Abstract 01:

Platelet Heterogeneity in patients with chronic coronary syndrome: mature and reticulated platelet profiling using mass cytometry

Authors:

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Abstract:

Reticulated platelets (RPs) are prothrombotic RNA-rich platelets suggested to be detrimental in patients with chronic coronary syndrome (CCS). In addition, circulating RPs levels are independent predictor for adverse cardiovascular events in CCS patients and other pathological settings. However, RPs biology still need to be investigated. CyTOF allows the exploration of receptors expressed on the platelet membrane and can detect differences in platelet subgroups. In this study, we sought to investigate the RPs proteome on single-cell level at rest and after activation using time-of-flight mass cytometry.

Thrombocytes from peripheral blood of 11 CCS patients were isolated, prepared and stained with a custom-made CyTOF-antibody panel of 20 antibodies targeting important transmembrane platelet proteins. According to previous experiences and common practice, we detected RPs and mature platelets (MPs) based on their RNA content. We analyzed the results with a custom bioinformatic pipeline available under https://exbio.wzw.tum.de/cyanus/ comparing marker expression of RPs and MPs. Earth mover's distance (EMD) was assed as a measure of differential expression. Interestingly, the four markers with the highest calculated EMD are all key regulators of platelet activation and aggregation: the collagen receptor GPVI,

the collagen integrin receptor unit CD29 (ITGB1), the adhesion protein CD9 and the von Willebrand receptor unit CD42b (GPIbα). Regarding the activation marker expression upon TRAP stimulation, RPs show higher median signal intensities of all four activation markers compared to MPs. Especially the markers CD107a (LAMP-1) and CD154 (CD40L) are expressed in MPs only to a low extend, whereas there is a clear overexpression in RPs.

This dataset provides the first deep analysis of the RP proteome at rest and upon stimulation. The shown pro-thrombotic profile of RPs explains their hyperactivity and could offer the first biomolecular explanation of the detrimental role of RPs in CCS patients. In addition, this study adds high resolution biomolecular information which could be useful to personalize antiplatelet therapy in patients with high RPs levels.

Abstract 02:
High-dimensional immune mapping aiming at understanding the immune-driven regenerative potential of CD34$^+$ stem cells in multiple sclerosis therapy

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Abstract

Background: Multiple sclerosis (MS), an inflammatory disease of the central nervous system, is characterised by a relapsing and remitting course (relapsing-remitting multiple sclerosis; RRMS) at disease onset, for majority of patients (80-90%). Early disease-modifying therapy is the cornerstone of treatment of RRMS. Recently there has been increasing interest, in autologous hematopoietic stem cell transplantation (AHSCT) as a potential high-efficacy immune reconstitution treatment of RRMS.

Aims: To gain improved understanding of the underlying immune-driven regenerative potential of CD34$^+$ cells in AHSCT treated RRMS patients using Cytometry by Time of Flight (CyTOF).

Methods: CyTOF enables multi-dimensional phenotypic characterization of single cells. The 42-marker panel designed, required 17 in-house antibody conjugations. Antibody titrations were done on a barcoded sample mixture including blood and stem cell samples from both healthy donors and RRMS patients. The 42-marker panel was validated using samples from RRMS patients treated with AHSCT

Results: We have successfully developed a 42-marker CyTOF panel that can reliably detect and identify both immune- and stem cell phenotypes within heterogeneous samples from RRMS patients. The panel will further be utilized to characterize samples from larger cohorts of RRMS patients treated with AHSCT.

Conclusion: We aim to distinguish the immune-driven regeneration in AHSCT treated RRMS patients with unprecedented depth using this novel 42-marker panel established. This deeper immune mapping would facilitate defining biomarkers for treatment response to tailor treatment regimens in individual patients more precisely.

Abstract 03:
Tin sulfide nanoparticles (SnS-NP) as novel reporter tags for mass cytometry

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Current mass cytometers can detect up to 135 different isotopes in the range of 75 - 209 amu. However, this potential is only partially exploited, so that current applications are limited to approximately 60 parameters. Tin (Sn) has 10 natural, stable isotopes, six of which do not overlap with other reporter isotopes already used in mass cytometry. Despite its promise to increase the analytical capacity of mass cytometry, tin-based reagents remain unexplored until today. Here, we describe pilot studies on the application of elemental tin (II) sulfide nanoparticles (SnS-NP) as antibody tags in mass cytometry.

Stable SnS-NP, with sizes ranging from 60 - 80 nm, were synthesized and functionalized with FITC-derivatized carboxymethyl dextran, followed by conjugation to an anti-human CD8 antibody. Mass cytometric analysis revealed that the SnS-NP antibody conjugate bound specifically to its target cells in PBMC, with only minimal background binding to non-target cells. The specific signal intensities observed ranged from 100 to 10,000 for the ^{120}Sn isotope, and the SnS-NP antibody conjugate was compatible with standard mass cytometry reagents and surface staining protocols.

In summary, we here describe for the first time the application of SnS-NP-based antibody conjugates suitable for mass cytometry. SnS-NP conjugates produce strong and specific cell staining patterns, rendering them ideal for the expansion of existing antibody panels and mass cytometry assays.

