Ph.D. thesis

Development of myeloid cell-based immune profiling on microarrays

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Introduction

Humoral immunity is mediated by various macro-molecules, mainly proteins, found in our extracellular fluids, most importantly in the blood plasma and serum. The state of humoral immunity determines an organism's capability to protect itself against various microorganisms, yet in some cases, as in autoimmunity or allergy, can cause harm as well.

Nowadays the typical approach in diagnostics investigating antigen specific immune response focuses on the detection of one molecule binding to the antigen, despite the fact that when immune complexes (ICs) form many different components: antibodies, complement components and pentraxins from the serum can react and bind to the antigen. In this typical setting monoclonal antibodies, produced in animals different from the investigated sample, specific to the given serum component may be detected through fluorescence, radioactivity or enzyme activity.

In our body these antigen-antibody binding derived immune complexes, may mediate various effector functions. The main effector functions mediated by antibodies are Antibody Dependent Cellular Cytotoxicity (ADCC), Antibody Dependent Cellular Phagocytosis (ADCP), Antibody Dependent Cytokine Release (ADCR), and Antibody Dependent Respiratory Burst (ADRB). Not all antibodies are equal. While some isotypes may be better in mediating one aspect, the same antibody can be weaker in another. Therefore this thesis investigates the hypothesis that the detection of serum components alone may be further improved by detecting multiple reactions in parallel by using myeloid cells as detectors. These cells with receptors for immune complex components presumably can be applied to detect immune complexes, and through their binding, or by detecting their inflammatory activation, activating immune complexes can be identified.

Multiplex diagnostic systems allow simultaneous detection of multiple antigen specific reactions, simply by spatially encoding the different antigens or compounds, by fixing them at given locations of an array to form micro-array or protein chip of features. Such systems show their strength in analyzing immune-reaction, from serum derived components of the humoral immunity and the fixed antigens, simultaneously. These capabilities combined with fluorescent detection offers great advantage for bio-marker identification, and diagnostic purposes.

The diversity of humoral immunity and the effector functions triggered by the immune-complexes calls for novel methods to provide a more biological readout, compared to secondary antibodies. This work presents our results in this field, with the emphasis on cell based diagnostics for autoimmune diseases, in a multiplex system, presenting how monocytoid U937, as a model for myeloid cells, can be applied for immune profiling.

1 Aims of the study

The aim of this study was to investigate how cells can be applied to detect immune-complexes. We hypothesized that cellular detection may hold advantages in this field, as myeloid cells with their set of receptors are the main effector cells reacting with immune complexes in vivo. As a model we picked monocytoid U937 cells and tested how components of immune complexes determine the cellular adhesion and how this adhesion differentiates healthy and autoimmune donors. Moreover we planned to modify the U937 cells to apply them as detectors for NF-κB translocation mediated inflammation. Finally we tested the adhesion based model for immune complex and IgG detection in a label-free manner through applying imaging SPR.

Our specific goals were as follows:

1. to compare U937 cell adhesion to ICs, and find correlations between antigen specific antibody isotypes and cell adhesion
2. to show that FcR based detection is susceptible to IgG glycosylation, removal of the core glycan will lower the cell binding to ICs
3. to generate a reporter U937 cell line to detect inflammatory activation of these cells, through stable transfection with an Nf-κB reporter element GFP plasmid, selection and cloning of reporter cell line
4. to test the cell Nf-κB reporter cell line activation induced GFP production by immunoglobulins and LPS
5. to demonstrate the application of U937 cells in label-free detection of immune complexes and immunoglobulins with imaging SPR

Methods

- Flow cytometry (cell surface receptor expression analysis)
- protein microarrays (detection of antigen specific components from serum samples)
- Cellular microarray (to detect cell binding)
- EndoS endoglycosydase treatment of antigen bound IgG
- ELISA (to detect antigen specific IgG subclasses from serum samples)
- Cell culture of U937 cells, electroporation, stable transfection, by G418 selection and cloning of resistant U937 cells by fluorescence activated cell sorting (FACS)
- in vitro activation of monocytoid U937 cells (by IgG subclasses and LPS)
- imaging SPR – U937 cell and serum binding to IgG subclasses and on-sensor formed antigen specific immune complexes

Results and conclusions 1.

Fluorescently labeled U937 cells can be applied as qualitative detectors in nitrocellulose based antigen and antibody microarrays

- We showed that monocytoid cells expressing receptors for components for immune complexes can be applied to detect activating complexes in nitrocellulose-based protein microarray format. Binding profile of monocytoid U937 cells, presumably through their Fc receptors showed that they differentiate between printed human immunoglobulin subclasses. IgG3 was the most effective in the activation of cell binding, while IgG1 and IgG3 were less effective, and IgG2 triggered no or very little cell binding. We also showed the cell binding modulatory effect of complement deposition.
- We found that treatment of antigen specific IgG with endoglycosidase on the microarray, to remove the N-linked carbohydrate side chain, resulted in decreased binding to these complexes in antigen microarray format.
- We compared cell binding and anti-human IgG and anti-human IgM results obtained following serum incubation of Systemic lupus erythematosus (SLE) specific autoantigens with normal healthy and SLE patient serum samples, and found that U937 cells can detect immune complexes and differentiate the healthy and the SLE sample group, moreover that the cell binding signals correlate with the anti-human IgG signals and not with those obtained with anti-human IgM.

