A UV-C LED based reactor for continuous decontamination of the sheath fluid in a flow cytometric cell sorter

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Aseptic cell sorting is challenging, especially when a cell sorter is not operated in a sterile environment. The fluid system of a cell sorter may be contaminated with germs such as bacteria, yeast, viruses, or fungi. Therefore, a regular cleaning procedure is required to prepare a sorter for aseptic cell sorting by flushing the fluidic system with sodium hypochlorite or ethanol. However, this procedure is time consuming, and most importantly, the researcher can never be sure that the cleaning process was successful. In addition, residues of cleaning agents in the fluidics system are toxic to cells.

Here we present a method based on a UV-C reactor for flow-through irradiation of the sheath fluid. The reactor containing 6 UV-C LEDs with an emission wavelength of 275 nm was installed in a BD Influx cell sorter. Using the reactor, we were able to decontaminate sheath fluid contaminated with *pseudomonas aeruginosa* to enable long-term cell culture.

Endothelial cell apoptosis perturbations during oxygen-induced lung injury: Implications for the pathogenesis of bronchopulmonary dysplasia

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Introduction

Apoptosis is known to play a pivotal role during both prenatal and postnatal development of the lung. The importance of apoptosis in lung development has been highlighted in in vitro and in vivo studies, but the regulation and cellular sites of apoptosis in the developing lung remains poorly understood. Perturbations to signaling pathways that regulate apoptosis are associated with deregulated alveologenesis and lung vascular development, which are hallmarks of bronchopulmonary dysplasia (BPD), the most common consequence of oxygen supplementation for respiratory failure in preterm infants. To date, no study has fully addressed the impact of oxygen exposure (hyperoxia) on the apoptosis of endothelial cells in the developing lung.

Aim of the study

The aims of the study are to investigate which molecular pathways are involved in the modulation of endothelial cell apoptosis during oxygen-induced lung injury in the postnatal mouse lung.

Methods

In order to detect which apoptosis pathways are deregulated in endothelial cells in immature mouse lungs exposed to hyperoxia in vivo, endothelial cells were first sorted by fluorescence-activated cell sorting (FACS). To do so, CD31 was employed as positive marker, CD45 for exclusion for the immune cells, and 4',6-diamidino-2-phenylindolen(DAPI) as live/dead cells discriminator. The FACS-sorted endothelial cells where used to quantify the effect of hyperoxia on the expression of the early apoptosis marker annexin V by flow cytometry, while the he dynamics of genome accessibility was assessed by ATACseq, and steady-state mRNA transcript levels were assessed using bulk RNA-Seq.

Results

Flow cytometry analyses revealed a reduction in the early apoptosis signaling in lung endothelial cells subjected to hyperoxia, compared to normoxia-exposed controls. Oxygen exposure led to a significant decrease in the chromatin accessibility of genes known to play a pivotal role in initiating the intrinsic pathway of apoptosis in lung endothelial cells. RNAseq data suggest that this modulation was induced by reduced expression of mRNA transcripts encoding key signaling molecules in the intrinsic pathway of apoptosis.

Conclusion

These data highlight the prop-apoptosis effects of hyperoxia exposure on endothelial cell apoptosis in developing mouse lungs

Induction of robust immunity against SARS-CoV-2 after a third dose of BNT162b2 vaccine in previously unresponsive elderly

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Vaccination protects against fatal courses of SARS-CoV-2 infection, also in the elderly. Induction of neutralizing serum antibodies was observed after two intramuscular applications of BNT162b2 mRNA vaccine in people >80 years of age. However, recent outbreaks among elderly vaccinees, and antibody responses inferior to those observed in younger vaccinees, prompt discussion on the necessity of a third vaccination.

Herein, we studied vaccine-induced humoral and cellular immune responses to SARS-CoV-2 in a cohort of 51 elderly individuals aged >80 years, vaccinated twice with BNT162b2. Analysis of spike-specific IgG and CD40L+IFNgamma+ CD4 T cells in peripheral blood on day 35 revealed a striking inter-individual variability in the immune response. Remarkably, five individuals retained both very low specific IgG and T cell frequencies, despite normal reactivity to the staphylococcal superantigen SEB. With the aim to enhance SARS-CoV-2 immunity, all five were again vaccinated using BNT162b2 during week 16 after the first dose. The third vaccination was well tolerated. Two weeks later four out of five vaccinees demonstrated robust spike-specific T cell and antibody responses, comparable to that detectable in responders after two-dose vaccination. In a healthy man without obvious morbidities, specific serum IgG and T cells also increased, however only to low levels. He is scheduled

for a fourth vaccination. No COVID-19 infections were recorded in the cohort until August 2021.

