3.375 mL of precooled triple-distilled water), blended with the same volume of Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) to complete emulsification (ice bath). The mouse tail roots were subcutaneously injected (150 μL) the same day and after 48 hours. Pertussis toxin (200 ng, Sigma-Aldrich) was administered intraperitoneally to boost immune reactions. After that, 60 mice were randomly split into 2 groups, 1 for the RA model (30 mice) and the other for cell therapy (30 mice; inject iNKT2 cells through the tail vein after 8 days of model establishment, 3×10^6 cells/each). The treatment effects were observed, and mice were sacrificed at 8, 14 and 21 days after model establishment, by cervical dislocation for sample collection.[22,23]

Acquisition and Infusion of iNKT2 Cells

α-GalCer (100 ng/g weight, ENZO Life Sciences, NY, USA) was injected intraperitoneally into 30 male DBA/1 mice. After 3 days, the mouse spleens were

removed to prepare a single-cell suspension, and the lymphocytes were isolated using lymphocyte isolation medium (Solarbio, Beijing, China). PE-labelled CD1d Tetramer (MBL International Corporation, Woburn, MA, USA) and Anti-PE MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) loaded with α-GalCer were added. Purified iNKT cells were isolated by mass spectrometry (MS) columns (Miltenyi Biotec) and obtained using magnetic-activated cell sorting (MACS). The cell therapy group (30 mice) was infused via the tail vein with iNKT cells (3×10^6 each).[23,24].

Detection of Cytokine Contents by Cytometric Bead Array (CBA)

Collect serum from each mouse group. The levels of tumor necrosis factor alpha (TNF-α), IL-6, IL-4, IFN-γ, IL-2, IL-17A, and IL-10 were measured by the CBA cytokine kit (BD Biosciences Pharmingen, CA, USA). Flow cytometry was utilized to detect 50 μL of each sample, and the data were analyzed using fluorescence-activated cell sorting (FCAS) software (BD).[25]

Detection of Th1/Th2/Th17/Treg Subsets by Flow Cytometry

FITC Rat Anti-Mouse CD4 and APC Rat Anti-Mouse CD25 (BD Biosciences Pharmingen, CA, USA) were added to spleen lymphocyte suspensions, and cells were collected after stimulation with phorbol-12-myristate-13-acetate (PMA, 50 ng/mL), ionomycin (IO, 1 μg/mL) and golgi blocker (4 μL/6 mL, Cayman Chemical, MI, USA). In addition, precooled CytofixTM buffer, 1×Perm/WashTM buffer, and cocktail (BD) were added, and flow cytometry was used to detect the Th1/Th2/Th17/Treg subsets using the CFlow software (BD).[23]

Detection of cDC Frequency and Expression of cDC Surface and Intracellular Related Molecules by Flow Cytometry

On the 11th, 14th, and 20th days after modeling, lymph node tissues from mice were separated, and lymph suspensions were obtained using a lymphocyte separation medium. Lymphocytes (1×10^6 cell/tube) were taken in a flow tube and PE Hamster Anti-Mouse CD11c and FITC Rat Anti-Mouse I-A/I-E (BD) were added, followed by PerCP-Cy™5.5 Hamster Anti-Mouse CD80, APC Rat anti-Mouse CD86, BV421 Rat Anti-Mouse CD274 and BB700 Rat Anti-Mouse CD273 (BD). Then add Fix Buffer (eBioscience, CA, USA) to fix the cells for 10min, and add Perm Buffer III (eBioscience, CA, USA) to permeabilize for 30min. Cells were resuspended by adding Alexa Fluor 647 Mouse Anti-Signal transducer and activator of transcription 3 (STAT3) (pY705) and PerCP-Cy™7 Mouse anti-p38 mitogen activated protein kinase (p38MAPK) (pT180/pY182), BV421 Mouse Anti-ERK1/2 (pT202/pY204) and PE Mouse anti-IκBα (BD). Flow cytometry was used for detection.

Statistical Analysis

SPSS 26.0 software was employed for analysis. The mean ± standard deviation (SD) was used to express the experimental data. The *t*-test was used for 2 groups with normal distribution and uniform variance. For comparison among the three groups, if the variance was satisfied, the one-way analysis of variance (ANOVA) was used. The least significant difference (LSD) test was used for further comparison. If the variance was not uniform, the Kruskal-Wallis H test was used. The data with $p<0.05$ was considered statistically significant.

