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Adoptive immunotherapy of iNKT cells in glucose-6-phosphate isomerase (GPI)-induced RA mice
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1   **TITLE:**
2   **Adoptive Immunotherapy of iNKT Cells in Glucose-6-Phosphate Isomerase (G6PI)-Induced RA**
3   **Mice**
4

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27   **KEYWORDS:**
28   rheumatoid arthritis, adoptive immunotherapy, iNKT cells, cytokines, glucose-6-phosphate
29   isomerase (G6PI), in vivo imaging system (IVIS)
30

31   **SUMMARY:**
32   This protocol uses G6PI mixed peptides to construct rheumatoid arthritis models that are closer
33   to that of human rheumatoid arthritis in CD4^{+} T cells and cytokines. High purity invariant natural
34   killer T cells (mainly iNKT2) with specific phenotypes and functions were obtained by in vivo
35   induction and in vitro purification for adoptive immunotherapy.
36

37   **ABSTRACT:**
38   Rheumatoid arthritis (RA) is a complex chronic inflammatory autoimmune disease. The
39   pathogenesis of the disease is related to invariant natural killer T (iNKT) cells. Patients with active
40   RA present fewer iNKT cells, defective cell function, and excessive polarization of Th1. In this
41   study, an RA animal model was established using a mixture of hGPI325-339 and hGPI469-483
42   peptides. The iNKT cells were obtained by in vivo induction and in vitro purification, followed by
43   infusion into RA mice for adoptive immunotherapy. The in vivo imaging system (IVIS) tracking
44   revealed that iNKT cells were mainly distributed in the spleen and liver. On day 12 after cell

45 therapy, the disease progression slowed down significantly, the clinical symptoms were
46 alleviated, the abundance of iNKT cells in the thymus increased, the proportion of iNKT1 in the
47 thymus decreased, and the levels of TNF-α, IFN-γ, and IL-6 in the serum decreased. Adoptive
48 immunotherapy of iNKT cells restored the balance of immune cells and corrected the excessive
49 inflammation of the body.
50
51 **INTRODUCTION:**
52 Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic, progressive
53 invasiveness with 0.5–1% incidence[1–2]. The underlying pathogenesis is attributed to the abnormal
54 proliferation of autoreactive CD4^{+} and CD8^{+} T cells, manifested by an increase in the proportion
55 of CD4^{+}IFN-γ^{+} and CD4^{+}IL-17A^{+} T cells, and the reduced number of CD4^{+}IL-4^{+} and
56 CD4^{+}CD25^{+}FoxP3^{+} T cells. Therefore, the secretion of inflammatory cytokines is increased, and an
57 excessive inflammatory reaction destroys the native balance and tolerance function of the body's
58 immune system. Moreover, the helper T lymphocyte (Th) 1 cells that penetrate the joint
59 aggravate the inflammatory response and joint damage. Therefore, the inhibition of excessive
60 inflammatory response and restoration of immune tolerance and immune balance are key to the
61 treatment of RA[3,4].
62
63 The iNKT cells have both NK cell and T cell functions and characteristics. The iNKT cells harbor a
64 distinct, invariant T cell receptor (TCR) α-chain with limited TCR β-chain repertoires[5] and
65 recognize the glycolipid antigen presented by the major histocompatibility complex (MHC) class
66 I molecule CD1d on the surface of the antigen-presenting cells. Mitsuo et al.[6] detected a large
67 number of iNKT cells and functional defects in many autoimmune diseases, including RA. Aurore
68 et al.[7] demonstrated that iNKT cells have a positive effect on maintaining autoimmune tolerance
69 and that when the number and function of iNKT cells are restored, the disease is alleviated. In
70 addition, Miellot-Gafsou et al.[8] found that iNKT cells not only abrogated the disease but also
71 increased the progression of the disease. These contradictory results suggest that iNKT cells are
72 heterogeneous T cells, and the function of different subsets may be reversed. In a clinical study
73 of RA, the frequency of iNKT cells correlated with the score of the disease activity[9]. The results
74 also confirmed that the frequency of iNKT was decreased in RA patients, the number of CD4^{+}IFN-
75 γ^{+} T cell subsets increased, and the secretory levels of inflammatory cytokines IFN-γ and TNF-α
76 increased[10–11]. In addition, Sharif et al.[12] investigated type 1 diabetes (T1D) and found that
77 selective infusion of iNKT cells upregulated the expression of the inflammatory cytokine IL-4,
78 maintained immune tolerance, and prevented the development of type 1 diabetes. Therefore,
79 adoptive infusion of specific iNKT cells or targeted activation of iNKT cells increases the level of
80 iNKT cells in RA patients, which can be a breakthrough in RA treatment.
81
82 Cellular immunotherapy is currently of great interest and has been widely used in cancer therapy.
83 However, iNKT cells are rare, heterogeneous immunoregulatory cells (only 0.3% of the total
84 number of PBMCs)[13], which limits potential clinical applications. These cells are mainly divided
85 into three subpopulations: 1) iNKT1 cells, which have a high expression of promyelocytic
86 leukemia zinc-finger protein (PLZF) and T-box transcription factor (T-bet); 2) iNKT2 cells with
87 intermediate expression of PLZF and GATA binding protein 3 (GATA3); 3) iNKT17 cells with low

88 expression of PLZF and retinoid-related orphan nuclear receptor (ROR)-γt that secrete IFN-γ, IL-
89 4, and IL-17[14]. Activated iNKT cells secrete Th1, Th2, and Th17-like cytokines, which determine
90 the different immunomodulatory effects of iNKT cells[15]. The immunomodulatory and
91 immunotherapeutic effects of specific activation of various subpopulations of iNKT cells are
92 different. Therefore, the selection of specific phenotypes of iNKT cells (mainly iNKT2) with anti-
93 inflammatory functions to regulate the immune response of the body can correct the immune
94 imbalance and immune disorders in RA.
95
96 The establishment of an ideal animal model is of great significance for the treatment and study
97 of RA pathogenesis. Presently, the most commonly used and mature animal models include
98 collagen-induced arthritis, adjuvant arthritis, zymosan-induced arthritis, and polysaccharide-
99 induced arthritis[16–17]. However, there is no model that can fully simulate all the features of
100 human RA. Type II collagen-induced arthritis (CIA) is a classic arthritis model. The CIA is induced
101 by immunization of mice with type II collagen-specific monoclonal antibodies, reflecting the
102 antibody dependence of this disease model. Benurs et al. described a model with a systemic
103 immune response to glucose-6-phosphate isomerase (G6PI), which induces peripheral symmetric
104 polyarthritis in susceptible mouse strains[18,19]. In this model, the development of arthritis depends
105 on T cells, B cells, and innate immunity[18–20]. Horikoshi[21] found that RA models resulting from
106 immunization of DBA/1 mice with G6PI polypeptide fragments are more similar to human RA in
107 terms of CD4^{+} T cells and cytokines (i.e., IL-6 and TNF-α) than the CIA models. In order to increase
108 the stimulating effect on the TCR recognition site, the mixed polypeptide fragments of G6PI
109 (hGPI325-339 and hGPI469-483) were used to immunize DBA/1 mice to construct the RA mouse
110 model. The success rate of this approach can high because hGPI325-339 and hGPI469-483 are
111 immunodominant for I-A q-restricted T cell responses. Therefore, this model can simulate the
112 overproliferation of CD4^{+} T cells and iNKT cell defects in RA patients[22]. The basic research of RA
113 immunopathology laid the foundation for our further in-depth investigation.
114
115 **PROTOCOL:**
116
117 All experimental mice (150 in total) were healthy male DBA/1 mice, 6–8 weeks old (20.0 ± 1.5 g),
118 reared in a specific pathogen-free (SPF) environment. There is no special treatment before
119 modeling. The experiment was divided into a healthy control group (15 mice), a model control
120 group (15 mice), and a cell therapy group (55 mice). This study was approved by the Animal
121 Welfare and Ethical Committee of Hebei University.
122
123 **1.   Constructing the disease model**
124
125 1.1.   Duplicating the RA animal model
126
127 1.1.1.   Weigh 1.75 mg of both hG6PI 325-339 and hG6PI 469-483 fragments and dissolve them
128 in 5.25 mL of 4 °C triple distilled water.
129
130 1.1.2.   Dissolve complete Freund's adjuvant (CFA) in a 50 °C water bath, draw 5.25 mL into

131 another 10 mL centrifuge tube, and cool it for use.

