Title

Divergent and dominant anti-inflammatory effects of patient-derived anti-citrullinated protein antibodies (ACPA) in arthritis development

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Abstract

About two thirds of rheumatoid arthritis (RA) patients develop anti-citrullinated protein antibodies (ACPA), which are a characteristic of the disease and sparsely observed in other clinical settings or in the healthy population. ACPA are used as a diagnostic criterion and often develop prior to diagnosis. Therefore, ACPA titers are important in the identification of individuals at-risk of developing RA. Interestingly, the titers and target cross-reactivity of ACPA increase by time of diagnosis, suggesting a causality between anti-citrulline reactivity and the development of RA. However, not all ACPA-positive at-risk individuals will progress to a clinical RA diagnosis. This observation suggests that there are different types of ACPA and that their relationship with RA development is more complex than presently assumed.

To address the biological effect of ACPA in the establishment and development of inflammatory arthritis, we made use of the collagen antibody-induced arthritis (CAIA) model of passive arthritis. Using seven unique patient derived monoclonal ACPA, we observed that ACPA are predominantly anti-inflammatory, with some clones (C03 and BVCA1) completely inhibiting disease development. We have also identified a clone (C04) as having a disease-prone effect, by increasing and sustaining disease prevalence. This clone had previously been reported as being pro-inflammatory in a different model of joint inflammation. The anti-inflammatory effects of C03 were dependent on FcγR, since neither F(ab')2 or a mutated Fc-null GRLR-C03 clone could mediate disease protection. Most importantly, mice receiving polyclonal ACPA enriched from RA sera were equally partially protected from disease. Looking at the cell and tissue of origin of the ACPA clones, their V(D)J sequences, their glycosylation pattern and their fine-specificities, we could not identify a common feature between anti-inflammatory ACPA distinguishing them from those without the properties Together, our data demonstrates that circulating ACPA in RA patients are predominantly anti-inflammatory, emphasizing the need to study ACPA repertoires in order to determine their influence on the clinical outcome of the patient or at-risk individual.

Letter

The presence of anti-citrullinated protein antibodies (ACPA) is currently used in RA diagnosis and distinguishes the major subsets of patients. The demonstration that ACPAs occur before onset of RA and more rarely after disease onset[1] has been used to advocate a causal role of ACPAs in disease development. However, not all ACPA-positive individuals progress to clinical RA, suggesting a complex relationship between ACPAs and arthritis development, where ACPAs displaying different inflammatory aptitudes may exist. To address such questions, we and others have isolated single B cells from RA patients and re-expressed monoclonal ACPAs[2,3]. These monoclonal ACPAs display different properties regarding immune-mediated processes in vitro, as well as in vivo phenotypes such as pain and bone erosion[4,5]. Using the collagen antibody-induced arthritis (CAIA) model of passive arthritis, we assessed the properties of different monoclonal ACPAs in vivo concerning their ability to modify the arthritic process (figure 1A). To address this question in an unbiased manner, we tested seven monoclonal ACPAs expressed as murine chimeric IgG2a and displaying unique profiles of citrulline-directed fine-specificities (figure 1B and supplementary figure 1). In line with previous evidence, ACPAs per se did not induce arthritis (supplementary figure 2A). Interestingly however, we observed several monoclonal ACPAs inhibiting (clones mC03 and mBVCA1) or ameliorating arthritis (mB09 and mA01; figure 1B, 1C and 1D), whereas other clones showed no effect in the model (mX1604, m17D08; figure 1C and 1D), and only one clone provided a slight enhancing effect on arthritis prevalence (mC04; figure 1C). Using a different model of joint inflammation, the mC04 monoclonal ACPA was previously shown to have an arthritis-accelerating effect[6]. Strikingly, mice receiving the mC03 clone at the peak of disease recovered almost completely from joint inflammation 48h post ACPA transfer (figure 1E). Similar results were observed with mB09, although less dramatic (supplementary figure 2B). When combining mC03 and mC04 ACPAs, the anti-inflammatory effect of mC03 ACPA prevailed, i.e. no arthritis developed (supplementary figure 2C). The inhibitory effect