RESULTS

Successful Replication of the Rheumatoid Arthritis Model

The RA mouse model used in this study exhibited clinical symptoms, pathological manifestations, inflammatory responses, and immune cell alterations consistent with established RA criteria, as reported in previous literature.[22,23]

Acquisition and Infusion of iNKT2 Cells

Following MACS isolation and purification of spleen lymphocytes, the iNKT cell purity was confirmed to be >85%. Notably, iNKT2 cells, which are known to primarily secrete the anti-inflammatory cytokine IL-4, accounted for more than 92% of the isolated cells. The safety profile of the transferred cells was favorable. For further details, please refer to the previous publication.[24]

iNKT2 Cell Therapy Restored Th1/Th2 and Th17/Treg Balance in Rheumatoid Arthritis Mice

The cell-treated group exhibited significantly higher frequencies of spleen Th2 (Figures 1C and 1D) and Treg (Figures 1H and 1I) subsets compared to the RA model group during the three stages of inflammation progression (day 11), peak inflammation (6 days of treatment, day 14), and inflammation remission (12 days of treatment, day 20) ($p<0.05$). Furthermore, there was a significant decrease in the ratios of Th1/Th2 (Figure 1E) and Th17/Treg ($p<0.05$, Figure 1J). Moreover, the

frequency of Th1 (Figures 1A and 1B) decreased on days 11 and 20, while the frequency of Th17 (Figures 1F and 1G) reduced on days 11 and 14 ($p<0.05$). This suggested that iNKT2 cell therapy could be a treatment option for restoring immune system disorders and correcting Th subset imbalances in RA mice.

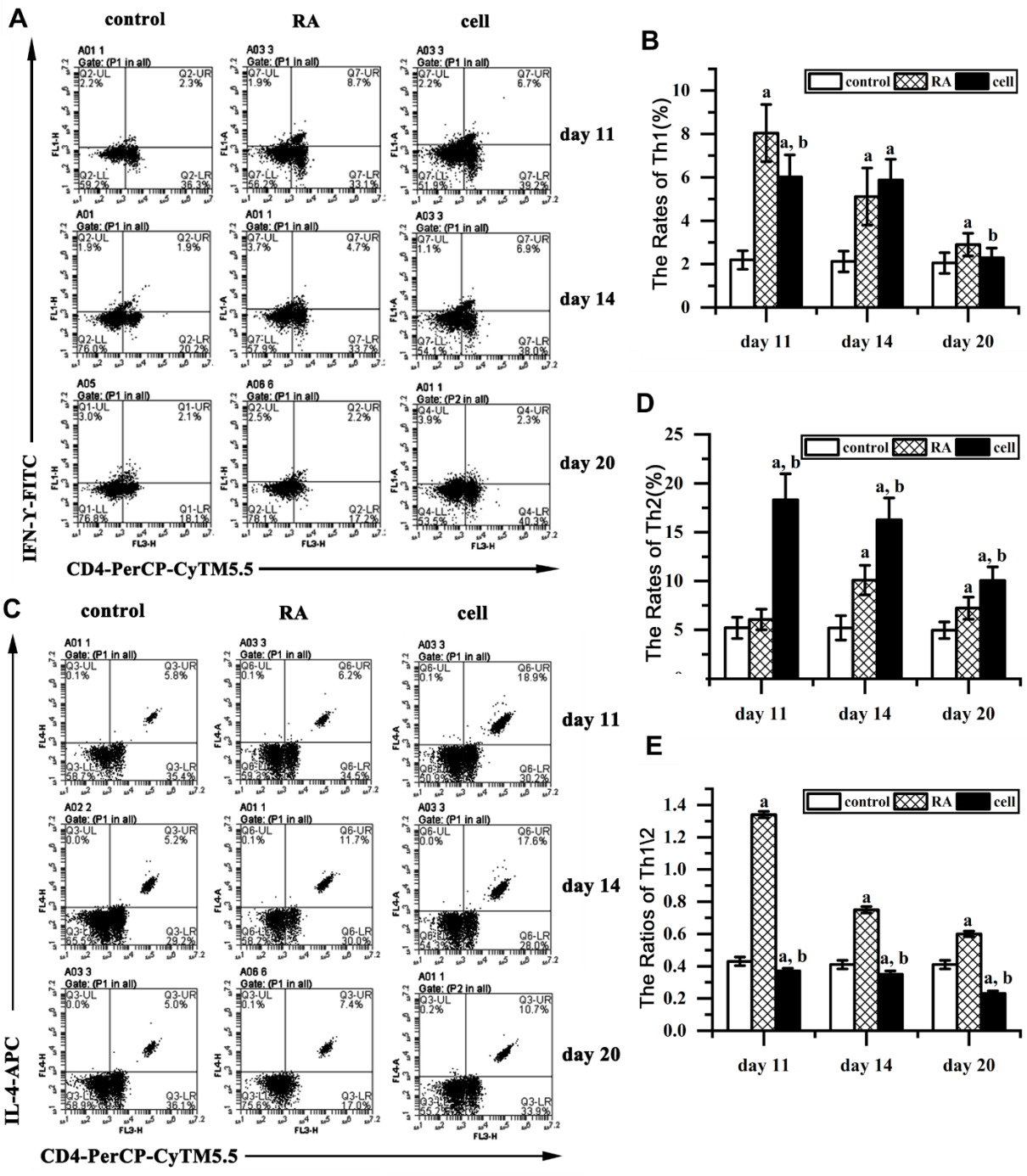

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Figure 1: Continued.

