**DOMINANT B-CELL RECEPTOR CLONES IN PERIPHERAL BLOOD PREDICT ONSET OF ARTHRITIS IN INDIVIDUALS AT RISK FOR RHEUMATOID ARTHRITIS**

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Supplementary methods

Supplementary results

Supplementary figures and tables

**SUPPLEMENTARY METHODS**

Synovial biopsies were snap-frozen in liquid nitrogen and cryopreserved until use. RNA was isolated using a polytron tissue homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in the presence of STAT60 RNA reagent (Tel-test Inc, Friendswood, TX) according to the manufacturer’s protocol. After isolation RNA was purified using the RNeasy Mini System (Clean-up-protocol (#74106, Qiagen).

RNA quality was checked using the Bioanalyzer 2100 system (Agilent) and quantified using the Qubit 1.0-platform (#Q32857, Invitrogen Life Technologies, Breda, the Netherlands). cDNA was synthesized using Superscript RT-III and oligo-dT primers according to the manufacturer’s protocol (#18080-051, Invitrogen). Linear amplification (LA) of the complete B-cell receptor (BCR) repertoire was performed as described earlier (1). In the first step of LA, cDNA was amplified using a custom primer set. All Vheavy-primers contain the primerB sequence needed for sequencing according to the 454-titanium protocol for amplicon sequencing (version 2010) (Roche Diagnostics, Mannheim, Germany). In the first step of LA the cDNA was amplified in the presence of 5 pmol of each of the V-primers, 1x buffer B (Solis BioDyne, Tartu, Estonia), 1 mM MgCl2, 0.1 mM dNTPs and 3U of Hotfire (Solis BioDyne) in a volume of 20 uL using a T-Professional thermocycler (Biometra, Goettingen, Germany) (96°C (900s), 40 cycles (96°C (30s), 60°C (60s), 72°C (60s)), 72°C (600s)). Amplified products were purified using AMPure XP SPRI-beads (#A63881, Agencourt-Bioscience, Beverly, MA) in a template:bead ratio of 0.9. After the first amplification step, a generic PCR was performed to prepare the samples for sequencing. In this second reaction primerB was used as generic forward primer and a generic primer specific for the BCR heavy-joint gene segment or the constant region was used as reverse primer. The reverse primer contains a multiplex identifier and primerA as described in the amplicon-sequencing manual (Roche). The PCR was performed with 50% of the purified LA product in the presence of 10 pmol of each of the primers, 1x buffer B, 1mM MgCl2, 0.1 mM dNTPs and 3U of Hotfire in a volume of 40 uL using a T-Professional thermocycler (96°C (900s), 35 cycles (96°C (30s), 60°C (60s), 72°C (60s)), 72°C (600s)). After amplification, samples were again purified using the AMPure beads and quantified using fluorospectometry (Quant-iT dsDNA HS Assay Kit (#Q32851, Invitrogen). Samples were prepared for sequencing according to the manufacturer’s protocol for Amplicon Sequencing. NGS was performer on a Roche Sequencer FLX using the Titanium platform. For each sample > 60,000 (bead-bound) BCR heavy sequences were analyzed.

**SUPPLEMENTARY RESULTS**

**Dominant peripheral blood BCR clones are predominantly IgG1+ and have autoimmune features**

In an earlier study in a different cohort of rheumatoid arthritis (RA) patients we described features of BCR clones dominant in synovial tissue (1). To analyze whether the dominant peripheral blood clones in *at-risk* individuals who develop arthritis share these same features we compared them to the top25 peripheral blood clones in both control groups (*at-risk* individuals who that did not develop arthritis, and autoantibody-negative healthy individuals).

First, we analyzed the isotypes of the BCR clones and found that the dominant clones in at-risk individuals who developed arthritis were significantly more switched to the IgG isotype compared to at-risk individuals who that did not develop arthritis, and autoantibody-negative healthy individuals (both p<0.0001) (**supplementary figure 2A**). Accordingly, the number of IgM+ clones was significantly decreased compared to the control groups (*at-risk* individuals who that did not develop arthritis, and autoantibody-negative healthy individuals, both p<0.0001). The number of IgA+ and IgD+ clones was comparable between the 3 groups. More detailed analysis of the IgG isobtype showed that all subtypes contributed to the IgG predominance, but this was only statistically significant for the IgG1 subtype (p<0.0001) (**supplementary figure 2B**).