on arthritis was not linked to any of the ACPA clones' fine-specificities, nor to a previously reported histone epitope associated with this effect (supplementary figure 1E)[7]. The anti-inflammatory effects induced by mC03 were clearly FcγR-dependent, with both its F(ab')2 fragments and FcγR null (GRLR-mutated) variants incapable of suppressing arthritis development (figure 1F and 1G; comparison between murine and human C03 ACPA in supplementary figure 2D). However, no parallel differences in terms of expression of activating or inhibitory FcγR in blood immune cells throughout the course of disease could be observed (supplementary figure 3). Importantly, using IgG ACPAs purified by affinity chromatography from a pool of sera from RA patients, we demonstrate that polyclonal ACPAs seem to be dominantly anti-inflammatory in the CAIA model, contrasting to the respective non-ACPA fraction of the sera (figure 1H).

The results that ACPAs display a beneficial phenotype in inflammatory arthritis will change the way researchers and clinicians interpret the presence and characteristics of ACPAs in patients and individuals at risk of developing RA. Whereas some of the here used monoclonal ACPA have previously shown to induce symptoms such as arthralgia, bone loss or tenosynovitis that often precede onset of RA, the present demonstration of a dominant anti-inflammatory effect by mono- and polyclonal ACPAs calls for a re-evaluation of the presence of these antibodies. We acknowledge that the complete molecular mechanism mediating these effects is currently unknown to us and admittedly CAIA is not a citrullination-dependent arthritis model, but the striking anti-inflammatory effects warrants further investigations. The availability of the monoclonal ACPAs used here will enable such urgent investigations to take place.

Figure Legends

Figure 1 – Dominant anti-inflammatory properties evidenced by distinct ACPA clones in the development of inflammatory arthritis. (A) Schematic representation of the animal model used. (B) Anti-citrullinated peptide and protein reactivity by monoclonal ACPAs used in the study. (C-I) CAIA was induced in mice by i.v. transfer of arthritogenic anti-CII antibody cocktail. Monoclonal ACPAs (C, D and E), C03 F(ab')2 fragments (G), mutated FcγR-null GRLR-C03 ACPA (H) or polyclonal CCP-pool and respective flow-through (I) were transferred simultaneously at time of disease induction. Therapeutic effect of ACPAs was assessed by transfer of C03 ACPA at the peak of disease, day 8 (F). Boosting and synchronization of disease symptoms was done by i.p. administration of LPS 3 days post disease induction. Statistical analysis calculated by non-parametric repeated-measures Friedman test with Dunn's multiple comparison test. Disease curves from mC03 and mE02 reference groups are identical in panels C and D due to splitting of the data into two panels for better visualization. Cit: citrullination; KAc: acetylated lysine; Carb: carbamylation; TNC: tenascin C; Fib: fibrinogen; Vim: vimentin; Fil: fillagrin; His4: histone 4; mC03: murine IgG2a C03; hC03: human IgG1 C03; FT: flow-through; IVIG: intravenous IgG preparation.

Supplementary figure 1 – Citrullinated antigen reactive fine-specificities by monoclonal ACPA do not associate with observed anti-inflammatory effects. Reactivity to citrullinated and native full protein fibrinogen (A), vimentin (B), alpha-enolase (C) and type II collagen (D) by monoclonal ACPAs and respective associations with observed anti-inflammatory effects in CAIA. (E) Reactivity of tested ACPA clones against the citrulline 3 peptide from histone 4 (His4) identified by Chirivi and colleagues [*R. Chirivi et al (2021); Therapeutic ACPA inhibits NET formation: a potential therapy for neutrophil-mediated inflammatory diseases; Cellular and Molecular Immunology 18:1528*]. Nat-Fib: native fibrinogen; cit-Fib: citrullinated fibrinogen; nat-Vim: native vimentin; cit-Vim: citrullinated vimentin; nat-ENO1: native alpha-enolase; cit-ENO1: citrullinated alpha-enolase; nat-CII: native type II collagen; cit-CII: citrullinated type II collagen.

