SUPPLEMENTARY MATERIAL

Longitudinal follow-up reveals peripheral immunological changes upon tick bite in α-Gal sensitized individuals

METHODS

Tick extract and IgE quantification against tick extract

Adult *Ixodes ricinus* ticks from Insect Services (Berlin, Germany) were crushed in phosphate buffered saline (PBS) with a Retsch Mill. Extract was centrifuged for 20 min at 20000g at 4°C and the protein concentration of the supernatant was determined with Bradford assay.

Deglycosylation of tick extract (TE) was performed by adding 1U/5µg TE of α-galactosidase from green coffee bean (Sigma-Aldrich). For IgE quantification, TE and deglycosylated TE (dTE) were coated at 5µg/ml in PBS overnight at room temperature (RT) in 384 well plates (High Binding, Greiner Bio-one), followed by 1 hour incubation with human serum albumin (HSA, Sigma-Aldrich) 1% (w/v in Tris buffered saline with 0.05% Tween-20). Sera were incubated at a dilution of 1/10 for 2 hours at RT on shaker (300 rpm). IgE was detected after 90 min incubation (300 rpm) with biotin labelled anti-human IgE (Southern Biotech) diluted 1/2000, followed by 30 min incubation (300 rpm) with alkaline phosphatase labelled streptavidin (Southern Biotech) diluted 1/2000. Signal was developed with para-nitrophenyl phosphate solution (Sigma-Aldrich) and optical density (OD) was read at wavelength 405 nm on Spectramax 384Plus ELISA reader (Molecular Devices). IgE concentrations were calculated by extrapolating OD values from a standard curve, which consisted of serial dilutions of a patient serum with a known titer to cat serum albumin [33].

Cytokine measurements

Cytokine levels were measured in undiluted sera with the U-PLEX custom biomarker electrochemiluminescence assay from Meso Scale Discovery (MSD). The following cytokines were quantified by multiplex assay: interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukins (IL) IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13. The protocol was strictly followed as provided by the manufacturer. Data were acquired with Methodical Mind software and analysed with MSD Discovery Workbench software.

Sample preparation for mass cytometry (CyTOF) acquisition

PBMCs were thawed in a waterbath at 37°C and washed with 10ml serum-free medium (TheraPEAK™ X-VIVO™ 10 Medium, Lonza), followed by 2 washes with 5ml phosphate buffered saline (PBS) without Mg2+/Ca2+ (Lonza). Cells were incubated 5 min in 1 µM Cell-ID^{TM} cisplatin-195Pt (Fluidigm, now Standard Biotools) at a concentration of 10 x 10^6/mL cells. After two washes with flow cytometry staining buffer (2% bovine serum albumin (BSA) in PBS), cells were incubated 10 min with Human TruStain FcX Fc Receptor Blocking Solution (BioLegend) at a concentration of 6 x 10^7/mL cells, followed by a 30 min incubation in antibody staining mix, which was composed of 31 markers from the Maxpar® Deep Immuno-Profiling Assay (Standard Biotools), 2 additional ready-to-use antibodies and 5 self-labeled antibodies (Maxpar® X8 Antibody Labeling Kit, Standard Biotools) (Table E1). After two washes with FACS buffer, cells were incubated overnight at 4°C in 50nM Cell-ID^{TM} Intercalator-Ir (Standard Biotools) at a concentration of 6 x 10^6/mL cells. Cells were washed twice with PBS and twice with cell acquisition solution (CAS buffer, Standard Biotools). Before acquisition on a HELIOS mass cytometer (Standard Biotools), cells were suspended in 0.1x Maxpar Four Elements EQ Beads (Standard Biotools) at a concentration of 0.5 x 10^6 cells/mL.

Data analysis

Normalized and cleaned CyTOF data were manually gated in FlowJo v10.8.0 software and 66 cell populations were identified (Fig. S2 and S3). Cell population frequencies of single live CD45+ cells (gate L2) were calculated in Tableau Desktop software (version 2021.4) [34] and statistical tests were performed with GraphPad Prism (version 9.3.1). Patient group and control group were compared at corresponding time-points with multiple Mann-Whitney tests, setting alpha threshold to 0.05 and applying Holm-Šídák method for correction of multiple comparisons. For longitudinal analysis, time-point V1 was compared to V2, V3 and V4 with Friedman test, setting family-wise alpha threshold to 0.05 and including Dunn's correction for multiple comparisons. The Friedman test could be performed on paired samples (V1 to V4) of a group of 8 patients for cell frequencies, resp. 10 patients for IgG subtyping, sIgE quantification and cytokine profiling. As not all participants were bitten and some others enrolled only upon a bite, a paired sample analysis starting at V0 was not possible. Comparisons between V0 and V1 were therefore performed with a Mann-Whitney test (confidence level 95%). The same strategy for statistical analysis was applied for IgG subtyping, cytokine profiling data and blood cell frequencies.

