Establishment of a whole blood ELISA to quantify T-cellular cytokine release in response to *Aspergillus fumigatus* antigens

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Objectives

Depending on the host immune status, the ubiquitous mould *Aspergillus fumigatus* can cause a range of disease manifestations from invasive infection to severe hypersensitivity syndromes. Reliable bio-effect monitoring tools to efficiently track the multifaceted T-cell response to *Aspergillus* antigens are lacking. Flow cytometry or ELISPOT have been established to monitor *Aspergillus*-specific T-cells as a supportive biomarker for environmental exposure and invasive aspergillosis yet are complicated by their time- and resource-intensive nature and pre-analytic difficulties [1,2]. Cytokine release assays are successfully used as a T-cell-driven diagnostic marker for tuberculosis and cytomegalovirus (CMV) infections and are easy to use with minimal hands-on time. We therefore sought to develop a T-cell optimized whole blood-based interferon gamma (IFN-γ) and interleukin (IL)-17 release assay for *A. fumigatus* antigens.

Methods

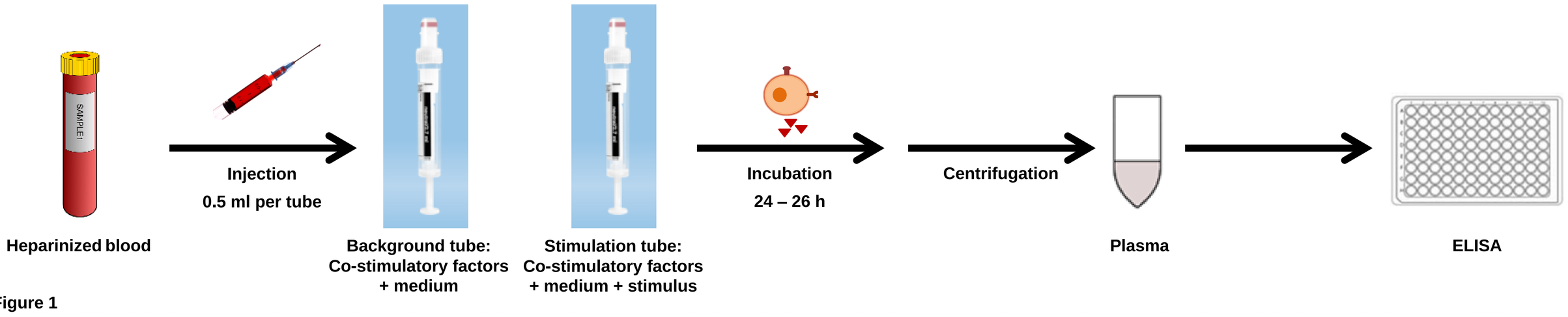

Figure 1
0.5 ml heparinized blood was injected into stimulation tubes containing 0.5 ml RPMI medium (1:1), costimulatory molecules αCD28 and αCD49d plus CMV phosphoprotein (pp) 65, *A. fumigatus* mycelial lysate (50 µg/ml final conc.), or recombinant *A. fumigatus* antigens (30 µg/ml final conc.) Aspf4, Aspf6, or Crf1. Phytohemagglutinin (PHA) was used as a positive control. After incubation for 24-26 h, plasma was obtained by centrifugation and analysed by IFN-γ and IL-17 ELISA. The cytokine concentration determined in the background tube was subtracted from antigen-reactive cytokine concentrations in the stimulation tube.

Optimization of stimulation conditions for whole blood T-cell ELISA and assay validation in immunocompromised patients