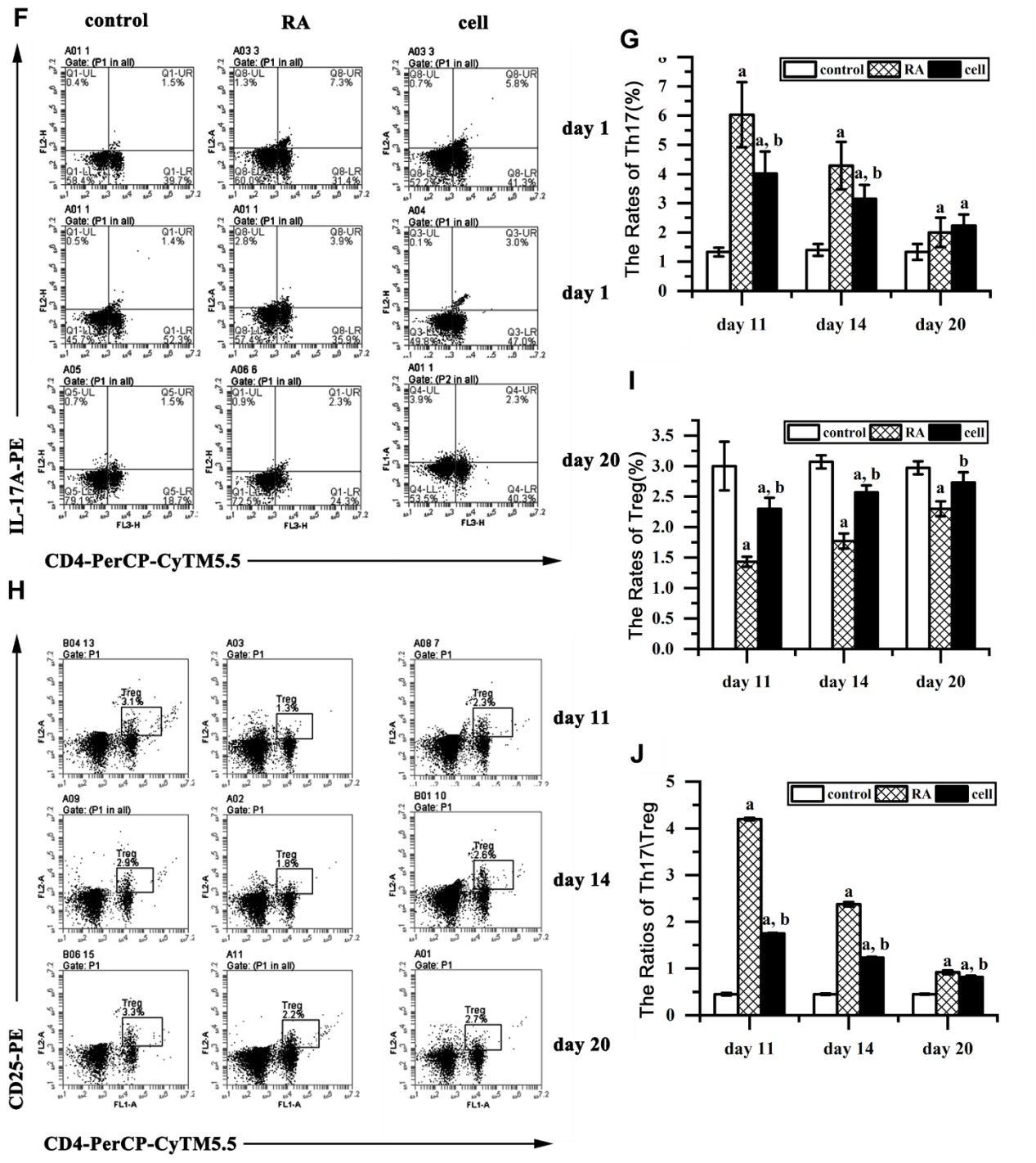

Figure 1. Invariant natural killer T 2 (iNKT2) cell therapy reduced the ratios of helper T cell 1 (Th1) /Th2 and Th17/ regulatory T (Treg). To explore the impact of iNKT2 cell therapy on the frequency of Th subgroups andTreg cells, flow cytometry was utilized to identify the variations the frequencies of Th1 subset (A, B), Th2 subset (C, D), Th17 subset (F, G), Treg subset (H, I), Th1/Th2 ratio (E), and Th17/Treg ratio (J) in the spleens of mice from each group at 11, 14, and 20 days. [a]*p*<0.05 vs. control, [b]*p*<0.05 vs. rheumatoid arthritis.

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iNKT2 Cell Therapy Increased IL-4 and IL-10 in RA Mice

The impact of iNKT cell adoptive therapy on cytokine levels was examined (Figure 2). The RA group had higher levels of IL-17A, TNF-α, IFN-γ, IL-6, and IL-2 compared to the control group. Cell therapy considerably lowered these levels (p<0.05). IL-4 and IL-10 were significantly lower in the RA group compared to the control group but elevated after cell therapy (p<0.05). These findings suggested that iNKT2 cell adoptive therapy suppressed excessive inflammatory responses by decreasing the secretion of pro-inflammatory cytokines and boosting the secretion of anti-inflammatory cytokines in the serum of RA mice.

iNKT2 Cell Therapy Reduced the Frequency of cDC in Lymph Nodes of Rheumatoid Arthritis Mice

We compared the frequency changes of cDC in the lymph nodes of different groups of mice at various time points. As shown in Figure 3, the frequency of cDC in the cell therapy group was significantly lower than in the RA model group on day 14 (p<0.05). It suggested that iNKT2 cell therapy reduced RA inflammation by decreasing the frequency of cDC and limiting self-antigen presentation by cDC during the peak of inflammation (day 14).