133 1.1.3.   Put the mixture of hG6PI solution and CFA solution in an artificial emulsification unit with
134 two glass syringes connected.

136 1.1.4.   Push the syringe at a constant speed and frequency of 10–20x per min to completely
137 emulsify the mixed peptide solution and CFA solution. Perform the operation in an ice bath, and
138 keep the emulsion droplets in the water for 10 min after the completion of the emulsification
139 without dispersing.

141 1.1.5.   Inject 150 μL of emulsified hG6PIs into the mouse's tail root subcutaneously.

143 1.1.6.   Inject 200 mg of Pertussis toxin into the mouse intraperitoneally at 0 h and 48 h after
144 the hG6PI injection.

146 **1.2.   Experimental verification of the RA model**

148 1.2.1.   Measure the thickness of the mouse's paw with a Vernier caliper (2x a day).

150 1.2.2.   Observe and mark the degree of redness and swelling of the foot. Use the following
151 scoring criteria: 1) toes with mild swelling; 2) dorsum pedis and foot pad with clear red swelling;
152 3) ankle with red swelling.

154 1.2.3.   Euthanize the mice under deep anesthesia by intraperitoneal injection of 1% sodium
155 pentobarbital (50 mg/kg body weight) 14 days after modeling and remove the paws for HE
156 staining.

158 1.2.4.   Determine the secretion levels of serum IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, and IFN-γ
159 using a commercial cytometric bead array (CBA) assay according to the manufacturer's
160 protocol.

162 **2.   Obtaining iNKT cells with adoptive cellular therapy**

164 **2.1.   Directional induction of iNKT cells**

166 2.1.1   Inject normal mice intraperitoneally with α-GalCer (0.1 mg/kg of body weight).

168 **2.2.   Isolation of iNKT cells**

170 2.2.1.   Three days after modeling, inject mice intraperitoneally with 1% sodium pentobarbital
171 (50 mg/kg of body weight) for anesthetization. Adequately anesthetized mice do not show a
172 hind paw withdrawal response to a toe pinch.

174   2.2.2.   Isolate the spleen of a DBA/1 mouse after injecting it intraperitoneally with α-GalCer.
175   Prepare a single cell suspension by cutting and grinding the spleen in a 200 mesh sieve.

176

177   2.2.3.   Wash the cell suspension with PBS, centrifuge at 200 x g for 5 min, and discard the
178   supernatant. Repeat.

179

180   2.2.4.   Resuspend the cells with 1 mL of whole blood and tissue dilution solution. Add 3 mL of
181   mouse lymphocyte separation medium, and then centrifuge the cells for 20 min at 300 x g at
182   room temperature.

183

184   2.2.5.   Collect the layer of milky white lymphocytes (i.e., the second layer from the top), wash it
185   2x with PBS, and count with an automated cell counter.

186

187   **2.3.**   **Magnetic activated cell sorting (MACS) positive selection strategy for purification of**
188   **iNKT cells**

189

190   NOTE: For the pretreatment of CD1d tetramers, 1 mg/mL of α-Galcer was diluted to 200 µg/mL
191   with 0.5% of Tween-20 and 0.9% of NaCl, and 5 µL of the resulting solution was added to 100 µL
192   of the CD1d tetramer solution. The mixture was incubated for 12 h at room temperature and
193   placed at 4 °C for use. TCR β was diluted 80x with deionized water. All other antibodies were
194   used as a stock solution.

195

196   2.3.1.   Resuspend 10^7 cells with 100 µL of 4 °C PBS, add 10 µL of α-GalCer-loaded CD1d
197   Tetramer-PE, and incubate them at 4 °C for 15 min in the dark.

198

199   2.3.2.   Wash the cells 2x with PBS and resuspend them in 80 µL of PBS.

200

201   2.3.3.   Add 20 µL of anti-PE-MicroBeads and incubate them at 4 °C for 20 min in the dark.

202

203   2.3.4.   Wash them 2x with PBS and resuspend the cells with 500 µL of PBS.

204

205   2.3.5.   Place the sorting column in the magnetic field of the MACS sorter and rinse with 500 µL
206   of PBS.

207

208   2.3.6.   Add the cell suspension from step 2.3.4 to the sorting column, collect the flowthrough,
209   and rinse 3x with PBS buffer.

210

211   2.3.7.   Remove the magnetic field and collect the cells from the sorting column. At this point,
212   add 1 mL of PBS buffer to the sorting column, and quickly push the plunger at a constant
213   pressure to drive the labeled cells to the collection tube and obtain purified iNKT cells. Count
214   with an automated cell counter.

215

216   **2.4.**   Identification of the iNKT cell phenotype

2.4.1. Take 1 x 10^6 cells from steps 2.2.5 and 2.3.7, respectively, and resuspend them in 50 μL of PBS.

2.4.2. Antibody incubation: Do not add the antibody to the negative control tube, add 0.5 μL of α-GalCer-PE-CD1d Tetramer or 10 μL of FITC-TCR β in the single positive control tube. Add 0.5 μL of α-GalCer-PE-CD1d tetramer and 10 μL of FITC-TCR β in the sample tube. Incubate them at 4 °C for 30 min in the dark.

2.4.3. Wash the cells in PBS and then centrifuge at 200 x g for 5 min.

2.4.4. Discard the supernatant, add 1 mL of Foxp3 Foxation/Permeabilization working solution, and incubate the cells for 45 min at 4 °C in the dark.

2.4.5. Add 1 mL of 1x Permeabilization Buffer working solution and centrifuge the cells at room temperature for 500 x g at room temperature for 5 min.

2.4.6. Discard the supernatant. Add 1 μL of Alexa Fluor 647 mouse Anti-PLZF and 1 μL of PerCP-Cy 5.5 mouse anti-T-bet (or 1 μL of PerCP-Cy 5.5 mouse anti-RORYt) for 30 min at room temperature in the dark.

2.4.7. Add to 2 mL of Permeabilization Buffer working solution for cleaning.

2.4.8. Discard the supernatant, resuspend the cells in 500 μL of PBS, and measure by flow cytometry.

2.5. Functional identification of iNKT cells

2.5.1. Take 3 x 10^6 iNKT cells from step 2.3.7 and resuspend them in 12 well plates with 1.5 mL of RPMI-1640 incomplete medium (i.e., without serum).

2.5.2. Add phorbol ester (PMA, 50 ng/mL) and ionomycin calcium (IO, 1 μg/mL) and place in a CO_2 incubator for 24 h.

2.5.3. Collect the cell supernatant and detect the secretion levels of IL-2, IL-17A, TNF-α, IL-6, IL-4, IFN-γ, and IL-10 using a commercial CBA assay according to the manufacturer's protocol.

2.6. Experimental study on the migration pathway of iNKT cells in RA mice

2.6.1. Dissolve DiR dye (2.5 mg/mL) in DMSO.

2.6.2. Resuspend iNKT cells in 6 well plates with RPMI-1640 incomplete medium. The density is 1 × 10^6 cells/mL.

260

261   2.6.3.   Add DiR (5 μg/mL) solution and incubate in a CO_2 incubator for 25 min.

262

263   2.6.4.   Wash with PBS and resuspend the cells (3×10^6/300 μL) to obtain DiR-labeled iNKT cells
264 (DiR-iNKT).

265

266   2.6.5.   Inject 1% sodium pentobarbital (50 mg/kg body weight) intraperitoneally to anesthetize
267 the mice. Adequately anesthetized mice do not show a hind paw withdrawal to toe pinch. Apply
268 veterinary ointment to the mouse eyes to prevent dryness while under anesthesia for imaging.

269

270   2.6.6.   Inject DiR-iNKT cells 3×10^6 per mouse into the tail vein with the RA model for 8 days.
271 Monitor the iNKT cells in mice postinjection for 0 min, 10 min, 30 min, 60 min, and day 0 (after
272 3 h), 1, 3, 6, 12, 26, 34, 38, and 42 days using a small animal in vivo imaging system (IVIS). The
273 excitation wavelength used was 748 nm, the emission wavelength was 780 nm, and the
274 exposure time was automatic.