Supplementary figure 2 – Supporting observations on how ACPAs affect inflammatory arthritis in vivo. (A) Transfer of anti-CII antibody cocktail or a combination of two monoclonal ACPAs i.v. day 0, followed by LPS administration i.p. day 4. (B-D) CAIA was induced in mice by i.v. transfer of arthritogenic anti-CII antibody cocktail. Boosting and

synchronization of disease symptoms was done by i.p. administration of LPS 3 days post disease induction. (B) mB09 ACPA transferred at day 0 or 7 to assess therapeutic potential. (C) Co-administration of anti-inflammatory mC03 and disease-prone mC04 ACPAs at day 0, or mC03 day 0 followed by mC04 day 7. (D) Comparison between C03 ACPA expressing a murine IgG2a or human IgG1 constant domain. Statistical analysis calculated by non-parametric repeated-measures Friedman test with Dunn's multiple comparison test.

Supplementary figure 3 – Frequency of circulating immune cells and their expression of FcγR during CAIA is not affected by administration of ACPA. CAIA was induced in BALB/c mice followed by LPS administration day 3**.** mE02 or mC03 ACPA were transferred at day 0. Blood was collected at days 0, 3, 6, 9 and 13 for evaluation of immune cell frequencies in the blood, as well as their corresponding expression of FcγR1, FcγR2b, FcγR3, FcγR4. (A) Gating strategy. (B) Representation of FcγR expression in mast cells (FcεR+ c-kit+), (C) monocytes (Ly6C+Ly6C–),(D) inflammatory monocytes/macrophages (Ly6Ghi F4/80+), (E) neutrophils (Ly6G+Ly6C+), (F) DCs (CD11c+ CD19–) and (G) B cells (CD19+ CD11c–). No differences in mean fluorescence intensity (MFI) of any of the FcγR were detected between mice receiving mE02 or mC03.

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Competing Interests

The authors declare no competing interests.

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Material and Methods

Origin and re-expression of patient-derived monoclonal antibodies

Single B cells from RA patient were isolated using different methodologies and the immunoglobulin variable regions were amplified by reverse transcription and multiplex PCR and sequenced as previously described[1]. For in vitro binding studies the antibodies were subcloned as human IgG1 with human γ1, κ/λ constant regions by an off-site gene synthesis service (IDT). For in vivo studies the mAbs were subcloned as murine chimeric IgG2a constructs with murine γ2a/c, κ/λ constant regions. Monoclonal antibodies were recombinantly expressed in Expi293F cells (ThermoFisher Scientific), purified using protein G Sepharose (Cytiva), and extensively quality controlled using SDS-PAGE, ELISA, size exclusion chromatography, and endotoxin testing[1]. All clones were initially identified as ACPAs by screening using antigen microarray binding to citrullinated peptides and CCP2 by CCPlus ELISA (Svar Life Science) at 5 μg/ml. No clones had any reactivity to native peptides or unspecific polyreactivity by soluble membrane protein (SMP) assay[2]. All ACPA clones were derived from CCP2 positive RA patients. The clones 1325:01B09 and 1325:04C03 were derived from synovial plasma cells from the same ACPA+ RA patient (ref Steen et al). The cells were isolated based on antibody secretion based using the FOCI method. The BVCA1 clone is derived from a blood memory B cell using and immortalization protocol[3]. The clones 37CEPT2C04 and 37CEPF1C40 were derived from memory blood B cells from the same ACPA+ RA patient using tetramer antigen staining and flow cytometry sorting for reactivity to citrullinated alpha enolase (CEP1 peptide)[4]. The clone L204:01A01 was derived from a lung plasma blast from an early untreated RA patient by flow cytometry sorting BAL B cells. 254:17D08 and 254:C7X1604 were derived from bone marrow from the same RA patient using either flow cytometry sorting of CD138+ cells or 10X Genomic single cells transcriptomics.