We reported before that dominant synovial tissue clones are enriched for IGHV4-34 (1), a Vheavy gene previously associated with autoreactivity (2). In this study we found that the number of IGHV4-34+ clones was significantly overrepresented in dominant clones in at-risk individuals who developed arthritis compared to *at-risk* individuals who that did not develop arthritis, and healthy individuals (p=0.008), albeit at low numbers (**supplementary figure 2C**). This was not the case for IGHV3-23 or IGHV1-69 (p=0.334 and p=422 respectively; **supplementary figure 2D/E**). We did not observe an increase in the (amino acid) length of the third complementary determining region (CDR3), which also has been associated with autoantibodies (p=0.89; s**upplementary figure 2F**).

Also, we compared the ACPA and IgM-rheumatoid factor serum titres during the preclinical phase in at-risk individuals, using the cut-off of ≥5 dominant clones present in peripheral blood. This showed comparable titres in both groups (434 vs 130 kAU/L (median) for ACPA (p=0.345), 30 vs 28 kU/L (median) for IgM-RF (p=0.403) and did not show a correlation with the number of dominant clones found (for ACPA r2 = 0.004 (p = 0.615), for RF-IgM r2 = 0.014 (p = 0.324); **supplementary figure 2G/H**).

Taken together, dominant clones that are present in the pre-symptomatic phase of RA are class-switched to the IgG1 isotype and enriched for IGHV4-34, while CDR3 lengths are comparable between at-risk individuals that did and did not develop RA or healthy individuals.

**SUPPLEMENTARY FIGURES AND TABLES**

Supplementary table 1



Supplementary figure 1



Figure 1 (A) ROC curves of the number of dominant clones. Follow-up was divided in 5 different groups, and depicted separately. The black dots show the sensitivity and specificity for the presence of 5 dominant clones.

Supplementary figure 2 



**F**

Figure 2 (A) The percentage of the 25 most dominant clones occupied by the different immunoglobulin isotypes in peripheral blood during the preclinical phase, in 21 *at-risk* individuals, of which 11 developed arthritis (*at-risk arthritis developed*), and 10 didn’t developed arthritis yet (*at-risk no arthritis*), and 10 healthy individuals as controls (*healthy individuals*). Bars show mean and SD, \*\*\* p<0.0001 using two-way ANOVA. (B) Percentage of the IgG+ clones occupied by the 4 subtypes in peripheral blood during the preclinical phase. Bars show mean and SD, \*\*\* p<0.0001 using two-way ANOVA. (C/D/E) Percentage of total clones that are IGHV4-34 (C), IGHV3-23 (D) and IGHV1-69 (E) bearing in the 25 most dominant clones in peripheral blood during the preclinical phase. Mean and IQR are depicted, \* p<0.05 using 1way-ANOVA). (F) CDR3 length of dominant clones in peripheral blood. All bars show mean and SD (\*p=0.05, \*\*p<0.01, \*\*\*p<0.001 using 1way-ANOVA). (G/H) The absence (*clone-*) or presence of ≥5 dominant clones in peripheral blood (*clone+*) compared to the serum titres of ACPA (G) or IgM-rheumatoid factor (H). The red line depicts the cut-off for positive serology.

Supplementary table 2



Two-digit HLA typing was performed using sequence specific primers (PCR-SSP). In at-risk patient 1 and 2 who did not develop arthritis was no DNA available for typing.

Supplementary figure 3

**B**

**A**

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**Figure 3 (A/B)** The absence (*SE-*) or presence of the HLA-DBR1 shared epitope (*SE+*) compared to the number of dominant clones recovered in blood **(A)** or synovial tissue **(B)**. The red line depict the cut-off of 5 dominant BCR clones present in peripheral blood.

**REFERENCES**

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