275

276   2.6.7.   Place each mouse in a separate cage after each observation and maintain sternal
277 recumbency. Observe until recovery from anesthesia.

278

279 **3.   Evaluation of adoptive immunotherapy of RA mice with iNKT cells**

280

281   3.1.   iNKT cell adoptive immunotherapy for RA mice

282

283   3.1.1.   Inject 3×10^6 cells iNKT cells per mouse through the tail vein. Randomly select 15 mice
284 that were modeled 8 days prior and obtain iNKT cells without DiR labeling from step 2.3.7 by
285 the tail vein infusion.

286

287   3.2.   Evaluate the efficacy of adoptive immunotherapy for iNKT cells.

288

289   3.2.1.   Measure the thickness of the paw of the mouse, quantify the swelling of the ankle joint,
290 and systematically score after the infusion of iNKT cells as described in steps 1.2.1–1.2.2.

291

292   3.2.2.   Observe the inflammatory cell infiltration and joint changes of the mouse joints as
293 described in step 1.2.3.

294

295   3.2.3.   Determine the secretion levels of IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, and IFN-γ as
296 described in step 1.2.4.

297

298   3.3.   Determine the frequency of iNKT cells and subsets.

299

300   3.3.1.   Isolate the mouse thymus and prepare a single cell suspension.

301

302   3.3.2.   Separate the lymphocytes with lymphocyte separation fluid.

303

304   3.3.3.   Determine the iNKT cell frequency and subgroup frequency as described in step 2.4.

305

306   3.4.   Statistical analysis

307

308   NOTE: All data are presented as mean ± SD. Values of $P < 0.05$ were considered statistically
309   significant.

310

311   3.4.1.   Use one-factor analysis of variance (ANOVA). If the variance is satisfied, use the LSD test
312   for further comparison.

313

314   3.4.2.   If the variance is not uniform, use the nonparametric test. Use the Kruskal-Wallis H test
315   for further comparison[31].

316

317   **REPRESENTATIVE RESULTS:**
318   The arthritis index score and paw thickness increased after modeling. Compared with the control
319   group, the toes of the RA model group began to show red swelling at 6 days after modeling, with
320   gradual aggravation. At 14 days, the red swelling in the ankle joint peaked, followed by gradual
321   relief. The thickness of the paw changed similarly ($P < 0.05$) (**Figure 1**).

322

323   The inflammatory cell infiltration increased significantly after modeling. The pathological results
324   showed that the infiltration degree of inflammatory cells in the ankle synovial tissue of the RA
325   model mice was different at different stages. Peak inflammation occurred on day 14 post
326   modeling (**Figure 2**).

327

328   Inflammatory cytokines increased and anti-inflammatory cytokines decreased in the serum. In
329   the RA model group, the serum levels of pro-inflammatory cytokines (TNF-α, IFN-γ, and IL-6)
330   significantly increased ($P < 0.05$), while anti-inflammatory cytokines (IL-4 and IL-10) significantly
331   decreased ($P < 0.05$) (**Figure 3**).

332

333   The iNKT cells obtained by in vivo induction and in vitro purification mainly consisted of iNKT2
334   cell subsets, which secrete anti-inflammatory cytokines. Intraperitoneal injection of α-GalCer
335   increased the frequency of iNKT cells in the body, predominantly the iNKT2 subgroup. The
336   frequency of spleen iNKT cells in normal DBA/1 mice was about 2% of the lymphocytes, (iNKT2
337   was about 5%, iNKT1 about 15%, iNKT17 about 10%). Three days after the intraperitoneal
338   injection of α-GalCer, the frequency of iNKT cells was about 6% of the lymphocytes, (iNKT2 was
339   about 82%, iNKT1 about 1.5%, and iNKT17 about 0.5%). After purification by MACS, the purity of
340   iNKT cells was over 85%, of which iNKT2 was about 92%, iNKT1 about 0.4%, and iNKT17 about
341   0.2% (**Figure 4**).

342

343   The harvested iNKT cells secreted more anti-inflammatory cytokines and fewer inflammatory
344   cytokines. The iNKT cells were isolated from the spleens of normal mice and intraperitoneally
345   injected with α-GalCer 3 days after the mouse spleen (α-GalCer group) and the cytokine levels in

346 the cell culture supernatant were examined. Compared with the control group, the inflammatory
347 cytokines (IL-17A, TNF-α, IFN-γ, and IL-6) of the α-GalCer group significantly decreased ($P < 0.05$),
348 and the anti-inflammatory cytokine IL-4 level significantly increased ($P < 0.05$). There was no
349 significant difference in IL-10 ($P > 0.05$). The IFN-γ/IL-4 ratio significantly decreased ($P < 0.05$)
350 (**Figure 5**).

352 IVIS tracing confirmed that DiR-iNKT cells were adoptively infused into RA mice and immediately
353 appeared in the lungs postinjection. Fluorescence was detected in the liver at 10 min and in the
354 spleen at 60 min (**Figure 6A I, 6A II, 6A III**). In the isolated organs, there was no fluorescence in
355 the thymus and inguinal lymph nodes within 1 h. Fluorescence was detected in the lungs at 0
356 min, the fluorescence intensity was the greatest at 10 min, and then gradually weakened. There
357 was weak fluorescence in the liver at 0 min, and then it gradually increased. The fluorescence in
358 the spleen was detected at 30 min and then gradually increased (**Figure 6AIV, 6C**).

360 After the infusion of DiR-iNKT cells into RA mice, fluorescence was mainly concentrated in the
361 liver and spleen (**Figure 6B I, 6B II, 6B III**), but there was no fluorescence in the thymus and
362 inguinal lymph nodes. The spleen and liver had the highest fluorescence intensity on day 1 after
363 cell infusion, but it gradually weakened. On day 34, the surface fluorescence disappeared. On day
364 42, the fluorescence of the isolated organs disappeared. The average fluorescence signal
365 intensity of liver after the cell infusion was higher than that of the spleen (**Figure 6B IV, 6D**).

367 Adoptive infusion of iNKT cells into RA mice can alleviate disease progression and improve clinical
368 symptoms. The iNKT cells improved the clinical symptoms of RA mice after adoptive infusion.
369 Compared with the untreated RA model group, the swelling of the ankle joint was relieved in the
370 cell treatment group, and the scores significantly decreased from day 10 to day 20 postinjection.
371 In the same period in the cell treatment group, inflammatory cell infiltration in the synovial tissue
372 was reduced in comparison to the RA model group (**Figure 2**).

374 The success rates of thymus iNKT cells increased significantly ($P < 0.05$). Compared with the
375 healthy control group, in the RA model group the rates of iNKT cells in the thymus decreased at
376 the progress (day 11), peak (day 14), and recovery (day 20) stages. At peak inflammation, these
377 values were minimal and rebounded in the remission phase. The cell therapy group showed
378 significantly increased rates of iNKT cells at the peak (day 14) and recovery (day 20) stages in
379 comparison with the RA model group ($P < 0.05$) (**Figure 7**).

381 After iNKT cell infusion, the success rate of iNKT1 and iNKT17 in the thymus decreased and iNKT2
382 increased. Compared to the control group, in the RA model group on day 11 iNKT1 and iNKT17 in
383 the thymus significantly increased ($P < 0.05$), and iNKT2 significantly decreased ($P < 0.05$). On day
384 14, iNKT1 and iNKT2 in the thymus significantly increased ($P < 0.05$) and iNKT17 significantly
385 decreased ($P < 0.05$). On day 20, iNKT1 in the thymus significantly increased ($P < 0.05$), iNKT2 did
386 not significantly change ($P > 0.05$), and iNKT17 significantly decreased ($P < 0.05$). The iNKT1/iNKT2
387 ratio significantly increased during all three stages ($P < 0.05$).