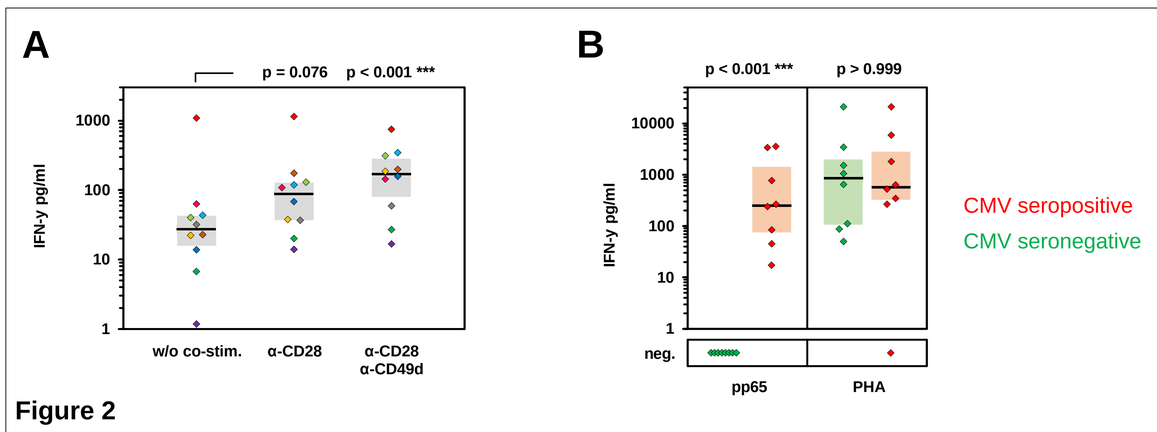

Figure 2
(A) Whole blood from 10 highly mold-exposed subjects [1] was stimulated with an *A. fumigatus* mycelial lysate and 1 µg/ml αCD28, 1 µg/ml αCD28 + 1 µg/ml αCD49d, or no co-stimulatory factors. Friedman test and Dunn's multiple comparison test. (B) Samples from 8 CMV seronegative (green) and 8 seropositive (red) stem cell transplant recipients (60-180 days post-transplant) were stimulated with pp65 or PHA. Medians (black bars), inter-quartile ranges (boxes).

Whole blood ELISA to detect *Aspergillus*-specific T-cell activation in subjects with occupational mold exposure

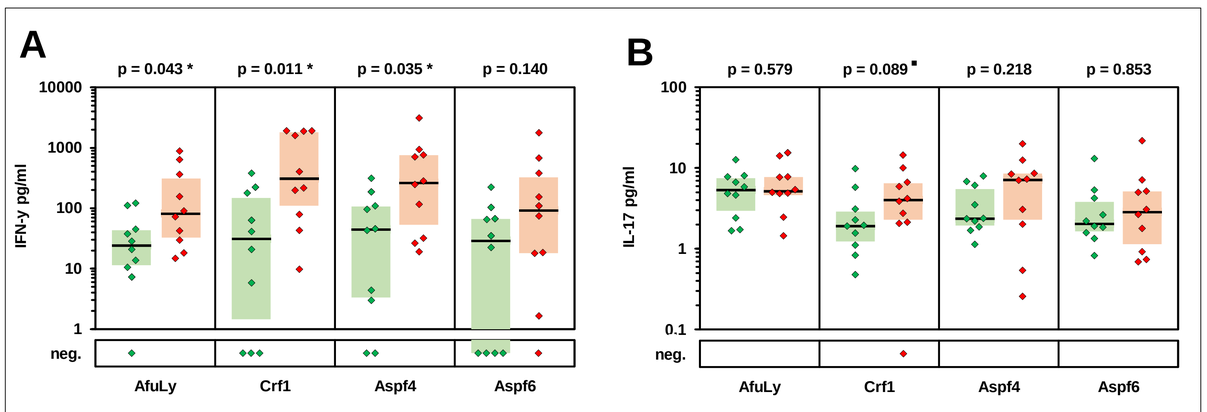

Figure 3
Whole blood from 10 organic farmers (red) and a control group of 10 non-occupationally exposed subjects (green) was stimulated with *A. fumigatus* mycelial lysate as well as antigenic proteins Crf1, Aspf4, or Aspf6. IFN-γ (A) and IL-17 (B) concentrations were determined by whole blood ELISA. Medians (black bars), and inter-quartile ranges (colored boxes) are shown. Mann-Whitney test.