Engineered human FcγR null IgG1 construct with G236R/L328R mutations (GRLR) which has previously shown not to interact with any FcγR[5], was generated by gene synthesis (IDT) and megaprimer PCR of the γ1 vector[6]. Unaffected citrulline reactivity was confirmed by CCP2 and cit-peptide ELISA.

Supplemental Table 1. Human monoclonal ACPA

Clone	Patient	B cell	Method of isolation	CCP2 reactivity#	Fab glycosylation sites
1325:04C03	Established ACPA+ RA (patient A)	Synovial plasma cell	FOCI[7]	1500 AU/ml	Yes – 2 VH; 0 VL
1325:01B09	Established ACPA+ RA (patient A)	Synovial plasma cell	FOCI[7]	1170 AU/ml	Yes – 1 VH; 1 VL
BVCA1	Established ACPA+ RA (patient B)	Blood memory	Immortalization[3]	795 AU/ml	Yes – 0 VH; 1 VL
37CEPT2C04	Established ACPA+ RA (patient C)	CEP1+ blood memory	Antigen-specific flow cytometry sorting[4]	3004 AU/ml	Yes – 2 VH; 1 VL
37CEPF1C40	Established ACPA+ RA (patient C)	CEP1+ blood memory	Antigen-specific flow cytometry sorting[4]	1884 AU/ml	No
L204:01A01	Early untreated RA (patient D)	Lung plasmablast	Flow cytometry sorting CD19+ cells BAL fluid	3200 AU/ml	Yes – 1 VH; 1 VL
254:17D08	Established ACPA+ RA (patient E)*	Bone marrow plasma cell	Flow cytometry sorting CD19+ CD138+	432 AU/ml	Yes – 3 VH; 1 VL
254:C7X1604	Established ACPA+ RA (patient E)*	Bone marrow plasma cell	Single cells transcriptomics	566 AU/ml	Yes – 1 VH; 2 VL
1362:01E02	ACPA- RA (patient F)	Synovial memory B cells	Flow cytometry sorting CD19+ CD27+	0 AU/ml	No

BAL: bronchoalveolar lavage
* patient undergoing hip arthroplasty
# CCPlus (Svar Life Science) at 5 µg/ml hIgG1

Collagen antibody-induced arthritis (CAIA) and antibody transfers

Unless described otherwise, all CAIA experiments were conducted in 7 weeks old male BALB/c mice. Mice were acquired from The Jackson Laboratory (USA) or Charles River (France) and maintained in a SPF animal facility with access to water and rodent chow *ad libitum*, in environment-enriched cages. Induction of CAIA was done by i.v. transfer of 1.5 mg per mouse of a 5 monoclonal anti-type II collagen (CII) antibody cocktail (Chondrex, USA). 3 days later, mice were challenged i.p. with 25 µg LPS (*E. coli* strain O55:B5). Wet food was provided to animals in the days following LPS administration, due to expected diarrhea and weight loss. Animals reaching a scoring > 0.4 according to the Karolinska Institutet humane endpoint guidelines were removed from the experiment. Macroscopic signs of arthritis were assessed via a 0-60 scoring system based on the number of inflamed joints: 1 point per toe, 1 point per knuckle and 5 points for an inflamed wrist or ankle, totaling a maximum of 15 points per paw, or 60 points per animal. Patient-derived monoclonal ACPAs (1.5 mg per mouse) were co-transferred at day 0 together with the anti-CII antibody cocktail or at the peak of disease (day 7-8) to assess their inflammatory properties. Similarly in other experiments, CCP-pools from RA patients, the respective flow-through (FT) pool or a control IVIG were transferred to mice (2 mg per mouse) at the time of CAIA induction. All animal experiments were conducted under ethical permits approved by the local ethical committees (Stockholm, Sweden).