389   Compared to the RA model group, on day 11 in the α-GalCer and the cell therapy groups, iNKT1
390   and iNKT17 were significantly lower ($P < 0.05$) and iNKT2 significantly increased ($P < 0.05$). On
391   day 14, in the α-GalCer group iNKT1 and iNKT17 significantly decreased ($P < 0.05$), and iNKT2 did
392   not significantly change ($P > 0.05$); in the cell therapy group iNKT1 and iNKT17 significantly
393   decreased ($P < 0.05$) and iNKT2 significantly increased ($P < 0.05$). On day 20, in the α-GalCer group
394   the iNKT1 significantly decreased ($P < 0.05$), iNKT2 did not change significantly ($P > 0.05$), and
395   iNKT17 significantly increased ($P < 0.05$); in the cell therapy group iNKT1 and iNKT17 significantly
396   decreased ($P < 0.05$) and iNKT2 significantly increased ($P < 0.05$). The ratio of iNKT1/iNKT2
397   significantly decreased during all three stages ($P < 0.05$) (**Figure 7**).
398
399   The levels of inflammatory cytokines were increased in serum and the anti-inflammatory
400   cytokines were decreased after iNKT cell infusion. In the RA model group, the levels of TNF-α,
401   IFN-γ, and IL-6in serum significantly increased (*P < 0.05*), while IL-4 and IL-10 showed markedly
402   decreased amounts (*P < 0.05*) in comparison with the control group. In the iNKT cell therapy
403   group, the levels of TNF-α, IFN-γ, and IL-6 in serum significantly decreased at the progress and
404   peak stages of inflammation (*P < 0.05*), while IL-4 and IL-10 significantly increased (*P < 0.05*) in
405   comparison with the RA model group (**Figure 3**).
406
407   **FIGURE AND TABLE LEGENDS:**
408   **Figure 1. The joint swelling score and paw thickness change in mice.** (**A**) Swelling of the ankle
409   joint in mice. (**B,D**) Paw thickness in different groups. (**C**) Clinical score changes in different
410   groups. The mouse arthritis score and the thickness of the paw were significantly reduced in the
411   cell therapy group on days 10–20 (i.e., 2–12 days after treatment) after modeling. **P < 0.05* vs.
412   control, ***P < 0.05* vs. RA.

413
414   **Figure 2. Histopathological changes of the ankle joint.** The infiltration of inflammatory cells was
415   significantly reduced in the cell therapy group and was significantly increased in the RA group on
416   day 14. ◊ = inflammatory cells. (**A**) 100x (**B**) 400x.
417
418   **Figure 3. The levels of serum cytokines in each group**. (**A**) Serum cytokines levels in mice on day
419   11 after modeling (pg/mL). (**B**) Serum cytokines levels in mice on day 14 after modeling (pg/mL).
420   The levels of TNF-α, IFN-γ, and IL-6 significantly decreased, and the levels of IL-4 and IL-10
421   significantly increased in the cell therapy group. [a]*P < 0.05* vs. control. [b]*P < 0.05* vs. RA.

422
423   **Figure 4. The rates of iNKT cells and proportion of iNKT cell subsets.** (**A,B,C**) The rate of iNKT2 in
424   normal mice is ~5%. (**D,E,F**) The rate of iNKT2 is about 82% after in vivo induction. (**G, H, I**) The
425   rate of iNKT2 is more than 92% after MACS purification.
426
427   **Figure 5. Cytokine levels in culture supernatant of mouse spleen-derived iNKT cells**. The level
428   of IL-4 significantly increased, and the levels of IL-17A, TNF-α, IFN-γ, and IL-6 significantly
429   decreased. [a]*P < 0.05* vs. control.
430

Figure 6. Distribution and metabolism of iNKT cells traced by caliper IVIS lumina II. (**A**,**B**) Migration path of iNKT cells. (**C**) The change of the average fluorescence signal intensity in the spleen, liver, and lung. (**D**) The change of the average fluorescence signal intensity in the spleen and liver. The fluorescence was detected in the lungs and liver at 0 min, and then gradually increased. The fluorescence intensity was strongest at 10 min in the lungs and then decreased. The fluorescence of the spleen was detected at 30 min, then gradually increased. The fluorescence of all organs disappeared on day 42. The average fluorescence signal intensity of the liver is higher than the spleen after cell infusion (I: supine; II: lateral lying; III: prone; IV: isolated tissue; a: control group; b: cell infusion group; 1, 2, 3, 4, 5 are thymus, spleen, liver, inguinal lymph nodes, lungs).

Figure 7. The rates of iNKT and its subsets in the mouse thymus. (**A**) The rates of iNKT cells at 11, 14, and 20 days after modeling. (**B**) The ratio of iNKT1/iNKT2. (**C,D,E**) The rate of iNKT1, iNKT2, and iNKT17. On days 11, 14, and 20 (days 3, 6, and 12 after cell therapy), the rates of iNKT cells significantly increased, the iNKT1 and iNKT17 of the thymus significantly decreased in the cell therapy group, and iNKT2 was significantly increased. [a]$P < 0.05$ vs Control. [b]$P < 0.05$ vs RA.

DISCUSSION:

iNKT cells are special T cells that bridge innate and adaptive immunity and are mainly developed from CD4^{++}/CD8^{+} thymocytes. iNKT cells have diverse immunoregulatory functions and interact with other immune cells by direct contact and secretion of different cytokines[23], affecting dendritic cells (DCs), macrophages, neutrophils, B cells, T cells, and NK cell differentiation and development[24]. α-GalCer is a classical iNKT cell-specific activator extracted from sponges. Several studies indicate that spleen iNKT cell frequency reaches a peak after a single intraperitoneal injection of α-GalCer for 3 days[25]. Our experimental results demonstrated the predominance of the iNKT2 subset in mice spleen for 3 days after an intraperitoneal injection of α-GalCer, which mainly secretes anti-inflammatory cytokines IL-4 and IL-10. We also found that the abundance of iNKT2 cells decreased and that of iNKT1 and iNKT17 cells increased in the RA model mice during the inflammatory phase. Therefore, we isolated the iNKT cells of the mouse spleen by intraperitoneal injection of α-GalCer for 3 days. These were used to treat the RA model mice. The rate of the iNKT2 subset was 82% after in vivo induction. After purification by MACS, the rate of iNKT2 exceeded 92%.

The specific activation of iNKT cells is employed as a novel biological treatment for RA. Horikoshi et al.[21] demonstrated that intradermal injection of α-GalCer inhibited GPI peptide-induced arthritis by significantly inhibiting the number of CD4^{+} T cells. Chiba et al.[26] showed that repeated injections of the synthetic iNKT2 selective activator OCH inhibited CIA, while α-GalCer showed a slight inhibitory effect. We injected spleen-derived iNKT into RA model mice, and the results showed that the degree of swelling of the ankle joints in the cell therapy group and the number of inflammatory cells infiltrating into the joints was reduced. The level of serum anti-inflammatory cytokines (e.g., IL-4 and IL-10) increased, and the secretion of pro-inflammatory cytokines (e.g., IFN-γ and TNF-α) decreased. the targeted activation of iNKT cells was able to alleviate the progression of RA and inhibit the inflammatory response. In addition, we detected

474   the frequency of iNKT in the thymus and found that the number of iNKT cells in the RA model
475   group was significantly reduced, while the frequency in the thymus increased after infusion of
476   the iNKT cells. Due to the presence of the blood-thymus barrier system, we did not consider the
477   adoptively infused increase in the level of iNKT in the thymus that was confirmed in later
478   experiments. Further detection of the subpopulations of iNKT cells in the thymus revealed that,
479   compared to the healthy control group, the number of iNKT1 cells in the RA model group
480   increased significantly during the three stages of inflammation and maximally during the peak of
481   inflammation, while the proportion of the iNKT2 subset began to increase at the peak of
482   inflammation. Notably, the iNKT1 subset might be involved in the early inflammation of RA, and
483   the iNKT2 subset may play an important role in inhibiting the inflammation. Compared to the RA
484   model group, the iNKT1 subset in the cell therapy group significantly decreased during the
485   inflammatory phase, and the iNKT2 subset significantly increased in the early and remission stage
486   of inflammation. These results indicated that adoptive infusion of specific phenotypes and
487   functional iNKT cells significantly increased the frequency of iNKT cells in RA and altered the
488   proportion of iNKT cell subsets.