Our data shows that the elderly initially hardly responding to two-dose vaccination can mount a vaccine-specific immune response after three doses of BNT162b2. While the reason for primary unresponsiveness remains unclear, BNT162b2 unresponsiveness is not fateful, and can be overcome by repeated vaccination even in the elderly.

Overall, we show that 90% of individuals aged >80 years establish adaptive SARS-CoV-2 specific immunity after two-dose vaccination with BNT162b2. Nevertheless, our data suggest routine screening for spike-specific immunity in this population, to assess its extent after two doses of BNT162b2. Screening should be unbiased and not limited to conditions of immunodeficiency or targeted immunosuppression. Should such tests reveal lack of specific immunity, re-vaccination should be considered.

The worm-specific immune response in multiple sclerosis patients receiving controlled *Trichuris suis* ova immunotherapy

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Abstract

Parasitic helminths are known to display potent immunomodulatory properties. More than a decade ago innovative approaches initiated the use of helminth-based therapy to treat autoimmune and inflammatory diseases like Inflammatory Bowel Disease (IBD) and multiple sclerosis (MS). One such approach utilizes the porcine whipworm *Trichuris suis*, closely related to the human-infecting parasite *T. trichiura*. Oral administration of *T. suis* eggs/ova (TSO) typically results in a self-limiting infection, where larvae fail to develop into sexually mature adults. Studies over the years have shown varying clinical outcomes and largely inconclusive results for the efficacy of helminth-based therapy for treating MS. Nevertheless, the development of helminth-specific immune responses in TSO-treated patients remains scarcely understood. Here, we therefore addressed the cellular and humoral immune responses targeting parasite antigens in individual MS patients receiving controlled TSO treatment as part of a small-scale monocentric, prospective, randomized, placebo-controlled Phase II trial.

Mass cytometry analysis of peripheral blood mononuclear cells (PBMC) revealed an increase in the frequencies of activated HLA-DR^{high} plasmablasts in TSO-treated versus placebo-treated patients. This was consistent with continuously increasing levels of TSO-specific IgG and IgE observed in serum samples in the TSO group. In the T cell compartment, placebo and TSO-treated patients displayed comparable frequencies of total CD4$^+$ and CD8$^+$ T cells, as well as Foxp3$^+$ Treg. Nevertheless, we could show a mild increase of activated HLA-DR$^+$CD4$^+$ T cells in the TSO group, compared with placebo-treated controls. Finally, using a *T. suis* antigen-specific T cell expansion assay we detected notable patient-to-patient variation in antigen-specific T cell recall responses and cytokine production. Overall, our study highlights that MS patients receiving experimental TSO treatment establish unimpaired nematode-specific T- and B-cell responses. In summary, varying degrees of cellular functionality were present in this patient cohort, an immunological phenomenon potentially contributing to the miscellaneous clinical efficacy of helminth-based therapy documented for autoimmune conditions like MS.

FlowSoFine - an analyzing tool for microbial pattern changes detected by flow cytometry

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The gut-liver axis is important in the pathogenesis of many liver diseases. An essential part of this axis is the gut microbiota. Detection of changes in microbial composition is therefore of high interest. Currently, deep sequencing methods are gold-standard to assess the bacterial microbiome. However, there are alternative methods available. Recently, flow cytometric profiling has been described, where bacteria are discriminated based on DNA-content and size, creating unique and robust microbial fingerprints for each sample. The advantages of this method are its low-cost, fast sample preparation and measuring. Unfortunately, the subsequent data processing and statistical analysis can be a challenge due to the lack of easily accessible analysis tools. Our **aim** is to develop a bioinformatics tool for statistical readout and visualization of microbial pattern changes measured by flow cytometry.

All flow cytometry data files of the experiment are merged into a single data set, on the basis of which a template in form of a multi-dimensional grid is created. This template is then laid over the scatter plots of the individual samples one by one. In this way, a data set is created which contains the proportion of the measured events for each grid section. This information is used for nonparametric statistics to explore differences between and characteristic features within sample groups. We implemented this bioinformatic approach in the web-application called FlowSoFine.

FlowSoFine is a freely accessible, user-friendly analyzing tool, enabling scientists without in-depth programming knowledge to process data from data collection to statistical evaluation. Additionally, a non-metric multidimensional scaling plot is used for visualization of differences in microbial patterns between groups (e.g. treatment). Automated heatmap clustering is applied to the samples and highlights areas with similarities within groups. Furthermore, a series of plots is created to identify bacterial populations of interest.