Generation of F(ab')2 fragments

Preparation of F(ab')2 fragments from the C03 monoclonal antibody was performed with a Pierce F(ab')2 preparation kit according to the manufacturer instructions (ThermoFisher Scientific). Briefly, a solution of C03 monoclonal antibody was initially desalted using a Zeba Spin Desalting Column. Digestion was achieved after 6h incubation of the monoclonal antibody with a freshly immobilized pepsin column. Undigested IgG and F(ab')2 were

separated via a Nab protein A Plus Spin column, with confirmation of the purified F(ab')2 fragment by SDS-PAGE.

Patient-derived CCP- and flow-through-pools

The collection of patient-derived CCP-pool and their respective flow-through pools were prepared as previously described[8]. Briefly, plasma was obtained from peripheral blood samples of RA patients (n=37; ≈10 ml/patient) and centrifuged at 3000 g for 5 min. IgGs were purified on HiTrap Protein G HP columns (Cytiva), according to the manufacturer's instructions. After low pH elution and neutralization, IgG was dialyzed against PBS and applied to a CCP2 affinity column (kindly provided by Euro-Diagnostica, Sweden). ACPA were then obtained by elution of the column with 0.1 M glycine–HCl buffer (pH 2.7), and the pH was immediately adjusted to 7.4 with 1 M Tris (pH 9). The flow-through (FT) containing the unbound IgG fraction was also collected and equally dialyzed against PBS. Both ACPA and FT fractions were concentrated and buffer exchanged to PBS using 10 kDa spin Amicon centrifugation units (ThermoFisher Scientific) immediately prior to in vivo usage. ACPA was quality controlled by anti-CCP2 ELISA (Immunoscan CCPlus assay, Euro-Diagnostica, Sweden), SDS-PAGE/ Coomassie Brilliant Blue staining, size exclusion and LAL endotoxin testing.

Flow cytometry analysis

Mice undergoing CAIA were bled one drop of blood every 3rd day. After lysis of the red blood cells, the remaining cellular components were stained with a viability dye (Zombie NIR fixable dye; Biolegend) for 15min on ice. Cells were washed with PBS and resuspended in a Brilliant Stain buffer solution containing an antibody cocktail of fluorescently labelled monoclonal antibodies (Pacific Blue anti-FceR1a, clone Mar-1; BV510 anti-CD11b, clone M1/70; BV605 anti-CD64, clone X54-5/7.1; BV650 anti-Ly6G, clone 1A8; BV711 anti-CD16.2, clone 9E9;

BV785 anti-Ly6C, clone HK1.4; FITC anti-CD32, clone S17012B; Spark Blue 550 anti-mouse CD19, clone 6D5; PE anti-CD169, clone 3D6.112; PE-Dazzle 594 anti-CD16, clone S17014E; PerCP anti-CD45, clone 30-F11; PE-Cy7 anti-CD11c, clone N418; APC anti-F4/80, clone BM8; Spark NIR 685 anti-CD117, clone 2B8; Alexa Fluor 700 anti-I-A/I-E, clone M5/114.15.2; all from Biolegend). After a 15min incubation on ice, cells were washed with PBS containing 0.5% bovine serum albumin and 2mM EDTA. Cells were fixed with eBioscience Foxp3/transcription factor fixation buffer according to the manufacturer instructions (ThermoFischer Scientific), acquired in a 3-laser spectral flow cytometer (Cytek Biosciences) and analyzed with FlowJo software (BD Biosciences).