490   We used IVIS to observe the distribution of iNKT cells in mice after adoptive infusion and found
491   the highest fluorescence intensity in the lungs 10 min after infusion, which faded gradually. The
492   liver showed weak fluorescence, which increased gradually and decreased gradually after 2 days.
493   Fluorescence was detected in the spleen at 30 min, which increased gradually and decreased
494   after 2 days. The average fluorescence intensity of the liver was stronger than that of the spleen.
495   However, no fluorescence was detected in the thymus and inguinal lymph nodes. The detection
496   of fluorescence in the lungs might be attributed to the infusion of iNKT cells into the tail vein.
497   These cells are circulated to the lungs with the blood. The liver fluoresces earlier than the spleen,
498   and the average fluorescence intensity of the liver is stronger than that of the spleen. This
499   preferential accumulation of the iNKT cells may be due to the abundant blood vessels in the liver,
500   the main metabolic organ. Fluorescence was not detected in the thymus. This might be due to
501   the hematological barrier, which could hinder the entry of iNKT cells. Fluorescence was never
502   detected in the inguinal lymph nodes, perhaps because fewer iNKT cells entered the lymph nodes
503   and did not reach the minimum level of detection. Also, it could be speculated that the iNKT cells
504   infused into the tail vein might not enter the lymph nodes. Therefore, we hypothesize that the
505   development and differentiation of iNKT cells in the thymus might be regulated through cytokine
506   pathways after adoptive infusion into the mice. This needs to be elucidated further.

508   GPI is present in the serum and synovial fluid of most RA patients and is a commonly used test
509   for clinical RA diagnosis[27]. Bruns et al.[28] used peptides of different lengths from the GPI sequence
510   and immunized the DBA/1 mice to identify six immunodominant T cell epitopes. Of these, three
511   were arthritogenic. The peptides with >95% incidence of arthritis are hGPI 325-339 and hGPI469-
512   483. Our previous studies demonstrated that the use of a mixture of the two peptides to establish
513   an RA model is better than a single peptide. In the resulting RA models, the toes and joints of the
514   mice began to appear red on day 6 and reached a peak of inflammation on day 14. The
515   inflammatory cell infiltration was accompanied by tissue hyperplasia in the synovial tissue of the
516   joints, and the inflammatory cell infiltration was most severe at day 14. The number of iNKT cells

517 was decreased significantly in the thymus at the peak of inflammation, which was consistent with
518 the trend of iNKT cells in RA patients[29]. Further detection of iNKT cell subsets revealed that the
519 frequency of iNKT1 and iNKT17 in the thymus increased and the frequency of iNKT2 decreased
520 during the progression of inflammation (day 11). In addition, the levels of serum cytokines IFN-γ
521 and IL-17A increased during the progression (day 11) and the peak of inflammation (day 14),
522 suggesting a similar polarization of the Th1 and Th17 subgroups in RA model mice[30]. Therefore,
523 the RA mouse model induced by hGPI325-339 and hGPI469-483 mixed polypeptide fragments
524 exhibited the characteristics of CD4$^+$ T cell hyperproliferation and iNKT cell defects, which was
525 similar to that of the RA patients, and could be used as an ideal animal model for investigating
526 the immunity of the RA cells. These results were in agreement with those from our previous
527 studies and demonstrated the stability of the hGPIs-induced RA model[31].
528
529 In general, the RA mouse model induced by the mixed G6PI peptides can simulate the changes in
530 CD4$^+$ T cells, iNKT cells, and related cytokines in RA patients. This provides a good model for in-
531 depth investigation of RA. Consequently, iNKT (mainly iNKT2) induced by intraperitoneal
532 injection of α-GalCer and purified in vitro was used in the treatment of RA. It can correct the
533 immune imbalance caused by abnormal proliferation of Th subsets, relieve the progression of
534 RA, and in the future may provide novel methods for the clinical treatment of RA.
535

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544

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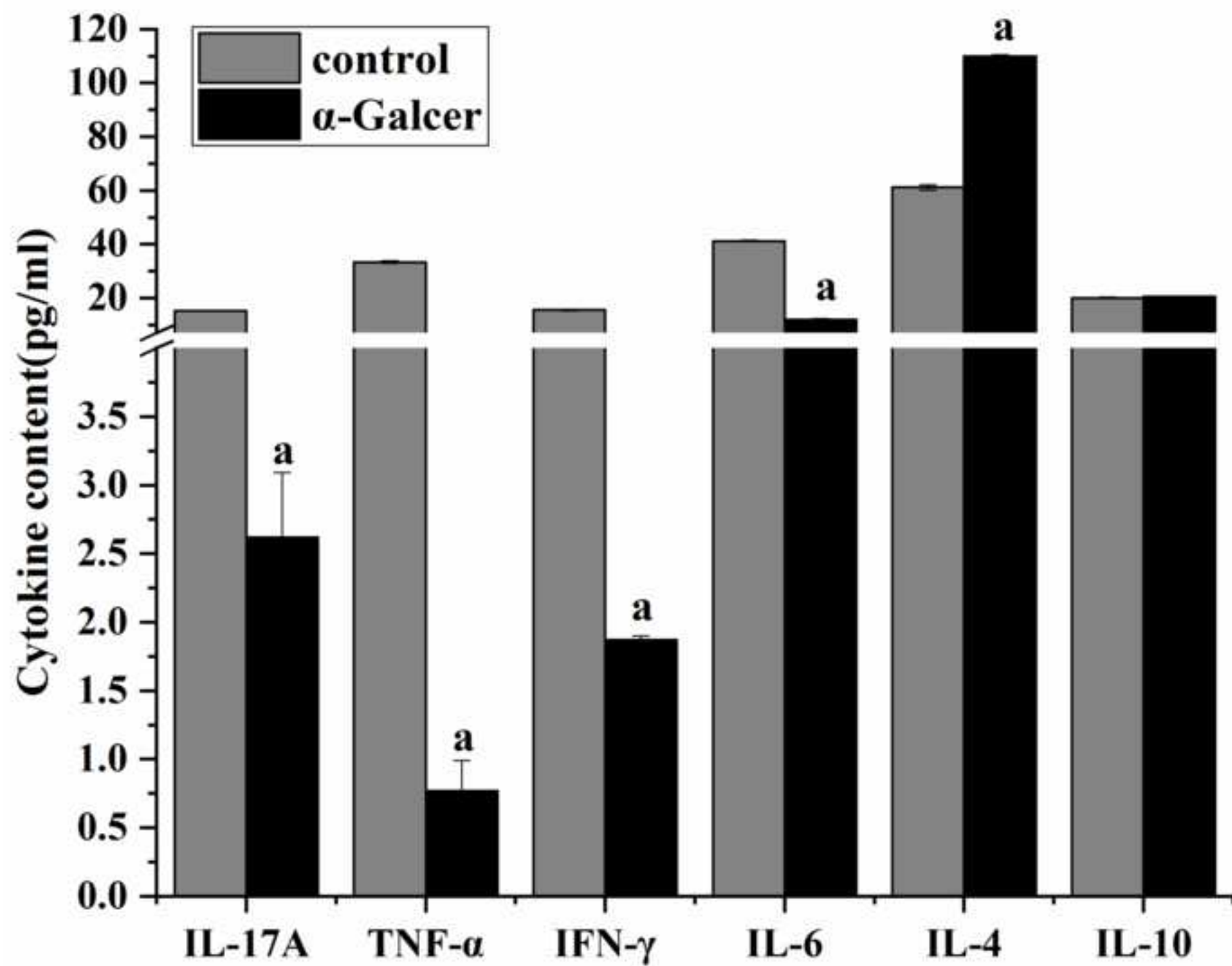621   1120–1125 (2015).