With FlowSoFine, we provide researchers the option to quickly follow the heterogeneity and dynamic changes in the gut microbiome after experimental interventions.

Development of functional *in vivo* fluorescence lifetime microendoscopy of the femoral bone marrow

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1. BACKGROUND

Immune cells adapt their function in dependence on their individual metabolism [1]. The complex interactions between shifts of intracellular metabolic pathways and immunity remain elusive, mainly due to the lack of technologies to analyze local metabolism *in vivo* over long periods [2]. With the development of our Fluorescence-lifetime Longitudinal Intravital Microendoscopy of the Bone marrow (FLIMB) approach, we aim to study the cellular immunometabolism in the distinct bone marrow niche environments during health and disease.

2. RESULTS

We adapted LIMB[3] - our method for intravital two-photon fluorescence microendoscopy of the femoral bone marrow – by designing new gradient index lenses and re-engineering our implants to set it up for fluorescence lifetime imaging of the metabolic cofactors NADH and NADPH, hereafter NAD(P)H.

Subsequently, we validated our system by performing *ex vivo* experiments with different fluorescence-lifetime reference dyes and NADH in MOPS buffer.

In first *in vivo* experiments, we wanted to characterize the metabolic immune cell activity during the inflammation phase after femoral osteotomy [4]. We could proof that we are able to analyze the cellular metabolism of phagocytic cells in LysM:tdRFP reporter mice after an osteotomy over the course of up to a three weeks. Based on our data, we could observe cell populations with different phenotypes and metabolic profiles. The respective glycolysis-OXPHOS balances - which modulate cellular bioenergetics and –sythesis[2] – were notably shifted.

We will quantify the spatiotemporal parameters and enzymatic activity profile of the pro- to anti-inflammatory switch reflected in the cellular metabolism *in vivo*.

3. CONCLUSION

FLIMB allows deeper insight into spatiotemporal and functional aspects of bone marrow biology: the cellular self-organization after injury (e.g. re-vascularization)[3, 4], dynamic analysis of cellular niches and the parallelized functional imaging of their respective NAD(P)H-dependent metabolic enzyme activity. With this, we aim to parametrize the bone regeneration progress and correlate immune cell dynamics and functions with their metabolic pathway alterations.

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Bone Clearing-Imaging pipeline enables comprehensive 3D insights at cellular level and reveals interactions of the myeloid compartment and the vascular network during bone regeneration.

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Background: Bone regeneration is a highly orchestrated process based on interactions between the immune cell compartment, mesenchymal cells, the vascular system and their microenvironment. This cascade of events results in a scar-free and complete regeneration, which is a unique characteristic of bone in contrast to other soft tissues. However, 5-10% of all cases do not heal properly and bone healing is either delayed or stays incomplete. Refining our understanding of basic principles in the bone regeneration can serve as a role model for the development of new regenerative strategies in both bone and other tissues. In previous studies, our group already analyzed the immune cell compartment and the vascular network via intravital microendoscopy of murine long bones following osteotomies. We were able to show that $CX_3CR_1^+$ macrophages precede vascularization, are present throughout the regeneration process, but localize in certain areas of the fracture gap and have an influence on vessel sprouting and endothelial remodeling (Stefanowski et al., 2019). We now want to analyze the interactions of myeloid cells with the vascular network during bone regeneration comprehensively in 3D, without the limitations of a narrow field of view.

Method: We present here an adapted clearing and light-sheet imaging pipeline that enables us to analyze the entire bone in 3D in an intact environment with cellular resolution. Our pipeline preserves endogenous reporter fluorescence in the bone and diminishes light scattering and autofluorescence up to the deep bone marrow. We achieve decolorization of autofluorescent bone marrow by adding a new step to our clearing protocol. We used our pipeline to detect endogenous fluorescence of the myeloid compartment via CX_3CR_1-GFP^+ cells and the vascular network via Cdh5-tdTomato fluorescence. We were able to not only analyze the interactions of myeloid cells with the vasculature in homeostasis but also integrated a model for intramembranous ossification, via a drill hole injury, to our pipeline.

Conclusion: By using an adapted light-sheet imaging pipeline, which is modified to meet the challenges of long bone clearing, we are able to preserve different fluorescent reporter proteins and to detect their signals throughout the whole bone in a non-limited 3D stack and in a model of bone regeneration. In doing so, we can comprehensively analyze interactions of myeloid cells with the vascular network in bone healing.