Reactivity against citrullinated peptides and proteins

ACPA mAbs were screened for binding to citrullinated, native control peptides and control antigens using a custom-designed antigen microarray (ThermoFisher Scientific, Immunodiagnostics)[9]. ELISA reactivity to modified citrullinated vimentin peptides (Cit-Vim58-69 GRVYAT-Cit-SSAVR) were evaluated using plates from Orgentec Diagnostika, and citrullinated Histone 4 using biotinylated peptides (Cit3-His4 bio: SG[Cit]GKGGKGLGKGGAKRHGSGSK-biotin) on streptavidin-coated high-capacity pre-blocked plates (Thermo Fisher Scientific) and hIgG1 at 5 μg/ml in RIA buffer (1% BSA, 325 mM NaCl, 10 mM Tris-HCl, 1% Tween-20, 0·1% SDS) and detection with HRP conjugated Fab'2 goat anti-human IgG(γ) (Jackson Immunoresearch) and TMB substrate (Biolegend). Antibody binding to full-length citrullined protein was evaluate by on-plate modification and ELISA. Briefly, antigen were diluted to 3 μg/ml in 8M Urea and coated to high-bind half area plates (Corning). Plates were blocked with 5% low-fat milk in PBS and antigens citrullinated with 280mU/ml recombinant human PAD4 (Cayman chemicals) in 50 mM Tris 10 mM $CaCl_2$ 1 mM DTT for 3h at 37C. Binding was assayed at 5 μg/ml hIgG1 to citrullinated vimentin,

alpha enolase (in-house expressed human) and collagen II (bovine purified, chondrex) and at 0.5 μg/ml hIgG1 to citrullinated fibrinogen (human purifed, Sigma Aldrich).

Supplemental Table 2 – Citrulline peptides analyzed on array

Peptide	Protein	Sequence*	Reference
Fibα 36-50	Fibrinogen α-chain	GP(cit)VVF(cit)HQSACKDSK	[10,11]
Fibβ 36-52	Fibrinogen β-chain	NEEGFFSA(cit)GHRPLDKK	[12]
Fibβ 60-74	Fibrinogen β-chain	(cit)PAPPPISGGGY(cit)A(cit)	[10,11]
Fil 307-324	Filaggrin	HQCHQEST(cit)GRSRGRCGRSGS[cyclic]	[13]
TNC1 2026-2040	Tenascin	VFLRRKNG(cit)ENFYQNW	[14]
TNC5 2176-2200	Tenascin	EHSSIQFAEMKL(cit)PSNF(cit)NLEG(cit)(cit)K(cit)	[14]
Vim 60-75	Vimentin	VYAT(cit)SSAV(cit)L(cit)SSVP	[12]

*The peptide amino acid sequence displayed where arginine R is substituted with citrulline in (cit). Both citrulline containing peptides and native arginine containing peptides were also analyzed on the array.
Besides native arginine peptides the following control autoantigens were included on the array: CENP B, collagen II, fibrillarin, Jo-1, Mi-2, PCNA, PM-Scl 100, Rip P0. Rip P1, Rip P2, RNA Pol III, RNP-70, RNP-A, RNP-C, Ro52, Ro60, Scl-70, SmBB, Sm, SSB/La
The ACPA fine-specificity array is further described in[9,15].

Experimental testing and statistical analysis

All animal experimental settings were repeated at least twice, with a total number of 10 to 50 animals assessed per condition, depending on the ACPA clone used. A minimal of 5 animals per group was used at any given time given the prevalence of arthritis being generally 80-100%, thus allowing statistical calculations with a good degree of power, and assuming a non-normal distribution of the data. Each cage contained no more than two animals of the same experimental group, with all experimental groups assigned to as many cages in order to minimize potential cage effects. Statistical analysis of arthritis development and prevalence between groups was conducted by non-parametric repeated-measures Friedman test with Dunn's multiple comparison test. When comparing two or more groups in single observation assays, a non-parametric Mann-Whitney or Kruskal-Wallis test was used. Prism GraphPad version 9.3.1 was used to calculate statistics and statistical significance was considered when $p < 0{\cdot}05$ for a 95% confidence interval.