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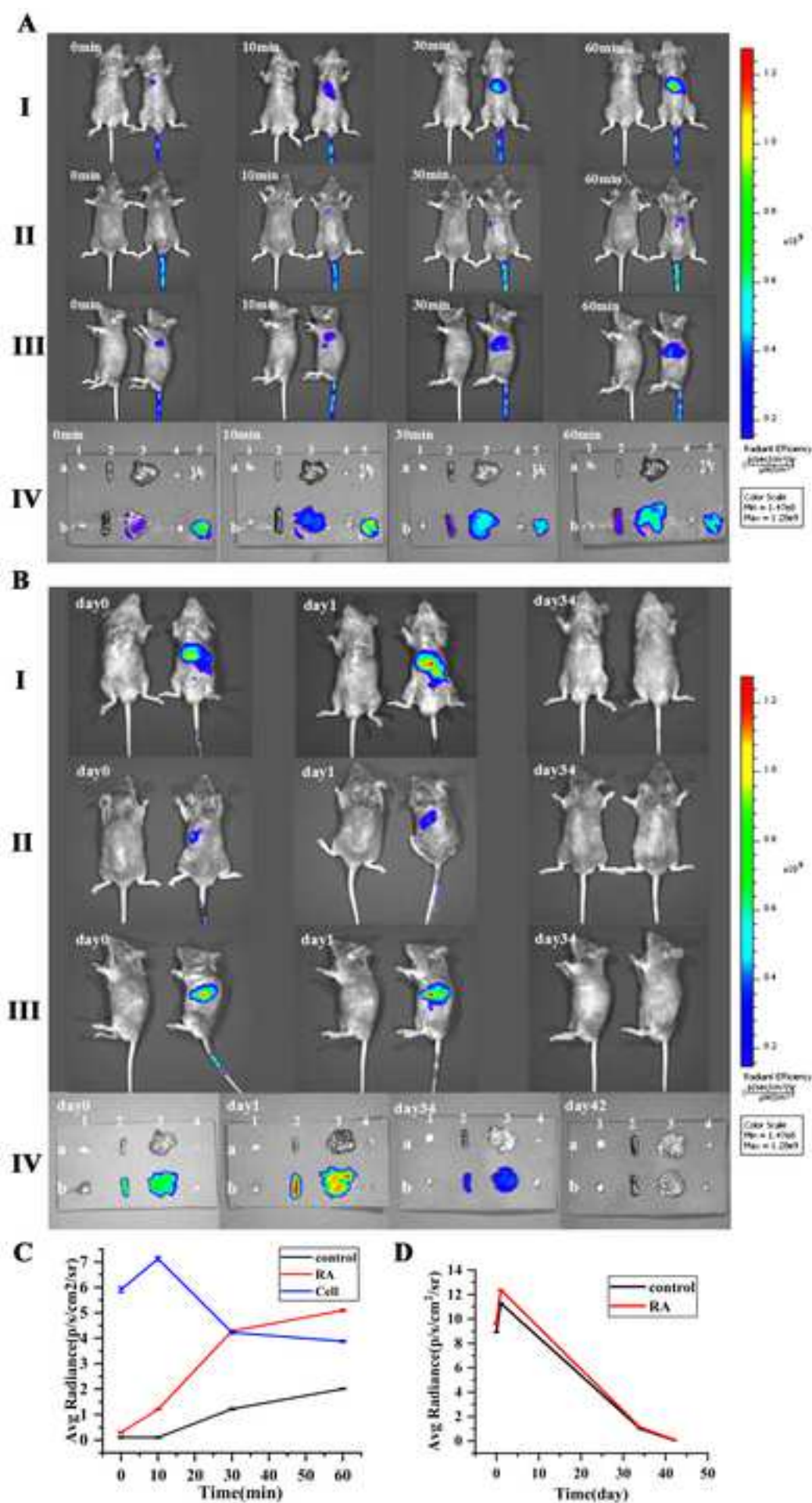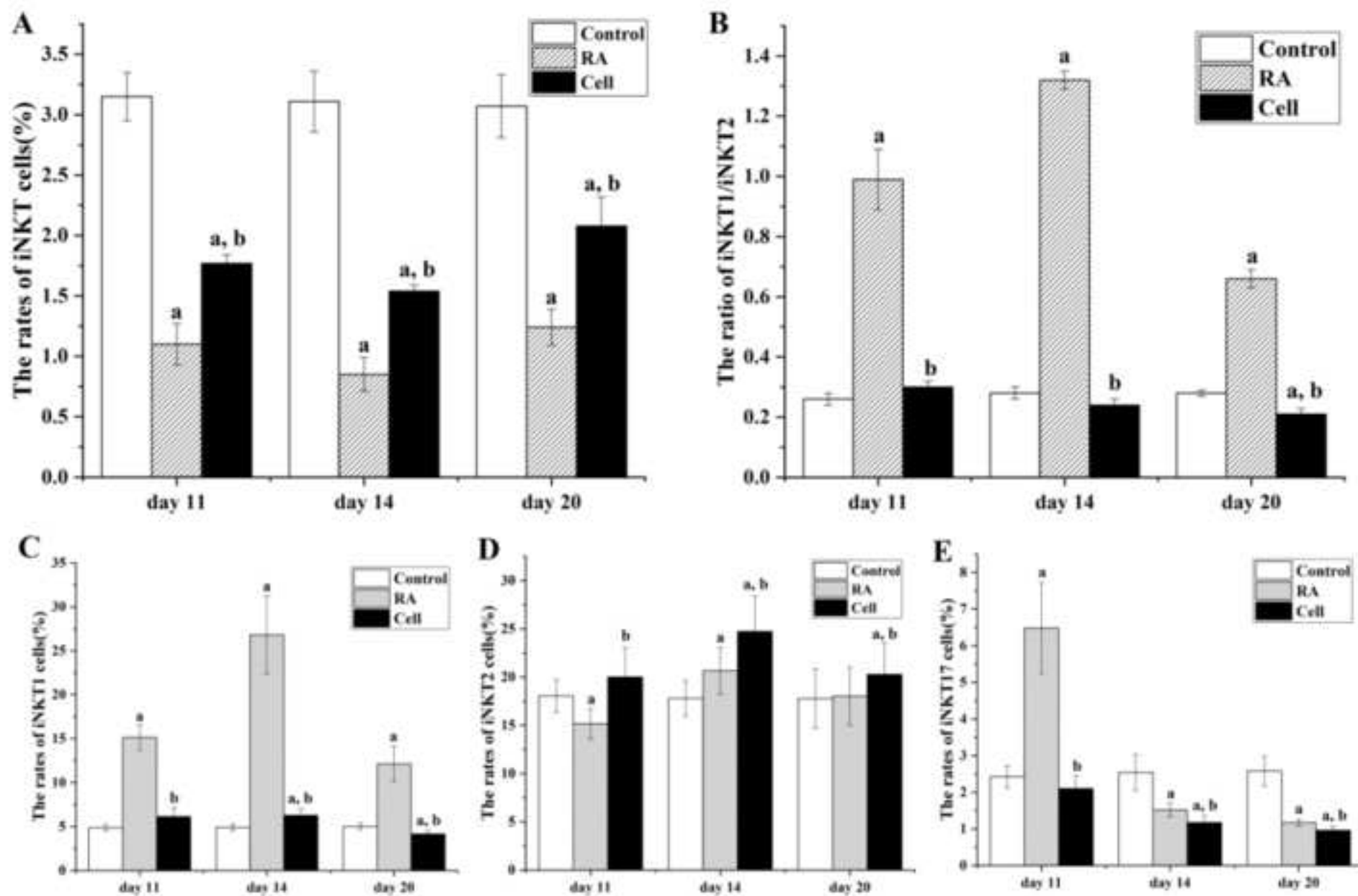624   (2019).

