White Paper

Immunohistochemical staining to investigate antibody binding to cartilage *in vivo*

Keywords: Immunohistochemistry, Rheumatoid arthritis, Cartilage, Animal models, Antibodies, Complement

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory joint disease in which the circulating autoantibodies, among them especially the anti-citrullinated protein antibodies (ACPAs), are characteristic for the classical form of the disease.

Cartilage-antibody induced arthritis, CAIA, is a model mimicking the acute phase of arthritis in which arthritogenic antibodies initiate arthritis. CAIA may be enhanced by either lipopolysaccharide (LPS) or mannan, to induce lpsCAIA and mCAIA, respectively. Both can also be used as a model of chronic relapsing arthritis when re-enhanced with LPS or mannan later during the disease course. [1, 2] CAIA may be induced in several mouse strains with the novel Cab3 and Cab4 cocktails, which may be used to induce arthritis even in the relatively resistant but commonly used C57BL/6J strain [3]. The CAIA model is dependent on intact innate immune defense, including activating functional Fc-receptors and complement [4]. Complement activation has been detected in synovial tissue in both RA and murine models [5], and arthritogenic antibodies bind to human and mouse cartilage [6].

This white paper describes the procedure to investigate the fate of intravenously administered cartilage-binding antibodies *in vivo* and the method to visualize complement deposition on the cartilage.

2. Methodology

In vivo administrations to investigate antibody binding to cartilage

To investigate antibody binding to cartilage *in vivo*, biotin-labelled antibodies are injected to mice for immunofluorescence staining by streptavidin-conjugates. Adult, preferably 8–12-week-old, mice are injected (i.v.) with antibody cocktails containing cartilage or collagen antibodies, at least 0.5 mg/mouse. Cab3 cocktail (containing M2139, L10D9, and 15A11 antibodies), anti-Col2 antibody

cocktail (containing M2139, UL1, CIIC1, and CIIC2 antibodies) and mock cocktail (containing G11, L243, and Hy2.15 isotype control antibodies with irrelevant specificities) have been tested and reported in [3].

After 48 hours, limbs are collected for preparation of sections. For sectioning, the limbs are snap frozen, dissected to 10–15 µm thick sections, and stored in −80 °C until used. Pls note, they cannot be decalcified as the tissue bound antibodies will be released. Due to bone in the tissue, it is difficult to make good sections. It is easier with small joints and using diamond knifes with broader angles. To make sectioning easier neonatal mice can be used.

Visualization of *in vivo* administered antibodies on the cartilage

The i.v. administered, biotin-labelled antibodies on the cartilage may be visualized by confocal microscopy. For that, the tissue sections are incubated with streptavidin-conjugated fluorescent dye. Result from studies using streptavidin-conjugated Alexa Fluor 568 (Thermo Fischer Scientific, Waltham, USA) at 1:300 dilution, and incubation for 60 min in RT have been reported in [3]. After incubation, tissue was mounted using VECTASHIELD® Mounting Medium with DAPI (Vector, CA, USA) to stain nuclei. The slides were dried for 30 min before scanned under confocal microscope. Representative results are shown in Figure 1, panel A.

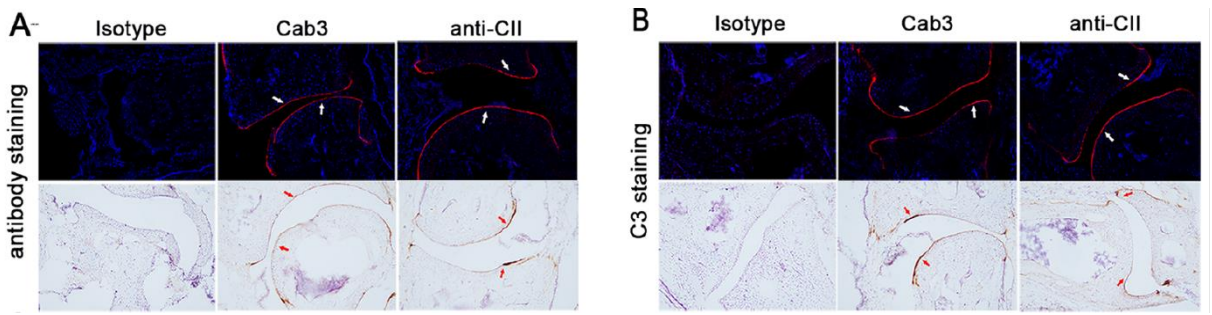

Figure 1. Immunohistochemistry to visualize antibody cocktail binding to cartilage in vivo (A) and the locally deposited C3 immune complexes on the surface of cartilage 48 hrs after antibody cocktail injection (B). Cab3 is the novel cartilage antibody cocktail and anti-CII is the classical Col2-antibody cocktail. Figure is from Li et al, published in Arthritis Research & Therapy (2020) 22:120; (Link to the Creative Commons Licence: http://creativecommons.org/licenses/by/4.0/) [3].