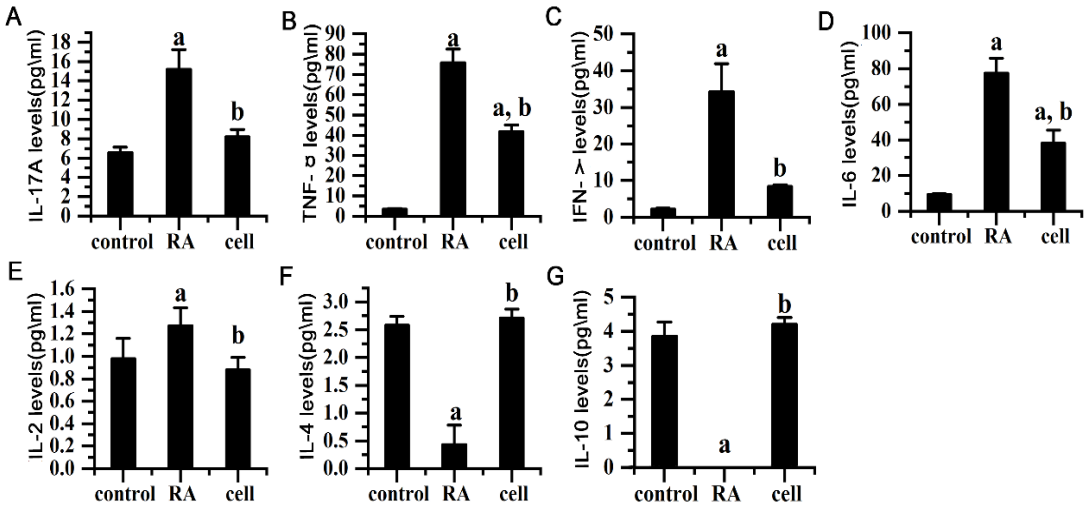

Figure 2. Invariant natural killer T 2 (iNKT2) cell therapy increased serum levels of interleukin-4 (IL-4) and IL-10. To evaluate the effect of iNKT2 cell therapy on cytokine levels, flow cytometry was performed to identify the variations in serum levels of IL-17A (A), tumor necrosis factor alpha (TNF-α) (B), interferon-gamma（IFN-γ）(C), IL-6 (D), IL-2 (E), IL-4 (F), and IL-10 (G) at the peak of inflammation (day 14). [a]p<0.05 vs. control, [b]p<0.05 vs. rheumatoid arthritis.

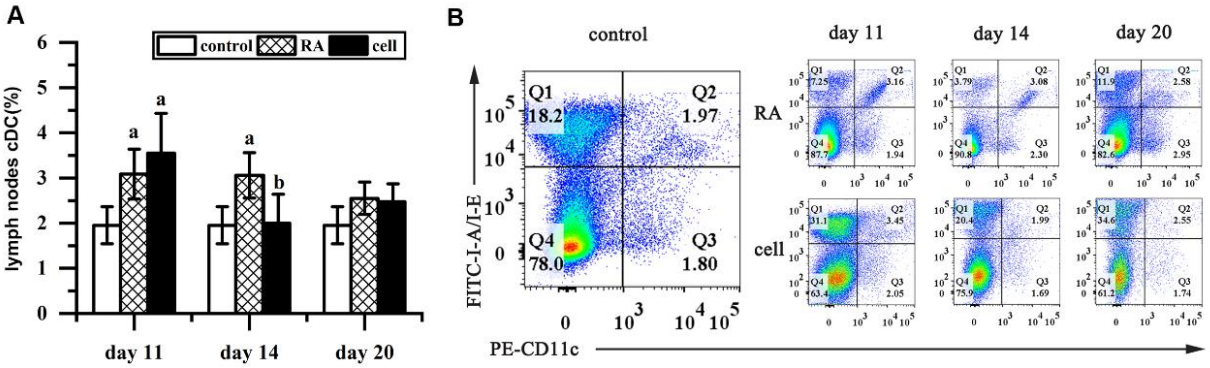

Figure 3. Invariant natural killer T 2 (iNKT2) cell therapy decreased conventional dendritic cells (cDC) frequency in lymph nodes. To investigate the changes in the frequency of cDC in lymph nodes, flow cytometry was used. Representative bar graph (A) and dot plot (B). [a]p<0.05 vs. control, [b]p<0.05 vs. rheumatoid arthritis.

iNKT2 Cell Therapy Decreased the Expression of CD80 and CD86 Molecules on cDC in Rheumatoid Arthritis Mouse Lymph Nodes