Abstract 04: Subtyping Patients with Immunoglobulin A Vasculitis using Mass Approach

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Immunoglobulin A vasculitis (IgAV) is a small vessel leukocytoclastic vasculitis, characterized by vascular IgA deposits and large neutrophil infiltrates. While IgAV might be self-limiting with complete recovery, renal involvement is common especially in adults. Currently, markers used for assessing IgAV renal involvement inaccurately estimate the risks and poorly guide clinicians in clinical management of IgAV patients. This could lead to undertreated patients that progress to dialysis and have increased risk of morbidity and mortality. Our leukocyte RNA sequencing data revealed differential expression of HLA-I and HLA-II genes in all patients with IgAV, suggesting involvement of innate, as well as acquired immune system in IgA vasculitis. Interestingly, genes specifically involved in NK-cells mediated cytotoxicity were downregulated in patients with renal involvement, whereas interferon-stimulated genes were up-regulated in patients with skin-limited disease. Implication of immune cell types in IgAV remains elusive. Our aim is to perform mass cytometry (CyTOF) to characterize immune cell phenotypes in IgAV and to confirm our RNA-sequencing results at the cellular level.

We collected whole blood samples from treatment-naïve adult IgAV patients at time of diagnosis, from specifically the following subgroups: 1) renal complications (n=3), 2) skin-limited disease (n=3), and age-/sex-matched HC (n=3), at the University Medical Center Ljubljana. Samples are matched with those already used for RNA sequencing and stored in Smart Tubes (SMART TUBE Inc., USA).

CyTOF would provide crucial knowledge into adult IgAV pathology and set the basis for biomarker studies on larger patient cohorts. We would confirm the decreased number/activity of NK-cells in IgA patients with renal involvement, as well as define the NK-cell subtypes, as they have already been reported to be deregulated in other autoimmune diseases. Additionally, the presence of interferon signature in one patient group could be confirmed through the analysis of immune cells, responsible for the production of interferons. All the markers identified as important will be further confirmed on a validation cohort, consisting of fresh IgAV patient samples by flow cytometry.

Abstract 05:

Challenges in measuring phosphoproteomics with CyTOF in CLL

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Analyzing cell signaling upon stimulation of specific pathways is a key element to identify new aspects of development and maintenance of chronic lymphocytic leukemia (CLL). The broad availability of different phosphomarkers and the ability to combine a huge set of markers in single CyTOF experiments renders this technique a promising tool for uncovering new aspects of cell signaling.

We established a 35-marker panel to analyze phosphorylation patterns, cell cycle states, and viability of murine CLL cells, B cells, and T cells upon different cell stimuli and treatments. To improve sample quality we implemented sample barcoding, batch normalization, and spillover compensation. Additionally, we compared sample and staining quality upon different timepoints of cell fixation.

As phosphorylation changes very rapidly, we found that immediate fixation after the stimulation step is fundamental for reliable results and prevents changes of the phosphorylation pattern upon antibody surface staining. Consequently, we analyzed if binding of the surface stain antibodies was affected by prior fixation and excluded some antibodies (e.g. PD-L1 and CD80) from our panel. As expected, sample barcoding in combination with batch normalization as well as spillover correction further improved sample quality and comparability between different samples and batches.

With our newly developed phospho and cell cycle panel we will now be able to reliably and reproducibly characterize phosphorylation patterns and, thus, signaling capacities of different cell types from murine CLL samples. We aim to compare cells from mice with or without specific aberrations in key pathways affecting disease progression and/or under various treatment conditions.

Abstract 06:

Store-Operated Calcium Entry Controls Innate and Adaptive Immune Cell Function in Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC) and Crohn's disease (CD), both representing major clinical challenges in need of new treatment modalities to improve patient care. Store-operated calcium entry (SOCE) is the predominant calcium influx pathway in immune cells regulating many functional properties. Conditional knockout mice, in which SOCE signaling components are deleted in T cells, revealed that SOCE is required to induce intestinal inflammation in mouse models of colitis. However, it is currently unknown whether the pharmacologic inhibition of SOCE is a suitable drug target in IBD, and we aimed to investigate the effects of SOCE inhibitors (SOCEi) on lymphocytes and epithelial cells isolated from IBD patients.

Peripheral blood (PBMCs), lamina propria lymphocytes (LPMCs) and epithelial cells were isolated from IBD patients undergoing colon resection. First, Ca^{2+} influx measurements were performed to assess the metabolic status of immune (PBMC/LPMC) and epithelial cells after SOCE blockade. Second, LPMCs (UC: *n* =6, CD: *n* =6, Non-inflamed: *n* =4) were 4h *ex-vivo* stimulated with ionomycin/PMA ± SOCE-inhibitor BTP2 and subsequently stained with a panel of 37 immunological markers for mass cytometry

acquisition. Finally, murine models of colitis were implied to investigate the effects of SOCE blockade on the induction of intestinal inflammation *in-vivo*.

Data on B, T, NK, and myeloid cells revealed that treatment with SOCEi caused a dose-dependent inhibition of SOCE, demonstrating that BTP-2 is a potent inhibitor of SOCE in human gut-resident lymphocytes and PBMCs. Furthermore, by applying mass cytometry, we investigated how a gradual pharmacologic inhibition of SOCE affects the activation and functions of various immune cell subsets obtained from colon specimens of IBD patients. Particularly, we observed that both UC and CD patients were characterized by the accumulation of $CD4^+$ effector T cells, IFNγ-producing $CD8^+$ T cells and IL-17-producing innate lymphoid cells (ILCs) compared to non-inflamed specimens. Remarkably, inhibition of SOCE attenuated the production of pathogenic cytokines including IL-2, IL-4, IL-6, IL-17, TNF-α and IFNγ by T cells and ILCs, reduced the activation of B cells and decreased the production of IFNγ by myeloid cells without affecting the viability of primary human epithelial cells. Finally, oral treatment with SOCEi attenuated the development of intestinal inflammation in mice where transfer colitis was induced.

Our data revealed for the first time that the cytokine production and the activation of several immune cell subtypes could be modulated by SOCE blockade in human intestinal inflammation, identifying SOCE as a novel therapeutic target in colitis.