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Immunkomplexek hatásainak jellemzése bioszenzorokkal, fehérje mikromátrixokon

Characterization of immune complex effects by biosensors on antigen microarrays

záró beszámoló - final report

Development and application of protein arrays for antibody function characterization

The first stage of our project focused on the development of antigen microarrays suitable for qualifying complement activation events on the chips. We identified molecules and materials that initiated complement activation in a controlled manner and thus helped the analysis complement activation pathways and extent of activation. Single-stranded DNA was identified as a highly efficient classical pathway activator that binds C1q both directly and via IgM but the complement cascade is downregulated by inhibitors also binding to this target. As a result complement activation proceeds to C4 cleavage but not farther in healthy individuals. In strong contrast, the presence of ssDNA specific IgG molecules in serum promoted the progress of complement fragmentation and resulted in the deposition of higher amounts of C4, C3 and the soluble membrane attack complex C5b9. Surface adsorbed IgG isotypes all induced complement deposition, with IgG4 being the least potent activator as expected. The adsorption of mannan or acetylated albumin triggered the lectin pathway, identified by the presence of C4 and lack of C1q on the chip surface. Activation here efficiently progressed till the C5b9 formation stage. Alternative pathway activation was initiated by known activators such as LPS but the contribution of other pathways could not be ruled out as antibodies were also binding and C4 was present in these complexes. Bacterial superantigen protein G and protein A efficiently captured serum immunoglobulins and triggered classical pathway activation, producing the highest densities of complement fragments on the chips.
These observations were exploited in the project GAPAID (Genes And Proteins in AutoImmune Diagnostics, FP7 SME supporting grant) where healthy and autoimmune subjects (systemic lupus erythematosus and rheumatoid arthritis patients) were examined with respect to autoantibody profile, complement activation profile, genetic background and clinical phenotype. Our key observations confirmed the susceptibility and severity association of the complement receptor type 3 (CR3, ITGAM gene polymorphism) and showed that complement activation was strong ex vivo in spite of decreased serum complement levels in patients with increased IgG autoantibody levels ***(Prechl et al. 2016, PloS ONE)***. These observations also lead to the development of a new hypothesis on the pathogenesis of systemic lupus erythematosus ***(Prechl and Czirják, 2015, F1000Research)***.

Development and application of reporters cell for immune complex biosensing

In contrast to the efficient initiation of on-chip complement deposition by antibodies binding to surface-immobilized antigens, the modulatory activity of complement on cell binding and activation was minimal or insignificant. The key role in cellular responses against opsonized antigens bound to solid surface was played by antibodies. We examined several different strategies for assessing cellular function triggered by adsorbed antibodies.
In general, protein microarrays comprising immunoglobulins or antigens were either directly treated with suspensions of cells or were first incubated with the serum to be tested and then treated with cell suspensions. Treatment of surfaces with cell suspensions required finely controlled liquid flow conditions in order to register sensitive cell binding events. For this reason we developed microluidic systems and devices that allowed autonomous, capillary-driven flow and liquid transport on the chips. The binding, adhesion and activation of the cells were detected by fluorescent labeling, optical detection (SPR, OWL), genetic or chemical reporters (NFkB-driven GFP, reactive oxygen sensitive dehydro-rhodamine).

Using high-throughput, multiplex surface plasmon imaging technology (iSPR) we analyzed the kinetics and quality of binding of human monocytoid U937 cells to antigen-bound immunoglobulins. As a model system we attached citrullinated peptides to the chip and treated the surface with serum from RA patients. Our results showed that cell binding showed a correlation with the presence and quality of citrullinated peptide specific serum IgG and IgA. Moreover a synergism between IgG and IgA was observed. The technology was thus found suitable for detecting autoantibodies in a label-free manner and combining IgG and IgA measurement, isotypes both responsible for disease pathology (***Szittner et al, 2016, Clinical and Experimental Immunology***).

The human monocytoid cells U937 were genetically modified, cloned and selected for LPS sensitivity to identify cells with the highest GFP production upon transcription factor NFkB activation. Following characterization of GFP response kinetics we examined the response of these cells to surface-bound immunoglobulins. This technology again showed a correlation of activation with FcgammaR binding affinity of IgG isotypes, with the exception of IgG4. IgG4 was efficiently activating reporter cells probably via CD64 that is highly expressed by these cells. Reporter cells were also capable of detecting pathogenic anti-citrullinated peptide antibodies from RA patient sera ***(Kecse-Nagy et al., 2016, PloS ONE***).