Technical and intra-individual variation of whole blood T-cell ELISA

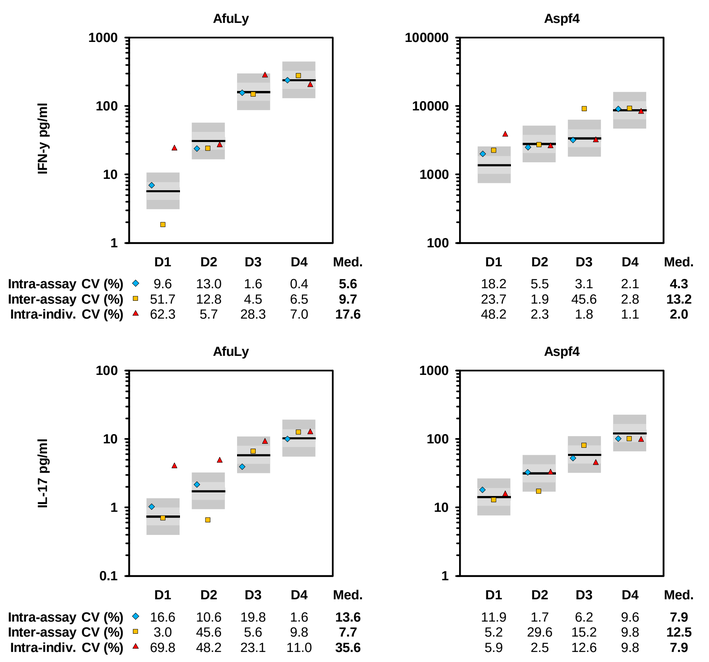

Figure 4
Whole blood from 4 highly mold-exposed healthy subjects was stimulated with *A. fumigatus* lysate and Aspf4 (black bars). The grey-colored boxes represent 15% and 30% coefficients of variation (CV).

To determine the intra-assay CVs, a second tube was injected by the same operator (blue diamonds).

For each donor and antigen, an additional set of tubes was injected by a different operator using blood from the same venipuncture to determine inter-assay CVs (yellow squares).

Another blood draw was performed after 4 weeks to determine intra-individual CVs (red triangles).

Robustness of whole blood T-cell ELISA to pre-analytic delays

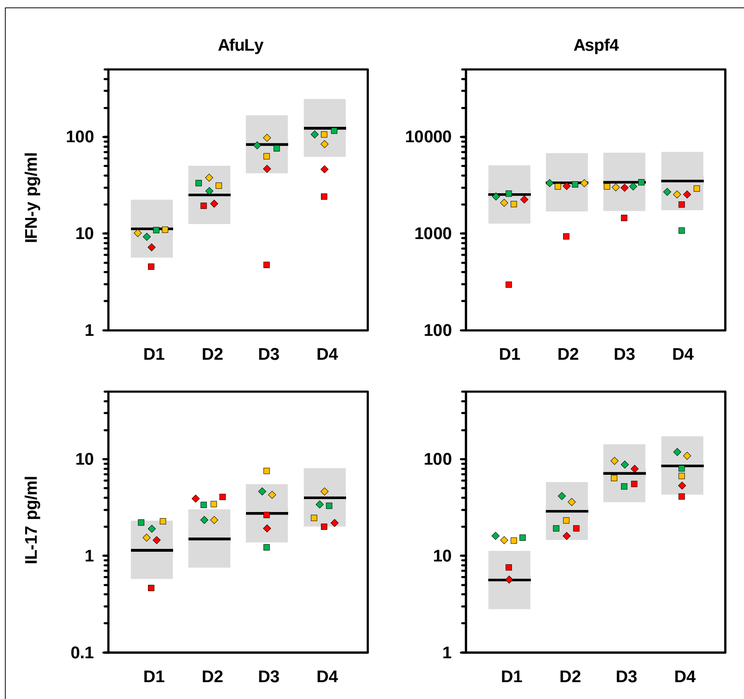

Figure 5
Whole blood from 4 highly mold-exposed healthy subjects was stimulated with *A. fumigatus* lysate (AfuLy) or Aspf4 either immediately or after a 4 to 24 h pre-analytic storage period at ambient temperature. The grey-colored error range represents a +/- 2-fold deviation compared with the immediately incubated samples (black bars).

Conclusions

- Optimized whole blood T-cell ELISA with enhanced co-stimulation is a fast, simple, and resource-efficient assay to track T-cell responses to *A. fumigatus* antigens, providing a differentiated representation of individual T-cell subsets by their signature cytokines.
- The whole blood ELISA protocol is technically reliable, reproducible, and robust to pre-analytic delays.
- The assay is currently undergoing clinical validation in different patient cohorts including chronic pulmonary aspergillosis.

Funding

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■ $p < 0.1$ ⋆ $p < 0.05$ ⋆⋆ $p < 0.01$ ⋆⋆⋆ $p < 0.001$

References: [1] Wurster S et al. Mycoses. (2017); [2] Potenza L et al. PlosOne (2013)