To investigate whether iNKT cells regulate DC maturation status in RA, we examined CD80 and CD86 molecules on cDC in RA mouse lymph nodes. Compared to the RA group, cell therapy significantly decreased the expression of CD80 on days 14 and 20 (Figure 4A) and CD86 at all stages (p<0.05, Figure 4B). iNKT2 cell therapy decreased CD80 and CD86 expression on cDC surfaces, promoted immature cDC production, inhibited cDC maturation, reduced autoimmune response, and induced immune tolerance, thereby further inhibiting inflammation development and alleviating disease progression.

iNKT2 Cells Increased PD-L2 Expression in Lymph Node cDC by Activating the STAT3 Pathway

The iNKT cell therapy group displayed dynamic changes in lymph node cDC PD-L1 and PD-L2 at various stages of inflammation (p<0.05, Figures 5A and 5B). We further examined the level of pSTAT3. The level of pSTAT3 in the RA group was considerably lower than that of the control group on days 11 and 14 (p<0.05, Figure 5C), which was consistent with PD-L2. It was indicated that cDC in lymph nodes at the onset of RA may regulate PD-L2 expression by decreasing pSTAT3 expression to relieve the suppression of T cells. Conversely, cell therapy increased the expression of cDC pSTAT3, thereby upregulating PD-L2 molecules and reducing autoimmune response.

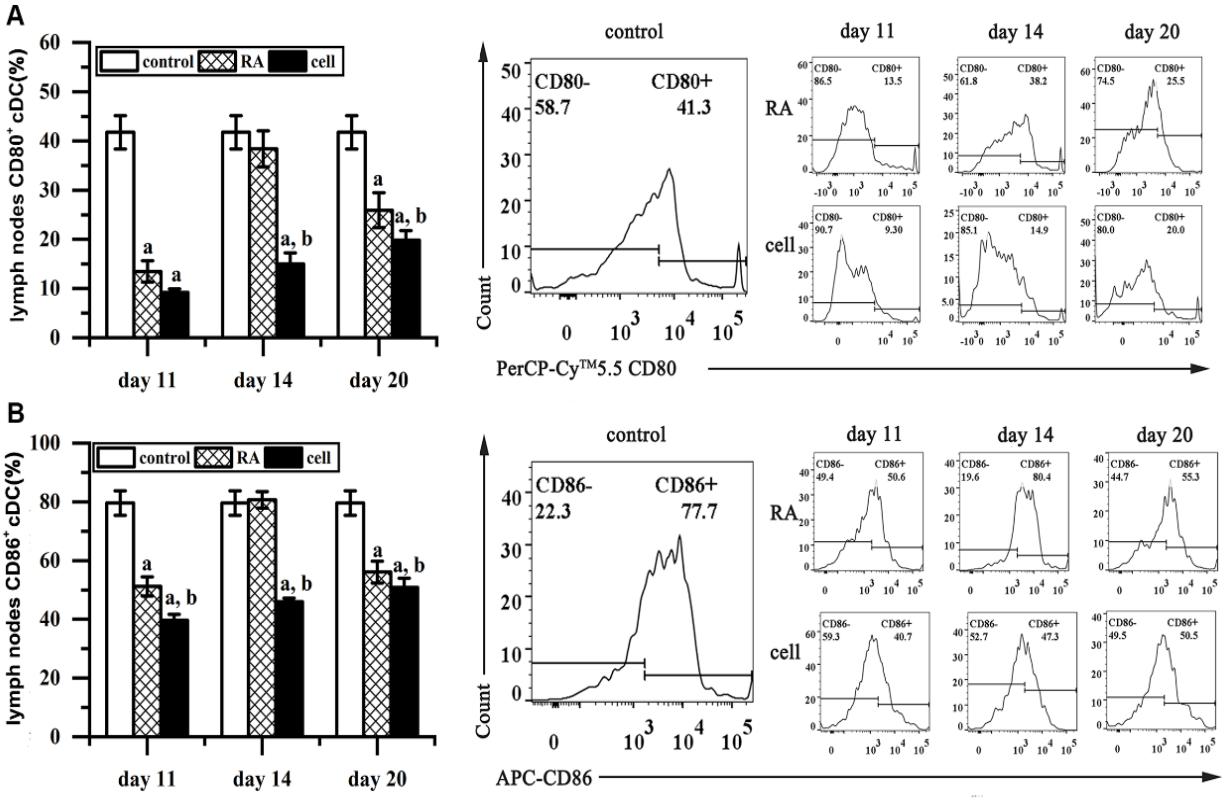

Figure 4. Invariant natural killer T 2 (iNKT2) cell therapy reduced the expression of CD80 and CD86 on conventional dendritic cells (cDC) in lymph nodes. To evaluate the impact of iNKT2 cell therapy on cDC maturation, we used flow cytometry to measure the frequency of CD80 (A) and CD86 (B) molecules on the surface of cDC in lymph nodes. [a]p<0.05 vs. control, [b]p<0.05 vs. rheumatoid arthritis.