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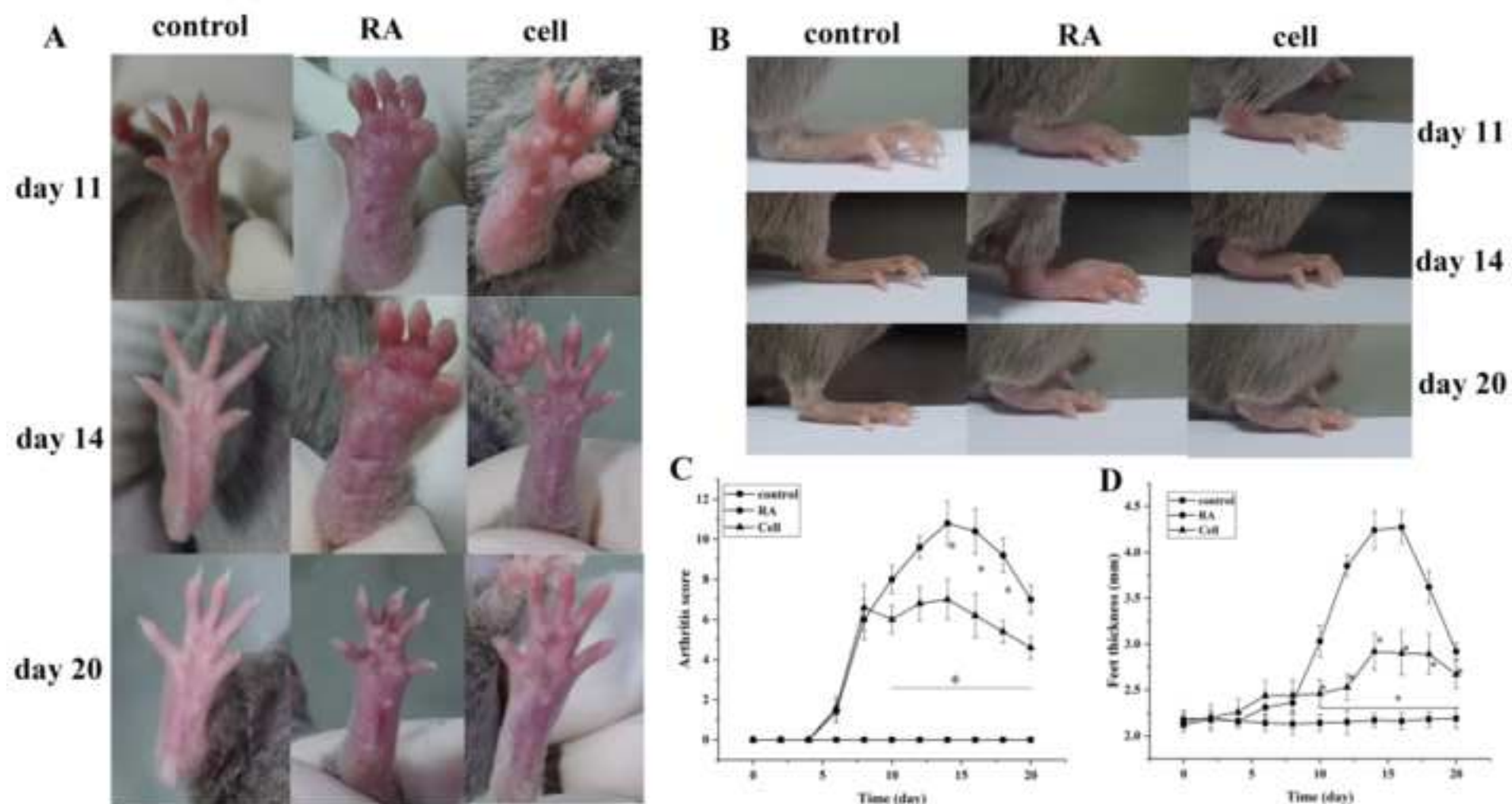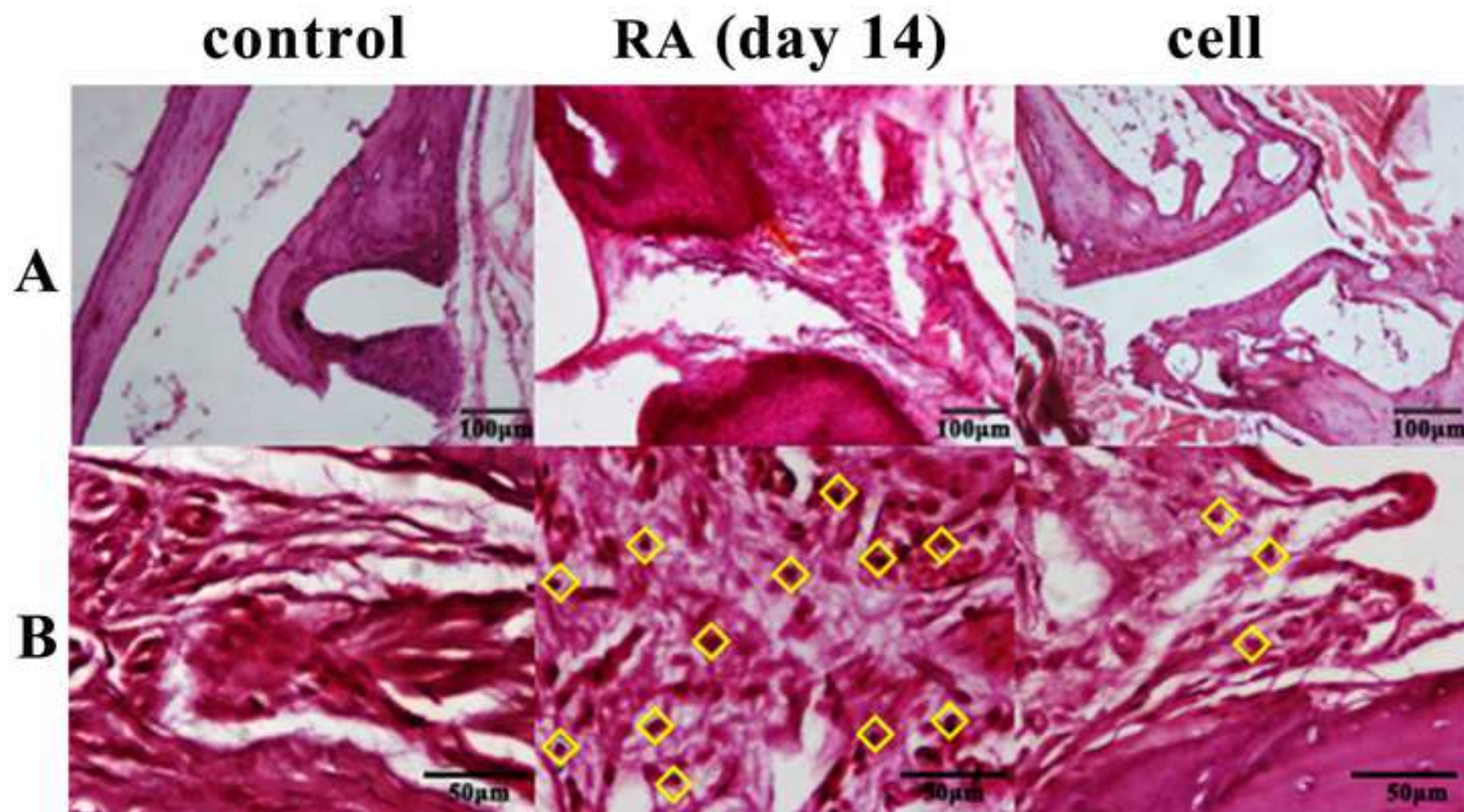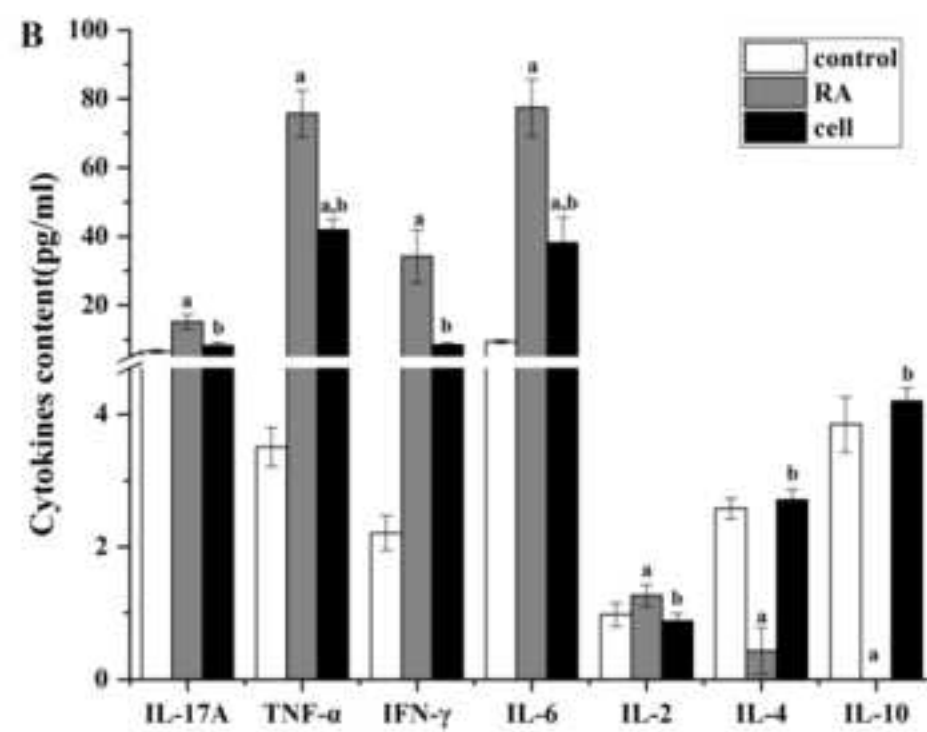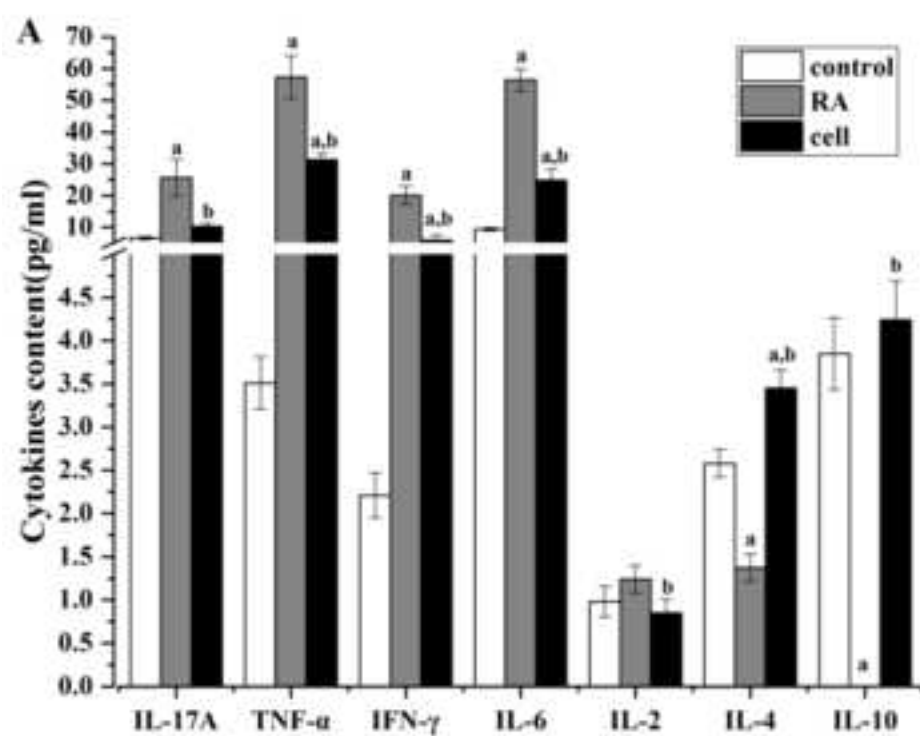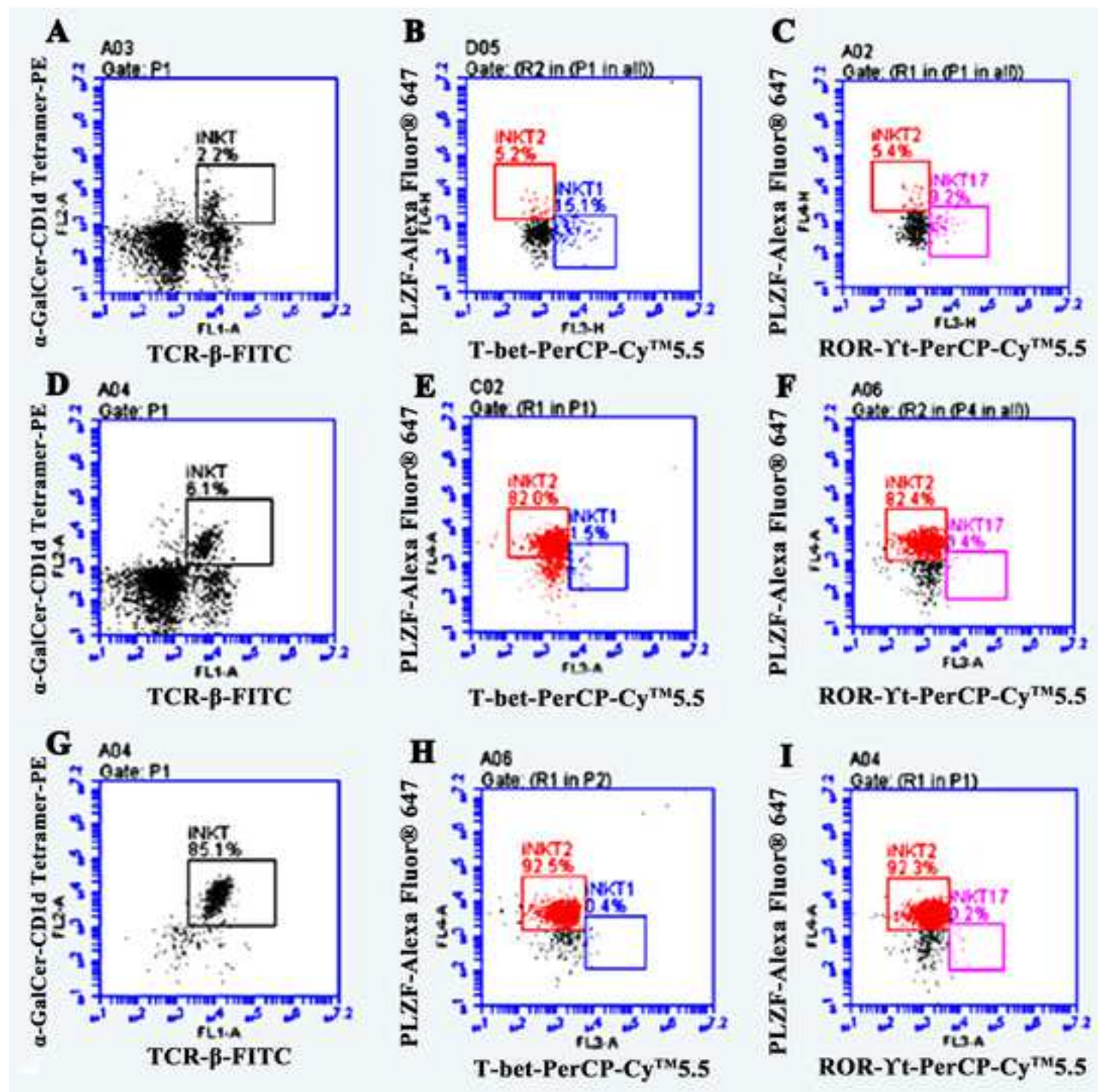
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FITC Hamster Anti-Mouse TCR β Chain	BD
Flow cytometer	BD
Freund's complete adjuvant	Sigma
hGPI325-339 （IWYINCFGCETH/	Karebay Biochem
hGPI469–483 （EGNRPTNSIVFTI	Karebay Biochem
In Vivo Imaging System	PerkinElmer
Ionomycin Calcium	Cayman
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Dear Dr. Nam Nguyen,

Thank you very much for your letter comments on the manuscript that named "Adoptive immunotherapy of iNKT cells in glucose-6-phosphate isomerase (G6PI) - induced RA mice". We appreciate the valuable and helpful comments. We have provided point-by point responses to all comments and modified accordingly the manuscript. We have also additionally improved the language of the manuscript. We uploaded the revised manuscript in the submission system, including the clear version and the markup version, and responded to and modified your comments in the markup version. Thank you again.

Kind regards,

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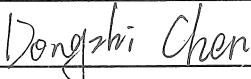
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