Table S1: Mass cytometry marker panel. Maxpar© Direct™ Immune Profiling panel of 31 markers and 7 additional markers were used to identify 66 cell populations of single live CD45+ cells. Two additional markers were ready-to-use (*) and 5 additional markers were self-labeled (**).

Table S2: **Median values and range for specific α-Gal IgG antibodies and subclasses.** Median values and range (minimum to maximum values), expressed in µg/mL, for specific α-Gal IgG antibodies and subclasses in controls and patients per time-point. Lower limit of detection (LLOD) were 0.01 µg/mL for sIgG, 0.1 µg/mL for sIgG1, 0.1 µg/mL for sIgG2, 0.01 µg/mL for sIgG3, 0.02 µg/mL for sIgG4. Measured concentrations below the LLOD were set to 0.01 µg/mL.

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Figure S1: Standard curves for IgG, IgG1, IgG2, IgG3 and IgG4 and cross-reactivities. Human IgG and IgG subclass antibodies were coated in serial dilutions and incubated with monoclonal anti-IgG detection antibodies. The standard curves are fitted to a 4-parameter logistic regression model. Monoclonal antibodies directed against one subclass did not cross-react to the other IgG subclasses.

Figure S2: List of gating names. Full names and short names of the gates defined by the gating strategy.

Figure S3: Graphical overview of the gating strategy (illustrated on one representative sample). Normalized CyTOF data were cleaned with several steps like excluding normalization beads, gating on single and live cells. The gate L2 (in red) was used as reference to calculate cell frequencies of single live CD45+ (indicated as number in percent below the gate name), leading to the identification of 66 cell populations within the main immune cell families, such as B cells and plasmacells, innate lymphoid cells, natural killer cells (NK), basophils, monocytes and dendritic cells (DC), NKT-like cells, CD8 and CD4 T cells, granulocytes.

Figure S4: List of samples available and analyzed per subject and time-point.
Open symbols could not be included for flow cytometry analysis, either because the acquired sample did not fulfill the inclusion criteria (< 50% mortality rate and/or CD45+ cells >50000 events, open circle), or because the PBMC sample was not available (open diamond). Serum was available for open symbols to measure immunoglobulin levels and cytokine concentrations.

Figure S5: **Longitudinal anti-α-Gal immunoglobulin profiles showing individual kinetic curves.** IgE (right Y-axis), IgG and IgG subclasses (left Y-axis) were plotted for each individual subject. Sera were diluted 1/50 for IgG and 1/20 for all IgG subclasses.

Figure S6: Proportions of sIgG1 to sIgG2 is higher in patients compared to controls. (A): The weight of sIgG1, resp. sIgG2 is expressed as percent (%) of all 4 IgG subclasses (median with interquartile range) for patients and controls. Multiple Mann-Whitney tests between patients and controls at each

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time-point showed only 1 significant difference (** p-value < 0.01). (B): The ratio of sIgG1 to sIgG2 is higher for patients than controls, all samples combined. The line represents the median for each group, Mann-Whitney test shows a significant difference between groups (**** p-value < 0.0001).

Figure S7: Longitudinal analysis of blood cell frequencies shows no significant variations.
Main cell types from total blood count, expressed as frequencies of total leucocyte counts for patient (blue line) and control group (grey line), are plotted as median with interquartile range. Friedman tests were not significant.

Figure S8: Plasmacytoid dendritic cell (pDC) frequencies are higher in controls regardless of the time of blood withdrawal. Frequencies of pDC cell are expressed as percent of single live CD45+ cells and divided into 2 categories of blood withdrawal before (am) or after noon (pm) for control (grey) and patient (blue) group. Boxes are delimited by interquartile range with median line and whiskers from minimal to maximal value. Pair-wise comparisons with Mann-Whitney tests show significant differences (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001).

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