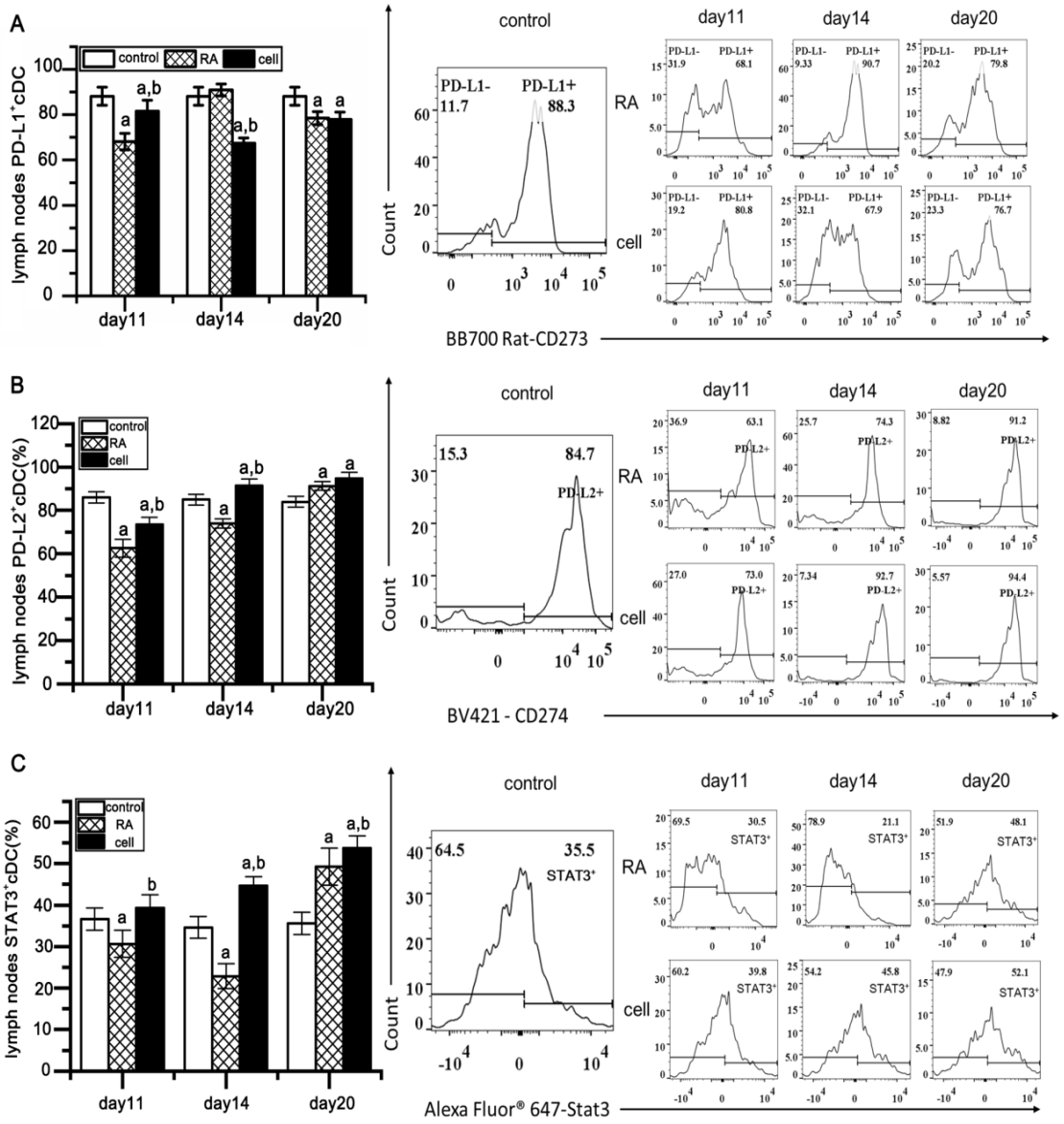

Figure 5. Invariant natural killer T 2 (iNKT2) cell therapy increased the expression of conventional dendritic cells (cDC) cDC phosphates signal transducer and activator of transcription 3 (pSTAT3) in lymph nodes and thus upregulated programmed cell death ligand 2 (PD-L2). PD-L1 (A), PD-L2 (B), and pSTAT3 (C) expression in lymph node cDC were detected by flow cytometry. [a]*p*<0.05 vs. control, [b]*p*<0.05 vs. rheumatoid arthritis.

iNKT2 Cells Activated the Lymph node cDC ERK1/2 Pathway while Inhibiting the NF-κB Pathway Mediated by p38

The MAPK and NF-κB pathways have been recognized for their pivotal roles in the maturation of DC.[26,27] To elucidate the potential impact of iNKT cells on the status of CDC through these pathways, we examined the expression of pERK1/2, pIkappaB-alpha (pIκB-α), and p38MAPK (Figure 6). Compared to the RA group, cellular therapy markedly augmented the phosphorylation levels of ERK1/2 while attenuating the phosphorylation levels of IκB-α and p38MAPK molecules (p<0.05). These findings suggested that iNKT2 cell therapy may foster the generation of tolerogenic cDC while repressing excessive inflammatory responses by activating the ERK pathway and suppressing the p38-mediated NF-κB pathway.