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In-depth phenotyping of ILCs in a systemic inflammation model using multiplexed histology

In organisms, cells do not occur isolated but are embedded in a complex network of neighboring cells, cellular factors and extracellular matrix components. Depending on internal and external stimuli, cells can react by regulations on the transcriptional level triggering changes in their cytokine profile, marker expression and migratory behavior. Innate lymphoid cells (ILCs) are tissue-resident innate immune cells that are potent sensors for cytokines and cellular factors. Studying the localization of ILCs and their microenvironment in the tissue has been challenging as they are low abundant (<1%) and require a variety of markers (>7) for a clear distinction from other cell types.

We aim to correlate functional and phenotypical adaptations of ILCs and their micro-anatomical location during inflammation. Therefore, we established multi epitope ligand cartography (MELC) in our lab, a fluorescence microscopy-based technique for multiparameter analysis with spatial information. This allows for a deep characterization of hematopoietic and stromal cells in tissue context. Our image analysis pipeline enables cell segmentation and extraction of single cell features. By measuring the mean fluorescence intensity on a subcellular level (transcription- & membrane markers) per cell for each marker, we were able to identify ILCs and other rare as well as abundant cell populations in inflamed human tonsils.

We are currently extending our approach to detect ILCs in mouse models. We are using a systemic inflammation mouse model and characterize murine ILC phenotypes and their neighborhood in different immunological-relevant organs (gut, lung, bone marrow). Preliminary results show that RORgt^{+} intestinal ILC3s seem to locate in fibronectin-enriched tissue areas. Surprisingly, these ILC3s also express podoplanin, a marker for lymphatic but not blood endothelial cells. To our knowledge, podoplanin has not been described to be associated with ILCs and, therefore, might show the potential of our technology to reveal new markers for immune profiling.

NAD(P)H fluorescence lifetime imaging of live nematodes Heligmosomoides polygyrus in murine intestine reveals metabolic cross-talk between host and parasite

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Background

The modulatory effects of nematode infection with *Heligmosomoides Polygyrus* (HP) on the host immune system and on its microbiota has been extensively investigated but less is known about the enzymatic activity, energetics and metabolism of the nematodes themselves, which rule over the way the parasites persist, proliferate and defend themselves in the intestinal environment. Intravital two-photon microscopy extended by **NAD(P)H** fluorescence lifetime imaging (**FLIM**) provides tools to make spatially resolved statements in the tissue about the NAD(P)H bound **enzymes** involved in the metabolic process, as well as the **activity** of the enzymes involved. Infection occurs in several stages, the first of which is when the larvae are still outside the lumen, distributed throughout the intestine, and diffuse into the lumen during the course of disease around day 8 post infection later migrate collectively into the duodenum. The measurement of metabolic activity and the enzymes involved at different time points, both of the host animals and the parasites, provides information about the immunological interactions and a possible **feetback loop** between worm and mouse and can significantly contribute to both therapy optimization and a better understanding of autoimmune gastroendrological diseases.

Results

The measured metaolic activity and NAD(P)H bound enzymes show an opposite response of host and parasite. Whereas host metabolic activity decreases steadily and linearly over the course of infection (Helthy 77%, d14 71%), parasite activity increases almost exponentially (d6 58%, d10 61%, d12 64%, d14 73%). Similarly, the metabolism of the worms shifts from anaerobic glycolysis to oxPhos with sharply increasing oxidative bursts, presumably in response to the host microbiota. In the mice, on the other hand, an immunological flare-up followed by calming down can be observed. The behavior of metabolism as well as activity in the host animals is consistent with previous studies.

Conclusion

The establishment of the measurement method for *HP* has been successfully and reliably demonstrated, further investigation of a possible feetback loop is thus possible.

Dysregulated CD38 Expression on Peripheral Blood Immune Cell Subsets in SLE

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Given its uniformly high expression on plasma cells, CD38 has been considered as a therapeutic target in patients with systemic lupus erythematosus (SLE). Herein, we investigate the distribution of CD38 expression by peripheral blood leukocyte lineages to evaluate the potential therapeutic effect of CD38-targeting antibodies on these immune cell subsets and to delineate the use of CD38 as a biomarker in SLE. We analyzed the expression of CD38 on peripheral blood leukocyte subsets by flow and mass cytometry in two different cohorts, comprising a total of 56 SLE patients. The CD38 expression levels were subsequently correlated across immune cell lineages and subsets, and with clinical and serologic disease parameters of SLE. Compared to healthy controls (HC), CD38 expression levels in SLE were significantly increased on circulating plasmacytoid dendritic cells, CD14++CD16+ monocytes, CD56+ CD16dim natural killer cells, marginal zone-like IgD+CD27+ B cells, and on CD4+ and CD8+ memory T cells. Correlation analyses revealed coordinated CD38 expression between individual innate and memory T cell subsets in SLE but not HC. However, CD38 expression levels were heterogeneous across patients, and no correlation was found between CD38