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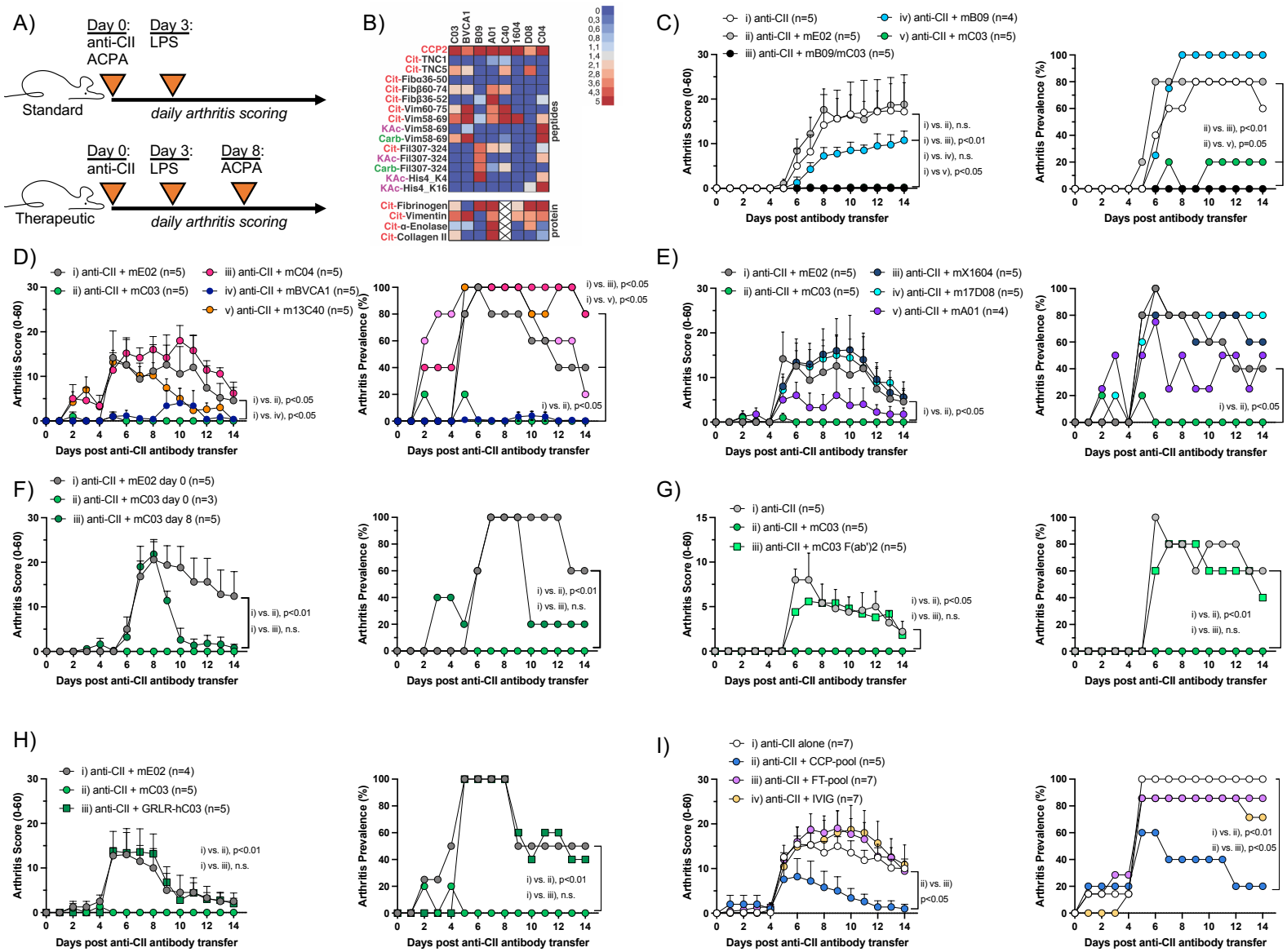

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