***In vivo* administrations to detect complement C3 on the cartilage**

To visualize that the antibody cocktail forms immune complexes with complement C3 on the cartilage surface, mice are injected (i.v.) with antibody cocktails containing cartilage or collagen antibodies (not labeled with biotin), 2 mg/mouse [3]. Cab3 cocktail (containing M2139, L10D9, and 15A11 antibodies), anti-Col2 antibody cocktail (containing M2139, UL1, CIIC1, and CIIC2 antibodies) and mock cocktail (containing G11, L243, and Hy2.15 isotype control antibodies with irrelevant specificities) have been tested and reported in [3]. After 48 h, the paws are collected for preparation of sections. For sectioning, the limbs are snap frozen, dissected to 7–15 µm thick sections and stored in −80 °C until used.

Similar procedure may be adopted to investigate not only complement C3 but also other proteins and their activation and/or expression changes that result from i.v. administration of Cab cocktails.

Immunohistochemical (IHC) staining to visualize complement C3

For immunohistochemical (IHC) staining of complement C3, the sections are fixed in acetone on ice, or alternatively at -20°C, for 10 min followed by air drying for 10 min and rehydration in 1× PBS for 3 times, for 5 min each time in RT. After peroxidase blocking with 3% H_2O_2 for 10 min, the sectioned tissue is further blocked with 1× PBS containing 5% BSA and 2% rat serum for 45 min at RT. Next, the sections are incubated with biotin-conjugated goat anti-mouse-C3c antibody (Nordic MUbio, Heerhugowaard, Netherlands) for 1 h at RT. Cryosections are washed in 0.05% (vol/vol) Tween-20 in PBS (PBS-T). Then the sections are incubated with ExtrAvidin ™-Peroxidase (Sigma-Aldrich) in PBS-T supplemented with 2% FCS (fetal calf serum) for 30 min. C3 is visualized with diaminobenzidine (Vector, CA, USA) according to manufacturer's instructions. Briefly, the sections are incubated with the diaminobenzidine substrate working solution at room temperature for 8–10 min and then washed with water. Optimal development times should be determined by each investigator, though. Representative results have been reported in [3] and are shown above in Figure 1, panel B.

3. Conclusive remarks

The method described here, is a powerful tool to follow the timing and extent of antibody binding on cartilage *in vivo*. Since the antibodies are allowed to settle on the tissue *in vivo*, the bound antibodies detected on tissue sections reflect the physiological condition very well, in contrast to methods when cartage-binding antibodies are incubated with tissue *in vitro*.

Complement staining method presented here is a tool to visualize and quantify, the complement deposited on cartilage during development of arthritis.

4. References

1. Kelkka T, Hultqvist M, Nandakumar KS, Holmdahl R. Enhancement of antibody-induced arthritis via Toll-like receptor 2 stimulation is regulated by granulocyte reactive oxygen species. Am J Pathol. 2012;181(1):141–50.
2. Hagert C, Sareila O, Kelkka T, Nandakumar KS, Collin M, Xu B, Guérard S, Bäcklund J, Jalkanen S, Holmdahl R. Chronic Active Arthritis Driven by Macrophages Without Involvement of T Cells: A Novel Experimental Model of Rheumatoid Arthritis. Arthritis Rheumatol. 2018 Aug;70(8):1343-1353.
3. Li Y, Tong D, Liang P, Lönnblom E, Viljanen J, Xu B, Nandakumar KS and Holmdahl R. Cartilage-binding antibodies initiate joint inflammation and promote chronic erosive arthritis. Arthritis Research & Therapy (2020) 22:120. (Link to the Creative Commons Licence: http://creativecommons.org/licenses/by/4.0/)
4. Kagari T, Tanaka D, Doi H, Shimozato T. Essential role of Fc gamma receptors in anti-type II collagen antibody-induced arthritis. J Immunol. 2003;170(8):4318–24.
5. Holers VM, Banda NK. Complement in the Initiation and Evolution of Rheumatoid Arthritis. Front Immunol. 2018; 9: 1057.
6. Ge C, Tong D, Liang B, Lönnblom E, Schneider N, Hagert C, Viljanen J, Ayoglu B, Stawikowska R, Nilsson P, Fields GB, Skogh T, Kastbom A, Kihlberg J, Burkhardt H, Dobritzsch D, Holmdahl R. Anti-citrullinated protein antibodies cause arthritis by cross-reactivity to joint cartilage. JCI Insight. 2017 Jul 6; 2(13): e93688.

Abbreviations

ACPAs: Anti-citrullinated protein antibodies; Col2: Type II Collagen; Col11: Type XI Collagen; Cab3: Three cartilage-binding antibodies; CAIA: Collagen or cartilage-binding antibody-induced arthritis; lpsCAIA: Cab-induced LPS-enhanced arthritis; mCAIA: Cab-induced mannan-enhanced arthritis; i.v.: Intravenously; IHC: Immunohistochemical; LPS: Lipopolysaccharide; mAbs: Monoclonal antibodies; PBS: Phosphate-buffered saline; RA: Rheumatoid arthritis.