expression on immune cell subsets and the disease activity index SLEDAI-2K or established serologic and immunological markers of disease activity. In conclusion, we identified widespread changes in CD38 expression on SLE immune cells that highly correlated over different leukocyte subsets within individual patients, but was heterogenous within the population of SLE patients, regardless of disease severity or clinical manifestations. As anti-CD38 treatment is being investigated in SLE, our results may have important implications for the personalized targeting of pathogenic leukocytes by anti-CD38 monoclonal antibodies.

Permeabilization-free detection of nucleated cells in mass cytometry using iodinated Hoechst

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Cell identification in mass cytometry relies on their sufficient labeling with a metal-labeled probe, which is routinely achieved by post-permeabilization staining with an iridium-containing DNA intercalator. Hoechst dyes are commonly used in flow cytometry to detect live nucleated cells due to its cell membrane permeant properties. Here, we introduce the permeabilization-free detection of nucleated cells using iodinated Hoechst-33258 (iHoechst) by mass cytometry. iHoechst specifically stained live nucleated cells, and was successfully combined with antibody staining and dead cell detection reagents in a single incubation step. We show that iHoechst can be applied to human whole blood as well as to peripheral blood mononuclear cells (PBMC). Thus, iHoechst reduces hands-on time for the preparation of mass cytometry assays. Preliminary data indicate that frequencies of major leukocyte subsets, signal intensities resulting from staining with metal-labeled antibodies, and numbers of cell events retrieved in immune phenotyping experiments involving iHoechst were similar to those observed when using conventional, iridium intercalator-based protocols. Fluorescence microscopy confirmed that Hoechst maintains its fluorescent properties after iodination. Therefore, iHoechst can potentially be used for cross-platform identification of nucleated cells in flow and mass cytometry. Together, we propose iHoechst as an alternative to iridium-based detection of nucleated cells in mass cytometry.

Transition of adherent to suspension state: relevance to cell mechanical properties

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Adherent cells often detach from their native surface as a result of important physiological changes such as those, for example, found in cancer. While many studies have examined the mechanical properties of cells in their native adherent or suspended state, few studies have addressed the consequences associated with the transition between these two states. We have addressed this problem by using atomic force microscopy (AFM) to study the mechanics of adherent cells and surface-tethered transiently suspended cells while cells fully in suspension were mechanically probed using real-time deformability cytometry (RT-DC). To this end, we use HEK293T cells and use surface-tethering molecules to mimic the transition of cells from adherent to fully suspended state. Our results indicate that cell detachment is associated with increased stiffening of cells. Interestingly, surface-tethered transiently suspended cells and fully suspended cells differ in their mechanical properties. Confocal laser scanning microscopy data further indicate that these changes are accompanied by morphological differences and in F-actin distribution.

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Dynamic real-time deformability cytometry to study the temperature-dependency of blood cell rheology for bats

Bats are asymptomatic reservoir hosts for several highly pathogenic viruses and are capable of limiting excessive or inappropriate virus-induced inflammation. To date, in-depth analysis of immune responses in bats has been hampered by the paucity of immunological reagents. In this work, for the first time, we have established an approach that enables the study of the mechanical phenotype of bat blood for label-free identification of specific sub-populations. We used real-time deformability cytometry (RT-DC), a microfluidic technology for mechanical single cell classification in real time with a high throughput of up to several hundred cells per second.

Bats experience up to 41ºC during flight and can lower their body temperature to ≤10ºC during torpor, which may impair immunity whilst hyperthermia could improve pathogen clearance. To understand the impact of heterothermy on cellular stiffness, we performed dynamic real-time deformability cytometry (dRT-DC) which is an extension of RT-DC to capture elastic and viscous properties of single cells for up to 100 cells per second at three different temperatures. Our novel label-free immune cell identification method had clearly distinguished different cell populations in bat blood (Figure 1). Also, we could observe changes in biomechanical properties of blood cells at different temperatures. Comparing blood cells of different bat species and humans exhibited variations in temperature-dependent viscoelastic properties.