Application of cellular biosensors in microdevices

Besides cytofluorimetric, SPR and imaging HTS analysis we also applied simple cell-detecting techniques as a direction towards bed-side, point-of-care fast diagnostic methods. We developed

autonomous microfluidics systems for stepwise filling-washing-labeling microarray-bound cells. Polymer-coated glass slides were used as substrate for the generation of simple microarrays containing reference materials and diagnostic antigen. A polymeric (PDMS) microstructured device was attached to the slides and cell suspensions were passed over the microarray area, then washed and labeled with simple vital dyes. We proved that such a simple system is suitable for sensitively identifying serum samples containing anti-citrullinated antibodies (***Papp et al, 2017, Sensors and Actuators B***).

The observations in these microfluidic devices lead us to hypothesize that interactions between cells and antibodies could influence the properties of fluid flow in the device. As a proof-of-concept approach we used red blood cells and blood group specific antibodies attached to the chip surface. Here the orientation is opposite to the previously described methods, as surface-bound antibodies capture specific target molecules on target cells. We found that blood-group specific antibodies captures RBC passing over the functionalized surface, and these interactions slowed the movement of RBC and the fluid as well. A simple microfluidic device is therefore suitable for identifying cell-surface antigens of RBC by monitoring the properties of blood flow (***Sautner et al, Scientific Reports, 2017***).

In addition to the use of monocytoid cells, we also tested primary human neutrophil granulocytes as reporter cells. A simple and fast lytic method was found suitable for enriching neutrophils from whole blood without pre-activating or damaging these sensitive cells. Neutrophils were either labeled with dehydro-rhodamine, a fluorogenic dye that is sensitive to the presence of radical oxygen species, or left unlabeled. Immobilized antibodies of distinct classes and subclasses, serum treated antibodies or serum treated antigens were then contacted with these cells. The binding of cells was detected by the changed optical properties of the surface (EPIC instrument) or by measuring the fluorescence of the cells (microscopy, HTS imaging, fluorescent-reader, flow cytometry).
We observed that neutrophil granulocytes basically showed a behaviour similar to monocytes despite the different expression pattern of Fc receptors. Thus, IgG3 was found to be most efficient in initiating cell adhesion and activation, with IgG1 and IgG4 being next in the row and IgG2 being inert or least efficient. IgA molecules, especially IgA2 were very efficient in triggering cell adhesion and activation. The presence of complement fragments did not significantly alter cell behaviour, as mentioned above (***manuscript in preparation***).

The utilization of antigen microarrays also allowed us to compare the traditional approach to autoantibody titration by serum dilution to titration by antigen density dilution on the chip surface. These measurements lead to the description of a novel approach to polyclonal serum antibody

quantification, which is faster (no need for serum dilution series) and can provide real quantitative results, expressed as molar binding capacity (***manuscript in preparation***).

The experimental observations described so far, along with earlier experiences of the grantee, lead to the development of a novel quantitative model of antibody generation and function. The properties of antibodies, with respect to specificity and affinity, isotype and effector functions, can be displayed as a relationship between the dissociation constant, antigen concentration, antibody concentration and immune complex concentration. Theoretical inclusion of cross-reactivity leads to the generation of an interaction space and a quantitative antibody-antigen interaction network (***Prechl, 2017 Clinical and Translational Immunology***). Likewise, effector functions are also closely related to the binding energies of antigen-antibody-antibody receptor complex and show continuity from silent homeostatic removal to aggressive inflammatory elimination of antigen (***manuscript*** submitted to Clinical and Translational Immunology; ***retrievable*** from bioRXiv[search:Prechl]). All these events can be projected into the thermodynamic energy landscape defined by Gibb's free energy and entropic penalty of binding (***manucript*** submitted to F1000Research; ***retrievable*** from bioRXiv[search:Prechl]).

In summmary, the project allowed us to develop novel cell-based biosensing strategies for serum antibody measurements in humans. These developments led to several important biological and biomediacal observations in the field of autoimmunity, to technologies and devices that are being patented for commercial immunodiagnostic applications, and to theoretical models that approach antibody function in a quantitative systems biological perspective.