Our results suggest that monocytes can be applied to detect immune complexes or printed antibodies in a microarray format, and their binding is based on the quality and density of the activating antigen specific serum components or printed antibodies.

Results and conclusions 2.

Cell binding to sensor coupled antibodies and on-sensor formed immune complexes can be detected with imaging SPR

- We verified our previous results regarding IgG subclass preference of U937 cells as we found that IgG3 triggers the cell binding most effectively followed by IgG1 and IgG4, while IgG2 once again showed weak activating properties.
- We demonstrated in proof-of-concept study that cells can bind to on-sensor formed immune complexes, generated by incubating rheumatoid arthritis (RA) specific diagnostic peptide autoantigens (VCP2 and HCP2) with RA and healthy serum samples.
- When compared cell binding measurements in iSPR to measurements with anti-human IgG, IgA and IgM in antigen microarray, and we found correlation between the VCP2-specific IgG and IgA content of the serum samples and the measured u937 cell binding response.
- When compared to VCP2 specific IgG subclasses measured by ELISA IgG3 showed correlation with U937 cell binding.
- Finally we found a significant agreement between results of RA diagnostic CCP2 ELISA tests and our cell binding measurements following serum incubation of VCP2.

In agreement with our previous results we verified the applicability of U937 cells to detect antigen specific immune complexes. Importantly we demonstrated that cells can as well bind to on-sensor formed immune complexes binding in imaging SPR measurements.

Results and conclusions 3.

IgG subclasses differ in their ability to trigger NF-κB translocation in NF-κB reporter U937 cells

- We generated and cloned an NF-κB U937 reporter cell line B12 by stable transfection with a plasmid encoding GFP downstream of NF-κB responsive elements.
- We demonstrated that B12 cells produce GFP upon stimulation with lipopolysaccharide endotoxin, in a dose and time dependent manner.
- We found that while coated IgG1 is a weak activator of NF-κB translocation, and IgG2 triggers no GFP production, IgG3 and IgG4 are potent activators of NF-κB translocation.
- We found coated IgA and IgM alone to be insufficient to trigger response, while hIgG and IVIG, presumably containing the naturally occurring ratio of the IgG subclasses, activated the GFP production in B12 cells.
- We verified our findings regarding blocking the antibody Fc parts by goat anti-human IgG F(ab')2 fragments prior to the incubation of cells, and also found that by adding the antibodies in solution to the cells instead of applying them in coat, results the lack of GFP production.

These results suggest that IgG subclasses differ in their ability to trigger NF-κB translocation and therefore may have different inflammatory potential. We found that from the IgG subclasses triggering cell adhesion IgG1 activates NF-κB weakly compared to IgG3 and IgG4.

Summary

We applied U937 cells to detect immune-complexes. These cells express receptors for immunoglobulins and complement components. We showed that U937 cells can differentiate between human IgG subclasses in agreement with the expressed FcγRs' affinity towards these molecules. We verified that the binding is mediated by the antibodies Fc-part by masking them with IgG specific F(ab')2 fragments and as well by hydrolyzing the N-linked glycan of IgG, thus our results suggest that the cell binding is mediated by FcγRs. We found that cellular signals can differentiate the healthy and the SLE group based on binding to on-microarray generated immune-complexes and that cell binding correlates with IgG signals. We also tested another platform, imaging SPR, and verified our results regarding U937 cell binding to IgG subclasses. We tested this approach with rheumatoid arthritis specific peptides, and showed for the first time as a proof-of-concept that cells can recognize on-sensor surface deposited molecules in SPR measurements. When compared to physician based RA diagnosis and RA diagnostic CCP2 ELISA U937 cell binding showed good agreement with both.

Finally to test the inflammatory activation we transfected U937 with a plasmid encoding an NF-κB Responsive Element with Green Fluorescent Protein in a downstream position, thus coupling inflammatory activation to fluorescently detectable protein production. We found that IgG3 and IgG4 were equally potent activators of this pathway; IgG1 was less effective while IgG2 triggered no activation, just as IgM and IgA. These results suggest that the cell binding activating properties of the IgG subclasses does not necessarily mirror their NF-κB mediated inflammatory activating properties. Altogether our results suggest that cellular detection in many ways can add to the conventional immune-complex detecting methods

Publications connected to the thesis

1. Kecse-Nagy C*, Szittner Z*, Papp K, Hegyi Z, Rovero P, Migliorini P, Lóránd V, Homolya L, Prechl J. Characterization of NF-κB reporter U937 cells and their application for the detection of inflammatory immune-complexes. Manuscript under revision.
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4. Papp K, Szittner Z, Prechl J. Life on a microarray: assessing live cell functions in a microarray format. Cell Mol Life Sci. 2012 Mar 4

Other Publications

1. Prechl J, Papp K, Hérincs Z, Péterfy H, Lóránd V, Szittner Z, Estonba A, Rovero P, Paolini I, Del Amo J, Uribarri M, Alcaro MC, Ruiz-Larrañaga O, Migliorini P, Czirják Serological and Genetic Evidence for Altered Complement System Functionality in Systemic Lupus Erythematosus: Findings of the GAPAID Consortium. PLoS One. 2016 Mar 7;11(3):e0150685.
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