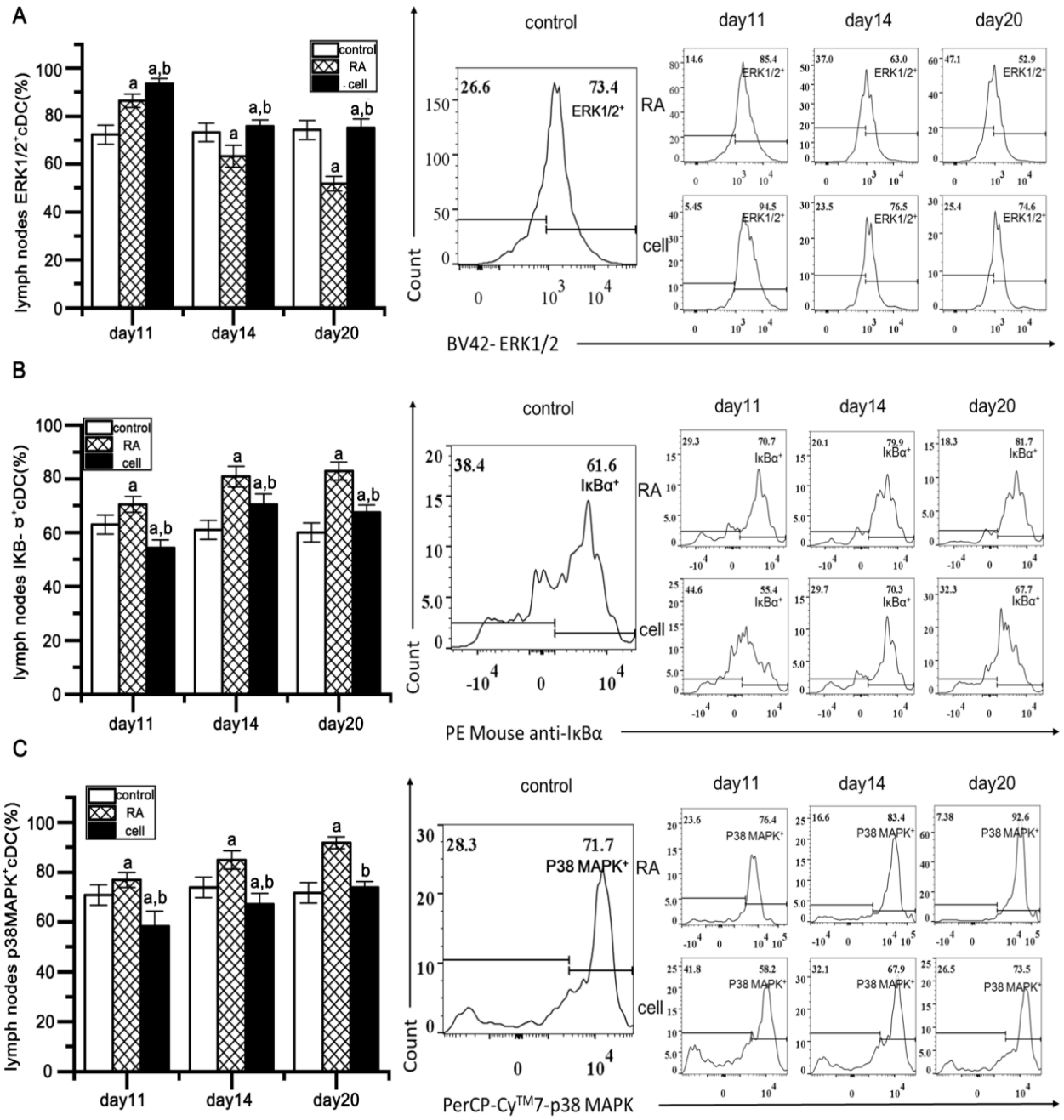

Figure 6. Invariant natural killer T 2 (iNKT2) cell therapy activated the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and inhibited the p38-mediated nuclear factor kappa B (NF-κB) pathway. We examined the relevant pathways involved in the regulation of cDC maturation. The expressions of pERK1/2 (A), pIkappaB-alpha (B), and p38 mitogen-activated protein kinase (p38MAPK) (C) in lymph node conventional dendritic cells (cDC) were assayed by flow cytometry. [a]*p*<0.05 vs. control, [b]*p*<0.05 vs. rheumatoid arthritis.

DISCUSSION

iNKT cells are classified into different types: iNKT1, iNKT2, iNKT17, and iNKT10. Among these, iNKT1 cells, characterized by their secretion of IFN-γ, promote Th1-mediated inflammation, while iNKT2 cells, known for their secretion of IL-4, foster Th2-mediated anti-inflammatory responses.[6] In the realm of RA research, iNKT cells are closely associated with this disease. Mitsuo et al,[28,29]revealed the constructive role of iNKT cells in autoimmune diseases, as restoration of their quantity and functionality resulted in disease remission. Within our laboratory, we have devised an in vivo method to induce specific phenotypes and functions of iNKT cells, which are subsequently administered to mice via intravenous infusion. Previous investigations have shown that transfusion of iNKT2 cells significantly alleviated redness and swelling in the ankle joints of RA-afflicted mice, diminished inflammatory cell infiltration within joint tissues, restored iNKT cell frequency, rectified imbalances in Th subsets, and repressed excessive inflammatory responses.[22-24] Hence, iNKT2 cell therapy is expected to be a safe and efficacious therapeutic approach for RA.

Abnormal presentation of autoantigens by APCs is a major factor in RA pathogenesis. Among the various APCs, DC stands out as the most functionally potent, and crucial for maintaining immune tolerance. In this study, it was observed that the frequency of cDC in RA mice rose during the progressive inflammatory phase (day 11), accompanied by a drop in CD80 and CD86. However, at the peak of inflammation (day 14), CD80 and CD86 exhibited an upsurge compared to the progressive inflammatory phase. This phenomenon can be attributed to the influx of immature cDC into the lymph nodes as inflammation advances, gradually maturing under the influence of inflammatory stimuli, and ultimately reaching their peak maturity during the zenith of inflammation.

Upon stimulation with α-GalCer, iNKT cells produced IFN-γ while activating APCs through CD40:CD40L interactions, specifically inducing DC maturation and upregulating CD80 and CD86. Notably, the interaction between iNKT cells and immature DC increased immunological tolerance, but interaction with mature DC favored T-cell immunity.[30]

Our findings unveiled that the administration of iNKT2 cells in RA mice resulted in a rebalancing of Th1/Th2 and Th17/Treg ratios. iNKT2 cells may interact with DC to modulate their maturation status, thereby rectifying the imbalance of Th subsets and suppressing excessive inflammatory reactions. To substantiate this assertion, we tracked the frequency and maturity status of cDC in the lymph nodes. Treatment with iNKT2 cells led to a reduction in the frequency of lymph node cDC during the peak of inflammation (day 14), which may effectively inhibit the presentation of autoantigens and the ensuing inflammatory response. Moreover, it curtailed CD80 and CD86 of cDC throughout the progressive (day 11), peak (day 14), and remission (day 20) phases of inflammation, thus inhibiting cDC maturation and reinstating immune tolerance.

Further investigation was conducted to delve into the intricate mechanisms governing the regulation of cDC maturation by iNKT2 cells in the setting of RA. Negative co-stimulatory molecules, such as the cytotoxic T-lymphocyte-associated protein 4 (CTLA4)-B7) pathway and the PD-1/PD-L1-2 pathway, assume a crucial role in controlling T cell responses.[31,32] We postulated that DC orchestrate and maintain T cell tolerance via the PD-1-PD-L1/PD-L2 pathway, which may be involved in the pathogenesis of RA. Consequently, we assessed the expression of PD-L1 and PD-L2 molecules on the surface of cDC. Lymph node cDC exhibited a decline in their immunosuppressive function before the peak of RA inflammation. However, following cell therapy, PD-L2 expression increased, thereby restoring the immunosuppressive capacity mediated by cDC through PD-L2. The changes in PD-L1 expression were not statistically significant, possibly because PD-L1 and PD-L2 have different functions in the regulation of the immune response across various cells and diseases.[33]

The pro-inflammatory maturation of DC-mediated by TLRs is generally linked to the activation of p38 MAPK and the NF-κB pathway. However, the activation of ERK1/2 is involved in the signaling pathway generated by tolerogenic DC.[21] Additionally, STAT3, which binds to the promoter of the CD274 (PD-L1) gene, is necessary for the expression of PD-L1/2.[34]

To gain further insight into the impact of iNKT2 cells on the status DC, we investigated the expression of pSTAT3, pERK1/2, pIκB-α, and p38MAPK. This observation suggests that lymph node cDCs regulate PD-L2 expression by downregulating pSTAT3, thereby disrupting T-cell suppression during the onset of RA. Furthermore, the enhanced expression of pERK1/2 after cell therapy implies that iNKT2 cells may facilitate the generation of tolerogenic cDC by activating the ERK pathway, thereby inducing immune tolerance. Reduced levels of pIκB-α and p38MAPK indicate that iNKT2

cells could produce pro-inflammatory cDC through the NF-κB pathway, thus attenuating the autoimmune response and suppressing inflammation.

Since all the experimental results in this study were obtained at the animal level, further investigations at the cellular level will be conducted to demonstrate the development of tolerance in terminally differentiated cDC after iNKT cell therapy. In addition, the interactions between signaling pathways are highly intricate and context-dependent, yielding diverse effects in distinct cell types and states. The impact of iNKT cell and DC interactions on alterations in the MAPK (p38, ERK1/2) and NF-κB signaling pathways will be further investigated.

In conclusion, adaptive treatment of RA model mice with iNKT2 cells resulted in an elevation of PD-L2 expression through the stimulation of pSTAT3 in lymph node cDC. Moreover, it effectively modulated cDC maturation and facilitated the generation of tolerant cDC by activating the ERK1/2 pathway while inhibiting the NF-κB pathway. Thus, peripheral immune tolerance was restored, Th subset imbalance was corrected, and inflammation was suppressed. These findings present novel perspectives on the treatment of RA.

STATEMENT OF ETHICS

All animal experiments were conducted according to the Chinese guidelines for experimental animal welfare and approved by the Animal Welfare and Ethical Committee of Hebei University (Approval Number: IACUC-2018017).

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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